ENCYCLOPEDIA OF Environmental Microbiology

Gabriel Bitton

ENCYCLOPEDIA OF

ENVIRONMENTAL MICROBIOLOGY

VOLUMES 1 - 6

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TABLE OF CONTENTS

A

Acetylene Reduction Assay
Acid Mine Drainage
Acidophiles
Actinomycetes, Airborne
Actinomycetes in Activated Sludge
Actinomycetes in Soils
Actinomycetes: Role in Foaming of Activated Sludge
Actinorhizal Symbiosis
Activated Carbon
Activated Sludge, Filamentous Bacteria in
Activated Sludge — Foaming
Activated Sludge, Methodology
Activated Sludge — Microbiology of Nitrogen Removal
Activated Sludge Models: Microbiological Basis
Activated Sludge — Molecular Techniques for Determining Community Composition
Activated Sludge — Sequencing Batch Reactors
Activated Sludge — The Floc
Activated Sludge — The "G-Bacteria"
Activated Sludge — The Microbial Community
Activated Sludge — The Process
Activated Sludge: Use of Molecular Tools
Activity and Carbon Transformations in Biofilms
Adenoviruses
Adhesion, Immobilization and Retention of Microorganisms on Solid Substrata
Adhesion (primary) of Microorganisms onto Surfaces
Adhesion to Surfaces
Adsorption of Microorganisms to Surfaces
Adsorption of Viruses to Surfaces
Aerobic Alkaliphiles
Aerobic Endospores
Aerobic Respiration, Principles of
Aggregates and Consortia, Microbial
Agroterrorism
Air Pollution Control
Air Sampling
Air-Water Interface
Airborne Bacterial Pathogens
Airborne Fungi
Airborne Microorganisms
Airborne Toxigenic Molds
Algae
Algae Biotechnology
Algae, Blue-Green
Algae: Eutrophication
Algae, Polar
Algae: Streams
Algae: Toxicity Testing
Algae, Use as Biological Indicators in Paleolimnology
Algae: Waste Stabilization Ponds
Algal Blooms
Algal Blooms — Impact on Treatment, Taste, and Odor Problems
Algal Pigments as Indicators
Algal Turf Scrubbing: Potential Use for Wastewater Treatment
Alkaline Enzymes
Alkaliphiles
Alkaliphiles: Alkaline Enzymes and Their Applications
Alkalithermophiles (Anaerobic)
Allergens, Fungal
Ammonification
Amoeboid Protozoa
Anaerobic Digestion of Biosolids
Anaerobic Granules
Anaerobic Granules and Granulation Processes
Anammox
Anhydrobiosis
Anthrax
Antibiotic Resistance
Anydrobiosis
Aquifers, Atlantic Coastal Plain
Arbuscular Mycorrhizae
Archaea, Detection of
Archaea: Detection Methods
Archaea in Biotechnology
Archaea in Marine Environments
Archaea in Soil Habitats
Archaea in Soils
Archaea, Thermophilic
Assessing Microbial Proteomes in the Environment
Assimilable Organic Carbon (AOC) in Drinking Water
Assimilable Organic Carbon (AOC) in Treated Water: Determination and Significance
Astroviruses
Attachment of Microorganisms

B

Bacteria: Streams
Bacterial Cell Structure
Bacterial Contaminants in Residential Environments
Bacterial Pathogens in Waste Stabilization Ponds
Bacterial Phytostimulators in the Rhizosphere
Bacterioneuston
Bacteriophage as Indicators
Bacteriophage: Biology and Genetics
Bacteriophage Detection Methodologies
Bacteriophage of Enteric Bacteria: Occurrence and Persistance in the Environment
Bacterioplankton
Benthic Algae
Benthic-Associated Primary Productivity
Bioaerosol Sampling and Analysis
Bioaerosols in Agricultural and Outdoor Settings
Bioaerosols in Industrial Settings
Bioaerosols: Modeling
Bioaerosols: Transport and Fate
Bioaerosols, Virus
Bioaerosols: Wastewater Treatment Plants
Bioaugmentation
Bioaugmentation: Cold-Adapted Microbes

Bioavailability of Organic Substrates
Biochip-Based Devices and Methods in Microbial Community Ribotyping
Biocontrol, Microbial Agents in Soil
Biocorrosion: Role of Sulfate Reducing Bacteria
Biodegradability: Methods for Assessing Biodegradability Under Laboratory and Field Condition
Biodegradable Dissolved Organic Carbon (BDOC)
Biodegradable Dissolved Organic Carbon in Drinking Water
Biodegradation: Composts
Biodegradation: Fuel Oxygenates
Biodegradation, Halogenated Aromatics
Biodegradation: Landfills
Biodegradation: Oxygenase Enzymes
Biodegradation: Reductive Dehalogenation and Metabolism of Chlorinated Organics by Anaerobes
Biodegradation: Wetlands
Biodeterioration
Biodeterioration of Mineral Materials
Biodiversity in Soils: Use of Molecular Methods for its Characterization
Bioemulsifiers
Biofilm Detachment
Biofilms, Activity in
Biofilms, Algal Biofilms
Biofilms: Bacterial-Fungal Biofilms
Biofilms, Carbon Transformations in
Biofilms, Conditioning Films
Biofilms: Extracellular Enzymes
Biofilms, Extracellular Polymeric Substance
Biofilms, Formation Rate in Water Distribution Systems
Biofilms, Growth Kinetics of Microbes in
Biofilms in Natural and Drinking Water Systems

TABLE OF CONTENTS

B

Biofilms in the Food Industry
Biofilms, Methodology
Biofilms, Modeling of
Biofilms, Pathogens in
Biofilms, Sorption Properties of
Biofilms: Suspended Biofilms
Biofiltration
Biofiltration and Bioodors
Biofouling: Chemical Control of Biofouling in Water Systems
Biofouling in the Marine Environment
Biofouling of Industrial Systems
Biogenic Trace Gases
Biogeochemical Cycles
Biohydrometallurgy
Bioleaching
Bioleaching of Metals
Biological Control, Use of Biosurfactants in
Biological Warfare
Biology of Cryptosporidium
Bioluminescence, Methodology
Biomarkers, Lipid
Biomass, Algal
Biomass, Bacterioplankton
Biomass: Soil Microbial Biomass
Biomineralization by Bacteria
Biomining
Biomining Using Archaea
Bioodors Control in Waste Treatment Facilities
Biopesticides
Bioplastics
Bioremediation: An Overview of How Microbiological Processes Can Be Applied to the Cleanup of Organic and Inorganic Environmental Pollutants
Bioremediation: Aquatic Ecosystems
Bioremediation, Aquifers
Bioremediation, Biosurfactants Use in
Bioremediation, Cold-Adapted Microorganisms in
Bioremediation, Methanotrophs in
Bioremediation of Hot Desert Soils
Bioremediation of Soils
Bioremediation, Pesticides in Soils
Bioremediation, PLFA Analysis
Bioreporters
Biosolids: Anaerobic Digestion of
Biosolids, Bioaerosols from
Biosolids, Granules in
Biosorbents for Metals
Biostability of Drinking Water
Biostimulation, Aquatic Ecosystems
Biostimulation, Aquifers
Biostimulation: Cold-Adapted Microbes
Biostimulation, Hot Desert Soils
Biosurfactants: Types, Screening Methods, and Applications
Biotechnological Applications of Biosurfactants and Bioemulsifiers
Biotechnology, Fungi in
Biotechnology, Methanotrophs in
Bioterrorism
Biotoxins
Biotrickling Filters for Air Pollution Control
Blue-Green Algae
Borreliosis, Lyme
Bottled Water
Bottled Water, Microbiology of
Brown Rot Fungi
Brown Tide
Bulking Control
Bulking in Activated Sludge
Bulking of Activated Sludge

C

Caliciviruses
Campylobacter jejuni and Other Enteric *Campylobacter*
Capillary Electrophoresis in Genetic Analysis and Ribotyping of Microbiota in the Environment
Carbon Monoxide
Caves and Mines Microbiological Sampling
Caves and Other Low-Light Environments: Aerophitic Photoautotrophic Microorganisms
Cell Viability, Methodology for
Chaperone and Chaperonins
Chemical Weapons, Biodegradation of
Chemolithoautotrophy
Cholera
Chrysophytes
Ciliated Protozoa
Ciliates in Freshwater Ecosystems
Cisterns
Climate Change, Use of Chrysophytes as Indicators of
Cloning
Clostridium
Coagulation in Water Treatment Plants: Pathogen Removal
Cold-Active Enzymes
Cold-Adapted Microorganisms: Adaptation Strategies and Biotechnological Potential
Cold Deserts
Cold-Induced Proteins
Cold Shock
Cold-Shock Proteins
Coliform Bacteria as Indicators of Water Quality
Coliform Bacteria—Control in Drinking Water Distribution Systems
Coliform Regrowth in Water Distribution Systems
Coliphage: Detection Methodologies
Cometabolism
Commercial Use of Microorganisms, Regulation of
Compost: Biodegradation of Toxic Organic Compounds
Conditioning Films in Aquatic Environments
Confocal Laser Scanning Microscopy (CLSM)

Consortia, Microbial
Controlling the Microbial Quality of Drinking Water in Distribution Systems
Corrosion
Cosmetics, Use of Biosurfactants in
Crude Oil, Biodegradation of
Crude Oil Spills
Culturable Subsurface Microbial Communities
Cyanobacteria
Cyanobacteria in Soils
Cyanobacteria-Toxins in Drinking Water
Cyanobacterial Mats
Cyanobacterial Mats and Nitrogenen Fixation
Cyanophages
Cyclospora: Basic Biology, Occurrence Fate and Methodologies

D

Data Analysis and Modeling
Dehalogenation of Haloorganics
Denaturing Gradient Gel Electrophoresis (DGGE)
Denitrification
Denitrification in Activated Sludge
Denitrification in the Marine Environment
Desert Environments
Desert Environments: Biological Soil Crusts
Desert Environments — Soil Microbial Communities in Cold Deserts
Desiccation, Adaptation of Microorganisms to
Desiccation by Exposure to Space Vacuum or Extremely Dry Deserts: Effect on Microorganisms
Desiccation of Microorganisms
Desulfurization of Fossil Fuels
Detection of Airborne Microorganisms
Detection of Enteroviruses
Diatoms in Biofilms
Diazotrophs
Dinitrogenase
Dinoflagellates, Toxic
Dioxygenases
Disinfection: Chlorine, Monochloramine, and Chlorine Dioxide
Disinfection, Effect on Viruses
Disinfection of Legionellae
Disinfection of Protozoan Parasites
Disinfection, Ultraviolet (UV)
Dna Chips
Domestic Microbiology
Drinking Water Biofilms
Drinking Water, Cyanobacteria in
Drinking Water Distribution Systems, Invertebrates and Protozoa in
Drinking Water Distribution Systems, Pathogens in
Drinking Water, Nitrifying Bacteria in
Drinking Water, Sulfur Bacteria in
Drinking Water, Viruses in

E

Ecological Significance of Subsurface Microorganisms
Ecology of Marine Microbial Biofilms
Ecology, Pathogenicity, and Systematics of *Aeromonas* in the Environment
Ectomycorrhizal Fungi
Electrophoresis
Emergency Water Supplies
Endolithic Microorganisms in Arid Regions
Endosymbiosis in Ecology and Evolution
Endosymbiosis Involving Amebas
Endosymbiosis Involving Cyanobacteria
Endotoxins in Bottled Water
Enhanced Biological Phosphorus Removal (EBPR) in Activated Sludge
Enhanced Detection of Airborne Microbial Contaminants
Entamoeba histolytica / Entamoeba dispar
Enteric Adenoviruses
Enterococci, Fecal
Enterohemorrhagic E. Coli (EHEC)
Enteroinvasive E. Coli
Enteropathogenic E. Coli (EPEC)
Enterotoxigenic E. Coli (ETEC)
Enteroviruses: basic Biology and Diseases
Enteroviruses in Water: Concentration and Detection

TABLE OF CONTENTS

E

Enteroviruses: Occurrence and Persistence in the Environment
Entomopathogens (Bacteria, Fungi, Prototozoa, Viruses)
Environmental Genomics
Environmental Release of Microorganisms
Enzymatic Toxicity Tests
Enzyme Inhibition
Enzyme Kinetics
Enzymes, Archaeal
Enzymes: Biotechnological Applications
Enzymes, Cold-Active
Enzymes, Industrial
Enzymes in Soils
Enzymes: Oxygenases
Epifluorescence Microscopy
Epilithic Microorganisms
EPS
Eutrophication and Algae
Evolution of Metabolic Pathways for Degradation of Environmental Pollutants
Exobiology
Extracellular Enzymes in Biofilms
Extracellular Polymeric Substances (EPS)
Extracellular Polymeric Substances (EPS): Structural, Ecological and Technical Aspects
Extremophiles: Life in Extreme Environments

F

Fate and Microbial Degradation of Halogenated Aromatics
Fate of Viruses and Protozoan Parasites in Aquatic Sediments
Fecal Contamination, Sources of
Fecal Streptococci/Enterococci in Aquatic Environments
Field Release of Genetically Engineered Microorganisms (GEM)
Filamentous Bacteria in Activated Sludge: Current Taxonomic Status and Ecology
Filamentous Bulking in Activated Sludge, Control of
Filtration (High-Rate): Removal of Pathogenic Microorganisms
Filtration: Occurrence of Protozoa in Spent Filter Backwash Water
Fingerprinting Techniques
Flagellated Protozoa
Flocs, Microbial
Flooded Soils
Fluorescence Microscopy
Fluorescent in situ Hybridization (Fish)
Fluorescent in situ Hybridization (Fish): Use in Activated Sludge
Fluorescent Probes for in situ Analyses of Microbial Communities
Fluorochromes
Foaming in Activated Sludge
Food Contamination
Food Industry
Food Preservation
Food Processing and Bioaerosols
Fossil Fuels Desulfurization
Free-Living Amebas Present in the Environment Can Cause Meningoencephalitis in Humans and Other Animals
Freeze-Drying
Freeze Drying: Preservations of Microorganisms by Freeze-Drying
Fuel Additives, Biodegradation of
Fumonisins
Fungal Allergy and Allergens
Fungal Biofilms
Fungal Contaminants
Fungi
Fungi and Indoor Air
Fungi and Pollutant Biodegradation
Fungi, Culture Media for
Fungi, for Biotechnology
Fungi in Freshwater Ecosystems
Fungi in Marine/Estuarine Waters
Fungi in Soils
Fungi in Streams

G

G-Bacteria in Activated Sludge
Gallionella ferruginea: An Iron-Oxidizing and Stalk-Forming Groundwater Bacterium
Gases, Trace
Gene Chips
Gene Exchange in Biofilms
Gene Probes
Gene Transfer in Biofilms
Genetic Ecology of Soil
Genetic Manipulation of Fungi
Genetically Engineered Microorganisms (GEMs)
Genetically Engineered Microorganisms for Biodegradation of Recalcitrant Compounds
Genetically Modified Microorganisms (GMM) in Soil Environments
Genomics, Environmental
Geochemical and Geological Significance of Subsurface Microbiology
Geochemical Aspects of Subsurface Microbiology
Geostatistics for Determining the Distribution of Soil Microorganisms
Giardia: Basic Biology, Genetics and Epidemiology
Giardia: Detection and Occurrence of in the Environment
Granular Activated Carbon, Bacteriology of
Granular High-Rate Filtration: Removal of Pathogenic Microorganisms
Granular Sludge
Green Fluorescent Protein (GFP)
Greenhouse Effect, Role of Microorganisms
Groundwater, Protistan Communities in

Groundwater Sampling
Groundwater, Sulfur Bacteria in
Groundwater, Viruses in
Growth, Diauxic
Growth, on Mixed Substrates

H

Haber-Bosch Process
Halogenated Compounds, Bioremediation of
Halogenated Hydrocarbon (Gases)
Haloorganics Dehalogenation
Halophiles
Halophiles: Aerobic Halophilic Microorganisms
Halophiles: Anaerobic Prokaryotes from Hypersaline Environments
Halophilic Archaea
Halophilic Microorganisms
Halotolerant Bacteria
Harmful Algal Blooms (HAB)
Heat-Shock Genes
Heat-Shock Proteins
Heavy Metal Toxicity
Helicobacter pylori
Hepatitis Viruses (HAV-HEV)
Heterotrophic Bacteria as Indicators of Water Quality
High Hydrostatic Pressure: Microbial Inactivation and Food Preservation
Home Treatment Devices — Microbiology of Point of Use and Point of Entry Devices
Hot Desert Soil Microbial Communities
Hot Tubs Microbiology
Human Caliciviruses: Basic Virology and Epidemiology
Humic Substances in Aquatic Environments
Hydrocarbon Biodegradation in Cold Environments
Hydrocarbons, Biodegradation of
Hydrocarbons, Bioremediation of
Hydrodesulfurization
Hydrophobicity of Microorganisms: Methodology
Hydrothermal Vents: Biodiversity in Deep-Sea Hydrothermal Vents
Hydrothermal Vents: Prokaryotes in Deep-Sea Hydrothermal Vents
Hyperthermophiles

I

Ice Environments
Ice Microbial Communities
Identification of Airborne Fungi
Igneous Rock Aquifers Microbial Communities
Image Analysis of Microorganisms
Imaging
Immobilization of Microorganisms on Solid Substrata
Indicators of Water Quality: Aerobic Spores
Indicators of Water Quality: Bacteriophage
Indicators of Water Quality: Clostridium
Indicators of Water Quality: Coliforms
Indicators of Water Quality: Fecal Streptococci/Enterococci
Indicators of Water Quality: Source Water Protection
Indoor Exposure to Fungi
Indoor Health
Industrial Enzymes
Industrial Settings, Bioaerosols in
Infectious Airborne Bacteria
Inorganic Nutrient Use by Marine Microorganisms
Insecticides, Microbial
Invertebrate-Associated Microorganisms in Deep-Sea Hydrothermal Vents
Invertebrates and Protozoa (Free-Living) in Drinking Water Distribution Systems
Ion Exhange in Water Treatment Plants: Pathogen Removal
Ionizing Radiation, Effect on Microorganisms
Iron Cycling
Iron Oxidation
Iron-Reducing Microbes in Petroleum Reservoirs
Isospora
Isotopic Fractionation

K

Kinetics (Microbial): Theory and Applications
Kinetics of Microbial Processes and Population Growth in Soil

TABLE OF CONTENTS

L

Labelling of Microbial Cells (Enzymatic, Fluorescent, Immunological, Phylogenetic, and Physiological Labels)
Lakes, Periphyton in
Landfilling of Municipal Solid Wastes: Microbiological Processes and Environmental Impacts
Laser Scanning Microscopy in Combination With Fluorescence Techniques for Biofilm Study
Leaf Decomposition By Fungi in Freshwater Ecosystems
Legionellae
Legionella in the Environment: Persistence, Evolution, and Pathogenicity
Legume Inoculation
Leptospirosis
Lipid Biomarkers in Environmental Microbiology
Lipids, Archaeal
Lithotrophic Microbial Ecosystems in the Subsurface
Low Light Environments
Luciferase and Green Fluorescent Protein as Bioreporters in Microbial Systems
Lyme Borreliosis

M

Machining and Bioaerosols
Macrophyte Colonization by Fungi
Magnetotactic Bacteria
Manganese Cycling
Manganese Oxidation
Marine Biotechnology
Marine Methanogens
Mats, Microbial
Membrane Filter Procedure for Heterotrophic Bacteria
Mercury Cycling
Meroplankton
Messenger RNA-Targeted Probes
Metabolic Pathways
Metabolism of Mixtures of Organic Pollutants
Metals
Metals, Bioremediation of
Metals in Soils
Metals: Interaction with Biofilms
Metals, Microbe Resistance to
Metals: Microbial Processes Affecting Metals
Metal Stressed Environments, Bacteria in
Metal Transformations
Metal (U,Fe,Mn,Hg) Cycling
Methane
Methanogenesis
Methanogenesis in the Marine Environment
Methanogens
Methanogens: Biotechnological Applications
Methanogens in Petroleum Reservoirs
Methanogens in Soils
Methanotrophic Bacteria
Methanotrophic Bacteria: Use in Bioremediation
Methanotrophs
Methods for Flow Cytometry and Cell Sorting
Methods for the Identification of Microbial Isolates
Methylation of Metals
Microarrays
Microarrays: Applications in Environmental Microbiology
Microbial Degradation of Explosives
Microbial Degradation of Fuel Oxygenates
Microbial Enhanced Oil Recovery (MEOR), Use of and Biosurfactants in
Microbial Flocs Suspended Biofilms
Microbially-Influenced Corrosion (MIC)
Microbial Removal by Pretreatment, coagulation and Ion Exchange
Microbial Starvation Survival in Subsurface Environments
Microbial Toxicity Tests
Microbiology of Atlantic Coastal Plain Aquifers and Other Unconsolidated Subsurface Sediments
Microbiology of Cretaceous Shales and Sandstones of the Southwestern United States
Microbiology of Deep High Temperature Sedimentary Environments
Microchips
Microgravity Effects on Microorganisms
Microorganisms in Soil: Factors Influencing Their Activity
Microspheres as Tracers in Groundwater
Microsporidia: Basic Biology
Microsporidia: Occurrence, Fate and Methodologies
Mine Tailings
Mines
Mixotrophs
Modeling of Biofilms
Modeling of Virus Transport and Removal in the Subsurface
Modeling the Transport of Bioaerosols
Modeling
Models, Activated Sludge
Models: Role of Protozoa
Molecular Methods for Soils
Monooxygenases
Mustard Gas, Biodegradation of
Mycobacterium avium Complex
Mycorrhizae
Mycorrhizae: Arbuscular Mycorrhizae
Mycorrhizae: Ectomycorrhizal Fungi
Mycotoxins

N

Natural Organic Matter (NOM) in Aquatic Environments;
Neural Networks
Neuston Microbiology: Life at the Air–Water Interface
Nitrification
Nitrification in Activated Sludge
Nitrification in Aquatic Systems
Nitrification in Soils

Nitrification in The Marine Environment
Nitrifiers Typing using Microchips
Nitrifying Bacteria in Drinking Water
Nitogen Cycle
Nitrogen Cycle in Aquatic Systems
Nitrogen Cycle in Soils
Nitrogen Cycle in the Marine Environment
Nitogen Fixation
Nitrogen Fixation in Methanotrophs
Nitrogen Fixation in Soils — Free-Living Microbes
Nitrogen Fixation in Soils (Symbiotic)
Nitrogen Fixation in the Marine Environment
Nitrogen Fixation Measurement
Nitrogen Gases
Nitrogen in Marine Microorganisms
Nitrogen Removal in Activated Sludge
Norwalk-Like Viruses: Detection Methodologies and Environmental Fate
Norwalk-Like Viruses (NLVS)
Nosocomial Infections
Nuclear Waste Respository in Yucca Mountain: Microbiological Aspects
Nucleic Acids Extraction from Soils
Nutrient Cycling: Role of Bacterioplankton
Nutrients Use By Marine Microorganisms

O

Occurrence of Protozoa in Spent Filter Backwash Water
Ochratoxins
Oil Reservoirs
Oligotrophic Bacteria
Opportunistic Pathogens
Organic Matter in Water
Organic Matter Removal in Water Treatment
Organic Substances: Interaction with Biofilms
Organophosphorus Nerve Agents, Biodegradation of
Osmoprotection of Microorganisms
Oxygen: Effect on Marine Microbial Communities
Oxygenase Enzymes: Role in Biodegradation

P

Paleoecology
Paleoenvironmental Reconstruction
Paleolimonology
Paleolimnology: Subfossil Algae Other than Diatoms and Chrysophytes
Paleolimnology: Use of Algal Pigments as Indicators
Paleolimnology: Use of Siliceous Structures of Chrysophytes as Biological Indicators in Freshwater Systems
Panspermia Thesis
Paralytic Shellfish Poisoning
Parasitic Protozoa: Fate in Wastewater Treatment Plants
Pathogen, Bacterial
Pathogenic *Escherichia coli*
Pathogens, Bacterial
Pathogens (Bacterial) in Waste Stabilization Ponds
Pathogens in Environmental Biofilms

TABLE OF CONTENTS

P

Pathogen Survival in Aquatic Environmnents
Periphyton
Permafrost
Pesticide Degradation in Soils
Pesticides, Bioremediation of
Pest Mangement
Petroleum and Other Hydrocarbons, Biodegradation of
Petroleum Reservoirs, Influence, Activity and Growth of Subsurface Microflora in
Petroleum Reservoirs, Microbial Diversity in
Pfiesteria: The Toxic Pfiesteria Complex
Phage: Detection Methodologies
Phosphorus Cycling in Aquatic Environments: Role of Bacteria
Phosphorus in Marine Microorganisms
Photosynthetic Bacteria
Photosynthetic Bacteria in Caves
Photosynthetic Bacteria in Drinking Water
Photosynthetic Bacteria in Soils
Photosynthetic Bacteria in Waste Stabilization Ponds
Photosynthetic Pigments in Marine Algae and Bacteria
Phototrophic Purple and Green Bacteria in Marine and Hypersaline Environments
Phycotoxins
Phyllosphere
Phylogenetically Based Methods in Microbial Ecology
Phylogenetics, Archaea
Phylogeny
Phylogeny of Aquifer Communities
Phytoneuston
Phytoplankton
Phytoplankton, Polar
Phytostimulators, Bacterial
Pigments of Algae as Paleolimnological Indicators
Planktonic Algae
Planktonic Algae in the Marine Environment
Planktonic Microorganisms: Bacterioplankton
Plant–Microbe Interactions in the Marine Environment
Plfa Analysis
Point-of-Use Treatment Devices for Drinking Water
Polar Marine Phytoplankton
Polymerase Chain Reaction (PCR)
Polymers in Activated Sludge
Population Growth in Soils, Kinetics of
Preservation of Algae for Toxicity Testing
Pretreatment in Water Treatment Plants: Pathogen Removal
Primary Productivity
Primary Productivity in the Marine Environment
Probes
Probes: Rrna-Targeted Probes for Activated Sludge
Processing of Subsurface Samples
Prochlorococcus
Productivity, Bacterioplankton
Protection of Source Water
Protistan Communities in Groundwater
Protozoa (Free-Living) in Drinking Water Distribution Systems
Protozoa in Activated Sludge
Protozoa in Marine and Estuarine Waters
Protozoa in Streams
Protozoan Ciliates in Freshwater Ecosystems
Protozoan Parasites
Protozoan Parasites, Disinfection of
Protozoan Parasites in Food
Protozoan Parasites in Sediments
Pseudomonas
Psychrophiles
Psychrophilic Bacteria
Psychrophilic Bacteria: Isolation and Characterization
Psychrophilic Microorganisms
Psychrotolerant Microorganisms
Psychrotrophic Bacteria
Psychrotrophic Microorganisms
Pulp and Paper Industry: Microbiological Aspects of

Q

Quality Assurance/Quality Control in Subsurface Sampling and Processing
Quality, Soil Quality
Quantification of Microbial Biomass
Quorum Sensing in Biofilms

R

Radiation Effect on Bacteria
Radiation Effects on Microorganisms
Radioactive Waste Disposal, Geomicrobiology of
Rainwater Roof Catchment Systems, Microbial Quality of
Red Tides and Other Harmful Algal Blooms
Reedbeds for Wastewater Treatment
Regrowth of Bacteria in Water Distribution Systems
Regulation of Bottled Water
Regulation of the Commercial Uses of Microorganisms
Regulations for Drinking Water
Removal of Pathogenic Microbes by Granular High-Rate Filtration
RESIDENTIAL ENVIRONMENTS Retention of Microorganisms on Solid Substrata
Rhizosphere
Rhizosphere, Archaea in
Rhizosphere Microbiology
Rhizospheric Effect
Ribosomal RNA-Targeted Probes.
Ribotyping
Ribotyping Methods for Assessment of in situ Microbial Community Structure
Ribotyping of Microbial Communities
Rice Agriculture
Rice Soils
Risk Assessment
Risk Assessment of Environmental Exposure to Viruses

Riverine Biofilms
Rivers, Periphyton in
Root Exudates
Root Secretions
Rotaviruses
Rural Water Supplies

S

Salinity Effects on the Physiology of Soil Microorganisms
Salinity Tolerance
Salmonella in Aquatic Environments
Salt Production
Salt Production, Involvement of Microorganisms in
Sampling in Subsurface Environments
Sampling of Bioaerosols
Sampling Techniques for Environmental Microbiology
Screening Chemical Toxicity in Soils
Seagrasses Communities
Sea Ice Microorganisms
Sediments
Sediment Sampling
Sediments and PLFA Analysis
Sediments, Archaea in
Sediments, Bioremediation of
Sediments: Nitrification in
Sediments, Nitrogen Fixation in
Sediments: Sulfate Reduction in Marine Sediments
S Gases
S-Layer, Archaeal
Septic Tank Systems
Sequencing Batch Biofilm Reactor (SBBR)
Sequencing Batch Reactor (SBR) Technology
Shelf Storage of Bottled Water
Shellfish, Salmonella in
Shigella
Siderophores
Siderophores in Marine Bacteria
Siliceous Structures of Chrysophytes
Silicon in Marine Microorganisms
Sludge
Sludge, Granules in
Snow and Ice Environments
Soil Archaea
Soil Bacteria
Soil Bioremediation (Pesticides)
Soilborne Diseases, Biocontrol of
Soil Crusts, Biological
Soil Distribution of Microorganisms
Soil Enzymes
Soil Formation, Role of Microorganisms
Soil Fungi: Nature's Nutritional Network
Soil Genetic Ecology
Soil Microbial Biomass
Soil and Soil Microorganisms
Soil Nitrogen Cycle
Soil Quality
Soil Quality: The Role of Microorganisms
Soil Sampling
Soil Slurry Sequencing Batch Reactor (Ss-Sbr)
Soils, Biodegradation in
Soils, Biodiversity in
Soils, Distribution of Microorganisms in
Soils, Kinetics of Microbial Processes
Soil Toxicity Tests
Soils, Virus Survival in
Solar Power: Disinfection of Cistern Water
Solid Wastes
Solid Wastes Microbiology
Solvents, Effects on Microorganisms
Solvent-Tolerant Bacteria
Sorption Properties of Biofilms
Sorting of Microbial Cells.
Source Water Protection: Microbiology of Source Water
Sources of Fecal Contamination
Spa and Hot Tub Microbiology

TABLE OF CONTENTS

S

Space Microbiology: Effects of Ionizing Radiation on Microorganisms in Space
Space Microbiology: Microgravity and Microorganisms
Space: Survival in Space
Space Vacuum
Spatial Distribution of Microorganisms in Soils
Spores As Indicators
Stalk-Forming Bacteria
Starvation of Microorganisms in Subsurface Sediments
Statistical Analysis
Storage of Environmental Samples
Storage Polymers: Role in the Ecology of Activated Sludge
Stream Microbiology
Streams, Periphyton in
Streptococci, Fecal
Stress
Stress Genes
Stress Proteins
Stress Response in Archaea
Stress Response in Bacteria
Stress Response in Bacteria: Heat Shock
Stromatolites
Subaerial Communities
Subsurface Microbial Communities: Diversity of Culturable Microorganisms
Subsurface Microbiology
Subsurface Samples: Collection and Processing
Subsurface Sampling
Subsurface Sedimentary Environments
Subsurface Sediments
Sulfate Reducing Bacteria
Sulfate-Reducing Bacteria: Environmental and Technological Aspects
Sulfate Reducing Bacteria in Petroleum Reservoirs
Sulfate Reduction in Marine Sediments
Sulfur Bacteria in Drinking Water
Sulfur Cycle
Sulfur Cycle in Soils
Sulfur Oxidizing Bacteria
Surfaces
Suspended Biofilms
Sustainable Agriculture
Sustainable Agriculture: Role of Microorganisms
Sustainable Solid Waste Management
Symbiotic Nitrogen Fixation

T

Textile Industry and Bioaerosols
Thermophiles
Thermophiles: Anaerobic Alkalithermophiles
Thermophiles, Diversity of
Thermophilic Alkaliphiles
Tnt Biodegradation
Toxicity of Metals to Microorganisms
Toxicity of Organic Solvents to Microorganisms
Toxicity Testing in Soil, Use of Microbial and Enzymatic Tests
Toxicity Testing in Wastewater Treatment Plants Using Microorganisms
Toxigenic Molds
Toxins, Microbial
Toxoplasma gondii
Trace Elements in Marine Microorganisms
Trace Gases Soil
Tracers in Groundwater: Use of Microorganisms and Microspheres
Transport of Bacteria Through Soils
Transport of Pathogens in Surface Waters
Trophic State and Algal Community Structure

U

Unbalanced Growth Conditions
Uranium Cycling
Use of Cold-Adapted Microorganisms in Biotechnology
Use of Microscopic Algae in Toxicity Testing
UV Disinfection — Theory to Practice

V

Vadose Zone Microbiology
Viable But Not Culturable (Vbnc)
Viable But Not Culturable (VBNC) Microorganisms
Viable Counts in Environmental Samples
Viral Aerosols
Viral Diseases
Viral Disinfection
Virus Survival in Soils
Virus Transport in The Subsurface
Virus Transport Though Soils
Viruses and Protozoan Parasites in Food, Including Methodology
Viruses: Astroviruses
Viruses: Caliciviruses
Viruses, Concentration and Detection
Viruses: Enteroviruses
Viruses: Hepatitis Viruses
Viruses in Drinking Water and Groundwater
Viruses in Food
Viruses in the Marine Environment
Viruses in Waste Stabilization Ponds
Viruses: Rotaviruses
Volcanic Tuffs: Deep Subsurface Microbiology of

W

Wastewater and Biosolids as Sources of Airborne Microorganisms
Wastewater Stabilization Ponds
Wastewater Treatment and Bioaerosols
Wastewater Treatment and Giardia
Wastewater Treatment Microbiology

Wastewater Treatment, Microbiology of
Wastewater Treatment-Septic Tank Systems
Water Activity
Water Distribution Systems
Water Fungi as Decomposers in Freshwater Ecosystems
Water Sampling
Water Treatment Plants: Protozoa in Spent Filter Backwash Water
Weathering
Weathering, Microbiol
Weathering: Mineral Weathering and Microbial Metabolism
Wetlands
Wetlands and Readbeds for Wastewater Treatment
Wetlands: Biodegradation of Organic Pollutants
Wetlands, Fungal Activity in
Wetlands, Periphyton in
White Rot Fungi
White Rot Fungi in Bioremediation
Wood Decomposition By Fungi
Wood Degrading Fungi
Wood Processing and Bioaerosols

X

Xerotolerant Microorganisms

Z

Zoonotic Transmission of Parasites
Zoonotic Transmission of Pathogens

PREFACE

Environmental microbiology was born at the dawn of the "environmental era" at the beginning of the 1970s. Thirty years of maturation have led to an exciting and vibrant field that has attracted countless numbers of productive and enthusiastic scientists and students at universities, research centers and government agencies around the world. The wealth of environmental microbiology literature has made it imperative to make a pause at this time and summarize our knowledge in an encyclopedia format. For the sake of organization, we have identified 14 areas within environmental microbiology. These areas are discussed in more details in the introductory chapter.

The rising tide of molecular biology has led to the use and adaptation of modern molecular techniques along with sophisticated equipment to the study of microorganisms in their environments, especially extreme environments. The study of extremophiles has increased our knowledge on the limits and origin of life on our planet.

Over 420 authors from 25 countries contributed 320 entries to this encyclopedia. We have witnessed a small reduction in the planned coverage of topics, due to the inability of some authors to deliver their manuscripts. Despite this slight setback, some on-board authors have courageously agreed to "plug the holes" by expanding their own contribution to cover a missing topic or by authoring another entry. In addition to the print version, an expanded version of the encyclopedia will be available in the near future on-line to cover those missing topics. Cross-reference titles (orphan entries) or key words have been included in the encyclopedia to help readers retrieve a given topic, and an author index is also provided.

The *Encyclopedia of Environmental Microbiology* will serve as a quick reference work to be used by professors, undergraduate and graduate students, researchers in the public and private sectors, research organizations, environmental and patent lawyers, and government officials for a quick introduction to a given topic in this vast microbiology field.

The preparation and completion of this encyclopedia is a complex undertaking that involved the participation and cooperation of several individuals. The authors are the "soldiers" without whom this work would not have been possible. They contributed their expertise unselfishly despite their busy schedules. I am thankful and grateful to all of them for sharing their knowledge with anybody interested in this fascinating field. I am indebted to my colleagues and co-editors on the editorial board who have patiently and expertly helped me in orchestrating this major endeavor. They have helped tremendously in selecting suitable authors and in participating in the review of the manuscripts. Their names and affiliation appear on a separate list in this encyclopedia. I am grateful to my colleagues and my students at the Department of Environmental Engineering Sciences for their support and encouragement.

The quality of manuscripts is greatly enhanced by the participation of expert reviewers. I am indebted to the hundreds of reviewers who offered many useful suggestions for improving the manuscripts. Their names do not appear in this encyclopedia because many of them expressed the desire to remain anonymous. Thank you for a job well done.

This encyclopedia involved the participation and cooperation of several individuals at John Wiley and Sons. I would like to thank the team at the Encyclopedia Department who has worked tirelessly to see the successful completion of this work. I thank Glenn Collins who recruited me and convinced me that the job of editor-in-chief of the encyclopedia was a worthwhile endeavor. I extend special thanks to Laurie Claret, the assistant managing editor, for her expert assistance as she undertook this job without missing a beat, following the departure of Glenn Collins. I thank Surlan Murrel, editorial assistant, for her patience and, along with Laurie, for shielding me from the massive amount of correspondence and tedious record keeping. I thank them for graciously adapting to my academic lifestyle which sometimes consists of reviewing manuscripts in the Luxembourg Garden and sidewalk cafes in Paris.

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ABBREVIATIONS USED IN AN OVERVIEW OF IMAGING SCIENCE

Abs	absorption
AgX	silver halide
ATC	air traffic control
B	magnetic
β	beta particle (electron)
$\beta+$	positron
Bio.	biologists
BW	bandwidth
CCD	charge-coupled device
Chem	chemical
CID	charge-injected device
CP	charged particle
CRT	cathode-ray tube
CT	computed tomography
DIP	digital image processing
E	electric
e^-	electron
ECG	electrocardiogram
EE	electrical engineers
EEG	electroencephalogram
ESR	electron spin resonance
EMG	electromyogram
EMR	electromagnetic radiation
fMRI	functional magnetic resonance imaging
FT	Fourier transform
ϕ	phase
γ	gamma particle
HC	hard copy
θ, t	angle, time
$h\nu$	photon, radiation
Impe.	electrical impedance
Intel.	intelligent
IR	infrared
IS	intelligent system
LC	inductive capacitive
LCD	liquid crystal display
MEG	magnetoencephalography
MIS	multispectral image segmentation
MRA	magnetic resonance angiography
MRI	magnetic resonance imaging
MW	microwave
n	neutron
ν	frequency
NMR	nuclear magnetic resonance
obj.	objects
org.	organic
PET	positron emission tomography
PMT	photomultiplier tube
Polar	polarization
PIXIE	proton-induced X-ray emission
P	proton
RF	radio frequency
Refl	reflection
Scint.	scintillator
SIMS	secondary ion mass spectroscopy
SC	soft copy
Scat.	scattering
SQUID	superconducting quantum interference device
SSDA	solid-state detector array
Surf.	surface
Syst.	system
t	time
tan	tangent
Tomo.	tomographic
Tran.	transmission
T_2^*	inhomogeneous nuclear spin–spin relaxation time
UV	ultraviolet
x, y	two perpendicular spatial dimensions
2-D	two-dimensional

CONVERSION FACTORS, ABBREVIATIONS, AND UNIT SYMBOLS

SI UNITS (Adopted 1960)

The International System of Units (abbreviated SI), is being implemented throughout the world. This measurement system is a modernized version of the MKSA (meter, kilogram, second, ampere) system, and its details are published and controlled by an international treaty organization (The International Bureau of Weights and Measures) (1).

SI units are divided into three classes:

BASE UNITS

length	meter[†] (m)
mass	kilogram (kg)
time	second (s)
electric current	ampere (A)
thermodynamic temperature[‡]	kelvin (K)
amount of substance	mole (mol)
luminous intensity	candela (cd)

SUPPLEMENTARY UNITS

plane angle	radian (rad)
solid angle	steradian (sr)

DERIVED UNITS AND OTHER ACCEPTABLE UNITS

These units are formed by combining base units, supplementary units, and other derived units (2–4). Those derived units having special names and symbols are marked with an asterisk in the list below.

Quantity	Unit	Symbol	Acceptable equivalent
*absorbed dose	gray	Gy	J/kg
acceleration	meter per second squared	m/s^2	
*activity (of a radionuclide)	becquerel	Bq	1/s
area	square kilometer	km^2	
	square hectometer	hm^2	ha (hectare)
	square meter	m^2	
concentration (of amount of substance)	mole per cubic meter	mol/m^3	
current density	ampere per square meter	A/m^2	
density, mass density	kilogram per cubic meter	kg/m^3	g/L; mg/cm^3
dipole moment (quantity)	coulomb meter	C·m	
*dose equivalent	sievert	Sv	J/kg
*electric capacitance	farad	F	C/V
*electric charge, quantity of electricity	coulomb	C	A·s
electric charge density	coulomb per cubic meter	C/m^3	
*electric conductance	siemens	S	A/V
electric field strength	volt per meter	V/m	
electric flux density	coulomb per square meter	C/m^2	
*electric potential, potential difference, electromotive force	volt	V	W/A
*electric resistance	ohm	Ω	V/A
*energy, work, quantity of heat	megajoule	MJ	

Quantity Unit Symbol Acceptable equivalent

[†]The spellings "metre" and "litre" are preferred by ASTM; however, "-er" is used in the *Encyclopedia*.

[‡]Wide use is made of Celsius temperature (t) defined by

$$t = T - T_0$$

where T is the thermodynamic temperature, expressed in kelvin, and $T_0 = 273.15$ K by definition. A temperature interval may be expressed in degrees Celsius as well as in kelvin.

	kilojoule	kJ	
	joule	J	N·m
	electronvolt†	eV†	
	kilowatt-hour†	kW·h†	
energy density	joule per cubic meter	J/m^3	
*force	kilonewton	kN	
	newton	N	$kg·m/s^2$
*frequency	megahertz	MHz	
	hertz	Hz	1/s
heat capacity, entropy	joule per kelvin	J/K	
heat capacity (specific), specific entropy	joule per kilogram kelvin	J/(kg·K)	
heat-transfer coefficient	watt per square meter kelvin	$W/(m^2·K)$	
*illuminance	lux	lx	lm/m^2
*inductance	henry	H	Wb/A
linear density	kilogram per meter	kg/m	
luminance	candela per square meter	cd/m^2	
*luminous flux	lumen	lm	cd·sr
magnetic field strength	ampere per meter	A/m	
*magnetic flux	weber	Wb	V·s
*magnetic flux density	tesla	T	Wb/m^2
molar energy	joule per mole	J/mol	
molar entropy, molar heat capacity	joule per mole kelvin	J/(mol·K)	
moment of force, torque	newton meter	N·m	
momentum	kilogram meter per second	kg·m/s	
permeability	henry per meter	H/m	
permittivity	farad per meter	F/m	
*power, heat flow rate, radiant flux	kilowatt	kW	
	watt	W	J/s
power density, heat flux density, irradiance	watt per square meter	W/m^2	
*pressure, stress	megapascal	MPa	
	kilopascal	kPa	
	pascal	Pa	N/m^2
sound level	decibel	dB	
specific energy	joule per kilogram	J/kg	
specific volume	cubic meter per kilogram	m^3/kg	
surface tension	newton per meter	N/m	
thermal conductivity	watt per meter kelvin	W/(m·K)	
velocity	meter per second	m/s	
	kilometer per hour	km/h	
viscosity, dynamic	pascal second	Pa·s	
	millipascal second	mPa·s	
viscosity, kinematic	square meter per second	m^2/s	
	square millimeter per second	mm^2/s	
volume	cubic meter	m^3	
	cubic diameter	dm^3	L (liter) (5)
	cubic centimeter	cm^3	mL
wave number	1 per meter	m^{-1}	
	1 per centimeter	cm^{-1}	

†This non-SI unit is recognized by the CIPM as having to be retained because of practical importance or use in specialized fields (1).

In addition, there are 16 prefixes used to indicate order of magnitude, as follows:

Multiplication Factor	Prefix	Symbol	Note
10^{18}	exa	E	
10^{15}	peta	P	
10^{12}	tera	T	
10^{9}	giga	G	
10^{6}	mega	M	
10^{3}	kilo	k	
10^{2}	hecto	h[a]	[a]Although hecto, deka, deci, and centi are SI prefixes, their use should be avoided except for SI unit-multiples for area and volume and nontechnical use of centimeter, as for body and clothing measurement.
10	deka	da[a]	
10^{-1}	deci	d[a]	
10^{-2}	centi	c[a]	
10^{-3}	milli	m	
10^{-6}	micro	μ	
10^{-9}	nano	n	
10^{-12}	pico	p	
10^{-15}	femto	f	
10^{-18}	atto	a	

For a complete description of SI and its use the reader is referred to ASTM E380 (4) and the article UNITS AND CONVERSION FACTORS which appears in Vol. 24.

A representative list of conversion factors from non-SI to SI units is presented herewith. Factors are given to four significant figures. Exact relationships are followed by a dagger. A more complete list is given in the latest editions of ASTM E380 (4) and ANSI Z210.1 (6).

Conversion Factors to SI Units

To convert from	To	Multiply by
acre	square meter (m^2)	4.047×10^{3}
angstrom	meter (m)	1.0×10^{-10}†
are	square meter (m^2)	1.0×10^{2}†
astronomical unit	meter (m)	1.496×10^{11}
atmosphere, standard	pascal (Pa)	1.013×10^{5}
bar	pascal (Pa)	1.0×10^{5}†
barn	square meter (m^2)	1.0×10^{-28}†
barrel (42 U.S. liquid gallons)	cubic meter (m^3)	0.1590
Bohr magneton (μB)	J/T	9.274×10^{-24}
Btu (International Table)	joule (J)	1.055×10^{3}
Btu (mean)	joule (J)	1.056×10^{3}
Btu (thermochemical)	joule (J)	1.054×10^{3}
bushel	cubic meter (m^3)	3.524×10^{-2}
calorie (International Table)	joule (J)	4.187
calorie (mean)	joule (J)	4.190
calorie (thermochemical)	joule (J)	4.184†
centipoise	pascal second (Pa·s)	1.0×10^{-3}†
centistokes	square millimeter per second (mm^2/s)	1.0†
cfm (cubic foot per minute)	cubic meter per second (m^3/s)	4.72×10^{-4}
cubic inch	cubic meter (m^3)	1.639×10^{-5}
cubic foot	cubic meter (m^3)	2.832×10^{-2}
cubic yard	cubic meter (m^3)	0.7646
curie	becquerel (Bq)	3.70×10^{10}†
debye	coulomb meter (C·m)	3.336×10^{-30}
degree (angle)	radian (rad)	1.745×10^{-2}
denier (international)	kilogram per meter (kg/m)	1.111×10^{-7}
	tex‡	0.1111
dram (apothecaries')	kilogram (kg)	3.888×10^{-3}
dram (avoirdupois)	kilogram (kg)	1.772×10^{-3}

†Exact.

‡See footnote on p. xxi.

To convert from	To	Multiply by
dram (U.S. fluid)	cubic meter (m^3)	3.697×10^{-6}
dyne	newton (N)	1.0×10^{-5}†
dyne/cm	newton per meter (N/m)	1.0×10^{-3}†
electronvolt	joule (J)	1.602×10^{-19}
erg	joule (J)	1.0×10^{-7}†
fathom	meter (m)	1.829
fluid ounce (U.S.)	cubic meter (m^3)	2.957×10^{-5}
foot	meter (m)	0.3048†
footcandle	lux (lx)	10.76
furlong	meter (m)	2.012×10^{-2}
gal	meter per second squared (m/s^2)	1.0×10^{-2}†
gallon (U.S. dry)	cubic meter (m^3)	4.405×10^{-3}
gallon (U.S. liquid)	cubic meter (m^3)	3.785×10^{-3}
gallon per minute (gpm)	cubic meter per second (m^3/s)	6.309×10^{-5}
	cubic meter per hour (m^3/h)	0.2271
gauss	tesla (T)	1.0×10^{-4}
gilbert	ampere (A)	0.7958
gill (U.S.)	cubic meter (m^3)	1.183×10^{-4}
grade	radian,	1.571×10^{-2}
grain	kilogram (kg)	6.480×10^{-5}
gram force per denier	newton per tex (N/tex)	8.826×10^{-2}
hectare	square meter (m^2)	1.0×10^{4}†
horsepower (550 ft·lbf/s)	watt (W)	7.457×10^{2}
horsepower (boiler)	watt (W)	9.810×10^{3}
horsepower (electric)	watt (W)	7.46×10^{2}†
hundredweight (long)	kilogram (kg)	50.80
hundredweight (short)	kilogram (kg)	45.36
inch	meter (m)	2.54×10^{-2}†
inch of mercury (32°F)	pascal (Pa)	3.386×10^{3}
inch of water (39.2°F)	pascal (Pa)	2.491×10^{2}
kilogram-force	newton (N)	9.807
kilowatt hour	megajoule (MJ)	3.6†
kip	newton (N)	4.448×10^{3}
knot (international)	meter per second (m/S)	0.5144
lambert	candela per square meter (cd/m^3)	3.183×10^{3}
league (British nautical)	meter (m)	5.559×10^{3}
league (statute)	meter (m)	4.828×10^{3}
light year	meter (m)	9.461×10^{15}
liter (for fluids only)	cubic meter (m^3)	1.0×10^{-3}†
maxwell	weber (Wb)	1.0×10^{-8}†
micron	meter (m)	1.0×10^{-6}†
mil	meter (m)	2.54×10^{-5}†
mile (statute)	meter (m)	1.609×10^{3}
mile (U.S. nautical)	meter (m)	1.852×10^{3}†
mile per hour	meter per second (m/s)	0.4470
millibar	pascal (Pa)	1.0×10^{2}
millimeter of mercury (0°C)	pascal (Pa)	1.333×10^{2}†
minute (angular)	radian	2.909×10^{-4}
myriagram	kilogram (kg)	10
myriameter	kilometer (km)	10
oersted	ampere per meter (A/m)	79.58
ounce (avoirdupois)	kilogram (kg)	2.835×10^{-2}
ounce (troy)	kilogram (kg)	3.110×10^{-2}
ounce (U.S. fluid)	cubic meter (m^3)	2.957×10^{-5}
ounce-force	newton (N)	0.2780
peck (U.S.)	cubic meter (m^3)	8.810×10^{-3}
pennyweight	kilogram (kg)	1.555×10^{-3}
pint (U.S. dry)	cubic meter (m^3)	5.506×10^{-4}

pint (U.S. liquid)	cubic meter (m^3)	4.732×10^{-4}
poise (absolute viscosity)	pascal second (Pa·s)	0.10†
pound (avoirdupois)	kilogram (kg)	0.4536
pound (troy)	kilogram (kg)	0.3732
poundal	newton (N)	0.1383
pound-force	newton (N)	4.448
pound force per square inch (psi)	pascal (Pa)	6.895×10^3
quart (U.S. dry)	cubic meter (m^3)	1.101×10^{-3}
quart (U.S. liquid)	cubic meter (m^3)	9.464×10^{-4}
quintal	kilogram (kg)	1.0×10^{2}†
rad	gray (Gy)	1.0×10^{-2}†
rod	meter (m)	5.029
roentgen	coulomb per kilogram (C/kg)	2.58×10^{-4}
second (angle)	radian (rad)	4.848×10^{-6}†
section	square meter (m^2)	2.590×10^6
slug	kilogram (kg)	14.59
spherical candle power	lumen (lm)	12.57
square inch	square meter (m^2)	6.452×10^{-4}
square foot	square meter (m^2)	9.290×10^{-2}
square mile	square meter (m^2)	2.590×10^6
square yard	square meter (m^2)	0.8361
stere	cubic meter (m^3)	1.0†
stokes (kinematic viscosity)	square meter per second (m^2/s)	1.0×10^{-4}†
tex	kilogram per meter (kg/m)	1.0×10^{-6}†
ton (long, 2240 pounds)	kilogram (kg)	1.016×10^3
ton (metric) (tonne)	kilogram (kg)	1.0×10^{3}†
ton (short, 2000 pounds)	kilogram (kg)	9.072×10^2
torr	pascal (Pa)	1.333×10^2
unit pole	weber (Wb)	1.257×10^{-7}
yard	meter (m)	0.9144†

†Exact.

ABBREVIATIONS AND UNIT SYMBOLS

"When a new discipline such as imaging science evolves from several existing scientific and engineering disciplines, it is difficult to establish an agreed upon list of abbreviations and unit symbols that will be used by all the disciplines. Therefore, intead of presenting here a comprehensive list of abbreviations and unit symbols used in this encyclopedia, each encyclopedia entry has a list of abbreviations and unit sybols used in the entry. Included in this section are a set of rules for writing unit symbols."

Rules for Writing Unit Symbols (4):

1. Unit symbols are printed in upright letters (roman) regardless of the type style used in the surround text.
2. Unit symbols are unaltered in the plural.
3. Unit symbols are not followed by a period except when used at the end of a sentence.
4. Letter unit symbols are generally printed lower-case (for example, cd for candela) unless the unit name has been derived from a proper name, in which case the first letter of the symbol is capitalized (W, Pa). Prefixes and unit symbols retain their prescribed form regardless of the surrounding typography.
5. In the complete expression for a quantity, a space should be left between the numerical value and the unit symbol. For example, write 2.37 lm, *not* 2.37 lm, and 35 mm, *not* 35 mm. When the quantity is used in an adjectival sense, a hyphen is often used, for example, 35-mm film. *Exception*: No space is left between the numerical value and the symbols of degree, minute, and second of plane angle, degree Celsius, and the percent sign.
6. No space is used between the prefix and unit symbol (for example, kg).
7. Symbols, not abbreviations, should be used for units. For example, use "A," not "amp," for ampere.
8. When multiplying unit symbols, use a raised dot:

N·m for newton meter

In the case of W·h, the dot may be omitted, thus:

$$Wh$$

An exception to this practice is made for computer printouts, automatic typewrier work, etc, where the raised dot is not possible, and a dot on the line may be used.

9. When dividing unit symbols, use one of the following forms:

$$m/s \quad or \quad m \cdot s^{-1} \quad or \quad \frac{m}{s}$$

In no case should more than one slash be used in the same expression unless parentheses are inserted to avoid ambiguity. For example, write:

$$J/(mol \cdot k) \quad or \quad J \cdot mol^{-1} \cdot K^{-1} \quad or \quad (J/mol)/K$$

but *not*

$$J/mol/K$$

10. Do not mix symbols and unit names in the same expression. Write:

$$\text{joules per kilogram} \quad or \quad J/kg \quad or \quad J \cdot kg^{-1}$$

but *not*

$$\text{joules/kilogram} \quad nor \quad \text{joules/kg} \quad nor \quad \text{joules} \cdot kg^{-1}$$

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R.P. LUKENS
ASTM Committee E-43 on SI Practice

INTRODUCTION

Environmental Microbiology encompasses numerous levels of organization ranging from microbial genes to microbial ecosystems. It is the study of the activity of indigenous microorganisms in their habitats and their interactions with other microorganisms or higher organisms. It is also the study of the fate of microbial pathogens and microscopic parasites outside their hosts in natural environments such water, sediments, soils, air, and engineered systems. Environmental microbiology also deals with the applied aspects of microbiology as regards the environment, agriculture, food and water quality, resource recovery, water and wastewater treatment, and human and animal health.

This Encyclopedia was designed to provide the most comprehensive coverage of the various fields within Environmental Microbiology. We have identified 14 areas within this discipline. These areas are illustrated in Figure 1 (the names of associate editors for each area are indicated in the figure).

IDENTIFIED AREAS IN ENVIRONMENTAL MICROBIOLOGY

Aeromicrobiology is the study of microorganisms found in the air and the public health implications of airborne microbes (fungi, bacteria, viruses), their metabolites (e.g., mycotoxins), and cell components. Several entries deal with the public health aspects (e.g., allergies) of airborne fungi, especially in indoor environments. Airborne microbes under agricultural and industrial settings are also covered. Other entries deal with models to predict the dispersion and fate of microorganisms in the airborne state. A current preoccupation of public health officials around the world is bioterrorism which concerns the intentional release of pathogenic microorganisms in the air (e.g., *Bacillus anthracis*) for harmful purposes.

The ***aquatic microbiology*** area covers various types of aquatic environments, including freshwater, estuarine and marine waters, groundwater, wetlands, and aquatic sediments. Some of these environments (freshwaters, marine waters) have long been studied by microbiologists and limnologists while others (groundwater, wetlands) have been investigated only during the past 20 years. This vast area necessitated the enlisting of three associate editors to tackle the task.

Freshwater microbiology entries included some on bacteria, cyanobacteria, fungi, algae and protozoa and their activities in lakes, rivers, streams and wetlands. Paleolimnology is also addressed through the use of paleolimnological indicators such as algal pigments and remains (e.g., diatoms) to reconstruct past environmental changes in aquatic environments.

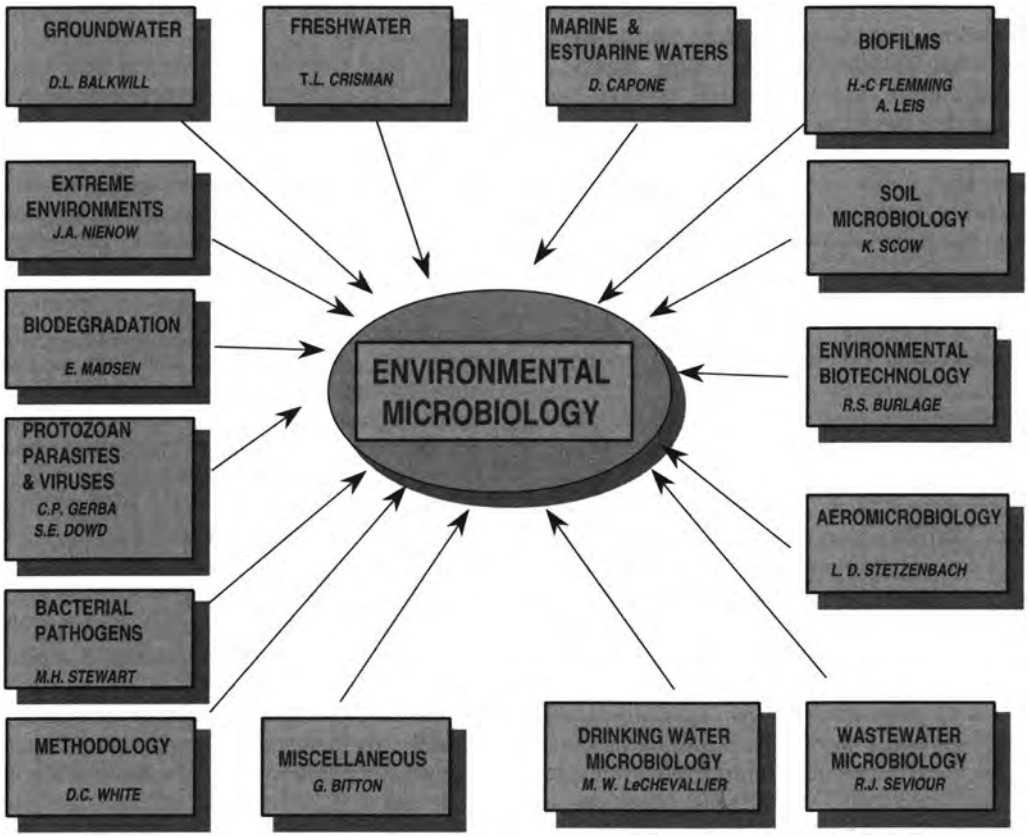

Figure 1.

Marine waters represent approximately 97% of the aquatic environment on planet Earth. This explains the attention given to this environment by marine microbiologists around the globe. Planktonic microorganisms in the oceans and seas serve as food for higher organisms and are the base of the food chain. The marine plankton includes algae, bacteria (eubacteria, cyanobacteria, photosynthetic bacteria), fungi, viruses and protozoa. Algae are responsible for primary productivity in the water column while bacteria play a crucial role in nutrient (N, P, S) and metal cycling in both the water column and the sediments. This encyclopedia also covers chapters on archae including methanogens, red tides and other harmful algae, plant-microorganisms interactions and seagrass microbial communities. The marine environment is the home of a wide range of microorganisms, many of which can be tapped for biotechnological applications which include pharmaceuticals, biomaterials, enzymes, nutritional supplements and other useful products.

The goal of **subsurface microbiology** is the study of the microbiology of subsurface sediments and formations as well as groundwater. Prior to the 1970s, research on this hidden resource was focused on groundwater supply. In the 1970s, researchers directed their attention to groundwater quality, leading to continuing studies on contamination of this precious resource by microbial pathogens and chemical contaminants. The 1980s saw the focus shifting to subsurface sediments with studies on characterization of subsurface microorganisms, their concentrations, and their activity. Cultivation techniques and molecular biology tools showed the existence of a wide range of bacterial types. The phylogenetic characteristics of culturable bacteria from diverse subsurface environments has been undertaken in the U.S., but only a small fraction of approximately 13,000 isolated strains has been characterized. We know now that microorganisms exist in subsurface environments extending to a depth of approximately two miles and possibly beyond this depth. Energy sources, both organic and inorganic, occur at relatively very low concentrations, enough to sustain the microorganisms in this oligotrophic environment. Subsurface microorganisms are stressed, starved, display very low metabolic activity and remain in a dormant state for very long periods of time (thousands to possibly millions of years).

Soil microbiology is one of the oldest subdiscipline within environmental microbiology. Soils play a key role in the life support system on planet Earth. A combination of physical, chemical and biological factors play a role in their formation. Soil microorganisms (bacteria, fungi, protozoa) interact with organic matter, mineral particles, water and gases, the major components of this complex ecosystem. They play a key role in nutrient cycling and affect soil quality by mineralizing plant nutrients, improving soil structure, processing plant residues into organic matter, and degrading toxic compounds in soils, particularly chemical pesticides used in modern agriculture. Rhizosphere microbial communities enter into symbiotic or mycorrhizal associations with plant roots, leading to promotion of plant growth. On the other hand, fungal and bacterial pathogens are the cause of plant diseases with the subsequent decrease in the yield of agricultural crops. Due to serious concerns for human and animal health and for the environment, attempts are being made to use microbial control agents or biopesticides in lieu of chemical pesticides to control plant diseases, nematodes or insects. The most successful biopesticide marketed for insect control is *Bacillus thuringiensis* which produces a toxin that is lethal to insect pests. Unfortunately, soils are becoming the receptacles of a wide range of toxicants, pathogens and parasites as a result of human activities which include agricultural practices, industrial and mining operations, and disposal of liquid and solid wastes. As regards soil contamination by hazardous wastes, current research is addressing soil bioremediation which consists of enhancing the biodegradation capacity of indigenous soil microorganisms or using genetically engineered microorganisms.

Advances in molecular genetics have helped launch the field of soil genetic ecology with the goal of studying gene exchange, microbial community fingerprinting, analysis of functional genes that code for key enzymes, and the phylogenetic diversity of microorganisms in soils. It is estimated that there are approximately 10,000 bacterial species in one gram of soils. Culture-based techniques yield only a very small fraction (<1%) of the total number of species uncovered by using molecular biology techniques such as amplified rDNA restriction analysis (ARDRA), polymerase chain reaction (PCR), denaturing gradient gel electrophoresis (DGGE) or terminal restriction fragment length polymorphism (T-RFLP). Soil genetic ecology may benefit from the use of microarrays (gene chips). All of these efforts may lead to some potential industrial applications.

Dr. Rick Cavicchioli, from the university of New South Wales, characterized the extremophiles as the *"bungi jumpers "* of the microbial world. There are 36 entries covering this theme in the Encyclopedia. These microorganisms are able to survive and grow in ***extreme environments*** with regard to environmental factors such as temperature (psychrophiles, hyperthermophiles), pH (acidophiles, alkaliphiles), salt concentration (halophiles), hydrostatic pressure (barophiles), water activity (xerotolerant microorganisms), microgravity, ionizing radiation, light (e.g., growth under low-light in caves), interfaces such as the air-water interface, nutrient concentration (oligotrophs), and in the presence of toxic chemicals (toxitolerant microorganisms). This area is important to environmental microbiologists who want to gain knowledge on the limits and the origin of life on Planet Earth and potentially in Space. Some of these extreme environments (e.g., permafrost) could serve as models for potential life in other planets and for attempting to answer the question of life preservation on Earth. An active research area concerns the biotechnological applications of these extremophiles and their products.

Research on ***biodegradation*** in water, soil, sediments, wastewater treatment plants is now receiving increased attention by microbiologists around the world. Microorganisms play a key role in biogeochemical cycles, particularly the carbon cycle. During the last few decades, an array of foreign compounds called xenobiotics (i.e.,

foreign to biological systems) was introduced into the environment. These compounds are generally resistant or recalcitrant to degradation by microbial action. The topics addressed in this area include the biodegradation of halogenated aromatics, petroleum and other hydrocarbons, reductive dehalogenation and metabolism of haloorganics, or fuel oxygenates, as well as the methodology to assess the biodegradability of these recalcitrant compounds. Some chemicals such as the halogenated organic compounds (halogenated hydrocarbons, halogenated aromatics, pesticides, PCBs; dioxin, polychlorinated dibenzofurans) are quite resistant to microbial action. Biotechnological applications of this research include mainly the bioremediation of soils and aquifers.

Public health microbiologists deal with the fate (transport and survival) of pathogenic microorganisms and parasites in the environment, and with the development of the appropriate methodology for their detection in environmental matrices. This information is useful for assessing the risk they pose to humans and animals, and for indicating preventive measures for avoiding or reducing human exposure to these harmful agents. During the past three decades we have witnessed dramatic advances in the methodology for detection of pathogenic microorganisms and parasites in environmental samples, including wastewater, drinking water, soils, biosolids, freshwaters, marine waters, sediments, and air. Three associate editors have been assigned to this vast subdiscipline in environmental microbiology. One editor dealt with bacterial pathogens and the two others dealt with viruses and protozoan parasites. Most of the "classical" bacterial pathogens are included (e.g., *Vibrio cholera*, *Salmonella*, *Shigella*, *E. coli*, *Pseudomonas*, *Mycobacterium avium* Complex, *Leptospira*, *Legionella*, *Campylobacter*) as well as emerging pathogens such as *Helicobacter pylori* for which there are indications that it may be transmitted via the waterborne or food-borne routes. The biology, methodology (concentration and assay), risk assessment, and fate in the environment of a wide range of pathogenic viruses are discussed in this encyclopedia. The list include enteroviruses, hepatitis viruses, human caliciviruses including the Norwark virus, rotaviruses, astroviruses, and adenoviruses. In recent years, research has focused on the epidemiology, methodology, fate in the environment, including food, and risk assessment of protozoan parasites which produce hardy cysts or oocysts which persist in the environment. Furthermore, they are not completely removed following passage though water and wastewater treatment plants and are generally resistant to disinfection. The list of parasites covered in this work include *Cryptosporidium*, *Giardia*, *Entamoeba histolytica*, *toxoplasma gondii*, *Isospora*, *Cyclospora*, microsporidia, and free-living amebas. The detection of bacterial and viral pathogens, and protozoan parasites discussed above requires costly and time-consuming methodology as well as well trained labor. The use of microbial indicators of fecal contamination is thus required. These indicators include total coliforms, fecal coliforms, *E. coli*, fecal streptococci/enterococci, *Clostridium perfringens* and bacterial phage.

Public health microbiologists, in collaboration with environmental engineers, must also work towards reducing the pathogen and parasite load in domestic wastewater, biosolids, and drinking water.

Drinking water *microbiologists* seek to understand the mechanisms behind the removal/inactivation of microbial pathogens and parasites, cyanobacterial toxins, algae, and microorganisms responsible for taste and odor problems, by various processes involved in water treatment plants. These processes include coagulation-flocculation, filtration (sand, activated carbon), water softening, disinfection (e.g., chlorination, ozonation, U.V irradiation) and, more recently, biological treatment of water. It is now realized that organic compounds, present at very low concentrations (μg/L levels) in water distribution systems, are responsible for the formation of trihalomethanes microbial regrowth in distribution pipes, and chlorine demand with the subsequent reduction of free available chlorine. Special topics such as home treatment devices, cistern water and bottled water are also covered in the encyclopedia.

The objectives of **wastewater treatment** are the reduction of organic compounds (i.e., BOD reduction) and suspended solids in wastewater, removal/reduction of nutrients (N, P) and toxic trace organics and metals to avoid or at least pollution of receiving waters,, and removal/inactivation of pathogenic microorganisms and parasites. Microorganisms (bacteria, fungi, protozoa) play a crucial role in the biological treatment of wastewater, particularly in BOD and nutrient removal. Several chapters in this encyclopedia are devoted to the microbiology of activated sludge, trickling filters, waste stabilization ponds, biofiltration, biosolids treatment and disposal, and to the use of wetlands, reed beds, algal turf scrubbers, septic tanks or landfills to treat domestic wastewater and municipal solid wastes. The activated sludge process is often prone to solid separation problems such as filamentous bulking and foaming. The physiology, growth kinetics, and control of filamentous bacteria are covered in a few chapters.

Biofilms develop on biological and non biological surfaces immersed in water and wastewater. They are ubiquitous in natural aquatic environments (e.g., stream beds) and in engineered systems (e.g., fixed-film bioreactors). While they are beneficial in fixed-film bioreactors (biological treatment of drinking waster and wastewater) or in groundwater (biodegradation of organic pollutants), their accumulation leads to problems in water distribution pipes, biofouling which adversely affect the performance of artificial structures, and to medical problems such as dental plaques or colonization of artificial implants, leading to increased infection in patients.

The colonization process starts with chemical conditioning of the surface, followed by microbial colonization and finally by macroorganisms. Bacteria, the first colonizing microorganisms, excrete extracellular polymeric substances that help "cement" the biofilm onto the surface. Eukaryotes (algae, fungi, protozoa) are also part of the biofilm microbial community. The members of this community display increased exchange of nutrients

and metabolites, facilitated gene exchange, protection from grazing, and increased resistance to toxic chemicals. Members use quorum sensing to communicate with one another. Signaling chemicals consist of homoserine lactones in gram-negative bacteria. Various techniques have been developed for studying biofilm microorganisms. Molecular techniques (e.g., rRNA-based methods, fingerprinting techniques) and other techniques such as laser scanning microscopy are now available for identification of biofilm microorganisms. Other methods are useful for determining the metabolic activity of these microorganisms. Specific biofilms covered in the encyclopedia include diatom and bacterial-fungal biofilms, and biofouling in industrial systems and its control. A few other important topics related to biofilms include biodeterioration of mineral surfaces, bioleaching, biocorrosion, microbial weathering, biofilms in natural and drinking water distribution pipes, and biofilms in the food industry.

Humans have exploited microbes and their products for thousands of years. Wine is the classical example of a biotechnological product, although the involvement of microscopic yeast cells in the process was not known until the pioneering work of Pasteur. Today, biotechnologists have developed a myriad of modern techniques and processes that use microorganisms and their products (e.g., enzymes, biosurfactants, compatible solutes, ice nucleating proteins) for the benefit of humans, animals and the environment. The microorganisms covered in this encyclopedia include archaea, bacteria (e.g., methanotrophs, sulfate reducing bacteria), algae, and fungi. The advances made in **biotechnology** are benefiting the fields of medicine, agriculture and environmental remediation. Much hope is now placed on marine microorganisms and extremophiles (especially those belonging to the archaea domain) for biotechnological breakthroughs. A wide range of useful products are now derived from hyperthermophiles, psychrophiles, halophiles, alkaliphiles and acidophiles, benefiting medicine, molecular biology, bioremediation, as well as the food, pharmaceutical, pulp and paper or the oil industries.

The encyclopedia also covers the biotechnological aspects of processes such as bioleaching of metals, desulfurization of coal, treatment of waste and air or bioremediation of contaminated soils and aquatic environments.

The achievements of researchers in all the areas of environmental microbiology discussed earlier would not have been possible without the development of adequate **methodology** to tackle the various tasks. This area is extremely important to our understanding of the role of microorganisms in their natural settings, including those inhabiting extreme environments. We have witnessed significant advances in our ability to determine the numbers, biomass, activity, injury, and death of microorganisms in environmental samples. The development of genetic probes, such as the 16S rRNA-targeted probes, has increased our knowledge of phylogenetics and microbial community structure in water, sediments, soils or biofilms, and has helped tremendously in the detection of microbial pathogens and parasites in complex environmental matrices. Methods covered detail in several entries of this encyclopedia, deal with the identification of microbial isolates, flow cytometry, biochip-based devices (i.e., microarrays) for microbial community ribotyping, image analysis, biophotonic imaging, bioreporters, fluorescent in situ rRNA probes, lipid biomarkers, phylogenetics, **ribotyping, capillary electrophoresis, and methods for specifically detecting microbes such as archaea or psychrophiles.**

It is hoped the information presented in this encyclopedia will be the catalyst for more exciting research on the role of microorganisms in the environment, the control of pathogenic and parasitic microorganisms by engineered treatment systems which serve as safeguards against disease occurrence in humans and animals, a better understanding of life at the edge, and the beneficial use of microorganisms for restoring the environment, and improving our quality of life.

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Gainesville, Florida
October 2001

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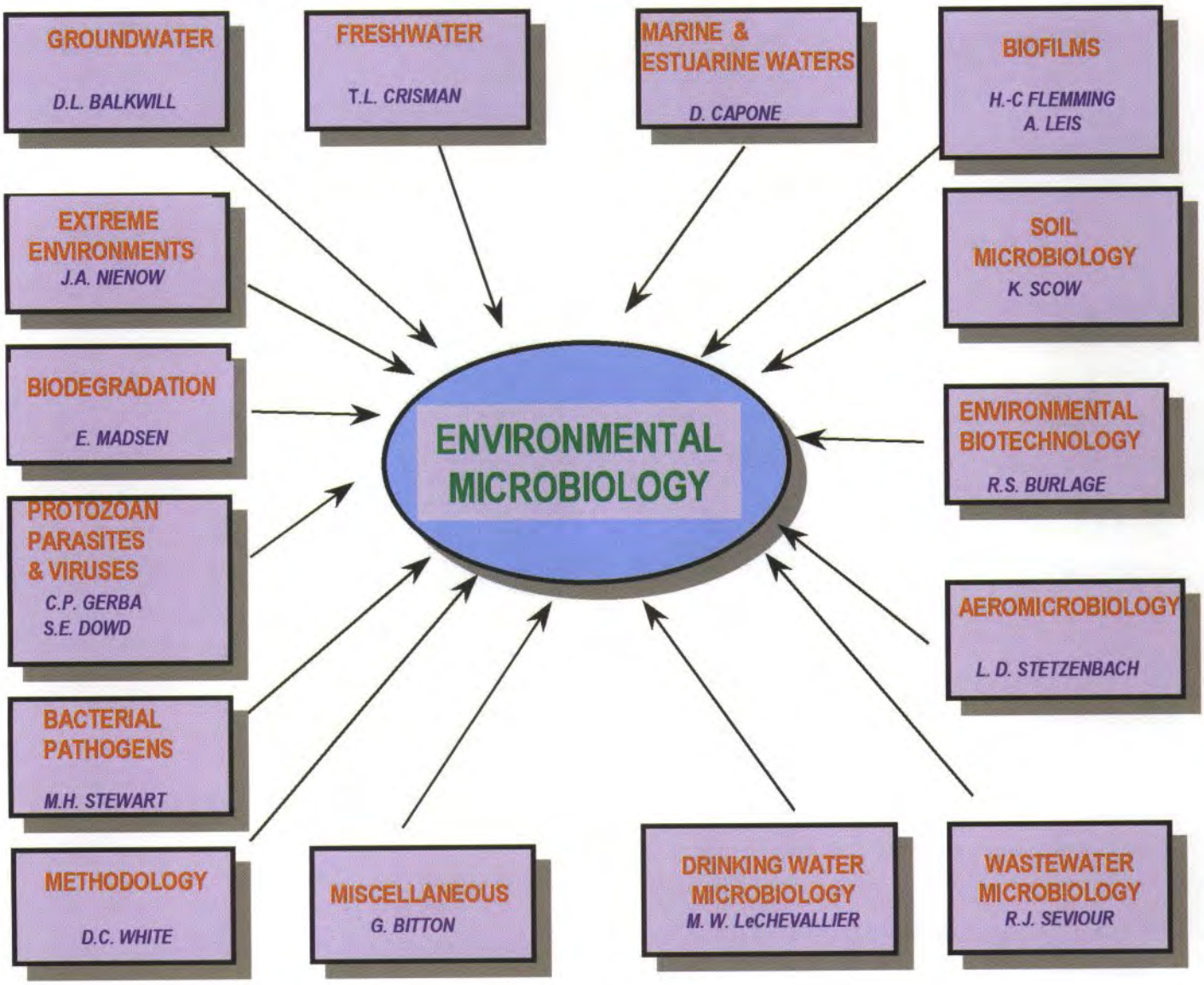

A

ACETYLENE REDUCTION ASSAY. See Nitrogen Fixation in Soils — Free-Living Microbes

ACID MINE DRAINAGE. See Biomineralization by Bacteria

ACIDOPHILES. See Extremophiles: Life in Extreme Environments

ACTINOMYCETES, AIRBORNE. See Infectious Airborne Bacteria

ACTINOMYCETES IN ACTIVATED SLUDGE. See Filamentous Bacteria in Activated Sludge: Current Taxonomic Status and Ecology

ACTINOMYCETES IN SOILS. See Soil Bacteria

ACTINOMYCETES: ROLE IN FOAMING OF ACTIVATED SLUDGE. See Activated Sludge — Foaming

ACTINORHIZAL SYMBIOSIS. See Nitrogen Fixation in Soils (Symbiotic)

ACTIVATED CARBON. See Granular Activated Carbon, Bacteriology of

ACTIVATED SLUDGE, FILAMENTOUS BACTERIA IN. See Filamentous Bacteria in Activated Sludge: Current Taxonomic Status and Ecology; Filamentous Bulking in Activated Sludge, Control of

ACTIVATED SLUDGE-FOAMING

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One of the major solids separation problems in activated sludge plants is the occurrence of a thick stable viscous scum on the surface of aeration tanks or sedimentation tanks (Fig. 1). This scum, or foam, a result of overgrowth of certain microbes in the activated sludge, causes a number of operational problems (extra maintenance, reduction in oxygen transfer, poor effluent, foam in anaerobic digesters) and is a potential health hazard (spread of pathogens) as well. It occurs often in activated sludge plants [e.g., 50% of plants surveyed in Italy recently exhibited foaming (1)]. Some consider that foaming has become the most serious operational problem in activated sludge systems today (2). In this review, I will discuss the organisms that cause the problem, factors affecting foam formation, and methods currently used to control the problem. There is more emphasis on recent issues. For an examination of earlier studies on the microbiology and control of foaming, see References 3 and 4. Other types of foam are sometimes experienced due to nonbiodegradeable detergents and foaming at plant start-up, but these foams are not stable (5) and are not considered here.

WHAT IS FOAMING?

Foam formation has been described as a flotation process involving interaction among gas bubbles (produced by the aeration system), surfactants (these can be produced by many microbes in the plant and be present in the incoming waste), and hydrophobic particles, principally the "foam-forming" microorganisms (4). The foam is made stable through the presence of surfactants and hydrophobic particles. Nonfilamentous microbes may also contribute to foam formation, particularly through their emulsifying ability (6).

WHICH MICROBES CAUSE FOAMING?

The principal method used for identifying the causative organisms in activated sludge mixed liquor and foam have been the microscopic methods based on early work by Eikelboom (7,8). These have been published in a number of manuals (5,9,10). Although foam formation was originally attributed to an actinomycete called "*Nocardia*," surveys using these methods (11–14) have shown that the main foam-forming organisms belong to a number of different morphological types, including branched and unbranched filaments and nonfilamentous forms.

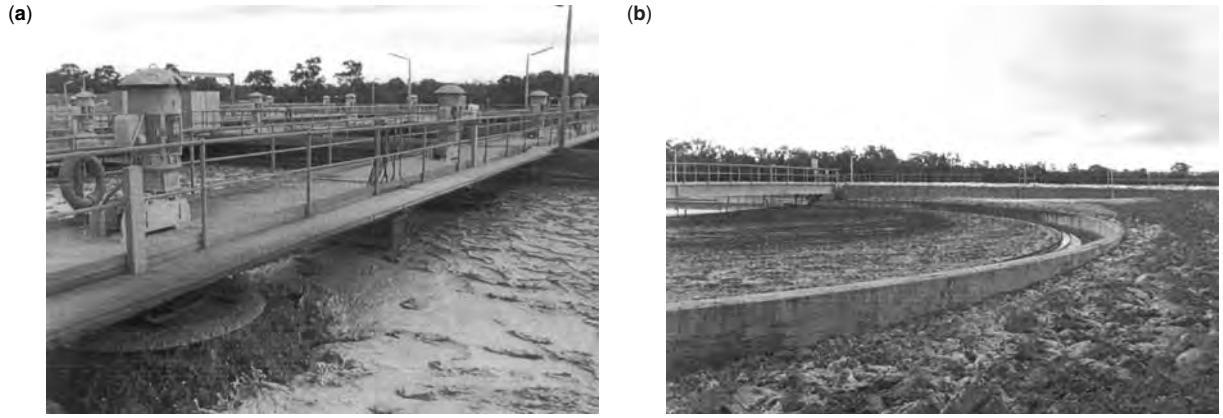

Figure 1. (**a**) Foam on surface of aeration tank, (**b**) major foam overflow. Photos: Wayne Murdoch, Coliban Water, Bendigo, Australia.

Microthrix parvicella

Microthrix parvicella is a long coiled unbranched gram-positive filamentous actinomycete (Fig. 2a). It has been the major cause of foaming in Europe (1,2,15), Australia (13,14), and South Africa (11,12), but less common in published reports from the United States. It appears to be more prominent in biological nutrient removal plants (12), and its appearance may be temperature related because it appears in larger numbers in cooler months in Europe

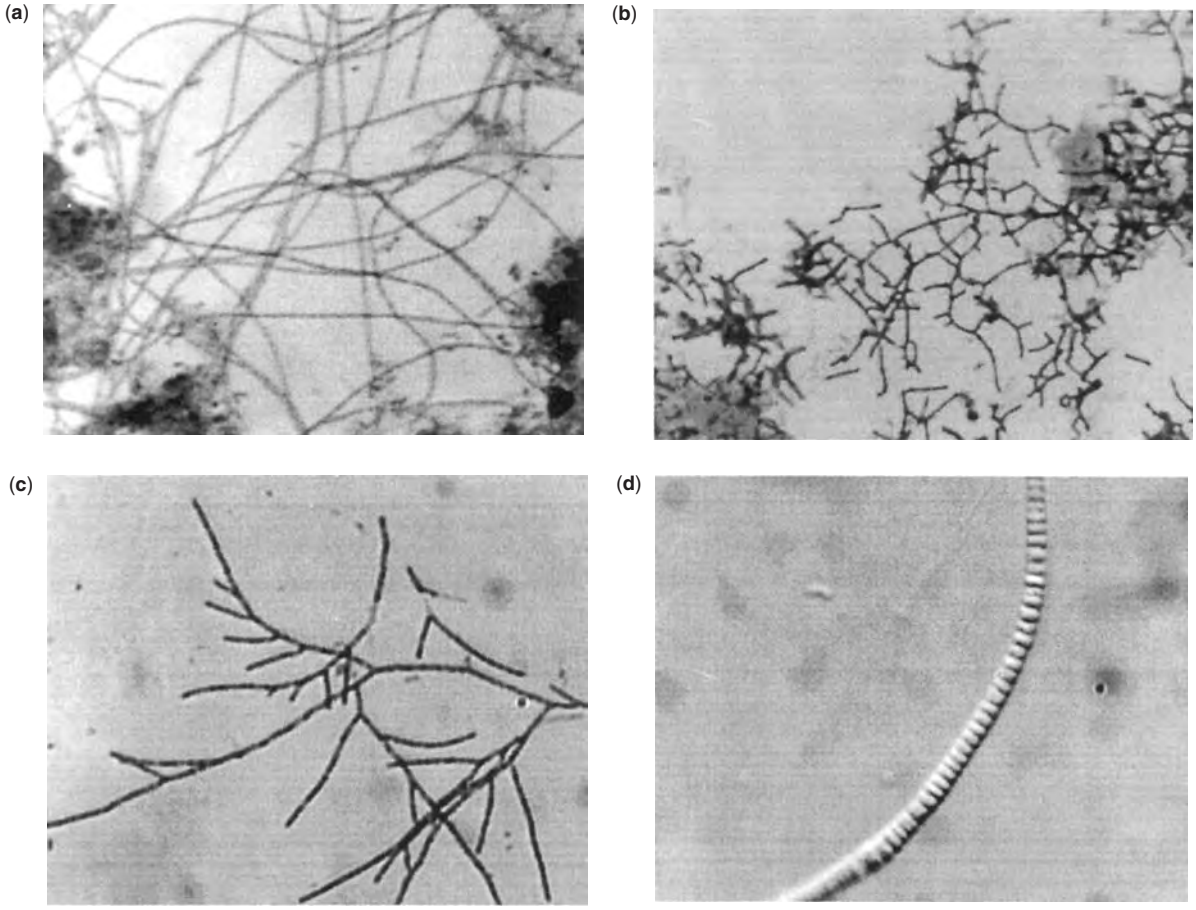

Figure 2. (**a**) Long coiled unbranched filaments of *M. parvicella*, (**b**) GALO (*Gordonia amarae*-like organism) or Nocardia showing typical right-angled branching, (**c**) *Skermania piniformis*, with its tree-like branching, (**d**) *Nostocoida limicola* II. Photos: Beth Seviour, Biotechnology Research Centre, Bendigo, Australia.

(15,16) and is more prominent in cooler regions of Australia (13). It is also responsible for bulking - another solids separation problem in activated sludge.

The taxonomy of *M. parvicella* has now been resolved despite difficulty with growing the organism in the laboratory (17) — it is a deep-branching member of high percentage molecular G + C group of actinomycetes closest to *Acidimicrobium ferrooxidans*. Organisms related to *M. parvicella* have been found in diverse environments, for example, a peat bog in Germany, geothermally heated soil in New Zealand, soil in Australia, and various marine environments in Japan and Finland. However, *M. parvicella* still has *Candidatus* status as it has not yet been fully characterized due to poor growth on conventional media (17).

Growth of *M. parvicella* occurs when aeration is intermittent, but is suppressed under continuous oxygen supply (18). This may be related to its requirement for reduced sulfur and nitrogen compounds, which may not be present in adequate quantities in fully aerated systems (18). It also explains why the organism is commonly found in biological nutrient plants (BNR), which have both anaerobic and anoxic stages.

Recent studies using microautoradiography (MAR) confirmed earlier studies by Slijkhuis, which showed that *M. parvicella* will only grow on long-chain fatty acids (or their Tween esters) but not on simpler substrates (19). However, another study contradicted these studies in finding some growth on acetate (20). The MAR studies (19) showed that uptake of long-chain fatty acids occurred under aerobic, anaerobic, and anoxic conditions, but there was no proof that they were actually metabolized under anoxic and anaerobic conditions (they may only have been incorporated as storage compounds). This rapid uptake of lipids probably explains the high lipid content of *M. parvicella*, which can approach 35% dry weight (21).

A property of the organism that has implications in foam control is its very high resistance to chlorination compared to other filamentous microbes found in activated sludge (22,23).

Although its significance is not known, there appears to be a link between dominance of sludge by *M. parvicella* and high levels of uronic acids produced in the extracellular polymers in the sludge (24).

Gordonia amarae-like Organisms (GALO)

These bacteria are shorter branched gram-positive filaments with branches at approximately 90° (Fig. 2b). They are commonly reported as *Nocardia*, but are sometimes called NALO (*Nocardia amarae*-like organism) because their morphology is similar to *N. amarae* (25) and because other mycolic acid–containing actinomycetes may also have similar morphology under certain conditions. Since *N. amarae* was reclassified as a *Gordonia* (26), our laboratory now uses the term *G. amarae*-like organisms or GALO to describe these morphotypes.

Although organisms such as *M. parvicella* and *S. piniformis* can be readily identified using microscopy, the morphotype described as GALO (*Nocardia*) represents many related organisms and hence cannot be identified to genus level by microscopic morphology alone. Recent advances in the taxonomy of these microorganisms have clarified that organisms with GALO morphology belong to a number of genera: *Nocardia*, *Gordonia*, *Tsukamurella*, *Rhodococcus*, and *Dietzia* (4). These are all actinomycetes, which contain mycolic acids in their cell walls (together with *S. piniformis*, but not *M. parvicella*). The following have been isolated in *pure culture* from foam, illustrating the diversity of mycolata found in activated sludge: *G. amarae*, *Gordonia terrae* and *Gordonia sputi*, *Nocardia asteroides Nocardia farcinica*, *Nocardia otitidiscaviarum*, *Rhodococcus coprophilus*, *Rhodococcus equi*, *Rhodococcus erythropolis*, *Rhodococcus globerulus*, *Rhodococcus rhodochrous*, *Rhodococcus ruber*, *Rhodococcus rubra*, *Tsukamurella paurometabola*, *Tsukamurella spumae*, and *Dietzia maris* (4). In addition, there are many new species belonging to most of these genera, and *Mycobacterium* (27–29) and maybe even new genera (29).

The mycolic acid containing actinomycetes are aerobic organisms and are stimulated by increased dissolved oxygen concentrations (30). Hence they can be controlled by biological selectors, which reduce dissolved oxygen. Although these organisms have traditionally been thought to occur in plants with long sludge age, some GALOs have particularly fast growth rates and may not be washed out by reducing sludge age.

Many wastes from food and chemical industries contain hydrophobic substrates such as vegetable oils, greases, and hydrocarbons, and these may cause problems in activated sludge plants (31). The mycolic acid–containing actinomycetes readily use such substrates, but with varying growth rates and affinities (29,32–34). The hydrophobic nature of foam-formers with GALO morphology allows them to attach to hydrophobic substrates rather than to grow in the aqueous phase. This may give them a means of competing with faster growing organisms present in the aqueous phase of activated sludge (34,35).

The mycolic acid–containing actinomycetes grow at a range of temperatures (36). Those growing at lower temperatures (5 °C) are principally rhodococci. Therefore, the so-called *Nocardia* foams at low temperatures are more likely to be caused by the *Rhodococcus* species. Some foam isolates can grow at 40 °C or higher, suggesting these are more likely to occur in plants treating warm wastewater, or possibly could grow in the foam itself when ambient air temperatures are high (36).

Skermania piniformis

Skermania piniformis is a branched filamentous actinomycete with a treelike appearance (Fig. 2c), and is the only *Nocardia* with morphology sufficiently different to allow identification in its own right (37,38). However, most surveys do not differentiate it from *Nocardia* or GALO. Hence its true incidence outside Australia, where most studies have been carried out, is unknown. It was originally called PTLO (pine treelike organism) because of its treelike branching morphology, but was then classified as *Nocardia pinensis* (39). More recently, these PTLOs were reclassified as a new genus, with only one member, *S. piniformis* (40). *Skermania piniformis* is usually associated with plants operating at sludge ages greater than 20 days (14), and grows over a relatively narrow

range of temperatures (15–30 °C), but this is still sufficient to cause problems in many plants (36). *Skermania piniformis* grows better on hydrophobic substrates such as olive oil and Tween 80 than on glucose (34) as well as other hydrophobic substrates (29).

Nostocoida limicola

Nostocoida limicola is an unusual filamentous bacterium containing chains of rounded cells (Fig. 2d). Recent European surveys suggest it appears in foam more commonly than previously thought (2,6,41). Although there are three morphotypes, these surveys did not differentiate among *N. limicola* types I, II, and III, which are now known to belong to separate, unrelated genera (42,43). The foam-former is probably *N. limicola* II, which is now recognized as Candidatus *N. limicola* II (44), belonging to the Intrasporangiaceae. It is phylogenetically closely related to and probably a member of the same genus as cocci in the genus *Tetrasphaera*, also isolated from activated sludge (45). *Nostocoida limicola* II is a low F : M organism (5). Increasing the F:M ratio causes it to lose its competitive advantage (30).

Others Filamentous Foam-Formers

Other filamentous microbes have also been associated with foam formation. Eikelboom Type 1863 is one of major causes of foam (after *Nocardia* and *Microthrix*) according to Jenkins and coworkers (5). Types 0675/0041 have a high incidence in some surveys, especially in biological nutrient removal plants (6,46). Other organisms such as Eikelboom Types 0092, 0041, 0803, 0413, 1851, 021N, 0914, 0581, 1701, *Haliscomenobacter hydrossis*, *Sphaerotilus* sp, and cyanobacteria have been reported as the dominant organism in foam, but their incidence is usually low (3,4).

Nonfilamentous Foam-Formers

Closely related taxonomically to the branching filaments are nonfilamentous forms of some of the mycolic acid–producing actinomycetes, called "actinomycetes," as revealed in one survey (14) to differentiate them from GALO and *S. piniformis*. These "actinomycetes" were defined as gram-positive rods (often slightly branched or not at all) without the distinctive branching patterns of GALO or *S. piniformis*. Other studies support their importance (6,47). These nonfilamentous foam-formers have possibly been overlooked in earlier studies of foam as they are less obvious than the branching organisms we call GALO or "*Nocardia*."

Cocci have also been reported in nonfilamentous foams in some plants. These include gram-positive cocci in Australian plants (13,48), which may also be mycolata, as some of these undergo a coccus stage in the so-called rod-coccus life cycle. The gram-negative coccobacillus *Acinetobacter* sp. have also been described as the dominant organism in the foam of a nutrient removal plant (49), but its more important contribution to foaming is probably through the emulsifiers it produces (6), hence encouraging the growth of organisms such as *G. amarae* (50).

The Need to Identify?

Although in some cases simple identification based on morphology is adequate for monitoring an activated sludge plant, there are occasions when more precise identification could be important: (a) To assist with foam control of GALOs: Since GALOs have different growth rates, correct identification may indicate whether manipulation of sludge age will be successful as a control measure. For example, some rhodococci grow relatively fast (38) and reduction of sludge age may not be sufficient to eliminate these, although it will eliminate slower growing rhodococci and other GALOs. (b) Some foam-formers are pathogens: Although *G. amarae*, *S. piniformis*, and *M. parvicella* are not known to be pathogens, others organisms isolated from foam (e.g., *N. asteroides*, *N. farcinica*, and *R. equi*) are potential pathogens. Since identification of pathogens is not possible on morphological grounds, identification using molecular probes is likely to become important.

USE OF MOLECULAR PROBES IN FOAM

While probes have made in situ identification possible without the need to isolate an organism, the wide range of mycolic acid producing organisms present in activated sludge means that probes will probably still remain a research tool rather than a routine quality assurance tool for a few years yet, as the number of different probes necessary to make a complete analysis is fairly large. However, microarray/biochip probe technology (51) may eventually simplify such an analysis.

To date, probe studies have not explored the range of mycolata likely to be present in activated sludge. Most studies have concentrated on *Gordonia*, especially *G. amarae* (52–54) and *Rhodococcus* (47). It was concluded that *G. amarae* makes up only a small proportion of gordoniae found in foam, strains previously identified as *G. amarae* may represent two different species of *Gordonia*, and that a *Rhodoccoccus* probe detects both branching organisms and short rods. The use of confocal laser scanning microscopy is recommended to improve detection of organisms within flocs (47,55).

A probe based on an Australia isolate of *M. parvicella* has also been designed and successfully detected *M. parvicella* in European studies (56). However, unless the *M. parvicella* probe detects some unusual morphotypes (not to date), it is less likely to be more useful than conventional microscopy, which readily identifies *M. parvicella*.

MONITORING FOAM

Quantification is a prerequisite for rational investigation of foaming in activated sludge (57) and various quantification techniques for monitoring the level of organisms in foam and mixed liquor have been reported. These include measuring the abundance of filaments (5), immunofluorescence (58,59), use of a selective medium containing n-octodecane and nalidixic acid (60) and quantitative in situ hybridization (57).

However, many of these methods have problems, and choosing a meaningful method is difficult. Levels of

foam-formers in the mixed liquor are only meaningful if foaming has not occurred. Once foaming occurs, it separates to varying degrees. The foam-forming organisms from the mixed liquor and the level in the mixed liquor is not necessarily then meaningful. Another problem with quantification is that the mycolic acid–containing actinomycetes may be present as short rods or cocci, and branching filaments, and some methods do not address this problem, counting only filaments. Lack of specificity can also be a problem with the immunofluorescent techniques described.

Foaming potential of a biomass can also be measured through foam production by aeration under laboratory conditions (61) and hydrophobicity measurements.

HYDROPHOBICITY AND FOAM

Mixed liquor biomass is more hydrophobic in foaming plants than in nonfoaming plants, and onset of foaming incidents is often correlated with increase in cell surface hydrophobicity (CSH) (62,63). Therefore, there is interest in the hydrophobic properties of foam-forming organisms and their role in foam formation, particularly the hydrophobic mycolic acids present in the cell walls of GALOs and *S. piniformis*.

Pure culture studies with foam isolates of mycolic acid–containing actinomycetes show that the CSH varies with culture age, nutrient sources, and temperature (64), but variations in the mycolic acid composition have little influence on their CSH or foaming ability (Stratton and coworkers, 1999). However, using cell-water contact angles as a measure of CSH, studies of *Corynebacterium*, *Rhodococcus*, *Gordonia*, and *Mycobacterium* revealed a tendency for CSH to increase with mycolic acid size. An exception was *Mycobacterium*, which was less hydrophobic than *Gordonia* (65). Possibly other cell wall or excreted components neutralize the hydrophobic nature of the mycolic acids (66). Thus, although foam-formers are hydrophobic, the relationship between CSH and mycolic acids is complex.

There is also some evidence that *M. parvicella*, which does not contain mycolic acids, is also strongly hydrophobic (21).

FOAM CONTROL

There are many inconsistencies and disagreement as to which methods are the best, and many are based on anecdotal data. The best methods to use may depend on which organisms are causing the foam, but this is not possible if microscopic examination is not carried out. Microscopic examination will easily identify *M. parvicella*, *S. piniformis*, and *N. limicola* II, and action can readily be instigated based on our knowledge of the physiology of these organisms. However, if the foam-former is identified as a GALO, identification to species is more difficult. Consequently, choosing the right course of action may be more difficult (4). The most common approaches to foam control are summarized as follows.

Manipulation of Loading Rate

Washout of foam-forming organisms by reduction of sludge age or increase in F:M (Food:Microorganisms) ratio is commonly practiced. Reduction of sludge age should wash out slowly growing organisms but this does not always work since different foam-formers have different growth rates (4). *Microthrix parvicella* and *S. piniformis* are slow-growers and can be controlled this way, but this may be at the expense of nitrification, which requires a long sludge age. However, organisms with the morphology of GALO may represent both fast- and slow-growing actinomycetes, and therefore reduction of sludge age may not successfully control the foam.

Use of Selectors

Selectors, also known as contact zones (67), are separate reaction vessels added to the system to allow kinetic and/or metabolic selection of certain groups of microbes before the waste enters the main treatment basin. They can be aerobic, anoxic, and anaerobic (4). However, these are less successful for foaming control than for bulking control (2). Anoxic selectors favor growth of floc formers at the expense of *Nocardia*, but are not useful for *M. parvicella* foams because *M. parvicella* grows well anoxically.

There has recently been interest in two-stage selectors. One method (68) involves a first stage, which is aerated, but the second stage is capable of variable aeration. When the second stage was subjected to an aeration period of between 9 and 12 hours, the population of *M. parvicella* was reduced sufficiently to prevent stable foam formation in the aeration tank, but had no effect on *N. limicola*. Another approach is to have separate "feasting" and "fasting" units (69), which capitalize on the sensitivity of filamentous bacteria to F : M ratio, with the combined effect of both units resulting in the suppression of the overgrowth of filamentous bacteria without adversely affecting the organic treatment efficiency of the modified process.

Physical Methods

Some feel that physical methods rather than biological methods are more successful in treating foaming problems (2), and emphasize the proper design and construction of secondary clarifiers to avoid the escape of floating biomass to the final effluent. A number of different physical approaches have been used. Water sprays are used to physically break down the foam, but with mixed results, probably because of the stability of the foam (3). Physical removal of scum as it accumulates is also practised. A variation on this is to use a so-called classifying selector (70) to encourage flotation so that foam-formers rise to surface more rapidly, and then remove them. This reduced *Nocardia* levels, particularly when nonionic detergent was added to the system. It is important not to recycle any trapped foam back into the activated sludge biomass — this recycles the organisms that cause the problem.

Chemical Additives

The addition of chemicals to control foaming is always a consideration, but is in most cases an emergency

control method rather than a fundamental solution (23). A number of approaches have been described, with various degrees of success.

Chlorination. This kills filaments protruding from flocs but does not kill flocs unless too much chlorine is applied. Chlorination is useful, but care must be taken not to over chlorinate. *Microthrix* is possibly more resistant to chlorine than "Nocardia" (22,23). However, *G. amarae* is more sensitive to chlorine than nonfoaming organisms such as *Pseudomonas aeruginosa* and *Escherichia coli* in pure culture and in a laboratory scale plant (71).

Ozonation. Like chlorine, ozone is a strong oxidizing agent. Recent studies suggest that it can be useful in suppressing scum without affecting the rest of the biomass (72,73) added it to aeration tank at slightly higher levels and found that it eliminated foaming in the aeration tank. Ozone also improves sludge settleability and accelerates nitrification (72).

Antifoam. The use of antifoams is not often successful (46,74), probably because the foams they were developed against are usually much less stable than those experienced in activated sludge plants (75). They are also very expensive.

Bioaugmentation. These are commercial mixtures of microbes, sometimes supplemented with enzymes. They are expensive and often do not work, or need to keep adding for continued effect (therefore, expensive). In both lab-scale and full-scale experiments such mixtures do not appear to affect foaming, although in some cases, they resulted in a change of bacteria involved (from "*Nocardia*" to *M. parvicella*) (76).

Iron Salts. The dosing of mixed liquor with iron salts has also been successful in some instances, but the response appears to be variable (77). Iron salts have been found to be useful for a Rhodococcus induced scum, but laboratory trials with different cultures of mycolic acid containing actinomycetes showed a variable response to iron (78).

Addition of Polymers. Addition of cationic polymer to the clarifier to combat poor settling activated sludge resulted in the elimination of "*Nocardia*" foam after three days (79), probably by neutralizing and precipitating the foam-stabilizing anionic surfactants present in the wastewater. Successful suppression of foaming by *M. parvicella* through the addition of quaternary ammonium-based antifilament polymer has also been reported (23).

Manipulation of Cell Surface Chemistry. Addition of clay compounds such as bentonite, talc, zeolite, and montmorillinite can remove foam-forming organisms (63,80).

Appropriate Design

It is thought that subsurface culture withdrawal encouraged the growth of dispersed (i.e., free floating) *G. amarae* filaments rather than filaments within flocs; and these dispersed filaments are more likely to produce stable foams (81). This is equivalent to the situation in vessels with foam-trapping, which tend to have dispersed growth of "Nocardia" (79). It is now possible to design plants to minimize scum formation by eliminating any chance of surface trapping of scum in a reactor. There should be a free-surface flow in the reactor, with the whole surface layer moving through the reactor with essentially the same retention time as the bulk of the mixed liquor. This ensures that solids trapped on the surface do not have retention periods in excess of the liquid retention period, particularly the sludge age (82).

FOAMING IN ANAEROBIC DIGESTERS

Recent investigations into foaming in anaerobic digesters suggest that this problem is related to that in activated sludge plants. Digesters fed with sludge containing GALO or *M. parvicella* end up with the same organisms dominating the digester foam (52,53,83–87).

The operating strategy to prevent foam in the anaerobic digesters at these plants is to control the growth of *M. parvicella* in the activated sludge tanks by increasing the sludge load. Top installed stirrers and the addition of polyaluminum salt have also been used to prevent foam formation (86).

Pilot scale studies show that heat pretreatment of excess activated sludge with a high content of *M. parvicella* (87) and "*Nocardia*" (84) reduces its foam potential, but chlorination increases its foaming potential (85) when placed in an anaerobic digester.

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ACTIVATED SLUDGE, METHODOLOGY.

See Activated Sludge — Molecular Techniques for Determining Community Composition

ACTIVATED SLUDGE — MICROBIOLOGY OF NITROGEN REMOVAL

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The elimination of inorganic nitrogen compounds plays an important role in wastewater treatment, because these compounds contribute significantly to the eutrophication of aquatic environments and are toxic to fish. Ammonium occurs as the main nitrogen component of untreated wastewater. Organically bound nitrogen is also released as ammonium. Generally, ammonium nitrogen is removed by biological treatment such as activated sludge systems or trickling filters. Removal is also effected by several chemical and physical methods such as precipitation, stripping, or ion exchange. The first step of biological ammonium elimination is nitrification. The oxidation of ammonium to nitrite is catalyzed by the ammonia oxidizing bacteria. The subsequent oxidation of the nitrite to nitrate is catalyzed by the nitrite oxidizing bacteria. In the course of denitrification, the second step of biological nitrogen removal, nitrite and nitrate are reduced to gaseous N-compounds such as nitric oxide, nitrous oxide, or dinitrogen.

THE BIOLOGICAL NITROGEN CYCLE

Nitrogen (N) is an element with oxidation states from −III to +V (Table 1).

Nitrogen is an important element in proteins, genetic material, and further essential organic molecules. In the atmosphere, nitrogen mainly exists as dinitrogen gas (N_2), where it constitutes about 78% of the atmosphere. Nitrogen is fixed by bacteria in primary production and is liberated as ammonium in the biological food chain (N-cycle). Part of the ammonium is returned to primary production and the bacterial processes of nitrification and denitrification recycle the surplus to the atmosphere as N_2.

Table 1. Oxidation States of Nitrogen

Compound	Formula	Oxidation State
Ammonium	NH_4^+	−III
Hydrazine	N_2H_4	−II
Hydroxylamine	NH_2OH	−I
Dinitrogen	N_2	0
Nitrous oxide	N_2O	+I
Nitric oxide (Nitrogen monoxide)	NO	+II
Nitrite	NO_2^-	+III
Nitrate	NO_3^-	+V

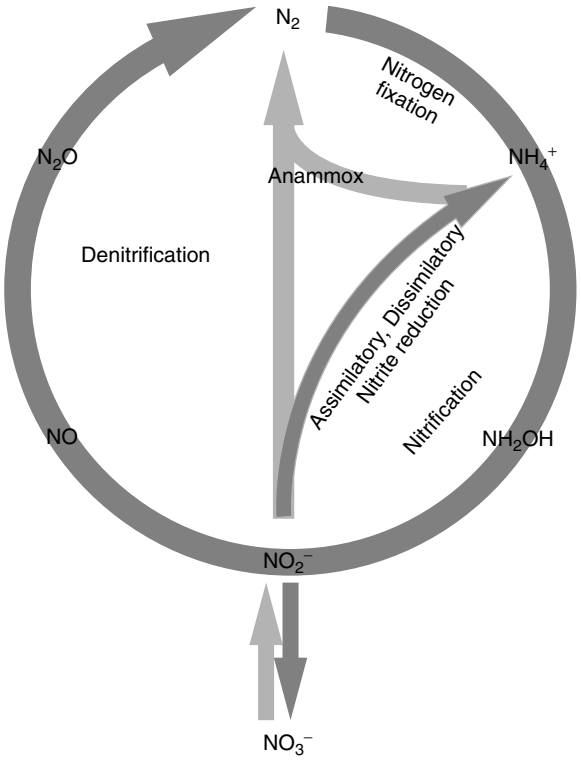

Figure 1. Biological nitrogen cycle.

The biological nitrogen cycle is shown in Figure 1. After fixation, nitrogen is available to other organisms in the form of ammonia/ammonium (NH_3/NH_4^+). It can subsequently be assimilated into biomass, or it can be used for energy generation by specialized bacteria in the process of nitrification.

Two groups of microorganisms are responsible for nitrification. Ammonia oxidizers catalyze the first step, the oxidation of ammonia to nitrite, and nitrite oxidizers catalyze the second step, the oxidation of nitrite to nitrate. Nitrate can be reduced to ammonia via nitrite. Further, nitrite and nitrate can be used as terminal electron acceptors during denitrification (anaerobic respiration) to form N_2. N_2O and NO may occur as intermediates in the process.

From a microbiological point of view, the nitrogen cycle (Table 2) is made up of four catabolic reactions (oxidation of ammonia, oxidation of nitrite, denitrification, and

dissimilatory nitrite reduction) and three anabolic reactions (ammonium assimilation, assimilatory nitrite reduction, and nitrogen fixation). Oxidation of ammonia can be divided into aerobic (O_2-dependent), anaerobic (NO_2-dependent), and anaerobic (nitrite-dependent, Anammox) oxidation of ammonia.

OXIDATION OF AMMONIA

Physiology of Ammonia Oxidation

The gram-negative ammonia oxidizers, for example, members of the genera *Nitrosomonas* and *Nitrosospira* (1) are lithoautotrophic organisms using carbon dioxide as the main carbon source. Species of the genera *Nitrosomonas* reveal extensive intracytoplasmic membrane (ICM) systems (Fig. 2). In *Nitrosococcus oceanus*, ICMs are arranged as a centrally located stack of parallel, flattered vesicles, and in other species, as flattened vesicles in the peripheral cytoplasm (e.g., *Nitrosomonas eutropha*). Recently, molecular tools that infer the presence of ammonia oxidizing bacteria in the environment have been supplemented by PCR primers for specific amplification of the ammonia monooxygenase structural genes *amoA*. Environmental 16S rRNA and *amoA* libraries extend the knowledge on the natural diversity of ammonia oxidizing bacteria. Comparative 16S rRNA sequence analyses revealed that members of this physiological group are confined to two monophyletic lineages within the *Proteobacteria*. *Nitrosococcus oceanus* is affiliated with the gamma-subclass of the *Proteobacteria*, whereas members of the genera *Nitrosomonas* and *Nitrosospira* form a closely related grouping within the beta-subclass of *Proteobacteria* (2).

Ammonia oxidizers are widespread in nature as well as in artificial ecosystems. Strains of different species have been isolated from fresh, brackish, and salt waters and soils. Some ammonia oxidizers are adapted to special, sometimes extreme environments. For example, strains of *Nitrosospira* have been isolated from acid tea soil at pH values of 4.6, *Nitrosomonas* strains from marine sediments with low temperatures, and from alkaline soda lakes with pH values above 10.0 (3).

Ammonia is oxidized in two steps. First, ammonia is oxidized to hydroxylamine by the ammonia monooxygenase (Eq. 1). This enzyme is unspecific and also oxidizes

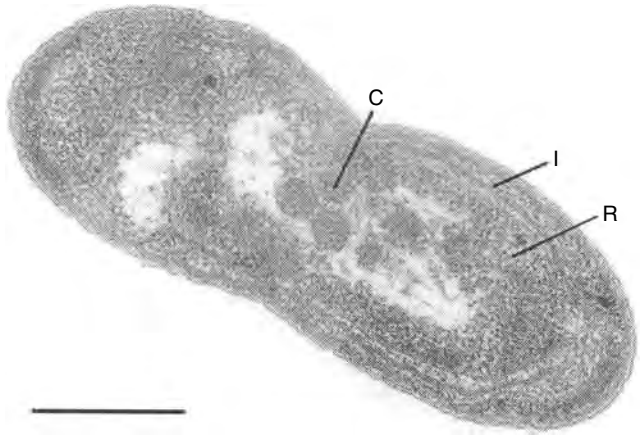

Figure 2. Electron micrograph of ultrathin sections of *N. eutropha*. C: carboxysome, Cy: cytoplasm with ribosomes (R), I: intracytoplasmic membranes. Bar: 0.5 μm.

several apolar compounds such as methane, carbon monoxide, or some aliphatic and aromatic hydrocarbons. These compounds can act as competitive inhibitors of ammonia oxidation. The second step (Eq. 2) in this metabolism is performed by the enzyme hydroxylamine oxidoreductase (HAO). This enzyme oxidizes hydroxylamine to nitrite. Two of the four electrons released from this reaction are required for the AMO-reaction and the remaining are used for the generation of a proton motive force in order to regenerate ATP and NADH.

$$NH_3 + O_2 + 2H^+ + 2e^- \rightarrow NH_2OH + H_2O \quad (1)$$

$$NH_2OH + H_2O \rightarrow HNO_2 + 4H^+ + 4e^- \quad (2)$$

$$NH_3 + O_2 \rightarrow HNO_2 + 2H^+ + 2e^- \quad (3)$$

ANOXIC METABOLISM (ANOXIC AMMONIA OXIDATION)

Recently, an anoxic metabolism (denitrification and oxidation of ammonia) in ammonia oxidizers (*Nitrosomonas*) was discovered (4,5). Furthermore, a new autotrophic

Table 2. Catabolic Reactions of the Nitrogen Cycle

Reaction		Equation
Ammonia oxidation	O_2-dependent	$NH_3 + O_2 \rightarrow HNO_2 + 2H^+ + 2e^-$
	NO_2-dependent	$NH_3 + N_2O_4 \rightarrow HNO_2 + 2NO + 2H^+ + 2e^-$
	Anammox	$NH_3 + 1.3HNO_2 \rightarrow 0.3HNO_3 + 1N_2 + 1.7H_2O + 0.6H^+ + 0.6e^-$
Nitrite oxidation		$HNO_2 + H_2O \rightarrow HNO_3 + 2H^+ + 2e^-$
Denitrification		$HNO_3 + 2H^+ + 2e^- \rightarrow HNO_2 + H_2O$
		$HNO_2 + H^+ + e^- \rightarrow NO + H_2O$
		$2NO + 2H^+ + 2e^- \rightarrow N_2O + H_2O$
		$N_2O + 2H^+ + 2e^- \rightarrow N_2 + H_2O$
Dissimilatory nitrite reduction		$HNO_2 + 6H^+ + 6e^- \rightarrow NH_3 + 2H_2O$

member of the order *Planctomycetales* was identified, oxidizing ammonia under anoxic conditions to N_2 with nitrite as electron acceptor via the intermediate hydrazine (6).

Denitrification by Ammonia Oxidizers

During oxic oxidation of ammonia by ammonia oxidizers, small amounts of nitric and nitrous oxide are released. Both gases are also produced in the course of aerobic denitrification by ammonia oxidizing bacteria. Additionally, the formation of dinitrogen is observed (5,7). In the absence of dissolved oxygen, *Nitrosomonas* is capable of anoxic denitrification (Fig. 3) with molecular hydrogen (4) or simple organic compounds serving as the electron donors, whereas nitrite is used as the electron acceptor. However, high aerobic denitrification activities were only obtained when the cells were grown under extremely oxygen-limited conditions. But under these conditions ammonia oxidation rates are low.

Anaerobic NO_2-Dependent Oxidation of Ammonia by *Nitrosomonas*

In the absence of dissolved oxygen, oxidation of ammonia has recently been observed in cultures of *N. eutropha* (5). These cells were able to replace molecular oxygen by nitrogen dioxide or dinitrogen tetroxide, respectively, in the course of ammonia monooxygenase reaction (Eqs. 4–6).

$$NH_3 + N_2O_4 + 2H^+ + 2e^- \rightarrow NH_2OH + H_2O + 2NO \quad (4)$$

$$NH_2OH + H_2O \rightarrow HNO_2 + 4H^+ + 4e^- \quad (5)$$

$$NH_3 + N_2O_4 \rightarrow HNO_2 + 2NO + 2H^+ + 2e^- \quad (6)$$

Hydroxylamine and nitric oxide were formed in this reaction. Although nitric oxide was not further metabolized, hydroxylamine was oxidized to nitrite, as shown, under oxic conditions. The nitrite produced was partly used as an electron acceptor, leading to the formation of dinitrogen (Eq. 7).

$$HNO_2 + 3H^+ + 3e^- \rightarrow N_2 \quad (7)$$

Equation 1 to 6 indicate that there are only a few differences between the anaerobic and the aerobic oxidation of ammonia by *N. eutropha*. Instead of O_2 in the course of aerobic oxidation of ammonia, N_2O_4 is used as electron acceptor and NO, an additional product, is released.

Effect of Nitrogenous Oxides on Oxidation of Ammonia

Ammonia oxidizing bacteria of the genus *Nitrosomonas* are inhibited when gaseous nitric oxide is removed from laboratory-scale cultures by means of intensive aeration. Nitrification starts again when nitric oxide is added to the gas inlet of the culture vessels (8). Using a laboratory-scale fermenter with complete biomass retention, it could be shown that nitrogenous oxides such as nitric oxide and especially nitrogen dioxide have a significant promoting effect on pure cultures of *N. eutropha* (9). Compared with cultures grown without these externally added nitrogenous oxides their addition resulted in a pronounced increase in nitrification rate, specific activity of ammonia oxidation, growth rate, maximum cell density, and aerobic denitrification capacity. Maximum cell numbers amounted to $2 \cdot 10^{10}$ *Nitrosomonas* cells per ml. Further, about 50% of the nitrite produced was aerobically denitrified to dinitrogen when nitrogen dioxide was present.

Recently, a new hypothetical model for oxidation of ammonia by *N. eutropha* was developed (10). A new complex role for nitrogen oxides in the metabolism of these organisms was proposed. Anaerobic oxidation of ammonia (Eqs. 4–6) is dependent on the presence of the oxidizing agent N_2O_4. NO is produced in stoichiometric amounts and released into the atmosphere. The situation is more complex under oxic conditions. In the presence of O_2, the produced NO can be oxidized to NO_2. According to the new model (Fig. 4), N_2O_4 is the oxidizing agent under oxic conditions. During the oxidation of ammonia,

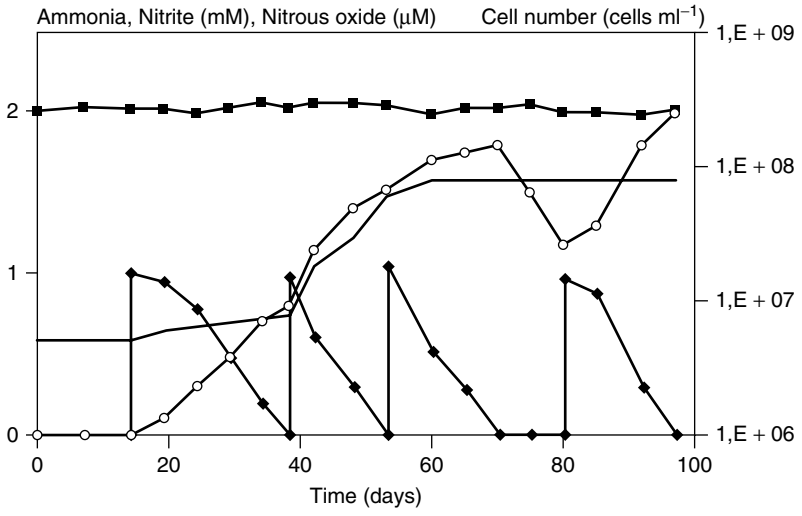

Figure 3. Denitrification and cell growth of *N. eutropha* in a hydrogen atmosphere with 20% carbon dioxide. Hydrogen, not ammonia, was used as electron donor indicated by decreasing the H_2 concentration (not shown), whereas the ammonia concentration remained unchanged. (■), ammonia; (♦), nitrite; (◯), N_2O; (−), cell number.

hydroxylamine and NO are produced as intermediates. Although hydroxylamine is further oxidized to nitrite, NO is (re)oxidized to NO_2 (N_2O_4).

The new hypothetical model is in agreement with the reaction described earlier for aerobic oxidation of ammonia. Although the total consumption rates (ammonia, oxygen) and production rates (hydroxylamine as intermediate) remain unchanged, the mechanism of the reaction is different.

ANAEROBIC AMMONIUM OXIDATION (ANAMMOX)

Engelbert Broda (1977) predicted the existence of chemolithoautotrophic bacteria capable of anaerobic ammonium oxidation (Anammox).

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \quad (8)$$

Only recently the experimental confirmation of Broda's prediction was described (6). The biological nature of the process was verified and nitrite was shown to be the preferred electron acceptor. Hydroxylamine and hydrazine were identified as intermediates. Bacteria were enriched in a mineral medium containing ammonia and nitrite, and bicarbonate was the only carbon source. The growth rate of the cultures was extremely low (doubling time three weeks), and reactor systems with very efficient biomass retention had to be used. The enrichments were dominated by a bacterium with a conspicuous distinctive morphology. The bacterium was physically purified from the enrichment by density gradient centrifugation (11). The purified cell suspension had high anammox activity and fixed carbon dioxide. The 16S rDNA gene of the bacterium was shown to branch very deep within the planctomycete lineage of descent. The anaerobic ammonium oxidizing *planctomycete*-like bacterium was named "*Candidatus* Brocadia anammoxidans." The 16S rDNA sequence information was used to design specific oligonucleotide probes for application in fluorescence in situ hybridization (FISH), and these probes were used to survey the presence of *B. anammoxidans* and related bacteria in several wastewater treatment systems with a very high nitrogen load and limited air supply. Indeed "*Candidatus* Brocadia anammoxidans" and the closely related "*Candidatus* Kuenenia stuttgartiensis" could be detected in many of these systems throughout the world (12).

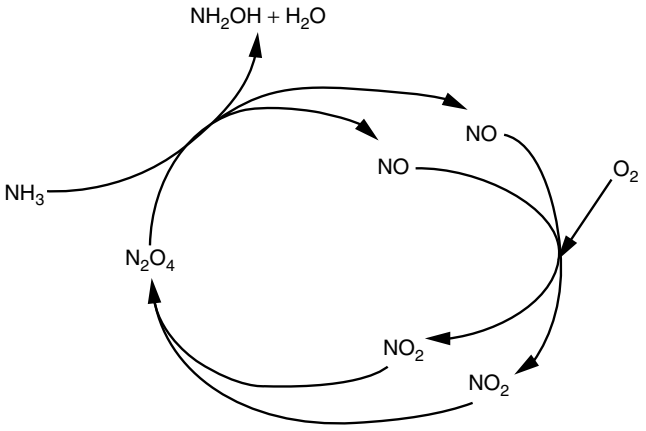

Figure 4. NO_x-cycle: New hypothetical model of ammonia oxidation by *Nitrosomonas*. According to this model, N_2O_4 is the oxidant for oxidation of ammonia. Under oxic conditions oxygen will be used to reoxidize NO to NO_2 (N_2O_4). Hydroxylamine will be oxidized to nitrite.

For the application of the Anammox process, it is important to know how *B. anammoxidans* coped with oxygen. Batch experiments showed that oxygen as low as 2 μM completely, but reversibly, inhibited the anammox activity. The obligately anaerobic nature of *B. anammoxidans* is in sharp contrast with the more versatile aerobic *Nitrosomonas*-like ammonium oxidizers.

As a result of this inhibition by oxygen, it seems likely that the aerobic ammonium oxidizing bacteria and *B. anammoxidans* are only able to coexist under oxygen limiting conditions. The ammonia oxidizers would oxidize ammonia to nitrite and keep the oxygen concentration low, and *B. anammoxidans* would convert the toxic nitrite and the remaining ammonium to nitrogen gas. By gradually supplying more and more air into an anammox sequencing batch reactor, it was indeed possible to establish such a cooperation. In the reactor, *Nitrosomonas*-like bacteria consumed the oxygen effectively, so that the actual oxygen concentration remained below the detection limit of 2 μM. Nitrite concentrations never exceeded 1 mM, indicating that *B. anammoxidans* was active as well. After five months wastewater with an ammonia concentration of 30 mM was converted into dinitrogen gas and some nitrate, according to Equation 9 (13).

$$2.5NH_4^+ + 2.1O_2 \rightarrow 0.2NO_3^- + 1.15N_2 + 3.6H_2O + 2.8H^+ \quad (9)$$

The microbial composition of the biomass in this reactor was analyzed with FISH, using *B. anammoxidans* (Amx820) and *Nitrosomonas* (Neu653) specific probes. Initially *B. anammoxidans* dominated (70%) but over time more and more *Nitrosomonas*-like bacteria were detected. Aerobic nitrite oxidizers (*Nitrobacter* or *Nitrospira*) were never detected, consistent with the absence of nitrite oxidizing activity in oxic batch tests.

The symbiosis of aerobic and anaerobic ammonium oxidizing bacteria is relevant for wastewater treatment. The experiments showed that ammonium can be removed in a simple, single oxygen-limited step. The process was named CANON (completely autotrophic N-removal over nitrite). Figure 5 shows images of CANON biomass after hybridization with labeled probes.

The introduction of anammox to N-removal would lead to a substantial reduction of operational costs. The process would be suitable to treat waters that contain high ammonium and little organic COD. The Anammox process would replace the conventional denitrification step completely and would also save half of the nitrification aeration costs.

The feasibility of anammox to treat sludge liquor was investigated in combination with the SHARON (single reactor system for high ammonium removal over nitrite)

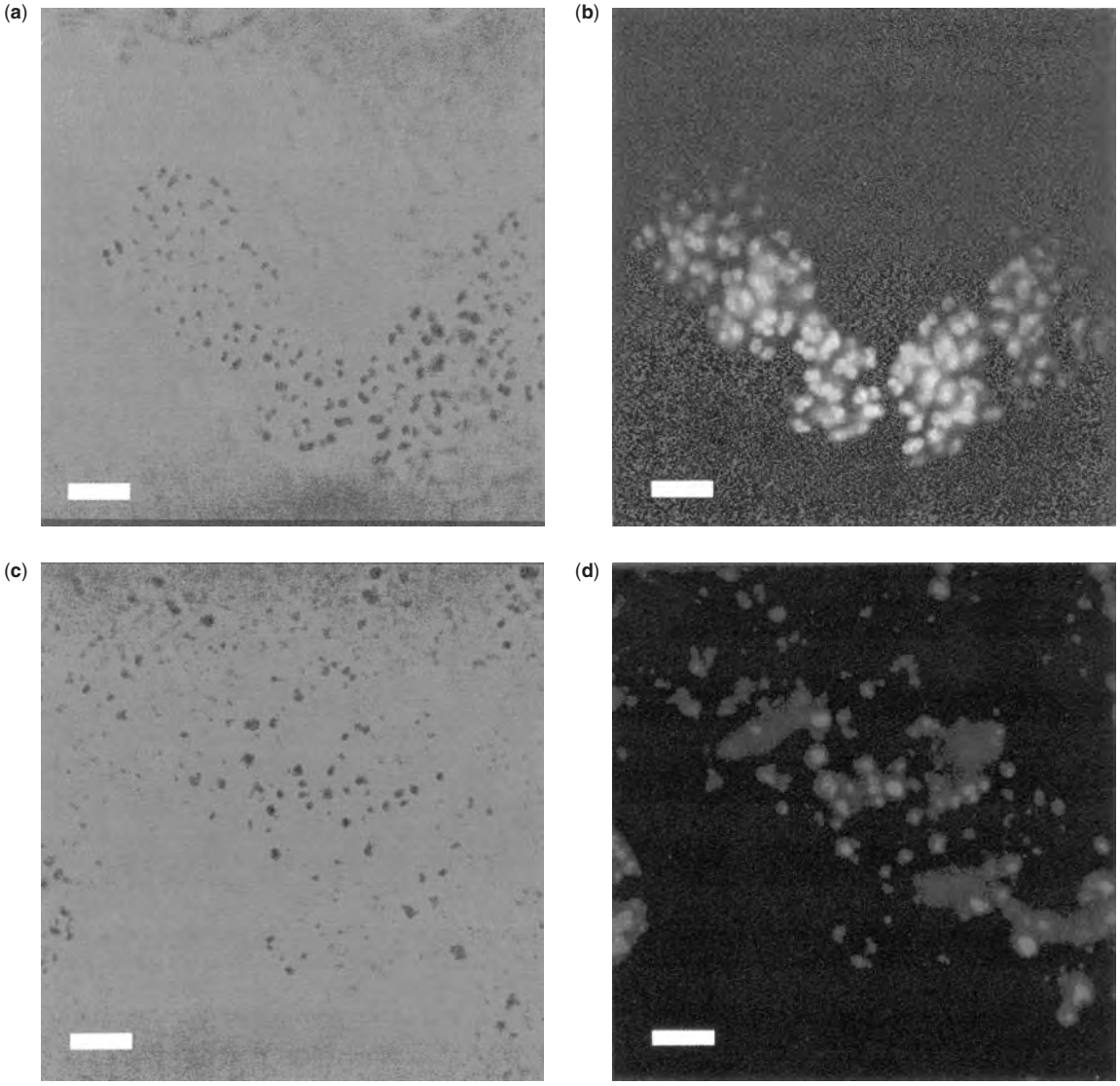

Figure 5. Phase contrast (a,c) and epifluorescence (b,d) images of biofilm aggregates from a CANON reactor after hybridization with Cy-3 labeled probes PLA46 (b, specific for planctomycetes) and BET42 (d, specific for beta proteobacteria, mainly nitrifiers in this case). The figure shows that both aerobic and anaerobic ammonium oxidizers are present in this reactor system for nitrogen removal from wastewater. Bar equals 5 micrometer. See color insert.

process. SHARON was developed recently for the removal of ammonium via the so-called nitrite route (14). It was tested for two years in the laboratory and successfully scaled-up to full scale (1,800 m^3) (15). Anammox needs ammonium and nitrite in a ratio of about one to one. This ratio can be achieved without control using the SHARON process as a prenitrification step. When half of the ammonium is converted, the alkalinity of the sludge liquor is depleted, leading to a pH drop and preventing further nitrification (Eq. 10).

$$NH_4^+ + HCO_3^- + 0.75O_2 \rightarrow 0.5NH_4^+ + 0.5NO_2^- + CO_2 + 1.5H_2O \qquad (10)$$

The feasibility of SHARON for the production of ammonium and nitrite (1 : 1) was demonstrated in a 20-L laboratory system (16). The ammonium was oxidized for 53% to nitrite at 1.2 kg N m^{-3} per day without pH control. The effluent of the SHARON reactor was fed directly to an anammox reactor system. This reactor removed all nitrite, and left some ammonium. During the test period the nitrogen load was 0.75 kg N m^{-3} per day. The specific activity of the anammox biomass was very high: 0.8 kg N (kg dry weight)$^{-1}$ per day.

The combined SHARON-Anammox process is presently evaluated for full-scale implementation. On the basis of the design of the combined SHARON-Anammox process, a cost estimate of 0.75 Euro kg^{-1} N was calculated. This is

very low compared with the 2 to 5 Euro kg^{-1} N estimated for other processes that have been tested on a pilot plant scale for N-removal from sludge liquors.

HETEROTROPHIC AMMONIA OXIDATION

The oxidation of ammonia, hydroxylamine, or organic nitrogen compounds, for example, oximes, to nitrite by various chemoorganotrophic microorganisms is called heterotrophic nitrification. The latter is a cometabolism, which is assumed not to be coupled to energy conservation. Heterotrophic nitrifiers are found among algae, fungi, and bacteria. Compared with those of autotrophic nitrifiers, nitrification rates of heterotrophic nitrifiers are low. Therefore, heterotrophic nitrification was thought to occur preferentially under conditions that are not favorable for autotrophic nitrification, for example, acidic environments. However, recent research has revealed that heterotrophic nitrification only contributes to a minor extent to overall nitrate production, even in acid soils. Recently, heterotrophic nitrification has received some attention, because it frequently occurs while accompanied by aerobic denitrification. In principle, this combination could lead to the elimination of dissolved nitrogen compounds in a single-step reactor system.

OXIDATION OF NITRITE

Physiology of Nitrite Oxidation

The second step in nitrification, the oxidation of nitrite to nitrate, is performed by nitrite oxidizing bacteria, for example, members of the genera *Nitrobacter*, *Nitrospira*, or *Nitrococcus*. Several strains of *Nitrobacter* and one strain of *Nitrospira* are the only nitrite oxidizers that are not restricted to marine environments (17).

The key enzyme of nitrite oxidizing bacteria is the membrane bound nitrite oxidoreductase, which oxidizes nitrite with water as the source of oxygen to form nitrate. The electrons released from this reaction are transferred via a- and c-type cytochromes to a cytochrome oxidase of the aa_3-type. However, the mechanism for energy conservation in nitrite oxidizers is still unclear. No electron transport chain linked to proton translocation could be found, a factor that is necessary to maintain a proton motive force for ATP regeneration. Thus, NADH is thought to be produced as the first step of energy conservation. The overall process deserves further research for complete elucidation.

Growth Characteristics of Nitrite Oxidizers

Nitrite oxidizers are generally lithoautotrophic organisms. Carbon dioxide is fixed as the main carbon source using RuBisCO, which is in part carboxysome-bound. Higher growth rates are obtained when the cells are maintained mixotrophically. In contrast to ammonia oxidizing bacteria, several strains of *Nitrobacter* are capable of heterotrophic growth under oxic as well as anoxic conditions. Heterotrophic growth is significantly slower than lithoautotrophic, although, 10- to 50-fold higher cell densities are obtained.

Oxidation of nitrite occurs under obligately oxic conditions. The involved organisms are much more sensitive to oxygen limitation than ammonia oxidizers are. At dissolved oxygen concentrations of about 0.5 mg l^{-1}, oxidation of nitrite is inhibited. Additionally, *Nitrobacter* is inhibited at high oxygen concentrations. Thus, the oxygen content of a nitrite oxidizing nitrification tank has to be maintained carefully to avoid accumulation of nitrite. With sufficient oxygen supply oxidation of nitrite proceeds at a faster rate than conversion of ammonia to nitrite. Therefore, high nitrite concentrations are rarely found in either natural environments or in wastewater treatment plants.

Denitrification by Nitrite Oxidizers

Some strains of *Nitrobacter* have been shown to be denitrifying organisms as well. Nitrate can be used as an acceptor for electrons derived from organic compounds to promote anoxic growth. Because the oxidation of nitrite is a reversible process, the nitrite oxidoreductase can reduce nitrate to nitrite in the absence of oxygen. Further, the nitrite oxidoreductase was purified together with a nitrite reductase, which reduces nitrite to nitric oxide. Evidence is given that the subsequent oxidation of nitric oxide to nitrite is involved in NAD-reduction in *Nitrobacter hamburgensis*. However, because denitrifying cells of *Nitrobacter* grow very slowly, it seems improbable that denitrification by nitrite oxidizers might have any significance in the treatment of wastewater.

DENITRIFICATION

General Aspects of Denitrification

Denitrification is the reduction of oxidized nitrogen compounds such as nitrite or nitrate to gaseous nitrogen compounds. This process is performed by various chemoorganotrophic, lithoautotrophic, and phototrophic bacteria and some fungi, especially under oxygen-reduced or anoxic conditions. Denitrification can be described as anoxic respiration. Electrons originated from, for example, organic matter, reduced sulfur compounds, or molecular hydrogen are transferred to reduce nitrogen compounds instead of oxygen in order to establish a proton motive force usable for ATP regeneration. Enzymes involved are nitrate reductase, nitrite reductase, nitric oxide reductase, and finally nitrous oxide reductase. Dinitrogen is the main end product of denitrification, whereas the nitrogenous gases nitric oxide and nitrous oxide can occur as intermediates at low concentrations (18). However, these gases are also released as end products when denitrification enzymes are not completely expressed, for example, when the concentration of dissolved oxygen is too high.

Substrate Requirements of Denitrifiers

The common denitrifiers in municipal wastewater treatment plants are chemoorganotrophic bacteria, for example, *Paracoccus denitrificans* or *Alcaligenes eutrophus*, which require an electron donor of an organic

nature. Hence, complete denitrification requires a sufficient supply of organic matter. In detail, a specific C : N ratio is required to provide an adequate amount of electron donors for the reduction of a distinct amount of nitrogen oxides. Wastewater with an inadequate COD loading should therefore be supplemented with an external organic electron donor–like acetate or methanol to avoid exceeding outlet concentrations of nitrate or nitrite. Recently, denitrification activity could be demonstrated in pure cultures of ammonia oxidizers such as *N. eutropha* (4). Under anoxic conditions *N. eutropha* is able to denitrify with molecular hydrogen as electron donor and nitrite as the only electron acceptor in a sulfide-reduced medium producing nitrous oxide and dinitrogen. Cell growth is directly coupled to nitrite reduction.

Aerobic Denitrification

Denitrification also occurs in the presence of oxygen. The range of oxygen concentration permitting aerobic denitrification is broad and differs from one organism to another (19). The onset of aerobic denitrification does not depend on the oxygen sensitivity of the corresponding enzymes but rather on regulation of oxygen- or redox-sensing factors involved in the regulation on a transcriptional level. This is further indicated by denitrifying enzymes expressed under anoxic conditions that remain active in the presence of oxygen (20). Generally, the ability to denitrify under oxic conditions seems to be the rule rather than the exception among denitrifiers. However, in the treatment of wastewater denitrification commonly proceeds under anoxic and not under oxic conditions because in the presence of oxygen large amounts of organic matter would be "wasted "on respiration and biomass production. Hence, denitrification would cease or run incompletely as a result of a lack of electron donors.

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ACTIVATED SLUDGE MODELS: MICROBIOLOGICAL BASIS

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The activated sludge (AS) process is the most common wastewater treatment process. It was primarily designed for the removal of organic material or carbon (C) (see ACTIVATED SLUDGE — THE PROCESS), but has been modified to remove nitrogen (N) and phosphorus (P) as well. From an engineering point of view, it is necessary to model the behavior of the process mathematically and to predict, for example, the effluent water quality or the amount of excess sludge produced. The main mechanism for the substrate removal is biological. Therefore, mechanistic and structured mathematical models are needed for describing the major biological events occurring within the system or the behavior of the microorganisms responsible for the treatment. Among the mechanistic mathematical models available, the IWA (International Water Association) AS models (1) have been recognized as the worldwide standard. In the present article, the fundamental concepts and the underlying microbiological bases of the IWA models are critically discussed.

HISTORY OF IWA AS MODELS

A pioneer work on AS modeling was carried out in the late 1970s to early 1980s by the group of G. v. R. Marais from University of Cape Town, South Africa. They proposed basic ideas for general structures of AS models (2), chemical oxygen demand (COD) fractionation for modeling purpose (2), application of oxygen uptake rate measurements for determination of model parameters (3), and so on. Their work has been the basis for the development of the IWA AS models.

In 1983, a task group was organized within the International Association on Water Pollution Research and Control (IAWPRC, later renamed as the International

Association on Water Quality [IAWQ] and then as the IWA) to formulate general mathematical tools for describing the behavior of the AS process. In 1987 they published a mechanistic mathematical model called the *IAWPRC Activated Sludge Model No. 1 (ASM 1)* that incorporated carbon and nitrogen removal (4). This model became very popular and was adopted as the standard AS model worldwide. In the present article, ASM 1 will not be discussed in detail because newer models have since been developed. In 1991, the IAWQ task group was reorganized, and they attempted to formulate an updated model that included enhanced biological phosphorus removal (EBPR) as well as removal of carbon and nitrogen. Thus, ASM 2, describing carbon, nitrogen, and phosphorus removal, was published in 1995 (5). In 1999, two additional models were proposed: ASM 2d (6) was a partial extension of ASM 2, and ASM 3 (7) was developed to overcome many defects in ASM 1, 2, and 2d and was designed as the core model that formed the basis for further extension. As IAWPRC or IAWQ has been now changed to IWA, all these models presented by the task group are hereafter called the "IWA" models. The main characteristics of these IWA AS models are summarized in Table 1.

Apart from the IWA AS models, a new approach was proposed by the group of van Loosdrecht and Heijnen from Delft University of Technology, the Netherlands (8–10) for modeling the EBPR processes. They developed a structured metabolic model for the EBPR processes based on bioenergetics and stoichiometry of known metabolic reactions. All relevant metabolic reactions underlying the EBPR metabolism were described, and their reaction rates were correlated with each other by applying stoichiometry-based linear relations between these reaction rates. This approach allowed for significant reduction in the number of kinetic or stoichiometric parameters to be determined: it was possible to reasonably describe the dynamic behavior of EBPR processes with a set of 14 parameters. On the other hand, the apparent complexity of the model increased and much biochemical knowledge is required to understand the behaviors of the model. This Delft model is too complicated to be used for practical purposes. However, as this Delft model describes more detailed biochemical structures of the EBPR metabolism in a scientific way, it is considered to be a promising research tool.

Currently, commercial software for running AS models are available in the market, and many attempts have been made to use AS models for either practical or scientific purposes (11,12). In such modeling practices, the IWA AS models are recognized as benchmark models.

BASIC CONCEPTS OF IWA AS MODELS

Mechanistic Models

There are several essential objectives of AS modeling. These include: (*1*) prediction of effluent water quality in terms of key water quality indexes, such as COD, nitrogen, and phosphorus species; (*2*) estimation of excess sludge production; (*3*) estimation of oxygen consumption in the system; and (*4*) prediction of the behavior of concerned microbes in the system. Recently, the importance of mechanistic mathematical models is increasing significantly because of the progress of computer technology. Huge computations can be performed within a short period of time, which allows these AS models to be complex, without limitations of computation time, and to be applicable not only for academic or educational purpose but also for more practical uses such as design, process control, and operation support. Increasing demand for nitrogen and phosphorus removal (see ACTIVATED SLUDGE — THE PROCESS, ACTIVATED SLUDGE — MICROBIOLOGY OF NITROGEN REMOVAL, ACTIVATED SLUDGE — MOLECULAR TECHNIQUES FOR DETERMINING COMMUNITY COMPOSITION) has facilitated the increased application of such mechanistic AS models in process control. Precise process control is not necessarily needed for carbon removal, but it is extremely important for nitrogen and phosphorus removal.

The AS process is a biological process in which carbon removal is carried out by heterotrophic organisms, nitrogen removal is achieved through biological nitrification and denitrification, and EBPR is made possible because a group of bacteria are able to accumulate intracellular polyphosphate (poly-P) (13). The more these biological processes have been understood, the more complex, but more generally applicable, the models developed have become. The use of AS mathematical models allows wastewater engineers to test many different possible designs or operational strategies by simulation, without carrying out expensive and time-consuming pilot plant studies. Therefore, once conceptual understanding of the AS process had expanded sufficiently, attempts were made to incorporate microbiological and biochemical information of relevant biological processes into AS models. Mechanistic and structured mathematical models have thus been formulated that can describe in general terms the major events occurring within the system or the behavior of the microorganisms responsible for the treatment. The IWA models are the most commonly accepted models of such kind.

Processes and Components

In AS systems, different groups of microorganisms perform various biological reactions, such as carbon oxidation, nitrification, denitrification, and poly-P accumulation. These biological reactions actually determine the concentrations of different system components. In AS modeling, the term "process" is used to define an event that occurs independently in the system (4) and is concerned with the transformation or conversion of one or more system components such as the microorganisms, COD, and ammonia. So, the identification of important processes affecting the system behavior and the selection of key components relevant to the identified processes are two major tasks required for AS modeling. From a modeling point of view, microorganisms are classified into a few groups, each of which should reasonably represent a functional microbial group and be responsible for at least one process (4). The system components and the processes in the model must

Table 1. Characteristics of IWA AS Models

Abbreviation	Year Published	Target Pollutant	No. of Processes	No. of Components		Microbial Groups Described	Characteristics, Advantages, and Constraints of Model
				Soluble	Particulate		
ASM 1	1986	C, N	8	7	6	Heterotrophs, nitrifiers	The first model proposed by the IAWPRC Task Group, which became a standard AS model and the common basis for further research A structured/mechanistic model simulating carbon and nitrogen removal Peterson's matrix format is adopted for the presentation of the model, offering the best opportunity to the users to trace all the interactions of the system components Heterotrophs are assumed to perform not only the aerobic growth with the oxidation of organic substrates, but also denitrification It is assumed that hydrolysis of high-molecular compounds is done only by heterotrophs As a general rule, the Monod type kinetic expression is used
ASM 2	1995	C, N, P	19	9	10	Heterotrophs, nitrifiers, PAOs	Biological phosphorus removal is modeled in addition to carbon and nitrogen removal, thus PAOs are introduces as bacteria responsible for EBPR Cell internal storage components, PHA, and polyphosphate, are introduced to describe the behavior of PAOs The COD fraction is assumed to carry a defined amount of nitrogen and phosphorus. The ammonification of organic nitrogen, which is adopted in ASM 1, is replaced by hydrolysis of nitrogen-containing COD The hydrolysis process eventually involves not only true hydrolysis of complex molecules but many other processes such as degradation of stored substrates and predation by higher animals
ASM2d	1998	C, N, P	21	9	10	Heterotrophs, nitrifiers, PAOs	An extension of ASM 2: denitrifying capability is given to PAOs so that anoxic phosphorus uptake can be realized in the model Basic structure is the same as that of ASM 2
ASM 3	1998	C, N	12	7	6	Heterotrophs, nitrifiers	An extension of ASM 1: phosphorus removal not included, and only carbon and nitrogen removal modeled Developed by revising some defects in ASM 1 and 2, and designed as the core model for further extension The concept of biomass decay in ASM 1 and 2 is replaced by the endogenous respiration concept All heterotrophs are assumed to be able to store cell internal storage compounds

Note: PAO–polyphosphate accumulating organisms, EBPR–enhanced biological phosphate removal.

be defined as simple as possible, but should be complex enough to describe the system behavior in such a way as to fit the model user's purpose (4). The components in the model do not always represent something identifiable in reality. In the IWA models, there are several variables clearly defined on the basis of certain modeling concepts that are neither measured by chemical analyses nor separated by physical means. Similarly, the processes in the model may not always reflect the corresponding reactions in reality. For example, "hydrolysis" in the IWA models should not only mean true hydrolytic biochemical reactions but may also include other processes such as predation by higher animals and utilization of intracellular storage polymers (see details a later section). The processes and microbial groups adopted in ASM 2, 2d, and 3 are listed in Table 2.

Stoichiometry and Kinetics

Then, the stoichiometry (quantitative relationship between components in a chemical reaction) and the kinetics (dependency of process rate on the concentrations of relevant components) should be defined for each process. When the stoichiometry of a process is determined, the continuity of the process must be strictly maintained. Continuity is a term that carries the mathematical equivalence to the principle that in chemical reactions, elements, electrons (or COD), and net electrical charges may neither be created nor be destroyed (4,5). For the

Table 2. Biological Processes in ASM 2, 2d, and 3

Model	Microorganisms Responsible for the Process	Biological Processes
ASM 2	Heterotrophs	Aerobic hydrolysis
		Anoxic hydrolysis
		Anaerobic hydrolysis
		Aerobic growth of heterotrophs on fermentation products
		Aerobic growth of heterotrophs on fermentable readily biodegradable organic substrate
		Anoxic growth of heterotrophs (denitrification) on fermentation products
		Anoxic growth of heterotrophs (denitrification) on fermentable readily biodegradable organic substrate
		Fermentation
		Lysis of heterotrophs
	PAOs	Storage of PHA
		Storage of poly-P
		Aerobic growth of PAOs
		Lysis of PAOs
		Lysis of poly-P
		Lysis of PHA
	Autotrophs (nitrifiers)	Aerobic growth of nitrifiers (nitrification)
		Lysis of nitrifiers
ASM 2d	Heterotrophs	Same as ASM 2
	PAOs	Storage of PHA
		Aerobic storage of poly-P
		Anoxic storage of poly-P
		Aerobic growth of PAOs
		Anoxic growth of PAOs
		Lysis of poly-P
		Lysis of PHA
	Autotrophs (nitrifiers)	Same as ASM 2
ASM 3	Heterotrophs	Hydrolysis
		Aerobic storage of cell internal product
		Anoxic storage of cell internal product
		Aerobic growth of heterotrophs
		Anoxic growth of heterotrophs (denitrification)
		Aerobic endogenous respiration of heterotrophs
		Anoxic endogenous respiration of heterotrophs
		Aerobic endogenous respiration of cell internal storage product
		Anoxic endogenous respiration of cell internal storage product
	Autotrophs (nitrifiers)	Aerobic growth of nitrifiers
		Aerobic endogenous respiration of nitrifiers
		Anoxic endogenous respiration of nitrifiers

kinetics, the Monod model (14) is generally adopted as the rate expression for the biological growth process in the IWA models:

$$\text{Process Rate} = \frac{\upsilon XS}{(S + K)}$$

where υ is the rate coefficient for the process, X the concentration of biomass that performs the process, S the limiting substrate concentration, and K the saturation coefficient.

The Monod model is generally used, not because it may either best fit the experimental data or have a definite microbiological basis. Generally speaking, the growth rate of an organism is dependent on the limiting substrate concentration in relatively low substrate concentration ranges, whereas its growth rate is not affected by substrate concentration at higher concentrations. The Monod model can express this general dependency of the process rate on the substrate concentration reasonably well in simple terms. Only for a few specific processes other types of kinetic expressions are adopted, because of several specific reasons; for example, a kinetic model for a surface-limited reaction is used for hydrolysis processes (4) and a saturation equation is used for poly-P storage (5).

After both the stoichiometry and the kinetics of the concerned system are defined, the mass balance for each component may be created for a given system boundary (e.g., a completely mixed AS reactor). Various processes can affect the concentration of a specific component, and all the processes affecting the concentration of that particular component are taken into account in the mass balance. From the mass balance, the concentration of each system component can be calculated and the system behavior can then be simulated (4). In order to simulate carbon oxidation, nitrogen, and phosphorus removal, COD (measured using potassium dichromate as the oxidizing reagent), nitrogen and phosphorus are selected as the basis for mass balance in the IWA models (4,5). Instead of COD, carbon itself can be used for the mass balance for organic substances (7). The advantage of using COD (or actually the oxygen equivalent in the oxidation-reduction reaction) for the mass balance is that the oxygen balance can be calculated and thus used to predict the oxygen requirement in the system (4). Because the AS process is an oxygen-dependent system, it is extremely useful, in practice, that the simulation model can predict reasonably the oxygen concentration in the system and its oxygen requirement.

ESSENTIAL PROCESSES IN IWA MODELS

General

Processes mainly adopted in ASM 2 are discussed in this section. For modeling purposes, microorganisms in the AS are usually classified into several functional groups. In ASM 2, only three groups of microorganisms are recognized and represent a broad range of different microbial populations. These three groups are: heterotrophs (microorganisms that live on organic substrates as carbon and energy sources), nitrifiers (autotrophs that oxidize ammonia to nitrite and nitrate), and phosphorus accumulating organisms (PAOs, microorganisms that have a capability to accumulate poly-P and are responsible for EBPR). Functions of these three biomass fractions in ASM 2 are schematically shown in Figures 1 to 3.

Heterotrophs are modeled as "almighty" microorganisms that can grow aerobically, denitrify, hydrolyze complex organic compounds and carry out fermentations. Nitrifiers are autotrophs and do not contribute to organic carbon removal. They are involved in the oxidative part of biological nitrogen removal. PAOs are a group of heterotrophs, but they are considered as a separate group when EBPR is modeled. The present classification of microorganisms is too simple, in some cases, to describe the complicated and poorly understood behavior of AS processes. However, it is sufficient to simulate major events that actually affect fundamental system design or control strategy of the AS process.

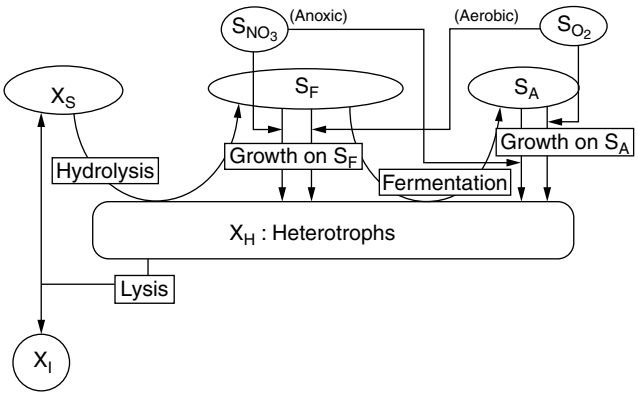

Figure 1. Transformation of organic substrates by heterotrophs in ASM 2. S_{NO_3}: nitrate and nitrite nitrogen, S_{O_2}: dissolved oxygen, S_F: readily fermentable biodegradable substrates, S_A: fermentation products, X_S: slowly biodegradable substrates, X_H: heterotrophic biomass, X_I: inert organics.

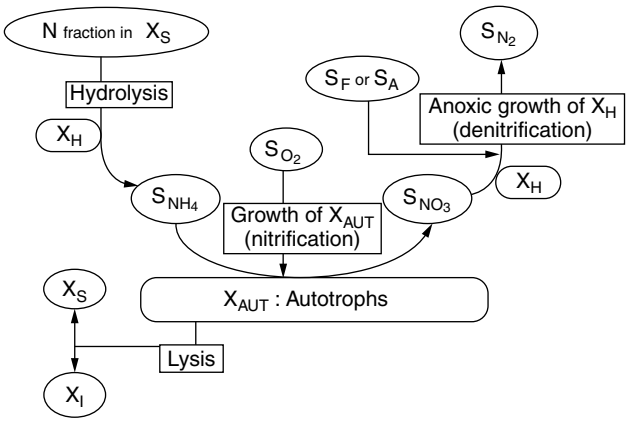

Figure 2. Function of autotrophs (nitrifiers) in biological nitrogen removal in ASM 2. S_{NH_4}: ammonia nitrogen, S_{N_2}: nitrogen gas, S_{NO_3}: nitrate and nitrite nitrogen, S_{O_2}: dissolved oxygen, S_F: readily fermentable biodegradable substrates, S_A: fermentation products, X_S: slowly biodegradable substrates, X_H: heterotrophic biomass, X_{AUT}: autotrophs (nitrifiers), X_I: inert organics.

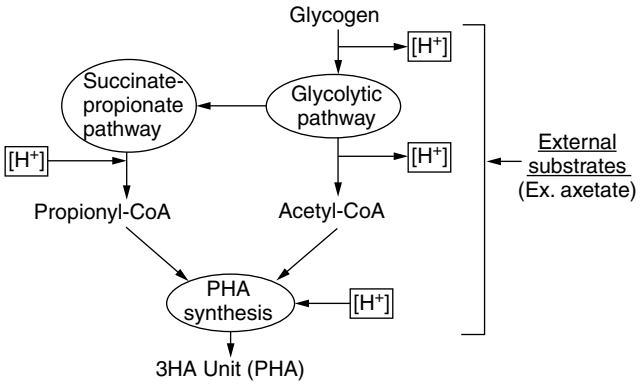

Figure 3. Anaerobic carbon metabolism of PAOs for enhanced biological phosphate removal. Glycogen functions as regulator of redox balance in the cell.

Aerobic Growth of Heterotrophs and Carbon Removal

The major mechanism of carbon removal in the AS process is by the growth of heterotrophs on organic substrates. These heterotrophs include diverse phylogenetic, taxonomic, and functional groups (15), but all, except for PAOs as discussed later, are assumed to be one functional group of microorganisms in ASM 2. Their common nature is that they all utilize organic substrates as the carbon and energy sources for growth.

In heterotrophic growth, organic substrates are used in two ways: one for the energy-generating metabolism (adenosine triphophosphate [ATP] production) and the other for assimilative metabolism (biosynthesis of the cell). Some substrates in the wastewater are oxidized completely to carbon dioxide and utilized for energy (ATP) generation. This is an oxidation process and requires a terminal electron acceptor. The AS reactors are usually aerated and kept aerobic so that oxygen serves as the electron acceptor. The rest of the substrates are used for the synthesis of biomass and withdrawn from the AS system as excess sludge. The ratio of this carbon distribution is represented by the yield (Y, the ratio of mass of produced biomass to mass of the substrate utilized) in the AS models (16). This yield is assumed to be a constant, an assumption that is generally acceptable for wastewaters with similar compositions.

Wastewaters may contain a large variety of organic compounds that are utilized by microorganisms at different rates. Therefore, an important question to be asked when heterotrophic growth is modeled is: how many fractions of organic substrates should be defined in the model to satisfactorily describe the substrate removal profiles in the AS system? In the IWA models, the organic substrates are principally divided into two fractions, namely, *slowly biodegradable substrate* (SB-COD, denoted as X_S) and *readily biodegradable substrate* (RB-COD, denoted as S_S). This is because the oxygen uptake rate (OUR) during aerobic substrate removal from a municipal wastewater showed (3) a two-stage response, which implies that organic substrates present in municipal wastewaters can be divided into two fractions with two different OURs. It is instructive to think that SB-COD represents the high-molecular or particulate fraction and that RB-COD represents the low-molecular or soluble fraction (4,5).

From a modeling point of view, SB-COD is defined as the COD fraction that is hydrolyzed to smaller molecules before being utilized, and RB-COD is defined as the COD fraction that can be directly utilized by heterotrophs with no prior hydrolysis (4). In other words, SB-COD need to be first hydrolyzed to RB-COD and only then is utilized for heterotrophic growth. The conversion of SB-COD to RB-COD is referred to as "hydrolysis" in the IWA models (5). The two observed rates in the profile can be interpreted as the hydrolysis rate and the aerobic growth rate of heterotrophs. However, the distinction between SB- and RB-COD is not always done clearly in practice. Some soluble COD fractions may contain rather complex molecules that cannot be utilized directly by heterotrophs and should be classified as SB-COD even though they are soluble (5). The present COD fractionation is simple enough for practical use and complex enough to reasonably describe the behavior of AS systems under most situations.

In ASM 2, the RB-COD faction is further divided into two fractions, "fermentation products (S_A)" and "fermentable readily biodegradable substrate (S_F)." Thus,

$$S_S = S_F + S_A$$

The process of conversion from S_A to S_F is defined as fermentation. This fractionation as well as the process of fermentation are necessary when EBPR is modeled, as discussed later. The transformation of organic substrates by heterotrophs, including the fermentation step, is shown graphically in Figure 1.

In the real world, many wastewaters do not show the typical two-stage response in the OUR test. For example, OUR profiles of some municipal wastewaters show a gradual decreasing trend along with time when organic substrates are removed in a batch test (17). Such results imply that a wastewater may contain many compounds with different utilization rates. However, it is not recommended to increase the number of COD fractions, because determination of each COD fraction as well as the parameters associated with it becomes increasingly difficult as the numbers increase. On the other hand, the composition of industrial wastewaters depends totally on the type of industry and the industrial processes used, and a few major organic components can be often identified from the raw materials or from the processes. Then, each major organic component can represent a COD fraction that has a single process rate. Specialist microorganisms can also be defined, which thrive on certain particular components. When the wastewater composition is defined clearly enough and the process rates for the identified components are measurable, it is possible to assign a process rate to each relevant component and, in more limited cases, a specialist microorganism that utilizes it.

Denitrification and Nitrification

Biological nitrogen removal is achieved by the combination of autotrophic nitrification and heterotrophic denitrification (see ACTIVATED SLUDGE — MICROBIOLOGY OF NITROGEN

Removal). The nitrogen conversion processes modeled in ASM 2 are shown graphically in Figure 2.

Under anoxic conditions (i.e., where oxygen is not available but nitrate or nitrite is), nitrate or nitrite can be used as electron acceptors in heterotrophic growth (15). This anoxic growth is referred to as denitrification. Denitrifying heterotrophs that grow anoxically usually grow aerobically utilizing oxygen as electron acceptor. Thus, denitrifiers should be a part of aerobic heterotrophs. In ASM 2, heterotrophs are defined as one biomass fraction and modeled in such a way that all heterotrophs can denitrify. But, this is not true in reality.

Aerobic and the anoxic growth of heterotrophs are very similar to each other in terms of stoichiometry and kinetics, but differ in that the observed specific growth rates of heterotrophs are usually lower under anoxic conditions than under aerobic conditions (18). A factor (the reduction factor for denitrification) is introduced to allow for this reduced growth rate under anoxic conditions (4,5). This factor is defined as the ratio of the specific growth rate under anoxic conditions to that under aerobic conditions, which actually implies the portion of denitrifiers within the total heterotrophic biomass (5). An advantage of the introduction of this factor is that most actual situations can then be simulated without increasing the number of processes. However, this factor may not be a universal constant but a parameter that should be calibrated for each specific system or situation, because the ratio of denitrifiers may vary according to different environmental conditions. It is of course possible to create a model that involves two heterotrophic biomass fractions, one with and the other without denitrification capability. The dual-heterotroph model may describe denitrification behavior of systems more sensitively than the IWA models. However, the complexity of the model will increase and the identification of stoichiometric or kinetic parameters will become more difficult.

Nitrification (19) is a process in which ammonia is biologically oxidized to nitrite and nitrate in the presence of oxygen as the terminal electron acceptor. Nitrifiers are autotrophic bacteria and use inorganic carbon (carbon dioxide) as the carbon source. Nitrification involves two steps: oxidation of ammonia to nitrite, and further oxidation of nitrite to nitrate (see Activated Sludge — Microbiology of Nitrogen Removal). These two steps are carried out by phylogenetically different groups of bacteria. The ammonia oxidizers include *Nitrosomonas* and *Nitrosospira* and nitrite oxidizers, including *Nitrobacter* and *Nitrospira* (20). However, the IWA models do not distinguish between these two groups of nitrifiers, but only one biomass fraction is used for all bacteria that carry out complete nitrification. There are two major reasons for this. First, the growth rate of nitrite oxidizers is usually higher than that of ammonia oxidizers. Therefore, nitrite does not accumulate in the system, but is consumed by nitrite oxidizers immediately after its production by ammonia oxidizers (21). So, in most cases, nitrification takes place as if it were a single process. Secondly, nitrite can be produced not only as an intermediate product in nitrification but sometimes as a by-product of denitrification (22). Production of nitrite through nitrification has been studied in detail, but there is very little kinetic information available about the nitrite production through denitrification (5). This means that the nitrite concentration is not satisfactorily predictable.

Nitrous oxide (N_2O) is a nitrogenous species that can be produced both in nitrification and denitrification and should not be neglected in constructing mass balance of nitrogen (22). N_2O is of concern these days from an environmental point of view, because it is a greenhouse gas that may contribute to global warming (23). However, N_2O production is not taken into account in the IWA models. The mechanism and the conditions for N_2O production are still poorly understood. In practice, it is almost impossible to determine satisfactorily all the kinetic parameters involved in these complex processes affecting its behavior.

EBPR

Phosphorus is an essential element for biomass growth, and the sludge produced as excess sludge will contain a certain amount of phosphorus. Thus, phosphorus can be removed from wastewater by withdrawing the excess sludge from the AS system. However, municipal wastewaters usually contain more phosphorus than is required for the biomass growth, so phosphorus in the influent may not be completely removed only by normal microbial growth equivalent. On the other hand, the phosphorus content of the sludge may increase up to 5 to 6% or even more than 10% in extreme cases, whereas the phosphorus content of a typical AS may be only about 1.5 to 2% of phosphorus (on dry weight basis) (13). Such high phosphorus content is caused by an excess accumulation of poly-P in the sludge and may lead to much higher phosphorus removal efficiency than usual (see Activated Sludge — Molecular Techniques for Determining Community Composition). This high phosphorus removal by biological means is referred to as EBPR. The bacteria that can achieve the high phosphorus content through the accumulation of poly-P and are thus responsible for EBPR are called PAOs. It is necessary to describe the behavior of PAOs in mathematical terms to incorporate EBPR into the AS model.

A high phosphorus content of the sludge from EBPR can be achieved by introducing an anaerobic phase into the influent end of the AS process, followed by conventional aerobic phase (see Activated Sludge — The Process; 24). In such EBPR processes, the AS passes through anaerobic and aerobic conditions alternately, and the influent wastewater (or actually the organic substrates) is introduced into the anaerobic phase. In practice, this anaerobic-aerobic alternation can be achieved either by spatial configuration of anaerobic and aerobic zones in series with sludge recycle in continuous flow reactors or by temporal arrangement of anaerobic and aerobic periods in sequence batch reactors (see Activated Sludge — Sequencing Batch Reactors). Such EBPR processes are called anaerobic-aerobic or anaerobic-oxic processes. By exposing the sludge to these alternative anaerobic and aerobic conditions, PAOs are selected and grow to become dominant in the process. To achieve high and stable EBPR performance, it is essential to maintain PAOs in the system (25,26).

The mechanism of proliferation of PAOs in the EBPR process has been described as follows (25–27; also see another contribution of this Encyclopedia, ACTIVATED SLUDGE — MOLECULAR TECHNIQUES FOR DETERMINING COMMUNITY COMPOSITION). Because the AS is mixed with the influent wastewater in the anaerobic phase, organisms that can utilize organic substrates more rapidly in an anaerobic environment have an advantage. PAOs are the organisms that utilize the energy generated by the hydrolysis of polyphosphate for the uptake of the carbon substrates in the anaerobic phase of the EBPR process and can proliferate over other microorganisms under such conditions. The carbon substrates taken up by PAOs in the anaerobic phase are converted to polyhydroxyalkanoates (PHA) and stored inside the cell (28). Because PHA is a reduced polymer, its synthesis requires a source of reducing power, which is obtained by partial oxidation of intracellularly stored glycogen to carbon dioxide (29). The glycogen is partially converted via acetyl coenzyme A (CoA) to polyhydroxybutyrate (PHB). Thus, uptake of organic substrates and concomitant PHA synthesis by the sludge, degradation of stored poly-P and the consequent release of orthophosphate to the bulk solution, and utilization of stored glycogen are typical metabolic characteristics of the anaerobic phase of EBPR processes (25). In the subsequent aerobic phase, PAOs grow aerobically, recover the glycogen level, and assimilate orthophosphate to resynthesize poly-P, by using the stored PHA as their carbon and energy sources. Such typical EBPR metabolism is shown graphically in Figure 3.

Anaerobic metabolism of PAOs may involve glycogen being metabolized via propionyl-CoA to 3-hydroxyvalerate-rich PHA (the propionate-succinate pathway). This conversion does not lead to carbon dioxide production and consumes reducing power. This means that glycogen can either produce reducing power if metabolized through acetyl CoA, or consume reducing power if metabolized through propionyl-CoA. Glycogen stored in the cell should function as a regulator of the redox balance in the cell (25). The generation of reducing power should occur when more oxidative substrates are available, whereas its consumption should occur when more reductive substrates are utilized (30). It is essential for PAOs to take up carbon substrates at a faster rate than the other bacteria in the anaerobic stage, no matter what kinds of substrates are available in the influent wastewater. The mechanism described earlier can provide PAOs with a capability to assimilate different kinds of both reduced and oxidized organic substrates in the anaerobic phase without disturbing the redox balance in the cell. Glycogen storage appears to be the key strategy for PAOs to maintain the redox balance in the anaerobic uptake of various organic substrates, and hence to emerge dominant in the face of the selective pressures existing in EBPR processes (25).

This EBPR mechanism is partially adopted in ASM 2. Conceptual structures of the PAO metabolism adopted in ASM 2 are schematically shown in Figure 4. A prominent difference between this model structure and the generally accepted scientific mechanism is that glycogen is not introduced in the model, whereas glycogen probably plays an essential role in the real world. Only one

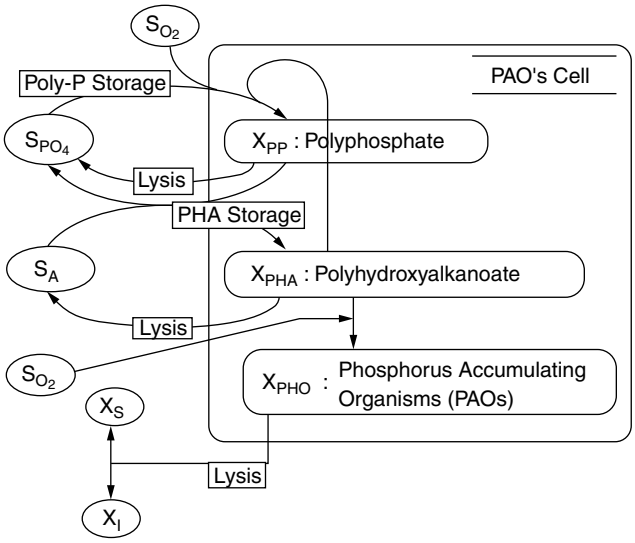

Figure 4. Metabolism of PAOs in ASM 2. S_{PO_4}: orthophosphate, S_{O_2}: dissolved oxygen, S_A: fermentation products, X_S: slowly biodegradable substrates, X_{PAO}: phosphorus accumulating organisms, X_{PP}: polyphosphate associated with XPAO, X_{PHA}: polyhydroxyalkaonates associated with XPAO, X_I: inert organics.

component, X_{PHA} (cell internal storage product of PAOs) is defined as carbon storage material in the model. This simplification is made in ASM 2 because the actual metabolism of the PAO is so unfamiliar to practitioners that they may have difficulty in understanding the model behavior fully. Glycogen and poly-P are the two key storage polymers that enable PAOs to take up organic substrates under anaerobic conditions. The former either supplies or consumes reducing power to maintain the redox balance in the cell and the latter generates energy necessary for the uptake of organic substrates and for their storage in the form of PHA under anaerobic conditions. Both are essential factors for PAOs to survive in the anaerobic-aerobic process. If glycogen is excluded from the model, situations in which glycogen, as the limiting factor, determines the system behavior may not be simulated properly. However, the relative size of the glycogen pool is usually almost the same as that of the poly-P pool (31). This means that most of the real situations in the operation of the EBPR processes, in practice, can be described with only one of the two parameters: either glycogen or poly-P. In ASM 2, poly-P is selected as a model component, and glycogen, which is not familiar to practitioners, is not adopted.

From the viewpoint of reaction stoichiometry, the component X_{PHA} in the model should represent all carbon storage materials in PAOs, including PHA and glycogen. Therefore, the model implies that the substrates anaerobically taken up by PAOs are converted to PHA without any loss or supply of carbon. However, a part of PHA is actually synthesized from glycogen that has already accumulated in PAOs (32,33). This argument should lead to an important warning: X_{PHA} in the model does not necessarily represent the analytically measured PHA.

It is believed that some organic substrates are selectively utilized by PAOs. As short-chain fatty acid such as acetate are favored as the carbon source for EBPR (34), the fraction of RB-COD that can be utilized by PAOs is defined as S_A in ASM 2. The fraction of RB-COD that can be utilized by heterotrophs other than PAOs but not by PAOs is defined as S_F. For the growth of heterotrophs, S_F and S_A have no difference: both are utilized by heterotrophs exactly in the same way in terms of stoichiometry and kinetics. In the modeling context the term "fermentation" is defined as the process in which S_F is transformed to S_A and is introduced to describe the possible selective preference of PAOs for certain substrates. Fermentation in ASM 2 does not exactly imply its microbiological meaning, as discussed later.

ASM 2d (6) is an extended version of ASM 2. It has been shown that some PAOs have denitrification capability (35). In other words, some PAOs can utilize nitrate or nitrite as the electron acceptor, and so they take up orthophosphate with a consumption of nitrate or nitrite under oxygen-free conditions. To describe this anoxic uptake of orthophosphate, this denitrification capability is attributed to PAOs in ASM 2d ("d" stands for "denitrification") (6). PAOs with denitrification capability (DN-PAOs) are very common in EBPR systems (35,36), so without the introduction of DN-PAOs, the EBPR processes incorporating recycling of nitrified liquid cannot be modeled properly. When nitrification is significant in the system, ASM 2d not ASM 2 should be used for its simulation. An anoxic reduction factor (the factor expressing the ratio of the anoxic growth rate to that of the aerobic) is again introduced to account for the reduced growth rate under the anoxic conditions (6).

Hydrolysis

Hydrolysis in the IWA models is defined as the process in which complex molecules not metabolized by microorganisms (SB-COD) are degraded to relatively simple molecules that can then be assimilated and metabolized directly by heterotrophic bacteria (RB-COD) (4,5). Hydrolysis is not an oxidation-reduction reaction and theoretically should not involve any electron transfer. However, the hydrolysis process in the model may include not only the real "biochemical" hydrolysis caused by hydrolytic exoenzymes, but also processes such as predation by higher animals or any other processes contributing to breakdown of polymers to low-molecular compounds. In addition, carbon storage may be strongly correlated with the hydrolysis process. As modeled in ASM 3, external substrates can be polymerized and stored inside the cell when they are utilized by microorganisms. Subsequent breakdown of these internal storage products to smaller metabolic intermediates cannot be distinguished from the extracellular hydrolysis of SB-COD in the oxygen balance–based models, because neither upsets the oxygen balance.

In ASM 2, the hydrolysis rate is considered to be greatest under aerobic conditions, medium under anoxic (denitrifying) conditions, and lowest under anaerobic conditions (5). If the same hydrolysis rate is applied under anaerobic, anoxic, and aerobic conditions, too much S_S is supplied through hydrolysis and too much denitrification (under anoxic conditions) or phosphate release (anaerobic conditions) emerges from the model compared with that actually observed in the AS systems. Differences in the hydrolysis rates under different electron acceptor conditions are allowed for by introducing anoxic and anaerobic hydrolysis reduction factors. As true hydrolysis is not an oxidative or reductive process, the availability of electron acceptors should not affect the process rate. However, several possible factors may contribute to the apparent reduced hydrolysis rates under anoxic or anaerobic conditions in the model as follows:

1. The activity of predators (protozoa and higher animals) may be less under anaerobic and anoxic conditions than under aerobic conditions.
2. Carbon storage may take place under any of these conditions, but the utilization of the storage products should be more under aerobic and anoxic conditions than under anaerobic conditions.

These may lead to an underestimation of the hydrolysis rate in the model. The hydrolysis rate in ASM 2 is one of the most difficult parameters to estimate. This problem has now partly been solved by the introduction of the storage process in ASM 3, where this anoxic or anaerobic hydrolysis reduction factor is no longer used (7).

Municipal wastewaters usually contain organic nitrogen compounds. After their hydrolysis, ammonia nitrogen is released. In the ASM models, it is assumed that SB-COD should include a nitrogen fraction and that ammonia is released when SB-COD is hydrolyzed to RB-COD. This approach is extended to other COD fractions. Once the nitrogen content of each COD fraction is determined experimentally, the model describes the behavior of organic nitrogen compounds quite well. The same philosophy is also applied to describe the behavior of phosphorus bound to organic matters, namely, each COD fraction include a phosphorus fraction.

Fermentation

The process "fermentation" was introduced in ASM 2 to describe EBPR metabolism, and is different from fermentation in the microbiological sense. In ASM 2, a fraction of RB-COD utilized by PAOs (S_A) is distinguished from the rest of RB-COD that cannot be directly taken up by PAOs (S_F). The conversion of the latter to the former is defined as fermentation. The process is called fermentation, because compounds that are likely to be classified as S_A include fermentation products such as acetate and other short-chain fatty acids.

In a microbiological context, fermentation is a growth generating process. However, fermentation in ASM 2 is not modeled as such, but it is assumed to be a conversion process in which S_F is simply converted to S_A without any production of biomass. There are two major reasons for this assumption. First, the biomass yield in fermentative growth processes is small. Second, two yield values for the same heterotrophs (X_H), one for aerobic and anoxic growth processes and the other for fermentation processes, are confusing in the modeling context.

Decay, Lysis, and Endogenous Respiration

When microorganisms are put into an environment in which no substrates are available, a gradual decrease in biomass level will occur. Several possible mechanisms can be proposed to account for this biomass reduction. They include decay or lysis and endogenous respiration (37). Conceptual schemes of decay and endogenous respiration are given in Figure 5.

The term "decay" may be used to describe degradation of biomass in general, in which the biomass is degraded and converted either to substrates that are then reutilized by heterotrophic biomass or to inert organic substances that are biologically inactive. Decay may occur partly because of activities of lytic enzymes produced by the biomass and partly because of external factors such as mechanical or physical stress, chemical attack, or predation activities (37). The indigenous enzymatic decay is often called lysis. When the biomass reduction is modeled allowing for this decay, the decay rate does not correspond to the biomass reduction rate, because some biomass is regenerated by the secondary substrates produced by the decay process itself. The first two IWA models (ASM 1 and 2) adopt this approach. However, the complex nature of this decay-regeneration model structure often leads to difficulties in evaluating relevant kinetic parameters. Therefore, ASM 3 adopts a different approach by incorporating endogenous respiration. In this approach, it is assumed that the biomass is continuously oxidized to carbon dioxide under aerobic or anoxic conditions, thus generating the necessary energy for endogenous metabolism or for cell maintenance. The essential advantage of this endogenous respiration approach is its simplicity. No regeneration of substrate is considered and the endogenous respiration rate then directly expresses the biomass reduction rate. On the other hand, biomass reduction under anaerobic conditions cannot be described by this approach because no inorganic electron acceptors are available under anaerobic conditions. In reality, the decay rate seems to be very small and can be usually neglected under anaerobic conditions. However, hydrolytic decay can theoretically occur even under anaerobic conditions and its significance in actual wastewater treatment processes still remains unclear.

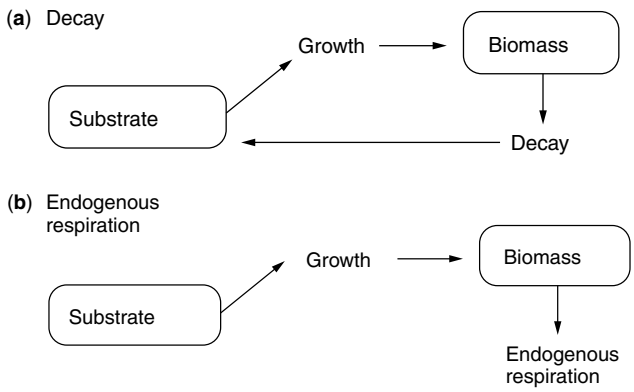

Figure 5. Conceptual scheme of decay and endogenous respiration.

Carbon Storage

It has been pointed out that the carbon storage capability of microorganisms plays a key role in the ecological selection in biological wastewater treatment (38). Microorganisms in AS are exposed to carbon-rich and -deficient conditions alternatively. For example, either spatial or temporal gradients of substrate concentration are formed in many AS reactors, such as sequence batch reactors, plug flow reactors, systems with selector, and contact-stabilization processes (39). Also, carbon loadings from influent wastewater tend to fluctuate dynamically from time to time. These dynamic conditions, in terms of the availability of carbon sources, will create highly competitive environments, in which substrate uptake rates during the carbon-rich periods will actually determine the survival and proliferation of certain microorganisms (39,40). So, some microorganisms adopt a strategy to store carbon sources in the cell under carbon-rich conditions by achieving a high substrate uptake rate. They use this stored carbon sources under carbon-deficient conditions. In fact, PAOs have the capability to store PHA under anaerobic conditions, and this explains their dominance in anaerobic-aerobic EBPR systems (25). This strategy for carbon storage seems to be adopted by aerobic or anoxic heterotrophs as well, because storage seems to be an essential capability of microorganisms to outcompete others for substrates under dynamic conditions (39). Therefore, the process of carbon storage is introduced in ASM 3 (7).

In ASM 3, a single heterotrophic biomass (X_H) is defined as being responsible for carbon and nitrogen removal, and this X_H stores RB-COD (S_S) in the form of cell internal storage products (X_{STO}). It is assumed in the stoichiometry of ASM 3 that X_{STO} is exclusively PHB. PHA, including PHB, are also likely to be major storage products, and possibly other polymers, such as lipids, glycogen, and polypeptides, exist (16). At present, little information is available about the presence, importance, and characteristics of these carbon storage products in AS. The storage process is modeled as an energy-requiring process, and the ratio of utilized S_S to stored X_{STO} is defined as the yield for the storage process. This yield should be much higher than yields of growth processes, because the energy requirement for storage is much smaller than that for growth (41). Storage occurs both under aerobic and anoxic conditions (36), and anoxic storage is identical to aerobic storage, but denitrification rather than aerobic respiration provides the energy required. As more energy is produced in aerobic respiration than in denitrification, a higher yield is applied to aerobic storage than to anoxic storage.

An assumption that may not be true in the real world but is incorporated into the models, is that all S_S taken up by X_H is first converted to and stored as X_{STO}, which is then utilized by X_H as the substrate for growth. This means that X_H accumulate and utilize X_{STO} at the same time during their growth. Heterotrophs utilize external substrates not necessarily via storage products but directly for growth. However, it is not wise, during modeling, to provide two heterotrophic growth processes (one on S_S and the other on X_{STO}), because more kinetic parameters

must then be identified. To avoid such a complexity in the model structure, the foregoing assumption is made in ASM 3.

LIMITATIONS AND FUTURE PERSPECTIVES OF AS MODELS

It is important to note that microbiological information is not always incorporated into these AS models. There are many processes that are known to microbiologists but that have not yet been introduced to IWA models for several reasons. It is assumed by the AS models that processes with engineering significance should be modeled. In other words, only those processes that affect the concentrations of system components relevant to practical objectives should be considered. Thus, the activities of absolute anaerobic bacteria, for example, are neglected in the IWA models, despite the fact that various strict anaerobic bacteria, such as sulfate-reducing bacteria, can be detected even in fully aerobic AS processes (42). However, their activities are usually considered to be negligible in terms of the COD balance and they are not introduced in the AS models. As their metabolisms are well understood, it would be relatively simple to model them if necessary.

As far as nitrogen removal is concerned, the model structures are far behind the microbiological information, and so the following are not considered in ASM 2 or other IWA models, as discussed previously.

1. Nitrite production, either through nitrification or denitrification, is not taken into account. Nitrite accumulation and its inhibitory effects on microbial activities are not predicted.
2. N_2O production is not considered. It is known that under certain conditions part of nitrogen can be released to the air as N_2O in the courses of both nitrification and denitrification (22). This leads to additional loss of nitrogen from the system, and to an incomplete mass balance of nitrogen, unless it is taken into account.
3. There are several other nitrogen-related metabolic reactions, such as those involved in anaerobic ammonia oxidation known as ANAMMOX (43), in which ammonia is oxidized by nitrite to produce nitrogen gas, autotrophic denitrification (44) (denitrification by inorganic compounds as electron donor), and so on. They will disturb the nitrogen balance, but are not considered at all in the IWA models, because it is considered unlikely that these metabolisms are significantly active in the AS systems.

Nitrite production during nitrification is more common than that in denitrification, and more microbiological and kinetic information is available about the former than the latter. Therefore, nitrite production during nitrification can be modeled satisfactorily and such models may be applied to situations in which nitrite production through denitrification is not significant.

Bulking is a situation in which AS does not settle in the secondary clarifier because of excessive growth of filamentous organism (45). This is a serious and critical problem in the operation of the AS process, because solutions to it have not yet been established in practice. From a practical point of view, there is a high demand for models to describe the bulking phenomena, but widely accepted bulking models have not been established yet. The main reason is that the mechanism of bulking is not clearly understood. Many different filamentous organisms can cause bulking and each of them may have different microbiological and kinetic natures (46). It is also very difficult to quantify filamentous biomass reliably. The conditions responsible for bulking are not always known. All these difficulties lead to lack of definite information about the mechanism of bulking, and thus to lack of acceptable bulking models. It is essential to obtain more detailed microbiological and kinetic information on different causative filaments to establish reliable bulking models.

Predation by protozoa or higher animals commonly occurs in the AS process (see PROTOZOA IN ACTIVATED SLUDGE). Predation is not included in the IWA models but may contribute to decay and hydrolysis in the modeling context. Several different types of predators are present in the AS, and some of their characteristics have been studied. They can be modeled relatively easily from a structural point of view. However, predator populations cannot be quantified easily, and thus identification of related parameters may be difficult.

Soluble microbial products (SMP) are often considered as important COD fraction in wastewater treatment systems (47). When decay or lysis of the whole cell or a part of the cell occurs, secondary substrates will be released into the bulk solution. This process is modeled as decay or lysis process in ASM 1 and 2. It is assumed in ASM 1 and 2 that the biomass is degraded to SB-COD and inert COD. This means that the secondary substrates produced through decay or lysis are exactly the same as other SB-COD fractions from a modeling perspective. This is not likely: secondary substrates produced by the biomass will almost certainly have different kinetic characteristics. Therefore, SMP should be defined as a separate component representing the secondary substrates produced by the lytic activities of the cells. Contribution of the SMP to the COD balance is more significant in biofilm systems than in AS systems, and SMP is sometimes introduced in the modeling of the biofilm process (47).

It is easy to allow a model to become more complex based on microbiological information. For example, we have only one heterotrophic biomass in ASM 1 and 3, but this can be divided into many different heterotrophic groups, including those with and without denitrifying capability or with and without storage capability. One may think that such a multiple heterotroph model should have a higher capability to describe more comprehensive situations, but this is not always true. Parameter identification becomes more difficult as the complexity of the model increases, and the predictability of models may decrease. As stated before, it is evident that cell internal glycogen plays an essential role in the EBPR metabolism, but glycogen is not incorporated into ASM 2 so that the structure of the model may not be too complicated for practitioners. In the practical application of the AS model, most of the PAO

behavior can be described with only one of the two storage polymers: poly-P or glycogen. It is reasonable, therefore, that poly-P, which can be easily understood by wastewater engineers, is adopted in ASM 2 to reduce its complexity. However, glycogen in PAOs is sometimes essential and critical if the AS models are to be used for scientific purposes. In fact, several AS models with glycogen have been proposed (48).

The concentration of hydrogen ion (H^+) or pH is not considered as a state variable in the IWA models. This is simply because pH is not exactly predictable. To describe the behavior of pH (H^+), it is necessary to be able to model the acid-base equilibrium. However, many chemical species can affect this in wastewater or in the AS systems in general. It is impossible to include all these species into the model. On the other hand, the acid-base equilibrium is well understood theoretically and it is easy to create a pH prediction model once the species that are involved in the this equilibrium are identified. For example, the carbonate equilibrium can be introduced into the AS model to predict pH. In oligotrophic water environments, the carbonate equilibrium actually determines the pH in the system. So, a carbonate-based pH prediction module of the AS model can be developed as a first step. For this purpose, ASM 3c (49), an adapted version of ASM 3, where organic components are expressed in terms of organic carbon rather than COD, may be useful. If carbon is used as the basis for mass balance, carbon dioxide should be accounted for in this balance and the pH prediction model based on the carbonate equilibrium will become realistic. Because pH is a very important factor affecting microbiological activities, the development of AS models containing the effect of pH will be useful both from a scientific and a practical point of view.

CONCLUSION

In the present article, various microbiological issues associated with the IWA AS models are discussed in terms of modeling. The IWA models have been developed on the basis of evidence accepted by the majority of microbiologically oriented wastewater engineers. Therefore, the IWA models are not the most advanced AS models, but rather give a fundamental and common basis for the further development of revised, detailed, or innovative models. Extension and improvement of the present models will surely be made according to practical demands in the future.

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ACTIVATED SLUDGE — MOLECULAR TECHNIQUES FOR DETERMINING COMMUNITY COMPOSITION

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Wastewater treatment is one of the most important biotechnological processes that is used worldwide to treat polluted sewage and to ameliorate anthropogenically induced damage to the environment. Depending on the treatment goals, different types of sewage treatment plants are used (see ACTIVATED SLUDGE — THE PROCESS). This article focuses on the microbiology of the activated sludge process, which is most commonly used and in which the microbial biomass is aerated and kept in suspension during the treatment process. In addition, we also cover activated sludge and biofilm nutrient removal plants in which anaerobic and aerobic treatments are combined to allow for complete nitrogen and/or biologically enhanced phosphorus removal (EBPR). It is common knowledge that in all types of wastewater treatment plants (wwtps), prokaryotic microorganisms dominate and represent the "causative agent" responsible for the observed conversions. On the other hand, certain microorganisms cause the most frequently encountered problems in wastewater treatment (see FILAMENTOUS BACTERIA IN ACTIVATED SLUDGE: CURRENT TAXONOMIC STATUS AND ECOLOGY). Thus, the efficiency and robustness of a wastewater treatment plant mainly depend on the composition and activity of the microbial communities present in its different stages (see ACTIVATED SLUDGE — THE MICROBIAL COMMUNITY). Although biological wastewater treatment has been intentionally used for more than a century, because of methodological limitations, knowledge on the microbiology of this process was scarce until a decade ago. Consequently, these microbial communities were considered as a "black box," and progress in the design and control of plants was derived mainly from empirical research in civil and process engineering. Only after the introduction of molecular techniques in microbial ecology, it has become possible to determine the composition and dynamics of microbial communities in these systems and to identify the microbial key players for the different process types. It is the aim of this article to review these new insights and to provide some guidance on how this knowledge could be extended by implementing newly developed methods and be used for future improvement in wastewater treatment.

METHODS FOR MICROBIAL DIVERSITY ANALYSIS IN WASTEWATER TREATMENT PLANTS (WWTPS)

This article briefly summarizes established methods for microbial community analysis. It is not the goal to provide an encompassing overview on technical details of each method but rather to discuss the advantages and limitations of each approach for its use in wastewater treatment microbiology.

Light Microscopy and Cultivation

Traditionally, two approaches to investigate the microorganisms in wwtps were applied. Most frequently, obtained samples were analyzed by standard light microscopy to obtain an overview of the abundance of floc-forming and filamentous bacteria. Because of the importance of the latter group of organisms for sludge bulking and foaming (Activated sludge — bulking; Activated sludge — foaming), keys were developed for a provisional identification of filamentous bacteria using (*1*) their reaction to gram- and Neisser-staining and (*2*) morphological characteristics (1,2). However, recent molecular approaches revealed that microorganisms affiliated with different bacterial domains are identified as the same filament type using these keys (see FILAMENTOUS BACTERIA IN ACTIVATED SLUDGE: CURRENT TAXONOMIC STATUS AND ECOLOGY). Furthermore, polymorphism of certain filaments has been described (3), which further complicates morphology-based identification.

The second approach is based on cultivation and isolation of bacteria from wwtps. The number of active bacteria can be estimated by most probable number (MPN) techniques and/or total plate counts. After isolation, bacteria are identified using either physiological parameters or chemotaxonomic markers such as cellular fatty acids or respiratory quinone profiles. The latter two biomarkers can also be used directly for profiling activated sludge microbial communities (4–6) but provide only relatively low-resolution information. Numerous bacteria were isolated and identified using cultivation-based techniques, leading to the perception that, for example, pseudomonads, enterobacteriaceae, and acinetobacters are key components of the microbiota of these systems (see ACTIVATED SLUDGE — THE MICROBIAL COMMUNITY). After the introduction of molecular techniques for community analyses it became obvious that only less than 16% of the microorganisms can be isolated from activated sludge by standard cultivation and that those bacteria forming colonies on the respective media are generally of minor numerical importance in situ (7–10). Therefore, cultivation offers a very biased and incomplete view of the bacterial diversity there. On the other hand, directed cultivation of in situ important microorganisms is still a prerequisite for their encompassing physiological and genetical analysis.

Immunofluorescence

A more direct identification and quantification of bacteria is offered by the use of fluorescent antibodies (FA). However, the production of these antibodies, generally, still requires the prior isolation of the target organism, restricting the method to culturable bacteria. Once available, the FA technique can be used to identify and quantify defined microorganisms within activated sludge or biofilms. For example, *Sphaerotilus natans*, *Acinetobacter* sp. (11), and *Thiothrix* sp. have been detected by this technique (12). The specificity of antibodies cannot be adjusted and is usually below the species level. This is an advantage if, for example, the fate of specific strains should be monitored. On the other hand, the high serological diversity of many bacterial genera and even species (13,14) would require the simultaneous use of a huge number of FA for their detection, thus rendering the method cumbersome. Furthermore, FA are relatively large molecules and thus do not easily penetrate through dense extracellular polymeric substances of flocs and biofilms, although this problem can be avoided if the FA technique is applied to cryosections. Another difficulty of the FA technique is that it often suffers from a high background fluorescence caused by unspecific binding of the FA to, for example, detritus (14,15) and filament sheaths. In addition, the expression of the detected epitopes on the cell surface can vary with changing environmental conditions, a fact that can cause false-negative results. Despite these limitations, the FA technique has the unique advantage that detection does not require the killing of the cells. Thus, antibodies can be used to enrich target cells, for example, via flow cytometry, coated microtiter plates, or magnetic beads for subsequent cultivation.

The 16S rDNA Approach

The comparative sequence analysis of environmentally retrieved 16S rDNA sequences has become the gold standard for cultivation-independent assessment of bacterial diversity in natural and engineered systems (16). Current 16S rDNA databases contain more than 20,000 entries (17) and thus provide a high-resolution framework for the assignment of those sequences obtained in 16S rDNA libraries from environmental diversity surveys (18). The approach consists of DNA extraction, subsequent PCR amplification of (a fragment of) the 16S rDNA gene using primers targeting regions conserved in the bacterial domain, cloning, and sequencing. The obtained sequences are analyzed together with adequate reference sequences to infer their phylogenetic affiliation. A meaningful phylogenetic analysis requires the use of almost full-length 16S rDNA sequences and of different treeing methods applied to different data sets (19).

As a result of the development of premanufactured kits (e.g., for cloning) and automated sequencers, the analyses of relatively high clone numbers per library has become possible. It is however important, but rarely done in published studies, to determine whether the number of analyzed clones does sufficiently well represent the diversity in the established library. For this purpose, the clones should be grouped into operational taxonomic units (OTUs) according to their 16S rDNA similarities with each other. We recommend to use a 97% similarity threshold for OTU assignment, because it has been demonstrated that two organisms with a lower 16S rDNA similarity also have DNA-DNA similarities below 70% and thus represent different genospecies (20,21). However, organisms with highly similar or even identical 16S rDNA sequence might nevertheless represent two different species (20), and thus the OTU concept leads to underestimation of the actual species richness. Once the clones have been assigned to OTUs, rarefaction analyses (22,23) or coverage estimates (24) should be performed to determine whether the analyzed number of clones represents a sufficient sample size. Figure 1 shows rarefaction analyses of 16S rDNA surveys of wwtps, the respective coverage estimates are depicted in Table 1.

The rarefaction analyses and coverage estimates show that most studies did not adequately harvest the diversity of their 16S rDNA libraries. This is understandable, because the number of identical or highly similar clone sequences increases with the number of sequenced clones and thus extensive clone sequencing creates highly redundant information. Therefore, screening of the 16S rDNA clones with fingerprinting techniques (discussed later) for selection of different clones for sequence analyses is recommended.

However, it is important to realize that even if the complete diversity of an environmental 16S rDNA clone library is harvested, the obtained species inventory might not represent the naturally occurring diversity. In other words, not all, and sometimes even not all, numerically important bacterial populations of an environmental sample will be found in a respective 16S rDNA library. This failure might be caused by (*1*) inefficient DNA extraction (27) (e.g., certain gram-positive bacteria are difficult to lyse), (*2*) inadequate coverage of the selected PCR primers, (*3*) kinetic and stochastic biases introduced by the PCR amplification (28–30), and (*4*) cloning biases. For example, the high coverage but low overall OTU number obtained in the 16S rDNA library analysis of activated sludge from a municipal high-load wastewater treatment basin (31) does not necessarily reflect low species richness but was probably caused by the omission of a dedicated DNA-extraction protocol before PCR amplification.

For establishing a most representative 16S rDNA library, one might consider to (*1*) use different DNA-extraction techniques and to combine the isolated nucleic acids before PCR amplification, (*2*) to use more than one primer set for amplification, and (*3*) to use different vectors for cloning. Regarding the PCR conditions best suited for a general diversity survey, extremely stringent annealing conditions should be avoided to allow binding of the primers to organisms with nucleotide exchanges in the target position. This procedure might lead to the amplification of undesired PCR products and the necessity to purify the expected PCR amplificate by agarose gel electrophoresis and band excision before cloning. Furthermore, the PCR cycle number has a profound

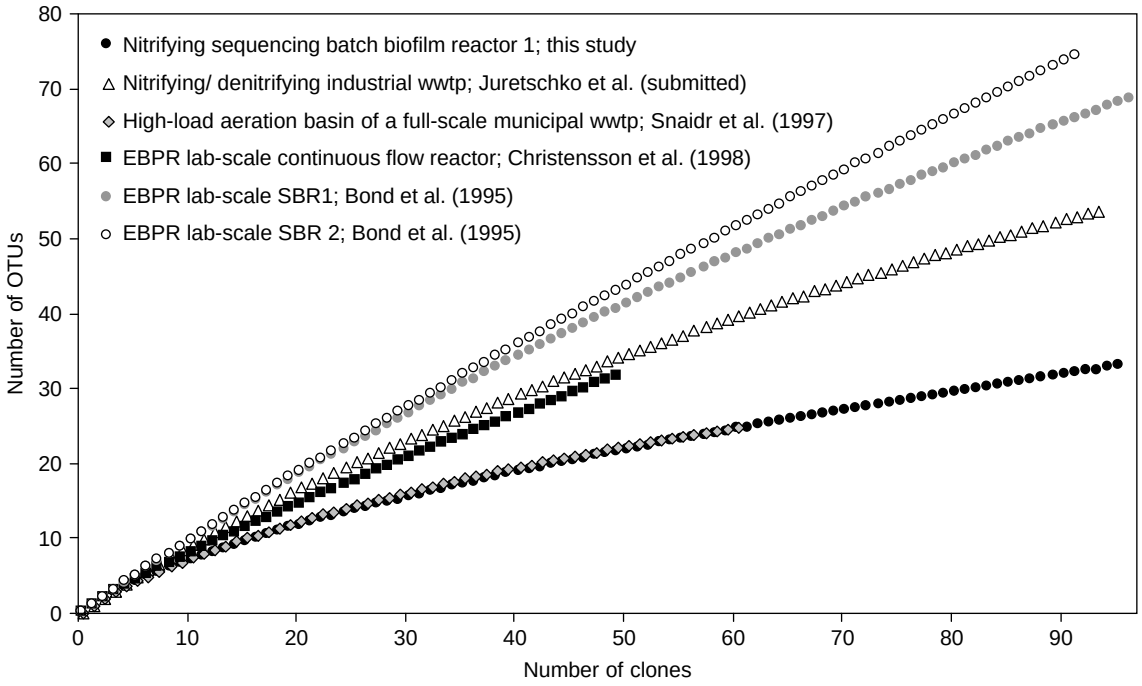


Figure 1. Rarefaction analyses of 16S rDNA inventories of different wwtps. A 97% similarity threshold was used for assignment to an operational taxonomic unit (OTU). The studies of Liu and coworkers (25) and Dabert and coworkers (26) were not considered because only one sequence per OTU has been deposited in public databases.

influence on the composition of the PCR amplificate if complex template mixtures are used (29). If high cycle numbers are used, highly abundant and less abundant organisms are more likely to be represented in comparable numbers in the PCR product, an effect caused by template reannealing at high concentrations (29). In contrast, the 16S rDNA composition of PCR products generated with low cycle numbers more accurately represents the composition of the 16S rDNA sequences in the isolated nucleic acids.

Additionally, the 16S rDNA approach can also indicate or even create artificial diversity. Small sequence differences among multiple rRNA operons of a single bacterium, the so-called microheterogeneities (32), might be interpreted after amplification and cloning as microdiversity, which, however, does not exist at the organism level. Furthermore, the formation of chimeric sequences via in vitro recombinations (33,34) and the introduction of point mutations via the thermostable DNA polymerase (35,36) leads to the retrieval of sequences that differ from their natural counterparts. Although chimeric sequences composed of distantly related sequence fragments can be recognized easily by standard software tools (37) or comparative phylogenetic analyses of different sequence regions (27), it is virtually impossible to identify chimeras among closely related organisms. Polymerase-induced mutations can only be detected with a certain probability in highly conserved sequence regions by database comparison.

In essence, the 16S rDNA approach described earlier is an essential tool for microbial diversity analyses in wwtps. However, because of the numerous biases inherent to this approach quantitative data on the microbial community composition can only be obtained if it is combined with quantitative dot blot or in situ hybridization techniques, which are described later.

16S rDNA-Based Fingerprinting Techniques

16S rDNA-based fingerprinting techniques nicely amend the 16S rDNA approach described earlier. The common principle of these methods is to separate PCR products of the same length but different sequence to visualize the diversity within the amplificate by a banding pattern. Many of these techniques were invented to analyze mutations in medical research and were later adapted to environmental microbiology. The most frequently applied fingerprinting technique in wastewater microbiology is the denaturing gradient gel electrophoresis (DGGE) (6,38–40), but also terminal restriction fragment length polymorphism (T-RFLP) (41), gelretardation (42), and single strand conformation polymorphism (SSCP) (43,44) have been used. The main advantage of the fingerprinting techniques is that high sample numbers can be processed in a relatively short time to gain an overview of the dynamics of complex microbial communities in wwtps. DGGE and gelretardation bands of interest can be excised, cloned, and sequenced for subsequent identification. DGGE offers a higher resolution compared with gelretardation but is, in contrast to the latter method, limited to fragments smaller than 500 nucleotides that are not well-suited for phylogenetic analyses. The organism represented by a T-RFLP or SSCP band can only be identified if a 16S rDNA clone library is also established, and if the obtained clones are sequenced

Table 1. Summary of 16S rRNA-Based Diversity Surveys of Wastewater Treatment Plants and Reactors. EBPR = Enhanced Biological Phosphorous Removal. SBR = Sequencing Batch Reactor. SBBR = Sequencing Biofilm Batch Reactor. Clones Were Assigned to Different Operational Taxonomic Units (OTUs) If They Shared Less Than 97% 16S rDNA Sequence Similarity with Each Other

	No. of			Bacterial Divisions[i]																		
				Proteobacteria[h]																		
WWT Plants and Reactors	Clones Sequenced	OTUs	Coverage C[j] [%]	α	β	γ	δ	ε	*Cytophagales*	*Acidobacterium*	Low-G + C Gram positives	High-G + C Gram positives	*Nitrospira*	*Verrucomicrobia*	*Planctomycetes*	Green-sulfur	Green-non-sulfur	*Fibrobacter*	*Fusobacteria*	OP11	Unaffiliated
High-load aeration basin of a full-scale municipal plant[a]	62	25	77	3	52	18		15			10			2			2				
Nitrifying/denitrifying industrial plant[b]	94	53	64	26	31		2			5	1	1	2	3	12	1	16				
Nitrifying SBBR1[c]	96	33	78	5	51	22	4	1	2	5		1	8								
EBPR lab-scale SBR1 + sodium acetate[d]	97	69	48	13	33	8	3	2	5	3	1	4	3	1	13		4		3		3
EBPR lab-scale SBR2 unsupplemented[d]	92	75	28	17	25	10	1	7	13	7		2	2	1	9		3		2		
EBPR lab-scale continuous flow reactor[e]	51	30	51	16	8	8	4	4	6	2		37			8	2	6				
EBPR lab-scale SBR[f]	92	50	46	4	17	5	3		39	9		4	3	4	3	3				1	
EBPR lab-scale reactor[g]	150	16	93	5	14	7	1		50		5	9						9			

[a] Snaidr et al., (1997);
[b] Juretschko et al., (submitted);
[c] This study;
[d] Bond et al., (1995);
[e] Christensson et al., (1998);
[f] Dabert et al., (2001);
[g] Liu et al., (2001);
[h] *Proteobacteria* are present at the subdivision level because of the extensive representation of this division.;
[i] Relative incidence of division-level representatives in respective studies,
[j] Coverage was calculated according to the formula $C = 1 - (n1 \times N^{-1}) \times 100\%$, n1 = No. of OTUs consisting of only one sequence, N = No. of all sequences in the 16S rDNA clone library.

for identification and are used also as references in the respective fingerprinting protocol. In general, fingerprinting techniques are affected by the same DNA-extraction and PCR-amplification biases as the 16S rDNA approach and thus cannot provide quantitative data.

Hybridization Techniques Using rRNA-Targeted Probes

Dot blot and in situ hybridization techniques using rRNA-targeted oligonucleotide probes allow us, in contrast to all other methods discussed in this article, to quantitatively determine the composition of complex microbial communities. The specificity of the probes can be adjusted to different phylogenetic levels. Specialized software tools have been developed to assist in probe design and evaluation (17). After design, for each probe optimal hybridization conditions have to be carefully determined using target and nontarget microorganisms. This evaluation requires different procedures for the dot blot (45) and in situ format (46,47). Today a considerable number of ready-to-use domain-, division-, genus-, and species-specific probes are available for identification of the respective target microorganisms within their natural habitat (for a review see Amann and coworkers (16,48)). For high-resolution analyses of a certain environmental sample, additional probes must be designed against cloned sequences obtained with the 16S rDNA approach. Application of these probes in the dot blot or in situ format allows the detection and quantification of the corresponding microorganisms within the habitat for which the 16S rDNA library was established. The complete procedure including the 16S rDNA gene library analysis, clone-specific probe design, and quantitative hybridization experiments is referred to as the *full-cycle rRNA approach* (16).

In the dot blot format, total RNA is extracted from the environment and is immobilized on a membrane together with dilution series of RNA of reference species. Reference rRNA of uncultured microorganisms can be obtained by in vitro transcription of the respective cloned 16S rDNA sequences. Subsequently, the membrane is hybridized with a radioactively labeled probe, and after a stringent wash step the amount of target rRNA is quantified with a Phospho-Imager. Results are expressed as ng of target rRNA per weight or volume of the environmental sample. Alternatively, the membrane can be rehybridized with a bacterial or universal probe, and the amount of population-specific rRNA detected with the specific probe is expressed as fraction of the total (bacterial) rRNA. For details of the method the reader is referred to Raskin and coworkers (49). Quantitative dot blot hybridization measures the abundance of the target population via its rRNA content. Because the rRNA content of a microbial cell varies with its activity and physiological history (50), and prevailing environmental conditions, the obtained data cannot be translated to cell numbers. Furthermore, the method might be biased by a varying efficiency of RNA-extraction from different microbial populations and degradation of the rRNA (which might affect different probe target sites in a different way) during the procedure. Quantitative dot blot analyses have been used in wastewater microbiology (45,51–53) but are more widely applied on environments such as soil and microbial mats (54,55), which because of their high background fluorescence are not well-suited for fluorescence in situ hybridization (FISH).

In situ hybridization with fluorescently labeled, rRNA-targeted oligonucleotide probes is perfectly suited to identify the target organisms within activated sludge flocs or biofilms by specific staining. For this purpose, samples are pretreated with chemical fixatives to kill the cells, to preserve their morphology, and to make them accessible to oligonucleotide probes. For gram-negative bacteria, a paraformaldehyde-based fixative fulfills these requirements and allows storage of the samples at $-20\,^{\circ}C$ for several years (16). However, a fixation protocol permeabilizing all gram-positive bacteria still has to be developed. Many, but not all, gram-positive bacteria can be permeabilized efficiently for FISH by addition of 50% EtOH (56). Others, for example, the mycolata containing mycolic acids in their cell walls, require additional or alternative pretreatments including the use of cell wall lytic enzymes (57–59). Fixed samples are hybridized with probes labeled with fluorescent dyes. For environmental studies, the dyes FLUOS 5 (6)-carboxyfluorescein-N-hydroxysuccimide ester: green fluorescence), Cy3 (orange fluorescence), or Cy5 (infrared fluorescence) are well-suited and permit the specific detection of the target cells with up to three simultaneously applied probes (60). If more than one probe is used in a hybridization experiment and if the probes require different conditions to ensure specificity, subsequent hybridization reactions from high to low stringency have to be performed (3). In contrast to dot blot hybridization, simultaneous binding of multiple probes to the same target cells can be proven by FISH through microscopic observation of the hybridized cells and the use of different probe labels. Therefore, sets of probes with hierarchical or identical specificity can be used to increase the reliability of the identification.

For many years, quantitative FISH data on the bacterial community structure of activated sludge or biofilms required microscopic counting of stained cells, a time-consuming procedure that is relatively inaccurate in samples containing densely clustered cells. Generally, the abundance of a certain population of interest is expressed as the percentage of all cells that can be stained with either bacterial probes (7,47) or a DNA-binding dye such as DAPI (61). The combination of FISH and confocal laser scanning microscopy (62) not only dramatically improved the image quality by exclusion of blurring out-of-focus fluorescence, but also allowed the development of semiautomatic quantification protocols by use of digital image analyses (42,63). These methods measure the specifically stained biovolume of a target population and refer it to the volume of those microorganisms stained by the bacterial probe set or a DNA-binding dye. Although using the manual technique only a few hundred or thousand cells are counted, the semiautomated protocols detect more than 100,000 cells per measurement and thus are much more reliable and reproducible.

However, it is important to realize that numbers obtained by this technique or its manual counterpart cannot be used directly to compare the abundance of detected bacterial populations among different samples, because differences in biomass among the samples are not considered. Thus, for intersample comparison, the respective numbers need to be normalized by taking into account biomass differences estimated, for example, via volatile suspended solids (VSS) determinations (64) or total DAPI counts per sample volume on a membrane filter (8).

Confocal laser scanning microscopic analyses of FISH results may also be used to investigate the spatial distribution of microorganisms in activated sludge flocs and biofilms and to study colocalization of microbial populations (27,62,65). However, such analysis can only be performed with high accuracy, if the architecture of the flocs or biofilms is preserved by embedding (for embedding protocols see (66) or cryosectioning (65,67)) and is thus not destroyed during fixation and hybridization.

One potential disadvantage of FISH is its relatively high detection limit of approximately 10^3 to 10^4 target cells ml^{-1}. Furthermore, only cells with a ribosome content of more than about 1,000 copies can be detected by probes without additional signal amplification (68). Because microorganisms with a low cellular ribosome content are probably not active, this requirement does not hamper the in situ analysis of physiologically active cells in wwtps. This is also reflected by the fact that about 90% of all DAPI-positive cells can be detected with the bacterial probe set in a typical plant (47). On the other hand, it is frequently assumed that a high cellular ribosome content of a microbial cell leading to a bright FISH signal is an indicator of its physiological activity at the time of sampling. This interpretation can be misleading because nitrifying bacteria, for example, maintain high ribosome levels even after complete

inhibition for several days (14) or starvation for a month (69). More direct in situ analyses of the activity may be achieved by the use of oligonucleotide probes targeting the 16S/23S intergenic spacer region as demonstrated for *Acinetobacter* sp. (70) and the anaerobic ammonium-oxidizer *Candidatus* "Kuenenia stuttgartiensis" (71).

In summary, a variety of molecular methods is available for investigating microbial diversity in wastewater treatment plants. In particular, the full-cycle rRNA approach allows to precisely measure the composition and dynamics of microbial communities in these systems. The following section provides an overview on the current status of knowledge that has been accumulated by application of these research approaches in wastewater microbiology.

GENERAL MICROBIAL DIVERSITY IN WASTEWATER TREATMENT PLANTS

FISH with Group-Specific Probes

Group-specific rRNA-targeted probes (Table 2) have been widely applied in the so-called top-to-bottom approach for the investigation of activated sludge to obtain a fast but relatively low-resolution analysis of the respective microbial communities (7,8,10,25,72). These studies showed the dominance of *Proteobacteria* and revealed that the β-subclass is the most abundant subclass in these systems. α- and γ-subclass *Proteobacteria* are found in lower but generally still considerable numbers. Subsequently, the widespread distribution of members of the *Cytophagales* (8,73), the *Planctomycetales* (74), and the high-G+C gram-positive bacteria (8,25) in activated sludge was demonstrated. In addition, application of group- and genus-specific probes showed that the numerically important bacterial groups in activated sludge are dramatically underrepresented after cultivation and that certain bacterial groups with a low in situ abundance are best adapted to laboratory cultivation and thus are isolated most frequently. For example, γ-subclass *Proteobacteria* generally occur in relatively low numbers in activated sludge but clearly dominate the heterotrophic flora obtainable on nutrient rich agar plates (7–9).

16S rDNA-Based Diversity Surveys

The most detailed knowledge on species richness of microbial communities in wastewater treatment plants can be obtained by phylogenetic inventories via 16S rDNA clone libraries established using bacterial or universal primers. However, probably because of the tediousness of the method, such surveys have only been published for six treatment systems, including five laboratory scale reactors (25,26,75,76), and one high-load basin of a full-scale municipal wastewater treatment plant (31). Results of these surveys are summarized in Table 1. In addition to published results, we have included in this table two studies from our laboratory dealing with a pilot-scale nitrifying, sequencing batch biofilm reactor (partially published by Daims and coworkers (77)) and an intermittently aerated nitrifying/denitrifying industrial activated sludge plant connected to a rendering plant (78). It is obvious that the number of plants for which 16S rDNA libraries have been established is too low to provide an encompassing overview of the entire microbial diversity of such systems. Although several SBRs with enhanced biological phosphorus removal have been investigated, for the other plant types only single surveys were performed. Nevertheless, some general conclusions can be drawn already. Of the 36 divisions recognized for the bacterial domain (18), members of 13 divisions were detected in the surveys, indicating considerable microbial diversity in wastewater treatment plants. Consistent with the FISH results mentioned earlier, *Proteobacteria* are abundant in each library and represent more than 50% of the clones in five of the eight surveys. With one exception, β-*Proteobacteria* are the most frequently retrieved members of this division. Apart from the *Proteobacteria*, the *Cytophagales*, the Green-nonsulfur bacteria and the *Planctomycetes* were detected in significant numbers. One library of a plant designed for enhanced biological phosphorus removal in a continuous flow system is dominated by high-G+C gram-positive bacteria, consistent with the proposed importance of this group for phosphorus removal (discussed later) (8).

Fluorescence In Situ Hybridization and the Full-Cycle rRNA Approach

So far the microbial community structures of activated sludges from only two wastewater treatment plants have been investigated using the full-cycle rRNA approach. Snaidr and coworkers analyzed a high-load aeration basin of a large municipal wastewater treatment plant (31,60), whereas Juretschko and coworkers studied an intermittently aerated industrial wastewater treatment plant designed for simultaneous nitrification and denitrification (27,78). The results from the respective 16S rDNA clone libraries are depicted in Table 1. Snaidr and coworkers designed probes for a few selected clones and showed that a high microdiversity of bacteria of the beta-1 group of the β-subclass of *Proteobacteria* was present in the municipal activated sludge. Furthermore, *Sphingomonas*- and *Arcobacter*-related populations were detected with a relative abundance of 3 and 4% of all cells, respectively.

The composition of the microbial community in the industrial plant was investigated in more detail using semiautomatic quantitative FISH. Hybridization with group-specific probes demonstrated that the β-subclass of *Proteobacteria* made up almost half of the total biovolume of those bacteria detectable with the bacterial probe set (Fig. 2a). Other in situ important groups were the α-subclass of *Proteobacteria*, the *Nitrospira*-phylum, the *Planctomycetes*, and the green nonsulfur bacteria. Figure 2a also depicts differences between the in situ abundance of the groups and their representation in the clone library.

The composition of the β-subclass of *Proteobacteria*, the numerically most important group within this system, was further analyzed using OTU-specific probes for quantitative FISH (Fig. 2b). Bacteria related to *Zoogloea*

Table 2. Domain and Group-Specific rRNA-Targeted Probes Suitable for FISH Coverage of the Main Prokaryotic Lines of Descent by Group-Specific, rRNA-Targeted Oligonucleotide Probes. Sensitivity and Nontarget Hits of the Probes Were Determined by Using the Arb "Probe Match" Module on Current Arb Databases of Almost Complete 16S and 23S rRNA Sequences

Probe	Sensitivity[a] (%)	Nontarget Hits[b]	Reference	Target Group and Total Coverage (%)[c]
ALF1b	37.6	1,105	46	*α-Proteobacteria* 84.7
ALF968	76.6	132	79	
BET42a[d]	92.6	22	46	*β-Proteobacteria* 92.6
GAM42a[d]	90.8	45	46	*γ-Proteobacteria* 90.8
LGC A	41.1	9	59	Low-G+C gram-positive bacteria 52.1
LGC B	29.1	7	59	
LGC C	10.9	0	59	
DHP1006	100	0	80	*Synergistes* 100
TM7905	100	2	81	TM7 100
GSB532	75	0	82	Green sulfur bacteria 75
CFB286	49.3	0	83	*Cytophagales* 90.5
CFB563	27.2	0	83	
CFB719	31.1	4	83	
CFB972	27.2	96	83	
CFB1082	39.2	2	83	
CF319	41.9	28	73	
BAC303	33.1	0	73	
Fibro	100	0	84	*Fibrobacter* 100
Pla46	92.6	6	85	*Planctomycetales* 95.1
Pla886[d]	82.7	2,561	85	
EUB338-II[d,e]	75.3	1	47	
EUB338-III[d,e]	70	31	47	*Verrucomicrobiales* 70
Ntspa712[d]	85	0	77	*Nitrospira* 85
IRog1	38.4	1	86	*Acidobacterium/Holophaga* 44.2
IRog2	44.2	0	86	
HGC	82.5	10	56	High-G+C gram-positive bacteria 82.5
EUB338	90.4	0	87	domain *Bacteria* 91.8
EUB338-II	0.8	0	47	
EUB338-III	0.6	0	47	
CREN499	30.6	0	88	*Crenarchaeota* 30.6
EURY498	50	2	88	*Euryarchaeota* 50
Arch915	88.3	0	48	*Archaea* 88.3 domain *Archaea* 89.8

[a]The fraction of the sequences within the target group that have not more than 0.4 weighted mismatches to the probe sequence.
[b]The number of nontarget sequences that have up to 0.4 weighted mismatches to the probe sequence.
[c]Total coverage of the target group by a combination of all listed probes that are specific for this group.
[d]The probe specificity is improved by a competitor oligonucleotide as detailed in the publication cited.
[e]The probe targets parts of the specified group and additional bacteria, because it belongs to the *Bacteria*-specific probe set.

ramigera and the *Azoarcus/Thauera* cluster were the most abundant members of this subclass in situ, and accounted for 36% and 34% of the biovolume of the *β-Proteobacteria*. In addition, significant numbers of the ammonia-oxidizer *Nitrosococcus mobilis* (which was not present in the clone library), *Alcaligenes latus*-like bacteria, and *Brachymonas denitrificans*-related microorganisms were recorded. In total, only 6.5% of the bacteria of the *β-Proteobacteria* detectable in situ could not be assigned to a specific genus.

FUNCTIONAL GROUPS OF MICROORGANISMS IN TREATMENT PLANTS

From an applied perspective, identification and characterization of those bacteria responsible for specific

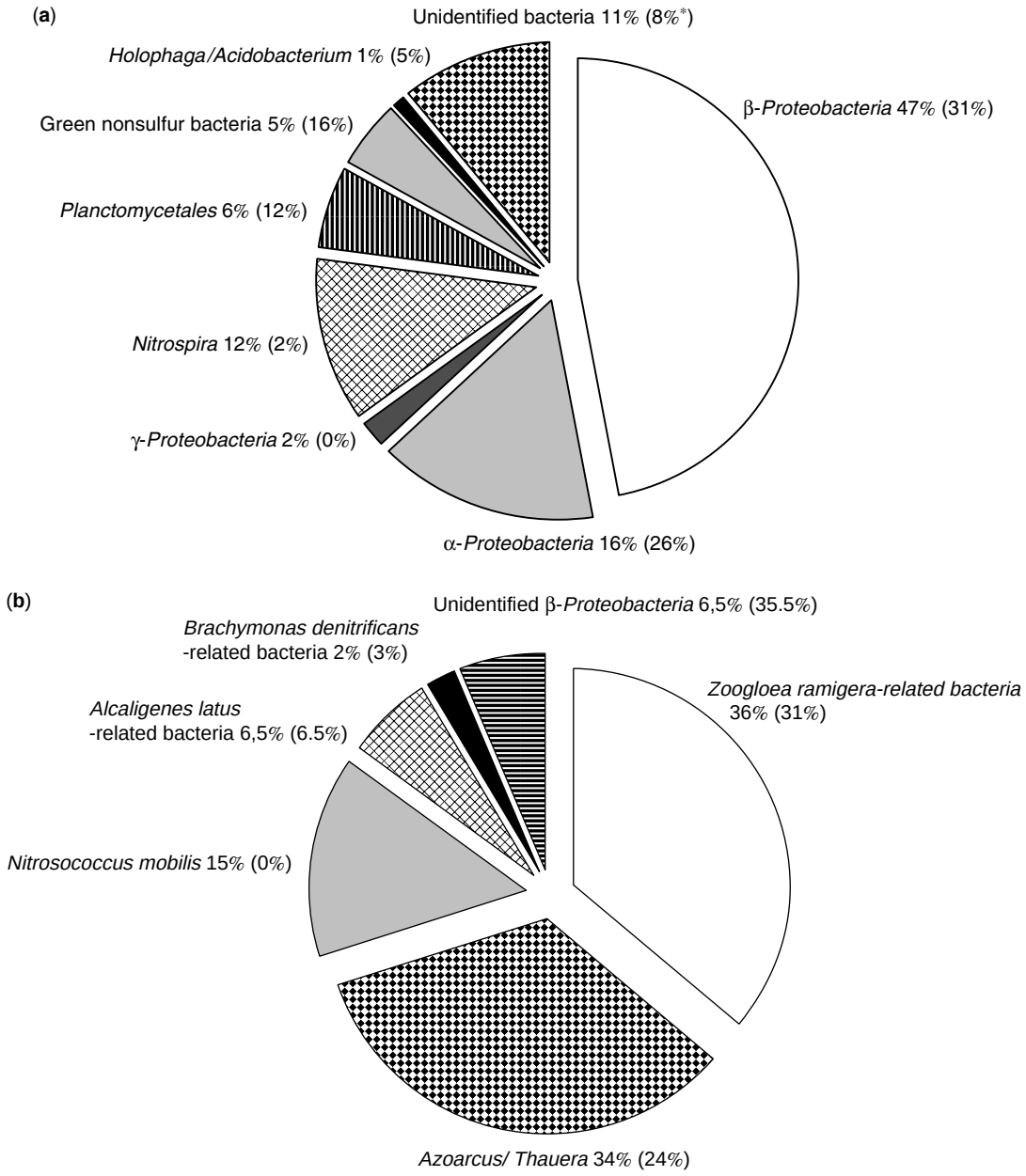


Figure 2. Microbial community structure of an industrial nitrifying/denitrifying activated sludge analyzed by FISH using group-specific (panel a) or β-subclass OTU-specific (panel b) probes. The biovolume of the specifically stained cells was determined using confocal laser scanning microscopy and digital image analyses and expressed as percentage of the biovolume of those cells detectable by in situ hybridization with the bacterial probe set. For panel B the biovolume of the β-subclass *Proteobacteria* was set to 100% and the other groups shown in this panel were scaled accordingly. Numbers in brackets show the abundance of the respective target organisms in the 16S rDNA clone library established for the same sample. ∗sequences not affiliated with the depicted groups.

transformations of sewage compounds are of primary importance. In particular, the organisms responsible for nitrogen and phosphorus removal are of interest. However, 16S rDNA sequence-based identification of a microorganism generally does not allow to infer its functional properties. Phylogenetically closely related microorganisms may possess different metabolic potentials. For example, certain obviously nonphotosynthetic bacteria that have been implicated with enhanced biological phosphorus removal (EBPR) are closely related to the phototrophic β-subclass proteobacterium *Rhodocyclus purpureus* ((89); mentioned later also), which is not capable of performing the required phosphorus transformations. On the other hand, several physiological traits important for wwtps, for example, denitrification, are found dispersed in many different phylogenetic lineages. Only for a few physiological

groups does an unambiguous linkage between phylogeny and physiology exist. One example is the lithoautotrophic ammonia oxidizers that form two monophyletic clusters in the β- and γ-subclasses of *Proteobacteria*, respectively (90). All known organisms affiliated with these clusters gain their energy by oxidation of ammonia to nitrite.

One way to identify microorganisms capable of performing a certain function in a wwtp is to use lab-scale reactors inoculated with activated sludge or biofilm material, which select for the respective functional microbial group. Using this approach, enrichments of yet uncultured nitrite oxidizers (91), anaerobic ammonium oxidizers (92), and bacteria catalyzing EBPR (89,93) were obtained. Although these studies provided important new insights, enrichment in reactors might favor the growth of organisms not important in a full-scale wwtp by inducing biases comparable to those caused by cultivation approaches. This effect is probably pronounced, in particular, if the reactor is fed with artificial sewage or if the sewage is amended with nutrients such as acetate. Therefore, the identification of organisms catalyzing a certain function in a reactor enrichment always has to be complemented with investigations on the in situ abundance and function of these bacteria in full-scale wwtps.

A major current research challenge is the development and validation of techniques for in situ analyses of the function of microorganisms within their environment. For example, selected genes coding for key enzymes of certain metabolic pathways (often referred to as *functional genes*) can be used both as phylogenetic and functional markers for the respective organisms. Using this approach, specific primers are used for PCR amplification of a fragment of the respective gene from a wwtp sample. After cloning and sequencing, the affiliation of the environmental gene fragments is determined by comparative sequence analyses with reference cultures. Although this approach also suffers from DNA extraction, PCR- and cloning-biases (discussed earlier), it combines, in contrast to the 16S rDNA approach, identification with assignment of a metabolic potential. In wastewater microbiology, the *dsrAB* genes coding for the dissimilatory sulfite reductase of sulfate-reducers (94) and the *amoA* gene encoding the active-site subunit of the ammonia-monooxygenase of ammonia-oxidizers (95,96) have been used to investigate the diversity of both functional groups (90,97). However, the mere detection of a functional gene does not prove that this gene was transcribed, translated, and functionally active in the respective organism.

Another way to link in situ identification of microorganisms in wwtps with a specific function is to combine FISH and microelectrodes (98). For example, zones of nitrification or denitrification in a biofilm can be determined using the appropriate microelectrodes. Equipped with this information, the bacteria living in the respective zones can be identified using FISH (65,67,99,100). If quantitative FISH data are available, this sophisticated approach can even be used to estimate substrate conversion rates per cell and K_S values (65). Nevertheless, the combination of FISH and microelectrodes has several limitations. Microelectrodes are not available for all important substances (e.g., a microelectrode suitable for phosphate determinations has not yet been developed), and those that exist do not function under all environmental conditions. Microelectrode measurements are one-dimensional and thus are heavily influenced by spatial heterogeneity of the sample. This is particularly important because it is currently not possible to detect the injection channel of the microelectrode in a subsequent FISH analysis in order to investigate the microbial communities surrounding this site. Therefore, FISH-microelectrode measurements are much easier to interpret in biofilms with a layered distribution of microorganisms than in heterogeneous and structurally dynamic activated sludge flocs. Furthermore, the spatial resolution of microelectrodes of 10 to 50 μm is clearly above the single-cell level and does not allow to directly investigate the function of single microbial cells in an environment.

The in situ physiology of microorganisms in wwtps can be investigated using a combination of FISH and microautoradiography (101,102). A small volume of the native sample is incubated with radioactively labeled substrates under suitable environmental conditions. After incubation the samples are fixed, and substrate not incorporated by the cells is removed by a series of wash steps. Subsequently, samples are cryosectioned and transferred to a cover slip for FISH analyses. Finally, the samples are covered with an autoradiographic emulsion, developed, and viewed with an inverse confocal laser scanning microscope. Cells are identified by their probe-conferred fluorescence, and silver grain formation on top of the cells (in the emulsion) indicates physiological activity and substrate uptake under the selected environmental conditions. If combined with an appropriate study design, most functionally important groups of microorganisms can be identified and quantified using the combination of FISH and microautoradiography (101). It should be stressed that radioactive labeling of cells in the FISH-microautoradiography procedure is unlikely to be caused by simple substrate uptake because the cells are permeabilized for oligonucleotide penetration and therefore unbound substrate is removed during the multiple wash steps. Consequently, microautoradiographic detection requires incorporation of the substrate into a macromolecule by the enzymatic apparatus of the cell. The major disadvantages of FISH-microautoradiography are that it is relatively expensive and time-consuming, and that radioactive derivatives of many substances are not easily available. Furthermore, the spatial resolution of microautoradiography depends on the isotope used for substrate labeling. Although tritium-labeled substrates often allow single cell resolution in cryosections, lower resolution on a cell cluster level is achieved using ^{14}C- or ^{33}P-labeled substrates.

In the following sections, the current knowledge on the diversity of important physiological groups of bacteria in wwtps is reviewed.

Nitrifying Bacteria

The nitrifying bacteria encompass two groups of microorganisms, the ammonia- and the nitrite-oxidizing bacteria, which catalyze the oxidation of ammonia to nitrite and of nitrite to nitrate, respectively. Because most of the

nitrogen in the influent of a wwtp is present either in form of urea (which is hydrolyzed to ammonia) or ammonium/ammonia, the nitrifying bacteria play a central role in nitrogen removal in wwtps. It is important to lower the ammonia concentrations in the effluent of wwtps because this compound is toxic to aquatic life and promotes eutrophication in the receiving water. Nitrifying bacteria are extremely slow-growing microorganisms and are recalcitrant to cultivation attempts. Because of the sensitivity of nitrifying bacteria to disturbances such as pH and temperature shifts, breakdown of the nitrification process is frequently reported from municipal and especially industrial wwtps.

According to microbiology and civil engineering textbooks, the model ammonia-oxidizer is *Nitrosomonas europaea*. However, FISH analyses in nitrifying activated sludge and biofilms showed that other ammonia-oxidizers are more important. In an industrial nitrifying/denitrifying plant, the dominant ammonia-oxidizer was *N. mobilis*, a bacterium that was previously considered to occur in brackish water only (27). Subsequently, *N. mobilis* was also detected in significant numbers in a nitrifying sequencing batch biofilm reactor (77). In contrast, *Nitrosospira*-related ammonia-oxidizers were found to be dominant in situ in a laboratory scale-fluidized bed reactor (103). Although *Nitrosospira* was also reported in a PCR-based study as important ammonia-oxidizer genus in wwtps (104), this finding could not be confirmed by FISH analyses of various wwtps and by a large *amoA*-based ammonia-oxidizer diversity survey in wwtps (90). Today it is generally accepted that nitrosomonads (including *N. mobilis*), and not nitrosospiras (encompassing the genera *Nitrosospira*, *Nitrosolobus*, and *Nitrosovibrio*), are important for ammonia oxidation in wwtps. This perception is also reflected in Figure 3 that shows the affiliation of 178 *amoA* clones retrieved from various nitrifying wwtps. Only 10 *amoA* clones from these systems cluster with the nitrosospiras, whereas the remaining 168 clones are affiliated with the nitrosomonads. Figure 3 also shows that almost all recognized lineages of β-subclass ammonia-oxidizers can be found in wwtps. Numerically, the *N. europaea/Nitrosomonas eutropha*-lineage, the *N. mobilis*-lineage, and the *Nitrosomonas marina/Nitrosomonas oligotropha/Nitrosomonas urea* cluster are most frequently detected.

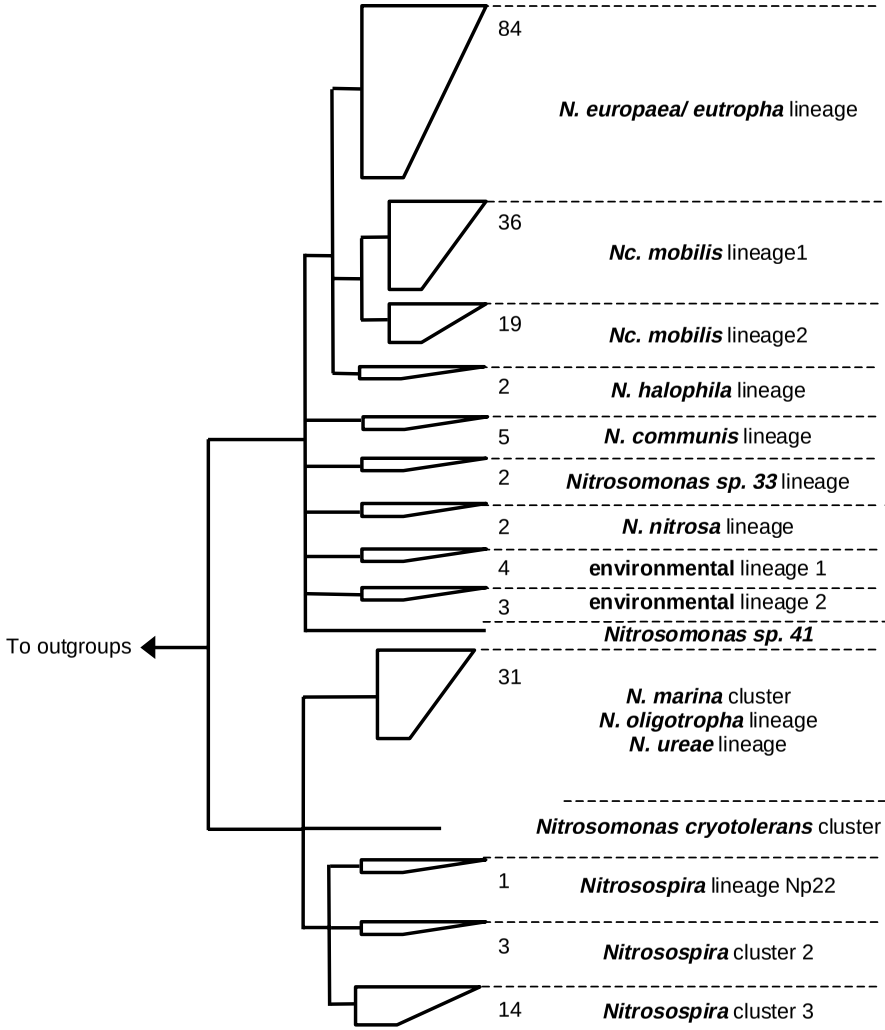
In conclusion, wwtps harbor a diversity of ammonia oxidizers of the β-subclass of *Proteobacteria*, which was enormously underestimated previously. Most of these ammonia-oxidizers are, on the basis of comparative *amoA* sequence analyses, relatively close relatives of described ammonia-oxidizer species. Interestingly, quantitative FISH results indicate that some nitrifying wwtps are dominated by a single ammonia-oxidizer species (27), whereas other plants harbor at least five different coexisting ammonia-oxidizer populations that are present in significant numbers (77).

Traditionally, members of the genera *Nitrobacter* were considered as the most important nitrite-oxidizers in wwtps (105). Therefore, the finding that *Nitrobacter* could not be detected by FISH with specific 16S rRNA-targeted probes in various nitrifying wwtps came as a surprise (106). Using the full-cycle rRNA approach, the occurrence of novel, yet uncultured, *Nitrospira*-like nitrite-oxidizing bacteria in nitrifying wwtps could be demonstrated (27,77,100,107,108). The importance of these microorganisms for nitrite oxidation in wwtps was also confirmed by reactor enrichment studies (91). Today four different phylogenetic lineages, two of them containing 16S rDNA clones of wwtps, within the genus *Nitrospira* have been recognized (Fig. 4) and phylum- and genus-specific probes suitable for FISH are available (109). Combination of FISH and microautoradiography showed that the *Nitrospira*-like nitrite-oxidizers in activated sludge fix carbon dioxide and can also grow mixotrophically using pyruvate but not acetate, butyrate, and propionate (109).

It has been postulated that the predominance of *Nitrospira*-like bacteria over *Nitrobacter* in most wwtps is a reflection of their different survival strategies. Although *Nitrospira*-like nitrite oxidizers are, according to data extracted from microelectrode-FISH analyses, K-strategists and thus may possess a low μ_{max}, but they are well-adapted to low nitrite and oxygen concentrations; *Nitrobacter* was postulated to be a relatively fast-growing r-strategist with low affinities to nitrite and oxygen (65). Because nitrite concentrations in most reactors from wwtps are low, *Nitrospiras* will outcompete *Nitrobacter* in these systems. In plants with temporally or spatially elevated nitrite concentrations, for example, in nitrifying sequencing batch reactors, both nitrite-oxidizers should be able to coexist. Consistent with this hypothesis, co-occurrence of *Nitrobacter* and *Nitrospira*-like bacteria has been observed by FISH in a nitrifying sequencing batch biofilm reactor (109).

Denitrifying Bacteria

Denitrification, the anaerobic respiration with nitrite or nitrate as electron acceptor, is used in wastewater to convert the product(s) of nitrification into gaseous nitrogen compounds (mainly dinitrogen), and thus to remove them from the sewage. Most attempts to identify and enumerate denitrifiers in activated sludge were based on cultivation-dependent approaches. Members of the genera *Alcaligenes*, *Pseudomonas*, *Methylobacterium*, *Bacillus*, *Paracoccus*, and *Hyphomicrobium* were isolated as part of the denitrifying microbial flora from wwtps (110–114). The latter genus was also detected microscopically by its typical cell morphology in denitrifying activated sludge (115,116). However, little is known about whether the aforementioned listed bacterial genera are representative for the in situ active denitrifiers in wwtps. Neef and coworkers, using specific FISH probes, detected significant numbers of *Paracoccus* spp. and *Hyphomicrobium* spp. in a denitrifying sand filter supplemented with methanol as reduced carbon compound for nitrate reduction. But both genera were present in numbers below 0.1% of the total cell counts in a nondenitrifying sand filter run in parallel without addition of methanol, indirectly suggesting an active participation of both genera in the denitrification process (74). Molecular studies of the community composition of denitrifying bacteria are difficult to perform because the denitrifying phenotype cannot be inferred from the



		Isolates/ described species	Isolates obtained from	Wwtp clones	No. of wwtps in which the respective clones were detected
84	***N. europaea/ eutropha*** lineage	5	Wwtp, soil	79	10
36	***Nc. mobilis*** lineage1	4	Wwtp, seawater	32	5
19	***Nc. mobilis*** lineage2	0	-	19	4
2	***N. halophila*** lineage	2	Seawater, alkaline lake	0	0
5	***N. communis*** lineage	1	Soil	4	2
2	***Nitrosomonas sp. 33*** lineage	1	Soil	1	1
2	***N. nitrosa*** lineage	1	Wwtp	1	1
4	**environmental** lineage 1	0	-	4	2
3	**environmental** lineage 2	0	-	3	1
	Nitrosomonas sp. 41	1	Soil	0	0
31	***N. marina*** cluster ***N. oligotropha*** lineage ***N. ureae*** lineage	6	Soil, sandstone, seawater, brackish water	25	7
	Nitrosomonas cryotolerans cluster	1	Arctic seawater	0	0
1	***Nitrosospira*** lineage Np22	1	n. d.	0	0
3	***Nitrosospira*** cluster 2	2	Soil	1	1
14	***Nitrosospira*** cluster 3	5	n. d.	9	3
	Σ	30		178	

Figure 3. Diversity of *amoA* clones retrieved from different nitrifying wwtps. A schematic AmoA–based phylogenetic classification of the β-subclass ammonia oxidizing bacteria is shown. Multifurcations connect branches for which a relative order could not be unambiguously determined by applying different treeing methods. The cluster designations were adopted from those of Purkhold and coworkers (90). The height of each tetragon indicates the number of sequences in the cluster. γ-subclass ammonia oxidizers are not included because they currently have not been detected by FISH nor by *amoA*-analyses in nitrifying wwtps.

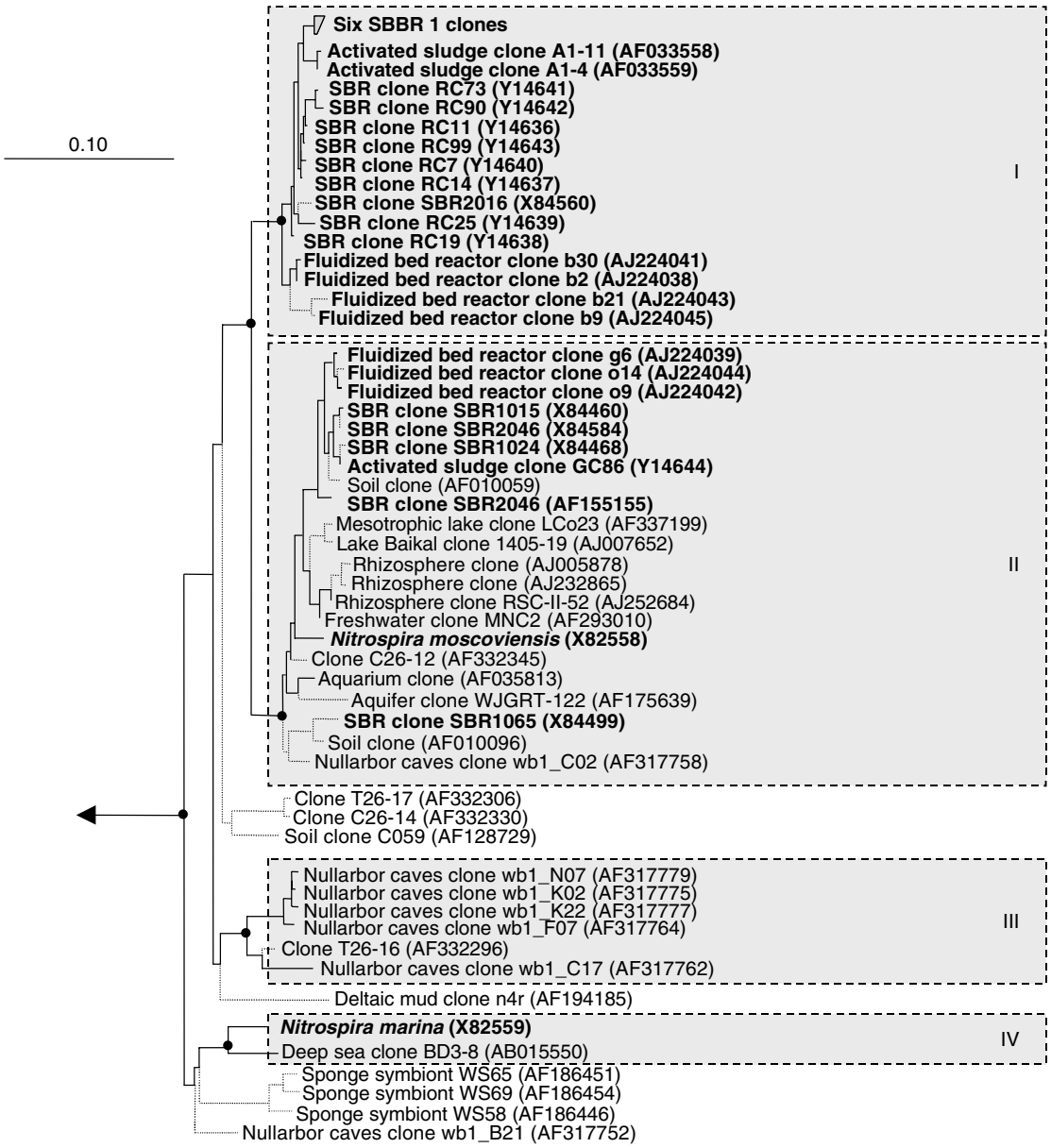


Figure 4. Phylogenetic tree of the genus *Nitrospira* based on comparative analysis of 16S rRNA sequences. The basic tree topology was determined by maximum-likelihood analysis of all sequences longer than 1,300 nucleotides. Shorter sequences were successively added without changing the overall tree topology. Branches leading to sequences shorter than 1,000 nucleotides are dotted to point out that the exact affiliation of these sequences cannot be determined. Black spots on tree nodes symbolize high parsimony bootstrap support, above 90%, based on 100 iterations. The scale bar indicates 0.1 estimated changes per nucleotide. Clones from wwtps and reactors and sequences that belong to isolated strains are depicted in bold. The four sublineages of the genus *Nitrospira* are boxed in gray and marked by the numbers I to IV. Figure modified from Daims and coworkers (109).

phylogeny of a microorganism. The capability to denitrify is found in many bacterial divisions and is not confined to defined phylogenetic clusters within these divisions. However, the combination of FISH and microautoradiography (101) allows to identify denitrifiers in situ. For this purpose, two types of experiments need to be performed. In the first experiment, the wwtp sample is incubated under anaerobic condition in absence of nitrate or nitrite with radioactively labeled substrates that are typically used as electron donors for denitrification. In the second experiment, the sample is incubated with the same labeled substrates under anaerobic conditions but in the presence of nitrate or nitrite. Bacterial species, identified by FISH, that take up substrate under anaerobic conditions exclusively in the presence of nitrate or nitrite are most likely denitrifiers. The use of this technique in combination with

the full-cycle rRNA approach revealed that novel, still uncultured *Azoarcus*-related bacteria are important denitrifiers in an industrial nitrifying/denitrifying wwtp (117).

Anaerobic Ammonium Oxidizing Bacteria

Unexpected nitrogen losses are frequently observed in wastewater treatment plants. In these plants, ammonia was obviously converted to gaseous nitrogen compounds in the absence of organic electron donors excluding the activity of conventional denitrifiers. Using the full-cycle rRNA approach, we could demonstrate that novel planctomycetes distantly related to the recently described anaerobic ammonium oxidizer *Candidatus* "Brocadia anammoxidans" (92,118) did occur in high numbers in these systems (42,118) and were thus probably responsible for the nitrogen removal. Consequently, the new candidatus genus "Kuenenia stuttgartiensis" was proposed (42). Using specific 16S and 23S rRNA-targeting probes for both anaerobic ammonium oxidizers (42,71), a predominance of *Candidatus* "*Kuenenia stuttgartiensis*" over *Candidatus* "Brocadia anammoxidans" in wwtps was observed.

The anaerobic ammonium oxidizers are a perfect example that completely unexpected organisms are hidden in wwtps. Both anaerobic ammonium oxidizers form a monophyletic deep-branching lineage within the *Planctomycetales* (Fig. 5) and oxidize ammonium anaerobically with nitrite to dinitrogen gas (42,92,119). Anaerobic ammonium oxidation in contrast to classical nitrification and denitrification allows to remove ammonium in a one-step process saving space, energy, and money because no aeration or addition of organic carbon for denitrification are required. Anaerobic ammonium oxidation is a very promising method for the treatment of sewage with a high ammonium and low organic carbon content. However, this process needs a mixture of ammonium and nitrite and thus must be coupled to partial nitrification for nitrite production (42,118,120). Furthermore, establishment of anaerobic ammonium oxidizing activity in a wwtp takes a very long time, and once established the process is sensitive to perturbation.

Bacteria Important for Enhanced Biological Phosphorus Removal

Phosphorus removal from wastewater is important to prevent eutrophication, and is therefore an integral part of modern municipal and industrial nutrient removal wwtps. Phosphorus can be precipitated by the addition of iron or aluminum salts and subsequently be removed with the excess sludge. Chemical precipitation is a very reliable method for phosphorus removal but increases significantly the sludge production and thus creates additional costs. Furthermore, the use of chemical precipitants often introduces heavy metal contamination into the sewage and increases the salt concentration of the effluent. Alternatively, phosphorus removal can be achieved by microbiological mechanisms in a process called *enhanced biological phosphorus removal* (EBPR) (121–124). This process is characterized by cycling the activated sludge through alternating anaerobic and aerobic conditions. In the anaerobic stage, the bacteria responsible for EBPR are supposed to gain energy from polyphosphate hydrolysis accompanied by subsequent P_i release for uptake of short-chain fatty acids and their storage in the form of polyhydroxyalkanoates (PHA). Two different models were postulated for the production of the reducing equivalents for this anaerobic metabolism (125,126). In the subsequent aerobic stage, the polyphosphate accumulating organisms (PAOs) possess a selective advantage compared with other microorganisms that were not able to take up fatty acids under the preceding anaerobic conditions by using the stored PHA in an otherwise carbon-poor environment. In parallel, PAOs restore their polyphosphate pools by aerobic uptake of available phosphate from the wastewater. After sedimentation in the secondary clarifier, a part of the biomass is recycled to the anerobic stage and mixed with new wastewater, whereas the excess sludge containing the intracellular polyphosphates is removed from the system.

In contrast to chemical precipitation, EBPR plants have been frequently reported to fail. This raised interest in the microbiology of the process. Traditionally, on the basis of cultivation experiments γ-subclass *Proteobacteria* of the genus *Acinetobacter* were believed to be the only PAOs (127–129). However, today it has become apparent that *Acinetobacter* can accumulate polyphosphate but does not possess the earlier-described PAO metabolism (122). Furthermore, cultivation-independent methods such as fluorescent antibody staining (11), respiratory quinone profiles (4), and FISH with a genus-specific probe (8,9) demonstrated that the relative abundance of *Acinetobacter* in EBPR systems was dramatically overestimated because of cultivation biases, further confirming that *Acinetobacter* is not of importance for EBPR.

Several other bacteria isolated from EBPR reactors have been suggested as PAO candidates. *Microlunatus phosphovorus*, a high-G+C gram-positive bacterium accumulates aerobically polyphosphate and consumes it under anaerobic conditions, but fails to take up acetate or accumulate PHA under anaerobic conditions (130,131). FISH, with a probe specific for *M. phosphovorus*, demonstrated the presence of this organism in an EBPR plant (2.7% of the total bacteria) (132), but no direct indications for the importance of this bacterium for EBPR are available. Furthermore, *Lampropedia* spp. were shown to possess the basic metabolic features of a PAO (133), but their acetate-uptake phosphate-release ratio was much lower than what EBPR models predict, and no additional data regarding the abundance or activity of these morphologically conspicuous bacteria in EBPR systems have been published.

Compared with these cultivation-based attempts, the hunt for PAOs was more successful using molecular tools for analyses of EBPR systems. β-subclass *Proteobacteria* and high-G+C gram-positive bacteria (actinobacteria) increased in number after addition of acetate to the raw sewage of a EBPR full-scale wwtp suggesting that these groups benefit from the enhanced availability of short-chain fatty acids in the anaerobic basin and thus represent candidates for PAOs (8). Additional support for the importance of both groups for EBPR stems from FISH experiments in an efficient EBPR laboratory-scale

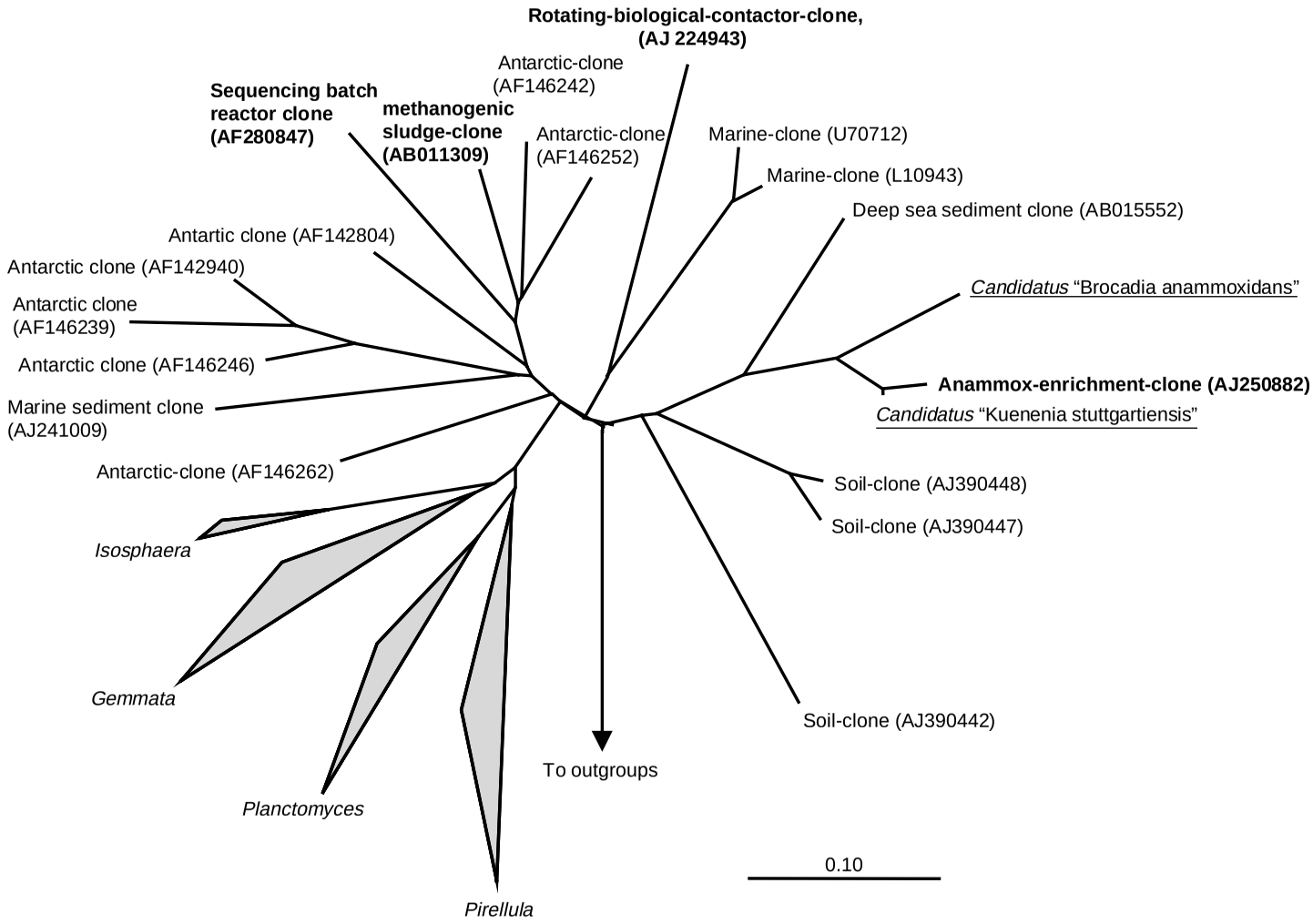


Figure 5. Phylogenetic 16S rDNA tree showing the deep-branching of the anaerobic ammonium oxidizers within the *Planctomycetales*. In addition to the described anaerobic ammonium oxidizers *Candidatus* "Brocadia anammoxidans" and *Candidatus* "Kuenenia stuttgartiensis," several environmental clone sequences, including sequences from wwtps depicted in bold, are unaffiliated with cultured members of the division. The physiology of the organisms represented by these sequences still needs to be clarified. Figure modified from M. Schmid et al. (71).

sequencing batch reactor (72) and respiratory quinone profiling in a lab-scale EBPR system (6). Recently, high-G+C gram-positive bacteria related to *Terrabacter tumescens*, *Tetrasphaera japonica*, and *Tetrasphaera australiensis* were reported to be abundant in EBPR systems and thus might be important for EBPR (25,76,93). Although the function of actinobacteria as PAOs still has to be proven, evidence is available that β2-subclass *Proteobacteria* related to *Rhodocyclus* and *Propionibacter* are important PAOs in many EBPR systems. These bacteria, for which the name *Candidatus* "Accumulibacter phosphatis" was suggested (89), were detected in high numbers in EBPR systems (26,75,89,93,134). Furthermore, phosphorus accumulation by these bacteria in the aerobic phase was demonstrated by sequential FISH and polyphosphate staining (25,93). In addition, acetate uptake in the anaerobic phase and phosphorus uptake under aerobic conditions could be demonstrated for *Candidatus* "Accumulibacter phosphatis" using FISH and microautoradiography (134).

A potential reason for the failure of EBBR plants is the presence of bacteria that use previously stored compounds such as glycogen (also referred to as *glycogen accumulating organisms* (GAOs)) to compete with the PAOs for substrate uptake under anaerobic conditions (41,135,136). Cech and Hartmann (135) described gram-negative cocci in clumps and packages of tetrades in activated sludge and called these morphotypes *G-bacteria*, because their numbers increased after glucose addition. Mino and coworkers (123) suggested that bacteria with a similar morphology are GAOs. However, recent molecular and physiological analyses of various G-bacteria isolated from wwtps revealed a high phylogenetic diversity of this morphotype and provided no support for their role as GAOs (Fig. 6; 137). Molecular community analyses of deteriorated EBPR reactors revealed the predominance of a novel bacterial group within the γ-subclass of *Proteobacteria* (40). The phylogenetic affiliation of this deeply branching group cannot unambiguously be resolved (Fig. 6). These bacteria are good GAO candidates because they occur in deteriorated EBPR systems, probably accumulate PHA, and store little or no polyphosphates (25,40).

CONCLUSION

During the last decade, the application of molecular tools in wastewater microbiology has revolutionized our view on the microbial ecology of these systems. Different groups of still not culturable bacteria were identified and shown to be responsible for nitrite oxidation, denitrification, and enhanced biological phosphorus removal. Surprisingly, the model organisms described in text books for these processes and for ammonia oxidation were shown to be generally not of importance for wastewater treatment. Furthermore, a new process, anaerobic ammonium oxidation, was detected in wwtps and the responsible (still not cultured) microorganisms were identified. It is important to note that significant diversity exists in each of these functional groups of bacteria. In the next research phase in wastewater microbiology, more detailed knowledge on the biology of the aforementioned noncultured bacteria needs to be gained. Therefore, an increased effort on the development of suitable cultivation strategies for these bacteria is needed. In parallel, the use of techniques referred to as *environmental genomics* should allow to investigate the genome composition of these bacteria without the need of cultivation (138).

Although researchers interested in microbial ecology will probably find the results summarized in this article interesting and important, engineers might ask what practical value can be extracted from the accumulated knowledge. Regarding application, the most obvious benefit of the progress described is that it provides a basis for a more knowledge-driven treatment of wwtp failures. One strategy to improve a particular aspect of process performance in a wwtp, for example, during start-up or after its breakdown, is the addition of specialized microorganisms or activated sludge from another wwtp (139). This operational tool that is called *bioaugmentation* does however frequently fail ((63) and references therein). Such failure is typically caused by addition of the "wrong" microorganisms, for example, the model organisms for nitrification, which cannot compete successfully with the autochthonous bacteria in the plant and are thus eliminated or washed-out. Therefore, it is important to identify those microorganisms responsible for nitrogen or phosphorus removal in a functioning wwtp. If problems arise, these results can be used as guidance to select the appropriate bacterial additive (for culturable microorganisms) or a well-suited activated sludge from a neighboring wwtp containing a comparable microbial flora. Once the appropriate bacteria have been selected, they need to be protected, for example, by polymer embedding from grazing by protozoa (63).

Compared with curing failure of a certain process in a wwtp by bioaugmentation, protecting the plant from process deterioration is a more sustainable strategy. For this purpose we need to understand the links between the diversity of a functionally important bacterial group and the stability of the catalyzed process. Preliminary observations indicate that plants with low functional redundancy caused by the presence of a low diversity of bacteria of a certain functional group are more sensitive to failure of the respective process than plants harboring a high diversity of the same bacterial group. If increase in diversity can indeed be proven to cause process stabilization, then it will be important to learn how plant design and process parameter control can be optimized to increase the diversity of functionally important bacterial groups. To answer these ecologically and economically important questions it will be necessary to determine the microbial community composition of a large number of different samples obtained from tightly controlled reactor studies and full-scale wwtps. Because of the tediousness of many of the established molecular methods, this kind of research will greatly benefit from the implementation of modern high-throughput techniques such as DNA microarrays for measuring microbial community composition in complex samples (140).

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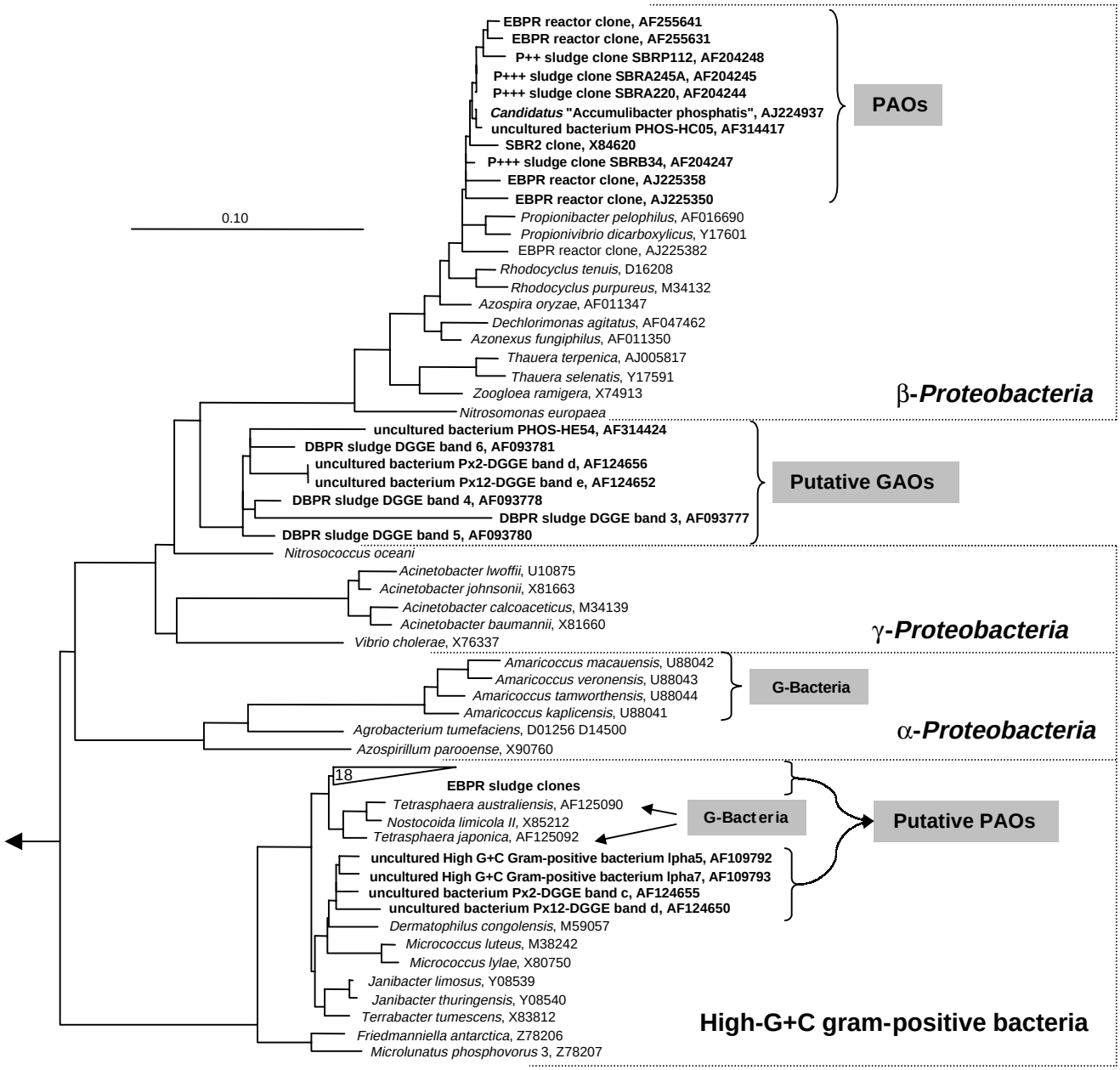

Figure 6. Phylogenetic 16S rDNA tree showing the affiliation of putative polyphosphate accumulating organisms (PAOs), glycogen accumulating organisms (GAOs) and G-bacteria. Environmental sequences of putative PAOs and GAOs derived from wwtps are depicted in bold. The tree was calculated using the maximum likelihood method with a 50% bacterial conservation filter. The bar corresponds to 10% estimated sequence divergence.

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ACTIVATED SLUDGE — SEQUENCING BATCH REACTORS

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The sequencing batch reactor (SBR) belongs to a broad class of treatment systems that use a flocculated suspension of microorganisms referred to as activated sludge. This microorganism suspension is mainly composed of many different species of bacteria (i.e., a mixed rather than a pure culture that is spatially organized in the form of clusters and microcolonies) that destroy, transform, or simply remove unwanted contaminants present in liquid streams. Within this frame of reference, the objective of the SBR and virtually all other biological reactors is to select and enrich a mixed culture of microorganisms that convert biodegradable contaminants into new cellular material, carbon dioxide, water, and other stable innocuous organic and/or inorganic products. Although they are sustained by the consumption of readily biodegradable

energy-rich compounds called *contaminants*, the resulting microbial community also removes nonbiodegradable and some slowly biodegradable contaminants by physical, chemical, or physiochemical means. In such instances, insoluble contaminants and soluble contaminants that precipitate or sorb become part of a flocculated mass of microorganisms (i.e., the "flocs") and are thus removed from the waste stream that is being treated. The SBR differs from conventional continuous-flow activated sludge systems because it is a periodically operated, time-oriented system, with flow, energy input, and tank volume varying according to a predetermined operating strategy. As a result, the SBR is a time-oriented, periodic process that readily and easily develops controlled unsteady state conditions that are required to meet discharge limits dictated by the regulators. These distinguishing features and others are described in this article.

BACKGROUND

Contaminants

Unwanted contaminants are present in liquids (e.g., municipal sewage, industrial wastewater, landfill leachate, and contaminated groundwater), solids (e.g., soils after a gasoline spill), and gases (e.g., odors from a composting plant). They are organic or inorganic, more or less dense than water, and either soluble or insoluble in water. Some are emulsions in water while others are not; some are volatile and some nonvolatile. Those contaminants that are sparingly soluble in water are termed *hydrophobic* or *nonaqueous phase liquids* (NAPLs) and those that are not are termed as *hydrophilic*. Contaminants that are biodegradable serve as electron donors for microorganisms that catalyze a plethora of conversions that range from the minor modification of a contaminant's structure (e.g., through the addition or removal of a hydroxy group) to the complete mineralization or oxidation of the contaminant to carbon dioxide, water, and other inorganic compounds.

The primary conversions that take place in biological reactors involve soluble organic compounds that provide carbon and energy for the growth of a mixed culture of heterotrophic microorganisms present in the biomass. As a result, the success of a biological treatment system is often judged first by its ability to remove soluble biodegradable organic compounds. When combined with powdered activated carbon (PAC), the "partitioning power" of the floc (i.e., the flocculated mass of microorganisms, PAC, and other associated materials) is appreciably enhanced because soluble nonbiodegradable organic compounds are removed more aggressively. Biological systems are also often operated to minimize the discharge of the nutrients, namely, nitrogen and phosphorus. The success of such treatment systems is then judged by their ability to remove soluble biodegradable organic compounds and the nutrients of interest.

Biological treatment systems are categorized in a number of ways:

1. *The microbial community's ability to use exogenous electron acceptors*. Some use oxygen (i.e., in aerobic zones of the treatment system); others use nitrite-nitrogen or nitrate-nitrogen (i.e., in anoxic zones); and some use none at all (i.e., in anaerobic zones). It is noted that strict aerobes only grow in the presence of oxygen; strict anaerobes only grow in the absence of oxygen; and facultative anaerobes grow in the presence or absence of oxygen.
2. *The ability of bacteria to grow attached to a surface as a biofilm or unattached and in suspension as dispersed organisms or in clusters or flocs.* The most common treatment systems distinguish organisms that grow attached as fixed films (e.g., in trickling filters or rotating biological contactors) from those that grow in suspension as "activated sludge." In both these systems, the mixed cultures of microorganisms convert the contaminants to end products that depend on the nature of the contaminants and the organism distribution that is present.
3. *The mixing pattern established in the reactor*. Two mixing patterns are most commonly employed: completely mixed or plug flow. Intermediate mixing intensities are often employed but are difficult to model effectively.
4. *The type of reactor used*. These include the completely mixed batch reactor (CMBR), the completely mixed flow reactor (CMFR), the plug flow reactor (PFR), the plug flow reactor with dispersion (PFDR), and the sequencing batch reactor (SBR). Microorganisms in these systems are either in suspension or attached to a bed of support medium (e.g., packing material such as rocks, sand, ceramic, styrofoam, etc.) that is fixed, fluidized, or expanded.
5. *The control strategy used to operate the reactor (i.e., periodically operated or used under continuous flow conditions)*. Continuous-flow reactors normally recycle settled biomass but can also be operated with no-recycle.

A typical process schematic of a continuous-flow activated sludge system is shown in Figure 1. The schematic represents one of the typical "controlled unsteady state systems" that are in use today. As shown, the biological wastewater treatment system is depicted as a cascade of three CMFRs with biomass (or sludge) returned (i.e., recyled) from the third CMFR in the cascade to the first CMFR after settling in the clarifier, which is sketched in Figure 1 as a cone-shaped vessel. In this system, the microorganisms circulating in the system are periodically exposed to different substrate concentrations with the highest concentration in the first CMFR and the lowest in the last CMFR. As a result, biomass settled and recycled to the first tank is repeatedly subjected to high and low growth-rate environments as it moves from reactor to reactor and back again into the cascade.

In order for the sludge to concentrate well in the clarifier, the rate at which flocs settle must be sufficient. Unfortunately, the rate of sedimentation is slowed when the relative abundance of microorganisms that grow head to tail within long sheaths as filaments is

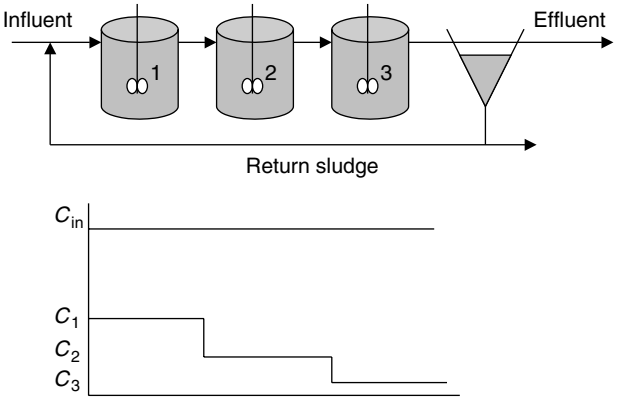

Figure 1. Process schematic of a continuous-flow activated sludge system with the aeration basin designed as a reactor cascade (top) and a profile of the substrate concentration from the influent port of the cascade to the discharge from the cascade (bottom). Microorganisms circulated in the system are repeatedly exposed to high and low substrate concentration.

greater than some minimum acceptable level. When this happens, poor sedimentation results and the microorganism (or activated sludge) shown in the shaded zone of the clarifier in Figure 1 extends upward toward the overflow weir causing the "clarified" effluent to contain unacceptable quantities of suspended solids (i.e., the flocs). Various researchers (2,3,10) demonstrated experimentally that the repeated exposure of activated sludge to feast (e.g., in the first CMFR in Fig. 1) and then famine (e.g., in the third CMFR) conditions is a very effective means of controlling the excess growth of filamentous organisms. The frequent shifting of activated sludge between aerobic, anoxic, and anaerobic zones establishes the conditions needed to enrich for microbial communities that carry out nitrification, denitrification, and enhanced biological phosphate removal. Frequent shifts of certain process conditions and the resulting controlled unsteady state conditions have significant and very important long-term effects on microorganism selection and enrichment and on the physiological state of the microorganisms enriched. Accordingly, these short-term unsteady state conditions, if properly selected and controlled, are an effective tool to maintaining long-term quasi–steady state conditions that control the structure and function of the microbial communities on which the performance of activated sludge systems is so greatly dependent.

In practice, the factors known to be effective in controlling the structure and function of microbial aggregates (e.g., activated sludge or fixed films) are difficult to maintain in continuous-flow systems. The frequency and amplitude of the changes needed to control variations in rate and time (e.g., from feast to famine) are not easily applicable to continuous-flow systems as they are to periodically operated systems such as the SBR. Because of inflow rate variations, biological treatment systems are often subjected to suboptimal control conditions even when they are designed to operate as controlled unsteady state systems.

THE CONCEPT

The SBR technology is used to gain control over the structure and functions of the microbial community in a multipurpose bioreactor that is exposed to varying influent conditions. An SBR system differs from a continuous-flow activated sludge systems in the following manner

1. The influent and effluent streams are uncoupled.
2. Biomass separation occurs in the biological reactor and not in a separate clarifier, as was shown in Figure 1.
3. The unit operations and unit processes that take place in a reactor follow each other in a time sequence that is progressively repeated in a "periodic" or "cyclic" manner and not from tank to tank as is done in space-oriented systems.
4. A portion of the treated water is periodically discharged from each tank to make room for a new batch of wastewater (see Fig. 2).

Because of these features, SBRs are referred to as periodic processes, single-tank systems, fill and draw reactors, or as variable volume reactors.

As represented in Figure 2, the SBR process is characterized by a series of process phases (e.g., fill, react, settle, draw, and idle), each lasting for a defined period. During the fill phase, the SBR behaves as a semicontinuous CMFR (if the reactor is well mixed and/or aerated during fill) that receives flow but has no discharge. As is described later, the fill phase may be unmixed, mixed, or mixed and aerated. During the react phase, the SBR behaves as a CMBR. Sludge wasting as a means to keep the biomass concentration in the reactor at a certain level normally takes place after the settle phase but may take place near the end of react phase or during the settle phase and may take place weekly, daily, or during each cycle.

As originally defined, the idle phase was included as a "buffer" phase in the SBR (4,5) (Fig. 2). No wastewater enters the reactor during the idle phase and, unless sludge is wasted during this phase, there is no discharge. It is usually neither mixed nor mixed and aerated. As such, the name "idle" is suitable. However, the time in the idle phase has an important function in the SBR as originally defined. Idle phase provides the excess capacity in the system so

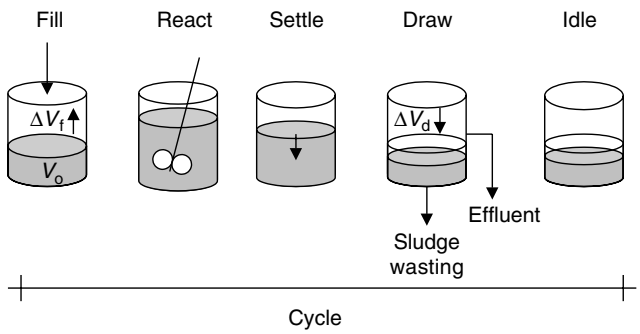

Figure 2. Schematic of a typical SBR cycle. The Idle phase is optional if an alternative means for handling excess inflow is provided. A cycle may include aerobic, anoxic, or anaerobic phases.

that permit limits are met during periods of high flow. The time for idle phase seamlessly reduces toward zero when high volumetric flow rates are observed and it increases when there are low volumetric flow rates. The idle phase can only be eliminated when an equalization tank or holding tank is available or when there is some other means for handling the excess inflow (e.g., continuous influent as in the intermittent cyclic extended aeration system depicted in Table 1).

In some of the variable-volume activated sludge systems, there is no distinct react phase, and the settle and draw phases occur while the influent enters the system. These differences are briefly described in Table 1 for selected variable-volume activated sludge systems.

In addition to the descriptions given earlier, the fill-and-react phases can have several subphases based on the energy input to the system that results in various aeration and mixing operating strategies. These subphases have been labeled in various ways as follows

- static fill — no energy input to the system allows the accumulation of unreacted substrate.
- mixed fill — mixing without forced aeration typically allows either anoxic or anaerobic reactions.
- aerated fill — mixing with forced aeration typically allows aerobic reactions.
- mixed react — mixing without forced aeration allows anoxic and possibly anaerobic reactions.
- aerated react — mixing with forced aeration allows aerobic reactions.

Contaminants in the wastewater enter the reactor during the fill phase. Unless a temporary influent storage volume is provided, more than one SBR is required to handle a continuous inflow of wastewater. When a holding tank for the influent is used, the time for the fill phase is often only a small fraction of the total cycle time and the wastewater constituents accumulate, thus providing the conditions required for "feast" and for the control of filaments. Without a holding tank, feast conditions are established during static fill for the following reasons

1. The reaction rates are reduced in systems with little or no mixing. The only energy supplied to the system during this static fill comes from the introduction of the wastewater. The highest rates of reactions occur in well-mixed systems.
2. Because of the lack of mixing, contact between the microorganisms and contaminant is minimal.
3. The extent of oxidation is limited by the availability of exogenous electron acceptors. Accordingly, although some electron donors (i.e., contaminants) and electron acceptors are utilized, and despite some fermentation reactions occurring in the absence of exogenous electron acceptors, anoxic and/or anaerobic reactions take place at a much greater rate during the mixed fill than during the static fill.

Similarly, initial aerobic reactions take place during aerated fill. In general, the extent of the aerobic reactions depends on the mass flow rate of the readily biodegradable substrates and the specific fill strategy employed. For example, anoxic reactions also occur during the aerated fill if oxidized forms of nitrogen are present, the concentration of dissolved oxygen is sufficiently low, and an adequate supply of electron donors (i.e., oxidized forms of nitrogen) is available.

The reactions initiated during the fill phase are completed during the react phase by initiating the proper mixing and/or aeration strategy. The settle (separation of the biomass from the treated waters), draw (withdrawal of the treated water, i.e., the supernatant that forms during the sedimentation), and idle (time between draw and fill that reflects the excess hydraulic capacity of the system) phases complete the cycle.

The generic cycle arbitrarily commences with the fill phase and terminates at the end of the idle phase. The volume of wastewater introduced into the reactor is ΔV_f (Fig. 2). It is added to the volume of water and sludge that remains in the reactor at the end of the past cycle (V_o). At the end of the fill phase, the reactor contains $V_R = V_o + \Delta V_f$. Once the react phase has been

Table 1. Distinguishing Features of Selected Variable-Volume Activated Sludge Systems (6)

Named Systems	Influent	Distinct React Phase	Distinct Settle Phase	Effluent	Distinct Idle Phase
Cyclic Activated Sludge Technology[a]	Periodic	No	Yes	Periodic	No
Intermittent Cyclic Extended Aeration System[b]	Continuous	No	No	Periodic	No
Intermittent Pasveer Ditch[c]	Continuous	No	No	Periodic	No
SBR With Equalization Tank	Periodic	Yes	Yes	Periodic	No
SBR without equalization tank	Periodic	Yes	Yes	Periodic	Yes

[a] CAST Cyclic Activated Sludge Technology (7)
[b] ICEAS Intermittent Cycle Extended Aeration System (8)
[c] Pasveer ditch carousel ditch with no external clarifier (9)

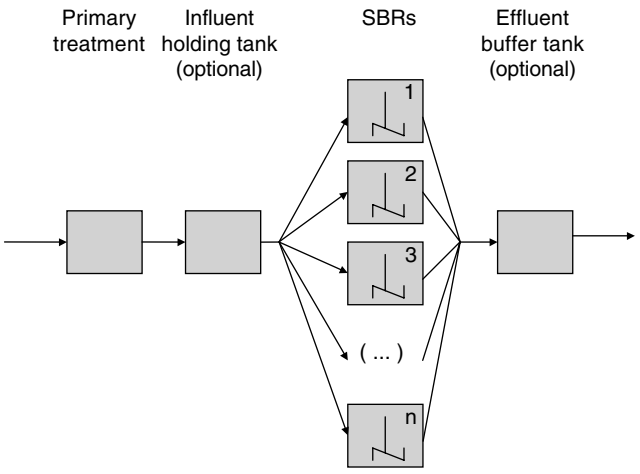

Figure 3. Schematic of an SBR plant.

completed and the mixing energy has been dissipated, the activated sludge starts coagulating and settling. After wasting excess sludge (ΔV_w) and discharging the treated supernatant (ΔV_d), the reactor is available to receive a new supply of wastewater.

Thus, a SBR process is characterized by the following set of parameters:

t_i	Time for the ith phase
t_c	Total time of one cycle ($t_c = \Sigma t_i$)
FTR	fill time ratio, t_f/t_c, where t_f is the time for fill
VER	Volumetric exchange ratio, $\Delta V_f/V_R$
HRT or τ	Hydraulic residence time, $n \bullet V_R Q^{-1}$, where n is the number of tanks and Q is the volumetric flow rate of the influent to the treatment plant

In addition, process parameters that are typical for activated sludge or biofilm systems apply. For instance, the design and operation of an activated sludge SBR usually includes considerations of key factors such as HRT, sludge age, and sludge loading.

DESIGN AND OPERATION OF SBR PLANTS

SBR plants typically consist of a number (n) of reactors. Pre- and posttreatment facilities may be added. A general layout of an SBR plant is shown in Figure 3. Four generic groups of SBRs can be distinguished (6). The specific fill strategy used and/or the inclusion of a react or idle phase characterize each of them. The generic groups are as follows:

(A) Systems with a periodic influent, a react phase, and an idle phase
(B) Systems with a periodic influent, a react phase, and no idle phase
(C) Systems with interrupted influent, a selector, and no react and idle phases
(D) Systems with a continuous influent.

Each of these generic groups is described in detail in the following section (Fig. 4).

Group A. The SBR as originally defined (4–6) is shown in Figure 4a. Two or more tanks are normally used. The influent is diverted from the tank being filled when its water level reaches a predetermined maximum value. The fill time ratio (FTR) for each tank is less than one. The time for the fill phase is defined by

$$t_f = \frac{t_c}{n}$$

where t_f is the fill time, t_c is the total cycle time, and n is the number of SBR tanks available.

Group B. SBR systems with a holding tank in front (Fig. 4b) are often used in Germany (3). The influent enters a holding tank before being distributed into the SBR tanks. Two or more tanks are normally used. The configuration of tanks allows the system to be operated with a variable fill strategy including instantaneous fill to control filaments or extended fill strategies to avoid the accumulation of inhibitors (e.g., as could be present in industrial wastewater). Therefore, an idle phase is not required to control the system during variable flow conditions. The holding tank in Group B provides the same function as the idle phase in group A.

Group C. Figure 4c describes SBR systems with an internal or external constant volume selector basin. In these systems, fill is interrupted either during the draw phase (e.g., as in the Cyclic Activated Sludge System) or during the settle and draw phases (e.g., as in the Cyclic Activated Sludge Technology). All of these systems have a recirculation line that returns mixed liquor from the main reactor to the selector. The Cyclic Activated Sludge System has a chamber that follows the selector to buffer the flow during settle.

Group D. Figure 4d describes SBR systems that have a continuous inflow of wastewater that is not interrupted during the settle and draw phases. These SBRs can be operated as single tanks or as sets of tanks operated in parallel. Once the water level in a tank reaches a predetermined maximum value, the aerators and mixers are switched off, the activated sludge is allowed to settle, and the supernatant is withdrawn from the reactor. To minimize cross-contamination of the treated effluent with the influent wastewater during the draw phase, the reactor is divided into two zones separated by a baffle. The first of the two zones limits cross-contamination and serves as a selector. Normally, SBRs such as the Intermittent Cyclic Aeration System operate with a completely aerated fill. In contrast to the operation of Groups A and B, static fill occurs at the end of the cycle and is termed *settle* when there is no discharge, and *draw* when the treated effluent is discharged.

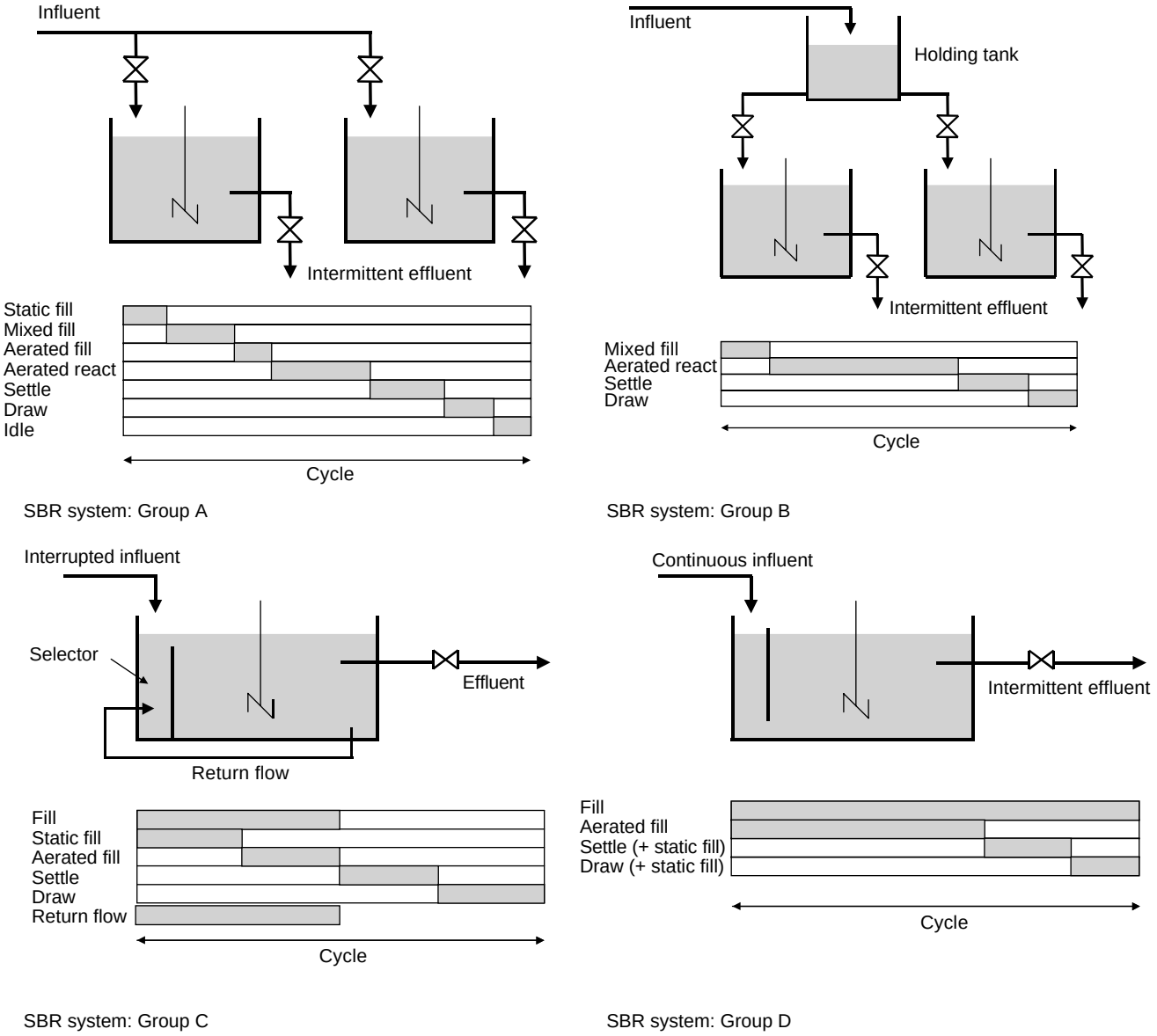

Figure 4. Four different groups of SBR systems currently applied in practice (6).

Each of these modifications provides specific advantages and disadvantages. Selection of a specific SBR type must be made on a case-to-case basis after taking into account the conditions under which the SBR will be operated and the treatment goals that must be achieved (6). Care should be taken, however, to select a system that maximizes the difference between the "intensity" of the feast and the "depth" of the famine. Doing so will provide protection against bulking sludge while maximizing the potential for biological nutrient removal.

SIZING OF SBRS

Whenever possible, design calculations for an SBR plant for tank size and cycle program (i.e., the operating strategy that defines the sequence and duration of phases) should be based on results obtained from treatability studies conducted at either bench or pilot scale. Treatability studies are especially recommended when the wastewater to be treated is unique with respect to composition, concentration, and variability as is the case for virtually all industrial wastewaters and landfill leachates. In these cases, design is based on a mathematical description of the facility that integrates projections of future flow and loading conditions with the data collected during the treatability studies (e.g., stoichiometric, kinetic, settling, oxygen transfer, nutrient requirements, etc.).

The design of SBRs for municipal wastewater treatment facilities is often done without the aid of treatability studies because it is assumed, sometimes incorrectly, that municipal wastewater varies little from location to location. When the characteristics of municipal wastewater

is "typical," the design and operating conditions that are selected are based on experience using methods that simply set the hydraulic residence time (HRT) and organic loading to size the system. In other cases, especially for the design of large facilities and/or when nutrient removal is required, engineers often combine experience with one of the many commercially available versions of computer models based on International Water Association (IWA, formerly IAWQ) (10) to test and refine possible design. These simulation programs use default values for stoichiometric and kinetic coefficients. An intermediate approach to these two extremes is used in Germany where the Waste and Wastewater Association (ATV) issued guidelines (11) for the design of SBR plants treating municipal wastewater. This guideline is based on established design procedures for continuous-flow systems. Table 2 describes the various assumptions and calculations that must be made when using these guidelines.

It is assumed that the mass and the activity of the activated sludge in the SBR will be the same as in a continuous-flow activated sludge system with plug flow characteristics (i.e., a cascade of three CMFRs). It is further assumed that the order and the duration of the SBR process phases are proportional to the design setting and to the mean volumetric retention time in the various zones of the continuous-flow systems (e.g., anoxic zone and aerobic zone).

One of the several possible design procedures directly based on mass balances is presented in Table 3. The example chosen is based on COD as a measure for the organic fraction of the wastewater constituents.

In determining an appropriate θ_X needed for nitrogen removal, nitrification capacity (N_{OX}) and denitrification potential (N_{DP}) are two important factors that were first defined for recirculation-type continuous-flow processes (12,13).

After selection of the relevant sludge age (θ_X), the total biomass present in the SBR reactors is calculated on the basis of the mass of influent COD removed. The water volume needed in the reactor after the draw phase, V_0, is calculated by determining the volume occupied by the biomass after settling for 30 minutes. The value is multiplied with a safety factor (SF_V) to cover any uncertainties with respect to the ability of the sludge to settle and growth of the respective groups of microorganisms.

The exchanged volume (ΔV_f) depends on the selected cycle time. The total volume of the SBRs (V_R) is obtained by adding V_0 and ΔV_f. Further information on using mass balances for the design of activated sludge systems can be found in Orhon and Artan (14) and Artan and coworkers (15).

EXPERIENCES

Unlike conventional technology, SBRs tend to be designed and marketed by equipment suppliers. To date there is little well-documented information on how existing SBRs are performing, including their cost-effectiveness, reliability, optimum design and operation, cost, and performance associated with different SBR configurations and equipment suppliers. The growth and acceptance of SBRs appear to be based on their ability to meet permit limits and at their competitive capital and operating costs.

During the past three years, several surveys were conducted in various countries to study the performance of SBR facilities (6). Survey results revealed that designer and operators were often not aware of the full range of control that is provided by SBR technology. Despite these problems, effluent values were well below permit limits.

In rural areas, the combination of a high-technology system (i.e., an SBR) and a natural system (e.g., effluent polishing ponds) was very effective with respect to chemical oxygen demand (COD) and five-day biological oxygen demand (BOD_5) removal. Only small variations

Table 2. Design of an Activated Sludge SBR Plant Based on the German ATV Guidelines (11)

1. Input: Design information from continuous flow system, operating strategy for the SBR	
2. Calculation of the time (t_a) during which biological reactions may take place	$t_a = t_c - t_s - t_d - t_{idle} - t_{staticfill}$
3. Effective mass of sludge in the SBR equals the mass of sludge in the continuous-flow system	$M_{X,SBR} = M_{X,Conti.} \cdot \frac{t_c}{t_c - t_s - t_d}$
4. Select MLSS in SBR after fill and, thus, $n \cdot V_R$	$n \cdot V_R = \frac{M_{X,SBR}}{C_X}$
5. Select V_0 depending on the volume of the settled sludge	$\min(n \cdot V_0) = M_{X,SBR} \cdot SVI \cdot SF_V$ or $VER = 1 - C_X \cdot SVI \times SF_V$
6. Select exchange ratio or cycle time	
7. Select number of SBRs (n) and calculate missing value (volumetric exchange ratio (VER) or cycle time)	$\frac{t_c \cdot Q}{n \cdot V_R} < VER$
8. For a system with nitrogen removal, select mixed and aerated process phases according to mixed and aerated volumes of the continuous-flow system	$\frac{t_D}{t_D + t_N} = \frac{V_D}{V_D + V_N}$
9. Check t_s and t_d based on settling velocity and maximal draw flow rate, respectively	

Note: Table prepared by E. Morgenroth, University of Illinois at Urbana-Champaigne, U.S.A.

Table 3. Design Procedure Based on Mass Balances

1. Input: operating strategy for SBR	
2. Select solids retention time (θ_X)	
3. Estimate effective fraction of t_c from t_s and t_d	$\text{effective fraction} = \frac{t_c - t_s - t_d}{t_c}$
4. Calculate mass of sludge in all reactors	$M_{X,SBR} = Y_{H,net} \cdot Q \cdot \Delta C_S \cdot \theta_X \cdot \frac{t_c}{t_c - t_s - t_d}$
5. Calculate volume of settled sludge in all reactors	$n \cdot V_0 = M_{X,SBR} \cdot \text{SVI} \times \text{SF}_V$
6. Select exchange ratio or cycle time	
7. If exchange ratio was selected, then calculate cycle time from	$t_c = \frac{n \cdot V_0 \cdot \text{VER}}{Q \cdot (1 - \text{VER})}$
8. Calculate exchange volume of all reactors	$n \cdot \Delta V = Q \cdot t_c$
9. Calculate total volume of the SBRs	$n \cdot V_R = n \cdot V_0 + n \cdot V_f$
10. Select number of SBRs (n)	
11. Calculate minimum aerated fraction of reaction time on the basis of minimal aerobic solids retention time, for example, nitrification	$\text{min .aerated fraction} = \frac{\min \theta_{X,a}}{\theta_X} \cdot \text{SF}_X$
12. Choose number of fill phases and distribution of the influent on the fill phases depending on the wastewater composition (e.g., TKN/COD ratio)	
13. For a system with nitrogen removal, check nitrification capacity and denitrification potential depending on cycle design	$N_{nit} = TKN_0\text{-}NH_{4,e}\text{-}N_{org,e}\text{-}N_{SL}$ $N_{DP} = (1 - Y_{H,net}) \cdot C_{S,in} \cdot \frac{1}{2.86} \cdot f_{DN} \cdot \eta$
14. Check react time for nitrogen removal using denitrification rates	$t_D = \frac{N_{DP} \cdot n \cdot V_f}{r_{DN,X} \cdot M_{X,SBR}}$
15. Check t_s and t_d based on settling velocity and maximum draw flow rate, respectively	

Note: Table prepared by E. Morgenroth, University of Illinois at Urbana–Champaign.

are found in the effluent despite the wide range of influent concentrations. Even very low effluent standards such as 40 mg L^{-1} COD were safely met.

A study funded by Ontario Ministry of the Environment, Environment Canada, and the Water Environment Association of Ontario evaluated 75 SBR-like installations in Ontario and the United States Great Lakes Region (16). A telephone and mail survey was conducted and 10 SBR facilities were visited in 1998 to define possible design and operational problems. The average daily design flow for the 67 of the 75 SBR plants providing such data was approximately 3,600 $m^3\,d^{-1}$ and ranged from a low of 7 $m^3\,d^{-1}$ to a high of 22,710 $m^3\,d^{-1}$. Results obtained from the 75 SBR plants were prioritized according to operating costs, plant capacity, effluent quality, and frequency of occurrence. No effort was made to subcharacterize the findings in terms of the four generic groups of SBRs described earlier.

The average daily flow for the 49 of the 75 SBR plants reporting actual flow data was nearly 2,600 $m^3\,d^{-1}$. Forty-six of the plants reported effluent readings for BOD_5; 58 for SS; 52 for ammonia-nitrogen; and 33 for total phosphorus (as P). The average results for those reporting were less than 6.5 $g\,m^{-3}$, 9 $g\,m^{-3}$, 1.5 $g\,m^{-3}$, and 1.4 $g\,m^{-3}$ for BOD_5, SS, NH_3, and phosphorous, respectively. These results are quite impressive for facilities located in either warm or cold regions. In addition, a preliminary cost comparison between SBRs and continuous-flow activated sludge plants indicated that for similar effluent requirements, SBRs-like facilities were more economical than continuous-flow activated sludge plants.

The responses received during the survey were used to generate a list of concerns, which, if addressed, would likely improve the performance of these SBR-like installations. The top ten of these are summarized in the following text:

1. Operators have no formal training on SBR operation or process control.
2. Mechanical equipment located outdoors may freeze or malfunction (e.g., air valves, solenoid valves, decanter arms, and level monitoring floats).
3. Decanters were unable to meet some specific treatment requirements (e.g., the discharge of floatables).
4. Discontinuous SBR effluent flow have an impact on downstream treatment processes (e.g., ultraviolet (UV) disinfection and the backwashing of filters).
5. Lack of online DO monitoring and control instrumentation eliminated the possibility of increased energy savings.
6. No specific control strategy was provided for the solids residence time.
7. The pretreatment systems (e.g., bar screens and comminutors) were inadequately designed.
8. Lack of automation for the selection of the time needed for sludge wasting.
9. Potential secondary phosphorus release in aerobic digesters.
10. Partial failure of the SBR control program during peak flows.

Most of the concerns listed earlier could easily be corrected if the operators were given a formal training program. Unfortunately, there is not a simple solution because the term *SBR* is used to include many different types of periodically operated reactor configurations, even more than the four generic types described earlier, and the specific training for each type would be different. The SBR is also a new technology that is being implemented by some who have little basic understanding of how to make it work best.

A few of the concerns listed previously are specific to the equipment used for SBR facilities. These are difficulties that can be overcome by using the equipment specified by qualified engineers and manufactured and installed by reputable vendors. The remaining concerns were independent of the type of treatment facility installed and are just as likely to cause difficulties at a conventional continuous-flow activated sludge plant as they are at an SBR system. It is worth noting that the effluent data compiled showed that the effluent criteria were consistently met, and in most cases, exceeded.

The evaluations indicate that SBRs are a cost-effective wastewater treatment technology (17,18). Unfortunately, limited historical data have been compiled comparing the cost of SBRs with other types of activated sludge treatment systems. Clearly, the lack of need for an external secondary clarifier and its associated hardware and the return sludge pumping system offers potential savings in construction costs. In addition, primary clarification is not normally employed (none of the 75 plants evaluated had primary clarifiers).

NOVEL APPLICATIONS FOR BIOFILM AND SLURRY SYSTEMS

The concept of implementing short-term unsteady state conditions to control the long-term performance of biological reactors is applicable to either aerobic or anaerobic systems, or to suspended growth or biofilm, or fluidized bed systems that treat wastewater, contaminated soil, or gases. Over the past years, a wide variety of innovative batch treatment technologies have been described in literature. The common basis of these novel approaches is application of well-defined sequences (i.e., operating strategies) that alter process conditions in a controlled manner. The common goal is control of the composition and activity of microbial communities by targeting the microorganisms that should be selected, enriching these organisms to the levels needed, and then adjusting their physiological state so that permit limits are achieved. Two of those systems, the Sequencing Batch Biofilm Reactor (SBBR) and the Soil Slurry-Sequencing Batch Reactor (SS-SBR), are described briefly here.

Gonzales and Wilderer (19) and Wilderer (20) proposed the sequencing batch operation of a biofilm reactor. The schematic of a SBBR plant is presented in Figure 5. The sequence of process phases is sketched in Figure 6.

Kaballo and coworkers (21) compared the response of continuous-flow and SBBR reactors to peak loading.

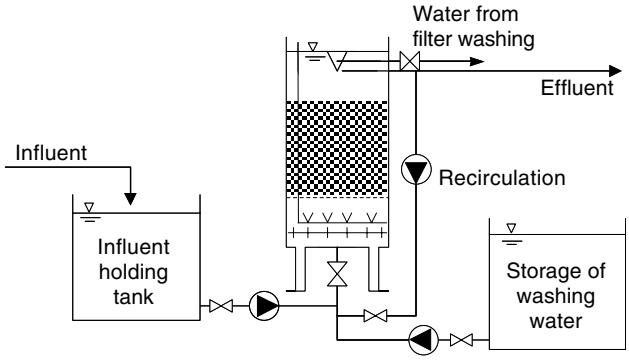

Figure 5. Schematic of a SBBR plant (6).

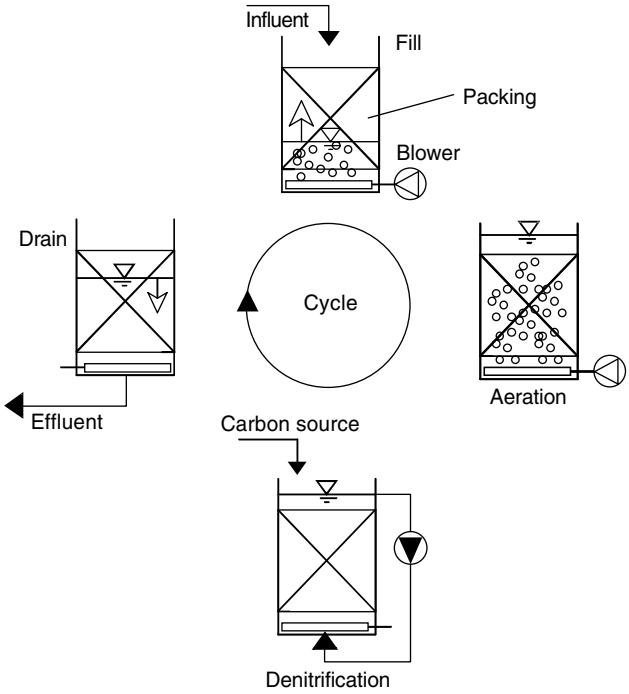

Figure 6. Schematic representation of the SBBR process.

Breakthrough events can be successfully avoided by temporary prolongation of the duration of the react phase.

Jaar and coworkers (22), Kolb (23), Chozick and Irvine (24), and Irvine and coworkers (25) packed the reactor with granular activated carbon. By means of adsorption processes during the fill phase, the concentration of critical pollutants (e.g., volatile organic compounds or inhibitory organic substances) were reduced in the bulk liquid. During the react phase, biodegradation and desorption became the dominant processes. The wastewater was effectively treated and the activated carbon was regenerated biologically.

Irvine and Montemagno (26) proposed the sequencing batch operation of a soil slurry reactor in 1989 for the remediation of trinitrotoluene-contaminated soils. In 1993, Irvine and coworkers (27,28) reported on how the SS-SBR can be used to treat soil contaminated with the plasticizer bis-(2-ethylhexyl) phthalate and petroleum products.

The schematic of an SS-SBR facility is presented in Figure 7.

The SS-SBR is operated on a fill and draw basis in a manner similar to that described for the SBR. Each tank is filled during a discrete period (fill phase), and then operated as a batch reactor during the react phase. Just as for the SBBR, the cycle for the SS-SBR does not include the settle phase (Fig.7). After the desired contaminant levels have been reached, a fraction of the slurry is removed from the SS-SBR (draw phase). A certain fraction of the treated slurry remains in the reactor to provide acclimated microorganisms for the next batch of untreated slurry. The optimum recycle fraction in the SS-SBR must be determined for each contaminated soil (27–29).

As can be seen in the bioslurry system illustrated in Figure 7, the soil is first screened to remove rocks, branches, and other debris. Water is added to the screened soil to make a slurry in a small mixing tank. The concentration of solids in a bioslurry reactor can range from 5 to 50% (weight/volume) depending on the contaminant concentration and the capacity of the mixing and aeration equipment. Nutrients and other possible amendments (e.g., microbes and surfactants) are also added to the mixing tank if needed. The slurry is then transferred to the bioslurry reactor via a slurry pump. Compressed air diffusers or surface agitators can be used to provide oxygen. Bioslurry reactors are easily covered and equipped with an emissions recovery system. Fugitive emissions may require a separate treatment before discharge or they can be recycled back into the slurry reactor for biodegradation. After the soil is treated, solids are separated from the slurry water using standard dewatering techniques.

The SBR has also been shown to be a cost-effective and energy-efficient means of removing hazardous organic compounds found in industrial wastes (30) and leachates from landfills (31–33). Periodically operated biofilters have recently been proposed for the treatment of air contaminants (33,34–36).

CONCLUSION

SBR technology defines a controlled unsteady process that imposes selective pressures and manipulates both the community structure and expression of a heterogeneous microbial consortium in a multipurpose bioreactor so that the impact of varying influent conditions is minimized. The SBR concept can be applied to the treatment of wastewater in both activated sludge and biofilm systems and to the treatment of contaminated soils and gases.

In contrast to the continuous-flow activated sludge systems, biomass separation occurs in the biological reactor and not in a separate clarifier. The unit operations and unit processes that take place in each reactor follow each other in a time sequence that is progressively repeated in a "periodic" or "cyclic" manner and not from tank to tank as they do in space-oriented systems. A portion of the treated water is periodically discharged from each tank to make room for a new batch of wastewater. Because of these features, SBRs are referred to either as periodic processes, single-tank systems, fill-and-draw reactors, or as variable volume reactors.

The SBR process is characterized by a series of process phases (e.g., fill, react, settle, draw, and idle) each lasting for a defined period. Four generic groups of SBRs were distinguished, each characterized by the specific fill strategy utilized, or the inclusion of a react or idle phase. Each generic group provides specific advantages and disadvantages. Selection of a specific SBR type must be made on a case-to-case basis after taking into account the conditions under which the SBR will be operated and the treatment goals that must be achieved. Care should be taken, however, to select a system that maximizes the difference between the "intensity" of the feast and the "depth" of the famine; this will protect against bulking sludge while maximizing the potential for biological nutrient removal. The technical report on SBR technology issued by the International Water Association (IWA Publishing) was written to provide a comprehensive description of the information needed by designers and operators (6).

NOMENCLATURE

BOD_5	Five-day biological oxygen demand	$g\,m^{-3}$
COD	Chemical oxygen demand	$g\,m^{-3}$
C_S	Substrate concentration	$g\,m^{-3}$
C_x	Biomass concentration	$g\,m^{-3}$
FTR	Fill time ratio, t_f/t_c, where t_f is the time for fill	–

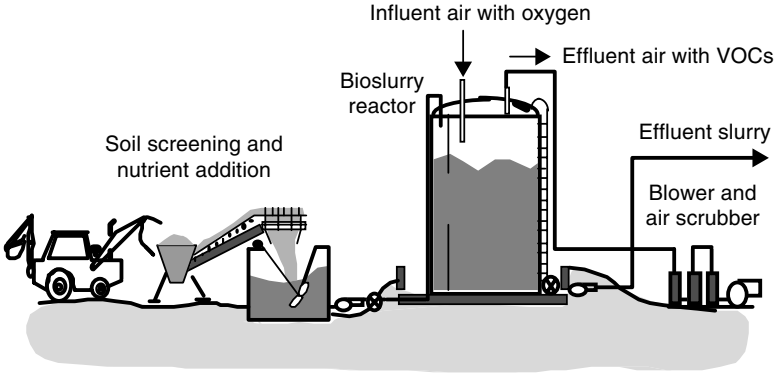

Figure 7. Schematic representation of a SS-SBR.

f_{DN}	Fraction of electron acceptors potentially associated with anoxic reactions	–
HRT	Hydraulic residence time, $n \cdot V_R Q^{-1}$	h
M or M_x	Mass of sludge	g
MLSS	Mixed liquor suspended solids	$g\,m^{-3}$
N	Concentration of nitrogen compounds	$g\,m^{-3}$
N_{DP}	Denitrification potential	$g\,m^{-3}$
N_{nit}	Nitrification potential	$g\,m^{-3}$
N_{SL}	Nitrogen concentration of the waste sludge	$g\,m^{-3}$
N	Number of tanks or reactors	–
PAC	Powdered activated carbon	–
Q	Flow rate (plant influent)	m^3h^{-1}
$r_{DN,X}$	Denitrification rate	$g\,g^{-1}h^{-1}$
SVI	Sludge volume index	$ml\,g^{-1}$
t_c	Total cycle time ($t_c = \Sigma t_i$)	h
t_d	Time of the decant phase	h
t_D	Time of the denitrification phase	h
t_i	Time of the ith phase	h
t_{idle}	Time of the idle phase	h
t_f	Time of the fill phase	h
t_N	Time of the nitrification phase	h
t_s	Time of the sedimentation phase	h
$t_{staticfill}$	Time of the no-mix/no-aeration fill phase	h
SF_v	Safety factor	–
TKN	Total Kjeldahl nitrogen	$mg\,L^{-1}$
VER	Volumetric exchange ratio, $\Delta V_f / V_R$	–
V_0	Liquid volume at low water lever	m^3
ΔV_f	Fill volume	m^3
V_R	Total liquid volume at high water level	m^3
V_D	Volume of the denitrification zone (continuous-flow plant)	m^3
V_N	Volume of the nitrification zone (continuous-flow plant)	m^3
ΔV_w	Volume of the wasted sludge	m^3
ΔV_d	Volume of the decanted effluent	m^3
Y	Cell yield	$g\,g^{-1}$
θ_X	Sludge age (HRT)	d
$\theta_{X,a}$	Aerobic sludge age	d
η	Correction factor for oxic respiration	–
τ	Hydraulic retention time (HRT)	h

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ACTIVATED SLUDGE — THE FLOC

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Use of activated sludge is the most common process worldwide for treatment of numerous types of wastewaters. The main process is the biological conversion of organic matter, nitrogen, phosphorus, and other compounds into biomass (sludge) and gaseous compounds (1,2). Another key process is the separation of the biological sludge flocs from the treated water, the solid-liquid separation. This process relies entirely on the formation of good, activated sludge flocs to ensure a proper effluent quality and a good dewaterability of the sludge (1,3,4).

The solid-liquid separation involves several steps (Fig. 1): floc formation in the process tanks and flocculation, clarification, and settling in the final clarifier to retain the majority of the sludge. A large percentage of the retained sludge (typically 80–97%) is recycled in order to keep a sufficiently high sludge concentration in the process tanks. In a typical activated sludge treatment plant, the amount of suspended solids in the process tanks, the mixed liquid suspended solids (MLSS), is typically 3 to 5 g suspended solids/L (gSS/L). To meet effluent standards of less than 20 to 30 mgSS/L, it is obvious that the entire floc formation and the separation process must work well. Typical problems are often encountered because of poor flocculation or poor settling of the sludge flocs (1,3,4).

The dewatering and further treatment of the surplus sludge is also an important step in the solid-liquid separation (5). This step is usually costly, and special care must be taken to handle sludge with a high content of pathogenic bacteria, heavy metals, micropollutants, and other unwanted substances. The dewatering process is strongly affected by the floc properties, and problems with the dewatering process caused by poor floc properties are often encountered (5).

Activated sludge flocs, like other biological aggregates such as biofilms, are complex heterogeneous structures in which bacteria and other chapter provides an overview of the physical, chemical, and microbial composition of the flocs. Furthermore, the principles of flocculation and possible correlations between floc composition, floc properties, and functional sludge properties are described.

PHYSICAL AND CHEMICAL STRUCTURE OF FLOCS

An activated sludge floc consists of many different components as shown in Figure 2. The components are bacterial cells, various types of extracellular polymeric substances (EPS), adsorbed organic matter, organic fibers, and inorganic compounds. The bacteria can be single, growing in microcolonies (sometimes termed clusters or microflocs) or growing as filaments. This basic composition is common to all flocs, but the relative proportion of the components and the exact types of chemical compounds or types of microorganisms vary from plant to plant. In plants treating domestic wastewater, the cell biomass typically constitutes 10 to 20% of the organic matter, whereas the size of other fractions is not certain. Typical values from various sources are shown in Figure 3 (6–8).

The size of activated sludge flocs ranges from very small aggregates of only a few cells (few μm) to large flocs of more than 1 mm. In most activated sludges, the flocs are typically 40 to 100 μm in diameter, relatively strong, and not easy to break apart (3,4,9). Usually, this fraction also constitutes the largest part of the sludge as measured by volume, whereas very small flocs that are numerous and single cells are less important in terms of mass or volume. An example of size, surface area, mass, and volume distribution of activated sludge flocs for five conventional treatment plants is shown in Table 1 (10). Under certain circumstances, very dense flocs or granules

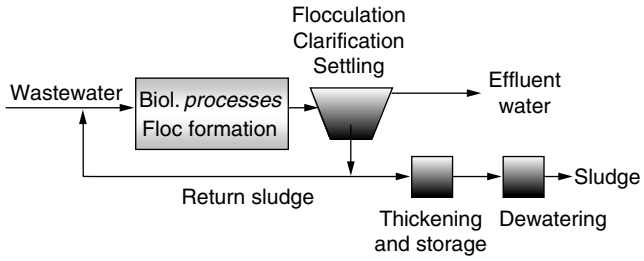

Figure 1. Floc formation and solid-liquid separation in an activated sludge treatment plant.

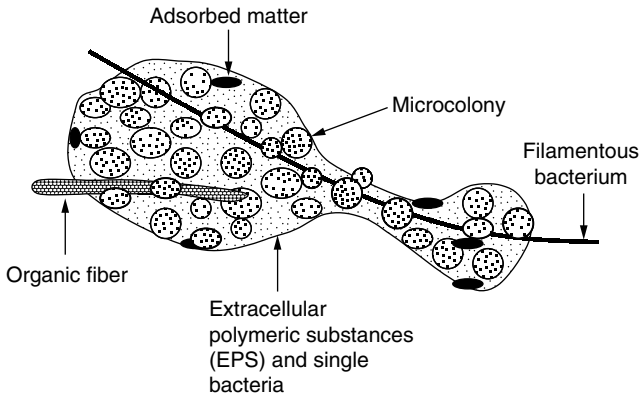

Figure 2. Schematic illustration of the constituents of activated sludge flocs.

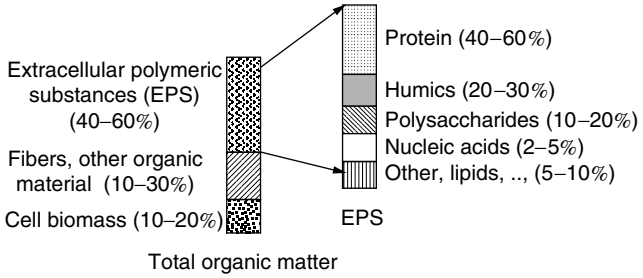

Figure 3. Composition of the organic part of sludge (EPS: Extracellular polymeric substances).

with a diameter of 300 to 500 μm can be formed in aerobic activated sludge systems. So far, however, aerobic granule formation has only been reported from laboratory-scale reactors (11).

The individual flocs can undergo three main types of transformation when they are subjected to some stirring (shear): erosion, fragmentation, and flocculation (Fig. 4). Fragmentation, during which flocs are split into a few large new flocs, is not considered important for activated sludge flocs (4). Erosion takes place when small particles, typically bacteria or EPS fragments are sheared off the main floc because of several processes that are described later.

Flocculation of the individual flocs into loosely connected, voluminous floc-aggregates takes place in the process tanks and in the final clarifier because of the high content of mixed liquor suspended solids. In the clarifier, the floc-aggregates have sizes up to several mm in diameter. Although these may have a lower density than smaller flocs, they settle faster on account of their greater size and lower friction (Fig. 4). The very loose structure of large floc-aggregates appears to allow a flow of water through the porous structure during the movements in the process tank and during settling (12).

Morphology of individual flocs is highly variable, ranging from dense, strong flocs to very irregular, loose structures (Fig. 5). The organization of the various structures, microcolonies, bacteria and the like is very heterogeneous (8,13). It is possible to quantify the floc shape by its fractal dimension. Typical values of 1.9 to 2.5 (14) have been reported, showing the loose structure and high porosity of flocs. The floc size, shape, and other properties can be described qualitatively (3,15) according to simple microscopic investigations and related to common operational problems in activated sludge plants (3):

1. Dispersed growth, where hardly any true flocs are formed and cells are freely suspended in liquid;
2. Pinpoint floc, where the flocs are small and fragile without a proper macrostructure or backbone from filamentous bacteria;
3. The ideal floc, where the filaments are largely restricted to the floc interior with limited extension into bulk water;
4. Filamentous bulking sludge, with extensive growth of filamentous bacteria inside and between the flocs.

Table 1. Examples of Distribution in Activated Sludge Flocs in Size, Surface Area, Volume, and Mass (10)

	Distributions			
Size (μm)	By Number	By Area	By Volume	By Mass
<2	30–80%	<2%	Negligible	2–8%
2–16	17–70%	1–8%	Negligible	5–28%
16–128	1–4%	20–70%	10–80%	50–80%
128–256	<1%	5–50%	10–70%	5–25%
>256	Negligible	0–50%	0–60%	0–30%

The Chemical Composition of Flocs

On the basis of weight, the organic matter (the volatile suspended solids, VSS) is usually the largest fraction, typically 60 to 80% of the dry matter weight (16). The inorganic fraction consists of various ions, some within the bacteria and some (mainly Mg^{2+}, Ca^{2+}, Fe^{3+}) adsorbed in the EPS matrix, and attached minerals, such as clay. Most of the organic matter in activated sludge is protein

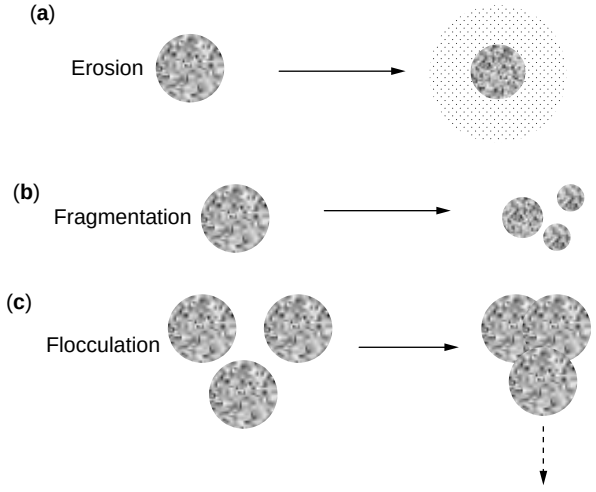

Figure 4. Flocculation behavior of activated sludge flocs.

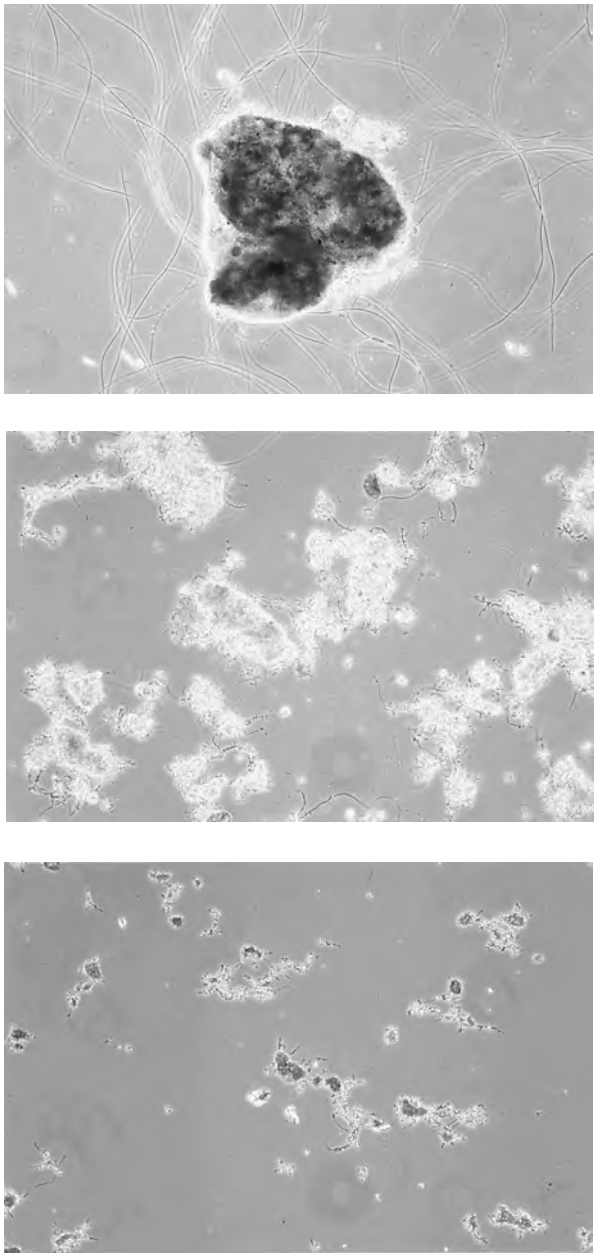

Figure 5. Examples of activated sludge flocs. (**a**) Large strong flocs form a nutrient removal plant, (**b**) Typical activated sludge from nutrient removal plant, (**c**) Small, dispersed flocs.

(40–60%), and polysaccharide material, humic substances and other compounds (e.g., lipids, nucleic acids) each constitute 10 to 20% (6). Only a minor part of these compounds represents the living cell biomass. Therefore, these compounds arise mainly from the EPS fraction, the adsorbed organic debris, and the fibers.

The EPS matrix consists of various macromolecules, such as proteins, polysaccharides, nucleic acids, humic substances, various heteropolymers, lipids, and so on. (Fig. 3). The macromolecules are partly exopolymers produced by bacterial activity and lysis and hydrolysis products, but they are also adsorbed from the wastewater. Because the EPS matrix is widely acknowledged to be very important for the floc properties (see the following section), several attempts have been made to quantify and characterize this fraction in greater detail. However, today there is no single universal extraction method that effectively extracts all EPS components without cell lysis (17). The most efficient extraction method at present is based on a combination of shear and ion exchange (17) and shows that protein is the predominant compound (Fig. 3). The total amount of EPS has been estimated to constitute 40 to 60% of the organic matter in activated sludge flocs (17). The exopolymers may be very different from one microcolony-forming species to another, and because part of the EPS components seems to be loosely attached to the flocs as a "cloud" (18), it is difficult to extract EPS that is representative of the entire floc.

It is important to note that polysaccharides, which are assumed to be an important part of bacterial exopolymers, are not present in large amounts in typical sludge flocs (6,17). Instead, protein seems to act as the most important "glue" component. The exact function of the large protein pool is not well understood, but exoenzymatic activity is present (19,20) and lectin-like proteins may have a structural function (21). Humic substances can be a large fraction in systems in which these substances are present in the wastewater and in which the sludge age is long.

As described in the next section, the EPS fraction is important for the formation of a three-dimensional gel-like network that keeps all the cells and other constituents together. The surface charge of the EPS-covered entities is generally assumed to be important for the gel properties and can be characterized by the zeta potential (or electrophoretic mobility), pH titration, and colloid titration. Typical values for the surface charge of activated sludge floc components are -0.5 to -1 meq/g SS (22,18).

Floc Microbiology

Most bacteria are known to grow in aggregates (flocs or biofilms) in natural and engineered systems, which provide a number of advantages for the bacteria when compared to suspended growth. In particular, the presence of EPS components ensures a well-buffered local chemical environment that provides substrate and important ions and protection against predators and toxic compounds. Furthermore, close proximity to other cells improves interspecies substrate and gene transfer. Considerable effort has been made in recent years to gain knowledge about the most important mechanisms controlling floc and biofilm formation. This has been supported by the recent development of tools, such as light microscopy, epifluorescence microscopy, and confocal laser scanning microscopy (CLSM) (23), for in situ studies. Use of microsensors has provided valuable knowledge about chemical gradients (24); fluorescence in situ hybridization (FISH) with gene probes and other molecular methods have revealed information about the identity and phylogeny of important microorganisms (25,26, FILAMENTOUS BACTERIA IN ACTIVATED SLUDGE: CURRENT TAXONOMIC STATUS AND ECOLOGY), and microautoradiography (MAR) provides information about their ecophysiology (27,28).

The bacteria within the flocs are present as single cells, as microcolonies, or as filamentous bacteria (Fig. 2). The filamentous bacteria are, in general, well known and well described in municipal treatment plants (3,15, ACTIVATED SLUDGE — MOLECULAR TECHNIQUES FOR DETERMINING COMMUNITY COMPOSITION), but much less is known about filamentous bacteria in industrial plants. Little is known about single cells in the flocs. Some are probably attached and incorporated into the floc matrix from the incoming wastewater and may not be active. However, several species, for example, *Spirochaetes* and *Spirillum*, can be observed by microscopy to be moving around in some sludge types (15), showing that not all active bacteria grow in microcolonies.

Microcolony formation is described among several bacterial groups in activated sludge, including *Zoogloea* spp., often forming very typical finger-like colonies easily recognizable by microscopy (15,29). *Zoogloea* spp. were originally believed to be the most important species forming flocs in activated sludge, but it appears that they are mainly present in large amounts in high-loaded conventional systems and hardly present in advanced treatment plants with N- and P-removal (30,31). They are also often present in treatment plants with plug flow or selectors to control filamentous bulking (1). Bacteria belonging to the genus *Thauera* can form zoogloeal clusters in industrial wastewater treatment plants (31). Little is known about other important microcolony-forming heterotrophic bacteria with regard to both their phylogeny and physiology. Several species have been isolated from flocs (2), but their importance remains unknown. In activated sludge from treatment plants performing biological phosphorus removal, microcolonies of phosphorus accumulating organisms (PAOs) are present and seem to form strong colonies of importance to the floc properties. However, these bacteria have not yet been properly identified (ACTIVATED SLUDGE — MOLECULAR TECHNIQUES FOR DETERMINING COMMUNITY COMPOSITION). In many plants the so-called "G-bacteria" are also present, and they form small, distinct microcolonies (ACTIVATED SLUDGE — THE "G-BACTERIA").

Autotrophic nitrifying bacteria can form large microcolonies within the flocs in nitrifying treatment plants. Several studies using FISH have clearly shown that ammonium oxidizers (mainly *Nitrosomonas*, *Nitrosococcus*, and *Nitrosospira*) as well as nitrifiers (mainly *Nitrospira*) grow in colonies, often in close proximity to each other (32: ACTIVATED SLUDGE — MICROBIOLOGY OF NITROGEN REMOVAL). These colonies appear to be strong and difficult to break apart.

The total number of bacteria in activated sludge, as determined by DAPI-staining using epifluorescence microscopy, is reported to be approximately 10^{12} per g VSS (6). Living or active cells can be identified using fluorescence in situ hybridization (FISH) with gene probes, by reduction of the redox dye CTC, or by microautoradiography (26,33,34). This fraction typically constitutes 80 to 90% of all cells in activated sludge although CTC active cells are often fewer (33). Most of the bacteria seem to be heterotrophic (34). In nutrient removal plants, the nitrifiers typically constitute 1 to 10% of the total bacterial population. Less is known about the other groups such as Fe(III)-reducers and sulfate- reducers, but they are always present (35).

Mechanisms of Floc Formation

Formation of activated sludge flocs (bioflocculation) is controlled by several processes as illustrated in Figure 6. Formation is partly determined by adsorption or flocculation of various components (e.g., of bacteria from the wastewater) and partly by growth and other biological processes taking place within the floc. Among the dominant physicochemical processes are adsorption-desorption of bacteria, EPS-components, and other organic and inorganic compounds. The desorption process, that is, when the components are detaching, is usually termed deflocculation, disintegration, or erosion of the flocs (4).

It is also important to note, as illustrated in Figure 6, that bacteria that do not properly adhere to the flocs will not be retained in the treatment plant and will leave with the treated effluent from the treatment plant. Thus, an accumulation and selection of bacteria (and other compounds) only takes place if they adhere well to the flocs or if they grow within the flocs.

Physicochemical Factors. The different constituents of the floc are embedded in an EPS matrix (or gel) kept together by various intermolecular forces. The forces are the DLVO interactions (van der Waals and electrostatic forces) and non-DLVO interactions (hydrogen bonds, hydrophobic forces, steric forces, and bridging of EPS by means of multivalent cations) (36,37). Furthermore, physical entanglement of the long EPS macromolecules may be important in the formation of the network structure of the floc. In particular, electrostatic forces and bridging are believed to be important. Most floc constituents are negatively charged overall because of the presence of proteins, polysaccharides, and humic substances with net negative charges at neutral pH (e.g., carboxylic groups). These are largely cross-linked by divalent (Mg^{2+}, Ca^{2+}) and trivalent cations (Fe^{3+}, Al^{3+}). The hydrophobic forces are probably also important, but less understood. It is known, however, that when relatively hydrophobic bacteria are added to activated sludge, they adhere better to sludge flocs than more hydrophilic bacteria (38).

Figure 6 deals with adsorption-desorption phenomena and consequently with various equilibria. These are determined by the turbulence in the system (e.g., aeration, stirring, and pumping) and by the interactive forces of the entities, which are largely determined by the ionic strength, ionic composition, and pH mentioned earlier. As described in the following section, changes in these parameters will affect the floc composition and the floc strength.

Biological Mechanisms. A main process in floc formation in the activated sludge is growth and activity of the bacteria within the floc. In addition, bacteria and particles from the incoming wastewater are adsorbed by existing flocs. Most bacteria entering the treatment plant are already aggregated in the sewer system or

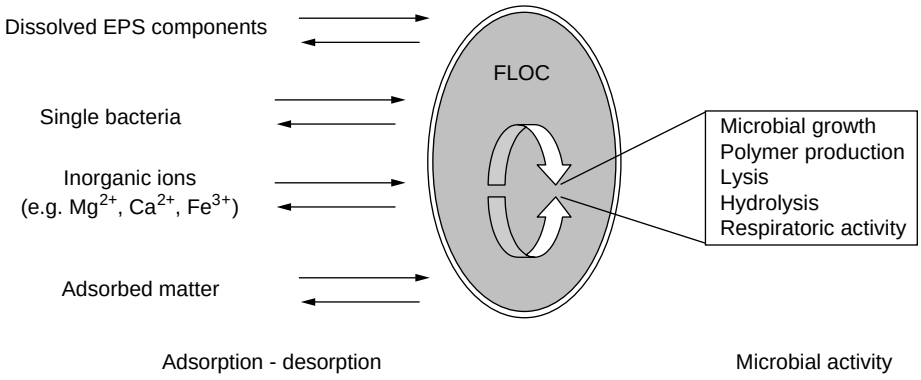

Figure 6. Formation of activated sludge floc.

present as detached sewer biofilm components. Therefore, the aggregation mechanisms known from pure culture, including specific adhesion and aggregation of a number of single cells (39), rarely occur in activated sludge systems.

Biological activity within the floc encompasses cell growth, exopolymer production, hydrolysis, death, and lysis (Fig. 6), as is also described for biofilm systems (40). Cell growth depends on the availability of soluble substrates, which is controlled by their diffusion into the floc, and by hydrolysis of the particles adsorbed from the wastewater. Often most of the organic substrate in wastewater is present as particles and colloids, and hydrolysis is required before it is available for consumption. This is reflected by the high activity of extracellular enzymes in the floc matrix (19,41). Very little is known about the controlling factors for general exopolymer production within the flocs. From pure culture studies it is known that a number of factors, such as bacterial species, their growth stage, type, concentration of substrate, and limitation of N, P, or micronutrients, and so on (40), affect the production of exopolymers (mainly proteins and polysaccharides). In activated sludge, bacteria are generally starved and exposed to dynamic conditions (1). These conditions seem to promote floc formation, perhaps as a result of an increased exopolymer production compared to cell production.

Flocs and other aggregates are characterized by high cell densities, which induce inter- and intraspecific interactions between the cells. An example is the production of low molecular weight, diffusible signaling molecules (autoinducers). These compounds can be involved in the induction of various genes that are responsible for aggregation behavior, EPS-production, disaggregation, and so on. Thus, cell-to-cell communication may be of fundamental importance to the dynamics of aggregation in flocs and biofilms and needs more attention in the future (42,43).

Stability of Activated Sludge Flocs. If activated sludge in a specific treatment plant is exposed to changes in certain environmental parameters, changes in the stability of the flocs also take place. A change typically causes a weakening of the floc and thus an undesired deflocculation, in particular if it is exposed to shear forces. This is illustrated in Figure 7, where the floc strength is characterized by the development in suspension turbidity with time when sludge is stirred under

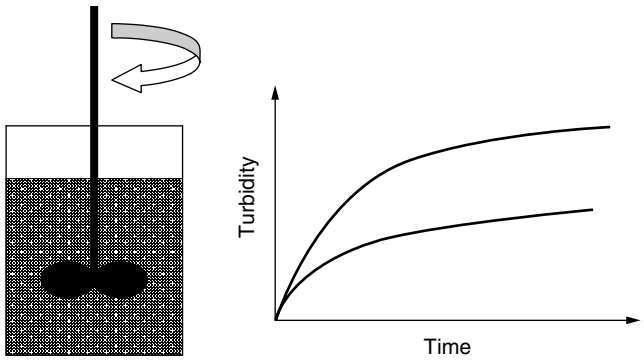

Figure 7. Measurement of the floc strength of activated sludge flocs. The sludge is stirred under defined conditions for two to three hours, and the increase in small particles in suspension (measured as turbidity after centrifugation) reflects the strength, that is, a high turbidity reflects a weak floc (44).

defined conditions (44,45). The floc weakness or the shear sensitivity can be quantified using the initial slope (45) or the ratio between the developed turbidity and total mass in the system after equilibrium (44). A more fundamental approach and more comprehensive modeling can be applied where the interaction energies and enthalphies for flocculation-deflocculation of activated sludge particles can be determined (44). The model by Parker describes the production of small particles by floc-breakup (4).

Strong flocs are believed to be formed from strong microcolonies, a large amount of exopolymers with a high charge density, many cations that effectively cross-link the matrix, and the presence of a macrostructure (filamentous bacteria or fibers). Changes in floc strength may be due to changes in the electrostatic interactions among exopolymers and particles in the floc, which can be induced by changes in environmental parameters such as ionic composition or pH, or to microbial activity, either directly or indirectly (Table 2).

Changes typically observed are a decreased floc strength when pH increases and when ionic strength is lowered, both a result of increased electrostatic repulsion. Changes can also be induced indirectly by microbial activity, for example, by denitrifying activity (which increases the local pH) or by the activity of Fe(III)-reducing bacteria, in which Fe(III) is reduced to a poorer flocculant, Fe(II) (46,47). The presence of sulfide, either produced by

Table 2. Examples of Changes in Floc Strength Due to Short-Term Changes in Environmental Factors or Microbial Activity

	Increased Deflocculation (decreased floc strength)
Environmental Condition	
pH	Increased pH (>8–9)
Ionic concentration	Change from high to low concentration
Ionic composition	Reduced ratio: (trivalent + divalent)/ monovalent ions
Detergents	Increased concentrations (>10–20 mg/l)
Microbial Activity	
Lack of oxygen	Lack of EPS production?
Denitrification	Increased pH (>8–9)
Fe(III)-reduction	Removal of the floc-forming Fe(III)
Sulfide production	Precipitation of Fe(III) to black FeS

sulfate-reducing bacteria or present in the wastewater, can seriously damage the flocs because of precipitation of the Fe(III) as ferrous sulfide (48). Furthermore, the active aerobic metabolism of the bacteria is essential to maintain strong aggregates on a short-term basis so that any lack of oxygen may induce some deflocculation of activated sludge flocs (49). The floc strength has been shown to have a seasonal variation in a certain treatment plant, with the strongest floc present during the summer period (50). The reason for this is yet to be ascertained.

Influence of Floc Structure on Functional Sludge Properties

The important functional sludge properties in the activated sludge process are flocculation and clarification, settling, and dewatering. Possible parameters that are believed to lead to floc formation with certain sludge properties are shown in Figure 8. Different environmental factors determine the microbial population as well as other components present in the floc. All these components, with their inherent properties, build up the flocs, determining size distribution, shapes, density, and strength. Ultimately, these floc properties determine the functional sludge properties that largely determine the operation of the plant.

Today, only little is known about the cause-effect relationship between the external factors that determine the various floc properties, whereas slightly more is known about which floc properties determine certain sludge properties. This mainly is due to (*1*) lack of knowledge about the composition of the microbial community, (*2*) lack of reproducible and comprehensive measurements of floc and sludge properties, and (*3*) difficulties in comparing the large diversity of wastewater types, process designs, and plant operations. Some of the generally accepted correlations are presented below.

Flocculation and Clarification. If the floc formation is working well in the process tanks and in the inlet to the clarifier, relatively large flocs are formed with an irregular shape that can collect smaller flocs during the initial flocculation and formation of floc-aggregates. The flocs must be strong to prevent breakup and production of many small particles. A number of factors are known to be important for flocculation (4). Among the physical factors are the level of turbulence (shear rate) and the design of the tanks. Important biological parameters include the F/M ratio (food to microorganisms, kgCOD/kgSS day) or sludge age (defined as total sludge mass divided by daily sludge production). At high F/M ratio or low sludge age (5–10 days), production of large but weak flocs has been reported, whereas smaller and stronger flocs are produced at lower organic loading and longer sludge ages (20–30 days). However, conflicting observations are reported in the literature, undoubtedly because other factors are important as well. For instance, large amounts of humic substances in the incoming wastewater or large variations in salinity can be expected to affect the floc composition and the floc properties.

Settling. A high settling velocity is observed if the flocs are spherical and large with a relatively high density and without too many filamentous bacteria extruding from the flocs (1,3). A high density is obtained if the inorganic fraction is high and if the porosity of the flocs is low. It is also reported that often flocs from plants with biological removal of phosphorus settle well, despite the presence of a high number of filamentous bacteria, probably because of high-density microcolonies of phosphorus-accumulating bacteria or precipitates. Low floc density often appears due to formation of conglomerates of flocs and filaments or due to extensive amounts of water-binding EPS, either adsorbed from wastewater or produced by certain bacteria (e.g., *Zoogloea* spp., (51)). Lack of inorganic cations has been reported to cause deflocculation and production of flocs with low density (52).

Dewatering. Good sludge dewatering properties are defined by low consumption of conditioning agents (organic

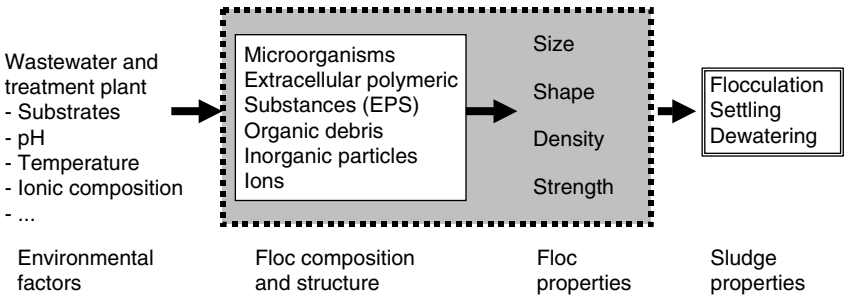

Figure 8. Overview of factors leading to floc formation with certain functional sludge properties.

polymers or iron/lime), a high dewatering rate, and a high dry matter content in the dewatered sludge cake (5). Conditioning agents are used to flocculate small particles, often by neutralizing the negative surface charges of the particles. Therefore, it is important to minimize the number of small particles (and thus the surface area) and, if possible, only have large flocs with a narrow floc size distribution. It is also important that the flocs are strong and do not break apart during dewatering, when a high shear is applied. This comment also applies to the rate of the dewatering, which is affected by many small particles because of filter clogging. Examples of poor dewatering are often observed when the floc size distribution is broad with many small particles resulting from low floc strength (53). A high dry matter content requires sludge with a low water-binding capacity, that is, a relatively low content of highly charged EPS (54). The presence of an extensive amount of water-binding EPS in zoogloeal bacteria can deteriorate the dewatering (31).

The Ideal Floc. It is not simple to define the "ideal" floc, one that is optimum for clarification, settling, and dewatering. For instance, strong flocs are good for flocculation and clarification but not necessarily for settling and dewatering. Regarding dewatering, the high water-binding capacity of most strong flocs (caused by many charged EPS components and cations) reduces the obtainable sludge dry matter content during dewatering (54). Furthermore, this high water-binding capacity may reduce the density of the flocs and thus, potentially, the settling velocity. The best compromise for floc properties that ensure good sludge properties for the entire solid-liquid separation process is probably for it to be relatively large, irregularly shaped with some filaments present, not too strong, and high density. However, it is obvious that an ideal floc with respect to solid-liquid separation is different from the ideal structure for biological transformations because for the latter, small flocs with low diffusional resistance would be desirable.

CONCLUSION

The activated sludge floc is a heterogeneous, complex structure consisting of a multitude of bacteria, extracellular polymeric substances, and other organic and inorganic components. Floc formation is determined partly by biological activity (growth-related processes, lysis, and hydrolysis) and partly by physicochemical, adsorption-desorption processes. Little is presently known about the identity of the dominating bacteria involved in these processes and about what controls their activity, whereas more is known about the dominating physicochemical factors. The most significant sludge properties for a well-working treatment plant (flocculation, settling, and dewaterability) are determined by the floc properties (mainly floc size, shape, density, water-binding capacity, and strength). Future investigations of both microbiological and physicochemical aspects of the flocs, for example, by using novel in situ methods, will improve our knowledge about cause-effect relationships between (*1*) wastewater characteristics, plant operation, and floc composition or structure, (*2*) floc structure and floc properties, and (*3*) floc properties and sludge properties. Such knowledge can be used for troubleshooting and optimization of the solid-liquid separation in existing treatment plants and to facilitate the development of new concepts, including active control of important floc properties.

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ACTIVATED SLUDGE — THE "G-BACTERIA"

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Many enhanced biological phosphorus removal (EBPR)–activated sludge systems (ACTIVATED SLUDGE — MOLECULAR TECHNIQUES FOR DETERMINING COMMUNITY COMPOSITION; ACTIVATED SLUDGE — THE PROCESS), where the biomass is recycled alternatively through anaerobic and aerobic regimes, contain large numbers of bacterial cells with a distinctive morphology. In the absence of any detailed physiological or biochemical information about them, they are defined here as a morphotype, appearing as cocci in tetrads or clusters, often of different sizes and usually closely associated with the flocs (ACTIVATED SLUDGE — THE FLOC), as shown in Figure 1. They are also seen in large numbers in sequencing batch reactors (ACTIVATED SLUDGE — SEQUENCING BATCH REACTORS) operating under aerobic–anaerobic conditions. An early report (1) suggested that they occurred more commonly in systems-fed glucose and not acetate, and so they were referred to as *G-Bacteria* (glucose-bacteria). Bacteria with the same appearance were reported earlier by Takii (2), and have now been recorded in plants all over the world (3).

Unfortunately, most of these have probably not been obtained in pure culture as they are very difficult to grow (3), although now enough have been cultured to demonstrate that the term *G-Bacteria* describes a group of phylogenetically quite different organisms, all sharing the same microscopic morphological appearance. Because their taxonomy is so different, it is likely that their physiologies and ecologies also vary. Hence their significance in activated sludge systems will probably differ and interpretations of their impact on plant performance need to be made with caution. This article briefly discusses the systematics of these "G-Bacteria," what is known and would like to be known about their biology, and how such information might help in understanding activated sludge systems better.

TAXONOMY OF THE "G-BACTERIA"

Taxonomy of the Gram-Negative "G-Bacteria"

It is now clear that the term *G-Bacteria* embraces both gram-negative and gram-positive bacteria, all capable of growing in this distinctive manner. The gram-negative "G-Bacteria" named by (1) were initially wrongly "identified" on their morphology only as members of the genus *Methanosarcina*, an Archaea. It is now known from 16S rRNA gene sequence data obtained from pure cultures, most of which possess the same distinctive morphology as seen in plant biomass, that these belong to a novel genus, *Amaricoccus* in the *α-Proteobacteria* (4). Some (e.g., *Amaricoccus kaplicensis*) often appear pleomorphic and produce large amounts of extracellular capsular material (Fig. 2a), which on drying appears to join the cells together in a microfibrillar network (Fig. 2b). Other *Amaricoccus*

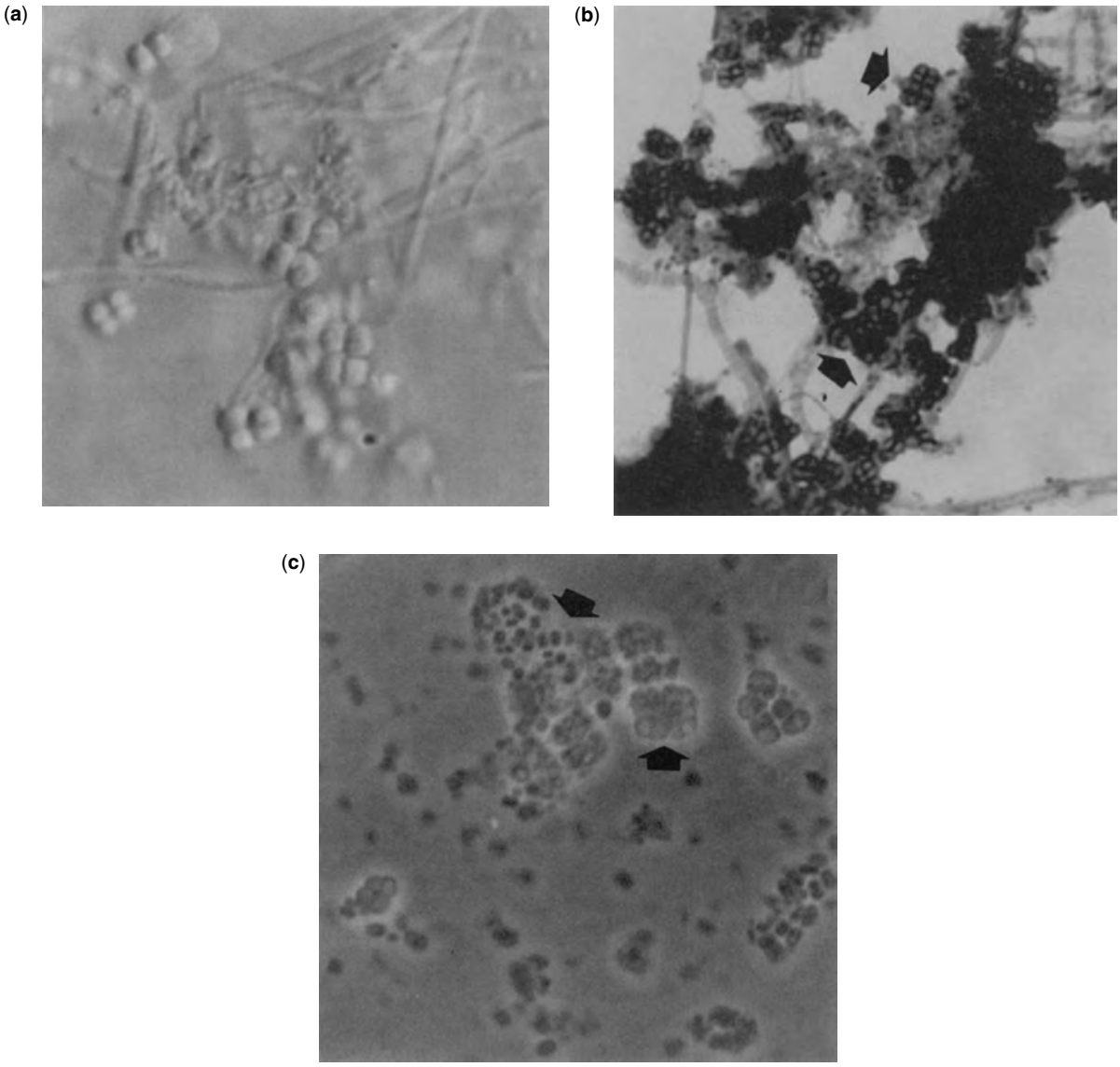

Figure 1. (**a**) Light microscope view of biomass showing "G-Bacteria" as distinctive cocci in tetrads and clusters associated with the floc. (**b**) "G-Bacteria" showing distinctive staining reaction with Neisser stain for polyphosphate, where cell contents do not stain but cell walls do. (**c**) Size variation in "G-Bacteria" from same biomass sample, where small and large cocci are visible (arrowed).

isolates are more regular in appearance in axenic culture (Fig. 2c). It also seems that isolates from different parts of the world represent several different species of *Amaricoccus*, and these have been named after their places of isolation, that is, *A. kaplicensis*, *A. veronensis*, *A. tamworthensis*, and *A. macauensis*. Some of the same isolates characterized by (4), that is, *A. kaplicensis* were also sequenced in a parallel study by (5) who named them *Tetracoccus cechii*. Under the rules of the Code of Bacterial Nomenclature, the genus name *Amaricoccus* has precedence, although the name *Tetracoccus* still appears in the literature. *Amaricoccus kaplicensis* and *A. tamworthensis* have been isolated from plants in countries other than where they were initially grown (Maszenan, 2000).

Once the 16S rRNA sequence data are available, it is then possible to design oligonucleotide probes that are specific for organisms at different taxonomic levels, that is, genus, species, and so on for fluorescent in situ hybridization or FISH (ACTIVATED SLUDGE — MOLECULAR TECHNIQUES FOR DETERMINING COMMUNITY COMPOSITION). A genus-specific probe for *Amaricoccus* has been designed (AMAR 839) and used to survey the occurrence of this organism in several plants (Fig. 3). There was no clear evidence from this survey that it was seen more commonly in EBPR plants with poor P-removal performance, although high numbers were detected, but not exclusively, in aerobic–anaerobic SBR systems and plants treating carbohydrate-rich wastes (6). These bacteria were also seen in many conventional systems, and in some plant biomass samples, the probe

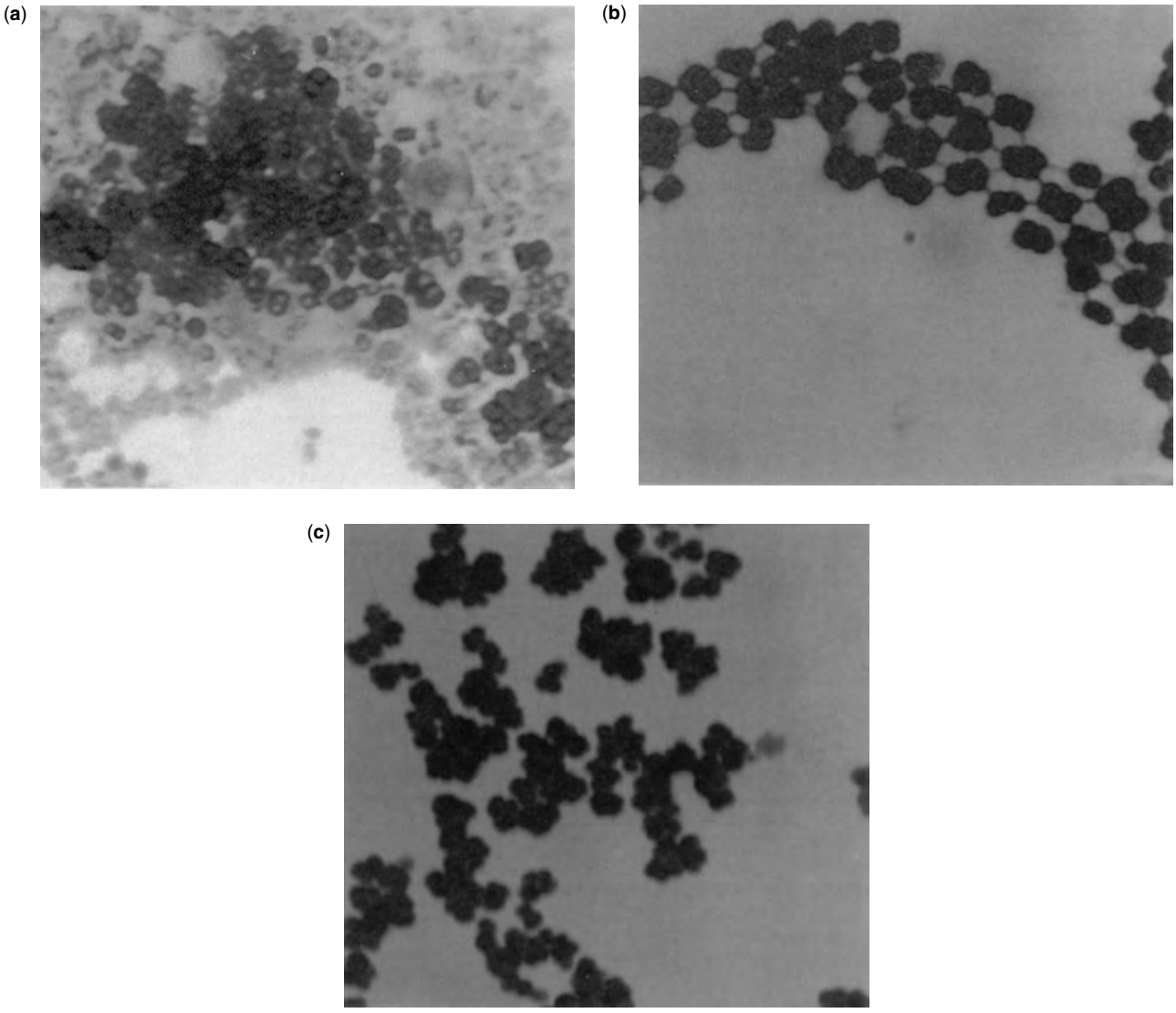

Figure 2. Light microscope of pure cultures of *Amaricoccus*, the gram-negative "G-Bacteria" (**a**) isolated originally by (1), showing the cells associated with large amounts of extracellular capsular material. (**b**) Dried capsular material in gram stained pure cultures of the isolate from Tamworth, NSW appearing as an interconnecting microfibrillar matrix between cells. (**c**) The strain from Macau showing no capsular material associated with the tetrads.

AMAR 839 was able to confirm that the "G-Bacteria" visible under the microscope but never successfully recovered into pure culture, were in fact *Amaricoccus* spp. (see following section).

It is now clear that *Amaricoccus* is not the only gram-negative coccus associated with activated sludge systems. Thus, molecular techniques using 16S rDNA fragments separated by DGGE (ACTIVATED SLUDGE — MOLECULAR TECHNIQUES FOR DETERMINING COMMUNITY COMPOSITION) detected bacterial sequences in a laboratory reactor showing poor P-removal, which emerged on sequencing as phylogenetically novel *α-Proteobacteria* and *β-Proteobacteria* (7). After FISH probing based on only their partial sequences, they appeared in biomass samples as large cocci (i.e., "G-Bacteria"). Neither of these has been cultured and so whether they do accumulate PolyP or another storage polymer is not known. Two other gram-negative "G-Bacteria" have been grown in an laboratory, which after 16S rRNA gene sequence analysis appear as novel genera. One, an unusual *α-Proteobacteria* has been placed in the genus *Defluvicoccus* (8), whereas the other, strain Ben 117, a *β-Proteobacteria*, has been allocated to the new genus *Cardococcus* (9). These data clearly demonstrate that these gram-negative coccal "G-Bacteria" are phylogenetically diverse (Fig. 4), and it is highly probable that many more await description.

Taxonomy of the Gram-Positive "G-Bacteria"

It is now clear that the gram-positive coccal "G-Bacteria" are also taxonomically diverse, but until more have been

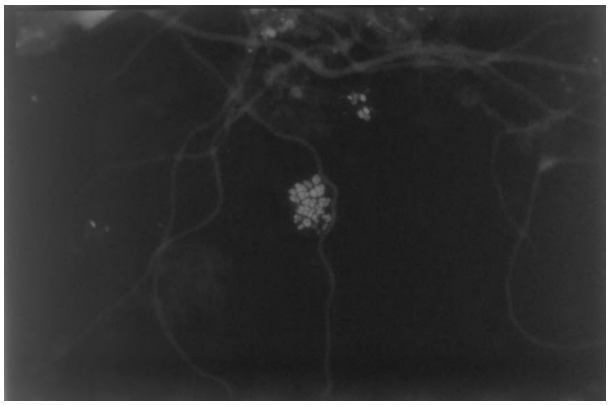

Figure 3. In situ identification of *Amaricoccus* by FISH using the fluorescein labeled AMAR 839 probe, showing the distinctive cell arrangement of tetrads and clusters in a sample of biomass. See color insert.

grown in pure culture and fully characterized, the true level of their biodiversity remains unknown. Those that have been grown axenically in the laboratory, and for which 16S rRNA sequence data (Fig. 5) have been obtained include

- possibly novel *Micrococcus* strains (10),
- two new species of *Friedmanniella*, *F. spumicola*, and *F. capsulata* (11), which grow in clusters of tetrads in a mucilaginous mass (Fig. 6),
- two novel genera, *Tessaracoccus* (as *T. bendigoensis*) (12) and
- *Tetrasphaera* (as *T. australiensis* and *T. japonica*) (13).

The taxonomies of *Friedmanniella* and *Tetrasphaera* are particularly interesting. The only other *Friedmanniella* species currently known, the slow growing *F. antarctica* was isolated, not from activated sludge, but from antarctic sandstone, a nutritionally stressed habitat (14). *Tessaracoccus japonica* is the coccus isolated from activated sludge after prolonged starvation by (15) and incorrectly "identified" then as a *Micrococcus*. Totally unexpectedly, it also emerges as a member of the same genus as the bulking activated sludge filamentous bacterium *"Nostocoida limicola"* II (FILAMENTOUS BACTERIA IN ACTIVATED SLUDGE: CURRENT TAXONOMIC STATUS AND ECOLOGY), which is known to grow as cocci in clusters (16), even though *Tetrasphaera* has never been seen to grow as filaments in pure culture. Such an outcome serves to emphasize the dangers inherent in relying solely on morphology to "identify" bacteria (17), especially those in activated sludge.

These strains are in addition to those gram-positive cocci so far isolated only from Japanese activated sludge plants and not described originally as "G-Bacteria." These are also in the high mol% G + C gram-positive bacteria and include the novel genera

- *Microlunatus* (as *M. phosphovorus*) (18) with a distinctive cell morphology
- *Microsphaera* (as *M. multipartita*) obtained from a plant treating a sugar-rich synthetic waste (19).
- *Micropruina glycogenica* (20) closely related to *Friedmaniella* and *M. phosphovorus* and obtained from an EBPR system.

The phylogenetic relationships based on 16S rRNA sequence analysis between these and the other gram-positive "G-Bacteria" are shown in Figure 5. The questions of how widely occurring these gram-positive "G-Bacteria" are and what operating conditions might favor their presence in activated sludge systems await the development of 16S rRNA-targeted probes for FISH of each of these. Probes have been described for *Microlunatus* (21) and *Tessaracoccus* (Maszenan, unpublished) so their population dynamics should now become clearer. Clone library data have shown that organisms very similar to *Tetrasphaera* occur elsewhere (22), but surprisingly no reports of the presence of any of the other "G-Bacteria" in activated sludge biomass have appeared when molecular techniques of community analyses have been used.

PHYSIOLOGY OF "G-BACTERIA"

Evidence (largely anecdotal) suggests that these "G-Bacteria" are favored in activated sludge and SBR systems operating with alternative anaerobic–aerobic regimes. Thus they possibly share the same ecological niches as some of the so-called polyphosphate accumulating bacteria (PAB) (ACTIVATED SLUDGE — MOLECULAR TECHNIQUES FOR DETERMINING COMMUNITY COMPOSITION), but there is insufficient information on their physiology so far to really explain why. The physiology and ecology of the few pure cultures available have not been examined in detail, and so the information comes largely from using mixed liquor communities enriched with these organisms. The taxonomic status of the bacteria present in these is usually not known, and given the earlier discussions on the phylogeny of these "G-Bacteria," they may in fact represent additional novel organisms. The reader is directed to (ACTIVATED SLUDGE — MOLECULAR TECHNIQUES FOR DETERMINING COMMUNITY COMPOSITION) for a critical discussion on current understanding of how these EBPR plants are thought to work. The roles of polyphosphate (PolyP), poly β-hydroxybutyrate (PHB) and glycogen in aerobic–anaerobic systems in providing the PAB with a selective advantage in EBPR, and the crucial importance of anaerobic substrate uptake by them are also discussed.

It has been suggested that the "G-Bacteria' are undesirable in EBPR systems because of their ability to synthesize intracellular storage carbohydrates such as glycogen aerobically and to use these to provide the energy to assimilate readily utilizable substrates anaerobically (1). Thus, the PAB were considered in danger of being outcompeted in the anaerobic stage, and instead of PolyP accumulation occurring aerobically, cells store polysaccharide material instead.

This attractive idea has never received any direct support from pure culture work (23), and although none of the *Amaricoccus* isolates or *Defluvicoccus* that were examined store PolyP in pure culture (4), *Cardococcus* does. Whether these non-PolyP accumulators can

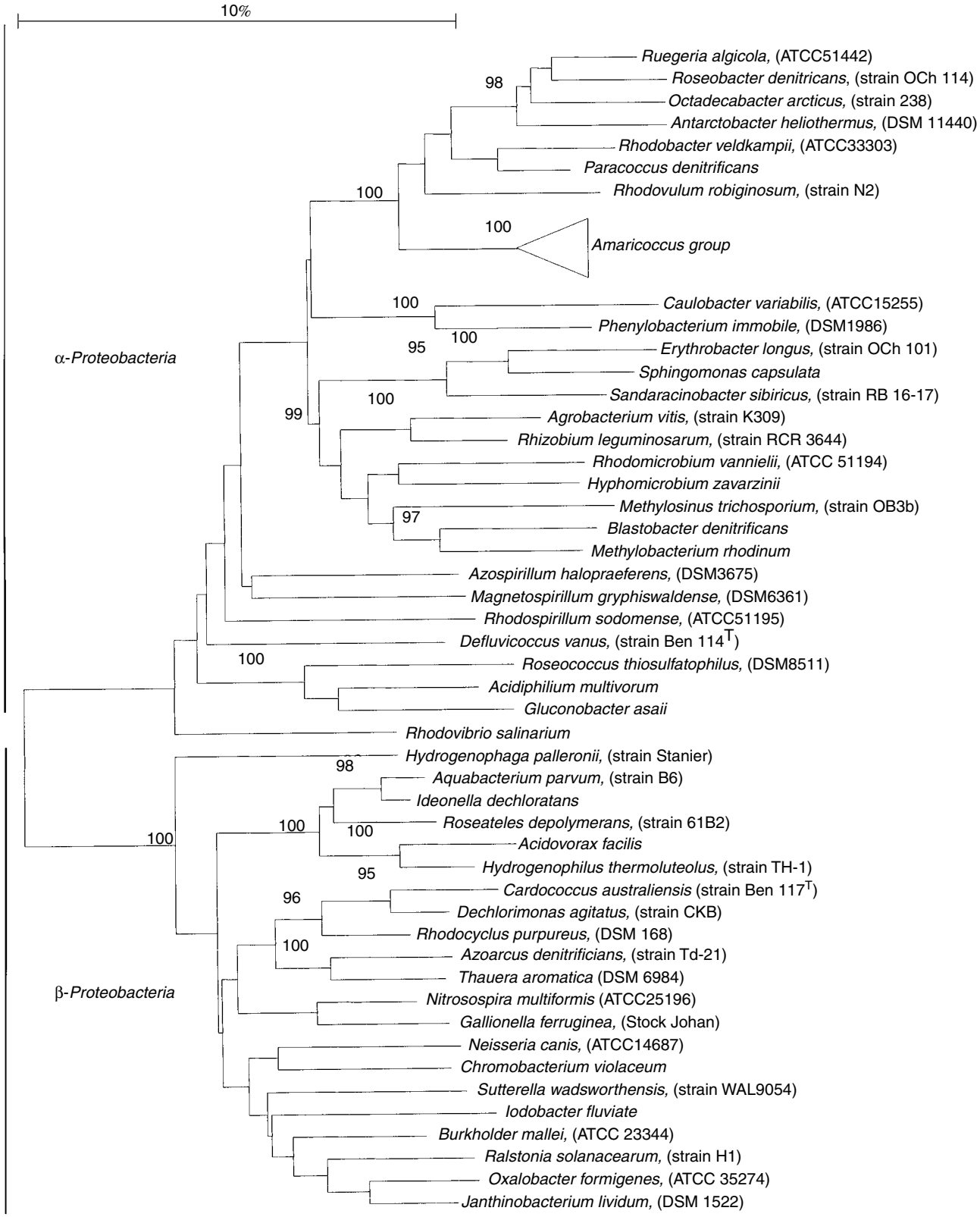

Figure 4. Phylogenetic tree (6) based on 16S rDNA sequence analysis of the cultured gram-negative "G-Bacteria" (in bold) among the *α-Proteobacteria* and *β-Proteobacteria*. Bootstrap values are given for the major clades and the bar marker indicates 10 nucleotide substitutions per 1,000 nucleotides.

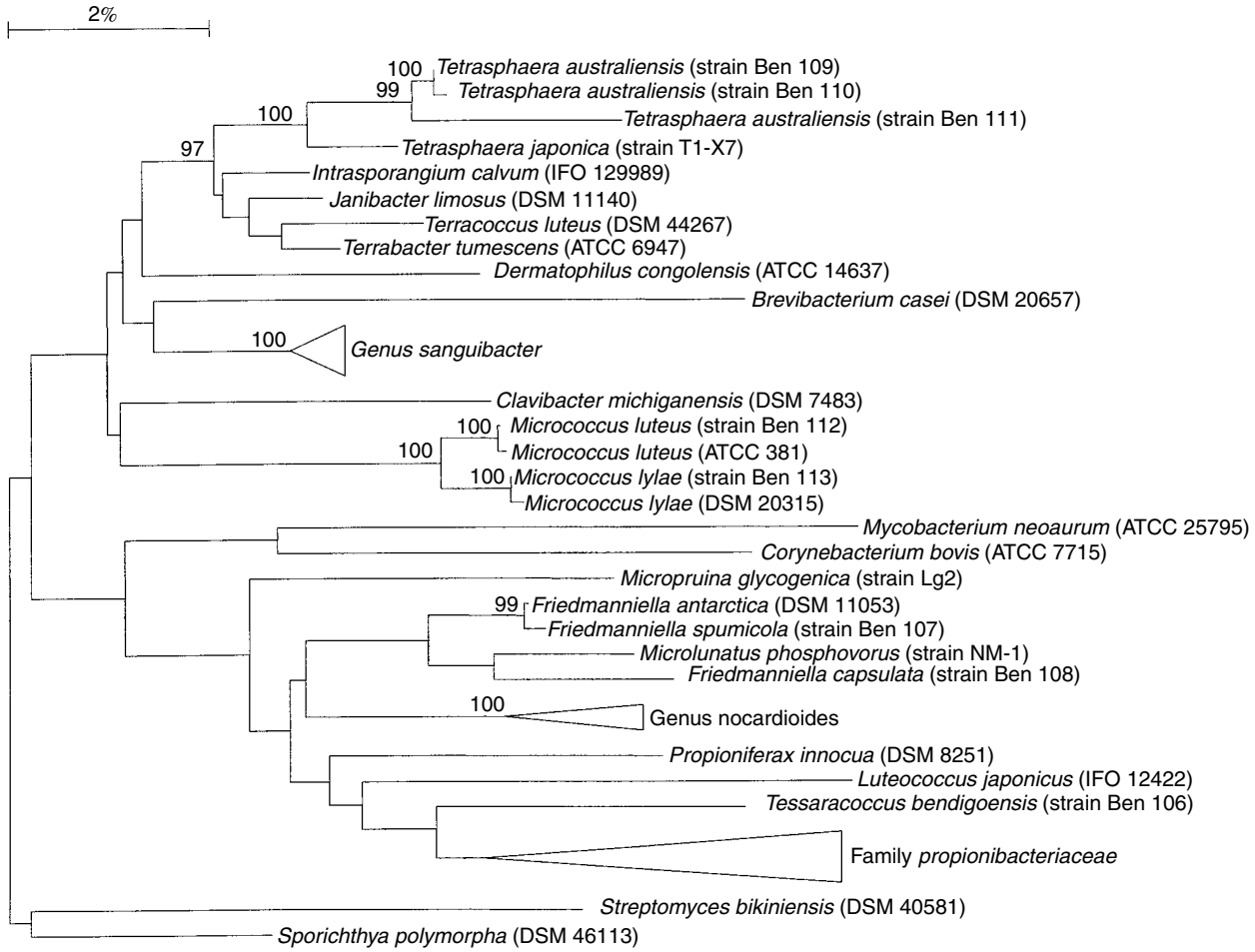

Figure 5. Phylogenetic tree (6) based on 16S rDNA sequence analysis of the cultured gram-positive "G-Bacteria" (in bold) among the high G + C gram-positive bacteria. Bootstrap values are given for the major clades and the bar mark represents 10 nucleotide substitutions per 1,000 nucleotides.

synthesize glycogen (either aerobically or anaerobically) instead is known only for *Amaricoccus*, where glycogen in high amounts (about 20% of cell dry weight) is stored under aerobic conditions from glucose (Kong, unpublished). PHB is also synthesized from acetate and several other carbon sources (23). Most of the gram-positive "G-Bacteria" that have been observed can accumulate PolyP in pure culture, although with the exception of *M. phosphovorus* (18,24), nothing is known about which conditions might influence its synthesis. This bacterium was described as behaving in pure culture in a manner close to that expected for the PAB, except that it could not assimilate acetate anaerobically. In fact, NMR studies (25) have shown that this organism fits little of the expected behavior of a PAB. Unable to grow anaerobically, it could still ferment glucose to accumulate anaerobically intracellular reserves of free acetate without synthesis of any PHB. Under aerobic conditions, glucose was respired without intracellular storage polymer (e.g., glycogen) production. However, it could assimilate and release phosphate at high rates in pure culture, similar to those measured in EBPR activated sludge samples. Similar experimental protocols applied to the other "G-Bacteria" are likely to be rewarding in the efforts to understand how these organisms function.

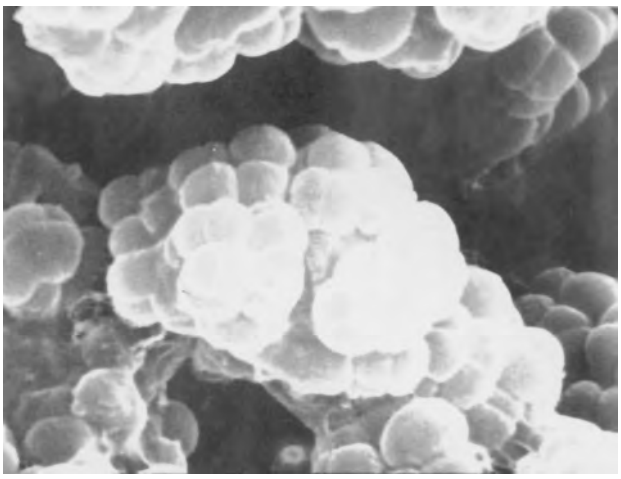

Figure 6. Scanning electron micrograph of *Friedmanniella spumicola* showing the coccal tetrads embedded in mucilage (bar marker = 2 μm).

MIXED CULTURE STUDIES WITH THE "G-BACTERIA"

Much of what is thought to be known about how these "G-Bacteria" behave in activated sludge comes from studies using mixed culture communities of unknown taxonomic status. Often the biomass in these experiments where conditions for very poor phosphorus removal have been established, has been dominated by morphotypes of the "G-Bacteria," although they were not always described as such, instead being referred to as the glycogen-accumulating bacteria, or GAO (25). As these bacteria have not yet been cultured, it is difficult to interpret the chemical transformations reported in terms of the organisms actually carrying them out. However, these experiments did attempt to explain why the PAB seemingly were replaced by GAO in EBPR systems-fed glucose and carbohydrate-rich substrates, and not acetate (1). The main proposition is that the GAO under these conditions could assimilate glucose anaerobically at a faster rate than the PAB could (who were thus disadvantaged), to synthesize PHB using glycogen as the source of reducing power and energy. Aerobically, glycogen was then replenished in the biomass from the PHB, and the PAB in the absence of PHB were no longer advantaged and able to accumulate PolyP. An acetate feed favored the PAB according to the models for EBPR (Activated Sludge — The Process). It was suggested (26) that the energy demand for acetate transport into the cells is high and cells need an energy source such as PolyP to support it, whereas glucose transport has a comparatively low energy demand that can be met from glycogen catabolism. However, no other convincing evidence has been produced to support such claims. Unfortunately, as we do not know yet which organisms are the GAO in these studies, and FISH-based identifications would help us here, it can only be explained in simplistic terms what might be going on. However, anaerobic glucose assimilation and aerobic glycogen synthesis may be a physiological adaptation of many of these "G-Bacteria," although some biomass samples dominated by *Amaricoccus* in fact remove phosphate very well (27).

ECOLOGY OF "G-BACTERIA"

Can the little that is known about these slow growing bacteria help understand why and how they survive in such highly competitive habitats as activated sludge systems, especially those removing phosphorus? They seem to possess the physiology to tolerate the wildly fluctuating availability of nutrients and the inevitable periods of nutrient stress and starvation encountered in such systems. An ability to synthesize energy storage polymers such as PolyP, PHB and/or glycogen appears to be a common attribute of these, and there is some evidence that they are able to withstand prolonged periods of starvation better than other activated sludge bacteria (15). It has also been shown (23) (albeit not for all these "G-Bacteria") that they can access any substrates when they do become available, immobilizing them almost exclusively into these storage compounds and not using them to increase what appears from pure culture evidence to be very slow growth rates. It could be argued from the organisms' perspective that this makes sound ecological sense because an "r-strategy" based on a high growth rate in activated sludge is certain to fail. Most of the 'G-Bacteria" that were observed appear to produce often large amounts in pure cultures of polysaccharide capsular material. Probably as a consequence of this material imparting hydrophobicity to their cell surfaces, the tetrads are usually seen closely associated with the activated sludge flocs. This may serve to

- protect them from possible predation from the protozoa feeding on suspended bacteria (Protozoa in Activated Sludge)
- provide protection against potentially harmful abiotic influences in the biomass
- ensure that they stay in the system in the return activated sludge and not leave the plants suspended in the bulk liquid

Their frequency of occurrence and predominance in biomass samples containing them together make them worthy of study.

CONCLUSION

- The cocci referred to as the "G-Bacteria" and often seen in activated sludge systems in large numbers are phylogenetically diverse and contain mainly novel gram-positive and gram-negative bacteria
- This finding reinforces the dangers in relying on morphology to "identify" bacteria and the risks in using this "identification" to interpret their possible role in communities such as activated sludge
- Generally, the literature seems to agree that these "G-Bacteria" appear to favor activated sludge systems where the levels and durations of periods of nutrient stress are particularly high, that is, SBR and EBPR processes
- These "G-Bacteria" appear to be found in niches shared by the PAB, although it is known how widely distributed each "G-Bacterium" is or the influences that promote or discourage their growth
- Although these organisms are difficult to obtain in pure culture, the molecular methods now available (e.g., FISH) provide the tools for studying their ecology and population dynamics
- The general understanding of their physiology is poor and so their role in EBPR plants is still not certain. All seem to possess an ability to synthesize intracellular storage polymers but the conditions affecting their production await clarification
- However, all seem to possess many of the required traits for a slow growing organism to survive in an activated sludge system that is able to cope with feast or famine conditions and remain in the system indefinitely. Therefore, with some confidence they might be described as true "K-strategists"

- It is likely that there are many more "G-Bacteria" in activated sludge systems and probably other stressful habitats (i.e., *Friedmanniella antarctica*) waiting to be described, and so views on what these bacteria might be doing will probably need to be revised.

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ACTIVATED SLUDGE — THE MICROBIAL COMMUNITY

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The activated sludge process (see ACTIVATED SLUDGE — THE PROCESS), the most popular process for treating both domestic and industrial wastes has been used around the world for almost a century, yet it is still very much a "black box." Some of the major chemical changes that are taking place can be readily measured (Fig. 1), but still, little is known about the microbes that are responsible for these and how their activities are affected by variables such as influent composition, process configuration, and process operation. The reasons for such ignorance are probably many, but until quite recently the methods available to gather such information from systems designed and run largely by engineers were not available. This has now begun to change. The application of molecular methods to the activated sludge ecosystem (see ACTIVATED SLUDGE — MOLECULAR TECHNIQUES FOR DETERMINING COMMUNITY COMPOSITION) has not only revealed a previously unsuspected level of microbial diversity, but has also questioned many earlier ideas as to which bacteria were responsible for important processes such as phosphorus (see ACTIVATED SLUDGE — MOLECULAR TECHNIQUES FOR DETERMINING COMMUNITY COMPOSITION) and nitrogen (see ACTIVATED SLUDGE — MICROBIOLOGY OF NITROGEN REMOVAL) removal. However, it could be argued that such important advances in the understanding of these systems are yet to help operators run them better or engineers to achieve improved designs. This will take some time to happen, but it is clear that it will not occur until the microbiology of activated sludge is far better understood than it is now.

WHY THIS IGNORANCE?

Applying traditional or classical microbiological techniques to activated sludge is beset with difficulties (1,2). These are often a consequence of the biomass being organized into discrete complex aggregates called flocs (see ACTIVATED SLUDGE — THE FLOC). Their chemical and physical structures and how they are formed are still not well understood, but unless these flocs settle rapidly in the clarifiers, the treatment process will ultimately fail. Activated sludge is also a highly complex ecosystem, so microbiologists with a preference for working with pure cultures have not been attracted readily to its study. The impact of applying molecular methods to overcoming some, if not all, of the problems associated with culture-dependent methods has been considerable. These include

- *Sampling*. Floc structure and organization mean that representative samples are difficult to obtain,

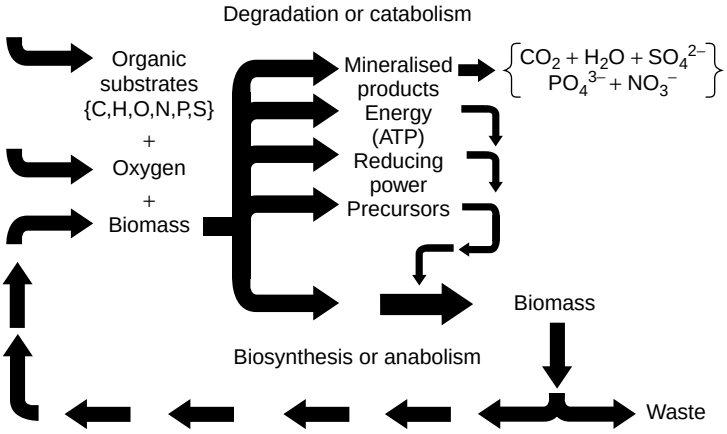

Figure 1. Summary of main chemical transformations thought to occur in the activated sludge process (2).

since the floc-associated microbes have to be suspended first. Various physical and chemical methods have been developed for this (2,3), but none are without problems, and all give serious underestimations of the bacterial numbers in quantitative studies (1) as well as the true level of bacterial biodiversity present in this system. Furthermore, it is not surprising that considerable heterogeneity can exist within individual flocs, where anoxic zones with different active bacterial populations have been detected in flocs in fully aerobic systems (4). Individual cells with a distinctive morphology (e.g., filamentous bacteria) can be recovered by micromanipulation (2), a technique with considerable advantages over other culture dependent methods, since the organism(s) of interest can first be "identified" under the microscope and can be selectively and physically separated from the rest. Flow cytometry allows for bacteria recovery but not for the growth of specific populations (5,6) that have been distinguished with fluorescently tagged (FISH) probes. (see ACTIVATED SLUDGE — MOLECULAR TECHNIQUES FOR DETERMINING COMMUNITY COMPOSITION).

- *Culturing and quantifying isolates*. Even the most nonselective microbiological media are unlikely to support the growth of more than a small subsample (<1%) of the bacteria present in activated sludge. Various molecular methods, which themselves are not without criticism (1,2,7,8), have all strikingly exposed the biased nature of culture-dependent techniques and the confusion that the data obtained from them have created (see ACTIVATED SLUDGE — MOLECULAR TECHNIQUES FOR DETERMINING COMMUNITY COMPOSITION). It is the view of the authors that there is still an important role for pure cultures in any study of activated sludge microbiology, especially if physiological and kinetic data are sought from individual populations (9). Unfortunately, without prior knowledge of the nutritional requirements or taxonomy of the bacteria sought (e.g., the bulking and foaming filamentous bacteria), it is not easy to design media rationally for their culture (2).
- *Measuring what they do*. One particularly difficult question to answer is what role these microbes are performing in the treatment process from the data using 16S rRNA targeted probes and FISH, it seems likely that most of the bacteria present are metabolically active and not dead or moribund (10) as once thought and thus probably contribute to the measurable chemical changes occurring. Only recently have attempts been made to find the specific microbial populations that are responsible for them in the hope that clearer structure–function relationships emerge. FISH probing directed at locating specific functional genes within particular populations is one approach. For example, the nitrate reductase gene in denitrifying bacteria (11,12), the sulfate reductase gene for dissimilatory sulfate reducing bacteria (4), and the ammonia monooxygenase gene for the nitrifiers (13) have all been used for this purpose. Respirometry via oxygen uptake rates (OUR) and substrate utilization and product formation rates for nitrifying and denitrifying bacteria (see ACTIVATED SLUDGE — MICROBIOLOGY OF NITROGEN REMOVAL) are popular general methods (2), especially for modelling purposes (see ACTIVATED SLUDGE — THE PROCESS). Dye reduction enzyme assays with CTC (14) and INT (15) for detecting respiratory active (i.e., viable) cells microscopically have been applied to activated sludge systems. However, when results obtained with all these different macrotechniques are compared, the data do not always correlate well (2). Determining carbon utilization patterns with the Biolog system to fingerprint heterotrophic metabolic activity, especially for systems treating industrial wastes, has also provided promising data, but these are not always easily interpreted (16).

  The fate of specific populations with metabolic activities of interest has been followed using green fluorescent protein tagging (17), which seems to be a technique of promise for the elucidation and assessment of their contributions to particular chemical events (18). Recent application (19,20) of microautoradiography (MAR) in combination with FISH probing to activated sludge

(see ACTIVATED SLUDGE — MOLECULAR TECHNIQUES FOR DETERMINING COMMUNITY COMPOSITION) has already provided invaluable information on the in situ physiology of bacterial populations of interest. A good example of how valuable this technique can be is seen in *Microthrix parvicella* (20). This bulking and foaming filament (FILAMENTOUS BULKING IN ACTIVATED SLUDGE, CONTROL OF; ACTIVATED SLUDGE — FOAMING) was shown to be selectively favored in aerobic : anaerobic systems such as enhanced biological nutrient removal (EBNR) plants (see ACTIVATED SLUDGE — THE PROCESS) because of its ability to assimilate hydrophobic substrates, such as oleate, anaerobically and anoxically as well as under aerobic conditions. Uptake of trioleate by *M. parvicella* was in fact best under anaerobic regimes and could be maintained under the starvation conditions inevitably encountered in such systems (see STORAGE POLYMERS: ROLE IN THE ECOLOGY OF ACTIVATED SLUDGE). As more bacterial populations are studied in this way, our current understanding of activated sludge microbiology will change dramatically, and such information should allow plant function to be related meaningfully to community structure.

- *Identifying the microbes present*. The classical approach with activated sludge has been to use phenotypic characters to identify those isolates that are obtainable in pure culture. For those not culturable (e.g., many of the filamentous bacteria and protozoa), "identification," by necessity, is based on their diagnostic microscopic appearance in biomass samples. Neither approach is appropriate if the aim is to understand which microbes are really present in activated sludge. Even if they can be cultured, most microbes, as is the case of many filamentous bacteria (see FILAMENTOUS BACTERIA IN ACTIVATED SLUDGE: CURRENT TAXONOMIC STATUS AND ECOLOGY), are novel organisms (21,22). Therefore, conventional identification methods are difficult, and morphology is a notoriously unreliable phylogenetic indicator with bacteria. Again, molecular RNA-based methods have provided the means for the in situ identification of many bacterial groups in the absence of any need to culture them, independently of their morphology, using FISH (see ACTIVATED SLUDGE — MOLECULAR TECHNIQUES FOR DETERMINING COMMUNITY COMPOSITION). The number of FISH probes available for detecting an increasingly diverse range of bacteria in activated sludge continues to grow rapidly and their advantages over both fluorescent and monoclonal antibody techniques, which have also been employed for the same purpose, are well recognized (10). Again, FISH techniques are not above criticism (7,23), but they have already supplied vital information about the role of the bacterial populations in processes such as nitrogen and phosphate removal (see ACTIVATED SLUDGE — MICROBIOLOGY OF NITROGEN REMOVAL). In combination with MAR, they appear to be even more powerful experimental tools, and it is not hard to predict that their application to activated sludge systems will continue to expand.

SO WHAT IS KNOWN SO FAR ABOUT THE MICROBIAL COMMUNITIES IN ACTIVATED SLUDGE PLANTS?

It is quite clear from recent studies applying these rRNA-based methods that perceptions of what were previously considered as more important (physiologically and numerically) populations in activated sludge systems are largely wrong. Organisms that are revealed using culture-dependent methods are a reflection more of these methods than the populations actually present. Several independent studies have shown that while the γ-Proteobacteria dominate cultured gram-negative bacterial populations, those revealed by using culture-independent methods are mainly the β-Proteobacteria (24–27). The relative importance of the high mol% G+C Bacteria differs between studies, which may reflect different operational conditions of the systems studied or variations in efficiencies of the individual methods of DNA extraction used, which are usually not checked for possible biases (28). Such observations also emphasize the well-known risks associated with trying to assess data quantitatively from these rRNA-based molecular approaches (7). The information available on which organisms are present in activated sludge is inevitably skewed. More effort has been directed at understanding those considered responsible for particular changes such as those involved in nitrogen and phosphate removal (see ACTIVATED SLUDGE — MICROBIOLOGY OF NITROGEN REMOVAL) or the bulking and foaming filamentous bacteria (see FILAMENTOUS BULKING IN ACTIVATED SLUDGE, CONTROL OF; ACTIVATED SLUDGE — FOAMING). Even so, these populations are still not always well understood, and often the picture is much more complex now than once thought likely. A brief overview of some recent additions to the view of the microbial composition of the activated sludge community follows. This community is likely to change in response to many factors in ways that are not yet understood, and so any generalization made here is risky.

- *Viruses*. As might be expected, activated sludge systems contain many viruses of animal and human origin, but because of problems in their isolation and characterization, their diversity is poorly understood, and many more different types may be present about which little is known. Molecular methods (e.g., probes) may eventually provide this information. These viruses are known to include the HIV virus, which is unlikely to survive the process, and more robust viruses including the rotaviruses, many of which will survive (29). Bacteriophages are readily isolated. Although their possible roles in bacterial population density control have not been fully assessed, it seems that some may have broad host ranges making such a function likely.
- *Bacteria*. Unquestionably, applying a battery of molecular rRNA-based techniques has changed views on the true extent of biodiversity among activated sludge bacterial populations, and many populations

previously unknown or ignored, or considered to represent unimportant members of this community, now emerge as numerically significant. Often, the populations detected in large numbers have no known function, and most of these are yet to be cultured in the laboratory and it is usually not known why they predominate. Activated sludge systems will certainly contain pathogenic bacteria of human fecal origin such as *Salmonella* spp., and some will survive the process. These will need to be removed by disinfection later because it is important to recognize that these processes are designed primarily to remove nutrients and not bacteria.

a. Chemoheterotrophs. The process relies on aerobically respiring bacteria to degrade and mineralize organic compounds present in raw sewage that are often complex and chemically diverse. Yet, little is known about the main bacteria that are responsible or how the communities of these might change with different operating conditions. Fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE) (30) and terminal restriction length polymorphism (T-RFLP) (31) (see ACTIVATED SLUDGE — MOLECULAR TECHNIQUES FOR DETERMINING COMMUNITY COMPOSITION), which allow changes in community composition to be followed with time, may help provide answers. Substantial progress has been made in properly identifying some of the distinctive bacterial morphotypes readily seen under the microscope. These include the bulking and foaming bacteria (see FILAMENTOUS BACTERIA IN ACTIVATED SLUDGE: CURRENT TAXONOMIC STATUS AND ECOLOGY) and the "G-Bacteria" (see ACTIVATED SLUDGE — THE "G-BACTERIA"). Both contain bacteria that were not described previously, and emphasize how collections of bacteria indistinguishable morphologically can be phylogenetically quite unrelated to each other. Spiral bacteria, often recognized in biomass samples because of their characteristic corkscrew pattern of movement, are not spirochaetes as they have often been referred (2). Instead, on the basis of molecular characteristics, they were previously undescribed members of the *Cytophaga-Flavobacterium* division (6), a group long known to exist in activated sludge systems (2). Novel isolates readily detected among populations of *Aeromonas*, *Acinetobacter*, *Acidovorax, Sphingomonas*, and *Hyphomicrobium* (32–36) confirm activated sludge as a relatively unexploited natural source of bacteria. In addition, some bacteria that were never considered previously as important members of this community now appear to be present in significant numbers. These include the Planctomycetes (25,37,38) and oligotrophs such as *Hyphomicrobium* (36). What the reason or reasons for their presence might be are not understood, but such data imply that appreciation of the total spectrum of the metabolic changes occurring in activated sludge must also be simplistic.

   Molecular approaches have also forced us to reappraise anaerobic respiration in activated sludge systems. For example, the ANAMMOX process illustrates how much is still to be learned about the diversity of denitrifying bacteria (39), and their ability to reduce nitrate under aerobic conditions now seems to be a common one (12,40) (see ACTIVATED SLUDGE — MICROBIOLOGY OF NITROGEN REMOVAL). Which bacteria are the more active denitrifiers is not clear although unexpected candidates such as *Hyphomicrobium* may dominate in some systems (41). Bacteria that are capable of reducing Fe^{3+} during anaerobic respiration are readily detected in activated sludge systems in which their activity may lead to floc destabilization (42) and one isolated strain is closely related to *Geobacter sulfurreducens* (43). Sulfate reduction may also occur in anoxic microniches in flocs, even in fully aerated systems (4).

   The microbes responsible for phosphate removal, often appearing in large clusters, have proven elusive despite a great deal of directed effort, with the early favorite candidate *Acinetobacter* increasingly dismissed from molecular evidence (44) (see ACTIVATED SLUDGE — MOLECULAR TECHNIQUES FOR DETERMINING COMMUNITY COMPOSITION). The puzzle is still not satisfactorily resolved in the absence of pure cultures that behave as the models require but the β-Proteobacteria seem to be more likely participants (45) than yeast spores (46). Many different populations, each possibly achieving importance under different operational conditions, may be involved in phosphate removal, which will only complicate the story.

b. Chemoautotrophs. Although it was thought that the microbiology of nitrification in activated sludge is quite well understood, molecular data have shown this not to be the case, and doubts are now raised about a role in activated sludge for the genera that was once considered to be the major bacteria involved in NH_3 oxidation (13,47). What FISH has elegantly shown is that the Nitrosobacteria and the Nitrobacteria are closely and physically arranged in distinctive clusters in the biomass, allowing syntrophism to occur readily between the NO_2-producing and NO_2-consuming organisms (48) (see ACTIVATED SLUDGE — MICROBIOLOGY OF NITROGEN REMOVAL). Nitrifying bacteria are not the only autotrophic bacteria present in activated sludge. Thus, Fe^{2+} oxidizers have been detected (42) and some bulking filamentous bacteria such as *Thiothrix* may obtain energy from H_2S oxidation. However, whether these are strict autotrophs or mixotrophs that are unable to use carbon dioxide as a carbon source is uncertain (2).

- *Fungi*. The importance of fungi in activated sludge is not well understood, although their role as predators in consuming nematodes (49) deserves further study. Most of the fungi that have been cultured appear to be the Deuteromycotina that is commonly seen as part of the air microflora or in soil

samples (50), but experiences with activated sludge bacteria might suggest that many more that are currently nonculturable may be present.

- *Protozoa*. Because of their occurrence in such large numbers, the protozoa have attracted much interest, and it is quite clear that they play a vital role as predators in reducing the levels of suspended particles (e.g., bacterial cells) in the bulk liquid, thus reducing its turbidity (see PROTOZOA IN ACTIVATED SLUDGE). Most studies have looked at the ciliates, especially their ecology and value as indicators of plant performance. Less is known about the roles of the flagellates and ameboid protozoa, and it may turn out that their importance has been underestimated so far. Current interest in pathogenic protozoa such as *Giardia lamblia* and *Cryptosporidium parvum* is high, and many sensitive molecular and other methods are now available for their detection and enumeration at low levels in water and wastewater samples. Activated sludge seems to be more effective at removing cysts of *Giardia*, which could be detected in influents at high levels, than *Cryptosporidium* oocysts (51). How selective the predatory bacteriovorous protozoa are in their feeding habits is uncertain, but an increasing body of evidence suggests that feeding is not random. Thus, cell size (52), hydrophobicity (53), and floc-forming abilities of the bacteria have all been suggested to directly determine their fate in activated sludge. Yet, some ciliated protozoa appear to prefer filamentous bacteria to unicells (54).
- *Metazoa*. Under the microscope, activated sludge biomass samples from plants operating well contain nematodes, rotifers, and oligochaete worms (55). All probably act as predators contributing to the removal of suspended bacterial cells, although how rapidly they feed and the selectivity or otherwise of their feeding are less well understood than with those of the protozoa.

THE ACTIVATED SLUDGE FOOD CHAIN?

Despite a poor understanding of how individual populations might interact, models based on simple food chains have been proposed, and these often fit experimental data quite well. Most of these are simplified and based on predator–prey relationships. They recognize several trophic levels, with the activated sludge bacteria (both freely suspended and associated with the flocs) representing the base level. These bacteria are the main degraders of the soluble and colloidal organic substrates present and are then fed on by the flagellate and free-swimming, crawling and sessile, ciliated protozoa, which then serve as prey to the carnivorous ciliates (Fig. 2). The population dynamics of such a complex process is not easy to detail until the understanding of the basic microbiology of activated sludge is improved.

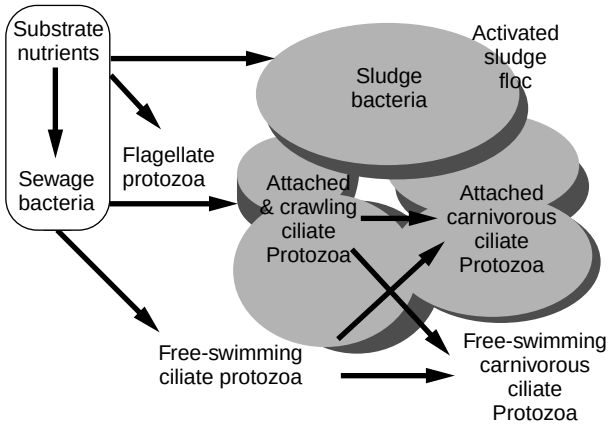

Figure 2. Proposed food chain for the activated sludge community (2).

WHICH FACTORS DECIDE THE FATE OF THESE MICROBES IN ACTIVATED SLUDGE?

An activated sludge process in simple terms is a continuous culture system with biomass recycling (56). Therefore, the microbial community there is subjected to considerable selective pressures. These will (at least partly) decide whether they remain in the system, die, or leave in the treated effluent. Some of the more influential selective factors are assumed to be the following:

- Growth rate of a population will decide how successfully an organism can compete for limiting resources, and the chemostat theory states that an organism with a higher μ_{max} and a lower k_s will outcompete one in which the μ_{max} is lower and the k_s is higher for a growth-limiting nutrient (57). Whether this happens in an activated sludge is doubtful. Little is known about how fast different populations can grow in activated sludge, and with such a diverse range of available substrates that are possibly utilized preferentially by different populations, any kinetic analysis of activated sludge becomes a daunting task. Furthermore, the opportunity for populations to interact both negatively and positively is considerable. Such interactions will certainly influence their fate, but what these are and what effect they might have on population dynamics is still completely unknown.
- Ability to tolerate the prevailing abiotic factors will affect an organism's μ, and, possibly, its ability to survive. These factors include pH, pO_2, redox potential, and temperature, as well as toxic chemicals including heavy metals (2). There is little information on how any of these might influence community composition and activity, and because of the physical heterogeneity of flocs, many microbes will experience conditions quite different from those that can be measured in the plant biomass as a whole (2). However, temperature may affect the communities of foaming bacteria (see ACTIVATED SLUDGE — FOAMING), and a low pH may favor fungi in bulking episodes (see FILAMENTOUS BULKING IN ACTIVATED SLUDGE, CONTROL OF).

The composition of the protozoan community may also be affected by heavy metals (58).

- Becoming part of the floc means that a population will be recycled in the return activated sludge (RAS) and thus survive longer. This ability probably is more important than a high μ, since fast-growing freely suspended cells will still leave in the bulk liquid. How cells might achieve this is simple to detect microscopically. Many apparently slow-growing bacteria (see ACTIVATED SLUDGE — THE "G-BACTERIA") often produce extensive capsular material enabling them to become integrated into the floc, and probably at the same time resisting predation by the floc-grazing protozoa (2). The sessile protozoa, also probably slow growers, attach to flocs with holdfasts, and many of the filamentous bacteria that certainly grow slowly in the laboratory are part of the floc matrix.
- Accumulate storage compounds such as glycogen, polyphosphate, and poly β-hydroxyalkanoates to provide them with the biochemical equipment to survive the inevitable periods of famine, which will be encountered in an activated sludge system. These are especially acute in anaerobic : aerobic systems such as EBNR plants in which only cells that are able to assimilate substrates into these polymers in the anaerobic stage can then grow in the subsequent aerobic stage in which available nutrients are no longer present. Many slow-growing bacterial isolates possess this ability, and the evidence suggests that cells provided with readily metabolizable substrates use them very rapidly not to maintain an increased μ but to synthesize one or more of these storage polymers.

CAN OUR COMMUNITIES BE MANIPULATED TO IMPROVE PLANT PERFORMANCE?

The idea of adding populations of microbes with specific, desirable, metabolic, or other properties to activated sludge is not a new one (59). Yet, when this has been attempted, the results have generally been unimpressive (60). It is not too surprising from an ecological perspective that most of the niches in activated sludge would already be occupied, and any introduced strains would struggle to overcome the prevailing selective factors. However, some success has been achieved in laboratory studies with genetically engineered bacterial strains carrying plasmids, which by conjugation may spread through the activated sludge community (61). It may be that we are trying to add the wrong organisms to the system, and once we understand better what populations do, the more success will be achieved with this aproach.

CONCLUSION

Understanding the composition of the microbial communities of activated sludge systems has been revolutionized by the increase in the interest shown in them by molecular microbial ecologists and by the techniques they have used in their studies. However, much is still to be learned. Obtaining this information will be slow and fragmentary and will tax the experimental skills of the microbiologist. The real challenge then is to translate this basic microbiological information into improved engineering outcomes in both plant design and operation.

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ACTIVATED SLUDGE — THE PROCESS

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Although recorded history and surviving archaeological sites show that the Babylonians and Assyrians, and later Romans, used quite sophisticated technology to collect and dispose off their wastes, this environmental awareness was not adopted by later societies, until the Industrial Revolution in Britain in the 19th century forced political intervention (1–3). Poor quality, densely packed housing with no provision for proper sewage disposal led to overbearing odor problems, polluted river systems, and inevitably epidemics of fatal diseases caused by pathogenic bacteria of faecal origin such as *Vibrio cholerae* and *Salmonella* spp. dispersed by them. Eventually, authorities were forced to take action and after several royal commissions, the Rivers Pollution Prevention Act was brought into effect in 1876. In fact, this act was fairly ineffectual, and achieved little except that it led to another Royal Commission on Sewage Disposal in 1898, with the function of coordinating evaluation of new treatment systems. Those that had been developed following the disease epidemics in Britain such as biological trickling filters, land effluent percolation, and septic tanks (see Septic Tank Systems) suffered from two major disadvantages. They were slow and inefficient and/or operated on a relatively small scale.

One important outcome of the Commission was the stipulation of standards for treated wastes, and in 1908, it recommended that treated effluent should meet the 30:20 standard (for BOD_5 and Suspended Solids, respectively), which was adopted in Britain in 1912. This requirement provided further impetus for developing more satisfactory treatment systems that could meet this standard, and undoubtedly played an influential role in the development of the activated sludge process in 1914.

In fact, this process grew from a trip Dr. Gilbert Fowler of the University of Manchester made in 1912 on behalf of the Manchester Corporation to the Lawrence Experimental Station in Massachusetts to look at different systems of treating sewage, including aerated processes under study there. On his return to Manchester, he passed on his experiences to Edward Arden and William Lockett at the Manchester-Davy Hulme wastewater treatment plant. They both understood the importance of the American work, and were inspired to carry out laboratory scale research during 1913 and 1914, which led to the activated sludge process. Their brilliantly original idea was to retain the sedimenting biomass (sludge) produced from the waste and reuse it to treat further waste material, instead of discarding it as had been the standard practice until then. By adopting this strategy, they showed that the aeration times needed to degrade the organic compounds in the sewage and achieve complete nitrification were spectacularly shortened over those in existing wastewater treatment systems.

The solids were perceptively called "activated sludge" by Arden and Lockett, the results of their work were published (4,5) and their process, very similar in configuration to current conventional activated sludge systems, was tested on a pilot plant scale in 1914. Both continuous flow and fill-and-draw (the Sequencing Batch Reactors Technology, which is discussed elsewhere) configurations were so successfully tested that a full-scale continuous plant was installed in Worcester, England, in 1915, the first one of its kind in the world. It used a mechanical aeration system (which is still preserved and functioning at a plant in Stockport, England) since the submerged diffusers used in the pilot plant system became rapidly clogged, a problem still encountered in many plants today and one which led to development around the world of a variety of aeration systems. The attractions of the activated sludge system soon spread outside of Britain, and by the beginning of World War II, plants had been built in many European and Commonwealth countries (2,3).

Since then the activated sludge process, which was developed originally mainly to remove carbonaceous materials and produce an effluent meeting the 30:50 standard, has undergone many modifications to its design and operation to increase its efficiency and flexibility so that many plants built now will also effectively remove both nitrogen and phosphorus (6,7).

FEATURES OF THE PROCESS

In terms of the volume of material handled, the activated sludge process probably ranks as the largest biotechnology process in the world (8). Its features (Fig. 1) include (9–11) the following:

- It uses a complex community of mixed populations of bacteria and protozoa (ACTIVATED SLUDGE — THE MICROBIAL COMMUNITY).
- This community has to cope with an uncontrollable diverse range of organic and inorganic compounds, some of which will be toxic to these microbes.
- The microbes are organized into discrete aggregates called flocs (ACTIVATED SLUDGE — THE FLOC), which are maintained in suspension in the aeration tank by mechanical agitation/aeration or by the mixing action of air bubbles from submerged aeration systems.
- The flocs must have good settling properties so that separation of solids (biomass) and liquid phases, upon which the success of the process ultimately depends, can occur efficiently and rapidly in the clarifier.
- In many plants this separation is not achieved because of the operational problems of bulking (FILAMENTOUS BULKING IN ACTIVATED SLUDGE, CONTROL OF) and foaming (ACTIVATED SLUDGE — FOAMING).
- Most of the settled biomass, the activated sludge, is recycled as "return activated sludge" or RAS to inoculate the incoming raw sewage, with the understanding that it will contain a selected community of microbes well adapted to treating that particular raw sewage.
- Some of this sludge is discarded or wasted, and although rich in nutrients, valuable minerals, and a possible food source, the sludge is viewed as a liability that is disposed of, often at great expense.

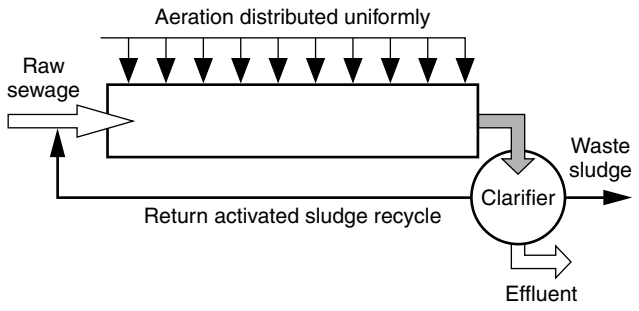

Figure 1. The activated sludge process.

Because of all these uncontrollable operational variables, it seems remarkable that activated sludge systems work as well as they do. However, because they are so versatile and adaptable, it is very likely that they will continue to represent the most widely used methods for aerobic waste treatment into the foreseeable future and evolve as they have in the past to meet additional requirements and process stringencies (7,11).

CONFIGURATIONS FOR ACTIVATED SLUDGE SYSTEMS

Many activated sludge configurations are in use around the world, differing in their design features and their performance criteria. It is convenient here to categorize them on their abilities to remove different organic and inorganic chemical components in the influent, a feature that reflects their design and operation into:

- conventional (those plants designed to remove primarily organic carbonaceous compounds) and
- nutrient removal (where the plants remove nitrogen and/or phosphorus either chemically or microbiologically) (7,12).

CONVENTIONAL ACTIVATED SLUDGE SYSTEMS

These were the early activated sludge systems introduced. Some of the first ran as semicontinuous "fill-and-draw" systems, the basis for the increasingly popular sequencing batch reactor configurations (7), while most conventional systems now run continuously and operate either as "plug flow" or "completely mixed systems", although the distinction between them when operating is not always clear (2,3,13).

Plug Flow Systems

These are systems in which the incoming sewage is thought to move as a discrete plug through the reactor(s), although some mixing will inevitably accompany aeration, either from submerged diffusers or surface agitators. The general view is that these plug flow systems are less prone to filamentous bacterial bulking (FILAMENTOUS BULKING

IN ACTIVATED SLUDGE, CONTROL OF), although they also operate relatively inefficiently because of their hydraulic characteristics that generate an uneven load distribution along the reactors in terms of their oxygen demand (7,11). Engineering approaches to this problem include adjusting the aeration systems to redistribute the oxygen to meet these different requirements, through modifications such as tapered aeration (Fig. 2), step aeration (Fig. 3), or altering the feed regime as in the step feed (Fig. 4; 6,7,11).

Completely Mixed Systems

Such systems evolved to overcome the problems with "plug flow" systems, in which all the components, including the RAS, are rapidly mixed (Fig. 5). There is a price in terms of loss of nitrification efficiency if underaeration or plant overloading occurs, short circuiting may be a problem, and the sludge usually settles less well than with "plug flow" systems (2,3). Completely mixed systems were found to be less susceptible to toxic materials in the feed due to the rapid dilution of the incoming waste.

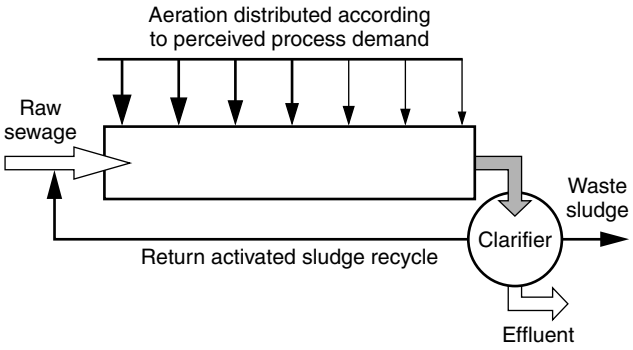

Figure 2. The activated sludge process with tapered aeration.

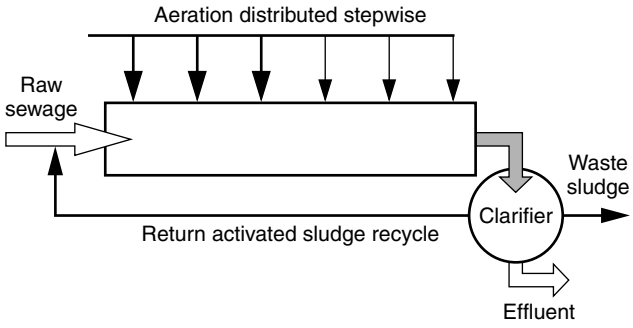

Figure 3. The activated sludge process with step aeration.

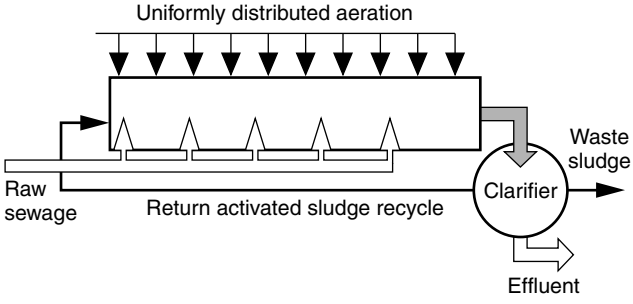

Figure 4. The activated sludge process with step feed.

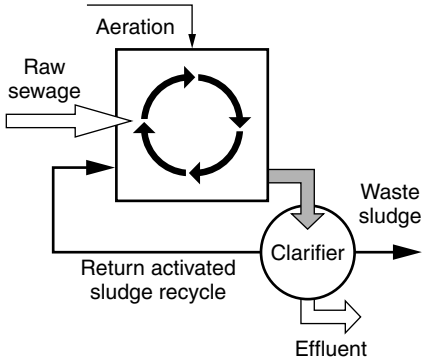

Figure 5. The complete mix activated sludge process.

Extended Aeration Systems

As the name suggests these processes operate best with low loadings, and long sludge ages (2,3). These systems were designed as inexpensive processes for small rural communities and were called Pasveer ditches after the Dutch Engineer who introduced them. These small plants were operated intermittently ("fill-and-draw") but the later-introduced Carrousel plants, which borrowed and developed the original concept, operated continuously and used separate clarifiers (Fig. 6). Surface agitation provides aeration and circular mixing, in which high dissolved oxygen levels close to the agitators encourage nitrification and denitrification then follows in the anoxic regions, which develop as the liquid moves further away from them (ACTIVATED SLUDGE — MICROBIOLOGY OF NITROGEN REMOVAL). Less sludge is produced because of their long sludge ages and they need little maintenance as long as the mixing is sufficient to keep the solids/sludge in suspension.

Stimuli for Improving Performances of Conventional Plants

Most attempts at improving performances of these plants have been directed at increasing their operational rates, especially with potentially toxic industrial wastes and reducing the volumes of sludge produced. Modifications include "contact stabilization," high rate systems including processes such as the Deep Shaft process in which pure O_2

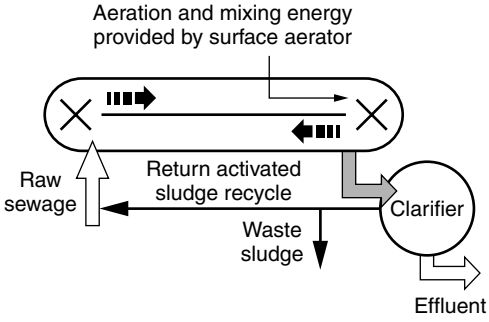

Figure 6. The Carrousel activated sludge process.

is used instead of air. Gray (2) discusses these processes in detail. Also, conventional systems fail to remove sufficient levels of inorganic nutrients such as nitrogen and phosphorus, both of which are serious environmental hazards, to prevent eutrophication and formation of "algal" or blue-green bacterial (Cyanobacterial) blooms in receiving rivers and lakes. So, many of the changes to conventional activated sludge processes have been in developing systems to remove these nutrients. Only microbiology-based activated sludge systems are discussed here, and the reader is directed to the reviews of References 6 and 14 for discussions on chemical nutrient removal systems, which pose their own environmental problems (15). Some plants using microbes often have facilities for chemical polishing when they fail to perform adequately to satisfy discharge license requirements (6).

ACTIVATED SLUDGE SYSTEMS DESIGNED TO REMOVE NITROGEN

Some nitrogen (N) removal will always occur in conventional plants since cells in the microbial community require nitrogen to grow, and some of these will be removed in sludge wasting. Furthermore, nitrification and denitrification leading to N removal will occur as long as conditions for these processes are met (ACTIVATED SLUDGE — MICROBIOLOGY OF NITROGEN REMOVAL). Because the amount of N removed by conventional processes is often small, some plants are deliberately designed and configured to achieve much higher rates and levels for its removal. Several conditions need to be met and all plants deliberately designed to remove N incorporate these features. These (ACTIVATED SLUDGE — MICROBIOLOGY OF NITROGEN REMOVAL) and their operational requirements are (10,16,17):

- an aerobic zone in which nitrification by the chemoautotrophic nitrifying bacteria can occur. This step is essential to generate nitrate from the oxidation of ammonia as the levels of nitrate in domestic sewage from prior nitrification are generally too low to encourage microbial activity and permit the chemical transformations needed for N removal;
- an anoxic zone in the reactor, with high levels of nitrate from the nitrifying bacteria, which acts in the absence of dissolved oxygen as the terminal electron acceptor for the anaerobically respiring chemoheterotrophic denitrifying bacteria. Many taxonomically diverse bacteria can denitrify, although it is still unclear as to which ones are important in activated sludge systems. It is known that many of these can also denitrify under aerobic conditions, although the impact of these aerobic denitrifiers on nitrogen removal in activated sludge systems is again unclear (16);
- most plants designed to remove N use the same biomass for both the nitrification and denitrification steps. In the aerobic zones of many such plants, simultaneous nitrification/denitrification (SND) is thought to occur (16), raising the tantalizing possibility of manipulating SND to make future N removal plants simpler in configuration and enabling a reduction in the size of the anoxic zone;
- an organic carbon and energy source to allow the denitrifying microbial community in the anoxic zone to reduce the nitrate to nitrogen gas. Deliberate addition of chemicals such as methanol for this purpose was once common, but as their costs increased, plants were designed to use substrates already present for this anaerobic respiration. Many plants, for reasons not yet understood, probably only partially reduce the nitrate to nitric oxide and nitrous oxide, both serious long-term air pollutants, in the anoxic zone (16);
- in plants in which the aerobic zone precedes the anoxic zone, as seen in the early Wuhrmann (18) design (Fig. 7), these organic substrates originate from lysis of the biomass as most readily biodegradable organic substrates would already have been utilized in the aerobic zone. Consequently, denitrification rates are low;
- later designs (19) placed the anoxic zone in front of and in partial contact with the aerobic zone (Fig. 8). Variable plant performance with this configuration and a better comprehension of the process requirements led to the suggestion by Barnard to modify it by physically separating the anoxic and aerobic zones and providing a recycle between the two into a two-stage process (Fig. 9). However, this also has an inherent weakness since nitrate removal will always be incomplete because the liquor recycled to the anoxic zone and the clarifier feed are derived from the same source (the aeration basin);
- Barnard (20) recognized these faults and came up with the four-stage Bardenpho process (Fig. 10) by incorporating a secondary anoxic reactor after the aerobic zone, with the dual advantage of maintaining a higher denitrification capacity and a nearly nitrate-free effluent. It also included a secondary aerobic reactor (reaeration reactor) to allow nitrification of any ammonia produced in the secondary anoxic zone;
- this configuration should be regarded as the direct ancestor of most of the nutrient removal systems that have followed, and required only minor modification to successfully remove P (6,7,11,20,21).

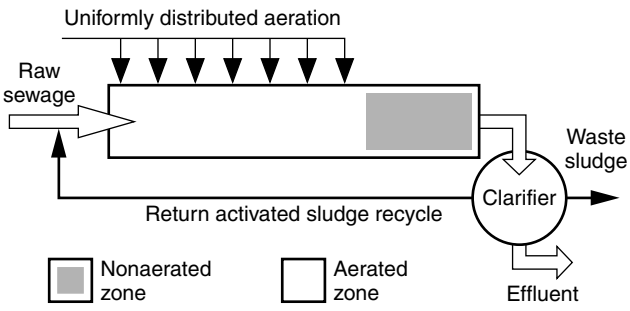

Figure 7. The Wuhrmann process.

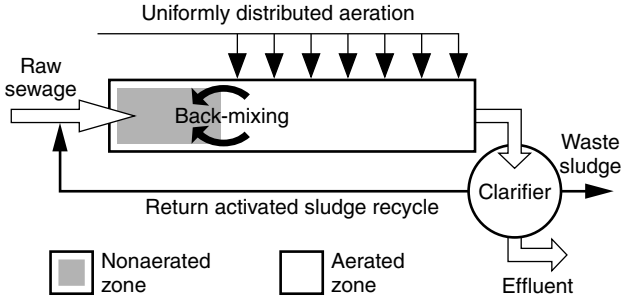

Figure 8. The Ludzack-Ettinger process.

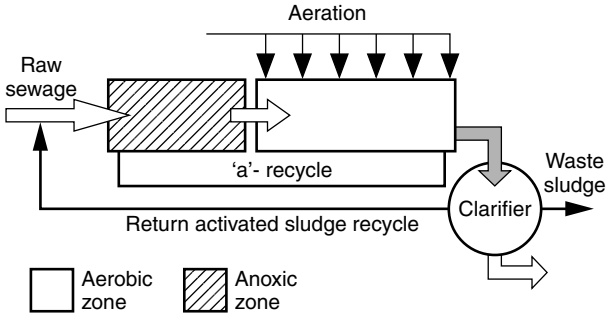

Figure 9. The modified Ludzack-Ettinger process (MLE).

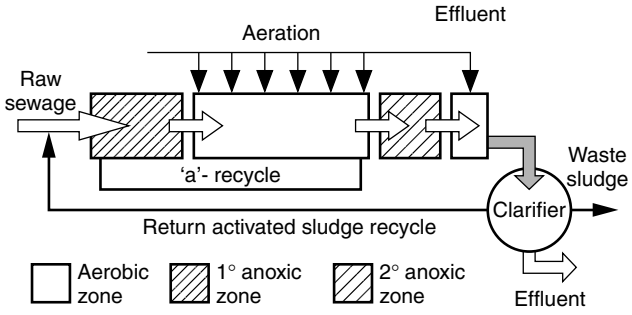

Figure 10. The Bardenpho process.

ACTIVATED SLUDGE SYSTEMS DESIGNED TO REMOVE PHOSPHORUS

Conventional activated sludge systems remove very little of the phosphorus that enters in the raw sewage, except that required by the biomass to grow. Thus, their effluents are usually rich in P and they are recognized as one of the major point sources for environmental P pollution (22). P-removing activated sludge systems work because the microbes assimilate more P than is needed for their growth, and so are also called enhanced biological phosphorus (EBPR) systems (7,20,21). Their detailed microbiology is discussed elsewhere (ACTIVATED SLUDGE — MOLECULAR TECHNIQUES FOR DETERMINING COMMUNITY COMPOSITION). Most agree that current EBPR systems, if properly designed, should yield a final effluent P level of less than 1 $mg\,l^{-1}$ (21).

Much of the development of these processes occurred in South Africa and most of the plants currently in

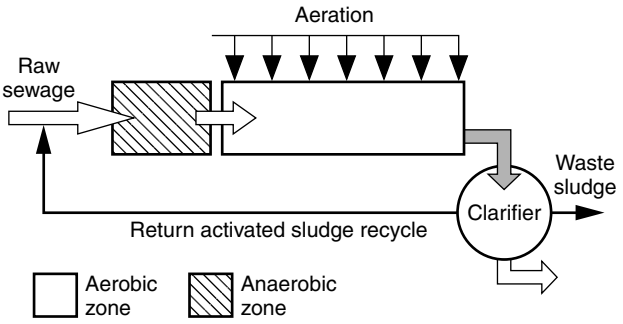

Figure 11. The two-stage or high rate Phoredox process.

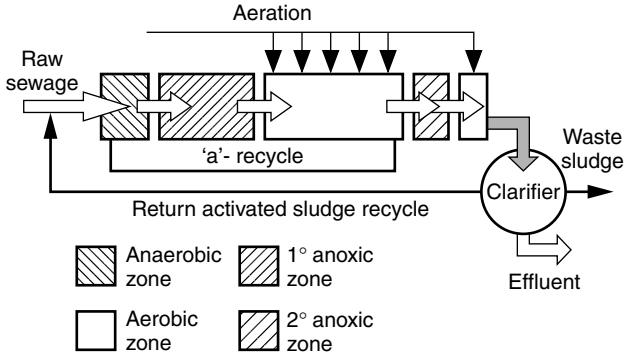

Figure 12. The five-stage Bardenpho or Phoredox process.

use have evolved from there. Barnard's pioneering work in the 1970s helped to strictly define the operational requirements for EBPR, although this phenomenon of EBPR was reported much earlier (23). He showed empirically that adding an anaerobic zone to the head of a conventional plant gave a process (the high rate Phoredox system, Fig. 11) that would remove P when the system was operated at a low enough sludge age to avoid nitrification. This process, in combination with the four-stage Bardenpho (Fig. 10) configuration, gave rise to what is known as the five-stage Bardenpho or Phoredox system (Fig. 12), a plant configuration capable of removing both N and P.

His and other studies elucidated the main design features needed for successful P removal. These include

- an initial anaerobic zone or reactor, which is an essential component of all EBPR systems. This zone appears to selectively favor bacteria [including the P accumulating bacteria (PAB) responsible for EBPR] that are capable of anaerobically assimilating the organic substrates present there (mainly short-chain volatile fatty acids, SCVFA, such as acetate). Cells store these substrates as intracellular reserves of poly β-hydroxyalkanoates (PHA) to be used as sources of energy and carbon for their growth in the aerobic zone (see later), where now other metabolizable substrates are no longer available.
- the stored polyP that is used as the energy source for PHA synthesis and the phosphate produced is released into the bulk liquid. Strictly anaerobic

conditions are essential here to eliminate any aerobic respiration of these organic substrates, reducing their availability to the PAB.

- a prefermenter using an anaerobic digestion of settled sludge that is installed in many EBPR plants before the anaerobic reactor to guarantee a supply of high levels of SCVFA to cells in the anaerobic zone (24). In some designs, acetate itself is added to the anaerobic reactor. Claims are that these modifications both improve and stabilize EBPR in systems in which they have been introduced, although odor can become problematic. However, the size of the anaerobic reactor can now be reduced.
- an anoxic zone where denitrification is encouraged (in plants that nitrify) so that the levels of nitrate entering the anaerobic zone are insufficient to permit any anaerobic respiration of these organic substrates, reducing their availability to the PAB. Consequently, most of the later designed plants removing P also remove N, although the presence if not the importance, of denitrifying PAB in activated sludge systems has been well documented (25) and these may make a contribution to denitrification in the anoxic zone.
- an aerobic zone, where the nitrifying bacteria function and those with stores of PHA produced in the anaerobic zone can grow and use the energy made available from them to assimilate phosphorus from the bulk liquid and store it as polyP granules. As more P is assimilated aerobically than released anaerobically, net P removal occurs, and this assimilated P immobilized in the biomass is wasted with it.
- alternating the biomass between the anaerobic and aerobic zones in the plants, although this was not a deliberate design feature of some of the earlier plants.

Stimuli for Improving Performances of EBPR Systems

Many of these improvements have been based on a better understanding of the microbiology of these systems, which is a trend that is likely to continue. These were introduced in attempts to (26):

- simplify plant configuration, reduce the size of the reactors (see the preceding text) making them inexpensive to build and run
- cope with high sudden flow rates, which would dilute SCVFA levels below those capable of supporting good EBPR
- overcome the perceived operational problems in many of the earlier design changes caused by returning nitrate back to the anaerobic zone, a stimulus that led to many new plant designs, some of which are operating successfully around the world. Thus, the three-stage Phoredox process evolved from the five-stage system by increasing the size of the primary anoxic zone to increase denitrification levels and reduce recycled nitrate, so that the protecting secondary anoxic zone and reaeration reactor were no longer needed (Fig. 13).

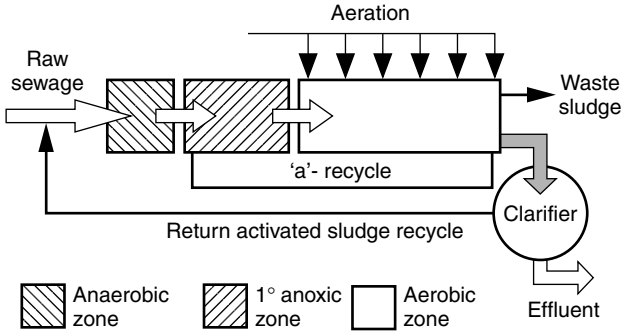

Figure 13. The three-stage Phoredox process.

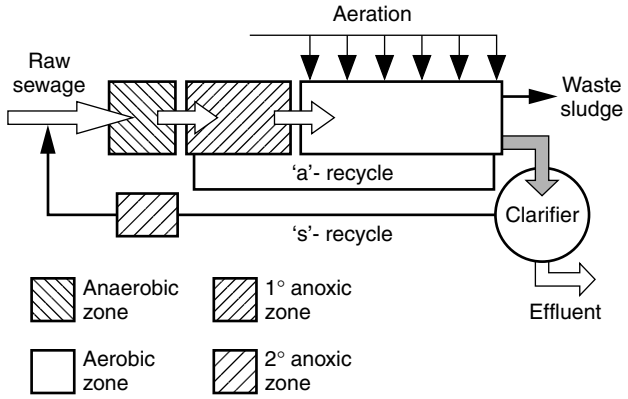

Figure 14. The Johannesberg process.

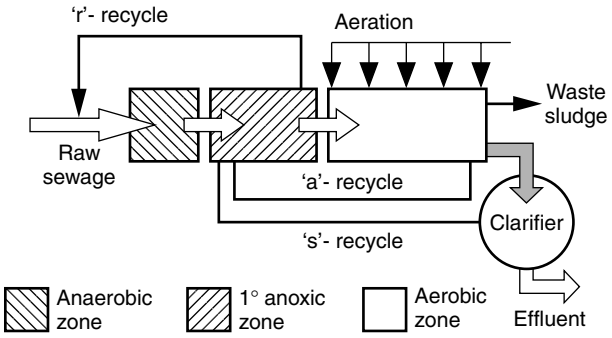

Figure 15. The University of Cape Town (UCT) process.

This design has in turn produced several progeny with characteristics to cope with difficulties encountered over time with their use. A few examples are given here. The Johannesburg process (Fig. 14) incorporated a secondary anoxic zone in the RAS line to the anaerobic reactor to ensure complete nitrate removal, but not without operational consequences, which were addressed by the University of Cape Town (UCT) process that recycles the RAS back into the anoxic rather than the anaerobic zone first before another recycle then transfers it from the anoxic to the anaerobic reactor (Fig. 15). Additional improvement was achieved when the anoxic zone was split into two separate reactors to provide further control, as seen with the Modified UCT (MUCT) process (Fig. 16). The

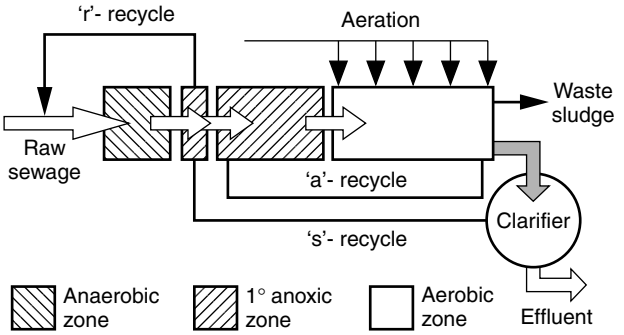

Figure 16. The modified UCT process.

MUCT process sacrifices some of its overall denitrification capacity to insure that nitrate is completely removed from the recycle to the anaerobic basin, thus protecting this zone and optimizing the P removal process.

- prevent P release (secondary release) from the settled sludge in the clarifier and the RAS by avoiding establishment of anaerobic conditions anywhere in the sludge handling processes (e.g., by avoiding prolonged sludge storage).

THE FUTURE FOR ACTIVATED SLUDGE PROCESSES?

What activated sludge plants might look like and how they might operate in 50 or 100 years from now is difficult to predict, but a few speculations are allowed.

- Methods and instrumentation for automatic monitoring and control of these systems will rapidly continue to increase.
- Single "add-on" units containing communities of bacterial populations with specific metabolic traits to treat particular wastes (e.g., toxic and industrial) will become common.
- Advances in membrane technology will revolutionize solids separation so that clarifiers will become archaeological oddities and problems such as bulking will become simple to handle.
- Membranes will be used increasingly in plant design so that most will use them to polish treated effluent water for reuse as potable water.
- Our knowledge of the bacteria responsible for N and P removal and the factors that affect these processes in these organisms will undoubtedly increase significantly, and this information will lead to the design of novel configurations with input from both engineers and microbiologists, ensuring that these populations and their pertinent metabolic properties are particularly encouraged.
- Sludge reuse will also become more sophisticated and less costly.

It is possible that few or none of these changes will occur, and future developments in the treatment of wastewater will use totally different technology. In either case, the costs of building the systems and running them will increase and additional demands will be placed on the operational staff who will need to be highly skilled.

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ACTIVATED SLUDGE: USE OF MOLECULAR TOOLS.

See ACTIVATED SLUDGE — MOLECULAR TECHNIQUES FOR DETERMINING COMMUNITY COMPOSITION

ACTIVITY AND CARBON TRANSFORMATIONS IN BIOFILMS

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Biofilms are an important sink of the carbon-associated biomass in aquatic and terrestrial ecosystems. Much of this biomass comprises eubacteria, although archaebacteria, cyanobacteria, fungi, and various forms of algae can be quantitatively important or perhaps, dominant in biofilms under favorable circumstances. The superior growth rates of prokaryotes enables a rapid turnover of usable substrates generated by primary producers, thus establishing the critical role of these organisms in the cycling of organic matter. It is an apparent paradox that biofilms form in natural waters, which are considered to be nutrient-depleted or "oligotrophic." While a reduction in the supply of nutrients in technical systems below a certain threshold level can minimize the formation of biofilms (1), this principle cannot be extended to the fundamental complexities of natural environments. A complete description must take into account the diversity of substrates available for use, the fluxes of substrates to and from biofilms, the taxonomic diversity of biofilm organisms, and the numerous metabolic pathways, which have evolved to enable the transformation of structurally dissimilar and dilute carbon sources into potentially utilizable forms. The majority of abiotic carbon compounds in soils, sediments, and natural waters are decay products known as humic substances, which bear only a superficial resemblance to their molecular precursors. The quantitative dominance of humic substances in the dissolved phase is a consequence of the relative recalcitrance of these compounds and the fact that most assimilable carbon compounds are immobilized as cellular biomass in biofilms. The use of humic substances as a source of carbon and energy is not necessarily restricted by an absence of suitable degradation pathways; at least, part of the explanation for the dominance of these compounds in natural systems is related to the inefficiency of microbial degradation pathways. Microorganisms have evolved diverse and efficient pathways for the utilization of those carbon substrates that warrant a metabolic investment.

DIVERSITY OF STRATEGIES FOR CARBON METABOLISM BY BIOFILM ORGANISMS

Environmental biofilms contain both eukaryotic and prokaryotic organisms, which act as primary and secondary producers. Primary production is the process by which photosynthetic organisms synthesize organic carbon compounds from carbon dioxide. Secondary producers convert the products of primary production into biomass (2).

By convention, metabolism of carbon substrates is divided into two closely linked processes.

- Dissimilatory metabolism involves catabolic (degradative) activities performed to provide energy for cellular respiration.
- Assimilatory metabolism describes anabolic reactions, in which energy is expended for the assimilation of low-molecular-weight substrates into macromolecules, which have structural or physiological functions.

Most of the energy obtained from catabolic reactions is used for growth, although biofilm cells spend a disproportionate part of their energy budget on maintenance and the production of the biofilm hydrogel matrix (extracellular polymeric substances, EPS). Planktonic bacteria seem to have adopted a strategy for maximum substrate utilization, rather than efficiency (3). The major transformation carried out by microorganisms in biofilms can be summarized as the metabolism of inorganic and organic electron donors and acceptors to produce soluble by-products, EPS, carbon dioxide, and/or methane, and water (4). The mechanisms used to achieve these goals are considerably more diverse, and all major metabolic classes of microorganism are represented in biofilms. In fact, it is often the biofilm that creates the potential for a metabolic group of organisms to become established. Recent studies employing either microelectrodes or fluorescent in situ hybridization (FISH) in combination with confocal laser scanning microscopy (CLSM) have provided direct evidence for the existence of different metabolic groups within discrete areas of the biofilm matrix (5). In general, classifications of biofilms according to metabolic pathways include the designations aerobic, anaerobic (sulfate-reducing bacteria, methanogens), anoxic (denitrifying), and fermentative, depending on the type and concentration of the electron donor and acceptor (6). Microorganisms can also be described in terms of carbon utilization:

- Autotrophs assimilate single-carbon (C-1) compounds, including CO_2, CH_4, and methanol.
- Photoautrophs harness electromagnetic energy to generate ATP and the reducing power required for the assimilation of carbon dioxide.
- Chemoautotrophs (lithotrophs, *lithos* Gr. for rock) obtain cellular carbon by assimilative carbon dioxide reduction or from reduced C-1 compounds and energy from oxidation of inorganic compounds such as NH_4^+.
- Heterotrophs (chemoorganotrophs) obtain energy from the oxidation of complex organic carbon sources.

The terms autotrophy and heterotrophy can apply to both dissimilatory and assimilatory metabolism. Autotrophs may require additional carbon in the form of growth factors or vitamins (6), but they assimilate all necessary elements as inorganic forms. The energy expenditure required for synthesizing macromolecules

partly accounts for the lower growth rates of strict autotrophs compared to most biofilm bacteria, which are (chemo)heterotrophic — they depend on organic compounds to generate energy and the precursors used to synthesize cellular biomass. A low-molecular-weight substrate may serve as a source of carbon for building macromolecules and as a source of cellular energy, producing carbon dioxide as a by-product (6). Phototrophic microorganisms use different catabolic and anabolic substrates. The complete metabolism of carbon substrates to CO_2 or CH_4 is called "mineralization." Confusingly, some of the inorganic carbon in biofilms is converted to mineral forms such as calcium carbonate, partly by abiotic processes and partly as a result of microbially-induced increases in pH (7). The biotic mechanisms are known as "biomineralization." Together with various geologic processes, these reactions form sedimentary deposits known as microbialites, which constitute fossilized biofilms. More specifically, stromatolites and thrombolites are formed by a consortia of bacteria and filamentous cyanobacteria, and bacteria and unicellular cyanobacteria, respectively (7).

Energy-yielding processes (catabolism) are further categorized as fermentation, respiration, phototrophy, and methanogenesis.

Fermentation

Fermentation is often used in a general sense to describe the utilization of a substrate by a microbial culture (heterotrophic fermentation). In a metabolic sense, fermentations are anaerobic processes in which the substrates are sequentially transformed by reduction-oxidation (redox) processes in the absence of an external electron acceptor. The redox levels of the substrate and metabolite(s) remain the same, and the energy yields are low. Fermenting bacteria may be:

- strict (obligate, O_2-sensitive) anaerobes, for example, *Clostridium* spp.;
- facultative anaerobes, which are capable of oxidative phosphorylation (production of adenosine triphosphate, ATP) in the presence of O_2, for example, *Pseudomonas* spp.; or
- aerotolerant anaerobes, for example, *Lactobacillus* spp., some *Streptococcus* spp.

Facultative anaerobes are especially suited to the gradients, which develop suddenly within biofilms as a consequence of the rapid depletion of oxygen in the vicinity of immobilized cells. Anaerobic fermentation of simple sugars yields little energy compared with aerobic metabolism. This type of fermentation is exploited in industry, but it is rare in environmental biofilms because the heterotrophic microorganisms usually ensure that high concentrations of sugar substrates do not accumulate. Amino acids can be fermented via the Stickland reaction, whereby one kind of amino acid is used to oxidize another to acetate and ammonia (8). This process is important when high concentrations of proteins are available for anaerobic degradation. Planktonic microorganisms can encounter relatively high-protein concentrations on suspended particles (9), where anaerobic zones form within flocs (see Microbial Flocs Suspended Biofilms, this Encyclopedia).

Respiration

The primary goal of respiration is the production of ATP (phosphorylation) by any combination of organic or inorganic electron donor and electron acceptor. Aerobic respiration of an organic substrate is described most simply as:

$$(CH_2O) + O_2 \rightarrow CO_2 + H_2O \qquad (1)$$

This process yields the most energy of all catabolic processes, so it is preferred by facultative microorganisms when oxygen is available. Many microorganisms are able to perform oxidative phosphorylation of organic substrates. Most respiratory organisms use the citric acid (or tricarboxylic, TCA) cycle for the oxidation of acetate to carbon dioxide, whereas phototrophic green sulfur bacteria and some archaea use the reverse of this cycle to assimilate carbon dioxide. Purple nonsulfur bacteria use the same electron transport system for both respiration and photophosphorylation. Anaerobic respiration is characterized by the use of exogenously derived terminal electron acceptors other than oxygen. Energy yields are always lower than those obtained by oxidative phosphorylation. Ecologically important anaerobic respiration processes include the reduction of sulfate, nitrate, iron, and manganese, although some low-molecular-weight organic compounds may also serve as terminal electron acceptors. Nitrate or nitrite reduction is performed by many facultative anaerobes, including members of the ubiquitous genera *Bacillus* and *Pseudomonas*. In these organisms, O_2 inhibits synthesis of the key enzyme, nitrate reductase (6).

Sulfate-reducing organisms are common in biofilms due to the depletion of oxygen by respiring organisms and subsequent diffusion limitation within the EPS matrix. Sulfate reduction is an important contribution to anaerobic metabolism in both freshwater and marine sediments. The principal carbon substrates for this process are low-molecular-weight organic acids (acetate, butyrate, propionate, lactate), alcohols, and H_2. Sulfate reducers can be subdivided into two further groups on the basis of carbon utilization. Organisms such as *Desulfovibrio* spp. do not use a citric acid cycle. They can use H_2 or lactate as substrates. The use of lactate is inefficient in that the carbon product is acetate (rather than methane), but this substrate is readily assimilated by heterotrophs within the biofilm matrix. Other organisms such as *Desulfobacter* spp. make use of a citric acid cycle to oxidize acetate completely. Members of the genus *Desulfuromonas* use elemental sulfur rather than sulfate for the complete oxidation of acetate, but also produce CO_2 and HS^-. The methanotrophs in soil and aquatic environments are gram-negative bacteria, which oxidize methane or other C-1 compounds, such as methanol or methylamine. They act as biofilters for methane produced in anaerobic environments. Methanotrophs are classified into three groups, partly according to their ability to assimilate the key intermediate, formaldehyde (10). Anaerobic oxidation of CH_4 takes place in sediments and soils; when oxygen

is present, soil methanotrophs can oxidize atmospheric methane. Some methanotrophs can synthesize a soluble methane monooxygenase capable of oxidizing priority pollutants such as trichloroethylene (10).

Methanogenesis and Acetogenesis

Methanogens belong to the archaea domain and reduce carbon substrates to methane, using H_2 as electron donor. The carbon substrate is usually carbon dioxide, which also constitutes autotrophy. Carbon dioxide, carbon monoxide, and formate comprise one group of potential substrates for methanogens. A second group of substrates comprises compounds with methyl groups, which can act as both electron donor and acceptor. The cleavage of acetate (acetotrophy) is a third mechanism, producing methane and carbon dioxide. Methanogenesis is important in anaerobic wastewater treatment, during which soluble organics are degraded to fatty acids and subsequently converted to methane and carbonate. The fermentation of acetate is responsible for the bulk of the methane formation (11). This process is performed by acetogens and homoacetogens.

Phototrophy and Photoautotrophy

Phototrophy and photoautotrophy are terms to describe carbon dioxide fixation, which is mediated by photosynthesis:

$$nCO_2 + 2nH_2O \rightarrow n(CH_2O) + O_2 + nH_2O \qquad (2)$$

Light energy is converted to chemical energy (ATP and the reduced form of nicotinamide adenine dinucleotide phosphate, NADPH) in the "light" reactions, and subsequently used to reduce carbon dioxide to organic compounds in the "dark" reactions. Phototrophs and some other microorganisms contain various forms of chlorophyll and accessory pigments, including carotenoids and phycobilins. Carotenoid pigments can transfer energy, but not directly through photophosphorylation. Phycobiloproteins are the principal light-harvesting pigments of cyanobacteria and red algae. They are arranged as high-molecular-weight aggregates called phycobilisomes. The energy transfer from the biliprotein complex to chlorophyll *a* is very efficient, enabling growth under low-light conditions. The phycobilisome content correlates with light intensity.

The two modes of phototrophy are oxygenic photosynthesis and anoxygenic photosynthesis. Oxygenic photosynthesis can be performed by organisms that contain chlorophyll *a*, including various microalgae. Light energy is used to synthesize ATP and NADPH by noncyclic photophosphorylation, whereby O_2 is produced from water. Cyanobacteria are the only prokaryotes capable of oxygenic photosynthesis (12). Filamentous cyanobacteria dominate shallow sediments and microbial mats, which are essentially thick, photosynthetic biofilms. The unicellular cyanobacteria (e.g., *Synechococcus*) are poorly represented in microbial mats, but they are important as planktonic primary producers (13). Oxygenic phototrophs have two, spectrally distinct forms of chlorophyll *a*, enabling the operation of two separate reaction systems, Photosystem I and Photosystem II. The so-called purple and green bacteria carry out photosynthesis, but do not produce oxygen. This form of photosynthesis involves the production of ATP by cyclic phosphorylation. Anoxygenic photosynthesis occurs in illuminated sediments, microbial mats, and anoxic zones in (other) biofilms, in which H_2S acts as proton and electron donor, and elemental sulfur is produced. The exposure of oxygenic, sediment-dwelling cyanobacteria to sulfide inhibits Photosystem II; however, the active Photosystem I allows anoxygenic photosynthesis with HS^- as the electron donor. In dark and anaerobic conditions, some cyanobacteria seem to be capable of fermentation or sulfate reduction (6). Four, unrelated groups of eubacteria can perform anoxygenic photosynthesis, and different groups have different combinations of carotenoid pigments. The purple bacteria contain bacteriochlorophyll *a* or bacteriochlorophyll *b*. The purple sulfur bacteria (e.g., *Thiocapsa*, *Chromatium*) use reduced sulfur as an electron donor, and also store elemental sulfur. The "purple nonsulfur bacteria" (e.g., *Rhodospirillum*) do not store elemental sulfur, and they are more sensitive to high sulfide concentrations. The designation is a misnomer because these organisms are also capable of using HS^- as electron donor. Purple nonsulfur bacteria are versatile in that they can use some carbon compounds (and Fe^{2+}) as electron donors, and they can assimilate low-molecular-weight compounds such as acetate. The green sulfur bacteria (e.g., *Chlorobium*) have bacteriochlorophylls *c* or *d*. They are autotrophic, strictly anaerobic organisms, which use sulfide as an electron donor, but they only oxidize it to S^0, which is excreted. The green nonsulfur bacteria (e.g., *Chloroflexus*) are also erroneously named, being able to use HS^-. These are filamentous gliding forms found in microbial mats, and they can live as photoautotrophs using HS^- and CO_2 or as aerobic heterotrophs. The heliobacteria are strict phototrophic anaerobes, which have been isolated from anoxic soils. Halobacteria are a group of archaebacteria, which usually act as aerobic heterotrophs, but under conditions of anoxia and light, they revert to phototrophy. A "purple" membrane consisting of a photosensitive pigment, rhodopsin, facilitates proton export from the cell. The proton motive force is then harnessed by ATPase in the cell membrane for ATP synthesis.

SYNTROPHY AND ANTAGONISM IN BIOFILMS

Ultimately, the establishment of different organisms within a biofilm is dependent on competition, and many interactions may be antagonistic (14). There is no convincing, direct evidence for antagonistic interactions within a biofilm, as can be observed frequently in the case of inhibition zones surrounding colonies of microbial soil isolates on laboratory media. It can be inferred, however, that sulfate reducers and methanogens will compete for carbon dioxide. Also, methanogens and homoacetogens use the same precursors, CO_2 and H_2, to produce methane and acetate, respectively. Unless carbon dioxide is subject to considerable diffusion limitation within a biofilm, its availability is far better than the relatively

insoluble O_2, which is depleted rapidly by respiring organisms. Competition is especially relevant for the limited supply of low-molecular-weight carbon substrates such as monosaccharides and amino acids, which constitute a "superlabile" carbon pool. The proximity of metabolically diverse neighbors and selection pressures, however, implies that neutral or even favorable interactions will allow particular metabolic groups to establish within the biofilm matrix. This is especially important for photosynthetic biofilms, in which the carbon and oxygen cycles are intimately related. Algal exudates in epilithic biofilms provide heterotrophic bacteria with significant quantities of organic and inorganic substrates (15,16). Syntrophy occurs when two or more organisms catabolize substrates in a mutually-dependent manner. In freshwater systems, purple nonsulfur bacteria commonly form syntrophic associations with heterotrophic sulfur reducers. Methanogens depend on other organisms for a supply of the few compounds, which can act as precursors for methane production, including some products of respiration. Respiring organisms can hydrolyze polysaccharides, proteins, and other macromolecular organic substrates. Low-molecular-weight organic acids produced by fermenting bacteria can be used by sulfate reducers, accounting for the presence of these organisms in consortia (6). Fermentation of organic substrates proceeds only as far as acetate + H_2, but the acetate can be respired by other organisms. Thus, complete mineralization depends on the supply of substrates by actively fermenting bacteria. In the case of phototrophs, energy derived from the oxidation of a noncarbon substrate, such as sulfide, requires the assimilation of some other carbon source, such as carbon dioxide produced by fermenting organisms. The expected carbon transformations in a mixed phototrophic/heterotrophic biofilm are depicted in Figure 1. The fermentation of glucose to acetate and H_2 by obligate acetate-producing bacteria requires the recycling of hydrogen by reoxidation of NADH. In thermodynamic terms, this process is only possible when the hydrogen tension is low (less than 10^{-4} atm). The close proximity of H_2-consuming biofilm bacteria such as sulfate reducers or methanogens facilitates "interspecies hydrogen transfer" (6), and the equilibrium hydrogen tension largely determines the dominant organisms.

The diversity of microbial metabolism is exemplified by the fact that some classes of microorganisms use more than one metabolic strategy. The most obvious and important example is the switch between autotrophy and heterotrophy under different conditions. Purple nonsulfur bacteria are strictly autotrophic during the day, when they use H_2 or HS^- as reductants for photosynthesis and for assimilating carbon dioxide. At night, they can also respire oxygen by using low-molecular-weight organic substrates, such as acetate. Some chemoautotrophs can assimilate organic substrates, and under anaerobic conditions, some sulfur bacteria can either reduce sulfur or perform heterotrophic fermentations. Autotrophy is an ambiguous term for biofilm organisms because the energy cycle is intimately related to other cycles occurring in close proximity. For example, purple sulfur bacteria carry out photosynthesis using HS^- as an electron donor, thereby assimilating carbon as carbon dioxide, but the reducing agent is often supplied by organisms performing oxygenic photosynthesis and subsequently degraded under anaerobic conditions. When referring to the use of HS^- as a substrate, the designation of autotrophy is only justified if the HS^- is provided abiotically, for example, geothermal vents or acid mine drainage. In the water column of a deep lake, temperature gradients induce "thermal stratification," in which the lower strata are colder and oxygen-depleted (reducing conditions). This prevents admixing among the lower, hypolimnion, and other layers. Photosynthesis is limited to the upper (euphotic) zone, which is defined by the penetration depth of solar radiation, and commonly measured by the Secchi disk method. Particulate matter absorbs much of the radiation, such that when POC levels in natural waters are high, the euphotic zone is extremely small. Within biofilms, absorption or other attenuation of incoming radiation confines some types of photosynthetic activity to the upper strata (17). Significant physicochemical gradients also exist in sediments and soil microcosms. Thus, substrate and physicochemical gradients become established within other gradients. Biofilm organisms are ultimately affected by the trophic status of the bulk fluid compartment.

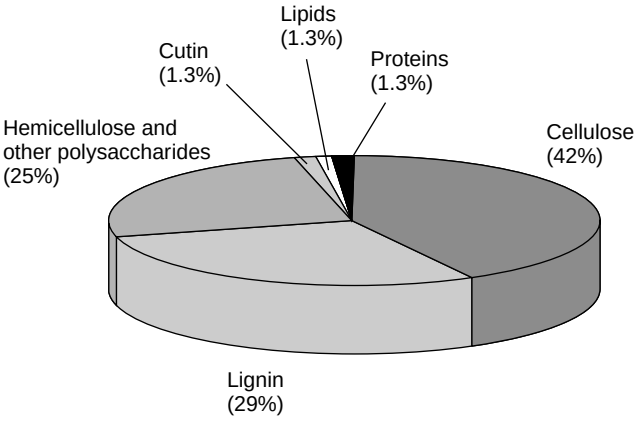

Figure 1. Schematic diagram showing the syntrophic metabolic interactions in a mixed phototrophic/heterotrophic biofilm. Note the concentration gradients within the biofilm, and the dependence of O_2 and CO_2 gradients on diurnal and nocturnal cycles. Carbon dioxide is buffered to hydrogen carbonate and is approximately 5 times more soluble in water than oxygen.

SOURCES OF NATURAL ORGANIC CARBON SUBSTRATES

Carbon substrates belong to the dissolved or particulate organic carbon pools (DOC and POC, respectively). Colloid scientists use the thermodynamic concept of speciation, where the term "dissolved" refers to chemical potential (18). In this sense, DOC is the fraction of greatest significance to surface phenomena because it adsorbs onto surfaces to form conditioning films (see CONDITIONING FILMS, this Encyclopedia). This molecular diffusion is considerably more rapid than the adsorption of colloids, including bacteria, as described by Fick's First Law of molecular diffusion. Thus, a continuous flow of (albeit limited) substrate replenishes nutrients within a porous biofilm matrix. These nutrients can be in the form of

trapped particles or macromolecules, which are degraded if the appropriate exoenzymes are available. Sorption of kaolin particles to a *Pseudomonas fluorescens* biofilm caused an increase in microbial activity compared to biofilms grown in the absence of clay particles, as explained by the greater biomass in the former (19). Nutrient sources are described as allochthonous or autochthonous, depending on their origins. Allochthonous carbon sources are produced in locations remote from the environment of interest and reach the location by some physical process. The leaves of vascular plants, for example, provide an exogenous source of lignin to lakes and rivers. This may be a direct event, such as in the case of the leaf falling into a stream, or a consequence of a more indirect route, such as the leaching of soluble components into the water from an adjacent soil matrix, in which case the microbial decomposition processes are initiated prior to reaching the water. Anthropogenic carbon sources (i.e., derived from human activity) are mostly allochthonous. Autochthonous carbon sources, as the name suggests, are produced in situ. Examples include cell lysis products and exudates from planktonic algae. The availability of autochthonous substrates is determined by the rates of incorporation into biomass, but also by the remobilization of carbon from particles. This is especially important when sediments are mixed by advection. Most of the DOC in oceans is autochthonous, especially in coastal regions and estuaries, due to the photosynthetic activity of phytoplankton (20). The broad range of potential substrate compositions (Table 1) is dependent on the extreme variability of spatial and temporal parameters.

The low DOC of groundwater may be due to any combination of limited leaching from aquifer solids (kerogen), complete mineralization of carbon substrates by heterotrophic microorganisms, and adsorption-immobilization (22). Like humic substances, kerogen is thought to be derived from the decay of plants and microorganisms (23). Ultimately, the major source of terrestrial organic matter is vascular land plants. Some of these carbon compounds, such as lignin, appear to degrade more readily in the ocean than would be expected from laboratory-based studies. Some of the most important findings were derived from the use of specific biomarker compounds, which can be traced with some level of certainty; however, only a small fraction of the organic matter dissolved in seawater and preserved in marine sediments appears to be terrestrial in origin, despite the fact that annual exports from rivers are more than sufficient to account for deposition in sediments (24). This anomaly is the most elusive aspect of the global carbon cycle. Similar difficulties apply to soil ecosytems, in which percolation of water-soluble organic carbon through the soil matrix is relatively small in comparison to inputs from leaf material, roots, and root exudates. It is possible, however, to estimate the above-ground organic matter prior to its incorporation into the soil matrix (Fig. 2). Microorganisms in some groundwater systems live in conditions that are completely remote from aquatic or soil inputs. The substrates in these environments are the products of microbial metabolism and lysis and kerogen. These two different scenarios emphasize the ability of microorganisms to live under conditions of "feast and famine," in which the latter predominates.

Table 1. Concentrations of Dissolved Organic Carbon in the Major Types of Natural Waters

Type of Water	[Dissolved Organic Carbon] (mg/L)
groundwater	0.2–15 (0.7[†])
marine	0.5–3.0
lake	2–10
—oligotrophic	1–3
—eutrophic	2–5
—dystrophic[‡]	20–50
rivers	5–60[§]

Source: Data from E. M. Thurman, Organic Geochemistry of Natural Waters, Martinus Nijhoff/Dr W. Junk Publishers, Dordrecht, Netherlands, 1985 and R. L. Malcolm, in A. J. Beck, K. C. Jones, M. H. B. Hayes and U. Mingelgrin, eds., Organic Substances in Soil and Water: Natural Constituents and their Influences on Contaminant Behaviour, Royal Society of Chemistry, Cambridge, U.K., 1993, pp. 19–30.

[†] mean concentration.

[‡] Dystrophic lakes have high levels of fulvic acids (21).

[§] "uncolored" water.

Sources and Classes of Proteins, Carbohydrates, and Lipid Substrates in Natural Environments

Hydrolysis of proteins and protein conjugates from seawater produces both dissolved free amino acids (DFAA) and dissolved combined amino acids (DCAA). DCAA derived from marine organisms constitute the largest, well-defined form of all DOC; however, studies of the amino acid composition of DCAA have yielded little information because proteins from living organisms have similar amino acid compositions (25). Carbohydrates are quantitatively dominant over proteins in soils, freshwater, and marine environments, accounting for up to 20% of seawater DOC. Most of this is associated with photosynthetic activity in irradiated surface waters. All major classes of carbohydrates occur in marine DOC, including amino sugars, uronic acids, and aldoses (26). Like amino acids, carbohydrates in natural waters exist both as free monomers and as polymers. Polysaccharides can account for 80% or more of the total dissolved carbohydrates in seawater. Fibrillar polysaccharides may account for up to 25% of the entire pool of natural organic matter (NOM) in fresh waters (27) and therefore constitute the second largest pool of aquatic DOC after humic substances. The major polysaccharides in soils are cellulose and hemicellulose. The most abundant protein in photosynthetic organisms is Rubisco (ribulose-1,5-bisphosphate carboxylase). The turnover time of this enzyme is thought to be rapid (28), although incubation with the nocturnal Rubisco inhibitor 2'-carboxy-D-arabitinol-1-phosphate was shown to confer protection against proteolytic enzymes (29).

Lipids constitute a small fraction of the total dissolved organic carbon. The major classes of lipids in DOC are hydrocarbons, chlorins, and their esters (i.e., pigments), alcohols, sterols, triacylglycerols, free fatty acids, and phospholipids (30), which can usually be regarded as

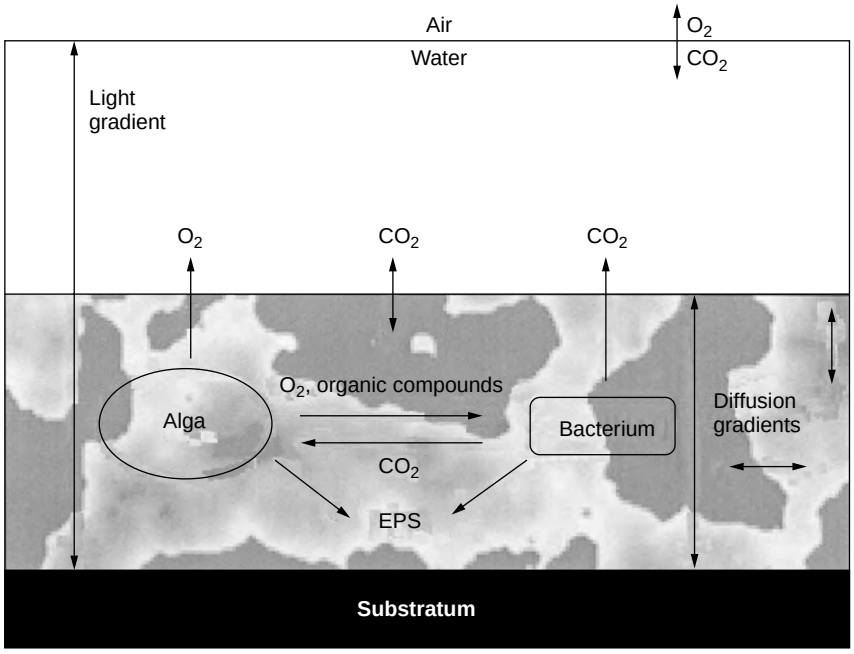

Figure 2. Carbon inventory of aboveground, terrestrial plant matter (data from A. M. Romani and S. Sabater, *Freshwater Biol*. 41(4), 729–736 (1999)).

autochthonous. The aqueous solubilities of many lipids are in the range of milligrams per liter (31); however, because they are relatively hydrophobic, most lipids tend to partition to air-water and solid-water interfaces, including particles. Alternatively, the lipids may be released as particles or micelles. Lipids represent 10 to 25% of POC in marine surface waters. Interestingly, a large proportion (approx. 70–90%) can be found in the dissolved fraction (less than 10 kDa) (31). Free fatty acids are thought to be liberated by microbial degradation of particulate substrates, whereas the phospholipids are considered to be derived almost exclusively from cell membranes (32). Some lipids are more refractory than others, and migrate through the water column relatively intact, where they are converted into alkanes at the sediment-water interface (30). A predominance of odd-carbon-number *n*-alkanes greater than $C_{23}H_{48}$ has been used as a marker for inputs of terrestrial plant waxes to sediments. The uncommon predominance of even-carbon alkanes is considered to be an indication of specific microbial inputs, degradation of algal detritus, and the reduction of alkanoic acids or other lipids (33). The degradation of lipids and triacylglycerols from algae and terrestrial plants produces long-chain (nonvolatile) fatty acids, such as palmitic acid and stearic acid. These compounds are poorly soluble in water and thus poorly bioavailable. Long-chain fatty acids predominate over short-chain forms in surface waters (oxidizing conditions), especially in the hydrophobic air-water slick. Conversely, microbial oxidation of POC and DOC produces volatile fatty acids, which accumulate only under reducing conditions. These conditions establish significant gradients within the biofilm matrix, but also more generally, such as in hypolimnetic waters and sediment cores.

Humic Substances

The bulk of DOC compounds are described as humic substances, which are complex and variable polyhydroxycarboxylates. These compounds account for 50 to 75% of aquatic dissolved organic carbon (34) and differ from other polymers in that they are the products of random chemical synthesis rather than intentional biological processes. The long-standing assumptions of soil humic chemistry were changed to reflect the fact that aliphatic, rather than aromatic, compounds form the major carbon reservoir (35). Aquatic humic substances have always been considered predominantly aliphatic and comparatively low in molecular weight, as inferred from solubility characteristics. Much recent information on the aliphatic and aromatic nature of humic substances has come from Nuclear Magnetic Resonance spectroscopy (NMR). NMR spectroscopy of humic substances usually involves sequential extractions with sodium pyrophosphate. Pyrolysis in conjunction with mass spectrometry yields molecular fragments, which must then be reconstructed into a coherent network. It is difficult to make specific statements about the microbial degradation of humic compounds if their chemical identities cannot be determined with any degree of certainty, or if they cannot be separated from biochemical compounds without altering their original properties. Far from being inert and irrelevant to microbial metabolism, humic substances can hinder the hydrolysis of bound compounds and may act as electron donors for some metabolic reactions (36).

Anthropogenic Carbon Sources

Increasing amounts of environmental carbon compounds are misappropriated by human activity. These include vast quantities of untreated or partially treated sewage effluent, point inputs of pesticides and fertilizers, and (other) xenobiotic carbon compounds. Not all of these compounds are unique — for example, phthalate esters and halogenated organic compounds are often described as undesirable anthropogenic compounds (37), yet natural sources of these compounds can be significant. Phthalate

derivatives are abundant in natural humic substances, as are organohalogens in emissions from volcanoes and hydrothermal vents (38). Methane is produced in vast quantities from the fermentation of cellulose by the intestinal flora of termites and ruminant animals. Only very preliminary estimates are available to predict the total impact of anthropogenic carbon on total microbial activity in terms of the global carbon cycle. It should be noted, however, that the oceans act as a major buffer system for carbon as soluble carbon dioxide, and that biofilms act as efficient buffer matrices for (nonxenobiotic) point disturbances. This property is exploited in wastewater treatment and bioremediation.

ASSIMILABLE ORGANIC CARBON AND BIOAVAILABILITY CONCEPTS

Descriptions of metabolic pathways give the impression that all heterotrophic activities are dependent on a limitless supply of low-molecular-weight precursors, such as glucose. In aquatic environments, low-molecular-weight substrates have very low turnover times in the range of minutes to hours, and are therefore exceedingly rare (Table 2; 34). The major part of the dissolved organic carbon pool comprises high-molecular-weight compounds from primary production and decay processes. The utilization of high-molecular-weight organic compounds depends on the ability of an organism to degrade the polymer into its constituent oligomers or monomers. This, in turn, depends on the proximity of degradable substrate, the production of appropriate exoenzymes, and the optimum size for passive transport through the cell wall. Proteins and polynucleotides are easily hydrolyzed, and the constituent monomers are mineralized fully to CO_2 or CH_4. Simple sugars are also easily hydrolyzed. Hydrolytic activity is mostly confined to the biofilm matrix (39). Structural polysaccharides constitute a considerably larger pool of potential substrates, which is directly related to the resistance of these compounds to hydrolysis. The β-1,4 polysaccharide linkage is especially resistant to degradation. Interestingly, this linkage may be the key to the cohesiveness in some biofilms (40), and is an intrinsic part of the holdfast structure of at least one, stalked bacterium (41). Suggestions that microorganisms produce humic substances independently of lignin-type mechanisms are not convincing, since infrared spectra of aged extracts from laboratory cultures (42) resemble the spectra of bacterial isolates (43).

The use of a compound as a metabolic substrate is linked intrinsically to the concept of bioavailability. In general terms, "bioavailability" describes the accessibility or otherwise of a substrate to living microorganisms, and can therefore be independent of the physiological capability of the organism to degrade the substrate. Physical accessibility is governed by parameters such as pH, ionic strength, temperature, and water activity. These parameters have a pronounced effect on factors such as solubility and partitioning. For example, an increase in temperature enhances the solubility and hydrolysis of macromolecular substrates, thereby increasing their availability (44). Even for common macromolecular substrates such as proteins, the presence of appropriate enzyme systems is not a trivial issue. Some bacteria are apparently unable to utilize high-molecular-weight substrates under any conditions, suggesting the name "dissipotrophs" (45). Especially in aquatic environments, dissolved organic matter can become more bioavailable by the action of solar radiation. Photochemical degradation of NOM produces carbonyl compounds, including carboxylic acids and aldehydes, and probably generates assimilable amino acids and carbohydrates (46,47). Ozonation has a similar effect on high-molecular-weight organic compounds during the treatment of drinking water. The activity levels of humic bound enzymes are also partially restored by UV photolysis (48). If particulate organic carbon in the euphotic zone of natural water contains chromophores, which strongly absorb solar radiation, then photolysis will have a major influence on bioavailability. A dual effect is observed when extensive absorption by humic compounds is sufficient to significantly raise the temperature in shallow, brown-colored lakes and surface soils. Chemical accessibility for a particular compound must also be considered. Different isomers or functionalized forms of molecules can be comparatively labile or nonlabile. For example, chitin is generally less bioavailable than the deacetylated form, chitosan.

Particles are less easily degraded than individual macromolecules; physical disintegration of particles increases the sites available for enzyme action. Adsorbed macromolecules must be hydrolyzed before assimilation by attached organisms. Surface immobilization may promote access of bacterial enzymes to the substrata, thereby facilitating utilization. In some circumstances, immobilization can have the opposite effect. High-molecular-weight substrates with extended conformations can have multiple binding sites that hinder hydrolysis, and the activities of bacterial enzymes can be reduced or even inhibited if they are immobilized by adsorption (49). Nucleic acids and enzymes can be preserved through interactions with humic substances, organic sediment, or clay minerals (50).

Table 2. Concentrations and Turnover Times for Dissolved Free Amino Acids (DFAA), Dissolved Free Carbohydrates (DFCHO) and Recalcitrant, "Humic" Substances in Natural Waters (34)

	Marine		Freshwater	
Substrate	Concentration (nM)	Turnover Time	Concentration (nM)	Turnover Time
DFAA	3–1,400	1.4 hrs–948 days	2.6–4,124	2 hrs–51 days
DFCHO	0.4–5,000		14–1,111	
recalcitrant	70–90% of compounds, with turnover time of 2,000–6,000 years			

Some inorganic particles, such as three-layered clay minerals can reduce the availability of citrate and glucose (51). Similarly, bacterial degradation of herbicides such as (2,4-dichlorophenoxy)acetic acid (2,4-D) or insecticides such as diquat can be inhibited by adsorption onto particles. In other words, some substrates must desorb before they can be assimilated, a phenomenon described as "desorption limitation" (52). Ogram and coworkers (53) showed that in one system, the degradation of 2,4-D could only take place if the substrate was in solution. Of special relevance to biofilm organisms is the fact that solid substrata may also act as a metabolic substrate. Some common examples are the bacterial degradation of agar, cellulose, wood, and poly (vinyl) chloride (PVC) in drinking water distribution systems. Alternatively, the substrate may leach from the solid phase and percolate through, for example, a soil column. The water-soluble organic matter from leaf litter is a major source of biodegradable carbon. Removal of leaf litter at a test site yielded soil respiration rates one-third lower than at control sites (54). Cycling of interstitial carbon by biofilm organisms undoubtedly contributes much of the remainder, but the relationship between POC and DOC in the soil matrix is not clear.

The major concept for bioavailability is the recalcitrance of the bulk pool of NOM. Humic substances, according to classical definitions, are refractory to both chemical and biological degradation. This definition is problematic in that some humic compounds may be artifacts of the harsh extraction procedures. Structurally related, but immature compounds, such as lignin and cutin have been shown to degrade extensively during incubation experiments (55), and a measurable percentage of the operationally defined humic substances are readily biodegradable. Studies that show "surprisingly" high levels of humic substance degradation by biofilms seem to contradict the classical definitions. At least in freshwater environments, this discrepancy can be explained by the strong affinities of carbohydrates and amino acids for the truly recalcitrant, humic compounds (56), resulting in humic fractions, which contain large amounts of relatively assimilable substrates. Unfortunately, it is difficult to abandon classical definitions without comprehensive information on chemical structure, which is clearly unfeasible given the demands on analytical techniques, and the extensive variability of humic substances in different environments. More useful terms such as "refractory organic carbon" are becoming more common, although it would be desirable if these terms discriminated between biotic and abiotic processes. Measurements of assimilable organic carbon (AOC) and biodegradable organic carbon (BDOC) are based on bioassays (57), which therefore give operationally useful data concerning the substrate. Conversely, these parameters are of limited benefit to the understanding of natural environments if the microbial inoculum is not representative of the system (AOC), or if the physical and chemical parameters represent "ideal" conditions for degradation, such as an absence of adsorptive mineral phases.

It has, only recently, been established that many of the recalcitrant compounds in freshwater sediments and possibly marine sediments are a distinct class of aliphatic products produced by the degradation of phytoplankton. These nonhydrolyzable and insoluble "algaenans" are components of the cell walls of many algae. Algaenan-like compounds reportedly present in cyanobacteria, and some bacteria are probably artifacts resulting from the hydrolysis procedure used in their isolation (58). Thus, algaenans have so far only been reported from a relatively few species of photosynthetic microorganisms. The comparative abundance of algaenan-containing organisms has not been investigated, so the cumulative effect of this class of compounds on biogeochemical cycles is mostly unknown. It seems likely, however, that aliphatic products from planktonic algae contribute much of the humiclike substances in marine environments. Table 3 shows a summary of these waxy compounds and their origins. It is postulated that algaenans comprise polymethylenic (*n*-alkyl) chains cross-linked via ether bridges (23). The persistence of these compounds in aquatic sediments has led to the suggestion of a humification route known as the "selective preservation" pathway (59). Although, it might appear from these discoveries that the humic substances in marine environments are exclusively aliphatic, and therefore somewhat unrelated to freshwater or soil systems, many recalcitrant aromatic compounds are present in these environments, and the origins of these compounds have not been established fully. Lignin phenols are easily detected in estuaries, but quickly become diluted in the marine environment (60). Other peculiar but common forms of carbon are the condensed residues from incomplete combustion processes, known as "carbon black" or "black carbon." Highly aromatic compounds can be produced from oxidized black carbon (61), suggesting that humic substances in some environments can be produced from charred biomass. Black carbon is extremely resistant to biodegradation (62), which partly explains its accumulation in many soil environments, and freshwater and marine sediments.

Table 3. Classes of Poorly Hydrolyzable, Aliphatic Compounds and Their Origins

Compound(s)	Origin
Algaenans	Freshwater and marine algae
Cutans	Cuticles of higher plants
Suberins	Periderm tissue of Higher plants
Tegmens	Inner seed-coats of Freshwater plants
Sporopollenins	Spores and pollen grains
Polycadinenes	Resins

MICROBIAL TRANSFORMATIONS OF DIVERSE AND DILUTE SUBSTRATES

The biofilm mode of existence demonstrates a dependence on transfer processes, or flux, to and from the biofilm. The ecology of biofilm organisms is unique in terms of the proximity of the attachment substratum, the comparatively high cell density, and the long residence time of the cells (63). There are several means by which biofilm cells can obtain nutrients: from the attachment

substratum, which can be degradable, but always contains an adsorbed conditioning film (but see Schneider and Leis, CONDITIONING FILMS, this Encyclopedia); from sorption of compounds (DOC or POC) onto biofilm cells and EPS; or from degradation of adjacent biofilm components. Geesey (64) suggested that an organism will not degrade its own EPS. Biofilms treated with dissolved organic carbon derived from decomposing macrophytes contained 10 to 57% less EPS than control biofilms (65), suggesting that the amount of EPS, which is produced by biofilm organisms is closely related to the type and amount of DOC. Classical microbiology has centered on the concept of a "limiting" nutrient, and the ability of microorganisms to use only one carbon source at any one instant, as exemplified by the diauxic growth of *Entamoeba coli* on glucose and lactose. In natural systems, assimilable, low-molecular-weight substrates are rarely present in high concentrations. Under these conditions, the formation of a biofilm would not take place if all cells were specialized for the utilization of a single substrate. Furthermore, biofilm organisms cannot translocate to nutrient sources as readily as planktonic organisms. Carbon starvation or slow growth induces the expression of many catabolic systems, despite the absence of the appropriate carbon source. This "catabolite repression" gives microorganisms an almost instantaneous ability to utilize a substrate should it become available, and in general, serves to optimize an organism's growth rate. In most bacteria, the enzymes involved in sugar transport and phosphorylation are important for signal transduction, although the regulation mechanisms vary widely between organisms (66). In one study, it was found that a strain of *Pseudomonas aeruginosa* was able to form a biofilm in tap water with a mixture of 45 carbon compounds, each present at just 1 $\mu g\ C\ L^{-1}$, whereas any one of these compounds in isolation could not produce growth at this concentration (67). The sorption of particulate organic matter by biofilms represents a major input of nutrients. These abiotic particles and flocs contain a relatively small proportion of easily biodegradable matter, but newly attached cells, and their metabolites and lysis products contribute to the cycling of organic matter. The turnover time for total organic carbon (TOC) in marine "snow" is approximately eight to nine days, suggesting that flocs are "hotspots" for microbial respiration (68). The biofilm environment is therefore, a dynamic exchange interface, even when the activity levels of the biofilm organisms are observed to be low.

MEASUREMENT OF TRANSFORMATIONS AND ACTIVITY IN BIOFILMS

The ability of microorganisms to rapidly alter levels of metabolic processes explains their ability to resume activity after extended stages of dormancy. Microbial activity can be measured in terms of individual metabolic reactions, or as a cumulative effect, as in the case of biomass measurements. Approaches to determine microbial activity include measurements of substrates, metabolic intermediates, or products of primary and secondary production. Primary production is measured as reactants such as CO_2 or H^+ (pH), while the products are usually measured as O_2 and ΔpH. Direct measurements of carbon dioxide production are useful for estimates of metabolism in whole systems, but do not provide any insight into transformations or partitioning of key components. The use of radiotracers such as $^{14}CO_2$ is more specific. Radiolabeled, low-molecular-weight compounds are certainly not representative of the bulk of NOM, and the use of labeled, high-molecular-weight compounds depends on their commercial availability and their ability to disperse within a system (69). Clearly, the use of any standards for high-molecular-weight NOM is subjective. Microautoradiography can be used for the analysis of labeled carbon dioxide assimilated by photosynthesis (9), but the visualization of biofilm cells in photographic emulsions requires considerable technical effort to avoid artifacts.

The fundamental prerequisite for studies of biofilm activity is the ability to distinguish between the metabolic processes of biofilm and planktonic environments, which in turn depends on the development of appropriate techniques for in situ measurements. The inadequacies of culture-based methods for measuring growth processes have been appreciated for some time, especially for biofilms. The purposeful detachment of microorganisms from particles is a selective process, and the extrapolation from total cell number to biomass is problematic because it is necessary to assume a universal conversion factor to account for the variations in cell volume among species, and among nutrient-limited phenotypes of the same species (32). Estimates of primary producers by measurement of chlorophyll *a* requires the same assumptions for average biovolume. Measurements of specific biochemical components is one strategy to avoid culture-based techniques and microscopy, but there are a number of assumptions inherent to these methods. Universal indicators of metabolism, such as ATP, can persist after cell death (70), and the turnover times of excreted compounds, which become localized in the biofilm matrix can be totally dissimilar to the turnover time of these compounds in the bulk phase. Measurements of extracellular enzymes are complicated by the fact that the enzymes often bind to the EPS matrix, and therefore do not necessarily represent the presence of active cells (71). Humic compounds complex with microbial phosphatases, thereby reducing enzyme activity (48). The same applies for the coenzyme, F420, which is found in nonviable cells of archaea and some eubacteria, as well as extracellularly (72). Conversely, polar lipids such as the cell wall phospholipids are degraded rapidly after cell death, with the added advantage of diversity profiles obtained from fatty acid analysis (32). High levels of activity do not necessarily imply an actively growing population. Cells in immature biofilm populations can be outnumbered by planktonic organisms, yet the biofilm carbon demand and hydrolytic enzyme activity can account for close to 100% of the system's total (73). Protease activity can be measured with the protein substrate azocoll, which yields a blue product on hydrolysis. The incorporation of radiolabeled nucleotides into DNA allows an

estimate of DNA synthesis, and therefore an estimate of the rate of cell division. This is commonly measured by the incorporation of ^{3}H-thymidine (74), although measurement of protein synthesis from the incorporation of radiolabeled amino acids such as ^{3}H-leucine is useful as an independent validation, and is probably more sensitive because of the large amounts assimilated by growing cells (2,75). Both methods are specific for heterotrophic bacteria, and contain a number of implicit assumptions. Measurements of the rates of nucleic acid synthesis from the incorporation of adenine, and perhaps also $^{35}SO_4$ into bacteria and algae provide a measure of the entire microbial community. An alternative to radioactive isotopes is an immunoassay in which the uptake of 5-bromo-2'deoxyuridine is measured by chemiluminescence (76).

Measurements of transformations and microbial activity within biofilms constitute a unique challenge because of the microscale heterogeneity within the biofilm matrix. Total cell counts of biofilm populations are hindered by the presence of aggregates, although confocal microscopy is an indispensable tool in this respect. With the aid of suitable fluorescent dyes, it is possible to observe relatively undisturbed biofilm populations as three-dimensional reconstructions, and to probe for features such as membrane integrity and redox activity. Membrane integrity is conveniently tested with the Live/Dead™ kit (Molecular Probes, Oregon). A potential complication of this technique is that dye uptake could vary depending on the size and charge of the dye molecules, and the uptake systems of particular organisms. Further indications of actively metabolizing cells are provided by the cleavage of fluorogenic substrates such as fluorescein diacetate to fluorescein, providing an indication of total esterase activity (77), and the reduction of tetrazolium salts by cellular redox mechanisms, generating an insoluble, colored, and perhaps fluorescent product. Changes in intracellular components such as organic phosphorus and carbohydrate, or NADH, can be measured by NMR spectroscopy and fluorescence spectrophotometry, respectively (14,78). Measurements of small amounts of heat from metabolic activity are possible using microcalorimetry (79). Measurements of changes in the isotope compositions of carbon, nitrogen, and sulfur are possible using an isotope ratio mass spectrometer (IRMS), since microorganisms discriminate against heavy isotopes during metabolic processes (80). This approach is especially useful for determining the sources and fates of groundwater carbon, and its partitioning between biotic and abiotic phases. Isotope enrichment is especially important for methanogenesis, acetogenesis, and methanotrophy. Microbially produced methane represents the most ^{13}C-depleted carbon compound in nature (80).

Microelectrodes allow the measurement of metabolites, diffusion coefficients (81) and flow velocity (82) in situ. Specific microelectrodes have been developed for measurements of several important compounds, including nitrate, nitrite, ammonium, carbon dioxide, hydrogen sulfide, and methane, and fiber-optical sensors are available for measurements of O_2, pH and temperature (83). In *P. aeruginosa* biofilms, microelectrodes have shown that a lack of oxygen restricted limited active protein synthesis to the top 30 μm of a biofilm (84). Other studies have demonstrated stratifications in nitrifying and sulfate-reducing biofilms (85). Planar optodes are superior to conventional O_2 microelectrodes because the latter create hydrodynamic disturbances, which can affect an area of several millimeters surrounding the measurement site (86). Reporter gene techniques allow the identification of genetic changes, which are invoked on recognition of an attachment substratum (87,88). These changes include the production of exopolysaccharides (89), and presumably other components of the EPS. Observations relating to cellular morphology are less discriminate because they depend on an ability to recognize a particular species within a complex biofilm community. This is only feasible with biofilms containing one or a few organisms, preferably with simple, distinguishing characteristics. The best examples are given by the initial attachment and subsequent morphological changes of the prosthecate (stalked) bacteria belonging to the genera *Hyphomicrobium*, *Flexibacter*, *Caulobacter*, *Prosthecobacter*, and *Hyphomonas* (41). In studies of an *Acinetobacter* sp. attaching to solid substrata, it was found that a nutrient-rich medium induced a transition from coccoid morphology to a bacillary form, and the converse occurred when cells were supplied with a nutrient-depleted (starvation) medium (90). Figure 3 shows the variability in morphology of a single species of marine bacterium, which

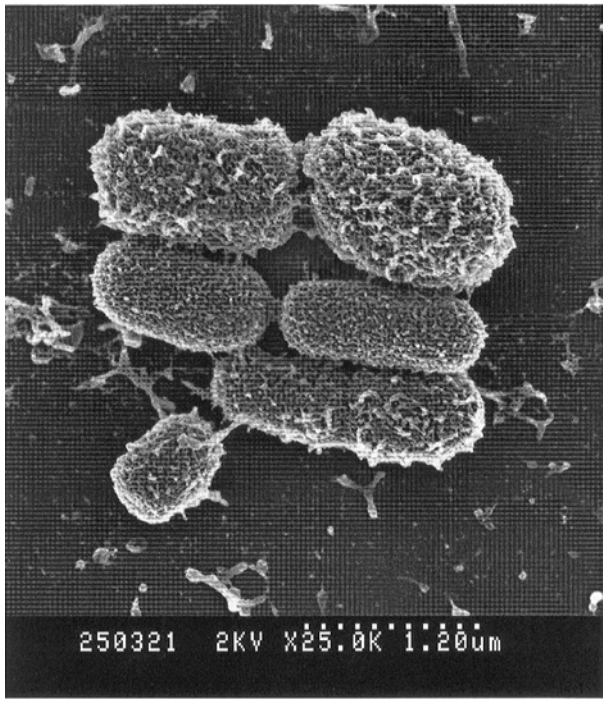

Figure 3. Differences in the morphologies of dividing bacteria (*Psychrobacter* sp. SW8) from a colony grown on minimal medium with acetate as the sole source of carbon. In continuous culture with carbon as the limiting nutrient, all cells of this isolate have a similar shape and indistinguishable surface morphology.

was isolated for its ability to attach to hydrophobic substrata, thus implicating it as a primary colonizer of immersed surfaces.

CONCLUSION

Sustainable, high levels of microbial productivity in low-nutrient environments are testimony to the diversity and adaptability of environmental microorganisms. Most of this growth is associated with biofilms. Microorganisms have evolved many mechanisms to utilize available substrates, only some of which are efficient. Biofilms can sometimes account for nearly all of the enzyme activity in a natural environment. The bioavailable natural organic matter is rapidly utilized, leaving a recalcitrant macromolecular skeleton. While much is known about the mechanisms of substrate utilization, and to a lesser extent, the genetic mechanisms regulating microbial activity, the largest knowledge gaps are the extent to which the poorly characterized pool of natural organic matter is available for microbial growth, and the mechanisms controlling this bioavailability. Further knowledge of the bioavailable organic carbon must consider the flux of particulate substrate to biofilms, the identification of biodegradable substrates using nondestructive techniques, and the measurement of metabolic activity in situ. Such fundamental knowledge requires the use of complex biofilms and substrates, monospecies biofilms and fractionated organic matter, and model systems capable of simulating key aspects of natural environments.

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ADENOVIRUSES

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As early as 1926, several outbreaks of swimming pool conjunctivitis had been documented in the United States (1). It is likely that some outbreaks were caused by adenoviruses, as no bacterial pathogens could be identified, and the description of clinical manifestations matched that of pharyngoconjunctival fever caused by adenoviruses (2–6). Strong epidemiological evidence of the waterborne transmission of these agents was obtained in 1955, when adenoviruses were isolated from patients suffering form conjunctivitis during an outbreak that originated in a swimming pool (3). Numerous waterborne

outbreaks of pharyngoconjunctival fever, caused by adenoviruses, have been documented since then (5,7–12). The first detection of adenoviruses during a waterborne outbreak of gastroenteritis was recorded in Finland in April 1994 (13), when approximately 25 to 50% of the population (1,500 to 3,000) of a municipality developed acute gastroenteritis. This outbreak was associated with contaminated well water. In addition to adenoviruses, a Norwalk-like agent, small round viruses (SRV), and group A and C rotaviruses were identified as the etiological agents, Norwalk virus being the most prevalent of the implicated viruses. In addition to gastroenteritis (adenovirus types 31,40, and 41) and pharyngoconjunctival fever (adenovirus types 3,7,8,11,14,19, and 37), adenovirus infections may cause other clinical conditions such as acute respiratory disease (adenovirus types 1–7, 14, and 21), acute hemorrhagic cystitis (adenovirus types 11 and 21), and acute respiratory disease (ARD) of military recruits (types 3, 4, 7, 14, and 21) (14).

CLASSIFICATION

Adenoviruses were first identified in 1953 as agents causing spontaneous degeneration of primary cultures of human adenoid tissue (15). In 1956, the name adenovirus was given to these agents denoting the tissue in which these viruses were discovered. Adenoviruses belong to the *Adenoviridae* family, which is represented by the *Mastadenovirus* and the *Aviadenovirus* genera (16). The *Mastadenovirus* genus contains human (49 serotypes) (14), simian (27 serotypes), bovine (10 serotypes), equine (1 serotype), porcine (4 serotypes), ovine (1 serotype), and canine (3 serotypes) viruses. The *Aviadenovirus* genus (11 serotypes) includes only bird viruses (4). The 49 human serotypes have been further classified into six subgroups (A through F) based on their ability to agglutinate red blood cells, capacity to cause tumors in rodents, relatedness of tumor antigens, electrophoretic mobility of viral proteins, genome homology, cross hybridization, and DNA digestion with restriction endonucleases (16).

STRUCTURE

Adenoviruses are nonenveloped icosahedric viruses of approximately 70 to 100 nm in diameter, composed of DNA (13% of mass), protein (87% of mass), and trace amounts of carbohydrates present in the virion fibers (16). Members of the *Adenoviridae* family possess a linear double-stranded (ds) DNA molecule with about 30,000 base pairs (17) and molecular weight of $1.5–1.8 \times 10^8$ (14). Virions consist of a protein shell (capsid) surrounding a DNA-containing core. This structure is composed by 252 subunits (capsomeres), 240 hexons, and 12 pentons. Each penton contains a projecting fiber that varies in length in different adenovirus serotypes. All human adenoviruses seem to possess a single fiber protein, with the exception of the enteric adenoviruses 40 (Eda 40) and 41 (Ead 41) that possess two fiber proteins (16).

GENETICS

Evolution and diversity of adenoviruses result mostly from recombination among members of the same subgroup; however, recombination among adenoviruses of different subgroups can also occur. In fact, adenovirus type 4 (the only member of group E) originated, apparently, from recombination between viruses from groups B and C (16).

BIOLOGY

Adsorption and internalization in adenoviruses is a two-step process. Attachment of adenovirus to susceptible host cells is mediated by the fiber protein; however, for internalization to occur, the penton base protein needs to attach also to a cell receptor of the family of "integrins." The penton–integrin interaction precipitates a receptor-mediated endocytosis. Once the virion enters the cell, it is disassembled by a systematic elimination of structural proteins, leading to the transport of the viral DNA into the cell nucleus (16). The adenovirus genome replicates by an unusual mechanism in which single-stranded (ss) DNA intermediates are formed, and a protein, but not short RNA molecules serves as a primer to initiate replication (17). Adenovirus DNA replication can start at one or both ends of the molecule. A similar general organization has been found in the genomes of different adenoviruses. The adenovirus ds-DNA molecule possesses two identical origins for replication, which is found in each of the virus short terminal repeats. Adenovirus mRNA synthesis takes place in the cell nucleus (16) adenovirus genes are expressed in two stages, early transcription (before viral DNA synthesis), and late transcription (after viral DNA synthesis); however, the functional distinction of the two stages is frequently obscure. A cellular RNA polymerase II transcribes each adenovirus gene into multiple mRNAs that are differentiated by alternative splicing (18). The accumulation of large quantities of viral proteins and DNA induces virus assembly. The formation of empty capsids seems to be followed by the internalization of the DNA. Virus release is mediated by disruption of cytoskeleton intermediate filaments, which renders the cell more susceptible to lysis (16).

EPIDEMIOLOGY AND CLINICAL DISEASE

Clinical Symptoms

Most adenovirus human diseases are associated only with one-third of adenovirus types. Although many adenovirus infections are subclinical, these viruses may cause acute respiratory disease (types 1–7,14, and 21), conjunctivitis (types 3,7,8,11,14,19, and 37), acute hemorrhagic cystitis (11 and 21), acute respiratory disease (ARD) of military recruits (types 3,4,7,14, and 21), and gastroenteritis (types 31,40, and 41) (Table 1; 14). Enteric adenoviruses 40 and 41 have been recognized as important etiological agents of gastroenteritis in children (2,19–25), second in importance only to rotavirus.

Table 1. Disease Caused by Human Adenoviruses

Subgenus	Serotype	Human Illness
A	12	Meningoencephalitis
	18,31	Gastroenteritis
B	3	Acute febrile pharyngitis; adenopharyngo-conjunctival fever; pneumonia; follicular conjunctivitis; fatal infection of neonates
	7	Acute febrile pharyngitis; adenopharyngo-conjunctival fever; acute respiratory disease with pneumonia; fatal infection of neonates; meningoencephalitis
	11	Follicular conjunctivitis; hemorrhagic cystitis in children
	21	Hemorrhagic cystitis in children; fatal infection in neonates
	14,16	Acute respiratory disease with pneumonia
	34,35	Acute and chronic infection in patients with immunosuppression and AIDS
C	1,2,6	Acute febrile pharyngitis; pneumonia in children
	5	Acute febrile pharyngitis; pertussis-like syndrome; acute and chronic infection in patients with immunosuppression and AIDS
D	8,19,37,9,10,13, 15,17,42,19, 20,22–29	Epidemic keratoconjunctivitis
	30	Fatal infection in neonates
	32,33,36,38	Asymptomatic infections
	39,42–47	Acute and chronic infection in patients with immunosuppression and AIDS
E	4	Respiratory infection
F	40,41	Diarrhea

Adapted from Maier, Pepper, and Gerba (107)

Children infected with enteric adenoviruses can shed up to 10^{11} particles/g in feces (26).

An electron microscopy survey of stool samples in London, from patients suffering from viral gastroenteritis, showed that enteric adenoviruses were found in 14% of the examined specimens (27). Enteric adenoviruses are ubiquitous, and antibodies to these viruses have been found throughout the world (28). An investigation in Guatemala (25) showed that Ead 40 and Ead 41 were associated with diarrheal episodes in ambulatory children three times more often than rotaviruses.

Adenovirus in AIDS patients

Adenovirus may cause opportunistic infections in immunocompromised patients, including individuals suffering from AIDS (29). Although uncommon, these infections may result in a high mortality (30). Several clinical conditions associated with adenovirus infections in immunocompromised patients have been described. These conditions include encephalitis (31,32), gastroenteritis (33,34), liver necrosis (35), nephritis (36), parotitis (29), hemorrhagic cystitis (37,38), and fatal pneumonia (39–41).

Modes of Transmission

Adenoviruses can replicate in the respiratory tract, the eye mucosa, the intestinal tract, the urinary bladder, and the liver. Adenoviruses can gain access into susceptible individuals through the mouth, the nasopharynx, or the conjunctiva. Although initial infection may occur through the respiratory route, the fecal-oral transmission accounts for most adenovirus infections in young children due to prolonged shedding of large numbers of viruses in feces (14). This may explain the high rate (close to 50%) of adenovirus secondary transmission in households (42). In addition to the type of infection, the duration of adenovirus shedding depends on the age, and immune status of the infected individual. Adenovirus can be isolated for one to three days from the throat of adults suffering from the common cold, and for three to five days from nose, throat, stool, and eye of adults with pharyngoconjunctival fever. At a younger age, adenovirus excretion in throat or stool is more prolonged than in adults. In children suffering from respiratory or generalized infection, adenoviruses can be recovered from throat or stool for three to six weeks, and in immunocompromised patients for up to 12 months or longer from urine, stool, throat, or organ biopsies. In some individuals, intermittent shedding of adenoviruses may occur for months or even years (14).

Most, if not all, waterborne outbreaks of viral conjunctivitis are caused by adenoviruses. Documented waterborne outbreaks of conjunctivitis, by adenovirus type 3 (3,8,43) and type 4 (5) have been reported; however, adenovirus types 1,7, and 14 have also been associated with family outbreaks of pharyngoconjunctival fever (44). According to the Centers for Disease Control (45), the only viral waterborne outbreak between 1991 and 1992 in the United States, associated with recreational waters, was caused by adenovirus type 3. In this outbreak, conjunctivitis, pharyngitis, and fever were the most common clinical manifestations among 595 affected individuals (8).

The leading routes of transmission of adenoviruses are direct contact via host-to-host, and indirect contact via fecally contaminated water, food, and fomites (46). Intact conjunctival mucosa seem to be resistant to infection by adenoviruses.

The enteric adenoviruses 40 and 41 have been recognized as the second most important etiological agents of gastroenteritis in children (21). These viruses, in contrast to other adenoviruses, are not shed in respiratory secretions (47,48), thus their transmission is likely to be limited to the oral-fecal route. It has been suggested that among

the adenoviruses, Ead 40 and 41 are the most likely to be transmitted by water (49), probably through recreational or drinking water (50). This idea was supported by findings by researchers from the United Kingdom (51), who reported a gastroenteritis outbreak in which a common unknown source of enteric adenoviruses affected several children from a long-stay ward at the same time.

Not only are the enteric adenoviruses a significant cause of gastroenteritis among the enteroviruses, adenovirus type 31 has been increasingly detected during the last few years as an important cause of infant gastroenteritis (52).

Contaminated inanimate surfaces may also play a significant role in adenovirus transmission because of their resistance to drying. At room temperature, adenovirus 2 survives for 8 and 12 weeks under low (7%) and high (96%) relative humidity, respectively. This virus was more stable under these conditions than poliovirus 2, vaccinia virus, coxsackievirus B3, and herpesvirus (53).

Reservoirs

Although adenoviruses are common in many types of animals, including simian species (14), adenoviruses infecting humans seem naturally limited to humans (46).

OCCURRENCE AND PERSISTENCE IN THE ENVIRONMENT

A greater number of adenoviruses than enteroviruses has been consistently found in raw sewage around the world (50,54–58). Results of a comparative study of cytopathogenicity, immunofluorescence and in situ DNA hybridization as methods for the detection of adenoviruses from water have suggested that 80% of infectious adenoviruses in raw sewage may be enteric adenoviruses (50). Adenoviruses are also commonly found in primary sewage sludge, where they have been found in concentrations ten times greater than that of the enteroviruses (59). In contrast to these reports, a five-year study of river water in Japan (60) indicated that adenoviruses were present throughout the duration of the study, but in lower number than enteroviruses or reoviruses. The peak concentration of reoviruses and enteroviruses was observed in winter and summer, respectively, whereas the number of adenoviruses remained constant throughout the year.

Adenoviruses seem to be relatively stable in the aquatic environment. Adenovirus type 5 survives longer in tap water, at either 4 or 18 °C, than either poliovirus 1, or echovirus 7 (61). In contrast to the survival patterns observed with enteroviruses, the enteric adenoviruses do not survive significantly longer in wastewater. The increased survivability of the enteric adenoviruses in tap and seawater and their faster inactivation in sewage may indicate that these viruses are inactivated by mechanisms that are different from those affecting the enteroviruses. The enteric adenoviruses are more thermally stable than poliovirus type 1 (polio 1), which is inactivated faster at temperatures above 50 °C (62).

Differences in survival among members of the same adenovirus type have been reported. The survival of two clinical isolates of adenovirus type 5 in tap water at room temperature was longer than that observed for a laboratory strain (61).When the same study compared the survival of adenovirus type 5 with that of poliovirus 1 in tap water at 4 and 22 °C, it was found that poliovirus 1 was inactivated faster at both temperatures.

Adenoviruses seem to be more stable than enteroviruses under different relative humidity conditions. A comparative study on the survival of several viruses at different temperatures and relative humidity values, reported that adenovirus type 2 survived longer than poliovirus 2, vaccinia virus, coxsackievirus B3, and herpesvirus, at relative humidity values ranging from 3 to 96%, at either 25 or 37 °C (53). In a study that compared the stability of enterovirus 70 and human adenovirus type 19 at room temperature (63), it was observed that the drying of these agents on ophthalmic instruments resulted in a 5-$\log_{10}$ reduction of the titer of enterovirus 70, whereas the titer of adenovirus 19 decreased by less than 1 $\log_{10}$ after 11 days. A study on the survival of hepatitis A virus (HAV), human rotavirus (HRV), polio 1, and Ead 40 on porous and nonporous materials showed that when dried on these materials, HAV and HRV were more stable than either polio 1 or Ead 40; however, Ead 40 survived longer in porous surfaces such as paper or cotton cloth than in nonporous surfaces such as latex, aluminum, china, and glazed tiles (64). The presence of fecal matter affected the survival of Ead 40, depending on the type of material. On nonporous surfaces, this agent persisted longer in the presence of fecal matter, whereas in porous materials the presence of feces resulted in a diminished infectivity of this agent (64).

When the survival of the enteric adenoviruses 40 and 41 was compared with the survival of poliovirus type 1 (polio 1) and HAV (in tap water at 4 °C, and at room temperature, and with the survival of polio 1 in primary and secondary wastewater at 4 and 15 °C, and with seawater at 15 °C), it was observed that the survival of Ead 40 and Ead 41 in primary and secondary wastewater was slightly greater than that of polio 1. However, in tap and seawater, the enteric adenoviruses were substantially more stable than either polio 1 or HAV (65).These results are in agreement with previous research in which it was reported that adenovirus type 5 survived longer than both poliovirus 1 and echovirus 7 in tap water, at either 4 or 18 °C (61), and that HAV in groundwater was more stable than polio 1 at different temperatures (66).

Additional evidence of the stability of the enteric adenoviruses at higher temperatures was obtained in an experiment in which both Ead 40 and polio 1 were held at 50,65, and 80 °C in phosphate buffered saline. After incubation at 50 °C for six minutes, polio 1 lost 0.88 $\log_{10}$, whereas Ead 40 lost less than 0.2 $\log_{10}$. Similarly, at 65 °C, polio 1 lost more than 2.5 $\log_{10}$ in 30 seconds, whereas Ead 40 lost only 1 $\log_{10}$ after the same incubation time. When the thermal inactivation was conducted at 80 °C polio 1 lost more than 4 $\log_{10}$ in 30 seconds, in contrast, adenovirus 40 lost only 1.8 $\log_{10}$ during the same period (62).

In addition to thermal stability, Ead 40 shows some resistance to extreme pH levels. No reduction of its infectivity in 0.1 M glycine buffer was observed after

Table 2. UV Doses for 90% and 99.99% Inactivation of Test Viruses

Virus Type	Inactivation Dose (mW sec/cm^2) 90%	99.99%	Relative Sensitivity
Ead 40	30	124	1.0
Ead 41	23.6	111.8	1.16
MS-2	14	65.2	1.8
PRD-1	8.7	31.6	4.08
Polio 1	4.1	21.7	5.6

Adapted from Meng and Gerba (69)

45 minutes at either pH 3.5, 9.5, or 10. However, after 45 minutes at pH 10.5, Ead 40 underwent a titer reduction of 2.7 $\log_{10}$. These results are in contrast to those reported earlier (67) in which the infectivity of adenovirus type 5 decreased steadily at pH higher than 9.0, with rapid inactivation at pH 10.

ULTRAVIOLET DISINFECTION

Differences in the stability to inactivating factors among adenovirus types were first reported in 1962 (68). Research on the inactivation of several adenoviruses by ultraviolet irradiation showed that the adenoviruses represent a very diverse group. Adenovirus 4 and 20, for instance, were more resistant to inactivation by ultraviolet (UV) light than adenovirus 1. Adenovirus 4 and 20 had a titer reduction of approximately 2.5 $\log_{10}$. In contrast, adenovirus type 1 lost almost 3 $\log_{10}$ during the same period. In another study, when the enterovirus 70 and human adenovirus type 19 were exposed to UV irradiation, it took less than 10 mW/cm^2/min to cause a 4.5 $\log_{10}$ reduction of the enterovirus 70 titer, whereas a UV dose of 60 mW/cm^2/min was necessary to reach similar levels of inactivation of adenovirus 19 (63). These results agree with a previous investigation that compared the resistance of enteric adenoviruses to UV disinfection with poliovirus type 1 and coliphages MS-2 and PRD-1 (Table 2; 69). Purified stocks of the viruses in deionized-sterile water were exposed to collimated UV irradiation in a stirred reactor for a total dose of up to 140 mW sec/cm^2. The doses of UV to achieve a 90% inactivation of adenovirus 40, adenovirus 41, coliphages MS-2 and PRD-1, and poliovirus type 1 were 30,23.6,14,8.7, and 4.1 mW sec/cm^2, respectively. Although MS-2 is considered to be much more resistant to UV disinfection than enteroviruses and enteric bacteria, and has been suggested as a good model for enteric viruses disinfection (70), adenovirus 40 was significantly more resistant to UV irradiation than this coliphage. Therefore, adenoviruses appear to be among the most resistant waterborne enteric viruses to UV light disinfection (69).

CHEMICAL DISINFECTANTS

Differences in the susceptibility to chlorine among members of the adenovirus group have been reported (71). A 99% reduction of human adenoviruses 3 and 7 was achieved with a concentration of free chlorine of 0.69 and 0.89 mg/L, respectively. In contrast, a similar disinfection level of the simian adenoviruses M2,M3, and M4 was only obtained with 0.93 mg/L of residual chlorine. Disinfection experiments with HAV, human rotavirus, poliovirus 1, and adenovirus 5 in tap water by copper and silver ions and reduced levels of free chlorine (1. 0.5 or 0.2 mg/L) showed that adenovirus type 5 was more resistant to all disinfection treatments than poliovirus 1 but less resistant than either HAV or human rotavirus (72). Similar results were reported in a study that estimated the time required to eliminate adenovirus 40 from artificially contaminated mussels in a continuous flow of ozonated marine water. This investigation showed that it took much longer to eliminate Ead 40 than poliovirus 1. However, it was depurated faster than either HAV or human rotaviruses (73).

ENVIRONMENTAL STABILITY OF ADENOVIRUSES

The increased resistance showed by enteric adenoviruses when compared with other enteric viruses may be associated with the double-stranded nature of their DNA, which if damaged, may be repaired by the host cell DNA-repair mechanisms, which in human cells, in addition to repairing pyrimidine dimers can also repair a wide range of DNA damages (74). In addition, both adenovirus DNA strands may serve as template for replication (75). Thus, if one strand is damaged by environmental factors, the other may still serve as template for replication of progeny DNA. This mechanism would not be effective on ssRNA-genome viruses such as polio 1 or HAV. It has been suggested that nucleic acids from DNA viruses would persist longer in the aquatic environment (76) because indigenous DNAases require cofactors to be active and are denatured by temperature more readily than RNAases. It could be speculated then, that the longer survival of the enteric adenoviruses in tap and seawater might have been associated with DNA damage that could have been repaired by the host cell, whereas the faster inactivation in sewage might have resulted from protein capsid damage, rendering the virions unable to attach and enter the host cell.

CONCENTRATION AND RECOVERY

Although Ead 40 and 41 are important human pathogens (19), methods for their concentration in water are very limited (77). However, some research has been conducted on the concentration of adenoviruses (67,78–82) and enteric adenoviruses (77).

The earliest attempts to concentrate adenoviruses from water were conducted in 1965 (78). Sanitary napkins were suspended for two to three days in sewage or sewage effluents. In the laboratory, the napkins were wrung, the liquid centrifuged, and the resuspended pellet inoculated into different combinations of primary cultures of African green monkey kidney cells (MK), human amnion cells (HA), and a continuous green monkey kidney

cell line (BCS-1). Using this procedure, 96 adenovirus isolates were obtained from 661 samples positive for viruses.

The concentration of adenovirus was improved by a protamine-sulfate precipitation procedure (79).Using this method, a 50- to 400-fold concentration of adenovirus was achieved. It was observed that adenoviruses were preferentially precipitated when mixed with enteroviruses, and it was pointed out that the protamine-sulfate precipitation method could be used to prevent overgrowth of adenoviruses by faster replicating enteroviruses. This methodology was used successfully to detect indigenous adenoviruses from raw sewage in Greece (55).

Electronegative epoxy-fiberglass filters and fiberglass textile filters have been used as primary adenovirus-adsorbing filters. Elution of adenovirus 5 from fiberglass filters was more efficient with 3% beef extract pH 9.0 than with 0.05 M glycine pH 11.5, which resulted in inactivation of this agent (67). An adenovirus high-pH sensitivity was observed in another study in which the simian adenovirus SV-11 infectivity was greatly reduced at pH 10 or above (83).

Elution of viruses from adsorbing materials is often achieved by the use of high pH solutions (28). However, when adenoviruses are exposed to an eluting solution of pH 11.5, more than 90% of the viruses are inactivated in less than one minute. Enteroviruses, on the other hand, can be reconcentrated with an overall efficiency of 15.4% (81). This suggests that the enteroviruses may not be good surrogates for adenovirus recovery from water, and that failure to recover adenoviruses from water samples may be due, in part, to virus inactivation during elution of filters with high pH solutions.

In a study to evaluate the ability of microporous filters to recover Ead 40 from different types of water, it was found that with electronegative filters the recovery of this virus from drinking water was of 36%, whereas with electropositive filters, the average recovery efficiency was 26.5% (77).These results suggest that adsorption Ead 40 to electropositive filter surfaces at neutral pH may be less affected by specific electrostatic characteristics of the virions than their attachment to electronegative surfaces at pH 3.5, and that the higher recovery of this virus with electronegative filters may have been associated with a more efficient elution of the virus from the electronegative filtering medium (77).

Reconcentration of Adenoviruses

Although the filter-adsorption-elution procedure allows for the processing of large samples of water (84), the volume of the eluate still needs to be reduced to be assayed in a practical way. Among the methods for second-step reconcentration, the organic flocculation procedure (85) continues to be the most efficient (86). The organic flocculation method (85) is widely used to reconcentrate viruses from environmental water samples. It has been used efficiently for the recovery of Simian adenovirus SV-11, with recoveries of 50 to 60% (80). A limitation of this technique, in addition to extreme pH changes, is that it is not equally effective with all viruses. The reconcentration efficiency by the organic flocculation method, may be different even among different serotypes of the same virus (86).

In a study that compared two aqueous-polymer-phase separation methods, as a second step of adenovirus reconcentration, it was demonstrated that a dextran-methyl cellulose-polyethylene glycol phase separation method was superior to a sodium dextran sulfate-polyethylene glycol sodium chloride procedure (67). Furthermore, the dextran-methyl cellulose-polyethylene glycol phase separation method selectively concentrated adenovirus 5 when mixed with poliovirus 2 (16 : 2 ratio).

As an alternative to the organic flocculation method, polyethylene glycol (PEG) precipitation has been used to concentrate Ead 40 from beet extract (BE). The reconcentration efficiency by this method (40%) was similar to that obtained by organic flocculation (38%). PEG precipitation may offer some advantages over the organic flocculation procedure to obtain high-quality RNA for RT (reverse transcriptase)-PCR analysis, especially when the number of viruses in the sample is low (87).

METHODS OF DETECTION

Several methods have been used to detect adenoviruses. Clinical specimens for detection may include feces (88), nasal washes, and pharyngeal, anal, and conjunctival swabs (42); whereas environmental samples consist mostly of water (58) and wastewater (58,59). Adenoviruses in these types of samples are relatively stable and will withstand freezing and storage at $-70\,^{\circ}C$ (14). Although adenovirus isolation by cell culture is the procedure used by most diagnostic and reference laboratories, it requires highly trained personnel and specialized facilities. Therefore, the enzyme immunoassay (EIA) is the method of choice for the detection of adenoviral soluble antigens in feces or respiratory secretions (42). Other methods used for the detection of adenoviruses include direct electron microscopy (89), immune electron microscopy (90), immunofluorescence (21), enzyme-linked immunosorbent assay (ELISA) (91), gel electrophoresis, and restriction analysis of viral DNA (92), nucleic acid hybridization (93,94), and polymerase chain reaction (PCR) (9,57,58,88). With the exception of PCR, detection by these techniques is only possible if a relatively large number of viral particles are present in the sample.

Enteric adenoviruses propagate poorly, or not at all, in conventional human cell lines in which other adenoviruses can be propagated effectively (95). The enteric adenoviruses 40 and 41 are even difficult to grow in fetal intestinal organ cultures (96). However, in 1992 the successful propagation of both Ead 40 and Ead 41 in PLC/PRF/5 human liver cell line was reported (97). Later, these agents were also propagated in a cell line derived from a human colon carcinoma (CaCo-2 cells) (98). The availability of these cell lines has provided a practical method for the propagation and detection of Ead 40 and Ead 41 to study these viruses in the aquatic environment (62,65).

Purification and Separation

Several continuous cell lines have been used in the propagation of many types of human adenoviruses. Some of these include Graham 293, HeLa, Hep-2, KB, and A549 cells (99). Propagation of the enteric adenoviruses 40 and 41 has been accomplished in PLC/PRF/5 human liver cell line (97) and CaCo-2 cells (98). Adenovirus cytopathic effect (CPE) is distinguished by large pycnotic cells that eventually aggregate into sheets that detached from the culture vessel (99).

Adenoviruses replicate in the nucleus of infected cells, and most virions remain cell-associated; therefore, the release of viral particles can be achieved by disruption of the nuclear membrane by freezing and thawing, sonication, or by extraction with an organic solvent such as Freon (100). Because of environmental concerns resulting from the use of Freon, other organic solvents have been proposed as an alternative for the extraction of nonenveloped viruses (101). After removing cell debris, virions can be purified by density gradient centrifugation (100). Depending on the adenovirus type, titration of can be accomplished by the plaque method or by an end point CPE method such as the tissue-culture-infectious dose fifty ($TCID_{50}$) (65). Although the plaque method is more accurate, it requires lengthy incubation periods and a very stable cell monolayer (100). Furthermore, not all adenovirus types respond to plaquing (97). In contrast, the end point CPE method is less sophisticated than the plaque method, but still sensitive and accurate enough for most applications (100).

Assay

Immune Assays. The enzyme immunoassay (EIA) is the method most commonly used for the detection of adenoviral soluble antigens in feces, or in respiratory secretions (42,91). Monoclonal antibodies (Mab) directed to a family-shared hexon epitope can be used in EIA to identify any member of the *Adenoviridae* family. If needed, a type-specific Mab can be utilized to further determine the precise adenovirus type (42). All known mastoadenoviruses share a family antigen that can be detected by the Complement-Fixation (CF) (102) and immunofluorescence (IF) tests (14). The hemagglutination inhibition (HAI) and serum neutralization (SN) tests can be used to further differentiate specific members of the *Adenoviridae* family (14,21).

Molecular Methods

PCR. The polymerase chain reaction (PCR) has been used to identify adenovirus DNA in tissue specimens and body fluids (14), diluted stool (52,88), air filters (103), swimming pool water (9), sewage, and polluted river water (58). Most primers for PCR have been selected from the hexon coding region because there is an extensive homology among adenovirus serotypes; whereas sequences from the early coding region E1B have been used for the detection of specific adenoviruses (88). Although PCR using hexon primers is more sensitive than culture methods, it may give false-positive results from stool samples of healthy patients. It has been suggested that PCR using these primers may be detecting latent adenoviruses of different types because EIB primers are usually negative in normal individuals (14).

Oligoprobe Hybridization and Restriction Endonuclease Digestion. Adenoviral DNA can be characterized by oligoprobe hybridization (59,94,104) and restriction endonuclease digestion (52,95). In contrast to the relatively small regions of the genome analyzed by either hemagglutination inhibition (HI) or serum neutralization (SN) tests, restriction endonucleases recognize and cleave DNA at specific sequences throughout the viral genome. The sensitivity of restriction endonuclease digestion can be increased by Southern blotting of the DNA restriction digests (105). Depending on the hybridizing probe, DNA from adenovirus isolates can be identified and classified by group or type (14). Hybridization of adenoviral DNA from fecal extracts by the direct-spot method has been successfully used to identify enteric adenoviruses from clinical specimens from patients suffering from gastroenteritis (106).

CONCLUSION

Adenoviruses are important human pathogens causing an array of clinical conditions ranging from asymptomatic infections to fatal illness. These viruses are ubiquitous in sewage-contaminated water and are relatively resistant to environmental stresses. Although their impact on human health, from the environmental point of view in particular as a waterborne organism, has not been fully assessed, evidence suggests that these agents may represent a significant hazard to public health. Further research on detection methods and disinfection procedures could provide the tools to assess the risk that adenoviruses pose for the population more objectively and also to implement actions to reduce their threat to public health.

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ADHESION, IMMOBILIZATION, AND RETENTION OF MICROORGANISMS ON SOLID SUBSTRATA

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Microorganisms occur in nearly all natural fluid environments on earth and have a strong tendency to become associated with surfaces (1). The interaction mechanism between microorganisms and substratum surfaces involves the physicochemical properties of the interacting surfaces (2) and, in fact, microorganisms can be considered as living colloidal particles with a density slightly greater than that of water, and size of about 1 μm in length or diameter (3). Stable suspensions of microorganisms in dilute electrolytes result, in part, from a mutual electrostatic repulsion between like charges on microbial cell surfaces. Virtually all microorganisms known at present possess a net-negative surface charge at the pH range found in most natural habitats (4), as revealed by zeta potential measurements. The pH dependence of four selected microbial strains are shown in Figure 1. Charge reversal upon association of acidic (carboxyl or phosphate) groups at low pH values is indicative of the presence of charged basic (amino) groups on the cell surface. Microorganisms also exhibit variation in surface-free energies, with some strains being hydrophilic and others being relatively hydrophobic (5). In contrast to inert colloidal particles, however, microorganisms are living microorganisms capable of metabolism, growth, and, in some instances, independent motion. An expression of the metabolism of the microorganisms is the production of various amounts of extracellular polymers (6) that together with flagella, pili, fimbriae, and fibrils (4,7) (Fig. 2) are important in the interaction between microorganisms and solid substrata. Microorganisms can interact with inorganic, living, or dead materials such as plants, animals, artificial structures, rocks, clays, sand grains, and organic remains of living microorganisms in various stages of decomposition.

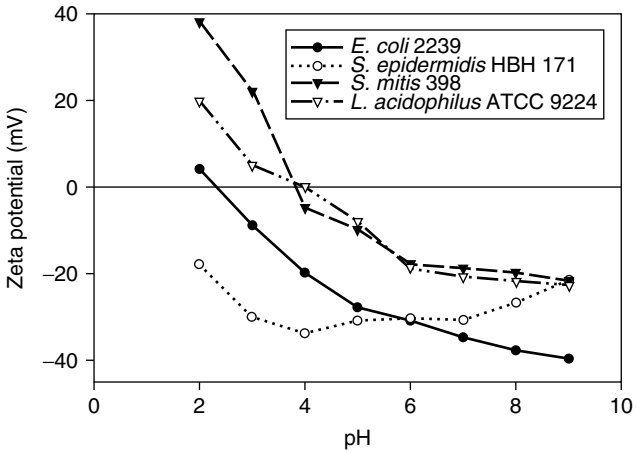

Figure 1. Zeta potentials as a function of pH for *E. coli* 2,239, *Staphylococcus epidermidis* HBH 276, *Streptococcus mitis* BA and *Lactobacillus acidophilus* ATCC 9,224 as measured in 10 mM potassium phosphate, with pH adjusted by addition of HCl or KOH. Data from H. C. van der Mei, R. Bos, H. J. Busscher, and P. S. Handley, in A. Baszkin and W. Norde, eds., Physical Chemistry of Biological Interfaces, Marcel Dekker, New York, 2000, pp. 431–458.

Once microorganisms are attached to a surface, a multistep process starts leading to the formation of a complex, adhering microbial community termed *biofilm*. A biofilm can be defined as a microecosystem in which different strains and species efficiently cooperate to protect themselves against environmental stresses and to facilitate more efficient nutrient uptake (8). Biofilms can be beneficial, for instance, in sewage treatment (9), bioreactors (10), and animal microflora (11). However, biofilms are most frequently unwanted as in food processing (12,13), on ship hulls (14), in drinking water (15) and dairy manufacturing plants (16), in pipelines (17), on biomaterial implants (18), or in the oral cavity (19). Although the function and appearance of biofilms in various environments may be different, all biofilms form from the same basic sequence of events (20), as depicted in Figure 3.

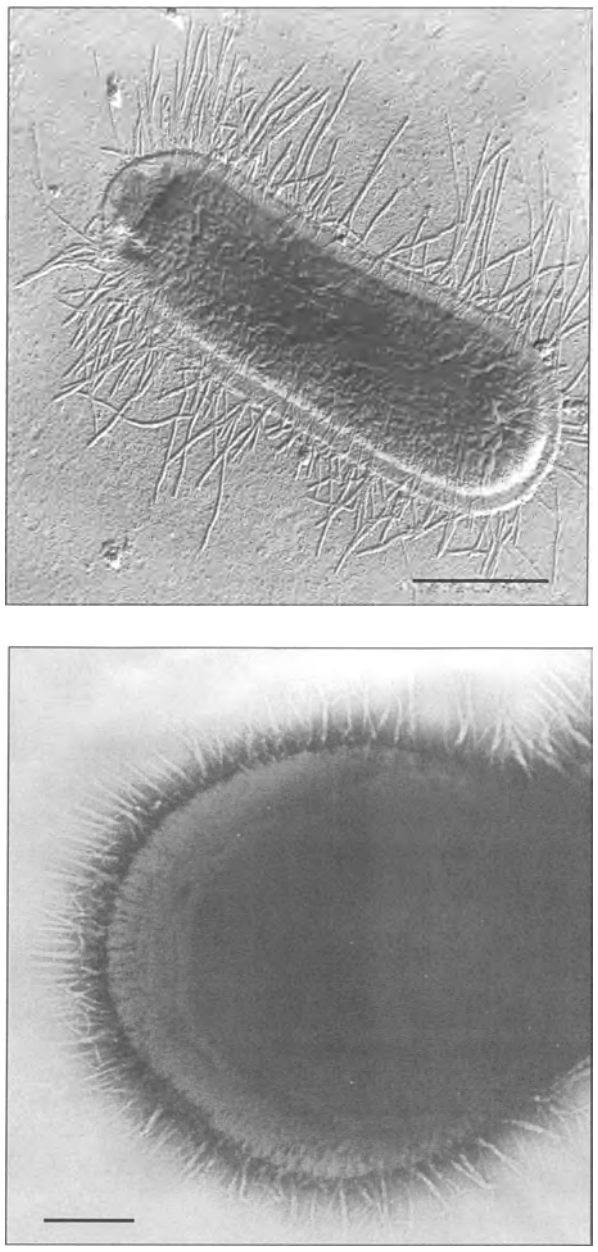

Figure 2. Electron micrographs of (**a**) *E. coli* with rigid, peritrichous fimbriae, approximately 7 nm in diameter and up to 1.0 μm length. Cells are shadowed with palladium. Bar marker represents 0.5 μm; (**b**) *Streptococcus salivarius* showing peritrichous clumped fibrils with a length of 191 nm. Cells are negatively stained with 1% methylamine tungstate. Bar marker represents 100 nm. Micrographs taken with permission from H. C. van der Mei, R. Bos, H. J. Busscher, and P. S. Handley, in A. Baszkin and W. Norde, eds., *Physical Chemistry of Biological Interfaces*, Marcel Dekker, New York, 2000, pp. 431–458.

When organic matter is present in an aqueous environment (e.g., blood, saliva, tear fluid, seawater, milk, or urine), a so-called *conditioning film* of adsorbed components (i.e., proteins and other organic molecules) is formed on the substratum surface before adhesion of microorganisms, because molecules diffuse much faster than microorganisms (21). As a second step, microorganisms are transported toward the solid surface through diffusion, convection, sedimentation, or by intrinsic motility. Alternatively, planktonic (suspended) microorganisms may be transported toward each other to form microbial coaggregates (22) and subsequently adhere, or a few sessile (already adhering) microorganisms may stimulate coadhesion of planktonic microorganisms (23). In later stages, microbial anchoring occurs through extrapolymer (i.e., polysaccharides) production (6,24) and eventually, adhering microorganisms start to grow, which is the main contributing factor for the accumulation of microorganisms in a biofilm.

The thermodynamic theory (25,26) considers the surface-free energies of the solid substratum, the microbial cell surface, and the suspending medium yielding the interfacial free energies between the interacting surfaces, as schematically illustrated in Figure 4. Accordingly, this comparison is expressed in the so-called free energy of adhesion

$$\Delta G_{\mathrm{adh}} = \gamma_{\mathrm{sm}} - \gamma_{\mathrm{sl}} - \gamma_{\mathrm{ml}} \qquad (1)$$

where γ_{sm}, γ_{sl}, and γ_{ml}, are the interfacial free energies, where "s" denotes the substratum, "m" the microbial cell surface and "l" the liquid surface. Adhesion is thermodynamically favorable if ΔG_{adh} is negative, because systems tend to minimize their free energy, whereas adhesion is energetically unfavorable when ΔG_{adh} is greater than zero.

A three-point hypothesis of microbial interaction mechanisms (27) related to the distance between the microbial cell and substratum surfaces is schematically illustrated in Figure 5. At large separation distances (>50 nm), only Lifshitz-Van der Waals forces are operative. Upon closer approach, both Lifshitz-Van der Waals and electrostatic forces (i.e., nonspecific interactions) occur for separation distances between 10 to 20 nm. Finally, at near contact, Lifshitz-Van der Waals forces, electrostatic and acid-base interactions, and so-called specific interactions dictate adhesion. Specific interactions may be defined as the interactions between stereochemically complementary surface components and act over extremely short distances allowing specific ionic, hydrogen, and possibly chemical bonds to form.

In this paper, a distinction is made among adhesion, immobilization, and retention of microorganisms on substratum surfaces, and the importance to make such a distinction is exemplified. A microorganism adheres to a substratum surface when it maintains the same separation distance from the substratum surface as a function of time through a balance of attractive and repulsive interactions homogeneously acting over an entire substratum surface. Consequently, adhering microorganisms are still free to move parallel to the substratum as opposed to immobilization. Immobilized microorganisms adhere to substratum surface but are kept at the same position by immobilization forces acting parallel to the surface. Often, both in natural habitats and in laboratory experiments, adhering microorganisms are challenged by external forces yielding detachment of all or subpopulations of adhering organisms. Retention will be explained to as the capacity of adhering microorganisms to remain

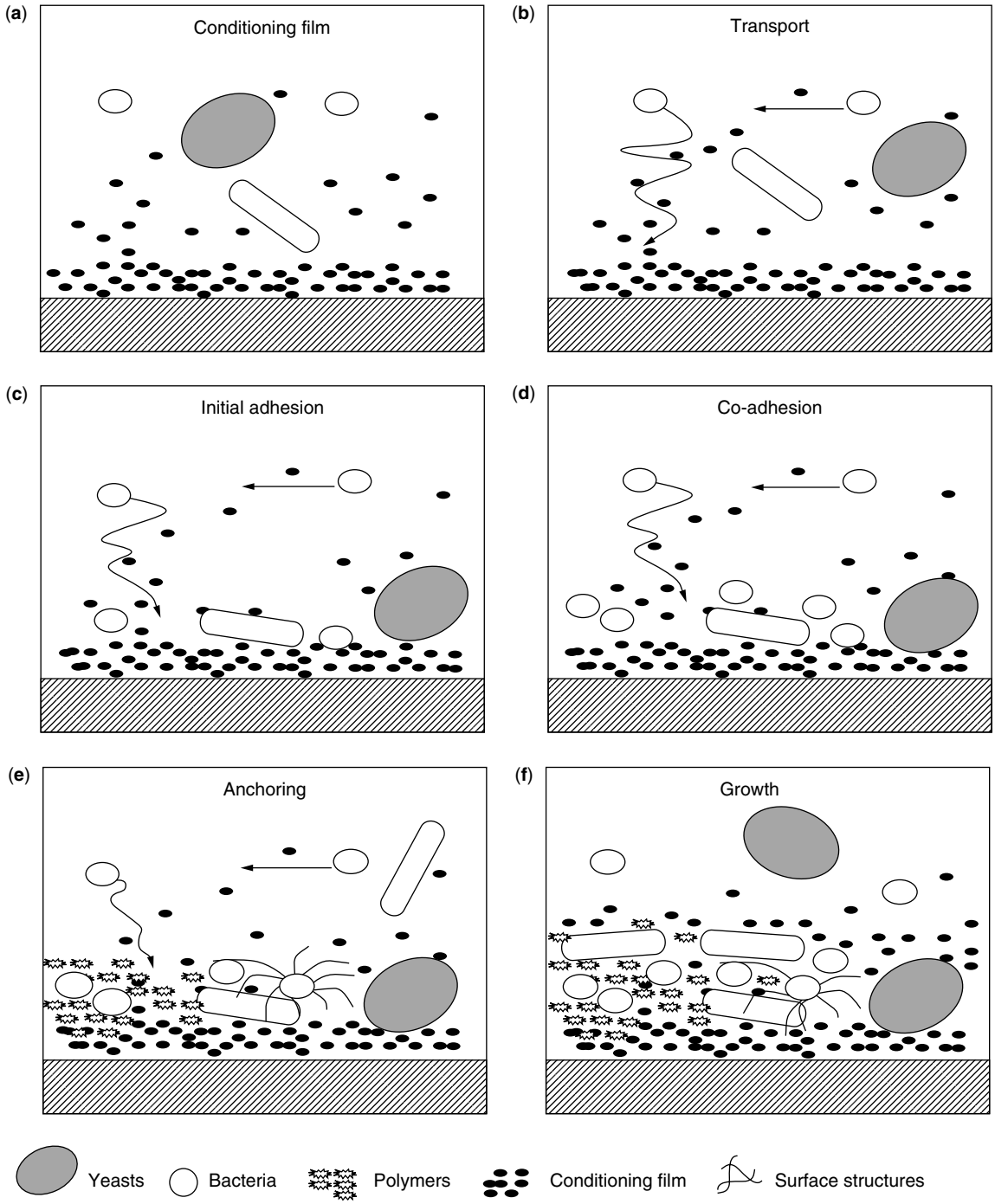

Figure 3. Sequential steps in the formation of a biofilm on a solid substratum. (**a**) Adsorption of conditioning film components. (**b**) Microbial transport and coaggregation. (**c**) Adhesion of single organisms and of microbial coaggregates. (**d**) Coadhesion between microbial pairs. (**e**) Anchoring yielding firm, irreversible adhesion through exopolymer production and surface structures. (**f**) Microbial growth and ingrowth.

adhering after having been submitted to an external force other than their mutual interaction.

ADHESION AND THE DLVO THEORY

The perpendicular interaction between microorganisms and a substratum surface consists of Lifshitz-Van der Waals, electrostatic, and acid-base components. In the original theory by Derjaguin, Landau, Verwey, and Overbeek (the classical DLVO theory), microbial adhesion is described as a balance between attractive Lifshitz-Van der Waals and repulsive or attractive electrostatic forces. Accordingly, the interaction energies between

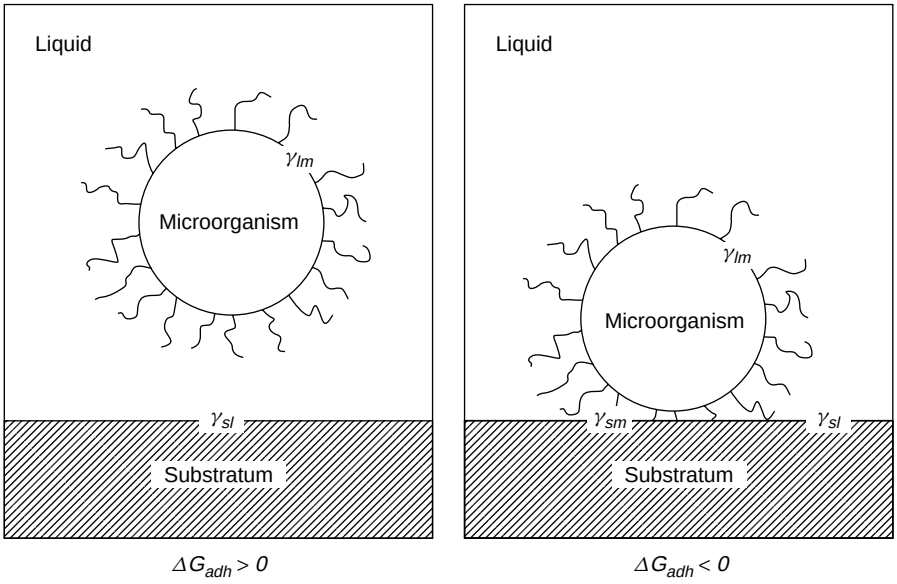

Figure 4. Schematic presentation of the interfacial free energies (γ_{ij}) involved in the adhesion of a microorganism to a solid substratum surface from a liquid suspension. The solid-microorganism (γ_{sm}), the solid-liquid (γ_{sl}), and the microorganisms-liquid (γ_{ml}) interfacial free energies are indicated.

microorganisms and substratum surfaces as a function of their separation distance (d) can be expressed as (28–30)

$$G^{TOT}(d) = G^{LW}(d) + G^{EL}(d) \tag{2}$$

where G^{TOT}, G^{LW}, and G^{EL} denote the total, the Lifshitz-Van der Waals, and the electrostatic interaction energies, respectively (31,32).

Lifshitz-Van der Waals Interactions

The Lifshitz-Van der Waals interaction between a microorganism and a solid surface at separation distances small enough to ignore retardation effects is given by

$$G^{LW}(d) = -\frac{A}{6}\left[\frac{a}{d} + \frac{a}{d+2a} + \ln\left(\frac{d}{d+2a}\right)\right] \tag{3}$$

where A denotes the Hamaker constant (i.e., an indication of the polarizability of the interacting surfaces) and a is the radius of the microorganism. The Hamaker constant depends on the nature of the interacting media. For the interaction of a microorganism (m) with a substratum (s) in a liquid medium (l), A is (29)

$$A_{msl} = (A_{ss}^{(1/2)} - A_{ll}^{(1/2)})(A_{mm}^{(1/2)} - A_{ll}^{(1/2)}) \tag{4}$$

In most cases, A is positive (attractive Lifshitz-Van der Waals forces), because in aqueous systems A_{ss} is greater than A_{ll} and A_{mm} is greater than A_{ll}, and consequently G^{LW} is less than zero.

Electrostatic Interactions

In aqueous media, electrostatic interactions are present in addition to Lifshitz-Van der Waals forces. These forces arise from the electrical surface charge of microorganisms and substratum surfaces, and can be influenced by introducing an electrolyte into the liquid phase, yielding changes in the electrostatic double layer at a surface (33,34). Electrostatic double-layer attraction or repulsion arises from the interpenetration of interacting double layers, and assuming constant potentials during interactions it is given by

$$G^{EL}(d) = \pi\varepsilon a(\zeta_m^2 + \zeta_s^2)\left\{\frac{2\zeta_m\zeta_s}{\zeta_m^2 + \zeta_s^2}\ln\frac{1+\exp(-\kappa d)}{1-\exp(-\kappa d)} + \ln[1-\exp(-2\kappa d)]\right\} \tag{5}$$

where ε denotes the permittivity of the medium, ζ_m and ζ_s the zeta potential of microorganism and substratum, respectively, and κ^{-1} is the double layer thickness, and constant potentials are considered. κ^{-1} depends on the total ion concentration in the solution and can be calculated from

$$\kappa = \left[\frac{e^2}{\varepsilon kT}\sum_i z_i n_i\right]^{(1/2)} \tag{6}$$

where e denotes the electron charge, k the Boltzmann constant, T the absolute temperature, z_i is the valence of the ions present and n_i is the number of ions per unit volume. For a symmetrical 1-1 electrolyte this equation reduces to

$$\kappa = 0.328 \times 10^{10}(z_i^2 M_i)^{(1/2)} \tag{7}$$

where M_i is the molarity (mol/L) of the ions.

Figure 6 illustrates the decay with the distance of the Lifshitz-Van der Waals and electrostatic energies between a charged microorganism and a substratum surface for repulsive electrostatic interactions (i.e., like charges) at different ionic strengths. It can be seen that at low ionic strengths (Fig. 6a), a high-energy barrier has to be overcome if the microorganisms are to come in close contact with the surface. At intermediate ionic strengths (Fig. 6b), the energy barrier, although still present,

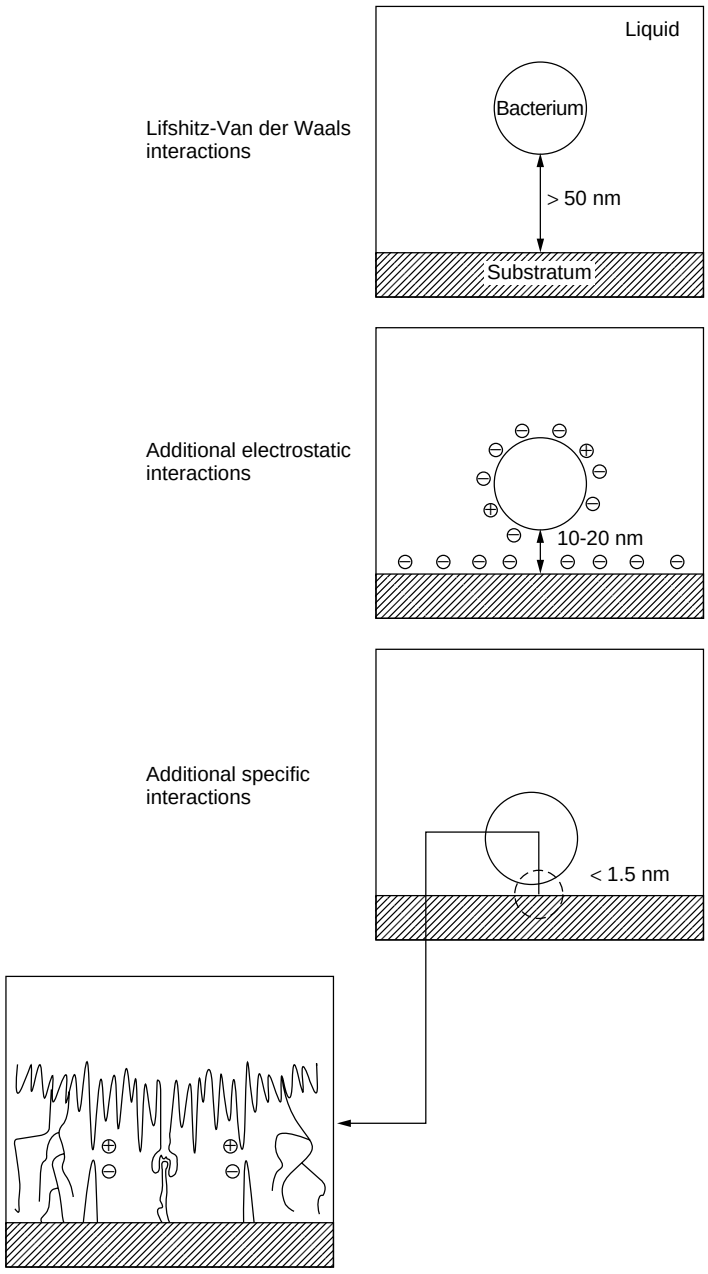

Figure 5. Three-point hypothesis of bacterial adhesion mechanisms related to the distance of the bacterium from the substratum surface. Adapted from H. J. Busscher and A. H. Weerkamp, *FEMS Microbiol. Rev.* **46**, 165–173 (1987).

is lower and microorganisms can become reversibly captured in a so-called *secondary minimum*. At high ionic strengths (Fig. 6c), the energy barrier disappears and a strong net attraction exists between microorganisms and the substratum surface at all separation distances. Microorganisms held in the deep primary interaction minimum are frequently considered to adhere irreversibly. Alternatively, Figure 7 depicts the interaction energies as a function of separation distance at different ionic strengths for unlike microbial and substratum surface charges. The net effect is a strong attraction regardless of separation distance, which decreases with the increasing ionic strength.

In summary, the Lifshitz-Van der Waals interaction between suspended microorganisms and a surface is not influenced by the ionic strength of the suspension, but both the range and the magnitude of the electrostatic interactions decrease with an increasing ionic strength.

The Extended DLVO Theory

The classical DLVO theory has been extended by Van Oss and coworkers (35) and recently related to the origin of hydrophobic interactions in microbial adhesion (36). The extended DLVO theory considers four fundamental, noncovalent interactions: Lifshitz-Van der Waals, electrostatic, Lewis acid-base, and Brownian motion forces.

Acid-Base Interactions

Acid-base interactions originate from electron-donating and electron-accepting interactions between polar moieties in aqueous solutions. The decay with separation

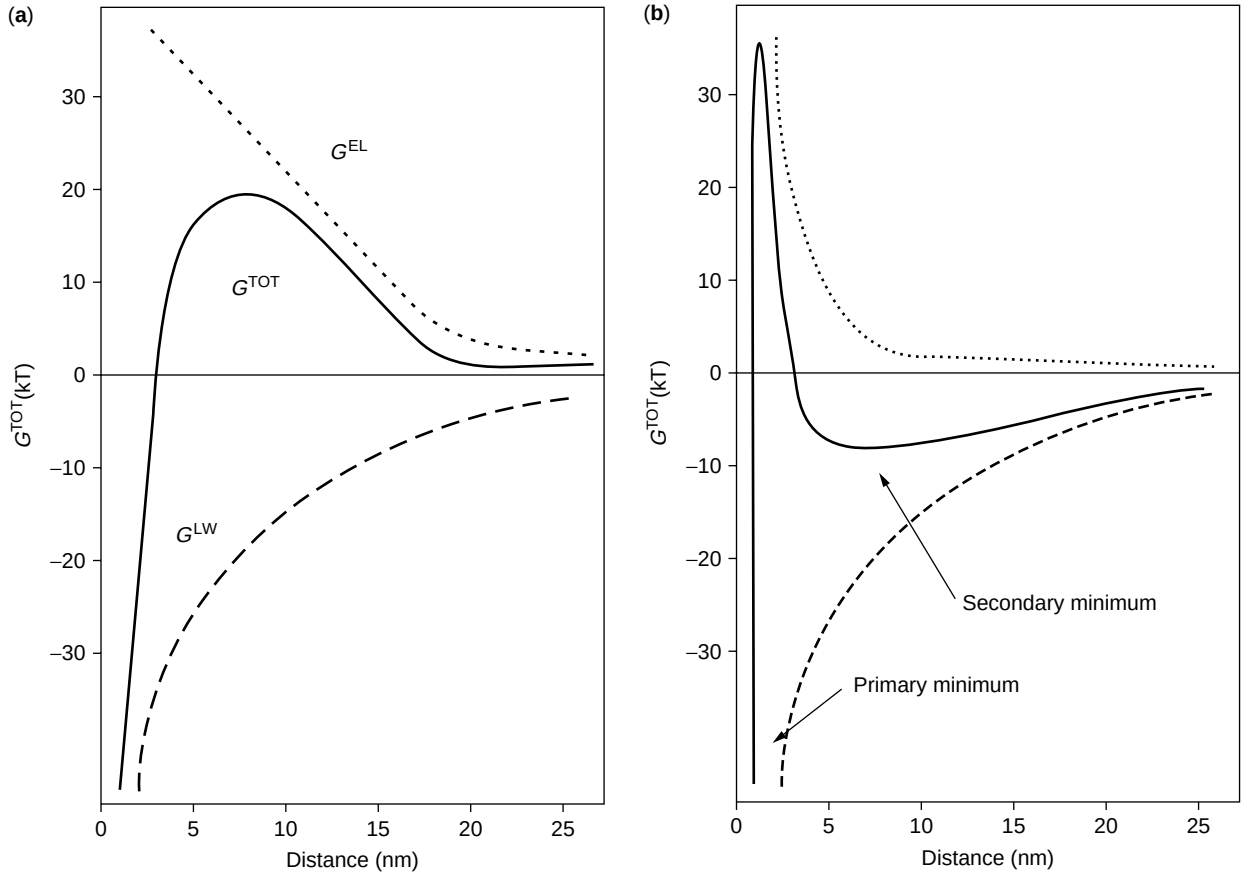

Figure 6. Interaction energy curves (classical DLVO theory) of a microorganism with a solid surface of like charge versus their separation distance for (**a**) low (i.e., one mM), (**b**) intermediate (i.e., 10 mM) or (**c**) high (i.e., 100 mM) ionic strength of the medium.

distance of the acid-base interaction energy (G^{AB}) between a microorganism and a substratum surface can be expressed as

$$G^{AB}(d) = 2\pi a\lambda\Delta G^{AB}_{adh}\exp\left[\frac{d_0 - d}{\lambda}\right] \qquad (8)$$

where ΔG^{AB}_{adh} is the acid-base interfacial free energy of adhesion, d_0 is the distance of closest approach between two surfaces 1.57 Å according to (37), and λ denotes the correlation length of molecules in a liquid medium and varies with the ionic strength and with the sign of ΔG^{AB}_{adh}. For ΔG^{AB}_{adh} less than zero ('hydrophobic attraction'), λ may be as large as 13 nm. For situations of *hydrophilic repulsion* ($\Delta G^{AB}_{adh} > 0$) λ equals 0.6 nm (38). The acid-base free energy of adhesion can be obtained from the following expressions

$$\Delta G^{AB}_{adh} = -2\left(\sqrt{\gamma^{AB}_{mv}} - \sqrt{\gamma^{AB}_{lv}}\right)\left(\sqrt{\gamma^{AB}_{sv}} - \sqrt{\gamma^{AB}_{lv}}\right) \qquad (9)$$

where γ^{AB}_{mv}, γ^{AB}_{lv}, and γ^{AB}_{sv} are, respectively, the microorganism-vapor, liquid-vapor, and substratum-vapor acid-base interfacial free energies, or from

$$\Delta G^{AB}_{adh} = +2\Big[\left(\sqrt{\gamma^{+}_{mv}} - \sqrt{\gamma^{+}_{sv}}\right)\left(\sqrt{\gamma^{-}_{mv}} - \sqrt{\gamma^{-}_{sv}}\right) - \left(\sqrt{\gamma^{+}_{mv}} - \sqrt{\gamma^{+}_{lv}}\right)\left(\sqrt{\gamma^{-}_{mv}} - \sqrt{\gamma^{-}_{lv}}\right) - \left(\sqrt{\gamma^{+}_{sv}} - \sqrt{\gamma^{+}_{lv}}\right)\left(\sqrt{\gamma^{-}_{sv}} - \sqrt{\gamma^{-}_{lv}}\right)\Big] \qquad (10)$$

where γ^- and γ^+ denote the electron-donating and electron-accepting parameters of the interfacial free energy, respectively.

Figure 8 illustrates the decay with the distance of the interaction energies in the extended DLVO theory between a microorganism and a substratum surface for apolar, monopolar, and bipolar surfaces under conditions of electrostatic repulsion. It can be seen that the influence of acid-base interactions is quite strong compared with electrostatic and Lifshitz-Van der Waals interactions. However, the acid-base interactions are only relatively short-ranged, and a close approach between interacting surfaces is required for these forces to become operative, which may well be impeded in microbial adhesion because of the different structural features on microbial cell surfaces (39).

IMMOBILIZATION: PERPENDICULAR AND LATERAL INTERACTIONS

In the case of adhesion, the main body of a microbial cell is held at a small, but finite, distance from the

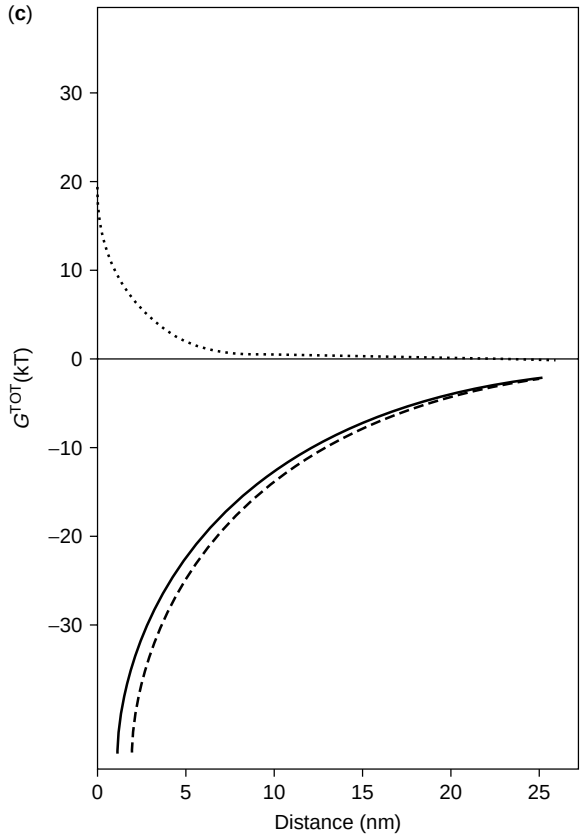

Figure 6. (*Continued*)

substratum surface by a balance between interaction forces acting perpendicular to the surfaces. Regardless of whether this position above the substratum surface is occupied in a reversible or irreversible fashion, the balance of forces acting perpendicular to the surface does not ensure immobilization of adhering organisms along the surface, which requires lateral interaction forces. As a consequence, mobile adhesion is not solely a property of the so-called mobile bacteria, but of all colloidal particles adhering on a surface in the absence of lateral interaction forces. The distinction between adhesion and immobilization is best illustrated by comparing the ease with which a magnet adhering on a smooth metal surface can be slid over the surface with the force required to pull the magnet off the surface (see Fig. 9).

Mobility of adhering microorganisms is due to forces on the adhering microorganisms acting laterally along the substratum surface and not due to perpendicular interactions between an adhering microorganism and a substratum surface (40). Accordingly, adhering microorganisms can be sheared, they can diffuse, or they can actively swim freely, laterally until they encounter a sticky patch on the surface (chemical heterogeneity) or become mechanically trapped in "rugosities."

Ideally Homogeneous and Smooth Substrata

On ideally homogeneous and smooth surfaces (see Fig. 10), the only lateral interactions operative result from a multibody DLVO interaction between all adhering bacteria, because the substratum surface cannot exert any lateral force. A lateral interaction minimum between microorganisms occurs at large separation distances (500 nm) and is extremely shallow (about 0.1 kT) as compared with the deep (i.e., 12 kT) perpendicular interaction minimum occurring at heights of around 6 nm above the substratum surface (see Fig. 10). Consequently, regardless of the strength of the perpendicular interactions, microbial adhesion is always mobile on ideally homogeneous and smooth surfaces.

Chemically Heterogeneous Substrata

Virtually all substratum surfaces contain chemical heterogeneities at a submicron level, which can be demonstrated by contact-angle hysteresis (41). Their importance in particle adhesion has recently been demonstrated by Wit and Busscher (42), showing that colloidal particles have a strong preference to deposit at the same locations on a substratum surface in repeated deposition experiments after detachment of the adhering particles. Different chemistries along a substratum surface yield different DLVO interaction energies with adhering microorganisms, as shown in Figure 11a, and secondary interaction minima with different depths are found for hydrophilic and hydrophobic regions on the surface, from which a lateral interaction force develops, driving adhering organisms to a location of minimum interaction energy, that is, the site with the deepest interaction minimum is occupied. The lateral interaction energy $dG = (G_2 - G_1)$ for a chemical heterogeneity in the example amounts to about -5 kT (see Fig. 11b), which is more than the lateral interaction energy on an ideally homogeneous and smooth surface (0.1 kT, compare with Fig. 10b).

Substratum Roughness

Because of rugosities on a substratum surface, macroscopically lateral interaction forces can arise from perpendicular interactions and stimulate immobilization. Microorganisms adhering near structural heterogeneities on a substratum surface have an energetically less favorable situation than when adhering on an overall smooth surface (43). Mobility on a rough surface is driven by a lateral interaction energy, which depends on the size of the rugosities and can be estimated to amount to several kTs in magnitude. In Figure 11b, it can be seen that lateral mobility of a microorganism adhering on a rough surface is inevitably associated with a change in the perpendicular position with respect to the overall substratum surface from which the majority of the interaction forces occur (44).

It is important to make a distinction between micrometer roughness and nanometer roughness, because new developed techniques (i.e., atomic force microscopy) permit the measurement of such values (45). A bacterial cell (size of about a micrometer) will interact laterally with a micrometer roughness and stipulate its mobility over the substratum. On the other hand, if the substratum surface has a roughness at a nanometer scale, the bacterial cell will not interact laterally with such rugosity and might even be repelled by it (46).

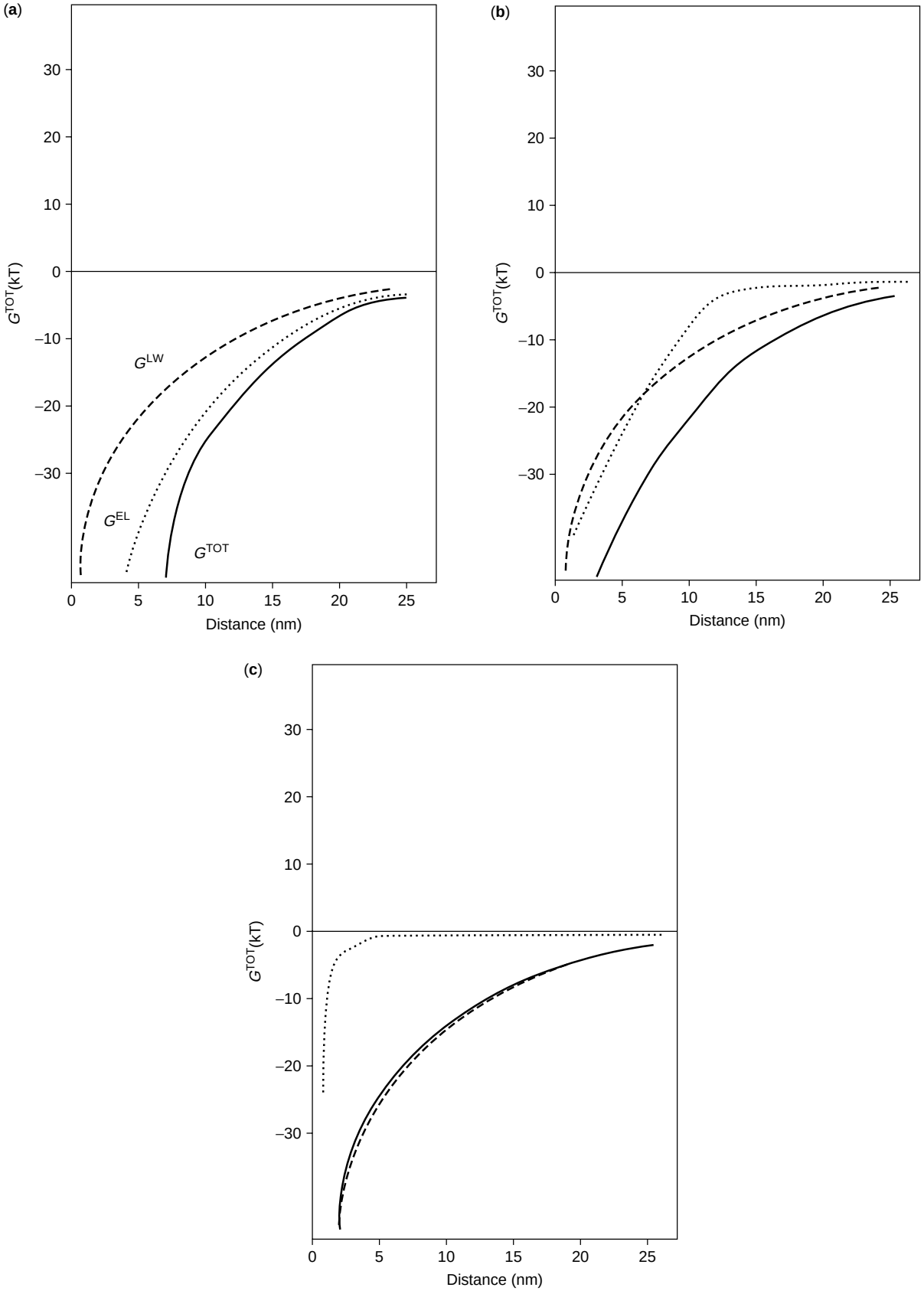

Figure 7. Interaction energy curves (classical DLVO theory) of a microorganism with a solid surface of unlike charge versus their separation distance for (**a**) low (i.e., 1 mM), (**b**) intermediate (i.e., 10 mM), or (**c**) high (i.e., 100 mM) ionic strength of the medium.

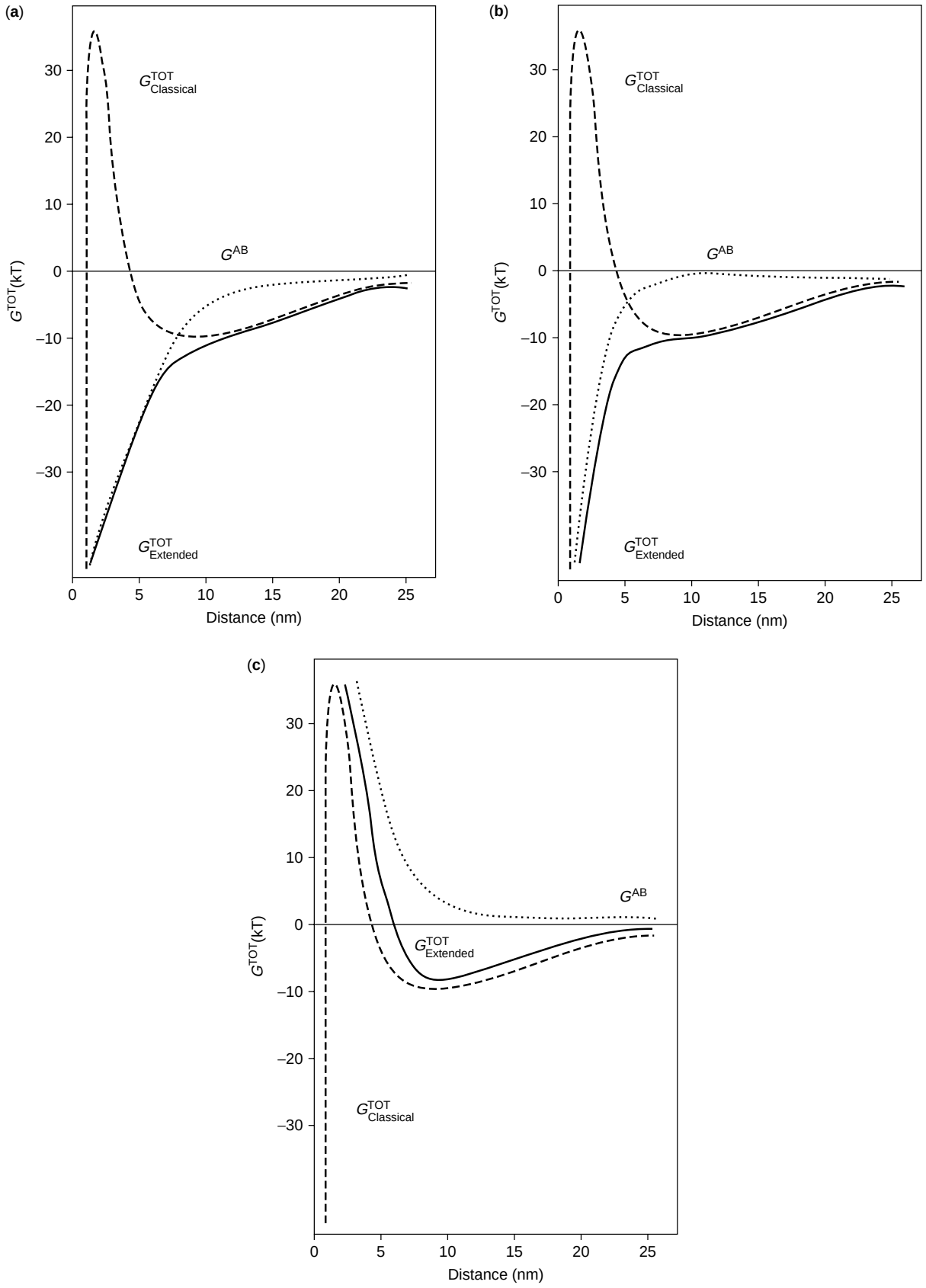

Figure 8. A comparison of the interaction energies according to the classical and extended DLVO theory between a microorganism and a solid substratum as a function of the separation distance for (**a**) apolar, (**b**) monopolar, and (**c**) bipolar surfaces.

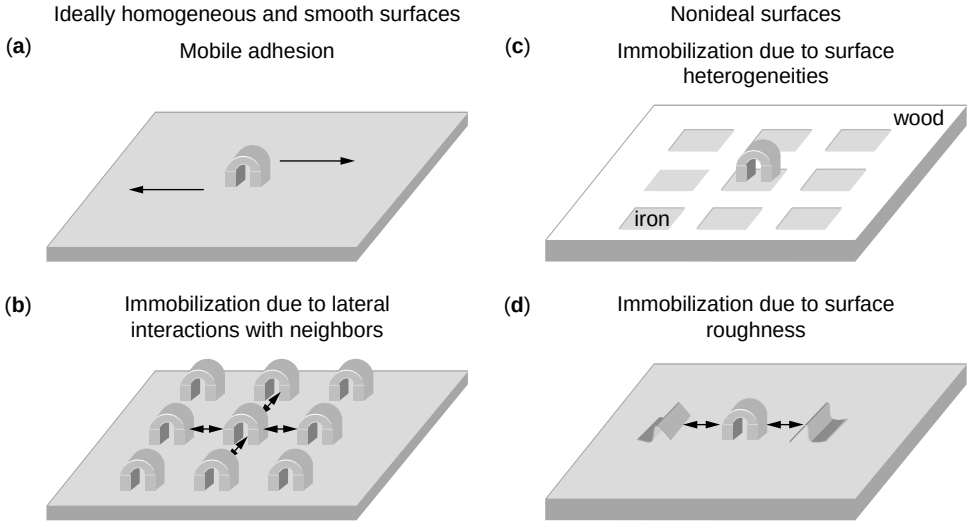

Figure 9. Motility of bacteria is, from a physicochemical point of view comparable with the interaction of a magnet with a metal plate. On an ideally homogeneous and smooth surface: (**a**) when a magnet can be easily slid over the surface of a metal plate, one speaks of adhesion without immobilization, (**b**) when a magnet is not free to move over the surface because of lateral interactions with neighbors, there is immobilization. On a nonideal surface: (**c**) Immobilization may result from lateral interaction forces as a result of heterogeneities on the metal surface, (**d**) surface roughness can provoke immobilization as a result of lateral interactions with the magnet.

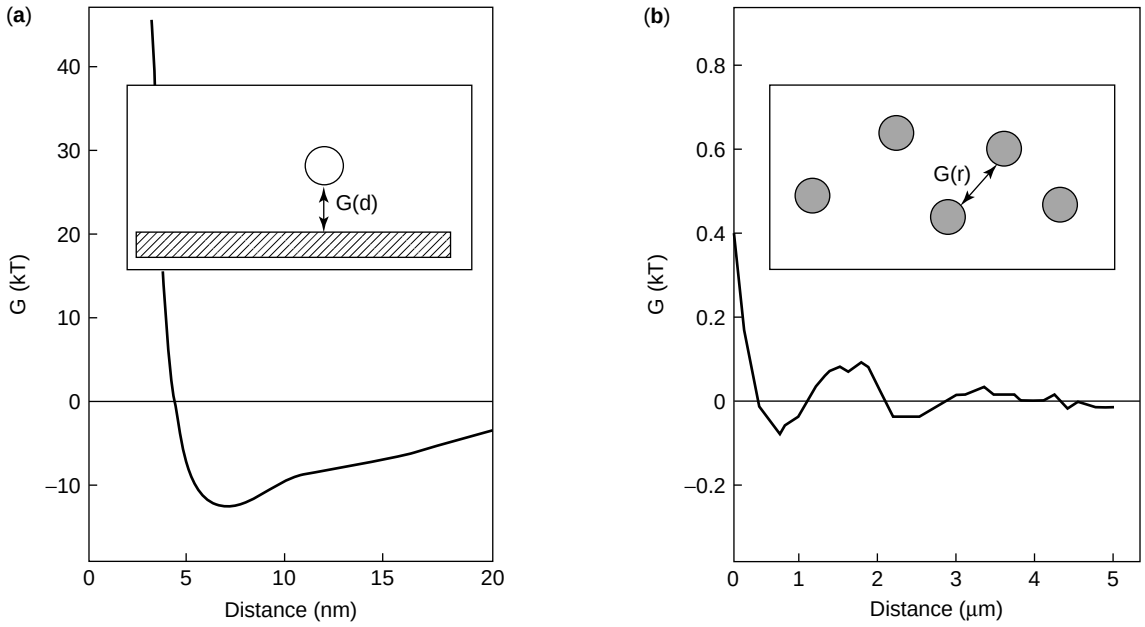

Figure 10. (**a**) The interaction energy $G(d)$ according to the extended DLVO theory as a function of separation distance between a microorganism and an ideally homogeneous and smooth, hydrophilic, and negatively charged model surface. (**b**) The lateral interaction energy $G(r)$ as a function of separation distance among microorganisms (r) is calculated from the radial pair distribution of the adhering microorganisms. With permission from H. J. Busscher, A. T. Poortinga, and R. Bos, *Curr. Microbiol.* **37**, 319–323 (1998).

Polymer and Other Short-Range Interactions

The surfaces of bacteria are covered with a variety of polymers, which differ from one species to another (47). The surfaces of gram-negative bacteria are mainly covered by lipopolysaccharides, surface proteins, or capsules of extracellular polysaccharides, whereas on gram-positive cells, lipoteichoic, teichoic, and teichuronic acids, mycolic acids, capsules of extracellular polysaccharides, and proteins can be present (48). Polymer interactions can be attractive or repulsive (49) and arise when bacterial

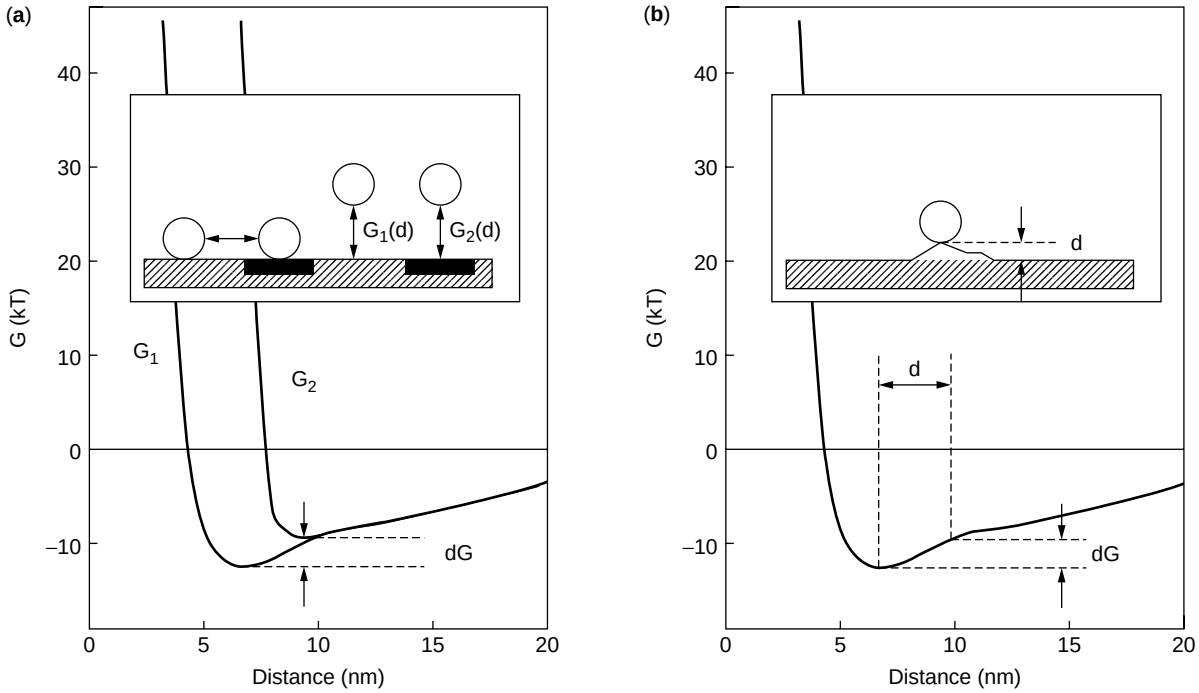

Figure 11. Interaction energies $G(d)$ of a microorganism, according to the extended DLVO theory, as a function of separation distance with different regions on a heterogeneous substratum surface. (**a**) Hydrophobic patches on a hydrophilic surface exert different interaction energies on an approaching microorganism, yielding a lateral interaction between the adhering microorganisms. (**b**) Microorganisms adhering on structural heterogeneities on a substratum experience lateral interaction forces on a macroscopic scale. *NB*. Organisms and heterogeneities are not drawn to scale. With permission from H. J. Busscher, A. T. Poortinga, and R. Bos, *Curr. Microbiol.* **37**, 319–323 (1998).

surface polymers have a high affinity for the solid surface and are long enough to reach and adsorb to the surface (see Fig. 12a). Such polymers may bridge the distance between a substratum surface and the microbial cell body, the latter of which is kept at a larger distance by electrostatic repulsion (30). If polymer interactions are site-specific on the substratum surface, they do not only assist in adhesion but also in immobilization. Excessive polymer production by bacteria may serve to effectively disperse bacteria in suspension and prevent them from adhering to a surface, as shown in Fig. 12b (50,51).

Short-range interactions arise upon direct contact between bacteria and solid substrata. Often these site-specific interactions involve covalent bonds, requiring 40 to 200 kT (52) to break, which is considerably more than, for instance, hydrogen bonds that can be disrupted at the expense of only 4 to 16 kT (53).

Motile Bacteria

Motility of adhering bacteria has been described so far from a physicochemical point of view, but motile bacteria constitute a special class of microorganisms in this respect. Depending on their orientation when approaching a substratum surface, bacteria (i.e., some strains of *Escherichia coli* and *Pseudomonas aeruginosa*) may exhibit a continuous gyratory motion around the pole of the cell or a propeller-like motion (54). Some motile bacteria near or adhering to a surface are able to overcome

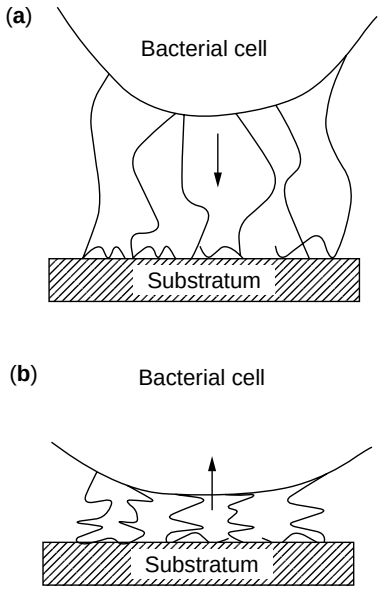

Figure 12. (**a**) Attractive polymer interaction occurs when bacterial polymers have affinity for the solid surface and are long enough to reach and adsorb to the surface. As a consequence, these polymers bridge the distance separating surface and bacterial cell. (**b**) On the other hand, when the bacterial cell produces an excessive amount of polymers, a repulsive polymer interaction occurs, which tends to keep the bacterial cell far from the substratum surface.

the attractive forces at the secondary minimum and swim away (55,56), often becoming reversibly attached at other sites. Gliding and near-surface swimming bacteria are a mobile form of microbial adhesion, as discussed earlier, with the major difference that the lateral interaction forces for mobile behavior now originate from the organism itself.

RETENTION

The balance between repulsive and attractive interactions in microbial adhesion is a delicate one and can be easily disturbed by dynamic factors in the natural microbial habitat or by experimental conditions in the laboratory. External forces other than the interaction forces between adhering microorganisms and a substratum surface frequently cause detachment of all or subpopulations of adhering organisms. Therefore, in natural habitats and experimental systems, microbial retention more readily occurs as a dominant factor in biofilm formation than microbial adhesion. Unfortunately, the distinction is not well made in microbial adhesion research (see section on Materials and methods, which mentions that "surfaces with adhering microorganisms were slightly rinsed to remove loosely adhering organisms.") Instead, a definition of "loosely adhering" should be given, along with a quantitative indication of the rinsing forces, although finally, such papers should be written in terms of microbial retention rather than adhesion. The reluctance of authors to do so greatly contributes to the inability of researchers in the field to compare microbial adhesion data, whereas, in fact, in many natural habitats, most notably the oral cavity, retention is more important than adhesion (57).

Methods to study microbial adhesive interactions (58,59) are summarized in Table 1, together with a critical evaluation of whether adhesion or retention is actually studied. In all the methods involving dipping, rinsing, and washing steps (60), adhering microorganisms are exposed to extremely large detachment forces provoked by the surface tension at the passing liquid-air interface (see Fig. 13). A significant detachment of adhering microorganisms can result from the passage of a liquid-air interface over adhering microorganisms depending on the velocity of the passing liquid-air interface, the surface tension at the liquid-air interface, the number of passing interfaces, hydrophobicity, the charge of the interacting surfaces, and the size of the microorganisms (60–62). As most of the methods summarized in Table 1 actually involve the passage of a liquid-air interface, retention of adhering microorganisms is measured in these assays rather than adhesion.

CONCLUSION

The interaction between microorganisms and substratum surfaces is mediated by different types of interaction forces. A distinction should be made with regard to the type of interaction according to:

1. adhesion, mobile or immobile, of microorganisms on substratum surfaces as a result of a balance between Lifshitz-Van der Waals, electrostatic, Lewis acid-base, and Brownian motion forces;
2. immobilization of adhering microorganisms on a substratum surface due to the lateral interaction forces, polymer bridging, or chemical bonds that keep an adhering microorganism at a certain location on a substratum surface;
3. retention of adhering microorganisms, denoting microorganisms that remain adhering on a substratum surface after application of an external force.

It is important to distinguish between adhesion, immobilization, and retention for a proper interpretation of experimental results. Real-life biofilm formation often

Table 1. Currently Employed Methods to Study Microbial Adhesive Interactions with Solid Substrata

Method	Relevant Details	Retention (R) or Adhesion (A)	References
Filtration	— High shears applied — Risk of bacterial clumping	R	Ofek and Doyle (58), Ofek and coworkers (59), Ellen and Gibson (63)
Differential centrifugation	— Shear forces depend on number of washings and centrifugations	R	Lowry and coworkers (64), Beachy and Ofek (65), Graham (66)
Beads	— No centrifugation needed — Washing steps included	R	Clark and coworkers (67), Wassal and coworkers (68)
Packed beads	— No washing steps included — Controlled mass transport	A	Lytle and coworkers (69), Elimelech (70)
Immobilization of macromolecules	— Washing steps included — Nonspecific adhesion	R	Ofek and coworkers (71)
Slide methods	— Bacterial enumeration after washing steps	R	Liu (72), Wirtanen and coworkers (73)
Critical forces tests	— Fluid-shear forces exerted	R	Taylor (74), Mohandas, and coworkers (75)

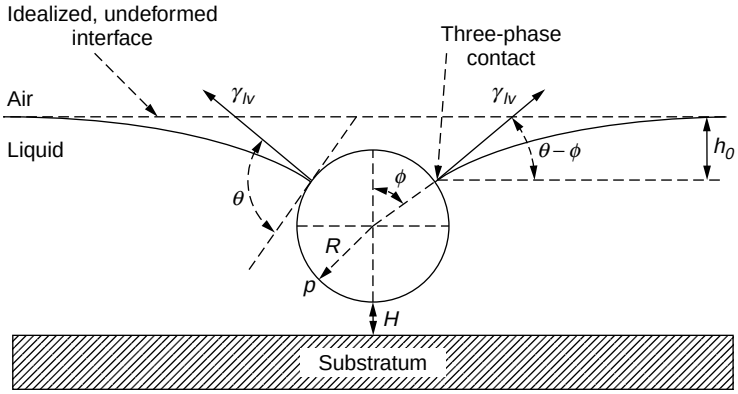

Figure 13. Schematic presentation of the three-phase contact between a liquid-air interface and a micron-sized particle. The surface-tension force can oppose the adhesion force between particle and solid substratum, depending on the three-phase boundary line as expressed by $F_\gamma = 2\pi R_p \gamma_{lv} \sin[\phi(t)] \sin[\theta - \phi(t)]$. With permission from A. F. M. Leenaars and S. B. G. O'Brien, *Philips J. Res.* **44**, 183–209 (1989).

involves adhesion, immobilization, or retention as a dominant contributing factor that should be identified before meaningful laboratory experiments can be set up.

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ADHESION (PRIMARY) OF MICROORGANISMS ONTO SURFACES

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Microbial adhesion and biofilm formation are widespread phenomena that play a crucial role in natural environments as well as in industrial processes. Natural surfaces on which microbial adhesion occurs vary from inanimate materials to living tissues. In aquatic habitats, microorganisms accumulate on other living organisms, suspended particles, rocks, and sediments. Soil particles, often coated by clay minerals, metal oxides, and organic matter, constitute another complex microbial habitat. Biofilm formation has a deleterious effect in many situations (commonly called biofouling): decrease of heat transfer on heat exchangers, increase of pressure drop in pipelines, corrosion enhancement, and source of bacterial contamination (1). Development of biofilms on medical devices often requires their surgical replacement (2). Removal of biofilms using biocides (3) or antibiotics (2) is often a difficult task due to the protective role of extracellular substances. On the other hand, biofilm formation may be beneficial in water and wastewater treatment (4–6), in

bioremediation processes (7,8), in the production of fermented food (9,10), or in establishment of symbiosis on plant roots (11).

The formation of microbial biofilms at the interface between a bare solid surface and a cell suspension may be seen as a succession of various steps, as illustrated in Figure 1 (2,12,13). The solid substratum is first conditioned by adsorption of organic (macro)molecules (proteins, polysaccharides, lipids; humic substances, natural organic matter,...) which diffuse faster than cells and are likely to modify the substratum physicochemical properties. Transport of the microorganisms to the substratum surface will occur by Brownian motion (i.e., diffusion), sedimentation, convection, and possibly also intrinsic motility. During this step, aggregation occurring between planktonic microorganisms (14,15) may considerably affect the "simple vision" of one microorganism approaching a surface. Attachment may occur between single or aggregated microorganisms and the conditioned substratum, which is referred to as the "primary adhesion" step. It is worth noting that coadhesion between planktonic and adhering cells may also be encountered (15). Subsequently, the production of extracellular substances may allow the anchorage of attached bacteria while bacterial growth leads to biofilm development. Later, the detachment of biofilm portions from the substratum may occur as a result of flow shear or chemical treatment (see BIOFILM DETACHMENT). It may also be induced by the enzymatic activity of entrapped cells (16,17).

The aim of this chapter is to provide an overview of (*1*) the various methods available for studying the primary adhesion of microbial cells, and (*2*) the physicochemical approaches, which help to understand and control this process. The importance of considering microbial cells as complex colloidal particles is emphasized. However, the role of specific (key-lock) interactions between the microorganism and a biological substratum is not considered in detail; more comprehensive details on this subject may be found in (18–20).

TERMINOLOGY

It has been proposed to use the term "deposition" to designate the attachment of microbial cells to a surface, and the term "adhesion" to refer to the force necessary to separate the adherents, that is, to express the strength of the adhesive force (21). On the other hand, "adsorption" was proposed to designate immobilization of an organism on a collector surface, and "attachment" to designate the consolidation of the interface between an organism and a collector (22); however, these distinctions are not of universal use. In this article, in order to avoid the confusion between the denomination of a phenomenon (attachment, deposition, adhesion) and the name of a physical quantity characterizing the phenomenon or its result (rate, amount, strength); the terms deposition, attachment, and adhesion are considered as synonymous. Adsorption is sometimes used as a synonym of attachment or deposition; in this chapter, however, adsorption will be reserved for the retention of molecules at a surface.

METHODS TO STUDY MICROBIAL ADHESION

Overview

Generally speaking, two types of approaches are used in experimental microbial cell attachment studies (23). In the first type, adhesion is evaluated in environments of practical significance. To this end, test surfaces are placed in the environment of interest (water delivery system, cooling system, soil, etc.), left for a period of time, retrieved, and the number of attached bacteria is evaluated. Another approach, typical of laboratory studies (24), is more appropriate for investigating mechanisms or establishing guidelines. The aim is typically to determine (*1*) the ability of one or several microorganisms to adhere, (*2*) the influence of particular environmental conditions (ionic strength, pH, substratum nature) on adhesion, and (*3*) the adhesion force between the microorganisms and the substratum. Usually, model materials are used; they may be chosen according to their hydrophobicity (hydrophilic such as glass, metal oxides, minerals; hydrophobic such as nonoxidized polymers), surface electrical properties, roughness, or mode of conditioning. The results obtained may be strongly influenced by the hydrodynamic conditions (turbulent, laminar, resting liquid) and the rinsing step.

Two limiting cases may be distinguished when considering the kinetics of particle attachment. On the one hand, in the bulk transport controlled regime, the surface interactions create no resistance and the kinetics is determined by convection, diffusion, and/or an external force field (sedimentation). On the other hand, in the surface barrier limited regimen, the bulk transport is fast and the resistance is caused by physicochemical interactions between the two surfaces (25). The former case will be mainly considered in this section; the latter case will be mainly treated in the next section.

The experimental systems will be presented here according to the hydrodynamic conditions. Therefore, it is worth recalling the types of flow which may be distinguished on the basis of the Reynolds number, Re,

$$Re = \frac{\ell v_o}{\nu} \tag{1}$$

where v_o is the fluid velocity, ν is the kinematic viscosity (Table 1) and ℓ is a reference length scale (26,27). Regarding the flow around colloidal particles (size ≤ 1 μm), Re is usually much lower than 1. In the absence of particular force fields (gravity, concentration gradients, interfacial interactions), particles with sufficient symmetry follow the fluid streamlines. For higher Re, a laminar flow is obtained, that is, the fluid streamlines are parallel to each other. This is the case of the Poiseuille flow through a circular tube or a parallel-plate channel; the fluid velocity is 0 at the wall, while the velocity gradient, also referred to as the shear rate, is maximal at the wall and 0 at the center. For Re above a critical value, typically 2,100 in pipes, unsteady and chaotic motion occurs, giving a turbulent flow. However, near the wall, no random fluctuation occurs, thereby providing the laminar or viscous sublayer. The concept of boundary layer finds its origin

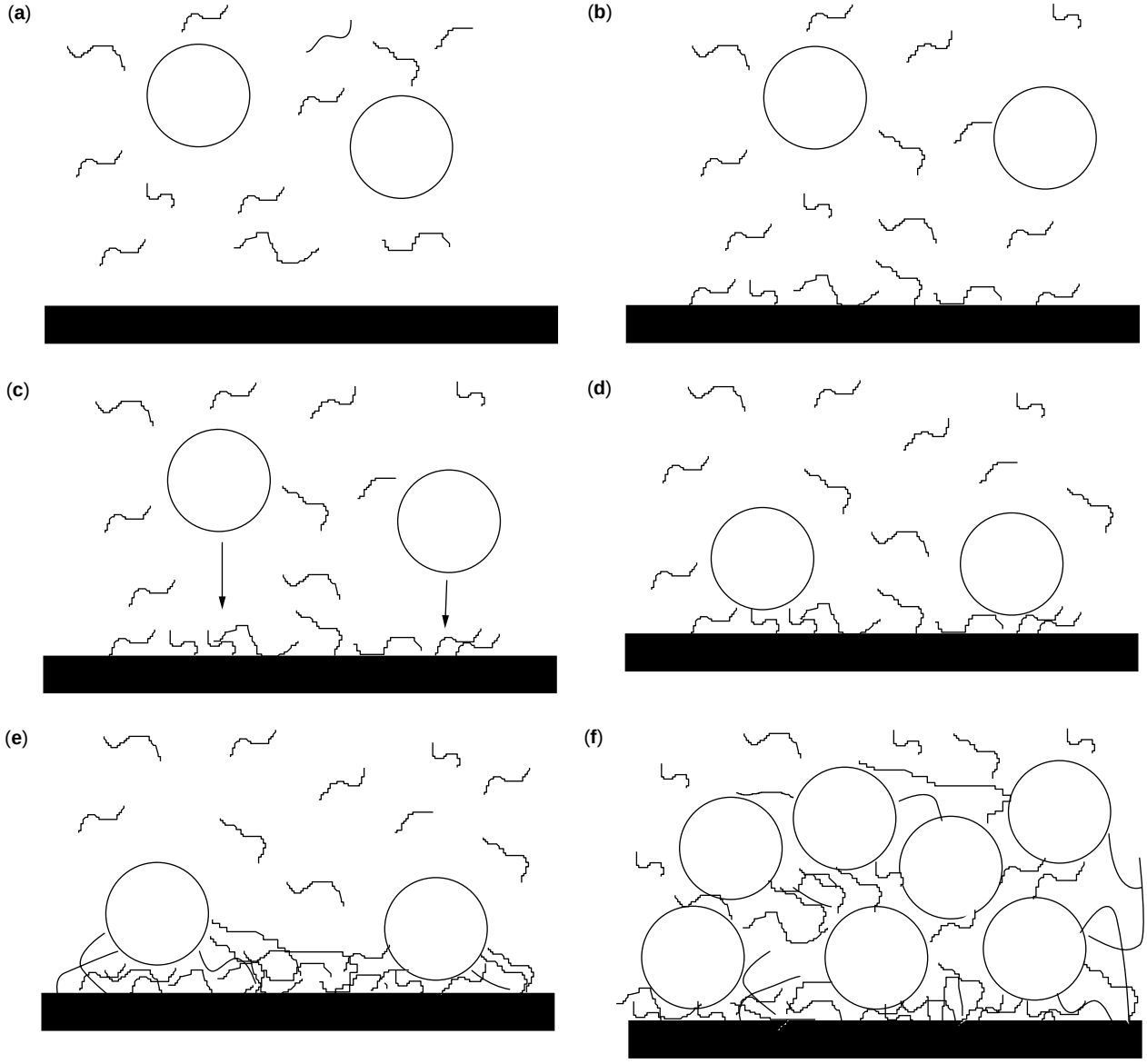

Figure 1. Schematic representation of the various steps involved in the formation of biofilms: (**a**), initial situation; (**b**), substratum conditioning; (**c**), cell transport; (**d**), primary adhesion; (**e**), anchorage; (**f**), growth.

in approximations, which involve a distinction between a fluid behavior near a wall and far from a wall.

Static Systems

In static tests, the microbial suspension remains stationary with respect to the substratum. Consider a quiescent liquid containing dispersed particles; the transport of which is governed by sedimentation and Brownian motion (diffusion). Table 1 presents the physical quantities, which influence the rate of transport according to the two mechanisms (26,27). It gives also indicative values for nonmotile bacteria. The characteristic distance L, that is, the distance along which the major concentration change takes place, may be evaluated as follows: a dense monolayer of bacteria represents about 5×10^7 cells/cm^2; with a suspension concentration of 5×10^8 cells/cm^3, the amount of cells present in a 1 mm layer of the suspension is needed to make a dense monolayer. The ratio of transport by sedimentation with respect to diffusional transport may be evaluated by the Peclet number, *Pe*, also defined in Table 1. For bacteria, *Pe* is about 300 (Table 1), indicating that transport caused by Brownian motion may be neglected compared to gravitational transport. The latter will control the transport to the upper face of a horizontal substratum immersed in a suspension of bacteria, but the cells will not contact the lower face.

A bacterial suspension may also contain macromolecules, which tend to be adsorbed onto the substratum. A deposition test performed using a closed cell with a rectangular section showed that *A. brasilense* cells were attached on the lower wall and not on the upper wall; however a surface analysis showed that the amount of

Table 1. Physical Quantities, with Indicative Values, Which Are Relevant to the Transport of Bacteria by Sedimentation and Diffusion (26,27)

Physical Quantity	Symbol and Definition	Indicative Values
Bacteria equivalent radius	a	0.8 μm
Acceleration of gravity	g	9.81 m/s^2
Viscosity coefficient	η	1.0×10^{-3} Pa s, for water
Kinematic viscosity	$\nu = \eta/\rho_{\text{liquid}}$	1.0×10^{-6} m^2/s, for water
Sedimentation velocity	$v_{\text{sed}} = 2\ g\, a^2 \Delta\rho/9\eta$	0.8 cm/day[a], 10^{-7} m/s
Difference of specific gravity	$\Delta\rho = \rho_{\text{cell}} - \rho_{\text{liquid}}$	0.07 g/cm^3[b]
Diffusion coefficient[c]	$D = kT/6\pi\eta a$	3×10^{-13} m^2/s[d]
Characteristic distance	L	1 mm
Rate of transport by diffusion	$v_{\text{diff}} = D/L$	3×10^{-10} m/s[d]
Particle flux[e]	$J = vC_0$	sediment.: 5×10^3 cells/cm^2 s diffusion: 15 cells/cm^2 s
Peclet number	$Pe = v_{\text{sed}}\ L/D$	300[d]

[a] close to the progression rate of the front of sedimentation measured in water for *Azospirillum brasilense* and *Pseudomonas* strains (non published).
[b] computed from v_{sed}
[c] k = Boltzmann constant, T = temperature (K)
[d] computed using the above data
[e] C_0 = initial bacterial concentration; indicative value of 5×10^8 cells/cm^3

adsorbed proteins was similar for the lower wall after cell detachment, and for the upper wall. This demonstrated that proteins were released by the cells into the solution and adsorbed by the substratum, and were not exclusively brought to the substratum by the cells (29,30).

A characteristic distance of 1 mm is still relevant for the migration of a macromolecule to a surface: consider a macromolecule with a molecular weight of 50 kDa, a specific gravity of 1 g/cm^3 and thus an equivalent radius of 2.7 nm; an adsorbed monomolecular layer represents about 6×10^{-8} mole/m^2 or 3 mg/m^2; this is equivalent to the amount present in a 1 mm thick layer of a solution at a concentration of 3 μg/cm^3. The diffusion coefficient of these macromolecules is about 8×10^{-11} m^2/s. It turns out that the ratio of the rate of sedimentation of bacteria (10^{-7} m/s) over the rate of diffusion of the macromolecules (8×10^{-8} m/s) is on the order of 1. This simple computation shows that, depending on the respective concentrations and properties of macromolecules and bacteria, different situations may occur: most of the bacteria may get in contact with the native substratum surface or with a surface fully modified by adsorption; the substratum surface may also be progressively modified by adsorption during the sedimentation process, which means that the substratum-cell interface will be different for bacteria contacting the substratum surface at different times.

By varying the cell concentration, the duration of the experiment and the height of the suspension, static tests allow the amount of cells brought in contact with the support to vary from a small fraction of a monolayer to a thick sediment. The thickness of the sedimented layer was found to influence the density of adhering yeast cells under conditions of low cell-support affinity; this was attributed to an increased pressure exerted on the bottom layer of cells or to a reorganization leading to the formation of a dense layer of cells in close contact with the substratum (31,32).

In practice, the cells are left to settle on the substratum and the nonattached cells are removed by rinsing. These tests are simple and quick, allowing rapid screening to be performed. However, the definition of adhering cells is strongly dependent on the conditions used for the washing procedure. Several rinsing procedures are used to remove microorganisms that are "unattached" or "loosely bound": successive dipping in the relevant fluid, mild shaking of the container with progressive replacement of the liquid, or washing under flow. All of these procedures may yield nonreproducible results due to the lack of control over the removal force (33). Progressive dilution and mild shaking avoid the passage of an air-water interface, and the possible influence of a water meniscus on the deposited cells. The hydrodynamic conditions may be controlled better by placing the test plates in a device, which ensures a laminar flow (31,34). The parallel-plate flow chambers described later can also be used to investigate bacterial adhesion under static conditions, while controlling the hydrodynamic conditions used for rinsing (29).

Evaluation of the Strength of Adhesion

An overview of earlier methods dealing with the determination of the strength of adhesion of small particles can be found in (35). Among the direct methods, a force is applied in a direction opposite to the adhesive force and the value required to cause cell detachment is measured. The centrifugal method is attractive due to its apparent simplicity; the applied force is given by

$$F_C = V_p(\rho_p - \rho_m)\omega^2 r \qquad (2)$$

where V_p is the particle volume, ρ_p and ρ_m are the densities of the particles and medium respectively, ω is the rotational speed and r is the radius of gyration. However, in the case of microbial cells, this method is strongly limited as a result of the small difference of density

between the cells and the usual types of surrounding liquid medium. Experiments performed with yeast cells adhering to glass in an aqueous medium showed that an acceleration up to 5,700 g did not cause cell detachment (unpublished). Centrifugation in air of a sample tilted with respect to the rotation axis allowed a cell detachment threshold to be determined. However, the interpretation of the data was not clear, because of possible interferences from capillary forces created by the water meniscus at the cell-substratum interface and of drying.

Indirect methods consist of measuring the force required to provoke cell detachment exerted by a liquid flow parallel to the surface (35,36); the force is thus of hydrodynamic origin. The drag force F_H exerted by a laminar liquid flow on a spherical particle of radius a adhering to a wall is given by

$$F_H = 32a^2\tau \tag{3}$$

where the wall shear stress τ is given by

$$\tau = \eta \frac{dv}{dz} \tag{4}$$

where v is the fluid velocity and z the direction perpendicular to the surface. Equations have been developed for the following systems: (i) flow between rotating concentric cylinders (21,35); (ii) flow created between two parallel discs with a narrow spacing, by injection of the fluid at the center of one disc (radial-flow chamber) (36,37); and (iii) flow in a rectangular pipe having a small thickness (38,39). In the latter case, provided a laminar flow is ensured in the pipe,

$$F_H = 96\nu v_{lo}\frac{a^2}{b} \tag{5}$$

where v_{lo} is the average linear flow rate and b is half the pipe thickness. However, the drag force required to detach an adhering cell has a clear meaning only when the cells are isolated and distributed with a low density on the substratum.

Dynamic Tests Under Controlled Hydrodynamic Conditions

Hydrodynamic conditions influence deposition tests in two ways: (i) the liquid flow governs the rate at which the suspended cells make contact with the substratum surface and, consequently, cell deposition; (ii) it also influences the shear stress near the substratum surface, thus the residence time of nonattached cells at a given spot of the surface and the drag force exerted on attached cells.

The transport of dissolved compounds or suspended particles in a moving liquid is governed by two mechanisms, convection and diffusion, the combination of which is called convective diffusion (26). The equations describing the convective diffusion have been solved when the collector is a smooth rotating disc immersed in an otherwise non agitated liquid (25–27). A fluid flow of well-controlled intensity is created by the rotation. At a distance from the disk larger than δ_o, the thickness of the hydrodynamic boundary layer, the liquid moves toward the disk and its velocity is v_z. Within the boundary layer, the radial and tangential velocity components are not 0. If the suspended particles are irreversibly captured when they arrive close enough to the collector surface ("perfect sink" model), the flux of particles to the disk surface J may be considered as being controlled by diffusion through a diffusion boundary layer. The thickness of the latter, δ, is constant over the entire disk surface, neglecting edge effects, and is much smaller than δ_o. Table 2 presents relevant physical quantities for two disk rotation frequencies. It gives also the apparent rate v_p at which the particles are transported to the surface. This is larger than the rate of diffusional transport in static conditions (Table 1), because δ is smaller than the characteristic distance used in the latter case. At a rotation frequency of 1 cycle/s, it becomes close to the sedimentation velocity that should no longer be neglected; note also that δ becomes close to the cell radius. The influence of particle size and density, and of Re is further discussed in (25). The rotating disk method has been used to study deposition of microorganisms associated with dental caries, namely, *Streptococcus mutans* (40), and with infections of biomaterials after blood contact, namely, *Staphylococcus epidermidis* (41).

The radial-flow chamber (36) mentioned earlier has been used under conditions of microbial growth, by pumping the culture fluid at a constant volumetric rate. The linear fluid velocity, and hence the surface shear stress decreases radially across the disc. With a suitable flow rate, a clear zone is found around the inlet, with attached cells beyond a certain distance from the inlet. This "critical radius" gives the critical shear stress below which attachment takes place. The radial-flow chamber was combined with automated videomicroscopy, which allowed the kinetics of bacterial attachment and detachment to be determined (24,42,43).

Deposition of colloidal particles has also been studied using the impinging jet method (25,44,45). The dispersion flows through a circular hole and the fluid impinges on the substratum. If the latter is transparent, the deposition process can be observed under the microscope. A stagnation point flow is created near the substratum surface facing the inlet hole. Mass transfer equations were solved for the stagnation point region, taking into account hydrodynamic, gravitational, and interfacial forces. The variation S of the density of deposited particles, as a function of time, can be approximated by

$$S = \frac{J_0}{\beta_m + \beta_e}[1 - \exp\{-(\beta_m + \beta_e)t\}] \tag{6}$$

where J_0 is the initial flux of particles towards the collector surface, β_m is a coefficient reflecting the surface blocked per particle, and β_e is an escape coefficient depending on the transport conditions near the collector surface and on the particle-support interactions. J_0 can be determined from the initial slope of S versus t; J_0, β_m, and β_e can be analyzed in relation to the characteristics of the system investigated, according to mass transport equations.

A theoretical analysis was made for the particle deposition kinetics onto walls of parallel-plate and

Table 2. Physical Quantities, with Indicative Values, Which are Relevant to the Transport of Bacteria to a Rotating Disc of Radius $r = 10$ cm at Steady State (26)[a]

Physical quantity	Symbol and definition	Indicative values		Units
Rotation frequency	—	10^{-2}	1	cycle/s
Angular velocity	ω	6.3×10^{-2}	6.3	radius/s
Rate of the liquid to the surface at a large distance from the disc	$v_z \approx 0.89\sqrt{\nu\omega}$	2.2×10^{-4}	2.2×10^{-3}	m/s
Thickness of the hydrodynamic boundary layer	$\delta_o \approx 3.6\sqrt{\nu/\omega}$	1.4×10^{-2}	1.4×10^{-3}	m
Thickness of the diffusion boundary layer	$\delta \approx 0.5(D/\nu)^{(1/3)}\delta_o$	4.7×10^{-5}	4.7×10^{-6}	m
Rate of transport of the particle to the surface	$v_p = J/C_o = D/\delta$	6.4×10^{-9}	6.4×10^{-8}	m/s

[a] ν taken for water, D and C_o taken for bacteria, as given in Table 1.

cylindrical channels through which a dilute suspension of spherical particles moves in laminar flow (46). Transport equations were formulated by taking into account surface forces as well as external forces. The parallel-plate flow chamber has been broadly used to investigate microbial adhesion (12,47). Therefore, the substratum of interest, which must be transparent, constitutes the main chamber walls and the cell deposition is observed directly under the optical microscope. The adhesion kinetics may be analyzed by using relations similar to Eq. (6) (48).

A flexible and frequently used device to study microbial adhesion is the modified Robbins device (49–51). Aseptically removable studs contain a dish of the substratum to be examined, which is exposed to the fluid passing through a pipe. This is a convenient method to compare different materials in the same conditions.

Table 3 shows a comparison of different systems regarding the possibility of examining a large substratum area, the possibility of direct observation, which facilitates the quantification of deposition kinetics, and the possibility of comparing different materials in the same run. Other systems consist of exposing several substratum samples in a flow chamber or on a rotating cylinder. They allow a comparison of different substratum samples to be made in the same conditions, but provide a less accurate control of the hydrodynamic conditions. The relation to biofilm reactors can be made using reference (52).

Batch Systems and Packed Beds

With batch systems, the amount of adhering cells is often determined by the difference between the initial and final concentration of microorganisms in the liquid phase. A direct quantification of adhering bacteria can be achieved by using cell labelling techniques, with fluorescent or radioactive probes (24,53,54).

In packed-bed systems, which are relevant to soil or to industrial bioreactors (55), the retention of bacterial cells, assumed to be caused only by attachment, is deduced from the concentration of cells remaining in the effluent and recording breakthrough curves (56–59). A disadvantage is that direct observation and localization of adhering microorganisms is not possible. In particular, microbial cells may be held in the bed by sedimentation in zones of low liquid velocity or by a mechanical retention of aggregates, which may erroneously be considered as attachment.

Rijnaarts and coworkers (60) compared bacterial attachment performed on glass and teflon surfaces, using packed-beds and batch systems, with a large collection of *Pseudomonas* and coryneform bacteria selected according to their environmental and biotechnological relevance. They showed that the transport of microbial cells from the bulk liquid to the substratum surface was faster in packed-bed columns (transport ensured by diffusion and convection) compared with batch tests (transport dominated by diffusion). The two methods were consistent for a

Table 3. Comparison of Different Methods to Study Microbial Deposition

Method	Large area	Direct observation*	Simultaneous study of different samples
Rotating disc	yes	no	no
Radial-flow chamber	yes	yes	no
Impinging jet	no	yes	no
Parallel-plate flow cell	yes	yes	no
Modified Robbins device	no	no	yes

*provided the substratum is transparent.

certain number of strain-substratum combinations. Deviations observed for other combinations could be attributed to method-dependent effects resulting from specific cell characteristics such as ability to aggregate, presence of capsular polymers, large cell sizes or detachment by an air-liquid meniscus.

PHYSICOCHEMICAL ASPECTS OF PRIMARY ADHESION

In the course of the approach of a substratum by a microbial cell, physicochemical aspects become important when the separation distance is smaller than the cell size. Depending on the nature of the surfaces involved, they may be the factor controlling the rate of attachment and even determining the possibility of attachment. Former reviews can be found in (21,61–63).

DLVO (Derjaguin, Landau, Verwey, Overbeek) Theory

Theoretical Frame. According to DLVO theory (28,64), the free enthalpy of interaction, G_T, when two surfaces approach each other, may be accounted for by the sum of two contributions, corresponding respectively to electrostatic and van der Waals interactions

$$G_T = G_E + G_V \tag{7}$$

The electrostatic contribution, G_E, is caused by the overlap of electrical diffuse double layers. Remember that two regions can be identified near a charged surface of potential ψ_0, as illustrated by Figure 2 (65). In the first region, called the Stern layer, the ions, chemically adsorbed or retained by localized electrostatic attraction, are in close contact with the surface and the potential is considered to fall linearly as a function of the distance from the surface. In the second region, called the diffuse double layer, the potential falls exponentially as a function of the distance, caused by the balance between electrical interactions of ions with the surface and molecular motion. The electrical potential at the inner limit of the diffuse double layer is called ψ_d. The zeta potential, ζ, is the potential at the plane of shear between the surface and the solution.

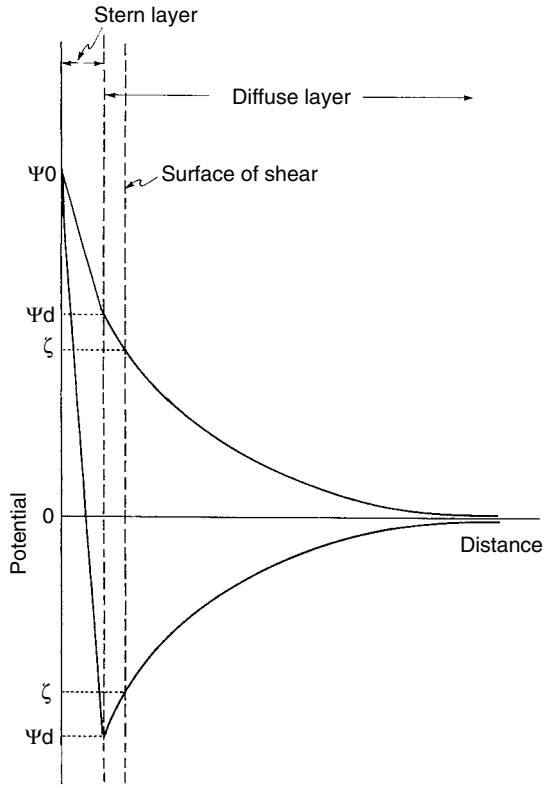

Figure 2. Potential decay curves for an electrical double layer associated with a surface of potential Ψ_0. The upper and lower curves arise, respectively, from weak and strong adsorption of counterions in the Stern layer.

Considering a sphere (cell) and a plate (substratum), G_E is expressed as follows for small values of the relevant electric potential ($\psi < 50$ mV) (62,66,67)

$$G_E = \pi\varepsilon a(\psi_M^2 + \psi_S^2)\left[\frac{2\psi_M\psi_S}{\psi_M^2 + \psi_S^2}\ln\left(\frac{1+\exp(-\kappa h)}{1-\exp(-\kappa h)}\right) + \ln(1 - exp(-2\kappa h))\right] \tag{8}$$

where ε is the permittivity of the suspending fluid; a is the cell radius; ψ_M and ψ_S are the values of ψ_d near the cell and the substratum surfaces, respectively; κ is the reciprocal of the Debye length; h is the separation distance between the interacting surfaces (28,68). For computing G_E, the value taken for ψ_d is usually the zeta potential (68). κ determines the rate of the variation of the electrical potential as a function of the distance within the diffuse double layer

$$\kappa = [(2{,}000Ie^2N)/(\varepsilon kT)]^{(1/2)} = 3.29 \times 10^9(I)^{(1/2)}(\text{at } 25\,^\circ\text{C}) \tag{9}$$

where I is the ionic strength of the bulk solution, e is the electron charge, N is the Avogadro number, k is the Boltzmann constant, and T is the temperature. At ionic strengths of 10^{-1}, 10^{-3}, and 10^{-5} M, κ^{-1} is close to 1, 10 and 100 nm, respectively. Note that the ionic strength is commonly in the range of 10^{-2} to 10^{-3} M for natural freshwater and near 10^{-1} M for most microbial culture media. Strictly speaking, h should represent the distance between the inner limits of the two diffuse layers facing each other. Considering that it is the distance between the two surfaces neglects the thickness of the Stern layer. For this reason and because of the uncertainties concerning the definition of ψ_d, computing G_E for distances shorter than 1 nm is meaningless.

The contribution of van der Waals interactions varies with the separation distance according to

$$G_V = -\frac{A}{6}\left[\frac{2a(h+a)}{h(h+2a)} - \ln\left(\frac{h+2a}{h}\right)\right] \tag{10}$$

which may be simplified for very small separation distance ($h \ll a$), giving

$$G_V = -\frac{Aa}{6h} \tag{11}$$

where A is the Hamaker constant (62,66,68). This is mainly determined by dispersion forces (surface-surface and surface-liquid interactions) and thus by the polarizability of the surface constituents. For bacterial cells suspended in water, A is estimated to be about 0.035, 1.54 and 3.58 kT for Teflon, glass, and iron oxide substrata, respectively ($kT = 4.1 \times 10^{-21}$ J/mol) (58,64,69).

Figure 3 presents the computed G_T vs h curves for microbial cells, considered as spherical particles of 1 μm radius, A = 1.54 kT, ψ_d of both cells and substrata = −20 mV, and $\kappa^{-1} = 10$ nm; the respective influence of variation of A, ψ_d, and κ^{-1} is also illustrated. The attractive contribution of van der Waals forces is shown in Figure 3 (center; $\Psi_d = 0$ mV). At physiological pH, most of biological and substratum surfaces have a negatively charged surface. As a consequence, an overall repulsion appears at a distance that may vary from 10 to hundreds of nanometers, depending on ionic strength (Fig. 3). The repulsion energy must be put in perspective, considering the kinetic energy associated with Brownian motion, equal to 1.5 kT. Figure 3 (left, center) shows that the chemical composition of the surfaces involved acts much more through the ionized functions, which determine the electrical potentials, than via the van der Waals forces. An increase of ionic strength from 10^{-5} to 10^{-1} M (reducing the Debye length from 100 to 1 nm) reduces considerably the magnitude of the potential energy barrier (Fig. 3, right). At sufficiently high ionic strength, the variations of G_E and G_V as a function of the distance bring about a secondary minimum, which may keep the cell near the substratum, although not in close contact. As illustrated in Figure 3 (center), a decrease of surface potential may favor microbial attachment by lowering the magnitude of the potential energy barrier. Such variation of surface potential can be encountered when comparing different bacterial strains, when comparing different substrata or when changing the pH of the surrounding solution.

Experimental data obtained during the last decades are in good agreement with trends predicted by DLVO theory. A more negative zeta potential of biotechnologically relevant microorganisms (70) and of soil bacteria (71), both hydrophilic, was shown to be unfavorable to attachment. Electrostatic repulsion may be reduced in different ways: (i) by increasing the ionic strength, (ii) by bringing the pH to a value closer to the cell isoelectric point, or (iii) by modifying the surfaces.

Surface Modification. The role of electrostatic interactions is illustrated by the influence of substratum modifications that confer a positive character to the surface. Figure 4 (left) presents a micrograph of a glass plate which had been partially immersed for one hour in a solution of 1.8 mM $Fe(NO_3)_3$ brought at pH 4, washed with distilled water and used as substratum for a static adhesion test of *Saccharomyces cerevisiae* suspended in water (unpublished). The cells did not attach to untreated glass but formed a denser layer on the modified substratum. Hydrolysis of ferric ions produces ferric hydroxide particles of about 10 nm size, which adsorb to negatively charged surfaces; this provokes a charge reversal that allows adhesion of a dense layer of yeast cells (72).

Figure 4 (right) presents data from static adhesion tests performed with *S. cerevisiae* and a glass substratum modified by particles of hydrous alumina with a 0.25 μm

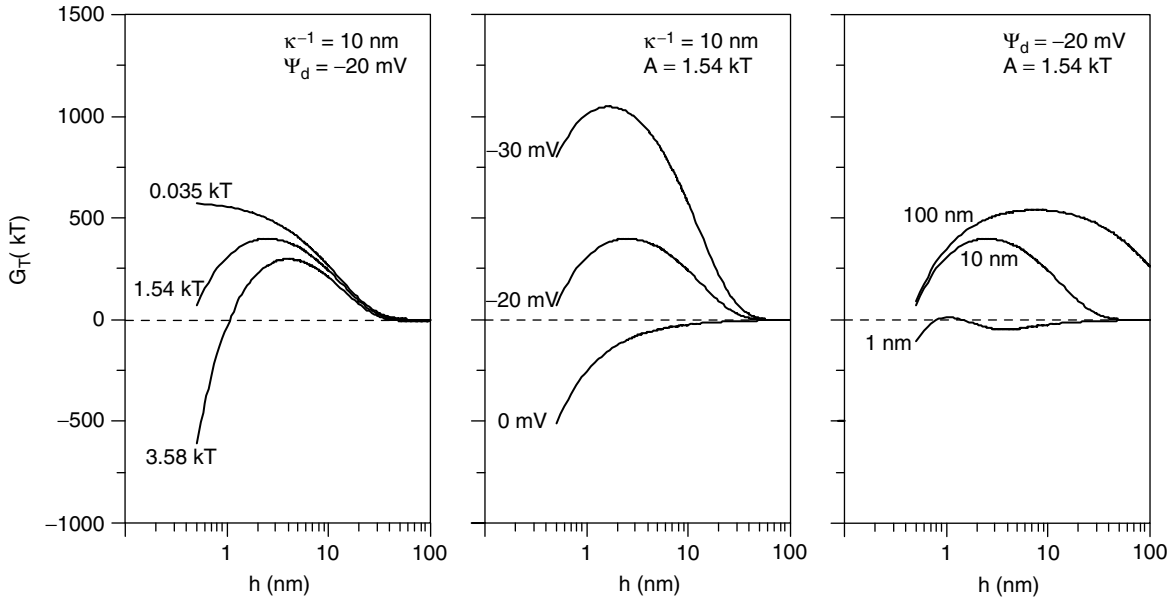

Figure 3. Variation of the free enthalpy of interaction as a function of the distance (h) between a sphere of 1 μm radius and a plane substratum, according to DLVO theory. Middle curve: Hamaker constant A = 1.54 kT; potential of the diffuse double layer $\Psi_d = -20$ mV; Debye length $\kappa^{-1} = 10$ nm. Left: $\Psi_d = -20$ mV, $\kappa^{-1} = 10$ nm, A varying from 0.035 to 3.58 kT; center: A = 1.54 kT, $\kappa^{-1} = 10$ nm, Ψ_d varying from 0 to −30 mV; right: A = 1.54 kT, $\Psi_d = -20$ mV, κ^{-1} varying from 1 to 100 nm.

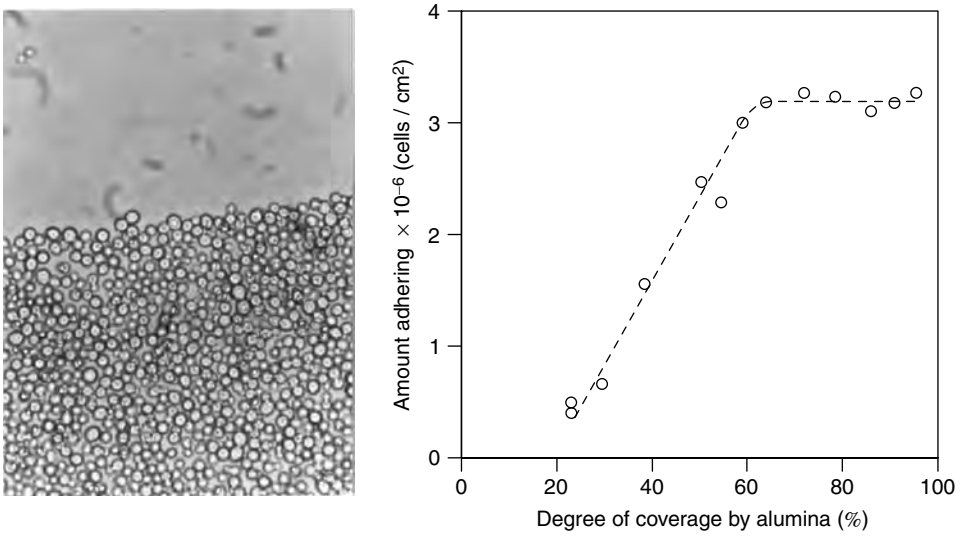

Figure 4. Left, micrograph of a glass slide partially immersed in a $Fe(NO_3)_3$ solution and subsequently submitted to a static adhesion test of *S. cerevisiae*. Right, variation of the amount of *S. cerevisiae* attached to a glass substratum as a function of the degree of coverage with hydrous alumina particles.

diameter and an isoelectric point at pH 9.3 (unpublished). Different surface coverages of the substratum surface with alumina particles were obtained by controlling the alumina concentration and were quantified by redissolution of the coating and chemical analysis. Below 20% coverage, the yeast cells were distributed in patches on the glass support. The amount of adhering cells increased with the degree of coverage by alumina up to 65% coverage, in which case it reached the amount typical of a densely packed cell layer.

Table 4 presents an overview of various systems in which treatments have been applied either to the cells or the substrata to promote primary microbial adhesion by decreasing electrostatic repulsion or achieving electrostatic attraction. These treatments include the use of positively charged colloidal particles (hematite, Fe_2O_3; hydrous alumina, $Al(OH)_3$), of cations (Fe^{3+}, Al^{3+}) and of polycations either adsorbed (chitosan, diethyl aminoethyl-dextran, polyethyleneimine) or grafted (aminosilane); further details may be found in (73). One may argue that these treatments may affect cell physiology. However, for various microorganisms, it was shown that the adhering cells retained their metabolic activity (34,74,75) or their germinating and growing capabilities (76).

It must be kept in mind that, when immersed in a solution, the positively charged substrata may tend to adsorb multivalent or organic anions, which will reduce or eliminate the effect of pretreatment. This can be examined by measuring the electrokinetic properties of the cells and of the substratum (34,65,72,85).

Influence of pH and Ionic Strength. The effect of ionic strength on adhesion may be easily understood from Figure 3 (right): the decrease of Debye length with increasing ionic strength causes a lowering of the potential barrier, which has to be passed by the cells in order to attach. In studies of the transport of soil bacteria through sand columns, an increase of ionic strength decreased the recovery of cells in the effluent, which may be attributed to an increase of cell attachment on sand particles (56,86). In similar experiments performed with negatively charged glass and Teflon beads, the retention of several negatively charged *Pseudomonas* and coryneform bacteria was enhanced by increasing ionic strength (57,59). Similar effects were reported for the attachment of these microorganisms to glass and Teflon plates (87), of several food-borne pathogens to stainless steel (88,89), of biomaterial pathogens to glass and to siliconized glass (50). The opposite was observed with a positively charged bacterium (57).

The effect of electrolytes may sometimes be more complex. Deposition of a *Pseudomonas* strain studied in sand columns was more efficient in solutions containing Mg^{2+} as the dominant cation compared with solutions of identical ionic strengths but containing Na^+. The authors showed, by electrophoretic measurements, that this could be explained by adsorption of Mg^{2+} within the Stern layer, reducing the zeta potential (90). Williams and Fletcher (91) showed that, although the attachment of *Pseudomonas fluorescens* to surface-treated polystyrene (water contact angle of 66°) was increased with a rise of ionic strength, the inverse effect was observed on native polystyrene (water contact angle of 90°). This apparent contradiction may be explained by the respective influence of electrostatic interactions (cfr. DLVO theory), which would dominate in the case of the more hydrophilic surface-treated polystyrene, and of other interactions (see below). The attachment of *Aureobasidium pullulans*, a fungus associated with the deterioration of polyvinyl chloride and painted wood surfaces was maximum at intermediate ionic strength (92). The explanation proposed by the authors was that the electrolyte inhibited attachment, either by screening short-range electrostatic attraction between oppositely charged groups, or by modifying the conformation of cell surface molecules.

In many instances, the isoelectric points of both cells and substratum are low (in the range of 1 to 4) and the zeta potential will generally become less negative when decreasing the pH, for a given ionic strength. Accordingly, the potential barrier preventing cell attachment will

Table 4. Immobilization of Microorganisms by Adhesion Through Modification of the Surface Charge of the Cells or of the Substratum

Microorganism	Substratum	Treatment of* Cells	Carrier	References
Saccharomyces cerevisiae	glass, polycarbonate	Al^{3+}, Fe^{3+}	—	(34,74)
			Fe_2O_3, $Al(OH)_3$	(74)
	glass, metals, organic polymers	—	Fe^{3+}	(70,72)
Kluyveromyces lactis, Klebsiella oxytoca	glass	—	chitosan	(77,78)
Arthrobacter simplex	glass	Al^{3+}	—	(75)
		—	Al $(OH)_3$	(75)
Acetobacter aceti	glass, mica, organic polymers	—	Fe^{3+}	(70,79)
Corynebacterium glutamicum	glass	—	DEAE-dextran, aminosilane	(80)
Xanthomonas campestris	glass	Al^{3+}	—	[c]
		—	Al^{3+}, Al $(OH)_3$	[c]
Escherichia coli	glass	Al^{3+}	Al^{3+}	[c]
Bacillus licheniformis	glass, organic polymer	—	Fe^{3+}, chitosan, DEAE-dextran, aminosilane	[c]
Syntrophomonas wolfei	glass	—	Fe^{3+}	(81)
Acetobacter	cotton cloth	PEI[a]	PEI	(82)
Lactobacillus casei	glass	—	PEI	(83)
Phanerochaete chrysosporium	glass, organic polymer	—	Fe^{3+}, PEI, PAA[b]	(76)
Baker's yeast	glass	PEI	PEI	(84)

*Al^{3+}, Fe^{3+}, chitosan, PEI, PAA, and DEAE-dextran refer to treatment by solutions of the indicated substance; Fe_2O_3 and Al $(OH)_3$ refer to coating by colloidal particles; aminosilane designates a polymer coating.
[a] polyethyleneimine.
[b] polyacrylamide.
[c] unpublished.

decrease or will be suppressed (Fig. 3, center). The density of attached cells has indeed been shown to increase when the pH of the suspending medium was brought near the isoelectric point. This was observed for *Moniliella pollinis* (a yeast — like fungus) (70), *A. pullulans* (92), and *A. aceti* (79).

Besides cell-substratum interactions, cell–cell interactions may be affected by the pH and the ionic strength of the cell suspension. In this context, an increase of ionic strength was shown to become unfavorable to attachment because of the formation of aggregates of *P. chrysosporium* conidiospores, the latter presenting higher susceptibility with respect to shear during rinsing (76). Also, flocculation of *M. pollinis* was found to compete with adhesion when decreasing the pH towards the cell isoelectric point (70).

Balance of Interfacial Free Energies

Conceptual Frame. This approach considers the adhesion process as the creation of a new microorganism-substratum (MS) interface, and the destruction of two preexisting interfaces, that is, the microorganism-liquid (ML) and substratum-liquid (SL) interfaces. Thus, this involves a molecular contact between the two adhering surfaces. The tendency of the surfaces to associate is expressed as a balance of the relevant interfacial free energies (γ)

$$\Delta G_{adh} = \gamma_{MS} - \gamma_{ML} - \gamma_{SL} \tag{12}$$

The interfacial free energy is the sum of several terms, each of which corresponds to different interactions: transient dipole-induced dipole interactions or dispersion forces (London), permanent dipole-permanent dipole interactions (Keesom), permanent dipole-induced dipole interactions (Debye), hydrogen bonding and ionic bonding (93). Like all systems in nature, the system made by these interacting surfaces will strive to reach a state of minimal free energy. Therefore, microbial attachment will be favored when ΔG_{adh} is negative.

Surface free energies are deduced by using the Young equation

$$\gamma_{LV} \cos\theta = \gamma_{XV} - \gamma_{XL} \tag{13}$$

where X stands for solid (either substratum, X = S, or microorganism, X = M) and V for the saturated vapor phase; θ is the contact angle measured on a lawn of microorganisms or on the substratum; γ_{LV} is the surface tension of liquid L. In this relation, two parameters can be experimentally assessed (θ and γ_{LV}). The other two parameters can be tentatively deduced using equations which relate γ_{XL} to γ_{XV} and γ_{LV}. Similar equations relate γ_{MS} to γ_{ML} and γ_{SL}, allowing ΔG_{adh} to be computed.

Several approaches have been used to establish these equations (32,94–96). One key point among the diverging hypotheses is whether γ_{XV} may be approximated by γ_X, the surface free energy of X under vacuum.

One approach assumes an Equation of State to relate the three interfacial free energies involved in the Young equation (97). It allows γ_X to be calculated from contact angles measured with one liquid. According to Spelt and coworkers (98), apolar and polar liquids of the same surface tension should give the same θ value for a given solid; however, conflicting results are reported regarding this aspect (99). According to this approach, if γ_{LV} is lower than γ_{MV}, ΔG_{adh} decreases with increasing γ_{SV}, predicting increasing bacterial adhesion with increasing surface tension of substrata. On the other hand, when γ_{LV} is higher than γ_{MV}, the opposite trend is observed (100).

In another set of approaches one assumes the additive contribution of dispersion and polar surface free energy components (γ^d and γ^p, respectively) in the total surface free energy ($\gamma = \gamma^d + \gamma^p$) (101). While γ^d is determined by London dispersion forces, γ^p comprises hydrogen bonding, Keesom, and Debye interactions. Moreover, it is considered that the dispersion and polar contributions of the interfacial energy γ_{XY} (where X and Y stand for M, S, or L) combine pairwise [$\gamma^d_{XY} = f\left(\gamma^d_X, \gamma^d_Y\right); \gamma^p_{XY} = f\left(\gamma^p_X, \gamma^p_Y\right)$]. In this frame, the methods differ according to whether the pairwise combination involves a harmonic- or a geometric-mean equation (102), and whether the vapor adsorption by the solids is neglected or not (103). The most frequently used approach, the Owens-Wendt method, is based on the geometric-mean equation and neglects the influence of vapor adsorption; it involves the use of two liquids, usually α-bromonaphthalene (apolar, $\gamma_L = \gamma^d_L$) and water (highly polar, $\gamma_L = \gamma^d_L + \gamma^p_L$). The γ^d_L value of water is determined from the water contact angle on a solid allowing only London dispersion forces (polyolefin). The dispersion component does not vary much with the nature of the substratum considered. As a consequence, if γ^p_M is lower than γ^p_L, adhesion will be favored if γ^p_S decreases (104). This fits the common understanding of hydrophobic bonding. A variant considers that γ_{XV} is different from γ_X, the difference being due to vapor adsorption by the solid (103).

Another type of approach proposed by van Oss (95) takes into account not only the polar character but also its electron-donor or electron-acceptor nature. It emphasizes that nonpolar interactions result from all three types of van der Waals interactions, lumped together in Lifshitz theory, giving γ^{LW} (LW for Lifshitz-van der Waals). Further, the polar interactions are designated as Lewis acid-base (γ^{AB}), resulting from the combination of electron-donor (γ^-) and electron-acceptor (γ^+) contributions. This method involves the use of at least three different liquids, such as water and formamide (two polar liquids), and α-bromonaphthalene or diiodomethane (assumed to be apolar).

Experimental Data. The validity of approaches on the basis of a balance of interfacial energies for predicting microbial adhesion has been tested. The adhesion of five bacteria (two strains of *E. coli*, *Staphylococcus aureus*, *S. epidermidis*, and *Listeria monocytogenes*), having a surface tension (γ_{MV}) lower than that of water (γ_{LV}), was studied on different solid substrata presenting a large range of surface tensions (γ_{SV}): as expected from the Equation of State approach, the adhesion density decreased with increasing γ_{SV}. Moreover, when dimethyl sulfoxide was added to water, the inverse correlation was observed when the added amount was sufficient to decrease the value of γ_{LV} below that of γ_{BV} (100). Similar results were reported by Fletcher and Pringle for *P. fluorescens* (105). More recently, Wang and coworkers (41) found that interfacial free energies, assessed by the Equation of State, are strong driving forces for adhesion of *S. epidermidis* to a large range of blood-contacting biomedical materials.

The Owens & Wendt approach was used to predict the adhesion of three bacteria encountered in implant infections (*E. coli*, *Pseudomonas aeruginosa* and *S. epidermidis*) to a large selection of orthopedic implant polymers (106). The comparison between computed ΔG_{adh} and experimental results revealed the following features: (*1*) as predicted by the negative values of ΔG_{adh}, significant adhesion was always detected whatever the microorganism and the substratum; (*2*) the least negative value of ΔG_{adh} obtained for *S. epidermidis* was related to a lower adhesion density; (*3*) when comparing the polymers, an inverse correlation between attached cell density and ΔG_{adh} was expected for the three bacteria, whereas it was observed only for *P. aeruginosa*.

Predictions based on the thermodynamic approach are not always in agreement with experimental data. According to the Owens-Wendt approach, a positive correlation between attached cell density and γ_S indicates that the polar component of the bacterial surface free energy M must be higher than γ^p_L (104). Nevertheless, the attachment of the teeth-colonizing *Streptococcus sanguis* bacteria ($\gamma^p_M = 52$ mJ/m^2) in phosphate buffered saline ($\gamma^p_L = 48$ mJ/m^2) was decreased with increasing γ_S (107). Adhesion of *L. monocytogenes* to relevant industrial surfaces (polypropylene, rubber, glass, and stainless steel) could not be correlated to the predictions when using the van Oss approach (108). Actually, the degree of success of the prediction of the adhesion of *Streptococcus thermophilus* and *Leuconostoc mesenteroides*, both at the origin of contamination in dairy processing, depended greatly on which thermodynamic approach was used to convert measured contact angles into surface free energies (94).

A more global but simpler approach is to consider the influence of surface hydrophobicity, as assessed from water contact angle measurements. The direct correlation between hydrophobicity, determined either on cell or substratum surface, and microbial adhesion was demonstrated in several studies. A study of eight strains of *Coryneform* and four strains of *Pseudomonas* showed denser attachment and stronger adhesion with increasing surface hydrophobicity of both bacteria and substrata, the latter being glass, polystyrene, and Teflon (58). The deteriogenic fungus *A. pullulans* adhered in higher amount on substrata of higher hydrophobicity (92). The incorporation of a biocide into polyvinylchloride provoked a reduced attachment of *P. fluorescens*, isolated from biofilm

developed on a swimming pool liner; this was partially attributed to a reduction of polymer hydrophobicity (109). The attachment of soil bacteria to sulfonated polystyrene, a hydrophobic substratum, was directly correlated with their water contact angle values, which were between 20 and 70° depending on the microorganism. It may be noted that, while electrostatic interactions were always repulsive, the effect of cell surface charge was visible only for more hydrophilic strains. Furthermore, the attachment of the same microorganisms to glass, a hydrophilic substratum, was shown to be controlled by electrostatic interactions, the cell surface hydrophobicity having only a small effect (71,110,111). Correspondingly, the attachment of hydrophilic microorganisms was found to be governed by electrostatic interactions (69). While these results demonstrate that water contact angles point to the role of hydrophobicity in the attachment mechanisms, they also illustrate that attachment results from a combination of hydrophobic and electrostatic interactions.

Limitations of Classical Physicochemical Approaches

Although the DLVO theory and the approach based on a balance of interfacial free energies have provided valuable insight into the mechanism of microbial adhesion, as illustrated earlier, they suffer from several limitations. In the DLVO theory, (i) the interacting surfaces are assumed to be chemically homogeneous, smooth, rigid, and nonpenetrable by ions; (ii) only long-range interactions, that is, electrostatic and van der Waals interactions, are considered, neglecting short-range interactions like hydrogen bonding. In the approach based on the balance of interfacial free energies, (i) the surfaces are considered as homogeneous and in molecular contact; (ii) the system is considered to be at equilibrium; (iii) the interfacial free energy computations rely on theoretical frames that lack accuracy; (iv) electrostatic interactions between approaching surfaces are not taken into account because the surface energies are determined from contact angles of liquids.

The extended DLVO theory includes an additional term G_{AB}, compared to classical DLVO theory, in order to account for short-range "acid-base" interactions (95). According to van Oss, these acid-base interactions form the basis of the hydrophobic or hydrophilic interactions. It is important to note that these acid-base interactions are short-range, that is, they become significant at small separation distance (5 to 10 nm) (12).

While examining the relevance of the extended DLVO theory, Bellon-Fontaine and coworkers (112) developed a partitioning method, called microbial adhesion to solvents (MATS), which is based on comparing microbial cell affinity to a polar solvent (electron acceptor or electron donor) and to a nonpolar solvent. The following pairs of solvents were used: (*1*) chloroform (electron acceptor) and hexadecane (nonpolar); (*2*) ethyl acetate (electron donor) and decane (nonpolar). Due to the similar Lifshitz-van der Waals components of the surface tension in each pair of solvents, differences between the results obtained with chloroform and hexadecane, on the one hand, and between ethyl acetate and decane, on the other hand, would indicate the electron-donor and electron-acceptor character of the bacterial surface, respectively. Recently, the adhesion behavior of *L. monocytogenes* to stainless steel was examined in the light of this method (89). The parameters γ^{LW}, γ^{+}, γ^{-} were assessed by the MATS method for bacteria and by the Van Oss approach for stainless steel: both surfaces had strong electron-donor and weak electron-acceptor characteristics. Nevertheless, a correlation between adhesion results to stainless steel and cell affinity for ethyl acetate was observed, indicating that Lewis acid-base interactions play an important role in the adhesion mechanism. It may be noted that this approach is equally open to criticism. Indeed, MATS is very sensitive to the surface area developed by the solvent droplets created during mixing of the two liquid phases, which can depend on the mixing conditions (temperature, type of mixing vessel ...). Moreover, an emulsion is frequently produced and the cells tend to accumulate at the interface between the two solvents. As a consequence there is no simple partition of the cells between two defined liquid phases and the measured decrease of the cell concentration in one liquid phase may be misleading. Moreover, the cells may have an amphiphilic character. Finally, the usage of organic solvents was reported to affect the bacterial integrity (113).

COMPLEXITY OF MICROORGANISM — SUBSTRATUM INTERFACES

This section is devoted to particular interfacial aspects, which may influence markedly the primary bacterial adhesion. Some features have already been pointed out as limitations for the classical physicochemical approaches.

Surface Roughness

Contrary to frequent assumptions made to model the cell-substratum interactions, cell and substratum surfaces are not atomically smooth. On the one hand, the presence of morphological heterogeneities on the substratum (roughness) may influence the biofilm formation. With regard to initial adhesion, a few reports have indicated that roughness has a minor effect. The adhesion of *S. thermophilus* and *L. monocytogenes* to stainless steel samples covering a range of finishes, representative of food industry equipment, did not vary significantly according to substratum roughness (88,114). In the same way, the density of *P. aeruginosa*, *E. coli*, and *S. epidermidis* attached on cast biomedical films was similar to extruded films, the latter being rougher than the former (106). However, surface roughness at the micrometer scale appears to be favorable by protecting the cells against shear stress or antimicrobial substances (115,116). Moreover, in conditions where the adsorption of a conditioning film plays an important role in the adhesion process, one may anticipate that substratum roughness at the nanometer scale will affect cell adhesion via changes in the organization of the adsorbed layer. It has indeed been shown that substratum roughness may affect the adsorption of macromolecules, such as proteins, and in particular, the supramolecular organization of the adsorbed layer (117).

On the other hand, microbial cells may bear surface appendages such as flagella, fimbriae, and fibrils (67). A relationship between the presence of flagella and cell behavior was pointed out for attachment of *P. aeruginosa* (colonizing contact lenses and catheters) to polyvinylchloride (118) and attachment of *P. fluorescens* in sand columns (119); this was made by comparing the behavior of a flagellated wild-strain and a nonflagellated mutant. However, interpretation of the data is delicate since the presence of flagella may affect both the transport step and the physicochemical interactions. To separate these effects, the attachment of the food-borne pathogen *L. monocytogenes* to stainless steel was studied by comparing the behavior of the wild-type strain possessing an inactive flagellum and a nonflagellated mutant: a higher adhesion was observed when the flagellum was present (120). The presence of a particular protein on fimbriae was shown to be necessary for the adhesion of the pathogen *S. epidermidis*, of concern in intravascular devices, to polystyrene spheres (53). Finally, the presence of fibrillar structures on the surfaces of *Streptococcus* strains has been linked to their ability to adhere to saliva-coated hydroxyapatite (121), to host surfaces (122), and to fibronectin-conditioned microwells (123).

The modes of action of cell surface appendages to allow bacterial attachment are still unclear. Firstly, cell surface appendages may influence bacterial adhesion through hydrophobic interactions. Water contact angles were significantly higher on fibrillated parent strains of *Streptococcus salivarius* and *S. sanguis* compared to their mutants devoid of fibrils (67). Similar observations were made for *Serratia marcescens*, a pathogen that is found in water, soil, sewage, and foodstuffs. Secondly, the interactions occurring between the cell and the substratum upon approach could be controlled by the physicochemical properties of surface appendages different from those of the overall cell surface. Handley and coworkers (124) used cationized ferritin to probe negatively charged sites, and colloidal gold to probe positive and hydrophobic sites on *S. sanguis* strains bearing fibrils. Their results suggested that these structures may have electrical and hydrophobic properties, which are different from the neighboring bald part of the cell surface (67,124). Thirdly, the radius of curvature ruling the DLVO interactions may be much smaller than the overall cell radius. In particular, it is conceivable that cells become bound via appendages through an electrostatic potential barrier, which results from average surface properties, as illustrated by Figure 5. The distinct role of the flagellum in bacterial adhesion is illustrated by the fierce rotation movements observed with attached *Pseudomonas putida* cells at low ionic strength, that is, when the overall electrical potential barrier is high (87). Finally, it must also be noted that appendages may be responsible for specific interactions with a biological substratum or with an inert material coated by substances of biological nature.

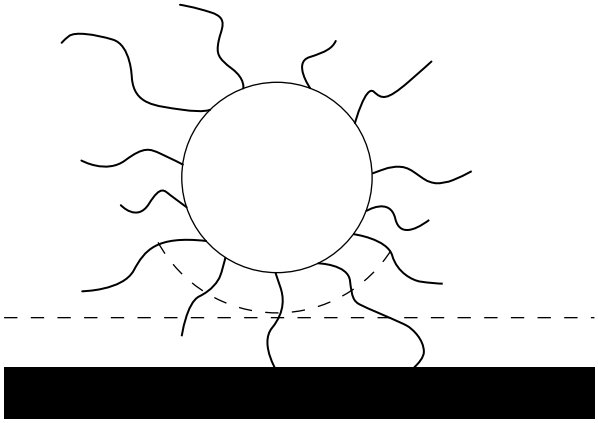

Figure 5. Schematic illustration of the penetration of surface appendages, allowing attachment of a microbial cell to a plane substratum, through a potential energy barrier (location represented by dashed line).

Surface Deformability

Although some microbial cells may be considered as rigid spheres, as assumed in DLVO theory, most of them should be viewed as soft and deformable. In recent years, atomic force microscopy (AFM) has made it possible to probe cell surface softness in a direct way (125–127). This is illustrated in Figure 6, which shows AFM force-distance curves recorded under water upon approach between a silicon nitride probe and the surface of fungal spores (Fig. 6a) and bacteria (Fig. 6b). For *P. chrysosporium* spores, no significant deviation from linearity was seen in the contact region, indicating that the sample was not deformed by the probe (128,129). Conversely, the curvature observed for both strains of *S. salivarius* may be attributed to surface softness (126). The difference in softness observed between the two strains was related to the presence or the absence of fibrils for *S. salivarius* HB and HBC12, respectively. Direct observation of bacterial surface deformability was provided after three-dimensional reconstruction of height AFM images of *Lactococcus lactis*, which revealed grooves induced by the scanning AFM probe (Fig. 7). While these grooves represent an alteration of the cell surface by the probe, they were informative about its nanomechanical properties (127).

Macromolecules at Interfaces

The presence of solvated macromolecules, either constitutive of surfaces or adsorbed, may generate interactions that may be either attractive or repulsive, depending on the properties of the macromolecules, the nature of the solvent and the degree of coverage (64). When two surfaces carrying nonionic (or uncharged) polymers approach each other, the segments of these macromolecules overlap, which leads to a repulsive force (Fig. 8a). The latter is caused by the decrease of entropy associated with a closer confinement of the polymer chains between the two surfaces and is known as steric repulsion. The extent of this repulsive force will be influenced by the nature of the solvent: in a good solvent, that is, in a solvent in which the polymer segments have a higher affinity for the solvent than for one another, the repulsion will occur at a large separation distance (Fig. 9). On the other hand, attractive forces may also be mediated by adsorbed macromolecules (Fig. 9). In a poor solvent, segment attraction

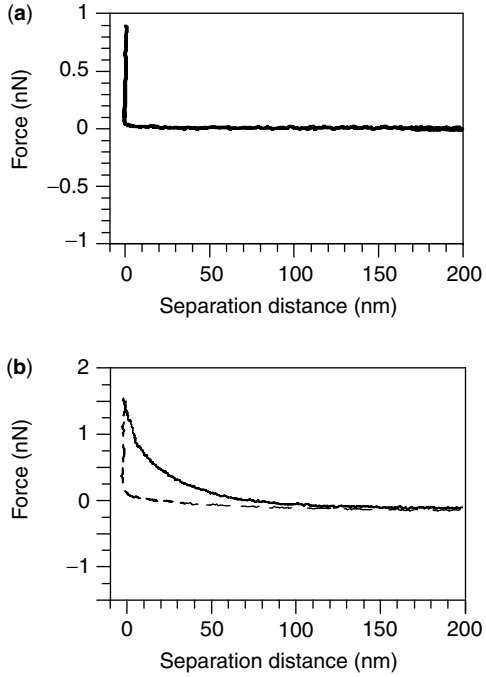

Figure 6. Representative AFM force-distance curves recorded, under water, upon approach between the silicon nitride probe and the surface of *P. chrysosporium* spores (a), *S. salivarius* HB (B; continuous line) or *S. salivarius* HBC12 (b; interrupted line) (126,128,129).

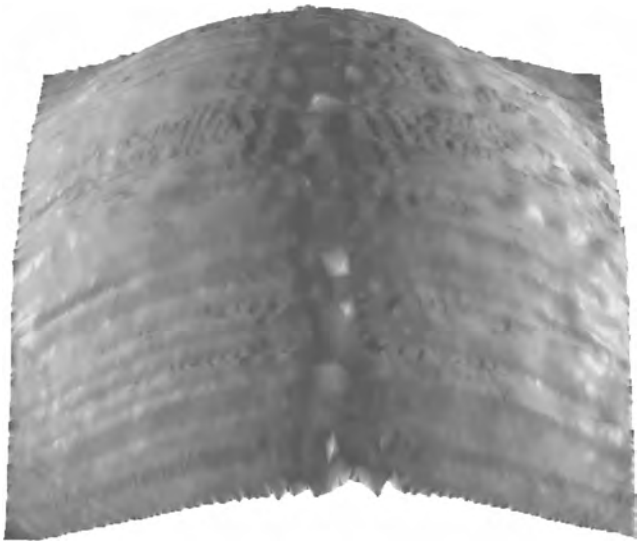

Figure 7. 3D reconstructed AFM height image (475×475 nm^2) obtained under water on *L. lactis* at an imaging force of about 7.5 nN; the groove direction was parallel to the scanning direction (127).

between molecules bound to opposite surfaces leads to overall attraction between the surfaces until a balance with steric repulsion is reached (Fig. 8b). When the degree of coverage is weak and the polymer is strongly adsorbed, the macromolecules may form bridges between the two surfaces (Fig. 8c).

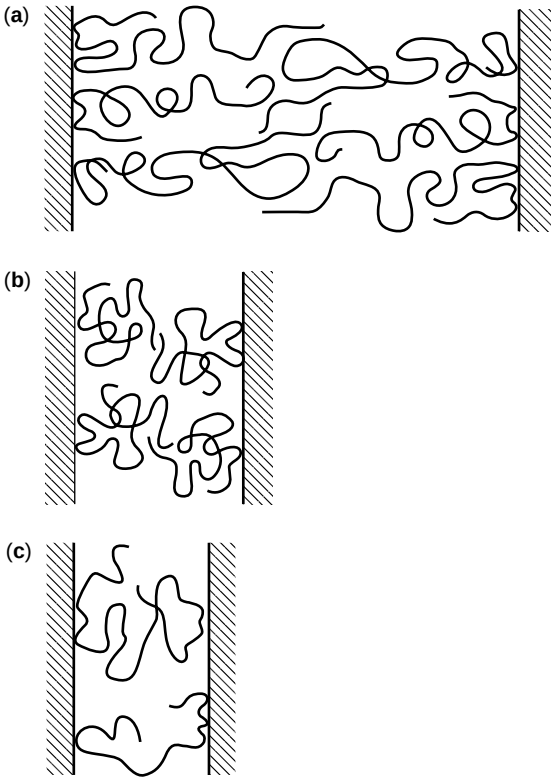

Figure 8. Illustration of steric forces generated by polymers present at an interface. Adapted from (64).

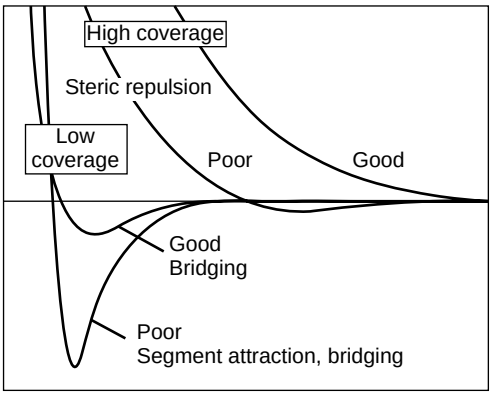

Figure 9. Variation of interaction free enthalpy as a function of the separation distance between two surfaces as a result of macromolecular interactions; influence of the polymer concentration at the surface (low or high coverage), and solvent quality (good or poor). Adapted from (64).

The influence of macromolecules at the interface will depend on the pH and on the ionic strength, which determine the electrostatic repulsion between the different partners. A microbial cell may be bound to a substratum if bridging macromolecules extend far enough from the surface to span the distance at which the electrical repulsion operates, as illustrated by Figure 10. On the left-hand side of the figure, the molecular mass or the

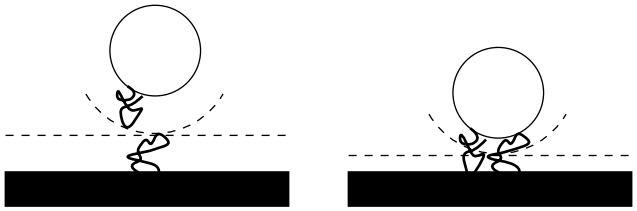

Figure 10. Illustration of the effect of ionic strength or surface potential on polymer bridging; the dashed lines represent the distance at which the electrostatic repulsion operates. The increase of ionic strength or decrease of surface potential from left to right allows polymer bridging to occur. Adapted from (130).

ionic strength are too low to allow bridging. On the right-hand side, the ionic strength is higher or the surface potential is weaker, the electrical repulsion operates at short distances compared with the thickness of the adsorbed macromolecules, and bridging occurs (130,131).

Conditioning Film

In most environments of practical relevance (natural waters, soil solution, biological fluids, industrial fluids, . . .), the substratum surface is rapidly conditioned by dissolved substances, particularly by dissolved macromolecules (proteins, polysaccharides, lipids). The formation of these conditioning films will affect both the physicochemical properties of the substratum surface and the adhesion of microorganisms. Nevertheless, explaining the change in microbial attachment is not straightforward (see CONDITIONING FILMS FROM ENVIRONMENTAL WATERS, this Encyclopedia).

The changes of hydrophobicity and electrical properties of silicone rubber, used in voice prostheses, could not be related to the systematic inhibitory effect of a salivary film on bacterial and yeast adhesion (132). The attachment (assessed after drying) of bacteria to natural (shale, sandstone, andesite) and industrial (stainless steel, polypropylene) materials was decreased or increased after the formation of a conditioning layer by dissolved compounds; however, no relation could be established with water contact angles determined on the conditioned substratum (133). The study of the influence of adsorbed milk-related products (skim milk, sodium caseinate, whey protein isolate and whey permeate) on *L. lactis* attachment revealed a beneficial effect of skim milk and whey permeate, which could not be attributed to the overall chemical composition, hydrophobicity, or electrical properties of the conditioning film. Force-distance curves recorded by AFM, combined with a study of dynamic wetting indicated that the mobility of adsorbed macromolecules play a key role in favoring attachment of *L. lactis* to skim milk-conditioned substratum (134). On the other hand, conditioning stainless steel with skim milk was found to reduce the adhesion of five food-borne pathogens; cross-linking adsorbed proteins partially reversed the inhibition of bacterial attachment (88). These two studies illustrate that adsorbed macromolecules of similar nature may condition substratum surfaces in a way that is favorable or unfavorable to attachment, depending on the partner surfaces involved. While the effect of conditioning film on attachment may be positive or negative, it appears from a recent compilation (12) that the force exerted by the liquid-air interface tends to detach a higher percentage of microorganisms attached via a conditioning film as compared with microorganisms attached to a bare substratum.

Microorganisms as Living Colloidal Particles

The study of microbial adhesion to living tissue (human or vegetal) indicated the role played by specific interactions, on the basis of highly stereochemically selective (key-lock) interactions between the microorganism and the substratum (18–20,135). The attachment of *Lactobacillus plantarum* to a human colonic cell line was shown to occur via a mannose-specific interaction (136). Another strain of *Lactobacillus*, that is, *L. crispatus*, had the capacity to attach to the collagen of human intestinal extracellular matrix via its S-layer protein (137). Specific interaction may also effect adhesion of microbial cells on vegetable surfaces. The attachment of the basidiomycetous yeast *Rhodosporidium toruloides* to barley leaf surfaces was mediated by the interaction between the mannose residues of yeast surface and a lectin (138).

Microbial cells may influence the adhesion mechanisms by modification of their surface properties or by releasing extracellular compounds into the surrounding liquid. The surface chemical composition of the soil bacterium *A. brasilense* was shown to become richer in protein and hydrocarbon-like compounds during its growth. These variations were related to an increase of both hydrophobicity and adhesion to glass and polystyrene (139). The chemical composition and/or the physicochemical properties of the surface of other bacteria were also shown to be influenced by the growth and environmental conditions (140–142). On the other hand, compounds released by microbial cells may influence in a positive or negative way their behavior at interfaces. Extracellular compounds released by *L. lactis* may affect its attachment by increasing the ionic strength locally, near the cell-substratum interface (143). Extracellular proteins released by *A. brasilense* adsorb on the substratum, allowing attachment to occur (29,30). In the latter case, the influence of cell physiology was demonstrated by the influence of temperature and of tetracycline (an inhibitor of protein synthesis) on both protein release and attachment (144).

Biosurfactants released by microorganisms have a distinct tendency to accumulate at hydrophobic-hydrophilic interfaces and to lower the interfacial tension (145) and were shown to inhibit microbial adhesion. *Streptococcus thermophilus*, regularly encountered on heat exchanger plates in dairy industry, was shown to be able to produce a biosurfactant which caused its own detachment (146). A small amount of attached oral *Streptococcus mitis* (1 to 4% of surface coverage) was able to prevent attachment of *S. mutans*, pointing to the potential protective role of *S. mitis* in the oral cavity against the cariogenic *S. mutans* (147). It may be noted that

conditioning the substratum alone with the same biosurfactant was less efficient, indicating that inhibition occurred by conditioning both substratum and cell surfaces. Attachment of *S. thermophilus* to voice prosthesis materials (silicone rubber) prevented yeast colonization by biosurfactant production, allowing the prosthesis indwelling lifetime to increase (148). Further, biosurfactants may be used to form a conditioning film that discourages microbial attachment, as for the uropathogen *Streptococcus faecalis* (149); such application presents a high potential to inhibit adhesion on catheter materials.

Are the microorganisms able to react upon contact with a solid surface by inducing a specific activity that leads to their attachment? Comparison of gene expression for *E. coli* cells, either planktonic or from a biofilm, showed that this microorganism is able both to repress the flagellum formation, which might destabilize the biofilm, as well as to activate the production of exopolysaccharide (colanic acid), which will reinforce the biofilm structure (150). However, this study does not answer the question. By comparing *E. coli* strains proficient or deficient in colanic acid production, Danese and coworkers (151) showed that exopolysaccharide production is not required for attachment, in contrast with biofilm development. The study of attachment of *P. aeruginosa* under flowing conditions revealed that the situation may be rather complex: (*1*) the biofilm development was related to the expression of the gene responsible for alginate synthesis, allowing bacteria to be retained by a glass substratum; (*2*) this expression was not a prerequisite for attachment, indicating that alginate synthesis was not necessary for attachment to glass; (*3*) in the absence of such expression, the bacteria were detached from the substratum as time passed (152). In the same way, synthesis of proteins was shown to be essential to the anchorage of *A. brasilense* to polystyrene (29,30). The production of quorum-sensing molecules (153) has been shown to be necessary to the differentiation step of *Pseudomonas* biofilm formation, but not to the primary adhesion step (154).

Heterogeneity of Cell and Substratum Surfaces

The hydrophobicity, electrical properties, and chemical composition of microbial surfaces, can be characterized by water contact angle measurement (155,156), electrophoretic mobility measurement (65) and X-ray photoelectron spectroscopy (157,158), respectively. While relevant information may thereby be obtained regarding cell behavior at interfaces, most of these techniques do not probe the cell surface in its native state. Moreover, all give information on overall surface properties and do not provide information at high lateral resolution.

Microsphere Attachment. Polystyrene latex microspheres were shown to attach preferentially to germinating tubes and hyphae compared with the parent cell, for yeast (159) and other fungal cells (160,161). The microspheres thus give information about the heterogeneity of the surface properties of these microorganisms. Nevertheless, the exact nature of the interactions (hydrophobic, electrostatic, steric) leading to the attachment of these microspheres (both negatively charged and hydrophobic) could not be specified (160). The meaning of the microsphere adhesion test must be therefore approached with caution.

In a similar way, the adhesion of gold colloidal particles (both hydrophobic and negatively charged) to a tufted streptococcal strain showed an asymmetry of surface properties (124). Electron microscopy revealed that colloidal gold did not attach to the bald part of tufted *S. sanguis* strains over the pH range 3.7 to 9.0 because of the hydrophilic nature of this portion of the cell surface. Adhesion of colloidal gold to the long tuft fibrils occurred irrespective of pH, confirming conclusions from contact angle measurements that these fibrils convey hydrophobicity to the cell surface. Adhesion of colloidal gold to short fibrils occurred at pH 3.7 but not at elevated pH, because of increased electrostatic repulsion between the gold and the negatively charged short fibrils with increasing pH. Accordingly, the short and long fibrils can be associated with carrying negative charges and hydrophobicity, respectively (67). It may be noted that, whether using polystyrene microspheres or gold particles, this approach does not account for the possible role played by macromolecular interactions.

Atomic Force Microscopy. Regarding the assessment of the heterogeneity of substratum and cell surfaces, exciting possibilities are now offered by atomic force microscopy (AFM) to investigate the molecular interactions and properties of surfaces on the nanometer scale. AFM, invented in 1986 (162) consists of measuring the forces acting between a sharp probe and the surface of a sample. It allows the nanoscale mapping of surface topography, as well as the direct measurement of mechanical properties, friction, and interaction forces (electrostatic, van der Waals, solvation, steric interactions). As opposed to more conventional surface analysis techniques, AFM can be operated in aqueous solutions, which makes it possible to study living cells under physiological conditions (125,163). A very challenging goal for future research is to directly map the surface properties of living cells in aqueous conditions.

AFM offers new perspectives concerning the nanoscale mapping of physicochemical properties (hydrophobicity, electrical properties, macromolecular interactions). Germinating spores of *P. chrysosporium* were shown to present a heterogeneous ultrastructure made of granular and smooth zones (128). Figure 11a shows a map of adhesion forces recorded on an area presenting both zones. A force-distance curve was recorded at each pixel of this map; lighter levels correspond to larger adhesion forces. The comparison of this map with the height image recorded on the same area (Fig. 11b) demonstrated the relevance of AFM to assess the heterogeneous character of microbial cell surfaces with regard to physicochemical interactions.

Recently, Dufrêne (164) described a new method for characterizing the physicochemical properties of

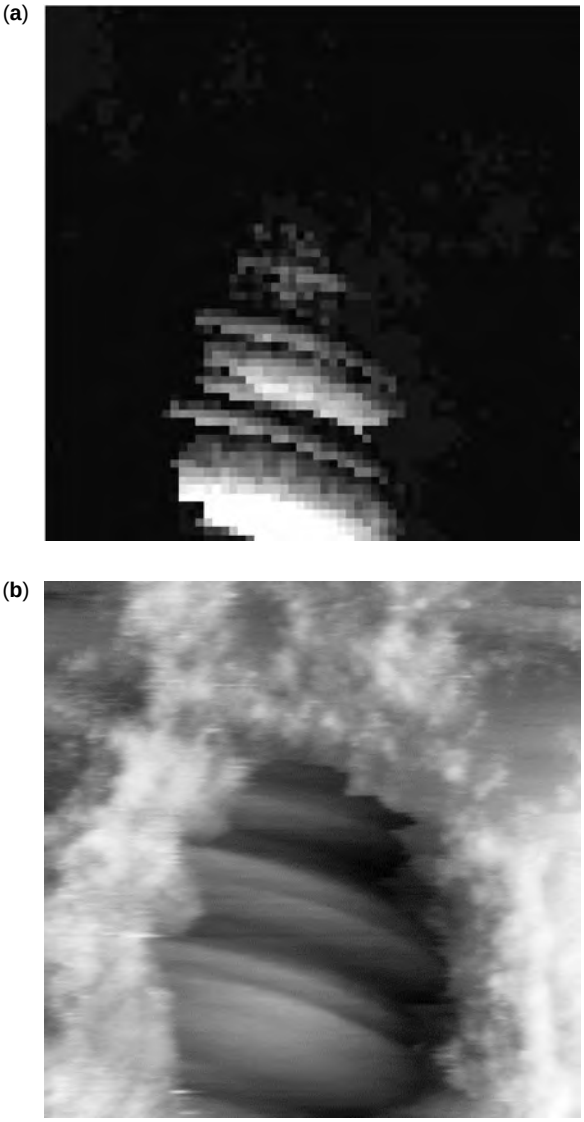

Figure 11. AFM adhesion force map (a) and height image (b) recorded with a silicon nitride probe on the same area ($2 \times 2\ \mu m^2$) of a germinating spore of *P. chrysosporium*. Each pixel of the map corresponds to a force-distance curve, the gray level corresponding to the adhesion force value (125). Z-range = 10 nN (a) and 100 nm (b).

native microbial cells by using AFM with chemically functionalized probes. Functionalization was shown to make the probe very sensitive to surface hydrophobicity, the magnitude of the adhesion forces measured between functionalized probes and substrata decreasing in the order $CH_3/CH_3 > CH_3/OH > OH/OH$. The surface of *P. chrysosporium* spores was homogeneously hydrophilic, as revealed by the lack of adhesion forces measured among the spore using CH_3- and OH-terminated probes. An AFM probe can also be functionalized to assess the specific interaction between a ligand and a receptor, as performed for streptavidin-biotin (165) or concanavalin-mannan (lectin-like) (166) interactions.

CONCLUSION

The methods used to study microbial adhesion differ according to two major characteristics that will influence the significance of the results: (i) the rate of transport of microorganisms to the surface; and (ii) the shear exerted near the substratum surface. The experimental conditions may also influence the physicochemical properties of the substratum surface, which may be affected by adsorption of substances present in the liquid or released by the cells. The choice of the methodology will differ depending on whether the interest is focused on the rate of attachment, on the strength of adhesion, on the study of the cell-substratum surface, or on the comparison of different substratum materials.

The role of electrostatic interactions and hydrophobic interactions in controlling microbial adhesion is well established. However, approaches attempting to quantify cell-substratum interactions and to predict cell attachment or adhesion strength are subject to severe limitations. The approach based on the balance of interfacial energies is limited by the impossibility to determine accurate values of the surface energy from contact angles of liquids or alternative methods.

This approach and the DLVO theory rely on hypotheses, which may be crude simplifications, particularly regarding the microbial cell surfaces. Of concern are the surface roughness, deformability, and heterogeneity. Solvated macromolecules or biosurfactants, whether present at the cell surface or adsorbed by the substratum, may also play a major role. Moreover, specific interactions may come into the play with biological substrata or substrata conditioned with compounds of biological origin. AFM offers promising perspectives regarding the possibility to probe directly interfacial interactions involving microbial cells and to map the physicochemical properties and adhesion forces over a cell surface.

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ADHESION TO SURFACES.

See CONDITIONING FILMS IN AQUATIC ENVIRONMENTS

ADSORPTION OF MICROORGANISMS TO SURFACES.

See ADHESION (PRIMARY) OF MICROORGANISMS ONTO SURFACES

ADSORPTION OF VIRUSES TO SURFACES.

See MODELING OF VIRUS TRANSPORT AND REMOVAL IN THE SUBSURFACE

AEROBIC ALKALIPHILES

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Recently, it has become clear that life exists in Milieux that were earlier thought to be incompatible with growth, for example, extremely high temperatures, very low temperatures, abyssobenthic conditions, strongly alkaline pH, and strongly acid pH. The "extremophiles" that grow in such conditions are the subject of current active research. Alkaliphilic microorganisms are extremophiles that actively grow in extremely alkaline Milieux, for example, pH 10, and generally require sodium ions for growth. Alkaliphilic microorganisms are classified as either facultative or obligate alkaliphiles, depending on their capacity to grow at neutral pH (Fig. 1). Obligate alkaliphiles are restricted to alkaline pH, whereas facultative alkaliphiles can also grow at neutral pH. Microorganisms called *alkalitolerant* typically have an upper limit of pH 9 for robust growth; they grow poorly in the pH ranges of true alkaliphilic bacteria. Alkaliphilic microorganisms are distributed widely in nature and include actinomycetes, eubacteria, archaea, and fungi. There are many interesting and unresolved issues with respect to how alkaliphilic microorganisms adapt to their extremely alkaline Milieux. The mechanism of this adaptation has been most extensively studied in the *B*. species. Data have been presented for the roles of Na^+/H^+ antiporters, which are present in the cell membrane, and of a barrier of charged cell wall–associated macromolecules in the accommodation of the bacteria to the alkaline Milieux. In addition to mechanistic studies, the applications of alkaliphile biology to industry are numerous. For example, extracellular enzymes produced by alkaliphiles are used in laundry detergents (e.g., alkaline cellulase and alkaline protease) and are used in the production of cyclodextrin, which can encapsulate large compound molecules (e.g., cyclomaltodextrin glucanotransferase). Thus, alkaliphilic microorganisms are very interesting from both the viewpoints of physiology and industrial applications.

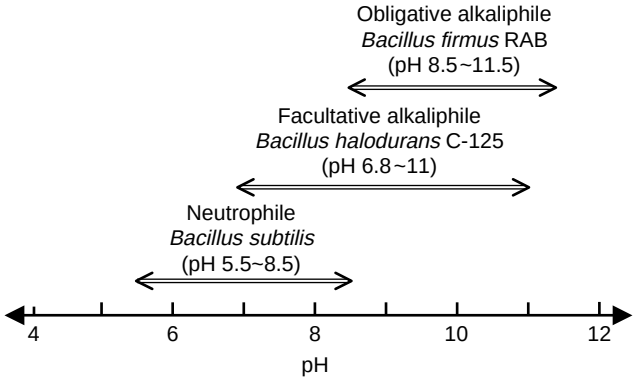

Figure 1. The relationship of pH to the growth of a typical neutrophile and two categories of alkaliphiles. The typical pH dependency of the growth of neutrophile, *B. subtilis*, and two categories of alkaliphiles, obligative alkaliphile, *B. firmus* RAB and facultative alkaliphile, *B. halodurans* C-125 is shown by two-way arrows.

In this article, I present an introduction to alkaliphilic microorganisms that includes (*1*) historical background, (*2*) diversity, and (*3*) physiology.

HISTORICAL BACKGROUND

Although alkaliphilic microorganisms are now well recognized, their existence was little noted until the 1970s. Koki Horikoshi, a leading investigator of alkaliphiles, was the first to initiate thorough studies of these bacteria, starting in the late 1960s. Horikoshi has commented that when he began these investigations, he found only 16 scientific papers referring to alkaliphilic microorganisms (1). Two of the earliest papers dealing with the growth of bacteria in alkaline environments were those of Meek and Lipman in 1922 (2) and Downie and Cruickshank in 1928 (3). However, both these reported on the growth of non-alkaliphiles when exposed to an alkaline environment. The earliest papers on true alkaliphiles were those of Gibson (4) on *B. pasteurii* growing at pH 11 and of Vedder (5) on *B. alcalophilus* growing well at pH 8.6 to 11. Early in the 1960s, Takahara and coworkers (6) demonstrated the industrial importance of alkaliphiles when he improved the process of indigo fermentation through the addition of the alkaliphilic strain *Bacillus* S-8. This was the first industrial application of alkaliphilic bacteria. Since then, more than 1,000 papers on alkaliphiles and their adaptations have been published (1).

DISTRIBUTION

Distribution of Alkaliphiles in the Extremely Alkaline Milieux in Nature

Research into the distribution of alkaliphiles in the extremely alkaline environments was developed by Grant and his colleagues (7). Natural soda lakes and soda deserts exist worldwide (e.g., Lake Magadi in Kenya (pH 10.9), Wadi Natrum in Egypt (pH 11), and Lake Sambhar in India (pH 9.0)), and they are summarized in Table 1. Some of them have NaCl concentrations of about 5% w/v, whereas others are hypersaline, with NaCl concentrations greater than 15% w/v. These lakes are strongly inhabited by prokaryotic and archaeal alkaliphiles, which prosper in these selective environments. Because Ca^{2+} and Mg^{2+} are precipitated as carbonates, the ionic concentrations of these important divalent cations are very low in these lakes. The major ions of the lake are Na^+, Cl^- and bicarbonate/carbonate. The alkaliphilic isolates from these lakes generally require Na^+ for growth. Most of them are haloalkaliphiles (7).

Artificial alkaline environments are probably selected for particular alkaliphiles from adjoining soils and waters. Isolates from such environments include *Bacillus* sp. No. S-8 from an indigo ball of indigo fermentation (6), gram-positive *Exguobacterium auratiacum* from potato processing waste (8), and a gram-positive facultatively alkaliphilic *Bacillus* sp. from the alkaline wash waters derived from the preparation of edible olives (9).

Table 1. Worldwide Distribution of Soda Lakes and Soda Deserts

North America	
Canada	Manito
United States	Alkali Valley, Albert Lake, Lake Lenore, Soap Lake, Big Soda Lake, Mono Lake, Searles Lake, Deep Springs, Rhodes, Marsh, Harney Lake, Summer Lake, Surprise Valley, Pyramid Lake, Walker Lake
Central America	
Mexico	Texcoco
South America	
Venezuela	Langunilla Valley
Chile	Antofagasta
Europe	
Hungary	Lake Feher
Yugoslavia	Pecena Slatina
Russia	Kulunda Steppe, Tanatar lakes, Karakul, Araxes plain, Chita, Barnaul, Slavgerod
Asia	
Turkey	Van
India	Lake Looner, Lake Sambhar
China	Qinhgai Hu, Sui-Yuan, Heilungkiang, Kirin, Jehol, Chahar, Shansi, Shensi, kansu
Africa	
Libya	Lake Fezzan
Egypt	Wadi Natrun
Ethiopia	Lake Aranguadi, Lake Kilotes, Lake Abiata, Lake Shala, Lake Chilu, Lake Hertale, Lake Metahara
Sudan	Dariba Lakes
Kenya	Lake Bogoria, Lake Nakuru, Lake Elmentieta, Lake Magadi, Lake Simbi, Lake Sonachi
Tanzania	Lake Natron, Lake Embagi, Lake Megad, Lake Manyara, Balangida, Basotu Crater Lakes, Lake Kusare, Lake Tulusia, El Kekhooito, Momela Lakes, Lake Lekandiro, Lake Reshitani, Lake Lgarya, Lake Ndutu, Lake Rukwa North
Uganda	Lake Katwe, Lake Mahega, Lake Kikorongo, Lake Nyamunuka, Lake Munyanyange, Lake Murumuli, Lake Nunyampaka
Chad	Lake Bodu, Lake Rombou, Lake Dijikare, Lake Momboio, Lake Yoan
Australia	Lake Corangamite, Red Rock Lake, Lake Werowrap, Lake Chidnup

The Distribution of Alkaliphiles in Nonalkaline Milieux in Nature

As for the broader distributions of alkaliphilic microorganisms, alkaliphiles are surprisingly distributed almost everywhere. Alkaliphiles are isolated from the soil of deep-sea trenches at 11,000-m depth (10) and from diverse soil and water samples from more conventional sites (1). Alkaliphiles can be found in soil at pH 4, but the highest frequency was observed in alkaline soil, especially above pH 8.3. The number of alkaliphiles found in soil was about 1/10 to 1/100 of that of neutrophilic bacteria. The number of organisms was to a certain extent correlated with the pH of the soil sample (Fig. 2).

Alkaliphiles Are Not Restricted to Any Taxonomic Group

Alkaliphiles are thought to change the pH of their Milieux to suit their optimum growth. In addition to prokaryotic alkaliphiles, various types of alkaliphilic microorganisms, including actinomycetes, fungi, yeast, and phages, have been isolated (1) and some of them are presented at Table 2.

A summary of the taxonomic group of prokaryotes isolated from soda lakes, an alkaline Milieux in nature, is reproduced from a review by Jones and coworkers (11). The enormous taxonomic diversity of extreme alkaliphiles is evident, and it is further reflected in the diversity of characteristics. Alkaliphilic cyanobacteria are among the primary photosynthetic organisms that produce oxygen; such organisms include *Spirulina*, *Cyanospira*, *Synechococcus*, and *Chroococcus*. Some of the soda lakes harbor many characteristic alkaliphilic phototrophic bacteria, for example, *Ectothiorhodospira* together with halophilic archaea (12). Zhilina and coworkers (13) isolated and characterized *Desulfonatronovibrio hydorogenovorans*, an alkaliphilic, sulfate-reducing bacterium, from a soda-depositing lake, Lake Magadi, Kenya.

Diversity of Alkaliphilic Bacillus Strains

Since Horikoshi and his colleagues began the screening and research of alkaliphilic microorganisms in 1968, many alkaliphilic microorganisms have been isolated. Most of them are gram-positive, spore-forming, motile, and aerobic rods, and so are classified in the genus *Bacillus*. *Bacillus* species are among the most commonly found aerobic and eubacterial alkaliphiles, both in alkaline and in nonalkaline Milieux. A very large number of

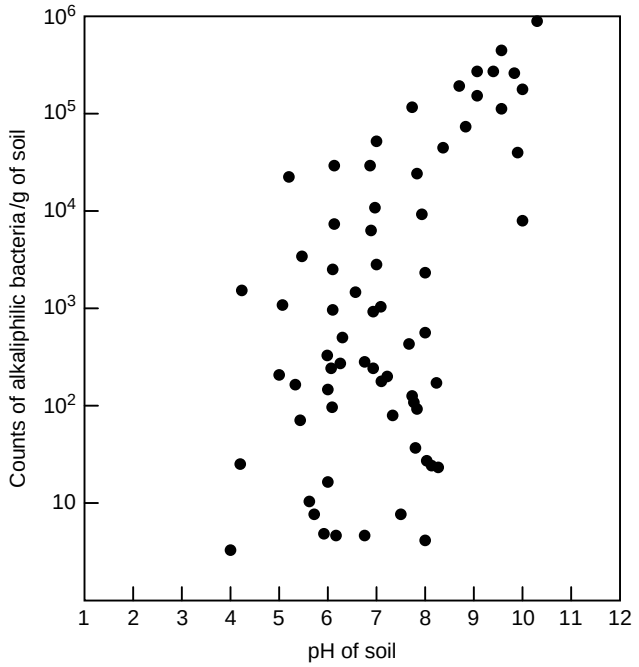

Figure 2. Distribution of alkaliphilic bacteria in soil (Reproduced with permission from Horikoshi and Akiba, *Alkalophilic Microorganisms: A New World*, p11, Japan Scientific Societies Press, Tokyo (1982)).

alkaliphilic bacilli have been isolated because of interest in the biotechnological application of useful alkali-resistant extracellular enzymes.

In the 1990s, Fritze and coworkers (14) and Nielsen and coworkers (15) classified alkaliphilic and alkali-tolerant *Bacillus*. They reported phylogenetic diversity of alkaliphilic *Bacillus* strains isolated from various sources and proposed that they were classified into 13 taxa and a number of unassigned single-membered clusters by using DNA-DNA hybridization, numerous physiological and biochemical characteristics, DNA base composition, and 16S rRNA analyses. Two taxa were equated with *B. cohnii* and *B. alcalophilus* and nine of the remainder were proposed as new species with the following names: *B. agaradhaerens*, *B. clarkii*, *B. clausii*, *B. gibsonii*, *B. halmapalus*, *B. halodurans*, *B. horikoshi*, *B. pseudoalcalophilus*, and *B. pseudofirmus*. Two taxa were insufficiently distinct to allow confident identification and these have therefore not been proposed as new species.

In a separate study, Agnew and coworkers (16) reported the isolation and characterization of novel obligate alkaliphiles from bauxite-processing waste. They are currently assigned to the new species *B. vdderi*. The new isolates appear to be members of the genus *Bacillus* but 16S rRNA sequence characters and other physiological and biochemical analysis indicate that the new isolates are not members of any validly identified *Bacillus* species.

Haloalkaliphiles

Oren (12) classified the Halobacteriaceae into six genera, *Halobacterium*, *Haloarcula*, *Haloferax*, *Halococcus*, *Natronobacterium*, and *Natronococcus*. *Natronobacterium* and *Natronococcus* are alkaliphilic archaea that grow only in specialized Milieux with the combination of extremely high salt concentrations (2.5–5.2 M NaCl) and high pH values (8.5–11.0). Such haloalkaliphiles have been isolated from soda lakes and soda deserts. The amount of glycolipid in the membrane hardly varied in haloalkaliphilic archaea when compared with that of haloneutrophilic archaea. A novel osmolyte, 2-sulfotrehalose, was discovered in several *Natronobacterium* species of haloalkaliphilic archaea (17). The concentration of sulfotrehalose increases with elevating concentrations of external NaCl, a behavior consistent with its identity as an osmolyte. Other common osmolytes (glycine betaine, glutamate, and proline) were neither accumulated nor used for osmotic balance in place of the sulfotrehalose.

Table 2. Taxonomic Group Containing Prokaryotes Isolated from Soda Lakes (Boldface Type). Reproduced from Jones, et al., (1998) with Permission from the Publisher

Eubacteria
Cyanobacteria
Chroococcales
Oscillatoriales
Spirulina
Firmicutes (gram-positive bacteria)
Actinobacteria (high G + C gram-positive bacteria)
Actinomycetales
Micrococcaceae
Nocardiform actinomycetes
Streptomycetaceae
Streptomyces
Bacillus/*Clostridium* group (Low G + C gram-positive bacteria)
Bacillaceae
Clostridiaceae
Haloanaerobiales
Proteobacteria(purple nonsulfur bacteria)
Beta subdivision
Delta subdivision
Gamma subdivision
Ectothiorhodospiraceae (purple sulfur bacteria)
Ectothiorhodosprira
Halomonadaceae
Pseudomonadaceae
Pseudomonas
Spirochaetales
Spirochaetaceae
Spirochaeta
Thermotogales
Thermopallium
Archaea
Euryarchaeota
Halobacteriales
Halobacteriaceae
Halorubrum
Natrialba
Natronobacterium
Natronococcus
Natronomonas
Natronorubrum
Methanomicrobiales
Methanosarcinaceae
Methanohalophilus

Kamekura and coworkers (18) studied the diversity of haloalkaliphiles on the basis of phylogenetic tree reconstructions, signature bases specific to individual genera, and sequences of space regions between 16 and 23S rRNA genes. They proposed the following changes: *Natronobacterium pharaonis* be transferred to a new genus, *Natronomonas* as *Natronomonas pharaonis* and *Natronobacterium magadii* to be transferred to a new species of the genus *Natrialba* as *Natrialba magadii*. Xu and coworkers (19) isolated and characterized novel haloalkaliphilic archaea from a soda lake in Tibet, China. They proposed to classify their strains in a new genus *Natronorubrum*. At present, microorganisms that belong to *Halobacteriaceae* have 16 genera registered in the taxonomy section of the National Center for Biotechnology Information (NCBI) (*http://www.ncbi.nlm.nih.gov/htbin-post/Taxonomyhome.html/*). These six genera include *Haloalcalophilium* in *Halobacteriaceae*, belonging to haloalkaliphilic archaea.

Romano and coworkers (20) isolated and characterized a haloalkaliphilic microorganism from hard sand of Lake Venere on Pantelleria Island, Italy. This haloalkaliphile is a gram-negative pleomorphic rod, strictly aerobic, capable of accumulating polyhydroxybutyrate, and growing optimally at pH 9.0 in the presence of 10% NaCl at 33–35 °C. They classified this strain in a new species of the genus *Halomonas*.

Yeast and Fungi

Most species of yeasts and yeast-like fungi can grow well in the acidic to neutral pH range. It has long been assumed that yeasts cannot grow at alkaline pH except for unusual strains. A few investigators have reported that certain yeasts could grow in initially alkaline media (21,22). However, these investigations were not carried out under appropriate conditions. The media used were poorly buffered and the change in pH was not followed.

Goto and coworkers (23) examined their microbial properties and proposed a new species, *Exophiala alcalophila* Goto et Sugiyama, with an accompanying new yeast morph *Phaeococcomyces alcalophilus* Goto et Sugiyama. These strains grow between pH 5.4 and 10.4 on YM medium (5 g peptone, 3 g malt extract, 3 g yeast extract, 15 g glucose, and 15 g agar in 1,000 ml distilled water). Aono (24) studied the taxonomic distribution of alkali-tolerant yeasts in his laboratory. Yeasts and yeast-like fungi (433 strains, 296 species, 53 genera) were examined to determine the upper limit of pH for growth. Among these, 135 strains of 86 species were found to be capable of growth at a pH above 10. These alkali-tolerant species belonged to 27 genera of yeasts, of which 10 genera contained only alkali-tolerant species. Furthermore, Aono (25) measured upper pH limits for growth of yeasts belonging to the genus *Hansenula* and some related strains. As shown in Figure 3, alkali-tolerant species occupied particular positions of a phylogenetic tree proposed for the genus.

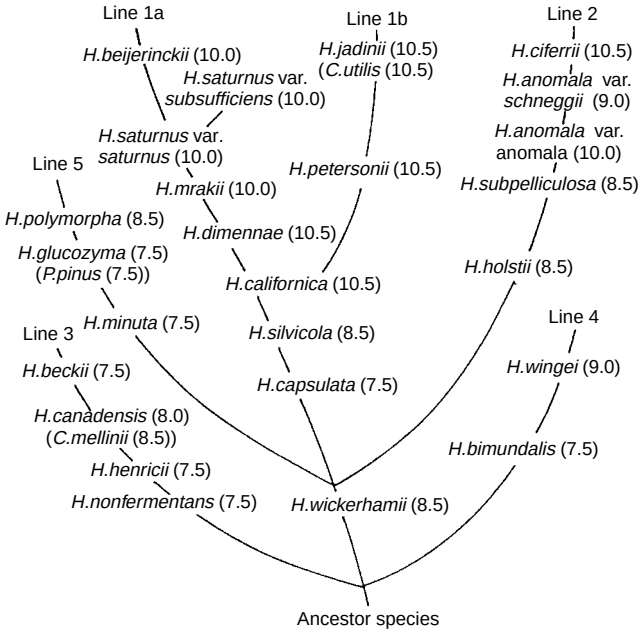

Figure 3. Alkali-tolerant yeasts on a phylogenetic tree of the genus *Hansenula*. The number in parentheses shows the upper pH limit for growth of a given strain. Species related to *Hansenula* are shown in parentheses. (Reproduced with permission from Aono, *Syst. Appl. Microbiol.* **15**, 589 (1992)).

Alkaliphilic Cyanobacteria

Gerasimenko and coworkers (26) reported the discovery of a wide variety of alkaliphilic cyanobacteria (16 genera and 34 species were found). Buck and Smith (27) reported an Na^+/H^+ electrogenic antiporter in alkaliphilic *Synechocystis* sp. Singh (28) partially purified a urease from an alkaliphilic diazotrophic cyanobacterium, *Nostoc calcicola*. Strain Z7935 (T) is an obligatory sodium-dependent alkaliphile, which grows in a sodium carbonate medium and does not grow at pH 7; the maximum pH for growth is more than pH 10, and the optimum pH is 9.5 to 9.7. The optimum NaCl concentration for growth is only 3% w/v (13).

Thermoalkaliphiles

Early work on alkaliphiles suggested that microorganisms adapted to the two extreme environmental conditions of high temperature and high alkaline pH did not exist. However, many kinds of thermoalkaliphiles have been isolated from different habitats since the 1980s. These include an obligate alkaliphilic *Clostridium* species isolated from sewage (29), an asporogenous, gram-positive ammonifying anaerobe from a soda lake deposit, *Tindallia mamadii* (30), a xylan-degrading anaerobic thermoalkaliphile designated as strain LB3A (31), and an actinomycete, *Thermoactinomyces* sp. HS682 (32). Stetter and colleagues (33) have even described a hyperthermophilic, alkaliphilic archaea, *Thermococcus alcaliphilus*, which grows on polysulfide at temperatures between 56 °C and 90 °C and with an optimum temperature around 85 °C. The pH range for growth was 6.5 to 10.5, with an optimum around 9.0.

Other Alkaliphiles

In contrast to thermoalkaliphiles, Kimura and Horikoshi (34) isolated a *Micrococcus* that was alkalipsychrotrophic in nature. This microorganism showed the highest growth rate at 0 °C and produced an amylase whose properties might be useful in food-processing circumstances.

Several alkaliphilic spirochetes were also isolated from Lake Magadi and from Lake Khatyn, Central Asia, by Zhilina and coworkers (35). Analysis of the genes encoding 16S rRNA indicated a possible fanning out of the phylogenetic tree of spirochetes.

PHYSIOLOGY

The Feature of Cell Surfaces in the Alkaliphilic *Bacillus* Species

Cell Walls. Aono and Horikoshi classified the alkaliphilic *Bacillus* species into three groups on the basis of cell wall composition and other physiological characteristics (36). As described in Table 3, the alkaliphilic *Bacillus* strains of group 1 contain large amounts of glucuronic acid and hexosamine in the cell walls and cannot grow at a neutral pH. The cell walls of group-2 strains contain large amounts of acidic amino acids and uronic acids. Na^+ is essential for their growth. The quantities of acidic compounds in the cell walls of both groups 1 and 2 are increased when cells are grown at elevated pH. In contrast, the cell walls of group-3 organisms contain phosphorus and neutral sugars in large amounts and are essentially similar to the walls of the neutrophilic *Bacillus subtilis* in chemical composition. Growth was observed in the presence of Na^+ or K^+.

The Role of the Cell Walls in Growth at Alkaline pH. Protoplasts prepared from alkaliphilic *Bacillus* are unstable at alkaline pH and regenerate only at neutral pH but not at alkaline pH (37). This suggests that some mechanisms of adaptation to high pH, "alkaliphily," might involve the cell walls, the part of the cell that is directly exposed to the extracellular environment. The cell walls of alkaliphilic *Bacillus* consist of peptidoglycan and non-peptidoglycan components. The peptidoglycan from all three groups is of the A1γ type, identical to that in the cell walls of neutrophilic *B. subtilis* (38). Cell walls groups 1 and 2 have specific acidic polymers called teichuronopeptide (TUP); TUP is a polymer in which an acidic polypeptide binds covalently to polyglucuronic acid. Cell wall concentrations of TUP increase with respect to peptidoglycan as the culture pH is elevated (39). While the mechanism is not clear, it is thought that the acidic fraction of the cell walls may act as an obstacle to the high extracellular concentrations of hydroxide ions or as a reservoir of hydrogen ions (39).

In other alkaliphilic *Bacillus* species, for example, *B. pseudofirmus* OF4, the uronic acid polymers are not found (40). Recent studies have shown that *B. pseudofirmus* OF4 has an acidic S-layer polymer produced from a gene that has strong homology with genes from neutrophilic *B. anthracis* and *B. licheniformis*. This protein was identified in studies of the 2D gel electrophoresis patterns of membrane-associated proteins of pH 10.5- and 7.5-grown cells of *B. pseudofirmus* OF4. It is a homogeneous, apparently processed, protein that is present in greater amounts at high pH. On cloning, sequencing, and disruption of the gene, the S-layer was found not to be required for alkaliphily in *B. pseudofirmus* OF4, but to confer an advantage for growth at pH 10.5. Cytoplasmic pH homeostasis, on a sudden shift from pH 8.5 to 10.5, is somewhat better in the S-layer-containing wild type than in mutant strains lacking the S-layer (41). *Bacillus pseudofirmus* OF4 may also have an acidic capsular layer partial sequence for genes that are likely to encode the synthetic enzymes for a polyglutamate capsule that were characterized in this species (42). It will be of interest to complete the characterization of the sequence and the role of this locus.

Table 3. Classification of Alkaliphilic *Bacillus* Strains

Group	Growth pH	Major Components of Cell Wall	Ion Requirement
1	No growth at pH 7	Glucuronic acid Hexosamine	Na^+ (essential)
2	Capable of growth at pH 7	Aspartic acid Glutamic acid Galacturonic acid Glucuronic acid	Na^+ (essential)
3	Capable of growth at pH 7	Phosphoric acid Glycerol Neutral sugar	Na^+ or K^+

Auxotrophy, Antibiotic Resistance

The majority of alkaliphilic microorganisms isolated from nature are apparently auxotrophic. Such alkaliphiles cannot grow in completely synthetic media (e.g., the M9 culture media in which *Escherichia coli* and *B. subtilis* can grow). However, some alkaliphilic *Bacillus* strains can; these are used for molecular biological manipulations. It is possible, in these strains, to introduce useful markers by chemical mutagenesis (1). Transformants are selected with the antibiotic resistance when plasmid DNA is incorporated into alkaliphilic microorganisms. In this case, common antibiotics such as kanamycin and tetracycline cannot be used because they are very unstable under alkaline pH (1). Chloramphenicol and erythromycin are stable at alkaline pH and can be used.

Lipid Composition

Clejan and coworkers (43,44) have reported that obligately and facultatively alkaliphilic bacteria exhibit significant differences in lipid constituents. They compared the lipid composition of several obligately and facultatively alkaliphilic Bacilli. High levels of cardiolipin were reported, that is, 13% and 25% of the polar lipids in *B. pseudofirmus* RAB and OF4, respectively. The obligately alkaliphilic *B. pseudofirmus* RAB had a much higher neutral/polar lipid ratio than the facultatively alkaliphilic *B. pseudofirmus* OF4. The *B. pseudofirmus*

RAB had 90% branched chain fatty acids as opposed to 72% in the *B. pseudofirmus* OF4. The lipids of obligate alkaliphiles contain a significant amount of unsaturated fatty acids, but these fatty acids are hardly present in the lipids of facultative alkaliphiles. Although the relationship of unsaturated fatty acid content to obligate alkaliphily has not been demonstrated, it may be hypothesized to involve membrane fluidity.

Flagella and Sodium Ion

Flagellar movement of neutrophilic, nonmarine bacteria is not caused by ATP but by H^+-driven motors (45). However, flagellar movement of alkaliphiles and marine bacteria is accomplished by Na^+-driven motors. These Na^+-driven flagellar motors are inhibited by amiloride (46). It has been reported that even on a significant shift to alkaline pH, Na^+-driven flagellar motors do not rotate without sodium ions (1). The structural difference of a flagellum motor of the H^+ type and the Na^+ type is not seen by observation at the electron microscope level. Even if there is a difference in the coupling ion between H^+- and Na^+-coupled flagella, the basal structure and the mechanism of the rotation are thought to be similar (1).

Membrane Transport

In the growing cell, nutrients can be taken up into the cell and metabolized, followed by secretion of the products. Uptake of the nutrient and secretion of the products involve passage through the cytoplasmic membrane. The cytoplasmic membrane also plays an active role in pH homeostasis (see section on Antiporters). Alkaliphiles have numerous transport systems that can take up nutrients efficiently from the highly alkaline environment (47). In bacteria, amino acids are usually taken up against a concentration gradient by active transport. Usually, the energy required for active transport is provided either by ATP or by the proton motive force. Under the high alkaline Milieux, the proton motive force decreases, and alkaliphiles tend not to utilize proton as a coupling ion. Alkaliphiles have Na^+-coupled uptake systems instead of proton as a coupling ion. Other monovalent cations such as K^+, Li^+, and NH_4^+ could not be substituted for Na^+, and different species of counteranions for Na^+ did not affect the uptake. The uptake of amino acids was stimulated by the addition of Na^+; this was especially true for glycine, L-alanine, L-serine, and L-asparagine (47).

Intracellular pH

Most alkaliphiles have an optimum growth pH at around pH 10, which is significantly different from that tolerated by well-investigated neutrophilic microorganisms. Therefore, the question arises as to how these alkaliphiles can grow in such an extreme Milieux. Are there any fundamental differences in the physiology of alkaliphilic and neutrophilic microorganisms? If intracellular pH is estimated by measuring the extra- and intra-cellular distribution of weak bases, which cells do not actively transport, the intracellular pH is found to be maintained at around 8, despite a high extracellular pH of 8 to 11, as shown in Table 4 (1). Therefore, one of the key features in alkaliphily is associated with the cell surface and its transporters, which must contribute to an intracellular neutral Milieux that is separate and distinct from the extracellular alkaline Milieux.

Na^+/H^+ Antiporters Involved in the Intracellular Acidification Relative to the Extracellular Milieux

Since alkaliphiles live in an extremely alkaline Milieux, their most challenging problem is keeping a cytoplasmic pH more acid than the outside pH, often by more than 1.5 pH units. In other words, they must actively bring protons into the cell. In contrast to research with neutrophiles, an especially strong case can be made for the acidification of the cytoplasm of alkaliphiles by Na^+/H^+ antiporters (48). When alkaliphiles are placed in a medium without Na^+ at pH 10, the intercellular pH quickly rises to the value of the outside pH. However, when Na^+ is present in the external medium, the intercellular pH does not rise on shifting to the more basic medium. Furthermore, mutants of alkaliphiles that cannot grow at pH values above 9 are defective in Na^+/H^+ antiporter activity. The antiporter is electrogenic ($H^+ > Na^+$) and driven by the membrane potential ($\Delta\Psi$), which is generated by the primary proton pumps of respiratory chains. The sodium ion circuit is completed when sodium ion enters the cell via Na^+/solute symporters that are also driven by the $\Delta\Psi$. The use of Na^+/solute symporters has the advantage that solute transport is driven by the sodium potential rather than the proton potential, the latter being low because of the inverted ΔpH (pH_{in}-pH_{out}).

Respiration-Dependent ATP Synthesis

Alkaliphiles always maintain their cytoplasmic pH lower than the outside pH, the opposite of most bacteria. This raises a serious conundrum for energy conservation. To

Table 4. Intracellular pH Values in Alkaliphilic Bacillus Strains at Different External pH Values

Microorganism	External pH	Internal pH
B. alcalophilus	8.0	8.0
	9.0	7.6
	10.0	8.6
	11.0	9.2
B. pseudofirmus OF4	7.0	7.7
	9.0	8.0
	10.8	8.3
	11.2	8.9
	11.4	9.6
Bacillus strain YN-2000	7.5	7.5
	8.5	7.9
	9.5	8.1
	10.2	8.4
B. halodulans C-125	7.0	7.3
	7.5	7.4
	8.0	7.6
	8.5	7.8
	9.0	7.9
	9.5	8.1
	10.0	8.2
	10.5	8.4

survive in an alkaline Milieux, alkaliphiles must adjust their strategies of energy conservation. One possibility is to change the coupling ion. For example, some anaerobic alkaliphiles use Na^+-coupled ATP synthases (49). However, this is surprisingly not adopted by the alkaliphilic aerobic *Bacillus* species. These microorganisms have respiratory chains that pump H^+ ion outward and H^+-coupled ATP synthases, apparently like those of neutrophilic microorganisms. For the present, the mechanism of ATP synthesis in these microorganisms remains obscure. Krulwich (47) presented several possible models of the proton translocated outward by respiratory chains during ATP synthesis. One possibility is that the protons in electron transfer reactions are not released into free solution but extruded directly by the respiratory chains to the ATP synthase. A diagram of these elements is shown in Figure 4. An Na^+/H^+ antiporter, which is the major mechanism for extruding Na^+ and which also functions to bring protons into the cell for pH homeostasis in alkaliphilic *Bacillus* species, is shown. The Na^+/H^+ antiporter creates the sodium potential necessary for the Na^+/solute symporters because a primary Na^+ pump is not present. The antiporter uses the proton motive force (Δp) as an energy source. Also shown are Na^+/solute symporters that use the sodium electrochemical potential to accumulate solutes. The flagellar motor is drawn to turns at the expense of the sodium electrochemical potential. The respiratory chain represents a hypothesis in which protons are transferred directly from this complex to the proton coupled F_1Fo-ATP synthase in a protein–protein interaction. The cell wall–associated layer reflects the finding that, in *B. halodurans* C-125 and *B. pseudofirmus* OF4, respectively, different negatively charged polymers play at least some role in pH homeostasis (47).

Genomics

Numerous alkaliphile genes have been sequenced and physical maps have been reported from *B. pseudofirmus* OF4, *B. halodulans* C-125, and *Micrococcus* sp. Y-1 (50–52). The first whole genome sequence project for an alkaliphilic *Bacillus*, *B. halodurans* C-125, was completed in 2000 (53) and the postgenome project, especially directed toward clarifying the mechanisms of alkaliphily, has begun. The vastly increasing database on sequences of alkaliphilic proteins will provide insights into global adaptations in cytoplasmic proteins or in functional cytoplasmic assemblies, namely, ribosome, secretory particles, and so forth.

CONCLUSION

Alkaliphiles are unique microorganisms with great potential for physiology and biotechnology. The aspects that have received most attention in the late 1990s include (*1*) extracellular enzymes and genetic analysis of their production, (*2*) mechanisms of "alkaliphily," and (*3*) taxonomy of alkaliphiles. Taxonomy of haloalkaliphiles and anaerobic alkaliphiles is a very active field, and the number of microorganisms keeps increasing. So far, studies of alkaliphiles belonging to the genus *Bacillus* have led to the discovery of many alkaline enzymes and mechanisms of "alkaliphily" under extremely alkaline pH. However, new findings and an even more diverse group of applications of alkaliphiles from genome analysis, proteome studies, and novel ecological, molecular, and biophysical approaches will soon provide greatly expanded developments.

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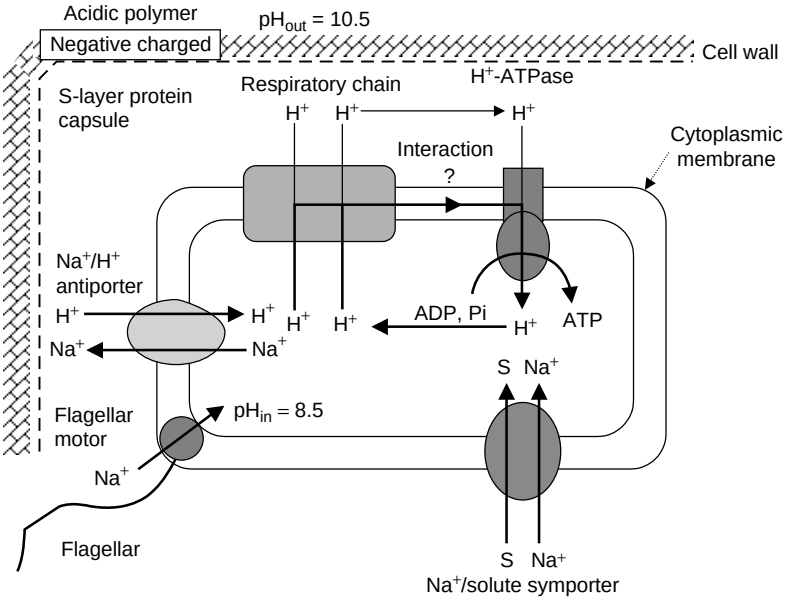

Figure 4. A model depicting the energy coupling mechanisms of alkaliphilic *Bacillus* species. The active pH homeostasis mechanism is shown as including respiration, Na^+/H^+ antiporter, and Na^+ re-entry pathway. The respiration chain extrudes H^+ (electron donors/acceptor not shown). The Na^+/H^+ antiporter is probably more Na^+-specific than those involved in pH homeostasis in neutralophilic bacteria. The Na^+ re-entry pathways are shown as a channel associated with the flagellum and Na^+/solute symporters (47). Other participants that have been suggested to function in the establishment and/or maintenance of an acidified cytoplasm relative to the medium are indicated and are discussed in the text. See color insert.

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AEROBIC ENDOSPORES

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Aerobic spores are not pathogenic to humans, which makes them useful as indicators of treatment effectiveness for drinking water. Because direct monitoring for waterborne pathogens such as protozoa and viruses is very inaccurate, time-consuming, and expensive, monitoring for aerobic spores can become a viable surrogate for the removal effectiveness of pathogens and parasites. Moreover, because most pathogenic organisms, even those found abundant in water sources, seldom are detected in treated drinking water, calculation of their removal through treatment is not possible. In contrast, aerobic spores are present in high concentration in most surface waters, and their presence can also be detected in treated water, thus allowing for an evaluation of treatment effectiveness.

The effectiveness of disinfection, especially the ultraviolet (UV) light treatment of both water and wastewater, can also be evaluated using aerobic spores as indicators. Being resistant to chemical disinfection and UV irradiation, aerobic spores can be used in development of disinfectant dose-response curve, thus allowing for a calibration and measurement of disinfection efficiency.

BACKGROUND

As we enter the new millennium, the drinking water industry is undergoing a major paradigm shift. This is because of increasing concerns over the potential presence of pathogens in potable waters and the large number of people with compromised immune systems. It has become clear that continuous process optimization based only on the current practice of turbidity monitoring (and to some extent particle counting) of treated water needs to be reevaluated (1). Although monitoring finished water quality is an essential step in the production of safe drinking water, traditional linkages between process monitoring and plant operation fail to provide an adequate margin of safety to enable operators to respond to temporal changes in source water quality (2). This monitoring paradox manifests itself periodically when cases of waterborne disease outbreaks occur even when finished water is in full compliance with regulatory requirements (3–5).

Water treatment regulations are becoming increasingly more stringent. However, emerging regulatory requirements do not provide utilities with a framework for optimizing treatment operations and processes to achieve finished water quality objectives. Alternative approaches for accommodating variability in source water quality are needed to provide plant operators with a sound basis for ensuring reliable plant performance.

Monitoring for microbial pathogenic contaminants presents a special challenge. Sampling and analytical methods that are available to detect *Giardia* and *Cryptosporidium* have limited sensitivity and accuracy (6). The time required for analyses does not allow for the practical use of the results in process control. Lack of "real-time" measurements of pathogens in water prevents utilities from being able to react to source water quality changes and provide proactive treatment optimization. In the absence of quick and reliable analytical methods for pathogens, pathogen indicators should be used.

The need for some measure that utility operators can use to determine how well their plants are optimized for the removal of pathogens is paramount if they are to achieve maximum treatment effectiveness. It has become clear that turbidity, as currently regulated, is an inadequate measure for this purpose. Recent waterborne outbreaks in the United States have been reported even though turbidity was within regulatory limits. Moreover, research has shown the presence of *Giardia* and *Cryptosporidium* even at turbidities of less than 0.1 NTU (4,7,8). A further complication to the turbidity issue is that optical turbidimeters are not accurate at readings less than 0.1 NTU (9).

In recent years, the use of on-line measurements by utilities to supplement routine sampling and analysis has increased substantially. Currently, the major types of on-line monitors in use at water treatment facilities include turbidity, pH, and ion-specific electrodes. Other options include particle counting and streaming current monitors. Relationships between on-line measurements and other monitoring parameters have not been developed. Correlation between suspended solids, particle counting, turbidity, and microbial content are highly source-specific (7), and most likely depend on the fraction of organic matter contained in the colloidal and suspended solids (10).

Common indicators are needed to establish a platform for comparison of water quality of various sources and their treatability, as well as to evaluate the efficiency of treatment achieved at various plants. Some indicators can be selected from the commonly used water quality parameters, such as turbidity, particle counts, and coliform bacteria (10–12). A critical assessment of microbiological parameters that could serve as surrogates for pathogenic microorganisms is needed to identify readily measurable analytes that can be quickly incorporated into the decisions made about water treatment plant operation.

The need for a surrogate has become increasingly apparent in the water industry in light of well-documented cases of disease outbreaks traced to drinking water as the route of transmission. Although the causative agent for a number of these outbreaks was identified as *Giardia* or *Cryptosporidium*, the affected water treatment plants did not have an effective way to monitor for the presence of the pathogen or to judge the treatment efficiency in eliminating the organisms from the finished water. Since these outbreaks, it has become even more apparent that the current methods for analyzing for *Giardia* and *Cryptosporidium* in watersheds and in finished water are inadequate. Besides, the reliability and reproducibility of the existing methods being an issue, there is a serious time lag between sample collection and data availability. Therefore, it is impossible to use even these flawed methods as a means to optimize treatment processes. Despite the water industry and the U.S. Environmental Protection Agency's (EPA's) attempts to improve this analytical method, the problems with its use remain. Although research into innovative and more rapid methods using such techniques as polymerase chain reaction and flow cytometry is promising, additional research is required before practical monitoring tools are available to the water industry.

SURROGATES FOR PROTOZOA IN DRINKING WATER

In recent years, a substantial effort has been focused on investigating the use of surrogates for optimizing treatment of drinking water and pathogen removal. Generally, it is agreed that an ideal surrogate would be abundant in water but not multiplying in treatment plant basins, resistant to treatment, and easy to detect and enumerate with cost-effective methods. The search for the surrogate includes both microbial and nonmicrobial parameters.

A research project on biological particle surrogates for filtration performance, performed at Colorado State University in 1995–1998 (13), has addressed the need for indicators that would serve as tools in plant optimization and was designed to evaluate a wide spectrum of microorganisms and their potential to serve as surrogates for monitoring of pathogen removal through filtration. This effort indicated that some bacteria, bacterial phages, and algae could represent *Giardia* and *Cryptosporidium* in removal mechanisms. In pilot tests, the use of various microbial surrogates was tested through seeding experiments. Significant correlation was established between the removal of *Giardia* and *Cryptosporidium* and some of the surrogates tested. However, because of a lack of supportive data, the significance of these correlations may be suspect. One suggested conclusion was that the strength of the correlations was dependent on the quality of measurements or enumeration of the surrogates. In fact, the strongest correlation was found with those surrogates that had the smallest deviation within the data set. A conclusion was also suggested that the deviation observed was due to error in measurement—the smaller the error, the more apparent the "true" relationship between pathogen and surrogate removal. The study demonstrated the difficulty in finding a reliable surrogate when there is so much uncertainty in the analytical methods for enumerating the pathogens.

Optical particle counting and sizing ("particle counting") has been considered a good candidate for a surrogate measure for pathogen occurrence in watersheds and treatment processes. Importantly, many utility operators and consulting engineers have begun to use this analytical technique in water production. A project evaluating data from 100 drinking water plants across the United States was initiated to assess particle removals by filtration (8). Specific objectives of this study included: distribution of

particle counts, factors that affect finished water particle counts, evaluation of the relationship between particle counts and pathogen concentrations in source and filtered water. Some relevant findings of this project indicated that particle counting can be used by utilities to optimize treatment for particulate removal, but a relationship between the two parameters is not straightforward. In full-scale sampling, a correlation between the occurrence of pathogens and the number and distribution of particles in watersheds was not substantial. Particle count was not found to be a good indicator of pathogen occurrence in watersheds. An exception to this observation was noted during storm and run-off events that resulted in higher levels of particles and in pathogens entering the source water intake. These data indicated that particle-counting measurement could be used as an alarm for unusual events, but not as a reliable indicator of the presence or absence of pathogens in a watershed. The uncertainty in the method used in enumerating the pathogen was indicated as a factor preventing the accurate comparison of particles and pathogen concentrations.

One of the important caveats when looking for a surrogate, however, is that the concept of log removal as a measure of filtration effectiveness is somewhat limiting in that the maximum log removal is governed by the detection limit of the analytical methods. Therefore, the maximum log removal that could have been obtained when considering the pathogen data is governed by the source water pathogen levels. Much higher levels of particles occurring in source water allowed calculation of higher removal effectiveness. It is almost numerically impossible to obtain similar log removals for particles and pathogens. However, because pathogens are "particles," it stands to reason that measuring particulate concentrations through a treatment process and optimizing the performance of that process to reduce particles will result in lower levels of pathogens. Particle counting is a good measure of these particulate changes. Measurements of finished water concentrations of particles or any microbial indicator of water quality could provide the assurance of consistency of plant operations. Water treatment systems should focus on the consistency of providing very good water quality, even during changes in source water quality. Consistently low concentration of particulates (and therefore microbial contaminants) in finished water may present a higher confidence in high water quality than the assurance of a specified log removal of these particulates. The direct monitoring of finished water quality may therefore be in conflict with the concept of log removal because when source water concentrations increase, so could the log removal increase even if the finished water quality deteriorates.

The database of the U.S. EPA's Information Collection Rule (ICR), (14), is an additional source of information about presence and removal of pathogens in treatment plants across the United States. The ICR data were collected and made available as a result of monthly monitoring for *Giardia*, *Cryptosporidium*, and enteric viruses conducted for 18 months (July 1997–January 1988) at more than 500 plants. Although samples were analyzed at various laboratories, making data comparison difficult, the resulting database concerning removal of these pathogens could be a valuable source of information concerning pathogen occurrence. The *Giardia* and *Cryptosporidium* occurrence data, collected during the ICR, show an overwhelming majority of sample results falling below the analytical detection limit, making the evaluation of national occurrence of these pathogens and an assessment of their treatment effectiveness more difficult and stressing a need for their surrogate more than before.

AEROBIC SPORES AS SURROGATES FOR REMOVAL OF PROTOZOAN PARASITES

A research effort, sponsored in 1995 by the U.S. EPA in Cincinnati, Ohio, focused on comparison between occurrence and removal of indigenous aerobic bacterial spores and particle counts (15). The project findings indicated that spores of spore-forming aerobic bacteria such as *Bacillus* could serve as a good surrogate of pathogen removal. Naturally occurring *Bacillus* spores can be easily and quickly detected in both the source and the treated water. The spores did not propagate in the various treatment processes. Removal of *Bacillus* spores correlated highly with removal of particles. The working relationship between the project team at EPA and the team involved in this proposed project allowed for an exchange of information and a continuation of efforts undertaken by other researchers.

In July 1994, the State of Utah and five major Utah water utilities organized the Utah Water Quality Alliance and initiated a Water Quality Enhancement Study to evaluate treatment effectiveness based on an increasing need to provide protection against pathogenic microorganisms (16). In this study, 10 water treatment plants were sampled for *Giardia*, *Cryptosporidium*, total aerobic spores, *Bacillus* spores, turbidity, and particle count. The project tasks included source and finished water quality data collection, development of water quality standards, search for surrogate analytical tools, and treatment optimization. A major conclusion of the study was that conventional monitoring practices do not provide sufficient information on source water quality variations to provide plant personnel with adequate response time to adjust plant operations. Thus, the ability to ensure a consistently reliable treated water quality, free of pathogenic organisms, is handicapped by inadequate linkages between monitoring and operating practices. A key issue that resulted from the study is the need for improved integration of source water quality parameters into strategies for operation of treatment unit processes.

To evaluate potential pathogen surrogates for treatment plant performance optimization, log removals for all measured parameters (*Giardia*, *Cryptosporidium*, total aerobic spores, *Bacillus*, particle count, and turbidity) were compared. The best correlation was seen between either total aerobic spores or *Bacillus* and *Cryptosporidium* followed by *Giardia*, particle counts, and turbidity. It was concluded that with lack of detectable amounts of *Giardia* and *Cryptosporidium* in filtered water, either total aerobic

spores or *Bacillus* spores proved to be a reliable measure for assessing plant performance effectiveness.

In 1996, the American Water Works Association Research Foundation sponsored a study to evaluate the applicability of pathogen surrogates to be used by treatment plant operators as a tool in continuous plant performance optimization (17,18). The target pathogens were *Giardia*, *Cryptosporidium*, and enteric viruses. Pathogen surrogates were selected among indigenous, nonpathogenic microorganisms that represent the target pathogens in removal through treatment. In undertaking this project, it was assumed that the detection of the surrogate should be quick, simple, and inexpensive. The selected surrogate could then become a tool to integrate source and finished water quality information with treatment plant operations. The implementation of a representative surrogate as a monitoring and operational tool should improve overall treatment process reliability and provide a sound basis for reducing the likelihood of microbial pathogen breakthrough. The project findings provided information on the source water occurrence of *Giardia*, *Cryptosporidium*, enteric viruses, total aerobic bacterial spores, *Bacillus* spores, total anaerobic bacterial spores, *Clostridium* spores, heterotrophic plate-count bacteria (HPC), *Escherichia coli*, fecal and total coliforms, bacterial phage, turbidity, and total particle count. The corresponding source water concentrations of *Giardia*, *Cryptosporidium*, and viruses were compared to these of other organisms to evaluate the potential of using surrogates to estimate the pathogen occurrence. Removal of the potential surrogates through treatment was examined, and compared to the removal of *Giardia*, *Cryptosporidium*, and viruses to evaluate the potential of using surrogates to estimate treatment plant performance. The effects of variables such as water source type, source water quality, and seasonal changes on the effectiveness of using surrogates to assess source water occurrence of pathogens were evaluated. Finally, the effects of treatment type, filter type, disinfectant type and application point, filtered water pH, as well as source water quality (turbidity and pathogen concentration) on the effectiveness of using surrogates to evaluate treatment plant performance were assessed.

Aerobic Spores Versus Other Surrogate Parameters for Protozoa in Drinking Water

Among possible microbial indicators of the removal of protozoa in drinking water treatment plants, aerobic spores could serve best because they meet most of the requirements of a surrogate measure. Surrogate parameters used in evaluating and improving water treatment plant performance should meet the following requirements: First, the organisms should be indigenous, nonpathogenic, and ubiquitous in an aquatic environment, especially in surface waters. They should be occurring naturally at concentrations greater than but corresponding to those of target pathogens. Next, their removal through treatment processes should be at levels comparable with removal of target pathogens. The surrogate parameters should be easy to analyze through techniques that are simple, quick, and economically feasible.

Table 1 contains a list of candidate surrogates and their capability to meet these criteria (19–26).

ANALYSIS OF WATER SAMPLES FOR AEROBIC SPORES

The twentieth edition of the *Standard Methods for the Examination of Water and Wastewater* (27) does not contain methodology for analysis of aerobic spores in water. Analysis of total aerobic spores and their major representative, *Bacillus*, is based on methods adopted by Rice and coworkers (14) from the dairy industry (28). The method has been further modified (18) to facilitate the adoption of the method by drinking water laboratories. On

Table 1. Candidate Surrogate Parameters for *Giardia* and *Cryptosporidium* in Water

Potential Surrogate	Natural Occurrence in Surface Waters	Removal Similar to Pathogens	Analysis Turn-Around Time, Simplicity, and Costs	Candidate Surrogate
Turbidity	Yes	Site- specific	Quick, simple, inexpensive	Yes
Particles >2 μm	Yes	Yes?	Quick, simple, relatively inexpensive	Yes
Streaming current potential	Yes	Treatment specific	Quick, simple, relatively inexpensive	Used only as baseline
Total and fecal coliform, and *Escherichia coli*	Yes	No	Quick, simple, inexpensive	Used only as baseline
Heterotrophic plate count	Yes	Treatment specific	Quick, simple, inexpensive	Yes
Micrococcus luteus	Yes, very low	?	Quick, simple, inexpensive	No
Anaerobic bacterial spores and *Clostridium perfringens*	Yes, very low	?	Quick, complicated, inexpensive	Yes
Aerobic bacterial spores and *Bacillus subtilis*	Yes	Yes	Quick, simple, inexpensive	Yes

the basis of the results of a study on methods comparison, the incubation temperature and time needed to kill all vegetative cells and to induce the spores to germinate were set at 60 °C for 15 minutes in a shaking water bath (150 rpm), (following pretreatment in a water bath at 37 °C for 30 minutes). The germinated spores in water samples should be filtered through 47-mm diameter, 0.45-μm pore-size membrane filters and plated on a nonselective nutrient agar also containing soluble starch (0.1%) and trypan blue dye (0.01 μg/L) and incubated for 21 hours at 35 °C in a humid environment. The total number of colonies on that agar represented the number of aerobic spores present in the sample. The number of colonies that have cleared zones around them (indicating the presence of amylase, an enzyme produced by *Bacillus* spores that degrades starch) was a presumptive representation of the number of *Bacillus* spores present in the sample.

OCCURRENCE OF PATHOGENS, AEROBIC SPORES, AND OTHER SURROGATES IN SURFACE WATERS

An example of the relative concentration of *Giardia* and *Cryptosporidium* and their microbial indicators, including aerobic spores, as well as some nonmicrobial indicators, is presented in Table 2, which was developed, based on the source water analytical results collected over one year of sampling at 24 water treatment utilities in the United States and Canada (17,18). Typically, the microbial content of surface source waters varies dramatically. Results of microbial analysis revealed that *Cryptosporidium* and *Giardia* are usually found in the least number of samples, whereas aerobic spore-forming bacteria were found in all source water samples tested. With the frequently observed exception of coliform bacteria and anaerobic spores, all the potential surrogate organisms occur more consistently in source waters than the pathogenic organisms, and at orders of magnitude greater concentration than those of the pathogenic organisms.

An example of the distribution of pathogens and their potential surrogates in source waters, based on the number of samples given in Table 2, is presented in Table 3.

Typically, *Giardia* and *Cryptosporidium* were detected in surface water samples at concentrations ranging from 1 to 10 per liter. In this study, only one sample revealed concentrations of *Giardia* in excess of 10 per liter. Anaerobic spores were usually detected at concentrations ranging from 1 to 10,000 per liter (0.1 to 1,000/100 mL). Aerobic spores were found to be more abundant, with typical concentrations ranging from 100 to 1,000,000 per liter (10 to 100,000/100 mL). Heterotrophic bacteria were found in a wide range of high concentrations as well, that is, from 1,000 to 10,000,000 per liter (100 to 1,000,000/100 mL). The physical surrogates (turbidity and particle count) vary significantly in various source waters.

It is very difficult, if not impossible, to establish a relationship between the *Giardia* and *Cryptosporidium* measurements and any of their potential surrogates, including aerobic spores. Figure 1 presents an example of the lack of correlation found between aerobic spores and the pathogens (17). Correlation coefficients (R^2) for occurrence among these pathogens to any of the intended surrogates in the source waters were less than 0.2 and showed no statistical significance. These results support a recommendation that the search for a *Giardia* and *Cryptosporidium* surrogate in natural waters should be put on hold pending the development of more sensitive and accurate analytical methods for these pathogens.

Factors Influencing Occurrence of Aerobic Spores in Surface Waters

Several factors can influence the concentration of aerobic spores found in waters. Because aerobic spores are soil-based organisms, their presence in water is associated with the presence of solids. Major factors responsible for the presence of spores in water are water quality (turbidity and particle concentrations), source water type (river

Table 2. Example of the Surface Water Microbial Quality Results (18)

Pathogen or Surrogate	Number of Samples	Percent Detection in All Samples	Median Concentr.	Twenty-Fifth Percentile Conc.	Ninety-Fifth Percentile Conc.
Giardia (/100L)	137	79	21	5	224
Cryptosporidium (/100L)	137	59	5	2	66
Enteric viruses (/100 L)	12	75	5	1	465
Aerobic spores (/100 mL)	242	100	1,820	528	25,280
Bacillus spores (/100 mL)	243	93	130	33	3,330
Anaerobic spores (/100 mL)	160	94	10	5	120
Clostridium (/100 mL)	160	92	6	2	78
HPC (/100 mL)	211	99	15,000	3,800	462,200
E. coli (/100 mL)	143	64	4	1	129
T. coliform (/100 mL)	176	85	39	6	4,496
F. coliform (/100 mL)	149	65	4	1	214
PhiX174 phage (/100 mL)	62	71	8	1	2,414
MS2 phage (/100 mL)	62	56	3	1	689
Turbidity (NTU)	195	100	3	1	33
Particles >2 μm (/mL)	68	100	8,937	5,675	360,633

Table 3. Distribution of Pathogens and Their Surrogates in Source Waters (18)

Range of Observed Number of Organisms per Liter	Number of Observations				
	Cryptosporidium	*Giardia*	Anaerobic Spores	Aerobic Spores	HPC
1–10	79	105	14	1	0
10–100	0	1	52	8	0
100–1,000	0	0	73	19	1
1,000–10,000	0	0	10	58	16
10,000–100,000	0	0	0	116	65
100,000–1,000,000	0	0	0	38	72
1,000,000–10,000,000	0	0	0	0	45
10,000,000–100,000,000	0	0	0	0	5

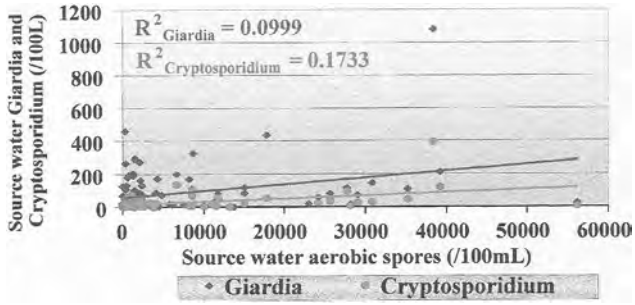

Figure 1. Correlation between occurrence of aerobic spores and parasitic protozoa in surface waters (18). See color insert.

or flowing stream and lake or reservoir), and seasonal changes.

A relationship between increasing aerobic spore concentrations and turbidity is presented in Figure 2.

PRESENCE OF AEROBIC SPORES IN TREATED DRINKING WATER

Aerobic spores can be found in treated drinking water samples. The median value, calculated after sampling at 24 drinking water plant effluents for 12 months (17,18) was 1,820 spores per 100 mL (the ninety-fifth percentile was 25,280 spores per 100 mL). Aerobic spores were detected in 84% of the filtered water samples. Because aerobic spores are not human pathogens, their presence in treated drinking water samples is an indicator of

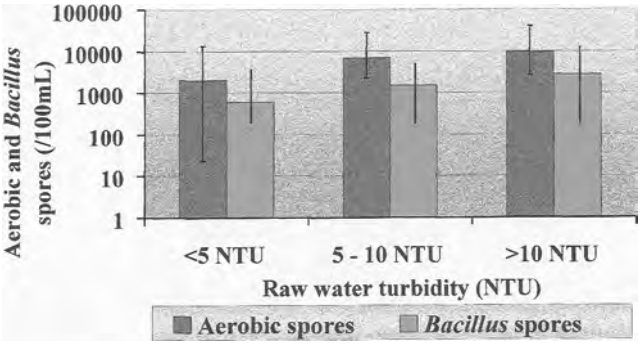

Figure 2. Comparison of aerobic and *Bacillus* spore occurrence to source water turbidity (18). See color insert.

a presence of particles that are soil-based, and not an indicator of water contamination that would pose a human health concern. They were found in sufficient concentrations in many of the source waters to be used as a direct indicator of treatment performance. Even for those waters with lower concentrations, aerobic spores can be used as relative indicators of treatment performance.

USING AEROBIC SPORES IN THE ASSESSMENT OF TREATMENT PLANT EFFECTIVENESS IN REMOVING PARTICULATES AND MICROBIAL CONTAMINANTS

The calculation of removal of microbial contaminants through treatment, and thus the ability to directly assess treatment plant performance, is controlled (or constrained) by the following factors:

1. The source water concentrations of microorganisms, or
2. The detection limits for microorganisms in finished water.

It has been documented that a well-operated water treatment plant can reliably remove more than 3 log of microorganisms and particles 2 μm in size (1,8–10,16). Therefore, it is necessary for the source water concentration and the filter water detection limit to be separated by at least 3 orders of magnitude to accurately assess a 3-log removal. Since most treatment plants are capable of greater than 3-log removal, even this range in source water occurrence and filtered water detection level limits an accurate assessment of performance.

To illustrate the relationship between source and finished water concentrations of *Giardia*, *Cryptosporidium* and aerobic spores, data in Figure 3 are plotted to show the frequency distribution of the organisms.

It can be noted that the range of concentrations of organisms in source water is significantly wider than the range of their observed concentration in filtered water. It can also be observed that the range between the median concentration of *Giardia* and *Cryptosporidium* in filtered water and the source water spans between 0.001 per liter and 0.1 per liter. The respective difference of median concentrations of aerobic spores in filtered and in source water ranges from 10 per liter to 100,000 per liter. This observation leads to an estimation of "a theoretically

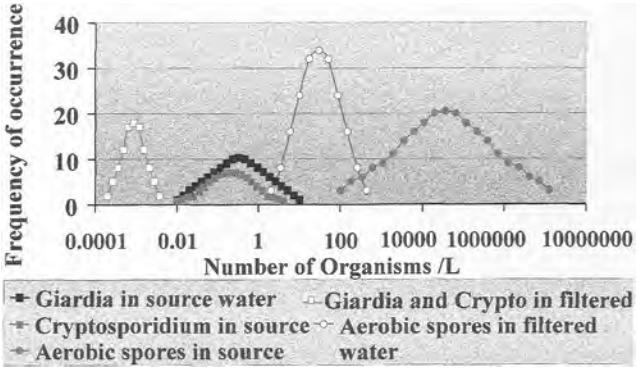

Figure 3. Distribution of aerobic spores and protozoan cysts in source and filtered water (18). See color insert.

probable removal capacity" that could be documented at a water treatment plant. Using naturally occurring aerobic spores detected in both the source and filtered water, up to 4-log removal can be documented at a majority of treatment plants. The removal of aerobic spores has been found to result from their physical removal through water treatment rather than inactivation (18). Inactivation of these organisms contributes little to their overall reduction in treated waters. In contrast, monitoring of *Giardia* and *Cryptosporidium* would only allow for documentation of 2-log removal through treatment in a typical plant.

Because of the limitations of the analytical procedures for *Giardia* and *Cryptosporidium*, no reasonable method has been found to date to develop a surrogate to predict treatment performance characterizing the removal of these pathogens. Thus, the search for a surrogate that correlates directly to pathogen treatment should be put on hold pending the development of sensitive and accurate analytical methods for the target organisms.

Practical Application of Aerobic Spores in Drinking Water Treatment

Aerobic spores and particle counts can be used as surrogate measures to evaluate or improve treatment plant performance. The spores present a more sensitive tool than particle counting as they maintain their size and shape, remaining intact through the treatment process. In contrast, particle counting does not reflect changes in the size of particles as they enter the plant, undergo coagulation into larger particles, and break apart when passing through the filters.

Among known indicators, only aerobic spores and particles are found in sufficient quantities in the source waters to provide a basis for assessing treatment plant performance, (i.e., removal through water treatment plant processes). HPC bacteria can also be found in abundance but cannot normally be used as a surrogate to directly evaluate treatment efficiency because they multiply in filters.

Limitations to Aerobic Spores as Microbial Surrogates

No "ideal" surrogate for predicting occurrence of protozoan pathogens (i.e., *Giardia* and *Cryptosporidium*) in source water was found to date due to the lack of accuracy in defining occurrence of these pathogens. The best relationship was found between turbidity and the pathogens. Even this relationship is only indicative of trends and not predictive in nature. All other comparisons had no perceptible relationship.

No correlation between surrogates and pathogens for removal could be derived in treated drinking water samples because it is not possible to calculate true pathogen removal. However, it is possible to use aerobic spores and particles to evaluate treatment performance based on direct removal calculations or on relative measurements of filtered water concentrations.

Benefits of Aerobic Spore Analyses to Water Treatment Plant Operations

Aerobic spores are sufficiently plentiful in the majority of source waters to allow for removal calculations that will demonstrate from 3- to 4-log treatment efficiency. In contrast, in most source waters only 1- to 3-log pathogen removal can be demonstrated. Future method development suggested that the sensitivity of the aerobic spore analysis can be improved, and its use as a monitoring technique could be extended.

In addition to calculating removal, evaluating finished water microbial quality could be performed by aerobic spore analysis. This type of microbial monitoring complements the physical measurements of turbidity and particles.

Efficiency of drinking water treatment will continue to be evaluated based on surrogates until a full range of pathogen analyses are developed that can easily test for all of the waterborne pathogens of concern. Even though an ideal pathogen surrogate has not been found, surrogates can and will continue to be needed to assist in assessing treatment efficiency. Several studies and drinking water utility practices have identified a number of factors that should be considered when selecting surrogates as predictors of treatment efficiency for pathogen removal.

The most important issue when selecting a surrogate is an understanding of the site-specific source water characteristics and treatment impacts on the surrogates. The surrogate parameter must be present in sufficient concentration to allow for an assessment of treatment efficiency. The concentration of the surrogate will dictate whether it can be used as a "direct indicator" of performance (e.g., removal of >3-log aerobic spores) or a "relative indicator" of performance (e.g., absolute value such as a turbidity value of 0.1 NTU).

Finally, understanding the impact of treatment on the surrogate concentration also is very important. For example, concentration of HPC bacteria may increase in a filter and obscure the actual removal efficiency of the process.

Using Aerobic Spores in Monitoring for Minimum Required Removal of Pathogens. To be used as a direct indicator of performance, the concentration of a surrogate in the source water must be greater than the detection limit in the filtered water by a factor equivalent to the removal to

be demonstrated and a safety factor. When this occurs, it is possible to monitor directly for the minimum required log removal by the treatment process.

For example, if aerobic spores are to be used as a direct indicator of performance for 3-log removal and the filtered water detection limit is 0.1 spores per 100 mL, the source water concentration must be at least 100 spores per 100 mL. A testing safety factor should be applied to assure reasonable accuracy, typically a factor of 2 (or 200 spores/100 mL should be satisfactory). Of the 24 utilities studied in the United States and Canada (18), more than 75% can use aerobic spores as a direct indicator of performance for 3-log removal. Over 50% of these utilities can use aerobic spores to demonstrate 4-log removal.

Particles greater than 2 μm are present in sufficient quantities in a number of source waters to allow for direct log removal determinations. More than 75% of the observed values were greater than 5,000 particles per milliliter. This allows for a 3-log removal calculation.

Probably the most important issue associated with monitoring to demonstrate minimum required assurance of log removal is an understanding that log removal is not appropriately assessed if the source water concentration is insufficient to calculate the desired log removal. As greater physical removal is achieved during treatment and there is a need to demonstrate better removal capabilities, it will be more difficult to demonstrate direct removal.

Monitoring of Treated Water Quality Through Monitoring of Aerobic Spores. Often, a surrogate is present in source water at a concentration below that necessary to allow for a direct determination of removal at a level that actually is achievable by the treatment process. A less than adequate source water concentration of the intended surrogate should not preclude its use as a parameter to assist in determining treatment plant performance. If this occurs, the surrogate might be used as a relative indicator of performance.

For example, source water may have variable particle concentrations from 1,000 to greater than 10,000 particles per milliliter. If the filtered water quality remains at 1 to 2 particles per milliliter, the actual log removal will be calculated at approximately 3 to 4 log. However, both calculations, and especially the 3-log removal calculation, are constrained by the source water concentration or the filtered water detection limit. It is not reasonable to imply that the calculated log removals are the true treatment efficiency of the facility. It is more reasonable to assign a given treatment efficiency for a given filtered water quality. This approach is being used to assure a 2-log *Cryptosporidium* removal in the U.S. EPA's Interim Enhanced Surface Water Treatment Rule. A 2-log removal will be credited to any facility achieving a filtered water turbidity of 0.3 NTU 95% of the time. Pilot-scale or demonstration-scale studies can be used to show that an absolute value for a surrogate in the filtered water indicates a predetermined level of treatment efficiency for pathogen removal.

A single surrogate measurement should not be considered sufficient to assess treatment. By measuring a number of surrogates in the filtered water (both physical and biological), it is reasonable to conclude that a specified level of treatment is being achieved. For example, a turbidity of less than 0.1 NTU 95% of the time, particles greater than 2 μm below 50 particles per milliliter, and aerobic spores below 2 per 100 mL may reliably indicate absence of pathogens. The actual level of treatment must be determined based on site-specific criteria and studies. Additional research in this area may lead to more universally applied treated water quality parameters.

Using Aerobic Spores in Challenge Studies. Challenge studies can be used to relate surrogate removal efficiency or a filtered water quality value to pathogen removal performance in a treatment facility. This is the basic approach that was used to produce the background data to support the establishment of a 0.3 NTU filter effluent quality being equivalent to a minimum of a 2-log *Cryptosporidium* removal.

Challenge studies can be conducted at pilot-, demonstration-, or full-scale. The studies can use viable pathogens, inactivated pathogens, bacterial spores, or other proven nonmicrobial pathogen surrogates such as microspheres. The selection of the appropriate constituent to use for the challenge study is site-specific.

The premise for a challenge study is that the surrogate and challenge constituent will be present in the source water at a concentration sufficient to measure the removal efficiency desired. Thus, seeding of the challenge constituent at a concentration sufficient to demonstrate the desired removal, and then comparing this to the surrogate treatment efficiency, can lead to the establishment of a treated water quality goal based on the surrogate measurement.

When there is a sufficient concentration of the intended surrogate in the source water supply, the challenge study can directly relate the removal efficiency of the surrogate to that of the pathogen. The treatment plant performance can then be determined based on the removal efficiency of the surrogate.

Where the concentration of the surrogate(s) in the source water is not sufficient, it will be necessary to relate the pathogen treatment efficiency to a filter water quality value, for example, aerobic spores. The result can be a set performance value that will relate to the level of pathogen performance desired. For example, it could be demonstrated that an aerobic spore concentration in the filtered water of less than 2 per 100 mL will assure a 3-log removal of Cryptosporidium oocysts. Turbidity and particle counts can be set in much the same way. The end result is the establishment of filtered water quality values for the surrogate(s) that relate to a desired pathogen removal.

For those source waters with highly variable surrogate concentrations, it may be desirable to establish both a filtered water value and a removal value. When the concentration in the source water is sufficient, a calculated removal can provide the desired treatment information. As the source water concentration drops below the value necessary to calculate the removal directly, a finished

water value may be sufficient to verify appropriate treatment. In most cases, there will be a maximum set for the finished water value, regardless of the source water concentration.

Each utility needs to determine which surrogates to develop and how the surrogates will be evaluated to verify the treatment efficiency of the treatment plant. The goal is to establish a number of surrogate measures that will assure appropriate treatment. Selection of the correct surrogates and verification of the surrogate measures as indicators of pathogen treatment will accomplish this goal.

CONCLUSION

Because removal calculations and the ability to directly assess water treatment plant performance is controlled (or constrained) by the concentration of pathogens in the source water and the detection limits for pathogens in finished water, it is difficult to accurately determine the rate of removal of parasitic protozoa such as *Giardia* and *Cryptosporidium* using the current analytical methods. Although sampling and processing significantly larger sample volumes results in lowering method detection limits and allows for calculation of higher log removals, it is very expensive and time consuming and not practical from the analytical standpoint. Aerobic spores are sufficiently plentiful in the majority of source waters to allow for removal calculations that would demonstrate from 3- to 5-log treatment efficiency. In contrast, only 1- to 3-log pathogen removal can be demonstrated based on natural occurrence of pathogens and analytical sensitivity. Aerobic spores can be used as effective surrogate measures to improve treatment plant performance. They cannot be considered, however, as direct surrogate measures of pathogen removal.

Because aerobic pores are nonpathogenic to humans and are not associated with fecal contamination, they could be used in challenge studies, being applied in a slug or at a step dose to water treatment plant unit processes and measured in the effluent to establish log removal. The analysis of aerobic spores is simple (using plate-count agar), quick (less than one day), and inexpensive ($25 per sample).

Evaluating finished water microbial quality can be performed by aerobic spore analysis. This type of microbial monitoring complements the physical measurements of turbidity and particles. The filtered water spore monitoring could be used as plant performance verification.

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AEROBIC RESPIRATION, PRINCIPLES OF

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Aerobic respiration is by definition the transfer of electrons from a donor compound through a series of carriers to molecular oxygen (O_2), which is reduced to water. Molecular oxygen serves as the terminal acceptor of electrons or terminal oxidant in this process. An oxidant is a compound that brings about the oxidation of other compounds and is therefore an acceptor of electrons. Likewise, a reductant is a compound that brings about the reduction of other compounds and is therefore an electron donor. Reactions involving the transfer of electrons are frequently termed redox reactions because they involve both oxidation and reduction reactions. An oxidation is the loss of electrons by an atom or molecule, whereas a reduction is the gain of electrons by an atom or molecule. The use of O_2 as a terminal electron acceptor differentiates aerobic respiration from "anaerobic" respiration reactions that use alternative oxidants such as nitrate, sulfate, or oxidized metals. Aerobic respiration is also distinct from fermentation in that carbohydrates serve as the terminal electron acceptors during fermentation. This article will focus on the mechanisms organisms have evolved to use O_2 as a terminal oxidant. A basic description of the structure and function of the components involved in aerobic respiration will be included. The organization of these complexes into functional respiratory chains will then be discussed. Examples of aerobic respiratory chains from well-studied model systems, along with a brief description of some of the mechanisms used by organisms to regulate the make-up of these chains, will also be provided. The primary focus will be on aerobic respiration in bacteria. However, the information discussed here is applicable to respiration by higher organisms because the mechanisms used by all organisms to couple respiration with energy production are essentially the same. Aerobic bacteria use a much broader range of respiratory strategies than higher organisms, so it is useful to discuss these strategies to help understand how bacteria can adapt to many different physiological niches.

BASIC FUNCTIONS OF AEROBIC RESPIRATION

Conservation of Energy

The most important function of respiration is energy conservation via the formation of ATP. This is termed *oxidative phosphorylation* because the oxidation of reduced substrates leads to the phosphorylation of ADP to produce ATP. The hydrolysis of phosphate from ATP, which is part of numerous reactions in the cell, releases a relatively large amount of Gibbs free energy (ΔG), a measure of the energy available for work. The "standard" value (ΔG^o) of the free energy available from the hydrolysis of ATP is approximately -7.7 kcal/mol. (Standard free energy values (ΔG^o) are calculated by setting the concentrations of both the products and the reactants at 1 M, and, according to convention, the larger the negative value of the Gibbs energy change the greater the energy available.) Because actively growing cells maintain a high concentration of ATP relative to that of ADP, the actual free energy (ΔG) of ATP hydrolysis is greater (more negative) than the standard free energy value. For example, it may be -10 kcal/mol. The biosynthesis of ATP requires the input of an amount of energy equal to that of the actual free energy of hydrolysis, ΔG. Respiration can provide this energy because the flow of electrons through the carriers that make up the respiratory chain to the terminal oxidant is energetically favorable. One form of the Nernst equation (Eq. 1) relates the amount of energy in electrons flowing through the electron transport chain to the amount of Gibbs free energy available to do work. In this equation, ΔG is the Gibbs energy change, n is the number of electrons transferred, F is the Faraday constant (23.06 kcal/V/mol) and ΔE_m is the difference between the redox potentials of the oxidation and reduction half-reactions of the reaction of interest.

$$\Delta G = -nF\Delta E_m \quad (1)$$

It can be seen from Equation (1) that, as long as mechanisms are available, cells can maximize energy production by transferring electrons from donors of low potential to terminal acceptors of high potential. The larger the overall voltage drop the greater the energy available, similar to a battery. The redox potential of the reduction of O_2 to H_2O is $+0.82$ V, which is one of the highest redox potentials of any biologically useful oxidant. This is one of the principal reasons O_2 is preferred as a terminal oxidant. This high midpoint potential also makes it possible to use a wide range of compounds as electron donors while maintaining an energetically favorable ΔE_m. The most common electron donor is the enzyme cofactor NADH, which is produced by the reduction of NAD^+ during many reactions in the cell involved in the oxidation of reduced carbon compounds. The redox potential of the NAD^+/NADH couple is -0.32 V. Using equation (1), the transfer of two electrons from NADH to O_2 yields a ΔG of -53 kcal/mol, which is sufficient free energy to drive the synthesis of several molecules of ATP.

Peter Mitchell first elucidated the mechanism, by which energetically favorable electron flow is coupled with energy requiring biosynthesis of ATP; the Mitchell theory of energy production and conservation is termed *chemiosmosis* (1,2). In the now proven chemiosmotic mechanism, the energy of electron transfer to O_2, via the respiratory electron transfer chain, is used to move a few negative and positive charges to opposite surfaces of a membrane, either by pumping protons to the positive surface or electrons to the negative surface (Fig. 1). The protein components of the respiratory chain are oriented with specific topology in the membrane to drive such charge separation events (Fig. 2). Lipid bilayers are relatively impermeable to charged species; this allows the transmembrane charge separation to persist as long as

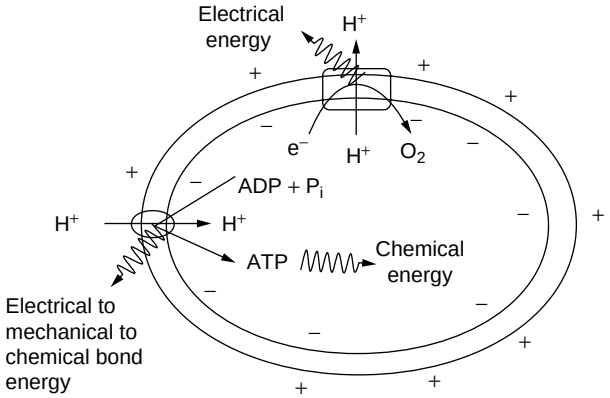

Figure 1. Schematic representation of the chemiosmotic circuit in a bacterial cell. The membrane bilayer is shown with the positive (+) and negative (−) charges that are closely associated with each face of the membrane during aerobic respiration. The redox energy of electron flow to oxygen within a terminal oxidase is used to pump protons through the oxidase to the outer face of the cytoplasmic membrane, thus establishing the separation of charges that is termed the transmembrane voltage gradient, or $\Delta\Psi$. $\Delta\Psi$ provides the driving force for proton flow through the F_0 portion of the F_1F_0-type ATP synthase, where the proton flow initiates a carousel-like rotation of a ring of proteins within the membrane. This rotary motion leads to the rotation of a protein spindle within the F_1 portion of the ATP synthase. Rotation of this spindle forces the active sites in F_1 that bind ADP and phosphate (Pi) to change shape, with the result being the formation of a chemical bond between ADP and Pi and the release of the product, ATP.

electron transfer continues. The charge separations create a transmembrane voltage gradient and this gradient attracts positively charged ions to the negative surface of the membrane. In fact, it is the flow of protons through the ATP synthase in the bacterial membrane, from the positive surface to the negative surface, which provides the energy for ATP synthesis (this is discussed in more detail later). Because only protons move through the ATP synthase, the transmembrane voltage gradient is also termed the *proton motive force* (PMF). A more detailed explanation of chemiosmosis can be found in (3).

Those components of the respiratory electron transfer chain that use some of the energy of electron flow to generate PMF via charge separations are termed *electrogenic protein complexes*. The respiratory electron transfer chain in all mitochondria contains three electrogenic complexes (4). The situation in bacteria, however, is considerably more complicated. Some bacteria modulate the number of electrogenic complexes in their electron transport chain depending on the environmental conditions as well as their physiological requirements. Moreover, many bacteria synthesize fewer electrogenic complexes than do mitochondria, although they use the same reductants and terminal oxidants.

Aerobic bacteria can also use a much wider variety of compounds as electron donors than higher organisms. In addition to carbon containing compounds, many inorganic compounds can serve as sources of electrons. Bacteria that can use inorganic compounds as sources of energy

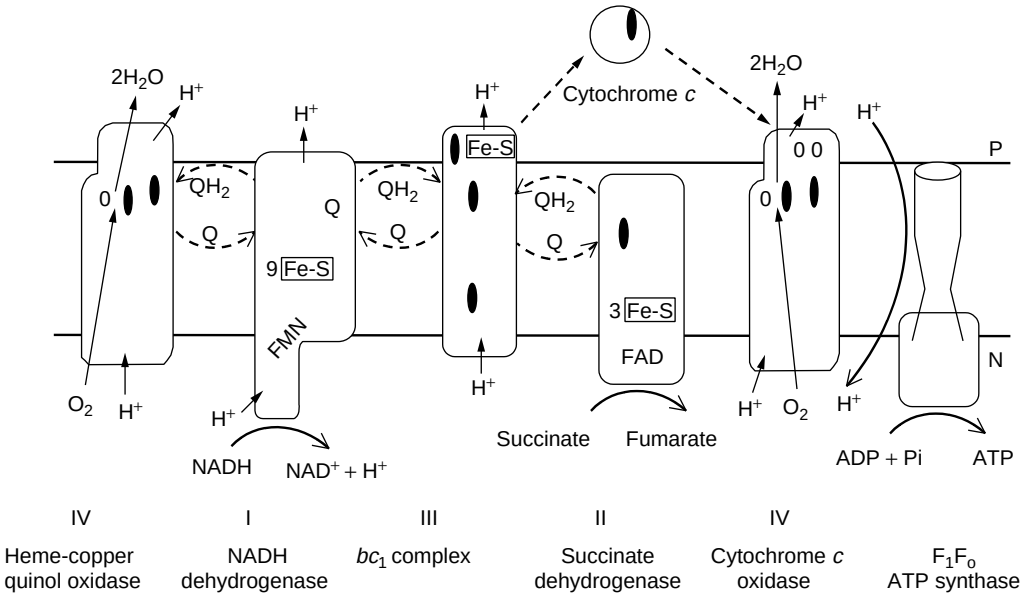

Figure 2. Representation of some of the protein complexes involved in aerobic respiration and the reactions they catalyze. The name of each complex is indicated below the complex along with its shorthand designation as a Roman numeral. Solid ovals represent heme centers and open circles represent copper. P and N represent the positive and negative surfaces of the membrane. Fe–S indicates complexes containing iron-sulfur centers and FAD and FMN represent flavin moieties. Dashed arrows within the membrane indicate the redox cycling of the quinone pool that occurs as NADH dehydrogenase and succinate dehydrogenase reduce quinone (Q), while the bc_1 complex and quinol oxidases oxidize quinol (QH_2). Redox cycling of the cytochrome *c* pool in the periplasmic space of the cell also occurs as cytochrome *c* is reduced by the bc_1 complex and oxidized by cytochrome c oxidases. The ability of four of the five electron transfer complexes shown to function as proton pumps is indicated by the entry and exit of H^+ from each complex.

are known as *lithotrophs*. For example, there are bacteria termed *nitrifiers* that use nitrite as their energy source by oxidizing it to nitrate. The relatively high midpoint potential of this reaction (+0.41 V) decreases the ΔE_h of electron transfer to O_2, as compared with the oxidation of NADH, thus reducing the amount of the Gibbs free energy that can be recovered (Eq. 1). Consequently, these bacteria must oxidize more reductant than is oxidized by bacteria that use NADH to produce an equivalent amount of ATP. The benefit of this strategy is that the development of an aerobic respiratory chain that oxidizes inorganic compounds allows lithotrophs to occupy a unique environmental niche.

The high concentration of O_2 in the atmosphere, along with the energetic efficiency of the aerobic respiratory chain, makes aerobic respiration the preferred mode of energy conservation for those organisms that can switch between aerobic and anaerobic modes of growth. Other modes of energy conservation, such as fermentation, are not as efficient in the production of ATP. In addition, fermentation and anaerobic respiration may result in the buildup of products that can be toxic at modest concentrations. Aerobic respiration by most organisms generates CO_2 and H_2O as its end products, neither of which is particularly cytotoxic. Accordingly, the regulatory circuitry of aerobic bacteria is designed to preferentially express those genes required for O_2 respiration at the expense of alternative modes of energy conservation whenever O_2 is present (5).

Detoxification

Aerobic respiration can fulfill other physiological functions besides ATP production. One of these alternative functions is to protect O_2-sensitive components of the cell. For example, the bacterium *Azotobacter vinelandii* uses the O_2-sensitive enzyme nitrogenase to fix nitrogen even under aerobic conditions. *Azotobacter* minimizes O_2 damage to nitrogenase by maintaining high rates of aerobic respiration (6). This results in low intracellular concentrations of O_2. Because these bacteria want to maximize O_2 consumption, they must also maximize electron flow through the respiratory chain. This can be accomplished by modifying the components of the respiratory chain. By definition, the processes of electron transfer and charge separation are linked, or coupled, in the electrogenic complexes of respiratory electron transfer. As a result of this situation, it becomes energetically more difficult to pump additional charges (protons or electrons) against the transmembrane voltage gradient as the magnitude of the gradient increases. This results in a slowing of electron transport. However, bacteria can adjust the components of the respiratory chain to include complexes that are less efficient at coupling electron flow to the generation of a voltage gradient. This makes it possible to maintain higher rates of electron transfer to O_2. As discussed later, *Azotobacter* uses a particular terminal oxidase that is less efficient at coupling electron flow to the generation of PMF.

Consume Reducing Equivalents

Another possible use for electron transfer to O_2 is to replenish the supply of NAD^+. Bacteria growing on highly reduced carbon sources, such as fatty acids or aliphatic hydrocarbons, produce NADH at such a rate that the supply of NAD^+ may become limiting for the metabolism of the cell. Under such conditions, a cell might modify its respiratory chain to ensure that NADH oxidation can keep pace with NAD^+ reduction. Some bacteria have been shown to reduce nitrate to nitrite in order to oxidize the NADH pool (7). Although never directly shown, it seems likely that O_2 also serves as a sink for excess reducing equivalents under certain conditions and may help explain the diversity of aerobic respiratory chains in many aerobes.

COMPONENTS INVOLVED IN AEROBIC RESPIRATION AND THEIR FUNCTION

Electron Transfer and Charge Separation

The process of oxidative phosphorylation can be viewed as a series of energy transformations (Fig. 1). The function of the components of the electron transport chain is to take high-energy electrons (those with a more negative redox potential) and transfer them stepwise to O_2, which is reduced to water. Proper arrangement of the respiratory components allows some of the redox energy released in the stepwise transfer of electrons to O_2 to drive charge separation events across the cytoplasmic membrane (Figs. 1 and 2). Charge separation is accomplished by moving a positive charge (a proton) to the outer, positively charged surface of the membrane or by moving a negative charge (an electron) to the cytoplasmic, negatively charged surface of the membrane. Thus, a transmembrane voltage gradient is created and maintained, which is another form of electrical energy (Fig. 1). The voltage gradient is used to pull protons through the membrane-bound ATP synthase, which is a nanomachine that converts the energy of the voltage gradient into mechanical energy. As the ATP synthase couples phosphate to ADP and forms ATP, mechanical energy is transformed into chemical bond energy. A good deal of Gibb's free energy is made available upon the hydrolysis of ATP to ADP and phosphate; for this reason the hydrolysis of ATP is incorporated into the mechanisms of numerous reactions in the cell to make them thermodynamically favorable.

Description of Basic Components of the Respiratory Chain

For aerobes that use carbon compounds as electron donors, the flow of electrons during respiration centers on a pool of lipid-soluble quinone (Q); each quinone molecule is a two-electron (and two-proton) carrier. In these bacteria, all of the possible electron transfer pathways of aerobic respiration can be described by defining which membrane-bound electron transfer proteins are adding electrons to the Q-pool and which protein complexes are removing electrons from the Q-pool (Fig. 2). The chemical structure of Q varies somewhat throughout the eubacteria and the archea, but all contain a simple quinone headgroup coupled to a long hydrocarbon tail that restricts the

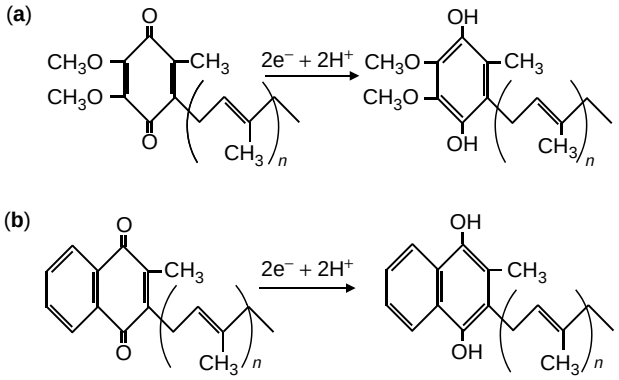

Figure 3. Structure of common quinones found in *E. coli* in their reduced and oxidized forms. Ubiquinol is shown in (**a**) and menaquinone is shown in (**b**). The length of the hydrophobic side chain (n) is variable but is typically 8 in *E. coli*.

quinone to the lipid bilayer (Fig. 3). Fully oxidized quinone and fully reduced quinol diffuse rapidly in the bacterial membrane to shuttle electrons between the membrane-bound electron transfer complexes. During the actual reduction or oxidation of each quinone, its headgroup binds to a specific site on the electron transfer complex. In this manner, the one-electron reduced quinone intermediates, which may be negatively charged, are sequestered from the hydrophobic lipid bilayer.

Eectrons enter the Q-pool in two ways: via the oxidation of NADH or via the direct oxidation of reduced metabolic intermediates, such as succinate. NAD^+ is a small, water-soluble two-electron carrier that is reduced to NADH in numerous cellular oxidations of reduced carbon substrates (see Table 1 for listing of respiratory complexes). When NADH is the source of electrons for respiration, it is oxidized by a NADH dehydrogenase. Many aerobic bacteria use a NADH dehydrogenase that is very similar to the mitochondrial enzyme (4). This enzyme is the largest of the membrane-bound electron transfer complexes of aerobic respiration, being composed of 13 to 14 separate protein subunits (8–11). NADH binds at a site on the internal extramembrane domain of the complex, where its electrons are transferred to a flavin prosthetic group (Fig. 2). Flavins are two-electron, two-proton carriers derived from riboflavin; they are always bound within proteins. The electrons then travel through a series of electron carriers known as *iron-sulfur centers* ([Fe–S]) spaced throughout the protein complex, and finally to a quinone that is transiently bound at a site accessible to the lipid bilayer. [Fe–S] centers are complexes of non-heme iron and acid-labile sulfur bound to particular residues in the protein complex (Fig. 4). These [Fe–S] clusters are of variable iron and sulfur stoichiometry and variable redox potential but are all one-electron carriers. The quinone reduced by the NADH dehydrogenase enters the lipid-soluble Q-pool. NADH dehydrogenase is one of

Table 1. Occurrence and Molecular Characteristics of the Protein Complexes of Aerobic Respiration in Representative Bacteria and Mitochondria

Respiratory Complex	Types	Number of Subunits	Complex Size (kDa)	Prosthetic Groups	Presence in: *Escherichia coli*	*Paracoccus denitrificans*	*Rhodobacter sphaeroides*	*Mitochondria*
NADH dehydrogenase (Complex I)	NDH I; *nuo*	13–14	~500–600	FMN, [Fe–S],Q	+	+	+	+[a]
	NDH II; *ndh*	1	~50	FAD	+	?	+	–
Succinate dehydrogenase (Complex II)		4	~115	FAD, [Fe–S], heme B	+	+	+	+
bc_1 complex (Complex III)		4	~110	hemes B and C, [Fe–S]	–	+	+	+[a]
Heme-copper terminal oxidases (Complex IV)	cbb_3-type cytochrome *c* oxidase	3	~120	hemes B and C, Cu	–	+	+	–
	aa_3-type cytochrome *c* oxidase	4	~120	hemes A, O, ±C[b], Cu	–	+	+	+[a]
	quinol oxidase	4	~120	hemes A, B, and O[b]	+	+	+	–
bd-type quinol oxidases (Complex IV)	*bd*-type	2	~100	hemes B and D	+	?	–	–
	bb-type	2	~100	heme B	–	?	+	–
F_1F_0 ATP synthase		20–23	~500	none	+	+	+	+[a]

[a]The mitochondrial enzyme has a greater number of subunits.
[b]Different hemes are present in various versions of these oxidases.

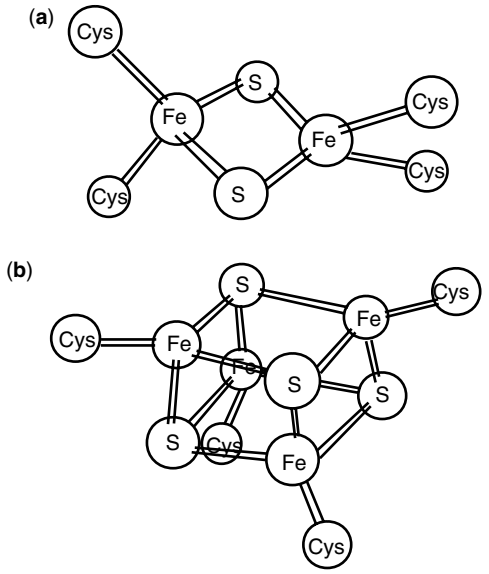

Figure 4. Examples of a [2Fe−2S] cluster (**a**) and a [4Fe−4S] cluster (**b**). The S represents acid-labile sulfur. One of the sulfur ligands for each iron (Fe) comes from a cysteine residue in the protein.

the three-electrogenic complexes that are used by aerobes, meaning that the enzyme contributes to the maintenance of the transmembrane voltage gradient. It does this by using a portion of the redox energy released during electron transfer from NADH to Q to pump protons from the negative surface of the cytoplasmic membrane to the positive surface. The mechanism of this proton pumping process is not fully understood (12,13).

Succinate dehydrogenase is a membrane-bound protein complex of three to four subunits that also transfers electrons from succinate to the Q-pool (Table 1, Fig. 2). This protein can be an important part of the respiratory chain of some aerobes if the cultures contain succinate or significant concentrations of tricarboxylic acid cycle intermediates. Like NADH dehydrogenase, the electrons flow through a flavin cofactor and a series of [Fe−S] centers to reach a lipid-accessible quinone-binding site (4,14,15). The energy released during electron transfer from succinate to Q falls short of that required to drive a charge separation event across the cytoplasmic membrane. Thus, the reaction catalyzed by succinate dehydrogenase is not electrogenic.

Electrons flow out of the Q-pool via two paths, both of which terminate with the reduction of O_2 to water through the activity of a terminal oxidase (Fig. 2). The first path requires a single membrane-protein complex that oxidizes quinol and reduces O_2; this type of terminal oxidase is referred to as a *quinol oxidase* (16,17). The second path from Q to O_2, commonly referred to as the *cytochrome c pathway*, involves two electron transfer complexes in the bacterial membrane. In the two-step pathway, quinol is oxidized by the bc_1 complex (18,19), which then reduces a mobile cytochrome *c*, a small electron transfer protein containing a single *c*-type heme (Fig. 2). Cytochrome *c* then shuttles its electron to a cytochrome *c* oxidase, another type of terminal oxidase that uses the electrons donated by successive cytochrome *c* molecules to reduce O_2 to water.

[Heme is composed of a conjugated ring of four pyrrole groups, termed *porphyrin*, with a central iron atom bound by one nitrogen from each pyrrole (Fig. 5). Hemes can be used as one-electron carriers; proteins that bind redox-active heme are termed *cytochromes*. Bacteria modify substituent groups of the porphyrin ring to produce at least four chemically distinct forms of heme, hemes B, O, A, or D. More than one type of heme may be present in multi-heme cytochromes.]

Electron transfer from reduced quinol to cytochrome *c* in the bc_1 complex occurs through a complicated dance that has been termed the *Q-loop* (18,20,21). The bc_1 complex is composed of three to four subunits (Table 1). One subunit contains two quinone-binding sites and two *b*-type hemes that are oriented perpendicular to the plane of the bilayer and span the membrane. A second subunit contains a *c*-type heme, and a third contains an [Fe−S] center in an extramembrane domain that moves back and forth on a flexible tether between the quinol oxidation site and the *c*-type heme (19,21). Some of the redox energy released by electron transfer from quinol to cytochrome *c* is used to push electrons through the *b*-type hemes from the positive surface of the cytoplasmic membrane (outside) to the negative surface (inside). Thus, electron transfer through the bc_1 complex is electrogenic, that is, it contributes to the transmembrane voltage gradient.

The third electrogenic component, present in all of the possible electron transfer pathways to O_2, is the terminal oxidase (4). Although the terminal oxidases of bacteria show the greatest level of diversity of any of the respiratory components (discussed later), basic structural and mechanistic similarities are shared by all of these enzymes (Fig. 6). All of the terminal oxidases are composed of two to four integral membrane proteins (16,22) (Table 1). Electrons from quinol or from reduced cytochrome *c* are passed by various means to a six-coordinate heme in the largest subunit of the complex. This heme transfers electrons, one at a time, to a nearby five-coordinate heme in the same subunit. Being five-coordinate, as opposed to the fully occupied six-coordinate geometry, allows one of the ligand binding sites of this heme's iron to serve as the O_2 binding site (23,24). Once O_2 binds, it is reduced by four successive electrons to two water molecules (25,26). The O_2 reduction site, or active site, of the oxidases is buried within the transmembrane region of the large subunit. The four electrons required to reduce O_2 follow a path from the outside to the buried active site (27,28); this path carries the electrons part of the way across the membrane toward its negative surface. Simultaneously, the four protons required to convert O_2 to $2H_2O$ are transferred from the cytoplasm to the buried active site; this path carries the protons part of the way toward the positive surface of the membrane (29–32). Because these electrons and protons combine at the active site to form H_2O, a neutral molecule, the partial electron and proton transfers add up to one charge separation event (i.e., the movement of one charge completely across the membrane) for each electron transferred to O_2. All

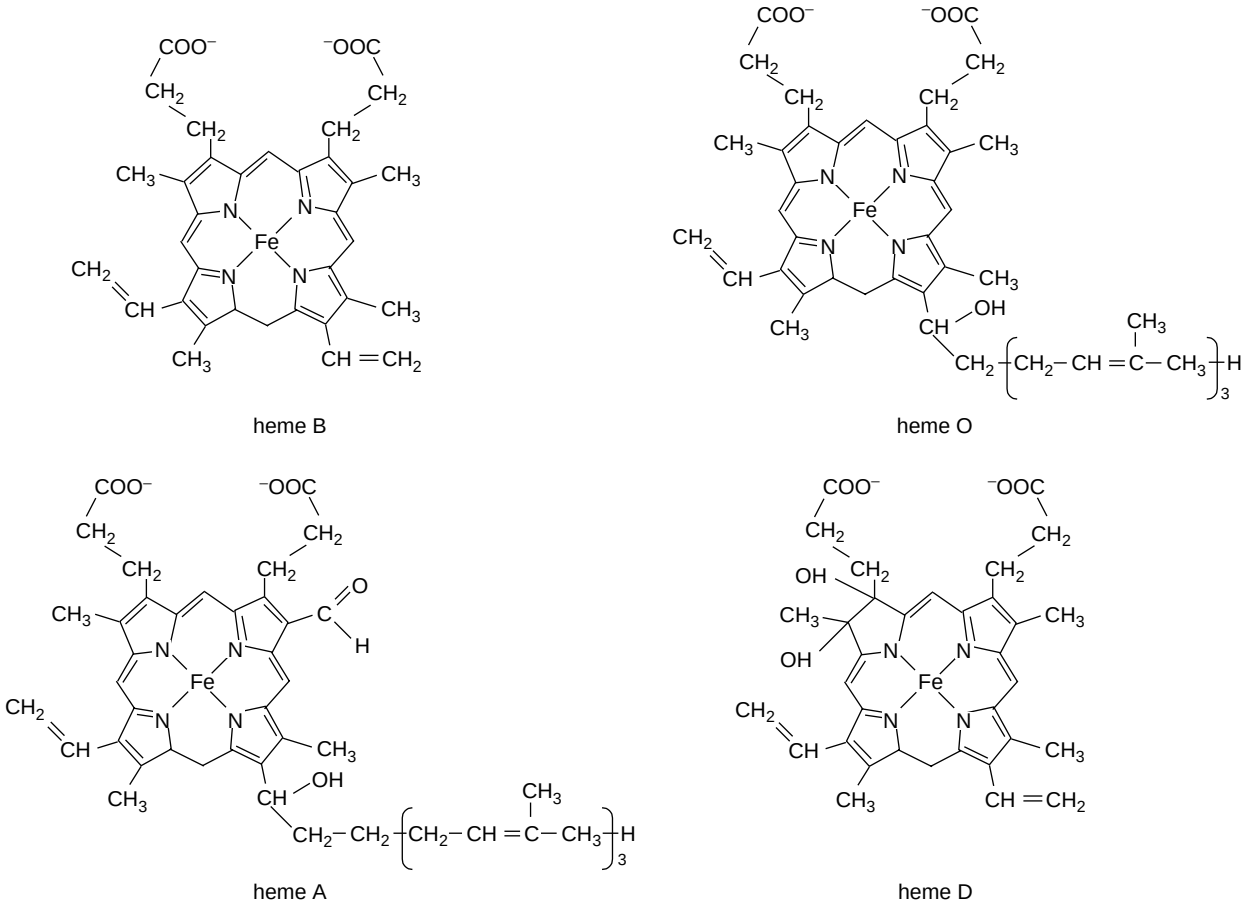

Figure 5. The different structures of the hemes found in common cytochromes of aerobic bacteria.

terminal oxidases are electrogenic in this fashion. The redox energy available in the oxidation of either quinol or cytochrome *c* by O_2 is substantial, however, and most terminal oxidases have evolved a second mechanism to convert more of this redox energy into the energy of the transmembrane voltage gradient. This is accomplished by pumping one proton, through the protein, from the negative to the positive surface of the membrane, for each electron transferred to O_2 (29). This proton is in addition to the charge separation achieved through the chemistry of O_2 reduction. It is not clear how the proton is transferred through the protein, but its transfer is obligatorily linked to electron flow through the oxidase complex (33,34).

Structural Diversity of the Essential Respiratory Complexes

The degree of structural diversity, as far as is currently known, varies considerably for each of the principal protein complexes. The structures and mechanisms of the bc_1 complex, succinate dehydrogenase, and the ATP synthase appear to be largely similar throughout the eubacteria and the archaea. Perhaps the greatest diversion known to date is the ability of certain marine bacteria, for example, *Vibrio alginolyticus*, to synthesize a NADH dehydrogenase, a terminal oxidase, and an ATP synthase that pump and use Na^+ rather than protons (35).

In addition to the proton-pumping NADH dehydrogenase, numerous eubacteria synthesize a simpler enzyme that oxidizes NADH and reduces Q (36). This simpler dehydrogenase consists of a single integral membrane protein containing a flavin prosthetic group; it does not use the energy of its electron transfer reaction to contribute to the transmembrane voltage gradient (Table 1).

The greatest degree of structural diversity is found in the terminal oxidases. Terminal oxidases are first divided into two groups on the basis of the structures of their active sites, in which O_2 is reduced to water. The first of these groups is composed of a family of quinol oxidases, the prototype of which is the *bd*-type quinol oxidase (Fig. 6). The *bd*-designation derives from the types of hemes associated with the complex. The *bd*-type oxidases are composed of two integral membrane subunits, one of which contains three hemes (26) (Table 1). Electrons from quinol are first transferred to a heme *b*, and then to the active site, which consists of a second *b*-type heme and a closely associated *d*-type heme. This two-heme center forms the active site. The *bd*-type oxidases have a high affinity for O_2 and thus allow respiration to continue under low ambient O_2 concentrations (37). This high affinity for O_2 allows some bacteria to use this oxidase for detoxification. For example, *Azotobacter* species use rapid electron flow through a

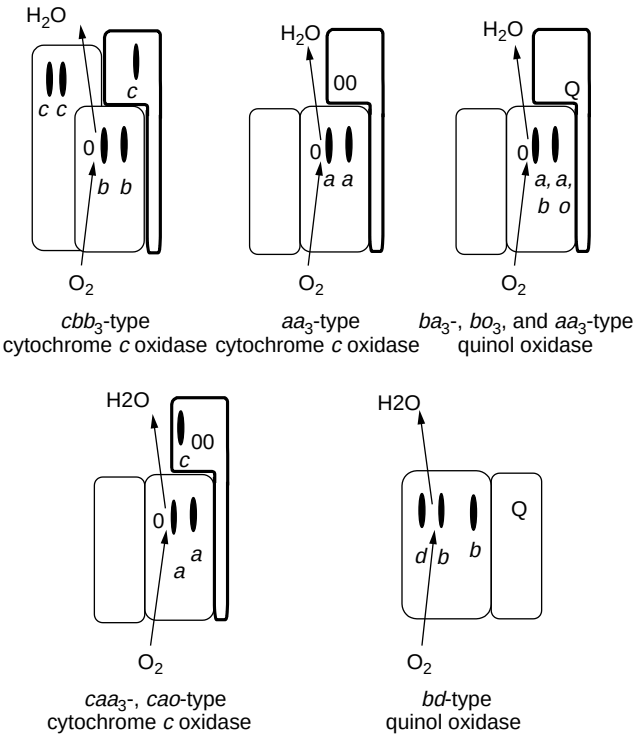

Figure 6. Schematic representation of the structural organization of the different type of bacterial terminal oxidases. Each complex is drawn such that the top of the figure would face the periplasmic side of the cytoplasmic membrane and the bottom would face the cytoplasmic side of the membrane. Closed ovals represent hemes and open circles represent copper. Q indicates the likely quinone-binding site in the quinol oxidases. The letters below the hemes indicate the possible hemes found in a particular type of oxidase. The subscript 3 is used to indicate those hemes that bind oxygen. This form of designation was developed to differentiate between the two chemically identical, but functionally distinct, a hemes in the aa_3 type cytochrome c oxidase. As additional variants of the heme- copper oxidase have been found the subscript 3 designation has been used primarily in those complexes that have chemically identical, but functionally distinct hemes. The catalytic site is indicated by the arrows indicating where O_2 is bound and water is produced.

bd-type oxidase to maintain the low intracellular O_2 concentrations required for nitrogenase activity (38). As discussed earlier. It appears that the *bd*-type oxidases do not pump protons, although their O_2 reduction mechanism is electrogenic.

The second, and more diverse, group of terminal oxidases is that of the heme-copper oxidase superfamily (39) (Table 1). In these enzymes, electrons are transferred from a six-coordinate heme to an active site composed of a five-coordinate heme, where O_2 binds, and a closely associated copper atom, termed Cu_B (Fig. 6). It appears that all of the heme-copper oxidases are capable of pumping protons. Because the heme-copper oxidases consume one cytoplasmic proton per electron as part of the O_2 reduction mechanism, as do all terminal oxidases, their additional ability to pump one proton per electron (as described earlier) makes them twice as efficient at energy conservation as the *bd*-type oxidases.

A wide degree of structural variation is found within the heme-copper oxidase superfamily (39,40). Some of these enzymes oxidize quinol, and thus bypass the bc_1 complex, whereas others oxidize cytochrome *c*. The hemes that are present vary considerably. In different enzymes, heme B, O, or A (Fig. 5) may be present in the heme-Cu_B active site, whereas heme B or A may be present as the six-coordinate heme that transfers electrons to the active site. There are no obvious mechanistic differences associated with the type of heme found in different heme-copper oxidases.

The oxidases of the heme-copper superfamily can be further divided into two groups. One group contains three core subunits that have obvious structural homology between species. In this group, electrons from quinol or cytochrome *c* enter through a protein designated subunit II; as in all of the heme-copper oxidases the six-coordinate heme and the heme-Cu_B active site are located in the subunit designated subunit I (39). Those enzymes of this group that oxidize cytochrome *c* contain an additional copper center in subunit II, termed Cu_A, close to the cytochrome *c* binding site. The quinol oxidases of this group appear to have evolved from the cytochrome *c* oxidases by losing the residues that bind Cu_A and cytochrome *c* and by gaining a quinone-binding site (40). One subset of this first group, the heme aa_3-type cytochrome *c* oxidases of the α subgroup of the proteobacteria (including *Rhodobacter* sp. and *Paracoccus* sp.), are the progenitors of the aa_3-type cytochrome *c* oxidase of mitochondria (40).

The second group of the heme-copper oxidases consists of the more "primitive" cbb_3-type (or FixN) cytochrome *c* oxidases (41) (Table 1). Only the largest subunit of this enzyme, containing the active site, is structurally homologous to the first group of heme-copper oxidases. Although this enzyme oxidizes cytochrome *c*, it does not contain Cu_A (42). The cbb_3-type oxidase has a higher affinity for O_2 than the other heme-copper oxidases, possibly because this enzyme arose when the O_2 concentration in earth's atmosphere was low (41,43).

Diversity of Respiratory Chains

Not surprisingly, given the diversity of respiratory enzymes, aerobes also exhibit a diversity of respiratory chains. Not only is there diversity between species, but because most aerobes are capable of synthesizing multiple terminal oxidases, the components of the respiratory chain of a single bacterium often changes in response to environmental conditions. Only a few well-studied examples of aerobic respiratory chains will be discussed. It should be emphasized, however, that the diversity of aerobic respiratory chains is a hallmark of bacteria. The presence of multiple respiratory complexes having similar functions allows electrons that are being transferred from reductant to oxidant to potentially flow through more than one path (Fig. 7). This "branched chain" structure of respiratory chains is common in bacteria. There are few examples of branched chain respiratory chains in higher organisms.

In some sense, the *Escherichia coli* aerobic respiratory chain is relatively simple (Table 1, Fig. 7). *Escherichia coli* encodes both a mitochondrial-type NADH dehydrogenase

and a single subunit, nonelectrogenic NADH dehydrogenase (44,45). Electrons from the dehydrogenase flow to one of the three types of quinones, ubiquinone, or either of two napthoquinones, menaquinone, or demethylmenaquinone (46). The quinones transfer electrons to either a *bo*-type heme-copper oxidase or a *bd*-type oxidase (16). The regulation of the expression of the various components of the *E. coli* chain is complex and not completely understood. In general, ubiquinone and the *bo*-type oxidase are preferentially expressed if O_2 levels are high (47). Menaquinone and the *bd*-type oxidase are more highly expressed under limiting O_2 concentrations (47). Regulation of the statement of the NADH dehydrogenases is not so clear-cut (48). The single subunit enzyme is apparently expressed aerobically and repressed during fermentation. Expression of the electrogenic enzyme is apparently stimulated by O_2 and nitrate.

The aerobic respiratory chain of *Paracoccus denitrificans* is similar to the mitochondrial respiratory chain and both more complicated than the *E. coli* chain (Table 1, Fig. 7). *Paracoccus denitrificans* apparently only encodes the mitochondrial-type NADH dehydrogenase (49). Ubiquinone is the major quinone. As in *E. coli*, there is a quinol oxidase of the heme-copper family that can directly oxidize quinol. This oxidase is designated the ba_3-type oxidase because of its heme content (50). Unlike *E. coli*, electrons can also flow from reduced quinol to the bc_1 complex. Electrons then flow from the bc_1 complex to one of the several *c*-type cytochromes that can either be located in the periplasm or are membrane-bound (49). *Paracoccus denitrificans* has two terminal oxidases that use reduced cytochrome *c* as a source of electrons. One of these oxidases is the aa_3-type oxidase, which has significant sequence similarity with the mitochondrial aa_3-type oxidase, consistent with the proposal that the bacterial progenitor of the mitochondria was closely related to *P. denitrificans* (23,51). The other oxidase is the cbb_3-type oxidase (52). The aa_3-type oxidase is preferentially expressed when O_2 concentrations are high. The cbb_3-type oxidase, consistent with it being a high-affinity oxidase, is more highly expressed when O_2 concentrations are limiting (53). The expression of the ba_3-type quinol oxidase is more complex and its role in the physiology of *P. denitrificans* is unclear (54).

In addition to multiple terminal oxidases, some bacteria also have the capacity to use electrons from

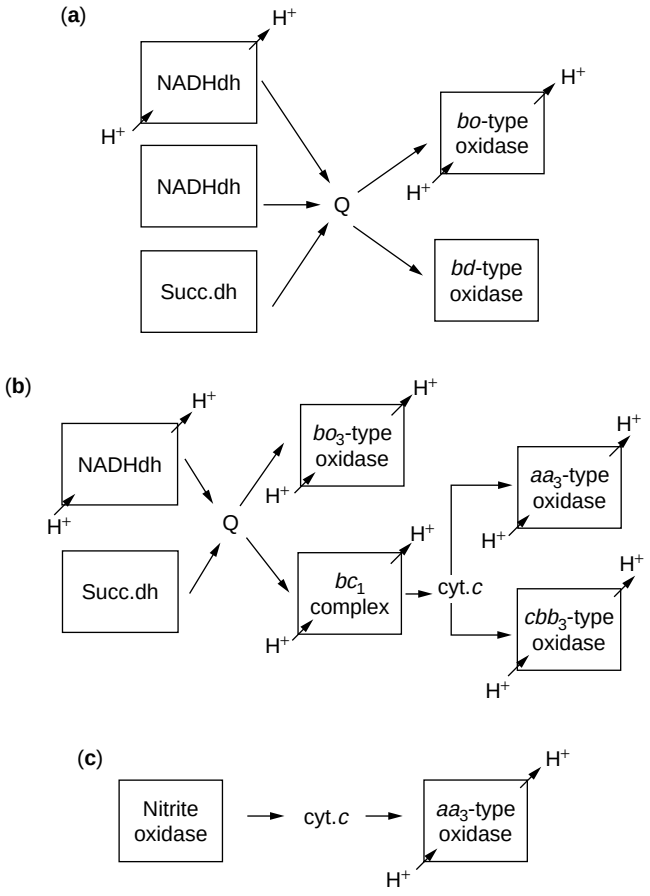

Figure 7. Representation of the aerobic respiratory chain of (**a**) *E. coli*, (**b**) *P. denitrificans* and (**c**) *Nitrobacter* species. Abbreviations are: Q, quinone pool, cyt. c, soluble cytochrome c, NADHdh, NADH dehydrogenase, Succ.dh, succinate dehydrogenase. Arrows indicate the direction of electron flow, and H^+ entering and exiting the boxes indicate electron transfer complexes that pump protons.

multiple substrates during aerobic growth. For example, some, including *P. denitrificans*, can use thiosulfate as an energy source (55). Others use the single carbon compounds methanol and methylamine as both a source of carbon and high-energy electrons (49,56). Oxidation of thiosulfate involves a periplasmic thiosufate-cytochrome *c* oxidoreductase, a periplasmic cytochrome *c*, and the aa_3-type terminal oxidase. Similarly, the oxidation of methanol or methylamine requires periplasmic dehydrogenases that feed electrons to the aa_3-type oxidase via periplasmic cytochrome *c*'s. In these pathways, the only electrogenic component contributing to the PMF is the terminal oxidase, because electron flow through the NADH dehydrogenase, the Q-pool and the bc_1 complex is bypassed. However, the oxidation of formaldehyde, one of the end products of methanol and methylamine oxidation, produces NADH (49,56). Because the oxidation of this NADH involves the entire respiratory chain, cells growing on methanol and methylamine still synthesize the NADH dehydrogenase and the bc_1 complex.

The lithotroph *Nitrobacter* provides another example of the versatility of bacterial aerobic respiration (Fig. 7). *Nitrobacter* obtains electrons from the oxidation of nitrite to nitrate; the redox potential of the nitrite/nitrate couple is about +0.4 V. This is too high a potential for the direct reduction of cytochrome *c*, as occurs in the examples given earlier. Thus, *Nitrobacter* places a nitrite-cytochrome *c* oxidoreductase within the cytoplasmic membrane in which it can take advantage of the PMF to help drive the transfer of electrons to the positive surface of the membrane in order to reduce a periplasmic cytochrome *c*. The reduced cytochrome *c* then transfers its electrons to an aa_3-type terminal oxidase (57). The entire PMF is generated through the electrogenic activity of the aa_3-type oxidase (3). The situation is further complicated by the fact that *Nitrobacter*, as an obligate autotroph, must generate NADH for CO_2 reduction. Using more of the PMF to drive electron transfer in reverse, such that the NADH dehydrogenase oxidizes quinol and reduces NAD^+ to NADH, does this. This strategy allows the cell to generate high-energy (low potential) electrons in the form of NADH from lower energy (higher potential) electrons from nitrite. To accomplish this, the cell must oxidize several nitrite molecules for each molecule of NADH produced.

As shown by the examples discussed earlier, aerobes can use many compounds as reductants and, by expressing a variety of oxidases, can grow over a wide range of O_2 concentrations. However, genomic sequence information suggests that some aerobes have an even larger respiratory repertoire than currently available biochemical evidence demonstrates. For example, it is known that the bacterium *Rhodobacter sphaeroides* expresses three terminal oxidases. These include an aa_3-type cytochrome *c* oxidase, a cbb_3-type cytochrome *c* oxidase, and an uncharacterized quinol oxidase (58–60) (Table 1). However, recent genomic analyses indicate that *R. sphaeroides* encodes at least five terminal oxidases: a *bd*-type quinol oxidase, a heme-copper quinol oxidase, and the three-cytochrome *c* oxidases. The quinol oxidase that is expressed is likely to be the *bd*-type quinol oxidase, in a modified form (58). It seems likely that *R. sphaeroides* encounters conditions in the environment in which the heme-copper quinol oxidase is functional, although it is not yet obvious what these conditions would be. Another example is provided by *E. coli,* which contains a set of genes that encode an alternative *bd*-type oxidase (61). The role of this oxidase is not clear, but it has been shown to be functional (62). It is likely that as more genomic sequences become available they will show that the true diversity of aerobic respiratory chains has been underestimated because standard laboratory culture conditions do not induce expression of the full set of respiratory enzymes.

The F_1F_O ATP Synthase

The protein complex that allows the electrochemical gradient to be coupled to ATP synthesis is termed the F_1F_O ATP synthase. Recent structural and mutagenesis studies have shown that the ATP synthase of the bacterial membrane is actually a molecular motor (63–66). The ATP synthase complex is composed of two domains: a transmembrane domain that contains a ring of small protein subunits and an extramembrane domain that is shaped like a mushroom (Table 1, Fig. 8). The stalk of the extramembrane domain consists of one elongated subunit (γ), which extends through the cap of the complex. The cap is a ring of six large subunits (3α and 3β); an active site where ATP is synthesized from ADP and phosphate is present in each of the three β-subunits. The proposed mechanism of this machine is as follows. Protons are pulled through the transmembrane domain, toward the inner, negatively charged surface of the membrane, using

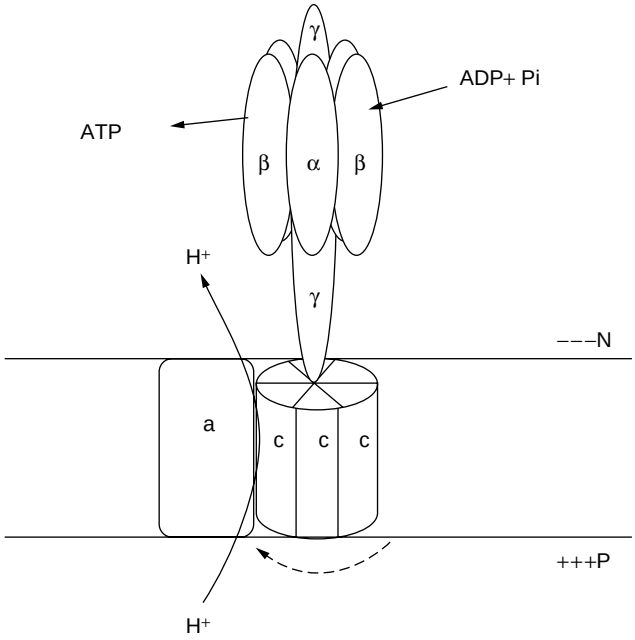

Figure 8. Structural organization of the F_1F_o-type ATPase. Not all subunits making up the complete complex are present. The F_o part of the complex resides in the membrane and is indicated by the a and c subunits. The F_1 part lies on the cytoplasmic side of the membrane. The dashed arrow indicates direction of rotation of the F_o complex during ATP synthesis.

the energy of the transmembrane voltage gradient or PMF. Through an electrostatic mechanism, the flow of protons causes the ring of small subunits in the membrane to rotate in a clockwise direction (64,67–69). This ring is connected to the γ-subunit, which also rotates, and the extramembrane ring of α- and β-subunits is prevented from rotating by additional subunits that act as a stator. As the γ-subunit rotates within the cap structure it forms different associations with each β-subunit, much the same way as a camshaft in a multicylinder engine forms different associations with each cylinder at any given second. As γ rotates, the active site on each β-subunit moves successively through three conformations: one that loosely binds ADP and phosphate, one that binds these substrates tightly and catalyzes the condensation reaction to form ATP, and a third conformation that opens the active site to release ATP. Thus, the overall result of continued proton transfer through the transmembrane domain of the synthase is constant consumption of ADP and phosphate by the extramembrane headgroup and the constant release of ATP.

REGULATION OF AEROBIC METABOLISM

As stated earlier, O_2 is the preferred oxidant for aerobic bacteria, and when it is available, aerobes will adjust their physiology to preferentially use this oxidant over all others. Bacteria have developed complicated regulatory circuitry to assess O_2 concentrations. A few of these have been studied in some detail and will be briefly described. These proteins are found in many aerobic bacteria; however, it should not be assumed that every protein would have the same function in every aerobe. Each species of bacteria has its own regulatory circuitry and physiological set points making it difficult to extrapolate from one organism to the next. One common theme among the regulatory proteins studied so far is that they are active when O_2 is absent. That is, they are used to activate genes whose products are required when O_2 is limiting. Regulatory proteins that are activated by increases in O_2 have not been studied in bacteria.

Oxygen Sensing Regulatory Proteins

The prototype of this type of regulatory protein is Fnr. Fnr apparently senses O_2 directly and binds to DNA when O_2 concentrations are low (Fig. 9). This is because Fnr contains an O_2 labile [Fe–S] center (70). When O_2 concentrations are high the [Fe–S] center is not completely assembled. Consequently, the protein does not dimerize and this prevents it from binding to its target sequence (71). As O_2 concentrations decline, conditions become more favorable for assembly of a stable [Fe–S] center. Once the [Fe–S] is formed, Fnr can dimerize and interact with its target sequence. In most cases, Fnr is a positive activator of gene expression. This protein is found in a wide range of bacteria and is responsible for the expression of many genes. It is now evident that Fnr is member of a large family of transcriptional regulators termed the *Fnr/Crp* family (72). All of these regulators have similar structural organization, with a

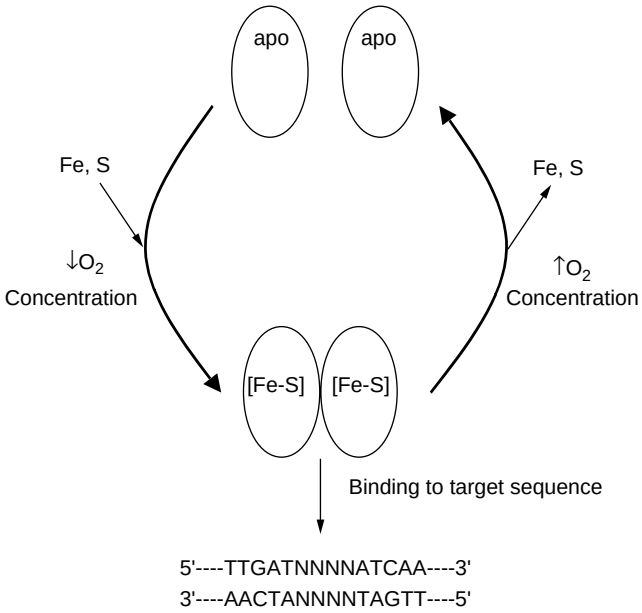

Figure 9. Model of how the transcriptional regulator Fnr responds to changes in O_2 concentration in the cell. [Fe–S] represent O_2 labile iron-sulfur centers found in Fnr. The consensus Fnr target sequence is given below the active [Fe–S]-containing Fnr.

DNA-binding domain at the C-terminus and an effector-binding domain at the N-terminus. There is little sequence similarity among the N-terminal domains among the various members of this family of proteins. However, most seem to be designed to use small molecules as effectors. For example, there are members of the family that detect the gas carbon monoxide and other members that respond to changes in cyclic AMP (73,74).

In *E. coli*, Fnr has been shown to repress the expression of the genes encoding the cytochrome *bd*-type oxidase under anaerobic conditions (75). A similar role for Fnr occurs in *A. vinelandii*, with Fnr repressing synthesis expression of the *bd*-type oxidase used to protect nitrogenase from O_2 (76). In *Pseudomonas aeruginosa*, inactivation of the gene encoding Fnr caused increased expression of an oxidase with high affinity for O_2, probably a cbb_3-type oxidase, along with a cyanide-insensitive oxidase that is probably a *bd*-type enzyme (77). Inactivation of a gene encoding an Fnr homologue in *P. denitrificans* also caused increased expression of a quinol oxidase, but repressed expression of the cbb_3-type oxidase (53). Expression of the genes encoding cbb_3-type oxidase in *R. sphaeroides* also requires Fnr (78).

Regulators That Indirectly Sense Changes in O_2 Concentration

Although Fnr appears to interact directly with O_2, other proteins appear to sense changes in O_2 concentration by indirect mechanisms. One of these systems is termed the *Arc system*, which has been extensively studied in *E. coli* and its relatives. The Arc system consists of two proteins, ArcB, which is a membrane-associated histidine kinase, and ArcA, which is its associated response

regulator (79). Together, these two proteins form a two-component regulatory system. ArcB apparently senses some aspect of respiration, perhaps the redox state of an intermediate in the respiratory chain. There is no evidence that ArcB directly senses O_2. When conditions limit electron flow through the aerobic respiratory chain, ArcB phosphorylates ArcA (80). ArcA then acts as either an activator or a repressor of its target genes. The activity of ArcB also seems to be influenced by the presence of metabolic intermediates that indicate O_2 is limiting, such as pyruvate and D-lactate (81). The ArcAB system functions as a positive activator of expression of the genes encoding the *bd*-type oxidase in *E. coli* (82).

A second example of a regulatory system that regulates anaerobically expressed genes, but does not directly sense O_2, is the PrrA/PrrB system (also termed *RegA*/*RegB*) in *Rhodobacter* species and related bacteria. Like the ArcBA system, the PrrBA system is a two-component sensor-regulator system, with PrrB being the membrane-associated histidine kinase and PrrA the associated response regulator (79). Recent work in *R. sphaeroides* indicates that PrrBA interacts with the cbb_3-type oxidase (83). When O_2 is present, the cbb_3-type oxidase is active and PrrB does not phosphorylate PrrA. However, when electron flow through the oxidase decreases because of O_2 limitation, PrrB phosphorylates PrrA, causing it to activate transcription of a number of genes. In this case, the activity of the oxidase is the gauge the regulator uses to determine ambient O_2 levels.

TAXIS RESPONSE TO O_2

In addition to using their regulatory systems to monitor environmental conditions, cells can use taxis to move themselves to positions that optimize aerobic respiration. Taxis toward terminal oxidants such as O_2 has been observed for many years. This taxis response to O_2, termed *aerotaxis*, has been extensively studied in *E. coli* and its relatives (84). Taxis toward O_2 is dependent on the ability of the cells to respire O_2, which suggests that O_2 is not directly sensed by cellular receptors. Rather, the cell appears to respond to electron flow to a particular oxidant. In *E. coli*, a membrane-bound flavoprotein termed *Aer* has been shown to be involved in the aerobic taxis response (85). It is likely that the redox status of the flavin allows Aer to monitor electron flow to O_2 (86). In the bacteria in which aerotaxis has been studied, O_2 is always the preferred oxidant and its presence will prevent taxis to alternative oxidants.

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AGGREGATES AND CONSORTIA, MICROBIAL

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Nearly a century of microbiological research indicates that structurally and functionally complex microbial communities commonly inhabit surface-associated environments in aquatic ecosystems. Surficial microbial communities often show higher metabolic activities and growth rates than their freely suspended counterparts. This has been linked to surfaces facilitating close metabolic coupling among community members, leading to enhancement of growth and nutrient cycling that exceeds what is achievable at the single species level. These synergistic, mutually beneficial associations are termed *consortia*. Consortia are common to aggregate and other surficial communities broadly distributed along geographic and trophic gradients, ranging from the tropics to the poles. Consortial activity, growth, and diversity are enhanced by biogeochemical (i.e., oxygen, E_h, pH, nutrients) gradients in surficial microenvironments, or microzones. In part, consortia themselves promote biogeochemical zonation and microbial diversification through localized production and mineralization of organic matter. This positive interaction is a requisite for biodiversification, which in turn enhances microzonal productivity and nutrient cycling. For these reasons, consortia are viewed as the functional units of biocomplexity in aquatic ecosystems.

The consortial "lifestyle" also appears to be a highly effective strategy for colonizing and exploiting nutrient and energy resources under environmentally extreme conditions at "the edge of life." Examples from oligotrophic planktonic, hypersaline, and lithifying benthic, Baltic Sea and Antarctic lake ice communities are used to exemplify the important roles aggregate and surface microbial

communities play in ecosystem, regional and global biogeochemical cycling and production dynamics. Because they are highly sensitive, exhibit rapid community growth, and compositional responses to environmental change and extremes, microbial consortia are excellent indicators of climatic and geochemical changes, as well as human pollutants and other perturbations in aquatic ecosystems.

SUSPENDED AND SOLID SURFACES AS MICROENVIRONMENTS

Microorganisms are the dominant producers and cyclers of organic and inorganic compounds in the biosphere. In aquatic environments, at least two-thirds of primary production is mediated by microorganisms, including bacteria (cyanobacteria and photosynthetic bacteria) and microalgae (1). A vast percentage of aquatic production is either sedimented or recycled via microbial mineralization (2,3). Therefore, environmental controls of microbial production, mineralization, and associated nutrient transformations are key determinants of aquatic ecosystem structure, function, and material flux. A vast proportion of the Earth's marine and freshwater ecosystems is nutrient- and energy-poor, and as such production and mineralization are tightly controlled by nutrient and energy availability. In natural waters, nutrient resources are heterogeneously distributed in time and space (4), with relatively high concentrations associated with particles and submersed surfaces (2,5), which can act as sources or concentration sites of nutrients. Microbial biomass and activities are generally higher on surfaces than in the free-floating state (2,5,6). As a result, microbial growth and nutrient transformation are often stimulated by surfaces (5,7). This has been attributed to the nutrient-rich "oasis" that surficial microenvironments provide in an otherwise dilute "desert" macroenvironment (2,8).

Relatively high rates of primary productivity and respiration associated with surfaces lead to localized regions of elevated organic-matter enrichment and consumption, accompanied by the development of oxygen gradients (2,9,10), which provide conditions for species enrichment along the resultant biogeochemical continuum. These are the essential ingredients for intensification and diversification of surficial biogeochemical cycling.

Owing to their rapid growth rates and small size (i.e., $\sim\mu$m), microorganisms are excellent exploiters of nutrient-enriched surficial microenvironments. Small cell size enables large numbers of cells to colonize these microenvironments. Small cell size also provides high surface to volume ratios, facilitating nutrient uptake at low concentrations. The metabolic activities (i.e., oxygen production and consumption, pH changes) associated with metabolically active microbial communities further enhance biogeochemical zonation, promoting microbial diversification. However, there is a price to pay for being small. The small cell size and prokaryotic (lack of organelles and nucleus) nature of bacteria restricts cellular biochemical specialization. This metabolic "packaging problem" is particularly problematic when considering the need for harboring aerobic (oxidized) and anaerobic (reduced) processes on the cellular level. Both types of processes are often required to fully metabolize and derive energy from organic and inorganic compounds. For example, cyanobacteria utilize oxygenic photosynthesis to obtain energy and carbon compounds to support growth. However, cyanobacteria also rely on highly reduced processes, such as nitrogen (N_2) fixation to obtain biologically available nitrogen in nitrogen-deplete waters. The oxygen evolved from photosynthesis is inhibitory to N_2 fixation; it can also exert a strong negative feedback control on photosynthesis itself (i.e., the Warburg effect, see Refs. 11,12). Some filamentous cyanobacteria are able to separate these cross-inhibitory processes by cellular specialization (e.g., the formation of heterocysts to which N_2 fixation is confined) (12,13). Such specialization requires multicellular compartmentalization, a luxury that may not be practical within the confines of micron-scale surficial habitats.

To circumvent the "packaging problem," physiologically distinct prokaryotes, eukaryotic microalgae, protozoans, and fungi participate in metabolically coupled associations (13–16), enabling them to colonize, optimize growth, establish redox gradients, and maintain effective nutrient cycling within the confines of a microenvironment. Under these conditions, smallness, metabolic specialization, and close proximity along micron-scale biogeochemical gradients are advantageous for (*1*) survival, (*2*) obtaining nutrient and energy sources, (*3*) establishing high biodiversity, (*4*) facilitating nutrient and genetic exchange, and (*5*) exploiting environmentally "extreme" habitats not colonizable by single species acting alone (14,17).

Microorganisms participating in the "lifestyle" described earlier operate as consortia. That is, populations have evolved to functionally specialize and diversify along micron-scale biogeochemical (redox, nutrients, organic matter supply, etc.) gradients, or "microzones," where effective exchange of metabolites leads to "one organism's trash being another's treasure." Consortia are defined as the operational units of biocomplexity in nature, as *several species or populations that function in a complementary fashion such that production and growth are enhanced above that which a single species or loosely-knit collection of populations (i.e., assemblage) can achieve independently* (14,17). On regional and global scales, consortial associations in aggregates, biofilms, and microbial mats play basic roles in production and nutrient cycling dynamics. The "microbial loop" in trophodynamics, energy flow, and biogeochemical cycling highlights the central role consortia play in ecosystem processes (3). Clarification and quantification of consortial components and their exchange rates are essential for developing process-based models linking biocomplexity to function in aquatic and terrestrial ecosystems.

Consortia develop within, and as a consequence of, an interactive matrix of geological, physical, and chemical parameters. Over time, consortia develop by interactions at some quasi "steady-state" whose determinants arise from the prevailing interplay of system parameters (Fig. 1). We will explore this operational definition of consortial life in aggregates and surficial microzones and its role in production and biogeochemical cycling dynamics in planktonic and benthic habitats.

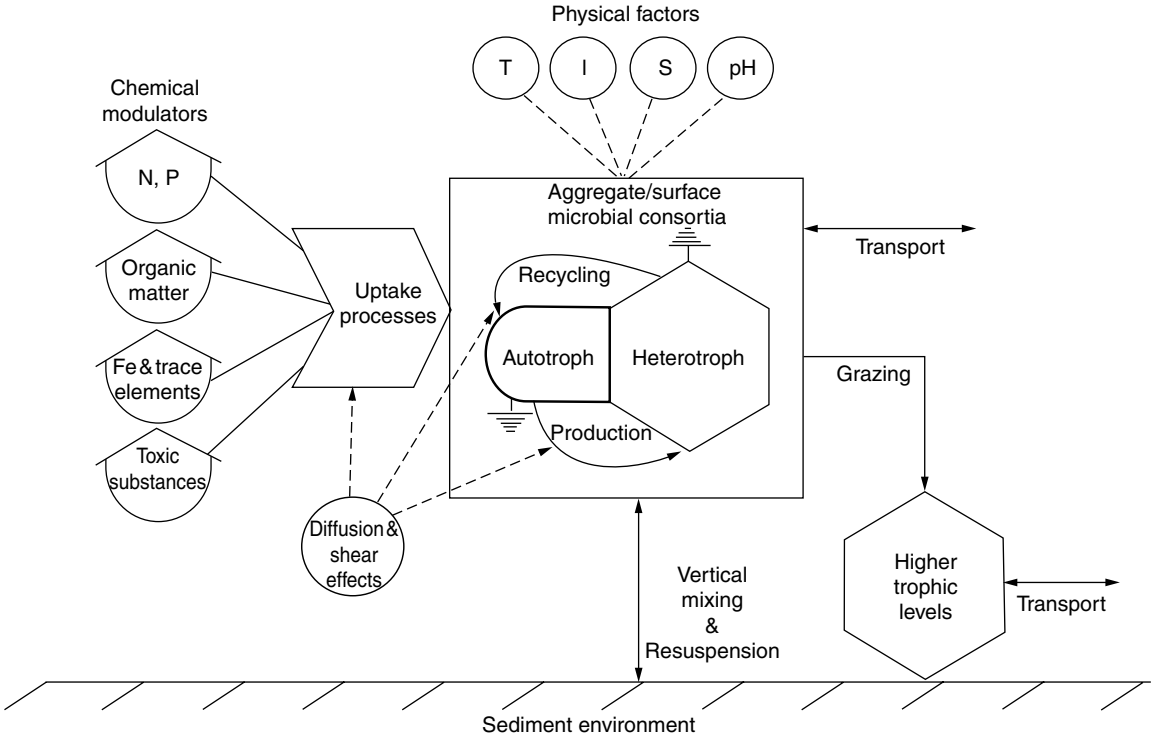

Figure 1. Conceptual diagram depicting influence of natural and anthropogenic environmental factors, and physical modulators and biotic interactions on aggregate/surface microbial consortial assemblages. Physical modulators include temperature (*T*), irradiance (*I*), salinity (*S*), and pH (pH).

FORMATION OF AGGREGATES AND CONSORTIA

Suspended Aggregates

Formation of visible, suspended macroscopic aggregates composed of microscopic particles is a common phenomenon in aquatic ecosystems (18–23). Microscopic particulate matter in surface waters consists of living organisms, inorganic particles, and nonliving organic matter, or detritus. Aggregation of these "particles" is a complex process involving physical, chemical, and biological interactions on different spatial and temporal scales (Fig. 1). Physical processes, such as shear (difference in fluid movements on small scales), Brownian motion (thermal movement of one particle toward another), and differential settlement (one particle sinking faster than the other, overtaking and colliding with it) bring particles together (Fig. 2) (24). The number of particles per unit volume of water is crucial for encountering other particles and forming aggregates. Frequently, physical discontinuities in the water column, such as the pycnocline, act as sites for enhanced aggregation.

Chemical and biological properties of colliding particles ("stickiness") determine whether they adhere and aggregate. The mucus sheaths and other organic surface materials, especially polysaccharide particles known as transparent exopolymer particles surrounding organisms, act as adsorption surfaces for additional particles (19,25,26). Attachment to surfaces stimulates bacterial exopolysaccharide synthesis, which further enhances aggregation (27). Aging microaggregates collide with each other and form visible macroaggregates, such as "marine snow" and "lake snow."

A two-state coagulation theory, in which coagulation is insignificant at low, but significant at high algal concentrations with a rapid transition between the two states has been suggested for diatom blooms (28–30). The critical cell concentration is dependent on cell size, cell surface stickiness, and turbulence, and may be reached rapidly, with the entire sequence of aggregation and sedimentation completed in 24 hours (31). In the Baltic Sea, such rapid transition from diatom growth to aggregation and sedimentation has been recorded during spring blooms (32–35). Nutrient depletion at the end of a bloom has been shown to increase diatom cell stickiness, enhancing aggregation (30,36,37). Despite its episodic nature, aggregation is significant and widespread enough to control ecosystem-level annual energy flow and nutrient dynamics owing to the transport of organic matter to the benthos by sedimentation.

Physical and biological processes may also decrease aggregate size (Fig. 2). Turbulence may tear particles apart or bring them together (38,39). Biological disaggregation by microorganisms or consumption by zooplankton can, at times, be an important factor regulating aggregate size (25,40). Grazing of aggregates by fish and zooplankton is an important shortcut in the food web, transferring organic carbon to higher trophic levels more efficiently

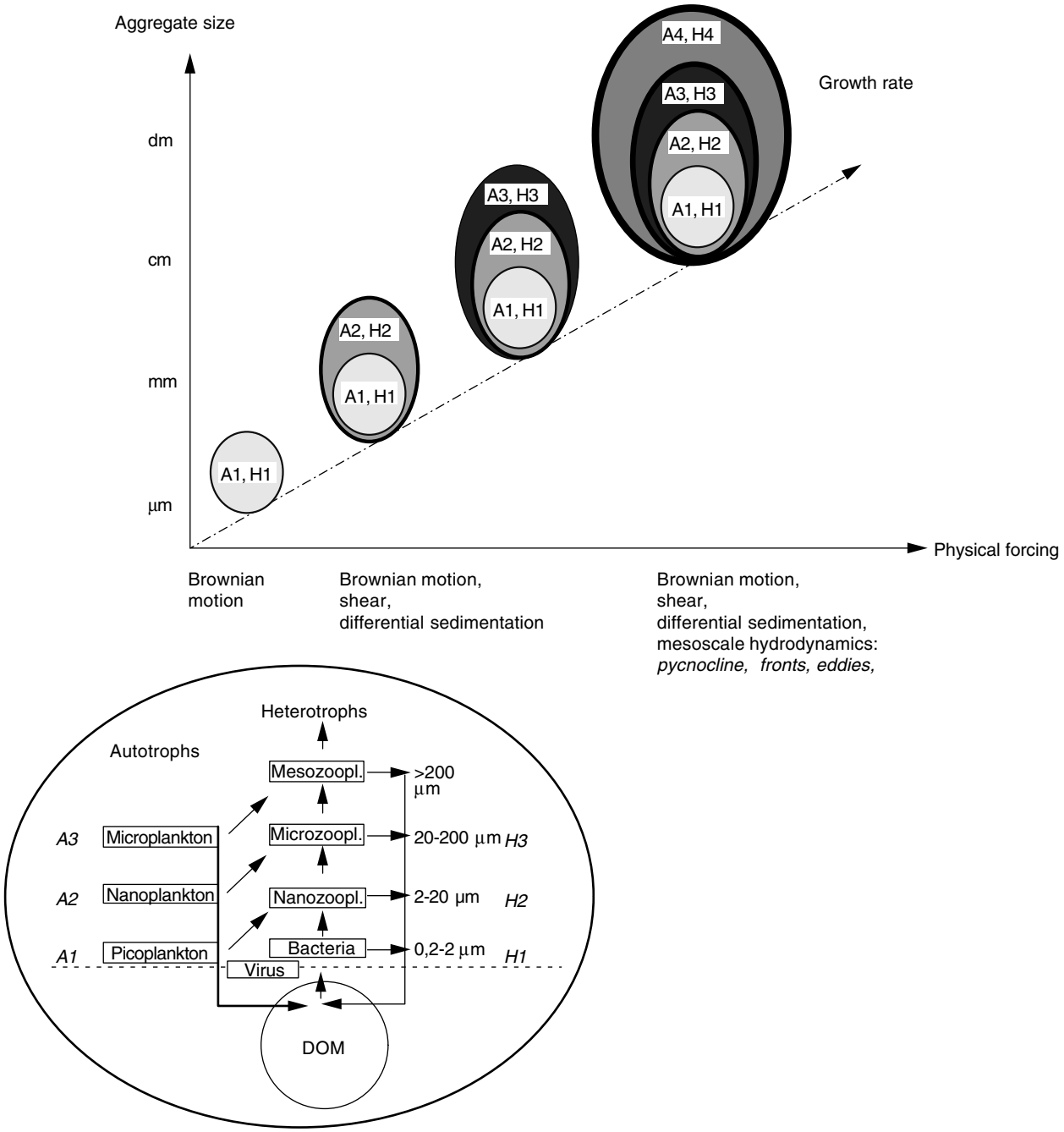

Figure 2. Aggregate size development in relation to physical forcing and time. A1-H3 and H1-H3 represent autotrophic (A) and heterotrophic (H) plankton in the classical size structured pelagial food web. Dashed line represents the artificial partitioning between DOM and particulate organic matter. Brownian motion is a relevant collision mechanism with particles smaller than 1 µm (A1, H1 particles) (38). Shear contact from laminar or turbulent processes is a common collision mechanism for particles larger than 1 µm (A2, H2, and above). The third collision mechanism is differential sedimentation (A2, H2, and above). The critical cell concentration for aggregation depends on cell size, cell surface properties, and environmental shear. Spherical particles have upper limits to their size as large particles become vulnerable to break up by the same shear that brings them together. Large aggregates (>cm A4, H4 particles) are formed when organisms have appendages, hairy structures, or grow in filaments. The physiological status of the cell adds to the "stickiness" of the particle.

than via the microbial loop (23,41). The relative importance of these mechanisms in aggregate dynamics depends on the origin and age of the aggregates (28,42,43).

Sea and Lake Ice Aggregates

Seasonal formation of sea ice traps organisms in organic-matter rich aggregates. Ice-associated microbial assemblages in the brackish Baltic Sea resemble those found in Antarctic and Arctic sea ice (44–46). The biotic assemblages in the Baltic Sea ice consist of diatoms, photo- and heterotrophic flagellates, cyanobacteria, heterotrophic bacteria, and metazoa (44–48). Diatoms and various autotrophic flagellates are the most important algal groups; hetero- and mixotrophic organisms (flagellates and ciliates) compose 2 to 10% of carbon-based biomass (49). The accumulation of inorganic nutrients and abundant heterotrophic organisms seasonally found in the Baltic Sea is indicative of active carbon mineralization in the interior parts of the ice. The interior layers of 2- to 3-month-old ice are also active sites for nitrogen transformations. The enrichment of denitrifying organisms in the middle layers of thick ice, together with elevated nitrite concentrations, indicate active nitrate reduction and potentially denitrification, that is, microbial reduction of NO_3^- to N_2 gas (50). Despite the low rates of N_2O and N_2 production from seasonal ice (50), the large aerial expanse of seasonal ice on the global scale renders sea ice microbial processes of high significance.

Microbial aggregates may also be embedded in the ice cover of lakes and marine systems for many years. The permanent ice cover of the glacial lakes in the McMurdo Dry Valley region of Antarctica is one example (51–53). Here, aggregates originate as windblown soil particles (originating in the surrounding exposed desert soils) harboring desiccated phototrophic (cyanobacteria) and heterotrophic microflora that are deposited on the ice cover. Solar heating of the soil particles enables them to melt into the ice cover, where they remain for several years. The ecophysiology of these "entombed" aggregates is discussed in detail later. As with sea ice, lake ice primary production and nutrient cycling rates are extremely low; however, on the ecosystem-scale, the aggregates are a major source of "new" production and nutrient input to these lakes (51).

Surficial and Benthic Habitats

The bottom (benthos) and other surficial attached environments provide diverse colonization sites for metabolically complex microbial consortia. These include, biofilms on submersed inorganic and organic nonliving substrates, including rocks, metals, glass, wood, plastics, and other synthetic materials (5,54). Consortia also inhabit the surfaces of living plants (epiphytic) and animals (epizoic). Benthic habitats supporting microbial consortia include submersed and intertidal mud- and sandflats, hard (rock) and soft (mud-silt) bottoms, marshes, reef, and shelf environments (55–57). Consortia inhabiting these environments range from ephemeral, loosely organized, flocculent, accumulations of microalgae, and associated bacteria, fungi, and metazoans, to well-developed laminated microbial mat communities (55). Microbial communities may physically modify the attachment substrates. Examples include, complex biofilms on submersed surfaces (54,58), adhesive mats that consolidate sediments (55), beach sands and lagoonal environments (55), providing protection against erosion from storm surges, and calcifying mats that form permanent lithified, layered deposits called stromatolites, the oldest known evidence (2 billion years ago) for microbial consortial life on Earth (59,60).

Microbial Modification of Aggregates and Biofilms

Once attached, microbial communities can physically and chemically modify the substrate. Recently divided "young" phytoplankton cells usually show the least amount of bacterial colonization (61,62). As the cells age, they are more readily colonized by microorganisms. Bacterial colonization may be quite intense along algal filaments surrounded by mucilage (23,62,63) and cyanobacterial heterocysts (61) (Fig. 3). These bacterial epibionts represent a key food source for protozoan grazers, leading to complex microbial food webs (19,21,39,62,64–66). Among aging aggregates, senescent algae provide degradable organic matter for large numbers of attached bacteria. The role of the recycling of organic matter via microzooplankton back to bacteria is enhanced as the aggregates mature (39,41,62).

Cyanobacterial aggregates that avoid sedimentation and grazing can maintain "steady-state" aggregation conditions for long periods owing to recycling of organic matter and mineral nutrients by attached microflora within the phycosphere (62,67,68). Under these conditions, "host" cyanobacteria can reveal high growth rates, frequently exceeding rates obtainable under axenic conditions (10). In fact, some bloom-forming cyanobacterial species are only culturable when bacterial epibionts are present (13). Among other cyanobacterial genera (e.g., *Oscillatoria, Lyngbya, Nodularia*), bacterially colonized strains revealed higher growth rates and were easier to maintain in culture than axenic strains. Gibson and Smith (69) reported that both axenic *Oscillatoria redekei* and *Oscillatoria agardhii* isolates "always appeared to grow better in the presence of contaminant heterotrophic bacteria." Similar findings were reported by Meffert (70), Herbst and Overbeck (71), Caldwell (72), and Lehtimäki and coworkers (73), suggesting consortial interactions.

Cyanobacteria excrete organic compounds, including organic and amino acids, peptides, alkaloids, carbohydrates, and lipopolysaccharides (74–76). Diverse excretion products chemotactically attract and support the growth of phycosphere-associated bacteria (77,78). In N_2 fixation and fate experiments, some of the ^{15}N-labeled N_2 fixed by host *A. oscillarioides* was rapidly transferred to heterocyst-associated *Pseudomonas aeruginosa* (79). Axenic isolates of *A. oscillarioides* exhibited optimal growth and N_2 fixation rates when reinoculated with *P. aeruginosa* (16). When released in axenic *A. oscillarioides* cultures, *P. aeruginosa* reestablished the heterocyst-specific association. These findings suggest close metabolic coupling and mutually beneficial relationships in cyanobacterial-bacterial aggregates.

Aggregation into large flocculates by filamentous cyanobacteria may be of major importance for prolongation

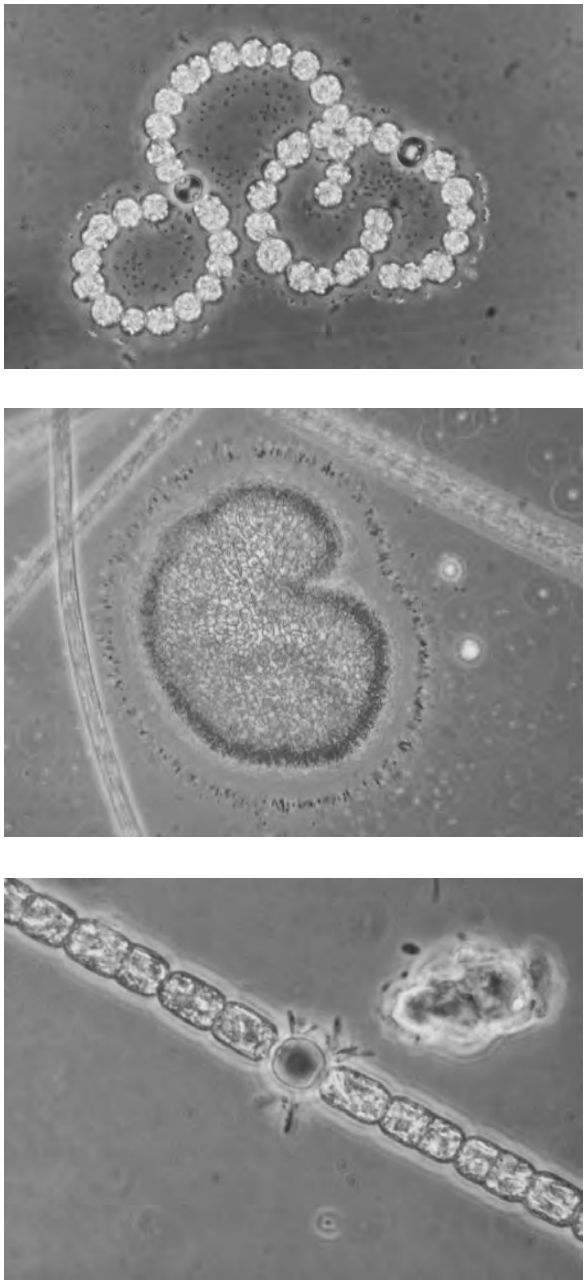

Figure 3. Microbial associations with bloom-forming cyanobacteria: (**a**) bacteria inside *Anabaeana spiroides* coils; (**b**) bacteria around *Gomphosphaeria* colonies; (**c**) bacteria on heterocyst of *Anabaeana oscillarioides*. See color insert.

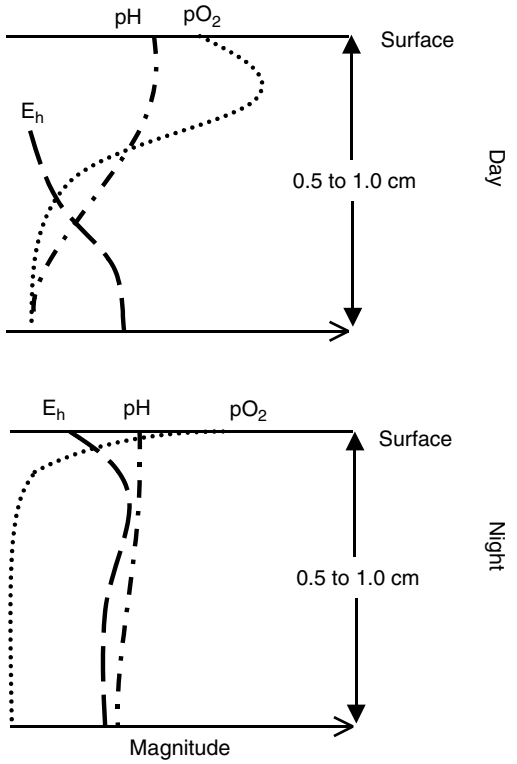

Figure 4. Idealized set of vertical profiles of redox potential (E_h), pH, and percent saturation of dissolved oxygen (pO_2) in a microbial mat. The differences among relative values for each parameter are shown for daytime (upper frame) and nighttime. Typically, the biogeochemical gradients show strongest vertical zonation in the upper 0.5 to 1.0 cm of the mat where light can potentially penetrate and photosynthesis takes place.

of bloom events. Further, the interior of these large flocculates can become anaerobic (80) like the microbial mats, support denitrification (81) and sulfate reduction (82), biogeochemical transformations not predicted by models that assume random distribution of organic matter and microbes in the pelagic environment.

Microbial Mats

Microbial mats are laminated microbial communities associated with a variety of benthic substrates, including sands, muds, rocks, metals, glass, concrete, wood, even plastics, and other synthetic substances. Mats are morphologically complex microecosystems that possess a high degree of phylogenetic and functional (metabolic) diversity compressed into only a few millimeters (55,83). Mats are widely distributed, with their range including polar deserts, glacial lakes and streams, lakes and marine ecosystems, the deep-sea, polar to tropical shallow seas, reefs, mud- and sandflats, saltmarshes, hypersaline, and hyperthermal ecosystems. In many of these systems, mats are among the most productive and diverse biotic communities. Mats are composed of microbial phototrophs (anoxygenic and cyanobacterial), heterotrophs, and chemoautotrophs (55,84–87). Characteristically, mats exhibit vertical biogeochemical zonation (10,88,89), which usually includes an upper oxic and lower anoxic zone (Fig. 4). In aphotic regions (i.e., deep sea), or under anaerobic (i.e., sulfidic hot springs) conditions, anaerobic processes prevail. In euphotic, oxygenated environments, photosynthetic production is usually conducted by a phylogenetically diverse assemblage of cyanobacteria and diatoms that dominate the upper oxic layer. Lower regions of well-developed mats are often O_2 subsaturated or anoxic, leading to sulfide accumulation generated by heterotrophic sulfate-reducing bacteria (SRB). These mats also have

steep vertical redox (E_h) and pH gradients. Gradients create a range of microzonal (micron to millimeter scale) habitats, in which metabolically diverse microbial consortia are located according to energetic, nutrient, and ecological needs and limitations. Consortia closely interact by exchange of metabolites, enabling major biochemical processes with different environmental requirements (e.g., oxygenic photosynthesis, N_2 fixation, denitrification, nitrification, sulfate reduction, methanogenesis) to function contiguously and contemporaneously (9,10,90–92).

Depending on environmental conditions, critical energy and nutrient transformation processes oriented along these gradients may be cross-inhibitory. For example, during sunny days, rates of oxygenic photosynthesis may be high, leading to a buildup of oxygen-saturated conditions in the mat matrix. This could prove inhibitory to anaerobic carbon, nitrogen, and sulfur transformation processes, especially near the mat surface. For example, daytime O_2 supersaturation presents a problem for N_2 fixation, denitrification, sulfate reduction, and other anaerobic processes (e.g., methanogenesis) that are reliant on photosynthetically produced organic matter as an energy source, yet are inhibited by even low ambient O_2 concentrations (91–93). Sulfate reducers account for a large fraction of the heterotrophs in some mats (90), and are also suspected of being significant contributors of fixed nitrogen (94). Although N_2 fixation can be observed during daytime, rates often increase at night or in the early morning when pO_2 is low. It is suspected that sulfate reducers may contribute to N_2 fixation at this time.

Denitrification can be an important process mediating nitrogen availability in microbial mats (95). Denitrifying bacteria include taxonomically diverse heterotrophs that are either facultatively anaerobic (*Pseudomonas* and *Thiobacillus*) or obligate anaerobic (*Bacillus* and *Spirillum*) genera. Molecular O_2 strongly inhibits denitrification because O_2 can outcompete NO_3^- as a respiratory electron acceptor. Oxygen can also be directly toxic to some denitrifiers. Therefore, denitrifiers are often most effective in anoxic zones near NO_3^- sources. Organically enriched surface sediments and mats harboring anoxic microzones overlain by NO_3^- rich bottom water are optimal conditions for denitrifiers (96,97).

Even though sulfate reduction, N_2 fixation, and denitrification are all inhibited by even low pO_2, they can co-occur during peak photosynthetic (i.e., high O_2 evolving) periods (56,90,97,98). Consortial syntrophic associations between presumed metabolically incompatible organisms, such as oxygenic cyanobacteria and obligate anaerobic purple sulfur bacteria, sulfate reducers and facultative aerobes (99,100), and sulfate reducers and methanogens (101) have been demonstrated. These results can be reconciled with the observation of small-scale (i.e., 1 to 100 μm) anoxic microzones in an oxygenated matrix (i.e., mats) as determined by localized reduction of tetrazolium salts (79,102) and the presence of active photosynthetic bacteria (103).

In mats, anoxic microzones are promoted and maintained by abundant supplies of organic matter (via sedimentation and high rates of primary production), which support high rates of respiration. If respiratory O_2 consumption exceeds inward O_2 diffusion, anoxic microzones can be formed even in the upper layers of mats. In addition, facultative anaerobic and aerobic bacteria associated with anaerobic bacteria provide removal of potentially toxic O_2 (79). Microscopic observations of mats treated with redox (tetrazolium) dyes reveal a highly heterogeneous matrix supporting anoxic microzones even within largely oxic regions (determined by microelectrodes) (103). This heterogeneity promotes high genetic and physiological diversity, needed to fully optimize production and nutrient cycling processes requiring a range of oxygen conditions. The production of mucilaginous exopolysaccharides (EPS) by resident cyanobacteria and associated bacteria promotes sediment stabilization and cohesion of the mat, acts as diffusional barrier, provides surfaces and substrates for growth (104), and selectively binds potential toxins such as heavy metals (105).

ECOLOGICAL AND BIOGEOCHEMICAL ROLES OF AGGREGATES AND CONSORTIA

By virtue of ensuring quasi-stable, small-scale biogeochemical gradients, planktonic aggregates and surficial microzonal habitats provide a protective set of niches for complex, highly interactive microbial production and nutrient cycling processes. These microzones harbor metabolically diverse microbes even in the face of severe environmental constraints. As a result, life in some of the most extreme environments on Earth largely operates in a consortial mode. We will explore the consortial "players," their metabolic and structural strategies for survival and growth in a range of environments, including extreme ones. We will also examine the implications for aquatic production and biogeochemical cycling in physically, geochemically, and ecologically diverse planktonic and benthic habitats. These include

1. Cyanobacterial aggregates in lakes, oceans, and in the brackish Baltic Sea
2. Nitrogen-fixing cyanobacterial (*Trichodesmium*) aggregates in the open ocean
3. Microbial mats in temperate and tropical ecosystems
4. Microbial mats in hypersaline waters
5. Aggregates embedded in the permanent ice cover of Antarctic lakes.

CYANOBACTERIAL AGGREGATES IN LAKES, OCEANS, AND IN THE BRACKISH BALTIC SEA

Planktonic diazotrophic cyanobacteria are common in eutrophic lakes, rare in oceans, and nearly absent in estuaries and coastal seas (106). Exceptions are the Baltic Sea and the Peel-Harvey estuary in Australia (107). Cyanobacteria are responsible for most of the planktonic N_2 fixation, but rates are high only when these organisms make up a majority of the biomass (107). In eutrophic lakes, planktonic N_2 fixation may account for 6 to 82% of the nitrogen input into the system. In the surface waters of the world's oceans and estuaries, including eutrophic estuaries, N_2 fixation provides a far lower percentage of nitrogen input (107). In the Baltic Sea,

however, planktonic N_2 fixation provides at least 15% of the nitrogen input (108).

The mass appearance of N_2 fixing cyanobacteria in eutrophic lakes has been linked to the ratio of nitrogen to phosphorus loading. An N/P ratio near or below the Redfield ratio of 16 : 1 provides a selective advantage for N_2 fixing phytoplankton species. However, planktonic N_2 fixation is low in many estuaries despite low N/P input ratios. One explanation for this conspicuous absence is iron or possibly trace element limitation (109,110). Another explanation is that persistent high turbulence negatively impacts N_2 fixing cyanobacterial growth and hence reduces their dominance (111). Lastly, organic matter enrichment may be a prerequisite for bloom formation (112,113). The cyanobacterial blooms in the Baltic Sea and Peel-Harvey estuary have been linked to high concentrations of dissolved organic matter, which increase the availability of iron and possibly other metals through chelation (114,115). With the exception of iron (discussed later), none of these factors appear to explain large blooms of the pelagic marine N_2 fixer *Trichodesmium*, which inhabit some of the most nutrient-deprived waters on Earth (116).

Nodularia spumigena is the dominant cyanobacterium in the Peel-Harvey estuary, Australia (117). In the Baltic Sea, *N. spumigena* is also abundant but other heterocystous cyanobacteria, such as *Aphanizomenon flos-aquae* and *Anabena* spp. coexist in blooms (118–121). *Nodularia spumigena* and *A. flos-aquae* blooms may be initiated in the open Baltic following deep-water phosphorus intrusions to the warm surface layer, providing a competitive advantage for N_2 fixers (121).

In the open sea, contrary to many enclosed coastal bays, hydrodynamically induced nutrient additions to the phytoplankton community are pulsed, episodic events. As shown by Rothhaupt and Güde (122) and Thingstad and coworkers (123), even minor amounts of nutrients may cause a shift from picoplanktonic (less than 2 μm) to larger organisms. The cyanobacterial blooms occurring in the open Baltic often start with *A. flos-aquae*, which, owing to high phosphorus storage ability, is favored by pulsed phosphorus inputs (124). Once the blooms are established, *N. spumigena* may cover large areas of the open Baltic Sea (Fig. 5) (121).

During their initiation, *N. spumigena* blooms start as straight filaments. During this early stage, phosphorus intrusions from deeper water can occur (121), enabling *Nodularia* to experience unrestricted growth conditions when it fixes N_2 (125,126). If phosphorus inputs to the planktonic community are intermittent, the development and persistence of a bloom must rely on regeneration of the stored phosphorus. In later growth stages, when *Nodularia* dominates over *Aphanizomenon*, formation of large aggregates of coiled *Nodularia* filaments has been observed (Fig. 5).

As a proposed survival strategy, coiling provides a means to engulf and support the development of an active microbial food web promoting phosphorus recycling within the confines of the aggregate. In contrast, *A. flos-aquae* forms tight flake-like aggregates that are not penetrated by other microbes. Rather, bacteria attach to the outside of these aggregates (127,128). In the absence of additional phosphorus pulses, *A. flos-aquae* loses its

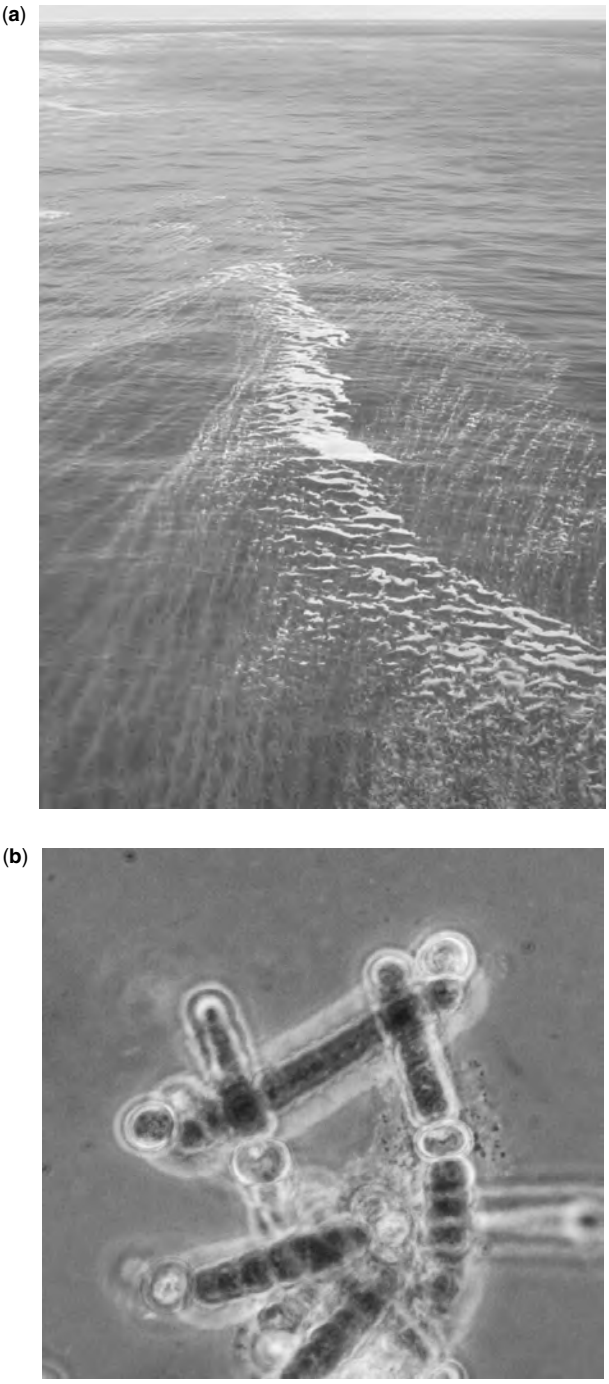

Figure 5. Observations of a bloom of the N_2 fixing cyanobacteria *Nodularia* spp. Left-hand side: a *Nodularia*-dominated cyanobacterial bloom observed in the Gulf of Finland, Baltic Sea (photo courtesy of Pia Moisander). Upper right-hand side: photomicrograph (400x) of several *Nodularia* sp. filaments, showing the nitrogen fixing heterocysts (round, light green cells), neighboring heavily (green) pigmented vegetative (photosynthetically active) cells, as well as associated bacteria. Lower right: coiled *N. spumigena* aggregates with associated bacteria and protozoans present inside the coils (400x). See color insert.

(C)

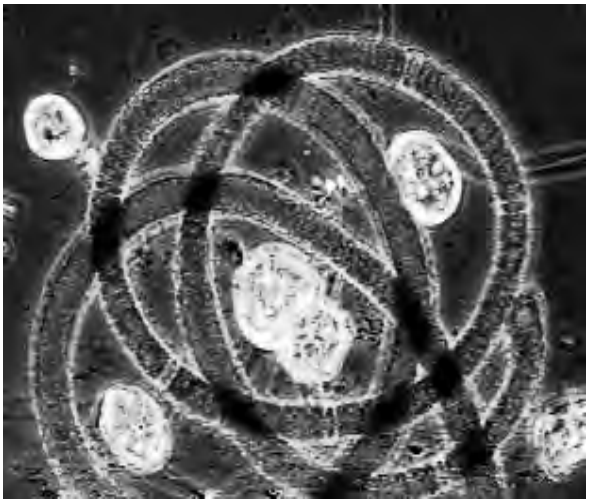

Figure 5. (*Continued*)

dominance owing to its reliance on this phosphorous supply mechanism. Under these circumstances, *N. spumigena* can persist because it is able to maintain effective phosphorus regeneration mediated by the microbial consortia in its coiled matrix. Thus, coiling-based aggregate formation provides a competitive advantage to *N. spumigena*, in part owing to its ability to "win the battle" against diffusive loss of nutrients. In addition, *N. spumigena* has a higher overall affinity for phosphorus than *A. flos-aquae*, which helps explain the successional sequence of the two species (129).

What is the biogeochemical contribution of cyanobacterial blooms to the Baltic ecosystem? Apart from the toxicity aspect of the cyanobacterial blooms, little is known about the contribution of blooms to, for example, carbon flux. Although visually impressive, the cyanobacteria may contribute only little to the pelagic food web in terms of carbon as the two dominant cyanobacterial species *N. spumigena* and *A. flos-aquae* are poorly eaten (only a few percentage of the biomass) by mesozooplankton (130,131). Little or no sedimentation of the summer plankton (including cyanobacteria) has been recorded in various basins (132–134). During the cyanobacterial bloom period, less than 1% of the suspended concentrations settled daily (133), which led the authors to conclude that the cyanobacterial bloom had disintegrated into dissolved and particulate organic pools incorporated into the food web via the microbial loop. Nevertheless, in *N. spumigena*-dominated blooms, a highly active microbial food web may develop within aggregates and thus contribute to pelagic planktonic production (135). In terms of the Baltic Sea's nitrogen budget, cyanobacterial aggregates appear to be important nitrogen transformation sites. N_2 fixation by cyanobacterial bloom-formers is a significant source of externally supplied or "new" nitrogen (108,136). It is suspected that aggregates may also be sites of denitrification (137); however, rates and overall contributions to nitrogen losses via this process have not been adequately assessed. It is possible that N_2 fixation and denitrification may co-occur in aggregates, complicating their roles in the nitrogen budget.

NITROGEN-FIXING CYANOBACTERIAL (*TRICHODESMIUM* SPP.) AGGREGATES IN THE SUBTROPICAL AND TROPICAL OCEAN

Among planktonic cyanobacterial aggregate-formers, the filamentous nonheterocystous N_2 fixing genus *Trichodesmium* is the most widespread and biogeochemically important contributor of "new" nitrogen to nitrogen-depleted subtropical and tropical oligotrophic oceans (138,139). *Trichodesmium* occurs as buoyant macroscopic (a few millimeters in length) radial (puff) or fusiform (tuft) aggregates (Fig. 6), which exhibit high rates of primary production and N_2 fixation, as well as associated microbial (bacterial, microalgal, and protozoan) and crustacean grazer populations (140,141). When calm seas prevail, buoyant aggregates accumulate as large (sometimes greater than 100 km^2) yellow-brown slicks covering the ocean's surface (139,142). *Trichodesmium* is one of the few planktonic nonheterocystous genera that can fix N_2 and CO_2 (and hence evolve O_2) simultaneously, exhibiting parallel maxima of these processes during daytime. Capone and coworkers (143) showed that even when supplied with light during nighttime, N_2 fixation (but not CO_2 fixation) ceased, suggesting strong daytime dependence and a diel "rhythm" in its diazotrophic behavior. Molecular studies indicate that the nitrogenase enzyme complex of *Trichodesmium* is structurally (i.e., amino acid sequence) conserved and similar to that found in other diazotrophs (144,145). As with all other diazotrophs, *Trichodesmium*'s nitrogenase is O_2 sensitive. However, unlike heterocystous cyanobacteria (e.g., *Nodularia*, *Aphanizomenon*), in which nitrogenase is protected in O_2-devoid heterocysts, *Trichodesmium* reveals no obvious cellular differentiation. A great deal of research and speculation has been directed toward clarifying how this enigmatic diazotroph manages to optimize these potentially cross-inhibitory processes in the open ocean. *Trichodesmium* fixes N_2 at highest cellular rates when present as surface-dwelling aggregates as opposed to single filaments (116,142,146,147). When viewed in situ, aggregates reveal highly-compacted, dense, dark-pigmented central cores, while their peripheral regions are often more diffuse and less compacted (148,149) (Fig. 6). Fogg (13) hypothesized that CO_2 and N_2 fixation were spatially segregated, with CO_2 fixation confined to high irradiance external regions and N_2 fixation confined to optically dense internal regions. Carpenter and Price (150) and Paerl (149) provided support of Fogg's hypothesis by microautoradiographically showing that external regions of individual trichomes were photosynthetically much more active than internal regions. Furthermore, numerous studies (148–151) have shown a positive relationship between calm sea state (facilitating aggregation) and rates of N_2 fixation in natural Caribbean Sea, Indian, and Pacific Ocean *Trichodesmium* populations. Bryceson and Fay (148) also showed a strong direct correlation between aggregate shape, size, and N_2 fixation potentials in *Trichodesmium* sampled off the east African coast.

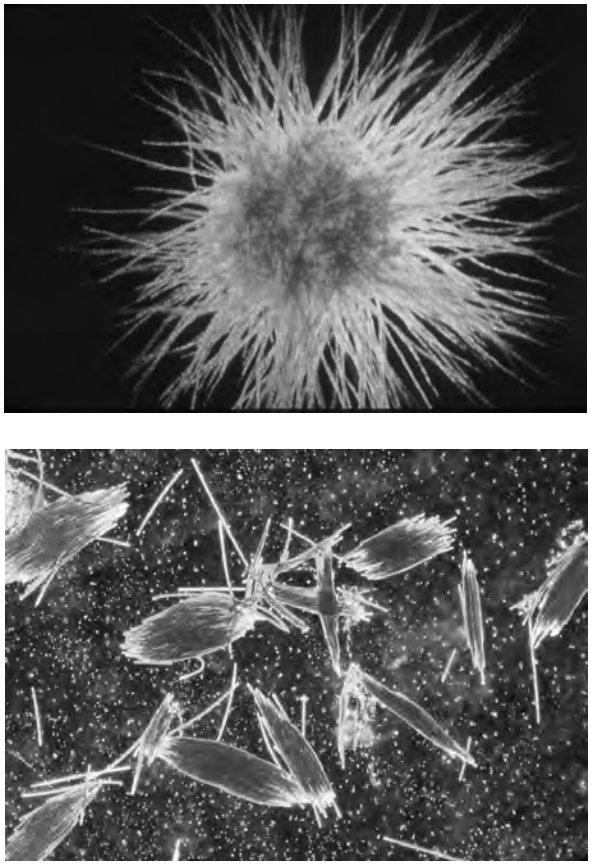

Figure 6. Surface-dwelling aggregates of the filamentous marine planktonic N_2 fixing cyanobacteria *Trichodesmium* spp. Upper frame: radial, or "puff"-shaped aggregates, sampled during a bloom in the Gulf Stream, W. Atlantic Ocean, off the coast of North Carolina. Lower frame: fusiform "tuft"-shaped aggregates sampled in the W. Atlantic Ocean near San Salvador Island, Bahamas. For more details on the N_2 fixing characteristics, and microbial ecology of these aggregates see Paerl (149). See color insert.

Paerl and Bland (102) demonstrated, using tetrazolium dye reduction, that highly reduced (O_2 depleted) regions were present in actively N_2 fixing *Trichodesmium* aggregates obtained from the western Atlantic Ocean (Gulf Stream). Using O_2 microelectrodes, Paerl and Bebout (147) directly measured small (μm) O_2-deplete internal regions in *Trichodesmium* aggregates. Collectively, these studies showed that photosynthetically active *Trichodesmium* aggregates could simultaneously exhibit high and low pO_2 microzones.

Recently, Fredricksen and coworkers (152) showed partitioning of nitrogenase and photosynthetic activities within single filaments of both naturally occurring and cultured *Trichodesmium* populations, suggesting spatial segregation of these processes without the aid of heterocysts. Microautoradiographic examinations of $^{14}C-CO_2$ fixation under both natural and artificial irradiance conditions (149) showed internal regions of aggregated filaments to be far less photosynthetically-active (and hence having low O_2 evolution rates) than the external regions. One explanation for this partitioning is self-shading, imposed by the dense aggregates. It is also possible that respiration is heterogeneously distributed in aggregates. Immunological studies did not show similar partitioning for nitrogenase, with this enzyme being scattered, possibly as groups of cells, throughout aggregates (140,153). These studies indicate a widespread potential for N_2 fixation throughout aggregates. Thus, while photosynthesis appears to be strongly partitioned between inner and outer regions, nitrogenase is not. The implications are that when inner regions of aggregates are photosynthetically inactive (and hence O_2 subsaturated), N_2 fixation can proceed there. Energy needed for supporting N_2 fixation would be supplied by photosynthetically active cells near the outer regions transporting reductant to the inner regions along the filaments, which was shown by autoradiography (149). These studies provide evidence for spatial partitioning of these processes, which is promoted by aggregation. Interestingly, Paerl (149) demonstrated that even single *Trichodesmium* filaments were able to partition photosynthesis along their length, with central regions having lowest rates. However, N_2 fixation and photosynthesis only co-occurred at very low irradiance levels (less than 100 $\mu E\,m^{-2}$ second) in single filaments, while aggregates were able to simultaneously conduct these processes at irradiances exceeding 500 $\mu E\,m^{-2}$ second. Aggregation therefore appears advantageous by enabling this genus to optimize these essential processes in radiant energy-rich, oligotrophic surface waters.

As observed among *Nodularia* aggregates in the Baltic Sea, *Trichodesmium* aggregates host diverse bacterial, microalgal, and protozoan epibionts (154,155). Bacteria associated with *Trichodesmium* are actively engaged in assimilating organic compounds, including amino acids and sugars known to be excreted by the "host" cyanobactria (139,155). Bacteria isolated from naturally occurring *Trichodesmium* aggregates are mostly aerobic heterotrophs. Some reveal chemotactic behavior, being attracted to nitrogen-containing amino acids (155), reflecting *Trichodesmium* excretion products (139). Culturing experiments show high *Trichodesmium* growth and N_2 fixation rates in the presence of these bacteria. These findings suggest mutualistic consortial associations, in which organic excretion products from *Trichodesmium* support the growth of associated bacteria, while these bacteria may promote *Trichodesmium* growth. Factors potentially involved in stimulating "host" *Trichodesmium* growth include localized respiratory O_2 consumption, nutrient (phosphorus, CO_2 recycling), and exchange of beneficial bioactive compounds, such as vitamins and chelators.

An additional functional role of surface-dwelling *Trichodesmium* aggregates is their possible ability to intercept atmospheric nutrient inputs. Iron (Fe) is a nutrient required as a cofactor in enzymes and energy transferring molecular mediating the synthesis and activities of the photosynthetic and nitrogen fixing machinery. This metal is known to be in short supply (i.e., limiting) in the tropical open oceans (156). Field and laboratory studies (116) indicated that iron supply controlled N_2 fixation

and growth in *Trichodesmium* blooms off the North Carolina coast (Gulf Stream). The dominant source of new iron in these as well as open ocean waters is atmospheric, either via dry or wet deposition (157,158). Rueter and coworkers (159) hypothesized that, by forming surface-dwelling aggregates, *Trichodesmium* enhances its ability to intercept iron associated with small particles (dust, aerosols) deposited on the ocean surface. Indeed, there appears to be geographic overlap between relatively high frequencies and densities of *Trichodesmium* blooms and areas of iron deposition downwind of iron-enriched African Saharan dust storms the Atlantic Ocean between 20°N and the equator (160). Alternatively, the concentrated blooms may simply arise from lengthy periods of calm seas state (i.e., "horse latitudes") periodically encountered in this region (160).

CYANOBACTERIAL MATS IN A TEMPERATE MARINE ECOSYSTEM: BIRD SHOAL, NORTH CAROLINA

Microbial mats are common features of shallow water marine environments. Coastal (Atlantic Ocean) North Carolina is blessed with expansive lagoonal, intertidal mud- and sandflat habitats supporting mats. Bird Shoal (~220 ha), located near Beaufort, NC (34°40′ N, 74°42′ W) forms the southern edge of the Outer Banks barrier island system ringing the major estuaries and Pamlico Sound, the second largest estuarine complex in the United States. Microbial mats on Bird Shoal are mainly located in the high intertidal on a protected, siliclastic sandflat. They are inundated twice daily with coastal Atlantic Ocean water. The climate is temperate to subtropical, with air temperatures ranging from an extreme low of −5 °C in the winter to temperatures often exceeding 30 °C in the summer. Water temperature ranges from 12 to 30 °C. Irradiance can reach up to 2,000 $\mu E\,m^{-2}\,s^{-1}$ during summer, when extended periods of low tide occur at midday, and evaporation greatly elevates the porewater salinity. Owing to this evaporation, mats may also experience desiccation. Mats in the supratidal may become desiccated for extended periods (days to weeks). The mats go through a dramatic diel cycle of O_2 supersaturation during the day to anoxia at night. Ambient waters are nitrogen-limited year-round (161). The impact of major storms such as hurricanes and nor'easters is unknown, but is likely to have a strong structuring effect.

The Bird Shoal mats are highly laminated, with resultant strong vertical biogeochemical (i.e., pO_2, E_h) zonation (91–93) (Figs. 4 and 7). The upper layers of the mat are dominated by non-heterocystous, filamentous cyanobacterial genera, including *Microcoleus*, *Lyngbya*, and *Oscillatoria*, and coccoid forms (e.g., *Synechocystis*, *Synechococcus*) (91,93). Other filamentous, nonheterocystous cyanobacterial genera, including *Phormidium*, *Arthrospira*, and unicellular morphotypes are present to a lesser extent. Diatoms are present in the surface layer, but they comprise less than 15% of total microalgal biomass (162). Purple sulfur bacteria are occasionally observed below the green cyanobacterial layer and pigment analyses indicate the presence of bacteriochlorophyll *a* (163,164). Ambient waters are nitrogen-deplete, leading to year-round nitrogen-limitation of planktonic and benthic primary production (93,161). Therefore, mats must rely on N_2 fixation to satisfy microbial nitrogen requirements (91,165). Recent estimates of nitrogen inputs by microbial N_2 fixation indicate that this process meets at least half of the phototrophic community's demand for nitrogen, the remaining nitrogen being supplied through efficient nitrogen recycling within the mat (166). *NifH* phylogenetic analysis of genomic DNA extracted directly from the mats and from cultured mat isolates has demonstrated

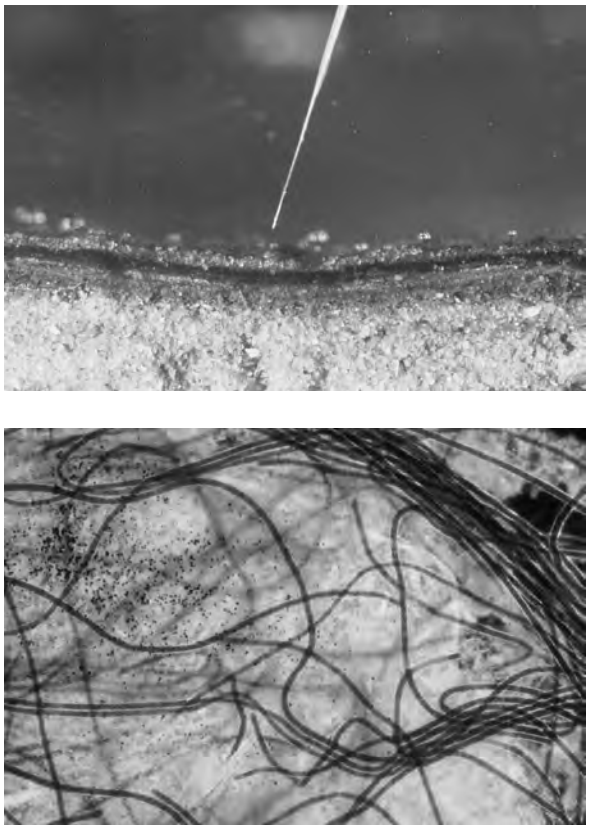

Figure 7. Laminated microbial mat obtained from the Bird Shoal, coastal North Carolina. As is the case with most marine intertidal mats, microalgal biomass and production are dominated by cyanobacteria. The cyanobacteria are concentrated in the upper, illuminated segment of the mat. The mat thickness is approximately 0.5 cm. Upper frame: cross-section of the mat, showing distinct microbial laminations. Phototrophic cyanobacteria dominate the upper, euphotic zone of the mat. Below that is a layer composed of photosynthetic bacteria, cyanobacteria, and heterotrophic bacteria. The lowest layer contains obligate anaerobic heterotrophs, including sulfate-reducing bacteria. Methanogenic bacteria can also be found in this layer. The mat is situated on highly porous sands, which allow for rapid exchange of sweater and oxygen. Hence, the lighter oxygenated appearance of the lower sand layer. The tip (~100 μm diameter) of an oxygen microelectrode is shown for size reference. Lower frame: photomicrograph of the mat matrix. Shown are nonheterocystous filamentous cyanobacteria (*Microcoleus chthonoplastes*) and a variety of bacteria, including microcolonies of purple photosynthetic bacteria (*Chromatium* sp.). See color insert.

a diverse assemblage of diazotrophs (86,167). These organisms include filamentous nonheterocystous cyanobacteria, microaerophilic beta/gamma proteobacteria, and anaerobic bacteria similar to delta SRB. The diazotrophic cyanobacteria genera include *Lyngbya* and *Oscillatoria*.

Interestingly, the numerically dominant filamentous cyanobacterium *M. chthonoplastes* does not appear to possess the genetic (*nifH*) ability to fix N_2 (87). Instead, it supports bacterial N_2 fixing epibionts that most likely utilize organic matter excreted by this cyanobacterial "host". In addition to being a major source of organic matter supporting consortial N_2 fixation, *Microcoleus* forms the "fabric" that consolidates the mats and maintains structural integrity, while providing numerous microaerophilic and anaerobic niches for metabolically diverse bacteria involved in nutrient cycling. In this manner, the presence of a highly productive non-N_2 fixer provides adequate organic carbon to support consortial N_2 fixation and complete nutrient cycling (regeneration of existing nitrogen and other nutrients), thereby enabling the mat to remain highly self-sufficient in a nutrient deplete, periodically hostile and environmentally extreme environment. The major N_2 fixing taxa in these mats can be found in other temperate shallow-water intertidal marine habitats having mats (Tomales Bay, California; Sippewisett Marsh, Massachusetts) (167). In coastal North Carolina, mats are present year-around, but reveal distinct seasonal patterns in community composition, photosynthetic performance, and diel patterns of N_2 fixation, indicative of both taxonomic and metabolic diversity (87,93).

The observed rates and patterns of N_2 fixation (nitrogenase activity) appear to be dependent on complex interactions between seasonality, mat development, and community structure. In the winter, NA rates are low, but exhibit maximum activity during the day. In the summer, average rates increase greatly. In less developed mats during the summer, substantial daytime NA may be observed, with maximum rates observed just after sunrise (93,166). In more developed mats during the summer, daytime NA is greatly repressed and maximum rates occur sometime during the night (Fig. 8). Results from the experiments designed to assess the inhibition of total photosynthesis, oxygenic photosynthesis, and protein synthesis suggest that different physiological strategies translate into contrasting diel NA patterns. Additionally, the results infer that multiple physiological strategies are utilized to sustain daytime NA during the summer. The inhibitory effect of O_2 on NA occurs very early. Evidence suggests that de novo protein synthesis is required to sustain daytime NA. Reverse transcriptase-polymerase chain reaction (RT-PCR) results demonstrate that organisms other than cyanobacteria are at least actively transcribing the functional genes for N_2 fixation (166). They further imply that transcription of nitrogenase for different organisms may occur as environmental conditions within the mat change.

Mats are an important source of organic matter (i.e., primary production) and fixed nitrogen in what

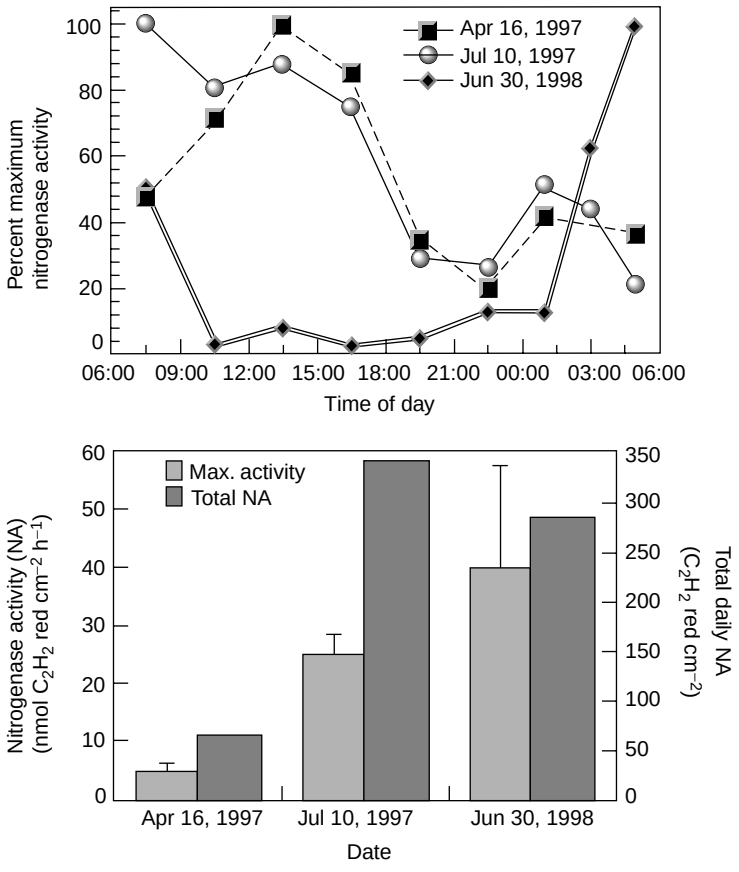

Figure 8. Observed diel variation in N_2 fixation (nitrogenase activity) in the Bird shoal microbial mats, Beaufort, North Carolina. The diel variation observed on April 16, 1997 depicts typical late wintertime observations. Highest specific nitrogenase activity is generally observed during midday. Diel variations observed on July 10, 1997 and June 30, 1998 depict diel nitrogenase activity variations observed from late spring through fall. Highest specific nitrogenase activity is generally observed during very low light periods near sunrise. Values are shown as percentage of maximum nitrogenase activity that was observed during diel experiments. (Data from 166)

are often very nutrient-deprived (oligotrophic) coastal regions (91,113,165). They represent an important nutritional source for micro- and macroinvertebrates that are critical food sources for a variety of fish species that utilize lagoonal marsh environments as nurseries and refugia from predators. The full biogeochemical and trophic importance of microbial mats is only now beginning to be understood.

STROMATOLITIC MATS OF HIGHBORNE CAY, EXUMA SOUND, BAHAMAS

Stromatolites are highly laminated, lithified ($CaCO_3$) rock formations that are at least partially formed by microbially mediated $CaCO_3$ precipitation (60). Fossil stromatolites, dating back 2.5 billion years, are among the oldest evidence for organized life on Earth (59). The stromatolites of Highborne Cay are subtidal along 2.5 km of an eastward-facing beach facing Exuma Sound, Bahamas. They form as ridges and columnar heads up to a half a meter height. The ambient waters range in salinity from 35 to 37 psu and are saturated with respect to calcite and aragonite (60). Surface irradiance can exceed 2,200 $\mu E\, m^{-2}\, s^{-1}$. Their depth at low tide is approximately one and a half meters. Water temperature ranges from 21 to 30 °C. The stromatolites are protected by a fringing reef complex, but are still subjected to substantial wave energy. Ambient nutrient levels (nitrogen, phosphorus, trace metals) are extremely very low. Laminated cyanobacteria-dominated mats cover the surface of the stromatolites (Fig. 9) and their metabolic activity is thought to be responsible for the formation of lithified laminae and subsequent buildup of the stromatolites (60). These mats constitute the dominant source of "new" production and nitrogen input to this nutrient deprived ecosystem. The internal microenvironment is characterized by vertical O_2 concentration gradients that are typical of microbial mats (168,169).

The phototrophic community is dominated by nonheterocystous, filamentous cyanobacteria *Schizothrix* spp., but the biomass is very low (Pinckney, unpublished). Other prevalent nonheterocystous cyanobacterial genera include *Phormidium*, *Lyngbya*, and *Microcoleus*. Unicellular cyanobacteria, such as *Solentia* spp. are also commonly observed. Diatoms are present, but not abundant. *NifH* phylogenetic analysis has yielded sequences most similar to unicellular cyanobacteria, filamentous, nonheterocystous cyanobacteria, including *Lyngbya* sp. and *Phormidium* sp., alpha proteobacteria, and anaerobic bacteria similar to SRB.

Rates of N_2 fixation show seasonality, but diel patterns do not. In both late winter and summer, most N_2 fixation is confined to nighttime, but summertime rates are much higher. However, the ratio of N_2 fixed to net primary production (CO_2 fixation) is relatively low. Net production values in Highborne Cay stromatolites for comparable times of the year range from one-third to a little over half the values for temperate Bird Shoal mats. Maximum N_2 fixation values are an order of a magnitude less than what has been observed in the Bird Shoal mats. Inhibition of protein synthesis by chloramphenicol had different effects on N_2 fixation rates depending on the season, suggesting contrasting physiological controls on this process at different times of the year.

Figure 9. Stromatolitic mat communities on the Exuma Islands, Bahamas. Upper frame: view of the subtidal stromatolites situated off a beach at Highborne Cay, Exumas. Middle frame: cross-sectional view of a laminated ($CaCO_3$) stromatolite, showing the cyanobacteria-dominated mat residing on the surface of the stromatolite. Lower frame: photomicrograph, showing the aggregated filamentous cyanobacteria (*Schizothrix* sp.) comprising a bulk of the surface mat (from 104). See color insert.

There are close associations between cyanobacteria and bacterial epibionts that appear to play an important role in the layered deposition of $CaCO_3$ that characterizes stromatolites. Microscopic and microautoradiographic observations indicate that the dominant cyanobacterial

taxa (most notably *Schizothrix* and *Lyngbya*) produce copious amounts of sheath and exopolysaccharide (EPS) materials (60,105). These materials can strongly bind calcium ions and by doing so prevent $CaCO_3$ precipitation and lithification (60). Bacteria that are embedded in the sheath and EPS decompose this material, thereby liberating Ca^{++} ions into solution. These Ca^{++} ions are then rapidly precipitated, allowing lithification to proceed. Microscopic investigation reveals bacteria encapsulated in $CaCO_3$ (aragonite), while the filamentous cyanobacteria remain free of precipitates as long as they are covered by sheaths and EPS (104). Thus, rather than being directly mediated by cyanobacterial precipitation, the lithification of stromatolitic mats is indirectly controlled by cyanobacteria, which produce organic matter that is mineralized by bacteria mediating precipitation (104). The ecological and biogeochemical significance (and underlying physiological mechanisms) of this consortially mediated lithification process require further clarification.

MICROBIAL MATS IN HYPERSALINE LAKES: CONSORTIA "AT THE EDGE OF LIFE"

The Bahamian hypersaline lakes are among the most extreme aquatic environments on Earth. These lagoonal ponds are isolated from the nearby oligotrophic oceans (Atlantic and Caribbean) by narrow dunes and carbonate ridges. They are exposed to hot (sometimes exceeding 40 °C), arid and extremely high irradiance (greater than 2,200 $\mu E\, m^{-2}\, s^{-1}$) conditions throughout much of the year. Annually, evaporative water loss greatly exceeds rainfall input, leading to persistent hypersaline conditions exceeding 150 psu (seawater salinity is ~35 psu). Because rainfall is extremely low and restricted to tropical storms of oceanic origin and surrounding carbonate soils are highly leached, nutrient (nitrogen, phosphorus, iron, and trace metal) inputs to these lakes are very small and restricted to aeolian sources (i.e., dust). As a result, the lakes exhibit a broad spectrum of environmental extremes and limitations. Despite these hostile conditions, the lakes support an abundant microbial community, largely confined to microbial mats lining the lake bottoms (164). The nitrogen requirements of these mats are largely met by N_2 fixation and nitrogen regeneration in the mat matrix (166). San Salvador Island, Bahamas (24°00′ N, 72°05′ W) has numerous hypersaline lagoonal systems, including: (*1*) Storr's lake (45 → 90 psu), which supports calcifying, stromatolitic mats dominated by the heterocystous cyanobacterial genus *Scytonema*, nonheterocystous *Schizothrix*, and coccoid forms *Synechocystis, Synechococcus*; and (*2*) Salt Pond (45 → 100 psu), which contain a highly laminated, noncalcified cyanobacterial mat dominated by nonheterocystous filamentous *Microcoleus, Lyngbya*, and *Oscillatoria* species (Fig. 10).

The hypersalinity is inhibitory to both photosynthesis and N_2 fixation (164,170), and may play a role in selecting for cyanobacterial species, because eukaryotic microalgae are confined to only a few diatom species that constitute less than 10% of the total microalgal biomass. Cyanobacterial biomass often exceeds 50 nmol Chl *a* cm^{-2}, which is very high by benthic microalgal standards (164,170). Biomass is highly compacted in the surface layer of the mat, causing rapid light extinction with depth. Photosynthetically active radiation (400 to 800 nm) decreases from greater than 2,200 $\mu E\, m^{-2}\, s^{-1}$ at the surface to undetectable levels at 4 to 5 mm depth. This leads to a highly compressed euphotic zone with strong vertical biogeochemical (O_2, E_h) zonation; typically, during daylight the upper 1 to 2 mm of the mat is O_2 supersaturated, while the lower 3 to 10 mm is permanently anoxic and sulfidic. A layer of anoxic photosynthetic bacteria (*Chromatium, Rhodopseudomonas*) is present at the oxic–anoxic interface. The O_2 gradients spanning oxic and anoxic zones are dynamic and light attenuation-dependent, varying with time of day and season.

Both light- and dark-mediated N_2 fixation occur in these mats. Rates are generally high (relative to photosynthesis (162,166,170). Both oxygenic (cyanobacterial) and anoxygenic (photosynthetic bacteria) photosynthesis contribute significantly to mat primary production, and are a dominant source of "new" carbon supporting the lake's unusual (extensive microbial loop and invertebrate grazing, but no fish) food web (170).

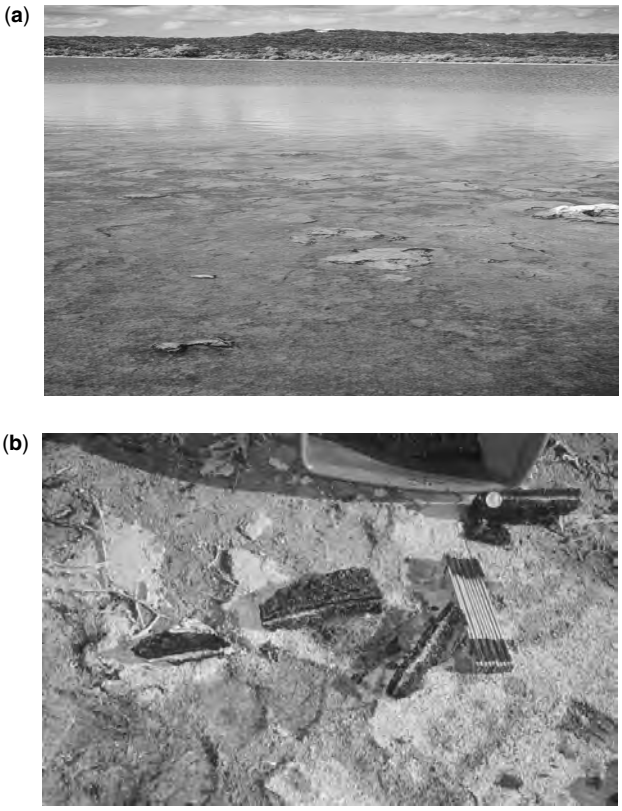

Figure 10. Upper frame: hypersaline (100 to >160 psu) Salt Pond, located on San Salvador Island, Bahamas. The entire bottom of this lake is dominated by laminated mats dominated by filamentous, nonheterocystous cyanobacteria (*Microcoleus* sp., *Lyngbya* sp., and *Oscillatoria* sp.). Lower frame: close-up side view pieces of laminated mat excised from the pond. See color insert.

AGGREGATES AND MATS IN THE PERMANENT ICE COVER OF LAKE BONNEY, MCMURDO DRY VALLEYS, ANTARCTICA

The McMurdo dry valleys of Southern Victorialand, Antarctica (160–164° E, 76°30′–78°20′ S) contain a series of glacial lakes having permanent ice covers varying in thickness from approximately 3 to over 6 m. The dry valleys are among the highest, driest, coldest, and most nutrient-deprived habitats on Earth, with wintertime air temperatures ranging from −40 to less than −60 °C, and summer temperatures ranging from −20 °C to near 0 °C. Because the dry valleys are surrounded by greater than 3,000 m mountains, they are in a "snow shadow," resulting in annual snowfall of only 2 to 4 cm. Under these conditions, evaporation (sublimation) greatly exceeds precipitation, leaving the soils of the valley floor exposed (51). As a result, the dry valley area forms the largest expanse of ice free soils (~4,000 km^2) on Antarctica. Liquid water is confined to a short summer period (December to early February), when air temperatures and solar heating are high enough to generate meltwater from permanent glaciers that reach the valley floor. Meltwater enters the lakes via narrow streams, which flow for only about 2 to 3 months. However, much of the rest of the valley floor is frozen, desiccated desert soil. Under these extremely cold, water-stressed conditions, prokaryotic life largely exists as cyanobacterial-bacterial aggregates and mats associated with the ice cover surface and embedded in the ice matrix (51–53).

Microbial consortia associated with the ice cover include desiccated mats around the edge of the ice cover, and aggregates associated with desert soil particles that are periodically dispersed over the ice surface by strong adiabatic and catabatic winds sweeping this region (42,43). Soil particles are very dark and absorb enough radiant energy to warm and melt their way down into the ice matrix during the austral summer (Fig. 11). These particles aggregate and tend to accumulate in distinct layers in the ice cover. The depths of the layers depend on the extent to which solar heating can maintain molten conditions around the particles. During relatively clear periods, more extensive melting and deeper layers result. A cyanobacterial community is associated with the soil particles (Fig. 11). This includes nonheterocystous genera (*Phormidium, Oscillatoria*), at least one heterocystous genus (*Nostoc*) and coccoid forms (*Synechococcus* spp.) (52,171). During at least nine months of the year (fall through late spring), ice-embedded aggregates exist in a desiccated, "freeze dried" state. Rehydration occurs during the summer period (late November to February) when localized solar heating of aggregates leads to localized melting, followed by activation of metabolic processes and growth (51,52). When desiccated aggregates are experimentally rehydrated, photosynthesis, heterotrophic activity, and N_2 fixation can be detected within a few hours.

The cyanobacterial-bacterial consortia that are associated with aggregates physically and chemically modify the aggregate microenvironment. Following rehydration, exudation of organic matter takes place and redox gradients

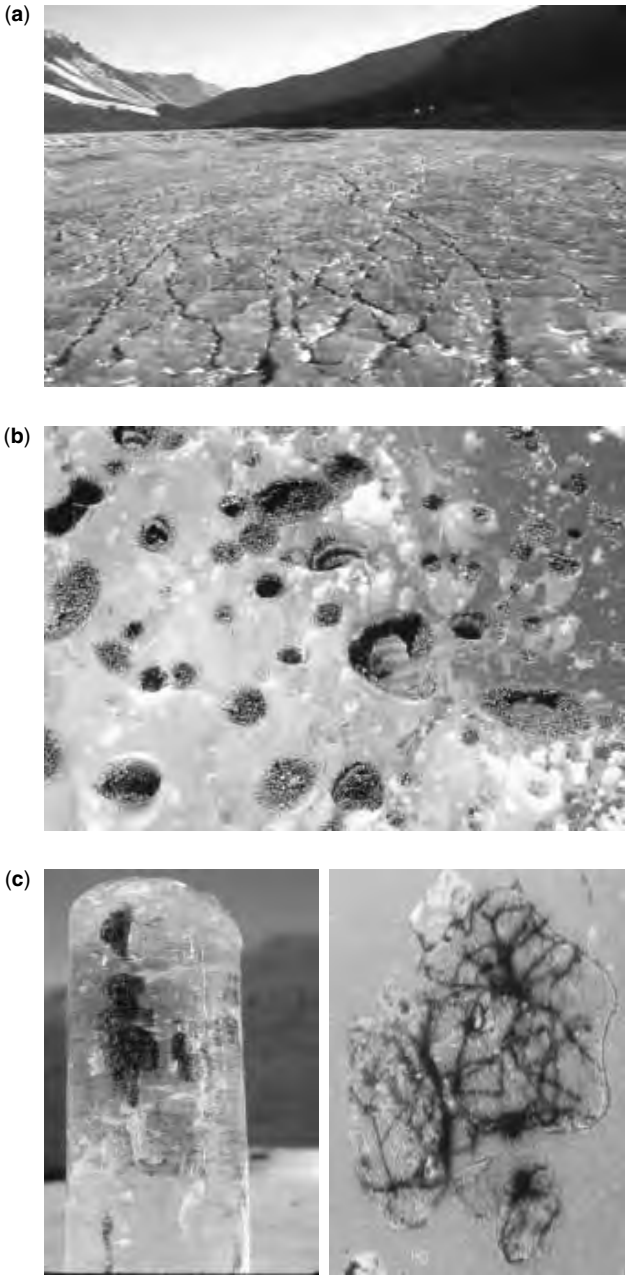

Figure 11. Soil-based microbial aggregates in the permanent ice cover of Lake Bonney, McMurdo Dry Valley, Antarctica. Upper frame: surface view of the ice cover, showing the cracks in which windblown soil accumulates. Middle frame: close-up view of the ice surface, showing localized melting of ice by dark soil aggregates. Eventually, this melting activity causes soil aggregates to settle into the ice matrix. Lower left frame: a core taken from the ice cover, showing soil aggregates embedded in the ice matrix. Lower right frame: microautoradiograph of $^{14}CO_2$ uptake by microorganisms associated with a soil aggregate sampled from the ice matrix. The darkened filaments are photosynthetically active cyanobacteria attached to a soil particle (from 52 and 103). See color insert.

are established in response to microbial photosynthetic O_2 production and heterotrophic O_2 consumption along microscale nutrient, light, and organic matter gradients. Ice aggregates represent structural and functional biocomplexity in the form of interactive production and nutrient cycling consortia ensuring microbial self-sustenance at the "edge of life" on Earth. Molecular studies of aggregate consortia (171,172) indicate a diverse assemblage, including some of the most ancient (i.e., Precambrian) known forms of life on Earth.

CHARACTERIZING AGGREGATE AND MAT CONSORTIAL STRUCTURE AND FUNCTION

Ecophysiological Approaches

The past two decades have seen significant developments and improvements in aggregate and mat consortial rate measurement techniques that are both appropriately scaled and sensitive. There have been breakthroughs in the areas of microsensing (i.e., microelectrodes), microchemical, and molecular techniques that have greatly expanded the "toolbox" microbial ecologists, chemists, hydrobiologists, geologists, and molecular biologists can deploy for examining aggregate and consortial structure and function. Consortial structure and function are most commonly analyzed in terms of (*1*) production and transformation rates (and their interactions), (*2*) community composition and structure, and (*3*) biogeochemical gradient and localization studies. Establishing diurnal and seasonal rate patterns of key production and nutrient cycling processes, such as CO_2 and N_2 fixation, denitrification, and sulfate reduction reveal how carbon, nitrogen, sulfur, and other essential elements are cycled and allocated in aggregates and mats in response to long- and short-term physicochemical influences. Microscale sensing and chemical characterization techniques are detailed by Jørgensen (9), Revsbech and coworkers (168), Canfield and Des Marais (90), Rysgaard coworkers (173), and Stal and Caumette (55), while molecular applications have recently been reviewed by Amann (174), Nielsen and coworkers (175), Zehr and Paerl (145), and Paerl and Zehr (113).

Aggregates and mats reflect broad taxonomic and physiological diversity and plasticity, which interacts with biogeochemical gradients. Gradients in large part reflect the combined product of O_2 production (P) versus consumption via respiration (R) in time and space (10). Gradients are controlled by the concentrations of dissolved O_2 and salinity in water surrounding aggregates and mats and the internally generated P/R ratios. O_2 gradients can be experimentally manipulated, allowing for examinations of the impacts of pO_2 on aggregate and mat structure and function.

Oxygen, pH, and H_2S gradient fluctuations can occur on temporal scales of seconds to minutes and spatial scales of microns to millimeters; spatial scales reflecting consortial dimensions. Microelectrode analyses and tetrazolium redox salt assays have been used to determine chemical gradients on these scales (89,92,102). In addition, microautoradiography allows visualization of those organisms responsible for primary production (CO_2 fixation) and dissolved organic matter (DOM) uptake (of radiolabeled compounds) at individual microorganism and biogeochemical gradient levels (57). Complementary techniques, such as process-specific (i.e., CO_2 and N_2 fixation, nitrification, denitrification) immunofluorescence assays can be used to visualize those cells actively expressing the structural genes (nitrogenase; *NifH*, ribulose 1,5-diphosphate carboxylase; *RubisCo*, nitrate reductase; *Nir*) and hence capacities for enzyme function (176–178).

Experimental approaches to examining the in situ impacts of O_2 tension on aggregate and mat production and nutrient cycling processes include the use of inhibitors of oxygenic photosynthesis (photosystem II). Among these, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) at greater than 10^{-5} M effectively blocks PS II activity, leading to a cessation of O_2 evolution (and CO_2 fixation) without altering respiration (i.e., O_2 consumption) (91,114). Studies (57,58,92,93) have shown that when mats are photosynthetically active (i.e., high rates of O_2 evolution), DCMU additions arrest O_2 production and CO_2 fixation (93), with concurrent stimulation of N_2 fixation rates, indicating that, within the aggregate and mat matrix, in situ O_2 evolution rates are high enough to negatively impact O_2-sensitive N_2 fixation. However, when photosynthetic rates are low (i.e., wintertime), or at nighttime when photosynthesis ceases, DCMU has little impact on diazotrophy. These results indicate N_2 fixation may be restricted by increased O_2 levels accompanying periods of active photosynthesis.

To compensate for this metabolic incompatibility, diazotrophs have evolved various morphological and physiological adaptations, including (*1*) temporally separating photosynthesis from N_2 fixation, by confining the former to daytime and the latter to nighttime; (*2*) spatial separation of the two processes, with photosynthesis concentrated near the illuminated surface of aggregates and mats and diazotrophy taking place in deeper, aphotic layers; (*3*) formation of localized O_2 deplete microzones throughout the matrix, in which R exceeds P; and (*4*) confinement of the N_2 fixation to O_2 devoid cells (heterocysts) or nonphotosynthetic regions of filaments and colonies. Despite the obvious advantage of forming heterocysts, many aggregate and mat systems in nitrogen deplete waters are devoid of heterocystous cyanobacteria (e.g., *Trichodesmium* aggregates, many intertidal mat systems) (55,113). This ironic situation is a puzzling aspect of cyanobacterial evolution, that is, why do microbial mats, biofilms, aggregates, and other surficial communities in nitrogen limited oxic waters and soils not contain more heterocystous genera? The resolution to this dilemma should yield key insights into ecophysiological controls of microbial N_2 fixing consortia.

Consortial composition has recently been assessed by bulk community analysis (DNA and photopigment) and specific gene expression (mRNA) (discussed later). Chemosystematic carotenoids and chlorophylls, quantified using high-performance liquid chromatography have been used as indicators of relative abundance of major phototrophic groups (162,179). 16S rRNA or rDNA sequences are considered accurate measures of microbial diversity (180). Genes encoding for biogeochemically important

and evolutionarily conserved processes, including N_2 fixation, nitrification, and denitrification are receiving increasing scrutiny for phylogenetic and physiological studies. Their application is discussed later. In the case of primary production (CO_2 fixation), the ribulose-1,5-carboxylase gene (RubisCo) is responsible for virtually all primary production in the world and the large subunit, *rbcL*, is evolutionarily well conserved (181). Examining *rbcL, nifH, amoA*, and *nirSK* expression and then correlating the expression with patterns of CO_2 fixation, N_2 fixation, denitrification, and nitrification will allow us to identify consortial members responsible for these processes, environmental factors controlling genetic expression of key functional genes, and how they are manifested in terms of community compositional or physiological responses (Fig. 12).

Molecular Approaches

A serious limitation to an improved understanding of the "players" involved in microbial consortia has been the lack of availability of quantitative identification and characterization techniques. Standard microbiological approaches to the identification of individual populations within microbial consortia, such as selective culturing, require the extensive knowledge of microzonal environmental conditions. This information is not easily determined along a natural gradient. Furthermore, environmental conditions are often transient, depending on the balance between oxygen production and consumption, nutrient uptake and release, and physical control processes, such as turbulence and diffusion, all of which operate on highly variable temporal and spatial scales. As a consequence, only a small portion (less than 1%) of microorganisms from the environment can be grown using routine culturing techniques (182–185).

Determination of functions, including rates of basic metabolic processes, such as photosynthesis, respiration, utilization of organic matter, and nutrient uptake/assimilation have traditionally been limited by the small scales and sample sizes needed to adequately assess microzonal consortial processes.

Small subunit rRNA (16S) sequence analysis is routinely used for the identification and phylogenetic placement of microorganisms without the need for cultivation. Members of the bacterioplankton that have not been previously described have been identified (186,187), and their diversity and distribution have been investigated (188–190). New members of the Archaea have also been identified in the water column (191,192) and in sediments (193,194), and their spatiotemporal variability has also been assessed (190,195). Functional genes encoding for enzymes directly involved in the transformation processes have also been used to characterize natural microbial assemblages. Target genes involved in denitrification (*nosZ*) (185), nitrification (*amoA*) (196), nitrogen fixation (nifH) (197,198), and the oxidation of methane (*pmoA*) (199) have been successfully used. Moreover, the sequences for the dissimilatory sulfate reductase genes (*dsr*) have been recently obtained from different organisms (200,201), and *dsr*-specific PCR primers have been developed to track sulfate-reducing microorganisms, which play a central role in the global sulfur cycle. Detection of target genes such as 16S, *amoA, nosZ*, and *nifH* can be coupled with high throughput techniques, such as terminal restriction length polymorphism analysis (TRFLP) to characterize consortia in a timely, quantitative, and unequivocal manner.

The utility of highly conserved functional genes for microbial consortial characterization studies can be readily demonstrated for *nifH*. Because studies of *nifH*-based diversity and phylogeny have been underway for at least a decade, a substantial database on taxa-specific sequence regions of interest has been established (86,87,145,167,172,197,198). This database can then be used to establish phylogenetic trees, which serve as references against which established as well as novel sequences derived from N_2 fixing consortia can be compared and characterized according to specific functional groups (i.e., heterotrophs, such as

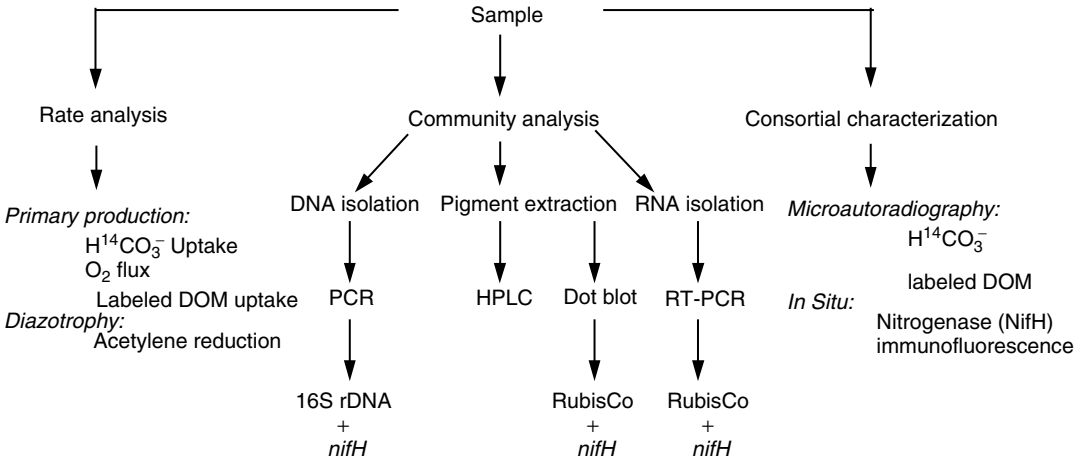

Figure 12. Schematic diagram showing complementation of rate measurements, diagnostic indicators of microalgal community composition with nucleic acid determinations of functional (i.e., *nifH*), phylogenically important (16s rRNA) genes for determining the structure and function of microbial consortia in natural samples.

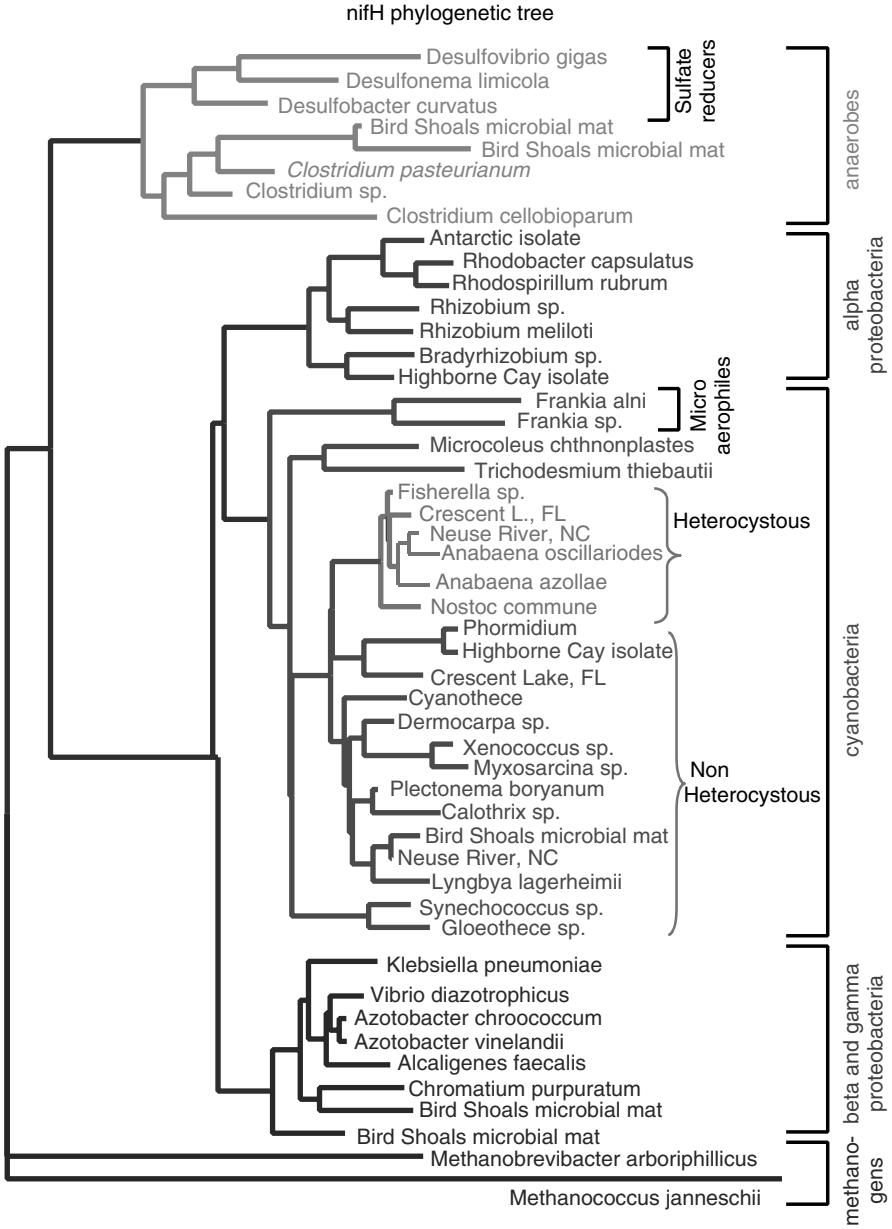

Figure 13. Phylogenetic tree of nitrogen fixing microbial groups, many of which participate in consortial associations. The tree was based on analysis of a 326 base pair nucleotide fragment of the *nifH* gene, which encodes for the highly conserved dinitrogenase reductase subunit of nitrogenase, the N_2 fixing enzyme complex. The tree was constructed by the neighbor-joining method and bootstrap values greater than 50% are given above or beside the corresponding nodes. See color insert.

sulfate reducers, chemolithotrophs, coccoid, filamentous-heterocystous, heterocystous cyanobacteria) (Fig. 13).

While molecular characterization of target genes, such as *nifH* and 16S rRNA, is a routine procedure in many microbial ecology laboratories, the procedure is slow, laborious, and potentially prone to biases. These biases are being overcome by developing highly reproducible extraction procedures (202) and quantitative nucleic acid characterization methods (202–204). Recently, rapid "fingerprinting" methods, utilizing fluorescent end-labeling of PCR product (target genes) and screening by terminal restriction length polymorphism (TRFLP; see Refs. 205,206) have greatly streamlined and improved the accuracy of these methods. Highly reproducible fingerprints can be obtained from complex samples. It is now possible to rapidly identify target genes for specific nutrient transformations using TRFLP analysis of clonal libraries to minimize the time necessary to identify specific target gene clones. For example, TRFLP analyses have been used to screen for denitrifying bacteria in sediments from salt marshes, fresh water marshes, and coastal sediments (207).

Specific target genes (and the nutrient transformations they catalyze) are available for characterizing aquatic microbial consortia. These biomarkers can be used to assess the numbers and activity of various consortial members, using TRFLP analysis. For example, one way to ascertain whether bacteria involved in denitrification are indeed active in an environment involves determining if a functional gene (like *nosZ*) is being transcribed (i.e., mRNA synthesis) (207). Rate measurements can then accompany transcript information to elucidate

consortia members that are actively growing and hence participating in nutrient transformations of interest (in this case denitrification).

CONCLUSION

Aggregates and other surficial microzones are ecophysiological, environmental, and evolutionary units of microbial biodiversity (i.e., community structure) and biocomplexity (i.e., community function) in aquatic and terrestrial habitats. There are commonalities and redundancies embodied in microzonal structure and function among the myriad aquatic and soil habitats. Using a variety of examples from geographically and geochemically distinct environments, we have shown that prokaryotic consortia, able to develop and colonize microscale biogeochemical gradients, are a common feature of these environments. Aggregate, mat, biofilm systems can be used as "models" to identify and functionally understand a basic set of process-level requirements or "ground rules" for microbial life to establish, maintain, and proliferate itself on a community organizational level.

Research on diverse marine, estuarine, and freshwater habitats points to aggregates and surficial microbial communities as biogeochemically and ecologically important nutrient transformation and trophic transfer sites. These sites are particularly significant in nutrient-poor and otherwise extreme environments, in which microbial production and nutrient cycling are severely restricted by nutrient and energy availability, as well as other environmental constraints (i.e., extremely low or high temperatures, desiccation, hypersalinity, high UV light). Changes in these conditions are likely to have a cascading effect on the structure, function, and overall production and nutrient cycling characteristics of these microzonal communities, with ramifications for biogeochemical cycling on larger ecosystem, regional and global scales. In particular, the biocomplexity and productivity of oligotrophic open ocean waters are likely to be affected. Contemporary issues of global change, including warming, altered amounts and patterns of rainfall and (conversely) drought, resultant changes in runoff and riverine discharge of nutrients and sediments, coupled to human land-use changes and associated nutrient and other pollutant loading patterns, will have profound impacts on the structure and function of aggregate/surficial microbial consortia.

Microzonal communities as described here are sensitive, metabolically active, and hence responsive indicators of environmental change, in essence serving as the microbial equivalent of the "canary in the mine." As such, microzonal communities may serve as excellent indices of environmental change spanning the size spectrum from specific habitat, ecosystem, regional-coastal to oceanic scales. Standard microbiological, molecular, physiological, and analytic assessments of aggregate and surficial genetic composition, function, and rates of relevant processes serve as the parameters of change. The advantage of using this suite of parameters for gauging environmental change over more conventional descriptive approaches (i.e., inventories of biotic diversity at higher organismal levels) is multifold. These microbial parameters: (*1*) simultaneously allow for measures of community structure (biodiversity) and function (rates of relevant processes, including primary production, mineralization, specific nutrient transformations, etc.); (*2*) can be coupled to large-scale survey and remote sensing tools, including aircraft and satellite imagery (of ocean color, turbidity, photopigments diagnostic of certain microalgal groups); (*3*) are amenable to microchip and other molecular-based, multiparameter indices of carbon, nitrogen, and other nutrient cycling processes, (*4*) have a role in the detection and characterization of microbial pathogens, harmful bloom algae, and other indices of environmental degradation of ecosystem and human health.

Now, at the beginning of the third millennium, we are facing unprecedented changes in Earth's biotic resources. One of the greatest and most important challenges is being able to detect, quantify, and differentiate human impacts form natural climatic and geological and geochemical changes facing our planet. For better or worse, microbially mediated changes are among the most fundamental and important in the biosphere. We have the opportunity and responsibility to judiciously apply the tools of microbiology, molecular biology, and ecology to gauge anthropogenic and natural change, using microbial consortia as key indicators.

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AGROTERRORISM. See BIOTERRORISM

AIR POLLUTION CONTROL. See VIRAL AEROSOLS

AIR SAMPLING. See SAMPLING TECHNIQUES FOR ENVIRONMENTAL MICROBIOLOGY

AIR-WATER INTERFACE. See NEUSTON MICROBIOLOGY: LIFE AT THE AIR–WATER INTERFACE

AIRBORNE BACTERIAL PATHOGENS.

See INFECTIOUS AIRBORNE BACTERIA

AIRBORNE FUNGI.

See IDENTIFICATION OF AIRBORNE FUNGI

AIRBORNE MICROORGANISMS.

See BIOAEROSOLS: TRANSPORT AND FATE; WASTEWATER AND BIOSOLIDS AS SOURCES OF AIRBORNE MICROORGANISMS

AIRBORNE TOXIGENIC MOLDS

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In the past 20 years, there is increasing recognition that an important factor in the health of people in indoor environments is the dampness of the buildings in which they live and work (1). Furthermore, it is now appreciated that the principal nonpathogenic biologics responsible for the health problems in such buildings are fungi rather than bacteria or viruses (2,3). Although traditionally fungi in this context have been viewed as allergens (and in some circumstances, pathogens), data have accumulated to show that the adverse health effects resulting from inhalation of fungal spores are due to multiple factors. One factor associated with certain fungi is the small molecular weight toxin (mycotoxin) produced by these fungi (1). Mycotoxins are held to be important in human and animal health because of their production by toxigenic fungi associated with food and feed (4). However, mycotoxins tend to concentrate in fungal spores (5,6) and thus present a potential hazard to those inhaling airborne spores. There are well documented cases of mycotoxicosis resulting from inhalation of toxigenic spores by agricultural workers handling moldy farm material (7,8), but until recently there have been few reports of such toxicoses in an urban setting (9).

MYCOTOXINS

The most potent toxin known is that produced by *Clostridium botulinum*, the bacterium responsible for botulism. Fungi also produce toxins that, like the botulism toxin, can enter our food and livestock feed. These fungal toxins are called mycotoxins, and they present both medical and economic problems to the community. Exposure to mycotoxin-producing (toxigenic) fungi has a long history and is a continuing threat to human and animal health (10), especially in developing countries where people do not often have the luxury of good agricultural practices. But even in more developed countries, contamination of food products results in significant economic losses, prompting governments to monitor levels of certain mycotoxins (e.g., aflatoxins) in food products to ensure that people do not consume unacceptable levels of these fungal toxins.

Mycotoxins are low molecular weight (generally $<1,000$ Daltons) and nonvolatile compounds; the odors associated with moldy buildings are due to microbial volatile organic compounds (mVOCs) that are typically low molecular weight alcohols, ketones, esters, and hydrocarbons of low inherent toxicity (11). Importantly, most mycotoxins are fairly stable compounds that can survive unchanged for long periods of time after the fungi are no longer viable. Thus, information obtained from culturing air samples (e.g., CFUs/m^3) needs to be evaluated with this in mind because low levels of viable (culturable) fungi do not necessarily reflect low levels of toxigenic fungi in the air. Furthermore, some airborne toxigenic fungal spores (e.g., *Stachybotrys*) have much shorter lifetimes in the dry state than do others, complicating the interpretation of CFU data from air sampling (12).

There is a great deal of information available on mycotoxins (4,13,14). In brief, mycotoxins are fungal secondary metabolites (15) that pose a risk to both human and animals that come into contact with these natural products, principally through ingestion of food and feed. They span an enormous range of chemical structures (Fig. 1), with diverse biological activities and are produced by a wide variety of fungal genera (16). Mycotoxins, like other secondary metabolites in general, tend to be idiosyncratic in that specific mycotoxins are often restricted in their production to a small number of fungal genera or even single species. Although some classes of mycotoxins may be restricted to a small number of species in a single genus (e.g., the aflatoxins to *Aspergillus*), some fungi are notorious for producing many different classes of mycotoxins within their genus (e.g., *Penicillium*). Additionally, there may be great variation in toxigenic potential within a single species. Most isolates of *Aspergillus flavus* are aflatoxin producers (although the percentage varies between areas of the country) (17), but only one-third of *Stachybotrys chartarum* isolates produce the satratoxins, highly toxic members of the trichothecene class of mycotoxins (18). Furthermore, there is a general lack of knowledge about the long-term chronic effects of most mycotoxins, especially in humans. With all this diversity in the biology, chemistry, and toxicology of mycotoxins, it is not surprising that there are no general methods available for mycotoxin analysis. The only useful analyses for mycotoxins are those for specific ones for which the analytical chemistry has been established.

From an agricultural view, the three most important genera of toxigenic fungi are *Fusarium, Aspergillus*, and *Penicillium*. The principal classes of mycotoxins of concern from ingestion are (fungal genera): the trichothecenes and fumonisins *(Fusarium)*, aflatoxins *(Aspergillus)*, and the ochratoxins (*Aspergillus* and *Penicillium*). Also, there are numerous other mycotoxins that intermittently cause problems. Some trichothecenes are acutely toxic (e.g., the

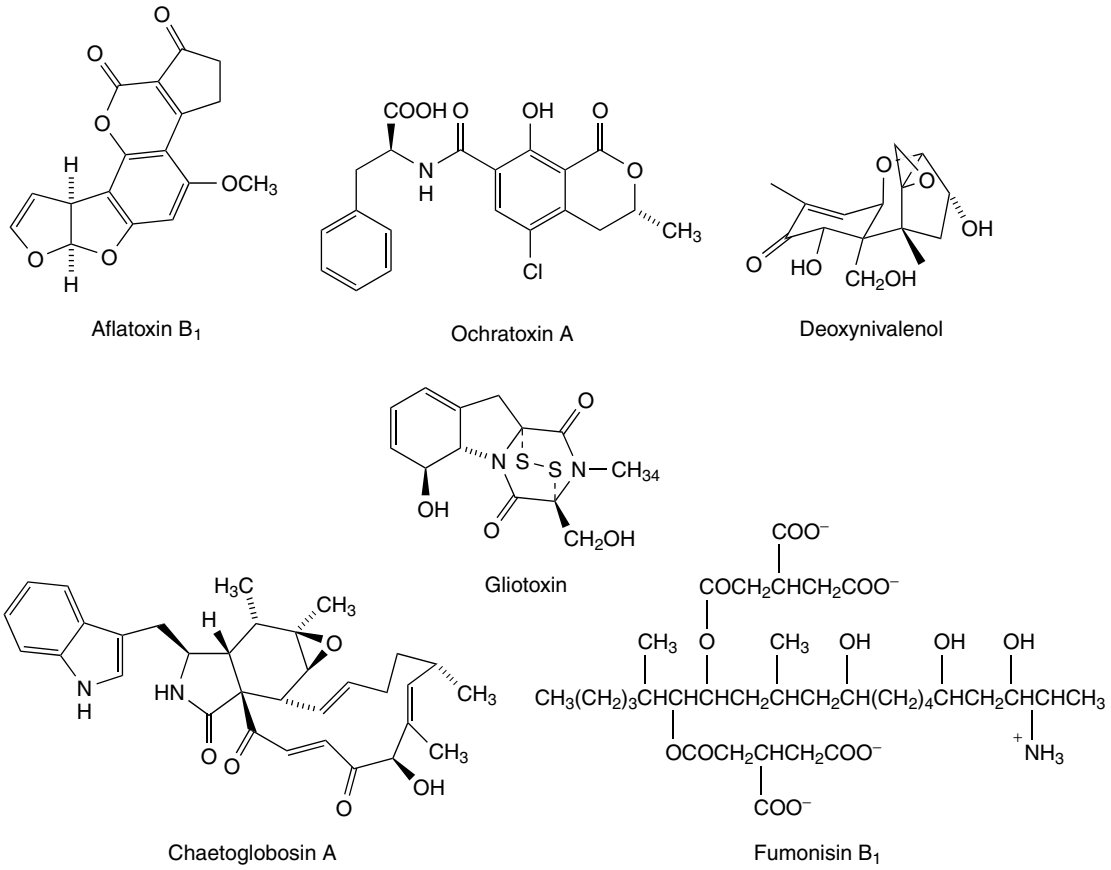

Figure 1. Selected mycotoxins.

satratoxins), whereas others are considerably less acutely toxic but are immunosuppressant or cause feed refusal in livestock (e.g., deoxynivalenol). Fumonisins, and especially some of the aflatoxins (e.g., B_1), are carcinogenic (10). There is now overwhelming epidemiological evidence that aflatoxin B_1 in the diet contributes significantly to the high incidence of liver cancer in many developing countries (10). Ochratoxin A is nephrotoxic and a possible cause of urinary tract tumors and Balkan-endemic nephropathy (10).

In an indoor environment, the toxigenic molds of most concern are *Penicillium, Aspergillus Chaetomium*, and *Stachybotrys*. However, the specific species of *Penicillium* and *Aspergillus* in indoor environments differ from those of an agricultural concern. For agricultural-based mycotoxicoses, *P. verrucosum, P. cyclopium, A. flavus*, and *A. parasiticus* (the latter two are aflatoxin producers) are of most concern; whereas, indoors, *P. aurantiogriseum, P. brevicompactum, P. chrysogenum, A. versicolor*, and the *A. nidulans* group are the most important toxigenic species (19). *Stachybotrys chartarum* (Ehrenb. ex Link) Hughes (*S. atra* Corda) has been focused on in recent years because of its association with acute bleeding in the lungs of infants (idiopathic pulmonary hemosiderosis, IPH), several of whom have died (20,21). However, the *Penicillium* spp. and *A. versicolor* are far more commonly encountered in water-damaged buildings than is *S. chartarum*. The basic reason for this is that the former species grow well on building material with water activities (a_w) in the range of 0.8; whereas *S. chartarum* requires a_w of 0.9 and higher and greater than 0.96 on building material to grow well (22). Generally, moisture levels this high are the result of local leakage (e.g., from a broken pipe) and are confined to a small area, but with leaking roofs, water intrusion can be quite extensive. Like all fungi, the growth of toxigenic fungi depends on substrate, temperature, and water activity (23).

AIRBORNE FUNGAL SPORES

The mycotoxins found in indoor air are most likely confined to the aerosolized spores (or more correctly referred to as *conidia*) of the toxigenic fungi (and building material on which these fungi grow) (24), with levels unlikely to approach those encountered in animal feed intoxications. However, mycotoxins are considerably more toxic when inhaled than when ingested (25,26). Furthermore, the effects of these toxins are multiple, and in particular, the immunosuppressant effects of these toxins on alveolar macrophage function is very pronounced (27,28). Studies of the effects of fungal spores on rat pulmonary alveolar macrophage cells have shown variability probably mediated by spore-borne toxins (29). Spores of five *Aspergillus, Penicillium spinulosum*, and *Cladosporium*

cladosporioides caused the release of leukotriene B4 and increased superoxide anion production and activation of complement. Several species of *Aspergillus* inhibited LPS-stimulated IL-1; whereas the other species had no effect (30). Data are available to support the role of the immunosuppressant mycotoxin, gliotoxin (Fig. 1), in the pathogenicity of *A. fumigatus* in animal lungs (31). Jakab and coworkers (32) demonstrated that inhalation exposure of aflatoxin in rats led to persistent reductions in phagocytosis, among other effects. In rats, inhalation exposure to aflatoxin-containing spores of *Aspergillus* has been demonstrated to be an effective route of exposure (33). Damage to clearance mechanisms would affect processing of antigen and lead to accumulation of material in granulomatous matter, leading to an immunotoxic effect (34–36). Low exposure to spores of toxigenic *S. chartarum* affects lung surfactant production in laboratory animals (37).

Toxigenic spores that strongly affect alveolar macrophage function pose a threat to workers handling mycotoxin-contaminated material. Occupational exposures to spores of *A. flavus*, a producer of the potent carcinogenic aflatoxins, have been shown to correlate to increased risk of liver cancer (7,8). A female agricultural worker suffered acute renal failure following exposure to grain dust in an enclosed granary, whose wheat was shown to be contaminated by *A. ochraceous* and its mycotoxin metabolite, ochratoxin A (Fig. 1). Although ochratoxin A was not demonstrated to be in air samples from the granary, caged experimental animals (guinea pigs and rabbits) experienced acute renal failure when exposed for eight hours to aerosols generated by their movement on moldy wheat (38). In this regard, a recent report of high concentrations (1,500 ppb) of ochratoxin A in dust from a home air-handling system in which occupants had complained of recurring health problems is noteworthy (39). Other reports have indicated that *S. chartarum* (40–42), *A. versicolor*, and several toxigenic species of *Penicillium* are potentially hazardous, especially when the air-handling systems have become heavily contaminated (28,43).

Epidemiological studies have connected the increased incidence of premature births in Norwegian farmers to their exposure to grain-borne toxigenic fungi (44). Researchers have shown that Finnish farmers are commonly exposed to relatively high levels of airborne spores (10^3–10^6 CFU/m^3), and that low to moderate (0.00–11 ppm) levels of *Fusarium* toxins (e.g., deoxynivalenol, see Fig. 1) are commonly found in the grain, although less commonly in air samples (45).

The literature on the growth characteristics of fungi in damp buildings is extensive (1–3,23,28). However, with respect to the production of organic compounds by these fungi, most of the literature has focused on the microbial-generated mVOCs (11). Although some of the mVOCs might be of health concern from a chronic perspective (e.g., styrene), mVOCs are not considered an acute toxic hazard (although the associated odors can present problems) (11). However, confounding factors such as the co-occurrence of ozone with mVOCs may result in a significant increase in volatile irritants (46).

MYCOTOXINS IN THE AIR

Although a great deal is known about the spectrum of mycotoxins produced by the relevant toxigenic fungi found indoors (20,21,47), there are few data available on the presence of the mycotoxins themselves in mold-contaminated buildings. In those few cases where mycotoxins have been found in mold-contaminated buildings (47–55), they have been found in bulk samples rather than in air samples. Following from this, there remains the critical question of the exposure of people in these buildings to the airborne toxigenic fungal spores, a problem that merits further discussion.

As noted earlier, many studies report the presence of toxigenic fungi in mold-contaminated buildings, but few actually determine the presence of the mycotoxins in the buildings. Nielsen and coworkers (47) showed that bulk samples from mold-contaminated building materials from water-damaged domestic residence contained both toxigenic fungi and the specific mycotoxins produced by these fungi. Although their data are complicated by these natural cultures being mixtures of two or more fungal species growing together (mixed cultures are the rule rather than the exception under natural conditions), the mycotoxins found are those expected from the fungi observed. Thus, the sterigmatocystin (an IARC 2A carcinogen i.e., a probable human carcinogen) and 5-methoxysterigmatocystin found in the bulk samples most certainly came from *A. versicolor* (which in fact produces these mycotoxins when grown on building materials) (54), and the chaetoglobosins from the *Chaetomium* sp. *P. chrysogenum* were commonly observed in these bulk samples, but no mycotoxins were found that could be ascribed to this fungus. In a similar study of mold-contaminated homes in Finland, workers showed that 34 of 79 bulk dust samples contained one or more of the 17 mycotoxins for which analyses were conducted. Of the 79 samples, 24% contained sterigmatocystin and 19% contained one or more of the trichothecene mycotoxins (55).

STACHYBOTRYS CHARTARUM — A MODEL?

As noted earlier, *S. chartarum* is less commonly encountered indoors than other toxigenic fungi (e.g., *A. versicolor*) but has nonetheless garnered more attention than the others because of its association with acute bleeding in the lungs of infants in Cleveland (20,21). This fungus was held responsible for serious health problems of a family living in a water-damaged home in Chicago (40) and has been implicated in several cases of building-related illness (39–41,56,57). A cluster of cases of acute bleeding in the lungs of infants was reported in Cleveland, Ohio where over 30 infants from homes that suffered flood damage became sick (12 deaths) with the illness beginning in January 1993 (21).

Stachybotrys chartarum was first described more than 150 years ago by Corda in 1837, who isolated the mold from damp wallpaper in a home in Prague. Although *S. chartarum*-related animal intoxications have no doubt existed for some time, it was not until 1931 that this

toxicosis was described and not until the late 1930s was the condition recognized as a mycotoxicosis: stachybotryotoxicosis (58). Horses are particularly sensitive to this mold that is a common contaminant of damp hay and straw (58). However, there are intermittent reports where major portions of a livestock herd have been killed following ingestion of *S. chartarum*–contaminated hay (58,59).

Stachybotryotoxicosis in humans is rare and has been reported most commonly in workers who handle moldy straw and hay (60). Workers handling *S. chartarum*–contaminated hay experienced bloody nose and nasal secretions, dyspnea, sore throats, and inflammation of the skin (61). More recently, severe dermatitis appeared on the hands of those workers who were handling decomposable flower pots made of recycled paper that had a heavy growth of *S. chartarum*; however, no respiratory distress was noted, although viable spore levels of *S. chartarum* up to 7,500 CFUs were found during the handling of the pots (62). Until 1986, there were no reports in the literature of stachybotryotoxicosis in North America. The first report of a toxicosis in North America attributed to *S. chartarum* occurred in a Chicago household where the home had suffered significant water intrusion from a leaky roof over a period of several years (40). The occupants of the home had suffered recurring maladies, including cold and flu symptoms, diarrhea, headaches, fatigue, dermatitis, sore throats, intermittent loss of hair, and general malaise. Examination of the home revealed extensive growth of *S. chartarum* on the ceiling of a bedroom and, more significantly, in the air-handling system. Ethanol extracts of the contaminated material were lethal (injected *per os*) to mice and rats within 24 hours. From these extracts were isolated a series of potent cytotoxic trichothecenes (40). After the home was thoroughly cleaned (protective clothing was necessary to prevent severe dermatitis in the workers), the residents returned, and their symptoms abated and within a few weeks, their health returned to normal.

The rare occurrence of *S. chartarum*–induced toxicosis in humans stems from the specific conditions this fungus requires for significant growth (e.g., this fungus is never found growing on food products). In damp buildings, high cellulose and fibrous surfaces are favored for growth if the moisture level is sufficient ($a_w > 0.9$). For example, if sufficiently moist, *S. chartarum* readily grows on gypsum board (63,64), wood fiberboard, wall paper, and dust-lined air-conditioning ducting (40,43,45–49,54). In the laboratory, *S. chartarum* is commonly grown on rice although toxin production is observed when the fungus is grown on building material as well (48,54,63,64).

Chemical investigation of *S. chartarum* has provided many highly toxic and novel compounds (65). *Stachybotrys chartarum* is known to produce the very potent cytotoxic macrocyclic trichothecenes (e.g., satratoxin H) along with a variety of immunosuppressants (65–67) and endothelin receptor antagonists (68–70). In fact, some of the most cytotoxic fungal metabolites ever discovered are products of *S. chartarum* fermentation (71), and by no means have all the active constituents been isolated and identified — there remains an uncharacterized hemolytic agent(s) found in several *S. chartarum* isolates from the Cleveland study (72). *Stachybotrys chartarum* can produce a diverse array of compounds, and the variation in individual and overall metabolite levels from one isolate to the next is remarkable — and troublesome to any who wish to draw toxicological inferences from levels of airborne *S. chartarum* spores. Similarly, the toxicity of two isolates of *S. chartarum* recovered from almost identical environmental conditions, and then grown in the laboratory, may exhibit widely different biological activity profiles (73).

It is instructive to see how chemically complex the profile of toxin production by this fungus is. Figure 2 illustrates members of the classes of mycotoxins reported to be produced by isolates *S. chartarum* and *Memnoniella echinata*, obtained from Cleveland homes involved in the IPH study (20,21,73–75). The latter fungus is closely related taxonomically to *Stachybotrys* (76) and is often found growing on the same substrates indoors with *S. chartarum*. The spectrum of compounds produced by *S. chartarum* in the laboratory depends not only on the particular isolate but also on the substrate. There are more than 10 each of the trichothecene (71) and atranone (77,78) classes of compounds produced by *S. chartarum* and they are the minor secondary metabolites. The major mycotoxins produced by *S. chartarum* and *M. echinata* are the benzodrimanes (Fig. 2: stachybotrylactone, stachybotrydialdehyde, and memnobotrin A), which are the "signature" compounds of this fungus (65,73). There are more than 20 members of this class, and unless steps are taken to separate these compounds from the others before chemical analysis by high performance liquid chromatography (HPLC), it is very difficult to even observe the presence of the other classes of mycotoxins (79). In addition, the more highly immunosuppressant dialdehydes (80), such as stachybotrydialdehyde (Fig. 2), are unstable and tend to readily rearrange to the corresponding significantly less active (80) stachybotrylactones (79,81).

As noted earlier, isolates of toxigenic fungi may differ significantly in their toxigenic potential. In the laboratory, isolates (approximately 40) of *S. chartarum*, grown on rice, from the Cleveland study (73), display cytotoxicities of their crude extracts that vary by five orders of magnitude. The most toxic isolates (about 1/3) are those that produce the macrocyclic trichothecene mycotoxins (e.g., satratoxin H). Of the remaining two-thirds of the isolates, some produce the considerably less toxic trichothecenes, trichodermol, and trichodermin; and most isolates yield the considerably less biologically active atranones (Fig. 2) (77,78). Although we have yet to find any significant biological activity associated with the atranones, these compounds are related in structure to the diterpenoid dolabellanes (two of whom have been isolated from *S. chartarum* cultures) (78), several of which do have appreciable biological activity (82). Others also have noted great variation in various biological activities of *S. chartarum* isolated from indoor environments (83), and thus this is no doubt a general characteristic of *S. chartarum*.

The cluster of cases of acute lung bleeding (idiopathic pulmonary hemosiderosis, IPH) in infants (two weeks to

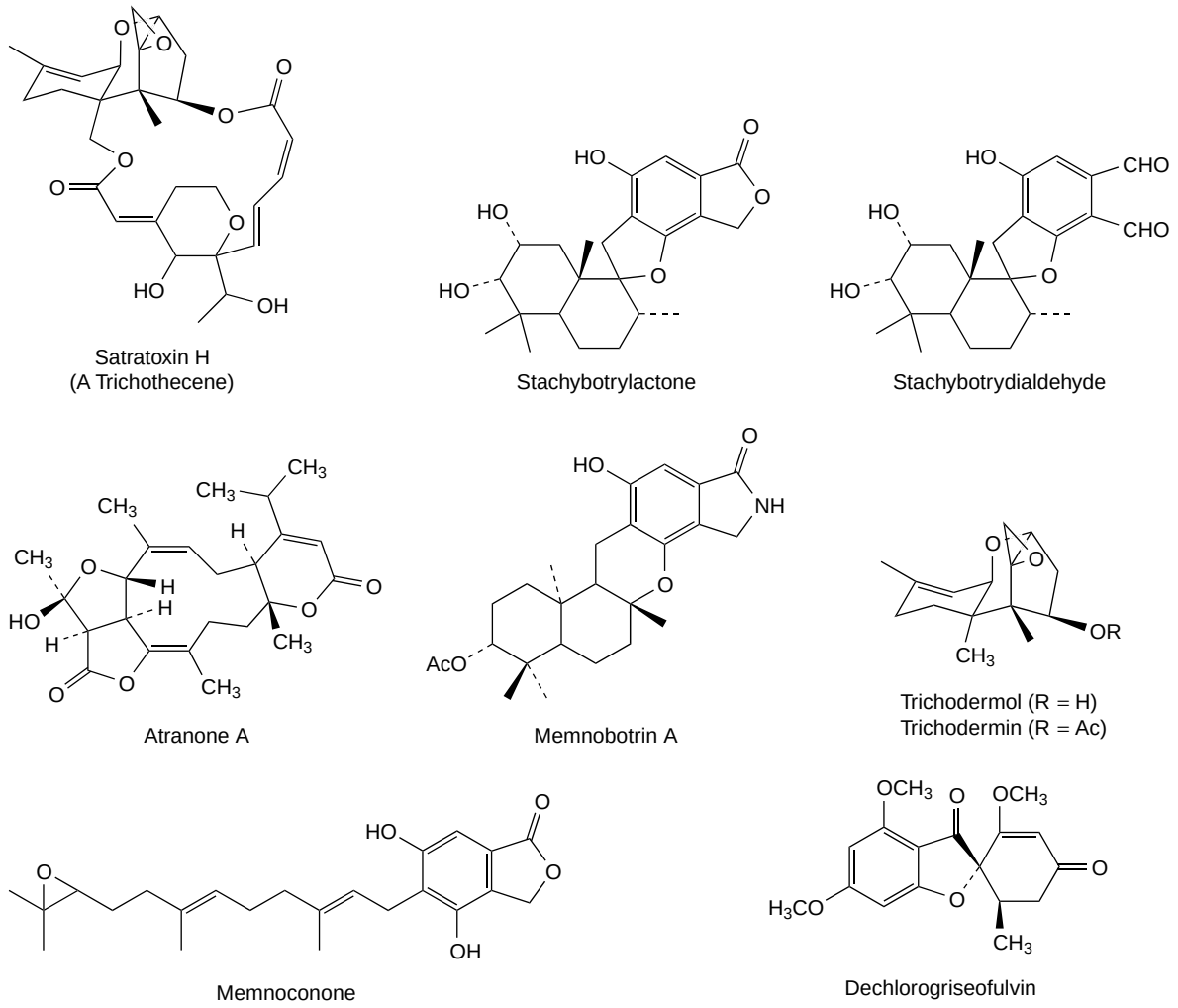

Figure 2. Mycotoxins isolated from *S. chartarum* and *M. echinata*.

six months of age) from Cleveland, Ohio has garnered a good deal of public attention. Between 1993 and 1998, there were 37 cases of IPH reported in the Cleveland area, a normally rare syndrome (20,21). Twelve of the cases resulted in deaths, including seven that had originally been diagnosed as sudden infant death syndrome. The researchers reported that all the case homes in the study had varying degrees of water damage and that *S. chartarum* was significantly more commonly found and at higher levels than in control homes (20,21). However, a strange observation in these cases is that occupants of the IPH homes older than six months of age appear not to suffer from any undue adverse health effects, although the investigators have offered a rationale for the hypersensitivity of infants to the effects of *S. chartarum* spores. In addition to the Cleveland IPH studies, there have been several recent reports of individual cases of IPH apparently associated with the presence of *S. chartarum* in homes (51,84), including one where culturable spores of *S. chartarum* were isolated from the bronchoalveolar lavage fluid of a child with pulmonary hemorrhage (85). However, "association" is not the same as cause and effect, and workers in this area (21) have taken some care in pointing out that other environmental factors (e.g., second-hand cigarette smoke) also may play important roles in the etiology of IPH. Nonetheless, others (86–90) have emphasized that the epidemiology of IPH vis a vis *Stachybotrys* is tenuous, and that reports such as the Cleveland studies may raise undue concern in the public mind. It is worth noting that it took 30 years (with hundreds of time-consuming and expensive studies) before the medical community accepted as fact the causal relationship between the consumption of aflatoxin-contaminated food and an increased incidence of liver cancer in humans.

THE FUTURE

There are presently a number of serious limitations in our ability to assess the role played by mycotoxins in the health of those exposed to toxigenic fungi in water-damaged buildings. Toxicologists prefer to study pure toxins, but mycotoxicoses always involve mixtures of chemicals and usually mixed fungal cultures as well. Furthermore, the spores appear to have noticeable

effects themselves apart from their mycotoxins. Intranasal exposure of mice to spores of a highly toxigenic strain of *S. chartarum* resulted in a remarkable deterioration of the lung tissue of the animals (91,92). In the case where animals inhaled pure trichothecene mycotoxins (25,26), little or no damage was observed in their lung tissue, even though the trichothecene toxin (T-2 toxin) administered in this manner was more than an order of magnitude more toxic relative to iv or ip injection. But spores in which toxins have been extracted are considerably less deleterious to lung tissue than are the spores prior to extraction of their toxins (93). Thus, it appears that the spores themselves play an important role in the responses observed in the lung tissue of animals who inhale the spores, strongly reinforcing data on the disregulation of pulmonary macrophages caused by toxigenic spores.

The critical question to be answered is that of the level of exposure. Without a reliable assessment of exposure, the risks to human health cannot be properly ascertained in individual cases. Although the epidemiological data clearly show that heightened exposure to indoor molds compromises the general health of those exposed (1), what cannot be gleaned from these data is the role played by mycotoxins. At present, few good methods for measuring exposure of individuals to indoor molds are available. The measurements of airborne mold levels indoors is problematic for a variety of reasons (12) and tend to be meaningful only when careful comparisons are made to the concurrent levels of molds in outdoor air. Indoor air sampling may be useful to demonstrate unacceptable amplification of indoor molds, but beyond that, these data are usually not statistically reliable (12).

Except for a small number of allergenic molds, no direct way of determining whether an individual has been exposed to a particular mold is currently available. In fact, the biochemical markers for general exposure to heightened levels of fungal spores are presently only of limited use, and certainly are such that one cannot distinguish exposure to toxigenic molds from exposure to nontoxigenic molds (12), let alone exposure to specific mycotoxins. What are needed are biological markers for exposure to both specific fungi and for their specific toxins. A few mycotoxins (e.g., ochratoxin A) are metabolically stable enough to give sustainable and measurable levels in blood (94), but most metabolize readily and few give metabolic products that have been characterized. An exception to this is aflatoxin B_1 that conjugates to genomic DNA and can be assayed as the guanine adduct, giving a measure of exposure to the mycotoxin (10).

Future advances in analytical chemistry coupled with the identification of biological markers for specific mycotoxins will be a great aid in addressing the fundamental questions of exposure to airborne toxigenic molds and their risk to people living and working in mold-contaminated buildings.

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ALGAE. See Meroplankton; Planktonic Algae in the Marine Environment; Primary Productivity in the Marine Environment

ALGAE BIOTECHNOLOGY

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In recent years there has been increased interest in the use of algae for biotechnology. Algal biotechnology represents an area of current and future development as new sources for products are being explored. Algae require minimal substances for growth and provide a rich source for bioproducts. Traditionally, seaweeds were harvested and used as a source of complex carbohydrates such as agar and carrageenan. People in several countries, particularly in Asia, have used algae as a food source for centuries. Yet, of more than 30,000 species of algae known, only a small number have been exploited for commercial purposes. *Spirulina* has been cultivated for several years as a health food product. Biotechnology for valuable biochemicals from algae is currently a reality, and growth of algae through aquaculture has developed into an important industry. Several countries produce carotenoids by growing *Dunaliella* and *Haematococcus* and harvesting the pigments. With the search for new natural products for use in pharmaceuticals, food materials, cosmetics, and other uses increasing, algae represent a likely resource to be explored for future development.

Algae, both micro- and macro-, are increasingly being sought as a means of bioremediation that can compete with other technologies in terms of efficiency, cost, and reliability. The application for bioremediation and for natural products derived from algae is even being combined, with algae being grown on wastewater and their proteins harvested for industrial use. Algal biotechnology represents an important area for current and future research.

COMMERCIALLY IMPORTANT ALGAE

Among the thousands of marine, freshwater, and soil microalgae, about 100 species have been shown to produce valuable compounds (1), a few of which have gained considerable significance commercially. *Spirulina, Chlorella, Dunaliella, Haematococcus*, and *Porphyridium* are five genera of microalgae with great economic relevance. Cultivation of these species is important for the health and pharmaceutical industries, and for the manufacturers of animal feed.

Spirulina is the most widely cultivated cyanobacteria (Fig. 1). It is a prokaryotic organism containing blue phycocyanin in addition to chlorophyll. It is found in lakes that are rich in salts in Central and South America and in Africa. It is characterized by forming helical strands of cells in aqueous environments and grows in alkaline media, surviving pH of 11. It can grow in temperatures as low as 18 °C, but the optimum is 35 to 37 °C. It grows well in mass cultivation in open, shallow racetrack ponds, or in closed tube systems. The media for growth was developed by Zarouk (2) but was modified to reduce the costs by substituting cheaper substances, such as ammonia or urea for nitrate. *Spirulina* is grown for its high protein content (71%) and is widely marketed as a health food product and for animal feed. In addition to its high protein content, it has essential fatty acids, minerals, and vitamins. Most health benefits to humans claimed for supplementation come from anecdotes and not scientific research. In vitro and animal tests have demonstrated a wide range of healthful benefits including anticancer (3), lipid lowering (4), and blood-iron status enhancing (5).

Chlorella vulgaris was the first microalgae to be isolated. It is a unicellular green algae and does not have flagella (Fig. 2). All strains have a cell wall, and asexual reproduction occurs within the parent cell wall producing four to 16 new cells. The cell wall then breaks to release the offspring. *Chlorella* has high tolerance for acidic pH ranging from 2.0 to 6.0. Its salinity tolerance ranges from 0 to 5%. Optimum temperature varies depending on the strain, ranging from 25 to 38 °C (2). Maximum growth rate can be induced by the use of mixotrophic cultures (supplementation with acetic acid), rather than strict reliance on autotrophic growth (6). *Chlorella* is grown commercially for its chlorophyll and protein content. It is used as a health-food supplement and for animal feed. *Chlorella* can be used for bioremediation of heavy metals in wastewater (Fig. 3).

Dunaliella are unicellular, flagellated, green algae that lack a cell wall (Fig. 4). There are halotolerant and

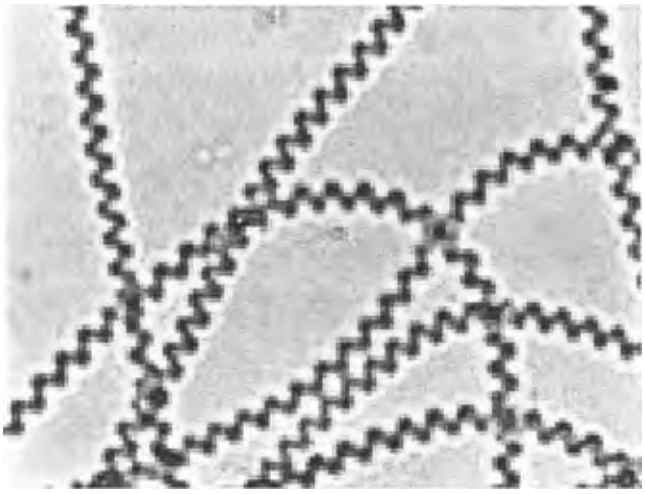

Figure 1. Cyanobacteria-*Spirulina* sp. *http://vis-pc.plantbio.ohiou.edu/algaeimage/pages/spirulina.html* See color insert.

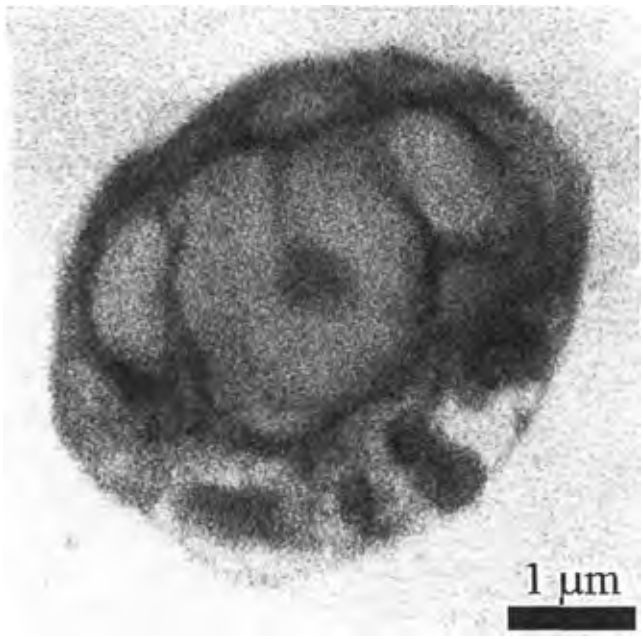

Figure 2. *Chlorella vulgaris*, *http://www.ifa.au.dk/~jabraham/algae.htm* See color insert.

halophilic strains; the species grows in a range from brackish to hypersaline waters. Optimum conditions for growth vary depending on the strain involved, and the interaction between the physical and chemical growth conditions. Several different media have been developed for growth, with commercially important strains adapted to high salt concentrations. These strains were isolated from environments such as the Dead Sea. Temperature requirements vary depending on the light intensity and salinity. With high salt concentrations, high temperatures, and intense light, halophilic varieties of *Dunaliella* produce significant quantities of beta-carotene. This is harvested for use in health-food supplements and as coloration in foods and cosmetics. In response to increasing osmotic pressure, *Dunaliella* produces glycerol to maintain osmotic balance, and this is also harvested for commercial distribution (2).

Haematococcus pluvialis is a unicellular green algae that has two anterior flagella (Fig. 5). It grows in coastal rock pools, water holes, and other similar small bodies of water. Its optimum temperature is between 15 and 25°C. *Haematococcus pluvialis* is able to respond to environmental stress by changing from the motile, vegetative green cell form to red stationary aplanospores. This occurs when nutrients become scarce or under hypersaline conditions, high temperature, or intense light. In the cyst phase, it produces high amounts of carotenoids and astaxanthin, 4 to 6% or more of their dry weight (7). Astaxanthin is a red pigment that is valued commercially for cosmetics and as an antioxidant. It is also used as a color additive in feed for salmon to enhance the pink color (Food and Drug Administration 21 CFR Part 73

Figure 3. Bioremediation of heavy metals in wastewater, *http://zobell.biol.tsukuba.ac.jp/~inouye/ino/g/chl/chl_picl.html* See color insert.

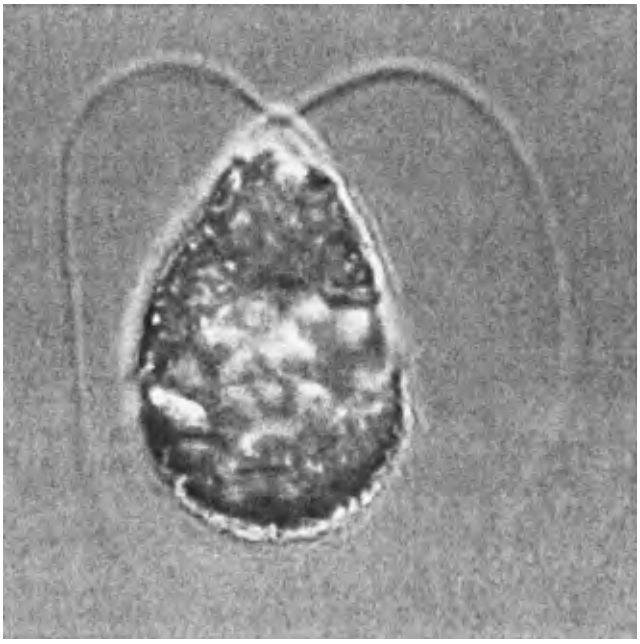

Figure 4. *Dunaliella*, *http://zobell.biol.tsukuba.ac.jp/~inouye/ino/g/chl/chl\picl.html* See color insert.

[Docket No. 98C-0212]). These characteristics have led to increased interest in developing culture methods for commercial exploitation.

Porphyridium is a unicellular red algae (Fig. 6). *Porphyridium* has been isolated from a wide range of environments, freshwater streams, brackish and marine water, and damp soils. It is characterized by a single large stellate chloroplast. It lacks a true cell wall, but is surrounded by a gel polysaccharide layer that causes clumping of the cells so that they form loose colonies. Its pigment, phycoerythrin, has commercial significance for cosmetics, medical diagnostics, and food coloring. *Porphyridium* is grown in large-scale open ponds and in closed tube systems in marine media.

CULTURE TECHNOLOGY

To produce sufficient quantities of algae for industrial purposes, methodologies for mass cultivation and harvest have been developed. Considerable technological research has been undertaken to determine the optimum conditions for growth and to remove problems with contamination.

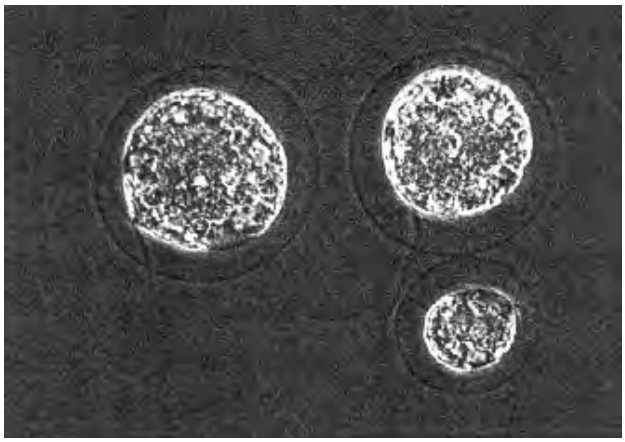

Figure 5. *Haematococcus*, *http://www.microscopy-uk.org.uk/mag/wimsmall/extra/haema.html* See color insert.

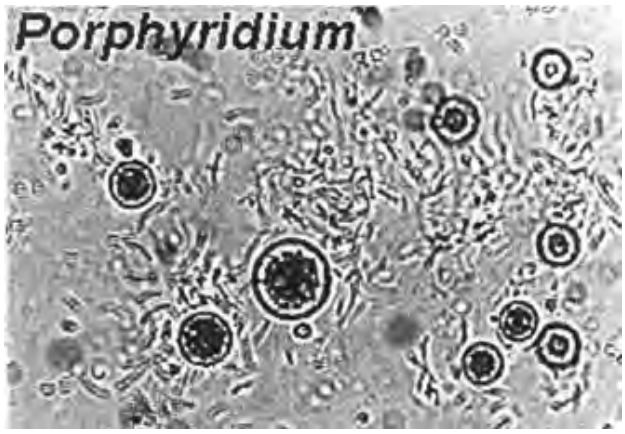

Figure 6. Rhodophyta-*Porphyridium purpureum http://vis-pc.plantbio.ohiou.edu/algaeimage/pages/Porphyridium.html* See color insert.

Cultivation of algae can occur in areas that are not suitable for conventional agriculture, but have high light availability and warm temperatures, making this suitable to thirdworld nations. Many species can be grown in saline water or in marine environments. All systems for mass production must include supply systems, culture systems, harvesters, and processing equipment (Fig. 7). Special technology is needed for each of the phases of the growing process: cultivation, enrichment of the required product, harvesting, and extraction of the products. The phases are dependent on each other and the process varies depending on the algae grown and the product to be harvested.

The standard method used for large-scale production is shallow ponds, 10 to 20 cm deep that are open or covered. The ponds are circular or oval and are stirred by paddle wheels to provide an even suspension and prevent the algae from settling out. The most common commercial structure is an open raceway, which is stirred by paddle wheels. There are several problems with large-scale cultivation, mainly concerned with low productivity and contamination by microorganisms and chemicals (8). Guterman and Ben-Yaakov proposed application of modified, simple, mathematical algorithms to computerized control systems to provide for optimum operating conditions by automatic systems in which the parameters of light intensity, optical density, pH, and temperature are included in the formula to calculate the conditions needed to increase yield (9).

Closed, long, polyethylene sleeves, first proposed by Trotta in 1980 (10), have been used to limit the problems found with open ponds. There are various types of horizontal or vertical closed systems including horizontally arranged glass or polyethylene tubes (11). The tube photobioreactors are arranged as vertical closed bioreactors that have limited contact with the external environment, especially compared to open ponds. These systems reduce the problems of contamination and increase control over temperature, evaporation, and light availability (12). Comparison of near horizontal straight tubular reactors and near horizontal flat panels in outdoor cultivation of *Spirulina* showed that the photosynthetic efficiency in the tubular systems was significantly higher because the curved surface reduced the light saturation

Figure 7. *http://members.nbci.com/_XMCM/rekel/ushn/helo/photo_daphnia.htm* See color insert.

effect (13). Comparison of curved versus straight tubes indicates that curved tubes are superior to straight ones (14) (Fig. 8).

Water is added by a system of tubes that connect the sleeves to a control center where the temperature and the densities of the materials are controlled and harvesting begins. Models have been developed to estimate the biomass productivity depending on the solar irradiance on the culture surface (15). The determination of the final product is subject to the environment present (16). Thus, changes in the composition of the medium, light intensity, and gas flow will affect the productivity of the algal cultures (17). Low-dose ultrasonic treatment can affect the growth rate and biomass yield of algae depending on the species of algae cultivated; the ultrasonic treatment either increasing or decreasing growth rates (18).

Microalgae can be grown by various technological means that are dependent on the needs and economics of the environment. Algae exist primarily in freshwater and marine habitats. They acquire the substances they need for growth and metabolism (water, carbon dioxide, and nutrients) directly from this environment. In Israel, *Dunaliella* and *Porphyridium* are grown in vertical sleeves in which the growth conditions are closely monitored and controlled. In these rows of vertical plastic sleeves, they are separated from the external environment, except for the sun (12). The advantage of the closed system is to limit contamination and fluctuations in environmental conditions. In other localities, where the growth of algae is linked to remediation of agricultural wastewater or similar environments, other technologies may be applicable (19,20). It is easier to commercially grow hardy organisms, such as *Chlorella, Spirulina*, and *Dunaliella* than *Haematococcus*, which cannot compete with contaminant organisms in open outdoor systems. *Haematococcus*, which produces the pigment astaxanthin in the cyst phase, is grown in a combination of enclosed outdoor photobioreactors and pond-culture systems (1) as well as other culture methods intended to increase yield (21).

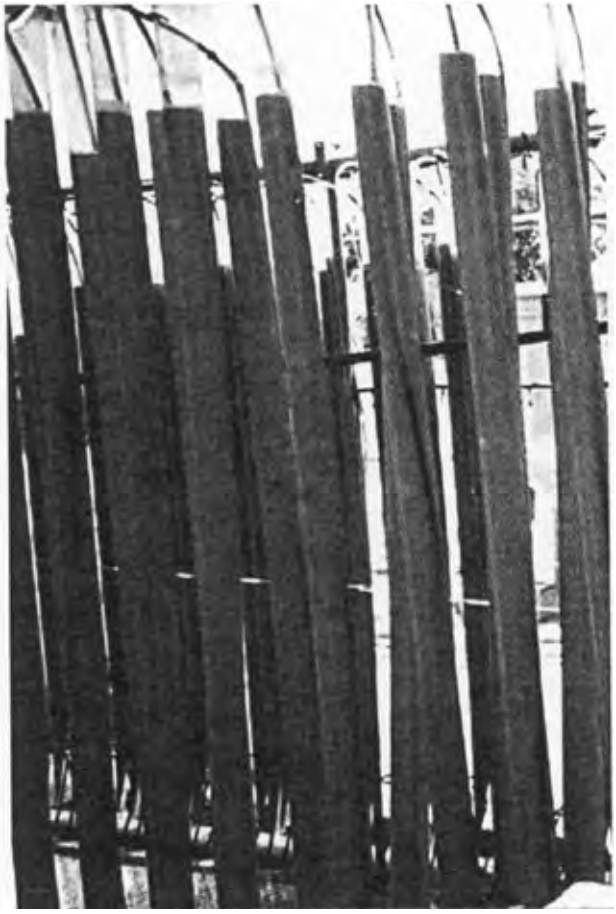

Figure 8. Polyethylene culture sleeves from Ben–Gurion University. Provided by Dr. Lustigman and Dr. Lee. See color insert.

The yield and type of product can vary depending on the nutrients and other growth conditions supplied to the algae. Nitrogen deprivation can increase the yield of polysaccharides of *Porphyridium* (22), but higher nutrient availability will increase lipid content of *Dunaliella tertiolecta* (23). High biomass protein production can be achieved by growth with high nitrogen content, such as swine wastewater (19). Temperature can be an important factor affecting the productivity and biomass in *Spirulina* grown outdoors (24). The growth stage can be followed by an enrichment stage aimed at the concentration of the desired product (25,26). The algae must be harvested from the culture medium, concentrated, and dried. The harvesting technique employed depends on the algae. For microalgae, harvesting occurs by centrifugation, filtration, flocculation and settling, or flotation, depending on the organism involved. Degradation of the product, as with beta-carotene, can occur because of exposure to light and air (27). Irradiation can change the proportions of the stereoisomers of beta-carotene in *Dunaliella* (28).

With the high costs involved in the cultivation of microalgae and harvesting the bioproducts, it is probable that in the future the identification of the genes responsible for the production of these products will take place. Genetic manipulation will then be used for enhancement of these products in organisms, not necessarily algae. Identification of some of the genes responsible for production of astaxanthin (29,30) and their transfer into tobacco plants has taken place (31).

NATURAL PRODUCTS FROM ALGAE

It is in the area of natural products that algae have become increasingly important as sources for the health and pharmaceutical industry and for use in animal feed. Algae have long been used as a source of commercial products, particularly for the food and pharmaceutical industry. These products such as agar, sodium alginate, and carrageenan are complex polysaccharides whose usage is well established. They are used as thickening agents, emulsifiers, and gelling agents in food products and paints, photographic films, and drug formulations. With increased consumer interest in natural products as opposed to synthetic ones, attention has been focused on algae as a source of these substances. Marine organisms including algae have been an important source of natural products with biomedical potential (32).

Protein

Although other algae also have high protein content, *Spirulina* is probably the most widely known algae grown for its natural products. Because its protein is complete, *Spirulina* is raised for use in protein supplements and for animal feed. In addition to protein, it produces essential fatty acids, minerals, and vitamins.

Pigments

Carotenoids. One of the most widely used products derived from algae are the pigments that are harvested from mass cultures. Microalgae are frequently grown for their pigments, particularly the lipid-soluble carotenoids. These are used for food coloration, cosmetics, and medicinal purposes. Carotenoids are found in a large number of organisms including bacteria, algae, higher plants, and animals. Carotenoids are lipid-soluble pigments made of isoprene units with a yellow-orange-red coloration. Beta-carotene is the most abundant of the carotenoids making up 10% of the dry weight of *Dunaliella bardawil* and *D. salina* (33).

In plants, carotenoids are secondary photosynthetic pigments that serve to absorb additional light energy for the photosynthetic process. They increase in concentration when the plant is undergoing stress such as high light intensity or high osmotic stress. This is true particularly for microalgae such as *Dunaliella*, which has been cultivated commercially for several years. Extensive cultivation of *Dunaliella* occurs in Israel, Australia, United States, Spain, and China (34–36). The amount of beta-carotene produced is directly related to the amount of light absorbed by the algae. Therefore this is a mechanism to protect against high light intensity (37). Increased salinity also increased the concentration of carotenoids, particularly beta-carotene (38).

Industrially, carotenoids have been used for food coloration, cosmetics, and as a health-food product (39). Beta-carotene is a vitamin A precursor and acts as an antioxidant (40). Exposure of algal cells to various environmental stresses causes an increase in the presence of antioxidative enzymes and beta-carotene production (41). Beta-carotene has been shown to scavenge reactive oxygen species generated in vitro (42) and to cause slight lowering of spontaneous and X-ray-induced chromosomal damage in bone marrow cells (43). Beta-carotene has long been considered as an anticarcinogen (44,45). Because of its antioxidant properties, it has been studied as a means of chemoprevention to prevent development of gastric carcinoma in high-risk populations with precancerous lesions (46). However, two large-scale studies, the Physicians' Health Study (PHS) and the Beta-Carotene and Retinol Efficacy Trial (CARET), question the benefit against heart disease or cancer (47). In fact, an increase in the risk of lung cancer among smokers who took beta-carotene supplements was reported in the alpha tocopherol, beta-carotene cancer prevention study (48), and among smokers and asbestos workers in CARET (49), but not among male physicians in the Physicians' Health Study (50). Studies using ferrets supplemented with high and low doses of beta-carotene show that high supplementary doses leads to precancerous lesions in ferrets exposed to tobacco smoke, whereas low levels were antiproliferative (51).

The pathway for biosynthesis of beta-carotene in *Dunaliella* has shown that several intermediates are produced, terminating as two stereoisomers (all-*trans* and 9-*cis*) found in equal concentration. The synthetic variety consists of the all-*trans* stereoisomer. In view of the question as to the possible greater value of natural versus synthetic beta-carotene, studies have been performed comparing serum and tissue concentrations of the all-*trans* and 9-*cis* isomers. The steroisometric mixture of beta-carotene in *Dunaliella* has been shown to be preferentially absorbed in animal tissues (34), but much of the 9-*cis* molecules are converted to all-*trans* (52,53). Serum analysis in humans shows that 9-*cis*-beta-carotene is more efficient as an in vivo lipophilic antioxidant than all-*trans*-beta-carotene, indicating that natural sources may be superior to synthetic ones (54). Comparative studies of natural and synthetic beta-carotene using in vitro tests indicate that natural, or a mixture of natural and synthetic beta-carotene, was more effective in antigenotoxicity than synthetic, and may be of value as a supplementary treatment in cancer prevention (55). Other carotenoids used commercially are xanthophylls, which are yellow-orange in color, used as food dyes for poultry skin, egg yolk, and salmon (56).

Astaxanthin. Another carotenoid is the red pigment astaxanthin produced almost exclusively by *Haematococcus pluvialis* cysts (Fig. 9). It has commercial value as a red colorant in cosmetics and foods, and enhances the pink color of salmon (57). The gene *CrtO*, responsible for the production of astaxanthin has been transferred to tobacco plants imparting red color in the manipulated plants (31). Astaxanthin present in cyst cells appears to function as an antioxidizing agent against oxidative stress (58). It increases in oxidative stress along with superoxide dismutase, an antioxidative enzyme (59). It is being evaluated for presumed cancer prevention (60). The development of methods for increased production and purification of astaxanthin is currently a very active area of commercial interest in several locations including Iceland, Israel, and Hawaii (1,61).

Phycobiliproteins. The water-soluble phycobiliproteins, produced by the cyanobacteria and red algae, are other important pigments. These pigments create the blue-green and deep red color, respectively. They have potential as food dyes and application in the cosmetic industry (62). Phycocyanin, which is the blue-green pigment obtained from cyanobacteria, has been patented and is commercially available as Linablue (Dainippon Ink and Chemicals Inc. 1980. Jap. Patent 8077890). It is used in the food industry for coloring dairy products, candy, and soft drinks. *Spirulina platensis* has been suggested for phycocyanin production (63). It has also been shown to have hepatoprotective effects against toxic substances (64). The red fluorescent phycobiliprotein, phycoerythrin, gives the red algae, Rhodophyta, their name and coloration. The phycobiliprotein from the red alga, *Rhodella reticulata*

Figure 9. Astaxanthin, *http://www.bioprocess.is/product.html* See color insert.

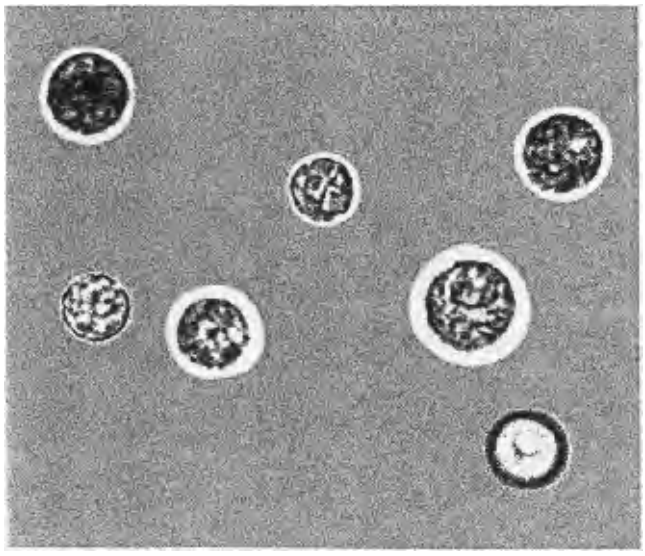

Figure 10. *Rhodella reticulata*, *http://www.cibnor.org/malgas/ifotrhr.html* See color insert.

(Fig. 10), have been purified and could be considered as a potential source of these substances for commercial purposes (65). They are also found in several microalgae including *Porphyridium* (66) and *Synechococcus*. The purification of phycoerythrin from *Synechococcus* sp. DC-2 has shown that two forms of phycoerythrin are produced (67). Heat-resistant C-phycocyanin has been purified from the red alga *Cyanidium caldarium*, and has potential for use in industrial processes because it does not denature up to 60 °C (68).

These pigments are used for diagnostic purposes in multiple-color-flow cytofluorimetric analysis. The phycobiliproteins and molecules with specific biological activity bind to target molecules forming fluorescent conjugates. The phycoerythrin conjugates are soluble in water, have a long shelf life, and are not affected by most natural molecules (69). Glazer and Stryer developed markers, phycoprobes, made of fluorescent phycobiliproteins covalently bound to biologically active molecules (70). These include phycoerythrin-immunoglobulins, phycoerythrin-biotin, phycoerythrin-avidin, and phycoerythrin-protein (71). The use of phycoerythrin-antibody conjugates allow for early detection of leukemia and other blood disorders (72).

Fatty Acids

Algae are also used commercially to obtain unsaturated fatty acids (73). Although fish oils are considered to be the source of unsaturated fatty acids, these acids are actually produced by the marine algae that the fish consume (74). Algae and cyanobacteria are rich in ω-3 fatty acid homologs (75), and if the fatty acids are harvested directly from the algae, the undesirable components, like the fishy smell and cholesterol, will not be present (76). These fatty acids are suggested to be of value in prevention of heart disease by reducing levels of plasma lipids and lipoproteins. They act to decrease serum cholesterol and triglyceride levels (77). High quantities of essential fatty acids such as arachidonic and docosahexaenoic acids are found in marine algae. Greater variation exists in the quantities of fatty acids in different algae than in the class of fatty acid produced, although within the class unique metabolites may exist (78). The Phaeophyta and Rhodophyta generally produce more fatty acids than the Chlorophyta, including eicosatetraenoic and eicosapentaenoic, and less linolenic acids (79). Arachidonic acid (5,8,11,14-eicosatetraenoic acid) is a natural precursor of a large family of structurally related C-20 compounds including prostaglandins, leukotrienes, and prostacyclins. Arachidonic acid and docosahexaenoic acid are important for neurological development in newborns (80). The tropical red marine alga, *Murrayella periclados* has been shown to produce high quantities of eicosanoids, including a leukotriene, usually found in animal sources (81). Other red algae including *Porphyridium*, are also rich in high unsaturated fatty acids, which is considered as a potential source of arachidonic acid (82,83). The green microalgae, *Chlorella* has been suggested for the commercial production of gamma-linolenic acid (84) as has *Spirulina* (85). Commercial fish feed enriched with fatty acids derived from algae produces better growth of fish larvae (86,87) and juvenile scallops (88). There is considerable difference in the success of the juvenile scallops, depending on the species of algae fed. There was greater growth when those species having high levels of ω-3 and ω-6 polyunsaturated fatty acids and carbohydrates were used (89).

Polysaccharides

The Rhodophyta are the traditional source of complex polysaccharides used commercially as gelling agents, stabilizers, thickeners, and emulsifiers for dairy products and other foods. They are also used in paints, photographic films, and pharmaceuticals. The most widely used are agar-agar and carregeenan. The insoluble carrageenan fraction extracted from *Stenogramme interrupta* has also shown antiviral and anticoagulant properties (90).

Polysaccharides derived from seaweeds are frequently used as fiber for nutritional supplements. Seaweeds such as *Ulva*, an edible green algae, have been raised in aquaculture for its polysaccharides and used as dietary fiber (91). For several years there has been an increase in the development of additional marine algal polysaccharides for pharmaceutical purposes (92). The polysaccharide derivative, calcium spirulan, from *Spirulina* has been shown to limit tumor metastasis (3).

Antimicrobials

Antibacterial. Because of the increase in the number of strains of bacteria that are resistant to antibiotics, there has been increased interest in the development of antimicrobial substances produced by algae. Most of the antibiotics currently used clinically are derived from substances produced by fungi or actinomycetes. However, the production of antibiotic substances from marine algae has long been known, dating back to Pratt in 1942 (93). Since the 1960s, many studies have been undertaken on the growth-inhibitory substances produced by marine algae (94,95). Several reviews have been published (32,96–98) on this topic.

Among the seaweeds, the Chlorophyta, Phaeophyta, and Rhodophyta have been surveyed for their activity. It has been shown that activity may be present, but it is species specific and varies depending on the season (99–102). Cyanobacteria have been found to produce antibiotic substances and other potentially pharmacologically active products (103,104). Green algae including Chlorococcum (Fig. 11; 95) and *Dunaliella* (105,106), have also been shown to produce antibacterial substances.

Many of the substances produced by marine microorganisms show a high degree of toxicity. The toxic nature of these substances, which include acrylic acid, phenols, terpenoids, and halogenated compounds, has made them unfit for, or have limited use in, commercial exploitation (107). However, the need to develop new antibiotics because of increasing resistance by clinical bacteria to traditional ones has led to renewed interest in the search for antibiotics from algal sources (108,109). A survey of 100 strains of marine microalgae from Japan determined that several strains showed activity, with a species of *Chlorella* displaying the greatest results, producing a light-induced substance (84). In a similar survey of 84 marine algae, *Dunaliella primolecta* showed the highest antibiotic activity (106). Nine extracts made from 16 marine algae from the Atlantic coast of Brittany, France, showed activity against isolates of marine fungi, bacteria, and yeasts (110).

Antiviral. Development of substances that show activity against viruses is another active area of research.

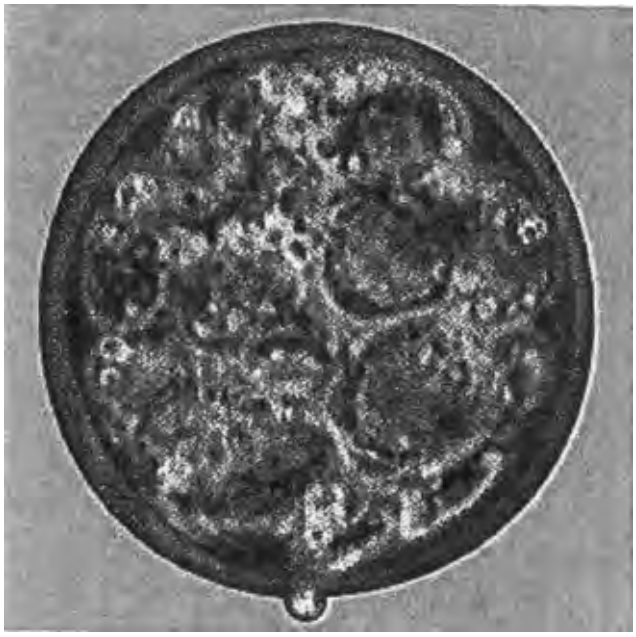

Figure 11. *Chlorococcum oleofaciens*, *http://www.cibnor.mx/malgas/efotcho.html* See color insert.

Screening of 89 seaweeds from British Columbia, Canada, and Korea for substances with antiviral activity has shown that 37% of the species tested were able to inhibit viruses (111).

Natural substances derived from algae that were active against HIV include terpenoids, xanthones, alkaloids, flavenoids, polyphenols, and polysaccharides and appear to be active against reverse transcriptase, protease, and integrase (112). In particular, the sulfated polysaccharides, such as dextran sulfate, are known to interfere with the adsorption and penetration of retro- and other viruses (113,114). Such substances have been extracted from *Spirulina* and *Cochlodinium polykrikoides* (115–117). Two mechanisms have been proposed: (*1*) Inhibition of retroviral reverse transcriptase (118) and (*2*) binding to CD4 receptors (119). The sulfated polysaccharide of *Porphyridium* (120) and that of *Spriulina* (116) have shown activity against *Herpes simplex* virus types 1 and 2 and *Varicella zoster* virus. The mechanism for action is the inhibition of production of new viral particles within host cells and/or prevention of adsorption of the virus into host cells (120).

The potential for the identification and development of antimicrobial substances from algae is an area of great potential for future development. With the increasing proliferation of resistant bacteria and the need for antiviral drugs, the exploitation of substances from algae is sure to increase.

Additional uses for algal products are the proposal of development for use as natural oral deodorants by members of the brown algae belonging to the Laminariales (121). Algae and other marine organisms have been suggested as a source of substances that are environmentally safe to control barnacles that live on ships' hulls, oil platforms, and pipelines (122). Methods, such as new assay gels containing extracts from these

organisms, are being developed to assist in the screening of such substances (123).

ALGAE AS FOOD SOURCES

As we search for new, safe, and less costly sources of feed for animals and humans, and for supplementary dietary products like protein, vitamins, and minerals, algae provide an important source for these substances. Algae have long been used as human food sources in cultures with access to the marine environment, especially in Asian cultures.

There is increased awareness of the potential for use of agar by consumers in countries outside of Japan (124). Aquaculture has become an important source of food for humans and animals. Technological advances have provided the ability to raise and harvest algae grown in controlled environments, with specialized media formulated to increase yield. The blue-green alga, *Spirulina*, has been effectively marketed as a healthfood supplement because of its high protein content (125). Although other species, including *Chlorella, Dunaliella*, and *Scenedesmus*, are also suggested to be used for their protein content (126), *Spirulina*, in particular, has been proposed for use in poultry feed, fish meal, and as a supplement in other animal feed (127–129). Since the cost of traditional protein sources such as fish, groundnuts, and soybean meals is expensive, especially for developing nations, the search for less expensive alternative sources such as algae, has increased (130,131).

Algae provide a source of high protein, comparable to those from conventional sources. *Dunaliella*, grown under optimum conditions, can produce up to 70% protein content (23). However, production costs are high and this limits use as animal feed, although many third world nations are exploring the cultivation of microalgae for protein and other biochemicals. This, in conjunction with wastewater treatment (128), or to clean liquid manure (129), provides dual benefits in terms of production and is ecologically sound.

TOXICOLOGY

Algae, as simple microorganisms, can be used as indicator species for environmental contamination and ecotoxicology (130). Large quantities of heavy metals are released into the environment mainly through industrial discharges. Agricultural runoff and sewage treatment are also sources of environmental contamination. The effect of these substances on algae will reflect in the entire ecosystem because they are the world's largest group of primary producers. Heavy metals exert their toxic effects by competing with essential metals for active enzyme or membrane protein sites, and by reacting with biologically active groups, thereby disrupting normal cell processing. The Environmental Protection Agency (EPA) has listed major toxic target contaminants including 13 heavy metals. Of these contaminants, the ones that have received the most attention are mercury(Hg), lead(Pb), and cadmium(Cd), although several others are of important environmental significance.

Because of the environment they inhabit, sediment microorganisms exhibit a higher level of tolerance to high heavy-metal concentrations. Aquatic microorganisms display the lowest tolerance to heavy-metal concentrations because the heavy-metal suspension in water is generally very low (132).

A microorganism's ability to tolerate specific heavy-metal toxicities is because of the organism's exposure to that substance. Thus, many bacterial strains show high tolerance to zinc (Zn) and copper (Cu), and few to silver and arsenic because of the greater percentage of Zn and Cu in the environment compared to silver and arsenic (133).

Collard and Matagne (134) showed that most tolerance appears to be physiological rather than genetic, but genetically caused tolerance to heavy metals was present in *Euglena gracilis* (Fig. 12), *Stigioclonium* (Fig. 13), *Chlorella vulgaris* (Fig. 2), and *Chlamydomonas reinhardtii* (Fig. 14). Cd tolerance in *Chlamydomonas* is achieved by alteration in the metabolic pathways associated with the chloroplast, causing reduced chlorophyll content and not by increased efficiency of a detoxification system (135). Pretreatment provides for increased tolerance. Preexposure of *Euglena gracilis* to low concentrations of Hg protected cells to concentrations up to 5 μM. Hg pretreatment also conferred tolerance against Cd exposure and vice versa. These algae were able to tolerate higher levels of Pb as well (136). Some algal species display cotolerance in which they can tolerate one or more additional metals to which they were not previously exposed (137). Exposure of *Dunaliella minuta* to Cu or Cd led to the acquisition of tolerance toward both Cu and Cd (138).

Anacystis nidulans, (Fig. 15) a unicellular cyanobacteria, and *Chlamydomonas reinhardtii*, a unicellular green algae, have served as indicator species for a long-term project that was undertaken to determine the inhibitory concentrations of the heavy metals identified by the EPA. In these studies, *Anacystis* with *A. nidulans* was treated with the heavy metals in media with and without the chelator, ethylene diamine tetraacetic acid (EDTA) (139). The results of this survey indicate a wide range of inhibitory concentrations of the heavy metals. These studies show the following order of inhibitory concentrations: Hg > Tl > Al = Cu > Ni > Co > Se = Zn > Cd > Mn > Ba = Pb with EDTA. A similar, but not identical pattern was seen when EDTA was not used in the medium. Without EDTA the results were: Hg > Cu > Tl > Ni > Co > Se = Zn > Cd > Mn > Ba. In this series, Pb was not studied and the results with manganese show a long lag period at lower concentrations before the onset of log phase (140–148).

There does not appear to be consistency based on toxicity. Matulova, working with the green alga *Scenedesmus quadricauda* (Fig. 16), and using lethality as the measurement found that the order was: Ni > Cr = Zn ≫ Pb (149). Rosko and Rachlin using *Chlorella vulgaris* cultures with EDTA found toxicity to heavy metals in the following order: Cd > Cu > Hg > Zn ≫ Pb (150) and Raichlin and colleagues using *Chlorella saccharophila* found that the order was Cd > Cu ≫ Zn ≫ Pb (151). However, Raichlin and colleagues using the freshwater

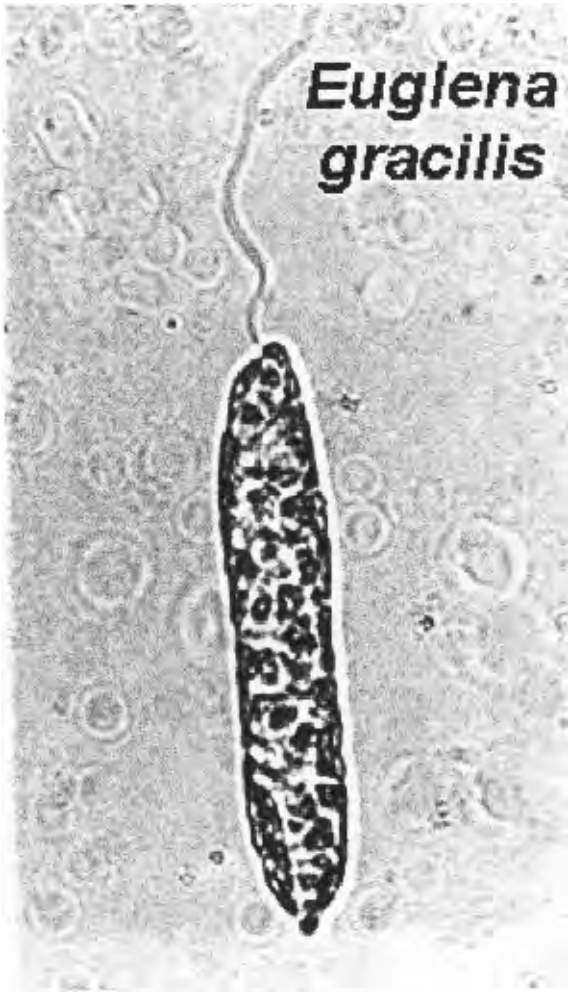

Figure 12. Euglenophyta-*Euglena gracilis http://vis-pc.plant-bio.ohiou.edu/algaeimage/pages/Euglena.html* See color insert.

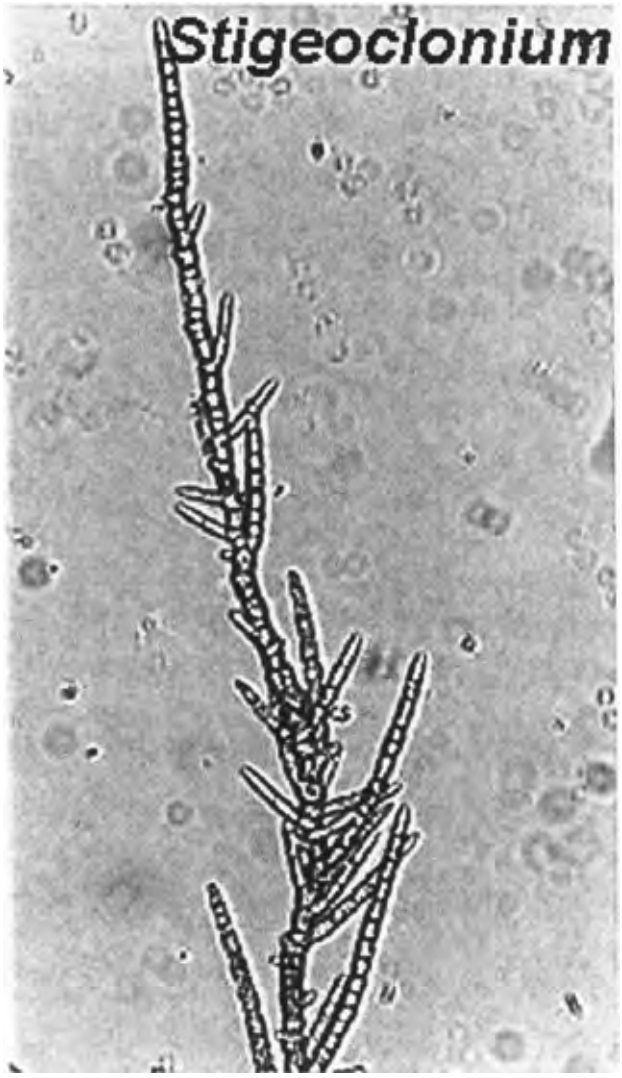

Figure 13. Chlorophyta-*Stigeoclonium* sp. *http://vis-pc.plant-bio.ohiou.edu/algaeimage/pages/Stigeoclonium.html* See color insert.

diatom *Navicula incerta* (Fig. 17) found the toxic hierarchy to be Cd > Pb ≫ Zn > Cu (152). Comparison in terms of toxicity to thallium shows a wide difference between *Anacysitis nidulans* and *Chlamydomonas reinhardtii*, with inhibitory concentrations at 10 mg/L and 0.25 mg/L, respectively (148).

These results contrast to environmental studies that contain a mixed phytoplankton population. Studies of the Saanich inlet showed the order of toxicity to be: Hg ≫ Cu = Pb > Cd > Ni = Cr in two experiments (153). Krock and Mason found the order of toxicity based on inhibition of photosynthesis in phytoplankton communities of San Francisco Bay to be: Hg > Cu ≫ Cd = Cr = Ni = Pb (154). It appears that the results depend largely on the type of algae used, and this constitutes an important factor in determining the effects of heavy metal toxicity.

Pb is the most abundant and widely used heavy metal, although recent use has declined with lead-free gasoline. It exerts its toxicity more in terms of chlorophyll a content than on cell division (150) and binds strongly to cell membranes (155). It appears to be the most widely tolerated of the major heavy-metal contaminants for phytoplankton (140–151).

On the other hand, phytoplankton appear to be highly sensitive to Hg; most organisms display greatest sensitivity to this metallic ion (153–156). Hg is one of the most lethal heavy metals studied, with no known biological function. There are three forms of Hg, elemental, inorganic, and organic compounds. Inorganic Hg is far less dangerous than organic methyl Hg. Because of limited solubility, Hg compounds are deposited in bottom mud of rivers, lakes, and so on. Through biological processes Hg compounds are converted into dimethyl and methyl Hg and thereby enter the food chain (157).

Cd has widespread industrial usage and is capable of bioaccumulation (158). It is released as a result of electroplating and alloy preparation. Plants absorb Cd more readily than Pb. Cd binds to organic molecules through sulfur and nitrogen (159), thereby inactivating proteins. The degree of toxicity to Cd appears to vary depending on the algae used as a model (150–154).

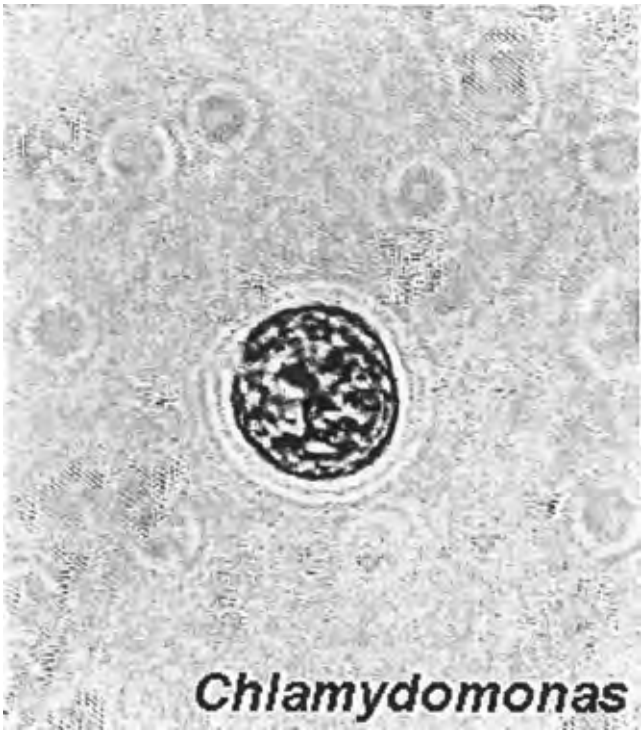

Figure 14. Chlorophyta-*Chlamydomonas* sp. *http://vis-pc.plantbio.ohiou.edu / algaeimage / pages / Chlamydomonas.html* See color insert.

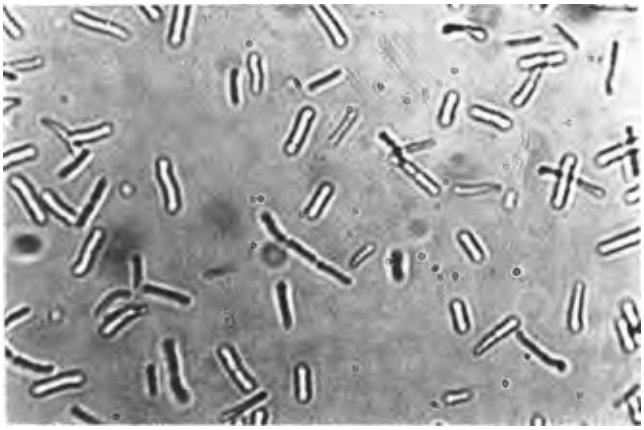

Figure 15. *Anacystis nidulans*, provided by Dr. Lee H. Lee. See color insert.

Resistance of algae to toxicants has significance for bioremediation. Elucidation of the mechanisms for resistance may produce more efficient means to remediate contaminated waters or reclaim the metals involved. Algal resistance to heavy metals may be due to several mechanisms. These include production of extracellular chelating factors (160), the presence of heavy metal–resistant genes present in plasmids, or as part of the genome (134,161,162). Specific enzymes have been identified that are capable of reducing toxic heavy metals to forms that are nontoxic (163,164). Metallothionein-like compounds have been identified in several types of microorganisms that function in reducing the toxicity

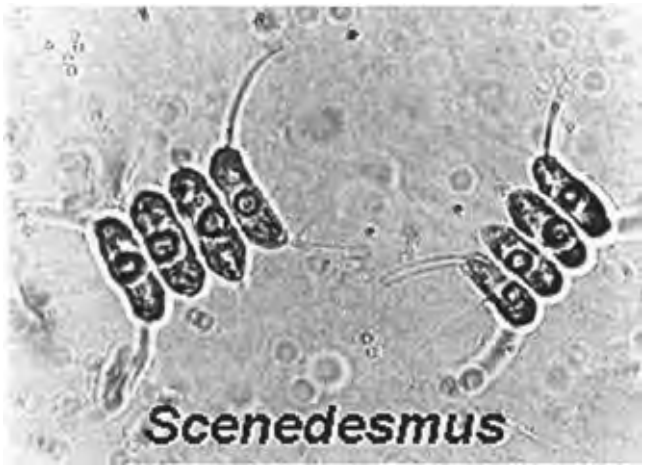

Figure 16. Chlorophyta-*Scenedesmus* sp. *http://vis-pc.plantbio.ohiou.edu/algaeimage/pages/Scenedesmus.html* See color insert.

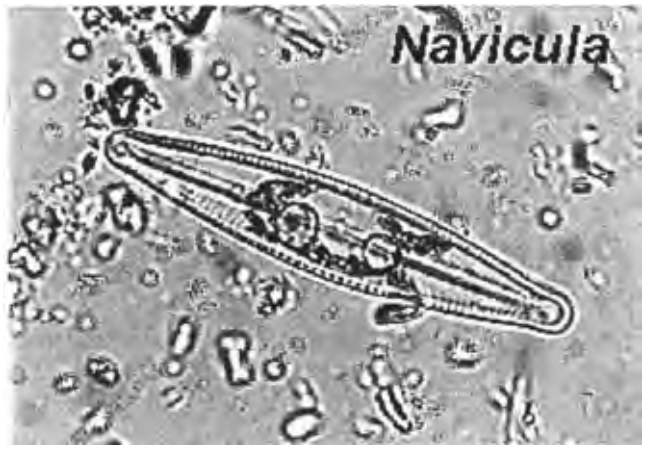

Figure 17. Bacillariophyceae-*Navicula* *http://vis-pc.plantbio.ohiou.edu/algaeimage/pages/navicula.html* See color insert.

of heavy metals. These compounds act by binding cysteine and lysine to heavy metals (165) thereby providing a mechanism by which the ions are incapable of adversely interacting with essential metabolic enzymes. Another detoxification mechanism is conformational changes in the cell wall that will trap the heavy-metal ions and prevent them from entering the cell (133). Most metals, when absorbed into the cytosol are concentrated in the vacuole and excluded from vital portions of the cell, such as the nucleus and chloroplast (166). Nassiri and colleagues showed that microalgae could develop a tolerance toward metallic pollutants by exclusion or by internal trapping (159). *Skeletonema costatum*, when exposed to Cd, Hg, or Zn forms electron-dense inclusions, multivesiculate bodies, and cytoplasmic tubules. Several trace metals including Cu, Pb, Hg, and Zn retard the flow of electrons in electron-transfer reactions of plant mitochondria and chloroplasts (167). It is currently possible to make use of some of these resistance mechanisms in products derived from algae. Thus dried biomass produced from *Chlorella* has been proposed for bioremediation (168) and polysaccharides from *Ascophyllum* can be used to remove

heavy metals (169). Similarly, genetic engineering may be used to produce strains of microalgae with greater resistance and ability to adsorb pollutants than those currently in use.

REMEDIATION

In view of increasing environmental protection control, the use of microorganisms has received attention in recent years as a means of removing soluble metallic ions from wastewaters. Existing treatment technologies may have problems with removal of the ions; therefore, alternative treatment techniques have been explored (170). Absorption of heavy metals by microbial cells has become recognized as an alternative to existing methods such as precipitation methods or use of synthetic ion exchangers. Several microorganisms have been studied, including bacteria (171), fungi (172), and algae.

Algae, like other aquatic microorganisms, have the ability to take up dissolved metals and other contaminants from the environment. Biooxidation by Cryptophyceae in lake water showed processes that can detoxify organic contaminants such as resorcinol (173). Polycyclic aromatic amines are promutagenic/procarcinogenic. Because they can accumulate and metabolize promutagenic pollutants, algae, in combination with other microorganisms, can be used in aquatic environments for the detection and conversion of environmental promutagens (174). Microalgae such as *Botryococcus-braunii*, can be used for hydrocarbon recovery, but results in lower cell viability (175,176). Bacteria and microalgae act symbiotically in wastewater treatment to provide for removal/metabolism of materials (177). The process, microalgae use the end-products of bacterial metabolism (carbon dioxide, ammonia, etc.,) and in turn, supply bacteria with oxygen for total degradation of organic compounds. The algae serve for reoxidation and mineralization, and contribute to the food chain (178). Filamentous *Oscillatoria* has been proposed as an agent to remove nutrients from secondary activated sludge effluent, and the proteins produced could be utilized commercially (179). *Spirulina maxima* grown in seawater has been suggested as a means of controlling pollution from anaerobic effluents of animal wastes and for harvest for high protein (19). Cultures of algae and *Daphnia* are raised in Holland on sewage effluent (Fig. 18).

Algal cell walls contain polysaccharides and proteins having amino, carboxyl, phosphate, and sulphate groups. The amino and carboxyl groups, and the nitrogen and oxygen of the peptide bond could be used for coordination bonding with metallic ions (180). Algae need not be living to act for biosorption. *Heterosigma akashiwo (hada) Hada*, which was inactivated by steam sterilization, showed biosorption of bivalent metal ions owing to binding to carboxylic- and phosphatic sites (181). Modification of carboxyl and amino surface functional groups has been shown to decrease the adsorption capacity of Cd (II) and Zn (II) binding with *Chlorella* (182) indicating the role these groups play with regard to metallic ion absorption. Carboxyl groups are considered to be the major metal-sequestering sites (104).

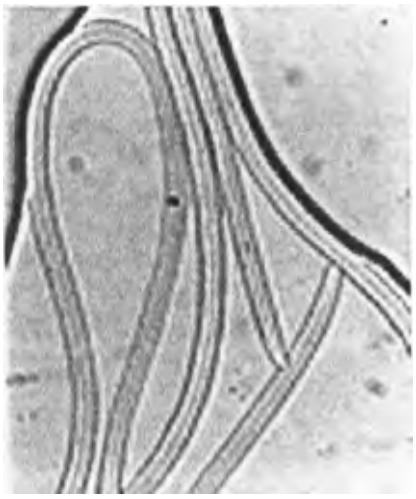

Figure 18. *Oscillatoria http://vis-pc.plantbio.ohiou.edu/algaeimage/pages/oscillatoria.html* See color insert.

pH plays an important role in the removal of metallic ions. The pH for optimum adsorption depends on the physiochemical conditions of the metal ions and the algae (168,180). The pH will affect the bond formation of the algal cell wall, particularly the amino groups, to the metallic ions. It has been found that higher pH provides for greater adsorption of Pb and (Ni) with *Chlorella* (168), and with the brown algae, *Fucus, Ascophyllum*, and *Sargassum* (183). However, at pH values above 5.0 for Pb and Cu, and 6.7 for Ni, the ions precipitate out of solution. pH may also affect the ionic form of the ions and therefore plays a role with regard to toxicity.

Many algae are known to be able to absorb and concentrate metal ions from dilute solutions and to accumulate them within the structure of the cells. The accumulation of trace metals can be of particular importance from several perspectives. Although accumulation of trace metals may not be of sufficient toxicity to the algae, magnification can occur up the food chain, disrupting the metabolism of higher organisms, thereby causing biomagnification. Similarly, absorption and accumulation can be used for metal reclamation with gold (Au), cobalt, and other ions (184–186) and remediation of industrial wastewaters (187,188). Use of algae for the biosorption of radioactive Strontium 90 and Yttrium 90 allows for concentrating and removal of these radionuclides (189). Acidophilic microorganisms including *Euglena* sp., have been proposed for the treatment of highly acidic minetailing waste to remove Ni–Cu sulphide minerals and other toxic heavy metals (190). Use of microorganisms including algae for the removal of Hg is less satisfactory because whereas the cells absorb the Hg, volatilization of significant quantities occurs (191).

Metal uptake by intact algal cells has been found to consist of two processes: (*1*) A rapid passive adsorption to the cell surface and (*2*) a slower metabolism-dependent uptake into the cytosol. Dead cells accumulate heavy-metal ions

to the same or greater extent as living cells. The passive process of adsorption is called biosorption (192). The sorption phenomenon is most frequently expressed by the Freundlich adsorption isotherm and is used for calculating the residual or adsorbed metal-ion concentration with application in wastewater treatment (180). The Langmuir isotherm is used to quantitatively determine sorption performance and reflects the attraction between the sorbent and the sorbate (168). The biopolymers involved include Na/Ca alginate, agarose, and cellulose-acetate. These biopolymers are nontoxic, selective, efficient, and inexpensive (193). When heavy metals are accumulated intracellularly, toxicity can occur that is not present in cell surface adsorption (194). Wehrheim and Wettern (195) compared dry weight of whole cells and isolated cell walls rather than definite surface area and found that isolated cell walls have 10-fold higher accumulation rates than whole cells for Cd, Cu, and Pb. Similar rates have been observed with *Vaucheria* (Fig. 19) (196), fungi, and bacteria (197–199). However, the amount of uptake varies depending on microbial biomass employed and the type of metal ion (200).

As a result, biosorption processes, which are the passive accumulation of metals by biomass, have been proposed as an efficient and cost-effective way of removing toxic heavy metals from industrial effluents (201). For cost-effectiveness, they can be manipulated for better efficiency and multiple reuse (202). Biosorption processes can be

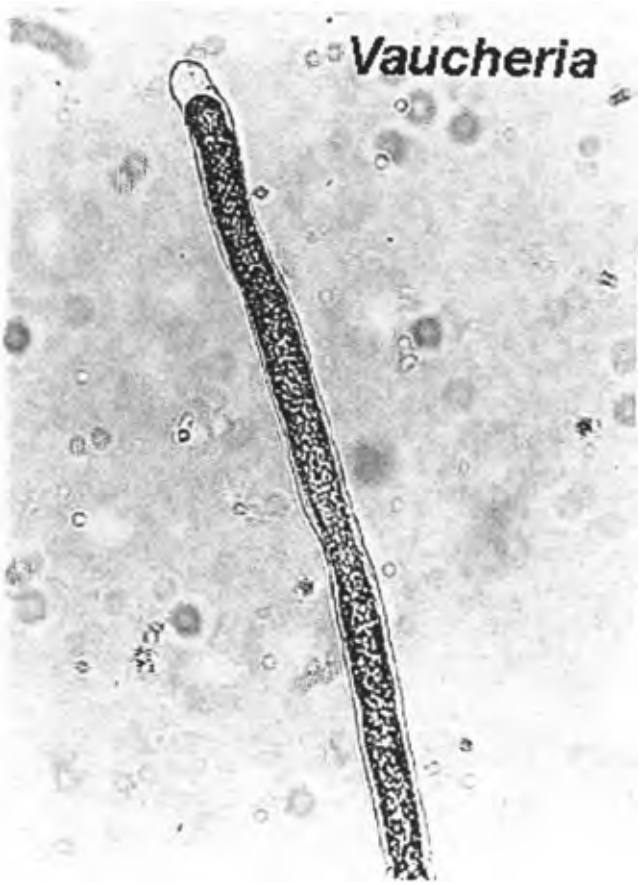

Figure 19. Xanthophyceae-*Vaucheria http://vis-pc.plantbio.ohiou.edu/algaeimage/pages/Vaucheria.html* See color insert.

produced from a wide range of algal and nonalgal sources, including dried biomass of *Chlorella* (168). Similarly, screening of 191 strains of marine microalgae showed that 24 of these were able to remove Cd from their growth media. *Chlorella* showed the highest rate of removal. Six strains out of 19 green algae and one out of five cyanobacteria removed more than 10% of total Cd from the medium. Although 12 days of incubation showed that most of the Cd was accumulated intracellularly, dried cells adsorb the ions more quickly than living cells (203). *Ascophyllum nodosum* produces a polysaccharide that provides it with the ability to remove and sequester high levels of Cd/g dry weight (169).

Biosorption of metals is not based on only one mechanism. It consists of several mechanisms that vary according to the species used, the origin of the biomass, and its processing (197). The mechanisms involve chelation, adsorption by physical forces, and binding to cell wall polysaccharides and amino, phosphate, sulfhydryl, and carboxyl groups in proteins. Within cells, heavy metals adsorbed strongly to biological membranes or to cytoplasmic polyphosphate bodies (152,204).

Combinations of ions can have a variety of effects in biosorption. Combination of Cr VI and Fe III indicate that these ions can act antagonistically with regard to levels of adsorption of Cr and Fe in *Chorella vulgaris* (205). Studies with reinforced *Ascophyllum nodosum* using two and three metal sorption systems containing Cd, Cu, and Zn have shown that Zn and Cu solutions showed an antagonistic effect on the uptake of Cd (197). Combinations of Cd, Cu, and Zn with formaldehyde cross-linked *Ascophyllum* showed that the total metal sorption increases at low concentrations, but each metal can inhibit the sorption of the others. At high metal concentrations the total metal sorption uptake is either constant or slightly decreases (206). However, there was little effect on uptake of Au by *Sargassum* biomass by other metal ions (207).

Chlorella has been found to be effective in adsorbing metallic ions (203). Dried cells showed high capacity for Pb adsorption and reached saturation within 10–15 minutes, with pH 5.0 being optimum (168). Comparison of whole cells and isolated cell walls indicated that whole cells accumulated more metal ions than cell walls using Cd, Cu, and Pb (195). Similarly, *Chlorella* was shown to have a stronger affinity for Cd II than Zn in uptake studies (182).

CONCLUSION

It is expected that future trends in algal biotechnology will involve the commercial development of additional products derived from algae. With improvements in culture methodology and harvesting of natural products, the cost for production of bioproducts from algae should make them competitive compared to production from synthetic sources. Similarly, the need for cheap, safe, animal feed will bring about increased aquaculture of algae for feed. Fish farming is increasing and therefore the need for high-quality feed. The search for antimicrobials, especially those that have antiviral properties or new

antibiotics to fight resistant bacterial strains represents an important area for future development.

Genetic engineering provides for a new area of biotechnology of algae. As genes are isolated and their functions determined, transfer of valuable genes from algae to other species will provide another avenue for bioproducts to be produced for commercial exploitation. Algal biotechnology is a field that has only begun to be developed and one with much promise for the future.

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ALGAE, BLUE-GREEN. See CYANOBACTERIA

ALGAE: EUTROPHICATION. See EUTROPHICATION AND ALGAE

ALGAE, POLAR. See POLAR MARINE PHYTOPLANKTON

ALGAE: STREAMS. See STREAM MICROBIOLOGY

ALGAE: TOXICITY TESTING. See USE OF MICROSCOPIC ALGAE IN TOXICITY TESTING

ALGAE, USE AS BIOLOGICAL INDICATORS IN PALEOLIMNOLOGY. See PALEOLIMNOLOGY: SUBFOSSIL ALGAE OTHER THAN DIATOMS AND CHRYSOPHYTES; PALEOLIMNOLOGY: USE OF SILICEOUS STRUCTURES OF CHRYSOPHYTES AS BIOLOGICAL INDICATORS IN FRESHWATER SYSTEMS

ALGAE: WASTE STABILIZATION PONDS. See WASTEWATER STABILIZATION PONDS

ALGAL BLOOMS. See RED TIDES AND OTHER HARMFUL ALGAL BLOOMS

ALGAL BLOOMS — IMPACT ON TREATMENT, TASTE, AND ODOR PROBLEMS

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Algae are not regulated in drinking water. However, algae can cause water quality to exceed secondary maximum contaminant levels (SMCLs) such as color, taste, and odor. Algae can increase the pH above the SMCL limit of 8.5 and they can increase the threshold odor number above the limit of three. SMCLs are nonenforceable guidelines under the Safe Drinking Water Act as set by the U.S. EPA; however, in some states they have been adopted as enforceable standards. In most cases, algae are a nuisance. They affect the water's aesthetics or the water's usability. Algae are also known for their interference with the treatment of water. More recently, concern has been raised over the production of toxins by cyanobacteria (blue-green algae).

Although algae themselves do not typically pass into drinking water, their by-products often do. These by-products can be (*1*) chemicals released upon death or cell lysis, (*2*) chemicals formed by treatment, that is, chemical reactions with algae materials, or (*3*) chemicals released by the active algae before treatment.

WHAT ARE ALGAE?

The terminology is alga for the singular form and algae for the plural form of the word. The study of algae is called algology or phycology. Algae are related to plants because they contain pigments that cause photosynthesis to take place. They are eukaryotic organisms (their cells have a true nucleus as do plant and animal cells). But algae have no roots or leaves like plants. (There is an exception with the blue-green algae or cyanobacteria, and they will be mentioned in more detail later.) The cell wall of most algae is made of cellulose. The diatoms have an outer protective shell made of silica. When dead diatoms accumulate they form deposits called diatomaceous earth.

Algae are typically photoautotrophic: they only require light, water, and carbon dioxide to produce their own food source. Photosynthesis is the process in which light is absorbed by chlorophylls (green photosynthetic pigments) and converted to chemical energy (sugars). Chlorophyll *a* and *b* and *c* are common pigments, giving algae their green color. Other carotenoids are also present, such as those, which give rise to the color of the golden-brown algae.

Algae in water can be found in a variety of places: benthic algae are found in sediments, planktonic are free-floating, and periphyton are attached to aquatic plants, rocks, submerged debris, and walls. They can be found in

various waters, from salt water to freshwater and from swamps to hot springs. Planktonic algae are most often associated with taste and odor and filter-clogging problems at water treatment plants.

Algae also vary in physical structure, which makes them relatively easy to identify and differentiate. Algae vary in size from microscopic forms to aquatic seaweeds or kelp:

- Picoplankton are of the size 0.2 to 2.0 μm.
- Nanoplankton are of size 2 to 20 μm.
- Microplankton are of the size 20 to 200 μm and are the types involved directly in water-treatment problems.

Finally, algae differ in the ways their cells associate with each other. For example, they can be unicellular (Fig. 1), filamentous (Fig. 2), or colonial (Fig. 3).

In clear or slightly polluted water, diatoms are often found as planktonic algae. They are an important source of food for fish and wildlife. Increasing the nutrient pollution load (nitrogen and phosphorus) to the water causes a shift in the predominant forms of planktonic algae to filamentous green algae, colonial green algae, unicellular green algae, and blue-green algae. There are many variations and many other secondary factors also affect algal growth and predominance. Blue-green algae are often indicators of organic pollution or excessive nutrient loading. They can form blooms that, upon death of the cells, cause a depletion in dissolved oxygen, which in turn can result in fish mortality.

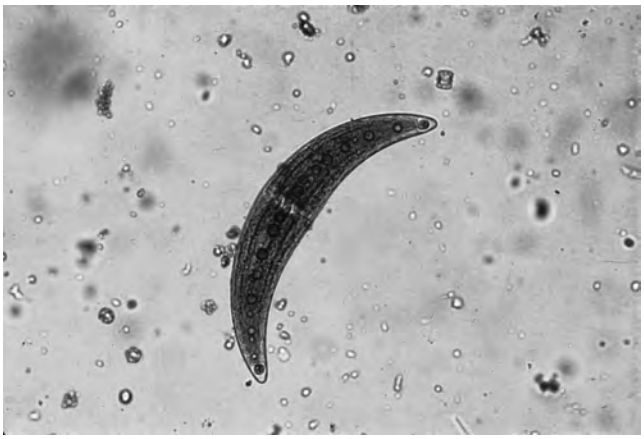

Figure 1. An example of a unicellular green alga (*Closterium* sp.). See color insert.

Figure 2. An example of a filamentous blue-green alga (*Oscillatoria* sp.) surrounding a single strand of a green alga (*Spirogyra* sp.). See color insert.

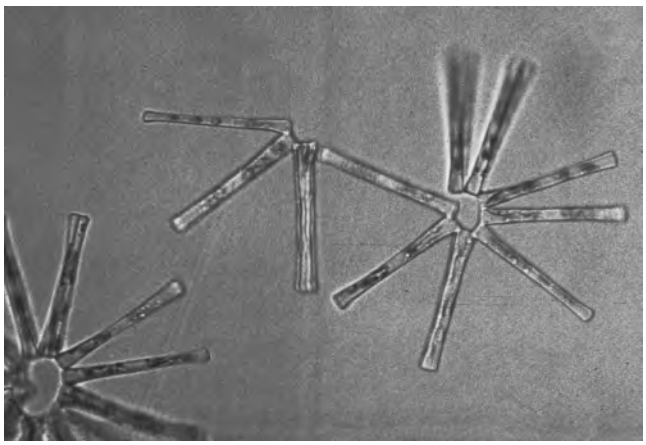

Figure 3. An example of a colonial diatom (*Asterionella* sp.). See color insert.

COMMON ALGAE THAT AFFECT DRINKING WATER

Waters in which algae are present are either surface waters (such as rivers, lakes, and reservoirs) or groundwaters under the influence of surface waters. These waters are required, in most cases, to be filtered to remove particulate matter such as algae. If treatment is insufficient, algae can pass into finished drinking water and have been found in water distribution systems. In general, water never contains a single alga specie, but always consists of some combination of algae species. Sometimes the alga of greatest dominance in water is not the alga causing the problem. Palmer's Guide to Algae (1) provided an empirical classification of algae according to their impacts on water supplies and treatment. Table 1 provides an overview of Palmer's guide. The following divisions of algae are of common concern for drinking water and its treatment (2).

Green Algae

Chlorophyta or the green algae are the largest and most diverse division of algae, which range from microscopic single-celled algae to colonial and filamentous algae (examples include the genera *Chlorella*, *Scenesdesmus*, *Spirogyra*, *Cladophora*, and *Ankistrodesmus*).

Dinoflagellates

Pyrrhophyta or the dinoflagellates are important components of plankton. They have flagella and, therefore, are motile and are typically single-celled in occurrence (examples include the genera *Peridinium* and *Ceratium*).

Table 1. Algae Associated with Certain Source Water and Treatment Conditions Based on Palmer's Guide (1)

Association:	Taste & Odor Problems in Tap Water	Clogging of Filters in Water Treatment	Nutrient Pollution of Natural Waters
Most often	Blue-green algae	Diatoms	Blue-green algae
Common	Pigmented flagellates	Blue-green algae	Pigmented flagellates
Infrequent	Diatoms	Green algae	Green algae
Least often	Green algae		Diatoms

Yellow-Green or Golden-Brown Algae

Chrysophyta or golden-brown algae include two classes of algae, which are important in drinking water treatment. The *Chrysophyceae* are pigmented flagellates (examples include the genera *Volvox*, *Pandorina*, *Synura*, *Peridinium*, *Chlamydomonas*). The *Bacillariophyceae* are the diatoms (examples include the genera *Cyclotella*, *Asterionella*, *Navicula*, *Melosira Diatoma*, *Fragillaria*, and *Synedra*).

Blue-Green Algae

Cyanophyta or blue-green algae are also known as cyanobacteria (examples include the genera *Oscillatoria*, *Microcystis*, *Anabaena*, *Aphanizomenon*). These algae are more closely related to bacteria than algae. They can be single-celled, colonial, or filamentous in occurrence, and can appear as floating scum or benthic mats, in eutrophic waters or stratified reservoirs. Blue-green algae are not plantlike in their cellular structure as are the other algae. They exhibit a gliding motility, have resting spores or endospores, have gas vacuoles to regulate their buoyancy, and can have a sheath surrounding the cells. They are diverse in their metabolism and are oxygenic photosynthetic bacteria. Some fix nitrogen and develop heterocysts for nitrogen storage. Some are found in symbiotic relationships in lichens.

Figure 4. Example of visible abundance of a blue-green alga as a surface scum on water. See color insert.

WHAT IS A BLOOM AND WHERE DOES IT OCCUR?

A bloom of an alga is commonly considered a visible abundance of plankton at the surface of water (Fig. 4). Algal blooms can also appear as carpetlike mats of algae attached to the bottom of a water body or to submerged matter (Fig. 5). Perhaps more accurately, in the context of water treatment, a bloom is a significant increase in the density of an alga to the point where the use of the water is negatively affected. For some algae a bloom could translate into a density of 100,000 cells per milliliter, whereas for others it could mean only 10,000 cells per milliliter of water or less.

An accepted cause of an algal bloom is the excess availability of nutrients such as phosphorus and nitrogen (3). Nutrients are considered very important in determining the types of algae that will grow. Nutrients can come from sources external to the body of water (farm runoff and wastewater discharges) or within the body of water (recycling from the sediment, aquatic vegetation). The three primary nutrients are carbon, nitrogen, and phosphorus. In most cases, there is no limit to the availability of carbon since carbon is derived by algae through photosynthesis from dissolved carbon dioxide. Phosphorus is most often the limiting nutrient. Sediments can contain insoluble phosphorus bound to iron, calcium, or aluminum. Under anaerobic or elevated pH conditions the bound phosphorus can become soluble and fuel an algal bloom. When phosphorus becomes the limiting nutrient, any addition of phosphorus can lead to green algae blooms. When nitrogen is limiting, this can select for blue-green algae, which can fix nitrogen from other sources. When a bloom occurs, an alga can deplete the nutrient that fueled the bloom and can, in turn, limit the bloom.

Although algal blooms occur most often during the warmer months of the year, clear lakes, which are enriched with nitrogen, during the late winter to early spring, can experience blooms of pigmented flagellates, diatoms, and green algae.

Depending on the characteristics of the lake or reservoir, the distribution both vertically and horizontally

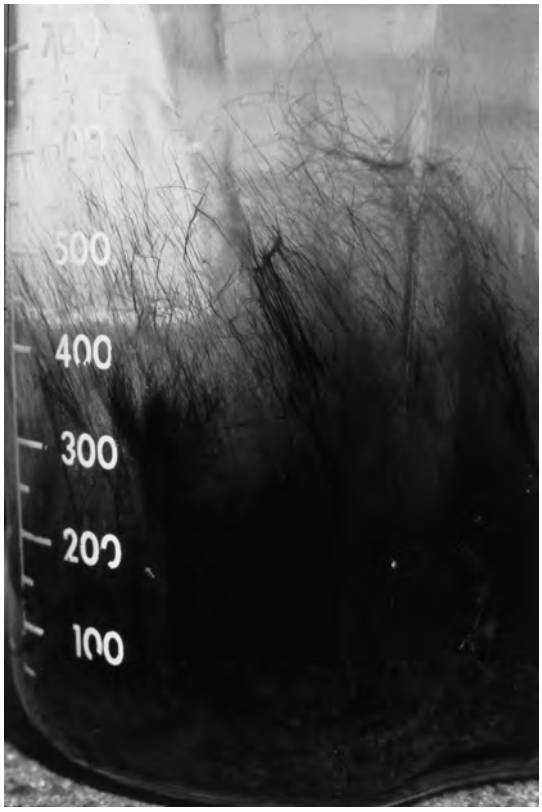

Figure 5. Example of how filamentous blue-green algae appear attached to bottom sediment. See color insert.

of nutrients, temperature, pH, light, and oxygen can result in different types of algae in different concentrations at different depths. As a result, the depth at which a treatment plant withdraws water from a reservoir can affect the types of algae that must be treated. It is important that a treatment plant manager develop an understanding of the source water. The ability to predict algal blooms to provide an early warning to water treatment operators depends on sanitary surveys of the watershed and measurements of nutrients such as total phosphate, nitrate, ammonia, total organic carbon, dissolved silica, dissolved oxygen, pH, water temperature, available light, and water turbidity.

OVERVIEW OF IMPACTS FROM ALGAL BLOOMS

There are two categories of impacts that algae can have on water treatment (2): direct impacts caused by presence of algae cells, and indirect impacts caused by cellular by-products. The direct impacts of algal blooms include filter clogging, surface scums, and change in color of the water. Indirect impacts include the production of odors, consumption of oxygen in the water when algae decompose, and change in the pH of the water.

Algae convert inorganic matter (carbon dioxide, nitrogen, phosphorus) to organic matter through photosynthesis. This organic matter makes up the algal cells. It can contribute to extracellular organic matter, which is excreted outside the cells. When algae cells are lysed, as by chemical treatment or stress, they release intracellular organic matter, which can affect water-treatment processes.

Perhaps the most dramatic impact caused by algal cellular products are caused by toxins (4,5). Freshwater blue-green algae or cyanobacteria can produce two important human and animal toxins. Hepatotoxins inhibit enzyme activity and can cause acute liver damage. Animal poisoning has resulted in animal mortality in one hour because of liver failure. They are produced by species of *Microcystis* (*M. aeruginosa* and *M. flos-aquae*), *Anabaena*, *Nostoc*, *Oscillatoria*. *Microcystis aeruginosa* is a commonly implicated cyanobacterium. Neurotoxins are nerve poisons and can cause death by respiratory distress. They are produced by such algae as *Anabaena flos-aquae* and *Aphanizomenon flos-aquae*. *Microcystis aeruginosa* can also produce a neurotoxin.

Toxins can have effects in two ways. Direct effects are where algae release their toxins into water, which is ingested, or the toxin is released after ingestion of the algal cells. Mediated effects are where the algae or toxin accumulate in a vector organism, such as shellfish, which is unaffected by the poison but causes illness when the vector is consumed. Human contact, rather than ingestion, can cause skin and eye irritation, whereas the ingestion of very low levels of toxin can cause gastrointestinal illness such as vomitting, cramps, and fever. (See also CYANOBACTERIA-TOXINS IN DRINKING WATER.)

OVERVIEW OF TREATMENT PROCESSES AFFECTED BY ALGAE

The following discussion explains the treatment stages of a conventional drinking water treatment system (6) (Fig. 6):

Presedimentation Basin

Some treatment works contain a presedimentation basin where treatment is achieved by gravity settling in a large basin at the head of the treatment plant. The primary purpose is for the removal of silt and sand and debris, which can damage pumps and processes. This basin also provides for additional emergency storage. Generally, presedimentation basins are ineffective at removing algal cells and may serve to increase algal production because of their long detention times.

Preoxidation

In the presedimentation basin or in the conduits that deliver water to the plant, an oxidant such as potassium permanganate, chlorine dioxide, or chlorine might be used to precondition the water or satisfy the oxidant demand. The demand can be caused by organic matter (algae, decaying plant matter, suspended natural organic matter) and inorganic matter (ammonia, iron, manganese). The achieving of this oxidant demand helps the plant operators achieve disinfection and produce stable water before it leaves the treatment plant. Aeration has at times been used to oxidize dissolved metals such as iron and manganese. Preoxidation can be effective at inactivating algal cells and at improving their removal in subsequent treatment processes.

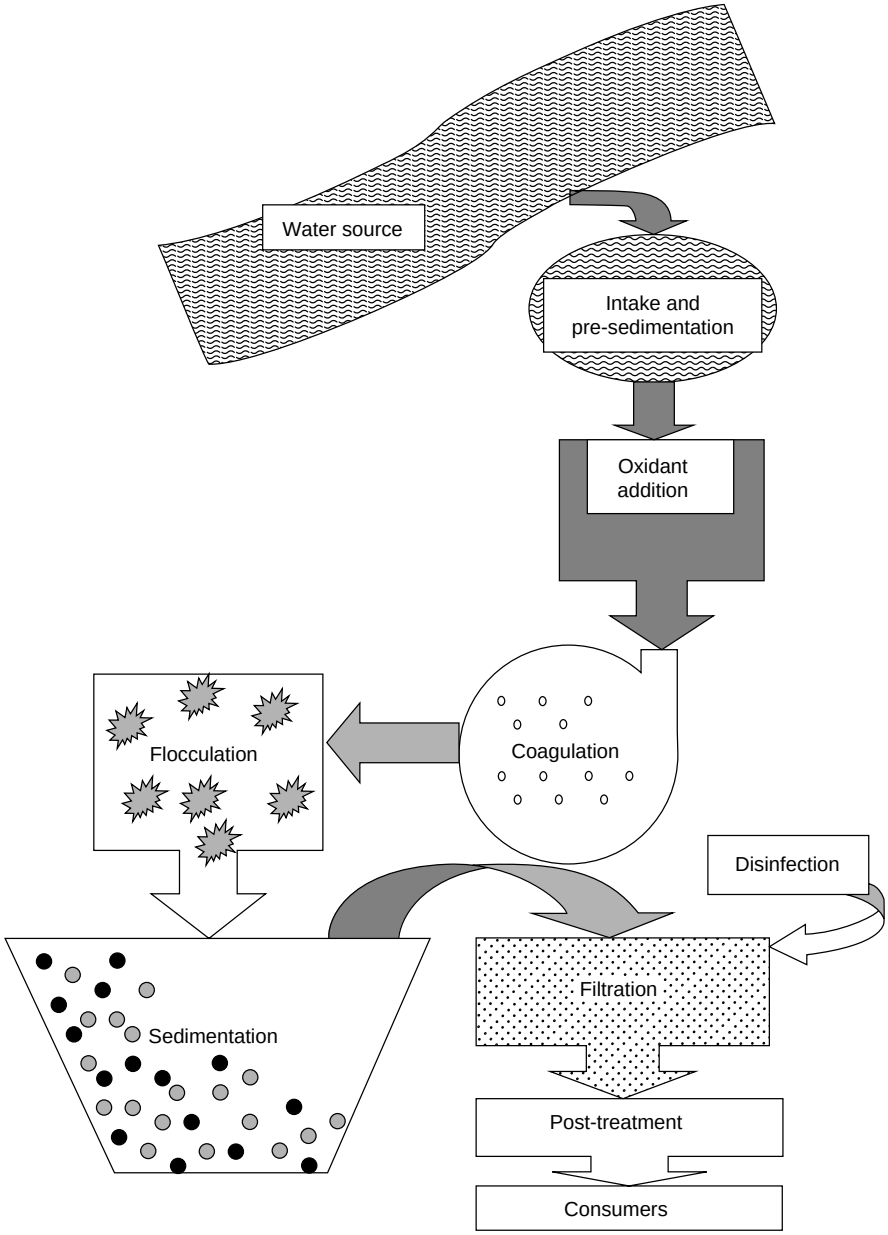

Figure 6. Stages of a conventional water treatment plant.

Coagulation/Flocculation/Sedimentation

Coagulation is the addition into water of coagulant chemicals to neutralize the surface charges of the remaining suspended matter. Aluminum salts (alum), and ferric salts such as ferric chloride and ferric sulfate, when added to water form hydroxide complexes in water. These positively charged complexes neutralize the negative surface charges of particles in the water, allowing them to stick together. This is followed by flocculation, which is the gentle mixing of water to cause the floc particles to collide and form larger particles that will settle out by gravity (sedimentation). Coagulation and flocculation are often followed by sedimentation and filtration. The combination of these processes constitutes a treatment train by which dissolved inorganic and organic matter, biological particulates, and suspended inorganic matter are removed from the water. This sequence of coagulant addition, rapid mixing, flocculation, and sedimentation has been commonly used in the treatment of surface waters and is effective at removing a large percentage of algal cells.

Filtration

Filtration by sand media, or a combined bed of sand and coal (dual media) has been commonly applied for the removal of suspended microscopic particulates and floc, which did not settle. Typical turbidity levels in well-run filter effluents can range from 0.1 to 0.3 nephelometric turbidity units or less. Turbidity is a measure of the light-scattering properties of water and, thus, is a relative measure of the suspended particulate matter in water. At

low turbidity levels, such as these, very few algal cells will have penetrated the filters.

Disinfection

Disinfection using chlorine, chloramines, or ozone is common. Disinfection renders the water free of microbial pathogens. Once the water has been adequately pretreated, the addition of a disinfectant to the water can effectively remove or inactivate most disease-causing organisms without interference from other organic and inorganic substances. Disinfection should also ensure that no viable algal cells are transmitted to the distribution system.

Posttreatment

Posttreatment can include fluoride addition, pH adjustment, corrosion control, and the addition of a disinfectant residual to make the water acceptable for distribution and storage in reservoirs. Posttreatment has little or no effect on the control of algal cells.

SUMMARY OF NEGATIVE IMPACTS FROM ALGAE ON TREATMENT

There are sources of information on the negative impacts of algae on water treatment processes (1,2). The size, shape, motility of algae, release of extracellular organic matter (EOM), and aggregation of algae affect the treatability of the algae. For example, algae that associate as dense rafts will more readily clog filters. Smaller, singly occurring algal cells can more readily break through the filters. Algae that have cell structures that prevent the proper formation of floc will interfere with the flocculation and settling processes. Motile algae can stay near the surface of settling basins and avoid getting caught in the floc that is settling out.

Control of Coagulant Demand

The amount of coagulant that must be added to water to achieve the process goal for coagulation, flocculation, and sedimentation is termed *the coagulant demand*. The process goal could be charge neutralization for the suspended particulates or it could be a reduction in total organic carbon or turbidity. Algae or their extracellular organic matter can induce an additional coagulant demand and interfere with the efficiency of the process.

Maintenance of Coagulation pH

The pH of the water is important for determining the actual mechanism by which particles settle out and the efficiency of the coagulation and flocculation processes. Algal blooms can increase the pH of the water above 9. Optimum coagulation pH is usually from 5.0 to 8.5 units. Treatment plants may not be able to counteract the pH increase. Water with an elevated pH then enters the coagulation and flocculation processes, thereby affecting the efficiency of the processes.

Performance of Settling and Clarification

Although algae can be effectively incorporated into the floc, the presence of the algae and their by-products such as extracellular organic matter and cellular oils may impair the settling of the floc. One effect is the carryover of algal cells and floc to the filters. The size and shape of the algae are two important factors in determining the extent of these effects to occur. It is generally believed that coagulation and flocculation should be preceded by oxidation to inactivate the algae.

Performance of Filtration

Algae that pass through the treatment processes and make their way onto the filters can cause the clogging of the filters. This requires more frequent filter backwashing, which increases turbidity and the quantity of wasted water. For example, rapid sand filters that are normally backwashed every forty hours into operation might have to be backwashed every twelve hours. Along with this effect on the filters comes a reduction in water quality. Increased backwashing of the filters and the penetration of algal cells through the filters can increase the turbidity of the finished water, but the largest effect of clogging is to limit the amount of water that the treatment plant can produce.

Disinfectant Demand and By-Product Formation

Algal cell products such as extracellular organic matter or products released upon cell lysis may constitute sufficient organic matter to react with oxidants such as chlorine to form disinfection by-products. The oxidation of algae cells causes cell lysis, which releases organic matter that itself will get oxidized and exert an oxidant demand. Chlorination by-products (trihalomethanes and haloacetic acids) are regulated under the Safe Drinking Water Act. Aldehydes are one other group of by-products. Sufficient levels of aldehydes can alter the odor quality of the finished water. Algae and their extracellular organic matter impart a chlorine demand, thus requiring increased use of chlorine in the treatment process, which increases disinfection by-product formation in general.

Overall Control of the Treatment Process

The treatment plant operator and manager must adjust chemical dosages and other process controls according to changes in water quality (such as during a rainstorm that washes excessive sediment into the source water and decreases alkalinity). Algal blooms that cause changes in chlorine demand, pH, coagulant demand, and filter operations require the plant operator to adjust treatment processes. Such changes impact other processes such as sludge management, storage of treatment chemicals as more chemical might have to be used, or the recycling of filter backwash water. In addition, adjustments to treatment to offset the direct and indirect effects of the algae can in turn increase turbidity, disinfection by-product formation, and change the chemical quality of the water. All of these issues must be carefully balanced.

Quality of Water Passed into Distribution and onto Customers

Changes in treatment or the inability of treatment to control the impacts of algal blooms can result in increased turbidity, lower chlorine residuals, increased disinfection by-products, and tastes and odors. These can negatively affect compliance with regulatory standards along with customer relations.

TASTE AND ODOR OF DRINKING WATER

One goal of the treatment process is to produce a drinking water that has minimal taste and odor (or flavor). One consequence is that customers become accustomed to the flavor of their tap water. Customers will complain if they detect a change in tap water flavor irrespective of whether that change is an improvement or not. Therefore, the flavor of water must be consistent. In the United States, the Safe Drinking Water Act requires that tap water contain a detectable residual of chlorine to control microbiological quality. Thus, the minimal flavor of tap water is a chlorinous flavor.

There are only four basic tastes (sweet, sour, salty, bitter). There are innumerable odor qualities (such as musty, earthy, fishy, grassy, rotten eggs, swampy, chlorinous) and their names are usually representative of associations or experiences (smells like fresh cut grass). Feeling sensations, produced at free nerve endings in the nasal and oral regions by chemical reactions, include drying, slick, metallic, and astringent. Changes in the inorganic and organic chemistry of drinking water have caused such off-tastes, off-odors and off-feeling-factor sensations.

IMPACTS ALGAE HAVE ON TASTE AND ODOR

There has been much research into the taste and odor of drinking water and the impacts that algae have on that quality (7–9). Findings of this research are summarized in the following section. Odors that have occurred in source waters include grassy, fishy, earthy, musty, cucumber, geranium-flowery, rancid, sulfide-like, decaying vegetation, aquarium, rotting hay, tobacco, and septic odors. The most common odors that affect drinking waters and cause customer complaints come from two naturally produced, compounds known as geosmin and 2-MIB. They impart earthy or musty odors to the water.

Geosmin ("earth odor") is an abbreviated name for trans-1,10-dimethyl-trans,9-decalol. This compound has been known since the 1960s. It is naturally produced, and blue-green algae are a common source, although not the only source. The odor of geosmin has been likened to earth, dirt, corn silk, and beets. It can be smelled at levels as low as about 5 nanograms per liter. Analytical detection methods exist for detection of this compound in water at these low levels. Typical levels in source waters are around 5 to 200 nanograms per liter.

2-MIB is an abbreviated name for 2-methylisoborneol. This compound has been known since the 1960s. It is naturally produced and blue-green algae are a common source though not the only source. The odor of 2-MIB is likened to earth, damp musty soil, and peat. It can be noticed as an odor at levels around 5 nanograms per liter. Analytical detection methods exist for detection of such low levels in water. Typical levels in water are around 5 to 100 nanograms per liter.

Cyanobacteria that have been reported to produce geosmin include *Anabaena*, *Aphanizomenon*, *Lyngbya*, *Oscillatoria*, *Phormidium*, *Schizothrix*, *Symploca*, and *Fischerella*. Cyanobacteria reported to produce 2-MIB include *Oscillatoria*, *Pseudanabaena*, *Synechococcus*, and *Phormidium* (8).

The decay of algae can produce sulfurous odors such as from dimethyldi- and dimethyltrisulfides (decaying vegetation, septic and fishy odors). The sulfides are decomposition products of proteins and amino acids. Other decay products include isovaleric and butyric acids, which give off rancid, sour milk, and dirty sock odors.

Grassy odors have been noticed during algal blooms and have been associated with *cis*-3-hexen-1-ol and *cis*-3-hexenyl acetate. The grassy odors sometimes are more noticeable after oxidation of the algae. Algae can produce linolenic acid, which has a watermelon type of odor, and this can degrade microbially to the grassy odorants *cis*-3-hexenal and *t*-2-hexenal.

A cucumber odor has been found to be produced by alga such as *Synura*. The responsible compound is *trans*,2-*cis*,6-nonadienal. This chemical is naturally produced and is also found in cucumbers and melons. The cucumber odorant can be detected as an odor down to levels under 10 nanograms per liter.

Fishy odors have been noticed during algal blooms. Fishy odors have been related to the compounds n-hexanal, n-heptanal, decadienal, and heptadienal. Some of these same compounds also produce fishy odor problems in fish oils and related products.

A hay-woody-tobacco-like or fruity-fragrant odor can be produced by other alga such as *Microcystis*. The odor comes from the chemical beta-cyclocitral. It is a degradation product of carotenes (which are cellular pigments).

The diatoms (*Asterionella*, *Tabellaria*, *Synedra*, *Melosira*) that bloom during the spring and fall, have been associated with grassy and fishy odors. The yellow-brown algae or pigmented flagellates that bloom sometimes in winter (*Dinobryon*, *Mallomonas*, and *Synura*) have been associated with cucumber and fishy odors depending on the stage of the bloom such as during the exponential stage of growth or during the death of the bloom. The green algae that bloom more typically during the summer have also been associated with grassy and fishy odors.

IMPACTS ON TAP WATER CONSUMERS

Consumers of tap water rely on their senses of taste and smell and sight to detect changes in water quality. Consumers can choose to install point-of-use treatment systems (such as faucet filters) or purchase bottled water to avoid consumption of water they do not trust or find

acceptable. Consumers can choose to register complaints with the water provider or with the local health agency and regulatory authority. Depending on the response they receive from the water provider and the attention given to the problem by the local media, their trust of the water provider can be greatly challenged.

EVIDENCE OF THE OCCURRENCE OF TASTE AND ODOR PROBLEMS

It is an unacceptable, but often unavoidable practice to wait until customers complain to know that an algae bloom has affected the taste and odor quality of the drinking water. There do exist some means by which to better predict the occurrence of algal blooms and better prepare for changes in treatment. For example, algal photosynthesis requires pigments such as chlorophyll. There are analytical techniques for measuring the chlorophyll content of water. On-line analyzers are available to help operators monitor trends in algal growths before the water turns green in color. Other predictors of algal blooms include water temperature, light transmittance, light availability, orthophosphate, ammonium nitrogen, nitrate nitrogen, dissolved silica, and pH. Finally, the odorous chemicals produced by algae can be monitored for increasing trends over time.

The actual source of a taste-and-odor problem may not be easy to find. The algal growth that is releasing an odorous compound could be in one of many coves of a reservoir, only in a certain bottom area of a body of water, only at a certain depth of a reservoir, or many miles up river from a treatment plant's intake.

OVERVIEW OF CONTROL OPTIONS

There are sufficient sources of information from treatment chemical manufacturers to treatment handbooks, which cover the options for control of algae and their associated problems (3,5,6–9). The following is a summary of these options.

Source Controls

Controls include chemical oxidation or inactivation, nutrient reduction, alteration of the ecological balance (in smaller reservoirs), and aeration or mixing. The average cost per year for algae control by treatment works ranged from 1,000 to 25,000 dollars during the 1980s (9). Controls are often initiated according to time of year, start of a taste or odor problem, occurrence of certain algae, and the temperature of the water.

If phosphorus is the limiting nutrient for a reservoir or slow moving river, it is possible that when the sediment becomes anoxic the bound phosphorus that had precipitated out redissolves into the water. With mixing or reservoir turnover this bottom concentration of dissolved phosphorus then becomes available to the algae. Attempts have been made to precipitate out the phosphorus with aluminum sulfate, aluminum silicate, and ferric chloride because phosphorus is tightly bound to aluminum hydroxides. The floc lays a blanket over the sediment and prevents recycling for a period of time.

Hypolimnetic aeration is the process of adding oxygen to the hypolimnion (colder bottom layer of water) without upsetting the stratification of the reservoir. This aeration is used in deep reservoirs that develop anoxic bottom layers. The aeration maintains the water density layers in the reservoir and helps prevent the recycling of phosphorus and the release of other nutrients, such as iron and manganese, from the sediment into the photic areas where algae thrive.

Artificial destratification is used in the late spring or early summer when reservoirs stratify. It attempts to mix the layers of water to prevent the bottom layer from becoming anoxic. It can be accomplished by hydraulically mixing the waters and by injecting air across the bottom. The objective is to keep the water uniform in temperature throughout the whole body of water. Mixing can also stir together the various types of algae growing at different levels, and thereby cause a shift to more favorable algae types. Other reservoir control options include removing sediment; harvesting aquatic vegetation; draining and cleaning the reservoir.

Algicides are chemical agents, which are toxic to algae. Copper sulfate has been the most common reservoir control option since about 1905. It is less effective for lakes, which have a high pH or high alkalinity or water temperatures below 15 °C. Algae vary in sensitivity and some can be quite resistant to copper sulfate such that its use can even encourage the growth of more problematic algae. Copper sulfate-type algicides can be fed to a reservoir as small crystals such as in a burlap bag filled with crystals and pulled behind a boat to dissolve the chemical into the water. Helicopters and boats can also apply larger crystals, which dissolve slowly and sink to reach the algae at lower depths or on the bottom of the reservoir.

Potassium permanganate has been used to control algae in the water source as well as algae coming into the treatment plant. However, it seems to work best as a pre-oxidant in the treatment plant. Potassium permanganate, in all cases, must be applied at a sufficient dosage and given sufficient time of contact.

Another control option is watershed management. This includes land use management, control of runoff and land development, use of agricultural practices that capture runoff, waterway preservation, and control of the use of chemicals in the watershed. Included is the reduction of nutrients by upgrading wastewater treatment plants to achieve better nitrogen and phosphorus removals.

A final option is avoidance of the source of water containing the nuisance algae. This includes providing multiple intakes in a reservoir, which allow the draw off of water from different depths, where algal blooms are not found. This also includes changing source waters.

Inplant Treatment Controls

Treatment can either physically remove or kill the algal cells. It is preferable to capture and settle out the algae, avoiding cell lysis and the release of intracellular toxins or organic matter, which could increase taste and odor,

chlorine demand, coagulant demand, or disinfection by-products. Chemical oxidation can kill the algal cells, or at lower dosages, immobilize motile algae so that they can be more readily trapped in floc. Oxidation also changes the surface of the cells, which can enhance or impede charge neutralization. This may aid or interfere with the removal of algal cells during flocculation.

Conventional particle removal treatment (coagulation, sedimentation, rapid sand filtration) is ineffective for soluble algal toxins. Adsorption onto activated carbon or oxidation can be effective in controlling the toxins. Lysed cells can release high levels of toxins, which may require the use of large chemical dosages. Therefore, it is preferable for treatment to remove the cells before cell lysis.

Jar testing is often used by treatment plant operators to determine the optimum dosages of treatment chemicals. The amount of coagulant added, the mixing energy, the settling time, the water temperature and the pH of the water all affect the efficiency of settling of the floc.

Treatment plant operators can measure charge neutralization with a streaming current detector, which gives a relative measure of the surface charge on particles in the water. One would, in the lab, add increasing amounts of coagulant until charge neutralization is achieved. Cationic, anionic, and nonionic polymers (flocculant aids) have been used to improve floc formation and settleability to get charge neutralization. Microsand enhanced flocculation and dissolved air flotation are other options for improving the physical removal of algal cells. This is important in preventing the loading of the filters with algae. The algal cells must be inactivated or immobilized and physically captured in floc and removal by sedimentation. The goal is to accomplish 90 to 99 percentage removal of algae before filtration without producing other negative side effects.

The predominant algae species in the surface water supply might not be the same species that cause filter clogging or that break through filtration into the drinking water. Algae vary in their propensity to resist different stages of treatment and even low counts of certain species can cause treatment or water quality problems.

Chemical oxidation attempts to accomplish a variety of tasks for algae control: inactivate the algae; oxidize taste and odor compounds; and prevent the biological formation of other odor compounds. Chlorine has been commonly used at dosages from 1 to 5 milligrams per liter and is effective for most algae and many algae odorants but causes formation of disinfection by-products. Chlorine dioxide has been used at dosages from 1 to 3 milligrams per liter in place of chlorine to reduce the formation of disinfection by-products. Ozone has been used at dosages from 1 to 3 milligrams per liter and has been effective for the control of geosmin or 2-MIB, which are not affected by other oxidants. Potassium permanganate at dosages from 1 to 5 milligrams per liter, though a relatively weak disinfectant, has been found to be effective for certain algae and certain odorants. For example, it works more effectively for the cucumber odor and does not form disinfection by-products.

Adsorption of By-Products

Although treatment can effectively remove algae from the water, algal intracellular and extracellular chemicals can still pass through into the treated water. Carbon has been used to adsorb algal by-products from the water. Carbon can be added to water in an activated powdered form (as a slurry that settles or filters out) or a filter can be made of granular activated carbon to adsorb the organic materials from the water. Since many algal blooms are infrequent or episodic in nature, powdered activated carbon has been the most commonly used form. Geosmin and 2-MIB have been removed up to 40 to 90 percentage of their initial levels. However, different types of carbon are more or less effective on different odorous compounds depending on the chemical quality of the water being treated.

CONCLUSION

Algae in source waters can affect drinking water quality. Typically, they affect the aesthetic quality of water (color, taste, odor) but some algae can impart toxins that have human health impacts.

Algae in source waters can affect drinking water treatment processes in a treatment plant. They can exert an oxidant demand, interfere with the removal of particulate matter, and clog filters.

The treatment of drinking water can be optimized to reduce the effects of algae on the treatment processes and the finished water quality. However, at times, source water treatments, watershed controls, selection of alternative water supplies, and advanced treatments have to be used because the problems caused by algae cannot be mitigated using existing treatment.

Much is known about algae, their impacts on drinking water quality and its treatment, and their control. Algal impacts can be documented and, at times, even predicted by water quality testing and algae monitoring. However, the solutions to problems caused by algae remain site-specific. Each water quality manager and treatment plant engineer must effectively apply existing experience and knowledge to secure a high quality drinking water for their consumers.

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ALGAL PIGMENTS AS INDICATORS.

See PALEOLIMNOLOGY: USE OF ALGAL PIGMENTS AS INDICATORS

ALGAL TURF SCRUBBING: POTENTIAL USE FOR WASTEWATER TREATMENT

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Algal turf scrubbing (ATS) is a novel algal technology that has been designed and engineered to promote natural wastewater treatment processes. Algal turf scrubbing improves water quality by passing a shallow stream of water over the surface of a gently sloped floway in a series of pulses. The floway is colonized by a natural heterogeneous assemblage of periphyton consisting of filamentous algae and symbiotic aerobic bacteria and fungi. Algal photosynthesis provides oxygen for aerobic breakdown of wastewater by heterotrophic bacteria. Pollutants are extracted from the wastewater by several processes including assimilation, adsorption, filtration, and precipitation. The algal turf is periodically harvested to remove the accumulated periphyton biomass and associated pollutants from the system.

DEVELOPMENT

ATS was developed by Adey and coworkers at the Smithsonian Institution, Washington, D.C., during their research on coral reef ecosystems (1–4). Adey found that the algal turfs growing on coral reefs have very high growth rates despite the low nutrient concentrations ($mg\,m^{-3}$) in the surrounding seawater (3). The high growth rates were attributed to two main factors. First, the constant surging of seawater across the turf, which by turbulent mixing breaks down boundary layers and provides a constant supply of nutrients to the algae. Second, the efficient grazing of the algal turf by herbivorous invertebrates and fish maintains the turf species growth in exponential phase.

By using a screen to mimic the reef surface, a dump bucket to provide a wave of turbulent mixing, artificial lighting, and periodic manual scrapping of the screen to remove accumulated turf biomass, the algal turf scrubber can simulate these natural conditions (Fig. 1). Adey and Hackney (3) found that ATS provides a much more natural means of maintaining water quality and community structure in model coral reef ecosystems than bacterial filters and protein skimmers that are traditionally used in aquarium systems. Bacterial filters and protein skimmers are not suitable for use in ecosystem models as they filter out the zooplankton and larval stages of many aquatic organisms.

Following the success of the ATS system as part of the coral reef ecosystem model, ATS was used in several other ecosystem models at the Smithsonian Institution, including a salt marsh estuary, a northern temperate littoral system, and a tropical mangrove (2,5,6). ATS was used as an integral part of the 3,400 m^{-3} mesocosm of the Space Biosphere 2 project in Arizona (4,7). Algal turf scrubbers have been used to remove nutrients and maintain water quality in the aquarium systems of several cities in the United States, including Indianapolis, Pittsburgh, and St. Louis, and in the 2,500 m^3 Great Barrier Reef Aquarium in Townsville, Australia (4).

The use of ATS systems to treat aquaculture wastewater at pilot-scale has been demonstrated (3,4) and a full-scale Tilapia aquaculture facility is now operating in San Antonio, Texas. The system uses 1.6 hectares of parallel algal turf scrubber floways to treat the aquaculture wastewater, and the harvested algae biomass is used as a feed supplement for the fish (8).

In terms of wastewater treatment, small-scale laboratory experiments have indicated the potential of ATS systems to remove both refractory organic compounds and heavy metals from polluted water (9). The capability of

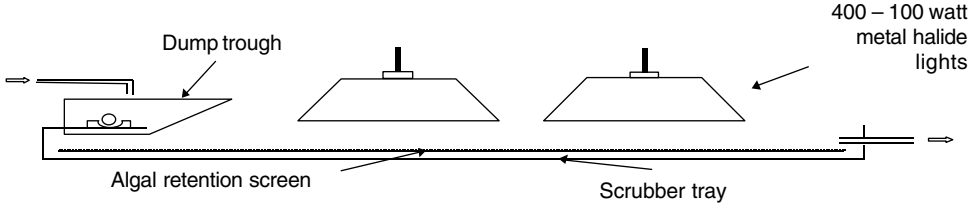

Figure 1. Schematic diagram of the component of ATS.

ATS to remove nutrients from agricultural runoff has also been demonstrated at pilot-scale using a 15.0-m long, 0.75-m wide algal turf scrubber floway (10).

The first large-scale ATS system for the treatment of domestic wastewater was constructed in Patterson, California (Plate 1). The capability of ATS to polish secondary treated wastewater at several influent flow rates (ranging from 108.9 $m^3 d^{-1}$ to 1,336 $m^3 d^{-1}$) was evaluated over a three-year period (1994–1996) (11,12). The system consisted of a single floway that was 152.4 m long, 6.5 m wide, and had a surface area of 1,012 m^2.

A second trial to assess the potential of an ATS floway to polish domestic wastewater was conducted at Fruitland, Maryland. The system consisted of 10 parallel, 91.4-m long floways, which were connected so that the effluent from the first floway was pumped to the top of the second and so on, until the wastewater was treated by all 10 floways. The system was evaluated over a one-year period (1998–1999) for its ability to treat secondarily treated wastewater at a flow rate of 284 $m^3 d^{-1}$ (8).

At the time of writing, the United States Department of Agriculture (USDA) in Beltsville, Maryland in association with the University of Florida, is evaluating ATS systems for treatment of cow manure. The systems consist of 200-m long, 1-m wide floways. Their ability to remove nitrogen and phosphorus from raw or anaerobically digested cow manure diluted with water is being determined. Two pairs of 50-m by 1-m floways (one pair at a 1% slope and the other at a 2% slope) are also under construction and will be used to study seasonal variation in algal growth and nutrient uptake rates (8).

Plate 1. The ATS pilot system at Patterson, California. See color insert.

ATS WASTEWATER TREATMENT SYSTEMS

Algal turf scrubbers are low-cost treatment systems, which are simple in design and construction (Fig. 2). The floway is formed from graded (0.25% to 2.0% slope), compacted earth covered with an impervious liner and an overlying biomass retention screen between two raised sidewalls. The liner may be constructed from any impervious material including concrete, asphalt, high-density polyethylene, polyvinyl chloride, or sprayed on asphalt rubber surfaces. Geomembranes with low thermal expansion are particularly suitable as they do not wrinkle or retain manufacturing creases.

Pulsed Influent

The influent is normally delivered to the ATS by a surging device, which releases the wastewater in a series of waves. Various techniques have been tested to produce pulsed wastewater application including pulsed pumping, counter weighted dump troughs, and displacement troughs. Pulsed application was found to be important in maintaining the treatment efficiency of marine ATS systems (3,4,13). However, research at Patterson showed that surged flow may not be necessary for freshwater and wastewater treatment systems (12). Freshwater algae may be better adapted to the continuous turbulent flow that naturally occurs in streams and rivers.

Water Depth

Algal growth on ATS systems declines with increased water depth (3). Vymazal (14) found that periphyton growth was much higher at shallow depth of 6 cm than at 64 cm. Therefore, the water depth of ATS systems is maintained between 2 and 4 cm to allow optimum exposure of the algal turf to sunlight.

Residence Time

One factor influencing the treatment efficiency of ATS systems is the time that the wastewater is in contact with the algal turf (the hydraulic residence time). A simple

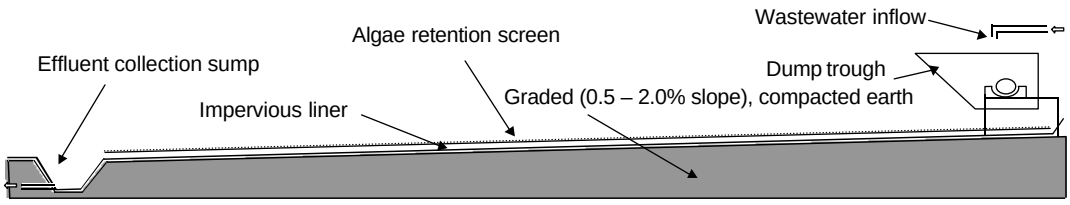

Figure 2. Schematic diagram of an ATS for the treatment of wastewater.

means of optimizing treatment is by controlling residence time, by changing the hydraulic loading velocity, or by passing the wastewater down a floway of variable length.

Hydraulic Loading Velocity. Reducing the hydraulic loading velocity of the Patterson ATS system from $1.36\ m\,d^{-1}$ to $0.44\ m\,d^{-1}$ increased removal efficiency and produced an effluent with lower concentrations of nutrients, total suspended solids (TSS), and biochemical oxygen demand (BOD) (11).

Floway Length. For a particular hydraulic loading velocity, a minimum length of floway is required for efficient treatment to be achieved. Some parameters (e.g., dissolved oxygen (DO), temperature and concentrations of all forms of nitrogen) have a linear correlation with floway length. Other parameters (particularly, pH, soluble reactive phosphorus concentration, hardness, and conductivity) change more rapidly at or after a particular floway length.

Algal Species

The algal species that typically occur on algal turf scrubbers can be divided into three ecological types: the mat-forming species consisting primarily of Cyanophyta, the green filamentous periphyton that grow through the mat to form a canopy (Plate 2), and the diatomaceous epiphytes that grow on the surface of the periphyton or are embedded within the mat (Fig. 3). The population density and algal species diversity of algal turfs in wastewater treatment systems are much lower than in systems treating agricultural drainage water and seawater (3,4,10,12). The dominant algal species is also strongly influenced by season. For much of the year they are cyanobacteria (specifically, *Oscillatoria* sp.) and diatoms (including *Navicula* sp., *Nitzschia* sp., and *Cyclotella* sp.). It is only in the summer that the green filamentous periphyton *Ulothrix* sp. and *Stigeoclonium* sp. are dominant with *Cladophora* sp., *Spyrogyra* sp., *Tribonema* sp., *Oedogonium* sp., and *Rhizoclonium* sp. present to a lesser extent.

The low population density and species diversity of algal turfs in wastewater treatment systems probably is due to the absence of algal spores in secondary treated wastewater compared to agricultural drainage water to seed the system (10) and the lack of recirculation, which

Plate 2. ATS filamentous algae canopy. See color insert.

provided a source of algal spores in the mesocosm and ecosystem model systems (4).

Seeding and Recirculation. Algal turf development and species diversity are much improved by seeding the floway with periphyton from nearby streams and rivers. This can be done either by placing the biomass retention screens in natural waterways and transferring them to the top of the floway once a turf has established or by collecting algal turf, breaking it up, and evenly distributing it over the floway surface. The latter method can bring a floway into full productivity after only a few weeks (12). Species diversity may also be maintained by recirculating a portion of the algal turf scrubber effluent to introduce algal spores with the influent.

Invertebrates

Several invertebrate species are found in wastewater algal turfs. The most abundant are amphipods and chironomid midge larvae. Chironomid infestation can be a significant problem because the larvae, which live in cocoons on the floway surface, dislodge the surrounding algal turf causing a reduction in algal turf standing crop. Chironomid problems have been observed in several other periphyton growth systems (15–17). A simple method of controlling chironomid populations on algal turf scrubber floways is by drying out the floway following harvest of the turf, especially as the productivity of the turf can be quickly restored by seeding.

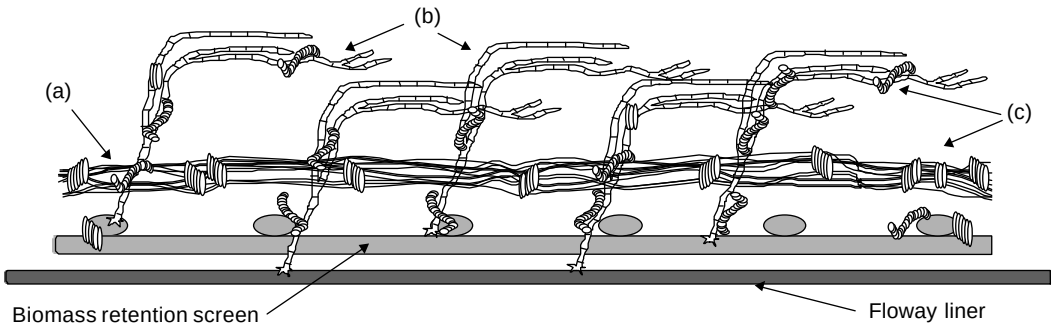

Figure 3. Schematic drawing of algal turf species growing on an ATS screen: (**a**) cyanobacterial mat, (**b**) green filamentous algae, (**c**) epiphytic diatoms.

Algal Standing Crop

Self-shading by accumulated turf biomass is one major factor limiting the growth rate and productivity of the turf algae. This can be prevented by frequent harvest, although a sufficient standing crop of algal biomass is required to maintain regrowth of the turf and effective treatment immediately following harvest. Several operational parameters influence the standing crop of ATS systems, and these are harvest technique and interval, and growth surface texture.

Harvest Technique. Harvesting the floway has several roles. It physically removes all the pollutants accumulated by the algal turf from the system before they naturally slough off. It simulates heavy grazing of the plant community, which has been shown to stimulate algal growth and nutrient removal (4,17,18). Harvesting also controls the population densities of most invertebrate grazers so that pollutants remain entrapped in the turf community and are not reintroduced into the water. Harvest is achieved by initially stopping the flow of wastewater and allowing the water to drain from the floway. Several harvest methods have been successfully employed, including both manual scraping or vacuum suction of the screen surface on the floway as well as removal and scrapping of a screen that is rolled up off the floway. Vacuum harvest, which was found to reduce the population density of filamentous algae, is not as suitable as harvest by scraping (12).

Harvest Interval. Because the growth rate of algal turf varies with season, treatment performance can be improved by varying the harvest interval over the year to maintain sufficient biomass on the ATS floway. A harvest interval of less than one week may be appropriate when the highest productivity is achieved during summer, whereas intervals of up to a month may be required during winter when productivity is at its lowest.

Growth Surface Texture. The texture of the floway liner seems to be of particular importance for the maintenance of algal species diversity and the promotion of turf productivity. Particularly, the addition of a biomass retention screen to the floway surface improves treatment performance by increasing the surface area available for turf colonization and retaining a larger standing crop of filamentous algae beneath the screen following harvest (Plate 3). Biomass retention screens were used in much of the original research conducted on ATS (3,4,9,13). The mesh becomes impregnated with algal holdfasts and rhizoids, which are not removed during harvest (4,19). Algal productivity has been found to vary with screen mesh size (optimum mesh size appears to be between 1 mm and 5 mm) and color (3). The lower turf productivity of black screens is possibly due to its greater absorbance of heat than white screens, which may inhibit algal growth.

Algal Turf Productivity

There is a gradual decline in the productivity of the algal turf down the length of the floway. Algal turf productivity (total solids dry weight) averaged for the whole floway can attain values of up to 61 $g\,m^{-2}\,d^{-1}$, although the annual mean productivity (24 $g\,m^{-2}\,d^{-1}$) is similar to that previously reported for periphyton water treatment systems (10,12,17). Sedimentation, filtration, and precipitation of particulates on the floway surface contribute to the accumulation of algal turf solids, such that nearly 50% of turf dry weight is nonvolatile and contains high concentrations of calcium (Ca) and magnesium (Mg).

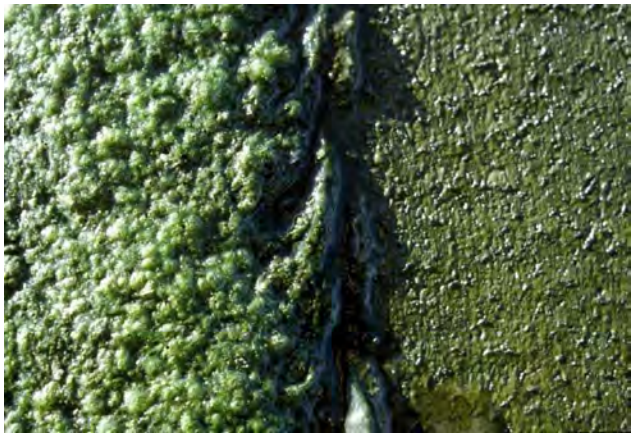

Plate 3. Unharvested (left) and harvested turf biomass (right) on an ATS floway without a biomass retention screen. See color insert.

WASTEWATER TREATMENT CAPABILITIES

Algal turf scrubber wastewater treatment systems have been demonstrated to improve water quality in many ways. They increase dissolved oxygen concentrations to between 100% and 300% saturation during the day and reduce alkalinity, conductivity, and hardness (11).

BOD, TSS, and turbidity of wastewater can be removed by ATS treatment. Removal is due to filtration of particulates by filamentous algae and bacterial oxidation. Particulate filtration is increased when the predominant turf algae are filamentous rather than the mat-forming cyanobacteria and diatoms (12). Bacterial oxidation of BOD varies diurnally, correlating with dissolved oxygen concentration. Mean annual concentrations in Patterson secondary treated wastewater, BOD (10 $g\,m^{-3}$), chemical oxygen demand (COD) (45 $g\,m^{-3}$), and total organic carbon(TOC) (10 $g\,m^{-3}$) were minimally affected and are probably close to background levels for ATS systems. Removal of BOD and COD by ATS systems was shown by Adey and coworkers (9) although the operational parameters of the recirculating mesocosm system used in that study are very different from those of a single pass wastewater treatment floway. The capability of ATS floways to significantly remove BOD remains to be evaluated.

Pathogen and Indicator Removal by ATS

Because of the shallow water depth and large surface area of ATS systems, wastewater is exposed to high amounts of

solar-ultraviolet (UV) radiation, which can result in a high removal rate of the fecal indicator bacteria *Escherichia coli* (11). However, because of the very short residence time (typically 20 to 40 minutes) of wastewater on the ATS floway, there is little (<1 log) overall reduction in fecal coliform bacteria concentrations by this system.

Nutrient Removal

ATS systems have been shown to reduce nutrient concentrations to low levels in agricultural runoff and marine systems. In coral reef mesocosms, nitrate can be maintained at levels between 7 and 14 $mg\,m^{-3}$ (4). This level is limited by the nitrogen fixation of cyanobacteria present in the system. Phosphorus can be reduced to levels below limits of detection.

ATS wastewater treatment systems are effective at removing nitrogen and phosphorus from secondary treated wastewater, with greater removal achieved at higher influent concentrations. Nutrient removal is a result of several microbiological and physical processes (Fig. 4) that are affected by a number of parameters, including hydraulic loading, the seasonal change in turf growth and species, and attainment of a pH greater than 9.0 in the floway effluent. Reduction of nutrient concentrations to $mg\,m^{-3}$ levels is limited in wastewater treatment systems as there is not enough available organic carbon to enable the nutrients to be assimilated into algal turf biomass. Addition of a carbon source to the wastewater on the floway when carbon is limiting may enhance nutrient removal by the system.

Nitrogen Removal

(see also ACTIVATED SLUDGE — MICROBIOLOGY OF NITROGEN REMOVAL.)

All forms of nitrogen can be removed by ATS wastewater treatment systems. Particulate nitrogen removal is related to the dominance of filamentous species on the floway. The simultaneous removal of both ammoniacal-nitrogen and nitrate is due to the mixed assemblage of algal species including both cyanobacteria that generally prefer nitrate and filamentous green algae that prefer ammonium as a nitrogen source. However, as nitrate is the principal form of inorganic nitrogen in secondary treated wastewater, algal species that prefer nitrate tend to dominate the turf. Annual mean inorganic nitrogen levels in the Patterson influent and effluent were 9.2 $g\,m^{-3}$ and 6.1 $g\,m^{-3}$, respectively. The longer Fruitland ATS system reduced inorganic nitrogen levels from 20 $g\,m^{-3}$ to 4 $g\,m^{-3}$ and could probably be enhanced if additional carbon was added.

Phosphorus Removal

(see also ACTIVATED SLUDGE — MICROBIOLOGY OF NITROGEN REMOVAL.)

Mean annual total phosphorus concentrations in the influent and effluent of Patterson ATS systems were 3.1 $g\,m^{-3}$ and 1.5 $g\,m^{-3}$, respectively. Periphyton can remove phosphorus from wastewater by a combination of filtration, adsorption, assimilation (including luxury uptake), and precipitation (20). Most of the phosphorus removal in ATS wastewater treatment systems is by assimilation into algal biomass or by precipitation of soluble reactive phosphorus (SRP), which is the main form of phosphorus in secondary treated effluents. Precipitation of SRP with cations such as Ca^{2+}, Mg^{2+}, and Al^{3+} is known to occur at pHs between 8.9 and 9.5, depending on the buffering capacity of the water (21). SRP removal therefore is dependent on the wastewater pH reached while the wastewater is on the floway. The increase in the pH of the ATS effluent is a result of carbon limitation of the turf algae and their subsequent use of bicarbonate as a carbon source for photosynthesis (22). At Patterson, a threshold pH of approximately 9.0 was required before SRP removal occurred to any great degree.

Reducing the hydraulic loading velocity of the ATS system from 1.4 to 0.11 $m\,d^{-1}$ increases the residence time of the wastewater on the floway and the pH attained in the floway effluent, hence SRP removal by precipitation is improved. SRP precipitation on the algal turf scrubber floway is indicated by the reduction of alkalinity, hardness, and conductivity that occurs as the pH of the effluent increases. Maintenance of the pH of the ATS system effluent above the level at which phosphate precipitates may provide an effective means of controlling SRP removal by ATS systems. The threshold pH may be easily achieved by controlling the length of time the wastewater is in contact with the algal turf. This may be done either by altering the hydraulic loading rate of the floway or by

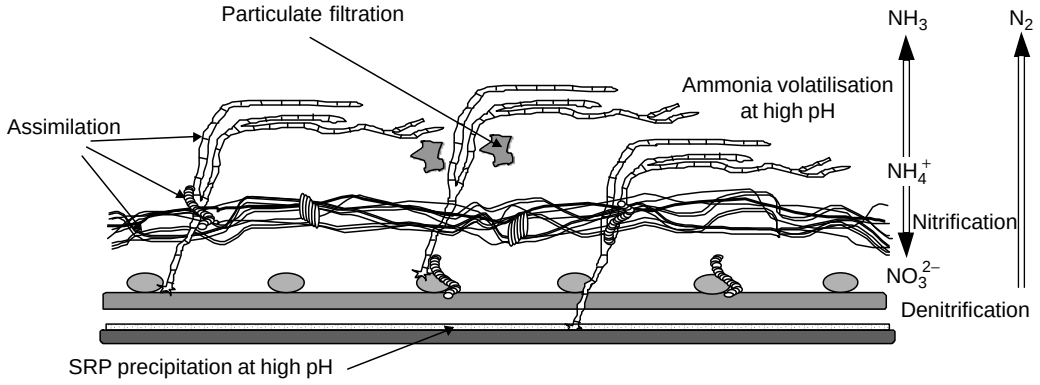

Figure 4. Schematic drawing of nutrient removal processes that occur on an ATS.

passing the wastewater down different lengths of the floway. However, other factors need to be considered in determining the residence time of the floway, such as the temperature attained in the ATS effluent, which may restrict algal growth (12).

Nighttime decline of the ATS system effluent pH to below the threshold pH results in the dissolution of some of this precipitate and the release of SRP back to the ATS effluent. Discharge of this released SRP can be prevented either by recirculating the ATS effluent at night or by greatly reducing the flow at night such that little or no water is discharged until the effluent pH has risen above the threshold again.

ATS systems could be designed to enhance phosphorus removal from wastewater by a two-stage process (11). The first floways would be only operated during the day and have low hydraulic loading to promote pH-mediated precipitation with cations. This would be followed by the second floways with high hydraulic loading and perhaps recirculation to promote rapid algal growth and assimilation of phosphorus into the algal biomass. The efficiency of treatment under both of these modes of operation is affected by the amount of biomass on the floway (11). Frequent harvesting would maintain an exponential growth of the biomass in the second floways, whereas a longer harvest interval would maintain a large standing crop to raise pH in the first floways.

Algal Turf Nutrient Content

The mean annual percentages of nitrogen and phosphorus in the accumulated solids of the ATS floway at Patterson were 3.96% and 1.83%, respectively (11). The values for nitrogen are typical of algal biomass. However, those for phosphorus are higher than that (<1%) normally associated with periphyton biomass (17,23–25). Adey and coworkers (10) found a phosphorus content of 0.4% in periphyton grown on agricultural runoff. The high phosphorus content of the algal turf of ATS wastewater treatment systems further indicates the role of pH-mediated SRP precipitation in phosphorus removal.

On the basis of percentages of nitrogen and phosphorus in the accumulated solids and the mean annual turf solids accumulation rate ($24\ g\,m^{-2}\,d^{-1}$), the mean removal of nitrogen was $0.95 \pm 0.48\ g\,m^{-2}\,d^{-1}$ and the mean removal of phosphorus was $0.44 \pm 0.22\ g\,m^{-2}\,d^{-1}$. The pilot ATS system treating agricultural runoff achieved phosphorus removal rates of $0.12\ g\,m^{-2}\,d^{-1}$ (10). Mass balance calculations of the wastewater treatment system show that more nitrogen is removed than is accumulated in the algal turf and indicate that denitrification does occur, perhaps in anoxic micro environments of the algal turf.

Algal Turf Biomass

The solid content of harvested turf biomass usually does not exceed 5%. However, the fibrous material may simply be dried using drying racks or sand beds and is otherwise suitable for dewatering by filter press or drum and spray dryers. Finding a use for the turf biomass remains unresolved, although there are several potential applications (11):

Fertilizer: Dried or composted algal turf biomass is very suitable for use as a high-quality soil amendment and slow-release fertilizer.

Energy: The turf biomass could also be used to produce energy through fermentation to alcohol or methane gas.

Feed: Turf algae have a high protein content and thus could be used as a feed additive for ruminants, poultry, and fish.

CONCLUSION

Algal turf scrubbers are capable of removing nitrogen and phosphorus from secondary wastewater without added chemicals and with minimum energy expenditure. When covered with filamentous algae, ATS systems are capable of filtering significant amounts of suspended solids and the BOD, TSS, nitrogen, and phosphorus associated with these suspended solids. Inorganic nitrogen removal is due to assimilation and some denitrification, whereas soluble reactive phosphorus removal is a result of assimilation and precipitation. Maintenance of the pH of the ATS system effluent above the threshold at which precipitation occurs could provide a simple means of controlling phosphorus removal by ATS. Control of phosphorus removal by ATS systems may be achieved by altering the length of time the wastewater is in contact with the algal turf, either by reducing the hydraulic loading velocity of the floway or by passing the wastewater down a longer floway. Stopping overnight flow until the threshold pH is regained the following day also prevents resolution of precipitated phosphorus at night. A comparison of the hydraulic loading velocities used for the Patterson ATS wastewater treatment system with those for pond and wetland treatment systems (26–28) indicates that a hydraulic loading velocity of as low as $0.25\ m^3\,m^{-2}\,d^{-1}$ would remain economical (Table 1).

ATS treatment systems have good potential for the removal of nutrients and other contaminants from wastewaters. The simplicity of ATS treatment systems and the ease with which configuration and operational parameters such as hydraulic loading, floway length, and harvest period can be changed should enable process

Table 1. Comparison of the Typical Hydraulic Loading Rate of ATS Floway Wastewater Treatment Systems to Those of Other Wastewater Treatment Methods

Treatment Method	Hydraulic Loading Rate ($m^3\,m^{-2}\,d^{-1}$)
Activated sludge	10–50
Oxidation ditch	1.0–1.5
Algal turf scrubber	0.25–0.5
Stabilization pond	0.1–0.2
Rotating biological contactor	0.08–0.2
Rapid infiltration	0.02–0.3
Overland flow	0.02–0.2
Wetland	0.01–0.05
Irrigation	0.002–0.02

control and optimization of the system for treatment of different wastewaters. Appropriate use of ATS technology may be for the remediation of eutrophic reservoirs and lakes, for polishing secondary effluents, and for algal biomass production.

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ALKALINE ENZYMES. See ALKALIPHILES: ALKALINE ENZYMES AND THEIR APPLICATIONS

ALKALIPHILES. See EXTREMOPHILES: LIFE IN EXTREME ENVIRONMENTS

ALKALIPHILES: ALKALINE ENZYMES AND THEIR APPLICATIONS

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There are no precise definitions of what characterizes an alkaliphilic or an alkali-tolerant organism. Several microorganisms exhibit more than one pH optimum for growth, depending on the growth conditions, which are influenced by nutrients, metal ions, and temperature. Therefore, in this article, the term *alkaliphile* is used for microorganisms that grow optimally or very well at pH values above 9, often between 10 and 12, but cannot grow or grow only slowly at the near-neutral pH value of 6.5.

The discovery of alkaliphiles occurred fairly recently. Only 16 scientific papers on alkaliphiles could be found when the author started experiments on alkaliphilic bacteria in 1968 (1). Since then, the author and his colleagues have isolated a great number of alkaliphilic microorganisms using a variety of different media. Alkaliphiles are a diverse group, including representatives from the three major domains of life; many of these have been proposed as new taxa. They can be isolated from nominal environments, but viable counts of alkaliphiles are higher in alkaline environments. The particular adaptations of alkaliphiles to their environment are not completely understood, but it is thought that the cell surface plays a key role in maintaining the intracellular pH between 7 and 8.5.

The industrial use of alkaliphilic microorganisms has a long history in Japan. From ancient times, indigo

from indigo leaves has been reduced by the particular bacteria that grow under the highly alkaline conditions of the traditional process called "indigo fermentation." The most important factor in this process is the control of the pH value. Formerly, this could only be accomplished by the skill of the craftsmen. It was not until we rediscovered alkaliphiles that the process could be studied and understood from a microbiological point of view. Even then, alkaliphiles remained little more than interesting biological curiosities, and no further industrial application was attempted or even contemplated. The situation has changed since then. The first paper concerning an alkaline protease was published in 1971 (2). Now, biological detergents contain enzymes, notably proteases and cellulases derived from alkaline bacteria; 60% of the total world production of enzymes is destined for the laundry detergent market. The industrial production of cyclodextrin using cyclomaltodextrin glucanotransferase is another important application of alkaliphile-derived enzymes. This enzyme reduced the cost of production and paved the way for the use of cyclodextrin in large quantities in foodstuffs, chemicals, and pharmaceuticals. It has also been reported that alkali-treated woodpulp could be biologically bleached by xylanases produced by alkaliphiles. Other applications are also contemplated.

GENETIC MAPS OF CHROMOSOMAL DNAS OF ALKALIPHILIC *BACILLUS* STRAINS

How alkaliphiles have adapted to their alkaline environment is one of the most interesting and challenging topics facing microbiologists. In addition, the genes of alkaliphiles are potentially a valuable source of information waiting to be explored and exploited by biotechnologists. Physical maps of the chromosomes of two alkaliphilic *Bacillus* strains, *Bacillus pseudofirmus* OF4 (3) and *Bacillus halodurans* C-125 (4), have been published; the complete sequence of the genome of *B. halodurans* C-125 has also been determined (5). The physical map of the chromosome in *B. pseudofirmus* OF4 is consistent with a circular chromosome of approximately 4 Mb, with an extrachromosomal element of 110 kb (3,6). Although the analysis is still in progress, several open reading frames for Na^+/H^+ antiporters that may be involved with pH homeostasis have been detected. The physical map of the chromosome in *B. halodurans* C-125 is consistent with a circular chromosome of approximately 4.20 Mb. As can be seen in Figure 1, many open reading frames show significant similarities to those of other microorganisms, such as a fragment of an intracellular serine protease from *Thermoactinomyces* sp. HS682 or a G6-amylase from the

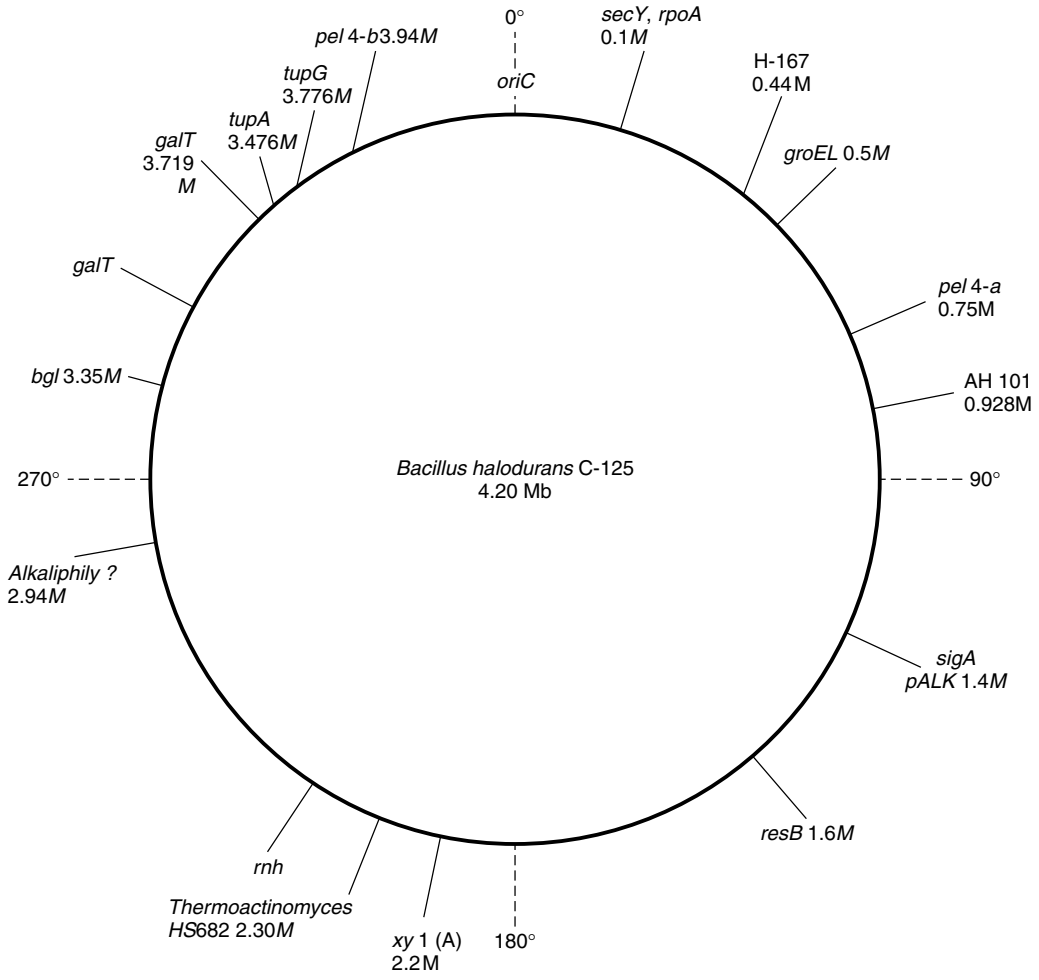

Figure 1. Genetic map of the chromosome of *B. halodurans* c-125.

alkaliphilic *Bacillus clausii* H-167. These DNA fragments do not code for functional enzymes because they are truncated, but they do suggest that there must be some degree of horizontal gene transfer between *B. halodurans* and other microorganisms (5,7–11).

MECHANISMS OF CYTOPLASMIC pH REGULATION

The cells of *B. halodurans* C-125 have two barriers to reduce pH values from 10.5 to 8.

The plasma membrane must be kept below pH 9 because it is very unstable at alkaline pH values much below the pH optimum for growth (12). It has been suggested that the cell wall may play a role in protecting the cell from alkaline environments. In addition to peptidoglycan, alkaliphilic *B. halodurans* C-125 contains certain acidic polymers, such as teichuronopeptide, composed of polyglucuronic acid and a polypeptide of acidic amino acids. The negative charges on the acidic non-peptidoglycan components may act as a barrier to sodium and hydronium ions and repulse hydroxide ions, and consequently, may assist cells to grow in alkaline environments (13,14). Aono and coworkers reported that teichuronopeptide (TUP) is one of the major structural components of the cell wall of *B. halodurans* C-125. A mutant defective in TUP synthesis grows slowly at alkaline pH. An upper limit of pH for growth of the mutant was 9.4, while that of the parental strain C-125 was 11.0. A gene *tupA*, directing synthesis of TUP, was cloned from the parental C-125 chromosomal DNA. Introduction of the *tupA* gene into the TUP-defective mutant complemented the mutation responsible for the pleiotropic phenotypes of the mutant, leading to simultaneous disappearance of the defect in TUP synthesis, the diminished ability for cytoplasmic pH homeostasis, and the low tolerance for alkaline conditions. These results demonstrate that the acidic polymer TUP in the cell wall plays a role in pH homeostasis in this alkaliphile (15).

Plasma membranes may also maintain pH homeostasis by using Na^+/H^+ antiporter system ($\Delta\Psi$-dependent and ΔpH-dependent), K^+/H^+ antiporter and ATPase-driven H^+ expulsion. Recent works in several laboratories on the critical antiporters have begun to clarify the number and characteristics of the porters that support active mechanisms of pH homeostasis (see AEROBIC ALKALIPHILES).

The author's group isolated a non-alkaliphilic mutant strain from *B. halodurans* C-125 as the host for cloning genes related to alkaliphily. A 3.7-kb parental DNA fragment (pALK fragment) from the parental strain restored the growth of an alkaline-sensitive mutant 38,154 at alkaline pH. The transformant was able to maintain an intracellular pH that was lower than the external pH and contained an electrogenic Na^+/H^+ antiporter driven only by $\Delta\Psi$ membrane potential, interior negative. Membrane vesicles prepared from the mutant 38,154 did not show membrane potential $\Delta\Psi$-driven Na^+/H^+antiporter activity. These results indicate that the mutant 38,154 affects, either directly or indirectly, electrogenic Na^+/H^+ antiporter activity. This was the first report of a DNA fragment responsible for a Na^+/H^+ antiporter system in the mechanism of alkaliphily (16–22).

Independently, Aono and coworkers isolated an alkali-hypersensitive mutant AS-350 from *B. halodurans* C-125 as a host for protoplast transformation. A gene that restored the alkaliphilic growth of the mutant was cloned from the parent strain (23). The mutation in the AS-350 was complemented with a 1.0-kb fragment expressed as "Alkaliphily" in Figure 1. However, role of this fragment is not yet clear.

Krulwich and her coworkers have focused their studies on the *B. pseudofirmus* OF4, which is routinely grown on malate-containing medium either at pH 7.5 or at pH 10.5. Current work is directed toward clarification of the characteristics and energetics of membrane-associated proteins that must catalyze inward proton movements (see the previous section by M. Ito).

ALKALINE ENZYMES

Studies of alkaliphiles have led to the discovery of many types of enzymes that exhibit interesting properties. The first report concerning an alkaline enzyme published in 1971 described an alkaline protease produced by *Bacillus* sp. 221 (2). More than 100 new enzymes have been isolated and purified in many laboratories (24). Some of these are summarized in Table 1.

Alkaline Proteases

In 1971, Horikoshi (2) reported the production of an extracellular alkaline serine protease from alkaliphilic *B. clausii* No. 221. This strain, isolated from soil, produced large amounts of alkaline protease that differed from the subtilisin group. The optimum pH of the purified enzyme was 11.5 with 75% of the activity maintained at pH 13.0. The enzyme was completely inhibited by diisopropylfluorophosphate or 6 M urea but not by ethylenediamine tetraacetic acid or *p*-chloromercuribenzoate. The addition of a 5 mM solution of calcium ions was reflected in a 70% increase in activity at the optimum temperature (60 °C). The gene encoding this enzyme was cloned in *E. coli* and expressed in *Bacillus subtilis*. An open reading frame of 1,140 bases, identified as the protease gene was preceded by a putative SD sequence (AGGAGG) with a spacing of 7 bases. The deduced amino acid sequence had a peptide of 111 residues followed by the mature protease comprising 269 residues (25). Subsequently, two *Bacillus* species, AB42 and PB12, which also produced an alkaline protease were reported (61). These strains exhibited a broad pH range of pH 9.0 to 12.0, with a temperature optimum of 60 °C for AB42 and 50 °C for PB12. Since these reports, many alkaline proteases have been isolated from alkaliphilic microorganisms (62–66). Fujiwara and coworkers (67) purified a thermostable alkaline protease from a thermophilic alkaliphilic *Bacillus* sp. B18. The optimum pH and temperature for the hydrolysis of casein were pH 12 to 13 and 85 °C, both of which are higher than those of alkaline proteases. Han and Damodaran (68) reported the purification and characterization of an extracellular endopeptidase from a strain of *B. pumilus* displaying high stability in 10% (w/v) sodium dodecyl sulfate and 8 M urea. Following these studies, many alkaline proteases for

Table 1. Major Extracellular Alkaline Enzymes Produced by Alkaliphiles

Optimum pH*	Stable pH	Applications	References
Alkaline Proteases			
B. clausii No. 221	11.5–12.0	4–11	Detergent additives (2,25)
B. halodurans AH-101	12–13		5–13 (26)
Bacillus sp. D-6	10.0–11.0		4–12 (27)
Alkaline Amylase			
Bacillus sp. A-40-2	10.5	6–9	α-amylase type (28)
B. clausii H-167	10–11	6–11	G6 forming** (10,29–31)
Bacillus sp. 38-2	4.5–9	6–9	CD forming*** (32–35)
Alkaline Pectinase			
Bacillus sp. P-4-N	10.0	5–9	(36,37)
Alkaline Pullulanase			
B. halodurans No. 202-1	9.0	6–10	(38)
Micrococcus sp.	207	7.0–8.0	Food processing (39,40)
Alkaline Cellulase			
Bacillus sp. N-4	6–11	5–11	Detergent additives (41,42)
Bacillus sp. No. 1,139	9	5–11	(42–47)
Alkaline Lipase			
Pseudomonas sp.	9.5		Detergent additives (48)
Bacillus sp.	9.5	9.5	(49)
Xylanses			
B. halodurans C-59-2	5.5–9	5–9	Biobleaching (50)
B. halodurans C-125	6–10	4–12	(51)
Bacillus sp. TAR-1	5.0–9.5	9	(52)
Alkaline Chitinase			
Bacillus sp. BG-11	7.5–9.0	6.0–9.0	(53–55)
Alkaline Mannanase			
Bacillus sp. AM001	9.0–9.5	8–9	Food processing (56–58)
β-1,3-gucanase			
Bacillus sp. K-12-5	6–9	6–8	(60)
Bacillus sp. AG-430	9.0–10.0	4–10	

*Optimum pH for enzyme action.
**Maltohexaose forming.
***Cyclodextrin forming.

laundry detergent additives were isolated from various alkaliphilic *Bacillus* strains, although their properties are almost the same as those reported previously (69–71).

Some of the enzymes are now commercially available as detergent additives.

Takami and coworkers (26) isolated a new alkaline protease from alkaliphilic *B. halodurans* No. AH-101. The enzyme was most active toward casein at pH 12 to 13 and stable under 10-minute incubation at 60 °C and pH 5 to 13. The optimum temperature was about 80 °C in the presence of 5 mM calcium ion. The alkaline protease showed a higher hydrolyzing activity against insoluble fibrous natural proteins such as elastin and keratin in comparison with subtilisins and proteinase K (72–76). Cheng and coworkers (77) reported a keratinase of a feather-degrading *Bacillus licheniformis* PWD-1. This enzyme was stable from pH 5 to 12. The optimum reaction pHs for feather powder and casein were 8.5 and 10.5 to 11.5, respectively. Zaghloul and coworkers also reported isolation, identification, and keratinolytic activity of several feather-degrading bacteria isolated from an Egyptian soil. These isolates could degrade chicken feathers (78).

Other Proteases

Several alkaline proteases produced by alkaliphilic actinomycetes have been reported. Tsuchiya and coworkers (11,79,80) isolated thermostable alkaline protease from alkaliphilic *Thermoactinomyces* sp. HS682. The strain grew in alkaline media between pH 7.5 to 11.5. Maltose gave the highest productivity of protease at a concentration of 10 g/L. The protease had the maximum proteolytic activity around pH 11.0 and at 70 °C. In the

presence of Ca^{2+} ions, maximum activity was observed at 80 °C. An intracellular alkaline serine protease gene of alkaliphilic *Thermoactinomyces* sp. HS682 was cloned and expressed in *Escherichia coli*. Yum and coworkers (81) purified an extracellular alkaline serine protease produced by *Streptomyces* sp. YSA-130. The optimum temperature and pH for the enzyme activity were 60 °C and 11.5, respectively. The enzyme was stable at 50 °C, and between pH 4 and 12. A keratin-degrading serine protease of *Streptomyces pactum* DSM 40,530 was purified by casein agarose affinity chromatography by Bockle and coworkers (82). The proteinase was optimally active in the pH range from 7 to 10 and at temperatures from 40 to 75 °C. After incubation with the purified proteinase, a rapid disintegration of whole feathers was observed. Extracellular proteolytic activity was also detected in the haloalkaliphilic archaeon *Natronococcus occultus* as the culture reached the stationary growth phase (83). Proteolytic activity was precipitated with ethanol and subjected to a preliminary characterization. Optimum conditions for activity were attained at 60 °C and 1 to 2 M NaCl or KCl. Gelatin zymography in the presence of 4 M betaine revealed a complex pattern of active species with apparent molecular masses ranging from 50 to 120 kDa. Subsequently, several haloalkaliphilic proteases have been isolated from haloalkaliphilic archaea. These enzymes were dependent on high NaCl concentrations in order to express protease activity and stability (84,85).

Industrial Applications of Alkaline Proteases

1. Detergent Additives.

   The main industrial application of alkaliphilic enzymes is in the detergent industry, and detergent enzymes account for approximately 30% of the total worldwide enzyme production. Not all of these are produced by alkaliphilic bacteria. However, almost all alkaline proteases have been produced by alkaliphilic *Bacillus* strains and are commercially available.
2. Dehairing.

   Alkaline enzymes have been used in the hide-dehairing process, in which dehairing is carried out at pH values between 8 and 10. These enzymes are commercially available from several companies.
3. Others.

   An interesting application of alkaline protease was developed by Fujiwara and coworkers (66,67,86,87). They reported that an alkaline protease was used to decompose the gelatinous coating of X-ray films, from which silver was recovered. Protease B18′ had a higher optimum pH and temperature, around 13.0 and 85 °C, respectively. The enzyme was most active toward gelatin on film at pH 10.

STARCH-DEGRADING ENZYMES

The first alkaline amylase was produced in Horikoshi-II medium by cultivating alkaliphilic *Bacillus* sp. No. A-40-2 (28). Several types of alkaline starch-degrading enzymes were observed. No alkaline amylases produced by neutrophilic microorganisms have so far been reported. Recent studies revealed that the starch-degrading enzymes α-amylase and cyclodextrin glycosyltransferase (CGTase) are functionally and structurally closely related.

α-Amylases of Alkaliphilic Bacillus Strains

The production of the alkaline amylase was first achieved in an alkaliphilic *Bacillus* species strain No. A-40-2 (ATCC21592) that was selected from about 300 colonies of bacteria grown in Horikoshi-II medium (28). The enzyme is most active at pH 10.0 to 10.5 and retains 50% of its activity between pH 9.0 and 11.5. The enzyme is not inhibited by 10 mM EDTA at 30 °C and is completely inactivated by 8 M urea. The enzyme can hydrolyze 70% of starch to yield glucose, maltose, and maltotriose and is a type of saccharifying α-amylase. Boyer and Ingle (88,89) reported an alkaline amylase in the strain NRRL B-3881, which was the second report of an alkaline amylase. The B-3881 amylase had its optimum pH at 9.2. The enzyme yields maltose, maltotriose, and a small amount of glucose and maltotetraose, all of which have a β-configuration. Considerable diversity of α-amylases has been reported. Kim and coworkers (90) reported that an alkaliphilic *Bacillus* sp. Strain GM8901 produced five alkaline amylases in a culture broth. McTigue and coworkers studied the alkaline amylases of three alkaliphilic *Bacillus* strains (91,92). *Bacillus halodurans* A-59 (ATCC 21591), *Bacillus* sp. NCIB 11203, and *Bacillus* sp. IMD370 produce alkaline α-amylases with maxima for activity at pH 10.0. One of the strains isolated, *Bacillus* sp. No. H-167, produced α-amylases that yielded maltohexaose and the main product from starch. The nucleotide sequence of the G6-amylase gene from alkaliphilic *Bacillus* sp. H-167 was determined. The open reading frame of the gene consisted of 2,865 bp encoding 955 amino acids. The DNA sequence and the deduced amino acid sequence of the G6-amylase gene showed no homology with those of other bacterial α-amylases, although the consensus amino acid sequences of the active center were well conserved (10,29–31). It is of interest that this nucleotide sequence, which is fragmented, was well conserved in the DNA of *B. halodurans* C-125, although no enzymatic activity was expressed.

Kelly and coworkers (93) found that the alkaline amylase of *Bacillus* sp. IMD 370 could hydrolyze raw starch. The enzyme digested raw cornstarch to glucose, maltose, maltotriose, and maltotetraose. The maximum pH for raw starch hydrolysis was pH 8.0 compared with pH 10.0 for soluble starch hydrolysis. It is of interest that degradation of raw starch was stimulated sixfold in the presence of β-cyclodextrin. Lin and coworkers (94) have recently reported the production and properties of a raw-starch-degrading amylase from the thermophilic and alkaliphilic *Bacillus* sp. TS-23. Activity staining revealed that two amylases with molecular masses of 150 and 42 kDa were produced. The 42-kDa amylase only hydrolyzed raw starch. Igarashi and coworkers isolated a novel liquefying α-amylase (LAMY) from cultures of an alkaliphilic *Bacillus* isolate, KSM-1378 (95). The enzyme had a pH optimum of 8.0 to 8.5 and displayed maximum

activity at 55 °C. The structural gene for the amylase contained a single open reading frame 1,548 bp in length, corresponding to 516 amino acids that included a signal peptide of 31 amino acids. The four conserved regions were found in the deduced amino acid sequence. Essentially, the sequence of LAMY was consistent with the tertiary structures of reported amylolytic enzymes, which are composed of domains A, B, and C. Furthermore, Igarashi and coworkers (96) improved the thermostability of the amylase by deleting an arginine-glycine residue in that molecule. Then they reported that thermostability of the enzyme was also improved by proline substitution (Arg124 to Pro124) (97).

Another modification to improve stabilization was reported by Villalonga, and coworkers (98). Carboxymethylcellulose activated by periodate oxidation was covalently linked to α-amylase. The thermostability and pH stability were improved for α-amylase by this modification. The conjugate was also more resistant to the action of denaturant agents such as urea and sodium dodecylsulfate. They concluded that modification of enzymes by the anionic polysaccharide carboxymethylcellulose might be a useful method for improving enzyme stability under various denaturing conditions.

Other α-Amylases

Kimura and Horikoshi (39,40,99–102) isolated a number of starch-degrading alkaliphilic psychrophilic microorganisms from the environments. Recently, a gene coding for a new amylolytic enzyme from *Pseudomonas* sp. KFCC 10818 was cloned, and its nucleotide sequence was determined (103). A deduced amino acid sequence contained four highly conserved regions of α-amylases. A haloalkaliphilic *Natronococcus* sp. strain Ah-36 produced an extracellular maltotriose-forming amylase (104). The amylase exhibited maximal activity at pH 8.7 and 55 °C in the presence of 2.5 M NaCl. Kobayashi and coworkers (104) have cloned this α-amylase and expressed it in *Haloferax volcanii*.

Cyclomaltodextrin Glucanotransferases (CGTase)

Nakamura and Horikoshi discovered several alkaliphilic *Bacillus* strains producing CGTases. A crude enzyme of *Bacillus* sp. No. 38-2 was a mixture of three enzymes: acid CGTase having optimum pH for enzyme action at 4.6, neutral CGTase at 7.0, and alkaline CGTase at 8.5 (32–35). In 1975, Matsuzawa and coworkers established the industrial production of cyclodextrin by using the crude CGTase of *Bacillus* sp. 38-2 (105). Since then, several alkaliphilic microorganisms producing CGTases have been reported (35,105). Georganta and coworkers (106) isolated CGTase producing alkaliphilic psychrophilic bacteria from samples of deep-sea bottom mud. The isolate No. 3-22 grew at 4 °C and showed activity in both broad temperature and pH 5 to 9 ranges. The CGTase produced predominantly β-cyclodextrin (β-CD), with minor amounts of α- and γ-CDs. The formation of various cyclodextrins was observed after accumulation of maltooligosaccharides with degrees of polymerization more than seven (107). Salva and coworkers isolated sixty-eight CGTases from alkaliphilic bacteria from among 400 soil bacteria (108). An enzyme of isolate No. 76 was partially purified by starch adsorption and some of its properties were investigated. The enzyme properties seem to be quite similar to those of *Bacillus* sp. 38-2 enzyme (33). Chung and coworkers isolated a thermostable CGTase from alkaliphilic *Bacillus stearothermophilus* ET1 (109). The optimum pH for the enzyme-catalyzed reaction was pH 6.0, and the optimum temperature was observed at 80 °C. A 13% (w/v) cornstarch solution was liquefied and converted to CDs solely using this enzyme. The cornstarch conversion was 44%, and α-, β-, and γ-CDs were produced in the ratio of 4.2 : 5.9 : 1. Terada and coworkers studied the initial reaction of CGTase from an alkaliphilic *Bacillus* sp. A2-5a on amylose (110). Cyclic α-1,4-glucans with a degree of polymerization ranging from 9 to more than 60, in addition to α-, β-, and γ-cyclodextrin, were detected at an early stage of enzymatic reactions. Subsequently, large cyclic α-1,4 glucans were converted into smaller cyclic α-1,4-glucans. The final major product was β-CD. From the industrial point of view, γ-CD is of practical interest because it is rare and can encapsulate larger compound molecules. Recently, Parsiegla and coworkers reported that mutation of *Bacillus circulans* Strain No. 8 was effective in increasing the γ-cyclodextrin production (111).

Yamane's group has extensively investigated molecular structure of the CGTase of alkaliphilic *Bacillus* sp. 1,011. Kimura and coworkers isolated the enzyme, purified, and cloned its gene (112,113). The enzyme, consisting of 686 amino acid residues, was crystallized and subjected to X-ray analysis. The molecule consists of five domains, designated A to E, and its backbone structure was similar to the structure of other bacterial CGTases. The molecule had two calcium binding sites where calcium ions were coordinated by seven ligands, forming a distorted pentagonal bipyramid. Three histidine residues in the active center of CGTase participate in the stabilization of the transition state. His-327 is especially important for catalysis over the alkaline pH range (114). Three-dimensional structures of cyclodextrin glucanotransferases (CGTases) revealed four aromatic residues, which are highly conserved among CGTases but not in α-amylases, are located in the active center (115). Ishii and coworkers analyzed the crystal structure of asparagine-233-replaced enzyme. The enzyme that was a site-directed mutation of histidine-233 to asparagine changed the nature of the enzyme such that it no longer produced α-cyclodextrin. The neighborhood of asparagine-233, maintaining the architecture of the active site cleft, seems to be responsible for the change in molecular recognition of both substrate and product of the mutant CGTase (116,117).

Industrial Production of Cyclodextrins

In 1969, Corn Products International Co. in the United States began producing β-CD using *B. macerans* CGTase. Teijin Ltd. in Japan also produced β-CD using the *macerans* enzyme in a pilot plant. However, there were several serious problems in both the production processes: (*1*) The yield of CD from starch was not high, and (*2*) toxic organic solvents such as trichloroethylene, bromobenzene,

and toluene were used to precipitate CD because of the low conversion rate.

The use of CGTase of alkaliphilic *Bacillus* sp. No. 38-2 overcame all these weak points and led to the mass production of crystalline α-, β-, γ-CD at low cost without using any organic solvents. The yield of CD was 85 to 90% from amylose and 70 to 80% from potato starch on a laboratory scale. Owing to the high conversion rate, CDs could be directly crystallized from the hydrolyzate of starch without the addition of organic solvents (105). These simple methods reduced the cost of β-CD from 200,000 yen to 1,000 yen/kg, and that of α-CD to within 3,000 yen/kg. This has paved the way for its use in large quantities in foodstuffs, chemicals, and pharmaceuticals. Several other processes to produce CDs have been reported after Matsuzawa's original paper (105). Kim and coworkers reported an enzymatic production of cyclodextrins from milled cornstarch in an ultrafiltration membrane bioreactor to reduce product inhibition (118). As compared with an operation without ultrafiltration, the conversion yield was increased by 57% in a batch operation with ultrafiltration. Okada and coworkers also developed a bioreactor system with the enzyme immobilized on a capillary membrane (119). The percentage of CDs to total sugar obtained was slightly more than 60% under most operating conditions. Abraham (120) immobilized CGTase for the continuous production of cyclodextrins. The immobilized enzyme had a pH optimum shifted to the alkaline side (from 6.5 to 7.5) and had a reduced temperature optimum (from 60 °C to 50–55 °C). Lima and coworkers developed a unique cyclodextrin production process (121). A 10% (w/v) solution of cassava starch liquefied with α-amylase was incubated with CGTase using only the enzyme, added ethanol (from 1 to 5%) followed by the addition of yeast, *Saccharomyces cerevisiae* (12% w/v), plus nutrients. However, production costs were not reported.

Pullulanases

In 1975, Nakamura and coworkers discovered an alkaline pullulanase of *Bacillus* sp. No. 202-1 (38). The enzyme had an optimum pH for enzyme action at 8.5 to 9.0 and was stable for 24 hours at pH 6.5 to 11.0 at 4 °C. The enzyme was most active at 55 °C and was stable up to 50 °C for 15 minutes in the absence of a substrate. Kelly and coworkers (122) found that alkaliphilic *B. halodurans* No. A-59 (ATCC21591) produced three enzymes, namely, α-amylase, pullulanase, and α-glucosidase, in culture broth. These three enzymes were separately produced, and the levels of α-glucosidase and pullulanase reached maxima after 24-hour cultivation at the initial pH 9.7. Although this pullulanase was not purified, the indicated pH optimum was at 7.0.

Two highly alkaliphilic pullulanase-producing bacteria were isolated from Korean soils (123,124). The two isolates were extremely alkaliphilic since bacterial growth and enzyme production occurred at pH values ranging from pH 6.0 to 12.0 for *Micrococcus* sp. Y-1 and pH 6.0 to 10.0 for *Bacillus* sp. S-1. The enzyme displayed a temperature optimum of around 60 °C and a pH optimum of around pH 9.0. The extracellular enzymes of both bacteria were alkaliphilic and moderately thermoactive; optimum activity was detected at pH 8.0 to 10.0 and between 50 °C and 60 °C.

In screening alkaline cellulases for detergent additives, Ito's group isolated a novel alkaline pullulanase from alkaliphilic *Bacillus* sp. KSM-1876, which was identified as a relative of *B. circulans* (125,126). The enzyme had an optimum pH for enzyme action of around 10.0 to 10.5. This enzyme is a good candidate for use as an additive to dishwashing detergents. Furthermore, another alkaline amylopullulanase from alkaliphilic *Bacillus* sp KSM-1378 was also found. This enzyme efficiently hydrolyzed α-1,4 and the α-1,6 linkages of amylose, amylopectin, and glycogen at alkaline pH values. The kinetic studies revealed two independent active sites for the α-1,4 and α-1,6 hydrolytic reactions. Incubation of the enzyme at 40 °C and pH 9.0 caused complete inactivation of the amylase activity within 4 days, but the pullulanase activity remained at the original level under the same conditions (126). This alkaline amylopullulanase can therefore be considered to be a "two-headed" enzyme molecule. Limited proteolysis with papain also revealed that the α-1,6 and α-1,4 hydrolytic activities were associated with two different active sites (127). Furthermore, amino acid sequence and molecular structure analysis of the enzyme showed two different active sites (128). The enzyme was observed by transmission electron microscopy; it appeared to be a bent dumbbell-like molecule with a diameter of approximately 25 nm.

Takagi and coworkers reported diversity in size and alkaliphily of thermostable α-amylase pullulanases produced by recombinant *E. coli*, *B. subtilis*, and the wild-type *Bacillus* sp. XAL601 (129). It was revealed that the noncatalytic C-terminal region may be responsible for the high optimum pH of the enzyme activity. These observations were also reported in CGTase (130,131) and alkaline CMCase (132).

Lin and coworkers purified a thermostable pullulanase from thermophilic alkaliphilic *Bacillus* sp. strain TS-23. This purified enzyme had both pullulanase and amylase activities. The temperature and pH optima for both pullulanase and amylase activities were 70 °C and pH 8 to 9, respectively. The enzyme remained more than 96% active at temperatures below 65 °C, and both activities were retained at temperatures up to 90 °C in the presence of 5% SDS (133).

In some food industries, enzymes showing activity at lower temperatures have been requested for food processing. Psychrophilic bacteria are thought to be potential producers of these enzymes. Kimura and Horikoshi (39,40) reported that an alkalipsychrophilic strain, *Micrococcus* sp. 207, produced amylase and pullulanase extracellularly. The pullulanase of *Micrococcus* sp. 207 was purified to an electrophoretically homogeneous state by conventional ways. The purified enzyme was free of α-amylase activity. The enzyme had a pH optimum at 7.5 to 8.0 and was relatively thermostable (stable up to 45 °C). The enzyme could hydrolyze the α-1,6-linkages of amylopectins, glycogens, and pullulan. Although many alkaline pullulanases

have been reported as described earlier, no industrial application has been developed yet.

CELLULASES

Alkaline Cellulases of Alkaliphilic Bacillus Strains

Commercially available cellulases display optimum activity over a pH range from 4 to 6. No enzyme with an alkaline optimum pH for activity (pH 10 or higher) had been reported before the rediscovery of alkaliphiles. Horikoshi and coworkers found bacterial isolates (*Bacillus* sp. No. N4 and No. 1,139) producing extracellular alkaline carboxymethylcellulases (CMCases) (41,43). One of these alkaliphilic *Bacillus* sp. No. N-4 (ATCC21833) produced multiple CMCases that were active over a broad pH range (pH 5 to 10). Sashihara and coworkers cloned the cellulase genes of *Bacillus* sp. No. N-4 in *E. coli* HB101 (42). Another bacterium, *Bacillus* sp. No. 1,139, produced one CMCase, which was purified and shown to have optimum pH for activity at pH 9.0. The enzyme was stable over the range of pH 6 to 11 (24 hours at 4 °C and up to 40 °C for 10 minutes). The CMCase gene of *Bacillus* sp. No. 1,139 was also cloned in *E. coli* (43–47). Nakamura and coworkers (132) and Park and coworkers (134) constructed many chimeric cellulases from *B. subtilis* and *Bacillus* sp. N-4 enzyme genes in order to understand the alkaliphily of N-4 enzymes. Despite the genes having high homology, the pH activity profiles of the two enzymes are quite different; *B. subtilis* (BSC) has its optimum pH at 6 to 6.5, whereas *Bacillus* sp. N-4 enzyme (NK1) is active over a broad pH range from 6 to 10.5. The chimeric cellulases showed various chromatographic behaviors, reflecting the origins of their C-terminal regions. The pH activity profiles of the chimeric enzymes in the alkaline range could be classified into either the BSC or the NK1 type, mainly depending on the origins of the fifth C-terminal regions.

Alkaline Cellulases from Other Alkaliphiles

Park and coworkers and Damude and coworkers (135,136) studied a semi-alkaline cellulase produced by alkaliphilic *Streptomyces* strain KSM-9. Dasilva and coworkers (137) reported two alkaliphilic microorganisms, *Bacillus* sp. B38-2 and *Streptomyces* sp. S36-2. The optimum pH and temperature of the crude enzyme activities ranged from 6.0 to 7.0 at 55 °C for the *Streptomyces* and 7.0 to 8.0 at 60 °C for the *Bacillus* sp. B38-2. However, their results indicated that the properties of these enzymes were not sufficient for industrial purposes.

Cellulases as Laundry Detergent Additives

The discovery of alkaline cellulases created a new industrial application of cellulase as a laundry detergent additive. Ito (personal communication) mixed alkaline cellulases with laundry detergents and studied the washing effect on cotton underwear. The best results were obtained by one of the alkaline cellulases produced by an alkaliphilic *Bacillus* strain. However, the yield of enzyme was not sufficient for industrial purposes. Consequently, Yoshimatsu and coworkers (138) isolated an alkaliphilic *Bacillus* sp. No. KSM-635 from a soil and succeeded in producing an alkaline cellulase as a laundry detergent additive on an industrial scale.

Besides the KSM-635 enzyme, Shikata and coworkers isolated three strains, alkaliphilic *Bacillus* KSM-19, KSM-64, and KSM-520, producing alkaline cellulases for laundry detergents (139). Their activities (pH optima: 8.5 to 9.5) were not inhibited at all by metal ions or various components of laundry products, such as surfactants, chelating agents, and proteinases. With a view to increasing industrial production, Sumitomo and coworkers (140,141) overexpressed alkaline cellulase of alkaliphilic *Bacillus* sp. KSM-64 by using *B. subtilis* harboring their vector pHSP64. By this process, they produced 30 g of alkaline cellulase in one liter. After the discovery of the industrial application of alkaline cellulase as a detergent additive, many microbiologists have extensively studied alkaline cellulases (142–147). Further details are reviewed by Ito (148).

Alkaline Lipases

Although the initial motivation for studying alkaline lipases was its application to detergents, many alkaline lipases were significantly inhibited in the presence of either alkylbenzene sulfate or dodecyl benzene sulfonate.

Watanabe and coworkers (48) conducted an extensive screening for alkaline lipase-producing microorganisms from soil and water samples. Two bacterial strains were selected as potent producers of alkaline lipases. These were identified as *Pseudomonas nitroreducens* var. *thermotolerans* and *Ps. fragi*. The optimum pH of the two lipases was 9.5. Both enzymes were inhibited by bile salts such as sodium cholate, sodium deoxycholate, and sodium taurocholate at a concentration of 0.25 %.

A thermophilic lipase-producing bacterium was isolated from a hot spring area of Yellowstone National Park (49). The organism characterized as *Bacillus* sp. grew optimally at 60 to 65 °C and in the pH range of 6 to 9. The partially purified lipase preparation had an optimum temperature of 60 °C, at an optimum pH of 9.5. It retained 100% of the original activity after being heated at 75 °C for half an hour. The enzyme was active on triglycerides containing fatty acids with a carbon chain length of 16 to 22 as well as on natural fats and oils. Bhushan (149,150) found a lipase produced from an alkaliphilic *Candida* species in a solid-state fermentation. The lipase from this microorganism had temperature and pH optima of 40 °C and 8.5, respectively, and was stable at 45 °C for 4 hours. Enzyme activity was stimulated by Ni^{2+} and Ca^{2+} ions whereas Fe^{2+} and Fe^{3+} ions inhibited the activity.

Large-scale industrial application such as laundry detergent additives has not been reported yet.

XYLANASES

Xylanases of Alkaliphilic Bacillus Strains

The first paper describing a xylanase from alkaliphilic bacteria was published in 1973 by Horikoshi and Atsukawa (50). The purified enzyme of *Bacillus* sp. No. C-59-2 exhibited a broad optimum pH ranging from 6.0

to 8.0. In the culture broth of *B. halodurans* No. C-125, two xylanases were found (51). Xylanase A had molecular weight of 43,000 and xylanase N had a molecular weight of 16,000. Xylanase N was most active at pH 6 to 7 and xylanase A was most active at a pH range of 6 to 10 and had some activity at pH 12. The xylanase A gene was cloned, sequenced, and expressed in *E. coli* (151–155). Four thermophilic alkaliphilic *Bacillus* strains (W1 (JCM2888), W2 (JCM2889), W3 and W4) produced xylanases (156,157). The pH optima for enzyme action of strains W1 and W3 was 6.0 and that for strains W2 and W4 was between 6 to 7. The enzymes were stable between pH 4.5 and 10.5 at 45 °C for 1 hour. The optimum temperatures of xylanases of W1 and W3 were 65 °C and those of W2 and W4 were 70 °C. The degree of hydrolysis of xylan was about 70 % after 24-hour incubation.

After the demonstration that alkali-treated woodpulp could be biologically bleached by xylanases instead of the usual environmentally damaging chemical process using chlorine, the search for thermostable alkaline xylanases has been extensive. Dey (158) isolated an alkaliphilic thermophilic *Bacillus* sp. (NCIM 59) that produced two types of cellulase-free xylanase at pH 10 and 50 °C. Khasin and coworkers reported that alkaliphilic *B. stearothermophilus* T-6 produced an extracellular xylanase that was shown to optimally bleach pulp at pH 9 and 65 °C (159). Nakamura and coworkers also reported that an alkaliphilic *Bacillus* sp. strain, 41M-1, isolated from soil produced multiple xylanases extracellularly (160–162). One of the enzymes, xylanase J, was most active at pH 9.0. The optimum temperature for the activity at pH 9.0 was around 50 °C. Then, another alkaliphilic thermophilic *Bacillus* sp. strain TAR-1 was isolated from soil (52). The xylanase of *Bacillus* sp. strain TAR-1 was most active over a pH range of 5.0 to 9.5 at 50 °C. Optimum temperatures of the crude xylanase preparation were 75 °C at pH 7.0 and 70 °C at pH 9.0. Many thermostable alkaline xylanases have been also produced from various alkaliphiles isolated from geothermal areas (163–165).

Recently, biobleaching by xylanases of *Streptomyces thermoviolaceus* and *Staphylococcus* sp. SG-13 have been reported besides *Bacillus* sp. Xylanase (166–170). These xylanases did not act on cellulose, indicating a possible application of the enzyme in biological debleaching processes.

PECTINASES

The first study on alkaline *endo*-polygalacturonate lyase produced by alkaliphilic *Bacillus* sp. No. P-4-N was published in 1972 (36). The optimum pH for enzyme action was 10.0 for pectic acid. Recently, the gene for this alkaline pectate lyase, Pel-4A of *Bacillus* sp. No. P-4-N was cloned, sequenced, and overexpressed in *B. subtilis* cells (37). The deduced amino acid sequence of the mature enzyme (318 amino acids, 34,805 Da) showed moderate homology to those of known pectate lyase in the polysaccharide lyase family 1. The purified recombinant enzyme had an isoelectric point of pH 9.7 and a molecular mass of 34 kDa and exhibited very high specific activity compared with known pectate lyases reported so far. The enzyme activity and stability were stimulated by the addition of NaCl at an optimum of 100 mM. Fogarty and Kelly (171,172) then reported that *Bacillus* sp. No. RK9 produced *endo*-polygalacturonate lyase. The optimum pH for the enzyme activity toward acid-soluble pectic acid was 10.0. Ito and his colleagues isolated two excellent pectate lyase producers, *Bacillus* sp. strains KSM-P7, KSM-P15 (173–175). This high-alkaline pectate lyase, Pel-7 from strain KSM-P7 was purified to homogeneity and its molecular weight was about 33,000. The isoelectric point was close to or higher than pH 10.5. It exhibited optimum activity at pH 10.5 and around 60 to 65 °C in glycine-NaOH buffer. The gene was cloned and sequenced, and the deduced amino acid sequence of mature Pel-7 (302 amino acids) was obtained. The Pel-7 is basically grouped into the Pel super family, although the enzymatic and molecular properties are different. Another high molecular weight enzyme, Pel-15W from strain KSM-P15, had a molecular weight of 70,000, the pI was around pH 4.6. Pel-15 HHrandomly trans-eliminated polygalacturonate in the presence of $Ca2^{+}$ ions, and the maximum activity was observed at pH 11.5 and at 55 °C in glycine-NaOH buffer. The gene for Pel-15 HHwas cloned and sequenced, and the structural gene contained a 2,031-bp open reading frame that encoded 677 amino acids including a possible 28-amino acid signal sequence.

Several papers on potential applications of alkaline pectinase have been published. The first application of alkaline pectinase-producing bacteria in the retting of Mitsumata bast (*Edgeworthia papyrifera*, plant for Japanese paper making) was reported by Yoshihara and Kobayashi (176). The pectic lyase (pH optimum 9.5) produced by an alkaliphilic *Bacillus* sp. No. GIR 277 has been used in improving the production of a type of Japanese paper. A new retting process produced a high-quality, nonwoody paper that was stronger than the paper produced by the conventional method. Tanabe and coworkers (177,178) tried to develop a new waste treatment by using an alkaliphilic *Bacillus* sp. No. GIR 621-7. Cao and coworkers (179) isolated four alkaliphilic bacteria, NT-2, NT-6, NT-33, and NT-82, producing polygalacturonase and xylanase. They selected high-producers of polygalacturonase from mutants resistant to rifampin in alkaliphilic *Bacillus* sp NT-33. One strain, NT-33, had an excellent capacity for degumming ramie fibers.

CHITINASES

Tsujibo and coworkers (53) isolated chitinases from an alkaliphilic *Nocardiopsis albus* subsp. *prasina* OPC-131. The isolate produced two types of chitinases. The optimum pH of chi-A was pH 5.0 and that of chi-B was pH 7.0. Suresh and Chandrasekaran (180) isolated a chitinolytic fungus, *Beauveria bassiana*, from a marine sediment. The organism was strongly alkaliphilic and produced maximum chitinase at pH 9.20. The NaCl and colloidal chitin requirements varied with the type of moistening medium used. The addition of phosphate and yeast extract resulted in the enhancement of chitinase yield. This is the

first report of the production of chitinase from a marine fungus.

Recently, Bhushan and colleagues isolated an alkaliphilic, chitinase-producing *Bacillus* sp. BG-11 (54,55). The purified chitinase exhibited a broad pH and temperature optima of 7.5 to 9.0 and 45 °C to 55 °C, respectively. It was stable between pH 6.0 to 9.0 and 50 °C for more than 2 hours. The pH and thermostability of immobilized chitinase were enhanced significantly. The chitinase immobilized on chitosan was stable between pH 5.0 and 10.0, and the half-life of chitosan-immobilized enzyme at 70, 80, and 90 °C was 90, 70, and 60 minutes, respectively. No industrial application has been investigated.

METABOLITES PRODUCED BY ALKALIPHILES

Antibiotics

The first report was published in 1980 by Sato and coworkers (181), in which they recorded the isolation of *Paecilomyces lilacinus* No. 1,907 from soil using an alkaline medium with a pH of 10.5. New antibiotics were produced only under alkaline conditions (pH 9 to 10.5). *Paecilomyces lilacinus* No. 1,907 produced one major product, 1907-II, and a minor product, 1907-VIII, which had antibacterial and antifungal activities. Subsequently, an alkaliphilic *Nocardiopsis dassonvillei* that produced phenazine antibiotics under alkaline culture conditions was isolated (182). Bahn and coworkers isolated a novel aldose reductase inhibitor from alkaliphilic *Corynebacterium* sp. YUA25-1. Compound YUA001 was purified from the supernatant of culture broth by successive silica gel column chromatography. The compound had no antimicrobial activity against some gram-positive and gram-negative bacteria, fungi, and yeasts (183).

Since alkaliphiles have been rediscovered, many Japanese pharmaceutical companies have tried using alkaline media to discover new microorganisms producing new antibiotics. Although several have been found and reported, none are as yet commercially available.

2-Phenylamine

Hamasaki and coworkers found that a large amount of 2-phenylethylamine was synthesized by cells of alkaliphilic *Bacillus* sp. strain YN-2000. This amine was secreted in the medium during the cell growth (184).

Carotenoid of Alkaliphilic Bacillus Strains

Aono and Horikoshi reported that alkaliphilic *Bacillus* sp. No. A-40-2, No. 2B-2, No. 8-1, and No. 57-1 produce yellow pigments in the cells (185). These are triterpenoid carotenoids. However, A-59, M-29, and Y-25 white strains when grown in Horikoshi-II medium did not produce carotenoids. The physiological role of the yellow pigments was not reported.

Siderophores

Gascoyne and coworkers isolated a siderophore-producing alkaliphilic bacterium that accumulated iron, gallium, and aluminum (186,187). Enrichment cultures initiated with samples from a number of alkaline environmental sources yielded 10 isolates. From this group, selections were made on the basis of growth at high pH and the gallium-binding capacity of the siderophores. It was found that some isolates grew well and high concentrations of siderophore were detected, whereas others grew well in the presence of much lower concentrations of siderophore. The effect of iron, gallium, and aluminum on growth and siderophore production batch culture was investigated for six isolates. The presence of iron greatly decreased the siderophore concentration in these cultures, whereas the response to added gallium or aluminum was dependent on the isolate.

Cholic Acid Derivatives

Kimura and coworkers isolated an alkaliphilic *Bacillus* strain from soil that grew well in media containing cholic acid (CA) at 5% (w/v) or higher concentrations (188). The 7- and 12-hydroxyl groups of CA were efficiently converted into keto groups, with the conversion rate for both hydroxyl groups reaching 100% after 72 hours of cultivation. The strain also converted a 3α-hydroxyl group to a keto group, but the conversion rate was about 5% after 72 hours. The strain neither affected any other part of the CA molecule nor oxidized 7β-or 12β-hydroxyl groups. By nitrosoguanidine (NTG) mutagenesis, Kimura and coworkers isolated five mutants that selectively produced the following compounds from CA at high yield (closed 100%): Strains M-4 and M-5 produced 7,12-diketolithocholic acid; strain No. 250 produced 7-ketodeoxycholic acid; strains No. 124 and 336 yielded ketochenodeoxycholic acid from CA. Furthermore, strains M-4, M-5, and No. 250 produced only 7-ketolithocholic acid from chenodeoxycholic acid.

Organic Acids

During the cultivation of alkaliphiles, pH values of the culture media often decrease sharply owing to the production of organic acids, which are produced by growth on carbohydrates. Paavilainen and coworkers (189) reported comparative studies of organic acids produced by alkaliphilic bacilli. Four bacilli, *Bacillus* sp. 38-2 (ATCC21783), *B. alkalophilus* subsp.*halodurans* (ATCC27557), *Bacillus alcalophilus* (ATCC27648) and *Bacillus* sp. 17-1 (ATCC31007) were cultured in the presence of various 1% (w/v) sugars and related compounds such as sugar alcohols. All these alkaliphiles produced acetic acid (4.5–5 g/L at the maximum) while formic acid was produced by only one of the strains. In contrast to neutrophilic bacilli, acetoin, butanediol, or ethanol were not detected. Moderate amounts of isobutyric, isovaleric, α-oxoisovaleric, α-oxo-β-methylvaleric, α-oxoisocaproic, and phenylacetic acids were generated with three of the alkaliphiles.

CONCLUSION

Since the rediscovery of alkaliphilic bacteria, more than 1,200 studies have been published on many aspects of alkaliphiles and alkaliphily. The alkaliphiles are unique microorganisms with great potential for microbiology

and biotechnological exploitation. The aspects that have received the most attention in recent years include (*1*) extracellular enzymes and their genetic analysis; (*2*) mechanisms of membrane transport and pH regulation; and (*3*) the taxonomy of alkaliphilic microorganisms. It is unclear what the next development will be, but it may be the wider application of enzymes. Alkaline enzymes should find additional uses in various fields of industry, such as chiral synthesis, biological wood pulping, and more sophisticated enzyme detergents. Furthermore, alkaliphiles may be very good general genetic resources for such applications as signal peptides for secretion and promoters for hyperproduction of enzymes.

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ALKALITHERMOPHILES (ANAEROBIC).

See THERMOPHILES: ANAEROBIC ALKALITHERMOPHILES

ALLERGENS, FUNGAL.

See FUNGAL ALLERGY AND ALLERGENS

AMMONIFICATION.

See SOIL NITROGEN CYCLE

AMOEBOID PROTOZOA.

See PROTOZOA IN MARINE AND ESTUARINE WATERS

ANAEROBIC DIGESTION OF BIOSOLIDS.

See BIOSOLIDS: ANAEROBIC DIGESTION OF

ANAEROBIC GRANULES.

See ANAEROBIC GRANULES AND GRANULATION PROCESSES

ANAEROBIC GRANULES AND GRANULATION PROCESSES

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In the 1970s, Lettinga and his coworkers developed the upflow anaerobic sludge blanket (UASB) reactor to allow anaerobic treatment of a variety of high-strength wastewaters at relative high loading rates (1). An important feature of the UASB system is the ability to retain a high biomass concentration, which makes it possible to operate at high loading rates. Since the first development of the UASB system, a variety of modifications of this process have been developed, but all consist of an upflow-type reactor. Examples of modified UASB systems include the expanded granular sludge blanket (EGSB) reactor (2), compartmentalized UASB reactors (3), and upflow sludge bed filters (USBF) (4). The biomass in UASB and related reactors typically develops into separate spherical entities of a few millimeters in diameter, which are called anaerobic granules. Therefore, the biomass as a whole is referred to as granular sludge. Although the formation of granules is not necessary for the operation of a UASB reactor (5), granulation is desirable to allow operation at high loading rates. Recently, the formation of granular sludge has also been observed in a few other types of reactors, in particular in the anaerobic migrating bed reactor (AMBR) (6) and the anaerobic sequencing bed reactor (ASBR) (7).

Various aspects of granular sludge characteristics and granulation processes are reviewed in this article. We start with a review of studies that determined the abundance of various microbial populations in anaerobic granules. The research reviewed in this first section used techniques that did not allow preservation of granular structure. In the second part, we summarize the results of studies on techniques that preserved granular structure. In a third section, we review research that studied granulation processes with the general objective to minimize the start-up period for granule formation. Finally, the recommended start-up procedures for UASB reactors are summarized.

MICROBIAL COMPOSITION OF ANAEROBIC GRANULES

Various anaerobic microorganisms exhibit wide-ranging abilities to utilize different substrates and have variable affinities for similar substrates. Furthermore, growth and death rates, and requirements for nutrients and microenvironments vary greatly for different anaerobes. Therefore, it can be expected that wastewater characteristics, reactor configuration, and reactor operating conditions determine the microbial composition of anaerobic granules. However, as discussed later, the microbial composition of granules obtained from widely varying systems is often quite similar, suggesting key roles for certain populations present in granules.

Wu and coworkers (8) used enrichment methods and microscopy to identify microorganisms in granules from a UASB reactor treating brewery wastewater (with 0.6 to 1.2 mM sulfate present). It was found that *Methanobacterium*-like cells were the prevalent H_2-CO_2 and formate utilizers, whereas *Methanospirillum*-like cells were occasionally found. *Methanosaeta*-like cells were the most abundant aceticlastic methanogens. In addition, *Desulfovibrio*-like and *Desulfobulbus*-like cells were found in enrichments prepared with ethanol and propionate (with or without sulfate), respectively. In a subsequent study, enrichments were obtained from granules fed a medium containing acetate, propionate, and butyrate (with 0.15 mM sulfate) (9). Using most probable number (MPN) estimates combined with microscopy, the authors found that fermentative *Clostridium*-like spore-forming rods were present at 10^9 cells/g suspended solids (SS), that syntrophic propionate-degrading bacteria were detected at 10^{10} to 10^{11} cells/g SS, and that syntrophic butyrate-degrading bacteria were found to be present at 10^{11} cells/g SS. The observed hydrogenotrophic methanogens included *Methanobacterium formicicum*-like cells (10^{12} cells/g SS) and *Methanospirillum*-like cells (10^9 cells/g SS). The aceticlastic methanogens *Methanosarcina mazei*-like and *Methanosaeta*-like cells were observed at levels of 10^8 cells/g SS and 10^{12} cells/g SS, respectively.

These two studies demonstrated that the most abundant aceticlastic and hydrogenotrophic methanogens were similar in granules from UASB reactors fed different

wastewaters. *Methanosaeta* spp. and *Methanobacterium* spp. dominated granules from both systems. It was also observed that *Methanobacterium formicicum*-like cells were dominant in the MPN test using acetate (9). This might indicate that a significant amount of acetate was first converted to CO_2 and H_2 by acetogens, which was then converted to methane by *M. formicicum*-like cells. So far, only two strains have been isolated that can convert acetate to CO_2 and H_2, the thermophilic strain AOR (acetate oxidizing rod) and *Clostridium ultunense* (10). Other researchers also found that the indirect conversion of acetate to methane by acetogens and hydrogenotrophic methanogens can be significant in granular sludge (11–13).

Several methods were used to reveal the microbial composition of granules obtained from a UASB reactor treating sugar refinery wastewater (original granules) and after they were adapted to ethanol and propionate for 6 and 36 months, respectively (14). Direct counting based on morphology and autofluorescence showed that 20% of the total cells in the original granules were *Methanosaeta*-like organisms, 10% were *Methanosarcina*-like, and 15% were hydrogenotrophic methanogens. After six months of adaptation to ethanol, *Methanosaeta*-like cells made up less than 1% of the community, *Methanosarcina*-like cells increased to 20%, and hydrogenotrophic methanogens decreased to around 10%. On the other hand, granules adapted to propionate for 36 months contained 30% of *Methanosaeta*-like cells, less than 1% *Methanosarcina*-like cells, and 40% hydrogenotrophic methanogens. MPN methods were used to estimate the abundance of populations in these three sets of granules using various enrichment media and antibodies were used to identify the cells present in the positive highest dilution tubes of the MPN test. Both *Methanosarcina barkeri* and *Methanosaeta concilii* were detected in the original granules, but only *M. barkeri* was present in the ethanol-adapted granules and *M. concilii* in the propionate-adapted granules. Similarly, both *Methanospirillum hungatei* JF1 and *Methanobrevibacter arboriphilus* AZ were found in the original granules, but only *M. hungatei* JF1 was present in the ethanol-adapted granules and *M. arboriphilus* AZ in the propionate-adapted granules.

Visser and coworkers (15) used monoclonal antibodies to show changes in the microbial composition of granules, which were obtained from a mesophilic UASB reactor treating a potato-processing wastewater and were exposed for 20 days to a medium containing acetate, propionate, and butyrate. The authors found no changes in population patterns (in terms of the degree of antigenic relatedness to different methanogens) after 20 days of adaptation to the VFA-based medium. However, the total number of methanogens detected dropped from 12% in the original granules to 5%. The authors documented changes in the population profiles for different groups of immunologically related methanogens after the reactor was operated at thermophilic conditions for four months. The abundance of *Methanobrevibacter smithii* PS, *M. smithii* ALI, *M. hungatei* JF1, *Methanogenium cariaci* JR1, and *Methanosarcina thermophila* TM1 related cells increased immediately after the rise in temperature, and gradually decreased to lower levels, which persisted throughout the observed period. The populations of methanogens related to *M. concilii* Opfikon and *M. arboriphilus* AZ increased, and reached a stable level after 21 and 76 days, respectively. The population of *Methanobacterium thermoautotrophicum* ΔH related organisms continued to increase over the observed period.

Several research groups have used oligonucleotide hybridization probes targeting ribosomal RNA (rRNA) to determine the microbial composition of anaerobic granules. Zheng (16) applied 16S rRNA targeted oligonucleotide probes to analyze the microbial composition of granules sampled at different heights from a UASB reactor treating wastewater from a corn processing plant, which produced ethanol as its major product. The levels of archaeal (methanogenic) rRNA ranged from 18.4 to 30.6%, with 4 to 5.9% of *Methanosaeta* spp., 3.4 to 4.3% *Methanomicrobiales*, and 10.5 to 13.6% *Methanobacteriaceae*. The predominant sulfate-reducing bacteria (SRB) were *Desulfovibrionaceae* (28.6 to 53.3%), but *Desulfobulbus* spp. (2.7 to 6.2%), and *Desulfobacter* spp. (1.3 to 2.6%) were also present.

The same method was used to show the population changes after granules formed in potato-processing industry wastewater adapted to different substrates (17). The inoculum had very low levels of *Desulfobulbus* spp. (around 2% of total 16S rRNA) and of the syntrophic propionate degrader, strain SYN7 (less than 1% of total 16S rRNA). After adaptation, the *Desulfobulbus* spp. increased significantly (up to 35%) in the granules fed propionate and sulfate, whereas SYN7 increased to around 10% in the granules fed propionate only.

Granules from two laboratory-scale UASB reactors also were characterized using 16S rRNA targeted oligonucleotide probes (18,19). The feed for one of the UASB reactors contained glucose (Reactor NP), whereas the second reactor was fed glucose and propionate (Reactor P). Despite these differences in feed composition, the methanogenic composition of granules from the two reactors were very similar. Granules from both reactors contained around 40% archaeal (methanogenic) rRNA. *Methanosaeta concilii* was the predominant methanogen in both reactors. Species within the family *Methanobacteriaceae* were the dominant hydrogenotrophic methanogens in Reactor NP, whereas species belonging to the *Methanobacteriaceae* and *Methanomicrobiales* were equally abundant in Reactor P.

Sekiguchi and coworkers (20) evaluated the diversity of mesophilic and thermophilic granular sludge that had been maintained in UASB reactors treating a sucrose/propionate/acetate-based artificial wastewater by constructing 16S ribosomal DNA (rDNA) clone libraries using prokaryotic primers. Partial sequencing of the clones showed that about 20% of the clones were *Archaea*, all of which were close relatives of known methanogens and most of them were affiliated with *M. concilii* and *M. thermophila* for the mesophilic and thermophilic granules, respectively. An important difference between the two clone libraries was that a major group in the mesophilic clone library (27% of clones) belonged to the delta subclass of the *Proteobacteria* and contained close relatives of SRB (*Desulfovibrio* spp. and *Desulfobulbus* spp.)

and syntrophic bacteria (strain SYN7 and *Syntrophobacter fumaroxidans* [strain MPOB]). In contrast, none of the clones in the thermophilic clone library was affiliated with the *Proteobacteria*, but clones that were closely related to gram-positive syntrophic bacteria (*Thermosyntropha lipolytica* and propionate-oxidizing spore formers A and B) were identified. Using fluorescence in situ hybridizations (FISH), they further determined that approximately 40% and 45% of total cells (as determined using the intercalating dye 4',6-diamidino-2-phenylindole [DAPI]) were labeled with archaeal and bacterial oligonucleotide probes, respectively. This discrepancy between FISH and cloning results lead Sekiguchi and coworkers (20) to believe that their DNA extraction method did not result in sufficient lysis of all cells, which may have limited their estimate of diversity.

In summary, the studies reviewed earlier have shown that the microbial composition of granules depends on the type of substrate, and can change substantially when substrates or environmental conditions are changed. However, there are similarities, especially with regard to methanogen representation. This suggests that the presence of certain methanogens is critical for the anaerobic degradation of organic compounds in general and for systems depending on granulation in particular. Because different microbial characterization techniques were used in the various studies, results need to be interpreted with care. For example, cultivation-based methods, such as MPN estimates, may underestimate the abundance of *Methanosaeta* spp. and syntrophic bacteria as pointed out by Grotenhuis and coworkers (14). When using oligonucleotide probes for population quantification, only those organisms that are targeted by the probes are included in the quantification. Furthermore, rRNA targeted oligonucleotide probe hybridizations provide estimates for rRNA levels, and not for biomass levels or cell numbers. Techniques based on cloning have the potential to reveal microbial diversity, but can be biased when DNA is not uniformly retrieved.

STRUCTURE OF GRANULAR SLUDGE

Layered Structure

To study the structure of granules, MacLeod and coworkers (21) used mesophilic granular sludge that had been fed a sucrose-based wastewater for one month (after transfer from a UASB reactor treating cheese whey wastewater). Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) determined that the granules consisted of three layers:

- Organisms in the external layer were of diverse morphology: chain-forming coccoids, long thin filaments (*Methanospirillum*-like), small rods and cocci, clustering coccoids (*Methanococcales*-like), and chain-forming rods (*Methanosaeta*-like). Some of the large coccoids were surrounded by extracellular polymers (ECP).
- The middle layer was tightly packed with ECP and rod-shaped bacteria similar to *Methanobrevibacter* spp. and *Syntrophobacter* spp.
- *Methanosaeta*-like organisms were dominant in the center. In addition, the center contained many cavities, which might help to release produced biogas.

On the basis of these observations Guiot and coworkers (22) proposed a model for anaerobic granules formed on carbohydrate-based substrates (Fig. 1), consisting of:

- an external layer, in which fermenters are predominant, but which also contains some hydrogenotrophic microorganisms;
- a middle layer that contains syntrophic consortia;
- a core that contains mainly aceticlastic methanogens, usually *Methanosaeta* spp.

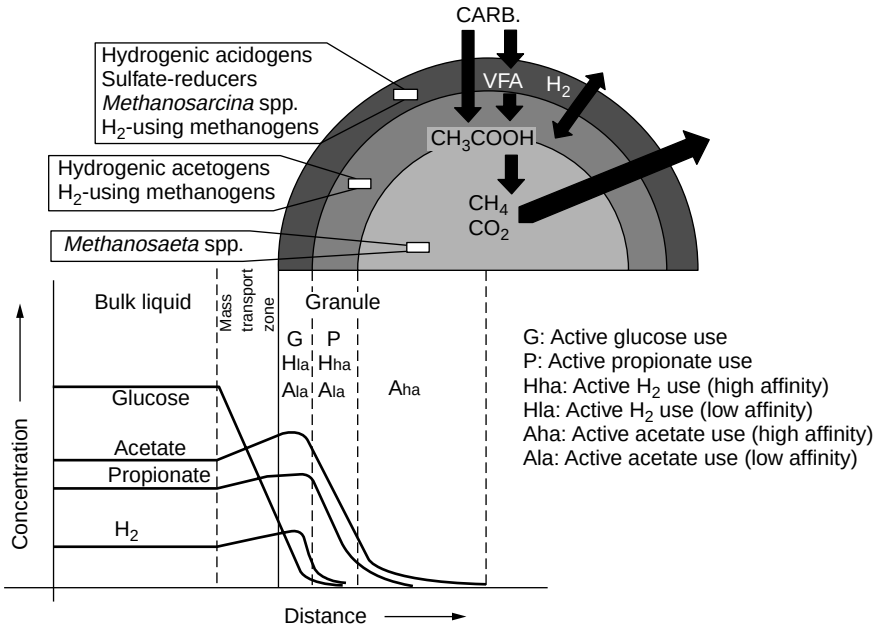

Figure 1. Proposed structure of arrangement of populations in glucose-fed granules. Reproduced with permission from. S. R. Guiot et al., *Water Sci. Technol.* **25**, 1–10 (1992).

As discussed by Guiot and coworkers (22), the layered structure develops because of diffusion limitations. Because the substrates in the wastewater can only diffuse for a certain distance into the granules, fermenters stay in the external layer. Volatile fatty acids (VFAs) produced by the fermenters diffuse further into the granules and are utilized by syntrophs. Acetate produced here diffuses into the core and is used by aceticlastic methanogens. Because H_2 is produced in the external layer and the middle layer, hydrogenotrophic microorganisms are present in both layers.

The model was supported also by results from metabolic activity tests for granules formed on carbohydrate-based substrates (22). First, the metabolic activity on formate, acetate, and propionate was greater for larger granules. This observation was explained by the low surface-to-volume ratio of larger granules and by assuming that glucose can only diffuse for a certain distance into the granules. Second, metabolic activity tests were performed on the three fractions that resulted from abrasions of anaerobic granules. The external layer had the highest specific activity on glucose, but the lowest specific activity on propionate, whereas the core exhibited the opposite effect. However, the specific activity on glucose was relatively high for all three fractions and the specific activity on acetate was similar for all three fractions. Thus, each layer was not exclusive of other trophic groups.

Similar layered granular structures with *Methanosaeta* spp. dominant in the center were also reported for mesophilic granules treating sucrose (23), paper factory wastewater (24), paper-mill wastewater (25), and sugar refinery wastewater (25). These observations were made using SEM, TEM, phase contrast microscopy, or epifluorescence microscopy on sectioned granules.

Using FISH with rRNA targeted oligonucleotide probes, Harmsen and coworkers (17,26) were able to show the microbial structure of granules in more detail. They observed three layers in granules that were originally obtained from a system treating sugar beet wastewater and were adapted to sucrose for six months (Fig. 2; 26):

- The external layer contained mainly bacterial cells.
- The middle layer consisted of syntrophic microcolonies that contained propionate degraders and *Methanobrevibacter* spp. (as suggested by positive hybridization signals with a probe for the family *Methanobacteriaceae* and morphological similarity to *Methanobrevibacter* spp.). This layer also contained

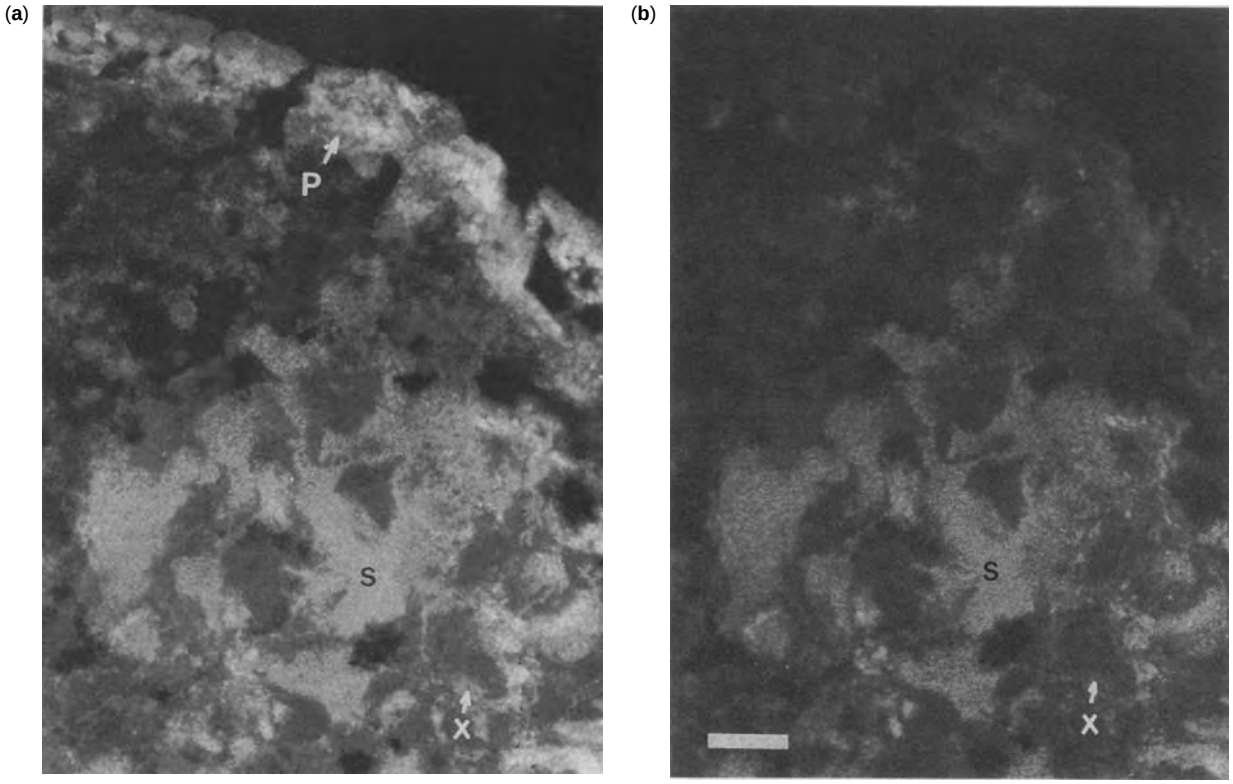

Figure 2. FISH of a sucrose-fed granule viewed by epifluorescence microscopy at a 200-fold magnification. (**a**) A fluorescein-labeled bacterial probe detects bacterial cells mainly located in the outer layer of the granule (P, peripheric location) and the syntrophic microcolonies more toward the inside of the granule (S, syntrophic microcolony). (**b**) A rhodamine-labeled probe specific for a syntrophic population detects only the microcolonies inward of the granule. X, autofluorescence. Bar, 50 μm. Reproduced with permission from H. J. M. Harmsen et al., *Appl. Environ. Microbiol.* **62**, 1,656–1,663 (1996). See color insert.

microcolonies of *Methanosaeta* spp. (as revealed by both TEM and FISH), which were usually located close to the syntrophic microcolonies.

- The core contained inorganic material with large cavities and some methanogens (positive hybridization signal with an *Archaea*-specific probe, but not with a *Methanosaeta*-specific probe).

Thus, in contrast with the observation made by MacLeod and coworkers (21), Harmsen and coworkers (26) found that the core did not contain large numbers of *Methanosaeta* spp., but that *Methanosaeta* spp. formed microcolonies near the syntrophic microcolonies in the middle layer. Granules that were originally obtained from a system treating sugar beet wastewater and adapted for six months to a mixture of butyrate, propionate, and acetate, had an additional thick layer rich in microcolonies of *Methanosaeta* spp. and *Methanosarcina* spp. between the external layer and the middle layer (as detected by FISH and based on the morphology of *Methanosarcina* spp.) (26). The authors explained the presence of this extra layer by the high acetate concentration in the feed. High concentrations of acetate are inhibitory to syntrophic propionate degradation caused by the unfavorable thermodynamics of propionate conversion. *Methanosaeta* spp. and *Methanosarcina* spp., present in the thick layer, removed the acetate before it reached the syntrophic microcolonies. Because the feed contained only butyrate, propionate, and acetate, the authors suggested that the *Bacteria* in the external layer were mainly butyrate degraders.

A similar structure was observed also in granules from a thermophilic UASB reactor treating alcohol distillery wastewater using SEM (27). This reactor was inoculated with mesophilic granules treating fruit-juice wastewater. The surface of the granules was covered with filamentous bacteria, presumably fermenters. The second layer was dominated by *Methanosaeta* spp., whereas the core contained *Methanobacterium*-like cells.

The same mesophilic and thermophilic granules that were characterized in terms of diversity by constructing clone libraries by Sekiguchi and coworkers (20) (see previous discussion) were recently analyzed using FISH (28). Granules from both reactors consisted of outer and inner layers dominated by *Bacteria* and *Archaea*, respectively (Fig. 3). The center did not show hybridization signals with

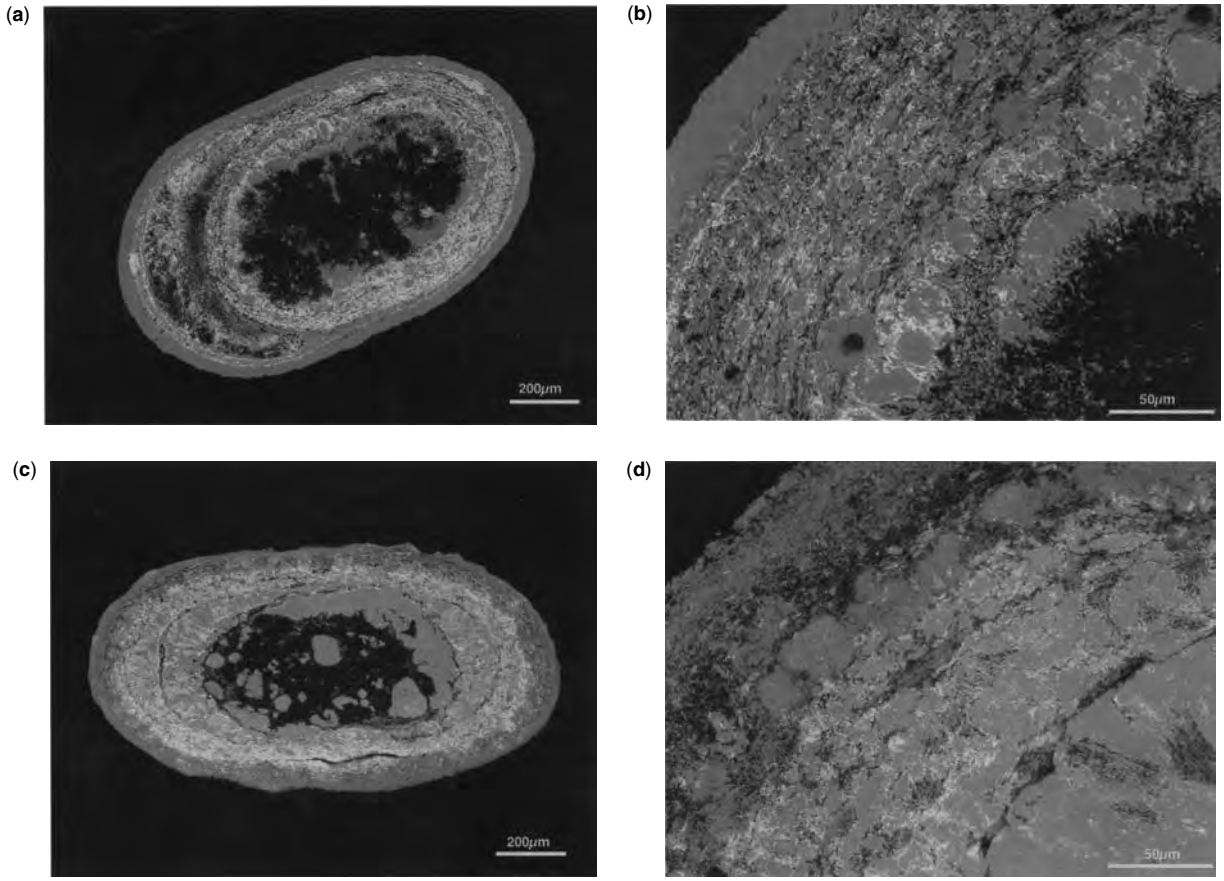

Figure 3. FISH of sections from mesophilic (**a** and **b**) and thermophilic (**c** and **d**) granules viewed by confocal laser scanning microscopy. The sections were simultaneously hybridized with a Cy-5-labeled bacterial probe (green) and a rhodamine-labeled archaeal probe (red). Reproduced with permission from Y. Sekiguchi et al., *Appl. Environ. Microbiol.* **65**, 1,280–1,288 (1999). See color insert.

a bacterial probe or with an archaeal probe. *Methanosaeta* spp. were the predominant *Archaea* in both types of granules. In the mesophilic granules, *Methanobacteriaceae* cells juxtaposed with *Bacteria* and some *Methanomicrobiales* cells were distributed in the granules. In the thermophilic granules, *Methanobacteriaceae* and *Methanosarcina* cells were detected. The authors also tried to locate the microorganisms that were found to be significant in their earlier study using clone libraries (20,28). For mesophilic granules, *Desulfobulbus* cells were found in the outer layer of the granules. Cells that were closely related to *Syntrophobacter* were shown to form microcolonies together with *Methanobacteriaceae* cells in the mesophilic granules. FISH with a probe that targets clones associated with green nonsulfur bacteria revealed filamentous cells on the surface of the thermophilic granules.

In another recent study, FISH revealed a layered structure for granules used to treat a methanol-containing wastewater (29). *Methanosarcina barkeri* were found in the outer layer, and *M. concilii* cells were located in the inner layer and core. Layered structures were also observed using FISH for granules from two lab-scale UASB reactors treating glucose- and glucose/propionate-containing wastewaters (18,19). In both reactors, granules contained large cores that consisted almost exclusively of *M. concilii*. Granules from both reactors exhibited a thin outer layer of *Bacteria*, most of which had a filamentous morphology. For granules treating glucose/propionate-containing wastewater, a layer surrounding the core consisted of both *Bacteria* and*Archaea*. Finally, Santegoeds and coworkers (29) used molecular techniques (denaturing gradient gel electrophoresis and FISH) and microsensors for hydrogen sulfide and methane to study anaerobic granules originally obtained from three full-scale UASB reactors. They observed a layered structure and found that the localization of sulfidogenic, methanogenic, and syntrophic populations determined by FISH and microsensor data corresponded well.

Nonlayered Structure

Nonlayered granules were observed under a number of conditions. A random distribution of an ethanol-degrader (strain EE121), *M. hungatei* JF1, and *M. mazei*-related organisms were found in granules adapted to ethanol (14). The same study also demonstrated a nonlayered structure for granules fed propionate. These granules were dominated by *M. arboriphilus* and *M. concilii* together with a bacterium, presumably a propionate oxidizer. Recently, Rocheleau and coworkers (29) found that granules treating protein-rich wastewater also exhibited nonlayered structures in which *M. concilii* were randomly distributed.

On the basis of observations that granules fed propionate, peptone, or glutamate exhibited nonlayered structures, Fang (30) proposed that the initial degradation rate of the substrate determines the granular structure. If the initial step for degradation of a substrate is the rate-limiting step, some of the substrate can diffuse into the granules before it is degraded by the microorganisms at the surface, resulting in nonlayered granules.

Temperature can also play a role. A layered structure was only observed for granules treating low-fat milk at 5 °C, but not at 15 °C and 25 °C (7). As discussed by the authors, the nonlayered structure at 15 °C and 25 °C was probably related to the low hydrolysis rate of the substrate. At 5 °C, the diffusion rate was also low, possibly allowing the fermenting bacteria to degrade the substrate before it diffused into the granules.

A nonlayered structure also can develop when the substrate can be fully degraded by a single population. For example, granules obtained from a methanol-fed UASB reactor were dominated by *Methanosarcina* with small numbers of rods and filaments in the center (31).

Microcolonies

Microcolonies often have been observed in granules. Wu and coworkers (8) observed microcolonies in mesophilic granules from a full-scale UASB reactor treating brewery wastewater. The wastewater contained mainly ethanol, some propionate and acetate, sulfate, and trace amounts of glucose, butyrate, lactate, and succinate. Three types of microcolonies were found:

- type 1: *Methanosaeta*-like cells mixed with small amount of *Methanobacterium*-like cells;
- type 2: *Methanobacterium*-like cells juxtaposed with *Desulfobulbus*-like cells;
- type 3: *Methanobacterium*-like cells combined with *Desulfovibrio*-like cells.

Type 2 and type 3 were believed to be syntrophic microcolonies, which were responsible for syntrophic degradation of propionate and ethanol, respectively.

Two types of microcolonies were found in propionate-fed granules (14). One was solely made up by *M. concilii*-related organisms, and the other one contained *Methanobrevibacter arboriphilicus* AZ and a possible propionate degrader, which was not closely related to *Syntrophobacter wolinii*.

Similarly, Harmsen and coworkers (17) found two types of microcolonies in propionate-fed granules using FISH. One microcolony was dominated by *Methanosaeta* spp.; the other one was a syntrophic microcolony that contained the syntrophic propionate degrader, strain SYN7, and a methanogen from the order *Methanomicrobiales*, morphologically similar to *Methanospirillum* spp. Harmsen and coworkers (26) also found two types of microcolonies in both sucrose-fed granules and VFA-fed granules (discussed earlier), that is, *Methanosaeta* dominated microcolonies and syntrophic microcolonies containing syntrophic propionate degraders and *Methanobrevibacter* spp.

Three types of syntrophic microcolonies were found in granules treating carbohydrate-containing wastewater (32). Two types consisted of acetogen cells juxtaposed with hydrogenotrophic methanogens (*Methanobrevibacter*-like), whereas the other type consisted of clusters of acetogens in close proximity to clusters of *Methanobrevibacter*-like cells.

Using wax-embedded sections and histogram stains, Morgan and coworkers (25) observed that the diameters of microcolonies increased from the outer layer to the center. To explain this observation, they suggested that inner microcolonies were older than those at the outside.

In summary, studies on granular structure generally have found two types of microcolonies. One type consists of syntrophic consortia, and the other type contains mainly *Methanosaeta* spp. The mechanism of microcolony formation seems to be obvious. Once a microorganism colonizes, it starts to grow and its offspring stays in the same area. This results in the formation of a single-species microcolony. The juxtaposition of syntrophic partners is probably the result of internal mixing of the offspring (10).

Other Studies Related to Granular Structure

Distribution Patterns of Methanogens. Macario and coworkers (33) observed that thermophilic VFA-fed granules consisted of a two-layered structure with an outer layer and a core. Both the outer layer and the core had a thin, dense outer layer. In some granules, this dense layer separated the core into several parts. Using antibody probes, the authors showed the distribution pattern of different groups of methanogens (methanogens immunologically related to *M. thermoautotrophicum* ΔH, *M. thermophila* TM1, *M. concilii* Opfikon, *M. arboriphilus* AZ, and *M. smithii* ALI) located in a spongy matrix formed by other organisms or by intercellular material.

The immobilization patterns of *Methanosarcina* and *Methanosaeta* cells in autoclaved granules were studied using polyclonal antibodies (34). *Methanosaeta* was immobilized on the surface and in the center of the granules, and all immobilized *Methanosaeta* cells were single short rod-shaped. *Methanosarcina*, on the other hand, stayed on the surface or in the cavities. They grew as single cells, small clusters, or large clusters (especially when the acetate concentration was high).

Metabolic Activity Zones. Lens and coworkers (35) observed metabolic activity zones in granules using pH and glucose microelectrodes. For example, in granules (diameter 0.5 to 2.0 mm) from a full-scale UASB reactor treating potato starch wastewater that were acclimated to glucose or diluted molasses for about 10 days, they identified an outer, glucose-converting zone of 200 to 300 μm and an inner methanogenic activity zone. The authors were not able to correlate these two activity zones to the granular structure. *Methaonsaeta*-like cells were found throughout the granules, even in the surface layer. In addition, coenzyme F_{420} was found to be distributed homogeneously in the granules.

Hydrophobicity. Three layers of different hydrophobicities were observed in gray colored granules fed sucrose (36). The outer layer was hydrophilic, the second layer was very hydrophobic, and the core was moderately hydrophobic. In general, fermenting bacteria are hydrophilic, whereas acetogens and methanogens are hydrophobic. This finding again supported the structured model proposed by Guiot and coworkers (22).

Extracellular Polymers (ECP). ECP were found around some clusters of microorganisms in the surface layers of granules from two full-scale UASB reactors treating paper mill and sugar refinery wastewater (25). There was very little ECP in the area where *Methanosaeta* was dominant. In addition, lots of empty sheaths of *Methanosaeta*, which are rich in proteins, were observed. This might explain why other researchers have found proteins to be dominant in the center of granules (see following section).

Quarmby and Forster (37) used gram staining, as well as staining for carbohydrates, proteins, and lipids to show the structure of granules taken from UASB reactors treating different industrial wastewaters. The surface contained mainly gram-positive cells with some colonies of gram-negative cells, whereas the center mainly consisted of gram-negative cells. Carbohydrates were dominant in the outer layer and decreased inwards, whereas proteins were present at relatively low levels in the outer layer and increased inwards. Lipids were evenly distributed throughout the granules. The structures of granules treating the same types of wastewater were observed to be different, demonstrating that other factors such as organic loading rate, pH, and the strength of the wastewater may also affect the structures.

In summary, the structure of anaerobic granules can be either layered or nonlayered, depending on substrate characteristics. In addition, diffusion factors and degradation rates play important roles in determining the structure. Microcolonies are observed in both layered and nonlayered granules. They fall into two groups, one group consists of juxtaposed syntrophs and hydrogenotrophic methanogens; the other one contains mainly *Methanosaeta* spp. *Methanobrevibacter* spp. also are observed frequently in syntrophic microcolonies.

GRANULATION PROCESSES

Because it is generally accepted that granules are merely spherical biofilms, Schmidt and Ahring (38) used a modification of biofilm formation theory to explain that the granulation process consists of four steps: (*1*) transport of cells to the surface of uncolonized inert material or other cells (nucleus), (*2*) initial reversible adsorption to the nucleus by physicochemical forces, (*3*) irreversible adhesion of the cells to the nucleus by microbial appendages and/or polymers attaching the cells to the substratum, and (*4*) cell growth and development of granules. Thus, the granulation process starts with the colonization of cells onto a nucleus (inert material or other cells). The cells then grow into granules of visible size.

Importance of Nuclei

Nuclei can be particles consisting of inert material and/or cells present in the original inoculum, or they can be particles added to a reactor together with inoculum to enhance the granulation process. Precipitates, such as iron sulfide can also act as nuclei (39).

Hulshoff Pol (40) demonstrated that particles with dimensions between 40 and 100 μm are important for granulation. He showed that granulation still happened after particles larger than 100 μm had been removed by sieving of the inoculum. However, when the inoculum was sieved using a 40-μm sieve, no granulation was observed.

Several studies demonstrated that the addition of particles resulted in a higher rate of granulation.

Addition of 100-μm hydroanthracite particles (with good adhesion properties) reduced start-up time and increased granulation rate (40). Using a water-absorbing polymer (WAP, made mainly of an acrylic compound with particles between 100 and 200 μm), Imai and coworkers (41) were able to enhance the start-up process of UASB reactors using anaerobic digester sludge as the inoculum. Laboratory-scale reactors fed with glucose showed that the granulation time reduced from 60 days to 40 days when WAP was added. For a laboratory-scale reactor fed with acetate, propionate, and butyrate, granulation was observed after 70 days when WAP was added, and no granulation had taken place in the control reactor after four months of operation. In pilot-scale studies with acetate, propionate, and butyrate as substrates, however, granulation enhancement was less significant (65 days with WAP addition versus 75 days without). SEM showed that WAP particles appeared in the center of the granules and were gradually decomposed.

Not all inert particles can enhance granulation. Four different carriers, powdered activated carbon (PAC), granular activated carbon (GAC), gamet sand, and silica sand were tested for the enhancement of granulation in ASBRs treating synthetic wastewater containing mainly sucrose (42). All average particle diameters were 0.5 mm. Only the addition of GAC and PAC showed significant enhancement of the granulation process, suggesting that GAC and PAC provided sites to which microorganisms could adhere and thus be retained.

The addition of 30- to 100-μm gravel particles to digested sewage sludge did not show significant enhancement of granulation for a thermophilic UASB reactor fed acetate as the major substrate (43). *Methanosaeta* was believed to be more important as a nucleus than the inert material. Attachment of *Methanosaeta* filaments to the inert particles was considered a requirement for granulation and the lack of significant enhancement of granulation in this case was believed to be caused by the low numbers of *Methanosaeta* in the inoculum.

These results are consistent with observation reported by Yoda and coworkers (44). Powdered zeolite (50 to 100 μm) was used in an anaerobic expanded microcarrier bed reactor. A thin biofilm developed on the zeolite powder, which was later transformed into granules. *Methanosaeta* spp. were the predominant organisms in the thin film.

Beeftink and Staugaard (45) demonstrated that sand grains were important during the start-up of an anaerobic gas-lift reactor. Later, granules were found to have no sand center and the sand was gradually lost in the system. This indicates that once granules have been formed, there is no need for further addition of inert particles to maintain the granules.

The nucleus can also consist of small aggregates of microorganisms. For example, by recycling small microbial aggregates back to a UASB reactor, Thiele and coworkers (46) found that granule bed growth was stimulated. Microorganisms or groups of microorganisms believed to play important roles in the granulation process are discussed below.

Fermenting Bacteria. On the basis of observations that granulation was promoted by wastewater containing carbohydrates but not by preacidified wastewater, Vanderhaegen and coworkers (47) suggested that fermenting bacteria that produce ECP act as "nucleation centers" and that other microorganisms subsequently associate with this nucleus. Filamentous fermenters also were believed to be responsible for promoting granulation in UASB and anaerobic fixed film reactors treating partially acidified wastewater (48).

Methanosaeta. Wiegant (49) proposed the "spaghetti theory" by analyzing available data from other researchers. This theory states that as a result of the upflow velocity in a UASB reactor, *Methanosaeta* cells are forced to grow as filaments and/or attached to inert material in order to be retained in the system. These filaments serve as precursors of granulation for "healthy" UASB reactors. Because granulation usually is much faster than the maximum growth rate of *Methanosaeta* spp., Wiegant suggested that aggregation of *Methanosaeta* with or without inert material is very important during the initial stage of start-up. Once the nuclei are formed, they grow out to granules by individual growth of trapped microorganisms and entrapment of nonattached microorganisms.

On the basis of their observation of layered, sucrose-fed granules, MacLeod and coworkers (21) proposed that the granulation process started with *Methanosaeta* aggregation. Then, acetogens (syntrophs) and hydrogenotrophic methanogens colonized outside the *Methanosaeta* aggregates. Finally, fermenting bacteria and hydrogenotrophic methanogens colonized the surface. A similar process was also proposed by Morgan and coworkers (21) after they observed layered granules treating paper mill and sugar refinery wastewater. They proposed that the granulation process started with a nucleus consisting of aggregates of *Methanosaeta* spp. and other microorganisms. As *Methanosaeta* filaments grow, they form bundles. The bundle sizes increase and eventually grow into each other and exclude other microorganisms from the center.

Methanosarcina. During a granulation experiment with defined species (including a butyrate-degrading strain BH, a propionate-degrading strain PT, *Methanosaeta* sp. strain M7, *M. formicicum* T1N, and *M. mazei* T18), *M. mazei* T18 was found to be in the center of the granules (50). Obviously, the aggregation of *M. mazei* T18 served as the nucleus for the granulation. However, the authors were not convinced that *Methanosarcina* was essential for granulation and suggested that inert particles may be able to function in a similar manner.

Some *Methanosarcina* spp. (usually isolated from nonmarine environments) can form aggregates up to several millimeters during their life cycle (51). A pure culture of *M. barkeri* formed granules in a UASB reactor with recycling (52). It is possible that small aggregates of *Methanosarcina* form and serve as nuclei for granulation because the acetate concentration can be sufficiently high (>200 mg/liter) to favor the growth of *Methanosarcina* during start-up (10). Later, when

the acetate concentration decreases, the growth of *Methanosarcina* aggregates will stop.

Although it is possible to use *Methanosarcina* aggregates as nuclei, *Methanosarcina* dominated granules are usually of less practical significance because this type of granules are easily washed out of UASB reactors (43,49).

Unidentified Filaments. A UASB reactor fed with methanol was inoculated with sludge (fine floc) from another UASB reactor treating methanolic waste (31). Electron micrographs showed that granules contained rod-type and long filaments in the core and *Methanosarcina* at the surface. The rod and filament cells likely served as nuclei for the granulation. Thin filaments (possibly SRB) also may have served as nuclei for granulation during start-up of a UASB reactor treating wastewater high in sulfate (5 g/L) (53).

Syntrophic Consortia. In a study by Grotenhuis and coworkers (52), granulation of a coculture consisting of a propionate degrader and *Methanosaeta* proceeded very fast (11 days) in a UASB reactor with recycling. A few other studies also compared the effectiveness of syntrophic consortia as nuclei with other possible nuclei. For example, El-Mamouni and coworkers (54–56) compared the influence on granulation of nuclei that contained mainly *Methanosaeta* spp., *Methanosarcina* spp., syntrophic consortia, or fermenters. The characteristics of four types of nuclei enriched in four different USBF reactors are summarized in Table 1. The fermenter nuclei had the smallest size, lowest settling velocity, and lowest ash content, whereas they had the highest ECP content and were rich in carbohydrates. The *Methanosaeta* nuclei had the highest ash content, which might be due to the precipitation of calcium carbonate, magnesium carbonate, and iron sulfide. Together with suspensions of municipal anaerobic sludge, the four types of nuclei were used to inoculate four USBF reactors treating sucrose (54). The mass ratios of municipal anaerobic sludge to nuclei were 1 : 4 g VSS/g VSS. At the end of the experiment (on day 120), the granules in the reactor seeded with syntrophic nuclei reached their plateau of stabilization and had the largest size (mean diameter of 3.1 mm). The granules in reactors seeded with *Methanosarcina* or *Methanosaeta* nuclei were still in the exponential phase at this point, and the mean sizes were 2.4 and 2.7 mm, respectively. In the reactors seeded with fermenter nuclei, granules were still in the lag phase and had a mean diameter of 1.1 mm. The authors suggested that the following two factors led to the fast start-up of the reactors seeded with the syntrophic, *Methanosarcina*, and *Methanosaeta* nuclei. First, syntrophic bacteria and methanogens have relatively low growth rates. The presence of these organisms in the nuclei secured a fast start-up. Second, the nuclei with syntrophs and aceticlastic methanogens effectively promoted the attachment of fermenters and prevented flocculant growth.

In another study, the potential for *M. concilii* and propionate-degrading syntrophic consortia to serve as nuclei for granulation was tested by monitoring granulation in two laboratory-scale UASB reactors inoculated with nongranular sludge and treating synthetic wastewaters containing glucose (Reactor NP) and glucose and propionate (Reactor P) (19). The effluent acetate concentrations were controlled to be below 200 mg/L. Quantitative membrane hybridizations and FISH with oligonucleotide probes targeting 16S rRNA were used to monitor changes in microbial communities and to study cell aggregate structures during granulation. *Methanosaeta concilii* cells were present at high levels and demonstrated good settling ability. Increases in their abundance were associated with significant increases in cell aggregate sizes at the early stage of granulation. *Methanosaeta concilii* cells were found to serve as backbones in small cell aggregates with other archaeal cells and bacterial cells attached to them, and they remained dominant in large cell aggregates and mature granules. These observations support the hypothesis that *Methanosaeta* cells serve as nuclei for granulation. On the other hand, syntrophic propionate-oxidizing bacteria, namely *Syntrophobacter* spp., exhibited poor settling ability and were easily washed out from the system. Their contribution to granulation was probably minimal.

In summary, studies have shown that nuclei can be inert particles or aggregates of microorganisms. Particles with diameters ranging from 40 to 100 μm are important for granulation and the addition of certain types of inert particles can increase the granulation rate. Also, several studies pointed to the importance of *Methanosaeta*, which is believed to attach to inert particles first after which granulation is started. Filamentous microorganisms (e.g.,

Table 1. Characteristics of Four Types of Microbial Nuclei for Granulation (55)

Nucleus Type	Syntrophic Consortia	*Methanosaeta* spp.	*Methanosarcina* spp.	Fermenters
Enrichment medium	Ethanol/Acetate	Acetate[a]	Methanol	Sucrose[b]
Settling velocity (m/h)	10.5	11.3	8.7	3.2
Size (mm)	up to 1.2	up to 1.2	up to 1.2	<0.6
Ash content (%)	30	60	40	16
Extracellular polymers (mg/g VSS)	35.2	42.2	26.6	216.5
Carbohydrates: ECP (%)[c]	12	19	21	84
Proteins: ECP (%)[d]	41	43	64	7

[a] Effluent acetate concentrations were controlled to be below 150 mg/Liter;
[b] Reactor was overloaded (3 to 5 g COD/g VSS·day) and pH was 5;
[c] Percentage of carbohydrates in ECP.
[d] Percentage of proteins in ECP.

Methanosaeta, fermentings filaments) or small aggregates (e.g., syntrophic consortia and *Methanosarcina*) can serve also as nuclei. Finally, syntrophic consortia, at least in some studies (54,55,56), appear to be better nuclei than *Methanosaeta*, *Methanosarcina*, and fermenters.

Colonization

Once the nuclei are formed, microorganisms need to attach to them in order to be retained in the system, which results in the formation of granules. The following factors can affect the adhesion process.

Divalent Cations and Cation Polymers. Divalent cations such as Ca^{2+} and Mg^{2+} can promote granulation in two ways. First, they condense the diffusive double-layers resulting in a stronger effect of van der Waals attractive forces. Second, they may directly neutralize the negatively charged cell surface. Mahoney and coworkers. (57) confirmed this experimentally. They shortened the start-up time of a process treating a synthetic wastewater consisting of acetate, propionate, and butyrate through the addition of 2.5 mM Ca^{2+}. Compared to a system without Ca^{2+} addition, the granules formed in the system with Ca^{2+} addition were larger and exhibited a settling ability three to four times higher. Similarly, addition of 0.2 mM Mg^{2+}, 0.34 mM Ca^{2+}, and trace elements enhanced the granulation in a mesophilic UASB treating sucrose (58). However, too much Ca^{2+} can deteriorate the granulation process. For example, Thiele and coworkers (46) demonstrated that the addition of 25 mM Ca^{2+} caused washout of the biomass.

Schmidt and Ahring (59) studied the importance of Mg^{2+} concentration (0 to 100 mM) for the formation of stable granules (using acetate as the substrate at 55 °C). At very low Mg^{2+} concentrations (0 and 0.5 mM), the biomass was washed out at high rates (50% and 20%, respectively). On the other hand, high Mg^{2+} concentrations (100 mM) resulted in breakage of *Methanosarcina* cell aggregates and adversely affected the granulation process. In addition, the presence of high Mg^{2+} concentrations (30 and 100 mM) resulted in biofilm formation on the reactor wall rather than enhancement of granulation. The authors recommended that the Mg^{2+} concentration should be higher than 0.5 mM but lower than 30 mM to promote granulation.

Analyses of ECP indicated that Co^{2+} and Fe^{2+} were found in extracted ECP (60), in which they might bind to polymers and play important roles in linking ECP, which is usually negatively charged. Furthermore, calcium and phosphorus were found to be the dominant elements in ashed ECP (39). They might also contribute to the stability of granules.

Adding cationic polymers during the start-up of an ASBR reduced the granulation time from four months to one month (42). The cationic polymers were believed to enhance granulation through bridging negatively charged bacterial cells and through the formation of large biomass aggregates via sweep-floc mechanisms.

Hydrophobicity. From the point of view of surface thermodynamics, the free energy change during adhesion of two identical bacteria is determined by liquid surface tension (γ_{LV}) and the surface tension of the bacteria (γ_{BV}) (61). Calculations demonstrated that the adhesion of hydrophilic cells was favored for γ_{LV} less than 50 mN/m, and adhesion of hydrophobic cells was favored for γ_{LV} greater than 55 mN/m. When γ_{LV} was between 50 and 55 mN/m, neither process was favored. Contact angle measurement of pure cultures showed that, in general, fermenters are more hydrophilic than acetogens and methanogens (62). Granules treating sucrose wastewater consisted of three layers, an outer hydrophilic layer, a middle hydrophobic layer, and a core with an intermediate level of hydrophobicity. When the surfactant linear alkylbenzenesulfonate was added to a UASB reactor fed with sucrose to lower the γ_{LV}, the outer layer became more hydrophilic and the middle layer more hydrophobic (62). These granules had the advantage that biogas bubbles did not adhere strongly to their more hydrophilic outer layers and thus were less susceptible to washout than granules with less hydrophilic outer layers, resulting in higher reactor stability when the volumetric loading rate was high (36,61). It appeared that microorganisms in the granules were able to change their position according to the γ_{LV}. When the feed contained higher levels of VFAs, the surface of the granules became more hydrophobic because of the presence of fewer fermenters.

In addition, protein degraders are very hydrophilic compared to other fermenters (62). Their high γ_{BV} might explain why it is difficult to form granules in protein-rich wastewater. Daffonchio and coworkers (62) demonstrated that the addition of a polycation, which reduces the γ_{BV} of the cells, to a wastewater consisting of 20% molasses and 80% peptone with an intermediate liquid surface tension ($\gamma_{LV} = 55$ mN/m) increased the yield of granules significantly (60 to 75%).

Modification of hydrophobicity can help in certain reaction systems, but does not promote granulation per se because granules should contain both hydrophilic fermenters and hydrophobic acetogens and methanogens. Changing the level of surface tension will only favor one type of cell. Two lab-scale UASB reactors were started with sludge from a full-scale anaerobic digester in a yeast production plant (63). Linear alkylbenzenesulfonate was added to one of the reactors to reduce the liquid surface tension to 48 mN/m. No granulation was observed in either of the reactors 50 days after start-up. When the sugar level in the wastewater increased from 7% to 27%, granules formed in both reactors at the same time.

Species-Specific Interactions. Some species prefer to form aggregates with certain species rather than with others. This type of aggregation may be determined by the production and consumption of intermediates, for example, in the case of syntrophic interactions.

In batch experiments with pure cultures, butyrate-degrading bacteria (strain BH) formed strong aggregates with *M. formicicum*, but not with *M. hungatei* (50). In addition, propionate-degrading bacteria (strain PT) only formed strong aggregates with *M. formicicum* when a

Methanosaeta sp. was present. Wu and coworkers (50) demonstrated that *M. formicicum* played a very important role during granulation when a number of defined species (strain BH, strain PT, *Methanosaeta* sp. strain M7, *M. formicicum* T1N, and *M. mazei* T18) were used. *Methanobacterium formicicum* covered the surface of *Methanosarcina* aggregates and served as a link between the nuclei and the other cells. This study also demonstrated the importance of a *Methanosaeta* sp., which encouraged the aggregation between *M. formicicum* and strain PT and thus helped with syntrophic degradation.

Extracellular Polymers (ECP). ECP is an alternative name for glycocalyx, which is defined as the polysaccharide-containing structures from bacterial (or archaeal) origin located outside the cell wall (64). It contains polymers of saccharides, proteins, lipids, phenols, and nucleic acids (38). ECP can be present in forms of slime layers or capsules and appears outside of almost all types of prokaryote cells (64). ECP is sometimes also referred to as extracellular polymeric substances (EPS). However, EPS also stands for extracellular polysaccharides. Here, we use ECP to represent extracellular polymers, and EPS to designate extracellular polysaccharides.

As discussed previously, ECP play an important role in granulation. They help to form strong bonds between nuclei and cells. Also, they help to trap the offspring of attached cells to form microcolonies. This theory was supported by MacLeod and coworkers (21) who found ECP in all three layers of sucrose-fed granules.

The ECP content in granules ranges from 0.6 to 20% of VSS (38), and is much lower than in activated sludge (39). The main components of the ECP extracted from granules are proteins and polysaccharides, and a typical ratio between these two components is 2 : 1 to 6 : 1 (w:w). The lipid contents are usually very low, only 0.02 to 0.05% of VSS.

The production of ECP depends on the growth conditions and characteristics of wastewater. At high substrate concentrations and thus, high growth rates, the ECP content of anaerobic sludge is high, and vice versa (65). Excessive production of ECP can have a negative effect on granulation because the negative surface charge increases with increasing ECP yields (39) in a linear relationship (65). When the substrate is propionate or butyrate, the increase of negative surface charge is linearly correlated with the increase of protein content of ECP. However, when the substrate is glucose, the negative surface charge increases with both protein and carbohydrate content (65).

Schmidt and Ahring (66) determined the ECP content of six types of granules treating different wastewaters at different temperatures. It was found that granules treating complex substrates have higher total ECP and higher polysaccharide and protein contents, but lower lipid contents than those fed acetogenic and methanogenic substrates. Methanogens and acetogens seemed to have a limited ability to produce ECP, particularly polysaccharides, possibly because the production and excretion of ECP require too much energy. On the other hand, fermenters were believed to be the major organisms, which produce polysaccharides (38). For granules grown on acetogenic or methanogenic substrates, proteins may play an important role in granulation, and lipids might be compensating for the lower amounts of polysaccharides and proteins. The authors also pointed out that high ECP levels are not necessary for the production of active granules.

Thermophilic granules were found to have a lower total ECP yield as well as a lower content of polysaccharides and proteins, but higher lipid levels (66). Similar observations for thermophilic granules were reported by other researchers (67,68). This indicates that the production of polysaccharides and proteins may be limited, or that they are degraded faster under thermophilic conditions. It was also found that thermophilic granules were not as strong as mesophilic ones (68), which might be due to the lower total ECP in thermophilic granules.

In general, fermenters are believed to play an important role in producing ECP and enhance the granulation process. Using vinasse as the feed for a UASB reactor, Vanderhaegen and coworkers (47) demonstrated that preacidification of the feed resulted in a growth yield of granules ten times lower than that fed with fresh vinasse. The authors believed that the presence of high-energy carbohydrates favors granulation by enhancing the growth of populations of fermenters, which produce ECP. First, VFAs produced through the degradation of high-energy carbohydrates provide selective conditions for fermenters, which are insensitive to VFAs. The insensitiveness is acquired by secreting ECP. Second, because of the production of hydrogen, fermenters that do not produce hydrogen are favored, such as *Propionibacterium*, which can effectively produce ECP. In another study (discussed earlier), granulation only happened when the sugar content in the feed increased to 27% (in terms of COD) (63). Yoda and coworkers (44) found that when a reactor was fed VFAs (acetate, propionate, and butyrate), only 10 to 40% of the biomass changed to granular sludge, whereas in the reactor fed with glucose or molasses, all the sludge became granular. The authors believed that the presence of fast-growing fermenters produced large amounts of ECP, which was beneficial for granulation.

However, the addition of fermenters to a UASB reactor resulted in flotation of granules (69), suggesting the need of the right types of fermenters (i.e., fermenters with suitable surface characteristics). In another study by Brito and coworkers (70), granulation only started in a UASB reactor inoculated with digested activated sludge when the VFA content of a low strength wastewater (1,000 to 1,500 mg/L COD) changed from 15% to 85%. The authors found that only fluffy flocs were formed when the feed contained 15% VFA and 85% glucose. The polysaccharide ECP was very high in the fluffy flocs as a result of the predominance of fermenters, and it decreased after granules formed. The same changes were observed for protein ECP. The electrophoretic mobility of the biomass was less negative after granulation. This could relate to the predominance of *Methanosaeta*, which has a less negative surface in comparison with other anaerobic microorganisms isolated from granular sludge (71).

Beside fermenters, some other microorganisms are also found to be important in producing ECP for granulation. For example, *M. arboriphilicus* can synthesize all amino acids but cysteine. When hydrogen and nitrogen supply is high and cysteine is limiting, *M. arboriphilicus* cells secrete extra amino acids as peptide polymers. These polymers are believed to be important for granulation (72). TEM observation of granules fed VFAs showed that cells in the granules were surrounded by ECP and attached to each other by fibrous polymers (73). Chemical analysis of the components of EPS of the granules demonstrated that they were the same as EPS of *M. mazei* and *M. formicicum*. The authors concluded that these two microorganisms, especially *M. formicicum*, contributed significantly to the production of the ECP of anaerobic granules, and facilitated granulation.

The importance of the effect of polymers on granulation was also demonstrated by El-Mamouni and coworkers (74). UBF reactors were started up with syntrophic consortia as inoculum, or disintegrated biomass with natural or synthetic polymers. The granulation rate of the reactor with natural polymer was the highest, whereas the one with syntrophic consortia was the lowest. Metabolic activity tests on the three types of granules showed that polymer enhanced granules had higher substrate activities on acetate and ethanol, but demonstrated lower substrate activity on glucose compared to granules developed from syntrophic consortia.

In summary, ECP contributes to granulation by allowing microorganisms to stick together. The proper amount of ECP can make granules stronger. In addition, it can make the granules more hydrophilic and prevent flotation (75). Fermenters can produce large amounts of ECP with high-carbohydrate content. Their presence could enhance granulation. However, excessive ECP (as in the flocs) causes repulsion between cells and prohibits granulation (38). Moreover, *Methanobrevibacter* spp., *Methanobacter* spp., and *Methanosarcina* spp. also can be important in secreting ECP for granulation.

Colonization involves the attachment of different organisms to the nuclei as a mechanism to be retained in the reactor. In addition to interactions between different organisms, cell surface characteristics play an important role at this stage. Granulation can be enhanced by stimulating the production of ECP by selecting for populations that produce significant amounts of ECP and by the addition of chemicals such as surfactants, cations, or synthetic polymers.

START-UP PROCEDURES FOR REACTORS THAT UTILIZE GRANULAR SLUDGE

The ultimate goal when studying granular sludge and granulation processes is to change reactor designs and operational strategies to improve the start-up and performance of anaerobic wastewater treatment systems. There are two types of start-up procedures for UASB reactors, namely, primary start-up and secondary start-up. A primary start-up indicates that the inoculum consists of nongranular sludge (e.g., anaerobic digester sludge and crushed granular sludge). Some general guidelines for primary start-up of UASB reactors were summarized by Lettinga (5). These guidelines reflect the current understanding of the mechanisms involved in granulation. For instance, given the importance of nuclei, it is recommended to use inert carrier particles or crushed granules to promote start-up (40). Similarly, based on the fact that *Methanosaeta* spp. usually are present at high levels in various granules and play an important role in granulation, it is generally recommended to maintain the acetate level below 200 mg/L to promote the growth of *Methanosaeta*. Furthermore, given that *M. arboriphilicus* secretes ECP and can enhance granulation, Wentzel and coworkers (72) suggested that a UASB should be operated in plug-flow or semi plug-flow pattern with high hydrogen partial pressure (P_{H2}) in the lower zone of the reactor. This should enhance the growth of *M. arboriphilicus* and promote secretion of ECP, thus enhancing granulation. In addition, because fermenters can produce large amounts of ECP, wastewaters containing high-energy substrates (e.g., sugars) should be ideal for granulation. However, because of the high growth rate of fermenters, it may be necessary to control the levels of fermenting bacteria (40). For example, an excessive abundance of fermenters can result in fluffy granules as observed in a UASB reactor fed with 85% glucose and 15% VFAs (acetate and propionate) at low COD concentrations (1,000 and 1,500 mg/L) (70). The mixing level and/or the upflow velocity can be adjusted to selectively remove some of the fermenters from fluffy granules (19).

As an alternative to primary start-up, a reactor can be started up with (nonadapted) granular sludge (76). This is called secondary start-up. With more and more full-scale UASB reactors in operation, excess granular sludge is becoming available. As a result, secondary start-up is becoming more popular.

As discussed in the previous sections of this article, the microbial composition and granular structure change when feed composition and operating condition change. These changes are likely to cause problems when nonadapted granular sludge is inoculated into a new reactor. Problems often associated with secondary start-up have been summarized by Lettinga in several reviews (2,5,76):

- Dispersed fermenters present in wastewater can act as carrier material for methanogens, or can attach to granules. Both phenomena result in flotation.
- Flotation can also be caused by the formation of hollow spaces inside granules and/or the formation of poorly gas-permeable scales of organic (e.g., lipids) or inorganic (e.g., $CaCO_3$) scales, which retard or completely obstruct the escape of biogas bubbles.
- Granules may disintegrate, which can result in a serious drop in the amount of granular sludge biomass. Disintegration can be caused by changes in loading rate, substrate types, or level of acidification of the substrate.
- Granules can become too big, which may lead to serious substrate transport limitations.

- It may also be difficult for organisms with low growth rates to develop to high levels in nonadapted granular seed sludge. For example, it may take a long time to increase the levels of organisms required for the degradation of specific compounds present in a new wastewater or of thermophilic organisms when starting up a thermophilic reactor using mesophilic granules.

Because of these problems, in particular problems associated with granular structure, crushed granules may be a better inoculum than intact granules (76). Crushed granules can provide nuclei and the specific microbial populations necessary for granulation.

CONCLUSION

Three decades have passed since the development of the UASB reactor and the use of granular sludge to treat wastewater were first reported. During this time period, the UASB reactor and its derivatives have demonstrated their potential as high-rate anaerobic wastewater treatment systems, and our understanding of granules and granulation processes has improved significantly. This article reviews achievements in understanding the microbial composition and the architecture of anaerobic granules, the mechanisms of granulation, and the role of different microorganisms in granulation processes. These achievements in turn have contributed to the improvement of start-up and operating strategies for reactors, which utilize granular sludge.

Some of the recent findings can be attributed to the development and application of new technologies for investigating complex microbial communities. In particular, nucleic acid based techniques (e.g., rRNA targeted probe hybridizations, PCR, and cloning) and techniques based on immunology (e.g., antibody techniques) have made it possible to study microbial community composition and structure without the limitations and biases of cultivation. However, our current understanding of granules and granulation processes is far from complete. For example, we still need to answer questions such as what are the metabolic activity levels within granules, how are substrates transferred into granules, and how do the characteristics of microorganisms influence the granulation process and the properties of granules. The ability to answer these questions largely depends on the availability of suitable techniques, such as in situ metabolic activity assays, in situ measurement of concentration profiles, and in situ microbial identification and determination of microbial characteristics.

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ANAMMOX. See ACTIVATED SLUDGE — MICROBIOLOGY OF NITROGEN REMOVAL

ANHYDROBIOSIS. See DESICCATION BY EXPOSURE TO SPACE VACUUM OR EXTREMELY DRY DESERTS: EFFECT ON MICROORGANISMS

ANTHRAX. See BIOTERRORISM

ANTIBIOTIC RESISTANCE. See HETEROTROPHIC BACTERIA AS INDICATORS OF WATER QUALITY; NOSOCOMIAL INFECTIONS; FECAL CONTAMINATION, SOURCES OF

ANYDROBIOSIS. See FREEZE DRYING: PRESERVATIONS OF MICROORGANISMS BY FREEZE-DRYING

AQUIFERS, ATLANTIC COASTAL PLAIN. See MICROBIOLOGY OF ATLANTIC COASTAL PLAIN AQUIFERS AND OTHER UNCONSOLIDATED SUBSURFACE SEDIMENTS

ARBUSCULAR MYCORRHIZAE. See MYCORRHIZAE: ARBUSCULAR MYCORRHIZAE

ARCHAEA, DETECTION OF.

See ARCHAEA IN MARINE ENVIRONMENTS

ARCHAEA: DETECTION METHODS

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During the last twenty years, our understanding of the phylogeny of living things has undergone significant change. The once commonly held notion that there were two basic cell types, the complex eukaryotes and the more simple, ancestral prokaryotes, was tested when molecular biochemical techniques became available to study an integral part of the protein synthesis machinery, Ribosomal RNA (rRNA) (1). Because rRNA is ubiquitous in nature and the product of highly conserved genes, comparing the sequence of nucleotides in rRNA of different organisms provided an excellent means for assessing the relatedness of two organisms. The relative length of time since the two organisms diverged from a common ancestor could also be estimated once the molecular clock is adequately calibrated against evolutionary events. Early studies showed that prokaryotes and eukaryotes had evolved from a common ancestor by separate pathways (2).

Closer examination of prokaryotes revealed a group as distinctly different, biochemically and structurally, from other bacteria as it was from eukaryotes (2,3). These microorganisms formed the third phylogenetic Kingdom and were named *Archaeabacteria* (1) to reflect their primitive evolutionary status; they are at least as old as the other major bacterial groups. The term *Archaeabacteria* was changed subsequently to the archaeal domain (4). The domain is divided into two Kingdoms (5). The first, *Euryarchaeaota*, contains members that are physiologically diverse and includes the methanogens that produce methane from hydrogen and carbon dioxide or other simple carbon compounds in a strictly anaerobic environment (6), extreme *halophiles* that grow aerobically in highly saline environments and maintain an extremely high internal salt concentration (7), and *thermophiles* found in close proximity to terrestrial and shallow water–hot springs and deep-sea hydrothermal vents. The second kingdom, *Crenarchaeaota*, includes members of the extremely thermophilic sulfur-metabolizing phenotype. Originally known as *thermoacidophiles*, these bacteria have also been well studied (8).

In addition to their ability to inhabit specific "extreme" environments and the presence of identifying 16S rRNA sequences, there are several other characteristics held in common by the Archaea studied thus far. Archaeal cell walls are complex structures but do not contain muramic acid common to bacteria (9). Cell membranes of the Archaea are made of lipids containing ether linkages and phytanyl chains (10). Transfer RNA lacks ribothymidine in one very specific loop in the secondary structure of the molecule (11). The subunit structure of RNA polymerase is unique (12), and several unique coenzymes have been found among the methanogenic Archaea (11). Many of these characteristic molecular or biochemical traits can be exploited to detect Archaea, and methods to do so are described in this chapter.

CULTURING METHODS

Classical microbiological techniques have been applied extensively to the culturing of Archaea. Isolation media for Archaea are selective in themselves, and growth is a direct method of detection as few other bacteria share the growth optima common among the Archaea. However, there is some overlap, and selective culturing methods have been used to detect Archaea in mixed communities. Many Archaea are enriched in media containing antibiotics such as kanamycin, penicillin, or vancomycin, which inhibit the cell walls of bacteria.

Bile Salts and Antibiotics

Halophilic Archaea grow at salt concentrations between 1 M and 4.5 M (7). Halophilic bacteria can also exist at the lower end of this range, although some, such as members of the family *Halomonadaceae*, do live in saturated brines. Low concentrations of bile salts such as sodium deoxycholate or sodium taurocholate have been shown to lyse *Halobacterium* and related Archaea, whereas both halophilic bacteria and *halococci* remain intact. Antibiotics such as anisomycin behave in a similar fashion (13). In this way, halophilic Archaea can be detected indirectly and population sizes can be estimated among mixed communities (14,15).

Most Probable Number (MPN)

Methanogens produce methane by metabolizing carbon substrates and hydrogen within reduced environments (Eh below −330 mV). Although most methanogens are *mesophiles*, marine species include *psychrophiles, thermophiles*, and *hyperthermophiles* and they are ubiquitous despite their special growth requirements (16).

The concentration of viable cells from these various environments can be roughly calculated using MPN techniques. This involves the mathematical estimation of the viable count from the fraction of multiple cultures that fail to show growth in a series of dilution tubes containing a suitable medium (17). The procedure involves preparing several replicate dilutions in a growth medium selective to the particular methanogen under study and noting the fraction of tubes showing bacterial growth. Tubes with no growth are assumed to not have received even a single cell capable of growth. Accuracy in detecting and estimating methanogenic biomass using the MPN technique increases with the use of increasing numbers of culture tubes. This, combined with the use of anaerobic media, can result in

this approach being a cumbersome technique when applied to methanogenic Archaea.

Despite these drawbacks, the MPN method has been applied to many anaerobic environments, such as rice fields (18), where selective media enabled the enumeration of the populations from four major methanogenic trophic groups. In another study, MPN counts showed that viable methanogens coexisted with homoacetogens at depths of 45 m to 446 m below sea level in a deep-granite aquifer (19). In a third study, MPN of methanogens from a rumen were used to elucidate the relationship between Archaea and ciliate protozoans in the gut (20).

MOLECULAR TECHNIQUES

Despite the search for new strains, all Archaea isolated using classical microbiological culturing techniques fall into one of the three basic phenotypes. Traditional laboratory enrichment and culturing methods identify microorganisms based on metabolism, morphology, and physiology (16). The current opinion is that this method of detection uncovers less than 0.1% of microbes in the environment (21–23). Molecular techniques have provided a means by which to determine the presence of Archaea and to characterize them while circumventing the need for culturing (16,24). However, the physiology and ecological role of many of the "uncultivatable" Archaea still requires elucidation.

Molecular Cloning

The advent of nucleic acid sequencing techniques have opened up the potential to study archaeal members of microbial communities from environments in which they were never before known to exist (25,26). Short segments of deoxynucleotides have been used as probes to bind very specifically to targeted sequences of 16S rRNA. This allows the total chromosomal DNA of a specific group to be detected in a complex microbial population. Sections of archaeal 16S rRNA genes bound by Archaea-specific primers are amplified by PCR (see RIBOTYPING METHODS FOR ASSESSMENT OF IN SITU MICROBIAL COMMUNITY STRUCTURE, this Encyclopedia). Individual fragments are then cloned and sequenced, and the sequences are then compared to known Archaeal rDNA sequences. In this way, the phylogeny of Archaeal members of a population can be determined quickly and without the need for classical culturing techniques (16).

These molecular techniques were applied to marine plankton (25). Sequences that were speculated to be from a previously undescribed Archaeal group were found. These microorganisms may have diverged from ancestors of other previously characterized microorganisms very early in evolution. More recently, these techniques have been used to detect Archaea in cold Antarctic marine surface waters, where they were estimated to comprise 34% of the prokaryotic biomass (26). Archaea were found to be widespread in the deep sea when this method was applied to samples taken at great depth (1,000–3,000 m) from both the Atlantic and Pacific Oceans (27). These researchers concluded that previously unknown and uncultured Archaea may be among the most abundant microorganisms on earth. The microbial community of Deep Lake in Vestfold Hill, Antarctica, was found to be made up almost entirely of halophilic Archaea, including several novel lineages (28). In the past, conventional culturing methods had resulted in the isolation of one halophilic Archaeon from Deep Lake (29).

Denaturing Gradient Gel Electrophoresis (DGGE)

Another technique that avoids the need for cloning has been developed. It involves the fractionation of PCR-amplified 16S rDNA fragments on polyacrylimide gels containing a denaturing reagent consisting of formamide and urea. The level of denaturation of individual sequences is used to resolve mixtures of DNA fragments. Gel patterns generated can be used to compare different environmental samples and individual bands can be directly analyzed by sequencing. This method has been applied to study microbial communities in deep-sea sediments (30) in which archaeal 16S rRNA represents 2.5–8.0% of the total prokaryotic DNA.

Fluorescent In Situ Hybridization (FISH)

Chemical staining has been used in the past to detect cells in culture and in environmental samples. These methods tend to be nonspecific (31,32). Fluorescent antibodies used as stains can be highly specific (33–35), but the strain specificity is dependent on the use of well-studied microbes (24). A staining method that involves hybridization of oligodeoxynucleotide probes (24) has been described. The probes binding to complementary regions of 16S rRNA sequences (17–34 nucleotides in length) are chosen because they are specific to a particular phylogenetic group (e.g., Archaea). This molecular probe–staining method is highly specific, but requires no previous knowledge of the organisms under study, and has successfully been applied to archaeal cells (see FLUORESCENT PROBES FOR IN SITU ANALYSES OF MICROBIAL COMMUNITIES). The probes are tagged with a fluorescent label for microscopic examination. Cells are harvested from a culture or environmental samples, resuspended in buffer, and fixed with formaldehyde. Cells are then smeared on a slide coated with gelatin, dried and fixed again (36). Smeared slides are treated with buffered fluoro-labeled probes, incubated in hybridization mixture, and then viewed.

Optical Tweezers

An extremely sensitive microscopic technique has been used to complement the accuracy of these molecular detection methods. A highly focused infrared laser is coupled to a computer-controlled inverse microscope and this system, called "*optical tweezers*" is used to micromanipulate single Archaeal cells from a mixed microbial population for culturing. Using this method, it can be demonstrated that cultures obtained subsequently from this single cell correspond to the one detected in situ (16,37). In the past, such detection and description of Archaea would have been possible only by in situ phylogenetic analyses. This new method was used to isolate anaerobic Archaea, which, at 83 °C, produce

grapelike aggregates of coccoid cells that were previously overgrown during enrichment by filamentous cells.

CELL ENVELOPE — CHEMICAL TECHNIQUES

The cell envelope includes the cell wall and cell membrane constituents. The latter includes lipid in the membrane bilayer and quinones. With reference to Archaea, these specific constituents and methods to detect them are described in the following section.

Cell Wall

Methods to detect Archaea by measurement of cell wall components are neither direct nor as conclusive as either molecular (previous section) or signature lipid techniques (following section). Nevertheless, a range of protocols exist for measuring cell wall constituents and methods to detect them are described in the following paragraphs.

In bacterial cell walls, a polymer of peptidoglycan (or murein) provides strength and rigidity to the cell. Although it differs slightly from one bacterial species to the next, three main components form the basic structure: (*1*) *N*-acetylmuramic acid (NAM), (*2*) *N*-acetylglucosamine (NAG), and (*3*) a tetrapeptide containing D-amino acids (38).

Archaea possess cell walls that are markedly different from those of bacteria (39–41). Changes in chemical composition of the constituents are reflected in structural differences. Cell walls of the Archaea, when they are present, do not contain peptidoglycan as muramic acid, and D-amino acids are absent. Instead, glycoproteins or polysaccharides and proteins give rigidity to the cell wall. The composition of the cell walls of the various members of the Archaeal group reflects the heterogeneity of the group. No common cell wall component, such as muramic acid found in bacteria, is present in the Archaea (42). In Archaea such as *Thermoplasma*, the cell wall is completely absent (43). The gram reaction is highly variable among the Archaea, and is dependent on the combination of cell wall components involved in this structure (44).

Pseudomurein

The cell wall components of Archaea have not been as thoroughly studied as those of bacteria, but there is enough evidence to support their use as taxonomic markers (44). Archaea of the genus *Methanopyrus* and those of the order *Methanobacteriales* have cell walls composed of pseudomurein (45). This polymer consists of peptide subunits (Lys, Glu, Ala or Thr) that cross-link glycan strands composed of an N-acetylated amino sugar (glucosamine or galactosamine) that is common to bacteria and an *N*-acetyl-L-talosaminuronic acid that is unique to Archaea (42,44).

Although there are many different variations in the peptide subunit of murein in bacteria, only one structure for pseudomurein has been found so far in Archaea. However, substitution in the peptide subunit can occur. For taxonomic purposes, elucidation of the chemical composition of pseudomurein is sufficient (45). Initially, the cells are mechanically disintegrated and then digested with trypsin (40,46,47). Purified cell walls can be further degraded into constituent amino acid and amino sugar residues by acid hydrolysis. Identification and quantification of amino acids and sugars are carried out with an amino acid analyzer. Talosaminuronic acids may be qualitatively determined after short-term hydrolysis (4 M HCl, 30 minutes, 100 °C) (40,45). After isolation by thin-layer chromatography, the acid and lactone forms of the acid are determined by an amino acid analyzer (41). Neutral sugars are converted to their alditol derivatives, which are then identified by gas chromatography (GC) (48).

S-Layers (Surface Layers)

Protein S-Layer. Methanogens of the order *Methanococcales* have a cell envelope consisting of the cell membrane and a layer of nonglycosylated protein subunits arranged hexagonally. This outer layer is referred to as an S-layer, and differences in the molecular weights of S-layer proteins are useful taxonomic markers (49). Methods for the isolation of S-layers and S-layer proteins of methanococci have been described (44). After mechanical disruption, cells are treated with DNAase and then a surfactant. The S-layer is extracted by a modified Bligh-Dyer (50) extraction. Dialysis is followed by gel electrophoresis to obtain the S-layer protein. Protein is determined by measuring absorbance at 750 nm (51).

Glycoprotein S-Layers. The surface layer of *Halobacterium halobium* is made up of glycoprotein subunits that maintain the shape of the cell (52). This S-layer dissolves in salt concentrations below 12% in contrast to S-layers of other cells, which tend to aggregate in water.

In *Methanothermus* spp., S-layer glycoproteins and glycoprotein sugars are characteristic. Extraction with trichloroacetic acid followed by reversed-phase chromatography yields the purified glycoprotein (53). Molecular weights obtained through SDS–polyacrylamide gel electrophoresis together with other features can be useful taxonomically (44,54).

The glycoprotein of the S-layer in *Sulfolobus acidocaldarius* contains predominantly the polar amino acids serine and threonine. To isolate and quantify these components the acidic culture is neutralized, and after centrifugation the buffered pellet is digested with DNAase. Cell envelopes are disintegrated with heat (60 °C) at pH 9, and the resultant glycoprotein is purified by molecular-sieve chromatography (55). After acid hydrolysis, amino acid residues are identified and quantified using an amino acid analyzer (44).

Methanochondroitin

Methanochondroitin is a cell wall component unique to *Methanosarcina*, with a few exceptions (*M. acetovorans* and *M. frisia*) that have only a single S-layer. A polymer of uronic acid and *N*-acetylgalactosamine residues present in a 2 to 1 molar ratio gives cell aggregates their cuboid appearance (56). Methanochondroitin is isolated according to methods used for the isolation of pseudomurein. Cells are mechanically disintegrated and then digested

with trypsin (40,46,47). Uronic acids are subjected to methanolysis and then derivatization before identification by capillary GC (48).

Heteropolysaccharide

Methods have been described for the isolation and identification of heteropolysaccharide and constituent components (41,57). To date they have not been demonstrated to be taxonomically useful and their use for detecting Archaea may therefore be limited. Of interest is the observation that *Haloccocus morrhuae* has a rigid cell wall of a complex heteropolysaccharide that prevents cell lysis at low-salt concentration.

Microscopy

Electron micrographs of thin sections of Archaeal cells reveal a variety of profiles, which give rise to two basic gram reactions. Gram-positive Archaea are most similar to gram-positive non-Archaea cells. The cells have a pseudomurein layer surrounding the cytoplasmic membrane and in some cases a heteropolysaccharide or a single layer (S-layer) of protein or glycoprotein as well. Most Archaea are gram-negative and contain an S-layer surrounding the cytoplasmic membrane. Cells containing mitochondroitin and an underlying S-layer may gram stain positive or negative.

Thin sections of Archaea are prepared after cells are fixed in gluteraldehyde, washed, dehydrated, and embedded. After cutting, thin sections are contrasted with lead citrate, washed, dehydrated, and then contrasted again. Cells may also be subjected to platinum shadowing or negative staining before electron microscopy (58).

Membrane Lipids

General Considerations. Along with the cell wall, the cell membrane is a major component of the cell envelope. A number of terms have been used to describe molecules used for the measurement of lipid components in cultured Archaea and environmental samples. These include molecular marker, biomarker, lipid biomarker, signature lipid, signature lipid biomarker, and chemical fossil. In this chapter, the term signature lipid is used as it is more often the term preferred by microbial ecologists. The main criteria for an ideal signature lipid are: (*1*) source specificity and (*2*) conservative behavior.

Source specificity refers to the link between a signature lipid and its source. Under ideal conditions, this link should be direct and unique (59). In showing conservative behavior, a signature lipid should be stable over timescales relevant to the processes under study. Most signature lipids will be subject to microbially-mediated transformations and/or breakdown in the environment. To assess the potential use of a signature lipid as a quantitative tool, we need to understand all aspects of its behavior in the environment.

The recognition that Archaea contain unique lipid profiles comprising ether-linked isoprenoid alkyl side chains relative to bacteria and eukaryotes has encouraged the assay of ether lipid distribution in pure isolates and field samples. Specific structures of selected archaeal signature lipids are shown in Figure 1. The isoprenoid side-chains are usually of C_{20} and C_{40} chain length. When two C_{20} isoprenoid side chains are linked to a single glycerol moiety, the structure is termed a diether (DE) lipid (diphytanylglycerol, archaeol). When two C_{40} side chains are linked to two glycerol units, the structures are termed tetraether (TE) lipids (bidiphytanyldiglycerol, caldarchaeol). The diether and tetraether "lipid cores" refer to the hydrophobic or lipid portion of the membrane lipids.

C_{15} and C_{25} side-chains may be present in the diether lipids from selected Archaea, as may be a hydroxy group at the C_3 position of the isoprenoid side chains. A variation to the DE core lipid occurs with the macrocyclic DE present in deep-sea methanogens (60); a single C_{40} side chain is linked at both ends to one glycerol molecule. Variations to the tetraether lipid structure include the presence of between zero and eight cyclopentane rings.

Other variations to the tetraether structure include the presence of a cyclic $C_6H_{11}O_5$ unit at the second glycerol, calditoglyceroarchaeol (61,62). In Archaea, the diether and tetraether lipids are generally present in phospholipid and glycolipid structures.

Within the Archaea, the compositions of the lipid cores, phospholipids, and glycolipids can be used to distinguish between the three subgroups, extreme *halophiles*, methanogens, and extreme *thermophiles* or *thermoacidophiles*, and to some extent between members of the different genera within each subgroup (63). The analysis of pure cultures provides taxonomic information as the distribution of specific ether lipids may be restricted to certain subgroups of Archaea (Table 1). Application of the signature lipid assays to field samples then allows the detection of Archaea by microbial ecologists, organic geochemists, and other scientists interested in understanding the role of Archaea in the environment.

In their use of signature lipids, microbial ecologists are often more interested than organic geochemists in the viable component of the community. As all living cells are surrounded by a membrane containing polar lipids, the component fatty acid and ether lipid side

Table 1. Listing of Main Archaeal Groups and a Summary of Their Ether Lipid Composition (adapted from reference 63). See Table 2 for explanation of abbreviations.

Group/Genus	Ether Lipids Present DE, Diether; TE, Tetraether
Halophiles *Halobacterium, Haloferax, Haloarcula, Halococci*	DE (diphytanylglycerol, archaeol)
Haloalkaliphiles	DE
Methanogens *Methanococcus, Methanosarcina, Methanobacterium, Methanothermus*	DE and TE (bidiphytanyldiglycerol, caldarchaeol). 3-OH group may be present in the isopranyl side chains.
Thermophiles *Desulfurococcus, Thermoplasma, Sulfolobus, Thermoproteus*	DE and TE. TE may contain cyclopentane rings in the isopranyl side chains.

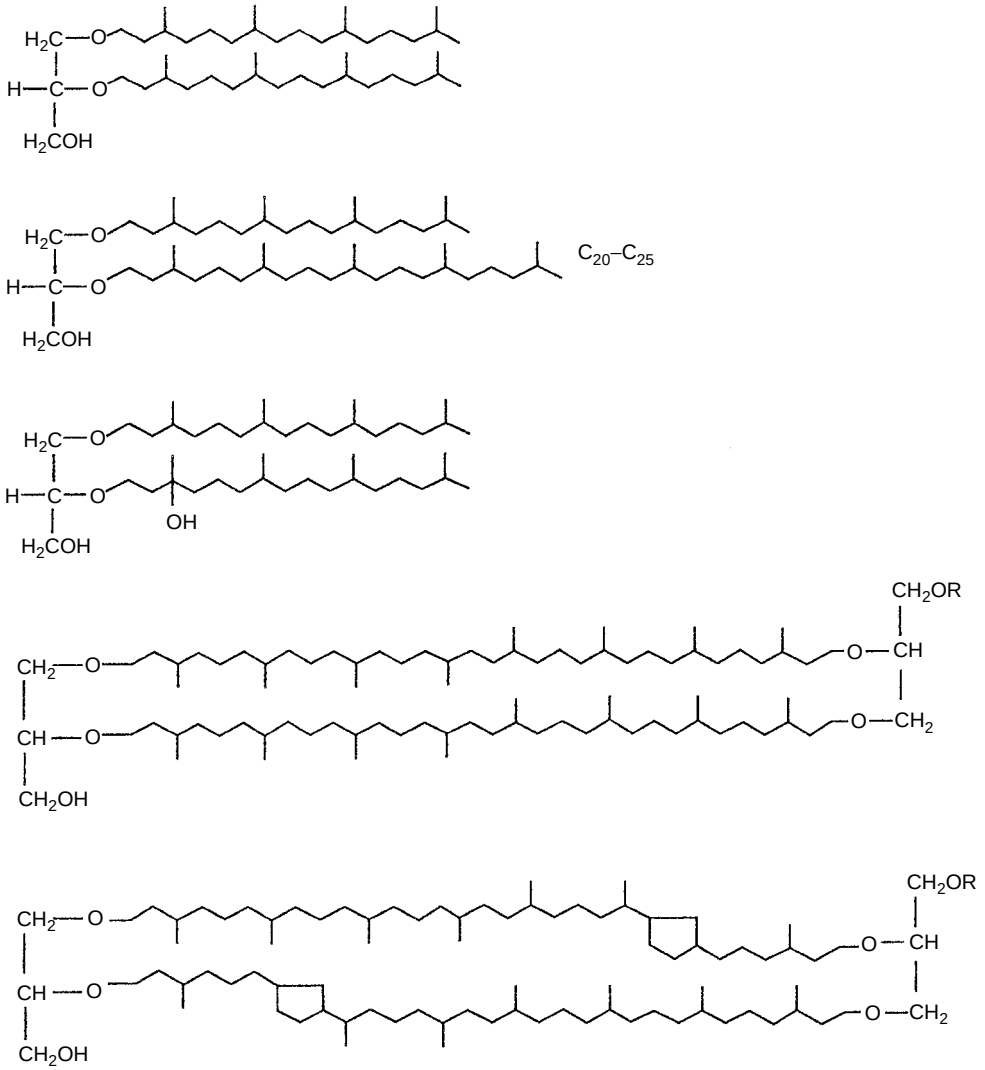

Figure 1. Structures of ether lipid cores. Upper three diether (DE) core lipid structures: 2,3-O-diphytanylglycerol, archaeol; 2-O-sesterterpanyl-3-O-phytanylglycerol; 3-hydroxy-2-O-phytanyl-3-O-phytanylglycerol. Lower two tetraether (TE) core lipid structures: bidiphytanyldiglycerol, caldarchaeol; an example representative of a cyclopentanyl-containing caldarchaeol.

chains are considered to be several of the more important signature lipid classes (64). Unlike many other lipids, phospholipids are rapidly degraded within hours of cell death. Therefore, the examination of phospholipid-derived fatty acids of eubacteria and eukaryotes and phospholipid ether lipids of Archaea allows the viable community to be investigated (65,66). The concept and use of signature lipids is discussed more fully in the entry LIPID BIOMARKERS AND IMMUNOLOGICAL METHODS IN ENVIRONMENTAL MICROBIOLOGY.

Lipid Extraction and Fractionation

Total lipids are extracted quantitatively from most Archaea using a modified Bligh-Dyer one-phase (chloroform-methanol-water, 1/2/0.8, v/v/v) extraction procedure (50,63). *Halophiles* with rigid cell walls (e.g., *Halococcus* spp.) are subjected to sonication before extraction, and acidic conditions may be needed with certain methanogens (see following section). The lipid-containing chloroform layer is obtained by addition of chloroform and water to the Bligh-Dyer extraction (final solvent ratio, chloroform-methanol-water, 1/1/0.9, v/v/v). A representative scheme for the preparation of ether lipids is shown in Figure 2.

A combined lipid and DNA extraction method for environmental samples has been recently reported (67). At the phase separation stage of the Bligh and Dyer procedure, lipids partitioned into the organic phase and DNA partitioned into the aqueous phase. The DNA was not degraded during the lipid extraction procedure, although DNA recovery was 40 to 50% compared to samples treated by conventional DNA extraction techniques alone. An advantage of the Bligh and Dyer procedure was that it also resulted in DNA extracts from field samples containing lower amounts of humic material, compared to conventional DNA extraction. The method shows utility for the co-recovery of both signature lipids and DNA from a single sample; this is particularly useful when only

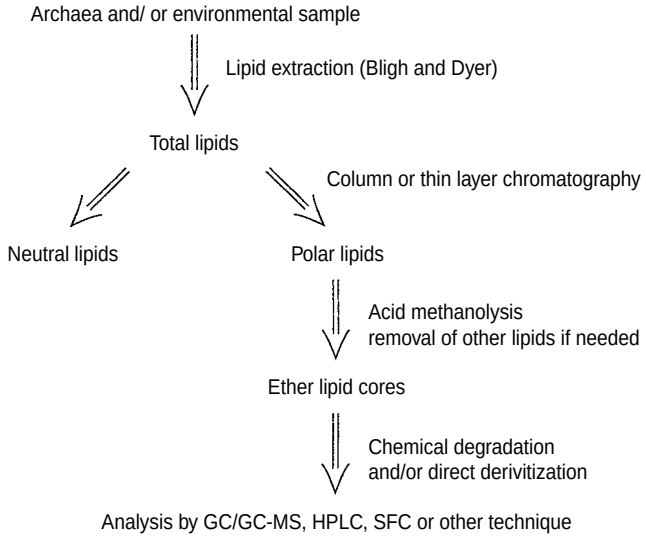

Figure 2. Flow diagram for the preparation and separation of total lipids from bacteria, Archaea, and environmental samples.

Table 2. Listing of Selected Abbreviations Used with Archaeal Components and Detection Methods

Abbreviation	Description
	Chemical Structures
DE	Diether
MDE	Macrocyclic diether
OH	Hydroxy-containing structure
PLEL	Phospholipid ether lipid
TE	Tetraether
	Instrumental and Other Methods Used to Separate Archaeal components
DGGE	Denaturing gradient gel electrophoresis
FISH	Fluorescent in situ hybridization
FTIR	Fourier transform infrared spectroscopy
GC	Gas chromatography
GC–MS	Gas chromatography — mass spectrometry
HPLC	High performance liquid chromatography
MPN	Most probable number
PCR	Polymerase chain reaction
rRNA	Ribosomal ribonucleic acid
SFC	Supercritical fluid chromatography
TLC-FID	Thin layer chromatography

small samples are available. This procedure was used with samples containing bacterial lipid (i.e., phospholipid fatty acids, PLFA), and is yet to be applied to archaeal-containing samples.

Total lipids are separated into lipid classes (neutral lipid, glycolipid, and phospholipid) by silicic acid column chromatography (68), acetone precipitation, or other procedures. TLC can also be used to separate individual components (see following section).

Degradative Procedures

Following extraction and column chromatographic fractionation of total lipid material, chemical breakdown of the complex lipids is undertaken to produce the diether and tetraether lipid cores. A standard strong acid methanolysis treatment of the complex lipids with 0.6–2 N methanolic HCl at 80–100 °C for 1–3 hours has been often used to produce the diether and tetraether side-chains.

Identification of the hydrocarbon type and chain length of diether and tetraether lipid cores is performed by cleavage of the ether linkages with $BCll_3$, BBr_3, or HI. The ether lipid is made to react with the reagent for 4 to 12 hours, typically at 100 °C (69). The alkyl halides or derived acetates produced by treatment with glacial acetic acid in an excess of silver nitrate or hydrocarbons produced by treatment with $LiAlH_4$, are then analyzed by GC. The analyses of ether lipids by these cleavage techniques are essential for their structural elucidation. However, such analyses involve many steps and generally result in low recoveries and therefore have been deemed as not appropriate for routine or quantitative analysis of environmental samples (70).

Thin Layer Chromatography (TLC)

Preparative TLC has been used to fractionate both polar lipids (e.g., individual phospholipids) and ether lipid cores (diether and tetraether). For separation of the polar lipids, the chromatography is generally by silica gel, and mixtures of chloroform/methanol/acetic acid/water (e.g., 85/30/15/5, v/v) (71) are used as the solvent system. In two-dimensional developments, ammonia replaces the acetic acid in the first development (71). Following preparation of the ether lipid cores by acid methanolysis, TLC has also been widely used to separate the ether lipid cores. Again, silica gel is the standard plate used with solvent systems composed of light petrol or hexane/ethyl ether/acetic acid mixtures (e.g., 50/50/1, v/v; 70/30/1, v/v) (71).

A simple TLC procedure has been used to resolve mixtures of tetraether lipid cores containing up to eight cyclopentane rings (72). Silica gel F254 was used with hexane/ethyl acetate (7/3, v/v) as the solvent system. For most TLC systems, components are qualitatively observed by using iodine vapor or dichlorofluoroscein spray (all lipids), or more selective sprays (e.g., ninhydrin, amino groups; acid molybdate, phospholipids; α-naphthol, glycolipids).

A TLC–flame ionization detector (TLC–FID) analyzer has been used to quantitatively analyze lipid mixtures from a range of sample matrices (73). The analyzer uses silica-gel coated glass rods with conventional development of the rods in solvent tanks for component separation. Detection of the separated lipid classes is by the sensitive FID; sensitivity is within the submicrogram to the 10-mcg range. The TLC-FID analyzer has not, to our knowledge, been used for the separation of ether lipid cores. This technique may be useful for both qualitative screening and quantitative analysis of both archaeal isolates and environmental samples, including determination of the concentrations of diether and tetraether.

Issues with Extraction and Other Processing Procedures

A few studies have reported the occurrence of unsaturated diethers in Archaea. The halophilic *Halobacterium lacusprofundi* isolated from an Antarctic lake was first reported to contain unsaturated diether lipid (74). Based on this finding, *Methanococcoides burtonii* was subsequently noted to also contain unsaturated diethers (75). Subsequently, it was also reported that novel 3-OH hydroxydiether lipids (hydroxyarchaeol) were present in a number of methanogens (76,77), and that the standard strong acid methanolysis treatment (0.6–2 N methanolic HCl) of these lipids produced various products including a methoxy derivative, 3-mono-O-phytanyl-sn-glycerol (78), and an unsaturated diether lipid (79). This finding was confirmed in further studies examining the ether lipid composition of *M. burtonii* in which it was noted that the unsaturated ether lipids were most likely produced during sample preparation (80,81). Based on these findings, we believe that the identification of the unsaturated diether in *H. lacusprofundi* may also be doubtful.

Production of the artifacts from the 3-OH hydroxyether lipids can be avoided by use of milder acid methanolysis conditions. The core ether lipids may be obtained without degradation, and in good yield, by mild acid methanolysis in 0.18% HCl (0.05 N) in methanol (71,79) or in 5% methanolic HCL/chloroform (1 : 27, v/v) at 50 °C for 24 hours (82).

Low recovery of ether lipids using the Bligh and Dyer procedure has been noted for *Methanobacterium thermoautotrophicum* and other methanogenic species containing highly polar and acidic lipids (82,83). These studies demonstrated that recovery of the ether lipids was increased by first extracting with the standard Bligh and Dyer procedure and then rapidly with a mildly acidic Bligh-Dyer one-phase solvent, chloroform/methanol/5% aqueous trichloroacetic acid (1/2/0.8, v/v/v). This method was also applied to environmental samples containing methanogens. The total lipid in the chloroform layer is washed using methanol and water to remove any traces of acid.

Compared with the considerable literature available on PLFA abundance in bacteria, only limited data are available for phospholipid ether lipid (PLEL) concentrations in Archaea. Conversion factors are routinely used to translate PLFA concentration results into either number of cells/g or number of cells/L; the average Bacterium is generally assumed to contain 100 μmoles of PLFA/g cells (dry weight) (64,84). However, the as-yet-limited quantitative data available for Archaea has shown large variations between species. Therefore, care may now be needed in the use of PLEL concentration data and conversion factors as is routinely performed with PLFA data. Notwithstanding this qualification, PLEL analysis will still prove to be a reliable tool for determining and comparing viable archaeal populations in environmental samples.

High Performance Liquid Chromatography (HPLC)

Several research groups have used HPLC procedures to separate the diether and tetraether core lipids. HPLC was first used to separate the diether core lipid derived from methanogens (85). The diether core lipids were initially obtained by acid methanolysis. The *p*-nitrobenzoyl ester of the diether core lipid was separated using a silica column and gradient elution (hexane to hexane-diethyl ether, 80/20, v/v) with detection at 254 nm. The tetraether core lipids were not separated using this system.

A further HPLC separation of the diether core lipids was obtained using 9-anthroyl derivatives (82,86). The derivatives were formed using 9-anthroyl nitrile with purification needed. Separation was then obtained using a C_8-bonded silica column employing a spectrofluorometric detector. The solvent was an isocratic acetonitrile/isopropanol mixture (80/20 v/v). The technique was also capable of separating the 3-OH diether core lipids.

The HPLC systems noted above only separate the diether core lipids. Several other procedures have been developed that enable simultaneous separation of both the diether and tetraether core lipids. The first HPLC system developed to separate both ether lipids used an amino silica column, with an isocratic solvent, hexane/n-propanol (87). Unlike the HPLC systems that separate the diether core lipids alone with detection of derivatized components, this system employed refractive index (RI) detection of underivatized diether and tetraether core lipids. An advantage of this system was an absence of impurities derived from the derivatization procedure. However, the RI detector is prone to baseline drift restricting the analysis to use of an isocratic solvent. The sensitivity of RI detection is also lower than for other common HPLC detectors. This system was shown to also separate the macrocyclic diether derived from *Methanococcus jannaschii*, a thermophilic deep-sea methanogen. Separation of the diether, tetraether, and macrocyclic diether obtained using this system is illustrated in Figure 3. Component identification was further confirmed by FT–IR (DRIFT mode) analysis of HPLC fractions. The various ether lipid cores were differentiated by Fourier self-deconvolution of infrared spectra. This HPLC technique has been applied in environmental studies to measure methanogenic biomass in an Antarctic lake and in digestors (87–89).

A second HPLC system capable of separating the diether and tetraether core lipids has been reported (90). The system comprised an Inertsil ODS-2 column and UV detection at 254 nm of dinitrobenzoyl (DNB) derivatives of the core ether lipids following gradient elution (acetonitrile/2-propanol, 40/60–20/80, v/v). The technique claimed to have greater sensitivity than the first HPLC system developed (87). However, based on the chromatograms presented, the diether was not adequately resolved from the internal standard (1,2-di-O-hexadecyl-rac-glycerol), and impurities introduced by the derivatization procedure tailed into both the internal standard and diether core lipid peaks. Furthermore, the preparation of DNB derivatives involved three additional steps, and verification by TLC of derivatization was also required. Several of the advantages claimed for this HLPC procedure have to be weighed against possible disadvantages.

Considerable advances have occurred in recent years with evaporative light-scattering detectors (ELSD), including application with a range of lipid classes (91). To

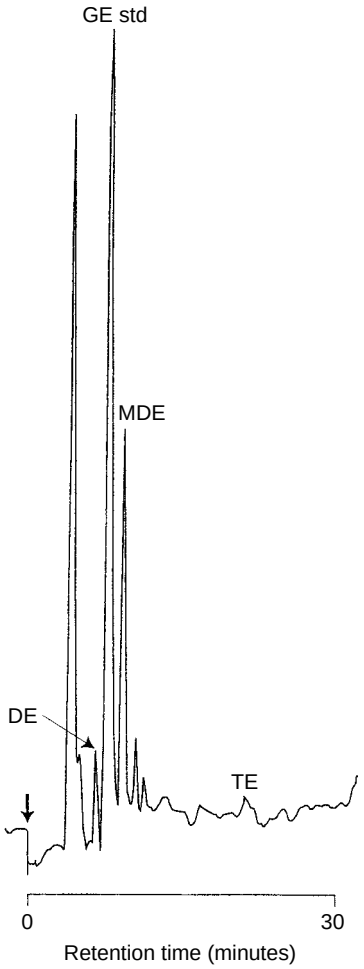

Figure 3. HPLC chromatogram showing separation of the ether lipid cores derived from the glycolipids of *Methanococcus jannaschii*. Solvent, hexane/isopropanol, 99/1 v/v. Abbreviations: Std, internal standard; DE, diether; MDE, macrocyclic ether; TE, tetraether.

our knowledge, the use of HPLC with ELSD has not been applied to ether lipid analysis. Such an application has potential for routine analysis of ether lipids and is worthy of investigation. Similarly, it would be particularly useful if HPLC procedures capable of resolving both diether and tetra ether core lipids could be routinely interfaced to mass spectrometers to further facilitate the identification of the tetra ether core lipids in particular. With recent developments in HPLC-MS, it is foreseen that such analyses will be increasingly used by researchers.

Gas Chromatography (GC)

GC is the most widely used instrument employed by lipid researchers for the analysis of fatty acids and other lipid classes, including hydrocarbons and sterols. Many studies on ether lipids have used GC analysis to measure the C_{20} and C_{40} hydrocarbon or other products derived from the ether lipid alkyl chains obtained using chemical degradation procedures. Typical operating conditions for analysis of the C_{20} and C_{40} hydrocarbon and other products, such as alcohols or alkyl chlorides, include use of a nonpolar column (e.g., SE30, OV17, HP1) with the GC oven initially run isothermally, followed by temperature programming to between 300 and 340 °C (92–94). Detection is usually by flame ionization detection (FID) or GC–mass spectrometry (GC–MS).

A number of researchers have analyzed diether core lipids, derived from cultured Archaea and environmental samples, by GC. In the environmental samples, measurement of the concentration of diether core lipids has been used to estimate the biomass of methanogens (69,95) and other Archaea (96). The protocol usually involves solvent extraction of the total lipid, chemical breakdown of the complex lipids to produce the diether lipid core, derivatization, purification by TLC (this step may be excluded), and analysis by capillary GC. Measurement of diether core lipids is related to cell numbers, methane production and turbidity (69), and to production of organic matter (95).

Conditions for the GC analysis of the diether core lipids are similar to those noted earlier for the C_{40} hydrocarbon. In particular, a high final oven temperature is required (e.g., 300–340 °C) to ensure that the diether core lipids elute from the column. A number of internal standards have been employed in diether core lipid analysis. These are generally phytanyl acetate prepared from phytol (69) and 1,2-di-O-hexadecyl-rac-glycerol (as the O-trimethylsilyl ether), following treatment with N,O-bis(trimethylsilyl)trifluoroacetamide(BSTFA) (96). An issue with the analysis of the 3-OH ether core lipids is their incomplete derivatization when using silylating reagents such as BSTFA. Complete derivatization of hydroxyarchaeol to the di-TMSi derivative occurs on using BSTFA in pyridine/dichloromethane (1/2 v/v) (97).

For cases in which GC has been used to determine diether core lipid composition and content, the diethers have been usually purified first. This removes the fatty acids that, in environmental samples, are derived from bacteria, eukaryotes, and other sources. A capillary GC procedure capable of simultaneous estimation of microbial phospholipid-derived fatty acids and diethers was recently reported (98) and applied to the microbial consortia of wetland sediments. See Figure 4 (99). The fatty acids (as methyl esters) and diethers were not separated before analysis. In particular, a slow oven temperature-heating rate (from 110–220 °C at 1 °C/minute) was employed to gain separation of the novel signature lipids, C_{16} and C_{18} monounsaturated fatty acids derived from methanotrophs (e.g., 16 : 1ω6c, 16 : 1ω8c, 16 : 1ω5c & t, 18 : 1ω8c), in the same chromatogram as the more conventional C_{16} and C_{18} monounsaturated fatty acids and the diether lipid core. The slower oven-heating rate was used to achieve separation of novel fatty acids that are generally not resolved when using more standard oven-heating programs (e.g., from 150 °C to 250 °C at 2 or 3 °C/minutes).

Archaeal diether and tetraether lipid cores were analyzed simultaneously by high-temperature capillary

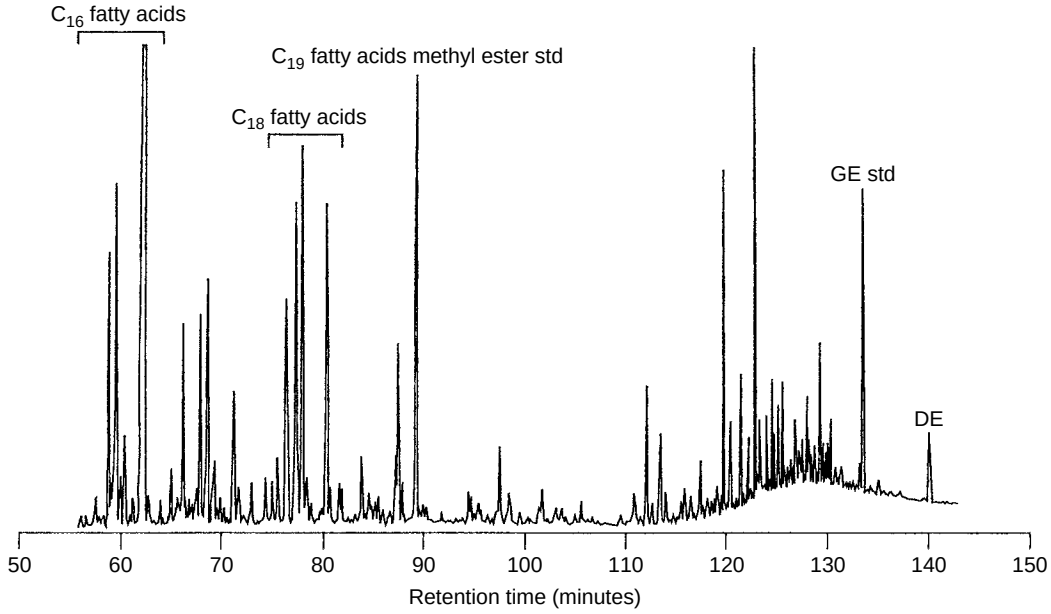

Figure 4. Partial gas chromatogram (HP1) showing phospholipid fatty acids (as methyl ester) and diether lipid (as OTMSi ether) from Billabong sediments. Abbreviations: GE std, glyceryl ether internal standard; DE, diether. Adapted from P. Virtue, P. D. Nichols and P. I. Boon, *J.Microbial. Methods* **25**, 177–185 (1996).

GC (80). The high-temperature GC achieved separation of diether, tetraethers, and cyclopentane-containing tetraethers. A short BPX5-fused silica capillary column (3 m × 0.25 mm) and on-column injector were used for the analyses. Initial research on high-temperature GC separation of diether and tetraether core lipids used novel Aluminum-clad capillary columns. Separation was obtained, but the columns were brittle and their use was not pursued. With other columns tested, loss of tetraether was observed; this was most probably due to the breakdown of this component by active sites on the column. The use of the BPX5 phase on a short capillary column overcame these problems and the tetraether recovery remained stable for continual running of the column over a period of several months. The GC oven was temperature-programed from 90 °C to 190 °C at 30°/minutes and then to 380 °C at 10 °C/minutes. The final temperature was maintained for 15 minutes. Using FID, the instrumental limit of detection for individual ether lipids was approximately 1–2 ng. Representative GC traces illustrating the separation of ether lipids obtained using the high-temperature capillary column are shown in Figures 5 and 6.

It was recommended for high-temperature GC analysis of samples containing both di- and tetraether core lipids that appropriate standard mixtures containing known proportions of the two ether lipid cores be run routinely. Alternately, internal standards that elute close to the di- and tetraether core lipids can be used to assess recoveries.

GC is widely used in many research laboratories. Therefore, the use of high-temperature capillary GC for measurement of Archaeal diether and tetraether lipids provides considerable potential for routine analysis of these microbial lipids in taxonomic, microbial, ecological, and organic geochemical studies. It was reported that the high-temperature GC procedure was at least comparable to and generally better than other techniques in use, and that these features provide new possibilities for measuring Archaea in industrial projects (80). It would be particularly useful if the high-temperature GC procedure could be interfaced to a mass spectrometer to further facilitate the identification of tetra ether core lipids.

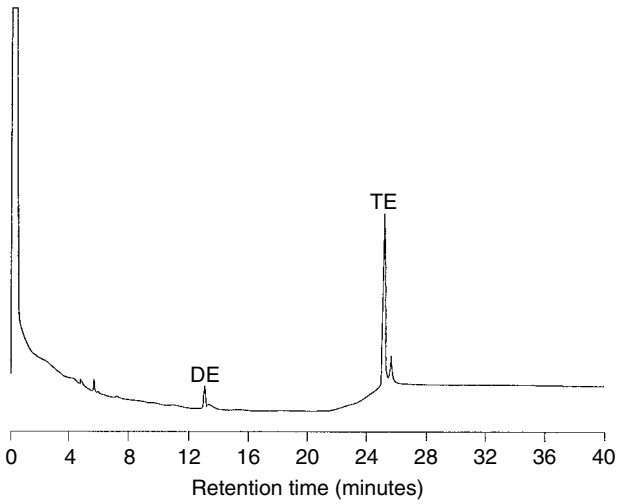

Figure 5. Capillary gas chromatogram (BPX5) showing phospholipid-derived ether lipid distribution of *Methanobacterium thermoautotrophicum* strain Hverigerdi. Abbreviations: DE, diether; TE, tetraether. Components eluting between four and six minutes are fatty acid methyl esters. Adapted from P. D. Nichols, P. M. Shaw, C. A. Mancuso and P. D. Franzmann, *J. Microbiol. Methods* **18**, 1–9 (1993).

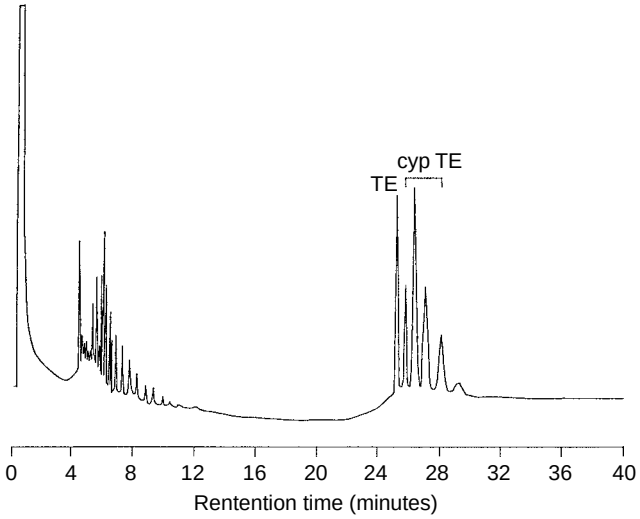

Figure 6. Capillary gas chromatogram (BPX5) showing ether lipid distribution of *Thermoplasma acidophilum*. Abbreviations: TE, tetraether; cypTE, cyclopentane-containing tetraethers. Components eluting between 4 and 10 minutes include phytadienes and monophytanyl glyceryl diols. Adapted from P. D. Nichols, P. M. Shaw, C. A. Mancuso and P. D. Franzmann, *J. Microbiol. Methods* **18**, 1–9 (1993).

In a specific application, highly sensitive gas chromatography combined with negative chemical ionization mass spectrometry (with selected ion-monitoring) enabled the detection, for the first time, of C_{15} and C_{25} homologs of the normal C_{20} isopranyl side chain in several strains of methanogens (100). These methods would be applicable to field samples for determining archaeal community structure.

Mass spectral data is often not reported for studies that examine the distribution of core ether lipids in Archaea and environmental samples. The mass spectra of the diether lipid core (as the trimethyl silyl ether) derived from the methanogen, *Methanoccoides burtonii*, is shown in Figure 7. Further details on formation of the major mass fragments observed have been reported (96).

Recent developments with GC analysis include multidimensional GC and cryo-focusing. Both procedures can be used to enhance component resolution. Should these and other techniques become more widely used, they could be readily applied to obtain increased resolution of the various ether lipid components when analyzed by GC.

Supercritical Fluid Chromatography

Several researchers have used supercritical fluid chromatography (SFC) to analyze archaeal lipids. SFC is a separation technique similar to GC and HPLC. GC and HPLC employ a gas and liquid phase respectively as the mobile phase, but a compressed gas is used in SFC; carbon dioxide is the gas employed in most SFC separations. It has been stated that the versatility of SFC and its range of applications is directly related to the unique physicochemical properties of the supercritical fluid.

The first report of SFC applied to ether lipids demonstrated separation of a range of tetraethers from *Thermoplasma* and also tetraether-derived isopranyl hydrocarbons (101). Complete separation of the tetraether core lipids that differed only by the number of cyclopentane rings present in the isopranyl side chains was achieved. With the phased used, the tetraether core lipids eluted in order of increasing cyclopentane content. The method was suitable for qualitative- and quantitative-screening for ether lipids in living organisms and sedimentary organic matter.

A strategy was developed for use of SFC to separately analyze the polar lipid, glycolipid, and lipid-extracted residue fractions, and the approach was applied to seven strains of Archaea (70). The methodology was suitable for integration with a protocol for the analysis of bacterial lipids. Currently, the SFC methodology does not have sufficient resolution for the analysis of the more complex fatty acid mixtures. The limited literature results available to date for SFC assay of individual strains of Archaea show that separation of individual ether lipid structures is comparable to other chromatography (GC

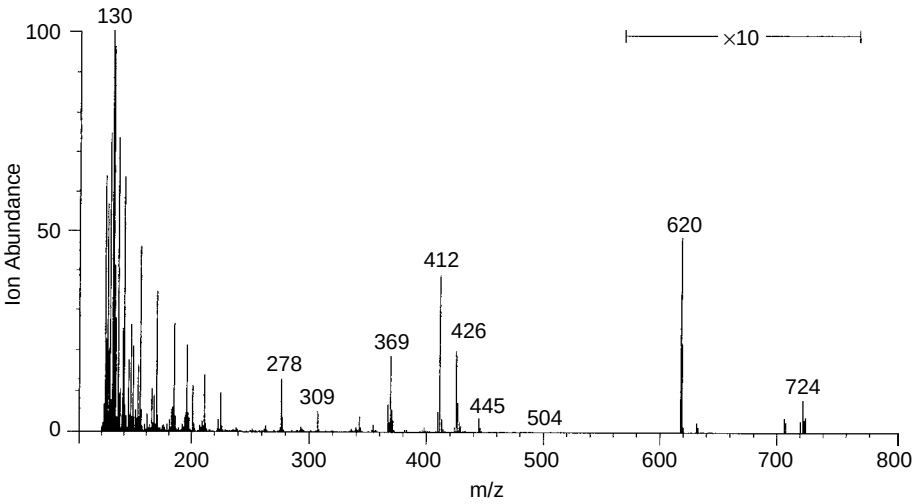

Figure 7. Mass spectrum of the diether lipid (as the trimethyl silyl ether) derived from the methanogen *Methanoccoides burtonii*. Instrument: VG Autospec Ultima mass spectrometer. Mass spectrum kindly provided by Dr. Noel Davies and Ms. Jenny Skerratt, University of Tasmania, Hobart, Tasmania, Australia.

and HPLC) procedures. However, the use of SFC has generally not been widespread and, to our knowledge, the technique is not used in many laboratories performing microbial lipid assays.

Other Signature Lipid Procedures

Carbon isotopic fractionation has been used to determine carbon sources, including the contribution from methanogens, in environmental samples. It is generally considered that methanogens produce biomarkers, including ether lipids and pentamethyleicosane (PME) that are ^{13}C-depleted compared to their carbon source. The advent of GC–isotope ratio mass spectrometry (GC–IRMS) as a research tool now permits more routine examination of the isotopic composition and fractionation for cultured microorganisms and environmental samples. In a recent study, the finding of highly ^{13}C-depleted ether lipid and polyisoprenoid moieties, compared to the ^{13}C-content of biomass of cultured methanogens suggests that there is significant isotopic fractionation inherent in the lipid biosynthetic pathways of some Archaea (81).

Other instrumental procedures have been used in the structural elucidation of ether lipids. These include NMR (both ^{13}C and ^{1}H), probe MS, and fast-atom bombardment MS (FAB MS). These procedures have been invaluable for the determination of precise structural details (102). For example, in a report on the extremely halophilic Archaea *Halobacterium cutirubrum*, FAB (tandem) mass spectrometry was used to analyze intact polar ether lipids (103). Simple positive and negative ion mass spectra of the three major polar lipids extracted from *H. cutirubrum* were obtained that contained ions with masses corresponding to cationized or deprotonated lipid molecules. Structural information was then obtained on individual components of the polar lipid mixtures by discrete selection of ion masses when the instrument was operated in tandem mass spectrometric mode.

These procedures are generally not routinely used to detect Archaea in environmental studies. However, their use is noted here, although they are not covered further in this section. Further information on many of these methods is available (102).

Quinones

The structure and distribution of the various types of isoprenoid quinones in Archaea has been reviewed (102). Isoprenoid quinones are recovered from the neutral lipid fraction of total lipid extracts obtained from aerobic Archaea (Fig. 2) and their structures are based on naphthoquinone or benzothiophenquinone chromophores. Separation of components has been performed by TLC and HPLC procedures, with structural elucidation by NMR, MS, UV, and visible spectroscopy. Studies of archaeal quinones have mainly focused on thermophilic and halophilic strains; detailed studies of the quinone composition of methanogens are yet to be performed (102).

Thermophilic Archaea are divided into two groups with respect to their quinone composition. Members of the genera *Acidianus, Desulfurolobus, Metallosphaera, Stygiolobus and Sulfolobus* are, with few exceptions, based on the benzothiophene nucleus, whereas members of the genera *Archaeoglobus*, *Thermoplasma*, and *Thermoproteus* contain only unsaturated or fully saturated isoprenoid naphthoquinones. The application of quinone methodology to environmental samples for measuring Archaea remains to be explored.

OTHER PROCEDURES

Methane Production

Several studies that have used ether lipids or other techniques to estimate methanogenic biomass have also measured methane. In marine sediments (86), a direct correlation was found between methane concentration measured by a headspace procedure (104) and diether core lipid measured by HPLC.

In water samples, methane is measured by purge and trap procedures. Such techniques have been applied to ocean waters (105). The increased recognition of the presence of methanogens in oxygenated surface oceanic waters has occurred over the past decade. This observation was even termed *the ocean methane paradox* (106). Methane measurements in northern Pacific Ocean waters showed that methane was supersaturated with respect to its equilibration with atmospheric methane (107). To gain further insight into this interesting phenomenon, application of the signature lipid approach in combination with methane measurements and molecular probes would be extremely fruitful.

The direct measurement of methane production rates can be used to detect archaeal activity. This is achieved by measurement of $^{14}CH_4$ formed from $NaH^{14}CO_3$, [^{14}C] — formate and [^{14}C-2] — acetate in time-course experiments using the gas chromatography–gas proportional counting (GC–GPC) procedure (108). In an application of this method in a unique Antarctic lake (109), the sensitivity of the method was demonstrated and a maximal methane production of 2.5 $\mu mol\,kg^{-1}\,day^{-1}$ was estimated.

A signature lipid and radiotracer analysis approach has been used to determine anaerobic conversion of biomass to methane (110). Methane was measured by GC — GPC, and both PLFA and PLEL (the latter by SFC) were measured to determine the bacterial and archaeal biomass, respectively, as well as the community structure. In addition, radiolabel incorporation into PLFA and PLEL fractions was also determined.

CoEnzymes

Methanogens — Coenzyme F_{420}. Coenzyme F_{420} is the N-(NL-lactyl-γ-L-glutamyl)-L-glutamic acid phosphodiester of 7,8-didemethyl-8-hydroxy-5-deazariboflavin 5′-phosphate (111). The enzyme functions as an electron carrier in methanogens for anaerobic respiration and cell carbon synthesis (112). On exposure to UV light, fluorescence emitted by this molecule allows for tentative ID as either coenzyme F_{420} or its analog has been found in all methanogens analyzed to date (113). After purification

by HPLC (114), quantification of the coenzyme has been used to measure methanogenic biomass in mixed systems (115). However, recent studies have shown that the levels of this cofactor and others varied significantly among methanogens as well as within individual methanogens, according to growth substrate (113).

Fourier Transform–Infrared (FT–IR) Spectroscopy

FT–IR spectroscopy has been used by microbial ecologists to examine microbial biomass and nutritional status, including exopolymer production (116,117). Use of the FT–IR technique offers the potential for rapid and nondestructive analysis of bacteria and bacteria-polymer mixtures. The protein-derived amide I band at 1,680 cm^{-1} defines biomass, and bands at 1,740 cm^{-1} — derived from the storage product polyhydroxybutyrate — and 1,150 cm^{-1} are indicative of exopolymer-containing carbohydrate.

The ratio of the various IR bands provides a comparative tool for use in environmental studies. Differences in IR spectra for a range of bacteria have also been reported, suggesting that the FT–IR technique may be capable of providing information on community structure. This possible application of the technique is certainly less developed than its use in examining biofilms of the key constituents just noted.

To our knowledge, FT–IR has not been used to directly examine members of the Archaea. Given the promising findings to date for bacterial communities and noting the distinctive biochemical properties of Archaea with respect to their lipid and cell envelope composition, use of this instrumental approach may offer potential for examining this unique kingdom.

CONCLUSION

The routine measurement of Archaea in taxonomic and environmental studies is now possible, based on developments in the past two decades. In environmental samples, in particular, the use of modern molecular and signature lipid techniques enables the detection and quantification of these important microorganisms. The choice of equipment available to the researcher will influence the selection of a suitable protocol with the signature lipid approach. Further research on molecular techniques is necessary before such methods can become routine and quantitative. It is expected that further developments will undoubtedly occur with these procedures and will increase their resolving power and widen their application. For example, it should be possible to detect methanogens during climate change and in other research programs in which modeling studies of the role of this key group will increasingly need validation via field measurements.

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ARCHAEA IN BIOTECHNOLOGY

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The Archaea represent one of the three fundamental domains of life. Genotypically, they are as distinct from Bacteria because they are from higher organisms (the Eukarya). The domain Archaea is composed of at least three phenotypically distinct groups, each dominated by members inhabiting extreme environments (high temperature/low pH, anoxia, hypersalinity). The unique biochemistry of some members of the Archaea (such as the methanogens), coupled with the molecular and physiological adaptations of others to physical and chemical extremes, has endowed this group of microorganisms with significant biotechnological potential. The use of enzymes in diagnostics and biocatalysis, fermentations in methane production and metal ion recovery, and cellular bioproducts as food additives and separation membranes are all examples of the enormous capacity for biotechnological exploitation of this diverse group of organisms.

ARCHAEAL PHYLOGENETICS

The Archaea comprise one of the three fundamental groups (domains or superkingdoms) of living organisms (Fig. 1). This fundamental tripartite topology is derived from small subunit rRNA sequence comparisons (1) and is rooted by the inclusion of pairs of paralogous genes (related genes that duplicated and diverged before the three domains separated) as out-groups (2,3). The rooting of the phylogenetic tree places the Archaea as a very deep branch on the Eukaryotic lineage, linking these two more closely in evolutionary terms than the Bacteria and the Archaea. Notwithstanding the importance of genetic transfer between members of the three domains, the close evolutionary relationship between the Archaea and the Eukaryotes has considerable significance in terms of Biotechnology, for example, in the potential use of Archaea as primitive models for eukaryotic cells.

Until relatively recently the Archaeal domain has been clearly separated, on the basis of the genotypic relatedness of cultured organisms, into two kingdoms, the Crenarchaeotes and Euryarchaeotes (Fig. 1). The crenarchaeote kingdom was thought to be phenotypically homogenous and composed largely of the so-called sulfur-dependent thermoacidophiles (Table 1). The euryarchaeote kingdom was more phenotypically diverse and included the methanogens, the extreme halophiles, and various thermophiles, including the fermentative sulfate reducers. For in-depth discussions of the phylogeny of the Archaea, the reader is directed to some excellent reviews (4,5).

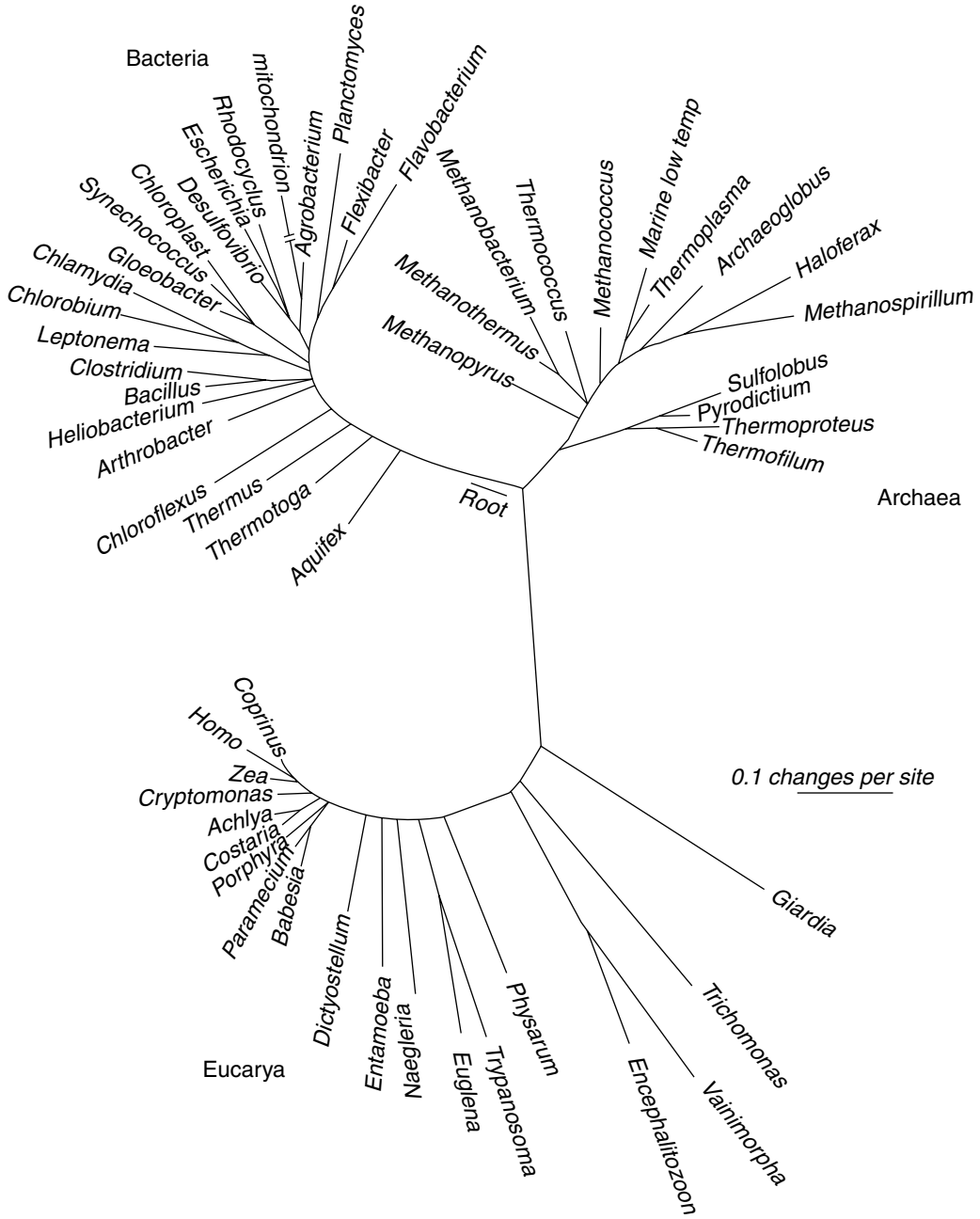

Figure 1. The universal phylogenetic tree. (Reproduced with permission from pace [18]).

The clear-cut twin kingdom structure of the domain Archaea has been substantially blurred, over the last decade of the twentieth century, by the application of molecular phylogenetics to microbial population analysis. The direct extraction of microbial community DNA, coupled with PCR amplification of 16S rRNA genes using universal and Archaea-specific primers (14), has led to a multitude of phylotypes positioned between the two kingdoms (15,16), generating a more homogeneous single domain. Molecular phylogenetic analysis of community DNA has also added apparently new phenotypic and genotypic groups to the domain. One of the startling revelations of this method has been the appearance of numerous low temperature marine and terrestrial derived phylotypes (17,18) in the hitherto exclusively thermophilic crenarchaeota. The discovery of several sequences that branch very deeply in the crenarchaeote lineage has led some to propose a third archaeal kingdom [the "Korarchaeota" (19,20)] and others to speculate on the generation of treeing artifacts.

ARCHAEAL DIVERSITY

The intense interest in all aspects of the Archaea has led to the isolation and characterization of hundreds of different genera and species. Tables 1 to 3 summarize the current

Table 1. The Thermoacidophilic Archaea

Order	Genus	Species	Habitat[a]	Upper Growth Temperature (°C)
"Archaeoglobales"	*Archaeoglobus*	*A. fulgidus*	D,S,O,B	92
		A. profundus	D,S	92
		A. veneficus	D	85
	Ferroglobus	*F. placidus*	S	95
"Igneococcales"	*Desulfurococcus*	*D. amylolyticus*	D,T	97
		D. mobilis	T	95
		D. mucosus	T	97
		D. saccharovorans	T	97
	Hyperthermus	*H. butylicus*	S	108
	Pyrodictium	*P. abyssi*	D	110
		P. brockii	D,S	110
		P. occultum	D,S	110
	Staphylothermus	*S. marinus*	D	98
	Sulfophobococcus	*S. zilligii*	T	95
	Thermodiscus	*T. maritimus*	S	98
	Thermosphaera	*T. aggregans*	T	90
Sulfolobales	*Acidianus*	*A. ambivalens*	T	95
		A. brierleyi	T	75
		A. infernus	T	95
	Metallasphaera	*M. sedula*	T	80
		M. prunae	T	80
	Stygioglobus	*S. azoricus*	T	89
	Sulfolobus	*S. acidocaldarius*	T	85
		S. metallicus	T	75
		S. shibatae	T	86
		S. solfataricus	T	87
	Pyrolobus	*P. fumarii*		
Thermococcales	*Pyrococcus*	*P. furiosus*	D,S	103
		P. horikoshii	D	102
		P. kodakaraensis		
		P. woesii	D,S	103
	Thermococcus	*T. alcaliphilus*	S	90
		T. acidaminovorans	S	93
		T. aggregans	D	
		T. barossii	D	>88
		T. celer	S	93
		T. chitonophagus	D	93
		T. fumicolans	D	>90
		T. hydrothermalis	D	>85
		T. gorgonarius	D	>88
		T. guaymasensis	D	
		T. littoralis	S	98
		T. pacificus	D	>88
		T. peptonophilus	D	85
		T. profundus	D	90
		T. stetteri	D,S	98
Thermoproteales	*Aeropryum*	*A. pernix*	T	100
	Pyrobaculum	*P. aerophilum*	T	104
		P. islandicum	T,S	103
		P. organotrophicum	T	103
	Thermofilum	*T. librum*	T	95
		T. pendens	T	95
	Thermoproteus	*T. neutrophilus*	T	97
		T. tenax	T	97
		T. uzoniensis	T	97

[a]D, deep marine hydrothermal vent; S, shallow marine hydrothermal vent; T, terrestrial; O, subsurface oil reservoir, B, subsurface biosphere

Source: Data from References 6–13; *http://www3.ncbi.nlm.nih.gov/htbin-post/Taxonomy/*

Table 2. Halophilic Archaea

Order	Genus	Species	Habitat[a]	Optimum Salt Concentration (% w/w)
Halobacteriales	*Halobacterium*	*H. salarinarium*	SF	25
		H. cutirubrum	SF	37
		H. halobium	SF	37
	Halorubrum	*H. saccharovorum*	SL	20
		H. sodomense	SL	15
		H. lacusprofundii	SL	20
		H. trapanicum	SL	20
	Haloferax	*H. volcanii*	SL	15
		H. denitrificans	SL	15
		H. gibbonsii	SL	15
		H. mediterranei	SL	15
	Haloarcula	*H. vallismortis*	SL	20
		H. marismortui	SL	20
		H. hispanica	SL	20
		H. japonica	SL	20
	Halobaculum	*H. gomorrense*	SL	20
	Halococcus	*H. morrhuae*	SL, SP	20
		H. saccharolyticus	SL	20
		H. salifodinae	SM	20
	Natronobacterium	*N. pharaonis*	SD	20
		N. gregoryi	SD	20
		N. magadii	SD	20
		N. vacuolata	SD	20
	Natronococcus	*N. occultus*	SD	20

Source: Data from Reference 13; *http://www3.ncbi.nlm.nih.gov/htbin-post/Taxonomy/*

status of archaeal species diversity, divided as the three primary phenotypic groupings, the Thermoacidophiles, the Halophiles, and the Methanogens. The reader should be aware that this list represents only cultured archaea, that it will be out of date before publication as a result of taxonomic revisions and new isolations, and that the true archaeal diversity includes the hundreds or thousands of new species and higher orders so far seen only as 16S rRNA sequences or not yet observed. Given that some of the uncultured phylotypes represent major phenotypic groups (such as the low-temperature crenarchaeotes), the "mining" of the genomic resources of the Archaea has barely started.

The Archaea show a wide metabolic diversity, with a number of unique pathways. For example, the methanogenesis pathway of all methanogens (Fig. 2), reviewed in detail by Daniels (21), contains completely novel sets of enzymes and cofactors. Many of the cofactors are structural analogs of coenzymes in either bacteria or Eukarya, but some are unique to the methanogens. Among the thermoacidophiles and halophiles, a variety of chemolithoautotrophic, fermentative, and chemo-organotrophic modes of metabolism have been identified, for example, see Reference 6. Most are not unique to the Archaea, although certain central catabolic pathways such as the modified Entner-Doudoroff pathway (in halophiles) and the nonphosphorylated Entner-Doudoroff pathway (in thermoacidophiles) seem to be common in the Archaea but very rare outside this domain (22). Other key enzymes, such as the thermostable hydrogenases of the chemolithoautotrophic thermoacidophiles, have unique properties of considerable potential application (shown later). For more comprehensive reviews of the evolution, ecology, physiology, and biochemistry of the Archaea, the reader is directed to more specialized articles (6–13).

UNCULTURED ARCHAEA

The number of known and cultured Archaea is now far exceeded by the number of "new" species and higher taxa known only through their 16S rRNA sequences (phylotypes). Phylotypic analyses of microbial community DNA from established "archaeal" habitats such as thermal pools (19) and hypersaline lakes (23) inevitably show numerous novel phylotypes, in which each represents a new species, genus, or higher order of thermoacidophile or halophile. Even more significantly, phylotypic analyses of many other terrestrial, freshwater, marine, and other habitats have shown that entire groups of Archaea await isolation. Table 4 shows a selection of the "uncultured" phylotypes that have been revealed in recent years. Some of these phylotypes appear to be virtually ubiquitous and in many, such as the low-temperature crenarchaeotes (Table 4), not a single example organism has been isolated. It is perhaps ironic that one such organism, a psychrophilic symbiont of a marine sponge (24), is presently a target for genome sequencing (Table 5) despite never having been cultured.

ACCESSING UNCULTURED GENOMES

There is obviously a great need for new technologies to isolate and culture novel microorganisms. However, in

Table 3. The Methanogens

Order	*Genus*	Species
Methanobacteriales	*Methanobacterium*	14 (3 thermophilic)
	Methanobrevibacter	7 (0 thermophilic)
	Methanosphaera	1 (0 thermophilic)
	Methanothermobacter	3 (3 thermophilic)
	Methanothermus	2 (2 hyperthermophilic)
Methanococcales	*Methanococcus*	11 (6 thermophilic)
Methanomicrobiales	*Methanocorpusculum*	4 (0 thermophilic)
	Methanocalculus	2 (1 halotolerant)
	Methanoculleus	5 (1 thermophilic)
	Methanofollis	2 (0 thermophilic)
	Methanogenium	3 (0 thermophilic)
	Methanomicrobium	1 (0 thermophilic)
	Methanoplanus	3 (0 thermophilic)
	Methanospirillum	1 (0 thermophilic)
Methanopyrales	*Methanopyrus*	1 (1 hyperthermophile)
Methanosarcinales	*Methanomicrococcus*	1 (0 thermophilic)
	Methanococcoides	3 (0 thermophilic)
	Methanohalobium	1 (1 halotolerant)
	Methanohalophilus	4 (4 halotolerant)
	Methanolobus	5 (2 thermophilic)
	Methanomethylovorans	2 (0 thermophilic)
	Methanophilus	1 (0 thermophilic)
	Methanosaeta	2 (1 thermophilic)
	Methanosarcina	8 (1 thermophilic)

Source: Data from *http://www3.ncbi.nlm.nih.gov/htbin-post/Taxonomy/*.
Note: Only validly named species are included: Many named genera have numerous unnamed species and uncultured phylotypes.

recent years, methods have been developed that partially circumvent the current limitations of microbial isolation. On the basis of direct extraction of total "community" DNA and the generation of multigenomic libraries (43,44), this technology (Fig. 3) accesses a much higher proportion of the prokaryotic genomes present in any environmental sample than can be obtained from classical microbial isolation. A second advantage is that any number of gene products may be targeted, limited only by the ingenuity and design of the assay method.

Current limitations in these methods, including uncertainties in extraction efficiency, biases introduced during cloning and transformation, and low expression efficiencies due to the presence or absence of upstream and downstream control sequences, will clearly be reduced by further developments in recombinant technology. Although still relatively new, multigenomic cloning will undoubtedly be adapted and optimized for eukaryote genomes (e.g., via cDNA technologies), for expression screening of libraries, and for the targeting of components of the "metabolome" (the complement of metabolites generated by an organism) via specialized vectors accepting large DNA inserts and capable of expressing the components of partial or entire synthetic pathways.

ARCHAEAL GENOME SEQUENCES

The complete sequencing of an organism's genome provides a comprehensive blueprint of the metabolic and functional characteristics of the organism. A genome sequence is also a valuable biotechnological resource for the rapid acquisition, via PCR cloning, of known genes and for the identification of novel or hitherto unidentified genes. The latter requires powerful computational tools, but must always be confirmed by traditional biochemical methodologies.

Given the relatively small number of known Archaeal species compared with known bacteria, the number of Archaeal genomes being sequenced (Table 5) is disproportionately high. This preoccupation with the Archaea, particularly the hyperthermophiles, reflects the biochemical and physiological novelty of the domain and the widespread belief that hyperthermophile genes and gene products will yield substantial rewards for basic science (such as new molecular mechanisms of protein stabilization) and for biotechnology.

The novelty of the Archaea is evident in the proportion of their genomes constituted by unassigned open reading frames (ORFs) (Table 5). These hypothetical protein sequences (identified computationally by the presence of putative start and stop signals) are categorized either as *conserved hypothetical* proteins (observed in another genome, but function unknown) or *hypothetical* (not seen elsewhere, function unknown) proteins. Although the proportion of the latter decreases with each new genome sequence, functional annotation of the former is slow because it is primarily dependent on more detailed biochemical or computational characterization.

The mining of information from new genomes is clearly hampered by the limitations in assigning structure and/or function to hypothetical protein sequences. New computational tools, such as more sophisticated and

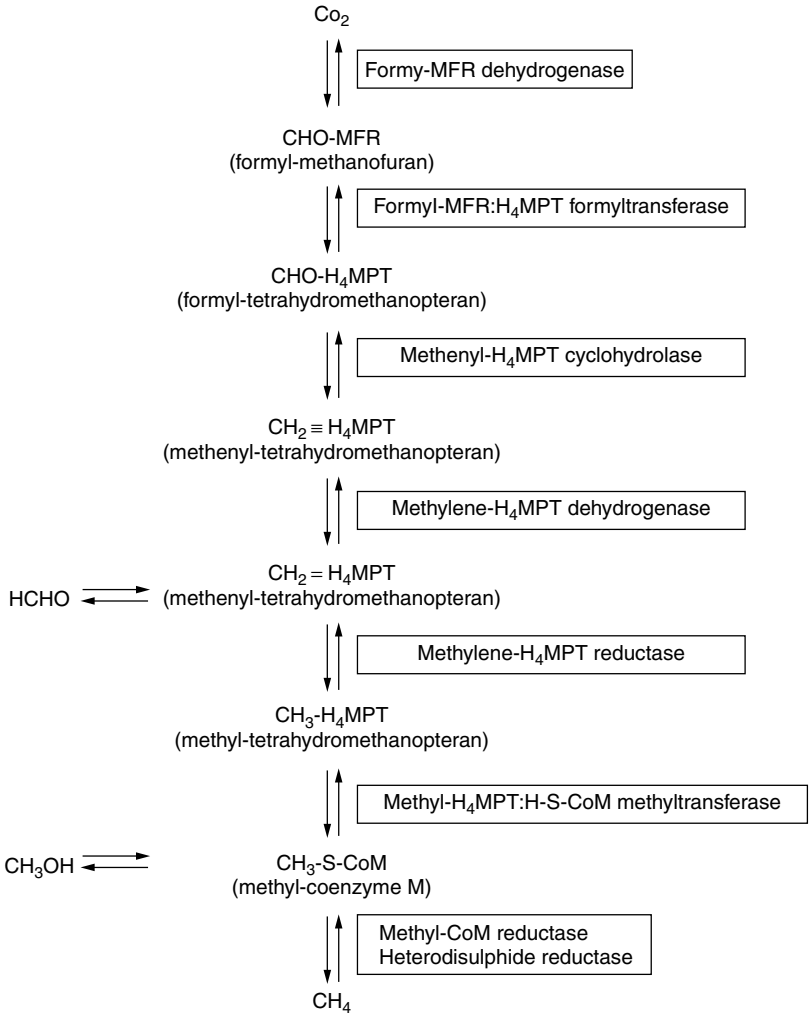

Figure 2. The methanogenesis pathway.

Table 4. Diverse Habitats Harboring Uncultured Archaeal Phylotypes

Habitat	Putative Organism Type/Phylogeny	Reference
Marine surface and aphotic picoplankton	Low-temperature (Group I) crenarchaeotes; low-temperature (Group II) euryarchaeotes/methanogens	25
Marine abyssal water	Unknown (group III) euryarchaeotes	26
Marine sediments	Low-temperature (Group I) crenarchaeotes; low-temperature (Group II) euryarchaeotes/methanogens	27
Fish midgut	Low-temperature (Group II) euryarchaeotes/methanogens	28
Rice paddies	Low-temperature (Group I) crenarchaeotes; low-temperature (Group II) euryarchaeotes/methanogens; new phyla	29
Terrestrial soils	Low-temperature (Group I) crenarchaeotes	30
Freshwater lakes	Low-temperature (Group I) crenarchaeotes	31
Deep subsurface biosphere	Low-temperature (Group I) crenarchaeotes	32

Table 5. Archaeal Genome Sequences (from *http:/linkage.rockefeller.edu/wli/seq/*)

Organism	Metabolism	Group Responsible for Sequence	Genome Size (Mb)	Full Sequence Completed/Available?	% Conserved Hypothetical ORFs	% Hypothetical ORFs	Reference
Aeropyrum pernix	Aerobic heterotroph	Biotechnology Center, National Institute of Technology and Evaluation, Japan	1.67	Yes/Yes	—	—	33
Archaeoglobus fulgidus	Anaerobic heterotroph	TIGR, U.S.A.	2.18	Yes/Yes	27	26	34
Cenarchaeum symbiosum	Unknown	TIGR, U.S.A.	?	No/No	—	—	24
Halobacterium salinarum	Halophilic heterotroph	Max-Planck-Institute for Biochemistry, Germany	4	No/No	–	—	—
Halobacterium sp. NRC-1	Halophilic heterotroph	University of Massachusetts/ University of Washington, U.S.A.	2.57	Yes/Yes	—	36	35
Methanobacterium thermoautotrophicum	Anaerobic autotroph (methanogen)	Genome Therapeutics & Ohio State University, U.S.A.	1.75	Yes/Yes	28	27	36
Methanococcus jannaschii	Anaerobic methanogen	TIGR, U.S.A.	1.66	Yes/Yes	6	56	37
Methanococcus maripaludis	Anaerobic methanogen	University of Washington, U.S.A.	?	No/No	—	—	—
Methanogenium frigidum	Anaerobic methanogen	UNSW / AGRF, U.S.A.	?	No/No	—	—	—
Methanosarcina mazei	Anaerobic methanogen	Goettingen Genomics Laboratory, Germany	2.8	No/No	—	—	—
Pyrobaculum aerophilum	Anaerobic chemolithoautotroph	Caltech / UCLA, U.S.A.	2.2	Yes/??	—	—	—
Pyrococcus abyssi	Anaerobic heterotroph	GENOSCOPE, CNRS, France	1.8	Yes/Yes	—	—	38
Pyrococcus furiosus	Anaerobic heterotroph	Center of Marine Biotechnology / Univ. Utah, U.S.A.	1.91	Yes/No	—	—	39
Pyrococcus horikoshii OT3	Anaerobic heterotroph	Biotechnology Center, National Institute of Technology and Evaluation, Japan	1.74	Yes/Yes	—	—	39
Sulfolobus solfataricus	Aerobic heterotroph/ anaerobic autotroph	European and Canadian consortium	3.05	Yes/Yes	—	—	40
Thermoplasma acidophilum	Microaerophilic heterotroph	Max-Planck Institute for Biochemistry, Germany	1.56	Yes/Yes	29	16	41
Thermoplasma volcanium GSS1	Microaerophilic heterotroph	Osaka University and AIST, Japan	1.58	Yes/Yes	—	—	42

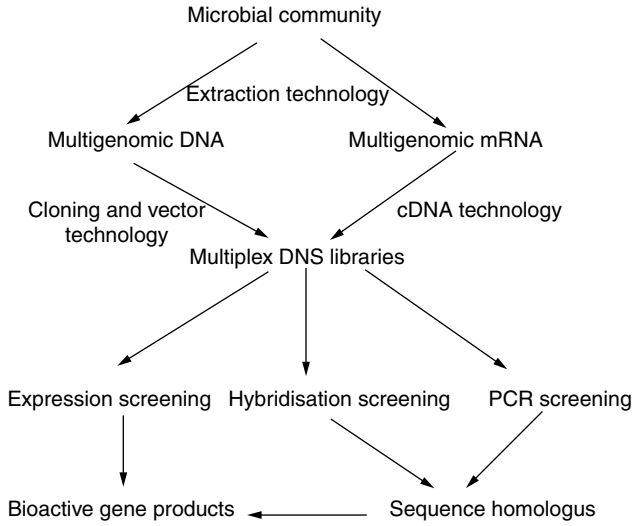

Figure 3. Schematic representation of the components of "multiplex cloning."

reliable methods for searching for structural or functional motifs, are required. Rapid methods for identifying the functional properties of expressed ORFs are also essential. The development of automated microarray technologies for monitoring binding or catalytic functions (45) would be a significant advance.

FERMENTATION AND PRODUCTION

The successful and economically feasible exploitation of Archaeal products, such as enzymes, structural proteins, lipids, pigments, compatible solutes, and so on, will, in many cases, be dependent on the ability to generate high biomass yields in fermentation. Alternatively, for primary gene products, the ability to express Archaeal genes in heterologous hosts at high levels is equally important. Table 6 outlines some of the current limitations associated with Archaeal fermentations and production of recombinant Archaeal products (47).

One major problem associated with generation of high biomass yields of two of the three groups of Archaea, the hyperthermophiles and the methanogens, is that many of these organisms are autotrophs. Chemoautotrophic metabolism is generally energy inefficient, and typically results in low cellular doubling times and culture growth rates and poor biomass yields. In addition, many of these organisms grow optimally at temperatures above 85 °C. Apart from the practical and physical disadvantages of high temperature growth (Table 6), it is probable that high temperatures impose a significantly greater metabolic burden on cellular constituents than low temperatures. The higher requirement for molecular repair and regeneration in hyperthermophiles is probably a significant factor in low biomass space-time yields. Thermal degradation of key nutrients, such as vitamins, may also be a limiting factor.

In consequence, biomass yields for Archaeal fermentation are generally very much lower than for many other microorganisms, as indicated by consensus figures from various batch fermentations (Table 7; 48). These figures are only broadly representative, because the degree of fermentation development varies between organisms, and the mode of fermentation (e.g., batch vs. fed-batch vs. continuous) has a marked effect on space-time yields (49). Recent developments in fermentor technology, including the use of gas-lift and membrane-dialysis fermentors, have resulted in substantially increased biomass yields (48).

For production of primary gene products (enzymes, proteins), the obvious answer to problems of low native biomass yields is the cloning and expression of the relevant gene in a suitable heterologous host. Yields of recombinant gene product are then dependent on expression levels and biomass yields of the host organism.

Despite predictions that hyperthermophilic proteins might not fold correctly in mesophilic hosts, there is ample evidence that this is not the case. Many thermophilic and hyperthermophilic protein genes have now been expressed in a variety of mesophilic hosts, including *E. coli*, *Pichia pastoris*, Saccharomyces cerevisiae and insect cells, and, in most cases, the recombinant enzymes are functionally identical to the native protein. There is some evidence, largely anecdotal that some recombinant hyperthermophilic proteins are significantly less thermostable than the purified native proteins, but no structural or mechanistic basis has yet been established.

The production of recombinant extreme halophile proteins in heterologous hosts is severely limited. Many proteins from the haloarchaea have an obligate requirement for 1 to 2 M intracellular salt concentrations for correct folding and stability and are normally expressed as insoluble, inactive inclusion bodies in nonhalophilic hosts. Halophilic host-vector systems for expression of salt-dependent proteins are currently under development (50).

PRODUCTS OF ARCHAEA

Enzymes

The Archaea present a unique source of novel enzymes because growth in extreme environments requires unique enzyme properties with respect to both activity and stability. The metabolic rates, and hence biochemical reaction rates, in extremophilic cells are not significantly different from those in mesophilic cells, yet the extreme environmental conditions impose unusual constraints on the protein structure-function relationships of extremophilic enzymes. Various studies, using techniques such as genome sequencing and protein crystallography, and comparisons with mesophilic enzymes, have demonstrated that archaeal enzymes have certain structural properties that allow them to maintain their catalytic activity under conditions of extreme temperature, pH, salinity, or chemical environment. These properties make archaeal enzymes particularly attractive as candidates for industrial bioconversion processes, in which stringent reaction conditions frequently prevail. Enzymes possessing enhanced stability and selectivity, unusual or novel reactions and/or substrate specificities, are well-established goals in the field of biotransformations. Furthermore, the technologies of molecular biology now allow us to manipulate genes and

Table 6. Limitations in the Production of Archaeal Biomass and Gene Products

	Hyperthermophiles	Methanogens	Extreme Halophiles
Archaeal fermentations	High temperatures increase wear and tear on steel vessels, bearings, lines, and detectors	Methanogenic metabolism is inefficient	Very high chloride concentrations are detrimental to steel vessels
	Operating costs are high	Methane end-product may pose safety issues	Native enzymes are unstable in low-salt buffers
	Large-scale culturing of chemoautotrophs is difficult	Many low-temperature methanogens have very low doubling times	Some halophilic archaea are genetically unstable
Expression of Archaeal genes in heterologous hosts	No high-level *extremophilic* host-vector expression systems are yet available.		
	Hyperthermophilic genes expressed in mesophilic hosts are not always as stable as the wild-type gene products	No known major problems for mesophilic methanogenic genes	Most mesophilic hosts are incompatible with correct folding of extremely halophilic proteins, which typically require molar intracellular salt concentrations

Table 7. "Laboratory" Biomass Yield Ranges for Various Organisms

Organism	Growth Temperature (°C)	Metabolism	Maximum Batch Fermentation Biomass Yields (g/L w.w.)
Streptomyces	25	Heterotrophic, aerobic	20–100
Bacillus	30	Heterotrophic, aerobic	20–50
Escherichia coli	37	Heterotrophic, aerobic	20–100
Natronobacterium	37	Heterotrophic, aerobic	5–15
Bacillus stearothermophilus	60	Heterotrophic, aerobic	5–10
Thermus	70	Heterotrophic, aerobic	2–5
Methanothermus	85	Autotrophic, anaerobic	1–5
Sulfolobus	85	Heterotrophic, aerobic	2–10
Methanococcus	95	Autotrophic, anaerobic	0.1–1
Pyrococcus	98	Heterotrophic, anaerobic	2–10
Pyrodictium	98	Autotrophic, anaerobic	0.1–0.5

gene products to the extent that enzymes with desirable extremophilic properties may be fine-tuned for specific biotechnological applications.

Enzyme Properties

Thermostability. The remarkable activity and stability of thermophilic enzymes at high temperature suggests numerous advantages with regard to their biotechnological application. Many industrial processes are ideally run at elevated temperatures, in which, for instance, increased reactant solubilities or equilibrium ratios may be favored. Higher enzyme stability and resistance to inactivation reduces the need for replacement or regeneration of the biocatalyst. Structural thermostability also confers resistance to inactivation by chemical denaturants (detergents, chaotropic agents), oxidizing agents, proteases, and organic solvents, and hence to the harsh conditions that may prevail in many industrial process situations (51,52). Conversely, of course, the high temperature-optima of thermophilic enzymes constrains reaction conditions to the extent that the process proceeds less efficiently at lower temperatures.

Thermophilic enzymes have evolved to control cellular metabolic activity optimally at the elevated temperatures of their environment, generally by means of mechanisms causing the proteins to have less conformational flexibility than homologous mesophilic enzymes, and more resistance to the unfolding processes that initiate denaturation (53). Recent research has indicated that a number of relatively minor modifications to the higher order structures of the proteins are responsible for these differences (Table 8), and protein sequences do not appear to vary significantly between functionally homologous thermophilic and mesophilic enzymes (54,55). In particular, the presence of extended networks of ionic interactions on the surfaces of the proteins, increased hydrogen bonding, reduced sections of random loop structure, and structural mechanisms resulting in compact protein packing, such as hydrophobic interactions and subunit association, have all been observed in thermophilic enzyme structures. In addition, low proportions of thermolabile amino acids enhance

Table 8. Structural Modifications Leading to Increased Thermostability in Archaeal Proteins

Structural Property	Modification	Example	Reference
Primary structure	Reduced number of thermolabile amino acids, e.g., Met, Cys, Asn, Gln	*Pyrococcus furiosus* citrate synthase	11
	N- and C-terminal amino acid residues immobilized into protein structure	*Methanothermus fervidus* histone	56
Polar and ionic interactions	Extended ion-pair networks acting over extensive portions of protein	Glutamate dehydrogenases from a range of thermophiles including *P. furiosus*	57,58
	High concentration of hydrogen bonds	Glutamate dehydrogenase from *P. furiosus*	59
	Binding of calcium ions	Extracellular protease Archaelysin from *Desulfurcoccus* strain $Tok_{12}S1$	51
Protein folding structure	Increased hydrophobic interactions giving close packing and excluding solvent	*Staphylothermus marinus* surface protein	58
	Increased inter-subunit interactions and oligomer formation	—	—
	Extended secondary structure	—	—
	Few and /or short random loops	Citrate synthase from *P. furiosus*	11
	Solvent-filled hydrophilic cavities	*S. solfataricus*	—

stability in the primary structure and the absence of many glycine residues reduces peptide chain flexibility. Extrinsic factors such as binding of calcium ions also contribute to thermostabilization in some proteins (51).

Wherever thermophilic enzyme genes are expressed in mesophilic hosts, the thermostability of the recombinant enzyme confers the advantage of facile purification, in which contaminating mesophilic proteins can be removed by heat shock.

Halotolerance. The defining feature of the enzymes of halophilic microorganisms is resistance to inactivation in high ionic strength (low water activity) solutions with salt concentrations of 2 to 5 M. Conversely, the presence of a high ionic strength environment is mandatory for the correct folding and biological activity of many extreme halophile proteins. One strategy for the survival of halophiles is the intracellular accumulation of inorganic ions, such as K^+ and Cl^-, which means that intracellular proteins must be halotolerant to the same extent as extracellular enzymes. Significant adaptations of the protein structures are required to give the proteins the unusual resistance to the unfolding and aggregation processes that typify mesophilic proteins exposed to high ionic strength conditions. The halophilic proteins are commonly characterized by a high proportion of acidic amino acids (glutamate and aspartate), which contribute a high concentration of negative charge on the protein surface. This facilitates the binding of hydrated counterions from solution (halophilicity), maintaining the protein surface hydration and decreasing the surface hydrophobicity that would otherwise lead to aggregation (54).

A high salt environment is an absolute requirement for many extreme halophiles, and their enzymes are frequently observed to be unstable in the absence of high ionic strength media. This can be a serious disadvantage in the successful expression of extreme halophile protein genes in common hosts, in purification procedures, and in the development of biotechnological applications.

Organic Solvent-Tolerant Enzymes. The advantages in biocatalysis of using organic or aqueous-organic media for enzyme catalyzed reactions (e.g., increased substrate solubility and/or stability, reduced water-mediated side reactions, reduced microbial contamination) have provided the incentive for seeking and characterizing solvent-tolerant enzymes. The stability of halotolerant enzymes originates at least partly from the ability to withstand very low water activity; such adaptations can also confer solvent tolerance. The solvent tolerance of several enzymes from halophilic Archaea has been reported (Table 9). Similarly, the mechanisms leading to enzyme thermostability, particularly reduced conformational flexibility, also confer resistance to solvent-mediated denaturation (53,60).

Cold-Tolerant Enzymes. Numerous potential applications of cold-tolerant enzymes, particularly in the food, detergent, and textile industries, have stimulated interest in psychrophiles (64,65). Advantages in the use of cold-tolerant enzymes include the facility to inactivate them by moderate increases in temperature, increased reaction yields with unstable substrates, and energy cost savings. Among the very few archaeal cold-tolerant enzymes that have been reported is a psychrophilic DNA polymerase from *C. symbiosum*, isolated by direct genomic DNA cloning (60). The structural features that apparently facilitate activity at low temperature included extended charged surface loops, reduced numbers of hydrogen bonds and disulfide bridges, and increased ion-pair content, all of which would lead to increased protein flexibility (66). This increased flexibility has potential value in biocatalysis applications in which low water activity is used, but broad substrate selectivity must be maintained.

Table 9. Examples of Solvent-Tolerant Enzymes from the Archaea

Archaeal Group	Enzyme	Potential Application	Reference
Halophiles	*Halobacterium cutirubrum* catalase	Removal of H_2O_2 from reaction mixtures	61
	Haloferax mediterranei protease	Peptide synthesis	60
	Halobacterium halobium extracellular protease	Peptide synthesis with advantage of broad substrate specificity and very high activity	59
Thermophiles	*Sulfolobus solfataricus* malate dehydrogenase	Biocatalytic redox reactions	60
	Sulfolobus solfataricus alcohol dehydrogenase	Biocatalytic redox reactions	62
	Thermococcus stetteri proteinase	Stereospecific peptide or amide synthesis	63

Pressure-Tolerant Enzymes. High pressure is generally regarded as a denaturing condition for proteins. However, in the case of thermostable archaeal proteins, high pressure has been shown to increase the stability and even reaction rate, as demonstrated for a protease from *M. jannaschii* (11). Pressure-stabilization is thought to be due to increased packing of hydrophobic core regions. The DNA polymerase of *Pyrococcus* strain ES4 was used as an example of a barophilic enzyme with high thermostability in a study that indicated that pressure-tolerance is related to restricted volume changes during protein unfolding (67).

Applications — Low Specificity Enzymes

Glycosyl Hydrolases. Thermostable glycosyl hydrolases derived from Archaea act on intracellular and extracellular polysaccharides, facilitating the utilization of external energy sources and maintaining a dynamic equilibrium in the formation and break down of carbohydrate osmolytes (68). Heterotrophic hyperthermophiles that can grow on starch or β-linked carbohydrates are common and have been well documented (Table 10; 69,70). The functions of the glycosyl hydrolases are not always clear, as in the case of the autotrophs such as *M. jannaschii* (71). Hyperthermophiles produce numerous glycosyl hydrolases with varying activities and exhibit both α- and β-hydrolyzing specificities. Typically, extracellular hydrolases are more stable than the intracellular enzymes (53).

Enzymes that are stable and active at the high temperatures required to gelatinize starch form one of the largest-scale industrial applications of biocatalysis. The glycosyl hydrolases of importance in the starch industry include amylases exhibiting both α-1, 4- and β-1, 4 specificity, β-glucosidases that hydrolyze nonreducing terminal 1,4-linkages, and pullulanases (debranching enzymes) required to release glucose monomers from 1,6-linked branches in the polymers (Fig. 4). Biotechnological applications of bacterial thermostable glycosyl hydrolases are well established, and as the availability of bulk, low-cost archaeal enzymes improves, their potential should be realized (Table 11). For example, the halotolerant amylases produced by the halophilic Archaea *H. halobium* and *Natronococcus* could provide useful alternatives to the commercial enzymes currently in use (72). In addition, more specialized reactions may be developed, such as transglycosylations catalyzed by *P. furiosus* β-glycosidase for the production of modified chiral sugars used in pharmaceuticals (53).

Proteases. Thermostable proteases have particular application in protein hydrolysis because the elevated temperatures accelerate the unfolding of a protein polymer and increase accessibility of the peptide to protease activity. Conversely, proteases that are resistant to denaturation by organic solvents find application in peptide synthesis carried out in low water activity media. Thermostable proteases have been isolated

Table 10. Thermostable Glycosylases Produced by Archaea (66,67)

Enzymes			Properties
α-specific	Amylase	*Pyrococcus furiosus*	T_{opt} 100 °C Recombinant enzyme cloned into mesophilic host, expressed extracellularly
	Glucosidase		T_{opt} 115 °C
	Amylopullulanase		T_{opt} 100 °C Extracellular recombinant enzyme
	Glucosidase	*Sulfolobus solfataricus*	T_{opt} 105 °C
	Amylase		T_{opt} 115 °C
	Pullulanase	*Pyrococcus woesei*	T_{opt} 120 °C Recombinant enzyme
β-specific	Glucosidase	*Pyrococcus furiosus*	T_{opt} 105 °C Recombinant, expressed in *E. coli*
	Glucosidase	*Sulfolobus solfataricus*	T_{opt} 105 °C
	Mannosidase	*Pyrococcus furiosus*	T_{opt} 105 °C
	Lamminarase		First endoglucanase found in the Archaea

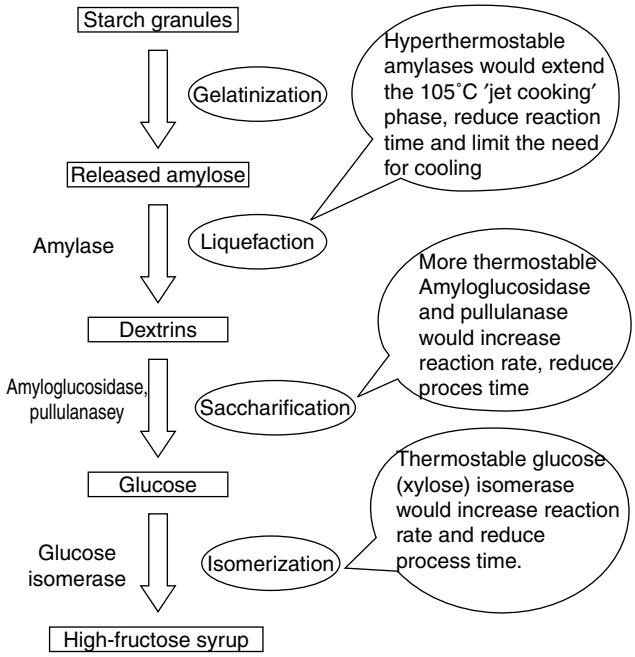

Figure 4. Enzymes used in carbohydrate conversions.

and/or cloned from a number of thermophilic Archaea (Table 12).

Halotolerant proteases, produced by the halophilic species, are utilized for proteolysis reactions in the high-salt (up to 25% w/v salt) fermentations used to convert certain foods (commonly fish or vegetables) into strongly flavored sauces such as *nuoc mam* and soy sauce. Members of the Archaeal *Halococcus* and *Halobacterium* genera have been isolated from such fermentations, having originated, putatively, from the salt added to the fermentation (72). Halotolerant exoproteases are also produced by *H. salinarum* and other *Halobacteriaceae* strains.

Applications — High Specificity Enzymes

DNA Polymerases. DNA polymerases catalyze intracellular reactions involved with the replication and repair of DNA. The biotechnological applications of DNA polymerases, particularly in thermal cycling reactions for amplification of DNA (the polymerase chain reaction), have led to very significant developments in molecular biology. Although the first used and perhaps best known DNA polymerase, Taq (from *Thermus aquaticus*), is not a product of an archaeal microorganism, thermostable DNA polymerases from archaeal thermophiles represent an important potential source of variety. The heat-stable "Vent" and "Deep Vent" polymerases from *Thermococcus litoralis* (73) and Pfu polymerase from *P. furiosus* have been characterized, cloned, and successfully expressed, and are widely used in molecular biology.

Table 11. Potential Biotechnological Applications of Glycosyl Hydrolases

Enzymes	Sources	Potential Application
Xylanases	*Thermococcus zilligii*	Xylans associated with lignin in wood pulping effluent treatment (74)
Hemicellulases	*Thermatoga neopolitana*	Decreasing viscosity of mannan solutions, e.g., guar gum (70)
Cellulases		Cellulose degradation to release glucose Laundry detergent additives (68) Stonewashing cloth
Amylase	*Natronococcus* sp.	Conversion of starch to maltotriose (75)
Amylase + pullulanases + glucosidase	*Pyrococcus furiosus*	One-step starch-to-glucose process (70)
Pullulanases	*Pyrococcus furiosus*, *P. woesei*	Glucose polymer 1,6-linkage debranching in starch hydrolysis
Amylase	*Halobacterium halobium*	Complete starch conversion to glucose
β-glycosidase	*Pyrococcus furiosus*	Transglycosylation of chiral sugars (70)

Table 12. Thermostable Proteases from Archaea

Archaeal Source/Protease	Properties	Ref
Aeropyrum pernix 'Pernilase'	Intracellular;T_{opt} 90 °C; $t_{1/2}$ 12 minutes at 110 °C	74
Aeropyrum pernix "Aeropyrolysin"	Extracellular;T_{opt} 100 °C; $t_{1/2}$1.2 hours at 125 °C	75
Sulfolobus acidocaldarius "Thermopsin"	—	76
Sulfolobus solfataricus neutral protease	T_{opt} 87 °C	77
D. mucosus "Archaelysin"	T_{opt} 100 °C; purified protease active up to 125 °C	78
Pyrococcus furiosus protease "Pyrolysin"	T_{opt} 115 °C; protease in cell-free extracts still active after 24 hours boiling with 1% SDS	79
Pyrococcus furiosus protease Pfp1	T_{opt} 95 °C; $t_{1/2}$19 minutes, 95 °C	80
Pyrobaculum aerophilum "Aerolysin"	—	81
Methanococcus jannaschii proteosome	700 kDa complex;T_{opt} 100 °C	82

These enzymes have functional advantages over Taq polymerase, with higher thermostability and proofreading (3′-5′ exonuclease) activity. More recently, other DNA polymerases from *T. acidophilum*, *M. thermoautotrophicum*, *S. acidocaldarius*, and *S. solfataricus*, have been reported (53). Although none of the aforementioned show these properties, any thermostable polymerase with particularly high fidelity DNA amplification or extended (e.g., megabase) read-through will almost certainly be commercializable.

Restriction Endonucleases. Restriction nucleases with unusual site specificity are of particular value in molecular biology, for use in the cloning and/or the restriction analyses of DNA. Such a nuclease, produced by *Halococcus acetoinfaciens*, was reported by Obayashi and coworkers (76). Although many Archaea possess Type 2 endonuclease activities, few have unique (and therefore particularly valuable) recognition- and/or cut-site specificities. In addition, there are currently no significant applications in which high thermostability in restriction endonucleases is perceived to be an advantage. In most instances, the simple and effective removal of activity after a restriction digest is paramount.

Inteins. Inteins (protein introns) are internal portions of protein sequences that are post-translationally excised although the flanking: regions are spliced together, making an additional protein product. Inteins have been found in a number of homologous genes in yeast, mycobacteria, and hyperthermophilic Archaea such as *P. furiosus* and *M. jannaschii* (86). Inteins are probably multifunctional, autocatalyzing their own splicing and, in some cases, exhibiting endonuclease activity.

Yeast intein genes have been used in the development of an innovative expression-purification system. The IMPACT™ system, developed by New England Biolabs, Inc. U.S.A., used a chimeric *E. coli* expression vector incorporating fused genes encoding the yeast intein and a *Bacillus* chitin-binding protein. The heterologous protein gene is inserted into a multiple cloning site at the N-terminal autocleavage site of the intein. After capture of the expressed fusion protein (using a chitin affinity column), the heterologous protein can be released autocatalytically after addition of a reducing agent.

This novel expression-purification system has many possible applications. The potential use of a thermophilic archaeal intein gene rather than the mesophilic yeast gene may provide an alternative approach to the rapid purification of heterologous thermophilic proteins

DNA Ligases. The DNA ligase reaction system (ligase chain reaction) allows defects in DNA to be detected through mismatching during hybridization of DNA samples with specifically selected primers. The reaction depends on thermostable ligase activity catalyzing the annealing of previously heat-denatured oligonucleotides. Such a reaction has potential application in detection of genetic defects (87) or specific pathogens (88). Although a thermostable DNA ligase from the bacterial *Thermus thermophilus* has been cloned and made available, there is potential value in the search for additional, more thermostable, ligase enzymes from the Archaea.

Specialist Enzymes for Biocatalysis. With the rapid expansion of biotransformation technology, the need for biocatalysts with very specific characteristics now drives the search for novelty in enzyme function. The exquisite stereospecificity of many enzymes is not readily matched in conventional synthetic chemistry, and enzymes possessing both high selectivity and stability under extreme conditions potentially have a high-value market. For example, the alcohol dehydrogenases of *S. solfataricus* and *H. butylicus*, which have thermal stability and solvent tolerance, have good stereoselectivity in the catalysis of secondary alcohol oxidations or carbonyl compound reductions (53). Similarly, esterases with solvent tolerance have application in stereochemical resolution and in transesterifications and ester hydrolyses. Thermostable and halotolerant archaeal enzymes may well provide new sources of these activities. Biocatalytic redox reactions may also impose unusual constraints, such as specific redox potential or cofactor recycling. Enzymes with high stability and activity and able to function in organic solvent media, would be especially valuable in cofactor regeneration. For example, pyruvate dehydrogenase from *P. furiosus* (T_{opt}>95 °C), glutamate dehydrogenase from *P. furiosus* (T_{opt}95 °C), glutaraldehyde-3-phosphate dehydrogenase from *P. woesei*, and formaldehyde oxidoreductase from *T. litoralis* (T_{opt}95 °C) (50) may all be candidates for developing such applications.

Lipids

The "core" lipids of the Archaea are largely based on saturated isoprenoid chains linked to a glycerol backbone by ether bonds. Common structures include the monomeric diphytanylglycerol ethers and the dimeric dibiphytanyldiglycerol tetraethers and dibiphytanyl glycerol nonitol tetraethers (a generic structure is shown in Fig. 5). The former, termed archaeols, are found in all Archaea, whereas the latter, termed caldarchaeols and nonitolcaldarchaeols, are found only in the thermophilic Archaea. The caldarchaeols and nonitolcaldarchaeols exhibit further modification by containing up to four cyclopentane rings in each of the C_{40} biphytanyl chains. The addition of cyclic structures in the transmembrane portion of the lipid appears to be a thermoadaptive response, resulting in enhanced membrane packing and reduced membrane fluidity (89).

Although few, if any, studies have been carried out on the chemical stability of purified ether lipids, the stability of artificial ether-lipid membranes (liposomes) has attracted considerable attention. Membranes composed of

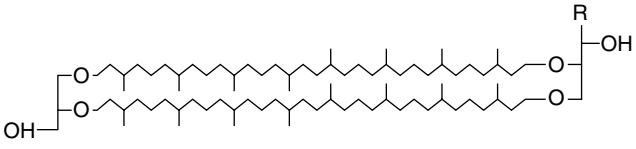

Figure 5. Biphytanyl tetraether core lipid structure (where R = H, calditol, etc).

C_{40} membrane spanning (boliform amphiphilic) tetraether lipids maintain a constant thickness of 25 to 30Å (89), somewhat thinner than typical C_{18} phosphodiester bilayer membranes. Nevertheless, these archaeal membranes are much more physically stable than those formed from phosphodiesters. For example, large (600 nm) vesicles generated from *T. acidophilum* ether lipids were found to be more resistant to physical disruption by high temperature and surface-active agents such as phenol, alcohols, and detergents than dipalmitoyl phosphatidylcholine vesicles (90). In a more extensive study of liposome stability, Sprott and coworkers (91) compared liposomes prepared from the ether lipid extracts of a number of archaea with egg phosphatidylcholine and dipalmitoyl phosphatidylcholine liposomes. In virtually all instances, the archaeal ether lipid liposomes showed higher levels of stability to temperature, pH, serum proteins, and long-term oxidative effects than the ester lipid vesicles. Not surprisingly, the former were also highly resistant to the addition of phospholipases. The use of fluorescent probe techniques to measure the thermal stability of *S. acidocaldarius* lipid liposomes over a temperature range of 25 to 85 °C showed little variation in proton permeability across the temperature range (92).

The remarkable physical and chemical stability of archaeal ether lipid liposomes, attributed to the presence of the ether linkage, to the intimate packing of the phytanyl chains, and to the reduced degree of molecular translational freedom in a boliform structure, has provided a significant incentive to the practical utilization of such liposomes. A number of applications, including drug delivery systems (91) and bioelectronics components (93), have been proposed.

The recent demonstration that Archaeal lipid fractions could be formed into stable liposomes (termed archaeosomes) has led to a resurgence of interest in these structures as vaccine and drug delivery systems (94). The uptake of archaeosomes by phagocytic cells can be up to 50-fold greater than normal liposomes (95), and toxicity trials have not shown any adverse reactions. Mice treated with archaeosome preparations containing antigenic peptides have been shown to give immune responses of the same order as when using conventional adjuvants (96). In addition, tissue distribution data following administration of archaeosomes by different routes suggests that some degree of organ targeting may be possible (97).

S-Layers

The cell walls of most Archaea are characterized by the presence of a regular arrangement of paracrystalline, homogenous protein, or glycoprotein subunits, the Surface-layer (S-layer), as the outermost surface barrier of the cell. The protein subunits are usually of high molecular weight (40–200 kDa) and are arranged in lattices with characteristic patterns of symmetry and complexity, conferring varying degrees of porosity (98). In some archaeal species, such as *H. salinarum*, *H. halobium*, and *S. acidocaldaricus* (99), the cell wall is composed only of an S-layer, a simple protein layer outside the plasma membrane. However, the composition and complexity of the S-layer varies considerably, with some species possessing multiple layers with higher order structures, and/or layers incorporating unusual glycoprotein polymers.

The S-layer proteins of Archaea have a high proportion of hydrophobic amino acids and are often highly glycosylated, sulfated, and/or acidic. The glycosyl components involved frequently have complex composition, with unusual methylation or acetylation of amino groups on the polysaccharide backbone. For example, in *H. halobium*, 50 sulfate groups are present per molecule, on acetylated glucuronic acid and acetylated *N*-aminogalactosyl residues (100). In some methanogens, the S-layer peptidoglycan subunits also contain the unusual polymer pseudomurein, which contains the amino acids L-lysine, L-glutamic acid, L-alanine, and sometimes ornithine, in addition to certain unusual N-acetylated amino sugars. The cell walls of *Methanosarcina* spp. contain methanochondroitin, another specialized glycopeptide, in which the carbohydrate residues are N-acetylated glucosamine and galactosamine (101). Such glycosylation and chemical diversity patterns are far less common in the bacteria.

The functions of S-layers are believed to include maintenance of shape, protection, cell adhesion, molecular surface recognition, and molecular binding to the cell surface (102). The well-defined pore-size, 30 to 40 kDa (103), is likely to confer a molecular exclusion function, protecting the cell against attack by lytic enzymes, immunogens, and biocides. Pseudomurein, for instance, is resistant to cell-wall antibiotics, lysozyme, and proteases and methanochondroitin is resistant to cellulolytic activity. Whatever the structure or function of the S-layer, continuous recrystallization occurs, allowing the dynamic processes of protein turnover and cell expansion and division (98). The highly active expression systems required for continuous in vivo S-layer production are of considerable interest in the development of vectors for high-level expression of heterologous proteins.

Biotechnological Applications of S-Layers. The capability of S-layer proteins to self-assemble and recrystallize correctly and consistently is also observed in vitro, even after disruption due to chemical treatment, which suggests a number of potential biotechnological applications for S-layers. The cloning and overexpression of bacterial S-layer proteins has facilitated their production for reassembly on foreign (non cell-surface) surfaces, in amounts sufficient for industrial applications.

Porous monolayers can be obtained by allowing S-layer proteins to self-assemble on a variety of different type surfaces, including inorganic (e.g., silicon) and metallic (e.g., gold). The orientation of the protein subunits is such that there is asymmetry in the monolayer, with respect to hydrophobicity of the component molecules, so that the layers assemble with a more hydrophobic face presented to the more hydrophobic portion of any biphasic environment such as an air-water or water-solvent interface or on solid or liquid supporting surfaces (104). Thus, selection of the medium permits some control over the nature of the monolayer that forms and hence its function.

The exploitation of archaeal S-layers has been less commonly reported, possibly because of difficulties in the

large-scale fermentation or expression of archaeal proteins. Nevertheless, the progress of production technology is sufficiently rapid to justify the assumption that archaeal S-layer proteins will soon be accessible in quantities that allow the industrial exploitation of these more complex monolayers. The discussion in the following text therefore reports largely on results achieved with bacterial S-layer proteins, but may be extrapolated to the application of archaeal S-layers.

Ultrafiltration Membranes from S-Layer Proteins. Self-assembled monolayers obtained from S-layers have regular and well-defined dimensions (5–15 nm thick, with pores of 2–8 nm), making them particularly suitable for use as ultrafiltration membranes for high-selectivity molecular exclusion, with molecular cutoff of 30 to 40 kDa (103). The consistency of their chemical composition and charge distribution, as well as physical structure, give S-layer membranes more uniform characteristics than conventional synthetic ultrafiltration membranes. The proteins are allowed to assemble on synthetic microfiltration membranes, and may be cross-linked chemically to provide physical resilience. Further chemical modification of the protein monolayers can be used to produce customized membranes with specific functions such as antifouling properties (105).

Immobilization of Biomolecules on S-Layers. The functional groups of S-layer proteins and their associated carbohydrate moieties provide a variety of potential reaction sites for immobilization of other biomolecules, such as enzymes, antibodies, or lipids (106). Such immobilized systems can be formulated on a micro- and nanoscale, as in the case of immobilization of enzymes for use as microscale biosensors in the detection of sucrose (98,107). Coimmobilizing multiple biomolecules allows application of the technology to biochemical reactions of considerable complexity, for example, in immunoassays (106). More sophisticated surfaces, formed by combining S-layers with bimolecular lipid membranes and incorporating various biochemical systems such as proton pumps, provide much potential for new applications (107).

Nanotechnology. In more advanced applications, recrystallized monolayers can be laid down in predesigned patterns on a silicon surface and etched by use of ultraviolet radiation, resulting in practical nanoscale semiconductor components. Coatings of S-layer proteins can also be used to mask areas of silicon surfaces before etching. Alternatively, nanometer scale metallic particles (3–5 nm) and lattices can be formed with preselected symmetry, by using S-layer assemblies as templates (104).

Compatible Solutes

An important characteristic of halophilic and hyperthermophilic microorganisms is the accumulation of a range of low molecular weight compounds in cells, serving as osmoprotectants of cellular components, including proteins (68,108). The chemical nature of these "compatible solutes" varies between species, but all are hydrophilic, water-compatible molecules. Although significantly more information is known regarding the bacterial solutes, hyperthermophilic members of the archaeal group are known to accumulate di-myo-inositol-1, 1′-3, 3′-phosphate, cyclic 2, 3-bisphosphogycerate, or trehalose (109). In thermophilic Archaea, these compatible solutes are considered to have a role in protein thermostabilization, and in halophiles, in osmoregulation and stabilization against high ionic strength denaturation. In certain halobacteria, production of compatible solutes such as biodegradable polyalkanoates and ectoines has been developed for commercial applications, but no such applications have yet been developed for archaeal sources. Nevertheless, an understanding of the role of these solutes in protein stabilization is likely to provide mechanisms whereby biotechnological and pharmaceutical products can be stabilized.

ARCHAEAL PROCESSES

Methane Production

The generation of methane through the mesophilic (and sometimes thermophilic) anaerobic metabolism of methanogenic Archaea is a naturally-occurring and widespread process and is particularly evident in swamps, rice paddies, landfill sites, and in ruminant and mammalian digestive processes. Many of the consequences of this process may be considered to be detrimental, particularly where unwanted methane production generates an explosion hazard (as in landfill sites) or through the supposedly deleterious effects on the ozone layer. However, under "controlled" conditions, Archaeal methanogenesis (biogas) has been exploited as an inexpensive and effective source of energy for many centuries. The first biogas production plant was constructed in India in 1859 and some 10 million small-scale digestion plants are now in use worldwide for local heating and lighting. Large-scale energy production employing methanogenesis is now commonplace in Denmark.

Biogas production is based on the digestion of inexpensive renewable organic material, including animal manure, organic industrial waste, and municipal solid waste. On a larger scale, the biogas production process is frequently integrated into the treatment of industrial, commercial, and municipal wastewaters and effluent streams. For additional information, the reader is directed to the following reviews (110,111).

H_2 Production

An innovative application of hyperthermophilic archaeal enzymes to molecular hydrogen production has recently been proposed. Because of its high energy content, hydrogen is a particularly favored fuel, although biological production methods have received relatively little attention. The discovery (112) of an NADPH-dependent hydrogenase in the hyperthermophilic Archaeon *P. furiosus* has provided an in vitro mechanism for H_2 production. By coupling NADPH production to an enzymic generation system, such as a thermophilic glucose dehydrogenase, a process that uses an inexpensive feedstock (glucose) and generates

two high-value products (glucuronic acid and H_2) can be developed. Preliminary results that show high conversion efficiencies are promising (113).

Biomining: Biological Extraction of Metals

The ability of iron- and sulfur-oxidizing acidophilic microorganisms to enhance the extraction of metals such as copper, gold, and uranium (known as biomining or bioleaching) has been employed commercially for some years. Piles or dumps of low-grade mineral ores are typically acidified by addition of mineral acid, stimulating the natural populations of Fe^{II}- and S-oxidizing organisms (primarily the mesophilic bacteria *Thiobacillus ferrooxidans*, *Thiobacillus caldus*, and *Leptospirillum ferrooxidans*; 114). Where natural heating occurs, populations of the moderately thermophilic bacterium *Sulfobacillus* and archaeon *Thermoplasma* may become significant. At higher temperatures (e.g., >60 °C), the extremely thermophilic Archaea, *Sulfolobus, Acidianus*, and *Metallosphaera*, dominate. The process of metal recovery is dependent on oxidative solubilization of copper and iron sulfides (chalcopyrite and iron pyrite), releasing soluble metals (e.g., copper) and insoluble metal particles (e.g., gold and uranium).

For recovery of high-value metals, it is economically feasible to leach mineral sulfide concentrates rather than low-grade ores and to employ bioreactor technology (typically simple but large stirred tank reactors) rather than ore piles. A number of pilot plants and large-scale demonstration plants are currently operating in Brazil, South Africa, Ghana, and Australia (115,116), with throughputs of a few tens to more than a thousand tonnes of ore per day (Table 13).

The interest in thermophilic bioleaching is derived from advantages in process economics, mineral solubility, and reaction rates from operating at elevated temperatures (117). Norris and coworkers (118) have demonstrated that efficiency of extraction increases proportionally with temperature up to around 80 °C. Furthermore, when operating an exothermic batch reaction in a hot climate, the use of thermophilic organisms precludes the requirement for costly cooling technology. Possible disadvantages lie in the sensitivity of archaeal cells to physical damage in stirred reactors (116) and in the sensitivity of some archaeal strains to high concentrations of soluble metal ions (118). However, the recent identification of *Sulfolobus* strains capable of tolerating concentrations of copper ions up to 40g/L (Fig. 6) suggests that these organisms may be well suited to large-scale metal ion recovery.

Table 13. Operating Mineral Processing Bioreactors (100,116)

Location	Throughput (tonnes ore/day)	Reactor Capacity (m^3)
Fairview, South Africa	40	900
São Bento, Brazil	110	1,160
Wiluna, W. Australia	152	4,240
Obuasi, Ghana	1,152	21,360

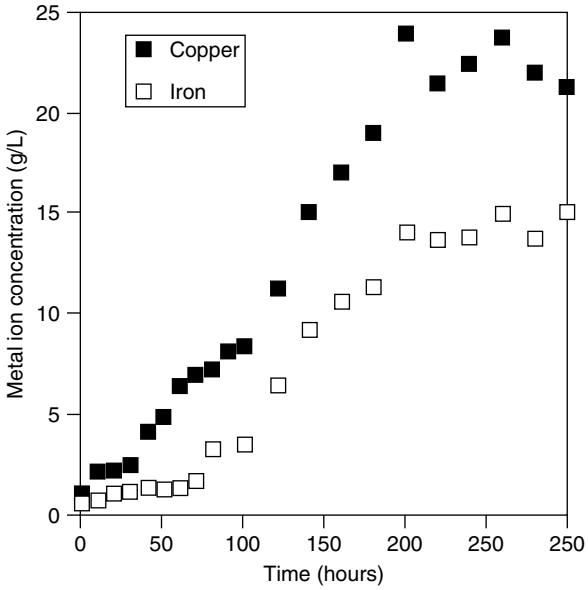

Figure 6. Solubilization of chalcopyrite ore at 78 °C using thermophilic archaea (redrawn with permission).

CONCLUSION

The reader will note that one of the most used terms in the entry with regard to the value of Archaea in biotechnology is the term *potential*, reflecting the reality that while much information is available and more is being generated very rapidly, the number of real commercial applications is limited. This situation is strangely at odds with the level of interest in both academic and industrial sectors with respect to extremophiles in general and Archaea in particular. One factor contributing to this is limited success in developing efficient expression systems for the purposes of enzyme production in recombinant hosts.

Additionally, the operating conditions required for fermentation processes for generating Archaeal bioproducts are substantially different from those of conventional industrial processes and require novel approaches in terms of reactor development, process engineering, and economy. Both of these problem areas are currently being addressed by modern technology developments. The section of the entry describing Archaeal products, whether potential or real at this stage, provides justification for the attention the field is receiving.

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ARCHAEA IN MARINE ENVIRONMENTS

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Archaea were originally thought to exist solely in extreme anoxic, hot, or hypersaline environments. However, technological advances in the past 20 years have led to a significant revision of this view. Archaea are now recognized to be abundant in the largest habitable regions on earth, the ocean, and in underlying sediments, at times rivaling the abundance of the bacteria (1–3). Marine Archaea have been detected as free-living plankton associated with suspended particles in oxic and anoxic zones of marine sediments, in the guts of fish and holothurians, and as symbionts of marine sponges. Phylogenetic diversity of the marine Archaea is somewhat limited in comparison to the diversity of marine organisms in the bacterial domain, although it is evident that we do not currently know the extent of archaeal diversity, as new groups are continually being discovered (4–6). Although most of these newly described Archaea remain to be cultivated, promising results from environmental genomic, stable isotopic, and microautoradiographic analyses suggest that at least some phenotypic characterization of these important organisms is possible. Evidence suggests that the Archaea are probably important to present day biogeochemical cycles and to past conditions on earth.

NEWLY RECOGNIZED ARCHAEAL DIVERSITY

Recent developments in the study of molecular evolution have led to a unified view of life on earth in which all life falls into three evolutionarily distinct forms, Bacteria, Archaea, and Eucarya (Fig. 1; 7,8) as opposed to five kingdoms (Monera, Protista, Fungi, Plantae, and Animalia) or two forms (Eucaryotic and Prokaryotic) as suggested by other theories based on morphology. Biochemical, genetic, phenotypic, and now genomic data collectively suggest that there are two kinds of prokaryotes, the bacteria and the Archaea, and that the Archaea are as distinct from the bacteria as they are from the Eukaryotes. Archaea were formally recognized to be distinct from bacteria in the late 1970s following the work of Carl Woese (7). There are a number of distinctions on which these divisions have been based, supporting

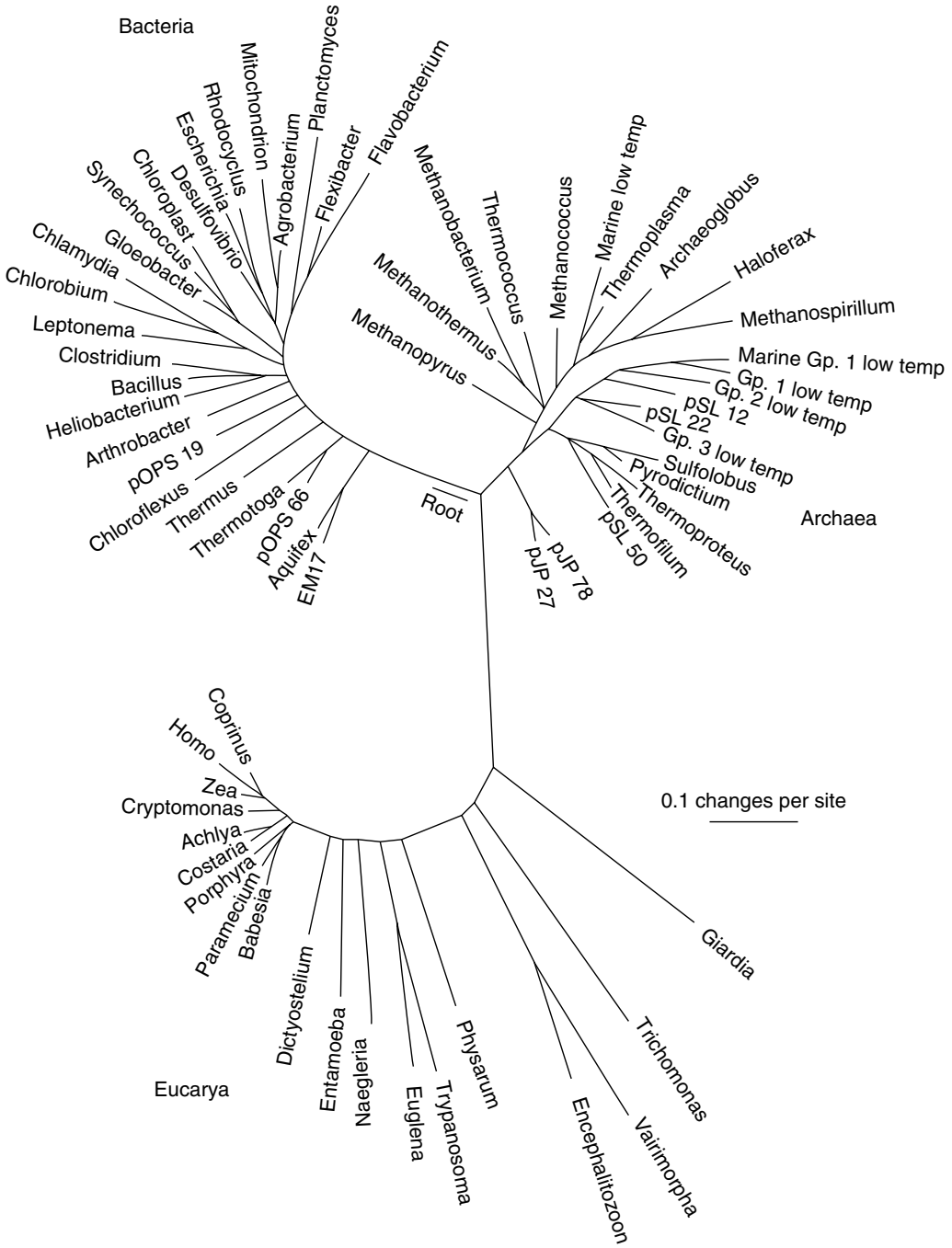

Figure 1. Universal phylogenetic tree based on small subunit rRNA sequences for the three domains of life. (Reprinted (abstracted/excerpted) with permission from N. R. Pace, A molecular view of microbial diversity and the biosphere, *Science*, copyright, 1997, American Association for the Advancement of Science.)

their unique properties. For example, the Archaea harbor the replication machinery of eukaryotes, and the basic metabolic attributes of the bacteria. Initially only thought to occur in extreme (hot, saline, or anaerobic) environments representing ancient forms of life, representatives of the Archaea have been found globally in both terrestrial and marine environments, in oxygenated and anoxic habitats. The phylogeny of the archaeal division strongly supports the existence of two deep branching lineages, the Crenarchaeota (traditionally thought of as the sulfur-dependent hyperthermophiles), and the Euryarchaeota (thought to comprise the methanogens, some thermophiles, and halophiles, which require salt at concentrations greater than 1M).

From the perspective that scientists have been studying marine bacteria for more than 200 years and the deep sea for 120 years, scientists may have unknowingly been studying marine Archaea since the establishment of

marine microbiology. This is a result of the fact that under the microscope most prokaryotes basically look the same and Archaea cannot be readily distinguished by direct cell counts. The history about the existence of Archaea (as they are now formally recognized) in marine environments dates back to at least 1951 with the isolation of a marine methanogen, *Methanococcus vannielii* from San Francisco Bay sediments (9). Marine methanogens, thermophiles, and hyperthermophiles have been isolated subsequently from extreme environments such as deep-sea hydrothermal vents, firmly establishing that Archaea occupy marine ecosystems (10,11). Since then, a number of significant revelations about the extent of marine archaeal diversity, distribution, and abundance have irreversibly transformed the perception of the importance of these organisms in marine environments, with the most revolutionary discoveries following introduction of modern molecular biology to the study of marine environments. Before 1992, all Archaea were thought to exist solely in extreme environments, in which they harbored unique metabolisms and performed specialized biogeochemical transformations for survival. On the whole, Archaea were not considered to be important in marine environments because the habitats in which they flourished were relatively scarce and geographically isolated. However, after molecular tools enabled the discovery that there are mesophilic and psychrophilic Archaea affiliated with both the Crenarchaeota and Euryarchaeota in seawater and marine sediments (12–15), this view has changed dramatically.

In recent years, molecular surveys have repeatedly indicated that cultivated microorganisms represent only a minority of the naturally occurring microbial diversity (16 and references therein); the planktonic Archaea are exemplary of this observation. Today Archaea are often found in molecular surveys of marine environments. Cultivation of the mesophilic and psychrophilic Archaea remains to be accomplished, although many experts have extended efforts to do so. Although the closest relatives of the uncultivated marine crenarchaeotal psychrophiles are the cultivated hyperthermophiles, there are a number of lines of evidence suggesting that these organisms belong to a phylogenetically distinct lineage. The marine crenarchaeotes appear to have a common ancestor with the hyperthermophiles and may have adapted to the colder habitats in which they have been detected. The approach for sampling organisms directly from the environment is tremendously valuable for detecting organisms and establishing relationships in an evolutionary framework, yet the molecular signatures do not provide much in the way of physiological potential, role, or capability.

This chapter addresses the topic of Archaea in marine environments from a global biological diversity and ecological perspective that has risen largely as a result of technological advances made with modern biology. Because the salinity of seawater does not approach the levels required by the extreme halophiles, they are rarely recovered from marine samples (although they are common in ancient hypersaline seas), and thus will not be covered in this article. A key finding from these studies is that the cultivated Archaea numerically represent a minority of the naturally occurring Archaea.

DETECTION OF MARINE ARCHAEA

Microorganisms are inherently difficult to recognize and study because of their small size and limited morphological characteristics. In fact, it is thought that microbiologists are able to cultivate less than 1% of the microbial diversity on this planet (17). At times, sophisticated equipment and media are required for microbial cultivation. Methanogenic members of the Euryarchaeota were the first marine Archaea to be isolated. They are widely distributed in marine environments (mostly sediments), and are thought to be the most commonly cultivated archaeal groups in marine environments (18). They are typically associated with low-temperature anoxic sediments in which they carry out the final step in carbon oxidation to methane. Isolation of marine methanogenic archaea was followed by characterization of hyperthermophilic methanogens; these discoveries precipitated a number of cultivation-based efforts at hydrothermal vents (see HYDROTHERMAL VENTS: BIODIVERSITY IN DEEP-SEA HYDROTHERMAL VENTS and HYDROTHERMAL VENTS: PROKARYOTES IN DEEP-SEA HYDROTHERMAL VENTS, this Encyclopedia).

Cultivation approaches by necessity limit the diversity sampled because the organisms are taken out of their environment. Thus, a major trend in the study of microorganisms living in the environment in the past 15 years has turned to approaches that directly sample the diversity of organisms in natural ecosystems. Significant advances in recognizing archaeal diversity can be attributed to recognition that small subunit ribosomal rRNA (SSU rRNA) sequences can be used to study microbial diversity in natural environments (19). Thus far, it is thought that Archaea in the Crenarchaeota division harbor single ribosomal operons, whereas Euryarchaeota can have as many as four. There is no single genomic organization of these genes, because a variety of patterns (tandemly linked and randomly distributed) have been identified (20).

A number of SSU rRNA-based approaches have been adapted for studying marine Archaea. (*1*) SSU rRNA amplification and clone library generation and DNA sequence analysis was performed in initial studies of marine Archaea (i.e., 12,13). SSU rRNA sequences provide robust data for biological diversity and phylogenetic studies. (*2*) Denaturing gradient gel electrophoresis (DGGE) analysis has been useful for assaying diversity in a comparative framework. This approach was recently used to describe archaeal diversity in deep marine sediments (4). (*3*) Terminal fragment length polymorphism (T-RFLP) analysis of SSU rRNA gene fragments is not as sensitive as DGGE, although it is useful for discriminating between marine crenarchaeotal and euryarchaeotal sequences (21). A recent study demonstrated the utility of this approach for detecting marine Archaea only after a 10-cycle amplification (22) in which the different phylogenetic clusters could be resolved easily, and more closely related

archaea could be resolved following multiple digestions. Amplification-based approaches are typically not very useful for quantifying specific prokaryotic groups, although recent work in this area shows promising results for estimating the abundance of groups of organisms in natural populations using highly sensitive nucleic acid detection techniques such as the 5′ nuclease assay (Taqman assay, 23,24).

For quantification, quantitative membrane hybridizations and fluorescently labeled in situ hybridization (FISH, 25,26) that was modified to increase sensitivity (1,2,27), have proven to be quite effective for estimating natural abundances of marine Archaea. The first quantitative marine Archaea data was determined using oligonucleotide probes designed to be complementary to archaeal SSU rRNA sequences (12). Application of oligonucleotide hybridizations has enabled the detection of Archaea in numerous samples because it is amenable to multiple sample analysis and is relatively quick to perform. Membrane-based oligonucleotide hybridizations to marine community rRNA has proven to be quite accurate in comparison to direct counts of whole cells that are fluorescently labeled (1), and to gene copy numbers estimated by quantitative PCR methodologies (23), although owing to intricacies with probe design, secondary structure conformation, and sequence biases, overestimation, and underestimation are known to occur.

Another recent technological advance has enabled phylogenetic identification of microbial cells (FISH) to be combined with metabolic activity (microautoradiography, 28,29). The cells then are triple stained with 16S rRNA probe, DAPI (binds DNA), and the radiolabeled substrate (incorporated into proteins, DNA, or membranes), which can be visualized under phase contrast following emulsification and developer treatment. This approach has enabled researchers for the first time to show incorporation of a substrate into planktonic marine archaeal cells (30).

One of the unique and unifying characteristics of all Archaea is that archaeal membranes are derived from ether linked isoprenoid-based alkyl chains, as opposed to acyl-ester membrane lipids of all Bacteria and Eukaryotes. The major membrane lipid present in methanogens and halophiles is diphytanylglycerol (archaeol), whereas hyperthermophiles and a few methanogens contain caldarchaeol, a dibiphytanyl-diglycerol tetraether (31). Detection of archaeal lipids in marine waters and sediments (32–34) has confirmed that (*1*) the Archaea are present in abundance in planktonic marine environments, (*2*) their lipids are similar to membranes of other archaea, and (*3*) because of the recalcitrant nature of the ether-linked compounds, marine planktonic Archaea probably contribute significantly to lipids in marine sediments, and hence, are present in the geologic record. Four major lipid compounds found exclusively in Archaea possessing a C_{40} biphytane carbon skeleton containing zero to three cyclopentane rings have been detected from seawater and sediment samples collected from diverse locations (Fig. 2).

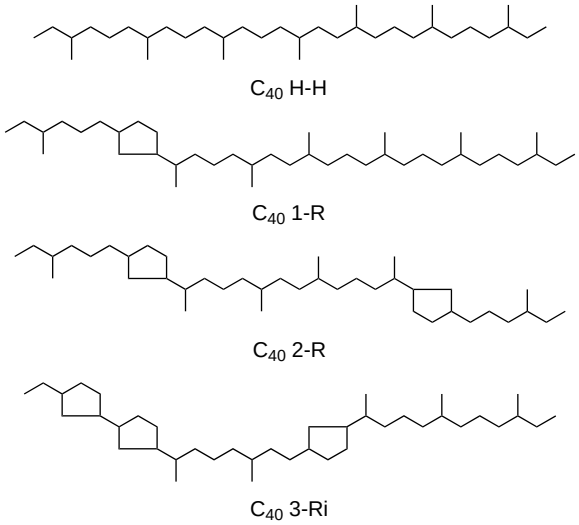

Figure 2. Caldarchaeol-derived acyclic and cyclic biphytane structures derived from Antarctic picoplankton lipid extracts. Symbols: R, pentacyclic ring; i, isomer.

MARINE ARCHAEAL HABITATS, ECOLOGY, AND DIVERSITY

Archaea have been isolated from or detected in a growing number of marine environments. The extremophiles (methanogens, thermophiles, or hyperthermophiles) are not considered to be numerically dominant in earth's ocean systems as they are typically associated with hydrothermal vents, anoxic marine sediments, or associations with guts of marine organisms. They are covered to some degree later in the text and in other articles in this Encyclopedia. Refer to Table 1 for a synthesis of the various studies that have been completed in recent years reporting diversity of marine Archaea using small subunit ribosomal RNA sequences.

Marine Plankton

Biological Diversity and Environmental Distribution. Planktonic Archaea have been detected in both coastal and open ocean ecosystems worldwide in which only four divergent groups (Marine GI-IV) have thus far been identified. All four groups remain uncultivated to date and are distantly related to known thermophiles and methanogens. Cumulative evidence from studies of planktonic archaeal diversity indicate that the most commonly recovered sequences are phylogenetically closely related in a cluster known as the marine group I (Marine GI, 12), in fact, these organisms might be one of the most abundant microbial species on the planet (3).

The marine GI Archaea were originally detected by phylogenetic analysis of environmentally-derived sequences in 100 mm and 500 mm waters off the coast of California (13). At the same time marine GI and GII sequences and archaeal rRNA (using small subunit rRNA-targeted oligonucleotide hybridization) were detected in coastal waters of the Santa Barbara Channel and at Cape Cod (12). Interestingly, at that time, no archaeal sequences were recovered from surface

waters of the central Pacific or Atlantic Ocean gyres. The GI sequences were distantly related to known sulfate-reducing hyperthermophiles grouping with the Crenarchaeota, whereas the GII sequences were most closely related to the *Methanomicrobiales*, a diverse group of methanogens (12). Since then numerous studies have most commonly detected close relatives of marine GI Archaea in midwater to deep ocean depths (Table 1).

Investigations of the extent of diversity of the marine planktonic Crenarchaeota, in particular the marine GI cluster, have indicated that the diversity of this group is fairly restricted, perhaps only being represented by a few closely related species (52). Comparisons of 16S rDNA (Fig. 3a) and ribosomal spacer (internal transcribed spacer, ITS) region suggest that there is a population level structure (ecotypes) in this group that defines two surface-associated clades, an Antarctic clade, and a deep-ocean clade, which harbors the greatest diversity. Diversity of the marine planktonic Archaea supports the view that prokaryotes can have global distribution. It remains to be shown how different the genomes and physiologies of such closely related ecotypes will be.

Although it was initially thought that the planktonic archaeal diversity was restricted to the marine GI and GII, this view has also changed. A cluster of euryarchaeaotal sequences classified as the marine GIII was reported from sequences recovered from deep-sea samples collected in the Pacific and Atlantic Oceans (36). Representatives from this apparently deep-sea-adapted cluster have since been detected in the Aegean Sea (22), and at 3,000 m in the Antarctic Polar Frontal region (Fig. 3b). Similarly, another marine archaeal lineage, referred to as marine GIV, was detected in deep waters (3,000 m) in the Antarctic polar front region. These deeply branching Euryarchaeota are affiliated with the haloarchaea clade (Fig. 3b). Surveys of various regions across the Drake passage, in the Mediterranean, and North Atlantic confirmed the presence of these organisms using primers specific to the marine GIV cluster in samples collected at depth (occasionally at 100 m, and more commonly below 500 m), and never in the upper water column [<100 m (6)]. Recent detection of the marine GIV cluster is probably due to the divergence of this group, which would not have been discovered with several commonly used primer sets. Also, detailed phylogenetic investigations of the deep sea are scarce, and it appears that although the marine GIV organisms are commonly present in deep-sea samples, they might not be very abundant (6).

Several studies have indicated that planktonic archaeal populations are vertically distributed in the water column and that this distribution has phylogenetic coherence. Studies in the Santa Barbara Channel (36,53) and Monterey Bay (1,23) have established the ecological distribution of the two groups of the marine Archaea. The marine GI affiliated sequences appear to be more frequently isolated from deeper in the water column, whereas marine GII Archaea have been typically detected intermittently in the surface waters (Table 1). Planktonic archaeal ecotypes have been shown to be associated with specific environments, such as a biogeographical region studied in the Columbia River Estuary (CRE, 39) or in the anoxic zone of the Cariaco Basin (42). Most reports of marine Archaea suggest that they are free-living, although studies from the Pacific coast, Mediterranean coast of Spain, and North Sea have found archaeal 16S rRNA sequences to be associated with particles (12,21,39,51,54).

An important confirmation that the planktonic archaea were indeed significant residents of the plankton resulted from the gas chromatographic (GC) and GC-mass spectroscopy (GC-MS) lipid analyses. Archaeal lipids containing a biphytane carbon (C40) skeleton, with zero to three cyclopentane rings have been detected in seawater from Arthur Harbor, Antarctica, the Santa Monica Basin, Cariaco Trench, and the Black sea, (Fig. 4), and the Cariaco Trench (34). Acyclic and cyclic biphytanes were the most dominant lipids found in the Arabian Sea at water depths between 450 and 1,500 m (34). The marine picoplankton and *Cenarchaeum symbiosum* were found to contain caldarchaeol and derived acyclic and cyclic dibiphytanes, in which the acyclic head to head biphytane was the most abundant isoprenoid identified (Fig. 4). The value of these lipids as biogeochemical tracers is just beginning to be realized, indicating that these organisms contribute significantly to carbon deposition in the sea (33).

Marine Archaeal Abundance. Several studies have reported direct counts of Archaea in marine coastal and open ocean environments from surface waters to the deep sea where values ranged from 1×10^3 to nearly 2×10^5Archaea $\times$ mL^{-1} seawater, representing 1 to 60% of the DAPI-stained cells (Table 2). There is substantial variability in archaeal abundance (as well as community diversity) with depth (Table 2). In the coastal and continental shelf studies, it appears that the GI Archaea dominate below 60 m, whereas the GII cells are mostly restricted to the upper 40 mm with seasonal peaks in surface waters (1,53). In the open ocean, the GI Archaea peak between 150 and 500 mm and persist in high relative abundance throughout the mesopelagic and bathypelagic zones to the ocean bottom (5,000 mm Fig. 5). In a study at Station Aloha in the north pacific, the abundance of marine GI Crenarchaeota below 1,000 mm equaled the numbers of bacteria and approached up to 40% of the DAPI-stained cells (Fig. 5). The marine GII cells at Station Aloha represented at most a few percentage of the DAPI-stained cells, although sporadic peaks in abundance were detected in the upper water column. Abundance of marine GII declined throughout the mesopelagic zone then increased again in deeper samples. The abundance of marine GI and GII planktonic Archaea have also been estimated using a Q-PCR Taqman assay, in which the marine GI and GII sequences could be detected at the level of 2.5×10^3 to 2.5×10^7 gene copies per reaction (sensitive enough to detect archaeal in one milliliter of seawater, in many locations, 23).

Seasonal Variation. Most molecular-based studies have described archaeal abundance by single point surveys or described distributions of the Archaea down the water column, leaving far less known about the seasonal dynamics of the Archaea. Four temporal studies exist, which

Table 1. Summary of Marine Archaeal Groups Detected by Environmental rRNA Cloning and Sequencing Studies

Habitat/Location	Water Depth (m)	Sediment Depth (cm)	Temp. (°C)	Archaeal Division/Group	Reference
Seawater					
North East Atlantic	500			Marine GI	35
North Atlantic off Scotland	0–500		7–13	Marine GI	35
Atlantic Ocean	1,000		7	Marine GI, GII	36
Atlantic Ocean	coastal waters		15–20	Marine GI and GII	12
Atlantic Ocean	1,000		3–5	Marine GI	36
Pacific Ocean	500 and 3,000		6.8 and 1.6	Marine GI, GII, GIII	36
Santa Barbara Channel	coastal waters		10–15	Marine GI and GII	12
Santa Barbara Channel	10–20		10–15	Marine GI	27
Santa Barbara Channel	0–200			Marine GI and GII	37
Pacific Ocean	100		7–9	Marine GI	38
Pacific Ocean	500		5–7	Marine GI	36,38
Pacific Ocean	3,000		3–5	Marine GI	36
Western Pacific Ocean	100		14	Marine GII	38
Western Pacific Ocean	500		5.5	Marine GI	38
Columbia River Estuary	coastal waters			Marine GI, GII, + others	39
Antarctic Peninsula	0		−1.8	Marine GI, GII	40
Antarctic	10–200			Marine GI	41
Antarctic, Polar Front	3,000			Marine GII, GIII, GIV	6
Southern Ocean	coastal waters		−1.8	Marine GI and GII	40
Mediterranean	5–25			Marine GI	41
Mediterranean, Biscane Bay	5–50			Marine GI	41
Mediterranean (Atlantic, Pacific)	150–450			Marine GI	41
Eastern Mediterranean (Agean Sea)	10		13	Marine GI, GII, GIII	22
Cariaco Basin	500–1,310			Marine GI, ANME	42
Sediment					
Mariana Trench	11,000			Marine GI	43
NW Atlantic	1,500–4,500	0–27		Marine GI, marine benthic groups (MBG) A–E	4
Buzzard's Bay, Cape Cod		15		Marine benthic group B, D	15
Coastal Lagoon, SW France		1–2		Marine GI, distantly related GI type, and euryarchaeotal types	44
Eel River Basin, Pacific	521	13–15 and 22		*Methanosarcinales*, ANME-1	5
Hydrothermal Vent					
Tachibana Bay, Japan	22		128	Korarchaeota, Crenarchaeotal hyperthermophiles	45
Tachibana Bay, Japan		1–10	25–75	Marine GI	45
Suiyo Sea Mount, Myojin Knoll, Iheya Basin	972–1,398		100–300	Korarchaeota, Crenarchaeotal hyperthermophiles	18
Suiyo Sea Mount, Myojin Knoll, Iheya Basin				Marine GI	18
Peles Vent, Loihi Seamount, Hawaii				Marine GI, Marine GII	46
North Gorda Ridge	2,000–2,800			Hyperthermophiles	47
Milos, Greece		0.2–11.7	21–100	Hyperthermophiles	48
Mid-Atlantic Ridge			112 at vent, 20–70 in chamber	Hyperthermophiles, Euryarchaeotal Marine sediment GII	49
Associated with other organisms					
Holothurian				Marine GI	50
Axinella mexicana, sponge				Marine GI	27
Flounder, grey mullet				Marine GI, GII, Methanococcoides	21,51

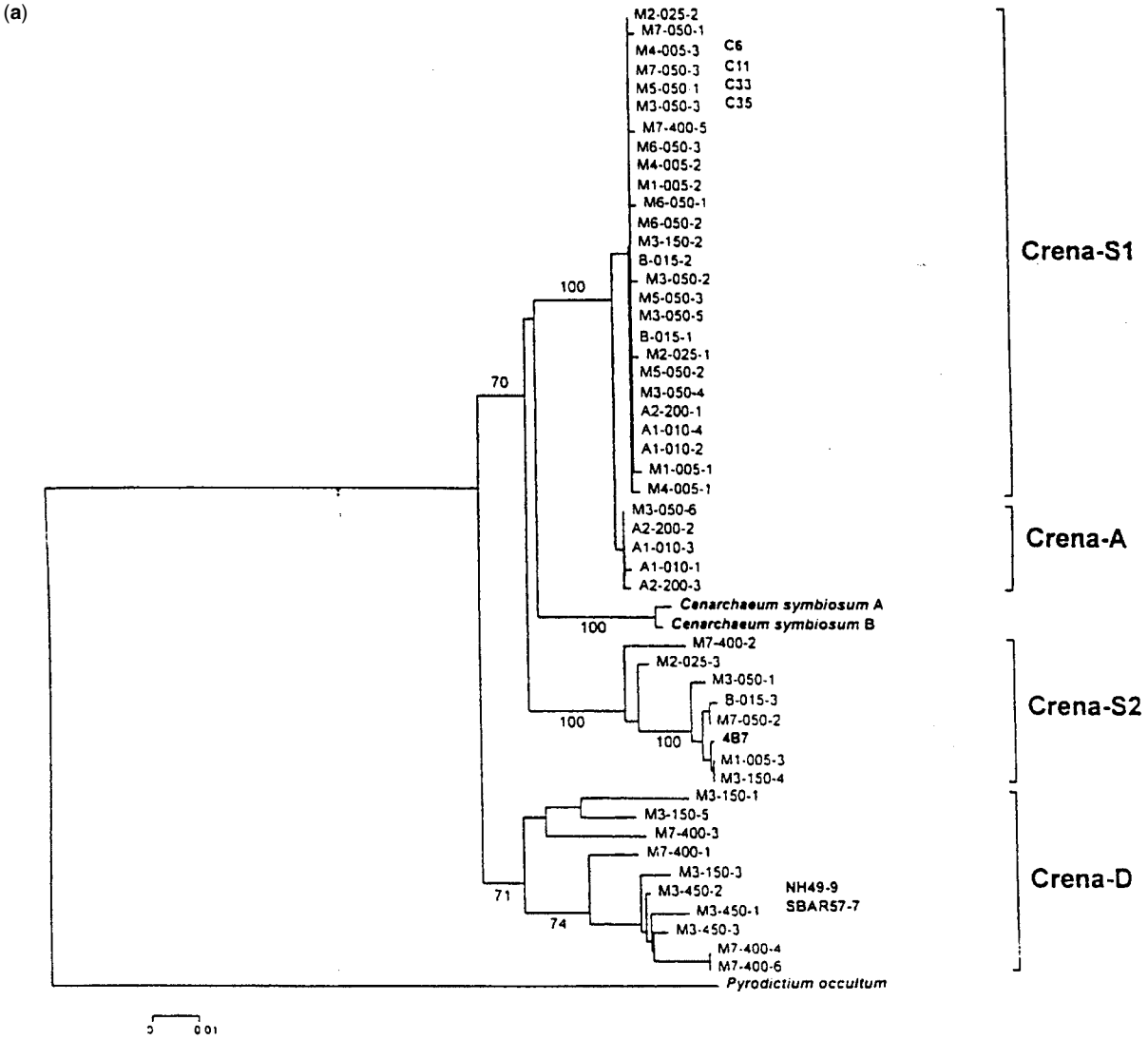

Figure 3. Phylogenetic relationships among the marine archaeal clusters based on SSU rRNA sequences. (**a**) Phylogeny of the marine GI sequences that group into a surface-associated cluster (Crena-S1 and Crena-S2), an Antarctic cluster (Crena-A), and a deep-sea cluster (Crena-D). Relationships for aligned sequences (600 bp) were determined by neighbor-joining analysis with distances estimated using the Jukes-Cantor parameters, bootstrap values above 50% are indicated. Reprinted (abstracted/excerpted) with permission from Garcia-Martinez and Rodriguez-Valera Microdiversity of Uncultured Marine Prokaryotes: the SAR11 Cluster and the Marine Archaea of Group I, *Molecular Ecol.*, Copyright, (2000) Blackwell Publishers. (**b**) Phylogentic relationships among the marine GII, GIII, and GIV (GIV shown in the circle). Maximum likelihood analysis based on 1,047 aligned positions was used to determine the relationships of the sequences. Representatives with full-length sequences are in bold. The scale bars correspond to 5 substitutions per 100 positions. Reprinted (abstracted/excerpted) with permission from Lopez-Garcia et al., A novel haloarchaeal-related lineage is widely distributed in deep oceanic regions, *Environ. Microbiol.*, copyright, (2001), Blackwell Publishers.

paint partially divergent stories about the persistence of these organisms in marine waters. Antarctic archaeal vertical distribution differs from the patterns described earlier, because the planktonic Crenarchaeota dominate the surface waters of the coastal Antarctic Peninsula. The pattern of seasonal abundance is striking in these waters, where the surface archaeal signal (approaching 25% of the total rRNA signal), which is high in the austral winter to early spring, drops significantly with the onset of spring, and stays low, (1 to 2%) until fall (Fig. 6). A similar observation was made during the late winter to spring transition (October to November) at a station in coastal waters of the Gerlache Strait, Antarctica where relative proportions of the Archaea diminishing from 5 to 1% in surface waters, and 20 to 10% in deep (500 m) waters (56). The GI Archaea were dominant in

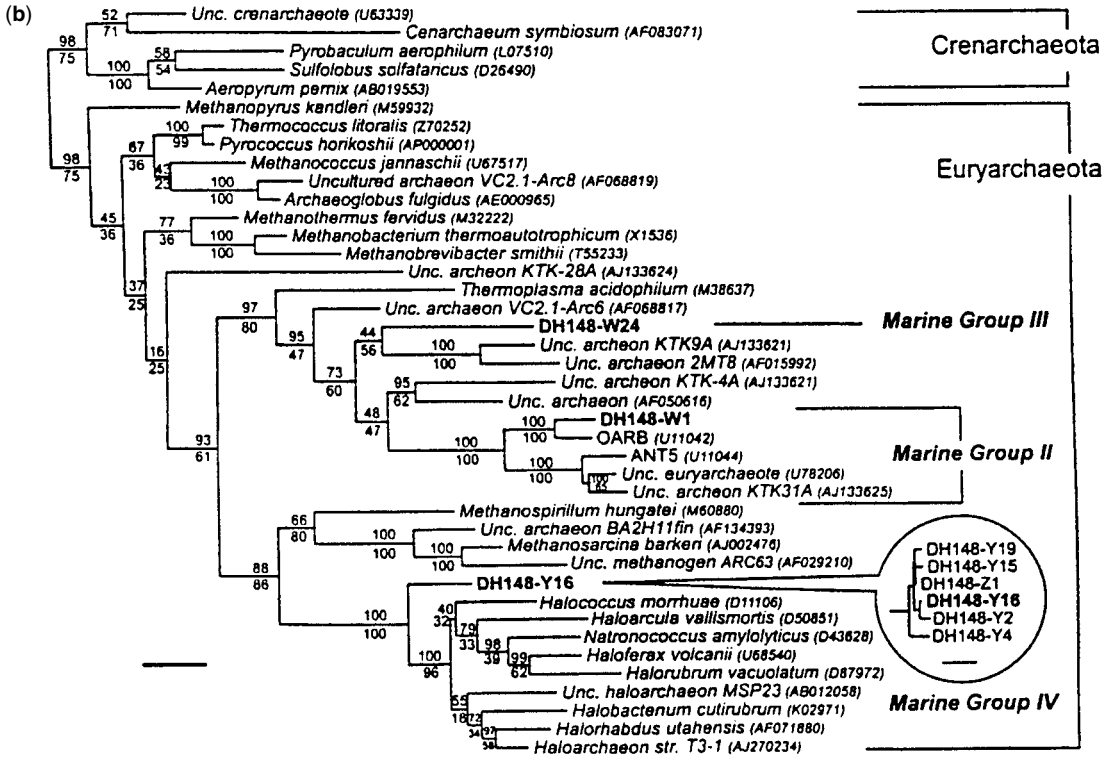

Figure 3. (*Continued*)

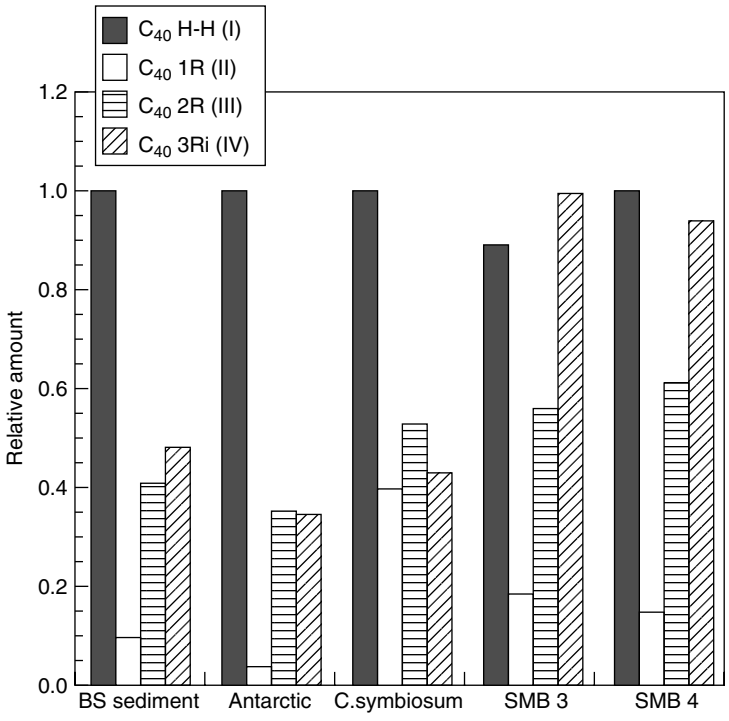

Figure 4. Relative abundances of acyclic and cyclic biphytanes in marine samples from the Black Sea sediment (BB sediment), Antarctic picoplankton lipid extract (Antarctic), *C. symbiosum*, the marine GI sponge symbiont (*C. symbiosum*), and the Santa Monica Basin suspended particulate lipid samples (SMB3 and SMB4). Structures for these compounds are shown in Figure 2. Reprinted (abstracted/excerpted) with permission from DeLong et al. Dibiphytanyl ether lipids in nonthermophilic Crenarchaeotes, *Appl. Environ. Microbiol.*, copyright, 1998, American society for microbiology.

the Gerlache Strait waters (69% of the archaeal signal), although a surface-associated GII rRNA hybridization signal was also detected. In the Antarctic studies, negative correlations were apparent between chlorophyll (and Eukaryal hybridization signal) and the planktonic archaeal hybridization signal (56,57,60). Hypotheses to explain the difference in Antarctic archaeal vertical distributions include increased biological competition in the

Table 2. Summary of Studies in Which Archaeal Abundances Were Characterized Using Various Molecular Techniques in Marine Environments. The GI and GII Percentages are Related to the Total Archaeal Signal. Abbreviation for the Methods used are as Follows: In situ (rRNA Targeted In situ Hybridization), RFLP Band (Restriction Fragment Length Polymorphism Band Fragments), Q-hyb Membrane-Based Quantitative rRNA-Targeted Hybridization, Q-PCR (Quantitative Polymerase Chain Reaction, Taqman Assay), RCA (Relative Clone Abundance Derived From Clone Library)

Location	In situ - % of Total or Cells/mL	Molecular Technique	Reference
Plankton			
Mediterranean 0 m	17%	In situ	2
Mediterranean, 10 m	75 bands	RFLP Band	22
Mediterranean 200 m	61%	In situ	2
Mediterranean, 200 m	GI and GII: 43%	In situ	30
Woods Hole, 0 m	Arch: 0.1% of total signal	Q-Hyb	12
Atlantic central ocean, 0 m	Arch 0%	Q-Hyb	12
North Atlantic, coast of Scotland, near-shore	Arch: 8%, Near-shore Arch 30%	Q-PCR, RCA	35
North Atlantic, coast of Scotland, off-shore	off-shore Arch: 28%	RCA	35
North Sea, 1 m	3%	In situ	55
Sargasso Sea, near Bermuda 10 m (28.C)	0	In situ	38
Monterey Bay (vertical profiles to 3,400 m)	GI: $5 \times 10^3 - 1 \times 10^5$ (1–20% max at 60 m)	In situ	1
Monterey Bay (vertical profiles to 3,400 m)	GII $9 \times 10^3 - 1.8 \times 10^5$ (5–8% max at 0–20 m)	In situ	1
Monterey Bay, 200 m	GI and GII: 14.3%	In situ	30
Santa Barbara Channel 0 m	Arch: 2.3% of total signal	Q-Hyb	12
Santa Barbara Channel, 0 m	Arch: 8.01; GI: 0.63%; GII: 49%	Q-Hyb	53
Santa Barbara Channel, 20 m	Arch: 11.47%; GI: 1.95%; GII: 48%	Q-Hyb	53
Santa Barbara Channel, 75 m	Arch: 31.02%; GI: 22.27%; GII:12%	Q-Hyb	53
Santa Barbara Channel, 150 m	Arch 34.20; GI: 23.59%; GII: 8%	Q-Hyb	53
Santa Barbara Channel, 300 m	Arch 39.17%; GI: 30.49%; GII: 8%	Q-Hyb	53
Playa del Rey, Coastal Pacific Ocean, 0 m	2%	In situ	54
San Pedro Channel, California 100 m	<5%	In situ	2
San Pedro Channel, California 400 m	30%	In situ	2
San Pedro Channel, California 600 m	40%	In situ	2
Western Pacific, 100 m	Arch: 23%	RCA	38
Western Pacific, 500 m	Arch: 60%	RCA	38
North Western Pacific, Alaska	Arch: 13.5%	Q-Hyb	40
North Pacific, off Hawaii (0–5,000 m)	Arch: 3×10^3–5×10^4,	In situ	3
Central Pacific, 0 m	Arch 0%	Q-Hyb	12
Palmer Station, Antarctic, 0 m	Arch: 18.5–26.2%, GI: 64%; GII: 36%	Q-Hyb, RCA	40
Gerlache Strait, Antarctic Peninsula	GI: 69% (average)	Q-Hyb	56
Gerlache Strait, Antarctic Peninsula, 0 m	Arch: 0–11%	Q-Hyb	56
Gerlache Strait, Antarctic Peninsula, 75 m	Arch: 5–21%	Q-Hyb	56
Gerlache Strait, Antarctic Peninsula, 250 m	Arch 7–30%	Q-Hyb	56
Drake Passage	Arch: 20–55%, GI: 93.4%; GII 2.9%	Q-Hyb	57
Marine Sediments			
Estuarine sediments, Japan, 15 cm	Arch: 13.1%, Arch: 9.6%	Q-PCR, RCA	24
Svalbard, Arctic sediments (0–15 cm)	Arch: 0.4–6.4%; up to 1.9×10^8, Arch: 0.6–1.7%	In situ, Q-Hyb	58
Svalbard, Arctic sediments (0–31 cm)	Arch: 1–4%	Q-Hyb	59
Baltic Sea	Arch: 1–12%	Q-Hyb	59
NW Atlantic (0–2 cm)	Arch: 2.5%	Q-Hyb	4
NW Atlantic (8 cm)	Arch: 8%	Q-Hyb	4
NW Atlantic (0–13 cm)	GI: 68%	RCA	4
Eel River Basin, Pacific Ocean (22 & 13–15 cm)	ANME: 82% at 22 cm & 55% at 13 cm	RCA	5
Hydrothermal Vent			
Effluent vent water, Iheya Basin, Japan	Arch: 30%, Arch: 30%	Q-PCR, RCA	24
Shallow vent sediments, Milos Greece	Arch: 1–22% increasing with depth	Q-Hyb	48

Antarctic summer, reaction to increased light irradiance with the onset of summer, or other responses to the onset of summer, because Antarctic surface winter water is analogous to temperate and tropical deep water.

In comparison with the dramatic seasonal variation seen in Antarctic archaeal rRNA abundance, studies in temperate waters off the coast of Santa Barbara and at Station Aloha in the North Pacific reported little correlation between archaeal rRNA abundance and season (3,53).

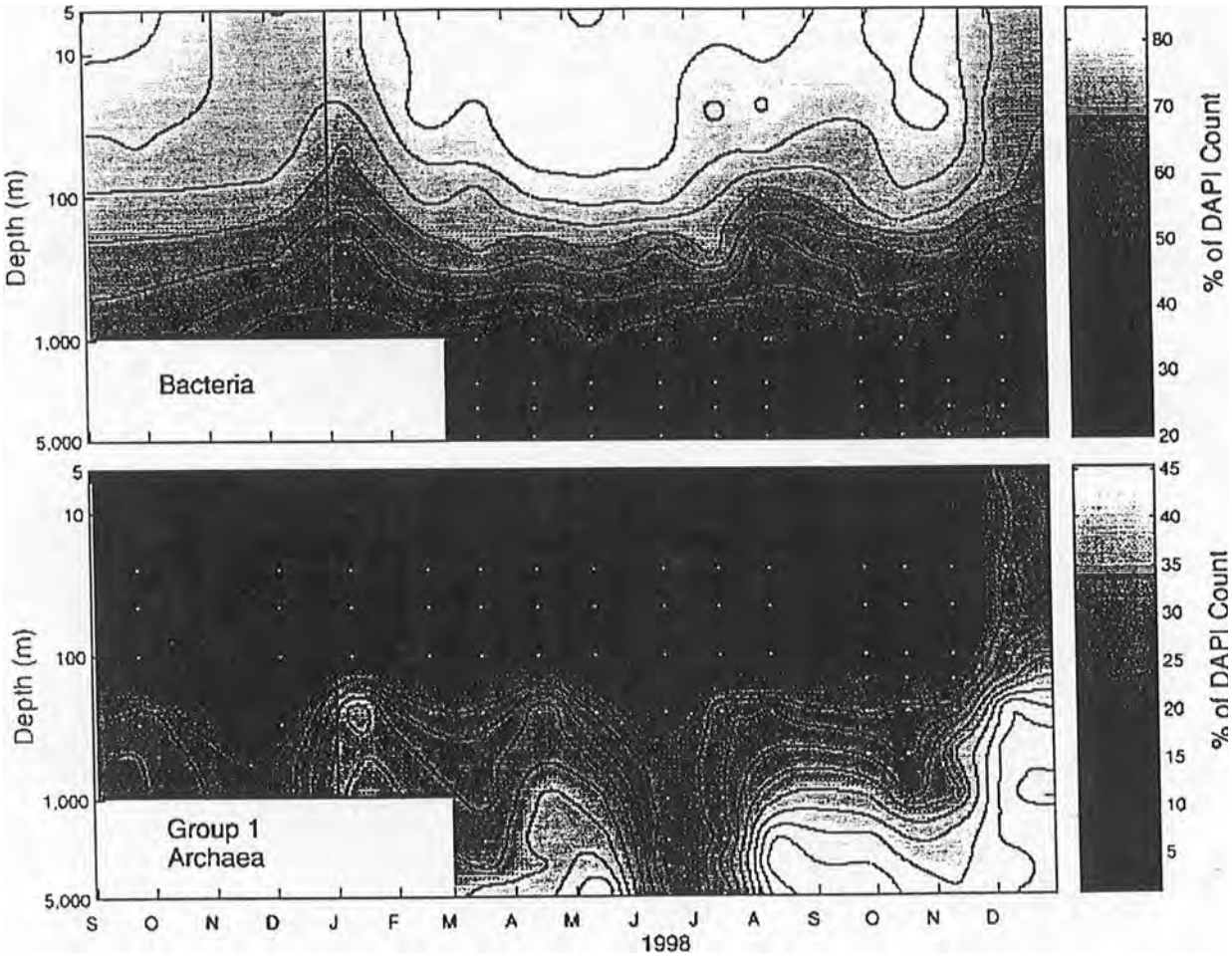

Figure 5. Temporal distribution of bacterial and crenarchaeotal cells indicated by contour plots at the Hawaii Ocean Time-series station, ALOHA, in the North Pacific subtropical gyre. White dots indicate sample depths. Contours represent percentages of the bacterial or crenarchaeotal cells hybridizing with fluorescently labeled polynucleotide probes, in comparison to the numbers of cells stained with the DAPI nucleic acid stain. Reprinted (abstracted/excerpted) with permission from Karner et al. "Archaeal dominance in the mesopelagic zone of the Pacific Ocean," *Nature*, copyright, 2001, Macmillan publishers Ltd.

The differences in the latitude between the polar and more subtropical-temperate environments may partially explain this discrepancy. In polar environments, the influence of strong periodicity in season has immeasurable impact on biological organisms and processes. In the Santa Barbara Channel (SBC) and the North Pacific, mesoscale processes might play a more important role in influencing planktonic archaeal distribution and abundance. The marine GI Archaea appear to consistently inhabit the waters 75 mm or below, although during upwelling periods in the SBC when deep, nutrient-rich water is brought to the surface, GI archaea were detected in surface waters. The marine GII were detected on occasion, typically in SBC surface waters (<20 m). Peaks in the marine GII rRNA signal succeeded phytoplankton blooms four out of nine times, suggesting a potential biological connection to their distribution. One trend seen with studies in the Antarctic and in the SBC is that the GI Archaea are rarely dominant in conditions of high biological activity or prokaryotic abundance (53,60).

Marine Sediments

Originally, methanogens were thought to exist only in anaerobic marine sediments. However, following the isolation of *Methanococcus jannaschi* at a marine hydrothermal vent and other thermophilic methanogens in subsequent studies, it is apparent that the marine methanogens are widely distributed in anoxic marine habitats, where they have since been isolated from psychrophilic, thermophilic, and hyperthermophilic marine environments. It is now thought that methanogens are the most cosmopolitan of the presently cultured Archaea (61). Methanogens have been isolated from seawater particles (62,63), suggesting the existence of anoxic microzones within the particles.

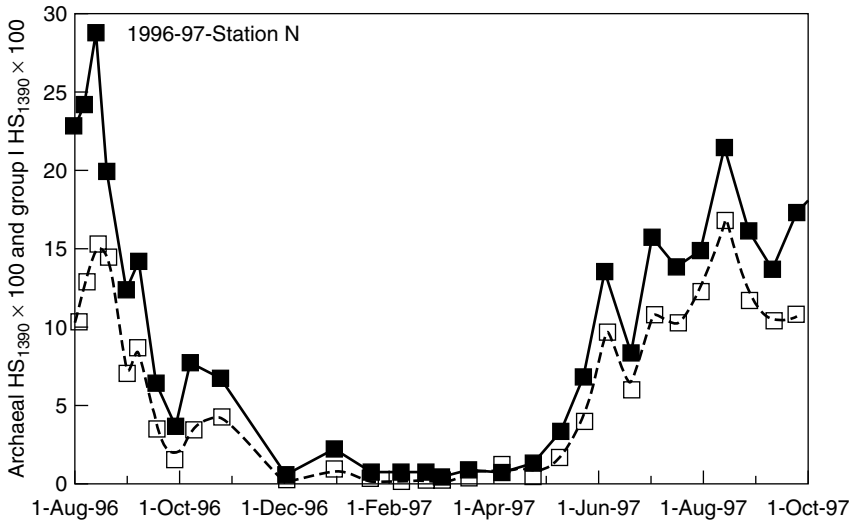

Figure 6. Temporal variation in the planktonic archaeal rRNA hybridization signal (black boxes) and marine Group I hybridization signal (open boxes) in the near-shore waters of Arthur Harbor, Antarctica. Reprinted (abstracted/excerpted) with permission from Murray et al. "Seasonal and spatial variability of bacterial and archaeal assemblages in the coastal waters near Anvers Island, Antarctica," *Appl. Environ. Microbiol.*, copyright, 1998, American Society for Microbiology.

Archaea have been detected in marine sediments around the world in a number of molecular surveys in the past six years. Sediment-associated Archaea consist of a ubiquitous diverse consortium in which several previously unknown lineages have been repeatedly found. Similar to early findings in the plankton, molecular detection suggests that the cultivated groups of methanogens probably do not represent the abundance or diversity of the benthic archaeal microbiota, although several studies have detected some methanogen-related sequences. One of the first such studies conducted in salt marsh sediments detected a diverse assortment of Euryarchaeal sequences including some that clustered with different methanogenic groups (*Methanococcoides* and *Methanolobus*) that either reduce carbon dioxide, or a suite of noncompetitive substrates (14). Sequences affiliated with a halophilic clade, which is surprising because halophiles that require at least five times the amount of salt that is found in the ocean, were detected along with sequences clustering with the marine GII sequences found in the plankton.

Kato and coworkers (43) sampled perhaps the deepest marine sediments on the planet in the Marianas Trench (11,000 m), and reported finding sequences that grouped with the Marine GI cluster. Because the marine GIs are thought to be planktonic in origin, the investigators presume that the Archaea arrived from the middle ocean with settling planktonic particles. Marine GI affiliated sequences were also the most common phylotype found in cold-seep sediments sampled in the Japan Trench [6,400 m (64)]. Diversity of Archaea in deep-sea sediments sampled in the NW Atlantic appears to have higher complexity, including four Crenarchaeota and two Euryarchaeota-affiliated sequences that are distantly related to each other (with 70 to 80% sequence divergence, 4). Both RFLP analysis of clone libraries and DGGE analysis indicated that community diversity increased with sediment depth.

Using 16S rRNA-targeted oligonucleotide probes, reports of archaeal abundances in Arctic sediments of Svalbard showed a trend of increasing archaeal rRNA with depth (Table 2, 65). In the same region, abundance of marine benthic Archaea was determined by FISH (58). Although generally a small fraction of the community, the Archaea were detectable mostly in the surface sediments (up to 6.4% of DAPI-stained cells), and decreased rapidly in abundance, although they represented about 1% of the DAPI-stained cells even at 15 cm; however, difficulties with detection of cells decreased substantially below 10 cm, probably because of low activities and rRNA amounts in those cells.

The same archaeal lipids detected in the plankton have also been found in abundance in marine sediments in the Cariaco Trench, Indian Ocean, Black Sea, Arabian Sea, and other geologic formations (32–34,65). Arabian sea sediments had significant concentrations of acyclic and cyclic biphytanes at depths between 0 to 141 cm (34). In comparison to lipids detected in the plankton, marine sediments off California and Antarctica contained higher lipid concentrations, probably indicating their stability and recalcitrant nature rather than the abundance of organisms in sediment environments, although this has yet to be quantitatively determined (33). It may also be possible to estimate the abundance of Archaea in marine sediments from lipid concentrations, as was suggested by a study conducted in Tokyo Bay in which high concentrations of archaeol, and caldarchaeol were detected in bay sediments (66). The Tokyo Bay study that focused on quantifying archaeol, (to relate concentrations to methanogens) also noted that dibiphytanyl glycerol and cyclophytanyl glycerol were detected in appreciable concentrations in relation to the archaeol.

Methane Hydrates. Methane hydrates consist of a frozen combination of methane and water that can be found in continental margin subduction zones, the deep sea, and in the Polar Regions. Studies have found that active areas of microbial activity are associated with methane hydrates, although the rates of carbon remineralization are thought to be slow. These habitats have been known to harbor dense assemblages of chemoautotrophic sulfide-oxidizing communities composed of *Beggiatoa* mats, tubeworms, and clam fields (*Calyptogena*). The sulfide

source supporting the chemoautotrophic community has often been puzzling because sulfate-dependent carbon mineralization was not occurring in these sediments at appreciable rates. A relatively new, and very interesting story has unfolded in recent years regarding the diversity and metabolic capabilities of the microbial consortia associated with this habitat. Biogeochemical evidence has suggested that methane is being consumed in anaerobic habitats before contacting the aerobic water column or atmosphere. However, no organisms have been isolated that perform this process (67). To get a cultivation-free assessment of the microbial assemblage, Hinrichs and coworkers (5) characterized the small subunit rRNA phylogenetic diversity and stable isotopic signatures of lipids found in methane hydrate deposits off California in the Eel River Basin. This study indicated the existence of a microbial community containing sequences from known anaerobes including methanogens (*Methanosarcinales*), sulfate-reducing bacteria, gram-positive bacteria, and a novel lineage distantly related to the *Methanosarcinales* and *Methanomicrobiales* (5). The stable isotopic signatures of methanogenic archaeal lipid biomarkers (archaeol and hydroxyarchaeol) were strongly depleted in ^{13}C, indicating that they were derived from methane, which has less ^{13}C than any other biologically produced compound. Currently, effort is being extended to cultivate these organisms because they may be a key link in the global methane cycle and past climates.

Rapid progress has led to a much better view of the microbial processes occurring at methane hydrates and in other anoxic marine sediments in general. Results from several studies have detected *Methanosarcinales*-related sequences in methane-enriched environments (5,68–71) suggesting that these organisms, which were thought only to perform methanogenesis (using acetate as a carbon source), may be operating methanogenesis in reverse. A key piece of evidence supporting the existence of a methane-oxidizing sulfate-reducing microbial consortia was demonstrated using FISH in several independent studies, in which cells hybridizing to a *Methanosarcinales*-targeted archaeal probe (ANME-probe) were aggregated in a cluster (1–11 mm in diameter) that was surrounded by cells hybridizing to probes for sulfate-reducing bacteria (70–73). These studies and others have also suggested that there are probably more archaeal species involved in the system because not all archaeal cells hybridize to the ANME-probe (72), the diversity of lipid structures and/or rRNA sequence diversity found indicate the presence of other archaeal groups (70,74), and recently, a study combining FISH with the ANME-probe and secondary ion mass spectrometry presented striking evidence that the non-ANME archaeal cells had significantly higher d13 CCratios than the *Methanosarcinales*-hybridizing clusters (71).

Hydrothermal Vents

Archaeal diversity has been studied in both deep (>4,000 m) and shallow (<100 m) hydrothermal vents using a combination of isolation, enrichment, and cultivation-independent approaches. There are 18 marine archaeal hyperthermophilic genera, 16 of which have exclusively marine origins (75). These groups all are salt requiring, and do not appear to have a pressure requirement. The predominant metabolism thus far known involves the use of elemental sulfur as an electron acceptor, and organic carbon (amino acids/peptides, carbohydrates) as electron donor, and thus is heterotrophic. However, autotrophic Archaea such as the methanogens (growing at temperatures from 2 to 100 °C), and a couple of crenarchaeotal genera including *Pyrolobus* and *Ferroglobus* have also been isolated (75).

Molecular surveys have also turned up new microbial diversity in hydrothermal environments (Table 1). One study at a shallow hydrothermal vent in Tachibana Bay, Japan (45) recovered organisms related to commonly cultivated hyperthermophiles in vent water samples, as well as members of the *Korarchaeota*, a newly recognized third division of the Archaea, described by Barns and coworkers (76). Korarchaeotal sequences were also recovered from a study at a deeper vent site (18). Marine GI archaeal sequences have been detected in the sediments associated with a hydrothermal vent (45), in microbial mats associated with a venting sea mount (46), in black smoker vent water, and a nearby chimney (18). The explanation for this is either that the members of this group actually can reside, and perhaps thrive, in mesophilic-thermophilic sediments, or that because the marine group I have been thought to dominate the archaea found in the plankton, the sediment-derived sequences are from planktonic origin. At many hydrothermal vents, the bacterial diversity exceeds that of the archaeal diversity, although within the Archaea, diverse sequences have been reported (18,45,48). Between 3 to 26.5% of universally primed clone libraries were represented by archaeal sequences in a survey of several deep hydrothermal vent environments (Table 2), and archaeal rRNA abundances from 0.7 to 22.2% were detected from shallow vent sites in sediments sampled in Milos, Greece (48). In situ hybridization analyses confirmed cloning and sequencing results that the black smoker chimney and hot sediments had high cell numbers, and relatively high levels of archaeal diversity (Table 2).

Although it is logistically challenging to do experimental research at the hydrothermal vents, Reysenbach and coworkers (49) succeeded in conducting short-term growth experiments in an in situ growth chamber at a vent site on the Mid-Atlantic Ridge. This study reported substantial archaeal diversity in which representatives from the *Thermococcales* dominated the community (71% of the clones), followed by a significant representation from the *Archaeglobales* group (22%). *Thermococcales* are known to be widespread at deep-sea hydrothermal vents. A couple sequences affiliated with the *Thermoplasmales* (thermoacidophiles) were detected, which were not previously known to exist in hydrothermal vent ecosystems, and a sequence that was in the marine GII lineage that appears to group with other deep-sea sediment Archaea (4).

One of the questions stemming from hydrothermal vent research has been the origin and source of the hyperthermophilic microorganisms. In one case, Summit and Baross (47) sampled a recently venting plume and were able to isolate thermophiles from 8 of 9 samples

at one plume, and 9 of 13 samples in a second plume, in contrast to background samples from the same area where they were unable to cultivate any thermophiles. Their conclusion was that there is a subsurface reservoir of thermophilic organisms, and suggested that these subsurface microbes likely thrive in the vicinity of mid-ocean ridges because of circulation and chemical energy sources.

ECOPHYSIOLOGICAL ATTRIBUTES OF MARINE ARCHAEA

Planktonic Archaeal Metabolism

Determining the metabolic role of the planktonic Archaea has been the subject of many investigations, although few if any have made headway in accurately measuring growth rates, or even determining definitively their basic metabolism. It is likely that the planktonic Archaea do influence oceanic biogeochemistry and elemental cycling because of their shear abundance (20% of the global picoplankton population). Regardless of their metabolism, it is agreed that they contribute significantly to planetary carbon biomass. It was suggested by Karner and coworkers (3) that the GI Archaea are better adapted to the oceanic environment than most pelagic prokaryotes, such that they have probably adopted a common adaptive strategy that has enabled their expansive radiation. It is also the default assumption that, because of the environmental conditions in which the marine GI Archaea prevail, they have aerobic heterotrophic lifestyles, although the evidence for this is scant (27,36). Scientific clues are mounting in a couple of directions, although until these organisms have been cultivated, or enough of their genome sequence is determined, this may remain largely guesswork. Stable isotopic analyses of membrane lipids suggest that the archaeal lipids have ^{13}C ^{12}C ratios ranging between 19.7 to 23 parts per mil (32,34). Hoefs and coworkers (32) proposed two alternative hypotheses to explain the results; (*1*) that the Archaea use dissolved inorganic carbon as their carbon source, and do not discriminate against ^{13}C as other primary producers do, or (*2*) the Archaea carbon and energy source are acquired from low-molecular weight organics such as acetate and methylated amines derived from algal carbohydrates and proteins. The archaeal lipid signatures in the Arabian Sea were typically enriched in ^{13}C compared to algal sterols (34).

One of the facets of Archaea is that they are resistant to many antibiotics that inhibit bacterial activity. On the basis of this premise, experiments conducted on Antarctic planktonic microbiota set out to measure the activity remaining following erythromycin treatment of concentrated plankton in which the Archaea represented between 1 to 9% of the assemblage (<1.6 μm fraction, (56), Massana and coworkers 1998). Although these investigators failed to detect any activity signal higher than background in those experiments, they suggested that the marine Archaea could be sensitive to erythromycin, perhaps do not incorporate leucine at appreciable rates, were damaged in the concentration procedure, or simply were not as active as their biomass indicated.

Probably the most promising evidence that the planktonic GI Archaea are active came from a study in which FISH was used in tandem with microautoradiography. The results of this study suggest that 60% of the planktonic Archaea were active, and that they incorporated dissolved amino acids (30). This finding led the researchers to conclude that the archaeal cells were heterotrophic, and perhaps as active as the heterotrophic bacteria in the same sample (by comparisons of the staining intensities). However, more work in this arena will be necessary to determine whether the amino acids are being used in assimilatory or dissimilatory pathways.

It is possible that the marine GII, GIII, and GIV have different metabolic properties than the GI, given the differences in their phylogenetic groupings as well as their close relationship to the methanogens and halophiles. Fuhrman and Davis (36) postulated that the marine GII and GIII could be methanogens — because oceanographers have reported on midwater methane signals (77,78), but little proof exists to support this hypothesis and some reports have found the marine GIIs to bloom in surface waters, although these occurrences have been shown to occur following periods of upwelling (53). The potential that GIV phylotypes are halophilic is possible on the basis of their close relationship to cultivated halophiles (6).

Methanogenesis in Marine Environments

Methanogenesis has been thought to occur in marine sediments either where there are low concentrations of sulfate, so that they are not in competition for substrates with sulfate reducers, or when the methanogens can use non-competitive substrates such as methanol, methylamines, or methionine. Both physiological and molecular data support both conditions. Methanogen-related sequences have been isolated from surface sediments. The topic of methanogenesis will be covered elsewhere in this Encyclopedia and will not be covered in more detail here.

Sulfur-Dependent Anaerobic Methane Oxidation

Anaerobic oxidation of methane (AOM) not only occurs at methane hydrate sites, it is also suspected to occur in areas with substantial methane production and sulfate availability, including marine sediments, anoxic waters, deep continental margin sediments, and soda lakes (79). Theoretical and field-based lines of evidence indicate that AOM occurs in the presence of sulfate reduction by a consortium of organisms. Thermodynamics suggests that AOM would only be favorable if there was a removal process for hydrogen, which could occur if sulfate reducers were consuming the hydrogen. Lipid analyses from a well-known methane-enriched mud volcano in the Eastern Mediterranean, methane hydrates in the Eel River basin and Santa Barbara Channel, and seeps in the Black Sea have indicated that *archaeol* and *hydroxyarchaeol* are present along with a number of other archaeal and sulfate-reducing bacteria derived lipids (68,70,74,80). These studies showed that not only were the archaeal lipids strongly depleted in ^{13}C, but glycerol ethers and fatty acid products from Bacteria were abundant, and showed similarly depleted ^{13}C values in comparison to

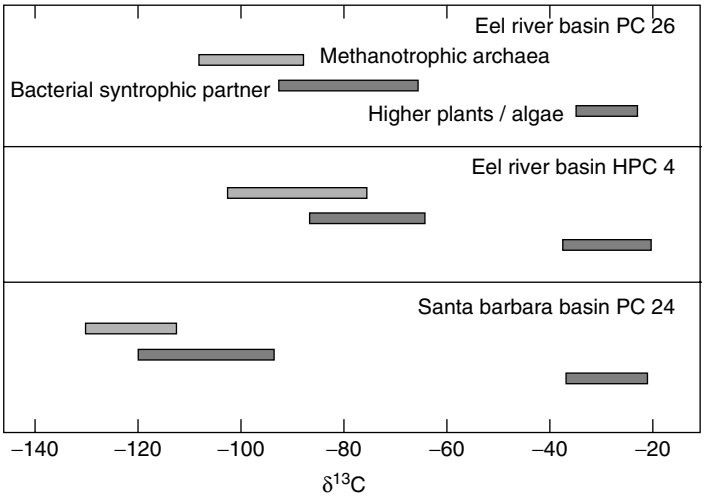

Figure 7. Carbon isotopic concentrations of sedimentary lipids assigned to methanotrophic archaeal, bacterial syntroph, and plant/algal sources from three different methane seeps. Note the similarities in ^{13}C depletion for the methanotroph and bacterial syntrophy in comparion to the relatively ^{13}C enriched algal lipids, indicating that the carbon source for the methanotroph and syntrophy was methane, and not algae or terrestrially-derived carbon. Reprinted (abstracted/excerpted) with permission from Hinrichs et al. "Molecular and isotopic analysis of anaerobic methane-oxidizing communities in marine sediments", *Org. Geochem.*, copyright, 1999, Elsevier science.

control sites (Fig. 7). In addition, algae and plant lipids from the nearby sites was significantly enriched in ^{13}C in comparison to either the methanotrophs or bacterial syntrophs, indicating different carbon sources (CO_2 versus CH_3, Fig. 7). Control sites with similar carbon input as the hydrate sites consistently had less recalcitrant carbon in the sediments in comparison with methane seep sites that had significant levels of residual carbon in the sediments, suggesting that sulfate removal processes, rather than remineralization, are occurring at methane seeps (68). A recent review proposed a mechanism for the sulfur-dependent methane oxidation process, in which the following reaction provided the highest energy yield of the different reactions examined (79), and produces twice the amount of energy predicted from a reverse methanogenesis reaction.

$$\text{Methane Oxidation: } 2CH_4 + 2H_2O \rightarrow CH_3COOH + 4H_2 \quad (1)$$

$$\text{Sulfate reduction: } 4H_2 + SO_4^{-2} + H^+ \rightarrow HS^- + 4H_2O \quad (2)$$

$$\text{Sulfate reduction: } CH_3COOH + SO_4^{-2} \rightarrow 2HCO_3^- + HS^- + H^+ \quad (3)$$

$$\text{Net reaction: } 2CH_4 + 2SO_4^{-2} \rightarrow 2HCO_3^- + 2HS^- + 2H_2O \quad (4)$$

Whether this reaction or something similar is actually occurring as a result of an archaeal-sulfate-reducing bacterium syntrophy remains to be seen.

Archaeal Metabolism at Hydrothermal Vents

Hyperthermophilic microbes contribute significantly to geochemical cycling in hot environments in present day environments and in early earth conditions. There are three major metabolic processes currently thought to dominate hydrothermal vent habitats, reduction of sulfur compounds, methanogenesis, and iron reduction. Sulfate reduction rates measured in hydrothermal sediment samples collected in the Guaymas Basin had optimum of 70 °C and 82 °C in different cores, with an upper limit determined to be 102 °C, where Archaea such as *Archaeglobus profundus* have been previously isolated (81). Dissimilatory reduction of iron is a relatively new discovery (82), and was recently shown to occur in the tandem with humic substance reduction (83). In this case, the extracellular quinones in the humics are reduced and the electrons are abiotically transferred to neighboring iron oxides, thus regenerating the humics.

ARCHAEAL ASSOCIATIONS WITH MARINE ORGANISMS

Endosymbiosis has been a common feature of methanogenic euryarchaeotes (*Methanomicrobiales* and *Methanobacteriales*) including associations with ciliates, insects, and a large number of vertebrates including humans. The discovery that psychrophilic Crenarchaeota were found in associations with marine organisms was initially quite surprising. Marine GI archaeal sequences were first found to be associated with the midgut of a deep-sea deposit feeding holothurian (50). Perhaps not as surprising in light of recent molecular surveys of marine sediment habitats in which the Archaea appear to be common constituents (although the GII-affiliated sequences and other euryarchaeotal benthic Archaea appear to be more common), this was an important finding because this was the first nonmethanogenic archaeal group to be characterized in association with another organism. Since then a true symbiosis has been described for the marine sponge *A. mexicana*, which hosts the marine GI symbiont *C. symbiosum* in its tissues (27). The rationale for determining that the association was symbiotic was based on the high archaeal cell numbers, active cell division, and persistence of the association. *Cenarchaeum symbiosum* appears as a rod, with a specific nuclear region, which can be discriminated when using DNA and rRNA stains. Archaea have been detected in other marine sponges off the California coast, in the Antarctica, and in the Great Barrier Reef (84,85), although the associations have not been described in detail. The exact nature of nutrient exchange, secondary metabolite production, or

other benefits from the archaeal-sponge interaction have not been elucidated as of yet.

Marine GII archaeal sequences have also been found in the guts of marine fishes, flounder, and grey mullet, as well as flounder fecal pellets (21). Marine GI clones (86) were also recovered from the flounder digestive tract, although marine GII sequences were more frequently represented (22 of 29 clones). In a later study, *Methanococcoides*-related archaeal sequences were detected in the flounder digestive tract using a nested-PCR approach (51). The sequences from the fish digestive tracts were quite similar to those detected on suspended particles in the surrounding water column, suggesting that a potential habitat for the anoxic methanogens lies in marine fish or other animal digestive tracts. Fecal pellets appear to be the marine particulate source of the methanogens detected, although the alternative that the fish acquire the Archaea via seawater ingestion cannot be discounted (51).

ARCHAEAL GENOMICS AND BIOTECHNOLOGICAL APPLICATIONS

A promising future for discovery in archaeal biology lies in genomics. The hopes entail developing an understanding of the basic biology of extremophiles in which comparisons to mesophilic and psychrophilic genomes will help determine how these organisms cope with the extreme conditions they inhabit. Similarly, for the uncultivated Archaea, environmental genomics may be one of the best ways to advance knowledge concerning the basic metabolic properties of these widespread yet poorly understood groups of organisms.

Approximately ten archaeal genome sequences are present in public databases, in which six are from organisms isolated from marine environments (Table 3). Genome organization is not well conserved among those archaeal genomes sequenced. However, comparative genomics studies of these genomes are presently taking place (87). The genome sizes of all archaeal autotrophs are reduced (approximately 1.6 Mbp), in comparison with bacterial heterotrophs (approximately 2.5 to 6 Mbp). The first archaeal genome to be sequenced was *M. jannaschi*, a methanogenic hyperthermophile, isolated from a hydrothermal vent at a depth of 2,600 mm. The novelty of finally having a whole genome sequence validated suspicions that archaea are composed of bacterial and Eukaryal genes. Although 56% of the *M. jannaschi* genome coded for hypothetical proteins, not matching any sequences in sequence databases, the genes associated with central metabolism and cell division matched sequences of bacterial origin, whereas genes coding for information processing, such as DNA replication, transcription, and translation were more eukaryotic like (88).

One of the key features of an environmental genomics approach is that it provides access to the genomes of uncultivated organisms. Several large archaeal DNA fragments have now been sequenced from environmentally derived samples (92–95). The first environmental contig was assembled from a 40 Kbp sequence prepared from a library assembled with DNA collected from a depth of 200 m off the coast of Oregon. The results of this study confirmed that the genome fragment containing an archaeal ribosomal operon contained several genes that had the highest similarity to archaeal sequences in the database, to the exclusion of the Bacteria or Eucarya (92).

Evidence for the thermal sensitivity of enzymes from the psychrophilic marine GI archaeon, *C. symbiosum*, resulted from a study in which the DNA polymerase gene discovered by sequence analysis of a 42.4-kbp fosmid was expressed in *E. coli* (93). The results indicated that the DNA polymerase from *C. symbiosum* was inactivated at 40 °C, which lent support to the nonthermophilic phenotype of this symbiont. Further screening of the *C. symbiosum*-sponge genomic library indicated the presence of two highly related, but nonidentical rRNA sequence variants with 28 kbp of overlapping sequence (94). Results

Table 3. Archaeal Genomes Sequences from Completed Genomes and Environmentally Derived Large DNA Fragments. Some Were Not Published (n.p.) at the Time of Printing. The *Pyrococcus Furiosus* Genome is being Sequenced by the Center of Marine Biotechnology/University of Utah, and the *Pyrococcus abyssi* Genome is being Sequenced by Genoscope

Species/Clone Name	Environmental Origin of Organism/Sample	Reference
Complete Genomes		
M. jannaschii	Submarine hydrothermal vent; East Pacific Rise, Pacific Ocean	88
Archaeoglobus fulgidus	Marine hydrothermal sediment; Vulcano island, Italy	89
Pyrococcus horikoshii	Marine hydrothermal vent; Okinawa Trough vents, Pacific Ocean	90
Aeropyrum pernix, K1	Marine solfataric vent, Japan	91
Pyrococcus furiosus	Marine sand surrounding sulfurous volcanoes	n.p.
Pyrococcus abyssi	Deep-sea hydrothermal vent, South-east Pacific	n.p.
Environmental Genome Fragments		
Fosmid 4B7	Marine plankton, crenarchaeotal marine group I	92
Cenarchaeum symbiosum (Fosmids 60A5, 101G10)	Marine sponge *A. mexicana*, symbiont	92,93
Fosmid 37F11	Marine plankton, euryarchaeotal marine group II	94

indicate that there is microheterogeneity in the GI archaeal symbiont population within the sponge host and suggest that heterogeneity will be a complicating factor in genomic studies of natural populations.

A 60-kbp marine GII archaeal insert was detected from a BAC library prepared from surface water picoplankton of the Monterey Bay (95). Sequence analysis of this large insert resulted in identification of 38 genes, with the majority of characterized genes having archaeal homologs, although eight genes appeared to have bacterial homologs, suggesting the potential for lateral gene transfer. Structural analysis of protein domains of unknown genes suggests the potential for a membrane-associated proteolytic system to be encoded by a couple of the open reading frames, thus demonstrating the potential for gene discovery in uncultivated organisms and the potential for predicting phenotypes from gene sequences.

The marine Archaea are a valuable resource for applications in the biotechnology field, providing enzymes active from -2 to $+130\,^{\circ}C$, biopolymers, and the ability as whole cells to perform chemical manipulations that currently require application of a number of toxic chemicals. Hyperthermophilic archaeal enzymes are being developed and used for their properties of high-temperature activity and tolerance for activity in organic solvents (96). Recent studies indicate that marine hyperthermophilic diversity is still untapped, and the potential for these organisms as a biotechnological resource, for example, in producing enzymes for research applications (polymerases, restriction enzymes, etc.), for the food industry, and in "green chemistry" is just being realized (97,98). Through the study of homologs in genomic fragments of the psychrophilic Archaea, important clues to enzymatic temperature adaptation are likely to unfold (16,94). One of the areas of future research, in particular regards to extremophilic genomics and developing an understanding of molecular adaptations to high temperatures will be predictive modeling and structural biology. Using signature 3-D structural themes in microbial proteins, modelers will be primed to apply this approach to the understanding of enzyme structure for hyperthermophiles and other extremeophiles.

ORIGINS AND EVOLUTION OF THE MARINE ARCHAEA

The "source" of the Archaea in marine planktonic environments has been debated since their discovery nine years ago. Theories that they arise from hydrothermal vent plumes, from anaerobic gut environments in marine fish or invertebrates (copepod guts) have mostly been put to rest because their widespread distribution, abundance, and occurrence in aerobic cold habitats is commonplace (16). However, hyperthermophilic archaea can survive in low-temperature oxic seawater (99), and have been found in hydrothermal vent plumes, which could potentially lead to the dispersal of these organisms far from the vent habitats (100,101). Similarly, methanogens could be resuspended from anoxic sediments, and have been isolated from oxic seawater (62,63). Evidence including the *C. symbiosum* symbiosis and other sponge-archaeal associations, thermolability of the *C. symbiosum* DNA polymerase, G + C content of the GI crenarchaeotal rRNA operon, detection of terrestrial GI Archaea, and so on indicated that the newly described, nonextremophilic Archaea belonging to the Crenarchaeota and Euryarchaeota branches of the Archaea are likely adapted to their cold, aerobic environments.

The cold-adapted Archaea have probably been long-term residents of marine planktonic and sediment environments, and have potentially played key roles in these present day and potentially ancient ecosystems. Archaeal lipids have been detected in sediments from the Eocene Era [50 million years ago, (32)], suggesting their long-term existence in marine environments. Furthermore, new studies investigating deep cores samples in the North Atlantic provide compelling support for dominance of non-thermophilic Archaea in sediment horizons correlating with oceanic anoxia (approximately 112 mya). Stable isotopic analysis of the archaeal lipids indicated that they had chemoautotrophic lifestyles (102). In addition, the recognition that anaerobic methane oxidation is a significant archaeal-mediated biogeochemical process also suggests that the same process could have occurred on early earth when the atmosphere may have consisted primarily of methane in which the anaerobic methane oxidizers could have played an important role in earth's climate (79).

Theories regarding the origins of life and early earth environmental conditions that led to the formation of life have been an active area of research in the past decade. One popular theory postulates that the earliest forms of microbial life existed in hot anaerobic environments. It is likely that the origin and evolution of many metabolic reactions and pathways occurred in the thermophiles and hyperthermophiles. For example, work by Lovley and coworkers (83) demonstrating the ability of hyperthermophiles to reduce quinones in extracellular humics may suggest a significant impact on early life on earth because extracellular quinones and iron oxides may have both been present in early earth conditions. The origins of the cold dwelling Crenarchaeota remain a mystery as to the history of life on earth, since the discovery of these organisms opened up the potential for psychrophilic origins from what was once argued to be the hot origin of life for the Archaea. However, a recent study conducted in Yellowstone appears to have recovered sequences that have a common ancestor (pSL12) with the cold Crenarchaeota (76).

CONCLUSION

Marine Archaea have been isolated or detected from environments with temperatures ranging the spectrum found on this planet, from more than $100\,^{\circ}C$ to less than $0\,^{\circ}C$ representing hyperthermophilic (>80 to $110\,^{\circ}C$), thermophilic (45 to $80\,^{\circ}C$), mesophilic (15 to $45\,^{\circ}C$), and psychrophilic ($<15\,^{\circ}C$) lifestyles. Archaea have been found to exist as free-living organisms, attached to particles, and associated with other marine organisms in a variety of habitats. Planktonic Archaea can be found throughout the water column from surface regions in coastal areas, to the deep abyss, and appear to be globally distributed among the world's oceans, including

the Southern Ocean, Atlantic, Pacific, North Sea, and the Mediterranean. The planktonic Crenarchaeota may be one of the most abundant bacteria on the planet (3). Archaea associated with marine sediments comprise a diverse consortium in which the diversity is largely defined by the geochemistry of the sediments. In contrast to the plankton, several members of sediment-associated archaeal communities have been cultivated, although molecular methods have revealed new diversity in nearly all phylogenetic archaeal lineages. Diversity recognized thus far includes methanogens, relatives of halophiles, anaerobic methane oxidizers, hyperthermophiles, and psychrophilic Crenarchaeota and Euryarchaeota.

The future holds promise for revealing detailed information about the presently uncultivated GI and GII planktonic lineages through applications of genomic technologies, which could eventually uncover mysteries of their physiological lifestyles, and the adaptations that these organisms have undergone for survival in cold environments. These organisms may hold the clues to understanding differences between thermophily and psychrophily because the nearest cultivated relatives of the GI Crenarchaeotea are hyperthermophiles. Along similar lines of discovery, the interdisciplinary approaches being applied to the anaerobic methane oxidizer consortia (69) through combining stable isotopic signature analysis with molecular identifications promises to be a highly valuable approach that will likely be applied to increasingly more microbial-biogeochemical investigations.

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ARCHAEA IN SOIL HABITATS

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Archaea comprise a heterogeneous group of microorganisms, many of which contribute substantially to terrestrial biosphere processes through their unique metabolisms. Their "extremophile" image in popular literature as well as in scientific circles, derives from the fact that archaea, traditionally, have been isolated from "extreme" habitats, that is, high sulfide, hypersaline, anoxic, or hyperthermal. Today, molecular evidence shows that soils and aquatic environments of a "nonextreme" nature are also habitats of novel archaea with as yet unknown metabolic properties.

Here, we present an overview of soil as a habitat and a review of what is known about archaea in

soils. We describe what is known or deduced about the ecological properties of archaea found in both extreme and nonextreme soil habitats. Research on ecological roles of archaea in soils is scarce except with regard to methanogenesis, which is a globally important process carried out exclusively by members of the Archaea. We give an overview of the ecology of methanogenic archaea; however, this topic is more thoroughly discussed by R. Conrad (Flooded Soils) in another article in this Encyclopedia on the microbiology of flooded soil habitats.

SOIL AND THE BIOSPHERE

Soil is a major reservoir of microbial biomass, comprising from 10 to 50% of the Earth's terrestrial, prokaryotic carbon, with an estimated 26 Pg (1 Pg = 10^{15} gram) of organic C in 2.6×10^{29} cells (1). The global impact of prokaryotic metabolism in soil habitats is significant. For example, symbiotic N_2 fixation provides approximately 80% of the biologically fixed reduced nitrogen on land (2). Microorganisms in both water-saturated, anoxic, and drier, well-aerated soils are also important influences on atmospheric chemistry; they make a net contribution of about 60% of the CH_4 present (3) and contribute significantly to the cycling of carbon monoxide (CO), carbonyl sulfide (OCS), nitrous oxide (N_2O), and nitric oxide (NO) [(4) and refs. within]. A complete account of the contribution of archaea to the biomass and metabolism of soil prokaryotes awaits results and future studies on the prevalence, metabolic repertoire, and microhabitats of soil archaea.

Complexity of Soil

Although familiar and seemingly simple, soil is so complex as a microbial habitat that many of its secrets remain well guarded as the twentyfirst century dawns. Soil is a heterogeneous mixture of living organisms, decomposing organic materials, and inorganic substances (Fig. 1). Differences in particle composition, size, shape, density, and charge, combined with climatic variation, form the basis for diversity in colonization of soil by different life-forms. Plants, animals, and fungi, the latter two mainly microscopic, inhabit soil. Billions of prokaryotes also live in each gram of soil. Physical, chemical, and biological heterogeneity in soil creates a network of microenvironments; these are strongly influenced by temperature and can vary widely in ionic strength and composition, pH, and water and redox potential. The diverse prokaryotes that adapt to and exploit these microniches match this diversity in habitat with equally diverse metabolic capacities.

Soil Microorganisms and Biosphere Functioning

Potential productivity in both natural and cultivated systems is directly related to soil organic matter concentration and turnover, which in turn supplies most macronutrients required for plant growth. Through their roles in the decomposition of soil organic matter, soil microorganisms are largely responsible for terrestrial ecosystem development and functioning. Both prokaryotic and eukaryotic metabolisms influence the temperature regulation of the Earth through CO_2 turnover, whereas CH_4 cycling (3) and the recycling of crucial minerals such as N, S, and P are largely dependent on prokaryotic activities (4).

Figure 1. Electron micrograph of soil (scale bar 5 μm). Bacteria (B) are usually associated with organic matter that still contains carbohydrate (e.g., cell walls, W). Other highly lignified and convoluted organic matter (O) does not support bacteria, but there are numerous microorganisms (0.3 μm diameter) scattered throughout the clay (*). *P* is a pore, approximately 1 μm in diameter. Reproduced from R. C. Foster, *Quaestiones Entomologicae* **21**, 609–633 (1985).

Diversity of Soil Microorganisms

Soil is an immense reservoir of microbial diversity. The rich diversity of soil microorganisms can be glimpsed through use of the agar plating technique; a broad

array of different colony morphologies can be recovered upon plating a soil sample. Knowledge of the microbial composition of natural environments was, for a long time, based mainly on those organisms that were amenable to growth in vitro using such standard approaches. However, it was also known that a large discrepancy existed between culturable and total microscopic cell counts in natural habitats, and soil habitats in particular (5,6). This observation suggested many unknown and not easily cultured microorganisms were present. Torsvik and coworkers elegantly demonstrated this concept using DNA-DNA reassociation kinetics, showing that thousands of prokaryotic genomes are present in a gram of soil, compared with a relatively less complex genome mixture derived from the culturable subset (7,8). A staggering diversity of microorganisms is also inferred from the results of culture-independent methods that exploit direct recovery of genomic DNA and analysis to derive phylogenetic types (phylotypes) from sequences of ribosomal RNA; such analysis reveals that the majority of phylotypes recovered from soil are novel (9–14).

The Archaea serve as an example to illustrate the discrepancy between culture-based and nonculture-based knowledge derived from studies of microorganisms in the environment. Most archaea have not been cultured from habitats considered to be nonextreme, such as mesophilic soil or oxygenated marine surface waters. On the basis of culture-independent studies, it now appears that archaea are not only present in such habitats, but are reasonably (and in some cases very) abundant members of the microbial assemblages there (Table 1).

AN OVERVIEW OF ARCHAEA IN SOIL

Archaea: The Third Domain

The discovery of *Archaea* as a third domain of life revolutionized evolutionary thinking by establishing that prokaryotes are not monophyletic (23). Although archaea are prokaryotes and resemble bacteria in morphology (single cells lacking internal compartmentalization), in evolutionary lineage they are closer to the Eukarya than to bacteria. Archaea, in fact, share some characteristics with eukaryotes, for example, the homology of archaeal and eukaryotic RNA polymerases (24), but in other ways are similar to bacteria, for example, gene composition and order (24). Examples of characteristics exclusive to archaea include the sequence and structure of particular regions of ribosomal RNA (rRNA) (25) and the composition of membrane lipids and lipid linkages (26).

Table 1. Estimates of Abundance of Nonthermophilic Crenarchaeota

Habitat	Abundance	Ref.
Oxygenated coastal waters	2% total rRNA	15
Antarctic surface waters	34% prokaryotic biomass	16
Santa Barbara channel	20% total rRNA	17
Antarctic waters	1–24% total rRNA	18
Pacific mesopelagic zone	30–50% prokaryotic biomass	19
Axinella mexicana (marine sponge)	60% prokaryotic cells	20
Michigan soil	1.4% total rRNA	21
Tomato roots	3.3–16% prokaryotic cells	22

Taxonomy and Phylogeny of Archaea

There are two phylogenetic divisions of the domain *Archaea* represented by cultured isolates, *Crenarchaeota*, and *Euryarchaeota* (27), and a third proposed division, *Korarchaeota* (28), for which only rRNA sequences have been recovered (28–30). All cultured representatives of the Crenarchaeota are extremely thermophilic sulfur metabolizers. Examples include members of the genera *Sulfolobus* and *Thermoproteus*. The Euryarchaeota comprise cultured members with a more diverse metabolic repertoire, including methanogens, for example, *Methanothermus* and *Methanosarcina*, extreme halophiles ("salt-loving") such as *Halobacterium* and *Natronococcus*, and the thermophilic, wall-less acidophile *Thermoplasma*, in addition to sulfur-metabolizing hyperthermophilic archaea, for example, *Archaeoglobus* and *Thermococcus*.

Archaea in Soil

In this article, we describe the distribution of archaea in what are considered conventional soils, both extreme and nonextreme (see also METALS: MICROBIAL PROCESSES AFFECTING METALS). Archaea typically have been isolated from extreme (high sulfide or saline, elevated temperatures) and anoxic environments, resulting in the generally accepted idea that they represent a primitive group exiled from the (now) more common aerobic and nonextreme environments of contemporary earth. Until recently, this view extended to soil habitats as well. Archaea were previously and almost exclusively cultured from soils of an extreme nature, such as acidic hot soils heated by solfataric gases (a solfatara is defined as a volcanic fissure that gives off sulfurous vapors and steam) and evaporative saline soils. An exception to this observation is cultured isolates recovered from anoxic soils. Archaea isolated from these environments encompass diverse groups and physiologies. More recently, however, the known diversity among Archaea has been broadened considerably more by the inclusion of new members represented solely by small subunit (SSU) rRNA gene sequences recovered from temperate habitats, including mesophilic and low-temperature soils that have not previously yielded isolates of Archaea.

Distribution of Archaea in Soil

Archaea have been isolated by culturing from extreme and anaerobic soils located around the globe (Table 2). Examples include solfataric fields in Italy, the Azores, United States, Iceland, and New Zealand, and rice paddies in Japan and Italy. Even more novel SSU rRNA sequences of uncultured Archaea have now been recovered from soils all over the globe. Flooded soils and wetlands yield sequences representing members of both Euryarchaeota and Crenarchaeota (11,31), whereas only sequences representing the Crenarchaeota have been recovered thus far from dry soils (9,10,21,32,33). The phylogeny of archaeal sequences from the various

Table 2. Examples of Archaea Isolated from Soils

Division	Order	Family	Isolate Type	Soil Habitat	Reference
Euryarchaeota	Methanobacteriales	Methanobacteriacea	culture, SSU rRNA sequence.	Italian rice paddy soil, bog, pasture, and marsh	11,34–38
Euryarchaeota	Methanobacteriales	Methanothermaceae	culture,	solfataric mud, Iceland	36,115
Euryarchaeota	Methanococcales	Methanococcaceae	culture, SSU rRNA sequence	black shore mud, San Francisco Bay	37,39
Euryarchaeota	Methanomicrobiales	Methanomicrobiaceae	SSU rRNA sequence,	rice paddy soil, Italy	11,34,36
Euryarchaeota	Methanomicrobiales	Unknown	SSU rRNA sequence,	rice paddy soil, Italy	11,34,36
Euryarchaeota	"Methanosarcinales"	Methanosarcinaceae	culture, SSU rRNA sequence,	marshes, wetlands, Italian rice paddy, and garden soil	11,34,36–38,40
Euryarchaeota	"Methanosarcinales"	"Methanosaetaceae"	SSU rRNA sequence,	rice paddy soil, Italy	34,36
Euryarchaeota	"Methanosarcinales"	Unknown	SSU rRNA sequence,	rice paddy soil, Italy	11,34,36
Euryarchaeota	Unknown	Unknown	SSU rRNA sequence,	rice paddy soil, Italy	11,34,36
Euryarchaeota	"Thermoplasmatales"	Unclear	culture	solfataras, coal refuse piles	41,42
Euryarchaeota	Halobacteriales	Halobacteriaceae	culture	hypersaline soils in Spain & former USSR	43–45
Crenarchaeota	Sulfolobales	Sulfolobaceae	culture	acidic hot soils	46–48
Crenarchaeota	Thermoproteales	Thermoprotaceae	culture	Uzon caldera, Russia	41,49
Crenarchaeota	Thermoproteales	Desulfurococcaceae	culture	solfatars, Indonesia	41
Crenarchaeota	Unknown	Unknown	SSU rRNA sequence	agricultural, boreal forest, rice paddy soil	9,11,33,34,36,50

different soil habitats ranges from having close affiliation to members of known groups of Archaea, such as *Methanobacterium* and *Methanosaeta* (11), to being highly divergent from any cultured archaea (9).

GROUPS OF SOIL ARCHAEA

Methanogens

Methanogens are a phylogenetically congruent group of microorganisms that play critical roles in recycling elements through the biosphere. They provide the only significant biogenic source of the hydrocarbon methane (CH_4), the second most abundant carbon-containing gas in our atmosphere and a major greenhouse gas (3). Although very diverse, both morphologically and physiologically, methanogens have a limited substrate range and are all strictly anaerobic, obligate methane producers (51). Methanogenesis is important in anoxic environments with low redox potentials, in which the reduction of alternative oxidants such as nitrate, Mn(IV), Fe(III), and sulfate has occurred (52). Waterlogged soils are among the most common habitats from which methanogens are isolated, along with anaerobic sediments, sludge digesters, insect guts, and mammalian rumina and intestines. Soil-dwelling methanogens from wetlands, rice paddies, and landfills are major contributors to the global CH_4 cycle, contributing about 60% of atmospheric methane (3).

Members of four of the five methanogenic orders (Methanosarcinales, Methanococcales, Methanobacteriales, and Methanomicrobiales) have been isolated by culturing from soils. SSU rRNA gene sequences from members of the families Methanosarcinaceae, Methanococcaceae, and Methanobacteriaceae, as well as SSU rRNA gene sequences representing members of previously unknown groupings, have been recovered from soils (Table 2).

Morphology and Metabolism of Methanogenic Archaea

Methanogenic archaea exhibit diverse morphologies: rods, cocci, spirals, pseudosarcinae, and multicellular aggregates. They are either motile or nonmotile and they stain either gram-negative or gram-positive. Carbon and energy sources include $H_2 + CO_2$, formate, acetate, methyl compounds (methanol, methylamines, methsulfides, and possibly methylselenides), methanol + H_2, or alcohols + CO_2. A number of isolates are obligate or facultative autotrophs. Ammonia is the typical nitrogen source (although some isolates can use amino acids or fix nitrogen) and sulfur or sulfide is a source of sulfur (51).

Soil Habitats of Methanogenic Archaea

Soil macroenvironments can be divided into the zones above and below the water table (51). The zone below the water table is saturated with water, whereas the vadose zone above is not. O_2 diffuses rapidly through soil and thus anoxic vadose zones are extremely rare, although anoxic microenvironments can occur. Because O_2 diffuses more slowly through water than air, the water-saturated zone is often anoxic, and representative environments are waterlogged soils such as rice paddies, stagnant marshes, and bayous. Groundwater, which contains low amounts of O_2 even when air-saturated (less than 0.3 mM O_2 at 20 °C), can easily become anoxic and suitable for the growth of methanogens, particularly in polluted areas. Although methanogens are extremely sensitive to O_2 in culture, they are protected in oxic zones in nature by the activities of O_2-utilizing organisms (51).

Methanogens produce methane in three major ways, with acetate and H_2/CO_2 being the most important methanogenic substrates in soils (52). Some produce methane by the reduction of CO_2, using H_2, formate, or certain alcohols as an electron donor and CO_2 as the electron acceptor. This method is common among rumen methanogens. Methane production through the oxidation of C-1 compounds is another, less common, process. Some of the substrate molecules are typically oxidized to carbon dioxide and the remaining methyl groups are reduced to methane. Methanogenesis from C-1 compounds occurs in environments that are enriched for these substrates, such as marine sediments. A third manner of methane production occurs by an aceticlastic reaction, in which the methyl carbon of acetate is reduced to methane and the carboxyl carbon is oxidized to carbon dioxide. The ability to catabolize acetate among the known methanogens is limited to species of *Methanosarcina* and *Methanosaeta* ("*Methanothrix*"). Methane production from acetate is found in habitats such as freshwater sediments where other anaerobes are limited for acetate catabolism by the availability of alternate electron acceptors.

In nature, methanogens typically play a role in part of a degradative microbial consortium, performing the final anaerobic step in the decomposition of organic matter in soils (51). The major methanogenic precursors in the complete decomposition of organic matter to CO_2 and methane are acetate, formate, and $H_2 + CO_2$. Initially, bacterial fermentation converts organic matter into volatile organic acids. The longer-chain volatile organic acids (with three or more carbon atoms), such as propionate and butyrate, must be further metabolized to suitable methanogenic substrates. Interspecies electron transfer, an association involving methanogens with other, specialized groups of microorganisms, often helps accomplish this.

Anthropogenic Increases in Methane Production

A steady increase has occurred in the amount of atmospheric methane in conjunction with the rise in human populations (53–55). Both agricultural practices and landfilled waste are major sources of methane and contribute to erosion of the ozone layer. Over the last decade, the atmospheric content of methane has been measured at about 1.7 ppm, and is estimated to increase by 0.5 to 1% a year (see also TRACE GASES SOIL). Terrestrial environments, particularly forests, are the major sources of atmospheric methane. Although they are low-flux systems relative to waterlogged soils, their contribution of methane to the biosphere is significant because of their vast coverage area (56). Although methane production in terrestrial ecosystems has not been well studied, two major terrestrial habitats of methanogens are termite guts and the rotting heartwood of trees. Better studied are high-flux systems, including wetlands, rice paddy soils, and freshwater sediments, which account for between 15 and 45% of the total biogenic methane (54).

Thermoplasmales

Variable-shaped, wall-less members of the genus *Thermoplasma* have been isolated from solfataric fields in many geographic locations (Table 2). Originally found in smoldering coal refuse piles, solfataras appear to be the primary soil habitat of *Thermoplasma* species. Isolates have been cultured from these environments in wide geographic locations, including Italy, the Azores, United States, Iceland, and Indonesia; the two characterized species are *Thermoplasma acidophilum* and *T. volcanium* (42). In culture, these microorganisms grow heterotrophically as facultative anaerobes by sulfur respiration. They grow aerobically with yeast or other similar, complex extracts. Growth temperatures range from about 45 °C to 63 °C for *T. acidophilum* (42) and from about 33 °C to 67 °C for *T. volcanium* (41,42). Neither natural growth factors nor ecological roles are currently known for *Thermoplasma* spp. found in conventional soils.

Halobacteriales

General Characteristics. Cultured isolates of halophilic archaea are currently placed in a single family, Halobacteriaceae, in the order Halobacteriales (57). Halophiles comprise a variety of morphological types, including rods, pleomorphic forms, angular cells, and cocci. Isolates from saline soils, like other halophilic archaea, are red colored because of the presence of cellular pigments such as β-carotene and bacterioruberin (58). Some halophilic isolates are chemoorganotrophs and use only amino acids or organic acids as carbon sources, but many have the ability to grow on carbohydrates. Originally reported as obligate or facultative aerobes, there is a growing body of evidence suggesting that most species are facultative anaerobes (58). Certain species also synthesize ATP using bacteriorhodopsin as a light-driven proton pump.

Habitats and Ecophysiology. Halophilic archaea are found in hypersaline environments in which salt concentrations exceed 150 to 200 g/liter. Most exhibit optimum growth at NaCl concentrations of between 2 and 4 M, whereas extremely halophilic species can grow even at the saturation point for NaCl in water (5.5 M) (57). Halophiles fall into a number of ecophysiological groups on the basis of their response to salinity, magnesium concentration,

and pH (58,59). There is a reasonably clear division, for example, between alkiliphilic and neutrophilic members of Halobacteriaceae (no acidophiles have been reported). Halophilic archaea are most commonly found in aquatic environments, but representatives have also been isolated from saline soils (Table 2). Isolates have been recovered from saline soils of arid areas such as the Mediterranean coast of Spain (43) and in soils of various regions of the former Soviet Union (45). Examples include, among others, members of the genera *Halobacterium, Haloarcula*, and *Halococcus*. The ecology of halophilic soil archaea has not been studied, but by analogy to hypersaline aquatic environments, the concentration of divalent cations, especially magnesium and calcium, may be important (57,58).

Outward-directed sodium pumps in the cytoplasmic membrane of halophilic archaea are essential for regulating pH and for maintaining suitable intracellular ionic conditions. In addition, members of the Halobacteriaceae maintain osmotic balance in hypersaline habitats by the accumulation of inorganic ions to reduce internal concentrations of Na^+ (59). K^+ appears to be the main compatible ion used. Adaptations to highly ionic environments are also observed in the particular composition of halophilic membranes and proteins (58). (See also SALINITY EFFECTS ON THE PHYSIOLOGY OF SOIL MICROORGANISMS).

Thermoproteales

General Characteristics and Habitats. Members of Thermoproteales are strictly anaerobic gram-negative rods, discs, or spherical cells of varying sizes. These hyperthermophilic microorganisms, typically, have optimum growth temperatures between 85 °C and 105 °C. Members of Thermoproteales that can be found in soils include those from the genera *Thermoproteus*, *Pyrobaculum*, *Desulfurococcus*, and *Thermofilum*, all of which can be isolated from mud holes and soils of continental solfataras in Italy, the Azores, Iceland, United States, and New Zealand (Table 2). Many members of these and other genera are adapted for life in high saline, thermal submarine environments.

Physiology and Ecology of Thermoproteales. Unfortunately, there are no studies so far that describe the physiological ecology of members of Thermoproteales. In culture, all members are capable of sulfur respiration using various organic substrates and of producing CO_2 and H_2S (60). Sulfur can be replaced by various sulfur components under heterotrophic growth conditions. Some Thermoproteales are strict organotrophs, for example, *Pyrobaculum organotrophum* and members of the genus *Thermofilum*. Others, such as *Thermoproteus tenax, Thermoproteus neutrophilus, Pyrobaculum islandicum, Pyrodictium occultum*, and *Pyrodictium brockii*, are able to grow chemolithoautotrophically with CO_2 as the sole carbon source (61), reducing sulfur with molecular hydrogen and producing H_2S. In *T. neutrophilus*, an unusual reductive citric acid cycle for carbon assimilation was found with characteristics similar to those of pathways in eukaryotic microorganisms (62,64,114). Studies are needed to determine the relevance of the physiological characteristics of Thermoproteales members in culture to their ecology in situ.

Sulfolobales

Taxonomy and Habitats. The order Sulfolobales comprises a number of genera, including *Sulfolobus; Metallosphaera; Acidianus; Desulfurolobus*; "*Stygiolobus*" and, potentially, *Sulfurococcus*. Commonly isolated from continental solfataric water and mudholes of broad geographic distribution, members of the order Sulfolobales are all extremely thermoacidophilic aerobic organisms. The naturally geothermally heated terrestrial environments in which they have been isolated tend to be acidic (as low as pH 0.5) with low salinity (0.1 to 0.5% salt). *Sulfolobus and Acidianus* spp. occur in diverse locations such as the United States (46,65–67), Italy (68), the Azores (46), New Zealand (69), Japan (70), the West Indies (66), and Iceland (46,66,71). The typical irregular, lobed cells of members of the order can be present in quite high numbers (approximately 10^8 cells/ml) in these habitats, and are frequently recognized by the presence of an oily iridescent layer floating on the surface of muddy pools. *Sulfolobus* strains have been isolated as well from hot acid soils (72); such soils, in fact, being the dominant feature of solfataric fields (66).

In contrast to the cosmopolitan distribution of *Sulfolobus* and *Acidianus*, *Metallosphaera,* and *Stygiolobus* isolates have been recovered on a much more limited basis. *Metallosphaera* isolates have been found only within Neapolitan solfataras (73), and *Stygiolobus* exclusively from the Azores (74,75). It is presently unclear whether their limited detection is due to geographic isolation or selective cultivation.

Physiology of Sulfolobales. In culture, Sulfolobales possess diverse metabolic capabilities, spanning the biological spectrum from autotrophy (76,77) to heterotrophy (66–79) on a wide variety of complex organic substrates, sugars, and amino acids, to possibly even mixotrophy (77). All species are able to oxidize molecular sulfur to sulfuric acid (73,76,80), with the exception of *Stygiolobus*. MoO_4^{2-}(81) and Fe^{3+} (82) can both serve as alternative electron acceptors; S^{2-} (66), $S_4O_6^{2-}$, and Fe^{2+} (65,82) can all replace sulfur as an energy source. The ability to oxidize sulfur and ferrous iron results in the formation of soluble sulfates and subsequent mobilization of metals from sulfidic ores by some *Sulfolobus* species (65). This and other characteristics make these organisms important candidates for microbial leaching (83). See Brierley and Brierley (81) for a review of copper leaching studies performed with *Sulfolobus*.

Acidianus and *Desulfurolobus* can both oxidize and reduce molecular sulfur (74,84). Growth and sensitivity profiles suggest that strict and facultative aerobic species of Sulfolobales are microaerophiles. *Acidianus*, *Desulfurolobus,* and *Stygiolobus* species are capable of growth anaerobically by $S0/H_2$-autotrophy, with H_2S being produced (61).

Ecology of Sulfolobus. Although isolated from hot acid soils, *Sulfolobus* could not typically be observed directly in the soil or attached to soil particles (66). The organism was therefore quantified in such soils by microscopy, using most-probable-number dilutions in an

autotrophic medium containing elemental sulfur at pH 3.0 (72). *Sulfolobus* numbers measured across thermal gradients at two sites in Yellowstone National Park reached approximately 10^2 to 10^3 per gram of soil at temperatures ranging from about 50 °C to 70 °C (72). The uptake of $^{14}CO_2$ was measured in these soils, and a good relationship was found between cell counts and rate of CO_2 fixation for higher numbers of sulfur oxidizers. Two peaks were observed for $^{14}CO_2$ incorporation, one around 70 °C with a preliminary assignment to *Sulfolobus*, and another larger peak at around 30 °C, which was cautiously attributed to *Thiobacillus*. *Thiobacillus*, the only other organism detected by Fliermans and Brock (72) in these soils, demonstrated a growth peak at around 30 °C and overlapping growth with *Sulfolobus* at around 55 °C. There was not an exact correlation between CO_2 fixation and numbers of sulfur oxidizing microorganisms because no CO_2 fixation peak was observed corresponding to peaks of microbial growth at around 55 °C.

Distribution of Sulfolobales and Thermoproteales in Solfataras. The distribution of Sulfolobales and Thermoproteales differs within the solfataric habitat. Two niches have been identified in solfataric regions: (*1*) an upper, oxic zone that is approximately 30-cm thick, highly acidic (pH < 2) because of both biotic and abiotic oxidation, and rust-colored because of the presence of ferric iron (66,85), and (*2*) a deeper, highly anaerobic zone reduced by volcanic gases that is higher in pH (pH > or = 4) and blackish-gray in color because of the presence of heavy-metal sulfides (85). *Sulfolobus* are present in both niches, whereas members of Thermoproteales are found exclusively in the reduced zone. Unfortunately, there is no distribution data available for other archaeal members in solfataric habitats.

NONCULTURE-BASED APPROACHES TO MICROBIAL SAMPLING

The view that archaea are strictly extremophiles is now changing because of a "molecular revolution" (23) in microbial ecology that has led to the discovery of nucleic acid sequences and in situ evidence for previously unknown members of the Archaea (as well as for bacteria and Eukarya). The development and application of a variety of molecular techniques not reliant on culturing to detect and discriminate microorganisms has led to a restructuring of our view of microbial ecosystems. The most extensively used phylogenetic tools are those based on the exceptional conservation of SSU rRNA gene sequences among different organisms. Such approaches are powerful because they enable the recovery of sequences representing specific microbial phylotypes from natural environments, without cultivation of the corresponding microorganisms (5). Use of this approach has demonstrated the existence and abundance of novel archaea across a broad array of nonextreme terrestrial and aquatic habitats (9–11,15,16,19,21,22,31–33,86).

Because all techniques have biases, multiple approaches are often utilized when analyzing the microbial diversity of a particular habitat. Initial approaches leading to the discovery of microbial complexity in the environment involved DNA-DNA reassociation kinetics after strand dissociation of the DNA in a sample (7,8). Control experiments demonstrated that reassociation kinetics were affected by the diversity of genomic DNA in a sample. Subsequently, approaches taking advantage of phylogenetic conservation among the rRNA of various groups of microorganisms have involved the use of polymerase chain reaction (PCR). Typically, DNA is extracted from the environment of interest and SSU rRNA gene sequences are amplified from the sample by PCR and analyzed by various methods. The most informative approach for using rRNA information to study the microbial members of an ecosystem involves cloning and sequencing (87) and is also the most labor-intensive.

Less time-consuming, but also less informative, are methods such as amplified-ribosomal-DNA-restriction analysis (ARDRA, 112), denaturing-gradient gel electrophoresis (DGGE, 90), temperature-gradient gel electrophoresis (TGGE, 111), single-strand conformational polymorphism (SSCP, 63), and terminal-restriction fragment length polymorphism (T-RFLP, 113) analyses. These methods rely on size and/or nucleotide composition of the native or PCR-generated DNA fragments for discrimination, but do not reveal precise phylogenetic information without sequencing of specific bands or the use of quite specific primer sets.

Some methods, such as in situ hybridization (5,88), reverse-transcription PCR (89,90), TGGE or DGGE, and T-RFLP, are used to identify metabolically active or numerically dominant populations. However, all but the first method rely on PCR, which is known to demonstrate strong bias under certain circumstances (91–94). Attempts to avoid PCR bias have been made, for example, with a recent approach that takes advantage of the ability of the bacterial artificial chromosome (BAC) vector to accept large fragments of DNA (95,96) and act as an expression vector for environmental DNA in a surrogate host (97). Although molecular methods also have limitations, the evidence from their concerted use is uncontestable. Our knowledge of natural microbial populations was previously severely limited through use of culture-based techniques only.

UNCULTURED SOIL ARCHAEA

Research by Bintrim and coworkers serves as one example of a study examining the diversity of Archaea in a mesophilic soil environment (9). Phylogenetic analysis of sequences recovered from a Midwestern (fallow) agricultural soil placed all 35 of the archaeal clones recovered (designated SCA) in a clade that diverged deeply within the division *Crenarchaeota*. This and other such discoveries (10,21,32,33,98) were surprising because archaea had never before been suspected to be present in mesophilic dry soils.

Crenarchaeota SSU rRNA sequences have now been recovered worldwide from terrestrial and aquatic low- to moderate-temperature environments. The sequences belong to at least four distinct subgroups and appear

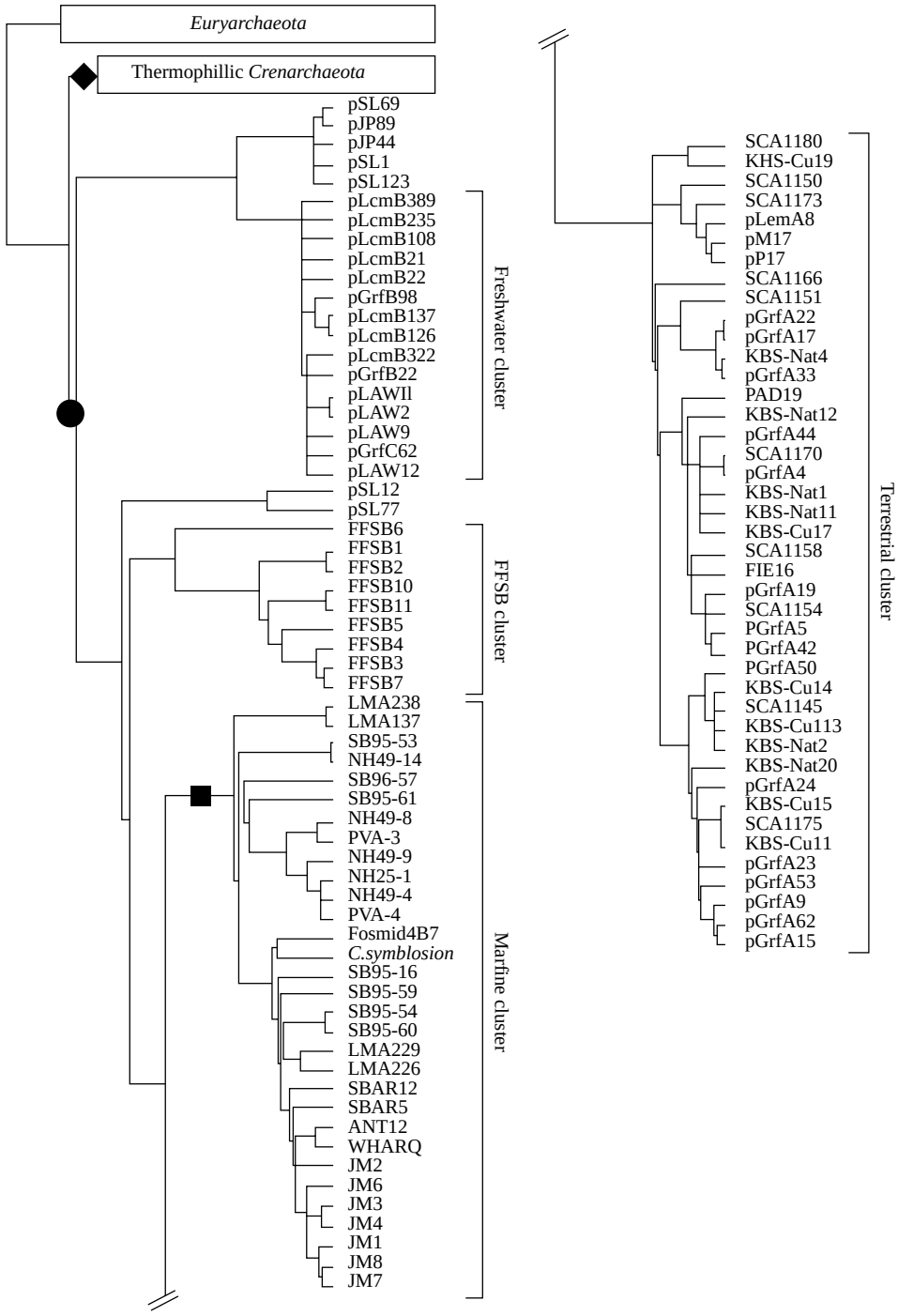

Figure 2. Phylogenetic tree showing the relationships of nonthermophilic Crenarchaeota 16S rDNA sequences. The symbols indicate the specificities of Crenarchaeota probe Cren499R (diamond), probes Cren667 and GI-554 (♦), and probe Cren745 (●). *Cenarchaeum symbiosum C. symbiosum*. Reproduced with permission from Buckley et al. (1998).

to have a common ancestry (Fig. 2; 21). The majority of archaeal sequences recovered from soil cluster together in one clade — with the exception of a group of sequences recovered from a single study of boreal soil from Finland (32) — which cluster in a separate group. Quantitative estimates of the total RNA of crenarchaeota in agricultural and undisturbed soils range from 0.37 to 1.4% relative to total community RNA (21) (Table 1). The closest affiliation of uncultured soil archaea is to a clade of marine, planktonic crenarchaeotes, designated as "Group 1 archaea" (15). Studies of the biomass contribution of planktonic archaea ranged from 2% (15) up to 34% (16) (Table 1). Thus, in both terrestrial and oceanic nonextreme habitats, archaeal members constitute a significant proportion of microbial assemblages. Similar trends of novelty, diversity, and abundance are also observed for SSU rRNA gene sequences representing uncultured members of the Euryarchaeota (15,16,18,99).

The Rhizosphere (See also RHIZOSPHERE MICROBIOLOGY)

Soil archaea thrive in another habitat previously thought to be the domain of bacteria and eukaryotes, plant

roots and the rhizosphere. The rhizosphere extends from the surface of the root (the rhizoplane) into the surrounding soil a few millimeters. It is a rich ecological niche abundantly exploited by soil microbes, in terms of both population numbers and activity. Roots and rhizosphere habitats are nutrient-rich relative to those of bulk soil. This is in part due to the fact that roots deposit from 5 to 30% of the total photosynthate of the plant into the surrounding soil in a process known as rhizodeposition (100), and in part due to sloughed, injured, or senescing plant cells that leak nutrients.

Although a far more nutrient-rich habitat than bulk soil, the rhizosphere consists of networks of microenvironments similar to those found in soils. Extreme niches exist in the rhizosphere as certainly as they do in soil, because the rhizosphere environment is subjected to dramatic climatic events resulting in fluctuations of water and osmotic potential, salt concentrations, pH, and soil particle structure (101–103). The question remains to be answered, however, as to whether archaea occupy extreme niches in soil and rhizosphere, or if, instead, the archaea there compete successfully against bacteria within nonextreme niches. Specialized metabolic activities such as methane production or nitrogen fixation may allow archaea, which typically grow more slowly than most bacteria, to overcome competition by making essential trophic contributions within the ecosystem.

Archaea in the Rhizosphere

Root-Associated Methanogens. The relative contribution of root-associated methane production to the atmosphere can be very important for flooded soil environments. For example, root-associated methane production in rice paddy soils varied between 4 and 52% in one study (104). In another study, removal of aboveground vegetation from natural wetlands resulted in more than an order of magnitude reduction in methane emissions, without a concurrent decrease of methane stored in soil (105).

Root Colonization by Archaea of Unknown Phenotypes. Additional evidence for archaeal associations with the rhizosphere comes from studies in both flooded (11) and dry (22) soils. SSU rRNA gene sequences were recovered from these environments using total rhizosphere and/or rhizoplane DNA and primers hybridizing to the SSU rRNA genes of archaea in PCR reactions. In keeping with the results from previous studies in soil, rhizosphere samples from rice plants grown in flooded soils harbored SSU rRNA sequences from both Euryarchaeota and Crenarchaeota (11), whereas rhizosphere samples from plants grown in dry soils, thus far, have yielded evidence for Crenarchaeota only (22). rDNA clones recovered from tomato rhizoplane (*Lycopersicon esculentum*) samples (designated TRC clones, with 91 to 99% sequence identity over 1,280 nucleotide positions) clustered with uncultured soil Crenarchaeota from dry, mesophilic environments. Phylogenetic analysis also indicated that TRC sequences were distinct from sequences representing uncultured Crenarchaeota clones from rice rhizospheres and rice paddy soil (designated ARR/ABS, 11). Direct sequence comparisons supported divergence of these two rhizosphere groups, yielding sequence similarity of 76 to 84% between the TRC and ARR/ABS sequences, compared with a range of 91 to 99% sequence similarity between the TRC and uncultured clones from West Madison (designated SCA, 9) and Michigan (designated KBS, 21) soils. These studies, like those on mesophilic soil archaea, indicate that rhizosphere Crenarchaeota comprise a phylogenetically coherent, but quite diverse, group of microorganisms.

Abundance of Crenarchaeota on Tomato Roots. In the first study to document colonization of plant surfaces by members of the Archaea, we showed that members of the nonthermophilic Crenarchaeota colonize the roots of tomato plants grown in soil (Fig. 3; 22). Our results demonstrated the recruitment of the crenarchaeotes from soil and their growth, and apparent persistence, on roots. The abundance of crenarchaeotes was documented on tomato roots by fluorescence in situ hybridization (FISH) assays, using probes designed to be specific for the SSU rRNA gene sequences of Crenarchaeota recovered from soil and tomato rhizospheres. We found Crenarchaeota broadly distributed on both young and senescent roots. Their abundance was determined to be about 3.3% relative to the number of total probe-positive cells (bacteria and crenarchaeotes) on young roots and about 16% on senescent roots of plants seven to eight weeks in age. Although they were present on all roots examined from plants spanning one to eight weeks of age, their abundance increased 10-fold on senescent roots compared with younger roots of about the same age, with a concurrent increase in the frequencies of cells in pairs (presumed to be dividing cells) and in colonies. By contrast, bacterial abundance increased only fewfold on senescent roots. Greater abundance of crenarchaeotes was also observed in the older regions of nonsenescent roots, whereas bacterial numbers decreased in these regions. These results suggest that rhizosphere Crenarchaeota may have roles in mediating later stages of root biology, a function previously unsuspected for members of the Archaea.

CONCLUSION

One of the great mysteries of the world lies beneath our feet in the guise of the mundane, a substance known simply as soil or dirt. Through time and considerable effort, humankind continues to push back the frontiers of science in the fields of chemistry, physics, and biology. We have yet to fully understand, however, the dynamics of these forces and how they interact to form that enduring interface between the organic and inorganic components of the terrestrial biosphere. Although scientific knowledge of the chemical and physical properties of soil is substantial, our understanding of microbial composition and contribution to soil ecosystem dynamics is incomplete. The recent discoveries regarding archaea in soil and other conventionally nonextreme habitats dramatically illustrate this point.

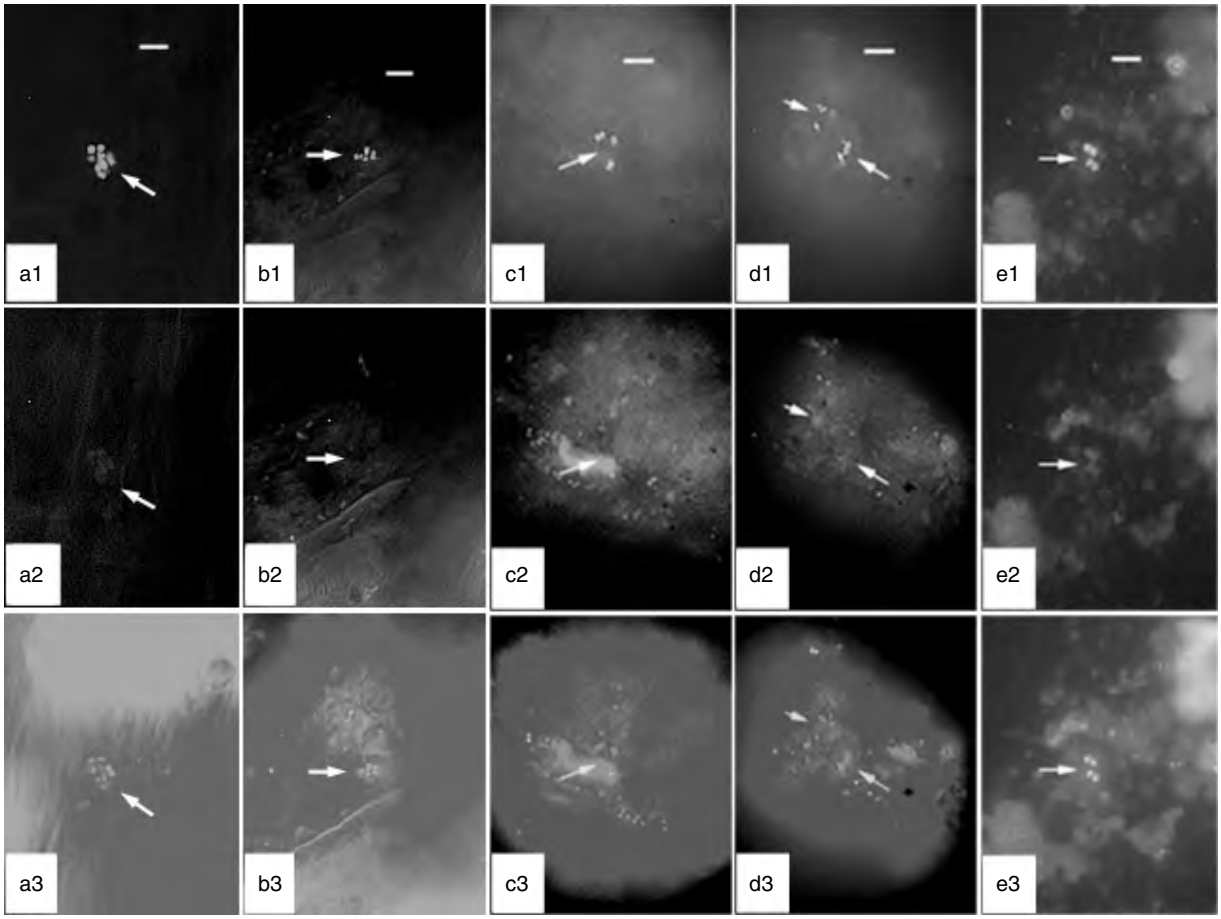

Figure 3. Fluorescence in situ hybridization of the tomato rhizosphere for Crenarchaeota. One-week-old (A) tap and (B) lateral rootlets, (C and D) senescent rootlets, and (E) rhizosphere soil. Oligonucleotide probes used were designed to be (1) specific for nonthermophilic Crenarchaeota and (2) Bacteria. Images in row (3) show results of DAPI staining of the same fields shown in rows (1) and (2). Scale bar represents 5 μm. Reproduced with permission from Simon et al. (2000). See color insert.

Extreme or Not Extreme — That Is the Question

The fact that isolation of archaea was initially successful in primarily extreme or uncommon environments implied the failure of archaea to compete with bacteria in more common habitats. Following this line of thought, archaea are successful because of their specialization. For example, adaptation to extreme environments, in which growth of bacteria and eukaryotes is limited, results in reduced competition. Thus, archaea may survive by avoiding direct competition with other microorganisms. On the other hand, the discovery of archaea in mesophilic habitats poses the question — do archaea thrive only in highly specialized niches, as dogma holds, or is our knowledge of the extent of their ecological range limited by our inability to culture them? Soil is an excellent case in point. It is well known that microscopic counts of microbial cells in soil far exceed, by as much as two orders of magnitude, the number of cells that can be cultured on typical agar medium (5,6). A hypothesis to explain this result states that a portion of cells in soil environments are in a starvation state and that makes them recalcitrant to growth in vitro. Such a "viable but nonculturable" state is known to exist for, at least, some culturable microorganisms under certain conditions (106). This leads to the prediction that we may have mislabeled mesophilic soil habitats as "nonextreme," and suggests that archaea thrive in soil precisely because it contains niches of an extreme nature. The recent discovery of archaea in other nonextreme habitats, such as low-to-medium temperature marine environments, can also be rationalized using this hypothesis. It can be argued that marine planktonic archaea are in relatively extreme environments as well, because the majority of the marine ecosystem is oligotrophic (contains 1–10 mg of carbon per liter), with microniches created, for example, by pockets of suspended detrital matter (107). The answers to the questions of what makes a habitat extreme (from a microbial point of view) lie, in part, within the physiological mysteries of the as-yet uncultured archaea found thriving, unexpectedly, in these so-called nonextreme environments. When we unlock the metabolic secrets of these archaea, we will gain a better understanding of the niches they inhabit.

The Future

Although molecular techniques have added substantially to our knowledge of the composition of microbial assemblages in soils and expanded our knowledge concerning the ecological range of archaea, there is more to learn in this regard as the list of habitats harboring these organisms lengthens. There are also a number of challenges involved in describing microbial dynamics in soil habitats that remain unsolved. For example, many questions remain relating to sampling; spatial heterogeneity and scale are both extremely complex problems that demand integrative and interdisciplinary approaches for their solution. Approaches monitoring chemical and physical parameters, in combination with microbial analyses at the micro- as well as macroscale level, are needed to address these issues and others.

Advances in the areas of genomics and high throughput monitoring of changes in microbial populations in response to ecosystem fluctuations will further illuminate microbial composition, metabolic potential, and dynamics in soil environments. Examples of such advances include obtaining genetic and functional information from soil microorganisms without culturing using metagenomics (97) and other direct access methods (50,108–110) and the use of oligonucleotide arrays to monitor detection, abundance, and gene expression of populations and assemblages of microorganisms.

Progress in defining ecological roles of archaea and other microorganisms will be realized best by using classical culturing and molecular approaches in concert. For example, sequences recovered from previously unknown and numerically abundant archaea in soil environments provide information for the design of specific oligonucleotide primers and probes hybridizing to the SSU rRNA gene sequences of those microorganisms. The oligonucleotides can then be used to monitor the growth of previously uncultured organisms in enrichment cultures. Growth of these archaea in pure culture will, in turn, potentially lead to an understanding of their metabolic capabilities and potential impact on ecosystem dynamics, predictions about which can be tested using phylogenetic and substrate-level probes in situ.

In conclusion, many questions remain regarding the ecological distribution and roles of archaea. We predict that archaea have far greater global impact on biosphere composition and function than previously thought. Archaea will be found to perform fundamental roles in conventionally nonextreme environments, as they do in typically extreme habitats. Their early evolution and history placed them in these environments as fundamental nodes in the microbial network; their unique physiological properties keep them there.

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ARCHAEA IN SOILS.

See Soil Bacteria

ARCHAEA, THERMOPHILIC.

See Thermophiles, Diversity of

ASSESSING MICROBIAL PROTEOMES IN THE ENVIRONMENT

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Proteins are generally recognized as the molecular wheels of cellular machinery. Their functions include enzymatic catalysis, maintenance of the integrity of membrane receptors and general cellular architecture, and the mitigation of stressful environmental conditions. The profile of proteins synthesized by microorganisms under indigenous conditions offers a window through which investigators may glimpse complex environmental modulation of multilevel interactions between genetic endowment and natural selection. The term *proteome* was coined by Wilkins and Williams and their coworkers to describe a postgenomic field of research aimed at understanding the disposition of genetic potential in the strategic survival of organisms in variable environments (1–8). Microbial proteomics is an integrative approach toward the analysis of protein molecules in microbial communities, with particular emphasis on understanding how genetic potential is transformed into the observed array of diverse but compatible structures and functions in both natural and engineered environments (9–24). Proteomics research encompasses both the genetic and environmental underpinnings of microbial community integrity. Application of proteomics in environmental microbiology research is still evolving, but it is so far dominated by axenic culture studies. However, investigations of microbial community proteins are emerging in the literature (17,25–34). Early reports focused on methods of quantitative recovery of protein molecules from uncultivated but densely populated microbial communities (22,24), the radiolabeling of target proteins in heterogenous populations (21), and the direct assay for enzymatic proteins in situ (21). These research programs fulfill the need for methods to complement the remarkable progress that has been made toward the elucidation of phylogenetic diversity and microbial community structures, made possible by lipid-based and nucleic acid–based techniques for analyzing heterogenous microbial systems such as soils and groundwater (35–39). The methodological complementation is essential because knowledge of microbial community structure does not necessarily lead to useful information on microbial community functions such as metabolic capacity, control of population dynamics for native and invasive species, sensitivity to variable environmental conditions, and intrinsic niche diversity (40).

APPLICATIONS OF PROTEOME-BASED ASSESSMENTS IN ENVIRONMENTAL MICROBIOLOGY

The practical rationale for proteome analysis in aquatic microbiology is the desire to understand and harness the roles performed by protein molecules in a given sample of water for the improvement of environmental and public health. However, it is generally recognized that only about 10% of the microbial diversity in natural samples is extractable through traditional techniques involving laboratory cultivation. Additionally, methods developed for direct nucleic acid assessments such as DNA and rRNA fingerprints do not illuminate the response of the diverse microbial world to real-life environmental variations (Fig. 1). Similarly, assessment of bacterial mRNA is generally too temporary for its use in constructing a correlative map between environmental conditions and microbial response (41).

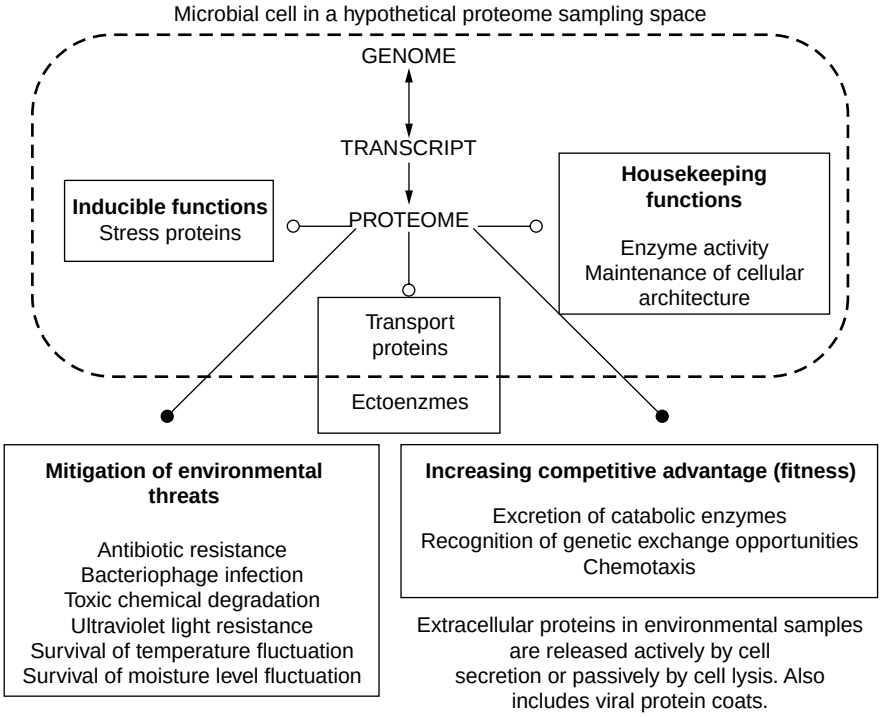

Figure 1. Conceptual diagram showing the transformation of genomic information into an array of proteins molecules that may be assessed within the framework of responses to influential environmental parameters. In theory, all species in a given environmental sample contribute to the collective proteome pool but responses by particular species present in large numbers may dominate the outcome of assessments limited by the stringency of molecular resolution.

Microbial community proteome analysis has been useful for understanding the ecotoxicological effects of chemical pollutants, the activity of catabolic enzymes in environmental bioremediation, the tracking of indicator species and pathogens in potable water distribution systems, and the tracking of genetically engineering microorganisms and their protein products following deliberate release into natural ecosystems.

Questions pursued by environmental microbiologists engaged in proteome assessments include the following:

1. Are specific pathogens present in a given environmental sample recognizable by defined protein profiles? For example, several waterborne pathogenic viruses are detected in polluted water samples through antigen–antibody reactions that are based on polypeptides in the structural backbone of the capsid protein. Similarly, pathogenic bacteria can be detected through screening for proteinaceous toxins (12,17).
2. When, and in what quantities, are certain enzymes capable of detoxifying chemical pollutants produced in contaminated environments? For example, direct assessment of mercuric reductase is possible in protein extracts from mercury-contaminated freshwater systems (18).
3. How do specific microorganisms respond to specific environmental stimuli through inducible gene expression? For example, the production of specific "head-shock" or stress proteins by bacteria cultivated under relatively uncomfortable environmental conditions has been a major application of proteome assessment in environmental microbiology (34). In some cases, microbial stress proteins have been designed to serve as biosensors of environmental change.
4. How does microbial community structure change in response to changes in biotic and abiotic modifications to their habitat? For example, protein-profile assessments have been used to monitor the status of the microbial community necessary for successful wastewater treatment in engineered processes and to diagnose microbial cellular states (42,43).
5. What is the evidence for evolutionary divergence and population disequilibrium in highly conserved niches that are inhabited by distinct phylogenetic groups of microorganisms? Investigators frequently find that different versions of an enzyme (allozymes or isozymes) have evolved over time despite the conservation of relevant catalytic function. For example, at least two versions of δ-aminolevulinate dehydratase, the key enzyme in porphyrin synthesis, are known to respond differently to toxic metal exposures (25,26). At least eight versions of β-glucosidase have been identified in marine microbial communities (33). The protocol of multilocus enzyme electrophoresis discussed later facilitates a rapid assessment of these physiological ecology questions through the analysis of protein molecules in their natural states.

EXTRACTION OF PROTEINS FROM AQUATIC ENVIRONMENTS

Several protocols have been developed to facilitate the recovery of proteins directly from environmental samples. The paramount consideration in selecting a method for recovery of proteins from environmental samples

is whether the study is a general survey of protein molecules or whether it focuses on a few targeted proteins assayed by function. In the former case, there may be no need to preserve the structure or catalytic function of recovered proteins and relatively harsh but thorough extraction techniques may be used. When emphasis is placed on preserving protein activity, conservative cell-lysis techniques are preferred. For the recovery of virus-associated or other cell-free proteins from aquatic samples, a combination of ultracentrifugation, adsorption, and/or precipitation is used (17) (Fig. 2).

Protein extraction methods have been developed for a variety of environmental sample materials including sediments, freshwater, seawater, and wastewater (20). In general, sonication is the preferred method for cell-lysis when the preservation of protein structure and function is essential. Chemical cell-lysis techniques including the use of detergents and alkali or boiling are used when the resolution of proteins requires denaturation (44–46). Because large numbers of polypeptides are expected in complex microbial communities, sample fractionation is usually required before the attempt to separate proteins on the basis of molecular mass, ionic charge, or both. Sample fractionation can be achieved by differential centrifugation, molecular sieves, and precipitation (7).

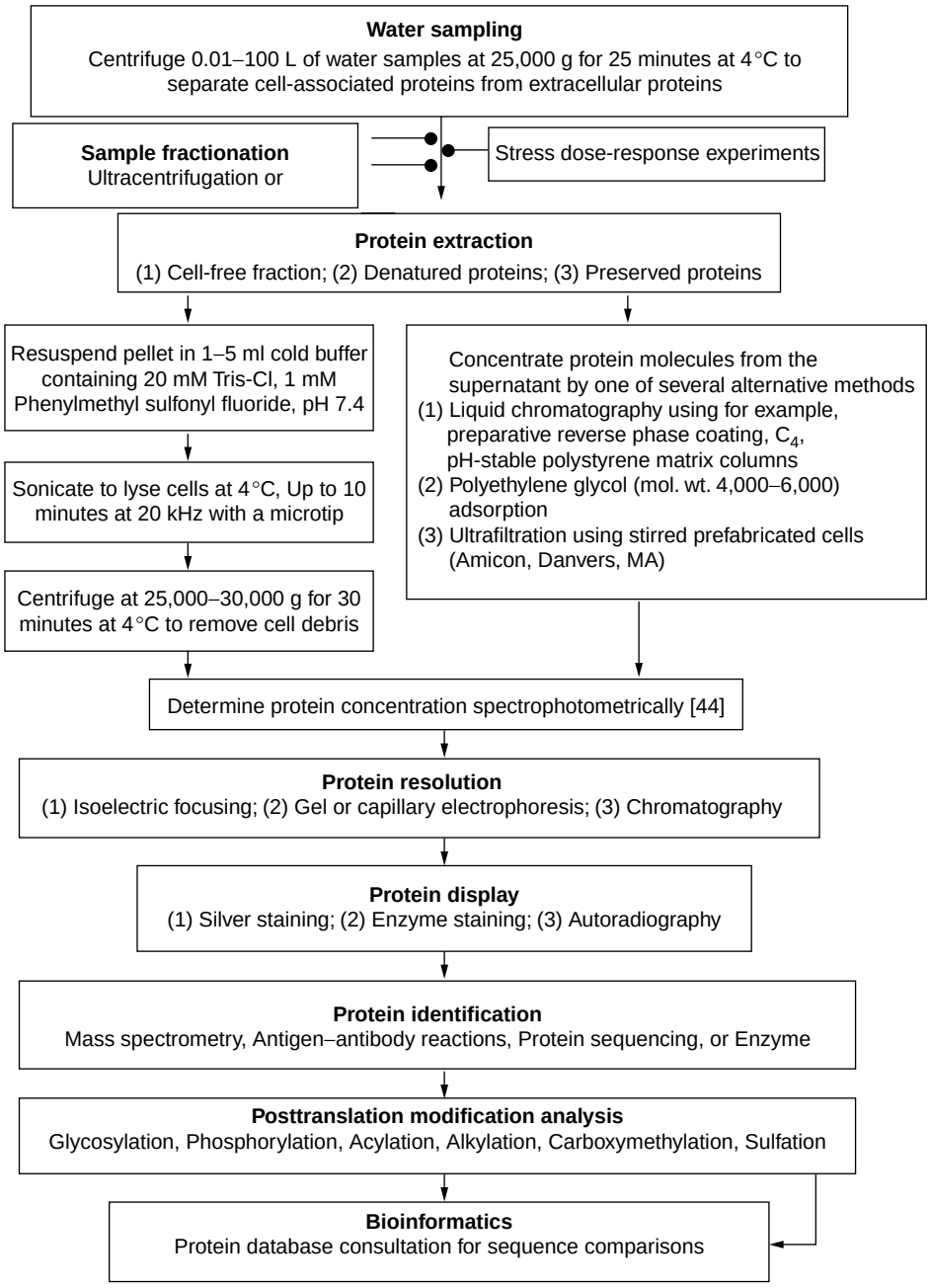

Figure 2. Flow diagram of procedures used in the construction of microbial protein profiles in environmental assessments. The initial volume of water sample is determined based on preliminary determinations of cell density and potential contaminating materials that may interfere with protein extraction and resolution.

RESOLUTION AND DISPLAY OF PROTEINS EXTRACTED FROM ENVIRONMENTAL SAMPLES

Gel Electrophoresis

Polyacrylamide gel electrophoresis is the most widely used method for protein resolution. The resolution power of one-dimensional electrophoresis is limited to less than 50 polypeptides, but it remains the most widely used method when highly specific methods are available to select from for the polypeptide(s) of interest. In theory, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is capable of simultaneous separation of thousands of proteins and is uniquely suitable for resolving complex and highly diverse preparations (43,47). In practice, however, several factors can reduce the resolving capacity of 2D-PAGE, particularly with respect to sample preparation and solubilization. The configuration of the interface between the first dimension (isoelectric focusing (IEF) to resolve polypeptides according to ionic charge) and the second dimension (polypeptide resolution according to molecular size) is also important. It is now possible to analyze single spots from a 2D-PAGE gel partly because the availability of immobilized pH gradients have eliminated the problem of gradient instability during IEF. In addition, the reproducibility of electrophoretic conditions and the results have improved with the commercial availability of ready-made gels in a variety of prescriptions.

A few precautions enable a relatively trouble-free PAGE analysis. First, it is important that proteins remain soluble throughout the electrophoresis process. Therefore, chaotropes and surfactants such as thiourea and sulfobetaine are often used to enhance protein solubility (8,46). Secondly, nucleic acids must be removed from samples before electrophoresis to reduce the viscosity of samples. A combination of carrier ampholites and ultracentrifugation may be used to remove nucleic acids if the extraction is conducted at high pH. Alternatively, endonuclease digestion is recommended when sample preparation is performed at high pH (40 mM Tris). Thirdly, it is important to eliminate disulfide bonds that act to reduce protein solubility. Therefore, chemical-reducing agents such as β-mercaptoethanol, dithiothreitol, or tributyl phosphine are used before isoelectric focusing. Finally, when working with environmental samples, humic and fulvic substances are usually coextracted and must be removed. Several commercially available chromatographic matrix columns (for example, from Biorad, Hercules, Calif.) may be used to reduce the concentration of these compounds in samples in which they present a problem.

Capillary Electrophoresis

The use of capillary electrophoresis equipment to resolve complex preparations of biomolecules is increasing (48). Capillary electrophoresis of protein molecules is performed in very thin (internal diameter of 25–100 μm) fused silica tubes ranging in length from 25 to 100 cm. The dimensions of the capillary allow the use of high electric fields, leading to very fast separation of proteins with a protocol completion time of less than one hour compared with the typical four to ten hours required for slab gel electrophoresis. In general, capillary electrophoresis resolves polypeptides according to ionic charge, but the system is adaptable to the use of various detectors, including conductivity, ultraviolet and visible light absorbance, radioactivity, and fluorescence. It is also possible to couple capillary electrophoresis with mass spectrometry for measurement of the molecular mass of resolved polypeptides (49). Capillary electrophoresis has been used to study various aspects of proteome assessment in environmental microbiology, including the construction of protein profiles for phylogenetic differentiation (51), changes in bacterial outer membrane proteins in response to toxic chemicals (51), and enzyme polymorphism in marine bacterial communities (33).

Radioisotopes

Radioisotopes have been used extensively in proteome research involving single microbial species and their use is increasing in in situ investigations. The radioisotope tracer approach takes advantage of the fact that many microorganisms are able to incorporate externally supplied amino acids into polypeptide chains. For these organisms, ^{35}S-labeled amino acids, typically methionine, is added to the growth medium. For organisms that engage in de novo protein synthesis, radioisotope is supplied as $H_2{}^{35}SO_4$. When working with natural environmental water samples, it is better to use a combination of radiolabeled amino acids and sulfuric acid so as to cover the entire range of microbial protein synthesis preferences. The radioactive isotope tracer technique has been used to detect the synthesis of specific proteins in the immediate aftermath of exposure of freshwater microorganisms to toxic chemicals (22).

For most applications, cells pooled from aquatic samples are usually incubated for up to an hour with radioactive precursors of protein synthesis. During the incubation period, cells may be exposed to a variety of stressful environmental conditions (e.g., toxic pollutant exposure, temperature shift, high salt concentrations, or virus infection). Proteins are then extracted from the cells and resolved electrophoretically. The radiolabeled polypeptides are subsequently detected by exposing gels to an X-ray film for autoradiography, or if capillary electrophoresis is used, detection is achieved by direct measurement of radioactivity. Control experiments with cells incubated under relatively normal environmental conditions allow the detection of inducible proteins synthesized in response to specific environmental stress stimuli. Quantitative assessment of radioactivity incorporated into each polypeptide band is achieved with radioactive image analyzers. Such quantitative data provide valuable cross-scale information on the maintenance of homeostasis in microbial communities under variable environmental conditions. Intensely labeled polypeptides of interest can be isolated, sequenced, and compared with existing protein sequence databases for possible identification of function (5).

Immunofluorescence and Enzyme-Linked Immunosorbent Assay

Antigen–antibody reactions targeted against "signature proteins" are widely used for immunological detection of microorganisms in environmental samples (52). The increasing availability of genomic sequences is contributing to rapid revision and developments in the use of antisera for tracking and identifying microorganisms through immunofluorescence and enzyme-linked immunosorbent assays (ELISA) (52). Furthermore, because monoclonal antibodies recognize very specific protein targets, their use have virtually eliminated traditional limitations of immunologically based techniques involving polyclonal antibodies. Monoclonal antibody-based protocols are also amenable to quantitative assessment. Immunofluorescence and immunomagnetic extraction of proteins from aquatic sources are also undergoing rapid development, but are currently limited by the fact that microbial antigens exhibit different ranges of immunological specificity. In addition, the type-specific antigens may be shared by related microorganisms (52).

Enzyme Electrophoresis

Microbial enzymes are invariably of central importance to the major research issues in environmental microbiology. Hence, the influences of environmental conditions on enzyme activity in nature have implications for the understanding and controlling of desirable processes ranging from bioremediation of polluted local environments to the impact of global environmental change on ecosystem functions sustained by the microbial community. It is generally well understood that enzyme activities depend on assay conditions. Therefore, the forms and functions of specific enzymes in their natural habitat can be expected to vary somewhat from assays conducted under laboratory conditions. Therefore, it is important to develop methods useful for understanding enzyme diversity in microbial populations present in the environment. The fact that many enzymes depend on redox reactions for effective catalysis has been exploited to produce colorimetric assays amenable to rapid assessment of enzyme kinetics and enzyme polymorphism in environmental microorganisms. Such assays are linked to sensitive indicator dyes such as formazan that assume color simultaneously with the progression of enzyme-catalyzed reactions (19,30,45). The technique can be accomplished on the same gel or membrane used for protein resolution, thereby producing a documented zymogram.

Because small variations in protein properties can influence the course of microbial adaptation and evolutionary innovation, techniques such as multilocus enzyme electrophoresis (MLEE) have been developed to capture the occurrence and distribution of allozymes (multiple enzymes with similar functions encoded by different alleles at the same genetic locus) and isozymes (multiple enzymes with identical functions encoded by different genetic loci) in microbial populations (31). Hence, MLEE yields direct information on niche diversity and selective pressure at the molecular level in a given microbial community. MLEE has also been used extensively to investigate the determinants of population dynamics in single microbial species, but not much work has been done on the application of MLEE to whole microbial communities. In general, conservative extraction of total proteins present in cells pooled from 1 to 100 L of water samples is achieved by sonication and centrifugation. Protein extracts are then resolved on gels made with starch or very thin slabs of polyacrylamide under nondenaturing conditions. After electrophoresis, the location of enzymes that depend on the redox reactions can be ascertained by incubating the gel in solutions such that the enzymatic reaction is coupled to the electrochemical production of colorful dyes (45). A quantitative method has also been developed for routine screening of enzyme activity in natural microbial communities by immobilizing proteins on nitrocellulose membranes before the development of staining reactions (21). The membrane method is rapid, but precludes the detection of multiple bands that indicate molecular diversity MLEE.

Another approach toward assessing microbial enzyme profiles in aquatic environments involves the use of capillary electrophoresis coupled with the detection of fluorescent products of enzyme activity. For example, investigation of β-glucosidase isozymes in the microbial population present in 100 L of seawater revealed unsuspected isozyme polymorphism (9,33). Pooled protein extracts from aquatic microorganisms can be loaded onto capillary tubes filled with buffer containing fluorogenic substrates, for example, resorufin β-D-glucoside for glucosidase activity. During electrophoresis, isozymes separate according to electrophoretic mobility. The electrical current through the capillary is then interrupted for approximately 10 minutes to allow interaction between enzyme and substrate. Fluorescent products are then detected at the anodic end of the capillary.

Mass Spectrometry

The coupling of gel or capillary electrophoresis to mass spectrometry for the identification of polypeptides is a rapidly evolving protocol (5,6). Standardized protocols using this approach have generated reproducible maps of 2D gels that allow comparisons across multiple experiments. Consequently, the identification of polypeptides through mass spectrometry can be achieved by matching profiles across phylogenetic boundaries. Both matrix-assisted laser desorption-ionization mass spectrometry (MALDI-MS) and electrospray ionization mass spectrometry (ESI-MS) represent technological advancements in this category. Mass spectrometric analyses of peptides require enzymatic digestion of proteins. Therefore, not every routine gel-staining technique can be used for primary resolution of protein molecules. For example, traditional silver-staining methods are generally not compatible with enzymatic digestion unless they are modified to avoid the use of cross-linkers and strong oxidizing and reducing agents. A compilation of protein-staining methods that are compatible with mass spectrometry is available from Protana (Table 1). Mass spectrometric fingerprinting of protein molecules allows rapid collection of information on a large number of proteins in a given sample, but ultimately, several pieces of data derived by using different methods must be integrated to identify a given

Table 1. Selected World Wide Web Resources for Environmental Microbial Proteomics Research

Category	Web Address
Protein Sequence Databases	
SWISS-PROT	*http://www.expasy.ch/sprot/sprot-top.html*
NCBI	*http://www.ncbi.nlm.nih.gov/*
Protein Patterns and Profiles	
PROSITE	*http://www.expasy.ch/sprot/prosite.html*
Prowl	*http://prowl.rockefeller.edu/*
Protein prospector	*http://prospector.ucsf.edu/mshome.html*
Metabolism Databases	
ENZYME	*http://www.expasy.ch/sprot/enzyme.html*
EcoCyc	*http://www.ai.sri.com.ecocyc/ecocyc.html*
Two-dimensional Analysis	
ECO2DBASE	*http://www.pcsf.brcf.med.mich.edu/eco2dbase*
SWISS-2DPAGE	*http://www.expasy.ch/ch2d/ch2d-top.html*
WORLD-2DPAGE	*http://www.expasy.ch/ch2d/2d-index.html*
Three-dimensional Analysis	
Swiss-SDImage	*http://www.expasy.ch/sw3d/sw3d-top.html*
PDB	*http://www.pd.bnl.gov*
Some Proteomics Companies	
Ciphergen	*http://www.cyphergen.com*
Kendrick laboratories	*http://www.msn.fullfeed.com/~-kendrick/*
Large scale biology corporation	*http://lsbc.com*
Protana	*http://www.protana.com*
Proteome Inc.	*http://quest7.proteome.com/*
Proteome-systems	*http://www.Proteomesystems.com*
Proteometrics	*http://www.proteometrics.com*
Scanalytics	*http://scanalytics.com*

protein unequivocally. A major follow-up aspect of polypeptide mass fingerprinting is the search for characteristic matches in established protein databases (Table 1).

The Proteinchip™ Array

The convergence of microchip electronics and high-fidelity biosensor technology continues to revolutionize environmental microbiological research. Microarray technology involves the immobilization of large numbers of biological molecules on tiny chip platforms for subsequent screening with molecular probes. This approach was initially developed to support large-scale genome sequencing efforts to facilitate the screening of large numbers of distinct DNA or mRNA molecules (for example, the GeneChip™ system developed by Affymetrix). Similarly, sophisticated technology is now available for proteome assessments. The surface enhanced laser desorption-ionization (SELDI) ProteinChip™ manufactured by Ciphergen (Palo Alto, CA) is designed to facilitate high throughput protein resolution and identification (49).

The ProteinChip™ array is typically made of metallic or plastic trays containing up to a thousand wells of 1 mm in diameter. The wells are chemically treated (e.g., with copper (II)) to capture trace quantities of unknown proteins (e.g., copper-binding proteins) in a given sample. Wells may also be coated with a known protein to be screened with biomolecules from unknown samples for the determination of protein function. Laser-based mass detection is then employed to measure the molecular mass of proteins. The stringency of molecular recognition in the array can be adjusted by washing and reading procedures to optimize signal-to-noise ratios. Potential applications of the protein microarray in environmental microbiology include the screening of proteins extracted from a natural microbial community for interaction with a given toxic metal. For such a task, metal ions are immobilized in the microarray wells and protein extracts will be added, followed by a stringent wash and read process. For example, molecular diversity in enzymes such as δ-aminolevulinate dehydratase and mercuric reductase that interact with lead and mercury, respectively, can be rapidly assessed (18,25,26). Other biomolecules that can be immobilized in microarray wells include membranes, receptor molecules, and antibodies.

Neural Networks and Cluster Analysis for Protein Profile Analysis

On the basis of the molecular size of microbial genomes, a prediction can be made about the expected number of independent proteins present in a cell at a given time. Although the one gene–one protein conjecture has proven to be inexact, it can be surmised that between 500 and

5,000 protein molecules are extractable, in theory, from each microbial component of environmental samples. The expected total number of proteins increases dramatically in environmental samples with highly diverse population of species. The development of highly sophisticated pattern recognition techniques is required for optimizing the information generated by protein profiles in natural microbial communities. Early attempts to identify microorganisms on the basis of protein profiles depended on statistical cluster analysis for recognizing matches between different protein profiles (42). More recent approaches to the interpretation of profiles from complex heterogenous microbial communities rely on strategies based on neural network computing (53,54). Artificial neural networks, also known as *parallel distributed processing*, have an aptitude for comparative assessment and retrieval of experiential information concerning large databases. Protein-profile management techniques should be commensurate with the molecular resolution capacity (Fig. 3). For example, profiles constructed using one-dimensional polyacrylamide gel electrophoresis can be managed with visual or linear image analysis programs, but two-dimensional electrophoresis requires more sophisticated pattern recognition techniques. A profiling database constructed with three-dimensional information, for example, on protein hydrophobicity, ionic charge, and molecular mass, on large numbers of organisms would be facilitated with neural network computing for pattern recognition.

INTERNET RESOURCES FOR ENVIRONMENTAL MICROBIAL PROTEOME ASSESSMENT

A comparative analysis of protein characteristics is central to proteomics research. Therefore, access to databases available on the World Wide Web for protein conformation, sequence, and function are becoming indispensible. In general, web sites for proteome researches specialize in one of six activities, namely, sequence depository and retrieval, comparative analysis of patterns and profiles, metabolic functions, posttranslational modification, two-dimensional maps, and three-dimensional structure databases. Table 1 shows selected web site addresses that are useful for consultation by both beginners and experienced proteomics researchers.

CONCLUSION

Accurate assessment of genetic expression is fundamental to all applications of biotechnology that involve the activities of viable cells. Furthermore, in biotechnological processes with an environmental dimension, the need for consistent analysis of gene expression is paramount because of the extensive range of environmental parameters that are known to influence the transformation of genetic potential to metabolic and ecological advantage. The goals of assessing microbial proteomes in the environment include (*1*) monitoring the expression and activity of specific enzymes or structural proteins in targeted populations that inhabit natural ecosystems, (*2*) identifying specific indicator organisms present in complex heterogenous microbial communities, (*3*) monitoring changes in the population structure of microbial communities as they respond to various environmental stimuli, and (*4*) tracking the tempo and mode of molecular evolution in microbial populations. With imminent technological advancements in protein extraction and analysis and in the robustness of protein characteristics databases, these already ambitious goals will undoubtedly be expanded dramatically to address the increasingly multifaceted research questions in environmental microbiology.

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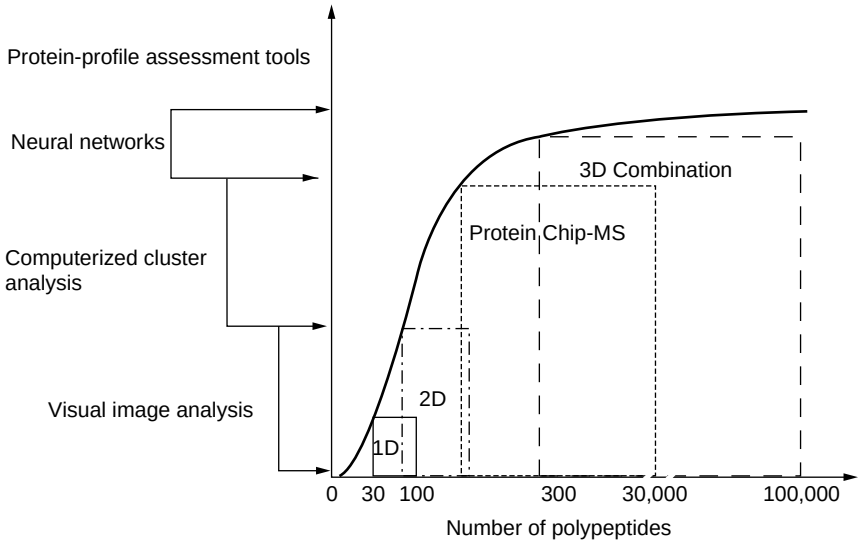

Figure 3. Protein-profile management tool requirement is proportional to the power of molecular resolution. MS = mass spectrometry. 1D, 2D, and 3D refer to one-, two-, and three-dimensional resolution, respectively. The pattern recognition capacity of protein-profile management tools increases linearly on the vertical axis. Techniques higher on the vertical scale can also accommodate tasks performed by lower, less sophisticated techniques.

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ASSIMILABLE ORGANIC CARBON (AOC) IN DRINKING WATER.

See BIODEGRADABLE DISSOLVED ORGANIC CARBON IN DRINKING WATER

ASSIMILABLE ORGANIC CARBON (AOC) IN TREATED WATER: DETERMINATION AND SIGNIFICANCE

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CONTROLLING MICROBIOLOGICAL WATER QUALITY

The quality of drinking water at the consumer's tap depends on a chain of processes, namely, the effects

of water treatment on the raw water, the effects of distribution on the composition of treated water, and the effects of the household plumbing system, respectively. Water quality criteria related to health are defined in national and international regulations and the water industry has the obligation to provide a safe drinking water. Microbiological safety is of major importance because ingestion of pathogens can have a direct impact on the health of the consumer. For this reason, multiple barrier approaches are used in water treatment in combination with frequent monitoring of indicator bacteria in treated water and pathogens (protozoa and viruses) in raw water, respectively. A multiple barrier approach is also used to ensure the safety of drinking water in the distribution system (1). Barriers against contamination of drinking water in the distribution system include (*1*) a constant high pressure in the mains, (*2*) cross-connection prevention measures, (*3*) safety procedures for activities in the distribution system, (*4*) reliable materials and construction techniques. Also, appropriate water quality monitoring is important to check the effectiveness of these preventive measures. Maintaining a disinfectant residual in drinking water during distribution is considered as an additional safety factor (2), but in a few European countries it is allowed to distribute drinking water without disinfectant residual (3–5). The main disadvantages of maintaining a disinfectant residual are the effect on taste and odor of the water, and/or the formation of disinfection by-products, including trihalomethanes with toxic properties (6–9).

A main function of the disinfectant residual is the prevention of multiplication of microorganisms in drinking water distribution ("regrowth"). Controlling regrowth is important to the water industry for the following main reasons (*1*) multiplication of coliform bacteria results in noncompliance with regulations (10), (*2*) the multiplication of opportunistic pathogenic bacteria, including species of *Legionella*, *Mycobacterium*, *Pseudomonas, Aeromonas*, and *Flavobacterium* poses a potential health threat to the consumer (11–15), (*3*) heterotrophic plate count values may exceed those defined in national legislation and may cause problems in the food industry, (*4*) microbial activity and biomass may affect turbidity, color, taste, and odor of the water (16), (*5*) microbiological processes may accelerate corrosion (Microbiologically Induced Corrosion, MIC) of pipe materials (17,18), (*6*) invertebrates multiplying on bacterial biomass may cause consumer complaints (19,20).

In the absence of a disinfectant residual regrowth is controlled by distributing biologically stable water, as obtained by a far-reaching removal of biodegradable compounds from water during treatment (21). Assessment of the degree of biological (in)stability requires a method for determining the concentration of growth-promoting compounds in water or the microbial growth potential. This entry describes the assimilable organic carbon (AOC) method and its significance for defining biological stability.

REGROWTH PROCESSES AND GROWTH KINETICS

Regrowth is a complex phenomenon affected by many variables. Key processes in regrowth are the uptake of biodegradable compounds by microorganisms, formation of biofilms on the exposed surface detachment of (micro-)organisms and dead biomass from the pipe wall and accumulation of sediments, respectively. The presence of biodegradable compounds is a major driving force in these regrowth processes but other environmental conditions (e.g., temperature, system hydraulics, and retention time) and physicochemical processes (adsorption, oxidation/reduction, and coagulation/sedimentation) also have significant effects on the extent to which microorganisms multiply. When a disinfectant is added to water before distribution, the situation is even more complex because the concentration of disinfectant generally changes in time and differs among various locations in the system (22).

Bacteria present in the biofilm are released into the water and may be detected in routine monitoring of the microbiological quality of drinking water. Most bacteria predominating in regrowth processes do not seem to constitute a direct hygienic or aesthetic problem (23,24), but their ability to produce biomass under the prevailing conditions is a main cause of the enables Parts of the biofilm sloughing from the surface may settle in pipes at low flow conditions and contribute to the formation of sediments. Biofilms and sediments of decaying biomass are niches for undesirable bacteria including coliforms (25), aeromonads (12), *Legionella* spp. (13,15), *Mycobacterium* spp. (11,14), but also fungi and yeasts may be present (26). Certain invertebrates use biofilms and sediments as a food source (19,20). (See also this Encyclopedia Invertebrates and protozoan in drinking-water distribution systems, Van Lieverloo et al.).

The rate and extent of multiplication of microorganisms in water depend on the concentration and the composition of the available food sources. These food sources provide energy and the elements needed for the biomass synthesis. Heterotrophic bacteria utilize organic compounds as a source of energy and carbon. In addition, certain inorganic compounds are required, in particular nitrogen and phosphorus, and a number of other elements at much lower amounts, for example, sulfur. The need for carbon, nitrogen, and phosphorus can be derived from the gross composition of microbial biomass, $C_5H_7NO_2P_{1/30}$, and the proportion (50%) of organic carbon used for dissimilation (27). Hence, organic compounds serving as a source of energy and carbon are needed in much larger amounts than the inorganic nutrients nitrogen and phosphorus ($C : N : P = 100 : 10 : 1$). Nitrate, a common compound in drinking water, is a good nitrogen source for many aquatic bacteria, although certain types (e.g., *Aeromonas* spp.) require ammonia-nitrogen for growth (28). Phosphate is also a common compound in the aquatic habitat, and it is only needed in very low quantities relative to the carbon source. Hence, the availability of suitable organic compounds will be the growth-limiting parameter for heterotrophic bacteria in most water types. Some studies suggest that phosphate may be growth limiting in certain types of groundwater, which contain a relatively high concentration of organic compounds (29).

The amount of biomass produced on a certain amount of substrate depends on the yield (Y) values of the bacteria for the present compound(s). Generally, 1 mg of substrate

carbon yields 1 mg of biomass (dry weight) during aerobic growth (27). Expressing the yield value as number of bacteria (colony forming units, CFU) is more appropriate for describing regrowth processes. Typical yield values for compounds serving as favorable growth substrates for heterotrophic bacteria (low molecular organic compounds) of 4×10^6 to 2×10^7 CFU have been reported (see later). Hence, utilization of 1 μg of C/L theoretically corresponds with a maximum colony count value of 10^4 CFU/mL. The substrate concentration also affects the rate of growth. This relationship is described by the Monod equation (30):

$$V = V_{max} \times S/(K_s + S) \quad (1),$$

where: V = the growth rate (doublings/hour) in the exponential growth phase at substrate concentration S; V_{max} = maximum growth rate (h^{-1}); K_s = the substrate saturation constant, is the concentration of S at which $V = 1/2V_{max}$. An example of such a relationship is presented in Figure 1, which clearly demonstrates that the increase of the growth rate is less than proportional to the increase of the substrate concentration. However, at very low values for S, that is, $S \ll K_s$ then:

$$V = V_{max}/K_s \times S \quad (2)$$

and the growth rate is linearly related with the substrate concentration S and also depending on the values for V_{max} en K_s, which are constants for specific combinations of organism and substrate. The quotient V_{max}/K_s ($h^{-1} \ldots \mu g^{-1} \ldots L$, the substrate affinity constant is numerically identical to the growth rate calculated for a substrate concentration of 1 μg C/L.

Bacteria representing the indigenous bacterial flora of drinking water have K_s values for easily degradable low molecular weight compounds as low as a few micrograms of C/L. Relatively low K_s values have been also been observed for the high molecular weight compounds amylopectin and amylose (Table 1). *Escherichia coli*, coliforms, and also *P. aeruginosa* have higher K_s values than the typical aquatic bacteria for identical substrates. Still, the K_s values of 13 to 72 μg of glucose-C/L (31,32), and 50 to 150 μg of yeast extract/L (38) as reported for *E. coli* are also relatively low. Despite these kinetics, *E. coli* and *P. aeruginosa* do not belong to the indigenous bacterial community of drinking water. This may be due to the relatively slow growth of these bacteria at temperatures below 15 °C (34) and the very low concentrations of easily available compounds in treated water.

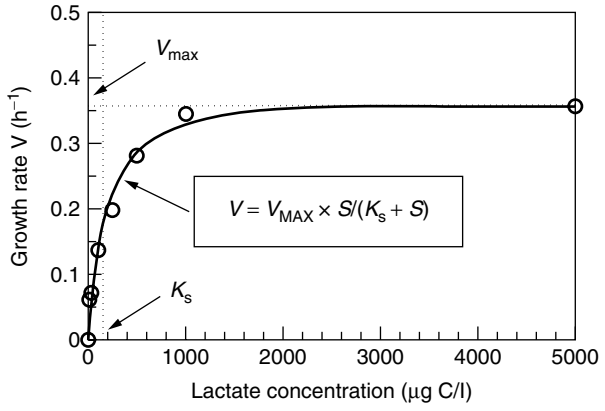

Figure 1. The growth rate of an *Escherichia coli* strain (isolated from river water) at various concentrations of lactate in autoclaved slow sand filtrate ($+PO_4^{3-}$P and NH_4^+-N) incubated at 25 °C. The constants of the Monod relationship (K_s and V_{max}) are given in Table 1.

As a result of maintenance energy requirements, a certain minimum substrate concentration (S_{min}) is required for survival without growth (35). Typical maintenance rates for bacteria due to endogenous substrate utilization range from 0.005 to 0.02 h^{-1} (36), but a much higher value (0.3 h^{-1} at 37 °C) has been reported for *E. coli* (31). The values for the substrate affinity of a number of bacteria presented in Table 1, are below the value of maintenance rate. Consequently, these bacteria are not able to grow at a concentration of 1 μg of C/L of the specified compounds. In practice the situation is more complicated because in most situations, several compounds will be present. Most of the bacteria described in Table 1 are able to utilize a number of substrates simultaneously when present at an individual compound concentration of 1 μg C/L. A mixture of amino acids stimulated the multiplication of *A. hydrophila* at an individual compound concentration as low as 0.05 μg C/L (28).

The growth kinetics as presented in Table 1 demonstrate that low concentrations of easily available organic compounds can cause a rapid and significant growth of bacteria present in treated water. For this reason, the method for assessing the regrowth potential of treated water as developed in the Netherlands was mainly directed at determining the concentration of such compounds.

Assessment of the Microbial Growth Potential of Treated Water

Already at the end of the nineteenth century, studies were conducted on bacterial multiplication in treated water (44). It was observed that the number of culturable bacteria was much higher in stored samples of a high-quality deep-well water with a low concentration of organic compounds (up to 5×10^5 CFU/mL) than in river water samples (4,300 CFU/mL). The assumption was made that the well water obtained from the chalk contained a relatively high concentration of biodegradable compounds because of the absence of bacteria in this water directly after abstraction. On the other hand, it was known at that time that the relatively strong growth in treated water stored in flasks with a cotton was related to compounds present in the air. Also, the difference in growth between well water and river water was most probably the result of the multiplication of a few types of bacteria contributing to the HPC values in well water, whereas many of the bacteria present in river water were not able to grow on a solid medium. These observations show that determining the growth potential using colony counts of the indigenous bacterial community has a number of limitations. In the Netherlands, Beijerinck (45) suggested in 1891 that the growth promoting properties of drinking water be studied by inoculating selected pure cultures in sterilized

Table 1. Growth Kinetics of Bacteria Isolated from Drinking Water*

Organism	Compound	Temp. (°C)	K_s (μg C/L)	V_{max} (h^{-1})	V_{max}/K_s (h^{-1}). μg^{-1}.L	Reference
Aeromonas hydrophila	Acetate	15	11	0.15	0.013	28
Aeromonas hydrophila	Glucose	15	16	0.28	0.018	28
Aeromonas hydrophila	Amylose	15	93	0.26	0.0028	37
Aeromonas hydrophila	Oleate	15	2.1	0.23	0.109	28
Citrobacter freundii	Glucose	15	95	0.17	0.0018	38
Enterobacter sp.	Glucose	15	60	0.21	0.004	38
Escherichia. coli	Lactate	25	142	0.37	0.0026	This paper
Flavobacterium sp.	Glucose	15	3.3	0.21	0.063	39
Flavobacterium sp.	Glucose	15	109	0.15	0.001	40
Flavobacterium sp.	Maltose	15	23.7	0.37	0.016	40
Flavobacterium sp.	Maltopentaose	15	5.7	0.44	0.077	40
Flavobacterium sp.	Amylose	15	26	0.50	0.020	40
Flavobacterium sp.	Amylopectin	15	11	0.48	0.044	40
Klebsiella pneumoniae	Maltose	15	51	0.49	0.0096	41
Klebsiella pneumoniae	Maltopentaose	15	92	0.41	0.0045	41
Pseudomonas fluorescens	Acetate	15	4.	0.18	0.045	42
Pseudomonas fluorescens	Glucose	15	57	0.22	0.004	42
Pseudomonas aeruginosa	Acetate	15	28	0.09	0.003	34
Spirillum sp.	Oxalate	15		0.24	0.016	43

Note: * growth measurements conducted in slow sand filtrate

samples of the water that was to be tested. He also pointed out the necessity to prevent contamination by volatile compounds. However, no results were presented and it seems that this method did not attract much attention. In 1928 a completely different approach was reported by Heymann (46) who determined the effect of a series of passages of water through sand columns on the concentration of organic compounds, measured as permanganate value. However, this method also did not gain wide application.

Interest in the regrowth phenomenon strongly increased in the Netherlands in the 1970s as a result of the introduction of ozonation and granular activated carbon (GAC) filtration in water treatment for the removal of persistent organic pollutants. In 1978 a method for determining the heterotrophic growth potential of treated water was described, which was based on determining the maximum level of growth of a selected pure culture in samples of water collected and contained in thoroughly cleaned glass-stoppered Erlenmeyer flasks (47,48). Subsequently, this method, which was designated as the AOC method, was improved by including a bacterial strain with the ability to utilize certain carboxylic acids, as produced by ozonation (49). Regrowth phenomena gained also attention in other European countries and in the United States, and a series of methods for assessing the growth potential of drinking water have been developed (Table 2).

Table 2. Characteristics of Methods for Assessing the Microbial Growth Potential of Treated Water

Method*	Mode	Organisms	Parameter	Reference
AOC	Batch	Pure culture	CFU	43,47,48,53
AOC	Batch	Indigenous bacteria	ATP	54
AOC	Batch	Pure cultures	ATP	55
BGP	Batch	Indigenous bacteria	Turbidity	56
BDOC	Batch	Indigenous bacteria	DOC	57,58,59
BDOC	Column	Indigenous bacteria	DOC	60,62
BFR	Column	Indigenous bacteria	ATP	5,51,52,62

*AOC, easily Assimilable Organic Carbon; BGP, Bacterial Growth Potential; BDOC, Biodegradable Dissolved Organic Carbon; BFR, Biofilm Formation Rate.

Batch tests are most commonly used because such test can be conducted under controlled conditions in the laboratory. These tests give information about the growth potential but the test conditions do not reflect the processes occurring in the distribution system. Another limitation of the AOC and BDOC tests is that the growth potential of treated water may also be affected by the presence of inorganic compounds (e.g., ammonia and sulfides) and methane, which are not included in these tests. A variety of techniques and devices is available to simulate the biofilm formation as occurring in distribution systems, for example, Rototorque system (49), Robbins device, coupon test (50), and a biofilm monitor (51,52). A combination of growth tests, determining the (effects of) concentrations of rapidly and more slowly available compounds, as well as chemical analysis (e.g., for ammonia and methane), may be needed to assess the biostability of treated water. In the Netherlands biostability assessment of water is conducted by using the AOC test and the BFR test (5).

AOC METHOD

Principle of the Test

As explained earlier, easily biodegradable organic compounds may cause significant growth at a level of a few micrograms per liter. Therefore, a test was developed aiming at determining low concentration of these compounds, which were designated as assimilable organic carbon (AOC), derived from earlier descriptions where the terminology "assimilable compounds" was used (46). Assessment of the concentration of easily assimilable

organic carbon (AOC) is based on growth measurements of a mixed culture of two select bacterial strains in a sample of pasteurized water collected and contained in a thoroughly cleaned glass-stoppered Erlenmeyer flask. On the basis of the maximum colony counts of these organisms, the original concentration of substrates is calculated using the yield values of the bacteria for acetate. Hence, the AOC concentration is expressed as μg of acetate-carbon equivalents/L.

Test Strains

The strains used in the AOC test are *P. fluorescens* strain P17, which is capable of utilizing a wide range of low molecular weight compounds at very low concentrations (42) and a *Spirillum* sp. strain NOX, which only utilizes carboxylic acids (43). The general properties of these organisms are described in Table 3, together with another organism specialized in the utilization of carbohydrates (63).

P. fluorescens strain P17 originates from tap water and belongs to one of the most commonly occurring biotypes of the fluorescent pseudomonads in drinking water (64). Growth measurements conducted in drinking water supplemented with mixtures of compounds revealed that the organism can utilize most or all amino acids, a number of carboxylic acids, some carbohydrates and a number of aromatic acids, at a concentration of 1 μg of C/L when present as a mixture (Fig. 2a; 42). In addition, the organism requires a simple nitrogen source and multiplies rapidly on agar media. For these reasons, strain P17 was selected for determining the concentration of easily AOC in (drinking) water (48). The yield factor of strain P17 for acetate-carbon (4.1×10^6 CFU/μg carbon) is used for calculating the AOC concentration from the maximum colony counts (N_{max}, CFU/mL) obtained in the water types tested.

Spirillum species strain NOX had been isolated from slow sand filtrate enriched with 25 μg C/L of oxalate, glyoxylate and formate, respectively (43). The strain is specialized in the utilization of carboxylic acids, including formate, oxalate, glycollate and glyoxylate, which are not used by strain P17. Growth tests conducted with mixtures of substrates with an individual compound concentration of 1 μg of C/L clearly revealed that only carboxylic acids were utilized at this low concentration (Fig. 2b). As a result of its preference for carboxylic acids, the N_{max} value of strain NOX gives information about the concentration of carboxylic acids in the water tested. Yield factors have been determined for acetate and for oxalate, respectively. The yield (CFU/μg of carbon) of strain NOX for acetate is about four times higher than for oxalate-carbon. This difference can be explained by the low energy content of oxalate (27). The organism is used in a mixed culture strain P17 for determining the concentration of easily assimilable organic carbon (AOC) in (drinking) water.

Flavobacterium species strain S12 was obtained from slow sand filtrate incubated at 15 °C after enrichment with 100 μg of starch-C/L (63). The organism is specialized in utilizing carbohydrates, including amylose, amylopectin, maltose, and maltodextrins, which are not used by strain P17 nor by strain NOX. Growth tests conducted with mixtures of substrates with an individual compound concentration of 1 μg of C/L clearly revealed that only certain carbohydrates were utilized at this concentration. N_{max} values of strain S12 therefore give information about the concentration of maltose- and maltodextrin-like compounds in the water tested. Yield factors of strain S12 for starch, maltose, and maltodextrins range from 2.0×10^7 to 2.3×10^7 CFU/μg C (40). Concentrations of

Table 3. Properties of *P. fluorescens* Strain P17, Spirillum sp. Strain NOX, and Flavobacterium sp. Strain S12, Respectively (42,43,63)

Characteristic	*P. fluorescens* strain P17	*Spirillum sp.** strain NOX	*Flavobacterium* sp. strain S12
Origin	Tap water	Slow sand filtrate	Slow sand filtrate
Shape	Rod	Curved rod	Rod
Motility	+	+	−
Gram stain	−	−	−
Oxidase	+	+	+
N-sources	NO_3/NH_4	NO_3/NH_4	NO_3/NH_4
Max. growth temperature (°C)	30	30	30
Arginine dihydrolase	+	+	−
Denitrification	+ (N_2)	−	−
O/F test with glucose	+/−	−/−	−/−
Hydrolysis of:			
− Gelatin	+	−	−
− Tween-80	+	−	−
− Starch	−	−	+
− Chitin	−	−	−
Preferred substrates	Versatile organism	Carboxylic acids	Carbohydrates

*a recent 16SRNA sequencing analysis indicated that the organism is related to the genus *Ultramicrobium* (unpublished observation).

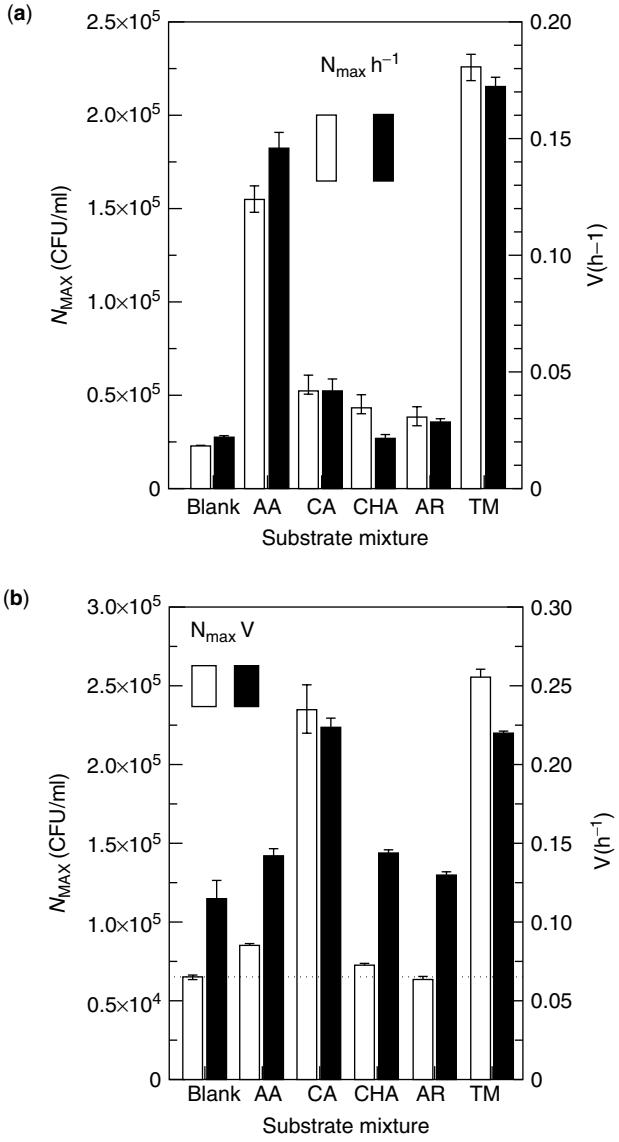

Figure 2. (**a**) Maximum colony counts and growth rates of *P. fluorescens* strain P17 in the presence of mixtures of compounds at an individual compound concentration of 1 μg C/L. Blank: slow sand filtrate without added substrate; AA, 19 amino acids; CA, 14 carboxylic acids; CHA, 6 carbohydrates/alcohols; AR, 7 aromatic acids; TM, total mixture (46 μg C/l). Error bars indicate duplicate measurements. Data adapted from Fig. 3. (**b**) Maximum colony counts and growth rates of *Spirillum* sp. strain NOX in the presence of mixtures of compounds at an individual compound concentration of 1 μg *C*/*L*. Error bars indicate duplicate measurements.

carbohydrates available for strain S12 in drinking water usually are very low (<1 μg of C/L) and therefore, the organism is not included in the test for determining the concentration of easily assimilable organic carbon (AOC) in (drinking) water. In certain water types however, a significant concentration of carbohydrates may be present, for example, when algal growth occurred in the raw water and/or when a starch-derived coagulant aid is used in water treatment (40).

Other strains can be used for growth measurements to determine the utilization of present or added substrates or to determine the growth potential of the water for a specific organism. Typical examples of such strains include *Aeromonas* species (28), representatives of the coliform group, including *E. coli* (65,66; Fig. 1) and also *P. aeruginosa* (43).

Test Conditions

Sampling and transportation of the samples and sample treatment are essential to obtain an accurate AOC value. For this reason, representative samples of the water to be investigated are collected in duplicate in thoroughly cleaned (including heating at 550 °C for four hours) Erlenmeyer flasks. In these flasks the water samples are transported, pasteurized, and incubated, respectively. Hence, contact with surfaces and air is restricted as much as possible. Pasteurization aiming at inactivating the indigenous bacterial community as present in the water samples is conducted by placing the flasks in a water bath at 90 °C. The flasks are removed from the water bath when water temperature has reached 60 °C and subsequently are placed in an incubator at 60 °C for 30 minutes. After cooling with cold tap water, the test strains are added to

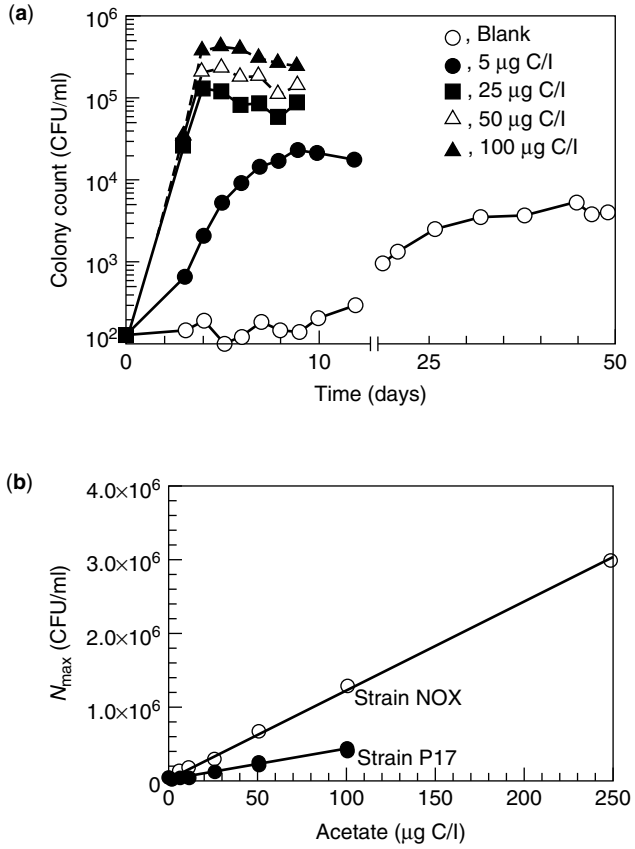

Figure 3. (**a**) Growth curves of *P. fluorescens* strain P17 in pasteurized slow sand filtrate (DOC = 2.3 mg/L) supplemented with different acetate-C concentrations (at 15 °C); (**b**) calibration curves of strain P17 and strain NOX for acetate. Yield values are presented in Table 4.

the flasks. Cultures of the test strains grown in tap water at an initial concentration of 1 mg of acetate-C/L, in which the maximum colony count had been reached are used as inoculum. The inoculum gives is about 50 to 500 CFU/mL in the pasteurized samples. The flasks are incubated at $15 \pm 1\,^{\circ}C$ in the dark, without shaking.

Membrane filters have been used to remove the indigenous bacteria from the samples. However, organic compounds can release from: (*1*) the filters, (*2*) the equipment used for membrane filtration, and (*3*) the flasks in which the filtered water is collected. Furthermore, membrane filtration does not always result in sterile samples because of the presence of very small bacterial cells (ultramicrobes). Hence, application of membrane filters may give unreliable results. Also, autoclaving is not appropriate because it causes a strong increase (three- to fourfold) in the AOC concentration, depending on the water type (unpublished results).

In certain samples, detoxification of the water is required, for example, in the presence of a disinfectant residual, or in the presence of copper. Growth measurements with thiosulphate, used to neutralize a disinfectant residual showed an AOC increase of about 5 μg C/L at a concentration of 10 mg/L. EDTA and NTA do not affect the AOC concentration as determined with strains P17 and NOX (unpublished results). The use of NTA is preferred because this compound is more easily biodegradable in the environment than EDTA.

Growth Measurements and Calibration Curves

The growth curve is determined by periodic colony counts for which purpose water volumes of 0.05 mL are spread in triplicate over the surface of agar medium plates, followed by incubation at 25 °C for 2 (P17) to 3 (NOX) days. A broth agar medium (Lab Lemco Agar, Oxoid) is used routinely, but the R_2A medium 67 has the same recovery for strain P17 and strain NOX (data not shown). Colonies of strain P17 are larger than those of strain NOX and produce a fluorescent pigment on R_2A medium.

The yield (Y) values of strain P17 and strain NOX for acetate are needed to calculate the AOC concentration from the maximum colony counts. Typical growth curves of strain P17 in slow sand filtrate supplemented with different acetate concentrations are presented in Figure 3. N_{max} values were reached after five to seven days at acetate concentrations greater than or equal to ≥ 10 μg C/L, and after about 10 to 14 days at an initial acetate-carbon concentration of 5 μg C/L. Similar experiments have been conducted with strain NOX 43. The relationship between N_{max} values and the acetate concentrations is used for calculating the yield values (Fig. 3b) At acetate-carbon concentrations above 100 μg C/L, the N_{max} value of strain P17 remains below the level expected on the basis of the values observed at lower concentrations (data not shown). This is probably because the cells are larger when grown at higher substrate concentrations. This phenomenon has not been observed with strain NOX. Yield values of a number of test strains are given in Table 4.

AOC concentrations are determined using a mixed inoculum of strain P17 and strain NOX. The AOC concentration is determined from the N_{max} values of each of the strains, using their *Y* value for acetate (Fig. 4). Next to the AOC concentration, information also is obtained about the AOC composition because strain NOX can only grow on carboxylic acids (40).

Figure 5 shows the contributions of strain NOX and strain P17 to the AOC concentration when grown simultaneously in pasteurized slow sand filtrate supplemented with yeast extract. Growth of strain NOX was not enhanced by the addition of yeast extract. The *Y* value of strain P17 for yeast extract is 0.35 μg AOC/μ g YE.

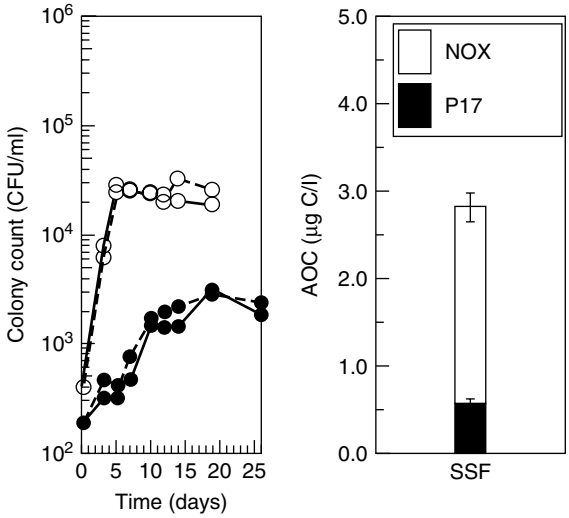

Figure 4. Growth curves of test strains P17 and NOX in slow sand filtrate (SSF) and the AOC value calculated from the N_{max} values of the strains. In these samples the concentration of AOC compounds utilized by strain P17 was less than 1 μg C/L.

Table 4. Yield Values of a Number of Bacterial Strains Used in Growth Experiments (28,40,42,43,48)

Organism	Y value (CFU/μg C) Acetate	Oxalate	Glucose	Starch	Lactate
P. fluorescens strain P17	4.1×10^6	No growth	Nd*	No growth	Nd*
Spirillum sp. strain NOX	1.18×10^7	2.9×10^6	No growth	No growth	Nd*
Flavobacterium strain S12	No growth	No growth	1.8×10^7	2.2×10^7	No growth
A. hydrophila strain M800	6.8×10^6	No growth	8.0×10^6	Nd*	Nd
Escherichia coli strain 8872**	Nd*	Nd	4.5×10^6	Nd*	6×10^6

Note: *nd, not determined; **tested at 25°C, see Fig. 1.

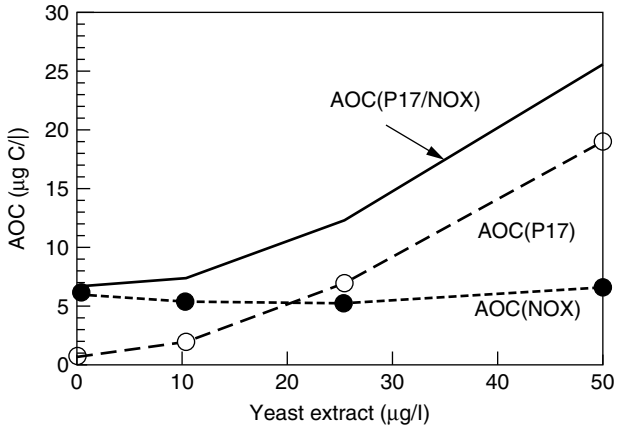

Figure 5. AOC concentrations at various concentrations of yeast extract added to slow sand filtrate. AOC-P17 is the AOC concentration derived from the N_{max} values of strain P17 grown in the presence of strain NOX; AOC-NOX is the AOC concentration calculated from the N_{max} values of strain NOX. AOC-P17/NOX is the total AOC concentration.

Effect of Separate Growth of Test Strains

Both strain P17 and strain NOX are able to utilize carboxylic acids. In a mixed culture, there is competition for these substrates. When grown separately, compounds available to the two strains are used by both strains. Figure 6 shows the AOC concentrations determined in two different water types by the individual strains and by growth as a mixture, respectively. In both water types, the AOC concentration mainly consists of compounds available to strain NOX (carboxylic acids). From these results, it can be calculated that 45 to 66 % of the compounds potentially available to strain P17 were utilized by strain NOX when grown as a mixed culture. In addition, a small fraction of the compounds available to strain NOX is utilized by strain P17 when grown as a mixed culture.

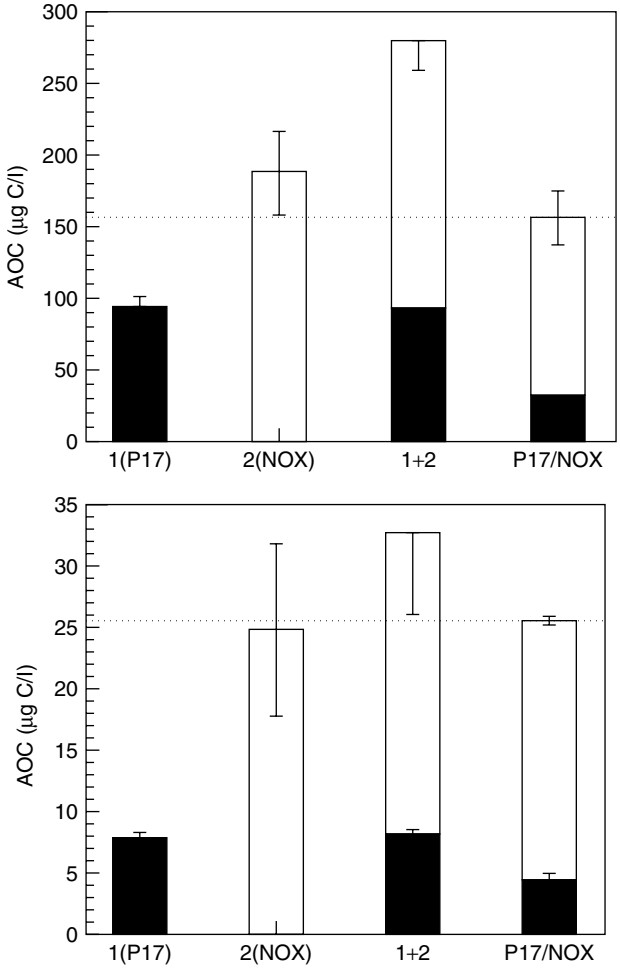

Figure 6. Effect of separate growth of the test strains in two water types. (**a**) water after ozonation; (**b**) ozonated water after GAC filtration. 1(P17), strain P17 alone; 2(NOX) strain NOX alone; $1+2$, AOC calculated from 1 and 2; P17/NOX, strains P17 and NOX grown as mixed culture. Error bars give standard deviations.

Reproducibility and Repeatability of AOC Determinations

A large number of AOC determinations have been conducted since the development of the method. In all cases, the test was done in duplicate sample flasks. Hence, information is available about the reproducibility of the test. Figure 7 shows the Relative Standard Deviations (RSD, %) of a total of 430 AOC tests, conducted within a one-year period, most of which with an AOC value below 25 µg C/L. The STD values were below 10% for 60% of all samples tested. In 10% of the samples STD values were greater than 35% (Fig. 7b). The repeatability of the test is shown by the constant yield values of the test strains for acetate and the constant AOC values obtained for one particular slow sand filtrate over a period of more than 15 years (with values <5 µg C/L) (results not shown).

Comparison of Described AOC Procedures

The AOC test as described earlier, has been adapted by a number of researchers to improve its use as a monitoring tool (55,60). A major adaptation is the use of small-capped containers, in combination with a single measurement in a container (in triplicate) after a defined incubation period. Table 5 compares the conditions of the AOC test as described in standard methods (6g) with the AOC test that was described earlier. A further adaptation to the procedure described in standard methods is the use of ATP for determining the growth of the test strains. With the ATP analysis, the AOC test can be conducted within two to four days (55).

AOC CONCENTRATIONS IN WATER

AOC Concentrations in Raw and in Treated Water

The AOC test had been developed for assessing the biostability of treated water. However, it is also used in raw water and in water in various treatment stages. A problem observed with the use of the AOC test in raw water is the presence of spore-forming bacteria

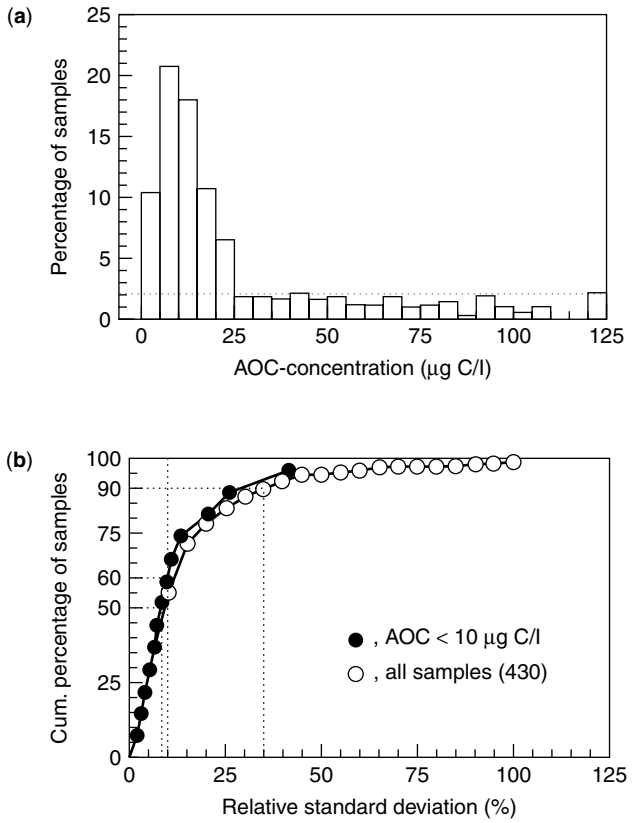

Figure 7. (**a**) AOC concentrations as measured in 430 samples and (**b**) the relative standard deviation (RSD) of the AOC concentrations (relative to the average value of duplicate measurements).

or other types of bacteria surviving the pasteurization procedure. These organisms hamper the AOC test either by growing on the plates and/or by growing in the water samples and thus consuming an unknown part of the substrates potentially available to the test strains. Pasteurization at a higher temperature (e.g., 70 °C for one to two hours) can be applied to eliminate this problem.

Typical AOC concentrations in river (Meuse) water ranged from about 50 µg C/L to 400 µg C/L, depending on the season, with the highest concentrations in spring and in late summer (Fig. 8a). About 70 to 90% of these AOC concentrations was available to strain P17, indicating that the substrates available to strain P17 (e.g., amino acids, peptides, carbohydrates, etc.) were present in much higher concentrations than the carboxylic acids. The concentration of dissolved organic carbon (DOC) was 3.2 to 4.2 mg/L and the AOC concentrations were 1.5 to 10% of the DOC concentration.

In a survey conducted in 1986 to 1987 (53), AOC concentrations observed in drinking water in the Netherlands ranged from 1 to about 60 µg acetate-carbon equivalents per liter (Fig. 8b). The highest values were observed in surface-water supplied with ozonation a treatment ste. In all types of treated water the fraction available to strain NOX was the largest proportion of the AOC concentration, indicating that carboxylic acids were the predominating compounds. The AOC concentration utilized by strain P17 was less than 1 µg C/L in most samples of treated water. The data presented in Figure 8b and many observations conducted since then clearly demonstrated that the AOC concentrations in treated groundwater water in the Netherlands usually are below 10 µg of C/L. Also, in surface water supplies with slow sand filtration as the final treatment step such low AOC concentrations were found.

AOC concentrations in treated water are only a small fraction (<1.7%) of the DOC concentration (Fig. 9). The lowest AOC/DOC ratios have been observed in groundwater supplies, in which AOC concentrations usually were below 10 µg of C/L. Minimum values for the AOC/DOC quotient were all close to 0.1%.

AOC values in treated water observed in the Netherlands are low in comparison to values reported in other countries. Several surveys conducted in the United States revealed that AOC concentrations in treated water

Table 5. Comparison of AOC Procedures

Test Characteristic	AOC (Original Method)	AOC in Standard Methods (69)
Containers	Borosilicate glass Erlenmeyer (1 L); glass stoppered	Borosilicate glass vial (45 mL) with TFE-lined silicone septa
Sample volume	600 mL	40 mL
Surface/volume (cm^{-1})	0.63 (equivalent to pipe diameter of 6 cm)	1.5 (equivalent to pipe diameter of 2.7 cm)
Test strains	P17 and NOX	P17 and NOX, separate growth
Biomass parameter	Colony count	Colony count or ATP (55)
Sample treatment	30 minutes at 60°C	30 minutes at 70°C
Addition of chemicals	none	Thiosulphate (50 mg/L)
Incubation period	≥14 days	9 days
Calculation of AOC	N_{max} values in duplicate flasks	Average of colony counts in triplicate vials on days 7,8, and 9
Result (units)	µg of acetate-c equivalents/L	µg of acetate-C equivalents/L

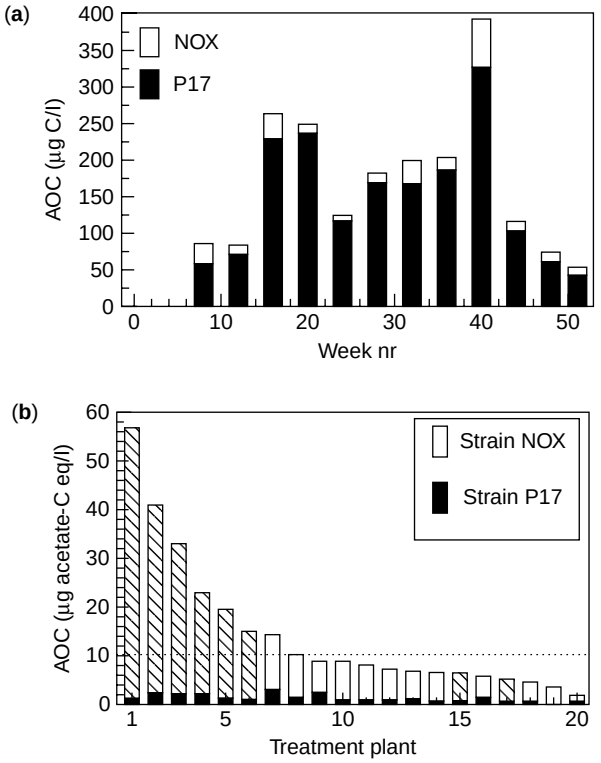

Figure 8. (**a**) AOC concentration in river water in a one year period. (**b**) AOC concentrations in treated water of 20 different treatment plants. Hatched bars represent treated surface water; open bars represent treated groundwater. Groundwater was treated without chemical disinfection. Nrs. 15 and 17 are slow sand filtrates. Adapted from D. Van der Kooij, *J. Am. Water Works Assoc.* **84**(2), 57–65 (1992).

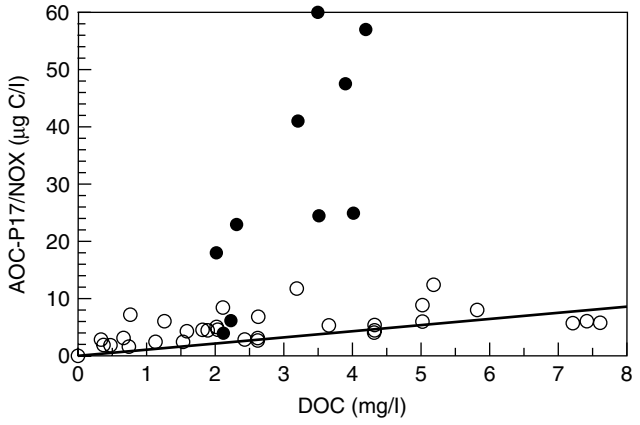

Figure 9. AOC concentrations in treated water as a function of the DOC concentration. Open circles: groundwater supplies; closed circles: surface water supplies. Line shows Lowest AOC/DOC ratios. Data (adapted) from D. Van der Kooij, *J. Am. Water Works Assoc.* 84(2), 57–65 (1992).

ranged from about 20 to 200 or more than 300, respectively (70,71). Median AOC values observed in these studies were about 100 µg C/L. In one of these surveys, the fraction of AOC available to strain P17 exceeded 50 µg C/L in a number of water types. Hence, AOC values in treated water in the United States are much higher than those in treated water in the Netherlands. These differences in AOC levels may be due to differences in water treatment (less biological treatment and addition of chlorine). Also, the AOC test as used in the United States is not identical to the one described in this report (Table 5), but a few comparisons between the test methods did not show large differences between the results (72). Still, the separate growth of the test strain will have an effect in certain water types (cf. Fig. 6).

Effects of Water Treatment

From the differences between the AOC concentration in raw river water and the AOC concentration in treated water, it can be concluded that surface water treatment as applied in the Netherlands can cause an AOC reduction of more than 90%. A variety of processes have different effects on the AOC concentration Fig. 10. The AOC concentration is reduced by biological processes in filter beds when these processes are applied properly and also during soil passage physicochemical processes such as coagulation/sedimentation and adsorption also can reduce the AOC concentration of water. On the other hand, oxidation processes clearly cause an increase of the AOC concentration. The effect of ozonation is related to the degradation of large molecules of natural organic compounds, such as fulvic and humic acids, into low molecular compounds, as has been demonstrated in studies, using advanced chemical analytical techniques. AOC concentrations increase with increasing ozone dosage (74,75,76).

The effect of biological filtration processes on the AOC concentration is of major importance in achieving a biologically stable drinking water. In the Netherlands such processes are included in all water treatment plants, either as rapid sand filtration, GAC filtration, slow sand filtration, or soil passage of surface water (river bank filtration, artificial dune recharge). Fig. 11 shows

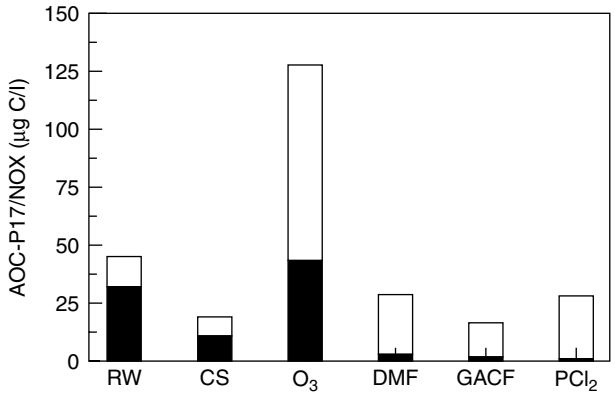

Figure 10. The effects of water treatment on the AOC concentration. RW, raw water river Meuse water after storage in open reservoirs; CS, coagulation/sedimentation; O_3, ozonation; DMF, dual media filtration; GACF, granular activated carbon filtration; pCl2, postchlorination. The water is distributed after postchlorination. Black bar represents the AOC fraction utilized by strain P17.

that the AOC reduction decreases with decreasing AOC concentration in the influent.

From the data shown in this figure, it can be calculated that the AOC reduction in this type of process is negligible at AOC concentrations below 6 μg of C/L. Similar observations have been reported by other investigators (77). Figure 11 also shows that the presence of a chlorine residual in the influent of rapid sand filters decreased the AOC removal, most probably by hampering the biological processes.

Membrane filtration processes have a huge potential in water treatment because of the variety of available membrane types, and the capacity of removing undesirable particles (including bacteria) and dissolved compounds without side-effects on water quality. Ultrafiltration cause an AOC removal up to 84% (from 25 to 3.8 μg C/L) in a surface water treatment, probably by removing particulate compounds of biological origin (78). Reverse osmosis is capable of reducing the AOC concentration to a level below 1 μg C/L. In this water, inorganic nutrients were added to conduct the AOC test (unpublished result 5).

Effects of Distribution

Observations on water sampled from a number of distribution systems have revealed that AOC concentrations may decline rapidly in the distribution system, depending on the initial AOC concentration (Fig. 12). Most likely, biofilm processes play an important role in AOC uptake (53,79). Figures 12 and 13 demonstrate that AOC reduction is very limited at values close to and below 10 μg C/L.

Effect of Storage of Water Samples on AOC and HPC Values

Water samples collected in thoroughly cleaned Erlenmeyer flasks were incubated without pasteurization to obtain information about the effect of the multiplication of the indigenous population on the AOC concentration. Figure 14 shows that the AOC concentration of river water (Fig. 9) collected from a storage reservoir initially followed an exponential decrease. After three days at an AOC concentration of about 10 μg C/L the rate of decrease

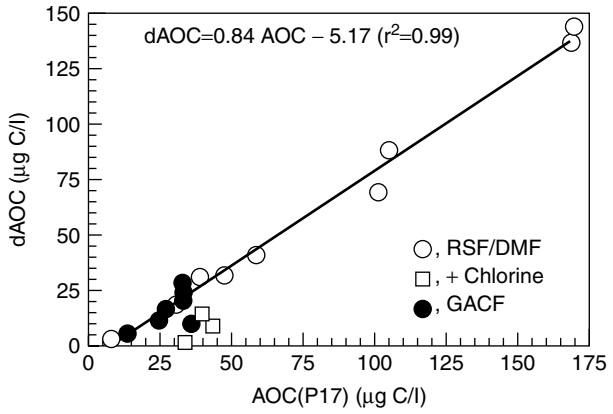

Figure 11. AOC reduction (dAOC) as achieved with biological filtration. RSF, rapid sand filtration; DMF, dual media (anthracite/sand filtration); GACF, granular activated carbon filtration. Adapted from Van der Kooij, 1984 (73).

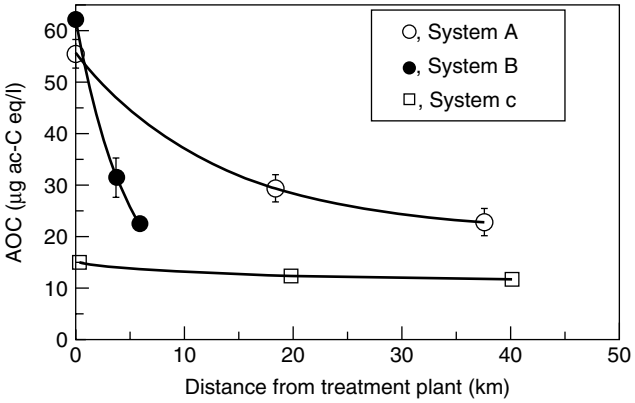

Figure 12. The effect of distribution on the AOC concentration in three different distribution systems. In all cases, ozonation was used as a treatment step. From: D. Van der Kooij, *J. Appl. Microbiol. Symp. Suppl.* **85**: 39S–44S (1999).

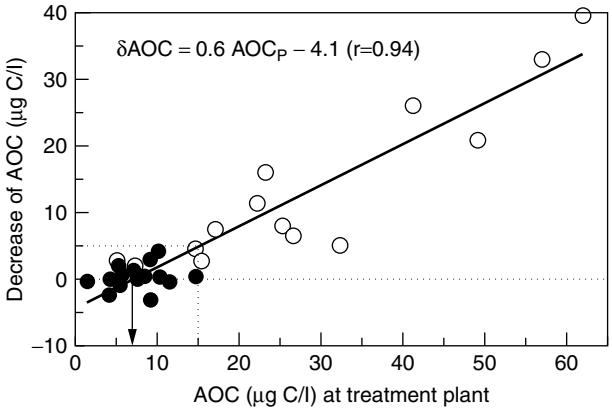

Figure 13. Maximum AOC decrease observed in distribution systems as a function of the AOC concentration of treated water. Open circles: surface water supplies; closed circles: groundwater supplies. Adapted from D. Van der Kooij, *J. Am. Water Works Assoc.* **84**(2), 57–65 (1992).

declined sharply and followed a linear function with time in the test period up to four weeks. In ozonated water, the AOC concentration rapidly declined to a value of 15 μg of C/L. Thereafter the decline became linear with time (Fig. 14(b)). In the stored river water, the fraction of AOC utilized by strain P17 constituted 72% of the initial AOC concentration. In ozonated water the fraction available to strain P17 initially was less than 10% of the total AOC concentration. Obviously, biomass compounds (amino acids, peptides, etc.) predominated in stored river water, whereas carboxylic acids were the major AOC fraction in ozonated water.

The following equations can be used to describe the observed AOC decrease:

Phase 1 (up to five days); exponential decrease with residual:

$$AOC_T = AOC_0 \text{ x } e^{-kT} + AOC_R, \quad (3)$$

Phase 2 (after about 5 days); linear decrease:

$$AOC_T = AOC_5 - Ux(T - 5) \quad (4)$$

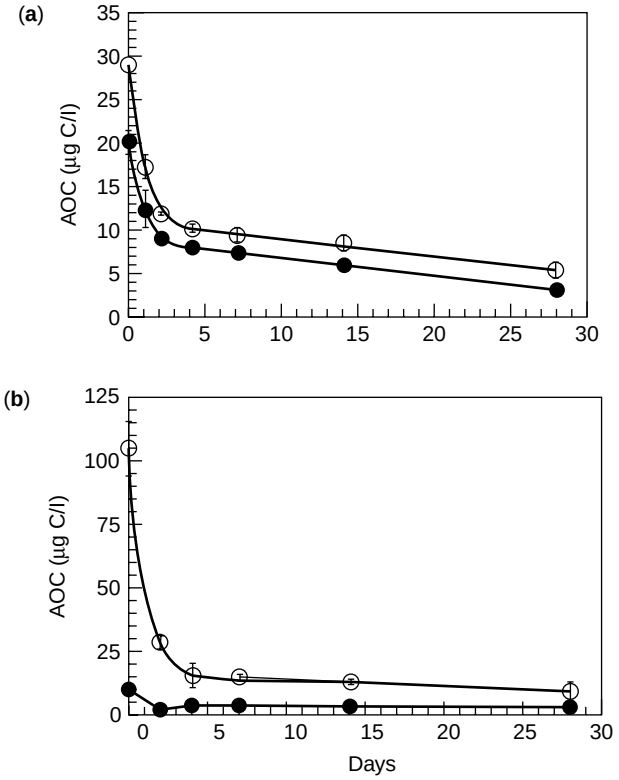

Figure 14. AOC decrease in water samples incubated at 15 °C in thoroughly cleaned Erlenmeyer flasks. (**a**) river water from an open storage reservoir; (**b**) water after ozonation. Symbols: ○, AOC P17/NOX; ●, Fraction of AOC available to strain P17. Error bars give standard deviation of the AOC value (duplicate flasks).

In these equations AOC_T is the AOC concentration at time T, AOC_0, the AOC concentration at the start (zero time), AOC_5 the AOC concentration at day five, and AOC_R, is the apparent residual AOC concentration, respectively. K is the decay constant (d^{-1}) in the exponential phase, and U is the uptake rate ($\mu g \ldots l^{-1} \ldots d^{-1}$) in the linear phase. The constants of the equations for the two water types are given in Table 6.

Simultaneously with the AOC decrease, the numbers of bacteria increase in the water samples during incubation. This increase can be determined with several techniques, including the heterotrophic plate count (HPC), the total direct (microscopic) count (TDC), or the concentration of adenosinetriphosphate (ATP). These techniques all have their specific advantages and limitations. The main limitations of these parameters include: (*1*) the HPC value of a mixed population of aquatic bacteria is always a (small) fraction of the total active population, and requires a long incubation period; (*2*) the TDC values include active and inactive cells, which may also differ greatly in size; (*3*) the ATP test is fast, but the ATP concentration declines rapidly once the maximum level has been attained. For a number of samples, maximum HPC values have been determined in relation to the initial AOC concentration. The results presented in Figure 15 clearly show that there is a good correlation between the AOC concentration and the maximum HPC values, with an average yield of 7.37×10^6 CFU/µg C. Obviously, the compounds serving as sources of carbon and energy for the indigenous population are those compounds used by the AOC test strains P17 and NOX. However, differences between the bacterial communities in the various water types result in different yield values and differences in the fraction contributing to the HPC value influence the N_{max} values.

BIOLOGICAL STABILITY

Comparison of AOC and BDOC

The AOC and BDOC methods are used to assess the biological (in)stability of treated water. Table 2 gives an indication of the difference between these two approaches. AOC values are a small fraction of the DOC concentration of river water and even lower (0.1 to 1%) in treated water. BDOC values may constitute a significant proportion (19 to 54%) of the DOC concentration (3.5 to 13.3 mg/L) of river water (80). Surveys revealed that BDOC concentrations of drinking water from 79 utilities in the United States ranged from 0.01 to 2.4 mg/L and were 0.4 to 52.8 % of the DOC concentration (70). In another study, BDOC values for treated water of 31 plants averaged by site ranged from 0.03 to 1 mg/L and constituted 5 to 21% of the DOC concentration (71). In both studies significant correlations were found between concentrations of AOC and BDOC, respectively. However, the correlation coefficients were

Table 6. Kinetics of the AOC Decrease in Samples (600 mL) of Two Water Types Stored at 15 °C in Thoroughly Cleaned Erlenmeyer Flasks

Parameter*	River Water from Open Storage Reservoir	Water After Ozonation
DOC (mg/L)	3.4	2.9
AOC_0 (at time zero) (µg C/L)	29.1 ± 6.1	105 ± 11
AOC_R (µg C/L)	9.6 ± 0.7	13.2 ± 0.8
K (day^{-1})	0.98 ± 0.11 ($r^2 = 0.99$)	0.90 ± 0.04 ($r^2 = 0.99$)
Uptake coefficient (µg C/L.day)	0.2 ± 0.01 ($r^2 = 0.99$)	0.27 ± 0.02 ($r^2 = 0.99$)

Note: *DOC, dissolved organic carbon; AOC_0 = AOC concentration at time zero; AOC_R = residual AOC concentration; K = exponential decay rate (d^{-1}).

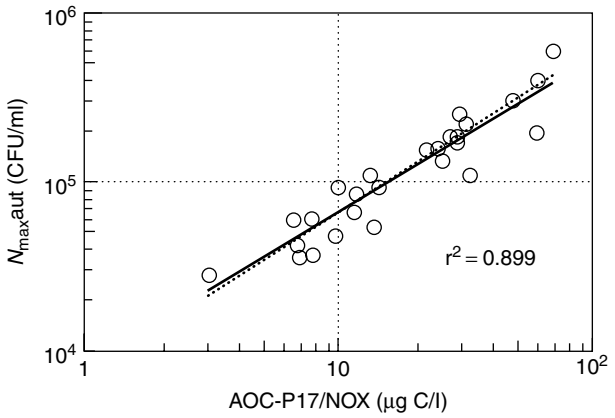

Figure 15. The relationship between the AOC concentration and the maximum colony count (N_{max}) of the autochthonous bacterial community in water samples stored at 15 °C in thoroughly cleaned Erlenmeyer flasks. HPC values were determined using diluted broth agar medium (spread plate method), incubated at 25 °C for 10 days.

relatively low, as a result of the wide range of AOC to BDOC ratios. On average, the AOC concentration was about 20 to 30% of the BDOC concentrations in treated water.

The main reasons for the difference between AOC and BDOC concentrations include:

- the use of an adapted microbial community in the BDOC test;
- the use of a large amount of biomass (bacteria attached to sand) in the BDOC test.

Also, the basis of the methods differ. The AOC concentration gives information about the production of biomass, which is the parameter of concern. The difference between the *Y* values for acetate and oxalate (cf Table 4) implies that the relationship between the amount of utilized organic carbon (BDOC) and biomass formation may differ between water types. In fact, using the *Y* values for oxalate in the AOC test gives much higher AOC values, some of which being close to the BDOC value (70).

From various studies a BDOC concentration of 0.1 to 0.2 mg/L has been derived as a reference value for biological stability (81,82).

The data about growth kinetics (Table 1), suggest that the concentrations of easily biodegradable compounds in treated water should be less than 1 µg of C/L to prevent multiplication. However, complete growth inhibition cannot be achieved and is also not required. The goal is to limit microbial growth to such an extent that water quality deterioration does not occur. Defining an acceptable level of growth is complicated, and may be different for different systems and seasons.

In the Netherlands an AOC value concentration of 10 µg of C/L has been derived as the reference value below which treated water has a very limited regrowth potential. This value is based on the following observations:

1. Samples collected from drinking water distribution systems revealed that AOC concentrations did not decrease at values close to or below 10 µg of C/L (cf. Figs. 12 and 13). Such values are observed in slow sand filtrate and in groundwater supplies in the Netherlands, where rapid sand filtration is the final treatment step;
2. AOC reduction in biological filtration (e.g., rapid sand filtration) is very limited at values below 10 µg C/l. (Fig. 11);
3. AOC concentrations in samples of stored water decline rapidly to a level of about 10 µg C/L (Fig. 14);
4. A statistically highly significant relationship was observed between the AOC concentration and the heterotrophic plate counts as determined on a nutrient poor medium (diluted-broth agar). At AOC values below 10 µg of C/L, the increase of these colony counts during distribution remained limited (53).
5. In a number of water types, the AOC/DOC ratio was as low as 1 µg of C/mg C. Values below this level have not been observed, suggesting that this level is indicative for organic compounds with a high degree of biostability. With DOC values below 10 ppm, this also gives AOC values below 10 µg of C/L.

For limiting regrowth of coliforms in chlorinated supplies AOC concentration of 50 to 100 µgC/l have been derived from extended studies, which indicated that many factors affected regrowth of these organisms (83).

Biofilm Formation Rate

Practical experience in the Netherlands show that the 90-percentile values of the heterotrophic plate counts (22°C, 3 days incubation) remain below 100 CFU/mL in water types with AOC levels below 10 µg of C/L. In groundwater supply this level of AOC is achieved with traditional treatment processes, namely, aeration followed by biological (rapid sand) filtration. In most supplies in the Netherlands the level of 10 µg of C/L is attained, but defining biological stability was found to be more complicated. Even at AOC values below 10 µg C/L and at low heterotrophic plate counts, aeromonads were found to increase in numbers (expressed as CFU/100 mL) (5). Observations indicated that these organisms multiplied in biofilms and in sediment 28. Therefore, a biofilm monitor had been developed for determining the Biofilm Formation Rate (BFR) values of treated water (25,55). Biofilm concentrations are determined by assessing the concentration of adenosinetriphosphate (ATP) with the luciferin-luciferase test. In the Netherlands, the BFR values typically range from less than 1 pg ATP $cm^{-2}\,d^{-1}$ to a value greater that 100 pg ATP $cm^{-2}\,d^{-1}$ (5). Values below 1 pg ATP $cm^{-2}\,d^{-1}$ have been observed in slow sand filtrate and in drinking water prepared from aerobic groundwater. These water types, with AOC values clearly below 10 µg Cl^{-1} represent drinking water with the highest degree of biostability. Higher BFR values have been found in drinking water prepared from anaerobic groundwater, but also these water types had AOC values below 10 µg C/L.

Dosage of acetate at a concentration of 10 μg of C/L to a biofilm monitor gave a BFR value of about 360 pg ATP/cm^2.d. This and other observations showed that a concentration of 1 μg of acetate-C/L causes a BFR value of 35 pg ATP/cm^{-2} . . .d^{-1} (84).

A significant relationship has been observed between the level of regrowth of aeromonads in groundwater supplies and the BFR value of the water leaving the treatment facility. At a BFR value of 10 pg ATP cm^{-2} d^{-1}, the risk of exceeding the guideline value for *Aeromonas* (90-percentile value of 200 CFU 100 mL^{-1}) is 20% (5).

Based on these observations the definition of biostability has been extended and a two dimensional approach is used. Hence, AOC concentrations below 10 μg of C/L in combination with BFR values below 10 pg ATP/cm^2 day represent treated water with a high degree of biostability. Only in a few water types (slow sand filtrate and aerobic groundwater) lower values can be achieved with biological processes.

Materials in contact with treated water

Materials in contact with drinking water can also affect bio(in)stability by releasing biodegradable compounds into treated water. For this reason methods have been developed to test the growth promoting properties of materials. In the United Kingdom the MDOD (mean dissolved oxygen difference) test is used (85), and in Germany a test based on measuring slime production is used (86,87). In the Netherlands the Biofilm Formation Potential (BFP) test is applied, which determines the biofilm concentration (pg ATP/cm^2) on the material as a function of time in a batch test in slow sand filtrate (88). Typical BFP values are below 10 pg ATP/cm^2 for glass and 20 to 50 pg ATP/cm^2 for PVC, whereas values between 500 to 3,000 have been observed for polyethylene materials. Such BFP values can directly be compared with biofilm concentration values as observed on the surface of pipe segments collected from the distribution system. In addition, BFR values as observed in the biofilm monitor also give information about the biofilm concentration, which can be attained on the pipe wall in contact with the water tested. In this way a simple framework is obtained, which enables the assessment and evaluation of the biostability of both water and materials with a consistent approach, the Unified Biofilm Approach.

CONCLUSION

- The AOC method described above has proven to give highly reproducible results. Contamination of the water with biodegradable compounds during sampling, transportation, handling and analysis is a main concern, with glassware and air as potential sources. The described procedures aim at preventing such contamination.
- The two test strains are not capable of utlizing all biodegradable compounds present in a water type. The compounds, which are utilized by the test strains are ubiquitous in water and low AOC concentrations in treated water demonstrate that easily available compounds have been removed.
- AOC concentrations in treated water in the Netherlands are below 10 μg C/l in the groundwater supplies and in most supplies as the result of the application of multiple biological processes in water treatment. The much higher AOC values (median: 100 μg C/l) as reported for treated water in the USA may be ascribed to the absence of biological filtration and the effect of the disinfectant on the AOC concentration;
- A reference AOC value of 10 μg C/l has been derived from effects of biological filtration on the AOC concentration, the AOC decline in distribution systems and the relationship between AOC and HPC values, respectively in unchlorinated supplies. AOC values below 100 or 50 μg C/l have been suggested for limiting coliform regrowth in chlorinated supplies in the USA.
- Even at AOC values below 10 μg C/l, certain microorganisms may multiply in the distribution system. Assessment of the Biofilm Formation Rate in combination with AOC values gives more complete information about the biological stability of treated wate;
- Materials of pipes and reservoirs may release biodegradable compounds into treated water. Assessment of the growth-promoting properties contributes to the selection of appropriate materials.

Acknowledgments

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ASTROVIRUSES

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Astroviruses are 28-nm diameter, icosahedral viruses that have a characteristic five- or six-pointed star-like surface structure when viewed by electron microscopy (EM) (Greek. *astron*, star; Fig. 1). Madeley and Cosgrove coined the term astrovirus in 1975 to describe 28-nm diameter round viral particles they observed by direct EM in the stools of infants with diarrhea (1,2). Along with the *Picornaviridae* and the *Caliciviridae*, the *Astroviridae* comprise a third family of nonenveloped viruses whose genome is composed of plus-sense, single-stranded RNA. In addition to humans, astroviruses have been isolated from numerous animal species, including lambs (3), calves (4), deer (5), piglets (6,7), kittens (8), mice (9), dogs (10,11), and turkey poults (12,13).

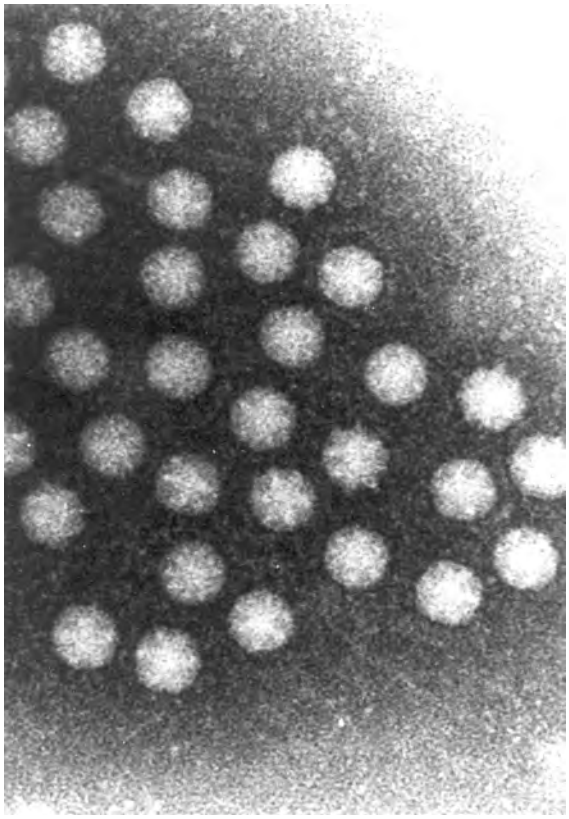

Figure 1. Electron micrograph of human astrovirus in a fecal specimen. Courtesy of W. D. Cubitt.

CLASSIFICATION

Structure

The average diameter given for astroviruses, 28 nm, is based on the measurement of approximately 1,000 negatively stained (phosphotungstic acid) astroviruses in stools (14). The diameter of astrovirus particles may vary, depending on the source of virus and the method of preparation for EM. For example, bovine astrovirus serotype 2 (US2) propagated in primary NBK cells had an average diameter of 34 nm (range 30 to 37 nm) after fixation in glutaraldehyde/1% osmium tetroxide and staining with uranyl acetate (15). Purified preparations of cell culture-adapted human astrovirus serotype 1 particles evaluated by electron cryomicroscopy and image analysis demonstrated a rippled solid capsid shell (330 Å in diameter) with 30 dimeric spikes extending 50 Å from the surface (16). Most of the data regarding the buoyant density of astrovirus particles have been collected using cesium chloride density gradients. However, separation of fecal astrovirus in this medium and further concentration by pelleting disrupts virus morphology (17). Highly purified viral particles are needed for evaluation by electron cryomicroscopy. The inclusion of divalent cations (Ca^{++} and Mg^{++}) has found to stabilize viral particles undergoing isopycnic centrifugation in cesium chloride (16). A gradient of potassium tartrate progressively diluted with glycerol yields a positive-density, negative-viscosity gradient in which virus morphology is maintained and from which viral particles may be pelleted without deleterious effects (17). Peak density for astroviruses in this medium was 1.32 g/mL (17). Buoyant densities of human astroviruses in cesium chloride have ranged from 1.35 to 1.40 g/mL (18–23).

With cell culture-adapted astroviruses, four structural proteins (36.5, 34, 33, and 32 kDa) were identified in astrovirus serotype 4 that was propagated in cell culture in the presence of trypsin (20). It was postulated that the faint 36.5-kDa band might represent a precursor protein that undergoes subsequent processing, in a manner similar to the VP0 protein of enterovirus (24). Two additional proteins with 24 and 5.2 kDa were found by Kurtz (25) in cells infected with astrovirus serotype 1. In CaCo-2 cells infected with astrovirus serotype 1, three astrovirus-specific polypeptides (33.5, 31.5, and 24 kDa) were identified by reactivity with astrovirus serotype 1-specific antiserum (23). In some preparations, a 27-kDa polypeptide was also noted, and it was hypothesized that this may be derived from one of the larger proteins by proteolytic digestion. In studies of human serotype 5 isolated from volunteer fecal specimens, a single 30-kDa structural protein was immunoprecipitated (22).

In astrovirus-infected LLCMK2 cells in the absence of trypsin, a single approximately 90-kDa protein was immunoprecipitated from infected cell lysates with hyperimmune antiserum to purified human astrovirus serotype 2 (26). Pretreatment of the lysates with trypsin resulted in immunoprecipitation of three proteins, a predominant 29-kDa protein as well as a 31- and a 20-kDa protein. These results suggested that the approximately 90-kDa protein may be proteolytically cleaved to yield at least three smaller proteins. In cells infected with human astrovirus serotype 2, grown in trypsin-containing media, Sanchez-Fauquier and coworkers (27) used a neutralizing monoclonal antibody (PL-2) to determine that an approximately 86-kDa protein (P86) is the precursor to VP26, a major component of viral particles. VP29, a minor component of virions and the product of alternative processing of P86, and intermediate proteins ranging from P74 to P35 were also identified in infected cells by PL-2. Although these results suggest processing of P86 to P26 and/or P29 occurs within infected cells, the presence of trypsin in the media impairs optimal interpretation of the data.

Using partially purified preparations of human astrovirus reference strains (serotypes 1 to 7) grown in cell culture, astrovirus types 1 to 4 consisted of three proteins, P1 (~33 kDa), P2 (~33 kDa), and P3 (25 to 28 kDa, depending on serotype), that were immunoprecipitated with homologous rabbit antisera (28). For reference types 5 to 7, three infection-specific proteins were observed in radiolabeled samples, but only P2 and P3 reacted with type-specific rabbit serum (28). In a relatively trypsin-free system, Bass and Qiu (29) found evidence for intracellular processing of the full-length ORF 2 product to a 79-kDa moiety that was incorporated into viral particles. Infectivity was significantly enhanced by treatment of these particles with trypsin, which also resulted in cleavage of the 79-kDa protein to 33-, 29-, and 26-kDa subunits.

Genetics

The genome of astroviruses is a plus-sense, single-stranded RNA of approximately 6,800 nucleotides (nt) in length, excluding the poly(A) tail at the 3′-end. RNA extracted from virions is infectious (30). Infectious RNA also can be transcribed from a full-length cDNA clone of human astrovirus serotype 1 [AVIC (30)]. BHK cells, previously thought not infectable with astrovirus (31), have been shown to support astrovirus replication and maturation when transfected with RNA extracted from virions or transcribed from AVIC. Astrovirus produced in these BHK cells have been shown to infect monolayers of Caco-2 cells in trypsin-containing media.

Complete nucleotide sequences of four strains of human astrovirus are currently available: (*1*) the serotype 1 Oxford reference strain propagated in LLCMK2 cells (32), (*2*) a serotype 1 Newcastle strain recently isolated and maintained in CaCo-2 cells (33–35), (*3*) the serotype 2 Oxford reference strain propagated in LLCMK2 cells (36), (*4*) a serotype 3 strain (GenBank accession AF141381), and (*5*) a serotype 8 strain (37).

During infection of susceptible cells, it has been noted that both the full genomic (6.8 kb) and a subgenomic (2.4 kb) RNA are produced (21,26). The viral genome comprises three open reading frames (ORFs). The two ORFs at the 5′-end of the genome, designated ORF 1a and ORF 1b by the International Committee on the Taxonomy of Viruses [ICTV (38)], encode nonstructural proteins. The third ORF, designated ORF 2, encodes a structural protein and is found at the 3′-end of the genome (32,34,38). This ORF is common to both the genomic and subgenomic

RNA. Sequences of the capsid-encoding ORF 2 are available in GenBank for human serotypes 1 through 8 and for feline, porcine, and turkey astroviruses.

A schematic representation of the astrovirus serotype 1 Oxford reference strain's genome is shown in Figure 2. A 5′-untranslated region of 85 nt precedes ORF 1a, which is 2,763 nt in length. The length of this ORF can vary depending on the method by which the virus was initially isolated. For example, ORF 1a of the Newcastle serotype 1 strain, isolated in Caco-2 is 45 nt longer than the Oxford serotype 1 strain, isolated in primary HEK cells (39). A 73-nt overlap exists between ORF 1a and ORF 1b and contains the sequences critical for (−1) ribosomal frameshifting and translation of ORF 1b. There is an 8-nt overlap between ORF 1b and ORF 2. ORF 2 varies in length among human astroviruses from 2,316 nt for a serotype 4 strain to 2,391 nt in the Oxford serotype 2 strain (36), but for serotype 1 (Oxford) is 2,364 nt. An 80-nt 3′-untranslated region is found between ORF 2 and the poly(A) tail at the extreme 3′-end of the genome. The length of the astrovirus 3′-untranslated region and its predicted secondary structure are similar to those of certain picornaviruses (33), but there is minimal sequence identity between these two viral groups. In a separate study, the terminal 19 nt of ORF 2 and adjacent 3′-untranslated region were found to be highly conserved among all eight human astrovirus serotypes (40). Of note, similarities in sequence and folding of the 3′-untranslated region of human, sheep, pig, and turkey astroviruses, avian infectious bronchitis virus (a coronavirus) and equine rhinovirus serotype 2 (a picornavirus) were observed (36) and it has been suggested that these features may have arisen from recombination events.

The polypeptide sequences of ORFs 1a and 1b were the first indication that nonstructural proteins are encoded by these ORFs. The ORF 1a-encoded protein contains a viral serine protease motif that has features consistent with chymotrypsin-like proteases of other plus-stranded RNA viruses, including RHDV and feline calicivirus (36). An important difference found when comparing the astrovirus protease motif with that of the caliciviruses is the substitution of a serine for a cysteine at the third catalytic amino acid residue. This substitution is seen in several viruses, sobemoviruses, luteoviruses, and arteriviruses, but the putative astrovirus protease bears stronger overall resemblance to the calicivirus protease.

A bipartite nuclear localization signal is encoded downstream of the viral protease motif in ORF-1a (35,36). Recent studies of human astrovirus serotype 1 (Newcastle) have indicated that this signal is functional and directs ORF-1a-encoded proteins to the nucleus (41). Of note, a prior study of cell culture-adapted bovine astrovirus detected viral proteins in the nucleus of infected cells by immunofluorescence (15). Further studies are required to determine the role and importance of nuclear localization in the astrovirus life cycle.

Analysis of ORF 1a sequences of reference serotypes 1 to 7 indicates two distinct genogroups that correlate with serotype (42). Types 1 to 5 (42) and 8 (43) are found in one genogroup (designated A), whereas types 6 and 7 fall into a separate genogroup (B). These phylogenetic associations for ORF 1a sequence are different from that observed for ORFs 1b or 2 in which nearly equidistant clustering of serotypes is found with unrooted phylogenetic trees. Whether these differences are attributable to recombination remains to be studied.

ORF 1b contains a motif consistent with an RNA-dependent RNA polymerase. This motif is highly conserved among astrovirus serotypes 1 to 8 (32,37,42). The astrovirus polymerase motifs are most similar to those of plant viruses, bymovirus, and potyvirus, and can be aligned with other polymerases of Koonin's Supergroup I (44) that includes picornaviruses, caliciviruses, and certain plant viruses (36), although similarities between astrovirus and plant viruses in this region do not necessarily imply a common origin (36).

Regions that may encode an RNA helicase, methyltransferase, or papain-like protease have not been identified in the astrovirus sequences that are currently available. It is unusual for a plus-strand RNA virus with a genome length of greater than 6,000 nt to lack a helicase domain (36,45). Astrovirus may encode a VPg, as suggested by the absence of a methyltransferase-encoding region and the similarity of its RNA-dependent RNA

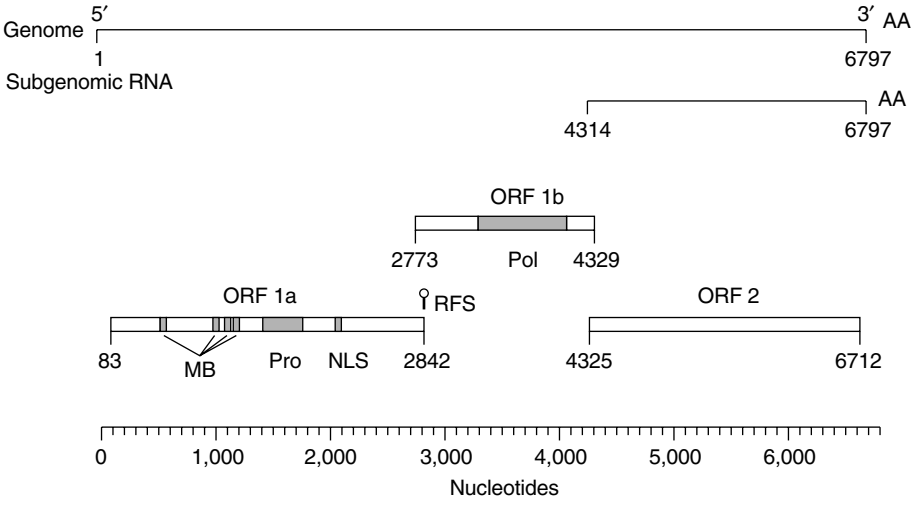

Figure 2. Genome organization of human astrovirus serotype 2 (36). Genomic and subgenomic RNA are depicted in the upper half of the figure. Below this, the three ORFs with predicted locations for transmembrane helices (MB), protease (Pro), nuclear localization signal (NLS), ribosomal frameshift structure (RFS), and RNA-dependent RNA polymerase (Pol) are shown schematically. Nonstructural proteins are likely encoded in ORFs 1a and 1b. At least one structural protein that is most likely a precursor to smaller capsid proteins is encoded by ORF 2.

polymerase with primarily VPg-containing viruses. A convincing VPg domain has not been identified, but it may be located between the putative protease motif and upstream transmembrane alpha-helices, where a serine at aa position 420 that could link the VPg to genomic RNA is found. ORF 1a also encodes an immunoreactive epitope (aa 757 to 899) identified by antisera produced to purified viral particles, but its significance is not known.

The greatest sequence variability is found in ORF 2, which encodes a viral structural protein. A high degree of conservation of the N-terminal 415 (or so) amino acids is observed among the human serotypes (46). In this region, the amino acid sequence for the feline and porcine strains share certain conserved stretches of amino acid residues with the human strains and can be aligned with minimal gaps, whereas the avian strains cannot be aligned easily with the human and other mammalian strains. Beyond this region, there is considerable variability in sequence among the serotypes, requiring gaps to be introduced for optimal alignment. The C-terminal 8 amino acids are highly conserved, particularly among the human strains.

Information regarding processing of the nonstructural proteins is very limited. A preliminary report by Gibson and coworkers (47) noted a "lack of processing" in vitro of ORFs 1a and 1a/1b translation products containing the 3C-like serine protease motif. By contrast, Willcocks and coworkers (41) studied nonstructural protein processing in astrovirus-infected Caco-2 cells by Western blot analysis with antisera specific for the C-terminal 298 aa of the ORF 1a product. They detected proteins of 74, 34, 20, 6.5, and 5.5 kDa, but no full-length ORF 1a product (p101).

In a separate study (48), processing of the ORF 1a product was studied in a cell-free expression. ORF-1a products identified by astrovirus-specific and N-terminal tag-specific antibodies included the full-length 101-kDa protein (p101), an N-terminal cleavage product (p64), and a C-terminal cleavage product (p38). Mutation and deletion analyses indicated the important role of the catalytic triad of the viral 3C-like serine protease (Ser-551, Asp-489, and His-461) in processing of p101.

In summary, the astrovirus genome is organized with the primarily nonstructural proteins encoded by the 5′ two-thirds (ORFs 1a and 1b) and the structural protein encoded by the 3′ one-third (ORF 2). This arrangement resembles the genome organization of caliciviruses (49). However, several features distinguish these two viral families, including differences in the size, number, and processing of structural proteins, the lack of a helicase domain in astroviruses, the use of ribosomal frameshifting to translate the RNA-dependent RNA polymerase, and distinctive morphologic features described in the preceding text. Astroviruses differ from picornaviruses in genome organization, lack of a helicase domain, and use of translational strategies, such as ribosomal frameshifting and subgenomic RNA.

Biology and Antigenic Properties

Astroviruses appear to incorporate specific features of a number of different viruses without strictly resembling any one group and therefore have been accorded a separate viral family, the *Astroviridae* (38). The type species is the human astrovirus. It has recently been proposed by the Astrovirus Study Group of the International Committee on Taxonomy of Viruses that the Family *Astroviridae* be subdivided into two genera, Mammoastrovirus and Aviastrovirus, on the basis of phylogenetic clustering of capsid sequence, host of origin, and target organs. Mammoastrovirus would include astroviruses that infect mammals and primarily cause gastroenteritis. Aviastrovirus would include astroviruses that infect avian species and may cause intestinal as well as extraintestinal illness.

Astroviruses are resistant to many common chemical agents, including chloroform, a variety of detergents (nonionic, anionic, and Zwitterionic) lipid solvents, and acid conditions (pH 3) (20). Human astrovirus retains activity after 5, but not 10, minutes at 60 °C. Viral particles appear to be stable when stored at ultralow temperatures (−70° to 85 °C) for 6 to 10 years (50), but may be disrupted by repeated freezing and thawing. Methanol has been shown to be more effective in reducing astrovirus infectivity than equivalent concentrations of isopropanol or ethanol (51).

When astroviruses were first described during the 1980s, five serotypes of human astrovirus were identified by immunofluorescence, neutralization, and immune EM (52–54). Each strain was isolated from natural infections in the United Kingdom and adapted to growth in cell culture (31). Serotypes 6 and 7 were identified in the United Kingdom in 1989, 1991, and 1992 (55). An eighth serotype is also now described (56).

By serotyping isolates, astrovirus serotype 1 has generally been the most common type found (20,53,55–63), but the predominant serotype may vary depending on location (64). In community-acquired astroviruses studied in Oxford, United Kingdom, between 1975 and 1987 (20), 72% of the astroviruses detected were serotype 1. Serotypes 2 to 5 each accounted for 6 to 8% of the astrovirus strains. A later survey by Lee and Kurtz (55) examined the astrovirus serotypes found in the Oxford region between 1976 and 1992. Serotype 1 was again found most frequently and accounted for an average of 65% of the cases 1991 was the only year in which the number of serotype 2 cases exceeded the number of serotype 1 cases. A third study from the United Kingdom also found a predominance of astrovirus serotype 1 (63). By contrast, a longitudinal study of diarrhea in a cohort of Mexican children found that astrovirus serotype 2 was most common (35%) and serotypes 1 and 5 were least common (4% each) (64).

Animals hyperimmunized with each of the human astrovirus serotypes 1 to 7 raise antisera that appear to react type-specifically in immunofluorescence tests and IEM (20,55,65). Also, rabbit polyclonal antiserum neutralizes astrovirus in a serotype-specific manner by plaque assay (65). All eight serotypes of human astrovirus are recognized by a monoclonal antibody produced by Herrmann and coworkers (55,66). This monoclonal antibody, which does not neutralize virus, is directed at a viral structural protein (57), most likely an epitope within the highly conserved N-terminal half of the ORF-2 product (Geigenmüller and Matsui, unpublished data). An EIA in which the monoclonal antibody serves as a capture

antibody and polyclonal antiserum is used as a detector antibody has been developed (66) and employed in large-scale seroepidemiologic studies described in the following text. A typing EIA that uses serotype-specific reference antisera for antigen capture and the group-reactive monoclonal antibody as detector has been developed to assess the antigenic types of astroviruses found in clinical samples (66). Molecular methods for genotyping clinical isolates by reverse transcriptase polymerase chain reaction (RT-PCR) may also be used, using primers from the more conserved N- or C-terminal regions of ORF-2 (11,59). A high concordance between results of genotyping and serotyping has been observed.

EPIDEMIOLOGY AND CLINICAL DISEASE

The medical importance of astrovirus in humans was established initially in studies conducted in Thailand and Guatemala where astroviruses were found to be the second most common cause (after rotavirus) of viral diarrhea in young children (67,68). This was made possible by the development of a monoclonal antibody EIA that can detect all known human astrovirus serotypes (64,66). This and other improvements in detection techniques, along with increased awareness of astrovirus infection have helped to define the epidemiology of this illness. Astrovirus infections have been found worldwide, primarily, but not exclusively, in young children with diarrhea (19,67–104). Sporadic outbreaks of gastroenteritis because of astrovirus have been reported among elderly patients (79,86,97,105) and military recruits (106). Several studies associate astroviruses with diarrhea in immunocompromised adults (73,81,107,108). Large, foodborne outbreaks, affecting thousands of individuals in Japan, have occurred among otherwise normal school-age children and adults as well (109,110).

Most astrovirus infections are detected in the winter months in temperate regions and in the rainy season in more tropical climates, a pattern that resembles rotavirus infections (20,68,111). Both community-acquired (1,67,82,89,95,112,113) and nosocomial (69,70,76,77,82,83,87,90,95,103,112,114,115) infections have been described. In Australia, astrovirus is the second most common cause of gastroenteritis in children, after rotavirus (61). Infection with serotype 1 predominates, particularly in the winter months when gastroenteritis is common (61,116,117). In a population of Mayan infants, astrovirus was the most common enteric pathogen identified in stool samples collected during a prospective study of oral poliovirus immunogenicity (118).

Astroviruses have been shown to be an important cause of outbreaks of diarrhea in childcare centers among children age three years or less (85,91). In eight outbreaks of astrovirus diarrhea in six childcare centers, 20% of the children with diarrhea shed astrovirus (92). Although each outbreak was associated with a single astrovirus serotype, two distinct serotypes were identified in sequential outbreaks during one winter diarrhea season.

Serological studies indicate that astrovirus infections are common in the United States (111,119), Japan (19), the United Kingdom (60,120), and other areas of the world (63,78,121). In samples of gamma globulin pools collected in the United States, antibodies to all five of the originally identified serotypes of astrovirus are detectable (111). Antibodies to astrovirus tend to be acquired in early childhood. A survey of 87 children less than 10 years old in the Oxford region of the United Kingdom revealed that antibody prevalence rises rapidly from 7% in 6- to 12-month-old infants to 70% by school age. Astrovirus antibodies could be detected in 75% of the 10-year-old children studied. Among young adults (nursing students), 77% had antibodies to astroviruses.

Clinical Symptoms

Astroviruses are a cause of gastroenteritis worldwide. Young children, especially those under six months of age, are the ones primarily affected. However, all ages may be affected. Older children and adults have been involved in outbreaks (109,110), and astrovirus infections have been recognized in elderly, institutionalized patients (63,79,89,105) and in immunocompromised patients (20,73,75,81,122). Human astrovirus infection induces a mild, watery diarrhea that typically lasts for two to three days, associated with vomiting, fever, anorexia, abdominal pain, and various constitutional symptoms that last for up to four days (20,111,123–126). Protracted diarrhea and viral shedding have been observed in some studies (20,107,108). In children, it may not be possible to distinguish diarrhea owing to astrovirus from that owing to rotavirus on clinical grounds alone (20,67,68) and studies of hospitalized children have found astrovirus to be the second most common viral pathogen, after rotavirus (61,116). In general, astrovirus diarrhea is milder and does not lead to significant dehydration or hospitalization (16,64,67,83,85,91,95,126). However, in a significant percentage of rural Egyptian children with astrovirus gastroenteritis severe dehydration was seen (127). In addition, severe infections have been noted in young adults infected with astrovirus serotype 4 (74). Deaths associated with astrovirus infections are extremely rare but have been reported (100,105).

The role of astrovirus in persistent diarrhea remains to be established (103). Prolonged lactose intolerance wand, less commonly sensitivity to cow's milk, have been problems for some patients (76,83,95). Intravenous immunoglobulin may be a useful adjunct in severely immunodeficient patients who fail to respond to conservative measures (107,128), but larger prospective studies are needed to establish the efficacy of this approach.

In animals, as in humans, astrovirus has usually been identified in association with diarrhea, but in ducklings has also been associated with a rapidly fatal hepatitis (129). Avian astroviruses have also been associated with nephritis in chicks (130) and immunosuppression compounding enteritis in turkey poults (131).

Viral Pathogenesis

Pathogenesis of astrovirus infections in humans has been limited to a report of two children with diarrhea that correlated fecal shedding of astrovirus with identification of astrovirus particles in intestinal epithelial cells. This

suggested that virus replication occurs in intestinal tissue in humans (99). Astrovirus particles in the biopsy specimen were localized to the epithelial cells in the lower regions of the villus in a patient with sucrase isomaltase deficiency and in the "exposed surface epithelium" of a second patient who had a severe enteropathy because of sensitivity to cow's milk formula. The significance of astrovirus infection in these patients was not definitive because they had underlying gastrointestinal problems and fecal shedding of *Escherichia coli* 086 or rotavirus. Human volunteer studies have not examined the histological effects of astrovirus-related diarrhea (22,132).

In animals, gnotobiotic lambs given ovine astrovirus showed that mild transient diarrhea was caused by infection of mature enterocytes in the apical two-thirds of villi and subepithelial macrophages, followed by transient villus atrophy and crypt hypertrophy (3,133). Infected intestinal cells were detected by immunofluorescence between 14 and 70 hours after inoculation. Most infected cells were found 14 to 38 hours postinoculation (133) and during this time, aggregates of virus particles along the microvilli or in lysosomes and autophagic vacuoles were observed by EM (133).

Bovine astrovirus infection, which does not result in symptomatic illness, causes infection of M cells and absorptive enterocytes overlying the dome villi of Peyer's patches in the small bowel (134,135). Infected cells were sloughed, replaced by cuboidal cells, and formed an exudate with inflammatory mononuclear and eosinophilic cells above the dome villi. The lamina propria was infiltrated with neutrophils and contained cells with degenerate nuclei. Lymphoid cell depletion was noted in the central region of germinal centers beneath infected dome villi.

These two studies with mammalian astrovirus infections suggest that astroviruses enter cells through the apical surface. In polarized CaCo-2 cells, however, human astroviruses isolated from wild-type infections appear to enter through the basolateral surface (23). Whether or not this result reflects the arrangement of astrovirus receptors in differentiated intestinal epithelial cells in vivo remains to be determined (136).

Entry of astroviruses into cells has also been studied using Graham 293 cells, a transformed line of primary HEK cells (137) that has been frequently used for isolation of enteric adenoviruses. The effects on astrovirus infection of lysosomotropic agents (ammonium chloride, methylamine, and dansylcadaverine) and the ionophore monensin were investigated. All inhibited viral infection, suggesting that a functional endocytic pathway is necessary for delivery of infectious astrovirus into the cytoplasm.

Bovine astrovirus infection in cell culture has also been studied. Bovine astrovirus was propagated in primary NBK cells and the expression of viral antigens was followed by fluorescent antibody probes (15). Immunofluorescence was first detected at 7 hours postinfection in the cytoplasm of infected cells. Shortly thereafter, immunofluorescent granules were identified in the nucleus in a pattern suggesting nucleolar involvement. This was followed by dense immunofluorescent granules appearing in the perinuclear region and diffuse staining of the cytoplasm. This pattern of infection has also been seen in cell cultures infected with human astroviruses (Herrmann, unpublished data).

Modes of Transmission

Human adult volunteer studies indicate that astroviruses can be transmitted through the fecal-oral route, (22,132) although few of the volunteers developed diarrhea with fecal shedding of virus. It appeared that person-to-person spread was responsible for an outbreak of gastroenteritis in Marin County, California, in 1978 (97), that was subsequently shown to be owing to astrovirus type 5 (138,139). Astrovirus diarrhea does not usually develop in adults, but adult caregivers of infected children, including parents, teachers and medical personnel, may become ill (20,109,125,140). An outbreak among military troops has also been described (106). This suggests that these adults may have been exposed to a larger dose of astrovirus or through a variable route (e.g., fomites, contaminated food, or water). Astrovirus has also been detected in water from an area where an outbreak of astrovirus gastroenteritis occurred (141), and 93% of surfers in the United Kingdom had antibodies to human astrovirus serotype 4, compared to 22% of age-matched controls (142). A study of sewage and environmental samples from a water treatment facility in the United Kingdom failed to detect astrovirus RNA by RT-PCR (143). A case of astrovirus gastroenteritis was diagnosed at the end of the sampling period, but occurred outside the surveyed area.

Large outbreaks of astrovirus gastroenteritis occurred in Japan that were thought to be food-borne (109,110). Food handlers should be instructed that shedding of astrovirus in the feces may begin a day before symptoms and continue for several days after diarrhea resolves. In immunocompromised individuals, viral shedding may last for weeks after resolution of symptoms (107,108). In addition, foods, such as shellfish that have been implicated in outbreaks of astrovirus gastroenteritis should be carefully selected and prepared.

RESERVOIRS

Astrovirus infections appear to be species-specific, which suggests that animals are not a reservoir for human infections. Where antigenic relationships among astroviruses of different animal species have been examined, there has been no evidence for cross-reactivity between species (5,6,54,135,144). In general, infection of animals and cell culture is also species-specific. BHK-21 cells support propagation of human astrovirus type 2 (145), and astrovirus isolated from red deer has been shown to infect BEK cells, but serial propagation was not achievable (5). It is likely that not all the animal astroviruses have been extensively tested as serial passage has not been established for astroviruses from lambs (3), red deer (5), kittens (8), dogs (10,146), turkeys (12), and ducks (129). To date, only

bovine (15) and porcine (7) astroviruses have been propagated in cell culture.

DETECTION, OCCURRENCE, AND PERSISTENCE

Methods of Detection

Electron Microscopy. Before immunoassay or PCR methods for astrovirus detection were developed, EM or immune electron microscopy (IEM) techniques were the major assays used, and although insensitive, are still used by some for detection of astroviruses where other assays are not available. EM may also be important for standardizing new detection methods as they are developed. Patients with diarrhea owing to astrovirus frequently shed large numbers of viral particles, 10^{10} per milliliter, or 10^8 viable particles per milliliter (20). IEM techniques are useful to detect lower levels of astroviruses (19,147,148). The sensitivity of EM has been estimated to be 10^6 to 10^7 virus particles per gram of stool.

Diagnosis by EM requires experience, as only 10% of the particles in a given specimen may display the distinctive astrovirus surface star (149). Oliver and Phillips conducted a retrospective analysis of fecal viruses that were originally identified as small, round, featureless viruses by EM alone (96). When complementary immunologic techniques were applied to careful reexamination of the specimens by direct EM, 14 of the 53 samples (26%) first classified as small, round, featureless viruses were shown to be astrovirus. Two caliciviruses and a Norwalk-like virus were also found to be misclassified. Another example of misclassification by direct EM is the Marin County virus, originally classified in 1978 as a "Norwalk-like" virus (97). Subsequent analyses, however, proved that the Marin County virus was an astrovirus (serotype 5) (138,139).

Isolation in Cell Culture. Serial passage of astroviruses in cell culture (primary HEK cells) was first described in 1981 by Lee and Kurtz (31). This was made possible by incorporating trypsin (10 μg/mL) in serum-free growth media (31). After six passages in HEK cells, primary baboon kidney (PBK) cells and a continuous line of LLCMK2 cells could also be infected, but not by direct inoculation of PBK or LLCMK2 cells with fecal derived astroviruses. All five of the serotypes of human astrovirus first described (53) were adapted to growth in LLCMK2 cells.

Direct isolation of an astrovirus from stools in a continuous cell line was first described by Willcocks and coworkers (23) for astrovirus serotype 1. The cell line used, CaCo-2 cells, a continuous line of human colon carcinoma, has been subsequently found to allow isolation of all astrovirus serotypes, including the more recently identified types 6, 7, and 8 (43,55,145). Use of this cell line may allow more rapid isolation and subsequent propagation of wild-type astroviruses (150). Growth in CaCo-2 cells may be also a way to distinguish astrovirus infection from infection with human caliciviruses, viruses that have not been successfully isolated or propagated in cell culture.

Propagation of serotypes 1 to 4 was accomplished in a hepatoma cell line, PLC/PRF/5, a line that was also used to isolate human astrovirus directly from fecal samples (four isolates) (151). Several other cell lines have been tested for astrovirus isolation and cultivation (145). When laboratory-adapted human astrovirus serotypes 1 through 7 were tested for growth in 15 human, 7 simian, and 10 other nonprimate mammalian cell lines, propagation of all seven serotypes was successful in the human cell lines Caco-2, T84, HT-29, and in the African green monkey kidney cell line MA-104. Both primary and secondary African green monkey kidney cells were more effective than Rhesus monkey kidney cells for cultivation of astrovirus. Except for human foreskin cells, all of the other human and simian cell lines supported growth of at least one astrovirus serotype. The only nonprimate cell line that permitted sustained passage of astroviruses was BHK-21 (C13) cell line for astrovirus serotype 2. BHK cells transfected with RNA transcripts derived from a genomic cDNA of human astrovirus serotype 1 have been shown to support viral replication and morphogenesis (30). Of 17 human stool specimens that had previously been shown to be astrovirus positive by ELISA, Caco-2 cells (13 isolates), T84 cells (12 isolates), and PLC/PRF/5 cells (12 isolates) were the cell lines most effective for isolation of human astroviruses from these stool specimens (145).

Enzyme Immunoassay. A group reactive monoclonal antibody (8E7) was developed by Herrmann and coworkers and was used in an enzyme immunoassay (EIA) to capture viral antigen in stools (65,66). In this assay, polyclonal antiserum is used as the detector antibody in an indirect EIA. A modification of this EIA uses a biotinylated polyclonal detector antibody (93). Both EIAs are comparable to IEM in sensitivity (91%) and specificity (98%). EIAs have been useful in rapidly detecting astrovirus antigen in studies where a large number of samples must be assayed (2,67,68,85,112). EIA- and RT-PCR-based methods to type human astroviruses found in clinical samples have been described more recently (59). An astrovirus antigen EIA that uses a group reactive monoclonal antibody [8G4] for both capture and detection (biotinylated) has also been developed (112). The sensitivity of EIA is estimated to be 10^5 to 10^6 viral particles per gram of stool (66,93).

A commercial enzyme immunoassay based on the group reactive monoclonal antibody 8E7 (59) is available for the detection of astrovirus antigen in clinical specimens (IDEIA™ Astrovirus EIA, DAKO Diagnostics). In one survey from Australia, this qualitative assay was found to have a sensitivity of 100% and specificity of 98.6% (117).

RNA Probes and Reverse Transcriptase-Polymerase Chain Reaction (rt-PCR)

Hybridization. The use of nucleic acid probes for astrovirus identification was first described in 1991 (93,152). The sensitivity of RNA probes is approximately equal to that obtained by EIA (93,152), thus for routine diagnosis there is no advantage of probes compared with monoclonal antibody immunoassays for astroviruses in gastroenteritis. The sensitivity is similar, but the probe techniques

are considerably more complex and require more time to obtain results.

Polymerase Chain Reaction. Amplified techniques, such as the polymerase chain reaction (PCR), offer a more sensitive approach than direct hybridization, and PCR applications have been described for astroviruses (81,153,154). The sensitivity in detecting astrovirus infections is improved, and is estimated to be 10 to 100 particles per gram of stool (62). In one study of an outbreak of astrovirus gastroenteritis at a day care center (155), the prevalence of astroviruses determined by EIA varied from 50% in affected rooms to 20% in rooms with older children. Analysis by RT-PCR indicated nearly all children were infected and viral shedding was detectable for weeks despite resolution of symptoms. In addition, RT-PCR detected astrovirus in 32% of the samples tested, compared to a detection rate of 10% by EIA.

The complete sequences of four strains of astrovirus (two serotype 1, one serotype 2 and one serotype 3) are currently available. Oligonucleotide primers selected from sequences at the 3′-end of the genome can amplify astrovirus-specific products from all five reference serotypes (156). Primers derived from the highly conserved RNA-dependent RNA polymerase motif may also be suitable candidates for amplification of different human astrovirus serotypes by RT-PCR (32). RT-PCR has been used also to confirm astrovirus-positive samples detected by EIA (73,81,91,118). Good correlation has been observed between these two methods for detection (78).

Recovery and Concentration. As was discussed earlier, the mode of transmission of astroviruses is considered to be similar to that of other enteric viruses, which includes potential transmission in environmental samples. The environmental samples most commonly assayed are those in which other enteric viruses have been isolated or detected, including surface waters, drinking water, shellfish, and sewage samples. The stability of astroviruses in water also suggests the possibility of transmission by the water route. In dechlorinated water, astrovirus survival was comparable to rotavirus serotype 3 and enteric adenovirus 40/41, both of which can be transmitted through water. Astrovirus and adenovirus were more resistant than poliovirus to inactivation by the addition of free chlorine to the water, but less resistant than human rotavirus or hepatitis A virus (157,158).

Although studies on detection of astroviruses in environmental samples have been very limited in comparison to those for other enteric viruses, the techniques for recovery of astroviruses are similar and follow the same general procedures; extraction, concentration, purification (or removal of inhibitors), and detection. For water samples, this entails adsorption of virus to filters followed by elution and concentration. For the one study on naturally occurring astroviruses in foods (shellfish) (159), virus was extracted from a food homogenate with organic solvents, followed by virus precipitation and nucleic acid extraction for RT-PCR.

Recovery from Water Samples. The first description of recovery of astroviruses naturally occurring in an environmental source was described in 1996, in a sample of sewage polluted water (141). The virus was recovered by passage of 500 L of water through positively charged filters (Zeta plus MKII filters) followed by elution with 0.05 M glycine buffer plus 3% beef extract, and organic flocculation. The final volume of the sample was 50 mL. Viral nucleic acid could not be directly detected in the concentrated samples, but was detected after passage of the samples in CaCo-2 cells. It was estimated that for the wild-type astrovirus strain detected, there was a minimum of 20 astroviruses per liter of in the unconcentrated sample. A second report was published in 2000 (160). Surface water samples were processed using filtration through cartridge filters (type 1 MDS Zetapor cartridge filters) followed by elution with beef extract and concentration by organic flocculation according to the method described for enteric viruses by the Environmental Protection Agency (161). Portions of concentrated samples were inoculated onto monolayers of CaCo-2 cells and tested for astroviruses by RT-PCR. Of 29 water samples analyzed, 15 were positive for astroviruses.

Recovery from Sludge. Because astroviruses have a different capsid structure than enteroviruses, Chapron and coworkers (162) compared the prevalence of human astroviruses in sludges as compared to enteroviruses, using an EPA method designed for other enteric viruses. Using 500 mL of sludge samples collected from sewage plants in the United States from January to June 1999, viruses were concentrated from sludges according to the EPA CFR part 503 regulations (EPA 1992). This procedure uses beef extract elution followed by organic flocculation, and results in a final volume of 20 mL. For detection of viruses, CaCo-2 cells were inoculated and virus detected by RT-PCR/nested PCR. Of 16 sample sites analyzed, 15 were positive for astroviruses and 16 were positive for enteroviruses. This study not only demonstrated that astroviruses can be recovered from sewage sludge, but also showed that astroviruses can be detected in sludge samples by standard EPA procedures for the recovery of enteric viruses.

Recovery from Shellfish. Methods have been described for recovering astroviruses from samples of mussels (163) or mussels and oysters (164) seeded with laboratory strains of viruses, but to date only one report has been published for naturally occurring astroviruses in shellfish. In a three-year study of enteric viruses in shellfish, Le Guyader and coworkers (159) detected astroviruses in 17% of oyster samples and 50% of mussel samples. The mussels were collected from beds frequently contaminated by fecal coliforms. Astroviruses were recovered more frequently from mussels than either hepatitis A virus (13%) or Norwalk-like viruses (35%), both of which have been implicated in shellfish-borne outbreaks of disease. Recovery of astroviruses was accomplished by extracting nucleic acid for PCR from dissected tissues (stomach and digestive diverticula) of shellfish, a method that has been described for recovery of Norwalk virus and Hepatitis A (165). To concentrate virus, the tissues were homogenized in buffered saline, extracted with chloroform-butanol, and precipitated with polyethylene glycol. Viral

nucleic acids were extracted by the phenol-chloroform-water technique and ultimately precipitated with ethanol for use in RT-PCR. As is the case for other enteric viruses, the extracts need to be tested for the presence of PCR inhibitors. A commercial kit that uses a guanidine thiocyanate silica procedure to extract viral RNA directly, without mechanical homogenization of shellfish, is now available (Promega, Madison, Wis.). The virus of interest (astrovirus, Norwalk virus, hepatitis A virus) then can be amplified from the extracted RNA using virus-specific primers in RT-PCR assays (164). Methods for isolation or detection of astroviruses in foods other than shellfish have not been reported.

CONCLUSION

The medical importance of astroviruses as major causes of gastroenteritis has now been well established through clinical and epidemiological studies. The detection of astroviruses in water and shellfish samples, combined with epidemiological evidence for food-borne outbreaks of astrovirus gastroenteritis (109,110) suggests that astroviruses have been spread through environmental sources. With the techniques for detection of astroviruses that are now available, it will be possible to assess the impact of these viruses in food and waterborne gastroenteritis.

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ATTACHMENT OF MICROORGANISMS.

See ADHESION (PRIMARY) OF MICROORGANISMS ONTO SURFACES

B

BACTERIA: STREAMS. See STREAM MICROBIOLOGY

BACTERIAL CELL STRUCTURE. See SOIL BACTERIA

BACTERIAL CONTAMINANTS IN RESIDENTIAL ENVIRONMENTS

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Over the last thirty years, scientists have drawn attention to a number of actual and potential infection concerns related to the presence of biocontaminants in the home. The list of infections associated with the home includes food-borne infections, shigella dysentery, viral gastroenteritis, common cold virus infections, skin infections, respiratory allergies, and zoonoses associated with domestic pets. In addition, there are specific infection control issues related to the care of infants and young children, the elderly, and other immunocompromised persons in the home. This has prompted an interest in the study of microbial contamination at environmental sites and surfaces in residential settings. Most studies have investigated bacterial contaminants, and there are very few reported studies on fungi or viruses in residential environments.

This article focuses on bacterial contamination at environmental surfaces in domestic settings and the different approaches that have been employed to elicit data on this topic.

BACTERIAL CONTAMINANTS

Studies of domestic bacterial contaminants have largely been conducted under one of the following approaches: (*1*) baseline studies of either multiple or single sites that collect information on a broad spectrum of bacterial species, (*2*) searches focused on collecting data on specific pathogens at environmental sites in the absence of illness in the home, (*3*) pathogen searches at environmental sites related to the presence of a known human illness, (*4*) cross-contamination studies designed to elicit information and understanding on the potential for the transfer of potential pathogens from and via environmental sites, or (*5*) disinfectant studies that attempt to evaluate household disinfectant products under "in-use" conditions in the home.

BASELINE STUDIES

A number of multiple site baseline studies have been conducted in volunteer homes in the United States and in the United Kingdom. In some of these studies, sites and surfaces throughout the home have been examined (1,2), whereas in others, kitchens and bathrooms (3) or kitchens only (4,5) have been examined. There does not appear to be any significant difference in the data based on the regional or national distributions of the homes in these surveys. This is perhaps not surprising as, in general, the lifestyles are relatively similar in the developed world, and most of the homes in these surveys probably fall into a broadly similar socioeconomic distribution. Climatic conditions do not appear to have made a significant impact on the microbiology of surfaces inside the home.

Sampling Techniques

A number of different sampling techniques have been employed. Hard, flat surfaces such as counter tops have been sampled with a combination of Rodac plates (1,2), contact slides (2), and moist cotton swabs (1–5). A "sweep plate" method has been reported for dry fabrics (1) and for premoistened swabs. Kitchen rags and sponges have also been sampled by wetting them with a sterile solution and then collecting a small quantity of this fluid by squeezing it out of the rag or sponge (3,4). Settle plates have been used for air sampling (2) and liquid samples from toilets and waste traps have been collected by pipette and transferred to contact slides in their containers.

The different sampling techniques that have been employed make it difficult to draw conclusions from specific comparisons between the studies. However, as the following sections will show, it is possible to see common trends in the results. While sampling techniques have changed little, laboratory techniques have become much more sophisticated over the period in which these studies took place. In addition to searching for evidence of contamination with *Salmonella* species, more recent studies have specifically looked for pathogens such as *Yersinia, Listeria*, and *Campylobacter*. The role of these organisms in food-borne illness and their culture requirements was not widely known before the last decade.

Bacterial Species

The range of organisms examined in baseline surveys of all sites and surfaces in the home is given in Table 1.

BACTERIAL CONTAMINATION IN DOMESTIC KITCHENS

In total, results for 28 different kitchen sites have been reported, as shown in Table 2. A factor that seems to have a major impact on the type of species and levels of contamination, at any given site, is whether the site is wet

Table 1. Bacterial Species Isolated from Sites and Surfaces in the Home

Enterobacteria
E. coli
K. pneumoniae
Klebsiella spp.
Proteus mirabilis
Salmonella spp.
Citrobacter freundii
Citrobacter spp.
Ent. Cloacae
Ent. Agglomerans
Enterobacter spp.
Serratia spp.
Pseudomonads
P. aeruginosa
P. maltophilia
P. cepacia
P. putrifaciens
P. fluorescens
Pseudomonas spp. (nontypable)
Aeromonas hydrophila
Others
Yersinia enterocolitica
Campylobacter spp.
Listeria monocytogenes
S. aureus
Bacillus cereus
Bacillus spp.
Streptococci
Micrococci

Table 2. Sites and Surfaces Sampled in Domestic Kitchens

Wet Sites
Sink surface and drain rim area
Sink drain
Dishcloth/sponge
Sink drainer
Sink brush and sink bowl
Cleaning utensils
Soap dish
Dry Sites
Food Contact Surfaces
Worktop and cutting board
Refrigerator surfaces
Cooker top/kitchen table
Cutlery and crockery
Food shelf and vegetable rack
Hand Contact Surfaces
Sink taps/faucet handles
Tea towel/hand towel
Handles
General Surfaces
Kitchen floors
Trash barrels
Window sill
curtains
radiator
Ceramic tiles

or dry. Dry sites can be further divided into food contact surfaces, hand contact surfaces, and general surfaces.

For all kitchen sites, species of *Bacillus* and coagulase-negative micrococci are almost universally present. Results for wet sites indicate some common trends although differing sampling, culturing, and counting techniques are probably responsible for some of the variations in the frequency of occurrence and the levels of contamination for any particular species. Other parameters such as the occurrence of food preparation or cleaning and disinfection before sampling may also impact the data.

Highest recovery for *Escherichia coli* and other coliforms is associated with sinks, sink drains, and to a lesser extent, draining boards. High total and fecal coliform counts of 10^5-10^7 per sample area also have been commonly recorded at sink surfaces (5) and around the rim of the sink drain (3). Counts as high as 10^{11} per sample area for *E. coli* have been recorded from sink surfaces (1). Overall, the recovery of *E. coli* from sink surfaces ranged from 4% to 81% of samples. Recovery of *E. coli* from drains has been somewhat more consistent, ranging from 39% to 65% of samples, probably reflecting the influence of moisture on bacterial survival and recovery.

High total and fecal coliform counts of 10^7 to 10^9 also are associated with sponges, with more than 65% of samples yielding these organisms on occasion (5). The presence of *E. coli* and *Pseudomonas aeruginosa* also has been recorded in baseline studies of these items (1,2,4).

Data for dry sites show a different picture with lower recovery of Enterobacteria and generally, lower coliform counts. Even so, for food contact surfaces, total and fecal coliform counts range up to 10^6 per sample area on counter tops and cutting boards, refrigerators, and kitchen tables. The presence of *E. coli* is recorded on worktops and cutting boards, refrigerator surfaces, cookers, clean cutlery/crockery, and on food shelves. Fecal coliforms have also been recovered from 24% of cutting boards, 20% of kitchen tables, and 10% of refrigerator surfaces in one study (5). *Staphylococcus aureus* was recovered from 7.4% of cutting boards in one study (2) and *Pseudomonas* spp. have been recovered from 37% of refrigerator surfaces.

For hand contact surfaces, sink taps are often found to be contaminated with Enterobacteria. Fecal coliforms have been isolated from about 32% of sink taps with average counts of 10^3 to 10^4 and *S. aureus* has been isolated from up to 48% of kitchen towels (1). Although some studies have found that hand contact surfaces such as handles showed only species of *Staphylococci* and *Bacillus*, average total and fecal coliforms of 10^6 and 10^2, respectively, per sample area have been recorded from the refrigerator handle (3).

General sites such as kitchen floors and kitchen trash barrels generally show low levels of contamination with a range of different organisms and high levels of *Bacillus* and *Micrococci* spp. *Pseudomonas* species have been recovered from 37% of uncarpeted kitchen floors (2) and total and fecal coliforms counts of 10^4 and 10^2 per sample area, respectively, have been recorded (3).

Bacterial Contamination in Domestic Bathrooms and Toilets

Data are available for 25 bathroom and toilet sites, as shown in Table 3. As with sites in the kitchen, the influence of moisture on the occurrence of Enterobacteria and on high levels of contamination is also observed at bathroom and toilet sites. Highest counts have been observed at diaper buckets, face cloths, cleaning cloths, bath surfaces, bath and basin drain rim areas, and drain pipes. Interestingly, toilet water samples generally yield low counts, indicating the effectiveness of toilet flushing as a means of reducing counts here. *Escherichia coli* and other Enterobacteria were most often isolated from the potty, the diaper bucket, the toilet brush, toilet water and toilet bowl, bath surface, bath and basin drain rim areas, and face cloth. One study reported finding *S. aureus* on 44% of towels and 20% of bathroom floors (1).

Bacterial Contamination in General Living Areas

There is little published in the literature on bacterial contamination at general living area. A total of 11 sites have been reported from only two studies (1,2), comprising the following: living rooms (carpet, curtains, upholstery, table, window sill, stair rail, and telephone mouthpiece) and bedrooms (bed sheets, blankets or duvets, carpet, and dressing table).

The predominant organisms at sites in living rooms and bedrooms are species of pseudomonads, micrococci, and bacillus. In general, the incidence of *E. coli* and other Enterobacteria and also *S. aureus* is low at these sites and contamination levels are also low.

Table 3. Sites and Surfaces Sampled in Domestic Bathrooms

Wet Sites
Toilet bowl
Toilet water
Toilet brush
Diaper bucket
Basin surface
Bath surface
Bath and basin drains
Bath and basin drain rim area
Facecloth
Toothbrush
Bathroom cleaning cloth
Dry Sites
Hand Contact Surfaces
Toilet seat
Toilet handle
Toilet door handle
Potty
Basin and bath/faucet handles
Soap dish
Towels
General Surfaces
Floors
Countertop
Drapes
Window sill

Sponges, Rags, and Other Fabrics

A number of bacterial surveys of household sponges, rags, and other fabrics have been published over the last 30 years. It has long been established that dishcloths, tea towels (6) and hand towels (7) can support high levels of bacterial contamination.

Bacterial counts, carried out by both contact plate and serial dilution methods on reusable kitchen rags, have shown that such items are contaminated on average with more than a billion bacteria per rag after only one day's normal domestic use (8). After further use, counts can reach more than 10 billion per rag. Different bacterial species have been isolated from sponges and dishrags (9) including species of *Salmonella*.

Investigations have shown that soiled clothes are heavily contaminated with potentially pathogenic microbes (10,11). Various studies have looked at the potential for biocontaminants to survive the domestic laundry wash processes. One study (12) described the spread of staphylococcal skin infections among families sharing laundry facilities in a closed student community. The machine wash temperatures of up to 149 °F/65 °C were found to be inadequate for disinfection. A review (13) of the changes that have been made to household laundry practices in recent times indicates the impact that these changes have had on textile cleaning and hygiene assurance. Measures to reduce the environmental impact of household washing machines have stressed the ability to reduce bacterial contamination to safe levels.

PATHOGEN SEARCHES

A number of surveys have incorporated isolation methods targeted at specific primary and opportunistic pathogens. Further, some of the organisms isolated in baseline surveys (1–5) may be considered as pathogens, although they were not in that category at the time of sampling. Pathogens isolated in the baseline surveys are *S. aureus, Salmonella* spp, *P. aeruginosa*, *Bacillus cereus*, *Listeria monocytogenes*, *Yersinia enterocolitica*, and *Campylobacter*.

Species of *Listeria* have been detected in 47.4% of 213 houses investigated (14) with dishrags and the area around the bath drain being most frequently contaminated. *Listeria monocytogenes* was present in 21.1% of homes. Highest numbers (10^4 per object) were found in dishrags and washing-up brushes. Lower numbers (up to 10^3 per object) were obtained from kitchen sinks, refrigerator vegetable compartment samples, and toothbrushes.

CROSS-CONTAMINATION STUDIES

For an infection to result from contact with contaminated environmental surfaces, a number of criteria must be in place. These include the presence of an infectious

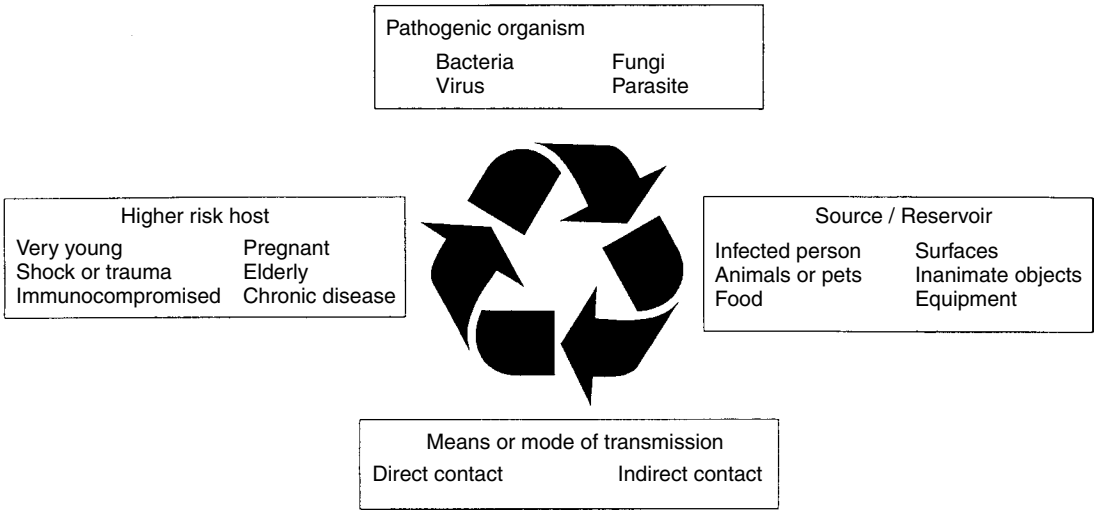

Figure 1. Factors necessary for the transmission of infectious microorganisms via contaminated surfaces.

agent, an environmental source or reservoir for pathogenic contaminants, a means or mode of transmission, and the presence of a host. Although anyone may potentially acquire an infection from a contaminated surface, more often than not, the host is likely to be characterized as at higher risk for infection. When linked together, these criteria increase the probability that disease will occur via contaminated surfaces, as shown in Figure 1 (15).

A number of experiments designed to assess the potential for cross-contamination in the domestic environment have been performed in homes. It should be noted that many other experiments on cross-contamination have been performed in laboratories, under conditions that mimic the home environment and that these are not reported here. Such studies have been reviewed by Scott and coworkers (15).

The microbiological hazards of household toilets droplet production was assessed in the mid 1970s (16). Detection of seeded bacteria and viruses on surfaces in bathrooms after flushing indicated that such organisms had become aerosolized and may present an aerosol infection hazard in older toilet designs. Subsequent studies of domestic toilets and bathrooms have not detected large numbers of toilet contaminants on surrounding surfaces, suggesting that under normal domestic circumstances, flushing is an effective means of removal of bacterial contaminants.

The potential for cross-contamination in kitchens resulting from the preparation of frozen chickens has been examined (17). Raw chickens were seeded with *E. coli* K12 and prepared in 60 kitchens. Cross-contamination occurred in a high proportion of the kitchens examined and contaminants were found on surfaces such as chopping boards, dishrags, and sinks, even after rinsing and washing-up actions occurred. The routine preparation of chicken that is naturally contaminated with *Salmonella* and *Campylobacter* spp. has been found to cause wide dissemination of these organisms to hand and food contact surfaces throughout the home kitchen (18).

ILLNESS STUDIES

Studies in homes with active *Salmonella* infections indicate that environmental contamination may play a role in the development of *Salmonella* infections, especially in young children (19,20). Infecting *Salmonella* serotypes have been isolated not only from human occupants, but also from pets, kitchen sinks and other kitchen surfaces, floors (19,20), and from dishrags (personal communication). In addition, serotypes of *Salmonella* similar to those that have caused infection in the home have been detected in biofilms under the toilet rim several weeks after the infectious outbreak occurred (21). An outbreak of *Staphyloccus aureus* skin infections among families sharing laundry facilities demonstrated the potential cross-contamination risk posed via household fabrics (22).

DISINFECTANT BENEFIT STUDIES

With the growing interest in the positioning of disinfectant products in the home, a number of studies have attempted to evaluate the potential benefit of disinfectants and disinfection processes in reducing microbial contamination at environmental surfaces in the home. This is an area of consumer-related microbiology that is likely to continue to develop in order to fulfill requirements for product development, testing, and support.

In-use tests performed in homes show that the targeted use of household disinfectant products on a range of surfaces can produce substantial reductions in bacterial contamination (3,5,18,23) but that the maximum protection afforded by disinfection is relatively brief; three to six hours after disinfection, contamination levels are only marginally less than those observed at pretreatment (23), indicating that the timing of disinfectant application is a critical factor. In addition, results for the use of detergent and hot water cleaning at fixed surfaces such as counters show that reductions in contamination levels cannot be reliably achieved (18)

and that there may even be an apparent increase in contamination due to surfactant or mechanical break-up and redistribution of cell aggregates (23).

In addition to simple disinfection benefit studies, the data on bacterial contamination and cross-contamination in the home is now being used to develop hygiene practice models for surface cleaning and disinfection, using similar concepts to those employed in the food industry, using the hazard analysis and critical control point concept (HACCP) (15).

FUNGAL CONTAMINATION

There have been a small number of studies investigating the fungal species that are present in the indoor air of houses (24–27). Species of Penicillium, Cladosporium, and Aspergillus are predominant and there is considerable variation concentrations between houses. Studies have indicated a link between the presence of both airborne fungi and airborne bacteria in the home with the incidence of respiratory allergies such as asthma (28,29). In addition to generally damp conditions, factors such as textile floor coverings and indoor storage of organic household waste have been linked to the presence of increased levels of fungal markers in homes (30). Air-conditioning equipment, humidifier reservoirs, dehumidifier drip pans, and showerheads can all harbor molds and mildews. (For more details, see FUNGI AND INDOOR AIR.)

VIRAL CONTAMINATION

There are a number of published studies that indicate that various viral species survive for significant periods on dry surfaces and that contaminated surfaces can play a role in virus transmission in settings such as day-care centers, as reviewed by Scott and coworkers (15). However, at this time, there are no published studies indicating the prevalence of viral species at environmental sites and surfaces in the home.

HYGIENE PRACTICES IN THE HOME

It has been proposed that there are three general categories of sites and surfaces in the home where the risks of bacterial contamination and cross-contamination are highest (2). These are known as

- Reservoirs (toilets, diaper buckets, sinks, drains, etc.) where bacterial contamination is high and there is often the potential for bacterial multiplication.
- Reservoir-disseminators (wet cleaning utensils such as rags, sponges, and mops) where bacterial contamination is high and there is the potential for direct transfer of this contamination to other surfaces whenever these items are used.
- Hand and food contact surfaces (kitchen counters, cutting boards, faucets, handles, laundry, floors, etc.) where there are generally lower levels of contamination but there is a constant potential for cross-contamination to other crucial surfaces such as high-risk foods or the hands.

Effective hygiene practices should include steps to limit the potential transfer of pathogenic microbes from these areas to other more sensitive surfaces such as food and hands. This is especially important when there are people present who are at higher risk for infection (i.e., the young, the elderly, and those who are immunocompromised through existing illness and/or treatment) or when there is potential for increased levels of environmental contamination, such as might occur during incidents of enteric infection in the home. Encouraging the concept that home hygiene is a series of interrelated practices and procedures all based on the same underlying microbiological principles allow the opportunity for a rational approach based on risk assessment (15). The hygiene processes used in the home include the use of soap and detergents, the use of heat (especially for cooking and washing), the use of drying, the use of mechanical action as part of the washing process, and the use of chemical disinfectants. An effective hygiene strategy for the home targets opportunities to prevent or reduce the risk of cross-contamination using effective processes and an understanding of best practices.

CONCLUSION

The study of biocontaminants in the home is a relatively new field but one that is likely to continue to expand, as scientists try to better understand the impact that biocontamination in our immediate living environment has on our overall health. In an era that has already been described as "postantibiotic," there is a growing indication that more emphasis will be placed on effective hygiene practice in our daily life as a means of reducing the potential for infection. But in order to develop effective environmental hygiene practices, we need a better understanding of the nature of the microbial contamination in the environment. At this time, there is a body of data concerning bacterial contamination on surfaces, however, the data are not yet comprehensive and there are many gaps in the knowledge base. For example, there is still little information available on the indoor air quality of domestic dwellings or on the role that the many commensal bacterial species may play in cell-mediated disease and allergic responses. Further, there is even less information about the presence and impact of fungi and algae in domestic settings. There is almost no published data on virus sampling in homes. Viruses are a major cause of domestic infections, and data on the role of inanimate surfaces in the transfer of virus infections would be invaluable in further characterizing the risk posed by contaminated surfaces in the home. To expand the knowledge base in the emerging field of domestic microbiology, further research is needed to complete the picture of biocontamination in the home, to develop and refine risk analysis for environmental surfaces in the home, and to link the potential benefits of hygiene practice in the home, either directly with reduced infections or with other meaningful indicators.

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BACTERIAL PATHOGENS IN WASTE STABILIZATION PONDS.

See WASTEWATER STABILIZATION PONDS

BACTERIAL PHYTOSTIMULATORS IN THE RHIZOSPHERE

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From an agricultural point of view, "soil" refers to the broad environment from which plants obtain mechanical and nutritional support (1). That part of the soil, which is adjacent to the plant root and therefore under its influence is generally denominated as the rhizosphere. As a result of their activity, living roots secrete a wide variety of compounds that alter the physical and chemical properties of their surrounding environment.

Defining the rhizosphere is still a matter of debate, and even more of choice. Throughout the history of rhizospheric microbiology research, investigators have been arbitrarily determining what they consider to be the rhizosphere in their studies. In broad practical terms, the rhizosphere has been considered to be the soil attached to the roots when extracting them from their growth medium, whereas a more restricted view regards the rhizosphere as the relatively small amount of soil particles that remain attached to the roots after they are washed from the residual "bulk" soil. This aspect becomes even more complicated when considering that the qualitative and quantitative distribution of the secreted compounds from the roots is largely influenced by plant species and development, environmental conditions, and location along the roots (2). Hopefully, the increasing exploitation of modern molecular tools by microbial ecologists will be helpful in delimiting the rhizosphere "borders."

In the rhizosphere, bacteria can be enriched by a factor of 100 as compared with their number in the surrounding soil (3). The main factors responsible for this "rhizosphere effect" are the exudation of photosynthetically derived carbon sources (sugars, amino acids, organic acids, phenolic compounds) and the sloughing off of root cap cells and older sections of the cortex (3,4). In turn, these secretions affect bacterial activity and, therefore, the microbial composition of the rhizosphere. Thus, the bacterial population of the rhizosphere generally differs from that present in soil areas not influenced by the roots, not only quantitatively but also in its qualitative composition and distribution (3), although in some studies no differences were observed (5). Table 1 summarizes the effects caused by roots on the rhizosphere.

Among the bacteria living in the rhizosphere, some are able to affect plant growth, either positively or negatively. Rhizospheric bacteria that favorably affect plant growth and yield of many commercially important crops are designated plant growth promoting rhizobacteria (PGPR). PGPR are able to exert positive effects on plants by various mechanisms. These include those that can directly cause plant growth promotion (PGP) by improving seed germination, root development, mineral nutrition, and

Table 1. Factors Derived from the Activity of Living Roots That Influence the Composition of Rhizosphere Microbial Communities

Root Activity	Factors
Secretions/Exudates	Release of low- or high-molecular weight compounds used as nutrients
	Effects on soil solution pH and conductivity
	Release of extracellular enzymes
Lysates	Release of compounds from aging/dead epidermal and cortical cells
Synthesis of mucilage (mucigel)	Changes in hygroscopicity
	Increased root area available for bacterial colonization
	Protection of rhizosphere bacteria from bacterial predators
Respiration	Effects on air composition

Table 2. Modes of Action of Plant Growth Promoting Rhizobacteria (PGPR)

Effects	Mechanisms
Direct effects — Phytostimulation	Production of plant growth regulators (PGRs): auxins, gibberelins, cytokinins
	Biological nitrogen fixation (biofertilizers)
Indirect effects — Biological control	Production of antibiotics and bacteriocins
	Production of cyanide acid (HCN)
	Production of siderophores
	Competition for colonization sites and nutrients
	Systemic acquired resistance (SAR) of the plant against pathogens
	Production of chitinases and glucanases and hydrolysis of fungal cell walls

water utilization (phytostimulation). Others affect plant growth via indirect effects that involve suppression of bacterial, fungal, and nematode pathogens (biological control) (6). The main modes of action of PGPR are summarized in Table 2.

Growing interest in sustainable agriculture has encouraged PGPR research. Many groups are currently investigating possible modes of action of these bacteria at the molecular level using DNA recombinant techniques. However, because of the complexity of the rhizospheric environment and the infinite possibilities of plant-soil-microbial interactions, in most cases, the suggested mechanisms have not yet been fully elucidated. Nevertheless, substantial advances have been made in recent years, as reviewed later.

In this chapter, we will focus mainly on the ecology, modes of action, and utilization of agrobiotechnological inoculants of PGPR that positively affect plant growth by direct mechanisms (phytostimulators). Rhizospheric bacteria capable of improving plant growth by biological control of soilborne diseases, and those able to invade the plant and develop nitrogen-fixing symbioses such as *Rhizobium* and *Frankia*, will not be discussed. Rhizobia will be mentioned only in relation to their interactions with PGPR in the growth promotion of legumes.

MECHANISMS OF ACTION OF PHYTOSTIMULATING BACTERIA

Biological Nitrogen Fixation (BNF)

There are many free-living nitrogen-fixing rhizobacteria contributing fixed nitrogen from the atmosphere to plants, including agronomically important crops (7,8). Bacterial diazotrophs in the rhizosphere belong to a wide variety of genera (Table 3), the most investigated of which have been *Azotobacter* and *Azospirillum*, although many others have been reported to be associated with tropical grasses and rice (7).

Nitrogen fixation by rhizospheric bacteria is sometimes referred to as *associative symbiosis*, and has been extensively estimated by a wide variety of methodologies, including long-term nitrogen balances in soils, estimation of nitrogen contents by the Kjeldahl method, acetylene reduction assay, and the ^{15}N isotope dilution method (9). However, to date, quantification and interpretation of the results have been difficult and controversial. It is estimated that the contribution of nitrogen fixation by free-living rhizobacteria in the rhizosphere of crops such as wheat, sorghum, and maize is in most cases on the order of 1 to 10 kg N ha^{-1} yr^{-1} (10). It may be higher in rice because of the activity of nitrogen-fixing blue-green algae, and in some tropical grasses (7). Although positive, these amounts are, in fact, of minor importance when compared with the amounts of nitrogen fertilizers applied in modern agriculture, which are on the order of 150 to 250 Kg N ha^{-1} yr^{-1}.

Many agriculturally important grasses are associated with endophytic diazotrophic bacteria (Table 3). They have mainly been isolated from plants in which significant BNF has been demonstrated, particularly in some Brazilian sugarcane and rice cultivars (6,11) and in Kallar grass in water logged salty soil in Pakistan (12).

Production of Plant Growth Regulators (PGRs)

Evaluation of the contribution of BNF to plant growth is complicated by the fact that most diazotrophic rhizospheric bacteria and endophytes (Table 3), and many other soil microorganisms, produce a wide variety of PGRs such as auxins, cytokinins, gibberelins, ethylene, and abscisic acid that may alter plant growth (8,13).

A common observation following inoculation with bacteria from the genus *Azospirillum* is an increase in the number of root hairs and lateral roots. This leads to an improved uptake of water and nutrients, resulting in significant increases in crop yields (14). Although it has been proposed that changes in root morphology by *Azospirillum* are caused mainly by the

Table 3. Phytostimulating Bacteria and Their Modes of Action (6–8)

Organisms	Mechanisms
Rhizosphere diazotrophs	
Azospirillum brasilense *Azospirillum lipoferum*	Mainly through production of plant growth regulators (PGRs), mostly auxin. Proliferation of root hairs and branching. Improved mineral and water uptake. Increased nodulation and biological nitrogen fixation (BNF) (by *Rhizobium*) in legumes. BNF: 5 to 10 kg N ha^{-1} yr^{-1} in Gramineae. Increased crop yields in inoculated fields at intermediate fertilizer levels: 10 to 30%
Azotobacter chroococcum	BNF: 5 to 10 kg N ha^{-1} yr^{-1}. Yield increases of 10 to 20% (rhizosphere habitat controversial)
Azotobacter paspali	BNF: increased N-content of about 10% in *Paspalum notatum* cv batatais. Production of PGRs. Proliferation of root hairs and branching
Pseudomonas stutzeri	BNF in rice
Klebsiella pneumoniae and *Klebsiella oxytoca*	BNF in maize and rice. Root hair proliferation in *Poa pratensis*
Enterobacter	BNF in rice
Burkholderia	BNF in maize
Endophytic diazotrophs	
Acetobacter diazotrophicus	BNF in sugarcane (for some cultivars, 50 kg N ha^{-1} yr^{-1}), coffee, and sorghum
Herbaspirillum seropaedica	BNF in sugarcane
Azoarcus	BNF in Kallar grass and rice
Rhizobium leguminosarum bv trifolii	Plant growth promotion in rice
Nondiazotrophic	
Bacillus	Promotion of nodulation and BNF (by *Rhizobium*) in chickpeas and common bean
Serratia liquefaciens and *S. proteamaculans*	Promotion of nodulation and BNF (by *Bradyrhizobium*) in soybean
Pseudomonas fluorescens	Promotion of nodulation and BNF (by *Bradyrhizobium and Rhizobium*) in soybean and peas
Pseudomonas putida	Promotion of nodulation and BNF (by *Rhizobium*) in common bean. Improved phosphate uptake
Pseudomonas putida GR 12.2	Root elongation. ACC deaminase. Production of PGRs

bacterial production of indole-3-acetic acid (IAA) (14,15), production of gibberellins and cytokinins may also be involved (14). Most beneficial bacteria synthesize IAA via the indole-3-pyruvate pathway in which tryptophan is first transaminated to indole-3-pyruvic acid, then decarboxylated to indole-3-acetaldehyde, which is oxidized to IAA. Genes encoding indole-pyruvate carboxylase (*ipdC*) have been isolated from *Azospirillum* (16). An *ipdC* Tn5-mutant of *Azospirillum brasilense* that shows 10% residual IAA production in comparison with the wild type has been obtained and shown to possess reduced ability to promote root hair proliferation (15).

Involvement of ethylene in plant growth promotion has also been investigated. It was demonstrated that a small number of soil bacteria contain the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase. This enzyme hydrolyzes ACC, the immediate precursor of ethylene in plants, to yield ammonium and α-ketobutyrate. Bacteria possessing ACC deaminase activity may therefore grow using ammonium as the sole nitrogen source. The beneficial rhizobacterium *Pseudomonas putida* strain GR 12–2 stimulates root elongation of different plants. This bacterium was found to contain ACC deaminase. Mutants lacking ACC deaminase activity were not able to promote root elongation of canola seedlings, implying that this enzyme participates in root growth promotion by this bacterium (17).

Beneficial Effects of PGPR on the *Rhizobium*-Legume Symbiosis

The effects of PGPR on root hair proliferation and root branching, mineral, and water uptake and growth can also be observed in legumes (8). Positive effects were observed for several *Azospirillum*-inoculated legumes in the field and greenhouse, and under gnotobiotic conditions. The increases in dry-matter production and nitrogen content were attributed to early nodulation, increased nodulation, and higher nitrogen fixation rates, and to a general improvement in root development (18).

Rhizobium infection occurs via the formation of infection threads in the root hairs. Stimulation of nodulation following inoculation with *Azospirillum* may be due to the differentiation of a greater number of epidermal cells into infectable root hairs (19). In addition, *A. brasilense* was shown to cause an increase in the secretion of *nod* gene inducing flavonoids by common bean and alfalfa roots, resulting in the appearance of a greater number of upper nodules following coinoculation with *Rhizobium* and *Azospirillum* in comparison with inoculation with *Rhizobium* alone (20,21).

Several works have been published on the interaction of other rhizobacteria (e.g., *P. putida*, *Pseudomonas fluorescens*, *Bacillus polymyxa*, *Enterobacter* and *Serratia*) with the *Rhizobium*-legume symbiosis (Table 3). Despite data supporting positive effects on nodulation, nitrogen fixation, and yield attributes, little is known about the mechanisms involved in these interactions, but it is likely that in some cases they are similar to those described for *Azospirillum* (8).

Phosphorus Solubilizers

Phosphate deficiency in crops can be diminished by the use of bacteria that act directly as phosphate solubilizers in the rhizosphere, and indirectly by stimulating root excretion of organic acids and/or mycorrhizal associations. Enhanced excretion of organic acids results in increased phosphate solubilization and hence in increased phosphate uptake (22).

Insoluble inorganic phosphorus is largely unavailable to plants, but many microorganisms can bring the phosphate into solution. Species of *Pseudomonas*, *Mycobacterium*, *Micrococcus*, *Bacillus*, and *Flavobacterium* are active in that conversion. These bacteria grow in media with $Ca_3(PO_4)_2$ and apatite as sole phosphorus sources. Not only do the microorganisms assimilate the element, they also render a large portion of it soluble. The many phosphate-dissolving microorganisms found near roots may appreciably enhance phosphate assimilation by higher plants (22).

Inoculation with *A. brasilense* was shown to significantly enhance H_2PO_4 uptake by maize in hydroponic systems, on the order of 30 to 50% over noninoculated controls. In field-grown sorghum and wheat, the increase was in the range of 10 to 30%. In that case, the increases in phosphorus uptake could have resulted from increased root respiration (10).

BACTERIAL FEATURES FOR RHIZOSPHERE COMPETENCE

Inoculation with PGPR is becoming a common practice in agriculture. It is therefore important to understand what microbial traits are involved in rhizosphere competence, which is the relative root-colonizing ability of a rhizobacterium (23). The role of some intuitively defined properties has been not fully demonstrated, with contradictory results found in the literature. In this section, the focus will be on those bacterial features that have received the greatest attention.

Metabolic Versatility

In aerobic environments, most bacteria are highly versatile and can use a large variety of organic compounds as carbon and energy sources. One of the best-characterized examples of metabolic versatility are bacteria belonging to the genus *Azospirillum*. These are free-living nitrogen-fixing rhizobacteria found in close association with plant roots, where they exert beneficial effects on plant growth and yield of many agronomically important crops (14). A variety of carbon sources may be used by azospirilla, (Table 4) for example, salts of organic acids such as malate, succinate, lactate, and pyruvate, and various sugars or amino acids. Under aerobic conditions, oxygen is the preferred respiratory electron acceptor. However, in the absence of oxygen, most strains can use nitrate, nitrite, and nitrous oxide as electron acceptors of the respiratory chain (24,25).

In addition to the monomeric or dimeric carbohydrates, *A. brasilense* and *A. lipoferum* can degrade polymers such as xylan, starch, cellulose, and carboxymethyl cellulose, which are available in the rhizosphere of host plants (26,27). Furthermore, *A. irakense* has been found to grow with pectin as sole carbon source, and some *Azospirillum* spp. are able to grow on methanol or other C1 compounds (25).

Relatively high growth rates and the ability to effectively use a wide range of organic compounds seem to be important factors for rhizosphere competence. However, it was found that rhizosphere and soil bacteria do not significantly differ in their rates and extent of growth in the presence of exudates from soybean seeds and plants (28). In a large study searching for determinants of rhizosphere competence, Jjemba and Alexander (29) could not find a correlation between the growth rate of some of the tested bacteria and their ability to colonize the rhizosphere.

Accumulation of Storage Material

Microorganisms can accumulate a variety of storage compounds such as glycogen, polyphosphate, and sulfur compounds. It is thought that the accumulation of these storage materials enhances competition and survival of bacteria under stress.

A wide variety of microorganisms are known to produce intracellular energy and carbon storage compounds, generally described in the past as being poly-β-hydroxybutyrate (PHB). In recent years, it has been found that in most cases, the polymers are polyhydroxyalkanoates (PHAs)

Table 4. Utilization of Different Carbon Sources by *Azospirillum* spp. (25)

Carbon Source	*A. lipoferum*	*A. brasilense*	*A. amazonense*	*A. halopraeferens*	*A. irakense*
D-Glucose	+	−	+	−	+
Glycerol	+	+	−	+	−
D-Mannitol	+	−	−	+	−
Pectin	−	−	−	−	+
D-Sorbitol	+	−	−	−	−
Sucrose	−	−	+	−	+

Note: + indicates utilization of the carbon source.

comprising copolymers containing different alkyl groups at the β-position (30).

It has been proposed for diverse ecological systems that the accumulation, degradation, and utilization of PHAs by several bacteria may favor their establishment, proliferation, survival, and competition, especially in competitive environments in which carbon and energy sources are limiting factors, such as those encountered in soil and in the rhizosphere (31).

In situ studies of the role played by PHAs on bacteria in the rhizosphere are very scarce, because of the lack of an accurate methodology for measuring their levels. Although mutants unable to synthesize PHAs have been isolated and genetically engineered (32–34), most PHA-negative mutants were examined for their effects on symbiosis and cellular metabolism, whereas their role in rhizosphere competence was not addressed.

Electron microscopy studies of the rhizosphere have shown many bacteria containing PHAs. The C:N ratio of the rhizosphere is estimated at 20. This ratio favors PHA accumulation in *Azospirillum* and *Azotobacter*, and in other rhizospheric bacteria in culture (31).

The possible functions of PHAs in *A. brasilense* strain Cd were investigated, with PHB being the only PHA accumulated by this bacteria (31,35). Degradation and synthesis of the polymer occurred in a biphasic pattern under starvation conditions and were affected by the PHB content of the cells. Under starvation, the survival and respiration rate of bacteria containing about 40% PHB (of total dry weight) were higher than in bacteria containing 5% PHB. Polymer-rich cells fixed atmospheric nitrogen in the absence of exogenous carbon and combined nitrogen. Under stress and adverse conditions, such as UV-irradiation, desiccation, and osmotic pressure, PHB-poor cells died more rapidly than PHB-rich cells. It was concluded that PHB might provide *A. brasilense* with the ability to survive under conditions of starvation and stress, by serving as a sole carbon and energy source (35). Furthermore, in many cases, PHB accumulation is accompanied by flocculation (36), which renders cells highly resistant to desiccation. These properties could conceivably be important for the proliferation of *Azospirillum*, providing a competitive edge over other microorganisms in the rhizosphere (31,35).

In *Rhizobium* bacteroids, PHAs are mobilizable, energy-yielding reserves that provide endogenous carbon substrates for support of nitrogen fixation when exogenous carbon is not available. They may also serve as a carbon and energy supply outside the bacteria (37).

Production of Siderophores

Despite the fact that iron is only a minor nutrient for the growth of most soil microorganisms, it is essential. Although it is abundant in terrestrial habitats, its availability is limited as a result of the low solubility of the ferric ion (1). Therefore, many microbes have developed pathways to obtain it. Many microorganisms overcome this iron unavailability by producing siderophores, specific low molecular weight Fe^{3+}-chelates, and a complementary uptake system, the biosynthesis of which is regulated by the level of available iron (38). There exists a wide variety of siderophores, with organisms producing one or more different kinds. Siderophores are present in the soil and in the rhizosphere, and their utilization is important for the competitive growth of most microorganisms.

Fluorescent pseudomonads produce siderophores termed *pseudobactins*. They seem to play an important role in rhizosphere competition, enhancing their capability as biocontrol agents and as growth promoters (39).

Scher (40) gave indirect evidence of the role of siderophores in competing for iron in *Fusarium*-suppressive soils. In that work, biocontrol of *Fusarium* wilt by *P. putida* was lost when available iron was added, whereas when a Fe^{3+}-chelate with a high binding constant was added, biological control was enhanced. It was also shown that a siderophore-negative mutant of *P. putida* loses its suppressive property against carnation wilt (41). In potato stem cuttings treated with a siderophore-negative mutant with and without the parent strain, the mutant strain multiplied on the cuttings to higher levels when the parent strain was present (42). This indicates that the siderophore produced by the parent strain made iron available to the mutant strain and played an important role in its development.

Azospirillum lipoferum was shown to produce a catechol-type siderophore under iron-starved conditions. In addition to their established role in iron transport, the siderophores exhibited antimicrobial activity against various bacterial and fungal isolates, suggesting that the high-affinity iron uptake system of *A. lipoferum* may be of use in the competition among soil microbes for access to available iron, thereby enhancing its survival in the rhizosphere (43).

Production of Antibiotics

One of the mechanisms used by PGPR, which helps them ensure an ecological edge over other root-colonizing microorganisms, is the excretion of antibiotic substances, which exclude or reduce pressure from competitors on colonized niches. A large array of different compounds showing antibiotic activity against plant pathogens have been isolated from PGPR (44–46). Until now, the bulk of the research on antibiotics was focused on their role in biocontrol, with their significance in rhizosphere competition not being specifically examined.

Kloepper and Schroth (47) demonstrated that five PGPR fluorescent pseudomonads strains that exhibited antibiosis against the soft rot pathogen *Erwinia carotovora*, and several other bacteria isolated from the rhizosphere of radish plants, increased plant growth, but mutants that lacked antibiotic production had no effect. The parent strain, but not the mutants lacking antibiotic production, reduced the number of gram-positive bacteria and fungi in the rhizosphere.

It was shown that the higher the number of *P. fluorescens* on the root, the lower the number of take-all lesions found. When an antibiotic-negative mutant of *P. fluorescens* was tested, there was no suppression of the disease (48). Mazzola and coworkers (49) found that when a phenazine-negative mutant strain of *P. fluorescens* was added together with its parent strain to pasteurized soil to which the take-all fungus had been added,

populations of both the parent and mutant strain were maintained. However, when they were added to untreated soil, the phenazine-mutant population declined, whereas the parent strain proliferated, thereby illustrating the importance of antibiotic production for the establishment of an organism in the rhizosphere.

However, when mutants of *P. fluorescens* defective in in vitro antibiotic production and the parental strain were coinoculated at a 1:1 ratio, no differences in root establishment were found between the strains, suggesting that in this case, antibiotic production contributed little to rhizosphere competence (50).

Resistance to Protozoa

Protozoa are unicellular eukaryotic, generally motile microorganisms. They usually obtain food by ingesting other organisms or organic particles and are found in a variety of habitats, including the rhizosphere. These microorganisms are thought to play a major role in controlling the population of bacteria residing in the soil (51).

The extent of soil colonization by *Rhizobium phaseoli* was shown to be inversely related to the presence of large numbers of bacteria and protozoa. Colonization of *R. phaseoli* was improved upon suppression of protozoa with protozoa inhibitors (52). Casida (53) added representatives of several categories of bacteria to soil to determine which of them might elicit responses from soil protozoa. The protozoa did not respond to *Cupriavidus necator*, a potent bacterial predator, or to *Micrococcus luteus*, one of its prey species. *Cupriavidus necator* also had no effect on the protozoa, showing that in this case bacterial and protozoan predators do not interact. The soil protozoa did not respond to *Arthrobacter globiformis* or to *Bacillus thuringiensis.* Apparently, resistance of these microbes to protozoa enhanced their survival. The addition of *Bacillus mycoides* and *Escherichia coli* caused specific responses by soil protozoa (53).

In summary, addition of bacteria to soil did not cause a general increase in the numbers of protozoa. In other studies, the extent of colonization of a nonsterile rhizosphere by tested bacteria was not correlated to their densities in the rhizosphere containing large numbers of protozoa (29). In view of these findings, it seems that protozoa may reduce the abundance of some species of rhizospheric bacteria; however, its significance in controlling the population of bacteria in the rhizosphere is not yet well understood.

Root Adsorption and Colonization

Bacterial adherence to plant roots is thought to be an important trait in determining rhizosphere competence, and this property has been the subject of a large number of studies.

Attachment to plant roots by some rhizobacteria, including rhizobia, seems to occur in two distinguishable steps: a rapid, weak, and reversible adsorption and a slow, firm, and irreversible anchoring to the plant root surface (54). In the case of *A. brasilense*, it was suggested that an adhesin closely related to the polar flagellum is involved in the first step, and that the second step is mediated mainly by extracellular polysaccharides (54,55).

In the last decade, outer membrane proteins (OMPs) have been proposed to play a role as adhesins in invasion and adhesion processes in various gram-negative bacteria. The major OMPs of *P. fluorescens*, *Rahnella aquatilis*, and *A. brasilense* were suggested to be involved in the adsorption of these rhizospheric bacteria to plant roots (56–58).

Bacteria able to adhere to the root surface are closer to the source of root exudates than are bacteria at some distance from the root. Moreover, adhering cells are presumably more likely to be transported with the extending root. For adhering cells, the root may also serve as a basis of physical support for bacterial proliferation. However, studies carried out on root adhesion have been quite contradictory in supporting the importance of this trait for rhizosphere competence.

Chao and coworkers (59) showed that 82% of the bacteria isolated from the rhizosphere exhibit a positive agglutination reaction with pea roots, in contrast to only 30% of bacteria from bulk soil. However, it was suggested that root attachment by bacteria is, in most cases, a very widely occurring property of low specificity, being greatly influenced by the medium ionic composition (60). Studies carried out with single-gene mutants of *P. putida*, differing in root attachment ability, showed that some agglutinin-negative strains still possess good root colonization ability (61). Recently, Jjemba and Alexander (29) showed that the ability of introduced bacteria to colonize the rhizosphere of soybeans does not correlate with the capacity of low or high densities of these bacteria to adhere to the roots. The aforementioned examples suggest that the ability to attach to the roots is not a crucial determinant for rhizosphere colonization, especially when considering that only a very small percentage of the cells are firmly attached to the roots (62).

Motility and Chemotaxis

Most of the known bacterial species are flagellated by means of polar and/or lateral flagella. Flagella provide the motility needed for the bacteria to reach the most favorable niche and to compete with other microorganisms for these niches. It has been observed that in many cases when competition is absent, bacteria lose their motility and/or flagellation ability (63).

In *P. fluorescens*, many studies have suggested that motility does not affect root colonization following either seed or soil inoculation (64–66). In contrast, De Weger and coworkers (67) found that bacterial motility is required for root colonization of potato by *P. fluorescens*. A comparative study between a wild type and a nonmotile Tn5 mutant of *P. fluorescens* suggested that motility plays a role in the movement of cells along roots more than in the movement from bulk soil toward roots (50).

Many studies have been carried out on the importance of motility for nodulation of legume roots by rhizobia. Although some of them indicated that motility is not essential for root attachment or nodulation, some studies based on competition assays among wild-type and nonflagellated and/or nonmotile mutant strains

suggested that motility confers a selective advantage when competing with nonmotile strains (62,63).

In *A. brasilense*, horizontal and vertical movements in different soil types depend mainly on the presence of plant roots and on the availability of water films in soil (68). On the basis of observation of motile and nonmotile strains of *A. brasilense*, Bashan and Holguin (69) concluded that active motility is needed for interroot travel (movement among neighboring plants) by this bacterium.

Besides locomotive properties, adhesive properties have been attributed to bacterial flagella. As previously indicated, the first step in the root attachment process by *A. brasilense* is mediated by an adhesin closely related to the polar flagellum. *Azospirillum brasilense* cells lacking the polar flagellum were unable to adsorb, and purified polar flagella adsorbed specifically to wheat roots (55).

Strongly related to flagellation is chemotaxis, which allows bacteria to detect and move toward gradients of different compounds. In the rhizosphere, it may be advantageous to respond positively to chemoattractants released by plant roots that may increase metabolic rates.

Some of the most studied PGPR with respect to chemotaxis are those belonging to the genus *Azospirillum* that exhibit positive chemotaxis to a wide variety of compounds such as organic acids, sugars, amino acids, and aromatic compounds (70–72). These bacteria also possess a strong aerotactic response toward reduced oxygen pressure in the root zone when nitrogen fixation is required (73,74).

Using the β-glucuronidase (GUS) reporter system, various *A. brasilense* mutants were investigated for their capacity to initiate wheat root colonization at the root hair zones. Only nonflagellated mutants and a generally nonchemotactic mutant exhibited strongly reduced root colonization ability as compared with the wild type, thus demonstrating the requirement of bacterial motility in the establishment of the *Azospirillum*-plant root association (75).

Conversion into Forms with Increased Resistance Under Stress

Under adverse environmental conditions, some bacteria are able to convert themselves into forms that are more resistant than the vegetative form. For instance, *Bacillus* species, which are known to promote both phytostimulation and biocontrol effects in a wide range of plants (62), are able to form endospores that are highly resistant to desiccation and heat.

Species of *Azotobacter* (e.g., *A. chroococcum*) are known to form heat- and desiccation-resistant cysts endowed with a long life span. This property has been used to prepare nitrogen-fixing liquid bioinoculants useful for a variety of crops (76). Cyst formation was also reported for *A. brasilense* and *A. lipoferum* (77).

Cell aggregation is a widespread phenomenon in the microbial world. Rhizospheric bacteria such as *Rhizobium*, *Pseudomonas*, *Azotobacter*, and *Azospirillum* are able to aggregate and flocculate under both soil and laboratory conditions (58,78,79). There is evidence that this property positively affects bacterial survival under adverse environmental conditions (77,78). In addition, bacterial flocs can be produced on a large scale and be easily separated from the growth medium. This is of great interest for the production of bacterial inoculants.

On the basis of experimental evidence, Neyra and coworkers (80) proposed the generation of inoculants containing intergeneric coaggregates of *A. brasilense* and *Rhizobium leguminosarum* biovar phaseoli to improve the growth of common bean plants.

Despite much evidence indicating that the aforementioned traits improve bacterial survival under laboratory conditions, their real impact on bacterial survival in the rhizosphere has yet to be demonstrated. In addition, these resistant forms are less metabolically active than the vegetative forms, a fact that should be taken into account when preparing bacterial inoculants.

INTERACTIONS OF PGPR WITH BIOTIC AND ABIOTIC FACTORS

Because soil, the milieu in which roots develop, is inherently heterogeneous, and because of their impact on their immediate environment (see the introductory section), beneficial root-associated microorganisms meet a complex habitat. A major component of this habitat is the indigenous microbial populations that vary in structure, spatial distribution, physiology, and impact on the plant's health. Not one methodological approach for studying rhizospheric bacterial communities and their relationship is necessarily better than another, but each, within its own bias, offers a different vantage point from which a more complete and accurate picture of "life in the rhizosphere" can be reconstructed.

The physicochemical parameters that bacterial cells encounter in the rhizosphere are important factors in determining their fate. Such parameters, among others, include hydrogen and redox potentials, water tension and flow, atmospheric composition, viscosity, solution composition, nutrient fluxes, and charged surfaces. Reliable devices enabling precise acquisition of this type of data at the relevant micrometer scale have been successfully applied in microbial ecology (81) and are also being applied to study the rhizopheric environment.

Microsensors

A thorough description of microsensor devices can be found in another article of this book. Briefly, these are mostly microelectrodes responding to specific solutes or gases that, owing to their small tip diameter, are able to perform measurements at the micrometer level. A large variety of microsensors have been developed, such as ones for measuring pH, temperature, carbon dioxide, sulfide, nitrate, nitrite, oxygen, nitrous oxide, organic carbon, and methane (81–83). They have been used to study sediments, sludge, and plant rhizosphere, the latter mainly under flooded conditions (84–86).

Reporter Genes

Soil remediation has been an important incentive for the development of biosensors, namely, reporter genes, cloned under the regulation of a promoter sensitive to

specific deleterious organic or inorganic compounds. The type of genes employed usually yields a colorimetric (*lacZ, gus, xyl*, and *lux*) or a bioluminescent (*gfp*) reaction, or ice nucleation. This approach is also gaining acceptance in rhizosphere studies, with indicator organisms for iron, oxygen, sucrose, and tryptophan already being developed (87–89).

INOCULATION WITH PGPR IN A DENSE MICROBIAL BACKGROUND

Bacterial inoculants, phytostimulators, or biocontrol agents, insomuch as they colonize the rhizosphere and influence plant growth and health, certainly affect indigenous populations. Such effects may stem from direct competition for resources and/or from products of metabolic activity of the introduced microbe, or from indirect factors such as changes occurring in the root system and in exudate release following inoculation. In turn, these indigenous communities may also impede or promote the establishment of the introduced microbe. Although few such studies have been performed, this knowledge is important for increasing the performance of inocula and for evaluating the impact of the introduction of exogenous bacteria on root communities. It may also lead to the isolation of new, efficient PGPR.

Introduced Bacteria and Indigenous Rhizosphere Populations: Support, Interference, or Indifference?

The influence of natural populations on introduced inocula and vice versa is strongly dependent on the systems under study, that is, using PCR-based, culture-independent ribosomal interspacer analysis (RISA); Robleto and coworkers (90) were able to show that inoculation of common bean (*Phaseolus vulgaris* L.) rhizosphere with a trifolitoxin (TFX) antibiotic-producing *Rhizobium etli* leads to a detectable shift in bacterial populations, with bands originating from members of the α-*Proteobacteria* subgroup disappearing.

Plants inoculated with a nonproducing TFX derivative yielded a pattern identical to that of noninoculated plants. Conversely, soil bacteria can reduce PGPR root colonization; Ikeda and coworkers (91) showed that individual strains of soil fluorescent pseudomonads exhibit such deleterious effects on a fluorescent pseudomonad PGPR. Combinations of individually nonsuppressive gram-negative bacteria could significantly suppress subsequent colonization of tomato (*Lycopersicon esculentum*) roots with this particular PGPR, suggesting that less competitive strains may hinder colonization of a competitive strain. An inverse situation was found with *Frankia*, as Knowlton and coworkers (92) reported a significant increase in nodulation of *Alnus rubra* under nonsterile conditions as compared with gnotobiotic growth with *Frankia* alone. Moreover, inoculation of pepper (*Capsicum annum* L.) with a mycorrhizal fungus resulted in a strong reduction of ^{14}C root exudation, altering the composition of the rhizosphere, as detected using a bioluminescent pseudomonad (93).

Such studies are also important for assessing the impact of genetically modified organisms, microbes, and plants alike. Mahaffee and Kloepper (94) investigated whether the introduction of a bioluminescence gene in a rhizosphere isolate of *P. fluorescens* would cause detectable changes in microbial community composition. No such shift could be found by fatty acid methyl ester analysis of 7,200 colonies isolated over a two-year field study.

In contrast, a population shift was observed in the rhizosphere of *Lotus corniculatus* plants engineered to produce opines; increased levels of bacterial strains able to specifically grow at the expense of a particular opine were detected in the rhizosphere of the transgenic plants producing that opine as compared with the wild-type plants (95).

MOLECULAR METHODOLOGY FOR TRACKING INTRODUCED PHYTOSTIMULATORS

Although most studies have been conducted using classical, culture-based techniques, culture-independent modern molecular techniques are being implemented to track introduced bacteria, to monitor their physiological status in situ, to understand their relationship with the surrounding bacterial communities and to analyze these communities. More information about these techniques can be found in another article of this book and in Burdman and coworkers (8).

Reporter Genes

Inocula of engineered PGPR can be tracked using reporter genes such as β-galactosidase (96) or GUS (97,98), the latter being more adequate for direct detection on plant material because X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), the substrate of β-galactosidase, can be cleaved by root enzymatic activities. Newer approaches, combining both markers yielding stronger signals and more sensitive detection systems, such as coupled charge devices (CCD), result in greatly enhanced sensitivity. For example, a 1,000-fold increase in sensitivity was achieved using the bioluminescence *luxAB* gene system in rhizosphere-inoculated *Pseudomonas* spp. as compared with that achieved using the β-galactosidase gene (99).

The green fluorescence protein (GFP), product of the *Aequoria victoria* jellyfish *gfp* gene (100), has been demonstrated as perhaps the most versatile and efficient reporter system; it is highly stable and does not require external or internal reducing power, only illumination to fluoresce. A number of modified proteins have been engineered and exhibit absorption/emission spectral shifts resulting in a change of color, fluorescence intensity, or half-life (101,102). These genes have been successfully used in a number of studies aimed at tracking introduced bacteria on or in roots in various plant species (103,104). Although quantitative uses are possible, bioluminescent-tagged PGPR have mainly been employed for spatial, in situ studies of cell distribution along the root, in conjunction with epifluorescence or confocal laser microscopy. Moreover, the physiological activity of a particular introduced bacterium can be monitored at the single cell level using unstable variants of the GFP protein

in cells containing this particular gene under the control of a ribosomal promoter (105).

Antibodies

Antibodies raised against a phytostimulating bacterium can be particularly useful for quantifying colonization and for obtaining data on the spatial distribution of the inoculum, without relying on reporter genes, which may put a physiological burden on the cell.

The high specificity of monoclonal antibodies (mAbs) raised against specific strains of *A. brasilense* was demonstrated (106). These mAbs were then tested in situ in experiments in which the bacterial strains were coinoculated on wheat roots grown in soil where they could be specifically quantified in different root compartments by chemiluminescent ELISA (106). In situ detection and differentiation of coinoculated *A. brasilense* strains Wa3 and Sp7 was achieved on a background of root-associated bacteria concomitantly stained with 4',6-diamidino-2-phenylindole (DAPI) and rRNA-directed fluorescent oligonucleotides (107).

Polyclonal antibodies (pAbs) have been used to identify rRNA homology group I pseudomonad colonies grown from rhizosphere material (108) and for in situ tracking of each component of a mixed inoculum of *P. fluorescens* in barley roots grown under sterile conditions (109). Schlöter and Hartmann (110) used a monospecific pAb to quantify and track *R. leguminosarum* biovar trifolii R39 inoculated on roots of various legumes and nonlegumes using chemiluminescent ELISA and fluorescein isothiocyanate (FITC)-coupled antibodies, respectively.

rRNA Probes

Characteristic features of the rRNA molecules (16S and 23S) form the basis for the development of oligonucleotide probes designed to match various levels of specificity, from the universal to the species-specific level (111), rendering this tool instrumental in microbial ecology research.

Fluorescence-labeled rRNA-directed probes can be used in conjunction with scanning confocal laser microscopy to detect bacteria in the root system (85,112). They are useful for studying the spatial distribution of targeted groups when applied in conjunction with scanning confocal laser or epifluorescence microscopy, or for direct quantification when cells are extracted from the rhizosphere. However, because in most cases strain-specific probes cannot be designed, tracking a specific strain by this means in a dense bacterial background may prove difficult. When used along with other detection methods, such as specific antibodies directed toward the introduced bacterium, the relationship of the latter with surrounding rRNA-labeled bacterial populations can be observed, as Aßmus and coworkers (107) reported for *A. brasilense* (inoculated on soil-grown wheat).

Furthermore, and because the signal obtained is directly proportional to the rRNA cellular content (113), it is possible to assess the proportion of cells that remain physiologically active. This proportion appears to be greater in the rhizosphere (114,115) than in bulk soil (116).

COMMERCIAL PRODUCTS: PRESENT AND FUTURE

The use of PGPR inoculants has become rather common practice and is on the rise in many regions of the world. Inoculation with PGPR also aims at reducing the application of potentially polluting chemical fertilizers and pesticides (22,117). Various inoculants, referred to as *biofertilizers*, *phytostimulators*, and *biopesticides*, are commercially available under different brand names (10,118,119).

As an example, first-generation (wild-type) *Azospirillum* inoculants for maize (Azogreen®) are commercially available in Europe (Lipha, France). Results from field experiments have consistently shown better utilization of nitrogen fertilizer by maize plants inoculated with *A. brasilense* (P. Wadoux, Lipha, Personal Communication).

Another company, Soygro (Pty) Ltd. (Potchefstroom, South Africa), is producing liquid and peat-based *Azospirillum* inoculants for 150,000 and 12,000 commercial hectares of maize and wheat, respectively, and 500 experimental hectares of sorghum. Field experiments with maize carried out over the last six years have shown yield increases of 10 to 30% above noninoculated plants. In the last few years, *Azospirillum* inoculants have also been applied to legumes in South Africa. Average yield increases of 15 to 30% have been observed, following coinoculation with *Azospirillum* and *Rhizobium*, as compared with inoculation with *Rhizobium* alone (T. E. M. Odendaal, Potchefstroom, Personal Communication).

Large surfaces of maize and wheat in Mexico and Argentina are currently being inoculated with *Azospirillum*, leading to significant increases in yield (J. Caballero-Mellado, CIFN, Cuernavaca, Mexico, Personal Communication). Surprisingly, a company specializing in the improvement of golf courses in the United States (EcoSoil Systems, Inc.) is now using *Azospirillum* to obtain better quality of grass turf.

Important factors that can influence the efficiency of microbial inoculants are summarized in Table 5. Successful field inoculation experiments appear to be those in which researchers paid special attention to those factors, especially to the optimum number of cells in the inoculants and to the appropriate inoculation methodology, whereby an optimum number of cells remained viable and available for root colonization (120).

The main obstacle impeding more intensive and widespread use of PGPR inoculants has been the so-called inconsistency of results in field experiments, which could be explained by the complexity and the variability of the soil-plant-microflora relationship at any experimental site. However, such variability is also the lot of *Rhizobium* inoculants, which have been commercially utilized for about a century, and of chemical fertilizers and pesticides, which are not always efficient in the field.

Biotechnological research objectives aiming at improving bacterial performance should also aim at diminishing inconsistencies. To this aim, the following approach may be taken: first, a large number of strains need to be screened, using selected crop plants grown under laboratory or greenhouse conditions. In the screening assays,

Table 5. Factors Influencing the Efficiency of Microbial Inoculants

Factor	Description
Inoculant quality	Utilization of sterilized (gamma) carriers Optimum bacterial concentration Optimum physiological state of the cells Quality control
Soil cultivation	Cultivation practices leading to reduced natural microbial population Preparation of a weed-free seedbed Light irrigation just after planting/inoculation Tillage implements, prevention of soil compaction (aerobic conditions)
Fertilization	Nitrogen, phosphate: optimization according to the inoculant
Fungicides/Insecticides	Prevention of negative effects on microorganism survival (e.g., physical/temporal separation between application of chemicals and inoculation)
Environmental factors	Soil characteristics at planting: pH, temperature Flooded soil conditions, cool and hail storms that negatively influence bacterial activity

the different strains should be evaluated according to their capabilities to improve germination, seedling vigor, root elongation, root branching, nitrogen fixation, and nodulation in the case of legumes. However, this procedure does not ensure that similar performance will be obtained in soil. Therefore, selected strains need to be further tested in pots and finally in the field. It is necessary to demonstrate, using Koch's postulates, that the selected microorganism is responsible for the observed effect on the plant. One can then proceed toward the development of a commercial product with the best strains.

Another approach should aim at understanding the mechanisms involved in the production of the beneficial plant growth effects seen with certain bacterial strains. Such knowledge can provide a rational basis for the improvement of inoculant production and storage procedures, thus resulting in qualitatively superior products. However, this approach is so expensive that it is not feasible for most agroindustrial products.

Application of genetic engineering techniques for the development of better products with "superior" PGPR strains appears to be the most promising path. Extensive research efforts are being carried out to isolate, clone, and characterize genes involved in bacterial features and activities related to survival, colonization, and plant growth effects in the rhizosphere. Efforts are also being made to fuse reporter genes to those rhizosphere-related genes with the aim of following their activities and expression in situ. However, because of public disagreement and political developments with regard to the release of biologically engineered material to the environment, at the present time, this approach cannot be taken into account for applied purposes.

Development of PGPR products includes paying attention to survival and viability of the bacteria, not only in soil, but also during storage. The aforementioned properties (such as accumulation of storage material, encystation, aggregation, etc.) may be extremely relevant to the success of an inoculant. Carrier optimization also has to be taken into account, as it is expected that a suitable carrier will provide good support and encourage bacterial survival during storage. For the *Rhizobium* inoculants, the most "popular" carrier has been peat. Alternatives may be vermiculite, alginates, and liquid formulations (8). With regard to application technology, more extensive investigations need to be performed in order to compare the performances of seed and soil inoculation.

Growth and development of bacteria under laboratory conditions is now rather well understood. The challenge of the next decade will be to extend this knowledge to microbial growth, survival, and exertion of beneficial effects on the plants in situ, to understand the significance of factors limiting growth (e.g., nutrient limitation, toxic products, protozoa) in the rhizosphere, and the impact of abiotic factors (e.g., temperature, drought, oxygen pressure) on bacterial survival and activity. Moreover, the discovering of specific gene expression patterns in the rhizospheric environment and the understanding of the influence of environmental factors on the essential functions for survival, and on traits involved in colonization and in plant growth promotion, will provide answers necessary for the development of more reliable PGPR inoculants.

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BACTERIONEUSTON. See NEUSTON MICROBIOLOGY: LIFE AT THE AIR–WATER INTERFACE

BACTERIOPHAGE AS INDICATORS

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The use of indicator organisms to assess the microbiological quality of water is well established and has been practiced for almost a century. Early work focused on the detection of bacterial indicators of fecal pollution, but now it has been extended to bacterial indicators of sewage, indicators of nutrient pollution, and so on. In the last 50 years, bacteriophages have been investigated as indicators for different purposes. The most widely studied is the use of phages infecting enteric bacteria as models in water quality control. Other examples are the use of

bacteriophages infecting *Serratia marcescens* or *Staphylococcus aureus* as tracers of groundwater movement, or bacteriophages infecting *Vibrio parahemolyticus* as indicators of eutrophication in marine waters. The scope of this article is limited to a critical description of the relationship between the presence of bacteriophages of enteric bacteria in water and their possible application as model microorganisms in water quality control. Different authors (1,2) have listed the requisites for a model microorganism. However, some potential extra advantages of bacteriophages as indicators of water quality deserve comment. First, the limited importance, if any, of phenomena such as "stress," "injury," or "reactivation" that frequently lead to misinterpretation of environmental data on bacterial indicators. Secondly, the samples for phage analysis can be conserved for longer periods of time than samples for bacterial analysis before the performance of assays. Finally, the somatic coliphages allow results to be obtained in four hours.

MODEL, INDEX, AND INDICATOR CONCEPTS

The term indicator organism is not clearly defined, which sometimes leads to confusion. Therefore, the concept of model microorganisms as used by other authors (1,2) will be followed. According to this usage, the index and the indicator functions can be attributed to the model organisms.

An index organism is one that is related to the occurrence of the selected surrogate microorganism or microorganisms. The relationship may be direct, such as the index of human viruses or indirect, such as an index of fecal pollution, sewage pollution, or types of fecal pollution (i.e., human or animal). The criteria for an index organism are very similar to those commonly used for bacterial fecal indicators.

The model organisms with an indicator function have a much broader definition. An indicator is basically a model that has behavioral characteristics similar to those of the surrogate microorganism or microorganisms, and has the same or greater resistance to environmental stresses, but that does not necessarily originate from the same source as the pathogen (e.g., feces). This criterion, and others mentioned earlier, define an indicator as a marker for the efficiency of treatments or for the variation in numbers of the surrogate microorganisms in specific environments. In general, the criteria for indicator organisms are less restrictive than the criteria for index organisms (1).

Ideally, a perfect model organism will fulfill both the index and the indicator functions. However, the more is known about fecal pathogens and potential model microorganisms, the more unlikely it seems that a single fecal microorganism fulfilling all criteria under all circumstances, will be found.

BACTERIOPHAGES AS MODEL ORGANISMS IN WATER QUALITY ASSESSMENT

Three main groups of bacteriophages infecting enteric bacteria have been considered so far as potential model microorganisms for various aspects of water quality assessment: somatic coliphages, F-specific RNA bacteriophages, and bacteriophages infecting *Bacteroides fragilis*. Table 1 summarizes some of the features of the three groups of bacteriophages that is more extensively described in the following text.

Table 1. General Features of the Three Groups of Bacteriophages Proposed as Model Microorganisms for Water Quality Assessment

Feature	Somatic Coliphages	F-specific RNA-Phages	Phages of *B. fragilis*
Homogeneity of the group	+	+++	+++
Availability of standardized methods	+++	+++	+++
Occurrence and levels in human feces	++	+	+
Occurrence and levels in animal feces	+++	+	+
Occurrence and levels in urban sewage	+++	+++	+++
Occurrence and levels in abattoir sewage	+++	++	+
Probability of replication in environment	++	+	−
Resistance to inactivation in environment	++	+	+++
Resistance to disinfection			
Chlorination	+	++	+++
Ozonization	ND	+++	ND
UV irradiation	+	+++	++
High-energy radiation	++	+	++
Thermal treatment	+++	+	+++

Note: +++, high; ++, intermediate; +, low; −, very low; ND, no data available.

Somatic Coliphages

Somatic coliphages are bacteriophages, which consist of a capsid containing single-stranded or double-stranded DNA as the genome. The capsids may be of simple cubic symmetry or complex structures with heads, tails, and tail fibers. They are classified into the following families: Myoviridae (dsDNA, long contractile tails, and capsids up to 100 nm), Siphoviridae (dsDNA, long noncontractile tails, and capsids 50 nm), Podoviridae (dsDNA, short noncontractile tails, and capsids 50 nm), and Microviridae (ssDNA, no tail, and capsid 30 nm). All types are found in sewage, although the most abundant are Myoviridae and Siphoviridae (3,4). Somatic coliphages attach to the bacterial cell wall and may lyse the host cell in 20 to 30 minutes under optimum conditions. They produce plaques of widely different size and morphology. The methodology to detect them is very simple and results may be obtained in four hours.

Natural host strains of somatic coliphages include *Escherichia coli* and other closely related bacterial species. Some of these may occur in pristine waters, hence somatic coliphages may also multiply in these environments even in *E. coli* (5,6). Indeed, one of the drawbacks of somatic coliphages is their replication potential outside the gut. However, the contribution of this potential

replication outside the gut to their occurrence in natural environments has never been quantified.

The term *somatic coliphages* covers many types of phage with a wide range of characteristics, including differential resistance to inactivating factors. Moreover, different strains of *E. coli* and different assay media count, different numbers, and types of somatic coliphages (4,7). Consequently, the information available on both the presence and the behavior of somatic coliphages in water environments has to be interpreted very cautiously, because the data reported in the literature had been obtained with different host strains of *E. coli*, media, and assay conditions. To avoid confusion, the term *somatic coliphages*, unless otherwise indicated, will be restricted to phages infecting *E. coli* C as established by standard methods (8) or ISO (9). Bacteriophages frequently used to study somatic coliphage behavior are T-even and T-odd, ΦX147, and PRD-4.

F-Specific RNA Bacteriophages

F-specific RNA bacteriophages consist of a simple capsid of cubic symmetry of 21 to 30 nm in diameter and contain single-stranded RNA as the genome. They belong to the family Leviviridae. They infect bacteria through the sex pili, which are coded by the F plasmid first detected in *E. coli* K12. The F plasmid is transferable to a wide range of gram-negative bacteria. The pili coded by the F plasmid do not form below 25 °C (10). Therefore, the probability of these phages replicating in the environment is small (10). The infection process is inhibited by the presence of RNase in the assay medium, which can be used to distinguish between the F-specific RNA bacteriophages and the rod-shaped F-specific DNA bacteriophages of the family Inoviridae, which also infect the host cell, through the sex pili.

Strains (*Salmonella typhimurium* WG49 and *E. coli* HS) tailored to detect F-specific bacteriophages also detect small percentages of somatic phages. All phages detected by the tailored strains are usually referred to as F-specific bacteriophages. The number of F-specific RNA bacteriophages is the difference between the number of phages counted in the presence and in the absence of RNase in the assay medium. More than 90% of the phages detected in sewage by tailored strains are F-specific RNA bacteriophages. This percentage may be lower in receiving waters.

A standardized method for the detection and enumeration of F-specific RNA bacteriophages is now available (11). Results can be obtained in 12 hours.

F-specific RNA bacteriophages mentioned in this entry are f2 and MS2.

Bacteriophages Infecting *Bacteroides fragilis*

The most abundant bacteriophages infecting *Bacteroides fragilis*, one of the most common bacteria in the gut of warm-blooded animals, belong to the family Siphoviridae, with flexible tails (dsDNA, long noncontractile tails, and capsids up to 60 nm). Phages infecting *B. fragilis* attach to the cell wall of the host bacteria and may lyse the host cell in 30 to 40 minutes under optimum conditions. They produce clear plaques, which do not differ very much in size or morphology. *Bacteroides fragilis* strains differ widely in the numbers of phages that they recover from municipal sewage, but most strains recover bacteriophages that are very similar and belong to the family Siphoviridae. Bacteriophages infecting *B. fragilis* have not been reported to replicate under environmental conditions. The method for detecting these bacteriophages is slightly more complex than methods for detecting the other groups because of the anaerobic nature of the host bacteria. However, a relatively simple standardized method is now being discussed (12). Results can be obtained in 18 hours.

Bacteriophage B40-8 cited in the text belongs to this group.

BACTERIOPHAGES AS INDEXES

Bacteriophages as Indexes of Fecal Pollution

Many studies have been performed on the distribution of bacteriophages specific to enteric bacteria in human and animal feces. Somatic coliphages using *E. coli* C as the host have been isolated in variable percentages (up to 60%) of human stool samples, reaching numbers up to 10^9 plaque forming units (PFU)/g (13,14). The percentages of isolation reported in mammals and birds range from 100% in pigs to 38% in rabbits (14,15) with counts up to 10^7 PFU/g.

F-specific bacteriophages rarely have been isolated from human feces, irrespective of the host strain used. The maximum positive isolation described is approximately 30% (14–16). Such phages have been isolated inconsistently from domestic and feral animal feces, but incidences are in general higher than in humans (15,17), with densities up to 10^4 PFU/g, although usually lower.

Bacteroides fragilis RYC 2056 has been reported to recover phages from 28% of human stool samples, although it also allows the isolation of phages from animals (18), with the maximum incidence, 30%, in pigs with maximum values of 3×10^2 PFU/g. Bacteriophages infecting *B. fragilis* HSP40 have been isolated from 10 to 13% of human stool samples (15,19) with maximum values above 10^8 (19). However, they have not been isolated from animal feces (15,19).

Bacteriophages as Indexes of Sewage Pollution

Bacteriophages of the three groups mentioned earlier are consistently present in raw sewage and sewage effluents. Consequently, sewage pollution will lead to contamination by the three groups.

Most reports indicate that somatic coliphages are the most abundant in raw municipal sewage, with values ranging from 10^6 to 10^7 PFU/100 ml, approximately less than one order of magnitude lower than the numbers of fecal coliforms or *E. coli* (15,20,21). In addition, they are the most abundant in abattoir wastewater, with values that are similar to those found in municipal wastewater, or values that keep the proportion to fecal coliforms as in municipal wastewater (13,22,23). Values from slurries of different animals are also similar.

Numbers in sewage effluents depends on the wastewater treatment. However, they are present in the great majority of sewage effluents.

F-specific RNA bacteriophages rank second in abundance both in municipal raw sewage and in raw wastewater from abattoirs. The most frequent average values range from 5.10^5 to 5.10^6 PFU/100 ml, usually about one order of magnitude lower than values for somatic coliphages (13,17,18,20,22,24,30). The ratios between F-specific RNA bacteriophages and somatic coliphages are of the same order of magnitude in both raw municipal sewage and wastewater from most abattoirs. In some slurries (e.g., cattle) and specific abattoir wastewater, their relative abundance may be lower. Numbers in sewage effluent depend on the treatment, but these phages are always found in wastewater effluents.

Bacteriophages infecting *B. fragilis* RYC 2056 are found in municipal sewage (Europe, Africa, and America), frequently ranging from 10^4 to 10^5 PFU/100 ml, usually one order of magnitude less than F-specific RNA bacteriophages. Their ratio with respect to somatic coliphages and F-RNA bacteriophages is remarkably constant in municipal sewage (18,20). Host strain VPI 3625 has been shown to recover approximately 10^4 PFU/100 ml in the United States (25), whereas strain HSP40 detected approximately 10^4 PFU/ml in some geographic areas, but much lower values in other areas (18,25).

In abattoir effluents, phage numbers detected by host strain RYC 2056 range from 0 to 10^4 PFU/100 ml, but their ratios to somatic coliphages and to F-RNA bacteriophages are significantly lower when compared with urban sewage (18). Numbers in municipal sewage effluents depend on the treatment, but these phages are regularly found in wastewater effluents.

It can be concluded that numbers of the three groups of bacteriophages are fairly constant in raw sewage throughout the world, as are the numbers of bacterial indicators. Furthermore, bacteriophages of the three groups are consistently found in sewage effluents. Consequently, the three groups of phages may be considered as indexes of sewage pollution. On average, all the groups of phage are more abundant in raw sewage than most pathogens.

Studies on the abundance of bacteriophages infecting enteric bacteria in wastewater sludges (biosolids) are relatively scarce. The accumulation of bacteriophages in both primary and secondary sludges is similar to that of bacterial indicators, and the ratios of such indicators to phages do not differ significantly from those in sewage (26,27). The three groups of bacteriophages seem to accumulate in a similar manner (26). However, sludges are subjected to additional treatment before release to the environment. Phage concentrations in treated sludges depend on the treatment (26).

Bacteriophages as Indexes of Human Viruses

Somatic coliphages (28), F-specific-bacteriophages (22), and bacteriophages infecting *B. fragilis* (19) have been proposed as potential indexes of the presence of human viruses in the environment on the basis of their resemblance to human enteric viruses.

Because of the difficulty of enumerating human viruses, studies establishing a correlation between human viruses and bacteriophages are scarce. A certain degree of correlation has been reported between: (*1*) enteroviruses and somatic coliphages in water at the different stages of a water treatment plant (29), (*2*) F-specific RNA bacteriophages and enteroviruses in fresh water (31), (*3*) F-specific RNA bacteriophages and enteroviruses in shellfish (25), (*4*) F-specific RNA bacteriophages and calicivirus in shellfish (32), and (*5*) enteroviruses and rotaviruses and phages infecting *B. fragilis* HSP40 in marine sediments (33).

If bacteriophages are not isolated in all human stool samples tested and if they may originate in sites other than the human gut, there will not be an absolute and unequivocal correlation between the presence of viruses and the presence of the different groups of bacteriophages. However, taking into consideration that bacteriophages infecting enteric bacteria are always present in raw sewage that sewage constitutes the main input of fecal pollution to the environment, and that some phages behave similar to human viruses, the presence of certain phages in given numbers in waters may indicate the presence of human viruses in such waters.

Bacteriophages as Indexes of Human and Animal Pollution

Identifying the sources of fecal pollution is important for water management. One reason, although not fully supported by epidemiological studies, is the perception that there is a higher health risk associated with human fecal pollution than with fecal pollution of animal origin. But, irrespective of the health risk, tracking the origin of fecal water pollution will be a very useful tool for water managers. A range of microorganisms and chemical indicators have been examined as fecal source indicators, including phages.

F-RNA bacteriophages are divided in four main subgroups that can be recognized by serotyping. Studies with a number of isolates indicate that serotypes II and III are mainly isolated from human feces, whereas, serotypes I and IV are usually found in animal feces (34). More recently, it has been shown that the subgroups can be grouped in four main genotypes, which, with few exceptions, show overall comparability with serotypes. Probes for each genotype allow plaque hybridization, and then it is easy to study the distribution of subgroups in water samples. Subgroups II and III predominate in water samples contaminated with human fecal pollution and subgroups I and IV in animal feces and water samples contaminated with animal fecal pollution (25,35,36).

Bacteriophages infecting *B. fragilis* HSP40 are detected in samples with human fecal pollution and are practically absent in samples with animal fecal pollution (15,19), but they are very scarce in wastewater in some geographic areas (18,25). Bacteriophages infecting *B. fragilis* RYC 2056 are found at concentrations ranging from 10^4 to 10^5 PFU/100 ml in municipal sewage of very different geographic origins, and at significantly lower levels in wastewater from abattoirs (18).

More studies are needed on the ecology of these two groups of phages, but they seem to be one of the best ways

of distinguishing human from animal fecal pollution at present.

BACTERIOPHAGES AS INDICATORS

The indicator function requires that resistance to natural inactivating factors and to treatment of the indicator should be similar to or slightly greater than that of the surrogate microorganism or microorganisms. In addition, pathogens are assumed not to replicate in the aquatic environment. Model organisms should not replicate in the environment, and consequently, if there are no new contributions, their numbers in any aquatic environment are assumed to decrease.

The reduction of pathogens and model microorganisms in sewage and environmental waters, on their way from human or animal feces to humans through water and food is a complex process in which many factors interact. There are three major groups of processes:

1. Physical removal by mechanisms such as sedimentation, adsorption, and straining. These processes do not inactivate the microorganisms. They merely transfer the microorganisms to other environmental compartments (sewage sludges, sediments, soils, etc.) where they are inactivated, but from which they can return to the water.
2. Inactivation by direct or indirect effects of physical-chemical factors on the integrity of the organisms. Die-off of phages and viruses in aquatic environments is influenced by many factors, such as suspended matter, sunlight, temperature, pH, and ionic environment. The disinfectants used by man are included in this group.
3. Inactivations as a result of the biological activity of other microorganisms. Antagonistic activity and grazing by protozoa contribute to pathogen removal. The main factor that seems to affect bacteriophages and viruses is the production of antiviral substances.

The relative importance of each of these processes is determined by a great number of variables, which are also interrelated. Two major factors are time and temperature. Each of the earlier mentioned processes is directly and positively affected by time, and the great majority is also directly or indirectly affected by temperature. Removal and inactivation are more rapid at higher temperatures and increase with time.

Separate studies have indicated that there are great differences between the removal and the inactivation of seeded and naturally occurring bacteria, viruses, and bacteriophages. Hence, realistic comparisons of the behavior of the different groups of microorganisms can be made only on data with naturally occurring microorganisms. Unfortunately, extensive studies have not yet been carried out, and those already published are difficult to compare because of differences in detection methodologies. Many studies were performed with a single-seeded phage, which in addition to the shortcoming mentioned earlier may be neither the most abundant nor the most representative of a given group regarding resistance (e.g., the T-even and T-odd bacteriophages among the somatic coliphages). When possible, this article will refer to data based on changes of ratios among the different model microorganisms or simulation experiments with naturally occurring populations.

Removal and Inactivation in Nature

Sedimentation. Sedimentation of microorganisms depends on size, whether they occur singly, in clumps, or are associated with suspended solids, among other factors. In general, removal of microorganisms by sedimentation is more efficient for larger particles. But these then survive better in sediments, because they are bound to solids. However, they may be resuspended into the water column if the sediments are disturbed. Adsorption and aggregation of viruses may also occur. The tendency to be adsorbed to particles has been described to be similar for viruses and somatic coliphages (37). At least in waters with a high fecal pollution, the three groups of bacteriophages suggested as model microorganisms settle similarly (38). However, their survival in sediments is expected to depend on the type of microorganism.

Adsorption to Soil. Adsorption of pathogens and indicators to soil particles plays an important role in their removal from water as it moves through the soil. Soil type and composition, ionic environment, pH, moisture content, temperature, microorganism species, or strains all interact to affect the adsorptive capacity and die-off rate in soil of pathogens and model microorganisms.

Data on the presence of bacteriophages in groundwater and on the comparison of numbers of bacteriophages, viruses, and bacterial indicators are scarce, and most often only presence/absence is given, and consequently, results from different reports are very difficult to compare. Various studies performed across the globe indicate small and nonsignificant differences among the frequencies of isolation of the three groups of bacteriophages. Thus, in a nationwide study performed in United States, F-specific bacteriophages were more frequently isolated (18%) than somatic coliphages (7.6%) in concentrate equivalents of 4.5 L (39). F-specific coliphages were isolated 18.9%, *B. fragilis* phages 17.1%, and somatic coliphages 10.4% of time in 100-ml samples of groundwater in Spain (40).

Most model studies on the behavior of bacteriophages in soil were done with F-specific RNA bacteriophages, mostly MS2 and f2, both belonging to subgroup I. A number of studies have consistently shown that F-specific RNA bacteriophages adsorb soil particles poorly and survive better in groundwater than enteric viruses. As a result, the F-RNA phages have been recommended as an indicator of human virus transport in soil and of their presence in groundwater (41–43).

Inactivation. Susceptibility or resistance to inactivation in the environment can be estimated either by studying the ratios between the different microorganisms naturally occurring in sewage and water environments with remote pollution or through the performance of experiments that are modeled on what happens in nature.

Data available on phage inactivation in the environment frequently seem contradictory. This may be because of the fact that various phages have been studied under different experimental conditions and using diverse detection methodologies. Phages generally survive longer in aquatic environments than most bacterial indicators, with the exception of clostridia. Differences between the survival of phages and bacterial indicators seem to be greater in seawater than in fresh water, in warm water than in cold water, and in areas with abundant sunshine than in areas with scarce sunshine. Somatic coliphages and bacteriophages infecting *B. fragilis* survive better than F-specific RNA bacteriophages in most water environments, mainly in those with more inactivating power. These assertions are supported by data on relative abundance of the different groups of microorganisms in different aquatic environments (20,25,38,44–46) and by results of model experiments (24,47,48).

Data available from experiments with seeded cultured viruses and bacteriophages indicate that phage survive more similarly to human viruses than bacterial indicators (24,47).

Removal and Inactivation by Wastewater Treatment

Wastewater treatment processes, especially the biological processes, are diverse in design and operating conditions. If we consider the different bacteriophage detection methodologies used in different studies, it is not surprising that data in the literature on bacteriophage removal by wastewater treatment processes vary widely. Presumably, the bacteriophage removal rate is lower than that of bacterial indicators and closer to that of animal viruses. However, the proportion of phages removed depends on the treatment process and, in some cases, on the group of bacteriophages studied.

The removal of bacteriophages by primary treatment is usually inefficient and erratic, and data reporting ranges from 10 to 90% without significant differences in the removal rate of bacterial indicators and bacteriophages, or between the different groups of bacteriophages (49,50). However, treatment with lime shows a clear difference, because fecal coliforms, the most sensitive of the indicators suffer a 99.85% reduction, and phages infecting *B. fragilis*, the most resistant of the phages, display a 91.8% reduction (50).

Most data on secondary treatments are related to the activated sludge process. Again, available information is very variable. Pathogen and indicator reductions depend on many factors that may have a differential effect on the various pathogens and model organisms. Well-operated plants remove from 90 to 99% of bacteria and viruses (21,51). The different groups of bacteriophages are also removed in similar percentages (21,50,51).

The typical concentrations of the different indicator microorganisms in secondary effluents maintain the same relative proportion as in raw sewage.

Effluents from activated sludge plants may be further in, that is, lagoons, oxidation ponds, and wetlands; filtration, coagulation, and sedimentation; and disinfection. Lagoons, oxidation ponds, and artificial or natural wetlands may be particularly useful in warm and temperate climates to further treat secondary effluents. Again, information is very variable and partial, but in all these systems bacterial indicators are removed more efficiently than somatic coliphages (52,53). Seeded polioviruses and phage MS2 behave similarly (54). Data comparing the three groups of bacteriophages are very scarce, but all were inactivated more slowly than the bacterial indicators and the differences in their removal are smaller than differences between their removal and the removal of bacterial indicators (55).

Filtration, coagulation/filtration, and postprecipitation processes are frequently used to remove suspended solids from secondary effluents and precipitation (postprecipitation) to remove N and P compound from secondary effluents. Filtration alone has little effect on the removal of bacteria and viruses. But coagulation/filtration and (post)precipitation have a significant effect on the reduction of microorganisms, improving the removal obtained by activated sludge by 90–95%. The reduction of viruses, bacterial indicators, and bacteriophages appears to be similar (21,56).

Removal and Inactivation by Drinking Water Treatments

Most data on the presence of bacteriophages in drinking water refers to somatic coliphages. These have been isolated in tap water samples in the absence or in the presence of low levels of fecal coliform bacteria in both developing and developed countries. Many of these samples containing somatic coliphages contained residual-free and combined chlorine (1,57,58). After filtering nine samples of 20,000 liters of drinking water, no enteroviruses were isolated, somatic coliphages were isolated in two samples and F-specific RNA bacteriophages in one sample in Canada (59). In a three-year study performed in Israel on 1,136 tap water samples, the percentages of positive isolations were 0.7% for fecal coliforms, 5.6% for somatic coliphages, 7.1% for F-specific bacteriophages, and 5.0% for *B. fragilis* phages (40). In Spain, in 410 samples including finished water from a drinking water treatment plant and tap water samples the percentages were 0.5% for fecal coliforms, 1.5% for somatic coliphages, 2.2% for F-specific bacteriophages, and 5.2% for phages infecting *B. fragilis* (7,40).

Precise quantification of bacteriophage removal in drinking water plants is difficult to achieve, either because of the low levels of naturally occurring bacteriophages in source water or because of the treatment removes all the bacteriophages. Bacteriophages are more difficult to remove than coliform bacteria. Their comparative removal depends on the proportion of physical removal (coagulation, sedimentation, and filtration) and chemical disinfection. Physical removal procedures frequently used in drinking water treatment plants such as flocculation-sedimentation and filtration have little effect on removal of bacteriophages, less than 0.5 logs, in comparison with the removal of bacteria including *Clostridium* (29,58,59). Removal of bacteriophages by disinfection is greater than physical removal. Bacterial indicators are inactivated more efficiently than bacteriophages, with the exception of clostridia, which may be more resistant than phage.

Levels of the three groups of phages seem to be reduced similarly by physical removal. In contrast, their removal by disinfection depends on the procedure. The difference in the fecal coliform removal and the most resistant group of bacteriophages in a given plant may be of several orders of magnitude. Thus, prechlorination followed by flocculation removed phages infecting *B. fragilis* 1.5 logarithmic units, when fecal coliforms removal was 4.9 logarithmic units (60). The differences in the extent of removal of the different phages will be greater as the treatment plant increases in complexity and will depend on the type of disinfectant (7,59).

Bacteriophages are expected to behave more similarly to human viruses than bacteria, and many drinking water treatments provide water without fecal indicators that still contain infectious bacteriophages. It is suggested that the presence of bacteriophages of any of the three groups in drinking water indicates a potential risk of viral pollution, and bacteriophage tests have been proposed as additional criteria for drinking water safety (1).

Inactivation by Sludge (Biosolid) Treatment

Wastewater treatment generates huge amounts of biosolids, where pathogens and indicators accumulate. According to their destination, sludges receive different treatments, the most frequent being storage, digestion, and, to a lesser extent, disinfection by pasteurization, irradiation, or lime treatment.

The survival of viruses and bacteriophages in sludge during storage depends on the temperature, as occurs for human viruses. At 4 °C all survive quite well, whereas at higher temperatures F-specific bacteriophages inactivate more rapidly than somatic coliphages and bacteriophages infecting *B. fragilis*, the difference being significant at 37 °C (26).

Different kinds of digestion processes are used to reduce the amount and improve the quality of sludges. Bacteriophage f2 has been reported to survive more successfully than enteroviruses and rotaviruses to both mesophilic and thermophilic digestion (61).

Inactivation by Disinfection

Disinfection is used both in wastewater and drinking water treatment. It is in these processes that major differences in removal are given between pathogens, including different strains or types of the same pathogen, between pathogens and indicators, and between indicators themselves. Resistance of phages will only be compared with that of bacterial indicators because our knowledge of comparative resistance with respect to other pathogens such as *Giardia* and *Cryptosporidium* is very limited and because the methods to determine their viability are difficult to perform. The difference in sensitivity to a given disinfection process deduced from experiments with pure cultures, especially those seeded with the very different types of somatic coliphages, may not reflect what happens in the real world. Moreover, contradictory results comparing a given virus and a given bacteriophage, for example, f2 and poliovirus, appear in the literature. These inconsistencies probably reflect differences in the type of water, viral strain studied, and methodology used.

Chemical Disinfectants. The most widely used disinfectants for water and wastewater are chlorine, chloramine, chlorine dioxide, bromide, bromine chloride, iodine, ozone, and peracetic acid.

The available information on the effectiveness of these disinfectants are with laboratory-grown strains. Bacterial indicators, with the exception of spores of clostridia, are usually more sensitive to free chlorine than with viruses and bacteriophages. But viruses and bacteriophages differ in their sensitivity. Thus, among viruses, human rotaviruses and hepatitis A virus (HAV) rank among the most resistant and polioviruses and SA11 among the most sensitive (62). Among phages, B40-8 infecting *B. fragilis* is more resistant than MS2 and f2, which are more resistant than polioviruses (63). In the presence of 2 ppm of free chlorine, B40-8 is as resistant as HAV (62). Data on somatic coliphages are very variable, but this variability may depend on the morphological type. Thus, some of them are very sensitive and others significantly more resistant than f2 and similar to SA11 (64).

Comparative data on the chlorinated secondary effluents referring to naturally-occurring pathogens and indicator microorganisms are scarce, but in spite of the fact that they were obtained in different treatment plants and even on different continents, they show a similar pattern of inactivation. The difference of inactivation of coliform bacteria and fecal streptococci with respect to inactivation of phages is higher than in drinking water. This fact probably indicates than phages are more resistant to monochloramines than bacteria. Regarding bacteriophages, those infecting *B. fragilis* are the most resistant, followed by F-specific RNA and finally, somatic coliphages. Enteroviruses rank between F-specific RNA bacteriophages and bacteriophages infecting *B. fragilis*. Sulfite-reducing clostridia behave similar to the most resistant bacteriophages (24,65,66).

Available data on the effect of ozone on bacteriophages are scarce and contradictory. Since the aggregation state of microorganisms is a key factor in determining inactivation by ozone, data on naturally occurring viruses and phages are essential to assess the effect of ozone on these microbes. The effect of ozone in finished drinking water is difficult to measure, because usually ozone inactivates all microorganisms to such an extent that they are never detected.

Very few data exist on removal of phages from secondary effluents by ozonation and, again, they are contradictory regarding the comparative resistance of bacterial indicators, viruses, and phages. Thus, some studies indicate that bacteria are more sensitive than viruses and phages, but other studies indicate the contrary. These contradictions may be because of the fact that these studies were done with laboratory-grown microorganisms.

Radiation. Different types of radiation are used for disinfection. The most frequent are UV radiation, which is used for low-turbidity water (drinking water, secondary

effluents, shellfish depuration water, etc) and gamma and electron-beam irradiation, for water and sludges respectively.

UV radiation is extensively used for disinfecting drinking water and secondary effluents. Viruses and bacteriophages are less sensitive than bacterial indicators, although there are broad ranges of sensitivities between the strains of each group of microorganisms. Moreover, binding to suspended solids protects both viruses and bacteria. Therefore, data obtained with seeded drinking water cannot be extrapolated to secondary effluents. F-specific RNA bacteriophages are more resistant to UV irradiation than fecal coliforms, fecal streptococci, most human viruses, bacteriophages infecting *B. fragilis*, and selected somatic coliphages (63,67,68).

Few experiments have been performed with naturally-occurring phages. F-specific phages have been reported to be 2.3 times more resistant than *E. coli* in secondary effluent (67). Other studies reported removals from filtrated secondary effluents of between 2 and 3 logs for fecal coliforms, fecal streptococci and somatic coliphages, between 1 and 2 logs for F-specific RNA bacteriophages and intermediate values for *B. fragilis* bacteriophages (55); and between 1 and 2 logs for fecal coliforms, fecal streptococci and somatic coliphages from coagulated secondary effluents (69). F-specific RNA bacteriophages thus appear to be the most suitable indicator to evaluate UV radiation effects.

Gamma and electron beam radiation are slightly more effective in eliminating *E. coli* than in eliminating somatic coliphages in sewage (70). The only data comparing the different groups of phages are on phages seeded in distilled and tap water, which indicates that MS2 is more sensitive to ionizing radiation than phage B40-8 infecting *B. fragilis*, and this is more sensitive than somatic coliphage ΦX147. In this case, MS2 is as sensitive or more than *E. coli* (71).

Heat Treatment. Levels of fecal microorganisms in sludges are lowered mainly through thermal treatment, either by mesophyllic and thermophilic digestion or by even pasteurization. Bacterial indicators are more sensitive to heat treatment than viruses and phages, which in turn differ in their resistance/sensitivity to heat. Thus, bacteriophage f2 is more resistant than rotaviruses and enteroviruses, but less so than parvoviruses (61). Data comparing the resistance of naturally occurring phages of all groups to pasteurization are not available. Data available for thermophilic and mesophyllic digestion do not allow to differentiate the thermal effect from chemical effects (61).

Accumulation and Depuration of Fecal Pathogens by Shellfish

The presence of fecal pathogens and indicators in shellfish, providing there is no replication, depends on many factors, but, briefly, it is the result of accumulation and/or depuration.

There is no relationship between the levels of bacterial indicators and viruses in the water and their concentrations in shellfish, where most of them accumulate. The extent of accumulation depends on many factors, such as water characteristics, physiological status of the animal, and the identity of the microorganisms. Bacteriophages accumulate in shellfish in a similar manner (25,72,73).

Many studies have also shown differential rates of reduction of bacteria and viruses in depurating shellfish, with bacteria generally being reduced more rapidly. In depuration experiments with heavily contaminated oysters and mussels, F-specific bacteriophages are depurated much less efficiently than *E. coli* and similarly to polioviruses (74,75).

Numbers of fecal bacteria, viruses, and bacteriophages in shellfish collected in areas with different pollution levels are in agreement with the earlier assertions. All the three groups of bacteriophages are found in shellfish grown in waters with low levels of fecal pollution in amounts that indicate that they accumulate and survive longer than bacterial indicators, and more similar to human viruses (24,32,73). Data on somatic coliphages should be considered cautiously, because they may be able to replicate in shellfish (6).

CONCLUSION

Bacteriophages infecting different enteric bacteria have a number of characteristics that make them potential useful model organisms in water quality control and assessment. Three groups of bacteriophages that infect enteric bacteria are under consideration as potential model microorganisms. These are somatic coliphages, F-specific RNA bacteriophages, and bacteriophages infecting *B. fragilis*. Bacteriophages belonging to the three groups of phages are irregularly found in feces, but they are very consistently found in sewage, in numbers that usually exceed the numbers of pathogens by several orders of magnitude. Standardized methods for detecting and enumerating the three groups of bacteriophages are presently available. Methods for detecting and enumerating bacteriophages are simple and can be carried out by any well-trained microbiologist. They do not need sophisticated equipment and can be performed in standard routine microbiology laboratories. They are relatively inexpensive and definitive results can be obtained quickly.

Bacteriophages had been proposed as indicators of the presence of fecal pollution, sewage, human viruses, and human and/or animal fecal pollution. If bacteriophages are not found in all stool samples and if bacteriophages have a chance to originate in other sites than gut, there will not be an absolute and unequivocal correlation between the presence of bacteriophages and the presence of fecal pollution, sewage, human viruses, and human and/or fecal pollution. However, if bacteriophages are always present in sewage, and if sewage, raw or treated, constitutes the main input of fecal pollutants into the environment, the presence of given numbers of certain bacteriophages in waters will indicate the likely presence of the surrogate fecal microorganism or microorganisms.

Moreover, bacteriophages may be used to indicate the behavior of pathogens other than bacteria during the processes of removal and inactivation in nature, water,

and sludge treatment processes. In general, the behavior of bacteriophages resembles more to the behavior of persistent pathogens (i.e., animal viruses and protozoa) than the behavior of currently accepted bacterial indicators, although different groups of bacteriophages show different degree of persistence to different removing processes and resistance to different inactivating factors.

Each one of the three groups of bacteriophages proposed as model organisms has advantages and drawbacks. Somatic coliphages are the most abundant, and the method for their detection and enumeration is the most simple and fast, with results available in one working day. On the other hand, they are a heterogeneous group with important differences in resistance to inactivation among its members, and it has been reported that they may replicate outside the gut. This fact is without any doubt the main shortcoming of somatic coliphages. However, the contribution of this potential replication in the different water environments has never been quantified. Because of the ease of the method for their detection and their abundance, this potential contribution should be estimated before rejecting them as model organisms for water quality control.

F-specific RNA bacteriophages rank second in abundance and they are a homogeneous group. The method for detecting them is simple and fast, although not as much so for the somatic coliphages. They offer good perspectives as index organisms for viruses in groundwater and to monitor some water treatments, for example, UV disinfection. On the contrary, their persistence in surface waters, mainly in moderate and warm climates, seems low, and their resistance to some inactivating treatments is low or intermediate as compared with persistent virus or protozoa.

Bacteriophages infecting *B. fragilis* rank third in abundance. They are a homogeneous group. They are more resistant than the other groups of phages to most inactivating factors and treatments and they do not seem to be able to replicate outside the gut. The method for their detection requires anaerobiosis and results are available only after 18 hours. Their low numbers constitute their main drawback.

In view of the present knowledge, it seems unlikely that we can find a single group of bacteriophages fulfilling all the requirements to be used as an universal indicator, but this seems to be true for all the potential microbial indicators. Now that standardized methods are available, more research in the ecology of these groups of bacteriophages should be done to guarantee the best choice for the various potential applications of bacteriophages as model microorganisms.

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BACTERIOPHAGE: BIOLOGY AND GENETICS

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Bacteriophages (phage), by definition, are viruses that infect bacteria. In general, phages have a proteinaceous outer shell with an inner core containing their genetic material. They usually infect a host bacterium by attaching to the bacterial cell via specific host receptor proteins and injecting their nucleic acids into the bacteria. The phages then use the chemical energy and biochemical machinery of the host to produce new phage particles. The phage is ultimately released from the bacterial cell by budding or extrusion from the plasma membrane or even complete lysis of the host cell. Phages are in most instances, host-specific, with particular phage infecting only certain bacterial species. Since their discovery in 1915 by the British investigator Frederick W. Twort, and the introduction of the term *bacteriophage* by Felix Hubert d'Herelle (1), bacteriophage have become important tools, not only in the studies of bacterial genetics and cellular mechanisms but also in the field of environmental microbiology. Similar to other disciplines, phage are used primarily as models or surrogates to assess the fate of pathogenic human enteric viruses, as pathogen indicators, as vectors for transport of genetic information, and even as biocontrol agents.

Unlike molecular biology that makes use of the phage as tools for genetic engineering, environmental microbiologists more often use phage such as MS2, PRD1, and PhiX174, which structurally and biochemically resemble human pathogenic enteric viruses, such as *Poliovirus*. These spherical bacteriophage are used as indicators or model viruses not only because they have similar size and shape to important human pathogenic viruses but more importantly because they are much easier and less expensive to assay. The coliphage MS2, in particular, has been used extensively as an indicator and model for enteric viruses such as *Poliovirus*, which is a member of the *Picornaviridae* family. Table 1 lists

Table 1. Comparison of the Phage MS2 and the Enterovirus Poliovirus

Feature	MS2 *Leviviridae*	*Poliovirus* *Picornaviridae*
Particle		
Weight (Mdalton)	3.9	8–9
Diameter (nm)	24	27[b]
Isoelectric point	~3.9	~8.2[a]
Major capsid proteins, number	1	4
Symmetry	Icosohedral	Icosohedral
Enveloped	None	None
Triangulation number	T = 3	T = 1
RNA		
Sense (positive sense acts as mRNA)	Yes	Yes
Molecular weight (Mdalton)	1.2	2.5
Monocistronic	No	Yes
Overlapping genes	Yes or No	No
Replication form infectious	No	Yes
Multiplication		
Translation sequential	Yes	No
Cleaved precursor	No	Yes
RNA enters preformed capsid	No	Yes

Source: Adapted from H. Ackermann and M. S. DuBow, *Viruses of Prokaryotes*, vol. 1, *General Properties of Bacteriophages*, CRC Press, Boca Raton, Fla., 1987, pp. 1–202; H. Ackermann and M. S. DuBow, *Viruses of Prokaryotes*, vol. 2, *Natural Groups of Bacteriophages*, CRC Press, Boca Raton, Fla., 1987, pp. 1–242.
[a]Floyd and Sharp (4)
[b]Burke and coworkers (5)

some of the similarities and differences between MS2 and *Picornavirus*.

Many of the specific areas where phages are important in the field of environmental microbiology are covered in other entries of this encyclopedia and therefore will only be mentioned briefly in this entry. The main purpose of this section is to describe the basics of bacteriophage structures, taxonomy, and genetics. This section concentrates on the spherical coliphage MS2 and the tailed lambda phage. The lambdoid phage may be important in environmental exchange of genetic material between bacteria, which could be important in many disciplines within environmental microbiology including bioremediation, in which the transport and exchange of genetic material can help to increase the ability of naturally occurring bacteria to metabolize pollutants. The lambdoid phage may also prove to be important in the environment, where genes encoding virulence factors such as Shiga toxin and antibiotic resistance may be transferred from one bacterium to another. Such events could create a potentially pathogenic strain from an otherwise innocuous variety.

TERMINOLOGY

Basic terminology for virus and phage include the following:

Capsid	The protein shell that encloses and protects the nucleic acid of the phage
Structural units	The smallest functional equivalent building units of the capsid
Nucleocapsid	The capsid together with its enclosed nucleic acid
Virion	The complete infective virus particle

These structures are highlighted in Figure 1.

TAXONOMY

The International Committee on Nomenclature of Viruses was established in 1966 to develop classification schemes for viruses and phages (6). Since its establishment, there has been dispute over the taxonomic systems proposed by the committee. One such system acts as an all-embracing scheme for the classification of viruses and phages into subphyla, classes, orders, suborders, and families (Table 2). In this type of scheme, descending hierarchical divisions would be based on characteristics such as nucleic acid type, capsid symmetry, and presence or absence of an envelope. Although the debate over classification schemes continues, David Baltimore (7) devised a scheme for classification of viruses and phage, which is now coming to be widely accepted. This scheme is based on the relationship between the viral or phage genome and the viral or phage mRNA. Table 3 shows the major families of bacteriophage grouped according to the Baltimore scheme.

The taxonomic nomenclature used to describe bacteriophage is different from that used in the naming of animal and plant viruses. Animal and plant viruses are typically named in one of three ways: (*1*) after the disease; (*2*) after the discoverer of the virus; or (*3*) after the location where the virus was discovered. Viruses are also classified at the family (*-viridae*), genus (*-virus*), and species level.

Originally, bacteriophages were only classified into families. Exceptions now exist to this rule. Phages unlike plant and animal viruses are named exclusively with numbers and letters. For instance, PhiX174 was the 174th virus characterized in the 10th group of a large set of phage being classified, with phi (ϕ) being the symbol that stands for phage. For instance, three genuses *Chlamydiamicrovirus*, *Microvirus*, and *Spiromicrovirus* are grouped into the family *Microviridae*. The *Leviviridae* are not only broken down into genuses but subgroups as well. In the past, classification of the *Leviviridae* was based on serotyping, so classification schemes based on six serogroups also exist. In the National Center for Biotechnology Information's GenBank (October 1999), the genera designations *Allolevivirus* and *Levivirus* are listed under *Leviviridae* along with their serogroupings. Bacteriophage taxonomy continues to develop, mainly assisted by the rapid development of genetic sequencing technologies. For instance, the genome of a typical phage can reasonably be determined in a matter of

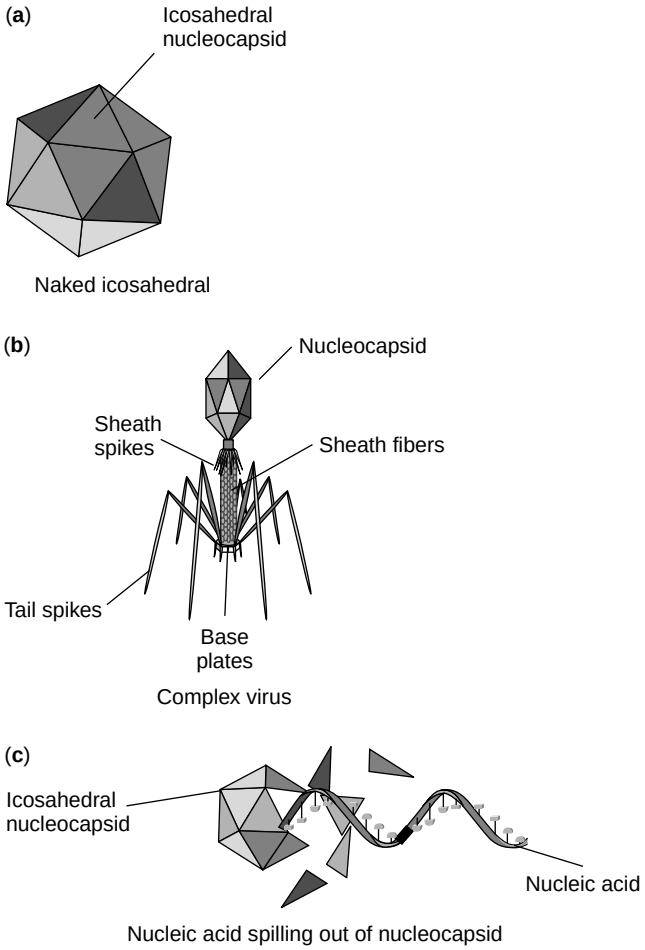

Figure 1. Schematic view of general bacteriophage and virus structures. (**a**) Naked icosahedral structure, which is the basic shape of many phage including *Leviviridae* and *Microviridae*. By definition, an icosahedron is composed of 20 facets, each shaped as an equilateral triangle, 12 vertices, 6 fivefold axes of symmetry passing through the vertices, 10 threefold axes extending through each face and 15 twofold axes passing through the edges of an icosahedron. Caspar and Klug (1962) later defined all possible polyhedra by determining that because an icosahedron has 20 equilateral triangular facets, each of which is identical, there must be 20 T structure units, where T is the triangulation number given by the rule: $T = Pf^2$, where P is any number of the series 1, 3, 7, 13, 19, 21, 31 and f is any integer. Typically, for viruses the P value is 1 or 3. Morphological units can be clustered as 20 T trimers, 30 T dimers, or considered individually as 60 T monomers. (**b**) Represents a complex structure such as *Myoviridae* possess. Indicated in this figure are the major features such as the tail spikes and base plates. (**c**) Represents a nucleocapsid with the nucleic acid spilling out.

days. Similarly, improvements in other molecular and biochemical techniques are continuing to provide tools that allow for structural and topological characterization of viruses and phages at the angstrom level of resolution.

STRUCTURE AND PATHOGENESIS

The architecture of bacteriophages and viruses in general is an elegant molecular structuring of protein subunits,

Table 2. Phage Families and Genera of Bacteriophage

Major Bacteriophage Families	Genus Example	Examples of Species (Host Organisms)
Myoviridae	*Myovirus*	• T4, T2, T6, Wphi, P2, 186, Ac3, Acm1 •(*Escherichia coli*), • PBSX, PBS2, beta22, 2C, (*Bacillus* spp.), • S-WHM1, S-PM2, N1 (Cyanobacteria), • PSP3 (*Salmonella* spp.), • Twort (*Staphylococcus* spp.), • RL1RES, RL2RES (*Rhizobium leguminosarum*), • HP1, S2, HP1 (*Haemophilus influenzae*), • A118, A500, A511 (*Listeria monocytogenes*),
Siphoviridae	*Siphovirus*	• Lambda, 21, T5−933W (*E. coli*) • A118 (*Listeria monocytogenes*) • B40−8 (*Bacteroides fragilis*) • FC1 (*Enterococcus* spp.) • phi-41 (*Lactococcus lactis*) • phi-C (*Staphylococcus aureus*) • psiM2 (*Methanobacterium thermoautotrophicum*) • SFI11 (*Streptococcus thermophilus*
Podoviridae	*Podovirus*	• T7 H-19B, N4, (*E. coli*) • phiAAU2 (*Arthrobacter aureus*) • B103, phi29, H1, NF, Phi-15, Phi-29, (*Bacillus* spp.) • Cf16 (*Xanthamonas campestris*) • Cp-1, CP-7 (*Streptococcus pneumoniae*) • K11 (*Klebsiella* spp.) • Kvp18 (*Kluyvera* spp.) • LP7, P22, PS119 (*Salmonella* spp.) • Phi-13 (*Staphylococcus aureus*) • Phi H (*Halobacterium salinarium*) • Phi-vML3 (*Lactococcus* spp.) • P1 *Mycoplasma* spp.) • phage V, Phage X (*Shigella flexneri*)
Microviridae	*Microvirus*	• PhiX174, G4, S13, ST-1, U3, alpha 3, G14, PhiK (*E. coli*, *Salmonella* spp., *Shigella*) • PhiCPG1 (*Chlamydia psittaci*)
Corticoviridae	*Corticovirus*	• PM2 (*Alteromonas* spp.) • O6N-58P (*Pseudomonas*)
Tectiviridae	*Tectivirus*	• PRD1 (*E. coli*, LT2, *Acinitobacter* spp., *Pseudomonas*, and *Vibrio*), • PR3, PR4, PR772, L17 (*E. coli*) • AP50 (*Bacillus anthracis*)

(*continued overleaf*)

Table 2. (*Continued*)

Major Bacteriophage Families	Genus Example	Examples of Species (Host Organisms)
		• PhiNS11 (*Bacillus acidocaldarius*) • Bam35 (*Bacillus megatarium*),
Leviviridae	*Levivirus*	• MS2, f2, fr, JP501, GA, JP34, Ku1, TH1, BO1, fr1, PP7, TW19, (*E. coli*)
	Allolevivirus	• Q-beta, M11, ST, VK, NL95, MX1 (*E. coli*) • PRR1 (*Pseudomonas aeruginosa*)
Cystoviridae	*Cystoviridae*	• Phi6 (*Pseudomonas phaseolicola*)
Inoviridae	*Inovirus*	• Fd, I2–2, If1, Ike, M13 (*E. coli*), • Pf1, Pf3 (*Pseudomonas aeruginosa*) • Cf1c, Cf1t, Cf (*Xanthomonas campestris*) • fs-2, v6, Vf33, Vf12 (*Vibrio cholerae*)
	Plectrovirus	• MV-L1 (*Acholeplasma* spp.) • Phage 1 (*Spiroplasma* spp.)

Source: Adapted from H. Ackermann and M. S. DuBow, *Viruses of Prokaryotes*, vol. 1, *General Properties of Bacteriophages*, CRC Press, Boca Raton, Fla., 1987, pp. 1–202.

which provides the basis for infectivity, specificity, and self-assembly. The requirements of self-assembly and host infection specificity have generated a remarkably wide array of phage viroid structural organization and geometric designs. Nevertheless, certain common features and general principles can be applied to most phages.

The description by Watson and Crick of the molecular structure of DNA (8) was followed by yet another macromolecular structural elucidation. Crick and Watson (9) reasoned that the small genomes of viruses could encode for only a few proteins from which they had to build their protein shells. They predicted that to use identical subunits to construct such a shell, it would, by necessity, possess a cuboidal symmetry. These predictions were later confirmed in principle and the basic symmetrical structure of viruses were shown more precisely to be icosahedral. Although not the only shape employed, an icosahedral structure is utilized by a wide variety of bacteriophage and animal viruses. The basic shapes of bacteriophage are schematically depicted in Figure 2, and, Table 4 lists the structural properties for the various phage families. In addition to the variety of structural types found among the various bacteriophage families, there is also a variety of nucleic acid types. Table 5 provides information on the nucleic acids for the various bacteriophage families.

Morphologically, phages can be classified into several groups:

1. Naked icosahedral such as MS2virus, and phiX174
2. Naked helical such as Xf2, Pf1, and v6
3. Enveloped icosahedral such as phi6
4. Enveloped helical of which there are no known bacteriophage
5. Complex such as T4 and lambda

PATHOGENESIS

Bacteriophage can be considered pathogenic to bacteria and thus they follow infection processes common to viral pathogens, including attachment, penetration, and release. Table 6 provides information on the pathogenesis variables of phage families.

Table 3. Baltimore Classifications for Bacteriophage

Kind of Genome	Single- or Double-stranded	Genome Sense (if single strand)	Naked or Enveloped	Families
RNA	SS	Positive	Naked	*Leviviridae*
			Enveloped	N/D
		Positive but reverse-transcribed	Enveloped	N/D
		Negative, nonsegmented	Enveloped	N/D
		Negative, segmented	Enveloped	N/D
	DS	N/A(segmented)	Enveloped	*Cystoviridae*
DNA	SS	Negative (most often)	Naked	*Inoviridae Microviridae*
DS	DS	N/A	Naked	*Tectiviridae Siphoviridae Podoviridae Myoviridae Lipothrixviridae Microviridae Fuselloviridae Corticoviridae*
		N/A	Enveloped	*Plasmaviridae*
		N/A, but with reverse transcriptase step in replication	Enveloped	N/D

Source: Adapted from D. Baltimore, *Trans. N. Y. Acad. Sci.* **33**(3), 327–332 (1971).

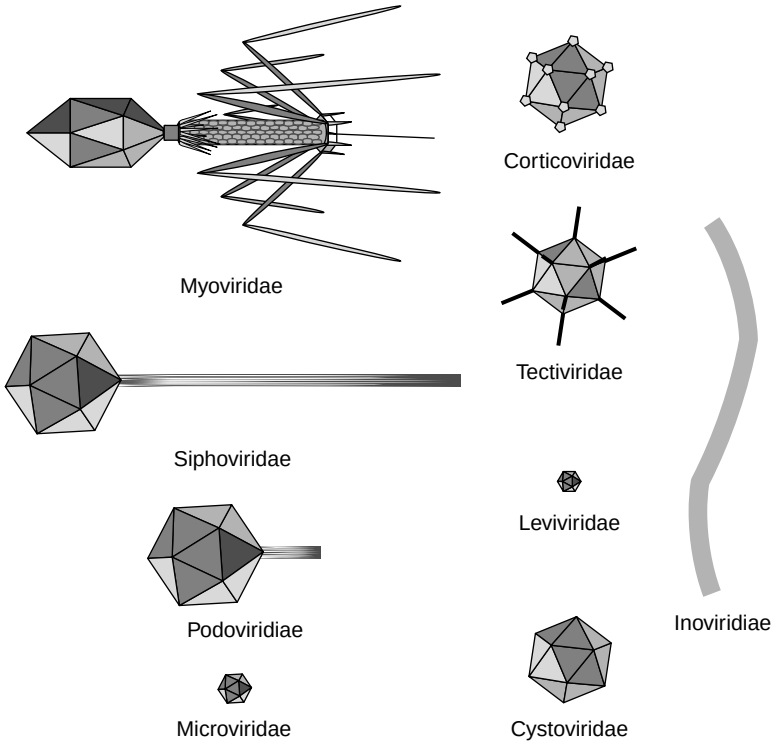

Figure 2. Schematic representations of the structure and relative sizes of major bacteriophage families. Indicated here are virus structures such as the complex structure of *Myoviridae*, the tailed phages *Siphoviridae*, simple icosohedral such as *Microviridae*, spiked icosohedral such as *Tectiviridae*, and filamentous such as *Inoviridae*.

Table 4. Comparative Structural Properties of Bacteriophage Families

Phage group	Symmetry of Nucleocapsid	Lipid (%)	Env[a]	Size	Buoyant density (g/mL,CsCl)	Mol.[b] Weight (MD)
Microviridae	Cubic, Icosahedron	–	–	27	1.41	6.7
Corticoviridae	Cubic, Icosahedron	13	–	60	1.28	49
Tectiviridae	Cubic, Icosahedron	16	–	63	1.28	70
Leviviridae	Cubic, Icosahedron	–	–	24	1.43	4
Cystoviridae	Cubic dodecahedron	23	+	75	1.27	90
Inovirus genus	Helical	–	–	760–1915 × 6	1.30	12–23
Plectrovirus genus	Helical	–	–	85–250 × 14	1.37	?
Plasmaviridae	Pleomorphic	12	+	70–90	?	?
Myoviridae	Complex-tailed	?	–	Capsid 84 Tail 158	1.48	140
Siphoviridae	Complex tailed	?	–	Capsid 62 Tail 195	1.50	84
Podoviridae	Complex tailed	?	–	Capsid 58 Tail 195	1.48	88

Source: Adapted from H. Ackermann and M. S. DuBow, *Viruses of Prokaryotes*, vol. 1, *General Properties of Bacteriophages*, CRC Press, Boca Raton, Fla., 1987, pp. 1–202;
H. Ackermann and M. S. DuBow, *Viruses of Prokaryotes*, vol. 2, *Natural Groups of Bacteriophages*, CRC Press, Boca Raton, Fla., 1987, pp. 1–242.
[a]Envelope.
[b]Molecular.

BASIC GENETICS OF RNA AND DNA PHAGE

Because of the relatively simple genomes, rapid replication, simple assay procedures, and ease of manipulation, early bacteriophages such as lambda became the favorite tool of many molecular microbiologists. Phages also allowed for genetic manipulation of their hosts, quickly becoming a necessary tool for molecular microbiologists. Because of their utility and the need for basic understanding, bacteriophage genetics was quickly unraveled and now serves as a model for genetic control and as a mechanism for protein expression. In this section, we will

Table 5. Properties of Bacteriophage Families Nucleic Acids

Family or Genus	Nature	Total (%)	Mol. Weight (Md)[a]	G + C (%)
Microviridae	D1C	26	1.7	44
Corticoviridae	D2C	13	5.8	43
Tectiviridae	D2L	14	9.7	51
Leviviridae	R1L	31	1.2	51
Cystoviridae	R2L	12	10.4	58
Inovirus	D1C	6–21	1.9–3	40–60
Plectrovirus	D1C		1.8	
F3 group	D2L		10	
Plasmaviridae	D2C		7.6	32
Myoviridae	D2L	43	108	45
Siphoviridae	D2L	49	35	50
Podoviridae	D2L	43	32	48

Source: Adapted from H. Ackermann and M. S. DuBow, *Viruses of Prokaryotes*, vol. 1, *General Properties of Bacteriophages*, CRC Press, Boca Raton, Fla., 1987, pp. 1–202;
H. Ackermann and M. S. DuBow, *Viruses of Prokaryotes*, vol. 2, *Natural Groups of Bacteriophages*, CRC Press, Boca Raton, Fla., 1987, pp. 1–242.
Note: D, DNA: R, RNA; 1, single-stranded, 2, double-stranded, C, circular: L, linear.
[a]Mega Daltons.

describe the genetics of two bacteriophages: MS2, a single-stranded RNA coliphage, and lambda, a double-stranded DNA coliphage.

RNA Phage: MS2

As noted earlier, MS2 is physically similar to the enteroviruses such as *Poliovirus*. Because of this and its easy assay procedures, MS2 soon became the classic model of choice of environmental microbiologists in disinfection and survival experiments such as aerosol or groundwater viral survival and UV exposure modeling. MS2, or more precisely the F+ coliphage of which MS2 is the model organism, have also been used extensively as indicator viruses. Indicators are nonpathogenic microorganisms whose presence in an environmental sample indicates that human pathogens may also be present.

As mentioned, MS2 is a small, icosahedral bacteriophage containing a single-stranded linear RNA molecule. The MS2 capsid is composed of a single protein except for a single copy of an accessory protein known as the A-protein (maturation protein). MS2 is a male-specific phage that usually infects F^+ or *Hfr* strains of *E. coli*. F+ refers to bacteria that possess the F-plasmid, which encodes for the F-pilus, a conjugation structure that allows for transfer of genetic material between two bacteria. MS2 attaches to the sides of the pilus using the A-protein and injects its genetic material into the hollow conjugation tube, allowing migration of the phage RNA up through the conjugation tube into the host bacterium.

Because the RNA of MS2 is coding sense (positive-stranded RNA), it serves as its own mRNA, and once it gains entrance to the cytoplasm of the host bacterium, it can immediately begin production of a new phage. MS2 codes for four known proteins, the coat protein, the A-protein (maturation protein), the replicase protein, and the L protein (lysis protein). Figure 3 shows the genetic map of MS2 indicating the relative positions of these genes. Two of the genes are coded within one reading frame, while the second two are coded in another. To further conserve on the size of the genome, the L-protein's reading frame overlaps with that of the coat and the replicase proteins.

Fiers and coworkers (10) were the first to determine the complete sequence of the 3,569 ribonucleotide bases of the MS2 genome. Even if the sequence information, mapping of open reading frames, and the RNA polymerase binding sites is given, the regulation of the genome and

Table 6. Pathogenic Characteristics of Bacteriophage

Phage	Host Adsorption Site	Virus Attachment Structure	Localization of Infection	Release Method
Tailed phage	Cell wall, capsule, pili, flagella, Surface protein	Tail	Nucleoplasm periphery	Lysis
Microviridae	Cell wall	Spikes	Nucleoplasm	Lysis
Corticoviridae	Cell wall	Spikes	Plasma membrane	Lysis
Tectiviridae	Pili, cell wall	Spikes, "tail"	Nucleoplasm	Lysis
Leviviridae	Pili	Apical protein	Cytoplasm	Lysis
Cystoviridae	Pili	Envelope	Nucleoplasm	Lysis
Inovirus	Pili	Virus tip	Nucleoplasm	Extrusion
Plectrovirus	Plasma membrane	?	Plasma membrane	Extrusion
Plasmaviridae	Plasma membrane	Envelope	?	Budding

Source: Adapted from H. Ackermann and M. S. DuBow, *Viruses of Prokaryotes*, vol. 1, *General Properties of Bacteriophages*, CRC Press, Boca Raton, Fla., 1987, pp. 1–202;
H. Ackermann and M. S. DuBow, *Viruses of Prokaryotes*, vol. 2, *Natural Groups of Bacteriophages*, CRC Press, Boca Raton, Fla., 1987, pp. 1–242.

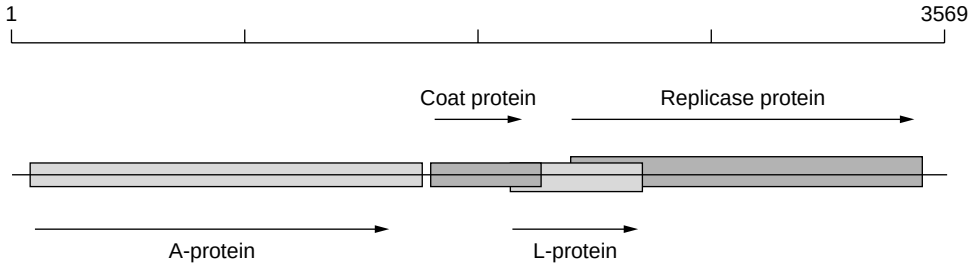

Figure 3. Schematic view of the MS2 genome showing the four encoded proteins. Note that there are two open reading frames that overlap. The replicase gene overlaps the lysis (L) protein and the L-protein overlaps the coat protein. The assembly (A) gene does not overlap with any of the other genes. The arrows indicate the reading direction of each gene and the scale at the top indicates the relative length of the genome.

its four encoded proteins is still difficult to understand without an understanding of the secondary structure of the MS2 genome. It is the secondary structure that actually controls expression of the four encoded proteins. By using secondary structure to control expression levels of individual proteins, the MS2 genome adds elegance and economy, forgoing the need to encode for additional regulatory proteins that would increase the size of the genome. Regulation is accomplished through a process known as translational coupling. Translational coupling refers to a gene arrangement in which the translation of one protein coding sequence on a polycistronic mRNA (coding for more than one gene) is required for the translation of the second downstream or upstream coding sequence.

To grasp the concept of how MS2 uses the secondary structure of its RNA, it is important to realize that ribosomes, which translate the MS2 RNA genome into proteins, have an inherent ability to relieve mRNA secondary structure (disrupt hydrogen bonds). However, this can only occur when the ribosome is bound to mRNA and actively moves along during scanning or translation. Thus, ribosomes are unable to relieve secondary structure before binding and scanning. Thus, nucleic acid secondary structure of mRNA can prevent initiation of macromolecular synthesis but does not inhibit initialized synthesis (11). This regulatory system based on secondary structure controls the production of the A-protein (attachment/maturation protein), the replicase protein, and the L-protein (lysis protein) of MS2. The fourth protein is the coat protein, which is for the most part freely transcribed as it is needed in higher concentrations than the other proteins.

In the case of the A-protein, its coding region is preceded by an untranslated leader of 130 nucleotides, which folds into a cloverleaf, (three stem-loop structures) covering the A-protein's translation initiation region. Thus, as the coat protein is translated and the ribosome moves into the coding region of the A-protein, the cloverleaf structure is dissolved exposing the A-protein ribosomal binding site, allowing binding of a ribosome and translation of the protein. Groeneveld and coworkers (12) indicated that translational starts of the A-protein gene only take place before reannealing of the cloverleaf structure and after translation of the capsid protein.

The replicase protein is required for the formation of the negative-strand template RNA. Replication of phage RNA into a negative strand is a necessary process for the phage to generate its genome. The replicase and three host-derived proteins combine to form a holoennzyme, which converts positive-strand RNA into negative-strand RNA (RNA-dependent RNA polymerase). These negative-stranded RNAs are then converted back into positive-stranded (original sense) RNA genomes, which are packaged into capsids during maturation of the phage. Similar to the A-protein, the expression of the replicase also is controlled by the RNA tertiary structure and translation of the coat protein, although the replicase is located downstream of the coat protein. Thus, as the coat protein is translated and the ribosome dissolves the secondary structure around the coat protein termination site, it exposes the ribosomal binding site of the replication enzyme, allowing for its translation.

The L-protein is encoded in a different reading frame than the coat protein. In addition, the reading frames of these two proteins overlap. Thus, after the ribosome completes translation of a coat protein and relieves the secondary structure that obscures the L-protein initiation site, it is required to release the mRNA, move backward (upstream) to the now exposed binding site of the L-protein, and initiate translation in a new reading frame. It is reasonable to assume that if the ribosome does not quickly find this new open reading frame, or it diffuses in the wrong direction after release from the stop site of the coat protein that the secondary structure (hairpin loop) that blocks translation, can quickly reform (13). Thus, RNA secondary structure regulates translation of the L, A, and replicase proteins and represents the primary form of repression, that ensures the proper balance protein expression.

This mechanism of genetic regulation may be more important for the L-protein than for the A or replicase proteins, as a premature accumulation of the L-protein would cause lysis of the host cell before sufficient coat protein has been made and all phage effectively packaged. In essence, overexpression of the lysis protein lowers the burst size (number of phage produced per bacterium). By nature, the binding affinity for the polymerase is relatively low at the L-protein ribosome binding site. Decreasing this affinity further can also delay or even

prevent accumulation of the L-protein, which in turn can inhibit subsequent lysis of the host cell (11).

Competition for ribosomes is another control method used during translation of A, L, and replicase. The ribosome leaving the coat protein gene can only bind to one of these additional protein genes. Thus, competition for ribosomes represents yet another level of control (11). Three levels of expression control in MS2 include the RNA secondary structure, consensus of ribosome binding sites, and competition for ribosomes during or after translation of the coat protein ensures that proper ratios of the other three proteins encoded by MS2 are achieved.

It is interesting to note that MS2 can self-assemble in vitro with no special enzymatic process involved (14,15). During this maturation process, the coat proteins accumulate around the positive-strand RNA, and the capsid shape begins to form automatically. Finally, the A-protein is added at one end and the virion is completed. The A-protein is required in the mature phage for attachment to the host pilus during infection and injection of the genetic material. At this point during the phage life cycle, sufficient L-protein has accumulated and the cell wall of the host is considerably weakened. As the strength of the wall is compromised, water tension causes the eventual bursting of the host cell and release of the now complete phage particles (11).

DNA Phage: Lambdoid Phage

Andre Lwoof discovered lambdoid phage at the Pasteur Institute, Paris, France. He discovered that certain isolates of *Bacillus* spp., when exposed to ultraviolet light, ceased dividing and then burst open, releasing hundreds of bacteriophage particles. Later in collaboration with Jacob and Monod, he showed that these bacterial strains carried the bacteriophage in a dormant form, termed a *prophage*, and that the phage could be induced to switch from the lysogenic (nonproductive) to the lytic (productive) cycles. Lambda has since become one of the most studied and best understood models for genetic control. Even today, many of the intricacies of the genetic control of lambda are still being elucidated. The genetics of lambda has been extensively studied and provides a valuable model for regulatory control of gene expression.

Similar to MS2, the lambdoid phage can infect *E. coli*, replicate, synthesize new phage, lyse the host cell, and release progeny phage as part of a lytic growth cycle. Unlike MS2, however, lambda can also infect the bacterial cell and its DNA and can then become integrated in the host cell's genetic material, where it is propagated as a part of the bacterial chromosome. Whether the lambdoid phage enters the lysogenic cycle or the lytic cycle is dependent on many factors including the health of the host cell and the multiplicity of infection (MOI). In the case of the discovery of the phage, Lwoff had exposed the host bacteria to UV light, which ultimately signaled the lysogenized phage that its host was in danger. In response to the signal, the lysogenized phage was induced into the lytic cycle. Many forms of stress send signals to the lysogenized phage causing it to leave its dormant stage and enter into a lytic cycle. In the event that the host cell is in a vegetative state, the lambdoid phage does not receive the required stress-related signals and remains incorporated in the host genome. In either case, whether the phage goes into a lytic cycle or stays in the lysogeny cycle, the phage is still able to ensure its own survival and propagation, making it a very successful parasite of *E. coli*.

Structure. The lambdoid phages are considered "complex," meaning that in addition to their icosohedral capsid (which is like MS2's capsid), they also possess an additional tail-like structure that is used for attachment of the phage to the host cell during infection. The more complex structure of a lambdoid phage can be contrasted to the simple structure of the MS2 capsid (Fig. 2). The lambdoid virion has an isometric head with two major capsid proteins, E and D, two minor proteins B and C, and portal proteins W and FII. The capsid is attached to a long flexible tail composed of several proteins, culminating in a single fiber of J, which recognizes the outer membrane, maltose porin protein LamB (2,3).

The genetics of bacteriophage lambda is well known, and most of this section will deal with the prototypical lambda phage genetics. Lambda has a genome of about 46 kb. In the capsid, the lambdoid chromosome is a linear dsDNA molecule usually with 12 nucleotide ssDNA cohesive ends. Table 7 provides the major genes found in lambda phage along with the function of the encoded proteins.

Early Infection. Relying on diffusion, as opposed to any directed locomotion or motility, the lambdoid bacteriophage attaches to the LamB receptor protein, which is located in the bacterial cell outer membrane and which was later discovered to be the maltose porin. LamB protein is responsible for transporting maltose from the environment into the cell. Following contact between the J-protein on the tail of the lambdoid phage and the host cells LamB protein, a conformational change results in the injection of the phage DNA through the core of the hollow tail structure, through the LamB core, and ultimately into the cytoplasm of the host cell. On entry into the bacterial host cell, the linear genome circularizes as the complimentary

Table 7. Major Proteins Encoded by Lambdoid Phage

Genes	Function
nul, A	DNA packaging
W, B, C, D, E, FII	Head formation
Z, U, V, G, T, H, M, L, K, I, J	Tail formation
int, xis	Integration, excision
exo, bet, gam	Recombination
cIII	Stabilization of cII
N	Early antitermination
cI	Repression (primary)
cro	Repression (secondary)
cII	Turn on cI, *int*
O, P	Replication
Q	Late antiterminator
S, R, Rz	Lysis

Source: Adapted from A. Campbell, in Frederick C. Neidhardt et al., ed., *Escherichia Coli and Salmonella: Cellular and Molecular Biology*, 2nd ed., ASM Press, Washington, D.C., 1996.

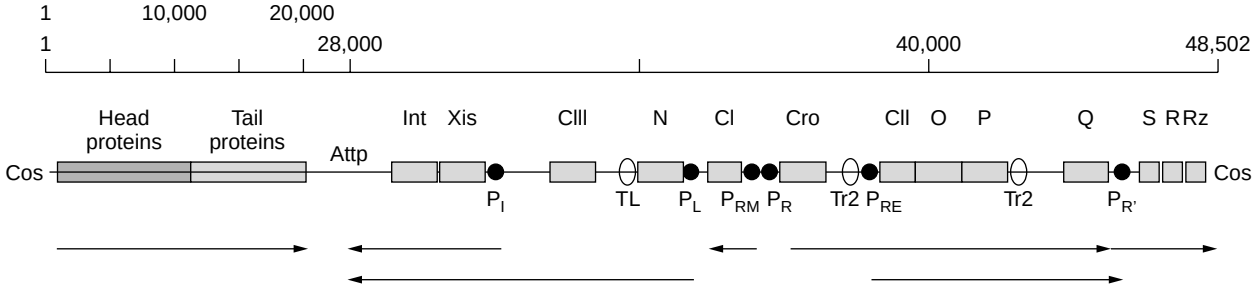

Figure 4. Schematic view of the lambda genome showing the major genes associated with the control of the lysogenic or lytic decision. To the left end of the genome, the bp scale indicated on the top of the figure is condensed to provide clarity to the early genes. Arrows toward the bottom indicate the direction of transcription, which originates from promotors (P). The genome is laid out as it would be in the nucleocapsid before the sticky ends (cos) join after entry into the host. Note the location of the attP site where integration with the host genome occurs.

3′ overhanging cos sites (sticky ends) present on each end of the phage genome hybridize with each other and are sealed by DNA ligase to form a circular genome.

During early infection, several components contribute to the decision of whether the phage will enter the lytic or the lysogenic cycle. These components include three promoters (P_L, P_R, and P_{Rm}), two operators (O_L and O_R), and a group of regulatory proteins (cI, cII, cIII, cro, and N). The location of these components on the phage genome can be seen in Figure 4. An introductory overview of these factors is as follows:

- P_L, P_R, and P_{Rm} are promoters responsible for transcription.
- O_L and O_R are regulatory protein binding sites.
- cI is the major regulatory protein and is transcribed from two different promoters (P_{Rm} and P_{RE}). cI represses transcription from P_L and P_R but allows transcription from P_{Rm}.
- cII and cIII encode activator proteins, which cooperatively bind DNA regulatory sites and enhance transcription of the cI gene from P_{RE}.
- cro is a repressor, which competes with cI to bind the O_R sites.
- N encodes an antiterminator protein, which acts by replacing rho factor for host cell RNA polymerase. It modifies host RNA polymerase activity and prevents termination at the termination sites (T_L, T_{R1}, and T_{R2}).

After circularization of the phage genome within the host cell, the bacterial genetic machinery, considering the newly introduced circular DNA to be "self," begins transcription and translation from the phage genome. As can be seen from Figure 4, this primarily results in the production of three proteins. Transcription and translation from P_R (rightward) leads to production of cro, with transcription termination at the right transcription terminator site (T_{L1}). Transcription and translation from P_L (leftward) leads to production of N and termination at the right transcription terminator site (T_R). There is also a lower level of transcription and translation of cI originating from P_{Rm} (leftward) because P_{Rm} is a weak promoter (16).

Thus, N, cro, and to a lesser extent cI are produced in the cell soon after injection of the phage DNA. The N protein is an antiterminator, which associates with the terminators T_L, T_{R1}, and T_{R2} and consequently allows transcription of longer mRNA. Additional phage proteins are transcribed once sufficient N is produced, including cII from rightward transcription and cIII from leftward transcription. The N protein performs its antitermination function through a complex interaction with several host proteins, RNA, and the host RNA polymerase. Thus, there was an increase in the concentration of N, cro, cI, and now the production of two new proteins, cII and cIII. These events lead the bacteriophage to the next stage in the Lambda infection termed the *decision-state*.

Making the Decision Between the Lytic and Lysogenic State. At this point in early infection, lambda must make the choice between a lytic and a lysogenic cycle. The decision is controlled by the regulators and operators of lambda, but is ultimately decided by the environment and consequently by the physiological state of the host. For example, if the host is experiencing any stress such as DNA damage, starvation, and so forth, the lytic pathway is favored. In other words, if the host has been damaged by radiation from ultraviolet light or is experiencing a limited supply of a growth factor such as tyrosine, the phage presumably decides to move onto a more suitable host. Recent research has revealed that the regulatory molecule responsible for the lytic-lysogenic signal may be Guanosine tetraphosphate (ppGpp) (17), which is a nucleotide that is synthesized by *E. coli* cells in response to amino acid or carbon source starvation. Too low and too high levels of ppGpp resulted in less efficient lysogenization. If the host is growing, and more importantly, dividing normally and has not been exposed to any harmful chemicals or DNA-damaging radiation, the phage considers the bacteria to be a good host, and the lysogenic cycle is more likely to be chosen. Lambda makes this decision using a very elegant mechanism of genetic balance among operators, promoters, and regulatory proteins, centered around the intracellular levels of the cI protein. If the phage can produce sufficient cI before too much cro is built up, it will

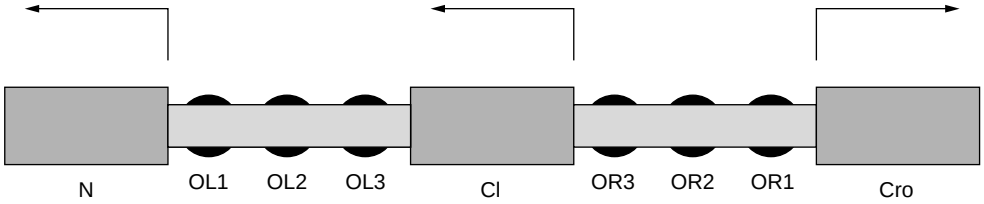

Figure 5. Schematic view of the focal area of the operator sites to which cro and cI bind. Arrows indicate the direction of transcription. O_L and O_R both have three binding sites for cI, and each binds one cro or cI dimer as described in the text. As indicated in the text, cI first binds to O_{L1} and O_{R1} and then to the other operator sites in a cooperative manner. The carboxy-terminal domains of the repressor (cI) dimers mediate this cooperativity. Cro has the opposite affinity binding first to O_{R3}, which represses transcription of P_{RM}.

enter lysogeny. The phage genome will be integrated into the bacterial genome as a prophage. If too much cro is produced before cI reaches the critical level, the cell enters the lytic cycle, resulting in expression of phage structural genes, replication of the phage genome, production of mature phage, and lysis of the host cell.

Cro and cI compete for binding sites on O_R. This is the operator that ultimately decides the fate of the bacteria and the bacteriophage. Two properties of the O_R region enable it to provide sensitive regulation of the phage life cycle. First the O_R region has three binding sites for cI or cro, which from left to right are termed O_{R3}, O_{R2}, and O_{R1} (Fig. 5). Secondly, cro and cI, while they bind to the same locations on the O_R, do so with different affinities for different binding sites. The cI repressor binds with affinity $O_{R1} > O_{R2}$, $>O_{R3}$; binding of cro is with an affinity O_{R3}, $>O_{R1}$, $>O_{R2}$. Occupancy of sites O_{R1} and O_{R2} by cI facilitates the constitutive production of cI from P_{RM} as required for lysogenic growth, while precluding transcription of cro from P_R. Similarly, cro bound to O_{R3} inhibits transcription of cI from P_{RM}.

Transcription of cI originating from P_{Rm} by itself cannot compete with the transcription of cro during early infection. If the levels of cro reach a threshold level before cI is able to turn off its synthesis by binding the O_R sites, then the phage becomes committed to the lytic cycle. There is, however, a very strong cascade of genetic events and kinetic reactions, which tip the balance back toward cI if cellular conditions are adequate (18). For instance, proteins cII and cIII assist cI in at least two ways: they stabilize and protect cI from degradation by host proteases such as RecA and they interact with two additional promoters on the lambda genome (P_I and P_{RE}) (19,20). P_I is the promoter in charge of the proteins needed for lysogeny (int and xis) and P_{RE} is important in the decision-state of lambda. P_{RE} is located just downstream to the left of the cII gene and controls leftward transcription of a cro antisense RNA and the cI mRNA (positive-sense). Thus, additional cI is transcribed from P_{RE}, which becomes a stronger promoter when associated with cII/cIII (21,22), which stabilizes cI and is thought to be the sensory molecule for multiplicity of infection (MOI). MOI is the number of phage that infect a cell at one time. It has been observed that a high multiplicity of infection enhances the expression of the cIII and cII genes, progressively delays lysis time, and increases the rate of lysogenization (23).

Lysogenic Decision. Lysogeny in lambda is characterized by events that lead to integration or incorporation of the lambda genome into the host chromosome. Integration is accomplished through site-specific recombination between the lambda genome and the host chromosome. A lambda-encoded recombinase enzyme known as integrase (Int) mediates the recombination event. Int, together with several host and phage accessory proteins, inserts the phage genome into the host chromosome using specific sites in both genomes known as attachment sites (att) (24). The attachment sites on both genomes, attB for the bacteria and attP for the Phage, share 15 bp of identical sequence. It is important to realize that even during optimum environmental conditions, a substantial fraction of lambda enter the lytic cycle. Some conditions, such as high multiplicity of infection or the presence of divalent cations or cyclic AMP can increase the proportion of cells that go toward lysogeny.

Thus, in what may be considered a rare occasion when the concentration of cII builds up, transcription of cI is enhanced and intracellular levels of the cI repressor protein rise in relation to cro lambda enters the lysogenic cycle. The cI protein binds to O_R and O_L, preventing transcription of all late infection (structural, reproductive, lysis) phage genes from the early promoters P_L and P_R. At this point, the level of cI protein is maintained automatically by a negative feedback mechanism at P_{RM}. Protein cII, during the time when it successfully upregulated expression of cI, also turns on the promoters that control the prophage *int* gene (25).

Integration is very efficient and requires no external energy sources. As the lambda DNA and the host chromosome come together, they form an integration complex, which mediates synapsis and strand exchange. The integration complex consists of the attB, attP, the bivalent DNA binding protein Int, and host proteins. The host proteins include DNA gyrase, which introduce negative supercoils into DNA (26), integration host factor (IHF) that bends attP DNA (27,28), and Fis, which assists in integration (29). Int cleaves each strand at attB and attP, creating strand exchanges by joining broken ends from each (30). Following the initial exchange, there is a "resolving" exchange between the other two DNA strands, resulting in an integrated phage genome.

At this point, the genome of the prophage is almost completely shut down. The cI protein has accumulated and is bound to the operator sites on the phage genome.

There is a balancing act between cI production from the P_{RM} promoter and the normal degradation of cI by the host. As long as cI is the only protein being expressed from the phage genome, the result is a lysogenized *E. coli* bearing an integrated lambda prophage (31).

Lytic Decision. In order to enter the lytic growth cycle, cro must bind to the three operator-sites in O_L and O_R, repressing all transcription from P_L and P_R. This binding ultimately prevents expression of N, which consequently prevents expression of cII and cIII. Thus, cro turns off all early gene expression. During early infection, the transcript from P_L, which produces cro and cII, also produces three other proteins, which accumulate in the host. O, P, and Q proteins are essential for the development of the lytic cycle. Q is an antiterminator, which allows RNA polymerase to override the termination signal downstream of $P_{R'}$ so that it can transcribe the lysis genes (S, R, and Rz) and the structural and accessory genes (Nu1, A, W, etc.). O and P are utilized for replication of the phage genome. Thus, at the point where the lytic decision for the phage genomic DNA replication is well under way owing to the background levels of the O and P proteins, the copy number of late genes available for activation is increased.

Replication of the lambda genome early in the lytic cycle is bidirectional (Theta-form replication) and initiates from an origin within the *O* gene. Protein O recognizes the four 19-bp palindromes of the origin and protein P binds to the host-derived primase DnaB, recruiting it into the replication complex. Ultimately during viral DNA replication, multigenomic tails of double-stranded DNA are spun out from rolling circles. These long pieces of DNA containing repeated lambda genomes become substrate for DNA packaging (16). During rolling, circle as the DNA is spooled out, it is cut into correctly sized pieces by a phage-encoded enzyme called *Ter*. Ter cuts at the cos sites releasing mature phage lambda DNA ready to be packaged into the phage head.

Morphogenesis is the process of phage particle assembly that takes place in the host cell nucleoplasm periphery and includes formation of the phage capsid, condensation of DNA, packaging of the DNA into the capsid, and attachment of the tail. Morphogenesis results in mature phages that are capable of infecting a new host. During morphogenesis of the phage particles, there is a concurrent buildup of lysis proteins, which ultimately results in lysis of the host cell and release of the infective lambda particles into the extracellular environment. Lambda has at least two lysis genes, *S* and *R* (32). Protein R is an enzyme that cleaves between N-acetylglucosamine residue bonds similar to the action of lysozyme. Protein S is a holin-like protein, which disrupts inner membranes of the host, allowing *R* access to the N-acetylglucosamine in the murein layer. Ultimately, the infection cycle results in the production and release of 50 to 100 infective phage particles per lysed cell (33).

Induction: The Switch from Lysogenic to Lytic. Lysogeny, once established, is relatively stable. The cI repressor continues to stimulate its own synthesis and the inserted prophage is passively replicated as part of the bacterial chromosome. A delicate equilibrium exists between stable lysogeny and induction. Induction is the stimulation of transcription resulting in excision of the phage genome from the host chromosome and, ultimately, movement into the lytic cycle. The health of the host and its responses to environmental stimuli determine whether the phage will remain within the host genome or enter the induction process. The primary mechanism that controls induction is the availability of cI. Physiological stress such as UV damage results in the activation of a host cell SOS response. The expression of the genes in the SOS regulatory network is controlled by a complex circuitry involving the RecA and LexA proteins (28,34–36). As indicated aRecA's normal substrate is LexA but it also nonspecifically degrades cI (lambda repressor), stimulating the dormant prophage to enter the lytic cycle.

The nonspecific cleavage of cI by aRecA renders it inactive and it falls off the operator sequences O_L and O_R. This allows *E. coli* RNA polymerase to transcribe the gene for cro and Xis and Int, which were under repression by cI. The Xis and Int proteins interact to form an excision complex, which more or less reverses the integration process and effects induction of the prophage from the host chromosome. From this point, the lambda genome circularizes and follows a lytic infection cycle as described previously. It should be noted that induction is irreversible. One probable explanation is that in a lysogen there is a MOI of only one (the prophage itself). After induction, the lytic cycle, as described previously, leads to morphogenesis, lysis of the host cell, and release of progeny phage particles.

CONCLUSION

Bacteriophages have been and will continue to be used by environmental microbiologists as indicators and model organisms. Simple and inexpensive assay procedures create a remarkably easy system that can provide a wealth of information not only about the environmental fate of the phage themselves but also as a model system for the study of eukaryotic viruses. From their use as airborne indicators of fecal pollution, as models for studying the subsurface transport of viruses in aquifers, as indicators of fecal pollution in surface and drinking water, as vectors for the transmission of genetic information between species of bacteria and as simple models to help unravel the complexities of genetic control, bacteriophage have been instilled into the core of most aspects of microbiology.

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BACTERIOPHAGE DETECTION METHODOLOGIES

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Bacteriophages are viruses that infect bacterial cells. A variety of different bacteriophages or phages (as they are commonly referred to) that infect different bacterial cells have been reported in the literature. Phages that infect the coliform group of bacteria are termed coliphages. Coliphages that infect coliform bacteria that contain the F-pilus or sex pilus are termed male-specific phages or F-specific phages. According to the International Standards Organization (ISO), the F-specific RNA bacteriophages are defined as, "bacterial viruses which are capable of infecting a specific host strain with F-or sex-pili to produce visible plaques (clearance zones) in a confluent culture lawn grown under appropriate culture conditions. The infectious process is inhibited, however, by concentration (40–400 μg/mL) of RNase in the plating medium" (1). The ISO defines somatic coliphages as "bacterial viruses which are capable of infecting selected *Escherichia coli* host strains (and related strains) by attachment to the bacterial cell wall as the first step of the infectious process. They produce visible plaques in a confluent lawn of host bacteria grown under appropriate culture conditions" (2). Bacteriophages that infect *Bacteroides fragilis* are defined by the ISO as "bacterial viruses which are capable of infecting selected *Bacteroides fragilis* host strains by attachment to the bacterial cell wall as the first step of the infectious process" (3). Various studies have shown the usefulness of bacteriophages as indicators of fecal pollution caused by viruses. Coliphages may be good indicators of enteric viral pollution because they are found in relatively high numbers in human and nonhuman feces, are persistent in the environment, do not multiply in the environment, and the cost of coliphages assay is only a fraction of an enterovirus tissue culture assay. Recently, coliphages also have been suggested as conservative indicators of enteric viruses because of their similarities in size, transport, survival, persistence, and high densities in sewage and septic samples. Bacteriophages that infect the *Bacteroides fragilis* also have been suggested as potential indicators of fecal pollution (4). *Bacteroides fragilis* is an obligate anaerobe found in high concentrations in human feces, and thus the presence or absence of these phages may be indicative of fecal pollution in a sample. One salient feature of using Bacteriodes phages is that because these bacteria are reportedly present only in human feces, the presence of these phages in a sample may be a strong indication of human fecal pollution. The use of bacteriophages such as coliphages as indicators of fecal pollution is based on the assumption that their presence in water samples denotes the presence of bacteria capable of supporting the replication of the phage (5). Bacteriophage as an indicator has an advantage. The detection method is simple and inexpensive and the results usually are available within 8 to 10 hours. This chapter provides an overview of some of the methods that have been used to detect and enumerate bacteriophages, especially the coliphages and the *Bacteriodes* phages in

environmental samples. The methods that have been described pertain to only those assays in which the primary purpose is to determine the microbiological quality of an environmental sample. The reader is advised to consult other entries in this encyclopedia for a detailed discussion on the classification of bacteriophages, their occurrence, their persistence, and the rationale for using phages as indicators of fecal pollution.

RECOVERY OF BACTERIOPHAGES FROM GROUNDWATER AND DRINKING WATER

One of the primary issues confronting the detection of bacteriophages, especially the male-specific coliphages in environmental samples is the potentially large volume of samples that needs to be analyzed. The rationale for employing large sample volumes is based on the premise that enteric viruses are normally present in very low numbers. There are various procedures for the recovery, detection, and enumeration of bacteriophages (2,6–8)). These protocols vary in terms of the recommended volumes to be analyzed, sample processing methods, and the recommended host bacteria. The terminologies used in these procedures are based on the distinguishing characteristics of the procedure, of the original publication, or of the agency that published it. For example, the "AWWARF method" is based on a membrane filtration (MF) approach developed by Sobsey and coworkers (9), whereas the "Environmental Protection Agency (EPA) methods" rely on the concentration of large-volume (500 gal) samples and smaller (100 and 1,000 mL) samples. The "ISO method" differs from the other methods primarily in the use of *B. fragilis* as the host bacteria.

MF Technique

The MF method of Sobsey and coworkers (9) was based on a field-tested protocol that compared the recovery of male-specific coliphages in several source waters with concentrations of fecal-indicator bacteria. Erb and coworkers (10) reported on the detection of low concentrations of male-specific coliphages in swimming pool waters using the MF method. However, they had to adjust the sample pH to 3.5 before filtration to achieve efficient recoveries. They have reported that humic acids in the water samples could interfere with adsorption of phages to the filter. The MF-based method is unique in that it relies on the use of widely available inexpensive membrane filters and filtration apparatus along with manageable sample volumes (1,000 mL).

Procedure

1. A water sample (1,000 mL) initially is supplemented with $MgCl_2$ to obtain a final concentration of 0.05 M $MgCl_2$.
2. The sample is filtered through a 47-mm (0.45 μm pore size), cellulose acetate membrane at a flow rate not exceeding 100 mL per minute.
3. The F-specific coliphages adsorbed to the membranes are eluted using 3 mL of a high pH buffer (3% beef extract V; 0.3% Tween 80; pH 9.5).
4. The sample concentrate is neutralized as soon as possible with 0.1 M HCl.
5. This sample concentrate then is used for the bacteriophage analysis using appropriate host bacteria.

In the original method, the host bacterium *Salmonella typhimurium* (strain WG 49) was employed for the detection of F-specific coliphages. This particular host strain is resistant to nalidixic acid and kanamycin and contains an *E. coli* plasmid (F′42lac : Tn5) that is responsible for pili production and thus susceptible to infection by F-specific coliphages (6). The host strain is grown in a medium containing 1% tryptone, 0.1% glucose, 0.8% NaCl, 0.03% $CaCl_2 \cdot H_20$, 0.015% magnesium sulfate, 100 mg/L nalidixic acid, and 20 mg/L kanamycin sulfate. The concentrations for the top and bottom agar were 0.7% and 0.5%, respectively. Although the original protocol suggested the use of *S. typhimurium* WG49 as the host bacterium, the authors indicated that there may be interferences by somatic Salmonellaphages. There is concern that the plasmid is not stable within the host bacterium and thus false negatives could arise if the WG49 strain is used. This has led to the use of alternate host bacteria such as *E. coli* 15597 as a tool to screen for male-specific phages. Theoretically, it is possible to use any other suitable host bacteria and exploit the advantageous features of this protocol. In laboratory studies, the observed decrease in phage recovery with increase in sample volumes was attributed to the accumulation of "deposits" on the filter surface. Sobsey and coworkers (9) recommended that sample volumes should be small enough to prevent deposit accumulations (or the use of 90-mm diameter filters). Sobsey and coworkers (9) suggested that the filters could be placed "face down" onto agar media containing lawns of the specific host bacteria. To aid in the detection of the enumeration of the plaques, the use of tetrazolium dye (0.03% final concentration) was suggested.

The MF method has been used to screen groundwater serving public water supply wells along the Texas-Mexico border region. In this study, 17 wells were sampled over a span of three months, however, none of the samples were positive for male-specific coliphages. The protocol was as follows:

1. Groundwater field samples (1,000 mL) were collected in sterile containers filled under optimum conditions. The samples were adjusted initially to 0.05 M $MgCl_2$ using sterile $MgCl_2$ and thoroughly mixed.
2. The samples were then filtered through a sterile 0.45-μm pore size filter at the rate of 100 mL per minute.
3. A sterile polypropylene tube was placed beneath the membrane and 5 mL of a beef extract elution solution (3% beef extract V; 0.3% Tween 80; pH 9.5) was added to the membrane. After five-minute incubation, this solution was filtered through the membrane (by vacuum application). To quickly bring the pH of the buffer to neutrality, 770 μl of 0.1 M HCl was added to the tube containing the eluate.

4. This concentrated solution was then used for the bacteriophage analysis. Aliquots (0.5 mL) of the solution were used in conventional double agar overlay techniques for phage enumeration using host bacteria specific for F-specific phages (*E. coli* ATCC 15597) and somatic phages (*E. coli* ATCC 13706). Parallel studies, using spiked groundwater samples, showed very high phage recoveries.
5. In this protocol, a filter-elution step (to elute the viruses off the filter) was employed, rather than "laying the filter facedown" on an agar plate. This avoided problems associated with the plaque visibility and the need to use tetrazolium dyes for plaque contrast purposes.

The U.S. EPA ICR Protocol

The U.S. EPA published a standardized procedure for the enumeration of somatic and male-specific coliphages in the implementation of the Information Collection Rule (ICR) (7). In this procedure, water samples are passed across and in between a positively charged 1 MDS filter (CUNO Inc., Meridien, CT). However, for source water, the total amounts of sample that can be filtered through the 1 MDS filter will depend on the water quality in terms of suspended solids. The recommended sample volumes are 200 L for source water and 1,500 L for finished water. However, samples up to 1,500 L were used for groundwater-monitoring purposes (11).

ICR Method

Sampling

The routine precautions that one employs normally when sampling for enteric viruses are followed such as flow rate (not exceeding 3 gallons per minute) (7). Also, if the water to be sampled contains a disinfectant such as chlorine, a 2% sodium thiosulfate as a neutralizing agent is injected continually into the sampling stream at a rate of 10 mL/gal (2.6 mL/L or 30 mL per minute at a flow rate of 3 gal per minute). The filter cartridge along with the filter is then aseptically packed in sterile foil and transported to the laboratory on ice, and processed as soon as possible.

Sample Processing

1. Filters are eluted initially using one liter of sterile beef extract glycine solution (1.5% beef extract V, 0.05 M glycine in water, pH 9.4). The filter is soaked in the elution solution for 15 minutes. The solution then is forced from the housing into a sterile 2-L beaker using nitrogen gas or positive air pressure. (It has been found that this particular step in the protocol is too cumbersome, prone to significant errors, and possibly could lead to cross-contamination). Currently in our laboratories, the elution solution is added to the filter holder and rocked back and forth for 15 minutes using a rocking platform.
2. The eluate is then poured into a sterile 2-L beaker, the pH adjusted to 7.0–7.4 using 1 M HCl, and the solution stirred for 15 minutes. Forty milliliters of the eluate is mixed with 4 mL of glycerol and the sample is stored at −80 °C until the sample is flocculated and further concentrated (details of these steps are presented elsewhere in the encyclopedia).
3. The bacteriophage assay is then performed on the concentrated sample using the appropriate host bacteria and the conventional double agar overlay method.
4. There is concern that the exposure of phage to high pH during the sample processing could lead to significant loss of phage recovery. This has forced researchers to explore alternate sample processing approaches.

ISO Method for Detection and Enumeration of Bacteriophages

The method for the detection of F-specific bacteriophages already is an official standard for the ISO, whereas the method for the detection and enumeration of somatic phages and *B. fragilis* phages currently is in the final stages of approval (Juan Joffre, Personal Communication). The detection and enumeration method for F-specific RNA bacteriophages can be applied theoretically to all kinds of water, sediments, and sludges where necessary after dilution. The ISO method recommends a preconcentration if necessary for samples that may harbor low numbers of bacteriophage.

ISO Method for the Enumeration of F-Specific RNA Bacteriophages. The sampling, transport, and storage of water samples are to be handled in accordance with the ISO, ISO 8199 (ISO 5667-1, ISO 5667-2 and ISO 5667-3 (1,11–13). The method recommends the use of *S. typhimurium* WG 49, *E. coli* K-12 Hfr, or *E. coli* HS (pFamp) R (14). The host cultures are grown in tryptone yeast extract glucose broth (TYGB) at 37 °C. The quality control check of the *S. typhimurium* strain (WG49) is performed by screening for the presence of lactose-negative colonies (which would indicate plasmid segregation) and inhibition zones around antibiotic disks impregnated with kanamycin and nalidixic acid. Less than 8% of the cells should be lactose-negative with no inhibition zone around the nalidixic acid disk and an inhibition zone around kanamycin disks of less than 20-mm diameter. The ISO method clearly details the protocol for samples containing high bacterial background, samples with low phage counts, and a presence/absence test. The salient feature of the protocol is that the method suggests confirmatory steps when sampling new sources, when there is an unexplained overabundance of F-specific bacteriophages, or when there is indication that somatic phages ("large, circular, clear plaques with smooth edges") are being isolated. For confirmation purposes, the protocol recommends plating aliquots of the sample (in parallel) on a series of plates containing RNase at a final concentration of 40 μg/mL. The appropriate volume of a stock solution is added to soft agar just before adding the top layer. The protocol suggests that at times the RNase concentration may need to be as high as 100 μg/mL for selectivity.

Protocol

1. Semisolid (soft agar) TYGA media (50 mL) in bottles are melted and placed in a 45 °C water bath. (Because nalidixic acid is heat-stable, this antibiotic could be added before autoclaving to prevent background flora interferences). To these bottles, 0.5 mL of calcium-glucose solution ($CaCl_2 \cdot 2H_2O$ 3 g; glucose 10 g; water 100 mL) and 2.5-mL aliquots are distributed into sterile capped tubes.
2. To each tube, one milliliter of the *undiluted or diluted* sample is added, thoroughly mixed, and the contents of the tube poured over a 9-cm bottom agar TYGA plate. The plates are incubated for 20 hours at 37 °C. The protocol recommends that each sample be tested at least in duplicate.

Samples with High Background Flora. For such samples, nalidixic acid is added to semisolid soft TYGB to a final concentration of 100 μg/mL. The conventional double agar layer method then is adopted.

Samples with Low Phage Counts. For samples in which the phage numbers are suspected to be low, the methodology calls for modifications as mentioned in the following text. Ten milliliters of semi solid TYGA is combined with one milliliter of host culture and 5 mL of sample dilutions (in duplicate) are poured over 50 mL of TYGA in a 14-cm petri dish. It is mentioned that this procedure is capable of detecting up to one phage particle per 50 or 100 mL if 10–20 plates are inoculated in parallel.

Confirmatory Test. Plates containing RNase (40 μg/mL) are prepared by mixing the appropriate volume of RNase solution to 2.5-mL semisolid TYGA in a tube and then pouring plates. Confirmatory tests are recommended when examining new sampling points, and when large circular clear plaques with smooth edges (probably somatic Salmonella phages) are routinely isolated.

Presence/Absence Test

Method

1. The host culture is prepared as described previously. Twenty-five milliliters (25 mL) of TYGB are added to a conical flask and prewarmed to room temperature.
2. The host culture (0.25 mL) is added along with the calcium-glucose solution (250 μL) to the TYGB-containing flask and incubated at 37 °C for three hours with shaking (100 rpm).
3. The water sample (1 mL) (prewarmed to room temperature) is added to the flask and incubated for 18 hours at 37 °C.
4. One milliliter (1 mL) of the culture is transferred to a centrifuge tube, and 0.4 mL of chloroform is added, thoroughly mixed, and centrifuged at 3,000 X g for five minutes.
5. The host culture is prepared as described previously.
6. Semisolid TYGA in bottles is melted and placed in a 45 °C water bath. Calcium-glucose solution then is aseptically added (500 μL/50 mL)
7. Aliquots (2.5 mL) of the TYGA media are placed into culture tubes with caps, placed in a 45 °C water bath.
8. To each tube, one milliliter of the host culture is added, mixed carefully to avoid bubble formation, and the contents are poured atop a TYGA plate in a 9-cm petri dish. Care is taken to make sure that the soft area is spread out evenly over the bottom agar plate and is allowed to solidify on a level surface.
9. The plates are dried in a laminar flow cabinet or in a 37 °C incubator for 30 minutes, while the plates are inverted and the lids are off.
10. A drop of the chloroform-treated culture is placed on the inoculated plate using a fine capillary pipette.
11. The spot is allowed to dry and the plates are incubated inverted (with the lids) at 37 °C for 18 hours.
12. The plates then are examined for clearance zones, which are indicative of F-specific phages.

The ISO (standard procedure) does mention that the protocol can be amended for use with larger samples (by using double-strength TYGB) and in a most probable number (MPN) format. Controls (both positive and negative controls, especially spiked positive controls) are strongly recommended. The results are expressed as plaque-forming particles per milliliter

$$(\text{pfp/mL}) = \frac{N - NRNase}{n} \times F$$

where pfp/mL is the confirmed concentration of F-specific RNA bacteriophages in milliliters, N is the total number of plaques counted on W49 plates, *NRNase* is the total number of plaques counted on WG49 plates with Rnase, n is the number of replicates, and F is the dilution factor.

ISO METHOD FOR THE ENUMERATION OF SOMATIC COLIPHAGES

The ISO method recommends the use of the *E. coli* strain c (ATCC 13706) for samples with low bacterial count such as drinking water and unpolluted natural waters. For samples containing large numbers of bacteria (e.g., wastewater and polluted source waters), the method recommends the use of the nalidixic acid–resistant *E. coli* strain CN (ATCC 70078) also known as WG5. (15).

Preparation of Host Culture. The host culture is grown in modified Scholtens' medium. The medium contains (per 1,000 mL) peptone 2 g, yeast extract 3 g, meat extract 12 g, NaCl 3 g, Na_2CO_3 solution (150 g/L) 5 mL, and $MgCl_2$ solution (100 g $MgCl_2 \cdot 6H_2O$ in 50 mL water) 0.3 mL. The pH of the medium after sterilization should be around 7.2.

Method

1. Molten semisolid modified Schölters' agar (MSA) medium is placed in a 45 °C water bath and 300 μL

of sterile calcium chloride solution (14.6 g/100 mL) is added to it.
2. Aliquots (2.5 mL) are placed into culture tubes with caps and placed in a 45 °C water bath.
3. To each of the culture tubes, one milliliter of the water sample or its dilutions (prewarmed to room temperature) is added.
4. The host culture (1 mL) is added to each of the culture tubes containing the media and the sample, and mixed well, taking care to prevent bubble formation. The contents of the tube are then poured over a bottom agar plate of MSA.
5. The plates are allowed to incubate for approximately 18 hours at 37 °C.

Samples with High Background Flora. For such samples, nalidixic acid at a final concentration of 250 μg/mL is added to the top agar (semisolid MSA).

Samples with Low Phage Counts. For such samples, the protocol suggests the use of 10 mL of semisolid media, 60 μL of calcium chloride solution, one milliliter of host culture, and 5 mL of sample in duplicate. The entire contents are poured over a 15-cm petri dish containing 50 mL of MSA.

Presence/Absence Test

Method

1. Modified Schölters broth (25 mL) is added to a sterile conical flask and prewarmed to room temperature.
2. Calcium chloride solution (1 M) (150 μL) is added aseptically to the flask.
3. Then, 0.25 mL of the host culture is added and followed by one milliliter of the sample (prewarmed to room temperature) and incubated for 18 hours.
4. An aliquot (1 mL) of the above culture is transferred to a centrifuge tube and 0.4 mL of chloroform is added, mixed well, and centrifuged at 3,000 x g for five minutes.
5. Bottles containing molten 50 mL of semisolid MSA are placed in a 45 °C water bath.
6. Prewarmed calcium chloride solution (300 μL) is added aseptically to the agar in the water bath and 2.5-mL aliquots are transferred to culture tubes with caps. The culture tubes are placed in a 45 °C water bath.
7. To each of these tubes, one milliliter of the host culture is added, mixed well, and the contents poured atop bottom agar plates containing MSA.
8. The plates are incubated for 30 minutes in a laminar flow hood or incubator at 37 °C and air-dried with the lids off.
9. One drop of the chloroform-treated sample culture is "spotted" on the above plates.
10. The plates are incubated at 37 °C for 18 hours.
11. The presence of phages is indicated by the appearance of clear zones near the spotted area.

The procedure can be used in an MPN approach to quantitatively estimate phage numbers. However, double-strength medium needs to be employed.

The number of pfp is computed as follows:

$$\text{pfp/mL} = \frac{N}{nvF}$$

where N is the number of plaques counted, n is the number of replicates counted, V is the volume employed, and F is the dilution factor

ISO METHOD FOR THE ENUMERATION OF BACTERIOPHAGES INFECTING *B. FRAGILIS*

The rationale for enumerating phages infecting *B. fragilis* is that the bacterium (*B. fragilis*) is an obligate anaerobe found only within the gastrointestinal tracts of warm-blooded animals. Thus, detecting such phages in source water or finished water is indicative that the sample has fecal contamination.

The primary difference between this bacterial host and the other hosts is obligatory anaerobic incubation requirements of the host bacteria. Although the bacterium is an obligate anaerobe, it does not require anaerobic handling conditions. Only the incubation has to be carried out under anaerobic conditions. Anaerobic cabinets, jars, or bags can be employed for this purpose. However, when using liquid media, it is critical that the containers are completely filled (to avoid oxygen-rich headspaces) and closed with a screw cap.

The specific host bacterial strain for the purposes of this methodology is *B. fragilis* RYC2056 (ATCC 700786) (16). The bacterium can be grown in (Bacteroides phage recovery medium broth) BPRMB. This medium contains (per liter) meat peptone 10 g, casein peptone 10 g, yeast extract 2 g, NaCl 5 g, monohydrated L-cysteine 0.5 g, glucose 1.8 g, $MgSO_4 \cdot 7H_20$ 0.12 g, and $CaCl_2$ solution (0.5g/mL) one milliliter.

Preparation of Host Bacterial Culture. A vial of the stock culture is allowed to equilibrate to room temperature. Using a cotton swab, the surface of the BPRM agar plate is streaked and incubated at 37 °C for at least 40 hours under anaerobic conditions. Using a cotton swab, portions of the colonies are transferred from the plate to a 10-mL tube containing 10 mL of BPRMB. This is incubated at 37 °C for at least 24 hours. The optimum cell density for plaque formation and visualization is between 1 to 4×10^8 CFU/mL. Appropriately designed side-arm flasks are used to obtain optical density readings.

Method

1. The host culture is prepared as described and allowed to equilibrate to room temperature.
2. Bottles containing molten 50 mL of semisolid BPRMA (top agar) is maintained at 45 °C in a water bath.
3. Ten milliliters (10 mL) of hemin solution (hemin 0.1 g, 1 N NaOH 0.5 mL, 99.5 mL H_2O), one milliliter of (0.05 g·mL) $CaCl_2$ solution, 25 mL of

1 M Na_2CO_3, one milliliter of 100 μg/mL kanamycin sulfate, and 4 m of nalidixic acid (100 μg/mL) are added to the medium already mentioned.
4. Aliquots (2.5 mL) are removed and placed into culture tubes with caps and placed in a 45 °C water bath.
5. To each of these tubes, 1 milliliter of water sample is added and mixed well, avoiding the formation of air bubbles.
6. The entire contents are poured on a bottom agar plate containing BPRMA in a 9-cm petri dish.
7. The plates are allowed to solidify, and incubated upside down under anaerobic conditions at 37 °C for 18 hours.
8. After incubation, the number of plaques is counted to estimate the phage concentration per unit volume of the sample.

Samples with High Background Flora. For such samples, it is recommended that kanamycin monosulfate at a final concentration of 300 μg/mL be added to the soft agar.

Presence/Absence Test. This test can be used to qualitatively determine the presence or absence of *B. fragilis*–specific phages in different samples volumes. The protocol given in the following text is for 100-mL sample volume.

Method

1. Double-strength BPRMB (100 mL) is added to a 250-mL screw cap glass bottle and prewarmed to room temperature.
2. The sample (100 mL) (also prewarmed to room temperature) is added to the bottle containing the medium.
3. The host culture in the exponential growth phase containing approximately 108 cells per milliliter is added to the bottle.
4. Additionally, medium is added to fill the bottle completely and the bottle is screwed tight. (To indicate anaerobiosis, resazurin can be added. Resazurin at a stock concentration of 0.025 g/100 mL is recommended. Anaerobiosis is indicated by a change of color from blue to straw color).
5. The bottles are incubated at 37 °C for 18 hours.
6. To 1 milliliter of this enrichment culture, 0.4 mL of chloroform is added, mixed, and centrifuged at 3,000 X g for five minutes.
7. Semisolid BPRMA (50 mL) is melted using a boiling water bath and incubated at 45 °C. Hemin, Na_2CO_3, and antibiotics are added as per the concentrations mentioned earlier.
8. Aliquots (2.5 mL) are placed in culture tubes with caps and the tubes are incubated at 45 °C.
9. Host culture (1 milliliter containing approximately 10^8 cells per milliliter) is added and mixed well with the media. Care is taken to make sure that no air bubbles form.
10. The contents of each tube are poured on to a 9-cm petri dish and allowed to solidify on an even surface.
11. One drop of chloroform-treated culture is placed on each plate and the plates incubated at 37 °C for at least 18 hours under anaerobic conditions.
12. The plates then are examined for characteristic clear zones or plaques.

QUALITATIVE DETECTION OF COLIPHAGES IN SOURCE WATER — U.S. EPA METHOD 1601

Method 1601, a two-step enrichment procedure, is a performance-based method for qualitatively detecting the presence or absence of male-specific and somatic coliphages in groundwater and source water (17). This protocol has been tested in a multilaboratory round robin testing protocol in the United States. Method 1601 specifies the host strains for isolating male-specific and somatic coliphages.

The attractive features of this protocol are that it can be used either with 100-mL or 1,000-mL sample volumes. The ability to use a large sample volume is advantageous because it increases the probability of detecting phages that may be present in the low concentrations. Somatic coliphages are screened using *E. coli* CN-13 as the host strain, whereas male-specific coliphages are screened using *E. coli* F_{amp} as the host strain. *E. coli* CN-13 is a nalidixic acid–resistant mutant of *E. coli* (ATCC 700609), whereas *E. coli* Famp is an ampicillin-streptomycin-resistant mutant of *E. coli*. *E. coli* CN-13 was originated at the University of Quebec in Canada, whereas *E. coli* Famp was originated by Victor Cabelli at the University of Rhode Island.

Method

1. Logarithmic phase host bacterial cultures are prepared in tryptic soy broth (TSB) amended with the appropriate antibiotics. *E. coli* CN-13 is grown in nalidixic acid–containing medium (10 mg/100 mL), whereas *E. coli* Famp is grown in streptomycin-ampicillin-containing medium (1.5 mg each antibiotic/100 mL).
2. The cultures are incubated at 37 °C on a shaker at 100–150 rpm for approximately four hours until the cultures are in log phase. The host cultures when ready can be placed at 4 °C for four hours until they are ready to be used in the protocol.
3. Spot Plate Preparation. Separate spot plates are prepared for the somatic and male-specific coliphages. Two flasks of tryptic soy agar (TSA) (100 mL) are autoclaved and cooled to 45 °C in a water bath. To one of the flasks, 2 mL of the host culture and one milliliter of 10 g/mL nalidixic acid solution are added. To the other flask, 2 mL of the host culture and one milliliter of 0.15-mg/mL ampicillin-streptomycin stock solution are added. Twenty-milliliter aliquots are poured into 100-mm petri plates. The plates are appropriately labeled as being either CN-13 or Famp plates depending on the type of antibiotic that was added. The plates

are allowed to solidify and could be used that day or stored at 4 °C for up to a week.

4. 100-mL Samples. Water samples (100 mL) are placed in sterile 125-mL bottles. To each of the bottles that contain the sample, 0.5 mL of the appropriate host bacterial culture, one milliliter of the appropriate antibiotic solution, 1.25 mL of a 4 M $MgCl_2 \cdot 6H_2O$ solution, and 5 mL of 10 X TSB medium are added. The bottles are tightly capped, mixed by inversion, and incubated for 24 hours at 37 °C with no further mixing.
5. 1,000-mL Samples. Water samples (1,000 mL) are placed in sterile 2-L bottles. To each of the bottles that contain the sample, 5 mL of the appropriate host bacterial culture, 5 mL of the appropriate antibiotic solution, 12.5 mL of a 4 M $MgCl_2 \cdot 6H_2O$, and 50 mL of a 10 X TSB are added. The bottles are tightly capped, mixed by inversion, and incubated for 24 hours at 37 °C with no further mixing.
6. Following incubation, 10 μL of the enriched sample is "spotted" on the appropriate spot plate that contains the appropriate host bacteria and antibiotic. For example, 10 μL of the sample bottle that contains the CN-13 host bacteria is spotted on the spot plate that has CN-13 and the nalidixic acid.
7. As many as 20 spots can be made on one 100-mm spot plate. The spots are allowed to absorb into the medium by incubating for approximately 30 minutes. Care must be taken to make sure that the spot inoculum is not allowed to "run" across the plate.
8. The plates are incubated at 37 °C for 16 to 24 hours.
9. Positive results are indicated when lysis zones (a circular zone of clearing) appear at the various spots. A positive result may also appear as one or more small plaques or clearings at the spots. If bacterial growth at the lysis zones interferes with an accurate determination of whether the spot is "positive" or "negative," aliquots of the enrichment sample can be centrifuged or filtered quickly and the supernatant can be spotted on a fresh spot plate.
10. Careful controls should be included in all experiments because the potential for phage cross-contamination is very high. The use of replicate spots on separate plates also is recommended.
11. Because this is a qualitative procedure the results can be expressed only as either positive or negative for each 100- or 1,000-mL sample.

QUANTITATIVE DETECTION OF COLIPHAGES IN SOURCE WATER — U.S. EPA METHOD 1602

Method 1602 is a quantitative method for the detection of coliphages in water samples (18). However, unlike the previously described Method 1601, this method allows the quantitation of the coliphage numbers that are detected and also employs *E. coli* CN-13 and *E. coli* Famp for somatic and male-specific coliphage detection, respectively. Method 1602 is only for 100-mL samples. The basic principle of the method is as follows: host bacteria, double-strength agar medium, and $MgCl_2$ are added to 100-mL water sample and the entire contents poured into 5 to 10 petri dishes. After overnight incubation, the total numbers of plaques are counted and tallied and the result reported as PFU(plaque-forming unit)/100 mL.

Method

1. The host bacteria and their respective antibiotic solutions are similar to the enrichment culture described under Method 1601.
2. The host bacterial cultures are prepared as described earlier. The cultures can be stored at 4 °C for no more than 48 hours.
3. Concentrated (2X) TSA (100 mL) is prepared and allowed to cool to 45 °C in a water bath. Separate flasks are prepared for the nalidixic acid amendment and for the ampicillin-streptomycin amendment.
4. Two 100-mL samples are placed in separate 500-mL flasks.
5. To each of these flasks, 0.5 mL of 4 M $MgCl_2 \cdot 6H_2O$ is added.
6. The flasks are placed into a 37 °C water bath for five minutes to prewarm the sample.
7. To each of the flasks, 10 mL of the appropriate host bacterium is added.
8. This sample/host bacterium mixture is then added to the 100 mL of the 2 X TSA that was cooled to 4 °C.
9. The contents of each of the flasks are poured into 100-mm petri dishes at 20 mL per plate. Care must be taken to make sure that the plates are labeled properly to designate whether they contain CN-13 or Famp host bacteria.
10. Positive and negative controls should be included with each assay.
11. The PFU/100 mL of either somatic or male-specific coliphages is calculated based on the total of the plaques obtained for all plates for that particular host bacterium.

OTHER METHODS

In addition to the protocols already mentioned, other methods have also been reported for the enumeration of coliphages. Armon and Kott (19) have reported on a simple, rapid, and sensitive presence/absence detection test for bacteriophages in 500 mL of drinking water. In this method, a probe filled with solidified soft agar and bacterial host cells was immersed in a glass jar containing the water sample and bacterial host cells (Fig. 1). The entire device was incubated in water bath at 36 °C. Following this incubation (which ranged from 90 minutes for *E. coli* CN13 to 8 hours for *B. fragilis*), the probes were removed and incubated further. Using this method, the authors report their ability to detect male-specific coliphages infecting *E. coli* Famp within six

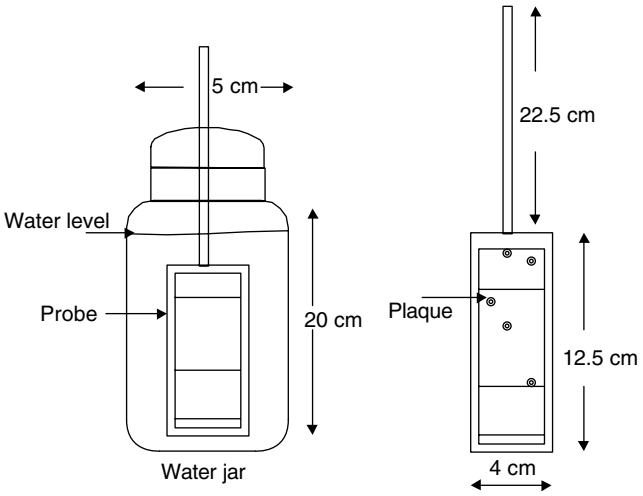

Figure 1. Presence/absence–based bacteriophage detection apparatus.

hours and phages infecting *B. fragilis* within eight hours. They successfully field-tested this approach on 45 different types of aquatic samples.

COLORIMETRIC DETECTION OF BACTERIOPHAGES

Ijzerman and coworkers (20) reported on liquid colorimetric presence/absence coliphage detection method. This methodology is based on the induction of β-galactosidase by *E. coli*. The release of this compound in the medium because of cell lysis as a result of coliphage infections permits the hydrolysis of a yellow chromogenic substrate that develops into a distinct red coliphage-positive sample. A coliphage-negative sample will remain yellow. The results can be obtained within 4.5 hours. The only apparent disadvantage of this method is the requirement that the water sample be concentrated to no more than 5 mL. Although the liquid colorimetric system was developed using *E. coli* strain C (ATCC 13706), the method has the potential to be adapted to any of the other bacterial host strains. According to the authors, in combination with adequate sample concentration methods, this protocol can detect 2 PFU/1,000 mL.

Colorimetric Method

1. *E. coli* (ATCC 13706) is inoculated into 25 mL of Luria broth supplemented with 5 mM of $CaCl_2$ and 1.25 mM of $MgSO_4 \cdot 7H_2O$.
2. The culture is incubated at 37 °C for one hour at 200 rpm on a rotary shaker (Lab-Line Orbit Environ-Shaker, Melrose Park, Illinois).
3. The culture is then aseptically inoculated with 25 μL of isopropyl-β-D-thiogalactoside (IPTG).
4. The culture is incubated for an additional 30 minutes at 37 °C.
5. Concentrated water sample (1.25 mL) (obtained through any virus concentration protocols) is added to the culture.
6. The culture is swirled and maintained at room temperature without shaking for 10–15 minutes and then returned to the shaker at 37 °C for an additional 105 minutes at 200 rpm.
7. The culture is placed in a sterile 50-mL centrifuge tube and centrifuged at 5,000 rpm (Beckman model TJ-6) at 6 °C for 15 minutes.
8. The supernatant is vacuum filtered through a 0.2-μm pore size low protein-binding filter into a sterile test tube.
9. In a clean tube, 9 mL of Z-buffer (20) was combined with one milliliter of the supernatant and 100 μL of chlorophenol red β-D-galactopyranoside and incubated at 37 °C for 30 minutes.
10. A positive coliphage test is observed by the immediate development of red color in an initially yellow solution that intensifies over a 15-minute period and reaches a maximum within 30 minutes. A negative test is indicated by an unchanged yellow color.

RECOVERY OF BACTERIOPHAGES FROM SOILS AND BIOSOLIDS

Mignotte and coworkers (22) tested eight virus extraction protocols for recovering phages and mammalian viruses from urban sludge. The three extraction techniques that resulted in the highest virus recoveries were as follows: beef extract (10%) solution at pH 9 combined with sonication, 0.3 M NaCl/7% beef extract solution at pH 7.5, and freon and 0.1 M borate buffer/3% beef extract solution (pH 9) combined with sonication. It is obvious that virus recoveries tend to be higher with organic buffers at elevated pH levels. Lasobras and coworkers (23) also have reported on high recoveries of somatic, male-specific, and *Bacteroides*-specific phages from different types of sludges encountered in water treatment plants. The protocol reported by Mignotte and coworkers (21) for optimum recovering phages from urban sludge is provided in the following text.

Method

Ten grams (10 g) of sludge is centrifuged initially at 1,500 g for 15 minutes.

The pellet is resuspended in 360 mL of 0.1 M borate solution containing 3% beef extract (pH 9.0).

The mixture then is stirred at 500 rpm (New Brunswick Scientific) for 15 minutes followed by sonication for one minute at a 100 W, 0.9s setting.

The sample is centrifuged at 10,000 x g for 45 minutes at 4 °C and the supernatant is neutralized to pH 7.2

Before bacteriophage analysis, the extract is decontaminated by adding one-third volume of chloroform and vigorously mixing for 30 minutes at 4 °C.

The sample is centrifuged at 1,500 x g for 10 minutes at 4 °C. The chloroform phase is removed and the remaining phase is used for the bacteriophage assay.

RECOVERY OF COLIPHAGES FROM SHELLFISH

Studies have shown that during depuration, FRNA phages are removed from the digestive tracts of a contaminated shellfish considerably more slowly than *E. coli* is removed. Because this relatively slow elimination has been shown to be similar to the elimination of enteric viruses (24), the presence of FRNA phages thus could be used as an indicator of enteric viruses. Dore and coworkers (25) recently reported on the isolation and use of male-specific RNA bacteriophages as candidate human enteric virus indicators for bivalve molluskan shellfish. They described a procedure for the detection of the FRNA phages using *S. typhimurium* WG49 as the host strain.

Method

1. Oysters are washed and scrubbed thoroughly under running potable water.
2. The oysters are opened aseptically with a flame-sterilized shucking knife and the meat and intravalvular fluid are removed, diluted in 0.1% (w/v) peptone water, and homogenized.
3. The diluted homogenates are centrifuged at 1,000 X g for five minutes at room temperature.
4. The supernatant is decanted into a sterile bottle and a 1 : 10 dilution with peptone water (pH 7.2) is made.
5. Ten milliliters of the undiluted supernatant and 4 mL of the 1 : 10 dilution are assayed for FRNA phages by using the standard double agar overlay method with 1 milliliter portions and 90-mm petri dishes. One milliliter of the sample is mixed with 2.5 mL of the molten soft agar and 1 milliliter of the host bacterium and poured over a prepared bottom agar plate.
6. The presence of RNA phages was confirmed using plates amended with RNase in parallel.
7. The reported sensitivity was 30 PFU of FRNA/100 g of shellfish.

RECOVERY OF BACTERIOPHAGES FROM AEROSOL SAMPLES

There have been a few studies reporting on the concentration of coliphages in bioaerosol samples. Most of these studies have centered on municipal waste handling operations such as biosolid land application sites and effluent spray irrigation sites. Although the survival of virus particles in bioaerosols is less than that of bacterial cells, detectable concentrations of both mammalian viruses and bacteriophages have been reported in the literature. There is no standard method for collecting and concentrating viruses in bioaerosols. A number of different samplers have been employed (26–28). Carducci and coworkers employed an impacting sampler (surface air system SAS, PBI, Milan, Italy) to collect the air samples. A Rodac plate made of a phage agar base was used as the impacting surface. A top layer of phage agar (7 mL) combined with 1 milliliter of host bacterial culture was poured over the Rodac plate to enumerate the phages. Pillai and coworkers (29) employed the impinger, AGI-30 samplers for 20 minutes at 12 L per minute using 0.1% peptone. The 20-mL sample was *concentrated* down to 7 mL using Centriprep-50 concentrators (Millipore, Bedford, Massachusetts). Aliquots of the concentrate were used in a conventional double agar layer format. It is extremely important to decide whether to employ impaction or impingement approaches because the method of sample collection will dictate the extent of sample processing and sample analysis.

MOLECULAR DETECTION AND CHARACTERIZATION OF BACTERIOPHAGES

The complete nucleotide sequences of a number of bacteriophages are currently available. The availability of this information has led to the development of oligonucletodie probe and primer sequences for the detection and characterization of bacteriophages. Molecular detection techniques are not being used primarily for the rapid detection of phages in environmental samples. These approaches are being employed to find relationships between the presence or absence of mammalian enteric viruses and phages.

Phylogenetically, F-specific RNA coliphages fall into four subgroups (30). Subgroups I and II are related and form the major group A. Subgroups III and IV are very similar and together make up major group B. Male-specific RNA coliphages are composed of serogroups I through IV. It has been shown (30,31) that strains isolated from human feces usually are group II and III, whereas groups I and IV usually are found in animal feces. Although serotyping of F + RNA coliphages can distinguish human fecal contamination from animal fecal contamination, antisera for F + RNA coliphages are not readily available and some isolates are difficult to stereotype (30,32). Given these reasons, the serotyping of phages is not being discussed in detail in this chapter.

Genotyping of male-specific coliphages with oligonucleotide probes is feasible (32,33). The oligoprobes were end-labeled with digoxigenin and used in DNA-RNA hybridizations and hybrids were observed by colorimetric, immunoenzymatic detection.

The nucleotide sequence of the oligoprobes used for genotyping is shown in Table 1.

The protocol for phage transfers and hybridization, which is provided in the following text, is based on the report by Hsu and coworkers (32).

Method

1. Two microliter volumes of the phage isolates and prototype strains that are representative of each group are spotted on a lawn of host bacteria and incubated at 37 °C overnight.
2. Plates are incubated at 4 °C for 30 minutes.
3. The bacterial lawn is covered with a nylon membrane for two minutes to adsorb phages from the plaques. Nylon membranes from Biodyne (Pall Biosupport, East Hills, New York.), Boehringer Manheim Corp, Indianapolis, Indiana, or Gene Screen (NEN Research Products, Boston, Massachusetts) show efficient transfer capabilities.

Table 1. Oligonucleotide Sequence Information for Group-Specific Probes (modified from F. -C. Hsu et al., *Appl. Environ. Microbiol.* 61, 3960–3966 (1995))

Probe	Sequence	Locus	Source Phage
I	CTA AGG TAT GGA CCA TCG AGA AAG GA	Maturation protein	MS2 (I)
II	CCA TGT TAT CCC CCA AGT TGC TGG CTA T	Maturation protein	GA(II)
III	ATA CTC AGT GAA (A/G)TA CTG CTG TGT	5′nontranslated region	Qβ
IV	GGC ATA GAT TCT CCT CTG TAG TGC G	5′nontranslated region	SP (IV)
A	AGC CCG ATC TAT TTT ATT GTT CTT CGG AAC	Replicase	MS2 and GA
B	TAA TTT TGC CAT GAT CGA ATT GAC CCA AAC	Nontranslated region and coat protein	Qβ and SP

4. Blotting buffer (7.5 X SSC buffer with 4.6 M formaldehyde) and heating at 65 °C for 15 minutes are used for the denaturation step.
5. The membranes are cross-linked using ultraviolet (UV) light treatment for five minutes and vacuum-baked at 80 °C for 15 minutes before hybridization.
6. Oligonucleotides are 5′ end labeled using dioxygenin by terminal transferase using the Genius labeling kit (Boeringer Manheim, Indianopolis, Indiana). Unincorporated nucleotides were removed using ethanol precipitation and probes are stored at −20 °C until use.
7. The membranes are prehybridized for two hours at 45 °C in prehybridization solution [6 X SSC buffer (0.9 M sodium chloride; 0.09 M sodium citrate), 5 X Denhardt's solution (100 x Denhardt's solution:for 500 mL: 10 g Ficoll 400; 10 g polyvinylpyrrolidone MW 360000; 10 g BSA fraction V), 16 mM tris-HCl (pH 8.0), 0.1% Sodium dodecyl sulfate solution (SDS), and 75 μg sheared salmon sperm DNA].
8. Hybridization is performed in the same solution using 5 pmol of DIG-UTP-labeled probe overnight at 45 °C.
9. The membranes are washed twice in 6 X SSC-0.01% SDS at 40 °C for 15 minutes.
10. The detection and color development is based on the labeling kit manufacturer's suggested protocol.

Using this approach, more than 80% of the phage isolates could be genotyped directly from plaques, providing evidence that genotyping is a much easier method of differentiating phages than sereotyping (which would have required extensive phage purifications). Hsu and coworkers (32) report that 17 isolates from piglet and porcine feces were classified as group-II phages (primarily human), suggesting that group classification will not always distinguish between human and porcine fecal contamination. Because the dietary and living conditions of pigs have historically involved exposure to human fecal wastes, this may account for the detection of group-II phages in pig feces (32).

GENE AMPLIFICATION–BASED METHODS

With the advent of gene amplification methods, the nucleotide sequence information that was available for the generation of oligonucleotide probes has been used to develop PCR primers. Primers have been developed against phages including MS2 and *B. fragilis* phages. Puig and coworkers (34) recently published a report describing the DNA amplification procedure for detecting *B. fragilis*phages in water. In this work, primers were designed from a 1.5-kb region that was specific to *B. fragilis* phages. The nested PCR assay was capable of detecting as few as 10–100 virus particles in seawater and sewage and 1–10 virus particles in river water.

CONCLUSION

A number of culture-based and molecular methods are available for the detection and characterization of coliphages. Many of these methods have been used successfully in multiple laboratories whereas some of them also have been rigorously evaluated as part of round robin multiple laboratory testing. Given the importance of male-specific coliphages to serve as indicators of fecal pollution, it is, however, necessary that these methods be tested, employed, and optimized using a variety of environmental samples under different environmental conditions. Molecular characterizations of male-specific RNA coliphages have demonstrated that it is possible to identify the sources of the male-specific coliphages once they are isolated. Thus, the ability to detect and characterize male-specific coliphages can assist in the detection of fecal contamination and in the identification of possible sources.

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BACTERIOPHAGE OF ENTERIC BACTERIA: OCCURRENCE AND PERSISTANCE IN THE ENVIRONMENT

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Bacteriophages, viruses that infect bacteria, are distributed ubiquitously in nature. Their potential use as indicators of fecal pollution and as models for the behavior of enteric pathogens has been studied for many years. This is due to the relative ease, cost, and speed of analysis for these organisms compared to that of pathogenic microorganisms. These uses of bacteriophages will be the focus of this article.

SURROGATES FOR HUMAN ENTEROVIRUSES

Human enteric pathogens include viruses, bacteria, and parasites that infect the gastrointestinal tract of warm-blooded animals. Exposure to these pathogens is through the oral route, and they are eliminated from the body in fecal material in the so-called "fecal-oral" route of transmission. The major source of pathogenic microorganisms in domestic wastewater is the fecal material of infected individuals; however, urine may also be a source of certain pathogenic viruses (1). There are hundreds of different types of microorganisms that may be present in domestic wastewater. The numbers and types of pathogens found in wastewater will vary both spatially and temporally, depending on the disease incidence in the population producing the wastewater, season, water use, economic status of the population, and quality of the potable water (2).

Between 1971 and 1998, there were more than 650 outbreaks of waterborne disease reported in the United States (3). These outbreaks resulted in more than 550,000 people becoming ill, and more than 125 deaths. The majority (90%) of these outbreaks were caused by microorganisms (see Fig. 1), most of them of enteric origin.

Indicators of Microbial Contamination

Because human pathogenic microorganisms have such an impact on public health, water microbiologists and regulators have searched for suitable compounds or microorganisms that can indicate microbial and fecal contamination of ground and surface water. Such a compound or organism is called an indicator.

Male-specific coliphages, such as MS-2, are often naturally present in sources of human wastes (e.g., septic tank effluent) and therefore their presence or absence in groundwater may be indicative of water quality. The suitability of a particular virus as an indicator is evaluated

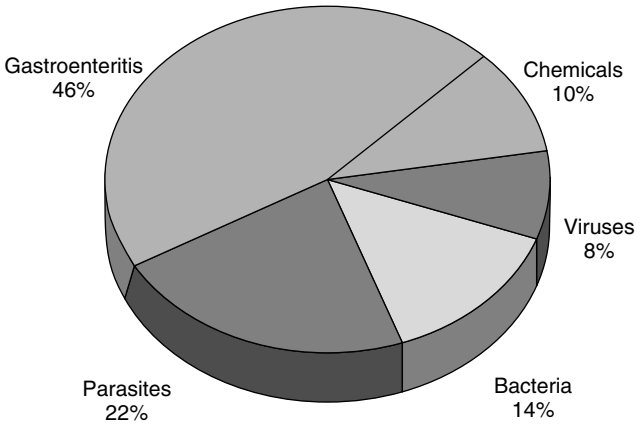

Figure 1. Causative agents on waterborne outbreaks (U.S.) 1971–1998.

on the basis of relative insensitivity to inactivation (4) and its ecological and morphological similarities to human viral pathogens (5).

There are numerous review articles on indicators available on this subject (6,7). Coliphages, particularly RNA phage, have been proposed as suitable indicators for human enteric viruses (6). Among the coliphages, MS2 appears to be the most suitable indicator (8,9).

Models for Pathogen Transport

When considering indicators to use as models for microbial survival and transport in the environment, a worst-case indicator for transport and fate should:

1. be unable to reproduce in the environment
2. show similar or less sorption and retention than pathogenic microorganisms in porous media under identical conditions
3. be at least as resistant to inactivation under natural conditions as pathogenic microorganisms
4. be nonpathogenic to humans (only if used as tracers in the field)

Several markers have been used as indicators for domestic wastewater contamination (10,11). Examples of such markers are coprostanol or surfactant related markers such as linear alkylbenzene. These markers have not been tested yet for their sorption and transport properties in porous media and no comparative studies with viruses are available at present.

Based on the mechanisms of virus sorption and retention and the experimental evidence of virus transport available, MS2 appears to be a reasonable candidate for a transport model at this time. MS2 was found to be a useful indicator for viruses because it showed similar or less sorption and retention than other viruses under identical conditions (12–14). However, this conclusion is based on the specific experimental conditions used in these studies, and there is no guarantee that MS2 behaves as a worst-case indicator under a variety of soil and water conditions. MS2 is not an ideal indicator in the sense that it can replace the exact behavior of a human enteric virus in the environment. However, because of the extremely complex sorption and transport mechanisms of viruses, recent studies have raised doubts regarding the applicability of any single indicator's ability to mimic the behavior of a specific virus. Jin and coworkers (15) have shown that MS-2 is relatively easily inactivated by the air-water interface in unsaturated systems and that it is sensitive to the presence of metal oxides (16). Penrod and coworkers (17) compared the deposition kinetics of bacteriophages MS-2 and λ and found that even subtle differences in viral surface structures could significantly influence the rate at which viruses were removed from the water phase by infiltration. Considering the complex sorption and retention mechanisms of viruses, it is unlikely that any single bacteriophage will adequately mimic the transport behavior of viral pathogens in the environment, particularly given that the surface structures of different waterborne human viruses are quite dissimilar.

ECOLOGY OF BACTERIOPHAGE

Coliphages, the viruses that infect coliform bacteria, are the most widely studied bacteriophages in terms of their use as surrogates. Coliphages are found in the feces of man and other warm-blooded animals, in domestic wastewater, and in wastewater-contaminated waters, thus suggesting the utility of these organisms as indicators of the presence of fecal material that may contain pathogenic microorganisms and thereby pose a threat to human health.

The distribution of coliphages in several hundred samples of feces from human and other warm-blooded animals is shown in Table 1. Reported concentrations of coliphages in various sources are shown in Table 2. Clearly, the concentrations and presence of coliphages in feces is extremely variable. In addition, these viruses are found more commonly in the feces of lower animals than in those of man.

However, in contrast to human feces, coliphages are consistently found in domestic raw wastewater. Raw sewage has been found to contain between 10^5 and 10^6 plaque-forming units (pfu)/L (Table 2). Septic tanks, which serve a smaller population than would be

Table 1. Distribution of Coliphage in Human and Animal Feces

Source	No. of Samples	No. Positive	% Positive
Birds (zoo)	25	23	92.0%
Fowl	30	9	30.0%
Mammals (zoo)	97	72	74.2%
Horses	30	3	10.0%
Cows	20	6	30.0%
Pigs	11	10	90.9%
Humans	597	140	23.5%

Source: Modified from (K. Furuse, Distribution of Coliphages in the Environment: General considerations, Chap. 3, in S. M. Goyal, C. P. Gerba, and G. Bitton, eds., Phage Ecology, John Wiley & Sons, New York, 1987.)

Table 2. Concentrations of Coliphages in Feces and Wastewater

Source	Typical Concentration	% Positive
Animal feces	$0–10^6$/g	12–75
Human feces	$0–10^7$/g	10–92
Gray water	$0–>10^{10}$L	12
Septic tanks	$10^3–10^6$/L	100
Raw sewage	$10^5–10^7$/L	100
Primary effluent	$0.2–10^6$/L	100
Secondary effluent (activated sludge)	$10^1–10^4$/L	100
Oxidation pond effluent	$10^3–10^7$/L	100

Source: Modified from (C. P. Gerba, Phage as Indicators of Fecal Pollution, Chap. 8, in S. M. Goyal, C. P. Gerba, and G. Bitton, eds., Phage Ecology, John Wiley & Sons, New York, 1987.)

served by a municipal system, have also been found to consistently contain coliphages. Concentrations in septic tank effluent have been reported to range between 10^3 and 10^6 pfu/L (Table 2). A recent report of coliphage analyses of septic tank effluent from a single septic tank revealed a mean concentration of 7×10^5 pfu/L (20). The ubiquitous presence of the coliphages in untreated domestic wastewater is the rationale behind the use of these organisms as indicators of fecal contamination of water.

Bacteriophages have been found in a variety of natural waters including lakes, streams, rivers, wetlands, and groundwater (19). In the majority of the studies that have been conducted, strains of *E. coli* have been used as the host organism. Thus most of the available data on phage occurrence in freshwater environments reflect coliphage occurrence. Table 3 lists concentrations of coliphages found at selected sites.

Phage that infect other enteric bacteria have also been isolated from freshwater. From rivers in the United Kingdom, bacteriophage that infect *Klebsiella pneumoniae* and *Salmonella typhi murium* have been isolated at concentrations ranging from approximately 1,000 to 1,000,000 per liter (21). Little work has been done on the occurrence of phage that infect indigenous freshwater bacteria. Phage that infect *Aeromonas hydrophila* have been recovered from river water by several investigators (21). Concentrations of these phage were on the order of a few thousand per liter of water. *Pseudomonas aeruginosa* phage have also been isolated from river water in the United Kingdom at a concentration of 3.5×10^3 per liter (23).

In addition to surface water, the occurrence of phages in groundwater systems has been studied. There has been a surge of interest in this area due to recent activity at the U.S. Environmental Protection Agency (EPA). In 2000, the EPA proposed the Ground Water Rule, the goal of which is to protect potable groundwater supplies from fecal contamination (23a). The difficulty associated with monitoring more than 150,000 water systems for enteric viruses spurred a great deal of interest in the identification of a suitable indicator for the presence of these microorganisms in groundwater. Due to the similarity to enteric viruses in terms of their size, shape, persistence in the environment, and transport behavior, bacteriophages are being considered as indicators for the purposes of this regulation.

One of the most extensive surveys of the occurrence of enteric microorganisms in groundwater involved the analysis of water samples from more than 400 groundwater wells in the United States. This survey revealed the presence of coliphages in 20% of the samples (24). In this study, three different hosts, *E. coli* C, *E. coli* C3000, and *S. typhi murium* WG49, were used to test for the presence of coliphages in concentrated groundwater samples. Of the 444 samples analyzed, 4.1%, 10.8%, and 9.5% were found to be positive using these three as the respective hosts. Only one sample was found to be positive for coliphages using all three hosts, while 92 of the samples (20.7%) were found to be positive using at least one host.

Coliphages have also been isolated from groundwater in an area influenced by recharge of the subsurface with reclaimed water (22). Coliphages were detected on at least one occasion in all twenty-six of the wells sampled. The shallowest of the wells had a perforated interval of 25.9 to 72.9 m; the deepest was perforated at 345.2 to 434.5 m. While some of the wells were located in close proximity

Table 3. Concentrations of Coliphages in Freshwater

Source	Host	pfu/L
pond, Florida	*E. coli* B	1.9×10^4
lake, Florida	*E. coli* B	$1.5 \times 10^1–1.1 \times 10^3$
lake, Florida	*E. coli* C	$1.5 \times 10^2–1.3 \times 10^4$
river, U.K.	*E. coli* W3110	$3.5 \times 10^3–6.5 \times 10^5$
river, U.K.	*E. coli* HfrH	1.8×10^4
river, South Africa	*E. coli* K12Hfr	1.7×10^5
river, New York	*E. coli*	5×10^1
river, Washington, DC	*E. coli* C	1×10^4
river, Czechoslovakia	*E. coli* B-39	2×10^4
river, California	*E. coli* HS12 (pFamp)R	$1.7 \times 10^1–1.5 \times 10^2$

Source: S. Farrah, Ecology of Phage in Freshwater Environments, Chap. 4, in S. M. Goyal, C. P. Gerba, and G. Bitton, eds., Phage Ecology, John Wiley & Sons, New York, 1987.

(20 to 40 m) to the recharge basins, others were located more than 100 m away; in one case the well was located more than 1,700 m from the recharge basins. Estimated travel times from the basins to these wells ranged from 24 to 5,852 days. Based on the data presented, it is clear that the presence of coliphages in some of the wells is not due to the presence of the recharge basins. The presence of other potential sources of coliphage, such as seepage from the domestic sewerage system, may be responsible for the presence of these viruses in the groundwater.

PERSISTENCE OF BACTERIOPHAGES IN THE ENVIRONMENT

There are a number of factors that affect the length of time that bacteriophages persist in the environment. The most important factors and the effects they have on persistence are summarized in Table 4. Some of these factors will be discussed in more detail in the following text. For a more complete description of the effects of these factors on the persistence of viruses in general, please see VIRUS SURVIVAL IN SOILS.

Temperature

Temperature is probably the most important factor influencing virus inactivation in the environment (25). In general, it has been found that the inactivation rate is significantly correlated with temperature, with faster inactivation rates occurring at the higher temperatures. Figure 2 presents a compilation of data (shown in Table 5) on the inactivation rate of MS2 coliphages as a function of temperature. This is the general trend, although there are clearly situations in which viruses are inactivated more rapidly at lower temperatures. Those exceptions can likely be explained by differences in other environmental characteristics, such as pH, microbial activity, etc.

The exact mechanism whereby temperature inactivates viruses in soils has not been determined, but several theories have been proposed. The inactivation may be due to denaturation of the viral capsid (25) as proteins are denatured at high temperatures. However, it has been shown that the RNA released during thermal denaturation remains infective as it is more resistant to heat inactivation than the protein capsid (26).

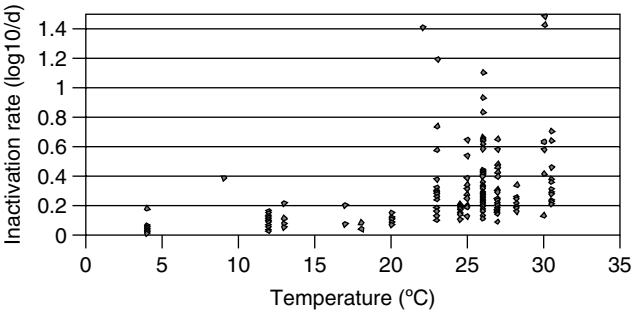

Figure 2. Survival of MS2 coliphage in groundwater as a function of temperature.

Microbial Activity

There are conflicting reports regarding the role of microorganisms in virus inactivation; many of these have been reviewed (27,28). The survival of MS2 viruses in groundwater that had been filtered to remove the indigenous bacteria compared to their survival in nonfiltered water is shown in Figure 3 (data shown in Table 5). There is no clear trend — in some cases the phage survived longer in the filtered water, in other cases survival was prolonged in the presence of the indigenous bacteria.

pH

The effect of pH on virus survival in soil has not been extensively studied. It has been suggested that pH indirectly influences virus survival by controlling adsorption onto soil particles, which, in turn, affects virus survival (29). The direct effects of pH on virus persistence have been studied by a few investigators. Hurst and coworkers (30) studied the survival of poliovirus 1 and

Table 4. Factors Affecting the Persistence of Viruses in the Environment

Factor	Effect on Persistence
Temperature	Viruses persist longer at lower temperatures
Microbial activity	Some viruses are inactivated more readily in the presence of certain microorganisms, and sorption to the surface of bacteria can be protective
Moisture content	Most viruses survive longer in moist soils and even longer under saturated conditions; unsaturated soil may inactivate viruses at the air-water interface
pH	Most enteric viruses are stable over a pH range of 3 to 9; however, survival may be prolonged at near-neutral pH values
Salt species and concentration	Certain cations may prolong survival depending on the type of virus
Virus association with soil	Viral association with soil generally increases survival, although attachment to certain mineral surfaces may cause inactivation
Soil properties	Effects on survival are probably related to the degree of virus sorption
Virus type	Different virus types vary in their susceptibility to inactivation by physical, chemical, and biological factors
Organic matter	Organic matter may prolong survival by competitively binding to air-water interfaces where inactivation can occur; organic matter may also retard viral infectivity

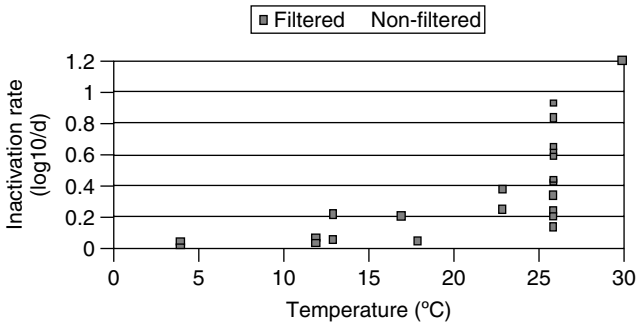

Figure 3. Survival of MS2 coliphage in groundwater in the presence and absence of indigenous bacteria.

two bacteriophages, MS2 and T2, in nine soils with pH values ranging from 4.5 to 8.2. They found that virus inactivation was significantly correlated ($p < 0.05$) with soil saturation pH, with longer survival at the lower pH values.

The exact mechanism whereby pH causes virus inactivation has not been fully elucidated, but results obtained by Salo and Cliver (31) suggested that virus inactivation involves alterations in the virus capsid. These investigators found that the RNA of the inactivated virus particles became sensitive to ribonuclease at all pH levels tested (pH 3 to 9), and at pH 5 and 7 the RNA was hydrolyzed in the absence of ribonuclease.

Loveland and coworkers (32) studied the effects of pH on the reversibility of virus attachment to mineral surfaces. Their experiments on the attachment of PRD1 to quartz and ferric oxyhydroxide-coated quartz indicated that attachment is controlled by the isoelectric point of the mineral surface. Attachment of PRD1 was observed at pH values 2.5 to 3.5 units above the isoelectric point of the mineral surface. Below this pH, the attachment of PRD1 was found to be complete and irreversible, whereas above this pH, the adsorption was minimal and reversible.

The mechanism(s) whereby pH affects virus adsorption to soil particles has been explained in terms of the electrochemical nature of the virus and soil surfaces (29,33,34). The outer surface of the enteric viruses is made of protein; therefore, the surface charge is influenced by the ionization of the carboxyl and amino groups in the capsid. The isoelectric point (pI) of many enteric viruses is below 7 (34); thus, at neutral pH, most viruses are negatively charged. Most soils are also negatively charged at neutral pH, and virus adsorption is not favored because of the mutual repulsion. If the pH of the environment is decreased, the surface charge of the virus will become positive (or less negative) due to increased ionization of the amino groups and decreased ionization of the carboxyl groups. While soils also become less negatively charged at lower pH values, soil pI values are generally lower than those of viruses, thus they may still have a net negative charge at acidic pH levels. This results in an electrostatic attraction between the virus particle and the soil, which leads to adsorption. In reality, the effect of pH on virus adsorption is not so clear-cut. There are many complicating factors that can interfere with the mechanism discussed earlier. One is that a given virus may have more than one pI: poliovirus 1 (Brunhilde strain) has pIs at pH 4.5 and 7.0 (34). The factors responsible for passage from one form to another are not known at this

Table 5. Survival of Coliphage in Groundwater

Virus	pH	Inactivation Rate (log10/d)	Temp. (°C)
f2	7.8	0.390	9
f2	7.6	1.416	22
MS 2	7	0.180	4
MS 2	7.9	0.014	4
MS 2	8	0.020	4
MS 2	8.1	0.032–0.064	4
MS 2	8.2	0.025	4
MS 2	8.3	0.012	4
MS 2	6	0.034	12
MS 2	7.3	0.037	12
MS 2	7.9	0.030	12
MS 2	8	0.093	12
MS 2	8.1	0.113–0.162	12
MS 2	8.2	0.040	12
MS 2	8.3	0.028–0.095	12
MS 2	7.7	0.114	13
MS 2	8	0.077	13
MS 2	8.1	0.075	17
MS 2	8	0.082	18
MS 2		0.069–0.149	20
MS 2	7.9	0.187	23
MS 2	8	0.244	23
MS 2	8.1	0.278–1.196	23
MS 2	8.2	0.325–0.738	23
MS 2	8.3	0.244–0.262	23
MS 2		0.103–0.16	23
MS 2		0.109–0.211	24.5
MS 2	7.3	0.129–0.207	25
MS 2	7.5	0.186–0.34	25
MS 2	7.6	0.313	25
MS 2	7.7	0.249	25
MS 2	7.8	0.39–0.54	25
MS 2	7.9	0.198–0.649	25
MS 2	6.8	0.293	26
MS 2	6.9	0.269	26
MS 2	7.2	0.211–0.254	26
MS 2	7.3	0.321–0.663	26
MS 2	7.4	0.278	26
MS 2	7.5	0.426–1.104	26
MS 2	7.6	0.132–0.836	26
MS 2	7.7	0.235–0.932	26
MS 2	7.8	0.286–0.648	26
MS 2	7.9	0.237–0.321	26
MS 2		0.113–0.325	26
MS 2	7.8	0.107–0.652	27
MS 2	7.9	1.870	27
MS 2	8	0.144	27
MS 2		0.153–0.581	27
MS 2		0.161–0.340	28.25
MS 2	7.8	0.132–0.637	30
MS 2	7.9	0.584–0.634	30
MS 2		0.214–0.706	30.5

Source: M. V. Yates, Report to EPA, 1992 (unpublished).

time. Other soil factors such as cations and humic and fulvic acids may also influence the net surface charge of viruses.

Salt Species and Concentration

The presence of certain chemicals may render a virus more or less susceptible to inactivation. Burnet and McKie (35) found that bacteriophage inactivation at 60 °C was partially prevented in the presence of 0.002 to 0.01 M $CaCl_2$, or $BaCl_2$. However, when the concentration was increased to 0.15 M or greater, thermal inactivation was increased.

Thurman and Gerba (36) studied the effects of modifying soil with several metals on the survival of MS2 and poliovirus. The addition of aluminum metal, magnesium oxide, and magnesium peroxide had a significant negative effect on the inactivation rate of MS2 when compared with unmodified control soils. The addition of unrefined substances such as zeolite, bauxite, limonite, and glauconite (which contain multivalent cations and oxides of iron and aluminum) did not have a significant negative effect on the inactivation rate of MS2. Rather, they seemed to have a protective effect, as indicated by the lower inactivation rate as compared with the unamended control soil. Further experiments by these investigators indicated that contact between the aluminum and virus was necessary for inactivation of the viruses. They postulated that a combination of electrodynamic van der Waals interactions and electrostatic double layer interactions promoted virus adsorption to the surface of the aluminum, where inactivation of the virus could then take place.

The role of soil cation exchange capacity (CEC) in virus adsorption has also been investigated. Burge and Enkiri (37) found that the CEC of four of five soils was correlated significantly ($p < 0.05$) with virus adsorption. In contrast, Goyal and Gerba (38) did not find a significant correlation between soil CEC and adsorption of 15 different viruses. Additionally, no correlation was found between virus adsorption and total phosphorus or between total and exchangeable iron, calcium, and magnesium.

Virus Adsorption to Soil

The adsorption of viruses to soils and other surfaces may prolong or reduce survival, depending on the properties of the sorbent. Murray and Laband (38a) found that poliovirus adsorption onto some inorganic substances, such as CuO, results in decreased infectivity of the virus. These investigators suggested that van der Waal's interactions between the virus and the particle surface induced spontaneous disassembly of the virus.

More commonly, however, adsorption to soils has been found to prolong virus survival. The mechanisms whereby adsorption to a solid surface prolongs or reduces virus survival have not been elucidated. However, Gerba and Schaiberger (39) have suggested several possibilities, including interference with the action of virucides, increased stability of the viral protein capsid, prevention of aggregate formation, and adsorption of enzymes and other inactivating substances.

Aggregation of Virus Particles

The formation of aggregates influences virus survival in natural waters. It has been suggested that this is because virus particles within the aggregates are highly resistant to destruction by environmental factors (25). It has been shown that aggregation renders virus particles more resistant to inactivation by chemical disinfectants such as bromine (40), free chlorine, and monochloramine (41). Although there are no definitive reports on the effects of aggregation on virus survival in soil, the results of studies in aqueous media would suggest that viruses in aggregates would survive longer in soils than would monodispersed viruses (33).

Soil Properties

The influence of soil properties on virus survival is probably related to the degree of adsorption. Hurst and coworkers (31) suggested that the correlation between pH and virus survival was probably mediated through its influence on virus adsorption. A positive correlation between virus survival and soil exchangeable aluminum and a negative correlation with resin-extractable phosphorus were also attributed to influencing virus adsorption onto the soil.

Virus Type

As is obvious from the previous discussions, different viruses vary in their susceptibility to inactivation in the subsurface environment. In a comparative study of the survival of poliovirus, echovirus, and coliphage MS2 in several different groundwater samples, no significant difference ($p < 0.01$) was found overall (6). There were, however, differences in the inactivation rates of the viruses in individual water samples.

The survival of poliovirus 1 and f2 coliphage in dry sand was compared by Lefler and Kott (42). Poliovirus survived for at least 77 days at room temperature. The f2 phage survived considerably longer, possibly for as long as one year under dry conditions. Based on the previous discussions, it is possible that the survival times of both viruses would be greatly increased if water were added to the soil.

Organic Matter

The influence of organic matter on virus survival has not been firmly defined. In some studies, it has been found that proteinaceous materials present in wastewater may have a protective effect on viruses; however, in others no effect has been observed (25).

The effects of natural humic material and wastewater sludge-derived organic matter on the transport of MS2 bacteriophages in unsaturated soil was studied by Powelson and coworkers (43). The transport of MS2 was found to be higher in a loamy fine sand column that had been treated with the organic material than in a parallel column that had not been treated. In a series of experiments, Bales and coworkers (13,44) studied the effects of hydrophobic organic material on the attachment of bacteriophages MS2 and PRD1 and

poliovirus 1 to silica beads. These investigators found that even very small amounts of hydrophobic organic material ($\geq$0.001%) can retard the transport of viruses in porous media.

Several investigators have found that organic material can act not only as a competitor for virus adsorption sites, but also as an eluting agent, that is, it can cause sorbed viruses to desorb from the soil (34). Pieper and coworkers (45) studied the effects of wastewater-derived organic matter on the transport of bacteriophage PRD1 in a sand and gravel aquifer on Cape Cod, MA. They found that the removal of the virus was greater in an uncontaminated area (83%) than in an area that had been contaminated by secondary wastewater effluent (42%). They concluded that the difference in removal was due to the higher content of organic matter in the contaminated zone (2.0 to 4.4 mg/L vs. 0.4 to 1.0 mg/L dissolved organic carbon), which blocked the adsorption of the PRD1. The attachment of the viruses to the soil surfaces was reversible, as demonstrated by the recovery of viruses upon addition of linear alkylbenzene sulfonates (LAS) to the system. In the contaminated zone, 87% of the attached PRD1 bacteriophages were remobilized. Jin and coworkers (14) also found that addition of a solution of 1.5% beef extract, which is high in organic matter, could remobilize ΦX174 bacteriophages from Ottawa sand.

Widespread use has been made of this property of organic matter in the area of removing viruses from filters to detect them in environmental samples. Mucks and other soils with high organic matter content are poor adsorbers of viruses (46) and may not be suitable for wastewater application sites (33).

In contrast to the other studies, Powelson and coworkers (46) found that the type of effluent (primary, secondary, and tertiary) had no significant effect on the transport of three viruses (poliovirus 1, MS2, and PRD1) through columns of an alluvial coarse sand.

CONCLUSION

Bacteriophages have been isolated from wastewater, surface water, and groundwater. There is a great deal of interest in the potential for the use of coliphages as indicators of the presence of human enteric pathogens, as well as of the potential for fecal contamination of groundwater. While bacteriophages show a great deal of promise as indicators, all scientific and regulatory communities have not yet accepted their use as such.

The interaction of factors affecting the survival of coliphage in the environment is a complex issue that has not yet been completely explained. The inactivation rates of two coliphages and the temperatures and pH values at which those inactivation rates occurred are shown in Table 5. Close examination of the data reveals that there are trends, as discussed earlier, but quantitation of these trends is not yet possible.

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BACTERIOPLANKTON.

See PLANKTONIC MICROORGANISMS: BACTERIOPLANKTON

BENTHIC ALGAE.

See MEROPLANKTON

BENTHIC-ASSOCIATED PRIMARY PRODUCTIVITY.

See PRIMARY PRODUCTIVITY IN THE MARINE ENVIRONMENT

BIOAEROSOL SAMPLING AND ANALYSIS

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Aerobiology is the study of airborne microorganisms and their effects on human health and the environment. Historically, aerobiology has been concerned with the airborne transmission of disease. However, in recent years, research in this field has expanded due to an increased awareness of the variety of health effects potentially arising from exposure to airborne microorganisms. Today the concentration and composition of airborne microorganisms is of interest in diverse areas, such as agricultural and industrial settings, medicine, home and office environments, and military research. Particular emphasis has been placed recently on the areas of indoor environmental quality and exposure assessment.

The term bioaerosol is used to describe living airborne particles or those that originate from living organisms (1). Microorganisms, including bacteria, fungi, viruses, parasites and algae, as well as microbial fragments, toxins and metabolites, may be present in bioaerosols. Bioaerosols can range in size from less than 0.01 μm to more than 100 μm in diameter. Bioaerosols in the respirable size range (≤10 μm) are of particular concern to human health.

Human exposure to bioaerosols can result in a variety of adverse health effects. Numerous microbial agents representing every class of microorganisms have been associated with adverse health effects resulting from bioaerosol exposure (2). Contamination and subsequent dispersal of biocontaminants in the workplace and living quarters may result in human exposure and adverse health effects, ranging from lost productivity to severe illness. Detection and measurement of biocontaminants in indoor environments are needed to assess contamination levels and estimate the resultant exposure of occupants. A wide variety of sampling and analysis methods have been used and new methods are being developed (3). However, several problems remain to be solved. For instance, no single sampling method is suitable for the collection and analysis of all types of bioaerosols and no standardized sampling protocols are currently available. Therefore data between studies are often difficult to compare because of differences in sampler design, collection time, airflow rate, and analysis method. In addition, there is currently no regulation limiting biocontamination or bioaerosol concentrations for home, office, and school environments. One reason for this is that human exposure limits have not been established for bioaerosols because of the lack of exposure, dose, and response data (1). This complicates the use of sampling results for risk assessment.

Conventional biocontaminant monitoring relies on the collection of airborne and surface microorganisms and analysis of samples by either culture on artificial growth media or microscopic assay. Although culture and microscopy methods are generally effective analytical tools, they are hampered by critical limitations. Culture analysis methods underestimate concentrations because only culturable cells are enumerated and identified, whereas nonculturable organisms go undetected (4). Microscopic assays are laborious and imprecise, and the level of identification is generally limited to the genus level at best. The inaccuracy of conventional methods and lengthy analytical time required to characterize bioaerosol concentrations emphasizes the need for developing new analytical techniques that can provide rapid and reliable data for bioaerosol exposure monitoring.

It is important for the investigator to consider carefully the objectives of sampling before any samples are taken. After determining what information is desired, an appropriate sampling and analysis method can be selected and incorporated into the monitoring design. The purpose of this chapter is to present various bioaerosol sampling and analysis methods to facilitate the selection of instrumentation and techniques. The collection methods used in bioaerosol sampling are presented with a description of sampling equipment currently available, including a discussion on sampling efficiency. Equipment calibration and sampling considerations such as collection times and the number of samples are also addressed. Analysis methods are presented in the second section of the chapter, beginning with traditional culture and total count methods and concluding with some recently developed biochemical and molecular techniques.

BIOAEROSOL SAMPLING

Bioaerosol sampling may be used to obtain useful data for a variety of applications, such as (*1*) measuring

the presence and concentration of airborne biocontaminants; (*2*) confirming dissemination from a biocontaminant source; (*3*) evaluation of bioaerosol exposure and health risks; (*4*) documentation of clearance to reoccupy a space, following remediation efforts; and (*5*) compliance monitoring in industrial environments. Regardless of the application, the objective of bioaerosol sampling is the efficient removal and collection of biological particles from the air in a manner that does not affect the integrity of the sample. This is dependent on the characteristics of the microorganisms and on the physical features of the sampling instrument (5,6). The selection of a sampler depends on a number of factors, such as sampler performance, expected bioaerosol concentration, and the analysis method. These factors are discussed later in the chapter.

Sampling Methods and Equipment

There is a wide variety of commercially available bioaerosol samplers (3,5,7). Bioaerosol samplers may be grouped according to their method of collection. The three main collection methods used in quantitative bioaerosol sampling are impaction, impingement, and filtration. Electrostatic precipitation has also been used for the collection of airborne microorganisms. Nonquantitative sampling by gravitational settling of particles will also be discussed. Several of the commercially available bioaerosol samplers are listed in Table 1.

Impaction. The majority of bioaerosol samplers utilize impaction as the collection principle. The impaction method uses the inertia of the particles to separate them from the air stream. The particles are deposited onto a collection surface that usually consists of an agar medium for culture-based analysis or an adhesive-coated surface for microscopic analysis. The impaction process depends on the inertial properties of the particle, such as size, density and velocity, and on the physical parameters of the impactor such as inlet nozzle dimensions and air flow pathway (7,8). The impactor sampler draws air through an inlet nozzle toward a collection surface, and particles with sufficient inertia impact while smaller particles remain in the air stream (Fig. 1a). Impactor samplers may be designed with multiple circular inlet nozzles; the sampler is then referred to as a multiple-hole or sieve sampler. If there are several stages with successively smaller nozzles, the sampler is referred to as a *cascade impactor*. For samplers in which air is drawn through a single nozzle, the shape of the nozzle is usually rectangular and the impactor is referred to as a *slit sampler*. Some impactors utilize centrifugal impaction, which also uses inertial forces to separate the particle from the air stream, but in a radial geometry (Fig. 1b).

Multiple-Hole Impactor Samplers. The Andersen impactor sampler (Fig. 2) has been widely used for culturable bioaerosol measurements (9). The sampler draws air at a flow rate of 28.3 lpm and is operated using an electric vacuum pump. The Andersen six-stage

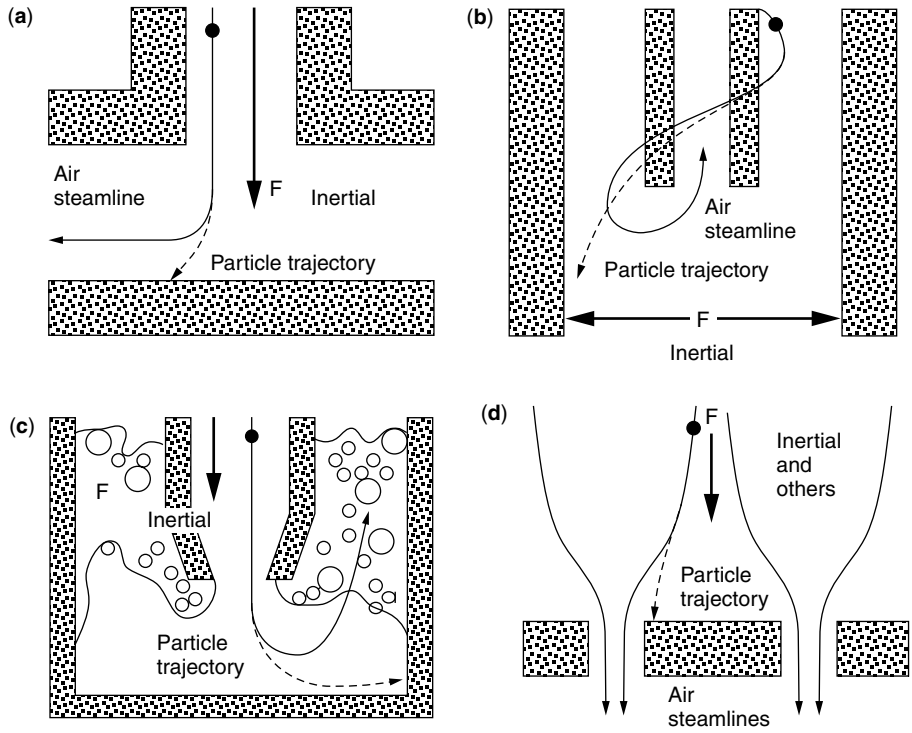

Figure 1. Mechanisms of particle removal used in bioaerosol sampling. (**a**) solid plate impaction; (**b**) centrifugal impaction; (**c**) liquid impingement; (**d**) filtration; $F_{inertial}$ = inertial force (Adapted from Nevalainen et al., 1993, with kind permission from Elsevier Science Ltd., The Boulevard, Langford Lane, Kidlington OX51GB, United Kingdom).

Table 1. General Characteristics of Several Commercially Available Bioaerosol Samplers (see text for Manufacturer Information; Adapted from Buttner et al., 1997, with kind permission from ASM Press, Washington, DC)

Sampler	Collection Medium	Air Flow Rate (liters/minute)	Sample Analysis Method(s)	Comments
		Impaction		
Multiple-Hole Impactors				
Andersen viable impactors 1-, 2-, or 6-stage	Agar; 100-mm Petri dishes	28.3	C	Particle size discrimination (2 and 6-stage models); vacuum pump required; positive-hole correction
Burkard portable air sampler	Agar; l00-mm Petri dishes	10–20	C	Battery operated; positive-hole correction
MicroBio MB1 and MB2	55 mm contact plates	100	C	Battery operated; positive-hole correction
SAS Super 90, compact and high flow	Agar; 55-mm contact plates 85-mm contact plates	90 (compact, super 90) 180 (high flow)	C	Battery operated; programmable for multiple samples; positive-hole correction
Slit Impactors				
Air-O-Cell sampling cassette	Adhesive-coated surface	12–30	M	Determination of total fungal spores and pollen
Allergenco MK-3	Adhesive-coated glass slide	15	M	Determination of total fungal spores and pollen; programmable for multiple samples; electric or battery-operated
Burkard spore traps, 24-hour, 7-day, and personal samplers	Adhesive-coated surface, tape, or glass slide	10	M	Determination of total fungal spores and pollen; time discrimination (24-hour and 7-day models); battery operated (personal model)
Mattson/Garvin 220 and P-320	Agar; 150-mm Petri dishes	28.3	C	Time discrimination up to 1 hour
New Brunswick slit-to-agar, STA-101, -203, and -303	Agar; 150-mm Petri dishes	50	C	Time discrimination up to 1 hour; vacuum pump required for some models
Rotorod Models 20 and 40	Adhesive-coated polystyrene rods	48	M	Determination of total fungal spores and pollen; programmable for multiple samples; electric or battery-operated
Centrifugal Impactors				
Biotest RCS, RCS plus and RCS high flow	Agar; plastic strips	40 (RCS) 50(RCS Plus) 100(High Flow)	C	Battery operated; high volume sampling (High Flow); strips available unfilled or with agar medium
SASS 2000	Liquid	225	C,M,B,P,I	High volume sampling; High and low bioaerosol concentrations; electric or battery-operated
		Impingement		
All-glass impingers (AGI-30 and AGI-4)	Liquid	12.5	C,M,B,P,I	High and low bioaerosol concentrations; vacuum pump required

(*continued overleaf*)

Table 1. (*Continued*)

Sampler	Collection Medium	Air Flow Rate (liters/minute)	Sample Analysis Methods	Comments
BioSampler	Liquid	12.5	C,M,B,P,I	High and low bioaerosol concentrations; short- and long-term sampling; viscous and nonviscous fluids; vacuum pump required
Burkard multistage liquid impinger	Liquid	20	C,M,B,P,I	High and low bioaerosol concentrations; particle size discrimination; vacuum pump required
		Filtration		
25, 37, or 47 mm filter cassettes	Filter membrane	1–50	C,M,B,P,I	Loss of culturable vegetative cells; portable, useful for personal monitoring; vacuum pump required
Sartorius MD8 air sampler	Gelatin membrane filter	42–133	C	Gelatin filter reduces desiccation stress; high volume sampling; virus collection
		Electrostatic Precipitation		
SCAEP PM-1B	Liquid	115–371	C,M,B,P,I	High volume sampling; High and low bioaerosol concentrations;

Note: C = Culture; M = Microscopy; B = Biochemical Assay; P = PCR; I = Immunoassay

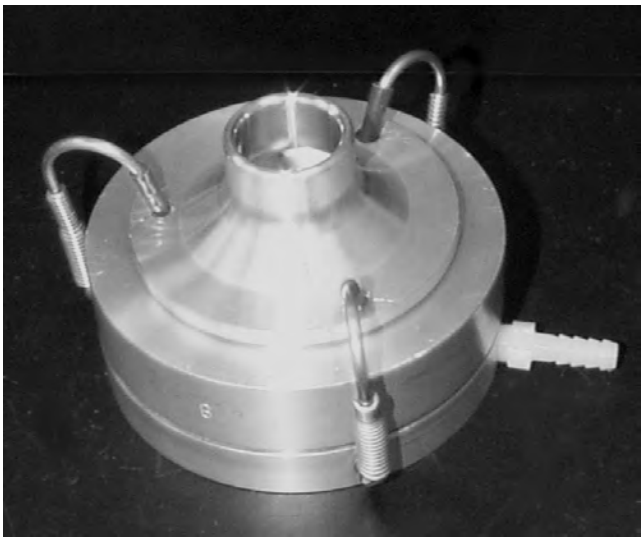

Figure 2. Andersen single-stage sampler (courtesy of Paula Jacoby-Garrett, UNLV).

impactor sampler (Andersen Instruments, Inc., Smyrna, Ga.) consists of six stages with decreasing nozzle diameter that collect progressively smaller particles onto agar plates. The number of colony-forming units (CFU) that form on the agar collection plates provides bioaerosol size distribution information (9). Single-stage and two-stage models of the sampler are also available. There are several portable, battery-powered single-stage impactor samplers designed for sampling culturable airborne microorganisms. These include the Surface Air System (SAS, International PBI, Milan, Italy, distributed by Bioscience International, Rockville, Md.); the Burkard portable air sampler (Burkard Manufacturing Co., Ltd., Rickmansworth, Hertfordshire, England); and the MicroBio MB1 and MB2 (Spiral Biotech, Inc., Bethesda, Md.) (Table 1).

Slit Impactors. Slit impactors are available for the measurement of either culturable or total airborne microorganisms. Many slit impactors have a moving collection surface that allows enumeration of bioaerosol concentration over time. Slit impactors that deposit the bioaerosol onto an agar surface for the estimation of culturable cells include the Mattson-Garvin air sampler (Barramundi Corp., Homosassa, Fla.) and the slit-to-agar sampler (New Brunswick Scientific Co., Inc., Edison, N.J.). Slit impactors that collect particles onto an adhesive-coated surface are generally used for the microscopic enumeration of fungal spores or pollen grains. These include the Burkard spore traps and the personal air sampler (Fig. 3) (Burkard Manufacturing Co., Ltd.; Spiral Biotech, Inc.), the Air-O-Cell sampling cassette, (Zefon International, Inc., St. Petersburg, Fla.), the Allergenco air sampler (Allergenco/Blewstone Press, San Antonio, TX) and the Rotorod (Sampling Technologies, Inc., St. Louis Park, MN) (Table 1).

Centrifugal Impactors. The Reuter centrifugal sampler (RCS) and RCS plus (Biotest Diagnostics Corp., Denville,

Figure 3. Burkard personal air sampler (courtesy of Paula Jacoby-Garrett, UNLV).

Figure 4. AGI-30 sampler (left) and SKC BioSampler (right) (courtesy of Paula Jacoby-Garrett, UNLV).

N.J.) are portable battery-powered samplers that centrifugally impact the microorganisms onto agar strips for culture analysis. The agar strips containing selected media formulations are available from the manufacturer. The Smart Air Sampler System (SASS) 2000 (Research International, Woodinville, WA) is a wetted-wall cyclone sampler that collects airborne particles in a thin liquid layer through centrifugal action.

Liquid Impingement. Liquid impingement samplers draw air through an inlet and particles are collected in a liquid (Fig. 1c). The particle is removed from the air by inertial force and impaction into a swirling or bubbling liquid. Aggregates of cells may be broken apart by the action of the collection fluid. The collection of bioaerosol particles in a liquid medium has several advantages, including the retrieval of bioaerosol particles over a wide range of airborne particle concentrations, the ability to divide the sample for multiple analyses, and the option of applying a variety of analysis methods as discussed in the following section. A liquid sample can be concentrated by filtration or diluted by liquid addition, depending on the concentration of collected microorganisms. In addition, several culture media can be inoculated with aliquots of the collection medium for the culture of groups of microorganisms with different nutrient requirements.

The AGI-30 all-glass impinger sampler (Fig. 4) (Ace Glass, Inc., Vineland, N.J.) is a widely used sampler that has a curved inlet tube designed to simulate the nasal passage, making this sampler useful for studying the respiratory infection potential of bioaerosols (10,11,12). For other applications the inlet tube is washed with a known volume of collection fluid to recover nonrespirable airborne particles. The AGI-30 has an impaction distance of 30 mm from the jet to the bottom of the sampler. The AGI-4 model features a shorter distance of 4 mm to improve particle collection efficiency over the AGI-30. However, added sampling stress may result from impaction against the glass bottom of the sampler, resulting in a loss of cell viability (3,7). The Burkard multi-stage liquid impinger (Burkard Manufacturing Co., Ltd.) is a stainless steel sampler that collects particles in three size fractions: $>10\ \mu m$, 4 to 10 μm, and $<4\ \mu m$. The BioSampler (Fig. 4) (SKC, Inc., Eighty Four, Penn.) combines impingement into a liquid with centrifugal motion (13). The BioSampler collects particles by drawing air through three nozzles that are directed at an angle toward the inner sampler wall and the liquid swirls upward on the inner wall of the sampler to remove collected particles. Although other impingers are designed for use with water-based collection fluids, the BioSampler can be used with viscous collection fluids (e.g., heavy white mineral oil) to minimize sampling stress and evaporation loss of the collection buffer.

Filtration. Filtration sampling is used to separate particles from the air stream by passing air through a

porous medium (Fig. 1d). Collection of particles depends on the aerodynamic diameter of the particle, the filter pore size, and the airflow rate (14). Inertial forces and other mechanisms such as interception, diffusion, and electrostatic attraction result in the collection of particles on the surface of the filter, including particles smaller than the pore size of the filter (12). Membrane filters housed in disposable plastic cassettes are typically used for filtration sampling (Table 1). The air sampling cassettes may contain 25-, 37-, or 47-mm diameter filter membranes and are available from a variety of manufacturers (e.g., Gelman Sciences, Ann Arbor, Mich.; Millipore Corp., Bedford, Mass.; Nuclepore, Corning Costar Corp., Cambridge, Mass.; Poretics Corp., Livermore, Calif.; SKC Inc., Eighty Four, Penn.). Filter material, including polycarbonate, cellulose mixed ester or polyvinyl chloride with a variety of pore sizes, may be used depending on the nature of the bioaerosol and the method of sample analysis (7,12,14).

The advantages of using filtration sampling for bioaerosol monitoring are its simplicity, low cost, and versatility. Filtration sampling is adaptable to a variety of assays but loss of viability of vegetative cells may occur, presumably due to desiccation stress during sampling (15,16,17). The MD8 air sampler (Sartorius AG, Göttingen, Germany) collects airborne microorganisms on a gelatin filter to reduce desiccation stress. The gelatin membrane is incubated on an agar medium of choice for culture analysis.

Electrostatic Precipitation. This method of collection uses electrical forces to separate particles from the air stream (18). The Space Charged Atomizing Electrostatic Precipitation (SCAEP) PM-1B sampler (Team Technologies, Newton Upper Falls, MA) operates by drawing air through a charged liquid spray that collects particles by electrical forces and concentrates them into a liquid.

Gravity Sampling. Gravity sampling, also referred to as *settling plate* or *depositional sampling*, is a semi-quantitative collection method in which airborne microorganisms are collected by gravitational settling onto an exposed agar plate. Although this method is simple and inexpensive, collection of airborne microorganisms using this method is affected by the size and shape of the particles and by the motion of the surrounding air (8). As a result, large particles are more likely to be deposited onto the collection surface (19). This can lead to misrepresentation of the prevalence of airborne microorganisms and the exclusion of smaller particles from collection (20). Furthermore, the number of colonies enumerated cannot be related to the bioaerosol concentration of the environment because the volume of air from which the particles originate is unknown. Gravity sampling has been compared with various volumetric culture sampling methods. The results show that the airborne concentrations derived from gravity sampling are not qualitatively or quantitatively accurate and do not compare favorably with those obtained by volumetric sampling methods (20–23).

Sampling Efficiency

No single sampler can be used for the collection and analysis of all types of bioaerosols. The selection of the appropriate sampler depends on a variety of factors including the sampling environment, the analysis methods used, and the monitoring objectives. Consideration of the theoretical aspects of air sampling efficiency and the experimental results of performance studies may assist in the selection of the appropriate sampling method for a particular monitoring situation. The performance of bioaerosol samplers can be divided into physical and biological components. The primary physical parameters that affect sampling efficiency are the inlet sampling efficiency and the particle collection efficiency. These criteria are presented briefly in the following section and have been discussed in detail in the literature (7). Biological sampling efficiency refers to the effects of sample collection on the culturabilty or integrity of the cells or cellular components for sample analysis.

Inlet Efficiency. The efficiency of the sampling inlet affects the ability of the sampler to extract airborne particles from the environment. Ideally the inlet should entrain particles from the air without bias due to their size, shape, or density. However, when sampling from a moving airstream, the air velocity and the orientation of the sampler may effect the inlet efficiency. The inlet characteristics of several bioaerosol samplers have been calculated for different types of bioaerosol particles sampled under various conditions (24). The results showed that certain sampling conditions could result in either overestimation or underestimation of the true bioaerosol concentration. Therefore it is important that while sampling in a moving airstream the samples be collected isokinetically, with the sampler inlet aligned with the airstream flow and with the velocity through the inlet equal to the airstream velocity (25).

Collection Efficiency. Collection efficiency, also termed *particle removal efficiency*, is the ability of the sampler to remove particles from the airstream and transfer them to the collection medium. Particle losses may occur between the sampler inlet and the collection device. The distance between the inlet and the collection device is therefore usually minimized to improve collection efficiency (7). For impactor samplers a useful parameter for evaluating collection efficiency is the "cut-off diameter" or d_{50}. The d_{50} refers literally to the particle diameter at which 50% of the particles are collected. However, because of the sharp cut-off characteristics of impactor samplers, the d_{50} is generally considered the particle diameter above which all particles are collected (26,27). The d_{50} values for several samplers have been calculated using the physical dimensions of the impaction nozzle(s) and the airflow rate (Table 2). For membrane filters, the collection efficiency is approximately 100% for particles larger than the pore size (28).

Biological Efficiency. Ideally a bioaerosol sampler should collect all airborne microorganisms without altering the culturability or the biological integrity required

Table 2. Calculated and Reported Cut Diameters (d_{50}) for Several Bioaerosol Samplers (Adapted from Buttner et al., 1997, with kind permission from ASM Press, Washington, DC)

Sampler	d_{50}(μm)[a] Calculated	Reported
Impaction		
Multiple-hole impactors		
Andersen 6-stage viable impactor		
Stage 1	6.24,6.61[b]	7.0
Stage 2	4.21	4.7
Stage 3	2.86	3.3
Stage 4	1.84	2.1
Stage 5	0.94	1.1
Stage 6	0.58	0.65
Andersen 2-stage viable impactor		
Stage 0	6.28	8.0
Stage 1	0.83	0.95
Andersen single-stage viable impactor	0.58	0.65
Burkard portable air sampler	4.18,2.56[c]	
MicroBio MB1 and MB2	1.8[c]	
Surface Air System (SAS)		
Super 90	1.94[c]	2.24[c]
Compact	1.97	2.0
High Flow	1.52,1.45[b]	2.0
Slit impactors		
Air-o-cell sampling cassette	2.3[c]	
Allergenco MK-3	2.0[c]	
Burkard spore traps		
24-hour and 7-day		
Standard nozzle	3.70,5.2[c]	
High-efficiency nozzle	2.17	
Personal sampler	2.52	
Mattson-Garvin 220 and P-320	0.53	
Centrifugal impactors		
Biotest RCS	7.5	3.8
Biotest RCS plus	6[c]	0.82[c]
Impingement		
All-glass impinger AGI-30	0.3	
Biosampler		<0.3[d]
Burkard multi-stage liquid impinger		
Stage 1		10
Stage 2		4
Stage 3		—

[a] values obtained from Jensen et al., 1994, unless otherwise noted.
[b] A. Nevalainen et al., 1992.
[c] K. Willeke and J. M. Macher, 1999.
[d] K. Willeke et al., 1998.

for the detection and quantification of the microorganisms or their metabolites. However, the infectivity and allergenicity of bioaerosols may be affected by numerous chemical, physical, and biochemical factors (29). Airborne microorganisms are subjected to a variety of environmental stressors, such as UV radiation, chemical pollutants, desiccation, and temperature extremes (4). In addition, the stress of sampling may injure the collected microorganisms, depending on their physiological characteristics (30). As a result, microorganisms may be in a nonculturable state.

Many sampling methods rely on culturing the collected microorganisms. Minimizing sampling stress is therefore critical. For agar or liquid collection media, it has been observed that culturability of cells can be improved by using certain culture media (31–33) or by the addition of certain compounds to the collection medium such as osmoprotectants, which aid in the resuscitation of stressed or damaged cells (34). Filtration sampling, although highly efficient for the collection of airborne microorganisms, has the disadvantage of viability losses of vegetative cells, presumably as a result of desiccation (15–17). Therefore filtration sampling in combination with culture analysis is generally used when bioaerosol concentrations are very high and sampling times are short. Filtration sampling is also used to monitor desiccation-resistant forms, such as fungal spores or bacterial endospores, or in combination with a total count method of analysis (35). The length of collection time, discussed in the following section, also plays a major role in the efficacy of air sampling for the retrieval of culturable microorganisms.

Sampler Performance. Numerous sampler comparison studies have been published, evaluating overall sampler performance and this work has been reviewed (3). The Andersen 6-stage viable impactor and the AGI-30 liquid impinger sampler have been suggested as reference methods (5) due to their efficiency and wide spread use, and many sampler efficiency studies have included these samplers in side-by-side comparisons. Some of the factors that can negatively affect sampler performance are presented here and in the following sections.

Agar impactor samplers collect microorganisms directly onto the culture medium. One of the disadvantages of this sampling method is that the nonculturable component of the bioaerosol is not measured and can be a significant percentage of the total composition (35). Another disadvantage of agar impaction is overloading when bioaerosol concentrations are high, resulting in agar plates or strips that contain colonies that overlap and are numerous to count (36). In addition, aggregates or clumps of microorganisms may impact at the same place on the agar surface and be enumerated as a single colony, resulting in underestimation of the bioaerosol concentration. Other problems that may occur with impaction onto either agar or adhesive-coated collection surfaces and can lead to erroneous results are particle bounce, where particles rebound off the collection surface and reenter the airstream (8), and electrostatic forces that attract biological particles to the surfaces of the collection device (9).

Impingement of microorganisms by impaction onto a liquid-wetted surface may also result in culturability losses through sampling stress (9,37). Another difficulty is that the microorganisms collected into a liquid may become reaerosolized from the bubbling collection fluid during sampling. In addition, the collection efficiency of liquid impingers may decrease significantly due to liquid evaporation over time (38).

Sampling Considerations

In the development of a bioaerosol sampling design, the investigator should have an understanding of the objectives of sampling and the types of data that will be obtained. The selection of the sampling and analysis method(s) is a primary concern. Numerous other factors should also be considered before sampling is initiated, including the length of the sample collection time, the number of samples to be taken, and calibration of the sampling equipment.

Collection Time. The goal of sample collection is to obtain a representative sample of the bioaerosol of interest. If sampling times are too short, too few microorganisms are collected to reliably estimate the bioaerosol concentration and composition. If sampling times are too long, the sampler may be overloaded with particles too numerous to enumerate and sampling stress may cause losses. In the selection of sample collection times, parameters that must be considered are the expected bioaerosol concentration, the quantitation range of the sampler, and the effect of sampling stress on the overall collection efficiency (3).

Bioaerosol concentrations can vary over several orders of magnitude, from $<10^2$ to $>10^6$ colony-forming units (CFU) per cubic meter of air, depending on the environment. Concentrations of airborne microorganisms can also fluctuate over time within the same environment. Therefore a single air sample provides only a brief glimpse of the environment in a particular time and place. Although the ambient bioaerosol concentration is an unknown quantity, estimates of its concentration can be used to determine the sample collection time as shown below.

The quantitation range of a bioaerosol sampler can be determined for a specific collection time using the sampler airflow rate (39). The lower detection limit (LDL) of a sampler for a particular collection time may be calculated assuming the detection of a single microorganism. Air samples collected near the LDL of a sampler can contain an insufficient number of particles to accurately represent the true bioaerosol concentration. There is no definite upper detection limit (UDL) for filtration or impingement sampling methods because the liquid collection medium or the liquid used to elute microorganisms from a filter membrane may be serially diluted prior to analysis. For impactor samplers the UDL is determined by the number of sampling nozzles or the area of the collection surface. Overcrowding of the impaction surface may result in errors in enumeration for microscopic and culture analysis methods (see Sample Analysis in the following section). For multi-hole impaction samplers, the UDL is reached when a CFU develops under each of the sampling nozzles. Other problems at or near the UDL include difficulties in resolving distinct particles or colonies (36) and inhibition of the growth of microorganisms as a result of competition for space and nutrients.

One method for establishing the collection time for a particular sampler is to determine the ideal surface density of microorganisms on the collection area and to estimate the order of magnitude of the bioaerosol. Nevalainen and coworkers (27) calculated the optimal sampling time for five bioaerosol samplers by using the formula $t = (\delta)(A)/(C_a)(Q)$, where t is the sampling time, δ is the desired surface density, A is the area of the sampling surface, C_a is the average expected bioaerosol concentration, and Q is the sampler flow rate. In this way the investigator determines the optimal number of colonies or cells on the collection surface and predicts the bioaerosol concentration. The drawback of this method occurs when the actual bioaerosol concentration is substantially different from the estimate. For this reason, more than one collection time is often employed for sample collection to increase the likelihood of obtaining useful data.

Another factor that influences the selection of the sample collection time is sampling stress, which can result in the loss of culturability of airborne microorganisms or degradation of the sample. It has been observed that increased sampling time has resulted in decreased viability for aerosolized vegetative bacterial cells (10,39–41). Therefore a doubling of sampling time may not result in a doubling of CFU or other analytes, depending on the stress tolerance of the bioaerosol components. In many cases, taking several consecutive samples of short duration is preferable to fewer samples with a long collection time. For further information on the selection of collection times, guidelines for the use of various bioaerosol samplers have been published (1,8).

Number of Samples. As previously stated, air sample collection periods are of relatively short duration and a single sample is of limited monitoring value due to the heterogeneity and variability of bioaerosols. Multiple samples for each sampling method used should therefore be taken for bioaerosol assessment. Determining the number of samples required for a particular situation is dependent on several factors, including the objective of sampling, the statistical approach used to interpret the data, the variability of the bioaerosol, the length of the sample collection period, and the available resources for sampling and analysis. The number of samples required should therefore be determined before sampling during the experimental design phase.

A high degree of variability has been shown between paired samplers of the same type (21,42). Duplicate samples are therefore often recommended, and the sample measurement is reported as the average of the replicate data points. Guidelines indicating the number of samples required for monitoring in the occupational environment have been published (1). For example, to estimate average worker inhalation exposure, the ACGIH recommends sampling in duplicate at least three times a day per site for at least three consecutive days for each sample type collected. Statistical methods can also be applied to calculate the number of samples needed to estimate confidence intervals around a mean or to compare mean values from sampling locations.

Sampler Calibration. The concentration of a bioaerosol is calculated by dividing the number of microorganisms or other analyte in the sample by the air sample volume (e.g., CFU per cubic meter). Therefore the volumetric airflow

rate of a sampler must be known and should be calibrated regularly. For impactors, the sampler's d_{50} is a function of the airflow rate through the nozzle(s). A decrease in airflow increases the d_{50} and the sample collection is shifted toward larger particles (26). Conversely, an increase in airflow decreases the d_{50}, resulting in increased small particle collection and potentially increased stress on the microorganisms. Various calibration methods that measure the flow rate through a soap bubble meter or an electronic calibration instrument are available (7).

SAMPLE ANALYSIS

A wide variety of sample analysis methods can be applied to air samples to provide information on the concentration and composition of bioaerosol components. The selection of an analysis method should be determined before monitoring is conducted because sampling and analysis methods are not necessarily compatible. Many impaction-sampling methods rely on the traditional analysis methods of culture or microscopy. Filtration and impingement sampling methods are more flexible with respect to sample analysis options. Limitations in the traditional analysis approaches have led to the development of alternative techniques, such as biochemical, immunological, and molecular biological assays. New methods are continuously being developed for the enhanced detection of bioaerosols (see ENHANCED DETECTION OF AIRBORNE MICROBIAL CONTAMINANTS).

Culture

The majority of bioaerosol data reported has been generated using culture analysis, and many of the currently available samplers are designed for collection onto a nutrient agar surface. Airborne bacteria and fungi collected in this way can be cultured directly, whereas samples collected by impingement or filtration are processed and transferred to a culture medium. The major drawback of culture analysis is that only those cells that survive and reproduce to form visible colonies are enumerated. Microorganisms that have been subjected to aerosolization and sampling stress may not respond to the artificial nutrient conditions in the laboratory. In addition, microorganisms exhibit a wide range of nutritional and temperature requirements for growth, and no formulation is capable of culturing every type of microorganism. For example, different media are required for isolation of airborne bacteria and fungi. Therefore a common approach in bioaerosol monitoring is to choose general media that promote the growth of the greatest diversity of species. For the isolation of specific microorganisms or groups, conditions such as pH, temperature, water activity, nutrients, antibiotics, light and aeration, can be manipulated to select for the growth of the target microorganisms (43). Consequently, it may be necessary to perform replicate sampling using multiple types of nutrient media or to divide the samples prior to inoculation.

Culture Media and Incubation. Several broad-spectrum media have been evaluated for their utility in the retrieval of culturable airborne fungi (31–33,44). Although results and interpretations vary, a number of media have been recommended. Malt extract agar (MEA) is a widely used fungal isolation medium. Of the formulations available, unamended 2% MEA was found to promote sporulation better than MEA amended with glucose and peptone (45,46). Rose bengal–containing agars (e.g., rose bengal-streptomycin and dichloran-rose bengal-chloramphenicol) have been used to inhibit the spread of rapidly growing fungal genera (e.g., *Rhizopus* and *Mucor*), allowing the enumeration and identification of other fungi in the sample. However, rose bengal is photoactivated in direct sunlight, forming cytotoxic products that may inhibit fungal growth (47). Dichloran glycerol-18 is a low water activity medium ($a_w = 0.955$) developed for the isolation of xerophilic fungi (48) and it compares favorably with other media for culturing mesophilic airborne fungi (33). Other commonly used fungal media include potato dextrose agar and Sabouraud's agar (20,49). It should be emphasized that many bacterial species are capable of growth on general fungal media and the use of antibacterial agents, such as chloramphenicol as an amendment to the medium, is recommended. Incubation periods for fungi typically range from 3 to 10 days and most airborne fungi are mesophilic and grow well at temperatures of 20 °C to 25 °C.

For the culture of bacteria, several broad-spectrum media, including tryptic soy agar, nutrient agar, and casein soy peptone agar, are commonly used (12,43). These media should be amended with antifungal agents such as cycloheximide to restrict the growth of fungi. Incubation temperatures from 28 ° to 35 °C for 1–7 days are usually used for environmental and human source bacteria. Important exceptions are the thermophilic actinomycetes, which are cultured at 55 °C.

Enumeration. After the appropriate incubation period, culturable microorganisms are determined by enumerating the colony-forming units (CFU). The concentration of culturable airborne microorganisms (CFU per cubic meter) is calculated by dividing the number of CFU per sample by the volume of air sampled. When microorganisms are collected using multiple-hole impactor samplers (Table 1), positive-hole corrections are generally applied to the data before calculations to avoid underestimation of the culturable bioaerosol concentration (9,21,50). These corrections are based on the probability of multiple impactions of microorganisms through the same sampling hole. The magnitude and the standard deviation of the correction increase with the number of CFU (7).

When air samples are collected by impaction onto an agar plate and the bioaerosol concentration is unknown, too few or too many colonies may be cultured, leading to enumeration errors. Colony counts that are too low can be nonrepresentative of the population and exhibit high variability. High colony counts can lead to errors because of overlap of colonies and inhibitory effects of microorganisms on one another. When a limited amount of overlap occurs, the true bioaerosol concentration can be statistically calculated from the number of colonies on the agar (36,50). Enumeration errors for agar impaction

samplers can be reduced by selecting multiple sample collection times as previously discussed. For filtration or impingement sampling, the wash solution or collection buffer can be serially diluted to obtain counts within an acceptable range.

Identification. Identification of fungal isolates is based largely on microscopic determination of morphological characteristics and requires training and expertise in mycological techniques. Bacterial isolates may be identified by several methods. Often culturable bacteria are differentiated according to their Gram stain reaction. Identification to the genus or species level requires the use of classical biochemical methods or various commercially available identification systems.

Microscopy

Microscopy is another commonly used method for the enumeration of airborne microorganisms collected by impaction onto an adhesive-coated surface or by filtration. Microscopy allows enumeration of total (culturable and nonculturable) microorganisms, unlike culture analysis that only measures the culturable bioaerosol components. This method can provide important exposure information because some microorganisms that are nonviable or nonculturable can still elicit an immune or toxic response when inhaled. However, identification of microorganisms beyond the genus level is usually not possible without utilizing a taxon-specific technique such as immunospecific fluorescence staining (described in the following section). If information on viability or culturability is desired, microscopy can be used in conjunction with culture sampling and analysis. In addition, a variety of stains are commercially available that differentiate respiring cells from nonrespiring cells.

Microscopic enumeration is generally used for the determination of total airborne fungal spores. Air samples collected with slit impactors (e.g., Burkard spore traps, the Allergenco, and Air-o-Cell samplers) are prepared by mounting and staining the collection slide or tape, and fungal spores and pollen grains are enumerated by light microscopy. The total number of spores or pollen grains enumerated in a sample is divided by the volume of air sample to estimate the total number of spores or pollen grains per cubic meter of air. When the sample is collected onto a moving surface, time discrimination of fungal spore concentration is possible by enumerating spores present between specified points along the collection surface that represent discrete time intervals. Several stains, including lactolphenol cotton blue, phenosaffranin, and basic fuchsin, are commonly used to facilitate discrimination of spores or pollen grains from debris (43). Data may be reported as the total spore concentration, the concentration of a specific target genus, or the percent composition of fungal genera. However, differentiation of certain fungal genera is not possible by spore morphology alone (e.g., *Aspergillus* and *Penicillium* spp.). Therefore these genera are grouped when reporting percent composition of bioaerosol samples.

For the determination of total airborne bacteria, liquid impingement or filtration sampling is generally used with microscopic analysis. Aliquots of collection buffer or the buffer used to elute cells from the filter membrane may be stained for epifluorescence microscopy by the acridine orange direct count method (35,51,52). Fungal spores may also be enumerated using the method. However, some fungal spore types may resist staining or have dark pigmentation that masks fluorescence (43). Cells and endospores may also be enumerated by bright-field or phase-contrast microscopy using a hemocytometer or some other cell counting chamber.

A disadvantage of microscopic analysis of air samples is the labor-intensive nature of microscopic enumeration. Computerized image analysis systems that overcome this limitation are available and can be used for enumeration. An alternative to microscopic enumeration of total cells is the use of flow cytometry, in which cell concentrations can be measured by light scattering and fluorescence emitted from fluorochromes bound to cells. Impinger samples labeled with DAPI yielded comparable results when enumerated by microscopy or flow cytometry (53).

Another difficulty of microscopic analysis is the level of expertise required to identify fungal spores and pollen. Misidentification and the inability to distinguish biological from nonbiological particles are common sources of error in this method (43).

Immunoassay

The limitations of traditional analysis methods and the need for human exposure data for bioaerosol risk assessment have lead to the development of enhanced detection methods for air sample analysis. Immunoassay methods have been recognized for their potential application to bioaerosol analysis (19). Immunoassays rely on the binding of antibodies to a specific target antigen. Target antigens may be cell surface–associated proteins, polysaccharides, or human allergens. The development of a specific antibody to the target antigen is required for these assays. The advantages of immunoassay methods for quantification of airborne allergens are their specificity and sensitivity. The major limitation of immunoassays is that specific antigens for microorganisms are difficult to define and standardize (54). Variations of this method that have been applied to bioaerosol analysis include fluorescence immunoassay, enzyme immunoassay, and radioimmunoassay.

For fluorescence immunoassays, samples are stained with a fluorescence-labeled antibody that binds specifically to the antigens on the surfaces of the target organisms. The samples are then analyzed by epifluorescence microscopy. Fluorescent labeling dyes include fluorescein, fluorescein isothiocyanate, and rhodamine isothiocyanate (12). Radioimmunoassays utilize radioactive-labeled antibodies and enzyme immunoassays utilize enzyme-labeled antibodies or antigens. The concentration of antigen is measured by radioactivity or enzyme activity. These methods have been applied to the measurement of airborne allergens, such as dust mite allergen, animal dander, and β-1,3-glucan (55–58). Impaction, impingement, and filtration samples may be analyzed using immunoassays (59).

Biochemical Assay

Numerous cellular components or metabolites, such as endotoxins and mycotoxins, may produce adverse health effects when inhaled (60,61). Biochemical assays have been developed to measure these compounds directly in an effort to relate environmental exposure to human responses.

Endotoxins are lipid polysaccharides (LPS) present in the cell walls of gram-negative bacterial cells. Elevated levels of endotoxin due to the presence of airborne gram-negative bacteria have been measured in bioaerosol samples from a variety of agricultural, industrial, and office environments (62). The concentration of endotoxin in a sample may be measured using the *Limulus* amebocyte lysate (LAL) test. The lysate of amebocytes from the horseshoe crab, *Limulus polyphemus*, gels in the presence of LPS, and a variety of test systems have been developed to quantify endotoxin using this method. Filtration sampling is most often used to collect airborne endotoxin; however, certain filter materials have been shown to inhibit the LAL assay (63). In addition, interference with the LAL reaction by inhibitors present in environmental samples must be accounted for in the measurement of endotoxin content (64).

Mycotoxins associated with dusts from fungus-contaminated grains are a known source of illness (65). Exposure to *Stachybotrys chartarum* mycotoxins has been associated with toxicoses in contaminated home and office environments (66–68). Filtration sampling using glass-fiber filters or polycarbonate membranes has been used to measure aerosolized mycotoxins of *S. chartarum* in the laboratory (69,70) and in the field (71). Other biochemical methods that may be applied to the analysis of airborne fungal exposure include ergosterol (72,73) and β-1,3-glucan (56,74,75) assays.

Polymerase Chain Reaction

Polymerase chain reaction (PCR) is an example of a molecular biological technique that has been applied to the detection of target microorganisms in air samples (76–78). PCR is an enzymatic procedure used to rapidly amplify specific DNA sequences (79). Following amplification, the target DNA is present in sufficient quantity to allow detection as low as the level of a single microorganism. The authenticity of the amplified DNA can be confirmed by sequencing, restriction analysis, or gene probe hybridization. The requirement for PCR detection of target microorganisms is that unique, non-cross-hybridizing sequences are available to serve as primers for amplification. Quantitative PCR methods have been developed and are commercially available for the measurement of the concentration of the DNA segments present in the original sample.

The primary advantages of using PCR for the analysis of bioaerosols are sensitivity and speed. Because PCR detection is not dependent on the culturable state of a microorganism, PCR can be used to detect microorganisms that are nonculturable, difficult to culture, or grow slowly. For example, positive results with the PCR technique were demonstrated for the detection of bacteria aerosolized in a greenhouse when culture data were negative (76). Using PCR, it is possible to obtain results within hours of sample collection, compared with days or weeks for culture methods. If there are multiple organisms of interest in a sample, multiplex PCR may be performed in the same reaction mix to simultaneously amplify several DNA targets of interest. Liquid impingement and filtration sampling methods have been used with PCR analysis for the detection of target microorganisms (76–78).

A limitation of the PCR assay is the inability to distinguish between culturable and nonculturable microorganisms. In addition, the presence of PCR inhibitors in environmental samples may reduce sensitivity or result in false negatives (80). A nucleic acid purification step or dilution of the sample may be required to remove environmental interference to PCR amplification (81).

Data Analysis

Data generated from air sampling studies can be used to test hypotheses and answer scientific questions pertaining to environmental biocontamination, biocontaminant dispersal, human exposure, and efficacy of remediation. For data to be useful, quality assurance and statistical considerations should be addressed before sample collection. A quality assurance plan should be developed to minimize errors in sample collection and analysis (82,83). Statistical considerations include determination of appropriate data reporting methods, statistical tests for data analysis, and sample sizes. Statistical tests can be used to compare means from various sampling locations and from different sampling or analysis methods.

There are several commonly used methods for data reporting (83). Often descriptive statistics, such as means, standard errors, and confidence intervals, are used for interpretation of bioaerosol concentration results. Expressing data as the percent composition of genera or groups is often helpful for population comparisons. There are several acceptable approaches for reporting and analyzing bioaerosol data, and consideration of these factors in advance will increase the likelihood of obtaining meaningful data.

CONCLUSION

There are many sampling and analysis methods available for bioaerosol monitoring. Increased awareness of the variety of health effects potentially arising from exposure to airborne microorganisms has led to the development of numerous new analytical techniques that can provide rapid, reliable data for bioaerosol exposure monitoring. Therefore it is important for the investigator to consider carefully the objectives of sampling before any samples are taken. After determining what information is desired, an appropriate sampling and analysis method can be selected and incorporated into the monitoring design. Reliable bioaerosol data are needed to further the understanding of the effects of human exposure to airborne microorganisms

and aid in the development of standardized monitoring protocols.

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BIOAEROSOLS IN AGRICULTURAL AND OUTDOOR SETTINGS

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Bioaerosols are biological agents carried in the air as large molecules, volatile compounds, single particles, or clusters of particles that are living or were released from a living organism. Included in this broad classification are viruses, bacteria, fungal spores, algae, pollen grains, arthropod particles, mammalian particles, complex metabolites, and volatile compounds. Particle sizes of bioaerosols range from approximately 0.05 to 100 μm and they occur both indoors and outdoors with the greatest diversity typically occurring outdoors.

A tremendous variety of bioaerosols in the atmosphere have been studied since the nineteenth century. Some of the early investigators include C.H. Blackley in England, who first described chest tightness and "bronchial catarrh" brought on by inhaling fungal spores on moldy straw and D.D. Cunningham, who attempted to relate airborne organisms to the incidence of disease in India. During the early twentieth century, advances in aerobiology (the study of bioaerosols) were made by plant pathologists interested in the spread of agricultural pathogens, specifically the movement of stem rust of wheat in the United States.

The development of the volumetric spore trap by Hirst in 1952 was probably the most important catalyst in the scientific study of bioaerosols because it made it possible to accurately estimate spore and pollen concentrations in the air at any given time. Nearly 50 years later, spore traps based on Hirst's design are among the most widely used instruments for studying bioaerosols. Analyses of air samples have shown that fungal spores are the most abundant types of bioaerosol particles (1). This entry will discuss fungal spores in the atmosphere and their importance in agricultural settings. Other bioaerosols are addressed in other sections of this volume.

SOURCES OF AIRBORNE FUNGI

Fungal spores are a normal component of the atmosphere, and they occur in large numbers whenever the ground is free of ice and snow. Atmospheric concentrations may exceed 200,000 spores/m^3 of air (2), although the levels are typically an order of magnitude lower. Spores are produced by fungal saprobes, pathogens, and symbionts; and they are dispersed into the atmosphere by various methods through the action of both wind and rain. The enormous number of spores that are produced has been well documented for many species (3–5).

Although fungi are everywhere in the environment, the major sources of the airborne spores are considered to be from fungi growing on living plants and those growing on leaf litter (1). *Cladosporium* and *Alternaria*, which frequently colonize both substrates, are typically the two most abundant genera of spores registered during atmospheric sampling. Lacey (6) found that natural and

cultivated grasslands were important spore reservoirs. He showed that spores from *Cladosporium*, *Alternaria*, and *Epicoccum*, which colonize senescent and dead grasses, reached high concentrations in the atmosphere (up to 10^6 spores/m^3) when a wheeled spore trap was drawn over an area of grass. Yeasts and yeast-like fungi are also abundant on leaf surfaces, and are the sources of yeasts in the outdoor air (7). Soil also contains many fungi; soil moisture and wind speed appear to be the critical environmental factors that control their dispersal. Spores will only become airborne when soils are dry and wind speeds high enough to entrain soil particles into the atmosphere (1).

DISPERSAL MECHANISMS

The entrainment of spores into the atmosphere is dependent on the method of discharge by individual types of fungi as well as meteorological factors such as temperature, humidity, and wind speed. Spores are discharged into the air either passively (relying on outside forces) or through active mechanisms (relying on drying or the development of osmotic pressure). Although both wind and rain play an important role in some dispersal mechanisms, wind appears to be the major factor (11). The literature on this topic is extensive. Lacey (8) provided a general review, and McCartney and Fitt (9) recently described the dispersal mechanisms for foliar fungal pathogens.

Passive Mechanisms

Wind dispersal is well studied and is the method resulting in the most abundant fungal spore types occurring in the atmosphere. Many wind-dispersed spores are produced on erect sporangiophores or conidiophores, which position the spores above the substrate and its boundary layer, thereby facilitating dispersal. Others are produced on leaf surfaces, which are well above the ground so that the spores are liberated directly into the turbulent layer of the atmosphere (8).

For many of these wind-dispersed spores, the atmospheric concentration depends upon the ease with which spores are detached from the reproductive structure or hyphae at different wind speeds and levels of turbulence. Minimum wind speeds needed to remove spores range from 0.2 to 2.0 m/second (8,10). Wind gusts facilitate their removal, as does the movement of the leaf itself. Aylor (11) and Pedgley (12) have suggested that strong gusts are able to disperse spores from leaf surfaces even when the average wind speeds are too low to accomplish this. Warm, dry weather promotes wind dispersal for both pathogenic and saprobic fungi, and the entrained propagules are referred to as the *dry air spora*. They include the most abundant fungi in the atmosphere and include conidia of *Cladosporium*, *Alternaria*, *Botrytis*, *Drechslera*, *Epicoccum*, smut teliospores, and rust uredospores. Spores of the *dry air spora* usually peak during the afternoon hours under conditions of low humidity and maximum wind speeds (5). Although there are similarities in the dry air spora worldwide, at any one time the air spora may be dominated by nearby sources of spores (2). During crop harvesting or mowing, remarkable levels of spores (up to 10^9 spores/m^3) are released into the atmosphere with *Alternaria*, *Cladosporium*, and *Epicoccum*, typically the most abundant taxa (2,8,13).

Rain can passively disperse spores by various mechanisms. Members of the dry air spora may be removed by raindrops, as they strike a leaf surface causing vibration and puffing (8). This generally occurs with the initial raindrops, and it may result in increased atmospheric concentrations of *Cladosporium*, *Alternaria*, and other conidia. This may explain some anomalous results of air sampling that show increases of these spores during rain events (14,15). A similar tapping mechanism occurs among puffballs. These fungi are unique among the basidiomycetes as their spores are passively dispersed. They are released in a puff when a raindrop strikes the peridium of the mature fruiting body.

Spores of many fungi are dispersed by rain splash (9,16–18). Splash-borne spores are usually formed in mucilage, which inhibits their dispersal by wind and protects them from desiccation. During rain, the first drops dissolve the mucilage and leave a spore suspension available for splash dispersal by additional raindrops. For example, in *Colletotrichum gloeosporioides* (19), the number of conidia was found to vary from drop to drop. Splash droplets from the first drop released some mucilage and only a few conidia; the largest number of conidia occurred in the fifth drop. The size of the droplet may also affect the number of conidia as the smallest droplets are unable to carry spores. Studies show that most spores are carried by droplets greater than 100 μm in diameter (8,12). In addition to precipitation, which is the most important medium for splash dispersal, overhead irrigation can also disperse fungal spores using the same mechanisms.

Many splash-dispersed spores have thin colorless walls and elongated shapes, such as conidia of *Fusarium* and *Pseudocercosporella*. Many frequently have adhesive properties that enable them to stick to the new host surface. Although these spores lack the protective features of thick, pigmented walls commonly found in members of the dry air spora, they are protected from desiccation by their mucilage during dry periods and by wet conditions during dispersal and deposition (16). For plant pathogenic fungi, splash dispersal is second to wind in importance for pathogen dispersal, but is only of significance for local dispersal (16).

Raindrops are also efficient spore collectors, and even a light rain can cleanse most spores from the atmosphere (20). The surface properties of the spore are important for determining the outcome of this washout. Spores with a wettable surface are incorporated within the raindrop, while those that are nonwettable remain on the surface of the drop. Wettable spores will stay with the drop and settle when the raindrop comes to rest, while the nonwettable spores may be left on leaves or other surfaces as the raindrop rolls over the surface (4).

Active Mechanisms

Spores of many fungi are dispersed by a variety of active discharge mechanisms that propel spores into

the turbulent layer of the atmosphere. The ballistics of some species are quite amazing, and these have been studied by many researchers throughout the twentieth century (3,4,8).

In the majority of ascomycetes, the explosive discharge of ascospores from the ascus is linked to available moisture. The absorption of water within the ascus leads to swelling and the development of high osmotic pressure (21). The resulting pressure causes the ascus to burst, explosively propelling the spores into the turbulent layer of the atmosphere. Characteristic bursting methods (apical slit, hinged lid, apical cap, or minute pore) are related to the taxonomic position of individual species (4). The osmotic force can be assessed by examining the distance the spore is propelled, which ranges from less than 1 mm to 40 cm. Because of moisture requirement, ascospores are usually abundant in the atmosphere during and after rainfall or even during times of high humidity. Diurnal rhythms among the ascomycetes are complicated by this water requirement. Provided the water supply is not limited, ascomycetes with a perithecium (flask-shaped fruiting body) tend to be either diurnal or nocturnal (4).

Many ascomycete fruiting bodies are xerotolerant but only release ascospores under wet conditions (4). During dry periods, the fruiting bodies dry out and become inactive. When wetted again, the fruiting bodies recover quickly releasing spores into the atmosphere soon after rainfall. This explains spectacular increases in spore levels soon after rainfall. The amount of moisture necessary for this recovery is highly variable, and in some ascomycetes such as *Mycosphaerella* moisture from dew is sufficient for spore liberation. In *Venturia inaequalis*, as little as 0.2 mm of rain will lead to the release of abundant ascospores, but dew is not effective. In other fungi more than 1.2 mm of rain are needed (4).

Ascomycetes also show differences in the speed of response following wetting. *Ophiobolus* begins spore release within 15 minutes (4). Release of *Mycosphaerella musicola* ascospores began within 10 minutes after wetting infected leaf tissue, with 85% of the spores being released from the perithecia within 2 hours (24). Similar results were seen in *Mycosphaerella citri* and *V. inaequalis* (25). Ascospore release in *M. citri* began a few minutes after rain and continued for about 1 hour. However, a second rain event 3 hours later released few spores, suggesting a depletion of mature spores after the initial rain. In *V. inaequalis*, ascospore release began 5 minutes after the start of rain and the maximum release was approximately 1 hour later. In addition to spore release directly related to wetting, other ascospores are released only as the fruiting body begins to dry following a rain event (4).

The moisture requirement can be met by several different sources of water. For example, Richardson (22) found that *Didymella* spores were present in the Edinburgh atmosphere following rain as well as during periods of high humidity, but spore release only occurred at night. *Leptosphaeria* is abundant in the atmosphere following rain (4); however, a background level of *Leptosphaeria* was found in the Tulsa, Okla., USA, atmosphere on rainless days as well (23). The average daily concentration of *Leptosphaeria* spores in the Tulsa atmosphere during 1994 and 1995 was 69 spores/m^3. On rainless days the average concentration was 44 spores/m^3 but on rainy days it increased significantly to 109 spores/m^3 ($t_{728} = -7.52, p < 0.01$). These spores were present on 607 days during the 2-year period, but rain only occurred on 271 days. The data suggest that dew is effective in stimulating spore release in some *Leptosphaeria* species. Water stored within the organism can also play a role in spore release. *Daldinia concentrica* fruiting bodies were shown to release ascospores during the night throughout the summer even in the absence of rain. In fact, when a mature fruiting body was detached from its substrate and brought indoors, spore discharge continued for weeks from water stored in the stromal tissues (4). Other species not dependent on direct wetting by rain include *Epichloe typhina* and *Bulgaria inquinans*. The former, a grass pathogen, is apparently able to obtain sufficient moisture for spore release from the transpiration stream of its host, whereas the later is able to obtain sufficient moisture from the mucilaginous layer of the apothecium to sustain spore release for several days (4).

Discharge of basidiospores from most basidiomycetes requires moisture but the mechanism is quite different than that found in the ascomycetes. Money (26) has recently reviewed this topic, which has been of intense interest to mycologists for over 100 years. Basidiospores form externally on basidia, with four basidiospores typically produced by each basidium. Each basidiospore develops asymmetrically and is attached to the sterigma by a hilar appendage. Moisture from the atmosphere condenses around a crystal of mannitol and hexoses excreted at the hilar appendage, and the resulting drop of fluid, called *Buller's drop*, quickly increases in size (26). The moisture in Buller's drop ultimately fuses with a film of water that surrounds the basidiospore and produces a shift in the center of gravity (27). The result of this transfer is that the spore is violently shot from the basidium. This mechanism has been described as a surface tension catapult. Although the discharge only propels spores a fraction of a millimeter, the spores are liberated from the basidial layer and able to fall free under gravity and be swept into the turbulent layer of the atmosphere.

The need for atmospheric moisture confines basidiospore discharge to periods of high humidity. The presence of rain, however, may be a deterrent because liquid water would disperse the solutes from the hilar appendage. Various studies have shown peak basidiospore concentrations during nighttime or early morning hours when humidities are high (14,28–30). Haard and Kramer (28) studied spore discharge in a number of mushrooms and bracket fungi and found three patterns of spore discharge. Many species exhibited a peak during the middle of the night, while others showed a double peak with spore release in the early morning and also in the evening. A third group composed of small mushrooms exhibited continuous spore release with the peak 24 to 48 hours after the onset of discharge.

The active discharge of both ascospores and basidiospores requires increasing atmospheric moisture; however, the active dispersal of some oomycete sporangia occurs during drying conditions. Rapid decreases in relative humidity during the morning hours are thought to be responsible for the hygroscopic twisting of the sporangiophores in species of *Phytophthora* and *Peronospora* (4,8). It is believed that during twisting, the sporangia from these fungi are flung into the air. Recently, it has been suggested that electrostatic repulsion may also be involved, as the leaves and the sporangia become charged and repel each other during rapid drying conditions (31).

TRANSPORT IN THE ATMOSPHERE

When spores are discharged from the parent hyphae or fruiting bodies, they pass through several layers of the atmosphere. An extremely thin film of still air exists at the surface of the ground and on the surface of objects; air in this zone is held to the surface by molecular forces. The laminar boundary layer occurs above this zone. Here, airflow is typically parallel to the surface. The laminar boundary layer is usually thin, 1 mm, but the thickness will vary with wind speed and topography (5). During the night when air is very still the thickness of the laminar layer increases exponentially; however, during high wind speeds the thickness of the layer decreases to less than 1 mm. Above the laminar layer is the turbulent layer in which air movement is constantly shifting and unpredictable. The turbulence depends on wind speed and direction, temperature, and the roughness of the terrain. Bioaerosols are carried in the turbulent layer and the dispersion is related to the intensity of the turbulence (5,32).

Plant pathogen spores within a crop must first pass from the laminar layer on the leaf surface into the turbulent layer within the crop. Wind gusts and turbulence enhance spore removal, but before spores reach the open air above the crop they must also pass through the boundary layer surrounding the crop (11). Under normal turbulence as much as 90% of the spores are deposited within 100 m with greater escape occurring under more turbulent winds (8). The location of lesions within the canopy will also affect escape of the spores. Spores produced high in the canopy will have greater chance of atmospheric entrainment as they are exposed to high winds and greater turbulence than spores lower in the canopy. Consequently, disease will spread more rapidly through the field if infection occurs in the upper canopy than if the pathogen is confined to the bottom or middle of the canopy (11).

Spores are transported vertically and horizontally by wind. Spores can be carried upward by thermals and have been recovered from various altitudes up to 5,000 m (5). Pady and coworkers (33) reported the recovery of rusts, smuts, *Alternaria*, *Helminthosporium*-type, and many unidentified spores on slides exposed during flights over Canada at 1,200 to 1,500 m. Gregory (5) reviewed the numerous studies from the 1920s through the 1960s that recovered a variety of fungal spores, especially plant pathogens, from various altitudes. Horizontally, bioaerosols can be carried downwind for thousands of kilometers. One of the best-studied examples of long-distance transport is the movement of *Puccinia graminis* uredospores from southern United States and Mexico to the wheat belt in the northern United States and Canada. This and other instances of long-distance dispersal of rust spores have been reviewed by Nagarajan and Singh (34) and Pedgley (12).

During airborne transport, fungal spores are vulnerable to various types of environmental damage. Exposure to harmful UV radiation and extremes of temperature and humidity can decrease viability of pathogenic species. Damage may be greater for species with thin-walled, colorless spores that are easily plasmolyzed and lack the protection provided by melanin found in pigmented spores (32). Despite the environmental hazards, a small percentage of spores are able to survive long-range transport and cause infection (34). Although pathogenic spores may lose viability during transport, it should be emphasized that spores probably retain their allergenic properties even when no longer viable.

DEPOSITION

Spores can be deposited by sedimentation, impaction, boundary-layer exchange, turbulent deposition, electrostatic deposition, and through rainfall (34). In still air, spores would fall to the ground in response to gravity at a rate (based on Stokes' law) that is proportional to the square of the spore radius (5). However, in the natural environment air is seldom calm, and the effect of sedimentation is insignificant at wind speeds at or above 2 m/second. Under normal atmospheric wind speeds, impaction and turbulent deposition are the most important mechanisms for deposition. Impaction occurs when air currents approach a leaf or twig or other objects and are deflected around it. Spores carried in the air diverge from the deflected air stream by amounts depending on the size of the spore and the size of the object. Impaction is most efficient when large spores impact on small objects at high wind speeds (8). Very small spores, at or below 5 μm, would require wind speeds of 25 m/second for impaction to occur. Impaction may be one of the most important methods for spore deposition in crops. Turbulent deposition occurs when air blowing over a horizontal surface deposits spores at a greater rate than would occur by gravity. Turbulent deposition increases at higher wind speeds, although some bounce may occur as well (5,8).

TYPES OF FUNGAL SPORES IN THE AIR SPORA

Many fungi are cosmopolitan; as a result, there are similarities in the air spora worldwide. From agricultural to urban areas, and from arctic to tropical areas, it is possible to find many of the same types of spores in the atmosphere whenever the ground is free of ice or snow. Although some spore types are common throughout the year, other taxa may peak in the spring, summer, or fall. In addition to seasonal variation, most fungi show a diurnal rhythm with peaks occurring at specific times of the day.

Examination of air samples from most areas of the world will show that many of the spore types are ones that potentially have an impact on humanity as allergens, potential human pathogens, and devastating crop pathogens. Approximately 20% of the human population suffer from respiratory allergies and many of these individuals are hypersensitive to fungal spores. While exposure to airborne fungal spores is typically associated with asthma and allergic rhinitis (hay fever), spores are also known to cause hypersensitivity pneumonitis (2). Allergens have been reported from every major group of fungi, but the most widely studied allergens are the asexual spores that are components of the dry air spora (35,36). In 1952, Gregory and Hirst (37) suggested airborne basidiospores as possible allergens, and in the past two decades, clinical evidence has been collected on the occurrence of basidiospore-induced allergies (36,38). Far less is known about the allergenicity of ascospores although anecdotal evidence has suggested these are also important triggers of allergic disease (39).

Although the focus of this entry is outdoor bioaerosols, especially related to agricultural applications, concentrations of fungal spores in farm buildings are also of interest as they may impact respiratory diseases in farm workers and their families as well as storage disease in crop plants. Wherever crops or hay are stored, airborne concentrations of storage fungi are high, with levels up to 10^{10} spores/m^3 recorded (2,40). *Penicillium* and *Aspergillus* are frequently the dominant fungi found; however, the taxa depend on the actual storage conditions. Pasenen and coworkers (41) studied the airborne spore load in urban and rural areas in Finland. They found that cow barns had levels up to 10^9 spores/m^3, with the highest levels associated with areas where hay was handled. Airborne concentrations up to 10^5 spores/m^3 were found in farmhouses; these levels were 10 to 1,000 times higher than levels measured in urban dwellings. Pasenen and coworkers (41) suggested that many of the fungal spores were introduced into the farmhouses on the person or clothing of the residents.

Many fungal spores are allergenic, but only a small group of fungi are considered human pathogens (42). The majority of mycoses (human fungal infections) are infections of the skin or mucous membranes, such as athlete's foot and thrush, with only a few fungi considered systemic human pathogens. However, many common airborne fungi can also cause serious human infections in immunocompromised-compromised individuals. Species of *Aspergillus* and *Fusarium* are two of the more common types of fungi in this category (36). These infections are of special concern because fungi do not respond to conventional antibiotic treatment, and mortality rates are high.

The majority of plant diseases are caused by fungi with approximately 10,000 fungal species recognized as plant pathogens. Although many important plant diseases are caused by soil fungi, these seldom cause sudden, widespread epidemics (43). By contrast, fungal pathogens with an airborne dispersal phase can quickly spread from the initial infection focus and cause widespread epidemics. The remainder of this section will examine the most prominent components of the air spora, especially focusing on those implicated in plant diseases.

Dry Air Spora

In dry weather the atmosphere in temperate areas is dominated by spores of fungal saprophytes and pathogens, which are passively dispersed by the wind. *Cladosporium*, *Alternaria*, *Epicoccum*, *Drechslera*, *Bipolaris*, *Pithomyces*, *Curvularia*, smut teliospores, and rust uredospores are among taxa typically found in the dry air spora (Fig. 1).

Cladosporium. *Cladosporium* is a large genus of anamorphic fungi with a wide variation in spore size and shape (44,45). Several species of *Cladosporium* are anamorphs of *Mycosphaerella* or *Venturia* spp. Spores are typically ellipsoidal to cylindrical and slightly pigmented (Fig. 2). Spores may be unicellular or have one or two septa. The size range of spores in the genus is seen in common species. Spores of *C. sphaerospermum* and *C. cladosporoides* range from 3–4.5 μm in diameter for the former to $3–7 \times 2–4$μm, for the latter; while those of *C. macrocarpum* average $15–25 \times 7–10$ μm.

Conidia of *Cladosporium* species are generally the most abundant airborne spore type in temperate areas of the

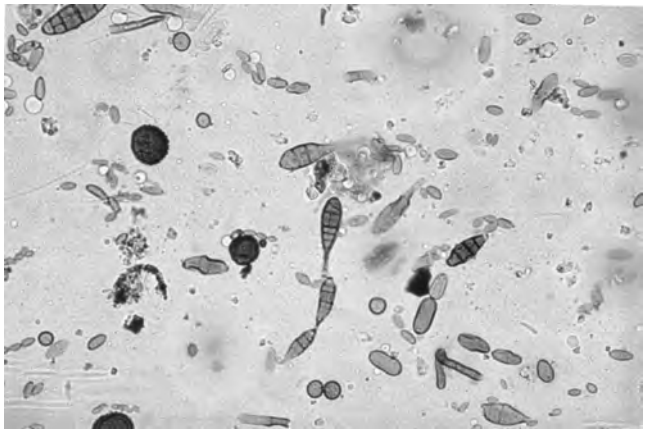

Figure 1. Components of the dry air spora from Tulsa, Okla., air sample.

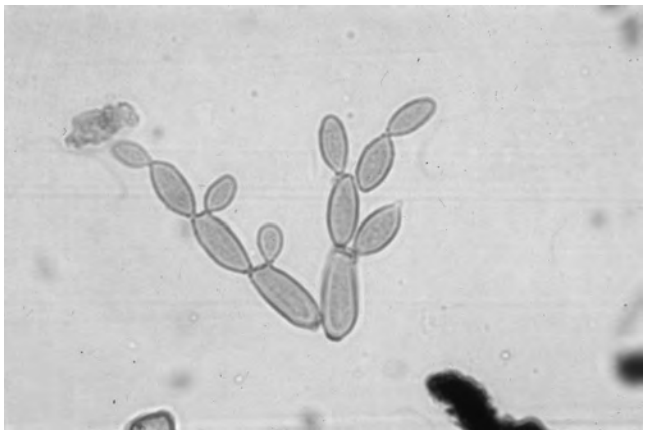

Figure 2. *Cladosporium cladosporoides* conidia from air sample.

world. *Cladosporium* species are common saprobes on leaf surfaces, dead herbaceous and woody plants, soil, foods, fabrics, and paint (44). Several species are plant pathogens causing various diseases such as leaf spots, scabs, and fruit rots. In addition, spores of *Cladosporium* are well known to be important aeroallergens (35). Aerobiological surveys including those conducted by the Aeroallergen Network of the American Academy of Allergy, Asthma, and Immunology (AAAAI) (46) routinely report the presence of *Cladosporium* conidia in the atmosphere. This network consists of a group of 84 certified air sampling and reporting stations, primarily in the United States. In the 1999 network report, airborne fungal spore concentrations were reported from 35 of the network sampling stations. *Cladosporium* spores were the most abundant airborne spore type reported at 23 stations and among the top six at all 35 stations.

In many areas, airborne *Cladosporium* spores can be detected year-round with the highest levels occurring from late spring though early fall. Summer peaks have been found in England (47), Sweden (48), and Colorado (49). However, Halwagy (50) showed year-round occurrence of *Cladosporium* in the atmosphere of Kuwait with highest levels found in the spring and secondary peaks occurring in the fall. Similar results were shown by Cosentino and coworkers (51) in Italy. These differences may result from moisture and other climatic growth factors. In the Tulsa, Oklahoma area, *Cladosporium* spores are also present in the atmosphere year-round with the highest concentrations typically occurring in the late summer or fall (46). At times, hourly concentrations of *Cladosporium* have exceeded 100,000 spores/m^3. Atmospheric concentrations of *Cladosporium* spores typically peak around noon (14).

Although generally considered a member of the dry air spora (8,47,49), various researchers have also described dispersal by mist droplets in this genus. Experimental studies with both mist-laden and dry air showed that mist was no more efficient in dispersing spores than the dry air for *Cladosporium herbarum* (4). In addition, studies have shown transient increases in the concentration after the start of rainfall (14,15). It has been suggested that an electrostatic release or the vibration and puffing from the first raindrops may be responsible for this increase (8). Others have suggested that washout is responsible for this increase in concentration (49). Wind gusts associated with the frontal system may also play a role in this increase; however, different species of *Cladosporium* may respond differently to precipitation.

Alternaria. *Alternaria* spores are frequently the second most abundant component of the dry air spora. *Alternaria* species are anamorphs of ascomycetes including members of the genus *Pleospora*. The genus is characterized by distinctive large multicellular dictyospores, which are beaked and produced in chains (Fig. 3). *Alternaria* spores are commonly found in atmospheric concentrations of several hundred to several thousand spores/m^3. In some areas, these spores may be present throughout the year; however, peak concentrations usually occur in late summer or fall (46).

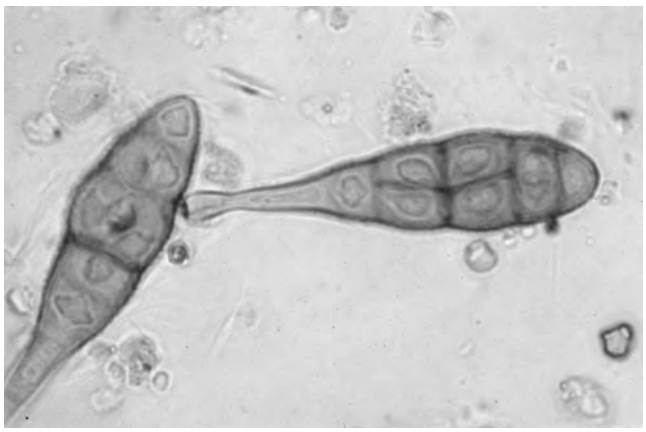

Figure 3. *Alternaria* sp. conidia from air sample.

Alternaria conidia are passively released from infected leaves or other substrates by moderate to strong gusty winds with minimum velocities of 2 to 3 m/second required. Dispersal typically occurs during midday when conditions are warm and dry with high wind speeds. Rotem (52) reported that the greatest dispersal occurs during dry weather, which immediately follows periods of rain or heavy dew. Dry windy periods can eventually deplete spore reserves on the leaves and inhibit sporulation, which requires moister conditions. Spores can also be dispersed into the atmosphere when washed or splashed from leaf surfaces by raindrops or irrigation. Timmer and coworkers (53) showed large numbers of conidia were released by simulated rainfall in an environmental chamber, but they found no correlation between spore concentration and rain during field studies. By contrast, high humidity inhibits the release of spores from wet leaves and airborne spores are washed from the atmosphere by rain and irrigation (52).

Species of *Alternaria* are pathogenic to a number of crop plants, causing early blight, black spots, brown spots, seedling blight, black rots, or leaf spot diseases. They also occur as saprobes on a large variety of organic substrates including leaf surfaces, stored foods, soil, and textiles (44). Most pathogenic species have a cosmopolitan distribution including *A. solani* on potato and tomato, *A. brassicae* on members of the Brassicaceae, *A. porri* on onions, and *A. alternata* on a variety of host species (52). *Alternaria* pathogens are often secondary invaders attacking plants under stress, especially those stressed by drought, insect infestation, senescence, or even other fungi.

Crop losses caused by *Alternaria* pathogens tend to be less than losses owing to more serious pathogens such as rusts or downy mildews; however, this genus is prominent in the aerobiological literature because it is also recognized as an important airborne allergen. Although there is no nationwide network of plant pathologists conducting air sampling for the detection of fungal pathogens, data from the Aeroallergen Network show that *Alternaria* conidia were among the top 10 spore types (in terms of atmospheric concentrations) reported from all 35 stations in 1999. At 19 stations *Alternaria* spores were among the top five spore types identified from the atmosphere (46). While some of

the *Alternaria* conidia may be from saprobic species, these spores are a significant component of the air spora and represent a ubiquitous threat to many crops.

Helminthosporium/Drechslera-Type Spores. Several genera of anamorphic fungi form similar thick-walled, pigmented, cylindrical conidia with rounded ends and a distinctive attachment scar at the base. The conidia are pseudoseptate but vary in shape with some being long (up to 160 μm) and tapering, while others are relatively short (44) (Fig. 4). For many years, fungi with this type of spore were classified as species of *Helminthosporium*; however, in the past 20 years the taxonomy in this group has undergone revision and several genera are now recognized (54). The best known genera in this group are now *Helminthosporium*, *Drechslera*, *Bipolaris*, and *Exserohilum*. Teleomorphs are species of *Cochliobolus* (for *Bipolaris*), *Pyrenophora* (for *Drechslera*), and *Setosphaeria* (for *Exserohilum*). The genus *Helminthosporium* is generally reserved for saprobic members of this group, and no teleomorph is recognized. Other genera are delimited by the shape of the conidium, morphology of the attachment scar, and the number and location of germ tubes produced by the conidium. It should be noted that the separation into these genera is generally acknowledged by mycologists and plant pathologists, but it is not universally accepted. Some books and journals still use the *Helminthosporium* binomials and others use *Drechslera* for all taxa (54).

This group of fungi is known to cause leaf spots and blights and other diseases on wild and cultivated members of the Poaceae; they are especially important pathogens on cereal crops (54). Some fungi in this group are able to cause sudden, devastating diseases that have resulted in major famines and great economic loss (55). Although many other host plants are infected by these fungi, the major losses have been to cereals. In addition to their role as plant pathogens, members of this group are allergenic and are the leading cause of fungal sinusitis. In addition, they are capable of causing fungal infections in livestock and are also toxigenic (42,54).

The great Bengal famine of 1943 was owing to the destruction of rice plants by brown spot of rice, which is caused by *Cochliobolus miyabeanus* (*Bipolaris*). During the famines that resulted from this plant disease, two million people died of starvation. Southern corn leaf blight caused by *Cochliobolus heterostrophus* (*Bipolaris maydis*) is another species in this group capable of causing devastating crop loss. The fungus causes small lesions on the leaf that may be so abundant they almost cover the entire leaf surface (55). An epidemic of one race of this species quickly destroyed approximately 15% of the U.S. corn crop in 1970. The fungus attacked all corn hybrids with the Texas male sterility gene and the resulting crop loss was estimated at $1 billion.

Tan-spot caused by *Pyrenophora tritici-repentis* (*Drechslera*) is a serious disease of wheat throughout the Great Plains and is considered the most economically important wheat disease in North Dakota (56). Epidemics are initiated by both ascospores and conidia that are found on overwintered crop debris. The conidia form the repeating stage, which can rapidly spread the disease when environmental conditions are suitable. Francl (56) showed that the greatest numbers of conidia were produced after rains as the wheat neared maturity. Conidia develop in the dark, but peak dispersal occurs in the afternoon after leaf surfaces have dried.

Conidia from these fungi are common in the atmosphere. They are reported among the top 10 spore types at 12 stations from the Aeroallergen Network in 1999 (46). Moderate winds disperse the conidia that can travel through microscale or mesoscale transport (9,56).

Curvularia is a closely related genus with a *Cochliobolus* teleomorph. Conidia have three or more transverse septa and are typically curved owing to an enlarged central cell (Fig. 5). Like the other genera in this group, *Curvularia* is responsible for a number of leaf spot diseases and is a common component of the dry air spora.

Smuts. Smut fungi are plant pathogenic basidiomycetes in the order Ustilaginales. There are approximately 1,200 species of smut fungi classified in 50 genera; however, the majority of species are classified into two large genera, *Tilletia* and *Ustilago* (57). About 4,000 species of host plants are attacked by smut fungi including both crops and native plants. They are especially serious pathogens on cereal crops.

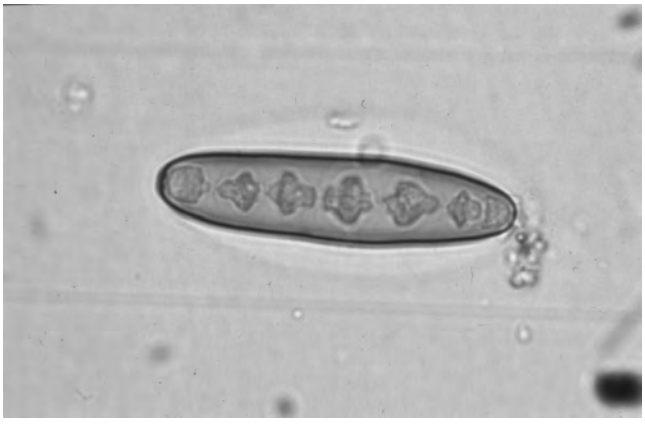

Figure 4. *Drechslera*/*Bipolaris* type spore from air sample.

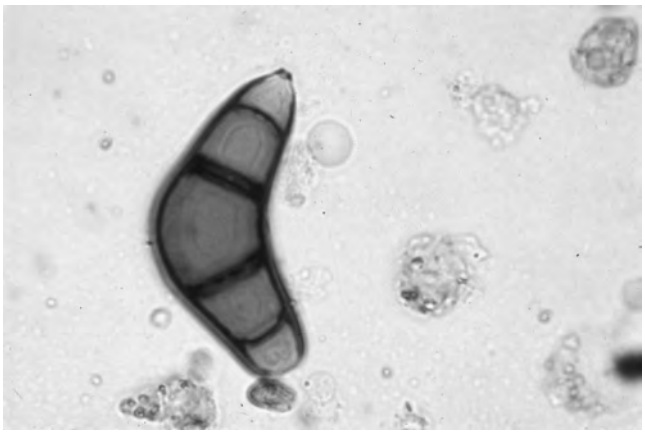

Figure 5. *Curvularia* sp. spore from air sample.

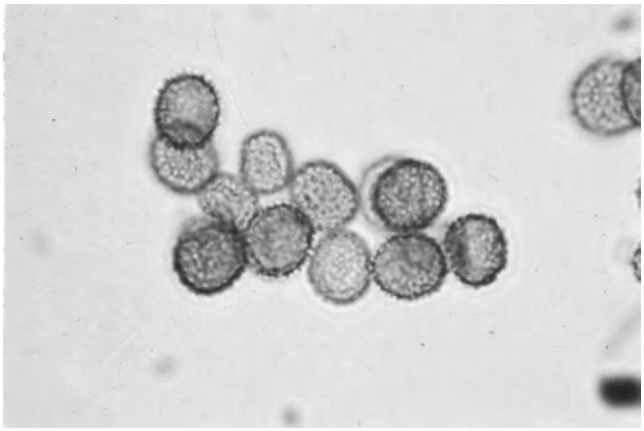

Figure 6. *Ustilago maydis* teliospores from infected corn.

The major dispersal phase of smut fungi is the asexual stage, which produces teliospores (55). Extraordinary numbers of teliospores develop and are passively dispersed by wind (Fig. 6). Smut fungi have no repeating stage; host plants produces only one generation of teliospores per growing season (55). Spores of various smut species are produced during different seasons. Hamilton (47) found that airborne spores of *Tilletia* showed highest levels in late August and early September in England, while *Ustilago* spores peaked from mid-June and late July. She also showed that the atmospheric concentrations of these genera depended on several meteorological variables. Levels decreased during rain or high humidity and increased during periods of abundant sunshine, strong winds, and high barometric pressure, typical condition for release of dry air spora (47). Smut spores typically show a mid-day peak but here the rhythm seems entirely owing to environmental conditions. Teliospores of an infected plant tend to mature all at one time; however, their release is spread over a long period and appears to be closely correlated with wind velocity, which is often higher around noon (4).

Various other studies have also reported the presence of smut spores in the atmosphere. Halwagy (50) showed that *Ustilago* spores were the second most common spore type identified in the Kuwait atmosphere. Misra (58) noted that smut spores peaked in India during dry conditions in December and January, while Rubulis (59) reported peaks during the late spring and fall in Sweden. In both of these studies, smuts were basically considered as one spore category with no attempt to differentiate the species involved. Likewise, the data from the Aeroallergen Network from the AAAAI also report smut spores as a single category. In their 1999 report (46), smut spores were among the top 10 spore types, in terms of atmospheric concentration, reported from 32 out of 35 sampling stations that provided counts for fungal spores.

Crotzer and Levetin (60) examined the atmospheric levels of smut spores in Tulsa, Okla., during 1991 and 1992 and attempted to identify the most abundant smut spores. The authors found that smut spores were present in the atmosphere every day from May through October in both 1991 and 1992. The average daily concentration generally ranged from 100 and 1,000 teliospores/m^3 during both seasons with, the mean daily concentrations at 291 spores/m^3 and 356 spores/m^3 for 1991 and 1992, respectively. Peak concentrations were 1,874 spores/m^3 on July 5, 1991 and 5,906 spores/m^3 occurring on May 12, 1992. Daily concentrations during the study period experienced many fluctuations owing to variable climatic conditions as well as the phenologies of both the host species and pathogens (60). This last factor was especially important because it resulted in different species of smuts occurring at different times of the year. In May and June, teliospores found in the atmosphere included those belonging to species, which infect Bermuda grass (*Ustilago cynodontis*), Johnson grass (*Sphacelotheca occidentalis*), oat (*U. kolleri*), and wheat (*U. tritici*). Smut spores identified during September and October included *U. maydis*, which infects corn, and *U. brumivora* and *U. bullata* pathogenic to several native Oklahoma grasses (60). Although this study was limited to the period of May to October for both years, other data from Tulsa show that atmospheric smut spores are not limited to this 6-month period (46).

In addition to the significance of smut fungi as plant pathogens, smut spores serve as important aeroallergens as they are abundant in the atmosphere for extended periods of time (35,61). However, the full extent of allergenicity to smut spores and the clinical significance of various smut species is not known (60). Data from the Aeroallergen Network showed that smut spores represent a significant component of the air spora and are, therefore, a significant source of aeroallergens as well as a significant source of plant pathogen inoculum (46).

Rusts. Members of the basidiomycete order Urediniales are commonly called *rust fungi*. About 6,000 species of rust fungi have been identified; these fungi attack a wide range of seed plants and can cause destructive plant diseases. Many rust fungi have complex life cycles with several spore stages. Probably the most important spore state is the uredial stage, which typically causes reddish (or rust-colored) lesions on plants and is the source of the common name for these pathogens. Spores produced in these lesions are known as *uredospores* (also called *urediospores* and *urediniospores*). Thousands of years before scientists knew that fungi could cause plant disease, rust epidemics were recognized and the reddish lesions noted on plants. The epidemics were described in the writings of Aristotle and Theophrastus who also realized that different plants varied in the susceptibility to these diseases. The rust fungi are responsible for some of the most important diseases of cereal crops, and it is estimated that 10% of the world grain crop is lost to rust fungi each year (55). In addition to the cereals, rust fungi have caused devastation to coffee, apple, pine trees, and other crops.

Because of the economic importance of these fungi, the long-distance transport of rust spores, especially wheat rust, has been studied more extensively than that of any other fungal pathogen (34). Worldwide, rust fungi are the most important and damaging diseases of wheat, with stem rust (*P. graminis* f.sp. *tritici*) and leaf rust (*P. recondita* f.sp. *tritici*) the most prevalent types. In North

America, leaf rust is currently the more important species, but historically major epidemics of stem rust have been more damaging. In recent decades, the widespread use of resistant wheat varieties has prevented major epidemics of stem rust. Nevertheless, approximately one million metric tons of wheat are lost to stem rust in North America each year, and in other wheat growing areas the losses are far greater (55).

Puccinia graminis f.sp. *tritici*, the fungus responsible for stem rust of wheat has a complex life cycle that involves five spore stages on two separate host plants, wheat and barberry (55,62). This fungus typically overwinters as teliospores that give rise to basidiospores in the spring. Basidiospores are capable of infecting young barberry leaves and giving rise to spermatia and receptive hyphae and later to aeciospores, also on barberry. Aeciospores become airborne and can transfer the infection to wheat on which the spores germinate, and hyphae enter the plant through stomata. Once established in wheat, the fungus produces uredia within 2 weeks; these appear on the stem as long narrow pustules, which produce dark red powdery masses of uredospores. Large numbers of spores are produced during the uredial stage (Fig. 7). It is estimated that a mature uredium is capable of producing about 10,000 uredospores per day over a period of several weeks and an even mildly infected plant with 50 uredia would produce 500,000 spores/day (63). Uredospores become readily airborne and infect new wheat plants, thereby forming the repeating stage of this fungus. Near the end of the growing season, the uredia turn black as two-celled overwintering teliospores replace the uredospores (55,62). Wheat plants infected by *P. graminis* are severely weakened, but not destroyed, and the grain yield is significantly reduced (55).

Efforts to eradicate barberry plants from wheat growing areas have reduced the risk of infection from stem rust of wheat. Also, the alternate host for leaf rust, meadow rue apparently does not occur in North America. However, for both pathogens the alternate host is less important than uredospores as a source of inoculum. Uredospores are easily carried by wind from one plant to another giving rise to rust epidemics. In fact, uredospores can be transported by prevailing winds for hundreds or thousands of kilometers (10,34). In southern Texas and Mexico where the climate is mild, the uredial stage can continue all winter and give rise to spring infections in northern states when the uredospores are carried by prevailing southerly winds. Uredospores can also be transferred back to southern areas in late summer and fall. Initially demonstrated in 1923 by Stakman, the movement of rust uredospores along the "*Puccinia* Pathway" in North America is one of the best known examples of long-distance dispersal (64). In addition to *P. graminis* and *P. recondita*, other fungal pathogens and the insect vectors of viral diseases are all linked to dispersal along this pathway (34). In a review, Nagarajan and Singh (34) also described the long-distance transport of *Puccinia* uredospores in other parts of the world including parts of Europe, India, and Australia. They also provided evidence for the transoceanic transport of uredospores between Australia and regions in southern Africa.

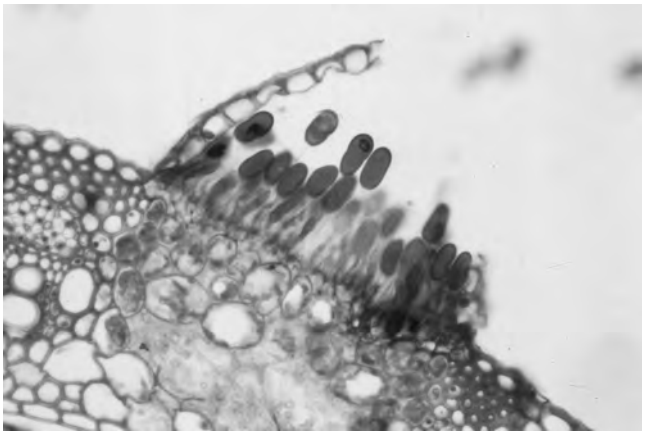

Figure 7. *Puccinia graminis* uredium with uredospores on infected wheat.

In addition to suitable wind patterns, successful long-distance dispersal of rust spores is also contingent on source strength and viability. Nagarajan and Singh (34) estimated that a field of wheat moderately infected with stem rust would produce 4×10^{12} uredospores/day/hectare (34). They suggested that uredospores must reach a suitable host within 5 days after discharge to remain infectious. However, Eversmeyer and Kramer (65,66) examined the survival of *P. graminis* and *P. recondita* uredospores under a variety of temperature conditions in the field and in environmental chambers. In field experiments, they found that during freezing or subfreezing temperatures spore viability quickly declined; but during the warmer temperatures in spring, 10 to 20% of the spores remained viable after 5 days. In addition, 1% of the spores remained viable for 19 days. In their chamber studies, spores exposed to constant temperatures between 10 and 30 °C remained viable for up to 36 days (66). While environmental conditions during transport may be severe, these studies suggest that at least a small percent of the enormous number of spores may survive long enough to reach a suitable host.

Other Members of the Dry Air Spora. Other fungal spores are also commonly found in the dry air spora. These include conidia of *Epicoccum*, *Pithomyces*, *Nigrospora*, *Botrytis*, *Torula*, *Stemphylium*, *Oidium*, *Aspergillus*, *Penicillium*, and many others. Although many of these genera are widespread saprobes, others are well known plant pathogens. The genus *Oidium* represents the anamorphic state of the powdery mildews, which are important pathogens on many crops, especially the cereals (55). *Botrytis* diseases are possibly the most common diseases of vegetable crops, responsible for gray mold and rots of fruits and vegetables in the field as well as in storage (55). *Epicoccum* is a cosmopolitan genus of asexual fungi that occurs as saprobes on a wide variety of substrates, but it is also a secondary invader on many types of plants and is frequently found on leaf spots (44).

Moist Air Spora

In damp or wet weather, the atmosphere is typically dominated by ascospores and basidiospores. As described

earlier, both spore types are actively released from the fruiting body through mechanisms that require moisture. *Leptosphaeria* ascospores are often abundant along with *Venturia*, *Didymella*, and spores from members of the family Diatrypaceae (39,67). Many ascospores found in the air spora are produced by important agricultural pathogens. Basidiospores that are typical components of the atmosphere include those from saprobic species of mushrooms, bracket fungi, and puffballs (38) although spores from a number of tree pathogens can also occur in the atmosphere (30). In addition to ascospores and basidiospores, asexual conidia of *Fusarium*, *Cercospora*, and *Colletotrichum* are also abundant in moist weather. This section will briefly consider two representatives of the moist air spora. For a fuller review of this topic, see Fitt and coworkers (16) and Huber and coworkers (17).

Apple Scab. The ascomycete, *V. inaequalis* Scab is responsible for apple scab, the most important disease of apples with all commercial varieties susceptible to infection (55). This pathogen occurs worldwide wherever apple trees are cultivated; similar scab diseases also affect pear and hawthorn. The principal symptoms are the development of scab lesions on both leaves and fruit; however, the disease can also cause premature defoliation, premature fruit drop, and poor bud development for the following year (55). Control of apple scab requires repeated applications of fungicides (8 to 20 times per season) to protect the crop. Without fungicidal protection, 70 to 100% of the crop would be unsalable.

In the spring, the primary inoculum consists of ascospores that develop in overwintered fruiting bodies. Immature fruiting bodies are found in dead leaves that overwinter on the ground in the orchard. They mature in the spring, and ascospores are actively discharged from these ascocarps during rain (16) (Fig. 8). *Venturia* ascospores are a major component of the air spora in orchards during the spring and are carried by wind to young leaves in which they cause a primary infection (68–70). Ponti and Cavanni (70) showed that the ascospores could typically be found in the air of Italian apple orchards during periods of rainfall from mid-March to mid-June. In the northeastern United States, ascospores were found in orchards from mid-April to early-June (71). Once the primary infection is established, conidia of the anamorphic stage (*Spilocaea pomi*) begin developing leading to further disease spread. The more primary inoculum present, the more rapidly the disease will build up and the more serious the epidemic will ultimately become. Several studies have recently focused on modeling the aerial dispersal of *Venturia* ascospores to help evaluate the risk of infection and optimize the application of fungicides to the periods when it is absolutely necessary (43,68,70,72).

Fusarium. *Fusarium* species are well known soil fungi with several species responsible for a variety of plant diseases. Vascular wilts and other diseases caused by *Fusarium oxysporum* have been recognized for over 100 years. Many subspecies of *F. oxysporum* attack a wide variety of crop plants. Although typically considered a soilborne pathogen, there is considerable evidence of airborne dispersal of conidia in many races and subspecies. Katan and coworkers (73) described the airborne collection of viable *F. oxysporum* f.sp. *lycopersici* conidia in greenhouses from diseased tomato plants. They also obtained similar results with a variety of other subspecies on other vegetables. Splash dispersal of *Fusarium* macroconidia has been generally accepted as the major vehicle of disease spread (8,74) (Fig. 9).

Fusarium head blight (FHB) of wheat and other small grains is caused by *Fusarium graminearum*. FHB, also known as *scab*, is increasing throughout the world and during the past decade there were several epidemics on wheat in the United States and Canada (74). *Fusarium graminearum* regularly forms its sexual stage, *Gibberella zeae*, in nature, and both conidia and ascospores are involved in disease spread. Ascospores are formed in perithecia and are forcibly shot into the air, while macroconidia are formed on sporodochia and are splash dispersed. Both stages occur on over-wintered crop debris and both require rainfall for dispersal. Spores infect the anthers on wheat inflorescences and the fungus spreads in the developing grains. Symptoms are evident in two to three weeks and include the premature senescence of spikelets (74). The fungus survives on the wheat residue

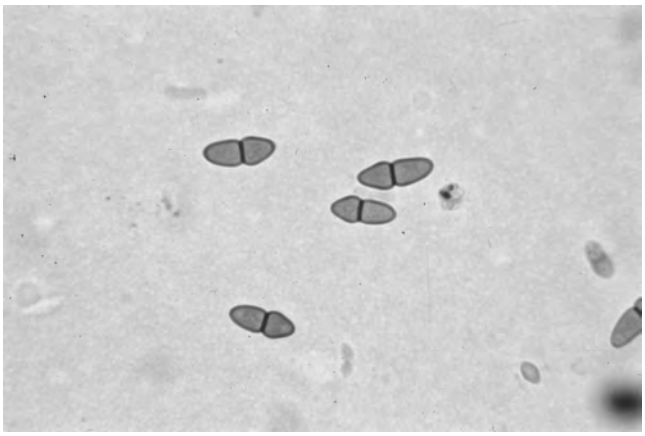

Figure 8. *Venturia* sp. ascospores from air sample.

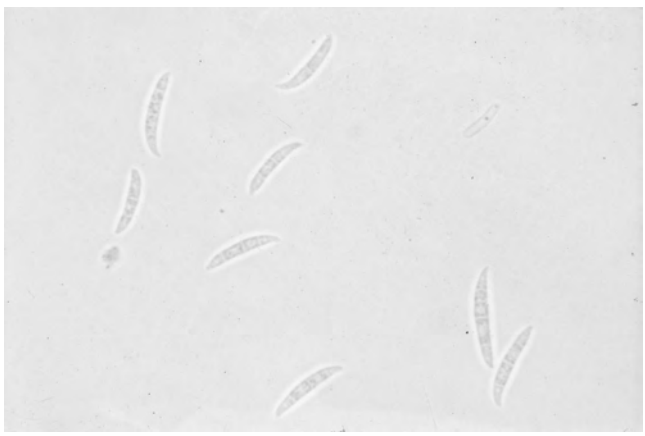

Figure 9. *Fusarium* sp. macroconidia from culture.

and gives rise to new infections the following spring. *Fusarium graminearum* also can exist as a saprobe on other residue especially corn stalks. The increased cultivation of corn in wheat growing areas and reduced tillage agricultural systems are both possible reasons for the recent epidemics.

Other Pathogens

The spore types described earlier are common components of the air spora and are often present in the atmosphere year-round. However, spores of other plant pathogens are not normally seen in the air spora unless the disease is epidemic in the area. Two diseases of this type are late blight of potato and tobacco blue mold.

Late Blight of Potato. *Phytophthora infestans* is the pathogen responsible for late blight of potato (and also tomato). This oomycete occurs wherever these crops are grown, and all cultivars are susceptible. Late blight of potato has been the most important disease of potato since the 1840s, when it caused the destruction of the potato crop in Ireland and the resulting widespread famine. *Phytophthora infestans* can invade all parts of the plant resulting in the rapid death of infected parts. Productivity of the potato plants is quickly reduced and tuber destruction frequently occurs as well. Sporangia are produced on aerial hyphae, which grow out of the stomata and are dispersed by wind, possibly carried for tens of kilometers (75). At low temperatures (10 to 15 °C) and high humidity, sporangia germinate by producing numerous zoospores, while at higher temperatures each sporangium gives rise to a single germ tube that develops into hyphae. As a result, during cool, wet periods, the production of zoospores leads to an amazingly rapid spread of the disease. Without fungicidal protection, a blighted field can be destroyed within a couple of days. Similar disease progression also occurs in infected tomato fields (70). Forecasting models have been used for over 40 years to help optimize the timing of fungicide application to periods when weather conditions promote the disease spread (76).

Generally, *Phytophthora infestans* overwinters as mycelium in infected tubers. In countries where both mating types have been identified, it can also overwinter as oospores in the soil. In the 1840s, when the disease first appeared in Europe and the United States, only one mating type (A_1) of the fungus was present. The second mating type (A_2) was identified in 1950 in central Mexico and was confined to Mexico until 1980 when outbreaks began occurring in Europe. It has been suggested that the A_2 mating type was introduced in a shipment of potatoes from Mexico to Europe in the late 1970s. In the past 20 years, the A_2 type has spread throughout the world (77,78). This has raised anxieties about the possibility of sexual reproduction occurring in the field leading to new strains developing at a faster rate. The implications for potato breeders searching for blight resistance are obvious. These concerns have been strengthened by the recent outbreaks of late blight in the United States and Canada. The strains of *P. infestans* involved were nearly all resistant to metalaxyl, the most widely used fungicide to control late blight (77,78).

Long-distance dispersal of *P. infestans* has been well documented by two different modes of transport (75). Intercontinental migration has been associated with the transport of infected plants or tubers by humans. This occurred in the 1840s and again before the 1980 outbreak of the A_2 mating type. Dispersal over tens of kilometers is attributed to airborne sporangia, which are able to survive for hours at the reduced humidity encountered during transport (75). The rapid progress of blight epidemics in the 1840s and in the 1990s illustrates the effectiveness of airborne dispersal.

Blue Mold. *Peronospora tabacina* (syn *P. hyoscyami* f.sp. *tabacina*) is an oomycete that is responsible for tobacco blue mold. This disease was first described in Australia during the nineteenth century, and the fungus was identified by Baily in 1890 (79). The first serious epidemic in North America occurred in 1979. Prior to this time the disease was confined to seedbeds. In 1979 and again in 1980, the infection rates were especially severe, with the epidemic advancing northward at rates of 10 to 32 km/day in the eastern United States to southern Canada and with crop losses estimated at $350 million.

Blue mold is able to attack both wild and cultivated species of tobacco. Host plants and pathogen exist year-round in tropical areas such as the Mediterranean and Caribbean basins. However, in temperate regions, tobacco is grown as an annual. Also, *P. tabacina* is not able to overwinter in temperate zones. As a result, reappearance of the pathogen is owing to either contaminated seedlings or the long-distance transport of inocula from tropical regions.

The asexual propagules of *P. tabacina* are the sporangia. Infection can begin within four hours after a sporangium lands on the leaf surface. A symptom-free incubation period, typically five to seven days, ends with the appearance of yellow lesions and the development of new sporangia. Unlike many other oomycetes, no zoospores are produced by *Peronospora* and the sporangia themselves (often referred to as *sporangiospores* or *conidia*) are the only asexual stage. As described earlier, sporangia are released from the sporangiophore by the twisting movements of the sporangiophore that occurs during the drying of the leaf in the morning as humidity decreases and temperature increases (11,80). Because the sporangia are subject to desiccation during transport, cool, wet, overcast weather, favors the rapid advance of the fungus, while clear, hot, dry weather halts disease spread (80).

Each spring in the eastern United States, weather conditions are favorable for the northward transport of *Peronospora* sporangia with two possible pathways of disease spread (80). Along one pathway, the pathogen can spread northward from Cuba into Florida and Georgia. In the other pathway, the source of inoculum is on wild *Nicotiana repanda* south central Texas or from cultivated tobacco in Mexico. From here the disease spreads to Kentucky and then east to North Carolina. These pathways show that long-distant transport of

sporangia can lead to blue mold outbreaks, and suggest that forecasting systems can potentially provide time for tobacco farmers to apply fungicides. This is the aim of the North American Plant Disease Forecast Center from the Department of Plant Pathology at North Carolina State University (81). Since 1995, this center has used the HYSPLIT trajectory model to predict outbreaks of blue mold in North America. Documented sources of blue mold are used in the model along with meteorological data from NOAA's ETA or AVN Models. This modeling system predicts the short-term weather and plots the trajectories of inoculum-laden parcels of air. The daily forecast produced with HYSPLIT trajectories describes weather conditions at the source and along the anticipated pathway with emphasis given to those atmospheric conditions that favor sporulation at the source, survival during transport, and deposition. An overall outlook describes the likelihood of blue mold spread over the subsequent 48 hours. The forecasters anticipate that the use of this model will continue to provide valuable information in the efforts to control blue mold epidemics (81). A recently developed numerical model encompasses the release, transport, and deposition of blue mold sporangia (82). Since 1996, the daily blue mold forecasts have been available on the Internet during the growing season (81) to provide on-line forecasts for tobacco blue mold outbreaks throughout continental North America. In 1998, the center was expanded to also include forecasts for downy mildew of cucurbits.

CONCLUSION

A tremendous variety of fungal spores are present in the atmosphere. While many of the spores originate from saprobic species, spores of plant pathogens are often a significant component of the air spora. These spores pose a constant threat to crops throughout the world. Control of plant diseases often requires extensive use of fungicides. To optimize the application of fungicides, timing of the applications should be tied to periods of peak spore dispersal. This requires a thorough understanding of the aerobiology of the pathogen. Aerobiological studies should be part of any effort to understand the distribution and epidemiology of agricultural pathogens that rely on air currents for dispersal. Unfortunately, no regional or national sampling network exists for agricultural pathogens. The Aeroallergen Network of the AAAAI maintains a national network of air sampling stations, and a similar network is needed for the plant pathology community. Studies during the last half century have provided an understanding of the dispersal mechanisms used by fungi; however, research is still needed on many aspects of fungal biology to fully understand the effects of meteorological conditions on dispersal, transport, and survival in the atmosphere. This knowledge will help in the development of better computer models for forecasting disease epidemics. These models are in place for forecasting of blue mold and downy mildew of cucurbits from the North American Plant Disease Forecasting Center. More systems of this type are needed.

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BIOAEROSOLS IN INDUSTRIAL SETTINGS

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Microorganisms are normal inhabitants of the environment; they exist in the soil on which we walk, in the air that we breathe, in the water that we drink, and even in the food that we eat. The saprophytic varieties (those utilizing the organic products of other organisms, living or dead, as a food source) generally predominate. Under appropriate conditions (optimum temperature, pH, and with sufficient moisture and available nutrients), saprophytic populations can be amplified. In the air, they can exist as discrete particles (e.g., as individual fungal spores) or they can be attached to other particles or substrates in a medium (solid, liquid, or gas). Many microbial species have been documented to tolerate various environmental extremes of temperature and pressure, allowing them to acclimate to the most extreme conditions. Bacteria have been identified in the lower depths of the ocean, growing amidst intense volcanic activity. Mycobacteria have been found in mummified tissues. It is clear that microorganisms

(fungi, bacteria, and viruses) are ubiquitous occupants throughout many types of diverse environments.

Many species of microorganisms are beneficial to the environment and to our existence. Microorganisms have been used for centuries to produce alcoholic beverages and to leaven bread. More recently, the metabolic systems of specific microbial populations have been used to produce pharmacologically important products (e.g., penicillin and citric acid) and a limited number of other chemical agents. Unique uses of the microbial community include the application of bacterial species in the decontamination of hazardous substances (i.e., oil spills in the ocean) and as an insecticide (i.e., *Bacillus thuringiensis*) to control cabbage worm, cotton boll worm, and chicken louse. Our everyday encounters with the microbial community generally do not pose a significant health risk to the immunocompetent population (healthy adults). However, select occupational environments present unique exposure concerns because of the nature of the microorganisms encountered, the microbial concentrations observed, and the susceptibility of the exposed population. Within the health care industry, attention has been focused on human infections or infectious agents, of which the obligate parasites and facultative saprophytes (including the primary pathogens and opportunistic pathogens) are primary concerns (1).

Exposure to bioaerosols (defined here as microorganisms and/or their products entrained in/on airborne particles) has been a well recognized safety and health issue in agricultural settings, and more recently, in nonindustrial indoor environments. However, bioaerosols are also important health and safety issues in nonagricultural industrial work settings, the topic of this article. Bioaerosols include microorganisms (both alive and dead) and their fragments or products. Among the specific components of bioaerosols that have been studied include microorganisms (such as bacteria, fungi, and viruses), endotoxins, and [1 → 3]-Beta-D-glucans. Endotoxins are lipopolysaccharide (LPS) compounds, which are part of the outer cell wall of all gram-negative bacteria. [1 → 3]-Beta-D-glucans are glucose polymers, the structural components of most fungal cell walls.

Occupational exposure limits have not been established for bioaerosols or for any of the specific components of bioaerosols (2), although several reports have proposed guidelines for occupational exposure limits to endotoxins (3–5). One factor that limits our ability to establish guidelines for exposure to endotoxins (and for bioaerosols in general) has been laboratory variation in analysis; this issue has recently been addressed for endotoxins in a collaborative study that found that both intra- and interlaboratory variation remain important problems (6).

We will now review several industries in which exposure to bioaerosols is an important safety and health issue.

BIOTECHNOLOGY

Biotechnology has been broadly defined as any technique that uses living organisms (or parts thereof) to make or modify products, to improve plants and animals, or to develop microorganisms for specific uses (7). Within the focus of this article, biotechnology will be presented in terms of the production of goods using industrial processes, specifically, fermentation technology. Aseptic fermentation techniques have been employed to produce a variety of commodities on a large scale, including single-cell proteins, antibiotics, acetic acid, citric acid, lactic acid, amino acids, vitamins, and enzymes. During industrial fermentation, microbial inocula (fungi or bacteria) are used to convert a substrate to a desired product typically using a batch processing system in three main stages: laboratory and inoculation, (or microbial preparation and growth), fermentation (or product biosynthesis), and process recovery (or product extraction and purification).

During the laboratory and inoculation stage, cultures of microorganisms are developed, prepared, and grown prior to the transfer to large-scale fermentation. All pertinent microbiological operations within the laboratory are conducted using sterile equipment with aseptic transfer techniques. To reduce the risk of contamination with foreign microbial strains, successive recultivations of cultures and numerous propagation steps are held to a minimum.

During the fermentation stage, multiple propagation steps are again held to a minimum to reduce the risk of contaminating large quantities of culture media and to optimize the use of process equipment. The seed tank, containing a sterile nutrient medium, is inoculated with the selected microbial culture prepared in the laboratory. The seed tank is designed to promote the growth of the microorganism population to a level necessary for proper inoculation of the fermentor tank. The batch mixture in the seed tank is aerated and mechanically agitated until the optimum level of culture growth is achieved. On completion of the cycle, the contents of the seed tank are aseptically transferred to a larger seed tank or, as is generally the case, directly into the fermentor tank. The fermentor tank is where "fermentation" occurs and the product of interest is biologically synthesized. A submerged, batch fermentation process is typically employed using a deep-tank reactor vessel with a top-mounted agitator and bottom air sparger. Proper temperature conditions are maintained with cooling coils inside (or a cooling jacket outside) the reactor vessel. The fermentor tank, containing the presterilized nutrient medium from a batching tank and the inoculant microbial culture broth mixture from the seed tank, is aerated and mechanically agitated for continued microbial growth and biologic synthesis of the product.

The mechanical equipment components used in the process recover stage are largely determined by the product and the microorganism used in the biosynthesis. Products that are produced extracellularly are extracted via solid/liquid separation techniques, which might employ the application of simple unit operations such as centrifugation and filtration. In some instances, concentration and purification (polishing) may be required and can be achieved with such processes as ultrafiltration, vacuum evaporation, or the precipitation of proteins. The separation of intracellular products is more complicated because the cells must first be disrupted. These processes

generate bioaerosols, which can be released to the surrounding area.

The etiologic agents of disease may be a contaminant that has found a suitable environment to proliferate (as in agricultural environments) or may be an active component of the manufacturing process. Topping and coworkers (8) documented sensitization among workers in a biotechnology plant producing citric acid using *Aspergillus niger*. The results of this study indicated that the risk was from exposures to not only fungal spores but also proteinaceous products found in the culture fluid. A follow-up longitudinal survey study of the same workforce indicated no new skin sensitizations believed to be the result of the implementation of control strategies, that is, process enclosure, the application of local exhaust ventilation, the use of respiratory protection, and worker/management education (9). In the pharmaceutical industry, Lagier and coworkers identified a case of occupational asthma in a worker exposed to penicillamine (10). The primary occupational biological hazards in the biotechnology industry is the potential for process microorganism and their metabolic products to produce an immunologic response in susceptible individuals (11). Intermediate processing chemicals used during the manufacture of specific products of biotechnology can also pose occupational exposure hazards (e.g., amyl acetate used to extract penicillin). It has also been suggested that although infections from microbial production systems based on the use of recombinant DNA technologies are plausible, the risk is considered extremely low (12).

The effective containment of the potential hazards in the biotechnology industry is dependent on the equipment designs employed in existing chemical process technology. The equipment designs must provide for containment of the microorganisms (viable and nonviable forms), biologically active products or intermediates, and processing chemicals such as extraction solvents. The level of containment is determined by the anticipated risks associated with these agents. Specific factors affecting containment include the selection of appropriate fermentor and associated equipment, suitable exhaust gas treatment, design considerations for vessel overpressurization and relief, suitable inoculation and sampling systems, and the collection and inactivation of condensate that may contain viable microorganisms (13). Consideration to these factors are categorized as primary physical containment, which offers protection from the microbial agent to the process operator through isolation. Protection of the general environment is achieved through the application of secondary physical containment, which includes consideration of the building design, the appropriate use of ventilation systems, and waste disposal concerns.

Anticipating this reliance on chemical process technology, a 1988 study characterized the engineering controls used in conventional enzyme fermentation processes (14). Sample locations selected to reflect worker exposures to process microorganisms included the laboratory (where culture transfers were conducted), inoculum and fermentor tanks (sample ports and agitator shafts), filtering operations, and background locations (Fig. 1). The study

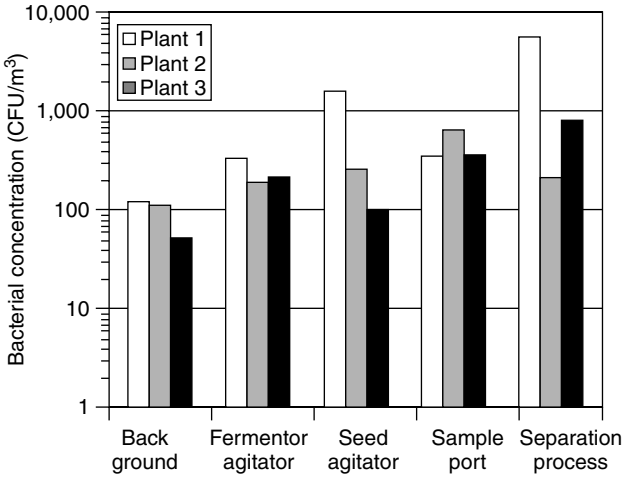

Figure 1. Sample locations reflect worker exposures to process microorganisms.

results indicated that controls are most needed around high-energy operations, including separation equipment (i.e., filters and centrifuges), fermentor agitator shafts, and manual sampling ports. These operations may not be amenable to complete sealing, enclosure, or isolation. Significantly lower bacterial concentrations were observed at a rotary vacuum drum filter compared with concentrations at the filter press (1). These differences appeared to have resulted from the application of local exhaust ventilation, the inherently better containment characteristics of drum filters, and operator work practices (dislodging filter cake from the filter press plates at the end of each cycle). Rotary vacuum drum filters have been reported to be the most widely used filters in the fermentation industry (15). In contrast, a centrifuge would be expected to produce large concentrations of microbial aerosols. However, as reported in the enzyme manufacturing study, effective process enclosure and the application of local exhaust ventilation at the biomass discharge point resulted in bacterial concentrations significantly below those of the filter process (although above those of the rotary vacuum drum filter).

Alternative methods of solids removal include precipitation, coagulation, floculation and chromatography, electrophoresis, and ultracentrifugation. These methods should be applied only after consideration of the agent risk and the inherent containment abilities of the technology. Rapid advancements in the biotechnology industry and use of microorganisms that pose significantly increased risks have resulted in large-scale equipment that offers improved containment capabilities. In a study of biohazards using a large-scale zonal centrifuge on moderate risk oncogenic viruses, it was determined that minimal risks to laboratory personnel existed during optimum operation (16). However, faulty seals did result in the detection of high concentrations of phage in the turbine air exhaust and the seal coolant system.

Exhaust gases from the fermentor tanks can be another major emission source for the production microorganisms. The aeration of fermentor broths produces a foam on

the surface of the liquid that results in the continuous production of bursting bubbles. It has been demonstrated that the droplets of dilute solutions formed by the bursting of bubbles can enrich the concentration of microorganisms by factors of 10 to 1,000 times (17,18). The control of vented bioaerosols within the fermentor tanks produced by this and other mechanical aerosolization processes is achieved primarily by the application of sterilizing (i.e., high efficiency) filtration systems preceded by "roughing" systems (e.g., cyclones, scrubbers, and/or condensers) (19). Data from the enzyme manufacturing study revealed that scrubbing systems alone may not be effective in controlling vented bioaerosols from the fermentors. The seals around the agitator shafts may be another emission source for the process microorganisms. Double mechanical steam seals appeared to provide inherently better containment than packed seals.

The work practices of the operators can also be a determining factor in the potential for occupational exposure. During the collection of fermentor tank sample volumes in enzyme manufacturing, operators were observed purging the sample port with a "blast" of pressurized steam prior to the collection of a broth sample. The steam served to clean and decontaminate the interior surfaces of the pipes to ensure a pure sample for subsequent analysis. However, the contact time between the steam and residual microbial populations in the sample line were not adequate to kill the microorganisms and therefore resulted in a dissemination of viable aerosols into the surrounding environment. Work practices are most reliable when used in combination with effective engineering measures such as isolation or automation. For example, microbial exposures during the separation of solids can be reduced by limiting operator interaction with those processes or, if this is not possible, the observance of proper and safe work practices.

Large-scale spills of fermentor tanks can be effectively controlled by concrete dikes constructed around the periphery of the tank. Spilled material is directed to a sump and subsequently pumped to a holding tank for inactivation of the microorganisms. In-line sterilizers may also prove effective prior to the disposal of the biological material. Bulk samples of the inactivated material should be microbiologically examined to validate the efficacy of the sterilization techniques. Spill responders should be equipped with personal protective equipment (PPE), including impervious clothing, gloves, autoclavable boots, and appropriate respirators (e.g., a self-contained breathing apparatus).

MACHINING

Machine tools use metalworking fluids (MWFs) during grinding, forming, treating, and machining operations. These fluids extend the life of the bits, blades, or other cutting components of the machine tool by cooling and lubrication of the machined surface. The application of the fluid also assists in the removal of the metal chips, swarf, fines, and other residues and may serve to protect or treat the material surfaces being machined. The fluids are disseminated from a dedicated sump located proximal to the machine or, as is found in larger machine shops, from a central sump that distributes fluids to multiple machines. Occupational exposures to the fluids or contaminants within the fluids are influenced by the fluid composition, the tool type and speed, the method of fluid application, the use of engineering controls (such as enclosure and local exhaust ventilation), and the fluid maintenance program.

There are four major types of MWFs — straight oils, water-soluble oils, semisynthetic, and synthetic. Straight oils (neat oils) are solvent-refined petroleum oils not designed to be mixed with water. The other three types are water-based. Soluble oil MWFs are emulsions or are composed in part of water-soluble oils (30% to 85% by concentration); the concentrate is diluted with water prior to use. Semisynthetic MWFs are composed of 5 to 30% petroleum oils. Synthetic MWFs contain no petroleum oils. MWF aerosol refers to the mist generated during grinding and machining operations, and may contain a mixture of substances. Water-based MWFs are suitable growth environments for many types of microbes owing to the abundance of water and organic substrates. As a result, many MWFs and MWF aerosols are routinely contaminated by bacteria and/or fungi.

Historically, microbial contamination of MWF has been a problem, primarily because it can affect fluid quality and performance. Fluid degradation from microorganisms can cause changes in fluid viscosity, and the acid products of fermentation may lower the pH of MWF, causing corrosion and leaks in the MWF system (20). The predominant microbial species routinely recovered from MWFs are frequently the same as those recovered from natural water systems (20). Although most bacterial species found in MWFs are gram-negative, the microbial populations within such MWF samples are continually changing (20,21). The species identified in MWFs are generally characterized as nonpathogens or opportunistic pathogens, of which the bacterial genus *Pseudomonas* is the most frequently isolated (22,23).

Bacterial concentrations in MWFs often range from 10^5 to 10^8 colony forming units per milliliter (CFU/mL), but they can be as high as 10^9 CFU/mL (24–28). It has been suggested that well-maintained MWF systems should have bacterial concentrations of less than 10^6 CFU/mL (29). Only recently have gram-positive bacteria and *Mycobacterium* species been identified as predominant species in the MWF of some plants (30). Data regarding airborne concentrations of mycobacteria from contaminated fluids are limited, but one study has reported concentrations ranging from 1,300 to 9,200 CFU/m^3 (31). Occupational disease syndromes have been suggested in workers exposed to metalworking fluids, some of which have been reported to be contaminated with mycobacteria (32,33). Similarly, recent evidence in the scientific literature suggests an association between the aerosolization of mycobacterial laden hot tub waters and the development of respiratory disease in the exposed population (34). However, although the acid-fast organism *Mycobacterium chelonae* has been found to be present in MWF associated with outbreaks of hypersensitivity pneumonitis (HP) (30), the significance of finding any

particular fungal or bacterial species in MWF is not clear at this time.

Due to the limited nature of microbial sampling, results of bulk sampling of MWFs at any given time may only represent a portion of the microecology in the MWF. Most water-based fluids have low concentrations of fungi, except when a bloom (which is often caused by a dramatic decrease in bacterial contamination) occurs (28,35). MWFs that have high concentrations of gram-negative bacteria frequently have high levels of endotoxin, which have been quantified in machining operations where water-based MWFs are used.

Exposure to MWF aerosols is known to be associated with increased prevalence of respiratory symptoms, decreases in pulmonary function, and the occurrence of occupational asthma and HP (22). The significance of reported respiratory and irritant symptoms among MWF-exposed workers in relationship to loss of pulmonary function or illnesses such as asthma or HP is unclear at this time. HP, which is an immunologically mediated inflammatory disease of the lung that occurs after repeated inhalation and sensitization to a wide variety of microbial agents (bacteria, fungi, amoebae), animal proteins, and low-molecular weight chemical antigens, has been associated with exposure to MWFs in several recent reports (30,36,37). Aerosolized endotoxins are suspect causative agents of occupationally related respiratory effects (e.g., chronic bronchitis, abnormal cross-shift declines in pulmonary function, and asthma) among workers exposed to MWF aerosol (22).

Limited information has been published regarding the concentrations of disseminated bioaerosols from MWF operations. However, questions regarding the development of occupationally related respiratory disease and exposure to MWF have prompted continued research focused on environmental characterization of the microbiological component. In one study evaluating worker exposures to MWFs (including synthetics, mineral oil, and rapeseed oil), airborne concentrations of gram-negative bacteria and endotoxins from contaminated fluids ranged up to 41,000 CFU/m^3 and 600 nanograms (ng) per cubic meter, respectively (38). Additionally, MWF workers responded with higher IgG antibody levels to *Stenotrophomonas maltophila*, *Pantoea agglomerans*, and *Comamonas acidovorans*, the most common bacterial contaminants in the fluids, than did controls. In a separate study characterizing MWF exposure indices and acute respiratory effects, a thoracic fraction sampler [that permitted the collection of personal breathing zone (PBZ) samples for bioaerosols] was used to enumerate airborne bacteria and endotoxin (39). PBZ bacterial concentrations of personnel working around MWF processes were reported to extend up to 26,600 bacteria/m^3. Sampling for endotoxins resulted in PBZ concentrations ranging to 234 endotoxin units per cubic meter of air (EU/m^3).

To prevent or greatly reduce the risk of adverse health effects due to MWF exposure, the National Institute for Occupational Safety and Health (NIOSH) recommends that airborne exposures to MWF aerosols be limited to 0.4 milligrams per cubic meter of air (mg/m^3) for thoracic particulate mass* as a time-weighted average (TWA) for up to 10 hours per day during a 40-hour week (22). The NIOSH recommended exposure limit was established primarily to eliminate or reduce respiratory health effects. Neither the Occupational Safety and Health Administration (OSHA) nor the American Conference of Governmental Industrial Hygienists (ACGIH) have exposure limits for MWF aerosols, although both have an eight-hour TWA limit of 5 mg/m^3 for mineral oil mist.

Currently, there are neither specific microorganisms in MWFs linked to specific, exposure-related health effects nor specific criteria concerning the level of total microbial contamination that may be related to potential health effects. Because the ecology of MWF systems fluctuates, documenting the microbial exposures at the time of symptom (or disease) onset is difficult. This points to the need for ongoing evaluations of the MWF environment, which may be correlated with ongoing surveillance of health effects among exposed workers. Proper management of the MWF and ventilation systems in machining areas, as components of an overall MWF health and safety program, plays an important part in reducing exposures to MWF aerosols and minimizing potential illness.

WASTE TREATMENT AND HANDLING

Because many types of waste inherently contain potentially infectious organisms, persons working in both wastewater and solid waste systems have potential exposure to bioaerosols. Important components of bioaerosols present in waste systems include a variety of microorganisms (viruses, bacteria, fungi, and protozoan) and components of microorganisms (endotoxin, [1 → 3]-Beta-D-glucan) (40). Airborne exposures that have most commonly been monitored include total and respirable dust (sometimes including analysis of particle size distribution), bacteria, and endotoxin. Recent data have shown that aerosols generated by various types of wastewater treatment processes include varying concentrations of microorganisms (including bacteria, fungi, and viruses) (41,42). Generally, the solids-handling aspects of waste systems (the typical household and industrial sewage treatment process) are thought to involve the most likelihood for bioaerosol exposure (40). However, the relationship between bioaerosols produced in sludge production, processing, and handling steps and potentially related health effects is not well described. In one study, a variety of clinical and laboratory effects among sludge workers were associated with aerosolized sewage sludge or a dust of dried sludge, both probably containing substantial concentrations of endotoxin (43).

Recently, the process of land application of biosolids (sewage sludge treated to significantly reduce pathogen levels) has received considerable attention. In the United States, land application of biosolids is a process regulated

* Thoracic particulate mass is the portion of MWF aerosol that penetrates beyond the larynx and may be deposited in the lung airways and/or gas exchange region.

by the Environmental Protection Agency (EPA) in which biosolids are applied to farmland, surface mines, and other lands, following specific requirements (44). However, potential occupational exposures during biosolid application are not directly considered by the EPA. One case study by NIOSH found that reports of gastrointestinal illness among five workers were temporally related to work in a land application process (45). In that survey, air concentrations of bacteria ranged from 412 CFU per cubic meter (CFU/m^3) to 2,356 CFU/m^3, with a small percentage of the organisms being enteric organisms. Endotoxin levels ranged from 23 to 39 endotoxin units per m^3 (EU/m^3). Another survey of bioaerosol concentrations at a land application site found concentrations of bacteria in the air of 10^5 CFU/m^3; those investigators noted that their data may not be relevant when considering occupational exposures in areas or processes in which workers are performing job tasks involving sludge agitation (46). Although direct contact with potentially infectious material is likely to be the greatest potential hazard for biosolid application workers, there is potential for respiratory exposure to bioaerosols. NIOSH is currently drafting recommendations, including eliminating potential hazards where possible and using appropriate engineering controls and personal protective equipment, to help minimize occupational exposures of workers exposed to biosolids (47).

Handling of solid waste also involves exposure to bioaerosols. A recent exposure study of workers at a refuse-derived fuel (RDF) plant found mean air concentrations of total bioaerosol (measured by epifluorescence microscopy) to range from 10^6 to 10^8 organisms per m^3, with endotoxin concentrations ranging from 5 to 346 EU/m^3 (48). Composting is another area of solid waste treatment that has been evaluated. In a case study evaluating exposure and respiratory illness of a worker handling composted wood chips and leaves, high levels of fungi (10^6 to 10^8 CFU/m^3), total bacteria (10^5 to 10^8 CFU/m^3), and endotoxin (636 to 16,300 EU/m^3) were documented (49). Another type of solid waste handling process has recently been found to present potential hazards to waste workers. As a component of an evaluation of exposures at a medical waste processing facility, where at least one employee was epidemiologically determined to have acquired tuberculosis due to occupational exposure, investigators conducted bioaerosol sampling. Air concentrations of total bacteria in the area of the process with the highest concentrations ranged from 183 to 256 CFU/m^3. Speciation of bacteria was limited to three organisms (*Pseudomonas aeruginosa*, *Escherichia coli*, and *Staphylococcus aureus*), which were considered atypical environmental organisms and utilized as indicators of aerosolization of waste materials. Although air sampling for *Mycobacterium tuberculosis* was not conducted, the investigators concluded that employees in that facility could be exposed to this pathogen as a result of occupational bioaerosol exposure; recommendations were made to minimize the potential for exposure and infection (50).

In summary, waste handling and treatment of a variety of types of waste involves potential exposure of workers to bioaerosols, which may contain a variety of potentially infectious or toxic substances.

WOOD PROCESSING

Various wood processing operations can place workers at risk for health effects due to bioaerosols. The degree of risk depends on the particular operation (logging, sawmilling, furniture manufacturing, carpentry), the types of woods utilized, the conditions in which the wood is stored before use, and the particular treatments applied to the wood (for example, fungicides) (51). A number of studies assessing the relationship between exposure of wood processing workers to bacteria, fungi, endotoxins, and (1 → 3)-Beta-D-glucan, and respiratory and mucus membrane symptoms were conducted by Alwis and coworkers (52–54). The workers in the studies worked in logging sites, sawmills, wood chipping mills, and joineries. Maintenance workers were used as controls. The lowest fungal concentrations were observed in the sawmills (3,000 CFU/m^3) and joineries (4,000 CFU/m^3). These process operations were equipped with the most efficient and well-maintained exhaust ventilation systems and used vacuuming to remove dust from machinery and the floor. Workers in the woodchipping mill were exposed to the highest concentrations of fungi (74,000 CFU/m^3). *Aspergillus fumigatus* and *Penicillium* spp. were the predominant genera at the logging sites where debarking was conducted; *Penicillium* spp. predominated in sawmills and the woodchipping mill. Joinery, sawmill, and chip mill workers showed significant correlations between mean personal exposure and work-related symptoms after controlling for smoking and age. The investigators pointed out that, although the exposures were considered low, they were still associated with symptoms. A dose-response relationship was shown between (1 → 3)-Beta-D-glucan exposure and the occurrence of chronic bronchitis, throat irritation, and regular ear inflammation and infections. A significant correlation was also found between mean personal exposures to endotoxin and chronic bronchitis. Pulmonary function testing showed that respiratory symptoms were correlated with cross-shift decreases in lung function.

Similar occupational exposures to microbiological agents have been described in related industries, that is, cellulose and paper manufacturing, which use timber as a raw material resource. Pulp logs absorb moisture during water-driven transport or during storage prior to processing. During storage, the ample supply of water and organic nutrient material provide an optimum environment for the development of microbiological communities, especially, thermotolerant species. The inner wood of logs are less likely to contain levels of fungi that would pose a significant biohazard; however, bacteria are more likely to develop in this "core" wood (55). Fungi appear to develop more prominently during the processing of waste wood and during removal of bark. The debarking of the pulplogs and subsequent storage of the resulting biomass can result in the release of bioaerosols, including fungi, bacteria, and endotoxins. A NIOSH study of a paper manufacturing process revealed elevated

concentrations of thermoactinomycete species during the debarking and subsequent transport and storage of removed biomass material (56). Concentrations of bacteria were two orders of magnitude greater in the transfer tower and the biomass storage area than those observed in outdoor control samples with thermoactinomycetes comprising greater than 90% of the genera identified. The lower concentrations of bacteria in the debarking process were attributed to the application of water, which reduce the dissemination potential of bacterial spores. Another reported study of a cellulose factory described elevated exposure concentrations of bacteria and fungi to barking workers of 46,000 and 5,900 CFU/m^3 (geometric means), respectively (57). Caterpillar drivers operating in wood chip piles were exposed to concentrations of bacteria averaging 1,500 CFU/m^3 and fungi averaging 45,000 CFU/m^3. Additionally, IgG antibody levels were significantly higher among the wood chip workers as opposed to the barking workers. It was suggested that dry wood chip material can penetrate to the lower parts of the lung more readily than wet aerosols generated in the barking process.

Exposure to bioaerosols have also been documented in the paper recycling industry. In a Danish study of six recycling plants, two plants were reported with very high counts of airborne microorganisms noting that several values exceeded the Danish threshold limit value of 10,000 CFU/m^3 (58). The highest microbial concentrations were observed in the effluent water treatment area with a mean concentration of 261,000 CFU/m^3; lesser mean concentrations of 34,000 and 8,700 CFU/m^3 were observed around paper machines and near winding and cutting machines, respectively. Samples for endotoxins at one plant near the paper machine revealed concentrations, which exceeded the Danish threshold limit value of 100 ng/m^3 (mean value of 294 ng/m^3). High endotoxin concentrations may have been the result of intensive reuse of process water.

Health effects associated with exposure to wood dust include occupational asthma, HP, organic dust toxic syndrome (ODTS), chronic bronchitis, and upper respiratory and mucus membrane irritation (59). Although exposures to the dusts of many different woods are thought to be associated with occupational asthma, a specific sensitizing agent directly related to the occurrence of asthma has been identified for only one type of wood (plicatic acid in the case of western red cedar) (59). A variety of fungi have be found to be associated with HP among workers in this industry, including *Cryptostroma corticale* (maple bark), *Aureobasidium pullulans* (redwood), and *Alternaria*, *Aspergillus*, and *Thermoactinomyces* (moldy wood chips) (59). *Penicillium*, *Graphium*, *Pullularia*, and *Trichoderma* species have also been found to be causative antigens (60). ODTS is characterized by the occurrence of a flulike illness (fever, chills, myalgia, malaise, nonproductive cough) within a few hours of inhalation of wood dusts contaminated with fungi, bacteria, and/or endotoxins. Although the clinical picture differs in some respects from HP (59,61), it has been postulated that HP and ODTS may represent parts of a spectrum of responses to exposure to bioaerosols (49).

Avoidance of exposure to the offending agent(s) is the primary means of prevention of these disorders and can usually be accomplished through product substitution or engineering controls. PPE may be suitable for occasional brief exposures. If symptoms persist despite engineering controls and PPE, an affected worker will need to be removed completely from the exposure situation (61). This has been shown to be particularly important for HP as repeated episodes of HP can lead to chronic lung disease and recurrent low level exposures can lead to the insidious development of chronic interstitial lung disease with fibrosis.

TEXTILE INDUSTRY

Recognition of adverse respiratory effects related to work in the textile industry dates back many years (62). Byssinosis is an occupational lung disease caused by inhalation of cotton, hemp, or flax dust, which in the early stage is characterized by distinctive chest tightness associated with decreased lung function. The relationship of respiratory effects and workplace exposures to cotton dust vary depending on the stage of processing workers are performing, with higher risk areas being those areas where the cotton is first processed (4,63). OSHA has regulations, directed primarily at the prevention of byssinosis that limit occupational exposure to cotton dust (64,65), and decreases in exposure to cotton dust in the textile industry has been associated with a decrease in the incidence of byssinosis (62). There has been ongoing research related to bioaerosol exposure in the textile industry. Important issues being addressed by some of that research include the issue concerning byssinosis occurring among persons with exposures below the limits set by OSHA and the issues of other respiratory disorders such as chronic bronchitis and long-term decrements in pulmonary function, which have been associated with exposure to cotton dust. Many investigators have evaluated the role of endotoxin as the etiologic agent within cotton dust, and acute pulmonary responses (decreased pulmonary function) have been related to the endotoxin concentration within the cotton dust (4,66). More recently, results of an 11-year longitudinal study indicate that chronic lung function decrements are more closely related to cotton dust exposure than exposure to associated endotoxins (67). This raises the question of whether exposure to bioaerosol components (e.g., endotoxin) and vegetable components of dusts such as cotton dust may lead to different health effects.

Studies designed to characterize occupational exposures to microbiological agents in cotton mills have been reported. In a study of two cotton mill plants in China, samples for airborne dust and endotoxins were collected using vertical elutriators and 5 micrometer (μm) polyvinyl chloride filters (68). Dust concentrations were similar for the two plants ranging from 0.15 to 2.34 mg/m^3 at the first plant and 0.19 to 2.50 mg/m^3 at the second plant. However, endotoxin concentrations between the two plants were different, with the first plant exhibiting higher levels, ranging from 20 to 920 ng/m^3, and the second plant, ranging from 2 to 550 ng/m^3. In a separate study of two

cotton mills in England (one mill was characterized as new while the other had been long established), air samples were collected for culturable fungi, actinomycetes, and bacteria and for spores counts of fungi, actinomycetes, and bacteria (69). Bulk samples of the cotton were also collected and microbiologically analyzed. Cotton dust samples revealed spore counts averaging 2,400,000 spores per gram (spores/gm) of material, with approximately two-thirds comprising "actinomycete + bacteria" classified spores. Culture techniques revealed predominance by bacteria with lesser numbers of actinomycetes, including *Faenia rectivirgula*, *Streptomyces albus*, *S. griseus*, and *Thermoactinomyces* spp., and fungi, including species of *Aspergillus*, *Penicillium*, and *Cladosporium*. As with the bulk sample analyses, "actinomyces + bacteria" classified spores were the most numerous spores identified with a mean concentration of 220,000 spores/m^3 for "actinomyces + bacteria" and 50,000 spores/m^3 for fungi in the new mill, and 2,770,000 spores/m^3 for "actinomyces + bacteria" and 50,000 spores/m^3 for fungi in the long established mill. This was consistent with the culturable air sample analyses, with bacteria being the most numerous on most culture plates.

FOOD PROCESSING

The relationship of bioaerosol exposure as a cause of health effects has been evaluated in a variety of food processing plants, including trout processing facilities (70) and beet sugar refineries (71). The latter survey documented exposure to fungal bioaerosol in a workplace where employees had been diagnosed with occupational asthma, and provided recommendations for detailed engineering controls designed to minimize bioaerosol exposure. Respiratory effects related to exposure to flours and grains have been researched for many years; it is clear that work-related respiratory symptoms and illnesses have been documented among workers in both agricultural and nonagricultural industries in which exposure to flours and grains occurs. A broad range of etiologic agents are thought to be involved with these health effects, including many types of microorganisms (such as bacteria, fungi, wheat weevils, and mites) and additives (such as enzymes and other proteins) (72). A series of evaluations have been performed concerning the potato processing industry (73,74) and a recent report evaluating workers in that industry demonstrated that decrements in lung function were associated with endotoxin exposure above 53 EU/m^3 (75). In addition to the components of bioaerosols mentioned earlier, another area of research relevant to the food processing industry includes evaluation of microbial volatile organic compounds as etiologic agents for respiratory and other symptoms.

CONCLUSION

The preceding discussions demonstrate that exposure to bioaerosols can occur in a wide variety of nonagricultural industrial settings and that occupational safety and health professionals in these settings must continually consider the potential for bioaerosol exposures. The discussions provided here are not meant to be an exhaustive review; for example, in-depth studies at other types of industrial sites have provided information useful in characterizing exposures and health effects related to bioaerosol exposure (76,77). Further research is needed to help clarify the health implications of exposure to bioaerosols in many industrial settings.

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BIOAEROSOLS: MODELING.

See MODELING THE TRANSPORT OF BIOAEROSOLS

BIOAEROSOLS: TRANSPORT AND FATE

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The study of bioaerosols (microorganisms aerosolized by natural or artificial means) and how they behave in the environment has long interested the scientific community. In the late 1800s, it was discovered that diseases could spread from person to person through the air. The process of how diseases were actually transmitted was not understood however, and the effort to elucidate that process was the birth of the scientific study of aerobiology. Aerobiology is defined as the study of the transport and fate of aerosolized microorganisms. Since then the study of bioaerosols has continued and much has been learned in the last century. However, as with many subjects of scientific interest, there is still much to learn and understand with regard to the transport and fate of bioaerosols. Within the last decade, interest in bioaerosol studies has grown considerably because of the increase in perceived threats of biological warfare and biological terrorism. Therefore, the study of bioaerosols has two purposes, the prevention and prediction of the spread of airborne contagious diseases and the detection of aggressive biological warfare/terrorism threats.

A large number of human pathogens are aerosolized from a wide variety of sources. Airborne human pathogens and common airborne microbes that cause opportunistic infections are listed in Tables 1 and 2, respectively.

The physics of biological aerosol stability and transport have long been established. Modern efforts are geared toward clarifying some of the more obscure effects the environment has on the bioaerosol as a whole. As the data from current efforts are collected and added to the body of knowledge it helps to increase our understanding of a very complex subject that has perplexed scientists for many decades.

Transport and fate are two fundamental parameters of bioaerosols that must be understood by aerobiologists. Transport involves the origin of the bioaerosol and meteorological influences, as well as physical obstacles that may affect the path the bioaerosol travels. The second parameter of interest is bioaerosol fate. This relates to the biological survival or viability of the aerosolized microorganism and all of the external factors that influence that survivability. These two parameters of biological aerosol study are interrelated. Both transport and fate are influenced by many of the same parameters including meteorology, forces involved in aerosol generation and dispersion, and time of day.

Table 1. Airborne Human Pathogens from Nonhuman Sources

Agent	Disease	Source
Bacillus anthracis	Woolsorter's disease, pneumonic and cutaneous anthrax	Contaminated hides, bone meals
Histoplasma capsulatum	Histoplasmosis	Soil enriched with bird droppings
Coxiella burnetii	Q fever	Contaminated meats, animal products
Chlamydia psittici	Ornithosis	Dried droppings from infected fowl

Source: Table Reproduced from Burrell (1991) with permission of the publisher (1).

Table 2. Common Airborne Opportunistic Infections from Nonhuman Sources

Agent	Disease	Source
Cryptococcus neoformans	Cryptococciosis	Pigeon dropping contamination
Acanthamoeba spp.	Various amoebic infections	Natural water sources
Legionella spp.	Legionaires' disease	Natural water sources
Atypical mycobacteria	Tuberculosis like illnesses	Water vegetation
Aspergillus spp.	Allergy, bronchopulmonary aspergillosis, mycetomas, hypersensitivity pneumonitis	Widespread in decaying vegetation

Source: Table Reproduced from Burrell (1991) with permission of the publisher (1).

In this article, the transport and fate of bioaerosols are investigated with an in-depth examination of their contributing factors, as they are currently understood. It is important to note that all of the studies cited in this chapter used culture-based analysis, and this assay method favors the results discussed.

TRANSPORT

According to Bartlett (2), there are three distinct stages involved with the transport of biological aerosols: dispersal, diffusion, and deposition. Dispersal is the aerosolization of the microbe, diffusion is the movement of the microbe over a distance, and deposition is the settling of the microbe onto a surface. All of these stages are influenced by meteorological conditions. In order to fully

appreciate their functions in aerosol transport, each of the three stages will be investigated.

Dispersal

Dissemination of microorganisms occurs when adhesion (i.e., electrostatic) forces attracting a particle to a surface are overcome by wind, mechanical, or other forces. This action is termed dispersal. Any activity or event that provides the energy necessary to elevate a microorganism into the atmosphere is capable of producing a bioaerosol.

Naturally occurring bioaerosols are generated by a variety of means. One example of natural aerosolization is injection into the atmosphere by a bubble traveling through a liquid and bursting on the surface (i.e., waves breaking on the shore, wastewater treatment plants). Aerosol particles generated by bursting bubbles have a higher concentration of microorganisms than a sample of the same volume taken from the liquid suspension that the bubble traveled through (3–8). Aerosol droplets containing *Serratia marcescens* generated by the bubbling action contain concentrations of bacteria greater than the source solution by a factor of 1,000 (4). Other studies indicated that viruses could be concentrated up to 50 times with respect to the source solution concentration (6,7). During the bubbling action the microbes collected in the resulting aerosol particle are concentrated in the surface microlayer and are drawn into the particle during formation (5).

Additionally, it was found that aerosols generated from five-day-old *Escherichia coli* cultures, showed an aerosol particle concentration 15 times greater than those generated from one-and-a-half-day-old cultures (5). However, the researchers did not observe this same effect with all of the bacteria used in their study. They felt that increased lipid content in the cell envelope could account for the organisms being present in the surface microlayer. They theorized that the lipid content of the cell envelope in *E. coli* might increase as the organism ages, which would account for the increase in particle concentration over time.

Bioaerosols can be dispersed by natural events including rain or snow impacting on the surface of pooled water (4) and wind blowing across a surface where microorganisms are present. For this last mechanism the force required to remove a particle from a surface increases as the particle size decreases (9). Bartlett (2) explains that almost all naturally occurring organisms are released within three to four meters from the ground with a majority being released less than 1 meter from the surface. It is this region that is most strongly affected by the daily cycle of heating and cooling. During the day this region can become very unstable so organisms will be rapidly mixed vertically through a substantial depth of the atmosphere. After dark, strong inversions develop and the movement of particles in the lower atmospheric layers is restricted. Material that finds its way above the inversion becomes entrained in a low-level jet of air and can be transported to substantial distances (2).

In a three-year study of the natural fluctuation of aerosolized microbes in four localities (agricultural districts, coastal areas, city parks, and city streets) several patterns were observed (10). The average counts of aerosolized bacteria in the four locations were: agricultural areas, 99 bacteria/m^3; coastal areas, 63 bacteria/m^3; city parks, 763 bacteria/m^3; and city streets, 850 bacteria/m^3. In all areas, the highest counts were observed in summer and autumn with the lowest counts observed in winter. Rain and low relative humidity (RH) corresponded to decreased bacterial counts.

Humans also generate natural bioaerosols as they go about their everyday activities. General movement (e.g., bed making, closing curtains, dressing, walking, talking, coughing, or laughing), can generate significant amounts of bioaerosols. A cough can generate 3,000 droplets, talking for five minutes may also generate 3,000 droplets, and sneezing can generate as many as 40,000 droplets (11,12). Table 3 lists a variety of sources for bioaerosols of health significance and the activities creating the aerosols.

Artificial bioaerosols can be generated using equipment specially designed to serve that purpose. Some examples and uses of aerosol generators include, atomizers used for the generation of mondisperse aerosol standards for the calibration of particle size analyzers (13) and atomizers used in the agricultural industry for spraying trees and plants for pest control (14). Other aerosol generators include the Collison nebulizer commonly used to generate aerosols used in respiratory studies (15), the vibrating orifice aerosol generator (16) and the spinning top aerosol generator (17), both used to generate monodisperse aerosols for calibration.

Artificial bioaerosol generation is sometimes an unfortunate by-product of industrial and agricultural activity. In one community, in which contaminated wastewater was used for spray irrigation, the number of enteric communicable diseases was two to four times higher than in communities that did not use wastewater for irrigation (18). It was determined that the bacterial levels in the air were directly related to the bacterial levels in the wastewater being sprayed. Wastewater treatment plants are also a significant source of bioaerosols. Aerators used to oxygenate the wastewater generate bubbles that burst creating aerosols (19) as described earlier.

Artificial bioaerosols are intentionally generated in the agricultural industry for use in pest and disease control. For example, *Erwinia* sp. are commonly sprayed onto fruit trees in an aerosol form to help control fire blight (20–23).

Generation of bioaerosols by either natural or artificial means exerts some degree of stress on the microbes. Strange and Cox (24) state that the injury to an aerosolized microbe results mainly from shear and impaction forces with the extent of the injury depending on the method of aerosol generation, the microbial species being generated, and the physiological state of the microbe. However, in a more recent study using *Pseudomonas syringae* and *Erwinia herbicola* and an aerosol generation method dissimilar to that discussed by Strange and Cox, it was found that the mechanism of aerosol generation itself did not seem to adversely affect the organisms being aerosolized (25). This information could lead one to conclude that the damage to an aerosolized microbe occurs after aerosolization; however, further study is needed in this area.

Table 3. Health Significance of Various Sources of Indoor Aerosols

Sources	Activities Creating Aerosols	Examples of Organisms of Health Significance	Potential Risk to Human Health
Human:			
Desquamated skin	Motion (clothed and unclothed), showering, bed making	Staphylococci	Low
Respiratory tract	Talking, coughing, sneezing, blowing nose	Staphylococci, streptococci, respiratory viruses, *Mycobacterium tuberculosis*, *Yersinia pestis*, *Haemophilus pertussis*	Moderate to high
Gastrointestinal tract	Toilet	*Escherichia coli*, enteroviruses	Low
Ventilation:			
Penetration from exterior	Air movement from cooling towers and other exterior aerosols	*Legionella* species, *B. anthracis*, and various others	Low to high
Interior systems	Operation	*Pseudomonas* species, staphylococci, fungi	Low
Humidifiers	Operation	*Pseudomonas* species, *Acinetobacter* species, *Serratia* species, *Actinomycetes* species	Moderate
Industrial:			
Wool, goat hair, cotton	Textile and furniture manufacture	*Bacillus anthracis*, *C. burnetii*, gram-negative organism	High
Slaughtered animals and birds	Meat packing and rendering plants	*Coxiella burnetii*, *Brucella* species, *Chlamydia psittaci*	High
Vegetables	Freezing, canning, drying	*Streptococcus* species	Low
Dairies	Milk processing	*Coxiella burnetii*, streptococci	Low to high

Source: Table Reproduced from Spenklove and Fannin (1983) with permission of the publisher (12).

Diffusion

After dispersion into the surrounding atmosphere, the aerosol diffuses by natural meteorological means. Diffusion is the spreading of aerosol particles caused by thermal energy possessed by all particles, expressed as Brownian motion, or by the action of meteorological conditions (e.g., wind mixing). Bioaerosols can be transported very long distances if the conditions are right. To enable long-distance transport of naturally occurring aerosols, it is necessary that the aerosol source be abundant, the microbes must be resistant or protected from the atmospheric environment, the aerosol particle size not too large (e.g., <10 μm), and the meteorological conditions be favorable (e.g., high wind speed and suitable stratification) (26).

Terrestrial-based bacteria have been found 160 km out to sea and marine-based bacteria found 54 km inland (27). There are also reports of various plant pathogens (28) and the causative agents of Foot and Mouth disease (FMD) (29–31) and New Castle disease (31) traveling great distances. Bacterial spores aerosolized during a sand storm near the Black Sea were found to have traveled 1,800 km to Sweden and Finland (32). Stem rust disease has been documented to travel from the Mississippi Valley to central and northern Canada (33).

One group of researchers (34) theorize that influenza viruses are transmitted over large distances from the far east because of seasonal atmospheric circulation patterns that may help explain the absence of influenza on the North American continent in summer. They feel this may also explain the ubiquity and persistence of the disease. Cold temperatures at high atmospheres, where the virus is theorized to travel, may increase the survival of the virus. An important factor in long-range transmission is the ability of the microbe to survive in the aerosolized state (26); this issue will be further explored in the fate section of this chapter.

The particle size of the generated aerosol is very important in the diffusion process. Not only does it determine how far a particle will travel but it influences the survival of the bioaerosol. As the aerosol particle increases in size, the survival rate of the microbes contained within the particle also increases (24). However, if a particle is too large it will not travel a great distance. A 100-μm particle has a gravitational settling velocity of 10 to 50 cm per second, whereas a particle less than 1 μm has a gravitational settling velocity of 0.003 cm per second (33). Small particulates of 1 to 2 μm can remain suspended in room air for more than 90 minutes (12). However, a large particle can remain airborne and travel a significant distance if the atmospheric turbulence has a velocity component with updrafts exceeding one meter per second (33). These updrafts will counteract gravitational settling of larger particles.

Cool humid weather, and overcast skies seem to favor the transport of bioaerosols. This effect may be due to the protection afforded microbes from the damaging conditions of desiccation, heat, and UV radiation (31).

Deposition

Deposition (the settling or impaction of an aerosol particle onto a surface) and terminal velocity (the rate at which a particle settles influenced by gravitational force) are both governed in part by the mass and size of the particle (9). Particles 1–5 μm generally follow the streamlines of the surrounding air, whereas larger particles have the momentum to deviate from the streamlines and impact surrounding surfaces resulting in deposition of the particle (9). When particles enter the region near a surface, van der Waals forces (weak attractions among electrically neutral molecules or parts of molecules) and electrostatic forces (electrical charge forces of attraction and repulsion) can influence deposition (9).

Deposition of bioaerosols is also influenced by meteorological conditions. For example, they will be restricted from deposition if vertical mixing and winds are strong enough to keep the particles aloft. Particles are washed from the atmosphere by precipitation, both rain and snow (10). It has been shown that the rate at which particles are removed by rain increases as the particle size increases (2). It was shown with FMD that when winds are blowing from infected areas, outbreaks tend to occur where rain has fallen (31). It is theorized that this may be a result of the FMD virus-bearing particles being washed out of the atmosphere.

Deposition in the respiratory tract depends on the size of the particle (27,35). 0.5–15 μm is the optimum size for deposition in the lungs (12,24,31). Respirable particles are considered to be in the size range of 1 to 5 μm (the size of most bacterial and fungal spores). These particles will penetrate as far as the distal alveoli and respiratory bronchioles (1). Therefore, it is easy to see that the infectivity of a given microorganism is in some ways dependent on the aerosol particle size. Particle size can also influence the dose of an organism required to cause infection. As the particle size increases, so does the dose required to cause disease (36). This may be due to the fact that the size of the particle determines where in the respiratory tract the particle is deposited (27,36).

FATE

Before 1965, there was little understanding of the survival of microbes in aerosols (33). Since then important research has been completed to explore the effects various factors have on bioaerosols. According to Theunissen, and coworkers (37) the fate of aerosolized microorganisms depends on five factors: (*1*) relative humidity (RH), (*2*) temperature, (*3*) oxygen concentration, (*4*) presence of UV radiation, and (*5*) constituents of the aerosol itself. In addition to these five factors, the type of microorganism being aerosolized and how much time the microbe spends in the aerosol are important. Interactions among these factors complicate interpretation making it difficult to ascertain the effect each individual factor has on the aerosol stability of the microbe. However, it is generally agreed that the factor that most influences the survival or viability of the bioaerosol is the RH level.

Relative Humidity

RH is defined as the ratio of the amount of water vapor actually present in the air to the greatest amount possible at the same temperature. In a study performed by Walter and coworkers (25), it was found that the RH level was more detrimental to bacterial survival than temperature or the physical stress of aerosolization.

Changes in water content represent the most fundamental potential stress experienced by airborne microbes. In general, aerosolized bacteria have an increased survival/viability at low and high RH levels than at mid-range humidities (24). The actual effect of desiccation on an aerosolized microbe is difficult to determine because of concurrent inactivation of the microbe by oxygen and other factors (33). In order to negate the effect of oxygen on aerosolized microbes, researchers conducted studies in enclosed chambers in which oxygen was replaced by an inert gas such as nitrogen. They were then able to observe the effect of different levels of RH on bioaerosols.

In the early to mid 1960s it was a common practice to evaluate aerosol survival at only three RH values corresponding to high (80%), medium (50%), and low (30%) RH. During a study using *E. coli*, it was observed that critical narrow RH bands exist where loss of viability of *E. coli* was much greater than in adjacent regions (24,33,38). Figure 1 displays the data from that study (33). The aerosols were generated in a nitrogen atmosphere and RH was increased in 2% increments. This phenomenon of narrow bands of instability was also seen with freeze-dried bacteria and viruses (24).

Many theories exist regarding the effect desiccation, caused by low RH, has on aerosolized microbes. In 1959, Webb (39) proposed that cell death, after aerosolization, was due to water molecule movement in and out of a cell in

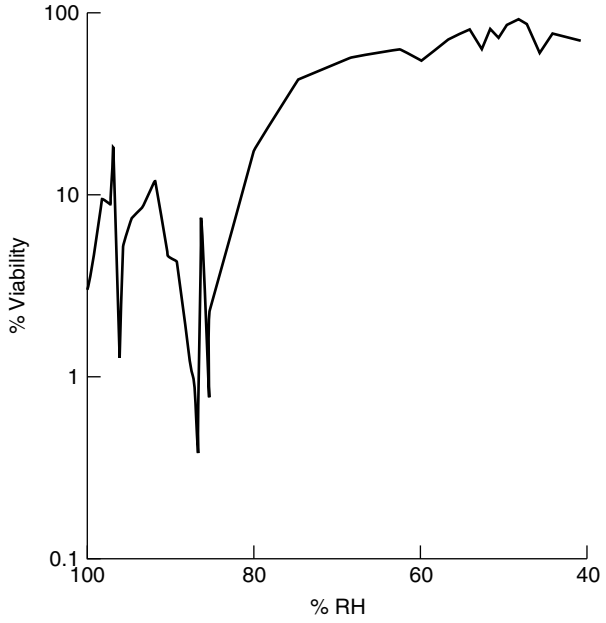

Figure 1. Aerosol survival of *E. coli* B sprayed suspension in distilled water and stored in nitrogen atmospheres. Reprinted from Cox (1989) with the permission of the publisher (33).

an equilibrium system resulting in the collapse of natural cellular protein structures. He also reported at that time that sensitivity of a bacterium to RH differed markedly from organism to organism.

In the 1960s, more specific knowledge regarding the effect of RH was attained. Webb and coworkers (40,41) suggested that the death of airborne bacteria was the direct result of the loss of water, bound to nucleoproteins. Anderson and Cox (27,42) published results supporting that theory. They went on to propose that the structures of macromolecules such as proteins, structural elements, and nucleic acids are affected by water loss. They theorized that the physical structure of proteins and deoxyribionucleic acid (DNA) and ribonucleic acid (RNA) depend on water content. However, Cox (27) stated that desiccation does not markedly denature nucleic acid but instead inactivates surface proteins. At low RH levels water loss might increase the concentration of toxic compounds inside the cell or crystallize them, potentially damaging the metabolic processes of the airborne microbe (27,37,42).

The reactions of bacteria and viruses to the relative humidity of the environment are different. This is most likely due to the difference in structural makeup of the bacterial cell wall and whether or not the viral envelope contains lipids. Envelopes on viruses, which are composed of a phospholipid bilayer, with inserted protein molecules, are analogous to the outer phospholipid membrane of gram-negative bacteria and mycoplasmas (33). Generally, these phospholipid membranes have an inherent thermodynamic instability that can lead to structural changes with little agitation (33). For bacteria, sensitivity to stress appears to be related to gram stain reaction. Gram-positive organisms are more stable in aerosols than gram-negative organisms. Gram-positive organisms are impermeable to ions and small molecules unlike gram-negative organisms, which may account for the differences in sensitivities between the two groups (41). Interestingly, in one study it was observed that culturable gram-positive cocci outnumbered gram-negative organisms of any kind in samples taken from urban air (43).

Gram-negative bacteria and viruses with envelopes containing lipids demonstrate phase changes in the outer phospholipid bilayer membrane when exposed to various RH levels (33). In viruses, those with envelopes containing lipids survive better at a low RH levels and those viruses without lipids in their envelopes survive better at a high RH(44–46) (Fig. 2).

Israeli and coworkers (48) studied freeze-dried microorganisms and concluded that for many bacteria, the phospholipid bilayer biomembrane undergoes conformational changes from the liquid-crystalline phase to gel phase as a result of water loss. The physical change of cell protein conformation leads to a loss of viability of the airborne microbe. Theunissen and coworkers (37) also state that the transition of lipids from a liquid-crystalline phase to a gel phase is a possible inactivation mechanism. They believe this phase change would occur when the airborne microbe is exposed to an environment of low temperature and low RH.

Cox (33) found that aerosolized gram-negative and lipid-containing viruses demonstrate phase changes in the outer phospholipid bilayer leading to a cross-linking reaction between protein moieties, thus enhancing inactivation. This occurs mainly at mid to high RH levels (27). Conversely, the reactions of the surface proteins of lipid free enveloped viruses occur most rapidly at low RH levels. In bacteria, Cox (27,33) proposed that the lipoproteins found in gram-negative organisms denature most readily at mid to high RH levels and other proteins denature most readily at low RH levels.

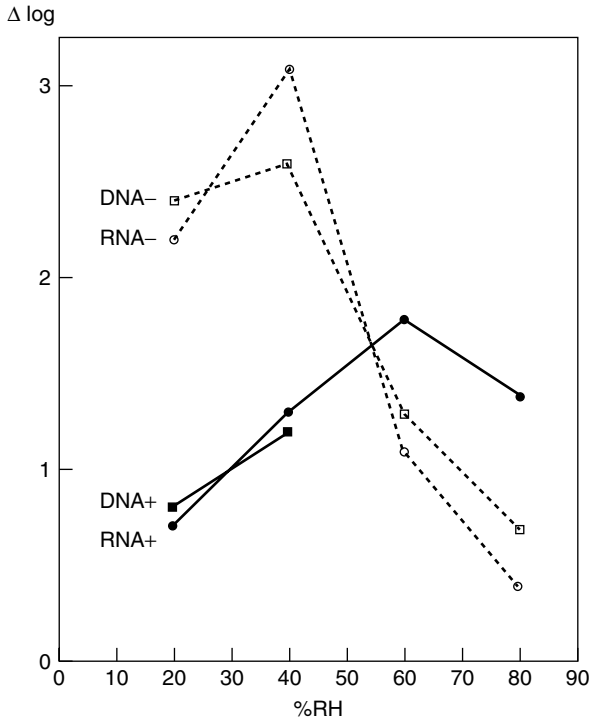

Figure 2. Inactivation of aerosolized viruses after one hour of storage in aerosol at a given RH. DNA– are lipid free-enveloped DNA viruses, DNA+ are lipid containing-enveloped DNA viruses, RNA– are lipid free-enveloped RNA viruses, and RNA+ are lipid containing-enveloped RNA viruses. Δ log: the difference between log of titre calculated and log of titre one hour after atomization. Reprinted from De Jong, et al. (1973) with the permission of the publisher (47).

Another inactivation mechanism is Maillard reactions (37). Maillard reactions are reactions between lipids and proteins or proteins and proteins and result in the removal of water. These reactions occur at low to medium (0% to 50%) RH levels and may possibly increase with increasing temperature.

Webb (39) reported that the death of a bacterial population in an aerosol proceeds in two stages. The first stage is a rapid initial kill within the first second after aerosolization and the second stage is a subsequent slower death. The research of many others (37,45,49–51) supports Webb's initial finding, in both aerosolized bacteria and viruses. Akers (45) expanded on Webb's theory by proposing that the quick initial death may be due to the osmotic shock resulting from the humidity change.

From the earlier discussion, it is apparent that RH directly plays an integral role in the survival and viability of airborne microbes. RH also affects the response of the

aerosol to other factors such as temperature and UV radiation (42). In a study with *Mycoplasma pneumoniae*, a high RH (95%) was observed to increase recovery by approximately ten percent after exposure to UV radiation (52).

Oxygen and Pollutants

In 1914, it was observed that oxygen could cause a loss of viability in freeze-dried bacteria (24). Enhanced bacterial survival was observed when oxygen was replaced with nitrogen (42) or helium (24), resulting in inert atmospheres.

Oxygen inactivation is only seen in some species of vegetative bacteria and algae (27). Oxygen susceptibility usually increases with the degree of desiccation of the aerosolized bacteria, increasing oxygen concentration, and the length of exposure time (27,48). The toxic effect of oxygen was observed to occur below 70% RH (53). Atmospheric oxygen is not toxic to viruses in the aerosol state (46). Figure 3 illustrates the results from a study conducted in which *Serratia marcescens* was aerosolized into different combinations of nitrogen and oxygen at 30% RH and 25 °C (54). As the amount of oxygen in the atmosphere decreased, the viability of *S. marcescens* increased. In this same study little or no toxic action of oxygen was detected at RH levels above 65%.

Lighthart and coworkers (55) observed that the sulfur dioxide (SO_2) found at urban air concentrations affects the survival/viabilility of *S. marcescens*. Exposure of *S. marcescens* to urban levels of SO_2 resulted in a marked decrease in viable bacteria. They also found that cells in recently generated aerosols (i.e., aerosols aged 0 to 1 hours) generally were more sensitive to SO_2, than cells in older aerosols (aerosols aged 1 to 5 hours). SO_2 at 3.6 ppm, in combination with simulated solar radiation, significantly reduced the viability of the Venezuelan Equine Encephalitis (VEE) virus in aerosol (56). It was observed in one study that inactivation due to the combination of RH and SO_2 was greater than the sum of the separate effects (45).

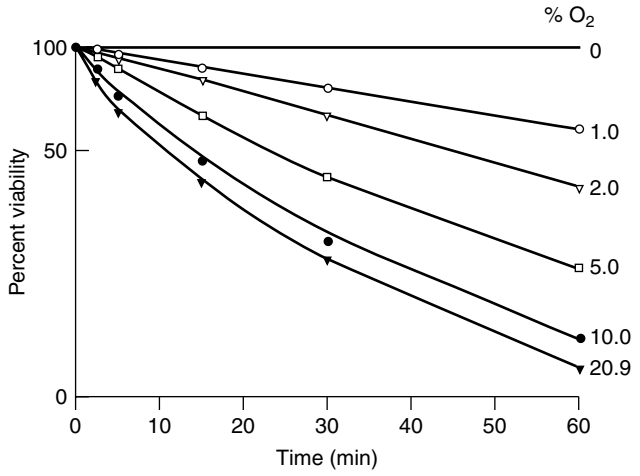

Figure 3. Aerosol survival (log scale) of *S. marcescens* 8UK sprayed different nitrogen + oxygen mixtures at 30% relative humidity and 25.0 °C. Points are experimental; lines are calculated. Reprinted from Cox, et al. (1974) with the permission of the publisher (54).

Carbon monoxide (CO) has been observed to provide protection to aerosolized *S. marcescens* at 88% RH and higher but at other RH levels, mainly below 75%, the death rate was increased (57). The protection by CO at high RH levels may be due to the inhibition of a small energy-requiring death mechanism or the rerouting of conserved energy to repair mechanisms, although the article did not describe or name the small energy-requiring death mechanism (57).

In a study with *Flavobacterium*, a dependence of nitrous dioxide (NO_2) toxicity on the RH level was observed (58). At lower RH levels, more NO_2 was needed to produce equivalent losses that were observed at higher RH levels. At 85% RH it is possible for atmospheric water to convert NO_2 to nitric or nitrous acids, both of which are known to denature proteins (45).

Open-Air Factor (OAF)

The term open-air factor (OAF) is derived from the fact that significant increases in biological inactivation were observed when aerosols were exposed to HEPA-filtered outdoor air compared with clean, inert laboratory-supplied air (9).

For many decades the causative agents of the OAF and the action of these agents have been speculated upon. The death action of the OAF is still not fully understood. Currently, the OAF is thought to be the result of olefins reacting with the ozone (24,27,33). Olefins are unsaturated open-chain hydrocarbons that come from a variety of sources. The question of why the OAF is observed on bioaerosols outside and not inside is addressed by Cox (33). However, the components of the OAF may require time to condense or buildup on the surface of the particle.

Druett (59) observed, through experimentation, that germicidal action arose from the reaction of the ozone with the olefin fraction of unburned fuel. Testing the OAF effect on aerosolized microbes is very difficult because the influence is rapidly lost once outside air is enclosed in a test chamber (60). This loss is presumably due to the adsorption of the ozone + olefin gaseous complex to the walls of the holding container (60). Ozone + olefin products have been reported (27,60) to have short half-lives that may explain loss of reactivity as outside air travels through pipes and particulate filters.

The concentration of OAF, at a given location depends upon wind direction, factors influencing vertical mixing of air masses, time of day, etc. (27). OAF toxicity is at its greatest in air masses that have crossed urban and industrial areas (27,59). In an OAF study of air passing over industrial areas, urban areas, and the sea it was found that the OAF was absent in air passing over the sea and the highest microbial death rates were observed when air passed over cities and industrial areas (61). Because of the many variables involved, the toxicity of OAF can vary from one day or minute to the next (62). The effect of the OAF is increased by high RH levels (33).

The toxic effect of OAF on some bacteria and viruses results in the inactivation of coat constituents and nucleic acids (27). The effectiveness of the OAF is due less to high

toxicity than to the readiness of the components of the OAF to condense on aerosol particles (27).

In some microbes, nucleic acids and coat proteins are seriously affected by the OAF (33). However, microbes that tend to be resistant to UV radiation also appear to be resistant to the OAF (33). Cox (33) believes this may be related to the lipid content, whereas, organisms without surface lipids have a greater resistance to OAF and UV radiation than those with surface lipids, namely, gram-negative bacteria and viruses with envelopes containing lipids. The causative virus of FMD, a lipid free enveloped virus, has been given as an example of a microbe that can travel great distances (at least 100 miles) while retaining its viability and infectivity. This is possibly because of its OAF resistance (33).

Temperature

The sole effect of temperature on aerosolized microbes is difficult to ascertain because RH levels are related to environmental temperature. However, several studies with airborne viruses, in which the RH level was held constant, show the rate of aerosol decay increasing with increased temperature (10,24,42,45). In general, as the temperature decreases the survival/viability of aerosolized microbes increase.

Repair

According to Cox (33), the ability of microbes to survive the airborne state depends on their ability to repair damage. This ability depends on the chemical environment as well as the genetic makeup of the microbe (27). Repair of damage caused by solar radiation may be activated by exposure to wavelengths of light longer than the damaging wavelength (photo reactivation repair) or spontaneously in the dark (dark repair) (63). This may account for the rise in the airborne bacterial concentration seen in the late afternoon or after sunset.

UV Radiation

Electromagnetic radiation can reduce the survival of microbial aerosols (42). Hypothetically, airborne inactivation of bacteria after UV light irradiation is due to ruptured hydrogen bonds among water molecules or between water molecules and cellular nucleoproteins or thymine dimers (42,45). UV radiation is also known to increase the mutation rate of bacteria in aerosols (42), possibly leading to inactivation.

Many factors determine whether or not an aerosolized microbe is sensitive to UV radiation. UV radiation effects are dependent on bacterial or viral species, physiological state, light intensity and duration, and wavelength (64). For example, many viruses (especially enveloped viruses) are sensitive to visible light of short wavelength (31), but a FMD virus is less sensitive (more resistant) to sunlight (30).

Larger particles survive solar radiation better than smaller particles (42). This may be because of the protective action provided to organisms located in the interior of the particle. Bacterial aerosols generated from the dry state are less sensitive to radiation than those generated from the wet state (42). No explanation for this effect has been determined. However, it has been shown that dry disseminated aerosols undergo a spontaneous reactivation when held in the dark after UV radiation. This may have something to do with the increased survival of bioaerosols generated from the dry state.

In a study in which airborne bacteria were collected continuously (64), microbes collected at night were more sensitive to solar radiation than those collected on cloudy days. Bacteria collected during sunny days had the lowest sensitivity to solar radiation. As the solar radiation intensity increased, the time to reach a lethal dose decreased and cells died more rapidly. The organisms that were the most resistant to solar radiation were found in the upper atmosphere where the least amount of atmospheric protection exists (i.e., ozone). However, these UV-resistant organisms are rare.

Bacteria with pigmentation survive UV radiation exposure better than those without pigmentation (43,65). When taking culture samples of indigenous background microbes, pigmented bacteria are more prevalent than nonpigmented bacteria (43).

Berendt and coworkers (56,66), in the course of their research, saw a reaction between sulfur dioxide and UV radiation. They found that under certain circumstances, sulfur dioxide (0.4 ppm concentration) reduced the virucidal properties of UV radiation. They theorized that this effect was due to the radiation and the sulfur dioxide acting in an antagonistic manner, thereby reducing a possible enhanced combined effect. Some of the wavelengths of solar radiation may be absorbed by sulfur dioxide or sulfur dioxide may react with UV radiation, leading to the production of sulfuric acid that has little influence on viral inactivation (56,66).

Protection

The survival of aerosolized microbes can be related to the method of propagation (11), the spray fluid (24,40,42,45,46,67,68), culture age (24,44,69–71), growth media (24), and suspending media (40,45,71). For instance, at high RH levels, it was observed that aerosolized *E. coli* survival was better if the organism was grown on agar rather than in liquid media (42). The survival response of an aerosolized virus to RH levels is dependent greatly on the composition of the spray fluid (45,46,71).

The stage of growth of an organism also affects the aerosol stability. *Escherichia coli* and *S. marcescens* both exhibit increased decay rates when the organisms are harvested in the transition from lag to log phase, during growth (42). Organisms harvested during the stationary phase of growth survive the best (38,69,72). Aerosols generated from *E. coli* harvested during the stationary phase displayed a survival rate of 40%–50% in contrast to cells generated during the exponential or declining growth stages in which survival rates of 6% and 1% were respectively observed (70). Aged organisms have a greater resistance to osmotic shock than log phase organisms (42,44). This may be because of the "mature" cells having low metabolic activity (70). Organisms that

grow slowly are more resilient than those that grow quickly (24,70).

Adding a variety of compounds to either the growth media or to the slurry before aerosolization, can affect the survival of an aerosolized organisms. At low RH levels, the addition of salts to the slurry has been shown to protect enteric viruses (73). Polyhydroxy and amino compounds (41,45) as well as proteins (45), and sugars (24,27,33,38,42) can enhance aerosol survival of bacteria and viruses. These compounds replace water in protein structure during periods of desiccation. Raffinose and trehalose stabilize desiccated phospholipid membranes and inhibit membrane fusion (27,33). Cox and coworkers (24,42) lists amino acids, antibiotics, aromatic compounds, dyes, metal-chelating agents, polyhydric alcohols, salts, spent growth media, and sugars as protective additives. They state that relatively simple compounds such as inositol and sugars (di- and trisaccharides) offer the best microbial protection over the widest range of conditions. Israeli and coworkers (74) observed that trehalose, added to drying media, protected biomembranes and proteins from inactivation during the freeze-drying process. In their study with *E. coli* it was found that trehalose protected bacteria from known environmental hazards such as visible light, air, and RH levels. They stated that trehalose imparts protection by stabilizing the liquid-crystalline phase of the outer bacterial membrane, probably replacing the water molecules in the phospholipids. This prevents these molecules from denaturing, thus inactivating the bacteria. In a similar study conducted by Larson (75), using *Francisella tularensis*, it was observed that the effect of RH levels on biological decay was essentially eliminated by the additives raffinose and dipyridyl. Inositol has been shown to provide a broad range of protection from RH effects, specifically in aerosols of *E. coli* and *S. marcescens* (76). Inositol in the absence of water can form strong reversible hydrogen bonds with cellular proteins, thereby stabilizing the protein structure (41). Catalase added to collection media caused a significant increase (>63%) in the colony forming abilities of airborne bacteria (77). Catalase acts by removing or degrading hydrogen peroxide that is lethal to cells. Cellular catalase activity is reduced by stress that results in the accumulation of hydrogen peroxide.

Webb found that some bacteria that possess antibiotic resistance have a protein alteration that affords them increased aerosol stability (41). He further discovered that challenging an aerosol-sensitive species to certain antibiotics resulted in adaptations in which the organism became considerably more stable during air storage. Antibiotics that produced this effect were those that interfered with protein synthesis.

CONCLUSION

Table 4 is a summary of bioaerosol stressors and most probable target molecules. It is generally agreed upon that the major stressor of any bioaerosol is desiccation but it is difficult to isolate the effect of this stressor because of the complicating factors that are invariably present.

Table 4. Bioaerosol Stressors and Most Probable Target Molecules

Stress	Most Probable Target Molecules
RH and temperature	Outer membrane phospholipids, proteins
Oxygen	Phospholipids, proteins
Ozone	Phospholipids, proteins
Open-Air Factor (O_3 + olefins)	Phospholipids, proteins, nucleic acids
γ-rays, X rays, UV radiation	Phospholipids, proteins, nucleic acids

Source: Table Reproduced from Cox (1989) with the permission of the publisher (33).

Although many of the processes involved with bioaerosol inactivation are unknown, some generalizations regarding these microbes can be made (37):

1. Gram-negative bacteria survive best in conditions of low temperature and high RH levels.
2. Gram-positive bacteria survive better and longer than gram-negative bacteria in aerosols.
3. The sensitivity of gram-negative bacteria to oxygen depends on species.
4. Viruses with lipids in the envelope are more stable than viruses without lipids in the envelope. Viruses with lipid-containing envelopes survive better at RH levels less than 50%, whereas viruses without lipid-containing envelopes survive better at RH levels greater than 50%.
5. Normal survival characteristics can be altered by the addition of certain compounds.

It is obvious that the interactions affecting the transport and fate of bioaerosol particles have many variables resulting in very complex relationships. Each of these variables affect the survival and viability of the aerosolized microbe making it difficult to determine exactly what effect each variable actually has on the bioaerosol particle. Even the mechanisms and affects of factors that have been studied extensively are, in many instances, not fully understood. Further research and study is necessary to add to the body of knowledge that currently exists. Advances in technology, which helps us to design equipment and execute tests, make attaining this information possible.

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BIOAEROSOLS, VIRUS.

See VIRAL AEROSOLS

BIOAEROSOLS: WASTEWATER TREATMENT PLANTS.

See WASTEWATER AND BIOSOLIDS AS SOURCES OF AIRBORNE MICROORGANISMS

BIOAUGMENTATION

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Bioaugmentation is the addition of exogenous organisms to an environment to complement or replace an indigenous population. In the context of remediating polluted environments, bioaugmentation typically involves the addition of bacteria that have been selected for their ability to transform a target contaminant. In the last several years, bioaugmentation for pollution remediation has become increasingly popular. Consequently, the relative conceptual simplicity of the technology has spawned an industry involved in the sale of mixed or pure cultures of bacteria with the promised ability to degrade nearly any contaminant in virtually any environment. In actuality, the rational selection of bioaugmentation as a remedial alternative for a given site requires a detailed evaluation of the pollution profile (i.e., chemicals and their concentrations) and the hydrogeology of the site, as well as nontechnical driving forces such as regulatory demands, property owner interests, and ultimately the cost of cleanup in terms of both money and time.

The remediation of sites contaminated with chlorinated volatile organic compounds (CVOCs) has been a focus area for the development of bioaugmentation technologies. CVOCs are a family of chemicals that have been used extensively as industrial solvents and cleaning agents, and include perchloroethylene (PCE), trichloroethylene (TCE), dichloroethylene (DCE), vinyl chloride (VC), trichloroethane (TCA) dichloroethane (DCA), carbon tetrachloride (CT), and chloroform (CF). The widespread use of CVOCs, improper disposal practices, and their chemical properties and stability have led to them becoming common groundwater contaminants throughout the United States (1).

REMEDIATION HIERARCHY

Within the group of in situ bioremediation technologies there has developed a hierarchy of alternative remedies based on ease of cleanup and cost. The first level of the hierarchy is intrinsic biodegradation whereby indigenous microflora destroy the contaminant of concern before it creates a significant risk to downgradient receptors. This has become a natural first choice of remediation alternatives where applicable because it requires no intervention; just monitoring of contaminant concentrations and modeling of the groundwater flow and natural degradation rates.

The second choice in the hierarchy, biostimulation involves the stimulation of indigenous microbial populations to allow them to destroy the target chemicals. In this case, the observation is made that a natural population exists within the contaminated zone; however, specific nutrients, growth substrates, inducers, and/or oxygen are insufficient for microbial activity. Thus, through the introduction of the correct co-substrate, the native degradative population can be stimulated to grow and destroy the target contaminant.

In cases in which natural attenuation or biostimulation do not work because of insufficient or unacclimated indigenous microflora, bioaugmentation of the subsurface is an option. Selected strains of bacteria with the desired catalytic capabilities can be injected directly into the contaminated zones along with any required nutrients to effect the biodegradation of the target chemicals. Two distinct approaches have developed in the area of bioaugmentation for remediation of CVOCs. In the first approach (Type 1), degradative organisms are added to complement the native microbial population. The added microorganisms can be selected for long-term survival and the ability to occupy a selective niche within the contaminated environment, and stimulants or selective co-substrates, can be added to aid survival. Thus, the goal of the Type 1 approach is to achieve prolonged survival and growth of the added organisms and the concomitant prolonged degradation of the target pollutants.

In the second approach (Type 2), large numbers of degradative bacteria are added to a contaminated

environment as catalysts that will degrade a significant amount of the target contaminant before becoming inactive or perishing. Attempts can be made to increase the expression of the degradative enzymes or to maximize catalytic efficiency or stability, but the long-term survival, growth, and establishment of the biocatalyst are not the primary goals of the treatment approach. Additional microbial catalysts can be added as needed to further the degradation process. Although this review focuses on bioaugmentation for the remediation of chlorinated solvents, in particular TCE, examples of field-scale applications of bioaugmentation for other compounds are discussed.

ORGANISMS FOR BIOAUGMENTATION

Several classes of microorganisms can degrade TCE under the proper conditions (2). TCE can be biodegraded by aerobic bacteria, which oxidize aromatic hydrocarbons (3–6), methane (methanotrophs; 7–10), ammonia (11), and propane (12). All of these bacteria initiate the degradation of growth substrates and CVOCs by incorporating atmospheric oxygen through the action of enzymes known as oxygenases, and in each case the degradation appears to be co-metabolic; that is, the degradative organism does not gain sufficient metabolic energy from the oxidation of the CVOC to support growth of the organisms, but rather, the CVOCs are fortuitously oxidized by enzymes that have evolved to degrade other substrates. Furthermore, the oxygenase enzyme systems typically are only induced by growth in the presence of the specific hydrocarbon (e.g., toluene-4-monooxygenase is induced by toluene).

Anaerobic microorganisms can reductively dechlorinate and ultimately mineralize many of the CVOCs. This can occur co-metabolically or by dehalorespiration, a process that involves the chlorinated compound acting as the electron acceptor (13). Dehalorespiration in particular can effect dehalogenation at relatively high rates (14). Some of the lower chlorinated CVOCs can also be used as sole sources of carbon and their degradation does not require a co-substrate. There is only one strain that has been identified to date, that can degrade PCE all the way to ethene (15). In general, the stepwise pathway of degradation is mediated by a number of different bacteria. A number of these consortia have been enriched from environments in which reductive dechlorination is occurring (16–19).

IMPROVING ORGANISMS FOR BIOAUGMENTATION

The successful application of bioaugmentation for remediation of contaminated aquifers is limited by several physiological characteristics of CVOC-degrading organisms. For example, many bacteria in nature produce a glycocalyx layer and/or a variety of surface structures or polymers that aid in their adhesion to surfaces (20). As a result, bacteria tend to be very adhesive and they resist migration through tight matrices such as those existing in aquifer soils. Also, the initial oxidation of CVOCs is an energy and reductant requiring process, and thus, cofactors such as NADH are required for degradation (2). Furthermore, because the oxidation of CVOCs does not allow regeneration of sufficient energy to continue the oxidation process, supplemental energy substrates are needed to prolong degradation. Finally, the production of oxygenase enzymes known to transform CVOCs is typically tightly regulated at the genetic level. That is, degradative genes must be induced, and the inducers are typically the natural substrate for the enzyme. For example, CVOC-degrading toluene monooxygenase genes are induced by toluene. Thus, in addition to supplying a substrate for energy production, an inducing substrate must also be available.

To circumvent these limiting characteristics of natural CVOC-degrading microorganisms, the organisms may be modified to improve their utility for bioaugmentation. These modifications can often be accomplished by chemical mutagenesis and/or natural selection, or they can be made by using recombinant DNA techniques.

CONSTITUTIVE DEGRADATIVE ACTIVITY

A significant breakthrough in the development of organisms for remediating CVOC-contaminated aquifers has been the selection of organisms that constitutively produce degradative enzymes. In these organisms degradation of the target pollutant is uncoupled from growth of the organisms, thereby allowing the organisms to grow on alternative substrates while degrading the pollutant. Problems of competitive substrate inhibition or the requirement for using toxic or explosive co-substrates can potentially be eliminated.

In the first example of creating organisms that degrade CVOCs constitutively, Shields and Reagin (1992) (21) used transposon mutagenesis to generate insertional toluene-*ortho*monooxygenase (TOM) mutants of *Burkholderia cepacia* G4, and then selected spontaneous revertants of the mutants that expressed TOM constitutively. TOM is an enzyme that catalyzes the degradation of certain CVOCs. One resulting variant of this selection process, *B. cepacia* PR1, degraded TCE in the absence of inducing substrates such as phenol and toluene, and was extremely stable over more than 100 generations of growth. In further studies by the same group (22) a similar variant, *B. cepacia* $PR1_{301}$, was developed by traditional chemical mutagenesis. The latter strain is more applicable for bioaugmentation because it is not considered genetically engineered, and it has been evaluated in a series of laboratory and field demonstrations of bioaugmentation (see below).

In similar studies, a constitutive soluble methane monooxygenase (sMMO) variant of *Methylosinus trichosporium* OB3b was developed by chemical mutagenesis and revertant selection (23). The resultant strain produced sMMO, a broad-substrate range CVOC-degrading enzyme, even in the presence of relatively high copper concentrations, and during growth on methanol. Constitutive variants of CVOC-degrading phenol hydroxylase-producing organisms (24), toluene oxidizers (25) and isopropylbenzyne-degrading organisms (26) have been developed by using various recombinant DNA techniques.

Like *B. cepacia* $PR1_{301}$, any of these mutants could potentially be added to contaminated aquifers and fed noninducing, noncompetitive, growth substrates to prolong their degradative activity in situ.

In related work, McClay and coworkers (1995) (27) showed that TCE could act as an inducer of TCE degradation activity in the toluene-oxidizing bacteria *P. mendocina* KR1 and strain ENVPC5. Likewise, if *P. putida* F1, *P. pickettii* PKO1, or *B. cepacia* G4 were grown in the presence of TCE, the toluene oxidation genes were induced (28), and a wild-type toluene dioxygenase-producing *P. putida* degraded TCE in the absence of inducing substrates (29). Either of these strains could potentially be used for bioaugmentation where noninducing substrates are added to support degradative activity and TCE acts as the inducing substrate. Again, competitive inhibition between the growth substrate and the target CVOCs should be limited, but the added substrate could increase competition from nondegrading organisms.

ENHANCED TRANSPORT

Adhesion-deficient variants of CVOC-degrading bacteria should allow improved distribution of the biocatalysts throughout contaminated aquifers, and minimize problems of wellhead plugging during injection of organisms. Previous studies have demonstrated the feasibility of generating adhesion-deficient variants of a number of different bacterial strains by transposon mutagenesis, chemical mutagenesis, or by selection of naturally occurring adhesion-deficient variants from a population (30–35). In one study, greater than 91% of a highly adhesive strain of *Pseudomonas fluorescens* (Pf0–1) was consistently retained by a 3 cm tall, 12 g sand column (36). With this degree of attenuation, in situ transport would be limited to approximately 0.2 m in an unconsolidated sand aquifer. However, an adhesion-deficient variant (Pf0–5) with 40% retention in the same sand column would be capable of traveling approximately 1 m in a sandy aquifer.

By using this simple adhesion assay, transposon-generated adhesion-deficient mutants and natural variants of *P. cepacia* G4, *M. trichosporium* OB3b, *P. cepacia* $PR1_{301c}$, *P. mendocina* KR1, and *P. cepacia* ENV BF1 have been selected (37). Some of the variants adhere to the sand columns at less than 10%, while the wild-type strains adhere at greater than 90%. Model aquifer studies have demonstrated that several of these strains migrate through a 25 cm sand column at approximately the same rate as a conservative tracer. Thus, these metabolically proficient, adhesion-deficient strains are more suitable for in situ applications than the wild-type organisms because they are less likely to plug the injection well during application and they should travel farther into the subsurface, thereby increasing the effective zone of remediation.

In a related approach, Rothmel and coworkers (1998) (38) used surfactant foams to distribute strain ENV435 throughout a model aquifer. Because of their viscosity and scouring properties, surfactant microbubble foams (a.k.a. colloidal gas aphrons) are useful for removing dense nonaqueous liquids (DNAPLs) from aquifers. The foams flow effectively around heterogeneities in the aquifers, and remove more DNAPL than either water or liquid surfactant. By incorporating strain ENV435 into the foam, the strain was distributed evenly throughout a model aquifer, and it resulted in significantly greater TCE removal than treatment with foam only. Incorporating growth substrates, important nutrients, inducing substrates, or oxygen-generating compounds into the foam, even greater degradation might be expected.

Liquid surfactants and low ionic strength solutions also have been tested for enhancing transport of bacteria for bioaugmentation. Early work using glass beads as a model porous media indicated that a significant increase (0.16 to 60 m) in bacterial transport would result from some of these chemical treatments (39). However, subsequent work using the same bacteria and chemical additions, but with soils as the porous media, resulted in only moderate increases in transport (40). These moderate increases in transport would be sufficient to create small bioactive zones (~1 m), but would not be sufficient for dispersion of the bacteria throughout a plume.

Other efforts to enhance bacterial transport have shown that under conditions of controlled starvation bacterial cells become significantly smaller than active vegetative cells. These "ultramicro" bacteria produce less of a biofilm than vegetative cells and are able to penetrate deeper into consolidated materials than full-size cells (41,42). Once in place, the cells can be revived by adding the appropriate nutrients and/or carbon source. A similar approach could be applied with spore-forming cells (43).

Others have evaluated the use of encapsulation to improve survival and transport of bacteria added to the environment. In a study by Crawford and coworkers, the encapsulation of a PCP-degrading *Flavobacterium* improved its survival in groundwater (44). In this study, encapsulation procedures were modified from previously used techniques in order to create much smaller capsules that would both improve oxygen diffusion to the cells and improve the transport of the encapsulated bacteria in the subsurface.

ENERGY ENRICHMENT

All known aerobic CVOC-degrading microbes require a cosubstrate for sustained enzyme activity and metabolic survival. However, adding a cosubstrate to an aquifer can stimulate the growth of other non-TCE-degrading organisms and create problems with biofouling and oxygen depletion. Furthermore, some of the desired cosubstrates for CVOC degradation may be explosive (e.g., methane, propane, or butane) or toxic (e.g., phenol, toluene, or isopropylbenzene) and their use may be regulated or require the use of expensive equipment or excessive monitoring.

An alternative approach for enhancing and maintaining biological activity in situ is to utilize biocatalysts that are enriched in energy reserves. The production of energy storage polymers, most commonly poly-beta-hydroxybutyric acid (PHB), by bacteria is a long-studied phenomenon (45). PHB is produced naturally by many bacteria, typically under conditions of a nutrient limitation

and the presence of excess carbon, and it may account for up to 80% of the bacterial cell dry weight. Utilization of PHB as a reducing power substrate by methanotrophic bacteria degrading TCE has been demonstrated (46,47). Likewise, cells containing PHB may survive longer in groundwater than cells without PHB (48). Thus, selecting a degradative organism that produces storage polymers may be advantageous over selecting an organism that does not produce such polymers. In work performed by Steffan and coworkers (1999) (49), *B. cepacia* ENV435 was grown to optical densities ($O.D._{550}$) of greater than 65 (100 g/L wet weight) and so they contained as much as 60% dry weight of PHB.

An additional advantage of using energy-enriched organisms for in situ remediation, versus feeding indigenous or introduced organisms, may be that it forces more efficient utilization of added oxygen and inorganic nutrients. The degradative organisms carry an internal food reserve with them into the aquifer. The food reserve is not available to less efficient indigenous organisms and thus should not stimulate increased oxygen demand or biofouling associated with feeding indigenous microbes. The only additional oxygen that may be needed is that required to support oxidation of target contaminants and cellular respiration of the added strain.

MIXED CULTURES AND CONSORTIA

Most commercial-scale bioremediation applications of bioaugmentation involve the addition of mixtures or consortia of bacteria. This is particularly true in the area of hydrocarbon treatment where a number of vendors market products composed of uncharacterized mixtures of bacteria that have been cultured and prepared for application in the field. Few mixed cultures or consortia of organisms have been developed for remediation of CVOCs. This may be due, in part, to the fact that bacteria are not known to use CVOCs as a growth substrate. Therefore, it is difficult to enrich degradative organisms by using target CVOCs as a selective substrate. It may be feasible, however, to create stable mixtures of CVOC-degrading organisms by enrichment on substrates known to co-metabolize CVOCs. Such stable mixtures have been maintained for extended periods on methane, propane, toluene, and phenol (50–52) in the laboratory. Likewise, the stimulation of indigenous microbial communities by adding selective substrates to a contaminated environment undoubtedly selects for the enrichment of stable consortia that can sustain prolonged co-metabolic degradation of CVOCs. The use of such mixtures in bioaugmentation may provide more stable populations of organisms to help prolong degradation in situ.

EXAMPLES OF TYPE 1 BIOAUGMENTATION

Type 1 bioaugmentation involves the addition of degradative organisms to a contaminated environment with the goal of establishing an active degradative microbial population. Although this technique has been used extensively for destroying contaminants that can act as growth substrates for the added organisms, it has been applied less often for destroying targets that are only degraded co-metabolically. In the latter case, an additional substrate must be added to support the growth and degradative activity of the added organism.

A laboratory study performed by Krumme and colleagues (53) compared the TCE degrading activity and survival of wild-type *B. cepacia* G4 (54), with that of a transposon-generated mutant, strain PR1 that constitutively expresses the TCE-oxidizing enzyme toluene-*o*-monooxygenase (TOM). Both strains survived for extended periods (>10 weeks) in sterile aquifer material, but the population of each strain decreased by about three orders of magnitude during the same period. When PR1 was added to microcosms at an initial cell concentration of 5×10^8 CFU/g of aquifer material, it degraded TCE from 60 μM to less than 0.1 μM within 24 hours. When PR1 was added at lower cell densities, it continued to degrade TCE over the entire 10-week incubation, but the observed degradation rates were low relative to those observed with the high cell density inoculum.

In a related laboratory study, Munakata-Marr and coworkers (1996) (22) coupled bioaugmentation with biostimulation in aquifer microcosms. Aquifer microcosms were augmented with either *B. cepacia* G4 or a chemically induced mutant of the strain, *B. cepacia* $PR1_{301}$, which constitutively produces TOM. The augmented microcosms were then supplied with either phenol or sodium lactate, and TCE degradation in the microcosms was compared to degradation in microcosms that did not receive the degradative organisms.

When strain G4 and phenol were added in small columns to sterile aquifer material containing 250 μg/L TCE, there was no breakthrough of TCE during the first 37 days of incubation and several exchanges of TCE-contaminated water. Without the addition of G4, TCE degradation was not observed until approximately 19 days after biostimulation with phenol. The TCE concentration in microcosms augmented with strain $PR1_{23}$ and lactate, however, were not significantly different than in sterile controls. These results suggested that bioaugmentation could be effective, but that in this system, the constitutive expression of TOM during lactate feeding was not an effective mechanism for maintaining degradative activity.

In related experiments, microcosms were augmented continuously with lower concentrations of degradative organisms (22). Bioaugmentation with phenol-grown G4 alone generated TCE removals similar to those observed in microcosms fed only phenol. However, if lactate was added with the phenol-grown G4, TCE degradation was 70% greater than in microcosms receiving only G4 or only phenol. Similarly, if strain $PR1_{301}$ was added to microcosms with phenol, TCE degradation was about twice that observed in phenol-only microcosms, and it proceeded without a lag period. In each case, the addition of organisms resulted in a significant oxygen demand that could potentially limit the effectiveness of the technology.

Field Application — TCE

In an early in situ bioaugmentation field study, *B. cepacia* G4, was injected directly into a TCE-contaminated sandy aquifer with tryptophane added as an inducing

substrate (55). Data from the study strongly suggested that, although the organisms degraded TCE, movement of the microorganisms was severely retarded by the aquifer material, thereby limiting the effective treatment area. Tracer test results estimated the hydraulic flow of the aquifer to be approximately 48 feet per day. Nonretarded organisms, therefore, should have appeared in the nearest down gradient recovery well (10 ft.) about 8 to 10 hours after injection, but none of the injected microorganisms were observed in the monitoring well until six days after injection. No injected organisms were detected at a monitoring well located 75 feet downgradient from the injection well. Thus, it appears as though the injected cells acted essentially as an in situ biofilter. Clearly more aquifer material could be treated if the organisms or the aquifer conditions were altered to reduce adsorption and retardation of bacterial movement.

Despite the poor distribution of the biocatalysts, TCE degradation by the injected strain G4 reduced groundwater TCE concentrations from 2,500 μg/L to 466 μg/L for a period of about eight hours. TCE concentrations increased, however, to 3,280 μg/L the following day, presumably due to a nutrient feed pump failure. After another eight hours, TCE concentrations again declined and remained below 300 μg/L with a mean TCE concentration of 135 ± 72 μg/L over the first five days following G4 injection. The mean TCE concentration during the following 10 days was 78 ± 64 μg/L.

Although the authors did not estimate the total amount of TCE degraded, their data suggests that degradation was significant. The groundwater flow rate was 48 ft/day and the receiving zone (area in which bacteria were injected) was 20 ft wide and 14 ft thick with a porosity of 25%. The authors thus concluded that approximately 25,000 gallons of groundwater went through the test area per day. The average decrease in TCE concentration during the first five days (2,500 μg/L to 135 μg/L) represents an apparent TCE removal of about 1,100 g TCE. However, because the authors estimated that the nutrient addition system diluted the groundwater by 30%, the apparent amount of TCE degraded was approximately 800 g. Applying the same calculation for data obtained during the subsequent 10 days during which TCE declined to an average of 78 μg/L, the apparent amount of TCE degraded during the 10-day period was 1,600 g. Thus, the total amount of TCE degraded during the 15 days could have been as much as 2,400 g.

Survival of Introduced Bacteria

The ability of an exogenous organism to survive and compete for resources against indigenous organisms has long been an area of interest in both macro- and microbial ecology, and in most cases added organisms do not fare well. When three well-characterized toluene-degrading bacteria, *P. putida* PaW1, *B. pickettii* PKO1, and *B. cepacia* G4 were added to a fluid bed bioreactor with toluene as a feed source, strain PaW1 became the predominant organism in the reactor (56). When groundwater strains were allowed to enter the reactor, however, even strain PaW1 was rapidly replaced. Thus, even precolonization of the reactor with the added strain did not prevent it from being rapidly outcompeted by native microbes.

The oxidation of CVOCs might further hinder the ability of an added strain to survive in a natural environment. The oxidation of CVOCs is known to result in toxicity to the degradative organisms, presumably due to the production of highly reactive epoxide molecules that can react with and harm cellular components causing cellular inhibition (50,57,58). When four strains of toluene-oxidizing bacteria were placed in a reactor with a low feed rate of toluene, each strain was maintained at relatively constant levels (59). When TCE was added to the reactor, however, *P. putida* mt-2, which does not degrade TCE rapidly, became the dominant organism in the reactor. Kinetic analysis of the three strains that perished revealed that their affinity for toluene was greatly reduced after exposure to TCE. Remarkably, this same selection did not appear to occur when toluene was used to stimulate TCE degradation during a prolonged field study, as indicated by continuous TCE degradation for more than one year (60). In a related study, the addition of toluene or phenol to a TCE-contaminated aquifer resulted in the selection of a native toluene oxidizing bacterial population in which 55% of the strains contained toluene-ortho-monooxygenase, which is known to oxidize TCE. A more detailed analysis of the native strains revealed that a significant number of the strains (20%) were poor TCE degraders, and none degraded TCE as well as strain G4 (61). Nonetheless, CVOC degradation in the aquifer was relatively constant through several months of biostimulation (62).

An area that has not been well investigated is the selection of organisms with an inherent selective advantage for long-term survival, or the development of organisms, which have a selective advantage or that can be provided with an environment that gives them a selective advantage. For example, carbon tetrachloride (CT) degradation by *Pseudomonas* sp. strain KC was found to be greater under denitrifying conditions than aerobic conditions (63,64). Furthermore, degradation was inhibited by iron, but by adjusting the pH of growth media to 8.3, conditions under which iron is precipitated, this inhibition could be overcome (65). Thus, strain KC had a competitive advantage over other aquifer organisms if grown under nitrate-reducing conditions at high pH. By adjusting the pH and redox potential of a contaminated aquifer, one could create a selective niche for added strain KC. These characteristics allowed Criddle and coworkers (66) to demonstrate the use of bioaugmentation for the remediation of a CT-contaminated aquifer in School Craft, Michigan.

In the School Craft demonstration, NaOH-amended groundwater was used to maintain slightly alkaline conditions (pH>7.6), KC was injected, and acetate was added in weekly pulses to maintain degradative activity. During a period of poor chemical delivery incomplete degradation of CT occurred and chloroform concentrations increased. However, data from downgradient monitoring wells indicated that as long as the appropriate amendments were maintained, bioaugmentation was effective for CT remediation (66).

In a related approach, Lajoie and coworkers (1992) (67) isolated a bacterial strain, termed "field application vector," that was resistant to a surfactant that it could also use as a carbon source. By cloning degradative genes into the resistant organism, the researchers could add surfactants to an environment to create a selective niche, and then add the resistant strain containing the degradative genes (68). Such strains may be of use in bioaugmentation of aquifers during or after surfactant or foam flushing designed to remove free product contaminants (69,70).

Field Application — MTBE

Type 1 bioaugmentation is most applicable to contaminants that can serve as a sole source of carbon and energy for the degradative strain so that the addition of a carbon source is not necessary for growth and maintenance of the added strain in situ. Methyl *tert*-butyl ether (MTBE), a component of reformulated gasoline, is a contaminant that can serve as a sole substrate for bacteria (71–73), but these bacterial strains are not ubiquitous. Biostimulation approaches for MTBE degradation have utilized propane to stimulate indigenous propane-oxidizing bacteria to degrade MTBE co-metabolically, but this approach is not applicable to every site (74). Bioaugmentation, therefore, has been utilized to remediate this highly soluble contaminant.

Three demonstrations of this technology have been conducted at Port Hueneme Naval Facility in Oxnard, California. In the first demonstration, injection of a microbial consortium (MC-100) along with oxygenation of the aquifer, was compared to oxygenation only and no treatment, in three adjacent test plots (75). Although oxygenation alone appeared to stimulate indigenous MTBE-degrading strains after a considerable lag time, the plot with added bacteria had a faster and greater decrease in MTBE concentrations than in the oxygen-only plot. Two other demonstrations are currently being conducted with pure cultures of MTBE-degrading bacteria (76,77). Strain PM-1 was injected into a test plot in November 1999 next to an oxygen-only plot (77). Difficulties in oxygen delivery and degradation of MTBE by indigenous populations stimulated by the oxygen injection have made data interpretation difficult, and the contribution by PM1 to degradation of the MTBE in this demonstration has yet to be determined. Strain ENV425 is a propane-oxidizing bacteria that degrades MTBE co-metabolically. Bioaugmentation with this strain coupled with oxygen and propane injection is also being tested at Port Hueneme (76).

Hydrogenophaga flava ENV735 is an isolate that degrades MTBE as a sole food source (73). This strain was injected into a fractured bedrock aquifer contaminated with MTBE that previous tests had shown did not a decrease in concentration during previous tests when only oxygen was added (76). The addition of ENV735 with oxygen was shown to effect immediate decreases in the concentration of MTBE throughout the injection zone (unpublished results).

Anaerobic Bioaugmentation

For some sites and contaminants anaerobic degradation is more applicable than aerobic. This may be because one or more of the contaminants only degrades anaerobically or because the site is already anaerobic and adding enough oxygen to reverse this condition is technically infeasible. PCE only degrades anaerobically to TCE and by successive dechlorinations these chlorinated solvents can be degraded to the innocuous product ethene. If PCE is present, or if the aquifer is already anaerobic, anaerobic biostimulation is a viable remedial option. Often the addition of an electron donor (substrate) will be sufficient to stimulate the complete degradation of the higher chlorinated solvents to ethene, especially if there is some evidence of dechlorination already occurring. In a previously aerobic aquifer at Dover Air Force Base, DE, the addition of a substrate (lactate) and nutrients (nitrogen and phosphorus) were successful in turning the aquifer at the test site anaerobic and also stimulating the anaerobic dechlorination of TCE to *cis*-1,2 dichloroethene (cDCE). Further dechlorination, however, was not observed until the site was inoculated with an ethene-forming microbial consortium (78). A lag period of 90 days occurred between injection of the bacteria and the first appearance of VC, the degradation product of cDCE, and an additional 60 days before complete conversion to ethene. This lag period can be attributed to the small amount of bacterial inoculum ($<$35 g dry weight) and the slow growth rate of this culture.

EXAMPLES OF TYPE 2 BIOAUGMENTATION

Type 2 bioaugmentation involves the addition of degradative organisms as biocatalysts that can degrade a significant amount of target compound and then perish. The assumptions are made that the added organisms will not compete well against native organisms, and/or that the added organisms can be grown more efficiently ex situ (e.g., in a fermentor) than in situ.

In Situ Biofilter

In one field-scale demonstration of Type 2 bioaugmentation (79), approximately 5.4 kg (dry weight) of the CVOC-degrading methanotroph *Methylosinus trichosporium* OB3b were injected into a TCE-contaminated aquifer near Chico Municipal Airport in Chico, California. The cells were used to establish an in situ biofilter (a.k.a. biocurtain or biobarrier) of attached resting state cells (i.e., the cells were not fed after injection) through which contaminated groundwater was directed. Analysis of groundwater extracted from the aquifer indicated that approximately 50% of the bacteria injected into the aquifer were extracted by the pumping action, whereas the other 50% presumably became attached to the aquifer solids surrounding the extraction well.

TCE degradation by the in situ biofilter was very limited. Initially, the TCE concentration in the extracted groundwater was reduced from about 425 μg/L to less than 10 μg/L, but it remained at these low concentrations for only about two days. The TCE concentration then increased until, after 21 days, it was near the initial

(untreated) concentration. Integration of the TCE concentration over time revealed that only about 20 g of TCE were degraded during the 40-day study. This represented approximately 40% of the TCE extracted through the in situ biofilter, and only about 4% of the calculated degradation capacity of the injected organisms.

One of the greatest apparent limitations of the in situ biofilter approach is that contact between the added organisms and the contaminant is limited by the same physiochemical properties that limit any pump-and-treat technology, namely, the TCE present in the aquifer must undergo repeated sorption and desorption as it moves toward the biofilter and its associated extraction system (80). The rate-limiting step, as in any pump-and-treat system, therefore, is transport of the TCE to the treatment system. The biofilter in effect becomes the treatment process for the pumping system, and in order to be a viable technology it must be as reliable and inexpensive as competing treatment technologies. Given the long treatment times associated with pump-and-treat systems, it is unlikely that in situ biological systems can become as reliable or cost-effective as a standard ex situ stripping tower and/or activated carbon adsorption system. In fact, in the demonstration reported by Duba and coworkers, regulatory treatment standards were met for only two days.

In situ biofilters are an attractive remedial option, however, because they rely on the natural tendency of bacteria to stick to solid surfaces, and because the bacteria are immobilized, addition of cosubstrates, nutrients, or oxygen and contact between the bacteria and the additives is easily implemented. One strategy that has been developed to overcome the pump-and-treat limitations of this technology is the use of an electric current to deliver the contaminant to treatment zones created by hydraulic fracture injection. In two field tests of this technology for TCE remediation (81,82) the treatment zones were composed of carbon and iron filings. However, bench scale studies have demonstrated that inoculation of these treatment zones with degradative bacteria is an effective technology for the biodegradation of the electrokinetically-delivered contaminants (83).

Field Application — Bedrock Aquifer

In another field-scale demonstration of bioaugmentation, strain ENV435 was injected directly into a bedrock aquifer (84). Strain ENV435, is a variant of the toluene/TCE-degrading strain *B. cepacia* G4 (54). The strain had been mutated to select a new strain, *B. cepacia* $PR1_{301}$ that constitutively expressed the toluene monooxygenase genes that were responsible for TCE degradation (22). An adhesion-deficient variant of strain $PR1_{301}$ was selected by successive passage of the culture over a sand column (85). Finally, before introduction into a contaminated aquifer, the strain was grown to high cell density under conditions that promoted the production of high levels of poly-β-alkonoates that can serve as a high-energy storage material to prolong degradative activity (46).

To facilitate injecting the strain into the aquifer, pneumatic fracturing was used to expand bedrock fractures. Approximately 550 L of a high cell density culture ($\sim 10^{10}$ CFU/ml) of strain ENV435 was injected into the aquifer formation during the fracturing process, and results of plate count analysis demonstrated that the organisms were dispersed throughout the aquifer in a radius of about 25 feet from the fracture/injection well. Cell numbers in groundwater collected from monitoring wells were as high as 10^8 CFU/ml almost immediately after injection.

TCE concentrations in the formation rapidly decreased from between 20 to 30 mg/L to less than 5 mg/L within a few days after injection. A decrease in TCE degradation rate with time correlated with decrease in the viable ENV435 population during the same two-week period. It was estimated that during that study approximately 825 g of TCE were degraded by approximately 46,000 g (wet weight) of ENV435 cells. This corresponds to an apparent transformation capacity (Tc) of about 0.018 mg TCE/mg cells, which is greater than that estimated for a toluene-degrading enrichment culture (0.0073) but lower than that reported for a phenol-degrading enrichment culture (0.031) (51,52).

Field Application — Porous Aquifer

In a related study, strain ENV435 was injected during four separate tests into a semi-confined CVOC-contaminated aquifer located in Central New Jersey (49). The groundwater within the treatment zone was contained by recirculating groundwater from a downgradient recovery well into a series of upgradient injection wells. A hydraulically-isolated control plot was operated identically to the test plot, but without the addition of microorganisms. Results of the study indicate that in situ bioaugmentation can be an effective treatment alternative for CVOC-contaminated aquifers.

In the first field experiment, ENV435 was injected into six injection wells located approximately 12 m upgradient of the recovery well. Groundwater samples were recovered from monitoring wells located 2, 5, and 8 m downgradient from the injection wells. Strain ENV435 was enumerated by selective plating, and CVOC concentrations were determined by gas chromatography. In another experiment, strain ENV435 was injected into each of nine monitoring wells (2 depths each) throughout the test plot.

During the first experiment, the adhesion-deficient strain ENV435 was distributed throughout the test plot aquifer in a pattern similar to the distribution of a bromide tracer. Sodium bromide was distributed throughout the test plot within about 13 days after injection, whereas strain ENV435 was distributed throughout the plot within 20 days. The calculated retardation factor for ENV435 in the fine sand layer of the aquifer was only 1.25. These results suggested that adhesion-deficient bacteria can be effectively dispersed throughout an aquifer. However, even better distribution is likely when the adhesion-deficient strain is injected into multiple injection points throughout the contaminated aquifer (e.g., experiment 2). In this case, the radius of influence of the strains can be calculated to determine the spacing required between injection points (49).

When CVOC concentrations were monitored in the ENV435-amended test plot, a rapid decrease in CVOC concentrations followed the injection of ENV435. An analysis of the ratio of degradable CVOCs in the groundwater (TCE, DCE, and VC) to nondegradable CVOCs (PCE and TCA) confirmed that the observed decrease in CVOC concentrations was caused by biodegradation and not other factors such as dilution. During the first experiment, this ratio was lowest at the first monitoring well and increased at the downgradient wells suggesting that the degradative activity of the injected cells decreased with time and distance during the 15-day travel time through the aquifer.

When the strain was injected into monitoring wells spaced across the entire test plot (experiment 2), CVOC concentrations were reduced throughout the entire test plot within only a couple of days after injection. Furthermore, the ratio of degradable CVOCs to nondegradable CVOCs decreased at every monitoring point. Thus, if economically and logistically feasible, it appears that the best treatment strategy for applying Type 2 bioaugmentation is to use multiple biocatalyst injection points spaced to allow rapid dispersion of the organisms throughout the contaminated zone of an aquifer.

The bacterial transport and CVOC degradation data gathered during the field demonstration allowed the development of treatment and cost models. The treatment model incorporated ENV435 transport characteristics to determine the required spacing of injection points, and knowing the spacing requirements, allowed the calculation of cost of system installation. For a site with the dimensions of 152 m × 152 m × 6 m, and a CVOC concentration of 1,000 μg/L, estimated cleanup costs would be approximately $\$7/m^3$ ($\$5/yd^3$) and it would require only about one year to remediate the site. These costs are comparable to or less than competitive technologies including air sparging/soil vapor extraction with off-gas treatment.

CONCLUSION

Bioaugmentation, like most remedial technologies, is site- and contaminant-specific. In other words, whether or not bioaugmentation will be effective and how it will be implemented depends of the hydrogeology and geochemistry of the site as well as the mixture and concentration of the contaminants. Extremely high concentrations and free product contamination can be toxic or inhibitory to bacteria and not amenable to bioaugmentation without pretreatment to lower concentrations. For contaminants that are strongly sorbed to aquifer solids, injecting bacteria that will disperse or grow throughout the contaminated zone would be more effective than an in situ biofilter because of the length of time, that it would take for desorption of the contaminant from the aquifer solids to occur. If bacteria are dispersed throughout the contaminated zone, the ongoing degradation will increase the concentration gradient and desorption will occur more rapidly. On the other hand, if there is an ongoing source, or if the contaminants are highly soluble, an in situ biofilter may be an effective application of this technology. As with all in situ remedial technologies, contact of the bacteria and any needed additions (cosubstrates, nutrients, oxygen, etc.) with the contaminants is key to the effectiveness of the remedy.

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See also BIOREMEDIATION: AQUATIC ECOSYSTEMS.

BIOAUGMENTATION: COLD-ADAPTED MICROBES.

See USE OF COLD-ADAPTED MICROORGANISMS IN BIOTECHNOLOGY

BIOAVAILABILITY OF ORGANIC SUBSTRATES.

See ACTIVITY AND CARBON TRANSFORMATIONS IN BIOFILMS

BIOCHIP-BASED DEVICES AND METHODS IN MICROBIAL COMMUNITY RIBOTYPING

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Studies in environmental microbiology are often limited by the inability to unambiguously identify and directly quantify the enormous diversity of natural microbial populations. Our perspective on microbial diversity has improved enormously over the past few decades. In a large part, this has been due to molecular phylogenetic studies of related organisms. Phylogenetic tree based on gene sequences are maps that are used to articulate the elusive concept of biodiversity. The phylogenetic classification of microbes based on rRNA gene (rDNA) sequences offers a well-defined framework, which can be used to develop molecular tools for microbial identification. The rDNA molecules typically consist of highly conserved sequences interspersed with regions of more variable sequences. Together with the development of the PCR technique and a number of high-resolution molecular typing systems, this knowledge has provided an approach for microbial community typing. Many devices and methods have been developed and applied for microbial community typing, which include biosensors (1) capillary electrophoresis (2), the biochip technology (3) and others (4). The traditional typing methods used in microbiology consist of (*1*) Culture followed by morphological and physiological analysis, (*2*) Molecular biology methods [such as polymerase chain reaction (PCR), restricted fragment length polymorphism (RFLP), amplification fragment length polymorphism (AFLP), and PCR-RFLP of polymorphic loci, etc.]. Compared with the newly developed biochip methods, the traditional methods are laborious and time-consuming. The advantages of the biochip-based systems are obvious: sensitive, specific, fast, portable, and parallel sample analysis capability. In this article, we will first present a brief overview of microbial community ribotyping in environmental microbiology and then focus on the application of biochip-based devices and methods in microbiology, especially in microbial community ribotyping in environmental microbiology.

BACKGROUND OF MICROBIAL COMMUNITY RIBOTYPING

Microbial Community Typing

Modern taxonomic approaches often employ technically more complex methodologies and are concerned with profiling the structural composition of bacteria. This often involves approaches based on "molecular biology" or "analytical chemistry." It is now recognized that many of the classical schemes for differentiation of bacteria provide little insight into their genetic relationships and to some extent are scientifically incorrect. The new information has resulted in renaming of certain bacterial species and, in some instances, in the complete reorganization of relationships within and among many bacterial families.

The aim of typing is to identify stable groups in a population of the same species using markers that are reproducible, accurate, and conveniently detected. The complex characteristics (phenotypic patterns) are intuitively recognized as good indicators of relationship. The characters of bacteria that are traditionally used for typing include biotypes, antigenic structure, phage susceptibility, and so forth. Genotypic information or sequence information is superior to phenotypic information in two ways for relating and classifying microorganisms: (*1*) the results can be more readily, reliably, and precisely interpreted, and (*2*) the results are innately more informative of evolutionary relationships than the phenotypic information. The elements of a sequence, nucleotides, or amino acids are restricted in number and are well defined. The subjectivity that goes into the judgments "same," "similar," and so forth at the phenotypic level is replaced by simple, more objective judgments and mathematical definitions.

In the past several years, sequence analysis of the entire genome of one representative (i.e., a strain) out of a few bacterial species has been achieved. For species identification, it would be ideal to compare the sequences of the entire bacterial chromosomal DNA. However, this is currently not a practical approach, because enormous amount of efforts has to be put in to sequence millions of nucleotides for each strain. An alternative way is to assess the genomic similarity by the content of guanine (G) + cytosine (C), usually expressed as a percentage (% G+C). This has lately been replaced by two different approaches, namely, hybridization or sequence analysis of the genetic coding segment for 16S rRNA.

Ribosomal RNA

The story of the ribosomal RNA (rRNA) begins in 1960s, but not much progress was made until the early 1970s, when Woese realized that rRNA is universal to life and that much historical information could be stored in it. Comparative analyses of small-subunit (SSU) rRNA (16S or 18S rRNA) and other gene sequences show that life falls into three primary domains — Bacteria, Eukarya, and Archaea (5). Since then, the three-domain theory based on rRNA has been widely accepted.

The ribosome is a molecular machine in which all the proteins are synthesized. In the past few years, sequence analysis of 16S rRNA has become the "gold standard" in bacterial taxonomy. This is due to its molecular ubiquitousness and the functional constancy. The 16S rRNA changes very slowly in sequence and can be easily traced through experimentation. Moreover, as the central component of the highly complex translation apparatus, rRNA is among the most refractory of molecules to the vagaries of horizontal gene flow and so was considered

likely to avoid the phylogenetic hodgepodge of reticulate evolution (6).

The nucleotide sequence of the 16S rRNA gene is about 1,500-letters long, and consists of many domains. The 16S rRNA changes very slowly in sequence, which makes it an accurate chronometer. The sequence of 16S rRNA provides a measure for genomic similarity above the level of the species and allows comparison of relatedness across the entire bacterial kingdom. Closely related bacterial species often have identical rRNA sequences. The technique thus provides complementary information to DNA-DNA hybridization. Exploiting this basic research in the clinical laboratory has allowed the development of probes for improved identification of bacterial pathogens.

A large collection of SSU rRNA-targeted DNA probes has been developed for studies in environmental microbiology. They have been designed to identify phylogenetic groups of different evolutionary depths, corresponding in general to the taxonomic ranks of species, genus, family, and higher. In particular, the use of group-specific DNA probes complementary to the SSU rRNA has provided a comprehensive framework for studies of microbial population structure in complex systems. However, to fulfil such studies, it is highly desirable to conduct independent hybridization of multiple environmental samples to multiple DNA probes. Biochip as a new platform can meet just this requirement.

BIOCHIP-BASED DEVICES AND METHODS

Biochip is a fingernail-sized solid device used for the analysis of biomolecules (e.g., DNA, RNA protein etc.) in parallel. A typical analytical system usually consists of three classical steps, for example, sample preparation, chemical reaction, and detection. The total integration of these three steps has been the dream for many years for both academic researchers and entrepreneurs. The marriage between molecular biology and the semiconductor industry for the first time brings this hope to the scientific community. The use of biochips will substantially increase the research speed in molecular biology and they will find their way in environmental microbiology, especially in bacteria community typing, which is now mainly based on the analysis of sequence diversity of 16S rRNA. Biochip-based systems can be made small and compact and thus suitable for field and point-of-care use for which hand-held analyzers are required.

Classification of Biochips

Passive Biochip. The passive biochip specifically refers to devices on which no functional elements have been fabricated to actively generate forces for molecule manipulation. The reaction takes place simply on the basis of the specific binding of complementary DNA or RNA and the specific recognition of antigen and antibody via random diffusion process. The results obtained from the passive biochip are displayed in an arrayed format. When compared with the traditional single-probe hybridization method, DNA microarray technology demonstrated its advantage of being able to perform nucleic acid hybridization analysis in a high-throughput and low-cost way.

Active Biochips. The active biochip (7) refers to devices on which elements with different functions have been fabricated to generate forces (i.e., electric force, magnetic force, acoustic force, etc.) for active manipulation of cells and molecules.

Most biological molecules are charged and can be moved and concentrated by electric fields. Cells or microparticles with molecules attached can be manipulated by inducing the dipole on the particles, which is independent of the native charges carried by these materials. Research reports on active biochips are currently limited. Companies such as Nanogen, Caliper technologies, Aclara, and AVIVA Biosciences are the leaders in this category. Among these, AVIVA is the only company making chips capable of producing multiple forces either sequentially or simultaneously depending on the actual needs of the experiment. The advantage an active biochip has over a passive chip is the speed, accuracy, and sensitivity in reaction and detection. Using active biochip-based analytical systems, the entire sample-to-result process could be completed in minutes.

Chip-Based Sample Preparation

Traditionally, the sample preparation step involves numerous operations (e.g., pipetting, centrifugations, extraction, precipitation, etc.) on a large quantity of samples. They are often labor-intensive and time-consuming. Recently, chip-based small disposable devices have been developed for handling biological samples. Many reports (8,9) have been dedicated to cell manipulation and nucleic acid extraction, that is, separation and transportation of cells and isolation of DNA and RNA molecules.

An effective microscopic sample preparation method should be sensitive to certain cell properties and selective for cell types to allow for discrimination and separation of target cells, requiring little or no sample pretreatment. So far, only a few microscopic-scale cell-manipulation concepts have been demonstrated including diffusion-based particle separation and detection; microtitration of cell samples that exploits the cell size and mechanical properties (10); and electronic separation of cells that makes use of the electrical properties of cells (11). Figure 1 shows the separation of *Escherichia coli.* from blood using a microfabricated dielectrophoretic chip.

The San Diego–based company AVIVA Biosciences is known for its unique multiple-force chips (Fig. 2). These

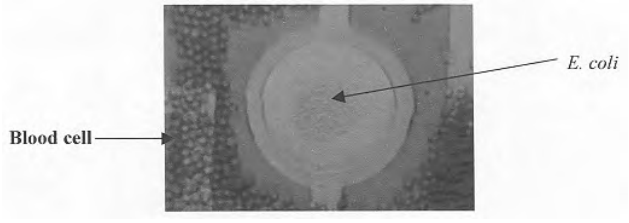

Figure 1. The separation of *E coli.* from blood using a microfabricated dielectrophoretic chip. See color insert.

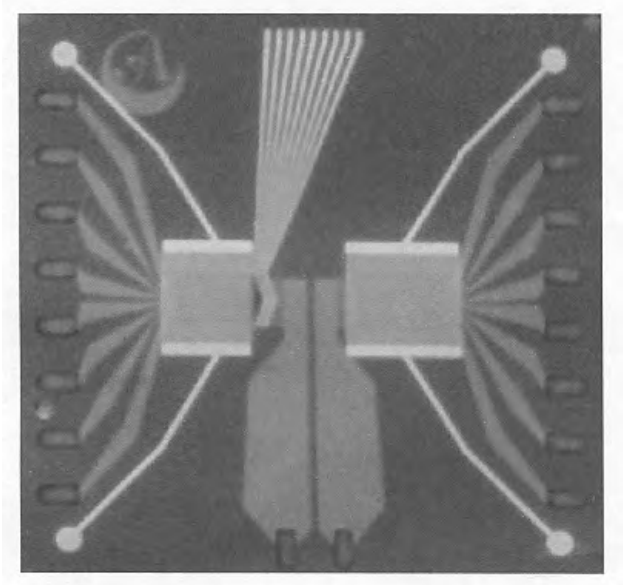

Figure 2. A microfabricated multiple-force active biochip capable of isolating various bioanalytes from a complex biological sample. With permission from AVIVA Biosciences. See color insert.

chips can produce a variety of physical forces and are useful for actively manipulating a variety of bioanalytes such as cells, proteins, DNA, and RNA. The combined use of AVIVA biochips with microbeads provides the capability for separating and manipulating diversified bioanalytes from crude biological samples. Extraction, purification, and concentration of nucleic acids from complex biological samples are the initial steps in any nucleic acid–based assay. This is necessary because the nucleic acids from live organisms are protected inside the living cell. Once the cell is broken open, nucleic acids will be released into solution and become accessible for analysis. However, other biological chemicals may also be present in the solution. Many of these biochemicals, such as proteins and metal complexes, tend to bind with the nucleic acids and therefore interfere with the followed DNA or RNA amplification reaction. Hence, to successfully carry out nucleic acid amplification, it is essential to eliminate those contaminating materials.

Oxidized silicon surface is useful for capturing nucleic acids. Christel described such a system for rapid nucleic acids analysis (12). The extraction, purification, and concentration of DNA from test samples have been accomplished using fluidic microchips with high surface area to volume ratios. Short (500 bp) and medium size (48,000 bp) DNAs have been captured, washed, and eluted using the silicon dioxide surfaces of these chips. DNA quantities approaching 40 ng/cm^2 of binding area were captured from input solutions in the 100 to 1,000 ng/ml concentration range. The extraction efficiency was about 50% with diluted samples of interest for pathogen detection.

Chip-Based Reactions

Chip-based reactions are usually represented by various enzymatic reactions such as PCR amplification reaction, or proteinase K digestion, chemical labeling reaction, and so on.

The nucleic acids extraction studies can be divided into two types: high-concentration inputs and low-concentration inputs (at or below 10^5 copies/mL). These two regimes have relevance to different clinical situations. Samples involving genomic DNA, such as whole blood, typically possess large quantities of DNA. On the other hand, samples for diagnosing infectious diseases often contain minute amount of pathogenic nucleic acids of interest. Serum, plasma, and urine are examples for the latter case. For studies on high concentration, the quantities of DNA are large enough to conduct direct fluorescence assay. For low concentrations, however, amplification reactions such as PCR must be used to first amplify a target sequence.

PCR chips shown in Figure 3 have been used to perform nucleic acid amplification (13). It has been assumed since the first illustration of microchip-based PCR in the early 1990s that features such as low reagent consumption, low-volume sample requirement, and rapid cycling would be a consequence of this technology. PCR reactions previously taking up to three hours were shown to be feasible with time as little as 20 minutes (14). It will require considerable effort to overcome many difficulties in fluidic control, efficient thermocycling, surface chemistry, specimen introduction, and quantitation of amplicons. However, it is quite clear now that microchip-based amplification will play a major role in molecular biology and find wide application in environmental microbiology.

Chip-Based Detection

The detection of nucleic acids is usually achieved by using two approaches. One approach is a "separation-based detection" such as electrophoresis, chromatography, spectrometry; the other one is a hybridization-based approach.

Chip-Based Electrophoresis Systems. Capillary electrophoresis (CE) is an attractive technique. In the last decade, chip CE has been made possible in separating and analyzing DNA samples within a few minutes or even seconds. It may eventually prove to be a valuable tool for microbiologists.

Different materials (e.g., silicon, glass, and plastics) have been used as substrata for fabricating chip CE devices. A variety of fabrication processes have been developed to accommodate the complicated requirements. Biochip-based CE systems have demonstrated their uses

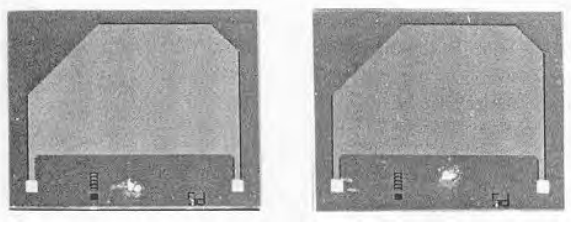

Figure 3. Microfabricated silicon-glass PCR chips. See color insert.

in diverse applications such as the separation of amino acids, DNA or RNA fragment sizing, DNA sequence analyses and CE-based laboratory-on-a-chip systems.

A fused silica CE chip has been fabricated and used for fast-DNA profiling (15) with a replaceable denaturing polyacrylamide matrix. Baseline resolution was achieved with four amplicons representing loci of the *CSF1PO*, *TPOX*, *THO1*, and *vWA* genes in less than two minutes. This represents a 10- to 100-fold increase in the speed of separation compared with the conventional capillary or slab gel electrophoresis systems. Ribosomal RNA samples were reportedly separated in an injection-molded plastic microchannel with a cross section of 100×40 μm and with an effective length of one centimeter.

Hybridization-Based Biochips. Hybridization analysis offers many advantages over other methods of DNA identification, such as comparative size or relative migration determination, by providing insight into the specific nucleotide sequence being investigated. Specifically, with hybridization, spatial position of sequence-specific probes the corresponding signal intensity generated by these probes provide the basis for analyzing complex genetic mixtures for sequence-specific characteristics.

Microchip-based nucleic acid arrays now permit the rapid analysis of genetic information by hybridization. Many of these devices take advantage of sophisticated silicon manufacturing processes developed by the semiconductor industry over the last 40 years. With these devices, parallel hybridization was made possible with immobilized capturing probes. Stringency and rate of hybridization are generally controlled by temperature and salt concentration of the solutions used for hybridization and wash. The passive microarray approaches have the following limitations: (*1*) All nucleic acids are exposed to the same conditions simultaneously — capture probes should be made with similar melting temperatures to achieve similar hybridization stringency; (*2*) The rate of hybridization is proportional to the initial concentration of the interrogated solution — high concentrations are therefore required to achieve rapid hybridization; (*3*) Hybridization conditions are difficult to control — single base discrimination is generally restricted to oligomers of 20 bases or less.

Many of the limitations with passive hybridization techniques can be overcome by using active biochips, which can generate desired forces, thus facilitating (*1*) the rapid transport and selective addressing of DNA molecules to the test on the chip surface; (*2*) speedy hybridization process; and (*3*) rapid discrimination of single base mismatches in target DNA sequences.

A research group at Yale University has produced an RNA-based microarray that promises to put a powerful diagnostic lab on a dime-sized chip (16). To create the prototype, they placed the RNA switches (a molecular switch is a molecule that can be turned on or off by another molecule or compound) on a gold-coated silicon surface and arranged them in clusters. Each switch was designed to bind only to a specific molecule — its "target" — and then release a signal that identifies the target molecule. They tested the array of RNA switches on a variety of complex mixtures. In one experiment, they successfully identified different strains of *E. coli* found in bacterial cultures. This array is able to simultaneously identify a potentially large number of compounds and, combined with the precise exclusivity of each switch, adds up to a recipe for a powerful and wide-ranging laboratory on a dime-sized slice of silicon.

Micro-Total Analysis System

The detection of microorganisms is important in disease diagnosis, in guaranteeing the safety of food and water supply, and in ensuring public safety from the threat of biological warfare agents. In many of these applications, the time taken to provide the result is a critical issue. A portable system that is easy-to-use, and yet would provide a rapid result, would be of great benefit. A fully integrated biochip-based micro-Total Analysis System (μTAS) or "lab-on-a-chip" possessing the aforementioned merits usually consists of (*1*) chip-based sample treatment, such as cell separation, cell fractionation, and nucleic acid extraction; (*2*) chip-based biochemical reaction, such as PCR, immunoassay, and fluorescent labeling; (*3*) chip-based detection, such as electrophoresis, chromatography, spectrometry, and "hybridization-based" approaches. However, to complete such a system will require the use of other microelectromechanic system (MEMS)-based devices including microheaters, valves, pumps, and detectors. Such a system, once constructed, would allow for the generation of very sophisticated biochemical and biomedical information with simplified steps. Figure 4 is the schematic of such a system.

The first Lab-on-a-Chip system was developed and reported by Cheng and coworkers (17). Using the same type of photolithographic and deposition techniques employed in the microelectronics industry, Cheng and colleagues have created a $1\,cm^2$ silicon chip that functions as a lab. The manufactured bioelectronic chip consists of an addressable array of 25 platinum 80-μm diameter microelectrodes covered by an agarose permeation layer in a 4.84-μl flow cell. The alternating current field that is set up in the chip separates cells by dielectrophoresis. Different cell types locate to different regions of the microelectrode array. Weakly held cells located in interelectrode regions of low field strength

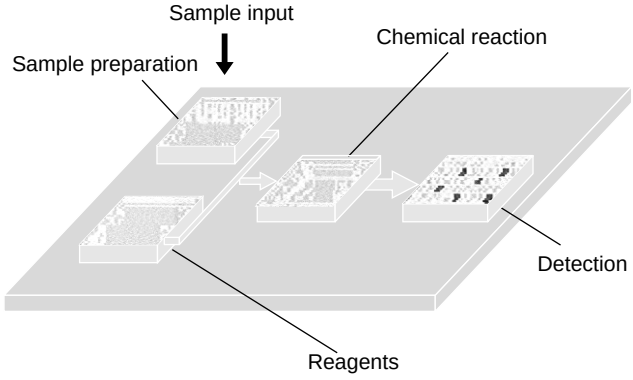

Figure 4. Illustration of a lab-on-a-chip system.

can be selectively removed by washing. The chip isolates *E. coli* cells from a whole blood sample in about 4 minutes through the use of dielectrophoresis, followed by electronic lysis and proteolytic digestion. The lysate contained a spectrum of nucleic acids including RNA, plasmid DNA, and genomic DNA and was further examined by electronically enhanced hybridization assay on a second bioelectronic chip. Cultured cervical carcinoma cells were similarly isolated from normal human blood cells by this group using the same device in a modified manner.

THE APPLICATIONS OF BIOCHIPS IN MICROBIOLOGY

Study the Relationships Between the Microbes and the Hosts

For more than a century, microbiologists studied pathogens in pure culture, whereas cell biologists studied mammalian cells in tissue culture. A few years ago, researchers realized that these laboratory methods of growing pathogens and their hosts were quite artificial and had very little in common with real life, wherein pathogens and hosts coexist, interact, and compete in conditions that are often far from optimum. To better mimic what happens in real life, the study of the interaction between microbes and host cells was proposed, taking advantage of technological progress that allowed cocultures of microbes and cells to be handled together. For this reason, a new discipline (cellular microbiology) was born (18) and a powerful new approach to cellular microbiology was described (19). Instead of studying one parameter at a time, microarray-based methods allow the study, within a single experiment, of all the host genes and those of the bacteria whose expression is modified during host–pathogen interaction: the global picture of the dialog between the pathogen and the host in one experiment.

High-density genome-wide microarrays of eukaryotic cells are commercially available. Homemade chips can also be printed when the genes are available. In the past few years, sequencing of the entire genome of one representative (i.e., a strain) of a few bacterial species has been achieved. These include *Helicobacter pylori*, *Neisseria meningitidis*, *Mycobacterium tuberculosis*, and *E. coli.* Chips containing all genes of the sequenced bacterial genomes are either commercially available or homemade, and many more will be available in the near future.

A typical microarray experiment is shown in Figure 5 (20). RNA is prepared from bacteria culture in standard laboratory conditions (a), from host cells grown under optimum conditions in tissue culture (b), and from cells that have been infected with bacteria (c). RNA is converted into fluorescent cDNA by incorporation of different fluorochromes during the reverse-transcription reaction (typically by using Cy3- and Cy5-dCTP). The labeled cDNAs are then hybridized to microarrays containing bacterial probes (d) or eukaryotic probes (e).

In the experiment shown in Figure 5e, the RNA prepared in (b) and labeled with Cy3 (green) is mixed with the RNA prepared in (c) and labeled with Cy5 (red). The two fluorescent cDNAs are then mixed together and hybridized to the chip. Sometimes, RNA prepared from a pool of control conditions or cells is used to prepare

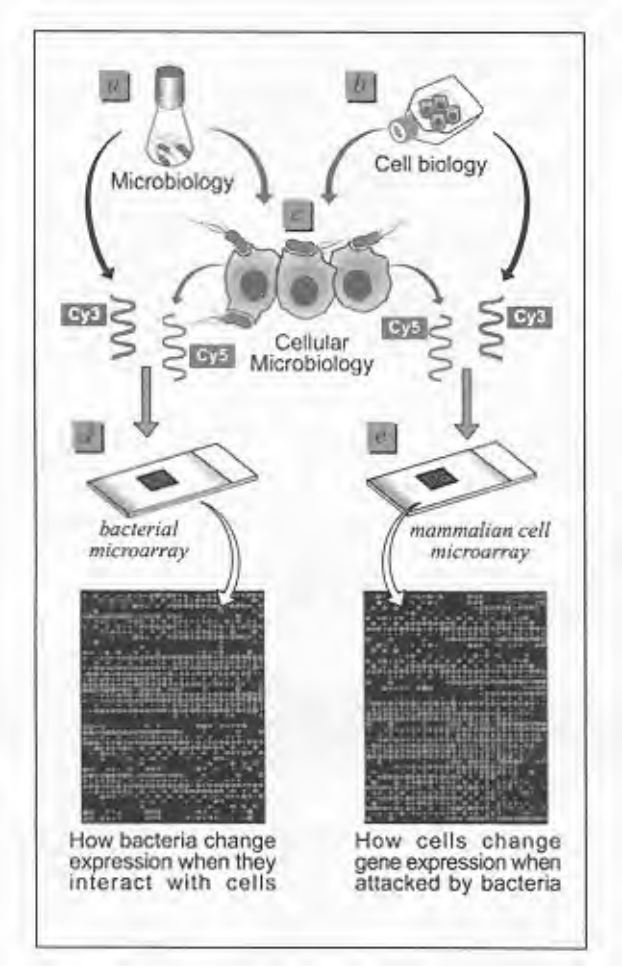

Figure 5. Schematic description of how microarrays can be used to study host cell–pathogen interactions (20). See color insert.

a Cy3-labeled reproducible control, which is then mixed with Cy5-labeled cDNA prepared from the experimental condition, including uninfected cells. This allows an invariant comparator, so that any experimental profile can be compared with any other. After hybridization, laser scanning, image detection, and analysis of each chip, the ratio of the targets in Figure 5b and c provides a precise indication of the relative expression of the genes of the mammalian cells grown in the conditions in Figure 5b and c. More specifically, for the labeling reaction [RNA in Figure 5b (Cy3) or RNA in Figure 5c (Cy5)], the red spot indicates gene activation after bacterial addition to the cells, and a green dot refers to the downregulation of the gene, whereas a yellow spot indicates no change in gene expression. The final results of the analysis are the expression ratios of Figure 5b or c obtained from the mean value of two labeling conditions.

Biochip may be used in the study of infected tissues and whole organisms. The possibility of studying pure cultures of pathogens while they interact with pure cultures of mammalian cells is exciting and represents a major step forward from traditional studies, in which pathogens and host cells were studied separately. However, even the in vitro infection of host cells is far from a real-life

scenario in which pathogens infect animals and their tissues. There is no doubt that the technology is available for studying host cell and pathogen-gene expression in whole organisms. For instance, bacteria recovered from infected tissues (blood, cerebrospinal fluid, etc.) should be suitable for sample preparation. Similarly, macrophages and lymphocytes from infected organisms and tissue from patients with chronic infections can be recovered and used for sample preparation.

So far, published studies describe only the host response to infection. Many studies regarding the bacterial response to host cell contact are in progress.

As an example (19), DNA microarray has been used for studying the transcriptional responses of respiratory epithelial cells to *Bacillus pertussis*. *Bacillus pertussis* is the causative agent of whooping cough, which has many well-studied virulence factors and a characteristic clinical presentation. Despite this information, it is not clear how *B. pertussis* interacts with host cells in disease generation. In this example, the authors examined the interaction of *B. pertussis* with a human bronchial epithelial cell line (BEAS-2B) and measured host transcriptional profiles by using high-density DNA microarrays.

The results showed that *B. pertussis* induces mucin gene transcription by BEAS-2B cells and then counters this defense by using mucin as a binding substrate. A set of genes is described for which the catalytic activity of pertussis toxin is both necessary and sufficient to regulate transcription. Host genomic transcriptional profiling, in combination with functional assays to evaluate subsequent biological events, provides insight into the complex interaction of host and pathogen.

Biochip-Based Microbial Community Ribotyping

Current analytical techniques used for the determination of microorganisms are rarely automated, usually require incubation in a suitable medium to increase the number of organisms, and generally employ visual and/or chemical methods to identify the strain or subspecies of interest. The inherent delay in such methods frequently necessitates medical intervention before definitive identification of the nature of an infection. In industrial, public health, or clinical environments, such delays may have unfortunate consequences. Therefore, there is a concrete need for any possible convenient systems that are capable of rapid detection of microorganisms. Ribotyping is mainly based on the sequence information of the bacterial 16S rRNA and, once combined with oligonucleotide microchip techniques, should have widespread applications in environmental and medical microbiology.

Oligonucleotide microchips are the widely used format for determinative and environmental studies in microbiology. Oligonucleotide microchips were originally developed for rapid sequence analysis of genomic DNA, that is, sequencing by hybridization with oligonucleotides in a matrix. For example, a single-stranded DNA fragment about 200-bases long could be hybridized in parallel with all the 65,536 possible DNA 8-mers. In principle, the DNA sequence of the fragment can be reconstructed from the pattern of hybridization to the chip. However, this method has not yet been adapted for large-scale sequencing projects because of the problems resulting from inefficient discrimination between perfect and mismatched duplexes, secondary structure within the single-stranded DNA (or RNA), and the presence of tandem repeats in the DNA to be sequenced. Yet, it has proven to be an excellent method for sequence analysis of mutations, gene polymorphisms, and other genetic changes and has been further extended to the studies in microbial ecology.

Nitrifying Bacteria Typing. Nitrifying bacteria have proved particularly difficult to study using cultivation techniques because of their long generation times and poor counting efficiencies. Thus, a rapid, culture-independent enumeration technique for nitrifiers could greatly facilitate research in their ecology.

Guschin and coworkers (21) described the utility of parallel hybridization of environmental nucleic acids to oligonucleotides immobilized in a matrix of polyacrylamide gel pads on a glass slide (oligonucleotide microchip). Oligonucleotides complementary to small-subunit rRNA sequences of selected microbial groups, encompassing key genera of nitrifying bacteria, were shown to selectively retain labeled target nucleic acid derived from either DNA or RNA forms of the target sequences. The utility of varying the probe concentration to normalize hybridization signals and the use of multicolor detection for simultaneous quantitation of multiple probe-target populations were demonstrated. The technique should provide a powerful format for the systematic exploration of natural microbial diversity.

Mycobacterium Species Identification and Rifampin Resistance Testing. One of the applications of high-density DNA probe arrays for bacteriology focuses on fastidious bacterium diagnostics. Using oligonucleotide as the probe, the potential of the microarray strategy for parallel testing of different targets has been demonstrated in this study, as the same hybridization conditions could be used for the three genes tiled on the mycobacterial array (16S rRNA, *rpoB, katG*).

Tuberculosis (TB), caused by members of the *M. tuberculosis* complex, is one of the most common human infectious diseases, causing three million deaths a year worldwide. Although the disease is associated with impoverished economic conditions, TB is on the rise in many industrialized nations. The spread of TB is due to immigration, the emergence of drug-resistant strains, and the AIDS epidemic. Reduced and compromised immune function as found in newborns, infants, and immunosuppressed individuals allows opportunistic infections caused by mycobacteria other than *M. tuberculosis*, such as *M. avium-intracellulare*, *M. chelonae*, *M. fortuitum*, *M. Kansasii*, *M. xenopi*, *M. marinum*, *M. scrofulaceum*, and *M. zxulgai.*

The increasing number of mycobacterial infections has made it clinically important to quickly identify mycobacteria at the species level. The diagnosis of a pathogenic versus a nonpathogenic species not only has epidemiological implications but also is relevant to the demands of patient management. Individuals with highly contagious infections may be isolated to prevent the spread

of the disease. Antibiotic treatments may vary according to the species encountered.

The emergence of drug-resistant *M. tuberculosis* has created additional concern in the event of TB diagnosis. Recently, a number of genetic changes leading to rifampin, isoniazid, pyrazinamide, and ethambutol resistance have been characterized and allowed for the development of probe-based assays for antibiotic resistance.

Traditionally, species identification within the genus *Mycobacterium* and subsequent antibiotic susceptibility testing rely on time-consuming, culture-based methods. Despite the recent development of DNA probes, which greatly reduce assay time, there is a need for a single-platform assay that is capable of answering the multitude of diagnostic questions associated with this genus.

Troesch and coworkers (22) described the use of a DNA probe array on the basis of two sequence databases: one for the species identification of mycobacteria (82 unique 16S rRNA sequences corresponding to 54 phenotypical species) and the other for detecting rifampin resistance (*rpoB* alleles). They designed an array that contained all the 16S rRNA polymorphisms over a 200-bp region present in a mycobaterial database. The array also contained 51 *M. Tuberculosis* rifampin resistance-causing *rpoB* mutations in a 200-bp region and 2.2 kb of the *M. Tuberculosis* wild-type *katG* gene—this fragment spanning codons in which mutations were previously shown to confer resistance to isoniazid. An experimental protocol was optimized to perform hybridization on the probe array, scanning, and data analysis in one hour. The hybridization-based probe array assay used here showed a specificity matching the sequence polymorphisms of the 16S rRNA marker. In this study, a total of 26 out of 27 species were correctly identified and so were all of the *rpoB* mutants, with the only discrepancy being an artifact because of an error in the reference sequence. This parallel testing format opens new perspectives in terms of patient management for bacterial diseases by allowing a number of genetic tests to be simultaneously run.

Portable System for Microbial Sample Preparation and Oligonucleotide Microoarray Analysis. The limitation with microarray technology is the lack of portability and inexpensive devices for the acquisition of hybridization patterns. The group headed by Mirzabekov has developed a three-component system and a rapid inexpensive procedure for analysis of different microorganisms based on microchip biotechnology (23).

The system consists of three main components: (*1*) a universal syringe-operated silica minicolumn for successive DNA and RNA isolation, fractionation, fragmentation, fluorescent labeling, and removal of excess free label and short oligonucleotides; (*2*) microarrays of immobilized oligonucleotide probes for 16S rRNA identification; and (*3*) a portable battery-powered device for imaging the hybridization of fluorescently labeled RNA fragments with the arrays.

The minicolumn combines a guanidine thiocyanate method of nucleic acid isolation with a newly developed hydroxyl radical-based technique for DNA and RNA labeling and fragmentation. DNA and RNA can also be fractionated through differential binding of double- and single-stranded forms of nucleic acids to the silica. The procedure involves sequential washing of the column with different solutions. No vacuum filtration steps, phenol extraction, or centrifugation is required. After hybridization, the overall fluorescence pattern is captured as a digital image or is recorded by a polaroid camera.

This three-component system was used to discriminate *E. coli*, *Bacillus subtilis*, *Bacillus thuringiensis*, and human HL60 cells. The procedure is rapid: beginning with whole cells, it takes approximately 25 minutes to obtain labeled DNA and RNA samples and an additional 25 minutes to hybridize and acquire the microarray image using a stationary image analysis system or the portable imager. This is the first passive chip-based total analysis system for environmental microbiology; the portable system may be further used in microbial identification in medical, agricultural, or environmental applications. Although the system is not perfect, it has demonstrated the promise of applying biochip technology in environmental microbiology.

PERSPECTIVE

It is increasingly evident that only a small fraction of the microbial world has been characterized, although there is little agreement about the full extent of undescribed diversity. The development of rRNA-targeted probes that selectively target characterized phylogenetic groups now provides a format to systematically address this question. These probes are fully compatible with the microchip format.

Biochip is one of the most promising biotechnologies for the twenty-first century, which has the advantage of combining a rapid, high-throughput and portable platform for nucleic acid hybridization. In addition to their use in identifying described phylogenetic groups, the great capacity of the microchip array could be used to evaluate sequence motifs of the novel microbial populations. Thus, we anticipate that the microchip will have application not only to microbial community ribotyping but also in ongoing studies of global diversity.

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BIOCONTROL, MICROBIAL AGENTS IN SOIL

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Plant pathogens affect crops throughout the world and are a major obstacle to effective food supplies worldwide. Losses due to plant diseases may be as high as $30 to $50 billion in cultivated and stored crops and 20% of total food production annually. The most efficient way of preventing crop diseases caused by these pathogens is to use chemicals to disinfect the soil against pathogens and pests. However, pesticides, common in agricultural practice, create a health hazard for many nontarget organisms. Their use becomes more limited every year because of concern of legislators for human and animal health, as well as for quality of life and the environment. In addition, excessive application of pesticides has led to an increased proportion of pathogens, which are resistant to the chemicals. Thus, each year, diseases cause millions of dollars worth of crop damage, despite extensive use of pesticides. Moreover, many nonspecific chemical pesticides currently widely used against soilborne diseases (e.g., methyl bromide) are soon to be taken off the market as a result of registration cancellation (1). Therefore, the development of safer, environmentally friendly control alternatives that promote sustainable agroecosystems is urgently required and has become a research priority in developed countries. Agricultural biotechnology offers a new approach to the problem: the development of alternative, efficient, and ecologically safer biological methods of plant disease control (2–6).

Biological control can be defined as "any conditions under which survival or activity of a pathogen is reduced through the agency of any other natural or modified living organisms and genes or gene products (delivered by organisms) as well, with the result that there is reduction in incidence of the disease caused by the pathogen" (2). Chemicals produced and delivered by living organisms represents biological control, whereas chemicals extracted from living organisms represents chemical control. A beneficial organism used to protect plants is referred to as a "biological control agent" (BCA), "biopesticide," "microbial pesticide" or, simply as "antagonist," because it "antagonizes," or interferes with, the target organisms that are damaging the plant.

Antagonists are generally naturally occurring, mostly soil microorganisms, which have some trait or characteristic that enables them to interfere with pathogen or pest growth, survival, infection, or plant attack. Usually, they have little effect on other soil organisms, leaving the natural biology of the ecosystem more balanced and intact than would a broad-spectrum chemical pesticide. Several plant diseases caused by soilborne plant pathogens have been shown to be inhibited by soils. Soil suppressiveness may be constitutive, an inherent property of the soil regardless of its cropping history, or adaptive, whereby soil suppression is only achieved after a specific cultural practice, such as monocropping, has been adopted. Soil suppressiveness of *Fusarium* wilt of melons (7) and of *Gaeumannomyces graminis* var. *tritici* (*Ggt*)-take-all of wheat (2) are examples of constitutive and adaptive soil suppression, respectively. Soil suppression is considered a rather complex phenomenon in which the antagonists establish themselves in the soil and interfere with the growth or survival of the pathogenic microorganism. Several microorganisms can be involved in soil suppression of a single disease. Studies of why some soils can naturally suppress plant diseases while others cannot have provided the basis of most research on BCAs.

Biological approaches to the control of soilborne plant pathogens were discovered more than 70 years ago in the classical works of G. Sanford and R. Weindling, however, it was only in the 1960s that biological control research took on a more practical approach (8). Since that time, many agrochemical and biotechnological companies throughout the world have increased their interest and investment in the biological control of plant diseases and pests. The current market for BCAs is estimated at only $500 million, which is about 1% of the world's total output for crop protection. The largest share of this market involves biopesticides marketed for insect control (mainly products based on *Bacillus thuringiensis* strains which produce a protein toxin with strong insecticidal activity) and these bioinsecticides represent around 4.5% of the world's insecticide sales. Other agents used for biocontrol exist on a much smaller scale commercially. However, the biopesticides market is expected to grow over the next 10 years at a rate of 10 to 15% per annum, versus 1 to 2% for chemical pesticides (1). Despite the fact that the total register of commercially available

biocontrol products is still small, their number has increased more than twofold in the last few years. For plant pathogens alone, the current list of microbial antagonists available for use in commercial disease biocontrol includes around 40 preparations (3,5). These are all based on the practical application of several species of nonpathogenic bacteria and fungi able to suppress plant pathogen growth (Table 1). The main topics discussed in this review are the following: the: biocontrol of soilborne diseases and nematodes by fungal and bacterial biocontrol agents, mechanisms of biological control, formulation, and delivery of biocontrol agents, combined usage of biocontrol agents, integrated pest management, and molecular approaches for improvement of biocontrol agents.

BIOCONTROL OF SOILBORNE DISEASES

Fungi as Biocontrol Agents

Fungi are relatively easy to grow and formulate for large-scale application. The first fungal antagonist to have been introduced commercially was *Peniophora* (Phlebia) *gigantea*, used for the control of *Heterobasidium* (*Fomes*) *annosum* root rot on pines (9). Since then several other fungi have been marketed as BCAs (Table 1). Among them, the necrotrophic mycoparasitic fungus *Gliocladium virens* Miller, Giddens, and Foster (=*Trichoderma virens* Miller, Giddens, Foster, and von Ark) is a common soil saprophyte and one of the most promising and studied fungal BCAs. *Trichoderma*, a genus of hyphomycetes and an anamorphic Hypocreaceae (class Ascomycetes) closely related to *Gliocladium*, is also common in the environment, especially in soils. Species of this genus have been used in the production of cellulolytic and hemicellulolytic enzymes, biological control of plant disease, biodegradation of chlorophenolic compounds, and soil bioremediation. The first report on a biological control experiment using *Trichoderma* spp. under natural field conditions came from Wells and coworkers (10), who used *Trichoderma harzianum* grown on an autoclaved mixture of ryegrass seeds and soil to control *Sclerotium rolfsii* Sacc. Since that time, more *Gliocladium* and *Trichoderma* isolates have been obtained from natural habitats and used in biocontrol trials against several soilborne plant pathogenic fungi under both greenhouse and field conditions (11,12). In particular, isolates of *G. virens*, *Gliocladium roseum*, *Trichoderma viride*, *T. harzianum* Rifai, and *Trichoderma hamatum* have been reported to be antagonists of many phytopathogenic fungi, including *Botrytis cinerea*, *Fusarium* spp., *Phytophthora cactorum*, *Pythium ultimum*, *Pythium aphanidermatum*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *S. minor*, *S. rolfsii*, and several other fungal pathogens that cause soilborne and foliage diseases in a wide variety of economically important crops under a wide range of environmental conditions. The antagonists kill the host by direct hyphal contact, causing the affected cells to collapse or disintegrate; vegetative hyphae of all species have been found susceptible. Thus, treatment of soil infested with propagules of *R. solani* or *P. ultimum* with *G. virens* resulted in a significant reduction in the number of viable *R. solani* sclerotia. Several isolates of *T. harzianum*, capable of lysing mycelia of *S. rolfsii* and *R. solani* by application to pathogen-infested soil, significantly reduced the diseases caused by these fungi in bean, cotton, tomato, and eggplant seedlings under greenhouse conditions. A seed treatment was developed to reduce the amount of *Trichoderma* added to the soil to control soilborne plant pathogenic fungi. *Trichoderma hamatum* conidia applied in the laboratory to seeds of pea and radish provided protection to seeds and seedlings from *Pythium* spp. and *R. solani*, respectively, almost as effectively as fungicide seed treatment. A *T. harzianum* isolate applied to either soil or rooting mixture efficiently controlled damping-off induced by *P. aphanidermatum* in several crops with disease reduction of up to 85%. Commercial preparations based on strain *T. harzianum* T-22 (Table 1) are sold to the greenhouse, row crop, and turf industries. Applied as a seed treatment, as a drench or incorporated into the soil as granules, the strains significantly increase yield of field corn, wheat, potato, and other crops. Granules of T-22 added to potting materials used in the production of vegetables and ornamentals reduced the incidence of diseases caused by *Fusarium* and other pathogens. The strain was shown to be highly rhizosphere competent, resulting in long-term root colonization and substantial advantages to plant growth and productivity (13).

Biological control of wilt pathogens that penetrate the vascular system of their hosts (e.g., *Fusarium oxysporum* or *Verticillium dahliae*) and that may attack the plant at various growth stages is considered to be less feasible than control of plant pathogens, which induce damping-off. In the latter case, the host is only susceptible to the pathogen for a limited time, at the early growth stage of the plant, after which it is not susceptible to infection. Thus, for biocontrol of damping-off, protection is required for only a limited time. This might be the reason for the slower progress in developing introduced antagonists for biocontrol of wilt pathogens. Nevertheless, *T. harzianum* T-35 isolated from the rhizosphere of cotton plants grown in fields infested with *Fusarium* significantly reduced the disease incidence caused by *F. oxysporum* f. sp. *vasinfectum*, *F. oxysporum* f. sp. *melonis* and *F. roseum* Culmorum in cotton, melon, and wheat, respectively, in greenhouse trials when applied as a peat-bran preparation, a conidial suspension or a seed coating. In trials over two successive growing seasons against *Fusarium* crown rot of tomato in fields naturally infested with *F. oxysporum* f. sp. *radici lycopersici*, this isolate provided a significant increase in the total yield of tomatoes (11). This and several other isolates of *Trichoderma* are produced commercially for the biocontrol of soilborne and aerial fungal diseases (Table 1).

Other fungal BCA, *Pythium nunn*, a necrotrophic fungus with a limited host range, was isolated from soil suppressive to a plant parasitic *Pythium* sp. The fungus efficiently suppresses growth of *P. ultimum*, *P. aphanidermatum*, *R. solani*, and *Phytophthora parasitica* by penetration and/or lysis of the host cell wall at the site of interaction. It can be used alone or in combination with *T. harzianum*. The fungus *Talaromyces flavus* (Klocker) A.C. Stolk and Samson is a mycoparasite of several soilborne plant pathogenic fungi, including

Table 1. Examples of the Microbial Products Containing Living Organisms Currently Employed for Biocontrol of Crop Diseases and Nematodes

Biocontrol Organism	Trade Name	Formulation	Application	Examples of Target Pathogens/Effects	Examples of Target Crop
Bacteria					
Agrobacterium radiobacter strain K84	Galltrol-A, Nogall, Diegall, Norbac 84 C	Culture grown on agar, culture suspensions	Bacterial suspension applied to seeds, seedlings, cuttings, roots, stems, and as soil drench	Crown gall disease caused by *A. tumefaciens*	Fruit, nut, and ornamental nursery stock
Bacillus subtilis strains GB03, FZB24 and others	HiStick N/T, Kodiak, Kodiak HB, Kodiak AT, Rhizo-Plus, Rhizo-Plus Konz, Serenade, Subtilex, System 3	Dry powder usually applied with chemical fungicides	Water-based slurry seed treatment; water-based suspension for in-furrow treatments, hopper box treatment, seed treatment, soil drench, dip, and addition to nutrient solutions	*Alternaria* spp., *Aspergillus* spp., *Fusarium* spp., *R. solani*, *Streptomyces scabies Verticillium*	Barley, beans, corn, cotton, peanut, pea, potatoes, rice, soybean, ornamental plants
Burkholderia cepacia	Dany, Intercept	Peat-based dried biomass from solid fermentation; aqueous suspension	Applied to seeds with a sticking agent in planter box (aqueous suspension formulation is for use in drip irrigation or as a seedling drench)	*Fusarium* spp., *Pythium* sp.; *R. solani*, nematodes	Alfalfa, barley, beans, clover, cotton, peas, sorghum, vegetables, wheat
Pseudomonas aureofaciens strain Tx-1	Spot-Less	Liquid	Over-head irrigation	Anthracnose, Dollar spot, Michrochium patch (pink snow mold), *P. aphanidermatum*	Turf grass
Pseudomonas chlororaphis	Cedomon	Seed treatment	Seed dressing	*Fusarium* sp., Leaf stripe and spot.	Barley and oats; potential for wheat and other cereals
Pseudomonas fluorescens A506	BlightBan A506	Wettable powder	Bloom time spray of the flower and fruit	Frost damage, *Erwinia amylovora*, and russet-inducing bacteria	Almond, apple, apricot, blueberry, cherry, peach, pear, potato, strawberry, and tomato
Pseudomonas syringae strains ESC10, ESC11	Bio-save 100, Bio-save 1000	Frozen cell concentrated pellets	Pellets added to water to produce liquid suspension, postharvest application to fruit as drench, dip, or spray	*Botrytis cinerea*, *Geotrichum candidum*, *Mucor pyroformis*, *Penicillium* spp.,	Pome fruit, citrus
Streptomyces griseoviridis strain K61	Mycostop	Powder	Drench, spray, or through irrigation system	*Alternaria brassicola*, *Botrytis* spp., *Fusarium* spp., *Phomopsis* spp., *Phytophthora* spp., *Pythium* spp., and	Field, ornamental, and vegetable crops
Fungi					
Ampelomyces quisqualis	AQ10	Water-dispersible granules	Spray	Powdery mildew	Apples, cucurbits, grapes, ornamentals, strawberries, and tomatoes

(*continued overleaf*)

Table 1. (*Continued*)

Biocontrol Organism	Trade Name	Formulation	Application	Examples of Target Pathogens/Effects	Examples of Target Crop
Coniothyrium minitans	Contans, Kony	Water-dispersible granule	Spray, granules incorporated into soil or soilless mix	*Sclerotinia sclerotiorum*, *S. minor*	Canola, sunflower, peanut, soybean, vegetables, and ornamental flowers in greenhouse production
Fusarium oxysporum, non-pathogenic	Biofox C Fusaclean	Spores, dust, or alginate granules	In drip to rock wool; incorporate in potting mix; soil incorporation; seed treatment	*Fusarium oxysporum*, *Fusarium moniliforme*	Asparagus, basil, carnation, cyclamen, gerbera, and tomato
Gliocladium catenulatum	Primastop	Wettable powder	Drench and incorporation	*Botrytis* spp., *Didymella* spp., *Pythium* spp., *Rhizoctonia* spp.,	Greenhouse crops
Gliocladium virens strain GL-21	SoilGard	Alginate granules	Granules incorporated in soil or soilless growing media prior to seeding	*Pythium* spp. and *R. solani*	Ornamentals, food crops
Paecilomyces lilacinus	Paecil	Dry spore concentrate	Seedling or soil drench	Various nematode spp.	Banana, wheat, and potatoes
Phlebia gigantea	Rotstop, P.g. Suspension	Spores in inert powder	Spray, chain saw oil	*Heterobasidium annosum*	Trees
Pythium oligandrum	Polyver-sum	Wettable powder	Root and stem drench, spray	*Aphanomyces* spp., *Alternaria* spp., *Botrytis* spp., *Fusarium* spp., *G. graminis* , *Pythium* spp., *Phytophthora* spp., *R. solani*, *S. cepivorum*, *Tilletia caries*	Canola, Cereals, fruits, forest nurseries, legumes, vegetables, and ornamental plants
Talaromyces flavus isolate V117b	Protus WG	Water-dispersible powder	Soil or seed treatment, soil drench, root dip application	*Rhizoctonia solani*, *V. dahliae*, *V. alboatrum*	Cucumber, tomato, and rapeseed oil
Trichoderma harzianum strain Y	Trichoderma-2000	Peat-bran preparation	Incorporated into soil or potting medium	*Fusarium* spp., *Pythium* spp., *R. solani*, *S. rolfsii*	Nursery and field crops
Trichoderma harzianum strain T-22 (KRL-AG2)	PlantShield, RootShield, RootShield Home and Garden, T-22 Planter Box, Turf Shield,	Wettable powder, granules, or dry powder	Granules added in-furrow, by broadcast application to turf, mixed with greenhouse soil, or by mixing powder with seeds in planter box, or in commercial seed treatment slurry	*Fusarium* spp., *Pythium* spp., *R. solani*, *Sclerotinia homoeocarpa*	Bean, cabbage, corn, cotton, cucumber, peanut, potato, sorghum, soybean, sugar beet, tomato, turf, greenhouse ornamentals
T. harzianum strain T39	Trichodex	Wettable powder	Spray	*Botrytis cinerea*, *Collectotrichum* spp., *Fulvia fulva, Monilia laxa, Plasmopara viticola, Pseudoperonospora cubensis, Rhizopus stolonifer, S. sclerotiorum*	Cucumber, grape, nectarine, soybean, strawberry, sunflower, tomato
T. viride Pers ex Gray	EcoSOM, Trieco	Powder	Dry or wet seed, tuber, or set dressing or soil drench, spread/broadcast over field	*Fusarium spp., Pythium spp.*, *Rhizoctonia spp.*	Tobacco, cereals, citrus, coffee, cotton, grapes, oilseeds, soybean, tea, tomato, vegetables, and others

(*continued overleaf*)

Table 1. (*Continued*)

Biocontrol Organism	Trade Name	Formulation	Application	Examples of Target Pathogens/Effects	Examples of Target Crop
Trichoderma harzianum other strains, *Trichoderma* spp.	Bio-Fungus, Root Pro, Supresivit	Granular, wettable powder, sticks, and crumbles	Applied after fumigation; incorporated in soil; sprayed or injected	*Armillaria*, *Botryosphaeria*, *Chondrosternum*, *Fusarium*, *Nectria*, *Phytophthora*, *Pythium* spp.,*Rhizoctonia*, *Sclerotinia*, *Verticillium*	Flowers, strawberry, trees, vegetables
Mixture of *T. harzianum* and *T. viride*	Trichopel, Trichoject, Trichoseal, Trichodo-wels				
Mixture of *T. harzianum* and *T. polysporum*	Binab T	Wettable powder and pellets	Spray, mixing with potting substrate, mixing with water and painting on tree wounds, inserting pellets in holes drilled in wood	Pathogenic fungi that cause wilt, take-all, root rot, and internal decay of wood products and decay in tree wounds	Flowers, fruit, ornamentals, turf, and vegetables

Modified from D. Fravel's list of commercial biocontrol products for use against soilborne crop diseases, updated on September 20, 2000 and available through the Internet (*http://www.barc.usda.gov/psi/bpdl/bpdlprod/bioprod.html*).

S. sclerotiorum, *R. solani*, and *Verticillium* spp. The mechanism by which *T. flavus* controls plant pathogenic fungi is not always clear and in some cases, it may involve a combination of antibiosis and mycoparasitism. The necrotrophic mycoparasite *Coniothyrium minitans* Campbell has been found to be a natural destructive mycoparasite of *S. sclerotiorum*, which kills both hyphae and sclerotia of this phytopathogen. Infection by *C. minitans* actually provided natural biological control of *S. sclerotiorum* and *Sclerotium cepivorum* under greenhouse conditions and in the field. *Coniothyrium minitans* could be recovered from the soil more than a year after its application, and during this time it spread throughout the greenhouse, indicating its potential for long-term control. The fungus *Fusarium chlamydosporum* was shown efficient against groundnut rust caused by *Puccinia arachidis*, whereas the soilborne fungal BCAs, *Trametes versicolor*, and *Pleurotus eryngii* were effective against *F. oxysporum* f. sp. *lycopersici* race 2 on tomato. The fungus *Penicillium purpurogenum* protected peach and tomato against the plant pathogens *Monilinia laxa* and *F. oxysporum* f. sp. *lycopersici.* Isolates of *T. flavus* and *Stenotrophomonas maltophilia* were shown to antagonize *S. rolfsii* and *P. ultimum*, respectively. The biotrophic mycoparasite *Sporidesmium sclerotivorum* Uecker, Ayers, and Adams is a dermatiaceous hyphomycete, which was isolated from field soil. The fungus has been found to be an obligate parasite on sclerotia of *S. sclerotiorum*, *S. minor*, *S. trifoliorum*, *Sclerotium cepivorum*, and *B. cinerea* under natural conditions (14). The fungus *Ampelomyces quisqualis* Ces. has been reported as a biotrophic mycoparasite and BCA of many fungi, which cause powdery mildew. An isolate of *A. quisqualis* M-10 obtained from an *Oidium* sp. infecting *Catha edulis* in Israel was developed as a BCA (preparation AQ10–Table 1) of several powdery mildew fungi belonging to the genera *Oidium*, *Erysiphe*, *Sphaerotheca*, *Podosphaera*, *Uncinula*, and *Leveillula* (15).

Bacteria as Biocontrol Agents

Many strains of soilborne and rhizospheric bacteria have been used for biocontrol of plant disease. The bacteria are easy to grow and can be applied as a seed treatment or as a soil drench, and mechanisms by which they reduce disease have been studied. The bacteria appear to protect plants against a wide range of pathogens and the potential for commercial utilization is promising. Bacterial BCAs can reduce disease by different modes of action, including production of antibiotics, toxins and lytic enzymes, direct parasitism of pathogen hyphae or propagules, competitive exclusion by occupation of infection sites and/or depletion of nutrients, plant growth enhancement and induction of host resistance mechanisms (3).

Most bacterial BCAs are gram-negative. Among them, *Agrobacterium radiobacter* strain 84 is probably the best example of the application of a bacterial antagonist for the biocontrol of an economically important plant pathogen *Agrobacterium tumefaciens*, a causal agent of crown gall disease in a large number of crops and considered one of the most destructive agricultural pathogens. Introduction of this strain into agricultural practice was the first case of successful commercialization of a BCA (16).

Rhizospheric bacteria belonging to the fluorescent *Pseudomonas* spp. are receiving increasing attention for the protection of plants against soilborne fungal pathogens (3). These bacteria, defined as root-inhabiting, plant growth-promoting rhizobacteria (PGPR) have been shown to improve plant health and increase yield. PGPR may exert their effect directly and/or indirectly by the suppression of plant pathogens, thereby providing biological control (17). Direct promotion of plant growth may be exerted by several mechanisms, for example, via the production of auxin and other plant hormones, synthesis of siderophores, biological nitrogen fixation, solubilization of minerals such as phosphorus, or via

enzymatic activities such as 1-aminocyclopropane-1-carboxylate (ACC) deaminase, an enzyme, which decreases the endogenous concentration of ethylene. PGPR strains producing this enzyme may have a competitive edge over other microorganisms in the rhizosphere because they can use ACC as a source of nitrogen (18). Several other mechanisms have been proposed to account for PGPR's disease-suppressive ability, including antagonism, and induced resistance. However, before the deliberate use of PGPR and other bacteria as biofertilizers and BCAs, it is necessary to know some key parameters, such as root colonization capacity, location of infection site, and degree of persistence of the inoculum. The most suitable techniques for studying these parameters are microscopical and immunological.

One of the most intensively studied biocontrol systems using rhizobacteria was generated after the isolation of *Pseudomonas fluorescens* strains from a soil naturally suppressive to Gtg-take-all of wheat. These fluorescent rhizosphere-colonizing bacteria, when applied as seed treatments to a soil conducive to the diseases, provided significant disease control. Other pseudomonad species with biocontrol activity have been described. Among them strains of *Burkholderia* (*Pseudomonas*) *cepacia*, *Pseudomonas aureofaciens*, *P. chlororaphis*, *P. corrugata*, *P. putida*, and *P. syringae* (3,5).

Besides *Pseudomonas* spp., several other gram-negative bacteria exhibit highly efficient biocontrol activity. For example, the bacterium *Enterobacter cloacae* was shown to be a capable BCA of different rot and pre-emergence damping-off diseases incited by *Pythium* spp., as well as of fusarium wilt and of some other plant diseases caused by fungal pathogens (19,20). Biocontrol potential was also demonstrated for strains of *Serratia marcescens* and *Enterobacter agglomerans* producing chitinolytic enzymes (21,22).

Gram-positive bacteria of *Bacillus* spp. are widely dispersed in nature, easy to reproduce, have a long shelf life when sporulated, and are nonpathogenic. The ability to produce endospores provides bacilli with a high tolerance to heat and desiccation. Therefore, they have a significant ecological advantage over other bacterial antagonists (23). Several strains of *Bacillus* are now produced commercially as BCAs (Table 1). Because *Bacillus subtilis* is a spore-forming organism, it is extremely tolerant of environmental stresses, including seed treatment pesticides, soil and seed pH, cultivar effects, edaphic factors, and long-term storage. It is readily produced with current fermentation technology. Other BCAs, such as *Pseudomonas* spp., do not readily adapt to large-scale production methods, and stability is a limiting factor.

BIOCONTROL OF NEMATODES

Nematodes are small, round worms, which are common inhabitants of many natural environments. Some of them are pathogenic to plants. Plant parasitic nematodes are major agronomic pests, with the annual global loss in agriculture of U.S. $100 billion worldwide caused by nematode damage to plants. The most important nematodes are root-knot (*Meloidogyne*), which have multiple generation per year and are the leading cause of crop loss due to plant parasitic nematodes, and cyst (*Heterodera* and *Globodera*) nematodes that have a single generation per year and cause damage mainly in the early root-infection process.

Natural enemies of nematodes include viruses, rickettsias, bacteria, and nematophagous fungi. Antagonists most likely to be receptive to management for the biocontrol of nematodes are trapping fungi, fungal egg pathogens or parasites, endoparasitic fungi, fungal pathogen or parasites of females, endomycorrizial fungi, plant growth-promoting bacteria, obligate bacteria parasites. The parasitoid endospore-forming bacterium *Pasteuria penetrans*is able to survive in the soil under extreme conditions and destroys nematodes by parasitic action. This bacterium is an obligate parasite of many nematode species thought to be responsible for the decline in nematode populations in some stable monocultures (e.g., sugarcane) and perennial cropping systems (e.g., grapes). *Pasteuria penetrans* has been an effective biocontrol agent against root-knot nematodes. An application of endospores to soil prevent juvenile females attacking roots. The limitation of the bacterium practical sense is that it can be cultured only in its nematode host or in association with host cells.

Other genera of bacteria known to reduce nematode populations mainly by colonizing the rhizosphere of the host plant include *Agrobacterium*, *Alcaligenes*, *Bacillus*, *Clostridium*, *Desulfovibrio*, *Pseudomonas*, *Serratia, Streptomyces,* and *Telluria.* spp. Application of some of these bacteria has shown very promising results (24). Fungi described as efficient for nematode biocontrol include mainly nematode-trapping species (e.g., *Arthrobotrys oligospora*) possessing the ability to capture, kill, and utilize nematodes by using specially adapted mycelial structures called traps, and egg-parasitic species (e.g., *Verticillium chlamidosporium*, *Paecilomyces lilacinus T. harzianum*, and *Fusarium* spp.) whose action in reducing the nematode populations is rather dramatic compared with other parasitic or predacious fungi. However, the endoparasitic fungi are most specific for their nematode host. They produce conidia or resting spores that are surrounded by adhesive material that allows the spores to attach to the nematode cuticle.

The most important obligate parasites of female cyst nematodes of the genus *Heterodera* are *Nematophthora gynophila* and *Catenaria auxiliaris*. Thus, *N. gynophila* takes up to seven days to kill female cyst nematodes. The resting spores can survive at least five years in the absence of cyst nematode females. Combined activities of *N. gynophila* and facultative parasite of females *V. chlamidosporum* are largely held responsible for natural reduction in *Heterodera avanae*, a cereal cyst nematode.

Endomycorrhizial fungi limit the densities of plant parasitic nematodes on a broad range of host plants. Mycorrhizial fungi *Gigaspora* and *Glomus* decrease damage caused by root-knot nematodes, especially where transplants are used in crop production. Endotrophic mycorrhizial fungus *Glomus mossea* was shown efficient toward

Meloidogyne incognita and *Rotylenchulus reniformis* in cotton, cucumber, tomato and so on (25,26).

MECHANISMS OF BIOLOGICAL CONTROL

Biocontrol strategies may be divided into two broad categories. One follows a fundamentally ecological approach. This strategy has been termed classical biocontrol or biocontrol with one-time introduction (27). The other uses microorganisms as biopesticides and resembles, in some important respects, the approach similar to chemical pesticide treatment (which aims at control for a limited period of time). This strategy has also been referred to as augmentative biocontrol. In the ecological approach, selected organisms must be able to function in the same environmental niche as that of the pathogen they are meant to control. Biological control of plant diseases can be achieved directly, through introduction of specific microbial antagonists into the soil or plant material, or indirectly, by changing the conditions prevailing in the plant's environment, and thus the microbiological equilibrium of its ecosystem. In some cases, a combination of indirect and direct approaches leads to the best biocontrol results.

Direct Biological Control

The direct approach includes four general direct mechanisms of biological control of plant diseases by soil antagonistic microorganisms: (*1*) "competition" with the pathogen for limited resources, such as space or nutrients, this is considered an important mechanism of biocontrol by bacterial and fungal antagonists; (*2*) "antibiosis," which is the inhibition or destruction of an organism by the secondary metabolites produced by another organism; (*3*) "production of lytic enzymes" (e.g., chitinases, glucanases, cellulases, and proteases) able to degrade the pathogen cell wall and/or to destroy its metabolic activities; (*4*) "predation or parasitism," the phenomenon that occurs when one fungus exists in intimate association with another from which it derives some or all its nutrients while conferring no benefits in return.

Competition. Many plant pathogens require exogenous nutrients to successfully germinate, then penetrate and infect the host tissue. In the rhizosphere, competition for space and nutrients is of major importance. Therefore, competition for limiting nutritional factors, mainly carbon, nitrogen, and iron, may result in the biological control of plant pathogens. Indeed, competition is one of the main mechanisms involved in controlling plant pathogens. Most work on bacterial competition has concentrated on iron (Fe). Many plant-beneficial rhizospheric bacteria, for example, those that belong to the pseudomonads (*P. fluorescens* and *P. putida*, to name a few) produce fluorescent siderophores, that is, low molecular weight microbial iron-transport cofactors. These high-affinity ferric iron (Fe^{3+}) chelators specifically enhance the bacteria's iron acquisition by binding to membrane-bound siderophore receptors. Siderophore production by the fluorescent pseudomonads leads to severe iron depletion in the rhizosphere and can limit the growth of some phytopathogenic fungi (e.g., *Fusarium* spp.) and bacteria (e.g., *Pseudomonas syringae*). As a result, these plant pathogens end up in an iron-deficient environment deleterious to their growth.

A competitive exclusion mechanism was shown responsible for the biological control of *P. ultimum* -incited seed infections by *E. cloacae* in which *E. cloacae* prevents the germination of the seed-rotting oomycete *P. ultimum* sporangia by the efficient metabolism of fatty acid components of seed exudate and thus prevents seed infections. It was conclusively demonstrated (a) that unsaturated fatty acids from seeds and roots are required to elicit germination of *P. ultimum* around planted seeds, and (b) that very rapid metabolism of unsaturated fatty acids that leach from seeds is the primary mechanism by which *E. cloacea* controls *Pythium* seed rot (28).

Antagonists capable of more efficiently utilizing essential resources (e.g., carbon, nitrogen, volatile organic materials, plant residues, iron, and microelements) effectively compete with the pathogen for its ecological niche and colonization of the rhizosphere and/or phyllosphere, leaving the pathogen less efficient to grow in the soil or colonize the plant. An important attribute of a successful rhizosphere BCA is the ability to remain at a high population density on the root surface, providing protection of the whole root for the duration of its life. The ability of a microorganism applied as a seed treatment to proliferate and establish along the developing root system has been termed "rhizosphere competence" (29). For example, competition for carbon and nitrogen in the rhizosphere, as well as rhizosphere competence, may be involved in the biocontrol of *F. oxysporum* by *T. harzianum* (11).

Antibiosis. This mechanism includes small toxic molecules, volatiles, and lytic enzymes that kill the target organism. Antibiosis is restricted for the most part to those interactions that involve a chemically heterogeneous group of low molecular weight (less than 1 kDa) diffusible organic compounds (known as antibiotics) produced by a microorganism, which at low concentrations, inhibit the growth or metabolic activities of another microorganism (30,31). In recent years, genetic and molecular studies, specific and sensitive assays, and detection systems have provided evidence of the contribution of antibiotics to pathogen suppression and disease reduction in vitro and in situ, under greenhouse and field conditions. Under natural conditions, antibiotics participate in soil suppressivenes and the antagonism of phytopathogens. However, even in cases where antifungal metabolite production by an agent definitely reduces disease, other mechanisms may also be operating.

Antibiotics Produced by Bacterial Biocontrol Agents. A large number of antibiotics produced by bacterial BCAs appear to be involved in the biocontrol of plant pathogens. Many individual strains can produce a set of antibiotics and secondary metabolites important in biocontrol. One of the best known is biocontrol of *A. tumefaciens* strains carrying the nopaline-type Ti plasmids, which are causative agents of crown gall disease in many dicotyledonous plants (16). The soilborne nonpathogenic

strain *A. radiobacter* K84 produces the bacteriocin agrocin 84, a low–molecular weight adenosine derivative with a very specific host range against pathogenic *A. tumefaciens*.

A large number of pseudomonads active as biocontrol strains are able to produce a diverse array of potent antifungal metabolites. The most well-characterized are simple metabolites such as phenazines (Phz), 2,4-diacetylphloroglucinol (Phl), pyoluteorin (Plt), pyrrolnitrin (Prn), hydrogen cyanide (HCN), and some others (30,32–35).

Pnz are nitrogen-containing heterocyclic molecules, two of the main ones being phenazine-1-carboxylic acid (PCA) and 2-hydroxy-phenazine-1-carboxylic acid (2-OH-PCA). The antibiotics have fungistatic effect on *G. graminis* var. *tritici* (take-all disease of wheat) growth. They act as electron donor or acceptors, which interfere with normal membrane function in target organisms resulting in increased level of toxic intracellular active oxygen products. Phz production by strain 30–84 of *P. aureofaciens* contributes to its ecological competence on plant roots in competition with indigenous microflora in natural soils. Therefore, Phz production was shown to be required for both BCA survival and pathogen inhibition.

Phl, a member of the phloroquinoline class of compounds, is a major factor in the biological control of *Thielaviopsis basicola* (black root rot), *G. graminis* var. *tritici* (take-all disease of wheat), and a range of other plant pathogens. Its antifungal activity may be realized via disruption of the fungal membrane. Phl was found in the rhizosphere of plants inoculated with Phl-producing bacteria and in the rhizosphere of wheat grown in suppressive soil. Production of Phl by bacteria may activate the host plant's defense systems.

Prn, or 3-chloro-4-(2′-nitro-3′-chlorophenyl)-pyrrole, synthesized from tryptophan, possesses wide range of antimicrobial activity. This secondary metabolite may interfere with normal membrane function in the target organism. Prn-producing strains of *Burkholderia cepacia*, *P. fluorescens*, *E. agglomerans*, and *Serratia plymuthica* were shown able to control *P. ultimum*, *R. solani*, and some other fungi. However, Prn is highly sensitive to sunlight and therefore was found to be unsuitable for use as an agricultural fungicide. Derivatives of Prn, called fenpiclonil and fludioxonil, with increased stability under light due to substitution of the chlorine on the pyrrole ring with a cyano group, were developed by Ciba-Geigy (Novartis) company as environmentally safe agricultural fungicides.

Plt, an antibiotic that consists of a resorcinol ring linked to a bi-chlorinated pyrrol moiety, is produced by biocontrol strains Pf-5 and CHAO of *P. fluorescens*, and has been shown to be most toxic to *Pythium* spp. However, the role of Plt in biological control varies depending on the patterns of its gene expression and production by the bacteria, which differ in the rhizosphere and spermosphere of various plant hosts.

HCN is a volatile compound shown to be enzymatically generated from glycine by several *P. fluorescens* strains and this trait is linked to the bacteria's ability to suppress the black root rot disease of tomato and tobacco, caused by the fungus *T. basicola*. Production of HCN is stimulated by iron. HCN has a direct effect on plant roots (stimulates root hair formation) and may induce plant defense mechanisms.

Antibiotics providing strain-producer biocontrol activity have been described in several gram-positive bacteria, mainly of *Bacillus* spp. Thus, *B. subtilis* strains with antifungal activity have been shown to produce the antifungal lipopeptides iturin A and surfacin. Antibiotics active against various plant pathogens have also been found in strains of *Bacillus cereus*, *Bacillus pumilus*, and *Bacillus licheniformis*. Many of these bacilli are generally soil-inhabiting bacteria or exist as epiphytes and endophytes in the spermosphere (23). For this reason, *Bacillus* species among other strains in the genera *Pseudomonas*, *Burkholderia*, *Enterobacter,* and *Trichoderma* spp. known as excellent root colonizers, both internally and externally, are good candidates for use as BCAs against soil borne pathogens. Examples of bacterial antibiotics that may be involved in disease biocontrol are presented in Table 2.

Antibiotics Produced by Fungal Biocontrol Agents. Species of *Gliocladium*, and *Trichoderma*, *T. flavus*, *Chaetomium globosum*, and some others produce a range of antibiotics, which are active against pathogens in

Table 2. Examples of Bacterial Antibiotics and Other Secondary Metabolites That May Be Involved in Crop Disease Biocontrol

Metabolite	Pathogen	Disease
Phenazines	*Gaeumannomyces graminis* var. *tritici*	Take-all of wheat
Pyrrolnitrin	*Alternaria* spp., *B. cinerea*, *Pythium* spp., *R. solani*, *S. sclerotiorum*, *Fusarium sambucinum*, *V. dahliae*	Seedling diseases, gray mold, root rot and damping-off, white mold, dry rot of potato, wilt
Oomycin A	*Pythium* spp.	Damping-off
Pyoluteorin	*Pythium ultimum*	Damping-off
2,4-Diacetylphloroglucinol	*Rhizoctonia solani*, *P. ultimum*, *T. basicola*	Root rot and damping-off, Black root rot of tobacco
Hydrogen cyanide	*Thielaviopsis basicola*	Black root rot of tobacco
Agrocin 84	*Agrobacterium tumefaciens*	Crown gall of fruit trees
Pseudobactin 10	*Fusarium oxysporum lini*	Flax wilt
Ammonia	*Pythium* spp.	Damping-off

Source: Modified from N. Dowling and F. O'Gara, *Trends Biotech.* **12**, 133–141 (1994), J. M. Ligon et al., *Pest Manage. Sci.* **56**, 688–695 (2000).

vitro and, consequently, antibiosis has been suggested to be involved in biological control by fungal antagonists (31,36). Several secondary metabolites produced by *Gliocladium* (= *Trichoderma*) *virens* were shown to be important in mycoparasite biocontrol activity. Thus, production of the antifungal antibiotics gliotoxin (an epidiothidiketopiperasine) and gliovirin (a diketopiperazine) has been associated with efficacy of *G. virens* as a BCA of seedling diseases incited by *Pythium* spp. and *R. solani*, which cause seedling damping-off and root rot, respectively, of many host plants. Gliotoxin appears to be the key factor in *G. virens* biocontrol activity against both these fungal pathogens, whereas gliovirin appears to kill the first fungus but not the last. Strains of *Trichoderma* produce more than 40 compounds with antibiotic activity. Many of them, including volatile alkyl pyrones, peptaibols, diketopiperazines, isonitriles, polyketides, and steroids are thought to be responsible for biocontrol activity of some *Trichoderma* isolates. Some antibiotics are synergistic with cell wall–degrading enzymes in their action against fungal phytopathogens. It is worth noting, however, that some highly effective biocontrol strains of *Trichoderma* may not produce the antibiotics shown to be important for the biocontrol efficiency of other *Trichoderma* strains. To illustrate, gliotoxin has been shown to be important for activity of the *G. virens* strain, which is the active component of SoilGard (Table 1), but it is not produced by *T. harzianum* T-22, which is described as one of the most efficient BCA (13).

Cell Wall Lysis. Most fungal plant pathogens have cell walls, which contain chitin as a structural backbone and laminarin (β-1,3-glucan) as a filling material. The other minor cell wall components are proteins and lipids. Chitin, an unbranched insoluble homopolymer consisting of β-1,4-linked N-acetylglucosamine (GlcNAc) units, is the second (after cellulose) most common biodegradable polysaccharide in nature, being the main structural component of cell walls of most fungi and arthropods (insects, nematodes, and other invertebrates) including many agricultural pests. Many species of bacteria, streptomycetes and actinomycetes, fungi, and plants produce chitinolytic enzymes, which catalyze the hydrolysis of chitin (37). Chitinolytic enzymes (chitinases) cleave a bond between two consecutive GlcNAc units. These enzymes play an important physiological and ecological role in ecosystems as recyclers of chitin, by generating carbon and nitrogen sources. In recent years, soilborne microorganisms that produce chitinases have become considered as potential BCA against fungal pathogens, insects, and nematodes, which cause diseases and damage in agricultural crops.

Lytic Enzymes Produced by Bacterial Antagonists. The ability to produce lytic enzymes is a widely distributed property of soil, marine, and rhizospheric bacteria. Many of these are potential BCA of chitin-containing plant pathogens. The list of chitinolytic bacterial antagonists includes *Aeromonas caviae*, *E. agglomerans*, a *Streptomyces* sp., *Pseudomonas stutzeri* and *P. fluorescens*, *S. marcescens*, and *S. plymuthica*, among others. Chitinase production may be part of a lytic system, which enables the bacteria to use living hyphae rather than chitin as the actual growth substrate, because chitin is an important constituent of most fungal cell walls. For example, a strain of *S. marcescens*, isolated from the rhizosphere of plants grown in soil infested with *S. rolfsii* Sacc., was found to be an effective BCA under greenhouse conditions against this pathogen and *R. solani* Kuhn. A chitinase(s) produced by the bacterium caused degradation of *S. rolfsii* hyphae in vitro, which provides evidence that this enzyme plays a role in biocontrol. Chitinolytic strains of *A. caviae* and *E. agglomerans* were able to control *R. solani* and *F. oxysporum* f. sp. *vasinfectum* in cotton and *S. rolfsii* in beans under greenhouse conditions. In many cases, however, the inhibition of fungal growth was not accompanied by bacterial chitinase production, indicating that other cell wall–degrading enzymes (β-1,3-glucanase and protease) and/or antibiotics may also be involved in the antagonistic activities of chitinolytic bacteria against fungi (38).

Lytic Enzymes Produced by Fungal Antagonists. The ability to produce lytic enzymes has been shown to be a crucial property of the mycoparasitic fungi *G. virens* and *Trichoderma* spp., and many bacterial antagonists of plant pathogenic fungi. In general, chitinolytic enzymes are not only involved in the destruction of the host cell wall; they may also play a role during the initial stages of mycoparasitism. However, the relevance and role of enzymes and toxic metabolite(s) of the mycoparasitic fungi's antagonism toward plant pathogens can vary among independent isolates and should be reinvestigated for each individual case. Moreover, the ability of lytic enzymes to provide biocontrol depends on the type of plant being protected and the fungal pathogen. Thus, the cell walls of basidiomycetes (e.g., *R. solani* and *S. rolfsii*) and ascomycetes (e.g., *Sclerotinia sclerotiorum*) contain mainly β-glucan and chitin, but no cellulose. In contrast, oomycetes (e.g., species of *Pythium* and *Phytophtora*) are composed of β-glucan (laminarin), cellulose and less than 1.5% chitin. Therefore, the cell wall lytic enzymes chitinase and β-1,3-glucanase have frequently been associated with biocontrol activity against the first groups of fungal phytopathogens, whereas β-1,3-glucanases and cellulases (β-1–4-glucanases and β-1–4-glucosidases) can provide biological control of the fungi belonging to the second group (38). Protease production is common in microorganisms, including fungi, among them *Trichoderma* strain T39 that serves as a BCA of the fungus *B. cinerea* (39) and is commercially produced as the biopreparation "Trichodex" (Table 1).

Chitinases and other lytic enzymes also play an important role in the biocontrol of plant pathogenic nematodes. The inhibition of *Globodera rostochiensis* egg hatch by the chitinase-producing bacteria *S. maltophilia* was suggested as a biocontrol strategy for the defense of potato crops against potato cyst nematodes (40). Another approach to controlling plant parasitic nematodes involves amending the soil with chitin that stimulates chitinolytic activity. This treatment drastically increases the populations of chitinolytic bacteria and fungi (41). The nematode's surface is composed of a multilayered cuticle, which consists mainly of collagen. Collagenolytic

activity was shown therefore to play a role in the antagonism of some fungi and bacteria toward plant pathogenic nematodes. Soil amendment by collagen sharply decreased the root-galling index of tomatoes inoculated with *Meloidogyne javanica*. This led to the enrichment of collagenolytic microorganisms, which drastically reduced the number of galls caused by *M. javanica*. The effect was even more pronounced when the amendment was supplemented with the collagenolytic fungus *Cunninghamella elegans* (42). The proteases produced by the fungus *P. lilacinus* and some others were shown to play a role in this fungal nematode-parasitic activity (43). Some of the fungal parasites of plant parasitic nematodes act by triggering induced resistance in the plant, by inhibition of the nematodes's host-recognition process or by producing toxins able to immobilize the nematode, following by penetration of hyphae through the cuticle (25,26).

Predation or Parasitism. This is a complex mechanism, which occurs when the BCA feeds directly on or inside the pathogen. Parasitism is defined as a direct attack on fungal cells followed by feeding by the parasite. When one fungus feeds on another fungus, it is generally called mycoparasitism. Competition for substrates already taken up by other fungi is an ecological feature shared by many mycoparasites. Briefly, mycoparasitism is a complex process, which involves "recognition" of the host, positive chemotropic growth, attachment, and de novo synthesis of a set of cell wall–degrading enzymes that aid the parasite in penetrating the host and completing its destruction. Lectins, the sugar-binding proteins or glycoproteins of nonimmune origin, which agglutinate cells and are involved in interactions between the cell's surface components and its extracellular environment, have been shown to play a role in the recognition and contact between necrotrophic mycoparasites of *Gliocladium* and *Trichoderma* spp. and soilborne pathogenic fungi. This contact, in turn, initiates a signal transduction cascade toward the second most important step of mycoparasitism, the induction of lytic enzymes able to degrade fungal cell walls (4).

Barnett and Binder (44) divided mycoparasitism into: (*1*) necrotrophic (destructive) parasitism, in which the relationship results in death and destruction of one or more components of the host thallus and (*2*) biotrophic (balanced) parasitism, in which the development of the parasite is favored by a living rather than dead host structure. Necrotrophic mycoparasites tend to be more aggressive, have a broad host range extending to wide taxonomic groups, and are relatively unspecialized in their mode of parasitism. The antagonistic activity of necrotrophic mycoparasites is attributed to the production of antibiotics, toxins, or hydrolytic enzymes in proportions that cause the death and destruction of their host. Necrotrophic mycoparasitic fungi, being more common, saprophytic in nature and less specialized in their mode of action, are easier to study. As a result, most mycoparasites (*P. nunn*, *T. flavus*, *C. minitans*, *Gliocladium* spp., and *Trichoderma* spp.), used as BCA in greenhouse or field trials to date have been necrotrophs. The biotrophic mycoparasites, in contrast to necrotrophic ones, tend to have a more restricted host range and produce specialized structures to absorb nutritients from their host. Because of their nature, only a few examples of biotrophic mycoparasites (*S. sclerotivorum* and *A. quisqualis*) as BCA exist (45).

Indirect Biological Control

Indirect approaches include: (*1*) use of organic soil amendments which enhance the activity of indigenous microbial antagonists against a specific pathogen and where changing conditions in the plant's environment may affect the parasite, its host, or the saprophytic microorganisms potentially involved in disease control (46); (*2*) plant growth promotion by root-colonizing bacteria and fungi which are able to stimulate plant growth and to increase tolerance to biotic and abiotic stresses through enhanced root and plant development (17); (*3*) induced resistance, which involves the stimulation of plant self-defense mechanisms against a particular pathogen by prior inoculation of the plant with a nonvirulent strain.

Successful induced resistance has been documented for both bacterial and fungal pathogens. Induced resistance is a plant response to challenge by microorganisms or abiotic agents, such that following the induced challenge, de novo resistance to pathogens is shown in normally susceptible plants. Induced resistance can be "localized" when it is detected only near the inducing factor, or "systemic," when resistance occurs subsequently at sites throughout the plant. Both localized and systemic types of induced resistance are nonspecific and can act against a wide range of pathogens. The difference between these two types of induced resistance is that localized resistance occurs in many plant species, whereas systemic resistance is limited to some plants. During localized resistance, the plant reacts to the environmental stimulus by activating a variety of defense mechanisms, which manifest themselves in various biochemical and physical changes. The general plant defense response consists of the induction of pathogenesis-related (PR) proteins and the deposition of structural polymers, such as callose and lignin.

Little is known about the distribution and/or the biochemistry of the interaction. Special emphasis has been placed on the accumulation of hydrolases such as chitinases and glucanases with antimicrobial potential. Another group of enzymes includes peroxidases, which play a key role in the plant resistance process, being involved in phenolic compounds synthesis and structural barrier formation. In some systemically protected plants (e.g., tobacco and cucumber), increases in newly formed PR proteins have also been recorded, and these may be chitinase-, glucanase-, or osmotin-like proteins. The induction of systemic resistance responses by sometimes only a single local lesion indicates involvement of an immunity signal(s), which are appeared at the induction site and then transduced to the target parts where it conditions the plant for resistance. Several endogenous plant compounds, salicylic acid, and jasmonic acid among them are promising candidate signals for induced systemic resistance. For example, application of exogenous salicylic

acid elicits PR protein patterns similar to those induced by pathogens, and the treated plants develop resistance to viral, bacterial, and fungal pathogens (47,48).

All known BCAs utilize one or more of these general indirect or direct mechanisms. At the product level, this includes the production of antibiotics, siderophores, and cell wall–lytic enzymes, and the production of substances that promote plant growth. Additionally, successful colonization of the root surface is considered a key property of prospective antagonists (17). The best BCAs may use two or three different mechanisms. Antagonists can also be combined to provide multiple mechanisms of action against one or more pathogens. Plant genes also play a role in biocontrol efficiency. Plant health depends, in part, on associations with disease-suppressive microflora, but little is known about the role of plant genes in establishing such associations.

Selection of Biocontrol Agents

Despite the increasing interest in studying the basic and applied aspects of biocontrol of plant pathogens, the commercial usage of biocontrol facilities is still very limited. The development of reliable biocontrol systems depends on an accurate assessment of the problems and on the limiting factors that interfere with them. One of the most prominent problems associated with biocontrol appears to be the inconsistent performance of microbial antagonists under field conditions. Several reasons can be attributed to this phenomenon, including screening techniques, ecological factors, mechanisms of control, and problems associated with formulation and large-scale production (11).

Different screening methods for the selection of BCAs have been described. Hence, using in vitro methods many strains of microorganisms with antagonistic properties have been isolated. In planta, screening methods have been developed to optimize selection of antagonists effective against a range of soilborne pathogens. Screening in the field follows screens in the laboratory or greenhouse. Ecologically adapted antagonists against seed and soilborne pathogens can be isolated from the surface of seeds and roots of the plants susceptible to the desired pathogen. A frequently used approach is to look for healthy plants in a field with diseased plants of the same species. Thus, effective isolates have been found in soils suppressive to take-all of wheat caused by fungus *G. graminis* var. *tritici* (49), or to *Fusarium* wilt (7). However, knowledge about the origin of an antagonist will always be relevant in connection with risk evaluation, and wild-type candidates isolated from natural locations can be expected to give less complications in the registration process (50).

Screening methods can be focused mainly on information about mechanisms of antagonism obtained in vitro or try to mimic greenhouse or field conditions. For example, two-component screening methods (e.g., dual cultures of a candidate antagonist and a pathogen on agar) are based on interaction studies. The potential antagonists are typically ranked according to their ability to inhibit the growth of the pathogen, as expressed by an inhibition zone. Dual cultures on agar are able to reveal the mycoparasitism of the pathogen (e.g., *R. solani*) by the antagonists (e.g., *T. harzianum*) (Fig. 1) or antibiosis causing the inhibition of the pathogen (e.g., *P. aphanidermatum*) by the antibiotics or siderophores produced by the antagonists (e.g., some bacteria) (Fig. 2). However, the results obtained with such plate tests do not necessarily indicate that a strain with good antagonistic activity in vitro will serve as an efficient BCA under natural environmental conditions. A soil plate method revealed a good correlation between the competitive saprophytic ability of a range of mycoparasitic *Trichoderma* isolates and their ability to destroy host (target) fungi in soil. In this method, an agar plate is first colonized by a host fungus, then particles of soil or plant material are placed on the surface. Only fungi that are able to derive nutrients from the precolonizing fungus and to tolerate its waste products will be able to grow. Mycoparasites isolated by this method include *G. roseum*, a *Papulaspora* sp., *Pythium* spp., and *Verticillium biguttatum* (50).

The introduced microbial antagonist will have to overcome competition from the indigenous soil microflora. Therefore, mechanisms other than those that can be

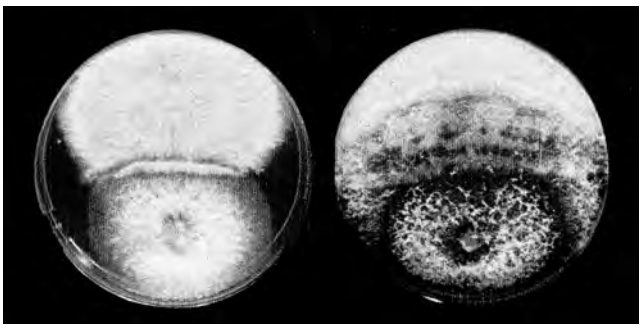

Figure 1. Dual cultures of mycoparasitic fungus *T. harzianum* (lower part) with the plant pathogenic fungus *R. solani* (upper part) growing simultaneously at 28 °C in a petri plates with potato-dextrose agar (PDA). Left and right plates are two and seven days after contact.

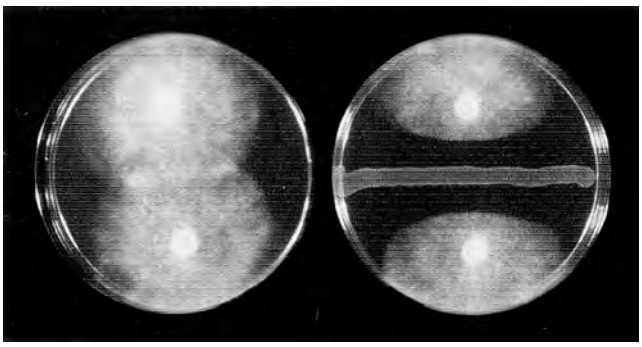

Figure 2. Assay of antifungal activity on petri plates. Two agar disks from an actively growing culture of *P. aphanidermatum* were placed on either side of the bacterial growth area, and the plates were incubated at 28 °C for two days. A suspension of bacterial strain *E. agglomerans* was seeded in a line through the center of a PDA plate and incubated at 28 °C for 24 hours (right plate). In a control plate (left plate) where sterile water was used instead of bacteria, the fungal mycelium covered the entire agar surface.

assessed in in vitro assays are important under field conditions. The ability of the antagonist to metabolize the exudate molecules produced by a protected plant, to colonize the plant rhizosphere and to protect infection sites in situ appears to be key factors in the biocontrol of soilborne plant pathogens. Soil pH may also be an important factor determining the success or failure of introduced antagonists. Competition and other antagonistic interactions (e.g., antibiosis and parasitism), between the introduced BCA and the pathogen may also take place in the rhizosphere. Other mechanisms, such as induced resistance and plant growth promotion, can also be taken into account by screening programs on the basis of approaches related to the plants.

FORMULATION AND DELIVERY OF BIOCONTROL AGENTS

Maintaining adequate viability of microorganisms throughout processing, storage, and application for biocontrol is critically important for commercial reasons. Unlike chemical pesticides that begin to degrade after application, BCA must survive and begin to proliferate. To compete with chemical pesticides, appropriate technology must be developed to improve the BCA stability, bioactivity, and shelf life. Therefore, formulating biocontrol organisms effectively is the key to their successful use. The formulation must be compatible with the requirements of the microorganisms, and even enhance their performance, and it should be compatible with regular agricultural practice, to be both acceptable and useful to the farmer. To be distributed through the normal commercial chain, the preparation should ideally have a shelf life of one to two years. The right formulation can help protect the microorganisms biologically and physically. To achieve this protection, the formulation should be able to induce low metabolic activity in the microorganism involved. This is usually brought about by reducing moisture potential by drying or by incorporating the agent into an oily microaerophilic environment. Surfactants, stickers, emulsifiers, and spreaders are added to formulations to improve their application to the target.

Commercial formulations of microbial agents have been used in the biocontrol of plant diseases and plant parasitic nematodes. Products aimed at soil pathogens can be formulated for bulk application in the plant growth media or for seed treatment, with or without a food base. The type of delivery system used to apply the BCA to its specific habitats important for its success. For example, a mixture of peat and wheat bran (1 : 1) was shown efficient medium for *T. harzianum* growth and sporulation. This formulation has been applied to seedlings before planting, to seeds in furrows, and in broadcast applications (51). The most extensively and successfully used bioencapsulation material is alginate. A method of forming alginate beads for encapsulation of biocontrol strains of *Trichoderma* and *Gliocladium* was developed. The fungal conidia, chlamydospores, or fermentation biomass are mixed in a 1% sodium alginate solution and added dropwise into a 2 to 5% calcium chloride solution. The formed pellets or granules are dried for use as a BCA. The resultant alginate-encapsulated cells or spores survive well in the soil. Encapsulation of microorganisms in an alginate matrix was considered to be an effective way of delivering useful microorganisms slowly into the soil for prolonged periods.

Various BCAs have been encapsulated in amylopectin (pregelatinized starch) to improve their survival under severe environmental conditions. Several other granular formulations, including 'Pesta' granules in which fungal propagules are encapsulated in a wheat gluten matrix, peat or alginate prills, vermiculite, and application onto crop residue were evaluated (52). Some formulations provide rapid release of bacteria early in the growing season whereas other formulations provide slow-release of bacteria, particularly for weeds that emerge later in the growing season. Industrially available products include formulated preparations of biocontrol fungi, bacteria, and streptomycetes (Table 1). For example, *G. virens*, formulated in alginate prill form (preparation GlioGard^{TM}) and incorporated into soilless potting media was shown effective for disease control of bedding vegetable plants and ornamental seedlings against *P. ultimum* and *R. solani*. Another example is alginate prill formulations of *T. flavus* with organic carriers for biocontrol of *V. dahliae*. In this case, pyrophyllite clay (Pyrax) and milled corncobs used to make alginate prill with ascospores of *T. flavus* consistently enhanced the biocontrol activity of the fungus.

COMBINED USAGE OF BIOCONTROL AGENTS

Most approaches for biocontrol of plant diseases use a single BCA as antagonist to a single pathogen. Indeed, microorganisms as BCAs typically have a relatively narrow spectrum of activity compared with synthetic pesticides and often exhibit inconsistent performance in practical agriculture resulting in the limited commercial use of biocontrol approaches for plant pathogens suppression (49). Usually, it is very difficult to find a single strain that is able to control a wide spectrum of pathogens under various rhizospheric and soil conditions. However, this goal can be achieved by genetic modification of the BCA by adding disease suppression mechanisms (e.g., genes encoding additional antibiotics or cell wall lytic enzymes), which operate against more than one pathogen, or by developing strain mixtures with superior biocontrol activity. Several strategies for forming mixtures of BCAs have been proposed, including mixtures of organisms with differential plant-colonization patterns; mixtures of antagonists that control different pathogens; mixtures of antagonists with different mechanisms of disease-suppression; mixtures of taxonomically different organisms; mixtures of antagonists with different optimum temperature, pH, or moisture conditions for plant colonization. Mixtures of antagonists are considered to account for the protection afforded by disease-suppressive soils. In the absence of cross-inhibition of antagonists, combining antagonists results in improved biocontrol. Therefore, successful development of strain mixtures requires compatibility of the coinoculated microorganisms (53).

Combinations of BCAs for plant diseases include mixtures of fungi, mixtures of fungi and bacteria, and mixtures

of bacteria. Thus, combined usage of bacteria and fungi for plant disease control have been shown in some experiments to be more efficient than a single antagonist. For example, *E. agglomerans* together with some *Trichoderma* spp. strains were shown to be the most promising antagonists from suppressive soils against *Phythophtora capsici* in pepper. *Enterobacter cloacae* and *Pseudomonas* spp. strain–induced suppression of *Rhizoctonia* damping-off were consistently more effective in combinations with *T. hamatum* relative to the fungal isolate alone. The integrated approach based on using binuclear *Rhizoctonia* spp. and *E. cloacae* strains in combination, led to protection of cucumber against the disease in greenhouse. Several bacterial and fungal BCAs, including *Trichoderma, Gliocladium, Pseudomonas, Bacillus, Agrobacterium, Enterobacter,* and *Serratia*, may protect plants against diseases via synergistic action. Therefore, it has often been stated that mixtures of BCAs are required for successful long-term control because the individual components that colonize different crops are adapted to different environments or have different functions (49). However, single isolates of *Trichoderma* and other microbial antagonists efficient for biocontrol of a number of plant pathogens also have been described (11,13).

INTEGRATED PEST MANAGEMENT

Biological control is just one component of a strategy termed integrated pest management (IPM). IPM is defined as the reduction of pest populations by natural enemies, and it typically involves an active human role. IPM strategy involves the coordinated use of pest and environmental information with available pest management methods to prevent unacceptable levels of pest damage by the most economical means, and with the least possible hazard to humans and the environment. In short, the goal of the IPM approach is to manage pests and the environment so as to balance costs, benefits, human health, and environmental quality. Thus, IPM is biologically based, cost-effective, and its site-specific risk-reduction system makes use of multiple methods in the decision-making process (54). Combinations of BCAs with low, otherwise inefficient doses of chemical pesticides are often considered an important IPM approach to achieving the most capable protection of crop disease. For example, under field conditions, a combination of lowered doses of methyl bromide (MB) and *T. harzianum* exhibited a significant synergistic effect on damping-off of carrot seedlings caused by *R. solani*, and its effect on growth, yield, and disease control was similar to that of the much higher recommended MB dosage. Integrated control of *V. dahliae* in potato by *T. harzianum* and the fungicide captan increased both marketable and total potato yield (36). Integration of induced systemic resistance into pest management systems can further lead to the decreased usage of the conventional pesticides (48). Combinating BCAs with soil solarization (solar heating) technique is an additional efficient way of using natural conditions to protect crops against pathogens and to enhance plant growth, even in the absence of known major pathogens. The rapid establishment of fluorescent pseudomonads was observed in the rhizosphere of plants that were grown in solarized soils and was found to be due to the improved capacity of these bacteria to compete for the plant exudates (55).

MOLECULAR APPROACHES FOR IMPROVEMENT OF EXISTING, AND CREATION OF NEW BIOCONTROL AGENTS

Improvement of Bacterial Biocontrol Agents

Some BCAs have been genetically modified to enhance their biocontrol capabilities or other desirable characteristics. Strain improvement is one of the most exciting ways of achieving highly efficient biocontrol of plant diseases. Molecular approaches and genetic engineering techniques have recently been applied to gain a better and more basic understanding of the mechanism of action of BCAs, and to develop superior and improved strains of BCAs with enhanced activity (18,32).

For example, synthesis of agrocin 84 by the BCA *A. radiobacter* K84 is directed by a gene located in the extrachromosomal DNA known as plasmid pAgK84. This plasmid also carries the genes needed for resistance to agrocin 84 and is able to transfer from one bacterium to another by a mechanism known as conjugation. Consequently, pAg84 may be transferred to pathogenic *A. tumefaciens*, which would then be resistant to agrocin 84. To prevent this resistance, a transfer-deficient mutant of strain K84 was constructed. *Agrobacterium radiobacter* strain K1026 is identical to the parental strain, except that the agrocin-encoding plasmid cannot be transferred from cell to cell (16).

In systems in which antibiotics play a primary role in the strains biocontrol activity, molecular approaches can be used to enhance the bacteria's biocontrol efficacy by increasing levels of antibiotic synthesis, either by increasing the copy number of the biosynthetic genes or by modifying the regulatory signals that control their expression. This is particularly interesting for bacteria in which the biosynthetic genes are arranged in either operons or clusters, as was shown for the genetics of Phz, Phl, and Prn production in *Pseudomonas* spp. strains (56). For example, increased production of Plt and Phl and superior control of *P. ultimum* damping-off of cucumber was achieved by increasing the number of antibiotic biosynthesis genes in *P. fluorescens* strain CHAO. Constitutive synthesis of oomycin A in *P. fluorescens* strain HV37a was achieved by insertion of a strong *tac* promoter from *Escherichia coli* upstream of the *afuE* locus encoding the antibiotic. The resultant recombinant strain produced a significantly higher level of oomycin A than the parental strain and provided greater control of *P. ultimum* infection (57). Another approach is to introduce biosynthetic genes into a strain deficient in antibiotic production, or into one that produces a different antibiotic, to increase the spectrum of activity. Thus, cloned Phz biosynthetic genes were transferred from *P. fluorescens* 2–79, which exhibits poor rhizosphere competence, into *P. putida* and *P. fluorescens* strains, which exhibit superior rhizosphere competence. The recombinant strains that synthesized

Phz in vitro are potentially superior BCAs of pathogenic fungus *G. graminis* var. *tritici* because of their ability to colonize the wheat rhizosphere. When genes conferring Phl synthesis were mobilized from a *P. aureofaciens* strain into a *P. fluorescens* strain that normally produces only Phz and inhibits this fungus, the obtained recombinant strain was shown to be additionally suppressive against *P. ultimum* and *R. solani* through expression of an additional antibiotic (49).

The chitinase gene *chiA* encoding one of the chitinases from biocontrol strains of *S. marcescens* and *E. agglomerans* was isolated and cloned into *E. coli* (58,59). *Escherichia coli* strains transformed with the *chiA* gene expressed and excreted the corresponding protein into the growth medium. Transformed *E. coli* was effective in inhibiting *S. rolfsii* on beans and *R. solani* on cotton, but to a lesser degree than the original chitinolytic strains. The genetically engineered *E. coli*, a nonsoil bacterium, served here as a model system to demonstrate the role of chitinase in controlling a chitin-containing plant pathogen. It is suggested that the introduction of such engineered genes into soil bacteria will increase control efficiency by combining high expression of a gene encoding a lytic enzyme with rhizosphere competence.

The current knowledge of regulatory mechanisms governing the expression of various antifungal substances may help in the construction of strains with enhanced biocontrol activity. Manipulation of regulatory systems responsible for the production of lytic enzyme and antibiotics has resulted in significant improvement of the bacteria's biocontrol potential. The advantages of this approach were demonstrated by increasing the doses of genes encoding the GacA-GacS system of global regulation or sigma factors of transcription in some biocontrol strains of *P. fluorescens* (35,56). The findings supported the idea that regulatory signals from the environment may contribute to the success of a bacterium in competition with plant pathogens and other rhizosphere inhabitants under natural conditions (33).

Improvement of Biocontrol Potency of *Trichoderma* spp.

Introduction of foreign genes that could potentially enhance the biocontrol capacity of *Trichoderma* (4). For example, genes encoding cell wall lytic enzymes were used as a tool to increase the chitinase activity of one of the most potent biocontrol species, *T. harzianum*. Two different strategies were used. According to one approach, the chitinase gene of *S. marcescens* was introduced into the genome of *T. harzianum* under the control of a constitutive eukaryotic promoter, providing a high level of expression of the foreign bacterial gene into a new fungal host. Following another strategy, a *T. harzianum* strain was transformed by a vector that contained the coding region of the 42-kDa endochitinase gene of this fungus under the control of a strong cellulase promoter from a relative fungus *Trichoderma reesei* (=*T. longibrachiarum*). A 10-fold increase in chitinase activity was achieved. Using the same the endochitinase gene of *T. harzianum* with its own regulatory sequences, transformants of *T. hamatum* with an approximately fivefold increase in chitinase activity were obtained. The 42-kDa endochitinase encoding gene of *T. harzianum* has been shown to be triggered in mycoparasitic interactions. Therefore, duplication of this highly conserved gene appears to be a potential means of improving the biocontrol capability of *Trichoderma* species.

Proteinases were also overexpressed in *Trichoderma* with the aim of improving the isolate's biocontrol activity. Under greenhouse conditions, *T. harzianum* transformants with an increased copy number of the basic proteinase gene *prb1* that were incorporated into pathogen-infested soil reduced the disease caused by *R. solani* in cotton plants up to fivefold as compared with the wild-type strain. In all these studies, the *Trichoderma* strains were genetically improved to serve as better BCAs of fungi, such as *R. solani*, that contain chitin and β-glucan as major cell wall components (4). A similar approach was approved for obtaining *Trichoderma* strains with high activity against plant pathogenic oomycetes, such as *Pythium* spp., containing cellulose as the main cell wall component. The difference in this case, however, was that the *Trichoderma* strains were designed to overproduce cellulytic enzymes instead of chitinolytic ones. Hypercellulolytic strains of *T. longibrachiatum* obtained by genetic engineering methods were shown to be significantly more effective in controlling *Pythium* damping-off disease on cucumber than the wild-type strain (60).

The major advantage of such genetic manipulations is the ability to isolate genes from one strain and to introduce them into other varieties of fungi or bacteria. This enhances the potency of BCAs and makes a single strain effective, stable, and consistent against more than one plant pathogenic fungus, without the hazardous effects of chemical pesticides.

The current strategies to improve BCAs activity through genetic engineering technology are summarized in Table 3.

CONCLUSION

Because of their beneficial activity, BCAs can serve as suitable alternatives to some commercial fungicides. However, more studies are required to develop screening techniques, appropriate formulations, and delivery systems to enhance application and survival, and to elucidate the molecular basis of their mode of action against plant pathogens. This, in turn, will yield genetic information to produce superior biocontrol strains, and give a better understanding of ecological factors that may interfere with biocontrol activity. Because these developments are time-consuming and often, do not produce immediate results, more emphasis should be directed to extending the use of integrated control as part of a comprehensive IPM program, thus introducing biocontrol into mainstream agriculture. The biocompatibility of these agents with fungicides is expected to enhance their efficacy, and there are a few examples available to demonstrate this.

Most microorganisms selected as potential BCAs never become products because of problems related to their production and downstream processing. The antagonistic activities in vitro or in vivo considered important for the

Table 3. Examples of Genetic Engineering Approaches to Improve Biocontrol Agents by Enhancement of Antimicrobial Compound Production

Strategy	Approach	Mechanism of Biocontrol Enhancement
Altered expression	Insertion of biosynthesis genes upstream of a stronger promoter; introduction of new/or additional regulatory genes, construction of regulatory mutants	Constitutive and/or increased production of antibiotics, siderophores, or lytic enzymes
Gene dosage	Increase copy number of gene(s) in wild-type strains	Overproduction of antibiotics and other antimicrobial compounds
Heterologous expression	Introduction of new genes encoding antimicrobial compounds	Conversion of non-producing strains to producing strains, expansion of assortment of antimicrobial compounds produced by a single biocontrol agent
Deletion	Specifically delete transfer (*tra*) genes from plasmid encoding agrocin 84	Eliminates potential transfer of agrocin 84 resistance gene to target pathogen

Source: Modified from N. Dowling and F. O'Gara, *Trends Biotech.* **12**, 133–141 (1994).

biocontrol potential of these strains are, in fact, often not sufficient to develop an efficient biocontrol product. To become a product, the microorganisms have to fit the following requirements. They must be easily produced in fermentors, that is, a high amount of cells produced per unit volume in a short time with low-cost culture medium; they must be easily converted to an appropriate form for longer shelf life; they must maintain biocontrol properties after large-scale production and drying. Because of their high production costs, biotechnological products require a large market to be beneficial. Therefore, it is worth being able to use the same product against a large number of diseases.

The greatest value of biocontrol agents versus chemical pesticides is that chemicals usually give a high level of protection but strongly rhizospheric competent organisms, in particular, can provide a somewhat lower level of benefit for extended periods, that is, up to several months. The point is that biologicals and chemicals both have strong points and neither can do what the other does best (13). The introduction or application of BCAs for pest and disease control will result in yields that are pesticide-free and hence biosafe. Pesticide-free products have already been introduced into the marketplace, particularly in developed countries. The market share of environmentally friendly fresh produce is constantly increasing, and pesticide-free fruits and vegetables are achieving being in higher prices than conventional produce. At present, biological control may lack, in some cases, the rapid protective effect exhibited by the best pesticides, and it may also result in somewhat lower efficiency and narrower host specificity than chemical control. Currently however, several BCAs with a rather wide range of biocontrol activity are available. The most extensively studied bacterial organisms, including *Pseudomonas* spp., *B. subtilis*, and *Enterobacter* spp., have been reported to reduce several soilborne and aerial diseases on various crop species. Fungal BCAs, including the extensively studied *Gliocladium* and *Trichoderma* species, in addition to other weakly virulent or saprophytic fungi, have been reported to reduce infection or reproduction of many pathogens. Most of the applications of these fungi are for soilborne diseases, with a few reports of reduction of foliar fungal pathogens. It is worth noting, however, that even the most efficient BCAs can be overcome by heavy disease pressure. BCAs cannot cure already existing infections; hence they must be used strictly as a preventive measure (13). There are several areas, in which additional research is required, and these include further improvement of the agents to achieve long-term effects and to enhance their survival and efficiency for routine application in agricultural practice for the benefit of the farmers.

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BIOCORROSION: ROLE OF SULFATE REDUCING BACTERIA

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In natural environments and artificial systems, corrosion reactions under a biological influence usually involve surface-associated microbial growth, namely, a biofilm. Terms such as microbially influenced corrosion (MIC) or biocorrosion are frequently employed to describe metal deterioration in the presence of microorganisms (1).

Corrosion under a biological influence frequently occurs as a localized attack, which results from the activity of physiologically diverse microbial species associated

with the metal substratum. Microorganisms implicated in biocorrosion have frequently been grouped by their metabolic demand for different respiratory substrates. Sulfate-reducing bacteria, sulfur-oxidizing bacteria, iron-oxidizing/reducing bacteria, manganese-oxidizing bacteria, as well as bacteria secreting organic acids and exopolymers or slime have all been named as culprits in MIC failures. These organisms coexist within biofilms, often forming synergistic communities able to affect electrochemical processes through cooperative metabolism, which individual species have difficulty initiating.

The first documented case describing biocorrosion dates to 1891. This report proposed that the increase of corrosion of lead cable could be due to bacterial metabolic products. However, it was not until 1934 that the corrosion of ferrous metal buried in anaerobic clay soil was solely attributed to the activity of obligate anaerobic microorganisms known as sulfate-reducing bacteria (SRB) (2). Since then SRB became the most investigated group of microorganisms implicated in corrosion damage of iron and ferrous alloys in a broad range of aquatic and terrestrial habitats varying in nutrient content, temperature, pressure, and pH values.

Biocorrosion of metals and their alloys is of great importance to many industries such as oil and gas, shipping, power, and water distribution sectors. In particular, the activity of sulfate-reducers can have considerable impact on oil and gas industry, leading to severe economical losses due to system failures and equipment deterioration and resulting in serious health and safety hazards and environmental risks (3,4). It has been proposed that approximately 20% of all corrosion damage to materials fabricated with metal is microbially influenced or enhanced (5). MIC is therefore the subject of intense research efforts aiming to develop environmentally acceptable prevention and protection measures and to improve the effectiveness of already existing control strategies.

Several theories have been presented to explain mechanisms by which sulfate-reducers can influence corrosion of metallic materials. In this article all proposed models of SRB-mediated corrosion are described along with the current hypothesis based on the identified reactions of primary importance leading to the coupled electrochemical and biotic electron transfer processes (6).

FUNDAMENTAL ASPECTS OF CORROSION

The term corrosion is defined as the physicochemical interaction between a metal and its environment, which results in changes in the properties of metal (7). These changes may lead to impairment of the function of the metal, the environment, or the technical system of which these form a part. Corrosion is an electrochemical process defined as a chemical reaction involving the transfer of electrons through a series of oxidation and reduction reactions.

For electrochemical corrosion to occur, the following components have to be present:

- An anode site
- A cathode site
- An electrolyte (a solution capable of conducting electrical flow).
- A cathodic reactant
- An anodic reactant
- Electronic contact between the anode and cathode

Schematic diagram of a basic corrosion cell for a ferrous metal under anoxic conditions is represented in Figure 1.

The process of corrosion consists of two half-reactions: an anodic reaction involving the ionization (oxidation) of the metal and a cathodic reaction involving the reduction of a chemical species in contact with the metal surface. At the anodic site the metal dissolution occurs and metallic ions are formed. A cathodic site is required as an electron sink for the return of current to the metal. This reaction is a reduction reaction or an electron-consuming reaction. The rate of corrosion is determined by the rate of the cathodic reaction, and as the electric current must migrate through the solution to the metal at the cathode; the cathodic current must be equal to the anodic current.

In aerated and oxygen-free solutions the anodic reaction is metal dissolution:

$$\mathrm{Me} \rightarrow \mathrm{Me}^{2+} + 2\mathrm{e}^- \tag{1}$$

For aerated solutions at neutral or alkaline pH, the main cathodic reaction is the reduction of oxygen

$$\mathrm{O_2} + 2\mathrm{H_2O} + 4\mathrm{e}^- \rightarrow 4\mathrm{OH}^- \tag{2}$$

In acidic or deaerated solutions, protons or, when present, hydrogen sulfide may act as electron acceptor, as demonstrated in reactions (3) and (4):

$$2\mathrm{H}^+ + 2\mathrm{e}^- \rightarrow 2\mathrm{H} \rightarrow \mathrm{H_2} \tag{3}$$

$$2\mathrm{H_2S} + 2\mathrm{e}^- \rightarrow 2\mathrm{HS}^- + \mathrm{H_2} \tag{4}$$

In an intermediate pH solution, that is, one of low pH but aerated, protons are more readily available than oxygen and, therefore, proton reduction will be the main electron-consuming route.

Although the anodic and cathodic reactions can occur at different locations on the metal, typically they are adjacent to each other. The sites of the anodic and cathodic reactions

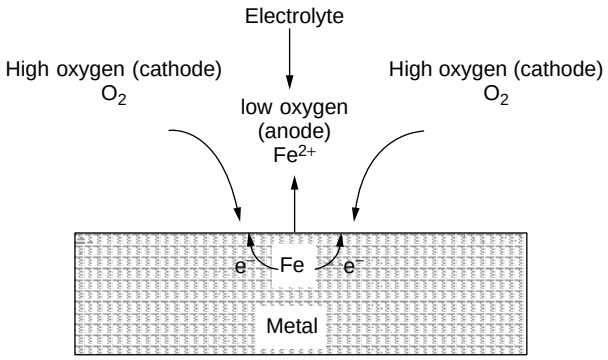

Figure 1. Basic corrosion cell for iron-based alloy under aerated conditions.

are referred to as the anode and cathode, respectively. The irregular distribution of corrosion cells across the metal surface causes localized attack in the form of pitting and/or crevice corrosion. When the half-cells are closely spaced, a general corrosion is observed.

As already stated, corrosion process causes materials fabricated of pure metals and/or their mixtures (alloys) to undergo chemical oxidation from ground state to an ionized species. The oxidation reaction, in most instances, slows to a low rate after a period of time because the oxidation products (corrosion products) adhere to the metal surface and form a layer, which serves as a diffusion barrier to other reactants. These products form a protective barrier to further oxidation of the underlying metal. Changes in the environmental conditions can affect the stability of the protective layers and, hence, the overall susceptibility of the metal to corrosion.

BIOCORROSION

Due to their presence on the surface and their ability to carry out specific biochemical reactions, microorganisms can alter the physical/chemical conditions at the metal/substratum interface and modify electrochemical reactions, which are fundamental to all corrosion processes. Microbial colonization of the surface is facilitated by the production of extracellular polymeric substances (EPS) that are composed of a mixture of macromolecules such as proteins, polysaccharides, nucleic acids, and lipids. EPS facilitate bacterial attachment to the substratum and form the biofilm matrix ((8) and references therein). The presence of exopolymers on the metal surface can modify both morphology and chemistry of corrosion products altering their corrosion characteristic, often rendering these products more aggressive.

The selective binding of different metal ions by EPS leading to the formation of metal concentration cells within the biofilm matrix has been proposed as one of the mechanisms of biocorrosion. The presence of such cells could promote electron transfer processes between the biofilm and base metal (9,10). A number of studies have demonstrated the involvement of exopolymers secreted by different genera of aerobic bacteria in metal deterioration (11,12).

Microbial metabolic processes, including the consumption of oxygen and production of acids and sulfides, promote the establishment of localized chemical gradients at the metal surface. The existence of such gradients leads to the formation of electrochemical cells, which stimulates anodic and/or cathodic reactions, the ultimate consequence being loss of metal from the discrete locations on the surface. A number of biocorrosion mechanisms have been identified that reflect the variety of physiological activities carried out by different types of microorganisms (13,14).

The interaction of a biofilm harboring many species of aerobic, facultatively anaerobic, and strictly anaerobic microorganisms and the underlying substratum produces a unique physical and chemical environment. Thus, conditions at the substratum surface can differ significantly from those in the bulk phase or at a biofilm-free surface. In the presence of biofilms, the following factors are likely to affect metal deterioration (1):

- Microbial activity and microbial metabolic products.
- The passage of charged entities through the exopolymer matrix.
- The degree of conductivity of the EPS matrix.
- The binding of metal ions by the EPS matrix.
- Destabilization of corrosion inhibitors.

The main importance to the corrosion process is the control the biofilm exerts on the transport of ions, namely, regulating the exchange of ions between the metal surface and the aqueous environment by either promoting or retarding metal ion transfer. Although in most natural and artificial environments, microorganisms and products of their metabolism, including EPS, are present in greater quantities at the metal surface compared to the bulk phase, water still constitutes 95% or more of the biofilm matrix. The biofilm can, therefore, still act as a modified electrolyte. The high growth rates, varied metabolic products, many of which are corrosive, and the high surface-to-volume ratio of microorganisms can result in interactions, which would modify physicochemical parameters at metal solution interfaces to such a degree that corrosion rates can be accelerated by factors of 10^3 to 10^5 (15). In contrast, certain types of biofilms may produce a barrier effect resulting in a reduction of the chemical activity and hence a substantial decrease in the corrosion rate of the metal (1).

SULFATE-REDUCING BACTERIA

Sulfate-reducing bacteria are readily isolated from biofilms formed on different type of surfaces in natural habitats and industrial systems. These bacteria thrive within anoxic niches, which exist within biofilms as a result of oxygen depletion due to the respiration of aerobic or facultatively anaerobic microorganisms.

Phylogeny

The family of taxonomically diverse SRB is currently distributed within two domains: *Archaea* and *Bacteria* (16). In the former domain, sulfate-reducers are represented by microorganisms of the *Archaeglobus* genus. In the Kingdom *Bacteria*, SRB are divided into three distinct groups, the gram-positive spore-forming SRB with *Desulfotumaculum* being a predominant genus, the gram-negative mesophilic SRB (δ-subclass of the *Proteobacteria*) of which *Desulfovibrio* is one of the better characterized genera and the thermophilic SRB represented by two genera *Thermodesulfobacterium* and *Thermodesulfovibrio*. Although SRB are currently divided into four phylogenetic groups, new divisions could be added, as several new genera of psychrophilic SRB were recently isolated from permanently cold Arctic marine sediments (17).

Physiology

Lactate, pyruvate, and malate are frequently used as carbon source and electron donors and are oxidized

to acetate and carbon dioxide (18,19). Some SRB can utilize high molecular weight fatty acids or simple aromatic compounds such as benzene or phenol and are known to participate directly or indirectly in the degradation of variety of substances, including saturated hydrocarbons (20). During their growth, SRB produce a large amount of hydrogen sulfide that assure the maintenance of an anoxic atmosphere.

Although all SRB species known to date have been described as strictly anaerobic, some genera are able to survive for long periods in the presence of oxygen, indicating the existence of enzymatic defense mechanisms against oxygen radicals (21,22). Indeed, the presence of enzymes catalase and superoxide dismutase, which prevent accumulation of H_2O_2 and eliminate toxic respectively, constitutively expressed during anaerobic growth of *Desulfovibrio gigas*, was recently confirmed (23).

Respiration

Sulfate-reducing bacteria are chemolithotrophic organisms that are able to carry out dissimilatory reduction of sulfur compounds such as sulfate, sulfite, thiosulfate, and even sulfur itself to sulfide by a series of energy-conserving reactions (Fig. 2; 18).

The initial step in the dissimilatory sulfate reduction is the transport of exogenous sulfate across the bacterial membrane into the cells. This process may be inhibited by selenate, which is the structural analog of sulfate. Once inside the cell, sulfate dissimilation proceeds by the action of adenosine triphosphate (ATP)-sulfurylase which combines sulfate with ATP to produce the highly activated molecule adenosine phosphosulfate (APS), as well as pyrophosphate (PP) that may be subsequently cleaved to yield inorganic phosphate.

$$ATP + SO_4^{2-} \rightarrow APS + PP$$

APS is rapidly converted to sulfite and AMP by the cytoplasmic enzyme APS reductase.

$$APS \rightarrow AMP + SO_3^{2-}$$

A number of different sulfite reductases have been identified in SRB (24). The most commonly recognized types, particularly among the genus *Desulfovibrio*, are the biosulfite reductases, desulfoviridin, and desulforubidin. Sulfite is reduced via a number of intermediates, including metabisulfite ($S_2O_5^{2-}$), dithionite ($S_2O_4^{2-}$), trithionate ($S_3O_6^{2-}$), and thiosulfite ($S_2O_3^{2-}$), to form the sulfide ion. The latter can be further converted to hydrogen sulfide (H_2S) in the presence of external hydrogen (H^+) ions.

Apart from sulfate, some *Desulfovibrio* species can grow with nitrate or fumarate as alternative electron acceptors releasing ammonia and succinate, respectively (20). At low dissolved oxygen concentrations certain SRB are able to respire with Fe(III) or even oxygen with hydrogen acting as electron donor (25,26). Although some gram-negative SRB can reduce Fe(III) there are no known SRB that are capable of performing this reaction using ferric ion as a sole electron acceptor.

Hydrogenase Enzymes

Hydrogen metabolism plays a central role in energy-generating mechanisms of sulfate-reducing bacteria. The capability of SRB to produce and consume H_2 means that they possess the enzyme hydrogenase, which catalyzes the reversible oxidation of hydrogen according to the reaction (5):

$$H_2 \leftrightarrow 2H^+ + 2e^- \quad (5)$$

Hydrogenase was first discovered by Stephenson and Stickland in 1931 (27). Since then hydrogenase activity was found in a large number of anaerobic and aerobic prokaryotes, as well as in eukaryotic cells.

Two mechanisms involving H_2-metabolism have been offered for energy coupling in *Desulfovibrio* during

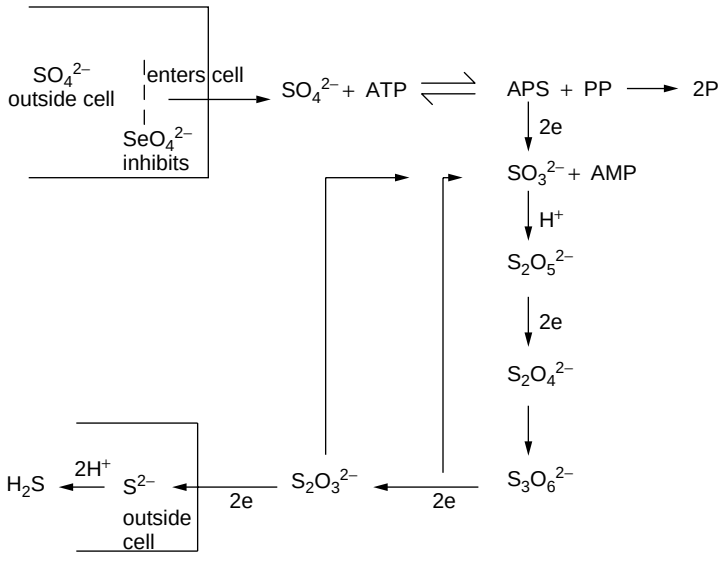

Figure 2. A proposed cyclic pathway for dissimilatory sulfate reduction (18).

growth on organic substances in the presence of sulfate: (*1*) generation of a proton gradient by the periplasmic hydrogenase itself linked to obligate H_2-cycling (28) and (*2*) proton translocation typically associated with a trace H_2-transformation model (29,30). Both models propose that bioenergetic mechanisms are dependent on the existence of at least two functionally distinct hydrogenases: one located in the cytoplasm involved in H_2-production and another in the periplasm involved in H_2-consumption.

Three types of hydrogenases have been isolated from the sulfate-reducing bacteria of the genus *Desulfovibrio*. According to the type of metal present, hydrogenases are generally divided into: the iron hydrogenase ([Fe] hydrogenase), the nickel-(iron-sulfur)-containing hydrogenase ([NiFe] hydrogenase) and the nickel-(iron-sulfur)-selenium containing hydrogenase ([NiFeSe] hydrogenase). They differ in their subunit and metal compositions, physicochemical characteristics, amino acid sequences, immunological reactivities, gene structures, and their catalytic properties (31). The [NiFe] hydrogenases have been found in all species of *Desulfovibrio* investigated to date. This class of hydrogenases can be localized in the periplasm, the cytoplasm, and/or the membrane depending on the SRB species and are particularly resistant to inhibitors such as CO and NO_2^- (32). [NiFeSe] hydrogenases, which contain nickel and selenium in equimolecular amounts, are only found in some species of *Desulfovibrio*. The [Fe] hydrogenase has the highest specific activity in the evolution and consumption of hydrogen and this enzyme is the most sensitive to CO and NO_2^-. It is not present in all species of *Desulfovibrio* (33).

The three classes of hydrogenases are not uniformly distributed in all *Desulfovibrio* and an individual species may contain one (e.g., [NiFe] hydrogenase in *D. gigas*), two (e.g., [NiFe] and [NiFeSe] hydrogenases in *D. baculatus*) or three hydrogenases (e.g., [Fe], [NiFe], [NiFeSe] hydrogenases in *D. vulgaris* Hildenborough). Most of the hydrogenases isolated from the genus *Desulfovibrio* are confined to the periplasmic space; however, membrane-bound and cytoplasm-located enzymes have also been described (34).

All three types of hydrogenases catalyze the same basic reaction, the reversible activation of the hydrogen molecule by a heterolytic mechanism. The proton-deuterium exchange reaction, the para-ortho hydrogen conversion, the hydrogen production from reduced methyl viologen and the hydrogen utilization with various artificial and natural electron acceptors can determine the activity of hydrogenases, which varies with pH and temperature (35,36).

Apart from their participation in energy production (i.e., the involvement in hydrogen cycling and the generation of a proton gradient), the hydrogenases of SRB are proposed to play an important role in biocorrosion (37).

MODELS OF SRB-INFLUENCED CORROSION

The role of SRB has been documented in pitting corrosion of iron-based alloys in both aquatic and terrestrial environments under anoxic, as well as oxygenated conditions (Figs. 3 and 4). The number of SRB detected in a system, as either suspended or attached populations, does not necessarily correlate with the extent or rate of corrosion and different types of SRB dissimilar in their physiology vary in the ability to influence metal deterioration (38–40). However, no clear consensus has yet been reached linking specific bacterial metabolic rates to observed corrosion rates.

Several key theories such as:

- The depolarizing effect of hydrogenase enzyme.
- Cathodic depolarization due to biogenically produced iron sulfide.
- Anodic depolarization due to the formation of iron sulfide corrosion products.

along with reports on the generation of corrosive phosphorous compounds and binding of metal ions by SRB exopolymers have been proposed to explain the mechanisms by which these bacteria can influence the corrosion reaction (41–43). It is now accepted that one predominant mechanism may not exist and that a number of processes are involved (44–46).

Hydrogenase Enzyme as Depolarizing Agent — Early Studies

In 1934, von Wolzogen Kühr and van der Vlugt proposed what is now referred to as the classical mechanism of anaerobic corrosion of ferrous metals in the presence of SRB, also known as the cathodic depolarization theory (2). The essential step in this theory involves the removal of hydrogen (cathodic depolarization) by the activity of the hydrogenase enzyme. The electron removal as a result of hydrogen utilization forces more iron to be dissolved at the anode. The main steps involved are listed in the following text (reactions 6 to 12) and depicted in Figure 5.

anodic reaction:

$$4Fe \rightarrow 4Fe^{2+} + 8e^- \quad (6)$$

dissociation of water:

$$8H_2O \rightarrow 8H^+ + 8OH^- \quad (7)$$

cathodic reaction:

$$8e^- + 8H^+ \rightarrow 8H \quad (8)$$

cathodic depolarization:

$$SO_4{}^{2-} + 8H \rightarrow S^{2-} + 4H_2O \quad (9)$$

corrosion products:

$$Fe^{2+} + S^{2-} \rightarrow FeS \quad (10)$$

corrosion products:

$$3Fe^{2+} + 6OH^- \rightarrow 3Fe(OH)_2 \quad (11)$$

overall reaction:

$$4Fe + SO_4{}^{2-} + 4H_2O \rightarrow 3Fe(OH)_2 + FeS + 2OH^- \quad (12)$$

Using cathodic polarization measurements, a range of studies demonstrated that the SRB species with

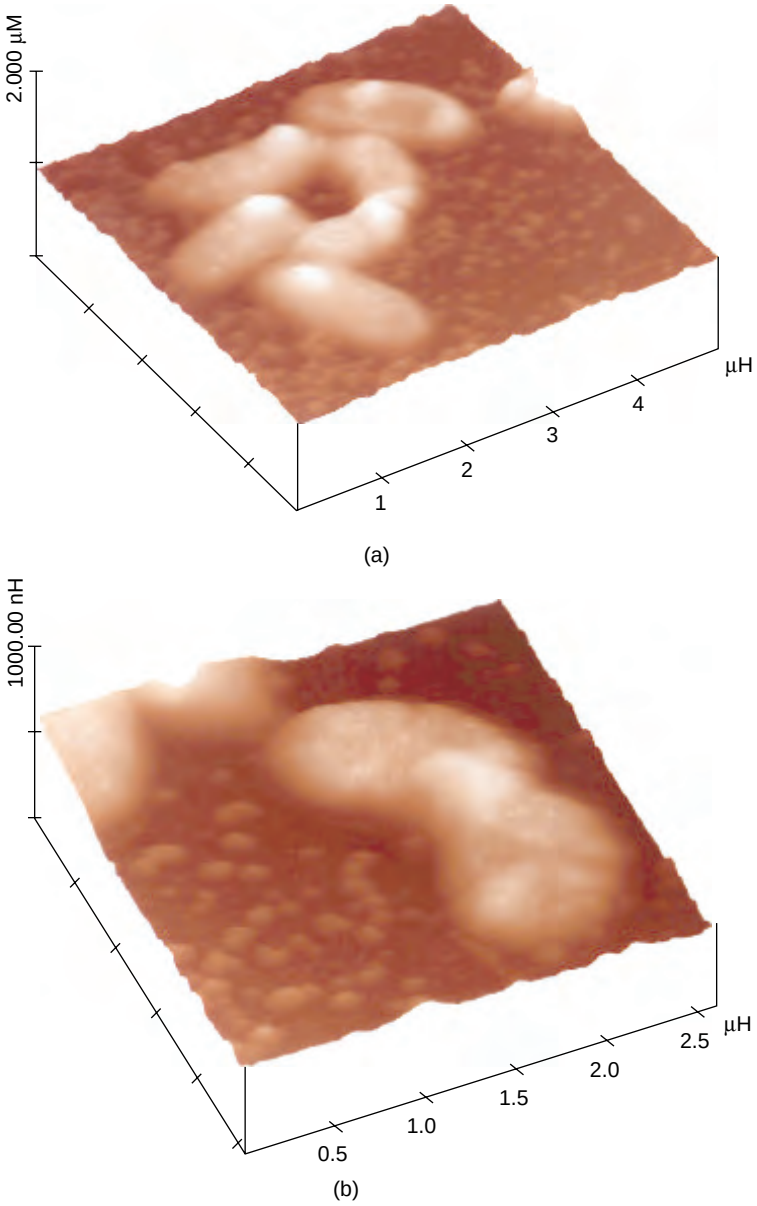

Figure 3. Atomic force microscopy image of a three-day-old biofilm formed by the marine sulfate-reducing bacterium *Desulfovibrio indonensensis* on AISI 316 stainless steel revealing (**a**) a group of cells forming a discrete colony on the steel surface and the abundance of irregularly distributed iron sulfide particles, and (**b**) detailed topographic features of a single SRB cell and its close association with FeS particles.

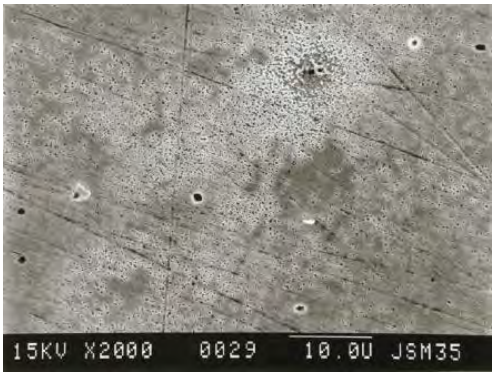

Figure 4. Scanning electron micrograph demonstrating localized corrosion on the surface of AISI 316 stainless steel following the removal of a 14-day-old biofilm formed in a pure culture of the marine sulfate-reducing bacterium *Desulfovibrio indonensensis*. Corrosion is visible as pitting attack of varying intensity.

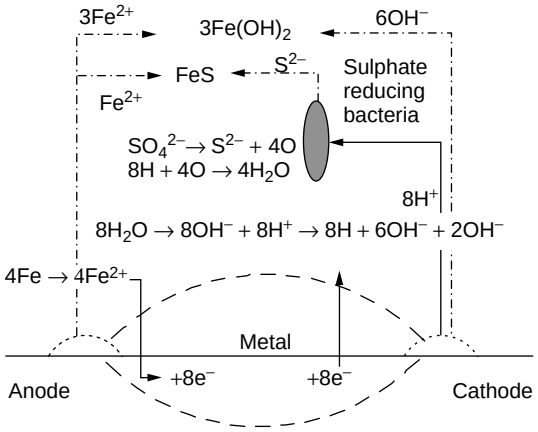

Figure 5. Schematic representation of the cathodic depolarization process of ferrous metal in the presence of sulfate-reducing bacteria.

detectable hydrogenase activity were able to depolarize the mild steel cathode. Depolarization did not occur in the cultures of SRB where hydrogenase activity was not recorded (47–49). Further support of the model was provided by demonstrating the dissolution of iron from a mild steel electrode electrically connected to a similar electrode in contact with a culture of hydrogenase-positive *Desulfovibrio* spp. on an agar surface containing benzyl viologen (BV), acting as electron acceptor. The SRB oxidized cathodic hydrogen and transferred the electrons to the redox dye, BV instead of sulfate (50,51).

However, the latter experiment met with criticisms since it was likely that hydrogenase-positive SRB catalyzed not only the consumption of hydrogen but also the liberation of hydrogen by reduced BV. The apparent hydrogenase-dependent cathodic depolarization was claimed to be an experimental artifact (15). A number of follow-up studies produced inconclusive or contradicting results leading to the revision of the proposed SRB corrosion model based solely on the hydrogenase activity.

Iron Sulfide as Depolarizing Compound

Low corrosion rates were measured in semicontinuous or continuous hydrogenase-positive SRB cultures grown with low ferrous iron concentration in lactate medium containing sulfate due to the formation of a protective iron sulfide film. When this film ruptured, much higher corrosion rates were recorded. Moreover, in the media with a high ferrous ion concentration, high corrosion rates (on the order of 1 mm y^{-1}) were observed for both hydrogenase-positive and -negative SRB strains. These results prompted investigation into the involvement of biologically generated sulfides in the SRB-mediated biocorrosion phenomenon (52). Detailed studies demonstrated that the initial sulfide film formed during corrosion was in the form of a continuous, adherent, and protective layer of mackinawite ($Fe_{1-x}S$). However, this film showed a degree of physical disruption in a time-dependent modification to greigite (Fe_3S_4). In the presence of higher soluble iron concentrations there was a similar loss of protective mackinawite film with the conversion to smythite (Fe_9S_{11}) and pyrrothite (FeS_{1+x}). In each case, this loss of the uniform protective mackinawite film generated active electrochemical cells between areas of unreacted and unprotected steel and deposits of the various ferrous sulfides, with the resulting corrosion occurring by cathodic stimulation.

Many sulfides under near-surface natural environmental conditions may only be produced by enzymatically catalyzed microbiological pathways on specific substrates such as metals. For example, formation and stability of the sulfide mineral, mackinawite, is dependent on a continuously acting hydrogen sulfide source. Although abiotic sources of sulfide may also lead to mackinawite formation in the absence of enzymatically governed biological reactions (53), the most common source of sulfide responsible for the vast majority of industrially related corrosion is that produced by SRB (54,55). Although mackinawite formation is not favored under standard thermodynamic conditions common to many corrosion scenarios, it is frequently found where SRB have been detected. On continued exposure to SRB, mackinawite alters to greigite and smythite and finally to pyrrhotite. Greigite is associated with generalized corrosion of iron, while smythite is indicative of pitting attack. The presence of mackinawite and greigite among corrosion products of ferrous materials is generally considered as proof that SRB participated in the corrosion reaction (56). Although pyrite (FeS_2) is not a common iron corrosion product, SRB can produce pyrite from mackinawite in contact with elemental sulfur.

Mineral signatures of both crystalline and amorphous iron sulfides indicative of SRB-mediated corrosion are commonly detected using X-ray diffraction (XRD) and energy dispersive X-ray analysis (EDX). Little is known about subsequent crystallization of amorphous sulfides, although biomineralization around SRB colonies, or within biofilm matrix, may be a key process.

The quantitative importance of cathode depolarization by solid FeS was demonstrated by the addition of chemically prepared FeS to mild steel test coupons in the presence and absence of *Desulfovibrio* spp. and replacing sulfate with fumarate as terminal electron acceptor for bacterial reduction processes. The extent of corrosion as assayed both by polarization and by weight loss measurements, was proportional to the FeS added and dependent on direct contact between the sulfide and the metal surface (57).

Comparison of the corrosion of mild steel by chemically prepared FeS and biogenically derived FeS indicated that, in media of high Fe^{2+} concentration, most of the corrosion in pure SRB cultures was attributed to the biogenically derived FeS (58–60). The corrosion caused by the biogenic FeS appeared identical to that caused by FeS from inorganic reactions (61).

Thus, a new model emerged, which proposed that corrosion of iron-based alloys in the presence of SRB proceeded under reduced conditions through cathodic stimulation of electrochemicals cells established between areas of unreacted metal (anode) and deposits of various biogenically generated reduced ferrous sulfide corrosion products (cathode).

Iron Sulfide and Hydrogenase as Depolarizing Agents

It has been noted that the presence of SRB is required to maintain the chemical integrity and electrochemical activity of iron sulfide species. In bacteria-free cultures the depolarizing capacity of FeS diminished with time, possibly as a result of the bonding of atomic hydrogen within the FeS crystals lattice. However, the corroding ability of the FeS was restored in active SRB cultures.

A theory was constructed proposing that the cathodic reaction, namely, H_2 evolution resulting from reaction of Fe^{2+} with bacterially produced S^{2-} occurred on the FeS deposits (41). It further postulated that SRB present as biofilms on the surface of FeS continually regenerated or depolarized the FeS by the removal of atomic hydrogen as a result of hydrogenase activity. This mechanism would explain the continued high rates of mild steel corrosion in biologically active, SRB-harboring systems.

Studies on physiology of SRB provided evidence that in oxygen-free environments bacteria were able to utilize

cathodic H_2 with sulfate acting as the sole source of energy (62–64). However, this process, which required the activity of hydrogenase, was influenced by the availability of organic electron donors. It was concluded that in the presence of SRB, anoxic aqueous environments rich in anaerobically degradable organic matter (e.g., interior of sewer pipes) should be more corrosive than environments that are mainly inorganic (e.g., in geothermal heating plants). These findings led to reevaluation of the role played by hydrogenase in the corrosion process.

Enzyme Hydrogenase as Depolarizing Agent — New Approach

Although the role of iron sulfide in the SRB-mediated corrosion process could not be dismissed, it has been argued that the oxidation of cathodically produced H_2 was the determining step. Experiments were conducted to demonstrate that the separation of bacteria from the surface by a dialysis membrane prevented the utilization of cathodic H_2 by the SRB (65). Furthermore, the current observed in the presence of biofilms formed by the hydrogenase-positive SRB did not appear when biofilms were developed by hydrogenase-negative SRB cultures. It has also been demonstrated that the hydrogenase activity did not depend on the presence of viable SRB cells and that the cell-free extracts of hydrogenase enzyme increased the corrosion of mild steel (66,67). Thus, it was determined that regardless of the presence of living cells, hydrogenase might still retain its depolarizing capabilities, which could be expressed under appropriate reducing conditions.

Studies on mixed populations of SRB in biofilms isolated from corroded and undamaged oil pipelines revealed that the biofilm with detectable hydrogenase activity was associated with a significantly higher corrosion rate (7.70 mm $year^{-1}$) than the noncorrosive biofilm (0.48 mm $year^{-1}$). Both biofilms had comparable number of SRB cells per unit area; however, no measurable hydrogenase activity could be found in the latter type of biofilm. It was therefore concluded that the susceptibility of a system to biocorrosion depended on the microbial makeup of the mixed population of SRB and the activity of the hydrogenase enzyme (68).

Subsequent investigations revealed that the hydrogenase activity of SRB is regulated by dissolved Fe^{2+} availability, which is subject to repression/derepression mechanism and depends on SRB species (69,70). It is therefore likely that apart from other parameters affecting enzyme performance, bacterial uptake of cathodically generated H_2 governed by the activity of hydrogenase, and hence the rate of cathodic depolarization, fluctuates depending on the ecology and physiology of SRB species within biofilms and the level of bioavailable Fe^{2+} ions. To date, hydrogenase contribution to the SRB-enhanced corrosion process has not been assessed in a quantitative manner.

The Influence of Oxygen

Numerous observations confirmed that the presence of dissolved oxygen played an important part in the SRB-influenced corrosion of ferrous metals (71–73). A corrosion rate recorded when the vessel containing SRB and steel foil was exposed to oxygen on an intermittent basis was 50 times higher than that reported under anaerobic conditions (74).

It is believed that under alternating aerated and anoxic conditions, the corrosion mechanism of mild steel is related to the large Fe-S pool, which provides both surface area for colonization and hydrogen to SRB, as well as potentially corrosive elemental sulfur and polysulfide (46). The latter can be produced as a result of sulfide reacting with oxygen. When anoxic sites with SRB become aerobic, new minerals are formed from the original corrosion products. The oxidation process yields lepidocrocite or goethite and rhombic or orthorhombic sulfur. Mixed reducing and oxidizing conditions, such as those commonly found in certain pipeline systems, often produce the partially oxidized iron oxide magnetite along with lepidocrocite and goethite (56).

It has been suggested that at the aerobic/anoxic interface, the corrosion reaction sequence due to accumulation of FeS might proceed in two stages. Firstly, the iron sulfide on the steel surface may form a protective film of mackinawite, thereby exerting anodic control of metal dissolution by limiting ferrous ion diffusion through the sulfide film. Subsequent breakdown of this protective film would create an electrochemical cell with sulfide-free steel acting as the anode, iron sulfide acting as the cathode, and sulfur serving as the electron acceptor to promote cathodic depolarization.

Anodic Depolarization

Careful analysis of cathodic depolarization theory and in particular the role of sulfides led to an alternative hypothesis. The latter proposed that SRB promoted corrosion by a mechanism of anodic depolarization, essentially dependent on sulfide production. This model postulates that an anode is first created by local H^+ production at a focus of SRB metabolic activity, with ensuing metal dissolution (75,76). A key element in generating the necessary kinetic conditions is a localized acidification at the anode resulting from the formation of iron sulfide corrosion products according to reaction (13):

$$Fe^{2+} + HS^- \rightarrow FeS \downarrow + H^+ \qquad (13)$$

The reaction also has the secondary effect of removing HS^-, hence reducing the effectiveness of the H_2S/HS^- buffer system, which would resist local acidification. The availability of sulfide and free iron would determine the nature of the corrosion products formed. If the local sulfide concentration was low, as would be the case with the reaction aforementioned, in the presence of high soluble iron concentration, the product formed would most likely be mackinawite, which, as already described, is considered to be nonprotective. Where sulfide was in excess, the product would be the more protective pyrite. Although pitting corrosion of steel can theoretically be explained by the mechanism of anodic depolarization, the processes under which locally acidic conditions are maintained needs to be confirmed.

Corrosive Phosphorus Compounds

It has been suggested that not only iron sulfides, but also a highly active phosphorus compound produced by SRB, could cause anaerobic corrosion of ferrous alloys (77). The corrosion reaction yielded iron phosphide as a corrosion product. Cathodic depolarization experiments on an agar surface in the absence of an electron acceptor resulted in the blackening of the agar at areas in contact with SRB. On extraction and analysis by X-ray diffraction, the black precipitate was found to contain iron phosphide (Fe_2P). It was concluded that the phosphorus compound was produced as a result of both the activities of SRB and the effect of H_2S on phosphate, phosphite, and hypophosphite. Although subsequent studies have excluded inorganic phosphate as the source of phosphorus metabolized by the SRB into the corrosive phosphorous product (78), it has been found that corrosive activity of hydrogenase can be augmented by the availability of inorganic phosphate (79). Organic phosphate in the form of phytic acid, a common metabolite of plants, was proposed as the form of phosphate metabolized by the SRB into the corrosive phosphorus product in the field. To date, however, the role of phosphorous compounds in SRB-influenced corrosion remains ambiguous.

The Role of Extracellular Polymeric Substances

The ability of SRB to produce EPS in batch cultures were first documented by Ochynski and Postgate (1963) who also reported the carbohydrate composition of exopolymer using paper chromatography (80). Subsequent investigations demonstrated that both freely suspended and surface-associated SRB are able to synthesize exopolymers varying in their chemical composition depending on SRB species and growth conditions (81–83). Electron and atomic force microscopy allowed visualization of EPS in SRB biofilms (84). Binding of metal ions, in particular Fe, Cr, Mo, and Ni by SRB exopolymers has also been documented (85). Additional study confirmed the direct involvement of a high molecular weight, thermostable protein-carbohydrate complex produced by a marine SRB in pitting corrosion of mild steel (86). Further investigation demonstrated SRB species specificity in binding of Fe^{2+} ions originating from the corroding surface of mild steel by exopolymers released into the planktonic phase of bacterial culture media (87). It is therefore conceivable that in addition to already listed processes, complex organic macromolecules excreted by SRB could contribute to corrosion at the biofilm/metal interface by mechanisms outlined in previous sections of this entry.

CONCLUSION

A hypothesis coupling electrochemical and physiological electron transfer processes has been presented to rationalize present understanding of the SRB-influenced corrosion mechanisms and to create a framework for future studies (6). The key features of this hypothesis are summarized below.

Phylogenetically and physiologically diverse anaerobic sulfate-reducing bacteria associated with mixed species microbial consortia thriving as biofilms on surfaces of metallic substrata can influence corrosion processes mainly due to the generation of sulfide ions. The production of the latter varies with SRB species and is linked to the type and activity of hydrogenase enzyme(s) present in all SRB. The enzymatically catalyzed reactions, which are influenced by bacterial ecology and physiological and environmental factors, facilitate oxidation of molecular hydrogen evolved at the cathode of the electrochemical corrosion cell, leading to cathodic depolarization. Metal that dissolves at the electrochemical anode can combine with the biogenically generated sulfide to form corrosion products such as metal sulfides. The latter will precipitate within the anoxic zones of the biofilm, causing further stimulation of either the cathodic reaction by serving as an electron sink and/or by influencing the anodic reaction, namely, further metal dissolution, due to local acidification at the anode. Depending on their physicochemical characteristics, the accumulating iron sulfides can be either protective or corrosive. A number of factors, for example, the traces of oxygen, levels of unbound iron ions, the concentration of soluble sulfide and the presence of other microorganisms such as sulfur-oxidizing bacteria, methanogenic bacteria, or iron-oxidizing/reducing bacteria determine both the metabolic activity of SRB and the type of metal sulfide species formed.

In the presence of SRB-harboring biofilms the highest corrosion rates were recorded when there are fluctuating aerobic-anaerobic (O_2/AnO_2) conditions. Under such conditions, sulfide ions and ferrous ions are prone to biotic/abiotic oxidation with oxygen acting as ultimate electron acceptor, which would lead to the development of corrosion products such as ferric oxides/hydroxides and elemental sulfur. A descriptive model of SRB-mediated corrosion is depicted in Figure 6 (46). The model demonstrates that corrosion in the presence of SRB occurs by a

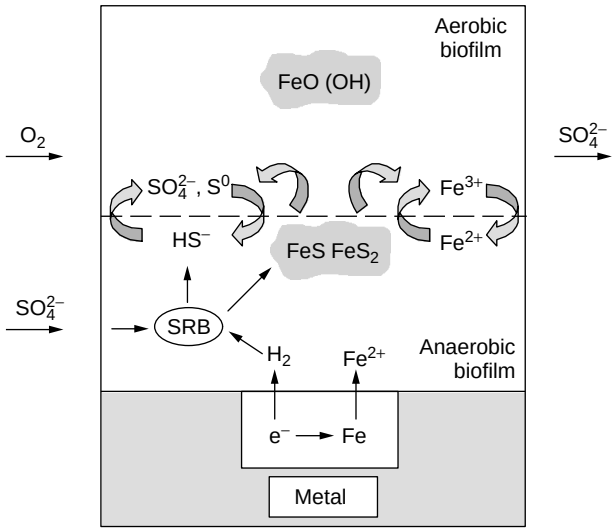

Figure 6. A diagram of the corrosion of ferrous alloys in an aerobic/anaerobic biofilm system due to the metabolic activity of sulfate-reducing bacteria. In this model oxygen acts as terminal electron acceptor (46).

process of electron transfer from the base metal to oxygen as ultimate electron acceptor, through a series of coupled redox reactions of electrochemical, biotic, and abiotic character.

It is unequivocally accepted that SRB-influenced deterioration of iron and ferrous alloys does not involve any new mechanisms of corrosion. The observed metal deterioration in SRB-active environments is due to microbially influenced changes in surface conditions of the metallic substratum that promote the establishment and/or maintenance of cathodic and/or anodic reactions not normally favored under otherwise similar conditions in the absence of these bacteria. Notwithstanding considerable research efforts undertaken to elucidate the role that sulfate-reducers play in corrosion, there is still a number of ambiguities related to the contribution of biologically catalyzed reactions, including the quantitative contribution of enzymic activities and significance of exopolymers, to the overall corrosion reaction. Undoubtedly, the better understanding of relationships, both direct and indirect, between the electrochemical corrosion reactions and spatial and temporal processes associated with metabolic activities of SRB within biofilms would aid in resolving such uncertainties.

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BIODEGRADABILITY: METHODS FOR ASSESSING BIODEGRADABILITY UNDER LABORATORY AND FIELD CONDITION

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Herein are described basic principles pertinent to the design, implementation, and interpretation of both laboratory and field determinations of biodegradability. The information is applicable to both organic and inorganic contamination problems whose solutions can be addressed microbiologically. However, the focus here is primarily on assessing the biodegradability of organic compounds.

DEFINITIONS OF BIODEGRADABILITY AND BIODEGRADATION

"Biodegradability" embodies qualities representing the susceptibility of substances to alteration by microbial processes (1). The substances may be organic or inorganic. The alteration may be brought about by (*1*) intra- or extracellular enzymatic attack that is essential for the growth of the microorganism(s) (e.g., the attacked substances are used as a source of carbon, energy, nitrogen, or other nutrients or as a final electron acceptor), (*2*) enzymatic attack that is beneficial because it serves some protective purpose (e.g., mobilization of toxic mercury away from the vicinity of the cells), (*3*) enzymatic attack that is of no detectable benefit to the microorganism (e.g., cometabolic reactions in which a physiologically useful primary substrate induces the production of enzymes that fortuitously alter the molecular structure of another compound), and (*4*) nonenzymatic reactions stemming from by-products of microbial physiology that cause geochemical change (e.g., consumption of oxygen, production of fermentation by-products, or an alteration in pH).

"Biodegradation" of organic compounds is the partial simplification or complete destruction of their molecular structure by physiological reactions catalyzed by microorganisms (2–7). Biodegradation is routinely measured by applying chemical and physiological assays to laboratory incubations of flasks containing pure cultures of microorganisms, mixed cultures, or environmental samples (e.g., soil, water, sediment, or industrial sludges). When attempting to measure biodegradation or judge the biodegradability of substances, the investigator must define the environmental context so that potential reactants and products can be identified. Microorganisms

can catalyze only reactions that are thermodynamically possible. Furthermore, reaction mechanisms are largely constrained by precedents set during the evolution of physiological and biochemical functions. Because of ongoing microbial evolution and biochemical research, our understanding of mechanisms by which microorganisms degrade substrates continues to expand.

LABORATORY METHODS

Principles for Measuring Biodegradability in the Laboratory

Biodegradation methodologies are designed to confirm, demonstrate, and explore both the net chemical changes and the associated intracellular details pertinent to how microorganisms influence the fate of contaminants. The procedures span a broad range of disciplines and sophisticated protocols. Figure 1 provides an overview of the variety of objectives, disciplines, and protocols that play key roles in biodegradation research. The two phases that serve as main divisions in Figure 1 result from the degree to which scientific detail is pursued. Phase 1 procedures treat samples of soil, sediments, water, or industrial effluents simply as "black boxes" that do or do not make contaminant compounds disappear, as judged by analytical chemical criteria. Phase 2 begins with the isolation of pure cultures of contaminant-degrading microorganisms. Once these have been obtained, refined physiological, enzymatic, and molecular biological assays may then be performed. As DNA sequences of genes that code for metabolic pathways become increasingly available, molecular procedures will continue to gain prominence in biodegradation protocols. One of the final goals of the procedures shown in phase 2 is understanding the molecular basis for gene expression and regulation.

Design and Implementation of Biodegradation Assays Using Environmental Samples and Pure Cultures

The traditional black-box approach to biodegradation assays asks the question, "Are microorganisms within this complex microbial community (e.g., derived from soil, water, sediment, or industrial sludge) able to metabolize the compound of interest?" To answer this question, one aseptically gathers samples from a given field location, dispenses known weights or volumes of the samples to replicated vessels, handles the samples in a variety of ways that include a treatment that

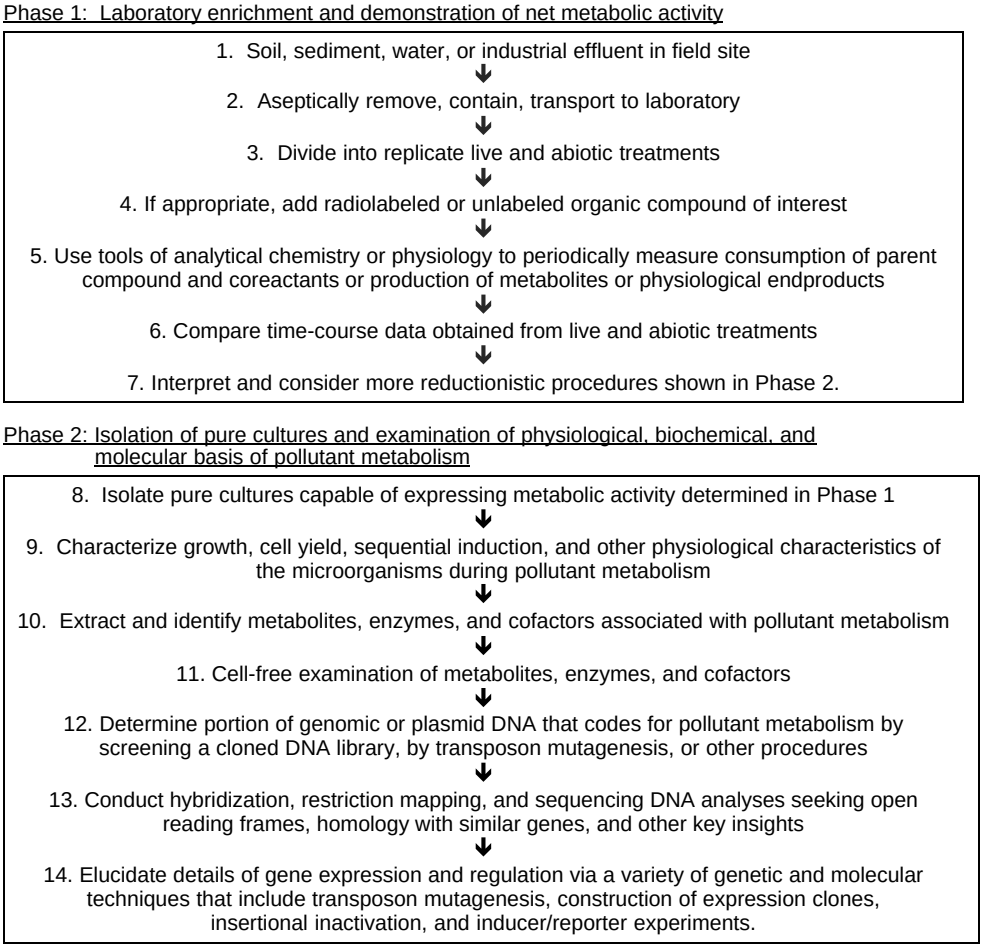

Figure 1. Two phases of procedures for understanding biodegradation processes. Phase 1 begins with environmental samples. Phase 2 proceeds through biochemical and molecular aspects of pollutant metabolism by single microorganisms (from Ref. 1).

has been either sterilized or poisoned, incubates the test samples under laboratory conditions, and employs over time both chemical and physiological assays that monitor the fate of the test compound within experimental vessels (Fig. 1; phase 1). The objective of this general experimental design for biodegradation procedures using environmental samples or pure cultures is remarkably simple, yet there is a substantial series of obstacles that must be overcome before one obtains clear data that truly test a given set of specific hypotheses. Every design parameter selected for inclusion in a biodegradation assay can influence the resultant data. Therefore, decisions made in implementing biodegradation assays should be well reasoned. Table 1 summarizes many of the practical and theoretical decisions that must be made in developing biodegradation protocols. Step 1, a background issue considering information use, is fundamental to all related experimental decisions. The degree to which experimental minutiae of a given testing protocol must be initially considered is commensurate with the scrutiny that the final data will undergo. Artifacts and biases in data are virtually unavoidable in biodegradation assays (see later); thus, it may be wise to simply accept methodological limitations rather than worry about initial potential technical design flaws that may later have no practical impact.

Once the reason for conducting the biodegradation assay has been put in perspective (Table 1, step 1), another background issue, that of physiological conditions, should be confronted. Step 2 appears in Table 1 to acknowledge

Table 1. Steps and Decisions Essential for Implementing Biodegradation Assays (After Ref. 7)

Step	Decisions
1. *Background*: Determine how the resultant data will be interpreted and used	Objectives range from information about crude "biodegradation potential" to tests of specific hypotheses about physiological or biochemical factors governing biodegradation processes
2. *Background*: Select the physiological conditions under which pollutant metabolism is to be measured	The pivotal physiological concern is defining the mechanism by which the compound(s) is metabolized. Of primary importance is discriminating among such possibilities as cometabolic reactions, its use as an electron acceptor, and its use as a carbon and energy source. Other concerns address conditions in experimental flasks such as nutrient sufficiency, which final electron acceptor regimen should dominate, what pollutant concentration ranges should be examined, and if conditions should change (batch culture) or remain constant (continuous culture) during the assay
3. *Practice*: Select and aseptically prepare or sample the microorganisms whose physiological activity is of interest	For assays using environmental or industrial samples, aseptic sampling techniques involve use of tools (such as flame-sterilized scoops, spatulas, and knives) and sample placement within sterilized glass or plastic containers. For assays using pure cultures of microorganisms, the microorganisms must be aseptically grown under conditions that carefully define the cell physiological status (e.g., stage of growth, cell numbers, induced enzyme systems, nutritional state) desired by the investigator
4. *Practice*: Select the physical apparatus and hence the physiological setting for biodegradation reactions to occur	Glass (or plastic) vessels must be assembled. These contain the test compound(s), the microorganisms being studied, and any accompanying components of soils, sediments, sludges, and water in various ratios. Experimental hardware may be fitted with a variety of gas and water exchange assemblies for maintaining physiological conditions and assaying reaction progress
5. *Practice*: Select a metabolic activity assay that is sensitive, effective, convenient, inexpensive, and compatible with experimental objectives	The general assay categories are physiological assays (e.g., respirometry or growth) and chemical assays (which include gas chromatography, gas chromatography-mass spectrometry, high-performance liquid chromatography, and radiotracer techniques)
6. *Practice*: Aseptically prepare stock solutions of ^{14}C-labeled organic compounds. Check radiopurity	The validity of the results from biodegradation assays using ^{14}C-labeled substrates is dependent on substrate radiopurity and aseptic preparation of stock solutions
7. *Practice*: Complete the experimental design parameters for the assay vessels and the assays themselves	a. Concentration of the test substrate(s) b. Number of replicated flasks per treatment c. Whether flasks can be sampled repeatedly or if they require sacrifice at each sampling period d. Frequency of sampling e. Method of preparing abiotic controls f. Methods for separating radioactive parent and product compounds from one another

the fact that biodegradation is only a small portion of the perhaps thousands of physiological reactions occurring simultaneously when both pure cultures and mixed microbial populations in environmental samples are incubated in the laboratory. These physiological processes feed one another, interact in complex ways, and can be governed by many of the sometimes inadvertent physical and chemical manipulations made while preparing, incubating, and sampling assay vessels. Uncertainties become particularly striking when one is attempting to troubleshoot failed attempts to demonstrate biodegradation activity. The interplay between fundamental knowledge of physiology and experimental design parameters demands that a variety of issues be confronted: (*1*) The mechanism by which the compound is metabolized (e.g., as a carbon source, as a nitrogen source, or as a cometabolic substrate whose transformation will occur only when another compound is supplied), (*2*) inclusion versus exclusion of potential growth-limiting vitamins and minerals, (*3*) inclusion versus exclusion of air in the headspace of the reaction vessel, (*4*) the solid-to-liquid ratios used in test vessels containing soil, sediments, or sludges, (*5*) the multiple roles of compounds in physiological reactions (for instance, nitrate can serve as both a nitrogen source and a final electron acceptor), and (*6*) the fact that the compound whose biodegradation is being tested may be toxic at high concentrations or fall below some minimum threshold value for uptake and cell growth at low concentrations. Background considerations raised in steps 1 and 2 guide most of the practical steps needed for completing the implementation of the biodegradation assays (Table 1, steps 3 to 7). Detailed considerations pertinent to steps 3 to 7 (Table 1) have been discussed previously (1,7,8).

FIELD METHODS

Assessing Biodegradation in the Field

There is a fundamental paradigm for verifying that the biodegradation processes we hope are occurring are actually occurring in field sites. The paradigm begins by modestly admitting that both microorganisms and their habitats are incomplete puzzles. Our task is to relentlessly find new ways to create the puzzle pieces describing microbiological processes and to assemble them logically. The scientific disciplines that contribute information and techniques toward creating the puzzle pieces include microbiology, geochemistry, hydrology, biochemistry, soil science, physiology, molecular biology, analytical chemistry, computer modeling, and both environmental and chemical engineering. It must be recognized that each of these disciplines is actively being advanced and therefore, contributes a dynamic spectrum of expertise to bioremediation, ranging from theoretical and basic knowledge to applied and practical instrumentation.

Verifying field biodegradation is perhaps best achieved in two mutually supportive ways (9): (*1*) succinctly using common sense and (*2*) using elaborate reasoning and analyses (see later). The succinct answer is "We know that bioremediation is taking place when all of the available information congeals as a coherent picture (it makes sense)." There needs to be consistency, redundancy, and convergence of all types of evidence from as many of the appropriate scientific disciplines as are available.

Because the key players in bioremediation are microorganisms, it is essential that the process makes sense to the microorganisms themselves, in the physiological and thermodynamic contexts where they reside. Contexts for bioremediation processes range from a variety of field sites in which organic contaminants have spilled accidentally (e.g., marine coastlines, desert soils, freshwater streams, or anaerobic deep subsurface sediments) to various aerobic or anaerobic engineered stirred and staged bioreactor systems. Regardless of the particular context, each must be scrutinized as a habitat for microbial metabolism in which individual cells can develop into populations and complex ecological communities whose fundamental physiological needs for adenosine triphosphate generation, carbon assimilation, terminal electron acceptors, other inorganic and organic nutrients, and dynamic intercellular interactions (competition, synergism, interspecies hydrogen transfer, commensalism, predation, parasitism, etc.) demand constantly improving sets of hypotheses aimed at refining our understanding of bioremediation. Once the fundamental thermodynamic, nutritional, and ecological bases for the sought metabolic functions are initially conjectured, a series of hypotheses will naturally unfold that provide a means for documenting the bioremediation process of interest on a site-specific and case-specific basis.

Table 2 contains four examples of contaminants in field sites whose physiological contexts dictate how microorganisms can metabolize the offending organic compounds. Knowledge from laboratory-based (using environmental samples, mixed cultures, and/or pure cultures) assays provides the biochemical basis for mechanisms operating in the field. Answers to key questions, such as "Are the contaminants suitable carbon and electron sources?," "Which physiological electron acceptors (oxygen, NO_3^-, Fe^{3+}, Mn^{4+}, SO_4^{2-}, carbon dioxide) are required coreactants?," "Are the contaminants, themselves, final electron acceptors?," and "What competing reactions may slow or prevent the sought biodegradation?," provide a framework that launches a broad array of possible assays that can argue for or against the successful establishment of a given biodegradation process (1,7,8). The example contaminated sites of Table 2 range from aerobic soil to aerobic and anaerobic aquifers. The assays range from field-based oxygen probes, to counts of contaminant-degrading bacteria, to laboratory biodegradation assays, and to molecular biological assays of DNA and ribonucleic acid. The following sections of this article elaborate on the reasoning and detailed analyses required for the generation and testing of hypotheses that allow bioremediation technology to be verified. Related aspects of bioremediation and biodegradation have been reviewed in several recent books (2,21–29).

A Three-Step Strategy for Verifying Bioremediation

The reasons for establishing sound scientific criteria for microbiological involvement in contaminant loss are (*1*) biodegradation processes are often unique in their

Table 2. Examples of Contaminated Sites, Hypothesized Key Bioremediation Processes, and the Corresponding Field and Laboratory Measurements that Allow Site-Specific and Case-Specific Verification of Microbiological Destruction of Contaminants (After Ref. 9)

Example Sites	Hypothesized Key Bioremediation Processes	Supportive Field and Laboratory Measurements
Aerobic soil contaminated with petroleum products	Heterotrophic microorganisms are growing using petroleum components as carbon and energy sources (10,11,12). Metabolism in this context relies on oxygen, both in the attack of aliphatic and aromatic compounds, and as a final electron acceptor in respiratory chains	• Coincident depletion of petroleum components and oxygen in the field • Corresponding production of carbon dioxide • High numbers of petroleum-degrading aerobic heterotrophs inside but not outside the contaminated areas • If petroleum has a distinctive $^{13}C/^{12}C$ ratio, this should be reflected in the carbon dioxide • Adding nitrogen or phosphorus fertilizer to replicated plots may relieve nutrient limitation, hence enhance loss from field plots compared to unfertilized controls • Genes involved in the catabolism of petroleum components should be expressed in high abundance inside but not outside the contaminated zone
Anaerobic aquifer contaminated with perchloroethylene	Dehalorespiring bacteria are using chlorinated aliphatic compounds as final electron acceptors (13,14). Dechlorination reactions are governed by: complex microbial and chemical interactions that generate physiological electron sources (especially hydrogen gas); the presence of alternative electron acceptors (NO_3^-, Mn^{4+}, Fe^{3+}, SO_4^{2-}, carbon dioxide) in site sediments and waters; and ecological and physiological competition among the microorganisms carrying out the metabolism that links electron donors and acceptors (15,16)	• Dechlorinated daughter products, trichloroethene (TCE), dichloroethenes (DCE), vinylchloride (VC) within contaminant plume • Products of complete detoxification, such as ethene, should be inside and not outside the plume • Adaptation of site microorganisms to dehalorespiration can be documented by finding dechlorination activity in site samples from inside but not outside the contaminant plume • Immunological or polymerase chain reaction–based data demonstrating the presence of dehalorespiring enzymes, genes, and characteristic bacteria inside but not outside the plume
Aerobic aquifer contaminated with TCE	TCE destruction is achieved cometabolically by aerobic microorganisms supplied with a primary carbon source. Oxygenase enzymes (involved in metabolizing primary substrates such as methane, propane, toluene, and phenol) fortuitously convert TCE to unstable compounds that spontaneously hydrolyze to nontoxic and/or readily biodegradable components (17–19)	• Microcosms prepared with site samples consume TCE only when supplied with both oxygen and the primary substrate • In recirculating field site waters, TCE loss is enhanced only when both oxygen and the primary substrate are supplied • Assays for oxygenase enzymes, genes, and appropriate metabolites (e.g., *trans*-dichloro-ethylene oxide) reveal high abundances inside but not outside the contaminated zone
Anaerobic aquifer contaminated with jet fuel	Aromatic fuel components, especially toluene, serve as growth substrates for anaerobic microorganisms that utilize sulfate as a final electron acceptor	• Microcosms containing site sediments incubated under sulfate reducing conditions produce $^{14}CO_2$ from ^{14}C-labeled toluene and benzene (20) • Sulfate is depleted along the groundwater flow path in the field site • Dissolved inorganic carbon (e.g., carbon dioxide) increases along the flow path in field sites (20) • Contaminant plume has ceased advancing despite a constantly dissolving reservoir of jet fuel (20) • A solute transport model accounts for dispersion, flow velocity, and adsorption, and produces biodegradation rate estimates that are consistent with microcosm estimates (20)

capacity to break intramolecular bonds of contaminant compounds; thus, contaminants can be destroyed and not simply transferred from one location to another, as is the case in many other pollution control technologies; (*2*) when the mechanism of pollutant destruction is certain, key site management decisions about process enhancement can be made; and (*3*) for bioremediation to meet pollution-control needs of the society, the industry must adopt some standards for uniformity and quality control so that credibility and reliability can be attained (25–27).

However, the question remains: What is an adequate proof of bioremediation? The legal system of the United States provides a variety of categories of certainty in interpreting evidence. The categories depend on the type of the case and the significance of the issues. Among the different burdens of proof are (*1*) proof beyond reasonable doubt, (*2*) proof in a clear and convincing manner, and (*3*) proof beyond a preponderance of doubt. This article neither intends, nor is able, to dictate to regulatory or legal agencies what level of proof should be deemed adequate for bioremediation technology practitioners. Nonetheless, approaches are discussed here that can be used to distinguish biotic and abiotic reactions affecting contaminants at field sites in which bioremediation technology is being applied (for additional discussion see Refs. 1,6,7,11,12,25,26,30–37).

The consensus of a National Research Council (NRC) (25) committee in recommending criteria proving in situ bioremediation is as follows:

1. Develop historical records documenting loss of contaminants from field sites.
2. Perform laboratory assays unequivocally showing that microorganisms in site-derived samples have the potential to metabolize the contaminants under expected site conditions.
3. Demonstrate that the metabolic potential measured under criterion 2 is actually expressed in the field. To achieve this, microbiological mechanisms of contaminant attenuation must be distinguished from abiotic ones. Evidence deemed suitable for these purposes will vary according to the contaminants and conditions found at each site.

Implementing Step One: Site Monitoring to Understand Site Biogeochemistry and Establish Historical Trends of Contaminant Behavior. It must be recognized that virtually all locations in the biosphere (from the poles to the equator, contaminated or pristine sites, engineered bioreactors, or the deep sea) are inhabited by microorganisms. Furthermore, whenever physiological resources are available, microbial metabolic activity will occur. Thus, site characterization is designed to assess the resources and guide the documentation of their exploitation by microorganisms. There is a critical need to relate results of geochemical measurements performed on field samples directly back to in situ processes and conditions. For details of completing in situ analyses, avoiding site-sampling artifacts, and understanding site biogeochemistry see References 7,38– 40.

All site characterization data must be interpreted in terms of the physiological processes that produce and consume geochemical constituents. Final electron acceptors that dominate the physiological reactions of field sites (or discrete zones therein) provide useful criteria for categorizing biogeochemical regimes as aerobic, denitrifying, iron reducing, manganese reducing, fermentative, dehalogenating, sulfate reducing, or methanogenic (41–43). These physiological regimes are often separated in space and/or time in field sites and largely determine the mechanisms of contaminant biodegradation. Information establishing the physiological regime(s) operating at particular field sites is provided by field measurements of the contaminants themselves, of concentration gradients of coreactant final electron acceptors (e.g., oxygen, NO_3^-, Fe^{3+} Mn^{4+}, SO_4^{2-}, halogenated compounds), and of end products of microbial metabolism (e.g., carbon dioxide, Fe^{2+}, Mn^{2+}, S^{2-}, N_2O, NH_4^+, organic acids, reductive dehalogenation daughter products, and methane) along site transects. In this regard, Lovley and coworkers (44) and Chapelle and coworkers (45) have devised a gas sampling bulb protocol for anaerobic groundwaters in the field, which, in combination with hydrogen gas determinations and Winkler titrations for oxygen (40), provides definitive information on dominant anaerobic redox couples.

The goal of establishing site-specific historical records of the behavior of contaminants, coreactants, and metabolic products is a simple one. Theoretically, compilation of such field data documents the effects of contaminant-attenuating processes over time. In conjunction with other assays (see later), field data can be interpreted in ways that may implicate biodegradation as a cause of pollutant losses. However, as in understanding in situ physiological regimes, obtaining robust, interpretable field-monitoring data may be an elusive goal if contaminant characteristics and site conditions are complex. The overall objective is to establish a site-monitoring regime using a network of consistent sampling locations, which affords the acquisition of contaminant concentration and other measurements that are comparable over time. If the distribution of contaminants at the site and factors influencing contaminant transport (e.g., climate, hydrology, commercial, or industrial activities) are erratic, then the pertinent database on contaminant behavior may be so noisy as to mask any trends. However, in many sites the type of contaminant monitoring protocols required by concerned regulatory agencies can be integrated over time and can sometimes produce a clear historical record of diminishing contaminant concentrations from year to year. When such data exist, they assist in meeting the first criterion for proving in situ bioremediation.

Implementing Step Two: Laboratory Assays Demonstrating That Microorganisms in Site Samples Have the Potential to Transform the Contaminants Under Expected Site Conditions. The biodegradation assays discussed in this article are designed to ask the question: "Are microorganisms within samples of soil, water, or sediment microbial communities able to metabolize the compound(s) of interest under conditions that are relevant to the specific field site of interest?" To answer this question, one gathers samples

Table 3. Strategies for Obtaining Evidence for Field Expression and Biodegradation Processes (After Ref. 9)

Type of Strategy	Principles and Examples	References
Internal conservative tracers	Assess loss of certain compounds relative to the persistence of less-biodegradable, but similarly transported, compounds. Examples include using ratios of straight-to-branched chain alkanes (C_{17}/pristane, C_{18}/phytane) and ratios of other compounds to hopane in crude oil; ratios of lower to higher chlorinated congeners in PCB mixtures (trimethyl benzene can serve as a conservative tracer in enzene-toluene-ethylbenzene-xylene (BTEX) plumes); ratios of nonchlorinated to chlorinated aromatics in mixed solvents; and selective metabolism of one stereoisomer of particular pesticides (e.g., σ-chlorocyclohexane)	47–57
Added conservative tracers	In some field sites, contaminant mixtures may lack internal tracers but be amenable to the addition of materials that provide a baseline measure of various transport processes. Examples include helium to assess oxygen loss or carbon dioxide production in groundwater, propane to assess toluene loss from a stream, and bromide to assess groundwater flow	25,58,59
Added radioactive tracers	In rare instances, regulatory authorities have allowed intentional field release of radioactive (e.g., ^{14}C-labeled) pollutants in field sites. Subsequent recovery of $^{14}CO_2$, ^{14}C-metabolites, and ^{14}C-parent compounds provide, definitive proof of metabolic and other field processes	60–63
Added stable isotopic tracers	Pollutant compounds that are nonradioactive, but isotopically labeled with deuterium or ^{13}C, have been released in field sites. Subsequent stable isotopic analyses of field samples for labeled CO_2, metabolites, and/or the parent compound provide proof of metabolic and other field processes	64,65
Stable isotopic fractionation patterns	CO_2 has different $^{13}C/^{12}C$ ratios depending on the $^{13}C/^{12}C$ signature of the substrates respired and the ^{13}C-enriching process of methanogenesis. When site-specific signatures of both inorganic and organic carbon reservoirs have been characterized, the relative contribution of pollutant biodegradation to the pool of CO_2 can be discerned. The radioactive (^{14}C) component of CO_2 is also revealing because petroleum contaminants contain no ^{14}C	49,66–70
Detection of intermediary metabolites	When sufficient biochemical knowledge of pollutant biodegradation has accrued, particular metabolites can be targeted using a combination of careful sampling and analytical chemistry. Detection of stable (dead-end) metabolites and transient metabolites (indicative of "real-time" biodegradation) has been reported. The metabolites include *trans*- dichloroethylene oxide, dihydrodiols of aromatic compounds, DDE, and hydroxylated pesticides	71–77
Replicated field plots	Some relatively homogeneous field sites are amenable to randomized block designs of treatments that stimulate microbiological activity. Comparing the loss of pollutants from plots with and without nutrients and/or inocula can demonstrate effectively the role of microorganisms in field biodegradation	47,53,78–81
Microbial metabolic adaptation	Naturally occurring microbial communities that grow in response to pollutant exposure have predictable characteristics relative to adjacent unexposed communities. Adaptation is reflected in laboratory or field measure of: qualitative pollutant metabolian or rates of pollutant metabolism; numbers of specific pollutant degraders; and enhanced concentrations of protozoan predators of bacteria inside but not outside contaminant plumes	16,82
Molecular biological indicators	Based on molecular biological characterization of pure cultures capable of pollutant metabolism, a variety of assays consistent with established genetic sequences and their expression can be devised. These include polymerase chain reaction (PCR) amplification of structural genes, messenger ribonucleic acid (mRNA) extracted from field sites, reverse-transcriptase PCR detection of mRNAs, nucleic acid sequencing, immunodetection of enzymes and metabolites, and 16S ribosomal RNA analysis of the composition of microbial communities	83–90
Gradients of coreactants and/or products	Ongoing *in situ* metabolism of pollutants consumes physiological final electron acceptors and generates metabolic endproducts that reflect site-specific pollutant metabolism. Chemical gradients in field sites should be apparent using measures that include oxygen, NO_3^-, Mn^{4+}, Fe^{3+}, SO_4^{2-}, CO_2, NO_2^-, N_2O, Mn^{2+}, Fe^{2+}, methane, hydrogen, pH, and alkalinity	20,91,92
In situ rates of respiration	A subset of the previous entry that has been effectively applied to engineered bioremediation of subsurface sites involves cessation of an oxygen (or air) sparging regime, followed by insertion of an oxygen probe that documents real-time oxygen consumption. This respiratory activity should be high inside but not outside the contaminated area. The conserved gas, helium, can be included in the sparging step to account for diffusional O_2 loss	93,94

(*continued overleaf*)

Table 3. (*Continued*)

Type of Strategy	Principles and Examples	References
Mass balances of contaminants, coreactants, and products (total expressed assimilative capacity)	Under well-defined hydrogeologic regimes, fluxes of water contaminants and physiological electron donors or acceptors can be quantified in a cross-sectional analysis of site sampling stations. The stoichiometry of all appropriate aerobic, anaerobic, isotopic fractionation, and inorganic equilibria reactions can serve to predict and distinguish biotic from abiotic processes and to identify contributions from a variety of microbiological groups	56,92,95–97
Computer modeling that incorporates transport and reaction stoichiometries of electron donors and acceptors	This approach considers quantitative aspects of fluid flow, dilution, sorption, volatilization, mixing, microbial growth, and metabolic reaction stoichiometries to achieve an integrated and predictive tool for understanding all processes influencing the fate of pollutant compounds. This approach resembles the previous entry, but is implemented on a larger scale and uses more sophisticated computations	92,98–101

aseptically from a given field site or bioreactor, dispenses known weights or volumes of the samples to replicated vessels, handles the samples in a variety of ways that include a treatment that has been either sterilized or poisoned, incubates the test samples under laboratory conditions, and employs both chemical and physiological assays that monitor the fate of the test compound within experimental vessels over time (Fig. 1, phase 1). These procedures have been described earlier.

Implementing Step Three: Evidence for Field Expression of Biodegradation Potential. Three sources of uncertainty must be confronted and overcome when demonstrating that microorganisms are the active agents of pollutant loss in bioremediation projects.

1. We must acknowledge that extrapolation from laboratory-based metabolic activity assays to the field is usually unwise because of the propensity of microorganisms in field samples to respond to laboratory-imposed physiological conditions that are unlikely to match those in the field perfectly (6,46).
2. The spatial heterogeneity of field sites may impede or completely prevent trends in the behavior of environmental contaminants from being discerned (12,20).
3. The action of a multitude of both abiotic and biotic processes may contribute simultaneously to pollutant attenuation (6,20).

To contend with these challenges, several strategies have been developed for verifying the success of field bioremediation efforts in truly activating pollutant-destroying microbial processes in field sites and bioreactors. These (comprehensively codified in Table 3) are simple, logical expressions of the fundamental paradigm for verifying bioremediation introduced previously and in Table 1. The strategies that appear in Table 3 are firmly based on the physiological principles that distinguish between biotic and abiotic contaminant attenuation processes. Four of the strategies involve tracers (internal conservative, added conservative, added radioactive, and added stable isotopic) that either account for or circumvent problems arising from abiotic changes in field concentrations of contaminants and related metabolites. Six of the strategies in Table 3 rely on detailed prior knowledge of specific microbiological processes (stable isotope fractionation, detection of intermediary metabolites, stimulation of microbial activity in replicated field plots, metabolic adaptation, in situ respiration, and gradients of coreactants and/or products) that are manifest as observable geochemical changes in the field. The molecular biological strategy in Table 3 is an elegant, emerging approach that is constantly strengthened by genetic links that are forged between information from pure cultures and real-world mixed microbial communities. The linkages are limited by the relatively small database of genetic sequences pertinent to pollutant metabolism and the uncertain metabolic diversity that may arise when genes of unrelated lineage may have converged on the same metabolic function. The final two strategies in Table 3 (computer modeling and mass balances of contaminants, reactants, and products) attempt to account quantitatively for both transport and metabolic processes within entire field sites or along distinct transects therein.

CONCLUSION

Understanding and proving biodegradation processes under laboratory and field conditions is a science of ongoing discovery. This discovery requires a close dialog among many disciplines. It must be recognized that only under relatively rare circumstances is a proof of field bioremediation unequivocal when a single piece of evidence is relied on. In the majority of cases, the complexities of contaminant mixtures, their hydrogeochemical settings, and accounting for competing abiotic mechanisms of contaminant loss make it a challenge to document biodegradation processes. Unlike controlled laboratory experimentation wherein measurements can usually be interpreted easily, cause-and-effect relationships are often very difficult to establish at field sites. Furthermore, certain bioremediation data that may be convincing for some authorities may not be convincing for others. Thus, in documenting bioremediation, the several approaches described previously should be independently pursued: a consistent, logical case relying on convergent lines of independent evidence should be built. The three-step strategy for verifying bioremediation described previously has been augmented recently by that of a new NRC Committee (26). What might be considered a fourth step and an overarching goal is assuring the

public that bioremediation in specific sites will be reliable, sustainable, and quantitatively complete.

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BIODEGRADABLE DISSOLVED ORGANIC CARBON (BDOC).

See ASSIMILABLE ORGANIC CARBON (AOC) IN TREATED WATER: DETERMINATION AND SIGNIFICANCE

BIODEGRADABLE DISSOLVED ORGANIC CARBON IN DRINKING WATER

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Natural organic matter (NOM) present in surface waters and sometimes in groundwaters can be the root cause of various problems in drinking water. Organic material can be responsible for undesirable water taste, odor, and color. Organic material lead to the formation of disinfection by-products (after reaction with the disinfectant during water treatment) and to bacterial proliferation within distribution systems. The presence of low concentrations of organic nutrients in water is sufficient to support biofilm growth within the pipe network, potentially leading to a deterioration of bacterial water quality.

Therefore, the control of organic matter concentration is becoming recognized as an important part of the operation of drinking water plants and distribution systems. The following section will list methods available for characterizing organic matter and its biodegradable fraction, present the impact of water treatment on organic materials, and describe the role of biodegradable organic matter (BOM) in bacterial regrowth. This section focuses on one type of measurement for bacterial substrate called *biodegradable dissolved organic carbon* (BDOC). The next section is devoted to a second type of measurement for bacterial nutrients named *assimilable organic carbon* (AOC). Limited AOC data is presented to compare both measurements.

MEASUREMENT OF ORGANIC MATTER AND ITS BIODEGRADABLE FRACTION

Chemical Characterization of Organic Matter

Most organic matter are naturally present in water, but one small portion is artificial and originates from pollution and human activity. Chemical characterization of organic matter is complex. To date, it has not been possible to identify all organic molecules in water. The difficulty of this approach arises from the great complexity of the organic matrix, constituted by a mixture of hundreds of simple molecules or heteropolymers, and the very low concentration of these compounds. Consequently, organic matter characterization is only possible through various fractionations and measurement techniques that give partial information on organic matter levels, chemical functions, or molecules, depending on the methodology used (1).

Measurement of Surrogate Parameters. The measurement of total organic carbon (TOC), dissolved organic carbon (DOC), ultraviolet absorbance at 254 nm (UV254), or fluorescence provides some insight into the organic levels and properties of water. TOC and DOC measurements are the most common techniques for monitoring organic matter levels in waters. Expressed as TOC, organic matter can be fractionated into DOC, colloidal compounds, and particulate organic carbon (POC). The dissolved and particulate fractions are distinguished on the basis of the porosity of the filters used to separate these two fractions. The typical exclusion limit of the particulate form of organic matter that has been adopted ranges from 0.2 to 0.45 μm. TOC is generally composed of 90% DOC and approximately 10% POC (2). Part of the colloidal carbon is categorized as dissolved, whereas the other part is quantified as particulate matter. TOC analyzers are common instruments used to assess organic matter levels. They are based on the following principle: organic compounds are oxidized to carbon dioxide using a combination of oxidizing agents (persulfate) and UV light or high temperature. The amount of carbon dioxide produced can then be measured using nondispersive infrared absorption or conductivity. As most waters contain carbonates and bicarbonates, the concentrations of these inorganic compounds are either measured or the samples are acidified and purged with an inert gas to remove the inorganic carbon, before TOC analysis of TOC. When these are purged, the remaining TOC is referred to as nonpurgeable TOC or as nonpurgeable DOC if the sample is filtered. Organic matter levels typically range from 0.1 mg/L to 10 to 20 mg/L. UV absorbance at 254 nm, measured using a spectrophotometer, provides some information on the aromaticity of the organic matter, because aromatic rings absorb UV light (1). Fluorescence indicates the level of polymerization of organic molecules present in water (1).

Extraction Methods Through Macroreticular Resins. Extraction techniques using selective resins fractionate organic matter into two parts: humic (humic and fulvic acids) and nonhumic substances, which correspond to hydrophobic and hydrophilic fractions, respectively (3). Humic substances are complex macromolecules obtained after polymerization of organic molecules, following biodegradation and chemical, biological, and photochemical oxidation of animal and vegetable wastes. They include aromatic structures linked together with aliphatic chains. The extraction method using the XAD-8 resin (3) is the most common technique for isolating aquatic humic substances. After acidification of a water sample to pH 2, humic substances adsorb to the XAD-8 resin, whereas hydrophilic acids pass through the resin. Half of the nonextractable chemicals includes hydrophilic acids and the other half represents well-identified chemicals such as carbohydrates, amino acids and proteins, carboxylic acids, and trace compounds. After an additional step of elution with caustic soda, humic substances are divided into two fractions: humic acids precipitating in acid (pH 1) and fulvic acids that remain soluble in acidic medium. Generally, fulvic acids have lower molecular weights and a higher number of carboxyl functional groups than humic acids. Fulvic acids represent the major part (80 to 85%) of the humic substances (3). Humic substances and hydrophilic acids are responsible for 50 and 30% of the DOC, respectively. The proportion of humic material in water can also be assessed by calculating the specific UV absorbance (SUVA). The SUVA value corresponds to the ratio between UV absorbance at 254 nm and DOC concentration (SUVA = UVabs [cm^{-1}] × 100/DOC [mg/L]). SUVA values higher than 4 to 5 generally describe a relatively hydrophobic DOC, mainly containing aquatic humic substances of high molecular weight and aromaticity. On the contrary, SUVA data less than 3 indicate that the DOC is hydrophilic, low in molecular weight, and low in charge density and aromaticity (4). Elemental analysis determination that defines the percentage of chemical elements such as carbon, hydrogen, nitrogen, oxygen, or sulfur within molecules showed that humic substances are mainly composed of carbon (average of 50%), oxygen (35 to 40%), hydrogen (5%), and nitrogen (1%) (5).

Identification of Functional Groups, Structural or Molecular Size (Weight) Characterization. Up to one-third of the organic molecules common in water can be identified using various technical analyses. Certain organic molecules can be characterized by spectrophotometric measurement (^{13}C nuclear magnetic resonance) or by a pyrolysis gas chromatography/mass spectrometry (GC/MS) technique. The

pyrolysis GC/MS method reveals fingerprints indicative of specific polymers such as polysaccharides, amino-sugars, proteins, and polyhydroxy-aromatic compounds (6). Amino acids (total and free), carbohydrates (total and free), aldehydes, and carboxylic acids can also be identified and quantified by specific chemical analyses (7–9). The apparent molecular weight of organic fractions can be assessed either by gel permeation liquid chromatography (Sephadex) (10) or after ultrafiltration fractionation (1).

Biodegradable Organic Matter Estimation (BOM)

Organic matter can be divided into two fractions. The first, called BOM, can be utilized by bacteria as a source of energy and carbon. The second is refractory to biodegradation (e.g., nonbiodegradable) and has little effect on bacterial growth (11). Several biological tests have been developed to assess the level of BOM in water (11,12). The specific experimental setting varies from one assay to another; however, they all include the following steps (13).

- *Preparation of Glassware*. Carbon contamination must be avoided when performing BOM measurements because water samples generally contain low carbon levels. The glassware is rendered carbon free after heat treatment at 550 °C for several hours or by washing in strong oxidant solution (e.g., sulfochromic acid and persulfate).
- *Pretreatment of Water Samples*. Depending on the methodology and sample type, pretreatment of samples is required to inactivate indigenous bacteria present in water, remove suspended matter, or neutralize any toxic chemical-like disinfectant residuals. Inactivation of microorganisms can be performed using pseudopasteurization. Water samples are heated at 60 to 70 °C for 30 minutes to inactivate autochthonous bacteria without drastically altering the organic matrix. Sample bacteria can also be retained after filtration using a 0.2-μm pore size membrane. Filtration is also performed for raw water samples containing suspended materials. Filtration membranes used to filter water samples are made of material such as fiberglass or polycarbonate that do not release organic compounds. Treated waters often contain disinfectants that could affect the microorganisms used in the bioassay. Disinfectant residuals are neutralized using sodium thiosulfate.
- *Preparation of a Bacterial Inoculum*. There is no standard inoculum. Inoculum can be composed of pure cultures of one or several bacterial strains used at certain concentrations or mixed natural flora. Pure culture inocula involve bacterial strains well adapted to low nutrient concentration conditions in water. Natural inocula are generally composed of many unknown species. In this case, it is difficult to assess the number of active cells, the microbial species involved, and their reactivity toward organic molecules. Inoculum may also be in suspension or attached to a solid media, such as biofilms attached to sand.
- *Inoculation and Incubation of Water Samples*. Inoculum density varies from a few hundred to millions of bacteria, depending on the method. Incubation periods, which allow test bacteria to grow and to degrade organic molecules present in the water sample, vary from a few hours to one month.
- *Measurement of the Test Parameter for Biodegradation*. Bacterial growth or carbon utilization is monitored during the incubation of inoculated water samples. Increase in bacterial counts can be evaluated using bacteria numeration on agar, microscopic numeration in epifluorescence, measurement of cell energy such as adenosine triphosphate (ATP), or turbidity monitoring. Carbon consumption by bacteria is assessed by monitoring DOC concentrations using a TOC analyzer.
- *Calculation of the BOM Concentration*. Depending on the parameter used to evaluate the BOM, results can be expressed using an index of biodegradability (micrograms acetate carbon equivalents per liter), cell production (growth yield or maximum growth), or carbon consumption (expressed in milligrams carbon per liter).

There are two established categories of biological tests: (*1*) AOC and (*2*) BDOC (Fig. 1). AOC refers to the fraction of TOC that can be utilized by bacteria for growth. The inoculum is a mixture of pure bacterial strains cultivated under laboratory conditions (*Pseudomonas fluorescens* P17, *Spirillum* NOX) (14–19) and environmental bacteria (20). Bacterial growth is monitored in the water samples using colony counts or ATP measurements. The maximum growth (N_{max}) observed during the incubation is converted into AOC using the growth yield of the bacteria from calibration curves performed with known concentrations of standard organic compounds (acetate and oxalate) (Fig. 1). When turbidity is measured for monitoring bacterial growth, two parameters are determined: the growth yield (μ) recorded during the exponential growth phase and the growth factor ($\log Y/Y_o$) corresponding to the increase in bacterial population (21). The concept of AOC has generated many different methods that are summarized in Table 1. In contrast, BDOC content represents the fraction of DOC that can be assimilated and mineralized by heterotrophic microbes. The inoculum consists of environmental bacteria suspended in water or fixed on a surface. BDOC is the difference between the initial DOC of the water sample and the minimum DOC observed during the incubation period (23–27) (Table 2, Fig. 1). In general, AOC molecules correspond to the "easily" assimilable organic compounds and generally represent a relatively small portion of the BDOC.

Microorganisms need various nutrients to grow in water. The ratio in which compounds of carbon, nitrogen, and phosphorus are required is 100 : 10 : 1 (C/N/P). Both AOC and BDOC assays quantify BOM (carbon), which is typically the limiting nutrient for bacterial regrowth in drinking water distribution systems. However, some nutrients other than organic carbon (phosphorus) may limit the regrowth of heterotrophic bacteria in the humus-rich water of some boreal regions such as Finland or

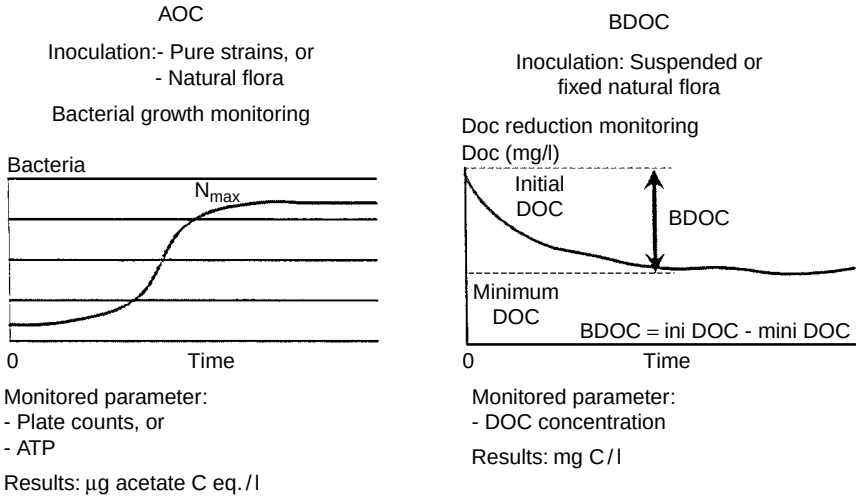

Figure 1. AOC and BDOC concepts for biodegradable organic matter determination.

Norway (28,29). In addition, a bioassay has been developed to determine if carbon or phosphorus is the limiting nutrient for regrowth (30).

BDOC Methodologies

BDOC Determination Using Suspended Bacteria. A filtered water sample (0.2-µm pore size filter) is seeded with suspended bacteria and incubated at $20 \pm 2\,^{\circ}C$ in the dark for 28 days (Fig. 2). Bacterial inoculum consists of indigenous bacteria contained in a small volume of surface water. Inoculum water is filtered through a 2-µm porosity polycarbonate membrane to remove particles and any protozoa that could graze bacteria. The inoculation ratio is 1 mL of surface water/100 mL of water sample (ratio of 1%, v/v). BDOC can be determined by two procedures. In the first one, bacterial biomass and mortality rate are monitored, the integrated flux of mortality (F) during the incubation period is then calculated and divided by the growth yield (Y) to give an estimate of BDOC (BDOC = F/Y) (24). This approach, which is very sensitive but also long, is used when BDOC levels are very low. The second procedure is simpler and is more commonly used. The decrease in DOC concentration is monitored until a plateau is reached and BDOC is the difference between the initial and final DOC values (24).

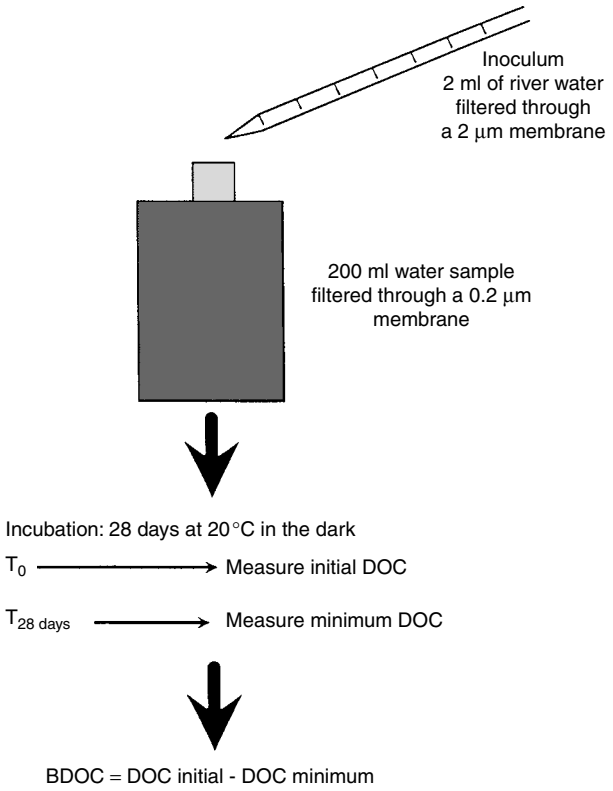

Figure 2. Experimental setup to measure BDOC levels with suspended bacteria.

BDOC Determination Using Sand-Attached Bacteria. This method is also based on the decrease in DOC concentrations, but the water sample is incubated at $20 \pm 2\,^{\circ}C$ in the presence of sand colonized by bacteria (23,31) (Fig. 3). Water samples can be aerated during incubation with organic carbon–free air. The inoculum is quartz sand (with a granulometry of 1 to 2 mm) colonized by bacterial biomass sampled from a sand filter of a water-treatment plant without a prechlorination stage (no oxidant residual present) or colonized sand from a freshwater fish tank. A sand/water sample ratio of 1 : 3 (w/v) is used for sample inoculation (e.g., 100 g of sand per 300 mL test water). Using such an abundant fixed flora allows a rapid response; the test is completed after an incubation period of a few days. The DOC values in the water sample are analyzed on a daily basis with a TOC analyzer. The incubation is continued until a minimum DOC value is reached. BDOC concentration is defined as the difference between the initial and minimum DOC values. The minimum DOC value observed after biodegradation, which corresponds to the nonbiodegradable DOC, is defined as the refractory dissolved organic carbon (RDOC).

BDOC Determination Using Bioreactors. The plug flow biofilm reactor was developed to measure BOM levels within a few hours (26,27). A BDOC reactor consists of

Table 1. AOC Methodologies (Adapted from Huck (11))

Authors (Reference)	Sample Preparation	Inoculum (Concentration)	Incubation Conditions	Parameter Measured	Result Expression
Vander Kooij and coworkers (10,11)	Pasteurization	Pure strains *P. fluorescens* P17 and *Spirillum* NOX (500 CFU/mL)	20 days 15 °C	CFU/mL	AOC calibration in known solutions of sodium acetate (μg C eq. acetate/L)
Kaplan and coworkers (12)	Pasteurization	*P. fluorescens* P17 and *Spirillum* NOX (500 to 1,000 CFU/mL)	9 days 20 °C	CFU/mL	AOC
LeChevallier and coworkers (14)	Pasteurization	*P. fluorescens* P17 and *Spirillum* NOX (10^4 CFU/mL)	5 days 22 °C	ATP	AOC
Bradford and coworkers (19)	Filtration	*P. fluorescens* P17	12 hours 20 °C	Cell elongation Microscopic counts	AOC
Kemmy and coworkers (15)	Filtration	Mixture of four strains: *P. fluorescens* + *Curtobacterium* + *Corynebacterium* + 1 species of coryneform type	6 days 20 °C	CFU/mL	AOC calibration in solution of mixed organic compounds (μg C/L)
Stanfield and Jago (16)	Filtration	Bacteria from			
		Raw water	Until max. growth 20 °C	ATP	AOC
		Sand-filtered water			Standard conversion factor (μg C/L)
Werner and Hambsch (17)	Filtration	Water sample bacteria retained on the filter (5×10^4 cell/mL)	60 hours 20 °C	Turbidity	Growth yield (μ), growth factor (log Y/Y_o)
Reasoner and Rice (18)	Filtration	*Enterobacter cloacae*, *Escherichia coli*, *Klebsiella oxytoca*	5 days 20 °C	CFU/mL	log N_5/N_0

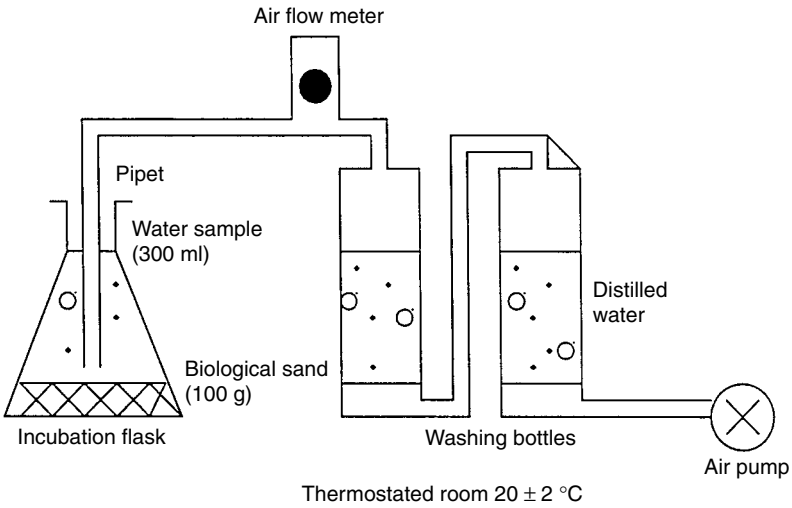

Figure 3. Experimental setup to measure BDOC levels with bacteria attached to sand.

two glass chromatography columns (2.5 × 60 cm, Kontes, Vineland, New Jersey) containing porous glass particles (Siran; 1- to 2-mm diameter, 60- to 300-μm pore size, Schott America, Yonkers, New York) (Fig. 4). The reactor column media is heavily colonized with bacteria from the environment. Bioreactors are continuously fed with water at a flow rate of 4 mL/minute. The total hydraulic retention time of the BDOC reactor is two hours for both columns. The BDOC test begins by loading the bioreactor reservoir with a water sample. Column inflow and outflow are sampled for at least two hours after the beginning of the test. BDOC concentrations are determined from changes in DOC concentrations from the reactor inflow and outflow.

Factors Affecting BOM Results

BOM levels can be estimated using various bioassays, and each test has specific strengths and weaknesses (11,12). Most of the methods have not been standardized, and

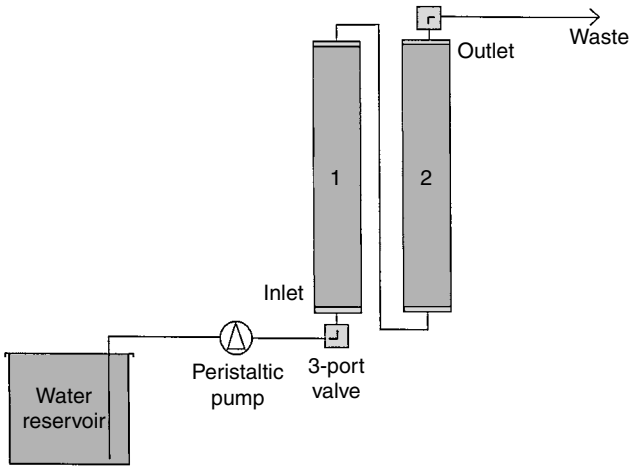

Figure 4. Experimental setup to measure BDOC levels with a biofilm reactor.

many factors such as the inoculum (pure strain versus mixed inocula from the environment), the glassware used, the monitored parameter (bacterial growth versus DOC), the use of suspended or fixed bacteria, and the experience of the laboratory performing the test can influence the final test results (12,32).

Nutrient Levels in Water. Organic matter and nutrient concentrations vary dramatically between water sources, over the seasons, and from one year to another. In general, DOC levels are low (<1 mg/L) in groundwater. Surface waters generally contain 2 to 10 mg/L of DOC (2); however, higher values (20 to 30 mg/L) have been recorded in some waters from Southeastern United States with high-humic content. BDOC concentrations typically range from less than 0.1 mg/L to 1 to 2 mg/L. For example, a study conducted on various water types showed that river waters had DOC values of 3.2 to 4.0 mg/L and BDOC concentrations above 1.2 mg/L (33). After treatment, BDOC levels ranged between 0.2 and 1.0 mg/L (DOC between 0.9 and 2.9 mg/L). Waters with low organic matter levels had BDOC levels lower than 0.1 mg/L (mineral, spring, and ultrapure waters with DOC levels less than 0.5 mg/L) (33). A large survey that evaluated BDOC concentrations in 31 surface water treatment plant effluents showed that the geometric mean of BDOC concentrations was 0.32 mg/L for all the sites (34). On an average, the BDOC represented 5 to 21% of the DOC. When the BDOC levels were tabulated site by site, the mean concentrations of plant effluents ranged between 0.03 and 1.03 mg/L, whereas the DOC levels ranged from 0.61 to 4.53 mg/L (34) (Fig. 5).

AOC concentrations typically vary from a few to several hundred micrograms per liter. Figure 5 shows AOC results of a survey conducted at 95 sites. AOC concentrations entering distribution systems were generally high. Fifty percent of the sites had plant effluent AOC levels above 100 μg/L. When data were averaged for each site, the mean AOC levels ranged from 18 to 214 μg/L, depending on the site (34). These finished water results are comparable to other AOC data collected from 53 source waters, including surface and groundwaters in North America (35). Raw water AOC concentrations varied from 18 to 322 μg C-acetate/L or from 2.4 to 44% of the DOC content. Sixty-two percent of the raw water AOC values were above 100 μg/L. Bradford and coworkers (19) reported AOC variations from 94 to 275 μg/L (mean of 168 μg/L) in a surface water reservoir and 75 to 731 μg/L (mean of 317 μg/L) in river water (Santa Ana River, California). The mean AOC concentration for three groundwater samples was 54 μg/L. Much lower AOC concentrations (<10 μg/L) were recorded after bank or slow sand filtration (14).

Parameters Affecting BDOC Results. BDOC concentrations vary, depending on the method applied and the operating conditions (inoculum size, incubation time, sample aeration) (Table 2). For the BDOC determination using bacteria fixed on sand, both inoculum size and aeration have been shown to affect the result of BDOC measurement (31,36) (Table 3). A sand/water ratio of 100 g : 300 mL (w/v) allows a rapid decrease in DOC concentration and optimum biodegradation of the organic matter. However, levels of BDOC tend to increase with the sand/water ratio, showing sorption of a low fraction of DOC (0.1 to 0.2 mg of DOC/100 g of sand). Aeration

Table 2. BDOC Methodologies (Adapted from Huck (11))

Author (References)	Sample Preparation	Inoculation	Incubation Conditions	Parameter Measured	Result Expression
Servais and coworkers (24)	Filtration	Suspended bacteria from river water	28 days 20 °C	DOC	$BDOC = DOC_i - DOC_f$
Joret and Levi (23), Volk and coworkers (31)	–	Bacteria fixed on sand	1 week 20 °C	DOC	$BDOC = DOC_i - DOC_{mini}$
Mogren and coworkers (25)	–	Bacteria fixed on sand	5 days 20 °C	DOC	$BDOC = DOC_i$ – DOC 5 days
Frias and coworkers (26), Kaplan and coworkers (27)	Filtration	Column with bacteria fixed on porous glass particles	2.5 hours 20 °C	DOC	$BDOC = DOC_{inflow} - DOC_{outflow}$

Note: DOC_i: initial DOC concentration, DOC_f: final DOC concentration, DOC_{mini}: minimum DOC concentration.

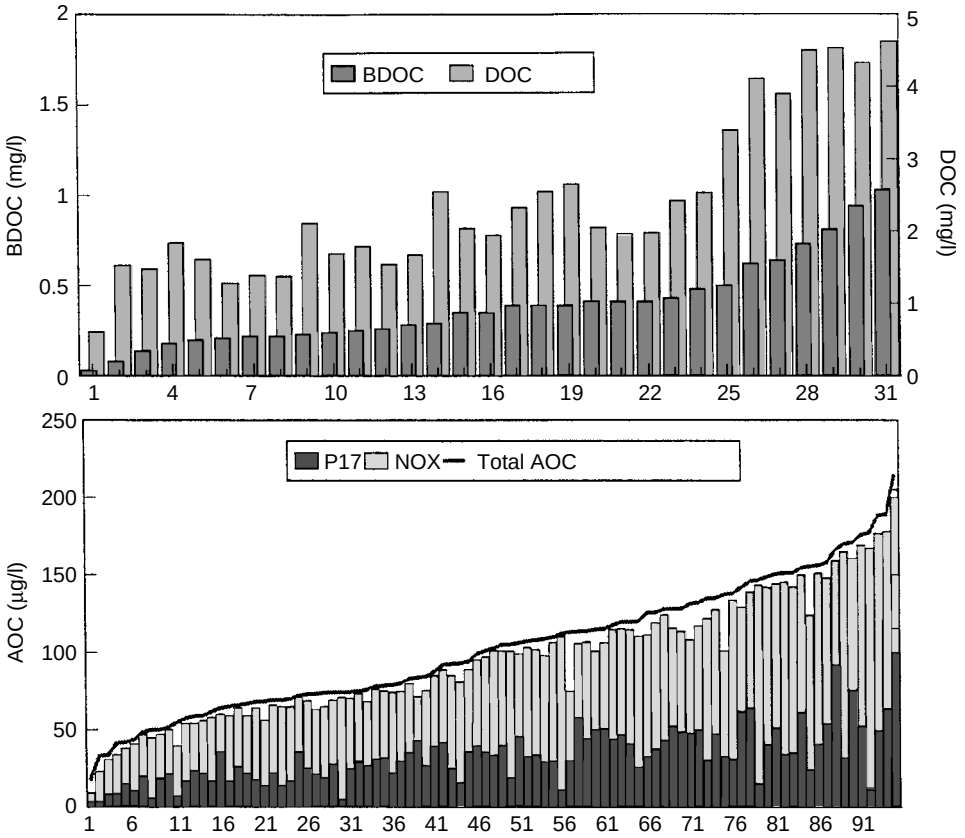

Figure 5. DOC, BDOC, and AOC concentrations for different plant effluents (from Volk and LeChevallier (34)). The *x*-axis represents the site number from 1 to 31 and from 1 to 95 for AOC, respectively, after ranking.

Table 3. Factors Affecting BDOC Results

Method	Parameter	Effects on Measured BDOC Concentration
Suspended bacteria method	Inoculum size	None
	Aeration	None
	Incubation period	BDOC level increased
	Disinfectant neutralizer	BDOC level decreased
Sand method	Inoculum size	BDOC level increased
	Incubation period	None
	Aeration	Faster test, possible increase in BDOC level
Bioreactor method	Contact time	Possible increase in BDOC level

$$BDOC_{\text{suspended bacteria}} < BDOC_{\text{sand}} = BDOC_{\text{bioreactor}}$$

mixes the water and provides a more uniform exposure to the sand, promotes faster kinetics of DOC consumption, increases dissolved oxygen concentrations, and sometimes leads to a greater consumption of DOC (31).

In the method using suspended bacteria, inoculum size (from 0.04 to 5%, v/v) and aeration (4 L/hour) have not been shown to have any effect on BDOC determination (31,36). On the other hand, incubation time affects the result of BDOC measurement (Table 3). For certain water types, biodegradation may still be incomplete after one month of incubation. BDOC levels increased by 0 to 125% when the incubation was extended to 85 to 120 days (31). Finally, precautions have to be taken regarding the dechlorination agent used to neutralize disinfectants. MacLean and coworkers (37) reported that sodium thiosulfate levels higher than 20 mg $Na_2S_2O_3$/L interfered with the 30-day BDOC test using suspended bacteria. During incubation, the presence of $Na_2S_2O_3$ promoted the growth of sulfur

oxidizing bacteria resulting in the production of sulfuric acid and a depressed pH. The low pH inhibited the activity of heterotrophic bacteria in utilizing organic carbon (37).

The BDOC column method is attractive for use in water utilities because by simply measuring the DOC at the inlet and outlet of the columns, BDOC can be determined rapidly on a continuous basis. The problem with the bioreactor method, however, is that a long period (between six and nine months) is required for the columns to be colonized with the variety and density of bacteria necessary to rapidly utilize trace levels of organic carbon. Bioreactors are also highly site-specific — changing feed water source may require an acclimatization period of several months. Columns that were colonized with water from the Mississippi River required up to seven months to equilibrate once shipped to new locations with different types of source waters (34). Consequently, bioreactors must be colonized and used to perform BDOC measurements with the same water matrix. NOM is composed of a mixture of hundreds of simple molecules and complex polymers such as humic substances that are highly specific to a particular watershed. When colonization occurs, organic matter leads to the development of unique and specific populations of microbial species. This high specificity of bacteria to the site's organic material was reported to be a limitation of the BDOC-column methodology (27,38). Bioreactors do not perform well as a stand-alone analyzer for samples from various origins. During interlaboratory experiments of bioreactors colonized with different water sources, maximum biodegradation was observed at the site in which the microflora was specific to tested water samples. At the other sites, when bioreactors were exposed for a few hours to the new water conditions, the reactor biofilm could not metabolize any of the organic matter. In addition, the bioreactor columns require constant attention and maintenance. After colonization, bioreactors can operate several years, but they require a continuous flow of low-turbidity water that does not contain disinfectants (feed water can be filtered river water, granular activated carbon (GAC)-filtered water, or dechlorinated tap water). Routine checks include pump function, absence of blockage, absence of leaks, and flow through the reactor. If a feed water reservoir is used, the water within the reservoir must be periodically replenished.

Variation Between the Different BDOC Methods. The estimation of BDOC concentration depends on the applied method. BDOC concentrations obtained with a suspended and a fixed/sand inoculum are well correlated. However, the BDOC values are generally higher (twice as high) while using attached (i.e., fixed/sand) bacteria (31). Mathieu (13) also observed that the BDOC values measured using bacteria attached to sand were 1.2 to 1.5 times higher than those obtained with suspended bacteria. Several hypotheses could explain discrepancies between the suspended and fixed/sand inoculum. The bacterial concentration in freshly inoculated water samples is 100 to 1,000 times higher when using colonized sand than when using river water as the inoculum (10^7 to 10^8 bacteria per gram of sand versus 10^6 bacteria per milliliter of river water). An attached inoculum might be able to degrade a larger range of organic compounds. Thus, molecules that are refractory to biodegradation by a suspended inoculum could be biodegradable by a fixed inoculum. This observation could be because of a greater bacterial diversity in the sand biofilm than in a river sample, a higher adaptation of sand bacteria (fixed species are selected according to their ability to degrade a large spectrum of organic compounds), and an advantage caused by the fixation because cometabolism and synergy mechanisms can occur on sand particle biofilms.

Both the bioreactor and the sand methods yield similar BDOC results. A study involving 52 samples showed that the differences between the BDOC levels obtained from the bioreactor and sand methods were less than 0.1 mg/L for 50% of the samples (34). The difference was between 0.1 and 0.2 mg/L for 17% of the samples. In comparison, Joret (39) reported differences less than 0.1 mg/L and between 0.1 and 0.2 mg/L for 49 and 30% of the samples, respectively. Kaplan and coworkers (27) and Prevost and coworkers (40) found that the bioreactor results were higher than the BDOC levels determined using suspended bacteria. This observation is not surprising, as the BDOC-sand method gives higher results than the BDOC-suspended bacterial method (31).

Variation Between AOC and BDOC Data. The AOC test measures a biological response to assimilable carbon, but unlike BDOC, it is not a direct measure of the carbon level itself. Correlations observed between AOC and BDOC can be strong in some cases and weak in others (12). Figure 6 shows a comparison of the AOC and BDOC values based on a monthly analysis of 31 plant effluent samples (34). Although the AOC and BDOC values were significantly correlated ($p < .01$), the variation between the test results was high (correlation coefficient of only 0.45). When the test results were grouped, a more clear relationship was observed between the two tests (Fig. 6). Volk and coworkers (33) observed an excellent correlation ($r = 0.996$) between the AOC and BDOC levels in a series of dilutions of a particular type of river water. However, the correlation between the tests was not as strong ($r = 0.77$) when performed on 31 different water types. Kaplan and coworkers (35) reported a significant but weak correlation ($r = 0.59$) between the AOC and BDOC data in a survey involving 109 samples from 79 drinking water supplies. AOC is an index of regrowth potential, which is strongly related to specific groups of organic molecules. Moreover, the origin and changes in the characteristics of organic matter might affect the relationship between these two methods of BOM measurement. Because NOM characteristics vary from one water type to another, and seasonally for a given water type, it is not surprising that waters with similar BDOC concentrations might have low or high levels of AOC, depending on the type of organic molecules present in the water constituting BDOC. BDOC concentrations are generally much higher than the AOC levels. This is probably due to a difference in the range of metabolic capabilities contained in the AOC test, which uses just two bacterial stains (P17 and NOX), versus the

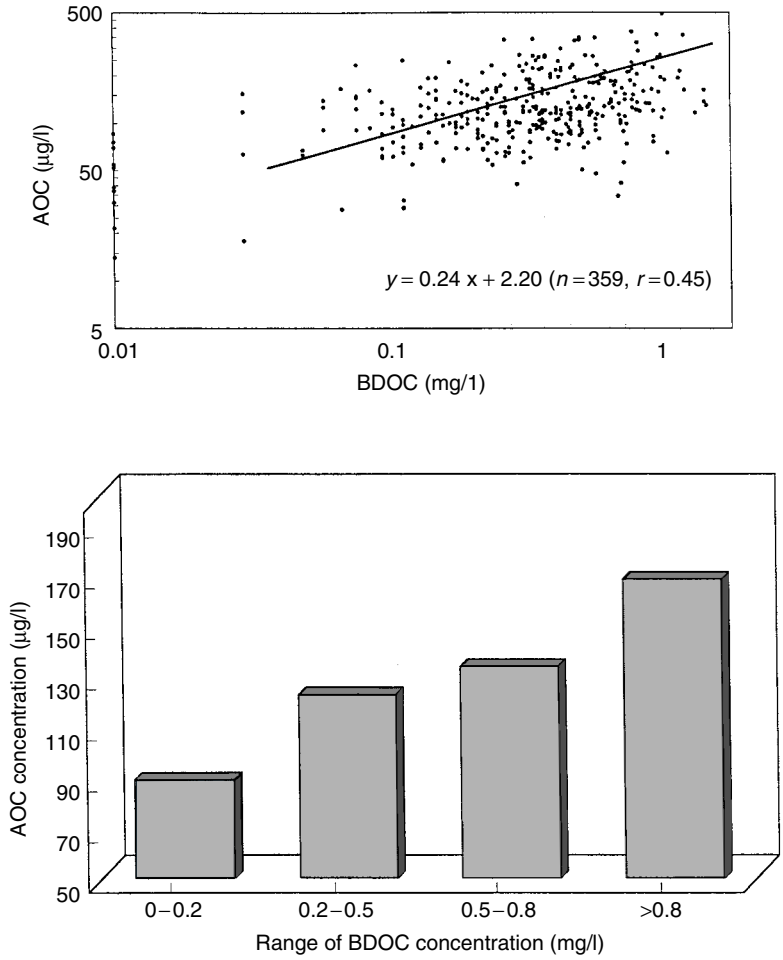

Figure 6. Relationship between AOC and BDOC concentrations (from Volk and LeChevallier (34)).

BDOC-sand bioassay with a wide variety and a greater density of microorganisms capable of degrading a large spectrum of organic compounds.

Composition of Biodegradable Molecules. AOC measures the "easily" assimilable carbon, whereas the BDOC fraction includes easily and slowly biodegradable compounds. Because of the complexity of the organic matter, it has not been possible to determine precisely the chemical characterization of the biodegradable fraction. Biodegradable organic materials have generally been considered to be carbohydrates and small molecular weight compounds, whereas complex molecules such as humic substances in water were assumed to be essentially nonbiodegradable. However, some studies have shown that this classification should be used with caution (41–43). It has been found that biodegradable compounds include both low and high molecular weight molecules. Some low molecular weight molecules can be directly utilized for metabolism. Other compounds, such as humic substances, which constitute most of the DOC, can be partially degraded by bacteria after enzymatic action. A study showed that 27% of humic substances in stream water were utilized by biofilm bacteria (43). On an average, BDOC was composed of 75% humic substances, 30% carbohydrates, 4% amino acids, and 39% of molecules greater than 100,000 Da (43). Finally, the BDOC fraction determined using the suspended bacteria assay (24) can be further fractionated into three pools of substrates (S, H1, and H2). The H1 fraction represents the macromolecular substrate that can be rapidly hydrolyzed by bacteria exoenzymes, whereas H2 includes polymeric organic molecules slowly hydrolyzed. The H1 and H2 fractions are transformed into the S fraction, including monomeric substrates directly available for bacteria. This partitioning is achieved by the use of a biomass flux technique (44) and by the use of the HBS Model (H: high molecular weight polymers, B: bacteria; S: direct substrates) (45). The polymeric BOM (H1 and H2 fractions) is hydrolyzed by the bacterial exoenzymes that are in concentrations directly proportional to bacterial biomass, according to Michaelis-Menten kinetics. The kinetics includes the maximum rates of exoenzymatic hydrolysis of H1 or H2 ($e_{1\max}$, $e_{2\max}$) and the half-saturation constants (K_{H1} and K_{H2}). The exoenzymatic hydrolysis rates for H1 and H2 are, respectively, equal to $e_{1\max}\mathrm{H1}/(\mathrm{H1} + K_{H1})$ and $e_{2\max}\mathrm{H2}/(\mathrm{H2} + K_{H2})$. The exoenzymatic hydrolysis of macromolecules yields monomeric substrates (S), such as amino acids, which are rapidly utilized by bacteria, according to another Michaelis-Menten kinetics. For substrate S, the direct substrate uptake is equal to $b_{\max}\mathrm{S}/(\mathrm{S} + K_S)B$. The kinetics is characterized by the following parameters: maximum rate of substrate uptake per

unit of bacterial biomass (b_{max}) and the half saturation constant of direct substrate uptake (K_H). The numerical values of the different kinetic parameters were determined for a large spectrum of aquatic environment and correspond to general physiological characteristics. These processes are used to describe BDOC removal during biological filtration (Model CHABROL) (46), or BDOC consumption in distribution systems (Model SANCHO) (47). These models are described later in this section.

While BDOC can include larger molecules, humic substances, and some humic bound compounds, it appears that for most water samples, the AOC is mainly composed of small (<1,000 Da) and nonhumic molecules (34).

REMOVAL OF ORGANIC MATTER DURING DRINKING WATER TREATMENT

Drinking Water Treatment and Water Quality

Most types of water require treatment to remove certain impurities and pollutants, add chemical substances, or change water characteristics in order to protect public health, improve water aesthetics, and comply with drinking water regulations. Overall, the quality of water is improved after treatment. Raw water type (ground versus surface water) and the type and levels of impurities present in the source water determine the treatment processes that need to be implemented. In general, groundwaters have a uniform quality and contain no turbidity (cloudiness), few microorganisms, and little organic matter. However, groundwaters often have high hardness and might contain various contaminants such as iron, manganese, hydrogen sulfide, and radionuclides. They can also be contaminated by synthetic chemicals, including organic solvents and herbicides/pesticides. Surface water supplies originate from creeks, streams, rivers, lakes, and reservoirs. Surface waters have the characteristic of varying quality after heavy rains or over the seasons. Surface waters must be treated to remove microorganisms and turbidity. In addition, they contain organic matter and numerous inorganic and organic contaminants related to natural or human activities (urban, industrial, and agricultural pollution). The kinds of treatment available to produce drinking water can involve aeration, adsorption, chemical oxidation and disinfection, settling, filtration, membrane filtration, softening, or ion exchange. Most surface waters are conventionally treated to remove high levels of microbes and particles. The basic conventional treatment train involves the following four steps: pretreatment, settling, filtration, and posttreatment before distribution (Fig. 7). Preoxidation is used to control bacteria and algae growth and oxidize iron, manganese, taste, odor and color molecules, as well as micropollutants. Primary oxidants include chlorine, chlorine dioxide, potassium permanganate, ozone, and hydrogen peroxide. Chlorine has been the most extensively used, but some concerns about the health effects of chlorine disinfection by-products have increased the use of alternative oxidants. Chemically enhanced settling allows removal of particulate and colloidal matter that would not precipitate within a reasonable time by gravity alone. After physicochemical reaction, the nonsettlable solids are converted into heavier and settlable solids that can be removed by sedimentation. Target elements include clay and silt particles, microorganisms, inorganic solids, as well as color

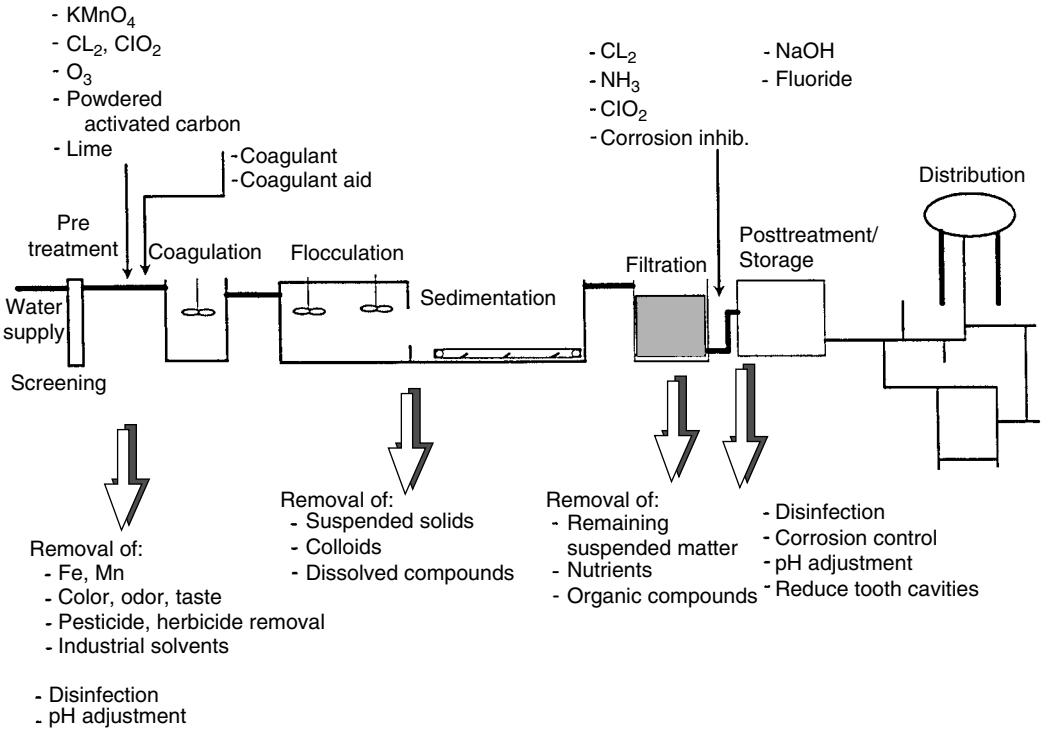

Figure 7. Conventional water treatment steps.

and organic matter. Settling involves three sequential steps of coagulation, flocculation, and sedimentation (Fig. 7). Coagulation consists of adding and rapid mixing of chemical coagulants into raw water. Alum salts, iron salts, and synthetic polymers are the most common coagulants used in water treatment. Particles in water are usually negatively charged and tend to repel one another. Positively charged coagulants neutralize particle charges and promote coagulation. Destabilized particles aggregate during flocculation. Slow mixing promotes particle aggregation and leads to the formation of bigger flocs. The flocs are allowed to precipitate out of the water by gravity during sedimentation (no mixing). Filtration is the final step in water clarification. Suspended matter remaining after settling is removed when passed through a bed of granular media filter at a certain velocity. The most common materials used in filter media are sand, anthracite, garnet, and GAC. Filter materials can be used alone (mono-media filter) or in combination (dual or multimedia filters). Following settling, a major goal of filtration is to physically remove suspended solids, remaining precipitates, and floc that did not settle, thus improving the finished water turbidity. Moreover, if media include GAC, dissolved organic compounds can be removed through adsorption and biodegradation. GAC is generally used to remove micropollutants through adsorption. However, after a few months, the adsorption capacity of GAC is exhausted and it must be regenerated or replaced. An alternative is to use GAC without regeneration, to use the benefit of microbial flora that colonize GAC grains in the absence of disinfectants. After filtration, a final disinfection (usually chlorine alone or in combination with ammonia to form chloramines) is fed to control bacteria levels in the distribution system. Additional treatment steps can be added to the basic conventional treatment train to deal with specific pollutants or improve water quality. Powdered activated carbon (PAC) can be fed during pretreatment to remove taste and odor compounds as well as micropollutants. A second-stage filtration (postfilter adsorber) may be required when waters contain high levels of organic compounds (nutrients, taste and odor, or synthetic organic compounds).

Organic Carbon Transformation During Water Treatment

Treatment of organic carbon is important because these materials can be responsible for water taste, odor, and color. Organic matter will affect oxidant demand, disinfectant by-product levels, micropollutant removal, residual disinfectant stability, and bacterial regrowth in distribution systems. The reduction of organic matter concentrations and its biodegradable fraction during water treatment is one strategy to control bacterial regrowth in the distribution system. A variety of treatment processes can be used to control BOM levels in drinking water. The degree of organic carbon removal during drinking water production depends on several parameters such as the treatment train design, operational conditions, and the seasonal fluctuations of the source water quality. In general, coagulation, biological filtration, or membrane filtration reduces BOM levels, whereas oxidation with ozone or chlorine forms biodegradable compounds from relatively nondegradable DOC.

Settling. Coagulation, flocculation, and sedimentation are critical steps in surface water clarification. Historically, most coagulation processes were primarily designed for particle and turbidity removal. However, some plants, especially those having to treat highly colored water, were designed to remove organic matter. NOM and BDOC levels can be reduced by coagulation through colloid destabilization, precipitation, coprecipitation, or adsorption onto floc (48,49). Organic matter concentration is generally reduced after settling. The elimination of organic material is impacted by many factors such as the characteristics of the NOM, coagulation conditions (coagulant type and dose, coagulation pH), the nature and concentrations of inorganic compounds, and the design and operation of the sedimentation basin. Consequently, the removal of organic matter by coagulation varies widely, and is generally between 10 and 90%.

Organic Matter Characteristics. In general, higher organic matter removal is observed for samples with high DOC values. For example, a study showed that water samples with DOC concentrations greater than 4 mg/L exhibited DOC and BDOC removals of 55 and 58%, respectively, versus 31 and 18% for waters with DOC levels below 4 mg/L (50). Lind (51) reported NOM removals as high as 90% for high-TOC water samples (>10 mg/L) from the southeastern United States. Coagulation mostly removes the hydrophobic organic fraction (humic substances) and large molecules. Hydrophilic compounds are little affected by coagulation (1,4,52,53). Moreover, the removal of NOM is affected by other physical and chemical properties of NOM, including the solubility of organic compounds, the charge density of molecules, or the functional group composition (1,4,54–58).

Coagulation pH. The pH of coagulation is also a very influential parameter governing organic removal. Typically, the best removals of humic substances have been obtained between pH 4 and 5 with ferric and between pH 5 and 6 with alum (59–61). A series of coagulation tests performed on a bench scale (jar tests) showed that the pH of coagulation for optimized organic matter removal with ferric chloride and alum ranged from 4.4 to 6.7 and from 5.6 to 7.1, respectively. Polyaluminum chloride (PACI), another type of coagulant, can be used at a higher pH (6.0 to 6.8). Lowering the pH of coagulation with sulfuric acid led to additional DOC and BDOC removal (versus coagulation without pH adjustment) of 6 to 17% and 0 to 13%, respectively (50). In comparison, Chowdhury and coworkers (62) reported a TOC removal of 9% under plant conditions, but TOC removals increased to 25% when carbon dioxide was added to lower the pH from 8.0 to between 7.0 and 7.3. Lind (51) also found that TOC removal was improved at lower pH values with alum and ferric chloride, except in cold, low alkalinity, and low TOC water. At lower pH, the dissolved organic matter precipitates and becomes particulate organic matter. Organic particles are then removed by sweeping or adsorption on floc.

Coagulant Type. In addition to pH, the amount of organic material removed is also dependent on the type of coagulant used. Studies reported in the literature are contradictory, suggesting that the performance of a particular coagulant is dependent on the specific characteristics of the organic matrix and the test conditions. Several studies have demonstrated that iron-based coagulants are superior to alum salts (50,52,63). Inorganic coagulants (ferric or alum) also seem superior to synthetic organic polymers for coagulation of organic matter (64). Researchers (57) compared the performances of ferric chloride versus alum or PACI for removal of DOC and BDOC in different surface waters. They showed that iron salts typically resulted in greater removal of DOC and BDOC, as high as 28 and 21%, respectively. PACI resulted in the lowest removal of DOC and BDOC. In other experiments performed on high-TOC water (Hillsborough River, Tampa, Florida), the average TOC removal was 47% with alum and 65% with ferric sulfate (65). Alternatively, a field study involving 46 different plants showed that TOC removal was best with PACI or alum with sulfuric acid (51). The average TOC removal was 39% with low basicity PACI, 32% with alum, and only 13% with ferric salts.

BOM Removal. As seen for DOC, reduction in BDOC levels varies from one water sample to another, and the characteristics of the biodegradable materials govern their removal during coagulation. Limited studies have been performed to assess biodegradable DOC removal by coagulation. Removal of BDOC ranged from 50 to 86% using alum at a full-scale treatment plant using alum and no preoxidation (66). Croué and coworkers (67) reported that removal of BDOC by ferric chloride and alum correlated to removal of DOC, and ranged between 38 and 88%. When coagulation was optimized or enhanced for removal of dissolved organic materials for various surface waters, the removal of DOC could be increased from 25 to 43% (50). Similarly, removal of BDOC could be improved from 30 to 38% through the application of enhanced coagulation. However, when coagulation is optimized, the additional NOM removal preferentially impacts the refractory (nonbiodegradable) fraction of DOC as opposed to the biodegradable DOC fraction (50). Some biodegradable compounds such as proteins are removed very well by precipitation. Hureiki and coworkers (68) reported that coagulation removed 34 to 72% of total amino acids. However, AOC is typically not affected by coagulation, probably because AOC target small molecular weight, nonhumic compounds that are not amenable to coagulation. AOC removal during settling could be attributed to phenomena other than physicochemical reactions. Huck and coworkers (69) found AOC removals ranging from 0 to 85% by clarification; however, the reduction in AOC levels may have been caused by biological activity because no oxidation step was applied before coagulation, and there were long residence times within the sedimentation basin (70).

Filtration. The removal of the biodegradable fraction of organic matter is one of the main goals of biological treatment. When there is no prechlorination stage before filtration, a large amount of biomass can accumulate on filter media in the form of a biofilm. This biofilm can absorb and assimilate biodegradable materials contained in the water. Organic removal during filtration is impacted by the organic matter's quality and quantity, and will vary by season, media type, contact time, and backwashing strategy.

Temperature. Water temperature can limit bacterial activity within biological filters. Moreover, season affects the composition and concentration of the organic matrix and its subsequent biodegradation kinetics. Huck and coworkers (69) suggested that biological contactors should be operated seasonally, depending on seasonal water characteristics and utility criteria. Bacterial density and biomass activity have shown differences between winter and summer periods (71). The removal of DOC, BDOC, and trihalomethanes (THMs) was found to be significantly greater during warm temperatures (71–73). Merlet and coworkers (72) showed that DOC removal ranged from 0 to 15% in winter and 25 to 30% in summer. In a pilot study (73), two filters containing similar bacterial biomass were loaded with the same water at 8 and 20 °C . Bacterial activity led to a 60% removal of BDOC at 20 °C and a 27% removal at 8 °C . Consequently, it was necessary to double the contact time to have similar BDOC removal efficiency.

Filtration Media. Various filtration media can be used alone or in combination; however, the medium type is critical. Filtration using biologically active carbon is more efficient than biological sand filters for the removal of biodegradable organic carbon (74). GAC also showed better removal of DOC, BDOC, oxalate, aldehyde, and glyoxalic acid than anthracite (75,76). This difference can be explained by higher densities of fixed bacteria (four times as much) on carbon filters. Compared to microporous activated carbon, macroporous carbon provides the bacteria with a larger number of attachment sites protected from the abrasive action of backwash. The more abundant biomass allows for stable BDOC removal as well as sustained nitrification, even in cold waters (72). First-stage filters that are designed to remove particles can also achieve significant BOM removal through biological activity. However, the accumulation of floc and particles could act as a diffusion barrier for BOM molecules and affect biomass activity, especially under cold-water conditions (76).

Contact Time. The water-filter media contact time influences the amount of organic compounds removed. The higher the contact time, the better the removal. DOC removal was doubled (from 7 to 15%) when empty bed contact time (EBCT) was increased from 5 to 20 minutes (72). Some organic compounds also require longer contact times to be removed by biological filtration. Biologically active filtration is very effective on aldehyde removal. However, dialdehydes such as glyoxal require a slower filtration rate for their removal than formaldehyde and acetaldehyde (77).

Backwashing. Effective backwashing is essential for long-term successful filter service and for avoiding the formation of mudballs or filter cracks. In the case of biological filtration, the role of backwash is of primary importance for removal of nonbiological and biological particles without removing all the fixed bacteria. Different filter backwash methods are available, using water alone or in combination with air. While using an upflow wash, water is introduced into the bottom of the filter, and the filter medium gradually assumes a fluidized state as the backwash water flow increases and the bed expands. Particles accumulated are washed out of the filter media. Backwash flow is continued with full fluidization until the wastewater is clear. The effect of backwashing on biofilter bacteria varies depending on the type of support media and the backwash strategy. Backwashing with chlorinated water may remove a significant portion of the fixed biomass and decrease the performance of the filter for removal of organic carbon (78,79). In the case of anthracite, backwashing with chlorinated water may remove a significant portion (24%) of the fixed biomass (79). This had a short-term effect on the removal efficiency of certain organic molecules such as aldehydes. Similarly, Characklis (78) showed that chlorinated backwash of pilot sand/anthracite filters decreased TOC removal and microbiological activity in the filter, as evidenced by the number of heterotrophic bacteria fixed to the media. As for GAC filters, chlorinated and nonchlorinated backwashes lead to the same amount of fixed biomass and similar TOC removals (80). Servais and coworkers (71) reported that in the case of activated carbon, the loss of biomass appeared to be negligible, and it was less than 5% in warm water. A combination of water and air can be used to improve backwash efficiency. Pilot studies showed that backwashes with a combination of water-air scour and with water only yielded similar postbackwash AOC and TOC removals (81).

Modeling. Several models were developed to predict the performance of biological filters. Biofiltration processes are based on biofilm process principles. Biofilm kinetics has been well studied in the wastewater field. Ritmann (82) applied steady state biofilm modeling to interpret field data from drinking water biofilters. The principle of steady state biofilm is that the growth of new biofilm caused by substrate utilization is balanced by the biofilm losses caused by decay, detachment, and predation. The application of steady state biofilm kinetics to drinking water filters requires additional considerations. First, under low nutrient conditions, the presence of soluble microbial products might also affect the quality of the filter effluent. Moreover, backwash effects must be included. Biofilm detachment is high during backwash, whereas it is relatively low between backwashes when biofilm growth may accumulate. Finally, biofilms composed of multiple bacterial species are exposed to a wide range of inorganic and organic nutrients (82–85). Huck and coworkers (86) predicted the removal of biodegradable materials such as AOC and BDOC, disinfection by-product precursors, and chlorine demand by first-order models. The removal rate of biodegradable substances is defined as the difference in concentration at the inlet and outlet of the filter divided by the EBCT. The removal rate is proportional to the influent concentration at the inlet of the filter. The slope of the relationship between the removal rate and the BOM level is defined as the average specific removal (r_{as}) expressed in units of inverse minutes. The relationship also allows one to determine a minimum concentration below which the biodegradable materials could not be reduced by biological filtration. This concentration was estimated as 20 to 25 μg/L and 3 to 4 μg/L following first-stage filters and GAC contactors, respectively (86). The CHABROL Model is another tool used to predict BDOC removal during rapid sand or GAC filtration (46,71,87) (Fig. 8). The model includes the following processes occurring during biological filtration:

- *Interaction Between Bacteria and Organic Matter*. Bacteria can directly assimilate monomeric substrates (S fraction) and utilize easily and slowly biodegradable organic compounds (fractions H1 and H2, respectively), after exoenzymatic hydrolysis.
- *Interaction Between Bacteria and the Filter Media*. Bacteria can be biologically fixed (population B1) or adsorbed to the solid support (population B2) and suspended in the liquid phase (B3).
- *Mortality and Grazing of Bacteria*. The model describes bacterial biomass fate and BOM removal during biofiltration. The relationship between experimental results collected in full scale or pilot treatment plants and data predicted by the deterministic model are generally satisfactory. The CHABROL Model can be used to design filters producing biologically stable water.

Ozonation. Ozone has been widely applied at different stages of the treatment trains in Europe and more recently in the United States. The use of ozone in pre- or posttreatment considerably improves the quality of drinking water. Ozone is a powerful disinfectant that effectively oxidizes many chemical pollutants, removes color, tastes, and odors, and enhances coagulation (88). However, ozonation of NOM leads to the production of a large number of biodegradable compounds such as carboxylic acids, keto-acids, and aldehydes. The oxidation of organic matter by ozone leads to an increase in molecular polarity, encourages the formation of low molecular weight compounds at the expense of larger molecules, and reduces the aromaticity (reduction in UV absorbance at 254 nm). Ozone reacts with organic contaminants in water via two major chemical pathways. It reacts directly as molecular ozone (O_3) through highly selective and fast reactions. Ozone acts directly to form carbonyl groups by acting as a dipole agent on C=C double bonds, as an electrophilic agent on aromatic molecules by ring hydroxylation, or as a nucleophilic agent on C=N double bonds. Ozone can also react indirectly as a free radical (hydroxyl radical $OH^{\bullet}$) arising from ozone decomposition to form carbonyl compounds. The radical action of ozone is less selective than the direct action. Many compounds are only partially oxidized by ozone or are refractory to the action of ozone alone. Several processes

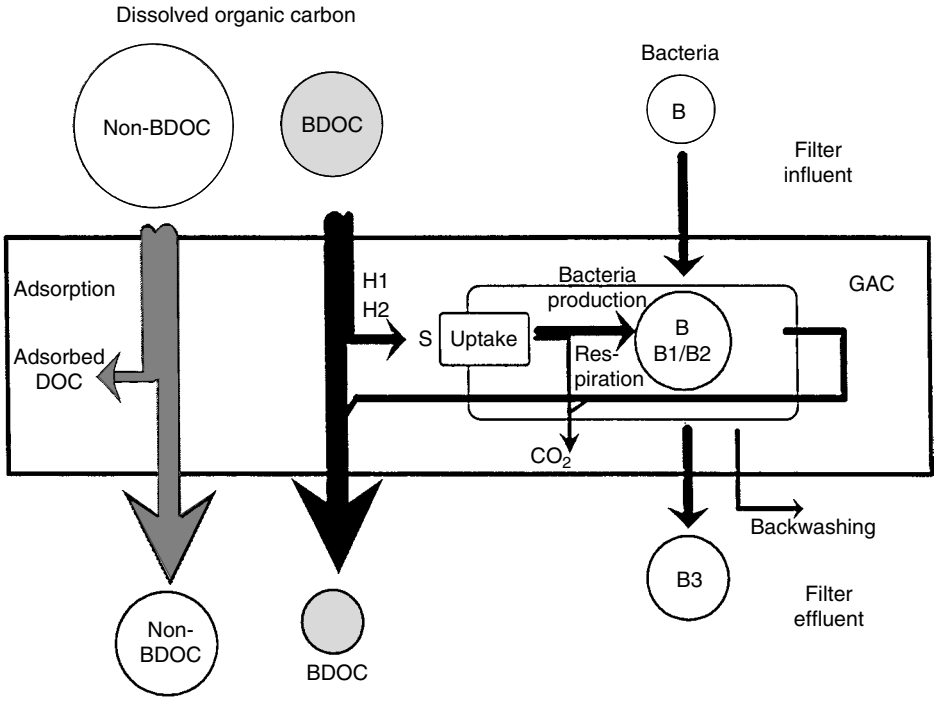

Figure 8. CHABROL Model and functioning of GAC filters (adapted from Servais and coworkers (71)). H1: Rapidly hydrolysable polymeric BDOC; H2: slowly hydrolysable polymeric BDOC; S: direct substrate; B: total filter bacteria; B1: biologically fixed bacteria; B2: adsorbed bacteria; B3: free bacteria.

have been combined with ozone to increase the oxidation level of molecules and to oxidize a larger spectrum of organic compounds (88–91). The ozone-hydrogen peroxide and ozone-UV systems provide nonselective degradation by enhancing free radical oxidation. The ozone-hydrogen peroxide combination leads to the degradation of alcohols, aliphatic acids, aldehydes, and some micropollutants such as atrazine and chlorinated solvents that are refractory to the action of ozone alone. The oxidation of these compounds with free radical action may be accompanied by significant TOC reduction when hydrogen peroxide-ozone is applied at an optimal ratio of 0.35 to 0.45 g/g in waters of neutral pH. Ozone has also been combined with a catalyst such as titanium dioxide in an attempt to mineralize a greater fraction of organic matter to CO_2 and H_2O (92).

Several authors have noted the increase in biodegradability of model substances or dissolved NOM following ozonation (88,91,93,94). BDOC concentrations of the ozonated water are a function of the raw water BDOC and ozone dosage. After ozone application, BDOC is formed within the first minute of treatment. The best ozonation conditions for producing BDOC are generally those that simultaneously provide maximum UV reduction measured as changes in absorbance at 254 nm (maximum changes in aromaticity) and leave traces of ozone residuals (Fig. 9). Ozone treatment increases the biodegradability of organic molecules in two stages: first, the rate of BDOC formation is high at low/intermediate oxidant levels. Then higher doses have little effect on the biodegradability of organic substances because all the molecules likely to be BDOC precursors have been transformed (93) (Fig. 9). Optimum BDOC formation is generally obtained with moderate ozone doses of 0.5 to 1.0 mg O_3 per mg DOC. Within the approximate range of 0 to 1 : 1 ozone to DOC ratio, several studies reported a 0.15 to 0.20 mg BDOC increase for each milligram of ozone added (93,94). At higher ozone doses, BDOC production is low (<0.05 mg BDOC/mg O_3). BDOC levels typically increase from 20 to 90% after ozonation of various river or sand-filtered waters. Applied ozone dose also shows a greater effectiveness than contact time. For equivalent applied ozone dose-contact time, a short contact time and a high ozone dose lead to higher BDOC formation than a long contact time associated with lower ozone dose (93). Figure 9 also compares the influence of ozonation used alone or in combination with hydrogen peroxide or a catalyst (titanium dioxide) on a fulvic acid solution. Maximum BDOC formation was obtained with ozone-hydrogen peroxide and ozone alone. BDOC formation was 30% lower in the presence of a catalyst than with ozone because catalytic ozone induced oxidation of ozone by-products into CO_2 (95).

Ozonation is often combined with biological filtration to remove biodegradable molecules produced after oxidation. When the filter influent water is ozonated, the biological degradability and thus filter biological activity are enhanced. Although ozone increases biodegradability by lowering the RDOC, ozone followed by biological filtration removes an additional DOC and AOC/BDOC fraction, reducing the number of chlorine-reactive sites, which is proportional to the risk of THM formation (71,96–101). By-products formed during ozonation, such as carboxylic acids, aldehydes, and peroxides (some of which are health concerns), can be removed through biological filtration (88,102–104).

Membrane Filtration. Different membrane technologies are available for the production of potable water, including reverse osmosis (removal of ions and organic matter), nanofiltration (NF) (removal of polyvalent ions

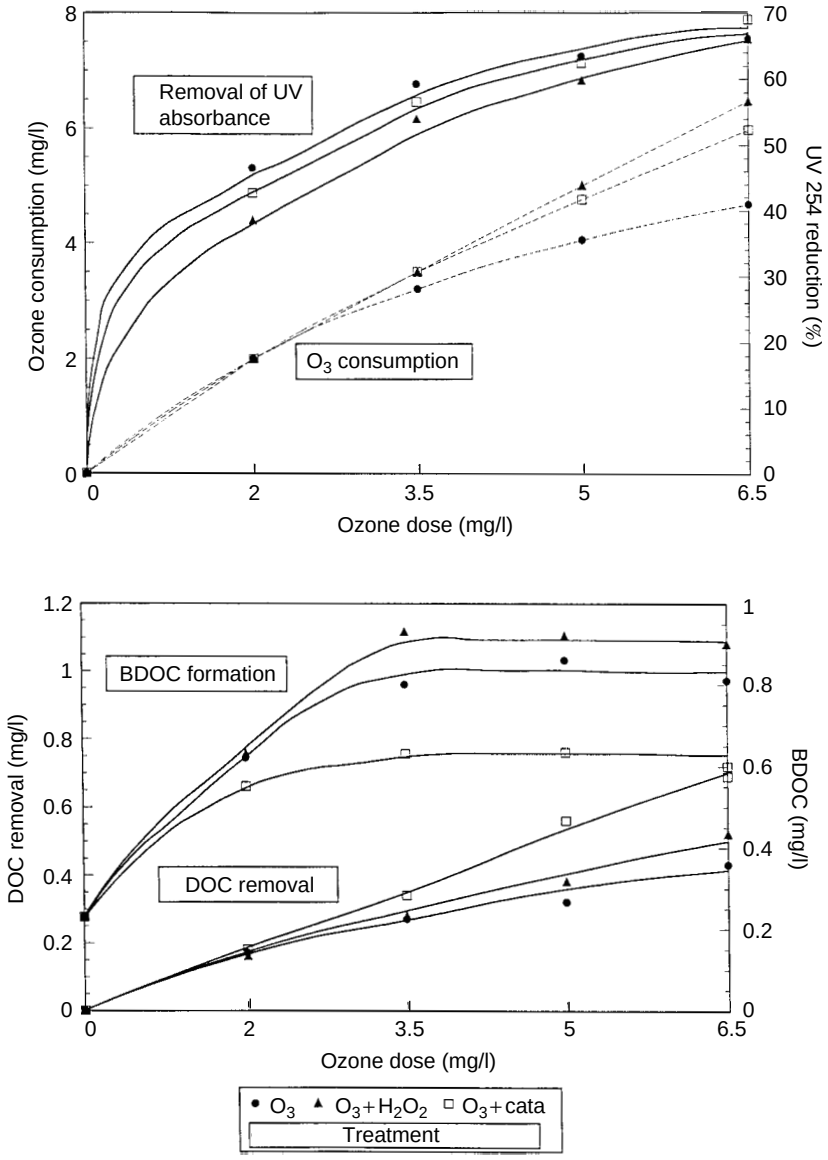

Figure 9. Effects of different oxidation treatments on UV absorbance, oxidant residual, BDOC, and DOC levels for a fulvic acid solution (O_3 + cata: ozone with a titanium dioxide catalyst).

and organic compounds larger than 400 Da), ultrafiltration (UF) (removal of colloids and molecules larger than 10,000 Da), and microfiltration (MF) (removal of particles greater than 0.2 μm, including turbidity, parasites, bacteria, and some viruses). MF and UF may not be suitable for organic matter removal unless pretreatments such as ozone and PAC are applied (105–107). The addition of PAC increased DOC removal efficiencies of ultrafiltration membranes to 40 to 50% (106). Ozonation improved DOC removal by the PAC/UF process up to 80% (108). NF membranes produce high removal efficiencies of DOC (from 50 to 99%), BDOC, precursors of chlorination by-products, pesticides/herbicides, color, and UV absorbance (109–111). However, main organic constituents in the membrane permeate following NF were identified as amino acids and sugars that are highly biodegradable. These data suggest that not all BOM molecules could be retained during membrane filtration treatment (111,112). Full-scale and lab data (112) showed that under high hardness and low pH conditions of feed water, NF allowed the passage of the AOC fraction even though DOC and BDOC rejection was near complete. This also highlights the complementary nature of AOC and BDOC as parameters.

Chlorination. Few studies documented the effects of chlorination on organic matter. It has been reported that biodegradability tends to increase after chlorination (96,100,113). Hambsch and coworkers (113) reported higher biodegradability of humic substances after chlorination.

BOM Changes During Conventional Treatment. BOM levels can be altered (increased or decreased) depending on treatment practices. Figure 10 shows the fate of organic compounds at different points within a typical

conventional treatment, before and after GAC implementation. Removal of biodegradable substrates is typically not favored during conventional treatment with sand/anthracite. While organic matter concentrations can be reduced, those of biodegradable substrates can be increased when using sand or sand/anthracite filters. Overall, after prechlorination and anthracite/sand filtration, the site recorded a DOC removal of 25% and an increase of AOC concentration of 50% through the treatment process (Fig. 10). Removal of DOC occurred during coagulation and settling, but removal was primarily of the refractory part (nonbiodegradable) of the DOC. Average raw water AOC level increased from 122 to 174 μg/L after preoxidation and settling processes, suggesting that chlorine pretreatment resulted in a large production of AOC. DOC, BDOC, and AOC were not removed by mixed media anthracite/sand filtration because a continuous chlorine residue was maintained through the filters. In contrast, GAC implementation modified organic fate during treatment and BOM was removed by biological GAC filtration. Overall, the plant recorded a reduction in AOC and BDOC levels after implementing GAC filtration (Fig. 10). AOC was increased by preoxidation; however, AOC levels were reduced to 58% by GAC filtration. BDOC concentrations also decreased after GAC filtration. Biological activity can take place during conventional treatment when chlorine levels are low on top of the GAC filters. Solutions for conventional treatment plants with high BOM levels could include the conversion of existing sand and sand/anthracite filters to sand/GAC filters and possibly delay the preoxidation step until after filtration, to limit BOM levels entering the distribution system.

BACTERIAL REGROWTH IN DISTRIBUTION SYSTEMS

Biofilm Formation and Bacterial Regrowth

BOM that is not removed during water treatment can result in the growth of biofilm bacteria in the distribution system (Fig. 11). The consequences of this bacterial growth can include: degradation of bacterial water quality, the acceleration of corrosion rates, the development of tastes and odors, customer complaints, and the development of a food chain leading to the presence of invertebrates (114–117). The degradation of bacterial water quality can be observed by growth of heterotrophic bacteria such as *Flavobacterium*, *Acinetobacter*, *Pseudomonas*, *Bacillus*, *Moraxella*, and *Staphylococcus* (118–120) and sometimes by the occurrence of coliforms (*Klebsiella*, *Escherichia coli*, *Enterobacter*, etc.) (121–124). Coliform regrowth can cause a violation of drinking water regulations. Suspended bacteria present in the water column come mostly from biofilms formed on the surface of distribution pipes, suspended particles, or sediments accumulated in storage tanks (124,125). Microbial accumulation in biofilms is governed by the three processes of adhesion, growth, and detachment (125–129) (Fig. 11). Bacteria adhering to the pipe surface have several origins. They may come from the source water through the treatment plant (130), regrowth of biofilm cells existing already within the distribution system, and introduction during main repair or cross connection. Growth of bacteria fixed on pipes is a combination of cell multiplication and mortality. Biofilm levels at steady state in pilot or full-scale distribution systems generally range between 10^5 and 10^7 bacteria/cm^2. The detachment of biofilm bacteria into the water column can be induced by fluid

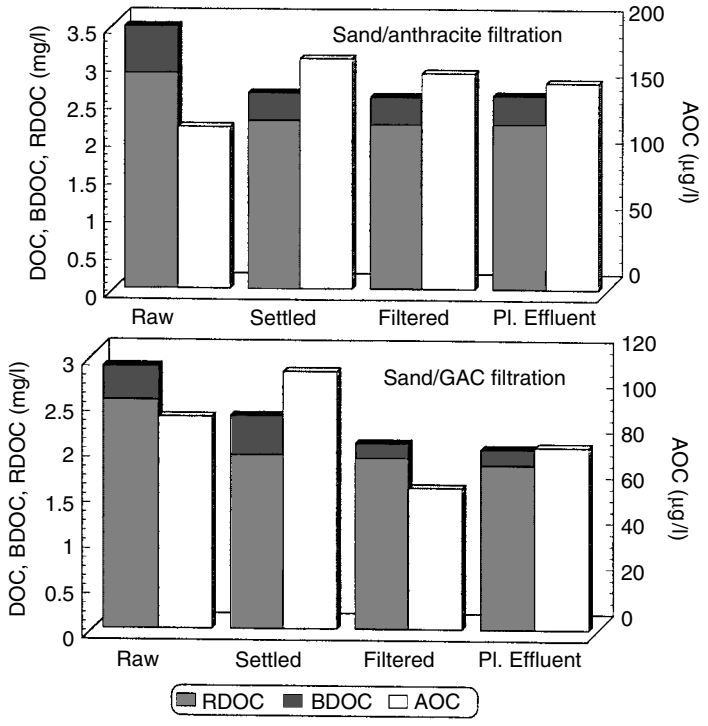

Figure 10. Changes in DOC, BDOC, and AOC concentrations during water treatment using sand/anthracite or GAC filtration. DOC = RDOC + BDOC where RDOC: refractory DOC.

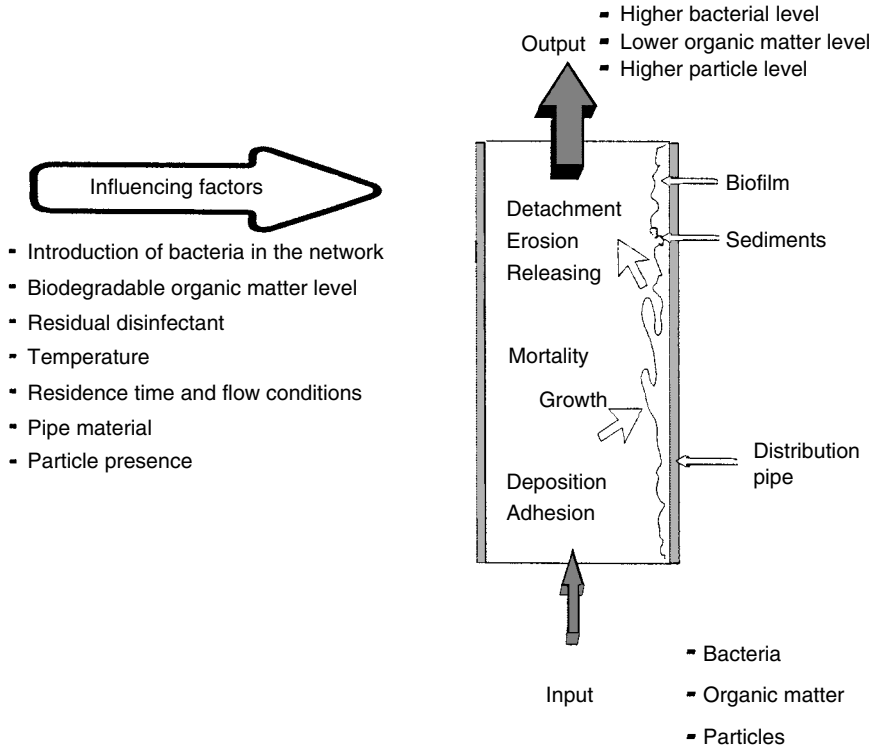

Figure 11. Biofilm in distribution systems.

shear stress at the biofilm surface or other water quality changes, such as increased disinfectant levels. It is possible to correlate the bacterial density in the biofilm to the dynamics of suspended bacteria in the bulk water phase. Servais and coworkers (131) suggested a ratio of approximately 60 between biofilm and suspended bacteria for small diameter pipes in several full-scale distribution systems. Consequently, a biofilm density of 10^7 bacteria/cm^2 could correspond to 5×10^4 bacteria/mL. Mathieu and coworkers (132) reported that a biofilm of 10^6 bacteria/cm^2 resulted in suspended bacterial concentrations of 10^3 to 10^5 bacteria/mL in pilot system studies.

BOM and Bacterial Regrowth

The level of BOM is an important parameter affecting bacterial regrowth because all heterotrophic bacteria use organic carbon for the production of new cell material or for utilization as energy (132–134). BOM is gradually consumed as the water travels through the distribution system. When both BDOC levels and temperatures are high in the water treatment plant effluent, BDOC consumption within the distribution system can be very rapid (36,131). There is generally a trend observed in biofilm detachment and organic loading.

The control of bacterial regrowth can be achieved when the amount of BOM entering the distribution system is limited. The BOM level required for biostability depends on the amount of disinfectant present. Block and coworkers (130) recommended an absence of biodegradable organic material after water treatment to limit bacterial regrowth. Servais and coworkers (131) associated biological stability (corresponding to no BDOC consumption within the distribution system) with a BDOC concentration of 0.16 mg/L or less in finished water in the absence of chlorine residue. Volk and Joret (135) indicated that BDOC levels should be less than 0.15 mg/L at 20 °C (warm temperature) and less than 0.30 mg/L at 15 °C for achieving biological stability in the Paris suburb an distribution systems. Coliform occurrences were related to the consumption of more than 0.10 to 0.15 mg/L of BDOC within the distribution system (135). Van der Kooij (134) showed that heterotrophic bacterial levels in nonchlorinated systems did not increase when AOC levels were lower than 10 μg/L. LeChevallier and coworkers (133) suggested that regrowth of coliform bacteria in chlorinated systems may be limited by AOC levels less than 50 to 100 μg/L. All the above objectives are difficult to consistently achieve and require very high levels of treatment. In addition to the amount of nutrients, the composition of biodegradable organic materials is also an important factor for controlling microbial growth. Amino acids are only a small fraction of the NOM, but they allow a high regrowth potential (high biomass production per unit of substrate) and are highly reactive with chlorine (104,136). Amino acids, carbohydrates, and humic substances were found to support heterotroph growth. However, coliform bacteria were able to persist in water containing amino acids or carbohydrates, but not with humic substances alone (104). The presence of disinfectant modifies the affinity of biofilm bacteria toward various organic substrates. In the absence of any disinfectant, biofilm cells preferentially utilize amino acids, then carbohydrates, and finally humic substances. However, in the presence of chlorine, biofilm affinity switches to amino acids, then humic substances, and finally carbohydrates. Humic substances may adsorb onto the biofilm and protect the microorganisms

from disinfection. In addition, some of the humic substances can be oxidized and converted into more easily degradable compounds after reaction with chlorine. Moreover, the presence of a disinfectant also affects substrate consumption and the regrowth of heterotrophic bacteria in the system. In the presence of chlorine, biofilms show greater carbon removal, higher specific biofilm growth rate, but lower biofilm yield, and lower biofilm densities than unchlorinated biofilms (137,138). The authors suggest that there is a protective mechanism of bacteria cells, which may require more substrate in the presence of a disinfectant. Bacteria may produce larger amounts of extracellular polymeric substances (EPS) to react with and neutralize chlorine (explaining the higher substrate uptake and carbon requirement). Acting as a sacrificial barrier, EPS are critical in limiting chlorine penetration into the biofilm (138).

Biofilm density and bacterial water quality are related to the amount of biodegradable material entering the system. Servais and coworkers (131) observed a relationship between biofilm bacteria and the concentrations of BDOC at the point of entry of several full-scale distribution systems. Another study compared bacterial water quality in two pilot distribution systems supplied with different levels of organic matter (139). The first system was fed with ozonated water, whereas the second was supplied with biologically filtered water. Lowering the nutrient levels with biological filtration led to lower biofilm densities. Biofilm counts were 10^6 to 10^7 CFU/cm^2 for the ozonated system, compared to 10^5 to 10^6 CFU/cm^2 for the system fed with biologically filtered water.

It is critical to produce low nutrient level waters. Several studies evaluated the effects of reducing nutrient levels on bacterial water quality after a treatment change. In two studies (140,141), bacterial water quality changes were monitored after implementation of NF in a pilot or full-scale system that was initially supplied with surface water treated with ozonation and biological filtration. In the pilot study (140), BDOC levels decreased from 0.25 mg/L in the ozonated/filtered water to less than 0.1 mg/L after NF. The authors did not observe drastic changes in the biofilm or suspended bacteria levels after the treatment change. At the beginning of the study, total and culturable counts were 4.9×10^6 cells/cm^2 and 4×10^5 CFU/cm^2 for the biofilm and 2.6×10^5 cells/mL and 10^3 CFU/mL in bulk water, respectively. Bacterial concentrations slightly decreased after 6 weeks of supplying nanofiltered water (suspended bacteria of 1.4×10^5 cells/mL and 259 CFU/mL; biofilm of 2.3×10^6 cells/cm^2 or 1.2×10^5 CFU/cm^2). After one year of supplying nanofiltered water, biofilm levels were 1.9×10^5 CFU/cm^2 and suspended bacterial levels were 50 CFU/mL. Similarly, bacterial water quality changes were not obvious when the same treatment conversion was performed in a full-scale distribution system supplying a suburban community (141) in Paris, France. Biofilm densities were low before the treatment change because of the high chlorine levels (0.85 mg/L) used to combat microbial growth caused by the high BDOC levels (0.75 mg/L). Following the application of NF, biofilm levels remained low, but a low disinfectant residual could be used because of the improved biostability (low BDOC) of the water. Another pilot study using annular reactors to simulate distribution systems evaluated the effect of reducing nutrient levels after biological filtration on bacterial water quality in drinking water (142). A system fed initially by conventionally treated water received biologically treated water. BOM levels were reduced approximately by half after biological treatment. On an average, biofilm densities were reduced by 1 log unit by biological treatment. Interestingly, the effect of the treatment change on bacteria levels was not immediate. It required approximately six months of biological treatment before there was an observable impact on bacterial water quality.

Field experience shows that distribution systems represent complex reactors and that a variety of parameters influence biofilm growth. Factors such as water temperature, disinfectant type and residual, selection of the pipe material, corrosion control, and hydraulic conditions may be more influential than the levels of organic matter for regulating the biological activity of the biofilm. These (and other) parameters probably influence microbial water quality data as much as the amounts of nutrients variable. Pipe characteristics strongly influence the density of fixed biomass. Iron pipe surfaces have been shown to stimulate bacterial growth (117,122,143,144). Camper (143) found that more heterotrophic bacteria grew on mild steel surfaces than on polycarbonate. Although growth rates were higher in the polycarbonate reactors, mild steel surfaces contained 10 times more heterotrophs and 2- to 10-fold more coliform bacteria. Biofilm levels are also governed by the pipe conditions. Corrosion, pitting, and tuberculation are fundamental to the presence of biofilms, their metabolic activity, and the release of bacteria into the water column (144). Pipe conditions also indirectly impact biofilm bacteria by affecting disinfection efficiency, especially with free chlorine. There is a relationship between the corrosion of iron surface and the protection of biofilm bacteria from chlorine disinfection (117,144). Rompre and coworkers (139) reported that free chlorine produced a rapid decrease in biofilm density (>1 log CFU/cm^2) in polycarbonate annular reactors, whereas the chlorine did not affect the biofilm in gray-iron reactors. Moreover, organic matter tends to adsorb to corrosion products on iron pipe surfaces and the elevated concentration of organic molecules can stimulate bacterial regrowth (137,138). A large survey conducted to understand coliform regrowth showed that coliform occurrence rates were higher in systems with a large proportion of unlined cast iron pipes subject to corrosion, suggesting that corrosion control is an important factor for limiting coliform regrowth (145). The use of phosphate-based corrosion inhibitors was associated with lower coliform levels in several systems. Coliform regrowth can also be related to several engineering and operational factors, including water filtration, the use of ozone without biological treatment, a large proportion of storage tanks, the presence of uncovered finished water reservoirs, corrosion control, and distribution system flushing (145). Removal of BOM through effective treatment and filtration is critical for controlling bacterial water quality. Coliforms occurred more

frequently in systems using unfiltered surface waters or using ozone without biological filtration. Finally, the configuration and management of the distribution system also influenced water quality. Increased levels of coliforms were observed in systems having a large proportion of storage tanks. Storage tanks are necessary to meet water demand, and provide adequate pressure and fire protection. However, the disinfectant can dissipate quickly and microbial water quality can deteriorate when water is stagnant in storage tanks. Systems with open finished water reservoirs are prone to animal contamination and algal bloom, and more likely to experience coliform regrowth (145). Annual flushing of the distribution is a maintenance practice performed to remove accumulated sediments, limit red water complaints, and reduce coliform occurrences.

Distribution System Modeling

Several models have been developed to predict water behavior and degradation of bacterial water quality during distribution. Although different factors may be listed and examined individually, in reality, all these factors are interrelated, sometimes with competing effects. For instance, increased water temperature favors microbial growth, but higher temperatures also improve disinfection efficiency (decreasing bacterial regrowth). Warm temperatures lead to higher reaction rates that may result in more rapid dissipation of disinfectant residuals and stimulate pipe corrosion. Higher disinfectant doses can reduce bacterial growth but increase nutrient levels and stimulate iron corrosion. Because of the complexity of the distribution networks and the variety of confounding effects, the existing models are overly simplified or apply only to a single site (146). Several models are available for predicting hydraulic flows and retention times (147). These models can incorporate a reactive component such as chlorine decay or bacterial growth rates. The EPANET Model simulates hydraulic and water quality behavior in distribution systems (148). The water quality modules include chlorine decay, trihalomethane propagation, or fluoride tracer analysis. PICCOLO software is another example of a residence time model that integrates the prediction of free chlorine decay within the Paris suburbs network (149).

Biofilm development on a surface is the result of several chemical, physical, and microbial processes, including an initial colonization phase of the pipe surface (transport and adsorption of organic material and bacteria from the bulk water to the surface and adhesion of bacteria), the growth of bacterial cells leading to a multilayer biofilm embedded in a polymer matrix, and the detachment of bacteria. Although fundamental aspects of these biofilm processes have been investigated (150), models for predicting bacterial water quality are limited. The Biofilm Accumulation Model (BAM) models the processes of nutrient (AOC) transport and consumption, biofilm development, and subsequent growth and decay of bacterial cells, detachment, and transport of microorganisms (148). The validation of the BAM has been performed in a pipe loop pilot system.

The SANCHO Model was adapted from biofilm processes observed during biological filtration (151). It is based on the relationship between bacteria, disinfectant, and BDOC levels in distribution systems (Fig. 12). The components of the model include the attachment of bacteria onto the surface pipe (reversible adsorption followed by biological attachment), the interaction between bacteria and organic matter (direct assimilation or exoenzymatic hydrolysis of organic material, growth of fixed and bulk bacteria, and bacterial mortality), and free chlorine behavior (chemical demand, disinfection of bacteria). The model successfully evaluates the spatial fluctuations of BDOC, chlorine, and suspended and fixed bacteria concentrations in a water mass circulating in a series of pipes with decreasing diameters. The model can be used to determine treatment goals to prevent the degradation of microbial water quality in any network (47,151,152).

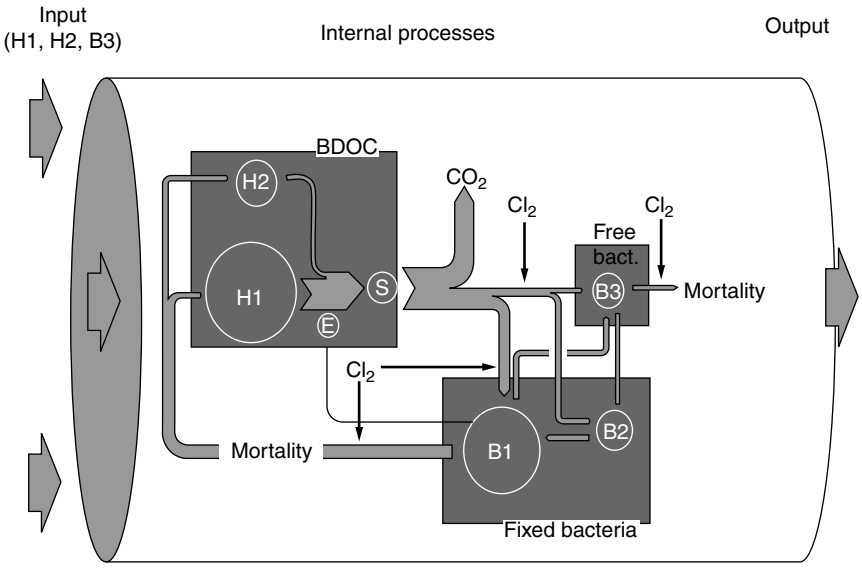

Figure 12. SANCHO Model (from Servais and coworkers (151)) H1: Rapidly hydrolyzable polymeric BDOC; H2: slowly hydrolyzable polymeric BDOC; S: direct substrate; B: total filter bacteria; B1: biologically fixed bacteria; B2: adsorbed bacteria; B3: free bacteria.

Because coliform occurrences are not related to a single variable, it is necessary to simultaneously examine the interaction of multiple parameters to predict the propensity for coliform regrowth events. Tree-based statistical models have been used to define predictor variables for coliform regrowth (146). A tree-based model is a descriptive summary of how a set of predictor variables relate to an outcome variable (e.g., presence or absence of coliforms). Coliforms can be predicted using a large set of variables, including AOC level, turbidity, temperature, disinfectant type, residual, pH, phosphates, TOC, and alkalinity. Although it may be possible to develop extremely sophisticated models using a large number of variables to predict coliform occurrences, such models are too complicated to be applied to most water utilities. Volk and Joret (135) developed the ALCOL Model to evaluate the risk of occurrences of total coliform bacteria in full-scale distribution systems in the suburbs of Paris, France, on the basis of temperature, BDOC consumption, disinfectant residual, and suspended bacteria counts (microscopic counts by epifluorescence) in the distribution network. The analysis of coliform occurrence data has shown no simple linear relationship between coliform-positive samples and these four water quality parameters. However, it is possible to determine a threshold value above which coliforms occur more commonly. The model uses the following four critical thresholds: water temperatures at or above 15 °C, BDOC consumption greater than 0.15 mg/L, a chlorine residual lower than 0.10 mg/L, and a logarithm of bacterial concentration higher than 5.2. The decision tree presented in Figure 13 graphically depicts the combinations of the threshold criteria. The sum of the positive criteria (number of threshold variables simultaneously exceeded) is indicated for each combination. Four positive criteria recorded for a distribution network lead to the most favorable situation for coliform occurrence (high temperature, low disinfectant residual, high nutrient consumption, and elevated bacterial concentration). Alternatively, a ranking of zero combines all the conditions for coliform control (low temperature, high chlorine residual, low nutrient consumption, and low bacteria levels). The probability of experiencing coliform bacteria when various threshold criteria are exceeded is shown in Table 4. The majority of positive coliform samples occurred when the four criteria were exceeded. The probability of coliform occurrence was 64% when the four criteria were positive, but nil when only one or none of the thresholds was exceeded. Such a model for the prediction of coliform episodes may be a useful tool to control bacterial regrowth. When a system is considered to be at high risk, various actions have to be undertaken to control bacterial water quality. Punctual actions such as increased flushing, boosting disinfection residuals, and decreased storage tank residence time can

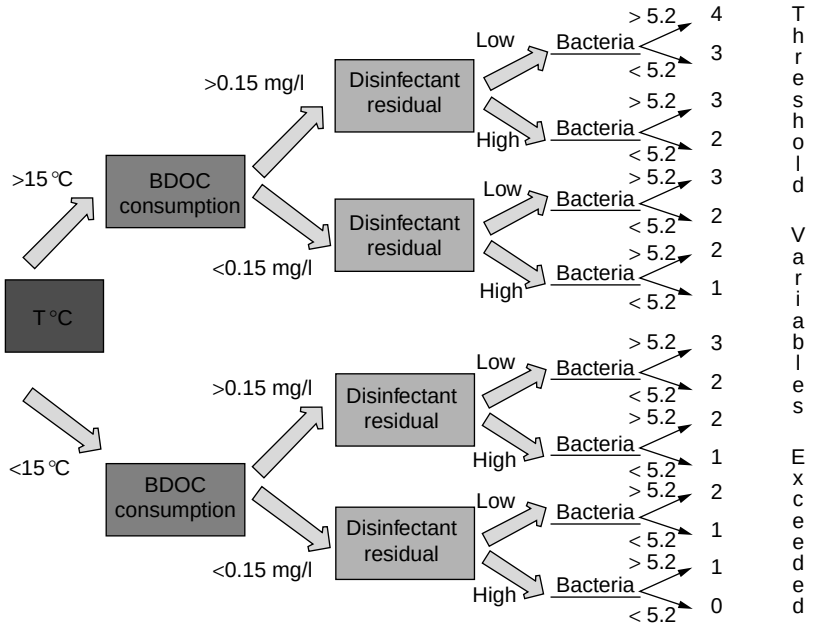

Figure 13. Decision tree for predicting coliform regrowth (ALCOL Model, 135).

Table 4. Risk of Coliform Occurrences in a Distribution System

Risk	Number of Positive Criteria	Total Number of Events	Number of Coliform Episodes	Frequency (%) of Coliform Observations
Exposed	4	14	9	64
Low risk	2 or 3	87	3	3
No risk	0 or 1	22	0	0

be effective in certain areas of the distribution system. Treatment modifications such as increasing plant effluent disinfectant residuals or improving BOM elimination can also be implemented at the production facility. However, the current model is site-specific and situations in which coliform regrowth does not occur while the model predicted coliforms are possible. Further refinement of the models may make these tools more accurate and reliable. Individual models for problem distribution systems may be warranted and could integrate hydraulic models, pipeline configurations, and operational characteristics of the network. Additionally, the weight of each parameter can be defined to refine the risk calculation.

CONCLUSION

Because a high BOM level is one factor related to bacterial regrowth, BOM removal should be emphasized during water treatment to improve water quality. Several bioassays are available to determine the levels of biodegradable organic materials in water. Measured BOM concentrations are often related to the applied methodology and the laboratory conducting the test. Each method has its own advantages and disadvantages. There is a weak correlation between AOC and BDOC concentrations. The BDOC test attempts to identify the entire pool of BOM, including both very labile constituents and slowly biodegradable molecules (requiring the successive steps of cell adsorption, exoenzymatic hydrolysis, and cell consumption), whereas the AOC test detects only easily assimilable organic compounds. Because both tests emphasize different fractions of BOM, both parameters should be monitored when studying nutrient changes during water treatment and distribution. Bacterial regrowth and coliform occurrences increase with temperature, the absence of disinfectant, and elevated levels of BOM. Beyond these three major factors, coliform regrowth is also dependent on other physicochemical, engineering, and operational parameters such as pipe material, hydraulic shear, and corrosion. Therefore, it is difficult to predict the effects of a treatment change at a specific site, depending on the weight of the different factors regulating bacterial growth. As a first step, a water utility can better limit biofilm problems when simultaneously addressing the following three issues: nutrient levels, corrosion, and disinfection. High organic matter removal during water treatment and effective corrosion control during distribution would improve disinfection and limit bacterial regrowth within the distribution system.

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BIODEGRADATION: COMPOSTS.

See COMPOST: BIODEGRADATION OF TOXIC ORGANIC COMPOUNDS

BIODEGRADATION: FUEL OXYGENATES.

See MICROBIAL DEGRADATION OF FUEL OXYGENATES

BIODEGRADATION, HALOGENATED AROMATICS.

See FATE AND MICROBIAL DEGRADATION OF HALOGENATED AROMATICS

BIODEGRADATION: LANDFILLS.

See LANDFILLING OF MUNICIPAL SOLID WASTES: MICROBIOLOGICAL PROCESSES AND ENVIRONMENTAL IMPACTS

BIODEGRADATION: OXYGENASE ENZYMES.

See OXYGENASE ENZYMES: ROLE IN BIODEGRADATION

BIODEGRADATION: REDUCTIVE DEHALOGENATION AND METABOLISM OF CHLORINATED ORGANICS BY ANAEROBES

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INTRODUCTION

Of the enormous number of different halogenated organic compounds that have been chemically synthesized, several have found widespread use, particularly as solvents and biocides. This use has led to their being accidentally or intentionally released into the environment, mainly to groundwater. Although there is good evidence for natural production of chlorinated organic compounds (1), many of these anthropogenic organic halogen compounds appear to be resistant to chemical and biological degradation and therefore persist in the environment. This chapter discusses the roles of anaerobic microorganisms in the biodegradation of these compounds and focuses mainly on chlorinated compounds, which are the most pervasive organohalogen contaminants and have received the greatest attention. Additionally, reactions carried out by microorganisms in pure culture are emphasized.

Chlorinated organic compounds of environmental significance (Fig. 1) include chlorinated methane, ethane, ethene, and benzene solvents; the biocide pentachlorophenol; the herbicide 2.4-dichlorophenoxyacetic acid (2,4-D); polychlorinated biphenyls (PCBs); the insecticide DDT [1,1,1-trichloro-2,2-bis (chlorophenyl) ethane]; and dioxin contaminants such as 2,3,7,8-tetrachlorodibenzodioxin (TCDD). The chlorinated solvents found widespread use because they are nonflammable and were once considered relatively nontoxic; however, many are currently considered to be carcinogens, teratogens, neurotoxins, or estrogen mimics. Moreover, because many chlorinated compounds are lipophilic, they tend to accumulate in fat deposits in members of higher echelons of the food chain including humans.

When chlorinated solvents are released into soil, they form dense nonaqueous phase liquids (DNAPLs) or, in combination with other compounds such as hydrocarbons can form light nonaqueous phase liquids (LNAPLs). The DNAPLs, in particular, tend to accumulate as neat solvent at the bedrock or soil interface and then slowly leach into groundwater forming a plume. Because the maximum

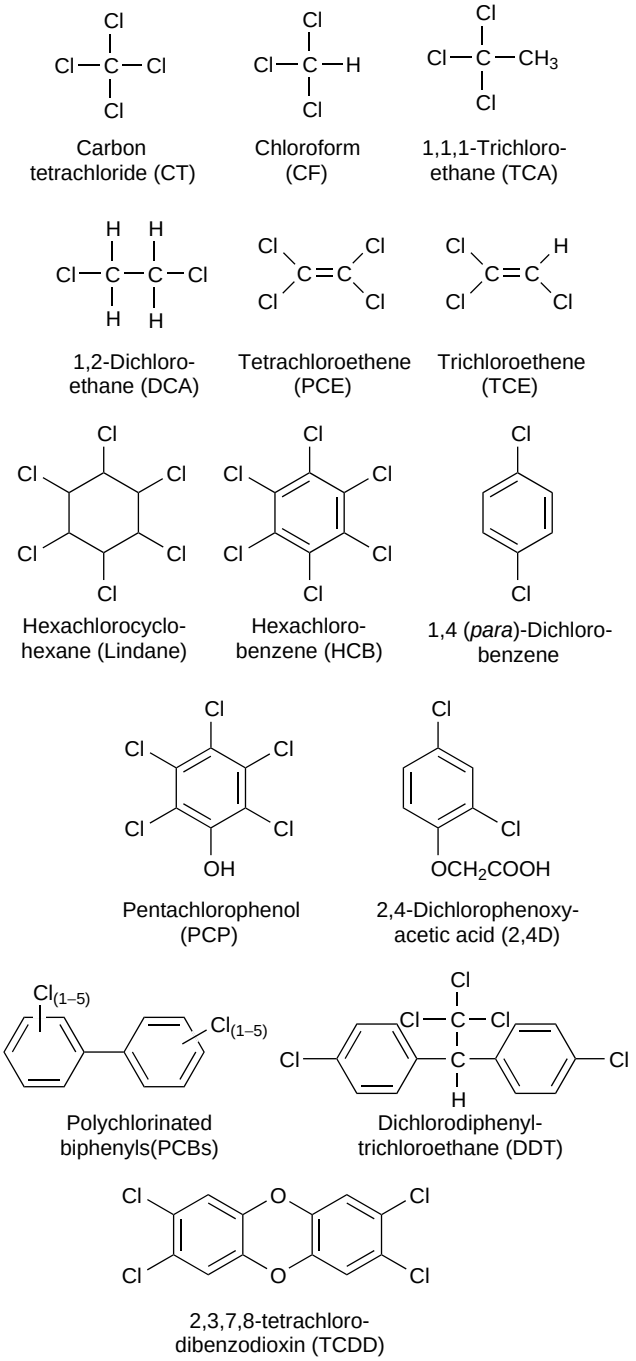

Figure 1. Examples of synthetic organochlorine compounds of environmental significance.

allowable concentrations of many of these solvents in drinking water are one to five parts per billion, a large amount of groundwater can be contaminated by a relatively small amount of these compounds.

Research on biodegradation of chlorinated compounds initially focused on aerobic metabolism, and much exemplary scientific data has been obtained about the organisms, pathways, and genes involved in aerobic degradation processes. However, it became increasingly apparent that many highly chlorinated compounds

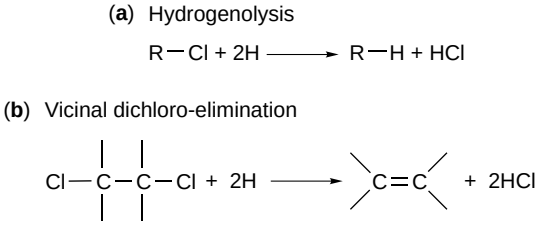

Figure 2. Reductive dechlorination of chlorinated organic compounds. (**a**) Hydrogenolysis. (**b**) Vicinal dichloro-elimination. If the carbon–carbon bond is double in part B, a triple bond is formed.

degrade poorly. Anaerobic processes, on the other hand, were originally considered slow, unreliable, and applicable to only a few compounds. Significant improvements in anaerobic techniques, however, have led to studies showing that there is a much greater metabolic diversity of anaerobes than was originally believed and that anaerobes were capable of utilizing highly chlorinated compounds, often better than more lightly halogenated ones.

Aerobic metabolism of chlorinated organic compounds generally proceeds via destabilization of the carbon–chlorine bond by addition of proximal hydroxyl or epoxy groups by oxygenases, or sometimes by replacing the chlorine with a hydroxyl group by hydrolytic enzymes. Under anaerobic conditions, electrons derived from the oxidation of organic matter are available for reductive reactions, such as reductive dechlorination. The most common type of reductive dechlorination is essentially a hydrogenolytic reaction replacing a Cl with an H, liberating HCl (Fig. 2a). In certain compounds containing chlorines on adjacent carbons, there can also be a vicinal dihalo elimination (Fig. 2b) in which two chlorines are released as 2HCl and another carbon–carbon bond is formed.

COMETABOLIC REDUCTIVE DECHLORINATION

Early studies showed that reduced transition metal complexes including biological tetrapyrroles such as hemes (Fe^{2+}), and vitamin B_{12} and other corrinoids (Co^{1+}) could carry out reductive dechlorination and other dehalogenations (2). Later, reduced cofactor F_{430}(Ni^{1+}) from methanogens (3) was also shown to carry out reductive dehalogenations. Thus it was not surprising that various reductive dehalogenation reactions were demonstrated in organisms rich in these cofactors, such as sulfate-reducers (hemes), acetogens (corrinoids), and methanogens (corrinoids and F_{430}). However, in all these cases, the reactions represented a low fraction of the organisms' overall metabolism and the organisms received no tangible benefit from them. These reactions are therefore considered cometabolic. Cometabolic reductive dechlorination may be quantitatively important in anaerobic habitats in which these organisms are naturally abundant such as in anoxic sediments.

A few examples of this type of dechlorination are given in the following section. Gantzer and Wackett (4) tested several compounds and found that hematin, vitamin B_{12},

and coenzyme F_{430} could dechlorinate tetrachloroethene (PCE), hexachlorobenzene (HCB), and carbon tetrachloride (CT), whereas ferredoxins, which contain iron-sulfur centers, and azurin, which contains copper, could not. Indeed, vitamin B_{12} and F_{430} could reduce PCE completely to ethene. All the dechlorinations followed first-order kinetics, and each subsequent dechlorination was slower by more than an order of magnitude than that preceding it. In another example, the enzyme carbon monoxide dehydrogenase from *Methanosarcina thermophila* could also reduce PCE to ethene (5), and a similar amount of reductive dechlorination was obtained from corrinoids in concentrations equal to that of the enzyme. Similarly, crude extracts of the methanogen *Methanobacterium thermoautotrophicum* were shown to reduce 1,2-dichlorothane(DCA) to ethene (vicinal dihalo-elimination) and to chloroethane (hydrogenolysis) (6). A large fraction of this activity could be attributed to the enzyme methyl-coenzyme M methyl reductase, which contains cofactor F_{430}. Interestingly, this enzyme could also slowly reduce to ethene bromoethane sulfonic acid, a molecule that is often used in ecological studies as a specific inhibitor of methanogenesis because of its inhibition of the methyl reductase.

It has been shown that a variety of obligate and facultative anaerobes can carry out reductive dechlorination of the pesticide Lindane (hexachlorocyclohexane, Fig. 1) (7). A culture of *Clostridium rectum* was shown to conserve more ATP from pyruvate oxidation when it reduced Lindane to 1,2,4 trichlorobenzene or pentachlorocyclohexane to 1,4-dichlorobenzene (8). In this case, the Lindane apparently was serving as an "electron dump," allowing greater oxidation of the pyruvate, rather than as a respiratory electron acceptor. Enhanced growth by *C. rectum* in the presence of Lindane could not be demonstrated, presumably because of toxicity.

REDUCTIVE DECHLORINATION AND DEHALORESPIRATION

A water shed paper in the field of reductive dechlorination was that of Suflita and coworkers (9) in 1982. It described enrichment cultures derived from lake sediments and sewage sludge that carried out the reductive dechlorination at relatively high rates of several chlorinated, brominated, and iodinated benzoates, but not fluorinated benzoates. This was the first description of reductive dechlorination of halogenated aromatic compounds. Perhaps even more significant, however, was their statement that a mixed culture that completely converted 3-chlorobenzoate (3-CB) to methane and carbon dioxide with benzoate as an intermediate had been maintained for two years. This was the first suggestion that reductive dechlorination could promote growth of a microorganism.

Further studies led to a better characterization of the microbiology of the 3-CB–utilizing consortium (Fig. 3) (10). A dechlorinating organism reductively dechlorinated 3-CB to benzoate, which was then converted to H_2, CO_2 by a benzoate oxidizer resembling *Syntrophus buswellii*. One of the moles of H_2 was used for the reductive dechlorination of 3-CB, whereas the other two moles

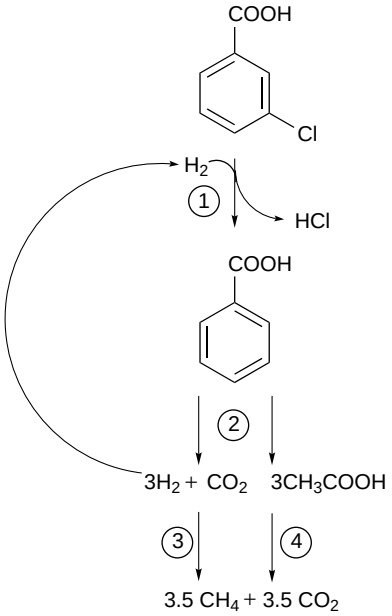

Figure 3. Conversion of 3-chlorobenzoate to CH_4, and CO_2 by a microbial consortium. Predominant microorganisms involved: (*1*) Reductive dechlorinator (*Desulfomonile tiedjei* (DCB-1)); (*2*) Benzoate oxidizer (*Syntrophus buswelii*); (*3*) H_2 consuming methanogens (*Methanobacterium formicicum*, and *Methanospirillum hungatei*); (*4*) Acetotrophic methanogen, (*Methanosaeta* (*Methanothrix* sp.). After Dolfing et al. (10).

were used by hydrogenotrophic methanogens to produce 0.5 moles of CH_4 from CO_2. Another three moles of CH_4 and CO_2 were produced from acetate by a *Methanosaeta*-like methanogen. This consortium must function as a unit because benzoate oxidation is only thermodynamically favorable if H_2 is removed by methanogens and the dechlorinator and acetate is removed by the acetotrophic methanogens (11). Moreover, the dechlorinator is dependent on benzoate oxidation to provide reducing power (H_2) needed for its dechlorination, whereas the benzoate oxidizer is dependent on the dechlorinator for its substrate.

Desulfomonile tiedjei strain DCB-1 was eventually isolated from the consortium (12). It grew only poorly, even in rich medium, until its nutrient requirements were determined to include nicotinic acid, 1,4-naphthoquinone, and thiamine. Once the organism grew well in a defined medium, it was clearly demonstrated to be able to use 3-chlorobenzoate as a respiratory electron acceptor for growth (13). *Desulfomonile tiedjei* also used sulfate, thiosulfate, and several metachlorinated benzoates as electron acceptors (Table 1).

Using reductive dechlorination as a respiratory anaerobic electron-accepting reaction has been called dehalorespiration as well as halorespiration (14), but because the latter term could also apply to reduction of compounds such as perchlorate, the former one is used here. A considerable amount of energy is available for conservation from reductive dechlorination. For example, the oxidation-reduction potential of chloroethene reductions varies from 0.37 to 0.58 v (2) and that for chlorinated aromatics varies

Table 1. Representative Organisms Capable of Respiratory Reductive Dehalogenation

Organism	Representative Dehalogenations	Electron Donors	Other Electron Acceptors	Phylogenetic Position
Desulfomonile tiedjei (12)	3-chlorobenzoate to benzoate	H_2, formate, pyruvate	Sulfate, thiosulfate	Delta *Proteobacteria*
Isolate 2CP-1 (50)	2 chlorophenol to phenol	Acetate	Oxygen	Delta *Proteobacteria*
Desulfitobacterium dehalogenans (27)	2, 4, 6-trichlorophenol to 2,4-dichlorophenol	H_2, butyrate, pyruvate	Sulfite, fumarate, nitrate,	*Firmacutes*
Dehalospirillum multivorans (74)	PCE to *cis*-DCE	H_2, formate, pyruvate	Fumarate, nitrate	Epsilon *Proteobacteria*
Desulfitobacterium frappieri (26)	Pentachlorophenol to 3-chlorophenol, PCE to TCE/*cis*-DCE	Pyruvate,	Sulfite, thiosulfate, nitrate	*Firmacutes*
Dehalobacter restrictus (25)	PCE to *cis*-DCE	H_2	None	*Firmacutes*
Enterobacter strain MS-1 (30)	PCE to *cis*-DCE	Formate, pyruvate, acetate	Oxygen, nitrate	Gamma *Proteobacteria*
Desulfuramonas chloro ethenica (29)	PCE to *cis*-DCE	Acetate, pyruvate	Polysulfide, fumarate, Fe(III)	Delta *Proteobacteria*
Dehalococcoides ethenogenes (31)	PCE to ETH 1,2-DCA to ETH(VC)	H_2	None	*Chloroflexi*
Desulfovibrio strain TBP-1 (53)	2,4,6-tribromophenol to phenol	H_2, formate, lactate	Sulfate, thiosulfate, S°	Delta *Proteobacteria*
Trichlorobacter thiogenes (47)	Trichloroacetate to dichloroacetate	H_2S (acetate)	S°, fumarate	Delta *Proteobacteria*
Dehalococcoides strain CBDB1 (56)	Tetra- and trichlorobenzenes to tri- and dichlorobenzenes	H_2	None	*Chloroflexi*

from 0.27 to 0.48 v (15), close to the nitrate/nitrite couple (0.43 v). Reductive dechlorination is therefore a much more favorable electron-accepting reaction than is sulfate reduction (−0.22 v) or methanogenesis from H_2/CO_2 (−0.24 v).

Desulfomonile tiedjei was officially described in 1991 and had been in pure culture for several years before that, yet it was not until the mid-1990s that several additional dehalorespiring organisms were isolated (Table 1). Nearly all were most closely related to the gram-negative sulfur compound reducers in the delta and epsilon subphyla of the *Proteobacteria* (purple bacteria and relatives) or to the *Desufotomaculum* group in the *Firmacutes* (low %G + C gram-positive bacteria). These groups contain organisms that are versatile at using electron acceptors.

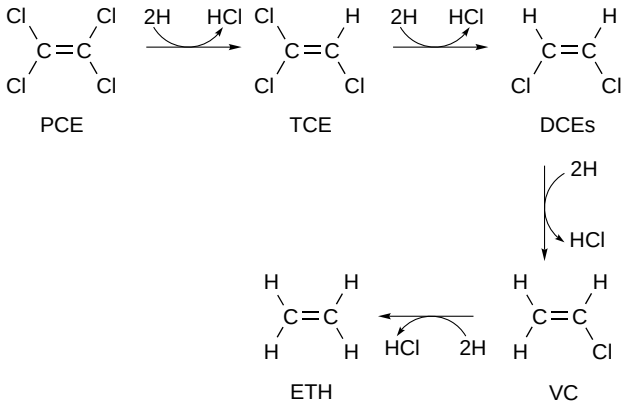

Figure 4. Reduction of tetrachloroethene (PCE) to ethene (ETH). Intermediates include trichloroethene (TCE), dichloroethene isomers (represented by the *cis* isomer), and vinyl chloride (VC).

CHLOROETHENE UTILIZATION

The solvents PCE and trichloroethene (TCE) were used extensively in the second half of the twentieth century, particularly for degreasing metal parts and dry cleaning clothes. They are among the most frequently encountered groundwater pollutants. Aerobically, TCE can be oxidized to an epoxide form by a variety of oxygenase enzymes such as methane monooxygenase or toluene dioxygenase (16), whereas PCE appears resistant to oxidative attack. Several studies showed reductive dechlorination by anaerobic microbial communities to the extent of vinyl chloride (VC) (17), a known human carcinogen. Freedman and Gossett (18), in their studies on an enrichment culture derived from a sewage digestor, first demonstrated complete reductive dechlorination of chloroethenes to ethene (ETH) (Fig. 4), and reduction as far as ethane has also been described (19). In the former system, it was shown that in the anaerobic enrichment culture, when methanol was fed as an electron donor, reductive dechlorination of PCE could occur at unprecedentedly high rates (20). It was subsequently demonstrated that H_2 was the direct electron donor for dechlorination even when methanol was being supplied to the culture (21). The dechlorination of PCE, TCE, and *cis*-DCE all followed zero-order kinetics in the

concentration range assayed, and rates of utilization of these chloroethenes were similar (22), in marked contrast to reductive dechlorination carried out by reduced transition metal cofactors (4). A more defined culture using H_2 as the electron donor was shown to require vitamin B_{12}, a cofactor that presumably was supplied by methanol-utilizing methanogens and acetogens in the methanol-PCE enrichment culture.

Several laboratories isolated organisms that could reduce PCE to TCE (23) or more often to *cis*-DCE. These include *Dehalobacter restrictus* (24,25), a gram-positive organism capable of utilizing only PCE or TCE as electron acceptors and H_2 as the electron donor (Table 1), hence its species epithet. *Dehalospirillum multivorans* is more metabolically versatile than *D. restrictus* and is a member of the epsilon subphylum of the *Proteobacteria*. Several strains of *Desulfitobacterium* are capable of dechlorinating PCE and TCE (23,26–28). *Desulfitobacterium* is a gram-negative endospore former and is phylogenetically related to the sulfate-reducer *Desulfosporosinus (Desulfotomaculum) orientis* and to *D. restrictus*. *Desulfuromonas chloroethenica* (29) is one of the few dechlorinators capable of using acetate as an electron donor and can also reduce polysulfide compounds. *Enterobacter* strain MS-1 (30) is a facultative aerobe and reduces PCE and TCE when utilizing nonfermentable substrates in the absence of another electron donor such as nitrate or oxygen. Interestingly, an *Enterobacter* strain from the American type culture collection that presumably was naive to any exposure to chloroethene solvents was capable of PCE reductive dechlorination. From these and other studies it can be concluded that organisms that reduce PCE as far as DCE are cosmopolitan, and at least some of them are relatively easy to culture.

The enrichment culture has been studied from the Gossett laboratory, eventually describing in 1997 (31) the isolation of an organism, tentatively named *Dehalococcoides ethenogenes* strain 195, capable of reductively dechlorinating PCE to etherene (ETH). *Dehalococcoides ethenogenes* had a very complex nutrition, requiring acetate, vitamin B_{12}, and extracts of mixed cultures containing it for its growth. Of many substrates tested, it used only H_2 as an electron donor and only chloroethenes or 1,2-dichlorothane (DCA), which it reduces primarily to ethene, as electron acceptors. Conversion of VC to ethene is the rate-limiting step, and subsequent results (32) showed that it cannot grow using VC or trans-DCE as electron acceptors. *Dehalococcoides ethenogenes* is a small disc-shaped organism that is resistant to peptidoglycan synthesis inhibitors such as vancomycin and ampicillin and apparently lacks a peptidoglycan cell wall. Initial phylogenetic analysis of its 16S rDNA showed that it was in the bacteria but not closely related to any isolated species. More recent analysis (33) shows that its 16S rDNA resides in a deeply branching subphylum of the *Chloroflexi* (green nonsulfur bacteria), most closely related to sequences that were derived from uncultured organisms in natural habitats (33). Interestingly, one of the most closely related sequences to that from *D. ethenogenes* was derived from an enrichment culture carrying out reductive dechlorination of PCBs (34).

Thus far, *D. ethenogenes* is the only isolate capable of reductive dechlorination of chloroethenes past DCE. Although *D. ethenogenes* cannot grow reducing VC to ethene, there is good evidence that other organisms in enrichment cultures are capable of growth from reductive dechlorination of VC (35,36).

CHLORINATED METHANE AND TRICHLOROACETATE UTILIZATION

Three of the chloromethanes, carbon tetrachloride (CT or tetrachloromethane), chloroform (CF), and dichloromethane (DCM or methylene chloride), are widely utilized solvents and are pervasive groundwater contaminants. Chloromethane is produced biogenically by several fungi and algae (1). Aerobically, CT and CF are barely utilized, whereas DCM can be utilized as the sole source of carbon and energy by several aerobic methylotrophic microorganisms (37). For anaerobic metabolism, one could imagine a reduction series for chloromethanes similar to that for chlorinated ethenes in which CT is sequentially dechlorinated to CF, DCM, CM, and finally CH_4. However, utilization of chloromethanes as respiratory electron acceptors has not been described, although it is thermodynamically feasible. Rather, these CT and CF compounds appear to be cometabolized by a variety of organisms, particularly those with high levels of corrinoids, that can chemically convert them to CO_2, CO, or formate (38–43). *Pseudomonas stutzeri* strain KC secretes a factor that converts CT to CO_2 along with small amounts of CS_2 and COS when grown under nitrate-reducing and iron-limited conditions. This factor has been identified as pyridine-2, 6-bis(thiocarboxylate) (44), a compound previously isolated from a *Pseudomonas* strain and considered to be a metal ion chelator.

Both DCM and CM are used as substrates by anaerobic acetogenic microorganisms. In both cases, the chloromethanes serve as substrates rather than as respiratory electron acceptors. *Dehalobacterium formicoaceticum* (45) converts DCM to acetate by the equation:

$$3CH_2Cl_2 + 2CO_2 \rightarrow 2HCOO^- + CH_3COO^- + 9H^+ + 6Cl^- \quad (1)$$

The DCM is at the same oxidation state as formaldehyde and feeds into the acetogenic pathway in a similar manner. It is to be noted that formate rather than H_2 was produced by this organism, which is unusual. *Dehalobacterium formicoaceticum* grew poorly without a methanogen or sulfate-reducer to remove the formate it produces. The only tested substrate that *D. formicoaceticum* could use was DCM. Dibromomethane was utilized in short-term assays but did not serve as a growth substrate, presumably because of toxicity. The organism's 16S rDNA sequence showed that it is a member of the *Firmacutes*.

Strain MC1 is a gram-positive organism that forms cocci in chains and ferments CM to acetate, CO_2, and HCl by the equation:

$$4CH_3Cl + 2CO_2 + 2H_2O \rightarrow 3CH_3COO^- + 7H^+ + 4Cl^- \quad (2)$$

Strain MC uses the CM as a source of a methyl group to ferment, much like it uses methanol (46). It was quite versatile at substrate utilization, growing on sugars, methanol, methoxylated aromatics, H_2/CO_2, lactate, or pyruvate. However, it did not utilize DCM.

An organism has been isolated that reductively dechlorinates the trichloromethyl group of Trichloroacetate (TCA) (47), which has been used as an herbicide. *Trichlorobacter thiogenes* has an unusual metabolism for its reductive dechlorination of TCA to dichloroacetate in that it uses sulfide as the source of electrons for the reduction, thereby oxidizing it to elemental sulfur, which it stores as intracellular granules. Sulfide was regenerated from the sulfur by electrons derived from oxidation of acetate, a metabolic mode similar to its close relative *Desulfuromonas* in the delta subphylum of the *Proteobacteria*.

CHLOROPHENOL AND BROMOPHENOL UTILIZATION

Chlorophenols have been used as biocides, and there is particularly widespread use and environmental contamination by pentachlorophenol (PCP). 2,4-Dichlorophenol is formed after hydrolysis of the common herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) (Fig. 1). Moreover, chlorophenols and related methoxylated compounds are formed during lignin degradation by fungi (48) and are, therefore, natural compounds found in soil. Aerobic organisms that can degrade lightly chlorinated phenols such as 2,4-dichlorophenol are relatively abundant, and there are aerobes that can degrade PCP (49).

Reductive dechlorination of chlorophenols has been found in anaerobic sediments and enrichment cultures and in at least two genera that are capable of conserving energy from the reductive dechlorination of chlorophenols. One of these, called *Isolate 2CP-1*, dechlorinated 2-chlorophenol to phenol (50). This organism was a facultative aerobe and is phylogenetically related to the fruiting myxobacteria, an unusual affiliation for a reductive dechlorinator, although in the delta subphylum of the *Proteobacteria*. *Desulfomonile tiedjei* could dechlorinate PCP to 2,4,6-trichlorophenol, but the activity needed a chlorobenzoate inducer and concentrations greater than 10 μM inhibited rather than stimulated growth on pyruvate, suggesting that the activity is fortuitous (51).

Several *Desulfitobacterium* strains can use chlorophenols (23,26,27). Most strains only affect *ortho* dechlorination of chlorophenols, whereas *D. frappieri* (26) is capable of converting PCP nearly stoichiometrically to 3-chlorophenol (Fig. 5). It attacks the *ortho* chlorines first, and then after a lag period, during which the organism is presumably inducing a different dehalogenase(s), it attacks the *meta* and *para* chlorines. *Desulfitobacterium frappieri* can dechlorinate many different chlorophenols except monochlorophenol and certain dichlorophenols. It also dechlorinates several chlorocatechols and chloroanilines and both dechlorinates and demethoxylates several methoxylated chlorophenols (52).

Bromophenols are produced by certain marine invertebrates, apparently as a means of controlling microorganisms in their burrows (1). Degradation of bromophenols by marine sediments has been documented, and the sulfate-reducer *Desulfovibrio* strain TBP was isolated from an anaerobic bromophenol-degrading enrichment culture derived from estuarine sediments (53) and was shown to completely debrominate 2,4,6-tribromophenol to phenol as a dehalorespiratory process.

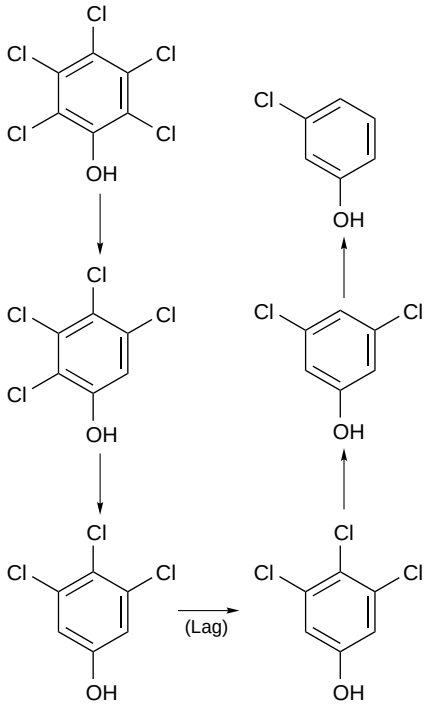

Figure 5. Reductive dechlorination of pentachlorophenol (PCP) to 3-chlorophenol by *Desulfitobacterium frappieri* (26). Addition of H, and removal of HCl as in Figure 3 are not shown for simplicity.

REDUCTIVE DEHALOGENATION OF OTHER ORGANOHALOGEN COMPOUNDS BY MIXED MICROBIAL POPULATIONS

There is good evidence of microbial reductive dechlorination of several other organochlorine compounds by anaerobic microcosms or by mixed microbial cultures. 1,1,1-trichloroethane (methylchloroform, TCA) (Fig. 1) was a commonly used solvent that has been shown to be transformed to 1,1-dichloroethane and chloroethane by mixed anaerobic microbial populations (2). It also underwent a slow abiotic elimination reaction leading to the formation of 1,1-dichloroethene, which was then biologically transformed to VC (2).

1,2-dichloroethane (DCA) (Fig. 1) is commonly used for making plastics and as a solvent and is readily biodegradable under aerobic conditions. *Dehalococcoides ethenogenes* could utilize DCA as an electron acceptor (32), converting it to approximately 99% ethene (dihalo-elimination) and 1% VC. Mixed cultures containing *Dehalococcoides ethenogenes* also carried out a rapid conversion of the fumigant 1,2-dibromoethane to ethene (22), but attempts to grow the pure culture using this compound as a respiratory electron acceptor failed. Another chlorinated ethane, the solvent 1,1,2,2-tetrachloroethane

(PCA), was dechlorinated mainly to *cis* and *trans* isomers of DCE and to VC by freshwater tidal sediments (54). 1,2-dichloropropane, an antinematode fumigant, was metabolized by mixed cultures to propene, with monochlorinated propenes detected only in early stages of enrichment (55), and apparently did not serve as intermediates in propene formation.

Chlorinated benzenes have been shown to be reductively dechlorinated, and reactions leading from hexachlorobenzene (Fig. 1) to monochlorobenzene have been demonstrated (14), although usually not all in the same sample. Often a large amount of 1,3,5-trichlorobenzene accumulates in cultures containing hexachlorobenzene, suggesting that dechlorination occurs most readily when two chlorines are adjacent to each other. *Dehalococcoides* strain CBDB1, which showed 98.3% identity in its 16S rDNA sequence with *D. ethenogenes*, could dechlorinate 1,2,3-trichlorobenzende, 1,2,4-trichlorobenzene, and some of the tetrachlorobenzene isomers (56). The organism did not dechlorinate PCE. Conversion of monochlorobenzene to benzene is slow, if carried out at all, by mixed cultures.

The insecticide DDT (Fig. 1) is no longer used in the United States but is still used elsewhere in the world to protect crops and to eliminate malaria-bearing mosquitoes. Through chemical and biological processes, it has been shown that DDT's trichloromethyl group under anaerobic conditions can be completely dechlorinated (57). The fate of the rest of the molecule under anaerobic conditions is not known. PCBs and dioxins (Fig. 1) are often resistant to aerobic biodegradation because of their high level of chlorination. Evidence for reductive dechlorination of these important environmental contaminants has been obtained using microcosms from contaminated sites (14,58–60), as is discussed in greater depth in another chapter in this volume.

BIOCHEMISTRY AND MOLECULAR BIOLOGY OF REDUCTIVE DECHLORINATION

With isolates in hand that carry out specific reductive dechlorinations, the enzymes that are responsible for the process and the genes encoding them can be studied. These studies are providing information about the molecular mechanisms of reductive dehalogenation and about the evolution of the process and the organisms carrying it out. It is conceivable that some day they may provide material for genetically engineering organisms for reductive dechlorination.

Because transition metal complexes are capable of catalyzing reductive dechlorination, they are good candidates for prosthetic groups of reductive dehalogenase enzymes, which can provide these complexes with a milieu that can accelerate their catalysis. This prediction has held true because the reductive dehalogenases characterized thus far contained either heme or corrinoid prosthetic groups. Moreover, the dehalogenases carry out the terminal reactions of a respiratory electron transport chains to develop a proton motive force, and research is being done on electron transport from the electron donors to the reductive dehalogenases (61).

Ni and coworkers (62) purified the membrane-bound 3-chlorobenzoate reductive dehalogenase (RD) from *Desulfomonile tiedjei*. The dehalogenase that was found only in cells grown in the presence of 3-CB was relatively oxygen tolerant and used reduced methyl viologen as an electron donor (as do all the RDs characterized so far), but not any known physiological electron donors. The protein had two subunits with molecular weights near 64 and 37 kDa. The spectrum of the purified protein indicated the presence of a heme group. In a subsequent study, evidence for an electron transport chain to the reductive dehalogenase involving a novel quinone and a low potential (-0.34 v) cytochrome c was obtained (63).

There have also been studies on the biochemistry of reductive dechlorination in chloroethene dechlorinating organisms. The PCE RD from *Dehalobacter restrictus* was isolated (64) and found to be membrane associated. It consisted of a single subunit with a molecular weight near 60 kDa. Both visible and electron paramagnetic resonance (EPR) spectral evidence showed the presence of iron-sulfur centers and a corrinoid, with oxidation-reduction potentials of 150 mv for Co(II) to Co(III) and -350 mv for Co(I) to Co(II). Incubation of the Co(I) enzyme with PCE yielded the Co(II) form. On the basis of the lack of reductive dehalogenation when whole cells were treated with reduced methyl viologen, which is considered to be unable to permeate the cell membrane, it was concluded that the PCE dehalogenase was on the inside face of the membrane and the hydrogenase was on the outside face (65). Evidence for the involvement of a quinone in the electron transport chain was also obtained.

The biochemistry of reductive dehalogenation in *Dehalospirillum multivorans* has also been intensively studied. The PCE RD from this organism was purified as a soluble cytoplasmic corrinoid-iron/sulfur-protein with a molecular weight near 58 kDa (61). The gene encoding this protein, *pceA*, was isolated and found to have little sequence similarity with previously described genes in the databases, including those for other corrinoid proteins. Cotranscribed with *pceA* was a second gene, *pceB*, that encoded a small (8.3 kDa) hydrophobic protein hypothesized to serve as the membrane anchor for the RD. The *pceA* gene was expressed in *Escherichia coli*, but no enzyme activity could be detected, probably because the expressed protein lacked correctly inserted prosthetic groups. Thus, if one wanted to transfer a reductive dehalogenase to another organism, it would need to be one that assembles the RD properly.

Two RDs were purified from a mixed culture containing *Dehalococcoides ethenogenes* (66). The first enzyme was a PCE RD that reduced PCE to TCE and had a molecular weight near 51 kDa, whereas the second enzyme had a molecular weight near 61 kDa and reduced TCE to DCEs, then to VC, and slowly reduced VC to ethene. Thus, PCE reduction to ethene is at least a two-step process in this organism. Inhibitor studies indicated that both RDs were corrinoid proteins.

An *ortho* chlorophenol RD was isolated from *Desulfitobacterium dehalogenans*. This membrane-associated protein had a molecular weight near 48 kDa. As for the haloalkene RDs, it contained iron/sulfur centers and a

corrinoid group. Its cloned gene, *cprA*, was approximately 30% identical at the amino acid level with *pceA* from *Dehalospirillum multivorans*, much closer than to any other proteins. Moreover, associated with it was a gene *cprB* that encoded a small protein homologous to *pceB*. Also of interest is that the cprA gene product had a signal sequence for secretion as well as a twin-arginine motif that is considered to be a signal for secretion of a cofactor-containing polypeptide into the periplasm. The *pceA* gene product in *D. multivorans* also has these periplasmic localization signals, in conflict with biochemical evidence placing it in the cytoplasm.

Thus, the RDs from *Dehalospirillum multivorans* and *Desulfitobacterium dehalogenans* form a distinct gene family that may have evolved for reductive dechlorination. A perusal of the BLAST microbial genomics site (*http://www.ncbi.nlm.nih.gov/Micro blast/unfinish-edgenome.html*) of the unfinished genome of *Dehalococcoides ethenogenes* indicates that it has several homologues of these corrinoid-containing RDs. A transposon mutagenesis system has been developed for *D. dehalogenans* (67) and should prove useful in defining genes involved in reductive dechlorination.

BIOREMEDIATION STUDIES

Bioremediation, especially in situ bioremediation, is considered a promising technology for treating groundwater sites contaminated with chlorinated organic compounds. There are both oxidative and reductive strategies for the bioremediation of chlorinated organic compounds. Many lightly chlorinated compounds degrade naturally and it is mainly a matter of ensuring that the right physiochemical conditions are present to allow for their biodegradation. Another aerobic approach takes advantage of the nonspecificity of certain oxygenases so that TCE, for example, has been treated in pilot studies by adding methane or toluene plus oxygen to groundwater aquifers to stimulate the appropriate organisms (68).

Approaches to anaerobic bioremediation of chlorinated organics involve both natural attenuation and accelerated bioremediation (69). In the former case, other compounds, such as hydrocarbons or organic solvents or natural organic compounds, are present along with chlorinated organic compounds. Degradation of these nonchlorinated compounds leads to anoxic conditions under which they can then be fermented to products, such as H_2, acetate, lactate, or butyrate, which are then utilized as electron donors by organisms capable of reductive dehalogenation. Lightly chlorinated products may migrate into aerobic zones in which they may be degraded. By careful analysis of a contaminant plume, one can sometimes document that the organohalogens are present at sufficiently low concentrations to meet regulatory guidelines by the time they migrate off site or reach a receiving body.

If natural attenuation is not rapid enough, steps can be taken, such as addition of an electron donor, and sometimes other nutrients, to accelerate the process. It is desirable to have an electron donor that supports reductive dechlorination but does not support a competing process, such as methanogenesis, as well. In the case of PCE and TCE biodegradation, it has been shown that because of the more favorable energetics of reductive dechlorination versus methanogenesis, reductive dechlorinators had a much lower threshold for H_2 than methanogens [Loffler, 1999 #415]. Moreover, electron donors that lead to low H_2 concentrations, such as butyrate or propionate, known to be poor substrates for methanogenic consortia (11), allowed much higher ratios of dechlorination to methanogenesis than other electron donors, such as lactate or ethanol, leading to higher concentrations of H_2 (70). It should be noted that in some cases, however, lactate or ethanol can be converted to propionate or butyrate, so that they may actually be good substrates, and it is always advisable to test dechlorination in microcosms before doing pilot or full-scale tests.

Early in situ studies, such as the one in which benzoate was added to a contaminated aquifer in Texas (71) and allowed complete conversion of PCE to ethene, indicated that stimulation of in situ anaerobic biodegradation was technically feasible. One important question is whether the appropriate dehalogenating organisms are present at the site. For example, it appears that organisms that reduce PCE and TCE to DCE are cosmopolitan, whereas those carrying out further reduction, such as *Dehalococcoides ethenogenes*, are not (72). Both microcosm studies and pilot scale studies for a contaminated site at Dover Air Force Base, Delaware, indicated that conversion of TCE did not proceed past DCE despite the feeding of large quantities of a lactate/yeast extract mixture. An enrichment culture developed from a site in Florida that carried out complete reductive dechlorination of TCE to ethene was added to Dover microcosms and complete dechlorination was effected (72). Several hundred liters of this culture were then added to the site (after pathogen tests and Environmental Protection Agency clearance), after which complete conversion of TCE to ETH was detected (73). This is a successful example of bioaugmentation, the addition of microorganisms to hasten biodegradation. It should be pointed out that often it is inappropriateness of the physiochemical milieu, such as pH or lack of nutrients, rather than lack of an appropriate organism that limits biodegradation of a pollutant.

It should be noted that reductive dehalogenation in contaminated aquifers requires the interactions between dehalogenators and other microorganisms in microbial communities, such as that depicted in Figure 3. The understanding of the diversity of reductive dechlorinators and the other members of the communities is still rudimentary. It will be fruitful to apply both cultural methods and molecular biological tools [Flynn, 2000 #457; Loffler, 2000 #437] to characterize these communities so that it will be possible to understand and manipulate them better.

CONCLUSION

It is now clear that many chlorinated organic molecules are susceptible to reductive dechlorination, including many highly chlorinated molecules that are resistant to aerobic attack. Particularly attractive for development are those

processes in which the chlorinated organic can serve as a respiratory electron acceptor because it then supports growth of the organisms carrying out the dechlorination. A greater understanding of both respiratory and cometabolic reductive processes and the organisms carrying them out will increase that ability to optimize the destruction of chlorinated organic molecules in the environment.

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BIODEGRADATION: WETLANDS.

See WETLANDS: BIODEGRADATION OF ORGANIC POLLUTANTS

BIODETERIORATION.

See WEATHERING, MICROBIOL

BIODETERIORATION OF MINERAL MATERIALS

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Mankind uses rocks, stones, and other natural and manufactured products as construction materials. Both in their natural state and when incorporated into a built structure, they are subject to the deteriorative and degradative action of the environment and microorganisms. These processes are normally referred to as *weathering*. It is often difficult to distinguish between nonbiological and biologically mediated weathering of mineral materials. The two processes can occur concurrently, each contributing to the overall deleterious effects. Mineral materials are used extensively in the construction industry and include a range of familiar building materials such as stone, bricks, roofing tiles and shingles, asbestos, mineralized felts, fascias (which are often natural stone or ceramic in nature), cement-based renderings, plasters and mortars, and adhesives and groutings. Concrete is used as reinforced structural elements and as building blocks.

BIOFILMS AND MINERAL MATERIALS

Biofilms are not confined to solid-liquid interfaces. They can also be found at solid-air interfaces (1). Almost all surfaces in nature, including mineral materials, may be colonized by microorganisms; this includes buildings (2) and monuments (3,4). Microorganisms are able to obtain calcium, aluminum, silicon, iron, and potassium from the substratum by biosolubilization, a process that involves the production of various organic and inorganic acids by microorganisms, bacteria, algae, lichens (symbiotic associations between fungi and algae), and fungi. These acids attack and degrade calcareous minerals by the dissolution of calcium ions and lead to the eventual formation of degraded products such as gypsum, calcite, glauconite, dolomite, ettringite, and quartz (5). In addition to liberating nutrients, the production of acids lowers the pH of the surrounding environment.

Microbial colonization of a surface can be associated with the formation of mucilaginous matrices, which may also be involved in degradative processes. Capsules and extracellular polymeric materials are produced by many bacteria and algae, but little attention has been paid to the role of the fungi in the structure or nutrition of biofilms (6). Fungi have been reported as members of natural biofilms (7) and their participation in these structures on mineral materials deserves more attention.

DETERIORATION PROCESSES

The processes involved in nonmicrobiological weathering of rocks are better understood than those that occur in the presence of microorganisms.

NONBIOLOGICAL WEATHERING PROCESSES

Apart from degradation by living organisms, rock, stone, and other constructional materials are weathered, that is, reduced to a more dispersed state, by rain, wind, gravity, ice, temperature change, and reaction with atmospheric gases. There are two general types of weathering: mechanical, involving only physical forces, and chemical, in which the chemical structure of the material is changed. In cold and/or dry climates, mechanical degradation is usually more important, whereas in the humid tropics and semitropics, chemical degradation is predominant.

The products of mechanical weathering include fragments of rock and materials formed by expansion and contraction of the rock, freezing and thawing cycles, and earth movements. Products of chemical weathering include the soil in its many forms; these chemical transformations follow mechanical weathering.

Chemical Weathering

Chemical weathering involves the migration of ions within the rock structure and within the surrounding environment. Ions may be mobilized by solution in water, which is enhanced by the presence of inorganic acids such as carbonic acid, sulfurous acid, sulfuric acid, and nitrous acid, and HNO_3, derived from the gases carbon

dioxide, sulfuric dioxide, nitrogen monoxide, and nitrogen dioxide, respectively. In the cases of iron and manganese, the chemical elements in the structure can be oxidized by atmospheric oxygen or reduced by volcanic H_2S. Silicate rocks are complex salts of silicic acids. Cations such as magnesium and potassium can be leached from the rock, leaving silica as the final product. Clays result from the weathering of aluminosilicates, and other silicon-containing minerals include quartz (SiO_2) and kaolinite ($Al_4Si_4O_{10}(OH)_8$). Iron oxides and bauxite are other resistant products of the weathering of siliceous rocks. Limestones and dolomites are degraded by the dissolution of calcium and magnesium carbonates in the structure, leaving behind the less soluble impurities such as clay and quartz (sand).

BIOLOGICAL WEATHERING — BIODETERIORATION AND BIODEGRADATION

Krumbein and Jens (8) quote the Bible as an early publication on the infectious nature of decay in houses (Leviticus XIV, 33–57), which also indicated the difficulty of treatment. Colonization and biodeterioration of building materials are determined by environmental conditions, the most significant being physical parameters (temperature, moisture, light intensity) and the chemical nature of the surface. It is generally accepted that microorganisms do not directly use the mineral components of building materials, but may solubilize them as a result of their normal metabolic activities, allowing the resultant ions to be absorbed by the cells in some cases. It is axiomatic that the only microorganisms able to colonize clean building surfaces are those that do not require organic materials for growth, namely, the autotrophs. These include the photosynthetic algae and cyanobacteria and lithotrophic bacteria. The literature usually cites photosynthetic organisms as the primary colonizers (9,10,11), but there is little direct experimentation to provide evidence for this.

Most of the information on the biological weathering of minerals comes from the literature on soil formation and describes processes at the soil/rock boundary. Estimates of the rates of weathering of rocks on the continental platforms give half-life values between 30 and 1,800 million years for complete weathering, depending on the site and composition of the rock (12). This is equivalent to 1 mm/y to 1 mm/1,000 y. Such processes may be of importance in the deterioration of structures built below the ground, but the literature in this respect is mostly restricted to the behavior of metal and concrete pipes. Above the ground, biodeterioration is the result of activities of superficial (epilithic) and deeper (endolithic) organisms. Colonizing organisms must be able to withstand dry conditions, the effects of high temperatures and light at the surface. Changes in acidity resulting from anthropogenic and natural inputs are also important. Microorganisms may increase the rate of transformation by their metabolic activities, such as the uptake of ions and the production of acids and chelating agents. They may also alter the water permeability of the minerals by the deposition of polymeric materials and surfactants. Water transport may be increased or decreased, according to the nature of the deposits, leading to localized dissolution or salting (13). Often, the end result of these processes is a catastrophic failure of the surface layer, known as *spalling*. Until such spalling takes place, the structure appears to be in sound condition, although the superficial rind or patina may hide a deep zone of rotten stone beneath the surface. After the initial catastrophic failure, the degraded mineral layer is subject to rapid and sustained erosion. Once this loose, friable surface is exposed, colonization by higher plants, with their extended root systems, may lead to more rapid deterioration.

SILICON AND SILICATES

Silicon is one of the most abundant elements of the earth's crust (about 28%) and is an important part of rock mineral structure, being present in most of the materials that comprise the crust; carbonates, phosphates, sulfates, sulfides, chlorides, and coal are some of the more common examples of rocks in which silicon is not significant. Sandstone, slate, schists, and granitic rocks, together with ceramics, mainly in the form of brick or flooring and roofing tiles, are siliceous materials widely used in buildings.

In view of the prevalence of silicon in the natural world, it is not surprising that there exist microorganisms capable of both degrading silicon minerals and absorbing silicon. Silicon is a structural element in diatoms, the cell walls of which consist of up to 95% hydrated, amorphous silica. They take up the element in the form of orthosilicate (H_4SiO_4) (14). Less well studied has been the uptake of silicon by bacteria, but this has been shown to occur from silica gel, quartz, or sodium silicate (15), whereas some fungi can accumulate silicon from organic silicon complexes (16). All these processes are linked to the biodegradation of silicon minerals.

Bacteria and fungi are able to solubilize silicon and are important agents in the weathering of rock silicates and aluminosilicates, both in nature and when used as building materials. The solubilization mechanisms include:

1. Chelation. Gluconate, for example, produced by various bacteria, can complex the cationic components of silica, solubilizing kaolinite, and quartz at neutral pH levels (17). Organic acids produced during bacterial and fungal metabolism can cause the dissolution of quartz by chelation, which is more rapid in neutral than in acid solutions (18). Silicates, as anions, may form soluble, hexacoordinate siliconate complexes with natural phenolics in soil (19).
2. Acid degradation. Many bacteria and fungi produce inorganic and organic acids that solubilize silicon minerals. Walch and Ullman (20) showed that organic acids are more effective than hydrochloric and nitric acid in solubilizing feldspars, but sulfuric acid produced by sulfur-oxidizing bacteria is extremely active against the aluminosilicates present in concrete (21).
3. Alkaline degradation. Silicates and aluminosilicates are readily mobilized under alkaline conditions because of the lability of the Al-O and Si-O bonds.

A number of bacteria make their surroundings more alkaline by the release of ammonia from proteins or urea and this has been shown, in the case of *Sarcina ureae*, to degrade nepheline, plagioclase, and quartz (22,23). Because silicates are relatively stable in dilute ammonia, it is reasonable to postulate that chelation is also important.

4. Reaction with extracellular polysaccharide. The extracellular polymers released by fungi, and especially bacteria, can react with siloxanes to form the more soluble organic siloxanes (16,24). The reaction seems to be pH-dependent and is favored by acid conditions (25). Extracellular microbial polymers on the surface of a mineral material may also cause increased water residence time, facilitating the chemical reactions that characterize low temperature silicate weathering (26).
5. Depolymerization of polysilicates (siloxanes, metasilicates). Two bacteria (a *Proteus* and a *Bacillus* species) have been shown to be capable of this transformation (27), but in view of the requirement of microorganisms to absorb silicon in its monomeric form, it seems likely that this activity is not restricted to these genera.
6. Where anaerobic conditions exist within the rock, both volatile and soluble alkoxysiloxanes may be formed (28).
7. Under dry conditions, many microorganisms produce osmotic protectants to resist desiccation. These are polyols such as glycerol, mannitol, alpha-glycerylglucose and sucrose, and other low molecular weight organic molecules including proline and betaines (29). Polyols cause the expansion of micaceous minerals by intercalation among the polysiloxane planes of the mica crystals. Such expansion will induce stress in the rocks, leading to physical weathering and spalling, and may increase the access of acids and chelating agents to ions deep in the crystals. Such polyols may also react directly with silanol groups present at the crystal edges to cause solution of silicon.

Chelation and transport of iron and manganese to the surface of rocks, where it is deposited as an oxide film, usually called a *patina*, create a barrier to the evaporation of water. Where this barrier is absent, the excess water evaporates and deposits salts, which can cause severe local spalling. Once this process is initiated, the protective film cannot be reformed, and severe pitting may result. Spalling on the underside of clay roofing tiles is a common problem, which does not become apparent until the entire area needs to be replaced; this is associated with the growth of microorganisms and of mosses and lichens.

LIMESTONE, SHALE, AND SANDSTONE

The three most abundant sedimentary rocks of the earth's crust, accounting for 95% of all sediments, are shale, sandstone, and limestone, formed from silicates, silica, and by the biological deposition of calcium carbonate. These rocks usually consist of a mixture of all three components with varying proportions of other minerals, such as iron oxides. Their properties and classification depend on their chemical constituents and the form of the mineral components. Only limestone and sandstone are commonly used as building materials. Shales are used in their metamorphic form, slate. Limestone is the most abundant of the nonclastic (nondetrital) rocks and is the largest reservoir of carbon at the earth's surface. It is the most important type of crushed stone used in the building industry, constituting about 75% of all the commercial products sold and is the principal raw ingredient used for Portland cement, in addition to being the source of lime used to make plasters and mortars. Sandstone, on the other hand, is a detrital sedimentary rock formed by the cementation of sand grains that are commonly composed of quartz, but which may include many other minerals. The overall stability of the rocks depends on the types of minerals and fragments of other rocks that are present and this will obviously affect their biodegradability.

Rock and stone may be biodegraded by bacterial, fungal, and algal acids (3,10,30) and by mechanical penetration by fungi and phototrophs (31,32). Microorganisms may be classified according to their mode of growth into epi- or endolithic, that is, growing upon or within the stone, respectively. Aesthetic biodeterioration is produced by surface discolorations, which occur on microbial colonization either on or within the stone.

Role of Fungi in the Biodeterioration of Mineral Materials

Fungi have been associated with the mechanical degradation of stone (33,34) and many fungi isolated from stone buildings have been shown to produce acids that could degrade calcareous structures (4,35–38). For instance, a dark-pigmented mitosporic fungus and the bacterium *Bacillus cereus*, both isolated from limestone buildings in Uxmal in the Yucatan peninsula, Mexico, have been shown to reduce an initial pH of 7.0 in a glucose/mineral salts medium to values between 3.0 and 4.0 pH units (39). A range of fungi have been shown to solubilize stone by organic acid production (40–43). Dark-pigmented mitosporic fungi can also actively penetrate limestone, causing "biopitting" (44) and have been shown to be particularly important in arid and semiarid environments of hot and cold deserts because of their ability to resist high temperatures, desiccation, and osmotic stress (45). However, the published literature does not suggest that fungi are present in high numbers on ancient limestone structures. Populations of 10^2 to 10^5 colony-forming units (cfu) g^{-1} in stone are common (38,46,47). It has been suggested that algae can contribute to fungal stone decay by providing organic substrates for the production of acidic metabolites (3,48), and fungal activity rather than numbers may be the more important parameter. Alternatively, the low fungal numbers may be explained by the detection techniques used. Slow growing fungi, which are considered to be important in decay processes, are outgrown in normal culture techniques by less relevant, rapidly growing species, such as *Aspergillus* and *Penicillium* (49), and thus are often not detected.

There is no doubt that fungi are important in the aesthetic biodeterioration of building surfaces. Melanotic fungi and actinomycetes, and cyanobacteria with dark-pigmented cells or capsules, are frequently reported on stone structures (47,50). Such growths cause much of the discoloration seen on stone buildings (Figs. 1–8). Warscheid and Krumbein (51) have discussed this phenomenon with respect to natural stone surfaces.

In the modern world, high levels of particulates in the atmosphere, particularly those contained in fumes from fossil fuels, adsorb to the outer surfaces of buildings and cause a gray-black discoloration (52). This can be mistaken for fungal growth. Winkler (53) discussed the apparent increase in the weathering rate of stone in urban locations after industrialization toward the end of the ninetienth century. It has been suggested that the layers of organic pollutants may act as nutrient sources for the growth of heterotrophic microorganisms (bacteria and fungi), thus accelerating both the aesthetic and physicochemical deterioration processes (30,54,55). The adsorbed pollutants are a diversity of organic materials, including fatty acids and aliphatic and aromatic hydrocarbons (54), and these obviously modify the nature of the stone surface.

Fungi will not grow, even on susceptible substrates, unless there is sufficient moisture. Fungi require water for the diffusion of nutrients into their cells and for the production of enzymes. Water activity (A_w) is numerically equal to RH/100 (e.g., 75% RH = Aw 0.75); on this basis an RH of 70% is near the lowest limit for fungal growth. For most environmental work the term *water potential* is preferred, and it is measured in megapascals (1 mMPa is equivalent to 9.87 atm, or 10 bar).

Fungi require water for the diffusion of nutrients into the cells and for the release of enzymes. However, water can be present in the environment and still be unavailable too because it is bound by external forces:

1. the osmotic potential (solute binding forces ϕ_π)
2. matrix potential (physical binding forces ϕ_m)
3. turgor (ϕ_p)
4. gravimetric potential (ϕ_g).

Their effects are additive, so they are denoted by the general term *water potential* ϕ.

$$\phi = \phi_\pi + \phi_m + \phi_p + \phi_g$$

Role of Cyanobacteria and Algae in the Biodeterioration of Mineral Materials

Cyanobacteria (blue-green bacteria, previously called *Cyanophyceae*, or *blue-green algae*) resemble the oldest fossil organisms on this planet and are found in an enormous variety of habitats. As photosynthetic microorganisms, along with the algae, they do not require organic materials for growth and are common inhabitants of all water bodies and soil. Cyanobacteria survive repeated cycles of drying and rehydration (56), making them particularly important on exposed surfaces and they are probably of greater ecological importance as pioneer organisms on building surfaces than any other class (10). It has also been suggested that they have the most important influence on the weathering of exposed natural stone and rock (31,57), penetrating rock in dry environments at the rate of 5 μm/y (58). In dry, exposed situations, the majority of the organisms are found within the rock.

Many cyanobacteria are able to withstand high levels of UV illumination (59–63), enabling them to exist at upper elevations. Nevertheless, most grow preferentially in shady situations, along with the algae. Chemical and microbiological analyses have shown that superficial growth is heavier on interior walls of ancient limestone buildings (47,64). This is due to the more sheltered conditions, reducing illumination below inhibitory levels and maintaining humidity, and due to the presence of organic nutrients supplied by the droppings of birds, bats, etc. In buildings of the ancient Mayan site of Uxmal, in Mexico, for example, the microbial biomass, expressed as total phospholipid fatty acids (PLFA) per gram of stone, showed that indoor surfaces had an average value of 73.7 nmol g^{-1}, compared with 18.4 nmol.g^{-1} for outdoor sites (47). The PLFA profiles indicated the presence of bacteria, actinomycetes, cyanobacteria, and fungi. Chlorophyll concentrations, showing the presence of phototrophic organisms, were similarly higher on interior rather than outdoor surfaces (1.17 mg.g^{-1}, as compared with 0.22 mg.g^{-1}), suggesting higher algal and cyanobacterial populations on interior walls. This was confirmed by direct examination and culture techniques, when it was found that the biomass was mostly that of cyanobacteria (47). The major genus detected was *Xenococcus*, with *Synechocystis* and *Gloeocapsa* the next most abundant. The latter two cyanobacterial genera have been stated to be capable of limestone boring activity (presumably penetration of the substratum effected by the dissolution of the limestone), along with *Stigonema* and *Schizothrix* (31).

Although there seems little doubt that cyanobacteria are frequently predominant on buildings in Latin America, algae have been cited as the dominant microorganisms in some other countries, with *Trentepohlia* frequently being detected in Singapore (65) and *Chlorella* and *Stichococcus* in Russia (66). It is difficult to know whether these varying results come from the use of different detection techniques in the various locations. However, the recent design of specific primers for the detection of cyanobacteria by molecular methods will assure that such information in the future is reliable and repeatable.

The growth of cyanobacteria and algae on the surfaces of buildings leads directly to aesthetic deterioration of the surface. Photosynthetic biofilms may be gray, black, brown, red, or green, depending on the predominant strain(s) of organisms and the environmental conditions (Figs. 3 and 6). Humidity, temperature, and the degree of exposure to light can all affect growth and pigment production in algal and cyanobacterial cells. Under drier conditions, the biofilms are generally gray in color, whereas more humid areas are more frequently green. The filamentous alga, *Trentepohlia*, found to be the most common colonizer of buildings in Singapore (65), stores orange/red carotenoids within its cells under appropriate

Figure 1. Discolored stone statue in Karlovy Vary, Czech Republic, showing growth of mainly mitosporic fungi and cyanobacteria. See color insert.

Figure 2. Mayan building in Chichen Itza, Mexico, showing blackening of limestone by epilithic and endolithic fungi and cyanobacteria. See color insert.

conditions, and this leads to the production of pink or orange biofilms. The presence of other microorganisms also affects the appearance of the biofilm. For example,

Figure 3. Fountain in Machu Picchu, Peru, showing intense growth of algae and cyanobacteria in the splash zone. See color insert.

a thick fungal growth on top of a cyanobacterial/algal layer will frequently be black (Fig. 8). However, the effects of cyanobacterial growth on building surfaces are not merely aesthetic. Danin and Caneva (57) suggest that cyanobacteria contribute to the decay of calcareous stones as follows:

1. Attachment of cyanobacterial cells in small fissures;
2. Growth within the fissure;
3. Water uptake and expansion of cell mass, exerting pressure within the structure;
4. Precipitation of carbonates and oxalates around the cells;
5. Opening of the fissure because of these internal pressures;
6. Entry of dust, pollen grains, etc.;
7. Partial death of cyanobacterial cells and establishment of heterotrophic bacteria, fungi, and small animals such as mites within the fissure;

Figure 4. Spalled soapstone on the external wall of a church in Ouro Preto, Brazil. Cyanobacteria and actinomycetes were the major microorganisms detected below the detached layer. See color insert.

Figure 5. Salting on the surface of mortar in the tomb of Sevilha, Necropolis of Carmona, Spain. See color insert.

8. Increasing internal pressure on the superficial layer of the structure leading eventually to its detachment (spalling) (Fig. 4).

Cyanobacterial cells on limestone buildings are often seen covered by calcareous deposits, suggesting migration of calcium from neighboring sites (47,67,68). The biological immobilization of carbon as carbonates, which can be deposited both intra- and extracellularly, occurs in some bacteria, fungi, algae, and some metazoa, and in cyanobacteria. Phototrophs deposit $CaCO_3$ in the light and solubilize it at night because of changing bicarbonate concentrations. This process has been well studied in

Figure 6. Intense algal growth behind a lamp attached to a building in Sao Joao del Rei, Brazil. Note black growth of mitosporic fungi in the rain runoff area below the lamp. See color insert.

the cyanobacterium *Synechococcus* GL24. These spherical cells possess on their external surface a so-called S-layer, which, in spite of its hydrophobic nature, is able to bind calcium ions at negatively charged sites (67). The bound calcium complexes with carbonate ions when the pH level is more than 8.3, and this high pH is readily produced as follows: bicarbonate ions are taken up by the cells and converted by the enzyme carbonic anhydrase into carbon dioxide and OH^- (69); the carbon dioxide is incorporated into the cell mass and the OH^- ions are released and concentrated around the cells (70), raising the local pH levels. The cells of *Synechococcus* can become encrusted with calcite within eight hours in a suitable environment and must continually shed patches of mineralized S-layer to remain viable (71). The whole procedure is then repeated, allowing the further uptake of calcium. Mobilization of calcium ions by such metabolic activity and ion transport, in addition to the trapping of released particles of calcite, either biotic or abiotic, in the gelatinous cyanobacterial sheath (72,73), is an important mechanism of stone degradation by cyanobacteria. The same processes occur with eukaryotic algae, and electron micrographs showing algal and/or cyanobacterial cells residing in micropits in limestone or mortar have been published (47,74). Siliceous rocks are also more readily degraded at high pH levels, as previously stated, because of the lability of the constituent cations under these

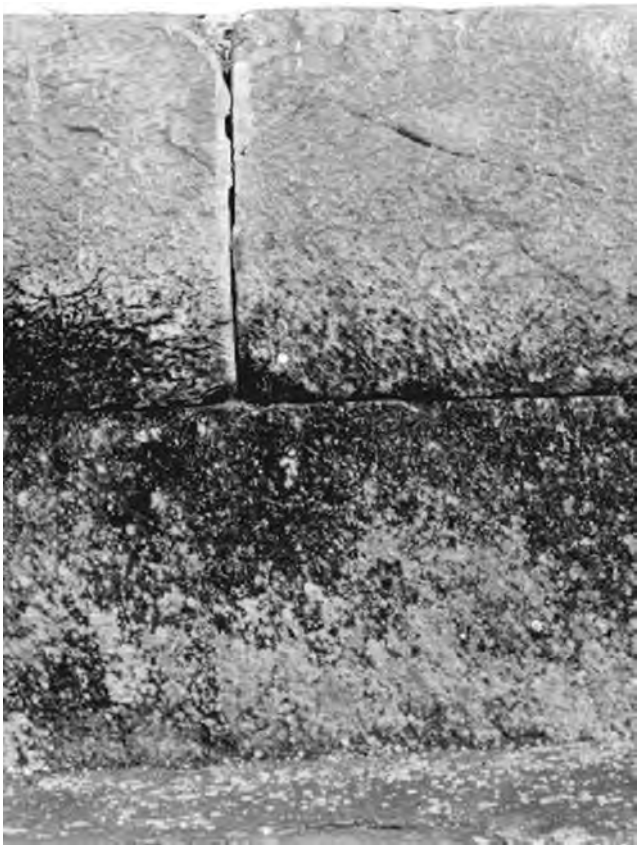

Figure 7. Growth of fungi (upper black layer) and lichen (lower pale green layer) on the quartzite course of a church in Tiradentes, Brazil. See color insert.

Figure 8. Intense black discoloration caused by growth of mainly mitosporic fungi and cyanobacteria on the east-facing painted wall of a church in Ouro Preto, Brazil. See color insert.

conditions and because the hydrolysis of polysilicates is favored at high pH levels.

The suggestion that algae and cyanobacteria may also dissolve stone by acid production (75) is more debatable. As discussed earlier, photosynthetic organisms actively concentrate carbon dioxide within the cells as bicarbonate, increasing the external pH level to as high as 10.5 within 48- hour laboratory growth in natural lake water (71,76). Nevertheless, Van der Oost and coworkers (77) showed that *Cyanothece* carries out mixed acid fermentation and could, therefore, induce acid degradation of stone. Mao-Che and coworkers (78) demonstrated the release of a carbonate-digesting liquid from the apical cells of one of the *Pleurocapsales* group; although the nature of the substance was not determined, it was suggested that it could have acidic properties. However, there is little evidence that cyanobacteria produce acids in situ, and Waterbury (79) suggests that acid decay of calcareous materials in the presence of cyanobacteria is due to the concurrent presence of heterotrophic bacteria.

Such associations of algal and cyanobacterial cells with heterotrophic bacteria can enhance the weathering of rock minerals through bacterial organic acid production (80). It is also known that fungal microflora can metabolize the organic matter secreted by phototrophic biofilms (81) and contribute to stone decay through the release of acidic metabolites (48). Urzi (46) has challenged the accepted view that phototrophic colonization of stone is followed by the growth of chemoorganotrophs, but Gaylarde and Gaylarde (82) demonstrated that the treatment of external walls with an algicidal substance (a copper-containing compound), used at concentrations too low for bacteriostatic and fungistatic activity, inhibited fungal and bacterial growth. This indicates that the inhibition of algal colonization reduces the growth of fungi and heterotrophic bacteria, and supports the accepted colonization sequence, although on chemically polluted buildings organic materials may act as nutrients for nonphototrophs. Under these conditions, heterotrophic bacteria could, indeed, be the primary colonizers of buildings (83).

Role of Lichens in the Biodeterioration of Mineral Materials

Lichens, symbiotic associations of fungi and algae or cyanobacteria, grow well on sun-exposed stone surfaces and in relatively unpolluted atmospheres (Fig. 7) (84). Ancient mortars are readily colonized by lichens because of their composition and physical structure, through the pores of which the hyphae may pass. Mortars, either ancient or modern, are generally the initial point of lichen colonization for other adjacent building materials, such as marble (85).

Lichens induce the biodegradation of mortar and stone by direct chemical attack of organic acids, principally oxalic acid (5,85–87). Large and small pits are formed on the surface of the material beneath the lichen thallus; hyphae and hyphal bundles produce micropits 5 to 200 μm in diameter, whereas the lichen fruiting bodies lead to pits of 0.5 to 1 mm in size (85). The penetration of the lichen structure into the rock or mortar produces a very strong association between the two and removal of the biological growths may damage the underlying material even further. However, some materials are little penetrated by lichens, the degree of internalization being determined by the mineralogical composition of the substratum (85). A discussion of the effects of lichen growth on various rock-forming minerals is presented in Jones and coworkers (88).

Lichen colonization does not necessarily lead to biodegradation of the structure and, in some cases, may actually protect the surface against the scouring action of wind and weather through the insoluble oxalate layer deposited beneath the thallus (89–91). Lichen growth may also be aesthetically desirable in some cases, covering ugly buildings and providing attractive coloration on otherwise drab constructions (92).

Role of Nonphotosynthetic Bacteria in the Biodeterioration of Mineral Materials

Bacteria have been implicated in the biodegradation of mineral building materials because of the high numbers of these prokaryotic organisms associated with deteriorated built structures (42,93–95). However, Somova and coworkers (66) reported that saprophytic bacteria were more common on undegraded, rather than degraded, masonry and consider that algae are more important.

The two groups of bacteria discussed in the literature are autotrophic and heterotrophic organisms.

Autotrophic Bacteria. Bacteria that oxidize inorganic sulfur and nitrogen compounds are able to degrade stone substrates by the production of inorganic acids (sulfate and nitrate). There is some debate about the overall importance of these microorganisms in the decay of stone, which is well discussed in the article by May and coworkers (30). However, the acceleration of decay by nitrifying bacteria has been demonstrated in the laboratory for limestone (96), ultrabasic rocks (97), and concrete (98). They have also been implicated in the deterioration of plaster (99) and asbestos cement roofs (100) in stables. Weight losses have been detected in concrete blocks incubated with sulfur-oxidizing bacteria (101,102). There is little doubt that these acid-producing bacteria can cause stone decay, but their role in the real world remains to be confirmed.

Chemoorganotrophic (Heterotrophic) Bacteria. The role of heterotrophic bacteria in the formation of soil by rock breakdown is well accepted (103). The rock is degraded by bacterial acids and alkalis and by chelation. There is little work, however, concerning the identity of organisms responsible for built stone decay, although many authors have reported high numbers of heterotrophic bacteria on decaying stone (40,93,104,105). Weight loss experiments have shown the involvement of such bacteria in the degradation of sandstone (95,106,107), and there are a number of publications on the staining of monuments by bacterial pigments (108–110).

Actinomycetes of various genera have been found frequently on ancient wall paintings and monuments (66,111–113). They contribute to biodegradation by the mechanical penetration of the mycelia into the structure. The genus *Geodermatophilus* has recently been considered to be of particular interest (66,113). The other bacterial genus that is frequently isolated from rocks and caves is *Bacillus* (114), although this group may not be important in decay processes (113).

Our knowledge of the bacterial populations present on stone surfaces is currently being modified by the results of molecular biological detection methods, which allow the identification of nonculturable organisms (115,116). Members of the family *Archaea* and many actinomycetes have been detected on degraded wall paintings (117) and the same techniques suggest that the major heterotrophic bacteria colonizing rocks and monuments are actinomycetes, mainly related to the genus *Geodermatophilus* (118,119).

CEMENT AND CONCRETE

It is perhaps inevitable that civilizations in their search for an acceptable substitute for stone as a building material eventually devised a medium with many of the characteristics and properties of natural stone. To the prerequisite properties of durability and strength were added the extra advantages of a material that was relatively inexpensive to manufacture and one that could be easily transported. Furthermore, in the hands of imaginative architects and competent builders, a new era of building was born in which the scale of building was no longer a major problem.

Cement can be described as a material that possesses both adhesive and cohesive properties that enable it to bond various mineral fragments into one complex whole. Since the introduction of Portland cement, concrete has become one of the most widely used synthetic materials within the construction industry (120). Portland cement (Table 1) is the most commonly used cement mixture used in construction. However, other types of cements exist and are widely used. The chemical composition of cement varies according to the role required by that specific batch. It is the unique chemical composition of cement that confers surface properties and tensile strengths peculiar to that specific formulation. In reality however, the silicates found in cement are not pure compounds; they contain minor oxides in solid solution that confer vital effects on the atomic arrangements, crystal form, and hydraulic properties of the silicates (120).

Concrete is composed of cement powder, water, and aggregates of various sizes, such as sand or gravel (121). The main constituents of cement powder are lime, silica, alumina, and iron oxide. The cement paste hydrolyses on curing to form hydrated calcium silicates (C-S-H gels) and Portlandite ($Ca(OH)_2$) (120,122–125), and the arrangement of these crystals allows channels to form within the concrete structure. The formation of these channels within the concrete permits the capillary action of water, which may contain microorganisms. Thus, as water permeates deeper into the concrete, fissures are formed, allowing the deposition of organic material and the further

Table 1. Main Compounds of Portland Cement

Name of Compound	Oxide-Composition	Abbreviation
Tricalcium silicate	$3CaO{\cdot}SiO_2$	C_3S
Dicalcium silicate	$2CaO{\cdot}SiO_2$	C_2S
Tricalcium aluminate	$3CaO{\cdot}Al_2O_3$	C_3A
Tetracalcium aluminoferrite	$3CaO{\cdot}Al_2O_3Fe_2O_3$	C_4AF

A. M. Neville, *Properties of Concrete*, 2nd ed., Pitman Publishing, London, U.K., 1977.

ingress of microorganisms into the concrete (126). In addition to the main compounds of cement, various minor components are present, for example, MgO, TiO_2, Mn_2O_3, K_2O, and Na_2O. These components may serve as important sources of nutrients for colonizing microorganisms.

In 1945, Parker (127) discovered that concrete was being broken down by the metabolic activities of microorganisms. He isolated *Thiobacillus* from areas of badly corroded concrete. This and subsequent studies (127–129) showed that *Thiobacilli* were the causative agents of microbially induced corrosion of concrete structures. This view has been substantiated in recent years by several others working in the same field (130–137).

Biogenic Sulfuric Acid Corrosion of Concrete

The decay of concrete by microorganisms is dependent on the production of corrosive metabolites that can solubilize minerals (138).

Freshly prepared concrete has a pH level between 12 and 13 compared with the highly acidic pH (2.5 to 0.5) of corroded concrete. The original pH must be reduced (by chemical carbonation) before microorganisms can begin to colonize and grow. The major organisms involved in acid concrete decay are the thiobacilli; their incidence and activity are described in Sand and Bock (2) and Milde and coworkers (130).

An example is their involvement in the deterioration of concrete in sewage pipelines. Parker (127) reported that the bacteria use hydrogen sulfide released from the sewage and oxidize it to sulfuric acid. Sand and Bock (2), reporting on the findings of a research project undertaken in Hamburg to establish the mechanism of corrosion found in several pipelines constructed between 1967 and 1971, recorded that thiobacilli were present in high numbers at the corrosion sites. The incidence of *Acidithiobacillus thiooxidans* was found to be high at sites that showed heavy corrosion. The bacterium was not detected at sites where corrosion was not detected. Thus, it was considered appropriate to adopt this microorganism as an indicator of corrosion rather than pH values resulting from bacterial sulfuric acid generation, which are partially buffered by different concrete types. Volatile sulfur compounds that are dissolved in the sewage escape into the atmosphere within the sewer. In this atmosphere, hydrogen sulfide is auto-oxidized to molecular sulfur, which adheres to the concrete walls of the sewage pipes where it is oxidized to sulfuric acid. This acid reacts with the calcitic binding material of the concrete thereby causing its destruction. These workers also detected two other sulfur compounds on the sewer walls, namely, sulfur dioxide and thiosulfate. Thiosulfate is a reaction product of sulfur dioxide with molecular sulfur and forms a good substrate for the growth of thiobacilli.

Biogenic Nitric Acid Corrosion

Although thiobacilli are considered to be responsible for the degradation of concrete above ground, two groups of nitrifying bacteria are considered to play a major role in the degradation processes below ground. They are responsible for the oxidation of ammonia via nitrous acid to nitric acid (2). The first group includes species of *Nitrosomonas* and *Nitrosovibrio*, which oxidize ammonia via hydroxylamine to nitrous acid. The second group that affects the oxidation of nitrous acid to nitric acid includes members of the genera *Nitrobacter* and *Nitrosovibrio* (139). The action of nitric acid upon the calcareous binding material of concrete results in the production of calcium nitrate. This is a soluble salt that is either lost from the concrete, resulting in the formation of corrosion pits, or remains, thus adding salt to the pore water.

Sulfate-Reducing Bacteria

The sulfate-reducing bacteria are usually associated with metal corrosion rather than with the deterioration of concrete, and therefore could be involved in the deterioration of the bars in steel-reinforced concrete. However, sulfate-reducing bacteria are widespread in nature and active in locations made anaerobic by microbial digestion of organic materials. In sewers, the involvement of sulfate-reducing bacteria in concrete decay is mediated by gas, produced as a result of the proteolytic action of sulfate-reducing bacteria living in anaerobic slimes or biofilms formed below the sewage level. (2).

Biogenic Organic Acid Corrosion

Fungi. Fungi isolated from the surfaces of monuments, facades of buildings, and degrading concrete include many genera that are representative of air and soil flora (140–143). Biodeterioration of stone by fungal organic acids has been described (141,144). Di- and trivalent cations are reported to be solubilized by chelate-forming organic acids (145). Krumbein and Schonborn-Krumbein (146) suggest mechanisms involving "siderophores" of fungi in the etching of microfractures, whereas Krumbein and Petersen (145) report on the biotransfer of cations involved in exfoliation induction in sandstone monuments. For concrete, however, this level of research activity is lacking.

Lichens. Oxalic acid has been one of the most studied acids produced by lichens (147). It is produced by the fungal partner of the lichen and is more prevalent in mature, calcium-loving species. (147,148). Lichens known to produce calcium oxalate are highly effective biodeteriogens in a range of rock types with oxalates formed at the thallus/substratum interface. These have closely related chemical compositions to the rocks that they colonize (148). Tiano (147) cites authors who discuss the importance of the pedogenic activity of lichens. The incidence of lichens on cement-based materials, for example, weathered roofing tiles, is common in the United Kingdom and elsewhere, but there is little evidence of biodeterioration problems.

Algae and Cyanobacteria. The hygroscopic sheath covering many algal cells growing on the surface of stones often seals moisture into the material, thus allowing for extended growth periods during dry spells (140). Repeated freezing and thawing of the hydrated algae contributes

further to deterioration. Lithophilous algae and cyanobacteria can be considered as the pioneering inhabitants of stone surfaces (147). The colonizers of such surfaces are closely related to those present in nonflooded soils (32). Controversy exists as to whether these microorganisms have a biodeteriogenic effect on the surfaces that they colonize (10,121). One point of view is that their deteriorative effect is negligible (149,150). However, there are reports of the positive role of these microorganisms on the weathering of stone in nature (151,152) and on monuments and sculptures (151,153,154). Such speculation applies equally to weathered concrete surfaces.

Chemoorganotrophic Bacteria. Chemoorganotrophs excrete organic acids, including acetic and benzoic acid (155), which can affect the structural properties of concrete. The metabolic activity of these microorganisms will depend on nutritional levels that prevail, in addition to a range of environmental conditions (147). However, there is little published work on their role in concrete degradation.

CONCLUSION

The deterioration of stonework, brickwork, and other mineral materials by microorganisms has an immediate impact on the construction and conservation industries because of the financial implications of remedial treatments. The incidence of lichens, cyanobacteria, algae, and fungi on stonework and monuments is often considered merely to be aesthetically undesirable, rather than severely damaging to these structures. However, there is little doubt that physical and chemical attack can also occur through the activities of organisms growing on the surface (epilithic) or in deeper layers (endolithic) of rock and stone structures. The role of bacteria in the breakdown of natural rock and the genesis of soils has long been accepted and the same processes can participate in the biodegradation of stone-built structures. The actinomycetes have recently been highlighted as important bacterial colonizers of rocks and buildings of cultural heritage and future research will certainly focus on the degradative activities of these organisms.

Biofilms, as regions where the metabolic activities of microorganisms are concentrated, have been accepted as of major relevance in the biodeterioration of materials, and this is an area of increasing importance in the study and control of biodeterioration of construction materials. Techniques recently introduced into biofilm research, such as atomic-force microscopy, environmental-scanning electron microscopy, confocal-laser-scanning microscopy, green fluorescent protein, fluorescent in situ hybridization (FISH), denaturing gradient gel electrophoresis (DGGE), and other molecular biology methods, are already being used on artificial and natural mineral materials. Rolleke and coworkers (156) using 16S rDNA analysis, showed the presence of the Archaea genus *Halomonas* on a mural in a German church, confirming the presence of extremophiles (in this case, salt-tolerant bacteria) on buildings. Recently, Saad and coworkers (157) have suggested the use of 17S rDNA analysis by DGGE to identify fungal genera on painted surfaces. Early results suggest that this is a promising technique for probing building surfaces and identifying changes in fungal biodiversity caused, for example, by the use of biocides. The future will see a huge increase in our knowledge of the types of organisms whose activities are really important in the deterioration and degradation of these structures.

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BIODIVERSITY IN SOILS: USE OF MOLECULAR METHODS FOR ITS CHARACTERIZATION

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Soil is a matrix of organic and inorganic particles formed by the combined action of biotic and abiotic processes. Geographic, geologic, hydrologic, climatic, vegetation, faunal, and anthropogenic factors together determine a soil's properties and, in turn, its resident microbial community (1,2). The soil mineral composition, salinity, pH, nutrient availability, organic content, temperature, and water content, among other factors, define which ecological niches are present. High spatial heterogeneity within a soil is a result of the vertical horizon structure and of heterogeneity in such factors as plant cover, microtopology, and perturbations by fauna. Therefore, just as plant communities show local variations in species composition within one vegetation type (3), so does the microbial community vary within one soil type (4). Plants, besides adding organic material to soil and affecting the microclimate, create the unique rhizoplane and rhizosphere habitats. The increased microniche complexity (5) at or near root surfaces selects for different microorganisms than those dominant in the bulk soil (6). Seasonal changes in weather and the concurrent vegetation cycles add a temporal heterogeneity to this complex system, so that no two-soil samples have the same microbial community. As a consequence, soil probably harbors most of the planet's undiscovered microbial biodiversity.

In one study of genotypic diversity in a forest soil, DNA renaturation kinetics were used to estimate that 4×10^3 to 7×10^3 differently sized microbial genomes were present in 1 g of soil (7). This represents as many as 13,000 species (8). Obviously, these species are not present in equal numbers, but vary from a single cell to as many as 10^8 cells per gram of soil. In a similar study employing DNA hybridization techniques, it was shown that the diversity varied with soil type (9).

Two qualities of microbial communities can be addressed in molecular microbial ecology studies: (*1*) diversity, and (*2*) community structure. Although diversity has been defined in a number of ways (3,10,11), it is often used in molecular microbiological studies, albeit incorrectly in a classical ecology framework, as a synonym for species richness (12). This is the number of different species or, more precisely, the number of different sequence types (phylotype richness) detected. Because of the special problems involved in quantitative analysis of molecular biology data, we shall adopt the convention that diversity studies needs to only include information on species presence. In contrast, analysis of community structure includes quantitative information on the abundance of different taxa or physiological groups, and is thus more related to classical definitions of ecosystem diversity such as Shannon's index.

The assessment of the diversity and structural composition of soil microbial communities is extremely dependent on the methodological tools used. Traditionally, characterization of microbial communities was based on cultivation of the respective members. However, this technique identifies only that tiny portion of the community which can grow on the media used, which is often less than 1% of the viable species present (13–15). Cultivation studies may fail to detect abundant or even dominant members of soil microbial communities and thus are strongly biased (16–20). This approach is also time consuming and labor intensive. As a consequence, the past decade has witnessed the development of a variety of molecular methods that bypass cultivation and instead base community analysis on DNA or RNA extracted directly from a given environment. This article will focus on the current state-of-the-art molecular ecology techniques available for cultivation-independent investigation of the phylogenetic and functional diversities of the prokaryotic domains Bacteria and Archaea in soils. It is organized into the following sections:

- Extraction of total nucleic acids (DNA, RNA) from soils
- Fractionation of total DNA based on mol% G + C content

- Phylogenetic diversity assessment by retrieval and comparative sequence analysis of small subunit (SSU) ribosomal DNA (rDNA)
- Retrieval and comparative sequence analysis of genes coding for key enzymes that perform defined functions
- Molecular profiling of soil microbial communities using phylogenetic and functional markers
- Microbial activity at the functional level
- Metagenome analysis

EXTRACTION OF TOTAL NUCLEIC ACIDS FROM SOIL

The extraction and purification of high-quality nucleic acids, either DNA or RNA, is a prerequisite for almost all molecular ecology studies on indigenous microbial communities (fluorescence in situ hybridization [FISH] is an exception). In principle, studies using extracted DNA assess the phylogenetic diversity (rDNA) or genetic potential (functional gene markers) of soil microbial communities. Studies on rRNA preferentially assess the active members of a community, because many species vary their ribosome content in positive correlation with their cellular activity (21). rRNA studies should therefore reveal adaptational shifts in community structure and activity more readily than DNA studies do. In addition, rRNA-based approaches have the advantage of a natural amplification of target numbers, as one cell can contain thousands of ribosomes. Although rRNA is the main component of total RNA extracted from natural environments, messenger RNA (mRNA) can also be extracted. Studies using mRNA are, however, technically far more problematic (see section Analysis of Microbial Activity at the Functional Level).

Extraction of Total DNA

DNA extraction should be carried out as soon as possible after soil samples have been collected from the field, as changes in community structure can occur during storage. In one study, storage of soil samples for three weeks at 4 °C significantly reduced the fraction of high molecular weight DNA retrieved (22). Two different procedures–indirect and direct–can be applied for the extraction of soil DNA. Indirect methods separate intact cells from the soil matrix prior to the lysis step (pioneered by Holben and coworkers (23)), whereas direct methods lyse cells within the soil (pioneered by Ogram and coworkers (24)).

Indirect Methods. Indirect methods use differential centrifugation steps to separate the cell fraction from the soil matrix. The cell fraction is then lysed, and total DNA isolated and purified (25–29). The main drawback of this method is the bias inherent in removing cells from soil particles. Although several physical and chemical dispersion techniques have been employed to efficiently desorb cells from soil or sediment particles (27,30–32), DNA obtained by these extraction procedures might not be representative of the entire microbial community, that is, might exclude DNA from microbes that attach strongly to soil particles (24,33–35). As a consequence, the use of direct DNA extraction techniques is usually preferred for studying soil microbial diversity. One advantage of the indirect approach is that uncultured bacterial cells might still be viable. This was observed to be especially true for a cell purification procedure involving lectin-mediated bead extraction (31). The applicability of cell extracts for parallel study of physiology and ecology has also been demonstrated for uncultured methane-oxidizing bacteria (36).

Direct Methods. In comparative studies, direct in situ cell lysis procedures have produced DNA yields that are higher by 60% (37), one order of magnitude (22) or 20 to 70 times (25), compared with yields obtained by indirect cell extraction procedures. Consequently, DNA extraction protocols reported in the last decade are primarily based on direct cell lysis within the soil or sediment (24,25,38–48).

A critical factor in determining the DNA yield by direct extraction methods is the choice of lysis protocol. Mechanical disruption of the cells should facilitate a quantitative extraction of environmental DNA (24,25) and allow the detection of the overall genetic potential in the sample, including dormant microorganisms. It may also recover DNA from moribund populations and even extracellular DNA from naturally lysed cells. On the other hand, some DNA released during the direct lysis procedure may bind to soil colloids and thereby reduce the overall recovery of soil DNA (49–52). This DNA adsorption is directly dependent on soil composition. A high content of clay minerals has an especially strong negative influence on the recovery of total DNA. Frostegård and coworkers (47) reported that this problem could be overcome by pretreating the soil with RNA to saturate the adsorption sites. RNA pretreatment of soils having a high clay mineral content increased the DNA recovery to levels obtained from soils with less clay content.

Of the lysis treatments available, those using the detergent sodium dodecyl sulfate (SDS) in combination with bead mill homogenization give the highest DNA yields from soil (41,45,48). Moré and coworkers (41) reported that the sequential application of SDS treatment and bead mill homogenization was more efficient than SDS treatment followed by three cycles of freezing and thawing. The improved extraction efficiency was attributed to a higher proportion of cells being lysed. Viable counts of sediment microorganisms revealed that 2 and 8%, respectively, survived the bead mill homogenization and freeze-thaw procedures. *Bacillus* endospores were not lysed by freeze-thawing treatment (94% survival rate), but were almost completely lysed by mechanical disruption (2% survival rate). Similarly, Kuske and coworkers (45) reported that only bead mill homogenization was effective for DNA extraction from *Bacillus* spores. However, the study by Moré and coworkers (41) also showed that small bacterial cells (0.3 to 1.2 μm) were more difficult to lyse than were larger bacteria (2 to 10 μm). After an SDS treatment and 10-minute bead mill homogenization of one sample, the remaining unlysed 6% of the original number of bacterial cells, as determined by direct counting with acridine orange, were all in an approximate size range of 0.3 to

1.2 μm. A major portion of soil bacteria (50 to 70% of the total bacterial numbers in nonrhizosphere soil) are less than 0.5 μm in diameter (53) and thus fall within the size range of unlysed cells in the study of Moré and coworkers (41).

Miller and coworkers (48) evaluated the effectiveness of various DNA extraction procedures on samples of varying organic mater content, including two silt loam soils and a silt loam wetland sediment. Again, bead mill homogenization in a lysis mixture containing chloroform, SDS, NaCl, and phosphate-Tris buffer (pH 8) was found to give the highest DNA yield and the most complete cell lysis. The recovery of high molecular weight DNA was greatest when using brief, low-speed bead mill homogenization. Under optimal physical lysis conditions, the maximum molecular size of the DNA extracted was 16 to 20 kb, which was similar to that obtained in previous studies using bead mill homogenization [e.g., 20 to 25 kb (37,44)]. More intensive use of bead mill homogenization increases the risk of severely shearing environmental DNA (24,33). Highly sheared, low molecular weight DNA might still be useful for polymerase chain reaction (PCR) amplification of SSU rRNA or functional marker genes, but there is an increased risk of chimeric gene fragments being formed during PCR (see section Analysis of SSU rRNA and Its encoding gene).

An alternative to bead mill homogenization is soil grinding (35,43,47,54). Frostegård and coworkers (47) suggested that this method is equally efficient as, or even more efficient than, bead mill homogenization. Using soil grinding only, 16 to 59 μg of DNA per gram of soil was obtained from different dried soils, a yield comparable to that obtained with extraction protocols involving bead mill homogenization (depending on the soil this ranges from 1.5 to 53 μg DNA per gram of dry soil (22,39,41,44,48,55).

Soft lysis techniques without mechanical treatment facilitate the retrieval of environmental DNA of high molecular weight (37,56). For example, a combined lysozyme and SDS treatment extracted soil DNA of size 40 to 80 kb (37). DNA of this size is suitable for metagenome analysis (see section Metagenome Analysis). However, DNA obtained by soft lysis techniques might be biased toward easily lysed microbial populations, especially gram-negative microorganisms (43), and thus might not be representative of the entire microbial community.

Purification of Crude Extracts. The extent to which purification is necessary depends upon the characteristics of the soil under investigation, especially its humic acid content. Watson and Blackwell (57) reported that a brown-tinted organic component of soil inhibits PCR and is tightly associated with the soil microorganism fraction. It was found to be distinctly different from humic and fulvic acids in both its solubility properties and in its Fourier transformed infrared and nuclear magnetic resonance spectra. This soluble inhibitor complexes with proteins, and thus the authors postulated that it inhibits PCR through interaction with *Taq* DNA polymerase.

In general, molecular detection of gene sequences from indigenous microbial populations demands a more intensive purification of extracted DNA than does the detection of artificially introduced bacteria (58). DNA extracted from crude cell fractions might also be more contaminated with humic acids than DNA extracted using in situ cell lysis (37). Procedures that have been applied, individually or in combination, to separate impurities from environmental DNA include: (*1*) adsorption of impurities on polyvinylpolypyrrolidone (PVPP) (23,44,59); (*2*) treatment with lysozyme (37); (*3*) cesium chloride density gradient centrifugation (22,24,25); (*4*) Sephadex G-75 (44), Sephadex G-200 (22,48,60,61), and Sepharose 4B (61,62) gel filtration; (*5*) precipitation of DNA with potassium acetate, cesium chloride, and spermine-HCl (39); (*6*) agarose gel electrophoresis (43,48,58,63); (*7*) reversible binding of DNA on silica-based support material (43,48); (*8*) a magnetic capture-hybridization PCR technique (64); and (*9*) affinity matrix purification (46). This last method is a sequential injection fluid system combining affinity purification with novel renewable-surface microcolumn technology. The affinity matrix used for purification is based on the universal SSU rDNA oligonucleotide 1392r (65), which is covalently attached to 60-μm microbeads by a dT_8 linker. Besides DNA oligonucleotide probes, peptide nucleic acid clamps have been assessed for their use in affinity purification of DNA and RNA from soils and sediments (66).

An evaluation of four different DNA purification methods (silica-based DNA binding, agarose gel electrophoresis, ammonium acetate precipitation, and Sephadex G-200 gel filtration) found that Sephadex G-200 gel filtration was the most effective at removal of PCR inhibitors from crude DNA extracts of a silt loam soil (48). However, CsCl/ethidium bromide density gradient ultracentrifugation was better than Sephadex G-200 gel filtration at removing humic acids from crude soil DNA extracts in a separate study (22).

Commercial kits for extraction of nucleic acids from soils also commonly include purification steps. Purified extracts from two commercial kits, the FastDNA Spin kit (Bio101, La Jolla, California) and the UltraClean Soil DNA kit (MoBio Laboratories, Inc., Solana Beach, California) gave similar PCR product yields (indicating similar coextraction of impurities) as soil DNA extracted with an SDS-bead mill homogenization method and purified on Sepharose 4B spin columns (62). However, there were differences in community fingerprints obtained by amplified rDNA restriction analysis (ARDRA) and ribosomal intergenic spacer analysis (RISA) (see section Microbial Community Fingerprinting) of these PCR products, indicating that different biases were inherent at some stage in the three different extraction/purification methods.

In summary, various protocols are available for cell lysis and for purification of crude extracts of soil DNA. Selection of the most appropriate methods depends on the aim of the study and on the characteristics of the soil being investigated, especially its humic acid, organic matter, and clay mineral content. If complete extraction of the gene pool for studies such as PCR-based assessment of microbial diversity is the main criterion, harsh techniques, which extract DNA fragments of up to 25 kb in size, should be applied. These include bead

mill homogenization and soil grinding. In contrast, if the focus of the study is metagenomic analysis, soft lysis techniques, which allow the extraction of large 40- to 100-kb DNA fragments, are more appropriate. Comprehensive manuals are available, which have detailed descriptions of different protocols for the extraction and purification of DNA from bulk soil (37,47,67,68) and from other habitats, such as aquatic systems, the rhizoplane, rhizosphere, and phyllosphere (67).

Extraction of Total RNA

Because RNA is more labile than DNA, more care has to be taken in both extraction and subsequent molecular analyses. All solutions and glassware have to be rendered ribonuclease (RNase)-free by diethyl pyrocarbonate treatment (69), and only certified RNase- and deoxyribonuclease (DNase)-free plasticware should be used. As a consequence, relatively few protocols have been published for extraction of total RNA from soils (32,70–75) or sediments (76).

Protocols have recently been developed for rapid coextraction of rRNA and DNA (32,75,76). Cell lysis is achieved by bead mill homogenization, either of soil samples directly (75,76) or of crude cell extracts obtained by using sodium pyrophosphate to disrupt cell-soil aggregates followed by several washing steps (32). Removal of humic acids and other contaminants from total nucleic acids is achieved using phenol-based extraction buffers in combination with various treatments already described for extraction/purification of total DNA, for example, inclusion of polyvinylpyrrolidone or hexadecyltrimethylammonium bromide in the extraction buffer, phenol/chloroform extraction, and Sephadex G-75 spin column filtration. Separation of rRNA from DNA is achieved either by treatment with RNase-free DNase (32,75) or by Sephadex G-75 spin column filtration using different concentrations of the elution buffer (76).

A protocol described by Felske and coworkers (74) recovers intact rRNA from soils by isolating ribosomes. After mechanical cell lysis, the glass beads, soil particles, and cell debris are removed by centrifugation at low speed (15,000 to 30,000x g), and then the ribosome fraction is pelleted by ultracentrifugation at 100,000x g for 2 hours at 2 °C. The inclusion of PVPP in the extraction buffer to adsorb humic acids was essential for high efficiency of RNA recovery. The RNA yield obtained by the ribosome isolation technique was 0.2 μg of highly purified RNA per gram (dry weight) of soil. Yields from direct RNA extraction methods are clearly higher, ranging between 0.25 and 5.0 μg RNA per gram (dry weight) of soil, depending on the extraction procedure and the soil type (32,75).

FRACTIONATION OF TOTAL DNA BY MOL% G-C CONTENT

Soil DNA extracts can be used directly for molecular community studies. Alternatively, the DNA can first be separated into fractions of different guanine plus cytosine (G + C) content (77). The rationale behind this fractionation is that the G + C content of prokaryotic DNA varies from approximately 24 to 76%, but that particular taxonomic groups vary in G + C content by no more than 3 to 5% (78,79). The G + C profiling percentage provides a coarse level of resolution in cultivation-independent community analysis, which is not achieved by the other available techniques (80). It allows a comprehensive and quantitative analysis of the structure and dynamics of complex microbial communities in soils (80–82) and other environments (29,83,84). However, the method requires a relatively large amount of DNA (50 to 100 μg) and an ultracentrifuge to separate various G + C fractions.

The separation is achieved through the binding of bis-benzimidazole (Hoechst reagent number 33258) to adenine and thymidine. The bound bis-benzimidazole changes the buoyant density of DNA, and this change is proportional to its A + T (hence, G + C) content (77). A gradient of DNA fragments sorted by G + C content is established by equilibrium density-gradient (CsCl) centrifugation, then DNA fractions with different G + C contents are collected and quantified by spectrophotometry. After purification, individual DNA fractions can be further analyzed using molecular ecology techniques. For example, comparative sequence analysis of SSU rRNA genes retrieved from DNA fractionated based on %G + C content allowed the characterization of dominant and rare members of a young Hawaiian soil bacterial community (81). Other molecular techniques that have been applied to G + C fractionated DNA include functional gene probing (80,85), SSU rDNA-based fingerprinting (81), and amplified rDNA restriction analysis (81,82).

ANALYSIS OF SSU rRNA AND ITS ENCODING GENE (rDNA)

Methodological Aspects

Comparative analysis of SSU rRNA sequences has become the central tool for determining phylogenetic relationships or, to put it otherwise, for reconstructing the history of life (86–91). It led to the proposal of the domains Archaea, Bacteria, and Eucarya as the three main divisions of the universal tree of life (92). Many properties of SSU rRNA make it an ideal marker biomolecule for the inference of phylogenetic relationships: (*1*) it is universal to life, (*2*) it is highly conserved structurally and functionally, (*3*) it is, as the central component of the highly complex translation apparatus, one of the most refractory biomolecules, and has probably never (or very infrequently) been transferred horizontally from one species to another. SSU rRNA–based trees therefore depict evolutionary lineages of organisms and not of the gene only, (*4*) it contains sufficient nucleotides to infer phylogenetic relationships, (*5*) different regions of the gene vary at different rates (93,94), thereby allowing the inference of phylogenetic relationships from the domain to the species level. Highly invariant, slowly evolving positions confer valuable information on widely separated groups or organisms, whereas positions of higher variability are useful for the elucidation of more recent branchings (89,94), and (*6*) highly conserved regions present targets for universal primers that allow PCR amplification of almost–full-length SSU rDNA from most members of either the domain Bacteria (65,95–98)

or the domain Archaea (99,100). Primers for amplification of partial SSU rDNA from most members of Bacteria and Archaea together have also been designed (65,95,97).

The ease with which SSU rDNA sequences can be studied has led to a rapid increase in the number of sequences now available in public-domain databases. For example, the SSU rDNA database of the Ribosomal Database Project (RDP) (101) currently contains more than 16,000 aligned prokaryotic SSU rRNA sequences (~75% are longer than 899 bp). Most of these have been retrieved directly from environmental samples during cultivation-independent studies into microbial diversity.

These cultivation-independent studies generally proceed by retrieval of total community DNA, PCR amplification of SSU rDNA, generation of an SSU rDNA clone library, and analysis of the clone library by comparative sequence analysis. PCR is carried out using the universal primer sets noted above, targeting Bacteria (102–106), Archaea (100,107–109), or both domains (110,111). Nowadays the most-widely used system for generation of SSU rDNA clone libraries is the TA cloning kit (Invitrogen Corp., Carlsbad, California). In this kit the linearized cloning vector carries single 3′ thymidine residues to take advantage of a particular characteristic of the *Taq* polymerase, the addition of a single adenosine overhang at each 3′ terminus of the double-stranded PCR products (112). This system avoids the need for endonuclease restriction of the PCR product prior to the ligation reaction, and results in a high cloning efficiency by avoiding the loss of information, which may occur because of restriction. The current state-of-the-art tool for administration and analysis of SSU rDNA sequence data is the ARB software package developed by O. Strunk and W. Ludwig (Technische Universität München, Germany). It is available online at *http://www.mikro.biologie.tu-muenchen.de/pub/ARB/*. The program includes various tools for phylogenetic analysis, including the phylogeny inference package (PHYLIP version 3.2 113) and maximum likelihood treeing methods (fastDNAml (101)). Details on how to construct phylogenetic trees with SSU rDNA sequence data and the various treeing algorithms available are reviewed elsewhere (94,114,115).

Total RNA, instead of DNA, can be extracted from soils, and the SSU rRNA reverse transcribed (116–119). Especially in soil environments, diversity assessment with rDNA is experimentally less problematic than with rRNA. However, organisms detected by retrieval of SSU rDNA may be ecologically unimportant because DNA persists in moribund and dead cells (120) and can be present as extracellular DNA adsorbed to mineral surfaces (24,121,122). In contrast, RNA is highly labile. Ribosome levels, and therefore rRNA levels, have been correlated with cellular activity (21,123–126). Thus, it can be argued that retrieval and analysis of SSU rRNA provides a more representative view of the metabolically active members of microbial communities (116–119).

Assessment of the SSU rDNA diversity in a clone library is usually carried out by partial or full-length sequence analysis of randomly selected clones, or by techniques which rapidly assign clones to operational taxonomic units (OTUs) (127) (the term phylotype is often used as a synonym to OTU (104)). One such technique is ARDRA. In ARDRA, the SSU rDNA clones are digested by hexameric and/or tetrameric restriction endonucleases and the resulting digests are separated into size fragments by agarose gel electrophoresis. Restriction fragment length polymorphisms (RFLPs) are used to assign SSU rDNAs to particular OTUs (80,82,104,128). Representative clones of individual OTUs can then be selected for sequence analysis (82,104). This approach is rapid, efficient, and thoroughly assesses the species richness in SSU rDNA clone libraries. However, a single OTU defined by ARDRA may consist of several diverse SSU rDNA sequences. Dunbar and coworkers (104) reported that partial SSU rDNA sequences (427 nucleotides analyzed), which had been grouped into the same OTU based on identical *Rsa*I-*Bst*UI RFLP patterns, had an average sequence similarity of only 87%. Thus, the screening of clone libraries by ARDRA provides only a broad, preliminary estimation of microbial diversity. McCaig and coworkers (105) grouped SSU rDNA sequences randomly sampled from clone libraries into OTUs defined by a 97% level of sequence similarity. Sequences exhibiting similarities above 97% may or may not correspond to microbial strains of the same genospecies, but sequence types below this similarity value never correspond to strains of the same genospecies (129). The number of unique OTUs retrieved in relation to the total number of randomly sampled sequences (collector curve), provides information on how well the species richness of clone libraries has been assessed (105).

It should be noted that the relative frequencies of defined OTUs in SSU rDNA clone libraries do not provide any quantitative information on community structure. Although the relative abundance of a single OTU relative to others is frequently compared across samples to show community differences in the relative importance of this OTU (see section Microbial fingerprinting techniques), one OTU cannot be directly compared to another because many biases lead to the preferential recovery of some sequences over others. Bias can occur at all methodological levels from environmental sampling to data analysis (130), but are especially severe during extraction of total soil DNA (47,62), during multitemplate PCR (131–136), and because of possible contaminant sequences (137).

Another problem in interpreting environmental SSU rDNA sequences is the formation of chimeric SSU rDNA (hybrids) during multitemplate PCR (136,138–141). Chimeric SSU rDNA sequences result in artificial lineages during treeing analysis, therefore any conclusion that a new microbial lineage has been detected in an environment must always be made with care. It should be checked by separate analysis of the 5′ and 3′ sequence halves (106,108,142), by use of the chimera check program of RDP (101), and by a careful secondary structure analysis.

Phylogenetic Diversity

The first cultivation-independent phylogenetic surveys of microbial diversity were published in 1990, regarding samples collected from hot springs of the Yellowstone National Park (143) and the Sargasso Sea (144).

The first studies on soil were published shortly thereafter, using samples from subtropical Australia (63,145). Since then, surveys of microbial diversity have been conducted on diverse soil environments including: tropical Hawaiian forest and pasture soil (82), mature forest soil and active pasture in Eastern Amazonia (111), arid pinyon pine-juniper woodland soils in Arizona (102,104), grassland soils in Scotland (105,146) and the Netherlands (119,147,148), agricultural soils in Wisconsin (110) and the Netherlands (149), German moorland soils polluted with zinc (150) or polychlorinated biphenyls (117), Italian rice paddy soil (106,118,142,151), and Siberian tundra soil (152).

These cultivation-independent studies have dramatically changed our view of microbial diversity (88,91,107,153,154). They have contributed to the tripling of identifiable bacterial divisions (also called *main lines of bacterial descent* or *phyla*) during the last 12 years (154–156). Only 23 of the 36 to 40 presently recognized bacterial divisions are currently represented by cultured species (154,155). The total number of divisions may in fact be well over 40, judging by various division-level lineages represented by only single or few known sequence types (142,154,157). Major bacterial groups commonly detected by cultivation-independent phylogenetic surveys of soils are: *Holophaga/Acidobacterium* (103,154,158); *Proteobacteria* (82,102,104,105,110,111,117,119,147,150,152,157); gram-positives with low G + C content (104), including clostridial sublineages (106,110,111) and *Bacillus* (102,106,110,111,119,147,152,157); *Actinobacteria* (102,105,116,117); the *Cytophaga/Flexibacter/Bacteroides* group (102,104–106,110,111,150); *Nitrospira* (104,149); *Planctomycetales* (102,110,111,117,152); and *Verrucomicrobia* (104–106,111,157,159).

The abundance and activity of each bacterial division depends strongly on physicochemical soil properties. For instance, the assessment of bacterial community structure along a vertical oxygen gradient in flooded paddy soil microcosms suggested that α- and β-*Proteobacteria* colonized the oxic soil especially, whereas clostridial subgroups predominated in the anoxic soil zone (118). Buckley and Schmidt (159) determined the relative abundance of rRNA from *Verrucomicrobia* in soil microbial communities by quantitative dot blot hybridization using a *Verrucomicrobia*-specific oligonucleotide probe. A total of 85 soil samples were taken to assess rRNA changes with soil depth and moisture content and in relation to plant community composition and soil management history over a period of 2 years. The authors concluded that *Verrucomicrobia* were significantly affected by environmental characteristics that changed in relation to time, soil history, and soil depth. A statistically significant amount of the variation in verrucomicrobial rRNA abundance could be correlated to changes in soil moisture.

A detailed description of the diversity and environmental range of all major bacterial divisions is beyond the scope of this article, so the *Holophaga/Acidobacterium* division will be used as an example. There are presently only three cultured representatives of this main line of bacterial descent: *Acidobacterium capsulatum* (160), *Holophaga foetida* (161), and *Geothrix fermentans* (162). However, members have been detected both by SSU rDNA (82,102–105,111,149,150,157,158) and by SSU rRNA (117,119,147) retrieval from various soil types and geographic regions (Fig. 1). Based on the three cultured representatives and on the many environmentally retrieved SSU rDNA sequences, up to eight monophyletic subdivision-level lineages within the *Holophaga/Acidobacterium* group have been identified. The three cultured organisms provide clues about the physiological versatility within the group: *A. capsulatum* is a moderately acidophilic aerobic heterotroph, whereas *H. foetida* and *G. fermentans* are strict anaerobes that ferment aromatic compounds and acetate, respectively. *Geothrix fermentans* is also able to grow by dissimilatory iron reduction. These diverse phenotypes, and the widespread occurrence of SSU rDNA sequences belonging to the *Holophaga/Acidobacterium* division in diverse environments, suggests that members of this group are active in many soil ecosystems. Besides terrestrial soil (103,154,158), species have been detected in a peat bog (163), acid mine drainage (164), marine samples (165), activated sludge (103), bioreactors (166), a contaminated aquifer (155), and a hot spring (153), as well as in sediments, water column and lake snow aggregates of freshwater lakes (103,167), and on sediment particles sampled from the permanent ice cover of an Antarctic lake (168).

Cultivation-independent molecular phylogenetic surveys have also helped to shape the present perception of diversity in Archaea. Cultivated members of the kingdom Euryarchaeota are physiologically diverse and include halophiles, thermophiles, and methanogens, whereas cultivated members of the kingdom Crenarchaeota are more homogeneous, consisting only of sulfur-dependent extreme thermophiles, such as *Pyrodiction occultum* and *Thermoproteus tenax* (91). Consequently, it was assumed at one time that Crenarchaeota are restricted to extreme high-temperature habitats (92). However, cultivation-independent retrieval of crenarchaeal SSU rDNA sequences has revealed this assumption to be false. Crenarchaota are widely distributed, inhabiting not only high-temperature habitats but also moderate- and low-temperature environments, such as marine waters, terrestrial soils, marine and freshwater sediments, and also living in association with metazoa (169,170). Pelagic crenarchaeota may even be one of the ocean's most abundant cell types (171). Crenarchaeota sequences from low-temperature habitats form several deeply branching clades clearly distinct from the monophyletic group of cultured thermophilic Crenarchaeota. Representatives of these groups have still not been obtained in pure culture.

Terrestrial soil environments in which SSU rDNA of nonthermophilic crenarchaeota have been detected include boreal forest soil in northern Finland (109,172); agricultural soils in Germany (173), Wisconsin (174,175), and Michigan (176); forest soil in Eastern Amazonia (111); and Italian rice paddy soil (177). Crenarchaeal SSU rDNA retrieved from soils form two distinct clades named the *FFSB cluster* and the *Terrestrial cluster* (176). Quantitative dot blot hybridization with an SSU rRNA–targeted

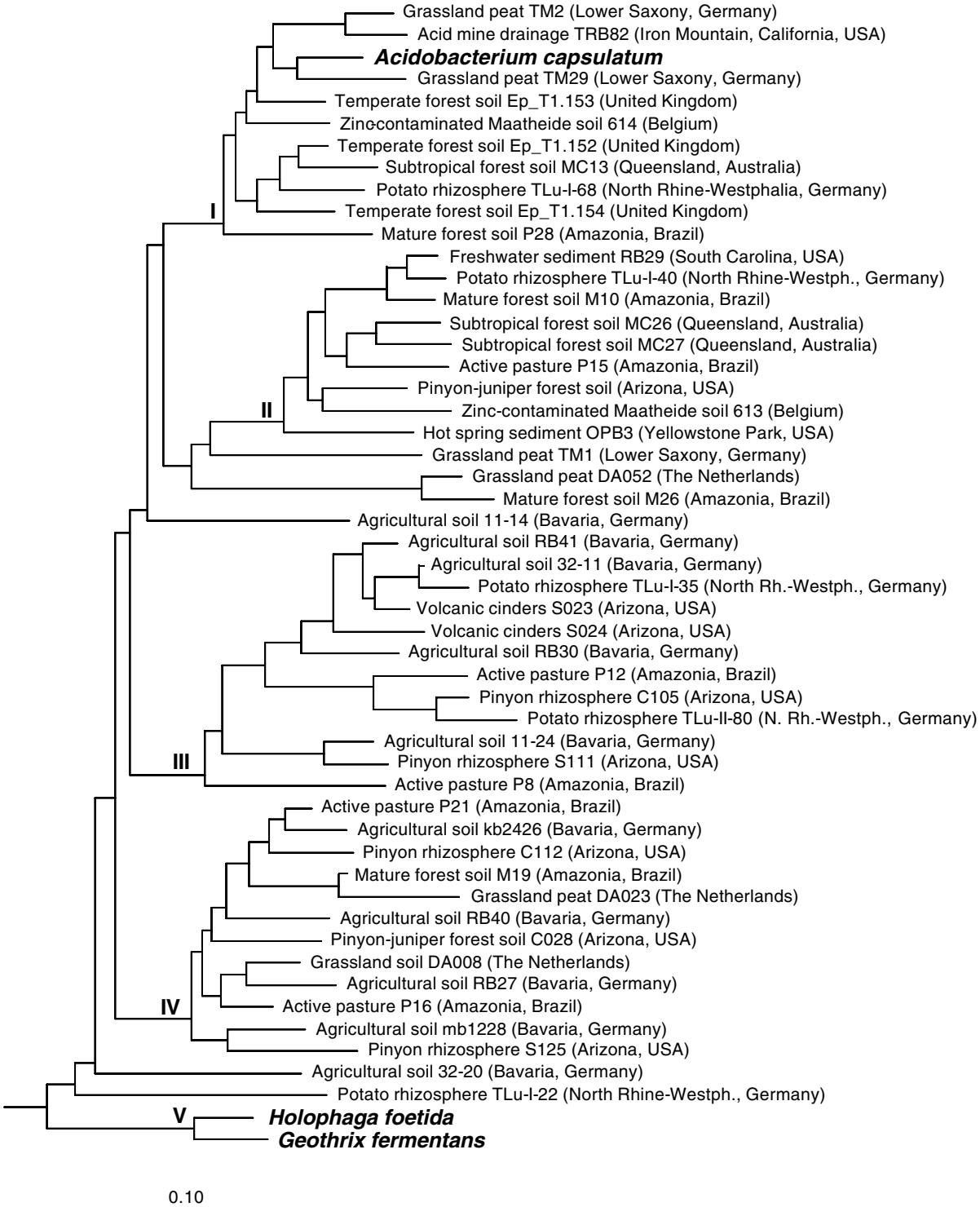

Figure 1. Phylogenetic dendrogram of the *Acidobacterium*/*Holophaga* division. Cultivated organisms are shown in bold. For each environmental sequence the habitat source, clone name, and geographic region from which the sequence was obtained are given. Subdivision-level lineages with bootstrap values of greater than 90% in 1,000 data resamplings are indicated by roman numerals (I to V). The tree was rooted using the SSU rDNA of *Escherichia coli* as the outgroup reference. The scale bar indicates 0.1 change per nucleotide.

oligonucleotide probe specific for all known nonthermophilic crenarchaeotal sequences assigned as much as 1.42±0.42% of the SSU rRNA extracted from agricultural soils to this group (176). FISH analysis with a set of SSU rRNA–targeted oligonucleotide probes specific for Archaea, Crenarchaeota, or nonthermophilic Crenarchaeota showed that Crenarchaeota colonize terrestrial plant roots at an unexpectedly high frequency (175).

Archaeal SSU rDNA directly retrieved from Italian rice paddy soil included, besides sequences from well-known methanogens, various novel lineages of Euryarchaeota (108). One of these lineages, termed *rice cluster I*, formed a distinct clade within the phylogenetic radiation of the orders Methanosarcinales and Methanomicrobiales and thus was considered a novel taxonomic group of methanogens at the family or order level. Members of rice cluster I also occur in close association with rice roots (108,178). A methanogenic phenotype for rice cluster I species was further supported by parallel phylogenies of SSU rDNA and sequences encoding the methanogen-specific gene marker methyl coenzyme M reductase subunit A (179) (see section Analysis of Functional Genes). Kim and coworkers (180) have also detected a novel clade of deeply branched Archaea in total DNA extracted from various Japanese rice paddy soils. This novel group could be assigned neither to the Euryarchaeota nor the Crenarchaeota.

Comparative sequence analysis of environmental SSU rDNA has therefore provided, and will continue to provide, insight into microbial diversity. It does not, however, allow conclusions about the metabolic capacities, phenotypes, or ecological niches of microorganisms. This is especially true for groups from which no representatives have yet been cultured (20,142,169).

ANALYSIS OF FUNCTIONAL GENES

Phenotypes of the organisms from which SSU rDNA sequences are retrieved cannot be known for certain, only inferred from the phenotypes of the closest phylogenetic neighbors that are available for study in pure culture. Consequently, assays have been developed, which target functional genes indicative of biogeochemically important groups. Ideally these genes code for an enzyme that is both universal and specific for the microbial group of interest, so that the presence of the phenotype is inherent from the recovery of the sequence. For example, the *amoA* gene is universal to autotrophic ammonium oxidizers, and possession of an *amoA* gene implies that the organism is an ammonium oxidizer (181,182). The sequence phylogeny of the functional gene also ideally corresponds closely to the SSU rDNA–based phylogeny, that is the selective pressure acting on the gene should be primarily neutral or negative and no interspecies gene transfer should occur.

Some functional genes that have been used as phylogenetic markers are listed in Table 1.

Further promising genes have also been identified, for example the hydroxylamine oxidoreductase gene (*hao*) (226). Many, on the other hand, such as the plasmid genes encoding for 2,4-dichlorophenoxyacetic acid–degrading enzymes (227) and the *nod* genes of rhizobia (228), have been shown through phylogenetic analysis to be very mobile across species, and the retrieval of these sequences from nature would not allow a clear deduction of the bacterial species from which they came. Several of the markers included in Table 1 also suffer to some extent from this drawback, but have nevertheless been applied for general phylogenetic deduction.

Examples of good functional phylogenetic markers are the *pmoA* and *amoA* genes. The phylogenies of these genes are excellent mirrors of SSU rDNA phylogeny, and allow identification to the species level (181–184). The *amoA* gene, originally applied phylogenetically by Rotthauwe and coworkers (181), codes for a subunit of ammonium monooxygenase, the enzyme that essentially defines the group of autotrophic aerobic ammonia oxidizers. Community analysis of *amoA* products amplified from environmental DNA has been performed with a wide

Table 1. Functional Genes Useful as Phylogenetic Markers

Gene	Enzyme	Target Group	Selected References
pmoA	Particulate methane monooxygenase	Methane oxidizers	183–190
mmoX	Soluble methane monooxygenase	Methane oxidizers	191–195
amoA	Ammonia monooxygenase	Ammonia oxidizers	181,182,196–203
mxaF	Methanol dehydrogenase	Methylotrophs	186,204–207
nifH	Dinitrogenase reductase H	Nitrogen-fixers	208–214
mcrA, mcrI	Methyl coenzyme M reductase	Methanogens	179,215,216
rbcL	Form I ribulose bisphosphate carboxylase/oxygenase	Autotrophs (phytoplankton)	217
nirK, nirS	Nitrite reductase	Denitrifiers	218,219
nosZ	Nitrous oxide reductase	Denitrifiers	220–222
narG	Nitrate reductase	Denitrifiers	223
dsrA, dsrB	Dissimilatory sulfite reductase	Sulfate reducers	224,225

combination of methods, including cloning followed by sequencing (181,196–200), denaturing gradient gel electrophoresis (DGGE) separation and hybridization analysis (201), RFLP of cloned sequences (202), and terminal restriction fragment length polymorphism (T-RFLP) (203). The *pmoA* assay targets subunit A of particulate methane monooxygenase, which is functionally and evolutionarily related to the *amoA* gene (183). This assay has been applied to identify methane-oxidizing species in various environments and enrichment cultures (185–188), and the discovery of novel *pmoA* sequences in forest soils has suggested the existence of not-yet cultivated species of methane-oxidizing bacteria (189,190). Unfortunately, there are pitfalls in the use of functional gene markers. The *pmoA* gene, for example, is neither completely universal nor completely specific. Although particulate methane monooxygenase was originally thought to be present in all methane-oxidizing bacteria, a species has recently been isolated, which seems to contain only the alternative, soluble form of the enzyme (229). Furthermore, the *amoA* gene can also be amplified by *pmoA* primers. This is not a problem if the sequences clearly fall within a known group such as the *Nitrosospira*. However, the interpretation of novel or unusual sequences (189,190,200) can be problematic.

MICROBIAL COMMUNITY FINGERPRINTING

Several molecular fingerprinting techniques are available to characterize and compare microbial communities. These analyses begin with a PCR product (either SSU rDNA or a suitable functional gene) obtained from complex community DNA, which is then separated into a pattern of different phylotypes based on some properties of the component DNA sequences. The most commonly used methods are DGGE, temperature gradient gel electrophoresis (TGGE), and T-RFLP.

DGGE is the electrophoretic separation of sequence types in an increasing gradient of chemical denaturant (urea plus formamide). The manner in which double-stranded DNA denatures in the gradient, and hence its electrophoretic mobility, is controlled by the arrangement of strong G–C bonds versus weak A–T bonds. TGGE is a variant in which the denaturation is controlled via a temperature gradient rather than a chemical gradient. The principles and technical aspects of these two methods have been well reviewed (230–232). A similar technique is single-strand conformation polymorphism (SSCP), in which separation is achieved in a polyacrylamide gel based on the different secondary conformations of different single-stranded DNA sequences (233).

Details of the T-RFLP method are covered by recent reviews (234,235). T-RFLP is a simplification of ARDRA, a technique used primarily to identify single DNA phylotypes by their unique patterns of restriction digests in an agarose gel. ARDRA can also give community-specific patterns for mixed PCR products (80,236), but these can be extremely complex for environmental samples and are therefore of limited use in phylogenetic deduction or assessment of species richness. In T-RFLP, one PCR primer is labeled with a fluorescent dye. After restriction digestion of the PCR product followed by gel or capillary electrophoreses, the terminal fluorescent fragments can be detected using a laser (237). In this way T-RFLP analysis detects a single-size fragment (T-RF) for any given sequence type. Typical T-RFLP patterns for SSU rDNA PCR products are shown in Figure 2. The comparison of DNA extracted from BIOLOG plates versus soil again demonstrates the greater complexity obtained with cultivation-independent analysis (238). Other, less-popular, size-based fingerprinting techniques include length heterogeneity PCR (239) and RISA (111,240,241), in which the length variability of SSU rRNAs, or of the intergenic region between the SSU and large subunit

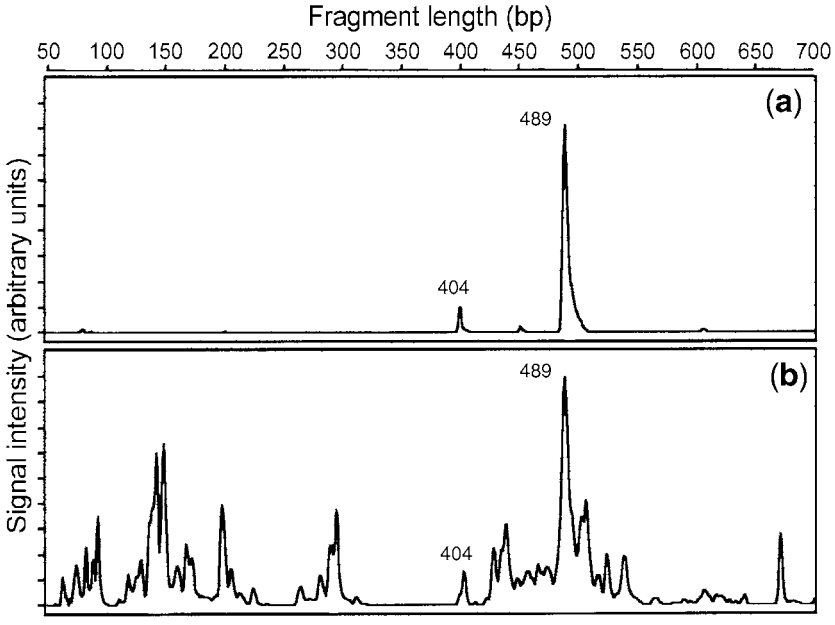

Figure 2. Comparison of SSU rDNA-based T-RFLP community fingerprint patterns obtained from a potato-rhizosphere soil sample after cultivation on a BiOLOG GN microtitre plate (**a**) or obtained by a cultivation-independent approach (**b**). The 404-bp T-RF corresponds to *α-Proteobacteria* and the 489-bp T-RF to *β-* and *γ-Protoebacteria*. The T-RFs indicative of bacterial groups detectable only by the cultivation-independent approach correspond to various major bacterial groups including the *Holophaga/Acidobacterium* cluster, the *Cytophaga/Flexibacter/Bacteroides* division, *Planctomycetales*, and *δ-Proteobacteria*.

rRNA genes, are the respective criteria for the separation of phylotypes.

The simplest analysis of the patterns obtained by community fingerprinting methods is visual: that particular species/phylotypes are detectable or not detectable in a community (190,213,242). This is the primary goal of studies analyzing single samples, and is generally sufficient when comparing few samples (142,202). For example, the disappearance or appearance of species with time has been followed with DGGE (186,243), and changes in bacterial community structure along a vertical oxygen gradient were clearly demonstrated with T-RFLP (118). Presence/absence data can also be used to calculate simple similarity coefficients between community profiles, which can be used in cluster analysis (244). However, better numerical quantification of the patterns facilitates more intensive analysis. Quantification is easiest and most reliable with T-RFLP, where the intensity of the fluorescence signal is used as a measure of the abundance of any product relative to the others, and a threshold signal/noise level can be set to score the presence or absence of a peak (236,245). Quantification in DGGE or TGGE is generally accomplished by image analysis software, and may or may not include a densitometric comparison of band strengths (246–248).

Relative abundances of T-RFs are often compared across communities, to conclude that particular T-RFs comprise a greater or lesser proportion of the total product in one sample compared to another (118,177,219). Of course, caution must be exercised in making conclusions because of well-known PCR biases, as well as the fact that data are relative and therefore all cross-correlated. If sufficient replicate data are available, a multivariate statistical analysis can determine whether the patterns observed significantly differ across samples or treatments (236). This analysis also takes into account the cross-correlation of the various T-RFs and allows the determination of which T-RFs contribute most to the overall variability of the patterns (236).

Phylotype richness–the total number of DGGE bands or T-RFLP peaks–is also often reported (142,235,242,249, 250). Combining this with relative abundance data then allows the calculation of classical ecological diversity indices such as Shannon's index and Simpson's index. This frequently give useful results (251–253), although because gel bands or T-RFs do not represent standard phylogenetic units, the resolution may not be fine enough to distinguish among very similar samples (245). Cluster analysis using matrices of pairwise correlation coefficients or any of a variety of similarity coefficients (244) is a popular and powerful method to determine whether certain classes of communities are distinguishable within a large group of samples (222,235,246–248,250–252,254). Cluster analysis of various marine bacterioplankton communities gave similar results with T-RFLP and DGGE data, although the T-RFLP detected more total phylotypes (255). T-RFLP analysis of a variety of soil samples also gave similar community clustering patterns as those obtained from analysis of SSU rDNA gene clone libraries (245). Different fingerprinting methods therefore seem to give consistent and comparable community profile patterns.

In choosing a fingerprinting method, one must consider the aim of the study. Of the methods described, T-RFLP best lends itself to quantitative analyses, but is least useful for phylogenetic deduction. T-RFs of SSU rDNA can be compared to the RDP to analyze possible phylogenetic affiliation (101). Unfortunately this is often not unequivocal, as many T-RFs are not species- or even genus-specific (256). This problem can be partially solved by comparing T-RFs instead with clone libraries generated from the same sample (142,177), but this procedure may defeat the desired simplicity of T-RFLP analysis. Alternatively, PCR assays specific to particular microbial groups can reduce the total diversity of the T-RFLP profiles. Assays have been developed for the functional genes *nifH* (212), *amoA* (203), *nirS* (250), and *mcrA* (179). Group-specific SSU rDNA primers may also be applied. Nevertheless, a major advantage of DGGE/TGGE and SSCP is still that individual bands can be excised from the gel and sequenced for direct identification. A problem with DGGE/TGGE is that they are often too sensitive for the determination of total community fingerprints, since nearly identical sequences can give separate bands. The situation is especially problematic for SSCP, where a single sequence can have multiple secondary conformations. The nonspecificity of T-RFs might therefore be considered an advantage when dealing with complex communities, since the overall redundancy of the fingerprint is decreased and broader OTUs are detected.

ANALYSIS OF MICROBIAL ACTIVITY AT THE FUNCTIONAL LEVEL

PCR-based retrieval of functional genes gives insight into the species composition of microbial communities, and their potential to carry out certain functions, but it does not provide information on in situ activity. This gap can be bridged by investigating gene expression at the transcription level. The hybridization of poly-A mRNAs to oligo(dT) probes allows direct extraction and purification of eukaryotic mRNA from complex matrices and has been performed for soils (257–259). However, bacterial transcripts lack polyA tails and are not detectable with these methods. In addition to the general methodological problems involved in the extraction of total RNA from soils (as mentioned earlier), the transient character of prokaryotic mRNA transcripts and their low cellular abundance relative to rRNA (260) account for the paucity of papers reporting the detection of prokaryotic mRNA transcripts from soils (72,73,260–264) and aquifer sediment (265).

Because of the present interest in biotechnology, most of these studies have focused on the detection of mRNA transcripts for genes coding biodegradation enzymes. They have included: (*1*) the detection of mRNA transcripts for naphthalene dioxygenase and mercury resistance genes by Northern blot hybridization (73), (*2*) quantification of mRNA transcripts of naphthalene

dioxygenase (261,262) and soluble methane monooxygenase (265) using an RNase protection assay, and (*3*) reverse transcriptase-PCR amplification of mRNA transcripts for β-glucuronidase (264). These methods of mRNA analysis require a priori information about the gene sequences of interest to design specific DNA probes (hybridization assays) or primers for mRNA detection and measurement. In this way they are simply an extension of the previously described techniques for detection and characterization of functional and catabolic DNA sequences (263).

Fleming and coworkers (260) combined the differential display (DD) technique (266,267) and the RNA arbitrarily primed PCR method (268,269) to detect cryptic or unknown RNA sequences transcribed under in situ conditions in soils. An arbitrary primer for the reverse transcriptase step and the same arbitrary primer in conjunction with a Shine-Dalgarno (SD) primer in the PCR step were used to monitor differential bacterial mRNA transcription between control and toluene-contaminated soil microcosms. The SD primer was used to amplify, by PCR, a wide range of prokaryotic mRNA sequences. The use of this arbitrary primer in combination with the SD primer for DD resulted in highly reproducible RNA fingerprints. However, one major shortcoming of the method was a high percentage of false positive bands. Of the putative clones screened, only one in 12 was confirmed to be unique and differentially expressed in the toluene-induced soil microcosms. Another limitation of this technique is the short lifetime of mRNA. Half-lives of mRNA molecules can be as long as 50 minutes, but typically range between 0.5 and 2 minutes. Thus, a large fraction of the prokaryotic mRNA pool may not be accessible for study, even after induction. Nevertheless, the DD technique has great potential for studying gene expression in soil environments, and potentially allows the detection of novel and differentially expressed gene sequences.

A promising alternative to mRNA-based techniques for analysis of microbial communities is the use of biomarkers, such as bromodeoxyuridine (BrdU) (270,271), or substrates labeled with stable isotopes (206), which are incorporated in the genomic DNA of active microbial populations only (206,240,270). Labeled DNA can be isolated from the target group of microorganisms and then characterized taxonomically by gene probing and sequence analysis.

Culture-independent identification of soil microorganisms that grow in response to specific stimuli can be performed using BrdU (270). After incubation of a soil with BrdU, total DNA is extracted and the newly synthesized DNA isolated by immunocapture of the BrdU-labeled DNA. This approach was used to measure bacterial functional redundancy in soils along a reclamation-revegetation gradient (240). Samples were taken on a transect from a mine site to a soil under a new-growth forest, and from an adjacent pristine forest. The diversity of bacterial groups responding to the substrates L-serine, L-threonine, sodium citrate, and α-lactose hydrate were determined by incubating soils with BrdU plus the various substrates, and then performing RISA of the BrdU-labeled DNA fractions. The major conclusion of this study was that bacterial functional redundancy increases with regrowth of plant communities.

Stable isotope probing takes this analysis a step further and directly links genomic DNA with the metabolism of defined microbial groups. With the BrdU method the soil can be manipulated to favor the growth of different bacterial groups (e.g., particular substrates can be added) and the actively growing groups identified, but with the stable isotope method the incorporation of a substrate can be followed directly. This technique is based on the capability of ^{13}C-DNA, produced during growth on a ^{13}C-enriched carbon source, to be resolved from ^{12}C-DNA by density-gradient centrifugation. Using this technique, methanol-utilizing microorganisms were investigated through the addition of $^{13}CH_3OH$ to a forest soil (206). Phylogenetic analysis of SSU rRNA genes recovered from the purified ^{13}C-labeled DNA fraction identified CH_3OH-utilizing bacteria from two distinct lineages, the α-subclass of the *Proteobacteria* and the *Acidobacterium* division. The conclusion that active methylotrophs belong to the α-subclass of *Proteobacteria* was also supported by a parallel analysis of *mxaF* sequences (see Table 1).

The BrdU and stable isotope probing techniques each provide cultivation-independent means of investigating the effect of environmental conditions, such as temperature, water potential, pH, and substrate availability on the identity of active microorganisms, and thereby allow analysis of functionally relevant ecological diversity. However, both techniques have methodological shortcomings that necessitate care in their application and in the interpretation of the data obtained. A major limitation of the BrdU strategy is the range of organisms capable of BrdU uptake and incorporation. In one study, only two of four different bacterial strains tested incorporated BrdU (271). One of these belonged to the α-*Proteobacteria* and the other to the γ-*Proteobacteria*, whereas failing to assimilate BrdU were a flavobacterium and a gram-positive bacterium. Stable isotope probing also has limitations that may render it unsuitable in certain situations. For instance, simultaneous growth of target microorganisms on an unlabeled (^{12}C) substrate will dilute the proportion of ^{13}C that is incorporated into their DNA. This will reduce the proportion of DNA that becomes isotopically labeled, making it more difficult to identify the microorganisms that are the primary users of a labeled substrate (206).

Other innovative cultivation-independent methods have been developed for linking the functional activities of microorganisms with their taxonomic identity. These include the combination of FISH with microautoradiography (272,273) and the coupling of radio- or stable-isotope analyses with characterization of microbial phospholipid ester-linked fatty acids (PLFAs) (189,274,275). For example, bacteria oxidizing atmospheric methane in soil samples from Greenland, Denmark, the United States, and Brazil were characterized by incubation of the soils under $^{14}CH_4$ and subsequent analysis of the radioactively labeled PLFAs (189,275), and in parallel by analysis of methane monooxygenase gene libraries (189). The ^{14}C-PLFA fingerprints of the soil methanotrophs were clearly different

from the PFLA profiles of the type I γ-proteobacterial methanotrophic bacteria, but similar to PLFA profiles of the α-proteobacterial type II methanotrophs. These ^{14}C-PLFA fingerprint results corresponded well to the results of monooxygenase sequence analyses. Phylogenetic treeing analysis identified a novel cluster of *pmoA* sequences possibly belonging to an unknown group of methane-oxidizing bacteria. These sequences were more closely related to those of type II α-*Proteobacteria* methanotrophs than to those of the type I γ-*Proteobacteria*.

METAGENOME ANALYSIS

Cultivation-independent surveys of microbial communities based on single gene phylogenies provide information on the diversity of microorganisms at the phylogenetic or functional level, but allow little inference about the detailed physiology or biochemistry of these organisms. Recent improvements in molecular analytical techniques and bioinformation sciences have provided methods suitable for the analysis of the metagenome–the total gene pool of a complex microbial community (276). Metagenomic analysis begins with the cloning of large fragments of DNA extracted directly from the microbial community. Analysis of these cloned fragments can provide a link between phylogenetic and functional information and allow for the characterization of total gene operons isolated directly from an environment. Pioneered by DeLong and coworkers, this new approach has been applied to characterize genome fragments of uncultivated nonthermophilic marine crenarchaeota (277), including *Cenarchaeum symbiosum* (278,279), a psychrophilic archaeon that lives in specific association with the marine sponge *Axinella mexicana* (280).

More recently, bacterial artificial chromosome libraries (BACs) from soil metagenomes have been constructed (56). BACs are modified plasmids that contain an origin of replication derived from the *E. coli* F factor. The replication of the BAC vector is strictly controlled, keeping the replicon at one or two copies per cell. BAC vectors can stably maintain inserts as large as 600 kb (281). It has also been suggested that BAC vectors display a low level of chimerism as a result of two genes carried on the plasmid (*parA* and *parB*) (282).

A critical step of soil metagenome analysis is the extraction of high molecular weight DNA suitable for the construction of BAC libraries (see section Extraction of Total Nucleic Acids from Soil). The total fragment mixture of DNA extracted from an agricultural soil was cloned into BAC library SL1, which had clone inserts ranging in size from 10 to 60 kb, but mostly between 20 and 30 kb (56). DNA fragments greater than 40 kb were then selected from the same extract by preparative gel electrophoresis prior to construction of BAC library SL2. This SL2 library had clone inserts ranging between 10 and 80 kb in size. The average insert size was 44.5 kb, and more than 60% of the inserts were larger than 40 kb (56).

A phylogenetic survey of BAC library SL1 identified seven genome fragments containing SSU rRNA gene sequences. These belonged to four different bacterial phyla: the *Proteobacteria*, low G + C gram-positives, the *Cytophaga/Flexibacter/Bacteroides* group, and the *Holophaga/Acidobacterium* cluster. Members of these phyla are typical inhabitants of agricultural soil. Complete sequence analysis of one BAC clone insert identified various open reading frames, including a gene cluster similar to the phosphate transport cluster (*pstCAB-phoU*) of *E. coli* (283). This finding demonstrated the potential of metagenomic DNA analysis to characterize complete, intact operons.

Screening the two BAC libraries identified clones expressing DNase, antibacterial compounds, lipase, and amylase of metagenomic DNA. This demonstrated that BAC libraries contain heterologous DNA sequences that can be expressed in *E. coli* at detectable levels. The analysis of metagenomic BAC libraries has the potential not only to provide insight into the genomic potential and ecological role of soil microorganisms–cultivated or not–but also to provide enzymes with biotechnological applications. This was demonstrated by comparative sequence analysis and expression in *E. coli* of biotin biosynthesis operons recovered from various soil enrichment cultures by direct cloning of total DNA (284).

FINAL COMMENTS

This article has attempted to provide an overview of the current cultivation-independent state-of-the-art methodological toolbox used to describe phylogenetic and functional diversity, structural composition and dynamics, and metabolic activities of soil microbial communities. New methods developed during the last few years or currently under development might soon be added to those described here. One candidate is quantitative PCR, which can be used to determine the exact number of functional or phylogenetic target genes in a sample (e.g., the TaqMan technique (285)). However, development of a bias-free application of quantitative PCR to environmental samples is still not complete (286,287). Another technique with great potential is the hybridization of fluorescently labeled total RNA to microarrays of immobilized rRNA-targeted oligonucleotide probes. This technique may soon allow a rapid and detailed assessment of the diversity and activity of microbial communities (288,289).

Various aspects of soil microbial diversity have been covered by other reviews, which we recommend because they nicely complement the present article. These reviews have focused on the exploration of bacterial diversity and functioning in relation to bioindication in agroecosystems (290,291) and on the impact of chemical pollutants on soil microbial diversity (290,292,293). Finally, an argument in favor of adopting a natural species concept for prokaryotes (294), that is, one that takes the ecological niche into consideration, is also recommended for further reading.

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BIOEMULSIFIERS. See BIOSURFACTANTS: TYPES, SCREENING METHODS, AND APPLICATIONS

BIOFILM DETACHMENT

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A biofilm consists of microorganisms attached to a solid surface called the substratum (1–7). Usually, the microbial cells are embedded in a matrix of organic polymers produced by the cells. Biofilms are ubiquitous in nature and are increasingly important in engineered processes. Some biofilms are viewed as "good," and we try to promote their accumulation. Examples of good biofilms include those that are exploited in fixed-film processes used to treat contaminated water, wastewater, and air (4,5,8), those that attach to stream beds and aquatic vegetation, leading to self-purification of water bodies (9), and those responsible for engineered or intrinsic bioremediation of contaminated groundwater (5,8,10). Other biofilms are viewed as "bad," and we try to remove or prevent them. Bad biofilms include those that foul ship hulls and pipelines, thereby increasing friction loss and corrosion, those that cause "souring" of oil wells, and those that cause medical problems, such as dental caries and infection related to implants (1,2,6,7).

Biofilms are highly diverse (1,3,4,11). Some are physically very thin, consisting only of small clusters of cells on a substratum. Other biofilms are quite thick, having a depth of as much as a few millimeters. While the physical structure of some biofilms is dense and homogenous, other biofilms are physically heterogeneous and have clusters or streamers of biomass intermingled with open channels (11). While single-species biofilms are useful for research studies, naturally occurring biofilms often show

a high degree of ecological diversity that can exhibit fascinating layering or sub-clustering.

One of the reasons that biofilms are so diverse is that many processes occur together as the biofilm forms (2,12): for example, microbial growth and death, attachment, and detachment. Furthermore, biofilms are characterized by strong gradients (2,5,8). Thus, the processes occur in very different ways at the outer surface of the biofilm, compared to near the substratum.

The subject of this article is biofilm detachment, which is the physical movement of microbial cells from the biofilm matrix. Detachment results in a loss of material from the biofilm, and these materials generally move to the liquid that is in contact with the biofilm. The article begins by describing the ways in which detachment can occur. Then, it discusses why detachment is such an important process for understanding and controlling biofilms. Finally, the article summarizes what is known about how forces acting on the biofilm and physiology of the microorganisms in the biofilm affect detachment rates.

Whether the goal is to encourage or discourage biofilm accumulation, detachment is one of the key determinants for how much biofilm accumulates, as well as its physical and microbiological characteristics. Biofilm detachment is an emerging research field. Although some broad concepts are coming into view, most of what needs to be known about what affects detachment rates will be discovered in the future. This article provides a structure for comprehending how physical forces and physiology interact to control biofilm determinant.

DETACHMENT PATTERNS

Detachment can occur with three broad patterns. The first pattern is erosion, which is a continuous process by which relatively small pieces of biofilm are removed from the biofilm's outer surface (2,8,12,13). Each erosion event has a small impact on the biofilm, but, because the events occur continuously, erosion can be a significant loss mechanism.

The second pattern, sloughing, is an abrupt, intermittent loss of a large segment of the biofilm (2,8,12,13). The sloughed segment extends deep into the biofilm, often all the way to the substratum. One sloughing event can cause a profound change to the physical and microbiological structure of the biofilm. Sloughing is associated with a breakdown of the structural integrity of the biofilm, allowing a "fault" to occur along lines of structural weakness. Figure 1 illustrates the difference between the erosion and sloughing mechanisms of detachment.

The third pattern can be termed scouring, and it involves removal of large segments of biofilm through the action of very strong physical forces, such as abrasion or scraping. Predation (sometimes called grazing) also removes biofilm mass, but we do not include it among the detachment mechanisms reviewed in this article.

IMPORTANCE OF DETACHMENT AND ITS PATTERN

Biofilm Accumulation

The most obvious impact of detachment is to control the total amount of biofilm present. The total mass of biofilm per unit of substratum surface area is called biofilm accumulation, and it can be represented mathematically by $X_f L_f$, in which X_f is the biomass density of the biofilm (mg biomass/cm^3) and L_f is the biofilm thickness (cm) (8,14). The accumulation results from a balance among gain by synthesis from substrate (food) utilization, loss by decay (or death), and loss by detachment. For a steady-state biofilm, that balance leads to (8,14)

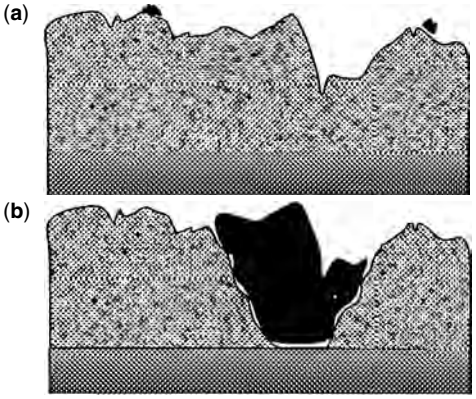

Figure 1. Schematic of two detachment mechanisms: (**a**) erosion and (**b**) sloughing.

$$X_f L_f = \frac{YJ}{b + b_{det}} \tag{1}$$

in which Y = true yield of biomass synthesized per substrate utilized (mg biomass/mg substrate), J = flux of substrate into the biofilm (mg substrate/cm^2 – d), b = specific decay rate (d^{-1}), and b_{det} = specific detachment rate (d^{-1}).

Equation 1 makes it clear that the amount of biofilm present on the surface ($X_f L_f$) increases if more food is available (making J larger) or if the detachment rate (b_{det}) is smaller. For situations in which biofilm accumulation is to be encouraged (say, to treat wastewater), b_{det} may have to be decreased. On the other hand, when biofilm accumulation is to be decreased (say, to minimize fouling or infection), it may be preferred to increase b_{det}.

In principle, biofilms are totally eliminated if the substrate concentration is so low that the positive growth rate from synthesis is always less than the sum of the loss rates from decay and detachment. This threshold concentration is called S_{min}, and it can be computed from (8,14):

$$S_{min} = K \frac{b + b_{det}}{Yq_{max} - (b + b_{det})} \tag{2}$$

in which K = the Monod half-maximum-rate concentration (mg substrate/cm^3), and q_{max} = the maximum specific rate of substrate utilization (mg substrate/mg biomass – d). The major trend is that increasing b_{det} makes S_{min} larger, thereby making it more difficult to sustain a biofilm.

Average Specific Growth Rate and Activity

Communities of slowly growing microorganisms accumulate a significant amount of inactive or inert solids because

low substrate concentration and endogenous decay lead to a residue of "dead" or "dormant" biomass (8). The inert solids also include extracellular polymeric substances (EPS) that hold the biofilm together and tend to accumulate most near the substratum. Therefore, slowly growing communities are less "active" than are fast-growing communities in which less inert biomass accumulates.

For steady-state biofilms, the average specific growth rate (μ_{ave}, d^{-1}) is equal to the specific detachment rate (8), or $\mu_{ave} = b_{det}$. Thus, the metabolic activity of a biofilm is controlled strongly by the detachment rate (8,15–21). Low detachment rates lead to biofilms having a low average specific growth rate and being enriched in inactive biomass.

Ecological Selection

Detachment rates and patterns affect ecological selection in profound ways. In multispecies biofilms, the most slowly growing species tend to accumulate deep inside the biofilm when erosion is the detachment pattern (15–21). Being deep inside the biofilm protects them from detachment losses that occur from the outer surface. Protection effectively lowers b_{det} for these slow growers, which increases their $X_f L_f$ (Eq. 1) and decreases their S_{min} (Eq. 2) (11).

Detachment by erosion leads to a layering of microbial types. The fastest growing species predominate near the outer surface, where they have the advantage of the best access to their substrate. On the other hand, the slowest growers predominate well away from the outer surface, where they have the advantage of protection from erosion. Examples of this sort of layering include heterotrophs protecting nitrifiers in aerobic biofilms (15,9) and sulfate-reducers protecting methanogens in anaerobic biofilms. The slowest growing "species" is the inert biomass produced as part of endogenous decay or dormancy; it predominates at the substratum (22).

In the short term, an increased rate of erosion removes the fastest growing, most active biomass, which resides near the outer surface. However, more erosion of active bacteria from the outer surface allows the substrate to penetrate deeper into the biofilm. This may activate dormant bacteria near the substratum, and it causes the active bacteria near the substratum to grow faster, which gradually pushes the inert biomass to the outer surface, where it erodes (15). Therefore, the long-term effect of erosion is most profound on the slowest growing species. Although it requires some time for the full effects to be realized, an increase to the specific erosion rate lowers the accumulation of slow growers (including the inert of biomass) most dramatically (15).

When detachment is by sloughing or scouring, the layering effects associated with erosion may be reduced or eliminated. Detachment that extends down to the substratum removes all types of biomass. These detachment patterns can be quite devastating to slow-growing species (such as nitrifiers), whose survival in the biofilm depends on protection from detachment.

Suspended Biomass Concentration

When biofilms dominate the biomass accumulation in a system, the concentration of suspended microorganisms is controlled by the rate of detachment from the biofilm. This is expressed mathematically as

$$\Delta X = b_{det} X_f L_f A / Q \tag{3}$$

in which ΔX = the steady state increase in suspended-biomass concentration caused by detachment (mg biomass/cm^3), A = the surface area of the biofilm (cm^2), and Q = the volumetric flow rate (cm^3/d). Equation (3) makes it clear that increasing b_{det} or A/Q makes biofilm detachment a more dominant influence on the concentration of suspended microorganisms.

Substratum Properties

As detachment decreases and $X_f L_f$ becomes larger, the physical and chemical characteristics of the substratum can be altered. Examples of changes to substrata are

- increased diameters and decreased density of substratum particles, such as sand and activated carbon (20,23–25);
- decreased flow-path sizes in pipes and channels;
- increased friction losses in pipes, channels, and porous media (26,27);
- decreased or increased surface roughness (2,27,28);
- changes to surface charge or hydrophobicity; and
- increased heat-transfer resistance (2,7).

FACTORS AFFECTING DETACHMENT RATES

What controls biofilm detachment is far from well understood today. Detachment is an active research area that generates conflicting points of view. Three characteristics of the field lead to today's tumultuous state.

1. Biofilms are incredibly diverse in terms of substrata, hydrodynamics, microbial ecology, and substrate loading. The controlling mechanisms surely differ widely from one system to another. Thus, one common "detachment theory" should not be expected.
2. Experimental methods to measure the biofilm properties relevant to detachment are primitive.
3. Theoretical models for linking observed detachment rates to biofilm properties are crude. So far, these connections rely mostly on statistical correlations, rather than mechanistic principles.

Despite much uncertainty, detachment is not totally mysterious. Certain factors that affect detachment rates are emerging, and they are the subjects of this section. The factors can be grouped into two categories: physical forces acting on the biofilm and the physiology of the microorganisms in the biofilm.

Physical Forces

The first factor recognized to affect the detachment rate is the tangential shear stress (29), τ (dyne/cm^2). Shear stress is proportional to the friction losses for water passing by the substratum, and it acts at the biofilm's outer surface. In general, a greater shear stress increases the detachment rate, although the effect is not necessarily linear. Bakke and coworkers (30) and Peyton and Characklis (31) found an approximately linear relationship between b_{det} and τ, although there was considerable variation. Rittmann (32) found that b_{det} was proportional to $\tau^{0.58}$ for thin biofilms ($L_f \leq 30$ μm), and Chang and Rittmann (28) found that this relationship was quantitatively accurate for biofilms on smooth surfaces. When the biofilm was thicker than 30 μm, the specific detachment rate declined, presumably because the biomass deep in the biofilm was protected from erosion (29). When the biofilm was grown on a highly irregular surface, b_{det} was essentially zero until the biofilm accumulated enough to fill the crevices that protected it from shear stress (28). Once the biofilm fully covered the surface, the detachment rate was similar to that of smooth surface (28).

The several studies that examined shear stress show that it is an important factor for determining b_{det} as long as the biofilm is actually exposed to the shear stress and other factors do not overwhelm it. Protection of all (28) or part (29) of the biofilm from the shear stress can result in substantially reduced detachment rates. Likewise, the effects of shear stress can be "swamped" by other factors. For example, Chang and coworkers (32) saw that b_{det} was inversely correlated to τ for fluidized-bed biofilm reactors. This correlation was the result of other factors (discussed in the following section) controlling b_{det}, $X_f L_f$, and τ.

The second factor that affects the detachment rate is the axial force acting on a biofilm. This force could be realized as tensile or compressive stress, σ (dyne/cm^2), which acts axially on the biofilm. Such stresses can occur through two main mechanisms: abrasion, caused by collisions with other particles, and pressure fluctuations, caused by turbulent eddies (Fig. 2).

Abrasion effects were studied by varying the carrier concentration (C_p, g/l) in a fluidized-bed biofilm reactor (32). Using C_p as a measure of the frequency of particle-to-particle contacts, Chang and coworkers (32) showed that b_{det} was strongly correlated to the abrasion intensity.

Turbulent effects can be gauged by the Reynolds number, $\mathrm{Re} = \upsilon\, L/\nu$, in which υ = the liquid velocity (cm^2/s), L = a characteristic size dimension, such as carrier diameter or pipe diameter (cm), and ν = the liquid's dynamic viscosity (cm^2/s). In the same fluidized-bed studies (32), b_{det} also correlated strongly with Re. Two other studies with fluidized beds confirmed a positive correlation between b_{det} and Re (24,25).

Turbulence effects can be greatly increased by adding energy inputs, such as from mechanical mixing or aeration. When fluidized-bed biofilm reactors were aerated to create a three-phase operation (20,23), the detachment rate increased significantly and was controlled primarily by the gas velocity, although C_p and Re were still significant.

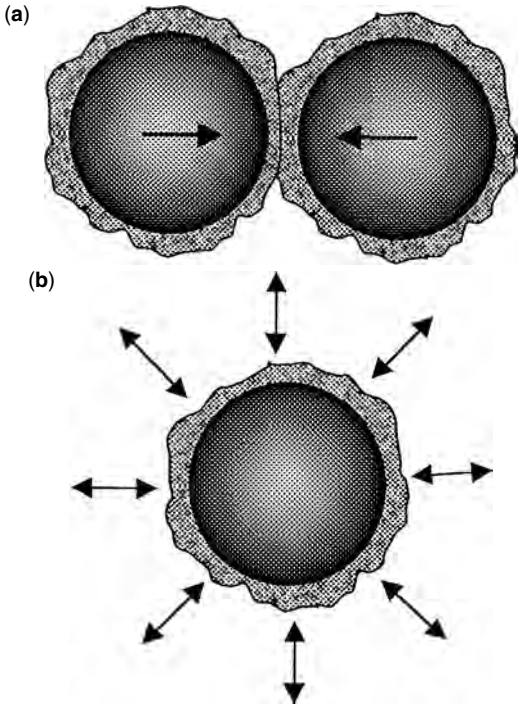

Figure 2. Graphic representation of the two main mechanisms through which axial stresses can occur on biofilm: (**a**) abrasion, shown as compressive forces acting on biofilm caused by the collision of two bioparticles, and (**b**) pressure fluctuations (represented by arrows) on a bioparticle caused by turbulent eddies.

The studies in which abrasion and/or turbulence created strong axial stress show that axial stress can overwhelm shear stress if the axial stress is large enough. For example, the specific detachment rate increased up to 25-fold over what was expected from shear stress alone in the two-phase fluidized bed (8,32). Aeration increased b_{det} by approximately another factor of 2 (8,23). Although the effect of abrasion can be profound, we have a weak basis for quantifying it or its impact on b_{det}.

Detachment is a structural failure of the biofilm matrix, which means that the stress at the point of failure exceeds the yield stress, σ_y. When the actual stress in the biofilm exceeds σ_y, the biofilm breaks. Several groups have measured yield stresses by different methods. Ohashi and Harada (33) applied tensile stress and found σ_y in the range of 0 to 1,750 N/m^2.

Two models have been proposed to explain the way in which the biofilm matrix behaves as the shear stress approaches σ_y. One model (33) views the biofilm matrix as a Bingham fluid (34), a useful concept in polymer science. A Bingham fluid exhibits non-Newtonian behavior. For stresses less than the Bingham yield stress, τ_0, it does not deform. When the stress reaches τ_0, the Bingham fluid flows with a viscosity that depends on τ_0.

The second model (35) views the biofilm matrix as a viscoelastic structure, which can be characterized by an elastic (Young's) modulus (E) and a shear modulus (G). E is the ratio of the applied axial stress to the strain (ε) caused by extension of the material (Fig. 3). G is the ratio of the

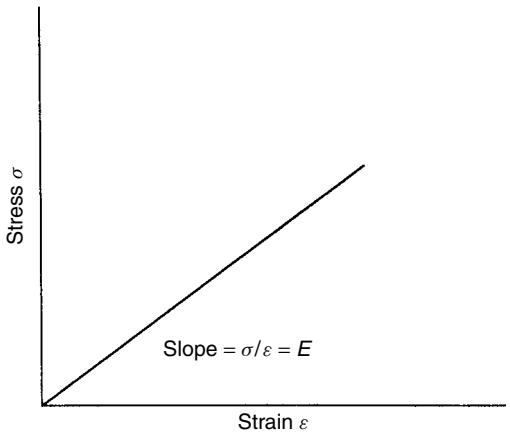

Figure 3. The stess-strain curve of a material, which defines its Young's modulus E.

applied shear stress to the shear strain caused by bending the material. Strain is the ratio of the distortion distance (ΔL) to the total length of the material (L), whereas shear strain is the gradient of deformation under shear. In an ideally elastic solid, strain is directly proportional to stress, and E or G is constant. If the stress is released, the solid returns to its original shape. Stoodley and coworkers (35) performed in situ deformation studies that gave average E and G values of 40 and 27 N/m^2, respectively.

Biofilms can also exhibit plastic behavior, which is a deviation from ideal elastic behavior. Plastic behavior can be viewed as the material "flowing" like a liquid that has a very large dynamic viscosity. Creep, an example of plastic behavior in biofilms (35), refers to the continued deformation of the material over time, although the applied stress is constant. On release of the stress, the plastic material that has undergone creep does not return to its original shape, but remains deformed. Creep probably occurs because intermolecular bonds between polymers in the biofilm matrix "unzip" gradually, allowing the molecules to slip past each other in viscous flow. When the stress is removed, the polymer strands contract and reaggregate with neighboring strands in a new position. Flemming and Wingender (36) have termed this sort of breaking and reforming of bonds "fluctuating adhesion points" and attributed it to the large number and widely ranging type of bonds formed by EPS. The formation of a new EPS may stabilize the new position further.

Physiology

The physiology of the microorganisms in the biofilm appears to affect the strength of the biofilm material and, therefore, detachment rate and pattern. Five physiological factors are reviewed: EPS, density, heterogeneity, specific growth rate, and quorum sensing.

EPS. The cells in a biofilm use EPS to adhere to each other and the substratum (37). The acronym EPS is sometimes used to mean extracellular polysaccharides, but this usage does not accurately represent the composition of the polymers. Although sugars are important in the EPS, proteins and nucleic acids are also key and may be more important in some cases (38,39). EPS formation is important in biofilm detachment because EPS forms the matrix that gives the biofilm structural integrity and its viscoelastic properties. It seems that EPS production depends on the substrate-consumption rate (40,41). Microorganisms seem to produce less EPS when rapidly consuming substrate. As the substrate concentration is higher near the outer surface of the biofilm, the EPS content may be lower there. As EPS is critical for cohesive strength, the outer part of the biofilm may be structurally weaker (e.g., a lower σ_y), and this may help explain how erosion comes about.

Biofilm Density. The density of the biofilm seems to play a crucial role in biofilm strength and detachment rate. In general, denser biofilms are stronger (e.g., a higher σ_y) and have smaller specific detachment rates (b_{det}). Ohashi and coworkers (42) found that the EPS content of the biofilm was directly proportional to the density. Kwok and coworkers (24), Chang and coworkers (32), and Nicolella and coworkers (25) found that thinner, denser biofilms had smaller b_{det} values. Stoodley and coworkers (35) found that E increased for biofilms exposed to increased shear stress.

Differences in biofilm density are often explained by assuming the presence of different types of microorganisms. However, evidence is strong that hydrodynamic conditions have a direct effect on biofilm density. Vieira and coworkers (43) showed that the density of a pure-culture biofilm increased with increasing shear stress on the biofilm. Chang and coworkers (32) and Kwok and coworkers (24) found that biofilms in fluidized-bed biofilm reactors became denser when the particle concentration (C_p), a surrogate for abrasion forces, was larger.

The evidence on the effects of shear stress and abrasion suggests that biofilms exposed to detachment forces adapt to become denser and stronger. How this adaptation occurs is not known. One hypothesis is that strong detachment forces remove low-density new growth from the outer surface (24). The continued removal of this less dense layer enables the substrate to diffuse into the inner biofilm layers, where microcolonies grow within the strong EPS matrix. A second hypothesis is that the microorganisms produce more and/or stronger EPS in response to the detachment force. This could occur by selecting for microorganisms that produce strong EPS (in multispecies biofilms) or by a regulatory response. A third hypothesis is that a denser biofilm is the result of a gradual displacement of loosely bound water out of the matrix.

Another factor that helps explain the differences in density across a biofilm is the buildup of inert solids, such as inactive biomass. Long-term operation with a long solid's retention time (i.e., a small b_{det}) allows inactive biomass solids to accumulate, particularly near the substratum. Mature biofilms, therefore, have a relatively large fraction of inactive volatile solids (20,23,42). Although inactive biomass cannot produce EPS, it may be well embedded in existing EPS, possible allowing a closer packing arrangement if loosely bound water can be squeezed out more easily. In fact, the EPS is probably part of the inert biomass.

Other inert solids that can build up deep in a biofilm are inorganic precipitates, including metal carbonates,

sulfates, sulfides, phosphates, and hydroxides. Often, these are formed because microbial reactions alter the chemistry within the biofilm and cause super-saturation for a solid phase (22,44–48). Precipitation of inorganic solids could create a very dense and strong structure (44), essentially "cementing" the biofilm in place. Alternately, inorganic deposits could make the biofilm brittle, perhaps making the biofilm susceptible to sloughing (35).

One very interesting adaptation finding (35) is on the importance of the shear stress applied during growth of the biofilm (τ_g). When exposed to other shear stresses, biofilms behaved as elastic solids when the applied τ was less than τ_g. However, they behaved like plastic fluids for $\tau > \tau_g$. In other words, τ_g was a good surrogate for the Bingham yield stress, τ_0. For, $\tau > \tau_g$, the elastic modulus (E) increased, indicating that the stiffness of the biofilm was increasing with strain once the biofilm became plastic.

Heterogeneity. Heterogeneity within the biofilm may affect the detachment mode. Ohashi and Harada (49) studied the in situ behavior of biofilm development and detachment using a video camera set up above an open-channel reactor in which they maintained uniform flow. They observed major sloughing events for mature biofilms. After 34 days of biofilm accumulation, massive sloughing events removed about 25% of the observable biofilms. They found that sloughing was associated with the formation of cavities within the biofilm.

Stoodley and coworkers (35,50) also used video imaging to evaluate the behavior of filamentous streamers that oscillate rapidly in the flow. These streamers cause increased head loss. The streamers exhibited hysteresis for high applied shear stress. Hysteresis was more evident near the base of the streamer than the tip. Streamers exposed to prolonged oscillations may experience fatigue, which involves thinning and finally detachment. Fatigue failure requires some time; as a result, it may be possible for the microorganisms to produce EPS that repairs small "cracks" or "thin regions." However, it probably will be unable to repair cracks or thin regions that result from high frequency oscillations.

As a biofilm grows on the substratum, it starts with sparsely distributed cell clusters. If the clusters grow together, they create a relatively planar biofilm. The shear and axial stresses acting on individual cell clusters will vary until the biofilm "fills in." If the clusters are far enough apart, the velocity and stress profiles around the clusters can reestablish between clusters so that each cluster experiences similar stresses. The roughness of the clusters may increase shear stress and turbulence. On the other hand, when the clusters are close together, clusters in the "wake" behind an upstream cluster may experience significantly different stresses than the upstream cluster. Once the biofilm fills in to become more or less planar, the surface may be hydrodynamically smoother, causing the shear stress and turbulence to decline. Therefore, shear and axial stresses depend on the degree of surface coverage by the biofilm and the distribution pattern of clusters in heterogeneous biofilms.

Specific Growth Rate. The specific growth rate (μ) of microorganisms in the biofilm has been implicated as a key factor affecting the detachment rate (31,51,52). This relationship appears reasonable based on the observation that EPS formation is inversely related to the substrate-utilization rate (40,41). However, conclusions about the role of the specific growth rate must be viewed cautiously because the average specific growth rate for a steady-state biofilm equals the specific detachment rate: $\mu_{ave} = b_{det}$. This equality opens up the possibility that observations of a relationship between μ_{ave} and b_{det} are a tautology, not cause and effect.

Some studies suggest that a high μ is not responsible for high detachment rates. Sawyer and Hermanawicz (53) report an inverse relationship between detachment rate and substrate availability. Gjaltema and coworkers (54) found that the effect of C_p on the detachment rate in fluidized beds differed depending on substrate availability. When substrate was available to give high biofilm growth rates, increasing C_p resulted in a slower detachment rate, a finding consistent with others (24,25,32). When substrate was not available for growth, a higher C_p caused a faster detachment rate.

Quorum Sensing. The final physiological factor is quorum sensing, which involves chemical signals by which microorganisms communicate with each other (55,56). Certain bacteria continually produce signal molecules, particularly homoserine lactones. When the density of bacteria is high enough in an aggregate (such as a biofilm), the signal molecule achieves a high enough concentration that it triggers a rapid community response. One known response is to control aggregation or disaggregation of biofilms of *Pseudomonas aeruginosa* (55). Although the range of applicability of quorum sensing is undefined, it could dramatically increase or decrease detachment for situations in which it acts.

CONCLUSION

Detachment is one of the most important phenomena affecting the accumulation, physical properties, and ecology of biofilms. The physical and physiological factors that control detachment are just beginning to come into view, and this article defines a framework for designing and interpreting the research that will illuminate detachment. Understanding detachment is at the core of answering fundamental questions about biofilms: for example, Why do some have an open structure, while others are dense? Why do biofilms sometimes experience massive sloughing events, while others only erode? How do physical forces interact with biochemical reactions to control where different microorganisms reside in a biofilm?

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BIOFILMS, ACTIVITY IN. See ACTIVITY AND CARBON TRANSFORMATIONS IN BIOFILMS

BIOFILMS, ALGAL BIOFILMS. See DIATOMS IN BIOFILMS

BIOFILMS: BACTERIAL-FUNGAL BIOFILMS

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Naturally occurring biofilms comprise a variety of different organisms including bacteria, fungi, algae, and protozoa. Costerton and coworkers (1) commented on the ubiquitous nature of bacterial biofilms, and although they are less studied, so too are bacterial-fungal biofilms. There are many definitions of a biofilm; however these definitions generally only refer to bacterial biofilms. Attempts have been made recently to account for the presence of other organisms. For the purpose of this review, a biofilm is defined as microbial cells immobilized at an interface, surrounded by an exopolymeric matrix of microbial origin. The simplest biofilm model to study is the monoculture or pure culture biofilm. This model alone offers numerous avenues for research, so the possibilities in studying mixed organism biofilms are vast. This in part may explain why bacterial-fungal biofilms are so little studied. Fungi play an important role in the biodeterioration of materials: They are implicated in several nosocomial infections and are associated with other health issues, and they are used in many industrial and biotechnological processes. The importance of understanding mixed organism biofilm formation, especially with the emergence of nosocomial infections and problems to industry, is apparent. The aim of this article is to discuss our current understanding of bacterial-fungal biofilms and to examine methods for their study. It is also the aim to highlight areas of bacterial-fungal biofilm dynamics that necessitate further investigation.

DEVELOPMENT OF BACTERIAL-FUNGAL BIOFILMS

In order to discuss bacterial-fungal biofilms, it is important to briefly mention the fundamental differences between bacteria and fungi. Fungi are eukaryotic organisms and can be differentiated from bacteria by the fact that fungal cells are normally larger and contain a nucleus, vacuoles, and mitochondria. Collectively, fungi are a large group of functionally diverse species and can be classified into three groups of major practical importance: moulds, yeasts, and mushrooms.

In spite of the variation in the definitions available to describe biofilms, and the inherent heterogeneity of biofilms, reports of biofilms always describe the following: A biofilm is always associated with a surface-interface at which cells accumulate and biofilm microorganisms produce an exopolymer matrix within which the cells are enclosed. There are a number of factors that will affect bacterial-fungal biofilm formation and development. To date it is assumed that the same factors that affect bacterial biofilm formation will also affect bacterial-fungal biofilm formation. These include biological factors, including competition, chemical factors, including the nature of the surface, and physical factors, including flow conditions. It is not the aim of this article to describe these factors in great detail but to describe the events that lead to bacterial-fungal biofilm formation (Fig. 1).

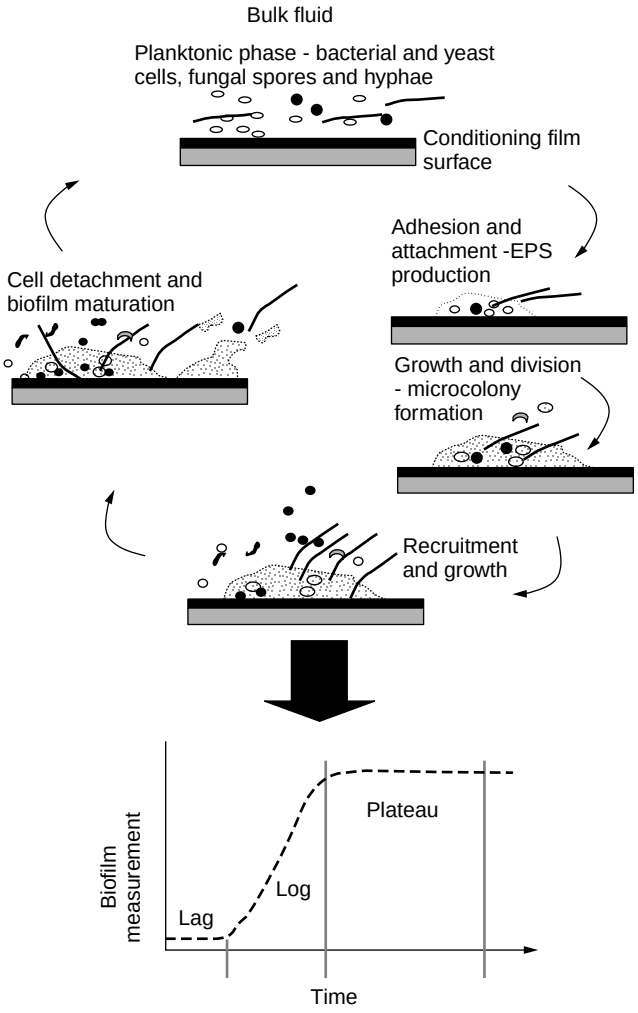

Figure 1. A schematic illustration proposing the life cycle and growth pattern, typically a sigmoidal growth curve, of a mixed organism biofilm.

The conditioning film is a crucial component in the attachment of cells to a surface. When a surface is exposed to the environment, for example, in aqueous systems, a layer of organic deposits, including proteins and humic acids, forms. This is termed the conditioning film. Molecules and small particles ($<0.01-0.1$ μm) rapidly adhere to the surface and can induce changes in the surface properties. These range from the acquisition of a net negative charge to changes in the hydrophobicity of the surface. The composition of the conditioning film will vary with the type of surface and the environment. It is generally assumed, though with no conclusive evidence that the conditioning film across the surface is uniform in both composition and coverage. It has been proposed that the strength of biofilm formation becomes dependent on the cohesiveness of the conditioning film to the surface, rather, than on the direct bacterial contact with the bare substratum surface (2).

Most surfaces and cells have a net negative charge; therefore, when a cell comes into contact with a surface,

a repulsion barrier is established between the cell and the surface. For attachment to take place this must be overcome. There are a number of attractive forces, including van der Waals forces and hydrogen bonding, which play a role in bacterial attachment but are less defined for fungal cells. The three step mechanism proposed by Busscher and Weerkamp (3) takes into account the different forces, which arise at different stages of attachment. This hypothesis is based on the distance between the cell and the surface. At distances greater than 50 nm only van der Waals forces operate; this stage is reversible. At distances between 10 and 20 nm both van der Waals forces and electrostatic repulsion forces are active. Adhesion at this stage is initially reversible but has the potential to become irreversible. In the third stage, where the separation distance is less than 15 nm van der Waals forces, electrostatic forces, and specific interactions, including the production of adhesive exopolysaccharides lead to irreversible bonding of the cell to the conditioning film/surface.

Fungi have been described as excellent colonizers of surfaces (4). Fungal adhesion has a number of stages that appear to be in common with bacterial biofilm formation. Spores, like bacteria, have a negative surface potential. Consequently, the adhesion of fungal cells to a surface is subject to the same electrostatic forces operating with bacteria. The initial contact of fungal spores to a surface may be described as passive, when spores are entrapped or adhere to a surface by appendages, and active when the spore is stimulated to produce adhesive mucilage (5). Spores may become entrapped on jagged surfaces where they are held by physical forces. Passive attachment may be mediated by a number of spore appendages including mucilaginous sheaths, hamate, or caplike appendages, which unfurl to form viscous threads, sticky appendages surrounding the spore, and tufts of fibrillar appendages.

Fungal spores, like bacteria, make repeated contact with surfaces. Fungi are thigmotropic, that is, they have the ability to respond to localized physical contact, and as a result the fungus is able to release spore adhesins on contact with a surface, which then attaches it to the surface. The chemical structure of the adhesins range from only carbohydrate or protein to a mixture of protein and carbohydrate. Once attached to a surface, the fungal spore swells by adsorption of water, and germ-tubes develop and elongate to form young hyphae. Both the germ-tube and hyphae are enveloped in a mucilaginous sheath. This mucilaginous sheath has been likened to the exopolymeric substances produced by bacteria, and is believed to play a similar role including anchorage, protection from desiccation, adhesion sites for other microorganisms and serving as a source of support and nutrition (5). Aquatic hyphomycetes have been shown to be specifically adapted to their habitat by having a high germination rate and the production of tetraradiate, branched, and filiform spores. The yeast *Candida albicans* produces pseudohyphae, which not only provide anchorage for the cells but also produce exoenzymes that aid in the penetration of the cell to tissue lining. It may be that all fungi have specific adaptations to their habitat but as yet many remain undiscovered. These habitat specific appendages may limit the colonization and attachment of the fungi to a specific environment and it may be that the habitat induces the expression of certain phenotypes. The success of biofilm formation, pure culture or otherwise, is therefore dependent on the success of the organism to adapt to its environment.

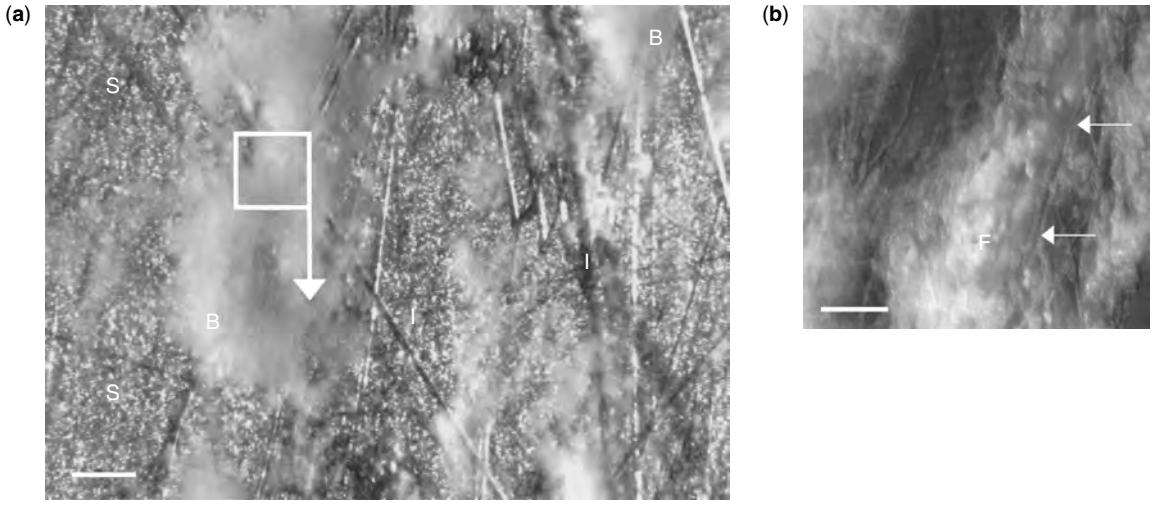

Figure 2. A mixed organism biofilm after 7 h growth on stainless steel using image analysis. The biofilm consisted of seven organisms isolated from an industrial system: *Stenotrophomonas maltophilia*, *Pseudomonas alcaligenes*, *Flavobacterium indologenes*, *Alcaligenes denitrificans*, *Rhodotorula glutinis*, *F. oxysporum*, and *F. solani*. (**a**) **B**, biofilm; **I**, interstitial channels; **M**, microcolony; **S**, stainless steel surface. Bar = 200 μm. (**b**) by varying the plane of focus different section of the biofilm microcolony can be visualised. **F**, fungal biomass; arrows indicate mycelial network. Bar = 50 μm.

Investigations into the development a biofilm has revealed that a typical three-stage sigmoidal growth curve occurs, much the same as a planktonic growth curve. The model proposed by Bryers and Characklis (6) consists of (*1*) initial biofilm formation (lag); (*2*) exponential accumulation (log) and (*3*) steady state (plateau phase) (Fig. 1). It is not known whether a hierarchical order of colonization exists between bacteria and fungi. However, the complexity of naturally occurring biofilms suggests that this would occur. It is recognized that the order in which cells attach to a surface may affect the attachment of other cells; it has been demonstrated that this is a species-specific event and would depend on the nature of the surface (7). However, once the biofilm has reached steady state conditions, the order of colonization may not be of great importance compared with its overall function.

BACTERIAL-FUNGAL BIOFILM ARCHITECTURE

The range of biofilm structures that have been identified vary from a thin layer of attached cells to more complex attached communities containing multiple species interacting with each other. Biofilm structure and architecture is strongly connected to the function required for the survival and maintenance of the microorganisms within and has been shown to be linked strongly to nutrient availability and flow conditions (8) (Fig. 3 and Fig. 4).

As yet, there is no consensus model for the structure of a typical biofilm. Initially a two-dimensional model for biofilm formation was proposed (9). With the increased knowledge of biofilm structure it has become apparent that biofilms are inherently heterogeneous, so three-dimensional models depicting biofilm structure have also been put forward (10). Research has shown that there are many conditions, which contribute to biofilm architecture and structure. The combination of species specificity dictating biofilm architecture and the different variables, which also influence biofilm structure, means that it is virtually impossible to obtain a consensus biofilm model. In practice different models are available for different growth conditions and a **consensus** of variables that influence biofilm architecture.

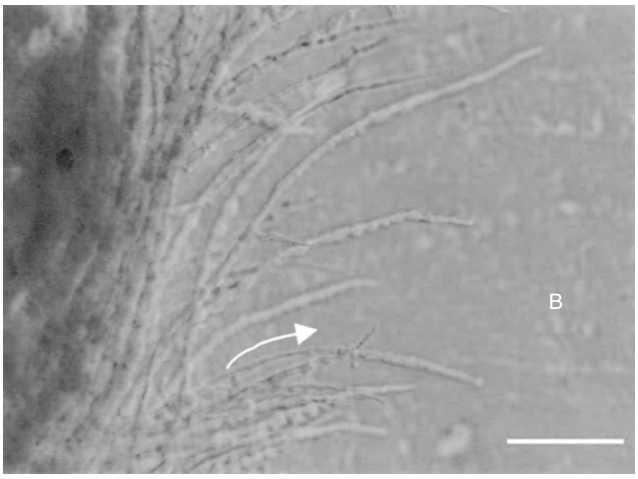

Figure 3. Filamentous fungal attachment (*Fusarium oxysporum* and *Fusarium solani*) to a PVC surface in flowing conditions. The fungal mycelia are positioned in the direction of flow (indicated by arrow). **B**, bulk fluid; **S**, PVC surface. Bar = 50 μm.

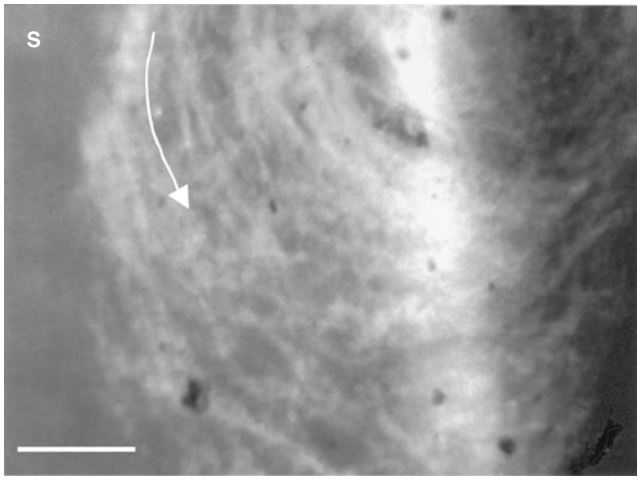

Figure 4. Filamentous fungal attachment (*F. oxysporum* and *F. solani*) to a PVC surface in flowing conditions. The fungal hyphae extend out into the bulk fluid and are positioned in the direction of flow (indicated by arrow). **B**, bulk fluid; **F**, fungal hyphae. Bar = 50 μm.

CHARACTERIZING BACTERIAL-FUNGAL BIOFILMS

Mixed organism biofilms can be described as diverse functioning communities. A community describes an association of interacting populations, usually defined by the nature of their interaction or the place where they live (11). The development of a mixed biofilm leads to a variety of complex relationships involving interspecies and intraspecies interactions. The number of interactions increases when there are more species within the mixed population. The simplest interaction model consists of a two-membered, or binary, population. In this scenario there are only three possible responses that a growing population can make to the presence of a second population, that is, beneficial, detrimental, or neutral (12). The interactions can occur in a reciprocal manner and the outcome consists of a maximum of six types of interaction: neutralism, mutualism, commensalism, amensalism, predator-prey relationships and competition (Table 1). However, another classification can be added to include a direct interaction, if it involves physical contact between individuals, or indirect if the nonliving environment is a necessary intermediary between the two populations (13). The binary system is useful for defining simple interactions; however, in natural communities, interactions will be more complex, and more than one type can occur with one species.

Although biofilms are considered in terms of microbial ecology, the complex nature of mixed organism biofilms necessitates descriptions, which are applied to macroecology. By measuring the diversity of a community, both the variety of species and the relative abundance of each

Table 1. Interaction Terms Used to Describe the Effect That One Population Has on Another

Interaction	Description
Neutralism	Occurs when neither population is affected by the presence of the other
Mutualism	Occurs when both members derive some advantage by the presence of the other population
Commensalism	Occurs when one member of the population benefits and the other member does not
Amensalism	Occurs when the growth of one of the populations is restricted and the second population is unaffected
Predator-prey	Occurs when one member gains directly at the expense of another
Competition	Occurs when both populations are limited in terms of growth or final population size by a common dependence on an external factor required for growth

species is taken into account. It has previously been discussed that many microorganisms exhibit certain traits that are particular to a habitat, and habitat specialization increases in direct relation to diversity. That is, the more diverse a habitat, the more diverse are the organisms. If a habitat is highly specialized, then diversity is decreased but habitat specialization is increased. Diversity indices have been devised to express the diversity of sample by using a single number. The most frequently used is the simple totaling of species numbers to give species richness (14). One of the indices that combine species richness with relative abundance is the Shannon diversity index (15). This method could be used to assess not only the diversity of a sample but to also to monitor the effect of disturbance to a system, for example, an antimicrobial regime or predation.

The reason that certain species grow together in a particular environment will usually be because they have similar requirements for existence in terms of environmental factors such as nutrient availability and temperature. If species do grow in association with each other, many of them should have similar responses and tolerances to environmental pressures. Over a period of time, community composition will often change according to the principles of succession. Succession involves the immigration and extinction of species together with changes in their relative abundances. Primary succession occurs on a bare surface. Organisms that are the first to colonize an area are termed *primary colonizers* and several successive groups of species, termed *secondary* and *tertiary colonizers*, will then establish. The end result of succession is known as a climax community. These stages are in common with the stages involved in microbial colonization of a surface. Populations within a community may cycle quite regularly and population cycles will vary from species to species. The degree of variation in the size of the population depends on both the magnitude of disturbance and on the inherent stability of the population. When a mixed organism biofilm reaches steady state or a climax community it could be said that if all parameters remain constant and there is no disturbance to the system that it is a stable community.

THE OCCURRENCE OF BACTERIAL-FUNGAL BIOFILMS

Most habitats contain a wide structural and functional diversity of microorganisms, which is in part related to the heterogeneity of the habitat and the evolution of species, which exist there. Presently there are limited reports describing bacterial-fungal biofilms, although this is changing rapidly. Bacterial-fungal biofilms have been isolated from a range of diverse locations including photoprocessing tanks (16), automobile air conditioning systems (17) and even the black slime that appears on the base of shower curtains is teeming with bacteria and fungi. There are many other habitats that are currently being investigated with respect to microbial adhesion and biofilms; These include soil particles, plant surfaces, and animal guts. Microbial adhesion and physiology are much more difficult to investigate in these habitats because of their diversity and range of conditions and knowledge of these areas should increase with advancing technology. The reader should also be made aware that in many examples describing bacterial biofilms the surrounding environment will invariably consist of other organisms and these will include fungi. It should also be noted that in the literature, bacteria and especially fungi are discussed without reference to a biofilm mode of growth when it is highly likely that in nature they will exist within a biofilm.

Biofilms play an integral role in the system in which they are present and are considered to either have a beneficial or detrimental effect on that system. The terms fouling and biofouling describe the presence of undesired surface associated microbial growth and are used frequently in the scientific literature. The uncontrolled and undesirable accumulation of biofilms in medical and industrial systems has three primary effects: physical damage, for example, corrosion and tooth decay; decrease in the function of the system, for example, reduced efficiency of heat exchangers, and the establishment of a reservoir for potential pathogens.

Medical Bacterial-Fungal Biofilms

Many recurrent infections are associated with bacterial biofilms and because of the nature of the medical environment, these infections are mainly attributed to a single species. Any medical implant ranging from a catheter to a replacement heart valve has the potential to be colonized by bacteria and fungi. Detachment of the organisms from the biofilm often results in septicaemia, which may be responsive to drug therapy; however the biofilm generally is not so responsive and therefore acts as a continuous source of infection. As a result, implant-associated infections are difficult to treat and in most cases the implant must be removed.

The occurrence of monoculture biofilms in the medical environment is well documented and there are now reports emerging concerning the infectious nature of fungal biofilms. Many fungi that are now increasingly responsible for nosocomial infections were previously thought to be of low virulence. The increase in the number of fungal-associated infections is related to the increase in the use of antibiotics. There are many examples of pathogenic fungi in the literature, for example, *C. albicans* and *C. neoformans*. It is unlikely, however, that in a medical environment the causative agent of infection would be attributed to bacterial-fungal biofilms. Infections are caused separately by bacteria and fungi, and presently it is considered extremely rare if found otherwise, although reports have demonstrated that *C. albicans* is the dominant and causative organism in a bacterial-fungal biofilm isolated from a voice prosthesis. However, with the increase in antibiotic resistance it may not be long before bacterial-fungal biofilms are the combined causative agents of infection.

Biofilm formation by *C. albicans* has generated much interest because it is a major fungal pathogen of humans and can be fatal to immunocompromised patients, for example, those with acquired immunodeficiency syndrome (AIDS). *Candida albicans* is a dimorphic, diploid fungus. The pathogenicity of this fungus is thought to be related to the production of pseudohyphae. The pseudohyphae produce exoenzymes, which penetrate cell tissue and medical implants. Analysis of *C. albicans* biofilms have shown that it consists of a dense network of yeasts, hyphae, and pseudohyphae (18). Another adaptive mechanism exhibited by *C. albicans* is the existence of different strains within the same location. The ability of the organism to achieve this is thought to be governed by its genetic makeup. There is no sexual stage associated with this fungus and it has what are termed as specific repetitive sequences, which contribute to the organism's ability to increase strain variation. It is likely that the biofilm mode of growth enhances the pathogenicity and recalcitrance of *C. albicans* by offering protection from antimicrobial agents and by facilitating the optimum utilization of nutrients.

Dental biofilms are also examples of mixed organism biofilms. Colonization of the oral cavity is a continuous process. The relatively smooth surfaces of the teeth are exposed to frequent mechanical cleaning by the tongue, cheek, saliva, or toothbrush; however, these actions have proved ineffective at preventing tooth decay/biofilm removal alone. The very structure of teeth, a nonshedding surface, favors the retention of food and thus provides numerous sites for harboring dental biofilms and it has been reported that there are between 200 to 500 resident bacterial species in the mouth. Species of *Candida* including *C. albicans* have also been isolated from dental biofilms. It has been found that the initial attachment of bacteria to the conditioning film is relatively species-specific and involves the cooperation and coaggregation of only a few gram-positive streptococci including *Streptococcus sanguis*, *S. sobrinus*, *S. mutans* and *S. mitis* (19,20). The growth of this microbial film encourages the proliferation of other organisms including filamentous gram-negative *Fusobacterium* species (*Fusobacterium nucleatum*) and spirochetes such as *Borrelia* species. Dental biofilms illustrate the complex nature of mixed organism biofilms and highlight some of the aspects covered in the literature.

Environmental and Industrial Bacterial-Fungal Biofilms

Biofilms that accumulate on suspended particles in rivers, lakes, and marine systems are considered beneficial to their environments because they play an essential purification role by removing suspended, settled, and dissolved organic material. Freeman and coworkers (21) described river biofilms as a trophic link between dissolved nutrients in the water column and the higher trophic levels of the ecosystem. These river biofilms possess the ability to trap nutrients by ion exchange processes and thereby increase the nutrient concentration within the biofilm. Natural biofilms are able to biodegrade organic compounds and transform inorganic compounds as part of their natural metabolic pathway. The role of bacterial-fungal biofilms in the degradation of environmental pollutants is not well understood. The ability of fungi to degrade environmental pollutants has been well documented but these studies rarely include a biofilm model. It is assumed that in a natural environment such as the soil habitat, bacteria, and fungi do form biofilms (22) and therefore the degradation of environmental pollutants occurs in a biofilm state where it is also assumed that there is a higher level of enzyme activity. The degradative capabilities of biofilms have been exploited in wastewater treatment systems including trickling filters and fixed or fluidized bed reactors. Such systems are based on particles that provide a large surface area for biofilm formation. The mixed biofilms that develop on these surfaces break down a wide range of chemical substances found in wastewater.

Bacterial-fungal biofilms have been recognized to play a role in the accelerated deterioration of buildings, including the well known and dreaded dry rot fungus. The degree of colonization and subsequent deterioration is dependent on the moisture content of the construction materials. The formation of bacterial-fungal biofilms in buildings has many serious health implications including the harboring of allergenic fungal and actinomycete spores. *Stachybotrys atra* grows on damp wallpaper and other domestic surfaces. It produces potent macrocyclic trichothecene toxins and is associated with chronic health problems. The building can also act as a reservoir for diseases including Legionnaire's disease. It is important therefore that the potential to form bacterial-fungal biofilms is assessed in the architectural design and that plans should include minimizing the ingress of water into the building construct.

In industrial systems, biofilms are particularly deleterious because of their ability to corrode the surface with which they are associated, a phenomenon known as microbial induced corrosion (MIC). The corrosion of metal ships, pipelines, and oil rigs is an extremely costly problem. Marine biofilms that form on ship hulls increase drag and frictional forces, which results in an increase in fuel consumption and a decrease in engine efficiency. In water distribution pipelines the formation of biofilms reduces

the pipe diameter, which affects flow and in cooling systems an estimated £500 million annually is lost due to an inefficient operating system.

CONTROL OF BACTERIAL-FUNGAL BIOFILMS

The control of biofilms has generated a huge commercial market in designing and developing new antimicrobial regimes. A wide variety of biofilm control strategies have been tested with varying degrees of success. There are two main approaches to the control of biofilms: the prevention of initial colonization and subsequent biofouling, and the development of removal/control strategies against an existing biofilm. Biocides and antibiotics have been incorporated into different surfaces in an attempt to prevent microbial colonization. However, to date these types of control strategies tend only to prevent biofilm formation for a limited period and subsequent biofouling occurs as if there was no antimicrobial agent in place. Present day control strategies of industrial biofilms are aimed at maintaining partial control and a minimum contamination level. In many cases, infection by antibiotic-resistant organisms, for example, methycilin resistant *Staphylococcus aureus* and vancomycin resistant *Enterococcus faecium*, biofilm eradication is not achieved and infection is fatal. Fatalities in these incidences are higher in immunocompromised patients. The ultimate aim is obviously to design an antimicrobial regime that will permanently prevent biofouling. This control strategy would be one that was entirely specific for the target organisms and would have no other detrimental effect. This is currently a problem that needs to be addressed. For example, infections caused by *C. albicans* can be treated with antifungal antibiotics including amphotericin B, however, the effect of the antibiotic on the patient often has more serious side effects than the original infection. Paints that have been used to protect ship hulls have been shown to have an irreversibly detrimental effect on marine life, namely, in generations of dog whelks, which have lost the ability to reproduce.

Antimicrobial agents may exert what are termed static or cidal effects although the mechanism of action of each may differ (23). The term static is used to describe the prevention or inhibition of growth by an antimicrobial agent measured under conditions in which growth would normally occur. The effect is reversible as removal or neutralization of the agent will allow the cell to reestablish growth. Cidal effects are the result of damage to cellular structures and function and cidal effects are irreparable and irreversible. In order for the antimicrobial compound to have its desired effect, the compound must be taken up by the cell, move to the target sites and then react with the target site. Different antimicrobial agents have different modes of action and it would be impossible to cover them all within this chapter. It is common practice to use a combination of antimicrobial agents to achieve a synergistic effect, for example, to use a combination of antibiotics, which will have a complimentary and enhanced effect without disrupting the mode of action of either of the antibiotic. This is referred to as combination therapy.

Once any type of biofilm is established in a system, complete eradication is very difficult and in some instances irremediable. The biggest problem associated with the control of biofilms is that biofilm cells are less susceptible to antimicrobial agents than their planktonic counterparts. The mechanisms for this increased resistance remains to be elucidated. The production of exopolymeric substances by biofilm cells are believed to play an important role in the protection of the cells from antimicrobial agents and was thought to be the basis of biofilm resistance (24). It is now believed by many research groups that the reduced penetration of antimicrobial agents through the extracellular polymers are insufficient to account for much of the observed resistance (24,25). There is no doubt that the exopolymeric matrix can function as an ion exchange column and excludes large, highly charged molecules; however, most solutes will equilibrate across it and access the resident population (24). The extent of penetration and concentration of the antimicrobial agent that reaches the population is dependent on the thickness of the biofilm. This is a transport limitation phenomenon common to control strategies, which aim to remove an established biofilm.

In addition to the physical barriers that a biofilm presents, individual cells possess structures, which prevent the biocide reaching its target(s). In the case of gram-negative bacteria the presence of the lipopolysaccharide cell envelope wall offers a supplementary barrier. This structure has a significant moderating influence on the penetration of both hydrophilic and hydrophobic molecules, establishing a molecular weight cutoff for the passage of molecules through water filled pores (porins) and requiring optimum lipophilic properties for the progress of hydrophobic biocides (23,26,27). The walls of fungal hyphae and spores also contain a variety of components, which help protect them from chemical treatment. Melanin components are chemically very stable and are frequently found in many surface moulds. The production of melanin in the cell wall in response to an antimicrobial regime has been shown to contribute to the resistance of fungal hyphae to lytic enzymes (28), oxidizing agents such as hypochlorite (29), and radiation (30). Regardless of cell wall structure, all cellular components offer opportunities for nonspecific binding of the antimicrobial agent, thereby depleting the chemical challenge (23).

Another problem that is of primary concern for the control of fungal biofilms is their location. Many types of fungi, such as the moulds, are found to grow on the interior and exterior of most buildings and the success of colonization by these fungi is related to the moisture content of the building construct. If a building is poorly insulated there will be an increase in the amount of condensation produced, which in turn will increase the diversity of the biofilm. Introducing equipment such as dehumidifiers into the home environment is not always a practical solution. Fungi are able to penetrate deeply into the building construct and emerge on wallpaper and wood, and it is not always possible because of safety reasons to provide a chemical solution for domestic purposes. The

areas of contamination may also be located in places that are not suitable for disinfection on a daily basis.

There are many other proposed mechanisms, which are thought to play a crucial role in the protection of biofilm cells from an antimicrobial regime. It is thought that because of the structure of the biofilm, nutrient gradients will exist where cells located in the upper sections of the biofilm will be exposed to higher levels of nutrients compared to those cells located at the surface, which are assumed to be exposed to less nutrient rich conditions. This will affect a number of physiological actions including enzyme activity. As a result, the growth rate of the biofilm is believed to be slower than planktonic cells, which have the high doubling-time capacity. Other defensive mechanisms activated by bacteria and fungi in response to stressful conditions include the viable but nonculturable state (bacterial) and the production of spores.

It is widely recognized that bacteria exposed to stressful conditions experience what is termed as sub lethal injury. This is when a cell maintains minimal metabolic activity but viability is still retained. The genes that are thought to regulate these responses are expressed on entry to stationary growth phase where a slow growth rate would allow the cell an adaptation period to many stressful conditions including antimicrobial regimes and heat shock. In order to monitor the success of an antimicrobial regime, an accurate recovery protocol must be established to take into account cells that are stressed. Quantification of cells in a stressed state by conventional methods, including plate counts, may lead to the underestimation of the level of contamination.

METHODS TO STUDY BACTERIAL-FUNGAL BIOFILMS

It has been common practice in the past to directly compare laboratory planktonic data directly to biofilm conditions based on the assumption that there was no difference between planktonic and biofilm cultures. It is now widely accepted that this is not the case. If the level of contamination in industrial systems was assessed by monitoring the number of planktonic cells, a serious underestimate of the level of contamination would ensue as it has been reported that typically for every one planktonic bacterium there are between 1,000 and 10,000 cells attached to the surface (31).

The ultimate aim in designing any experimental apparatus is to include the major components of the system under investigation so as to provide the most accurate model possible. Table 2 highlights some of the measurements that can be evaluated from experimental model biofilms. The results obtained would then be more reflective of in vivo conditions. In practice, most experimental biofilm models only represent certain aspects of a system under investigation and tend to fall into in to two main categories: replicative, which encompass a wide range of complex environmental variables, and investigative, which are usually simpler and enable the control of a variety of influencing factors (32,33). The techniques used to study biofilms can either be destructive, which involves the disruption and destruction of the biofilm, or they are nondestructive, which permits the monitoring of the biofilm in its natural state. There are currently a wide range of experimental models available to study biofilms and it is not our aim to discuss them all, but rather to discuss those that would be significant in increasing our understanding of bacterial-fungal biofilms.

The Modified Robbins Device (MRD, Fig. 5) was initially developed to study biofouling in industrial pipelines (34). Since then it has been used to study biofilms from a number of different systems. The MRD consists of an enclosed lumen with 25 replaceable sampling studs. Different surfaces can be attached to the studs and removed for analysis by a number of different techniques. The MRD can

Table 2. Examples of Some of the Measurements That Can Be Assessed from Experimental Model Biofilms

Measurement		Method
Microbial activity	Viable cell counts Metabolic activity	Culture techniques ATP levels Fluorescent staining Molecular techniques
Biofilm constituents	Exopolysaccharide	Protein content Total carbohydrate Fluorescent staining
Biofilm growth	Thickness Biomass Quantification Surface area	Image analysis Culture techniques Fluorescent staining
Biofilm formation	Structure Development	Microscopy — Image analysis — Scanning electron microscopy — Transmission electron microscopy — Confocal scanning laser microscopy — Environmental scanning electron microscopy — Fluorescent microscopy

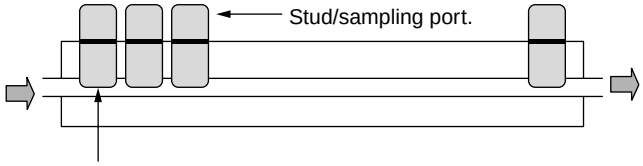

Figure 5. Diagrammatic representation of an MRD showing direction of flow (block arrows) and position of studs.

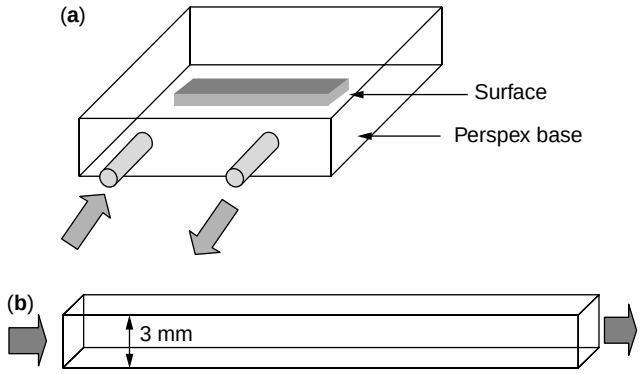

Figure 6. Diagrammatic representation of (**a**) a flat plate reactor and (**b**) a square glass tube reactor. Block arrows indicate direction of flow. The required reactor would be place on a microscope stage and images captured via a camera and analysed using the appropriate software.

be incorporated into a variety of experimental models such as a batch recirculating and flow through culture systems. It can be connected to a chemostat as a recirculating or flow through culture system, which would allow the control of growth conditions and thereby permit the assessment of growth rate as a function of biofilm resistance.

The main drawback with the MRD system is that it is a destructive technique and involves scraping of the biofilm for analysis of biofilm accumulation and metabolic counts. Other drawbacks with the MRD system are the inability to visualize the biofilm in situ, and possible nutrient gradients along the length of the lumen from the inlet to the outlet. Hydrodynamics around the sampling stud may also be compromised (35). Nevertheless, the information obtained from the MRD model is valuable and allows comparisons to be drawn with other techniques.

Although valuable information can be obtained from models that involve the disruption of the biofilm, the continuous and nondestructive analysis of biofilms is essential in understanding biofilm processes (36). In situ monitoring techniques have advanced with recent developments in image analysis technology. The advantages of this technique include the monitoring of the biofilm in a hydrated state, and real-time analysis. Using digital image capture, accumulation rates can be calculated by comparing captured images with those at the outset of the experiment, allowing quantification of the growth kinetics of the biofilm; qualitative information regarding surface colonization is also possible (35). The flat plate reactor (Fig. 6) was developed at the Center for Biofilm Engineering, Bozeman, Montana. In this apparatus, different surfaces can be examined and compared (35). The surface can also be removed for other types of analyses. The disadvantage of the flat plate reactor is that it is constrained by the channel thickness. This model of flow cell is limited by the nature of the working distances of the microscope objectives, resulting in a compromise between magnification and hydrodynamics (37). Use of the square glass tube reactor can overcome this problem and facilitates higher magnification of the biofilm, allowing the flow cell to be viewed from above (35). The square glass tube reactor flow cell (Fig. 6) was initially designed to replicate biofilm fouling in industrial pipelines. Stoodley and coworkers (38) have characterized the flow hydrodynamics of this system using the relationship between the friction factor and the Reynolds number, and found that they fit established equations describing laminar and turbulent flow through a smooth pipe. This system also permits visualization of biofilm formation under both laminar and turbulent flow concurrently (38).

Another technique that has also been instrumental in increasing our understanding of biofilm formation and structure is confocal scanning laser microscopy (CLSM). CLSM operates using a krypton/argon laser, which excites fluorophore dyes present within the sample. The resulting fluorescence is detected by photomultiplier tubes and a digital image is obtained. Alteration of the focal depth (z plane) and the subsequent acquisition of the x-y plane images (parallel to the surface) enables a series of optical sections to be collected that are then processed by a computer using image analysis software to create a three-dimensional image. CLSM is an effective tool to study a wide range of biofilm features, which include physiological profiles and structural heterogeneity that would be particularly pertinent to mixed organism biofilms. CLSM permits the visualization of biofilm formation in situ and therefore allows the study of the biofilm in its natural hydrated state. A number of other techniques could also be used in combination, including microelectrodes to monitor pH, and oxygen and nutrient gradients, resulting in a comprehensive profile of the biofilm. Biofilms that have been examined using CLSM are shown to be heterogeneous and consist of microcolonies or cell clusters separated by interstitial voids and channels (8). These channels have been shown to act as transport channels (39) and therefore the use of CLSM would allow the study of transport phenomenon within the biofilm, which would be relevant in assessing the delivery of an antimicrobial agent to the biofilm.

It has been previously discussed that microorganisms are not culturable by traditional culture methods. Recent advances in molecular biology have circumvented this problem. These methods include gene probing, DNA hybridization, polymerase chain reaction (PCR) and reporter gene technology. In 1994, a novel marker system, the green fluorescent protein (GFP) became available (40). The GFP is a 27-kDa polypeptide, which converts the blue

chemiluminescence of the Ca^{2+}-sensitive photoprotein, aequorin, into green light (40). This fluorescence was first observed in the jellyfish *Aequorea victoria*. GFP has been successfully used in eukaryotic systems and there are an increasing number of reports of the success that GFP has to prokaryotic systems. GFP is stable in the presence of many denaturants, high temperatures (65 °C) and a range of pH (6–12). It is therefore an attractive marker system to monitor individual cells within a biofilm. GFP is now commercially available in two other colors, red and yellow, and has also been applied to fungal cultures.

CONCLUSION

There still remains many aspects of bacterial-fungal biofilms to investigate. This paper has provided areas for discussion and has given insights into the complex nature of studying mixed organism biofilms. Characterizing bacterial-fungal biofilms in terms of macroecology may aid our understanding of such heterogeneous communities. Fungal colonization of a surface has many stages in common with bacteria, including the production of exopolymeric substances. Bacterial-fungal biofilms cause considerable problems in many systems, particularly within industry. They may act as a source of pathogens and because of their inherent resistance to many antimicrobial regimes are a serious health hazard. The treatment of bacterial-fungal biofilms is additionally problematic because the biofilm cells have an altered physiology and morphology, which promotes their success in a particular habitat. The advances in technology are aiding in our understanding of biofilms with the ultimate aim to control biofilm formation in the first instance.

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BIOFILMS, CARBON TRANSFORMATIONS IN.

See ACTIVITY AND CARBON TRANSFORMATIONS IN BIOFILMS

BIOFILMS, CONDITIONING FILMS.

See CONDITIONING FILMS IN AQUATIC ENVIRONMENTS

BIOFILMS: EXTRACELLULAR ENZYMES.

See EXTRACELLULAR ENZYMES IN BIOFILMS

BIOFILMS, EXTRACELLULAR POLYMERIC SUBSTANCE.

See EXTRACELLULAR POLYMERIC SUBSTANCES (EPS): STRUCTURAL, ECOLOGICAL AND TECHNICAL ASPECTS

BIOFILMS, FORMATION RATE IN WATER DISTRIBUTION SYSTEMS.

See ASSIMILABLE ORGANIC CARBON (AOC) IN TREATED WATER: DETERMINATION AND SIGNIFICANCE

BIOFILMS IN NATURAL AND DRINKING WATER SYSTEMS

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The concept of bacterial behavior and growth has long been influenced by studies of bacteria grown in liquid culture in the laboratory. In natural and engineered aquatic systems, however, microorganisms tend to grow preferentially in organized communities at almost all available interfaces. These organized structures, which are best defined as "cells immobilized at a substratum and frequently embedded in an organic polymer matrix of microbial origin" (1), are commonly referred to as biofilms. Costerton and coworkers (2) later broadened the definition to include microbial aggregates in the free-water phase ("matrix-enclosed bacterial populations adherent to each other and/or to surfaces or interfaces"), however, the widespread occurrence and importance of eukaryotic microorganisms in biofilms is neglected in this definition. Biofilms are ubiquitous in all aquatic habitats, ranging from thick cyanobacterial mats, biofilms covering stones and pebbles in rivers and lakes, immobilized cells in wastewater treatment reactors, surface-associated growth in drinking water distribution systems, to biofilms of medical relevance, such as dental plaque, bacterial growth on gut endothelial cells or on artificial implants.

Unlike bacteria multiplying as single cells in the planktonic phase, biofilm bacteria grow in a highly structured microenvironment, where diffusion of substances is affected by the properties of the biofilm and where metabolic interactions, cellular communication, and genetic exchange are facilitated by the close and ordered spatial arrangement of the cells (3,4). Biofilm bacteria are, to a certain extent, sheltered from harmful substances such as disinfectants, antibiotics, antibodies, toxins, and also from viruses. Bacterial growth rates are often enhanced within biofilms because of the accumulation of nutrients at interfaces and the cooperative mobilization of nutrients by different organisms of the microbial community. Thus, microbiologists have to study biofilm communities (1–4) to catch a glimpse of the true nature of microbial communities in the environment.

BIOFILM COMPONENTS

The structure and composition of biofilms can vary widely, depending on the environmental conditions and the population structure of the microbial community. The extracellular polymeric substances (EPS) excreted by the attached bacterial population, forming a slimy, highly hydrated matrix that acts as glue for all biofilm components, should certainly be regarded as a key constituent of biofilms. EPS generally consist of polysaccharides, proteins, nucleic acids, and heterogeneous polymeric compounds (5). The volume of the EPS matrix, in which the microorganisms are embedded, normally clearly exceeds the volume of the cells. Because of the reduced diffusion of substances, the EPS matrix acts as a protective barrier against harmful chemical compounds and viruses, retains extracellular enzymes, and leads to the establishment of gradients across the biofilm (6).

Bacteria generally form the dominant population, but eukaryotic organisms are also present in most biofilms, although they have been less extensively studied than the prokaryotic community. These protozoa have an important function as predators, influencing not only biofilm thickness and structure but also the species composition of the bacterial population (7). Apart from protozoa, fungi can occur in biofilms, as can algae in phototrophic biofilms. Although the EPS matrix was thought to prevent viruses from infecting biofilm bacteria, bacteriophages were recently found to be able to infect cells and multiply in single-species laboratory biofilms (8,9). Most biofilms are dominated by organic matter, namely microbial cells and their excretions, and usually to a minor extent by depositions of inorganic material like quartz grains and iron particles. In some cases, however, biofilms can also be largely dominated by inorganic compounds. In drinking water biofilms, for example, certain types of bacteria can be responsible for massive deposits of iron and manganese particles (7).

FORMATION AND STRUCTURE OF BIOFILMS

Colonization of Surfaces and Biofilm Development

The physicochemical properties of a surface submersed in an aquatic system not only influence the adsorption of organic molecules and ions to the surface, the so-called "antenna-effect," but also affect bacterial adhesion (10,11).

Bacteria in aquatic systems can reach a surface either by random Brownian displacement, by fluid dynamic forces transporting small particles to the viscous boundary layer at a surface, by random flagellar- or pili-based motility, or by active chemotactic movement toward the nutrient-enriched layer at the surface. The initial contact is followed by reversible or irreversible adhesion of the cell to the surface. To describe this process, the Derjaguin-Landau and Verwey-Overbeek (DLVO) theory, which was developed for colloid chemistry and describes changes in Gibbs energy of interaction as a function of the distance between two objects, has been invoked. In contrast to colloid particles, bacteria might play a more active role in adhesion because of cellular appendages such as fimbriae, flagella, pili, cell surface adhesins, and because of their motility. At small distances of separation, short-range forces such as hydrogen bonding and ion forces become effective. The physiological status of a cell can have a significant influence on its attachment behavior, although the effect may differ from species to species. Starved cells often show reduced motility, changes in cell surface hydrophobicity, and a reduction in cell size. For starved cells, both increased and decreased ability for adhesion has been reported (12,13), and these differences might be due to species-specific behavior and to differing starvation conditions generated in the experiments. Bos and coworkers (14) have discussed the initial interactions of microorganisms with surfaces and the various methods for studying adhesion, aggregation, and coadhesion.

Exopolymers produced by the adherent cells accumulate and act as glue between cells and the surface and lead to an irreversible attachment of the bacteria (6,15). Colonization of the surface may then proceed in a number of ways, depending on surface characteristics and on the colonization behavior of the attached species (16). For example, in streams, a range of colonization behaviors have been described, including formation of tightly packed, spreading, or branching colonies (17). In the marine environment, a *Psychrobacter* sp. initially formed microcolonies, followed by the development of compact, multilayered biofilms at hydrophobic surfaces but developed only long, multicellular chains at hydrophilic surfaces (18). Time-lapse microscopy was used and *Pseudomonas aeruginosa* was found to initially form a monolayer of cells on the surface, followed by formation of microcolonies through aggregation of cells from the monolayer, which led to a biofilm structure of dispersed microcolonies in the monolayer of cells (19). In highly oligotrophic systems, like drinking water or seawater, biofilms can be restricted to a monolayer of cells (20,21).

Structure of Mature Biofilms

The structure of mature biofilms in natural or engineered systems is as diverse as the habitats themselves, ranging from thin monolayers of dispersed cells in extremely oligotrophic habitats such as drinking water distribution systems, patchy arrangements of matrix-embedded cell clusters interspersed with water-filled channels observed in many natural ecosystems, and in laboratory model biofilms, to the layered, densely packed biofilms, forming cyanobacterial mats or dental plaque. The architecture of biofilms is controlled by many parameters. The kind and concentration of nutrients present in a habitat certainly has a tremendous impact on biofilm thickness and overall structure (22), as well as on the species composition of the microbial community. Mass transfer of nutrients, metabolites or inhibitors, diffusion of oxygen, salts, and ions create gradients across the biofilm and induce the formation of microniches, influencing microbial growth rates, production of extrapolymeric substances, and interactions between the microorganisms. Hydrodynamic forces can become important in shaping the biofilm architecture. When grown under turbulent flow conditions, where high-shear forces act on the biofilm, structural changes from loose cell aggregates toward streamlined filaments and ripples can be observed. It has also been shown that structural rearrangements occur within biofilms in response to environmental conditions, that is, turbulent flow (23). Moreover, the microorganisms themselves shape their environment. Eukaryotic predators, including amoebae and other protozoa, and bacterial parasites such as *Bdellovibrio* spp. may control the biomass and species composition of biofilms. Bacterial shape, motility, production of extracellular polymers, and cell signaling between bacteria, all play a crucial part for the architecture of mature biofilms. Substratum properties such as hydrophobicity, roughness, and electrostatic forces, on the other hand, play a substantial role in the initial stages of biofilm formation but appear to be less significant in established, steady state biofilms.

Detachment and Dispersal

(see Biofilm Detachment, this Encyclopedia)

Hydrodynamic forces, which influence the biofilm structure, are mainly responsible for the detachment and sloughing off of clumps of cells or small parts of the biofilm. These large particles will be transported by the flow and sediment or attach to a substratum when flow conditions permit it, thus contributing to the colonization of new surfaces. Biofilms, however, are not only subjected to passive detachment processes. Several studies have demonstrated that individual cells might actively leave biofilms. Cell division can cause the active detachment of cells, involving different dispersal strategies, with daughter cells leaving the biofilm, whereas the parental cell remains firmly attached (17,24) or detachment taking place during cell division, followed by dispersal or reattachment (25).

Genetics of Biofilm Formation

Attachment of bacteria is thought to induce an alteration of bacterial gene expression and metabolic activity. A positive effect of surfaces on bacterial growth had already been observed in the 1930s (26). Cells grown on solid surfaces or on agar show physiological properties and behaviors that are quite different from cells growing in the liquid phase; a striking example is the coordinated multicellular

behavior displayed in swarmer cell formation of bacteria such as *Serratia liquefaciens* (27), or induction of lateral flagella in *Vibrio parahaemolyticus* (28). Molecular tools applied in pure culture studies have helped to identify a number of genes required for biofilm formation and genes with altered levels of expression between sessile and planktonic growth. Genes whose expression is altered in biofilms can be identified by using gene reporter technology (29), whereas genes with an essential function in biofilm formation can be detected by random transposon mutagenesis, followed by screening for biofilm formation defective mutants and determination of the genes involved (30).

Altered gene expression has been reported for *Escherichia coli* by analysis with reporter genes. Expression of 38% of the genes was found to be affected by growth within biofilms. Some genes such as a gene involved in flagellin synthesis, were downregulated in sessile bacteria. Other cellular components showed enhanced expression; examples are the OmpC porin, the high-affinity transport system of glycine betaine, the colanic acid exopolysaccharide, tripeptidase T, and the nickel high-affinity transport system (31). These changes in gene expression show that bacteria rapidly adapt to the environmental conditions encountered in a biofilm, where osmolarity and cell density are increased, and oxygen less available.

Genes with an essential function for biofilm formation have been discovered by isolation of mutant bacteria and analysis of the affected genes. Biofilm formation can be triggered by external environmental cues and by bacterial cell-cell signaling, which is also important in the establishment of the complex three-dimensional structure of biofilms. For the initial stages of biofilm formation, motility mediated by flagella or pili is essential, whereas adhesins and EPS play a crucial role for interactions between cells and between cells and the surface (32).

The significance of cell signaling has been recently demonstrated for a *Pseudomonas aeruginosa* mutant, which had a mutation in the *lasI* gene. LasI directs the synthesis of an acylated homoserine lactone (AHL), which acts as a signaling molecule between cells and activates the transcriptional activator LasR. The mutant *P. aeruginosa* could only form flat, undifferentiated biofilms, which showed an increased sensitivity toward a biocide. When a synthetic signal molecule was added, biofilm growth appeared to be normal (33). In *Serratia liquefaciens*, swarming motility has been found to be controlled by two separate regulons, the *fhlDC* operon, a flagellar master regulator, and the *swrI* gene, a putative homoserine lactone synthase. It appears that the AHL cell-signaling system controls the production of an extracellular biosurfactant, which enables swarm cells to travel on top of surfaces and facilitates the movement of differentiated swarm cells, whereas swarm cell differentiation itself (i.e., cell elongation and hyperflagellation) is controlled by the *flhDC* operon (27). As mentioned in the preceding section, environmental cues such as nutrient availability, not only affect the metabolic status of cells but also biofilm formation. In *P. aeruginosa*, a mutation in the catabolite repression control (Crc) protein causes an inability to build biofilms: the mutant strain forms only a dispersed monolayer of cells and does not develop the dense monolayer punctuated by microcolonies typical of the wild-type strain. This phenotype had so far been observed in type-IV pilus mutants, which were defective for twitching motility. Apparently, the Crc protein acts as part of a signal transduction pathway that regulates, among other traits, pilus expression and thereby biofilm development (34).

Flagellar-mediated motility has been demonstrated as an important factor in biofilm development for a number of gram-negative bacteria including *E. coli*, *P. aeruginosa*, *P. fluorescens*, and *Vibrio cholerae*. For each of these organisms, mutations in genes involved in flagellar-mediated motility hindered biofilm formation. Apart from motility based on flagella action, twitching motility has been found to be essential for *P. aeruginosa* biofilm formation. Twitching motility is based on type-IV pili and seems to be widespread among gram-negative bacteria. Twitching plays a role in the establishment of biofilm structure, especially in the formation of microcolonies, whereas flagellar action apparently is necessary to generate cell surface contacts. Motility-defective mutants might, however, regain their biofilm formation capacity by mutations in other genes such as the *ompR* gene discussed in the following section, or they can be restored by addition of specific nutrients (32).

The ability to adhere to a surface or to another cell is crucial for the formation of biofilms. For example, cell-cell (intra or interspecific or multigeneric) coaggregation among biofilm bacteria has been shown to be significant in a number of systems including aquatic habitats (35). Mutant strains of *Staphylococcus epidermidis* have been isolated, which were affected in their primary attachment to polystyrene, but remained able to form multilayered cell clusters. These mutants lacked four cell surface proteins, which are postulated to function as adhesins. Other mutants were defective for intercellular adhesion and thus unable to form cell clusters and biofilms as a result of a failure to produce a specific polysaccharide (36). A mutant strain of *E. coli* has been isolated, which showed an increased biofilm-forming ability compared to classical laboratory strains. The mutant *E. coli* displayed a single point mutation in the regulatory protein OmpR, resulting in an increased expression of the particular fimbriae type Curli. Apparently, Curli acts as a cell surface adhesin and is under the control of the EnvZ-OmpR two-component regulatory system (37). Other examples are genes coding for the mannose-sensitive type I pilus and the pilus adhesin FimH, which are essential for *E. coli* biofilm formation or the mannose-sensitive hemagglutinin pilus in a *V. cholerae* strain (32). For *P. aeruginosa*, genes of the alginate synthesis pathway were found to be upregulated on attachment and biofilm formation, and expression of a putative alternative sigma factor required for expression of the critical alginate biosynthetic gene *algD* has been reported (38,39). Alginate is produced in large quantities by mucoid *P. aeruginosa* strains, which is a critical pathogenic determinant during chronic infections in cystic fibrosis patients. In a *V. cholerae* strain, production of an exopolysaccharide encoded by the

vps locus confers biofilm-forming capacity and chlorine resistance. Mutations within this locus result in an inability to form biofilms (40).

IDENTIFICATION OF BIOFILM BACTERIA

(see BIOFILMS: BACTERIAL-FUNGAL BIOFILMS, this Encyclopedia)

rRNA-Based Phylogeny and In Situ Hybridization

The most remarkable recent advance in microbiology was undoubtedly the development of molecular tools for the identification and in situ detection of bacteria based on their evolutionary history. Sequencing of ribosomal RNA (rRNA) genes from diverse organisms, which revealed the tripartite organization of life according to the domains Bacteria, Archaea, and Eucarya, eventually led to the design of short, labeled oligonucleotide probes complementary to specific target sites on 16S or 23S prokaryotic rRNA. Ribosomal RNAs contain highly conserved and variable sequence regions, which together with the natural amplification effect due to their high copy number in the cell, makes them ideally suited for in situ hybridizations with oligonucleotide probes, targeting phylogenetic groups ranging from domains to species (41,42).

For a first analysis of the phylogenetic diversity of a microbial biofilm community, in situ hybridizations with already existing probes are performed in a top-to-bottom approach, where oligonucleotides with increasing phylogenetic narrowness will be applied. If a complete analysis of the microbial community is attempted, bulk DNA is extracted from the habitat, rRNA amplified by PCR with universal primers, subcloned, subjected to DNA-sequencing, and finally compared to existing sequences in published databases. However, the data thus obtained have to be interpreted with caution because of the possibility of amplification and cloning biases, resulting in misjudgments of species abundances or the formation of chimeric artifacts (43). The sequencing data can subsequently serve for the design of new hybridization probes and their application in the microbial community, thus obtaining quantitative information on the population composition. Hybridization probes also allow the monitoring of enrichment cultures and enable the isolation of specific species of interest.

Fingerprinting Techniques: DGGE and RFLP

DNA strands of the same length but different sequences can be separated by exploiting differences in their migration behavior in denaturing gradient gel electrophoresis (DGGE). Genes coding for 16S rRNAs obtained by PCR amplification procedures from microbial communities can be subjected to this technique. The banding patterns or "fingerprints" thus obtained offer the possibility to rapidly detect changes in population structures of microbial communities. Apart from the visualization of such profiles, specific DNA bands of interest can be excised and further analyzed by DNA sequencing (44). DGGE has been successfully applied to investigate principles of microbial community ecology in a hot spring microbial mat (45), which is presented in more detail in the following section on examples of natural biofilms.

As an alternative to the DGGE technique, restriction fragment length polymorphism (RFLP) analysis of rRNA genes amplified from microbial communities can be applied to obtain specific fingerprints. After amplification and subcloning of the PCR products, RFLP patterns for the rRNA clones are determined with restriction endonucleases (46). Alternatively, RFLP patterns can be obtained for total 16S rRNA from a microbial community (47). A modification to the RFLP technique, termed *terminal-RFLP* (T-RFLP), consists of labeling one of the PCR primers, followed by restriction digestion and fragment detection by capillary electrophoresis, where only the end-labeled fragment of each PCR product is detected. DGGE and T-RFLP have been compared in a recent study, revealing that although both techniques show similar results, T-RFLP was more sensitive than DGGE (47). Because both techniques rely on PCR-procedures, they are subject to the same problems as cloning procedures with regard to chimeric sequences and quantification of species abundance.

Detection of Known Bacterial Strains

For the detection of known cultivated bacteria in microbial biofilm communities, the application of labeled antibodies or species-directed PCR procedures might be useful. Both methods, which depend on the prior cultivation of the species of interest, are mainly used for the detection of pathogenic bacterial species. Thus, *Legionella pneumophila* could be visualized by immunogold- or fluorescein-labeled monoclonal antibodies in drinking water biofilms (48). Problems with the use of antibodies in natural biofilms are posed by hindrance of antibody penetration into the biofilm and possible cross-reactivity with antigens presented by other species.

PCR procedures are helpful if the bacteria of interest are suspected to be present in such low numbers that detection by antibodies, in situ probing, or fingerprinting is impossible. Examples of this include detection of *Helicobacter* spp. or *Mycobacterium* spp. by rRNA amplification from microbial communities in drinking water systems. Specific PCR can be coupled with dot-blot hybridizations, reducing the possibility of false-positive detection of pathogens (49,50). For the differentiation of a known pathogen from other closely related bacterial species, PCR amplification of specific genes, differentiating these strains from others can be a very useful tool. In a recent study, a multiplex PCR approach was developed for the simultaneous identification of *Listeria monocytogenes* and five other species of this genus, based on species-specific amplification of the *iap* gene (51).

Cultivation Strategies

Cultivation of bacteria from natural microbial communities usually yields less than 1% of the bacterial population, even though the proportion of metabolically active bacteria in situ far exceeds this percentage,

a phenomenon described as the "great plate-count anomaly" (52). Comparison between cultivation and in situ detection methods in different habitats revealed drastic cultivation-induced population shifts; the composition of the isolates obtained did not reflect the in situ population composition (45,53,54). In one case, however, bacteria able to form colonies on solid media were reported to account for a large fraction of the community (55). Although PCR-based studies hold the potential to describe microbial diversity without cultivation-dependent biases, it has to be kept in mind that this technique is subjected to its own limitations (43,45). Furthermore, cultivation is indispensable to correlate phylogenetic information with physiological properties of the respective organisms. Cultivation of in situ relevant bacterial species can be feasible if it is combined with and monitored by molecular identification techniques. In young drinking water biofilms, isolation of the dominant species was successful, based on findings gained by in situ probing and activity measurements, which had revealed that most of the bacteria were affiliated to the β-subclass of Proteobacteria and that most cells could utilize the medium used for cultivation in modified direct viable count assays (54). Kane and coworkers applied a probe designed for an environmentally derived rRNA sequence for the enrichment and isolation of a sulfate-reducing bacterium (56). In cyanobacterial mats, an organism with little numerical relevance was found to competitively exclude other species that were more dominant in situ, when in laboratory culture. Only by dilution of the inocula to extinction before enrichment, could these specific cyanobacteria that were highly relevant in situ, be recovered. Enrichments from highly diluted inocula also allowed the isolation of in situ abundant chemoorganotrophic bacteria from this environment (45). Ward and coworkers stress the point that the incongruity between culture and molecular methods should not lead to the conclusion that many or most bacteria in natural environments are "uncultivable" (45). They should rather be regarded as "uncultivated" because the most likely explanation for cultivation failures might be our inability to understand and reproduce the real environmental niches of bacterial species.

Different methods of bacterial identification in biofilms are summarized in Table 1.

Table 1. Methods of Bacterial Identification in Biofilms

Method	Parameter Measured	Level of Measurement
Light microscopy	Transmission of light	Individual cell
Epifluorescence microscopy	Emission of light	
Confocal laser scanning microscopy (CLSM)	Transmission, emission, and reflection of light	
Scanning electron microscopy (SEM)	Transmission and reflection of short-wavelength light and electron beams	
Environmental scanning electron microscopy (ESEM)		
Total cell count determination:	Fluorescence emission after binding of nucleic acid–specific fluorochromes	Individual cell
DAPI (4',6-diamidino-2-phenylindole)		
AO acridine orange		
Hoechst 33258		
Hoechst 33342		
SYTO stains		
SYTOX stains		
SYBR Green		
PicoGreen		
Propidium Iodide		
FISH (fluorescence in situ hybridization)	Fluorescence emission after hybridization of DNA-probes to ribosomal RNA	Individual cell
GFP (green fluorescent protein)	Fluorescence emission after expression of the GFP	Individual cell
Microcalorimetry	Heat originated from microbial metabolism	Community
FTIR spectroscopy	Absorption spectra (amide I and II bands of proteins) obtained by their attenuated total reflection	Individual cell
RFLP (restriction fragment length polymorphism)	Fragment lengths after restriction of specifically amplified genes	Community and individual cell
DGGE (denaturing gradient gel electrophoresis)	Migration of specifically amplified genes of similar length along a denaturing gradient	Community and individual cell
Immunostaining	Fluorescence emission after binding of antibodies to their antigens	Individual cell

ASSESSMENT OF METABOLIC ACTIVITIES

Direct Viable Counts

Among microbiologists, it is widely accepted that "a single, viable microorganism is represented by a cell capable of dividing and forming at least one live daughter cell when it is placed in a favorable environment" (57). According to the definition given by Oliver (58), bacteria that show metabolic activity but are incapable of undergoing the sustained cellular division required for growth on a favorable artificial medium are termed *viable but nonculturable cells* (VBNC, see VIABLE BUT NOT CULTURABLE (VBNC) MICROORGANISMS, this Encyclopedia). To determine the metabolic potential for bacteria to undergo cell division, Kogure and coworkers (59) developed the direct viable count (DVC) method, where bacterial samples are incubated with appropriate nutrients in the presence of an antibiotic, which prevents DNA synthesis. Because other cellular functions are not affected, bacteria will elongate and can subsequently be enumerated by microscopy.

The difference between heterotrophic plate counts and elongated cells is commonly considered the portion of a given bacterial population in a VBNC state and according to this, DVC has become the most widespread method to determine the number of cells in a VBNC state. A major recent modification to this technique involves the application of a cocktail of five antibiotics, which greatly reduces the probability of cell growth due to the presence of bacteria with antibiotic resistance and allows longer incubation times (60). Another modification complements enumeration of elongated cells with in situ hybridizations, enabling one to monitor the metabolic potential of specific species or groups of organisms of interest (54).

Additional methods for determination of viable and nonviable cells include a commercial live-dead stain (Molecular Probes, Eugene, Ore.), and some measures of respiratory activity (see the following section) may also be used to assess activity. Because of the complexity of determining the status of bacterial cells, a combination of techniques may be required, particularly, in complex communities.

Respiratory Activity

Bacterial respiratory activity can be assessed on the basis of the reduction of tetrazolium salts to formazan crystals by the activity of cellular dehydrogenases. The tetrazolium salt (CTC) has replaced salts used in earlier studies because of the easy detection of its fluorescent-reduced form (61). CTC can be applied to biofilm samples, with or without addition of nutrients, yielding either the number of actively respiring bacteria under in situ conditions or the number of cells with the potential for respiratory activity when nutrient limitation is lifted. Some authors, however, reported that they could find no difference between incubation with or without nutrients (62). CTC has recently been shown to have a toxic effect on several bacterial metabolic processes. Samples subjected to a CTC-assay yielded lower heterotrophic plate counts, displayed lower bacterial production, and lower respiratory activity as measured by glucose incorporation and respiration, suggesting that the number of metabolically active cells in a microbial community might be underestimated by this method (63).

Microautoradiography

The uptake of specific substrates by microorganisms under in situ conditions can be studied by using radiolabeled substrates in combination with microautoradiography (64). This method has become widely used in ecological studies and allows conclusions about the metabolic activity of microbial populations. A correlation between active substrate uptake and identity of the organisms, however, is not possible based on microautoradiography alone. A combination with immunofluorescent-labeling of specific organisms was developed (65) but suffers from the drawbacks of antibody application in natural microbial communities mentioned in the preceding section. Recently, microautoradiography was successfully combined with rRNA hybridizations and applied for the characterization of seawater picoplankton (66) and activated sludge communities (67). For single-cell resolution in sludge samples, flocs were cryosectioned and examined by confocal laser scanning microscopy. Uptake of specific substrates could thereby be combined with the analysis of the phylogenetic identity of the organisms.

Other Methods

In recent years, a number of techniques have been developed for the assessment of the general metabolic potential of bacteria. Most of them can be grouped into methods detecting activities of enzymes, synthesis of macromolecules, or membrane potentials. They include detection of esterase activity (68), alkaline phosphatase (69), microcolony formation (70), membrane potential (71,72), membrane integrity (73), or DNA- and RNA-degradation (74).

Specific activities of bacteria can be visualized in situ by the detection of target mRNA sequences. The mRNA of interest is amplified by in situ PCR and subsequently visualized by labeled probes complementary to the amplified DNA (75,76). Reporter genes might be very useful to determine specific metabolic activities of genetically modified organisms in model biofilms. The reporter gene has to be fused to a gene or promoter of interest in a specific bacterial strain; the most commonly used reporter genes are the green fluorescent protein (*GFP*) gene, the *E. coli lacZ* gene, the *lux* gene coding for bacterial bioluminescence, or the firefly *luciferase* gene (77,78, see GREEN FLUORESCENT PROTEIN (GFP), this Encyclopedia).

At the level of activities of microbial communities, determination of the ATP content is one of the most frequently used methods, allowing, for example, study of the influence of water distribution pipe materials on bacterial activity and growth (79). For some applications, a combination of in situ probing and microelectrode measurements can be very useful. In trickling filters, the distribution of sulfate-reducing bacteria was correlated to gradients of oxygen and sulfide (80), and biofilms in a nitrifying bioreactor were examined for distribution patterns and activities of nitrifying bacteria and gradients of oxygen, ammonium, nitrate, and nitrite (81).

Table 2. Methods for Assessing Bacterial Metabolic Activities in Biofilms

Method	Metabolic Parameter Measured	Level of Measurement
RH-795 staining Rhodamine 123 staining Live/Dead Bacteria Light™ (Propidium Iodide/SYTO 9)	Integrity of cell membrane	Individual cell
ATP measurement	Overall adenylate content	Community
Microelectrodes	Redox potential, microbial activity	Community
Microautoradiography	Substrate utilization	Community and individual cell
FISH (fluorescence *in situ* hybridization)	Cellular ribosome content	Individual cell
FISH-MAR (fluorescence *in situ* hybridization microautoradiography)	Cellular ribosome content and substrate utilization	Individual cell
DVC (direct viable count)	Substrate utilization and elongation	Individual cell
PAC (probe active count)	Substrate utilization and cellular ribosome content	Individual cell
SFDA (sulfofluorescein diacetate) CFDA (carboxyfluorescein diacetate)	Hydrolytic enzymatic activity (esterases)	Individual cell
TTC (triphenyl tetrazolium chloride)	Cellular dehydrogenase activity (respiratory activity)	Individual cell
INT (2-*p*-(iodophenyl)-3-(*p*-nitrophenyl)-5-tetrazolium chloride) CTC (5-cyano-2,3-ditolyl tetrazolium chloride)		
gfp gene (Green fluorescent protein) *lacZ* gene (β-Galactosidase) *lux* gene (Bacterial luciferase)	Gene expression	Individual cell

Different methods for assessing bacterial metabolic activities in biofilms are summarized in Table 2.

COMMUNITY INTERACTIONS

Metabolic Interactions

In nature, many degradative processes cannot be performed by pure cultures of bacterial species, but require the metabolic interactions of different bacterial species. Biofilms, where bacteria are in close proximity, are ideally suited for interactions of their community members. Bacterial interactions in biofilms are not limited to competition for available substrates, but often involve a more cooperative behavior such as cometabolism or symbiosis. Well-known examples are the anaerobic degradation of organic matter, where at the end of the food-chain, H_2-producing acetogens and H_2-consuming methanogens interact as symbiotic partners; the nitrification process that requires the combined action of ammonia- and nitrite-oxidizing bacteria; or the degradation of many xenobiotic compounds. In a defined biofilm system in which a drinking water isolate was fed with benzoate as sole carbon source, growth of an added *E. coli* strain could be demonstrated within the biofilm, although the strain was unable to grow on benzoate in pure culture. Apparently, metabolites excreted by the drinking water bacterium allowed not only survival but also growth of *E. coli* (82). Another well-studied example for interspecies exchange of metabolites is the coexistence of *Legionella pneumophila* and *Flavobacterium breve*, caused by the release of cysteine by the *Flavobacterium* cells. Cysteine serves as a nutrient for *Legionella* and as a mild reducing agent (83).

Using GFP as a reporter system, metabolic interaction could also be demonstrated for degradation of benzyl alcohol in mixed culture biofilms. Gene fusions of *gfp* with either the upper-pathway promoter or the meta-pathway promoter of the toluene degradation pathway were generated and introduced into *P. putida*. GFP expression from the upper-pathway promoter could be induced in *P. putida* pure-culture biofilms and in mixed-culture biofilms by addition of benzyl alcohol. Expression from the meta-pathway promoter, however, was only induced when an *Acinetobacter* sp. was added to the biofilm community. Apparently, the *Acinetobacter* sp. degraded benzyl alcohol to benzoate, which leaked from the cells into the surrounding medium and could serve as substrate and inducer of GFP expression from the meta-pathway promoter in *P. putida* (84). In a flow cell-grown model system the effect of a commensal interaction on the spatial structure of the biofilm community consisting of two organisms was investigated using fluorescent in situ hybridization, confocal laser scanning microscopy, and a GFP reporter system (85). Depending on the nature of substrate given in the model system, different colonization patterns could be visualized. When the consortium was fed with chlorobiphenyl, which could only be utilized by one model organism, microcolonies consisting of that bacterium and the associated second organism occurred. In contrast to this, the two species formed separate microcolonies when fed with citrate, which could be metabolized by both organisms.

Cell Signaling

In recent years, it has become clear that to react to changes in the environment, bacteria must possess a form of intercellular communication. One of the important environmental factors for bacteria is the cellular density of their own population. Among many gram-negative bacteria, a "quorum-sensing" system has evolved, based on diffusible, membrane-permeant acylated homoserine lactone (AHL) pheromones that act as cell density cues or "autoinducers" and cause changes in gene expression. AHLs are constitutively expressed at a low basal level but expression is upregulated when the cell density, and hence AHL concentration, reaches a certain level. Quorum sensing has been discovered in *Vibrio fischeri*, where luminescence is controlled by two proteins, LuxI and LuxR. LuxI is an AHL-synthetase and LuxR is a transcriptional activator, which promotes transcription of *luciferase* genes when bound to the AHL-autoinducer. In *P. aeruginosa*, two analogous quorum-sensing systems *las* and *rhl*, have so far been discovered. LasI and RhlI direct the synthesis of specific AHLs, which induce the transcriptional activators LasR and RhlR, respectively. Genes regulated by quorum sensing include, among others, genes coding for an endotoxin, secretion proteins, a protease, and the stationary-phase sigma factor. Thus, production of virulence factors appears to be under the regulation of quorum-sensing systems in *P. aeruginosa*. Similar quorum-sensing systems have been found in numerous gram-negative bacteria, whereas in gram-positive bacteria, quorum sensing is based on peptide signal molecules, which are recognized by sensor kinases that interact with cytoplasmic response regulator proteins (86). AHLs not only serve as quorum sensors but some AHLs also stimulate their own production and can establish positive autoinducer feedback loops.

Apart from the obvious importance of quorum sensing for gene expression in biofilms, where cell densities are very high, defects in quorum sensing seem to have a crucial impact on the biofilm formation potential of bacteria. As already mentioned earlier, a *P. aeruginosa lasI* mutant formed only undifferentiated, flat biofilms, which were more sensitive to biocide action than the corresponding wild-type biofilm (33).

Recently, a marine red alga has been found to produce antagonists of AHLs. These halogenated furanones apparently inhibit AHL-mediated gene expression by displacing AHL from its receptor protein, the transcriptional activator LuxR in *E. coli*. By producing furanones, the alga controls bacterial colonization of its surfaces. Again, these findings demonstrate the importance of cell signaling for colonization and biofilm formation (87). Evidence has also been found for the role of AHLs in the formation of natural and urethral catheter biofilms (88,89).

Gene Transfer

(see GENE EXCHANGE IN BIOFILMS, this Encyclopedia)

Genes can be horizontally transferred between bacteria by conjugation, transduction, and transformation. Gene transfer by conjugation is thought to be one of the major mechanisms for the establishment of new genetic traits in microbial communities, and is also thought to play a considerable role in biofilms. Even though the probability of random encounters of cells is lower in biofilms than in the planktonic phase, the high cell density and stable spatial localization might facilitate intra and interspecies gene transfer. The frequency of gene transfer can be affected by a number of factors, such as cell densities (90), growth phase (91), temperature, pH, concentrations of nutrients, ions, and oxygen (57,92,93), as well as contact time (93) and spatial arrangement (90). Gene transfer methods are described in detail by Christensen and coworkers (78). The frequency of gene transfer is usually determined by plating on selective media, where only transconjugants can grow, raising the question whether the number of culturable transconjugants reflects the number of in situ occurring gene transfer events. In a recent study on interspecies gene transfer between *E. coli* and *Ralstonia eutropha* in biofilms, detection of transconjugants was achieved in situ by a combination of in situ probing and GFP expression. Interestingly, the rate of gene transfer was found to be thousandfold higher than the rate determined by classical plating techniques (93). However, if interspecies plasmid transfer was studied on floating filters, no difference between conjugations was found between in situ detection and traditional plating techniques (94). This discrepancy might be explained by different experimental setups or they could possibly hint at physiological differences between planktonic- and biofilm-associated cells (93). It must also be kept in mind that in situ detection of gene transfer does not allow any conclusions with regard to plasmid stability in the recipient. Because in situ monitoring of plasmid transfer is based on GFP expression from the transferred plasmid, it would be expected that only bacteria that keep the plasmid maintain green fluorescence. The method can be further refined by using unstable variants of the GFP.

Interactions Between Eukaryotes and Bacteria

Grazing of bacteria by various eukaryotic microorganisms is widespread in microbial communities. Moreover, predation was found to have a significant impact on bacterial morphologies and on the taxonomic composition of bacterial populations. In planktonic populations, protozoan grazing could be shown to trigger a population shift from small, fast-growing bacteria affiliated to the β-subclass of Proteobacteria and the Flavobacteria-Cytophaga phylum toward more grazing-resistant, larger cells affiliated the α- and γ-subclasses of Proteobacteria and filamentous bacteria of other phylogenetic groups (95). Grazing by protists may also influence the nature of the exopolymer matrix of biofilms as shown by Möller and coworkers (96).

Comparisons between grazing of planktonic versus attached bacteria showed protozoan preference for attached bacteria in some cases, in other studies, a protective effect of adhesion was reported (97). A recent study attempted to directly quantify protozoan feeding rates on biofilm bacteria by using unstained, viable prey. In this model system, the grazing rate of a small ciliate on *P. putida* cells was determined to be higher for suspended than for attached bacteria (97). Analysis of the

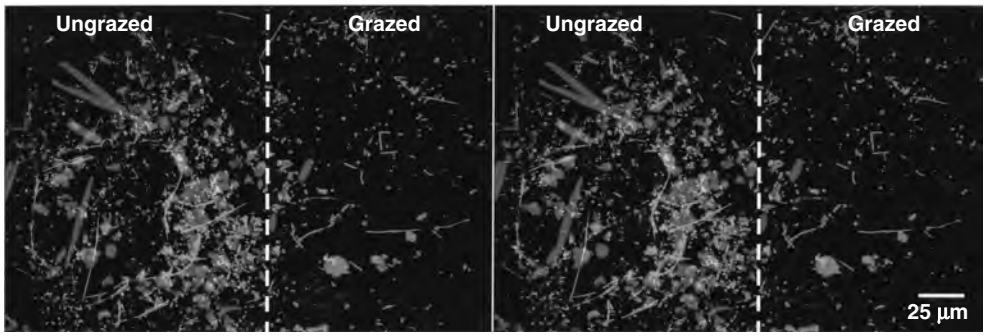

Figure 1. Stereo pair of false color confocal laser scanning micrographs, showing the effects of snail grazing on the distribution of bacteria (stained with Syto 9, green), algae (autofluorescence, red), and extracellular polymeric substance (stained with fluorescent *Triticum vulgaris* lectin, blue) in a river biofilm. See color insert.

bacterial community composition during a diatom bloom suggested that protozoan grazing, together with increased viral abundance, not only leads to a phylotype-shift of the bacterial community but also to a pronounced bacterial colonization of larger suspended particles (98). The study of Hahn and coworkers (99) showed that grazing by *Ochromonas* spp., resulted in the development of a floc-forming subpopulation in *Pseudomonas* sp. MWH1 that was interpreted as a successful grazing defense.

Interactions between eukaryotes and bacteria, however, are not restricted to grazing. A well-studied example for parasitic growth of a prokaryote within a eukaryotic host is represented by members of the family Legionellaceae. *Legionella pneumophila*, the causative agent of Legionnaires' disease, grows intracellularly in a number of amoeba species and in other protozoa-like ciliates. These hosts not only provide nutrients for *Legionella* organisms but also protect them from adverse environmental conditions. *Legionella* spp. present in drinking water systems may be enclosed in cysts of amoebae and thereby be protected from chlorination, ozone, and other adverse conditions (7). Recently, *Mycobacterium avium*, an opportunistic human pathogen that can survive and multiply within drinking water biofilms, has been shown to grow on metabolites excreted by *Acanthamoeba polyphaga*. *Mycobacterium avium* was even able to multiply within the outer walls of the double-walled cysts of the amoeba (100). Interactions between plants and bacteria also occur, as described above for a marine alga, which produces furanones to inhibit colonization by bacteria.

Interactions with micro and macroinvertebrates also influences development of microbial biofilms, through disturbance, sloughing, and active grazing of bacterial and exopolymer components of biofilms. For example, the impact of snail, grazing on the biofilm architecture, and abundance of bacteria, algae, and extracellular polymeric substance is shown in Figure 1.

EXAMPLES OF NATURAL BIOFILMS

Cyanobacterial Mats

Cyanobacterial mats are typical microbial biofilm communities in extreme environments such as hot springs or salt marshes. They are characterized by their layered structure, where production of organic matter through photosynthetic microorganisms is located in the uppermost layer, and aerobic and anaerobic decomposition of the organic substances takes place in deeper layers of the mat. The top layer of a microbial mat typically contains cyanobacteria and sometimes also diatoms and small algae, whereas anoxigenic photosynthesis takes place in the deeper levels. Here, purple phototrophs and filamentous green phototrophs can be found, together with aerobic and anaerobic chemoorganotrophs as well as sulfate-reducing and methanogenic bacteria. Many parameters vary vertically across the mats, including light intensity, oxygen, carbon dioxide, nitrogen, phosphate, sulfur, and organic nutrients (101). One of the most extensively studied systems are hot spring cyanobacterial mat communities, which have recently been covered in a review by Ward and coworkers (45). At the outset of investigations on the microbial ecology of these mats, studies based on microscopical observation and cultivation identified two main bacterial species, a unicellular cyanobacterium and a filamentous green nonsulfur bacterium. Other bacterial species cultivated from the mats were aerobic chemoorganotrophs, gram-positive fermentative bacteria, sulfate-reducing bacteria, and methanogens. Data gained by analysis of pigment and lipid biomarker abundances suggested that cyanobacteria and green nonsulfur bacteria predominated over chemoorganotrophic bacteria, and even fewer terminal members of the anaerobic food chain, such as methanogens or sulfate-reducers, were present. Direct retrieval of 16S rRNA sequences, using different molecular methods, however, did not yield sequences that could be ascribed to previously isolated organisms but only sequences that were 93% or less similar to their closest cultivated or uncultivated relatives. This divergence is thought to be because of competitive exclusion of species that are numerically dominant by bacteria, which perform well in laboratory culture but are not abundant in situ. As mentioned earlier, a solution for recovery of dominant strains may be dilution-to-extinction enrichment. DGGE analysis proved to be a very useful tool for the investigation of the spatial distribution of populations in the mat. Vertical distribution could be demonstrated for cyanobacterial populations and for green sulfur and green

nonsulfur bacteria. Although one cyanobacterial population (termed *type B*) could be detected throughout the top 0 to 0.5 μm layers, the other population (termed *type A*) was found to be restricted to a well-defined layer about 600 μm from the surface, suggesting a vertical stratification and adaptation to different light intensities of cyanobacterial populations. Mechanical disturbance of the mat, caused by removal of the top green cyanobacterial layer, allowed the investigation of recolonization behavior. DGGE analysis revealed that a new cyanobacterial population colonized the disturbed mat, rather than the previously dominant species that remained in adjacent undisturbed sites, indicating that as in macroecology, bacterial species might exist, which are effective at rapid growth rates (so-called r-strategists) and colonization. DGGE surveys also revealed cyanobacterial population changes along the thermal gradient that occurs in the effluent channel of the Octopus Spring location (45). The detected sequences appeared to be from dominant members of the population because they could also be recovered from cyanobacterial isolates obtained from highly diluted inocula. The detected sequences showed a high level of 16S rRNA sequence relatedness, suggesting that the species might have formed through adaptive radiation from a common ancestor (45).

Riverine Biofilms

In the global hydrological cycle from atmosphere to land and oceans, running freshwater systems (lotic systems) are the major linkages between terrestrial and aquatic habitats including surface and groundwater. The common feature of river ecosystems is the continuous movement of the bulk water phase. Microbiological investigations of lotic systems revealed the important role of the complex microbial river communities in global nutrient cycles. It has become apparent that microbial transformations (the so-called microbial loop, 102) have a serious impact on the flux of organic carbon in running waters (103). Consequently, attention is now focusing on the role of bacterial production and the microbial food web as well as their impact on the self-purification capacity of lotic systems.

In a recent study, the planktonic bacteria in the bulk water phase of the Japanese rivers Minoh and Neya were investigated as one distinct compartment of the lotic community, using rRNA-targeted fluorescent in situ hybridization (104). However, biofilm bacteria can comprise the majority of bacterial numbers (105) and most bacterial production and transformation in running waters occurs in biofilms or communities associated with suspended particulate matter (106,107), whereas relatively little production occurs in the free water column. The balance of planktonic, suspended floc, and biofilm communities in rivers is dependent on interactions between the nutrient status, flow regime, and order of the specific river under investigation.

Epilithic biofilms that grow on the surface of rocks or stones cover most substratum in the littoral zone (i.e., those parts of the water body that occur in shallower waters of unspecified depth) and the streambed. These complex mixed assemblages of bacteria, fungi, and algae even occur in the hyporheic zone (108,109), which means the interstitial layer of the river bottom that may be in contact with both the river water and the groundwater flow underneath. The continuously changing riverine ecosystem biofilms contribute significantly to the distribution and recycling of nutrients and inorganic substances (110), and heterotrophic activity is considered to be almost exclusively associated with sediments (111–113). Marxsen (114) discussed the significance of sediment biofilms for the carbon balance of streams. Dissolved organic matter (DOM) is converted by microorganisms in both hyporheic and epilithic biofilms (115–117). Naegli and Uehlinger (118) showed for a prealpine river that the epilithic and hyporheic biofilm community contributed 4 to 19% and 76 to 96% of the total respiratory activity as determined by oxygen consumption, respectively. Although most studies focused on the upper layers of the sediment microhabitat, even deeper sediment zones can be populated by metabolically active bacteria (106,108,113).

From a macroecological point of view, riverine biofilms play an important role as a trophic resource for macroinvertebrates in lotic systems (119,120). Biofilm bacteria and particle-associated microorganisms themselves are subjected to a top-to-bottom control by grazing protists (121,122), which have repeatedly been shown on surfaces in rivers (123).

The biofilm organisms are typically embedded in or associated with a complex heterogenous matrix of extracellular polymeric substances (EPS) secreted by the organisms themselves (124–127). The EPS matrix enhances the immobilization of dissolved organic carbon (DOC), and even particulate organic carbon, and allows the exchange of matter within syntrophic associations of microorganisms. Indeed, Freeman and Lock (128) demonstrated that river biofilms were remarkably resilient to a loss of available carbon in the overlying waters, indicating that river biofilms store energy that is subsequently available during starvation intervals.

Bacteria that can attach and multiply under the high-shear forces normally present in rivers are obviously adapted to live in this environment but only little is known about the physiological plasticity and the ecological impact of planktonic and attached bacteria within these ecosystems. It is generally accepted, however, that the activity of attached microbes dominates in small streams, whereas planktonic bacteria are of greater importance in large rivers because of the different ratios of the surface (of sediments or suspended particulate matter) to the volume of the floating water (110). However, the microbial components of lotic ecosystems have rarely been studied and it is not known to date whether there are specific bacteria in rivers that can be distinguished from those organisms that are passively transported downstream. The overall structure and bacterial composition of specific lotic biofilm communities was described by Lock (110). Nutritional relationships among bacteria and algae in river biofilms were studied by Haack and McFeters (129), suggesting a coupling of photosynthetic and heterotrophic populations. Leff and coworkers investigated the seasonal changes in planktonic bacterial assemblages of two Ohio streams by measuring the abundance of total

bacteria, colony forming units, and the population of *Pseudomonas cepacia* by hybridization with a species-specific probe (130).

Because of the obvious difficulties involved with biofilm investigations in situ within lotic systems, there is a strong need for suitable laboratory models that particularly mimic the development of attached communities under turbulent flow regimes and constant shear forces. Lawrence and Caldwell (17) used continuous flow cells to visualize the rapid colonization of the substratum by a diversity of lotic bacteria. Mohamed and coworkers (131) used flow cells to investigate the impact of nutrients that may control the growth of biofilm bacteria obtained from the Fraser River, Canada. As an important result of the study, the phosphorus limitation of heterotrophic river biofilm bacteria could be shown for the first time.

Rotating annular bioreactors (RAB) were applied for different studies including structural features and metabolism (132,133). Neu and Lawrence (134) used RAB for cultivation and structural examination of stream biofilms by confocal laser scanning microscopy (CLSM). The biofilms were generated in the reactor by feeding with natural river water from the South Saskatchewan River system as inoculum and sole source of nutrients and carbon. The reactor contained removable polycarbonate strips that allowed sampling of biofilm material at desired intervals. The architecture of the biofilms was in contrast to previous views of a homogeneous biofilm structure and more recent biofilm models with channels and inverted biomass distribution. There was a development of ridges, oriented to the flow direction, that were colonized by individual bacterial cells and microcolonies, as shown in Figure 2. As suggested by the authors, the differences may be attributed to the presence and coadsorption of humic and detrital material in natural lotic systems. After an initial phase of biofilm development, both the highest biomass and the numbers of putatively living cells were generally distributed within the outer regions of the biofilm. The heterogeneity of the biofilms was also visualized by the application of a panel of fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC)-conjugated lectins, indicating the presence of different carbohydrates within the EPS-matrix.

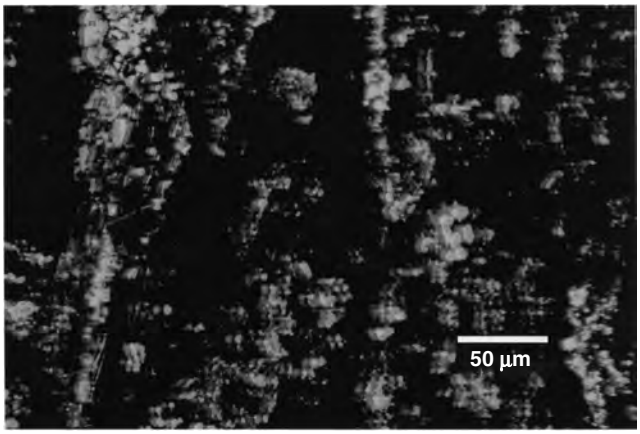

Figure 2. A red-green anaglyph stereo projection of a series of confocal laser scanning micrographs of a river biofilm stained with Syto 9, and TRITC-labeled *T. vulgaris* lectin. See color insert.

The same experimental setup was used in another study investigating algal, bacterial, and exopolymeric components of fully hydrated natural biofilm communities by Lawrence and coworkers (135). Multispectral imaging in conjunction with nucleic acid stains, fluor-conjugated lectins, and autofluorescence was performed in this study to evaluate biofilm community composition. Biofilms were treated with the nucleic acid stain SYTO 9 to quantify the bacterial biomass and fluor-conjugated lectins (i.e., *Triticum vulgaris* lectin) to identify and allow quantification of exopolymeric substances. Digital image analysis of confocal laser scanning sections in each of the channels was used to determine parameters such as biofilm depth, bacterial cell area (biomass), exopolymeric area, and algal biomass at various depths and locations. The results of multichannel imaging, showing an example of a rotating annular bioreactor generated river biofilm stained with fluor-conjugated lectins, a nucleic acid stain, and autofluorescence imaging of algae are shown in Figure 3.

In a further study, the phylogenetic composition, three-dimensional structure, and dynamics of the bacterial communities in river biofilms, generated in the RAB system were studied by a combination of fluorescent in situ hybridization (FISH) and confocal laser scanning microscopy. Digital image analysis was performed to measure specific cell numbers and specific cell area after in situ probing (136). During the different stages of biofilm development, individual cells within the α-, β-, and γ-Proteobacteria as well as the Cytophaga-Flavobacterium group could be visualized, demonstrating that they formed major parts of the attached community. During initial biofilm development, β-Proteobacteria accounted for the greatest part of the bacterial population. This was followed by a rapid increase of α-Proteobacteria and bacteria affiliated to the Cytophaga-Flavobacterium group. In mature biofilms, α-Proteobacteria and Cytophaga-Flavobacteria continued to be the prevalent bacterial groups. β-Proteobacteria constituted the morphologically most diverse group within the biofilm communities and more narrow phylogenetic staining revealed the importance of distinct β1-Proteobacteria for the composition of the microbial community. The importance of members of the β-Proteobacteria is also reported for sessile microbial communities that grew on glass plates exposed in a highly polluted river in Germany (137). In addition, Brümmer and coworkers showed the seasonal dynamics of Planctomycetes and Cytophaga-Flavobacteria that both showed maximum levels of relative abundance in July, and, in case of Cytophaga-Flavobacteria, a second peak in winter (137).

Besides the attached biofilm communities located at virtually all surfaces and interfaces in the rivers, bacteria, algae, and freshwater fungi also constitute a major part of the macroscopic mixed assemblages (suspended particulate matter) present in the bulk water phase. In accordance with microbial aggregates described in oceans and estuaries (107,138–140), in the pelagic zone of lakes (141–143) and rivers (144–146), the suspended particulate aggregates found in rivers have been recently termed *river*

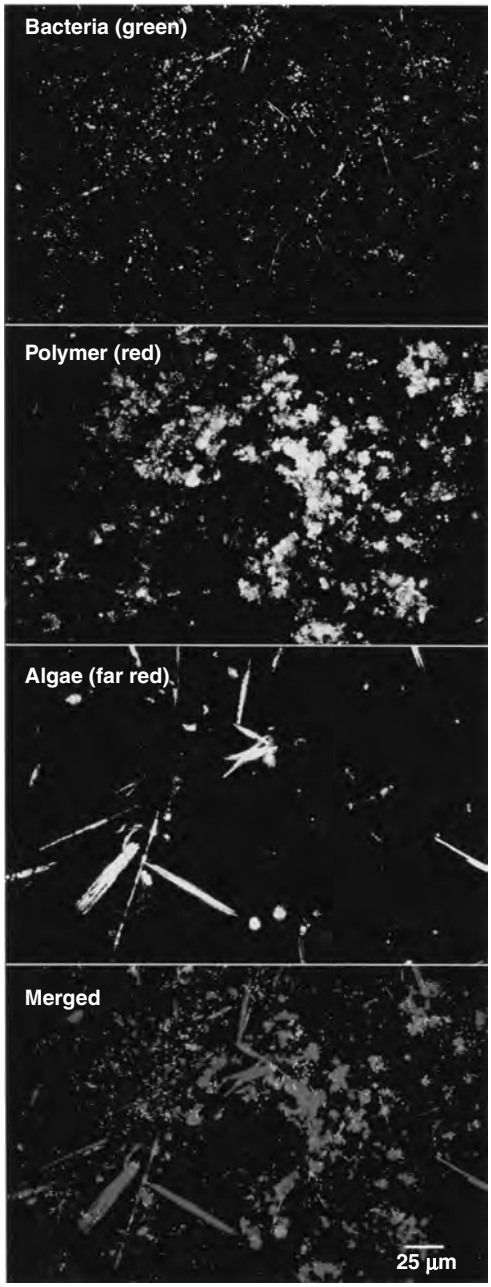

Figure 3. A series of confocal laser scanning micrographs, showing multichannel imaging of a single location in a river biofilm, visualizing Syto 9 stained bacteria recorded with the green channel (top image), *T. vulgaris* labeled extracellular polymeric substance recorded with the red channel (second micrograph), and the algae imaged by autofluorescence recorded with the far-red channel of the microscope (third micrograph). The bottom image shows the false color presentation of merging all three images, visualizing bacteria (green), algae (red), and extracellular polymeric substance (blue). See color insert.

snow (147,148). Like other biofilms, these river snow aggregates, which may be considered as mobile biofilms, consist of inorganic and organic compounds, extracellular polymeric substances (EPS), and the attached microbial populations. In comparison to the bulk water phase, river snow aggregates possess a higher nutrient concentration (149) and the associated bacteria show a higher rate of biological activity (150–152). Within suspended particulate matter, the formation of chemical gradients and anoxic microhabitats has been described (153). The river snow microhabitats are characterized by the occurrence of photosynthetic organisms (algae, cyanobacteria) and a broad range of different heterotrophic organisms including aquatic freshwater fungi (154,155). In the microbial aggregates, there is a pronounced patchiness of nutrients and consequently, these "hot spots" of high nutrient concentrations (156) and microbial life (157) show a high rate of heterotrophic metabolism and accelerate the mineralization process (158).

With regard to heterotrophic bacteria, the phycosphere of algae in which photosynthetic products are released is a particular hot spot of nutrients on a subcellular scale (159).

Because of the physiological plasticity of the mobile biofilm organisms, the river snow aggregates can also be regarded as miniaturized wastewater treatment plants and fulfill an important function within the self-purification processes in lotic systems. The high sorption capacity of the extracellular polymeric substances within the mobile river snow aggregates significantly contribute to the horizontal and vertical shift of inorganic and organic substances and link the pelagic and benthic compartments. Neu (147) examined the fully hydrated architecture of living river snow aggregates from the river Elbe, Germany, by multichannel confocal laser scanning microscopy. Simultaneous application of different fluorescently labeled lectins as carbohydrate-specific probes and general nucleic acid stains, visualized the heterogeneity of the EPS matrix in between the cellular constituents of the river snow community. CLSM performed in the reflection mode and in the far red channel gave additional information about the mineral content in the aggregates and the presence and spatial distribution of chlorophyll-containing organisms.

The phylogenetic diversity and physiological versatility of the Elbe river snow aggregates were investigated at the cellular level, using aerobic and anaerobic cultivation techniques, 16S rDNA based phylogeny, and fluorescent in situ hybridization (FISH) by Böckelmann and coworkers (148). FISH analysis of the river snow community revealed population dynamics to be governed by seasonal factors and more than 70% of the total bacterial cell count could be hybridized with a bacteria-specific probe, indicating the high physiological potential of the river snow community. During all seasons, β-Proteobacteria constituted the numerically most important bacterial group, forming up to 54% of the total cell counts. In contrast to this, the relative abundance of other major bacterial lineages ranged from 2% for the order Planctomycetales to 36% for Cytophaga-Flavobacteria. Cultivation of river snow bacteria under aerobic and anaerobic conditions with a variety of different media resulted in the isolation of 40 new bacterial strains. Phenotypic and phylogenetic analysis revealed them as mostly unknown organisms affiliated to different bacterial phyla. Some Elbe river snow isolates were affiliated to distinct freshwater phylogenetic clusters similar to organisms and environmental clones isolated from other

freshwater systems (160,161). The isolation of river snow bacteria with the potential for degradation of xenobiotics routinely monitored in the Elbe river water indicated the great physiological versatility of the river snow associated microorganisms.

Microbial community analysis by cloning and sequencing of 16S *rRNA* genes was conducted by Crump and coworkers to study the microbial ecology of free-living and particle-attached bacteria in the Columbia River and its estuary (162,163). Both studies showed the great diversity of bacterial sequences and the presence of putatively ubiquitous clades of bacteria within the aggregates. Interestingly, not only eubacterial sequences but also new clusters of archaeal 16S rRNA sequences could be identified, reflecting the great genetic diversity within the riverine microbial aggregates.

Drinking Water Biofilms

Drinking water distribution systems belong to the most oligotrophic aquatic habitats, and bacterial growth is severely limited by low contents of assimilable organic carbon (164) or by phosphorus availability (165). Nevertheless, basically all existing interfaces are partially covered by biofilms, from the waterworks through the distribution system to house installations. The existence of these biofilms was reported and documented several decades ago, based mostly on microscopic or electron microscopic examinations of pipe materials and encrustations from pipe surfaces (164,166).

In drinking water, biofilms are usually very thin and patchy, often consisting of a monolayer of cells. Thick biofilms can only develop if the colonized pipe material releases biodegradable dissolved organic carbon (BDOC), which is the substrate, allowing bacterial development in the drinking water. Apart from the availability of nutrients, the treatment of the raw water, use of disinfectants, temperature, type of surface material, and hydraulics of the system may affect the densities of biofilm bacteria (167–169). Bacteria growing in drinking water biofilms deteriorate the quality of the bulk water, and detachment of bacteria from the biofilms can additionally, negatively affect the water quality. Consequently, pipe and fitting materials used in drinking water systems have to be subjected to extensive testing. The unwanted growth of bacteria within drinking water distribution systems can be limited by distributing biostable drinking water that contains no easily degradable substrates and by applying biostable materials in contact with drinking water (170). The most often used methods for the assessment of the biostability of the materials are based on measurement of respiration activity in the presence and absence of test materials, on the detection of cell numbers by means of ATP content, and on scraping of the biofilm from the substratum and measuring its volume (7).

Drinking water biofilms can also contain high proportions of inorganic compounds, mostly consisting of iron and manganese. The deposits are caused, at least partly, by microorganisms (e.g., the genera *Hyphomicrobium*, *Siderocapsa*, *Gallionella*, *Leptothrix*, *Pedomicrobium*). These inorganic depositions and encrustations (e.g., flocs and tubercles) represent new habitats, where gradients for oxygen, redox potential, temperature, nutrients and toxins develop, thus offering growth conditions completely different from the free-water phase. Some bacteria can also promote corrosion by creating areas with different gradients in oxygen, minerals, and metals and also catalyze reactions associated with corrosion processes such as conversion of divalent iron to trivalent forms (171). In these new microhabitats, bacteria including pathogens may survive and even grow better, although the conditions in the flowing water are inhospitable (7,166,172).

Bacterial species isolated using conventional techniques from drinking water biofilms were typically identified as members of fast-growing genera such as *Pseudomonas*, *Acinetobacter*, *Alcaligenes*, *Flavobacterium*, *Moraxella*, *Arthrobacter* (164,166) mostly affiliated to the γ-subclass of Proteobacteria. In contrast to this, examinations with oligonucleotide probes in biofilms from different drinking water systems revealed the predominance of members of the β-subclass of Proteobacteria (20,173–175).

Recently, biofilm formation has been extensively studied in a house installation system in Berlin, Germany, with regard to population dynamics, species composition, and metabolic potential of the microbial community of young biofilms. Surfaces were found to be rapidly colonized, and a monolayer of rod-shaped β-Proteobacteria developed on the substrata. This young biofilm was grazed by amoebae, leading to a more diverse biofilm structure and population composition. The metabolic potential of the population, assessed by CTC-reduction and a modified DVC-assay (176), decreased with biofilm development. The in situ dominant species could be successfully isolated from the early biofilm formation stage and described as members of the new genus *Aquabacterium* within the β-subclass of Proteobacteria. The occurrence of the genus *Aquabacterium* could also be shown for other drinking water distribution systems (167), regardless of the raw water source used for the drinking water supply. The species composition in established biofilms, however, is expected to be far more complex (20,54,167).

CONCLUSION

- Biofilms are complex microbial communities and highly heterogenous in their structure, composition, and function.
- Biofilms are formed by bacteria, algae, fungi, protozoa, as well as organic compounds and inorganic materials.
- Biofilms are colonizing virtually each known interface of natural and engineered systems.
- The individual biofilm-associated cells within the biofilm communities are linked by extracellular polymeric substances (EPS).
- There is an complex exchange of genetic material between the individual members of the biofilm communities.
- The individual cells in the biofilm communities are communicating to each other by chemical transmitters

that regulate properties of the individual members (e.g., individual cell shape, motility) and of the whole bacterial population (e.g., colony shape, cell density).

- In nature, biofilm communities often form complex stratified microhabitats and foodwebs (e.g., photosynthetic cyanobacterial communities and heterotrophic sulfate-reducers).
- Biofilms in riverine systems play an important role in nutrient and energy metabolisms, can be regarded as hot spots of microbial activity, and are responsible for the self-purification ability of rivers.
- In drinking water distribution systems, biofilms are hygienically and technically important in terms of affecting both the quality of the drinking water and the materials used for pipes, reservoirs, and fittings in the distribution system.

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BIOFILMS IN THE FOOD INDUSTRY

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This entry deals with biofilm formation, detection, and elimination on surfaces in the food processing environment. Biofilms have been used in research on sanitation, that is, cleaning and disinfection, in the food industry since late 1980s. Microbes in microcolonies have a tendency to form protective extracellular matrices, which are called *biofilms*. The microcolony formation is the start-up in the biofilm formation. The biofilm formation occurs under suitable conditions on any — both inert and living — surface. Besides a surface, the microbes need only water to start-up this formation. The mechanisms of the microbial attachment and biofilm buildup can be divided into the following steps: (*1*) preconditioning of the surface by macromolecules, (*2*) transport of cells to the surface, (*3*) reversible and irreversible adsorption to the surface, (*4*) cell replication, (*5*) transport of nutrients and metabolism, (*6*) production of extracellular polymers, and finally, (*7*) detachment. The food hygiene legislation, the hygienic design of machinery together with increasing public awareness of product quality, make testing of process surfaces and equipment cleanliness important. Microbiological, physical, chemical, and microscopy methods can be used in studying the biofilm buildup and its elimination. Advanced molecular biology methods can also be used in the detection and enumeration of specific organisms on surfaces. Determination of disinfectant efficacy is, however, still performed in suspensions, which do not mimic the conditions on surfaces where the agents are expected to inactivate microbes within biofilms as well. The elimination of biofilm is a very difficult and demanding task because many factors such as temperature, time, mechanical, and chemical forces affect the detachment.

FORMATION OF BIOFILMS IN THE FOOD INDUSTRY

Biofilm formation causes problems in areas such as industrial water systems (1–3), medicine (4–6), and in the food-processing industry (7–12). Biofilm formation has both positive and negative implications in food related processes (13). It has been used effectively in the food industry in the production of vinegar (12) and beer (14).

Biofilms can generally be produced by any microbes under suitable conditions, although some microbes naturally have a higher tendency to produce biofilm than others. The most common food-borne biofilm producers belong to the genera *Alcaligenes*, *Bacillus*, *Enterobacter*, *Flavobacterium*, *Pseudomonas*, and *Staphylococcus* (10). It is somewhat alarming that bacteria such as *Listeria monocytogenes*, *Escherichia coli*, *Salmonella typhimurium*, and *Yersinia enterocolitica* produce biofilms readily, causing severe sanitation problems on surfaces in the food industry (15–21). Experiments have been carried out with *L. monocytogenes*, *Pseudomonas fluorescens*, and *Y. enterocolitica*, which cause contamination of materials used in gaskets (22). *Pseudomonas* spp. have also been found to attach easily to surfaces of hydrophobic materials (e.g., polystyrene. rubber- and teflon-based materials) used in gaskets. Some microbes are also able to decompose rubber and use it as a source of energy (23–24). Biofilm-bound bacteria can accelerate corrosion and material deterioration, for example, in sensors and detectors (25–28). Microbial corrosion has been observed in processing equipment, appearing either in combination with or without biofilm formation. Corrosion leads to huge losses in different industrial areas, for example, piping and cooling water systems (26,28).

In the food industry, equipment design plays the most important role in combating biofilm formation (29,30). The choice of surface materials is of great importance in designing and building equipment and processing lines for industrial use (31). Dead ends, corners, cracks, crevices, gaskets, valves, and joints are vulnerable points for biofilm accumulation (29,32–33). The process equipment is easy to clean if the surface materials are smooth and in good condition (Fig. 1). It should be cleaned frequently to avoid the accumulation of biofilm. The choice

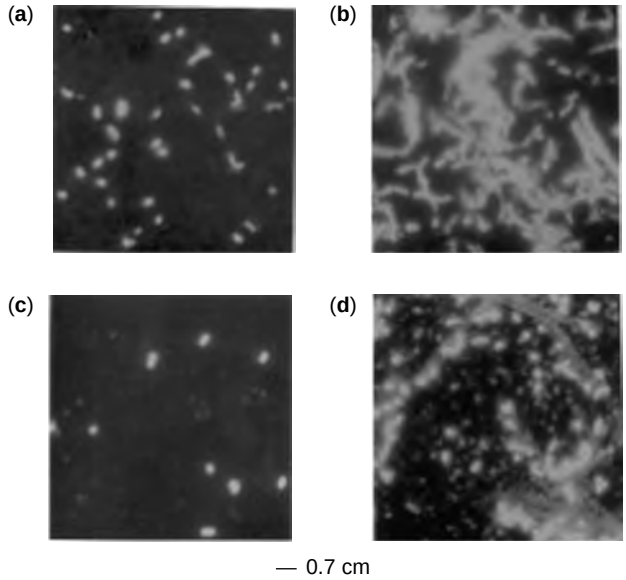

Figure 1. Epifluorescent microscopy photographs of 2 and 10 days old *L. monocytogenes* biofilms stained with acridine orange. The biofilms had been grown on standard as well as electrochemically polished 2B stainless steel surfaces (AISI 304): (**a**) 2-day-old and (**b**) 10-day-old *L. monocytogenes* biofilm on standard 2B, (**c**) 2-day-old and (**d**) 10-day-old *L. monocytogenes* biofilm on electrochemically polished 2B surfaces. The scale marker is equivalent to 0.7 cm. See color insert.

of materials and their surface treatments, for example, grinding and polishing, are important factors in inhibiting the formation of biofilm and in promoting the cleanability of surfaces (34). Treatments of surface materials to reject biofilms can be performed actively to remove or passively retard biofilm reoccurrence. The cleanliness of surfaces, training of personnel, and good manufacturing and design practices are the most important tools in combating biofilm problems in the food industry (30).

Besides causing problems in cleaning and hygiene (35), biofilm can cause energy losses and blockages in condenser tubes, cooling fill materials, water, and wastewater circuits and heat exchangers (36). Biofilms can also cause health risks by releasing pathogens in drinking water distribution systems. Biofilms may enter a food processing system by causing reduced effectiveness in ion exchange and membrane processes (37–38). In the food processing water supply systems, biofilms cause problems in granular activated carbon columns, reverse osmosis membranes, ion exchange systems, degasifiers, water storage tanks, and microporous membrane filters. The bacterial level of the purified water used and the defects found in microelectronic devices correlate directly. Conductance, electromigration, and corrosion in the oxide layers have been observed in devices manufactured with insufficiently pure water (2). Accumulation of mixed population biofilms containing sulfate-reducing bacteria causes corrosion in industrial water systems. A summary of biofilm effects in various processes follows:

- Reduced product quality in food production and processes producing goods for food handling, for example, package material
- Reduced water quality (e.g., pathogenic organisms) in cooling systems and in water distribution systems
- Contamination of immobilized systems, for example, loss of specific chemical transformations
- Material deterioration in metal condenser tubes, pipelines, and cooling systems
- Energy losses in heating of the process equipment
- Reduction in the effectiveness on remote sensors, sight glasses, etc.

Disinfection after removal of biofilms using suitable cleaning procedures is required in food plants where wet surfaces provide favorable conditions for the growth of microbes (22). In practice, a biofilm left on improperly cleaned surfaces is a barrier between microbes and the disinfectants, antibiotics, or biocides used against them (39–42). The permeability barrier of the biofilm considerably affects the efficiency of these agents. The effect of many antibiotics is based on inhibition of active growth. It has been said that most of the bacteria in biofilms are no longer growing actively and their resistance is therefore altered. The resistance of microbes in a biofilm cannot be proved without controlling their growth rate (43).

The food industries in which research on biofilms has been carried out are those producing canned products, meat and poultry products, pastries, biscuits, pizza, fish cakes, cheese, milk products, beer, spices, vegetable and salad products (20,42,44–49). Biofilms cause problems at various sites: air-handling systems (46,50–51), blancher extractors (46), conveyors (12,46), cooling systems (46,50), floors (12,52), floor drains (12,46), food contact surfaces of stainless steel (35,42,46), gaskets (45,48–49), heat exchangers (47,53), manufacture line for paper-based packaging material (28,54), milk-transfer lines (45,47), poultry-processing equipment (55), rubber-fingered pluckers (44), mixers, slicers, packaging machines (46), pasteurizers (47), ultrafiltration and reverse osmosis membranes (38,47), vegetable lines (46). We can see from the list that problems from biofilm can occur anywhere in the food process if the design and maintenance is improper.

Open Process Lines in Food Processing

Although there is a long history of biofilm impacting the food industry, actual biofilm research in this industrial area began in the late 1980s (8,56). The occurrence of slime-forming microbes is a major problem in the sanitation and disinfection of process equipment. Good surfaces, instruments, and equipment hygiene in the food industry promotes the quality of the products processed (55,57). Commonly found microbes on food contact surfaces are enterobacteria, lactic acid bacteria, micrococci, streptococci pseudmonads, and bacilli. Problems with the accumulation of particulates and cells will occur whenever cleaning is for any reason inappropriate (52). The most useful material in processing equipment is steel, which can be treated with, for example, mechanical grinding, brushing, lapping, and electrolytic or mechanical polishing. In electrolytic polishing, a preground surface is treated in an electrolyte bath to obtain an even surface. The surface structure of stainless steel is very important in avoiding biofilm formation (Fig. 2). It has been reported that although the grain boundaries of AISI (American Iron and Steel Institute) 316L acid-resistant stainless steel constitute 3 to 20% of the total surface area, over 90% of the adherent bacteria were found attached to the grain boundaries (58).

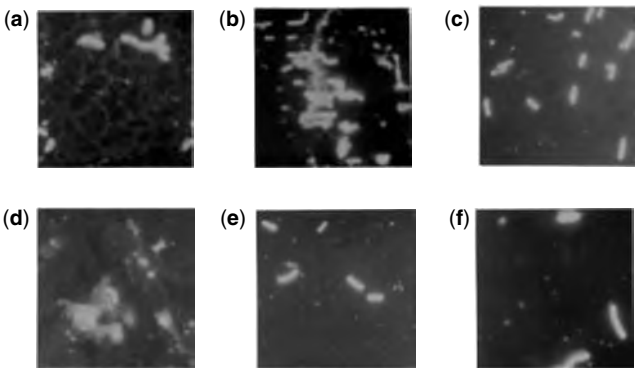

Figure 2. Epifluorescent microscopy photographs of 2-day-old *Bacillus subtilis* biofilms stained with acridine orange. The biofilms had been grown on various stainless steel surfaces (AISI 304): (**a**) standard 2B, (**b**) standard 4N, (**c**) standard BA, (**d**) glass blasted 2B, (**e**) brushed 2B and (**f**) electrochemically polished 2B surfaces. The scale marker is equivalent to 0.5 cm. See color insert.

Inadequate cleaning and sanitation of surfaces coated with biofilm cause contamination because the biofilm protects the microbes against sanitizers and disinfectants (22,59). Many equipment faults can be avoided by using good design practice guidelines, which are summarized in Table 1 (10).

The lubricants used in conveyors can function as a contamination source, especially in dairies and breweries. Oil-based lubricants containing water are very sensitive to microbial growth. *Acinetobacter* spp., *Alcaligenes* spp., *Pseudomonas* spp., and sulfate-reducing bacteria have been isolated from lubricants, and the biofilm formed in the lubricant can indirectly promote corrosion (60). *Listeria monocytogenes*, an opportunistic pathogen, has been isolated from lubricants in dairies (61). Problems with biofilms are also met in engineering works in which lubricants based on vegetable oils are used. Synthetic lubricants containing biocides have been developed, and the problems associated with contamination of lubricants has been decreasing (62).

Closed Equipment in Food and Drink Production

Poorly designed sampling valves can destroy an entire process or give rise to incorrect information because of biofilm effects at measuring points. Owing to their construction, valves are vulnerable to microbial growth and thus constitute a hygiene risk (29,63). Hoses, tubes, filters, etc. containing polyvinylchloride increase the risk of contamination (64). Biofilm is easily formed on measuring probes and the inner parts of equipment because these are usually difficult to clean (45,47). Microbial accumulation is known to be a problem on reverse osmosis membranes (65). When crust has fouled the probe no probe for measuring properties in biofilms can provide an accurate description of the process (29). The penetration of disinfectants and heat are hampered by the porous matrix on the surface, which diminishes the effect of sanitation and sterilization procedures. The bacterial slime of *Bacillus* sp. improved the heat resistance of the bacterium, extending the autoclaving time required for successful sterilization to several hours. Biofilm components can also alter the resistance to steam and formalin (10).

Table 1. Factors in Good Design Practice (10)

Design Area with Relevant Factors
• Organization and personnel responsibility functions, tasks, education, and courses
• Production and production control production: process parameters, installations, and maintenance of hygiene production control: working plan, material flow, and disturbance reports
• Equipment, pipelines, and equipment layout equipment: materials, surface finishing, accessories and joints, checking and cleanability, clearing and cleaning and insulation pipelines: accessories, materials, surface finishing, clearing and cleaning, consoles and insulation equipment layout: cleanability and cleaning of equipment, servicing of equipment, rationalization of operations and systematic layout
• Buildings and structures, electrification, and automation buildings and structures: materials, painting, sewerage, tipping, joints, cleanability of surfaces, layout of the connections of different production units and air-conditioning electrification: shielding of equipment and lighting automation: control of production and process reliability

Cooling Systems

Biofilm formation in process water systems has been known for a long time (3,66–68) and the adhesion of microbial cells to the surfaces in water distribution systems is also well known (1,69–71). The formation of biofilm takes several days at least, or weeks, in low-nutrient water systems. Biofilms formed in slow-flowing systems are structurally different from those formed in systems with high flow rates (72–73). The number of free planktonic cells in water does not necessarily correlate with the amount of biofilm on the pipe surfaces. The numbers of microbial cells adsorbed to surfaces may be as much as 500 to 50,000 times the numbers of planktonic cells in water (74). Biofilm formation appears in sinks, faucets, valves, and in different joint areas through the piping systems (3,75). It has been found that temperatures below 50 °C promote biofilm formation (72). The average temperature in cooling water systems is 35 °C, which is close to the optimum temperature for most microbial growth. From a hygienic point of view, biofilm can include organisms that cause infections and diseases. Problems with *Legionella pneumophila*, a pathogenic organism that is able to form biofilm, can occur in hot water systems (76–78). Biofilm formation can be decreased by more than 50% if the oxygen level in the water system is minimized, and by more than 80% when the amounts of both oxygen and nutrients are minimized (72). Anaerobic conditions favor biofilm formation by anaerobic organisms, which can increase corrosion (73,79).

Ventilation and Air-Handling Systems

Air quality in food production facilities is very important for the final product quality. The microbial population in the air channels depend on the environment, filtration membranes, and the sitings of airholes (51,80,81). Formation of biofilm in air-conditioning systems does not occur without a water reservoir of some kind. Normally, there is no water in the air-conditioning systems, but it can accumulate unintentionally through condensation. The pathogen *L. pneumophila* has been isolated from water systems that were connected to an air conditioner (82). When the air-conditioning system is to be cleaned and disinfected, it is very important that the disinfection medium penetrates the biofilm and does not simply flow through the system with the air (10).

A study carried out in various industrial ventilation ducts has shown that biofilms can accumulate

within the ducts. The occurrence of bacterial and fungal biofilms takes place within a few weeks. Biofilms form very easily in humid surroundings where oil has been left on the surfaces. Biofilm growth was prevented in clean room ducts by using effective filters and appropriate maintenance procedures (50). It is therefore very important to monitor clean room behavior, as the majority of particles in clean rooms originate from the personnel (81,83). The air filtration equipment must be chosen carefully to suit the processing environment (83,84). The membranes in the air-conditioning system and the walls in the air-conditioning channels are places where biofilms start to grow (80,85).

Production of Paper and Packaging Material

Biofilm formation causes major problems in paper machines and it affects the production of good quality paper-based packaging material for the food industry (28,54). The paper machines are connected to water-circulation systems, which are prone to rapid biofilm growth (86). The microbes, mostly bacteria and fungi, enter the process with the nutrients and from the air (87). The use of recycled fibers and changes in the acidity of the process to neutral or alkaline have increased the total amount of bacteria in the paper-making processes (88). It should be remembered that each paper-making machine has its own microbial flora, which depends on processing parameters such as pH and temperature, the season, and the additives used. The slime forming microbial flora can be divided into primary slime formers, for example, bacteria of the *Bacillus*, *Pseudomonas*, and *Enterobacter* genera and fungi of the *Aspergillus*, *Mucor*, and *Penicillium* genera, and secondary slime-formers, for example, bacteria of the *Alcaligenes, Flavobacterium, Klebsiella, Micrococcus*, and *Staphylococcus* strains, and fungi of the *Paecilomyces, Trichoderma*, and *Trichosporum* strains (89). Practical experiments show that biofilm in pulp- and paper-processing equipment detaches easily from the surface and the loose slime causes holes in the paper (90). Bacterial spores withstand the heat treatment during the production and therefore the *Bacillus* genera, which are spore formers, are the main source of contaminants in final paper and cardboard products (91). The slime in paper-machine biofilms contains 10^{11} to 10^{12} cfu/g dry mass. The microbial growth causes problems in corrosion, process flow, contamination, and paper quality (92). It also causes odor problems, which can destroy both the air quality in the process facilities and the quality of the end products (88). Bacterial biofilms of anaerobic sulfate-reducing bacteria, thermophilic bacteria, and bacteria producing acids and reducing compounds (5) cause corrosion of surfaces in the paper machines. Microbial corrosion causes more than 50% of all corrosion problems in pipelines in paper-processing machines. Efforts to combat microbial growth and biofilm formation in paper machines are important because the costs of maintenance of equipments are high (28,92).

DETECTION AND ELIMINATION OF BIOFILMS

Detection of Biofilm

Many reviews have been published on the formation and detection of biofilms in the laboratory (34,42,93,94). Methods of studying biofilm formation include microbiological, physical, chemical, and microscopical methods, and, methods commonly used in molecular biology (Table 2; 34,42,94–97). The biofilm consists of 85 to 96% water, which means that only 2 to 5% of the total biofilm volume is detectable on dry surfaces (4). Monitoring practices based on sampling of the liquid phase does not reflect the location or extent of microbes growing on surfaces. The counts of planktonic cell in the processing fluids should not be used in detecting biofilms because they do not represent the sessile organisms (98).

Inadequate cleaning and sanitation of surfaces coated with biofilms represents a source of contamination within the process (42). Practical methods for assessing microbes and organic soil on processing surfaces are needed to establish the optimum cleaning frequency of the equipment. These systems should, preferably, provide information online, nondestructively and in real time, about microbial growth required within the process. Online monitoring techniques transferred from various industrial fields, for example, glass fiber optics, infrared techniques, ion-mobility techniques, bioluminescence, microelectrodes, and heat transfer, could be used for assessing the quality of raw material and products and for control of microbial and chemical contaminants on processing surfaces. Advanced molecular biology methods can be used in the detection and enumeration of specific organisms on surfaces (34,99–101).

Both open and closed process equipment should be easily cleanable, and therefore, cleanability testing methods are needed. Development of practical detection methods for research on biofilm buildup is needed in order to use the knowledge of biofilm accumulation in the maintenance of process equipment. The natural luminescence of marine bacteria has been used to assess biofilms in marine systems and for on-line evaluation of biofilm formation and antifouling coatings (102,103). The transformation of luminescence-encoding *lux*-genes to food-spoiling bacteria or opportunistic pathogens makes it possible to use bioluminescence in cleanability studies (104). Further efforts are needed to optimize the microbial cleanability assay, using, for example, the *Photobacterium leiognathi* procedure (42), and to adapt the assay to both open and closed systems.

Chemical Agents Used in the Elimination of Biofilms

Efficacy of disinfectants and antimicrobial agents are usually determined in free-cell suspensions, which do not mimic the growth conditions on surfaces where the agents are required to inactivate the microbes (105–108). The agent must reduce the microbial population by 5 log units in suspensions in order to be considered effective. The goal for reduction of surface-attached bacteria with disinfectants is 3 log units (22). The standard suspension tests have proved sufficiently reliable because the

Table 2. Summary of Main Methods Used in Process Hygiene (34)

Detection Method	The Application Assesses
Conventional cultivation based on swabbing and contact agar techniques	Living bacteria on various types of growth media Limitation: sensitivity, time consuming
Impedance measurement	Activity of viable and injured bacteria directly and indirectly after reactivation in liquid growth media Limitation: sensitivity
ATP measurement	Total hygiene; large scatter in analysing biofilms because of different metabolic status of cells Limitation: sensitivity
Measurement of protein residues	amount of protein-based soil Limitation: sensitivity
Microscopical techniques using various staining procedures:	Limitation: not for routine use on-site
• epifluorescence microscopy	Area coverage; microbial distribution; cell type and cell viability; identification of microbes
• electron microscopy	Morphology of microbes; cell distribution; identification of microbes
• scanning confocal laser microscopy	Biofilm structures; ecology of biofilms; identification of microbes
• interference reflection microscopy	Adhesive interactions between surfaces and microbes
• differential interference contrast microscopy	Individual microbial cells (not for quantitative analysis)
Flow cytometry	Microbial cell densities Limitation: detachment of cells
Polymerase chain reaction (PCR)-based methods	Unculturable microorganisms; identification of bacteria in complex ecosystems Limitation: information on presence, but not on numbers and Viability of target organisms

variations of results are within acceptable limits when replication is adequate. There can, however, be problems with the repeatability and reproducibility of suspension tests if the organic load in the suspension varies. It is obvious that the surface tests are even more difficult to perform than suspension tests because of the carrier material used and the viability of dried cells on the surfaces (109,110).

Various disinfectants have been developed to destroy microbes (111). Microbes have, nevertheless, been found in disinfectant solutions, which means that microbial contaminants can be spread on the surface with the solution. Findings as early as 1967 reported that chlorhexidine mixtures were contaminated with *Pseudomonas* spp., which also have been found in concentrated iodine solutions (112). *Serratia marcescens* was found to be viable even after 27 months in a disinfectant containing 2% chlorhexidine. A concentration of 0.1% chlorhexidine is sufficient to kill the cells of *S. marcescens* if they are freely suspended in liquid (74). Microbial contamination has also been found in solutions of aldehydes and quaternary ammonium compounds (82). Other microbes that have been isolated from disinfectants include *Alcaligenes faecalis*, *Enterobacter cloacae*, *E. coli*, *Flavobacterium meningosepticum*, and *Pantoea agglomerans*.

Cleaning Agents. Cleaning agents are applied to remove soil, microbes, and biofilms from surfaces. The effects of the cleaning agents on biofilms have been thoroughly investigated (22,57,59,106). Removal of biofilm is important for the maintenance of the equipment, because the debris left on surfaces can act as nutrients for the buildup of new biofilm (36), or may corrode the equipment material. To minimize biofouling, it is best to clean the equipment at frequent and regular intervals, using suitable efficient cleaning procedures for the process before the biofilm has the opportunity to develop (113,114).

Efficient cleaning of surfaces depends on surface tension, viscosity, chemical reactivity, size of biofilm particles, solubility, and living microorganisms (115,116). The cleaning agents should be surface-active, soluble, easy-to-rinse, noncorrosive, and easy-to-use. They should also emulsify fats and oils, suspend insoluble particulates, and dissolve mineral deposits. Soaps and synthetic detergents clean surfaces in a similar manner, the surfactant molecule having hydrophilic and hydrophobic ends. The hydrophobic end dissolves oily soils (116). The alkaline and acidic detergents are mixtures of several classes of compounds: alkalis, acids, tensides, and surface-active agents (116). Currently used cleaning agents contain chelators, for example, EDTA, surfactants, detergent builders, antideposition agents, enzymes, and disinfectants (1,116,117).

The chelators, however, are not biocides and should therefore be combined with other antibacterial compounds (82). Chelators bind magnesium and calcium ions, thereby destabilizing the outer membranes of microbial cells and extracellular matrices. Chelators have been used in the breakage of the biofilm layers (118,119). Tensides

are large molecules with polar and nonpolar groups forming hydrophobic and hydrophilic parts, depending on the group. The tensides are divided into four types: anionic, cationic, nonionic and amphoteric tensides. Tensides are normally used in cleaning agents to achieve suitable cleaning and foaming effects. The amphoteric tensides contain both anionic and cationic parts and can therefore function both in acidic and alkaline environments or as double ions in the boundary region (120).

Disinfectants. Disinfection is required in food plant operations in which wet surfaces provide favorable conditions for the growth of microorganisms (57,121). The aim of disinfection is to reduce the surface population of viable microorganisms after cleaning, by destruction or removal, and to prevent microbial growth on surfaces during the interproduction period (122). Disinfectants do not penetrate the polysaccharide and glycoprotein matrix left on the surfaces after an ineffective cleaning procedure, and thus do not destroy all the living cells in biofilms (7,11). Disinfectants are most effective in the absence of organic material, for example, fat-, sugar- and protein-based materials (59). Microbes are less likely to survive disinfection after an effective cleaning. The efficiency of disinfectants is generally controlled by interfering organic substances, pH, temperature, concentration, and contact time (22,123). The desired characteristics of disinfectants are the same as for cleaning agents. They must be effective, safe, and easy-to-use, and easily rinsed off surfaces, leaving no toxic residues or residues that affect the sensory values of the product (116).

The use of disinfectants in food plants depends on the material used and the adhering microbes (116). It is therefore recommended that disinfectants used in the sanitation of food-processing equipment should be tested on surfaces in circulating systems. Disinfectant testing in a circulating system provides many replicates, which makes the evaluation easier and more rapid to perform. The variety in soil types, cleaners, and cleaning conditions makes it impossible to give an exact, overall statement on required temperatures and concentrations (124). Disinfectants approved for use in the food industry contain chlorine-based compounds, iodine compounds, alcohols, quaternary ammonium compounds or surfactants (1,116,125,126). The concentration of the disinfectant can be altered to provide shock treatments. It has also been suggested that the disinfectants should be changed continuously. In the maintenance of process hygiene, disinfectants should be chosen according to the process (127).

Chlorine or chlorine-based compounds that are approved for use in food plants, for example, gaseous chlorine, sodium hypochlorite, calcium hypochlorite, chloramine-T, and chlorine dioxide. The antibacterial active moiety is formed when chlorine gas or a hypochlorite compound is added to water and produces hypochlorous acid. Stabilized hypochlorites are used when disinfection of long duration is required (116). The range of microorganisms killed or inhibited by chlorine-based compounds is probably broader than that killed by any other approved sanitizer. Stressing factors such as chlorination can also induce biofilm buildup, for example, of *Pseudomonas* sp. (70). *Pseudomonas* spp. are relatively resistant to chlorine treatments and can even multiply when chlorine has been used. Free chlorine (1.0 mg/L) showed no effect on coliforms growing in biofilms, and even increased concentrations (2.0 mg/L) did not kill *E. coli* grown in biofilms. The material on which microbes form biofilm has a major influence on the effect of the disinfectants used. Capsulated *Klebsiella pneumoniae* grown on glass surfaces has been shown to have a 150-fold resistance to chlorine compound to those grown in suspensions. When a low-nutrient liquid is used, the resistance factor is increased to about 600. On metal and carbon surfaces, the corresponding resistance ratios were about 2,400 and 3,000 times the value in suspension, respectively (70). The effect of surfactant sanitizers against *L. monocytogenes* grown in biofilms was decreased (107,128); however, sodium hypochlorite and quaternary ammonium compounds have proved effective against 24-hour old biofilms (129).

Iodophors are used extensively in the food industry. Iodine oxidizes essential parts of the microbial cells. Like chlorine-containing products, iodophors are active against gram-positive and gram-negative bacteria, yeasts, and molds (116,122,125). Bacterial spores, however, are highly resistant to iodophors. Iodophors cannot be used in food plants in which starch-containing products are produced because iodine forms a purple complex with starch (116). Quaternary ammonium compounds are used as sanitizers in dairies and in the food industry because they have good wetting properties and are nonspecifically described as cationic surface-active agents in which the cationic part is hydrophobic. The greatest effect of quaternary ammonium compounds is observed against gram-positive bacteria, whereas gram-negative organisms, many of them significant in the contamination of food, may not be affected (116).

Hydrogen peroxide has been found to be effective in removing biofilms from equipment used in hospitals. The effect of hydrogen peroxide is based on the production of free radicals, which affect the polysaccharides and glycoproteins in the biofilm (130). The biocidal effect of peracetic acid on microbes in biofilms was shown to vary (131–133). Aldehydes did not disrupt the biofilm, but rather seemed to improve its stability. The biofilm must be disrupted in some way before chemical agents such as peracetic acid and aldehydes can be used effectively (131). The effect of ozone treatment has been found to vary depending on the processing circumstances and the microbes tested; for example, ozonation proved very effective in the treatment of cooling water systems (134,135). Physical disinfection can also be performed using ultraviolet irradiation, pulsed laser beams, or steam disinfection (116).

Elimination of Biofilms in Industrial Systems

General Aspects of Cleaning. Physical, chemical, and microbiological cleanliness are essential in food plants. Factors governing the selection of detergents and disinfectants in the food industry are that the agent should be efficient, safe, not damage or corrode equipment, be easy-to-rinse, and not affect the sensory values of

the product (59,113). Physical cleanliness means no visible waste, foreign matter, or slime on the equipment surfaces. Chemically clean surfaces are surfaces from which undesirable chemical residues have been removed, whereas microbiologically clean surfaces imply freedom from microbes (124). Attached bacteria or bacteria in biofilms can be a problem in food processing and grow and multiply after insufficient cleaning and contaminate the product (35). Once a biofilm is firmly established, cleaning, and disinfection becomes much more difficult (22,136). Caution should be exercised in selecting appropriate disinfectants for contaminated surfaces.

The key to the effective cleaning of a food plant is the understanding of the type and nature of the soil (e.g., sugar, fat, protein, and mineral salts) and of the microbial growth on the surfaces to be removed. The accessibility and type of equipment and accessories to be cleaned and the availability of suitable cleaning agents are also important (116). Intelligent integration and coordination between cleaning programs and manufacturing operations are essential to achieve both successful cleaning and business profit (116). An efficient cleaning procedure consists of a sequence of rinses and detergent and disinfectant applications in various combinations of temperature and concentration (113,131). This controls the development of biofilms on equipment surfaces without corroding the surfaces (113,114). An independent quality-control system to monitor the cleaning results for a food plant can be integrated in the program based on Hazard Analysis of Critical Control Points (HACCP). The goal to achieve a clean food plant must be desired by the plant management, which has to invest the necessary time and money to accomplish it. The personnel must be properly trained and responsible enough to maintain a good level of plant hygiene. The tools and materials must be properly designed for the plant equipment and the methods used must suit the process (122,124).

Factors affecting the cleaning process are based on mechanical and chemical impact, holding time, and temperature (22,59,116). The basic task of detergents is to reduce the interfacial tensions of soils so that the soil becomes miscible in water (116). The effect of the surfactants is increased by the mechanical effect of turbulent flow or water pressure, or of abrasives, for example, salt crystals. A prolonged exposure of the surfaces to the detergent makes the removal efficient. Detergents to be used in the cleaning of open systems are formulated to be effective at room temperature or at slightly elevated temperatures in the range 35 to 50°C (116). In closed systems, the detergents are formulated to be used at temperatures in the range of 55 to 80°C. Fats are easily removed at temperatures slightly above their melting point. Sugars and other carbohydrates are water-soluble at elevated temperatures, but temperatures causing caramelization should be avoided. Proteins are denatured at elevated temperatures and may adhere strongly to surfaces at high-temperatures. Cleaning and disinfection procedures can be optimized with pilot-scale equipment for both closed and open processes.

Elimination of Biofilm in Open Systems. Gross soil should be removed by dry methods, for example, brushing, scraping, or vacuuming, and visible soil rinsed off with low-pressure water. The cleaning effect is increased by using water of sufficient volume and temperature. However, a pure-water washing system is not practical because of its ineffectiveness and cost limitations. Pressure cleaning is used to remove the remainder of the soil and most of the microorganisms present on open surfaces (122). The elimination of biofilm from open systems, for example, surfaces in food-processing equipment, has not been widely reported. Biofilms were used in cleaning and disinfection studies for the food industry (8,56). After a production run, the equipment should be dismantled and the cleaned utensils should be stored on racks and tables, but not on the floor. The cleaning of open-process surfaces is carried out using either foam or gel cleaning. Foams are most effective in situations in which contact with the soil for an extended period of time is necessary. The foam-units are constructed to form foam of varying wetness and durability depending on the cleaning to be performed. The application of gels extends the contact time with a soiled surface and can be used with low-pressure systems (116). The cleaning is mostly carried out in combination with a final disinfection because there are likely to be viable microorganisms on the surfaces that could harm continued production (57,122,131).

Elimination of Biofilm in Closed Systems. In the cleaning regime applicable to closed systems, prerinsing with cold water is carried out to remove loose soil. The cleaning-in-place (CIP) treatment is normally performed using hot cleaning solutions (29), but cold solutions can also be used in the processing of fat-free products. The warm alkaline cleaning solution, normally of 1% sodium hydroxide (NaOH), is heated to 75 to 80°C and the cleaning time is 15 to 20 minutes. The equipment is rinsed with cold water before the acid treatment is performed at approximately 60°C for five minutes. The effect of chlorine-based agents can be divided into three phases: loosening of the biofilm from the surface, breakage of the biofilm, and the disinfecting effect of the active chlorine (137). The cleaning solutions should not be reused in processes aiming at total sterility because the reused cleaning solution can contaminate the equipment (29).

Studies on CIP cleaning showed that the "cleaning effect "decreased markedly when the washing time and the flow rate were reduced, indicating that the cleaning as a whole was affected by mechanical effects, time, temperature, and chemical parameters (57,59). Turbulent flow is therefore a decisive factor in combination with chemical compounds in the elimination of biofilms from closed systems. To ensure the removal of soil and to avoid biofilm formation and soil sedimentation, the minimum flow velocity in the CIP treatment must be at least 1.5 m/s, but 2.0 m/s is recommended. The flow should be turbulent with a Reynold's number from at least 10,000 to preferably 30,000, to ensure good radial mixing and heat, mass, and momentum transfer (138). Chelating agents in the cleaning solution enhance the removal of biofilms from processing surfaces. The CIP-systems

used today are based on a combination of alkaline-acid treatments and time-temperature treatments. Problems caused by equipment constructions, valves, and surface materials cannot be eliminated with CIP because the CIP treatment was not designed to eliminate biofilms. In normal operation, it is impossible to clean pipelines completely by mechanical treatment because there are always bends, corners, pockets, and cracks where biofilm remains (70). The depth of dead zones in the system should be less than two pipe diameters if the dead zone cannot be avoided, to ensure adequate cleaning throughout the system using CIP procedures. The maintenance programs should guarantee that excessive biofilm accumulation does not occur. Tanks should be cleaned by applying the cleaning solution through properly installed, removable spray balls or nozzles. The design should ensure that the part of the tank directly above the spray ball is also cleaned (29). Drainage, minimization of internal probes, crevices and stagnant areas, arrangement of valves, couplings, and instrument ports and instrumentation should be planned carefully so that the equipment is easily cleanable (29).

Legislation on food hygiene and the hygienic design of food machinery, together with the public awareness of product quality and manufacturers' desires to improve product safety, makes reliable cleanability testing an important issue. In this type of testing, it must be possible to assess the relative cleanability of various equipment components to facilitate the design, testing, and maintenance of hygienic food processing equipment. The assessment must use standardized test procedures with a sound scientific basis (139). The aim of the European Hygienic Equipment Design Group (EHEDG), which is an independent consortium of representatives from research institutes, the food industry, equipment manufacturers, and government organizations is to develop hygienic equipment on a scientifically and technologically sound basis.

CONCLUSION

Cleaning in the food industry should be on the basis of systematic planning. Biofilm formation in these systems is a symptom of disturbance in the process because biofilms can be produced under suitable growth conditions by any microbe. Biofilms are less likely to accumulate in well-designed systems, which are effectively cleaned. Detection of buildup on equipment surfaces at an early stage enables effective countermeasures and thus results in an improvement of the process hygiene. Methods used for monitoring the microbial load are based on conventional cultivation techniques using various types of growth media and for the total hygiene ATP (adenosine triphosphate). Careful thoughts must be given to the cleaning procedures, including the program, cleaning agents, disinfectants, and cleaning equipment. Results indicate that low-pressure cleaning is not sufficiently effective to remove biofilms, unless, the cleaning agent is effective. The efficiency of cleaning agents is assessed by their ability to remove biofilm from process surfaces together with their ability to kill the bacteria present in the biofilm. The cleaning effect in open systems can be enhanced using either increased chemical effect through double foaming or added mechanical forces through scrubbing. In closed processes, the removal of biofilms from surfaces can be performed using efficient flowing condition in combination with effective cleaning agents. Strong agents are used to combat microbial deposits on equipment surfaces in problem areas. Satisfactory elimination of biofilms using only disinfectant treatment cannot be achieved. The effects of cleaning procedures used in the food industry can be evaluated using life cycle assessment (LCA). All environmental aspects, including the process and energy consumption in producing the cleaning chemicals, transportation, properties of chemicals before and after cleaning, amount of water used, effects of organic and inorganic loads on the wastewater and on the recipient (e.g., nutrient load, pH, temperature).

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BIOFILMS, METHODOLOGY. See ACTIVATED SLUDGE — MOLECULAR TECHNIQUES FOR DETERMINING COMMUNITY COMPOSITION; LASER SCANNING MICROSCOPY IN COMBINATION WITH FLUORESCENCE TECHNIQUES FOR BIOFILM STUDY; MICROBIAL FLOCS SUSPENDED BIOFILMS

BIOFILMS, MODELING OF. See MODELING OF BIOFILMS

BIOFILMS, PATHOGENS IN. See PATHOGENS IN ENVIRONMENTAL BIOFILMS

BIOFILMS, SORPTION PROPERTIES OF. See SORPTION PROPERTIES OF BIOFILMS

BIOFILMS: SUSPENDED BIOFILMS. See MICROBIAL FLOCS SUSPENDED BIOFILMS

BIOFILTRATION

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Biofiltration is the removal and destruction of gaseous contaminants in air as the air flows through porous solids containing degradative microorganisms (1,2). In engineering nomenclature, it can accurately be called *ambient temperature catalytic oxidation*. Microbial enzymatic catalysis at ambient temperatures in biofilters achieves the same results as precious metal catalysts at high temperatures. Biofiltration can effectively and economically destroy most gaseous air contaminants. A rule of thumb is that if a molecule can burn in air, it can degrade in a biofilter.

Figure 1 shows a schematic biofilter. Moist-contaminated air flows slowly through a porous biofilter medium; microbes in the medium oxidize the contaminants to carbon dioxide, water, and calcium salts. The biofilter medium can be soil, compost mixed with bulking agents, composted wood chips or wood bark, peat, or synthetic media (activated carbon, porous ceramic, and plastic packing). Because the gaseous contaminants are molecularly subdivided and intimately mixed with oxygen, the oxidation rates are many-fold faster than the same compounds in liquid or solid state.

Biofiltration is the most common application of this air treatment process, which is also used by biotrickling filters and bioscrubbers. In biofiltration, the microbial medium and water are stationary, and air is the moving phase. In biotrickling filters, both air and water move over a stationary microbial support. In bioscrubbers, microbial slurry is sprayed into the air stream.

In contrast to other air pollution control techniques, biofiltration creates or emits no secondary pollutants (NO_x, CO, Cl_2, O_3, wastewater, or waste solids), consumes no fuel or chemicals, and minimizes carbon dioxide loading of the atmosphere.

The microbial population adapts to the input gas composition; the adaptation time depends on the gas composition and, secondarily, to gas concentration (3). For rapidly biodegradable gases (Table 1), the initial destruction/removal efficiency (DRE) is 80 to 95% of the final, steady state DRE. The final DRE is reached after one to three weeks as the microbial population adapts and the medium material settles. The DRE for more slowly biodegradable gases tends to be low initially, and the

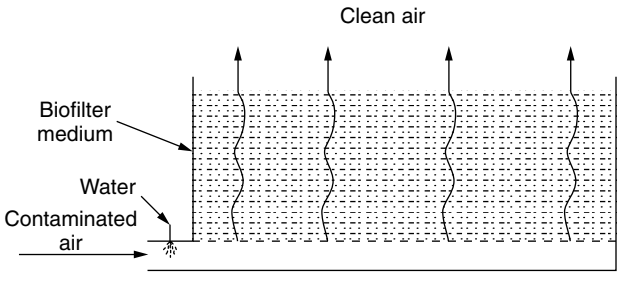

Figure 1. A schematic biofilter.

Table 1. Destruction/Removal (DRE) Rates of Air Contaminant Gases by Biofiltration

Rapid:
H_2S, HCl, SO_2, NO_x, Cl_2, O_3, silanes, alcohols, aldehydes, ketones, organic acids, ethers, esters, amines, mercaptans
Slow: (in approximate order of decreasing degradation rates): CO > terpenes > aromatic hydrocarbons > aliphatic hydrocarbons > CH_4 > monohalogenated and dihalogenated hydrocarbons
Unfeasibly Slow:
Siloxanes, tri-halogenated and higher-halogenated hydrocarbons

adaptation period may be as long as one month. The DRE, thereafter, is not affected by downtimes up to a week or two. Longer downtimes may require a short readaptation period.

The primary function of biofiltration is to destroy gases, but biofilters also filter out particles from air streams, even less than 2.5 μm particles. Particulates generally should be avoided in the input air, however, because they can plug the small pores of the biofilter. Mists are acceptable if the droplets are water or biodegradable liquids.

Although similar in appearance to sorption beds, biofilters function by oxidizing rather than by just adsorbing airborne contaminants. Biofilter media have little adsorption capacity for gases. The lack of any sorption capacity is an advantage in that, the media do not accumulate air contaminants in significant amounts and any adsorbate is quickly oxidized. Spent biofilter media is not a hazardous waste.

The potential benefits of biofiltration over other air pollution control techniques are

1. low to moderate capital costs,
2. low operating cost — no supplemental fuel or energy is needed, and the oxidant is atmospheric oxygen so no chemical oxidant (chlorine, ozone, and permanganate) is needed,
3. no secondary pollutants (CO, NO_x, Cl_2, O_3, dioxins, wastewater, and solid wastes) are created or released,
4. the catalysts (microbial enzymes) are free and continuously regenerate themselves,
5. low maintenance, and
6. inherent safety (low temperature and no combustibles nor hazardous chemicals).

In some cases, poor design and too high expectations have led to a lower than desired degree of pollution control, higher than expected maintenance requirements, and too frequent changeout of the biofilter medium. Biofilters, unfortunately, almost invite naive design because they are mechanically simple and because the air distribution system is not difficult. The difficulties of (*1*) maintaining an active microbial population, (*2*) maintaining low backpressure and uniform air distribution over the long term, and (*3*) moisture management are not apparent in Figure 1.

Some biofilter designs emphasize high effectiveness per unit volume or per unit weight of the biofilter, with penalties of higher initial cost, complexity, and maintenance. Other designs emphasize low initial and operating cost, ruggedness, long life, and low maintenance. Their penalty is larger size and mass.

In addition to the design problems, biofiltration has the inherent disadvantages:

1. Biofilters require longer reaction time than other techniques. Reaction rates at room temperature are slower than those at the temperatures of catalytic and thermal oxidizers and those with strong oxidants in chemical scrubbers.
2. Oxidation rates are compound-specific. Microbes generally oxidize gases containing oxygen, nitrogen and sulfur functional groups faster than hydrocarbon gases. Highly halogenated gases (tri-, tetra-, and higher) do not oxidize in biofilters. Monohalogenated and dihalogenated compounds oxidize slowly.

The air residence time in biofilters is seconds to minutes, depending on the biodegradability of the gas, the kind of medium, and the desired DRE. The residence time is usually expressed as the empty bed residence time (EBRT). The residence time in high-temperature oxidizers and adsorbent (activated carbon and zeolite) beds is fractions of seconds. Biofilters are, therefore, larger in size and area than other air pollution control devices.

Applications

The range of applications for biofilter treatment of contaminated air is potentially very broad (1,2,4). Biofiltration is most advantageous when the contaminant concentrations are low (<1,000 ppmv), at air temperatures of 10 to 55 °C, the air is humid and particulate-free, the contaminants are rapidly biodegradable, space constraints are absent, and recovering the contaminant is not economic, and when low maintenance is needed. The input air can often be made humid and particulate-free and brought to optimum temperature simply by water injection into the air stream. Biofilters are also advantageous where low maintenance, inherent safety, low visibility, and a green technology are important.

Wastewater treatment, food processing, and pharmaceutical, and some chemical manufactures have shown interest in biofiltration to date. This is largely because large airflow rates must be treated and the pollutant concentrations are low, and because their personnel recognize that microbes can influence the chemistry of their products.

In other industries, the responsibility for air pollution control is often given to mechanical and chemical engineers who may be unaware of the possibilities of microbial degradation. The trend toward size minimization also works to the disadvantage of biofiltration.

Potential industrial applications for biofiltration include treatment of ventilation air, process gases, storage tank vents, petroleum refining and handling, printing, solvents of all kinds, and relief valves. Because no start-up procedures are necessary and flow capacity is large, a

biofilter provides simple and useful treatment for storage tank and relief valve emissions. The Bhopal, India, accident reminds us that even simple treatment of accidental releases or safe containment in a biofilter is better than direct release into the atmosphere.

Because of the low cost of biofiltration and the large number of treatable pollutant gases, and because the moisture in the air is a benefit rather than a detriment, the extent of potential industrial applications is as wide or wider than the physicochemical alternatives — thermal oxidation, chemical scrubbing, or activated carbon or zeolite adsorption.

In an ideal world, air pollutants would be recovered for reuse rather than emitted. Recovery by adsorption or condensation is expensive, energy-intensive, incomplete, and sometimes hazardous. Hence, release is inevitable. Released gases are destroyed by oxidation. The choice of oxidative treatment — thermal, chemical, or biofiltration — is determined by economical and environmental constraints.

Economics

Figure 2 shows the applicability, based primarily on cost, of various air pollution control technologies. Over a wide range of airflow rates and pollutant concentrations — indeed the range of most air pollutant emissions — biofiltration has a strong economic advantage (the conversion factors are 1 cfm = 1.7 m^3 and mg m^{-3} = ppmv × molecular weight/24.06). The cost estimates are probably for the rapidly biodegradable gases as shown in Table 1. For hydrocarbons and other slowly degradable gases, the economic advantage of biofiltration is slightly less because the longer residence time requires larger biofilters.

Of the air pollution technologies, the installed cost of biofilters may be the most site-specific because of their size and weight. Urban locations may have too little available space to take advantage of biofiltration. In addition, biofiltration may require more ducting because the biofilter must be farther from the source. Biofiltration could find more applications if the constraints of biofilter weight and size were included in the initial plant design. Biofilter design has shown little imagination in adapting to the available space and configuration. Parking lot areas and rooftops are open invitations to innovative biofilters. In compiling Figure 2, the authors avoided some site-specific cost problems by using data for modular contained biofilters. Built-in-place, open biofilters tend to be less expensive.

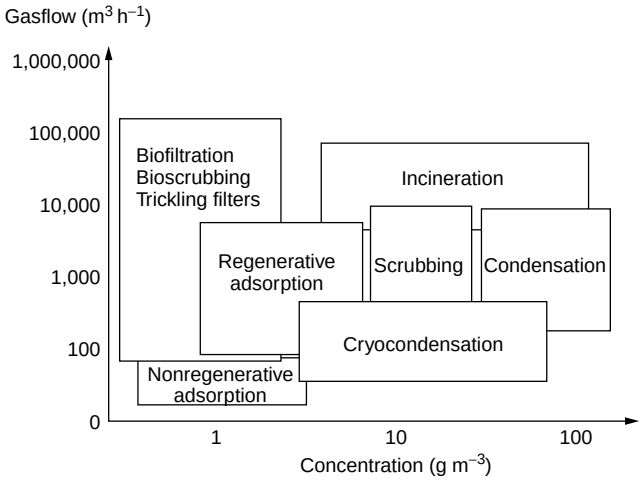

Figure 2. Applicability of various air pollution control technologies based on airflow rates and concentrations to be treated. (From Kosteltz, A. M. et al., in Proceedings of the 89th Annual Meeting and Exhibition of the Air and Waste Management Association, Pittsburgh, PA, 1996. With permission of KPMG Management Consultants, Ottawa, Ontario, Canada.)

Compound Specificity

The oxidation rate varies for each gas. Table 1 shows common gases grouped according to their relative biodegradation rate. If the molecule contains oxygen, nitrogen, or sulfur functional groups, it generally will biodegrade rapidly. A corollary is that if a molecule is water-soluble, it will biodegrade rapidly. Oxygen, nitrogen, and sulfur functional groups tend to increase both the water solubility and the biodegradation rate of organic gases.

Chlorine and bromine functional groups in organic molecules reduce biodegradation rates. Monohalogenated and dihalogenated molecules oxidize slowly under the highly aerobic conditions of biofilters. Biofilter control of these gases may be feasible under some conditions. More highly halogenated compounds need anaerobic conditions for the first stage of their biodegradation, reduction by substituting hydrogen atoms for halogens in the molecule. The reaction products are less halogenated and may be oxidizable in a biofilter. The reduction step is very slow, and a successful two-stage anaerobic–aerobic biofilter has yet to be devised.

Inorganic gases such as H_2S, NH_3, SO_2, and HCl react rapidly in biofilters, whereas NO_x (NO and NO_2–N_2 O_4) reacts more slowly. Nitrous oxide (N_2O) reacts even more slowly. The first step, the removal of the compound from the gas phase, is often chemical rather than biological and is very fast. The sulfur and the nitrogen gases are later oxidized microbially to their oxyacids, H_2SO_4 and HNO_3. The biofilter must neutralize this acidity or the microbes cease to function. The lower limit of acidity is conservatively about pH 2.5 for the sulfur gases and pH 4 for the nitrogen gases.

Table 1 gives no absolute numbers for oxidation rates or EBRT because the oxidation rates vary with the medium, temperature, water content; in some cases with nutrient conditions; and with the degree of DRE required. Laboratory measurements of degradation rates tend to be optimistic when compared with the field conditions, but the relative degradation rates obtained in the laboratory apply.

Mechanism

Under most conditions, where the contaminant gas concentration is less than 1,000 ppmv (ppmv is the volume fraction or mole fraction of the gas × 10^6), the kinetics of

biofilter oxidation are approximately first-order (5):

$$-dA/dt = kC_A \quad (1)$$

where A is a pollutant gas, k is a reaction coefficient related to the biodegradation rate of A, and C_A is its concentration. The oxidation rate increases with gas concentration, and the fraction of pollutant removed and destroyed increases logarithmically with reaction time t.

The DRE is

$$\mathrm{DRE}(\%) = \frac{C_{\mathrm{input}} - C_{\mathrm{output}}}{C_{\mathrm{input}}} \times 100 \quad (2)$$

Under first-order conditions, the DRE is independent of gas concentration, depends on reaction time (EBRT), and, therefore, depends on biofilter volume.

As gas concentrations increase above 1,000 ppmv, the reaction order gradually decreases to zero order:

$$-dA/dt = k \quad (3)$$

Then, the oxidation rate is constant and independent of gas concentration. Hence, the DRE decreases as the gas concentration increases at these high concentrations.

The first step of biofilter removal of air contaminants is chromatographic. The pore walls of the media are in equilibrium with clean air. The major components of air — dinitrogen, dioxygen, argon, and carbon dioxide — have already saturated the pore walls and, therefore, flow unimpeded through the biofilter. The contaminant gases, on the other hand, partition out on the moist pore walls in rough accordance with Henry's law and lag behind the major components of the air. The partitioning of a gas from air to the stationary phase increases with its polarity, water solubility, and molecular weight. The more tightly the gas is adsorbed, the longer the gas remains on the pore walls, the slower it flows through the biofilter, and the more likely it will be absorbed by a microbe and degraded or oxidized.

The chromatographic efficiency of biofilter media has not been measured under moist operating, conditions. In an air-dry sandy soil with low organic matter content, the retention volume (an index ratio of chromatographic efficiency and the reciprocal of the ratio of flow rate of contaminant gas relative to flow rate of air) ranged from one for methane to 10^5 for octane (6). The retention volume increased with increasing clay content in the soil. The retention volumes of the more polar gases methanol, dimethylketone, and diethyl ether were $>10^6$, too large to measure under the same experimental conditions.

The dryness was necessary to prevent appreciable biodegradation of the gases and to prevent large-scale changes of moisture content during the measurements. Biofilters, however, operate under moist conditions in which the water film on soil particles and microbes probably inhibits the partitioning of water-insoluble gases such as hydrocarbons onto the stationary phase and increases their relative flow rates. The presence of water increases the partitioning of water-soluble gases.

The chromatographic partitioning of gases has not been measured in organic, compost-based biofilter media. The greater organic matter content of these media might suggest greater chromatographic efficiency and greater retention of organic gases than in inorganic media. Because operating biofilters are very moist, however, the gases actually contact the moisture film on the particle surfaces rather than on the surfaces themselves. The water film greatly decreases the chromatographic differences between inorganic-based and organic-based biofilter media.

The sorption capacity of activated carbon mixed into biofilter media slightly increases chromatographic efficiency and buffers the effects of rapid changes of gas concentration in the input air (1). The benefit of activated carbon is lessened by its being saturated with water, which decreases its gas sorptivity. Microbes on the pore walls increase gas partitioning by degrading the gases and thereby reducing gas concentration in the water film.

The second and limiting step of contaminant removal and destruction is the rates of microbial oxidation, of chemical reactions, or of the gases on the pore walls. These rates are compound-specific (Table 1).

Biofilter modeling of degradation rates has emphasized the first step: dissolution of the gases into the water film and transport across that film. The shortcoming of this approach is shown by biofilter response to moisture content. Biofilter performance does indeed decrease when the moisture content increases beyond the optimum range. Biofilter performance, however, also decreases sharply when the water content is below this range (7). Although mass transfer across a thin water film is much faster, the important step is the microbial metabolic rate which has a maximum over a narrow range of moisture content.

The water films in biofilters are very thin. In an active biofilter, the water potential or hydraulic head is about -0.2 bar or -20 kPa (equivalent to a water activity of about 0.99 and equivalent to about 50–60% wet weight of water in compost and other organic media). At this water potential, pores with a diameter larger than about 10-μm in diameter are empty of water except for a water film <0.1-μm thick. "Pores" in biofilters are actually the network of interconnected cavities between the particles of the medium. The pores, which transmit the gas by convective flow, are tortuous and have nominal pore "diameters" ranging from perhaps 50 μm to several millimeters. Smaller pores may be dead ends or may have severely restricted openings so that diffusion is an important gas transfer mode in them.

Where the medium's particles contact each other at a water potential of -20 kPa, the water meniscus has a diameter of 10 μm. Between the contact points and on the surfaces of microbes, the water film is less than 0.1-μm thick. Because bacteria are on the order of 1 to 10 μm in diameter, this thin water film is a minor factor in gas uptake. The major function of water is to support microbial metabolism rather than to determine microbe-gas contact.

Gas adsorption and absorption are minor components of biofiltration. Like most solids, biofilter media adsorb only

minute amounts of gases. Porous solids can retain large amounts of liquids by capillarity, but this mechanism is absent in biofiltration. At biofilter temperatures and conditions, the gas concentrations are far below those that allow gases to condense to liquids.

Activated carbon is unique as a solid because its high surface area and nonpolar character help adsorb organic gases. Although compost and its mixtures also are organic, composts have a much lower sorption capacity than carbon because of higher polarity related to cellulose content and because of lower surface area. Silicates in soils are quite polar, a disadvantage for adsorbing nonpolar organic gases but an advantage for adsorbing inorganic gases. The organic coating on inorganic soil particles gives soils an appreciable nonpolar character.

Overloading of biofilters is evidenced by only partial oxidation of the input gases. Alcohols, for example, partially oxidize to aldehydes and organic acids rather than to carbon dioxide. A second indication of overloading is the buildup of microbial biomass that leads to plugging of the biofilter pores. This problem has been seen in laboratory experiments at gas input concentrations of more than 5,000 ppmv and more than 5 g/m^3.

Toxicity and Antagonism

Chemical toxicity to biofilter microbes is extremely unlikely. Microbes are so much more tolerant of biological toxins than higher organisms are that complete inhibition of microorganisms does not occur, although pulses of high concentrations of some pesticides might temporarily reduce degradation rates. Gas concentrations at even 10,000 ppmv are low in terms of mass per unit volume of biofilter media. Adding large amounts of toxic agents via the gas phase, therefore, is physically difficult. Biofilter slowdown usually is because of high or low moisture content or low temperature, rather than because of chemical toxicity. Herbicides for weed control on the surface of open biofilters are in too low a concentration to affect biofilter performance.

Antagonism, a decrease of gas removal or destruction related to the presence of other gases, is rarely seen in biofilters. Gas mixtures even at more than 1,000 ppmv concentrations show little evidence of antagonism; the presence of one gas does not inhibit the oxidation rate of other gases. Instead, the microbes degrade the more rapidly biodegradable gases first. More slowly biodegradable components are oxidized further along in the biofilter column.

Conditions at higher concentrations are more complicated, but antagonism is still small at 5,000 ppmv for some gas mixtures. These high concentrations are in the range where oxidation by synthetic catalysts may be more economical.

Some process gases may be low in oxygen so that, as in other oxidation techniques, dilution air is necessary. Microbes are efficient in their oxygen utilization, so only a little more than the stoichiometric amount of oxygen in air need be added. If the exhaust air contains some oxygen, the microbes in the biofilter are not oxygen-starved.

Biofilter Media

The essential criteria for biofilter media are that they support microbial growth and permit gas flow. Microbes can grow on many materials and some materials, such as soil, compost (alone and in mixtures), and peat, are natural supports for microbial growth. Other porous, synthetic media, including plastic packing, activated carbon, and porous ceramic, can be seeded to provide the initial microbial population.

Since many porous materials can serve in biofilters, the choice hinges on other features of the media, including cost, stability, hydrophobicity or hydrophilicity, density, nutrient supply, pH buffering, and maintenance requirements. The cost differences are large. Synthetic media cost dollars per kilogram, soil mixtures cost up to \$50/m^3, and compost mixtures cost from \$50 to \$500/m^3.

The stability of the medium affects the long-term flow uniformity through the biofilter bed and the frequency of bed replacement (changeout). Organic media are inherently unstable, heterotrophs feed on the media and on the input gases. As the medium is consumed, it shrinks, forms cracks, and pulls away from its container walls. In addition, the media particles become smaller and more mobile. Water flow tends to deposit them in the pores at the interface of the medium and the distribution layer so that this layer becomes impermeable.

Under careful management and in enclosures, some compost mixtures have a 10-year lifetime. Open compost beds can start developing cracks within several months; two years might be a maximum before backpressure builds up and the DRE is no longer satisfactory.

Inorganic media, such as siliceous soil, plastics, and ceramics, are not attacked by microbes, and impermeable layers are less likely to occur. Soils and distribution layers rich in $CaCO_3$, however, can clog severely within months.

Soil and other inorganic media are permanent, that is, no replacement is required and DRE and backpressure are stable. Native soils, however, usually are far too impermeable for industrial-scale biofiltration, and airflow is spatially nonuniform. An appropriate soil mixture is a mixture of several components to provide an acceptable trade-off between DRE, air permeability, and uniform flow.

Hydrophobicity and/or hydrophilicity affect moisture management in biofilters. The most common cause of biofilter failure is inadvertent drying of the medium. Organic substances tend to be hydrophobic when dry; inorganic substances tend to be hydrophilic. Rewetting a dry organic-based medium means taking out the medium and physically mixing in the water, which is a tedious, dirty, time-consuming, and often impossible task. Simply pouring water on the surface is ineffective because the water flows down through cracks and along wall boundaries rather than thoroughly and uniformly wetting the medium.

Hydrophilic substances, on the other hand, rewet easily and water flows to all parts of the biofilter bed.

Simple surface irrigation restores biofilter activity. Soils, ceramics, and pearlite materials are hydrophilic.

Synthetic media provide little or no mineral nutrients (nitrogen, phosphate, calcium, and so on) and hence require periodic supplementation. Microbes attach weakly to plastic media. Compost and the synthetic media are supplemented with a $CaCO_3$ source such as crushed seashells to maintain acidity at pH 6 to 8. Soil media have greater pH buffering capacity, but may require additional lime.

Media density is important when lightweight is important, and when biofilters are portable or placed on structures. Organic media have densities of about 400 kg/m^3 when wet and soil and ceramic media have densities of about 1,400 kg/m^3.

A lower maintenance requirement generally means a more successful and effective biofilter, and lower operating and maintenance (O/M) costs. Included in O/M costs are bed replacement, backpressure, moisture monitoring, and in some biofilters the cost of preheating the air. Since replacement is a high and recurring cost, good biofilter designs make replacement of nonpermanent media as easy as possible. Modular biofilters require replacement of the entire unit. Backpressure is lower initially in compost biofilters than in soil biofilters but increases with time. The backpressure remains stable in inorganic and plastic media. The initial backpressure of a large biofilter bed usually is 2 to 8-cm water head or 200 to 800 Pa. Small modular biofilters may have backpressures of 40 cm-water head, or 4,500 Pa.

Synthetic media are more homogeneous (thereby providing more uniform distribution of airflow through the medium), their DRE per unit mass is greater than that of natural media, and their density is low. As yet, these benefits rarely outweigh their much higher cost.

Moisture

Biofiltration is a microbial process, and the microbes function best over a rather narrow range of moisture content. Moisture content is usually expressed as percentage of wet weight of the bed medium. For organically based media, the range is 50 to 60%. At greater moisture contents, pores become saturated with water, whereas at lower moisture contents, microbial activity decreases. At less than 40%, the degradative capacity is severely limited (7). Rewetting the bed to 50 to 60% may not restore performance. The lack of recovery probably is due to the difficulty of thoroughly rewetting hydrophobic media.

Wet weight is convenient for measurement, but it depends on the density of the medium and the particle size distribution. For organic media, density and particle size distribution are similar enough that 50 to 60% suffices. The range for inorganic media (soil or ceramic), however, is 10 to 25%, depending on particle size.

Worse yet, mass units such as wet weight do not express the actual criterion — moisture availability — that affects microbial activity. Much of the water in biofilter media is too difficult for microbes to extract without slowing their metabolic (degradation) rate.

Availability of water can be expressed either as chemical potential (also called *chemical activity*, which is numerically equal to the relative humidity (RH) of the air at equilibrium) or as physical potential (also called *hydraulic head, water tension*, or *suction*) (8). The 50 to 60% wet weight moisture content in organic biofilters is a water potential or water activity around 0.99, with 0.95 being a lower limit for good performance. The same range of water availability expressed as physical potential is about -0.2 bars, with perhaps -3 bars as a lower limit. The equivalent SI units are -20 and -300 kPa. Measurement of water availability is, however, difficult and slow. Instruments that measure moisture content or availability that can be linked to irrigation devices are being developed.

Because drying out is the most common cause of biofilter breakdown and because the effluent air is at 99 to 100% RH, biofilters should ensure that the input air is at 100% RH. Fogger nozzles in the input air stream are common, indeed almost a necessity. Oxidation of the contaminants raises the biofilter temperature slightly. This decreases the RH of the air and increases evaporation in the medium. Surface irrigation is needed to overcome this internal evaporation and evaporation from the surface. This irrigation usually is triggered by a timer rather than by need.

Because water availability is in effect an index of the thickness of the water film on the medium's particles, water availability can be estimated visually. At water availability at the optimium moisture level, expressed as 0.99 activity, -0.2 bar, -20 kPa water potential, or 50 to 60% wet weight in organic media, the medium appears dark and moist. A visible sheen indicates that the water film is too thick and the moisture content is too high. If the medium is light-colored and darkens when water is added, the medium is too dry. Also, the water film at optimum moisture availability has sufficient surface tension to hold the medium's particles in a cast when the particles are squeezed lightly in the hand. These moisture properties should all be tested at greater than and equal to 15 cm below the surface of open biofilters. Moisture content at the surface reflects recent climate effects rather than biofilter moisture content.

Irrigation and rainfall are sometimes in excess of biofilter needs. Drainage must be provided to prevent water accumulation. Irrigation should be scheduled so that the amount of drainage water is kept to a minimum. The water phase may contain soluble gases, but these concentrations are low. Drainage water from compost biofilters contains brown-colored organic compounds similar to natural humic acids, and the water may be cloudy. The water is nonhazardous but unsightly. Drainage water from inorganic media is clear and colorless. The drainage water can be recycled to the biofilter, or the drainage water can flow slowly into the aerobic soil beneath the bed, where natural processes treat the water further. The risk of groundwater contamination is exceedingly small.

Impermeable liners under biofilter beds can complicate water management by inducing water buildup. Liners can be an additional liability because, despite careful

installation, they leak sooner or later, and all the drainage is concentrated at the leaks rather than spreading out the water for more effective treatment. Concentrating the flow makes natural water treatment much more difficult.

Temperature

The temperature operating range for biofilters is approximately 10 to 55 °C. The optimum is generally considered 37 °C, but some biofilters successfully utilize thermophilic bacteria at around 45 °C. Below 10 °C biodegradation still occurs at slower rates; biofilters can function successfully at very close to freezing temperatures. Input gases beyond the range of 10 to 55 °C can be warmed by steam injection or can be heated and cooled by water injection or mechanical cooling. Water and steam injection have the benefit of maintaining high RH. Water injection has the additional benefit of washing out airborne particulates that otherwise could plug biofilter pores.

The oxidation rates are less temperature-dependent than the typical $Q_{10} = 2$ (the reaction rate doubles with each 10 °C increase of temperature) of microbial reactions would indicate. At low temperatures, gas partitioning improves because gases are less volatile and are more soluble in the water film. Heterotrophs are adapted to function in natural soils in which the average annual temperature is 0 to 15 °C, except in very warm climates where soil temperatures may reach 25 °C.

CONCLUSION

Biofiltration is an effective means of removing and destroying air contaminants from many waste airstreams. Biofiltration is a low-cost technique over most of the range of industrial emissions because it requires no fuel or chemical and relatively little maintenance. In addition, biofiltration creates little or no secondary emissions or wastes, is inherently safe, and is environment friendly. Limitations of biofiltration are its compound specificity and relatively low oxidation rates, requiring relatively large reactor volumes. Good biofilter designs maintain high microbial activity over a wide range of moisture conditions and utilize the most appropriate biofilter medium.

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BIOFILTRATION AND BIOODORS

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With continuing population growth, it has become ever more common that residential developments are situated near industrial and utility facilities. At the same time, the public has become less tolerant of foul odors and is more likely to demand that release of odorous vapors into their neighborhoods be prevented.

Biological processes are commonly used for treatment of odorous wastes such as wastewater, sludge, compost, or garbage. Treatment systems may become sources of odors because vapors escape in their original form from the waste or because odorous compounds are created in the collection or treatment system.

Odors typically consist of low concentrations of contaminants in large volumes of air. This type of discharge is expensive to treat by thermal methods because of the large amount of fuel needed to heat the air. Adsorption often is also inefficient because the low concentrations mean only small amounts of material are adsorbed on a given mass of adsorbent. Biofiltration, in contrast, is well suited for treating odors.

There is a substantial body of literature on the generation and control of bioodors in wastewater and composting systems (1–4), and several summary treatments of sulfide generation in wastewater systems (5–7). There is a modest but rapidly growing body of literature on biofiltration for air contaminants (8) and its application to wastewater odors (9,10).

ODORS IN BIOLOGICAL TREATMENT SYSTEMS

Biological treatment systems are designed to transform waste materials to benign products. Organic compounds are converted to carbon dioxide and water, sulfides are oxidized to sulfate, amines are oxidized to nitrates, wastewater organics are converted to stabilized sludge, and yard waste becomes useful compost (11). However, the variable nature of the waste, the complexity of the waste collection and treatment systems, and the great diversity of microorganisms lead to the creation of transformation products that are offensive odorous compounds.

It is useful to classify odors as those that are released from aerobic environments and those that come from anaerobic environments. The odors from aerobic environments generally are less offensive, and often are described as “musty.” Odors from anaerobic environments are often extremely offensive. Humans undoubtedly evolved to perceive odors from anaerobic biological systems as disgusting because it causes them to avoid spoiled food and human waste that may be sources of diseases. The

Table 1. Odor Compounds in Wastewater Treatment

Reduced Sulfur Compounds	Reduced Nitrogen Compounds	Others
Hydrogen sulfide	Ammonia	Acetaldehyde
Allyl mercaptan	Dibutyl amine	Chlorine
Amyl mercaptan	Diisopropyl amine	Ozone
Benzyl mercaptan	Dimethyl amine	Sulfur dioxide
Dimethyl sulfide	Ethyl amine	
Diphenyl sulfide	Indole	
Ethyl mercaptan	Methyl amine	
Methyl mercaptan	n-Butyl amine	
Phenyl mercaptan	Pyridine	
Propyl mercaptan	Skatole	
Thiocresol	Trimethyl amine	

Source: Adapted from P. L. Schafer, Chair, Joint Task Force of the Water Environment Federation and the American Society of Civil Engineers, *Odor Control in Wastewater Treatment Plants* (ASCE Manuals and Reports on Engineering Practice No. 82 and Water Environment Federation Manual of Practice No. 22), American Society of Civil Engineers, New York, 1995.

dominant compounds in waste treatment odor problems are sulfides and amines (Table 1).

The distinction between aerobic environments and anaerobic environments is also useful because it is a key to some control methods. An adequate supply of oxygen can stop generation of the odors that arise from anaerobic systems. On the other hand, conditions that promote vigorous transfer of oxygen from the atmosphere to the microbial ecosystem also promote release of volatile compounds from the liquid. In some cases, it is best to suppress gas transfer until treatment of the odor compounds is complete.

Disposal of Odorous Compounds to Treatment Systems

Some of the compounds that cause odor problems in wastewater or composting systems are those that have been disposed of to the system. Human waste, of course, has odors at the time it is discharged, and manures smell bad when they are added to compost. Industrial wastes may also include odorous compounds, particularly volatile solvents.

Biological Odor Generation

Microorganisms transform chemicals for two purposes: to synthesize compounds that they use to make growing cells and to provide energy. The reactions that produce energy generally process far more material than the synthetic reactions do. Microorganisms need the energy to move and drive their metabolism and often to drive synthetic reactions. Because larger amounts of products are generated, catabolic reactions are more likely to produce odor problems. Further, the products of synthetic reactions are rarely gases, and only gases can become odors.

By definition, energy production requires reactions that are thermodynamically favored under the conditions the cell is experiencing: the products must contain less energy than the reactants. Under oxidizing conditions, the usable reactions are usually redox reactions in which oxygen acts as the electron acceptor and is added to the reactants. Hydrocarbons are converted to aldehydes, alcohols, or carboxylic acids. These compounds are further oxidized to carbon dioxide and water that are compounds with lowest energy under aerobic conditions. Although there is a multitude of metabolic pathways, depending on the starting compound and the microorganism, the ultimate products usually are carbon dioxide and water, which are odorless.

Microorganisms that oxidize organic compounds tend to dominate aerobic ecosystems because the energy available per mole of material processed is far greater than that for reactions possible without oxygen. However, other reactions are possible. Organic nitrogen or nitrite can be oxidized to nitrate by the nitrifying bacteria. Organic sulfide and hydrogen sulfide are oxidized by bacteria such as those in the genus *Thiobacillus*. Nitrate and sulfate have essentially zero vapor pressures, and so do not contribute to odor problems.

Ultimately, oxidative processes tend to convert odorous compounds to end products that are inoffensive (Fig. 1). Although some intermediate compounds such as short-chain fatty acids and amines may be troublesome, they tend to be rapidly biodegraded, so the general tendency is for wastewater, compost, or biosolids to become less odorous as aerobic treatment proceeds. Primary sedimentation basins and fresh solid waste must be covered, but secondary aeration basins and treated compost are far less offensive.

Oxidative processes will dominate as long as oxygen is plentiful. If the amount of oxygen-consuming material is small or if there is a mechanism by which oxygen is continuously supplied, the organic matter will be degraded and the sulfides and amines will be oxidized. However, oxygen is not highly soluble in water. Water in equilibrium with the atmosphere carries perhaps 7 to 9 mg/L of dissolved oxygen, depending on its temperature. A liter of wastewater typically contains material that will consume over 100 mg of oxygen, so the oxygen will soon be depleted in wastewater that is not vigorously

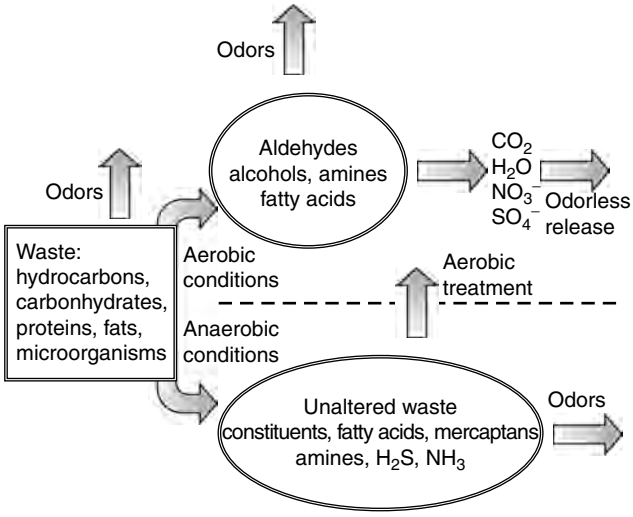

Figure 1. Pathways for odor production and release in biological treatment systems.

aerated. When this occurs, microorganisms that are capable of using other compounds as electron acceptors will proliferate. Denitrifying organisms will use nitrate as the electron acceptor to produce nitrogen gas or nitrous oxide. As nitrate becomes less plentiful, bacteria such as *Desulfovibrio* will oxidize organic matter using sulfate as the electron acceptor, thereby producing sulfides.

Hydrogen sulfide (H_2S) is produced in wastewater collection systems by anaerobic sulfate-reducing bacteria. Organic material contains approximately 1% sulfur (dry weight, 12). Sulfur as sulfate in wastewater ranges in concentrations from a few parts per million (ppm) to hundreds of ppm, depending on the source (5). Sulfate-reducing bacteria can grow only in the absence of oxygen, when the conversion is thermodynamically favored. The reduction rate of sulfate to sulfide depends on the wastewater BOD or COD, sulfate concentration, dissolved oxygen concentration, and a number of other growth factors (5).

Without the energy benefits of oxygen as an electron acceptor, degradation of waste organic matter by microorganisms is much slower and less complete. Under anaerobic conditions, odorous organic compounds that are present in the original waste are less likely to be degraded. Odorous intermediates such as fatty acids, mercaptans, and amines will accumulate.

Transfer of Odor Compounds to Air

The general strategies for control of biologically generated odors follow from Figure 1. The objective is to convert the waste to odorless carbon dioxide, water, nitrate, and sulfate, without releasing odorous intermediate compounds along the way. The best approach is to keep the waste aerobic and contained until it is converted to the odorless product. However, in complex collection and treatment systems, anaerobic zones will develop where force mains are required, where flow is unexpectedly slow, or where sediment or slime layers build up (2,5). Garbage left too long in the truck or compost at the bottom of a storage pile may consume all of a limited supply of oxygen. Some treatment processes intentionally are kept anaerobic. If anaerobic conditions do occur, the waste flow should be contained until aerobic treatment can complete the transformation to oxidized products. For air, this may be done in a downstream biofilter.

An odorous compound becomes a nuisance only after it is transferred to the air. It becomes a problem for the surrounding community only if it is released in sufficient amounts to overcome the dilution that occurs between the point of release and the neighbors and to arrive there in concentrations above the odor threshold.

Quigley and Corsi noted that three factors contribute to the release of volatile compounds from wastewater (13):

1. The amount of compound present in the wastewater. High concentrations of the odorous substance increase the rate of transfer of the compound from water to air.
2. The presence of conditions that create turbulence. Drops in collection system piping and treatment plant equipment such as weirs and flumes mix the water, promoting volatilization. For sulfide and many other compounds in wastewater flow, concentrations in the air above the water typically are far below equilibrium. The mixing constantly brings high concentrations of odor compounds near the surface where volatilization can occur.
3. Rates of ventilation of the headspace above the water. If the wastewater is within a pipe or some other enclosed structure, the rate of release of odor from the structure will depend on the rate at which the air in the structure is discharged. In some cases, it is possible to minimize this discharge by making the structures as airtight as possible. However, in any space that will be entered by workers, 20 to 30 changes per hour are required for safety (5).

Although Quigley and Corsi (12) described release of volatile organic pollutants that had been dumped in sewers, the same principles apply to any volatile compound in a wastewater handling structure. They emphasized that not all these factors may be present at the point where the compounds are discharged or created, and releases are highly variable along the path from wastewater discharge through treatment to treated effluent discharge. It is quite common that odorous compounds generated in one part of the collection or treatment system can be released from another part of the system.

Van Durme (1) made the same point with respect to odor releases at various points within a treatment plant, emphasizing that a compound may be produced in one part of the plant but dominantly released at some downstream point where there is turbulence and exposure to the atmosphere. She noted, for example, that wastewater flowing over weirs is commonly a major source of odor.

ODOR TREATMENT BY BIOFILTRATION

Principles of Biofiltration

Biofiltration removes air pollutants by passing the contaminated air through a damp, porous medium on which a mixed culture of microorganisms is immobilized. The contaminants are absorbed from the air phase into the water phase, which includes water and a biofilm, surrounding the medium (Fig. 2). The transfer rate between the air phase and water phase is dependent on the compound characteristics, the concentration of the chemical in the air and water phase, the concentration of other chemicals in the water phase, the type of medium, the surface area of the water/air interface, and other factors (8).

Biofilters may be characterized as completing the aerobic treatment pathway (Fig. 1). Heterotrophic bacteria in the water and biofilm that use organic compounds for their energy source are chiefly responsible for the oxidation of volatile organic compounds (VOCs). Hydrogen sulfide and ammonia are transformed by chemoautotrophs.

Under ideal conditions, carbon dioxide and water are the ultimate products of contaminant degradation. Treatment of certain VOCs or compounds containing nitrogen, chlorine, or sulfur may produce acids (14–19).

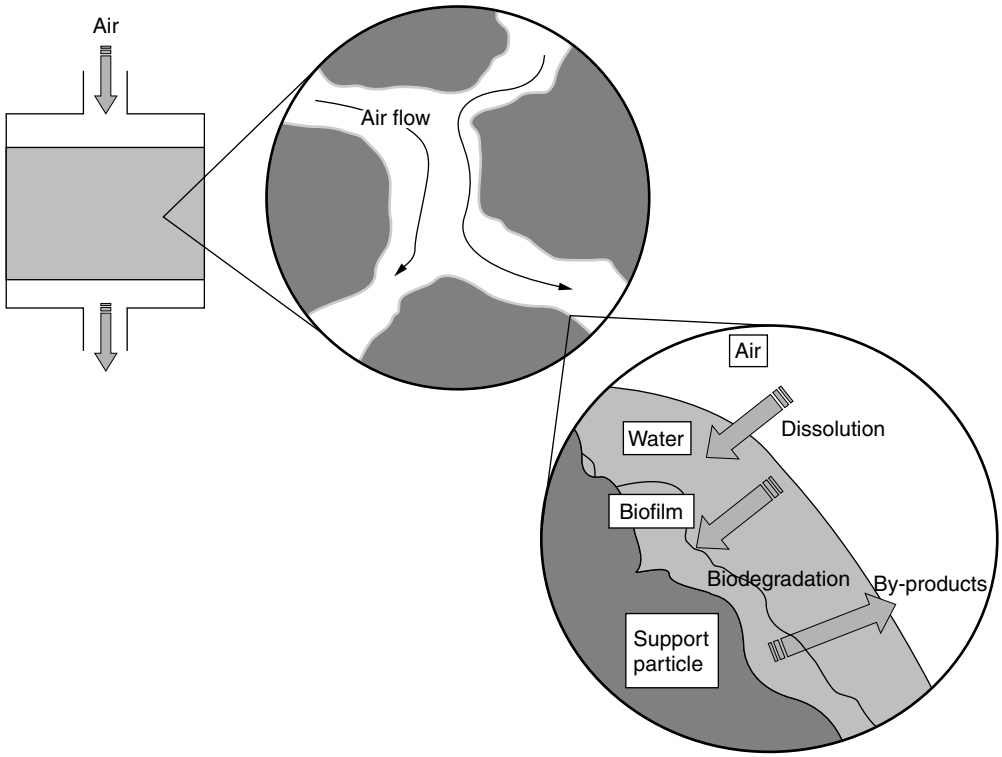

Figure 2. Detail of biofilter mechanisms.

Acid production is a common problem in Publicly Owned Treatment Works (POTW) biofilters because hydrogen sulfide usually is the dominant odor-causing compound, with typical concentrations ranging from the low ppm to the hundreds of ppm. The production of acid causes difficulties for POTW biofilters because most known heterotrophic bacteria capable of consuming VOCs prefer neutral pH (20). Researchers have assumed that removal efficiencies decrease with decreasing pH, and early data supported this (14,15) assumption. A second problem of acid production is that acid degrades organic packing such as compost, opening channels in some areas and compacting others.

Buffers, such as oyster shells (14) or dolomite (21,22), have been added to the packing to avoid pH decrease. However, the rates of acid production commonly seen in wastewater treatment plants consume packing buffer relatively rapidly. The spent minerals and acid-degraded compost often form small particles and contribute to biofilter clogging. Head loss rises, increasing operation costs, and efficiency declines. Elemental sulfur also may accumulate and may be inhibitory. This commonly limits compost to a lifetime of three or four years (15). The packing also may be washed with water to help control the pH (15). However, it is difficult to ensure uniform treatment because the water flows down through the packing in irregular patterns. In practical applications, it is difficult and expensive to control the pH, and the medium is usually replaced when its alkalinity is completely exhausted (20,23) and the pH falls below 6.

However, several recent studies have shown that biofilters are effective at lower pH where only a small amount of wash water is needed to stabilize the pH. The amount of acid discharged in biofilter leachate increases as pH falls because the concentration of protons in solution increases. Thus, the pH falls until acid production is equaled by the discharge of acid in biofilter drainage. Uncontrolled systems typically stabilize at a pH between 1 and 3, where treatment can continue indefinitely. Shinabe and coworkers (24) studied the removal of H_2S by *Thiobacillus thiooxidans*. They found that greater than 99% removal of H_2S was possible in their two-stage full-scale biofilter using a ceramic medium. The removal efficiency was not pH-dependent. Webster and coworkers (25) conducted a bench- and pilot-scale study at a POTW for 18 months. They used a variety of packing materials and compared pH-controlled and uncontrolled conditions. They found that their biofilters were effective at simultaneously removing hydrogen sulfide and VOCs at both bench scale and pilot scale irrespective of pH control. Chitwood and coworkers (26) operated a pilot-scale biofilter with a 1-cm diameter lava rock as the medium. The system was operated with an average hydrogen sulfide inlet concentration of 5 ppm and the bed at pH 4. They observed greater than 90% removal of hydrogen sulfide and 75% removal of total VOC at an empty bed residence time (EBRT) of 13 seconds. Each of these studies used inorganic medium that did not deteriorate at low pH conditions.

A practical limitation of biofilters is the required footprint. Traditionally, biofilters have been designed with an EBRT (calculated as the volume of packing divided by airflow) of 60 seconds. A loading rate of $15–25 \times 10^{-4}$ $m^3/s\text{-}m^2$ is typical. However, biofilters designed for removal

of H_2S from air can accept higher loads. Greater than 90% removal of H_2S has been achieved in several biofilters operated with an EBRT of 15 seconds or less (24,26,27). Under practical conditions for POTWs, transfer of hydrogen sulfide from the air phase to the liquid phase and maintenance of uniform airflow may be more important limitations than contaminant degradation rates.

Biofilter Designs

Conventional biofilters are single-stage, up-flow, and open-bed systems with an organic medium (Fig. 3). These biofilters require periodic watering with sprinklers but no nutrient addition, and require little maintenance. They are often constructed by excavating a pit, lining it with a plastic sheet, placing a perforated pipe, and adding gravel and compost. They are attractive because of their simplicity and low cost. However, closed systems (Fig. 4) that allow better system control and performance are largely replacing open systems in industrial applications (28).

A biofilter system generally consists of eight components. Ductwork collects the gases from the source. An air blower is provided to move the air through the system. The blower is usually located upstream of the biofilter vessel, but it may be placed after the biofilter if a closed bed is used. (With this arrangement, the biofilter operates under pressure slightly less than that of the surrounding atmosphere, so that any leaks will draw ambient air into the system rather than allow contaminated air to escape. This is especially beneficial when the biofilter is located inside a building or when the contaminants are strongly odorous or toxic. It also means that the blower will not be in contact with untreated air that may be corrosive.) A humidification chamber is used to increase the relative humidity of the air to approximately 100% before it enters the biofilter. This is necessary to prevent drying of the bed. Air distribution and ventilation piping provides homogeneous distribution of the air and then collects the treated air to be released. The biofilter bed can be single or multiple layers, depending on the treatment requirements and available space. An irrigation system is used to maintain proper water content in the bed. It can also be used to add nutrients and pH buffers. Although biofilters produce only a small amount of leachate, a leachate collection system is also required. Leachate is often recycled to the inlet of the irrigation system so that the nutrients in it can be reused. Periodic wasting of leachate is necessary to prevent buildup of salts or toxins in the system.

Application to Treatment Plant Odor Control

Application of biofiltration to odor-emitting facilities such as POTWs and compost plants is appropriate because biofilters are most suited for treating large volumes of air with low concentrations of contaminants characteristic of these facilities. Application of biofiltration may be more complicated at facilities that have a tendency to emit lower volumes with higher concentration, such as bakeries and rendering facilities. This problem can be solved in part by simple dilution of the inlet air.

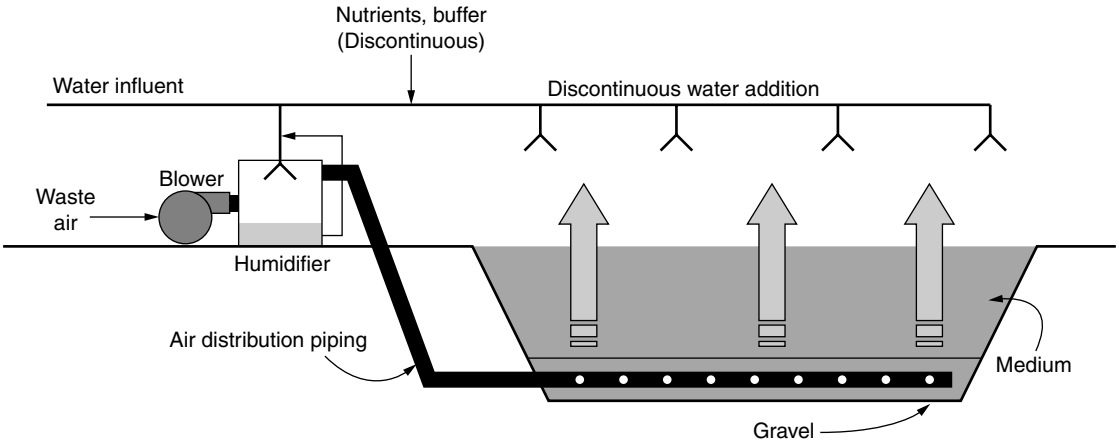

Figure 3. Conventional open bed biofilter design.

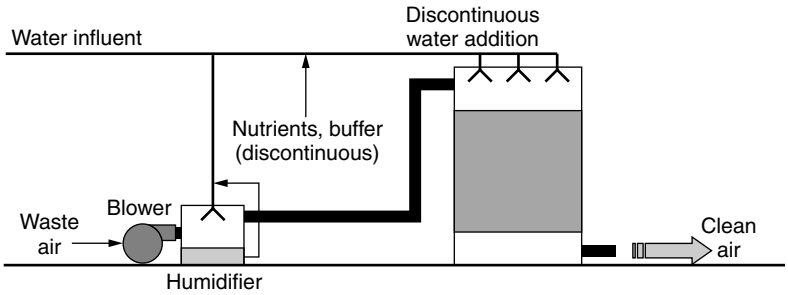

Figure 4. Closed bed system with downward flow.

Emissions from POTWs are characterized by high airflow rates with high moisture content. They are corrosive and may contain aerosols. Emission points include plant headworks (bar screens and grit chambers), primary clarifiers, aeration basins, and solids-handling processes (anaerobic digestion, biosolids dewatering, and composting facilities) (29). Emissions consist of various odor and organic compounds. Concentrations are low and vary greatly with time (30).

Treatment of Hydrogen Sulfide in Biofilters. The effectiveness of biofilters for removing hydrogen sulfide was investigated in numerous studies (15,19,23,31–46). Yang and Allen (15) conducted a comprehensive laboratory study of compost biofilters treating hydrogen sulfide. The relationship between H_2S removal efficiency and temperature, medium pH, water content, air retention time, sulfur loading rate, and sulfate concentration in the medium were experimentally determined. More than 99% removal efficiencies under a wide range of conditions were achieved with a maximum elimination capacity of 150 $g/m^3/hr$. However, lower pH, tested by the addition of hydrochloric acid, negatively affected removal efficiency, and the optimum pH was above 3.2. In long-term experiments, water was periodically added to the system for pH control. Addition of 0.57 liters of deionized water per liter of medium caused an increase in 0.2 to 0.5 pH units.

Treatment of Ammonia in Biofilters. Research has been conducted to determine the effectiveness of biofilters for removing ammonia from waste gas streams (47–50). Ammonia is a common odor problem from composting facilities at POTWs. The studies demonstrated that biofilters with an established nitrifying bacteria population could effectively oxidize ammonia to nitrite, nitrate, and organic nitrogen compounds. However, the major (68%) end product was nitrite (48). The optimum medium pH was approximately 8. Hartikainen and coworkers (47) demonstrated adequate removal of ammonia at pH 6. However, no removal occurred in their test at pH 4.

Treatment of VOCs in Biofilters. A wide range of studies has been conducted to determine the applicability of biofiltration to the removal of organic compounds from waste air. Applications have included bakeries (16), soil vapor extraction emissions (51,52), the print industry (53,54), pulp mills (55), plastics manufacturing (56), and various other industrial applications (8). In general, it was shown that chlorinated alkanes display the poorest removal efficiencies, followed by aldehydes and ketones, with the best removal for aromatics (25). Actual removal is compound-specific. A summary of elimination capacities of various compounds in past biofilter studies has been compiled (8).

POTW Biofiltration Research. Biofilters are an effective means of controlling odors and low concentrations of mixed VOCs from POTWs (14,23,24,31,37,57–59). The first biofilters constructed were used at POTWs to control odors (31). Ergas and coworkers (14) conducted a 10-month pilot-scale compost biofilter study at a POTW. They found that H_2S removal was greater than 99%, whereas aromatic VOC removal was consistently greater than 90%. However, they also controlled the pH at neutrality. Shinabe and coworkers (24) found greater than 99% removal of H_2S by *Thiobacillus thiooxidans* in their two-stage full-scale biofilter using a ceramic medium. The removal efficiency was not pH-dependent. They did not study simultaneous removal of VOCs. Webster and coworkers (25) conducted a 1.5-year bench- and pilot-scale study at a POTW. They used a variety of media and compared pH-controlled and uncontrolled media. They found that their biofilters were effective at simultaneously removing hydrogen sulfide and VOCs at both bench scale and pilot scale, irrespective of pH control. Recent work has confirmed that cotreatment of H_2S and aromatics at low pH is possible (60).

MICROBIOLOGICAL ASPECTS OF BIOFILTRATION

Microbial Ecology of Biofilters

Microorganisms in biofilters grow on the surface of the packing material. They first form colonies that spread to become patches, and the patches finally merge to create a continuous biofilm over the packing. To be active in the removal of contaminants, they must have adequate supplies of water, oxygen, and nutrients, as well as adequate contact with the contaminant.

Biofilter start-up requires an inoculum of microorganisms that are capable of growing under the conditions in the biofilter and obtaining their energy from the degradation of the contaminant. Many biofilters use partially composted materials, such as yard waste, as the packing material. These have the advantage that they carry a diverse and active ecosystem of microorganisms with a broad capability for degradation of organic compounds. It is very likely that microorganisms capable of degrading the contaminant will be present and ready to serve as the seed for rapid development of a dense culture for treatment. Biofilters that use inorganic media such as plastic shapes or stones must be provided with an appropriate inoculum. Biofilter operators commonly choose notably diverse inocula such as sewage sludge or extracts from previously active biofilters. In a few cases, pure cultures of chosen species known to degrade the contaminant have been used.

In any case, the microbial ecosystem will undergo sharp change and rapid development when it is exposed to the contaminant. The species that are capable of utilizing it will grow vigorously. Because of the large flow of air through the biofilter, new species are continually being introduced. In time, predators (e.g., protozoa) and parasites of the successful species will proliferate and then predators of the predators (e.g., nematodes) will grow, creating elaborate food chains. Insect larvae may grow. In one case, the headspace above a compost biofilter was colonized by thousands of spiders that were eating the insects. This process of ecosystem development may proceed for some time. Although a biofilter typically reaches a steady state with respect to treatment success in weeks, there have been reports of continuing ecosystem change after more than a year of operation (61,62). That

is, new species are still being introduced and the relative abundances of the species present are changing.

Factors Affecting Biological Activity

Water content is a key environmental variable for the microbial ecosystem in a biofilter. Drying is commonly the cause of declines in biofilter performance (63). The microorganisms do best when the packing is covered with a film of water. Indeed the microbial community helps hold this water by creating a biofilm composed of polysaccharides that has a significant water-holding capacity. Chitwood (26) showed, for example, that the water-holding capacity of an experimental biofilter using inorganic packing increased by 35% during the first three months of its operation.

Maintaining an ideal water content in the biofilter, however, is difficult. The porous medium water content at equilibrium with the atmosphere is a very sharp function of the RH of the air. Relative humidity values just a percent or two below saturation will cause the biofilter to gradually dry (8). Such slight declines in RH can in turn be caused by an increase in temperature by a few degrees, resulting from compression in the blower or heat gain through duct walls. For this reason, biofilters are often equipped with humidifiers on the incoming air and irrigation systems that spray the packing directly. Desiccation-resistant degraders, particularly fungi, have been investigated for use in biofilters (64), and may have valuable applications. However, even these systems require that the air be near 100% humidity.

An adequate oxygen supply is necessary for the great majority of biofilters that rely on oxygen as the electron acceptor in the degradation process. Unlike water treatment systems, biofilters for air are rarely short of oxygen. The gas being treated is air, and the contaminants are present at concentrations reaching at most a few thousand ppm, so the oxygen concentration is far above stoichiometric requirements. Oxygen availability within the biofilm is far less certain. Oxygen must diffuse from the surface of the biofilm to its depths. Oxygen concentrations and diffusion rates are far lower in water, especially if it contains a thick gel of cells and exudates. Biodegradation within the biofilm is constantly consuming oxygen, and in the deeper portions oxygen depletion is common. This is probable in biofilters treating high concentrations of contaminants that will cause development of a thick biofilm and rapid consumption of oxygen. The development of anaerobic regions at the base of the biofilm, or in the smaller and more remote pores of the packing are not desirable. First, the biomass in the anaerobic regions will not be active, reducing the effectiveness of the biofilter. Second, anaerobic activity may generate odorous compounds, contributing to the very problem the biofilter is intended to solve. Problems of inadequate distribution of oxygen will be more prevalent in deteriorating packing, where the airflow becomes less uniform and local volumes of packing are cut off from the oxygen supply.

Biofilter success requires that the contaminant be brought into close contact with the microorganisms. Like oxygen, it must dissolve in the water phase and diffuse into the biofilm. Degradation within the biofilm serves as a sink, so that the concentration of the contaminant declines with depth. If degradation kinetics are first order, degradation rates will decline where the concentration is low, and regions where concentration of contaminant is zero will contribute nothing to treatment success. In many ways, the challenge of providing contaminants to the microorganisms is much like that for oxygen. The major difference is that oxygen is present in high concentrations and has moderate solubility, whereas contaminants are present in relatively low concentration, and some are only slightly soluble. This means that it is much more likely that degradation rates will be limited by contaminant concentrations in the biofilm. Because contaminant concentrations fall as the air passes through the biofilter, this is especially true near the outlet.

Nutrients such as nitrate and phosphate must be supplied to the microorganisms. Biofilters that use compost or other organic packing may not require further addition of nutrients because the slow decomposition of the organic matter releases the necessary compounds to the biofilm.

Inorganic packing such as stones or plastic shapes does not provide nutrients, and these must be added from an external source. It is particularly important to add these with the inoculum, to ensure that an initial biofilm is rapidly established.

Although the general concepts of nutrient requirements are well established, the detailed dynamics in biofilters are not. The composition of biofilter biomass is probably much like other organic material, so that the mass of nutrient required for growth of a given amount of biomass can be calculated. However, the amount of biomass in biofilters, particularly compost biofilters, is poorly known. Furthermore, significant amounts of nutrients are presumably recycled. As cells die or are consumed by predators, these nutrients are released for reuse by other cells. Some nutrient is probably sequestered in dead or inactive biomass. Some will be lost in irrigation water that drains from the bottom of the bed. In compost biofilters, the rate at which nutrients are released by decomposition of the packing is not known and it is not certain that it is always sufficient to avoid nutrient limitation.

Given these uncertainties, it is not surprising that recommendations for nutrient addition vary widely in the literature. Although the observation that poorly performing biofilters can be improved by nutrient addition is common, the amounts necessary are not certain. Because nutrient addition is inexpensive, the common solution is to add them in excess.

Microbial Ecology of Sulfide Biofilters

Biofilters are quite effective for the control of hydrogen sulfide or organic sulfide odors, but special problems arise because the ultimate product of microbiological sulfide oxidation is sulfuric acid. As treatment proceeds, the pH of the biofilter packing declines. Rapid decline may inhibit the activity of sulfide-oxidizing microorganisms, and a sufficiently low pH will hasten the deterioration of compost packing (17).

One approach is to neutralize the acid and maintain the pH at 7. This can be done by adding buffer material to

the packing or adding base to the irrigation water. While this approach encounters some operational problems, as previously described, sulfide oxidation will proceed well. In addition, a neutral pH is ideal for a wide variety of heterotrophs, so that VOCs in the gas stream are also likely to be degraded.

A second approach is to allow the pH to decline. As pH falls, a successional change in ecosystem composition occurs, utilizing the sulfide and producing additional acid. This succession is better known than most in microbiology because it has been studied extensively in efforts to control sulfide corrosion in sewers (5,59). Ultimately, sulfide-oxidizing organisms tolerant of low pH dominate the ecosystem. These are presumed to be of the genus *Thiobacillus*, and the species *Thiobacillus thiooxidans* is probably common, but the species is rarely identified in practice. *Thiobacillus* produces short-chain fatty acids as metabolic by-products and these are self-inhibitory. Acidophilic heterotrophs that live in a symbiotic relationship with the *Thiobacillus* consume these acids. Some experiments have shown that many organic compounds can also be degraded in low-pH biofilters (60) and that some of the same acidophiles may be involved.

Most sulfide oxidizers are autotrophic and so obtain their carbon from carbon dioxide, which is a costly process in terms of energy. This may be the reason these microorganisms tend to release little polysaccharide and thick biofilms generally are not seen in sulfide biofilters.

Overall, biofilters are an excellent technology for removal of hydrogen sulfide from airstreams. Systems have removed up to 99% of sulfide in the tens of parts per million with an EBRT as low as 15 seconds (65).

CONCLUSION

An ideal biological treatment system uses microorganisms to convert waste materials to benign products. In most cases, the most efficient approach is to use dominantly aerobic systems to produce carbon dioxide, water, sulfate, and nitrate. Odors arise if waste materials or partially transformed intermediates escape to the atmosphere before oxidation is complete. Odorous compounds are generated profusely in anaerobic conditions that may arise from inadvertent stagnation in the waste stream or from anaerobic treatment processes.

Passing discharged air through a biofilter that completes the transformation to oxidized products can prevent the release of odors. Operation of biofilters requires attention to the needs of the microbial ecosystem, but well-operated units can be effective and economical solutions to the odor problems at treatment facilities.

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BIOFOULING: CHEMICAL CONTROL OF BIOFOULING IN WATER SYSTEMS

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Microbial biofilms are problematic in a range of industrial environments in which large areas of submerged surfaces are exposed to relatively high nutrient fluxes, providing niches for the formation of copious surface-associated growth (1,2). Bacterial colonization of surfaces in an aqueous environment is a basic strategy for survival in nature as nutrients are more available at the solid–liquid interface (3,4). The resulting aggregates form microcolonies that develop into biofilms (5). Biofilms increase fluid frictional resistance (5) and decrease the rate of heat energy transfer, collectively termed

biofouling. These biofilms also promote corrosion of ferrous and other metals by the concerted metabolic activity of a number of biofilm-associated bacterial types (6–8), a process collectively termed microbially-influenced corrosion (MIC). MIC encompasses a number of specific mechanisms relating either directly or indirectly to the metabolic activity of a variety of microorganisms, notably the action of sulfidogenic bacteria (9,10). As the costs attributable to MIC and biofouling are high, effective control of bacterial numbers in industrial aqueous environments is essential.

A range of bactericidal substances, commonly termed biocides or microbicides, are available, all of which are claimed by their producers to kill bacteria occurring in aqueous systems quantitatively. Biocides target a range of cellular loci, from the cytoplasmic membrane to respiratory functions, enzymes, and genetic material (11). However, different bacteria react differently to bactericides, either owing to inherent differences such as unique cell envelope composition and nonsusceptible proteins (12) or to the development of resistance, either by adaptation or by genetic exchange (13). Bactericides should therefore be evaluated against the organisms that they are chosen to control, that is, the dominant ones in the system to be treated. The composition of microbial populations in systems varies with the type of water used, and changes considerably following treatment with various biocides by selection for resistant strains (14). Bacteria growing as biofilms are also significantly more resistant to most antimicrobial agents known currently, so that methods for their control pose an ongoing challenge (2,15).

Successful biofouling control depends on rationally developed treatment strategies that are based on information about the specific system. The primary target should always be the biofilm-associated flora as it is the catalyst for MIC and impacts negatively on system operation.

Five approaches are currently available and may be used in combination:

1. Bacteria are chemically killed by application of biocides;
2. Cells are dislodged from surfaces by dispersants;
3. The biofilm structure is weakened by enzymes or chelating agents of divalent cations;
4. Biofilms are removed physically by a variety of processes; and
5. Biocide efficacy is enhanced by applying an alternating current or ultrasonic sound across the biofilm.

This entry focuses on the application of biocides to biofouling control.

BIOCIDES

Bactericides are antimicrobial agents employed in various spheres of human activity to prevent, inhibit, or eliminate microbial growth. They can be divided into two groups — those derived from naturally occurring antimicrobial agents (termed antibiotics) and those not occurring readily in nature (termed antiseptics, disinfectants, biocides, bactericides, sanitizers, and preservatives). Members of the second group are classified depending on their chemical nature, but more often on their specific field of application.

The use of biocides to control biofouling in water systems is still an accepted practice, although higher levels of environmental awareness and tighter legislation have placed increased pressure on the water treatment industry to seek alternative means of control (16,17). Nevertheless, biocides are still indispensable components for effective control of biofouling as they remain the core technology for decimating viable numbers whereas all other techniques merely aid in their efficacy.

The modes of action of a plethora of antibiotics have been investigated in detail. Much less is known about the mechanisms of action of the many biocides available for biofouling control. Biocides for water treatment target a range of specific cellular components and functions, from membrane permeability and electron transport to enzyme function.

Bactericides attack functional cell components, placing the bacterium under stress (18). At low concentrations, bactericides often act bacteristatically, and are only bactericidal at higher concentrations (19). Targets of bactericide action are components of the cytoplasmic membrane or of the cytoplasm (11). For bactericides to be effective, they must attain a sufficiently high concentration at the target site in order to exert their antibacterial action. In order to reach their target site(s), they must traverse the outer membrane of the gram-negative bacteria. Therefore, different bacteria react differently to bactericides owing to differing permeabilities of their respective cell walls (20). Bacteria with effective penetration barriers to biocides generally display a higher inherent resistance than those bacteria that are readily penetrated. The rate of penetration is linked to concentration so that a sufficiently high biocide concentration will kill bacteria with enhanced penetration barriers (11).

Water treatment bactericides fall into two categories, oxidizing (e.g., chlorine and hydrogen peroxide) and nonoxidizing. Nonoxidizing bactericides can be divided into five groups based on their chemical nature or mode of action, and these are discussed later.

Oxidizing Biocides

Oxidizing biocides are general chemical oxidants. They are not selective for living organisms, but react with any oxidizable matter. However, they are bactericidal because certain bacterial cell components can react readily with them, having a higher oxidation potential than most other chemicals present in water. Three classes of oxidizing biocides are available for bactericidal applications; oxidizing halogens, peroxides, and ozone.

Peroxides. Peroxides are unstable oxygen compounds that decompose to form free hydroxyl radicals. These react oxidatively with organic matter. The peroxides include hydrogen peroxide, peracetic acid, aromatic peroxyacids, persulfates, and calcium peroxide.

Hydrogen Peroxide. Peroxide has good antimicrobial properties and decomposes to water and oxygen, leaving no toxic waste. Hydrogen peroxide penetrates cells causing site-directed damage on account of metal-dependent OH formation (21). It causes DNA strand breaks and base hydroxylation. Guanine and thymine are the two main targets of peroxide-generated free radical attack. The resulting 7,8-dihydro-8-oxoguanine mispairs with adenine whereas thymine oxidation products stop DNA polymerase, halting replication (21). Most bacterial mutants cannot survive because of incoherent metabolism, so that peroxide treatment at low concentration leads to slow death. Hydrogen peroxide also inhibits mitochondrial ADP-phosphorylation.

The development of resistance to oxidizing bactericides has not been reported in the biofouling control literature. However, a variety of bacteria, mostly fermentative, exhibit oxidizing stress response by producing oxidant-degrading and repair enzymes. Stress response means that cells become more resistant to a deleterious factor within minutes of exposure to subinhibitory quantities of the factor. A variety of defense genes have been characterized in *Escherichia coli*, encoding various superoxide dismutases, catalases, alkyl hydroperoxide reductases and glutathione reductases, as well as DNA repair enzymes (22). In addition, various regulatory genes have been characterized, including *oxyR* and *soxR*. These regulators determine intracellular redox potential and activate stress response when cells are exposed to oxidizing agents.

Organic Peroxides. Peracetic acid is the best known of the organic peroxides. Like hydrogen peroxide, it forms free hydroxyl radicals, which react with various protein structures and DNA. In addition, the dissociation of peracetic acid leads to formation of acetic acid, which is mildly antibacterial. Application of peracetic acid to systems does not leave any toxic waste behind. The antibacterial activity of peracetic acid is not affected by water hardness or organic contamination.

Oxidizing Halogens. Hypochloric and hypobromic acids pose excellent antibacterial activity, although within a defined pH range.

Chlorine Compounds. Chlorine, chlorine dioxide, and hypochlorous acid (HOCl) are the most widely used biocides worldwide. Hypochlorite was first employed as a wound disinfectant by Hüter in 1831, and its bactericidal activity was confirmed by Koch in 1881 (23). Hypochlorite is used in industrial water systems to control biofouling.

The antibacterial mechanism of action of hypochlorite is not clear to date although much work has been done on the mechanism of action in eukaryotic cells (8). HOCl is a powerful oxidizing agent, oxidizing thiol groups and halogenating amino groups in proteins (24), as well as oxidizing lipids and proteins (25). Specific bacterial targets are cytochromes, nucleotides, and iron-sulfur proteins (26). Protein synthesis is impaired (data not published) and the uptake of nutrients is also affected (27). The stability and antimicrobial activity of hypochloric acid is dependent on pH. It dissociates at pH greater than 7, and the undissociated moiety is the antibacterial one (23). Above pH 7.5 it loses its antibacterial activity. It is excellent for biofouling control as it weakens the extracellular polysaccharide structure, leading to sloughing and removal of sections of the biofilm.

Bromine Compounds. Hypobromous acid works similarly to hypochloric acid. It is, however, stable at a pH up to 8.5. This makes it more suitable for application in cooling waters, which are often maintained at a slightly alkaline pH. Certain organic compounds release hypobromic and hypochloric acid slowly when in solution. An example is 3-bromo-1-chloro-5,5–dimethylhydantoin (28). Such compounds maintain a longer antibacterial level of hypohalous acid in the system treated.

Ozone. Ozone is a strong oxidizing material capable of killing bacteria and algae and of inactivating viruses. It is an unstable gas with a pungent odor. It further degrades the extracellular polysaccharide, holding biofilms together, so that treatment results in loosening of the biofilm. This leads to loosening of scale from the surface. Ozone has a very short half-life and therefore has to be generated on-site. In distilled water, the half-life of ozone at 20 °C is 25 minutes. Its solubility in water is 13 times that of oxygen. Upon reaction with organic material it decomposes to oxygen. It does, however, react with several cations and anions such as $Fe^{2}+$, $Mn^{2}+$, $MnO_4{}^{2-}$, $N0^{2-}$, and CN^{-}. Ozone is toxic to humans, and detectors should be installed together with ozone generators. Furthermore, ozone is very costly to produce when compared with chlorine. However, treated water is perfectly safe as ozone degenerates to oxygen whereas chlorine may react with humic substances in water to yield toxic trihalomethanes.

Electrochemically Activated Water

Principles of Electrochemically Activated Water Technology. Electrochemically activated water has recently become available, and has been found effective in biofouling control (29). Water of varying mineralization is passed through an electrochemical cell, the specific design of which permits the harnessing of two distinct and electrically opposite streams of activated water.

Aside from its distinctive attributes, the negatively charged antioxidant solution (catholyte) can also be channeled back into the anode chamber, thereby modulating the quality of the positively charged oxidant solution (anolyte) that is produced. Depending on the specifications of the required application, variations in the design of the hydraulic systems can be effected to meet the requisite objectives.

The design of the cell is such as to ensure a uniformly high voltage electrical field through which each microvolume of water must pass. This unipolar electrochemical activation created by potential gradients of millions of volts per cm^2 between the anode and cathode terminals results in the creation of solutions whose pH, oxidation reduction potentials (ORP) and other

physicochemical properties lie outside of the range, which can be achieved by conventional chemical means.

Properties of Activated Water. The properties of the activated solutions are dependent on a number of factors. These comprise the solution flow rate through the reactor cell, the current being applied, temperature, the degree of feedback of catholyte into the anolyte chamber and the degree of mineralization of the water.

During electrochemical activation, three categories of products within the solution are generated. They comprise:

1. Stable products that include acids and bases that influence the pH of the solutions,
2. Highly active, unstable products including free radicals and electrolytic gases in the form of microbubbles that influence the ORP of the solutions, and
3. Quasi-stable products comprising complexes of hydrated membranes that form clusters of water molecules, which impart the catalytic activity of the solutions.

Without maintenance of the activated state, these diverse products degrade to the relaxed state of benign water and the anomalous attributes of the activated solutions such as altered conductivity and surface tension similarly revert to preactivation status.

It is important to note that the level of mineralization of input water required to generate optimally metastable solutions is not significantly different from the composition of benign potable water. However, the heightened electrical activity and altered physicochemical attributes of the solutions differ significantly from the benign state, yet remain nontoxic to mammalian tissue and the environment.

Biocidal Properties of Anolyte. Earlier technologies that have employed electrochemical activation to generate biocidal solutions have not been capable of separating the output of the anolyte and catholyte solutions. In these cases the two opposing solutions have neutralized each other with regard to potential electrical activity.

The advantages of the current ECA technology has been confirmed, wherein the biocidal activity of hypochlorous acid generated by the current ECA technology is 300 times more active than the sodium hypochlorite generated by earlier systems. Additionally, a comparison of neutral anolyte (pH = 7), with alkaline glutaraldehyde (pH = 8.5), showed that the latter required a concentration of 2% versus 0.05% of the former, in order to achieve the same biocidal efficacy. Similarly, it has been shown that a 5% solution of sodium hypochlorite can only be used for purposes of disinfection whilst a 0.03% solution of neutral anolyte has both disinfectant and sterilizing properties. In general, the biocidal activity of nonactivated neutral anolyte (only stable products and no electrical charge) is 80 times the potential activity of the hypochlorite solution, but still exhibits only one-third of the full biocidal potential of the optimally activated ECA solution.

Thus, activated solutions have been conclusively shown to exceed chemically derived equivalents both in low-dosage effectiveness as well as physicochemical purity. This heightened biocidal capacity relative to traditional chemical solutions permits the incorporation of ECA solutions at substantially lower dose rates, therein obviating the risk of intoxication, adverse environmental impact, while providing cost-effective resolutions.

Nonoxidizing Biocides

Nonoxidizing biocides include a variety of organic chemical compounds that have antimicrobial activity. Their modes of action differ vastly, and their only common denominator is that they are nonoxidizing organic molecules. Most currently used biocides fall into five distinct categories, although a number of miscellaneous compounds are also useful.

Detergent-Type Biocides. Three groups of surface-active antimicrobial agents have been documented to date; anionic, cationic, and amphoteric (23). Anionic antimicrobials are only effective at pH < 3.0 and include the aliphatic acids such as sodium dodecyl sulfate. The cationic antimicrobial agents are the generally organic ammonium salts, commonly termed quaternary ammonium compounds (QAC). The best known is benzalkonium chloride (N-alkyl-N,N-dimethyl benzylammonium chloride) (23). Recently, a number of novel organic ammonium salts of general structure alkyl trimethyl and dialkyl dimethyl ammonium bromide were synthesized and evaluated (30). The authors reported extremely low minimum inhibitory concentrations to *Pseudomonas aeruginosa* of 12.5μg/ml where the second alkyl group was C_6 or longer.

Benzalkonium chloride adsorbs to the cell surface of negatively charged cells (pH > 7.0) in an irreversible way (11,31). The pH minimum for antimicrobial activity is 3.0. It is membrane active and induces leakage of cytoplasmic constituents (23). Upon exposure to benzalkonium chloride, membranes of *Pseudomonas cepacia* appeared irregular, indicating membrane damage (32). At 37 °C it is twice as active as at 20 °C. It is active against gram-positive as well as against gram-negative cells, but not against spores. Cations such as $Ca^{2}+$ and $Fe^{3}+$ decrease its activity, as does NaCl (23).

Biguanides. Biguanides are polymer derivatives of a general guanidine structure. Two biguanides are currently used as industrial bactericides. These are polyhexamethylene biguanide (PHMB) and 1,6-di-(4-chlorophenyldiguanido)-hexane, better known as chlorhexidine (23). Both are not corrosive and all are well-suited for application in cooling water (19).

Biguanides are bacteriostatic at low concentrations and bactericidal at higher concentrations, and have a wide spectrum of activity, especially against gram-negative bacteria (23). They are membrane active agents and attach rapidly to negatively charged cell surfaces (pH neutral or alkaline). By making use of ^{14}C-radiolabeled polyhexamethylene biguanide, it has been shown that polyhexamethylene biguanide absorbed into cells of *E. coli* within 20 seconds after exposure (33). Bactericidal action, however, requires a few minutes. Biguanides compete with divalent cations for negative sites at LPS, displacing these. PHMB then interacts by electrostatic interactions

with the charged headgroups of phosphatidyl glycerol and diphosphatidyl glycerol (negative), but not with the neutral phosphatidyl ethanolamine (34). By binding to phospholipids of the inner leaflet of the outer membrane and of the outer leaflet of the inner membrane, the two membranes attain net positive charges and are repelled from each other, causing membrane damage by distortion. This is supported by TEM studies on *P. cepacia* in which both membranes acquired a distinct irregular appearance after treatment with chlorhexidine (32). Cytoplasmic constituents start leaking out of the cell because of the rupture of the membranes, and the cell loses its viability.

Aldehyde-Based Biocides. Two aldehydes are commonly used as antimicrobial agents, that is, formaldehyde and glutaraldehyde. Further, there is a range of bactericides such as the hydroxyethyl- and ethyltriazine-bactericides available, all of which release formaldehyde (35). Formaldehyde has a high polarity and high nucleophilic reactivity, so that it reacts primarily with free primary amino groups, but also reacts with amines, amides, sulfides, purines, and pyrimidines (35). In water it hydrates to methylene glycol. Reaction with primary amino groups leads to the formation of methyloamines that react further with cellular components. Formaldehyde damages the transport properties of membrane porins, decreasing the rate of proline uptake and of enzyme synthesis. It is active over a wide pH spectrum (3.0–10.0) and is sporicidal (23).

Glutaraldehyde also reacts with amino and sulfhydryl groups (11). It is stable in acid solution but is only active at pH 7.5 to 8.5, so it must be alkalinified before application (23). A 2% solution at the correct pH is 10 times more bactericidal than a 4% solution of formaldehyde (23). Its reactivity is related to temperature; a 2% solution kills spores of *Bacillus anthracis* in 15 minutes at 20 °C, whereas it requires only 2 min at 40 °C. In gram-positive bacteria it reacts with, and binds to, peptidoglycan and teichoic acid and is also sporicidal (11). In gram-negative bacteria it reacts primarily with lipoproteins of the outer membrane, preventing the release of membrane-bound enzymes.

Phenol Derivatives. Phenol was the antimicrobial agent that revolutionized invasive surgery, and was pioneered by Lister in 1870 (36). It enters the cell by dissolving in the membrane, and upon entry into the cytoplasm, precipitates proteins. It is, however, harmful to humans, and its antibacterial activity is not very high (23). A range of halogenated phenols, cresols, diphenyls, and bisphenols have been developed from phenol, and have excellent antimicrobial activity, many being applied in the preservation of pharmaceutical products. Halogenation increases the antimicrobial activity of phenol, as does the addition of aliphatic and aromatic groups (23). Bisphenols have the highest antimicrobial activity of the phenol derivatives, especially the halogen substituted ones. Hexachlorophen and 2,2′-methylenebis(4-chlorophenol) (dichlorophen) fall into this group (13).

Phenol derivatives are membrane-active agents. They penetrate into the lipid phase of the cytoplasmic membrane, inducing the leakage of cytoplasmic constituents (11). 3- and 4-chlorophenol uncouple oxidative phosphorylation from respiration by increasing the permeability of the cytoplasmic membrane to protons (37).

Thiol-Oxidizing Biocides. Thiols on amino acids such as cysteine are important groups that influence the tertiary structure of proteins by forming disulfide bridges. Three groups of antimicrobial agents, isothiazolones, bronopol (2-bromo-2-nitropropane-1,3-diol), and mercury, and other heavy-metal compounds, react with accessible thiols, altering the three-dimensional structure of enzymes and structural proteins (38). Mercury interacts with sulfhydryl groups by complexing with sulfur (23). Bronopol oxidizes thiols to disulfides, reacting especially with the active center of hydrogenase enzymes (23).

Four water-soluble isothiazolones possess antibacterial activity; 5-chloro-2-methyl-3-(2H)-isothiazolinone (CMIT), 2-methyl-3-(2H)-isothiazolinone (MIT), 1,2-benzisothiazolin-3-one (BIT) and 2 methyl 4,5-trimethylene-4-isothiazolin-3-one (MTI) (23). MIT and CMIT are often supplied in a 3 : 1 ratio. Isothiazolones react oxidatively with accessible thiols such as cysteine and glutathione (39). These thiols are oxidized to their disulfide adjuncts, which, in the case of cysteine, leads to an alteration of protein conformation and functionality. Isothiazolone is thus reduced to mercaptoacrylamide, which in the case of CMIT tautomerizes to thioacyl chloride, the latter reacting with amines such as histidine and valine (38). Isothiazolones are primarily bacteriostatic and are only bactericidal at high concentrations.

Miscellaneous Biocides. The mechanisms of action of various antimicrobial agents, employed to control bacterial growth in cooling water systems, have not been formally published to date. These include tetra-alkyl phosphonium chloride, sodium diethyldithiocarbamate, methylene bisthiocyanate (MBT), 2-thiocyanomethylthiobenzothiazole, 2-thiocyanomethylthiobenzothiazole, hexachlorodimethylsulfone, tetrakishydroxymethylphosphonium sulfate (THPS) and a range of 2-arylthio-N-alkylmaleimides (6).

Phosphonium chloride probably has surfactant properties, damaging the bacterial cell envelope. It is active over an extremely wide pH range (2–12) and can be used in conjunction with oxidizing biocides. MBT is readily miscible with dispersants to aid the penetration into and the dispersion of biofilms. The mode of action of this nonmetallic organosulfur compound is as yet unknown. Thiocarbamates are used as agents for the extraction of trace metals such as Fe, Cd, Co, Cu, Mn, Ni, Pb, and Zn (40). This would imply that they chelate iron, a vital trace element of most bacteria. The nucleophilic sulfur atom indicates potential reactivity with accessible thiols. Thiocarbamates do react with accessible thiols and amines. Therefore, their antibacterial mechanism of action would rest partially on denaturation of surface proteins. We have found that the antibacterial mechanism of action depends on the alkyl chain length of the thiocarbamate. Sodium diethyldithiocarbamate is inactivated by free ferrous iron, indicating that it removes iron (a

growth factor) from the cell. Sodium dimethyl dithiocarbamate is not inactivated to the same degree, showing that its antibacterial activity does not rest as much on iron removal. 2-Thiocyanomethylthiobenzothiazole is also stable over a wide pH range and can be used synergistically with surfactants to aid in dispersion of and/or penetration into biofilms. Hexachlorodimethylsulfone is stable at high temperature and is an effective slimicide over a wide pH range, although it is not water-soluble and must be dosed as an emulsion. THPS is an amphoteric surfactant miscible with various other surfactants to improve action against biofilms. It is active in both acidic and alkaline conditions and at high temperatures, and remains water-soluble in oil-water mixtures.

The 2-arylthio-N-alkylmaleimides are extremely bacteriostatic, and many derivatives demonstrate an MIC against various bacteria except *P. aeruginosa* of 6.25 μg/ml (6).

Alternative Approaches of Chemical Control

Surface-Embedded Biocides and Catalysts. Various tertiary amines have been covalently linked to polystyrene (41), and quaternary amine compounds have been complexed into polyvinyl chloride and ethylene vinyl acetate latex (42). The broad-spectrum antimicrobial Triclosan (2,4,4′-trichloro-2′-hydroxy-diphenylether-5-chloro-2-(2,4-dichlorophenoxy)-phenol) has been impregnated into various polymers and shown to suppress surface-associated growth. It is active against most bacteria, excepting *P. aeruginosa*.

Recently, Wood and coworkers (43) reported a strategy for circumventing the resistance of bacteria in biofilms by generating biocidal species at the biofilm-substratum interface from less active agents using surface-embedded catalysts. They embedded the catalysts cobalt- and copper-sulfonated phthalocyanine in surface-associated resin. Both hydrogen peroxide and potassium monopersulfate are broken down into highly reactive oxygen species and significantly enhanced the killing of *P. aeruginosa* biofilms (44).

Enhancement of Biocidal Activity by an Electrical Field. Bacteria in biofilms are well known to be more resistant to biocides than are their planktonic counterparts. A select number of studies have reported that application of low-intensity electric fields in the range 15 $\mu A/cm^2$ to 2.1 mA/cm^2 and with a field strength of 1.5 to 20 V/cm can override the biofilm-associated resistance (45,46). All areas within the electric field are affected as the antimicrobial agent is moved by an electrophoretic effect.

Saccharolytic Enzymes. The biofilm is stabilized by an extracellular polysaccharide matrix, often interspersed by other extracellular polymers such as proteins. The extracellular polysaccharides are composed of homo- and heteropolysaccharides of mannose, glucose, fucose, galactose, mannuronic acid, guluronic acid, and pyruvate, giving rise to a complex array of polymeric structures (47). Saccharolytic enzymes have been used to degrade the polysaccharide matrix, thereby destabilizing the biofilm structure, but the heterogeneity of polymers dictates that mixtures be applied (47,48). Recently, Johansen and coworkers (49) demonstrated that the addition of lactoperoxidase to saccharolytic cocktails contributed not only to biofilm removal, but also to a decrease in the culturable count.

Dispersants (Surface-Active Compounds). More recently, surface-active compounds (surfactants) have been employed to prevent bacterial adhesion to surfaces. It is unlikely that surfactants will have any mutagenic effects on bacteria or that microorganisms would be able to become resistant to the action of surfactants, as can be the case with biocides (11,14). Unfortunately little published information is available on the efficacy of different biodispersants (surfactants) against bacterial attachment (50). According to Paul and Jeffrey (51), dilute surfactants completely inhibited the attachment of estuarine and marine bacteria. Surfactants result in both uniform wetting of the surface to be treated and have an additional cleaning effect (14,50). Whitekettle (52) found a correlation between the ability of a surface-active compound to lower surface tension and its ability to prevent microbial adhesion. White and Russel (53) classified surfactants according to the ionic nature of the hydrophilic group, viz. anionic, cationic, nonionic, and zwitterionic.

FACTORS AFFECTING EFFICACY OF TREATMENT PROGRAMS

The antibacterial activity of bactericides is determined by their chemical reactivity with certain organic groups. Bactericides do not select between free and cell-bound groups. Therefore, oxidizing bactericides react with any readily oxidizable organic compound, and not only with live cells. Bactericide activity is influenced by the chemistry of the environment it is used in (23). Factors affecting bactericide effectivity are the following:

- pH
- Water hardness
- Organic compounds such as proteins or saccharides
- Additives such as antiscaling agents or corrosion inhibitors

These factors affect different bactericides to different degrees. Some bactericides are not very stable in concentrated form and undergo changes. Formaldehyde polymerizes when exposed to polar compounds (acids or alkalis) or high temperature and oxidizes to formic acid when exposed to air (23). Isothiazolones are unstable at temperatures above 40 °C and chlorhexidine is unstable above 70 °C (23). A decrease in the efficacy of a bactericide treatment program can be due to a decrease in bactericide activity or due to inactivation by adverse conditions and does not always indicate bacterial resistance (1).

Chemistry of the Water

Chemicals that inhibit scaling and corrosion are also added to industrial water systems, and some of these chemicals interact with certain biocides. Chromates are used to

inhibit corrosion and also suppress microbial growth, acting synergistically with the biocide used. Glycolic acid secreted by algae can, however, reduce chromate, rendering it inactive. Dithiocarbamates reduce chromate, so the two substances are incompatible (40). QACs form insoluble chromate precipitates at high concentrations, so the two should not be added simultaneously to water. The careless application of chloride can lower the pH to a point at which the protective chromate film is solubilized. Na-2-mercaptobenzothiazole is a corrosion inhibitor that is oxidized by chlorine dioxide. Methylene bis-thiocyanate is hydrolyzed under slightly alkaline conditions (pH 7.5).

Where the chlorine demand of water is high, the large quantity of chlorine added leads to a high chloride level that increases the corrosion potential of the water. Chlorine and quaternary ammonium compounds increase the corrosion rate of copper alloys.

Bacterial Resistance

Bactericide treatment regimes for cooling water systems often fail, posing the question of bacterial resistance to the bactericide. Some authors have argued that the failure of treatment programs was on account of selection for resistant strains. We have, however, shown that susceptible bacterial isolates do acquire increased tolerance to bactericides following serial transfer in subinhibitory concentrations. Resistance has been defined as the temporary or permanent ability of an organism and its progeny to remain viable and/or multiply under conditions that would destroy or inhibit other members of the strain (31,54). Bacteria may be defined as resistant when they are not susceptible to a concentration of antibacterial agent used in practice. Traditionally, resistance refers to instances in which the basis of increased tolerance is a genetic change and in which the biochemical basis is known. Whereas the basis of bacterial resistance to antibiotics is well known, that of resistance to antiseptics, disinfectants, and food preservatives is less well understood. The basis of bacterial development of resistance to water treatment bactericides is little known.

As biocides are selective in their action, application of any one could result in selection for resistant bacteria. As cells in biofilms and planktonic communities are in continuous exchange, death of cells in the planktonic phase would influence the equilibrium and shifts would occur in both the planktonic and the sessile populations. Biocides attack targets of cell function, placing the bacterium under stress. It is well recognized that communities under stress have a lower species diversity and select for fitter species. Therefore, a more resistant community could develop. The concentration of a biocide is not related linearly to its activity; a concentration exponent is involved in the relationship. In many cases a small decrease in concentration will result in a notable decrease in activity.

In an in situ biocide evaluation study we found that the dominant planktonic survivor after 48 hours was a species most effectively killed by the relevant biocide under laboratory pure culture conditions. An example is dichlorophen, which killed 99.94% of *Pseudomonas stutzeri* at 50 ppm and yet left this species the dominant isolate after 48 hours (43%) in the cooling system. Also, thiocarbamate killed 99.87% of *P. stutzeri* at l74 ppm and left it the dominant planktonic survivor (62.5%) in the treated system. QAC-tin killed 100% of *Acinetobacter calcoaceticus* and left *Acinetobacter* spp. dominant (40%) in the system. Although the surviving strains could be different, the correlation is striking.

Two reasons explain why the efficacy of bactericide treatment programs can decrease at times. One is a decrease in the activity of the bactericide and the other is a decrease in the bacterial susceptibility toward the bactericide. Three mechanisms of resistance have been reported in the field of antibiotic study:

- Inaccessibility of the antimicrobial agent to its site of action,
- Absence of the susceptible site, or alteration to an insusceptible form, and
- Inactivation of the antibacterial agent.

Whereas antibiotics used in the medical field are rather specific in their modes of action, targeting specific cellular components or processes, biocides are far less specific so that the alteration of a reactive site or the substitution of an amino acid in a protein will not render bacterial cells resistant. Therefore, inaccessibility and inactivation are the two classes of possible mechanisms of resistance. Additionally, active removal of biocide is a third class of resistance.

Decreased Permeability. The initial stage of bactericide action is binding to the bacterial cell surface. It must then traverse the cell wall (gram-positive) or outer membrane (gram-negative) to reach its site of action at the cytoplasmic membrane or cytoplasm. In gram-positive bacteria there are no specific receptor molecules or permeases to assist or block bactericide penetration. The cell wall of *Bacillus megaterium* is permeable to molecules up to 30 kDa. Intrinsic resistance of gram-positive bacteria to bactericides is therefore low. The gram-negative cell envelope has, however, evolved to regulate the passage of substances into and out of the cell to a remarkable degree of specificity. All the components of the cell envelope, except peptidoglycan, play a role in the barrier mechanisms because peptidoglycan is spongy and therefore permeable. *Pseudomonas aeruginosa* is the most resistant nonsporeforming bacteria to most bactericides, as a result of the superior barrier properties of its outer membrane. In a recent study, the antimicrobial activity of a series of new 2-arylthio-N-alkylmaleimides were compared and many were found active against *Staphylococcus aureus*, *Bacillus subtilis*, and *E. coli*. Only one of the 51 derivatives tested was marginally active against *P. aeruginosa*.

The physiological state of cells and the nature of the habitat can lead to considerable variation in the susceptibility of bacteria to bactericides. The composition of the bacterial cell envelope does change as a response to available or limiting nutrients, so that the barrier properties of the envelope are affected. Exposure to subinhibitory concentrations of bactericides can lead to phenotypic adaptation, resulting in a resistant cell

population. In *E. coli*, certain proteins induced by heat or starvation stress also confer resistance to H_2O_2 and to UV light. Most bactericide-resistance is due to adaptation, and the resistant phenotype is mostly lost upon removal of the bactericide.

Efflux Systems. Bacteria can actively pump compounds out of the cell via membrane efflux systems. Only one type of bactericide-efflux system has been described to date, the QAC efflux system of *S. aureus*. This efflux system is coded by two gene systems. The genes *qacA* and *qacB* encode for a high level of resistance, and *qacC* and *qacD* encode for a low level of resistance. *qacC* and *qacD* are further identical to the *ebr* gene encoding for resistance to ethidium bromide in *S. aureus*, explaining why resistance to QAC is often concurrent with resistance to ethidium bromide. The *qacA* gene codes for a 50-kDa protein, which mediates energy-dependent efflux of both benzalkonium chloride and ethidium bromide. The *qacC* gene also mediates energy-dependent efflux of benzalkonium chloride and ethidium bromide. Two different but isofunctional gene systems appear to have evolved in *S. aureus*.

Enzymatic Degradation of Biocides. Resistance to antimicrobial agents can be caused by enzymes transforming the bactericide to nontoxic form. The phenomenon is usually investigated from the biodegradation point of view, that is, the biodegradation of toxic pollutants. A host of aromatic, phenolic, and other compounds toxic to many bacteria (some of which are employed as bactericides) can be degraded by certain bacteria. The topic has been reviewed by various authors and the current literature reports extensively on biodegradative pathways (55).

Two types of enzyme-mediated resistance mechanisms have been documented, that is, heavy-metal resistance and formaldehyde resistance. Resistance to heavy metals includes resistance to the following: mercury, antimony, nickel, cadmium, arsenate, cobalt, zinc, lead, tellurite, copper, chromate, and silver. Detoxification is usually by enzymatic reduction of the cation to the metal. Whereas some heavy-metal resistance genes are carried on plasmids, others are chromosomal. The resistant phenotype is usually inducible by the presence of the heavy metal. Some heavy metals induce resistance to a broader spectrum of heavy metals. Arsenate, arsenite, and antimony, for example, induce resistance to one another in *E. coli*.

The detoxification of formaldehyde by *P. aeruginosa* and *P. putida* has been studied extensively. Formaldehyde is reduced by an NAD+gluthathione-dependant dehydrogenase, giving formaldehyde NAD+ oxidoreductase. This enzyme is probably plasmid-encoded, and appears to be constitutively expressed. Resistance to most formaldehyde-releasing condensates of formaldehyde is therefore also caused by formaldehyde dehydrogenase activity.

Biofilm-Associated Resistance

Bacteria in biofilms are much more protected from bactericidal action than are planktonic bacteria. In a recent study, biofilm bacteria were found to be 150- to 3,000-fold more resistant to hypochlorous acid and 2 to 100 times more to monochloramine than were unattached cells. *Pseudomonas aeruginosa* growing as a biofilm has been found 20 times as resistant to tobramycin as are planktonic cells. A number of reasons for the increased biocide resistance of biofilm bacteria have been put forward, but none adequately explains the phenomenon. The current arguments are addressed adequately by Stewart (56) and Lewis (10).

CONCLUSION

Biofilms are problematic in a range of industrial environments in which large areas of submerged surfaces are exposed to relatively high nutrient fluxes, providing niches for the formation of biofilms. These biofilms also promote corrosion of ferrous metals by the concerted metabolic activity of a number of biofilm-associated bacterial types, a process collectively termed microbial influenced corrosion (MIC). The use of biocides to control biofouling in industrial water systems is still an accepted practice, although higher levels of environmental awareness and tighter legislation have placed increased pressure on the water treatment industry to seek alternative means of control. As the costs attributable to MIC and biofouling are high, effective control of bacterial activity and numbers in industrial aqueous environments is essential.

The primary target of a biofouling control strategy should always be the biofilm-associated microorganisms as these are the catalysts of MIC and impact negatively on system operation. The most common approaches currently available to control biofouling include the use of:

- Biocide to kill biofilm-associated microorganisms;
- Alternating currents to enhance the efficacy of biocides against biofilms;
- Dispersants to dislodge cells from the surface;
- Enzymes to hydrolyze biofilm-associated polymers;
- Physical processes for biofilm removal; and
- Ultrasonic sound to dislodge and kill microorganisms.

It is clear that an integrated biofouling management strategy is required to control biofilms and associated problems in industrial water systems.

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BIOFOULING IN THE MARINE ENVIRONMENT

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Biofouling has been defined as the modification of an immersed, artificial structure by organisms, which results in a deterioration in its performance (1). Its occurrence is a complex mixture of physical, inorganic, and organic chemical and biological phenomena (1). Basically, biofouling means that organisms (both microorganisms and macroorganisms) are acting out their normal life cycles, which include a sessile/attached phase, and doing it where humans do not want them to do so. Importantly, it has been observed that biofouling communities on artificial structures can be quite different from communities on natural surfaces (2). The consequences of biofouling are ultimately realized financially. In practical terms, they can be measured as increased fuel consumption by a fouled ship or submarine, deterioration of navigational buoys, blockage of pipes transferring seawater, length of downtime it takes to clean a fouled surface, or some other loss of performance. In addition, because fouling and corrosion are intimately related, the consequences of the former go beyond the simple accumulation of organisms on a surface to include the deterioration of the surface itself. Microorganisms play a variety of important roles in biofouling. They are both a component of a fouling community and may stimulate, inhibit, or have no effect on the subsequent attachment of other organisms. Both their ability to attach to surfaces and their interactions with other organisms are examined.

THE BIOFOULING "SEQUENCE"

Fouling has been traditionally separated into three types: molecular, microbial, and macrofouling (3). The classical and often reported view of biofouling has been described as a successional process (Fig. 1a; 4,5): first, involving a rapid (instantaneous) adsorption of organic and inorganic molecules to form a conditioning film (molecular fouling); second, a rapid formation of a biofilm (microbial fouling) involving formation of a bacterial film followed by algal and protozoan attachment; and third, invertebrate and macroalgal fouling (macrofouling). The idea of a succession in biofouling implies some sort of causality between the levels of fouling. On the one hand, unless causality can be demonstrated, it cannot be assumed (6), and on the other hand, it should not be assumed that causality between stages does not exist (7).

The aforementioned sequence of events in biofouling has been observed and reported many times largely due to the numbers of the components in the different levels and thus, their availability to attach to a surface (1,7). Thus, as an alternative to causal succession, biofouling can be considered to be seasonal or probabilistic (3). Probabilistic fouling (Fig. 1b) depends on the number of molecules and organisms that could attach to or colonize a surface. There are billions of organic and inorganic molecules per milliliter, around a million bacteria per milliliter, thousands of algae per milliliter, and possibly only a few larvae or spores per liter. For the most part, the number of molecules and bacteria per milliliter are "relatively" constant over time and so are readily available to attach to any new surface or one cleaned by a disturbance at

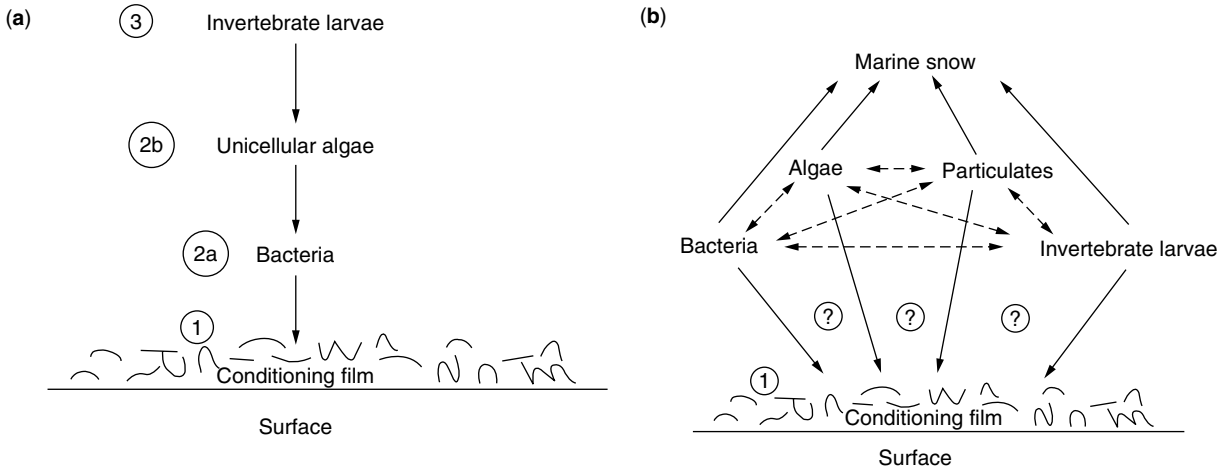

Figure 1. Diagrams of two models for biofouling. (**a**) The classical view of successional fouling on a substratum. Each fouling stage is a step that precedes the following stage and causality between levels is implied. (**b**) A diagrammatic representation of substratum fouling based on the probability that a particular fouling component will encounter the substratum. After formation of the conditioning film, further colonization will depend on which type of organism encounters the substratum. In the absence of a substratum, molecules and organisms may attach to each other and participate in the formation of marine snow. Adapted from A. S. Clare, D. Rittschof, D. J. Gerhart, and J. S. Maki, *Invertebr. Reprod. Dev*. 22, 67–76 (1992).

any time of the year. Although there should also be unicellular algae present throughout the year they will probably undergo seasonal quantitative and qualitative fluctuations. On the macrofouling level, some organisms reproduce throughout the year, but many reproduce seasonally (8). Thus, their arrival late in the fouling "sequence" may be more due to seasonal reproduction, and therefore a probabilistic relationship, than to any causal succession.

MOLECULAR FOULING

Molecular fouling forms what is called a conditioning film on a surface exposed to an aquatic environment (See CONDITIONING FILMS IN AQUATIC ENVIRONMENTS, this Encyclopedia). Macromolecules that adsorb to the surface include proteins, glycoproteins, proteoglycans, and polysaccharides (9,10). Specificity between the nature of the substratum and the chemical identity and quantity of adsorbed materials in the early stages of macromolecular adsorption also appears to occur (11–13). The surface free energy of the substratum can also affect the molecules. On high-energy surfaces the organic molecules are flattened and bind strongly, while on low energy surfaces the molecules are more weakly bound, but may have greater thickness (9). As pointed out in the preceding text, there are billions of organic and inorganic molecules in a milliliter of water. In the formation of the conditioning film on a surface exposed to the bulk phase water, not all organic species present in the water column initially adsorb (11). However, after a certain period, differences disappear. Because the air/water interface already contains a layer of surface-active organic molecules (14), molecular fouling that occurs on surfaces exposed to wave action is different and more complex (3).

The presence of a conditioning film can change both the charge and the free energy characteristics of the substratum (15,16). After the adsorption of the conditioning film, the substratum will assume the net surface charge and the surface fee energy characteristics of the outermost molecular groups of the film. However, the surface energy may not change much because the outermost molecules of the conditioning film reflect the nature of the underlying substratum (17).

The presence of a conditioning film can have two potential effects on attaching microorganisms. First, facilitation between adsorbed molecules and attaching microorganisms has been reported (18–20). However, adsorbed molecules have also been reported to inhibit attachment by microorganisms (12,21). Second, adsorbed molecules may provide attaching microorganisms (e.g., bacteria) with a carbon source for growth. Even molecules that are toxic in solution may become nontoxic when they are immobilized at a surface and provide a suitable substrate for bacteria (22). Attached bacteria on a surface may be able to metabolize adsorbing organic molecules very rapidly (23). In summary, the possibility exists that the nature of the substratum will influence the initial adsorption of components of the conditioning film, and these in turn may influence the types of microorganisms that initially attach.

MICROBIAL FOULING-BIOFILM FORMATION

Bacterial biofilms have been defined as matrix-enclosed bacterial populations adherent to each other and/or surfaces or interfaces (24). In the marine environment, biofilm communities contain a variety of other populations of microorganisms, which also colonize solid substrata including unicellular algae, fungi, and protozoa. Thus, the definition of a marine biofilm will be modified in this report to be matrix-enclosed *populations of microorganisms* adherent to each other and/or surfaces or interfaces. A biofilm should be considered to be more than a layer of cells and slime coating a solid substratum. It possesses dynamic structure and function. It is the nature of the various structures and functions and their changes, which may be involved in the attachment of other microorganisms and macrofoulers. Examples of some of the microorganisms found in marine biofilms follows.

Bacterial Colonization

Bacteria will be among the initial colonizers of a new surface for reasons stated earlier (Fig. 2a). Arrival at the vicinity of a surface will usually be by hydrodynamic means. Once within the viscous boundary layer of still water near the surface, positive and negative

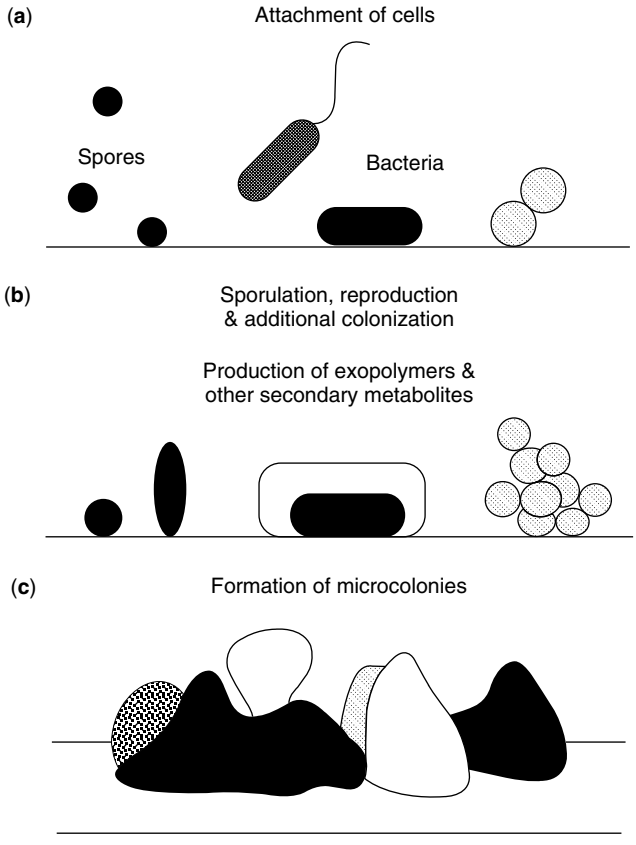

Figure 2. Steps involved in the formation of microbial biofilms in the marine environment. (**a**) Attachment of cells. (**b**) Sporulation, reproduction, and additional colonization. (**c**) Formation of microcolonies. See text for details.

chemotaxis may be important to motile bacteria for their transport to the surface. For nonmotile bacteria, transport through the boundary layer may involve Brownian motion or the hydrophobicity of the cell surface. As described by Zobell (25) and later defined by Marshall and coworkers (26), bacteria approaching a solid substratum have two phases of adhesion — reversible and irreversible or firm. Reversibly attached bacteria do not come in direct contact with the substratum because of electrostatic repulsion, but are capable of scavenging nutrients from the surface (27). The bacteria must overcome the repulsion to be firmly attached. Firm adhesion of a bacterium to a solid substratum is mediated by cell surface and extracellular polymers (28), including polysaccharides (29,30), proteins (31,32), and lipopolysaccharides (33,34). It is also likely that bacteria can utilize more than one mechanism to attach to different surfaces (31,32).

When a planktonic bacterium arrives in the vicinity of a solid substratum it is faced with a microenvironment that has different conditions. These conditions can act to stimulate changes in morphology (35), and turn on previously unexpressed genes (36–38). A bacterium may respond to more than one type of signal when near a surface (39). This may induce the production of new proteins (40) and stimulate the synthesis of exopolysaccharides (Fig. 2b; 41). Conditions that are associated with a surface and may be responsible for inducing genetic responses by an attaching bacterium, either individually or in concert, include changes in gas diffusion, pH, cell density, metabolite accumulation, nutrients, surfactants, viscosity, osmolarity, water activity, and inorganic ions (42).

The formation of a bacterial biofilm is a complex process and can be influenced by a number of factors (43). Once on the surface, factors including motility mediated by flagella and twitching, outer membrane proteins, and exopolysaccharides have been demonstrated to be important in establishing cell-cell interactions for biofilm formation by several bacteria in laboratory studies (44). Microscopic evidence has indicated that the three-dimensional structure of a bacterial biofilm can be quite complex (Fig. 2c; 24,43). In addition, bacteria may communicate between their own species and perhaps others through the use of N-acyl-L-homoserine lactones (AHL, 45–48). These small molecules act as extracellular signals that, when accumulated in the presence of increased cell density, activate transcription and modulate physiological processes. They have been demonstrated to influence the three-dimensional structure of a bacterial biofilm (49). A number of bacteria have been demonstrated to use this type of cell-to-cell communication, which raises the possibility of one bacterium receiving analogous signals produced by another species. These signals may actually be directed toward a different physiological response. AHL activity has been demonstrated to occur in natural biofilms (50). Thus, some biofilm physiology and phenotypic expression may be the result of intercellular communication between bacteria of same and/or different species. However, it is also apparent that some marine algae produce compounds, which interfere with AHL regulatory systems (51). Thus, in the natural environment, these regulatory systems may not function with the same efficacy in all habitats as they do in the laboratory.

Marine bacteria have been shown to produce a wide range of organic molecules that have antibacterial, antialgal, antifungal, antiviral, and anti-inflammatory activity, as well as enzymes, enzyme inhibitors, toxins, and polymers (52–54). Understanding how marine bacteria are adapted to life in the various habitats of the sea (including biofilms), and how these may affect the production of secondary metabolites, may help understand their biosynthetic potential (55). These secondary metabolites may have a wide range of effects on other potential fouling micro and macroorganisms.

Diatoms and Other Unicellular Algae

Of the unicellular algae that may be involved in biofilm communities, diatoms are usually among the primary algal colonizers of new surfaces (56–59). Because of their size and mass, diatoms (and many other unicellular algae) in aquatic systems are transported to surfaces through hydrodynamic means, except under completely calm conditions. Among the diatoms are a number of different attachment systems (Table 1), but all have one thing in common — the presence of mucilaginous material (56,60). The polymers have a high molecular weight, and are largely acidic, with lesser amounts of neutral polysaccharides and small amounts of protein in certain species (57,60). The extracellular polymers are involved in both gliding of diatoms and their adhesion (61,62).

Once it has arrived at a surface, a motile diatom (e.g., *Amphora coffeaeformis*) can move across the surface by means of its raphe and can respond chemotactically to nutrient gradients (58,59,63). Ca^{+2} plays a role in both the initial adhesion and the motility of diatoms (59,64,65). The role of Ca^{+2} is probably dual as it is involved intracellularly in transport and secretion of the adhesive and extracellularly by cross-linking the adhesive (63).

Adhesion of *A. coffeaeformis* can occur under either light or dark conditions and is not inhibited by the

Table 1. Characterization of Adhesion Mechanisms Used by Diatoms[a]

Adhesive Type	Description	Examples
Extracellular pads	Pads peripherally located or distinct pads at polar end or at corners of cells	*Synedra affinis*
Extracellular stalks	Adhesive secreted through slits in valve region	*Achnanthes* sp. *Licmophora* sp.
Extracellular encapsulation/Raphe secretions	Mucilage secreted through raphe fissures and large punctae	*Amphora* sp.
Extracellular sheaths	Sheath mucilage released all over cell through small punctae	*Navicula* sp.

[a] Adapted from Chamberlain (56) and Jones and coworkers (57).

photosystem II inhibitor, DCMU, but does require energy and protein synthesis (63). Once attached, the strength of the adhesion of diatoms to a surface is species-dependent and will increase over time (66). Stalked diatoms appear to stay attached to substrata at higher shear stress while diatoms attached by means of an adhesive pad do not (66).

The adhesion of the unicellular alga *Chlorella* has been reported to be stimulated by polymers recovered from *Chlorella* exudate, bacterial cultures, and seawater that were adsorbed to glass surfaces (18). This interaction appeared to be mediated by lectins or lectin-like activity (67). The growth of a number of diatoms and other unicellular algae was demonstrated to be inhibited by marine bacteria in laboratory studies (68,69). Thus, both positive and negative interactions between bacteria and algae may occur in biofilms.

Fungal Colonization

Fungi are widely distributed in the marine environment and include saprophytic and parasitic types (70,71). Marine fungi can also be members of biofilm communities. Colonization of surfaces by fungi usually takes place through attachment of their spores. Many marine fungal spores have appendages (72,73). The presence of appendages assists the ability of the spores to remain in the water column for longer periods of time than those without appendages (74). Because the spores are nonmotile, they are transported to surfaces through hydrodynamic forces. Once in the vicinity of a surface, spores may initially become entrapped (which may be assisted by the presence of appendages) or attach to the surface (75). Attachment is mediated by several methods (Table 2), all of which employ some mucilaginous adhesive (75,76). The strength of the initial adhesion is variable between species and can (*1*) remain constant, (*2*) increase and then level off, or (*3*) show variable increase with time (77). Eventually the spores will germinate and adhesion will be further improved by production of a mucilaginous sheath around both the spore and the germ tube (76).

Table 2. Mechanisms of Spore Attachment by Marine Fungi[a]

Type	Examples
Release of drop of mucilage from polar appendages	*Kohlmeyeriella tubulata* *Lulworthia medusa*
Disc or padlike attachment	*Ondiniella torquata* *Ceriosporopsis circumvestita*
Coiling of sticky appendages around objects	*Halosarpheia retorquents*
Straplike sticky appendages	*Haligena elaterophora* *Remispora maritima* *Remispora pilleata*
Hairlike sticky appendages or pads composed of fibrillar components	*Nereiospora comata* *Crinigera maritima*

[a]Adapted from Rees and Jones (75).

MACROFOULING: INVERTEBRATE LARVAE

Sessile invertebrates involved in fouling generally have a free-living planktonic stage for dispersal and development back into the adult stage (Fig. 3). The planktonic larvae of many sessile invertebrates are recruited to surfaces by various biochemical stimuli (78). For the purposes of this discussion, recruitment will be considered to be a two-stage process — first, an exploratory phase of repeatable behavior (settlement), and second, an irreversible developmental event that occurs once and includes secretion of a permanent adhesive (metamorphosis) (Fig. 3; 79–81). The stimuli can be broadly divided into two types — conspecific (or gregarious) and associative (79,82). Conspecific cues are those that induce the larvae to settle and metamorphose on or near their own species, while associative cues are those produced by other organisms (e.g., those in biofilms) and provide information to the larvae on the choice of habitat. Both positive and negative cues or stimuli are probably important for habitat choice. Although there is increasing evidence that some larvae can respond to soluble biochemical cues in either settlement or metamorphosis (83–91), many larvae will only metamorphose in response to specific stimuli adsorbed onto surfaces (92).

The length of time larvae remain in the planktonic stage of development varies among species. Some, including spirorbid polychaetes, are nonfeeding and generally only remain planktonic for some hours before settlement and metamorphosis (93). Others, including barnacles, have a number of larval feeding stages (naupliar) followed by a nonfeeding larval stage (cyprid) that undergoes settlement and metamorphosis, and can be in the planktonic stage for a considerable length of time (94). Once the larval stage responsible for settlement and metamorphosis is produced, hydrodynamics can affect its availability to arrive at a given substratum (95) and the settlement and attachment of larvae at a surface (96,97). For sessile organisms, such as fouling invertebrates, space is a resource (defined as something that can be consumed or made less available to other organisms, 98). As a result, it is something that can be competed for by organisms in the fouling community (98). Additionally, predation also plays

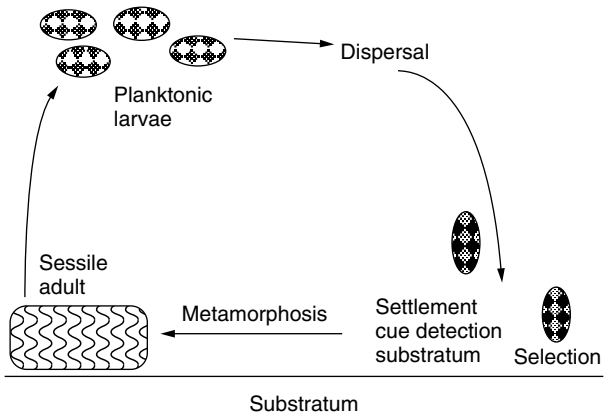

Figure 3. A generalized life history of a sessile marine invertebrate. Adapted from J. R. Pawlik, *Oceanogr. Mar. Biol. Annu. Rev.* **30**, 273–335 (1992).

a role in the community structure (98). Rittschof (3) has pointed out that it is important to remember that many invertebrate foulers have short life cycles. In fact, after they attach, the animals reach sexual maturity in about a month, and after four months the whole cohort is dead (3). After that, their remains on the substratum may act as a surface for other fouling organisms.

Biofilms and Invertebrates

The role of biofilms in invertebrate settlement and/or metamorphosis is complex (see reviews 99–101). Although a larva may not necessarily require a film of microorganisms to settle and metamorphose (102), it is likely to encounter a film on almost every surface that it comes in contact with during settlement. This occurs because although larvae may only be produced seasonally as stated earlier, microorganisms will attach continuously. As the previous information indicates, a marine microbial biofilm is quite complex, with potentially a wide variety of organic molecules that may act as associative cues for larval attachment. However, the abundance of any one cue will probably vary over space and time in any biofilm. The biofilm is particularly important for those organisms that respond to surface-associated stimuli.

Field studies have demonstrated that invertebrate attachment to substrata, with or without biofilms, is complex and potentially governed by a number of variables (103–106). The nature and beginning wettability of the substratum is important for some of the initial macrofoulers (105,106). As time passes, the wettability of the surface changes and is probably not a major influence on continued recruitment (106). Films of microorganisms can change the wettability of a substratum (107–109) and can modify the response of some larvae to surface energy (e.g., bryozoans; 107,108).

Recent field studies used natural films prepared by enclosing panels in a mesh that allowed development of a biofilm but prevented attachment of macroorganisms (110–112). Comparisons were made of filmed and unfilmed substrata, age of films, and films from different locales on the fouling of panels by macroinvertebrates and results showed that (*1*) filming generally enhanced larval attachment, although not for all type of organisms at all times (110); (*2*) some larvae responded positively to film age, some did not, and some preferred less filmed surfaces (111); and (*3*) generally, larvae could not distinguish between films developed in different areas, but were more attracted to more heavily-filmed substrata (112). From these data, it would appear that each type of invertebrate has its own response to biofilms, that there may also be variability in that response within any one group of larvae, and that biofilm density and/or age may play a role in the larval response.

Laboratory Observations

Many laboratory observations of larval interactions with biofilms have been performed without flowing water (although not always) to examine single strains of bacteria and their effects on the type of larva under study. This is particularly useful if the goal is to find bacteria and/or novel bacterial compounds that inhibit or stimulate larval attachment. However, it should be pointed out that stimulation or inhibition of larvae by a biofilm in the field may turn out to be the result of actions by a consortium of microorganisms, and focusing on films composed of a single bacteria may probably not duplicate that result. There is no way that the film produced by a single species can mimic the complex structure and function of a natural biofilm consisting of a number of microbial populations.

Natural films composed of a variety of microorganisms in laboratory tests have been reported to promote settlement and metamorphosis of spirorbid polychaetes (93,102,113), bryozoans (107,114–116), and barnacles (117,118). Factors such as composition of the biofilm (117) and its age appear to be of importance to some types of larvae (118). Natural biofilms have been demonstrated to have very different effects on the attachment of different types of larvae. For example, Crisp and Ryland (102) reported that while the bryozoan *Bugula flabellata* was inhibited by the presence of biofilms, the spirorbid polychaete *Spirorbis borealis* was stimulated. Similar results were reported for larvae of *B. flabellata* and larvae of the ascidian *Ciona intestinalis* (119).

Films composed of individual strains of bacteria can have varying effects on larval attachment (108,120–127). In fact, individual strains of bacteria isolated from surfaces may have an effect on larval settlement and metamorphosis, which was not expressed by the community it was isolated from. For example, the spirorbid polychaete, *Janua brasiliensis*, attaches to a variety of different substrata, and biofilms are an important component to the polychaetes' response to a surface (120,128). One surface that *J. brasiliensis* is commonly found on is the green alga *Ulva lobata*. When a number of bacteria were isolated from *Ulva* and films of these bacteria were tested for their effect on attachment of *J. brasiliensis* larvae, it was found that not all bacteria were effective inducers of larval attachment (120). This suggests that (*1*) the effect of a single strain of bacteria can be masked by the biofilm as a whole and that (*2*) just because a natural film does not show stimulation or inhibition of larval attachment, it does not follow that it does not contain strains of bacteria that could do either. Laboratory tests have also shown that a single bacterium can have a variety of effects on the settlement and metamorphosis of different invertebrate larvae (129). For example, the marine bacterium *Halomonas marina* (formerly, *Pseudomonas marina* and *Deleya marina*) has been reported to stimulate the settlement and metamorphosis of the spirorbid polychaete *J. brasiliensis* (120,130), inhibit or stimulate (see later) attachment of the barnacles *Balanus amphitrite* (109,123,131,132) and *Balanus improvisus* (127), inhibit the temporary adhesion of the barnacle *Elminius modestus* (133), and inhibit the attachment of the larvae of the bryozoan *Bugula neritina* (108). This implies that a single species of bacterium can possess different biochemical cues for larval settlement and metamorphosis. Some of these cues could be stimulatory to certain larvae, whereas other cues could be inhibitory to other larvae.

It has been suggested that there is an interaction between the substratum and the bacterium, which influences the response of the larvae (127,131–134). Films of single species of bacteria (e.g., *H. marina*) or their extracellular products attached or adsorbed onto one substratum are inhibitory, while on another substratum they are not inhibitory or may even be stimulatory compared to no-film controls. Bacteria have been demonstrated to have the capability of using separate mechanisms to attach to different surfaces (31,32). This may cause changes in the surface polymers that are expressed by an attached bacterium (i.e., production of a new extracellular polymer or rearrangement of an existing one to mediate adhesion). Polymers with distinct characteristics may be produced by a bacterium in response to attachment to different substrata (134). It is also important to remember that the substratum can cause new genes to be expressed (42). Thus, the response of the bacterium to the substratum before, during, or after adhesion may bring about a change in extracellular polymers, which in turn is detectable by the larval receptors.

In most cases, the stimulatory or inhibitory component of the individual strain of bacteria for larval settlement and metamorphosis has not been elucidated. A number of invertebrates, some of which are considered fouling organisms, have been shown to be stimulated to metamorphose by marine bacteria, and some of the inducing molecules have been isolated (Table 3). The marine bacterium *Pseudoalteromonas tunicata* has also been demonstrated to produce a number of compounds that are inhibitory to a variety of micro- and macroorganisms (125,135,136). In addition to the production of specific molecules, biofilms can influence larval settlement and metamorphosis in other ways. The extracellular polymers of bacteria in films provide sites for sorption or binding of metals or organic pollutants (28,137–139). For example, tributyl tin, a very toxic compound, has been demonstrated to be more concentrated in a bacterial biofilm and inhibits attachment and metamorphosis of oyster larvae (140). Thus, the cause of inhibition of larval attachment may be much more subtle than the presence or absence of a biofilm alone. Production of extracellular polymers can also been demonstrated to cause some larval types (e.g., ascidian) to become "trapped" in films, and these underwent metamorphosis and contributed to recruitment (124,119).

MACROFOULING: ALGAL SPORES

Macroalgae are also major fouling organisms in the marine environment. The macroalgae that contribute to biofouling belong to Chlorophyta (green algae), Phaeophyta (brown algae), and Rhodophyta (red algae). These may develop sequentially in the environment with green and brown algae occurring first followed by red (150). Mechanisms for the dispersion and colonization of new substrata by macroalgae in the marine environment include vegetative fragmentation (151) and young sporophytes (152). However, most of the dispersal and colonization of macroalgae occurs through the production of reproductive spore bodies formed in sporangia (153). There are two major types of spore bodies: (*1*) sexual spores (known as gametes) and (*2*) asexual spores (known as zoospores or swarmers for green and brown macroalgae and carpospores and tetraspores in red algae). The occurrence of two major types of spore bodies is the result of life cycles that contain both diploid (usually sporophytes) and haploid (usually gametophytes) vegetative phases. The distribution of sporophytes and gametophytes may depend on how rigorous the environmental conditions are (154), and their respective release of reproductive spore bodies is both seasonal and periodic (154,155). Both sexual and asexual spores are potential foulers, the latter as fused gametes or parthenospores (156).

The swarmers of green and brown algae are flagellated and motile and the length of time they exhibit motility varies among groups. In some cases they may swim for days (157–161). The asexual spores of red algae are nonmotile and colonize by sinking onto substrata (162). Their sinking rates are dependent on size, density, the amount of mucilage present on the spore surface, and hydrodynamic conditions. Water motion is a major force in the distribution of spores because the velocity of currents is generally greater than their sinking rates (163). The motility of green and brown algal swarmers may only be of value once the swarmer is out of turbulent hydrodynamic conditions and enters the boundary layer associated with the surface (164).

Motile spores go through a selection process in the search for substrata, which are suitable for colonization (160). Spores will settle on substrata of both high (hydrophilic, wettable) and low (hydrophobic, low-wettable) surface free energy (164,165). However, substrata that are more hydrophobic appear to be more

Table 3. Examples of Larvae That Have Metamorphosis Induced by Marine Bacteria

Invertebrate	Bacteria Involved	Nature of Inducer	Reference
Hydractinia echinata (hydrozoan)	*Alteromonas espejiana*	lipid material	141,142
Lytechinus pictus (sea urchin)	unidentified	unknown, <5, 000 MW	143
Cassiopea andromeda (scyphozoan)	*Vibrio* sp.	oligopeptides	144,145
Acanthaster planci (starfish)	unidentified	unknown	146
Aurelia aurita (scyphozoan)	Micrococcaceae	glycolipid	147,148
Janua brasiliensis (spirorbid polychaete)	*Halomonas marina*	unknown glycoconjugate	130
Crassostrea gigas	*Alteromonas colwelliana*	L-dopa mimetic	122
Crassostrea virginica (oysters)			
Hydroides elegans (polychaete)	unidentified	unknown, <7, 000 MW	149

attractive to some zoospores (e.g., *Enteromorpha*, 167). Before settlement, the spores switch from a random and erratic motion to a searching mode (166,167). This is followed by a spinning motion with the anterior of the zoospore down on the substratum (167,168). Initial contact with the surface was believed to be mediated by the flagella (153) but recent information using high-resolution video microscopy found no evidence to support this hypothesis (167). Once the zoospore is on the surface the flagella are adsorbed (159,169,170) and the spore flattens and contracts against the surface (167), followed by the secretion of a protein-polysaccharide adhesive (159,169,171,172). There also appeared to be gregarious settlement by *Enteromorpha* zoospores although no specific mechanism was elucidated (167).

Nonmotile spores generally have a coating of polysaccharide mucilage (164,173) and this mucilage is probably responsible for the initial spore attachment (153). After initial attachment the cell components undergo a rearrangement (174). Large quantities of a secondary adhesive is secreted and forms a pad beneath the spore (153,164,173).

Once the initial spore adhesion is completed, the spores germinate and primary and secondary rhizoid formation occurs. Primary rhizoids function to secure attachment during early development and secondary rhizoids grow in response to enlargement of the shoot. The attachment of the rhizoids is also mediated by secreted adhesives (175). The adhesion of at least one type of macroalgae (*Enteromorpha*) that were grown on substrata with a high surface free energy was found to be stronger than those grown on a low surface free energy substratum (176).

Algal Spores and Biofilms

Similar to invertebrate larvae because of the periodic and seasonal production of spores by macroalgae, the spores will usually encounter substrata, which possess a biofilm. However, little is known about how macroalgae develop on substrata with biofilms (101). *Enteromorpha* zoospores attached in greater numbers to surfaces with a natural multispecies film than to unfilmed surfaces (177,178). It was hypothesized that the presence of the film increased the surface free energy, which stabilized the adhesive of the zoospore (177). However, other studies have shown that zoospores will attach in greater numbers to hydrophobic surfaces (166), suggesting that a higher surface free energy is not the answer. Studies using individual strains of bacteria have demonstrated that some can be inhibitory to macroalgal spores while others may be stimulatory (179). The exact mechanisms for these results have not been elucidated.

CONCLUSION

The preceding discussion indicates the complex nature of biofouling. Marine biofouling occurs because humans place structures in this environment and the life cycle of many marine organisms includes a sessile phase, which ends up on these structures. Both microorganisms and macroorganisms are involved in this process. Microorganisms are participants in the biofouling process and may be sources of cues to other organisms arriving at a surface. Biofouling communities are dynamic both in their structure and function. A better understanding of these processes will be required in order to prevent or limit biofouling and its consequent deleterious effects on structures in the marine environment.

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BIOFOULING OF INDUSTRIAL SYSTEMS

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Biofouling is referred to as the undesired development of microbial layers on surfaces. Such microbial layers are termed *biofilms* (1) and the effect is called *biofouling*. The term has been adapted from the heat exchanger technology, wherein *fouling* is defined generally as the undesired deposition of material on surfaces (2). A distinction is made between different types of fouling, including the following:

Scaling, *mineral fouling*: Deposition of inorganic material precipitating on a surface

Organic fouling: Deposition of organic substances such as oil, fat, proteins and so forth.

Particle fouling: Deposition of particles from the water phase, such as humic substances, clay, silica, debris and so forth.

Biofouling: Adhesion and growth of microorganisms on surfaces.

In the first three kinds of fouling, the increase of a fouling layer arises from the transport and abiotic accumulation of the material from the water phase on the surface. What is deposited on the surface originates quantitatively from the water. In these cases, fouling can be controlled by eliminating the foulants from the liquid phase. However, this is different from biofouling — microorganisms are pseudo "particles" that can multiply.

Even if 99 to 99.9% of all bacteria are eliminated by pretreatment, there may be a large enough inoculum, which will enter the system to become protected, adhere to

surfaces, and multiply at the expense of biodegradable substances. Thus, microorganisms convert dissolved organic material into biomass locally. This is the same mechanism that supports biofilm technology — biofouling can be considered as a "biofilm reactor in the wrong place and at the wrong time." Substances suitable as nutrients, which would not act as foulants per se, will support fouling indirectly. As most antifouling measures target the microorganisms, the role of nutrients as a potential source of biomass is overseen and biocides tend not to decrease the nutrient level. On the contrary, nutrients supplied by the oxidation of recalcitrant organics can support rapid aftergrowth (3). As it is virtually impossible to keep a common industrial system completely sterile, microorganisms on surfaces will always be present, "waiting" for nutrients.

Usually, the different kinds of fouling occur together (4). The proportion of biofouling can be considerable. An example is the development of dental plaque, namely, mineral depositions on teeth that are favored by biofilms (5). In algal biofilms, precipitation of calcium carbonate is increased, mainly because of the rise in pH resulting from photosynthesis (6).

Generally, biofouling has to be considered as a biofilm-based problem. To understand the effects and dynamics of biofouling and to design appropriate countermeasures, it is important to understand the natural processes of biofilm formation and development.

In biofilms, microorganisms live in an environment that is entirely different from that experienced by planktonic organisms (7). They are more or less immobilized, embedded in a highly hydrated matrix of extracellular polymeric substances (EPS), which is commonly described as "slime." The cell density is some orders of magnitude higher than that in suspension. Owing to the long retention time of the cells next to each other, the development of interacting aggregates is possible, leading to so-called microconsortia.

A biofilm consists of 80 to 95% water. The organic proportion is dominated by the EPS, comprising mainly polysaccharides and proteins (8), whereas the cell material usually makes up only a minor fraction. Additionally, particulate matter such as clay, humic substances, corrosion products, and so forth can be included. Chemical analysis shows that this collected abiotic material can represent the major part of the dry matter, although it is deposited mainly because of the adhesive effect of the biofilm. Thus, the analysis of unwanted deposits can be misleading when the cause of a fouling layer development is investigated.

From an ecological point of view, life in a biofilm may offer important advantages to the cells (9). Among these are (*1*) the possibility to form microconsortia; (*2*) the facilitated exchange of genetic material; (*3*) the accumulation of nutrients from the bulk water phase (10); and (*4*) protection against toxic substances and against desiccation.

OCCURRENCE OF BIOFOULING

Biofouling, by definition, can occur in extremely diverse situations ranging from space stations (11,12) to profane explanations for religious miracles like that of Bolsena (13), which is attributed to the growth of *Serratia marcescens* on sacramental bread and polenta.

Not surprisingly, biofouling occurs in toilet bowls (14) but is commonly observed in water systems in general (15) and during the filtration of seawater (16). It also represents a serious problem in fish farms where the cage netting fouls rapidly (17). The submerged structural surfaces of offshore oil and gas production platforms are covered by biofilms (18), potentially giving rise to microbially influenced corrosion (19). Little (20) has given an excellent overview on marine biofouling. Microorganisms can contribute to calcareous deposits, adding scaling to biofouling (21). Massive deposition of manganese and iron minerals is frequently due to microbial activity (22). Boreholes and aquifers can be clogged by excessive biofilm growth (23), leading to considerable technical problems. A *Thiothrix* species resistant to 200-ppm free chlorine was identified as the cause of biofouling in groundwater systems in Florida (24).

Desiccation of biofilms can also lead to the deposition of minerals (25). In some instances, unexpected biofouling has led to problems such as the deterioration of pH electrode response due to biofilm formation on the glass membrane (26). Moored spectroradiometers were stricken by biofouling (27), and the list of other water-exposed sensors that may yield erratic data because they are affected by biofilms could easily be expanded. Biofilms growing on the walls of houses can influence the surface temperature of the building walls and thus increase mechanical weathering process due to differential thermal expansion. These biofilms can increase the heat uptake, leading to an increase of energy demand for air conditioning (28).

In medicine, implant devices such as catheters are prone to biofouling (29). Dental waterlines can be seriously contaminated by pathogens (30).

Biofouling in Drinking Water

The maintenance of the hygienic and aesthetic quality of water during transport in the distribution systems still challenges drinking water technology (31). Massive growth of cells and the occurrence of pathogenic organisms are observed (32). Both can originate from growth in the water phase but also from biofilms. Thus, all surfaces in contact with water have to be considered as potential supports for biofilm growth and as contamination sources. In particular, the surface to volume ratio in distribution systems leads to the fact that more than 95% of the entire biomass is located on the walls and less than 5% in the water phase. Fouling and corrosion in water filtration and transportation systems can therefore represent a serious problem (33).

Water that leaves the water works in a hygienically optimum state can contain substantial numbers of microorganisms after passing the distribution system. This is mainly due to contamination by biofilms (34,35). Biofilms release cells both continuously by erosion and discontinuously by sloughing events (36) and by swarmer cells (37), which deliberately leave the biofilm. The contamination rate, however, is unpredictable. Thus, there is no correlation between the cell concentration in the

water and in the biofilm. Data from the water phase give no information about the extent or the site of biofilm growth. In the same system, the cell concentration in a biofilm — as compared with the water phase and expressed in relation to volume — ranges from 10^7 to 10^{10} colony forming units (cfu) mL^{-1} (38,39), whereas the cell concentration in the water phase can be as low as 10^5 cells mL^{-1} and below 10 cfu mL^{-1}.

In drinking water technology, it is assumed that a massive development of microorganisms on surfaces only occurs if the support material releases biodegradable matter (40). Certain paints and coatings have led to increased cell numbers in drinking water (41) for this reason. However, similar incidents have been observed even when the support material did not leach nutrients (34,36,42).

Pathogenic and Potentially Pathogenic Organisms in Biofilms

It is now acknowledged that biofilms can provide a habitat for pathogenic microorganisms. In medicine, mycobacteria have been known for a long time as "trumpet bacteria" occurring in the mouthpieces of musical instruments of tuberculosic performers, and, less artistically, in telephone handles (43). *Klebsiellae*, *Mycobacteriae*, *Legionellae*, *Escherichia coli*, and coliform organisms have been found in biofilms (44–48). In the presence of corrosion products, the organisms seem to be particularly protected. This is the conclusion of the study conducted by Emde and coworkers (49), which identified a much higher variety of species in corrosion product deposits, called "tubercles," compared with the free water phase, even after extended periods of chlorination. The fate of viruses in biofilms is still in question. Schmitt and coworkers (50) showed that enteroviruses could be isolated from biofilms on sand filters of drinking water plants fed with surface water.

Biofouling in Purified Water

One of the major problems in the production of purified and ultrapure water is contamination from microorganisms. Processes that depend on ultrapure water suffer accordingly. In the production of microelectronic devices, in which ultrapure water is often used to wash printed circuit boards, microorganisms can cause severe quality losses. Dial and Chu (51) found a direct correlation between the cell content in ultrapure water and the defects in the microchips. Craven and coworkers (52) attributed failures in the metal coating process to the adhesion of microorganisms. Eisenman and Ebel (53) describe horizontal and vertical dislocations in the crystal lattice during the photoresist process, which are due to microbial contamination. Microorganisms can bridge electrical circuits where the distances are below 1 μm. Electromigration and corrosion of the oxide layers have also been observed. Sodium and calcium (Ca^{2+}) ions from lysed bacteria or from bacterial metabolism can diffuse into the surface of the silicon wafer and influence field effects (54). Bacteria are carbon-containing particles, and, thus, can increase the conductivity of semiconductors (55). These microorganisms are mainly released from biofilms. In a detailed study, Patterson and coworkers (56) could demonstrate that in the ultrapure water system (18 MOhm water) of the IBM plant in Burlington, Vermont, United States, all surfaces in contact with water carried biofilms. The piping material consisted of poly(vinylidene dichloride) (PVDF). The authors found that the contamination of the water was due to the biofilms. By sequential sampling, they showed that the cell numbers in water close to the walls were significantly higher than those in the bulk liquid phase.

What can these organisms live on? There are pioneer bacteria, which can multiply in water with less than 10 $\mu g\,L^{-1}$ glucose equivalents (57). If they form biofilms, they can sequester nutrients from the flowing water phase and accumulate them in the EPS matrix. This allows the organisms to live with an even lower concentration of biodegradable material, which is why biofilm formation is considered an ecological strategy in oligotrophic environments (58). These pioneers convert the organic materials into biomass, which represents spots of high concentrations of organic material. This offers a suitable habitat to other organisms, which may have invaded the system.

A common strategy to prevent microorganisms from entering a system is the use of so-called "sterile" filters. Nevertheless, "breakthroughs" occur repeatedly. An important cause is ultramicrobacteria (59). During starvation, the morphology of cells can change drastically, resulting in cells of less than 0.2 μm in diameter. This is demonstrated with *Pseudomonas fluorescens* rods within three weeks of nutrient depletion. Cells of that size have been shown to migrate through sterile filters (60).

Biofouling in Water Treatment Plants and Membrane Systems

Water treatment plants frequently include granular filters with a large surface area, which are predestined for biofilm growth. Biofilms can grow on piping material, insulations, fittings, elastic filling materials and so forth and develop significant tolerance against disinfectants. These biofilms contribute considerably to the overall purification process because they degrade diluted organic matter. A change in nutrient concentration, temperature, or other factors can cause either mass production or sloughing of biofilms, which leads to an increasing contamination of the water. In ion exchangers, biofilms clog the material and lead to an increased pressure drop, and in membrane processes such as reverse osmosis, ultrafiltration, and others, biofouling represents a serious problem (61–63).

Biofilms form a gel layer on top of the membrane and prevent direct tangential flow adjacent to the membrane surface. This leads to concentration polarization at the feed waterside, which increases the transmembrane pressure drop ($\Delta p_{\text{membrane}}$). Thus, both permeate production and salt rejection will decrease. The costs of biofouling of membranes can be summarized as follows (64):

- Loss of product quantity
  - — Flux decline due to increased $\Delta p_{\text{membrane}}$ (see Fig. 1)
- Loss of product quality
  - — Possible microbial contamination of permeate

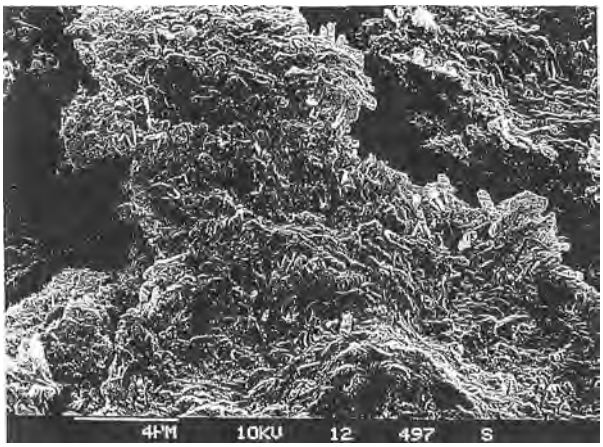

Figure 1. Biofilm on the feed side of a reverse osmosis membrane; bacteria are embedded in the slime matrix (39).

  - — Decrease of salt rejection because of concentration polarization
- Higher energy demand
  - — Increased p_{membrane} to overcome
  - — Increased concentration polarization
- Higher pretreatment and cleaning demand
  - — Chemicals (biocides, cleaners)
  - — Labor- Shutdown time
  - — Eventual treatment of effluent to meet legal regulations
- Higher replacement costs
  - — Damage by cleaning
  - — Damage by microbial attack
- Frustration and demotivation of the operating personnel

Figure 1 shows a scanning electron micrograph of the surface of an irreversibly biofouled RO membrane (39) with a biofilm that has survived many cleaning cycles.

Reverse osmosis (RO) membranes are theoretically impermeable for microorganisms as these membranes act as diffusion membranes and do not contain pores at all. However, microorganisms have been found at the permeate side of RO membranes (65). Figure 2 shows an example.

Even today, there has been no explanation for their occurrence at this site, although it has been observed in various cases. Permeate-side bound biofilm cells may seed the permeate, which will partially attach to the surfaces and form biofilms. Traces of nutrients will support their growth and, thus, further the contamination of the purified water. Indeed, this is also of great importance in the pharmaceutical industry. A detailed assessment of the costs of biofouling has been carried out for the reverse osmosis (RO) plant at Water Factory 21 in Orange County, California (64). The "bottom line" is US $730,000 spent each year to control membrane biofouling. This represents about 30% of the total operating costs for Water Factory 21.

Figure 2. Permeate side of a reverse osmosis membrane; bacteria are attached and can contaminate the permeate (Courtesy of H. F. Ridgway, in B. S. Parekh, ed., *Reverse Osmosis Technology*, Marcel Dekker, New York, Basel, 1988, pp. 429–481).

The costs of biofouling are increased by some common mistakes, which originate from the fact that biofouling is not understood as a biofilm process. Inadequate methods of detection and monitoring of countermeasures lead to delayed recognition and to suboptimal or ineffective biofouling control.

Investigations about the beginning of the fouling processes revealed that the microorganisms settle on the membrane during the first hours of operation (66). This means that every operating plant possesses a biofilm from its beginning. Only the extent of biofilm growth will decide whether biofouling occurs: only if a certain threshold of interference is exceeded does biofouling take place; thus, it is an operationally defined expression.

Biofouling on Heat Exchangers

The efficiency of a heat exchanger can be seriously decreased by biofilms (4,67). Forming a gel layer, they "insulate" the heat exchanger surface from the liquid phase. Although the heat-transfer resistance of biofilms is similar to that of water, the gel allows only diffusive heat transport. Thus, heat transfer by convective transport is inhibited. In addition, friction resistance is increased, which increases energy consumption; this aspect is

considered to be an even more costly problem in heat exchange (15). Similar to membrane systems, heat exchangers in aqueous systems operating in a temperature range between 5 and 60 °C will always carry biofilms, which remain unnoticed because their effects stay below the threshold level of interference. However, sudden biofouling problems do not arise from microorganisms that have suddenly invaded the system but are much more likely to be caused by an increase of nutrient concentration or an absence of inhibiting factors. The additives can represent such factors. As an example, chromate has been replaced by other corrosion inhibitors because of its environmentally undesirable properties. A new organic inhibitor that had excellent properties, including full biological degradability, was introduced. The system protected by this substance failed after three months. Biodegradation had started during operation and turned the exchanger into an activated biofilm sludge reactor (Fig. 3). Thus, an environmentally reasonable step has to be harmonized with all other effects in the system.

If light has access, substantial algal growth can occur. This is frequently the case in cooling towers.

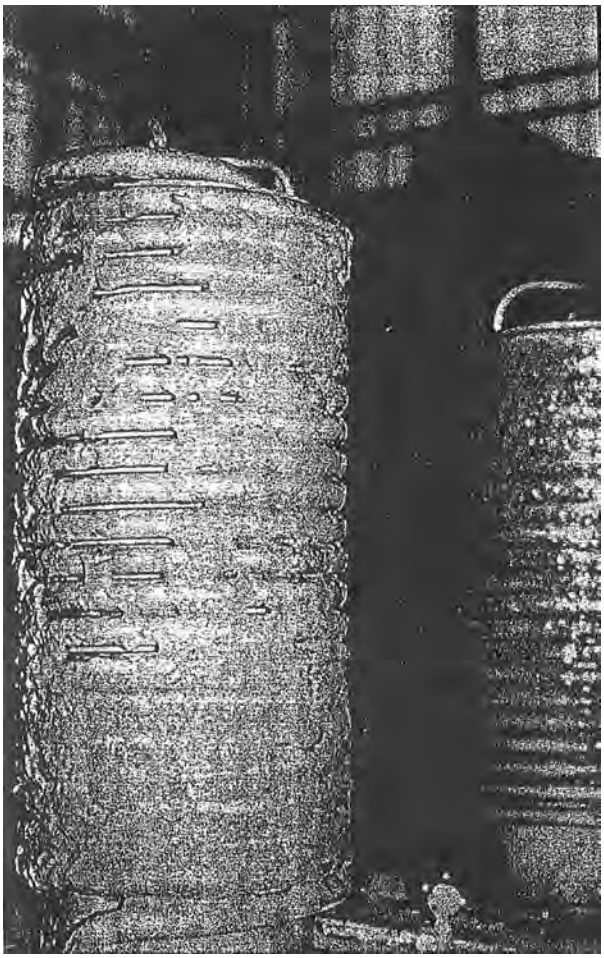

Figure 3. Heat exchanger in a cooling water cycle that was "protected" by a biologically degradable corrosion inhibitor after six weeks of operation.

The installations can be overgrown so heavily that they physically break down (4). In ocean thermal energy conversion (OTEC), biofouling is addressed as the most difficult problem, seriously limiting the use of an otherwise promising technology (68).

Biofouling in Oil Industry

The production, transport, storage, and processing of oil is stricken by severe biofouling problems (69,70). Sulfate-reducing bacteria (SRB) are of particular importance because they are responsible for souring of oil and for microbially influenced corrosion (MIC). A water content of only one percent is sufficient to support substantial development of SRB within only a few weeks (71). Microbial growth at the production sites can cause clogging, especially if water is used as replacement agent (70).

Biofouling on Ship Hulls

A classical case of surfaces prone to biofouling are ship hulls. As indicated earlier, the speed of ships can be significantly reduced even by thin biofilms. Before higher organisms such as mussels or others, a microbial biofilm develops (72). D.C. White (Inst. Appl. Microbiol., Univ. of Knoxville, Personal Communication) has assessed that the United States Navy has to spend more than US$ 500 million in additional fuel that is required to overcome the additional friction resistance caused by biofilms: a biofilm of only 100 μm thickness leads to a 10% increase in friction resistance (4). The attempts for effective antifouling coatings go back to the medieval ages when copper plating was introduced, without long-term effect. After a certain time, some organisms can tolerate the toxicity of copper, colonize it, complex the ions, and shield other organisms for which copper would be toxic. Later colonizing species do not encounter the copper surface but encounter the primary biofilm. The problem has been transiently solved by modern antifouling paints containing tributyl tin compounds. Unfortunately, they are so toxic and recalcitrant to biological degradation (73) that many countries have resorted to a partial ban. Thus, the problem remains unresolved.

Biofouling and Microbially Influenced Corrosion

In biofouling, the biofilm colonizes a given surface and creates problems by contamination of the adjacent medium or by impeding the function of the surface. However, it can also influence corrosion, as corrosion is an interfacial process, which is dependent on the physicochemical conditions at the interface such as pH value, redox potential, oxygen concentration, conductivity, and others. All of these factors are influenced by biofilms. The effect is called *microbially influenced corrosion* (MIC) and is basically a biofilm problem. Thus, biofouling and MIC are closely related (19).

COUNTERMEASURES

As biofouling often occurs far from the focus of attention, namely a product, a common practice is to ignore the

problem as long as possible. The next phase is to look for causes other than microorganisms and to test countermeasures. If none of the common causes proves to be valid and none of the countermeasures is effective, it is concluded that this must be a microbial problem. Certainly, this conclusion is true in many cases. Thus, an expert in the field of microbial deterioration is consulted. Unfortunately, this person encounters the consequences of all former attempts, and symptoms that would help identify the microbial origin of the problem are obscured by provisional repair actions. This can be a serious obstacle for the design and application of an effective counterstrategy. A checklist that links the symptoms to eventual microbially influenced corrosion (MIC) has been produced for this purpose.

If a problem is suspected to be of microbial origin, a three-step program has to be developed according to the following questions:

1. How can biofilms and biofilm-related damage be identified?
2. How can the system be sanitized?
3. What can be done to prevent future problems?

Detection of Biofilms

The presence of biofilms in a technical system is usually detected only indirectly. Operational parameters represent only the symptoms. In heat exchangers, heat transfer decreases while drag resistance increases. In membrane technology, both membrane resistance and drag resistance increase. In practice, identification of biofouling generally follows this pattern: a problem in plant performance occurs; all common countermeasures are applied; if they do not work, and there is no better explanation, the problem is attributed to biology, and in most cases, this is correct.

The Problem of Sampling

As soon as biofouling is suspected, water samples are taken and investigated microbiologically. Different methods are applied and they give different results. Cultivation methods detect only those organisms that are capable of multiplying on the supplied growth media. According to the cultivation temperature and time, the numbers of colony-forming units can vary by some orders of magnitude. The majority of bacteria occurring in natural and technical waters do not grow on common microbiological media (74). Microscopical methods give the number of all cells present in a sample but usually cannot discriminate between viable and nonviable cells.

A more fundamental problem in attempting to detect biofouling by sampling of the water phase is that there is no correlation between numbers of suspended cells detected by water sampling and the location or extent of biofilms. Although biofilms contaminate the water phase, they do so discontinuously. Cells can be eroded or leave the biofilm actively (37), and parts of the biofilm can slough off, leading to episodes of high cell numbers in the bulk liquid phase.

Even with low numbers in the water phase, considerable biofilm growth can occur, as has been shown with microbially contaminated ion exchangers: while 5 to 8 cFu mL^{-1} were found in the process water, sampling of the exchanger bed revealed cell numbers as high as 10^4 cFu mL^{-1} (75). The concentration of cells released from biofilms is usually diluted in the water body. However, in situations of stagnation, these cells can become concentrated — a frequently observed phenomenon. It does not arise because cells grow better under stagnant conditions. On the contrary, cells grow better under agitated conditions (all other conditions being favorable); this is the reason fermenters are vigorously mixed. The comparison of two ion exchanger columns, one left standing idle and the other operated with circulating water, revealed an identical extent of microbial growth (76). Similar observations were reported by other authors, who showed that the number of sulfate-reducing bacteria in the water phase was completely unrelated to the number of biofilm cells (71).

Thus, proper sampling of biofilms must be carried out on the surfaces in question or on representative surfaces and by adequate methods (77). These include either the removal of biofilms, or of biofilm-carrying surfaces, or new optical and spectroscopic methods. Special candidates for the occurrence of biofilms are crevices, holes, curves, and other areas protected from high shear stress.

Analysis of Biofilms

Biofilms in engineered systems have usually reached the plateau phase and are in most cases visible to the naked eye. Thus, optical inspection gives a first hint. In technical environments, biofilms tend to display a slimy consistency, which can be detected by wiping; thin layers can be made visible if a white tissue is used for wiping. In case of doubt about the biological origin of a deposit or layer, it is useful to take a small amount of the material and put it over a lighter until it smolders. A smell of burnt protein is characteristic of biological material. Presently, a test kit with simple dye reactions is under development to deliver reliable information about the presence or absence of biofilms and to semi-quantify them. The ultimate detection of biofilms is carried out in the laboratory. In Table 1,

Table 1. Examples of Biofilm Parameters

Parameter	Detection Method
Water content	24 hours, 110 °C
Organic carbon	TOC, COD, incineration loss
Protein	78
Carbohydrates	79
DNA	80
Lipids	81
Muramic acid	81
Polyhydroxybutyrate	81
Total cell number	82
Colony-forming units	Various standard methods
ATP	83
Hydrolase activity	84
Respiratory activity	85
Indolacetic acetic acid production	86
Catalase activity	87

some parameters suitable for the detection of biofilms are summarized.

For screening, the contents of water and organic carbon are indicative; if these are high, there is a high probability that the material is a biofilm. The presence of ATP or respiratory activity indicates living organisms. It is wise to use more than one parameter for the characterization of a biofilm. If the occurrence of a problem can be related to the actual presence of biofilms, the diagnosis "biofouling" is justified.

Sanitation

The most common practice to eliminate unwanted biofilms is the application of biocides. They are used to "disinfect" a system. This strategy is historically rooted in medicine: the germs that cause the disease are to be killed. Then, the immune system will dispose them. However, killing the organisms in an engineered system will not solve the problem because dead biomass remains. Unlike a living organism, an engineered system has no mechanism to discard dead microbes. This leads to an accumulation of readily biodegradable material and gives rise to rapid microbial regrowth. In addition, engineered systems generally cannot be run under sterile conditions (except for pharmaceutical or electronics water supply systems that are kept aseptic with great effort). Reinfection of the "disinfected" system is already assured by the water that is used to rinse the biocide. New cells encounter dead biomass that is readily biodegradable and will support rapid microbial regrowth. Thus, removal of biomass may be more important than killing. Rational cleaning measures include a step in which the physical stability of the biofilm matrix is weakened and a subsequent step in which it is removed from the system. The use of biocides can be justified in the framework of a combined strategy to minimize the biofouling potential. However, the considerations as discussed earlier must be kept in mind, particularly because "disinfection" and "cleaning" tend to be mixed up.

The variety of biocides has grown to such an extent (88–91) that it justifies the need for "biocide museums" (92) to avoid the reinvention of forgotten biocides and to collect experience and data. In the United States, Canada, England, and Australia, chlorine and chlorine-containing compounds make up most of the spectrum of biocides in industrial applications (4). Peracetic acid, the peroxide of acetic acid, has been widely applied in the former eastern block; its excellent disinfection properties were summarized by Flemming (93). Peracetic acid is an example of a disinfectant that was discovered twice, namely, in 1905 and 1947. The selection of a biocide suitable for a given system requires experience and should be supported by preceding laboratory screening. Natural product antifoulants seem to represent an interesting alternative (94). Some general criteria for the selection of biocides are summarized in Table 2. It is noticeable that they are contradictory. Economic advantages may cause increasing environmental damage or result in insufficient efficacy. Thus, a biocide that meets all of the criteria listed hereunder is not expected. It is impossible to discuss the vast range of biocides presently on the market.

Table 2. Requirements for an Ideal Biocide

Efficacy against the organisms in the system if they occur in biofilms
Compatibility with other conditioning chemicals
Satisfactory time, concentration, and temperature of action
Lack of corrosive effects on the system
Safety and health aspects concerning handling and storage
Stability
Biodegradability
Minimal effect in effluent water
Analytical detection
Low cost

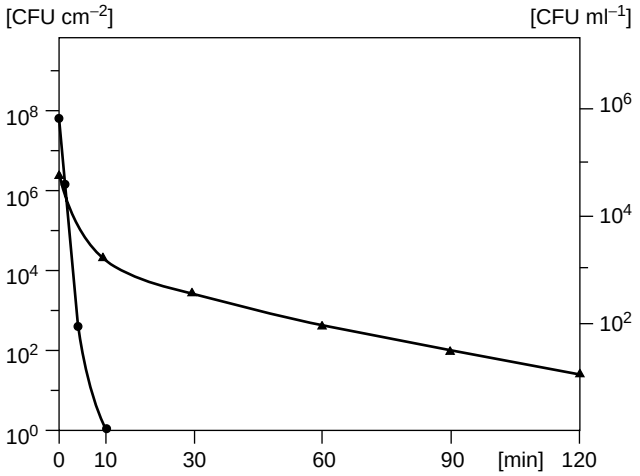

Figure 4. Survival of *P. fluorescens* in suspension (•) and in a biofilm (π) (after M. C. Mittelman, in H.-C. Flemming and G. G. Geesey, eds., *Biofouling and Biocorrosion in Industrial Water Systems*, Springer, Heidelberg, Germany, 1991, pp. 113–134).

The efficacy of biocides against biofilm organisms is known to be reduced compared with where the same organisms are suspended in the water phase (95,96). This is demonstrated by the different resistance of *P. fluorescens* shown in Figure 4 (97).

Organisms in biofilms are well known for increased tolerance toward biocides (95). Keevil and coworkers (96) reported that coliform organisms in a biofilm survived prolonged exposure to 12-ppm free chlorine. Living biofilms have been found on the inner walls of disinfectant pipes (98). In many cases, not all organisms in a biofilm are killed. They recover and metabolize the dead biomass. This leads to a rapid regrowth after a disinfection measure. Unfortunately, most data on biocides relate to suspensions of test organisms. Although this evaluation of biocide efficacy is reproducible, it does not reflect the effectiveness against biofilms. It is very difficult to obtain biofilms that can be used as reference systems with sufficient similarity to "natural" biofilms. Kinniment and Wimpenny (99) developed the "Cardiff constant depth biofilm fermenter" using *Pseudomonas aeruginosa* as the test organism and keeping the biofilm thickness constant by accurate abrasion with a razor blade. They could demonstrate that 200-ppm formaldehyde killed all planktonic cells while the

viable cell number in the biofilm was only reduced from 10^8 cells per square centimeter to 10^6 cells per square centimeter. Griebe and coworkers (100) successfully used an annular biofilm reactor to compare the efficacy of different biocides against biofilms. It was shown that chloramine was much more effective against biofilms than chlorine. However, in no case was biomass removed from the surfaces. Keevil and coworkers (96) suspected that the high nitrite levels in London tap water were caused by incomplete nitrification of monochloramine in biofilms, where ammonia oxidizers metabolized the amino groups only to nitrite.

The use of chlorine will be increasingly limited for reasons of sewage water pollution and tighter environmental regulations. Thus, ozone offers some advantages. Toxic by-products are formed to a much lesser extent and the method has been shown to be effective against biofilms (101,102). Ozone weakens the biofilm matrix and, thus, facilitates the removal of biomass by shear forces (103). A drawback is that the costs for ozone generation are about four times as high as that for chlorine treatment.

A potential problem in the application of oxidizing biocides is their effect on the bioavailability of recalcitrant organic matter. Refractory organic substances, for example, humic substances, have been shown to become biodegradable by chlorine and ozone treatment (3). The raw water was treated with 2.5 mg L^{-1} ozone for 15 minutes. Then, the water was filtered using granular activated carbon (GAC), and a concentration of 2.0 mg L^{-1} of free chlorine was maintained. The assimilable organic carbon (AOC) increased significantly after the application of ozone.

The essence of these considerations is that concentration, time of action, temperature, and interaction with dissolved substances and with biofilms must be increased if biocides are applied against biofilms.

Cleaning: Removal of Biofilms from Surfaces

For cleaning, the mechanical properties of biofilms are of fundamental importance. Although the biofilm matrix is kept together by weak physicochemical interactions (104), it is very difficult to remove it only by shear forces. Hydrogen bonds, electrostatic interactions, and dispersion forces add to an overall binding force between the EPS molecules, which is equivalent to several covalent chemical bonds per biopolymer. Oxidizing or dispersing chemicals are useful for the first step, while flushing, brushing, or similar physical techniques are useful for the second step. Of particular importance is an efficiency control in order to optimize the process.

Prevention of further microbial problems usually cannot be achieved by single measures but by a combined strategy, which can be called "good housekeeping." A very important element of this strategy will be the elimination of biodegradable material, as this represents potential biomass.

If an oxidizing biocide is used, it will weaken the biofilm matrix as a side effect. This is the case for chlorine, which is consumed by reaction with extracellular polysaccharides and proteins. As a result, the biofilm can be removed more easily from a surface. In this case, it is irrelevant whether the cells are dead — the "side effect" of decreasing matrix stability is much more important. Some biocides, however, can have the opposite effect. An example is formaldehyde that is used in microscopy as a preserving reagent because it cross-links proteins. In such a case, "disinfection" can make the problem worse, as has been reported many times in practice (105).

If a system is to be cleaned, the mechanical resistance of the slimy biofilm layer must be overcome. Although biofilms usually can be wiped off easily, they are surprisingly resistant to the shear forces of running water. This is the reason for the observation of biofilms in water pipelines with very high water velocity (4). They become thinner but more stable. Interestingly, an increase in performance after the application of cleaning substances does not necessarily have to be due to the removal of a biofilm. As an example: reverse osmosis membranes were cleaned with a commercial cleaning formulation (Ultrasil U 53, Henkel, Germany). Permeation performance increased, but biomass quantity on the membrane surface remained almost the same. The cleaning agent simply increased the permeability of the fouling layer (106), thus mitigating the original hydrodynamic resistance of the biofilm.

Time plays an important role in the mechanical resistance of a biofilm. "Young" biofilms are much easier to remove than "old" biofilms. Table 3 shows the rinsing efficacy of surface-active substances such as sodium dodecyl sulfate (SDS) and Tween 20. Cells were allowed to attach to a polyethersulfone membrane for either four hours or three days.

Subsequently, membrane samples were agitated for one hour with the surface-active agent. The number of cells per surface area was determined after this procedure. The results demonstrate that early action against an unwanted biofilm is more effective.

For an effective cleaning, the bond between the biofilm and the underlying surface must be broken. Although biofilms can develop on practically any surface, the binding force varies. A reasonable approach to better cleanability is the choice of materials or coatings with low adhesion force to biofilms (107). Low surface energy materials tend to display low adhesion forces to biofilms; this is exploited in Teflon coatings on ship hulls.

Another choice for cleaning of surfaces is ultrasound. It has been applied successfully in cleaning of medical devices, dental equipment, heat exchangers, and other surfaces. A critical appraisal of published material reveals that in almost all cases there is no specification of the ultrasound intensity to which the object is exposed. However, ultrasonic methods seem to be an attractive

Table 3. Influence of Biofilm Age on the Detachment of Cells, Expressed as Percentage of Detached Cells Compared to the Control (76)

Age of Biofilm	Control	1% Tween 20	1% SDS
4 hours	0	93	98
3 days	0	42	57

Table 4. Critical Ultrasonic Parameters for the Detachment of *Pseudomonas diminuta* from a Polyether Sulfone Membrane (95% Detachment of Cells)(108)

Variable	Value	Boundary Conditions
Intensity uptake (I)	4W	$t = 300$ s, $D = 2$ cm
Transducer distance (D)	4 cm	$t = 300$ s, $I = 4$ W
Exposure time (t)	60 s	$D = 3$ cm, $I = 4$ W

alternative to chemical cleaning. Thus, an ultrasonic bath was used to remove biofilms from separation membrane material. The sonification field was mapped with a hydrophone; this technique allowed an assessment of the energy, which arrived at the object. The critical values are summarized in Table 4 (108).

The application of ultrasonic energy seems to be feasible for the cleaning of surfaces from unwanted biofilms. However, it has to be considered that ultrasound may damage the surface on which the biofilm is growing. This was the case with membrane materials. In other cases, ultrasound may still offer a suitable method.

Cleaning Strategy

It seems wise not to rely on a single substance or method when cleaning a surface. Killing the cells, as effectively as this may be performed, and leaving them at the place will not solve the problem. Removal of biomass seems to be much more important. An effective strategy must be defined according to the system to be cleaned. If possible, facilitated cleaning should be integrated in the design of a system. In general, a cleaning strategy will include the following two steps:

1. Weakening of the biofilm matrix, mostly by chemicals such as oxidants such as chlorine, ozone, hydrogen peroxide, peracetic acid, or others; by alkaline treatment, tensides, enzymes (109), complexing substances (110), or by biodispersants (111). The latter are based on polyethyleneglycol and are supposed to weaken the interactions in the EPS matrix as well as the interaction between biofilm and support material. A combination of various agents may increase the efficacy. However, it is important to establish a system to quantify the success.
2. Removal of the biofilm by mechanical forces such as rinsing with water, air, steam, or a combination thereof; application of sponge balls, brushing, or ultrasound. Some methods are listed in Table 5.

The experience of the authors in the application of enzymes was not encouraging. This may be due to the specificity of enzymes, which are directed against certain bonds that may not be present in all matrix-forming molecules. Thus, microbial strains that form EPS against which the enzymes are not effective gain a selection advantage. Mechanical cleaning can also select those species, which form the most resistant EPS matrix, as has been reported in biofouling problems with the technique to recover ocean thermal energy (112).

Table 5. Physical Methods for the Removal of Biofilms

Method	Remarks
Rinsing	Simplest method; limited efficacy; thin biofilms are not affected; very effective if biofilm stability is first decreased, for example, by biodispersants
Backwashing	Similar to rinsing
Sponge balls, abrasive	Reasonable efficacy; may damage supporting material
Sponge balls, nonabrasive	Less effective than abrasive sponge balls; not suitable for biofilms, which display greasing effects
Sandblasting	Very effective, but difficult to focus the abrasive effect on the biofilm and not on the supporting material
Brushing	Very effective but limited to accessible surfaces; can shift biofilm population to species, which form extremely stable EPS
Hot water, steam	Especially suitable for purified water systems; efficacy against thick biofilms not yet evaluated; energy intensive
Ultrasonic energy	Good efficacy; restricted to surfaces accessible to ultrasonic energy; may damage underlying material
Ice crystallization	Application of ethyleneglycol (-12 C), destabilizes biofilm matrix (Costerton, 1983). No practical experience reported
UV irradiation	Poor efficacy against biofilms; abiotic particles may protect microorganisms; does not remove biofilm material

Control of Efficacy

In practice, the efficacy of a cleaning measure is assessed by the recovery of process parameters. However, this is an indirect method and not very well-suited to assess the success. As an example, the biofouling layer on a reverse osmosis membrane was removed by about 80% by chemical treatment. This led to a substantial improvement of the process parameters. However, the remaining 20% of the layer offered optimum conditions for the regrowth of the biofilm bacteria. Thus, after a very short time, the old situation was reestablished, which led to the well-known "saw-toothed curve" (39).

As biofouling is a biofilm problem, which in turn is related to surfaces, the latter have to be sampled for a proper effectiveness control. This can be generally achieved in several ways:

1. Integration of test surfaces, which are analyzed before and after the cleaning procedure, or,
2. Establishment of on-line, sensitive monitoring devices such as the continuous measurement of drag resistance, heat-transfer resistance, hydrodynamic resistance, weight of deposit, light transmittance, or reflectance.

Efficacy control allows the optimization of countermeasures against biofouling. Quite ineffective but widely practiced is the measurement of cell numbers in the water phase; these are relatively easy to measure but lead only to numerous data, which can never be interpreted.

PREVENTION OF BIOFOULING

Disinfection still remains the most common "prevention" practice. After considering the points discussed earlier, it is not surprising that disinfection tend to display transient effects. The disinfectant is usually rinsed out with nonsterile water, which leads to reinfection of the system. This explains why disinfection should not be expected to offer a suitable choice in prevention of biofouling.

As biofouling is a biofilm problem, prevention of primary adhesion seems to be the most reasonable strategy. The first steps in microbial adhesion are dominated by physicochemical forces rather than by physiological activity of microorganisms (114). Among the abiotic parameters, surface energy is considered to be of particular importance (115). Surfaces used in water technology will display different surface energies. When in contact with water, a conditioning film arises, formed by traces of macromolecules present in water. Among these are proteins, polysaccharides, humic substances, and others. Although they do not provide specific binding groups, many parts of a macromolecule can interact by weak forces such as van der Waals and electrostatic interactions and hydrogen bonds. However, the molecules possess many binding sites and will bind to a given surface. Thus, they will adhere irreversibly and result in a slightly negative overall surface charge (116). The conditioning film is not a prerequisite for microbial adhesion but only a result of the fact that macromolecules reach the surface faster than microorganisms. In some cases, the conditioning film may decrease the adhesion rate (117). Hydrophobic surfaces will become more hydrophilic, leading to a surface tension in the range of 30 to 40 $mJ\,m^{-2}$ (115). Slowest microbial adhesion is reported between 23 and 27 $mJ\,m^{-2}$; below these values, the adhesion rate is higher (118). Thus, efforts to use surfaces with very low surface energy will not succeed in preventing microbial adhesion. An example is given by leaves in autumn. The wax layer provides a high hydrophobicity, which allows the leaves to float on water. After a while, specifically hydrophobic microorganisms such as *Rhodococcus* sp. will colonize leaf surfaces, resulting in a hydrophilic biofilm, which causes the leaves to sink.

Primary adhesion occurs very rapidly, within the first hours of contact between water and surface. It is not necessary that the microorganisms are viable; it has been shown that adhesion to polysulfone membranes was equally rapid both with living and dead cells of *P. diminuta* ["passive adhesion," Flemming and Schaule (119)]. In other cases, cells excrete EPS in response to surface contact ["active adhesion" (114)]. In a biocoenosis of water, there will be various species present, some of them adhering even when dead and others developing contact-specific adhering substances.

Roughness of the surface will support colonization. However, smoothing of a surface will not completely prevent microbial adhesion. Electropolishing of metal heat exchanger surfaces only slightly prolonged the induction phase of biofilm development (4); EPS provide a "glue" that can stick to the smoothest surfaces. A certain success has been reported with anionic block copolymers (114,120). An interesting approach is coating with biocidal compounds (121). However, the coating can be dissolved or covered with dead microorganisms, which could nullify the effect. Practical applications of these approaches have not been reported. A similar case may be the use of plaque inhibitors, namely, substances that prevent microbial adhesion to teeth. Among these are quaternary ammonium compounds, biguanidines such as chlorhexidine, uncharged phenolic compounds ("Listerine"), enzymes, peroxides, and surface-active substances (122). All of these substances are candidates for the extension of the induction phase of colonization in closed systems.

Open systems such as ships in seawater are protected by antifouling paints. Success is always transient (123), implying that frequent cleaning is necessary. Tributyltin compounds are the only really effective substances known. However, they are perhaps too successful, as they belong to the most toxic materials (73) and are going to be banned worldwide because of their negative impact on the biosphere. Copper is widely believed to be bactericidal and copper plating belongs to the first antifouling measurements in naval history. However, colonization of copper surfaces is a well-known phenomenon (98,105) and once covered with a primary biofilm, even copper-sensitive organisms can survive in a biofilm on a copper surface. Copper still plays an important role in marine antifouling as an additive to Teflon coatings. Biofilm growth is not prevented but slowed down, and cleaning is easy.

Another so-called biocidal element is silver, to which an "oligodynamic effect" is attributed. In fact, most microorganisms are killed by very low concentrations of silver ions ($<10\ \mu g\,L^{-1}$), and silver is not known to be toxic to humans. Unfortunately, microorganisms develop a tolerance within a few weeks, allowing them to multiply in the presence of more than 1 $mg\,L^{-1}$ of Ag^{+} (76).

Highlighted by these observations, inhibition of primary adhesion is not a very promising strategy to prevent biofouling. Microorganisms are ubiquitous, and among these, there will always be some that can colonize in spite of the inhibitor. If UV light is used, the prospects are not much better. Only on surfaces that are directly irradiated, biofilm growth can possibly be prevented. As soon as abiotic particles adhere, microorganisms can be protected and can grow (Huber and Flemming, unpublished obs.). Additionally, UV light may oxidize humic acids and make

them more bioavailable, therefore creating more problems than what it solves.

The most common technical measure to prevent biofouling is the permanent dosage of biocides. This is usually performed with chlorine. Bott (102) showed that a concentration of at least 0.2 ppm had to be maintained in a technical system. Below this concentration, rapid biofilm growth was observed. In many cases, permanent dosage of biocides is undesirable for environmental reasons. Biocides and their toxic by-products contaminate the sewage water and require special attention in terms of storage and handling.

Living with Biofilms

Prevention of biofouling can only be achieved by a combined strategy. It must be based on a "clean system philosophy" that includes all surfaces in contact with water. It includes the minimization of biodegradable organic substances, good access of surfaces that must be kept clean, surfaces that are easy to clean, and no or very few crevices, fittings, edges, or other niches predestined for biofilm growth. Ultrapure water technology provides many experiences and solutions for less pure water systems. The elements of good housekeeping can be summarized as follows:

- Minimized nutrient concentration
- Monitoring of biofilm development on representative surfaces
- Strong shear forces
- Frequent cleaning (if possible, mechanical)
- Easy-cleaning design
- Effectiveness control and optimization of cleaning measures

The importance of effective monitoring systems must be pointed out clearly. Such systems allow for early intervention in a biofouling process and optimization of countermeasures.

All approaches to prevent biofouling as discussed earlier are based on killing and cleaning. A completely different strategy can be developed from some basic considerations. Almost all surfaces in contact with water carry biofilms, but not all water systems suffer from biofouling. It has been shown that biofilms are involved in the separation process on reverse osmosis membranes right from the beginning: after a few hours of operation, a biofilm develops and participates in separation (64). Biofouling occurs only when biofilm development exceeds a certain "threshold of interference." This is the case when performance parameters remain below certain limits (Fig. 5).

In technical systems, biofilms usually reach the plateau phase relatively rapidly. What happens is a natural phenomenon: microorganisms settle on surfaces and convert diluted nutrients into metabolic products and locally immobilized biomass. This is exploited in the design of a biofilm reactor. Biomass accumulates in the reactor, and the effluent water will support microbial growth, including the development of biofilms, to a lesser extent. Separation membranes, filter beds, pipe surfaces, and other technical environments offer large surfaces,

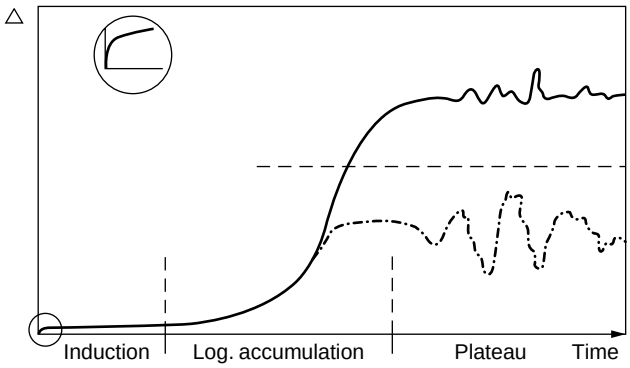

Figure 5. Schematic depiction of biofilm development. Dotted line: biofilm below the threshold of interference.

Table 6. Biofilm, Water, and Performance Parameters as Determined by Experimental Membrane Test Cells Before and After Sand Biofilter (After 125)

	Before Sandfilter	After Sandfilter
Biofilm		
Biofilm thickness	(27.3 ± 3.1) μm	(3.0 ± 0.5) μm
Protein content	77.7 $\mu g\,cm^{-2}$	3.6 $\mu g\,cm^{-2}$
Carbohydrate content	22.5 $\mu g\,cm^{-2}$	2.6 $\mu g\,cm^{-2}$
Uronic acid content	10.6 $\mu g\,cm^{-2}$	2.3 $\mu g\,cm^{-2}$
Water		
BDOC of water	0.325 $mg\,L^{-1}$	0.125 $mg\,L^{-1}$
Microbial content	1×10^7 cfu mL^{-1}	1.2×10^6 cfu mL^{-1}
Performance		
Flux decline	40% in 30 days	<2% in 30 days

encouraging the same phenomenon until the extent of biofilm growth exceeds the level of interference. This is why biofouling can be considered as a "biofilm reactor in the wrong place." The main factors, which govern biofilm growth, and biofouling potential, are the ubiquitous biofilms, the nutrients available, and the shear forces. As the occurrence of biofilms cannot be avoided without extreme efforts (e.g., as performed by the pharmaceutical and microelectronics industries), only nutrients and shear forces remain as variables. Shear forces can be increased quite easily in some systems but not in all. Thus, nutrient concentration represents a candidate for limiting biofilm growth: the lower the nutrient concentration, the less biofilm will form. This effect is well known in biofilters, where most biomass accumulates in the area with the highest nutrient concentration. The biofilm reactor can be "put in the right place" if positioned in front of the system to be protected from biofouling: the biofilm will develop mainly in the reactor. In the subsequent areas, biofilm growth will occur, but below the threshold of interference if the system is run properly. For example, river water was run through a sand bed as a biofilter. Before and after the filter, a reverse osmosis test cell had been installed, one operating with raw water and the other with water after the biofilter. Permeate production was taken as performance parameter (125). A BDOC of 0.125 $mg\,L^{-1}$ was sufficient to stay below the threshold of interference. Such a value can be reached easily with common biofilters.

Biofilm thickness was reduced to 10%. The microbiological data of the water reveal that the biofilter does not cause additional microbial contamination but rather removes cells from the process stream.

The results indicate that an antifouling strategy, which refrains from using chemicals and relies simply on nutrient depletion, can be successfully applied to technical water systems. Obviously, it is not necessary to kill *all* biofilms — it is only necessary to keep their development below the threshold of interference. Thus, it is possible to live with biofilms without biofouling. Of course, this is not feasible in all cases, but if it is applied where it is appropriate, unnecessary usage of biocides can be avoided. It may be pointed out that this approach is only one of a few that are based on the laws of biofilm development. We expect that in the near future more biocide-free strategies will be developed.

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BIOGENIC TRACE GASES.

See TRACE GASES SOIL

BIOGEOCHEMICAL CYCLES.

See PHOSPHORUS CYCLING IN AQUATIC ENVIRONMENTS: ROLE OF BACTERIA

BIOHYDROMETALLURGY.

See BIOLEACHING

BIOLEACHING

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Microorganisms are used worldwide for the extraction of valuable metals. The technique established in the last decades, is known as *bioleaching* (1). For this purpose, the world's largest bioreactors have been built. Stirred tank reactors of 900 m^3 have recently been constructed in Ghana (2) (Fig. 1) for treatment of sulfide concentrates containing a considerable amount of precious metals. In Uganda, reactors with a volume of 1,350 m^3 for cobalt leaching went into operation in 1998 (3). The leaching microorganisms dissolve the usually poorly water-soluble, metal-containing minerals due to an attack by biogenic compounds. The dissolution is achieved by complex interactions of biological, chemical (especially electrochemical), and physical processes. Nevertheless, the processes are not yet fully understood nor is their interdependence elucidated. Bioleaching of nonsulfidic minerals, that is, oxides, carbonates, and silicates, occurs by simple acid attack and/or complexation, whereas for dissolution of metal sulfides a combined acid and oxidative attack is needed. The products of these processes are metal cations dissolved in aqueous acidic solutions. The protons originate from biogenic acids. The most important acid is sulfuric acid, whereas relevant organic acids with good metal-complexing properties are oxalic and citric acid. Bacterially produced iron(III) ions are responsible for primary oxidation of the sulfur moiety of sulfides. In addition, intermediary sulfur compounds are oxidized by bacteria. Therefore, metal sulfide leaching bacteria are iron and sulfur compound–oxidizing species. The most important ones belong to the genera *Acidithiobacillus*, *Leptospirillum*, *Metallogenium*, *Acidianus*, and *Sulfolobus*.

Bioleaching occurs as a natural process in almost all climates, as long as water is available and the temperature does not exceed 114 °C, which is the the upper limit for microbial life (4). Bioleaching is applied in technical conditions for metal winning from raw materials that are not processable by conventional (usually pyrometallurgical) techniques. In most cases, low-grade ores are concerned, but increasingly even complex concentrates are treated by bioleaching techniques, such

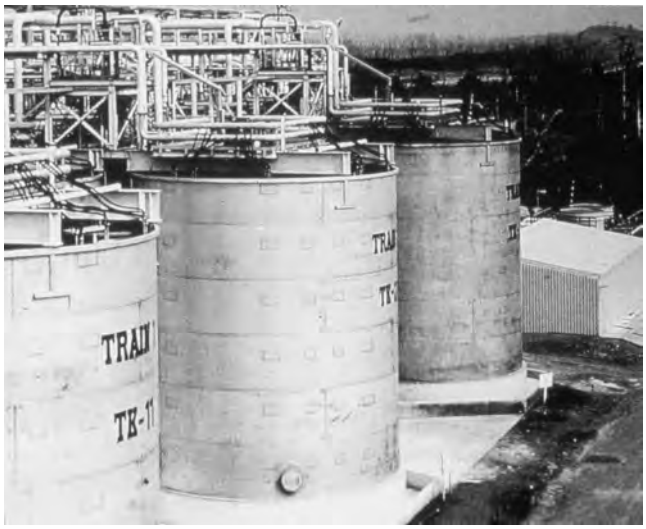


Figure 1. Stirred biooxidation tanks of the Ashanti's gold recovery plant in Obuasi, Ghana, for the bioleaching of refractory ores before cyanidation.

Figure 2. Dump bioleaching of copper ore in Peru. The dump is irrigated by sprinklers with acidified water.

as heap and dump leaching (Fig. 2) or tank leaching. However, anticipated bioleaching operations need to be empirically tested in the lab before realization, thereby requiring expensive scale-up. Especially the mineral itself, because of the occurrence of minor elements, plays an unpredictable role and causes many uncertainties in the design of the process and plant. It is well known, for example, that in heap leaching operations treating chalcopyrite ore, rarely more than 15% of the copper will be dissolved within a "reasonable" time of one to two years (5). The remaining part has to be deposited on waste dumps. However, the bioleaching process continues slowly and tends to pollute the environment with acidic solutions containing heavy metal ions. Similar uncontrolled bioleaching is encountered in abandoned mines, shafts, mine waste dumps, and so on and is known as "acid mine/rock drainage" (AMD/ARD). It is obviously caused by human activities in most cases but some natural bioleaching sites exist, too. In the past, spontaneous leaching has been used as an indicator of ore bodies because the colored waters attracted attention.

MINERALS

Technical applications of leaching processes for the extraction of valuable metals from natural raw materials must consider the quantitative description of the dissolution process. Physicochemical properties of the minerals subjected to dissolution and of the reaction products and reaction kinetics are of great importance for the economy of a process. Because pure minerals are not available in large quantities, an appropriate process design for technical applications has to consider the presence of invaluable constituents. More and more low-grade ores must be processed as high-grade ores are used up. Hence, because of impurities and/or gangue material, huge quantities of ore are mined, which consequently need large amounts of reagents for the extractive processes with all the inherent technical, economical, and environmental consequences.

Classical mineralogy is a descriptive science, which succeeded to identify and classify numerous minerals. Relationships between genesis, chemical composition, structures, and properties have been thoroughly described (6). Oxides and silicates are the most frequently encountered minerals but only few of them bear valuable metals that can or need to be extracted by (bio)leaching techniques. However, metal sulfides are a very important class of compounds because some of the base metals occur mainly in sulfidic ore deposits. Therefore, examples will focus on this class of minerals. The possible number of minerals having different compositions and properties is enormous because of traits classified as:

- *Polymorphism* (the same composition but a different crystallization system: pyrite-marcasite/FeS_2, sphalerite-wurtzite/ZnS),
- *Polytypism* (the same composition and the same crystallization system but different periodicity of crystal layers of the same arrangement: wurtzite-2H, -4H, -6H, -8H, -10H, -3R, -15R, and so on),
- *Isomorphism* (reciprocally unlimited exchangeable elements: sphalerite-hawleyite/ZnS-CdS, guadalcazarite Hg, Zn/S, Se; villamaninite Cu, Ni, Co, Fe/S_2, Se_2, irarsite Ir, Ru, Rh, Pt/AsS), and
- *Diadochism* (limited, nonreciprocal replacement of an element by another: up to 20% of zinc atoms replaceable by iron in sphalerite, but iron in pyrrhotite not replaceable by zinc atoms).

Additionally, chemical analysis revealed the existence of apparently nonstoichiometric compounds, that is, when calculating the chemical formula; the stoichiometry does not fit with simple valence states of the elements involved. Besides minerals, where the involved elements have quite well-defined valence states, such as FeS, FeS_2, ZnS, CuS, Cu_2S, $CuFeS_2$, and so on, minerals are known in which one element exists in several different valence states, for example, Cu_9S_5 or $Ag_3Fe_7S_{11}$. Moreover, different minerals may crystallize jointly to form intergrown crystals. Hence, obviously mineralogically approximately similar minerals present in ores from different sites may show differences in their physicochemical properties and a different behavior in technical processes such as flotation (7). Some minerals were also (re)produced experimentally (synthetically, in pure or deliberately impure state), showing that major differences in specific physicochemical properties may result from only minor compositional differences (8). Semiconducting properties (*n*- or *p*-conductivity) depend on the nature of minor amounts of impurities included in the crystal lattice, for example, *n*-pyrite contains Co and Ni ions, *p*-pyrite contains As-ions. These pyrite types show different behavior in the leaching process. Leaching of *n*-pyrite starts rapidly but slows down after a certain degree of dissolution is reached. On the contrary, leaching of *p*-pyrite starts slowly, but subsequently continues at an enhanced rate (9). Even in homeotypic minerals (different composition but the same crystallization system: pyrite-hauerite/FeS_2-MnS_2, molybdenite-tungstenite/MoS_2-WS_2, sphalerite-chalcopyrite/ZnS-$CuFeS_2$) electronic structures

are not identical. Different energy levels of the atomic and molecular orbitals cause different stability, reactivity, and even different reaction mechanisms. Hence, under certain conditions one mineral may dissolve and the other may not, or perhaps they may dissolve but by different pathways (10). Electrochemical properties like electrode potential and polarization also play an important role for the specific leaching behavior of a mineral complex. Intergrown particles of different minerals may form galvanic cells, causing preferential reaction sites at the solid interface (9). In modern materials sciences, sophisticated procedures have been established to obtain special materials with unique, useful properties. From this experience, it became understandable why naturally occurring mineral samples in many cases are also unique. Hence, it should also be clear that each ore deposit, in many cases each mineral vein, must be tested for technologically reliable process data. For practical purposes, a scale-up also must be done.

MICROORGANISMS

The bacterially catalyzed oxidation of metal sulfides (mediated by iron(III) ions) was discovered only half a century ago (11,12). Because of the rod-shaped morphology of the cell and its ability to oxidize both reduced iron and sulfur compounds, this first isolate of a bioleaching bacterium was named *Thiobacillus ferrooxidans*. This systematic name is no longer valid and the species is now called *Acidithiobacillus ferrooxidans* (13). Leaching bacteria have been detected all over the world and representatives are found in both bacterial domains (i.e., the *Archaea* and the *Bacteria*) (14). Bioleaching research and application focus on the metal and sulfur compound–oxidizing bacteria. In the following, the biology of these classic leaching bacteria is described. A short characterization of the only recently investigated metal-complexing chemoorganoheterotrophic microorganisms is also given.

Biology of Metal Sulfide Oxidizing Bacteria

At first sight, the biology of the acidophilic metal and sulfur compound oxidizing bacteria seems to be complicated. The bacteria can use the oxidation of metal sulfides as an energy source and thrive in heavy metal–containing solutions at pH 2 (14). However, the main metabolic pathways do not differ significantly from those found in other bacteria and the most investigated leaching bacterium, *A. ferrooxidans*, is a close relative of the most thoroughly characterized living organism, that is, the γ-proteobacterium and gut inhabitant *Escherichia coli*. Nevertheless, the adaptation to environments, such as natural, sulfur-rich hydrothermal springs and mining sites created by humans, gives the leaching bacteria a combination of several biotechnologically interesting physiological abilities. One of the major characteristics of these bacteria is their ability to oxidize inorganic sulfur compounds to sulfuric acid and/or iron(II) to iron(III) ions. In several bacteria, these abilities are found in combination, for example, *A. ferrooxidans*, *Sulfobacillus acidophilus*, and *Acidianus brierleyi* (14). These species can live chemolithoautotrophically with reduced sulfur or iron compounds and carbon dioxide as the sole electron, energy, and carbon sources, respectively. Furthermore, some strains can grow by oxidizing molecular hydrogen and are able to fix nitrogen from air. In leaching biotopes, these physiologically versatile species are often accompanied by less versatile bacteria. *Acidithiobacillus thiooxidans* (formerly *Thiobacillus thiooxidans*, 13) can only oxidize sulfur compounds, whereas for *Leptospirillum ferrooxidans*, iron(II) ions are the sole electron donors. External organic compounds are, if at all, used only to a minor extent and surprisingly, many organic substances are toxic to these bacteria (14). Therefore they are classified as strictly chemolithoautotrophic organisms. Besides these bacteria, mixotrophic species able to grow on both inorganic and organic substrates have been isolated from leaching biotopes. For instance, *Acidiphilium acidophilum* (formerly *Thiobacillus acidophilus*, 15) oxidizes sulfur and organic compounds such as sugars. *Acidimicrobium ferrooxidans* cannot only live autotrophically on iron(II) ion oxidation but also grow mixotrophically or even chemoorganotrophically, if yeast extract is added to the cultures. The oxidation of sulfur compounds and iron(II) ions is not limited to organisms growing in an aerobic environment but has also been observed in the presence of alternative electron acceptors others than oxygen. Some species of the genus *Acidithiobacillus* and *Acidiphilium* have been found to oxidize elemental sulfur and other sulfur compounds with iron(III) under anaerobic conditions (14). The reduction of nitrate can be coupled to bacterial oxidation of both reduced sulfur compounds and iron(II) ions (16,17), however, these latter processes have so far not been demonstrated in acidophilic leaching bacteria. Generally, for the characterization of the bacterially catalyzed reactions, concerning the sulfur and iron cycles in leaching biotopes, aerobic and anaerobic processes have to be considered. In addition to the anaerobic reactions mentioned earlier, the reduction of sulfate, tetrathionate, thiosulfate, and elemental sulfur may play an important role in anoxic zones. Furthermore, the disproportionation of elemental sulfur and other sulfur compounds is discussed as energy-yielding pathways to support bacterial growth. Bioleaching occurs in arctic regions with temperatures below 0 °C and in hot springs at temperatures around 100 °C. Although, leaching sites with cold and moderate temperatures (up to 40 or 50 °C) are dominated by the mesophilic, eubacterial genera *Acidithiobacillus* and *Leptospirillum*, other leaching bacteria thrive at elevated temperatures. Moderately thermophilic genera are the eubacteria *Sulfobacillus* and *Acidimicrobium* that oxidize metal sulfides at temperatures up to 60 °C. The upper temperature range of bioleaching is exclusively occupied by the extremely thermophilic archaebacterial genera *Sulfolobus*, *Acidianus*, *Metallosphaera*, and *Sulfurococcus* (14). The latter two groups of bacteria are of great interest for the design of new bioleaching plants and, therefore current research is focused on the metal sulfide oxidation potential of these thermophiles (18). If the operating temperature in stirred tank reactors could be permitted to increase to

60 or even 80 °C, cooling costs would be significantly reduced (see Section on Applications). Furthermore, recalcitrant metal sulfides such as chalcopyrite become more readily oxidizable at elevated temperatures. To date, commercial bioleaching processes operate at temperatures below 50 °C (2). The bacterial communities in these reactors are dominated by *Leptospirillum*-like bacteria and *Acidithiobacillus caldus* (formerly *Thiobacillus caldus*, 13) rather than by *A. ferrooxidans* and *A. thiooxidans* (19). For the latter, the conditions in the continuous-flow bioreactors with constantly high redox potentials above 700 mV (SHE), pH around 1.5, and temperatures above 40 °C are not conducive for growth. Defined inocula of pure or mixed bacterial cultures are not applied to industrial bioleaching processes. Mostly, poorly characterized enrichment cultures or endemic bacteria are used. More thoroughly selected or even genetically engineered organisms may have a potential to optimize leaching operations; however, the industrial applicability of these bacteria still remains to be demonstrated. Besides the bacteria described previously, leaching biotopes are inhabited by acidophilic, chemoorganoheterotrophic bacteria and fungi, as well as by protozoa, grazing on bacteria, and by photoautotrophic algae (14,20). The primary (biomass) producers in these generally oligotrophic biotopes are the chemolithoautotrophs because, in most cases, the biomass production of phototrophs is negligible. Interactions between chemolithoautotrophs and chemoorganoheterotrophs may beneficially affect leaching activity. As leaching bacteria usually excrete low molecular weight organic compounds, which inhibit their own growth, the presence of organotrophs can prevent the accumulation of these compounds and enhance leaching performance (14).

Biology of Chemoorganoheterotrophic Leaching Microorganisms

Bacteria, algae, fungi, and consortia such as lichens have the potential to extract metals of interest from nonsulfide ores or metal-containing industrial wastes such as fly ash and slag. A general characteristic of these organisms is the production and excretion of high amounts of organic acids and other metal-complexing compounds, basically because of an imbalance of their metabolism. Mainly organic acids are relevant, such as oxalic, citric, malic, succinic, pyruvic, acetic, glucuronic, and amino acids. Furthermore, they exhibit a rather high heavy metal resistance. These abilities are widespread among chemoorganoheterotrophic species and such diverse genera as *Bacillus*, *Pseudomonas*, *Penicillium*, and *Aspergillus*, are investigated in this context (21,22).

MECHANISMS OF MICROBIOLOGICAL METAL SULFIDE DISSOLUTION

Dissolution of metal sulfides is achieved by acid attack and the oxidation of the sulfur moiety. The first oxidation step is carried out by an electrochemical surface reaction of iron(III) ions with the sulfide species. Subsequently, the released intermediary sulfur compounds are further oxidized by iron(III) ions or oxygen, abiotically or enzymatically, to sulfate and protons. There is a long-standing debate in the literature about the mechanism of bioleaching, whether cells are able to oxidize the sulfur moiety at the metal sulfide surface enzymatically ("direct mechanism") or whether the primary oxidant are iron(III) ions and the role of bacteria is to reoxidize the resulting iron(II) ions and sulfur compounds ("indirect mechanism") (23). The differentiation between direct and indirect bioleaching was developed at a time when only *A. ferrooxidans* and *A. thiooxidans* were known as leaching bacteria. As soon as *L. ferrooxidans* (24) was discovered, a serious contradiction became evident: the cells of this bacterium dissolve metal sulfides exclusively by regeneration of the iron(III) ions; no sulfur compounds can be catabolized by this organism. The direct mechanism is considered disproved because of these and other contradictions (10,23) and, especially, because the required enzyme system has never been demonstrated. The most widely agreed scheme of bioleaching integrates findings from microbiological, chemical, electrochemical, and mineralogical studies. Together with images obtained by light microscopy, and scanning and transmission electron microscopy (SEM, TEM), as well as atomic force microscopy (AFM), a holistic theory on bioleaching of metal sulfides has been developed (10,25,26). Accordingly, observations regarding bacterial attachment, reaction pathways, and kinetics fit together.

Attachment of Bacteria to Mineral Surfaces

Leaching bacteria attach to metal sulfide surfaces (Fig. 3). From biofilm research it is known that attachment occurs via diffusion, convection, and chemotaxis. Although the first two are mostly random processes, chemotaxis is the active orientation of the bacterium in a chemical gradient. The bacterium is able to detect the site of active metal

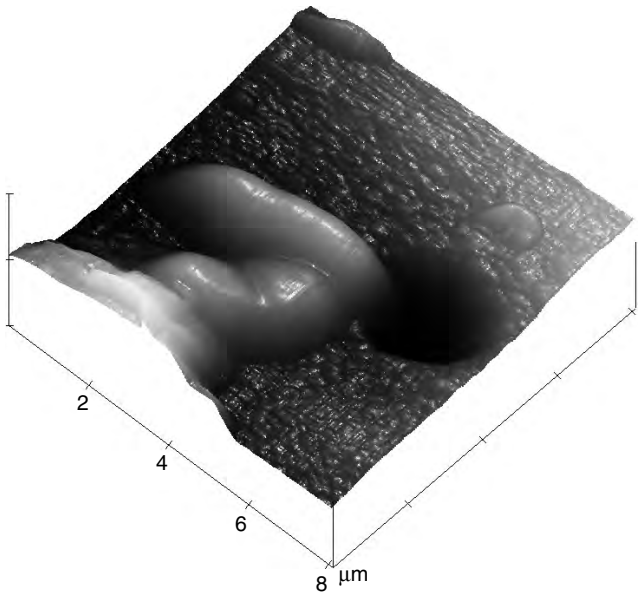


Figure 3. Atomic force microscopic image of a cell of *Acidithiobacillus ferrooxidans* attached to the surface of a pyrite crystal.

sulfide dissolution by sensing the resulting gradients (e.g., dissolved iron(II) ions and sulfate) and actively swimming in the direction of an increased concentration of the attracting ions (27). There, the bacterial cell attaches to the metal sulfide surface by mostly unknown processes. The extracellular polymeric substances (EPS), surrounding the cells play an important role in the attachment of the leaching bacteria. In the cases of *A. ferrooxidans* and *L. ferrooxidans* grown on pyrite, the exopolymers contain, besides neutral sugars and lipids as their major quantitative constituents, some glucuronic acid residues and complexed iron(III) ions (26,28). The stoichiometry of glucuronic acid to iron(III) ions amounts to a molar ratio of 2 : 1 for the strains of *A. ferrooxidans* and *L. ferrooxidans* tested so far (26,28). This indicates that regardless of the phylogenetic class of the leaching bacteria, the physicochemical characteristics of the environment enforced a similar adaptation. According to the stoichiometry, a net positive charge can be attributed to the EPS of the leaching bacteria. At the extremely acidic pH of the leaching environment, pyrite has a net negative charge. Thus, an attachment of a positively charged bacterium to a negatively charged surface may take place because of an electrostatic interaction between the two surfaces. Once the bacterium has attached, additional processes cause an intimate and tight adhesion to the respective mineral surface. These processes have not yet been elucidated. Besides, nothing is known about the specificity of the EPS for various metal sulfides. To date, only data for EPS of pyrite- and of sulfur-grown cells are available. They indicate that the bacteria change the composition of their EPS according to the substrate or substratum, which they use. In the EPS of *A. ferrooxidans* grown on sulfur, more lipids, less neutral sugars, and only traces of glucuronic acid were present as compared to those of pyrite-grown cells (26).

Reaction Pathways

Experimental results indicated that reaction pathways of metal sulfide dissolution are not determined by the crystal structure (10,25). The reactivity of the minerals with protons is the only relevant criterion. For instance, acid-nonsoluble monosulfides such as molybdenite and tungstenite are degraded by the same mechanism as the acid-nonsoluble disulfide pyrite. In contrast, the structurally similar disulfides pyrite and the acid-soluble hauerite are dissolved by different mechanisms.

Thiosulfate Pathway. The following reaction mechanism describes the degradation of acid-nonsoluble metal sulfides such as pyrite, molybdenite, and tungstenite (25,29). Pyrite is dissolved *via* electron extraction by hydrated iron(III) ions from the crystal lattice according to equation (1):

$$FeS_2 + 6[Fe(H_2O)_6]^{3+} + 9H_2O \rightarrow S_2O_3{}^{2-} + 7[Fe(H_2O)_6]^{2+} + 6H^+ \quad (1)$$

Thiosulfate subsequently yields via a series of reactions either sulfate and protons or higher polythionates

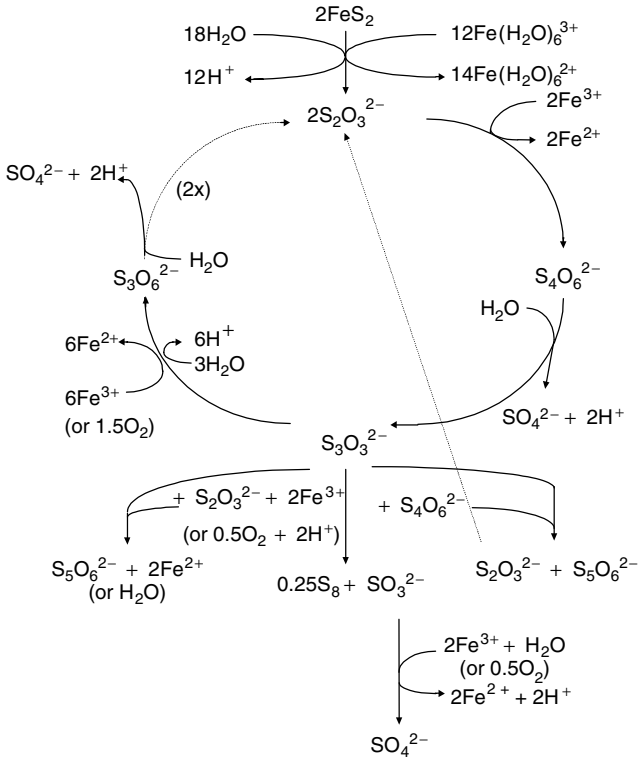


Figure 4. Cycle of oxidative pyrite degradation by bacterial and/or chemical leaching (29).

and elemental sulfur, as indicated in Figure 4. Some of the reactions occurring in the cyclic thiosulfate degradation have been described to be mediated by enzymes. For example, the enzyme for the oxidation of thiosulfate to tetrathionate (thiosulfate dehydrogenase) has been isolated from cells of *A. ferrooxidans* (30), as has been the enzyme for the hydrolysis of tetrathionate (tetrathionate hydrolase) from cells of *A. ferrooxidans* and *A. thiooxidans* (31–33). The extent to which enzymatic catalysis contributes to thiosulfate degradation has to be clarified. Yield calculations, using the amount of biomass produced in the course of pyrite dissolution, indicated that some of these reactions must release electrons at a level sufficient for cytochrome b/flavine reduction (34). This would consequently allow for two oxidative phosphorylations, whereas electrons at the cytochrome c level, as provided by iron(II) ions, would give only one ATP per mol of oxidized substrate. The increased growth yields, as measured, are explainable only with this assumption. The question whether the tetrathionate degradation is dominated by abiotic or by enzymatically catalyzed reactions is important because these reactions determine the end products: whether a considerable amount of elemental sulfur is formed or mainly sulfate and protons. In the first case, acidification in ARD could be reduced. In the second situation, full sulfuric acid production would enhance ARD problems. A regulation and control of this reaction is, furthermore, of high value for industrial application of pyrite bioleaching before gold extraction by cyanidation. The presence of elemental sulfur in the leach residue causes unnecessary

cyanide consumption as a result of the formation of isothiocyanates.

Polysulfide Pathway. The mechanism for metal sulfide degradation with the main intermediate polysulfide is valid for acid-soluble sulfide minerals, for example, hauerite, sphalerite, galena, chalcopyrite, or arsenopyrite (25). Here the attack on the crystal lattice (MS) is carried out by a combined action of protons and iron(III) ions. Protons induce polarization of a surface sulfide ion and enables its liberation from the crystal lattice concomitantly with an electron transfer to an iron(III) ion (25). The first free intermediate of this attack is a H_2S^+ cation radical, which after a series of reactions ends in a polysulfide, H_2S_n (35). The latter compound decomposes to H_2S and elemental sulfur (S_8 in the case of H_2S_9). The reactions may be summarized as shown in Eq. (2).

Subsequently, bacteria regenerate the protons by further oxidizing the elemental sulfur to sulfuric acid.

Hydrogen Sulfide Pathway. In the absence of iron(III) ions, readily acid-soluble metal sulfides such as sphalerite can be dissolved by simple acid attack, liberating hydrogen sulfide. Sulfur compound oxidizing bacteria oxidize it to sulfate and release protons, which can enter the cycle again to dissolve additional metal sulfide. Hydrogen sulfide is oxidized stoichiometrically to elemental sulfur in *A. thiooxidans* (36). The reaction is probably catalyzed by a sulfide : quinone oxidoreductase because the gene sequence of this enzyme has been detected in the genome of *A. ferrooxidans* (37).

Galvanic Influences

Besides the direct attack of iron(III) ions, protons, and complexing agents, in the case of intergrown crystals of different minerals, galvanic interactions have to be considered (9). Because of their different electrode potentials, a polarization can occur between different metal sulfides in an ore, if these are in direct electrical contact. The sulfide with the lower potential functions as the anode, whereas the metal sulfide with the higher potential represents the cathode. At the anode, the metal sulfide is dissolved by electron transfer to the cathode where iron(III) ions are then reduced without oxidation of the cathode itself. As long as the electrical contact between the different sulfides exists, the sulfide with the higher electrode potential is protected against oxidant attack and is dissolved at slower rates than the isolated (galvanically unprotected) sulfide and vice versa. For example, pyrite could be protected by intergrown chalcopyrite or sphalerite that have lower electrode potentials (38,39). Hence, these electrochemical effects may influence considerably the dissolution rates.

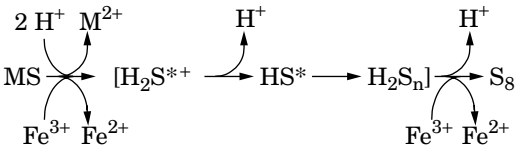
Kinetics of Metal Sulfide (Bio)leaching

The kinetics of metal sulfide dissolution in the presence of iron(III) ions is controlled by the charge-transfer reactions at the mineral surface (the shrinking-core model has to be considered). Reoxidation of the iron(II) ions in acidic solutions is primarily performed by bacteria because enzymatic catalysis is 10^6 times faster than abiotic oxidation (40). These two subprocesses are linked by the redox potential (41). Because in leaching experiments only a control of the bulk solution is possible, differences existing between the conditions in the bulk compared to the solid–liquid interface, for example, concentrations of iron species, pH, and oxygen, could be kinetically relevant. Such differences might occur if a delimited reaction space is assumed either because of solid reaction products remaining at the mineral surface (e.g., formation of a layer of elemental sulfur or iron precipitates) or because of the defined space, created by attached bacteria and the mineral surface (25,26). Solid–liquid, solid–solid–liquid, and solid–biofilm–liquid interfaces should be considered. Mainly concentration gradients of reactants and/or reaction products, as well as reduction of diffusion rates are the consequence but also other mechanisms of diffusion control can be imagined. Furthermore, in the case of intergrown sulfide minerals galvanic effects may cause preferential dissolution. Hence, dissolution kinetics is strongly dependent on the purity of the investigated materials.

Recent work focused on the comparison of abiotic and bioleaching of sphalerite and pyrite at electrolytically controlled redox potentials. Abiotic leaching was performed at similar high iron(III) to iron(II) ratios as are typical for bioleaching, where recycling of the iron(III) ions is enzymatically catalyzed. Comparative experiments without redox control showed that bioleaching rates were up to 20 times higher than in sterile controls started at the same initial amounts of iron(III) ions (42). However, in the absence of bacterial iron(III) reoxidation, the redox potential decreased rapidly as a result of the reduction of the iron(III) ions by the metal sulfide. Therefore, solution conditions in bioleaching experiments and sterile controls were not identical during the entire experiments and the data obtained are not comparable.

Leaching of Sphalerite in Acidic Iron(III) Solutions. The kinetics of sphalerite dissolution under potentiostatic conditions is determined by two processes. High leaching rates are achieved at high redox potentials where no significant difference between bacterial and abiotic leaching is observed. In this case, the dissolution kinetics is controlled only by the electrochemical reaction at the sphalerite surface (43). According to the polysulfide mechanism, this reaction consists of the proton-mediated electron extraction by iron(III) ions, which yields elemental sulfur as the first stable intermediate (Eq. (2), (25)). This could form a layer on the solid surface if no bacterial sulfur oxidation occurs. However, only at low leaching rates (low redox potentials) the kinetics becomes diffusion-controlled in the absence of sulfur-oxidizing bacteria (44). Consequently, sulfur compound–oxidizing bacteria enhance sphalerite dissolution at low redox potentials even under potentiostatic conditions by preventing the accumulation of elemental sulfur on the sphalerite (44). Generally, the formation of a diffusion barrier of elemental sulfur has to be taken into account in all cases in which the

polysulfide mechanism is involved (e.g., for the leaching of chalcopyrite).



(2)

Leaching of Pyrite in Acidic Iron(III) Solutions. In contrast to the dissolution kinetics of sphalerite, the rate-determining step of pyrite leaching is always the electrochemical reaction at the mineral surface. Because pyrite is oxidized via the thiosulfate pathway with water-soluble tetrathionate as the first detectable intermediate, no diffusion-controlling sulfur layer can develop on the mineral surface. The surface reaction is dependent not only on the redox potential, as it is for sphalerite oxidation in acidic solutions, but it is also influenced by the pH (45) because the oxygen incorporated into sulfate originates from water (46), probably via hydroxyl ions (46). In potentiostatic and pH-static experiments, bacterial pyrite leaching was enhanced over sterile controls by a factor of about 1.3 (48). As the reaction orders with respect to iron(III) ions are identical in biotic and abiotic experiments but differ with respect to pH, it was concluded that bacteria enhance pyrite dissolution by increasing the pH at the mineral surface, whereas the bulk pH is kept constant. This pH increase could be caused by the combined actions of bacteria and their EPS in the biofilm, that is, the proton consumption during bacterial iron(II) oxidation and the buffering properties of the EPS (48). Additionally, the positively charged EPS of the leaching bacteria (26) may obstruct influx of protons from the more acidic bulk solution.

APPLICATIONS

The industrial application of bioleaching for the winning of metals is often called biomining. At the end of the twentieth century, it has become one of the main processes used in biotechnology (1). The gigantic dimensions of the bioreactors at the Ashanti plant in Ghana for the pretreatment of gold ores (about 1,000 t ore concentrate are processed per day in 24 stirred tanks of 900 m^3 each since 1995, Figure 1), mentioned earlier (2), even surpassed the biological steps of sewage treatment plants as the largest bioreactors on Earth. It has been estimated that bioleaching is yielding up to 25% of the world's copper and about 10% of the uranium production (49,50). The total value of the products generated by this technology was assessed at U.S. $10 billion in 1998 (51). One of the major reasons for the increasing use of biomining is that conventional means such as ore dressing, followed by smelting techniques are uneconomical for low-grade ores. The oxidation efficacy of metal sulfides by bioleaching bacteria is nearly independent of the metal content, therefore extremely low-grade ores, in the case of copper, typically containing less than 0.3% of the metal, can be mined and processed besides concentrates. With the easy-to-mine rich surface ore deposits becoming exhausted, bioleaching is the alternative for metal winning from previously uneconomic ores. Secondly, in comparison with the nonbiological ore-processing techniques, bioleaching stands for low energy–consuming processes with reduced soil, water, and air pollution problems. Environmental laws increasingly regulate the emission of sulfur dioxide and heavy metals in the flue gas from smelters in many countries. Consequently, bioleaching or biohydrometallurgy turn out to be an important alternative to the conventional pyrometallurgy (1). In addition, no proper pyrometallurgical technique is available for processing of complex concentrates (Cu–Zn–Pb–Ni–Co), therefore hydrometallurgical alternatives to smelting are increasingly investigated. It is well known that zinc and lead contained in copper concentrates and copper contained in lead-zinc concentrates are undesirable because they generate major problems in the specific pyrometallurgical processes.

Current Biomining Techniques

Microbiological leaching is used for two different purposes. First, it is applied to mobilize the metal species of interest from insoluble compounds. For metal sulfides and uranium(IV) oxides, the formation of dissolved metal ions is achieved by oxidation processes. Furthermore, the metals can be extracted from nonsulfide ores and industrial wastes containing metal oxides, by microbially produced inorganic and organic acids. To date, the latter technique is not applied on an industrial scale, whereas the solubilization of base metals such as copper by bacterial oxidation of the corresponding sulfides plays an increasingly important role in commercial mining operations. Furthermore, bioleaching is applied to remove interfering metal sulfides before the conventional extraction of gold and silver from refractory ores by cyanidation or other solubilizing reagents. In refractory ores, the gold and silver are entrapped within the metal sulfide matrix such as pyrite and arsenopyrite. Because the precious metals must be reached by the leaching solution to become oxidized and subsequently complexed by cyanide, they need to be at least partially liberated from the surrounding compounds. As a result of the small size of the encased particles, even intensive grinding is often insufficient, and the interfering metal sulfides have to be oxidized by conventional pyrometallurgy (i.e., roasting, smelting, or pressure leaching), nitric acid oxidation, or bioleaching. In the last decade, the latter technique has turned out to be economically and environmentally the most beneficial option (2). The pretreatment of gold ores by bioleaching even allows to process ores from deposits that were previously considered uneconomical.

Several simple, low-cost techniques are applied for the winning of metals from low-grade sulfidic ores by bioleaching (1,9,52). Generally, an acid leaching solution with a pH around 2, containing the appropriate bacteria is pumped through an ore-bearing material. The metal-containing (pregnant) leachate is collected for recirculation or further processing. In underground and in situ leaching, the metal sulfide oxidation takes place directly in the

natural ore body. Leaching solutions are pumped into a fractured deposit. The pregnant leachate is collected at impermeable sites below the ore body or pumped from new boreholes back to the surface. This technique is often used for the solubilization and winning of uranium(VI) ions from underground uranium ore deposits. In dump leaching, the ore material is piled in heaps 10 to 20 m high on an impermeable base (liner, clay, and so on) and irrigated by sprinklers or flooded temporarily (Fig. 2). The drainage is collected and recirculated several times. Dump leaching is the oldest bioleaching technique. It is generally used to oxidize metal sulfides in waste rock (run-of-mine) that was deposited in the course of exploiting ore bodies for high-grade deposits. Furthermore, it was uneconomical to process this rock by conventional techniques. Both the underground and in situ and the dump leaching methods are rather inefficient processes with low oxidation rates and low total metal yields. The leaching solution often cannot uniformly percolate the underground deposit or the dump because of the low degree of fragmentation of the ore body or waste material. The conditions for bioleaching cannot be rigorously controlled, especially insufficient oxygen supply will limit the dissolution. A more sophisticated low-cost bioleaching technique is called heap leaching. The process is similar to dump leaching but the dimensions of the pile are limited to about 2 m in height, to facilitate oxygen diffusion. Furthermore, usually fine-ground ores or even concentrates are used. Before piling, the ore material is mixed with sulfuric acid to form an agglomerate. This process prevents the segregation of fine and coarse particles and causes a uniform permeability of the heap. For the circulation of the leaching solution, drip-type irrigation above and below the heap surface instead of sprinklers are installed to prevent cooling of the heaps by evaporation. The leachate is collected by a drainage tubing system above an impermeable liner. Heap leaching is mainly used for the winning of copper from chalcocite and covellite. Once concentrations of 1 g/L for copper ions have been achieved in this pregnant solution by continuous cycling, techniques such as solvent extraction and electrowinning are used to produce cathode copper. In contrast to the above-mentioned low-cost techniques for the winning of base metals from low-grade ores, more expensive methods can be applied where high-grade ores and/or precious metals such as gold and silver are concerned. For this purpose, stirred-tank reactors are used (1,9,52). Tank or reactor leaching operate with a sophisticated process control for optimum leaching conditions. In the newest tank bioleaching plants, several huge reactors with single volumes of about 1,000 m^3 are continuously fed with fine-ground, high-grade concentrates at pulp densities of up to 20% (w/v). The main operating costs result from the highly energy-consuming stirring and aeration of the leaching suspension. In addition, because of the high oxidation rates that are achieved with the sulfide-rich concentrates (>20%, w/w), cooling systems have to remove the evolving heat of the exothermic oxidation reactions to maintain a temperature below 50 °C. This is the temperature limit of the usually applied moderately thermophilic bacteria (2). Tank leaching is mainly used to decompose the metal sulfide matrix of recalcitrant gold and silver ores to facilitate the contact of the encased precious metal particles with the cyanide solution. If the interfering metal sulfides contain chalcopyrite, the tank leaching process could combine both the winning of the base metal (copper) and the extraction of the precious metals.

Research and Future Applications

Today, bioleaching research focuses on the optimization of the biooxidation processes in stirred tank reactors. With tank bioleaching becoming an accepted alternative for the pretreatment of recalcitrant gold ores in countries such as South Africa, Ghana, Brazil, and Australia, the mining industry also intends to use bioleaching tanks for the recovery of base metals such as copper, cobalt, nickel, and zinc (1). For instance, the Kasese Cobalt Project in Uganda represents the first large-scale cobalt bioleaching plant (3). The development of bioleaching plants, as with every large-scale biotechnological process, has scale-up problems. Laboratory experiments in shake flasks and percolation columns are often used to determine optimum particle size, ore composition, and other bioleaching parameters but cannot simulate the real conditions in a stirred reactor with 1,000 m^3 ore pulp. The conditions in the final design still have to be conducive for the bacteria, for example, agitation and aeration should balance the oxygen demand without killing the bacteria by detrimental shear forces. In addition, the bacterial process has to be integrated and linked to the other nonbiological units of the plant, for example, the upstream and downstream processing. Bacterial demands on the feed and the composition of the leaching product can have a significant influence on the total metal recovery process. For instance, if the leaching solutions are to be recycled to reduce process water demand, toxic substances added (e.g., in the course of solvent extraction) have to be sufficiently removed. Besides reactor design and total flow sheet development, one of the major current research topics deals with the operating temperature in bioleaching plants. To date, because of the generally applied mesophilic and/or moderately thermophilic leaching bacteria, bioleaching in stirred tanks is limited to temperatures of 40 to 50 °C. As a result of the high sulfide oxidation rates, and consequently, high heat evolution obtained in these reactors, cooling contributes significantly to the total operating costs. Therefore, the development of bioleaching processes that can operate at 60 to 80 °C are of great interest. Besides the reduction of cooling costs, bioleaching activities would be enhanced at these elevated temperatures and metal sulfides difficult to leach, such as the commercially important chalcopyrite, could be biooxidized with sufficient efficacy (53). In addition to the extraction of base and precious metals, bacterial metal sulfide oxidation could also be applied for the depyritization of coal before combustion, thus drastically reducing sulfur dioxide emission from power plants, especially in cases where expensive flue gas cleaning is not available. Despite several positive feasibility studies, this interesting technique has not been tested yet beyond pilot-scale dimensions (54).

In contrast to the biooxidation of metal sulfides, other bioleaching methods for the winning of metals are at present not applied commercially (21). Metals from nonsulfide ores, that is, metal oxides, carbonates, and silicates, and from industrial wastes, containing metal oxides such as fly ash, incineration slag, and contaminated soil, can be extracted by inorganic and organic acids and alcohols. For instance, sulfuric acid produced from the bacterial oxidation of inexpensive elemental sulfur has been used successfully to solubilize copper, cadmium, and zinc from solid-waste incineration fly ashes (55). Furthermore, the potential of heterotrophic bacteria and fungi to produce organic compounds with good metal complexation properties is under investigation (22,56).

ENVIRONMENTAL PROBLEMS CONNECTED WITH BIOLEACHING

The discovery of bioleaching resulted from research conducted on an environmental problem: the acidic drainage, containing heavy metals, which resulted from a waste heap of a coal mine (11). Consequently, bioleaching does not only help in an economic production of valuable and precious metals but also causes serious environmental problems known as AMD/ARD. Leaching bacteria are ubiquitous whenever metal sulfides become exposed to air and water. Most of these bacteria are chemolithoautotrophs, hence, only carbon dioxide from the atmosphere is needed for cell carbon generation (fixation). The degradation products of bioleaching, sulfuric acid and dissolved heavy metals, if not neutralized and precipitated by endogenous buffer material such as carbonates, will enter into the soil and/or the water paths and cause environmental pollution. In the case of a sufficiently high amount, all neutrophilic life will vanish, leaving an acidophilic ecosystem. In addition, the acidity may seriously reduce the fertility of the affected soils and waters. This has happened to the river Rio Tinto (Spain) as a result of mining activities for more than two thousand years, or naturally in the geothermal areas of Yellowstone National Park (U.S.A.) and similar environments. Soils, rivers, and lakes polluted in this manner serve as a source of heavy metals for incorporation into the food chain, either via plant uptake (and accumulation) or via predation, grazing, and so on. Finally, the heavy metals contaminate food products intended for consumption by humans.

Another connected problem is the generation of dust and fine particles. Because of the biological degradation of metal sulfides, the rock containing these compounds becomes fragmented and breaks down to small (microscopic) particles, especially under a combined action with water and freeze-thaw attack (57). These become wind-borne and because they may still contain some heavy metals, they may pollute the environment as dust. Often, tailings that have not properly been protected against wind and water erosion are located within inhabited areas or even within cities (e.g., Johannesburg, South Africa). The dust causes heavy metal pollution, in addition to respiratory diseases like false (mild) croup. In the case of radioactive wastes, another problem occurs: the liberation of usually entrapped radon gas into the atmosphere. Radon, formed as an intermediate of uranium (^{238}U) decay to lead (^{206}Pb), usually remains in the mineral in which the uranium is located. However, if the uranium is enclosed into pyrite or other metal sulfides, bioleaching may result in the liberation of this gas to the atmosphere. Because radon (^{222}Rn, half-life time of about 4 d) may be taken up for example, by humans and in the worst-case decay to a solid radioactive isotope of lead (^{210}Pb, half-life time 21 a), it may contaminate the lung for decades with an increased level of radioactivity. Besides these processes, which are directly and/or indirectly related to oxidative metal sulfide dissolution, reductive microbiological processes may also contribute to pollution with heavy metals. Manganese(IV) and iron(III) ion–reducing bacteria thrive under anaerobic conditions, oxidizing organic substances present and reducing the inorganic heavy metal ions to their soluble forms, manganese(II) and iron(II) ions, respectively. These tend to migrate. In addition, other coprecipitated metals become mobile too, causing a spreading of heavy metal pollution, in fact due to the availability of degradable organic matter. This natural process is believed to be the major process for mineralizing organic matter in anaerobic sediments (23,58,59,60) and thus cannot be avoided.

Countermeasures for problems connected with ARD/AMD deal mainly with a reduction of oxygen and/or water access to the metal sulfides because these are the two essential compounds needed for (bio)degradation. For this purpose, sophisticated systems have been developed, including multilayered covers, geotextiles, clay, limestone, organic substrates, antimicrobials, or underwater storage (also artificial dams). The use of reactive, neutralizing walls only stops the underground migration of unavoidably released acidity and heavy metals. Often, these systems function well for a while but fail unpredictably some time in the future. Too often, microbiologists have not been consulted in the design of these systems to improve their reliability. The interested reader can find plentiful information on these subjects (61–63).

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See also METALS: MICROBIAL PROCESSES AFFECTING METALS.

BIOLEACHING OF METALS

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Bioleaching is the term used to describe the microbial dissolution of metals from minerals. The commercial bioleaching of metals, particularly those hosted in sulfide minerals, is supported by the technical disciplines of biohydrometallurgy, hydrometallurgy, pyrometallurgy, chemistry, electrochemistry, and chemical engineering. The study of the natural weathering of the same minerals, above and below ground, is also linked to the fields of geomicrobiology and biogeochemistry. Studies of abandoned and disused mines

indicate that the alterations of the natural environment caused by human activities leave microbiological activity as remnants, which continues the biologically mediated release of metals from the host rock known as *acid-rock drainage* (ARD). A significant fraction of the world's copper, gold, and uranium is now recovered by exploiting native or introduced microbial communities. Although some members of these unique communities have been extensively studied over the past 50 years, our knowledge of the composition of these communities and the function of the individual species present remain relatively limited. Nevertheless, bioleaching represents a major strategy in mineral resource recovery, and its importance will increase as ore reserves decline in quality, become more difficult to process (because of increased depth and increased need for comminution), and as environmental considerations eliminate traditional physical processes such as smelting, which have served the mining industry for hundreds of years.

HISTORY OF BIOLEACHING

Microbial oxidation of iron and sulfur present in sulfide minerals is an important part of the natural iron and sulfur geochemical cycles. This biological weathering of sulfides, whether it occurs above or below ground, is frequently evident as surface seeps of acidic water, primarily sulfuric acid that is generated by microorganisms acting on these deposits.

Humans have exploited these biological phenomena for centuries. For example, cementation of copper with iron from naturally occurring acidic leachate in the Rio Tinto (so-called because of the deep red color resulting from dissolved ferric iron; Fig. 1) of Spain appears to have been practiced since the time of the Tartessians 5,000 years ago (1). The Chinese emperor Liu-An (177 to 120 B.C. (2)) describes this process in detail. Recent analyses of Greenland ice cores showed increased atmospheric copper deposition dating back 2,500 years, which is attributable to crude smelting technologies used by the Romans and Chinese (3). Similarly, vestiges of iron and copper mining during Roman times can be observed throughout Wales in the United Kingdom (e.g., Parys Mountain on Anglesey, North Wales; Fig. 2) and across Europe.

The Rio Tinto mine has played a prominent role in the history of hydro- and biohydrometallurgy. It was the first documented site to perform heap leaching on an industrial scale in the 1700s and the first large-scale open pit copper mine was excavated here. Until the connection between the chemolithotrophic bacterium, *Acidithiobacillus ferrooxidans* (formerly known as *Thiobacillus ferrooxidans*; (4)) and acid mine drainage in Pennsylvania (5,6) was established, it was generally thought that the Rio Tinto ore was uniquely suited to heap leaching, and this approach was not applied to low-grade copper ores in the United States. Today it is estimated that 25% of the world's copper is derived from dump and heap bioleaching.

With the association of *At. ferrooxidans* with sulfide mineral leaching, commercial exploitation of bioleaching was quick to follow. Researchers at the Kennecott Bingham Canyon mine near Salt Lake City, Utah, patented a process for biological dump leaching in 1958 (7).

Figure 1. The Rio Tinto, Spain, passing through the historic mining area. Note the ochre-colored water, which is reported to have dissolved ferric iron concentrations exceeding 2 g/L (1). See color insert.

Figure 2. A view of the Roman era iron mines at Parys Mountain, Anglesey, Wales. See color insert.

What began as a process used largely for the recovery of copper from low-grade mine refuse, or as a means of getting value from old tailings, is now a significant operation at virtually all open pit mines in the United States where billions of tons of low-grade ore are treated (8).

Heap leaching, in which ores are crushed to a defined size range, pretreated with acid, acidic ferric iron solutions containing bacteria, or other materials, and then stacked onto impervious pads, is employed to leach copper and recover gold from refractory ores. This strategy is selected to overcome a variety of economical considerations, which can include the low-grade nature of the ore, long distances from smelter facilities that would lead to prohibitive shipping costs, the small size of the projected mine, which restricts the total capital that may be invested at the site, and higher smelter costs (penalties) caused by contaminants in the ore (such as mercury). In some cases, the value of the metal recovered from low-grade reserves can be used to provide additional financial justification for a project (8).

Perhaps the most important development in the bioleaching of sulfide minerals since 1985 has been the

deployment and increasing scale of stirred tank biooxidation plants for the pretreatment of refractory gold ores. Biooxidation is distinguished from bioleaching in that it pertains primarily to the release of particulate gold and silver through the partial microbial oxidation of sulfides in pyrite or arsenopyrite ores hosting the precious metals. The first biooxidation plant for treatment of refractory gold ore was brought on-line at the Fairview site in South Africa by GENCOR Process Research (now Billiton Process Research; commercial operations are marketed by Gold Fields Ltd.) to treat 10 metric tons per day of flotation concentrate. The success of the initial operation led to the plant being expanded to treat the entire mine concentrate production of 40 metric tons in 1991, and the elimination of the previous roaster technology, with substantial environmental and economical benefit (9). Today one of the world's largest contained bioprocesses operates at the Ashanti Gold Fields Obuasi mine in Ghana, where 960 tons of ore concentrate per day have been processed since 1996 (9). The solid commercial success of these operations has established a momentum in the field of biohydrometallurgy, which is evidenced by the current consideration of bioleaching/biooxidation for a wide range of base metals, including copper, zinc, nickel, and lead. The benefits of the current commercial biooxidation plants include significantly reduced capital investment in physical plant, reduced operating costs when compared with smelting (10 to 15% estimated reduction), and stabilization of arsenic-containing waste (in the case of arsenopyrite ores). Knowledge gained from the long-term operating experience with these plants has also raised interesting scientific questions about the microbiology associated with these engineered environments such as what the actual microbial composition of the bacterial populations is during operation, whether thermophilic bacteria are more appropriate to use because of practical considerations such as cooling costs and many others. Most exciting to researchers in the field is the serious commercial consideration being given to bioleaching/biooxidation as an alternative to smelting in the recovery of metals besides gold from ore concentrates.

THE MICROBIOLOGY OF SULFIDE MINERAL LEACHING

Since the discovery of *T. ferrooxidans* (now *At. ferrooxidans*) and its role in the generation of acid mine drainage from pyrite in abandoned coal piles, extensive research has focused on the metabolism, physiology, and genetics of this microorganism. The recent reclassification of *T. ferrooxidans*, *Thiobacillus thiooxidans*, and *Thiobacillus caldus* into the new genus *Acidithiobacillus* (4) recognized sulfur-utilizing species of the genus *Thiobacillus*; these particular species inhabited a unique environmental niche of low pH and high dissolved metal concentrations. Much of our understanding of how inorganic substrates such as Fe^{2+} and sulfur are oxidized by chemolithotrophs has come from the study *of At. ferrooxidans* and *At thioxidans*. It is important to note that although *At. ferrooxidans* is able to oxidize both iron and sulfur, *At. thiooxidans* can only oxidize sulfur species.

The acidithiobacilli are gram-negative, obligately acidophilic, chemolithotrophic proteobacteria in the gamma subdivision of the proteobacteria. Genetically, they resemble other γ-proteobacteria such as *Escherichia coli* in terms of genetic organization and regulation. The physiology (10) and genetics (10,11) of *At. ferrooxidans* have been reviewed in detail recently, and the reader is directed to those excellent reviews for additional details. Although they must inhabit environments in which the pH is below 3.5, it has been determined that these bacteria maintain a circumneutral internal pH by actively pumping protons against the steep pH gradient between the cytoplasm and the external environment (12). Although these organisms have been the focus of intense study in relation to the microbial dissolution of sulfides, another phylogenetically unrelated eubacterium, *Leptospirillum ferrooxidans*, is perhaps even more important in natural and engineered leaching environments.

In 1964, Silverman and Ehrlich (13) published a comprehensive analysis of microbial metal interactions, and suggested for the first time that there must be both an indirect mechanism of microbial leaching of sulfide minerals (in which ferric ion plays a primary role, and where microorganisms accelerate the observed rate of mineral dissolution by regenerating ferric ion) and a direct mechanism, in which the microorganism attaches to the mineral surface, and enzymatically attacks the sulfide. These two mechanisms have till recently been universally accepted as the means by which sulfide minerals are leached, and many kinetic and mechanistic studies have explained observed results in terms of these mechanisms.

In the indirect mechanism, a metal sulfide, MS, is attacked by ferric ion:

$$2Fe^{3+} + MS \rightarrow 2Fe^{2+} + M^{2+} + S^0$$

The resulting ferrous ions could be oxidized by *At. ferrooxidans* (sulfur could also be oxidized by *At. thiooxidans*):

$$2Fe^{2+} + 0.5O_2 + 2H^+ \rightarrow 2Fe^{3+} + H_2O$$

(*At. ferrooxidans*, *L. ferrooxidans*, others)

$$S^0 + 1.5O_2 + H_2O \rightarrow H_2SO_4$$

(*At. ferroooxidans* and *At. thiooxidans*)

According to the direct mechanism, after attachment to the mineral surface, enzymatic oxidation of a metal sulfide occurs as follows:

$$MS + 1.5O_2 + H_2O \rightarrow M^{2+} + 2H^+ + {SO_4}^{2-}$$

Bacterial attachment to the sulfide mineral occurs rapidly, and several recent studies have provided ultramicroscopic examples of such interactions between *At. ferrooxidans* and synthetic pyrite (14,15) using scanning electron microscopy (SEM) or atomic force microscopy, and sphalerite (16) using SEM. In the case of sphalerite (ZnS), the SEM micrographs also show quite dramatically the difference in mineral surface appearance

when the bacteria are present, as opposed to when the mineral is leached chemically (substantial deposits of material "cladding" the mineral surface that were shown to be primarily sulfur, when examined by electron dispersive *x*-ray analysis, EDAX; 16). Attachment has been shown to be a function of the extracellular polysaccharide (EPS) layer of the bacterial cell, and ferric ion has been demonstrated to be bound within the EPS (15). In the direct mechanism, this ferric ion would be reduced to ferrous ion through interaction with the sulfide at the mineral surface, and then recycled by respiratory enzymes at the cell surface (rusticyanin is believed to be a key enzyme in the respiratory chain of *At. ferrooxidans* responsible for the extraction of energy from the oxidation of $Fe^{2+} \rightarrow Fe^{3+}$; 17,18).

Since 1996, a major debate has emerged concerning the validity of the direct mechanism of sulfide mineral bioleaching (16,19–21). Sand's group in Hamburg and other proponents have performed in-depth studies of sulfur intermediates formed during microbial attack of pyrite and other reduced sulfur minerals, and concluded that the sulfur compounds observed during microbial attack on sulfides substantiate only an indirect leaching mechanism that involves Fe^{3+} and the extracellular polysaccharide (EPS) layer of the microorganism. The nature of the sulfur compounds produced during this process are a function of the type of sulfide and whether the sulfide is soluble in acid (19,20). For acid-insoluble sulfides, including FeS_2, MoS_2, and WS_2, ferric ion extraction of electrons from the sulfide requires water to induce corrosion and release sulfur intermediates upon disruption of the metal-sulfur bond. Thiosulfate has been proposed as the initial sulfur compound released from the sulfide, which is rapidly oxidized further to sulfate (thiosulfate mechanism). Acid-soluble sulfides, such as sphalerite (ZnS), galena (PbS), hauerite (MnS_2), chalcopyrite ($CuFeS_2$), and realgar (As_4S_4), are argued to be readily susceptible to metal-sulfur bond dissociation by protons, with the resulting sulfur reacting with ferric ion to produce a reactive sulfhydryl radical that leads to the generation of polysulfides and ultimately, elemental sulfur (polysulfide mechanism). They propose that direct leaching does not actually occur, but rather, the indirect action of ferric ion facilitated by contact of the EPS of the microbe with the mineral surface accounts for the sulfide dissolution historically ascribed to the direct mechanism. Tributsch (22) has suggested that direct leaching should more accurately be termed *contact* leaching. Other researchers employing a suite of microscopic techniques to study the interactions of *At. ferrooxidans* and *At. thiooxidans* with colloidal sulfur and thin films of synthetic pyrite concluded that the chemolithotrophs present at the surface of the sulfides accumulate sulfur colloids within the EPS layer, and release excess sulfur species into the surrounding media that could be used by unattached bacteria (23). This has been termed *cooperative leaching*. Tributsch (22) and colleagues (24) provide a discussion of these various proposed mechanisms, integrated with electrochemical mechanisms associated with chemical and microbial interactions with the sulfide, and environmental conditions to explain the interactions leading to dissolution and the resulting products. There is still much support for the original direct mechanism of Silverman and Ehrlich, however, and ultimately, any postulated mechanisms must support the observed kinetics and energetics of microbial mineral leaching.

A schematic depiction of sulfide leaching, including key features of the direct and indirect mechanisms is shown in Figure 3.

Only since the 1990s has there been a widespread acknowledgment that the acidithiobacilli are merely part of a more complex acidophilic microbial community associated with the oxidation and weathering of pyrites, in which they may in fact only be minor players. From studies of acidic mine drainage (25,26), heap leaches (27–30), and commercial stirred tank bioreactors (31), it has become clear that the iron-oxidizing bacterium, *L. ferrooxidans* (32), is a dominant and important microbe that survives under conditions in which *At. ferrooxidans* is less active. In fact, there is certainly a succession of microbial populations that occur during the leaching of sulfide minerals. Acidophilic, heterotrophic bacteria of the genera *Acidiphilium* and *Acidocella* are often found in close association with *At. ferrooxidans*, *At. thiooxidans*, and *L. ferrooxidans*. The affinity of these organisms for one another is remarkable, and it has been noted several times in the literature that *At. ferrooxidans* cultures are often contaminated with these heterotrophic microorganisms (33–35). The first such contaminant, originally described as *Thiobacillus acidophilus* (33), has since been reclassified on the basis of 16S rDNA sequence and a variety of biochemical markers as another member of the genus *Acidiphilium*, *A. acidophilus* (36). It has long been thought that these heterotrophic species scavenge organic molecules that are metabolic by-products of the chemolithotrophic microbes in acidic environments, which have been shown to be detrimental to the growth of the chemolithotrophs (especially *L. ferrooxidans*; 35). More recently, it has been suggested that increased rates of iron oxidation observed when *L. ferrooxidans* and an *Acidiphilium sp*. are coinoculated on pyrite are due to the heterotroph consuming EPS on the pyrite surface, providing more sites for the chemolithotroph to attach and dissolve the sulfide (20,29). Although the acidophilic heterotrophic bacteria are nutritionally quite versatile as a group (37), it is still not clear what natural form(s) of organic carbon these microorganisms use.

As the temperature of acidic environments increase, *At. ferrooxidans* activity has been noted to decline (>45 °C). In this case, *L. ferrooxidans*, *At. thiooxidans,* and *Acidithiobacillus caldus* are the dominant leaching organisms. The impact of environmental conditions, including temperature, on the dominance of *L. ferrooxidans* in commercial leaching operations has recently been discussed in depth (24). *At. caldus* (38) had been known for some years as a moderately thermophilic sulfur-oxidizing species, which could complement *L. ferrooxidans* (iron oxidation capacity only) in the bioleaching of chalcopyrite concentrate (39). A similar synergistic effect has been seen between *At. caldus* and *Sulfobacillus thermosulfidooxidans* (40).

As the temperature increases beyond 50 °C, moderately thermophilic, gram-positive and gram-variable bacteria

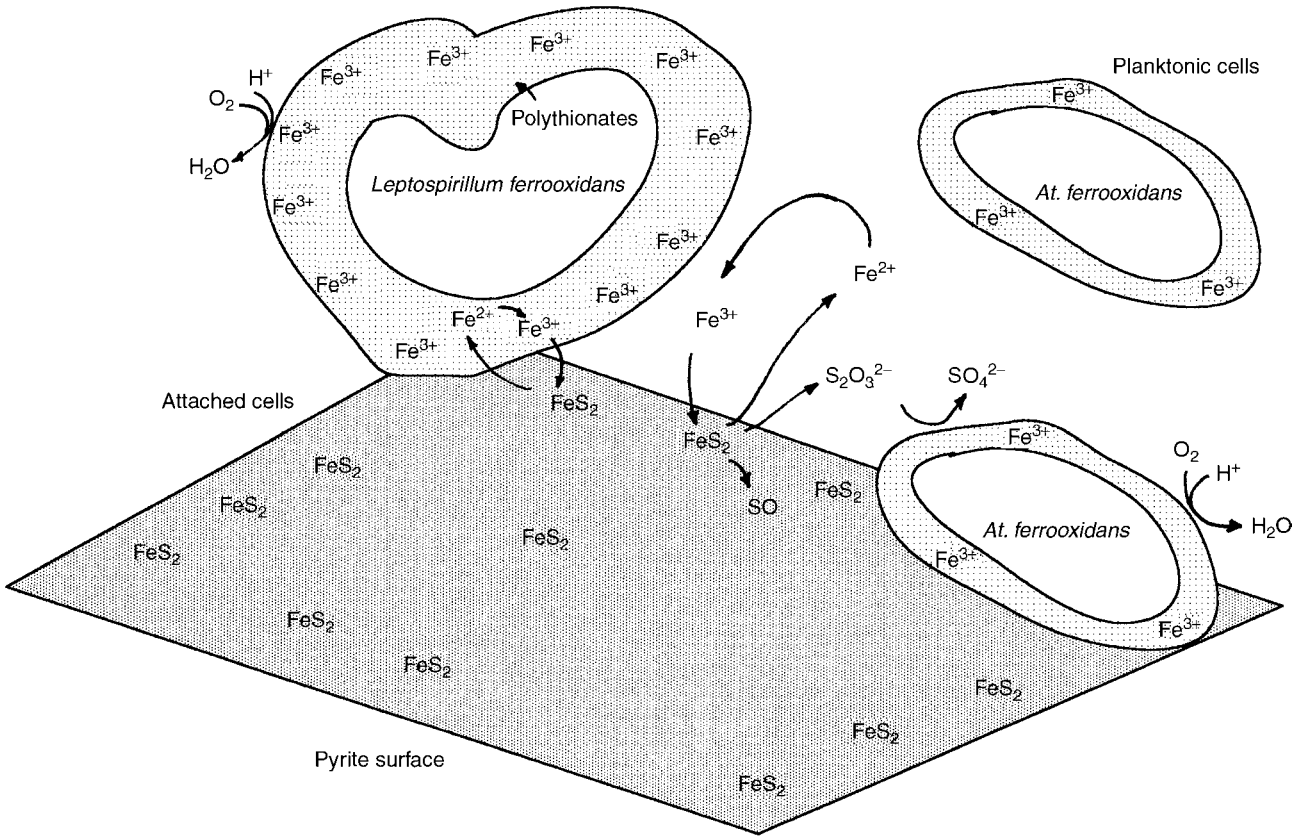

Figure 3. A schematic depiction of the mechanisms of microbial sulfide dissolution, including direct and indirect mechanisms. Cells attached to the surface of pyrite (shaded surface) are meant to depict not only the direct mechanism of sulfide leaching (consuming oxygen through the metabolic activity of the attached cell), but indirect leaching by attached and planktonic cells (such as *At. ferrooxidans*, *L. ferrooxidans* can also oxidize ferrous iron as a planktonic cell, but is not shown for clarity). Attack is either by attached cells, or bulk ferric ion. The lightly shaded cell walls of the various cells depict EPS embedded with polythionates and ferric ion. Note that although thiosulfate is indicated as an intermediate species of pyrite oxidation, it is unstable at pH less than 5, breaking down to sulfite and elemental sulfur.

are observed, including a range of gram-positive organisms comprising the *Alicyclobacillus* group of the high G+C% gram-positive bacteria. These organisms were first isolated from hot pools in Iceland and thought to be thermophilic relatives of *Thiobacillus* because they used sulfur (41). The original TH1 isolate has since been determined to be a strain of *Sb. thermosulfidooxidans* (42). *Sulfobacillus acidophilus* is another iron-oxidizing species in this group. The iron-oxidizing sulfobacilli are capable of growing autotrophically on pyrite, but require carbon dioxide supplementation to achieve reasonable growth rates. They can also grow mixotrophically using both carbon dioxide and glucose, and heterotrophically (43). Other heterotrophic thermophiles appear to be phylogenetically closer to the species *Alicyclobacillus acidoterrestris* and *Alicyclobacillus acidocaldarius*, first isolated by Brock in Yellowstone in the early 1970s. *Acidimicrobium ferrooxidans* is an unrelated eubacterial thermophile that grows readily on iron or pyrite without carbon dioxide supplementation, and which has been shown to promote the growth of *Sb. acidophilus* when both are grown together, presumably by providing organic nutrients to the latter organism (44) as a consequence of its own more rapid growth. There is currently substantial interest in the use of moderately thermophilic bacteria in heap and stirred tank leaching, as these organisms appear to be more efficient in the dissolution of complex sulfides such as chalcopyrite ($CuFeS_2$). Because cooling of stirred tank biooxidation reactors is one of the major costs in the operation of such plants, use of moderate thermophiles would also have an economic benefit, and this alternative is being pursued aggressively (see discussion later in this article).

Beyond 70 °C, chemolithotrophic and heterotrophic archaea dominate acidic mineral environments. Typical of these microorganisms are Crenarchaeota, such as *Acidianus brierleyi* (45; originally identified as *Sulfolobus acidocaldarius*; 46). There was immediate interest in evaluating the utility of these thermophilic microorganisms in bioleaching (47). Since that time, other thermophilic species have been identified and studied in the context of mineral leaching, including *Sulfolobus metallicus* (48,49) and *Metallosphaera sedula* (50). The application of these organisms, particularly in stirred tank bioreactors, has

been hampered by their inherent physiology. Because they lack a conventional cell wall, they are especially susceptible to physical damage from shearing by the mineral particles as the solutions are stirred, effectively limiting the amount of solids that can be introduced.

Figure 4 depicts the spectrum of acidophilic bacteria when considering changes in pH and temperature. There is considerable overlap in populations as environmental conditions change, but in both natural and artificial environments, the shifts in active iron- and sulfur-oxidizing species have been well documented. Organic carbon is indicated in Figure 4 as playing a role in the interactions of leaching microorganisms, whereby autotrophic species may provide substrates for the growth of strictly heterotrophic species. As mentioned earlier, iron oxidation by more rapidly growing species, as in the case of *At. ferrooxidans*, may also stimulate slower growing thermophilic iron-oxidizing species (such as *Sb. acidophilus*) when organic carbon is released into the environment. As was noted before, it is still not clear what form(s) of organic carbon may be involved in these microbial interactions.

Additional dimensions can be included in the consideration of how environmental conditions affect the microbial population of iron and sulfur-oxidizing species. For example, *L. ferrooxidans* has been noted to be more tolerant of high [Fe^{3+}] and lower pH (<1.5) than *At. ferrooxidans*, which explains the larger numbers of *L. ferrooxidans* found in the commercial BIOX® process, heap leaches, and in acidic mine drainage (24,26,27–30). In their comprehensive analysis of the factors that might influence the selection of *L. ferrooxidans* over *At. ferrooxidans* in commercial processes, Rawlings and coworkers (24) took into account the effects of redox potential (*L. ferrooxidans* favored at $> +700$ mV), pH (*L. ferrooxidans* is more tolerant of acidic pH), temperature (*L. ferrooxidans* is active beyond 35 °C), high [Fe^{3+}] (*At. ferrooxidans* is inhibited at ferric concentrations more than 10 times lower than that which inhibits *L. ferrooxidans*), and low [Fe^{2+}] (*L. ferrooxidans* demonstrates a K_m for ferrous iron six times lower than *At. ferrooxidans*).

Recently, Banfield and coworkers (26,51) studying the massive Iron Mountain site in California, have discovered that *L. ferrooxidans* is distributed widely in waters and sediments at the site, but were unable to find significant numbers of *At. ferrooxidans*. They concluded that *At. ferrooxidans* did not play a significant role in pyrite oxidation in this environment. It would be interesting to consider the microbial ecology of this extremely acid environment in light of the discussion of Rawlings and coworkers (24). Further, they have isolated a hitherto unknown species of Archaea, *Ferroplasma acidarmanus* (52), which appears to be a dominant microorganism at the extremely low pH (<0.5) found in the Iron Mountain environment. *Ferroplasma acidarmanus* is not an obligate chemolithotroph, however, because comparable growth rates were observed when the organism was grown on 0.02% yeast extract without added ferrous sulfate. Interestingly, although the total iron concentration measured at Iron Mountain is more than 20 g/L (some locations within the study site have measured iron concentrations exceeding 100 g/L), more than 90% is ferrous iron, which is quite different from many ARD sites, where most of the iron exists as dissolved ferric ion.

The preceding discussion has focused on the dissolution of sulfide minerals by chemolithotrophic acidophilic bacteria and archaea, and associated heterotrophic species. However, the field of biohydrometallurgy has not ignored oxide, silicate, and carbonate ores where heterotrophic microorganisms are likely to be most important to any eventual commercial application. Ehrlich (53,54) has recently provided excellent overviews of our state of

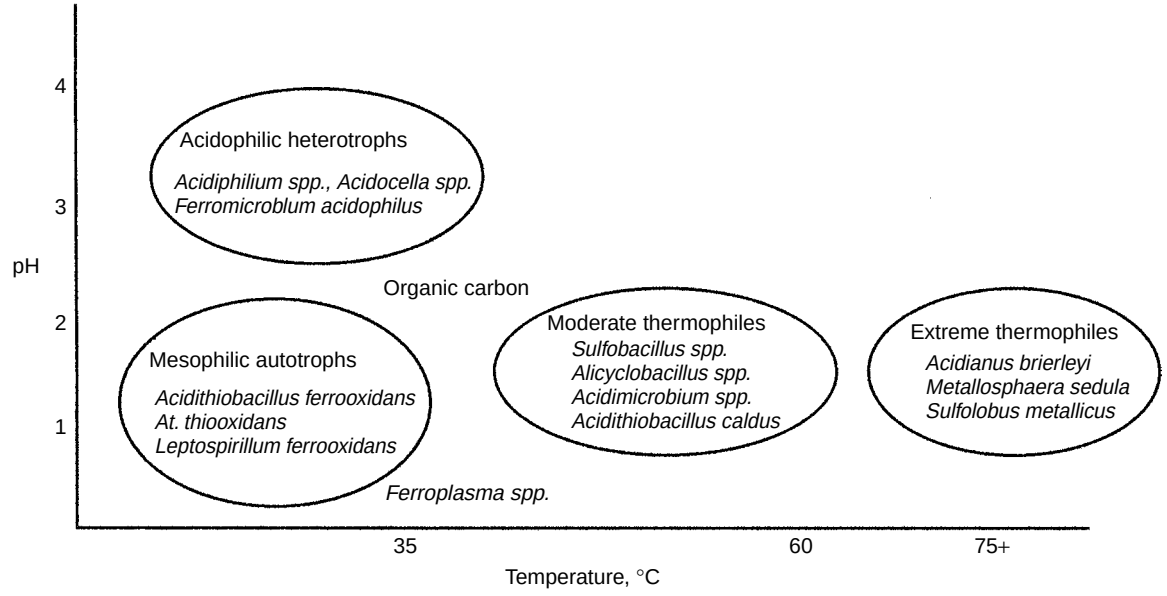

Figure 4. The microbial ecology of acidic environments as a function of temperature and pH.

understanding with respect to microbial attack of these minerals. In many cases, fungi producing organic acids, such as oxalate and citrate, have been demonstrated to leach aluminum ores (55) and lateritic nickel ores (56). Anaerobic bacteria may also be useful in the release of metals from metal oxides (57,58). Heterotrophic leaching is likely to be a more complex process because the ores cannot be sterilized, and environmental conditions are not highly selective as is the case in the leaching of sulfides by obligately acidophilic microorganisms. Inexpensive carbon sources will also be required to facilitate economically feasible microbial oxide leaching (53).

COMMERCIAL PRACTICE

BIOX®

Dew and coworkers (9) recently provided a comprehensive picture of the development of Billiton Process Research's BIOX® process. From a pilot study handling 10 metric tons per day (tpd) to the current full-scale operation in Ghana handling 960 tpd, considerable process knowledge has been obtained to characterize the operating parameters of these plants that have become an economically viable alternative to pressure oxidation and smelting.

The typical plant design is composed of a series of stirred tanks, with up to three being operated as primary reactors in parallel, and the downstream reactors functioning in series. Total residence time of the arsenopyrite flotation concentrate in the reactor train is four days. The ore concentrate is ground to 75 μm particle size (>80%, 100% <150μm) and the solids loading is 20 to 30%. Phosphorus, potassium, and nitrogen amendments are made to provide essential nutrients to the microbial consortium. The temperature of the process is controlled between 30 to 45 °C, with a desired average ceiling temperature of 40 °C to provide some cushion in the case of loss of cooling. Operating pH is maintained between 1.2 to 2.0. Aeration (maintained near saturation; 2.2 kg O_2/kg sulfide consumed) and agitation to maintain the ore particles in suspension account for 30 to 40% of the overall power consumption of the process. After biooxidation, the treated ore concentrate is washed extensively to remove iron and other cyanide consuming materials, and the dried ore is then removed for cyanidation to recover gold. Liquid effluents from the process, containing iron, arsenic, and other undesired metals, are treated with lime to neutralize, precipitate, and stabilize the waste. The solid precipitates are deposited in a tailings impoundment. The overall process is presented in a simplified process diagram in Figure 5.

BacTech

A similar process description has been made for the moderately thermophilic BacTech process (59). The BacTech process is distinguished from the BIOX® process in operating between 45 and 55 °C, where a different microbial consortium, dominated by sulfobacilli, *At. ferrooxidans*, and *At. caldus* have been described. The overall layout of such a plant is very similar to that of the BIOX® process, although special considerations must be made to the materials from which the reactors are constructed, owing to the elevated temperature and low pH of the operating environment. Special grades of stainless steel have been described to be more appropriate for construction of these systems, as compared with the 304-L stainless steel

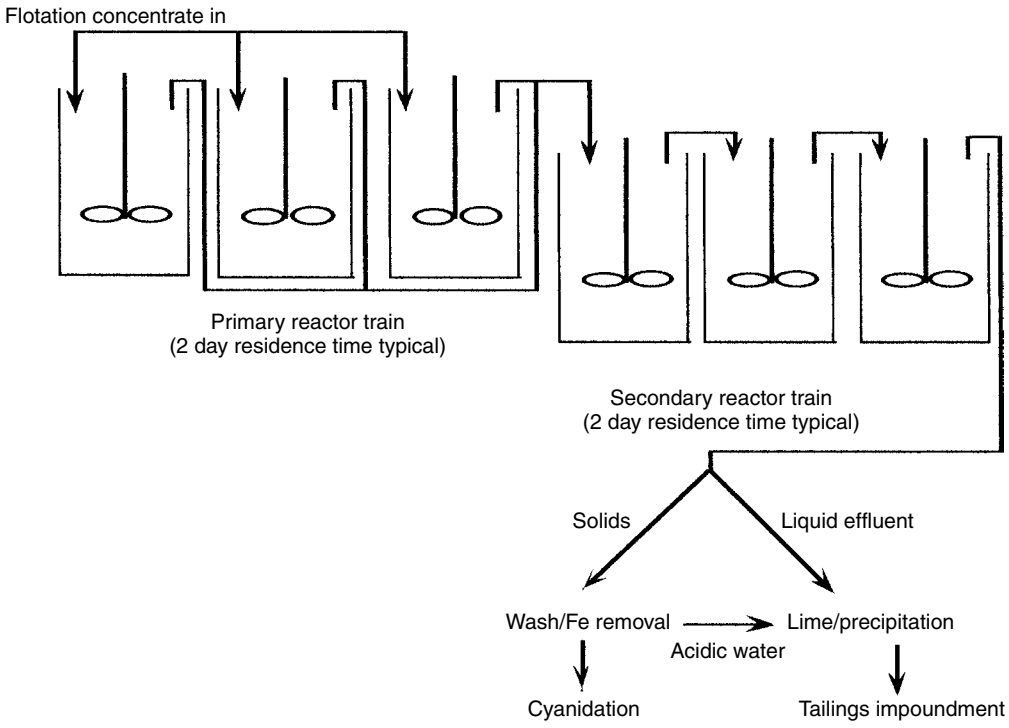

Figure 5. Schematic representation of commercial biooxidation plants in operation today.

commonly used for mesophilic processes. Corrosion resulting from elevated chloride ion appears to be a particular concern. As in the BIOX® process, aeration, mixing, and temperature control are major contributors to the overall power requirements of these plants. It is estimated that 3 to 12 MW of heat is produced in a typical 100-tpd plant. Interestingly, oxygen requirement does not appear to be higher in the BacTech plant, although the solubility of oxygen is lower in water at the higher operating temperatures of this process.

Heap Leaching

Heap leaching is without question the largest scale application of microbial mineral leaching now practiced (dump leaching, in which no attempt is made to optimize particle size is included in this statement). It has been noted that although the principles used in the commercial operation of these plants were developed in North America, virtually all commercial plants have been constructed in the Southern Hemisphere (60). Copper sulfide bioleach plants range from just over 1,000 tpd to over 17,300 tpd at Quebrada Blanca in Chile. At Lo Aguirre, Chile, 16,000 tpd were processed continuously from 1980 to 1996. The use of heap leaching, in which finely ground ore is agglomerated and placed on highly engineered heaps, is now being considered for the biooxidation of refractory gold ores. Brierley (61) has provided extensive details of such a plant that has been designed and tested by Newmont Gold Company. This state-of-the-art gold heap leach operation, with an idea of the scale of such operations, is depicted in Figure 6. The sheer scale of just a single such operation, in which millions of tons of ore are treated, substantiate claims that bioleaching of metals represents the largest bioprocessing application extant. In optimizing these heap leach operations in the future, more understanding of thermophilic bacteria and archaea and their ability to oxidize sulfides will be necessary because, unlike stirred tank reactors for the biooxidation of valuable gold concentrates, temperature control will be limited or nonexistent, and internal heap temperatures are known to exceed 65 °C.

Figure 6. One of the three biooxidation pretreatment heaps measuring approximately 1,000 by 500 feet, with ore stacked to 30 feet, containing 709,000 tons of low-grade refractory ore. Courtesy of Dr. J. Brierley, Newmont Mining Corporation. See color insert.

THE FUTURE

Since the first association of the acidophilic chemolithotrophic bacterium, *At. ferrooxidans*, with sulfide mineral leaching some 50 years ago, there has been a tremendous growth in our understanding of the microbes associated with natural and engineered bioleaching. Commercial practice of bioleaching has grown from a peripheral operation treating marginal ores and waste rock to highly optimized heap and stirred tank processes that handle thousands of tons of valuable ores and concentrates each day. The efforts of many scientists, engineers, and mine operators have resulted in the largest bioprocesses currently practiced. There is a great promise that bioleaching and biooxidation of minerals will increase the efficiency with which the Earth's metal resources are utilized, reduce the impacts associated with the extraction of metals from often deeply buried ores, and replace more energy intensive, polluting technologies. However, there is still much to be learned. Brierley (62) has noted that many of the environmental factors associated with bioleach operations that may influence microbial populations, including acid conditioning of ores, sulfide concentration, internal heat generation, dissolved solutes (Cl^-, NO_3^-, heavy metals, total dissolved solids), aeration, nutrients, and organic carbon, have rarely been considered from the context of the microorganisms operating in the commercial environment. Some modern heap leach operations now incorporate design features, such as aeration systems, to accommodate microbial activity, but there is still much to be learned with respect to optimizing these artificial environments for optimum activity of the biological catalysts present. Even our understanding of the mechanisms involved, let alone the genetic regulation of those mechanisms, is incomplete. The recent partial sequencing of the genome of *At. ferrooxidans* (63) raises the hope that our future understanding and control of these important biogeochemical and commercial processes will be extensive enough to permit the utilization of genomics and bioinformatics in the control of commercial operations.

TERMINOLOGY AND DEFINITIONS

Bioleaching

The liberation of metal values into solution from minerals and ores through microbiological action.

Biooxidation

The liberation of metal values (typically as microscopic particles) from ores. In this process, microbial oxidation of sulfides in the ore leads to deterioration of the rock matrix, and release of the bound metal (e.g., gold), which can then be recovered with increased efficiency through other physical and chemical treatments such as cyanidation and flotation. Often only partial oxidation of the sulfides

present is required to achieve efficient liberation of the metal values.

Dump Leaching

The leaching of run-of-mine ore placed in large piles. Because of environmental regulations, this frequently is done on impervious pads today to prevent escape of the leachate into the environment, but always involves run-of-mine ore.

Heap Leaching

The leaching of ores on prepared pads (geotextile membrane, concrete pad, sometimes including provisions for aeration, fluid collection, etc.) where the ore has been crushed to a particular size range, and may have been coated or mixed with microorganisms and/or nutrients to promote more rapid leaching.

Run-of-Mine

Ore as it is extracted from the mine, which can contain rock ranging from fine particles to hunks approaching 1 ton in weight, and perhaps a meter or more in linear dimension. No attempt is made to fractionate the ore further before other operations (such as dump leaching).

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I am indebted to Dr. James Brierley for the use of the photograph appearing in Figure 6, and to Dr. Henry Ehrlich for his willingness to read a draft of this manuscript and provide suggestions that have strengthened this contribution. The reader is directed to Dr. Ehrlich's authoritative book, *Geomicrobiology*, (4th edition, Marcel Dekker, in preparation) for a comprehensive treatment of this and other topics relating to microbe-mineral interactions.

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BIOLOGICAL CONTROL, USE OF BIOSURFACTANTS IN.

See BIOSURFACTANTS: TYPES, SCREENING METHODS, AND APPLICATIONS

BIOLOGICAL WARFARE.

See BIOTERRORISM

BIOLOGY OF CRYPTOSPORIDIUM

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TAXONOMIC CLASSIFICATION

Cryptosporidium is an obligate intracellular parasite belonging to the phylum Apicomplexa, order *Eucoccidiida*, suborder *Eimeriina*, and family *Cryptosporidiida*. *Cryptosporidium* was first diagnosed as an agent of disease in humans in 1976 (1,2). Since that time, it has been well recognized as a cause of diarrheal illness throughout the world (3). Currently, there are approximately 23 named species of *Cryptosporidium* infecting a wide range of vertebrates, including humans. However, many of these species are considered to be misidentified. There are 10 species commonly recognized today (Table 1; 4–6). These species include five mammalian (*C. parvum, C. muris, C. wrairi, C. andersoni, C. felis*), two avian (*C. meleagridis and C. baileyi*), two reptilian (*C. serpentis, C. saurophilum*), and one fish (*C. nasorum*). Speciation of isolates, in accordance with the practice for other coccidians, has primarily been based on host specificity, oocyst morphology, and site of infection. However, these traits have been shown to be insufficient to accurately identify separate species of *Cryptosporidium*. Much of the information, regarding host specificity has been based on a limited number of studies

Table 1. Named Species of Cryptosporidium and Their Hosts

Species	Hosts
Cryptosporidium baileyi	Chicken
Cryptosporidium meleagridis	Turkeys, immunocompromised human
Cryptosporidium muris	Mice
Cryptosporidium felis	Cats, immunocompromised human
Cryptosporidium serpentis	Reptiles
Cryptosporidium saurophilum	Skunk
Cryptosporidium nasorum	Fish
Cryptosporidium wrairi	Guinea pig, immunocompromised human
Cryptosporidium andersoni	Cattle
*Cryptosporidium parvum**	Cow, mouse, human

*Appears to be infectious for 152 species of mammals (6).

using a small number of *Cryptosporidium* isolates that render this information inconclusive (7,8). Finally, *Cryptosporidium* has been shown to infect numerous sites in the body including the upper and lower respiratory tracts, the uterus, bladder, and also the liver and pancreatic systems, rendering the site of infection a relatively useless tool to describe a species (9).

Two different genotypes of *Cryptosporidium parvum* have been shown to be infectious in humans (10). Genotype-1 isolates have been shown to be infectious only in humans, while genotype-2 isolates have been shown to be infectious in mice, calves, lambs, goats, and horses, as well as humans. This suggests the possibility that there are two distinct populations of oocysts cycling in humans with distinct transmission cycles; (*1*) zoonotic transmission from animal to human with subsequent human to human and human to animal transmission and (*2*) a transmission cycle exclusively in humans.

BASIC BIOLOGY

The environmentally stable stage of this organism is known as an oocyst. The oocysts of *Cryptosporidium parvum*, the species infecting humans, are 4 to 6 μm in size and have been shown to survive in the environment for extended periods of time, depending on temperature and water quality conditions because of the robust nature of the oocyst wall (11). The oocyst wall of *C. parvum* and related species appears to be composed of three layers when viewed by electron microscopy (7,12–14). The inner layer is relatively thick and is thought to be a filamentous glycoprotein (11,15). The middle layer is thought to be a more rigid layer composed of a complex lipid, whereas the outer layer is thought to be composed of acidic glycoproteins (11). Spanning one-third to half of the oocyst wall is a linear suture, which under the proper conditions opens to release the four internal sporozoites that are capable of initiating infection within the host.

REPRODUCTION

The reproductive life cycle of *Cryptosporidium* is complex, with both sexual and asexual reproduction cycles being completed within a single host (16, Fig. 1). Infection begins with the ingestion of an oocyst. Once ingested, excystation (release) of the four sporozoites from the suture line located along the side of an oocyst begins. Sporozoites once released from an oocyst may penetrate individual epithelial cells located in the region of the ileum and begin reproduction.

The initial phase of reproduction begins when the parasite attaches itself to the host cell by means of a feeder or attachment organelle that appears to be used to facilitate the exchange of materials. The sporozoites and all subsequent lifecycle stages are intracellular beneath the host cell membrane, but are extracytoplasmic. Once the sporozoite enters the host cell, it begins to differentiate into a spherical trophozoite with a prominent nucleus. The division of this nucleus signals the start of asexual reproduction known as *merogony* or *schizogony*.

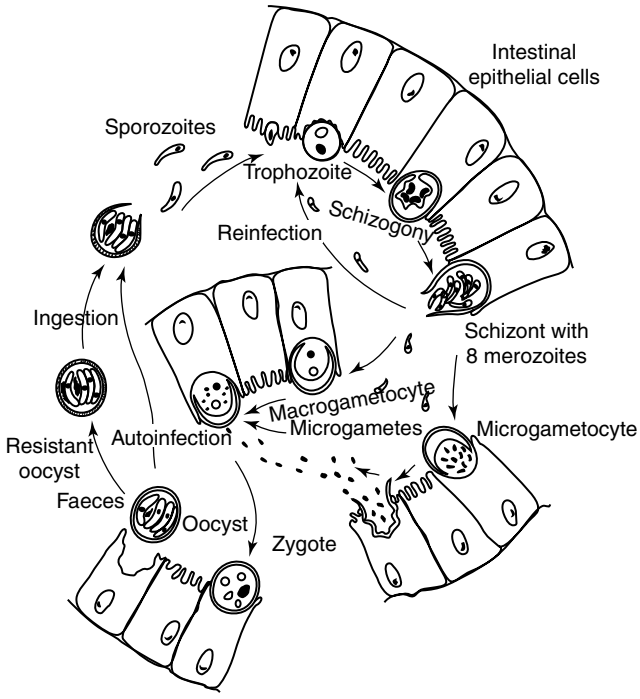

Figure 1. Reproductive life cycle of *Cryptosporidium parvum*.

Two morphologically different types of schizonts have been observed. Type-I schizonts contain six to eight nuclei. When the schizont matures, each nucleus becomes incorporated into a merozoite. Each merozoite can either develop into another Type-I schizont or a Type-II schizont, which will produce 4 merozoites. Merozoites formed from Type-II schizonts initiate sexual reproduction known as *gametogony*. During gametogony, the merozoite can invade other host cells where they may differentiate into either microgametes or macrogamonts (male and female stages). When the microgamont develops, it becomes multinucleate. Each nucleus, eventually, becomes incorporated into the equivalent of a sperm, which will fertilize the macrogamont. After fertilization, the macrogamont develops into an oocyst. After sexual reproduction is complete, the oocysts will be released in excreted feces. Approximately 80% of the oocysts excreted in the feces are thick-walled oocysts. These oocysts have multilayered walls that allow them to remain infective outside of the host. The remaining 20% of the oocysts produced are thin-walled oocysts that can rupture within the host, releasing their sporozoites and initiating an autoinfectious life cycle. Infected hosts may excrete between 10^9 and 10^{10} oocysts per day during the course of the disease (17).

CLINICAL ASPECTS

The incubation (or prepatent period) time between ingesting oocysts and shedding of oocysts varies among species. In humans, the average incubation period ranges from 5 to 10 days with a mean of 7.2 days (18–20). Oocyst shedding can last anywhere from 3 to 15 days and may continue well past the cessation of symptoms (18,19,21).

The severity and duration of the disease will vary greatly, depending on the status of the host's immune system (22). In immunocompetent individuals, symptoms including fever, malaise, nausea, and diarrhea typically lasts 10–14 days. Immunocompromised patients, such as patients with AIDS, patients undergoing immunosuppressive therapy, those with immunoglobulin deficiencies, or with concurrent viral infections affecting immunity may suffer a prolonged, life-threatening illness either because of dehydration or secondary complications or both.

Chemotherapy is currently not available for treatment of this disease, although more that 100 therapeutic agents have been studied. Supportive therapy, including the use of intravenous fluids is considered to be the treatment of choice. Treatment of HIV-infected patients may include the use of antiretroviral therapy, including the use of protease inhibitors to improve the immune status of the patient. This treatment regime has been shown to result in both clinical and parasitological improvement in the infected host (23).

Studies to determine the number of oocysts necessary to cause infection in humans have produced differing results. The first human-feeding study in healthy volunteers suggested that the infectious dose ID_{50} or the dose that is required to establish infection in 50% of the persons exposed was approximately 132 oocysts, although one recipient was infected with as few as 30 oocysts (24). A similar study using three geographically diverse isolates of genotype 2 showed a variance in the ID_{50} of 9–1042 oocysts (25). The variability between infectious doses is most likely because of the susceptibility of the host and the virulence of the particular isolate. Interestingly, the isolate with the lowest ID_{50} also had the highest illness attack rate (86% versus 50–55%) (25).

PREVALENCE OF CRYPTOSPORIDIOSIS IN HUMANS

Because cryptosporidiosis infections are generally self-limiting and often symptomatically similar to other diarrheal diseases, the disease may often be undiagnosed or misdiagnosed in the absence of a recognized outbreak. Incidence of *Cryptosporidium* infections in the population range from 0.6 to 20%, depending on the geographical locale with a peak incidence of the disease in children one to five years of age with no sex related disposition (26). Approximately 60% of all positive stool samples come from children with 30% coming from adults less than 45 years of age (26). Although clinical infection is uncommon over the age of 40, it does occur from time to time, and there is currently no evidence of increased incidence in the elderly.

Most early reports of human cryptosporidiosis were single sporadic cases or small clusters of cases. In 1983, population surveys began to be reported from Australia, Finland, the United Kingdom, and numerous other countries (23,27–33). Unfortunately, many of these surveys were not well controlled with data being derived from selected populations, using specimens that had been routinely submitted to the laboratory. Furthermore, accurate identification of the parasite could not be assumed in all studies. For these reasons, the information produced by these surveys may not provide an accurate assessment of the prevalence or incidence of the disease, and they should be viewed with caution. Further analysis of data compiled from 40 countries between 1983 and 1990, excluding outbreaks, suggests that the prevalence rates in industrialized countries of North America and Europe are between 1 and 3% and the rates in developing countries are approximately 5% in Asia and 10% in Africa (34).

It has been estimated that *Cryptosporidium* accounts for 13 to 16% of diarrheal cases in AIDS patients in developed nations, with that number climbing to 24 to 50% in developing nations (35,36,26,24,4). A prospective long-term study in Europe suggests that 3 to 4% of person with HIV will have cryptosporidiosis when diagnosed with HIV, and that an equal number of them will develop it, during the course of their disease (37). A study in Los Angeles in 1994 suggests that 3.4% of HIV patients in the USA will become infected with cryptosporidiosis during their symptomatic period (38).

Several studies have noted a temporal or seasonal peak in levels of cryptosporidiosis. However, these tend to vary from country to country, including summer in Australia, rainy season in Central America and India, spring or late summer in North America, and late summer in Germany (26). These trends may reflect rainfall patterns, farming events such as calving and lambing, or fertilization practices. However, outbreaks still remain unpredictable.

OCCURRENCE IN THE ENVIRONMENT

Cryptosporidium oocysts have been detected in 4–100% of surface waters sampled at levels of 0.0012–5800/L, depending on the impact from sewage and animals (39–41, Table 2). A survey performed by LeChevalier in 1991 found 87.1% of the surface water sites sampled positive for *Cryptosporidium* with maximum concentrations ranging from 10 to 484 oocysts/L, with the highest levels being found in the Mississippi, Ohio, and Missouri Rivers (43). A similar study by Rose in 1991 noted a tenfold difference in oocyst densities between urbanized and pristine watersheds (43). In pristine watersheds, concentrations ranging from 0.003 to 0.29/L have been detected (39).

Groundwater, previously thought to be a more protected water source, has shown between 9.5 and 22% of samples positive for *Cryptosporidium* (44). A similar survey of groundwater in the United Kingdom (258 samples) revealed a slightly lower percentage of positive samples (5.8%) (40). A U.S. survey of wells that were shown to be coliform-positive was also shown to be contaminated with *Cryptosporidium* oocysts at concentrations of 0.004–0.922/L (39). Outbreaks of cryptosporidiosis caused by the contamination of a drinking water well have been documented on four occasions in various geographical areas (Texas, Pennsylvania, Yakima, Washington, and Walla Walla, Washington). Two of the outbreaks (Pennsylvania and Yakima, Washington) were associated with shallow wells where the ground water was suspected of being under the influence of surface water. The outbreaks in Texas and Walla Walla, Washington, were due to the direct contamination from wastewater and irrigation water, respectively (45).

Table 2. Occurrence of Cryptosporidium in the Aquatic Environment. Adapted from H.V. Smith and J.B. Rose, *Parasitol. Today* 14, 14–22 (1998).

Country	Number of Samples	% Positive Samples	Oocysts/L
In surface waters			
USA	181	55	0.0025–44
USA	85	87	0.007–484
USA	35	97.1	0.18–63.5
USA	11	100	2–112
USA	101	24	0.005–252.7
UK	262	40.5	0.006–2.3
UK	403	15	0.0012–0.12
UK	375	4.5	0.07–2.75
UK	691	52.2	0.04–3
UK	375	4.4	0.07–2.75
UK	196	39.9	0.006–15.6
Germany	9	78	not stated
Spain	8	50	<0.01–0.31
Netherlands	52	11.6	0.002–0.018
Australia	114	46.5	0.1–14.3
Israel	16	68.8	0.006–0.52
Malaysia	76	10.5	2–245.6
In drinking water			
USA	36	17	0.005–0.017
USA	82	26.8	not stated
UK	15	7	0.006
UK	209	37	0.007–1.36
Spain	9	33	<0.01–0.02
Brazil	18	22.2	not stated
In wastewater effluents			
USA	11	100	4–3,960
USA	130	not stated	up to 0.05
USA	60	67	<0.6–120
UK	50	74	1–321
UK	70	37	0.03–2.3
UK	117	65	5–60
UK	94	25.5	10–60

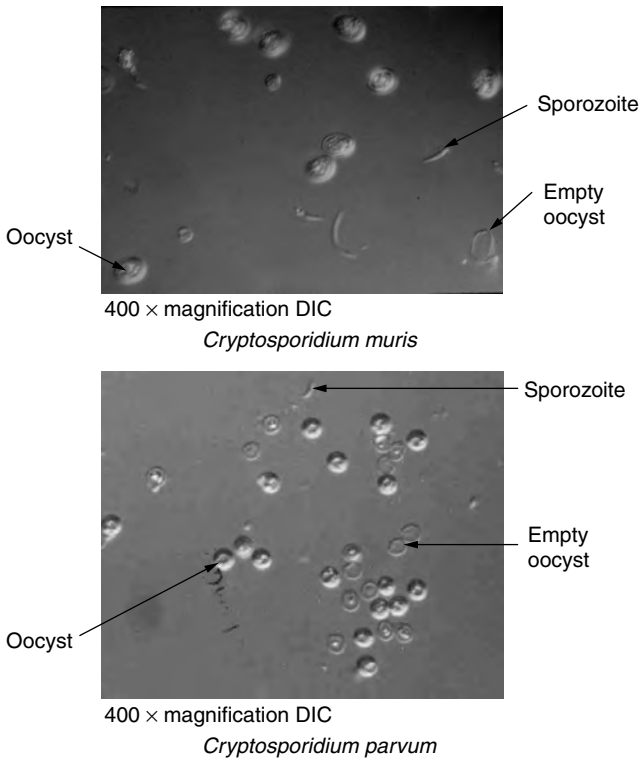

Figure 2

Cryptosporidium oocysts by nature are very robust and have been shown to survive for extended periods of time in the environment. They have also been shown to survive in both human and cattle fecal material, which can protect them from desiccation. Therefore, the practice of disposal of both human and animal waste, by land application may lead to indirect contamination of water supplies or food crops. Large amounts of contaminated fecal material can also provide a source of viable oocysts that may contaminate surface or groundwater through infiltration or run-off during periods of peak rainfall. Once deposited in receiving waters, oocysts have been shown to survive in river water up to six months with a die-off rate of 94% after 176 days (46).

The effects of both high and low temperatures on the survival of *Cryptosporidium* have been investigated using animal infectivity analysis (47). Inactivation greater than 99.9% can be achieved by raising the temperature to 70 °C for 1 min. These findings suggest that *Cryptosporidium* oocysts can be rendered noninfectious when held for relatively short times at temperatures far below boiling. A similar level of inactivation was shown at extremely low temperatures. When oocysts were held at −15 °C, they gradually became noninfectious and after a period of 7 days no infectious oocysts could be detected. At −20 °C and −70 °C a >99.99% inactivation could be achieved after 8 h and 1 h, respectively (48).

TRANSMISSION

Several factors related to the genus *Cryptosporidium* have contributed to their high incidence of disease in the population. These factors include the organism's ability to complete its lifecycle in a single host, the robust, environmentally stable infective oocyst stage and the large number of infectious oocysts excreted from infected hosts. These factors along with the broad host range for this organism increases the potential for waterborne, foodborne, person-to-person, and also zoonotic transmission.

Waterborne Outbreaks

Numerous waterborne outbreaks of *Cryptosporidium* both in the United States and throughout the world have been documented (6,40). Outbreaks have been attributed to all water types including contaminated rivers, lakes, springs, and groundwater sources. The first reported outbreak in the United States occurred in 1984, in Braun Station, Texas, with 368 cases identified (49).

This was followed by a larger outbreak in 1987 in Carrollton, Georgia, with an estimated 13,000 cases of cryptosporidiosis (50). The largest reported drinking water outbreak of cryptosporidiosis in the United States took place in Milwaukee, Wisconsin, in 1993, with an estimated 403,000 cases — 4,000 hospitalization and several deaths (51). The outbreak was caused by oocysts that had passed through the filtration system of the city's southern water treatment plant. It has been hypothesized that heavy spring rains and runoff from melting snowfall led to the contamination of the city's source water (Lake Michigan) with agricultural wastes (51,52). This outbreak was estimated to have cost the community $53 million in lost wages, lost productivity, medical bills, and emergency room visits. Further, the outbreak is said to have cost upward of $100 million in claims for loss of life (53).

Although treatment failures have been documented to have occurred during outbreaks situation, numerous waterborne outbreaks of *Cryptosporidium* have occurred in communities within the United States where evaluation of the water treatment plant during outbreak periods revealed no violations for turbidity, chlorine concentration, or coliform levels (54). Because of the small size of this organism (4–6 μm), the optimization of filtration for the removal of oocysts during drinking water treatment is extremely important. Similarly, the use of alternative disinfectants that have been shown to inactivate *Cryptosporidium* oocysts, such as ozone and ultraviolet light, are also important factors to be considered for the prevention of drinking water outbreaks.

Although the source of contamination during an outbreak often goes undetected, suspected sources of contamination include wastewater and heavy rainfall events, which can lead to increasing agricultural and other nonpoint source inputs. Although the agricultural runoff from cattle and sheep raising areas have been repeatedly implicated as sources of contamination during waterborne outbreaks both in the United States and abroad, only once has the bovine genotype been identified as being present, during an outbreak within North America. This outbreak occurred in Cranbrook, British Columbia, with approximately 2,000 cases of cryptosporidiosis. The bovine isolate (genotype 2) was detected in human fecal specimens, in cattle manure specimens found near the watershed, and in water samples collected from the reservoir (10).

Recreational Outbreaks

Several recreational outbreaks of gastrointestinal illness from *Cryptosporidium* have been reported during recent years, both in the United States and abroad (Table 3.) Fecal accidents from a person with current or recent diarrhea were suspected in most of the outbreaks. Infected individuals may continue to excrete large numbers of infectious oocysts for weeks after symptoms cease (55). Attack rates during outbreaks have been estimated to be as high as 78% (56). Although there have been documented outbreaks of cryptosporidiosis associated with freshwater sources, many more cases have been associated with swimming pool exposures (57). This is not surprising because a single fecal accident can input high number of oocysts into a confined body of water, causing an immediate risk to the pools occupants. Further, the oocysts are extremely resistant to chlorine levels used in conventional swimming pools and their small size enables them to pass through filter systems, which are not operating optimally.

Table 3. Outbreaks of *Cryptosporidium* Attributed to Recreational Exposure

Date	Number of Outbreaks/Facility	Reference
1988	2/pool	(58,59)
1990	1/pool	(56)
1992	2/pool, 1/wave pool	(60,61)
1993	4/pool	(62,63)
1994	2/ pool, 1/lake	(64–66)
1995	1/pool, 2/water park	(67)
1996	1/water park, 2/pool, 1/lake	(68)
1997	1/river, 2/pool, 1/fountain	(69)
1998	4/pool	(70,71)
1999	1/fountain	(57)

Foodborne Outbreaks

Although *Cryptosporidium* appears to be spread more often by a waterborne route, contamination of food products either by infected food handlers or through the use of contaminated processing or irrigation waters has been documented (72–75). It has been suggested that the high prevalence of *Cryptosporidium* species in some food animals increase the risk for contamination of other food products.

In nonindustrialized countries, the use of excreta as fertilizer or the use of irrigation water that has been contaminated with industrial, domestic, or agricultural wastes can also lead to increased risk of foodborne cryptosporidiosis, especially from consumption of raw vegetables (76). In a study in Costa Rica, oocysts from *Cryptosporidium* species were detected in 5.0% of cilantro leaves, 8.7% of cilantro roots, and 2.5% of lettuce samples (77). In Peru, a survey of vegetables collected from small rural markets detected the presence of *C. parvum* oocysts in 14.5% of the samples that were collected (78). Although these studies reveal the presence of oocysts in raw produce, it is often impossible to detect the presence of *Cryptosporidium* oocysts in food products because of the lack of sensitive detection methods and the potential for inhibition of detection when using molecular methods. Similarly, the long incubation period and potential for person-to-person transmission of the disease make epidemiological investigation of potential foodborne outbreaks of cryptosporidiosis extremely difficult.

The first foodborne outbreak of *Cryptosporidium* in the United States was reported in 1993. The outbreak was associated with fresh-pressed apple cider (72). An environmental survey revealed that the apples had been collected from uncultivated trees on the edge of a pasture where cows had recently been grazing. The apples were harvested both from trees and from the ground. The apples were then stored overnight and sprayed with municipal water from a hose on the morning that the

cider was prepared. Frozen samples of the cider revealed the presence of *Cryptosporidium* oocysts at levels of 375 to 750 oocysts per liter. There were 160 primary cases of cryptosporidiosis with a 15% rate of secondary household illness transmission.

Three other outbreaks have been reported since then, one was associated with chicken salad, one with green onions, and the third was also associated with apple cider (73–75). Suspected foodborne transmission of *Cryptosporidium* has also been reported from travelers visiting Mexico, the United Kingdom, and Australia. Suspected foods include salad, raw milk, sausage, and tripe. It has been proposed that livestock infection and water contamination are the two major causes of food contamination with *Cryptosporidium* oocysts (26).

Cryptosporidium oocysts have currently been detected in shellfish such as oysters, mussels, clams, and other bivalves that were harvested from both U.S. waters and abroad (79,80). Studies have shown that shellfish are capable of concentrating pathogenic bacteria, virus, and parasites from the large volumes of water that they pass over their gills. Because many of these filter feeders are consumed raw, they can present a significant public health risk to consumers. Currently, no outbreak of cryptosporidiosis caused by the consumption of raw shellfish has been reported.

Zoonotic Transmission

Zoonotic transmission of the organism is well recognized, and domestic cattle have been implicated in both direct (fecal–oral) and indirect (via water) outbreaks of human infection (24,27). Seasonal peaks in the detection of *Cryptosporidium* oocysts in the environment tend to coincide with lambing and calving, in spring and autumn. Although symptomatic infection in animals generally occurs only in the very young, adult animals such as deer, horses, pigs, and sheep may excrete low number of oocysts (81). These adult animals may provide a reservoir for transmission of this organism to the young of their own species and also to humans. Studies conducted by the U.S. Department of Agriculture, Animal Plant Health Inspection Service, and Veterinary Services (82) have found *Cryptosporidium* species commonly in dairy and beef calves in the United States. It has been estimated that this parasite is present in more than 90% of the dairy farms and nearly 40% of the beef operations.

Companion animals, such as cats and dogs, have also been implicated in the transmission of cryptosporidiosis to humans on rare occasion (5,26). However, they may represent an important reservoir for human infection (83). A recent study using genetic sequencing of the small subunit ribosomal RNA (SSU–rRNA) has identified the first human cases of infection by a canine and a feline species of *Cryptosporidium* in AIDS patients (84). Transmission by arthropods and by houseflies and wild-filth flies that were exposed to feces containing Cryptosporidium oocysts has been documented (85–87).

Secondary spread of cryptosporidiosis through person-to-person transmission has been well documented within families, in day care centers, and also in other institutional settings (88–91). Day care centers have also been linked to transmission of cryptosporidiosis through contact with contaminated toys, surfaces, and other fomites (89). Although the main route of infection remains the fecal-oral route, *Cryptosporidium* oocysts have been detected in sputum and vomit, and these may provide alternate modes of transmission.

CONCLUSION

Cryptosporidium has been endowed with a number of physical and physiological characteristics that have enabled it to become an important cause of waterborne disease throughout the world. Although our body of knowledge, concerning the biology and epidemiology of this organism continues to grow, we have only recently gone beyond the tip of the iceberg with regard to understanding this organism's place in the microcosm of waterborne disease.

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BIOLUMINESCENCE, METHODOLOGY

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Bioluminescence is the capacity of certain living organisms to emit visible light. The species that exhibit bioluminescence are found in many environments, but especially in the deep sea. They have achieved bioluminescence through different evolutionary processes. The responsible genes in bacteria, algae, coelenterates, beetles (fireflies), and fish are all unrelated to one another (4). The fundamental biochemistry of light production in these organisms consists of the oxidation of a luciferin, a cellular substrate, under the catalysis of luciferase enzymes, which produces, as a decay product of the chemical reaction, an almost heatless, bluish green light until the excited-state molecules return to the ground state. The luciferin substrates and the structures of the luciferase enzymes vary by organism, as do the mechanisms controlling the speed and intensity of the luminescence. Certain cofactors, such as the nucleotide ATP, may need to be present for the conversion of the substrate to take place (5).

Because these reactions can be replicated outside the organisms to which they are native, bioluminescence has proven extremely useful for research at the cellular and molecular levels (6). The availability of instrumentation that can measure the light emitted in these reactions with great sensitivity and dynamic range has made them powerful tools for biochemical and clinical analysis because the involved components can be detected at a very low level (7). Compared with other techniques, such as colorimetric or spectrophotometric indicators, bioluminescent analysis offers the advantages of high sensitivity, wide linear range, low cost per test, and relatively simple and inexpensive equipment (8). Because of their sensitivity, immobilized bioluminescent reagents have come into widespread use in biochemical analysis over the last 20 years.

Firefly and bacterial luciferases are the two most commonly used bioluminescent systems in research (5). The latter is the light-producing reaction of the North American firefly *Photinus pyralis*, which is also the most extensively studied bioluminescent system. Firefly bioluminescence, for example, allows the presence of the ATP nucleotide, which supplies energy to cells for many important biochemical processes, to be determined in reactions with extreme sensitivity (7). Although most enzyme assays yield either a product or the disappearance of a substrate, firefly luciferase acts as a quantifiable reactant rather than as a catalyst, whose most significant and easily measured product is light (9).

Bioluminescence has proven important for a wide range of disciplines central to the study of human disease, including hematology, immunology, bacteriology, and pharmacology, and clinical chemistry (8). More generally, it has stimulated studies in analytical biochemistry, molecular biology, cell-based assays, and various environmental applications (10).

Specific examples of bioluminescent-facilitated research include work with pathogens such as the *Mycobacterium tuberculosis*, which not only pose biosafety hazards but grow very slowly in laboratory conditions. An enhanced luciferase-expressing mycobacterial strain has been used to evaluate antimycobacterial activity in mice (11), permitting evaluation of drugs, with significant savings in time, labor, and expense. A pharmaceutical application, in vivo tracking of cells and monitoring of gene expression (12) has broad applications to the study of infectious disease. Currently, this technology has developed into widely applied methods for monitoring biological events noninvasively in living animals.

IN VIVO BIOPHOTONIC IMAGING

In vivo biophotonic imaging is a noninvasive means of tracking pathogens, tumor cells, or molecular events in live animal subjects that exploits the light-emitting properties of photoproteins such as luciferase enzymes. Typically, animal models for biological assessment represent late or end stages of a disease, usually require sacrifice of the animal, and employ cumbersome ex vivo assays to reveal the disease process. Moreover, much contextual information is lost that an in vivo analysis might yield, because late stage protocols, more distressful to experimental animals, are likely to miss important information about events in the early stages of a disease and from intact systems.

Biophotonic imaging holds great promise for the acceleration of data generation and analysis for the development of new drug candidates by the pharmaceutical industry.

This is because 80% of drug candidates fail to satisfy safety and efficacy requirements in clinical trials, and the need to conduct biological assessments in animal models to determine safety and efficacy is the principal obstacle in making the development process more efficient and effective. In vivo biophotonic imaging offers a rapid means of conducting in vivo analysis to improve the predictive quality of data used in the assessment of new drug candidates. The technology provides data sets from relevant, intact animal systems, increasing both the efficiency and the effectiveness of selecting new drug candidates for clinical development. Because it is an in vivo technology, fewer animals are needed, whereas time and costs are reduced and more data per protocol are obtained.

Before the advent of biophotonic imaging, noninvasive in vivo assays were based on magnetic resonance imaging (MRI), positron-emission tomography (PET), and X-ray (computerized tomography) technologies. Although these methods are useful for monitoring structural changes due to disease processes and, on occasion, the response to

therapy, they required relatively expensive devices that are not practical for use with most animal models and very long scan times. Moreover, specific contrast agents that could reveal the cellular and molecular changes relating to the disease processes were not readily available.

In vivo biophotonic imaging technology is based on the observation that light is transmitted through living tissue with efficiencies suitable for use in monitoring both structure and function. The external application of light as a biological monitor currently has both routine clinical uses (e.g., pulse oximetry) and novel experimental applications in medicine. Biophotonic imaging, however, employs photoproteins, internally emitted biological sources of light, to tag such biological functions as infection and gene expression (12–16).

Use of in vivo luciferase monitoring in living mammals was first described in 1995 with an infectious disease model, gastrointestinal infections by *Salmonella typhimurium* (12). This study indicated that the technique was not only feasible but that it yielded quantitative data that provided more information in less time than could be obtained from conventional assays. Because a large variety of bacterial species can be labeled with luciferase, this approach is generally applicable to microbiological investigations (see Fig. 1, Gastrointestinal infection with *S. typhimurium* and treatment with ciprofloxacin). Ongoing research is showing that mammalian cells can be labeled in the same way as bacterial species, indicating that the method is applicable to the evaluation of genetic- and cell-based therapies, anticancer drugs, studies of gene expression and development.

A significant advantage of in vivo luciferase monitoring is that bioluminescent tags allow for an integrated approach in which the same label can be used for in vitro, for quantitation and in cell culture correlates of in vivo processes, and then in vivo to test predictions made in vitro. In this way, a predictable animal model with highly correlative data can offer validation of an in vitro assay approach. Several parameters of drug efficacy and pharmacokinetics can be monitored in the same animal over time, yielding benefits for the discovery and development stages of drug evaluation.

In animal studies, the number of subjects required is reduced, and the issues of animal-to-animal variation are overcome by the zero time point functioning as an internal control, improving biostatistics. In bacterial models, bioluminescent tags are useful for distinguishing infecting pathogens from normal flora.

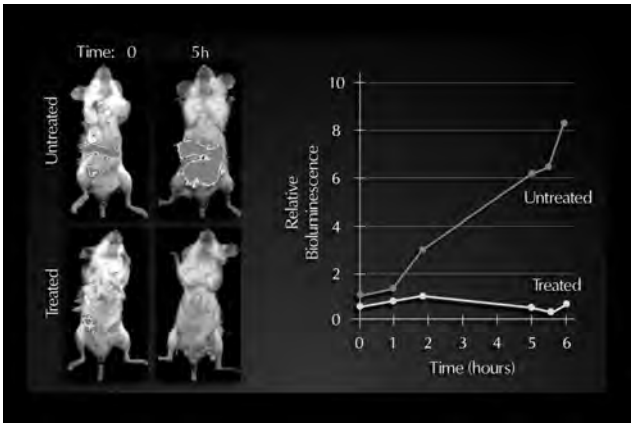

Figure 1. Gastrointestinal infection/Salmonella. The pseudocolor image is a color depiction of the intensity and quantity of photons with red being most intense and violet least intense. Untreated and treated mice are depicted at time zero (eight days after oral inoculation) with SL1344 lux *S. typhimurium*, but before treatment with ciprofloxacin. The same mice were imaged five and a half hours after treatment with ciprofloxacin (untreated control-received injection of phosphate-buffered saline). The graph shows relative bioluminescence intensity measured from the abdominal area at the time after treatment for both treated and untreated animals. See color insert.

METHODOLOGY

Labeling Bacterial Cells

Lux operons are ideal for labeling bacterial pathogens because they not only encode the luciferase genes but also the genes for the biosynthetic enzymes (fatty aldehyde synthases) for the substrate decanal. Use of these operons obviates the need for an exogenous supply of substrate. Few deleterious effects of substrate biosynthesis on bacterial metabolism or virulence have been observed. Among the *lux* operons from bioluminescent bacteria, which from *Photorhabdus luminescens*, previously known as *Xenorhabdus luminescens*, appears to be ideally suited for use in mammalian models (17,18), given that mammalian body temperatures lie within the temperature optimum for this enzyme (19–21), unlike beetle luciferases (*Luc*) and other characterized bacterial luciferases (from the genus Vibrio) that have low-temperature optimum.

Well-characterized vectors are used for the expression of the *lux* operons in gram-negative organisms. The original pUC-based plasmid used to clone the *lux* operon from *P. luminescens*, including the promoter region (vector designated pCGLS1) is well suited for expressing luciferase from common gram-negative organisms such as *Escherichia coli* and *Salmonella* (19,21,22). Standard methods of bacterial cell transformation for these organisms are used to introduce the *lux*-encoding vector into cells of these bacterial species.

Bacteria may be labeled with luciferases from either a bacterial or eucaryotic source (as shown in Fig. 2). Several arrangements for the expression of luciferase have been used, namely, the *lux* AB genes from firefly with luciferin as the substrate, the *lux* AB genes from *Vibrio* or *Photorhabdus* with decanal as substrate, or finally the entire *lux* operon from *Photorhabdus* with no exogenous substrate requirement.

Transposon-based vectors have been used to introduce *lux* genes modified for optimum expression in both gram-positive bacteria, such as *Staphylococci* and *Streptococci*, and gram-negative organisms outside the *Enterobacteriaceae*. The in vitro sensitivity for the labeled bacterial strains is approximately 10 to 100 bacterial cells (*Salmonella*, LB5000 lux) that yields a signal in culture over background, regardless of whether an absorbing and scattering biological medium such as blood is present. The lower limit of detection in some in vivo models is 1,000 bacterial cells.

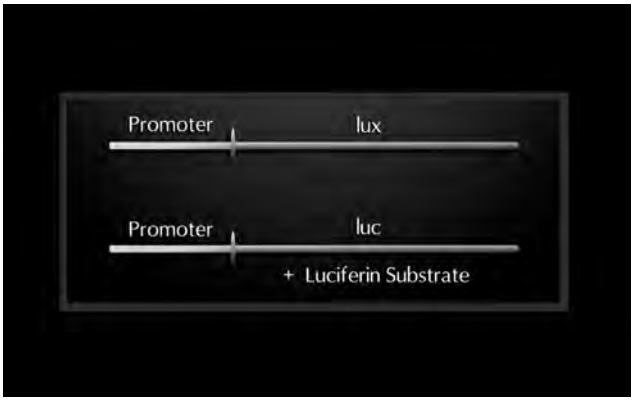

Figure 2. Prokaryotic expression. For expression of light from prokaryotic cells, a construct is used that includes the *lux* operon from *P. luminescens* in an appropriate gram-negative or gram-positive plasmid vehicle. More recent constructs include transposons for chromosomal integration. Expression of the *luc* gene from the North American firefly may also be used in prokaryotes as long as the luciferin substrate is administered. See color insert.

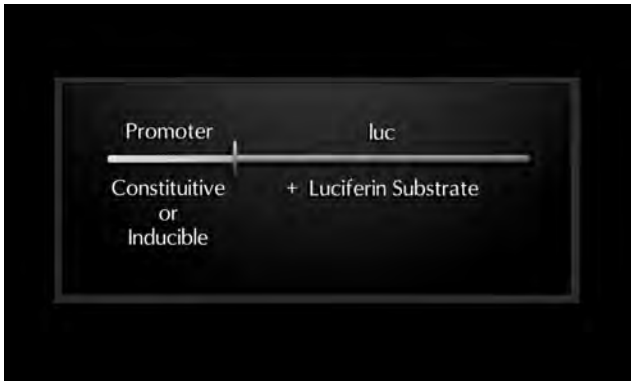

Figure 3. Eukaryotic expression. Expression of light from Eukaryotic cells may be accomplished with the *luc* gene of the North American firefly regulated by an SV40 viral promoter. Luciferin is administered introperitoneally as the substrate for the enzymatic reaction. See color insert.

Cell culture correlates for pathogenesis and expression can provide a rapid means of evaluating tagged events before introduction into living animals. Although correlates have not been established for some systems (e.g., propagation of human papillomavirus), when they are available, they can accelerate the development of in vivo assays and, in combination with luciferase tags, the same reporter can be used in vitro, in vivo, and finally as an ex vivo assay to confirm in vivo observations. In the case of bacterial infections, such assays have been described for many organisms, and some have been adopted for use with bioluminescent reporters, including for microscopic detection. In the case of mycobacterium, use of luciferase as a reporter can significantly accelerate in vitro assays and such labeled strains have also begun to be used in vivo.

Cultured Mammalian Cells

Reporter genes, such as firefly luciferase or variants of green fluorescent protein (GFP), in transformed cells can be employed to reveal the molecular and cellular features of cancer. In a recent study (23), tumor cells were labeled with pGL3, a luciferase gene driven by the SV40 viral promoter. Eucaryotic expression of the firefly luciferase in both cultured tumor cells and transgenic animals is accomplished by placing a constitutive or inducible promoter in front of luciferase, transfection of the vector into the chromosome, and then finally, the light from luciferase expression in stable transfectants is seen following the addition of luciferin (see Fig. 3).

Determination of Luciferase Activity Ex Vivo and In Vitro

A commercially available assay kit that includes acetyl CoA to prolong light emission can be used to determine luciferase activity for the firefly enzyme expressed in mammalian cells for tissue homogenates. Tissues are removed and homogenized with a tissue disrupter in minimal volumes. A lysis buffer containing luciferin, ATP, and acetyl CoA is added to samples and read in a standard luminometer.

For cells in culture, 10 μl of a 15 mg/mL stock solution of luciferin (per 1 mL of medium) is added to cells for a final concentration of 150 μg/mL. Cells are imaged immediately after substrate addition. Bioluminescent signals are apparent in these cultures hours later. Increased bioluminescent signals are obtainable by increasing the substrate concentrations. An ultrasensitive camera is used to measure the signals in living cells. The cells can be lysed and luciferase activity measured in a luminometer with the standard assay procedure if the signals produced, are too weak to be detected by camera.

Substrate Delivery to Tissues

An aqueous solution of the substrate, luciferin (50 mM), is injected into the peritoneal cavity 20 minutes prior to imaging for systemic delivery at a dose of 126 mg/kg. Alternatively, substrate can be applied topically (50 mM) in 100% DMSO for transdermal delivery to monitor luciferase expression in the skin. Luciferin has also been electroporated (ionophoresis) into the skin of the ear, using a caliper electrode connected to an electroporator. The duration of detectable luciferase activity in tissues varied with the different modes of delivery. With introperitoneal (IP) delivery, peak activity is detected at 20 minutes in the brain and at five minutes in other tissues with proximity to the peritoneal cavity.

Anesthesia

For imaging, mice and rats were anesthetized using pentobarbital (35–70 mg/kg body weight) (12). Low doses are used with neonatal mice and rats sensitive to the effects of pentobarbital. Animals are observed carefully while under anesthetic and their respiration is monitored. Following the imaging, the animals are kept warm and allowed to recover.

Imaging

Anesthetized animals need to be placed in a light-tight box. A gray-scale body surface image is collected

for reference in low light. Even the smallest light leaks can present significant background when using sensitive imaging devices and long integration times. The potential sites for light leaks are the door closure, camera mount (if camera is mounted externally), and the entry point for the cables (if camera is mounted internally).

Photons emitted from luciferase within the animal, and then transmitted through the tissue, are collected and integrated for 5 to 30 minutes. A pseudocolor image representing light intensity (generally blue representing least intense and red representing most intense light) is generated on an image processor. The images are transferred using a plug-in module for image processing software to a microcomputer. Gray-scale reference images and pseudocolor images are superimposed using the image software, and saved as PICT files. Superimposed images are opened within a graphics software package and composites made and annotations added. An intensified charge coupled device (CCD) camera, described in the following section, outfitted with a 50 mm f 1.2 lens and an image processor, has been successfully used in research studies.

Use of the CCD Camera

Low light imaging systems employ technologies that either reduce the background noise or increase the signal. By cooling the CCD chip in a video camera to temperatures of about $-30\,^{\circ}C$ the background caused by infrared irradiation (so-called *thermal noise*) can be reduced permitting detection of low light signals. An alternative is to specifically increase the signal using an image intensifier. Intensifiers are designed around a high voltage microchannel plate technology that amplifies signals in the form of electrons. A photocathode is used to convert photons to electrons that are then amplified over the microchannel plate; the increased number of electrons contact a phosphor screen generating photons that are then focused on a CCD video camera. The materials used in the photocathode determine the spectral sensitivity of the intensifier. To reduce thermal noise blue-sensitive materials can be employed. Alternatively, thermal noise on the intensifier is reduced by cooling intensifiers with photocathodes made of materials that are sensitive to longer wavelengths of light-cooled intensified cameras.

In a comparison between a cooled camera and an intensified camera, essentially equivalent signals are obtained from neonatal Tg mice expressing luciferase in their eyes and skin. In adult mice with bioluminescence originating from deeper tissues, differences have been apparent between cooled intensified systems (sensitive to light $\leq$850 nm) and the C2,400-32 ICCD camera (sensitive to light $\leq$600 nm). This is likely because of a suspected filter effect of the tissues in which shorter wavelengths of light are more readily absorbed by mammalian tissues, and the longer wavelengths of light (luciferases have rather broad spectral peaks of up to 50 mm bands) may preferentially pass through tissues and more red-sensitive instruments may be well suited for collecting this light.

Applications and Discussion

In vivo monitoring of luciferase is widely applicable for tracking pathogens and other disease processes, yielding more information in less time than conventional assays. Noninvasive monitoring of light emitted from within a living mammal, in which the light is constitutively expressed or is reporting fluctuations in gene expression, is a platform technology that results in increased information about specific physiological processes as well as integrated, whole biological systems. The monitoring of biological light requires less time and fewer animals, reducing the cost of analyses. This is especially relevant to the costly processes of drug discovery in which significant advances have been made in generating large numbers of potentially useful therapeutic compounds (e.g., combinatorial chemistries, genomics, and high-throughput screening).

In the absence of significant advances in in vivo analyses, the bottleneck in drug development is at the animal model step, and improvements in the earlier steps only accentuates this problem. In vivo luciferase monitoring addresses this limitation (16) with broad applications.

In studies of infectious disease, for example, the in vivo biophotonic monitoring technology can be superimposed over current methodology to perform faster experiments, and use fewer animals, while still extracting relevant data that provides more information relating to early stages of disease and patterns of disease.

In Figure 4, a lung model illustrates a high level of bacteria in the lung and nasopharangeal area that can be specifically characterized as pneumonia, with the lungs clearly outlined. In Figure 5, a typical model used to evaluate antibiotics, the soft tissue thigh model, can now be used as a fast screen for effective antibiotics. Biophotonic imaging allows this model to be run in half the time of the traditional thigh model. In Figure 6, labeled tumor cells allow researchers to follow the expansion and regression of tumors in response to therapy. This is especially important as tumor diagnostics leap ahead and

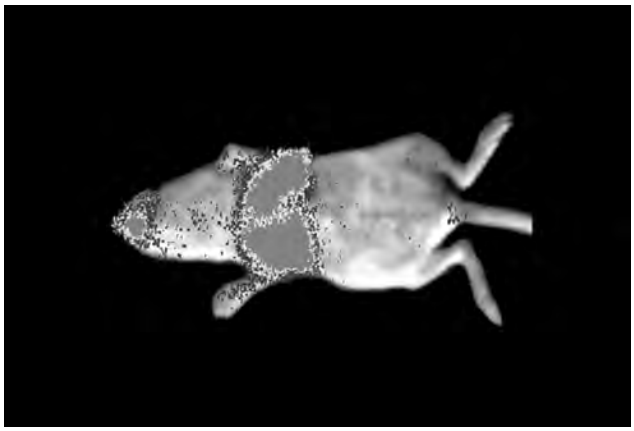

Figure 4. Lung model. Neonatal mice (under two weeks of age) are susceptible to lung infection following oral inoculation with *S. typhimurium* SL1344 lux. A nasopharyngeal infection is common in both adults and neonates from this mode of inoculation. See color insert.

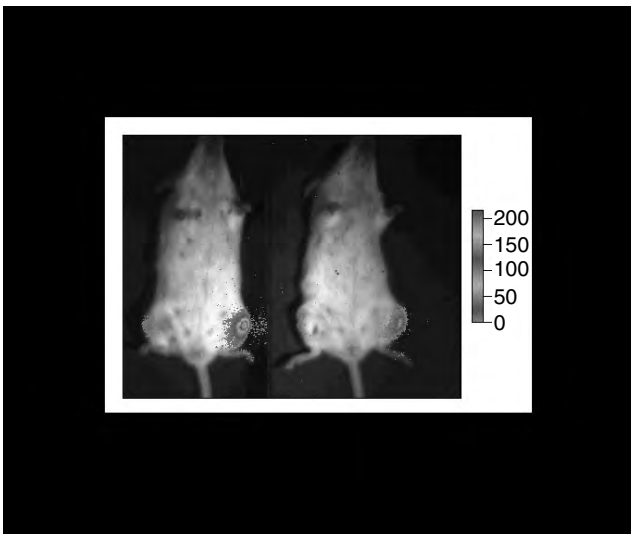

Figure 5. Soft tissue thigh model *Lux* tagged *E. coli* are injected at 10^4 bacteria/mL (right thigh) and 10^5 bacteria/mL (left thigh). Bacteria can then be quantified noninvasively with and without antibiotic treatment. See color insert.

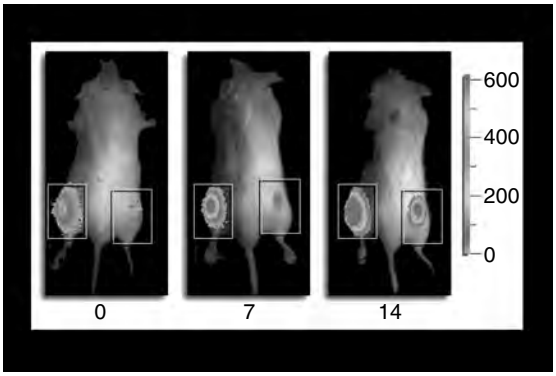

Figure 6. Labeled tumor cells. Labeled NIH 3T3 cells were injected with matrigel at zero time [10^4 cells (right thigh) and 10^5 cells (left thigh)] and mice were imaged between 7 and 14 days to monitor the increase in cell number, as demonstrated by the increase in red pixels of the pseudocolor image. See color insert.

we need tumor therapies that provide earlier intervention in the disease process. Virtually the first steps in the disease can be monitored, including metastatic disease.

Light-producing transgenic animals have allowed scientists to follow gene expression spatially and over time. The power of this technology rests in the ability to measure from baseline to maximum gene expression in one animal over time and during the changing conditions of disease state, therapy, and toxic events. We can now begin to understand the relationship between the level at which a gene is expressed in any given animal and the concomitant change in phenotype. Thus, it is possible to have surrogate markers of gene expression for specific biological events.

In a recent study, a transgenic animal was created using the promoter for hemooxygenase (also known as *heat shock protein* (HSP) (32)) placed in front of luciferase. Thus, because the endogenous gene was not altered, events that

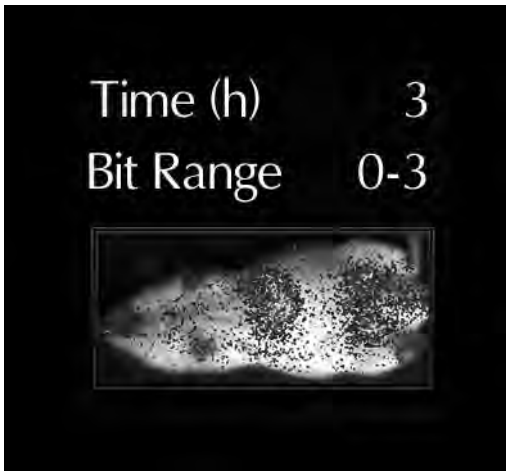

Figure 7. Transgenic animal exposed to heavy cadmium. A mouse line transgenic for a cadmium-regulated element, the hemooxygenase promoter, demonstrates in vivo monitoring of cadmium induction of *luc* in front of the *luc* gene. 10 mM cadmium was delivered IP and the mouse was imaged at zero and three hours. The three-hour time point with a camera sensitivity set at a bit range of zero to three is shown here. See color insert.

affect gene expression can be monitored noninvasively. This transgenic animal was exposed to the heavy metal cadmium, which is known to induce gene expression, and three hours after exposure, the liver and testes show a specific increase in gene expression (see Fig. 7). The changes in gene expression were monitored and it was demonstrated that they occurred in a dose-dependent fashion. By Northern analysis it was also demonstrated that HO protein was present.

CONCLUSION

It has been demonstrated that in vivo biophotonic imaging can deliver higher throughput animal models for a variety of pharmacological screens. The variety of animal models that this technology can be applied to is very broad and includes bacteria (gram-negative and gram-positive) and tumor models. Increasing the efficiency of the drug development process, although laudable, is only a small part of the story.

Gene sequencing has made it possible to assess the relatedness of species. More recently, there have been increases in our ability to group genes into families and to draw possible conclusions about relatedness of function. But what remains elusive is a true understanding of pathways, system connections, and the multifunctionality of genes in metabolism and development. This is because our in vitro assays are essentially two-dimensional; pathways may be connected but total systems, such as the central nervous system, and the immune system are not part of the assay. Current animal models are less robust than desired because of artifacts of ex vivo analysis and end points that do not reveal the biological process. In vivo biophotonic imaging, however, allows real-time data to be collected noninvasively; thus, the animal subject functions as its own internal control. This means that investigators

are no longer limited to a snapshot of biological data but now can watch the actual process of metabolism and disease.

Bioluminescent-based assays have also shown promise as a means of rapidly and reliably measuring biological and chemical contamination of the environment and for detecting pathogens that are undetectable by standard techniques, yet still threatening to human health (5). Other recent environmental applications include luciferase-based measurement of water contaminants (24), luminescence-facilitated detection of mercury in natural waters (25), and firefly luciferase-tagged bacteria in environmental samples (26).

The ability to collect quantitative data that preserves spatial and temporal context with extraordinary sensitivity opens new avenues for exploring in situ various environmental mechanisms of growth, colonization, and community structure and organization. Using a bacterial biosensor (27,28) that produces light in the presence of a particular signal would be very helpful in isolating crucial molecular communication among bacteria. We may speculate that this knowledge, along with two to three-dimensional organizational structure, would give us a clearer picture of microbial communities in nature.

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BIOMARKERS, LIPID. See LIPID BIOMARKERS IN ENVIRONMENTAL MICROBIOLOGY

BIOMASS, ALGAL. See PLANKTONIC ALGAE IN THE MARINE ENVIRONMENT

BIOMASS, BACTERIOPLANKTON. See PLANKTONIC MICROORGANISMS: BACTERIOPLANKTON

BIOMASS: SOIL MICROBIAL BIOMASS

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Soil microbial biomass is an important component of soil that regulates many processes associated with energy transfers and nutrient cycling. These functions are critical to maintaining ecosystem productivity at all levels of the food web. The soil biomass is composed of a wide range of microorganisms including viruses, bacteria, fungi, microfauna, and macrofauna. The soil microbial biomass expresses functions to take advantage of the multitude of soil niches composed of different habitats and substrates. These functions range from pathogenesis, symbiosis, and heterotrophic to chemoautotrophic activities.

The soil microbial biomass is a component of the soil that is considered to be part of the active fraction. The active fraction includes the microbial biomass, recently deposited plant residues, root exudates, and easily degradable portions of the soil organic matter such as light fraction, which are thought to play a prominent role in nutrient cycling and major energy transfers (1). In most soils, the soil microbial biomass comprises about 5% of total soil carbon and about 1% of total soil nitrogen (2). The microbial biomass is most active in the surface soil where most of the recent plant and litter inputs, mainly from above and belowground production and turnover, provide substrate (food) for microbial activity. Deeper soil horizons contain less plant input, and therefore a corresponding decrease in microbial population size and activity. However, in most soils, microbial biomass is present at all soils depths to the depth of the bedrock including deep sediments of 1,000 feet or more (3).

The most important function of the soil microbial biomass is decomposition of organic material. During decomposition, the microbial biomass releases nutrients from plant litter and gains energy for metabolic processes. Without this function, dead plant material would accumulate and limited nutrients would be available for plant uptake. Depending on the quality of litter, the microbial biomass either utilizes the nutrients (immobilize) or if there are excess of nutrients, they become available (mineralize) for other organisms or plant uptake. In this respect, the soil microbial biomass acts as a source and sink for nutrients in the soil. For these reasons, the soil microbial biomass is the main regulator of nutrient cycling in the soil, and therefore, regulates net primary production of ecosystems.

The importance of the soil microbial biomass goes beyond nutrient cycling. One of the important by-products of decomposition is the formation of stable organic matter. The soil organic matter, through its interaction with minerals, serves many functions that increase soil quality through enhancement of physical, chemical, and biological characteristics of the soil matrix. An important consequence of an increase in soil organic matter is the storage of vital plant nutrients, such as nitrogen, phosphorus, sulfur, and trace metal elements. The diversity of soil microbial biomass leads to other important biogeochemical processes, which regulate gaseous flux of carbon, nitrogen, and other nutrients. In contrast, other functions of the soil microbial biomass, such as the production of growth regulators, can serve to be detrimental or enhance plant growth. The amazing diversity of the soil microbial biomass is the foundation of a complex ecosystem component that regulates the productivity of the earth's biomes. This article will concentrate on the soil microbial biomass, which is composed mainly of bacteria and fungi and will emphasize carbon and nitrogen cycling in soil.

HABITAT OF THE SOIL MICROBIAL BIOMASS

Soils are formed through the dissolution of primary minerals and subsequent reformation of secondary minerals, such as clays and sesquioxides. The secondary minerals are rich in ion exchange activity and form stable complexes with organic matter. The interaction of minerals and soil organic matter lead to the formation of soil structure through the creation of aggregates and porosity. This complex three-dimensional matrix produces a wide variety of habitats in which the soil microbial biomass exist. The three-dimensional matrix is composed of solid, liquid, and gas phases. Characteristically, a well-developed soil contains 50% solids and 50% pores. The pores in the soil matrix contain the soil solution and air. The ionic exchange capacity of the minerals act to adsorb nutrients required for the growth of the soil microbial biomass and act as a surface to exist on. The wide array of habits in soil creates a complex predator-prey interaction, which is unrivaled compared to aboveground ecosystems (Fig. 1).

Soil microorganisms inhabiting larger pore areas are subject to grazing by larger organisms, such as protozoa and nematodes. However, soil organisms living in larger spaces normally have access to a greater food supply through the movement of the soil solution and exploration by plant roots. Microorganisms inhabiting smaller areas, such as capillary spaces, are protected from predation by larger organisms. However in these confined spaces, microorganisms are often subject to oxygen limitations

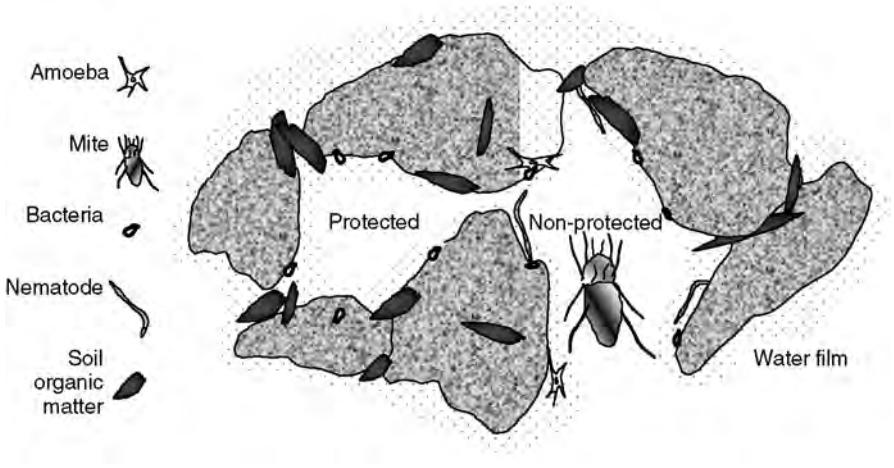

Figure 1. Depiction of the soil habitat showing soil structure and the interaction of organisms inhabiting various niches. The trophic level interactions depict protected and nonprotected regions of the soil matrix.

and substrate availability. The variety of habitats is directly responsible for influencing the immense diversity of the soil microbial biomass. Through long-term adaptive strategies microorganisms have adapted to these specialized niches in the soil.

The ecological theory that examines the distribution of species based on substrate availability and growth involves the concept of r and K selection (4). A soil microorganism adapted to bountiful energy and nutrient sources are designated as r-selected. Microorganisms existing under low energy and nutrient deprived conditions are termed K-selected. Selection pressures and physical environment would be unique to r- and K-selected organisms leading to diversity of function (5). The K-selected organisms would strive to produce a high growth- rate per unit of substrate because food supply would be erratic in the smaller capillary pores or protected spaces in the soil. The r-selected organisms on the other hand would put much energy into competitiveness to be able to survive the predation pressures, which exist in the larger soil pore spaces. This interesting ecological hierarchy has led to the notion of protected and nonprotected soil microorganisms (1). The concept has been widely used in simulation efforts to describe microbial activity in soil. It is believed that K-selected or protected organisms live mainly dormant existences until exposed to a new food supply. Predation pressure, substrate availability, and specialized habitats lead to selection of microorganisms with specific functions creating a complex nutrient cycle for all of the essential elements in soil.

THE COMPOSITION OF THE SOIL MICROBIAL BIOMASS

The soil microbial biomass is composed of a large number species that vary widely in their function. The soil microbial biomass is generally considered to be composed primarily of bacteria and fungi. Bacteria and fungi normally compose over 70% of the total soil biomass (Table 1). Faunal components make up a significant portion of the total soil biomass and also contribute to carbon cycling and other biogeochemical processes. The importance of the microbial and faunal components of the soil biomass to soil processes can often lead to a contentious debate among scientists. The size of soil microbial biomass and all of its major components is described in Table 1. (see SOIL BACTERIA and SOIL FUNGI: NATURE'S NUTRITIONAL NETWORK, this Encyclopedia, for more information on these soil organisms).

Table 1. The Mass of Soil Biomass Components in Soil

Soil Biomass Component	Tonnes Per Hectare
Bacteria	1 to 2
Fungi	2 to 5
Actinomycetes	1 to 2
Protozoa	Up to 0.5
Nematodes	Up to 0.2
Earthworms	0 to 2.5
Other fauna (collembola, mites, arthopods etc.)	Up to 0.5

Source: K. Killham, *Soil Ecology*, Cambridge University Press, New York, 1994.

Because they represent the majority of the soil microbial biomass, bacteria, and fungi are considered responsible for the majority of the energy flow and nutrient cycling that occur in soil. However, the faunal component of the soil biomass is responsible for influencing the size of the soil microbial biomass through predation thus influencing their ability to process plant litter and soil organic matter. Soil fauna also increases microbial substrate availability by physically burying plant litter in the soil. Larger soil fauna are also responsible for reducing the size of plant litter thus increasing its surface area, making it more accessible to the soil microbial biomass.

The functional diversity of the soil microbial biomass is required to be able to take advantage of the wide array of plant materials and habitats found in the soil. The soil represents an oligotrophic environment, becoming more nutritionally limited as the depth of soil increases. In the soil surface, soil aggregates also represent oligotrophic environments. For example, the interior of an aggregate may have extremes in pH, aeration, and redox potential. The soil has a wide variety of these niches, sometimes called hot spots, producing an array of microhabitats,

which maintain the immense diversity of the soil microbial biomass.

DISTRIBUTION OF THE SOIL MICROBIAL BIOMASS

The soil microbial biomass requires energy, nutrients, and habitat to exist. The soil horizon containing the most organic matter, nutrients, and plant influence normally contains the most soil microbial biomass. Soils are typically composed of distinct layers or horizons formed from the depositional, eluvial, and illuvial processes. The surface soil horizon, called the A horizon, is generally rich in organic matter as a result of plant litter deposition and root turnover. Eluvial and illuvial processes and plant root exploration deposits organic matter and nutrients in deeper soil horizons. Deep sediments and buried soils can also contain significant amounts of organic matter, however the bulk of the organic matter is usually located in the A horizon. Beneath the A horizon is the B horizon, which often contains appreciable amounts of eluviated clay or other amorphous minerals, depending on soil age. Beneath the B horizon is the C horizon containing unweathered parent material. Having additional or not well-defined horizons can often complicate soil horizon determination. The A horizon, because of the large organic matter input from the above- and belowground plant production, forms a friable structure because of a wider range of aggregates sizes promoting good aeration and moisture holding capacity. For these reasons, the greatest number of microorganisms exist in the A horizon (Fig. 2).

There are a number of exceptions in which the number of microorganisms can be influenced by soil characteristics such as the type of mineralogy, ecosystem, and water regime. A young or highly weathered old soil may influence the number of microorganisms because of low organic matter content. An example of this type of soil is a tropical soil, which is highly weathered and contains most of its organic matter in a quickly decomposed litter layer. Conifers forests and bogs, often produce acidic soil affecting the number of microorganisms in the upper soil layers. Extreme climatic conditions produce soils with prolonged dry (arid) or wet (aquic) regimes. Arid soils can accumulate salt and/or sometimes sodium causing high electrolyte potential in the soil solution thus affecting the number of microorganisms. On the other hand, in wet climates the landscape often contains areas with frequent or sustained high water tables leading to lower aeration and thus a decrease in the activity of microbial biomass.

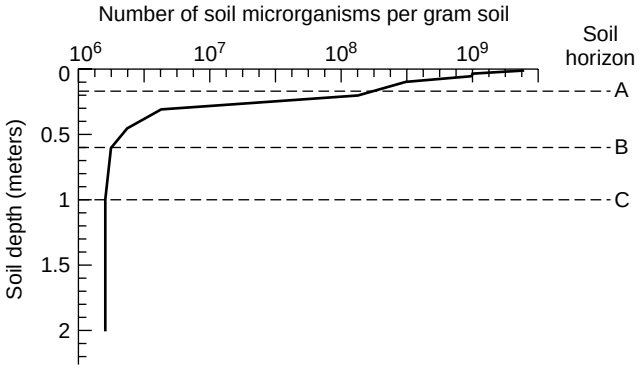

Figure 2. The carbon and nitrogen cycle in soil.

The rhizosphere effect or plant root effect dramatically affects the number of microorganisms distributed throughout the soil profile. In the vicinity of plant roots, high microbial activity exists due to increased deposition of compounds, such as amino acids and carbohydrates. The rhizosphere affect exists wherever plant roots explore different soil horizons. The rhizosphere effect is temporary and is maintained as long as the plant root is alive and immediately following its death during decomposition processes.

METHODS TO ASSESS MICROBIAL BIOMASS IN SOIL

The size of the soil microbial biomass is primarily dependent on soil type and ecosystems productivity. Finer textured soils and soils with more silt and clay tend to have a larger soil microbial biomass. Finer textured soils have more surface area and structure leading to greater number of niches for the soil microbial biomass to inhabit. Plant detrital input from aboveground production and root turnover regulates the size of the soil microbial biomass through substrate (food) availability. Other factors, such as soil temperature and moisture, also regulate the soil microbial biomass but manifest themselves more in regulating microbial activity and turnover. The following discussion will focus on methods to determine soil microbial biomass carbon and nitrogen. These two elements have been examined extensively especially in their relationship to nutrient cycling in soil. A multitude of other methods exist to describe specific microbial components and are described elsewhere in Quantification of Microbial Biomass, this Encyclopedia)

Collection of Soil Samples

The appropriate selection of soil samples dictates the kind of information needed to assess soil microbial biomass size and activity. Soils are not uniform and often vary dramatically on scales of less than a meter. Table 1 lists the physical, chemical, and biological factors, which influence the distribution in size of the soil microbial biomass.

One of the most important considerations when taking soil samples is the representativeness of the sample. Nontypical areas, such as low spots, steep slopes, eroded areas and so on should be avoided if they represent only a small fraction of the landscape of interest. Statistically speaking, soil sample collection should strive for reducing the error of the measurement to less than 20% and preferably 10% of the sample population mean. Depending on the uniformity of a landscape, 4 to 10 samples are required to determine statistical significance when comparing treatments or other experimental variables. Compositing a number of soil samples to produce up to five individual samples may often reduce the error of the measurement. Heterogeneous landscapes may require a form of geostatistics called “Kriging” to determine the dependence of soil properties on microbial biomass size or activity.

Soil samples taken under field conditions should be stored at 4°C until analyzed. If samples cannot be immediately transported to the laboratory, they should be stored at field soil temperatures taking care to avoid exposure to direct sunlight. Soils samples should be analyzed within one week after sampling and preferably in less than three days (6).

Microscopy

The oldest method used to examine soil microbial biomass size is microscopic examination. Dutch merchant A. Van Leeuwenhock first saw microorganisms through a microscope in the seventeenth century. The examination of a dispersed soil suspension is the most common procedure for counting soil microorganisms with a microscope. The advent of fluorescence microscopy utilizing ultraviolet light combined with flurogenic vital stains, which fluoresce under ultraviolet light enables the observation of living microorganisms in a soil dispersion preparation (7). Many stains have been developed to assist in identifying microorganisms in the dark background of a soil dispersion. Dyes that bind DNA (acridine orange) sulfhydryl groups in protein (5-(4,6-dichlorotriazin-2-yl) aminofluorescein) and helical DNA (4,6-diamidino-2-phenylindole-2-HCl) are available. Other dyes can probe metabolic activities, such as electron transfer, with the reduction of sensitive dye tetrazolium chloride. Immunofluorescent techniques using specific antigens are also available to count specific bacteria or fungal groups or species. The use of dyes and microscopic counting permits counting of both bacteria and fungi to estimate their mass in soil. Many of the methods to determine microbial biomass size and carbon and nitrogen content are calibrated using the microscopic counting techniques.

The volume of carbon and nitrogen and their content in the soil microbial biomass can be estimated using microscopic counts of bacteria and fungal hyphal length. Bacteria and fungal volumes and mass can be derived using average cell lengths and diameter. The number of microorganisms and their approximate carbon and nitrogen content can be determined as shown in the formulas presented in Table 2.

The carbon content of bacteria and fungi is assumed to be constant using the factors shown in Table 2. The nitrogen content of bacteria and fungi can be estimated assuming a carbon to nitrogen ratio of six to one for bacteria and eight to one for fungal biomass. The carbon to nitrogen ratio for bacteria and fungi were developed from pure culture studies. The carbon to nitrogen ratio under field conditions may vary substantially depending on substrate availability and species, leading to some uncertainty in the exact estimation of microbial carbon and nitrogen. Besides estimating true carbon and nitrogen contents, other limitations of microscopic counting include the tedious nature of counting microorganisms and soil colloidal interference. These shortcomings often lead to nonstandard approaches among users of microscopic counting techniques. Chemical analysis of soil microbial biomass carbon and nitrogen can avoid some of these problems; however, it must be stressed that these methods are calibrated with the microscopic counting technique.

Table 2. Soil, Environmental, and Organismal Factors Affecting the Distribution and Activity of the Soil Microbial Biomass

Soil Factor	Environmental Factor	Organismal Factor
Mineralogy	Rainfall	Plant cover
Parent material	Temperature	Net primary production
Soil age	Rain shadow	Vegetation history
Topography	Exposure (north vs. south)	Animal (grazing)
Soil pH	Elevation	Human influence
Water-holding capacity	Landscape depression	Animal (burrowing)
Water infiltration	Water springs	
Particle size(sand, silt, and clay)	History of burning	
Bulk density (g/cc)	Cultivation	
Soil organic matter content	Riparian influence	
Fertility	Pollution	
Erosion		

Chloroform Fumigation Incubation Method to Estimate Soil Microbial Biomass

A number of chemical techniques have been used to estimate soil microbial biomass and activity. The most popular approach to estimate soil microbial biomass is the use of chloroform vapor followed by incubation. Other methods utilizing chloroform with direct extraction techniques and metabolic approaches have also been utilized (6). Exposing the soil microbial biomass to chloroform vapor dissolves lipids in their cell walls allowing the cytoplasmic constituents to leak into the soil. For the determination of microbial carbon and nitrogen, the soil can be incubated for 10 days following exposure to chloroform vapor and the amount of carbon dioxide and ammonium determined (8). Because not all microorganisms are killed during the chloroform-fumigation procedure, the surviving biomass quickly consumes the labile components of the dead biomass mineralizing both carbon and ammonium. The fraction of the mineralized carbon and ammonium is used to estimate the original standing microbial biomass (9,10). Soil microbial biomass carbon is calculated as follows:

$$B_C = \frac{F_C - UF_C}{0.41}$$

Where B_C is soil microbial biomass carbon, F_C is the carbon dioxide produced from the chloroform treated soils after 10 days of incubation and UF_C is the carbon dioxide produced in an untreated control sample during a period of 10 days following a preincubation of 10 days (8). The proportion (0.41) used to convert the measured carbon dioxide into standing microbial biomass carbon was determined using radioactively labeled microbial biomass (9). The use of the 10- to 20-day control often leads to small or negative microbial biomass carbon values leading to the following equation, which subtracts

a partial control:

$$B_C = \frac{(F_C - F_C K_1) - (UF_C K_2)}{0.41}$$

Values for K_1 and K_2 are 0.29 and 0.23, respectively. The use of this formula strongly correlated to soil microbial biomass estimations obtained by microscopic counting (11). The preceding equation corrects for control soils having high background carbon dioxide production rates. These types of soils often have high organic matter content or recently added plant residues.

The determination of soil microbial biomass nitrogen is calculated with the following equation:

$$B_N = \frac{F_N - UF_N}{0.54}$$

Where B_N is soil microbial biomass N, F_N is the ammonium mineralized in the chloroform treated soils and UF_N is the ammonium mineralized in an untreated control sample incubated for 10 days in conjunction with the chloroform-fumigated sample. The proportion (0.54) used to convert the measured ammonium into standing microbial biomass nitrogen was proposed for samples with a F_C to F_N ratio of <6.7 (9). As with microbial carbon, the use of the 0- to 10-day control often leads to small or sometimes large microbial biomass nitrogen values, which can be corrected using the following equation:

$$B_N = B_C \left(K_C \left(\frac{F_N}{F_C} \right) + \frac{1 - K_C}{L} \right)$$

B_C is soil microbial biomass carbon, F_C is carbon dioxide produced in the chloroform treated soils, K_C is the proportion of microbial carbon mineralized after fumigation and L is the carbon to nitrogen ratio of the post chloroform-fumigated biomass (12). The chloroform fumigation of soils to estimate soil microbial biomass carbon and nitrogen has been used extensively to determine microbial biomass size and processes associated with nutrient cycling.

CARBON AND NITROGEN CYCLING

The soil microbial biomass is the "waste management" crew of an ecosystem. The enormous amount of plant detrital material produced annually is processed by the soil microbial biomass and other faunal components of soil. The processes that degrade and consumes plant litter, both leaves and roots, play an important role in maintaining the global carbon budget by balancing the carbon dioxide fixed through photosynthesis and released through decomposition activities. A by-product of the consumption and respiration of plant litter is the production of waste materials. These waste products, consisting of unpalatable plant litter components and microbial products, form soil organic matter, a relatively stable pool of soil carbon. Soil organic matter represents approximately two-thirds of the total terrestrial carbon pool (13). The importance of this large carbon pool lies in its ability to store nutrients and alter soil properties, which affects net ecosystem productivity. In addition, the large store of soil carbon acts as a buffer in regulating atmospheric carbon dioxide. Manipulating the soil carbon pool through increased storage of organic matter is thought to be a viable soil management approach to mitigate rising levels of atmospheric carbon dioxide and associated global warming effects. Therefore, the importance of the soil microbial biomass cannot be understated in its ability to influence ecosystem carbon and nutrient budgets.

Soil Carbon Turnover

Carbon inputs to soil come primarily from plant production. The heterotrophic soil microbial biomass and fauna consume plant production according to the following:

$$CH_2O + O_2 \rightarrow CO_2 + H_2O + \text{energy}$$

The oxidation of carbohydrates and other plant carbon provides the soil microbial biomass with an energy source for metabolic activity and growth. The effect of soil and crop management can greatly impact standing soil carbon pools. For example, soil tillage and reduced plant carbon input under conventional agriculture is suspected to have led to a 50% decrease in soil carbon over a 50-year period (14). Soil tillage is thought to affect soil microbial biomass activity through aeration and liberation of soil carbon from protected areas, such as within aggregates. For these reasons, it is thought that practices such as no-till and off-season cover crops can be used as management techniques to store more soil carbon and to mitigate rising atmospheric carbon dioxide levels. These management techniques may make it is possible to manipulate soil carbon storage through management of heterotrophic microbial biomass size and activity leading to the formation of stable soil carbon.

The turnover of soil organic carbon is often related to ecosystem productivity. Soil carbon normally turns over rapidly in productive ecosystems. The rapid turnover of carbon can often be attributed to higher temperatures and/or nutrient availability. In tropical rainforests, the soil carbon pool is smaller compared with temperate forests or grasslands yet ecosystem productivity is four times higher in the tropical forest as a result of the rapid turnover of nutrients from plant litter (5). Temperate grasslands store more soil carbon compared with other ecosystems in temperate and tropical biomes. In grasslands, the increase in soil carbon storage can be attributed to large belowground productivity leading to a rhizosphere effect that deposits more soil carbon leading to increased microbial biomass production and activity. Other ecosystems that can store more soil carbon than grasslands are peat bogs, tundra, and boreal forest. The storage of soil carbon in these ecosystems can often be attributed to anaerobic conditions and low temperature.

Rates of Soil Carbon Turnover

The rate of soil carbon turnover is intimately tied to the activity and turnover of the soil microbial biomass. The fate of soil carbon has become an important issue

in determining the role of terrestrial soil carbon in influencing the global carbon budget. Therefore, the importance of the soil carbon cycle and the factors affecting its stability are important global warming research topics.

The competitive and synergistic interactions among components of the soil microbial biomass may affect the fate of soil carbon by affecting the decomposer community composition and/or its activity. However, the inoculum potential or diversity of the soil microbial biomass is assumed to be rarely limiting unless the soil environment has been drastically disturbed (1). Examples of soil disturbance include erosion, fire, and volcanic deposition, which either remove surface soil or bury it. Other disturbance events that affect certain components of the soil microbial biomass such as pollution or flooding may have the same effect. Besides all the biological interactions of soil, soil physical properties, particularly those that affect temperature and moisture, will greatly influence the activity of the soil microbial biomass.

The quantity and quality of plant litter will determine how carbon becomes metabolized and stabilized in soil. Similarly, the potential to utilize and mineralize nitrogen in plant components is linked to the metabolism of carbon. The forms of plant litter entering the decomposition stream are a complex mixture of products with various degrees of biodegradability. Plants are composed of various labile (cytoplasm) and structural (cell wall) chemical components listed in Table 3. Each component has an inherent degradability based on its chemical structure. For example, proteins are much easier to degrade than polymeric substances such as cellulose and lignin. In addition, to the chemical recalcitrance of plant components, the nitrogen content of each component is also a good indicator of its biogradability. The lower the carbon to nitrogen ratio of the plant material the more likely it is easier to degrade. The carbon to nitrogen ratio or the nitrogen to lignin ratio has often been used to predict the degradability of plant litters. However, the carbon and nitrogen ratio of specific plant litter components is not a reliable index of decomposition (15). For example, because lignin contains no nitrogen it would be very difficult to degrade without an external nitrogen source. Therefore, nitrogen availability during degradation ultimately controls the decomposition of plant litter.

Table 3. Factors Used in Estimating the Carbon and Nitrogen Content of the Soil Microbial Biomass Using Microscopy

Bacterial Numbers

$N = nf\frac{A}{F}\frac{v}{V}\frac{W}{D}d$

N = number of bacteria per gram soil
n = bacteria per field of view
A = smear of filter area
F = counting field area
v = volume of sample applied to smear or filter
V = volume of dispersed soil
d = dilution factor
W = wet weight of soil
D = dry weight of soil

Bacterial Carbon Content

$B = NUeSC\ (10^{-6})$
B = bacterial biomass carbon (microgram (μg) per gram soil)
N = number of bacteria per gram soil
U = average bacterial volume (μm^3)r^2L; r = bacterial radius, L=bacterial length
e = bacterial density ($1.1 \times 10^{-3} mm^{-3}$)
S = bacterial solids content (0.4 in soil)
C = % bacterial carbon (0.45)

Fungal Biomass Carbon

$F = \pi r^2 LeSC\ (10^6)$
F = fungal biomass carbon (microgram (μg) per gram soil)
r = average hyphal radius (1.13μm)
L = hyphal length cm/g soil
e = density in soil (1.1)
S = solids content (0.3 in soil)

Source: (E. A. Paul and F. E. Clark, *Soil Microbiology and Biochemistry*, Academic Press, New York, 1996.)

Factors Controlling Nitrogen Availability During Decomposition

Nitrogen is an essential component of many cellular building blocks including nucleotides, protein, and cell walls. The competition for nitrogen in soil by plants and the soil microbial biomass is fierce. The large surface area and distribution of the soil microbial biomass compared with plant roots often gives them an advantage in competing for soil nitrogen. In general, plant roots explore approximately 1% of the total surface area of the soil. Because the soil microbial biomass is distributed widely in soil, it has a competitive advantage over plant roots in capturing nutrients in the soil solution moving by both mass flow and diffusion. The competition for nitrogen in soil among plants and the soil microbial biomass controls nitrogen availability during decomposition. The soil microbial biomass contains significant amounts of nitrogen often equivalent to the entire needs of plants during one growing season. The soil microbial biomass nitrogen content and that nitrogen becoming available during decomposition of plant residues and organic matter are the primary sources of available soil nitrogen for plants. As stated earlier, for this reason the soil microbial biomass is often considered the source and sink of nitrogen in soil.

The soil nitrogen cycle is complex and often the different components of the nitrogen cycle compete for different forms of nitrogen. The nitrogen cycle is a complex set of processes converting elemental nitrogen to organic forms and back to elemental nitrogen or other gaseous inorganic forms of nitrogen. The forms and availability of plant residue and soil organic carbon influence the formation of different nitrogen forms in the nitrogen cycle (Fig. 3).

Nitrogen mineralization is the primary nitrogen cycling process determining the availability of nitrogen for plants and decomposition processes. The availability of organic nitrogen to be mineralized to ammonium is the limiting process that controls nitrogen available to plants and the soil microbial biomass. Sources of organic nitrogen include soil organic matter, plant litter residues, and

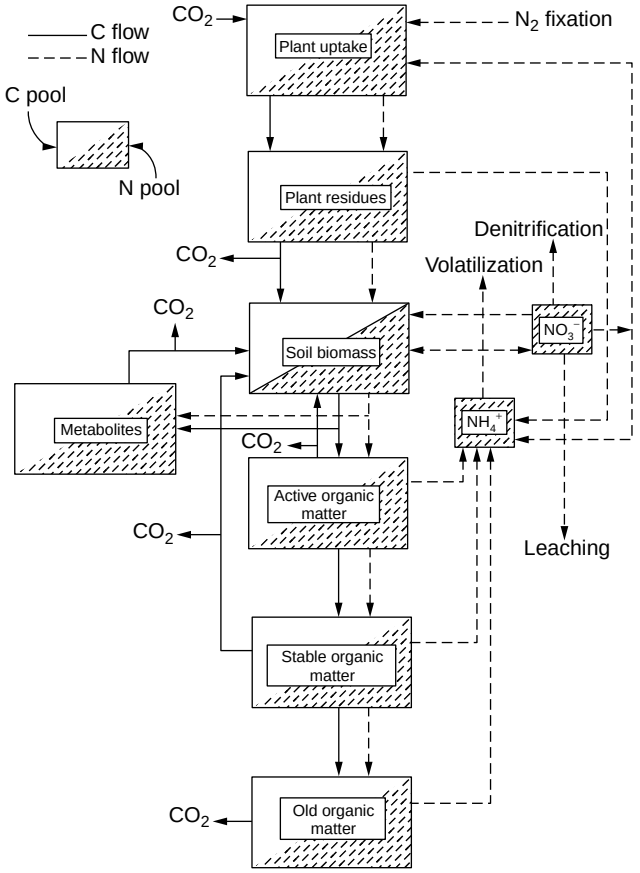

Figure 3

Table 4. Mass of Cytoplasmic and Cell Wall Components in Plants

Plant Component	% of Total
Waxes and pigment	1
Amino acids, sugars, nucleotides and so on	5
Protein	5–7
Hemicellulose	15–20
Cellulose	4–50
Lignin	8–20

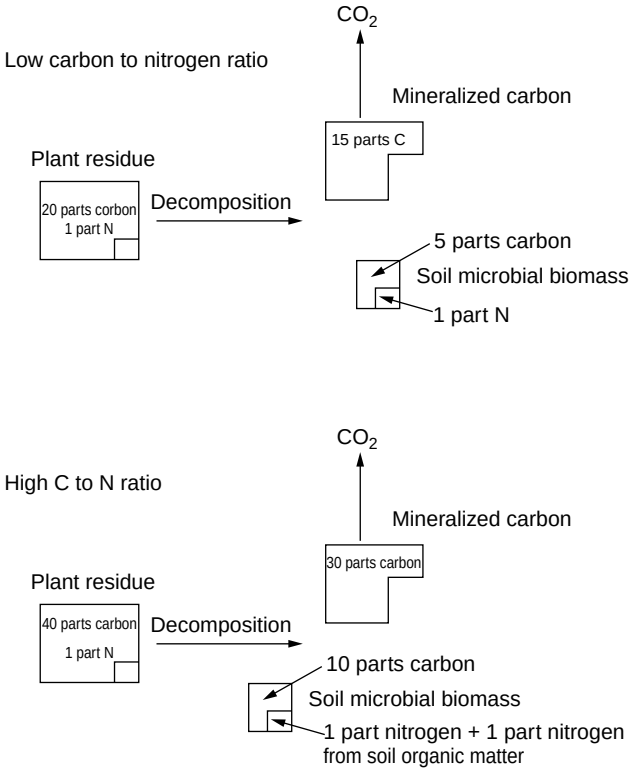

Figure 4. The influence of carbon to nitrogen ratio on the decomposition of plant litter. The scenarios assume that the soil microbial biomass will utilize 25% of the plant carbon and produce additional biomass at a carbon to nitrogen ratio of 5 to 1. Under low carbon to nitrogen ratio plant litter, 15 parts of carbon are mineralized leaving five parts of carbon and one part of nitrogen, thus no additional soil nitrogen is required. Under high carbon to nitrogen ratio, 10 parts of carbon and one part of nitrogen are used to produce new biomass thus requiring an additional one part of nitrogen from the soil. This is termed nitrogen immobilization. Nitrogen mineralization would occur when the carbon to nitrogen ratio is less than 20 to 1.

root exudation. Because extra nitrogen is often required for the decomposition of plant residues, other sources of nitrogen primarily from soil organic matter are utilized for the decomposition process. The amount of nitrogen made available from the mineralization of soil organic matter for degrading plant residues depends on the amount of nitrogen already present in the plant residue. When the plant litter has a carbon to nitrogen ratio of 20 to 25 to one the decomposition process has sufficient nitrogen and no nitrogen is required from the soil organic matter or other sources (Fig. 4). Plant litter carbon to nitrogen ratios above 25 to 1 require additional nitrogen from other sources to complete the degradation process leading to nitrogen immobilization in the soil. Carbon to nitrogen ratios of less than 20 to 1 often lead to mineralization of nitrogen to the soil from the plant litter. Although Figure 4 is simplistic it does show how the carbon to nitrogen ratio control decomposition process in soil and the availability of nitrogen for plant and microbial uptake.

Conversion of Plant Carbon into Soil Organic Matter

During the decomposition process, plant residues are converted into microbial biomass energy, microbial production, carbon dioxide, and water. Figure 5 shows how plant residue components disappear during decomposition and microbial production increases. As decomposition proceeds more microbial production occurs. Microbial production is the living soil microbial biomass and metabolic and turnover products that are difficult (recalcitrant) to process any further. The metabolic products contain carbon and nitrogen from plant, microbial, and faunal origin. The recalcitrant products are probably difficult to degrade

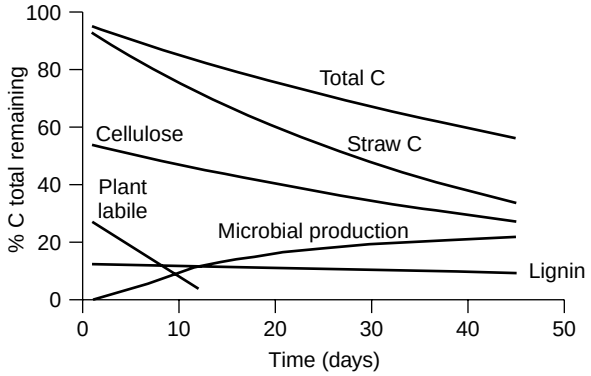

Figure 5. Loss of total carbon and plant components during decomposition. Microbial production increases during the decomposition process leading to the production of soil microbial biomass and soil organic matter (11).

or they are protected from degradation by their association with soil mineral colloids. The formation of soil aggregates is an example of a soil structure containing protected carbon.

The microbial production and resistant plant litter components are the source of soil organic matter. Soil organic matter formation is the result of life in soil. The formation and maintenance of soil organic matter creates habitats through the formation of soil structure and provides a source of nutrients for plants and decomposition processes. The formation of soil organic matter is a beginning and an end in itself because it creates a favorable habitat for both the soil microbial biomass and plants.

CONCLUSION

The soil microbial biomass plays an important role in ecosystem function primarily through the regulation of nutrient cycles. In their search for energy to grow, the soil microbial biomass relies on nutrients in plant litter and soil. Because of its competitive ability, the soil microbial biomass is often considered the source and sink of essential plant nutrients. As result of the heterotrophic activities, the soil microbial biomass greatly influences the terrestrial carbon cycle. The disturbance of ecosystems leads to net carbon losses to the atmosphere by exposing protected soil carbon and affecting the activity and turnover of the soil microbial biomass accentuating the greenhouse effect. Future management of ecosystems will need to consider the role of the microbial biomass in sequestering and producing stable soil carbon. The wide diversity and function of the soil microbial biomass exemplifies its role in a wide variety of processes including parasitism, pathogenesis, and symbiosis. This wide functionality has made the study of soil microbial biomass problematic and often leads to misinterpretation of its importance in ecosystem function.

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BIOMINERALIZATION BY BACTERIA

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Biomineralization is the process by which organisms form minerals; this ability is widespread among all taxa (1). The diversity of biogenic minerals is remarkable: more than 60 different minerals have been identified and the number continues to increase (1,2). Bacteria produce practically all types of biominerals including amorphous and crystalline varieties (e.g., carbonates, phosphates, silicates, sulfates, sulfides, oxalates, oxides, hydroxides, etc.). Moreover, because bacteria can inhabit extreme environments enriched with heavy metals, minerals

uncommon to eukaryotes are formed from elements such as Ag, As, Au, Cr, Me, Sb, Tc, Ti, V, and U. Microorganisms play principal roles in biogeochemical cycling and form some biominerals on a huge scale. Many sediments, sedimentary rocks, and ore deposits are formed by the direct participation of microorganisms.

MECHANISMS OF BIOMINERALIZATION

All biomineral-forming processes can be divided into two types, "biologically induced" and "biologically controlled" (3,4). When an organism influences solution chemistry and, thus, creates favorable conditions for mineral formation, it is biologically induced mineralization (BIM). Bacteria release different types of metabolites or ions, which change the oxidation state of metals and introduce charged surfaces (cell walls, capsules, etc.) that can induce mineral formation. The minerals produced, if they are crystalline, have a wide range of sizes and are usually randomly aggregated (1). For biologically controlled mineralization (BCM), the mineral nucleation site is separated from the external environment. Bilayer lipid vesicles (e.g., magnetosomes; Fig. 1) and proteins (e.g., ferritin) can serve as such sites and allow better regulation of the physicochemistry for mineralization (1). BIM is the predominant process among bacteria. Only a few examples of bacterial BCM are known; that is, the formation of magnetite (Fe_3O_4) or greigite (Fe_3S_4) in magnetotactic bacteria (5,6), magnetite and ferrihydrite in ferritin (7), and sulfide clusters in metalloproteins such as metallothioneins.

A necessary precondition for mineral formation is the development of supersaturation in the fluid phase. At this point, ions begin to associate into small unstable clusters. The rate of cluster dissociation prevails, however, over the rate of mineral growth. To exceed the critical nucleus size, above which further crystal growth is thermodynamically possible, certain energy barriers must be overcome. The level of supersaturation must be high enough to promote spontaneous crystallization (homogenous nucleation) or centers of crystallization must be introduced (heterogeneous nucleation). Particulate surfaces make excellent crystallization centers because their interfaces reduce the free energy necessary for mineralization at lower ion concentrations.

For this reason, bacteria, with their high surface area-to-volume ratio, make exceedingly good foci for mineral nucleation; their highly reactive surfaces are good templates to initiate and promote further mineral growth (Fig. 2). These surfaces consist of biopolymers (lipopolysaccharides, phospholipids, peptidoglycan, polysaccharides, proteins, etc.; see Reference 8 for more structural details) bearing important ionizable functional groups at physiological pH (e.g., amine, amide, carboxyl, carbonyl, phosphate, sulfhydryl, and hydroxyl groups). However, the most abundant are carboxyl, hydroxyl, phosphate, and amino groups (9). The ratio of acidic to basic groups in these biopolymers is generally greater than 1 and, overall, bacterial surfaces have an isoelectric point of 2 : 3. Above this point, bacteria are usually negatively charged and below it they are positively charged (10,11).

Because of this, the interaction of metal ions with bacterial surface polymers is strongly pH-dependent; pH not only dictates metal ion speciation but also activates surface functional groups for metal ion–polymer complexation. All metallic ions, as well as the lanthanides and actinides, have been implicated in these interactions (12,13). Generally, the sorption of the metal cations increases with pH, whereas that of anionic forms decreases. The sorption of Ag^+, Hg^{2+} and $[AuCl_4]^-$ does not, however, change much over a wide range of pH (14).

Metal sorption is facilitated by cation hydrolysis (hydroxo-complex formation) or protonation of oxyanions and negatively charged complexes. If ion concentration exceeds 10^{-3}–10^{-7} M, hydrolyzed and protonated metal ion species will polymerize. Compared to "free" metal ions these polymeric species are less hydrated and more hydrophobic. This should reduce the amount of free energy required for sorption, which appears to be noncoulombic (mainly via hydrogen-bonding and van der Waals cohesive forces). The amount sorbed exceeds the number of potential functional groups (13,15–17).

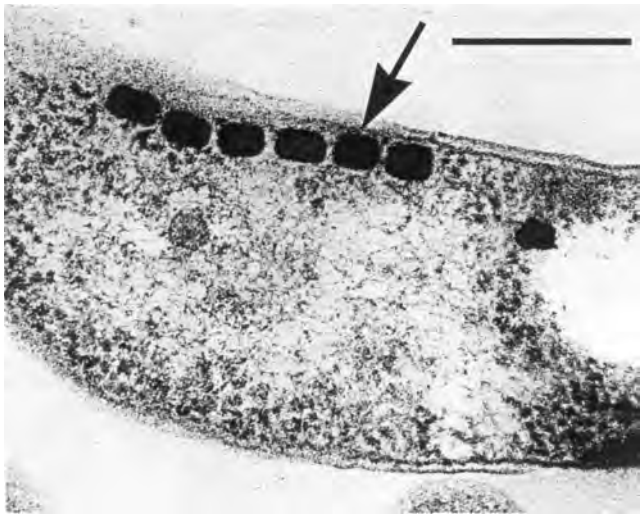

Figure 1

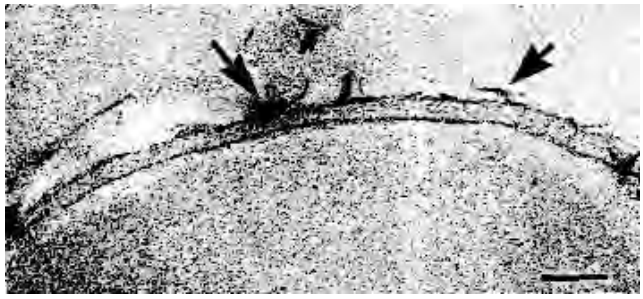

Figure 2

The strength of the metal–polymer bond is important because loosely bound metals can be more easily displaced. However, these can also attract anions to form ion pairs at the nucleation site. The anions at these sites can coordinate with secondary cations so that more and more metal is deposited. This is called *ionotrophy* and both high-binding capacity and low-binding affinity are necessary for this type of nucleation. Usually amorphous phases result, which are transformed (over time) through dehydration to crystalline phases (18,19). Strongly bound metals are not easily displaced and will frequently react with bacterial metabolites (e.g., HS^-, HPO_4^{2-}, and HCO_3^-), or undergo hydrolysis, or redox transformations to form mineral phases.

In addition to the de novo development of fine-grained minerals by surface polymer–metal ion interaction, bacterial surfaces also sorb particulate, finely dispersed mineral phases such as colloidal hydroxides and clays (20–22). These serve as new reactive surfaces for subsequent metal ion interaction (21,23) but they do not bind the ions as strongly as the biopolymers (24).

Carbonate Minerals

Bacteria are active agents of carbonate mineral formation because they release bicarbonate during respiration while at the same time increasing pH in their immediate surroundings (25). These factors, together with the processes mentioned in the preceding section, account for several biocarbonate formations. Hydromagnesite ($Mg_5[CO_3]_4[OH]_24H_2O$) and needlelike aragonite (orthorhombic $CaCO_3$) deposits have been found in association with mucous biofilms in a karstic cave (Altamira, Spain). Such mineral crystals are usually embedded in mucilaginous (producing hydromagnesite) or filamentous (aragonite) microbial films. In this case, abiotic hydromagnesite precipitation seems improbable because the cave waters are oversaturated with respect to calcite, aragonite, and dolomite ($CaMgCO_3$) but are strongly undersaturated with respect to hydromagnesite and other magnesium carbonates (26).

The crystal type and mineral composition are obviously dependent on solution chemistry and rate of precipitation. Laboratory simulations with pure cultures of different bacterial and cyanobacterial strains demonstrated that either calcite or aragonite can develop, but not simultaneously. In all cases, calcite was precipitated in the viscous medium close to cells, whereas aragonite was precipitated in the fluid phase. This is in good agreement with observations of bacterially mediated calcium carbonate deposition in natural environments (27,28).

Accordingly, slime-producing bacteria not only influence the viscosity of their immediate surroundings but also control the mineral composition of their precipitates.

Heterotrophic anaerobes frequently employ electron acceptors such as sulfates, nitrates, ferric iron, and manganite compounds, resulting in an increase in pCO_2 and pH. Deposition of a dolomite-ankerite ($CaFeCO_3$) phase was observed in the presence of sulfate-reducing bacteria (SRB; mostly *Desulfovibrio*) in Lagoa Vermella, Brazil. Geochemical solid field analysis showed that under the given conditions, inorganic precipitation of dolomite was impossible (29). Moreover, bacteria are able to release metal ions (Ca, Fe, and Mn) from insoluble forms thereby promoting carbonate deposition of these metals. For example, rhodochrosite ($MnCO_3$) and siderite ($FeCO_3$) deposition in the oligotrophic Punnus Yarvi Lake (Karelia, Russia) was attributed to the activity of manganese-reducing bacteria (30).

Cyanobacteria can produce significant carbonate deposits in carbonate-saturated waters (31). The giant towerlike microbialites (or bioherms) of Lake Van (Turkey) are a dramatic example of recent thrombolite formation, being up to 40 m high (32). Here, at sites where calcium-rich groundwater seeps into the alkaline (pH~9.7) soda lake water, inorganic calcite precipitation occurs. Microbial colonization of these crusts results in the formation of 10–40 μm biofilms, which are dominated by small coccoid cyanobacteria (*Pleurocapsa* spp.). Scanning electron microscopy (SEM) revealed that their gelatinous sheaths were heavily encrusted by aragonite that cemented and stabilized the soft porous calcite crust. Because groundwater is forced up and through this matrix, the tower grows. When the towerhead rises 8–10 m in height, noncalcifying cyanobacteria (predominantly *Oscillatoria*) begin to dominate the biofilm. This replacement makes further upward growth of the microbialites impossible (32).

Extensive studies by Schultze-Lam and coworkers and Thompson and coworkers on carbonate mineralization in Fayetteville Green Lake provided unequivocal evidence of this biogenic process (33–37). This lake contains Ca^{2+}-, Mg^{2+}- and SO_4^{2-}-rich waters with a pH of 7.9. *Synechococcus* GL24, a small cyanobacterium, dominates the mixolimnion because of high oligotrophic conditions. This cyanobacterium can be planktonic or it can form biofilms. In both cases, it contributes to an active biomineralization of calcium carbonate. As planktonic cells, it precipitates calcite so that a gentle but continuous rain of calcite contributes to the marl sediment of the lake. When it grows as a biofilm, it also precipitates calcite, which contributes to the size of the microbialites. Transmission electron microscopic (TEM) analysis showed that the surface of GL24 consisted of an S-layer of hexagonally arranged proteinaceous subunits. Strategically placed carboxyl groups on the S-layer interact with Ca^{2+}, thereby nucleating mineral formation; the mineral initially mimics the large lattice structure of the S-layer (Fig. 3). Here, there is a remarkable dependence on pH for mineral type. Because the solid field of the circumneutral pH lake waters is for gypsum, photosynthesis and respiration increase the pH immediately surrounding the cells so that a calcite solid field is produced. Laboratory studies in which Ca^{2+} is replaced by Sr^{2+}, Mg^{2+}, or a mixture of the two has revealed that strontianite, hydromagnesite, and celestite can be formed (33,34).

Silicate Minerals

Bacteria play a role in the formation of silicate minerals because their sorption of monosilicic acid at low Si concentrations (~0.5–3.0 mM) promotes silica polymerization (autopolymerization of monosilicic acid occurs at concentrations greater than 3.5 mM). Moreover,

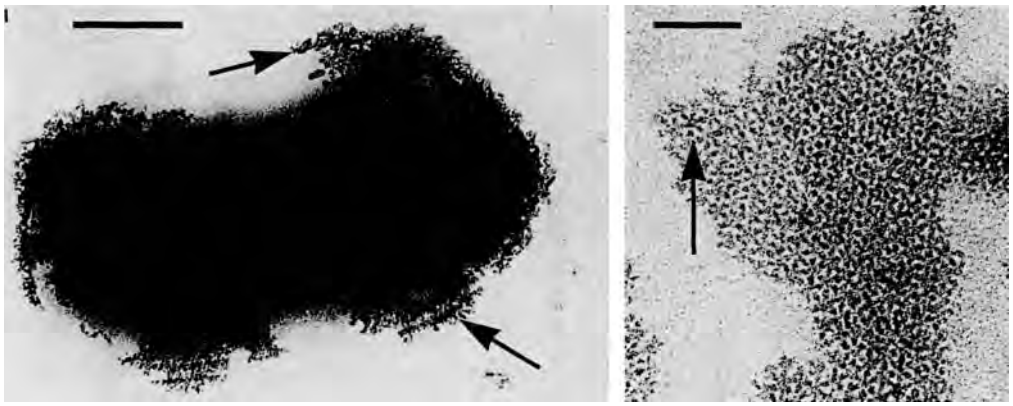

Figure 3

silica mobilized from quartz by *Bacillus mucilaginosus* or *Thiobacillus thiooxidans* was found to be mainly bound to cells and their exopolysaccharides (38). There are several ways in which silicate ions can bind to a bacterial surface, that is, through ionic condensation with phosphoryl, carboxyl, or hydroxyl groups (39), via H-bonding with =O, $-NH_2$, $-COOH$, $-OH$, and other groups (40,41), and by electrostatic interaction of silicate anions with positively charged groups (e.g., amines; 42,43). Covalent binding appears to have minor significance. Another mechanism, adsorption of multivalent metal cations on bacterial surfaces, results in reduced or reversed surface electronegativity, and facilitates silica sorption; silica-binding occurs through cationic bridging by preexisting wall-bound metal cations or via binding to surface hydroxyl groups of metal hydroxides adsorbed to the cell surface (42–44).

Numerous electron microscopy (EM) studies of environmental samples from river sediments, hot springs, mine tailings, and so forth have revealed bacterial cells to be partly or completely encrusted with different silicate minerals. Living cells have silicates associated with their surfaces or slimes, but when they die, silicates can be found within the cytoplasm (Fig. 4). These silicates exhibit a wide range of size (15 nm to >1 m), morphology, and chemical composition, and greatly differ from suspended minerals found in water samples. The mineral phases most frequently associated with bacteria are complex polymorphic silicates ranging from amorphous silica or poorly ordered chamositic clays [$(Fe_5Al)(Si_3Al)O_{10}(OH)_8$] to well-crystallized kaolinitic [$Al_4(Si_3O_{10})(OH)_4$] or glauconitic [$K(Al_{0.38}Fe_{1.28}Mg_{0.34})(Si_{3.7}Al_{0.3})O_{10}(OH)_2$] clays. Thus the most abundant elements of these minerals are Fe, Al, and Si, although Mn, Mg, K, and Ti are often present (44–52).

Interestingly, there seems to be no relationship between different taxonomic groups of bacteria and their silicate mineral type (46,47) and this has been verified by laboratory simulations using *Bacillus subtilis* cells to nucleate poorly ordered allophanes and serpentines. However, more crystalline kaolinite phases and (possibly)

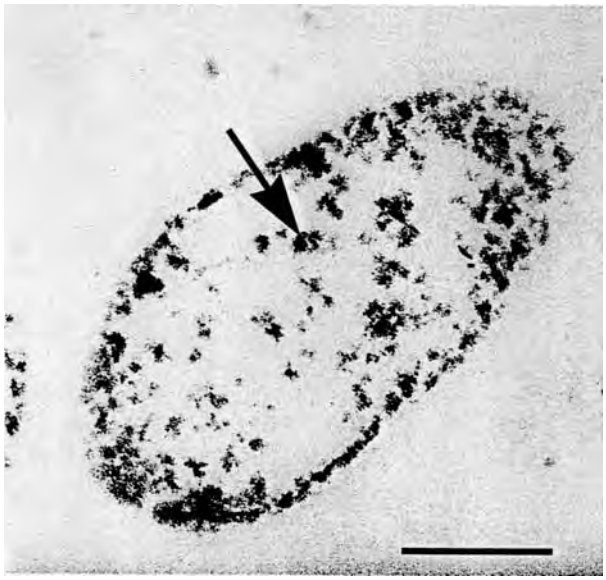

Figure 4

2 : 1 phyllosilicates have been detected as well (42,43). It seems that (Fe-Al) silicates, like other biominerals, mature over time from amorphous phases into crystalline forms by dehydration during which more Fe and Si are added (42–44,46,47,53).

Mineralization Coupled to Redox Transformations

Bacteria are able to oxidize or reduce metals such as Fe, Mn, As, Cr, U, and Au via enzymes (oxidases and reductases), metabolites (peroxides and thiols), inorganic substances (H_2S), and cellular redox potential (25,54,55). This can impact biomineralization processes because when a metal valency is changed, an insoluble

product can be formed by hydrolysis or anion interaction (e.g., with phosphates, sulfides, or carbonates). For instance, *Pseudomonas vanadiumreductans* and *P. isatchenkovii* can produce V_2O_3 from vanadate ions (56), *Desulfovibrio desulfuricans* and *Escherichia coli* deposit low-valence hydroxides and oxides (e.g., TcO_2, Tc_2O_5) from Tc(VII) (57–59), and several bacteria, for example, *Desulfovibrio* spp., *Geobacter metallireducens*, and *Shewanella* spp., reduce UO_2^{2+} to form insoluble uraninite ($UO_2{\cdot}nH_2O$) (60–63).

Reduction of Fe(III) (e.g., from iron oxides) by dissimilatory iron-reducing bacteria serves as a good example of complex interactions occurring between the solution and a solid phase. For example, simple solution chemistry dictates that as the concentration of Fe(II) increases, siderite ($FeCO_3$) and vivianite ($Fe_3(PO_4)_2{\cdot}8H_2O$) phases should result. Yet, because bacteria can modulate the Fe(II/III) couple as they grow with an iron oxide, ferrous ions often interact with amorphous ferric hydroxides resulting in the additional formation of crystalline magnetite (Fe_3O_4) and green rust $[Fe^{II}_{(6-x)}Fe^{III}_x(OH)_{12}]^{x+}(A^{2-})_{x/2}{\cdot}yH_2O$ (64–67). If Cr(VI) is also in the system, its reduction by bacteria leads to mixed Cr(III)-Fe(oxy)hydroxide phases (Fig. 5) (68,69). Such systems show that the dominant mineral phase depends on a complex interplay between bacterial reduction processes, the aqueous geochemistry, and the surface chemistry of an oxide (66,70).

Metal ions with high electrochemical potentials (>700 mV), such as those formed by Ag, Au, Hg, and Pt, can be readily reduced by bacteria. The resulting precipitates may grow on the cell surface and intracellularly as metal colloids. The metal ions easily damage the normal permeability of the plasma membrane; the membrane is breached, killing the cell and allowing intracellular mineral growth (Fig. 6) (71–73). The biosorption

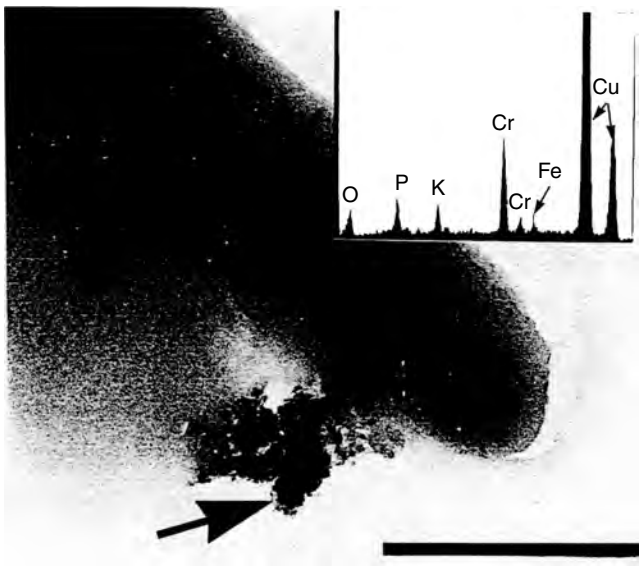

Figure 5

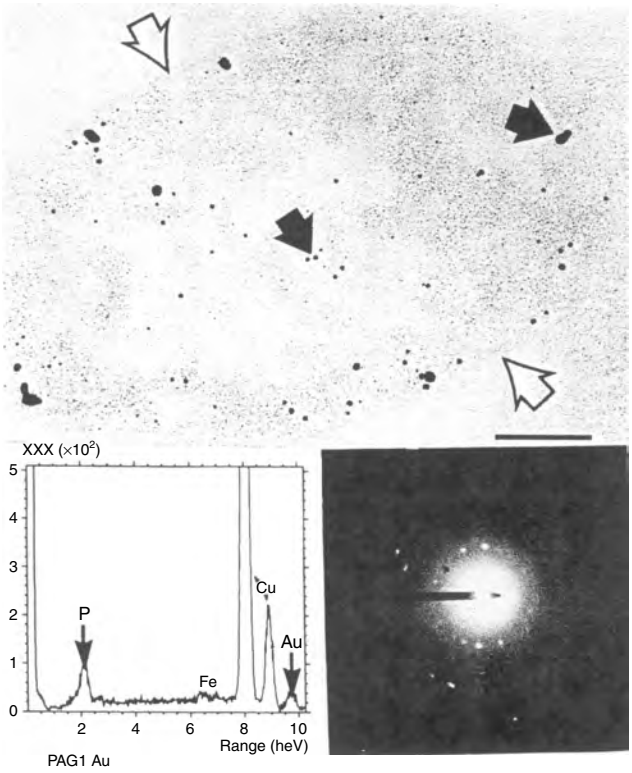

Figure 6

of Ag, Au, Hg, Pt, and so forth is a relatively quick process, occurring within 15 minutes in the *B. subtilis* system of Beveridge and Murray (74,75). Interestingly, once small metallic colloids are formed, they coalesce to form macroscopic crystalline phases (pseudotrigonal and hexagonal-octahedral forms) similar to placer gold (76). Similar results have been reported by Russian researchers using *Bacillus cereus* and *Micrococcus luteus* systems in which approximately 80% of the colloidal particles annealed into 0.2–0.8-mm-sized crystals after 8 to 10 months (77). The *M. luteus* culture was isolated from a gold ore deposit and is capable of sorbing up to 45% of its weight in gold, producing a crystal size similar to that found in the deposit. For these systems, the production of the gold crystals depended on the physiological activity of growing cells because metabolically inactive and dead cells sorbed the gold poorly (77–81). In field studies, bacterial numbers coincide well with the number of gold particles in oxic zones of gold deposits and in placers of Far East Russia (77). Sometimes gold deposits contain microfossils and these prokaryotic remnants possess golden castes in the shape of bacteria, suggesting that lithification developed over living cells (82).

If protein sulfhydryl groups are available (−SH groups are rare on bacterial surfaces but can be present in the periplasm), they will dominate the binding of gold ions, presumably via ion–ion interaction or complexation. It is also possible that gold and its analogs can bind

to oxygen- and nitrogen-containing functional groups of surface biopolymers (83,84).

Oxidation of Iron and Manganese

These oxidation processes deposit Fe and Mn oxides, have been found in gram-positive bacteria, gram-negative bacteria, and mycoplasmas, and are well studied. Fe–Mn-oxidizing bacteria are widespread in nature, being found in marinewaters, freshwaters, and groundwaters, sediments, and soils (85,86). The mechanism of Fe–Mn deposition involves both metabolic activity and sorption. As with other metal ions, anionic polymers of capsules, sheaths, and cell walls readily sorb ionic and colloidal forms of these metals. For example, distribution coefficients (K_d values), calculated as the ratio between sorbed Fe and Mn (on marine bacteria) and dissolved metal concentration, were as high as 10^7 (87). Metal accumulation on the bacterial surface structures is poised for subsequent oxidation and mineralization.

Ferrous iron is unstable at pH higher than 5.5 because of autooxidation, and it is difficult to distinguish between abiotic and microbial precipitates formed during the oxidation of iron. Microbial surfaces lower the free energy barrier for precipitation, but physiological processes seem to be necessary for large depositions. In this way although nonliving cells of *Leptothrix*, *Sphaerotilus*, and *Pedomicrobium* accumulate iron via sorption at autooxidation pHs (88,89), the amounts are low when compared to living cells because active oxidative processes occur at rates that are ~8 to 10 times higher than for dead cells (85). At neutral pH, Mn^{2+} is more stable than Fe^{2+} and the rate of its microbial oxidation can be ~500 times higher than abiotic oxidation. The processes of Fe–Mn oxidation are complex and are complicated by the fact that different bacteria can have distinctly different oxidative pathways; in some instances, oxidation occurs via enzymatic routes, whereas in others no enzymes are required.

Nonenzymatic Reactions. During their metabolism, bacteria will frequently excrete oxidants or bases while at the same time consuming organic anions. These processes will increase the pH and redox potential of the ambient solution, thereby promoting Fe–Mn autooxidation. It has also been shown that hydrocarboxylic acids, acidic exopolymers, and some proteins produced by the cells enhance this same reaction (25,86,90).

Enzymatic Reactions. The enzymatic mechanisms of Fe–Mn oxidation can be separated into three major groups:

1. Oxidation of free Mn–Fe ions by constitutive or inducible oxidases that transfer electrons to oxygen via cytochrome systems:

$$Mn2^{+} + 1/2O_2 + H_2O \Rightarrow MnO_2 + 2H^{+}$$
$$Fe^{2+} + 1/2O_2 + 2H^{+} \Rightarrow Fe^{3+} + H_2O$$

This pathway for Mn oxidation is found in *Pseudomonas* sp. S-36 (91), *Pseudomonas putida* MnB1 (90), *Citrobacter freundii* E_4, and *Pseudomonas* sp. E_1 (25). It has been shown that some anaerobic phototrophic purple and green bacteria (e.g., *Rhodobacter*, *Rhodomicrobium*, and *Chlorobium*) are able to oxidize Fe^{2+}, $FeCO_3$, and FeS, with concomitant reduction of carbon dioxide to produce cell material (92,93).

2. Oxidation of Mn bound to a manganese(IV) oxide:

$$Mn{\bullet}MnO_2 + 1/2O_2 + H_2O \Rightarrow 2MnO_2 + 2H^{+}$$

Some bacteria (e.g., *Arthrobacter*, *Vibrio*, and *Oceanospirillum*) are unable to oxidize free Mn^{2+} but oxidize the ion when it is bound to manganese(IV) oxides, ferromanganese, or some clays (e.g., montmorillonite and kaolinite). For a more complete summary of this work, see Reference 25.

3. Oxidation catalyzed by catalase during hydrolysis of metabolic H_2O_2:

$$Mn^{2+} + H_2O_2 \Rightarrow MnO_2 + 2H^{+}$$
$$2Fe^{2+} + 3H_2O_2 \Rightarrow 2Fe(OH)_3$$

This mechanism is widespread in various heterotrophic bacteria (85,94,95).

In acidic environments, chemolithoautotrophic and mixotrophic bacteria play a principal role in Fe(II) oxidation by enzymatically increasing chemical oxidation as much as six orders of magnitude. These acidophilic bacteria can be mesophilic or thermophilic and usually belong to the genera *Thiobacillus*, *Leptospirillum*, *Sulfobacillus*, and *Sulfolobus*. To obtain energy and reducing power for CO_2 fixation they can use as electron donors not only Fe(II) but also Cu(I), U(IV), Se(II), reduced sulfur species and sulfide minerals (96). It has recently been shown that acidophilic filamentous bacteria (similar to the *Leptothrix-Sphaerotilus* group) and acidophilic–heterotrophic unicellular bacteria can oxidize Fe^{2+} and pyrite (96–98). Bacterial Fe(II) oxidation results in extensive formation of jarosites, (crystalline basic iron sulfates), amorphous ferric hydroxides, ferrihydrite, lepidocrocite (γ-FeOOH), and magnetite. In the case of arsenopyrite oxidation, the deposition of iron arsenate takes place as well (99,100).

Depending on the conditions, Mn(II) oxidized by bacteria can be deposited as manganese(IV) oxides or as mixed manganese(II,III)/manganese(IV) oxides and oxyhydroxides. Thus in these precipitates the following minerals have been detected: vernadite, δ-MnO_2; todorokite, [Ca,Na,K][Mg,Mn]$Mn_5O_{12}{\bullet}H_2O$; buserite, [Ca,Na,K][Mg, Mn]$Mn_6O_{14}{\bullet}5H_2O$; feitknechtite, β-MnOOH; manganite, γ-MnOOH; hausmannite, Mn_3O_4 (101,102–104).

Localization of deposited Fe and Mn usually occurs on bacterial surface structures such as cell walls, capsules, sheaths and, in the case of mycoplasmas, on the plasma membrane. The most striking example is the sheath of *Leptothrix discophora*. Here, manganese is oxidized enzymatically on this outermost structure (105). The cells depart from old sheaths leaving them totally encrusted in manganese oxides (Fig. 7). Sometimes cells become so completely encrusted with metal oxides that the weight of the deposited mineral can exceed the cell weight

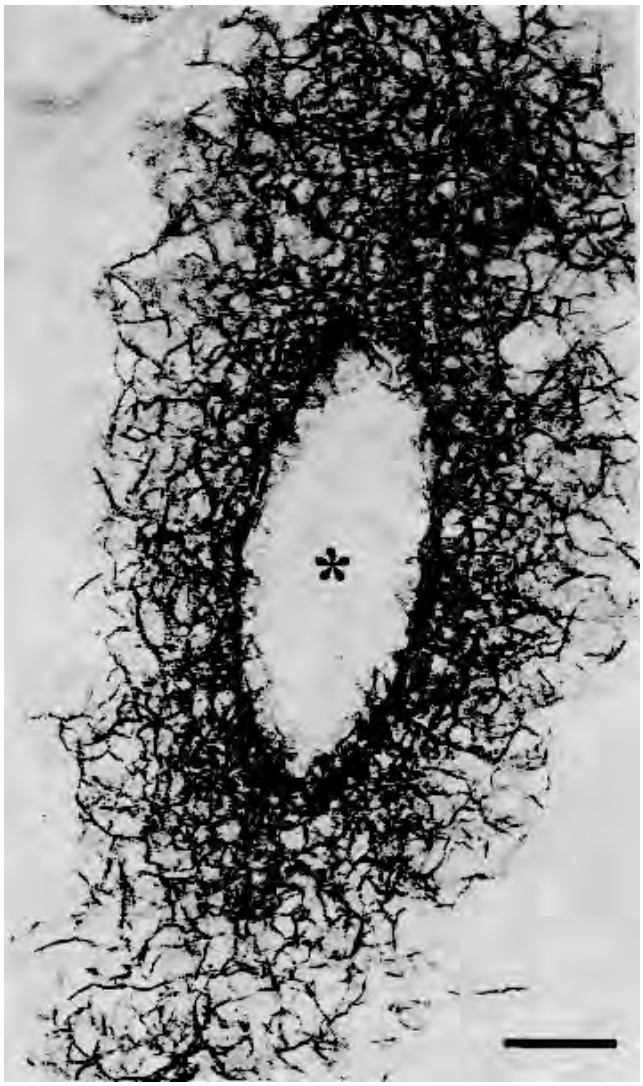

Figure 7

by an order of magnitude. However, deposition can also take place in the surrounding medium, especially when hydrogen peroxide is involved in the oxidation reaction (25,86).

Deposited Fe and Mn biominerals are finely dispersed and possess a high specific surface area. It has been reported that the specific surface area of *Leptothrix*-oxidized Mn is about four times the specific surface area of fresh abiotic precipitates and more crystalline manganese oxides. Biogenic manganese oxides have a high affinity for trace metals; the adsorption of Pb is more than four times that of abiotic crystalline oxides (106).

GEOLOGICAL EVIDENCE OF BIM

That bacteria can biomineralize under laboratory conditions has been shown for many combinations of bacteria and minerals (107); however, conclusive evidence in ancient sedimentary horizons for biogenic mineral formation is harder to find. Sometimes, the most that can be concluded is that biomineralization is the likeliest explanation for what is observed, given the difficulties in identifying remnants of bacteria in situ and relating their activities to a specific mineral or geological structure. The existence of an unusual mineral (in an environment in which it would not normally form) is a first clue that microbes have been active. Stronger evidence comes from electron microscopy studies of more recent horizons where visual observations have been combined with element analysis (energy dispersive X-ray spectroscopy; EDS) and selected area electron diffraction (SAED) (Fig. 6). These allow precipitates associated with cells to be characterized, and make it possible to identify those minerals around the cell perimeter, which have been mentioned in our previous sections. Isotope fractionation is another in situ method that has been used to identify past geological activity of microbes; it is based on the fact that life-forms (in this case early microorganisms) prefer to metabolize the lighter isotopes of elements such as C, H, O, N, S (25). Radioisotopes can be used for in situ studies of present microbial action (25).

Mineral Deposits

Iron Ores. Iron in ore deposits may be in the form of oxides, sulfides, or carbonates, each of which can be formed by bacteria. Clues that bacteria have been active include the presence of fossilized or living microbes possessing probable iron-metabolizing or iron-accumulating potential, or the existence of environmental conditions (at the time of formation) that enabled biogenic iron deposition (25). An example of modern iron ore deposits is found in some bogs overlying sediments rich in ferrous minerals such as glauconite or pyrite. Groundwater brings Fe^{2+} to the surface, where the rapid rate of oxidation is attributed to biocatalysis (see previous section on redox transformations); bacteria involved in the oxidation include *Leptothrix*, *Crenothrix*, *Siderocapsa*, *Metallogenium*, *Thiobacillus*, and *Gallionella*. Rivers and swamps in the Pine Barrens in southern New Jersey contain such oxidized iron deposits (108). The iron there was mined until it became economically unfavorable in the mid-1850s.

Banded iron formations are remarkable ancient stratified deposits that were formed throughout the world in the Precambrian era; most deposits formed around 2.2 to 1.9 billion years ago. The bands occur as continuous layers of alternating iron-rich and iron-poor minerals, with the total iron of a quality and quantity in some places to be a valuable ore. The microbial origin of these deposits is still controversial; one theory, based in part on fossil evidence, favors oxidation of ferrous iron by anaerobic photosynthesizing bacteria and algae (109). Another theory considers that bacteria oxidized carbon to produce the carbonate minerals characteristic of these formations, particulate Fe(III) serving as the electron acceptor (110).

The role of bacteria in forming ore deposits of other metals is still debatable but the evidence points increasingly toward biogenesis. The formation of uranium

ores is one example for which bacterial activity seems likely. The ore bodies develop when mobile hexavalent uranyl ions are reduced to insoluble tetravalent uranium forms. A likely reductant is hydrogen sulfide, given the close proximity of pyrite (FeS_2) to tetravalent ore minerals, and (at the generally low temperatures of sedimentary ore-forming environments) bacteria are the most likely source of H_2S. Indeed, sulfate-reducing bacteria (SRB) have been found in uranium ores in Hungary (111). Further support for biogenic formation of ores comes from laboratory studies showing that both gram-negative and gram-positive bacteria can concentrate silver and gold as particles in the cell wall (see previous section on redox transformations; 76,112).

Coal, Peat, and Oil. These share steps in their initial formation. Both coal and peat begin with large accumulations of biomatter that were most likely initially degraded by bacteria and fungi. Microbial activity stopped once all of the easily metabolizable compounds and some of the more refractory substances were consumed, the latter probably by anaerobic bacteria (25). Microbial evidence comes from remains of microorganisms identified in coal and from signature isoprenoid substances found in ancient oils. Further physical and chemical processes, such as heat and pressure, converted peat to coal or oil.

Mine Tailings and Their Leachates

Base metals (Cu, Zn, Fe, Ni, etc.) are generally mined from metal sulfide ores, most often pyrite. Exposure of sulfidic rock to air begins a slow oxidation process that is accelerated by native bacteria grouped together under the term *acidophilic chemolithotrophs*. The oxidation process generates acidity and dissolves the metal, creating acid mine drainage in the process. This natural process is exploited by mining companies to harvest metals from low-grade ores; however, higher-grade ores are extracted by more efficient means and the finely ground waste rock is deposited in tailings dumps (25). This mineral waste represents an oxidizable energy source to the bacteria, as exemplified by the activity of *Thiobacillus ferrooxidans*. These bacteria oxidize Fe^{2+} and sulfide, thereby generating Fe^{3+}, sulfuric acid, pH values as low as 1–2, and solutions with high metal concentrations (113). Drainage of acid leachates from the tailings represents some of the worst environmental problems worldwide. Hence, there is increasing pressure to understand the ecology and microbiology of these environments.

In addition to the *Thiobacilli*, researchers have shown that SRB play a significant role in tailings environments (107,114). These anaerobic, generally heterotrophic, bacteria require neutral to alkaline pH. Their numbers increase with depth in tailings deposits, although they are also found in the oxic zone where *Thiobacilli* are active. The H_2S generated by SRB reacts strongly with metal ions and the resulting precipitates are found both on the cell surface and throughout the environment surrounding the cell. The diagenetic minerals associated with SRB in Cu-Zn ore tailings from Kidd Creek Mine in Ontario, Canada, included amorphous FeS, mackinawite (FeS_{1-x}) and pyrite (114). Although the sulfate-oxidizing activity of bacteria dominates tailings, it is quite possible that *Thiobacilli* and SRB continuously recycle the sulfur in these minerals from soluble to insoluble forms. In another example in the oxic zone of tailings derived from a copper mine in Quebec, Canada, diagenetic iron phosphates and iron chlorides were detected associated with thiobacilli cell surfaces (76). The formation of minerals in the tailings piles shows the potential for the genesis of a mineral soil.

Freshwater Lakes and Rivers

Microbial populations in lakes tend to be much greater than those found in the open waters of seas; freshwater systems have populations approximately 10^2 to 10^5 per milliliter of water and approximately 10^6 per gram of sediment (25). The range reflects the wide variation in conditions that can be found in these environments, which are particularly susceptible to fluctuations in oxygen, temperature, and nutrient status. As mentioned previously (in the carbonate section), calcium mineralization at Fayetteville Green Lake, New York is mediated by *Synechococcus* GL24. Here the mineral phase alternates between gypsum and calcite, each being nucleated by the S-layer on the cyanobacterium. Because photosynthesis and respiration determine the pH of each cell's microenvironment, *Synechococcus* precipitates gypsum during the cold winter months (poor growth and circumneutral pH promotes gypsum) and calcite during the warmer summer season (good growth and a more alkaline pH promotes calcite). For more details on this study, see Reference 113.

Biomineralization can be a way to neutralize the toxicity of metals introduced to a lake. Lower Moose Lake in Ontario, Canada receives mine tailings drainage containing sulfate and other sulfur- and metal-containing compounds (44). Studies by TEM-EDS-SAED revealed that bacteria in the lake sediment served as nucleation sites for metal sulfides and (Fe,Al)-silicates. The formation of the minerals was explained by a combination of conditions including circumneutral pH, high metal concentrations, and low levels of sulfate reduction by bacteria, demonstrating again the specificity of the minerals to the environment that forms them.

In contrast to lakes, rivers tend to be uniform in temperature with depth and are usually well aerated. In addition, rivers vary in water velocity and turbulence. As with lakes, the nutritional status of rivers is variable and depends on the environmental setting. Researchers studying two rivers in the Amazon region of Brazil found that bacteria collected from the solute-rich Rio Solimões were encrusted with mineral precipitates, whereas bacteria sampled from the solute-deficient Rio Negro were not mineralized (46). The bacteria grew as biofilms on plant and sediment surfaces in the river and sequestered iron, as well as traces of Mn and Ni, in their capsules, suggesting a selective accumulation of these elements on the cell surface. Among the minerals found in these biofilms was a complex claylike (Fe,Al) silicate that has also been detected in other freshwater environments on other bacteria (115). Several characteristics point to a biogenic origin for these minerals, that is, the size of

the precipitates (~100 nm), their crystal structure, the tendency to form a crust around bacteria, and their chemical composition (which differed significantly from that found in other suspended material in the water). As discussed with respect to other minerals in the previous sections, an amorphous Al–Fe–Si phase forms, which dehydrates slowly and rearranges into a more crystalline form. In time, this process could form large deposits of minerals.

Hydrothermal Environments

The term *hydrothermal* encompasses a variety of natural terrestrial or marine environments that have exposure to water heated by geological activity. Often these regions have high concentrations of soluble silica and reduced metals. Although these are hostile environments, the metal-rich waters can provide energy — from reduced sulfur compounds or metals — to bacteria and large microbial populations may be found. Because silica and metals can embalm cells, preserving their natural arrangements in biogenic minerals, a fossil record is formed. Because of the extreme conditions [high temperature, high reduced metal concentrations, sulfide, salinity, and (sometimes) high pressure], these microorganisms often possess unique adaptations (116). They frequently belong to the *Archaea*. Three examples of biomineralization in different hydrothermal environments will be described — an ocean vent, a hot spring, and sediments from a shallow water hydrothermal system. For a review of microbiology in hydrothermal environments, see References 116 and 117.

Bacteria living near hydrothermal vents of the Southern Explorer Ridge in the Pacific Ocean provide nucleation sites for the growth of poorly ordered iron oxide, manganese oxide, and iron silicate particles, which coat cell surfaces and cell-associated filaments (118). A direct metabolic role for bacteria in mineral formation at ocean vent sites has not been shown. Researchers observed a similar preservation of bacterial cells in clay and mud sediments collected from an acidic hot spring at Yellowstone National Park (45). *Gallionella ferruginea* associated with hydrothermal brines in shallow waters around the Greek island of Santorini have a direct role in oxidizing Fe(III) bubbling up from vents, forming at least two meters of gellike sediments that contain poorly crystalline hydrous ferric oxide (HFO) precipitated around bacterial stalks (119). Transformations to more crystalline phases such as goethite (iron oxyhydroxide), siderite, and pyrite take place at greater depths, and pyrite formation is probably mediated by the activity of SRB.

Stromatolites

Stromatolites are ancient microbiolites that have been preserved by the minerals fixed in their biofilm communities as these communities grew. They are interesting because they record the presence of complex microbial ecosystems living up to 3.5 billion years ago during the Middle Archean period (120). The oldest known formations are the Onverwacht group in South Africa and the Warawoona group in Western Australia.

In cross section, stromatolites are finely laminated, with darker organic-rich cherts alternating with lighter carbonate-rich bands that have been cemented together over time. In form and shape they are similar to present-day microbial mats. Because they appeared at a time when the earth's atmosphere was reducing, it is thought that the organisms may have been lithoautotrophs or anaerobic photoautotrophs (120). These may have induced carbonate precipitation by creating an alkaline environment around the cell, although abiotic precipitation may also have contributed. For more information about stromatolites, see Reference 121.

Magnetotactic Bacteria

The term *magnetotactic bacteria* has no taxonomic significance and encompasses a diverse group of bacteria that display magnetotaxis. They are found throughout aquatic habitats, most often at the transition zone between oxic and anoxic conditions (6). Two regions where magnetobacteria have been studied in detail are Pettaquamscutt Estuary (Rhode Island, U.S.A.), and Salt Pond (Massachusetts, U.S.A.). Researchers studying magnetic properties within the water column at Salt Pond found that the peak in magnetic remanence (the magnetization recorded in the mineral) occurred in the oxic-anoxic transition zone. At this depth a great number of bacteria and the highest available Fe^{2+} (needed to form magnetite or greigite) were found (6).

Magnetotactic bacteria form unique structures (magnetosomes) that are aligned in rows to the cell axis and that contain membrane-bound single-domain magnetic particles of either magnetite or greigite (Fig. 1; 6). Magnetosomes can be preserved in sediments after these bacteria die and thus contribute to the paleomagnetic record if the concentration is sufficient. Some limitations are the detrital input of magnetic material, secondary formation of magnetic minerals, and mineral transformation of magnetosomes at depth. For example, Snowball (1994) found that the high concentration of biogenic magnetite in the upper portion of a Swedish lake sediment declined with depth (122). Bacteria can mediate chemical reactions leading to dissolution of magnetite or they can dissolve the mineral directly. The discovery that a Mars meteorite (ALH84001) contained magnetite particles similar in size and morphology to those produced by magnetotactic bacteria on earth has researchers speculating about interplanetary life (124). Such a discovery, if true, would considerably widen the geographic distribution of bacterial mineral formation. For a detailed review of magnetotactic bacteria, see Reference 6.

SIGNIFICANCE OF BIOMINERALIZATION

Iron Where It Is Not Wanted

Oxidation of reduced iron by bacteria is a worldwide nuisance. It results in plugged water pipes, wells, bore holes and field drains, as well as contaminated lakes and rivers. The slimy orange (although color and texture may vary) precipitates, consisting of iron oxyhydroxide or HFO, can be produced in copious amounts. Although the significance of Fe-oxidizing microbes in producing the deposits is controversial, researchers have

shown that both autotrophic and heterotrophic bacteria are responsible; the species depends on environmental conditions. *G. ferruginea* is found at neutral pH and low dissolved O_2 concentration. The characteristic twisted stalks of *Gallionella*, which extend from one side of the bean-shaped cells, become coated with HFO. *Thiobacillus ferrooxidans* is an example of a Fe-oxidizing chemoautotroph that also grows at the oxic–anoxic interface, although it prefers pH ~1 to 4. The bacterium, as previously described, produces acidity and ferric iron from pyritic ores. This iron leaches from the tailings to precipitate when it encounters higher pH, such as in a river or lake. See References 115 and 124 for more information on Fe-oxidizing bacteria.

Bioremediation

Bioremediation strategies that use bacteria exploit the affinity of bacteria for metals as well as their ability to reduce and oxidize metals. The latter requires living cells, whereas the former can use both living and dead biomass. Living systems include natural settings in which existing landforms such as ponds or streams are used to remediate metal contamination. These may already be in place, but are often simulated and have some kind of containment layer (e.g., a mine tailings leachate pond). In this case, immobilization of the metals is based on the interactions of an entire ecosystem, and contamination levels must be low enough that the microbes are not poisoned, although through natural selection heavy metal-resistant microorganisms often evolve. Over time, in aqueous systems, metals become part of the anaerobic sediments when the metal-loaded microorganisms die and sink to the bottom. Other kinds of living systems are those constructed to use organisms in an artificial environment for metals removal. One example is publicly owned treatment plants, which may incorporate a biological system to remove metals that remain after primary sedimentation; usually this is an activated sludge or trickling filter system. The metals are most often accumulated in the biopolymers produced by floc-forming microorganisms (usually bacteria) and not within the cells themselves (125). Another type of system uses cells colonized and immobilized (as a biofilm) on support material with the metal-loaded water flowing past. Good removals may be achieved with such systems, which can be tailored to immobilize specific metals (12).

Methods using sorption of metals by dead bacterial biomass are viable technologies for scouring metals from wastewaters and recovering valuable metals from ore-processing (126). Systems using dead biomass have the advantage that they are not susceptible to transient highly toxic concentrations of metals or other conditions; most waste streams are variable and can be toxic to living biomass. Furthermore, dead microorganisms can have higher metal-sorption capacities than living material (127). The dead biomass may be processed into a form in which it acts as an ion-exchange resin so that it can be regenerated by simple acid or alkali treatment. This makes such systems more economically competitive because the sorbed metals are easily recovered and the biomass can be reused (126).

Broad Implications

Biomineralization is a remarkably young science and has taken great strides in maturity over the last two to three decades. Who would have believed that the world's smallest life forms could have such a great impact on the earth's sedimentary fine-grained minerals? It is clear that one of the fundamental traits of microbial cells is to interact with the metal ions that surround them and, in so doing, profoundly affect geochemical conditions. Eh, pH, metal speciation, and interfacial chemistry are all affected, and sometimes enzymes act as catalysts for geochemical processes. It is imagined that since the dawn of life almost four Ga ago, prokaryotes have been reworking the earth, immobilizing metals with appropriate counter-ions and (presumably) even altering the primitive recipe of the oceans. Global carbon, hydrogen, nitrogen, oxygen, sulfur, phosphorus, and (now) metal cycling are all tied, at least partially, to microbes and their growth. With respect to mineralogy, bacteria have added their own particular flavor by instigating the production of a wide range of fine-grained minerals and by making possible the formation of some unusual minerals under geochemically unfavorable conditions. Exploring deep subsurface and (even) extraterrestrial microbiology may broaden the significance of bacteria to biomineralization.

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BIOMINING. See BIOLEACHING

BIOMINING USING ARCHAEA. See ARCHAEA IN BIOTECHNOLOGY

BIOODORS CONTROL IN WASTE TREATMENT FACILITIES. See BIOFILTRATION AND BIOODORS

BIOPESTICIDES. See INSECTICIDES, MICROBIAL

BIOPLASTICS

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Every living organism produces a tremendous variety of biopolymers. Each organism contains DNA, RNA, and proteins, but other important macromolecules found in living systems include polysaccharides (such as starch, cellulose, and xanthan gum), polyphosphates, polysulfates, polyphenols (such as lignin), isoprenoids (such as natural rubber), and polyhydroxyalkanoates (PHAs). PHAs are unique among the biopolymer groups in that they are all genuine thermoplastics — plastic materials that can be melted, formed, and reset. PHAs are unique among the plastics in that they are made inside living organisms, and thus they may be called "bioplastics." Sometimes materials such as polylactide are called "bioplastics" as well, but the polymerization is synthetic, using lactic acid produced by bacteria. This article focuses on PHAs because they are plastics made entirely by biological systems.

PHAs are water-insoluble polyesters that accumulate in the form of inclusions or granules inside microbial cells. The purified polymers have properties ranging from hard and brittle to soft and rubbery, depending on the composition. Many different kinds of monomers can be incorporated into PHAs, but the backbone of all of these monomers is essentially the same; they are all hydroxyalkanoic acids activated by linkage to coenzyme A. The polymerization is a process by which an enzyme called PHA synthase, or PHA polymerase, adds the activated monomer to the end of the growing polymer chain, yielding free coenzyme A and a polymer chain that has become one unit longer. The basic structure of PHAs is shown in Figure 1.

PHAs are made naturally by many species of bacteria as a carbon and energy storage compound, often under conditions where the carbon source is present in excess and the availability of some other element required for growth (such as nitrogen or phosphorus) is low. The polymer can accumulate to as much as 90% of the dry cell weight under controlled fermentation conditions, but generally much less under natural conditions. Table 1 shows some of the PHA-producing organisms and typical composition ranges for their PHAs. It is by no means an exhaustive list; more than 300 organisms are known to produce PHAs (1). Note that the absence of a composition in Table 1 does not necessarily signify the inability of the organism to produce that composition; these are only compositions that have typically been reported.

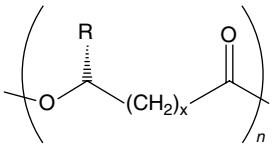

Figure 1. Chemical structure of PHAs. **R** represents a side chain having from 0 to 14 carbon atoms, **x** may range from 0 to 4, and **n** is an integer such that the molecular mass of the chain is higher than about 100,000 Da, typical of biologically produced PHAs. PHB: $\mathbf{R} = CH_3$, $\mathbf{x} = 1$. P(4HB): $\mathbf{R} = H$, $\mathbf{x} = 2$. PHO: $\mathbf{R} = (CH_2)_4CH_3$, $\mathbf{x} = 1$.

Table 1. Natural PHA Producers and Chain Lengths Found in Polymers

	Monomer Chain Length (Carbon Atoms)				
Organism	3	4	5	6	7+
Acinetobacter sp.		●	●		
Aeromonas caviae		●		●	
Agrobacterium sp.		●	●		
Alcaligenes latus	●	●			
Azotobacter vinelandii		●	●		
Bacillus megaterium		●	●		
Chromatium vinosum		●	●		
Chromobacterium violaceum		●	●		
Clostridium kluyveri		●			
Comomonas acidovorans		●	●		
Corynebacterium hydrocarboxydans		●	●		
Haloferax mediterranei		●	●		
Hydrogenophaga pseudoflava		●			
Legionella pneumophila		●			
Methylobacterium extorquens	●	●	●		
Methylobacterium organophilum		●			
Nocardia corallina		●	●	●	
Paracoccus denitrificans		●	●		
Pseudomonas oleovorans		●	●	●	●
Pseudomonas putida				●	●
Ralstonia eutropha	●	●	●		
Rhizobium meliloti		●	●		
Rhodobacter capsulatus		●	●		
Rhodococcus sp.		●	●	●	
Rhodospirillum rubrum		●	●	●	●
Sphaerotilus natans		●	●		
Spirulina platensis		●			
Synechococcus sp.		●			
Syntrophomonas wolfei		●	●	●	
Zoogloea ramigera		●	●		

The occurrence of PHAs in living cells has been known since the 1920s. Maurice Lemoigne of Institut Pasteur first discovered in 1925 that *Bacillus* spp. can synthesize 3-hydroxybutyrate (3HB) (2), the first reported production of 3HB by a microbe. In the two years that followed, he definitively identified a polymer of 3HB units having the formula $(C_4H_6O_2)_n$ and melting point 157 °C to be present as a reserve material in certain *Bacillus* spp. (3,4). He also observed that the polymer could be degraded by the organism under autolytic conditions to a material having a lower molecular weight, lower melting point, and different solubility characteristics.

The notion that PHAs are a carbon and energy storage material was solidified by the work of Macrae and Wilkinson, who set out to determine the physiological role of poly(3-hydroxybutyrate) (P(3HB), or simply PHB) formation in *Bacillus* spp. in 1958 (5,6). They observed the necessary conditions for demonstrating that PHB is a reserve carbon and/or energy source: that the accumulation of PHB increased as the carbon-to-nitrogen ratio increased, that PHB was rapidly degraded in the absence of a carbon/energy source, and that the degradation of PHB in the absence of a carbon/energy source could prevent cell autolysis and death. This degradation makes PHB not only useful to the cells as an energy source, but it also makes PHB useful to humans for its biodegradability.

Direct confirmation that PHB was the main constituent of the insoluble granules observed in bacilli occurred in 1958, when Williamson and Wilkinson (7) demonstrated that PHB within cells could be isolated and quantified by treating whole cells with sodium hypochlorite. Among their many analyses of the isolated material was the precipitation of a film of PHB from chloroform by evaporation, and they described it as "somewhat reminiscent of plastic sheeting." They also showed that the material underwent thermal decomposition, giving crotonic acid as a main product.

PHB was the only known naturally occurring PHA until 1974, when Wallen and Rohwedder (8) reported the isolation of a polyester containing 3HB, 3-hydroxyvalerate (3HV), and 3-hydroxyhexanoate (3HH) units from activated sludge. Nine years later, Findlay and White (9) found PHAs with monomer units having four to eight carbons in marine sediments and in *Bacillus megaterium* extracts, and in the same year, de Smet and coworkers (10) isolated a polymer composed predominantly of 3-hydroxyoctanoate (3HO) units from *Pseudomonas oleovorans* grown on octane. Presently, at least 100 different monomer types have been found in PHAs, ranging from three to fourteen carbons in length, and the position of the hydroxyl group has ranged from C-2 to C-6 (the α to ε carbon). The constituents found thus far have invariably been (*R*)-hydroxyacids; the (*R*) configuration, the hydroxyl group, and activation with CoA are characteristics to which no exceptions have yet been found. For a comprehensive review of the diversity of monomer types found in PHAs, the reader is encouraged to consult Steinbüchel and Valentin (11).

The thermoplasticity and biodegradability of PHAs has led to more than just academic interest in these compounds. W. R. Grace & Co. were the first to recognize the commercial potential of PHB. They used *B. megaterium* and *Rhodospirillum rubrum* for production, and in 1960 the company filed the first patent concerning efficient production of PHB (12). W. R. Grace eventually abandoned the project, but Imperial Chemical Industries (ICI), later Zeneca, revived it in the 1970s, with an important modification: the efficient introduction of monomers other than 3HB. They found that the crystallinity of PHB could be reduced in this way, thus making the polymer more flexible and commercially of greater potential (13). This led to the development of Biopol, a copolymer of 3HB and 3HV units in about a 4 : 1 ratio. Biopol first became commercially available in 1990 in the form of shampoo bottles in Germany (14). The Monsanto Corporation acquired the technology in 1996, but it discontinued production of Biopol, which had reached about 800 tons per year (15), blaming lagging market interest in renewable materials (16). The Biopol composition will become public domain in 2001 on the expiration of the original ICI patent.

The remainder of this article explores in more detail the following aspects of PHAs: metabolism (both naturally occurring and via metabolic engineering), material properties and applications, production and recovery, and market and economics.

PHA METABOLISM

There are three main modes of PHA production in naturally occurring organisms: (*1*) condensation of acetyl-CoA, (*2*) fatty acid β-oxidation, and (*3*) fatty acid biosynthesis. It is possible, of course, for these organisms to derive hydroxyalkanoyl-CoAs from closely related carbon sources such as the corresponding hydroxyacids, but the three modes discussed here are concerned primarily with PHA production from common carbon sources such as sugars and oils. The final PHA composition is affected strongly by the mode or modes employed for its synthesis. Condensation of acetyl-CoA generally leads to short-chain PHAs, commonly defined as having monomer units of up to 5 carbon atoms, whereas fatty acid β-oxidation generally leads to medium-chain PHAs having units of 6 to 14 carbon atoms. Long-chain PHAs have 16 or more carbon atoms per unit, and these have appeared in PHAs only in small proportions from β-oxidation of long-chain fatty acids (17). Metabolic engineering of PHA pathways is also discussed in the following sections because it provides both flexibility and novelty in polymer compositions, and it also can enable PHA production in nearly any organism desired.

Condensation of Acetyl-CoA

Macrae and Wilkinson (5) speculated in 1958 that PHB is synthesized via acetoacetate by the condensation of acetate and some other two-carbon compound, and that the active compound for polymerization is 3HB-CoA. A direct demonstration that 3HB-CoA is the species used for polymerization was given by Merrick and Doudoroff in 1961 (18). They also provided evidence that 3HB was not directly activated with CoA. On the basis of

assays of 3-ketothiolase and 3HB-CoA dehydrogenase in cell extracts, Schindler (19) correctly concluded in 1964 that the pathway shown in Figure 2 is the one employed by *Ralstonia eutropha*. The PHB pathway proceeds by condensation of two acetyl-CoA molecules to form acetoacetyl-CoA, followed by a reduction to 3HB-CoA and polymerization. The most common implementation of this pathway is that found in organisms such as *R. eutropha* and *Zoogloea ramigera*, where the reduction specifically produces the (*R*) isomer of 3HB-CoA. A more complex pathway exists in *R. rubrum*, however; the overall reduction to (*R*)-3HB-CoA appears to be accomplished first with a reduction of acetoacetyl-CoA to (*S*)-3HB-CoA, followed by the action of two enoyl-CoA hydratases with the intermediate crotonyl-CoA (20).

The first step in the PHB pathway, the reversible condensation of two acetyl-CoA molecules to acetoacetyl-CoA with the release of free CoA, is catalyzed by a 3-ketothiolase. The substrate specificity of these enzymes dictates that one reactant is always acetyl-CoA, but the other reactant can vary in length. Many organisms possess more than one of this type of enzyme. *Ralstonia eutropha*, for example, has at least two 3-ketothiolases that can participate in PHA synthesis. One will condense acetyl-CoA with acetyl-CoA or propionyl-CoA, and the other appears to accept all acyl-CoAs from acetyl-CoA to octanoyl-CoA (21). Condensation of acetyl-CoA with propionyl-CoA yields 3-ketovaleryl-CoA, which is reduced to 3HV-CoA. Propionyl-CoA, not nearly as common as acetyl-CoA in biological systems, is an end product of the catabolism of fatty acids with odd numbers of carbon atoms, but it is also derived in some organisms from succinyl-CoA via methylmalonyl-CoA (22,23). 3-Ketothiolase has a regulatory role in PHA synthesis by this route. In many cases, the condensation reaction is strongly inhibited by free CoA (21,24–27). In *R. eutropha*, the PHB pathway is constitutively expressed (28), so the regulation of 3-ketothiolase activity seems to be at the enzymatic level (25). The intracellular free CoA level does appear to be significantly lower during PHB-producing conditions (29).

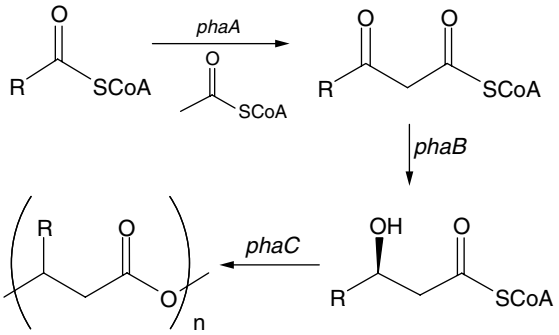

Figure 2. Pathway from acetyl-CoA and other acyl-CoAs to PHAs. When $\mathbf{R} = CH_3$, two acetyl-CoA molecules are condensed by 3-ketothiolase (EC 2.3.1.9), the product of the *phaA* gene. The resulting acetoacetyl-CoA is reduced to (*R*)-3-hydroxybutyryl-CoA by 3-ketoacyl-CoA reductase (EC 1.1.1.36), the product of the *phaB* gene, and finally PHA synthase (no EC number), the product of the *phaC* gene, incorporates an (*R*)-3-hydroxybutyrate unit into PHA with the release of CoA. When $\mathbf{R} = C_2H_5$, propionyl-CoA is condensed with acetyl-CoA, and ultimately an (*R*)-3-hydroxyvalerate unit is incorporated into PHA.

The reduction of acetoacetyl-CoA to (*R*)-3HB-CoA is accomplished in most cases by a single stereospecific reductase. Most of the reductases characterized to date have shown a preference for NADPH over NADH as cofactor (30–32), but there is at least one case where NADH is preferred (33). This second step of the pathway exerts some thermodynamic control over PHB synthesis. The 3-ketothiolase reaction is thermodynamically more favorable in the direction of cleavage, not condensation (34). Therefore, for PHB synthesis to proceed, the acetoacetyl-CoA concentration must remain low. This can be accomplished with a high NADPH/$NADP^+$ ratio, which will thermodynamically favor (*R*)-3HB-CoA formation. Provided that the PHA synthase activity is sufficient, the NADPH/$NADP^+$ ratio is instrumental in fueling the pathway (35).

PHA synthase is the final step of all the pathways to PHA, so the substrate specificity of this enzyme controls the polymer composition to some degree regardless of the pathway employed. Most synthases have a preference for either short-chain or medium-chain PHAs, although some recent exceptions have been reported. The *Aeromonas caviae* synthase accepts both 3HB-CoA and 3-hydroxyhexanoyl-CoA (3HH-CoA) (36), and the *Thiocapsa pfenigii* synthase accepts a broad range of substrates (37,38). The majority of synthases have only one type of subunit, but of the first 30 to be sequenced, four have two subunits (39).

Fatty Acid β-Oxidation

The β-oxidation pathway (Fig. 3) is the most common mode of fatty acid catabolism. Some bacteria, most notably the pseudomonads, can divert carbon flow out of β-oxidation to store the carbon as PHA. Fatty acids are first activated with CoA, then undergo repeated rounds of removal of two-carbon units, ultimately in the form of acetyl-CoA, by a thiolase. This is the same type of reaction catalyzed by 3-ketothiolase, but the reaction proceeds predominantly in the direction of cleavage. Hydroxyacyl-CoAs are intermediates in β-oxidation, but they appear in this pathway as the (*S*) stereoisomer. The diversion of these intermediates therefore requires the conversion of the (*S*) form to the (*R*) form. Alternatively, the (*R*) form can be formed directly by the action of an enzyme that is not part of the β-oxidation pathway.

The pathways that organisms actually use to divert carbon flow from β-oxidation to PHA synthesis have only recently begun to be explored. It has been known since 1983 (10) that *Pseudomonas* spp. can effectively synthesize medium-chain PHAs from fatty acid feeds. Precisely how they do this is still not known, although it is presumed to be via one of the three possible activities for the generation of (*R*)-hydroxyacyl-CoAs shown in Figure 3. Expression of *Pseudomonas aeruginosa* PHA synthase in *E. coli* fatty acid degradation mutants resulted in medium-chain PHA production (40). This suggests that *Escherichia coli* possesses at least one of these three activities, although it does not normally synthesize PHAs

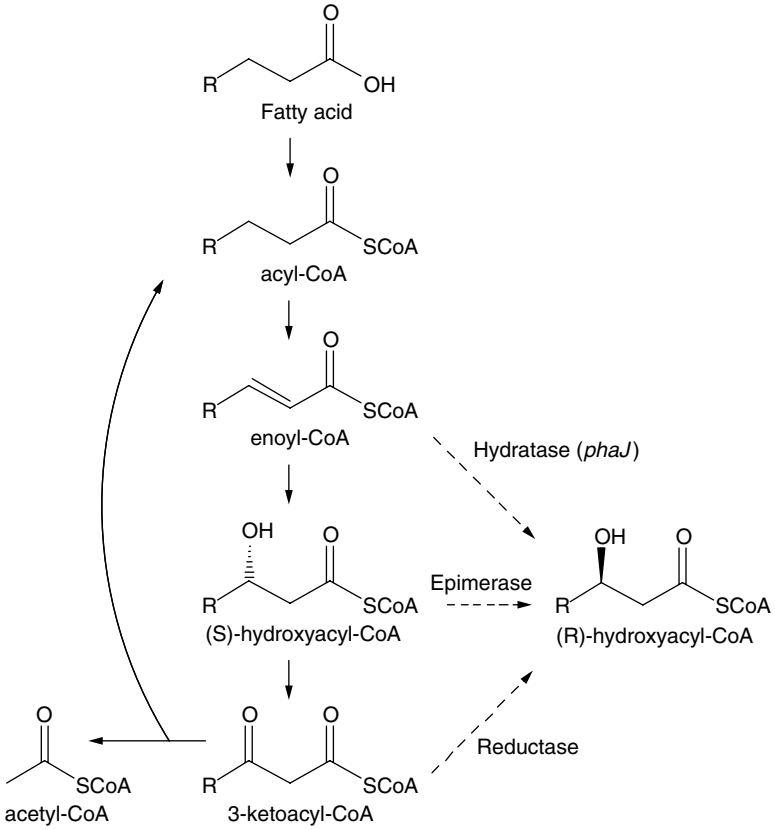

Figure 3. The derivation of PHA precursors from the β-oxidation pathway. The solid arrows represent reactions that are known to occur widely in biological systems. The dashed arrows represent reactions that can generate (*R*)-3-hydroxyacyl-CoAs, the precursors of PHA. In each round of β-oxidation, the alkyl group **R** becomes shorter by two carbons.

at all, and it also suggests that these additional activities are common in nature. Only one of the three activities has actually been found, but not in *Pseudomonas*: an (*R*)-specific enoyl-CoA hydratase. The first example of this activity was found in *A. caviae*, which can synthesize P(3HB-co-3HH) from fatty acids or oils (36,41). Another example of the activity was found in *R. rubrum* (42). Both genes have been cloned and are denoted as *phaJ*. Both of these hydratases accept enoyl-CoAs of four to six carbons, but not eight. The *R. rubrum* enzyme, when expressed in *E. coli* with the promiscuous PHA synthase of *Thiocapsa pfenigii*, produced P(3HB-co-3HH) when the carbon source was oleic acid. This shows how enzymes other than PHA synthase can at least partially dictate polymer composition.

Fatty Acid Biosynthesis

Both prokaryotes and eukaryotes synthesize fatty acids. Both use them for lipids to construct membranes. Many eukaryotes store energy by fatty acid synthesis in the form of fats and oils, but bacteria generally do not. Some bacteria, however, can use PHA synthesis to divert carbon flow from fatty acid synthesis to store carbon and energy (Fig. 4). In this sense PHAs can be thought of as "bacterial fat."

In 1990 it was found that many pseudomonads can synthesize medium-chain PHAs when fed gluconate (43,44), suggesting that the fatty acid biosynthesis pathway might be involved. Later it was shown in *Pseudomonas putida* grown on glucose that the degree of unsaturation in the PHA side chains decreased with temperature, as is generally also true of membrane lipid composition, offering more evidence that the fatty acid biosynthesis pathway can be used for PHA synthesis (45).

PHA synthesis using the fatty acid biosynthesis pathway, just as from the β-oxidation pathway, requires at least one additional activity for the synthesis of (*R*)-hydroxyacyl-CoAs. The intermediates of fatty acid biosynthesis are all of the (*R*) form, but the prosthetic group is the acyl carrier protein (ACP). Therefore, ACP must be exchanged for CoA, either directly by a transacylase or by two sequential activities, a thioesterase to remove ACP and a transferase or synthetase to activate with CoA. One development in this area was the discovery of the *phaG* gene in *P. putida* and the demonstration that it is a 3-hydroxyacyl-ACP : CoA transferase (46). The gene was also found in *P. aeruginosa* (47).

Metabolic Engineering

Naturally occurring PHA-producing organisms certainly generate a wide array of polymers. The diversity of PHAs and their numerous potential applications have motivated a great deal of effort in metabolic engineering of PHA production in both academia and industry. The main advantages that metabolic engineering offers in PHA production are that it can generate novel PHA compositions, allow PHA synthesis from less expensive substrates, improve the characteristics of the material, give better control over composition, improve the efficiency of production, and enable PHA synthesis in almost any

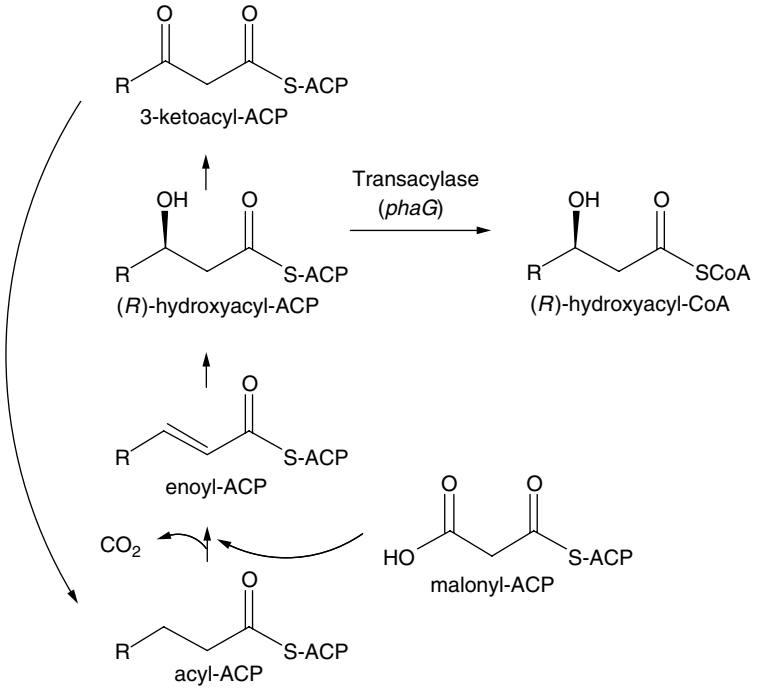

Figure 4. The derivation of PHA precursors from the fatty acid biosynthesis pathway. In each instance of condensation with malonyl-ACP, the alkyl group **R** becomes longer by two carbons.

desired organism. These advantages are summarized in Table 2, with an example of each.

Metabolic engineering of PHA production became possible in the late 1980s, when three independent groups cloned the PHA biosynthetic genes (*phaCAB*) from *R. eutropha* (48–50). Much progress has been made since then in assembling a library of cloned genes useful for PHA synthesis. For example, at least 30 PHA synthase genes have been cloned (39).

The host organism of choice for metabolic engineering of PHAs has most often been *E. coli*. PHAs with monomer units ranging from 3 to at least 12 carbons have been synthesized in engineered *E. coli* (42,51–54). The use of *E. coli* for PHA production is desirable for technical as well as economic reasons. The genome of *E. coli* has been sequenced in its entirety, and its metabolism and genetic techniques are well understood. Because *E. coli* does not naturally produce PHAs, it possesses no ability to degrade PHAs or synthesize unwanted PHA constituents. Unlike many natural PHA producers, *E. coli* grows at 37 °C and is relatively easily lysed. Furthermore, the use of a single organism to make an array of compositions necessitates only one general fermentation procedure, even if feeding strategies and operating conditions vary. Good control over composition allows the tailoring of material properties within a design space, a theme that is explored in the next section.

Table 2. Some Advantages of Metabolic Engineering in PHA Production

Advantage	Example	Ref.
Novel compositions	Poly(3-hydroxyhexenoate) with polyketide synthase	121
Inexpensive substrates	P(3HB-co-3HV) from glucose in *E. coli*, from CO_2 in plants	122
Characteristics of material	Molecular weight control	123
Control over composition	P(4HB) with no residual PHB	124
Efficiency of production	Reduction of *Pseudomonas putida* lysate viscosity	125
Any desired organism	PHB synthesis in cotton fiber	126

Properties and Applications

Because more than 100 different constituents have been incorporated into PHAs, and because these can be mixed in the same polymer chain, the number of compositions that can be generated is quite large. The constituents vary in length, position of the hydroxy group, and presence of functional groups and double bonds. As a result, the material properties and applications of PHAs are quite diverse. All of the possible combinations of monomer constituents make up the design space in which the engineering of PHA material properties can take place. The first conscious exercise of this nature was undertaken by ICI. They wished to develop PHAs commercially, but PHB by itself suffers from some disadvantages. Its melting point (about 180 °C) is very close to the temperature at which it begins to undergo rapid loss of molecular weight by thermal degradation, making melt processing somewhat difficult. When processed conventionally, it is also too brittle a material to be useful in many applications because of its very low nucleation density and high crystallinity. On adding propionate to an *R. eutropha* fermentation as a cofeed, it was found that significant amounts of 3HV were incorporated into the polymer. This increased the flexibility of the material, as measured by

modulus, elongation to break, and impact strength, and it significantly depressed the melting point (13). Thus began the endeavor to explore more fully the PHA design space.

Material Properties

Many PHAs have been evaluated for basic material properties over the last two decades. The short-chain PHAs tend to be more crystalline than the medium-chain PHAs, and hence generally the short-chain PHAs are more rigid, whereas the medium-chain PHAs are more rubbery. Table 3 gives an overview of the material properties of some PHAs. Its purpose is to illustrate the range of properties that exists; certainly many more compositions have been characterized.

Biologically produced PHAs can have molecular masses of more than 1 million daltons. Although the ring-opening polymerization of lactones to produce PHAs is a well-known procedure, its main drawbacks are that its products are generally of lower molecular weight and that, in cases where chirality is an issue (when the hydroxyl group is not terminal), its products are not stereoregular, and hence not isotactic (61). Molecular weight problems become more pronounced for the products of less strained lactones such as P(4HB) from γ-butyrolactone.

PHB does not have especially advantageous properties, as discussed earlier, but it is evident from Table 3 that copolymerization with another monomer type can improve the situation. In many cases, even a few percent of another constituent lowers the melting point and apparent crystallinity significantly. Sometimes the other constituent can cocrystallize with PHB to some extent, as has been reported for 3HV (56), so a somewhat higher percentage may be required to achieve the same plasticizing effect. PHB copolymers of this type are similar to commodity plastics such as polypropylene and high-density polyethylene, hence the first commercial application of P(3HB-co-3HV) as shampoo bottles (14). As the PHB comonomer fraction increases, material properties may change significantly. For example, P(3HB-co-15% 4HB) stretches and recovers like a rubber band but is translucent. P(4HB) homopolymer is extremely tough but also is very flexible. Medium-chain PHAs such as poly(3-hydroxyoctanoate-co-20%-3-hydroxydecanoate) are translucent and rubbery but somewhat stiff, and they can be tacky due to their low melting points.

Biodegradability and Biocompatibility

A distinguishing feature of PHAs is that they are fully biodegradable (can be reduced to carbon dioxide and water in a natural environment in a relatively short time) and often biocompatible (do not cause adverse reactions when present in living biological systems). Although they are degraded by microorganisms in composting, PHAs are stable in everyday environments; they do not break down on the shelf.

The environmental degradation of PHAs primarily is due to the action of microbially secreted depolymerases, which hydrolyze the polymer to compounds that are taken up by bacteria, fungi, and algae and converted ultimately to carbon dioxide and water. Methane can also result from anaerobic biodegradation. The depolymerases only hydrolyze ester linkages of monomers in the (*R*) configuration (63), and thus synthetic nonstereoregular PHAs are not completely biodegradable in this sense. The rate of degradation varies with composition and molecular weight, and it also varies with the type of natural environment. A bottle made from P(3HB-co-3HV), for example, will degrade completely in 5 to 10 years in a freshwater lake (64) but within 12 weeks in sewage sludge (65). P(3HB-co-4HB) degrades in seawater at about the same rate as P(3HB-co-3HV) (66) and somewhat faster than PHB and P(3HB-co-3HV) in soil (67). In sterile aqueous environments, polymers such as these hydrolyze very slowly. Doi and coworkers (68) showed that after 180 days at 37 °C in 0.01 M phosphate buffer at pH 7.4, PHB and copolymers with 3HV and 4HB had not lost significant mass, but their molecular weights (or average polymer chain lengths) began to decrease, apparently due to simple hydrolytic chain scission.

The biocompatibility of several PHAs has been evaluated. PHAs can perform very well in this capacity,

Table 3. Range of PHA Material Properties in Comparison with Those of Common Plastics

Composition	M_w (kDa)	T_m (°C)	T_g (°C)	ΔH_m (J/g)	Crystallinity (%)	Tensile Strength (MPa)	Elongation to Break (%)	Ref.
P(3HB)	1,020	177	4	97	60	43	5	127
P(4HB)	780	53	−48	9	34	104	1,000	128
P(3HP)	200	77	−19	74	37			129
P(3-hydroxy-4-pentenoate)	95	63	−11	15				130
P(3HB-co-20% 3HV)		130			35	20	50	131
P(3HB-co-25% 3HV)	336	132	0	40				13
P(3HB-co-17% 3HH)	1,122	120	−2	34	26	20	850	127
P(3HB-co-10% 4HB)	1,185	159	−3	54	46			132
P(3HB-co-10% 4HB)					45	24	242	133
P(3HB-co-8% C_8-C_{12})	1,392	133,146	−8	39		17	680	134
P(3HO-co-12% 3HH)	178	59	−37	19				135
Polypropylene		176	−10		50–70	38	400	136
Polyethylene terephthalate		267	69		30–50	70	100	136
High-density polyethylene		135	−80		80–95	30	30–1,000	137,138

provided they are extremely pure. Medium-chain PHAs, for example, have been shown not to elicit a significant inflammatory response after 40 weeks subcutaneously implanted in mice and not to elicit an allergic response on 48-hour exposure to the skin of guinea pigs (69). In many cases, PHAs are also resorbable in animal systems, meaning they can be completely metabolized over time by the host without production of toxins or adverse health effects. Short-chain PHAs such as PHB have been shown to be both biocompatible and resorbable in a relatively short time (70,71). PHB is actually present as low molecular weight oligomers at a few milligrams per liter in human blood plasma (72), and 3HB is a commonly encountered ketone body (73); significant toxicity of PHB hydrolysis products thus seems unlikely.

Applications

PHAs can be processed like commonly used thermoplastics. They can be useful in the form of containers and other molded items, disposable packaging and articles, medical implants and devices, coatings and paints, and in many specialty applications. Different compositions of PHA will, of course, find uses in different areas, especially when the distinction between the more rigid character of short-chain PHAs and the more rubbery character of medium-chain PHAs is considered.

PHAs can be recycled like many commodity-based thermoplastics, but because they are biodegradable, they lend themselves especially well to applications in which recycling is not practical, such as in single-use disposable food packaging and garbage bags, in which the plastic is mixed with food or garbage, and personal care items, in which the plastic is either contaminated or the article is made of different types of plastic that are too difficult to separate. Biodegradation can be a useful alternative to recycling in some cases. Mixtures of polyethylenes of different molecular weights, for example, often suffer deterioration of mechanical properties on repeated recycling and processing to the point when the material becomes unusable (74). P(3HB-co-3HV) did in fact appear in disposable applications during the 1990s, while Zeneca still produced Biopol. It was used not only in bottles in Germany, Japan, and the United States, but it also appeared in disposable razors and refrigerated meat trays (75). Compostable diapers and sanitary napkins made from PHAs are of current industrial interest (74,76–79). These are a good illustration of the utility of PHAs; diapers, for example, may be composed of nonwoven absorbable material, backsheet, and pressure-sensitive adhesive tape, made from PHAs and fully biodegradable.

The biocompatibility and in some cases resorbability of PHAs make them very useful as medical materials. Applications include tissue coatings, stents, sutures, tubing, prostheses, bone and tissue cements, tissue regeneration devices, wound dressings, drug delivery materials, and diagnostic and prophylactic uses (80). An active area of research with PHAs is tissue engineering (81), in which a resorbable material is used as a scaffold to "grow" human tissue from the patient's own cells. Recently, trileaflet heart valves were formed from P(3HO-co-3HH) and from P(4HB) and were seeded with mammalian cells, and each material showed a good level of cell attachment and collagen formation (82). These materials offer the added benefit of moldability and pliability; in the same study, a working valve could not be formed from polyglycolic acid, a traditional medical material. PHAs have already been successfully used to regenerate pericardial tissue and limit postoperative pericardial adhesions (83,84) and to correct atrial septal defects (70) in vivo. In drug delivery applications, PHAs can be used subcutaneously or in pill form in humans or as a stomach bolus in animals (85). In either case the composition of the PHA can be tailored so that the approximate release rate of the drug is appropriate without the need for frequent dosing. In any surgical or dressing application, bits of material left in the body are of no concern as they are simply resorbed (85).

PHA granules produced in biological systems are usually amorphous, either because of very low nucleation density, as with short-chain PHAs, or to low crystallization rates or lack of inherent crystallinity, as with medium-chain PHAs (62). Aqueous processing of these granules can yield a slurry of granules called latex. On spreading the latex onto a surface, it dries and can crystallize either spontaneously or when stimulated by heat or a nucleating agent. Medium-chain PHAs with unsaturated side chains can even cross-link during drying (86). Such latexes can be used to coat paper or plastic and as water-based paint ingredients (86,87) or to coat cheese and other foods (88). P(3HB-co-3HV) received food contact approval in Europe in 1995 (87).

Specialty applications of PHAs are too numerous to list here. PHAs can be used in agricultural applications such as planters and films, where biodegradability eliminates the need for removal, and they can be used as vehicles for controlled release of pesticides, with biodegradation being the means to liberate chemicals embedded in the polymer (85). They are useful as binders, where they hold powders or small pieces of another material together and are subsequently burned out cleanly (89). Intriguing food applications include chewing gum base (90) and fat substitute (91). PHAs with unsaturated side chains can be cross-linked to form materials that behave similar to rubber, yet are still fully biodegradable (62).

An often overlooked but potentially very important application of PHAs is not as polymers, but as chemicals derived from those polymers hydrolyzed chemically or biologically. (*R*)-hydroxyalkanoic acids can be used to synthesize antibiotics, vitamins, aromatics, and pheromones (92). (*R*)-3HB is used as a starting material for the Merck antiglaucoma drug Trusopt (93). Other chemicals with existing markets can be derived from PHAs by simple chemical reactions; for example, PHB can yield 1,3-butanediol, crotonic acid, β-amino acids, butyl esters, lactones, and other compounds (94). Analogous arguments can be made for P(4HB), P(3HO), etc.

Production and Recovery

The two types of organisms that are most favorable for industrial production of PHAs are bacteria and plants. Bacterial fermentation can be carried out as Zeneca

developed the process, with natural PHA producers such as *R. eutropha*. With the advent of routine bacterial genetic manipulation since that time, recombinant producers such as *E. coli* have been developed as well. The engineering of PHA production in plants is a longer-term solution because plant genetic manipulation is not routine. The reward, however, would justify the task; the resulting large scale and low cost of PHA production could make them a real alternative to commodity plastics derived from fossil fuels.

Fermentation and agricultural production of PHAs ideally coexist. Plants are suited to large-scale commodity applications without much complexity in composition. Crops are subject to variations in climatic and soil conditions, and they must synthesize all polymer constituents from carbon dioxide. Therefore, fine control of PHA composition and characteristics is likely not possible in plants. Bacterial fermentation is more expensive, relatively small-scale, and rather energy-intensive, making it questionable for long-term commodity production. However, bacterial fermentations can be controlled precisely, and any feedstock desired may be used. Medical and other specialty applications require this flexibility and do not require the immense scale of agriculture.

The most commonly used organisms in fermentation have been *R. eutropha* for short-chain PHAs and *Pseudomonas* spp. for medium-chain PHAs. Both types of cells are cultured aerobically in a fed-batch mode. Typically, the natural producers of PHA require limitation in some nutrient other than the carbon source to accumulate PHA to more than a few percent of the dry cell weight. Limitations in nitrogen and phosphorus are typically used in practice. This can be implemented by including in the medium all the nitrogen or phosphorus needed to achieve the desired cell density; when this is completely consumed, growth ceases and PHA accumulation begins. For example, *R. eutropha* accumulated 137 g/L of P(3HB-co-29% 3HV) at 67% of the dry cell weight in 65 hours with a feed of corn oil and propionic acid under phosphorus limitation at 34 °C (95). An interesting alternative to *R. eutropha* is *Alcaligenes latus*, which can accumulate PHA in significant amounts during exponential growth and can be cultured at up to 42 °C (96). *Pseudomonas putida* accumulated 73 g/L of medium-chain PHA, containing predominantly 3HO and 3HD at 51% of the dry cell weight in 38 hours on oleic acid and phosphorus limitation at 30 °C (97).

Transgenic PHA production has been developed primarily with *E. coli*, although genetic manipulations have been made on natural PHA producers as well. There are currently many advantages to the use of transgenic *E. coli*: genetic manipulations are routine, the metabolism is well understood, the genome has been completely sequenced, minimal medium can be used with many types of feedstocks, nutrient limitation is not necessary, the cells lyse easily, growth at up to 42 °C is possible, no depolymerases are present to degrade the product, and the choice of one organism eliminates the need to develop a markedly different fermentation process every time a new polymer composition is sought. *Escherichia coli* is capable of performance at least on a par with natural producers; a strain expressing the *R. eutropha* PHA synthesis genes from a plasmid accumulated 157 g/L of PHB at 77% of the dry cell weight in 49 hours with a glucose feed at 30 °C (98).

PHA production in plants has been implemented only on a very small scale. The first difficulty to be overcome with plants is the achievement of high, stable expression of multiple foreign genes in the same plant. The compartmentalization and complexity of metabolism is an added difficulty. However, there is reason to be optimistic; in 1994 it was reported that *Arabidopsis thaliana* plants could produce PHB at up to 14% of the dry cell weight and still develop normally when the *R. eutropha* PHA synthesis genes were targeted to the plastid (99).

The two main modes of PHA recovery from biomass are solvent extraction and aqueous processing. Solvent extraction consists of suspending biomass, usually dried, in a solvent that can dissolve the PHA of interest, removing solids, then precipitating the polymer either by the addition of a poor solvent, by evaporation, or by cooling. Aqueous processing is a general term for recovery techniques that begin with wet biomass, as from a fermentation or wet milling operation, and proceed in the presence of water to yield either an aqueous, largely amorphous latex or dry polymer with some degree of crystallinity, depending on its composition. An amorphous latex can be reconstituted from a crystalline PHA (100).

Large-scale application of solvent extraction suffers from the need to use large volumes of solvents; PHA solutions become viscous even at relatively low concentrations. The most useful solvents for short-chain PHAs are chlorinated solvents such as chloroform and 1,2-dichloroethane, which must be contained carefully. The latter problem has been addressed to some degree by the use of nonchlorinated solvents at higher temperatures (101,102). Medium-chain PHAs can readily be extracted with relatively benign solvents such as acetone, isopropanol, and hexane (103,104).

There are many variations of the aqueous processing theme, but generally aqueous processing consists of one or more of the following steps: separation of solid biomass from bulk liquid, mechanical or enzymatic cell breakage, solubilization of non-PHA biomass, and isolation of polymer. Usually the polymer remains in amorphous granule form throughout the procedure, although exceptions exist, such as melting and coalescence of granules followed by settling of bulk polymer (105). Initial reduction of the liquid volume is especially useful when bacterial cells are taken from a fermentation and the reduction is accomplished by centrifugation, filtration, or flocculation. Bacterial cells can be broken by heat treatment (106), homogenization (107), or chemical treatment with hypochlorite (108,109), alkali (110), surfactants or water-soluble copolymeric dispersants (111), or oxidants such as hydrogen peroxide (112) or ozone (113). Heat treatment may be followed by digestion with enzymes such as proteases, lysozyme, and DNase, which can become costly (106). Homogenization is rather energy-intensive (114) and does not effect any purification. The chemical treatments have the advantages of being inexpensive and facilitating purification by solubilizing non-PHA biomass (115). Recovery of the polymer granules from the resulting solution is generally done by washing and centrifugation, but other

methods such as sedimentation fractionation (116) and air classification (117) have been described.

Market and Economics

Above all else, the factor that will determine the fate of PHAs on the market is the price. There is no question about the usefulness and favorable properties of these materials, but the last sales of any volume to take place were of Biopol in the 1990s, for about $8/lb (118). Even with all the goodwill of biodegradability and derivation from renewable resources, bulk commodity applications certainly cannot take hold at this price. With larger scale and established markets, come lower costs, but these have not yet developed because plastic users in many cases have little experience with PHAs. At present only very small quantities of PHAs are available commercially, so market development is crucial.

One exception to the price issue is the medical use of PHAs. Here performance is much more important. The cost of the polymeric material in, for example, a heart valve replacement procedure is of little consequence, but the specifications on the material are quite exacting. The main hurdles to market acceptance in this area still lie in regulatory approvals and in confident acceptance by medical associations. The overall yearly demand for vascular grafts and heart valve replacements worldwide corresponds to approximately 1 million procedures (81).

A few analyses of expected cost of PHA production have been conducted recently by van Wegen and coworkers (107) and by Choi and Lee (118,119). These studies estimated the cost of producing short-chain PHAs using technology that is currently available. Both used a process consisting of fermentation followed by centrifugation, hypochlorite treatment, two more rounds of centrifugation, and finally drying. The main difference between the studies was that one used homogenization (three passes) and the other simply used a surfactant to complement the hypochlorite treatment. The studies agreed that the cost would be approximately $2.50 to $2.80/lb for a scale of 5 to 10 million lb/yr. These studies also agreed that improvements to the current pilot-scale process could readily be made, and that these could reduce the cost, especially at a larger scale, to $1.20 to $1.40/lb. The most sensitive parameter according to both studies is the PHA content as a percentage of the dry cell weight because fewer impurities means less energy expended on downstream processing. Other reasonable improvements cited were reduction of raw material cost (i.e., using hydrolyzed corn starch at $0.10/lb instead of glucose at $0.24/lb), efficiency of centrifugation, optimization of the chemical treatment, and higher productivity during the fermentation. Less-detailed studies were conducted to determine potential costs of medium-chain PHA production, and in the best case the estimate was about $2.30/lb, again with PHA content of cells being the most important parameter (120).

Citric acid and glutamate are produced by established large-scale fermentation processes at nearly 2 billion lb/yr, approaching that of smaller-scale bulk petrochemical processes. The selling price of these bioproducts tends to be in the neighborhood of $1/lb (121). These processes set an optimistic example for the foray of fermentation technology into the plastics market, and they demonstrate the legitimacy of the industrial effort to produce bioproducts on a large scale. Industry has become committed to the development of biodegradable plastics on a large scale as well. For example, Cargill Dow is expanding its capacity for polylactic acid (synthetically polymerized from fermentation-derived lactic acid) from 16 million lb/yr to 280 million lb/yr (122), and DuPont is developing Biomax, a polyethylene terephthalate with monomers vulnerable to hydrolysis incorporated (123). The most promising sectors for growth of biodegradables in the near future are predicted to be food packaging, compost bags, paper coatings (milk, juice, frozen food, etc.), dishes and cutlery (of which 100 million lb/yr can be replaced with biodegradables), and disposable hygiene products; forecasts for the 2003 biodegradable plastics market range from 50 million to 200 million lb/yr (122).

The maturation of transgenic PHA plant technology would bring about a sea change in the economics of PHA production. This is a long-term goal in comparison with fermentative production, so predictions are not as easy to make. Preliminary estimates put the price of plant-derived PHAs at $0.25 to $0.60/lb (94,124), competitive with petroleum-based commodity thermoplastics, which typically sell in this range (125). For PHAs to assume a volume of billions of lb/yr, however, certain milestones must be met. Starch or oil carbon flux must be diverted by metabolic engineering to achieve a polymer content of about 30 to 40% of the plant dry weight. Aside from technical issues, regulatory approval must be obtained for the transgenic plant to be used in the field as well as for the use of the particular polymer in applications such as food contact. Companies with the ability to develop applications on a large scale must be involved, and further development of composting infrastructure would be necessary if the biodegradability aspect of PHAs is to be very meaningful. If these milestones are met, the stage is set for the release of commodity plastic production from dependence on feedstocks such as crude oil, which is projected to run out during our century (126).

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BIOREMEDIATION: AN OVERVIEW OF HOW MICROBIOLOGICAL PROCESSES CAN BE APPLIED TO THE CLEANUP OF ORGANIC AND INORGANIC ENVIRONMENTAL POLLUTANTS

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BIOREMEDIATION, ECOLOGY, TECHNOLOGY

Bioremediation

Bioremediation relies on encouraging biological processes to minimize an unwanted environmental impact; usually it is the removal of a contaminant from the biosphere. A narrow definition of bioremediation might focus on the conversion of contaminating organic molecules to carbon

dioxide, water, and inorganic ions, and the oxidation or reduction of contaminating inorganic ions. A broader definition would include biological processes for ameliorating extremes of pH, concentrating contaminants so that they can be more easily removed by physical techniques, converting toxic species to less toxic or less bioavailable forms that pose less of a threat to the environment, and restoring functional ecosystems in contaminated or disturbed sites when the contaminants or disturbance cannot be removed. This article focuses on biological treatments for contaminants when they have escaped into the environment, particularly on the initial microbial reactions that remove or convert the contaminant.

Many approaches are being used effectively to catalyze bioremediation. Sometimes the simple addition of an otherwise limiting nutrient is appropriate, while at other times microbial inocula are needed. In other situations, it may be important to add readily degradable substrates to foster cometabolism of a contaminant or to drive an environment toward anaerobiosis to stimulate anaerobic degradation. Alternatively, it might be necessary to add surfactants or other chemicals to increase the bioavailability of a contaminant.

Bioremediation therefore overlaps several older biotechnologies. Municipal and industrial wastewater treatment is a well-established industry and while it can be distinguished from bioremediation in that the pollutants are under physical control during treatment, many of the fundamental biological processes are the same. Similarly, composting is a well-established phenomenon, currently gaining popularity in the municipal solid waste treatment industry, and the biofiltration of waste gases is becoming a useful technology. Development of these technologies, for cases in which the contaminant is already under physical control, will undoubtedly aid the development of bioremediation as an accepted tool for dealing with similar wastes when they have escaped our control.

Bioremediation is already a commercially viable technology, with estimates of aggregate bioremediation revenues of $2 to $3 billion for the period 1994 to 2000 (1). There are significant opportunities to enlarge upon this success. Bioremediation has applications in the gas phase, in water, and in soils and sediments. For water and soils, the process can be carried out in situ or after the contaminated material has been moved to some sort of contained reactor (ex situ). The former is generally rather less expensive, but the latter may be so much faster that the additional cost of manipulating the contaminated material is overshadowed by the time saved. Bioremediation may explicitly exploit bacteria, fungi, algae, or higher plants, although our focus here is on the microbes. Each, in turn, will be part of a complex food web, and optimizing the local ecosystem may be as important as focusing solely on the primary degraders or accumulators.

Bioremediation always competes with alternative technologies for achieving an environmental cleanup goal. Bioremediation is typically among the least expensive options, with the additional important advantage that in many cases bioremediation is a permanent solution to the contamination problem because the contaminant is completely destroyed or sequestered. Some alternative technologies, such as thermal desorption and destruction of organic contaminants, are also permanent solutions, but the simplest and most widely used remediation option, that of removing a contaminant to a dump site, merely moves the problem, and may well not eliminate the potential liability. Furthermore, by its very nature bioremediation addresses the bioavailable part of any contamination, and when biodegradation or bioaccumulation ceases this probably means that the bioavailable part of the contamination has been addressed. Residual concentrations of contaminants, while perhaps detectable by sensitive analytical techniques, may have only minimal residual environmental impact. The same cannot necessarily be said for nonbiological technologies, which may leave bioavailable contaminants at low levels.

Bioremediation also has the advantage that it can be relatively nonintrusive, and can sometimes be used in situations in which other approaches would be severely disruptive. For example, bioremediation has been used to cleanup hydrocarbon spills under buildings, roads, and airport runways without interfering with the continued use of these facilities.

Ecology

The biosphere plays a major role in the great elemental cycles of our planet (2), and bioremedation must be placed in this context if we are to appreciate its broadest ramifications. One of the underlying fundamental truths of biological diversity is that if there is free energy available in the metabolism of a substrate, there is probably a guild of organisms that has evolved to make use of it. This is particularly important for the biodegradation of organic molecules. For example, crude oil seeps, to land and water, have occurred for millennia, and as a consequence, aerobic oil-degrading microorganisms are ubiquitous. If biology does not yet take advantage of a source of free energy, then we can expect that there will be strong selection pressure in favor of any organism that develops an ability to exploit it. This has been seen with by-products of nylon manufacture, where *Pseudomonas aeruginosa* has gained the ability to degrade the novel compound 6-aminohexanoate linear dimer, a by-product of nylon-6 manufacture, as the sole source of carbon and nitrogen (3). The successful bioremediation of xenobiotic compounds, such as pesticides and herbicides, may sometimes represent a similar acquisition of genetic traits.

Not all organic molecules provide a source of free energy, however. Some, such as small halogenated solvents, provide no significant source of nutrients or energy, and their aerobic destruction can only occur cometabolically with the degradation of a more nutritious substrate (4) or anaerobically as a terminal electron acceptor (5). The white-rot fungi offer another variation on this theme. These organisms seem to be unique in their ability to degrade lignin, the structural polymer of higher plants. They may not gain any direct energetic benefit from lignin degradation, but it clearly allows them access to cellulose, which is a substrate for their growth. Lignin degradation is catalyzed by a group of extracellular peroxidases that generate nonspecific oxidants, and there

have been several proposals to use these systems for destroying contaminating organic compounds (6).

With successful bioremediation, organic compounds are eventually converted to carbon dioxide, water, and biomass. Similarly, nitrogenous molecules, such as excess ammonia or nitrate in groundwater, can be converted to gaseous nitrogen. Other nonorganic contaminants provide a different challenge for bioremediation. A few, such as mercury and selenium, are volatized by some biological processes, but it is not clear that this is always beneficial. In some cases, such as chromium and arsenic, there is a dramatic difference in environmental toxicity depending on the redox state of the contaminant. In these cases, bioremediation has sometimes focused on this detoxification by bacterial processes. A more satisfying approach would be to use a biological process to accumulate and concentrate the contaminant so that it can be removed for safe disposal; fungi, algae, and higher plants have all been used in these efforts.

Technology

Successful bioremediation hinges on the effective application of the biology discussed above. Sometimes the contaminant is on the surface, so access to it is reasonably simple. In these cases the required technology may be as simple as broadcast spreaders or sprayers to apply fertilizers, or tilling the soil to allow good aeration. Of course this is not necessarily as simple as it sounds, since contaminated sites are often very different from agricultural fields, and the technology has to be significantly stronger to "plow" the soil. Frequently the contaminant is below the surface, and applying even simple bioremediation strategies provides quite a challenge. Table 1 lists some of the technologies in use today.

This article addresses bioremediation in its broadest sense. The main sections address organic and then inorganic compounds. Key classes of contaminants are discussed, and for each I focus on the contaminants that can be treated, the underlying biological processes that can mitigate the contamination, and the technologies that have been used, or are being developed, to treat them.

ORGANIC CONTAMINANTS

Hydrocarbons

Constituents. Hydrocarbons are ubiquitous in the environment, from fossil, biogenic, and anthropogenic sources, and they provide readily digestible food for oil-degrading microbes (7). Bioremediation is thus an important potential technology for remediating hydrocarbons in oil spills and at contaminated creosote and town gas plants. Crude oils are very complex mixtures, primarily of hydrocarbons, although some components do have heteroatoms such as nitrogen (e.g., carbazole) or sulfur (e.g., dibenzothiophene). Chemically, the principal components of crude oils and refined products can be classified as aliphatics, aromatics, naphthenics, and asphaltic molecules, and representative examples are shown in Figure 1. The ratio of these different classes varies in different oils, but a "typical crude oil" might contain the four classes in a ratio of approximately 30 : 30 : 30 : 10. Most crude oils contain hydrocarbons ranging in size from methane to molecules with hundreds of carbons, although the lightest molecules are usually absent in oils that have been partially biodegraded in their reservoir. When crude oils reach the surface environment the lighter molecules evaporate, and are either destroyed by atmospheric photooxidation or are washed out of the atmosphere by rain, and are biodegraded. Some molecules, such as the smaller aromatics (benzene, toluene, etc.), have significant solubilities and can be washed out of floating slicks, regardless of whether these are at sea or on terrestrial water tables. Fortunately the majority of molecules in crude oils, and refined products made from them, are biodegradable, at least under aerobic conditions.

The other major sources of hydrocarbon contamination were the town gas and wood treatment industries. Town gas was made locally in many towns and cities by heating coal in the absence of air. Methane was produced and used, but the residue included coke and tarry material rich in polycyclic aromatic hydrocarbons. The production and use of creosote was quite similar, and again it gave rise to contamination with polycyclic aromatic hydrocarbons. The 16 polycyclic aromatic hydrocarbons on the U.S. EPA priority pollutant list are shown in Figures 2 and 3; all are abundant in creosote and coal tar, but only naphthalene, fluorene, phenanthrene, and chrysene are relatively abundant in petroleum.

Biodegradation

Methane and the volatile plant terpenes are fully biodegradable by aerobic organisms, and most refined petroleum products are essentially completely biodegradable under aerobic conditions. Estimates for crude oil biodegradability range up to 90% (7), and the least biodegradable material, principally polar molecules and asphaltenes, lack the "oily" feel and properties that we associate with "oil." Indeed they are essentially impossible to distinguish from more recent organic material in soils and sediments, such as the humic and fulvic acids, and appear to be biologically inert.

Numerous bacterial and fungal genera have species that can degrade hydrocarbons aerobically, and the pathways of degradation of representative aliphatic, naphthenic, and aromatic molecules have been well characterized in at least some species (7). It is a truism that the hallmark of an oil-degrading aerobic organism is its ability to insert oxygen atoms into the hydrocarbon, and there are many ways in which this is achieved (Figs. 4 and 5). Once a hydrocarbon possesses a carboxylate or alcohol functionality it is almost invariably a readily degradable compound that can eventually be attached to coenzyme A and mineralized in the ß-oxidation pathway. A simple example at the human level is the difference between oleic acid, present in olive oil, and octadecane, present in mineral oil, which is so inert that it serves as an intestinal lubricant!

For many years it was assumed that oil biodegradation was an exclusively aerobic process as any degradation must involve oxidation. Indeed, the very existence of oil reservoirs indicates that anaerobic degradation in

Table 1. Some Technological Definitions Relevant to Bioremediation

Technology	Description
Air sparging, Aquifer sparging, Biosparging	Injection of air to stimulate aerobic degradation. May also stimulate volatilization.
Air-stripping	Injection of air to stimulate volatilization. Contaminant is usually removed by subsequent adsorption.
Aquifer bioremediation	In situ bioremediation in an aquifer, usually by adding nutrients or cosubstrates through injection wells.
Aquifer sparging	Injection of air into a contaminated aquifer to stimulate aerobic degradation. May also stimulate volatilization.
Batch reactor	A bioreactor loaded with contaminated material, and run until the contaminant has been consumed. It is then emptied, and the process is repeated.
Bioactive Barrier, Bioactive Zone, Biowall, Wall-and-gate	A zone, usually subsurface, where biodegradation of a contaminant occurs so that no contaminant passes the barrier. Sometimes impermeable walls funnel contaminants to this reactive zone.
Bioaugmentation	Addition of exogenous bacteria with defined degradation potential (or rarely indigenous bacteria cultivated in a reactor and reapplied).
Biofilm Reactor	A reactor where bacterial communities are encouraged on a high surface area support. Biofilms often have a redox gradient so that the deepest layer is anaerobic while the outside is aerobic, allowing both aerobic and anaerobic processes to occur.
Biofiltration	Usually, an air filter with degrading organisms supported on a high surface area support, such as granulated activated carbon or compost.
Biofluffing	Augering soil to increase porosity.
Bioleaching	Extracting metallic contaminants at acid pH, perhaps while attempting to optimize fungal degradation of organic contaminants.
Biological fluidized bed, Fluidized bed bioreactor	Bioreactor where the fluid phase is moving fast enough to suspend the solid phase as a fluidlike phase.
Biological Plug	In-ground actively aerated bioreactors containing adapted microbial consortia to degrade contaminants of concern.
Biopile, Soil heaping	An engineered pile of excavated contaminated soil, with engineering to optimize air, water, and nutrient control.
Bioslurping	Vacuum extraction of the floating contaminant and water, and vapor from the vadose zone. The airflow stimulates biodegradation.
Biostimulation	Optimizing conditions for the indigenous biota to degrade the contaminant.
Biotransformation	The biological conversion of a contaminant to some other form, but not to carbon dioxide and water.
Biotrickling Filter	A reactor where a contaminated gas stream passes up a reactor with immobilized microorganisms on a solid support, while nutrient liquor trickles down the reactor.
Bioventing	Vacuum extraction of contaminant vapors from the vadose zone, thereby drawing in air to stimulate the biodegradation of the remainder.
Borehole bioreactor In well bioreactor	The addition of nutrients and electron acceptor to stimulate biodegradation in situ in a contaminated aquifer.
Closed-loop Bioremediation	Groundwater recovery, a bioreactor, and low-pressure reinjection to maximize nutrient use, and maintain temperature in cold climates.
Composting	Addition of biodegradable bulking agent to stimulate microbial activity. Optimum composting generally involves self-heating to 50-60°C.
Constructed Wetland	Artificial marsh for bioremediation of contaminated water.
Continuous stirred tank reactor (CSTR)	A bioreactor that is completely back mixed.
Digester	Usually, an anaerobic bioreactor that generates methane.
Ex-situ bioremediation	Usually, the bioremediation of excavated contaminated soil in a biopile, compost system, or bioreactor.
Fixed-bed bioreactor	Bioreactor with immobilized cells on a packed column-matrix.
Land-farming, land treatment	Application of a biodegradable sludge as a thin layer to a soil to encourage biodegradation. Tilling and fertilizing is usually required.
Natural Attenuation, Intrinsic bioremediation	Unassisted biodegradation of a contaminant.
Pulsed bioremediation	Alternating injection of a cosubstrate for the contaminant, and oxygen.
Pump and treat	Pumping groundwater to the surface, treating, and reinjecting.
Rhizosphere bioremediation	Stimulating the bacteria in the rhizosphere (root-zone) to carry out biodegradation of a soil contaminant.
Rotating biological contactor	Bioreactor with rotating device that moves a biofilm through the bulk water phase and the air phase to stimulate aerobic degradation.
Sequencing Batch Reactor	Periodically aerated solid phase or slurry bioreactor.
Soil vapor extraction	Vacuum assisted vapor extraction.

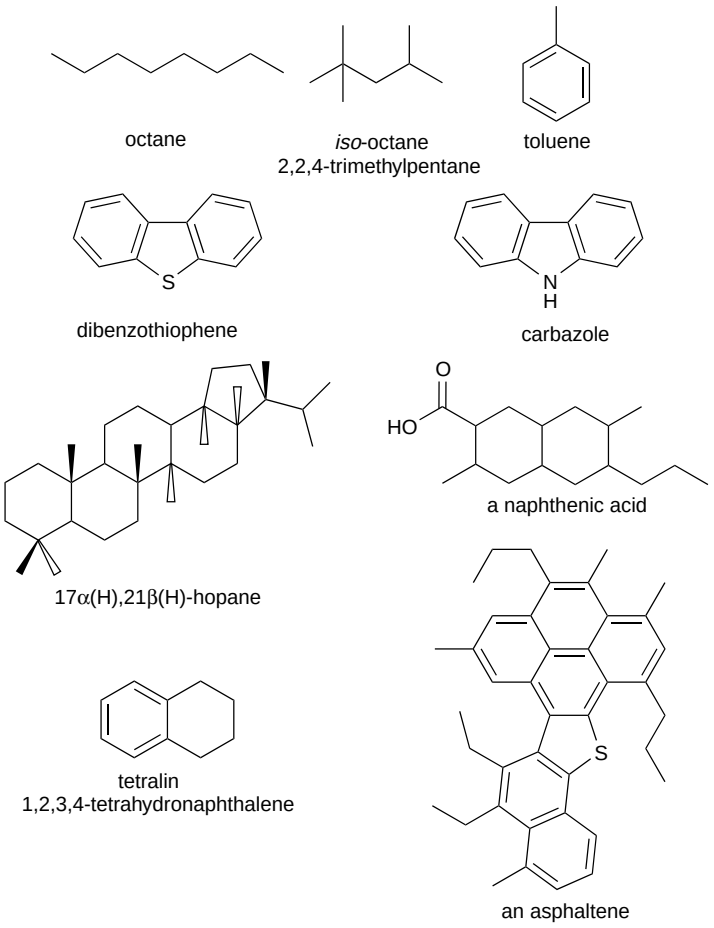

Figure 1. Some petroleum hydrocarbons. As in all figures in this article, hydrogens are omitted unless on a heteroatom. Octane is an example of a paraffin, iso-octane an isoparaffin, toluene is an aromatic. Benzothiophene and carbazole are heterocycles, but they are considered aromatics by the oil industry, as is tetralin. Hopane is an example of a biomarker that is recognizably derived from bacterial lipids, in this case from bacteriohopanetetrol. Naphthenic acids and asphaltenes are hard to characterize, but the structures shown are likely representatives.

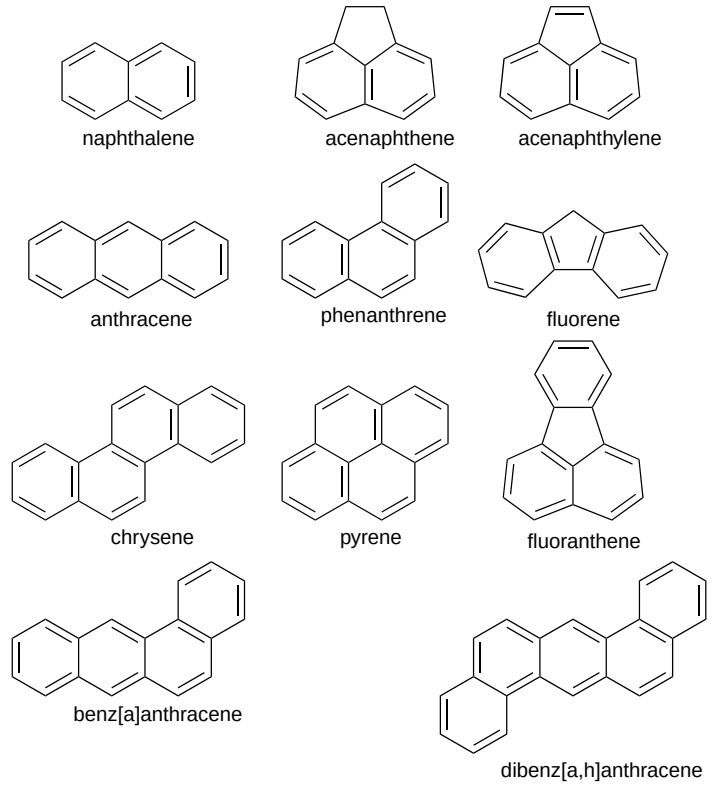

Figure 2. Some of the 16 polycyclic aromatic hydrocarbons on the U.S. EPA list of Priority Pollutants. Only naphthalene, phenanthrene, and chrysene are abundant in crude oils, the others are abundant in coal tars and other thermally treated materials.

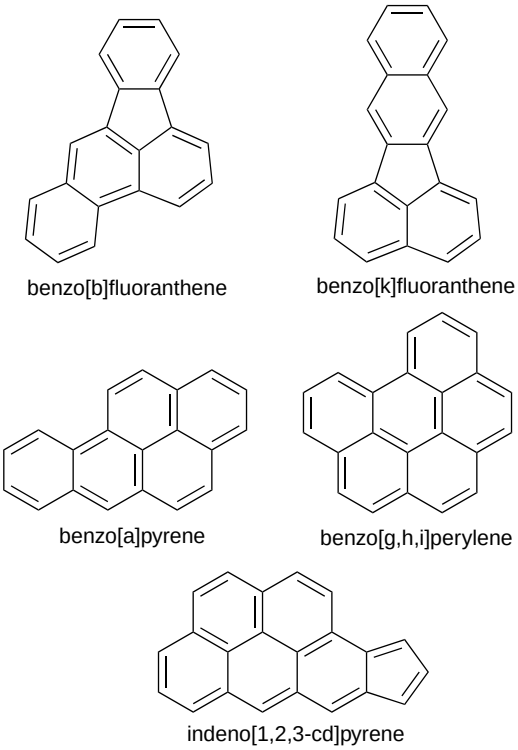

Figure 3. More of the 16 polycyclic aromatic hydrocarbons on the U.S. EPA list of priority pollutants. These are present in only tiny amounts in crude oils, but they are generated during incomplete combustion of carbonaceous materials, and can be abundant at coal gasification sites.

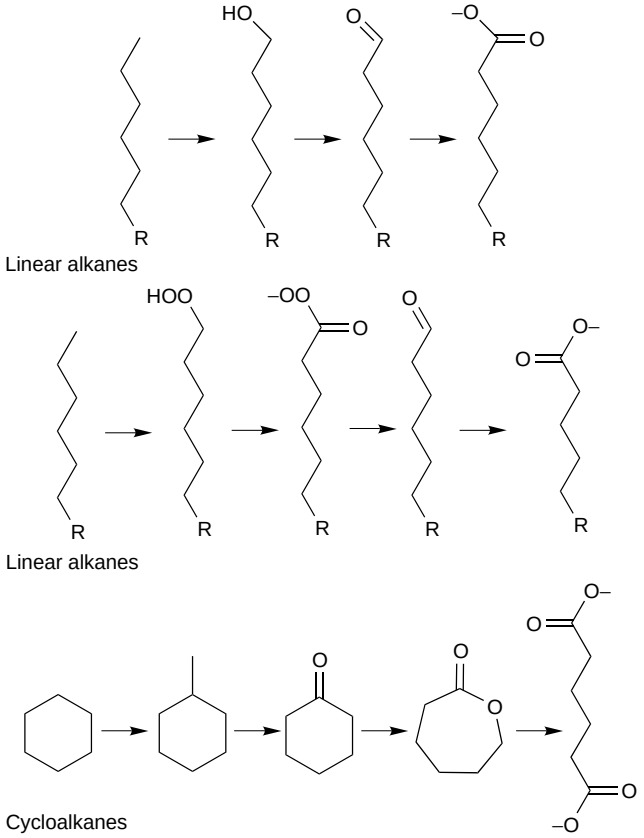

Figure 4. Schematics of the initial aerobic biodegradation steps for alkanes and cycloalkanes. The upper pathway is found in *Pseudomonas oleovorans*, the middle in *Acinetobacter* species, and the lower in *Nocardia* species.

such environments must be very slow. Nevertheless, in recent years it has become clear that bacteria under completely anaerobic conditions can oxidize many hydrocarbons (7). Hydrocarbon biodegradation has now been shown under sulfate-, nitrate-, chlorate-, perchlorate-, carbon dioxide- and ferric iron-reducing conditions (8). Pathways have not been characterized in detail, but current research suggests that a common motif in anaerobic hydrocarbon degradation is the addition of the hydrocarbon to the double bond in fumarate (Fig. 6) to produce alkyl- or aromatic-succinates (9,10). These are readily degraded after the addition of Coenzyme A. The anaerobic biodegradation of hydrocarbons, particularly of small soluble aromatics such as toluene, is now recognized as an important part of the natural attenuation of hydrocarbon spills, and is being exploited in active bioremediation protocols on a large scale.

While the majority of molecules in crude oils and refined products are hydrocarbons, the U.S. Clean Air Act amendment of 1990 mandated the addition of oxygenated compounds to gasoline in many parts of the United States. The requirement is typically that 2% (w/w) of the fuel be oxygen, which requires that 5 to 15% (v/v) of the gasoline be an oxygenated additive (e.g., methanol, ethanol, methyl tertiary butyl ether (MTBE) etc.). While methanol and ethanol are readily degraded, the degradability of MTBE remains something of an open question. The compound was previously very rare in the environment, but now it is one of the major chemicals in commerce. At first it seemed that the compound was completely resistant to biodegradation, but complete mineralization has now been reported (11,12).

Bioremediation

Crude oil and refined products are readily biodegradable under aerobic conditions, but they are only incomplete substrates since they lack any significant nitrogen, phosphorus, and essential trace elements. Bioremediation strategies for removing large quantities of hydrocarbon must therefore include supplementation with nutrients in a bioavailable form.

Air. Airborne hydrocarbon vapors are readily treated with biofilters. These are typically rather large devices with a very large surface area provided by bulky material such as shredded compost (see Biofiltration, this Encyclopedia). The contaminated air, perhaps from a soil vapor-extraction treatment or a factory using hydrocarbon solvents, is blown through the filter, and organisms, usually indigenous to the filter material or provided by a soil or commercial inoculum, grow and consume the hydrocarbons. Adequate moisture and nutrients must be maintained for effective operation. Alternatively, trickling biofilters with recycled water can be used. Bacteria and fungi readily colonize such filters, and they can be very

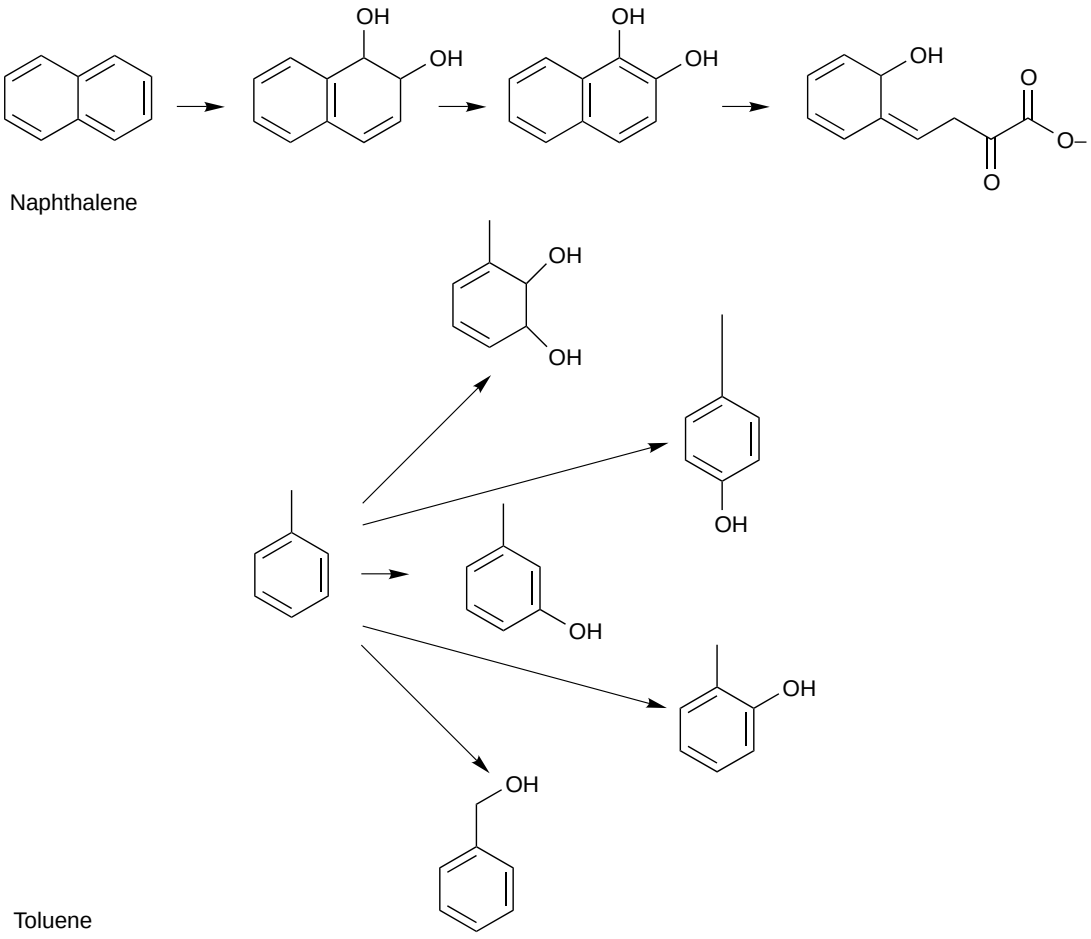

Figure 5. Schematics of the initial biodegradation steps for naphthalene and toluene. The upper pathway is found in many Pseudomonads, and the lower panel illustrates the many ways that toluene is activated in different Pseudomonads.

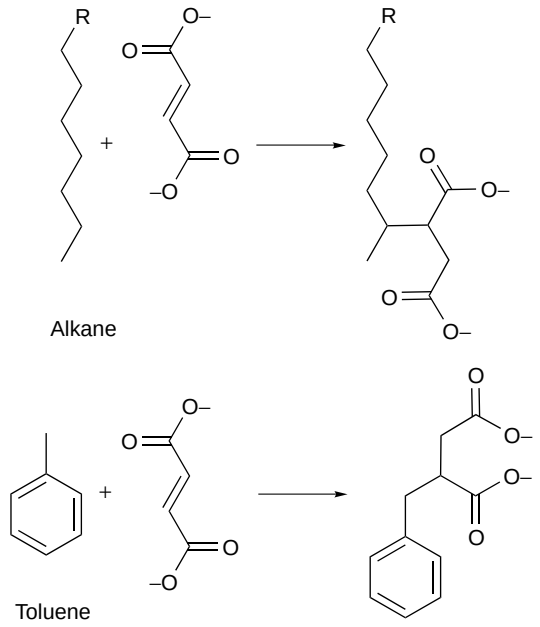

Figure 6. The initial reactions of anaerobic biodegradation of alkanes and toluene. In both cases the substrate is activated by addition of fumarate.

effective. Nevertheless, biofilters are usually equipped with a small granulated activated carbon "backup" filter to handle any sudden pulse loads that might overwhelm the biological capacity of the filter. Biofilters compete with granulated activated carbon filters, and are often less expensive because they minimize the cost of the granulated activated carbon and the energy required to destroy the contaminant and the granulated activated carbon when the latter is saturated (13). Potential problems include plugging and uneven air or water flow, but successful designs work for many years with minimal maintenance except for the occasional addition of nutrients and stirring of the bed.

Sea. Crude oil spills at sea are perhaps the most widely covered environmental incidents in the national and international media. Despite their notoriety, catastrophic tanker spills and well blowouts are fortunately rather rare, and their total input into the world's oceans is approximately equivalent to that from natural seeps; significantly more oil reaches the world's oceans from municipal sewers (14). Physical collection of the spilled oil is the preferred remediation option, but if skimming is unable to collect the oil, biodegradation, and perhaps combustion

or photooxidation, are the only routes for elimination of the spill. One approach to stimulating biodegradation is to disperse the oil with chemical dispersants (15). Patents have been issued for dispersant formulations that specifically include nitrogen and phosphorus nutrients, but the products are not currently commercially available.

Bioremediation by the addition of oil-degrading microbes is often promoted as a treatment option for floating spills, but this approach has not yet met with any documented success (16).

Shorelines. The successful bioremediation of shorelines affected by the spill from the *Exxon Valdez* in Prince William Sound, Alaska, was the largest bioremediation project to date; more than 111 km of shoreline was treated (17), usually after bulk oil had been collected. Residual oil had typically penetrated into the shoreline gravel, occasionally getting as deep as 30 cm into the sediment. Since the gravel was typically very permeable, oxygen availability was unlikely to be the limiting factor for biodegradation, and indeed this was subsequently shown to be correct. Bioremediation therefore focused on the addition of nitrogen and phosphorus fertilizers to partially alleviate the nutrient-limitation of oil degradation. Of course this was complicated by the fact that oiled shorelines were washed by tides twice a day. These tides would have rapidly removed any soluble fertilizer, so a strategy was sought that would provide nutrients for a significant length of time. Various approaches to applying fertilizers were tried, including standard and slow-release nutrients, oleophilic nutrients, and solutions of liquid fertilizers. Two fertilizers were used in the full-scale applications; one, an oleophilic product known as Inipol EAP22 (CECA, Paris, France), is a microemulsion of a concentrated solution of urea in an oil phase of oleic acid and trilaurethphosphate, with butoxyethanol as a cosolvent. This product was designed to adhere to oil and to release its nutrients to bacteria growing at the oil-water interface. The other fertilizer is a slow-release formulation of inorganic nutrients, primarily ammonium nitrate and ammonium phosphate, in a polymerized vegetable oil skin. This product, known as Customblen (Grace-Sierra, Milpitas, Calif.), released nutrients with every tide, and these were distributed throughout the oiled zone as the tide fell. Fertilizer application rates were carefully monitored so that the nutrients would cause no harm, and the rate of oil biodegradation was stimulated between two- and fivefold (17).

Subsequent work has shown that fertilizer application is likely to be a useful remediation approach in many situations, including in the Arctic. It is unlikely though that the addition of fertilizers will have a dramatic effect on situations in which agricultural or municipal runoff maintains elevated levels of nutrients, as happens in some estuaries and bays. Here, aeration is likely to be most effective.

Bioremediation by the addition of oil-degrading microbes has been promoted as a treatment for oiled shorelines, but the approach has not yet met with any well-documented success (16).

Areas where there are currently few remediation options include oiled marshes, mangroves, and coral reefs. These environments are generally easily damaged by human intrusion, and physical cleaning options may not provide any net environmental benefit. Bioremediation may provide some attractive options, and some success has been claimed, on a small scale, with fertilizer applications. Marshes and mangroves offer the additional complication that they are typically anoxic. Perhaps the anaerobic degradation of oil could be stimulated by inoculation with anaerobic hydrocarbon-degrading microbes or perhaps gentle aeration or the addition of slow-release oxygen compounds, such as some inorganic peroxides, might stimulate aerobic degradation without significantly changing the redox balance of these environments. This is an area where research is very much in its infancy, and there are no well-documented success stories to date.

Bioremediation also offers options for dealing with oiled material, such as seaweed, which gets stranded on shorelines; composting has been shown to be effective.

Groundwater. Spills of refined petroleum products on land and leaking underground storage tanks sometimes contaminate groundwater. Bioremediation is becoming an increasingly popular treatment for such situations.

Hydrocarbons typically have a density of less than 1, and refined products usually float on the water table if they penetrate soil that deeply. In the parlance of the remediation industry, such floating spills are often called NAPLs (nonaqueous phase liquids). Indeed they are sometimes known as LNAPLs (light nonaqueous phase liquids) to distinguish them from more dense materials such as halogenated compounds. While standalone bioremediation is an option for these situations, "pump and treat" is the more usual treatment, in which contaminated water is brought to the surface, free product is removed by flotation, and the cleaned water is reinjected into the aquifer. Adding a bioremediation component to the treatment, typically by adding oxygen and low levels of nutrients, is an appealing and cost-effective way of stimulating the degradation of the residual hydrocarbon that was not extracted by the pumping, and this approach is becoming widely used (18).

Hydrocarbons are not very soluble in water, but the most soluble components will leach out of a spill if there is continual flushing. Typically, only small aromatic molecules, the infamous BTEX (Fig. 7), are soluble enough to contaminate groundwater, although with the advent of oxygenated gasolines, these oxygenates [ethanol, methanol, MTBE (methyl tertiary butyl ether), etc.] are being found in groundwater (11,12). Remediation of such situations usually involves "pump and treat" methodologies, although these methods are slow and may leave behind reservoirs of contaminants in pockets that are poorly connected to the main water body. Of course the contaminant is biodegradable, and some biodegradation is probably already occurring when the contamination is discovered. The simplest approach to remediation is thus to allow this intrinsic process to continue. Evidence that it is indeed occurring can be found in the selective disappearance of the most biodegradable compounds in the

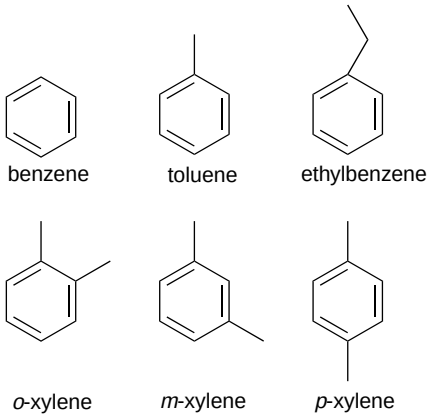

Figure 7. The principal water-soluble components of refined fuels; benzene, toluene, ethylbenzene, and the xylenes, commonly referred to as BTEX.

contaminant mixture, and the concomitant disappearance of electron acceptors from the groundwater. Thus, oxygen is depleted as the preferred terminal electron acceptor for metabolism, followed by nitrate, ferric iron, sulfate, and finally carbon dioxide for methanogenesis (19).

Intrinsic bioremediation is becoming an acceptable option in locations where the contaminated groundwater poses little threat to environmental health (20). Nevertheless, while it is appealingly simple, it may not be the lowest cost option if extensive monitoring and documentation is involved. In such cases it may well be more cost-effective to optimize conditions for biodegradation. One way to do this is to funnel the contaminated water through a reactive zone where biodegradation can be stimulated and monitored. The simplest intervention is a line of wells for "pump and treat," but including a biological component may be more cost-effective. This can range from the installation of a sparge line for aerating the contaminated plume (18), to installing some form of semi-contained bioreactor where nutrients can be applied with some modicum of control (21,22). Often these designs are combined with barriers to ensure that the entire contaminated plume passes through the reactive zone. These designs have a variety of names, including "biowall," "trench biosparge," "funnel and gate," "bubble curtain," "sparge curtain," and "engineered trenches and gates" (22). Both aerobic and anaerobic designs have been successfully installed. Another approach is to optimize the levels of electron acceptors in the contaminant plume. Oxygen can be pumped in as the pure gas (23), or as air, although this is relatively energy intensive since oxygen is so poorly soluble. Hydrogen peroxide has been used in some situations (24), but there have been problems with biomass plugging near the injection wells. Slow-release formulations of inorganic peroxides, such as magnesium peroxide, have recently been used with success (25,26). Nitrate may be added, although there are sometimes regulatory limitations on the amount of this material that may be added to groundwater (27). Ferric iron availability may be manipulated by adding ligands (28), and recent success at stimulating the biodegradation of benzene in groundwater by the addition of sulfate (29) suggests that stimulating the anaerobic biodegradation of hydrocarbons will become a broadly useful remediation technology.

If there are significant amounts of volatile and nonvolatile contaminants, remediation may be achieved by a combination of liquid and vapor extraction of the former, and bioremediation of the latter. This combination has been termed "bioslurping," where the act of pumping out the liquid contaminant phase draws in air at other wells to stimulate aerobic degradation (25). Such bioremediation requires that there be enough nutrients to allow microbial growth, and fertilizer nutrients are frequently added at the air injection wells. Bioslurping has had a number of well-documented successes (18).

Where there are large volumes of contaminated water under a small site, it is sometimes most convenient to treat the contaminant in a biological reactor at the surface. Considerable research has gone into reactor optimization for different situations, and a variety of stirred reactors, fluidized bed reactors, and trickling filters have been developed (21). Such reactors are usually much more efficient than in situ treatments, although correspondingly more expensive.

Of course, the presence of a liquid phase of hydrocarbon in soil gives rise to vapor contamination in the vadose zone above the water table. This can be treated by vacuum extraction, and the passage of the exhaust gases through a biofilter (mentioned earlier) can be an inexpensive and effective way of destroying the contaminant permanently.

Soil. Hydrocarbon contamination of soils runs the gamut from crude oils at production wells and pipeline spills to refined products at refineries, distribution centers, service stations and accident sites, and polycyclic aromatic hydrocarbons at wood-treatment facilities, town gas manufacturing sites, railroads, and their terminals, and various military bases. Sometimes the contamination is the result of leaking underground storage tanks and pipelines, leading to subsurface contamination, but surface spills also occur. Physical removal of gross contamination is an obvious first step at all locations, and bioremediation is an appealing option for remediating residual contamination in many of these sites (18,30).

Spills from production facilities and pipelines often involve both oil and brine, since most oil reservoirs float on top of concentrated brines and both are produced in later stages of production. The brine is typically separated from the oil and reinjected into the reservoir, but some is retained in many production pipelines. The environmental impact of spilled brine can be quite deleterious. Salt is not only toxic to most plants and inhibitory to many soil bacteria, but can also affect soil structure by altering the physical properties of clays. Successful bioremediation strategies must therefore include remediating the brine. In wet regions, salt is eventually diluted by rainfall, but in arid regions and to speed the process in wetter regions, gypsum is often added to restore soil porosity (31).

Many hydrocarbons bind quite tightly to soil components and are thereby less available to microbial degradation. The kinetics of binding seem to be complex, and the process of "aging" is only poorly understood. Nevertheless, it seems clear that hydrocarbons that have been in contact

with soil for a long time are not as available for biodegradation as fresh spills (30). Several groups of researchers have suggested the addition of surfactants to overcome this limitation, but this approach is not yet widely used. A significant potential concern is that the surfactant may be degraded in preference to the contaminant of concern.

Intrinsic biodegradation occurs, but it usually only removes the lightest refined products, such as gasoline, diesel, and jet fuel (20). Active intervention is typically required. Usually, the least expensive approach is in situ remediation (18,30), typically with the addition of nutrients and the attempted optimization of moisture and oxygen by tilling. Various approaches to applying fertilizers have been tried, including standard and slow-release nutrients, oleophilic nutrients, and solutions of liquid fertilizers. Oxygen is likely to be limiting in many cases, and soil tilling is widely practiced. This in situ bioremediation of hydrocarbon-contaminated soils is akin to the old practice of "land-farming" (32), wherein sludges and other refinery wastes were deliberately spread onto the soil and tilled and fertilized to stimulate biodegradation. Although this practice is now discontinued in the United States, it was quite widely used.

Deeper contamination may be remedied with bioventing, by which air is injected through some wells and extracted through others to both strip volatiles and provide oxygen to indigenous organisms (18). Fertilizer nutrients may also be added. This is usually only a viable option with lighter refined products.

When soil contamination extends to some depth, it may be preferable to excavate the contaminated soil and to put it into "biopiles" in which oxygen, nutrient, and moisture levels are more easily controlled (21). Biopiles can also be kept warm during winter months, increasing the amount of time available for biodegradation in colder climates. Since the soil is well mixed during the construction of the pile, there is an opportunity to add selected microbial and fungal strains in an additional attempt to maximize biodegradation.

Composting by the addition of readily degradable bulking agents is also a useful option for relatively small volumes of excavated contaminated soil (21). Since efficient composting invariably involves self-heating as biodegradation proceeds, this also offers an option for extending the bioremediation season into the winter months in cold climates. A potential drawback of composting is that it usually increases the volume of contaminated material, but if fully successful, the finished compost can be returned to the site as a positive contribution to soil quality.

Slurry bioreactors offer the most aggressive approach to maximizing contact between the contaminated soil and the degrading organisms (21). Both lagoons and reactor vessels have been used, but the former is often not optimally designed for all the soil to be partially suspended by the mixing impellers. Contained reactor designs include mixing tank, airlift, and fluidized-bed aeration. A major advantage of contained slurry bioreactors is the potential to continually optimize nutrients, aeration, and degradative inocula as fresh soil is added, and to control waste materials, including gases. Slurry bioreactors are usually the most expensive bioremediation option because of the large power requirements, but under some conditions this cost is offset by the rapid biodegradation that can occur.

In all these cases, it is important to bear in mind that while the majority of hydrocarbons are readily biodegraded, some, such as the steranes and hopanes, are very resistant to microbial attack. Estimates of oil biodegradation range from 60 to 95% for different crude oils, so fresh spills of crude oils are readily treated by bioremediation (7). Refined products, such as gasoline, diesel, jet fuels, and heating oils, are usually more biodegradable than typical whole oils, but the various heavy fractions of crudes, such as the asphalts, are far less biodegradable and are not such attractive targets for bioremediation. Some crude oils have already been extensively biodegraded in their reservoirs, and these are also poor targets for bioremediation. An example is Orimulsion, a heavy oil in water emulsion (70% bitumen) stabilized by low levels of surfactants, used as a fuel for electricity generation. Similarly, old spills may have already undergone significant biodegradation and the residue may be relatively biologically inert. It is thus important to run laboratory studies to ensure that the contaminant is sufficiently biodegradable and that cleanup targets can be met. On the other hand, even very heavy fuels contain some biodegradable compounds, and a case can be made that stimulating the biodegradation of this fraction will minimize the environmental impact of these compounds, even if it will not have a dramatic effect on the total amount of oil in the environment.

HALOGENATED ALIPHATIC COMPOUNDS

Constituents. Halogenated organic compounds are widespread in nature, and some, such as dichloromethane, are produced on an enormous scale (by fungi) and serve as carbon and energy sources for diverse microbes (33). Synthetic chlorinated solvents, especially chloroalkanes and chloroalkenes, have become widely used in industrial processes in the second half of the twentieth century, and some representative examples are shown in Figure 8. These solvents have been used for more than 50 years,

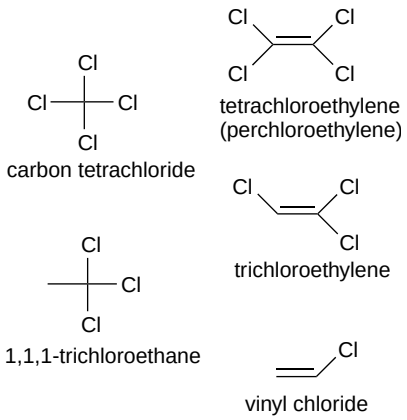

Figure 8. Some halogenated solvents.

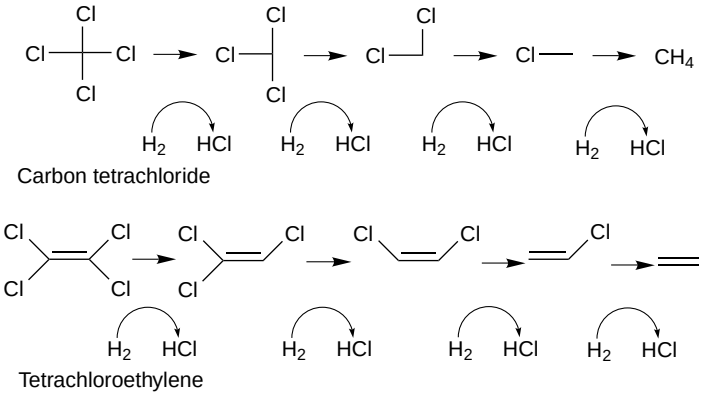

Figure 9. Sequential dehalogenation of carbon tetrachloride and tetrachloroethylene under anaerobic conditions.

and unfortunately they are now fairly widespread contaminants. Unlike the hydrocarbons, which usually float on water, the halogenated solvents typically have densities greater than one, and generally sink to the bottom of any groundwater and float on the bedrock. For this reason, they are sometimes known as DNAPLs (dense nonaqueous phase liquids).

Biodegradation

Halogenated solvents are degraded under aerobic and anaerobic conditions. The anaerobic process is typically a reductive dechlorination that progressively removes one halide at a time (Fig. 9). For example, carbon tetrachloride is sequentially dechlorinated to chloroform, dichloromethane, methyl chloride, and methane, and trichloroethylene is sequentially reduced to ethylene (34). The discovery of reductive dechlorination has led to halorespiration-based remediation technologies in which carbon sources are added to contaminated sites. Chloromethane and dichloromethane have been shown to be the sole carbon source for several anaerobic organisms (35), and it seems that we have much to learn about the microbial diversity of anaerobic microorganisms capable of dechlorinating solvents.

The simplest chlorinated alkanes, alkenes, and alcohols (e.g., chloromethane, dichloromethane, chloroethane, 1,2-dichloroethane, vinyl chloride, and 2-chloroethanol) serve as substrates for aerobic growth for some bacteria, but the majority of halogenated solvents do not seem to be able to support growth. Nevertheless, these compounds can be mineralized under aerobic conditions, albeit with no apparent benefit to the degrading organism. Indeed, the oxidation appears to be fortuitous, only occurring during the metabolism of a growth substrate. The phenomenon is therefore known as co-oxidation or cometabolism. Numerous bacteria are able to catalyze the oxidation of trichloroethylene; some use monooxygenases (e.g., methane and ammonia oxidizing species) while others contain dioxygenases (e.g., some toluene oxidizing species). The difference between these two classes of enzymes is the fate of the two atoms of molecular oxygen. Monooxygenases insert one oxygen atom into their substrate and reduce the other to water. Dioxygenases insert both atoms into their substrate (Fig. 10). In either case, biodegradation can proceed to complete mineralization.

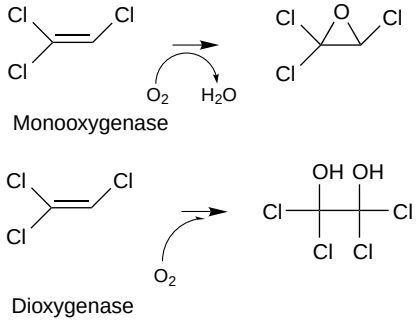

Figure 10. Comparison of the attack of monooxygenase and dioxygenase enzymes on trichloroethylene.

The biodegradation of trichloroethylene (trichloroethene) has been the most studied since this is probably the most widespread halogenated solvent contaminant. Several substrates drive trichlorethylene co-oxidation, including methane, propane, propylene, toluene, isopropylbenzene, and ammonia (36). The enzymes that metabolize these substrates have subtly different selectivities with regard to the halogenated solvents, and to date, none are capable of co-oxidizing carbon tetrachloride or tetrachloroethylene. Complete mineralization of these compounds can, however, be achieved by sequential anaerobic and aerobic processes.

Bioremediation

Air. Biofilters are an effective way of dealing with air from industrial processes that use halogenated solvents such as chloromethane, dichloromethane, chloroethane, 1,2-dichloroethane, and vinyl chloride, and that support aerobic growth (37). Both compost-based "dry" systems and trickling filter "wet" systems are in use. Similar filters could be incorporated into "pump and treat" operations.

Groundwater and soil. Soils and groundwater throughout the industrialized world have been contaminated with halogenated solvents, and remediation has a high priority. Since the solvents are so dense, they are typically found on the bedrock underlying aquifers. Pumping out the liquid phase is an obvious first step if the contaminant is likely to be mobile, but in situ bioremediation is a promising option (38). For example, the U.S. Department

of Energy has used anaerobic in situ degradation of carbon tetrachloride to treat an aquifer some 76 m below the Hanford, WA, site, with nitrate as electron acceptor and acetate as electron donor (39). Trichloroethylene is the most frequent target of remediation, and as discussed above, this only seems to be metabolized cometabolically. Remediation operations thus incorporate the addition of cometabolized substrate. Methane was used successfully at the U.S. Department of Energy site at Savannah River, near Aiken, South Carolina (40). This operation had both an air stripping and a biological component. Horizontal wells were used to pump methane and air below the contaminant, and an upper horizontal well in the vadose zone was used to withdraw these gases through the contaminated zone. Optimum biodegradation performance seemed to come from alternating injection of air and methane in air and the inclusion of nitrous oxide and triethylphosphate, both gases, to give a C : N : P ratio of 100 : 10 : 1. Success has also been achieved with a bioaugmentation strategy, using a toluene monooxygenase containing *Burkholderia cepacia* (and no cosubstrate) (41).

"Pump and treat" operations can also incorporate bioremediation; methane has been used as cosubstrate in aerobic bioreactors for halohydrocarbons including chlorofluorocarbons (42) and a sequential methanogenic-denitrifying bioreactor system has been developed (43).

Adding a slow release source of reductant to drive the reductive dechlorination of solvents in groundwater has also proved to be effective (26). One proprietary product is a polylactate ester that slowly hydrolyzes to release lactate, which in turn is metabolized anaerobically to release hydrogen. This in turn serves as the reductant of the chlorinated contaminants. A slow-release form of oxygen, magnesium peroxide, has also proved effective at stimulating the aerobic biodegradation of partially chlorinated species (26). Reactive barrier technologies, including biological barriers that exploit reductive dechlorination (22), are widely used for halogenated contaminants.

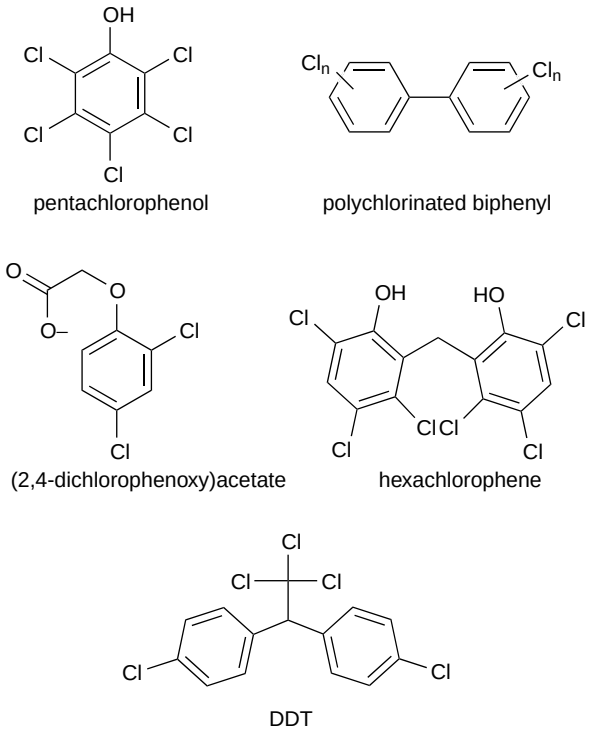

Figure 11. Some halogenated aromatic hydrocarbons.

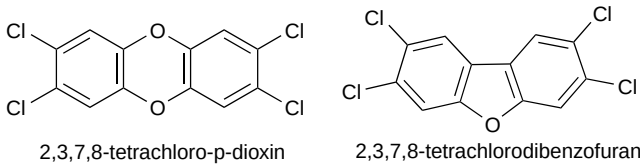

Figure 12. Dioxin and dibenzofuran.

HALOGENATED AROMATIC COMPOUNDS

Constituents.

Complex halogenated organic compounds have been widely used in commerce during the second half of the twentieth century. A few representative examples are shown in Figure 11; pentachlorophenol has been widely used as a wood preservative and also for termite control. (2,4-dichlorophenoxy) acetate (2,4-D) is widely used as a broadleaf herbicide, DDT is widely used as an insecticide, and hexachlorophene is widely used as a germicide. Polychlorinated biphenyls (PCBs) were sold with varying levels of chlorination for a range of purposes. They ranged from light oily fluids (with 2–4 chlorines) through viscous oils (5 chlorines) to greases and waxes (6 or more chlorines) and their names indicated the level of chlorination. Thus, Aroclor 1242 (Monsanto, U.S.A.), Kanechlor 300 (Kanegafuchi Chemical Industries, Japan), and Clophen A30 (Farbenfabriken Bayer AG, Germany) contained 42% chlorine by weight and an average of 3 chlorines per biphenyl. An important property that led to their broad use is their relative inertness, especially to biodegradation, so at first glance they may not seem a good target for bioremediation. Indeed, complex halogenated organic compounds were widely thought to be almost exclusively anthropogenic in origin so that there would have been little time for biodegradation pathways to evolve. This view is being corrected, for in fact a variety of organisms, particularly marine algae and some fungi, produce significant quantities of these compounds (44). Furthermore, it is now well established that polychlorinated dibenzo-*p*-dioxins and dibenzofurans (Fig. 12) are produced when biomass (or municipal waste) is burnt (45). There is thus good reason to expect that halogenated organic degrading organisms can be found in the biosphere, and this has been borne out in practice.

Biodegradation

Adriens presented a thorough description of the microbiology and technology of the bioremediation of chlorinated aromatic compounds in this volume. An important characteristic of degradation is the cleavage of carbon-chlorine bonds, and the enzymes that catalyze these reactions, the

dehalogenases, are being characterized (46). The reductive dechlorination seen with aliphatic chlorides (Fig. 9) seems to be a general phenomenon, and even compounds as persistent as DDT and the polychlorinated biphenyls are reductively dechlorinated under some conditions, particularly under methanogenic ones. Some compounds, such as pentachlorophenol, can be completely mineralized under anaerobic conditions, but the more recalcitrant ones require aerobic degradation after reductive dehalogenation.

Pentachlorophenol can be mineralized aerobically by bacteria (47) and fungi (48) and anaerobically by bacteria (49), and several bacterial isolates are able to grow aerobically using pentachlorophenol as the sole source of carbon and energy. Many of these grow rather well when supplemented with a more nutritious substrate, but unfortunately, in many cases it seems that the more vigorous growth with these substrates does not enhance the biodegradation of pentachlorophenol.

(2,4-dichlorophenoxy)acetate (2,4-D) has been one of the world's most popular herbicides, and although it is somewhat resistant to biodegradation, it is biodegraded by several bacterial isolates. It is a general truism that the more halogens on a molecule, the slower its biodegradation, and this is borne out with the related herbicide 2,4,5-T ((2,4,5-trichlorophenoxy)acetate). Nevertheless, bacterial degradation has been seen under both aerobic and anaerobic conditions, the latter involving reductive dechlorination via 2,4-D. Aerobic degradation removes acetate from (2,4-dichlorophenoxy)acetate to yield 2,4-dichlorophenol, which is subsequently hydroxylated to 3,5-dichlorocatechol, followed by ring cleavage and complete mineralization (50). There is good evidence that the genes for the degradation of 2,4-D have undergone interspecies transfer (51).

DDT (1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane) is a remarkably resistant molecule, which explains both its efficacy as an insecticide and its accumulation at the top of the food chain. Nevertheless, there are indications that it can be biodegraded, both anaerobically, with initial dechlorination (52), and aerobically, with initial ring hydroxylation (50). DDT and its partially degraded congeners are very hydrophobic and biodegradation seems to be stimulated by adding surfactants. White-rot fungi also degrade DDT under lignolytic conditions, although there is little mineralization to carbon dioxide (6).

The polychlorinated biphenyls are quite recalcitrant. Some lightly chlorinated biphenyls are readily mineralized under aerobic conditions (53), and indeed the structure of an enzyme that catalyzes the key ring-cleavage oxygenation has been determined by X-ray crystallography (54). More chlorinated congeners are resistant to aerobic degradation, but they are reductively dechlorinated under anaerobic conditions (55). Complete degradation of the commercial mixtures thus generally requires an anaerobic process, followed by an aerobic one. A major issue for the oxidative process is that biphenyl seems to be required for significant expression of the biodegradative system (53); the chlorinated compounds do not induce the enzymes that would degrade them. The biochemistry of the biodegradative process is only beginning to be unraveled, but already there are suggestions that there is considerable diversity in the enzyme systems able to degrade these compounds.

Bioremediation

Soil. Pentachlorophenol has been the target of bioremediation at a number of wood-treatment facilities, and good success has been achieved in several applications (56). It is rarely the sole contaminant and is often present with polynuclear hydrocarbons from creosote. In situ degradation has been stimulated by bioventing, wherein air is injected through some wells and extracted through others to both strip volatiles and provide oxygen to indigenous organisms (57).

The kinetics of such in situ degradation is rather slow, however, and more active bioremediation is usually attempted. For example, contaminated soil at the Champion Superfund site in Libby, MT, was placed into 1-acre land treatment units in 6-inch layers, and irrigated, tilled, and fertilized. Under these conditions, the half-lives of pentachlorophenol, pyrene, and several other polynuclear aromatic hydrocarbons, initially present at around 100 to 200 ppm, were on the order of 40 days (57). This success relied on the indigenous microbial populations in the soil, but many groups are focusing on the addition of organisms (58). Composting, and bioremediation focusing on the use of white-rot fungi, has also met with success at the pilot scale. Others have used fed-batch or fluidized-bed bioreactors to stimulate the biodegradation of pentachlorophenol. This allows significant optimization of the process and increases rates of degradation by 10-fold (57).

A major concern when remediating wood-treatment sites is that pentachlorophenol was often used in combination with metal salts, and these compounds, such as "chromated-copper-arsenate" (CCA), are potent inhibitors of at least some pentachlorophenol-degrading organisms (59). Sites with significant levels of such inorganics may not be suitable candidates for bioremediation.

The phenoxy-herbicide 2,4-D has been successfully bioremediated in soil contaminated with such a high level of the compound (710 ppm) that it was initially toxic to microorganisms (60). There were essentially no indigenous bacteria in the soil, and success relied on washing a significant fraction of the contaminant off the soil and adding bacteria enriched from a less contaminated site. Success was achieved in remediating both soil washwater and soil in a bioslurry reactor (60). 2,4-D is also effectively degraded in composting, with about half being completely mineralized, and the other half becoming incorporated in a nonextractable form in the residual soil organic matter (61).

The bioremediation of polychlorinated biphenyls in soils is receiving significant attention (53,56) because these compounds are quite widely distributed in the environment, either from leaking electrical transformers or because they were applied as part of road maintenance. In the latter case, the contamination usually includes petroleum hydrocarbons and unfortunately it seems that the two contaminants inhibit each other's degradation. Nevertheless, cultures are being found that

can degrade both polychlorinated biphenyls and petroleum hydrocarbons.

Groundwater. A successful groundwater bioremediation of pentachlorophenol is being carried out at the Libby Superfund site described earlier. A shallow aquifer is present at 5.5 to 21 m below the surface, and a contaminant plume is nearly 1.6 km in length. Nutrients and hydrogen peroxide were added at the source area and approximately half way along the plume, and pentachlorophenol concentrations decreased from 420 ppm to 3 ppm whenever oxygen concentrations were successfully raised. A membrane oxygen dissolution system was installed to replace the initial hydrogen peroxide additions, and costs were substantially lowered without an apparent decrease in remediation performance (62).

Pentachlorophenol is readily degraded in biofilm reactors (63), so bioremediation is a promising option for the treatment of contaminated groundwater brought to the surface as part of a "pump and treat" operation.

River and Pond Sediments. Much of the work on polychlorinated biphenyls has focused on the remediation of aquatic sediments, particularly from rivers, estuaries, and ponds (53). As noted earlier, a few of the most lightly chlorinated compounds are mineralized under aerobic conditions, but the more chlorinated species seem completely resistant to aerobic degradation, even by white-rot fungi. On the other hand, there is extensive dechlorination of highly chlorinated forms under anaerobic, particularly methanogenic, conditions. Bioremediation thus requires anaerobic and aerobic regimes. Intrinsic biodegradation of polychlorinated biphenyls can be recognized by the changing "fingerprint" of the individual isomers as biodegradation proceeds (53,64). The anaerobic dechlorination of the most recalcitrant congeners can apparently be "primed" by adding a readily dehalogenated congener, such as 2,5,3',4'-tetrachlorobiphenyl (65), but whether this is a realistic approach for in situ bioremediation remains to be seen. Harkness and coworkers (66) have successfully stimulated aerobic biodegradation in large caisons in the Hudson River by adding inorganic nutrients, biphenyl, and hydrogen peroxide, but found that repeated addition of a polychlorinated biphenyl degrading bacterium (*Alcaligenes eutrophus* H850) had no beneficial effect. Essentially no biodegradation occurred in the stirred control caissons, but losses on the order of 40% were seen in the caissons that received nutrients and peroxide, regardless of whether the stirring was aggressive or rather gentle. Whether this approach can be scaled-up for large scale use, with a net environmental benefit, remains to be seen.

NONCHLORINATED PESTICIDES AND HERBICIDES

Constituents. A vast number of herbicides, pesticides, fungicides, and so on, have been sold, and a few are shown in Figure 13. In order to be effective, they must have their effect before they are degraded in the environment, but on the other hand they must not be so resistant to degradation that they accumulate where they are used or in food chains. It is unusual for these compounds to become contaminants where they are applied correctly, but manufacturing facilities, storage depots, and rural airfields where "crop-dusters" are based have had spills that can lead to long-lasting contamination. Bioremediation is a promising technology to remediate such sites. There are also some locations where groundwater has become contaminated by these chemicals, and again, bioremediation may be a cost-effective remediation strategy.

Biodegradation

The majority of herbicides, pesticides, fungicides, and insecticides are biodegradable, although the intrinsic

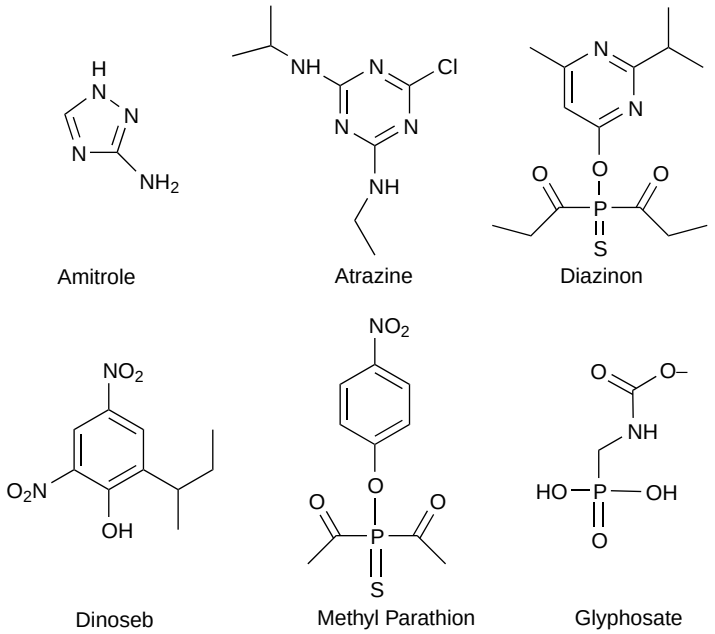

Figure 13. Some representative nitrogen-containing hydrocarbons.

biodegradability of individual compounds is one of the variables used in deciding which compound to use for a particular task. Some herbicides are acutely toxic to plants that absorb them, but are so readily degraded in soil that seeds can be planted at the same time as the herbicide application. Other herbicides are known to be effective at preventing plant growth for many months, and are used for long-term weed control. In some cases, the biodegradation of a pesticide is so rapid that it loses its efficacy, and pesticide rotation strategies must be used. Very few degradation pathways of these compounds have been worked out in detail, but some generalizations can be made.

Compounds with organophosphate moieties, such as diazinon, methyl-parathion, coumaphos, and glyphosate are usually hydrolyzed at the phosphorus atom (50,67). Indeed, several *Flavobacterium* isolates are able to grow using parathion and diazinon as sole sources of carbon.

Triazines pose rather more of a problem, probably because the carbons are in an effectively oxidized state so that little metabolic energy is obtained by their metabolism. Very few pure cultures of microorganisms are able to degrade triazines such as atrazine, although some Pseudomonads are able to use the compound as sole source of nitrogen in the presence of citrate or other simple carbon substrates. The initial reactions seem to be the removal of the ethyl or isopropyl substituents on the ring (50), followed by complete mineralization of the triazine ring. The nitrogen can be used as nitrogen source by some organisms (68), and as sole carbon and nitrogen source under anaerobic conditions (69).

Nitroaromatic compounds, such as dinoseb, are degraded under aerobic and anaerobic conditions (70). The nitro group may be cleaved from the molecule as nitrite or reduced to an amino group under either aerobic or anaerobic conditions. Alternatively, the ring may be the subject of reductive attack. Thus, while these molecules are sometimes quite long-lived in the environment, they can be completely mineralized under appropriate conditions (70). Alternatively, inert residues can be immobilized; for example, a *Clostridium bifermentans* anaerobically degrades dinoseb cometabolically in the presence of a fermentable substrate to below detectable levels, although only a small fraction is actually mineralized to carbon dioxide (71).

Carbamates such as aldicarb undergo degradation under both aerobic and anaerobic conditions. In fact, the oxidation of the sulfur moiety to the sulfoxide and sulfone is part of the activation of the compound to its most potent form. Subsequent aerobic metabolism can completely mineralize the compound, although this process is usually relatively slow so that it is an effective insecticide, acaricide, and nematocide. Anaerobically these compounds are hydrolyzed, and then mineralized by methanogens (72).

Bioremediation

Groundwater. Atrazine dominated the world herbicide market in the 1980s, and contamination of groundwater has been reported in locations worldwide. There are several reports that once in groundwater it is very recalcitrant, suggesting that atrazine-degrading organisms are not widespread. Nevertheless, successful biodegradation has been achieved with indigenous organisms in laboratory mesocosms after a lag phase, and activity remained once it was found (73). Interestingly, the degradation was somewhat slowed by the addition of low concentrations of readily assimilatable carbon, such as lactate, and it is not clear how biodegradation might be stimulated in the field. Nevertheless, it is clear that intrinsic remediation is likely to lead to the disappearance of atrazine from groundwaters once atrazine utilizers have become abundant, and perhaps inoculation with atrazine-metabolizing species will be effective.

If more active treatment is required, such as "pump and treat," it is possible that biological reactors will be a cost-effective replacement for activated carbon filters.

Soil. Herbicides and pesticides are of course metabolized in the soil to which they are applied, and there are many reports of isolating degrading organisms from such sites. Degradation activities are typically much higher at sites that have seen product application, indicating that natural enrichment processes occur. Much current effort is aimed at assessing the diversity of degradative pathways, and in many cases it seems that several different natural metabolic pathways can degrade individual pollutants. Little work has yet been presented in which the biodegradation of these compounds has been successfully stimulated, at field scale, by a bioremediation approach, but inoculation with active organisms may be a promising approach (74). The addition of genetically engineered organisms, albeit a killed recombinant *E. coli* that overexpresses atrazine chlorohydrolase, significantly increased atrazine degradation in a heavily contaminated soil (75).

MILITARY CHEMICALS

Constituents. The military use a range of chemicals as explosives and propellants, "energetic molecules," and by the nature of their use it is no surprise that there are now many areas in need of remediation. Generally speaking, modern explosives are cyclic, often heterocyclic compounds, composed of carbon, nitrogen, and oxygen. Perhaps the most well known is 2,4,6-trinitrotoluene (Fig. 14), but RDX (Royal Demolition explosive; hexahydro-1,3,5-trinitro-1,2,3-triazine, also known as cyclonite) and HMX (High Melting explosive; octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine) are even more powerful. N,N-dimethylhydrazine and perchlorate are used as solid rocket fuels. These compounds are sometimes present at quite high levels in soils and groundwater on military bases and production sites and need to be removed. One quite infamous problem at the latter is "pink water," a relatively undefined mixture of photodegradation products of TNT. Bioremediation is a promising technology for treating sites contaminated with such compounds.

Bioremediation may also be an appropriate tool for dealing with chemical agents such as the mustards and organophosphate neurotoxins (76,77), but little work on actual bioremediation has been published.

Biodegradation

Natural nitro-substituted organic compounds are quite unusual, and it was once thought that their degradation

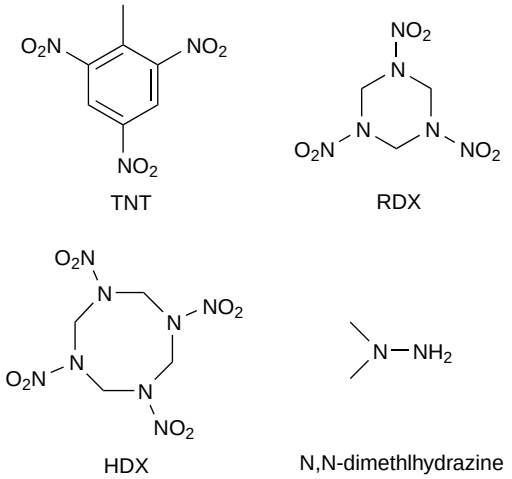

Figure 14. Some military chemicals.

was principally by abiotic processes. As we saw above in the case of dinoseb, however, nitro-substituted compounds are subject to a variety of degradative processes. The biodegradation of TNT is well established (78). Under anaerobic conditions it can be reduced to the corresponding aromatic amines and subsequently deaminated to toluene; the latter can be mineralized under anaerobic conditions, leading to the potentially complete mineralization of TNT in the absence of oxygen. Under aerobic conditions, a range of bacteria and fungi can mineralize TNT often cometabolically with the degradation of a more degradable substrate. However, there is also ample evidence that under some conditions the TNT is converted to insoluble large molecular weight compounds, probably by addition reactions to soil components such as humic and fulvic acids or cellular material in the case of plants. Lignolytic fungi also yield promising results for the degradation of both TNT and "pink water," particularly if the latter is pretreated with UV irradiation.

RDX and HMX are rather more recalcitrant, especially under aerobic conditions, but there are promising indications that biodegradation can occur under some conditions, especially composting (78). Several strains of bacteria able to use RDX (and triazine) as the sole source of nitrogen for growth have recently been isolated, and this is an area that is witnessing rapid progress.

Some work has been reported on the biodegradation of dimethylhydrazine (79), and there is a lot of current interest in perchlorate as a terminal electron acceptor for a range of heterotrophic bacteria (80). Perchlorate remediation is being achieved at the McGregor, Texas, Weapons plant site with a biological "reactive barrier technology" (81).

Bioremediation

Groundwater. Nitrotoluenes have been detected in groundwater in some areas, and intrinsic remediation seems to occur at some sites by anaerobic degradation. They are readily degraded in biological fluidized bed reactors (82), and research into whether this can be stimulated in situ with a net environmental benefit is proceeding. Rocket fuel-contaminated groundwater is also being treated at an experimental scale with bioremediation (83).

Soil. Optimum bioremediation of nitro compounds seems to require an anaerobic stage to encourage the reductive removal of the nitro groups, followed by an aerobic stage to encourage the mineralization of the carbon skeleton (78,56). Composting of soils contaminated by high explosives is being carried out at several U.S. Army Depot sites, including Umatilla in Oregon, Pueblo in Colorado, and Tooele in Utah (56). Bioslurry reactors are also in use (56).

OTHER ORGANIC COMPOUNDS

The majority of organic compounds in commerce are biodegradable, so bioremedation is a potential option for cleaning up after industrial and transportation accidents. For example, Flathman and coworkers (84) reported the successful bioremediation of 23,000 liters of vinyl acetate spilled from a railroad tank car in Albany, New York, at a cost approximately half that of excavation and disposal of the 1,100 m^3 of contaminated soil. They also report that in situ biological treatment has been used to remediate spills of other organics, including acrylonitrile, styrene, 2-butoxyethanol, and ethacrylate, at other railroad-accident sites. Bioremediation is thus already an important tool in remediating accidental spills of organic compounds.

Bioremediation is also an option when spills of such compounds contaminate groundwater. For example, bioremediation seems a feasible treatment for aquifers contaminated with phenol (85), alkylpyridines (86), sulfolane and diisopropanolamine (87). It has also been used to treat soils contaminated with the plasticizer bis(2-ethyl-hexyl)phthalate in a bioslurry reactor (88). "Biological plugs," actively aerated in-ground bioreactors, have been used to bioremediate a site contaminated with monoethanolamine (89).

INORGANIC CONTAMINANTS

Nitrogen Compounds

Constituents. Nitrogen-containing compounds are of concern for several reasons. Nitrate levels are regulated in groundwater because of concerns for human and animal health, while ammonia is regulated in streams and effluents as a potential fish toxicant, and any nitrogenous contaminant is a potential problem in water because of its stimulatory effect on the growth of algae. Other nitrogenous contaminants include cyanides in mine waters. Fortunately, all are amenable to biological treatment (90).

Biodegradation

The biological mineralization of fixed nitrogen is well studied; ammonia is oxidized to nitrite, and nitrite to nitrate, by autotrophic bacteria, and nitrate is reduced to nitrogen by anaerobic bacteria using nitrate as terminal

electron acceptor. Urea in sewage and industrial wastes is readily hydrolyzed to ammonia and carbon dioxide by many bacteria, and cyanides are used as sole sources of carbon and nitrogen by many aerobic organisms. Wastewater treatment facilities utilize these organisms in assuring that municipal and industrial effluents meet strict water quality standards.

Bioremediation

Surface Water. One example of exploiting biology to handle excess nitrogen in surface water arose at the USDoE Weldon Spring Site near St. Louis, MO. This site had been a uranium and thorium processing facility, and the processing involved nitric acid. The waste stream, known as raffinate, was discharged to surface impoundments and neutralized with lime to precipitate the metals. Two pits had nitrate levels that required treatment before discharge, but heavy rains in 1993 threatened to cause the pits to overflow. Bioremediation by the addition of calcium acetate as a carbon source successfully treated more than 19 million liters of water at a reasonable cost (91). The use of sulfur-based autotrophic denitrification ponds to remediate nitrate is also under investigation (92). Here the sulfur acts as electron donor to the nitrate, and competing sulfur oxidation by aerobes must be controlled by minimizing mixing and aeration.

Groundwater. One approach to minimizing the environmental impact of excess nitrogen in groundwater is to add a degradable substrate to the contaminated aquifer, in the absence of oxygen, to stimulate bacterial denitrification. Soluble compounds such as ethanol and acetate have been suggested (90), but insoluble substrates such as vegetable oil (93) or sawdust (94) seem to provide better control, especially for wells.

METALS AND METALLOIDS

A wide range of metallic and nonmetallic contaminants is present at industrial and agricultural sites throughout the world, both in ground and surface water and in soils. They pose quite a different problem from that of organic contaminants, since they cannot be degraded so that they "disappear." Some metal and metalloid elements have radically different bioavailabilities and toxicities depending on their redox state, so one option is stabilization by converting them to their least toxic form. This can be a very effective way of minimizing the environmental impact of a contaminant, but if the contaminant is not removed from the environment there is always the possibility that natural processes, biological or abiological, may reverse the process. Removing the contaminants from water phases is relatively straightforward, and the wastewater treatment industry practices this on an enormous scale. "Pump and treat" systems that mimic wastewater treatments are already being used for several contaminants and less complex systems involving biological mats are a promising solution for less demanding situations.

Immobilization and Toxicity Minimization

Adsorption. Biomass, often agricultural by-products but also including microbial products, have been widely used as adsorbents for metals and other contaminants in water (95), but they are outside the scope of this article.

Microbial Processes. Most elements display a range of solubilities and biological effects depending on their chemical form. For example, chromium, while it may be an essential micronutrient for many organisms, is known to be toxic at higher levels. Indeed, chromium has a range of available redox states that differ significantly in their environmental impact. Cr(VI) is generally more soluble than Cr(III), and more toxic, but a wide range of organisms, both bacteria and fungi, are able to reduce the former to the latter under both aerobic and anaerobic conditions, thereby reducing the environmental impact of the contaminant. Since Cr(III) precipitates as insoluble hydroxides under neutral and alkaline conditions, microbial reduction is a potential treatment for soils and waters (96).

Similarly, selenium is a micronutrient that is toxic at higher levels. It also has quite a different bioavailability in its different redox states, with the elemental form being the least biologically available. Many microorganisms, both bacteria and fungi, are able to reduce more oxidized species, especially selenite (Se(IV)), to the red elemental form, providing an appealing remediation option for this element (97). An alternative approach to remediating selenium contamination might be to encourage methylation to volatile dimethylselenide. Although the microbiology of this process is not yet very well understood, it seems to be the result of degradation of the selenium containing amino acid selenomethionine.

Arsenic is another element with different bioavailability in its different redox states. Arsenic is not known to be an essential nutrient for eukaryotes, but arsenate (As(V)) and arsenite (As(III)) are toxic, with the latter being rather more so, at least to mammals. Nevertheless, some microorganisms grow at the expense of reducing arsenate to arsenite (98), while others catalyze the reverse reaction (99). The element can be immobilized as an insoluble polymetallic sulfide by sulfate-reducing bacteria, presumably adventitiously because of the production of hydrogen sulfide (100). Indeed, many contaminant metal and metalloid ions can be immobilized as metal sulfides by sulfate-reducing bacteria. Sulfate-reducing bacteria can immobilize uranium quite effectively, but in this case it is precipitated as uraninite (UO_2) (101).

A rather more specific mechanism of microbial immobilization of metal ions is represented by the accumulation of uranium as an extracellular precipitate of hydrogen uranyl phosphate by a *Citrobacter* species (102). Staggering amounts of uranium can be precipitated — more than 900% of the bacterial dry weight! Indeed, even elements that do not readily form insoluble phosphates, such as nickel and neptunium, may be incorporated into the uranyl phosphate crystallites (103). The precipitation is driven by the production of phosphate ions at the cell surface by an external phosphatase, and while the process requires the addition of a phosphate donor, such as

glycerol-2-phosphate, it may be a valuable tool for cleaning water contaminated with radionuclides.

Microbial processes can also accumulate and immobilize mercury ions. *Escherichia coli* engineered to overexpress a Hg^{2+} transporter and metallothionein accumulate and immobilize mercury ions quite selectively (104). Alternatively, many microorganisms reduce mercury compounds to the elemental form, which is volatile (105). Although this certainly reduces the environmental impact of compounds such as methylmercury, such a bioprocess would have to include a mercury capture system (106) before it could be exploited on a large scale with public support.

Bioremediation

Water. Groundwater can be treated in anaerobic bioreactors that encourage the growth of sulfate-reducing bacteria, where the metals are reduced to insoluble sulfides and concentrated in the sludge. For example, such a system is in use to decontaminate a zinc smelter site in the Netherlands (107).

Bacterial remediation of selenium oxyanions in San Joaquin (CA) drainage water is under active investigation (108,109), but has not yet been commercialized. Agricultural drainage rich in selenium is also typically rich in nitrates, so bioremediation must also include conditions that stimulate denitrification (110).

Rhizofiltration and Other Forms of Phytoremediation. Rhizofiltration is the use of plant root systems to remove contaminating metal ions from water (111), and it now seems clear that microorganisms play an essential role on the roots (112). Nevertheless, little is known about the specific interactions of microbes with the plants, and this area is very much in its infancy.

Mine Drainage. Natural drainage waters that come into contact with active and abandoned metal and coal mines can become seriously contaminated with a range of heavy metal ions and/or often become quite acidic, with a pH near 2. While underground, the water is typically anoxic, and any iron is present as soluble ferrous species. When this mixes with aerobic surface water, the iron precipitates as bright orange ferric hydroxide, and this can have a serious environmental impact. In recent years, it has become clear that the environmental impact of acid mine drainage can be minimized by the construction of artificial wetlands that combine geochemistry and biological treatments. These systems are being designed for a range of wastewaters, most of which fall outside the scope of this section.

The precipitation of ferric hydroxide is typically biologically mediated, by iron-oxidizing bacteria, at acid pH, but is usually rather slow. Abiotic oxidation becomes more important at pH values above 5, and this is usually much faster than the biological process. Most constructed wetlands for treating acid mine waters thus start with a zone designed to raise the pH. A bed of crushed limestone often suffices to raise the pH significantly, and it is important that this be kept anoxic to prevent rust precipitation on the limestone, which would prevent further production of alkalinity. Once the pH is near neutral, the water is discharged into an aerobic wetland to encourage the precipitation of iron and aluminum oxides and the coprecipitation of arsenic, if this is present (113).

If heavy metals are present in mine water, the iron-free water can be made to flow into an anaerobic part of the constructed wetland, where organic material, such as compost, manure, or sawdust, provides reductants to sulfate-reducing bacteria that become established therein. These bacteria reduce sulfate to sulfide, which precipitates the heavy metal ions as insoluble sulfides. It is important that this part of the constructed wetland be kept anaerobic to prevent oxidation and remobilization of the precipitated metals. For this reason, this part of the wetland is typically kept flooded and free of aquatic plants that might introduce oxygen through their roots. (113). Finally, an aerobic algal mat can act as a polishing step to complete the removal of contaminants, particularly manganese (114). Mine drainage that is not acidic may not need such complicated systems, and individual parts of the treatment train described above may suffice in some situations.

Bacteria have been used to reduce Cr(VI) to Cr(III), thereby immobilizing it in soils and water (115). Reactive barrier technologies, including biological barriers (22), are being widely used for metal ion and metalloid contaminants.

CONCLUSION

Bioremediation is being successfully used to treat a wide range of contaminants in a broad range of environments. Contaminants amenable to bioremediation include crude oils and refined petroleum products, halogenated solvents, pesticides, herbicides, military chemicals, and mine waters, and they can be treated in air, soil, and water. In all cases it is clear that aiding and abetting natural selection is the key to success and that making small adjustments of the local environment to encourage the growth of remediating organisms may be all that is needed. For example, petroleum is biodegradable, but this degradation is typically nutrient limited; partially relieving this limitation can lead to stimulating the biodegradation severalfold with no adverse environmental impact. On the other hand, organisms growing on other substrates most effectively remove several halogenated solvents; adding these substrates, for example, methane, significantly increases the rate of removal of the contaminant.

The centrality of natural selection in designing a remediation strategy cannot be overestimated. One area where it sometimes seems to be ignored is the consideration of the need to add microbial inoculants to stimulate biodegradation (bioaugmentation). Some modern contaminants of concern (oil, many halogenated compounds) have been part of the biosphere for millennia, albeit usually at low levels. Microbes that can degrade them are probably ubiquitous, so adding organisms to stimulate biodegradation is unlikely to be cost-effective. If the environment is made hospitable for growth, the indigenous organisms are likely to prove sufficient for bioremediation. Perhaps the most startling demonstration of this was performed by the USEPA when testing potential inoculants for stimulating oil biodegradation

in Alaska following the Exxon Valdez oil spill (116). Eight products were tested in small laboratory reactors that allowed substantial degradation of a test oil by the indigenous organisms of Prince William Sound; all eight microbial inocula had a greater stimulatory effect on alkane degradation if they were sterilized by autoclaving prior to addition. This suggests that the indigenous organisms readily outcompeted the added products, but that autoclaving the products released some trace nutrient that was able to stimulate the growth of the endogenous organisms.

Of course, bioaugmentation may prove more effective with contaminants that have only recently entered the biosphere, such as methyl-*t*-butyl ether (11,12), or are toxic (58,60). It may also be effective if material is to be composted, where it is likely that composting organisms may take some time to become established. And modern molecular biology may offer opportunities for moving effective degradation pathways into organisms native to a contaminated site, improving biodegradation pathways by broadening the substrate range of degradative enzymes or by removing biochemical constraints to maximize degradative activities (75). There are, however, many technical and regulatory hurdles to be surmounted before this is likely to be widely used.

Another area where there is controversy is in the role of surfactants. Many contaminants of concern are hydrophobic and are adsorbed to soil material. Many bacteria, particularly those that degrade hydrocarbons, produce surfactants that are thought to help increase the bioavailability of the contaminant. Some release the surfactants into the medium, others incorporate them into their cell exterior, and there have been elegant experiments to show that inhibiting the production of these compounds inhibits the ability of the bacteria to degrade their substrates. Allen and coworkers (117) provide an example of how difficult it is to make broad generalizations; they examined the ability of a *Pseudomonas* and a *Sphingomonas* to degrade naphthalene and phenanthrene in the presence of a nonpolar, nondegradable (by these organisms) solvent (2,2,4,4,6,8,8-heptamethylnonane) or the nonionic surfactant Triton X-100. The heptamethylnonane increased the rate of degradation of both substrates for both microorganisms. The Triton X-100 increased the rate of degradation by the *Pseudomonas*, but inhibited that by the *Sphingomonas*. There have been many suggestions to add surfactants, either microbial or synthetic, to stimulate biodegradation. This seems to be beneficial in the case of some oil dispersants at sea (118), but the situation seems complicated in soils, and probably needs to be assessed on a case-by-case basis (119). Surfactants did not stimulate pentachlorophenol degradation in one recent study (120). Nevertheless, surfactants are clearly beneficial in the physical part of technologies such as bioslurping (18), so it will be important to ensure that, at least, they do not inhibit the biodegradation of residual contaminants.

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BIOREMEDIATION: AQUATIC ECOSYSTEMS

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Contamination of aquatic environments, both freshwater and marine, with petroleum hydrocarbons and a variety of xenobiotic compounds is a commonplace occurrence in the modern world. Oceans and rivers remain major avenues of transportation and commerce with tankers and supertankers traversing the globe. While spectacular oil spills, resulting in the contamination of hundreds to thousands of square miles, have left the most vivid impact on the general public, scores of smaller releases and spills have resulted in extensive contamination of open waters, shores, and sediments. One of the technologies that has found widespread application in the remediation and restoration of aquatic ecosystems is bioremediation. This chapter will review the basic principles involved in the use of bioremediation in aquatic (freshwater and marine) ecosystems, provide an overview of examples of successful bioremediation projects, and examine several case studies in more depth to present a snapshot of the current state of the art. In addition to other chapters in this work, the reader is referred to numerous recent books and review articles for additional information (1–13).

OVERVIEW OF BIOREMEDIATION

Bioremediation relies on the abilities of microorganisms to degrade and detoxify organic and inorganic compounds. In its simplest form, bioremediation involves no intervention at all and simply allows natural processes to reduce the concentration of the contaminant in an environment. This is usually referred to as natural attenuation or intrinsic bioremediation. In most instances, alteration or manipulation of physical/chemical properties of the contaminant or factors in the environment is used to enhance the natural processes. This is referred to as *enhanced bioremediation*. Fundamentally, there are two approaches to

this enhanced bioremediation: (*1*) biostimulation, which depends primarily on the modification of the environment and (*2*) bioaugmentation, which uses the addition of microbial cultures to increase biodegredation. Frequently, these two approaches are combined and microorganisms are introduced in conjunction with such environmental modifications as nutrient supplementation.

Bacteria capable of at least some transformation of all but the most exotic of man-made chemicals are probably present in all aquatic environments (1,2,5,6,14–16). Isolation and characterization of organisms capable of degrading petroleum and related hydrocarbons (17–22), polyaromatic hydrocarbons (19,23–28), alkylbenzenes (29–31), chlorinated solvents (31,32), polychlorinated biphenyls, dioxins, and related compounds (33–36), and other organic contaminants (37,38) under either aerobic or anaerobic conditions is commonly reported in the microbiological literature. Kimura and coworkers (39) isolated 96 strains of bacteria from seawater that were capable of degrading normal alkanes, branched alkanes, cycloalkanes, and aromatic compounds. A marine bacterium, isolated from intertidal crude-oil contaminated sediments, showed enhanced growth in the presence of added petroleum hydrocarbons compared to growth in unsupplemented marine medium (40). Oil degrading bacteria were isolated from 96% of the sites examined in the vicinity of Zhejaing islands. The abundance of oil degrading microorganisms was correlated with the concentration of petroleum hydrocarbons in the water (41). Considering that most (90–99%) microbial species in natural communities cannot be detected using standard laboratory culture techniques, it is likely that these reports reflect the barest minimum of the biodegradation capabilities of microorganisms (42).

Chronic release of low levels of contaminants into aquatic environments usually results in an enrichment of the microbial community for organisms capable of degrading the contaminant. Under unfavorable environmental conditions or in the absence of prior exposure to the contaminant however, both the numbers and activity of specific microbial populations may be extremely low, accounting for 1% or less of the total bacterial community (43–45).

In many instances a lag or acclimation period is observed before active degradation of the compound takes place. Acclimation is a general term that may refer to several changes in microbial populations, including (*1*) growth of an initially small population of organisms capable of degrading the contaminant, (*2*) changes in metabolic regulation including induction and/or derepression of key enzymes in the necessary metabolic pathways, (*3*) genetic change in the indigenous populations, and (*4*) the preferential use of alternative substrates by the degrader populations (8,46–48). Leahy and coworkers (49) concluded that lack of adaptation by microbial communities accounted for low petroleum hydrocarbon degradation rates in sediment samples from the Gulf of Mexico. Siron and coworkers (50) assessed the adaptation of an indigenous cold seawater microbial community to petroleum hydrocarbons by monitoring the ratio of oil-degrading bacteria to total heterotrophs within the community. They reported a 10-fold increase in this ratio following exposure of the community to crude oil. Delille and Delille (51) studied enclosures on nine different sub-Antarctic intertidal beaches to which crude oil had been added. Despite the initially pristine condition of all of the sites, indigenous populations of oil-degrading microorganisms were present.

If environmental conditions prevent the natural development of microbial populations capable of degrading a particular compound, or if the development of such populations occurs at too slow a rate, it may be possible to enhance biodegradation through the addition of supplemental microorganisms (52,53). This process, termed *bioaugmentation*, may be accomplished using either microbial cultures selected from the contaminated site, grown under controlled conditions, and then re-added at much higher concentrations to the site or, alternatively, a variety of commercially prepared microbial products each designed for degradation of specific contaminants.

Effective bioremediation depends upon extensive site characterization with a particular emphasis on factors that control or enhance microbial activity. Although there are a large number of factors that influence microbial populations and activities, only a limited number of factors are amenable to modification from a technological and economic perspective.

All biological systems are influenced by ambient temperature. Microorganisms, which can be broadly categorized in terms of their temperature range, are no exception. Within the range of temperatures in which a microorganism can grow, biological activity generally increases with increasing temperature with maximum activity occurring at a temperature just below the maximum temperature the organism can tolerate. As a general rule of thumb, microbial activity doubles with each 10 °C increase in temperature. Seasonal differences in temperature, therefore, can have profound effects on the rate of biodegradation (43,54,55). Wyndiam and Costerton (56) reported a 40-fold reduction in the rate of naphthalene degradation in river sediment during the winter (0 to 4 °C) compared to the summer (8 to 21 °C).

Aquatic environments are frequently at temperatures in which microbial activity is low. Manipulation of the temperature of aquatic environments usually is not feasible and, therefore, temperature can be a significant constraint in the use of bioremediation. Low environmental temperature was a significant factor limiting the rate of bioremediation during cleanup of the *Exxon Valdez* spill in Prince William Sound, Alaska. In some instances in which contamination is primarily associated with sediments, ex situ treatment in bioreactors may allow for modification of temperature.

The optimal pH for microbial activity is generally between 5.5 and 8.5. Environmental pH is not a significant factor controlling bioremediation in open waters, such as rivers or oceans in which pH is usually controlled by natural buffering systems; however, pH may be outside the optimal range within sediments. Incubation of salt marsh sediments under slightly acidic conditions (pH 5.0 and 6.5) was found to decrease hydrocarbon mineralization relative to incubation at higher pH (8.0) (57). As with temperature,

manipulation of sediment pH is usually confined to the treatment of excavated/dredged material in bioreactors.

Microorganisms require a terminal electron acceptor as a sink for electrons generated during the metabolism of organic compounds. Under aerobic conditions, oxygen is used as the terminal electron acceptor being reduced to water. As many organic contaminants such as petroleum hydrocarbons are less dense than water they usually are found on the surface of open waters. Under these conditions, oxygen concentration is not a limiting factor. Degradation of organic compounds is generally, but not always, faster under aerobic rather than anaerobic conditions.

Contamination of aquatic environments is not limited to the surface of open waters. Numerous mechanisms are responsible for the transport of contaminants to sediments. Some contaminants such as chlorinated solvents are heavier than water and sink in aquatic systems. As oil weathers, the formation of droplets that are heavier than water may result in the transfer of these compounds to the sediments underlying open water. High molecular weight fractions of petroleum, including polyaromatic hydrocarbons such as benzo(a)pyrene tend to sorb to particles within the water column and hence accumulate within sediments (14,58). Thus, contaminants within aquatic environments can be transferred downward in the water column and under conditions of thermal (or in some estuaries salinity) stratification may enter anoxic/anaerobic environments or accumulate in sediments with reduced or depleted oxygen. Finally, wind and wave action can carry floating contaminants to shore areas such as beaches and wetlands where they can be deposited in sediments. Under conditions of oxygen limitation, some populations of microorganisms that can use alternative compounds, such as nitrate, sulfate, and carbonate as terminal electron acceptors may be able to degrade the contaminants, including petroleum hydrocarbons and polycyclic aromatic hydrocarbons (26,58–60). Microorganisms from the New York/New Jersey estuary, a site with a history of pollution, were able to degrade benzene, toluene, ethylbenzene, and xylenes components in the absence of molecular oxygen. Enrichment cultures from the sediments with this ability were established under denitrifying, sulfidogenic, methanogenic, and iron reducing conditions (61). In the case of anoxic/anaerobic sediments, electron acceptors (and redox potential) can be controlled in bioreactor systems for the remediation of excavated material.

Carbon, nitrogen, and phosphorus are essential for microbial maintenance and growth (1,2,5,62,63). During biodegradation of organic contaminants, the microbe's carbon requirements usually are met by the contaminant itself. Nitrogen and phosphorus, on the other hand, are present at extremely low concentrations in aquatic environments. Microbial growth, therefore, is limited by the low nutrient levels (1,5). Fuentes and coworkers (64) reported that ambient phosphorus concentrations were important in the survival of petroleum degrading isolates in tropical bays. In some cases, there are sufficient inputs of inorganic nitrogen and phosphorus to support extensive microbial activity (65–68). In these cases, intrinsic bioremediation, the use of natural microbial biodegradation without environmental modification to enhance microorganisms, may be sufficient to remediate a contaminated site (67,68).

When degradation is limited by the available concentrations of fixed forms of nitrogen and phosphorus, fertilizer addition can be effective in stimulating microbial activity and enhancing biodegradation of the contaminating petroleum (69). Care must be taken in determining the type and amount of fertilizer that should be added to the site. The amount of supplemental inorganic nutrients can be estimated on a stoichometric basis. Several standard textbooks on hazardous waste treatment and bioremediation outline the methods for making such an estimation (6,70). A more accurate appraisal of the optimum amount of inorganic nutrients to be added can be made using microcosm studies. A variety of techniques for conducting these types of studies have been described in the literature (1,71,72).

The amount of inorganic nutrients is not the only factor that needs to be considered in designing a biostimulation system. Equally important is the form of nutrients used (73,74). Water-soluble nutrients such as inorganic salts rapidly dissolve and are dispersed and diluted (75). For example, Foght and coworkers (76) found that the addition of supplemental nitrogen in the form of ammonium to a marine enrichment culture resulted in decreased degradation of aromatic compounds while the use of nitrate, as a source of nitrogen, enhanced biodegradation. Oleophilic nutrients dissolve slowly and so their use can result in sustained release of nutrients necessary to support bacterial growth and activity. Santos and coworkers (77) compared two different oleophilic fertilizers for use in enhanced bioremediation. Both of the products increased the biodegradation of Iranian crude oil. Neither formulation was found to be clearly superior to the other; rather, each had its own advantage (faster biodegradation of alkenes versus quicker disappearance of the oil slick) and the authors concluded that a mixture of the two fertilizers would offer the best treatment option.

Microorganisms are unable to efficiently metabolize compounds that are not dissolved in water. The availability of a compound is influenced by both its solubility and its partitioning between the aqueous and organic phases (78,79). Sorption of organic compounds by sediments can have profound effects on the bioavailability of the compounds. Generally, these materials are less bioavailable than dissolved compounds. In addition, sorption to sediment particles may result in the transport of the sorbed compounds to environments not conducive to biodegradation. Microorganisms are known to produce surface-active compounds — bioemulsifiers or biosurfactants — that increase the solubility and availability of organic compounds (80). In addition, man-made emulsifiers can be used to enhance bioavailability (5,6).

Biosurfactants can increase the bioavailability of nonaqueous phase liquids, such as hydrocarbons by either increasing the aqueous solubility of the hydrocarbons or by increasing the hydrophobicity of the bacterial cell surface. Rosenberg and Ron (81) have recently reviewed the structure, biosynthesis, and possible uses of biosurfactants. Rhamnolipids produced by two strains of *Pseudomonas*

aeruginosa grown on glucose and hexadecane were found to cause release of LPS from the outer membrane of the organisms resulting in lower cell surface hydrophobicity even at concentrations of the biosurfactant below the critical micelle concentration (82). *Acinetobacter radioresistans* KA53, on the other hand produces a high molecular weight complex of polysaccharide and protein named *Alasan* that functions as a bioemulsifier (83). Passeri and coworkers (84) isolated a glycolipid biosurfactant from a marine bacterial isolate (MM1) that increased solubility of normal alkanes in water.

Effective bioremediation depends upon containment of the contaminant within a defined area in which environmental conditions can be modified and controlled to enhance microbial growth and metabolism. This is not possible within open waters. While a few studies have been conducted in open water, the effectiveness of bioremediation remains unproven. In contrast, bioremediation in coastal regions and sediments has been shown to be effective in the treatment of contaminating organic compounds (1,9,11,12,85).

AQUATIC ENVIRONMENTS

Marine environments — oceans, estuaries, shorelines, and tidal marshes — are the most extensive environments in the world. Oceans alone occupy a volume of 1.46×10^9 km^3, approximately 71% of the Earth's surface. They are highly diverse in terms of their chemical, physical, and biological characteristics. Marine water is characterized by salinity between 3.3 and 3.7%. If brackish waters are included within this definition, salinity ranges from 1.0 to 3.7% are possible. In addition to the variability in chemical composition, marine environments are subject to considerable physical mixing. Winds, waves, tides, and currents all interact to result in a constantly changing environment. Nor is the variability limited to surface waters. Vertical stratification of water layers results in the presence of multiple habitats that differ in terms of salinity, light, temperature, pressure, and other factors even within limited geographic areas. Finally, different marine environments show profound differences in the biological communities present. Open ocean waters have extremely low biological productivity analogous to terrestrial deserts, while estuaries and tidal marshes have some of the highest productivity in the world. Heterotrophic bacterial populations in open oceans are typically highest at the surface (neuston) and decrease rapidly with depth. Thus, while numbers of 10^7 to 10^8 cells/mL are commonly found in upper ocean waters, this number may decrease by as much as an order of magnitude with each 100 m of depth (86). In oligotrophic areas, microbial numbers within the water column may reach as low as 10^0 organisms/mL. In addition to the differences in microbial numbers within the water column, there are also differences in the typical number of microorganisms between coastal and open water areas. In general, coastal waters have higher concentrations of microorganisms than open waters (87,88). This difference primarily reflects the different nutrient status of the two habitats with coastal areas benefiting from the input of nutrients from the surrounding terrestrial systems.

In terms of the global water budget, freshwater environments make a miniscule contribution. In terms of human society, freshwater environments have an unparalleled importance. The limited global freshwater reserves serve as a medium for transportation, support agriculture and industrial processes, function as a repository for waste products, provide recreational opportunities, and sustain human life by providing potable water. Because of the necessity of water for human existence, much research has been devoted to developing treatment systems and other means to safeguard freshwater environments.

Freshwater environments are as diverse as marine environments. Fundamentally, a distinction can be made between standing (lentic) water bodies such as lakes and ponds and flowing (lotic) systems such as rivers and streams.

In contrast to marine environments, freshwater environments are scarce, accounting for less than 1/10,000 of the total water available on the earth. While sharing many characteristics with marine and estuarine environments, there are several key points to consider in addressing bioremediation of freshwater environments. First, because of their small size compared to marine environments, freshwater environments are less constant, less stable in terms of physical and chemical characteristics. Temperature fluctuation, diurnal cycles of dissolved oxygen, and sudden changes in silt and sediment load owing to storm water are among the variables significantly influencing the biota of lakes, streams, and wetlands. In addition, because of the drastically reduced ratio of water to sediments, benthic processes assume a more important role in freshwater environments than in marine systems. Sediments rather than becoming a geologic sink for materials as happens in the profoundal depths of the ocean, become a temporary repository with a constant exchange of materials occurring between the sediments and the water. This means that sediments can serve as a source of extended contamination to the surrounding water and biota by the slow desorption of organic contaminants. Furthermore, as benthic/detrital food chains dominate in freshwater environments, the transfer and subsequent accumulation of lipophilic organic compounds into upper levels of the food chain are likely.

BIOREMEDIATION OF PETROLEUM HYDROCARBONS IN AQUATIC ENVIRONMENTS

Contamination of aquatic environments is the result of both natural processes and human activities. The variety of compounds impacting marine environments is enormous; bioremediation of one category of contaminants — petroleum hydrocarbons — will be considered here. Oil spills from off-shore drilling, tanker accidents and lightings, accidental releases associated with maritime activity, industrial releases, atmospheric transport and deposition of products of incomplete combustion, runoff from surrounding land, and natural oil seeps are major sources of petroleum hydrocarbons into the marine environment (Table 1; 89–95). While oil spills such as the

Table 1. Global Input of Oil to Marine Environments

Source	Amount (estimated 1990) (thousand tons/year)	Percentage of Total
Offshore oil production	47	2
Natural seeps	259	11
Atmospheric deposition	306	13
Oil transportation and shipping	564	24
Urban runoff and discharge	1,175	50

Source: Ref. 89.

1989 *Exxon Valdez* spill into Prince William Sound are spectacular and immediately grab media attention, they are not the major source of oil pollution in the marine environment. Soclo and coworkers (91) documented the importance of waste oils from mechanics shops in contaminating sediments in coastal regions of Benin while petroleum discharges from tankers and other wastes associated with petroleum delivery and processing dominated in sediments in Aquitaine (France). Zakaria and coworkers (92) reported that runoff of lubricating oils from roadways was a major source of contamination in sediments in the Straits of Malacca (Malaysia).

Upon release into the environment, a variety of physical and chemical processes result in the transformation and dispersion of the oil. Weathering causes the spilled oil to break down and become heavier than water. At the same time, the action of wind, waves, and currents may result in dispersion of the oil spill (96–100). In addition, the turbulence resulting from wind and waves can result in the formation of oil droplets that can be further dispersed throughout the water, forming secondary slicks or sinking within the water column (98). The turbulence can also result in emulsification of the oil. Two types of emulsions are possible: (*1*) water-in-oil emulsions and (*2*) oil-in-water emulsions. Water-in-oil emulsions, frequently called *chocolate mousse*, are associated with strong turbulence, which forcefully mixes the spill with the surrounding water resulting in a product in which water becomes trapped within oil droplets. Chocolate mousse floats on the water surface and can be dispersed large distances from the original spill. It is highly resistant to degradation and may persist in the environment for months to even years after the spill has occurred. Unlike water-in-oil emulsions, oil-in-water emulsions are heavier than water and so sink to the bottom contaminating sediments.

Lighter substances (short-chain alkanes and monoaromatic compounds) within the oil may evaporate from the surface of the spill leaving the heavier oil components behind (101). In temperate climates, the majority of the light hydrocarbons can evaporate from a spill within 1 or 2 days. Photooxidation of compounds present in oil transform aromatic compounds into products associated with the polar and resin fractions of petroleum while aliphatic sulfur compounds are oxidized to sufoxides, sulfones, sulfonates, and sulfates (102). Partial oxidation of components of the oil, occurring primarily at the edges of the spill in which oil, water, and air interface, may result in the formation of tar balls, which sink to the bottom contaminating sediments. Finally, significant transformation of petroleum hydrocarbons results from the metabolic activity of indigenous organisms (1,5,8,9,11,12,102–105).

Dispersive forces frequently result in the transport of petroleum from open water to the shoreline. Because of the importance of coastal, shoreline environments to human activities, remediation of these areas has been the focus of most marine bioremediation. Coastal environments vary considerably depending upon climate, topography, and other local and regional factors.

Most experiments to evaluate the efficacy of bioremediation in the treatment of oil spills have been conducted either in laboratory micro- or mesocosms or in small in situ enclosures (1,8,11,12,106,107). While such experiments provide understanding of the physiology and ecology of microbial populations involved in the degradation of petroleum hydrocarbons, they are difficult to extrapolate to real-world situations. Field studies on bioremediation could provide the information needed. Unfortunately, field studies are technically difficult, expensive, and usually constrained by regulatory restrictions. Ideally, field studies would involve the controlled release of petroleum hydrocarbons into either open oceans or adjacent to shorelines. In reality, the vast majority is conducted during response to actual oil spills and lacks the control of planned studies. Despite these limitations, both laboratory studies and studies conducted coincidentally with oil spills have provided useful insight into bioremediation under field conditions. Because of the low nutrient concentrations and low microbial populations typical of open ocean environments, studies in this type of environment have focused on biostimulation (the addition of nutrients) and bioaugmentation (the addition of microbial cultures).

Bioaugmentation has been shown to have variable and often disappointing results when applied to marine oil spills. Tagger and coworkers (107) found that supplemental bacterial cultures rapidly declined when added to oiled seawater. There was no enhancement of hydrocarbon removal in the presence of the supplemental microorganisms compared to natural conditions. Fayad and coworkers (74) noted that many of the commercial bioaugmentation products proposed for use in the aftermath of the Persian Gulf War had no convincing evidence of increased petroleum removal. They concluded that testing of products in laboratory microcosms using conditions simulating in situ conditions is necessary before these products are used. Lin and coworkers (108) compared fertilizer addition with the use of bioaugmentation for the remediation of salt marsh sediments contaminated with oil. While fertilizer addition increased both microbial respiration and removal of normal and aromatic hydrocarbons, no such enhancement was found with bioaugmentation. Supplemental organisms, derived from both the indigenous microbial community and nonindigenous strains, were found to be unable to survive when inoculated into river sediments without the addition of supplemental carbon

sources to which the organisms were adapted (109). Even under these conditions, only some of the inoculated strains were able to persist. Neralla and Weaver (110) evaluated the efficacy of 10 commercial bioremediation products (bioaugmentation) in the treatment of crude oil contaminated marshes. The products did not increase the number of oil degrading microorganisms significantly, but did enhance numbers of total heterotrophic bacteria present. No product increased degradation of petroleum hydrocarbons compared to a fertilized control. On the other hand, Mochalona and Antonova (111) found the use of combined surfactants and supplemental bacterial cultures enhanced degradation of oil in aquatic environments.

Biostimulation through the addition of inorganic nutrients has generally been found to enhance removal of petroleum hydrocarbons from open seas. Much research has been devoted to developing an appropriate and efficient delivery system for inorganic nutrients because the direct application of these compounds in water-soluble form results in rapid dilution and dissipation of the added nutrients; the use of oleophilic nutrients octyl phosphate and paraffinized urea (112) and paraffin-supported $MgNH_4PO_4$ (113) increased biodegradation. Despite these encouraging results, other researchers have reported no increased biodegradation using supplemental nutrients (114).

Considerably more research has examined the influence of supplemental inorganic nutrients on remediation of contaminated shorelines. The application of a commercial fertilizer to shoreline contaminated with unweathered Forcados crude oil resulted in an initial increase in the rate of biodegradation compared to a control plot that did not receive fertilizer. The effect, however, appeared to be transient because there were no differences between the treated and control plots four years after the spill (115,116).

Bioremediation has been evaluated, albeit under less than ideal experimental conditions, during remediation of several recent oil spills. While the lack of appropriate controls and sufficient independent replicates preclude firm conclusions, the results of these studies indicate that bioremediation, primarily via biostimulation, is a worthwhile technology for oil spill *cleanup*s.

The best-designed and most extensive studies under actual spill conditions are those associated with the *Exxon Valdez* Spill in Prince William Sound, Alaska. The *Exxon Valdez* spill on March 24, 1989 released approximately 41,000 m^3 (11 million gallons; 258,000 barrels) of Alaskan North Slope crude oil into Prince William Sound, resulting ultimately, in the contamination of about 2,090 km of coastline. Initial response to the spill was hindered by a storm on March 26, 1989 that spread the oil to the west of Blight Reef where the tanker ran aground. Over the next several weeks, the oil continued to spread, eventually impacting approximately 15% of the shoreline in Prince William Sound and the Gulf of Alaska (117).

The contaminated shoreline was dominated by rocky areas (72% rock face, 24% mixed boulder and cobble, 3.5% mixed cobble and pebble, and 0.5% fine grain sand/mud or marsh) (10). Initial cleanup focused on removal of the heaviest concentrations of oil using physical methods (cold- and warm-water washing, steam cleaning, and manual oil recovery). Bioremediation was not employed as a remediation technique until after shoreline oiling had been significantly reduced by a combination of weather, natural processes, and physical remediation techniques (118–120). Therefore, evaluation of bioremediation focused on the use of this process as a final cleanup and polishing tool rather than as a first response technology.

Initial studies involved intense site characterization. While the distribution of petroleum hydrocarbons as well as chemical characterization of the changes in the oil owing to weathering and other natural processes was well established in conjunction with ongoing remediation efforts, several additional studies focusing on biological parameters were needed to evaluate the feasibility of bioremediation. Pritchard and coworkers (121) compared the number of oil-degrading bacteria on impacted beaches to unaffected controls and reported that the number of hydrocarbon-degrading microorganisms was as much as 10,000-fold higher than the controls. Other researchers conducted microcosm studies to determine if any easily modified environmental factors were limiting microbial numbers and activity (122–125). Because of the high probability that biodegradation was limited by the supply of inorganic nutrients, special attention was given to the effect of supplemental nitrogen and phosphorus. These laboratory feasibility studies demonstrated that the addition of inorganic nitrogen to the microcosms increased mineralization of weathered Alaskan Slope crude oil. Based upon these studies, biostimulation was proposed as a remedial option and two field scale trials were initiated, at Snug Harbor and Passage Cove.

The field studies tended to support the usefulness of bioremediation for the cleanup of oil beaches. As with open ocean tests, the heterogeneity of the samples coupled with the virtual impossibility of establishing true controls precluded drawing firm conclusions.

Both of these studies compared microbial and chemicals parameters for samples obtained from plots established within the study area. Although the plots were in close proximity to each other and were set-up to reflect comparable environments, difficulties in interpreting the data arose from both interactions between the study areas and unrealized differences between the plots. For example, in the Snug Island studies, analysis of beach sediment demonstrated the presence of significant amounts of inorganic nitrogen (ammonia and nitrate) and phosphorous (phosphate) in both the treated and the control plots at the end of the experiment. Because there were no preexisting data on nutrients in the beach sediments, it was impossible to determine if the increased nutrient levels were the result of leaching from the treated plots or if they reflected normal seasonal changes in beach sediment chemistry (123). In the Passage Cove studies, high within site variability coupled with the initial choice of an inappropriate marker of biodegradation (pristane and phytane) made it impossible to demonstrate statistically significant differences between treated and control sites (121,126). In both of these studies, the most consistent result was a visible clearing of the oiled beach

surface in plots with added nutrients (Inepol EAP 22). Based on the encouraging, but not definitive, results in the Snug Harbor and Passage Cove studies, additional field studies were conducted in 1990 on Knight Island. With the additional time to plan these studies, modifications in experimental design were made that addressed some of the limitations of the previous studies. Specifically, by sampling paired treated and control beaches in three different areas, the researchers were able to obtain data that could be analyzed statistically. In addition, monitoring of pore water nutrient levels allowed the movement of added nutrients to be determined.

The treated beaches received two types of nutrient supplement — Inepol EAP 22 and Customblen. Microbial numbers (heterotrophs and oil-degraders), ability of the microbial communities to use hexadecane and phenanthrene, and levels of petroleum hydrocarbons in sediments were monitored at each sampling. In addition, interstitial (pore) water samples collected at the same time were analyzed for dissolved oxygen, salinity, pH, temperature, and nutrient levels. There was an increase in microbial numbers in both treated and control beaches over the duration of the study; however, no statistically significant differences between the two were found. Neither were there statistically significant differences in the numbers of oil degraders between the treated and control beaches. The lack of sustained effect of the nutrient amendments on microbial numbers could have been the result of two factors: (*1*) the heterogeneity of the sediment that resulted in large within plot variability or (*2*) the rapid removal of the nutrients from the site of application. This possibility was supported by pore water nutrient measurement that indicated removal of the nutrients in 10 to 20 days from application.

Despite the lack of increase in microbial numbers in the treated beaches, the researchers found a significant increase in mineralization of hexadecane and phenanthrene in the treated sediments compared to the control (127). The increased mineralization of petroleum compounds provided clear support for the use of biostimulation as a remediation technology.

The apparent disagreement between the response of the microbial community to supplemental nutrients in terms of microbial numbers compared to overall mineralization, in part, reflect the heterogeneity of the study area. Actual changes in microbial numbers within the sample plots may have been masked by overall variability. More importantly, the conflicting results highlight the possibility that increased mineralization of the contaminants may have been the result of increased activity at the level of individual microorganisms rather than increased microbial numbers (10). This possibility is important to remember in evaluating the feasibility of bioremediation in other situations.

Results from the chemical analysis of the sediments, while not as clear cut, did supply evidence supporting the efficacy of bioremediation. The chemical analysis suggested that the polar content of the petroleum was a significant factor influencing biodegradation (122,128). Other important factors were the ratio of nitrogen to oil, the elapsed time since nutrient addition, and the extent of natural biodegradation. A multivariate regression analysis incorporating these factors was developed, which showed good correlation with removal of the oil.

The final two field studies associated with the *Exxon Valdez* oil spill were conducted on Elrington and Disk Islands in the summer of 1990 (129). These studies evaluated different types of aqueous nutrient additions for biostimulation and also separately evaluated bioaugmentation using commercially available inocula of oil-degrading microorganisms. Sample heterogeneity in these studies was further reduced compared to previous studies by mixing and sieving fine sediment material and placing it in mesh baskets prior to the start of the study, thereby reducing within plot variability. Two different nutrient application regimes, single application and multiple applications, were evaluated in the Elrington Island studies. In both cases there was a statistically significant decrease in the weight of oil present in the treated plots compared to the control plots (129). In both treatments, removal of oil followed a linear trend. Removal of oil was higher in the plots receiving a single addition of nutrients. In contrast, when biodegradation was measured in terms of alkane removal, the plots receiving multiple nutrient additions showed a greater decrease in C18 to C27 alkanes. This difference suggests that biodegradation was not the only process responsible for the removal of oil; although the researchers did not determine what other processes, such as production of biosurfactants, were involved. In agreement with the prior studies on Knight Island, there was no consistent effect of nutrient addition on the numbers of oil-degrading bacteria present, although microbial activity was increased.

Finally, the use of commercially available inocula for bioaugmentation was tested on Disk Island (42,130). Using a randomized block design, comparisons were made between control, nutrient addition, and two different inocula plus nutrient treatment plots. There were no significant differences found between the different treatments. While these results do not support the use of bioaugmentation they do not unequivocally rule it out either. In this study, nutrient addition alone did not result in increased biodegradation; however, prior studies at other locations within Prince William Sound indicated that biostimulation could enhance removal and degradation of oil. The investigators conducting the study proposed several possible reasons, in addition to the within treatment plot variability, which might account for the apparent failure of bioaugmentation. Most important among the possible reasons was the age of the spill and associated highly weathered condition of the oil and the short duration of the study.

Taken as a whole, the field studies following the *Exxon Valdez* oil spill indicated that bioremediation could enhance oil removal and thus shorten cleanup time in at least some field situations. More importantly, the results of the studies indicated several factors that should be considered in designing and implementing oil spill bioremediation. Specifically, the importance of nutrient delivery systems that ensured elevated nutrient levels in pore water was highlighted. Furthermore, the negative

impact of long lag periods between the spill and the implementation of bioremediation was reflected in the poorer results at sites in which the oil had been highly weathered and biodegraded before studies were initiated.

In the summer of 1994, the Environmental Protection Agency conducted a field study (The Delaware Bay Study) to evaluate bioremediation. Unlike all other field studies performed, the 1994 study, which took place on a shoreline beach of the Delaware Bay, involved a planned release of light crude oil. Therefore, adequate considerations of experimental design allowing for statistical analysis of the results were incorporated into the study. As much of the environmental variability and the lack of adequate controls that hampers the interpretation of field studies of accidental spills could be avoided, much stronger conclusions regarding the use and limitations of bioremediation could be drawn.

The Delaware Bay Study was designed to compare oil removal under unamended (background) conditions to removal using biostimulation and bioaugmentation (131). Five separate area blocks on the beach were studied using a randomized block design with four treatment plots per block. The treatments were: (*1*) no treatment, no oil control; (*2*) oil, no treatment; (*3*) oil, addition of inorganic nutrients (biostimulation); and (*4*) oil, addition of inorganic nutrients plus a commercial inoculum (bioaugmentation). Sand samples from the plots were obtained at 14-day intervals. The samples were analyzed for a variety of microbiological, toxicological, and chemical parameters. The results of the Delaware Bay Study confirmed many of the general observations and conclusions of the spill field studies. Hopane (a nonbiodegradable component of oil) was used as a marker compound so the loss of oil from the plots to be divided into physical and biological components. Loss of hopane from all of the oiled plots occurred at the same rate indicating that physical removal mechanisms, such as wave action, had the same impact on all of the plots. Degradation was significantly faster in both the biostimulation and the bioaugmentation plots compared to the oiled control. Interestingly, there was no difference between the two types of treatments indicating that the microbial inoculum did not have a significant effect in enhancing biodegradation. This observation is in agreement with the results of the Prince William Sound study in which the use of a commercial inoculum was not found to significantly enhance biodegradation. The data also indicated a rapid increase in the indigenous microorganisms in response to the added oil. This increase reflected the high background nutrient levels on the beach that allowed the microorganisms to rapidly use additional sources of carbon and energy when they became available. Subsequent evaluation of the microbial community structure in samples from each of the plots using molecular techniques showed that the inoculum was rapidly eliminated from the plots, indicating that the inoculated microorganisms may not have been able to compete with the indigenous microbiota (131). As with the studies in Prince William Sound, the results of the Delaware Bay Study indicated that differences in the microbial communities present and not a simple increase in the total number of bacteria account for the enhanced removal of oil with biostimulation. In addition, both sets of studies indicated that bioaugmentation is of little value in the remediation of shorelines.

Bioremediation of open waters and oil-impacted shoreline surfaces is best implemented as in situ bioremediation. Not only has this technology been demonstrated to be useful in the remediation of petroleum hydrocarbons, the use of in situ technology minimizes environmental disruption and the possible transport of contaminants associated with ex situ technologies. In some environments, however, in situ approaches are not feasible. This is particularly true for the remediation of sediments in which ex situ treatment has found a limited, but promising niche.

Dredging of sediments is a routine operation required for the maintenance of navigation channels. As a result of dredging operations, contaminated sediments are often removed from aquatic environments. These materials must be treated before they can be disposed of or reused.

The use of bioreactors in conventional waste treatment plants is a well-established procedure. Their use in the treatment of contaminated sediments (slurry reactors, primarily) is in its infancy. Applications of bioreactor technology have focused on anoxic and anaerobic systems (132–136) in which bench and pilot scale studies indicate that the technology should be applicable to remediation of pesticides, chlorinated compounds, and other recalcitrant molecules. Recently, a patent was filed for a bioreactor design for treating sediment and soil slurries that can be used for either anaerobic or aerobic treatment (137).

A pilot-scale bioreactor system has been applied in the remediation of petroleum hydrocarbon contaminated sediments in the Netherlands (138,139). Contaminated sediment was removed from the Petroleum Harbor in Amsterdam. The sediment was transported to a treatment site in which it was fractionated by hydrocyclone separation and froth flotation. These processes separate the silt fraction into which the PAH has been concentrated. The silt fraction was subsequently treated in a continuous-feed bioreactor. Using this treatment, both the concentration and associated toxicity of the PAHs in the sediment was significantly reduced.

CONCLUSION

Bioremediation at sea, on shorelines, and in sediments is an emerging technology that holds great promise. By enhancing the activity of microorganisms, degradation of a wide variety of contaminants in the environment should be possible. While there is extensive information concerning the metabolic activities of specific strains of microorganisms, additional information, particularly information on microbial communities and activity in real world environments, is needed before bioremediation can be used to its fullest. In addition to the research needs in microbiology, additional research into the fate and transport of contaminants in the environment and into the design of innovative methods to control and modify the environment are needed. Hopefully, future engineering developments addressing control of spill dispersion and

methods of nutrients addition will lead to enhanced use of this technology.

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BIOREMEDIATION, AQUIFERS.

See BIOAUGMENTATION

BIOREMEDIATION, BIOSURFACTANTS USE IN.

See BIOSURFACTANTS: TYPES, SCREENING METHODS, AND APPLICATIONS

BIOREMEDIATION, COLD-ADAPTED MICROORGANISMS IN.

See USE OF COLD-ADAPTED MICROORGANISMS IN BIOTECHNOLOGY

BIOREMEDIATION, METHANOTROPHS IN.

See METHANOTROPHIC BACTERIA: USE IN BIOREMEDIATION

BIOREMEDIATION OF HOT DESERT SOILS.

See HOT DESERT SOIL MICROBIAL COMMUNITIES

BIOREMEDIATION OF SOILS

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Bioremediation of soil involves decontamination by biological means. This process can be difficult to achieve because three main components must be in balance — the environment, contaminants, and microorganism(s). The environment must be able to support biological activity by having a suitable temperature, moisture content, pH, nutrient supply, and electron acceptors such as oxygen for aerobic microorganisms or redox potential for anaerobic microorganisms. The soil type is also important. The soil contaminant(s) must also have characteristics necessary for bioremediation. It must not be too toxic for the microorganism(s), in a concentration low enough that it does not inhibit microbial activity and is high enough to be bioavailable to the microorganism(s). The contaminant(s) must be available on the soil matrices and soluble enough in water so that it can enter the microorganism's aqueous environment. A microorganism must be metabolically capable of degrading the contaminant(s) and be present in sufficient numbers as either indigenous or added genetically engineered microorganisms (Fig. 1). This article discusses these major influences on bioremediation in detail. In addition, phytoremediation is presented to expand the ideas of bioremediation. This article is a general overview of bioremediation, and more specific details can be found elsewhere (1–8).

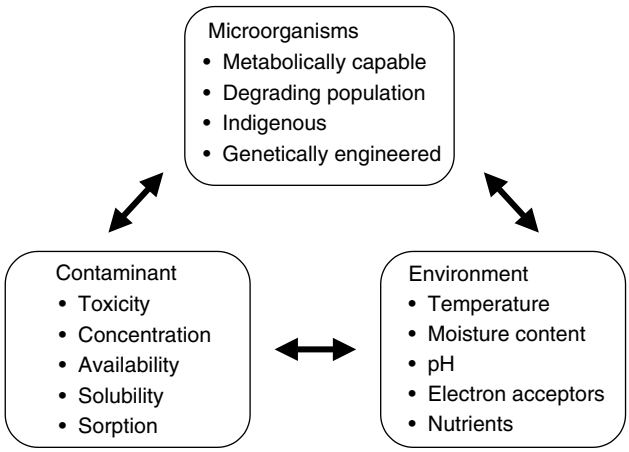

Figure 1. Three main interrelated components involved in bioremediation.

WHY BIOREMEDIATION?

Since the industrial revolution, human activities have resulted in large quantities of chemicals being eventually released into the environment, either deliberately for agricultural and industrial purposes or accidentally by mishandling of chemicals. It is estimated that there are about 50,000 contaminated sites in the United States alone, and the projected cost for cleaning these polluted areas in the next three to five decades using conventional physical or chemical methods (7,9,10) is estimated to be $1.7 trillion U.S. For a list of physical or chemical methods see Reference 7.

It is known that microorganisms are present in almost every environment on Earth and that they are capable of degrading and mineralizing a wide range of organic compounds. In general, biodegradation refers to a natural process that microorganisms use to break down organic compounds into less complex chemicals. When organic compounds of carbon, nitrogen, sulfur, and phosphorous are completely metabolized to inorganic compounds (e.g., pentachlorophenol degraded to carbon dioxide, water, and chloride ions), the process is referred to as mineralization (11). In most microorganisms energy is obtained from these processes. Typical microorganisms involved in biodegradation include aerobic bacteria, actinomycetes, cyanobacteria, anaerobic bacteria, fungi, and algae (7). Biodegradation is a natural process and costs associated with remediation technologies are less expensive than physical or chemical methods. In addition, bioremediation is more environment friendly and ecologically sound and has the potential to be more socially acceptable when compared to physical or chemical processes such as thermal treatment, incineration, soil washing, chemical treatment or extraction processes, supercritical fluid oxidation, and volatilization technologies (7).

NATURAL ATTENUATION

As a first step in determining if bioremediation is possible one should examine the efficacy of natural attenuation. Natural attenuation is defined as naturally occurring processes in the environment that act without human intervention to reduce the mass, toxicity, mobility, volume, or concentration of contaminants in those media (8). In some cases the reduction in contaminant concentration may not be biologically mediated. Reduction may be due to contaminant movement that includes dilution, dispersion, sorption, and volatilization, or because of abiotic transformation. Some of the advantages of natural attenuation are that the contaminant is normally transformed into less toxic by-products, the process is nonintrusive to the environment, there is no waste accumulation after excavating, the contaminant is not transported from its location, the process is less costly than conventional remediation strategies, and there is no need for specialized equipment. Some of the disadvantages associated with natural attenuation are that the time frames required for remediation are normally longer, someone must be responsible for monitoring the remediation process over the longer time frame, and natural and anthropogenic changes in the environment (i.e., hydrogeologic changes, reduction in electron acceptor concentrations, production in toxic biodegradation metabolites) may occur (8).

ENVIRONMENTAL CONSTRAINTS TO SOIL BIOREMEDIATION

One of the most important and fundamental factor in soil bioremediation is the condition of the environment. Temperature, moisture content, pH, nutrient supply, soil type, and electron acceptor or redox potential are all factors that contribute to providing a good environment for microbial growth, activity, and survival.

Temperature

Temperature is important for contaminant degradation because the rates of microbial enzyme reactions are temperature-dependent. An enzyme catalyzes a reaction at a specific temperature optimum; any temperature above or below that optimum decreases the reaction rate. Microbial degradation of hydrocarbons, for example, can occur at temperatures ranging from -2 to $70\,^{\circ}C$. Most microorganisms, however, are mesophiles and grow optimally between 25 and $35\,^{\circ}C$ (7). Temperature can also affect the properties of the contaminant. At higher temperatures, adsorption of the contaminant to soil particles is decreased; this in turn makes the contaminant more bioavailable. In conjunction with higher temperatures, however, shorter chain and lower molecular weight contaminants can evaporate and produce toxic effects to the microbial cell membranes and produce noxious odors from the soil site. Conversely, lower temperatures increase adsorption of the contaminant to the soil particles and therefore reduce the availability of the contaminant for microbial degradation (7).

Moisture Content

Moisture content is also an important factor in soil bioremediation. In general, bacteria must live in an aqueous environment. They depend on water for motility, gas exchange, transport of nutrients and wastes to and from the cell, and temperature regulation. In soil, water is held in different fractions of the soil — the saturated zone, and the vadose zone. In the saturated zone, soil particles are completely filled with water. In the vadose zone, soil particles are partly filled with water and partly filled with air (6). The amount of air and water in the vadose zone depends on the climate, soil composition, distribution of pore sizes, and depth of the groundwater and vegetation (6). At the soil–atmosphere interface, the soil gas and atmospheric air are relatively the same. As the groundwater is approached, the soil atmosphere changes due to microorganisms consuming oxygen and producing carbon dioxide. As the soil becomes more saturated with water, the gas exchange between the atmosphere and soil is reduced and the soil can become more anaerobic. Again there is a balance; if the moisture content is too low microbial activity is hindered, if too high a

suitable electron acceptor such as oxygen is decreased in concentration.

The ability of the soil to hold water is also important. Soil contains both mineral and organic fractions. The mineral fraction is classified according to the grain size of the mineral. In general, the larger the mineral the greater the permeability and aeration, whereas the smaller the mineral the lower the permeability and aeration. Therefore, clay (<2 μm) or silt (2–63 μm) particles will have a lower permeability than sand (63–2,000 μm) or gravel (2–63 μm). The organic fraction is composed of the living biomass (e.g., microorganisms, insects, worms, nematodes, arthropods, and plant material) and dead organic matter or humus. The dead organic matter is made up of dead microbial, plant, and animal cells that are not completely degraded. The humus content of soil can be as high as 20% in the case of peat soils and as low as 0 to 1% for poor sandy soils. The higher the organic content the higher the water holding capacity (WHC) of the soil (50–70% for aerobic soils and greater for anaerobic soils) (6,7).

pH

Microbial growth and activity is greatly affected by soil pH. Most soil bacteria have a pH range optimum between 6.5 and 8.5. If the soil is acidic, bacteria are poor competitors against acid tolerant fungi (7). The pH can also affect the solubility and availability of macro- and micronutrients, the mobility of toxic materials, and the reactivity of minerals (7). Toxic heavy metals are predominantly in an insoluble form near neutral pH. Toxic metals become mobile at lower pH values.

The surface charge on the soil colloidal particles is also affected by pH. For example, clays have a permanent negative charge but the charge of the organic or amorphous coatings around the clay material can change (7). A drop in pH can decrease the net negative charge and cause a greater adsorption of anionic material and vice-versa.

A common remedy to poor soil pH is liming to increase the pH or ferrous sulfate to decrease the pH. Liming involves the addition of any calcium- or magnesium-containing compound capable of reducing pH (e.g., calcium hydroxide, calcium carbonate, calcium magnesium carbonate, and calcium silicate) (7).

Electron Acceptors

Most electron acceptors are oxidized inorganic compounds, such as O_2, NO_3^{2-}, Mn^{4+}, Fe^{3+}, So_4^{2-}, and CO_2 (6). Many contaminants are degraded by aerobic pathways and therefore require oxygen. Aerobic reactions also yield the greatest amount of energy for microorganisms. In addition to its use as an electron acceptor, oxygen can be used as a substrate in oxygenase-catalyzed reactions (12). Thus, a well-aerated soil is important. As mentioned earlier, moisture content and oxygen diffusion in soil are closely related. The higher the soil moisture content, the lesser the oxygen diffusion from the atmosphere and therefore lesser the oxygen in the soil. In this situation the oxygen is consumed faster than it is replaced and the soil becomes anaerobic. Under anaerobic conditions, facultative and strict anaerobic microorganisms degrade the contaminants under denitrifying, methanogenic, sulfate-reducing, and iron-reducing conditions (12).

Nutrients

The three main nutrients, in addition to carbon (often the contaminant is a carbon source), required for microbial metabolism of soil contaminants are nitrogen, phosphorus, and potassium (7). When these are in limited supply, the ability of microorganisms to degrade the contaminant is reduced. Calculations on the bacterial cell indicate that there is a carbon, nitrogen, phosphorus ratio of 100 : 15 : 3. Other nutrients required in smaller amounts are zinc, calcium, manganese, iron, sodium, and sulfur (7). Many of the nutrients can be purchased from commercial suppliers.

CONTAMINANT VARIABLES THAT AFFECT THEIR DEGRADABILITY IN SOIL

The nature of the contaminant is another variable in the success or failure of bioremediation. If a contaminant is not biodegradable (i.e., recalcitrant), then any change in environmental or microbial factors will not help the remediation process. Other factors that one must be aware of are its toxicity, concentration, availability, solubility, and sorption of the contaminant to solid surfaces.

Toxicity

The toxicity of the contaminant to the microbial community in soil is a critical parameter. If the chemical is toxic toward the degrading microorganism then bioremediation will be inhibited. In some cases the toxicity of specific chemicals can be predicted based on chemical structure, substitution types and positions, solubility, and volatility (7). A problem with most contaminated sites, however, is that contaminants are usually present as chemical mixtures. Within a mixture, some of the chemicals may be more toxic to the microorganisms than others and therefore it can be difficult to predict what outcome the mixture will have on the degrading microbial population. In addition, degradation of the contaminant(s) can lead to products that are more toxic than the parent compound. Therefore, it is important to use toxicity tests during bioremediation to determine if biodegradation of targeted contaminant(s) and their intermediates have significantly decreased the toxicity of the treated soils. If the bioremediation program has been successful, a significant reduction in toxicity should ensue. A worst-case scenario is that no biodegradation occurs after treatment and either no reduction in toxicity or an increase in toxicity is observed. The possibility exists that the bioremediation program was successful in removing the targeted contaminant(s), yet the remediated soil remains as toxic or more toxic than before bioremediation. If this occurs, then the bioremediation program cannot be judged to have been successful.

Because reduction in toxicity is an important and desirable component of a bioremediation program, the problem arises as to what toxicity tests are suitable for use with soil samples that are heterogeneous by nature and

contain a multitude of inorganic and organic compounds as well as living prokaryotic and eukaryotic organisms that may produce toxic metabolites or extracellular products (i.e., proteases, nucleases, extracellular products or toxins, and capsular slime). The ideal test for a heterogeneous chemical mixture does not exist. Therefore, new tests need to be developed and existing testing protocols require modifications. It is likely that some tests suitable for certain soil types are not suitable with other types of soils (e.g., highly organic peat soil versus sandy soils).

From scientific and regulatory points of view, the ideal toxicological test should be simple, rapid, reproducible, inexpensive, amendable to standardization, highly sensitive, predictive, ecologically relevant, satisfy regulatory standards, and discriminating. However, neither does the ideal test that satisfies all the mentioned features exist at this time nor is one likely to be developed in the near future. Instead, for in vitro evaluation, it is necessary to use a battery of tests to ensure that the scientific and regulatory decisions are the best available at any point in time.

Toxicological tests that have been used to assess chemically contaminated soil include: Solid-Phase Microtox (13), Toxi-Chromotest (14), SOS-Chromopad test (14), Seed Germination Test using *Lathyris sativa* (modification of Greene and coworkers, 15), Earthworm Toxicity Test using *E. foetida* (modification of Goats and Edwards, 16), and Red Blood Cell Lysis assay (modification of Negrete and coworkers (17)).

With respect to soils, numerous toxicity studies have been undertaken to assess the effects of pesticides and other agrochemicals on microbial processes, population changes and numbers, bioaccumulation of pollutants by plants, and biodegradation of selected compounds added to soil as a result of industrial and/or agricultural practices (18,19). However, little information exists on the toxicity of soil before, during, and after bioremediation. Properly decommissioned soil sites may be used for numerous human activities and it is imperative that regulatory guidelines are established so as to eliminate significant risks from the site to humans or animals. The regulatory decisions will, therefore, be based on the best possible scientific knowledge available.

Concentration of Contaminant(s)

Contaminant concentration can affect the biodegradability and toxicity to the degrading microorganism(s). Higher molecular weight compounds appear to be more recalcitrant to degradation than lower molecular weight compounds, which may be related to the water solubility of the compounds (7). Once in contact with the degrading microorganism, concentration becomes a factor. In general, high concentrations inhibit microbial activity, however, the inhibitory concentration is dependent on the chemical. Some chemicals can be inhibitory at microgram concentrations per gram dry weight soil, whereas others may inhibit at gram quantities.

There also appears to be a lower concentration threshold for biodegradation. Microbial populations are unable to degrade chemicals at low concentrations. One reason may be the presence of microorganisms unable to grow or produce a sufficient population to degrade the contaminant (7). In some cases the only way to degrade a compound at a low concentration is to add a cosubstrate that is easily metabolized. Another reason could be that the chemical is unavailable for microbial degradation because of the adsorption to soil particles.

Availability

The bioavailability of the contaminant depends on the soil structure and the contaminant under study. Pollutants can be distributed among the solid, liquid, and gas phases of the soil. Many of the organic contaminants that enter soil are hydrophobic and therefore predominantly sorb onto the solid mineral and organic fractions. The longer the contaminant is in contact with the soil matrix the less accessible it becomes to the microorganisms (20). In most cases the rate of biodegradation is controlled by desorption of the contaminant from the soil matrix and not by microbial activity. Therefore, increasing desorption of the contaminant by use of surface-active agents (surfactants) can make the contaminant more available for biodegradation. Surfactants work by lowering the surface tension of the liquid.

Solubility

Chemical contaminants can differ significantly in their aqueous solubility. Polar compounds such as methanol are very soluble, whereas nonpolar compounds such as polychlorinated biphenyls (PCBs) have very limited solubilities. The greater the solubility of the chemical, the more available the chemical is to the microorganism and the greater the chance of biodegradation. Lower soluble chemicals do exhibit a mass transfer from the solid phase to the liquid phase, thereby supplying a limited concentration in the aqueous phase. Under these conditions it is the mass transfer rate that limits microbial growth and biodegradation rate (7). As mentioned in the previous section, surfactants can be used to increase the solubility and hence the bioavailability of the contaminant.

Sorption

Sorption of chemical contaminants to solid soil matrices affects the solubility and availability of the contaminant to the microbial population. Sorption includes both adsorption and absorption. Adsorption occurs on surfaces, whereas absorption describes uptake of the contaminant by the solid matrix beyond the surface (12). Adsorption may involve physical or van der waals forces, hydrogen bonding, chemisorption, or ion exchange (7). Cationic compounds are generally sorbed to cationic-exchange sites on clay minerals or humic surfaces, whereas anionic and nonionic compounds sorb to organic matter. The sorption of the contaminant to solid surfaces can depend on the concentration of the contaminant, type and quantity of the clay minerals, amount of organic matter in the soil, pH, temperature, and type of contaminant (7). The sorption of the contaminant to soil particles may not only make the contaminant unavailable for biodegradation but also make surfactant or chemical extraction difficult.

MICROBIOLOGICAL FACTORS

If the environment is optimum for microbial growth, activity, and survival and the contaminant is biodegradable, the last factor to consider is the population of catabolic microorganisms. Are there microorganisms or groups of microorganisms capable of degrading the contaminant? Is there a sufficient population present of indigenous, reintroduced or genetically engineered microorganisms (GEMs) to degrade the contaminant?

Metabolism of Soil Contaminants

The degradation potential of a contaminated site can be determined in the laboratory. Soil microcosms can be set up using the contaminated soil and the degradation or mineralization of the contaminant monitored over time. As this is performed in small scale and under optimum laboratory conditions (e.g., temperature, moisture content, pH, proper electron acceptor and nutrients) one would expect degradation to occur. If no degradation is observed, additional microbial metabolic barriers may need to be addressed. The first barrier to metabolism could be that the contaminant is structurally not amenable to biodegradation by the microbial population present in the soil (12). The halogen substitution pattern on many contaminants is usually the limiting factor for biodegradation. For example, there are 209 congeners of polychlorinated biphenyls (PCBs) ranging from one chlorine per biphenyl to 10 chlorines per biphenyl. Under aerobic conditions, bacteria have been shown to degrade various chlorine substitution patterns up to six chlorines per biphenyl. Under anaerobic conditions, reduction of higher chlorinated biphenyl molecules occurs, thus removing the inhibitory chlorine substitutions to enable further aerobic degradation to proceed (21).

Another metabolic barrier could be that the degrading microorganism requires a cometabolic substrate (12). Cometabolism is the transformation of a nongrowth substrate in the obligate presence of a growth substrate or other transformable compounds (22). In some cases the cometabolite may also induce production of catabolic enzymes necessary for degradation of the contaminant. For a more detailed review on cometabolism see Janke and Fritsche (23).

In some cases an alternate carbon source may be available in the soil that may limit the response of the degrading microorganisms to the contaminant. If an alternate carbon source is more easily degraded and provides microorganisms with sufficient carbon and energy, the microorganisms may preferentially utilize the alternate carbon source. For example, addition of easily degradable amino acids or glucose inhibits the aerobic mineralization of ethylene dibromide, *p*-nitrophenol, phenol, and toluene in subsurface soil samples (24).

Other factors that can inhibit biodegradation include toxicity of the contaminant or its metabolite to the microorganisms (as mentioned in the previous section on contaminant toxicity), protozoan predation, and genetic exchange. Predation by protozoa may not seem significant enough to hinder biodegradation of soil contaminants by bacteria, but documented evidence shows that it can occur (25). Genetic exchange between bacteria in the soil has been reviewed by Boyle (26) and Seech and Trevors (27). Evidence has shown that plasmid transfer can occur in nonsterile and sterile soil and the frequency of transfer depends on the population size and ratio of donor and recipient cells, along with several other factors.

Quantification of Degrading Microorganisms

Aside from metabolic constraints, the next question is whether there are contaminant degrading microorganisms in the soil and if the population is significant to effect bioremediation. Fertile soils contain approximately 1×10^9 bacteria per gram dry weight of soil. Isolation and quantification of contaminant degrading microorganisms from the soil microbial community by using classical microbiological techniques can be difficult. The best method is to enrichment for the contaminant degrading microorganism(s) from the natural soil community (7,11). Enrichment often requires that the specific target contaminant is present during the isolation. Soil is normally suspended in a minimal medium or agar plate containing the contaminant of interest. After an incubation period the culture is analyzed for growth and possibly contaminant loss. If microbial growth or loss of the contaminant is found, an aliquot of culture is removed and added to fresh minimal medium containing the contaminant. This process is repeated several times until a pure culture is obtained. This method does have its drawbacks because it only selects those microorganisms that can use the contaminant as a sole energy and carbon source. All other cometabolic isolates would not be found using this method (7). In addition, degradation of some complex pollutants may require a consortium of microorganisms and this creates difficulties in isolating individual microorganisms. Also, because an artificial environment is used when enriching the microorganisms, it could be possible that a specific nutrient required by the contaminant-degrading microorganism is missing. For example, various attempts to isolate polychlorinated biphenyl (PCB) dechlorinating strains from PCB contaminated sediment samples have been unsuccessful (28). One problem is that PCB dechlorination has not been obtained in a defined medium, implying a specific nutrient(s) in the sediment is necessary for the growth of the PCB-dechlorinating microorganisms.

Once a microorganism is known to degrade the contaminant it can be identified by several methods. Cellular morphology and gram-staining is often the first step. The second step can involve biochemical tests, cellular fatty acid profiles, or ribosomal ribonucleic acid (rRNA) analysis. Biochemical test kits such as the commercially available Biolog® System (biolog, Inc.) contain a series of carbon sources and a tetrazolium dye that changes colour when oxidized by the microorganism. The pattern of substrate utilization that results is representative of a specific species. Fatty acids present in the membrane of microorganisms work in the same way as a fingerprint. Fatty acids are extracted from the microorganism, methylated, and analyzed. The pattern that results is characteristic of the species. Using the same

fingerprint analogy, rRNA works in the same manner. The rRNA sequences 5S, 16S, 18S, and 23S can be used to identify microorganisms. Unique to rRNA is that the microorganism does not need to be isolated to be identified within a community of microorganisms (7,29).

Quantification of Contaminant Degrading Soil Bacteria by Most Probable Number (MPN)/Polymerase Chain Reaction (PCR)

Before the methods are discussed a brief description of PCR is necessary. PCR is an in vitro method for the amplification of defined DNA sequences. Via a repetitive series of cycles of template denaturation, primer annealing, and extension by DNA polymerase, PCR results in the exponential accumulation of an initial target DNA fragment. The essential components of a PCR mixture are the template DNA, primers, DNA polymerase, dNTPs, and buffer (30). The sensitivity of PCR has been theoretically stated to be as low as only one target DNA sequence per extractable soil sample for detection of a specific target sequence (31). For monitoring a contaminant-degrading bacterial strain, one needs to identify a unique DNA sequence for PCR amplification. This may be a partial sequence from a unique catabolic gene or in the case of genetically engineered microorganisms (GEMs), it may also be an artificial sequence not normally found in nature. Increasingly, PCR is being used to detect indigenous and introduced bacterial strains and their DNA from soil and water (32–39). DNA extraction protocols for environmental samples such as soil and water are becoming more rapid and efficient (32–34,40–42). Despite this, adsorption of bacterial cells and DNA to soil particles and interference by contaminants such as humic acids (43) during PCR often have a negative effect on the detection limit in soil samples. Although PCR detects the presence of specific genes, it does not indicate if cells are viable or if the gene is being expressed. To monitor expression one needs to use reverse transcription-PCR (RT-PCR). RT-PCR happens when reverse transcription of messenger RNA (mRNA) is combined with PCR to make complementary DNA from RNA, which is suitable for PCR. This method is very sensitive, highly specific, and efficient.

In addition to the classical method of viable plate counting, two major approaches have been used to quantify microbial subpopulations in soil using PCR. First, specific soil microbial subpopulations can be quantified by a most probable number (MPN)/PCR protocol. With this approach, Picard and coworkers (44) detected *Agrobacterium tumefaciens* ranging from 10^4 to 10^8 cells/g of soil. Recently, the authors modified and improved the sensitivity of the MPN/PCR method. With a week's enrichment of the Mpn-soil dilution samples prior to DNA extraction and PCR, the detection limit for a pentachlorophenol degrading *Pseudomonas* sp. UG30 was improved to 4 CFU/g dry weight of soil. This allowed for a unique study of the dynamics of a pentachlorophenol degrading bacterial species in a bioremediation process. The second approach is to quantify a target gene (or subpopulations of microorganisms) based on the quantity of the PCR products. To compensate for any differences that may affect the efficiency of PCR, a known amount of an internal standard DNA (i.e., DNA fragment amplified by the same primers of the target DNA) can be coamplified with the target DNA. As both the target and internal standard DNA are amplified with the same efficiency, the quantity of the target DNA can be calculated based on the amount of the amplified target and standard DNA. Automated quantitative PCR systems developed commercially are based on the principles of the competitive PCR protocol.

MONITORING BACTERIA INTRODUCED INTO SOIL

Once it is established that there is a degrading population in the soil the organism(s) can be introduced back into the soil in larger numbers or genetically modified to improve the catabolic capabilities and environmental fitness of the microorganism. If there were not a degrading population in the soil, nonindigenous microorganisms known to degrade the contaminant would need to be added. The above addition of microorganisms to soil is known as bioaugmentation. For bioaugmentation processes it is important to monitor the survival and persistence of the microorganisms.

Reporter Systems and Tracking Contaminant Degrading Soil Bacteria

Approximately 1% of the bacterial species observed and counted under a microscope from environmental samples can be recovered by conventional culture techniques (45). For example, contaminant-degrading bacteria expressing resistance to an antibiotic can be selected by plating on media containing the antibiotic. Antibiotic resistance can be and has been used as a reporter system; however, increasing occurrences of antibiotic resistant bacteria in the environment have raised serious concerns over introducing antibiotic-resistant genes into the environmental gene pool (46,47). These concerns have led to the construction of novel reporter systems.

Reporter systems are special genes that are either encoded on circular extrachromosomal DNA (plasmid) and then inserted into a microorganism or are inserted into the chromosome of a microorganism via a transposon. A transposon is a small piece of DNA that has the ability to jump from its source, usually a plasmid, to a recipient piece of DNA by a random event. When the reporter gene is on the plasmid, there is the possibility that the plasmid is lost when the cell divides. It can also be lost when the microorganisms are introduced into soil and the cells are no longer under selective pressure to maintain the plasmid. Some examples of reporter systems include the genes *xyle* (48), *lac*Zy (49), *lux* (50), and *gfp* (51). The *xyle* and *lac*Zy reporter genes are referred to as chromogenic markers because their gene products produce a color change in the bacterial colonies and this can be detected using selective or nonselective media. The *lux* and *gfp* gene products produce light and are therefore referred to as bioluminescent and fluorescent markers, respectively.

***xylE* Reporter Gene.** The *xyLE* gene codes for catechol 2,3-dioxygenase (C23O). Bacterial strains marked with

this gene can be detected and enumerated by incubating at 37 °C or 42 °C to enable expression of the enzyme C23O and then spraying plates with catechol. The enzyme converts colorless catechol to hydroxymuconic semialdehyde, a yellow product, causing the bacterial colonies to take on a yellow coloration that can be visually detected (47).

***lac*ZY Reporter Gene.** The *lac*Z gene codes for the enzyme β-galactosidase that cleaves lactose to produce D-galactose and D-glucose (49,52,53). The *lac*Y gene codes for lactose permease that transports lactose into the bacterial cell. In addition to cleaving lactose, β-galactosidase cleaves the chromogenic dye X-Gal (5-chloro-4-bromo-3-indolyl-β-D-galactopyranoside). Bacterial colonies expressing the *lac*ZY genes turn blue-green in color when exposed to X-Gal. Typical pseudomonad colonies are yellow-white in color and are lac^-, thus the *lac*ZY marker is very useful for pseudomonads.

***lux* Reporter Gene.** Bioluminescence is a phenotypic marker that is based on the introduction of genes for light emission. Originally cloned from *Vibrio fischeri* and *V. harveyi*, the *lux* operon includes *luxAB* coding for the luciferase enzyme, *luxCD* coding for reductase and transferase polypeptides of the fatty acid reductase, and *luxE* which codes for synthetase (50). The light-emitting reaction involves the oxidation of reduced flavin mononucleotide ($FMNH_2$) and a long-chain fatty aldehyde with the emission of blue-green light (50). Bacterial strains can also contain only the *luxAB* genes, and the long chain fatty aldehyde (e.g., *n*-tetradecyl aldehyde, dodecanal or decanal) can be added exogenously. Luminescence may be measured using a luminometer, scintillation counter, or photomultiplier. It may also be imaged directly using photographic or X-ray films and charge-coupled devices (32,47). *Pseudomonas* sp. UG14, a phenanthrene degrader was isolated from a creosote contaminated soil sample (54) and marked with *luxAB* genes (55). The background level of bioluminescence in soil was zero. One benefit of bioluminescent techniques for environmental application is the ability to measure light in real time without prior extraction of cells from the environment. Three disadvantages of using the *lux* reporter genes are the requirement for an aldehyde substrate such as *n*-decanal if the bacterium contains the *luxAB* genes only, the cost of a luminometer or photon imaging device, and the inability to obtain a specific activity. The requirement for an exogenous substrate can be overcome by cloning the *luxCDE* genes along with *luxAB* into the host (47). Although photographic or X-ray films may be used to image light-emitting colonies, colony resolution is reduced. Despite these drawbacks, bioluminescence is a versatile and reliable marker for monitoring bioremediating microorganisms.

***gfp* Reporter Gene.** A recent development in phenotypic markers is a fluorescent genetic tag called the green fluorescent protein (*gfp*) (51,56,57). The *gfp* genes were isolated from the jellyfish *Aequorea victoria* and when inserted behind a promoter they can be made to emit green light by exciting it with blue light. The chromophore responsible for *gfp* fluorescence is *p*-hydroxybenzylideneimidazolidinone, which is completely contained within the coding sequence of the protein and does not require any unusual cofactors. *gfp* also enables in vivo visualization of differential gene expression and protein localization when the *gfp* genes are fused directly to the genes encoding the protein of interest (51,56).

The *gfp* genes have been incorporated into several bacterial chromosomes, including *Pseudomonas* sp. UG14Gr (58) and *Moraxella* sp. strains G21 and G25 (59). *Pseudomonas* sp. UG14Gr was added to creosote-contaminated soil and was detected in the soil for up to 13 months after inoculation. In comparison, a non-*gfp* marked *Pseudomonas* strain was only detected up to 25 days, using antibiotic resistance as a selection marker (58). In the *gfp*-labeled *Moraxella* sp., insertion of *gfp* did not affect its biodegradative activity.

GFP is both advantageous and disadvantageous in environmental applications. Advantages include ease of detection, no requirement for an exogenous substrate, the possibility to observe single cells in real time, no requirement for fixing or staining of samples, the fact that GFP expression has no effect on the organism, absence of green fluorescent background in indigenous particles or bacteria, and its use in combination with other marker systems. The disadvantages are that there is variable GFP expression in different species, plasmids containing GFP may be unstable (chromosomal inserts are recommended), environmental influences are unknown, GFP may not work under anaerobic conditions, and GFP continues to fluoresce once the cell is dead or is lysed (60). The last problem has recently been solved by the production of unstable GFP mutants. The mutants contain a C-terminal oligopeptide extension that renders the *gfp* protein susceptible to degradation by intracellular tail-specific proteases (61). These *gfp* mutants can be used as transcriptional gene fusion products with other degradative genes to facilitate studies of real-time gene expression assays.

Nucleic Acid Probes and Immunological Techniques in Bioremediation

DNA hybridization technology is commonly used to detect the fate of introduced DNA sequences and avoid dependency on phenotypic expression of introduced genes. The technology is based on the formation of double-stranded DNA from complementary single-stranded DNA sequences. A homologous complementary labeled probe is applied to a targeted denatured nucleotide sequence bound to a filter. Probe labels may be radioactive or nonradioactive. Nonradioactive labels are preferred because radioactive probes have short half-lives, are hazardous, and require special waste disposal. Excess probe DNA is washed from the filter and the presence of the probe DNA is visualized using X-ray film or by chemical color production (62).

Using the appropriate probes, specific DNA sequences, such as the nitrogen fixation genes (*nif*), nodulation genes (*nod*), and mercury resistance genes (*mer*), can be detected in culturable cells using colony hybridization. The cells are recovered by viable plating and lysed directly on to a filter.

A most probable number-DNA hybridization method, originally described by Fredrickson and coworkers (63), can also be used and may provide higher sensitivity. For nonculturable cells, total community DNA extraction and analysis using dot or slot-blot hybridization or Southern hybridization can be employed. The sensitivity of gene probe assays can be greatly improved with the use of PCR.

Introduced microorganisms can also be monitored using immunological techniques (64). Immunological techniques involve the use of the microbial cell surface as a source of antigens for the preparation of serum containing antibodies, called antiserum. Microbial cells are injected into an animal whose immune system responds by producing antibodies to many different proteins present on the microbial cell surface. These heterogeneous antibodies are termed polyclonal. Monoclonal antibodies, specific to a single protein on the target bacterial cell, can also be produced by the hybridoma technique (65). This procedure involves the fusion of myeloma cells and B-lymphocyte cells. B-cells are responsible for the production of the antibody of interest. The myeloma cell has unlimited divisional properties and the B-lymphocyte cell produces the antibody. Fusion of the two nuclei forms a hybrid. Clones of the hybrid cells are referred to as hybridomas. The antibodies are labeled so that the microorganism of interest can be detected. Antibody labels include fluorescent dyes, radioisotopes, and enzymes (65). Monoclonal antibodies are more useful than polyclonal antibodies for monitoring specific introduced microorganisms because of the production of a single antibody specific to a single antigen. A newer technology using recombinant antibodies produced by bacteria is still in the early stages of development. However, this method has the potential to yield antibodies with greater specificity and affinity than either polyclonal or monoclonal antibody technologies. There is one limitation to immunological techniques, that is, the protein or antigen of interest must be expressed before the method can be employed.

ENHANCING BACTERIAL DEGRADING GENES THROUGH MOLECULAR BIOLOGY

Improving the degradative capabilities of microorganisms by altering bacterial degradation genes is important for developing efficient and quick bioremediation techniques. Genetic engineering, which is the use of *in vitro* techniques for the isolation, manipulation, recombination, and expression of a microorganism's DNA, is used to alter the genes. Ultimately, the changes incorporated into the DNA of a microorganism may be expressed in the proteins produced by it. Functional changes to a protein can be observed as alteration of its structure, rate of enzymatic activity, and/or an enzyme's affinity for its substrate in either a positive or a negative manner. Changes made to a DNA sequence often result in no observable change in enzyme activity. These types of neutral changes arise from the redundancy of the genetic code, that is, more than one codon code for the same amino acid, and/or from the incorporation of an amino acid with properties similar to the original one. Various techniques, including site-directed mutagenesis and DNA shuffling, have been developed to aid in the alteration of genes.

A common type of gene modification is in vitro oligonucleotide-directed (site-directed) mutagenesis. This method involves making specific base changes to a DNA sequence resulting in a known amino acid change in the resulting protein (66,67). The changes in DNA can be made through the use of mutagenic primers that contain the desired site-specific base change(s). Random mutagenesis involves the random incorporation of mutations using error prone PCR (68,69). The resulting amplified gene products will contain base changes with unknown effects on the resulting protein. The gene products are then screened for any differences in activity such as improved metabolism of xenobiotic compounds for the development of bioremediation techniques.

Although gene modification techniques have been in use for many years, they have some disadvantages. Oligonucleotide-directed mutagenesis requires some knowledge of where to make the specific changes in gene sequences. This means that some knowledge of the protein's structure-function relationship is essential. However, for the majority of enzymes, this knowledge is not available. As for random mutagenesis, it is an inefficient method, resulting in many nonfunctional mutations. The development of DNA shuffling, a relatively new technique described by Stemmer (70,71), has changed the way in which gene enhancements can be made. DNA shuffling is regarded as sexual recombination at the molecular level. Briefly, one or several homologous genes are digested with DNase I to yield a pool of random fragments. The fragments are reassembled using DNA polymerase, whereby homologous fragments recombine and prime one another until the gene is reassembled (70). The resulting products are screened for the desired functional properties. Individual proteins as well as entire pathways have been mutated using this technique to significantly improve their activity manyfold (Table 1).

Table 1. Some Selected Examples of Improvements of Proteins by DNA Shuffling

Example	Property to Be Modified	Increased Activity	Reference
β-lactamase gene	Antibiotic activity/enzyme activity	32,000 fold	71
Green fluorescent protein	Fluorescence	45 fold	72
β-galactosidase	Specific activity	66 fold	70
Arsenate Pathway	Arsenate detoxification	40 fold	73

One of the major problems associated with gene expression is determining which promoter will function in the environment and whether it will express the genes of choice (74). If a protein's affinity for a substrate is improved by oligonucleotide-directed mutagenesis and the promoter is left the same, regulation of the pathway would remain unchanged. Transcriptional regulation could then still be controlled by such mechanisms as substrate concentration inhibition or metabolite feedback inhibition. A solution would be to introduce the catabolic genes under a promoter that is unrelated to the contaminant but native to the environment. For example, place the catabolic genes under the regulation of a carbon source produced in a plant's root exudates (74).

ENCAPSULATION OF MICROBIAL CELLS FOR USE IN SOIL BIOREMEDIATION

The use of encapsulated microbial cells (i.e., cells distributed throughout a porous biopolymer) is being tested for bioremediation effectiveness in situ. Encapsulation may be a way for microorganisms to survive and maintain metabolic activity in a more controlled microenvironment within the heterogeneity of the soil environment. Biodegradable, nontoxic algal biopolymers such as alginate or κ-carrageenan may be the most practical and effective for environmental use (75). The encapsulation process is mild, and microbial cell viability is maintained for a wide range of microorganisms, including GEMs. Amendments such as nutrient sources or protective compounds can be added to the formulation, offering increased flexibility for diverse conditions that are found at different contaminated sites.

The ability of a microbial inoculum of either free or encapsulated cells to bioremediate contaminated soil has been investigated. Encapsulated microbial cells have been used effectively in bioreactors to detoxify or remediate liquid suspensions of toxic substances. Experiments with encapsulated cells in broth have demonstrated a significant increase (doubling) in tolerance to toxic compounds such as pentachlorophenol (76) and a significant increase in the ability to mineralize pentachlorophenol (75). Examples of similar results with other compounds have been reviewed (75).

Encapsulation of bacterial cells has been observed to significantly increase cell survival in soil compared to free cells (77,78). Encapsulated bacterial cells have demonstrated significantly increased survival through extremes in environmental abiotic conditions such as wet or dry cycles (77) and freeze or thaw cycles (79) when compared with free cells. Protection from potential biotic stresses such as predation has been demonstrated with bacteriophages (80) and protection from other predators is likely (75). Investigations of contaminant degradation in soil have shown that metabolic activity lasts longer in encapsulated cells than free cells (81), and significantly increased rates of degradation are possible using encapsulated bacterial cells. Cell movement through soil is shown to be reduced with the use of encapsulated cells (82,83); the cells remain within the bead in the soil matrix, whereas free cells move with vertical water flow around aggregates and through pores.

Dry encapsulated microbial cells may be easier to store, transport, and apply than liquid suspensions. The formulation also reduces the possibility of contamination during storage and transportation and reduces bioaerosol formation during application. Encapsulated cells have been kept in storage in the dark for up to one year at 4 °C without losing cell viability (84), and recent results have demonstrated encapsulated *Pseudomonas* sp. UG2Lr cells still maintaining viability after three years.

The application of microbial immobilization technology in bioremediation is still in its infancy but promises to offer enhanced effectiveness of microbial inoculum in environmental applications. Although there are limitations that need further exploration, including possible oxygen limitations, morphological alterations of cells inside beads (smaller cells), and effects of contaminant availability, the use of microbial inoculation on its own or in conjunction with other bioremediation strategies may prove to be more effective with this technique.

MONITORING OF BIODEGRADATION RATES

Gas Chromatography and High Performance Liquid Chromatography Analysis vs. Mineralization Studies

In laboratory experiments, two approaches have been used to determine the biodegradability of organic contaminants in soil: (*1*) loss of the parent compound as analyzed by either gas chromatography (GC) or high performance liquid chromatography (HPLC) and (*2*) mineralization experiments in which the ^{14}C-labeled parent compound is recovered as $^{14}CO_2$ (85). In the first method, the loss of a peak on a chromatogram makes it difficult to decide whether the parent compound is being partly degraded, mineralized, adsorbed to microbial biomass, or bound to soil particles or glassware. Even when experiments with killed-cell and abiotic controls are performed, the extraction efficiency and standard error can make data difficult to interpret (85). Conversely, recovery of significant amounts of $^{14}CO_2$ from a microcosm incubated with a ^{14}C-labeled substrate provides definitive proof of mineralization (85,86). Mineralization studies are generally simple to set up, and it is easy to collect samples that can be analyzed over a short period of time. In addition, information obtained from mineralization studies in microcosms can be used to predict trends on a larger scale field operation. The disadvantages of using ^{14}C-labeled compounds are that the compounds are expensive or may not be readily available from commercial sources, that studies can only be performed in laboratory soil microcosms, that the addition of the radiolabeled compound may not be a good indicator of the behaviour of a contaminant in aged soil, and the potential hazards of working with a radioactive material. Also, in soils with a mixture of contaminants, the radioactive compound would only be representative of a mixture and could not indicate the behavior of all compounds present. It is generally recommended that mineralization studies be accompanied by other analytical methods such as GC or HPLC (85).

METALS AND RADIONUCLIDES

The focus thus far has been on the biological remediation of organic contaminated sites, however, some mention should be made of metals and radionuclide contamination. Most of the metal contaminated sites are a result of industrial practices such as mining, nuclear processing, and manufacturing of batteries, electrical components, metal alloys, paints, preservatives, and insecticides (87). Toxic metals typically associated with contaminated sites include lead, mercury, arsenic, beryllium, boron, cadmium, chromium, copper, nickel, manganese, selenium, silver, tin, and zinc (87). Waste from nuclear power plants includes uranium (^{235}U), thorium (^{230}Th), and radium (^{226}R) (87,88). In comparison to the bioremediation of organic contaminated sites, metals and radionuclides are not degraded. Therefore, unless they are removed by human intervention, they will persist indefinitely. Thus, bioremediation in the case of metals and radionuclides predominantly involves immbolization to prevent further spread of the contaminant.

Immobilization of metals by microorganisms can occur due to changes in pH and redox potential and by complexation mechanisms. As mentioned in the pH section, pH has an effect on the mobilization metals. At an alkaline pH the metals form insoluble mineral carbonates or phosphates, whereas at a lower pH the metals exist as free ionic species or as soluble organometals (87). An example of a microbial environment in which the pH is dramatically decreased is in the case of acid mine drainage. The oxidation of iron sulfide ores by *Thiobacillus ferrooxidans* results in the formation of sulfuric acid. Although this organism would be useful in leaching of metals in the mining industry, its presence in a metal contaminated site would increase the mobilization of metals to groundwater.

The redox potential also plays an important role. This normally is controlled by the moisture content of the soil (see Moisture Content). Under a reduced or anaerobic environment (negative E_h), metals precipitate due to the presence of carbonates, ferrous ions, and sulfides (87). Metals become more soluble under oxidizing conditions (positive E_h) such as the situation with *Thiobacillus ferrooxidans* described in the previous paragraph.

Complexation of metals by microorganisms is a natural process. For example, microorganisms obtain metals that are essential for their viability, including sodium, potassium, copper, iron, and manganese. Unwanted or toxic metals may also be complexed to prevent them from affecting cellular processes. Essential metals such as sodium and potassium are important for the production of membrane potentials, whereas copper, iron, and manganese are used for metalloenzymes (87). Complexation of toxic metals can occur using extracellular polymers binding to cell surfaces, or they may be taken up intracellularly. Binding of metals to extracellular polymers and cell surfaces depends on the organism, metal, and pH (89). For example, algal cell walls are typically made up of a fibrillar layer and an amorphous layer. The fibrillar layer can be made of cellulose, mannan, or xylan, whereas the amorphous layer can contain alginic acid, fucoidan, agar, carrageenan, porphyran, or sulfated complex heteropolysacharides such as galactose, arabinose, xylose, rhamnose, and glucuronic acid (89). All of these compounds contain carboxyl sulfonated groups that are excellent in binding various metals. Bacteria on the other hand have a thick cell wall layer of peptidoglycan containing teichoic and teichuronic acids (gram-positive bacteria) or an outer cell membrane layer containing lipopolysaccharides and phospholipids (gram-negative bacteria) (89). The negative charges from the carboxyl and phosphonate groups are responsible for metal binding. Fungi have a cell wall composed mainly of chitin or cellulose (89). In addition, they have an outer amorphous layer containing α-glucans, mannan, galactans, chitosan, or glycogen (89). Like bacteria and algae, the phosphate and carboxyl groups are responsible for the negative charge, however unique to the fungi, a positive charge is produced by the amine groups in the chitosan (89). Therefore, depending on whether the metal is anionic or cationic, it will bind to the positive or negative charged groups, respectively, of the cell wall structures. pH of the environment also has an affect; at a lower pH cationic metal binding is reduced, whereas anionic binding is increased. For example, the anions chromate (CrO_4^{2-}) and selenate (SeO_4^{2-}) bind better at a lower pH. Intracellular uptake can take place either by passive diffusion or active transport. Once taken up some bacteria are known to sequester the metals using a cysteine-rich protein called a metallothionein. The metal can then be transported out of the cell or remain as an intracellular granule.

Radioactive waste is treated in the same manner as nonradioactive metals. An additional factor, however, is that radiation can cause lethal mutations in the DNA of microorganisms. For this reason biological products are most often sought, including tannins, melanins, bacterial chelating agents, microbial polysaccharides, metallothioneins, chitin, and whole cells (87). For an excellent review on the bioremediation of radionuclide-containing waste, see Lloyd and Macaskie (88).

PHYTOREMEDIATION

Phytoremediation has been defined as the in situ use of plants to stabilize, remediate, and reduce or restore contaminated soil, sediment, and surface or groundwaters (90,91). The prefix *phyto* means plant and *remediation* means to correct (92). Phytoremediation is an attractive remediation technology because it is cost-effective, causes minimal environmental disturbance, is suitable for a broad range of contaminants including metals, radionuclides, and organics, is aesthetically pleasing, has long-term applicability, and organic contaminants such as petroleum and pesticides can be completely degraded through mineralization (93–95). It relies on the plant's ability to act as "a solar-driven pumping and filtering system" and enhances or stimulates the natural tendency of ecosystem to restore itself (96).

There are limitations to phytoremediation and each site must be assessed individually to determine if phytoremediation is a feasible remediation option. If

the contaminant poses a direct hazard to humans or to the environment, phytoremediation is probably not the best remediation option because the clean-up time is longer than that for other remediation technologies. Other limitations of phytoremediation include the rate of plant development and depth of root systems. The contaminant must be in contact with the root and it follows that the contaminant concentration could be toxic to the plant or the microorganisms associated with the plant, there is a potential for entry into the food chain if appropriate control measures are not implemented, and the success of phytoremediation can be limited by soil and environmental conditions such as moisture and temperature (93–95).

Phytoremediation can be divided into six main categories: enhanced rhizosphere degradation, phytodegradation, phytoextraction, rhizofiltration, phytovolatilization, and Phytostabilization (Fig. 2). The two main contaminant degradation categories are enhanced rhizosphere degradation and phytodegradation. The former uses the bacteria in the root rhizosphere to degrade the contaminant, whereas the latter involves the plant degrading the contaminant by itself. All other phytoremediation techniques involve stabiliztin or extracting the contaminant from the environmental media that is then sequestered in the plant tissue or is transformed and/or volatilized. To keep with the theme of this encyclopedia only the enhanced rhizosphere degradation method is discussed later. For all other methods the reader is referred to Table 2.

Enhanced rhizosphere degradation is also referred to as plant assisted bioremediation and phytostimulation. It involves the stimulation by the plant through the release of exudates of the microbial community that is associated with the rhizosphere or root zone. It is the bacterial and fungal communities that are primarily responsible for degrading the contaminant rather than the plant itself. The interactions between the plant and the rhizosphere community are complex and not well understood.

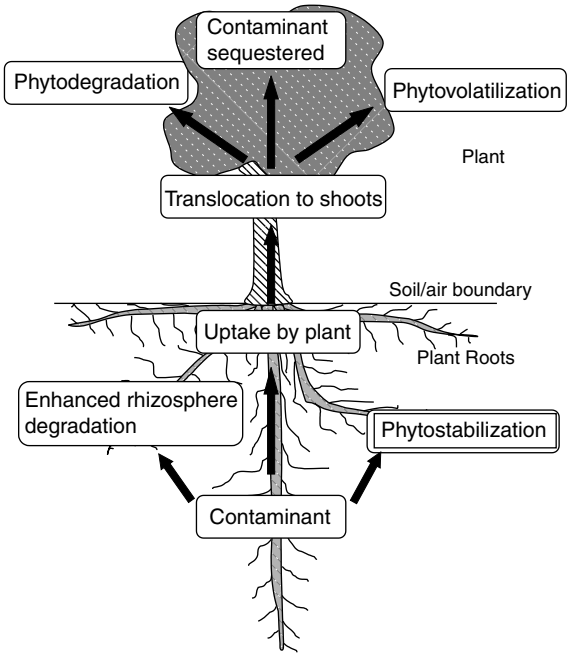

Figure 2. Schematic diagram showing the types of phytoremediation and where they occur in the plant.

Table 2. Types of Phytoremediation Available

Phytoremediation Categories	Technology	Media	Suitable Plants Tested	References
Enhanced Rhizosphere Degradation	Degradation of the contaminant by plant rhizosphere microorganisms	Mainly soil, sediment, and sludge, but also surface and ground water	Grasses, hybrid poplars, red-mulberry, alfalfa, cattails	93,97,98,99,100
Phytodegradation	The degradation of a compound through plant metabolism or through the release of enzymes by the plant	Soil, sediment, sludge, ground and surface water	Algae, stonewart, hybrid poplars, cattails, and rice	93,101
Phytoextraction	Plants accumulate metals and radionuclides and translocate them to harvestable plant parts	Soil, sediment, and sludge	Indian mustard, alpine pennycress, sunflowers, poplars	93,102–105
Rhizofiltration	Plants absorb or precipitate metals and radionuclides from aqueous solutions around the roots, therefore immobilizing the metals or radionuclides	Surface or groundwater	Sunflowers, Indian mustard, and water hyacinth	93,106–108
Phytovolatilization	The uptake and possible transformation of a compound by the plant and subsequent release into the atmosphere	Groundwater, soil, sediment, and sludge	Poplar, alfalfa, black locust and Indian mustard	93,109
Phytostabilization	Stabilization of the metal contaminated soil by plant roots reducing the movement off-site	Soil	Indian mustard, grasses, and hybrid poplars	93,102

Curl and Truelove (110) defined the rhizosphere as the soil subjected to the influence of living roots. It contains a diverse community consisting of microbiota, (bacteria, fungi, and algae) and micro and mesofauna (such as nematodes, insects, mites) (111). There are numerous factors that influence the composition and activity of this community, including plant species and age, root type, soil type, environmental conditions (carbon dioxide, light, pH, temperature, moisture, and nutrients) and exposure to historical contamination (97,98). Generally, the bacterial population is dominated by gram-negative, nonsporulating bacilli, with a small proportion of gram-positive bacilli, cocci, pleomorphic rods, and aerobic nonspore forming bacteria (111).

The rhizosphere has been studied extensively over the years, primarily for agricultural reasons (96). Attempts have been made to understand the interactions occurring in the hope of controlling plant pathogens, increasing crop yield, and optimizing symbiotic relationships such as *Rhizobium* and mycorrhizal fungi. Only recently has it been realized that the rhizosphere can be important in increasing the degradation of environmental contaminants (112–116).

Plants release compounds (exudates) into the rhizosphere (called rhizodeposition), which act as nutrient sources for microorganisms. Roots and root associated fungal hyphae provide surfaces for microbial colonization (96,117,118). Plants also alter the soil's physicochemical properties, making the soil environment more hospitable for microorganisms (112,118).

Exudates may be organic or inorganic and can result from active exudation, passive leaking, production of mucilage, or dead or sloughing off plant cells (119). Exudates can be up to 30–40% of the photosynthetically fixed carbon and include simple sugars, amino acids, organic acids, fatty acids, sterols, growth factors, nucleotides, flavanones, and enzymes (96,120). Some exudates are structural analogs to xenobiotics, so the rhizosphere community may already be enriched to degrade certain environmental contaminants. (121,122). Monomeric components of lignin are structural analogs to chlorinated aromatic acids (121), *p*-coumaric acid is structurally similar to benzenes (115), morin is similar to polycyclic aromatic hydrocarbons (PAH) (115), and biphenyl actually induces enzymes required for PCB degradation and acts as a growth substrate for cometabolism (115,123). Plants support a microbial community with densities of an order of magnitude that matches or is higher than that of bulk soil (97). This is termed the rhizosphere effect and is defined as the ratio of the number of microorganisms in the rhizosphere to that in the nonrhizosphere soil (R/S ratio) (96). R/S values typically range from 5 to 20 but can be as high as 100 or more (96).

Siciliano and Germida (114) reported three possible mechanisms that plants could enhance the degradation of soil contaminants: (*1*) the plant nonspecifically enhances the microbial community, that is, increases the R/S ratio (*2*) enhancement of microbial activity that protects the plant from the contaminant, and (*3*) the development of microbial communities that specifically degrade the contaminants.

The plant provides a niche that can support a wide diversity of microorganisms. Often, it is not a single strain responsible for the complete degradation of a contaminant, but rather the concerted effort of a community of organisms, each responsible for a sequential step in the degradation (122). By simply hosting a wide diversity of organisms, it is thought that the plant increases the probability of completing each step in the degradation process. Also, the release of exudates is thought to provide an environment conducive to cometabolism (96). Cometabolism is when one compound is degraded by an organism but is not used as an energy source by that organism. The organism must also degrade another compound at the same time that is used as a substrate and as an energy source. The rhizosphere also provides an environment favorable for gene transfer, including plasmids encoding degradative genes (122).

The second mechanism of the plant's role in phytoremediation is to enhance microbial communities that protect the plant. Interactions in the rhizosphere are complex and have often evolved to benefit both the plant and the microbial community. Walton and coworkers (111) proposed that plants exposed to contaminants alter their exudation patterns, thereby sending signals to microorganisms to detoxify the contaminant. The plant provides nourishment and oxygen in exchange for protection and/or detoxification. Siciliano and Germida (124) inoculated Daurian wild rye with two pseudomonads species. These inoculants enabled the plant to establish and survive in the contaminated soil, thereby reducing phytotoxicity. However, contaminant levels were not reduced. Increased microbial numbers and activity in the rhizosphere do not always correlate with increased degradation. Microorganisms may reduce the phytotoxicity of a compound by immobilizing the xenobiotic through humification or sequestration (97). Humification is the incorportation of organic compounds into soil organic matter. Humic material may also act as electron acceptors during low-oxygen conditions, thereby aiding in the degradation or transformation of organic substances when oxygen is limited (97).

The third possible role of plants is that they enrich a degrading microbial community. Vegetation has been shown to enhance degradation rates in a variety of environmental conditions of a wide range of organic contaminants such as total petroleum hydrocarbons (TPH), PAH, PCBs, pesticides, organic solvents, and surfactants (117,122,123). Nichols and coworkers (125) found an increase in microbes capable of degrading a mixture of organic contaminants, with the greatest increase in the rhizosphere. Microorganisms will typically adapt to a contaminant by selective enrichment of those capable of tolerating or degrading the contaminant. The plant provides further selection pressure, stimulates cometabolism, provides a niche for long-term survival of degradative plasmids and provides an environment conducive to gene transfer.

It is very difficult to develop a consistently effective plant-microbe system for phytoremediation because of the many possible plant–microbe–contaminant interactions and because of our limited understanding of the factors influencing rhizosphere population dynamics. It has been

shown, however, that inoculating or seeding a phytoremediation system with microbial degraders or plant growth promoting bacteria can be effective (114,115,121,126,127). Inoculation in bioremediation often fails because the introduced microorganisms fail to compete with indigenous soil organisms. It is thought that the plant provides a niche for the introduced bacteria, allowing them to compete with indigenous organisms. Also, the plant provides additional nutrients, increased moisture exposure through transpiration, and a surface area for colonization, and the roots provide a mechanism of delivering the organisms to the contaminants. In addition, land plants are often colonized by mycorrhizal fungi whose hyphal network acts as extensions of roots, exploiting a greater volume of soil. Hyphae also release photosynthetically fixed plant carbon into the mycorrhizosphere and provide extensive surface areas for colonization. Heinonsalo and coworkers (118) found the highest density of bacteria in the mycorrhizosphere of a contaminated soil.

Although phytoremediation is still in its infancy, it does hold considerable promise for the remediation of organic and inorganic contaminated soils, sediments, and water.

CONCLUSION

Bioremediation is increasingly being recognized as a promising environmental technology. It is possible to demonstrate bioremediation of chemically contaminated soils in relatively small-scale soil microcosms. Similar results may or may not be achieved in the open soil environment or in a bioreactor. Environmental conditions are often difficult to control in the open environment and the rate and extent of bioremediation of targeted chemicals may proceed slower than in the laboratory setting or possibly not at all.

Natural attenuation has been presented in this article followed by factors that are necessary for soil bioremediation to proceed. The first task is to determine what happens if the contaminant is left alone for a period of time (natural attenuation). Does intervention actually help in remediating the contaminated site or is spreading the contamination when disturbing the soil just exacerbating the problem? Once it is established that human intervention is necessary, then it must be determined if the environment, contaminant, and the degrading microorganisms are in balance or are present. If the environment is not optimum for microbial growth, activity, and survival, biodegradation of the contaminant is limited. Conversely, if the environment is optimum but the contaminant is toxic or not bioavailable, biodegradation and bioremediation is unsuccessful. The last important factor that must be present and active is the contaminant-degrading microorganism. Case studies of bioremediation taking into account the above variables are presented in Table 3.

Research on isolating, monitoring, and genetically engineering microorganisms has improved the prospects of using bioremediation as an alternative to chemical or physical remediation strategies. In addition, new technologies such as microbial encapsulation and

Table 3. Case Studies in Bioremediation

Case Study 1. Bioremediation of PCBs in the Hudson River
General Electric Corporation, Schenectady, New York, sponsored a bioremediation test site for the in situ bioremediation of PCBs (2,128). Six cylinders were placed in a shallow section of the Hudson River where sediment was contaminated with PCBs. Five cylinders received different treatments including oxygen, nutrient, and biphenyl amendment and the addition of laboratory PCB degrading bacteria. Over a 10-week test period, over one- third of the PCBs were destroyed. The loss of degradable PCBs and the presence of key microbially produced PCB intermediates indicated that microorganisms were responsible for the degradation. The bioaugmented cylinders did not have an additional effect on degradation.
Case Study 2. Bioremediation of Wood Preservatives
Grace Bioremediation Technologies, Ontario, Canada, used bioremediation technology to treat a sandy loam soil contaminated with polyaromatic hydrocarbons, petroleum hydrocarbons, and chlorophenols (129). Four treatment plots were constructed. Plot 1 was a control, whereas Plots 2–4 were tilled and irrigated to 70% water holding capacity twice a month, nitrogen and phosphorus were added to plots 3 and 4 to achieve a C : N : P ratio of 100 : 5 : 1, and plot 4 received additional amendment composed of agricultural crop residues. After 526 days, over 83% of each of the contaminants were degraded in plots 3 and 4, whereas less than 50% and 75% were lost in plots 1 and 2, respectively. The consensus between six toxicity tests (seed germination, earthworm survival, SOS Chromotest, solid-phase Microtox, and red blood cell haemolysis assay) revealed that the toxicity of the soil was reduced over the bioremediation period.
Case Study 3. Bioremediation of Soil Contaminated with Radionuclides
Agricultural land in Vromos Bay, southeastern Bulgaria, is contaminated with radionuclides (uranium, radium) and toxic heavy metals (copper, cadmium, lead) because of mining and mineral processing practices. Groudev and coworkers (130) set up a 400 m^2 test site to determine if the indigenous microflora could be used to bioremediate the soil. The soil was characterized to have a slight alkaline pH. To increase aeration in the upper soil horizon, the soil was ploughed periodically. Leach solutions containing carbonate ions plus dissolved organic compounds were used to help in the dissolution of the contaminants from the upper soil horizon (horizon A) to the lower soil horizons. In addition, water containing ammonium phosphate and organic compounds was injected into the lower soil horizons (horizon B_2, 55 cm) using vertical pipes to stimulate the sulfate reducing bacteria (SRB). Between 52 and 70% of the total radionuclides and toxic metals were removed from the upper soil horizon. The dissolution of these metals was predominantly attributed to the activity of the heterotrophic and chemolithotrophic aerobic microorganisms in the upper soil horizon. In the lower soil horizon the radium was found bound to clay particles, whereas uranium was precipitated as uraninite (UO_2). The metals were found as insoluble sulfides. The activity was attributed to SRBs.

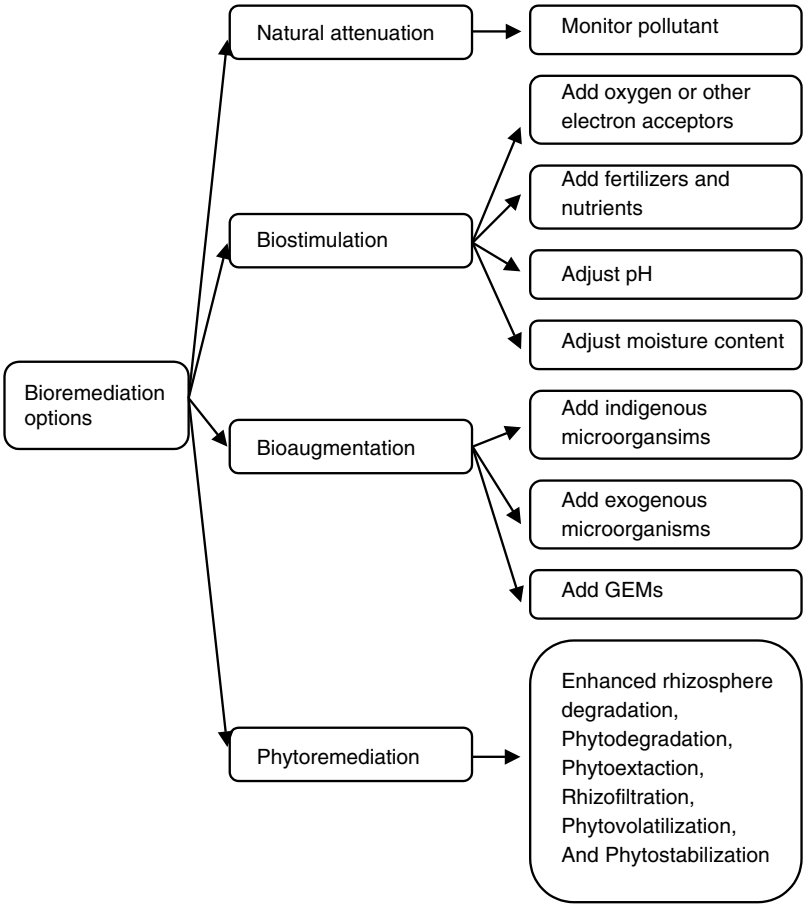

Figure 3. Bioremediation options.

phytoremediation have provided novel approaches to help existing bioremediation processes.

There is no standard protocol that can be uniformly applied to all sites as each bioremediation site is different. Each situation requires a careful examination of all options available for bioremediation (Fig. 3). In addition, each site needs to be assessed separately with all of the factors involved, including the environment, the contaminant and the degrading microorganism(s) considered as a whole.

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BIOREMEDIATION, PESTICIDES IN SOILS.

See PESTICIDE DEGRADATION IN SOILS

BIOREMEDIATION, PLFA ANALYSIS.

See LIPID BIOMARKERS IN ENVIRONMENTAL MICROBIOLOGY

BIOREPORTERS.

See GREEN FLUORESCENT PROTEIN (GFP)

BIOSOLIDS: ANAEROBIC DIGESTION OF

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The activated sludge process was developed in England in 1914 by Ardern and Lockett and was so named because it involved the production of an activated mass of microorganisms capable of stabilizing a waste aerobically (1). The activated sludge process is the most widely applied method for sewage treatment. Many versions of the process are in use, but fundamentally, they are all similar. The system shown in Figure 1 is a completely mixed activated sludge system, in which wastewater containing organic waste is first brought into a settling tank in which the solids are allowed to settle. Then the pre-settled wastewater is led into the aeration tank in which an aerobic suspended culture is maintained in suspension by the use of diffused or mechanical aeration. In the aeration tank the bacterial biomass carries out the conversions given in Equation (1) (1). In the aeration tank new biomass not only is formed but also is degraded through endogenous respiration Equation (2) (1). After a specific period the mixed liquor in the aeration tank is led to a settling tank in which the cells (or activated sludge) are separated from the treated wastewater. A portion of the activated sludge is recycled to the aeration tank to maintain the desired level of bacteria in the reactor. The remainder waste is called the secondary or waste activated sludge (WAS). Before WAS can be disposed off, it has to be stabilized to reduce pathogens, eliminate offensive odors, eliminate the potential for putrefaction, and improve the dewatering characteristics. The methods that can be applied to stabilize sludge can be divided into four categories. (*1*) biological reduction of the volatile content, (*2*) chemical oxidation of the volatile content, (*3*) addition of chemicals to render it unsuitable for the survival of microorganisms, and (*4*) application of heat to disinfect or sterilize the sludge (1).

The biological methods include composting, aerobic stabilization, and anaerobic stabilization. The latter is discussed in this article. Anaerobic digestion takes place under anaerobic conditions in an airtight reactor. Sludge, introduced continuously or intermittently, is retained in the reactor for a period of time, during which microorganisms, under anaerobic conditions, convert the volatile solids in the sludge to a variety of end products including methane (CH_4) and carbon dioxide (CO_2) (1).

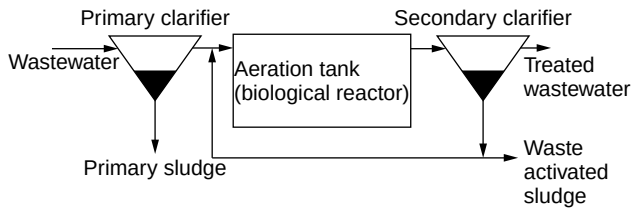

Figure 1. Schematic drawing of a completely mixed activated sludge system.

Table 1. Results of the Mesophilic Sludge Digesters of the WWTP Elburg, The Netherlands

Parameter	Primary Sludge Digester	Activated Sludge Digester
TSS reduction (%)	41	19
VSS reduction (%)	56	28
HRT (days)	27	23
Gas production (m^3/kg TSin)	0.25–0.65	0.10–0.16

In most wastewater treatment plants, the WAS and primary sludge are mixed and digested together in one reactor. However, when the amount of WAS in the reactor feed increases above 40% of the total amount of sludge, the performance of the digester decreases quickly (2). Moreover, when the primary and WAS are digested separately the production of methane and the reduction of volatile solids from WAS is much lower than compared to the primary sludge (Table 1). Apparently the volatile solids in the WAS are more difficult to degrade than those present in primary sludge.

$$\underset{\text{(organic waste)}}{\text{COHNS}} + O_2 + \text{nutrients} + \text{bacteria} \rightarrow CO_2 + NH_3 + \underset{\text{(new biomass)}}{C_5H_7NO_2} + \text{other end products} \quad (1)$$

Endogenous respiration:

$$\underset{\text{(biomass)}}{C_5H_7NO_2} + 5O_2 + \text{bacteria} \rightarrow 2H_2O + NH_3 + \text{energy} \quad (2)$$

THE STRUCTURE OF ACTIVATED SLUDGE

The structure/composition of the activated sludge is important for its biodegradability. In this section, information on the structure and composition of the activated sludge is presented.

A typical activated sludge floc is 80 to 120 m in diameter and consists of five components, namely, bacteria, water, organic fibers, inorganic particles, and extracellular polymeric compounds (ACTIVATED SLUDGE — THE FLOC). These substances arise from the wastewater or from the bacteria themselves (3–5). The backbone of the sludge is formed by the extracellular polymeric compounds, which are linked together by divalent cations (mainly Ca^{2+}), thus forming a gel-like structure in which all other components are embedded.

The bacteria in activated sludge comprise a viable and a nonviable fraction. A viable cell can be defined as a cell with an intact encapsulating structure that shows some kind of metabolism, that is, is capable of either metabolizing a substrate or reproduction, or both.

Owing to the complex structure of AS, the measurement of the number of viable cells is not as easy as it is for pure cultures. Many microorganisms are linked together in sludge flocs so that some kind of treatment to separate the

bacteria into individual reproductive units is necessary for counting procedures, such as plate counting or direct microscopic counting, possibly after staining. Staining procedures that are used are acridine orange, DAPI, and FITC staining (6). Respiring bacteria can be counted by INT staining, which is sensitive to a dehydrogenase enzyme in the electron transport system. A drawback of the plate counting techniques is that the actual number of microorganisms present can be easily underestimated as no single medium will support growth of all different kinds of viable bacteria present (7).

Microbial biomass consists of a variety of biopolymers. Stouthamer (8) determined that the dry weight of microbial cells is composed of protein (52.4%), polysaccharides (16.6%), lipid (9.4%), RNA (15.7%), and DNA (3.2%). It is important to note that the mentioned biopolymers contribute more than 95% of the total dry weight of microbial cells. However, the relative composition of the biomass is largely dependent on the types of microorganisms present in the sludge and the conditions under which the biomass has been grown. For example, under nitrogen, phosphorous, or iron limitation, microorganisms may produce and accumulate large amounts of polysaccharides or other biopolymers as storage compounds. This influences the relative polymer composition of the biomass. DNA concentrations are maintained in relatively constant proportions within microorganisms (9). Therefore, determination of the amount of DNA in the activated sludge is another method of determining the amount of viable cells. However, as DNA can also be detected in the extracellular polymeric components of the WAS, this method will lead to an overestimation of the number of viable bacteria in the WAS.

Several authors have tried to establish the viable biomass fraction in activated sludge. Weddle and Jenkins (10) derived activated sludge from laboratory reactors that were fed with autoclaved primary sewage. AS viability was determined by standard plate counting procedures on sonically dispersed sludge using activated sludge extract agar. The specific viability varied between 1.5 and 2.0×10^8 cells/mg VSS for net growth rates between 0.026 day^{-1} and 0.33 day^{-1}. Assuming an average VSS mass per viable microorganism of 1.0×10^{-12} gram, the viable fraction amounted to 15 to 20%.

Frolund (11) worked with sludge from two WWTPs with a sludge age of 7 to 9 days and 30 to 35 days, with (partly) chemical phosphate removal. They counted about 5×10^8 cells/mg VS in WAS with acridine orange (AO) staining in WAS from the high SRT plant. Only 10% of these cells appeared to be actively respiring as measured with CTC staining. From the average cell size measured from microscopic observations and a density conversion factor, the viable fraction was estimated at 10% of VS. They extracted a maximum of 16 mg DNA per gram VS from both the high SRT sludge and the low SRT sludge. Their sludge had a low VS content — 57% of TS. Taking a DNA content of 3.1% of dry weight for an average *Entamoeba coli* B/r cell (9) or $0.9 \times 3.1 = 2.8\%$ of VS, the viable fraction can be estimated at $160/2.8 = 57\%$.

The number of viable cells in the activated sludge depends, among others, on the sludge-loading rate of the activated sludge plant. This can be illustrated by simulation of a simple wastewater treatment plant (WWTP) consisting of one aeration tank and a secondary clarifier at different sludge-loading rates (12). The simulation of the WWTP was done with the activated sludge model no. 1 (13). This model has been widely accepted for the description of the activated sludge process. Inert particulate material plays an essential role in the process, which determines the fraction of viable microorganisms. This inert particulate material may be present in the influent but it is also formed in the death-regeneration cycle, because upon lysis of microorganisms, only part of the released material is biodegradable. In ASM 1 the inert particulate material becomes part of the sludge floc. Because the number of death-regeneration cycles increases with sludge age, the amount of inert particulate material also increases. This results in a lower viable fraction at higher sludge ages. In the model, therefore, the fraction viable particulate material, that is, viable microorganisms, depends on both the concentration of inert particulate materials in the influent and the sludge age.

In the simulation experiment the influent composition and oxygen concentration in the aeration tank was kept constant. Only the flow rate of the WWTP was varied in order to vary the sludge load. Each simulation was conducted over 40 days, which was amply sufficient to reach a steady state. Table 2 shows the viable fraction f_{bio}, depending on the organic loading rate (OLR) and solids retention time (SRT). The fraction of heterotrophic active biomass is seen to increase with increasing sludge-loading rate and decreasing SRT.

To reduce the growth of algae in surface water, many wastewater treatment plants (WWTP) have to meet very strict effluent standards for total phosphate and nitrogen. The removal of nitrogen in a conventional activated sludge plant requires a long sludge residence time to allow growth of the slow-growing nitrifying bacteria, *Nitrosomonas*, and *Nitrobacter*. Therefore, an ever-increasing amount of waste-activated sludge of a long sludge age is produced.

The exopolymeric compounds should be considered as the third major sludge component after water and bacteria (14). As aforementioned, these polymers are interconnected by divalent ions, mainly Ca^{2+} (15), through which a matrix that can be considered as the backbone of the activated sludge is formed. Two types

Table 2. Effect of the Organic Loading Rate (OLR) of a WWTP on the Fraction Viable Biomass (f_{bio}) in the Activated Sludge. Results were Obtained by Simulation of a WWTP with ASM 1 (12)

$Q_{influent}$ [m^3/day]	OLR [g COD/g TSS/day]	SRT [days]	f_{bio} [% of total solids]
120	0.167	25.8	27.9
240	0.298	12.2	42.4
480	0.499	6.2	55.1
960	0.857	3.1	64.6
1,920	1.522	1.6	70.8
3,840	2.789	0.8	73.5

of exopolymers are distinguished slime polymers and capsular polymers, respectively. The slime polymers are only loosely attached to the biomass and can be extracted by mild extraction methods. The slime contains all types of biopolymers, which are believed to be mainly autolysis products from the bacteria. The capsular polymers, on the other hand, are very strongly attached to the bacteria. Capsular polymers require much more rigid extraction methods than slime polymers. Capsular polymers are thought to play an important role in the dewatering of sludge, although publications on this subject are not unequivocal. This is probably because many different extraction methods are used and the reproducibility of most methods is low. Especially when using techniques for extraction of capsular polymers, lysis of biomass occurs often. This causes overestimation of the amount of extracted polymer. In Table 3 an overview is given of the methods used to extract slime and/or capsular polymers. The amount of slime and capsular polymers that were found in several investigations are listed in Table 4.

In summary, it can be seen that the composition of the volatile solids in activated sludge comprises more than 80% inactive biomass, less than 4% exopolymers and 7 to 19% active biomass.

The water is activated sludge can be divided into four fractions (23):

- free water
- interstitial water
- vicinal water
- water of hydration water

The free water is not bound to the sludge and can be removed by means of conventional techniques for dewatering such as thickening, centrifugation, and vacuum filtration. The interstitial water is situated in the floc structure and can only be removed when the floc is destroyed. If surface-active polymers are used in mechanical dewatering techniques it is possible to remove some interstitial water. The vicinal water is bound to the surface of the sludge and can only be removed by drying at 105EC. The water of hydration is water that is chemically bound to the particle. Removal of this water requires thermochemical destruction of the particles (24,25).

THE MICROBIOLOGY OF METHANOGENIC PRODUCTION

Anaerobic decomposition is initiated by hydrolysis of biopolymers by extracellular enzymes, which are formed

Table 3. Extraction Methods Reported to be Used for Extraction of Exopolymers. Division of the Methods Has Been Made on the Basis of the Type of Extracted Polymers

	Slime	Capsular	Both
Method 1			
Extraction of polymers:			
The polymers are extracted by centrifugation at 2,000 to 10,000 g. The supernatant contains the polymers and the pellet is discarded.	T		
Analysis of the polymers:			
The amount of polymers in the supernatant is determined by one of the following methods:			
— Extraction of polymers from the supernatant with, for instance, ethanol. The total amount of polymer is determined gravimatically after drying the extract or chemically after resuspension in water.			
— Direct measurement of the amount of protein, carbohydrate, DNA, RNA, and/or humic substances.			
Method 2			
Washing of the sludge			
Centrifugation at 2,000 to 10,000 g. The supernatant is discarded and the pellet is resuspended in a buffer solution.			
Extraction of polymers:		T	
The polymers are extracted by one of the following methods:			
— centrifugation at >10, 000 g			
— addition of ion exchange resin such as Dowex or XA			
— boiling upto one hour			
Analysis of the polymer:			
see method 1			
Method 3			
The same method as in method 2 are used. However the washing procedure is skipped.			T

Table 4. Composition of the Exopolymers Extracted from Fresh Activated Sludge. The Division Between Slime and Capsular Polymers Has Been Made based on the Extraction Method as Depicted in Table 3

Only Slime Polymers Extracted	Both Slime and Capsular Polymers Extracted	Only Capsular Polymers Extracted
1. 52 mg total slime polymers/g VS		
2.	212 mg protein/g VS 40 mg carbohydrates/g VS 101 mg humic substances/g VS 16 mg DNA/g VS	
3.	68 to 100 mg protein/g VS 71 to 127 mg carbohydrates/g VS 35 to 54 mg RNA/g VS 28 to 41 mg DNA/g VS	
4. 25 to 90 mg total polymers/g TSS		
5. 21 to 35 mg total polymers/g TSS		
6. 0.52 to 29.1 mg total polymers/g TSS		13.1 to 23.8 mg total polymers/g TSS
7.	9 to 90 mg carbohydrates/g TSS	

Note: References and extraction method:
1. centrifugation, 20 minutes, 2,000 g (16).
2. extraction with Dowex from unwashed sludge (method 3) (17).
3. centrifugation of washed sludge 15 minutes at 32,500 g (method 2) (18).
4. centrifugation (19).
5. centrifugation (20).
6. centrifugation 15 minutes, 13,200 g with and without addition of K_2HPO_4 (21).
7. settled sludge, washed with water subsequently boiled for one hour (22).

and excreted by fermentative microorganisms. Polysaccharides yield monomeric and oligomeric C6 and C5 sugars, while proteins are converted to single amino acids and small peptides. Proteins are composed of about 20 amino acids with different chemical structure. RNA and DNA are transformed to C5 sugars and nucleic bases purines (adenine and guanine) and pyrimidines (cytosine, thymine, and uracil). Lipids are degraded to glycerol and long-chain fatty acids. The general pattern of anaerobic mineralization of complex organic matter is that fermentative bacteria metabolize easily with degradable compounds such as sugars, amino acids, purines, pyrimidines, and glycerol to a variety of fatty acids, bicarbonate, formate, and hydrogen. Acetogenic bacteria degrade higher fatty acids to acetate, carbon dioxide, hydrogen, and formate. These compounds are the substrates for methanogens (26). All these processes, fermentation, acetogenesis, and methanogenesis (Fig. 2), will take place at the same time, but often, because of the different growth rates and activities of the different microorganisms, these processes are partially uncoupled. The whole sequence of anaerobic biodegradation is very dynamic in the sense that methanogens strongly influence the metabolism of fermentative and acetogenic bacteria by means of interspecies hydrogen transfer (27,28).

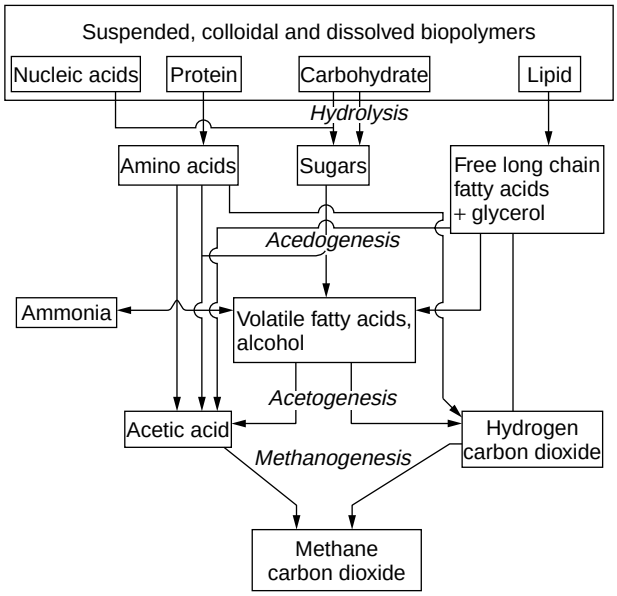

Figure 2. General scheme of the anaerobic digestion process.

Mineralization of Sugars

Sugars are substrates, which can be fermented by a variety of different microorganisms via different pathways leading to typical end products (29). Generally C6 sugars are degraded by glycolysis or the Entner-Doudoroff pathway to pyruvate, while C5 sugars are converted via a combined pentose pathway and the glycolytic or the Entner-Doudoroff pathway to pyruvate. Conversion of sugars to pyruvate is an oxidative process, which intracellularly results in the formation of NADH. The further metabolism of pyruvate depends on the organisms involved, and reflects the biochemical mechanisms, which the sugar-fermenting microorganisms use to dispose off reducing equivalents. Facultative aerobic microorganisms that are abundantly present in the sludge may perform a so-called mixed acid fermentation, in which a variety of reduced organic products are formed, including ethanol, lactate, succinate, formate, and butanediol. More specific fermentations by specialized microorganisms will occur as well. Alcoholic fermentation, lactic acid fermentation, homoacetogenic fermentation, and propionic acid and butyric acid fermentation are examples of

specific fermentations carried out by anaerobic microorganisms. The combined occurrence of all these individual fermentations will yield a variety of and products such as ethanol, lactate, acetate, propionate, butyrate, lactate, hydrogen, and bicarbonate. Other reduced organic compounds such as butanol, acetone, and butanediol may be formed, but are quantitatively of little importance. Except for butyrate, propionate, and acetate, the reduced compounds may be fermented further by specific microorganisms. For example, lactate and ethanol ($+CO_2$) will be fermented to acetate and propionate by *Desulfobulbus propionicus* and *Pelobacter propionicus* (30,31), *Clostridium kluyveri* can ferment ethanol (+acetate) to butyrate, while lactate and ethanol can also be fermented by the homacetogens to solely acetate. Succinate can be decarboxylated to propionate by *Propionigenium modestum* (32). However, propionate, butyrate, and long-chain fatty acids cannot be fermented. Propionate is oxidized to acetate, CO_2 and hydrogen formate, while butyrate and long-chain fatty acids are oxidized to acetate and hydrogen (see later). Instead of hydrogen, acetogens may also dispose off reducing equivalents as formate. The relative importance of hydrogen and formate is not clear (28). Acetate, $H_2 + CO_2$ and formate are finally converted by the methanogens to $CH_4 + CO_2$, CH_4 and $CH_4 + CO_2$, respectively. Physiologically, methanogens are very specialized. *Methanosaeta* species are only able to grow on acetate while *Methanobrevibacter* species can only use H_2 and CO_2 and *Methanospirillum* and *Methanobacterium* species use both $H_2 + CO_2$ and formate for growth. *Methanosarcina* species are most versatile; they can use $H_2 + CO_2$, acetate, methanol, and other methylated compounds for growth (33–35).

In methanogenic environments the utilization of hydrogen by methanogens affects the metabolism of those sugar-fermenting microorganisms that have the ability to use protons as electron sinks. A typical example is the fermentation of glucose by *Ruminococcus albus* (36). In pure culture this organism forms acetate, CO_2, hydrogen, and ethanol as end products, while in coculture, ethanol is not formed (Fig. 3). *Ruminococcus albus* degrades glucose via the glycolytic pathway. This results in the formation of NADH (glyceraldehyde-3-phosphate dehydrogenase) and reduced ferredoxin (pyruvate:ferredoxin oxidoreductase). The redox couples of $Fd_{(ox)}/Fd_{(red)}$ and AND/NADH are -398 and -320 mV, respectively, whereas the redox couple H^+/H_2 is -414 mV (37). The Gibbs free energy changes ($\Delta G^{0'}$ values) of the redox reactions can be calculated using $\Delta G^{0'} = -n.F.\Delta E^{0'}$, where n is the number of electrons transferred, F is the Faraday constant, and $\Delta E^{0'}$ is the difference between the redox couples:

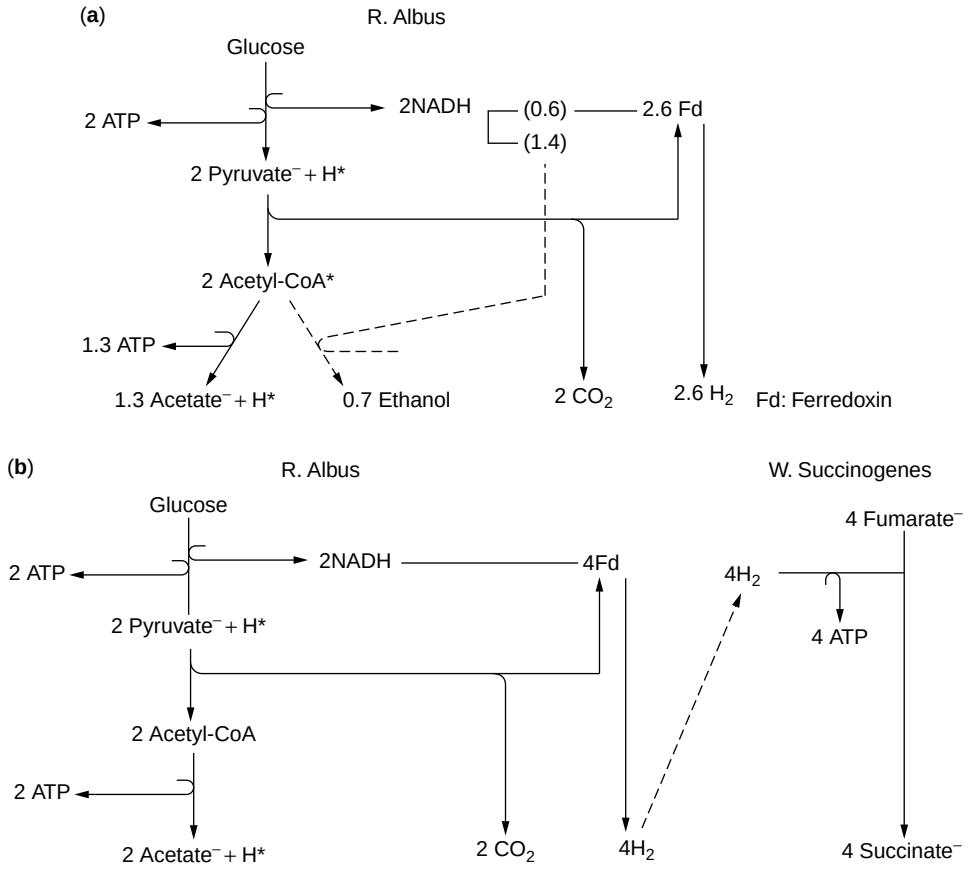

Figure 3. Sugar fermentation by *R. albus* in pure culture and in mixed culture, with a hydrogen-utilizing anaerobe.

$$2Fd_{(red)} + 2H+ \rightarrow 2Fd_{(ox)} + H_2 \quad \Delta G^{0'} = +3.1\ kJ$$

$$NADH + H^+ \rightarrow NAD^+ + H_2 \quad \Delta G^{0'} = +18.1\ kJ$$

The oxidation of reduced ferredoxin is relatively easily coupled to H_2 formation, while H_2 formation from NADH is only possible at a lower H_2 partial pressure. At a H_2 partial pressure of 1 Pa, which can be maintained by hydrogenotrophic methanogens the $\Delta G^{0'}$ of the two conversions is around -26 and -11 kJ, respectively. In the course of the fermentation by a pure culture of *R. albus*, H_2 accumulates, and consequently the organism is no longer able to couple the oxidation of NADH to the reduction of protons. As an alternative, acetyl-CoA or acetaldehyde is used as an electron sink to form ethanol. In the coculture, hydrogen is removed efficiently and ethanol is not produced. Because the organism forms ATP during acetate formation from acetyl-CoA, but not if ethanol is produced, the ATP yield per glucose is higher in the coculture than in the pure culture. Similar effects have also been observed with other sugar-fermenting microorganisms, which dispose off reducing equivalents by formation of reduced organic compounds such as ethanol, lactate, succinate, butyrate, or propionate (38–43). In general, the effect of the presence of hydrogenotrophic methanogens is that relatively fewer of these reduced organic compounds and more of the methanogenic substrate acetate are formed.

Mineralization of Amino Acids:

Proteins are composed of about 20 structurally different amino acids, which require different biochemical pathways for degradation. Therefore, anaerobic degradation of amino acids by the mixed methanogenic consortia is even more complex than described above for sugars. This degradation involves oxidation and reduction reactions of one or more amino acids. Some amino acids are degraded preferentially via reduction, others via oxidation. The combined oxidation and reduction of sets of amino acids (Stickland reaction) is a well-known way for many clostridia species to degrade amino acids (28). Table 5 shows some oxidative and reductive reactions involved in the anaerobic degradation of amino acids. In the Stickland reaction, an oxidative reaction with one amino acid is coupled with the reductive degradation of another. A classical and well-known Stickland mixture is alanine and glycine. Other couples that have been described are leucine, isoleucine valine, phenylalanine, and tryptophane, which are oxidized to 3-methylbutyrate, 2-methylbutyrate, 2-methylpropionate, phenylacetate, and indolacetate, respectively, and proline, phenylalanine, tryptophane, and leucine, which are reduced to 5-aminovalerate, phenylpropionate, indolpropionate, and 4-methylvalerate, respectively. Besides these other couples have been found or may occur. Some amino acids can be degraded both oxidatively and reductively (Table 5). This may result in fermentative growth of microorganisms on single amino acids. For example, trypthophane can be fermented to indoleacetate and indolepropionate, phenylalanine to phenylacetate and phenyl propionate, and leucine to isovalerate and 4-methylvalerate. Also, other compounds and intracellular intermediates may act as terminal electron acceptors in the degradation of amino acids. Leucine oxidation can be coupled to the reduction of acetate to butyrate as described for *Clostridium acetireducens* (44). A similar reaction takes place in the classical glutamate fermentation by, for example, clostridia, in which reducing equivalents formed

Table 5. Oxidative and Reductive Reactions Possibly Involved in the Anaerobic Conversion of Amino Acids

Oxidative Reactions:

1) $Serine + 2H_2O \rightarrow Acetate^- + HCO_3^- + NH_4^+ + H^+ + 2[H]$
2) $Alanine + 3H_2O \rightarrow Acetate^- + HCO_3^- + NH_4^+ + H^+ + 4[H]$
3) $Leucine + 3H_2O \rightarrow Isovalerate^- + HCO_3^- + NH_4^+ + H^+ + 4[H]$
4) $Isoleucine + 3H_2O \rightarrow 2 - Methylbutyrate^- + HCO_3^- + NH_4^+ + H^+ + 4[H]$
5) $Valine + 3H_2O \rightarrow Isobutyrate^- + HCO_3^- + NH_4^+ + H^+ + 4[H]$
6) $Phenylalanine + 3H_2O \rightarrow Acetate^- + HCO_3^- + NH_4^+ + H^+ + 4[H]$
7) $Tryptophane + 3H_2O \rightarrow Indolacetate^- + HCO_3^- + NH_4^+ + H^+ + 4[H]$
8) $Histidine + 3H_2O \rightarrow Glutamate^- + HCO_3x^- + NH_4^+ + H^+ + 4[H]$
9) $Glutamate^- \rightarrow Propionate^- + 2HCO_3^- + NH_4^+ + H^+ + 4[H]$
10) $Glutamate^- \rightarrow 2Acetate^- + HCO_3^- + NH_4^+ + H^+ + 2[H]$
11) $Aspartate^- \rightarrow Acetate^- + 2HCO_3^- + NH_4^+ + H^+ + 2[H]$
12) $Glycine \rightarrow 2HCO_3^- + NH_4^+ + H^+ + 6[H]$
13) $Threonine \rightarrow Propionate^- + NH_4^+ + H^+ + 2[H]$

Reductive Reactions

14) $Glycine + 2[H] \rightarrow Acetate^- + NH_4^+$
15) $Proline + 2[H] \rightarrow 5 - Aminovalerate$
16) $Leucine + 2[H] \rightarrow 4 - Methylvalerate^- + NH_4^+ + H^+$
17) $Phenylalanine + 2[H] \rightarrow Phenylpropionate^- + NH_4^+ + H^+$
18) $Tryptophane + 2[H] \rightarrow Indolpropionate^- + NH_4^+ + H^+$
19) $2HCO_3^- + H^+ + 8[H] \rightarrow Acetate^-$
21) $Acetate + HCO_3^- + 6[H] \rightarrow Propionate^-$
22) $2\ Acetate^- + H^+ + 4[H] \rightarrow Butyrate^-$
23) $2[H] \rightarrow H_2$
24) $HCO_3^- + 2[H] \rightarrow formate^-$

in the conversion to acetate are used to form butyrate from acetate (45,46). However, in this case no externally supplied acetate is needed for growth.

In methanogenic environments amino acid degrading microorganisms may also dispose off their reducing equivalents by formation of hydrogen and formate (47,48,28). This means that methanogens can take over the function of the reductive conversions listed in Table 5. There are several examples of syntrophic degradation of amino acids by methanogenic consortia. Nagase and Matsuo (49) observed that in mixed methanogenic communities the degradation of alanine, valine, and leucine was inhibited by inhibition of methanogens, while Nanninga and Gottschal (50) could stimulate the degradation of these amino acids by the addition of hydrogen-scavenging sulfate-reducers. *Clostridium sporogenes* (51,52), *Eubacterium acidaminophilum* (53), *Acidaminobacter hydrogenoformans* (54), and *Selenomonas (Thermanaerovibrio) acidaminovorans* (55) are examples of bacteria, which can grow syntrophically on one or more of the amino acids aspartate, alanine, leucine, isoleucine, or valine.

The initial step in the degradation of alanine, valine, leucine, and isoleucine is an NAD(P)-dependent deamination to the corresponding ketoacid. The $\Delta G^{0'}$ of this reaction when coupled to hydrogen formation is about +60 kJ/mol. Thus, methanogens are needed to pull this reaction in a fashion similar to that described for sugar fermentations. The ketoacid is converted further probably by a ferredoxin-dependent oxidative decarboxylation to a fatty acid, a reaction that energetically is much more favorable (the $\Delta G^{0'}$ is about −50 kJ/mol). Aspartate probably is not directly oxidized to oxaloacetate, but via fumarate and malate. In this conversion malate oxidation to oxaloacetate is most unfavorable; the $\Delta G^{0'}$ of this reaction is +47.7 kJ/mol.

The fermentation of glutamate to acetate and butyrate is well-known. The anaerobic oxidation of glutamate to acetate and propionate as shown in the following reactions is carried out by *A. hydrogenoformans* and some other glutamate-degrading microorganisms:

$$\text{Glutamate}^- + 3H_2O \rightarrow 2\text{acetate}^- + HCO_3^- + H^+ + NH_4^+ + H_2\Delta \quad G^{0'} = -33.9\text{kJ}$$

$$\text{Glutamate}^- + 4H_2O \rightarrow \text{propionate}^- + 2HCO_3^- + NH_4^+ + H^+ + 2H_2 \quad \Delta G^{0'} = -5.8\text{kJ}$$

These fermentations are less known, but in anaerobic environments with a high methanogenic activity, in particular, these glutamate degradation pathways seem to be rather common.

Mineralization of Nucleic Acids

Hydrolysis of RNA and DNA results in the formation of C5-sugars ribose and deoxyribose, respectively, and purines and pyrimidines. Purines and pyrimidines are easily fermented under anaerobic conditions (Gottschalk, 1986). Guanine, adenine, and a number of other heterocyclic compounds such as hypoxanthine, urate, and xanthine can be fermented by *Clostridium* strains, including *C. acidiurici*, *C. purinolyticum*, and *C. cylindrosporum* (56,57). The fermentation of these compounds is selenium-dependent and a glycine-reductase plays a key-role (58). A number of bacteria can ferment pyrimidines, although they are not as readily degraded as purines. Uracil is converted to β-alanine, CO_2, and ammonium by *C. glycolicum* and *C. uracillum*, while *C. sporogenes* can transform cytosine and thymine (59). So far no research has been conducted to investigate the influence of methanogens on the anaerobic degradation of purines and pyrimidines.

Mineralization of Fats

Fats are cleaved into glycerol and long-chain fatty acids. Glycerol is easily fermentable, and in a few enzymatic steps can be converted to intermediates of the glycolytic pathway. Therefore it may be fermented in a similar fashion as sugars. However, it should be noted that the carbon in glycerol is more reduced than the carbon in sugars. As a result, more reduced organic products are formed from glycerol than from sugars. For example, if glycerol is fermented by propionic acid bacteria, it mainly yields propionate.

Long-chain fatty acids are degraded via so-called beta-cleavage (47). In a series of reactions acetyl groups are cleaved off yielding acetate and hydrogen in the case of even-numbered fatty acids, while uneven-numbered fatty acids are degraded to acetate, hydrogen, and propionate. The biochemical mechanism is that oxidation of long-chain fatty acids is first activated to a CoA derivative before further conversion takes place:

$$R{-}CH_2{-}CH_2{-}CH_2{-}COO^- + ATP + HS{-}CoA \rightarrow R{-}CH_2{-}CH_2{-}CH_2{-}CO{-}SCoA$$

$$R{-}CH_2{-}CH_2CH_2{-}CO{-}SCoA \rightarrow R{-}CH_2{-}CH = CH{-}CO{-}SCoA + 2[H]$$

$$R{-}CH_2{-}CH = CH{-}CO{-}SCoA + H_2O \rightarrow R{-}CH_2{-}CHOH{-}CH_2{-}CO{-}SCoA$$

$$R{-}CH_2{-}CHOH{-}CH_2{-}CO{-}SCoA \rightarrow R{-}CH_2{-}C = O{-}CH_2{-}CO{-}SCoA + 2[H]$$

$$R{-}CH_2{-}C = O{-}CH_2{-}CO{-}SCoA + HS{-}CoA + H_2O \rightarrow R{-}CH_2{-}CO{-}SCoA + CH_3{-}CO{-}SCoA$$

The smallest fatty acid that is degraded with such a mechanism in butyrate. The energetics of this conversion is discussed later. Oxidation of long-chain fatty acids to acetate and hydrogen is energetically difficult and can only occur by syntrophic consortia of hydrogen-producing acetogens and methanogens. Microorganisms that degrade long-chain fatty acids generally are also able to grow with butyrate.

Syntrophic Degradation of Propionate, Butyrate, and Other Fatty Acids

Propionate and butyrate are important products of sugar fermentation, while branch-chain fatty acids

are formed during amino acid degradation. The fatty acids are degraded by obligate syntrophic consortia of bacteria.

The existence of obligately syntrophic degradation by interspecies electron transfer was recognized for the first time by Bryant and coworkers (60). It was observed that the originally believed pure culture of the ethanol-degrading *Methanobacillus omelianskii* consisted of two bacterial species, the S-organism that degraded ethanol to acetate and the methanogen strain M.o.H. However, as mentioned earlier, ethanol can also be fermented in other ways. Also, degradation of propionate and butyrate requires the syntrophic cooperation of acetogenic bacteria and hydrogenotrophic and aceticlastic methanogens.

Boone and Bryant (61) described *Syntrophobacter wolinii*, an organism that can only grow in syntrophic cooperation with methanogens or sulfate-reducing bacteria. Since then several other *Syntrophobacter* species have been described (62,63). Recently, *Smithella propionica* has been described (64). This organism forms butyrate during propionate degradation. The pathway of propionate degradation in this microorganism is different from the pathway in *Syntrophobacter* species (65).

McInerney and coworkers (48) enriched and characterized *Syntrophomonas wolfei*, a bacterium that degraded butyrate and some other short-chain fatty acids syntrophically. Several other butyrate-oxidizing bacteria such as the spore-forming bacterium *Syntrophospora bryantii* (former *Clostridium bryantii*) (66,67) and *Syntrophomonas sapovorans* (68) were described. The latter bacterium can oxidize butyrate and saturated and unsaturated long-chain fatty acids.

During propionate and butyrate degradation, methanogens are needed to remove the products, which negatively affect the energetics of their oxidation to acetate and hydrogen (Fig. 4).

$$\text{Propionate}^- + 3H_2O \rightarrow \text{Acetate}^- + HCO_3^- + H + 3H_2$$

$$\Delta G^{0'} = +76.1 \text{ kJ}$$

$$\text{Butyrate}^- + 2H_2O \rightarrow 2\text{Acetate}^- + H^+ + 2H_2$$

$$\Delta G^{0'} = +48.1 \text{ kJ}$$

$$4H_2 + 2HCO_3^- + H^+ \rightarrow \text{acetate}^- + 4H_2O$$

$$\Delta G^{0'} = -104.6 \text{ kJ}$$

$$\text{Acetate}^- + H_2O \rightarrow HCO_3^- + CH_4 \quad \Delta G^{0'} = -31.0 \text{ kJ}$$

The pathways of propionate and butyrate oxidation by *Syntrophobacter* and *Syntrophomonas* species have been resolved (27,28). During propionate oxidation via the so-called methylmalonyl-CoA pathway, reducing equivalents are formed in the oxidations of succinate to fumarate, malate to oxaloacetate, and pyruvate to acetyl-CoA; these reducing equivalents are formed at the level of $FADH_2$, NADH, and ferredoxin. Butyrate is oxidized via β-oxidation. In this pathway reducing equivalents are formed at the level of $FADH_2$ in the oxidation of butyryl-CoA to crotonyl-CoA and at the level of NAD in the oxidation of 3-hydroxybutyryl-CoA to acetoacetyl-CoA. In particular, coupling the oxidation of $FADH_2$ to proton reduction is energetically problematic in these organisms. The redox couple of $FADH_2$/FAD is -220 mV. The Gibbs free energy change of $FADH_2$ oxidation coupled to proton reduction is $+37.4$ kJ.

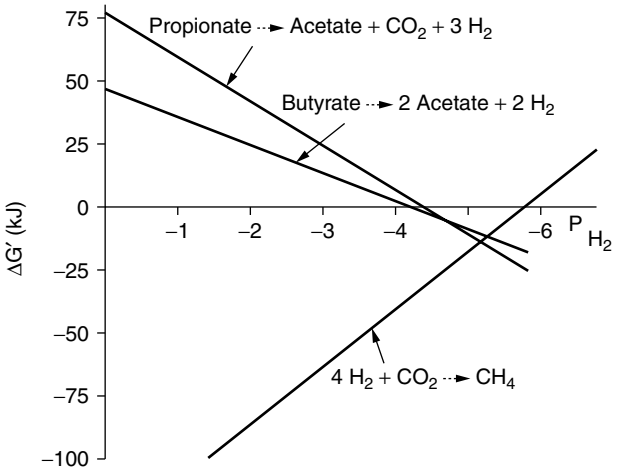

Figure 4. $\Delta G'$ values of propionate and butyrate oxidation and methanogenesis from $H_2 + CO_2$ at different PH_2 values.

$$FADH_2 \rightarrow FAD + H_2 \quad \Delta G^{0'} = +37.4 \text{ kJ}$$

Even at a hydrogen partial pressure of 1 Pa, a value which can be created by methanogens, this reaction is not feasible. The Gibbs free energy change is still positive. Therefore, propionate and butyrate-oxidizing bacteria have to invest metabolic energy to push this reaction. The biochemical mechanism involved has been explained later in detail (27,28).

Branched chain fatty acids such as isobutyrate and isovalerate are degraded by syntrophic consortia as well. The thermodynamic principles are the same as those described for butyrate, and the same types of microorganisms are involved in their degradation (66).

THE ROLE OF CELL DEATH AND CELL LYSIS DURING THE METHANOGENIC DIGESTION OF ACTIVATED SLUDGE

For anaerobic digestion of activated sludge, an additional factor has to be included because hydrolysis is preceded by the death and lysis of the aerobic biomass. This is illustrated by the conceptual model, which was proposed by Pavlostathis (69; Fig. 5), in which the biomass in activated sludge consists of viable and nonviable cells. When the cells are viable, the particulate and soluble biological oxygen demand (BOD) that resides in these cells, proteins, carbohydrates, and nucleic acids are not available for hydrolysis. Upon death of the cells the soluble BOD is either immediately released into the bulk fluid or leaks slowly from the cells by diffusion. This soluble BOD probably contains macromolecular components that require hydrolysis before they can be converted by acidogenic bacteria. However, the hydrolysis of soluble macromolecular components is very fast and in the model, soluble BOD is considered directly available for the acidogenic microorganisms. The particulate BOD

either is hydrolyzed intracellularly by the cell's own enzymes or is released to the bulk fluid where it is then hydrolyzed.

Because the conceptual model (Fig. 5) is very complex and difficult to verify it has been simplified (69,70; Fig. 6). For verification of this model Pavlostathis and Gossett (70) used a "biological" sludge. This "biological" sludge was generated in a fill-and-draw activated sludge reactor fed with synthetic wastewater. The wastewater contained nutrients and trace elements, and glucose and peptone were the carbon source. The reactor was operated at a sludge loading of approximately 0.25 ggCOD/g VSS/day, 20 °C, a hydraulic retention time of two days and a sludge retention time of 10 days. Several continuous and batch experiments were conducted with this sludge (69,70).

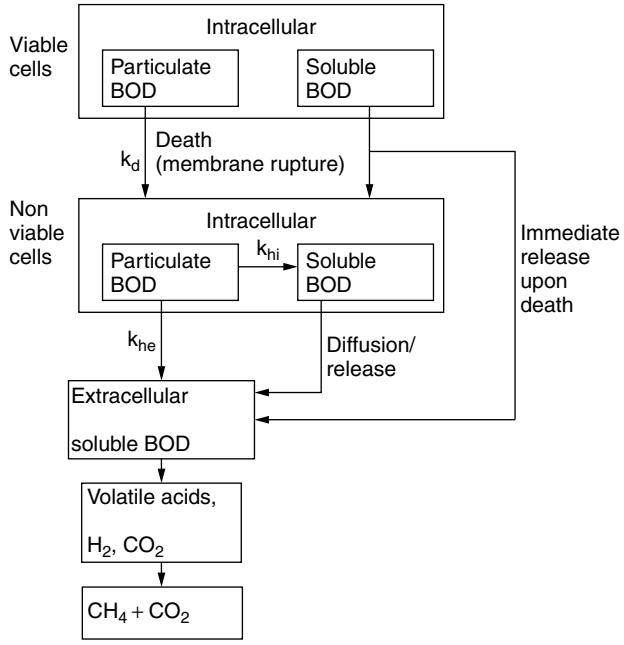

Figure 5. Conceptual model for activated sludge digestion (69).

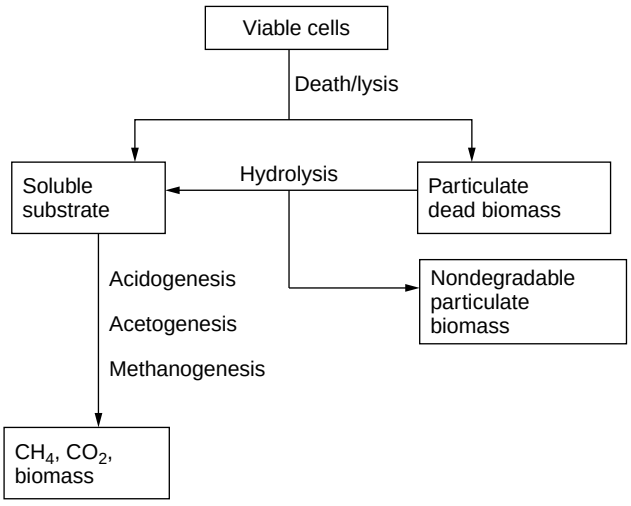

Figure 6. Simplified model for activated sludge digestion (70).

During continuous digestion of the sludge at 35 °C and a solid retention time of 1 to 23 days, degradability of the sludge was low. Only a maximum of 35% of the sludge could be converted to biogas. Moreover, from the validation of the model, it was shown that death/lysis (Fig. 6) was the rate-limiting step during the conversion of the biodegradable part of the sludge. Because the sludge was generated with a wastewater that it, all contained no refractory components it can be concluded that the refractory part of the waste activated sludge is formed during the activated sludge process. Incorporation of refractory material from the influent of the activated sludge plant into activated sludge seems to be of less importance.

During anaerobic digestion or anaerobic storage of activated sludge changes also occur with respect to the structure of activated sludge. Rasmussen and coworkers (15) observed an increase of divalent calcium ions in the water phase during the anaerobic storage of activated sludge. Moreover, Nielsen and coworkers (17) observed a decrease in the amount of capsular and slime polymers from 369 to 299 mg polymer/g volatile solids during a 12-day storage of activated sludge under anaerobic conditions at 20 °C. As the polymers and Ca^{2+} are believed to form the matrix that serves as the backbone of the activated sludge floc, it appears that the floc structure falls apart during anaerobic storage or digestion.

IMPROVING BIOGAS YIELDS

The usually applied one-stage mesophilic digestion of waste-activated sludge gives only a low biogas yield. Because most of the refractory material in activated sludge from low loaded WWTPs (Table 2) seems to be inactive, except unlysed biomass, attempts to improve the biogas yield have focused on lysis of this biomass. To destroy the cell wall of bacteria, different methods are used. Mechanical methods such as sonication and high-pressure homogenization, stirred ball mill or shear-gap homogenizer (71). Chemical pretreatment methods such as alkaline treatment (72) and thermal pretreatment have also been the topics of several investigations (73,74). Often combinations of chemical, mechanical, or thermal pretreatment have been investigated (75,72). Moreover, some authors report that a two-stage digestion might also improve the biogas yield from WAS, in which the low pH in the first stage (acidic) is believed to improve the death and lysis of the bacteria (76,77).

Mechanical Pretreatment

Müller and coworkers (71) investigated several mechanical disintegration methods to improve lysis of biomass in activated sludge. The specific energy input and the disintegration rate of a stirred ball mill, a high-pressure homogenizer, an ultrasonic homogenizer and a shear-gap homogenizer were compared. The specific energy input was defined as the amount of mechanical energy that disrupts a certain amount of sludge (in kJ/kg dry solids). The disintegration rate was defined as the oxygen consumption rate of the pretreated sludge relative to its consumption by an untreated sludge sample. Muller and

coworkers (71) reported that all disintegration methods, except the shear-gap homogenizer, gave a disintegration rate of more than 80% (this implied that more than 80% of the active biomass in the WAS was disintegrated efficiently). The high-pressure homogenizer and the stirred ball mill were selected as the most economical methods as they required the lowest specific energy input to achieve more than 80% disintegration.

All experiments were conducted with WAS with a sludge age of 3 and 13 days. After disintegration the WAS was digested at 35 °C in continuous flow experiments with a (HRT) of 2 to 23 days. The results of the digestion are depicted in Table 6. At a sludge age of three days the degradation of the disintegrated WAS was 8 to 10% higher than the untreated WAS, irrespective of the HRT of the reactor. At a HRT time of 17 days 55% and 63% of the WAS were hydrolyzed for the untreated and pretreated sludge, respectively.

The maximum degradation of the WAS with a sludge age of 13 days was much lower. The untreated and pretreated WAS both reached a maximum hydrolysis of approximately 48% at HRTs of 23 and 17 days, respectively. Moreover, the effect of the disintegration was dependent on the retention time of the digester. At a hydraulic retention time of 2.5 days the degree of degradation of the treated sludge was 15% higher than that of untreated sludge, but at a HRT of 17 days the effect of the disintegration was negligible. This indicated that, although mechanical disintegration destroys the active biomass in the activated sludge, most of the biomass still remains refractory.

Thermal Pretreatment

Pretreatment of the WAS at high temperatures can be applied to enhance death and lysis of the WAS. The method that has received most attention is the exposure of the WAS for short periods (20–60 min) to high temperatures (60–225 °C) and subsequent digestion at mesophilic temperatures.

Haug and coworkers (74) treated WAS for 30 minutes at 100 to 225 °C and subsequently digested it in a CSTR at 35 °C, organic loading rate 2.6 g/l.d, and an SRT of 15 days. At 175 °C, the highest WAS conversion was reached; a 56% higher degree of degradation was found than with the untreated WAS. At pretreatment temperatures higher than 175°C, methane production was inhibited, probably by toxic compounds from the pretreated sludge. Brons (73) investigated the effect of pretreatment at 80 to 175 °C on the batch digestion at 30 °C. After 32 days of digestion the highest gas production was observed in the digester with the WAS pretreated at 145 °C; 60% more methane was produced than that for untreated WAS. At 175 °C, gas production was only 51%, probably because of the same toxic effect as observed by Haug and coworkers (74) Hiroaka and coworkers (20) investigated lower pretreatment temperatures. After treatment of the WAS at 60 to 100 °C the sludge was digested at 35 °C. The highest methane production from WAS was gained at a pretreatment temperature of 60 °C, at which 30% more methane was formed, compared with untreated WAS. Hiroaka and coworkers concluded that at pretreatment temperatures below 100 °C, gas production from WAS was enhanced by a higher gas yield from the biodegradable fraction of the WAS. At pretreatment temperatures above 100 °C an increase in biodegradability of the sludge increased methane production.

Chemical Pretreatment

Alkaline pretreatment can be performed by sodium hydroxide or lime. It was reported that sodium hydroxide

Table 6. Results of Different Pretreatment Methods on the Solubilization and/or Subsequent Hydrolysis During the Anaerobic Digestion of WAS

Reference	Sludge	Pretreatment Method	Solubilization (%)	Digestion Conditions	Hydrolysis During Digestion (%)
71	WAS (age 3 d)	HPH or SBM		35 °C	55 to 60% (3d)
	WAS (age 13 d)			HRT 17 days	48% (13d)
74	WAS	175 °C, 30 minutes		35 °C	56% higher than untreated
				HRT 15 days	
73	WAS	145 °C,		30 °C, batch	60% higher than untreated, after 32 days
20	WAS	60 °C		35 °C	30% higher than untreated
72	1% WAS	40 meq/L NaOH	28 to 42		
		40 meq/L NaOH + Ultrasound*	70 to 89		
74	WAS	60 meq/L NaOH	15		
		115 to 150 °C, 5 minutes	15		
		180 °C, 5 minutes	30		
		34 meq/L NaOH +130 °C, 5 minutes	45		

*120 Watt, 20 KHz, 13.5 to 14.4 sec/ml.

Note: HPH = high-pressure homogenizer, SBM = stirred ball mill.

yields a greater solubilization efficiency than lime (78). A 12 to 24 hour pretreatment of 1% WAS with 40 meq/L NaOH resulted in a soluble COD over a total COD ratio of 28 to 42%. However, combined with a mechanical method, such as Ultrasound (120 W, 20 KHzKHz13.5 to 14.4 sec/ml) the solubilization of WAS could be enhanced to 77.9 to 89.0% (72).

DEWATERING OF ANAEROBICALLY DIGESTED ACTIVATED SLUDGE

In general, the two main reasons for digestion of excess sludge from WWTPs are stabilization and volume reduction. For volume reduction, it is assumed that not only is the amount of solids reduced but digestion also has a positive effect on the dewatering characteristics of the sludge. However, this assumption only seems to hold for the digestion of primary sludge. Lawler and coworkers (2) digested several mixtures of primary and activated sludge in CSTRs at a retention time of 10 days at 25 °C. The results indicated that the dewaterability, measured as the specific capillary suction time, became worse when the amount of activated sludge increased above 40% of the digester influent (Fig. 7). Lawler and coworkers (2) attributed this to the occurrence of particles between 2 and 9 μm. Increase of Ca^{2+} concentration in the water phase and degradation of the exopolymers are indications of the disintegration of the structure of the activated sludge floc (15,17). Because of the formation of small aggregates or suspended bacteria the flow of free water from the sludge during filtration tests, such as the measurement of the capillary suction time, is obstructed. Moreover, the results of Palmgren and coworkers (79) indicate that during oxygen-limiting circumstances the surface of the activated sludge becomes less hydrophobic. This effect could increase the amount of *vicinal* water in the sludge and as this cannot be removed by mechanical dewatering techniques. An increase in the amount of sludge will result in a lower dry weight of the sludge after dewatering.

When the activated sludge is pretreated before the digestion, the cells are lysed, the floc structure is destroyed, and numerous small particles arise. Kopp and coworkers (80) and Muller and coworkers (71) investigated

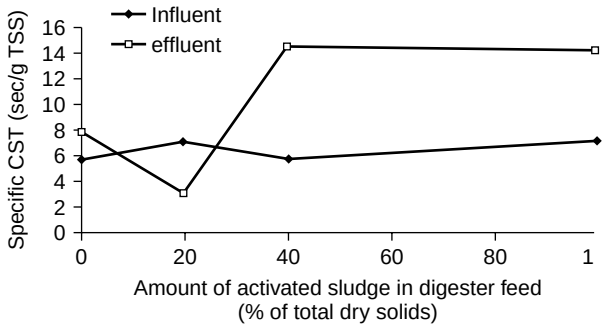

Figure 7. Effect of the fraction activated sludge in the digester feed on the dewatering characteristics of the digested sludge. (D. F. Lawler, Y. J. Chung, S. –J. Hwang, B. A. Hull, J. WPCF 58(12), 1,107–1,117 (1986)).

the relation among the degree of disintegration, the dry matter content of the dewatered sludge, polymer demand, and anaerobic digestion. They showed that sludge age of the WAS affected disintegration and subsequent digestion and the dewatering characteristics of the sludge. In experiments with WAS of 13-day sludge age, the dry matter content of the centrifuge cake decreased with increasing degree of disintegration. Moreover, more synthetic polymer was needed to obtain the maximum dry matter of the centrifuge cake. However, this negative effect of the disintegration decreased with increasing retention time in the digester. At an SRT of 17 days in the digester (35 °C) the dry matter content for both the untreated and disintegrated sludges became equal, although the disintegrated sludges still required twice as much synthetic polymer as the untreated sludge to reach maximum sludge concentration. Results obtained with three-day-old sludge were different from those obtained by using 13-day old sludge. With the former, a much higher dry solids content of the centrifuge cake could be obtained by disintegration. Immediately after disintegration, a dry solid content of about 5.2% could be achieved for the untreated sludge. For sludge that was pretreated by a high-pressure homogenizer, a dry solid content of about 9.5% could be obtained, although this required almost 10 times as much synthetic polymer (42 g/kg SS, Zetag-87 Allied Colloids). This trend persisted even when the sludge was digested, although these were fewer differences between untreated and disintegrated sludges became smaller. After 15 days of digestion, the dry solids content of the centrifuge cake was about 6% and 6.8% for the untreated and pretreated sludge, respectively. Polymer demand of pretreated sludge decreased dramatically, resulting in a demand of 10 g/kg SS and 15 g/kg SS for untreated and pretreated sludge, respectively.

CONCLUSION

In the anaerobic decomposition of waste activated sludge (WAS) microbial cells form the substrate of anaerobic methanogenic consortia. This process is applied to stabilize primary and secondary sludge from wastewater purification plants. Additionally, in this process a major part of the chemical COD is converted into biogas, which can be applied to operate the purification plant.

Technologically, key factors in this digestion process are the death and lysis of the cells, and the dewatering capacity of the sludge. Much of this depends on the sludge age of the activated sludge, as the viable fraction of the biomass seems to decrease with increasing sludge age (Table 2). The death and lysis of the biomass can be increased by chemical and mechanical disintegration of the sludge before the digestion. However, this pretreatment will result in a higher polymer demand during dewatering of the digested sludge.

Microbiologically, the decomposition of WAS is intriguing. In this methanogenic process complete degradation of organic biopolymers is achieved by a concerted action of a mixed microbial community consisting of fermentative bacteria, proton-reducing acetogenic bacteria, and methanogenic archaea. These microorganisms act in a

syntrophic food web. Metabolic interactions by means of interspecies hydrogen transfer are important in anaerobic digestion. Hydrogen consumption by hydrogenotrophic methanogens affects the metabolism of those sugar- and amino acid-fermenting anaerobes, which have the ability to dispose reducing equivalents as molecular hydrogen. In addition, the anaerobic oxidation of propionate, butyrate that are formed by fermenting bacteria, and the oxidation of long-chain fatty acids can only be carried out by hydrogen-producing acetic bacteria in coculture with hydrogenotrophic anaerobes. Stabilization of WAS is an important part of the conventional purification of municipal wastewater. It seems that in the near future aerobic purification of municipal wastewater remains the main purification process. However, in the long term it might be that other wastewater purification systems become more important. For the purification of industrial wastewater and for the treatment of municipal wastewater in tropical regions, an anaerobic treatment followed by an aerobic posttreatment seems the most appropriate technology.

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BIOSOLIDS, BIOAEROSOLS FROM.

See Wastewater and Biosolids as Sources of Airborne Microorganisms

BIOSOLIDS, GRANULES IN.

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BIOSORBENTS FOR METALS.

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BIOSTIMULATION, AQUIFERS.

See Bioaugmentation

BIOSTIMULATION: COLD-ADAPTED MICROBES.

See Use of Cold-Adapted Microorganisms in Biotechnology

BIOSTIMULATION, HOT DESERT SOILS.

See Hot Desert Soil Microbial Communities

BIOSURFACTANTS: TYPES, SCREENING METHODS, AND APPLICATIONS

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This article discusses biosurfactants and bioemulsifiers, microbially produced molecules that either reduce surface or interfacial tension, or act as emulsifying agents, or have both of these properties. Whereas certain physicochemical properties are shared among biosurfactants, this group of compounds exhibits great variety and uniqueness with respect to structure and properties. This combined with their environmental compatibility has created an interest in biosurfactants for application to many facets of the surfactant industry ranging from biotechnology to environmental cleanup. Currently the surfactant industry, which exceeds $ 9 billion per year, markets predominantly synthetic surfactants. Biosurfactants and bioemulsifiers are produced by a number of bacteria, yeasts, and fungi when cultivated on different carbon sources. The type of carbon source utilized ranges from simple sugars or

hydrocarbons, such as glucose or hexadecane, to complex mixtures, such as petroleum. It is important to point out that both substrate and growth conditions dictate the quantity and structure of biosurfactant/emulsifier obtained from a microorganism. In this article, we define the terms biosurfactant and bioemulsifier, and then discuss the types produced, methods used for screening and purification, synthesis, and potential industrial and biotechnology applications.

DEFINITION OF BIOSURFACTANTS AND BIOEMULSIFIERS

Biosurfactants

All biosurfactants have amphiphilic structures, containing at least one hydrophilic and one hydrophobic moiety. The hydrophilic moiety can be an ester, hydroxyl, phosphate, carboxyl, or carbohydrate group and is either neutral or negatively charged. There have been no cationic biosurfactants reported, presumably because cationic surfactants are quite toxic. The hydrophobic moiety is a fatty acid ranging in size from 8 to 18 carbons.

Because of their amphiphilic nature, surfactants tend to accumulate at interfaces (air–water and oil–water) and surfaces. As a result, surfactants reduce the forces of repulsion between unlike phases at interfaces or surfaces and allow the two phases to mix more easily. Specifically, surfactants can reduce surface (liquid–air) and interfacial (liquid–liquid) tension. For example, a proficient biosurfactant can reduce the surface tension between pure water and air from 73 to less than 30 mN/m. As surfactant monomers are added into solution, the surface or interfacial tension will decrease until the surfactant concentration reaches what is known as the critical micelle concentration (CMC). Above the CMC no further reduction in surface or interfacial tension is observed. At the CMC, surfactant monomers begin to spontaneously associate into structured aggregates such as micelles, vesicles, and lamellae (continuous bilayers) (Fig. 1). These aggregates form as a result of numerous weak chemical interactions between the polar head groups and the nonpolar tail groups, including hydrophobic, van der Waals, and hydrogen bonding. The CMC for any surfactant is dependent on the surfactant structure as well as the pH, ionic strength, and temperature of the solution. Further, the aggregate structure is dictated by the polarity of the solvent in which the surfactant is dissolved. For example, in an aqueous solution, the polar head groups of a micelle will be oriented outward toward the aqueous phase and the hydrophobic tails will associate in the core of the micelle (oil-in-water micelle). In contrast, in oil the polar head groups will associate in the center of the micelle, whereas the hydrophobic tails will be oriented toward the outside (water-in-oil micelle).

Bioemulsifiers

An emulsion is a stable mixture of two liquids. Normally an oil and water mixture forms separate phases. However, if the mixture is shaken vigorously, the oil will disperse into the water phase. When the shaking is discontinued, the phases quickly separate. One way to achieve a stable mixture or emulsion is to add a surfactant. The two most common types of emulsions are oil-in-water and water-in-oil. For an oil-in-water emulsion, the water phase is continuous whereas the oil phase is composed of discrete droplets. Since emulsions represent large interfacial areas,

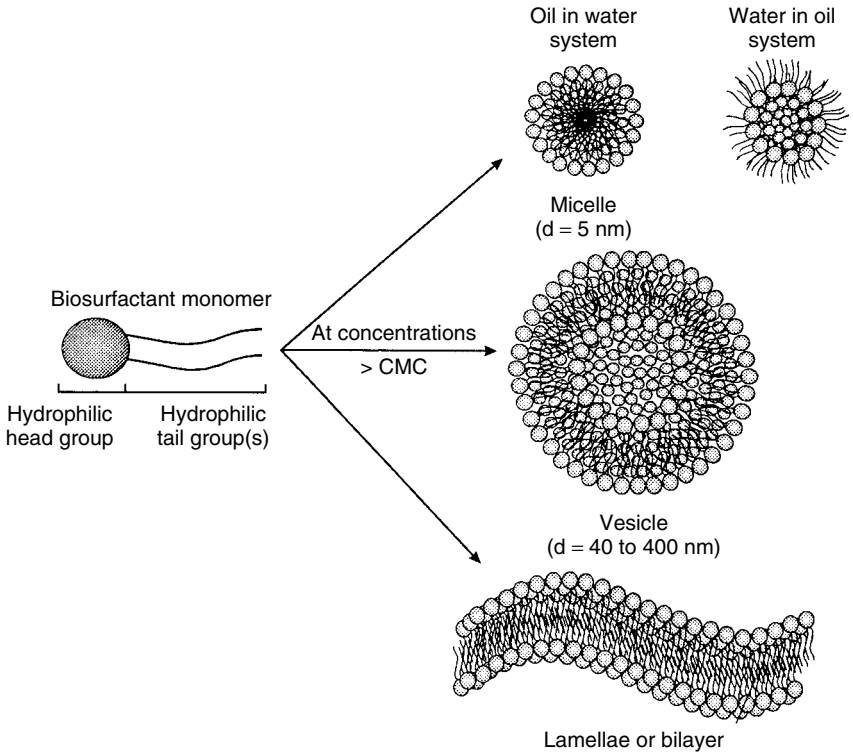

Figure 1. The types of aggregates formed by biosurfactants.

any reduction in interfacial tension caused by addition of a surfactant will promote stability of the emulsion. Although some bioemulsifiers decrease interfacial tension, others have little impact on interfacial tension. These emulsifiers are effective because they form films on the surface of the oil droplets in the aqueous phase. Once these films are formed, it is difficult to eject the emulsifier from the surface and therefore the oil droplets cannot coalesce, allowing a stable emulsion to be maintained. The length of time that an emulsion remains stable reflects the effectiveness of the emulsifier. After some period of time, the droplets in most emulsions will flock together and coalesce, ultimately causing separation of the two phases.

TYPES OF BIOSURFACTANTS AND BIOEMULSIFIERS

Biosurfactants and bioemulsifiers are classified based on their major structural features and the organism that produces them. The major groups include: (*1*) glycolipids; (*2*) phospholipids, neutral lipids, and fatty acids; (*3*) lipopeptides and lipoproteins; and (*4*) polymeric biosurfactants. There are many reviews of biosurfactants, including several recent ones (1–3).

Glycolipids

The most widely studied biosurfactants are the glycolipids. Glycolipids have a carbohydrate group attached to one or more long-chain aliphatic acid or hydroxy-aliphatic acid groups, and range in molecular weight from 500 to 1,500. The best-studied glycolipids include rhamnolipids, trehalose lipids, sophorolipids, and a new group, mannosylerythritol lipids. Other types of glycolipids, such as cellobiose lipid (4), glucose lipid (4,5), glycoglycerolipid (6), sugar-based bioemulsifiers (7–9), and many different hexose lipids (10–14), have been reported in the literature. These glycolipids are produced by a wide variety of microorganisms.

Rhamnolipids. The best-studied glycolipid is rhamnolipid (Fig. 2). Rhamnolipid was isolated from *Pseudomonas aeruginosa* 141 and characterized by Jarvis and Johnson in 1949 (15). Edwards and Hayashi (16) elucidated the rhamnolipid structure by identifying the α-1,2-linkage between adjacent rhamnose moieties. Rhamnolipid is a glycolipid that has been shown to have up to six different anionic forms ($pK_a \sim 4.6$) (7). The most abundant are the R1 and R2 forms, which contain one and two rhamnose sugars, respectively, and have two β-hydroxy fatty acid tail groups, most commonly β-hydroxydecanoic acid (18). Much less abundant are R1 and R2 forms with three β-hydroxy fatty acid tail groups (19). The R3 and R4 forms contain only one β-hydroxy fatty acid tail group (20–22). In addition to these anionic forms of rhamnolipids, there have been reports of neutral or methylesters of these rhamnolipid forms (23–25). Rhamnolipids reduce surface tension to less than 30 mN/m, and reduce interfacial tension to 1 mN/m in hexadecane/water (25–28). The CMC of rhamnolipid varies depending on the mixture of rhamnolipids produced, ranging from 20 to 100 mg/L. The average

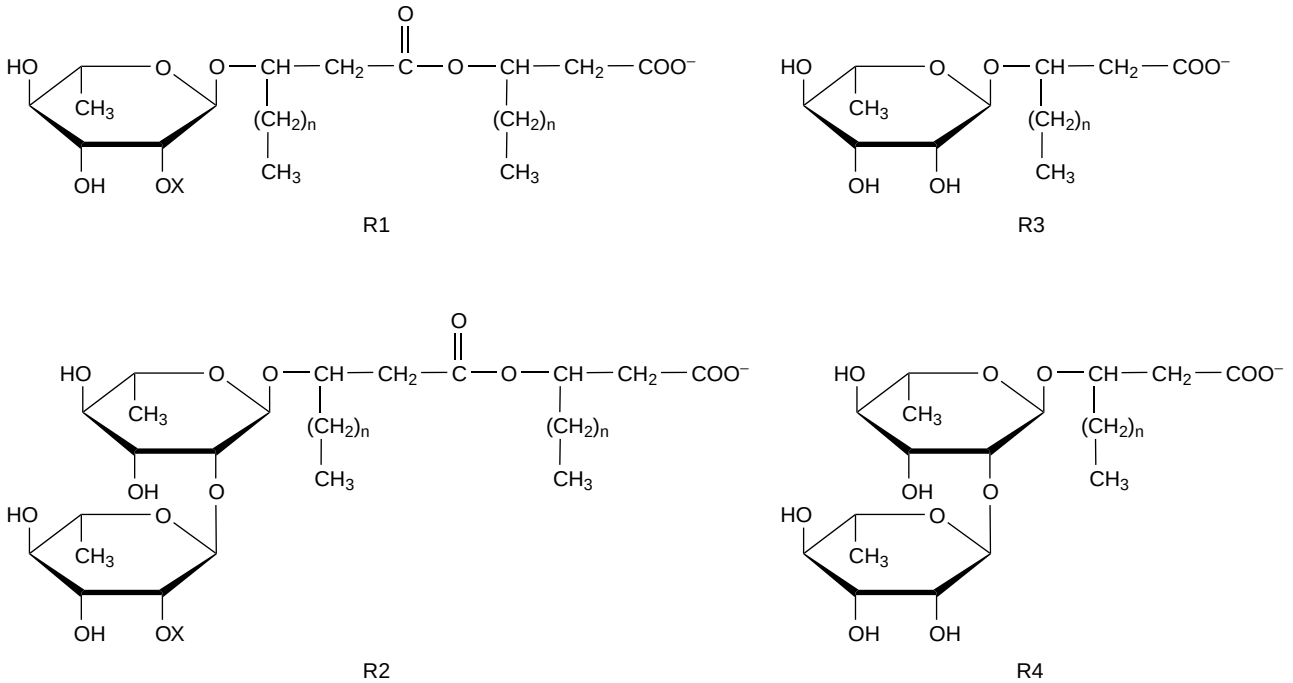

Figure 2. The six major types of rhamnolipid produced by *P. aeruginosa*. Most common are R1 (rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate) and R2 (rhamnosyl-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate) where $\mathbf{X} = \mathrm{H}$. There are two much less common forms of R1 and R2 wherein $\mathbf{X} =$ hydroxydecanoate. R3 and R4 are also less common, where R3 is rhamnosyl-β-hydroxydecanoate and R4 is rhamnosyl-rhamnosyl-β-hydroxydecanoate (29). In all cases $n = 4$ to 8.

molecular weight of rhamnolipid mixtures varies between 400 and 700 g/mol.

Rhamnolipid production is limited to *P. aeruginosa* strains. There are a few early reports that state that other *Pseudomonas* species produce rhamnolipid; however, it is likely that at the time these isolates were not accurately identified to the species level. Rhamnolipids are produced during the late log and stationary phases on a variety of carbon sources, including glucose (30–32), molasses (33), ethanol (34,35), vegetable oils (13,36,37), glycerol (21,38), *n*-alkanes (20,39–42), polyaromatic hydrocarbons (43,44), and mixtures of the above (45). Limiting addition of inorganic nutrients, including phosphate, nitrogen, and iron, has been reported to increase production of rhamnolipid (30,46,47). Reported yields of rhamnolipid range from 0.25 to 100 g/L (35,48). The highest yields have been obtained with continuous batch-fed or fermenter systems using mixed carbon sources such as glucose and hexadecane (35). Currently, rhamnolipid is commercially available from Jeneil Biosurfactant Co., LLC (Saukville, Wisconsin).

Parts of the rhamnolipid biosynthetic pathway and its regulation have been described (49,50). Rhamnolipid (R1) is formed from deoxy-thymidine-diphospho-L-rhamnose (dTDP-L-rhamnose) and β-hydroxydecanoyl-β-hydroxydecanoate (β-H) (Fig. 3). It is thought that the *algC* and the *rml* genes used to produce dTDP-L-rhamnose for the O-antigen in lipopolysaccharide (LPS) are also responsible for formation of dTDP-L-rhamnose used in rhamnolipid synthesis. The *rhlG* gene, which is homologous to reduced nicotinamide adenine dinucleotide phosphate–dependent ketoacyl reductase, is responsible for obtaining fatty acid precursors from the normal fatty acid biosynthetic pathway for synthesis of the β-H component of rhamnolipid (50). It is not yet clear how the *rml* and *rhlG* genes are regulated with respect to rhamnolipid synthesis. The *rhlA* and *rhlB* genes encode the enzyme rhamnosyl transferase I, which catalyzes the addition of dTDP-L-rhamnose to β-H forming R1. Rhamnosyltransferase II, encoded by the *rhlC* gene, adds a second dTDP-L-rhamnose to the R1 molecule to form R2. It is currently not known how *rhlC* is regulated or where RhlC is located in the cell.

The synthesis of R1 is under the regulation of the RhlR-RhlI quorum-sensing system (Fig. 4) which is coordinately regulated at both the transcriptional and posttranslational levels with a second quorum-sensing system, LasR-LasI (51,52). Together, these two quorum-sensing systems are responsible for expression of a number of genes at high cell density and in response to stress. Other exoproducts produced include alginate, elastase, exotoxin A, protease, and pyocyanin. Clearly, the genetic regulatory network involved in the production of rhamnolipid is very complex and a full understanding of the regulation of rhamnolipid production remains to be determined.

Trehalose Lipids. Trehalose lipids exhibit great structural diversity. They can be nonionic or anionic and contain two to eight sugar groups and one to two mycolic acids. The molecular weight of these compounds ranges from 800 to 3,000. The three major types of trehalose lipids are α-trehalose-6-monomycolate, α,α-trehalose-6,6′-dimycolate, and trehalose-2,3,4,2′-tetraester (Fig. 5). However, a mixture of trehalose structures and/or other glycolipid structures are normally produced depending on substrate source and growth conditions (54). *Rhodococcus erythropolis* trehalose mycolates are among the best studied of the trehalose lipids. Nonionic trehalose lipids from this species reduce surface tension to 36 mN/m and interfacial tension to 16 mN/m against hexadecane. However, when this isolate is grown in nitrogen-limiting conditions, it produces anionic trehalose tetraesters, which further reduce surface tension to 26 mN/m and interfacial tension to less than 1 mN/m (55–58). The CMC for trehalose lipids ranges from 3 to 15 mg/L (58,59).

Trehalose lipid structures have been isolated from several bacterial genera, including *Rhodococcus* (now separated into *Gordona* and *Tsukamurella*), *Mycobacterium*, *Arthrobacter*, *Nocardia*, *Brevibacterium*, and *Corynebacterium* (60–68). Trehalose lipids can be secreted from the cell as is the case for some *Rhodococcus*, but are often found as an important constituent of the cell wall in genera such as *Mycobacterium*. In *Mycobacterium*, α,α-trehalose-6,6′-dimycolate (Fig. 5) also known as cord factor, is found at the cell wall surface and causes the microbe to grow in serpentine cords. In this organism, the cord factor has been implicated in virulence, wherein the presence of cord factor is related to the ability of the organism to protect itself against macrophages and to induce delayed-type hypersensitivity (69,70).

Trehalose lipids are produced during growth on hydrocarbons, including alkanes or vegetable oils, such as sunflower oil. Yields for trehalose lipids range from 2 to 40 g/L, somewhat less than yields reported for other glycolipids (17,57,67,68,71–74). Yield is dictated by the carbon source used for growth, nutrient availability, the method of production, and the microbial isolate used for production. For example, the highest yield reported (40 g/L) was obtained using *Rhodococcus* sp. strain SD-74 with *n*-hexadecane as a carbon source in a jar fermentor. The following seven parameters were optimized in this system: hexadecane concentration, ionic strength, phosphorous concentration and form, nitrogen concentration and form, metal ion concentration (e.g., iron), temperature, and aeration (17,71,72). Increased yields are still needed to make trehalose lipids economically competitive with synthetic surfactants. However, trehalose lipids have the potential for a variety of applications under a wide array of conditions. For instance *Tsukamurella* sp. 26A produces trehalose lipids with good emulsification activity, and this biosurfactant is stable over a wide pH and temperature range (67).

Sophorolipids. Sophorolipids were first isolated from the yeast *Torulopsis mangnoliae* (75). Since then the taxonomical classification of *Torulopsis* has changed and several *Torulopsis* species now belong to the genus *Candida*, including *T. bombicola*, *T. magnoliae*, *T. apicola*, and *T. gropengiesseri*. Other species of *Candida* known to produce sophorose lipids include *C. apicola*, *C. magnolia*, and *T. petrophilum* (76–78). The chemical structure of sophorolipid is a sophorose sugar dimer linked to one of several fatty acids (Fig. 6). The molecular

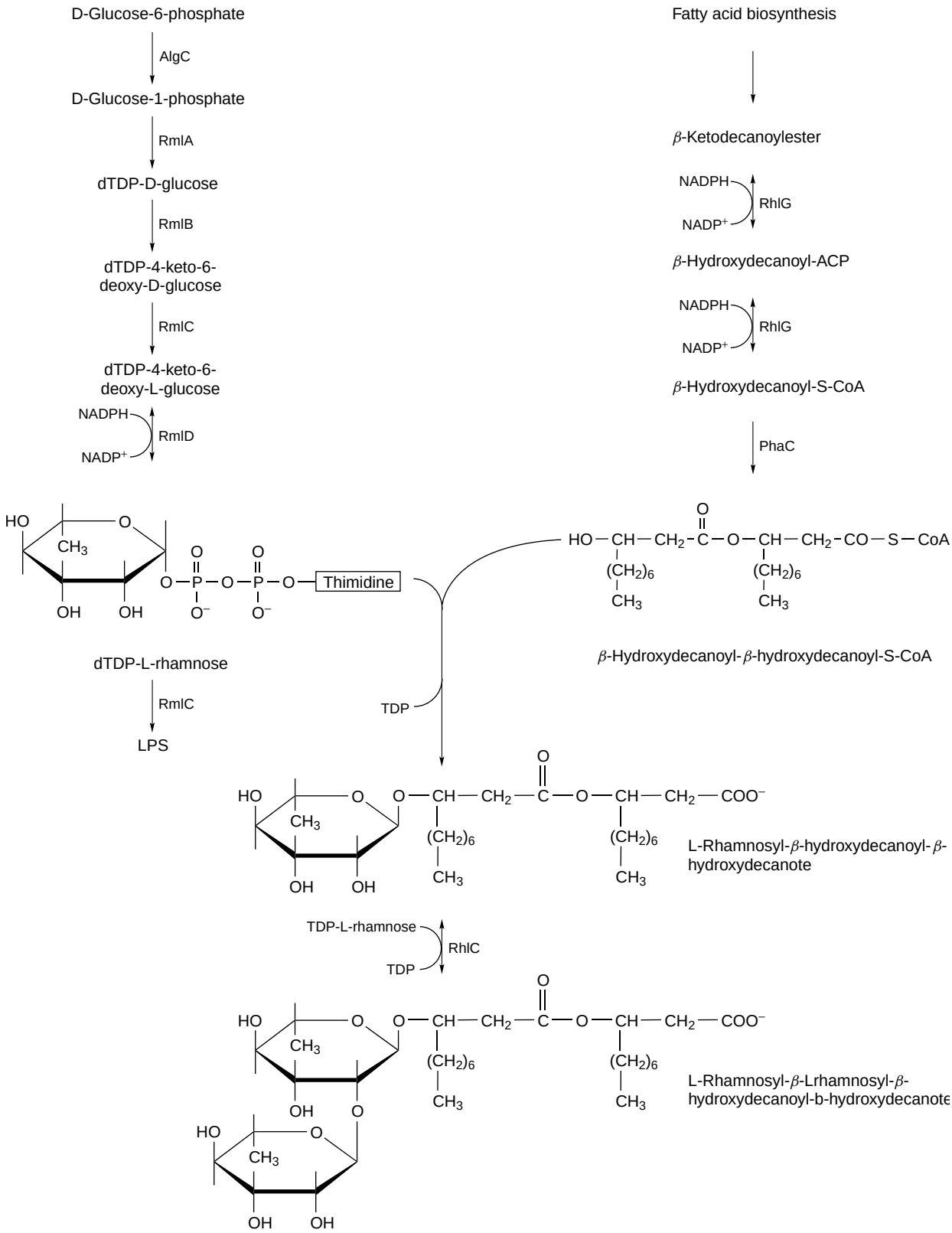

Figure 3. The rhamnolipid biosynthetic pathway. PhaC is polyhydroxyalcanoate synthase, AlgC is a phosphomannomutase that catalyzes the second step in the alginate biosynthetic pathway, the *Rml* genes are involved in synthesis of dTDP-L-rhamnose for LPS and rhamnolipid synthesis, and the *Rhl* genes are involved in synthesis of rhamnolipid (50).

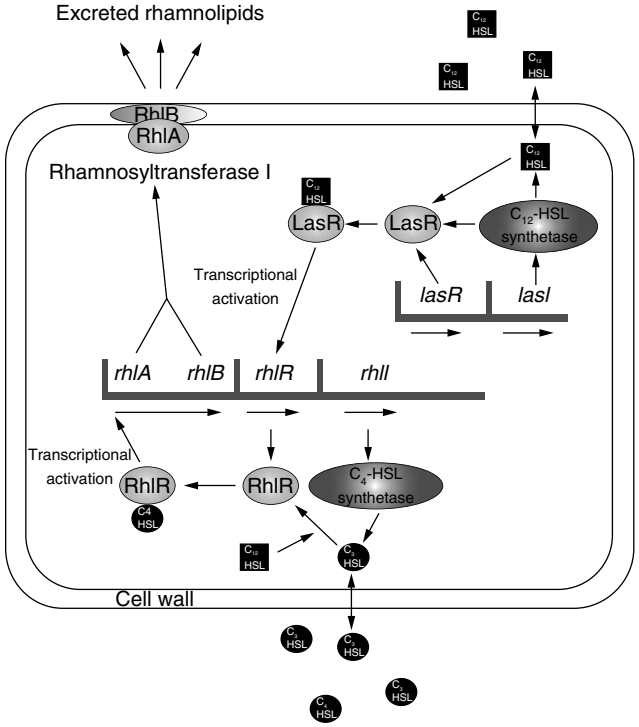

Figure 4. Quorum-sensing regulation of rhamnolipid (R1) synthesis. Rhamnosyltransferase I is composed of two subunits: RhlA, an inner membrane bound protein, and RhlB, a periplasmic protein. Together these catalyze the addition of dTDP-L-rhamnose to β-hydroxydecanoyl-β-hydroxydecanoate to form R1, which is then excreted from the cell (53). There are two signal molecules involved in R1 synthesis. The first is *N*-butryl homoserine lactone (C_4-HSL) encoded by *rhlI*. The C_4-HSL binds to the RhlR regulatory protein, and the HSL-RhlR complex causes transcriptional activation of the *rhlA* and *rhlB* genes that encode rhamnosyltransferase I. The second signal molecule is *N*-(3-oxododecanoyl)-L-homoserine lactone (C_{12}-HSL), which controls rhamnolipid synthesis in two ways. The C_{12}-HSL binds to the LasR protein, and the HSL-LasR complex causes transcriptional activation of the *rhlR* gene. However, at the posttranslational level, it has been shown that the C_{12}-HSL prevents binding of the C_4-HSL to RhlR and thereby prevents transcription of *rhlA* and *rhlB*. Pesci and coworkers (51) suggest that the latter control is more important at low cell density.

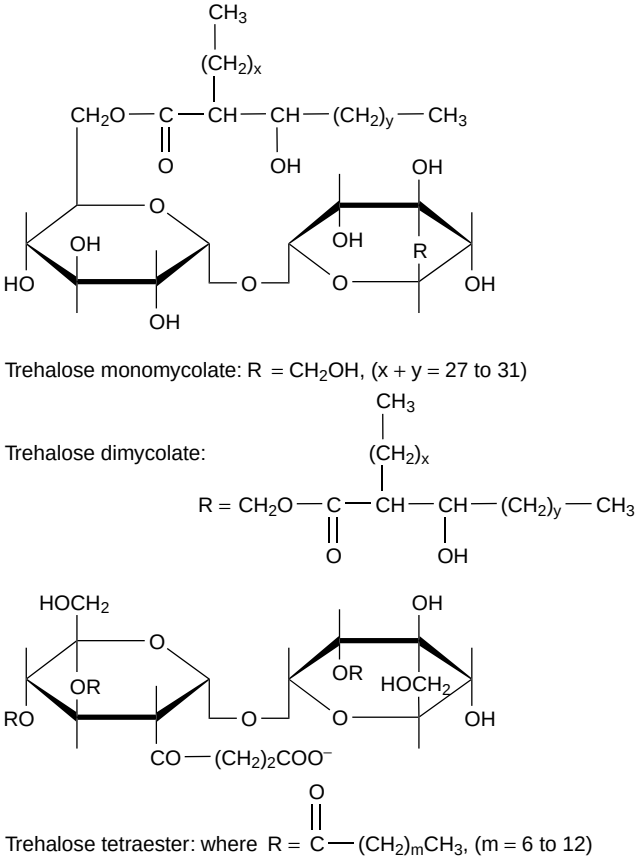

Figure 5. Structure of trehalose lipids produced by species of *Rhodococcus* (now separated into *Gordona* and *Tsukamurella*), *Mycobacterium*, *Arthrobacter*, *Nocardia*, *Brevibacterium*, and *Corynebacterium*. Nonionic trehalose monomycolate (56) and dimycolate also known as cord factor (55), and anionic trehalose tetraester (57).

weight of sophorolipids ranges between 460 and 705. Sophorolipids can reduce surface tension to less than 33 mN/m and interfacial tension between hexadecane and water from 40 to less than 1 mN/m (1,79). In contrast, they are generally poor emulsifying agents (77).

Growth conditions, including pH, temperature, resting cells versus growing cells (81), substrate(s), and method of cultivation (batch versus fed-batch), have been optimized for production of sophorolipids by *Candida bombicola* (82). As a result, yields of up to 250 to 300 g sophorolipid/L have been achieved (83). In general, the highest sophorolipid yields were obtained by fermentation using a continuous feed of a substrate combination containing a carbohydrate (glucose) and a lipid (rapeseed ester rich in C18 : 1 and C18 : 0 fatty acids) (83,84). Other carbon sources have been investigated, such as glucose, lactose, sucrose (85), canola oil, vegetable oil, deproteinized whey (86), sunflower oil, oleic acid, hexadecane, dodecanol with the hydroxyl group in the 1,2,3, or 4 position (87), and a mixture of the above. The substrate used dictates the composition of the sophorolipid fatty acid moieties as well as the number of sugars (83,84,88). Kim and Kim (89) determined that the carbon to nitrogen ratio impacts production yield for *C. bombicola*. Specifically, it was shown that nitrogen-limiting conditions are needed for good production. Finally, some sugars inhibit the production of sopohorose lipids even if a hydrocarbon is present (78).

The largest reported yield of sophorolipid is 422 g/L (86). This was obtained using a two-stage cultivation method. The first stage used *Cryptococcus curvatus* ATCC 20509 to deproteinize a whey substrate. In the second stage the deproteinized whey substrate was inoculated with *C. bombicola* ATCC 22214 and fed continuously with rapeseed oil. The high yields reported for sophorolipids make commercial production competitive with synthetic surfactants.

Mannosylerythritol Lipids. Mannosylerythritol lipids have been reported to be produced by several strains of the yeast *Candida* (90–93) and by the smut fungus Ustilago

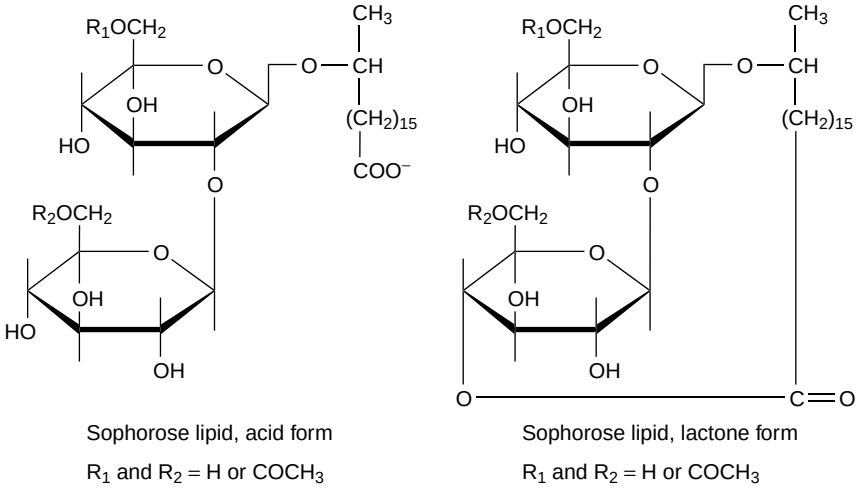

Figure 6. Structure of sophorose lipids produced by *Candida* sp. (80).

maydis (4). Mannosylerythritol lipids are anionic, with a structure that consists of a mannose sugar monomer linked to two fatty acids. There are three major forms of mannosylerythritol lipids reported, MEL-A, MEL-B, and MEL-C (Fig. 7). The molecular weight of this biosurfactant ranges from 577 to 745.

The mannosylerythritol lipid isolated from *Candida* sp. SY16 (MEL-B form) reduces surface tension to 29 mN/m and reduces interfacial tension against kerosene to 0.1 mN/m (93). The CMC of this surfactant was 10 mg/L. Reported yields of mannosylerythritol lipids from *Candida* sp. range up to 80 g/L. In this case high concentrations of resting cells were used with peanut oil as the carbon source (94). No nutrients (other than the peanut oil) or buffer were required in this system. Growing cells have also been reported to produce high levels of mannosylerythritol lipids (30 to 46 g/L) on a variety of carbon sources, such as soybean oil (90,95) safflower, coconut, cottonseed, corn, and palm oils (91), olive oil, triolein, and several long-chain alcohols (94). *Ustilago maydis* produces a surfactant

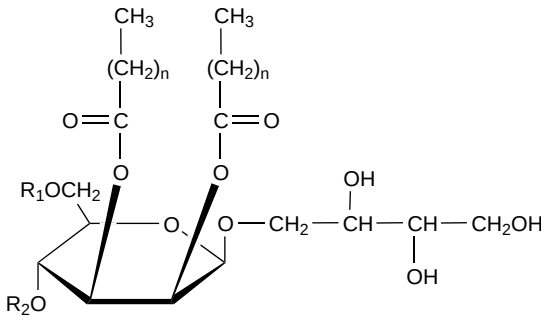

Figure 7. Structure of mannosylerythritol lipids produced by *Candida* and *Ustilago* (4).

mixture composed of mannosylerythritol and cellobiose lipids. Carbon and nitrogen sources dictate the mixture composition with oil-based substrates, favoring formation of mannosylerythritol lipids regardless of nitrogen source (ammonia or urea). In contrast, sugar-based substrates favor production of cellobiose lipids in the presence of urea and mannosylerythritol lipids in the presence of ammonia (4).

Other Glycolipids. A number of other glycolipids have been characterized, although information about these molecules and their properties is more limited. The fact that numerous different glycolipids have been identified illustrates the great structural variety possible in biosurfactants. This variety is especially important from the perspective of potential biosurfactant applications.

Several microorganisms have been reported to produce glucose lipids, including the yeast *Candida antaria* T-34 (4,91), the smut fungus *U. maydis* DSM 4500 and ATCC 14826 (4), and the bacteria *Alcanivorax borkumensis* (14,96,97), *Alcaligenes* sp. MM1 (98), and *Alcaligenes* sp. MM7 (99). Glucose lipids can reduce surface tension to less than 25 mN/m and interfacial tension against hexadecane to less than 5 mN/m (97). They have been shown to enhance the degradation of the polychlorinated biphenyl mixture Aroclor 1248 (14).

A number of bacteria can produce other glycolipids either singly or in mixtures. In some instances, the carbohydrate portion of the molecule can be dictated by the carbon source used. In particular, when a resting cell culture is used, it is possible to influence the glycolipid produced (100). The variety of glycolipids reported includes mannose, cellobiose, maltose, maltotriose, sucrose, fucose, galactose, and altrose lipids. The producing organisms include *Arthrobacter* sp., *Brevibacteria*, *Corynebacterium, Nocardia*, and *Rhodococcus* (12,100–103).

Phospholipids, Neutral Lipids, and Acidic Lipids

There are several lipid-containing molecules integral to cell structure that are also surface active and have activities normally associated with biosurfactants. These include phospholipids, acidic lipids such as fatty acids, and

neutral lipids such as triacylglycerol. For example, Beebe and Umbreit (104) isolated a mixture of phospholipids and neutral lipids from *Thiobacillus thiooxidans*, which was capable of wetting elemental sulfur. Phospholipid-rich and LPS-rich vesicles, produced by *Acinetobacter* sp. HO1-N during growth on hexadecane, were shown to function in enhancing the solubility of hexadecane (105). Similarly, *Arthrobacter paraffineus* KY 4303 produces a primary fatty alcohol when grown on paraffin that coats the paraffin in the medium (106). The surface-active compound produced by *Nocardia erythropolis* ATCC 4277 during growth on 4% hydrocarbon was determined to be a neutral lipid. This biosurfactant was able to reduce surface tension to 34 mN/m and interfacial tension against hexadecane to less than 1.0 mN/m (107,108).

One acidic lipid that is not integral to the cell structure is spiculisporic acid, a tricarboxylic acid produced by *Penicillium* (Fig. 8). Spiculisporic acid is produced during growth on glucose and yields of up to 110 g/L have been achieved (109). Whereas the surface activity of spiculisporic acid is low, derivatives of this molecule have good surface activity. For example, a disubstituted *n*-hexylamine salt of the S-form reduced surface tension to 27 mN/m (110).

Lipoproteins

Lipoproteins, also referred to as lipopeptides, have a hydrophilic protein moiety attached to a hydrophobic fatty acid. The protein moiety can be neutral or anionic and the amino acids are arranged in a cyclic structure. As a group, the lipoprotein biosurfactants are perhaps best known for their antibiotic activities. For example, gramicidin S is produced by *Bacillus brevis* (112) and the polymyxins are produced by *Bacillus polmyxa* (113). Both are cyclic lipopeptide antibiotics that have notable surface activity. The best-characterized lipoproteins, including surfactin, iturin, fengycin, and lichenysin, are those produced by *Bacillus* sp. These have molecular weights ranging from 1,000 to 1,500.

Surfactin, Fengycin, and Iturin. Surfactin is produced by *Bacillus subtilis*. The peptide sequence of surfactin's seven amino acids and β-hydroxy fatty acid were first characterized by Kakinuma and coworkers (114,115), and since then several isoforms have been detected and described. These isoforms vary in the composition of the anionic heptapeptide as well as the length and branching of the fatty acid tail (116,117) (Fig. 9). In many cases, the biosurfactant produced by *B. subtilis* is a mixture composed of surfactin and two other similar lipoproteins, iturin and fengycin (118,119). Iturin differs from surfactin in that an amino fatty acid is attached to the heptapeptide (120). Fengycin differs in that the β-hydroxy fatty acid is attached to a peptide containing 10 rather than seven amino acids (121). Like surfactin, there are isoforms of iturin and fengycin that vary in the amino acid composition of the peptide ring and in the length and composition of the fatty acid moiety (Fig. 8).

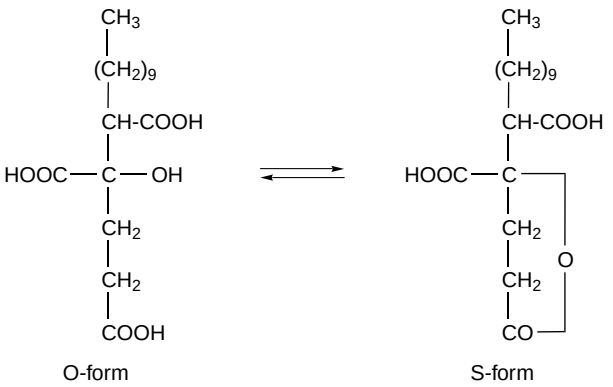

Figure 8. Structure of spiculisporic acid produced by *Penicillium* (111). Spiculisporic acid comes in two forms, the S or spiculiosporic form, and the O or open form.

Surfactin is a highly surface active molecule. It can reduce surface tension to 27 mN/m and interfacial tension against hexadecane to 0.1 mN/m (123–125). The reported CMC for surfactin is 10 mg/L, and the reported CMC values for iturin and fengycin are 20 and 11 mg/L, respectively (126). Efforts have been made to produce lipoproteins in high amounts for commercial use. The normal yield of lipoprotein is 0.1 to 0.4 mg/L. Cooper and coworkers (124) reported a doubling of surfactin yield to 0.8 g/L from *B. subtilis* strain ATCC 21332 using glucose as a carbon source. A fermentor was used for growth, and the surfactant was harvested continuously from the fermentor as a foam. Mulligan and coworkers (46) were able to increase surfactin yield to 1.1 g/L using a mutant *B. subtilis* strain. Iron enrichment was used to enhance surfactin production to 3.5 g/L (127). However, the highest reported yield, 7 g/L, was obtained under oxygen-limiting conditions (128). Surfactin has also been produced on unconventional carbon sources such as potato waste (129) and under extreme conditions. *Bacillus subtilis* is able to produce surfactin at 45 °C, under a wide range of pH (4.5 to 10.5) and at salt concentrations as high as 4% NaCl (130).

Surfactin biosynthesis and regulation have been recently reviewed (49,118). Thus far, three proteins have been identified as necessary for surfactin synthesis. One protein is surfactin synthetase, a complex multifunctional protein encoded by the *srfA* operon. The *srfA* operon contains four open reading frames, ORF1 (*srfA-A*), ORF2 (*srfA-B*), ORF3 (*srf A-C*), and ORF4 (*srfA-D*). These open reading frames encode the four subunits of surfactin synthetase, E_{1A}, E_{1B}, E_2, and a thioesterase thought to be involved in the lactonization of the molecule (Fig. 10). Subunits E_{1A}, E_{1B}, and E_2 are responsible for addition of surfactin's seven amino acids to the β-hydroxy fatty acid (131). E_{1A} and $E_{1B}$are multifunctional subunits that each add three amino acids to the β-hydroxyacylpeptide. E_2 adds the seventh amino acid. The second protein required for surfactin synthesis is the acyl transferase responsible for the initial transfer of the β-hydroxy fatty acid to surfactin synthetase. The gene encoding the acyl transferase has not yet been characterized. The third protein required is Sfp, a phosphopantetheinyl transferase that is required to activate the surfactin synthetase by posttranslational modification. The gene encoding Sfp is located several kilobases downstream of the *srfA* operon.

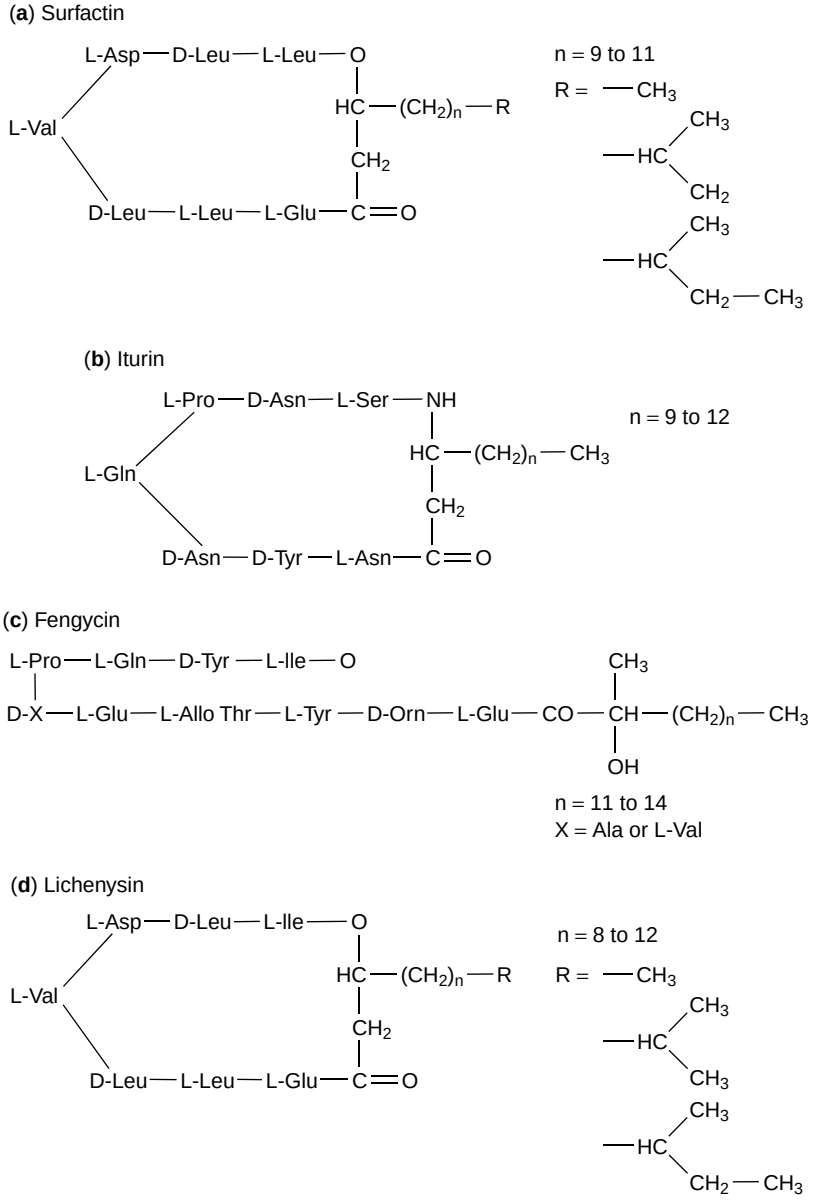

Figure 9. Structure of the lipopeptides surfactin, iturin, fengycin (produced by *B. subtilis*), and lichenysin (produced by *B. licheniformis*) (122). Surfactin and lichenysin are the best characterized of these surfactants. Both the fatty acid and peptide moieties can vary in structure. For example, for lichenysin, the amino acids shown in bold can be Gln or Glu (amino acid 1), Asn or Asp (amino acid 5), Ile or Leu (amino acid 7).

The synthesis of surfactin is under the regulation of the *com* operon, an operon required for development of competence of *Bacillus* (Fig. 11). This is because the *comS* gene, which is needed for competence, is embedded in the *srfA* operon. Therefore, in a quorum-sensing system similar to that described for rhamnolipid, a signal peptide, ComX, accumulates at high cell density and is responsible for initiating transcription of the *srfA* operon. Specifically, ComX interacts with a membrane bound protein, ComP. ComP then phosphorylates ComA, and ComA-P binds to the promotor of *srfA*. There is a second signal peptide, competence stimulating factor (CSF), which also influences *srfA* expression. CSF is the product of *phrC*, and it is processed into the active form of CSF as it is excreted through the cell wall (132). At low concentrations of CSF, such as the concentration present in midexponential phase (2 to 5 nM), CSF acts to stimulate expression of *srfA*. CSF stimulates *srfA* by preventing the phosphatase RapC from interacting with ComA-P (ComA-P $\rightarrow$ ComA). At stationary phase levels of CSF (approaching 100 nM), expression of *srfA* is inhibited. Currently the mechanism of inhibition is unknown.

Biosynthesis of fengycin is similar to that of surfactin. In this case, the operon responsible for synthesis of

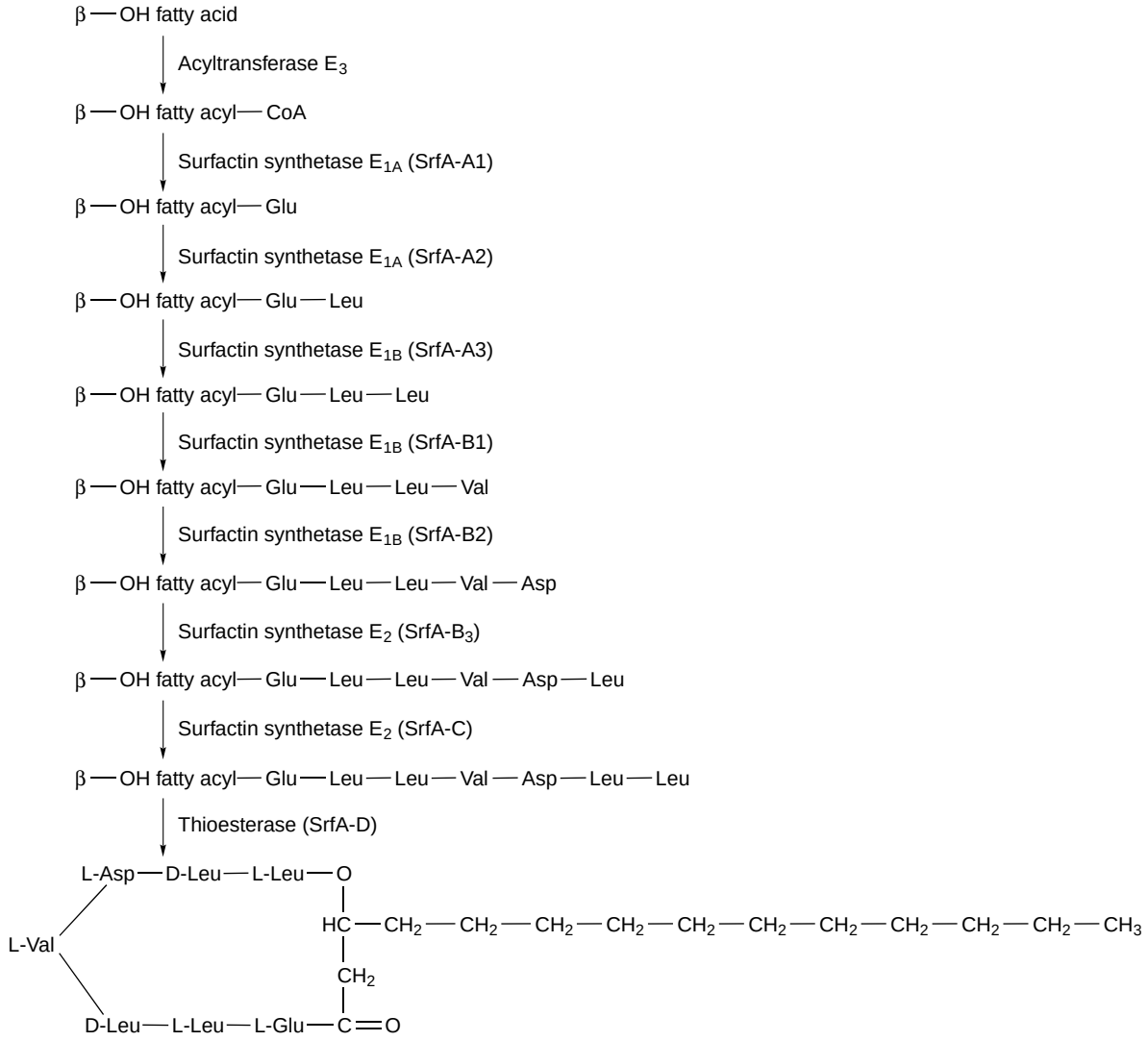

Figure 10. The surfactin biosynthetic pathway. An acyl transferase (E_3) activates and delivers β-hydroxy fatty acid to the surfactin synthetase complex. This complex is encoded for on the *srfA* operon, which contains four open reading frames (*srfA-A*, *srfA-B*, *srf A-C*, and *srfA-D*). SrfA-A is a multifunctional subunit of surfactin synthetase that adds the first three amino acid residues. SrfA-B is a multifunctional subunit that adds the next three amino acid residues, SrfA-C (E_2) adds the final amino acid and SrfA-D is a thioesterase involved in the lactonization of the molecule (131).

the peptide portion of the molecule is the *pps* or *fen* operon. There are five multifunctional peptide synthetases associated with fengycin (121). The *fen1, fen2*, and *fen3* genes encode three proteins of similar size (fengycin synthetase), which activate the first six amino acids of fengycin. The *fen4* gene encodes a multifunctional enzyme, which activates three additional amino acid residues. The *fen5* gene product activates the final amino acid, isoleucine, and is also involved with lactonization of the peptide.

Lichenysin. Lichenysin is a lipopeptide produced by *Bacillus licheniformis*, which is very similar in structure and properties to surfactin (Fig. 9). As described for surfactin, the peptide and the β-hydroxy fatty acid moieties are variable depending on growth conditions and the producing isolate (122,133–137). Lichenysin, depending on the isoform tested, can reduce surface tension to 27 mN/m and interfacial tension against hexadecane to 0.36 mN/m in a 4% salt solution (138). The reported CMC values are as low as 10 mg/L (138). Lichenysin is produced under both aerobic and anaerobic conditions during growth on a glucose medium containing 0.1% yeast extract and 0.1% sodium nitrate (139).

The biosynthesis of lichenysin is similar to that discussed earlier for surfactin and fengycin (137). The lichenysin operon is 26.6 kb and contains three open reading frames: *licA* and *licB* (both multifunctional subunits), and *licC*. All three open reading frames encode peptide synthetases. The *licTE* gene encodes a thioesterase that is homologous to the *srfA-D* gene.

Other Lipoproteins.. A surfactin-like lipoprotein with high hemolytic activity is produced by *Bacillus pumilus*,

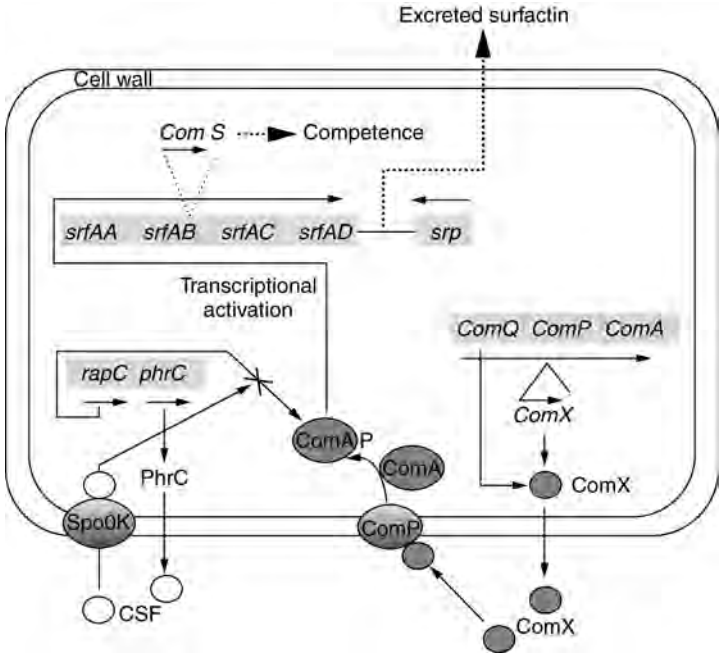

Figure 11. Regulation of surfactin synthesis is controlled by two quorum-sensing signal peptides, ComX and CSF (competence stimulating factor). ComX interacts with a membrane-bound protein, ComP, which phosphorylates ComA. ComA-P binds to the promotor of *srfA* and initiates transcription. CSF is the product of *phrC* and is activated as it is excreted through the cell wall. At low concentrations of CSF, expression of *srfA* is stimulated because the phosphatase RapC is prevented from interacting with ComA-P (ComA-P → ComA). This results in increased expression of *srfA* (132).

Table 1. Lipoproteins Produced by Genera Other Than *Bacillus*

Producing Organism	Lipoprotein	Remarks	Reference
Actinoplanes friuliensis sp.	Friulimicin β-Hydroxy-decenoyl-Asn-Dab[a]-Pip[b]-Me-Asp-Asp-Gly-Asp-Gly-Dab-Val-Pro-lactone	Antibiotic	141
Arthrobacter	Arthrofactin 3-Hydroxydecanoyl-D-Leu-D-Asp-D-Thr-D-Leu-D-Leu-D-Ser-L-Leu-D-Ser-L-Ile-L-Ile-L-Asp-lactone	S.T. = 24 mN/m	142
Pseudomonas fluorescens	Viscosinamide or viscosin β-Hydroxy-decanoyl-L-Leu-D-Gln-D-Allo-Thr-D-Val-L-Leu-D-Ser-L-Leu-D-Ser-L-Ile-lactone	Cell associated S.T. = 25 mN/m, CMC = 4 mg/L in phosphate buffer	143–145
Pseudomonas fluorescens	Tensin β-Hydroxy-decanoyl-D-Leu-D-Asp-D-Allo-Thr-D-Leu-D-Leu-D-Ser-L-Leu-D-Gln-L-Leu-L-Ile-L-Glu-lactone		146
Pseudomonas syringae	Syringomycin, nine amino acid residues	Phytotoxin	147
	Syringopeptin, 22 to 25 amino acid residues	Phytotoxin	147
Serratia marcescens	Serrawettin		148
Serratia rubidaea	Rubiwettin		148

[a] Diaminobutyric acid.
[b] Pipecolinic acid.

an organism associated with marine sponges (140). This lipoprotein is called bacircine. In addition, there are a number of lipoproteins similar to those shown in Figure 9 that are produced by genera other than *Bacillus*. These are summarized in Table 1.

Polymeric Biosurfactants

The polymeric biosurfactants are characterized by their high molecular weight, which ranges from 50,000 to greater than 1,000,000. These polymers can be carbohydrate- or protein-based, and in addition usually contain lipids. In some cases, the polymer may be a mixture of carbohydrate, protein, and lipid. These compounds, noted for their emulsification abilities, are produced by a number of bacteria, Archea, and yeast. Of great interest commercially is the fact that each polymeric biosurfactant has different hydrocarbon specificities with respect to degree of emulsification. In general, the polymeric biosurfactants do not lower surface or interfacial tension significantly (149). These polymers are quite heterogeneous and therefore have been difficult to categorize as precisely as the low molecular weight biosurfactants.

Emulsan. The best characterized of the polymeric biosurfactants is emulsan, produced by *Acinetobacter calcoaceticus* RAG1. The structure of emulsan is a heteropolysaccharide linked to fatty acids through *o*-ester and amide bonds. The fatty acid component comprises 15% (dry weight) of the polymer with average lipid molecular weight of 231. Three major sugars have been identified: *N*-acetyl-D-galactosamine (*20 to 30% dry weight of polymer), *N*-acetylgalactosamine uronic acid (*33% dry weight of polymer), and diamino deoxyhexosamine (*5% dry weight of polymer) (49,150). There is also a protein associated with this polymer, which is required for emulsification activity. A number of other *Acinetobacter* sp. produce polymers similar to emulsan, which also require a separate protein component for emulsification activity. These include *A. calcoaceticus* MM5 (151) and BD4 (152).

Emulsan is an effective emulsifier at concentrations of 0.001 to 0.01%. It initially accumulates on the cell surface as a capsule and is then released as the cells enter stationary phase. It emulsifies petroleum-like hydrocarbon mixtures best, showing little activity toward pure alkane, aromatic, or alicyclic compounds (3). All of these emulsifiers act to coat hydrocarbon droplets in solution, thereby stabilizing the oil-in-water emulsion. It is thought that the protein component associated with the polymer is required for mediating the initial association of the polymer with hydrocarbon droplets. Emulsan can reduce surface tension to 52 mN/m (153). Emulsan is commercially available from the Petroferm Company (154).

The biosynthesis and regulation of emulsan has been recently reviewed by Sullivan (49). Thus far two enzymes, an esterase and a lipase, have been identified as having a role in emulsan production. However, loss of their activity does not completely inhibit emulsan production. The esterase is an outer membrane-bound esterase encoded by the *est* gene, and is thought to have a role in releasing emulsan from the surface of the cell (155). The lipase is encoded by the *lip* operon and is involved in addition of fatty acids to emulsan. Specifically, a mutant in the lipase chaperone gene (*lipB*) was found to be defective in lipase activity. This mutant produces an emulsan that has a lower fatty acid content and reduced emulsification activity (155,156).

Other Polymeric Biosurfactants. There are quite a few other microorganisms that have been identified as polymeric biosurfactant producers (Table 2). These are of interest because they differ substantially from emulsan in their ability and specificity to emulsify hydrocarbons.

Table 2. Polymeric Biosurfactants from Representative Microorganisms

Producing Organism	Biosurfactant	Remarks	Reference
Acinetobacter radioresistens KA-53	Alasan	Anionic, MW = 9×10^5; complex polysaccharide covalently bound to alanine; emulsification ability is activated by heat treatment; emulsifies *n*-alkanes and aromatics separately	157,158
Acinetobacter calcoaceticus A2	Biodispersan	Anionic, MW = 51, 400; contains glucosamine, 6-methylaminohexose, galactosamine uronic acid, an amino sugar	159,160
Bacillus stearothermophilus VR-8		Contains protein (46%), carbohydrate (16%), and lipid (10%), emulsifies over wide pH, salt, and temperature range	161
Candida albicans		Contains 26% carbohydrate and 1% protein, minimum surface tension = 51 mN/m, increased yeast cell adherence to human intestinal epithelial cells and decreased adherence to buccal epithelial cells	162
Candida lipolytica ATCC 8662/IA 1055	Liposan	83% Sugar (glucose, galactose, galactosamine, galacturonic acid) and 17% protein, emulsifies between pH 2 and 5	163–165
Halomonas S-30	EPS	Salt tolerant, contains 41% carbohydrate, 5% protein, and 9% uronic acids	166
Methanobacterium thermoautotrophicum		Emulsifies over wide pH and temperature range	167
Phormidium J-1	Emulcyan	Cyanobacterium, MW greater than 1×10^5, polymer of sugar, protein, and lipid	168
Pseudomonas fluorescens 378	Protein/carbohydrate	MW = 1×10^6, CMC = 8 mg/L in 0.9% NaCl; minimum surface tension = 30 mN/m; interfacial tension against hexadecane = 0.7 mN/m	153
Pseudomonas maltophila CSV89	Biosur-Pm	Contains 45 to 50% protein and 12 to 15% carbohydrate; minimum surface tension −53 mN/m, CMC = 50 mg/L; demonstrates substrate specificity for 1-naphthaldehyde	169
Pseudomonas marginalis	PM factor	MW > 10^6, contains protein, LPS, and phospholipid (60 to 80% protein, 20 to 30% lipid, 3 to 7% polysaccharide)	170
Saccharomyces cerevisiae	Mannoprotein	44% Mannose and 17% protein, emulsifies over broad range of pH, produced in high yield (8% of wet weight)	171

Some of these microorganisms come from extreme environments, and their associated emulsifiers have activity under a broad range of pH, temperature, and salt conditions. Finally, some of these microorganisms, for example, *Saccharomyces cerevesiae*, are already used in the food industry and presumably emulsifiers from such organisms would have wide application in the food industry.

SCREENING FOR SURFACTANT AND EMULSIFICATION ACTIVITIES

Biosurfactants have a wide range of physical and chemical properties and there is little homology in the genes encoding various types of surfactants. Therefore, normal biochemical or molecular screening techniques are of little use. This has led to the development of several screening assays based upon either reduction of surface or interfacial tension or on emulsification ability.

Screening for Surface or Interfacial Tension Reduction

Drop-Collapse Test. A qualitative drop-collapse test was first developed by Jain and coworkers (172) and later modified by Bodour and Miller-Maier (173) so that it can be either qualitative or quantitative. In the test, a 5-μL droplet of culture is added to an oil-coated surface. If the droplet remains beaded, the sample contains no biosurfactant; if the drop collapses, the sample contains biosurfactant. The use of Pennzoil® as the coating oil allows this assay to be quantitative as long as a reference sample of the surfactant is available. A standard curve is constructed by measuring the diameter of a series of droplets containing known concentrations of the surfactant using 15x magnification. The advantages of this test are that it is rapid, requires very little sample, and uses no specialized equipment. Further, it can be used to give qualitative information.

Tilted Glass Slide. A qualitative tilted glass slide technique was used to screen *Pseudomonas* and *Vibrionaceae* sp. for surfactant production (174). In this test, the organism is first grown on agar plates. A colony is mixed with a droplet of 0.9% (w/v) NaCl on one end of a glass slide and the glass slide is tilted. If a biosurfactant is present, the droplet will travel down the slide when tilted. If the droplet remains motionless, the sample has no detectable biosurfactant. This test has the advantages that it is rapid, simple, and requires no specialized equipment. A potential disadvantage of this method is that in some cases an agar-grown colony can produce false negative results.

Hemolysis of Red Blood Cells. Some biosurfactants are known for their ability to lyse red blood cells (RBC), including the lipoproteins and some of the glycolipids (175). Singer (176) used this ability as the basis for a qualitative screening test for such biosurfactants. Commercial blood agar plates were used to screen a number of known biosurfactant producers as well as nonproducing isolates. Results were evaluated in terms of whether zones of hemolysis formed around colonies on the blood agar plates. In this study, zones of hemolysis were observed only with isolates that produced surfactants. Whereas this screening assay is simple to perform, the author notes that its use is limited to screening for microorganisms that produced water-soluble, diffusible, low molecular weight biosurfactants during growth on a complex medium (blood agar).

Axisymmetric Drop Shape Analysis by Profile. A modified axisymmetric drop shape analysis by profile (ADSA-P) technique was used by van der Vegt and coworkers (177) to assess cell-associated biosurfactant production by 11 *Streptococcus* strains. Of these 11 isolates, two were positive controls known to produce cell-associated surfactant, and two were negative controls that did not produce surfactant. Each isolate was grown overnight, harvested, and washed twice with potassium phosphate buffer. The cells were resuspended in buffer to a final concentration of 5×10^9 cells/mL. A 100-μL droplet of the resuspended cells was placed on a teflon surface, and the shape of the drop surface was digitized using ADSA-P for a 2-hour period. ADSA-P is a program that was developed to determine interfacial tension and contact angles from droplet profiles (178). Noordmans and Busscher (179) modified input and output terms of ADSA-P to incorporate parameters such as density, gravity, and magnification factors (input terms) and surface tension, contact angle, and droplet volume (output terms).

The results showed that the ADSA-P analysis predicted that the two positive controls were surfactant producers and the two negative controls were not. Of the remaining seven isolates, three were positive for cell-associated surfactant production according to the ADSA-P analysis. This technique can accurately predict surfactant production and most likely could be used to evaluate extracellular biosurfactant production as well. The disadvantage of this technique is that it requires video monitoring equipment that is interfaced with the ADSA-P program, which is not routinely available in most labs.

Colorimetric Assay. A colorimetric plate assay has been developed that is specific for anionic biosurfactants (180). This technique was originally developed to screen for rhamnolipids, but can be used to screen for any low molecular weight anionic biosurfactant. Mineral salts agar plates containing a carbon source (2%) and cetyltrimethylammonium bromide (CTAB)-methylene blue are prepared. Carbon sources that have been tested include glucose, mannitol, glycerol, peptone, succinate, and citrate. As the biosurfactant is produced it forms complexes with the CTAB-methylene blue. As a result, any colony that produces biosurfactant develops a dark blue halo.

Screening for Emulsification Activity

Emulsion Index. Cooper and Goldenberg (125) developed a simple technique to screen for emulsification activity based on the emulsion index (E_{24}). This index is obtained by adding a culture to kerosene in a 1 : 2/3 (vol/vol) ratio. The solution is vortexed for two minutes and allowed to stand. After 24 hours, the height of the emulsion (the layer between the aqueous and kerosene layers) is measured and used to calculate the emulsion index (E_{24}) using the following equation:

Emulsification ability can be expressed as an emulsion index but is also often expressed as activity units. For example, Trebbau de Acevedo and McInerney (167) expressed emulsifying activity as units per milligram where one unit of emulsifying activity was defined as the amount of emulsifier that gave an emulsification index of 20.

Turbidity Assay. An emulsification assay based on turbidity measurement was reported by Rosenberg and coworkers (149) and modified by Neu and Poralla (181). A 10-mL sample of the culture is filtered and the filtrate dried. The dry filtrate is added to 1.5 mL Tris-Mg buffer and the optical density (OD) measured at 446 nm. Hydrocarbon (0.2 ml) is added, the sample is vortexed for two minutes, allowed to stand for 10 minutes, and the OD is measured again. The emulsification activity is the difference between the initial and final OD reading. It is difficult to compare emulsification activities reported in the literature since emulsifiers show selectivity and a standard hydrocarbon is not always used. Therefore, it is important that both negative and positive controls be performed with this assay. As an example, the reported emulsification value for emulsan is 1.58 for corn oil (154). In this study, negative controls had values of approximately 0.1.

INDUSTRIAL AND BIOTECHNOLOGY APPLICATIONS

Some biosurfactants, for example, rhamnolipid, sophorolipid, and spiculosporic acid, can be commercially produced at levels exceeding 100 g/L (29,109,182). At this level, the cost of producing biosurfactants becomes competitive with the cost of producing synthetic surfactants. As the production cost becomes competitive and as the commercial availability of biosurfactants increases, one can expect the commercial use of biosurfactants to grow. It is important to note that only two biosurfactants have been studied extensively. However, the analytical and molecular tools now available make the tasks of identifying new biosurfactants, and understanding how and why biosurfactants are produced, more feasible and rapid. The following sections explore various uses of biosurfactants that have been proposed in the scientific literature.

Bioremediation

Bioremediation is the use of microorganisms and/or plants to aid in the cleanup of sites contaminated with organics, metals, or in some cases with a mixture of these two types of contaminants. According to a recent National Research Council report (183), the estimated cleanup cost for the United States alone exceeds $1 trillion. As a result, interest in cleanup alternatives, such as bioremediation, is increasing. Surfactants, both synthetic and biological, have been explored for their bioremediation potential, and show promise for application to sites impacted by both organic and metal contaminants. Biosurfactants have several potential advantages over synthetic surfactants for bioremediation applications. These advantages include low toxicity, better specificity for some applications, and the potential for in situ production of biosurfactants.

Biodegradation of Organics. There is an extensive body of literature documenting the effects of biosurfactants on biodegradation of hydrocarbons, both aliphatic and aromatic. The majority of research has involved glycolipids. Glycolipids can enhance solubility and biodegradation of a variety of alkanes, including hexadecane, octadecane, lubricating oil waste, and *n*-paraffins, as well as phenanthrene in solution or pure culture (25,31,40,42,66,80,184–190). Further, glycolipids have been shown to enhance degradation in soil systems containing hexadecane (191–193), tetradecane (191), pristane (191), creosote (194), a hydrocarbon mixture (195), crude oil (196), chlorinated phenols (65), and polychlorinated biphenyls (13,14). Polymeric biosurfactants have also been tested. Alasan (197) and the emulsifier produced by *A. calcoaceticus* MM5 (198), have been shown to enhance solubilization and biodegradation of polyaromatic hydrocarbons. Heptadecane uptake rate was increased by an unidentified emulsifier produced by *Pseudomonas nautica* (199).

While the success of biosurfactants is clearly documented, there are also reports involving both pure culture and soil systems, which suggest that the addition of biosurfactant can inhibit biodegradation. Whether biodegradation is enhanced or inhibited in pure culture seems to be dependent on the degrading genus, wherein the biosurfactant-producing genus is more likely to be stimulated than other genera (40). However, even biosurfactant-producing strains vary in their response to biosurfactant addition (185,193). For soils, it is unclear why biosurfactants cause inhibition, although recent findings suggest that biosurfactants can serve as a preferred carbon source (200–202).

Both low and high molecular weight biosurfactants enhance biodegradation. The mechanism of enhancement depends on the surfactant concentration. At high concentration (>CMC) low molecular weight biosurfactants increase the aqueous solubility of a contaminant by either incorporating the contaminant into a micelle (Fig. 1) and increasing the apparent solubility of the contaminant, or by increasing the rate of desorption of the contaminant from soil surfaces (42,203). High molecular weight biosurfactants similarly increase contaminant availability but they do so by increasing the available surface area through emulsification. At low concentrations (<CMC) low molecular weight biosurfactants induce alterations in cell surface properties to allow better contact between the cell and the contaminant (31,193,204). A recent study examined the interaction of the low molecular weight biosurfactant, rhamnolipid, with degrading cells. Results showed that rhamnolipid caused a loss of LPS, an important hydrophilic component of the gram-negative cell surface (204). Loss of LPS resulted in an increase in cell hydrophobicity, which in turn increased the interaction of the cell with the hydrocarbon, and ultimately increased the biodegradation rate. As cell surface changes occur at very low surfactant concentrations, there are several reasons for taking advantage of this phenomenon

for hydrocarbon bioremediation. First, because rhamnolipids are biodegradable, they can serve as a preferred carbon source in a mixed population. In this case, addition of high amounts of rhamnolipid will suppress degradation of hydrocarbons until a substantial portion of the rhamnolipid is degraded. Second, sub-CMC levels of rhamnolipid will not cause mobilization of hydrocarbons, which can result in undesirable spreading of a contaminant plume (192). Third, in situ production of sub-CMC levels of biosurfactant may be achievable (whereas it is unlikely that super-CMC levels can be produced in situ). Finally, if the biosurfactant is exogenously added to a contaminated site, it will be more economical to add sub-CMC levels.

Biodegradation of Organics in Metal-Organic Cocontaminated Systems. It is estimated that 40% of sites contaminated with organics are also contaminated with metals (205). The presence of toxic metals, such as lead, cadmium, or arsenic, can cause inhibition of biodegradation of organic compounds (206–208). Rhamnolipids have been demonstrated to enhance hydrocarbon degradation in systems cocontaminated-contaminated with organics and toxic metals. This has been shown both in pure culture with cadmium and naphthalene (205) and in two soils amended with cadmium and phenanthrene (201). In the soil tests, it was found that rhamnolipid biodegradation occurred over 8 to 12 days. Therefore, rhamnolipid was added in several pulses to each soil such that phenanthrene biodegradation in the cadmium-containing microcosms reached levels similar to the microcosms that contained no cadmium.

Biosurfactants as a Flushing Agent — Organics. Biosurfactants have been studied for their potential as soil washing or flushing additives for organics. In some instances, where biodegradation processes are too slow or infeasible, it may be necessary to remove organics from soil using ex situ soil washing or in situ soil flushing, also known as pump and treat. This pertains to two types of organic compounds: nonaqueous phase liquids (NAPL) (e.g., petroleum or chlorinated solvents), and solid phase organics (e.g., polyaromatic hydrocarbons [PAH]). In both cases the low aqueous solubility of the organic constrains removal by water alone. Additives that can enhance organic solubility have been researched including the use of surfactants.

For NAPL, addition of a surfactant to a flushing solution can enhance flushing efficiency by either mobilization of NAPL, which results from a decrease in interfacial tension between the NAPL and the surfactant solution, or by an increase in solubilization of the NAPL. Thus, to be effective, a surfactant must have a good solubilization capacity and/or be able to reduce interfacial tension. Rhamnolipid has been shown to have a solubilization capacity (expressed as a molar solubilization ratio [MSR]) for the model NAPL hexadecane, which is 20 times greater than the MSR for hexadecane-alkyl benzyl sulfonate (209). In studies examining the use of rhamnolipid for removal of residual hexadecane from soil, it was shown that rhamnolipid (20% removal) was more effective than either sodium dodecyl sulfate (SDS) (negligible removal) or Tween 80 (6% removal) (210). This experiment compared the surfactants on an equal mass basis (500 mg/L) and for an equal number of flushings (100 pore volumes). It was further shown that NAPL removal could be optimized by altering pH and ionic strength to achieve a maximum reduction in interfacial tension. Thus, in the same system, under conditions of pH 6 and 320 mM sodium, rhamnolipid removed 60% of residual hexadecane in only two pore volumes (211). A *Rhodococcus* biosurfactant has also been examined for its efficacy in removal of oil from sand (196). Crude biosurfactant extracts from 10 different *Rhodococcus* strains were tested with four different oils. Each strain performed slightly differently in terms of oil removal, with overall removal ranging between 9.8 and 98.0% (in comparison to the removal by water alone which ranged between 1.9 and 31.3%).

Similar promising results are reported for rhamnolipid solubilization of solid phase materials. Specifically, the rhamnolipid-octadecane MSR (203) was 10-fold higher than the MSR for Triton X-114-octadecane and fivefold higher than the MSR for Corexit 0600-octadecane (212). The MSR for rhamnolipid-phenanthrene ranged from 1.7 times higher to 2.8 times lower than that for 13 different synthetic surfactants that have been tested with phenanthrene (188,213,214). In a comparison of removal of a hydrocarbon mixture from soil, it was found that rhamnolipid performed more effectively than either Triton X-100 or Tween 60 for all hydrocarbon components. The hydrocarbon mixture contained undecane, pentadecane, hexadecane, octadecane, pristane, naphthalene, phenanthrene, and pyrene (215). Rhamnolipid-enhanced removal of phenanthrene (216,217), pyrene (218), polychlorinated biphenyls, and a variety of PAH (216) from soil have also been demonstrated.

Rhamnolipid as a Flushing Agent — Metals. Sites may be contaminated with metals alone or may be cocontaminated with metals and organics. In either case, in some instances it may be necessary to remove the toxic metals from the site. There are a variety of agents that have been shown to form complexes with metals. While the strength of the metal complexation is important, it is also requisite that these materials are environmentally compatible. The strength of the complexation is quantified by determining stability constants for the metal-organic ligand in question. Table 3 compares the stability constants for several different organic ligands that have been proposed for metal remediation. Whereas synthetic materials such as NTA, ethylenediamine tetraacetic acid (EDTA), and DTPA are extremely effective at metal complexation, as indicated by their high stability constants, their use in the field is questionable because of potential toxicity effects. NTA is a Class-II carcinogen (219) and DTPA is a potential carcinogen (Sigma Chemical Co., St. Louis, Missouri). Both EDTA and DTPA are toxic, as measured by invertebrate toxicity tests (220,221). These effects are suggested to be caused by formation of chelates with biologically important trace metals such as manganese, iron and zinc. Thus, these materials are such effective metal chelates that they remove required as well as toxic metals from the soil. A further concern is biodegradability.

Table 3. Stability Constants for Various Organic Ligands with Cadmium (Cd) and Lead (Pb)[a]

Organic Ligand	Naturally Occurring or Synthetic	Stability Constants[b]	
		Cadmium	Lead
DTPA	Synthetic	19.00	18.66
EDTA	Synthetic	16.36	17.88
NTA	Synthetic	9.78	11.34
Rhamnolipid	Naturally occurring	6.89	8.58
Oxalic acid	Naturally occurring	2.75	4.00
Citric acid	Naturally occurring	2.73	4.08
SDS	Synthetic	1.95	N.D.[c]
Acetic acid	Naturally occurring	1.56	2.15

[a]From Maier and Soberón-Chávez (50).
[b]Stability constants are expressed in log values. All stability constants are from Martell and Smith (223), except for those of rhamnolipid (224) and SDS (D. Gage and R. M. Maier, unpublished data).
[c]N.D. = not determined.

EDTA is a decontamination agent that was used in nuclear facilities and routinely buried with radioactive wastes. EDTA's limited biodegradability in the environment is demonstrated by its presence in groundwater near these nuclear facilities (222). These examples illustrate the importance of choosing environmentally compatible alternatives.

Sophorolipid is one such alternative. A 4% sophorolipid solution in 0.7% HCl has been used to remove copper (50%) and zinc (100%) from soil. This level of removal was achieved after several washings (225). Rhamnolipids have also been tested and show selectivity for metals of concern, including cadmium, copper, lanthanum, lead, and zinc (224,226,227). Rhamnolipid-facilitated metal removal was demonstrated in bench-scale column experiments for both cadmium (228) and lanthanum (D. Gage and R. M. Maier, unpublished data). Rhamnolipids were also used to demonstrate removal of zinc and copper from a contaminated soil with a 12.6% oil and grease content (229). Polymeric biosurfactants are also effective at metal complexation. Emulsan was found to bind up to 240 μg uranium (UO_2^+)/mg emulsan (230). Fatty acids have also been investigated for removal of metals from water. Tannic acid was able to remove both cadmium (99%) and lead (96%) from water samples (231).

Industrial Processes

A number of industrial applications have been investigated for biosurfactants. Helle and coworkers (232) report the use of sophorolipid and rhamnolipid to enhance the heterogeneous enzymatic hydrolysis of cellulose and steam-exploded wood. In this study, sophorolipid was capable of increasing the hydrolysis of steam-exploded wood by 67% and also decreased the amount of cellulase adsorbed onto the cellulose.

Another application proposed for biosurfactants is as an additive for microbially enhanced oil recovery (MEOR) and cleaning oily sludges from storage tanks. In a thorough review of this topic, Banat (233) describes several MEOR field trials that have been performed. In general, the results of these field trials are positive, with increases in oil production ranging from 11 to 250% following injection of microorganisms and nutrients. Similarly, injection of a combination of kerosene and biosurfactant resulted in a 500% increase in oil production. However, as Banat points out, it is difficult to provide controls for field studies that isolate each parameter tested. Therefore, it is difficult to conclusively say that addition of biosurfactants or biosurfactant-producing microbes were the cause of the increased oil recoveries observed. More recently, several groups have noted that isolates with emulsifying abilities can be isolated from environments that are extreme with respect to temperature, salinity, or pH (161,167). Such isolates may be applicable to MEOR as well as well bore and oil tank cleanups. As the price of petroleum continues to increase and petroleum reserves shrink, enhanced oil recovery will receive more attention. Clearly, the use of MEOR is an attraction option that will be worth pursuing in the future.

Fouling of heat exchanger plates in the dairy industry is a problem that can result in contamination of pasteurized milk. Busscher and coworkers (234) have studied the thermophilic dairy organism, *Streptococcus thermophilus*. This organism produces a mix of biosurfactants during growth, which seem to have a role in detachment of the organism from surfaces. Following detachment, a surface coating is left behind, which seems to prevent attachment of closely related isolates to the surface. The authors suggest that this ability could be used to retard colonization of heat exchanger plates in pasteurizers.

Medicine

Biosurfactants, in particular the lipoproteins, are known to have antibiotic properties. In some cases, this property has potential for medical research. For example, Vollenbroich and coworkers (235) showed that surfactin has antimycoplasma properties useful for protecting cell cultures from mycoplasma infection. Others have reported the antiviral activity of surfactin (236,237). In this case, surfactin effectiveness was dependent on its structure. Surfactin with fatty acid chains of 14 or 15 carbons inactivated an enveloped virus, whereas surfactin with a fatty acid chain of 13 showed little antiviral activity. Kim and coworkers (238) describe an anti-inflammatory effect of surfactin. This effect occurs through the selective inhibition of cytosolic phospholipase A_2 (PLA_2). The PLA_2s are a family of enzymes that catalyze the hydrolysis of the fatty acid ester bonds of membrane phospholipids. The result of the hydrolysis is the formation of inflammatory mediators such as prostaglandins and leukotrienes.

Busscher and coworkers (239) report that biosurfactants produced by *S. thermophilus* can be used to coat silicone rubber of voice prostheses and prevent adhesion of *Candida albicans*. Under normal conditions, colonization by *C. albicans* limits the life of the prostheses to 3 to 4 months. In this study, a single exposure to *S. thermophilus* prevented colonization by *C. albicans* for 4 hours. The authors suggest that regular consumption of this probiotic in yogurt could significantly extend the lifetime of the prosthesis.

The activity of seven different biosurfactants, including MEL-A and MEL-B, polyol lipid, rhamnolipid,

sophorolipid, succinoyl trehalose lipids 1 and 3, against human promyelocytic leukemia cell line (HL60) was investigated (240). Results showed that each of these biosurfactants, except rhamnolipid, inhibited the proliferation of HL60 cells.

Production of Fine Chemicals

Biosurfactants and chemically modified biosurfactants are being investigated as alternatives to high-value synthetic chemicals whose use may have toxic environmental impacts. For example, Ishigami and Suzuki (241) report the synthesis of a pyrenacylester of rhamnolipid (R-PE) for use in monitoring the polarity and fluidity of solid surfaces and the attendant impact of coatings on these surface properties. The R-PE was synthesized to facilitate the use of pyrene, which is one of the most effective fluorescent probes in monitoring the micropolarity and microfluidity of surfaces. However, pyrene alone is difficult to use in aqueous systems because of its extremely low aqueous solubility and its propensity to bind to hydrophobic surfaces.

Rhamnolipids are also a source of stereospecific L-rhamnose, a compound used commercially in the production of high-quality flavor compounds and as a starting material for synthesis of some organic compounds (242). Rhamnose can be obtained from a variety of sources, including quercitrin from oak bark, naringin from citrus peels, or rutin which is found in a variety of plants. Rhamnose can also be obtained from rhamnose-containing polysaccharides produced by either plants or microorganisms. However, processing rhamnose from these materials is difficult. Plant sources of rhamnose are bulky and generate unwanted waste products. Rhamnose in polysaccharides must be separated from other sugar components. An alternative to these rhamnose sources is the use of rhamnolipid produced by *P. aeruginosa*. Rhamnolipids are excreted by *Pseudomonas* in the late log and stationary phase allowing easy separation from cells. Once isolated, rhamnolipid can be hydrolyzed to produce a mixture of L-rhamnose and the fatty acid 3-hydroxydecanoic acid (242).

The cosmetic and health care industries use large amounts of surfactants for a wide variety of products, including: insect repellents, antacids, acne pads, antidandruff products, contact lens solutions, hair color and care products, deodorants, nail care products, lipstick, eyeshadow, mascara, toothpaste, denture cleaners, antiperspirants, lubricated condoms, baby products, foot care products, antiseptics, shaving and depilatory products, and moisturizers (243). Biosurfactants in general are considered to have some advantages over synthetic surfactants, the predominant type of surfactant used in this industry. These advantages are low irritancy or anti-irritating effects and compatibility with skin (243). Sophorolipids and rhamnolipids in particular are being used as cosmetic additives (1,244, Iwata Chemical Co., personal communication). There are currently patents for use of rhamnolipids to make liposomes (245) and emulsions (246), both of which are important in the cosmetic industry. Similarly, there are patents for the use of sophorolipid as a humectant in cosmetics (247,248).

Surfactants and emulsifiers are routinely used in the food industry. Torabizadeh and coworkers (249) found the mannoprotein emulsifier produced by *Saccharomyces cerevisiae* could be used as a mayonnaise additive. This emulsifier is found in the cell wall and can be obtained cheaply and in high yield (80 to 100 g/kg (g/g) wet cells). In addition, it is stable from pH 3 to 11, and the by-products can be used as animal feed supplements.

Biological Control

There are several reports on the efficacy of biosurfactants in biological control. Rhamnolipids have been shown to have activity against zoosporic plant pathogens, specifically *Pythium aphanidermatum*, *Phytophthora capsici*, and *Plasmopara lactucae-radicis*. In a study reported by Stanghellini and Miller (53), mono- and dirhamnolipid were found to cause loss of motility and lysis of zoospores in less than one minute. Effective concentrations ranged between 5 and 30 mg/L, depending on the rhamnolipid tested. The same group also demonstrated the efficacy of rhamnolipid in a near-commercial hydroponic recirculating cultural system. In this system, the recirculating tubs were inoculated with a *Pseudomonas* strain isolated from the greenhouse system and received inputs of olive oil as a substrate for growth. Therefore, rhamnolipids were produced in the system. Though positive results were observed, it was noted that results were not consistent in performance and/or sustained control.

Research indicates that surfactin and a similar lipopeptide, iturin A produced by *B. subtilis* RB14, play a role in the suppression of damping-off disease of tomato seedlings caused by *Rhizoctonia solani* (250). This group compared the effect of RB14 with a RB14 mutant, R*1, deficient in production of surfactin and iturin A, on prevention of damping off. Results showed that the wild type suppressed *R. solani* and prevented damping-off, whereas the mutant did not. This result was further confirmed using a transformant of R*1 in which the production of iturin A was restored and the production of surfactin was partially restored. This isolate was able to prevent damping off. Further, this group provides evidence that iturin A is stable in soil for one to two weeks and surfactin is stable for even longer periods of time.

CONCLUSION

Demand for new specialty chemicals in the agriculture, cosmetic, food, pharmaceutical, and environmental industries is steadily increasing. As these chemicals must be both effective and environmentally compatible, it is natural to turn to the microbial world to try to meet this demand. Microbes offer a largely unexplored variety of chemicals, such as biosurfactants, which have exciting potential for industrial application. However, to realize the application of any new chemical product is difficult. First and foremost, demonstrations of product efficacy have to be obtained. Then the new microbial product must be produced at an economical scale and yield, as well as regulatory testing requirements need to be met.

This article documents the types of biosurfactants that have been identified thus far, methods for screening for surfactant and emulsifying activities, and potential industrial and biotechnology applications for biosurfactants. Biosurfactants, including rhamnolipid, sophorolipid, and spiculisporic acid, can now be produced at high yield in large-scale fermenters and are becoming economically competitive with synthetic surfactant alternatives. Therefore, it seems likely that in the future, biosurfactants will be competitive for certain industrial and medical applications currently employing synthetic surfactants.

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BIOTECHNOLOGICAL APPLICATIONS OF BIOSURFACTANTS AND BIOEMULSIFIERS.

See BIOSURFACTANTS: TYPES, SCREENING METHODS, AND APPLICATIONS

BIOTECHNOLOGY, FUNGI IN.

See FUNGI, FOR BIOTECHNOLOGY

BIOTECHNOLOGY, METHANOTROPHS IN.

See METHANOTROPHIC BACTERIA

BIOTERRORISM

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History has illustrated the utility of biological agents as weapons of war. Although at least ten countries are suspected to have offensive biological warfare programs at the national level, the threat of biological attack from state and non-state-sponsored terrorists is a growing concern. Biological warfare may be defined simply as the use of a biological organism or biologically derived toxin or other substance to cause lethal or incapacitating effects. Agents may be used to target humans, crops or livestock, or nonliving but economically vital material such as an oil supply. An act of biological terrorism may be employed in a multitude of scenarios ranging from a small-scale attack on a restaurant salad bar incapacitating tens to hundreds, to the covert aerosolization of smallpox virus in a crowded auditorium that would kill an estimated 15,000 people causing confusion, hysteria, and civil unrest (1). Understanding the threat of bioterrorism is crucial in developing proper procedures and countermeasures to provide an effective response to an attack.

This article focuses mainly on the technical aspects of biological warfare as they apply to a terrorist attack. A history of biological warfare, employment scenarios, agent characteristics, aspects of dispersion, and countermeasures are covered in this review. Because of the sensitivity of this topic, some areas are covered more generally than others, so as not to provide helpful information to a would-be terrorist.

HISTORY OF BIOLOGICAL WARFARE

Early Development

Humans have known of the utility of biological organisms and toxins as weapons of war well before the germ theory of disease was understood. One of the first recorded events in which a biological agent was used in war was the siege of Kaffa, which occurred in the fourteenth century in what is now Feodosia, Ukraine. The attacking Tartar force took advantage of a plague outbreak among their ranks by catapulting bodies of their deceased into the city to create a plague epidemic. The ensuing spread of plague led to the fall of Kaffa to the Tartar force (2,3). For centuries to follow, diseased animals and humans were used to contaminate the water supplies of would-be aggressors in the hope of creating an epidemic that would affect the outcome of the battle. During the French and Indian war in 1763, the British successfully used smallpox-infected blankets to create epidemics among the native American tribes opposing the British (3).

World War I

Advances in microbiology through the nineteenth century allowed the isolation and identification of disease-causing microorganisms, which could be used to attack with some degree of specificity. World War I saw the use of biological agents to attack livestock. Strong evidence exists indicating that Germany used *Bacillus anthracis* and *Burkholderia mallei*, the causative agents of anthrax and glanders, to infect livestock during transshipment. Anthrax and glanders agents were reportedly used by Germany to infect Romanian sheep being transported to Russia. Additionally, German covert operators also may have used glanders to infect 4,500 mules in Mesopotamia and horses in France. They were also used in operations in the United States targeting horses bound for allied forces in Europe (2).

Extensive use of chemical weapons during World War I prompted the creation of the Geneva protocol of 1925, which called for the prohibition of the use in war of asphyxiating, poisonous or other gases, and of bacteriological methods of warfare. Although the Geneva Protocol prohibited the use of biological weapons, it did not prohibit research and development, production or storage of biological weapons. Many countries, while signing the protocol, maintained the right and capability to respond in kind to a biological attack. These countries included Belgium, Canada, France, Great Britain, Italy, the Netherlands, Poland, and the Soviet Union (2).

World War II

During World War II, Japan conducted extensive biological weapons (BW) research from 1932 to 1945 (2). The focal point of the Japanese BW program, called Unit 731, was located near the town of Pingfan and had a staff of over 3,000 scientists working in over 150 buildings and five satellite camps. Unit 731 conducted at least 12 biological weapons field trials and utilized prisoners of war as their test subjects (2). Agents tested include *B. anthracis, Neisseria meningitidis, Shigella species, Vibrio cholerae*, and *Yersinia pestis*. Japan used biological weapons on at least 11 Chinese cities during World War II. The tactics used in these attacks included contamination of food and water supplies as well as tossing cultures directly into homes and spraying agents from aircraft. The Japanese allegedly

used fleas fed on plague-infected rats to drop from aircraft over Chinese cities. As many as 15 million fleas were used per attack to infect the population with plague (2).

The use of biological weapons by the Japanese during World War II also made clear the dangers to the attacking force if they employed biological weapons. A biological attack on Changteh in 1941 led to a reported 10,000 Chinese casualties and 1,700 deaths among Japanese soldiers. Japan stopped field trials in 1942, but basic research in biological warfare continued until the end of the war (2).

The U.S. Program

The United States Biological Warfare Program began in 1942, and included a research and development facility at Camp Detrick (now Fort Detrick), Maryland, test sites in Mississippi and Utah, and a production plant in Terra Haute, Indiana. Experiments were performed on potential biological agents such as *B. anthracis* and*Brucella suis*. Experiments were also performed using nonpathogenic simulants such as *Serratia marcescens* and *Bacillus globigii* to test production, storage, and aerosolization methods for biological warfare agents (2). The United States also collaborated with its allies, including Canada and Great Britain, on biological warfare applications. Live fire tests by the allies in 1943 were conducted using anthrax bombs at Gruinard Island off the coast of Scotland. These experiments led to long-term contamination of the island due to the persistence of the anthrax spores tested. In 1986 and 1987 the island was successfully decontaminated using formaldehyde and was handed over to its original owners in May 1990 (2,4).

Expansion of the U.S. program during the Korean War included construction of a new production facility in Pine Bluff, Arkansas, and a countermeasures program for biological defense, which began in 1953. Animal studies using live agents were also carried out at Camp Detrick, remote desert sites, and on barges in the Pacific Ocean. Human tests were begun in 1955 using military and civilian volunteers to evaluate the effects of aerosol exposure to *Francisella tularensis* and *Coxiella burnetti* (2). By the late 1960s the United States had developed a substantial biological arsenal that comprises bacterial and toxin agents for antipersonnel use. This arsenal included both lethal and incapacitating agents such as *B. anthracis*, *F. tularensis*, *Brucella suis*, *C. burnetti*, botulinum toxin, and Staphylococcal enterotoxin B. The United States also developed plant pathogens as biological weapons, which would be used to target an enemy's food supply. These agents included rice blast, rye stem rust, and wheat stem rust (2).

In 1969 President Richard M. Nixon terminated the U.S. biological weapons program. Because of the already robust stockpiles of conventional, chemical, and nuclear weapons, it was believed that a biological weapons arsenal was not needed as a strategic deterrent. Furthermore, because of ethical and public health reasons, the true potential of biological weapons could not be tested. For this reason, BW was considered unproven as well as unpredictable and uncontrollable thus making them potentially hazardous to the attacking party (2,3). All stockpiles of biological weapons were destroyed by 1973, but research related to defense against biological warfare agents continues.

Former Soviet Union

The Soviet biological warfare program, which reportedly began during the early 1900s, was the largest of all national programs. After the signing of the Biological Weapons Convention in 1972, the Soviet biological weapons program continued under a clandestine organization called Biopreparat. Biopreparat operated over 40 research and production facilities scattered across the Soviet Union, employing up to 55,000 scientists. Many government agencies were involved in the BW program, including the Ministries of Defense, Agriculture, and Health, the Soviet Academy of Sciences, the Central Committee to the Communist Party, and the KGB (5).

The Soviet BW program considered scores of lethal bacteria, toxins, and viruses for use in its biological program, including anthrax, plague, ricin, ebola, marburg, and smallpox. Hundreds of tons of anthrax and dozens of tons of plague and smallpox were stockpiled for use against the United States and its allies (5). The Soviet Union also reportedly used biological agents as assassination weapons. For example, ricin toxin weaponized into a small pellet in an umbrella gun was reportedly used by the secret service of the Soviet Union to assassinate Georgi Markov, a Bulgarian defector living in London (2,5).

In April 1979, an outbreak of inhalational anthrax occurred in the city of Sverdlosk in Russia, which caused at least 77 clinical cases and 66 deaths. Persons working and living within 4 km from the suspected source were affected, whereas livestock were affected up to a distance of 50 km from the facility. Although an initial cover-up by the Soviet government claimed that the epidemic was caused by infected meat, suspicions that an accident occurred at a secret Soviet anthrax facility in Sverdlosk were prevalent (2,6). In 1992, Russian President Boris Yeltsin admitted that the outbreak at Sverdlosk was caused by the accidental release of anthrax spores from an offensive biological warfare production facility, specifically from the inadvertent removal of air filters on the exhaust stacks at the portion of the plant were the anthrax agent was dried (1,5). Since the fall of the Soviet Union, Russia claims that it has shut down all remaining remnants of its offensive biological warfare program.

Iraq

Before the Persian Gulf War, intelligence reports indicated that Iraq was engaged in an extensive offensive biological warfare program. When the Gulf war ended, the United Nations Special Commission on Iraq (UNSCOM) obtained information that confirmed these reports. Iraqi officials admitted to having an offensive biological warfare program, which included basic research on anthrax, rotavirus, camelpox, aflatoxin, and botulinum toxins, as well as T-2 mycotoxin, and the anticrop agent wheat cover rust (2). Some of these agents were reportedly weaponized in missile warheads and bombs. The Iraqi government claims to have destroyed its

biological arsenal after the war, and some research and production facilities were destroyed under the supervision of UNSCOM in the mid-1990s (2). The United Nations Monitoring and Verification Commission (UNMOVIC) is positioned to reenter Iraq and resume the monitoring and verification of the destruction of Iraq's weapons of mass destruction programs. UNMOVIC will replace the UNSCOM inspection regime, which was ousted by Iraq in late 1998. Iraq has not yet given permission to allow UNMOVIC to re-enter the country, and thus the status of the Iraqi biological warfare program remains unknown.

Aum Shinrikyo

Although the Aum Shinrikyo cult is most famous for its March 20, 1995 Sarin attack on the Tokyo subway system, subsequent investigations indicate that the cult was also involved in biological terrorism as well. In addition to producing chemical agents, Aum Shinrikyo reportedly performed research on botulinum toxin, anthrax, cholera, and Q fever. In 1992, a team of Aum cult members was detected in Zaire on an alleged medical mission; whereas the actual purpose of the trip was to obtain samples of the Ebola virus. Cult members also reportedly launched three biological attacks in Japan using botulinum toxin and anthrax, which were unsuccessful. Although many of the cults leaders are either in jail or still on trial, the Aum may still be capable of carrying out future attacks using biological agents (7).

BIOLOGICAL AGENT CHARACTERISTICS

Biological Versus Chemical Weapons

Although the terms "biological" and "chemical" weapons are used synonymously, there are profound differences between these two types of weapons of mass destruction. The most obvious difference is that chemical agents are nonliving poisons made by humans, whereas biological agents are infectious living entities that reproduce inside the host to cause incapacitating or fatal disease. Biologically derived toxins such as botulinum toxin or ricin share characteristics of both chemical and biological agents (8).

With regard to biological agent use, the phrase, "a little goes a long way" has direct meaning. Pathogenic organisms multiply within the host to cause disease, meaning that relative to chemical agents small quantities of biological agent, if properly disseminated through the air, have the ability to inflict casualties over a large area (8,9). The inhaled infectious doses for biological organisms are measured from tens to tens of thousands of cells depending on the organism (Table 1). "Weight for weight, biological and toxin weapons are hundreds to thousands of times more potent than the most lethal chemical warfare agents" (8). Figure 1 illustrates the lethal dose range in terms of quantity of agent for chemical, biological, and toxin weapons (8). Even a small-scale operation only producing a few hundred grams or agent may be sufficient to cause significant casualties. Some biological agents such as *Yersinia pestis* and Variola major (smallpox) have the added characteristic of being easily contracted from human-to-human contact. Thus, unlike chemical agents, biological agents have the potential to spread through human transmission from the area immediately affected to produce casualties of epidemic, or possibly pandemic proportions.

On a small scale, production of biological agents does not require a large amount of specialized equipment. All equipment necessary for production of biological agents is commercially available and procedures for their production are readily available in the scientific literature and require little specialized expertise outside of a basic knowledge of microbiology. Comparatively, some processes required for production of chemical agents are more complex and may require special knowledge

Table 1. Infective Doses (Aerosol) for Commonly Considered Biological Warfare Agents (10)

Agent	Infective Dose (Aerosol)
B. anthracis	8,000–50,000 spores
Brucella species	10–100 organisms
V. cholerae	10–500 organisms
Burkholderia mallei	Assumed low
Yersinia pestis	100–500 organisms
Francisella tularensis	10–50 organisms
Coxiella burnetti	1–10 organisms
Smallpox virus	Assumed low (10–100 organisms)
Venezuelan Equine Encephalitis Virus (VEE)'	10–100 organisms
Viral hemorrhagic fevers	1–10 organisms
Clostridium botulinum toxin	0.001 μg/kg in humans for *C. botulinum* type A toxin
Staphylococcus aureus enterotoxin B	0.03 μg/person for incapacitation
Ricinus communis toxin	3–5 μg/kg is the lethal dose for mice
Trichoethecene mycotoxins	Moderate

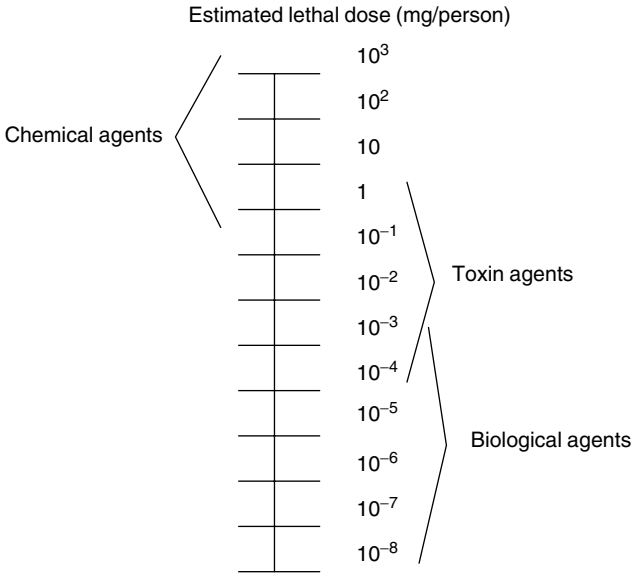

Figure 1. Toxicity of chemical and biological agents (8).

and equipment. Furthermore, production of chemical agents requires the procurement of some specialized and controlled precursor chemicals, which are not readily available (8). Because the production of biological agents does not require large amounts of chemicals or equipment, facilities used for production on a terrorist scale may be relatively easy to hide.

Although the production of biological agents appears relatively simple compared with that of chemical agents (Table 2), some obstacles exist. First only a small amount of pure starter culture is required, however, it may be difficult to acquire. Until recently, many infectious agents were accessible through commercially available culture collections and were fairly easy to obtain. Cases such as that of Larry Wayne Harris, who was arrested after obtaining cultures of *Yersinia pestis* through mail order in May 1995 and again in February 1998 after obtaining a *B. anthracis* strain have prompted tighter governmental regulation of those organisms that could be used as biological warfare agents (Table 3). Additionally, working with biological and toxin agents does pose a significant health risk to the operator if agents are not handled properly. Production of highly infectious or toxic biological agents will probably require some sort of personal protection or containment to be handled safely (8).

Table 2. Key Low-Technology Production Techniques for Biological and Toxin Weapon Agents (8)

Type of Agent	Low-Tech Production
Bacteria	Batch fermentation, production in animals
Rickettsiae and viruses	Cultivation in eggs, mouse brains, or tissue culture
Protein toxins	Batch fermentation and purification of a bacterial toxin, or extraction of toxin from a plant or animal source
Nonprotein toxins	Extraction from plant or animal source

Another major difference between biological and chemical weapons use is the timescale in which an attack may be detected. Generally, chemical agents produce symptoms of exposure in less than a minute to a few hours. These agents may have an acrid smell or taste, produce a colored smoke, or a burning sensation on the skin, lungs and mucous membranes (8). These factors combined will most probably lead to a relatively quick determination that a toxic chemical was involved. This was illustrated during the March 1995 Sarin gas attack on the Tokyo subway system by the Aum Shinrikyo cult, which killed 12 and injured almost 3,800. Authorities were able to assess within a short time that a chemical attack had occurred and were raiding Aum Shinrikyo facilities within 48 hours (7); however, this would have not been the case if this attack had been performed covertly with anthrax or plague.

The onset of symptoms from exposure to biological agents may vary from less than minutes to a few hours for toxin agents, to a range of a few days to weeks for

Table 3. CDC List of Critical Biological Agents (11)

Category A — high priority agents include organisms rarely seen in the United States. These agents pose a risk to national security because they can be easily disseminated or transmitted person-to-person, cause high mortality, with potential for major public health impact, might cause public panic and social disruption, and require special action for public health preparedness	Variola major (smallpox) *Bacillus anthracis* (anthrax) *Yersinia pestis* (Plague) *C. botulinum* toxin (Botulism) *F. tularensis* (tularemia) Ebola virus Marburg virus Lassa virus (Lassa fever) Junin virus (Argentine hemorrhagic fever) and related viruses
Category B — second highest priority agents include those that are moderately easy to disseminate, cause moderate morbidity and low mortality, and require specific enhancements of CDC's diagnostic capacity and enhanced disease surveillance	*Coxiella burnetti* (Q fever) Brucella species (brucellosis) *Burkholderia mallei* (glanders) *Venezuelan equine encephalomyelitis* virus (VEE) *Eastern equine encephalomyelitis* virus *Western equine encephalomyletis* virus *Ricinus communis* toxin (ricin) *Clostridium perfringens* epsilon toxin *Staphylococcus aureus* enterotoxin B
Category B subset — includes pathogens that are food and waterborne.	These pathogens include but are not limited to: *Salmonella* species Shigella dysenteriae *Escherichia coli* O157 : H7 *V. cholerae* *Cryptosporidium parvum*
Category C — Third highest priority agents include emerging pathogens that could be engineered for mass dissemination in the future because of availability, ease of production and dissemination, potential for high morbidity and mortality and a major health impact	Nipah virus Hantaviruses Tick-borne hemorrhagic fever viruses Tick-borne encephalitis viruses Yellow fever virus Multidrug-resistant Tuberculosis

infectious agents. Furthermore, for infectious biological organisms, and most biological toxins, the agent cloud is odorless, colorless, and produces no immediate sensation upon contact with the skin, lungs, or mucous membranes. These factors increase the chances that a biological agent may be covertly employed in a terrorist scenario with little or no warning until symptoms appear.

Biological agents used in a terrorist scenario may be endemic or exotic to a particular environment. A biological attack that utilizes a common or indigenous organism may be difficult to attribute to a terrorist attack because of the commonality of the organism, which was used. This was the case in a September 1984 outbreak of salmonellosis caused by a strain of *Salmonella typhimurium* in the community of Dalles, Oregon, which affected 751 people. Followers of the religious commune Bhagwan Shree Rajneesh, evaluated the use of *S. typhimurium* as an incapacitating biological agent in an attempt to influence a community vote affecting the construction of their new international headquarters. At least ten restaurants were affected in two phases spanning a total period of approximately four weeks (12). Although the *S. typhimurium* infections comprised the largest outbreak of food-borne disease reported to the Centers for Disease Control (CDC) in 1984, it took over a year after the outbreak had occurred to acquire sufficient evidence to attribute the unusual outbreak to the Rajneesh commune. Because salmonellosis is a common food-borne pathogen, the intentional nature of the outbreak in Dalles went initially unrecognized. An extensive epidemiological investigation was required to rule out the hypothesis that the outbreak occurred from "other factors" (12).

A biological attack that utilizes an exotic or nonendemic organism may be easier to identify as unusual once identified, however, a different set of problems exist regarding diagnosis. The initial symptoms of infection are similar for many pathogens, both lethal and nonlethal. Fever, chills, headache, fatigue, persistent cough, nausea, and vomiting comprise many of the initial symptoms of exposure to a biological agent by inhalation or ingestion. These symptoms are also prevalent in common, non-life-threatening illnesses such as respiratory infection, common cold, or influenza. Because they are similar to those of common illnesses, initial symptoms of exposure to a biological agent may not be properly diagnosed until symptoms appear that would discern from a common illness. The delay in proper diagnosis also causes delays in treatment and prophylaxis that may mean the difference between patient-survival and death. This is illustrated clearly when looking at the symptomology of inhalational anthrax exposure. Initial symptoms of anthrax exposure include fever, malaise, fatigue, cough, and mild chest discomfort. These symptoms are followed by respiratory distress and other severe symptoms. Shock and eventual death usually occur within 24 to 36 hours after onset of these severe symptoms and treatment at that point is largely ineffectual (6).

All of the factors that describe the nature of a biological terrorist event indicate that the emergency response may not be immediate, unlike the Aum Shinrikyo Sarin attack on the Tokyo subway. Because of the delayed onset of discerning characteristics of a biological attack, the first responders will probably not be hazardous materials teams, fire departments and police, but instead will be health care professionals working in emergency rooms, health clinics, and doctors offices. In the event of a biological attack, pressure will be placed on these individuals to have the ability to recognize a biological attack by symptomology and frequency of cases (13). Pressure will also be placed on the clinical laboratories to maintain the equipment, assays, and expertise to detect and characterize exotic or uncommon infectious agents rapidly and decisively. Moreover, disease surveillance and communication systems are required to quickly collate and analyze epidemiological data to recognize an outbreak of disease that may be attributable to a biological attack (13).

There are hundreds of biological organisms and toxins that can be used in a terrorist scenario to cause incapacitating or lethal disease, or produce toxic effects. The following comprises some of those that are most commonly considered biological warfare agents.

Bacteria

Anthrax (*B. anthracis*). *Bacillus anthracis*, the causative agent of anthrax is a gram-positive, spore-forming, rod-shaped, bacterium that is found primarily in herbivores. Cattle, sheep, goats, and horses are common domestic animal hosts. The disease may be contracted by humans from eating infected and improperly cooked meat, introduced through cuts or abrasions in the skin, or possibly by flies. Inhalational anthrax may be contracted by handling contaminated animal materials such as blood, flesh, hides, and bones (9,10). Anthrax was employed by both the United States and Russia as a biological warfare agent (5,10). Iraq has also admitted to performing research on weaponized anthrax before the Gulf War (9). *B. anthracis*, in its spore form is extremely resistant to environmental stresses. The sporulated form can survive for years in water and soil, and is also resistant to heat and ultraviolet light. This allows it to maintain viability during long-range travel through the air (9,14). Although the infectious inhaled dose for *B. anthracis* is higher than that of other biological agents (Table 1), its durability makes it desirable as a biological agent. The incubation period for anthrax varies from one to six days depending on the inhaled dose, and human-to-human transmission is rare. Initial symptoms include fever, malaise, fatigue, cough, and mild chest discomfort followed by severe respiratory distress, shock, and death (10). An effective FDA licensed vaccine does exist and inhalational anthrax is treatable with antibiotics. However, once the onset of severe symptoms has occurred, treatment is largely ineffective (9). In humans, the mortality rate of untreated cutaneous anthrax ranges up to 25%; in inhalational and intestinal cases, the mortality rate in untreated patients is virtually 100% (10).

Brucellosis (*Brucella* spp.). There are four organisms in the genus *Brucella* that are pathogenic to man. Although closely related, these bacterial organisms primarily infect different animal hosts (9). *Brucella melitenis* infects goats,

B. suis infects swine, *B. abortus* infects cattle and *B. canis* infects dogs. The severity of human disease from these organisms also varies with *B. melitensis* being the most severe followed by *B. suis*, *B. abortus*, and *B. canis*. Organisms of the *Brucella* species are small, slow-growing, gram-negative coccobacilli that are susceptible to heat and most disinfectants, but may survive for several weeks in dust, soil, or water. Most human infections are caused by contact or ingestion of contaminated raw meat or dairy products, and person-to-person transmission is rare (9,10).

The United States weaponized *B. suis* in the 1950s but stopped offensive work on this organism a decade later (9). Although *Brucella* species are non-spore-forming and may be considered relatively fragile, these organisms are extremely infectious by the aerosol route. It is estimated that as few as 10 to 100 inhaled bacteria are sufficient to cause infection in humans. The incubation period is highly variable ranging from 5 to 60 days and the symptoms are nonspecific. Symptoms include irregular fever, chills, headache, sweating, depression, and mental health changes (10). Respiratory symptoms may also occur in about 20% of those infected (9). The mortality rate for untreated brucellosis in humans is approximately 5%, and it is primarily considered a disabling or incapacitating type of disease (10).

Cholera (*V. cholerae*). *Vibrio cholerae* is a waterborne pathogen affecting multiple animal species. Cholera is usually contracted through ingestion of contaminated water and is endemic to many third world countries where poor sanitation and overcrowding are common. A recent and extremely large cholera outbreak in Peru produced over 250,000 symptomatic cases in Peru alone and spread to surrounding countries as well (9). *V. cholerae* affects multiple animal species including humans but is not readily transmitted from host to host. *V. cholerae* is easily killed by desiccation, steam and boiling, and is not viable in pure water. However, the organism may survive up to 24 hours in sewage and as long as six weeks in water containing organic, matter including sewage (10,14). *Vibrio cholerae* was reportedly considered by some countries for use as a biological warfare agent, however, because the amount of agent required to produce mass casualties is high, the organism is not considered to be effective. The use of *V. cholerae* on a small scale may still be effective in producing casualties in a terrorist scenario. *V. cholerae* is a short, motile, gram-negative, non-spore-forming rod. Two serotypes, O1 and O139, have been associated with disease in humans (10). The incubation period ranges from four hours to five days depending on the amount ingested and the ratio of symptomatic to asymptomatic cases is approximately 1 : 400. Symptoms include vomiting, headache, and intestinal cramping followed by severe and painless diarrhea that may produce fluid losses of 5 to 10 liters per day. Death may occur from severe dehydration, decreased amount of blood in the body, and shock. The mortality rates in humans for untreated cholera may be as high as 50%. Treatment includes continuous administration of fluids and electrolytes, as well as antibiotics. A licensed vaccine is available, but only provides 50% protection for as long as six months (10).

Glanders (*Burkholderia mallei*). *Burkholderia mallei*, the causative agent of glanders, is a gram-negative rod, which produces disease primarily in horses, mules, and donkeys. Human cases of glanders historically have been associated with veterinariansas well as horse and donkey caretakers. Although reports of human infection are rare, laboratory cultures of *B. mallei* have shown to be extremely infectious via the aerosol route. Because of the lack of a vaccine and effective therapy to treat the infection, *B. mallei* can be considered a potential biological agent (10). *B. mallei* was reportedly used during World War I to infect large numbers of horses and mules supporting allied operations in Russia. The Japanese also used *B. mallei* on horses, civilians, and prisoners of war during World War II. The United States and Russia also reportedly considered *B. mallei* as a biological warfare agent during the World War II era (2,10).

Glanders may occur in four forms in humans; an acute localized form, a septicemic and rapidly fatal form, an acute pulmonary form, and a chronic form. No form of glanders is readily transmissible from person to person. Aerosol infection may produce any of these four forms of glanders, or a combination of several. Incubation periods range from 10 to 14 days after exposure. Symptoms from inhalational exposure to *B. mallei* include fever, rigors, sweats, myalgia, headache, chest pain, swelling in the lymph nodes, and pustular eruptions. The mortality rate for septicemic infection is almost 100% if untreated. Recovery may occur for the chronic form, although this form may also turn septicemic (10).

Plague (*Y. pestis*). *Yersinia pestis*, the causative agent of plague is a nonmotile, gram-negative, non-spore-forming rod, which exists naturally as a zoonotic disease of rodents (rates, mice, and ground squirrels). Human plague is most commonly caused by bites from fleas, which live on infected rodents. Transmission to human by fleas produces the common bubonic form of the disease. Naturally occurring inhalational plague in humans is extremely rare. Although both the United States and Russia considered *Y. pestis* as a biological agent, only Russia chose to weaponize it. The Japanese Unit 731 reportedly inflicted heavy casualties by releasing plague-infected fleas of China during World War II (2,9). *Y. pestis* may remain viable in water and some moist environments for several weeks, and may survive for months to years at near freezing temperatures, but is susceptible to heat and ultraviolet light. Although *Y. pestis* is a relatively fragile organism, its low infectious dose and ability to spread from person to person make it a desirable biological agent capable of causing massive casualties if released in the aerosol form. The incubation for the pneumonic or inhalational form of plague in humans is two to three days. Symptoms include high fever, cough, chills, headache, and bronchial hemorrhaging, which progresses rapidly causing death by respiratory failure and circulatory collapse. The incubation time for the bubonic form of plague is approximately 2 to 10 days. Symptoms include fatigue, high fever, and tenderness in the lymph nodes, liver, and spleen (9,10). The bubonic form may also produce skin lesions and pustules containing virulent plague bacteria, and may spread spontaneously

to the blood stream, the central nervous system, lungs, and other organs. Although a licensed vaccine is available, it is not effective against exposure to plague through the aerosol route. The mortality rate for untreated pneumonic and bubonic forms of plague are 100 and 50%, respectively (10).

Tularemia (*F. tularensis*). *Francisella tularensis*, the causative agent of tularemia is a small, gram-negative, non-spore-forming cocco-bacillus. The disease is primarily zoonotic, and human cases are usually caused by contact with tissue or fluids from infected animals, or from the bites of infected insects. Human cases caused by inhalation or ingestion of *F. tularensis* are less common (9,10). Exposure by aerosol route, typical of a biological attack, would produce a pneumonic tularemia (10). The United States weaponized *F. tularensis* during its biological warfare program during the 1950s and other countries are suspected of its use as well. *F. tularensis* is easily killed by heat and disinfectants, but may remain viable for weeks in water, soil, carcasses and hides, and for months in freezing temperatures (10). As few as 10 to 50 organisms are sufficient to produce disease in man, which makes *F. tularensis* a desirable biological warfare agent. The incubation period for tularemia is 2 to 10 days depending on the dose. Characteristics of pneumonic tularemia include fever, headache, fatigue, weight loss, and pneumonia with nonproductive cough. The nonspecific symptoms of the pneumonic form of tularemia make it difficult to diagnose initially. Tularemia is treatable with antibiotics and an investigative live vaccine is available. The mortality rate for untreated pneumonic tularemia is approximately 35%. (9,10).

Viruses

Smallpox (Variola major and minor). Variola virus, the causative agent of smallpox occurs in at least two strains; variola major and minor. The last reported case of smallpox occurred in Somalia in 1977. In 1980, the World Health Organization (WHO) declared that smallpox has been eradicated from the planet (9,10). No animal reservoir for smallpox is known to exist, although monkeys are susceptible to infection (9). Although only two laboratories in the world (The Centers for Disease Control in Atlanta, Georgia and The Vector State Research Center of Virology and Biotechnology in the Novisibirsk region of Russia) are WHO-approved repositories for the variola virus, concerns still exist that the virus may be used as a biological weapon. The aerosol infectivity, stability, high mortality rate, contagiousness, and lack of vaccinated population would make the variola virus a desirable biological agent in a terrorist scenario (9). Russia reportedly weaponized and stockpiled smallpox as part of its biological arsenal (5). The average incubation period for smallpox is approximately twelve days. Initial symptoms from aerosol exposure include malaise, fever, rigors, vomiting, headache, and backache. Fifteen percent of patients also develop delirium (9,10). Approximately two to three days after initial symptoms appear, eruptions in the mucous membranes appear with a rash about the face, hands, and forearms. Lesions or pox, formed in the mucous membranes produce infectious secretions during the first few days of illness and are the primary means of transmission from person to person (9). The rash then spreads to the lower extremities, and finally to the trunk, producing lesions that progress to pustules. From 8 to 14 days after onset of initial symptoms, pustules progress to form scabs that may contain live variola virus. Currently, there is no effective treatment for smallpox virus. Although a live vaccine does exist, vaccinations for the general population were stopped in the early to mid-1970s leaving a large portion of the U.S. population susceptible (10). Furthermore, the smallpox vaccine, a live *Vaccinia* virus strain, may complicate vaccination because of its negative effects on immunocompromised patients (9). The mortality rate for smallpox in vaccinated and unvaccinated personnel is 3 and 30% respectively (10).

Venezuelan Equine Encephalitis Virus (VEE). Venezuelan Equine Encephalitis virus is an arthropod-borne alphavirus that is endemic to many areas in North and South America. Of the eight distinct viruses in the VEE complex that have been associated with human disease, the two most significant are variants A/B and C of the 1 subtype (10). Natural infections are caused by mosquito bites. and can also cause severe disease in horses, mules, burros, and donkeys. These animals serve as the host to VEE and are responsible for transmission of mosquito-borne infections. VEE was weaponized by the United States during its biological warfare program during the 1950s, and other countries may have also considered VEE as a biological agent (9,10). Besides being transmitted by mosquitoes, VEE is also highly infectious by aerosol, surviving best at low temperatures and relative humidity, but is easily killed by heat and disinfectants (10,14). The incubation time for VEE ranges from two to six days. Symptoms include malaise, spiking fever, rigors, headache, and light sensitivity. These symptoms may be followed by nausea, vomiting, cough, sore throat, and diarrhea. These symptoms may persist for 24 to 72 hours and full health is usually regained in one to two weeks. Four percent of children and few adults develop neurological complications because of infection. An investigational vaccine exists for VEE, and antiviral treatments have proven effective in postexposure treatment in animals, but clinical data do not exist to assess its efficacy on humans (10). The mortality rate for VEE is less than 1%, but is higher in the very young and very old (10). VEE is considered an incapacitating agent because of its low mortality rate.

Viral Hemorrhagic Fevers (Ebola and Marburg Viruses). Although the viral hemorrhagic fevers are caused by a number of virus families, Ebola and Marburg that are part of the *Filovirus* family are two of the most dangerous and have been considered for use as biological warfare agents in the past (5,9). The first cases of Ebola virus were recognized in Sudan and Zaire in 1976. These two outbreaks produced mortality rates of 53% and 92%, respectively (9). Subsequent outbreaks have occurred in Sudan in 1979 and in Zaire in 1995, which have produced a large number of casualties. In 1989, a strain of Ebola

determined to be nonpathogenic to humans was found in a monkey quarantine facility in Reston, Virginia. Marburg was first recognized in 1967 in Germany, where it produced 31 cases and 9 deaths. Human infection from these RNA viruses seems to be associated with contact with monkeys; however, the reservoir for these viruses in nature is not known. Human-to-human transmission of these viruses is not fully understood, but they definitely spread from direct contact with infected bodily fluids and organs (10). Russia reportedly performed extensive research and development on the production and weaponization of Ebola and Marburg (5). The high mortality rate, low infective dose, and lack of effective treatment would make these agents desirable for use as a biological warfare agent. The incubation times for viral hemorrhagic fevers may range from 4 to 21 days (10). Symptoms include fever, muscular pain, headache, vomiting, and diarrhea. Viral hemorrhagic fevers produce vascular damage, and changes in the permeability of the vascular system. Full blown viral hemorrhagic fever evolves into shock and hemorrhaging from the mucous membranes, and may be accompanied by neurological or pulmonary difficulties, or hemorrhaging on the skin (9,10). The mortality rate for Ebola may range between as high as 50 and 90%, depending on the strain. No vaccine or effective treatment is available for the Ebola and Marburg viruses, however, recent research is ongoing to develop a DNA vaccine for the Ebola using genes coding for virus proteins (9).

Toxins

Botulism (*C. botulinum* toxin). *Clostridium botulinum* toxin, the causative agent of botulism is comprised of seven related neurotoxins, A through G, which are produced from the anaerobic, gram-positive, spore-forming bacillus *C. botulinum*. The most common form of botulism in humans is caused by ingestion of contaminated foods and canned goods. However, the symptoms caused by food-borne exposure to *C. botulinum* toxin are very similar to those produced by aerosol exposure. *C. botulinum* toxin in extremely small amounts also has medicinal uses in treating muscle spasms and wrinkles. *C. botulinum* toxin has been researched by numerous countries for use as a biological weapon. Iraq admitted to The United Nations that it had performed research on and weaponized at least 100 munitions with *C. botulinum* toxin before the Persian Gulf War (9,10). The time from inhalation of botulinum toxin to onset of symptoms ranges from 24 to 36 hours to several days depending on the inhaled dose. Symptoms include bulbar palsies, blurred vision, and sensitivity to light. This is followed by skeletal muscle paralysis, which descends down the body as it progresses. Death is usually caused by respiratory failure caused by paralysis of respiratory muscles. With proper respiratory assistance, mortality rates due to exposure to *C. botulinum* may be less than 5% (9). The time taken from onset of symptoms to respiratory failure may be as little as 24 hours in food-borne botulism (9,10). An investigational vaccine is available for *C. botulinum* toxin types A through E, and has been shown to be effective in preventing botulism and in the treatment of botulinum exposure. Botulinum toxins are among the most toxic substances known (Table 1), and are 100,000 times more toxic than sarin gas, a well-known chemical warfare agent (10). Although it is not an infectious organism, the high toxicity and availability of *C. botulinum* would make it an effective agent in a bioterrorist attack.

***Staphylococcus aureus* enterotoxin B(SEB).** *Staphylococcus aureus* is a gram-positive cocci that produces a number of exotoxins including enterotoxin B. Exotoxins comprise those toxins that are released outside of the cell. The term enterotoxin indicates that the primary effects of the toxin occur in the intestines (9,10). SEB intoxication is caused by ingestion of improperly handled foods, and is extremely common, however, inhalation of SEB produces a distinctly different set of symptoms (10). Although SEB exposure through ingestion or inhalation does not normally produce death, 80% of those exposed may be incapacitated for 1 to 2 weeks (9,10). Symptoms may occur from 3 to 12 hours after inhalation. These symptoms include sudden onset of fever, headache, chills, muscular pain, and a nonproductive cough. Severe cases may also experience shortness of breath and chest pain. Inadvertent ingestion of the toxin may also produce nausea, vomiting, and diarrhea. Symptoms from SEB inhalational exposure are similar to those for common respiratory pathogens, which may complicate the diagnosis. Although several SEB vaccines are under development, no human vaccine is available, and treatment is supportive in nature, designed to alleviate the symptoms (10).

Ricin Toxin (*R. communis*). Ricin toxin is produced by the *R. communis* or castor plant, which is grown worldwide for the production of castor oil that is extracted from the castor bean. The waste product from castor oil production may contain as much as 5% ricin toxin by weight. The toxin is fairly persistent in the environment and is extremely toxic when inhaled, ingested, or injected (10). A few countries are suspected of performing research on ricin to be used as a biological weapon. Ricin injected from a specially designed umbrella gun was allegedly used to assassinate the Bulgarian exile Georgi Markov in London in 1978 (5,10). Because of its large availability and relative ease of production, ricin toxin could be used in a terrorist attack. Onset of symptoms may occur within 18 to 24 hours after exposure. Symptoms from inhalation of ricin toxin vary depending on the dose inhaled, but may include fever, chest tightness, cough, shortness of breath, nausea, and joint pain. Although data on human exposure to lethal doses of ricin are not available, animal models indicate that destruction of cells in the respiratory system may be sufficient to cause death if sufficient toxin is inhaled. Ricin toxin may cause severe gastrointestinal symptoms, followed by vascular collapse, and death when ingested, and circulatory and multiple organ failure when injected (10).

No human vaccine is available for Ricin exposure, and treatment is given to attempt to alleviate the symptoms (10).

Yellow Rain (trichoethecene mycotoxins). The trichoethecene of T-2 mycotoxins are low molecular weight

compounds produced by several types of filamentous fungi including *Fusarium*, *Myrotecium*, *Trichoderma*, and *Stachybotrys*. Mycotoxins may produce casualties when inhaled, ingested, or from contact with the skin, making them unique in that they are one of the only dermally active biological agents. Controversial reporting indicates that mycotoxins may have been used in biological attacks in Laos, Kampuchea, and Afghanistan during the 1970s and the early 1980s, producing an estimated 8,300 deaths resulting from exposure (10). The term "yellow rain" is derived from the characteristic yellow cloud that is created from aerosolization of the toxin in the liquid form. These toxins are resistant to inactivation by ultraviolet light, and are only destroyed by heat when exposed to temperatures of 1,500 °F for 30 minutes. Because of their stability in the environment and toxicity, the T-2 mycotoxins would be an effective biological agent for use in a terrorist scenario. The onset of symptoms may begin minutes after exposure and include burning skin pain, redness, and blistering. In lethal cases, blackening and sloughing of the skin occurs. Nasal contact from inhalation of T-2 mycotoxins produces pain, itching, sneezing, and nasal discharge. Lung exposure produces symptoms of shortness of breath, wheezing, and coughing, and ingestion produces symptoms of anorexia, nausea, vomiting, and bloody diarrhea. Because of the similarity in symptomology, exposure to T-2 mycotoxins may be misdiagnosed as a chemical weapon attack from an agent such as mustard gas. No vaccine, antidote, or therapy is available for exposure to mycotoxin and treatment is designed to alleviate the symptoms (10).

DISPERSION METHODS

As previously indicated, biological agents can be employed in a terrorist scenario using several different methods. These methods vary in levels of difficulty and effectiveness, but dispersion through aerosolization proves to be the most useful.

Waterborne and Food-Borne Application

History has shown that contamination of a water source can be an effective dispersion aerosol attack, contamination of a water source may provide a centralized means to reach a population and cause disease when ingested. Many bacterial agents have potential for use as a waterborne threat because of their stability in an aquatic environment. For instance, *B. anthracis* spores have been shown to survive for up to two years in pond water (14). *Vibrio cholera*, one of the most noted aquatic pathogens can survive for as long as six weeks in aquatic environment containing organic matter, and *F. tularensis*, primarily an epizootic disease of animals can survive for months in water or mud and may even multiply during that period (14). The viral diseases that are considered potential biological warfare agents, except the enteric viruses and possibly smallpox, have virtually no utility as waterborne threats (14). Toxin agents are fairly stable in aquatic environments, and given sufficient quantities, may be useful as potential waterborne threats. Although numerous biological agents may be suitable for waterborne contamination in a terrorist scenario, advances in water purification technology have significantly decreased the threat. Early use of biological agents to contaminate water sources proved effective primarily because of the absence of effective water treatment systems. A well-designed and well-operated water treatment system can be expected to remove significant amounts of bacteria and toxins from the water supply. Furthermore, disinfection of potable water using chemical additives such as chlorine decreases the threat of waterborne contamination (14). It should be noted that gaps do exist in the evaluation of the susceptibility of potable water supplies to biological attack, however, the dose of any biological warfare agent required to cause adverse affects is directly related to the proximity of the contamination to the consumer and the size of the body of water to be contaminated. Contamination of large bodies of water such as reservoirs would be impractical because of the immense magnitude of the agent required to produce disease (14). An effective biological attack through contamination of water sources would be most effective on small localized areas.

Similar to a waterborne attack, biological agents may also be applied to food sources to produce human disease. Contamination of food with the intent of infecting the consumer is a kind of terrorist scenario that has proved effective in the past. Advancements in food safety standards and packaging of food products will increase the chance that these attacks will be insignificant because of the need to place the agent in close proximity to the consumer, as was done in the Dalles Oregon incident. Intentional contamination of feed supplies to infect livestock and poultry may have serious economic consequences (15).

Vector-Borne Transmission

Vectors facilitate the transmission of disease from one species to another and apply mainly to zoonotic or epizoonotic diseases. Examples of natural vectors include deer flies and fleas with *F. tularensis and Y. pestis*, respectively. The use of plague-infected fleas by the Japanese illustrates the usefulness of vector-borne biological attacks (2). Although not attributed to biological terrorism, the August 1999 outbreak of West Nile virus in New York and its spread to points south in 2000 is a modern-day example of the affects of a vector-borne outbreak on a large population. West Nile virus taxonomically belongs to the Japanese encephalitis subgroup and is readily transmitted by mosquitoes, through which it can infect a wide variety of vertebrates including humans (16,17). During the period of August 23 (when the first case of West Nile Virus was identified), to September 28, 1999, there were 17 confirmed human cases, 20 probable cases, and 4 deaths in New York City and the surrounding counties of Westchester and Nassau (17). Although the incidence of infection is insignificant compared with the millions of people living in the affected areas, the outbreak sparked widespread fear in the population and necessitated extensive aerial and ground application of mosquito adulticides and larvacides. Other control measures that were instituted included the distribution of 300,000 cans

of mosquito repellent, and 750,000 public health leaflets with personal protection information. Local radio and TV networks, and newspapers were also used to distribute information (17). The New York City West Nile virus telephone hotline, set up on September 3, 1999 to address public inquires regarding the outbreak and pesticide applications, received more than 130,000 calls in a 25-day period (17). The West Nile virus outbreak may be considered an indicator of the public response that could be expected from a mild biological attack on a large population.

Aerosolization

Airborne infection has long been known to be a major factor in the spread of disease. The utility of aerosol spread of disease was readily illustrated in the investigation of respiratory disease experienced in recruit barracks at armed services training centers during World War II. This investigation showed that increasing the space between bunks substantially decreased the incidence of disease because the infectious aerosol particles had a further distance to travel to infect the adjacent individual (18).

Dissemination of biological agents via the aerosol route is the most effective means of dispersion in a biological attack. Dispersion of biological agents through aerosol dissemination has the potential to affect the largest area compared with waterborne, food-borne, or vector-borne dispersion methods. Biological agents that are disseminated as bioaerosols using a spray or explosive device in a terrorist scenario have the capability to travel long distances through the air covering large areas indiscriminately. For example, the hypothetical human health impact of 50 kg of anthrax sprayed by an aircraft along a 2 km-line upwind of a population of 500,000 would result in 95,000 dead, 125,000 incapacitated, and have a downwind travel distance of more than 20 km (15). Similar tests performed by the United States off the coast of San Francisco during the 1950s using *B. globigii*, an anthrax simulant, also illustrated the large area coverage potential for aerosol dissemination of biological organisms (1,19). Because of the nonspecific nature of an aerosol release of biological agent, the effects may be more widespread than other dispersion mechanisms. For example, an outdoor aerosol attack targeting a human population with anthrax may also kill livestock, contaminate food supplies, or cause vector-borne transmission to occur, as well as cause contamination inside buildings and homes because of transport of contaminated air. Additionally, biological attacks that occur indoors may also produce a large number of casualties because of modern-day heating ventilation and air conditioning (HVAC) systems that recirculate indoor air, requiring a smaller amount of "clean" makeup air from the outdoors (19). Decontamination of such a large area is also problematic, especially in the case of environmentally persistent spore-forming bacteria such as *B. anthracis*. For example, anthrax spores exploded on Gruinard Island from allied biological warfare testing during World War II, survived for at least 40 years in the soil before the area was decontaminated (4).

Some of the environmental factors that govern the effectiveness of an outdoor aerosol dissemination are wind patterns, turbulence, atmospheric conditions, temperature, and humidity (20). Another factor includes the efficiency of the dissemination device, which determines the size and amount of the particle disseminated. The dissemination device also produces shear forces during dispersion, which may damage the organisms to the point of nonviability. Other factors that affect the viability of the organism are ultraviolet radiation, atmospheric oxygen, and environmental pollutants such as ozone and other synthetic chemicals (21). The size of the particle disseminated is also an important factor as it controls the ability of the biological agent to travel downwind. For example, the sedimentation velocity for a 5-μm particle is 0.75 cm/s compared with 7.28 cm/s for a 50-μm particle (22). The increase in particle size leads to a decrease in the ability of a particle to remain airborne for an extended period, thus reducing its coverage area.

Particle size also plays a substantial role in infectivity. Small size particles (1 to 10 μm), if inhaled can penetrate into the distal bronchioles of the lung and alveoli to cause infection (9). Studies performed in the late 1950s using *B. anthracis* spores showed that the concentration of spores in the 12 micrometer particle size range required to infect 50% of exposed animals was 17-fold greater than those spores in the 5-μm size range. This relationship of particle size to infectivity is a consequence of the shape and structure of the upper and lower respiratory system (19). Larger particles are impacted onto the walls of the upper respiratory tract and are removed with the help of ciliated membranes and mucosal secretions in the lining of the nasal-pharyngeal region. Smaller particles evade the natural defenses of the respiratory system and deposit in the terminal alveoli of the lung where the infection occurs (8,19)

AGROTERRORISM: THE BIOLOGICAL THREAT TO PLANTS AND ANIMALS

Although one usually thinks of the threat to humans when considering a bioterrorist scenario, a vulnerable target remains in the plant and animal industry. The United States, like many developed nations is vitally dependent on its cash crops, livestock, and poultry to sustain its quality of life and provide economic stability. The utility of a biological attack on plants and animals was realized during the early 1900s and continued through the Cold War (2). Although the initial purpose of these weapons was to render crops or animals unfit for consumption, the terrorist threat is also motivated by economic factors (23). An outbreak of disease in livestock or cash crops not only renders it unfit for use but also has the added implication of industry restrictions on international trade and disruption of internal distribution caused by eradication efforts (23). Procedures for disease eradication in plants and animals may be severe. For example, an outbreak of avian influenza in Eastern Pennsylvania in 1984 almost eliminated the poultry population. Although 40 to 60% of these animals would

have survived the epidemic, containment procedures mandated that they be destroyed. Examples of antiplant and animal agents include: rice blight, corn blight, wheat stem rust, tobacco mosaic virus, foot and mouth disease of cattle, rinderpest or cattle plague, and Newcastle disease of poultry (24). Unlike most human biological warfare agents, many plant and animal pathogens are contagious and may be spread from host to host (15). The spread of a contagious disease among livestock may be amplified by large-scale, high-density husbandry methods, transportation practices, and centralized feed supply systems. Furthermore, many of the agents pathogenic to livestock have been eradicated from the United States, and vaccinations are not required, leaving the animal population increasingly susceptible to infection (15). Plant and animal pathogens may be dispersed in the same ways as those utilized for an attack on a human population. Although not attributed to a terrorist attack, the recent foot and mouth disease outbreak Great Britain illustrates the devastating nature of a widespread agricultural epidemic.

ENVIRONMENTAL SAMPLING

With regard to a bioterrorist scenario, environmental sampling may serve numerous purposes similar to those in an epidemiological, environmental, or public health investigation; its primary function is to identify the disease-causing biological agent. Sampling may be performed in response to a perceived attack, or in response to an unusual outbreak of disease. Sampling may be used to determine the source of exposure, determine the area of coverage, or to render an area free of contaminants, or determine if remediation is required. Types of sampling may include air, water, soil, or surface swipes. Methods of analysis may include live cultures, polymerase chain reaction (PCR), or immunoassays and vary depending on the organism being sought after, the sampling method, and the circumstances surrounding the event. Environmental sampling of air and water, for example, may also be used for continuous monitoring of the environment to serve as an alarm system for immediate notification that a biological attack has occurred. Detailed information on sampling methods and techniques used in environmental monitoring and detection directly applicable to the topic of bioterrorism, and are covered in other entries in this encyclopedia.

COMBATING BIOTERRORISM

The threat of domestic bioterrorism has captured the concern and attention of the American public and government officials alike. Concern for this issue is further amplified by the widespread belief that at the current time, the United States is not well prepared to respond to a biological attack on its own soil. Without considering the likelihood that an attack will occur, the social, economic, and public health consequences of a biological attack are severe enough to warrant a comprehensive plan to respond to a bioterrorist event. As the national center for disease control and prevention, the Centers for Disease Control (CDC) has been given the responsibility by the U.S. Department of Health and Human services to coordinate and lead efforts to upgrade national public health capabilities at the local, state, and national levels to effectively respond to a biological attack (25). The CDC has outlined five major areas that make up a comprehensive bioterrorism response plan.

1. *"Detection (Surveillance)."* An essential part of the response plan is recognition of unusual and unexplained illnesses. If an attack can be detected early, those exposed may receive prophylaxis, vaccination, or other medical treatment to minimize loss of human life (25).

2. *"Rapid Laboratory Detection."* Rapid diagnosis of unexplained and unusual outbreaks is dependent on the ability of diagnostic clinical laboratories to detect and characterize pathogenic agents that are likely to be used in a biological attack. The CDC is working with metropolitan health departments to enhance their capability to identify biological threats, as well as creating a "rapid response and advanced technology laboratory" to provide local and state health departments and bioterrorism response team analytical support 24 hours a day (25).

3. *"Epidemiological investigation and implementation of control measures."* Epidemiologists at the local and state levels will have the responsibility for investigating unusual disease occurrences to identify sources of exposure and route of transmission. The CDC is providing training at the state and local level to ensure that biological threat agents are considered when investigating an unusual outbreak (11).

4. *"Communication."* As in any large-scale domestic emergency, rapid and secure communications are critical in achieving a prompt and organized response as well as providing emergency-related guidance to the public through the media. The CDC is working to develop a national health alert network linking local health departments to each other, the CDC, other federal agencies, and local health care providers to ensure the efficient transmission of crucial information regarding a bioterrorist incident. (25).

5. *"Preparedness Planning and Readiness Assessment."* The CDC has enhanced its capabilities including new hires, pertinent training, and enhanced interagency collaborations to ensure that it is prepared to respond to a bioterrorist incident. The CDC is also providing support to state and local governments to develop similar public health response plans (25).

Other initiatives involved in developing a response to a biological attack include the creation of a national pharmaceutical stockpile for use in response to mass casualties from a bioterrorist incidents that overwhelm the local health systems. This program would provide the area affected with rapid access to vaccines, antitoxins, and therapeutic drugs used to treat a large number of biological casualties (25).

CONCLUSION

Biological warfare has shown its utility in causing disease, hysteria, and death, and is not limited to military applications or targeting only humans. The multitude of agents available for use and the variety of dispersion mechanisms that can be utilized increase the complexity in identifying and treating the intentional use of biological agents as weapons of mass destruction. Furthermore, the relative ease of obtaining and producing biological agents increases the chances that they will be used as a terrorist weapon targeting humans, plants, or animals.

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BIOTOXINS. See CYANOBACTERIA; CYANOBACTERIA-TOXINS IN DRINKING WATER; RED TIDES AND OTHER HARMFUL ALGAL BLOOMS

BIOTRICKLING FILTERS FOR AIR POLLUTION CONTROL

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Biological treatment of contaminated air is an emerging technology in air pollution control. The principle is relatively simple: a contaminated airstream is passed through a porous packed bed on which pollutant-degrading cultures are immobilized. Like all biological treatment processes, air biotreatment relies on microbial reactions for the degradation of waste compounds. Bioreactors used in air pollution control have found most of their success in the treatment of dilute, high flow waste gas streams containing odors or volatile organic compounds (VOCs). Under optimum conditions, the volatile or gaseous pollutants can be degraded completely to carbon dioxide, water, and excess biomass. In the case of contaminants such as H_2S, reduced sulfur compounds, or biodegradable chlorinated compounds, harmless sulfates or chlorides are additional by-products. Bioreactors for air pollution control are promising and could treat many contaminants in a wide spectrum of applications. The technology has a niche in commercial and industrial applications in which high airflows and low pollutant concentrations are encountered (Fig. 1). It offers several advantages over traditional technologies such as incineration or adsorption. These include lower treatment costs, reduced environmental impact, an absence of the formation of by-products such as nitrogen oxides (NO_x) or spent activated carbon, low energy demand, a lack of the need for fossil fuel burning, and low temperature treatment.

The two most promising bioreactors for air pollution control are biofilters and biotrickling filters. Biofiltration has been reviewed recently (1); hence, this article is only concerned with biotrickling filters.

- *Biofilters* work by passing a humid stream of contaminated air through a damp packing material, usually compost mixed with wood chips or any other bulking agent on which pollutant-degrading bacteria are naturally immobilized. Biofilters are simple and cost- effective. They require low maintenance and are particularly effective for the treatment of odor and volatile compounds that are easy to biodegrade and for compounds that do not generate acidic by-products. Biofilters are increasingly used in industrial applications. (See BIOFILTRATION and BIOFILTRATION AND BIOODORS).
- *Biotrickling filters* work in a similar manner to biofilters, except that an aqueous phase is trickled

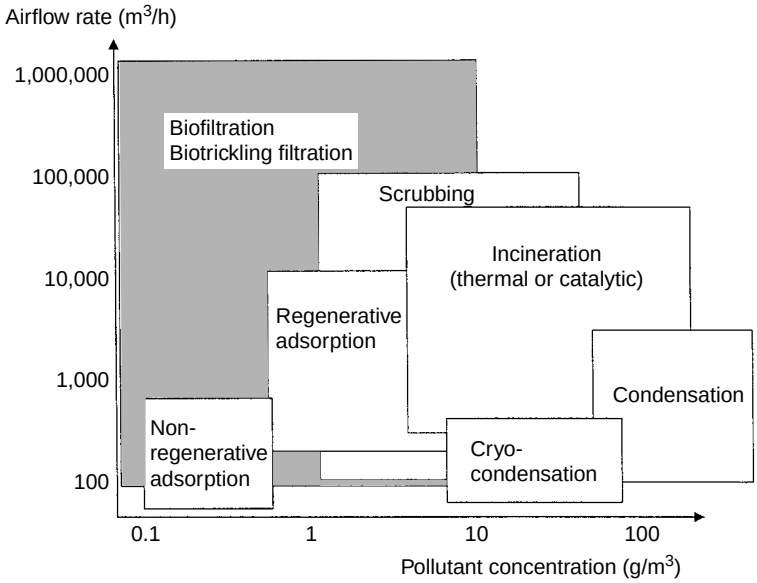

Figure 1. Applicability of the various air-pollution-control technologies based on airflow rates and concentrations to be treated (updated from reference 2).

over the packed bed and the packing is usually made of some synthetic or inert material, such as plastic rings, open pore foam, lava rock, and so on. The trickling solution contains essential inorganic nutrients such as nitrogen, phosphorous, potassium, and so on and is usually recycled. Biotrickling filters are more complex than biofilters but are usually more effective, especially for the treatment of compounds that generate acidic by-products such as H_2S. They can be built taller than biofilters. Biotrickling filters are more recent than biofilters, and have not yet been fully deployed in industrial applications.

BIOTRICKLING FILTRATION PRINCIPLE

The principle of biotrickling filtration is schematically explained in Figures 2 and 3, and typical characteristics of biotrickling filters are listed in Table 1. Biotrickling filters are biological scrubbers. At a first glance, the mechanism appears to be relatively simple: contaminated air is contacted with an immobilized culture of pollutant degrading organisms in a packed bed. A more detailed examination of the processes involved (Fig. 3) reveals that elimination of the pollutant is the result of a combination of physicochemical and biological phenomena. Understanding these phenomena is a key to the successful deployment of the technology.

In biotrickling filters, contaminated air is forced through a packed bed, either downflow or upflow. The packed bed is generally made of an inert material such as a random dump or structured plastic packing, or less often, an open pore synthetic foam or lava rocks. The packing provides the necessary surface for biofilm attachment and for gas-liquid contact. During treatment, an aqueous phase is recycled over the packing. It provides moisture, mineral nutrients to the process culture, and a means to control the pH or other operating parameters. The system is continuously supplied with essential mineral nutrients such as nitrogen, phosphorus, potassium, and

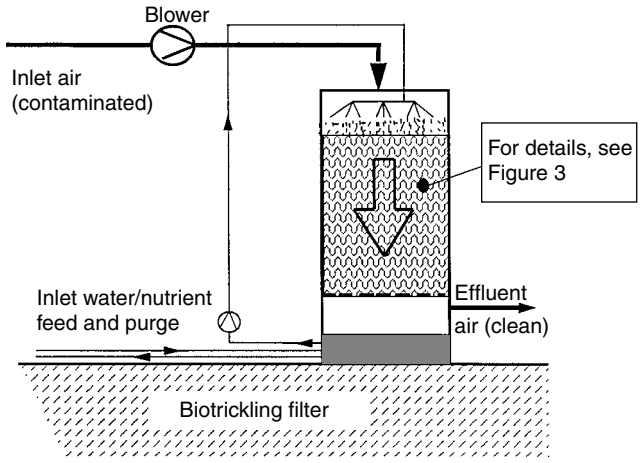

Figure 2. Schematic principle of biotrickling filtration; the cocurrent operation is shown here.

trace elements via a liquid feed. In general, most of the pollutant is biodegraded in the biofilm, but a part may also be removed by the suspended microorganisms in the recycle liquid (3). Possible biodegradation metabolites will leave the system via the liquid purge along with small amounts of biomass. Usually, less than 10% of the carbon pollutant entering the system leaves via the purge (3).

Biotrickling filters work because of the action of the pollutant degrading microorganisms. In the case of the removal of hydrocarbon vapors, the primary degraders are aerobic heterotrophic organisms that use the pollutant as a source of carbon and energy. For the removal of hydrogen sulfide or ammonia, the primary degraders are autotrophs, which will use the pollutant as a source of energy and use carbon dioxide as a source of carbon for growth. The removal of compounds such as dimethyl sulfide or dimethyl disulfide will require both autotrophs and heterotrophs to be present. In any case, the biotrickling filter will host a wide variety of microorganisms, similar

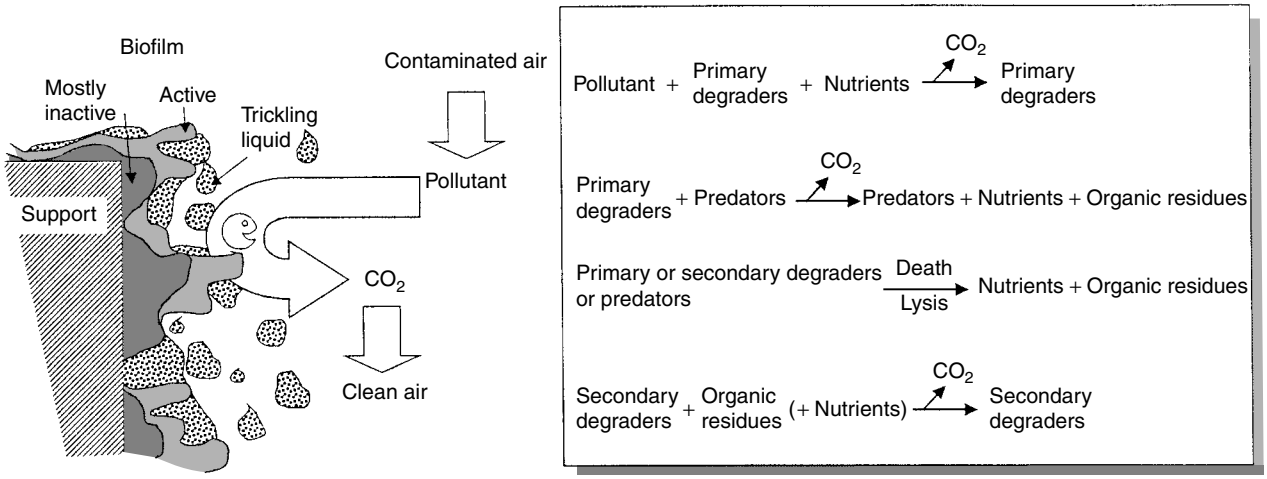

Figure 3. Mechanism of pollutant removal and the main biological processes involved in biotrickling filters.

Table 1. Typical Characteristics of Biotrickling Filters

Biotrickling filter bed height	1–5 m
Biotrickling filter cross section area	1–3,000m^2
Airflow treated	100–1,000,000 $m^3\,h^{-1}$
Packing void volume[a]	
— Plastic rings, foam, random or structured packing	90–95%
— Lava rock	~50%
Empty bed gas retention time[b]	2–60 s
Pressure drop	<1 cm of water column per meter of bed depth
Operating temperatures	15–50 °C
Trickling rates[c]	0.01–10 $m\,h^{-1}$
Liquid dilution rate[d]	0.1–2 per day
Usual pH of the recycle liquid	
— removal of VOCs or compounds difficult to degrade	~7
— removal of H_2S	1–2
Inorganic nutrient supply (N, P, K, traces)	Usually 0.05 to 1 times the amount calculated using biodegradation stoichiometry
Inlet pollutant concentration	
— VOCs	0.01–10 $g\,m^{-3}$
— Odors	500–50,000 odor units
Typical pollutant removal efficiencies	60–99.9+%

[a]Value at reactor start-up; over time, biomass growth will decrease bed porosity, typically by 10–30%
[b]The empty bed gas retention time (EBRT) is defined as the bed volume/airflow
[c]Trickling flow rate/bed cross-section area
[d]Liquid feed rate/recycle liquid volume.

to those encountered in wastewater-treatment operations. The microorganisms responsible for pollutant removal in biotrickling filters are usually aerobic because biotrickling filters are well-aerated systems. However, it has been proposed that the deeper parts of the biofilm (Fig. 3), where anaerobic conditions probably prevail, can be utilized to perform anaerobic biodegradation (e.g., reductive dechlorination, or NO_x reduction) for the treatment of pollutants that are otherwise recalcitrant under aerobic conditions (4). Anaerobic treatment in aerobic biotrickling filters is still an experimental area.

As illustrated in Figure 3, a major fraction of the biofilm becomes inactive (mostly because of mass transfer limitations) as the biofilm grows, and active primary degraders only constitute a minor fraction of the total population in the biofilm. Secondary degraders feeding on either metabolites, biopolymers, or predators feeding on the primary degraders include bacteria, fungi, and higher organisms such as protozoa, rotifers, even mosquito or fly larvae, worms, or small snails. The importance of higher organisms in the overall process should not be underestimated. They have been shown to play an important role in reducing the rate of biomass accumulation and in recycling essential inorganic nutrients (5,6). In fact, comparison of traditional mineral growth media with biotrickling filter

recycle liquid composition reveals that most biotrickling filters are operated under various degrees of inorganic nutrient limitation. The relationship between nutrient supply and biomass growth is discussed further in this article.

BIOTRICKLING FILTER PERFORMANCE

Definitions and Factors Affecting Performance

Operation and performance of biological reactors for air pollution control is generally reported in terms of removal efficiency, pollutant elimination capacity as a function of pollutant loading, or the gas empty bed retention time (EBRT). These terms are defined in Equations 1 to 4:

$$\text{Removal} = \text{RE} = \frac{C_{\text{in}} - C_{\text{out}}}{C_{\text{in}}} \times 100(\%) \quad (1)$$

$$\text{Pollutant Elimination Capacity} = \text{EC} = \frac{(C_{\text{in}} - C_{\text{out}})}{V} \times Q(\text{g m}^{-3}\,\text{h}^{-1}) \quad (2)$$

$$\text{Empty Bed Retention Time} = \text{EBRT} = \frac{V}{Q}(\text{s or min}) \quad (3)$$

$$\text{Pollutant loading} = \text{L} = \frac{C_{\text{in}}}{V} \times Q(\text{g m}^{-3}\,\text{h}^{-1}) \quad (4)$$

where C_{in} and C_{out} are the inlet and outlet pollutant concentrations (usually in g m^{-3}), respectively, V is the volume of the packed bed (m^3), and Q is the airflow rate ($\text{m}^3\,\text{h}^{-1}$). Pollutant concentrations are usually reported as mass per volume; conversion of volumetric to mass concentrations is done using the ideal gas law that reduces to Equation 5 at room temperature:

$$\text{Concentration}(\text{g m}^{-3}) = \frac{\text{Concentration}\,(\text{ppm}_v) \times \text{molecular weight of pollutant}\,(\text{g mol}^{-1})}{24,776} \quad (5)$$

It should be stressed that the elimination capacity and the loading are calculated using the volume of the packed bed and not the total volume of the reactor. Depending on the reactor design, the volume of the packed bed will be about 40 to 90% of the total reactor volume. In addition, the EBRT is calculated on the basis of the total volume of packed bed (Equation 3). The actual gas residence time will be lower depending on the porosity of the packing, the dynamic liquid holdup, and the amount of biomass attached to the packing. The porosity of packing ranges from about 50% (lava rock) to 95% (all random or structured packings), the liquid holdup is usually less than 5% of the bed volume, and biomass may occupy 5% to 30% of the bed volume. Hence, the actual gas residence can be less than half the EBRT.

A typical elimination capacity versus pollutant loading curve is shown in Figure 4. It is usual to report the performance as a function of the load, that is, inlet concentration × airflow, rather than the concentration. This enables comparison of systems of different sizes operated under different conditions. One underlying assumption is that the performance depends only on the pollutant load, hence, that low concentrations-high flow rates lead to similar elimination capacities as high concentrations-low flow rates. This assumption is generally valid because the pollutant concentrations commonly encountered in biotrickling filters are high enough for the microkinetics to be of zero order. This is no longer true at very low pollutant concentrations (typically below $0.05-0.1\ \text{g m}^{-3}$), particularly for pollutants with high Henry's law coefficients, because first-order kinetics will prevail in the biofilm, resulting in a reduction of the maximum elimination capacity.

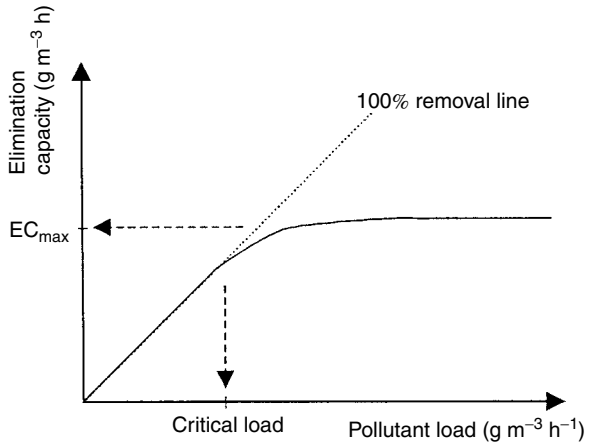

Figure 4. Schematic of a typical elimination capacity versus the load curve for a biotrickling filter.

Examination of Figure 4 reveals that there are essentially three operating regimes:

1. Low loading, also called first-order regime. The elimination capacity and the loading are identical and the pollutant is completely removed. The biotrickling filter is operated well below its maximum elimination capacity. The performance increases proportionally with the loading.
2. Intermediate range, where breakthrough of the pollutant occurs. With higher inlet concentration or higher airflow rates, the elimination capacity increases, but to a lesser extent than the loading.
3. High loading, also called zero-order regime. The biotrickling filter is operated at its maximum elimination capacity. Increases in pollutant concentration or the airflow rate do not result in further increases in elimination capacity. However, the removal efficiency decreases.

For the evaluation of biotrickling filter performance, both the maximum elimination capacity and the removal efficiency should be considered. For practical reasons, academic research is mainly concerned with the maximum elimination capacity or with high performance, which occur at relatively high pollutant concentration and often less than approximately 90% removal efficiency. On the other hand, reactor design for industrial application often needs to meet a certain discharge requirement or

achieve a high removal percentage. Thus, there might be some challenges in extrapolating research data for reactor design. In this context, the critical load defined as the maximum loading before the removal deviates significantly from the 100% removal line (Fig. 4) is a valuable parameter. Nevertheless, there are limitations to the use of the critical loading. It is relatively sensitive to the pollutant inlet concentration, thus extrapolation of low flow-high concentration to high flow low-concentration should be avoided.

Examples of Biotrickling Filter Performance

Research over the past 10 years has greatly broadened the range of pollutants that can be treated in biotrickling filters, including volatile organic compounds (VOCs), chlorinated hydrocarbons, reduced sulfur compounds, and compounds containing nitrogen. Typical examples are presented in Table 2.

Maximum elimination capacities generally are in the range of 5 to 200 $g\,m^{-3}\,h^{-1}$. Although many factors influence performance, a few general comments can be made. As biotrickling filters rely on microorganisms as the catalysts for pollutant conversion, biodegradability of the pollutant is of prime importance. Decreasing biodegradability causes lower elimination capacities and/or longer periods of adaptation. The use of specially acclimated or enriched microorganisms may be considered in these cases. Equally important is the accessibility of the pollutant to the microorganisms. The overall rate of pollutant removal may be limited by the mass transfer rate of the pollutant into the biofilm, which depends mainly on the pollutant's air-water partition, which is in turn best described by the Henry coefficient. Mass transfer limitation leads to a biofilm not completely saturated with the pollutant, hence pollutant concentrations in the biofilm are below those required for maximum biological activity. Means to improve the overall mass transfer rate in biotrickling filters include the selection of packing materials with a high specific surface area and intermittent trickling to reduce the thickness of the water film on the biofilm (Fig. 3).

As illustrated in Table 2, many different types of packing materials have been used in biotrickling filters, and research in this area is still in progress. The packing should combine a high porosity to minimize the pressure drop across the reactor and a high specific surface area to maximize biofilm attachment and pollutant mass transfer. Other factors to consider for a packing include water holding capacity, structural strength, surface properties, weight, stability over time, and cost.

Reaction conditions in the biotrickling filter can be optimized by controlling the pH, the concentrations of nutrients, and the metabolic end products in the recycle liquid. Many biotrickling filters are equipped with a pH control, with automatic water or nutrient addition to control ionic strength. The optimum pH depends on the process culture. Most VOC-removing biotrickling filters are operated at a near neutral pH. On the other hand, H_2S-oxidizing microorganisms such as *Thiobacillus* sp. are acidophilic and show maximum activity at low pH. In biotrickling filters treating H_2S vapors, pH values as low as 1 to 2 are common. Treatment of sulfur and chlorinated compounds will result in the accumulation of sulfate and chloride in the recycle liquid, respectively. These salts will inhibit biodegradation if certain concentrations are exceeded. Frequent supply of freshwater and purging of the recycle liquid are required to prevent accumulation of inhibitory concentrations. The dilution rate can be controlled by continuous measurement of the conductivity of the recycle liquid or by using ion selective electrodes.

BIOMASS GROWTH IN BIOTRICKLING FILTERS

Growth Kinetics

Clogging of biotrickling filters by growing biomass is one factor that has markedly slowed down the implementation of biotrickling filters at the industrial scale. A better understanding of biomass growth in biotrickling filters is warranted. In general, pollutants are used by the primary degraders to produce new biomass and to generate energy for its maintenance (Fig. 3). These processes have been extensively investigated in batch or continuous monocultures. The situation is much more complicated in biotrickling filters in which a complex ecosystem exists. In a first approximation, neglecting heterogeneities and mass transfer effects, it could be said that the rate of pollutant degradation depends on the intrinsic growth rate of the active fraction of the primary degraders (X_1) and their maintenance requirements as in Equation 6:

$$\mathrm{EC} = \left(\frac{\mu}{Y_{X/S} + m}\right) \times X_{1(\text{active fraction})} \tag{6}$$

where μ is the specific growth rate of the primary degraders, $Y_{X/S}$ is the biomass yield, m is the maintenance energy requirement, and $X_{1(\text{active fraction})}$ is the biomass content of active primary degraders per volume of reactor.

The specific growth rate of the active fraction of the primary degraders can be expressed using a modified Monod type equation:

$$\mu = \frac{\mu_{\max} \times S}{Ks + S} \times \frac{N}{Ks_{\mathrm{N}} + N} \times \frac{\mathrm{O}}{Ks_{\mathrm{O}} + \mathrm{O}} \times \frac{I}{1 + \dfrac{I}{K_{\mathrm{I}}}} \tag{7}$$

where S is the pollutant and substrate, N is any nutrient, O is the oxygen, and I is any inhibitor, and Ks, Ks_{N}, Ks_{O}, and K_{I} are the respective half-saturation and inhibition constants.

A similar equation can be written for all the species (or group of species) present in the system. Each will have one or several specific substrates, specific kinetic constants, and thus a specific growth rate. The overall rate of biomass accumulation is the sum, for all the different species, (designated by the indices i) of the growth rate minus death and lysis (d term), the predation by other species and the washout via the recycle liquid purge. This is expressed in Equation 8:

$$\text{Rate of biomass accumulation} = \sum_{\mathrm{i}} ((\mu_{\mathrm{i}} - d_{\mathrm{i}}) \times X_{\mathrm{i}} - \text{Predation}_{\mathrm{i}} - \text{Wash out}_{\mathrm{i}}) \tag{8}$$

Table 2. Laboratory Studies on Removal of Different Pollutants in Biotrickling Filters

	Methanol (7)	MTBE (8)	Hexane (9)[c]	Dichloromethane (10)	H_2S (11)	Nitrobenzene (12)
Compound classification						
Biodegradability	High	Low	Intermediate	High	High	Low
Water solubility	High	High	Low	Intermediate	Intermediate	Intermediate
Substrate profile	VOC; carbon and energy source	VOC; carbon and energy source	VOC; carbon and energy source	Cl compound; carbon and energy source	Inorganic; energy source	N compound; carbon and energy source
Operation						
Packing	NOR-PAC polypropylene packing	Lava rock/polypropylene Pall rings	Foam	Ceramic saddles	Foam packing	Perlite
Mode of operation	N/A	Cocurrent	Cocurrent/intermittent trickling	Cocurrent	Countercurrent	Cocurrent
Source of microorganisms	Activated sludge	Adapted microbial consortium	Two pure bacterial species	*Hyphomicrobium* sp. GJ21	NA	Adapted microbial consortium
Performance						
Start-up	5 days	7 months	50 days	7 days	2 weeks	4 weeks
EC_{max} ($g\,m^{-3}h^{-1}$)	100	42–50	7.5	150	100	50
Critical load ($g\,m^{-3}\,h^{-1}$)	~80	~40	~5	NA	~70	NA
-EBRT (s)	69	39–90	288		NA	
-Inlet concentration ($g\,m^{-3}$)	1.5	0.4–1	0.4		0.4–1.4	

Equations 6 to 8 are highly simplified since they do not take local heterogeneities into account. Still, they define a number of parameters that are impossible to determine. A possible solution is to split the process culture into large classes of organisms, such as primary degraders, secondary degraders, predators, and so on, and use lumped kinetic parameters. This is an area of current research. Even so, Equations 6 to 8 reflect the fact that the pollutant elimination and the observed biomass growth are interrelated in a complex manner. The equations further allow development of biomass control strategies for biotrickling filters. This is discussed in the next section.

Strategies for Controlling Biomass Growth

Examination of Equations 6 to 8 suggests several possible approaches to controlling biomass growth. Attempts can be made to reduce the overall rate of biomass accumulation (Equation 8) by either reducing the specific growth rate or increasing death and lysis. Several means have been investigated. Other options include increasing predation, washing-out or otherwise periodically removing the excess biomass. These are briefly discussed.

The first option to prevent clogging is the reduction of the biomass accumulation rate or of the specific growth rate (Eq. 7). The challenge is to reduce biomass accumulation while maintaining a high pollutant removal rate (Eq. 6) as growth and pollutant elimination are often tightly linked. This can be achieved by reducing the biomass yield coefficient ($Y_{x/s}$) and/or increasing the maintenance requirements (m). Growth, biomass yield, death and lysis, and activity and maintenance are interrelated parameters reflecting general cell metabolism and are as difficult to influence independently.

Table 3 reports various attempts to reduce the specific growth rate in biotrickling filters. These include limiting the supply of nutrients essential for growth (N or K), the use of nitrate as a nitrogen source instead of ammonium, the addition of compounds such as NaCl in concentrations that partially inhibit microbial growth, and so on. In general, these strategies also result in a reduction of microbial activity, thereby lowering reactor performance. Hence, larger reactors will be required to treat the same volume of waste gas, which will increase the capital costs. An interesting option is the use of organisms with lower biomass growth rates and yields, such as fungi. Interestingly, under similar conditions, fungi have shown a higher removal rate and a lower biomass accumulation rate than bacteria in toluene-degrading biotrickling filters operated under nutrient-limiting conditions (13).

The second option is to stimulate predation of the process culture by higher organisms such as protozoa (5), possibly even larger organisms such as larvae, small snails, or other biomass-eating organisms. This is a promising approach since it will not lead to a reduction in the performance, nor result in excess biomass to be disposed off, as per the methods discussed in the following paragraph. The challenge is that higher organisms may be difficult to control and/or to maintain in the biotrickling filter. This is an area of development, and advances are expected in the near future.

The last option to prevent clogging is to remove the excess biomass. This is usually done periodically rather than continuously, because shearing by the trickling liquid during normal operation is not sufficient to remove substantial amounts of attached biomass (20). Hence, the recycle liquid only contains a low concentration of biomass and increasing blow-down does not wash out much biomass. When periodic removal of biomass is chosen, the biotrickling filter is best operated at a high elimination capacity, and biomass is allowed to accumulate up to a given point where remedial action is required. From a cost perspective, the capital costs will be lower because a smaller reactor will suffice, but clogging will necessitate frequent cleaning, thus increasing operating costs (21). Removal of biomass can be done either physically or chemically (Table 4). Physical removal of biomass relies on biofilm detachment by high shearing forces. This can be done by backwashing the reactor, or by periodical stirring of the packed bed. Although these techniques result in prolonged, stable biotrickling filter operation, certain drawbacks exist (Table 4). Chemical removal of biomass is a simpler operation, as no major changes of the reactor configuration are required. In this procedure, a chemical solution is recycled over the packing using the existing system for liquid recycling. A stable toluene-degrading biotrickling filter was obtained by periodic washing of the packing with a NaOH solution for three hours (13). Posttreatment with HCl was needed to restore the pH to a neutral value. Other chemicals such as sodium hypochlorite and hydrogen peroxide may be more effective in removing biomass, but they are also more toxic to the microbial population (22). This could potentially slow down the restart of the reactor.

Unfortunately, all biomass control strategies have only been investigated in the laboratory and no experience is

Table 3. Options for Reduction of the Biomass Growth Rate in Biotrickling Filters

Option	Principle	Reference
Nutrient limitation	Reduction of the biomass yield	
— Nitrogen		13,14,15
— Phosphate		16
— Potassium		16
Use of $N\text{-}NO_3^-$ instead of $N\text{-}NH_4^+$	Reduction of the biomass yield	17,18
Use of specific microbial species	Selection of low biomass yield species	13
Addition of growth inhibitors	Reduction of the specific growth rate	17,19

Table 4. Options for Removal of Excess Biomass in Biotrickling Filters. Note that all methods were only tested at relatively small scale

Option	Advantages	Disadvantages	Reference
Backwashing	• Mild treatment • Possibly redistributes packing and may thus avoid formation of preferential paths or short circuits	• Requires larger reactors (+40% for packing fluidization • Requires packing that can be fluidized	18,23
Periodic stirring	• Probably low cost to perform • Easy to automate	• Complicated reactor design and construction • Higher capital costs • Not feasible with all packings	24,25
Chemical washing	• Effective removal of biomass • Does not require reactor modification	• Toxicity to microorganisms • Secondary waste	13,22

available from industrial-scale biotrickling filters. This is because most full-scale biotrickling filters have been designed for applications with low potential for clogging. In the future, design and operation of biotrickling filters will need to find the optimum between operation of large, low-performance biotrickling filters that do not require biomass removal and small, high-performance biotrickling filters with high potential for biomass accumulation (21). The perspective for progress in controlling biomass growth in biotrickling filters suggests that the latter option will be preferred.

BIOTRICKLING FILTRATION COSTS

Capital Costs

Capital costs for biotrickling filters vary a great deal depending on the size of the biotrickling filter and the material of construction. The size of the biotrickling filter is a function of the airflow, the nature and concentration of the pollutant treated, and the required removal efficiency. The presence of corrosive gases (e.g., H_2S) or solvent vapors will influence the choice of the construction material (polyethylene, fiberglass, steel, or concrete). The cost of the biotrickling filter will be further influenced by the presence of dust or fine particles, by excessively high or low temperatures, by highly fluctuating pollutant concentrations, and so on. Controls and ducting can also be a significant expense. Hence, before reactor design and construction, extended problem definition that includes a detailed characterization of the exhaust air is required.

Deshusses and Cox (21) have recently proposed a simple relationship (Eq. 9) to estimate the capital cost of a biotrickling filter on the basis of the volume of the bed. The costs include basic instrumentation (pumps, level switch) but no ducting and are for a simple biotrickling filter constructed out of inexpensive materials. For expensive materials such as stainless steel, a multiplication factor should be used. The cost obtained by Equation 9 is a rough estimation, with ±20% accuracy.

$$\text{Biotrickling Filter Capital Cost (\$)} = 13,000 \times \text{Bed Volume}^{0.757} \quad (9)$$

This equation covers bed volumes ranging from 5 to 1,000 m^3, where the reactor volume is in m^3. On the basis of the concentration of the pollutant, the target removal efficiency, and the airflow to be treated, the bed volume can be determined. Equation 9 is then used to estimate the capital cost (Table 5). Of course, vendor quotes are more appropriate for a detailed economic evaluation of the final installed costs.

Table 5. Estimated Costs, Footprint, and Treatment Capacity of Biotrickling Filters of Various Sizes

Bed Volume (m^3)	Capital Costs (Equation 1) (\$)	Approximate Footprint[a] (m^2)	Approximate Airflow That Can Be Treated[b] ($m^3\,h^{-1}$)
5	\$45 k	1–2.5	300–3,600
10	\$75 k	2–5	600–7,200
20	\$125 k	4–10	1,200–14,400
50	\$250 k	10–25	3,000–36,000
100	\$425 k	20–50	6,000–72,000
200	\$720 k	40–100	12,000–144,000
500	\$1.4 m	100–250	30,000–360,000
1,000	\$2.4 m	200–500	60,000–720,000

[a]Estimated using a 2- to 5-m bed height; to convert to sq. ft, multiply by 11.
[b]Calculated using EBRT of 5 s to 1 min; to convert to cfm, multiply by 0.59

Operating Costs

The determination of the cost of operating a biotrickling filter should include (*1*) nutrients and water expenses, (*2*) electricity for the blower and the recycle pump and miscellaneous electrical equipment, (*3*) maintenance, (*4*) costs associated with controlling the growth of biomass, and (*5*) capital costs (amortization). A detailed discussion of each of these costs is beyond the scope of this article. Specialized literature and vendor information can be referred to for more details (21). Even so, in general, the following applies:

- Nutrients, chemicals (e.g., for pH control), and water are usually a relatively small fraction (10–30%) of the total operating costs.
- Electricity for the blower is often a major fraction of the total operating expenses.
- Maintenance of biotrickling filters is minimal. A reasonable estimate is 2 to 4 hours per week. It is important is to inspect spray nozzles for possible clogging that would result in inadequate bed-wetting.
- If the biotrickling filter is likely to get clogged, the costs associated with controlling the growth of biomass must be included. These can be significant (21), up to half of the total operating costs. As discussed in the previous section, various approaches to control biomass growth exist. Unfortunately, there is only limited experience at the industrial scale. Careful evaluation of the various options is recommended.
- Since biotrickling filter operation is relatively inexpensive, capital cost amortization will be significant compared to other costs. An average fraction, assuming a plant life of 10 to 20 years, is between 20 and 40% of the total treatment costs. This stresses the importance of proper sizing and careful selection of the materials to minimize the actual capital costs.

A convenient way to compare the operating costs of biotrickling filters is to report the cost per thousands of cubic meter of air treated, that is, to divide the yearly costs incurred by the volume of air treated in a year (in thousands of m^3). Usual values for the operating costs range from \$0.05 to \$1.5 per 1,000 m^3 of air treated, not including capital costs, and from \$0.1 to \$3 per 1,000 m^3 when capital amortization is included. The wide range reflects the variety of possible applications and sizes of biotrickling filters. Typically, large biotrickling filters tend to be more economical per unit volume of air treated than small biotrickling filters.

CASE STUDIES

In this section, four cases of biotrickling filtration are presented. These case studies are reported to the best of our knowledge. Their description in this article does not constitute an endorsement of the design or of the vendor. Note also that the methods for calculating the treatment costs may be different from case to case. Hence, treatment costs may not be directly comparable. Nevertheless, they are included for information purposes, as an indication of the potential economic value of the technology.

H_2S and VOC Treatment at a Wastewater Treatment Plant in Los Angeles, California

Wastewater treatment plants have to control various odors and VOC emissions. The odor is generally from H_2S and from reduced sulfur compound emissions. Usually, the H_2S concentration is in the 3 to 100 ppm_v range and reduced sulfur compounds are in the ppb_v to ppm_v range. Some VOCs (aliphatics, aromatics, and some chlorinated) are also emitted, usually in the ppb_v range. Biotrickling filters have been proposed as one promising alternative treatment to the present use of chemical scrubbers. A pilot study was conducted in 2000 by the University of California, Riverside, at the Hyperion Treatment Plant in Los Angeles to evaluate the efficacy of biotrickling filters for the treatment of contaminated air from the headworks. The Hyperion plant treats domestic and industrial wastewater from the Los Angeles Basin. Odor nuisance and the growing concern about the potential toxicity of individual compounds require removal of H_2S as well as VOCs such as benzene and chlorinated compounds such as methylene chloride and chlorobenzenes. The main objective of this feasibility study was to evaluate combined treatment of H_2S and VOCs in a single-stage biotrickling filter. A particular emphasis was placed on determining the effect of pH on the elimination of the trace VOCs. The reactor used for this demonstration was a well-instrumented pilot unit (26) that included two vessels, but only one was used in this project (Table 6 and Fig. 5). The air was in an upflow mode and secondary effluent water ($2.2\ L\ m^{-3}_{reactor}\ h^{-1}$) was used as a nutrient source for microbial growth and to wash out sulfate. Despite great variations of the H_2S inlet concentration over the day (10–50 ppm), greater than 95% removal efficiency was continuously observed. Removal of VOCs and chlorinated compounds depended on their biodegradability, which was much higher at a near neutral pH than at a low pH (pH of 1–2). Moderate fractions of common VOCs (e.g., toluene, benzene) were removed. Typical results are presented in Figure 6.

Figure 5. Picture of the UCR biotrickling filter installed at Hyperion Wastewater Treatment Plant in Los Angeles.

Table 6. Characteristics of the UCR Biotrickling Filter Tested at Hyperion

Owner and present location	University of California, Riverside/Hyperion Treatment Plant, Los Angeles, California
Builder	University of California at Riverside and Environmental Biosystems
Type of airstream	Exhaust air from the primary headworks (slip stream taken prior to chemical scrubbing)
Year of construction	1996
Packing type and volume	3.87 m^3 of COOLdeck PVC Munters 12060 structured packing.
Height and number of layers of packing	7 layers of 0.30 m on top of each other. Total bed height is 2.1 m.
Biotrickling filter construction type	304 stainless steel cylindrical reactor of 1.5-m diameter and a total height of 3.35 m. The lower plenum of 0.77 m contains 800 L of recycle liquid and 0.30 m of void space. The upper plenum for liquid distribution is 0.45 m high. The reactor is mounted on a trailer.
Airflow rate	650 m^3/h (380 cfm), upflow. Airflow is variable depending on the application
Empty bed residence time	21 seconds
Pressure drop	4–8 mm of water gauge
Average bed temperature	15–35 °C
Pollutants treated	Hydrogen sulfide: 15–70 mg/m^3 (10–50 ppm_v); VOCs and chlorinated compounds 0–150 ppb, depending on the species
Biotrickling filter controls	Initial construction included 28 parameters monitored or controlled. Unit has been simplified since then to include only pH control and low-level switch. At Hyperion, pH control is either by purging sulfate with secondary effluent or by automatic addition of caustic soda to pH 7–8. Monitoring of various operational and performance parameters is by grab samples.
Biotrickling filter design and acceptance criterion	None. This is a feasibility study. The objective is removal of hydrogen sulfide to below 1 ppm and significant reduction of target VOCs and chlorinated compounds
Approximate investment costs	Estimated at $175,000 for the R& D unit, the cost include two reactors of 3.87 m^3 and many control and analytical devices for scientific purposes.
Approximate treatment cost per 1,000 m^3 off-gas treated	Electricity costs (recycle pump only): $0.02 per 1,000 m^3 of off-gas treated; pH control cost $0–0.04 per 1,000 m^3 of off-gas treated. Water costs: insignificant.
Typical performance	Reduction of hydrogen sulfide down to 0.25–0.5 ppm (i.e., about 98% removal efficiency), 50–70% removal of VOCs (toluene, benzene, xylenes), removal of chlorinated compounds is currently being studied.

H_2S and CS_2 Treatment in Monterrey, Mexico

Grupo Cydsa S. A. de C. V. is a large Mexican corporation with in-house needs for inexpensive air pollution control, mostly for treating H_2S and CS_2 emissions from cellophane film and rayon fiber manufacturing. After evaluating several technologies, a unique expertise was developed in the design and operation of biotrickling filters. So far, Cydsa has installed at least four full-scale biotrickling filters for the control of sulfur odors. The reactors usually use an inexpensive structured plastic packing of the type sold by Munters. They include a nutrient supply, a blow-down for the oxidized sulfur, and a pH control. The biotrickling filters have been very successful. The characteristics of one of their biotrickling filters are listed in Table 7 and a picture is shown in Figure 7.

Odor Treatment from Cigarette Manufacturing in Berlin, Germany

M + W Zander Facility Engineering GmbH (Germany) installed a large biotrickling filter for the treatment of odors at a tobacco company in Berlin. Several air pollution control technologies were evaluated, and biotrickling filtration was selected for its cost-effectiveness.

M + W Zander used open pore polyurethane foam as a packing material (27). The foam was very light (20 kg m^{-3}), had a large interfacial area for bacterial attachment (about 600 m^2 m^{-3}), and its open pore structure resulted in low pressure drops. Pilot tests were performed prior to the design of the full-scale unit to ensure that odor removal was satisfactory, and that no clogging of the bed occurred within a reasonable time frame. In the full-scale biotrickling filter, a small flow of water containing nutrients is recycled intermittently over the support. The pattern adopted in this case was sprinkling 5 to 15 minutes every hour. Thus, in a sense, this biotrickling filter was operated as a biofilter for most of the time. The reactor was remote controlled from an operator room via a modem. The characteristics of the system are reported in Table 8.

Start-up of the full-scale biotrickling filter required a two-month acclimation period, after which odor removal was continuously higher than 90%. Typical inlet and outlet odor levels were 5,400 and 400 odor units, respectively, and removal was consistently more than 90%. Because of the low pollutant loadings and the limited supply of nutrients, clogging of the filter bed was not an issue. The pressure drop was low and stable around 2 to 4 cm of water gauge.

Odor and VOC Treatment at a Naval Air Station

As part of a technology development or technology demonstration program, Envirogen, Inc. operated a small pilot biotrickling filter and then designed a full-scale biotrickling filter for the removal of low concentrations of VOCs from contaminated air vented from wastewater treatment tanks (28). The contaminants

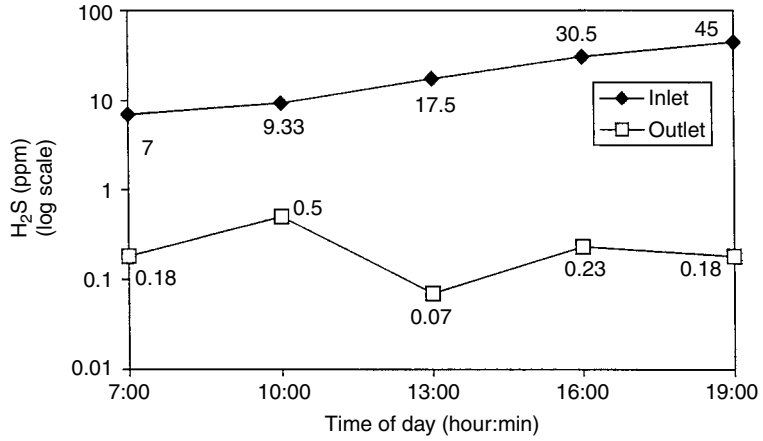

Figure 6. Typical performance of H_2S removal in the biotrickling filter. Note the fluctuating inlet concentration during the day (7 to 45 ppm). The labels show the measured H_2S concentrations in ppm_v.

Table 7. Characteristics of the Biocyd Celorey Biotrickling Filter

Owner and location	Grupo Cydsa S.A. de C.V. Ruiz Cortines Cydsa Industrial Complex, Celorey plant. Monterrey, NL. Mexico
Builder	DICOTEC (Design and Construction), a subsidiary of the Environmental Division of Grupo Cydsa.
Type of airstream	Exhaust air from a cellophane plant (viscose process).
Year of installation	1994
Packing type and volume	51 m^3 of PVC structured packing. Each layer is composed of a series of corrugated PVC sheets hot-welded together at an angle.
Height and number of layers of packing	Two sections of 2.44-m high on top of each other, separated by a 1-m air plenum. Each section is composed of 8 layers of corrugated PVC.
Biotrickling filter construction type	Cylindrical shape of 3.66-m diameter and 11.5-m total height. The bottom 3 m is used for waste gas distribution, for the foundation, and as a liquid reservoir; there is a 1 m of air plenum between the two beds and 2.5 m of space for water distribution an air exhaust above the top bed. The vessel is constructed of fiberglass reinforced plastic (FRP) that is coated on the inside and the outside with resin.
Airflow rate	Waste air from four production lines (viscose process), total flow of 44,200 $m^3\,h^{-1}$ (26,000 cfm), upflow
Empty bed residence time	4–10 seconds
Pressure drop	10 cm of water gauge of total pressure drop. 25 cm of water gauge when the maximum elimination capacity is reached.
Average bed temperature	18–34 °C
Pollutants treated	Carbon disulfide: 35–100 $mg\,m^{-3}$; hydrogen sulfide: 85–213 $mg\,m^{-3}$ (60–155 ppm_v)
Biotrickling filter controls	Continuous monitoring (gas flow rate, recycle liquid temperature, pressure drop, recycle flow rate and pH), no automatic control is performed, except for pH, which is maintained between 4 and 5 by the addition of a slurry of magnesium hydroxide.
Biotrickling filter design and acceptance criterion	The biotrickling filter was designed for odor control. Most of the odor comes from hydrogen sulfide, so the biotrickling filter was designed for 90% removal efficiency for hydrogen sulfide. There was a design criterion for carbon disulfide removal.
Approximate investment costs	$525,000
Approximate treatment cost per 1,000 m^3 off-gas treated	Yearly electricity and chemical costs are about $43,000; various indirect costs (personnel, various equipment maintenance) are $20,000. Total is $63,000 per year or about $0.18 per 1,000 m^3 of off-gas treated
Typical performance	Hydrogen sulfide is easy to degrade, and usual hydrogen sulphide removal efficiencies are in the range of 85–99%. Carbon disulfide–removal efficiency depends on the concentration of hydrogen sulfide. When the concentration of hydrogen sulfide is low, i.e., in the range of 100–150 $mg\,m^{-3}$, good removal of carbon disulfide is observed. At higher hydrogen sulphide concentrations, the removal of carbon disulfide decreases. Usually, carbon disulfide removal ranges from 40 to 70%. The combined elimination capacity for hydrogen sulfide and carbon disulfide is usually around 310 $g\,m^{-3}\,h^{-1}$

Table 8. Characteristics of the Reemtsma Biotrickling Filter

Owner and location	Reemtsma, Berlin, Germany
Builder	M + W Zander Facility Engineering GmbH, Nürnberg, Germany
Type of airstream	Cigarette production off-gas, odor treatment
Year of installation	1995
Packing type and volume	Polyurethane foam (cubes of 4 cm), total volume: 500 m^3.
Height and number of layers of packing	1 layer, 2.5-m high
Biotrickling filter construction type	6 container units.
Airflow rate	160,000 $m^3\,h^{-1}$, downflow
Empty bed residence time	11 seconds
Pressure drop	2–4 cm water gauge
Average bed temperature	40 °C
Pollutants treated	Odors: 800–1,200 OU.
Biotrickling filter controls	Continuous monitoring of temperature and pressure drop, water level is controlled.
Biotrickling filter design and acceptance criterion	90% odor removal or outlet air odor lower than 100 OU.
Approximate investment costs	4.3 million DM (1995, approximately $3.05 m) including ductwork cooling towers and heat exchangers.
Approximate treatment cost per 1,000 m^3 off-gas treated	Operating costs of 160,000DM per year ($93,000 per yr), i.e., 0.114 DM per 1,000 m^3 of off-gas treated (1997, $0.066 per 1,000$m^3$ of off-gas treated)
Typical performance	>90% odor removal

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Figure 7. The Biocyd Celorey biotrickling filter (Courtesy of Mauricio Acosta Grupo Cydsa S. A. de C. V.).

Figure 8. The North Island Naval Air Station biotrickling filter (Courtesy of Todd Webster, Envirogen Inc.).

of concern were common volatile paint solvents (ranging individually from 7 to 520 ppm_v), together with low concentrations (1–2 ppm_v) of H_2S resulting from sulfate reduction by anaerobic bacteria in the water tanks. The total VOC concentration in the air has relatively large fluctuations over the day, typically from 150 to 350 ppm_v (as methane equivalents).

The biotrickling filter design consisted of both air and water downflow operation through two media beds in series. A computer and various data loggers were

Table 9. Characteristics of the North Island Naval Air Station Biotrickling Filter

Owner and location	Naval Air Station-North Island (San Diego, CA)
Builder	Envirogen, Inc. (Lawrenceville, NJ)
Type of airstream	Effluent from industrial and oily wastewater treatment tanks
Year of installation	1999
Medium type, and volume of¡w¿medium	Random, dump packing. Approximately 31 m^3 (1,100 ft^3).
Height and number of layers of medium	Two beds in series, each 2.1 m (7 ft.) in height. Air is downward flow.
Biotrickling filter construction type	3.0 m (10 ft.) in diameter, 9.0 m (30 ft.) tall, cylindrical in shape, cast in fiberglass resin polymer.
Airflow rate	2,970 $m^3\,h^{-1}$ (1,750 scfm)
Empty bed residence time	37 seconds
Pressure drop	12.7 cm (5 inches) total of water column across both beds
Average bed temperature	18–27°C (65–80 °F) for recirculating water phase
Pollutants treated	Total sulfur compounds: 1–3 mg sulfur m^{-3} Total organic compounds: 75–175 mg carbon m^{-3} Identified compounds were hydrogen sulfide, benzene, toluene, xylene, trimethylbenzenes, acetone, methyl ethyl ketone, methyl isobutyl ketone, methylene chloride, heptane, cyclohexane, and numerous other aliphatics at lower concentrations
Biotrickling filter controls	Continuous monitoring of air and water flow, air and water temperature, pressure drop, and pH. All data is logged into a PLC for trend analysis. System conditions are monitored through the PLC. The PLC terminates system operation when alarm conditions occur (i.e. low airflow)
Biotrickling filter design and acceptance criterion	Design based on pilot-test studies on a site treating similar compounds, acceptance criterion: 80% removal of odor-producing compounds
Approximate investment costs	Not available
Approximate treatment cost per 1,000 m^3 of off-gas treated	Costs for water and chemicals were estimated at about $5,000 per year, i.e., $0.19 per 1,000 m^3 of off-gas treated. Electricity and maintenance costs were not available¡w¿but were estimated to be similar to those of a carbon adsorption system that would otherwise be used if the biotrickling filter was not available.
Typical performance	Greater than 99% removal of hydrogen sulfide Greater than 90% removal of aromatics Greater than 95% removal of ketones

used to monitor and control the reactor's operation (Table 9). The liquid recirculation rate was kept manually constant using butterfly valves. Approximately 230 to 380 L (60–100 gallons) of recycle liquid was removed from the system daily by a timer on a discharge valve located at the bottom of the reactor. Fresh water and nutrients were added automatically when the water level inside the system dropped below a specified height. The pH was controlled automatically. At reactor start-up and during initial operation, a large quantity of commercial microorganisms, cultured microbes from an in-house laboratory, and some activated sludge were used to inoculate the system. Which inoculum source proved to be most efficient is not known.

CONCLUSION

There is no doubt that reducing pollutant emissions at the source should always be attempted. However, zero emission is not always technically or economically feasible. For many of these cases, end-of-pipe treatment in biotrickling filters appears to be a promising alternative to conventional treatment technology. It is effective, environmentally friendly, and does not have many of the drawbacks of conventional treatment technologies. The field of biotrickling filtration is maturing. The number of full-scale biotrickling filters is rapidly increasing, and continuous research helps understand the fundamental principles of biotrickling filters. All together, this speaks for a rapid increase in the use of biotrickling filters in the twenty-first century.

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BLUE-GREEN ALGAE. See CYANOBACTERIA

BORRELIOSIS, LYME. See LYME BORRELIOSIS

BOTTLED WATER. See HETEROTROPHIC BACTERIA AS INDICATORS OF WATER QUALITY

BOTTLED WATER, MICROBIOLOGY OF

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"In all advanced ancient cultures water, especially running water, was associated with mythological ideas. Water was worshipped as a life-giving fluid of particular efficacy. Even in these days the Kastalia spring at Delphi is said to donate health and beauty to a great age. Both in religious thinking and in natural philosophy, water plays an important role. The spring is invariably the place of mysterious birth" (1). Since the majority of individuals no longer obtain their water at the source, the bottling process has replaced the journey to the spring. By 1999, bottled water sales in the United States alone exceeded 5.2 billion. Worldwide, the industry accounted for 35 billions in sales (2). In the United States, 54% of Americans regularly drink bottled water, although the cost of drinking bottled rather than tap water may be anywhere from 240 to 10,000 times more per gallon (3).

WHY DO PEOPLE DRINK BOTTLED WATER?

Many people drink bottled water because they are concerned about the quality of their tap water. In developing countries, the lack of quality-controlled tap water has led to the substitution of bottled water. Other individuals prefer the taste of bottled water to that of tap water. In general, bottled water drinkers believe that the water they drink, when compared with tap water, is free of microbes. This is, however, a misconception. Source springs are associated with soil formations, and the latter contain indigenous bacteria. The numbers of these organisms may be minimal, but they have the ability to proliferate during storage, that is, once the water has been bottled.

WHERE DOES BOTTLED WATER COME FROM?

Many of the older European springs that have served as source waters for hundreds (if not thousands) of years tend to have their distinct bacterial flora. In fact, Frenchmen have been known to believe that the taste of specific waters is dependent on the types of "normal" bacteria present, much as is the case with cheeses. Much of the bottled water that is purchased in the United States does not, however, come from springs. Springwater may be

defined as water that flows naturally to the surface of the earth from an approved underground source. Many bottlers obtain their water from boreholes, which may or may not be located in the proximity of natural springs, but which are actually wells dug into the ground. Twenty-five to 40% of bottled water is actually bottled tap water from municipal sources, which may or may not be subjected to additional treatment. Bottled municipal water actually has the added advantage of being regulated by the Environmental Protection Agency (EPA), as well as by the Food and Drug Administration (FDA). If any of these sources becomes contaminated, or if potential pathogens enter the water during the manufacturing process, the product has the potential of causing illness in the user. Although, with one exception (4,5), bottled water has not been implicated in confirmed outbreaks of disease, it is unlikely that incidents of gastroenteritis or related illnesses would tend to be reported by the consumer or be attributed to the water.

REGULATION OF BOTTLED WATER

In the United States bottled water is considered to be a food product and, as such, is regulated by the FDA. The quality of tap water, on the other hand, is overseen by the EPA. The rationale for this arrangement is mandated by the fact that bottled water may be purchased by choice, whereas access to tap water is a right, if not a requirement. EPA tap water regulations are identical in all states. FDA Regulations cover only bottled water sold in interstate commerce, so that unless a state has adopted its own standards and regulatory procedures, bottled water sold in-state may be totally unregulated. FDA rules also exempt many types of what the consumer would consider "bottled water," to wit, those labeled as "water," "carbonated water," "disinfected water," "filtered water," "seltzer water," "sparkling water," or "soda water." These are not considered "bottled water" by the FDA.

In the United States, microbial standards are based on total coliforms and the presence of either *Escherichia coli* or fecal coliforms. Coliform bacteria are referred to as "indicator" organisms since they indicate the presence of fecal contamination and thus the possible presence of pathogenic organisms, which may cause intestinal disease, although they themselves may not be disease producing.

There are other organisms that fall within the classification of "coliforms" as this type of organism is classified by biochemical tests. These may originate from the soil, rather than from the intestinal tract of humans or other warm-blooded animals. For this reason a differentiation is made between *E. coli* (which is a coliform organism characteristically found in the intestinal tract) and fecal coliforms, as opposed to coliforms in general. The EPA Tap Water standards do not allow for the presence of *E. coli* or fecal coliforms in drinking water. FDA standards, on the other hand, note that up to 1 of 10 bottles tested may contain one per 100 mL by the membrane filter technique of any type of coliform (6).

Coliforms have been used as indicators of water quality in the United States since the early twentieth century. They are correlated neither with the presence of certain viruses nor with *Giardia* or *Cryptosporidium*, which are waterborne intestinal parasites. In the United States "bottled water" is typically a flat water that may originate from a number of different sources. In Europe, individuals tend to drink *mineral water*. European regulations for these waters are considerably stricter than those promulgated by the FDA. In the United States mineral water is defined as "bottled water containing not less than 250 ppm total dissolved solids, coming from a source tapped at one or more boreholes or springs, and originating from a geologically and physically protected underground water source. Mineral water is distinguished from other types of water by its constant level and relative proportions of minerals and trace elements at the point of emergence from the source. No minerals may be added to this water" (7). There are few true mineral water springs in the United States. In the European Union (EU), mineral water typically comes from well-known springs that have, in some cases, been used since Roman days. The water must be officially approved. No disinfectant of any type may be added to the water for the removal of microorganisms (6). The EU regulations stipulate that, at the source, natural mineral water must be free of disease-producing organisms. This includes the total absence, in 250 mL samples of the water, of *E. coli*, coliforms, fecal streptococci, or *Pseudomonas aeruginosa*. Moreover, 50 mL samples may not contain sulfite-reducing, spore-forming anaerobic organisms (8). The normal habitat of fecal streptococci is the gut of humans and animals. *Pseudomonas aeruginosa* is an opportunistic pathogen, that is, although it is generally not pathogenic for a healthy population, it is typically problematic in immunocompromised patients. It has been shown responsible for the deaths of infants drinking water contaminated with the organism (9), although an effect of this severity is not typical. The organism is included in the microbial standards because it typically indicates contamination occurring somewhere during the bottling process. Spore-forming anaerobes are also common inhabitants of the intestinal tract, which on their elimination from the host, have the ability to produce spores, or protective structures, allowing for a long-term resting stage.

EU mineral water regulations also impose strict limitations on the number of colonies (populations of bacteria arising from a single cell) of normally-occurring springwater organisms present at the source and after bottling. Specifically, at source, water may contain no more than 20 colonies/ml when incubated for 72 hours at 20 to 22 °C and 5 colonies/ml when incubated for 24 hours at 37 °C. After bottling, the total colony count may not exceed 100 colonies/ml when incubated at 20 to 22 °C and 20 colonies/ml if incubated at 37 °C for 24 hours. Total colony count must be determined within 12 hours after bottling, with the water being maintained at 3 to 5 °C during this period (8).

GROWTH MEDIA FOR BACTERIA IN BOTTLED WATER

The twentieth edition of *Standard Methods for the Examination of Water and WasteWater* (10) defines

bacterial plate-count procedures for water, including the membrane filter technique, which allows for sampling volumes of water greater than 1 ml. It permits the use of a nutrient medium specific for the growth of organisms in the presence of low concentrations of organic matter. This is the so-called R2A medium, developed by Reasoner and Geldreich (11). The medium is specifically devised to allow for the growth of organisms that are oligotrophic or require only minimal amounts of organic matter. Heterotrophic plate count (HPC) measures those organisms that are normally found in springwaters. It is also used to quantify bacteria in the finished product and to determine the increase in numbers after bottling. There is no definitive limit placed on the numbers of HPC organisms in bottled waters, although suggested limits are generally on the order of not more than 500 per milliliter, which is in accordance with EPA limits. Individual states may set their own guidelines. Rhode Island, for example, had set a limit of 200 organisms/ml, which was subsequently dropped since it was legally unenforceable. Concern about high numbers of HPC organisms is based on the possibility of their masking disease-producing microbes in the water as well as indicating a problematic source water or contamination during the bottling process. In the United States, where disinfection is permitted, HPC may also indicate the effectiveness of the agent used and its extended effectiveness in the bottle. In the European Union, no disinfection is permitted.

TYPES OF BACTERIA NORMALLY FOUND IN SOURCE WATERS

Waters derived from springs typically contain a low number of autochthonous, or indigenous, organisms. These are generally gram-negative and capable of existing in environments with low nutrient levels, such as are characteristic of spring waters. Their origin is undoubtedly associated with the substrata from which the water arises. Most studies describing the qualitative presence of these organisms have concerned themselves with European mineral waters. Typically found are the group of organisms classified as pseudomonads. These bacteria are distributed through a number of different genera. They are widespread in nature, being found in soil and water environments, and are known for their ability to use an unusually large number of nutrient sources, many in trace quantities. Most of them are harmless, but a few are opportunistic pathogens, a term denoting the ability to cause problems in immunocompromised individuals. *Pseudomonas aeruginosa*, which can infect almost any body site, is one of the most problematic of the pseudomonads. It is rarely found in protected spring waters, rather being an indicator of contamination occurring from colonized equipment in the bottling plant or from plant personnel. The organism is not regulated in bottled waters in the United States, although concern has been raised as to its presence in waters used for mixing baby formula (see *Pseudomonas*, this Encyclopedia).

Many other organisms are normally found in spring waters. These include species of *Acinetobacter*, *Achromobacter*, *Flavobacterium*, *Alcaligenes*, and other usually gram-negative species (12–14). Typically, springs tend to have their own characteristic autochthonous flora. These organisms are present in typically minimal numbers at the source and do not present a problem to healthy individuals. The presence of allochthonous, or nonindigenous organisms, often indicates some type of problem, either during the manufacturing process or from a contaminated source. An unusual increase in the HPC count of source water also indicates grounds for concern. Schindler (15) reported the presence of noncoliform enterobacteriaceae in 41 of 54 manufacturing facilities that had hygienic problems and recommended the use of these organisms as a quality criterion for bottled mineral, spring, and table waters. Enterobacteriaceae are typically found in the intestinal tract of humans and other animals, although some are soil microorganisms.

TREATMENT OF WATER

Source water in the European Community may not be treated in any manner in order to remove microorganisms (8). For this reason, the purity of the source is of primary concern. In the United States, where bottled water may come from a variety of different sources, the water is typically treated in some manner to remove or decrease the number of organisms present. In the case of waters taken from municipal sources, that is, identical to tap water, chlorination has usually preceded other treatment and will have removed a large portion of the microbial population present. Water from springs and wells is not usually chlorinated and this fact is often used in marketing bottled water. The water may be filtered to remove small particles and/or bacteria.

In general, disinfection is accomplished through the use of ultraviolet irradiation and/or ozonation. Ultraviolet irradiation at wavelengths between 220 and 300 nm is effective primarily during multiplication and acts by forming dimers, or covalent bonds, among thymine molecules in the DNA of the organism. Resistant forms, such as bacterial spores, are minimally affected by this type of disinfection. Since glass and plastic containers are impervious to ultraviolet irradiation, disinfection of bulk waters, before bottling, may result in incomplete killing of organisms present, with subsequent regrowth within the container. Ozonation makes use of the principle that O_3, an unstable form of oxygen, acts as a strong oxidizing agent. In this regard its action in water is similar to that of chlorine, except that, whereas chlorination usually leaves a residual in the finished product, ozone, because it is unstable, decomposes quickly, leaving no residue. It is expensive and, as with ultraviolet irradiation, does not result in sterility, but it has the advantage of leaving no chemical taste in the treated water. Reverse osmosis, or hyperfiltration, is used to purify water by removing bacteria, salts, and other constituents having a molecular weight larger than 150 to 250 daltons. The water is pushed through a semi-permeable membrane, usually with the aid of a pumping mechanism. Contaminants are retained by the membrane and do not pass across it. A cleaning cycle, or backwash, programmed into the operation of the process, prevents a buildup of contaminants, which would

require increased pressure to force the water across the membrane.

In the absence of proper maintenance of plant equipment, bacteria may colonize membranes, filtration units, distribution lines, and other equipment, or contamination may occur through human error.

NOMENCLATURE OF WATERS

Labeling of water as other than "bottled" may result from various treatment methodologies. "Demineralized water" or "Purified Water" may be produced through distillation, demineralization, or reverse osmosis. "Sparkling waters" contain carbon dioxide, either from the spring or added during treatment. "Deionized water" is defined as water from which both anions and cations have been removed by an ion exchange process. "Distilled Water" is defined as water that has been purified by passage through an evaporation-condensation cycle. It may contain small amounts of dissolved solids.

BACTERIA IN THE FINISHED PRODUCT

Once water is bottled, unless it is sterile, quantitative changes appear in the microbial population. (Sterile water, such as that used for injection, is totally free of microorganisms, the latter having been removed by heat treatment or filtration.) Depending on the type and amount of organic matter present in the water, those bacteria present will begin to use the nutrients available to them (see Assimilable Organic Carbon (AOC) in Treated Water: Determination and Significance, this Encyclopedia). As noted previously, the organic content of bottled waters is usually low, and those organisms best adapted to living under these conditions will be at an advantage. In 1940, Heukelekian and Heller (16) showed that bacteria tend to attach to the walls of containers. Bacteria may live suspended in an aqueous environment, but it is much more economical for the organisms to utilize the nutrients in the water as they "flow by" than to have to utilize energy in moving through the liquid to reach the food sources. The attachment of bacteria to surfaces is generally referred to as biofilm formation. In either case, as suspended organisms or being attached to the sides of the container, bacteria will proliferate shortly after the container has been filled (17,18). At room temperature, it will take only a few days for the suspended organisms to reach concentrations as high as 10^4 to 10^5/ml. As the organic matter is depleted, the organisms will slowly begin to die-off, remaining viable for a period of months, with the more hardy forms utilizing the breakdown products from those no longer viable (19). Under conditions of refrigeration, there is a lag in multiplication, but organisms remain in the water for longer periods. However, their numbers do not reach those found in room-temperature stored containers (20). In contrast, tap water does not remain in a static state for extended periods since bacteria are flushed through the pipes whenever the faucet is turned on.

Heilwasser, or water from "Heilquellen" is the name given to water from springs with allegedly health-giving or curative properties, and is a product commonly sold in Europe. These waters may come from "heilquellen" or springs, which are rich in certain minerals. Heilwasser is usually recommended for treatment of various ailments including urinary, heart, and kidney disorders. Thus, immunocompromised individuals may be exposed to greater numbers of organisms than are typically found in other mineral waters (12).

PLASTIC AND GLASS CONTAINERS

All types of materials have been found to serve as solid substrates for bacterial attachment and biofilm formation. Mackenzie (21) used an agar-overlay method to determine adherence of *Staphylococcus epidermidis*, a common organism found on human skin, to polystyrene, polyvinyl chloride, polyethylene, and polytetrafluoroethylene, compounds often used in the manufacture of plastic containers for bottled water. Hamilton and Rosenberg (22) used an agar-overlay dye method as well as scanning electron microscopy to characterize the attachment of bacteria found in polyethylene bottled water containers obtained from the shelves of grocery stores. After storage at room temperature, containers of water with low initial bacterial counts (100 or less per ml. of water) were found to develop increasing levels of biofilm. Only low levels of attached bacteria were observed on containers stored under refrigeration.

Bottled water in plastic containers has been shown to contain higher numbers of bacteria than water in glass bottles (12,14,23,24). This may be due to nutrient release from materials in the container, as well as diffusion of oxygen through the thin container walls. Glass bottles tend to be reusable and are well cleaned before being refilled. Plastic containers may be produced in the bottling plant using an extrusion process, but are more often purchased from vendors. The caps for containers may come from other sources and lack of sterility may introduce organisms into the water when the containers are shaken or during transport by the consumer (25).

SHELF STORAGE

Many bottlers place an expiration date on the bottled water containers. This is often two years after bottling. From a microbiological standpoint, this makes little sense since most users of bottled water drink the product shortly after purchase when the numbers of bacteria tend to be highest. Multiplication of bacteria during early storage, that is, within a few days following bottling, may rapidly increase the number of organisms to a point where they exceed the regulatory limitations placed on water at the source, or within 12 hours of bottling. Water may adhere to EPA standards, or the more stringent European Union standards, when taken from a source, or immediately after the bottling process. Storage on the grocery shelf (without refrigeration), or at home, may increase the heterotrophic plate-count to as high as 10^5 or 10^6 CFU/ml (26). The bottler may have fulfilled his obligation in adhering to standards and thus cannot be held responsible

for bacterial multiplication "downstream." Naturally occurring organisms present little risk to the healthy individual under these conditions (27), but opportunistic organisms present in minuscule amounts in the source water may nevertheless multiply to numbers capable of producing potential problems in immunocompromised individuals.

PSEUDOMONADS IN BOTTLED WATER

The group of bacteria known as pseudomonads, which are commonly found in source waters, and thus in the finished product include opportunistic species capable of initiating nosocomial infections. *Pseudomonas aeruginosa* is known as the most prevalent *Pseudomonas* species in human disease. It is rarely found in pure spring waters and is usually considered to be associated with contamination during the bottling process. It can be considered a reliable indicator of manufacturing problems, although it has not been accepted as such in the United States. Other countries such as the European Union and Canada have recognized its importance and adopted stringent regulations concerning its presence. Pseudomonads have been shown to survive and multiply in bottled water for extensive periods. The fact that they require such miniscule amounts of organic matter and their potential to use breakdown products from other organisms in the water contribute to their longevity (28–30). *Burkholderia cepacia* and *Stenotrophomonas maltophilia* are commonly found in source waters. These organisms have been considered of particular concern to immunocompromised individuals (31,32). Their ability to grow on minute traces of carbon sources, including adhesives found in bottle cap-liners, contributes to their ubiquitous presence.

ANTIBIOTIC RESISTANCE OF PSEUDOMONADS

Pseudomonads are also known to be resistant to many of the commonly used antimicrobials. In a study of German mineral waters, Rosenberg and Hernandez-Duquino confirmed the resistance of pseudomonads associated with nosocomial infections to a variety of commonly used antibiotics. Of 12 antibiotics studied, the highest resistance among 81 organisms of 9 different species isolated was to cephalothin, ampicillin, and carbenicillin, in that order. Only one organism was resistant to tobramycin, and four to kanamycin and trimethoprim-sulfamethoxazole (12). In results obtained from studies in Greece, multiple-linked resistance was found to chloramphenicol, tetracycline, erythromycin, and nalidixic acid. *Pseudomonas aeruginosa* and *Pseudomonas stutzeri* were susceptible to the newer antibiotics such as ceftazidime and ciprofloxacin. *Stenotrophomonas maltophilia* showed the highest resistance (69.2%) to these newer antibiotics (33). In an Italian study, greatest resistance was found to nalidixic acid (34).

CARBONATED AND NONCARBONATED WATER

In 1974, during a cholera outbreak in Portugal, a strain of the bacterium *Vibrio cholerae* was isolated from two springs supplying mineral water to a commercial bottling plant in the Lisbon area (5). Seepage into a limestone aquifer was apparently responsible for the contamination. An investigation showed that cases of the disease occurred in individuals who ingested noncarbonated mineral water from the spring, whereas no cholera cases occurred in those who drank carbonated mineral water. In the case of *V. cholerae*, the organism is able to survive for less than a day in carbonated water (35), and prefers and tolerates conditions of high alkalinity. Several studies have characterized the sequence of bacterial growth in bottled uncarbonated mineral water. Morais and daCosta (36) noted an increase from low numbers to 10^4 to 10^5 CFU/ml within a few days after bottling, with fairly constant numbers remaining for a period of a year when incubated at 22 C. Gonzalez and coworkers (37) showed an increase from an initial population of 10^1 to 10^2 CFU/ml to 10^5 to 10^6 CFU/ml within three days storage. Similar results were shown by Rosenberg and coworkers. In this study, bottled water at room temperature and under refrigeration was sampled weekly over a period of six weeks. The increase in numbers of organisms at room temperature was basically identical to that shown in the study by Gonzalez and coworkers, with numbers beginning to decrease after six weeks storage. Where water was refrigerated, bacterial counts eventually reached those of nonrefrigerated waters, though this did not occur for about three weeks, and the decrease in numbers was prolonged over a period of several months (20). In their studies on German mineral waters, Rosenberg and Hernandez Duquino (12) showed that water in plastic containers, which typically has no carbon dioxide added, generally showed considerably higher counts of bacteria than that in glass containers with indigenous or added carbon dioxide. Noncarbonated waters are usually bottled in plastic, whereas carbonated waters require bottling in glass as well as screw caps to maintain carbonation.

ARTIFICIAL INOCULATION OF WATERS WITH BACTERIA

Several studies have measured the survival of pathogens in bottled water by inoculation. Warburton and coworkers (38) reported on the viability of *E. coli* O157:H7, a strain causing hemolytic uremic syndrome. Low levels of the organism (about 100 CFU/ml) were inoculated into mineral water and were shown to survive for up to >300 days at room temperature. Kerr and coworkers (39) terminated their experiment after 42 days, at which time the organism was still viable in natural, noncarbonated mineral water.

The artificial addition of organisms to a bottled water cannot be compared to the microbial flora and characteristics of natural spring or bottled waters. The equilibrium existing in such waters is bound to be upset by the intentional addition of a relatively large numbers of potentially pathogenic allochthonous species. Nevertheless, such studies show that bottled water will sustain the presence of medically important organisms and that frequent analyses and stringent regulations are of prime importance in maintaining the health of the consumer.

AEROMONAS SP.

Aeromonas sp. may be found in environmental water sources and are capable of causing diarrhea. Brandi and coworkers (40) noted the ability of *Aeromonas* spp. to remain viable for longer than 100 days in mineral water. However, neither Hunter (41) nor Massa (42) isolated *Aeromonas* spp. from natural mineral water in England or Italy. The Scientific Committee on Food, in an opinion given to the European Commission on March 19, 1998, concluded that "*Aeromonas hydrophila* and *Aeromonas caviae* in natural mineral water do not present a potential risk to human health" and that limits for these organisms cannot be justified (43). The U.S.EPA, on the other hand, has listed *Aeromonas* as a potential candidate for further regulatory consideration in drinking water.

VIABILITY OF BACTERIA WITHOUT MULTIPLICATION

Bacteria are capable of surviving in aqueous environments under conditions where multiplication does not occur. These organisms are referred to as being "viable but nonculturable." The induction of protective mechanisms allows the organism to enter a latent stage (see VIABLE BUT NOT CULTURABLE (VBNC) MICROORGANISMS, this Encyclopedia). This may occur under conditions of low nutrient concentration such as are found in bottled water. Plating on nutrient-poor media, such as those used to quantify HPC organisms, or on selective media that may contain toxic dyes or other substances, may prevent growth and thus, identification of these organisms. Typically these organisms are allochthonous, or foreign to the water source.

ISOLATION OF AMOEBAE FROM MINERAL WATERS

Free-living amoebae were isolated from Brazilian mineral water by Salazar and coworkers (44). A study from Mexico (45) describes the isolation, from the three best-selling brands of mineral water in the country, of *Naegleria* and *Acanthamoeba*, amoeba including species that have been associated with fatalities in humans. *Mycobacterium* and *Legionella* species also have the ability to replicate within *Acanthamoeba*.

FLUORIDE IN BOTTLED WATER

When municipal waters are used for bottling purposes, the presence of fluoride is generally equivalent to that found in tap water supplies from the same source. However, in the case of springs and other aquifers, fluoride is not usually added to the final product. The question of whether bottled waters may not give the same protection from cavities as treated municipal waters has been raised by members of the dental profession (46). In an Ohio study, Lalumandier and Ayers determined the fluoride content of fifty- seven samples of bottled waters and determined that only 5% of these samples, as compared to 100% of Ohio tap waters, fell within the required fluoride range recommended by the state (47).

BOTTLED WATER DISPENSERS

Many grocery stores sell water through "water dispensers" where customers bring their own bottles and purchase water from a machine dispenser. Potential problems include the lack of thorough rinsing of reusable containers used by the consumer and the proliferation of bacteria in the dispenser apparatus itself.

Water dispensers in offices, theaters, and other locales have become extremely popular in the United States. Most of these units dispense cold water, although some dispense hot water for tea and coffee as well. The carboys, often made of polycarbonate, usually contain five liters of water, identical to that sold in bottles by the distributor. Thus, the bacterial flora and its growth within the carboy are similar to that found in bottled water. The carboy is placed within the dispenser and rests on the neck of the bottle within a reservoir (Fig. 1). As water is removed for use, the reservoir is replenished by additional water flowing from the carboy. Bacteria attach to the walls of the reservoir and utilize organic matter present in the water that flows by. It has been shown that the greater the number of individuals in offices using water from dispensers, the higher the number of organisms present. 10^5 to 10^6 organisms/ml were routinely found in several dispensers. This is apparently due to the replenishment of organic matter from new bottles and subsequent growth of organisms attached to the reservoir walls within the unit (48). Proper cleaning of the units with a bleach solution will remove the bacterial biofilm.

USE OF BOTTLED WATER FOR CLEANING CONTACT LENSES

The use of bottled water for use with contact lenses is not recommended due to the presence of potential ocular pathogens (49). Coliforms, amoeba, and fungi, *P. aeruginosa*, and a variety of other potential pathogens were isolated from spring, drinking, and glacier bottled waters tested.

The lack of sterility allows for contamination of contact lenses following only brief exposure.

ENDOTOXIN IN BOTTLED WATER

When gram-negative bacteria die off the lysis of their cell walls results in the formation of endotoxins. The most important clinical effects are fever and shock, which result from the presence of endotoxins within the bloodstream of the host. The presence of endotoxins in bottled waters is not uncommon, due to the numbers of bacteria present in the water itself and attached to the sides of the container. Endotoxin can easily be detected using an assay using material from the horseshoe crab, *Limulus polyphemus*. Caramello and coworkers detected endotoxin levels higher than 0.5 ng/ml in 40 of 44 mineral water samples (50). Borders and Rosenberg (51) showed that endotoxin readily attached to bottled water container surfaces, approximately ten times as much attaching to the bottom of containers as to the sides. Potentially, diarrhea can result from endotoxin in bottled waters.

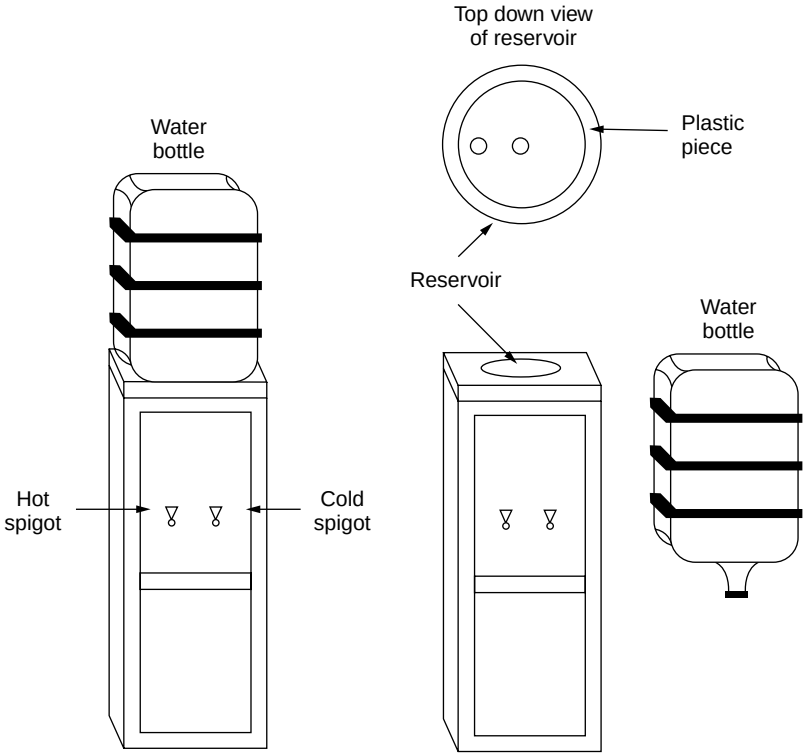

Figure 1. View of typical bottled water dispenser.

NEW TECHNIQUES IN BOTTLED WATER MICROBIOLOGY

A number of newer techniques have been adapted to determine the quantitative and qualitative characteristics of bacteria in bottled and mineral waters. Guillot and Leclerc (52), utilizing ribosomal RNA gene restriction patterns, showed a distinct specificity of bacteria isolated from four brands of French mineral water tested from the bottling lines of the companies, with few common patterns among springs. *Pseudomonas* sp. were shown to be the predominant culturable bacteria present.

The authors noted that only about ten percent of the bacteria present could be cultured on bacteriological media commonly used to grow mineral water isolates.

Using a LIVE/DEAD BAc Light™ VIABILITY kit, together with epifluorescence microscopy, Defives and coworkers (53) measured the variation in bacteria present in French mineral water taken directly from the spring. Viable but noncultivable bacteria were estimated by taking the difference between viable and viable culturable counts. Numbers of organisms increased from less than 10/ml., with only 20 to 30% of these being culturable on R2A medium, to approximately 3×10^5/ml after six days postbottling, and subsequently stabilized. Again, the majority of isolates belonged to the pseudomonads. Bottling stress and low amounts of organic material resulted in bacteria changing into the viable but nonculturable state.

The Food and Drug Administration has often considered the cost to the bottler of testing for *P. aeruginosa* as one of the reasons for not adapting this organism as an indicator of contamination, as is the case in Europe and Canada. A new methodology, using nucleic acid probes, has shown that a specificity of 100% for the identification of *P. aeruginosa* can be obtained by use of soybean peroxidase-labeled peptide nucleic acid probes, which are targeted to a species-specific sequence in the ribosomal RNA of the organism. The bacteria are identified directly on the membrane filter used to filter the sample (54).

Many viruses can be tested for in bottled water using the reverse transcription-polymerase chain reaction. An example is the Norwalk virus, a calicivirus (single-stranded RNA) that has been shown to cause diarrheal disease together with other systemic symptoms. It is the major cause of nonbacterial gastroenteritis in the United States and obtained its name during one of the first reported outbreaks, in Norwalk, Ohio, in 1969. Norwalk-like virus sequences were detected in 21 mineral waters imported into, or bottled, in Switzerland (55). The authors note that they are unaware of epidemics due to contaminated mineral water having been reported, but the possibility of nonreported individual cases cannot be disproved.

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BROWN ROT FUNGI. See PULP AND PAPER INDUSTRY: MICROBIOLOGICAL ASPECTS OF

BROWN TIDE. See RED TIDES AND OTHER HARMFUL ALGAL BLOOMS

BULKING CONTROL. See FILAMENTOUS BULKING IN ACTIVATED SLUDGE, CONTROL OF

BULKING IN ACTIVATED SLUDGE.
See FILAMENTOUS BACTERIA IN ACTIVATED SLUDGE: CURRENT TAXONOMIC STATUS AND ECOLOGY

BULKING OF ACTIVATED SLUDGE.
See FILAMENTOUS BULKING IN ACTIVATED SLUDGE, CONTROL OF

C

CALICIVIRUSES. See HUMAN CALICIVIRUSES: BASIC VIROLOGY AND EPIDEMIOLOGY; NORWALK-LIKE VIRUSES: DETECTION METHODOLOGIES AND ENVIRONMENTAL FATE

CAMPYLOBACTER JEJUNI AND OTHER ENTERIC *CAMPYLOBACTER*

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The members of the genus *Campylobacter* cause a wide spectrum of infections in both humans and animals, including gastroenteritis, systemic disease, and reproductive disorders in domestic animals. The organisms can be isolated from a wide variety of environments, including surface water, foods, and the intestinal tract of various animals and are accordingly considered to be among the most ubiquitous bacterial pathogens known to man. The remarkable prevalence of campylobacters makes them important from both clinical and economical perspectives. *Campylobacter jejuni*, in particular, is widely distributed in the intestinal tract of poultry, livestock, and warm-blooded domestic animals and is the leading cause of bacterial food-borne diarrheal disease throughout the world. In the United States alone this organism has a 4 to 35% isolation rate in patients with acute gastroenteritis (1). Given the high carriage rate of campylobacters in domestic and wild animals, large numbers of these organisms are continually excreted into the environment. As a consequence, the environment plays an important role in maintaining the contamination cycle of these pathogens. The following discussion focuses specifically on the major enteric pathogens of the *Campylobacter* genus, which are *C. jejuni* and *C. coli*. In addition, particular emphasis is given to the ability of these pathogens to persist in the environment and the role that the environment plays in promoting their contamination cycle.

GENERAL ASPECTS OF *CAMPYLOBACTER* AND *CAMPYLOBACTER JEJUNI*

Historical Perspective

The current impact of *Campylobacter* species on human health is indisputable, and currently they are considered to be among the most ubiquitous pathogens known to man. Compared with other bacterial pathogens, however, their association with human disease has been established only relatively recently. *Campylobacter*-like bacteria were first described in 1913 but were originally classified in the family *Vibrionaceae*. The genus designation *Campylobacter* (Campylo — curved, bacter — rod) was not introduced until 1963 (2).

The pathogenic campylobacters have specialized growth requirements, an optimum growth temperature of 42 °C, and a requirement for a microaerophilic atmosphere. Consequently, the first isolation of *Campylobacter* from humans suffering from acute gastroenteritis was not reported until 1972. For a time afterward, however, campylobacters remained obscure and merely generated medical curiosity as their fastidious nature hindered their isolation. The use of selective media for the detection of campylobacters in food and clinical samples in 1977 (3) marked a water shed in our understanding of these pathogens and provided for the first time a window that revealed the probable impact of these bacteria on human health. Having found these pathogens in more than 7% of diarrheal samples, Skirrow predicted that these microbes could pose a serious public health risk. Reports of enteritis caused by *Campylobacter* species have since then risen dramatically, and currently *C. jejuni*, in particular, is regarded as the leading cause of bacterial food-borne illness worldwide.

Taxonomy of the Genus *Campylobacter*

Today, the *Campylobacter* genus, which belongs to the epsilon subclass of proteobacteria, comprises 15 species (4), of which a particular number are of importance to human health (Table 1). The genus comprises slender, spirally curved, gram-negative rods with a characteristic corkscrew-like darting motility. In addition, campylobacters do not ferment or oxidize carbohydrates and generally are microaerophilic, although some species are able to grow aerobically and some grow under anaerobic conditions. A number of *Campylobacter* species are capable of causing disease in humans. However, *C. jejuni, C. coli*, and *C. lari* are considered to be the most important species causing human diarrheal disease, and whereas other species have been implicated as human pathogens in immunocompromised people, these species are isolated infrequently and generally represent only 1% of clinical *Campylobacter* isolations. However, the true impact of non-*jejuni*/*coli* species may not be fully appreciated as isolation techniques, routinely used for the detection of *C. jejuni* and *C. coli* in many diagnostic laboratories may not support the growth of these other species.

Campylobacter jejuni and *Campylobacter coli*

Campylobacter jejuni is considered to be closely related to *C. coli*, but whereas *C. jejuni* comprises 80 to 90% of all *Campylobacter* isolations in developing countries, *C. coli* infections represent only about 7%. The reason for this difference in incidence is not known. However, one study has suggested that the differential distribution of *C. jejuni* and *C. coli* in food does not account for this ratio of infection and thus has concluded that *C. jejuni* is more efficient in colonizing and causing enteritis in humans than *C. coli* and other *Campylobacter* species (5).

Table 1. Species of *Campylobacter* Associated with Human Disease That Can Be Isolated from the Environment

Organism	Reservoirs	Primary Vectors	Disease Association
C. jejuni	Man, mammals, birds, poultry	Food, poultry, raw milk, and water	Enteritis, systemic illness, GBS
C. coli	Mammals, pigs, birds	Food, poultry, and pigs	Enteritis
C. lari	Man, mammals, birds	Food and water	Enteritis
C. fetus	Cattle, sheep	Uncooked meat, beef, and pork	Systemic illness, bacteremia, meningitis
C. upsaliensis	Domestic pets	Dogs and cats	Rare, bacteremia, enteritis
C. hyointestinalis	Cattle, pigs, hamsters	Raw milk, hamsters	Rare, enteritis
C. mucosalis	Pigs	Meat, pork	Rare, enteritis
C. concisis	Man	Unknown	Periodontal disease, rare, enteritis
C. sputorum	Man, cattle	Unknown	Periodontal disease
C. curvus	Man	Unknown	Periodontal disease, enteritis
C. rectus	Man	Unknown	Periodontal disease

In terms of the incidence of human infection, *C. jejuni* is unquestionably the most important member of the *Campylobacter* genus, it being the most frequent cause of gastrointestinal illness in the developed world (1). Although the public at large is generally unaware of the impact of *C. jejuni* on human health, it causes infections more frequently than either *Salmonella* or *Shigella*, both of which command better public awareness. It is estimated in the United States that *Campylobacter* strains cause more than two million cases of diarrhea annually. In the United Kingdom, the incidence of *Campylobacter* enteritis has risen steadily during the last decade, and in 1998, more than 58,000 cases were laboratory confirmed. Remarkably, the true incidence of campylobacteriosis may be considerably higher because even for countries with sophisticated epidemiological surveillance systems, the true extent of *C. jejuni* infection may be underestimated. In the United Kingdom, for example, it has been established that for every case that is reported, approximately eight cases occur within the community but are not identified (6).

CLINICAL ASPECTS AND EPIDEMIOLOGY

Clinical Aspects

Campylobacteriosis is essentially a food-borne zoonosis, with *C. jejuni* commonly found in the gastrointestinal tract of a number of animals. The manifestation of the disease caused by this organism and other enteric campylobacters differs significantly between industrialized and developing nations. Although the incidence of *Campylobacter* infections in developing countries is much higher compared with that in industrialized nations, the actual rate of symptomatic illness generally is lower. In developing nations, the symptoms are mostly mild and characterized by a noninflammatory watery diarrhea. In contrast, in industrialized nations the usual manifestation of *Campylobacter* enteritis is acute inflammatory diarrhea that generally occurs one to seven days after infection. Symptoms include bloody diarrhea, fever, and abdominal pain persisting up to seven days, although the acute diarrhea lasts only two to three days. Although the acute symptoms may disappear in a matter of days, the pathogen can still be isolated at rates of between 10^6 and 10^8 organisms per gram of feces for up to two to three weeks after infection. The disease usually is self-limited, but on rare occasions the organism can cause appendicitis, bacteremia, meningitis, and other extraintestinal infections. Recurrence of symptoms can occur in 25% of cases.

Autoimmune Sequelae

A number of sequelae are associated with *Campylobacter* infection, and approximately 1 in 1,000 of the patients suffering from the acute form of gastroenteritis can subsequently develop acute inflammatory demyelinating polyneuropathy or Guillain-Barré syndrome (GBS). This is a serious neurologic complication that may result in severe and sometimes fatal paralysis. Although the risk of developing GBS following *C. jejuni* infection is low, in the United States as many as 1,300 cases of GBS may be attributable to prior infection with this pathogen (7). The pathogenesis of GBS and the role of *C. jejuni* in the disease process are not clear, but molecular mimicry between the bacterial LPS structures of certain serotypes and the ganglioside components of motor neurons may play an important role in the development of the disease (8).

Pathogenicity Mechanisms

Even today, the mechanisms that enable *C. jejuni* to cause disease remain largely unknown. Several reviews serve to condense the information concerning the pathophysiology of *C. jejuni* infection, in general terms (9–11), in terms of the interaction of the organism with eukaryotic cells (12), and focusing on the role of toxins in the disease process (13).

Animal Reservoirs for *Campylobacter* Species

Many domestic and wild animals such as poultry, swine, sheep, cattle, dogs, and cats carry campylobacters in their gastrointestinal tract without displaying overt symptoms, although the disease symptoms may arise in young animals (14). Consequently, the gastrointestinal tract of certain warm-blooded animals is considered to be the natural habitat of *Campylobacter* species. In particular, birds, and especially poultry, are regarded as the primary

reservoir for *C. jejuni*. For chicken and turkey, the organism is a commensal, it being able to permanently colonize the cecum in an asymptomatic manner. The intimate relationship between poultry and *C. jejuni* is further emphasized by a consideration of its temperature requirements for growth. In this respect, the optimum growth temperature of the organism (42 °C) mirrors that of the avian gut, which differs considerably from that encountered in the mammalian gut (37 °C). The high cecal carriage rates of commercially raised chickens of between 30 and 100% (15,16) are a further reflection of the intimate nature of the interaction between *C. jejuni* and poultry. Nevertheless, with the implementation of strict hygiene measures, including thorough cleaning and disinfecting procedures, disinfection of drinking water, change of footwear at the entrance to each broiler house, and the control of vermin, *Campylobacter* colonization of broiler flocks can be significantly reduced (17).

While poultry may be the primary vehicle for *C. jejuni*, other farm animals are also susceptible to colonization by campylobacters. For example, pigs from the day of birth are highly susceptible to enteric colonization, with the prevalence of campylobacters in commercial pig farms ranging from 76 to 100%, depending on the age or sex of the pig (18). Typically, *C. coli* is more often associated with swine that *C. jejuni*.

Transmission and Epidemiology

The epidemiology of *Campylobacter* infection demonstrates a number of interesting and notable features. First, while *C. jejuni* and *C. coli* are the leading causes of bacterial food-borne illness in developing countries, unusually for food-borne pathogens, the pattern of infection tends to be sporadic, with large outbreaks of infection caused by these organisms occurring rarely. Furthermore, the epidemiology of clinical cases of *Campylobacter* enteritis in temperature climates shows a striking seasonality. In the United Kingdom and the United States, for example, the rate of infection shows a marked and consistent increase in May that peaks in July. This variation in incidence may reflect warmer temperatures and concomitant changes in eating habits.

The infective dose for *C. jejuni* is generally considered to be low, and one report, which utilized human volunteers, demonstrated that oral ingestion of as few as 500 viable bacteria can result in clinical symptoms (19). In reality, the infective dose required for infection is likely to vary significantly from this figure for a number reasons. First, since not all persons exposed to the organism develop symptoms, the susceptibility to *Campylobacter* infection may vary from individual to individual. A further complication to consider is the fact that individual isolates of *C. jejuni* may differ significantly in their ability to establish infection and cause disease (20).

Given the high contamination rate of commercially raised chickens and the relatively low infectious dose, it perhaps is not surprising that the main infection route of *C. jejuni* to humans in most industrialized nations is through the consumption of undercooked poultry or food tainted by cross-contamination. The isolation rate for *Campylobacter* from poultry sold in many major outlets varies but generally is high. For example, in the United States, 69% of chickens bought from a local supermarket were found to be contaminated with *C. jejuni* (21). Once poultry harboring *C. jejuni* is introduced into the kitchen, it inevitably serves as a focal point for contamination. This aspect of *Campylobacter* epidemiology has been emphasized by a recent study that demonstrated that the preparation of chicken resulted in the contamination of multiple sites within domestic kitchens with campylobacters. Disturbingly, routine cleaning with detergent and hot water had no effect on the frequency of contamination (22).

Unpasteurized milk is also regularly implicated as a vehicle for infection. In addition, contaminated drinking water, in which *C. jejuni* can survive for extended periods, has been the cause of several large outbreaks of *Campylobacter* infection. In the United States, for example, between 1978 and 1986, 11 of 57 outbreaks of *Campylobacter* infection were waterborne and were associated with the consumption of unboiled surface water, contamination of groundwater with surface water, inadequate disinfection, or contamination of water by avian feces (23).

Other foods, including saladgreens, fruit, and fish, have also been implicated as vehicles for *Campylobacter* infection, but the risk associated with these is generally low (6). More unusual vehicles for food-borne campylobacteriosis include garlic butter (24) and milk that has been contaminated following the action of birds pecking through the aluminum bottle tops (25). Finally, domestic animals are also considered to be a possible source of infection. In particular, contact with puppies that have diarrhea has been shown to cause gastroenteritis in children (14), while a study centered on a health cooperative in the United States estimated that 6.3% of *Campylobacter* enteritis cases could be attributed to exposure to diarrhetic animals (26).

The Detection and Differentiation of *Campylobacter* Species

The development of selective media for the detection of *Campylobacter* in food and environmental samples in 1977 (3) marked a turning point in our understanding of these organisms. Today, however, traditional cultural methods, which involve preenrichment, selective culture (27,28), and final identification by biochemical or serological tests (29), are considered to be labor-intensive and take a minimum of four to five days for confirmation. In addition, given the limited metabolic capacity of campylobacters, the biochemical tests available for *Campylobacter* identification are generally regarded as unsatisfactory. Differentiation of *C. jejuni* strains has traditionally been achieved using serotyping, either based on heat-stable antigens (30) or using a rapid-slide agglutination technique (31). However, the serotyping of campylobacters is problematic because of its complexity, because of the labor investment required for antisera production, and because significant numbers of strains are nontypable. Consequently, the requirement for simple high-resolution identification systems for the epidemiological typing of strains has led to the development of a number of DNA-based methods (29,32).

ENVIRONMENTAL SURVIVAL OF CAMPYLOBACTERS

Factors Limiting Growth in Food and the Environment

The lower temperature limit for the growth of *C. jejuni* reported in the literature varies between 30 and 36 °C. This factor and the microaerophilic nature of the organism are generally considered to prevent its growth in food and the environment. Consequently, although *C. jejuni* is environmentally ubiquitous, having been isolated from river water, wastewaters (33), and sand on bathing beaches (34), it is not considered to be free-living, and therefore is capable only of survival, not of growth, in these environments.

Environmental Sensitivity of Campylobacters

Campylobacters' sensitivity to oxygen and inability to grow at temperatures below 30 °C are probably the major factors limiting the growth and survival of the organism outside of the host. However, *C. jejuni* is also susceptible to a number of other environmental conditions and is generally less able to tolerate environmental stress compared with other food-borne pathogens (Table 2). For example, the organism is very sensitive to desiccation and accordingly does not survive well on dry surfaces (35). In addition, campylobacters are also susceptible to raised levels of sodium chloride (36) and are killed readily at low pH (37). Despite being termed *thermophilic*, *C. jejuni* is also sensitive to exposure to high temperatures (Table 2), and consequently, the organism should not survive in food that has been adequately cooked.

In conclusion, campylobacters appear to be particularly susceptible to environmental stress, at least under laboratory conditions, and based on these observations, the organisms would not be expected to survive for long periods outside the host. Outside the laboratory, however, it is clear from the measured incidence of infection and from reports of the ubiquity of campylobacters in the environment that the organisms are able to persist to a greater extent than can be anticipated from laboratory-based studies.

General Aspects of Survival

The ubiquitous presence of campylobacters in the environment suggests that there are multiple and permanent sources for contamination and that large numbers of the organisms are excreted into the environment in a continuous fashion. In this respect, high rates of fecal shedding of *C. jejuni* have been detected in dairy cattle (38), sheep (39), and wild birds (40). In addition, campylobacters can also be introduced into the environment, following the discharge of sewage effluents into surface waters. In this situation, the campylobacters are thought to originate mainly from abattoirs and animal processing plants, because seasonal peaks in the frequency of *Campylobacter* isolation from sewage effluents parallel the incidence of zoonotic infections (41). As a consequence of these processes, surface waters are frequently contaminated with campylobacters. Again, because the minimal growth temperature for *C. jejuni* is between 32 and 36 °C, it can be assumed that *C. jejuni* survives rather than grows in aquatic environments.

Compared with other enteric bacterial pathogens, *C. jejuni* appears to be uniquely limited in its ability to grow at relatively low temperatures (Table 2). Because this deficiency places limitations on the ability of this organism to replicate in food and water, which are the primary vehicles for its transmission, the response of *C. jejuni* to low temperatures has been studied in some detail. The lower limit of bacterial growth is usually determined by the fluidity of lipids and membrane components, but whether these parameters are related to the temperature growth limitations of *C. jejuni* is not known at present. A feature that may give some insight into the limited temperature growth range for this pathogen, is the fact that, unlike other microorganisms, which show a gradual reduction in growth rate near the minimal growth temperature, *C. jejuni* shows a dramatic and sudden growth rate decline near the lower temperature limit (42).

Despite its failure to replicate at environmental temperatures, *C. jejuni* is metabolically active at temperatures far below its lower growth limit. For example, *C. jejuni* is able to perform respiration and generate ATP at 4 °C, which implies that the electron chain and substrate transport systems are active, despite the fact that this temperature is well below the minimum required for growth. Furthermore, *C. jejuni* is motile at 4 °C, indicating that, although replication at this temperature is not possible, the organism still retains the capacity to move toward favorable environments (42).

At even lower temperatures, viability is rapidly lost, and although campylobacters can still be isolated from frozen meats and poultry products, freezing significantly

Table 2. The Limits of Growth and Temperature Sensitivity of *C. jejuni* Compared with Other Food-Borne Bacterial Pathogens

Organism	Temperature °C			Typical D-Value at 55 °C	Minimum a_w	Minimum pH	Oxygen Requirement
	Minimum	Optimum	Maximum				
C. jejuni	30	42–43	45	1.0	0.987	4.9	5–10%
Salmonella typhimurium	5.2	35–43	46	4.7	0.93	3.8	Facultative
Escherichia coli	7–10	35–40	44–46	5.5	0.95	4.4	Facultative
Listeria monocytogenes	0	37	45	4.5	0.92	4.4	Facultative
Staphylococcus aureus	7.0	37	48	3.0	0.83	4.0	Facultative

Source: Data taken from reference 74.

reduces their survival. Although several factors including ice nucleation and dehydration have been implicated in the freeze-induced injury of bacterial cells, more recently oxidative stress has been shown to contribute to the freeze-thaw-induced killing of campylobacters (43).

Survival in Surface Waters

Although growth is not possible in the environment, surface waters play a vital role in maintaining the contamination cycle of *C. jejuni*. Data on the occurrence of campylobacters in surface waters vary widely, and this probably reflects the degree to which slaughterhouses, the presence of waterfowl, or waste treatment plants impact the quality of water within the cachement area. Isolation rates for river water, for example, vary from 53% in southern England to 37% in central Washington and 82% in Germany (44). Nevertheless, it is clear that campylobacters can persist for extended periods in surface waters. For example, at low temperatures and in nutrient-poor conditions survival can be as long as one to four months (33).

Generally, the survival of *C. jejuni* in surface waters is dependent on a number of environmental parameters such as temperature, the presence of oxygen, and the availability of nutrients. The rate of loss of viability in river water, a *microcosm* containing nutrients, is markedly less than that observed in deionized water (42,45). Temperature, however, appears to have the greatest influence on *Campylobacter* survival. Thus, although survival is poor at higher temperatures (37 °C), it is optimal at lower temperatures (5–15 °C), which do not promote growth and correlate to some environmental temperatures (45). The issue of environmental survival of campylobacters is further complicated by the fact that, certain strains of *Campylobacter* appear to be better equipped for environmental survival. In other words, not all strains of *Campylobacter* survive at equivalents rates in the environment. In general, strains of *C. coli* appear to be more sensitive to adverse environmental conditions (46) than *C. jejuni*. In addition, *C. jejuni* appears to be the more resilient strain in terms of survival in water, and for this reason *C. jejuni* is regarded as the main protagonist of water-mediated campylobacteriosis (45).

Survival of *Campylobacter* in Sewage and During Sewage Treatment

Wastewater is inevitably contaminated with fecal material and, as a consequence, is invariably contaminated with fecal pathogens. Thus, it is not surprising that wastewaters regularly contain campylobacters. The levels of these pathogens detected in wastewaters vary widely. This may reflect the presence of slaughterhouses or other sources of campylobacters in the collection area for the treatment plant (33).

The survival of campylobacters during the sewage treatment purification process, has been investigated by a number of different groups. The most significant reduction in *Campylobacter* numbers is most often associated with primary sedimentation, with reported reduction rates during this process varying between 56 and 99% (33). This reduction in numbers has been attributed to the attachment of *Campylobacter* to sedimentary particles (47) and to ingestion by protozoa (48). The survival and prevalence of campylobacters during wastewater treatment, however, was not correlated with water temperature, sunlight, or the intensity of rainfall (49). Surprisingly, given the microaerophilic nature of *C. jejuni*, there also appeared to be no correlation between the concentration of oxygen and the reduction in numbers of this pathogen. In conclusion, in the absence of a dedicated disinfecting process, it is clear that although aerobic biological purification of wastewater generates a substantial lowering of numbers, it does not result in the complete elimination of campylobacters (33). Consequently, these effluents may serve to contaminate surface waters.

In wastewater treatment, primary sedimentation brings about the settling of mainly fecal solids, and accordingly, a large proportion of the fecal bacteria including pathogens is carried into the primary sludge. In particular, campylobacters can be isolated from untreated sludge derived from primary sedimentation and the levels can be correlated with the incidence of infection within the community (50). In contrast, the organisms in activated sludge generally are those of aquatic origin rather than those of fecal derivation because the activated sludge process provides a hostile environment to the latter.

The treatment of sludge has a marked effect on the survival of campylobacters and thus significant implications for the safety of the treated biosolids if they are to be disposed of on arable lands. Although aerobic digestion has been shown to eliminate campylobacters completely (51), some studies have demonstrated that anaerobic digestion has little effect on reducing the numbers of viable campylobacters (52). The differential survival of campylobacters during these processes may reflect the microaerophilic nature of the pathogen and, in particular, its sensitivity to oxygen. In conclusion, although the disposal of aerobically digested sludge on terrestrial sites is not likely to contribute significantly to the environmental prevalence of campylobacters, the application of raw or anaerobically treated sludge to arable land may present possible hazards in terms of the presence of campylobacters.

The Role of Viable Nonculturable Stages in the Contamination Cycle

In aquatic and food environments, pathogenic bacteria, which most often grow optimally at temperatures that are equivalent to that of their host and which generally have fastidious requirements for nutrients, often encounter stress in the form of starvation, osmotic, and temperature stresses. As a consequence of these stresses, certain bacteria may enter a viable nonculturable (VNC) state. This novel concept of a bacterium that remains infectious but can no longer be cultured by conventional means was first proposed by Colwell following a study on the survival of *Salmonella* in aquatic systems (53). Such bacteria may retain metabolic activity, yet are unable under the prevailing environmental conditions, to undergo the cellular division required for growth. Furthermore, conversion from the VNC form has been shown to be

reversible with the advent of improved environmental conditions (54). Clearly, the presence of a VNC form of *Campylobacter* would have significant ramifications for the detection and epidemiology of this pathogen. In this context, a VNC form of *C. jejuni* was first reported by Rollins and Colwell (55), and since then there has been continuous debate as to whether a VNC form for *Campylobacter* actually exists.

Given the fastidious nature of *Campylobacter*, the loss of culturabilty in this organism is relatively easy to induce. For example, cultures rapidly lose viability, as assessed by plate counting, following exposure to oxygen, changes in temperature, and starvation. In particular, exposure to oxygen brings about a rapid decline in recoverable organisms (55), and generally, the higher the temperature and the more nutritious the media, the greater the loss of viability (56). The reduced metabolism of the cell at low temperatures and in conditions of limited nutrient availability may lessen the buildup of toxic metabolic products and thereby promote survival (56).

The viability of the VNC stage has been attributed largely to the ability of this form to modify chemical indicators of viability. For example, tetrazolium salts, which are colorless and water-soluble electron acceptors in the oxidized state, can be reduced by metabolically active cells to water-insoluble colored formazans. A number of studies have shown that VNC forms reduce certain tetrazolium salts, and this has subsequently been interpreted as an indicator of viability (57,58). In addition, the longevity of certain macromolecules within the VNC form has been correlated with the viability of this stage. For example, the chromosomal DNA of nonculturable cells has been shown to remain intact after 116 days storage at 4 °C (59).

Recovery or reversion of the VNC form has been reported by a number of groups, but these studies have often generated controversy. Accordingly, the recovery of VNC forms, which are not detectable by conventional culture techniques, has been reported in a number of different animal models (60,61) and more recently in embryonated eggs (62). In addition, VNC forms that were induced by oxygen starvation were reported to have been recovered following the provision of a microaerobic atmosphere. The authors of this study, however, could not exclude the possibility that at least some of the recovery could be attributed to the multiplication of residual viable cells (63). Further evidence for the existence of VNCs has been derived from the direct observation of nonculturable forms in untreated water supplies from a broiler farm by immunofluorescence microscopy and the subsequent colonization of *Campylobacter*-free chickens by these cells (64).

In contrast, a number of studies have failed to induce the recovery of VNC cells of *C. jejuni* in animal models (65,66), and these results have cast doubt on the existence of a VNC form and the role that it plays in the environmental transmission of campylobacters. Consequently, controversy concerning the existence of a VNC form for campylobacters still abounds. A recent study, however, may explain the basis for the disparate results concerning the VNC form. In this respect, Tholozan and coworkers (58) have demonstrated that only a limited number of isolates are able to form the VNC stage. In light of this study, it is possible that the contrasting results that concern the VNC form may simply be due to strain differences. Further emphasis for this contention can be derived from the fact that, of a total of 36 strains of *C. jejuni* of human origin examined, only three were able to enter the VNC state (58).

The Role of Coccoid Cells in *Campylobacter* Survival

The decline in viability and loss of culturability in campylobacters that occurs following exposure to certain unfavorable environments is often associated with a change in cell morphology from a spiral rod–like form into a coccoid form (55). This phenomenon has also been shown to occur with other bacteria including *Helicobacter pylori* (67). Because the rate of appearance of the coccoid form often parallels the loss in culturability, much research has focused on the possibility that the coccoid form is in fact the dormant VNC stage of *C. jejuni*.

A number of studies have suggested that the coccoid stage is merely a degenerative form that contains decreased levels of nucleic acids and peptides and also lacks cellular integrity (68,69). However, these conclusions that all coccoid forms are degenerate may be too simplistic because different types of coccoid forms, containing different fatty acid compositions, may be generated following exposure to different temperatures (56). Because the formation of cocci is not prevented by the inhibition of protein synthesis brought about by the addition of chloramphenicol or by irradiation, whatever the role of the cocci, the process must be a passive one rather than an active one, requiring de novo protein synthesis (56). More recently, it has been suggested that conversion to the VNC form and the transition to the coccoid form are two different but related phenomenon, with the real VNC stage to be found among the spiral rod–like population (59).

Oxidative Stress and Environmental Survival

The microaerophilic nature of *C. jejuni* and *C. coli* species implies an inherent sensitivity toward oxygen and its reduction products, particularly the superoxide anion. The reason for the lack of resistance to oxygen in atmospheric concentration is not known at present. However, it does not appear to stem from an absence of detoxification mechanisms. In this context, three pathways for the removal of oxidative radicals have been characterized in campylobacters (70). For example, the deleterious effects of exposure to superoxide radicals are counteracted by the activity of superoxide dismutase (SOD). *Campylobacter* mutants lacking this enzyme are hypersensitive to exposure to atmospheric oxygen during survival in a number of different environments. Consequently, the ability of *Campylobacter* cells to counter the effects of superoxide radicals is fundamental to their survival in the environment (71).

Analysis of the genome sequence indicates that the key regulators of oxidative stress defense enzymes found in *E. coli* and *Salmonella typhimurium*, namely, SoxRS and OxyR are not present in *C. jejuni*. Additional factors must

therefore be involved in the regulation of the oxidative stress response. In this context, a negative regulator termed *PerR*, which has considerable homology to the ferric uptake regulator *Fur*, mediates the response to oxidative stress (72).

Genetic Factors Influencing Survival in the Environment

The primary habitat of campylobacters is considered to be the gastrointestinal tract of warm-blooded animals. Consequently, when these microorganisms are released into the environment, they generally encounter conditions that are detrimental to their survival. Many bacteria, including enteric pathogens, are capable of surviving diverse environmental conditions. This ability is often essential for the survival of the bacterium and necessitates that the organism can sense its surroundings and modify its physiology to counter the effects of the stress imposed by the prevailing environmental conditions.

Until relatively recently, scientists knew little of the mechanisms governing the survival of campylobacters outside its host. However, some recent studies and the publication of the genome sequence for *C. jejuni* NCTC 11168 (72) have given them important insights into the mechanisms controlling the survival of this pathogen in the environment and how it recognizes environmental cues and is able to respond to them (Table 3; 70). Despite the ability of *C. jejuni* to survive in a diverse range of environments including the gastrointestinal tract of animals and humans, food, and oligotrophic environments such as surface waters, its capacity for regulating gene expression in response to changes in environmental parameters is very limited in comparison to other bacteria. A number of key global regulatory proteins that are important in the stress responses of other bacterial pathogens are absent (Table 3).

Initiation of transcription in eubacteria by the DNA-dependent RNA polymerase is regulated by a family of small proteins called *sigma-factors* (σ-*factors*). These associate in a reversible way with the core enzyme and provide bacteria with a mechanism for modulating gene expression in response to specific environmental stimuli. However, the capacity of *C. jejuni* to modulate gene expression in response to stress through the use of alternative sigma factors is very limited, as only two alternative σ-factors, σ^{54} and σ^{28} are present. Compared with *E. coli* and *Bacillus subtilis*, which possess six and 17 alternative σ-factors, respectively, this number is minimal. Notably, the alternative sigma factor *RpoS* is absent from *C. jejuni*. Generally, it is assumed that bacteria isolated from the stationary phase are more resistant to environmental stresses and toxic agents than cells in the exponential phase of growth and that this is a programmed adaptation mediated by RpoS in gram-negative bacteria. However, it has recently been shown that stationary phase cultures of *C. jejuni* do not enter an RpoS-mediated resistant state, as has been observed for a number of other gram-negative bacteria and that the tolerance of this bacterium to certain stresses is greatest in exponential phase and actually declines during early stationary phase (73).

Table 3. A Comparison of Key Proteins Involved in the Resistance to Environmental Stress in Other Bacteria with Those Present *C. jejuni*

Protein	Occurrence	Function	Presence in *C. jejuni*
Fur	*E. coli*, *S. typhimurium*, other bacterial species	Repressor of iron uptake, regulates iron uptake	+
SoxRS	*E. coli*, *S. typhimurium*, other bacterial species	Positive regulators of the response to superoxide stress	−
OxyR	*E. coli*, *S. typhimurium*, other bacterial species	Positive regulator of the response to peroxide stress	−
PerR	*B. subtilis*	Negative regulator of the response to peroxide stress	+
RpoS	*E. coli*, *S. typhimurium*, other bacterial species	Alternative sigma factor, regulates the response to stress, starvation, and stationary phase	−
RpoN	Numerous bacterial species	Alternative sigma factor, regulates nitrogen assimilation and other unrelated functions	+
RpoH	*E. coli*, *S. typhimurium*	Alternative sigma factor regulating the heat shock response	−
HspR	*Streptomyces albus*	Negative regulator of the heat shock response	+
HrcA	*Bacillus subtilis*	Negative regulator of the heat shock response	+
CspA	*E. coli*, *S. typhimurium*, *B. subtilis*	Major cold-shock protein	−
CsrA	*E. coli*, *S. typhimurium*,	Carbon storage regulator,	+
Lrp	*E. coli*, *S. typhimurium*	Global regulator of metabolism,	−
Crp	*E. coli*, *S. typhimurium*, other bacterial species	cAMP receptor protein, allows use of alternative carbon sources	?

Source: Data taken from reference 72.

The presence of a number of two-component regulatory systems suggests that these are a major regulatory family in this organism, and it is likely, therefore, that these play a central role in the physiological response of campylobacters to the environment (72). The lack of established regulatory mechanisms in *C. jejuni* is intriguing and suggests that alternative mechanisms for coping with environmental stress may operate in campylobacters.

CONCLUSION

Campylobacter jejuni is the leading cause of bacterial food-borne diarrheal disease throughout the world and is regarded as among the most ubiquitous bacterial pathogens known to man. Despite its incidence, in the laboratory at least, *C. jejuni* behaves as a relatively fragile organism compared with other food-borne pathogens and accordingly is sensitive to many environmental stresses. However, outside the laboratory it apparently is able to survive in aquatic environments and in food for extended periods and its ability to persist under these conditions is cardinal to its contamination–infection cycle.

Even today, more than 20 years since its role as a leading cause of enteritis was first brought to prominence, very little is known of the mechanisms that enable *C. jejuni* to survive in food and the environment. For example, we still await definitive answers concerning the existence of VNCs and the role of coccoid forms in its survival and transmission. However, the mechanisms that enable the organism to counter the effects of oxidative stress have recently been shown to be important for the survival of the pathogen in a number of environments. The development of strategies for the effective control of *C. jejuni* in the food chain are also hindered by a poor understanding of the physiology of this organism. However, the recent publication of the genome sequence of *C. jejuni* greatly increases our understanding of this pathogen, and during the next decade the application of postgenomic technology should greatly enhance our understanding of this important pathogen.

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CAPILLARY ELECTROPHORESIS IN GENETIC ANALYSIS AND RIBOTYPING OF MICROBIOTA IN THE ENVIRONMENT

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Among the main obstacles frequently encountered by microbiologists are the identification and taxonomic classification of new microbial entities or the identification and differentiation of microorganisms known for a long time but whose subspecific entities are difficult to determine. At sub-specific taxonomic levels, differences may be so reduced that traditional techniques are in some cases absolutely ineffective. In all these cases, the use of molecular markers, which exploit the natural variation present inside DNA to identify and differentiate pathogens, may help overcoming current limitations. Remarkable progress has been made in recent years in the development of new methods for elucidating relationships among groups of more or less related microorganisms. The advent of molecular biology techniques, mainly a consequence of developments obtained in the medical field, has allowed new and unexpected approaches to environmental microbiology, giving a great boost to systematic and diagnostic studies of communities in the environment.

GENETIC CLASSIFICATION BY DNA POLYMORPHISM

One more general approach used widely in both general and environmental microbiology involves the identification of DNA polymorphism located at specific sites. These analyzes are used to generate "genetic fingerprints" of the genome of individuals or families of related individuals or even unrelated or closely related members of a species for the purpose of identification of genetic markers as tools for the selection of organisms for environmental use, for diagnostic, commercial, or ecological purposes. These techniques have been applied to other fields of biology and were extremely successful in the rapid identification and differentiation of several genetically distinct groups of closely related organisms (1,2). The transfer of such methodologies to the study of microbiological communities has been rapid and has confirmed the high-resolution power of these techniques, as outlined in several recent reviews of the application of molecular technologies in molecular ecology and pathology (1–4). Although this review is intended to examine developments in microbiology, new developments in technologies employed for the study of microorganisms affecting human health and food have wide relevance to molecular microbiology and are included.

The great advance in the diagnostic microbiology is driven by the need to speed identification and differentiation techniques at the level of subspecies, variety (or pathovar), *formae speciales* and race beyond traditional morphological and physiological difference–based techniques. The identification of species based on culturability and on morphological or

biochemical differences (5–7) is slow and may underrepresent microorganisms requiring fastididous growth conditions. Surveys of many terrestrial and aquatic ecosystems indicate that 99% of microorganisms resist cultivation in the laboratory. Therefore, to study such unculturable organisms we have to rely on DNA-based technologies. Similar constraints affect both environmental microbiology and medical microbiology where the scoring of symptoms for infectious disease evaluation, especially of early symptoms, is difficult and still retains a subjective element (5,8–10). The infection process is strongly influenced by a range of environmental factors including temperature, moisture, light intensity, etc. For these reasons, it is quite variable in its initial symptomatic expression even in the same combination strain/clone or race/cultivar. The phenomena of symptom convergence, that is, when different pathogens cause the same type of alteration, and of symptom divergence, when the same pathogen causes different types of alterations, further complicates the problem (11–13). All these difficulties pose serious problems for the diagnosis of the causal agents of diseases as well as to the differentiation and taxonomic classification of many groups of microorganisms and frequently several independent methods such as ribotyping, repeat element polymorphism, random amplified polymorphis DNA (RAPD), and pulsed-field gel electrophoresis (PFGE) analysis of chromosomal fragments and plasmids are used (14–16). Recently, fluorescent in situ hybridization (FISH) analysis and image-analyzed microscopy have been applied for the rapid identification and quantification of particular microorganisms within environmental communities (17,18) and in health and clinical environments (5–19). Typically, the FISH probes are specific to characteristic ribosomal gene elements of particular microorganisms.

RIBOTYPING

Analysis of polymorphism of regions of the ribosomal gene complex is one of the most commonly used methods of differentiation of microorganisms (20). This is because of the ease in amplifying the ribosomal gene regions in many different bacteria and fungi by application of "universal" PCR primers to the highly conserved ribosomal gene repeats, and following DNA sequence analysis, the relative ease of defining highly specific PCR-primers to ribosomal gene loci for particular microbial and fungal species of interest, or by the definition of characteristic restriction fragment length polymorphism (RFLP) for particular microbial strains or species. Ribotyping is a general term applied to RFLP analysis of the internal transcribed spacer (ITS) regions located between the small and large subunits and encompassing the 5.8S rRNA gene (21–23) or to the nontranscribed intergenic spacer between the large subunit and the following small subunit, frequently encompassing the 5S rRNA genes (24,25). It is also applied to RFLP of the most part or all of the ribosomal gene repeat (26–29), or specifically to microbial 16S-18S small subunit rRNA (SSU rRNA) (4,5,8,30) and 23S large subunit (LSU) rDNA genes (7–19). Less frequently the tandemly repeated transfer RNA (tRNA) gene loci are used for microbial identification (31), reflecting the absence of suitable PCR primers for tRNA genes to most species and the lack of known characteristic polymorphism.

RIBOTYPING AND PHYLOGENY

One particular advantage of gene-specific "fingerprinting" methods such as ribotyping is that the results are absolutely reproducible and thereby suitable for compilation into databases for a comprehensive account of ribotyping. (see RIBOTYPING METHODS FOR ASSESSMENT OF IN SITU MICROBIAL COMMUNITY STRUCTURE, this Encyclopedia). Using ribotyping, the phylogeny of the fungi examined may be computed from the discrete character matrix for presence or absence of fragments by sequence parsimony programs such as the PHYLIP package and results in complete accordance to the phylogeny derived from ribosomal RNA sequence analysis (1–20). Thus, if sufficient different polymorphic loci are examined across the ribosomal genes in "long-range ribotyping," the accuracy of method may be equal to DNA sequence analysis of the same locus. However, a gene locus-specific method such as ribotyping may fail to distinguish between different bacterial or fungal strains, even when a large region of the ribosomal intergenic spacer or a number of different spacer regions between the various rRNA genes is examined (32,33). Therefore, parallel or complementary application of random genome-scanning methods such as polymorphic repeat element fingerprinting is advised for identification of strains and to detect a more complete picture of genetic diversity (34).

CAPILLARY ELECTROPHORESIS FOR HIGH-THROUGHPUT DNA POLYMORPHISM ASSAYS

Capillary electrophoresis (CE) is an analytical electrophoresis technique that offers high throughput and high resolution, automatic operation and on-line detection with automatic data acquisition (35) (Fig. 1). These features have particular stimulated application to the analysis of DNA polymorphism for genetic analysis, and medical diagnosis (35–37). The advantages provided by

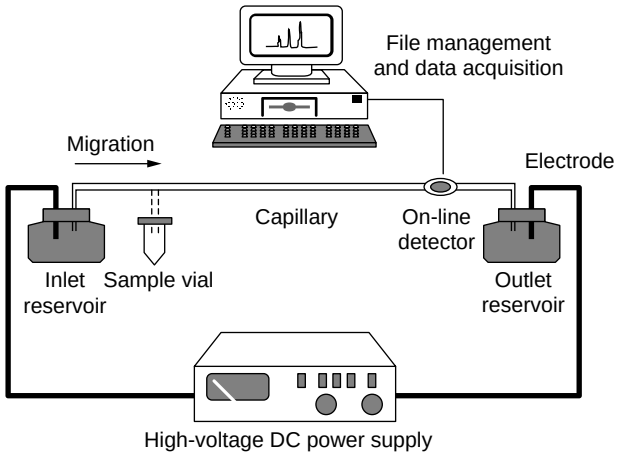

Figure 1. Schematic diagram of capillary electrophoresis (CE) apparatus. [K. R. Mitchelson, J. Cheng, and L. J. Kricka, *Trends Biotechnol.* **15**, 448–458 (1997).]

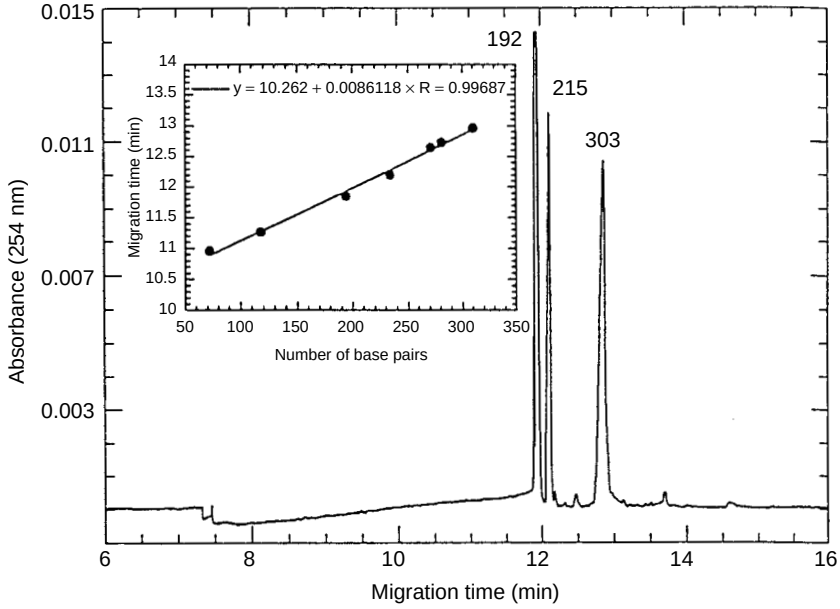

Figure 2. CE separation of *Rsa*I restriction fragments of the ribosomal IGS of the fungus *Laccaria bicolor*. Inset shows a calibration plot of migration time versus size of fragments of a ϕX174 *Hae*III digest. [F. Martin, D. Vairelles, and B. Henrion, *Anal. Biochem.* **214**, 182–189 (1993).]

the ability to automate CE operations have also given impetus to the miniaturization of capillary electrophoresis equipment to silicon-chip-based devices (38), which provide all of the earlier-mentioned facilities and a significant improvement in the speed of analysis and to the degree of automation. Particular development of other miniaturized electro-separation devices including molecular dielectric trapping using microelectrodes (39), may be integrated with CE to create microanalytical or micropreparative devices.

TOOLS FOR RIBOTYPING

Attempts to transfer capillary electrophoresis methods to microbiology have not been extensive, but several examples confirmed their high-resolution power and the potential of automation of these techniques. These techniques include capillary electrophoresis in conjunction with RFLP analysis of PCR-amplified ribosomal gene loci (40–43) (Fig. 2), direct linear DNA sequencing, and dideoxy fingerprinting (44). Characteristic repeated DNA polymorphisms that discriminate species and strains may be PCR-amplified using specific primers and assayed using CE. DNA polymorphisms include ribosomal gene regions (40) and bacterial repeat sequences (45) (Fig. 3). CE is also particularly useful for analysis of short tandem repeat (STR) loci (46).

SCANNING METHODS EMPLOYING CAPILLARY ELECTROPHORESIS

Capillary electrophoresis techniques have also been developed for DNA polymorphism scanning methodologies that detect polymorphism through alteration in the electrophoretic mobility of DNA fragments. These methods include single-strand DNA conformational polymorphism (SSCP) (47–49) and heteroduplex DNA analysis (HPA) (50) (Fig. 4). Although these methods can identify single base differences between short (typically

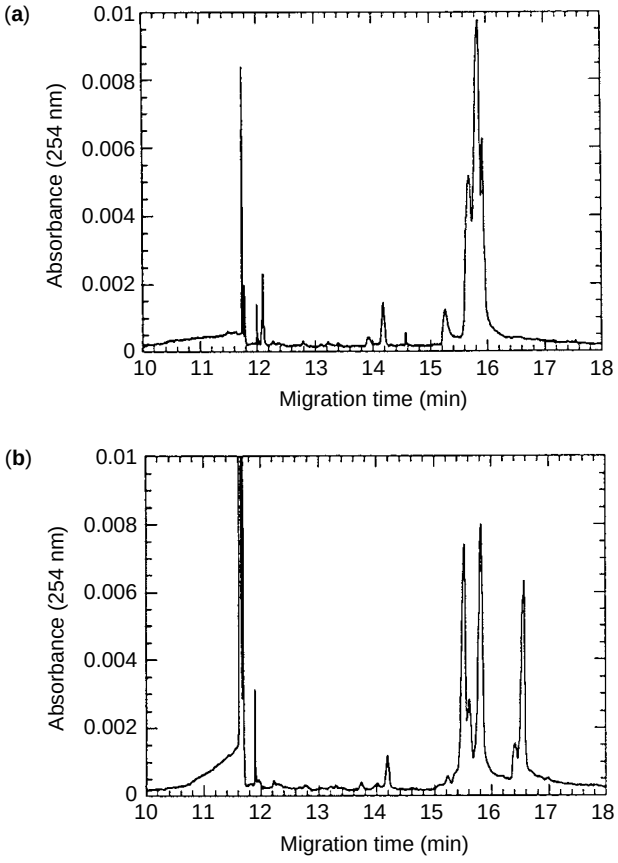

Figure 3. CE separation of heterogeneous undigested PCR products amplified from the ribosomal IGS from different strains of the fungus *Laccaria bicolor*; (**a**) strain 81306; (**b**) strain 83222. The heterogeneous IGS amplification products migrate past the detector from 15 to 17 minutes [F. Martin, D. Vairelles, and B. Henrion, *Anal. Biochem.* **214**, 182–189 (1993).]

<200 bp) fragments, the resolution between haplotypes requires optimization of the electrophoresis conditions

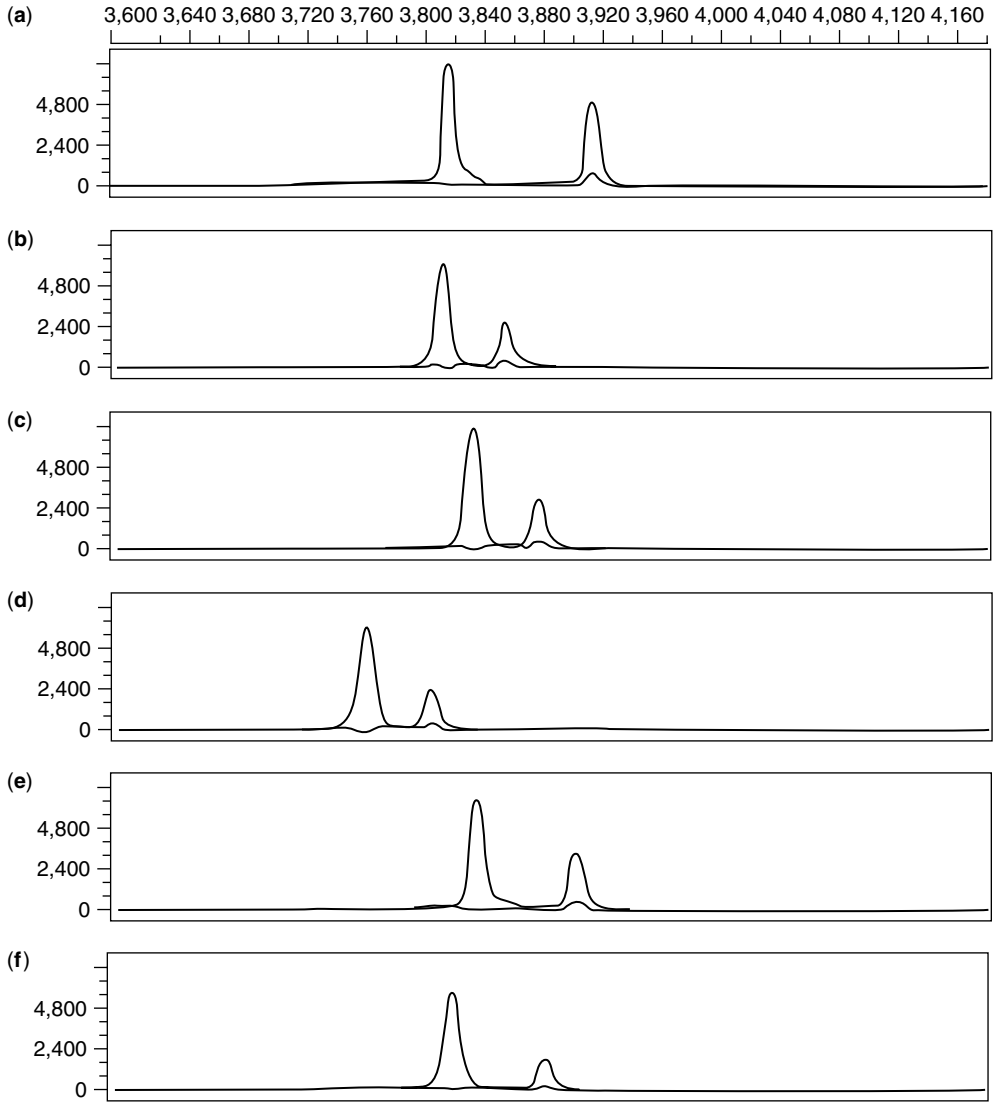

Figure 4. Single-strand conformation polymorphism assay by capillary electrophoresis (CE-SSCP) showing differentiation of *Pseudomonas aeruginosa* and other bacilli. (**a**) *P. aeruginosa* ATCC10145; (**b**) *B. cepacia* CIP80.24; (**c**) *C. acidovorans* ATCC15668; (**d**) *B. vescularis* ATCC11426; (**e**) *S. maltophilia* CIP60.77; (**f**) *A. xylosoxidans* CIP61.20. Samples were analyzed in the same run and aligned using GeneScan500 size standards. [R. Ghozzi et al., *J. Clin. Microbiol.* **37**, 3374–3379 (1999).]

(Fig. 5). Related sensitive methods that amplify the heteroduplex polymorphism effect include constant denaturant capillary electrophoresis (CDCE) (51), which is a modified version of denaturant gradient gel electrophoresis (52), and thermal-programmed capillary electrophoresis (TPCE) (53) in which a variable temperature is increased during a run using computer-controlled thermal ramping. These have typically been applied for detection of defined polymorphism in genes such as in "ribotyping" of ribosomal gene fragments. A comparable technology for the detection of single-base polymorphism is denaturing high-performance liquid chromatography (DHPLC), and separated single-strand DNAs (54). These techniques are both fast, about 5-minute analysis for DHPLC and about 15 minutes for CDCE, and can be used to identify low frequency mutations and to genetic screening of pooled samples for detection of rare DNA variants. Further, chemical mismatch cleavage which identifies heteroduplex molecules and cleaves the heteroduplex to size resolvable fragments can also be rapidly analyzed using CE (55). The high sensitivity and speed of CE analysis has been used in medical and diagnostic circumstances to detect post-PCR amplification virus-specific DNA fragments (56) using on-column dye-labeling (57) (Fig. 6). Dideoxy fingerprinting is a further application on CE in which both structural (SSCP) and sequence information from single dideoxynucleotide-extension tracking analysis is used to identify the location of gene polymorphism (58).

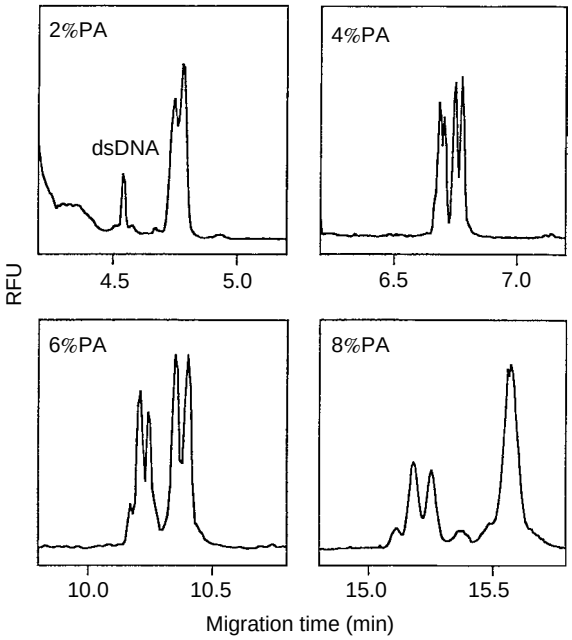

Figure 5. CE-SSCP assay is influenced by the choice of sieving matrix and by electrophoresis conditions. The "% PA" refers to the concentration of polyacrylamide sieving buffer. [J. Ren, A. Ulvik, P. M. Ueland, and H. Refsum, *Anal. Biochem.* **245**, 79–84 (1997).]

Ribotyping Using Capillary Electrophoresis

Species-specific ITS2 regions that are variable in amplicon length, may be amplified using primers for conserved sequences of the 5.8S and 28S ribosomal DNA (rDNA). The sequence variability of the internal transcribed spacer 2 (ITS2) region of fungi is potentially useful in rapid and accurate diagnosis of invasive fungal disease of immunocompromised patients (10). Terminal-restriction fragment length polymorphism (T-RFLP) of the 16S rDNA has been analyzed by capillary electrophoresis and used to characterize marine bacterioplankton communities for the detection of operational taxonomic units (OTUs) (52) (Fig. 7). Capillary electrophoresis-single-strand conformation polymorphism (CE-SSCP) analysis of PCR-amplified 16S rRNA gene fragments was used for rapid identification of *Pseudomonas aeruginosa* in patients with cystic fibrosis (49). Mycobacterium species have been identified based PCR amplification of polymorphic genetic regions with fluorescent primers followed by restriction analysis (PCR-RFLP) and by fluorescence capillary electrophoresis analysis of a 439-bp segment of the 65-kDa heat shock protein gene using *Hae*III and *Bst*EII digestion, and of a 475-bp hypervariable region of the 16S rRNA gene at the 5′ end using *Hae*III and *Cfo*I (43). The ability of CE to resolve DNA fragments efficiently has been employed for the analysis of bacterial growth rates in physiological situations by monitoring plasmid copy number — effectively a multicopy fragment analysis (59,60). Linearized plasmids were monitored off-line by CE, permitting both a rapid estimation of bacterial numbers and an assay of bacterial variants through the size of fragments (Fig. 8).

PULSED-FIELD CAPILLARY ELECTROPHORESIS

Pulsed-field gel electrophoresis formats have found wide application in the improved separation of large (20–100 kb) (61) and very large (several Mb) DNA

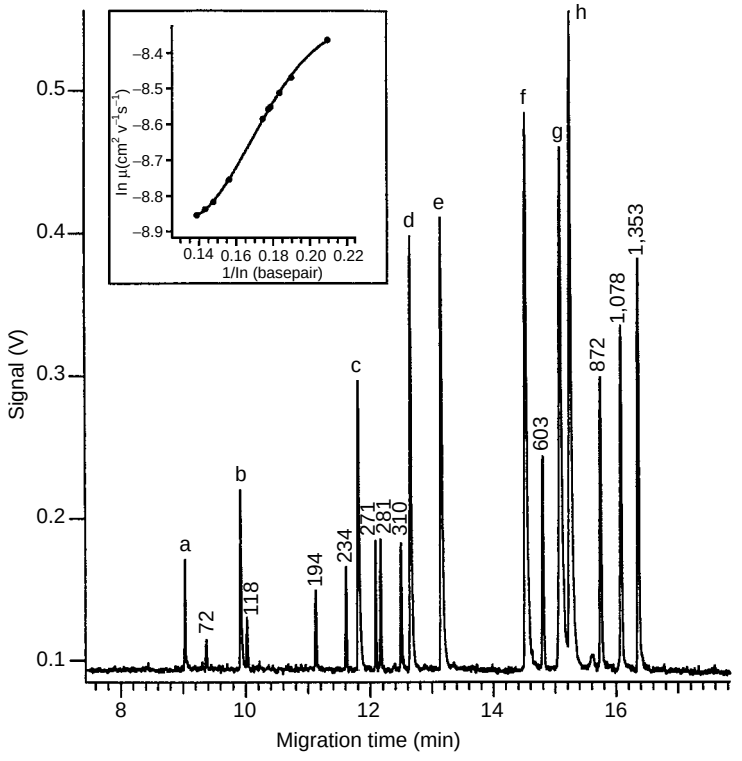

Figure 6. Detection of eight *Hpa*II digest fragments (a–h) of duck hepatitis viral DNA by capillary electrophoresis with internal size standards (base pair) and on-column dye labeling. Inset shows a calibration plot of migration time versus size of fragments of a ϕX174 *Hae*III digest. [W. G. Tan, D. L. Tyrell, and N. J. Dovichi, *J. Chromatogr. A* **853**, 309–319 (1999).]

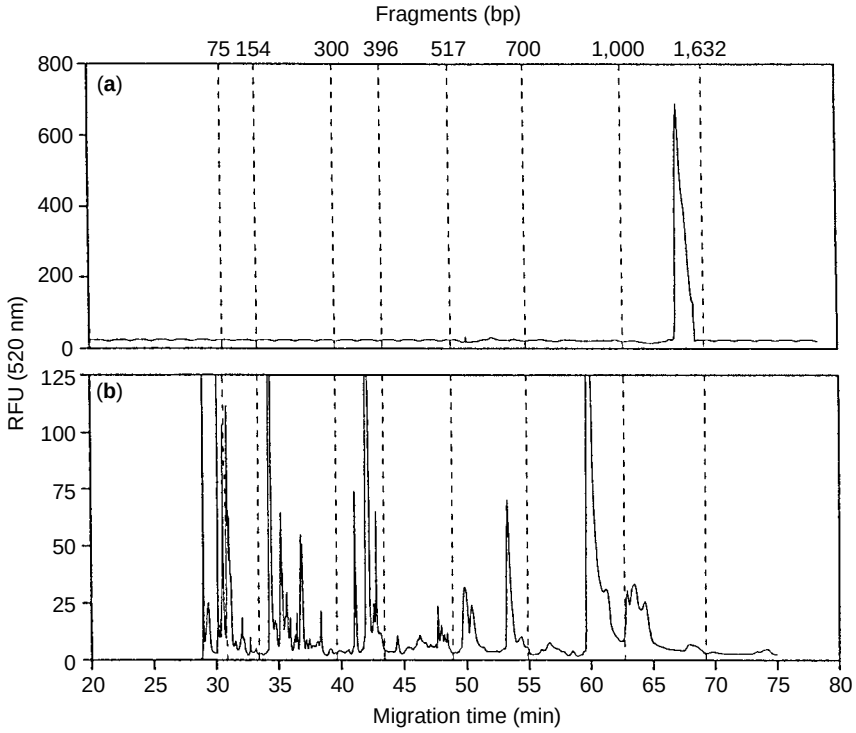

Figure 7. (**a**) Whole 16S rRNA PCR products and (**b**) T-RFLP fingerprinting by *CfoI* digestion of complex marine bacterial communities by CE. [M. M. Moeseneder et al., *Appl. Environ. Microbiol.* **65**, 3518–3525 (1999).]

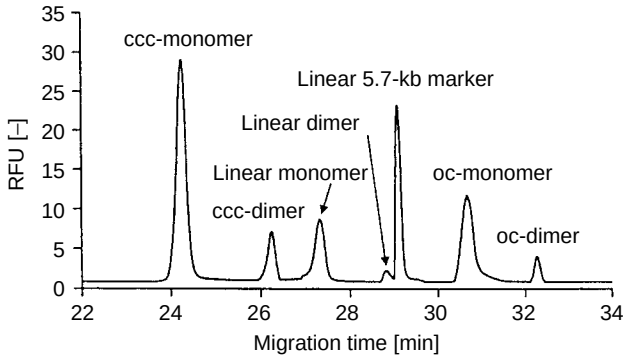

Figure 8. Quantitative analysis of a mixture of untreated, UV-treated (oc, open circle; ccc, closed concatameric circle), and linearized plasmid forms by CE. [T. Schmidt et al., *Anal. Biochem.* **274**, 235–240 (1999).]

molecules. However separations are slow because of the low field strength and low mobility of large molecules in solid gels. Pulsed-field electrophoresis with entangled solution-sieving media in a capillary format (61,62) and field-inversion capillary electrophoresis (FICE) (63) have been applied for separation of both large and very large DNA molecules with an improvement in speed by one to two orders of magnitude (64). Pulsed-field CE methods would be suited to a microdevice format, where very substantial gains in speed of separation would also be realized. Genetic diversity among bacteria may be characterized by PFGE pattern analysis. This analysis is applied to genomic fragments, plasmids and to ribosomal gene repeat fragments and is often performed in conjunction with ribotype analysis (65). Although some comparative studies of fingerprinting methods have found low information content from pulsed-field electrophoretic–RFLP studies compared to other PCR-based fingerprinting methods (RAPD's, REPs, PCR-RFLP) (16), other studies have found PGFE-RFLP to be complementary to ribotyping and for PFGE pattern analysis to subdivide the ribotypes into several PFGE genotypes, and further within a PFGE genotype, into subtypes following restriction with different enzymes (14,65–67). These PFGE studies would benefit from the large increase in speed by transfer to a capillary format (Fig. 9).

CAPILLARY ARRAY ELECTROPHORESIS

The recent development of capillary array electrophoresis (CAE) that offers all the advantages of conventional CE, but additionally provides very high throughput with up to 100 samples were simultaneously analyzed in parallel capillaries (68) with processing of multiple DNA fragments in under 70 minutes. Capillary array electrophoresis could conceivably be used for any other analytical procedure applicable to capillary electrophoresis (36,37) such as SSCP and HPA analysis. The advantage of high throughput could benefit direct sequence determination of sets of known characteristic polymorphic genes as capillary array electrophoresis apparatus is capable of running and analyzing 48 DNA sequencing samples simultaneously with runs of approximately 1 hour for about 500 bases as a throughput is on the order of 720 templates per day (44,69) (Fig. 10). As the cost and scarcity of such equipment falls, such rapid analysis increases the scope for epidemiological and taxonomic studies in fungi. In addition, small capillary array electrophoresis (CAE) chips have the capacity to rapidly analyze (2–3 minutes) differences in the mobility of DNA

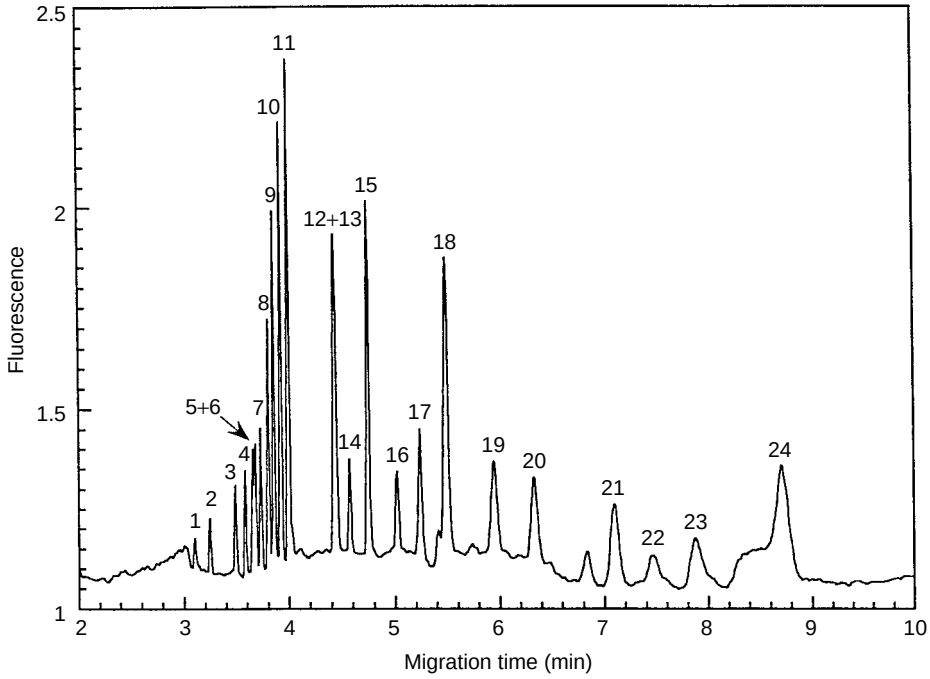

Figure 9. Separation of a mixture of ϕX174/*Hae*III fragments (72–1,656 bp), high molecular weight marker (8,271–38,416 bp) and Lambda-phage DNA (48,502 bp) by FICE in a 0.4% hydroxypropylcellulose sieving. Alternating peak sizes are in base-pair: (1) 72 bp; (3) 194 bp; (5) 271 bp; (7) 310 bp; (9) 872 bp; (11) 1,656 bp; (13) 8,612 bp; (15) 12,220 bp; (17) 17,057 bp; (19) 22,621 bp; (21) 29,942; (23) 38,416 bp and (24) 48,502 bp. [S. Magnúsdóttir, H. Isambert, C. H. Heller, J.-L. Viovy, *Biopolymers* **49**, 385–401 (1999).]

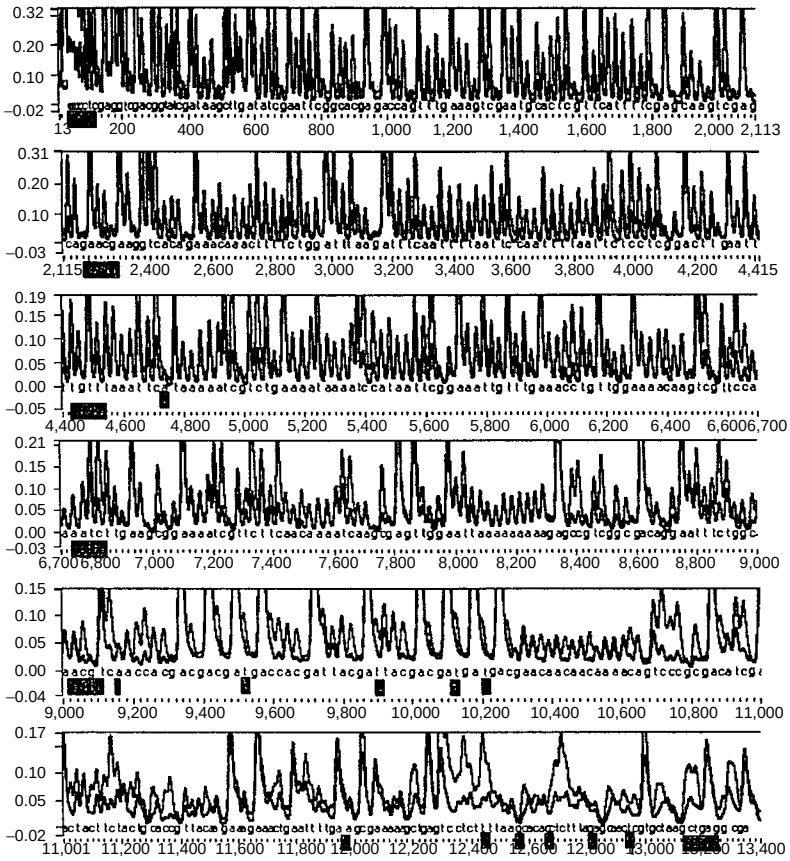

Figure 10. Multiplexed automated DNA sequencing directly from lysed single bacterial colonies by CE. [Y. Zhang, H. Tan, and E. S. Yeung, *Anal. Chem.* **71**, 5018–5025 (1999).]

fragments in parallel in multiple different samples and offer the potential for ultra-high speed, high-throughput genotyping by RFLP analysis (38,70) (Fig. 11). Microchip-based electrophoretic method for rapid detection of herpes simplex virus PCR products employed direct introduction into the microchip without a desalting step using a novel fluidic interface (71). The analysis achieves 100% sensitivity and specificity compared to the established gel methods with a detection of HSV DNA in less than 110 sec/sample (Fig. 12).

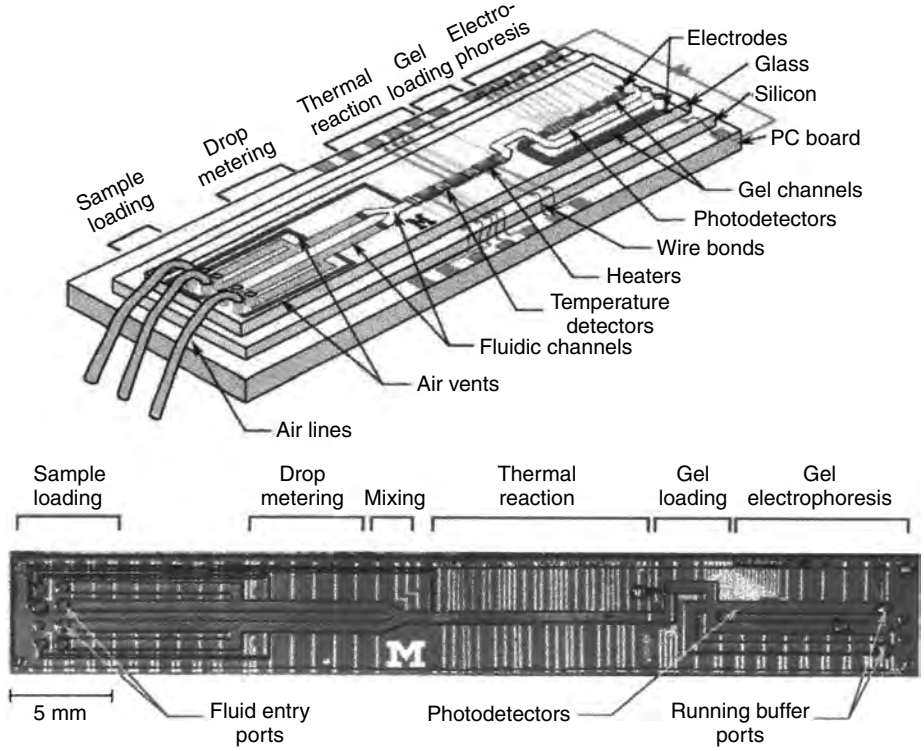

Figure 11. Schematic of integrated silicon device with two liquid samples and electrophoresis gel present (**a**) and an optical micrograph of the device from above (**b**). The microfabricated silicon chip has an integrated metering capability, a thermal pump, an isothermal reactor, a CE structure and an integrated photodetector. DNA amplification is achieved through an exponential isothermal "strand-displacement" amplification technique. The total analysis on the device from the metering of the reactants to the CE-based separation and detection took about 20 minutes [M. A. Burns et al., *Science* **282**, 484–487 (1998).] See color insert.

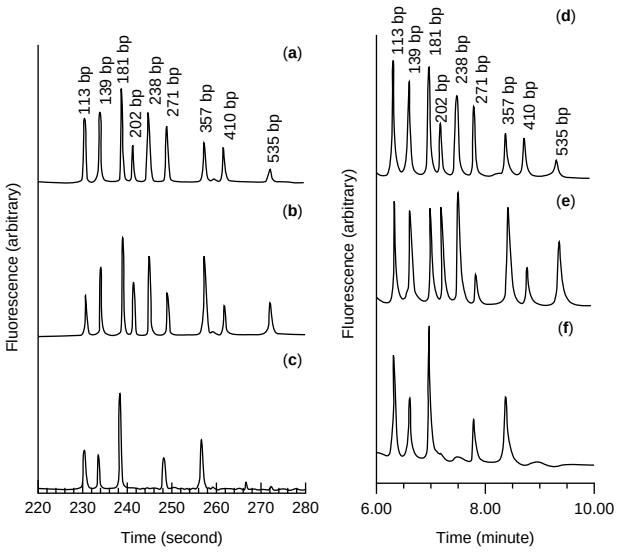

Figure 12. Comparison of chip CE electropherograms with conventional CE electropherograms. (**a**) control, PCR in a GeneAmp test; (**b**) PCR amplicons generated in a silicon-glass; (**c**) PCR amplicons from an affected patient generated in a silicon-glass chip; (**d–f**) conventional CE electropherograms. [J. Cheng et al., *Anal. Biochem.* **257**, 101–105 (1998).]

PARAMAGNETIC BEADS AND RIBOTYPING

Biotin-labeled DNA primers specific for gene regions can be used to amplify particular cDNA fragments from cellular mRNA using reverse transcriptase combined with PCR (RT-PCR), and these cDNA are collected specifically by binding to immobilized avidin (72). Both the quantitative level of gene expression and putative sequence variation within such cDNA fragments could be examined by SSCP analysis following release of a single DNA strands from the amplified dsDNA (73). One application is for the genotyping of bacteria (42) and fungi (74) in which length and sequence polymorphism analysis of rRNA-intracistronic and rRNA-intergenic regions are examined. The use of SSCP-CE analysis in combination with magnetic bead separation of the DNA strands allowed detailed CE migration profiles and creation of conformation isoforms for each isolate (74). An alternative approach might be a capture and detection system using biotin-labeled probes (75) to capture the DNA segments that contain specific regions of the genes by DNA-DNA hybridization, following PCR amplification or RT-PCR. Future use of DNA chips for genome-wide analysis of gene expression may stimulate the development of diagnostic applications of new technologies such as dynamic DNA hybridization using paramagnetic beads for expression analysis (76) (Fig. 13).

FUTURE DEVELOPMENTS

Nubel and his associates, (8) used temperature gradient gel electrophoresis (TGGE) to detect small sequence heterogeneities in PCR fragments of 16S rRNA genes from individual strains of *Paenibacillus polymyxa* (Fig. 14). The different strains of *P. polymyxa* showed heterogeneity in 10 variant nucleotide positions in a 347-bp fragment of the V6 and V8 regions in the RNA molecules.

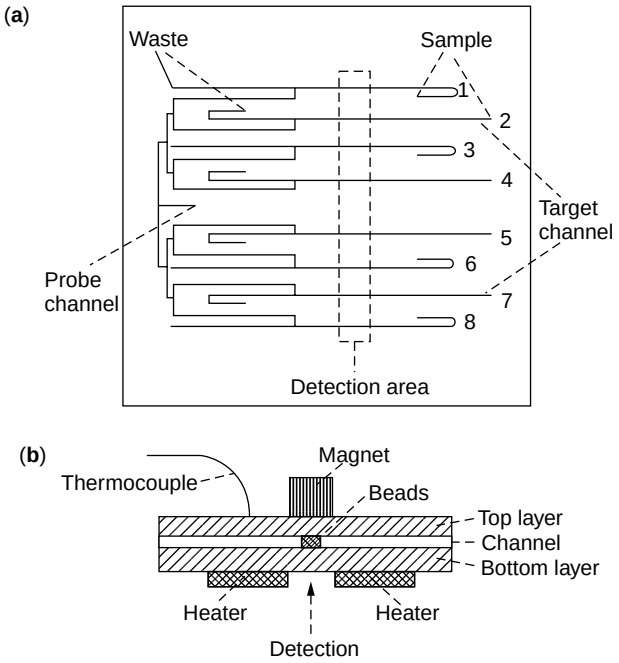

Figure 13. Dynamic hybridization of DNA on a microfabricated device using paramagnetic beads. (**a**) layout of the chip showing eight parallel channels for introduction of DNA and beads. (**b**) a cross-sectional view of the device. A magnet localizes beads at the detection area. Target DNA attached to the beads is assayed by successive hybridization against complementary probes which can be replaced by flushing. Selective hybridization is controlled by the thermocouple/ heater elements. [Z. H. Fan et al., *Anal. Chem.* **71**, 4,851–4,859 (1999).]

This analysis regime is well suited to capillary electrophoresis as the precise temperature control of capillary environments is superior to the conventional gel format (53). The ability to generate precise thermal gradients into the separating capillary columns is well established and TGGE is strongly established in the CE format (51,53). Amplifications of reverse-transcribed rRNA from ribosome preparations revealed a predominant representation of particular sequences in ribosomes of exponentially growing laboratory cultures (8). TGGE may be used for the structural analysis of heterogeneous rRNA genes and for their expression during stress conditions. Modern structure-specific cleavage of ribosomal genes (77) can also be directly assayed using capillary electrophoresis.

Remote Assay and Microanalytical Assay Systems

With the recent development of transportable and potentially "in the field" microsensors and microanalytical systems (71,78–80), the future holds promise for adaptation of these devices for the assay for the presence of specific target organisms and biological activities or processes under physiological conditions (80,81) or in situ conditions (18). These technical developments have parallels with field forensic systems (35,80) that amplify specific DNA fragments from target organisms, and may enable microbiologists to study the abundance, localization, and activity of microorganisms in situ. These systems may involve a

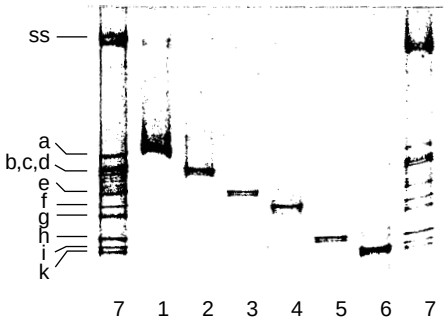

Figure 14. Temperature gradient gel electrophoresis (TGGE) separation of different 16S rDNA fragments from *P. polymyxa* strain DSM36, showing sequence polymorphism in gene copies. Lane 7 shows the mixed PCR products of the 16S rDNA gene, and lanes 1 to 6 show PCR products amplified from individual plasmid clones of the fragments. [U. Nubel et al., *J. Bacteriol.* **178**, 5,636–5,643 (1996).]

specific PCR or amplification reaction stage to increase the abundance of a specific target molecule, and an analysis stage to characterize the identifying amplicon. Typically, analysis can involve the determination of molecular size or DNA sequence that can be elegantly performed using capillary electrophoresis (60,82). The development of low-power microcapillary electrophoresis systems that provide analytical performance and plate efficiency comparable or better than conventional CE systems, with a separation efficiency on the order of 10^4 theoretical plates over a short effective separation distance of, say, 1.5 to 2.0 cm, in which analysis is completed within 5 minutes (83–85), will be of great benefit in future microbiological studies (Fig. 15).

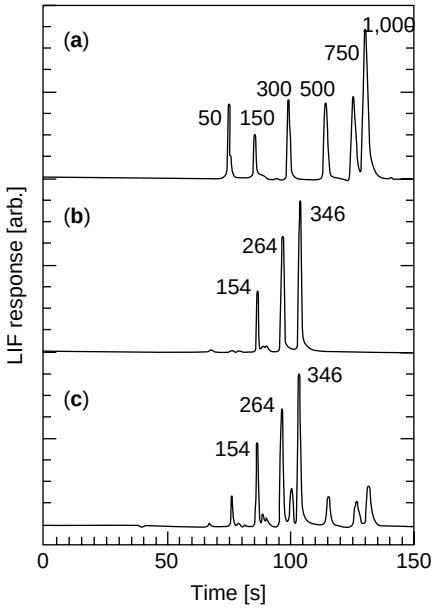

Figure 15. Chip CE electropherogram of: (**a**) a sizing ladder, (**b**) PCR products amplified from *E. coli* genomic DNA, and (**c**) PCR product mixed equally with the sizing ladder. [L. C. Waters et al., *Anal. Chem.* **70**, 5,172–5,176 (1998).]

WHOLE MICROBIAL ANALYSIS

A further advance in the application of capillary electrophoresis is in the fractionation of whole microbes in the manner of molecules, by microbial size and shape using polymer-based modulation of capillary electroosmotic flow, and separating by surface charge of the microbes by capillary isoelectric focusing (86,87). Potentially, pure microbia pools could be collected from the capillaries, allowing additional genetic analysis of each fractionated species peak by ribotyping techniques. Rapid, on-line identification of microbia could also then be made by direct physical measurements of microbial surface molecules, for example, a subset of microbial proteins, using Fourier transform-infrared (FT-IR) spectrography, or by mass spectrography (MS) (88). Small specific DNA fragments PCR-amplified from the microbial genome can also be readily analyzed directly, by electrospray ionization mass spectrometry (ESI-MS) or by time of flight mass spectrography (TOF-MS) (54). Both HPLC or CE instruments can be coupled directly to ESI-MS instruments; however, the outlets must be coupled with a sheath flow system to transport the molecular species into the MS (89). Sample stacking of CE peaks is also necessary before delivery into the MS device.

Another microfabricated device that allows the selective separation and purification of bacteria from other cells uses physical electrical forces such as dielectrophoresis and EEO (90). This exciting technology is an initial step toward the development of an integrated system that would combine elements for cell separation, DNA processing and signal detection. Other microfabricated devices that provide both DNA processing and signal detection elements have also been described (78,91). These separation and analysis microdevices could be applied together for the development of such an integrated total microanalysis system.

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CARBON MONOXIDE.

See Trace Gases Soil

CAVES AND MINES MICROBIOLOGICAL SAMPLING

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Microbial ecology has undergone a renaissance in recent years. The diversity of life on the surface of this planet has been probed more deeply than ever using new approaches. In contrast, the earth's subsurface remains comparatively unexplored. The spatial extent, collective biomass, phylogenetic diversity, and geochemical role of the subsurface biosphere remains conjectural (1,2). However, this milieu has received steadily increased attention in recent years (2–5). (see also Caves and Other Low-Light Environments: Aerophitic Photoautotrophic Microorganisms, this Encyclopedia). Yet, despite the emerging prominence of the field, the acquisition of meaningful samples remains a persistent obstacle to research (6,7). As an alternative to remote extractions (e.g., coring from surface), microbiologists have increasingly sought preexisting voids within the earth for direct sampling opportunities.

In the case of natural caves, biospeleological environments constitute an important and globally distributed biome in their own right (8). Underground mines permit directed passage into otherwise solid continental rock masses. In this article, we describe approaches to the microbiological sampling of these habitats. Natural caves may contain a variety of subsurface mineral habitats, including carbonates, quartzites, arkoses, sandstones, some varieties of volcanic substrata, and others (9). Many caves are influenced by surface microbial communities via streams, periodic flooding, groundwater, animals transporting materials in and out (trogloxenes), and air. Others are ancient in nature (10) and may house microbial communities isolated from direct outside influence for thousands to perhaps millions of years (10–14). Even here, the intricacies of karst hydrology can make prediction of recharge times tricky. Surface water input

into seemingly sealed caves may exhibit either slow or rapid movement along faults and fissures (15). Some caves may exhibit ongoing chemical excavation mediated by subsurface microbial communities, including acid-generating, autolithotrophic, sulfide-oxidizing bacteria (3,13,14,16–23). The very walls of the subterranean spaces that allow human access may thus represent exactly the right places to seek certain classes of subsurface microbes and to witness globally relevant biogeochemical processes in action (24,25).

Mines, by comparison, are artificial excavations. As such, they penetrate rock types not normally accessible to natural caves and often extend to greater depths. Mines may also represent conduits of potential surface-derived contamination into the subsurface biosphere. In any mine, all surfaces of potential microbiological interest will have undergone extensive alteration by the mining process. Material representative of the native subsurface may exist only centimeters beyond a given excavation face, but with each round of mining, a new contamination front is established between the microbiologist and the potentially pristine material. For this reason, great care must be taken in interpreting the significance of observed microbes and microbial activity in mines.

Microbially interesting cave systems and mining operations are often located in remote locations. Working underground in a cave or mine can be physically demanding and potentially dangerous. Equipment and samples may have to be carried over difficult terrain. The vertical relief typical of many wild caves necessitates specialized rock-climbing skill and equipment. Some cave atmospheres differ significantly from surface air and contain dangerous or lethal concentrations of various gases, including H_2S, CO_2, CO, NH_3, and others (8,22 and unpublished data). In underwater cave microbiology, the aquatic matrix itself imposes logistic and safety challenges. Only certified cave divers can conduct the sampling, and time limits are inflexibly constrained by air supply of the diver and blood gas physiology (26). Although in this case, underground mine research is also time-constrained mostly by blasting and cage schedules. In spite of these difficulties, underground samples must be collected with care to minimize contamination and postsampling changes.

SAMPLING AIR-FILLED CAVES

Cave Habitat Types

Air-filled caves present an array of potential microbial habitats, most of which parallel corresponding habitats of surface systems (Fig. 1). Habitats include running stream water, still pools varying from fresh to brine, and to alkaline, wet rock surfaces, flowstones and speleothems, dry rock surfaces, muds and cave soils, unconsolidated mineral deposits and pastes of various compositions, and highly organic microbial mats, streamers, and strings clinging to all surfaces of both the cave and the exoskeletons of

Figure 1. Terrestrial caves come in a large variety of types. As can be seen from the partial list of potential environmental variables earlier mentioned, the conditions under which sample taking is conducted will also vary tremendously. Every situation from walking-accessible caves where working is pleasant and easy to caves with poisonous atmospheres that make sampling potentially life-threatening can be encountered. Image courtesy of L. Hose.

cave invertebrates (8). Even stromatolitic microbial sediment structures are found in cave environments (27). Cave sites of potential interest can occur in the near-entrance twilight zone under skylights where varying degrees of attenuated light is present, or deeper into the cave where no light but the occasional caver's headlamp will ever reach. Many caves have invertebrates and vertebrates that access them occasionally (trogloxenes), frequently (troglophiles), or who live their entire lives in the confines of the cave (troglobites). Many of the invertebrates and some fish graze directly on microbial films. Some caves are sufficiently deep and isolated, such that they are primarily or exclusively microbial preserves (28). This can provide an ideal situation for microbiological study without the interfering effects of higher organisms.

Cave Conditions

Whatever the cave type, physical conditions (e.g., temperature and humidity) remain relatively invariant over time. Some caves experience greater fluctuations than others because of their specific three-dimensional structure, degree of closure, and hydrology, but they are more constant than most surface environments. Some caves allow entrance and pooling of cold air resulting in permanent ice deposits, even where summer surface temperatures are very hot. Caves in tropical environments reflect the higher annual average surface temperatures in their temperature.

Caves are highly mineralized and have mineralizing environments because they are essentially a subcomponent of the lithosphere. Additionally, the water-free portions of caves are good environments for preservation of organism remains and organic matter. Although humidity-saturated, caves under arid and desert surfaces can preserve organisms, their remains, and traces of organic matter for very long periods of time in the absence of episodic flooding. Cave air may contain significant concentrations of carbon dioxide (e.g., 7% carbon dioxide in a northern Arizona cave). Episodic levels of hydrogen sulfide and carbon monoxide in a Mexican sulfur cave have reached as high as 204 ppm hydrogen sulfide and 140 pm carbon monoxide (P. Boston, unpublished data).

Most caves are also oligotrophic environments with low levels of organic input derived primarily from the surface. In some cases, utilizable carbon sources may be contained within the parent rock housing the cavern or possibly enter as low molecular weight carbon compounds from deeper geological layers housing petroleum basins. In a few cases, large fluxes of hydrogen sulfide coming into caves via springs provide the basis for a greatly enriched food chain relying on primary productivity of sulfur-oxidizing chemolithotrophs. This additional nutrient base is reflected in noticeably greater biomass than is found in other caves (12,22,29,30).

Cave Microorganism Properties and Methodological Consequences

In general, the relatively constant environmental conditions in most caves endow their microbial inhabitants with a sensitivity to rapid change in parameters such as temperature and humidity. Unlike their surface relatives, cave organisms do not experience weather phenomena, diurnal temperature change, photic oscillations, or seasonal or periodic desiccation. They do not have to cope with ultraviolet radiation, but some caves do have to cope with periodic flooding. Caves with streams running through them from the surface have more organic input than drier caves. In the latter, inhabitants are highly adapted to low-nutrient conditions and highly susceptible to disturbance by the introduction of organic materials. Various minerals in caves, including iron oxidizers, manganese oxidizers, sulfur oxidizers, and others, can provide habitat for a variety of chemolithoautotrophic organisms (8,22,28).

These principles must be kept in mind when designing culturing techniques or other sample-collection methodologies. In general, we recommend performing as much analysis in situ as is practical. For example, metabolic tracer and uptake studies, exoenzyme and biomass assays, and staining with biological dyes can be performed at the site with sufficient redesign of well-known techniques (8). For culturing studies, greater success in recovery of diverse organisms can be achieved by in situ incubation of newly inoculated cultures for as long as possible. We regularly employ extended incubations from weeks to months before removing cultures, particularly in the case of slow-growing chemotrophs.

Dirt is a problem. Most caving involves becoming moderately to extremely filthy and caked with a variety of substances. This is not conducive to good aseptic techniques. We employ sterile nonlatex surgical gloves for most manipulations (Fig. 2). In the humid cave environment, glove-wrestling events can be avoided by using a size larger than normal. Use unpowdered gloves to avoid introducing an unintended contaminant. In addition to dirt, human contamination is a major problem in oligotrophic cave environments. The shedding of hair and skin cells produces a virtual blizzard of organics on a

Figure 2. Long-term in situ incubations of glass or parent rock slides in cave or mine habitats is a handy means of collecting biomass without making much of a sampling impact on the environment. Sterile gloved hand is shown placing a glass slide in Cueva de las Barrancas, New Mexico, a pristine unexplored science-only cave. Slides will be left in place from months to years before collection. Image courtesy of V. Hildreth-Werker.

scale that can be significant for obligate oligophiles. In addition, human-associated microorganisms can persist in the cave environment by using this nonnative nutrient source (A. Hunter and P. Boston, unpublished data). In cases where caver proximity may compromise asepsis, sterile tyvek clean room garments are useful (Fig. 2).

In tiny deposits or puddle-sized cave pools, only minute quantities of sample can be taken to avoid negative impact on the miniscule environment. Miniaturization of procedures facilitates both the conservation of unique microbial habitats and also helps investigators to maneuver their scientific equipment through difficult passages and descents! An excellent way to minimize the quantity of collected material is through the use of microbial "traps." These can be conventional glass slides (Fig. 3) or slides machined from parent materials preferably collected from the surface outcrops of the country rock (Fig. 4). Slides can be hung on sterile monofilament lines to study airborne transport and colonization of organisms or at thin waterfilm-transported organism colonization.

Another useful technique to avoid decimating small bodies of water can be termed the *blood plasma* model of sampling. In this case, volumes of water can be withdrawn from a pool using large (100 cc) sterile syringes with large needles. This fluid can then be injected into a sterile microfiltration assembly. The filter traps organisms and particles of interest, whereas the water can be captured into a sterile collecting vessel and returned aseptically back to the pool. Filter-trapped organisms can be frozen for later DNA analyses, scraped, inoculated into growth media, subjected to a variety of staining procedures performed in situ, or chemically fixed for later examination with SEM or TEM.

Ideally, no-impact techniques are perfect for applications in caves. Many techniques are being developed that greatly minimize the impact on sample sites. Culture-free molecular measures of biodiversity (31,32) are being applied successfully to cave microflora (28,33). Indicator substances like exoenzymes are detected in situ (e.g., 34 modified in 8), biomass inferred in insoluble materials (35); both visualization of microbes (36) and biomineralization (37) have cave applications. A variety of techniques are reviewed in Hirsch and coworkers (38) and modified for cavers in Boston and coworkers (8).

As with many environments, perhaps a majority of species present in cave samples are not presently culturable even with highly specific, selective, and enrichment media that we create for them (32). The use of molecular techniques, specifically 16S rRNA analysis,

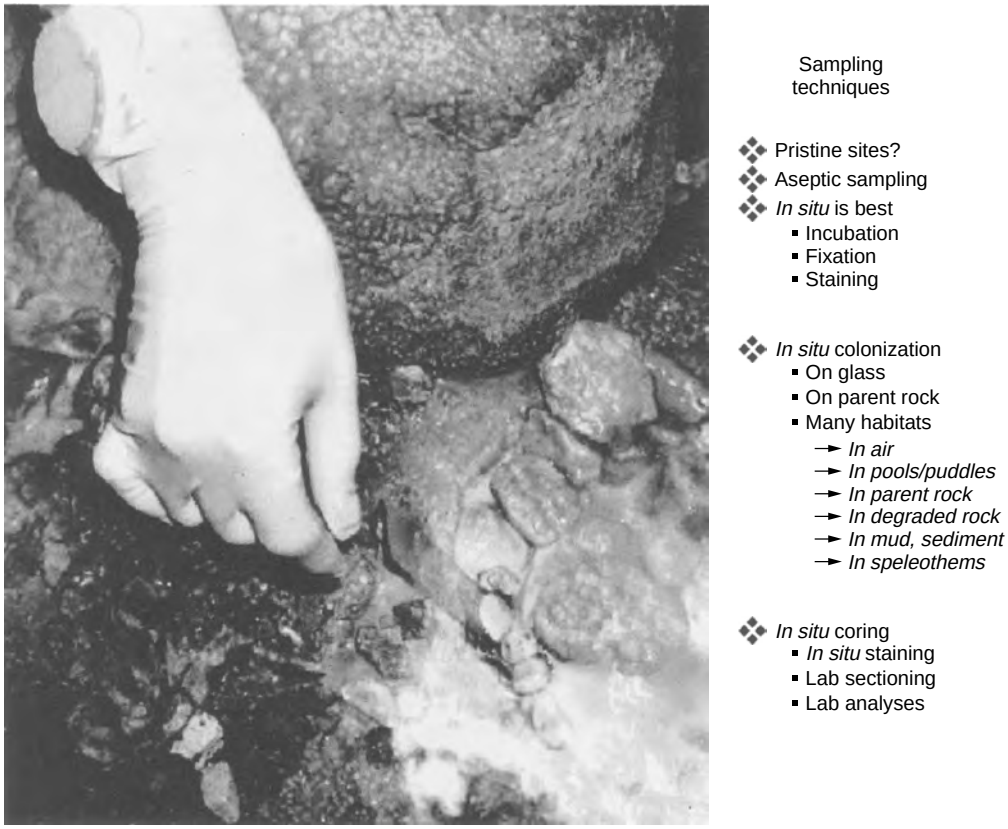

Figure 3. The hydrogen sulfide and carbon monoxide saturated atmosphere of Cueva de Villa Luz, Tabasco, Mexico, necessitates serious breathing protection and an emergency oxygen tank worn on the belt at all times to compensate for episodic dips of oxygen to levels as low as 9.6% (20.9% oxygen is standard at sea level). Sterile gloves and tyvek suit, and wiping helmet surfaces with an antimicrobial are precautions against contaminating the samples and provide some measure of protection for the skin against sulfuric acid dropping from the ceiling. Image courtesy of K. Ingham.

Figure 4. Long-term aerial colonization study is set in place by caver D. Hamer. Country rock machined into slides, polished, sterilized, and hung on a sterile apparatus will be left in place in an off-limits area of Lechuguilla Cave, New Mexico. This experiment is testing whether there is aerial transport of organisms through the cave air circulation cells and colonization on new rock surfaces. Experiments of this nature can run for many years before showing results. Image courtesy of V. Hildreth-Werker.

provides both a measure of biodiversity and a variety of closest taxonomic affiliations, thereby providing potential insight into the composition and potential metabolic characteristics of cave communities. Although the specifics of these analyses are beyond the scope of this discussion (see 8,22,28,33), requirements of the techniques impact the sampling procedures. Because we are trying to achieve the analysis of an unaltered sample, staging of the material on dry ice is the preferred method of field preservation. It should be remembered that cave organisms are adapted to an extremely uniform thermal environment and are more likely to be perturbed or destroyed by even small temperature fluctuations than their surface relatives. Dry ice can be taken into the cave-sampling area in a conventional dewar, provided the cave is not difficult to negotiate. For demanding sites requiring vertical-ascent techniques or extensive crawling, a more robust version fabricated from unbreakable materials can be obtained. Dry ice must be handled with care and sublimating gaseous carbon dioxide must not be allowed to build up if the samples are collected in small, closed areas.

Small-scale coring can be done into parent rock, degradation products, and deposits like elemental sulfur. For soft materials, a simple sterilizable soil corer is required. For rocky materials, battery-powered drills fitted with sterilizable core bits of various configurations are suitable. Any cooling gas used to reduce drill bit temperature must be sterilized. Typically, nitrogen is a good choice for small tanks fitted with in-line microfiltration devices. Significant problems may be encountered when drilling because of the strict power limitations imposed by the necessity to carry the heavy batteries to remote sites.

SAMPLING WATER-FILLED CAVES

The microflora of phreatic conduits are not accessible by ordinary environmental microbiology sampling techniques. A combination of sterile technique and technical cave diving is required to sample these habitats. Challenges such as sample container buoyancy, confined space, darkness, remoteness of sampling sites, and an aquatic environment can, however, be met by cave divers sampling bacterial colonies while hovering in the water column in underwater caves.

Cave divers have observed bacterial colonies large enough to be visible to the naked eye in springs and underwater limestone caves within the Floridan aquifer in Florida (3,39), in underwater caves and meromictic sinkholes on the Yucatan Peninsula in Mexico (40,41), and the phreatic, brackish water caves in the Nullarbor Karst Plain in Australia (42). Divers' observations of these colonies include thin white or gray mats or tufts on cave floors, filamentous masses in the water column, drooping threads or sheets of slime, and gelatinous masses attached to the cave floor (Fig. 5a). Underwater cave fungi have been observed in a Yucatan cenote (Brigmon and coworkers, unpublished data). Underwater cave bacteria from Florida have been identified with monoclonal antibodies and examined with electron microscopy (3,43,44). Bacteria that are found in underwater caves (such as sulfide-oxidizing *Thiothrix* spp.) can form aquatic biofilms that, in other settings, contribute to biofouling of municipal water storage tanks, sewage treatment facilities, private wells, and drip irrigation systems (45). The bacteria of underwater caves may constitute a valuable genetic resource (39).

Approaches for sampling aquatic bacteria in underwater caves have been described previously by Martin and coworkers (26) and are presented in the following text. These methods were developed for research on *Thiothrix* spp. (3,39,43,44). They are based on a combination of standard environmental methods originally developed for sediment (46) and macroinvertebrate sampling in underwater caves (47–50). Microbiologists cannot sample in underwater caves unless they have amassed several years of extensive training and experience in scuba diving, cavern diving, and cave diving. Thus, it is most likely that the microbiologist will remain outside the cave while a professional cave diver collects the samples.

Diving Techniques and Equipment

A helmet-mounted light (commonly used by European and Australian cave divers, but rarely in the United States) can greatly facilitate the sampling work of divers in caves. Kayaking and bicycling helmets are preferred to caving helmets because of the bulkiness and straps of the latter. Helmets should fit over the diver's head with or without a neoprene hood. Light-mounting brackets can be purchased or made from schedule 40 PVC pipes. Lights for helmet mounting should be as bright and lightweight as possible and designed for one-hand operation. Because bacterial sampling requires the use of both hands, handheld lights, cameras, and diver propulsion vehicles must be set aside or passed to a safety or assisting diver before sampling.

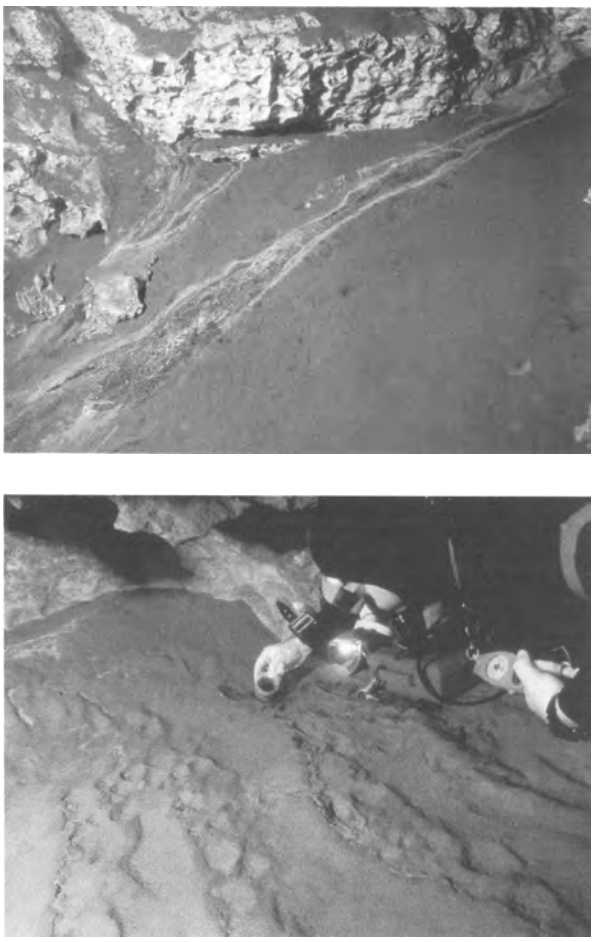

Figure 5. Sampling underwater caves. (**a**) Sulfur seep with Thiothrix spp. in Luraville Cave in northern Florida. The cave is entirely submerged at all times. Note troglobitic crayfish in upper center. (**b**) Martin collecting sample for sediment characterization from a sulfur bacteria biofilm at a groundwater discharge area in an underwater cave in North Florida. Sampling container nonsterile. Photos courtesy of Tom Morris.

This should be done carefully so as not to stir up bottom sediment. To accomplish this, the sampling diver needs to hover in the water column (low-flow areas) or crouch gently on the bottom downstream of the sampling site (practical only in high-flow areas) (Fig. 5b).

Sampling divers and assisting divers should carry at least one extra-underwater writing slate each. These slates should be formatted before the sampling dive with spaces for information that will be required to fill out a sample log sheet or field data notebook.

Sampling Containers and their Underwater Handling. Whirl-pacs (Nasco International, Fort Atkinson, Wisconsin) and many other commonly used sterile, environmental sampling containers cannot be used underwater because of their physical characteristics. The standard 50-mL polypropylene screw cap centrifuge tube is the only sterile sample container that has successfully been used in underwater caves.

During earlier underwater cave microbial sampling efforts, sampling containers were stowed in strapped-on pouch-pockets of a wet suit, or buoyancy-control vest pockets, or in water-filled "dry" boxes with handles that were clipped to a "D" ring. An improved method is the "Gomez tube," developed by the late cave diving microbiologist Peter Gomez. The Gomez tube is a thin-walled PVC pipe with the inside diameter of the sterile sampling tube wider than the outside diameter and of sufficient length to hold up to six sterile sampling tubes. Tire inner tube rubber is placed over each end of the PVC pipe and secured with a hose clamp. A slit is made in each piece of the rubber, allowing prelabeled sterile sampling tubes to be removed from the PVC pipe in a predetermined order. To remove the first prelabeled sterile sampling tube from the PVC pipe, an extrasterile sampling tube is inserted at one end of the PVC pipe, displacing the first. With the exception of the inner tube material, all of the materials required for constructing a Gomez tube can be obtained at a hardware store. If the entire Gomez tube is to be placed on ice after the sampling diver leaves the water, it should be short enough to fit in an ice chest.

Polypropylene tubes and sample bottles and their caps are positively buoyant, so the sampling diver must not let go of either underwater. To prevent cap loss, Velcro can be glued onto each cap and onto some area of the container (Dr. Jill Yager, Antioch College, personal communication). The ideal bacteria sample container for use in underwater caves is one that is slightly negatively buoyant underwater, water-filled, sterile, and easy to manipulate with gloved or cold hands.

Each diver can easily manage three or four air-filled tubes, or up to six when using a Gomez tube. Our experience in Florida caves has shown that divers have time to collect at the most four samples and still accommodate required decompression. In shallow caves such as those found in the Yucatan in Mexico, more than four samples per diver can be taken (Dr. Jill Yager, Antioch College, personal communication). The number of divers needed is a function of the size of the cave, the sampling tasks required, and requirements to minimize environmental disturbance. A two-diver team is desirable. In larger caves, a three-diver team may be necessary for safety and to handle the workload. We have observed no implosion of sterile 50-mL polypropylene centrifuge tubes at depths of 30 m or less, but some have imploded at greater depths. However, we have observed that at depths approaching 30 m, it was difficult to remove the lids from the sterile air-filled tubes because of the elevated pressure. For deep sampling (>30 m), sterile tubes can be filled with autoclaved water in a sterile laminar flow hood in the laboratory. Gas headspace must be close to zero, however, or caps will be difficult to remove at depth.

Sampling Technique

General Considerations. The collection of microbiological samples is a slow process for even the most efficient methods, so shallow sampling sites are most convenient. However, sites of interest may be deep. If samples are to be collected at sites of a depth greater than 30 m, be sure to factor adequate time into the dive plan. If possible,

water and bacteriological sampling should be done on the way into or down to the most distant/deepest sampling point during the dive (51). Careful region-by-region sampling, temperature measurements, and other operations should be done during the inbound part of the journey. In some caves, this is necessary to maintain the integrity of the water column stratigraphy and chemistry, which may otherwise be disrupted and/or contaminated by divers' movements and the upwelling of deeper, sometimes colder water by divers' air exhaust bubbles (51). Inbound sampling is particularly important in sinkholes. Whether it is best to sample bacteria in a siphon cave (caves where the entrance is upstream and divers must enter swimming downstream) on the way in or out depends on the penetration distance and the rate of cave water flow.

For some bacterial colonies, it is useful to collect one sample with and one without adjacent sediment. If the bacteria are growing on limestone, collection of a piece of substrate or adjacent limestone may be valuable. Rock samples are stored in labeled sterile bags. Some underwater cave bacteria are difficult to culture in the laboratory without springwater or other groundwater from the same or similar sites as an addition to growth media (3,43,44,52). Water for culturing should be collected from the aquifer where the bacterial colony is located. We have found that it is not necessary to use diving to collect these water samples if a surface spring or flooded sinkhole is present. Slurp guns are plastic suction devices designed for the live collection of fish and invertebrates in aquatic and marine settings. Assuming a slurp gun could be autoclaved or gas-sterilized, such a device may be used for the underwater collection of bacterial colonies.

We have found sterile 50-cc tubes to be the best container for sampling bacteria in underwater caves. The following procedures have proven successful (26). Uncap the tube while in direct proximity to the bacterial formation or sample surface. Try to get as much material as possible into the sample tube. Sample different morphologies in separate containers. In our experience, the best way to collect bacteria occurring as films on surfaces such as rock, clay, or iron oxide deposits is to scrape them off with the mouth of the tube, orienting the tube so that the bacteria (with a specific gravity slightly greater than water) sink into the tube. Flocculent bacteria, usually found in cave areas of low flow, can be collected by gently pushing the tube through the colony and, if necessary, prodding the sample into the tube with the cap. If it is necessary to obtain bulk in situ water for media preparation, collect as close as possible to the location of the original sample. Purge outside water from the sample container (prefilled with sterile water to minimize buoyancy and implosion pitfalls) with a compressed air nozzle while holding the jug upside down. Refill with cave water, purge, and fill again to ensure sample integrity (51). Where water flow is substantial, this can be done without stirring up cave-floor or ceiling sediment or contaminating in situ water samples with water of a different chemistry. Where cave water flow is limited, care should be taken to minimize the disturbance of ceiling sediment or mixed waters. Other methods for water sampling by scuba divers described by Miller (53) are not generally practical in the underwater cave setting.

On-Site Observations. Record pertinent measurements including water current on an underwater slate by the method of Wilson (54). If diving a Ghyben-Herzberg lens cave (an island or coastal aquifer cave with a lens of fresh water overlying seawater), such as those in the Yucatan, Bahamas, and elsewhere, indicate whether samples were taken in the fresh- or saltwater layer and the vertical distance from the halocline, if present. Note morphological and positional information of the sampled material.

Postcollection Activities. From the sampling site, the diver must swim the entire distance out of the cave, possibly picking up staged breathing tanks (stage bottles) and negotiating low or narrow restrictions where gear can be knocked about. The careful stowing of samples is critical. Before leaving the cave, the diver must often decompress for as long as an hour or more. During this time, samples will equilibrate to the local groundwater temperature. If the samples must be transported to the surface quickly, support divers can enter the cave and retrieve the samples from the decompressing divers and deliver them to the surface staff. Finally, the sampling or retrieving diver climbs out of the water, exposing gear and samples to more jostling. Carefully passing samples to surface staff before the divers exit the water can minimize disturbance. Some samples may require the addition of fixatives or concentrated acid if sampling for protozoans, sulfide, or soluble minerals. Treatment and processing of microbiological samples cannot be conducted until the sample containers have been removed from the water. This is a limitation not shared by microbiological sampling in air-filled caves or mines.

Sampling for Anaerobes. We have not sampled subaqueous cave anaerobes but Martin and coworkers (26) proposed the following modifications for sampling anaerobic bacteria in underwater caves: (*1*) Sterile sampling tubes should be configured for the sampling of anaerobes. The gas in sterile tubes should be nitrogen, and not air. Sterilized in-line microfiltration units are readily available in many connection styles for the sterilization of gas. If tubes are filled with sterile water, this must also be oxygen-free. (*2*) Do not use air from the scuba tank to purge the water sampling container. Rather, fill a "pony bottle" (tank with 25 or less cubic feet capacity) with pure nitrogen gas. If the tank is not a scuba cylinder, the valve must be modified with a fitting that allows attachment of scuba fittings. These fittings are available at some dive shops, particularly those catering to cave divers. Do not install a second stage regulator on this tank because breathing this gas could be fatal. To the first stage regulator on this tank, attach only a low-pressure hose with an air jet nozzle and a pressure gauge. Attach the nitrogen bottle to the double scuba tanks or wherever convenient to the diver and secure the nitrogen hose and nozzle where the diver can reach it and will not confuse it with an air nozzle secured elsewhere on the diver. (*3*) When collecting the sample, open and close the sample container only in

the anaerobic zone. (*4*) After the dive and after putting anaerobe samples in the cooler, purge the air from the cooler with nitrogen gas from the nitrogen tank. A separate cooler for anaerobic samples may be useful. Conduct all sample transfers "under" nitrogen gas to the extent possible under field conditions.

SAMPLING UNDERGROUND MINES

Mines may be thought of as portals into the subsurface, but to be useful for studies of the subsurface biosphere, a number of issues must first be addressed. Although the true extent of this habitat remains unclear, some minimal prerequisite conditions (moisture, pore volume, energy, and nutrient availability) within the mined country rock must be met for microbes to persist. These factors should be considered in the selection of study sites. Subsurface microbial communities are likely to be composed of nutrition with low collective biomass. Native biosignatures within freshly opened rock panels and groundwater intersections may be ethereal and competitively overwhelmed by those of mining contaminants. Thus, to detect indigenous microbial communities, process-induced contaminants must first be recognized or negated.

Choice of Mines

Mine excavations tend to be composed of multiple levels, one over another. Service or drain water and their associated microbes may move by cross connections only to be rediscovered at other locations and interpreted as indigenous. For this reason, newer workings penetrating virgin rock and lacking overlying mining activity are the most desirable for microbiological investigations (55,56). In some cases, however, the goal may be to investigate mine microbiota itself, and thus older workings can also be of interest (57,58). Mine microbial communities can have a direct relevance to excavations created for the deep burial of radioactive waste (58) where the corrosive effects of microbes, irrespective of origin, may represent a hazard.

Contamination Issues and Quality Assurance

Potential vehicles for the direct spread of microbial contamination in a mine include service and drain water, ventilation air, equipment, and miners and their wastes. Additionally, biostimulative changes that favor surface-derived organisms (e.g., aerobes) in the local environment surrounding mine workings may alter microbial ecology and further complicate interpretations. Mines most typically intersect quiescent, anaerobic strata rich in reduced or partially reduced minerals and infused with reducing gases such as methane, hydrogen, and hydrogen sulfide. Mines functionally serve as conduits for the injection of oxidizing equivalents (ventilation air and service water) into these zones. The oxidative nature of service water reflects not only saturating levels of dissolved oxygen but also accumulations of blasting and disinfection residuals (e.g., nitrate and chlorine). In the presence of water, the resulting geochemical gradients, often supplemented with fuel and lubricants from equipment, represent a novel energetic resource that can lead to luxuriant microbial blooms of mixed origin.

Every sample taken from a mine must be assumed potentially contaminated, at least on its outer surfaces. Although the mining process may alter the outer surfaces of rock samples, internal pore spaces often remain pristine. To track potential process-induced contamination, many workers intentionally dose drilling or service water with easily traced materials and monitor the movement of these as surrogates for contaminating microbes. Alternatively, the unique chemical and biological components of known sources of contamination can be tracked. The U.S. Department of Energy (DOE) Subsurface Science Program has used tracers for the evaluation of sample contamination (59, and references therein). Although not limited to mine research, many of these techniques are directly applicable to mine sampling. Natural caves, by contrast, generally represent biologically protected zones and thus disallow this potentially intrusive technique.

Tracers may be particulate or soluble. The most often used particulate tracers are fluorescent carboxylated latex beads (Polysciences Inc., Bay Shore, New York). Typically, 0.4- to 1.2-μm diameter beads are used to approximately estimate the size of a bacterium (60). The beads are tracked using a fluorescence microscope on 10 X objective (100 X final magnification) and can be detected at approximately $10^4\ g^{-1}$ in crushed rock samples (59) or $10\ mL^{-1}$ in liquid extracts (61). There are several caveats in the use of microsphere tracers that must be considered before this technique is elected. Many microbes are motile and may penetrate more deeply into a porous substrate than passively transported particles. Microbes possess varied surface charges and envelope characteristics that are difficult to mimic. Also, even trace levels of contamination by nonindigenous microbes may still lead to significant bioburdens after postsampling growth. These concerns aside, microspheres remain one of the best methods available and have received generally good acceptance as a measure of quality control.

Water-soluble fluorescent dyes such as Rhodamine WT (Crompton and Knowles, Green Hills, Pennsylvania, excitation/emission wavelengths 546/590 nm, respectively), can be detected in extracts from samples by fluorimetry at approximately $2\ \mu g\ L^{-1}$ final concentration (62). Inert perfluorocarbon tracers have been used in a variety of applications, including subsurface studies, and can be detected by pyrolysis gas chromatography at picogram per gram final concentrations (59). Dissolved ions can function as tracers as well. These may be intentionally added (e.g., lithium bromide, 63) or intrinsic to process fluids (ammonium, sulfate; 61,64). In mine service water, nitrate ion may be present in millimolar concentrations and thus the submicromolar detection limits possible from ion chromatography and flow injection techniques permit better than three orders of magnitude protection. Dissolved oxygen is another easily detected service water component. One caution for dissolved constituent tracking, however, is that not all are conservative. Oxygen, nitrate, and sulfate, for example, can be quickly consumed by microbial action under permissive conditions.

Field kits for the detection of dissolved oxygen, nitrate, and others can be obtained from various suppliers (e.g., Chemet kits, Chemetrics Inc., Calverton, Virginia). Assuming deep groundwater to be generally anaerobic and nitrate-free, positive field tests for nitrate and oxygen (and/or free chlorine) can be taken as evidence that a given sample is process-contaminated. Temperature, easily measured in situ, may serve as a physical tracer of water origins. In deep, hot mines, service water is chilled before being piped to depth. Water emanating from fractures at a temperature below the ambient temperature for a given depth is cause for suspicion. In the same way, anomalously warm drainage may indicate the upwelling of pristine water from deeper levels.

Bacterial membranes contain phospholipid fatty acids (PLFAs). PLFA profiles have been employed to differentiate microbial communities from drilling fluids and sediments, to provide information concerning overall physiological status, and to estimate total biomass of subsurface microbial communities (65,66). One PLFA class (the epoxides) can serve as a biomarker for oxidative stress in bacterial population. Chlorinated mine service water reliably contain epoxide biosignatures in their particulate fraction. The absence of detectable epoxide may be taken as an indication that a given sample contains little or no service water (67). Cultivable microbes may serve as either added or fortuitous tracers in their own right. Several authors, for example, have used coliform bacteria as tracers in drilling fluids (68,69). Aerobic bacteria and fungi can be cultivated from swabbings of mine surfaces and used to monitor the transfer of mine contaminants into samples (70). Dilution plate counts followed by comparison of colony morphology patterns have been used to compare samples and known sources of contamination (71). Community-level physiological fingerprinting (e.g., the Biolog system, Hayward, California) have been used to characterize drilling fluid microbial communities (65).

Molecular methods employing universally conserved ribosomal RNA genes (e.g., 16S rDNA in prokaryotes) are insensitive to culture bias because they detect environmental DNA directly (32). These approaches can be used for rDNA fingerprinting of the whole community [e.g., terminal restriction fragment length polymorphism (TRFLP) (72)] or rDNA clone libraries following amplification of target sequences from environmental DNA by the polymerase chain reaction (PCR). In our work in South African ultradeep gold mines, molecular biosignatures for service water and mine air proved diagnostic over time and distinct from putative deep groundwater intersections (73). Such prior knowledge of expected contaminant rDNA sequences can be employed to judge the quality of samples. Conversely, the presence of unusual rDNA signatures in samples can be indicative of sample quality as well. Samples dominated by rDNAs derived from known obligate anaerobes or hyperthermophiles, for example, would be inconsistent with service water origins.

Rock Sampling

Various types of rocks can be sampled in mines. For the purposes of the microbiologist, it is useful to divide mines into "hard rock" and "soft rock" because the methods for both mining and sampling differ. Hard rock mining (e.g., granite and quartzite) generally entails excavation by blasting, whereas softer materials (halite, coal, tuffs, etc.) can be mechanically worked. Hard rock mining uses water-cooled percussion drills to perforate advancing panels and allow the setting of charges. Approximately the outer half-meter is typically fractured following each blast cycle. Beyond this, a so-called *kick plate* boundary defines the outermost extent of unfractured, minimally contaminated pristine rock. Percussion drilling infuses the newly created fractured zone with service water before each blasting cycle. Subsequently, the act of blasting can drive water- and airborne contamination deep into microfractures.

For these reasons, precautions must be taken when sampling hard rock in mines. In this environment, samples will take the form of either blocks or cores. Core samples can be obtained by sidewall probings from well beyond the panel face assumed to be contaminated, but with a correspondingly high probability of acquiring surface contamination from pressurized drilling fluids. Even small diameter cores, however, will often contain pristine material inside and, with rigorous tracer testing, can yield provably uncontaminated subsamples (60). Freshly blasted mine panel grab samples afford some advantages of their own. First, they can be collected with minimal logistic commitment as soon as it is safe to reenter after blasting. Loosened wall blocks and even floor rubble present a potentially wide array of sample choices. A large block sample with a large diameter core will better insulate pristine inner material from contamination than could any other sample.

Although tracer addition before the blast cycle would be ideal, logistics generally mandate that tracers be applied afterward. The chosen rock sample is typically dosed with a concentrated solution of microspheres and/or fluorescent dye tracers, using a spray bottle, and then aggressively washed with a high-pressure mining hose. This treatment is intended to mimic the service water exposure that would be expected from panel drilling and dust suppression. After tracer addition, the sample is transported to the surface in a sterile autoclave bag (on ice if possible). The rock is coated with bright spray paint (e.g., blaze orange) to permanently mark all outer surfaces. Next, the sample is placed in an anaerobic chamber and the outer surfaces pared away using a hydraulic rock splitter and sterile hand tools (59,74; Fig. 6). We have found that wrapping the sample in clear plastic wrap (effectively sterile inside the roll) greatly enhances control and traps rock shrapnel. Outer parings are saved for comparative tracer studies and the inner subsample is stored or finely ground with an autoclaved Platner rock mill for cultivation studies.

In contrast, soft rocks can be excavated using an alpine miner or other boring machine. These devices employ rotary carbide bits to grind into the rock without the need of cooling water and thus minimize contamination from this source. Endolithic microbes were sampled by alpine miner at the Nevada Test Site (70), for example. The best alpine miner strategy is to enter the newly opened rock face as soon as possible after mining and collect with gloved hands and sterile hand tools. A number of researchers working in soft rock have reported that

(a)

(b)

(c)

Figure 6. Rock processing equipment. (**a**) Hydraulic rock splitter. (**b**) Diagram illustrating rock splitter jaws and a typical cut made during the paring operation. (**c**) Paring of rock sample in anaerobic chamber. Note that the sample is wrapped in clear food-grade plastic and the splitter in clean sheet plastic. Sterile gloves are worn and all surfaces that contact the sample are swabbed with 10% bleach solution between cuts. Moser operating rock splitter. Jim Fredricksom (left) and T. C. Onstott look on. Photo in panel C courtesy of Louise Gubb.

simply chiseling away the contaminated outer surfaces of tunnels with hand tools (55,58) is sufficient to obtain endolithic samples for microbial studies.

Water and Biofilm Sampling

Mine-wall seeps associated with water-bearing fractures and dykes are often extensively colonized with biofilms. Generally, sterile spatulas or even the lip of a sterile plastic tube is effective for scraping material into a waiting sample tube. At times, zonation can be seen within a biofilm and a sterile syringe, without the needle, and can be used as a mini "slurp gun" to collect subsamples with precision. A number of workers have obtained samples for microbiology by collecting water dripping from the ceiling (55), although, as with surface biofilm sampling, the potential for mine contamination is high.

Exploratory boreholes can be exceptional opportunities for microbial studies in mines. Often situated at the periphery of mining development, water derived from such boreholes probably represents the least impacted deep subsurface material currently available. After initial water contact, mines generally valve a borehole and then allow it to drain in a controlled manner. The high flow rates of such holes ensure correspondingly high dilution factors and thus reduce the possibility of detecting mining contaminants. Even so, in the case of extremely low biomass fissure water, borehole wall colonists derived from the service water and inoculated during drilling may ultimately bloom to become detectable by molecular methods (73). Drained boreholes often gradually reach equilibrium and drip at slow rates for years.

Mine boreholes are typically lined at the wall face with steel casings (Fig. 7c), which facilitates sampling. Anaerobic samples can be obtained from flowing borehole outlets after sealing them with an inflatable packer or expansion plug (Fig. 7b). Sampling systems can take a variety of forms but must incorporate a pressure-relief valve for safety (Figs. 7a, 7c) because mine waters can reach dangerous pressures. We attempt to make our sampling designs suitable for an autoclave. Although the prototype model (Fig. 7) contained some metal parts, subsequent versions have been entirely made out of plastic to negate the possibility of compromising geochemical analyses by metal leaching or artifactual hydrogen production from groundwater-steel interactions. Plastics such as delrin, polysulfone, and even nylon mill are easily available and tolerate autoclaving. Plastic fittings, tube stock, valves, and information about their thermal stability and gas solubility properties are available from scientific and speciality suppliers (e.g., Cole Parmer, United States, Vernon Hills, Iillinois, and U.S. Plastics Corp, Lima, Ohio).

Once sealed, all or part of a borehole's flow can be diverted by a sterile hose into a collection container. Small-scale samples can be taken directly into sterile serum vials preloaded with filtered argon or nitrogen using sterile syringe needles with the Luer end threaded into a sampling hose of the correct inside diameter. A small-bore vent needle must be inserted into the bottle septum to bleed the headspace off as the bottle fills. For larger samples, stainless steel soft drink canisters of the

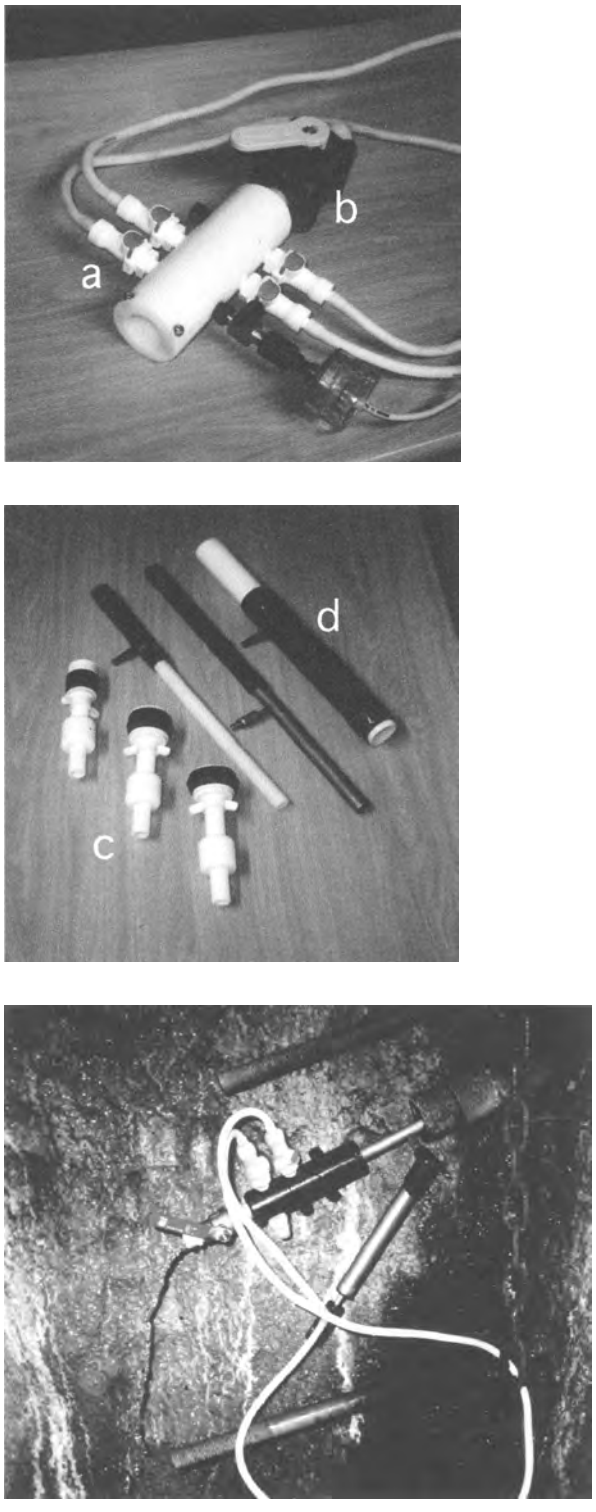

Figure 7. Sampling of flowing boreholes. (**a**) Sampling head, (**b**) quick connect fittings. Note pressure relief valve. Sampling head is securely affixed to standard diameter packer outlet by screw operated clamp and leaks prevented using grove-mounted O-rings inside delrin plastic manifold housing. (**b**) Borehole packers; (**a**) expansion drain plug design, (**b**) inflatable bladder design. (**c**) Borehole sampling in progress. Inflatable packer (note bicycle pump) is largely out of view inside the pipe casing. Water flow is controlled by throttling of pressure relief valve. Photos taken at Driefontein Mine, South Africa.

three gallon size (e.g., Vineland, syrup Inc., Vineland, New Jersey), with modifications (Fig. 8) have proven excellent for this purpose and are suitable for an autoclave. These canisters can be prefilled with argon for anaerobic sampling, and the anaerobic headspace vented during filling. Once filled, the canister is transported to the surface and, following the reestablishment of headspace pressure with inert gas, the sample filtered. Out of a variety of available membrane and housing assemblies, any one (0.1 or 0.2 μm) can be used to collect microbes for subsequent DNA extraction or cultivation (Fig. 8). Multiple subsamples can be obtained at once by splitting the flow using a manifold filtration. The major limitation of sampling with tanks is that only finite volumes of water can be collected and transported. In the case of low biomass samples, direct filtration would allow for the processing of much larger volumes.

An alternative method for microbiological sampling of boreholes is to collect cells on artificial substrates (glass, stainless steel, ceramic, polypropylene, etc.; 56,73). The flow from dripping boreholes can be directed through upflow cartridges filled with various physical substrates such as sand or rock chips (60, Fig. 9). This method allows the collection of considerable biomass under anaerobic conditions. After a suitable incubation period (typically months), the cartridge is sealed off, transported to the surface, and the entire assembly processed in an anaerobic chamber.

Mines cycle enormous volumes of ventilation air. This must be regarded as a major source of potential contamination. It is a good practice when sampling mines to obtain an air sample for comparative purposes along with each sample of interest. Battery-powered personal air-filtration units, generally used for lead and asbestos monitoring, are now widely available (Spectrex Inc, Redwood City, California, and F&J Specialty Products, Ocala, Florida). These can be fitted with membrane or glass fiber filters to capture particulates including airborne microbes and fungal spores. After sampling, filters can be either vortexed in buffer to release captured cells and spores for subsequent cultivation or extracted for DNA analysis. As an alternative approach, a number of air samplers (e.g., the Andersen N6 impactor type, Thermo Andersen Instruments, Smyrna, Georgia) disperse airborne microbes directly onto the surface of an agar plate for viable count analysis.

Transport and Storage of Samples

As with any environmental sampling, mine materials should be processed or fixed as soon as possible. Several studies have documented shifts in microbial populations in samples postsampling even with refrigeration (63,74). Haldeman (75) has recommended that sample analysis be initiated less than eight hours before collection. Biofilm samples can be fixed with preservatives such as glutaraldehyde solutions underground. More often, however, samples require surface processing. When feasible, samples should be transported on blue ice in a cooler. As an alternative to chilling, high-temperature samples (50 °C and greater) may be better protected by maintenance at ambient temperature. This approach

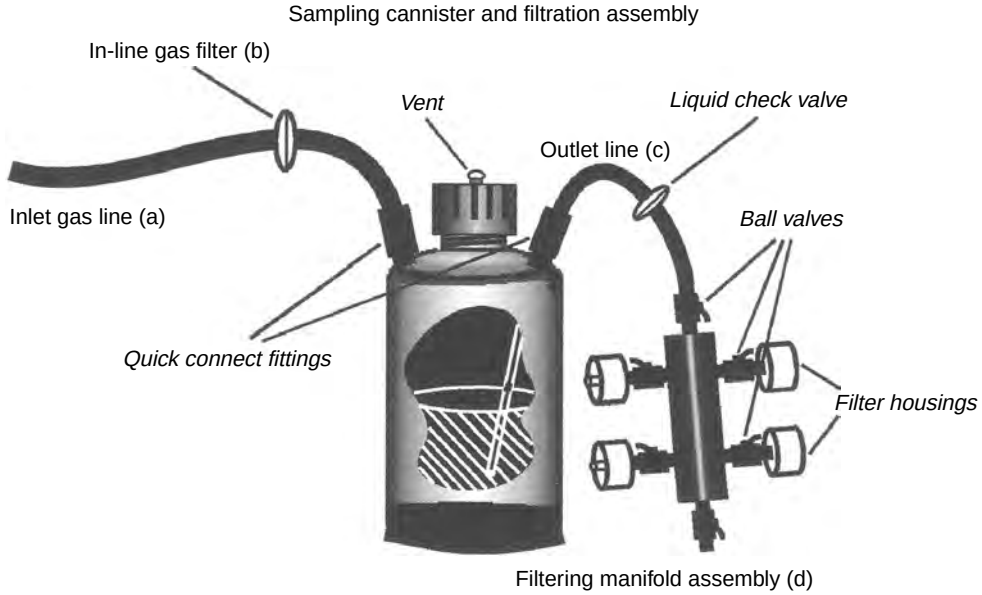

Figure 8. Stainless steel water sampling canister and laboratory setup of anaerobic filtration. A slight headspace overpressure is developed by (**a**) a compressed gas line charged with nitrogen and fitted with an (**b**) in-line filter. Sample is displaced because of gas pressure and extruded by straw and (**c**) outlet water line to (**d**) manifold filtering assembly. Filtrate is collected to measure the flow and can be used as a basal medium for cultivation.

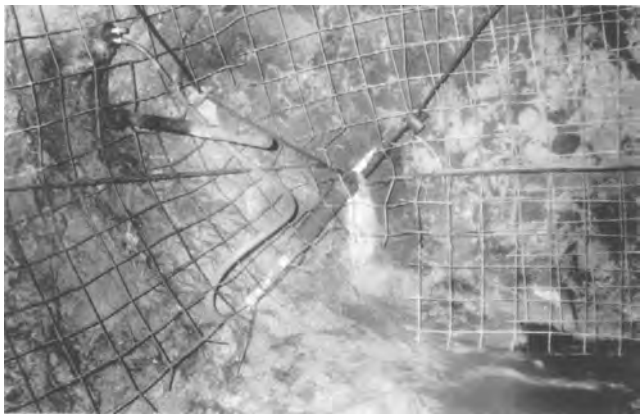

Figure 9. Up-flow substrate cartridge installed for the accumulation of biomass from a 3.2 km depth draining borehole in Driefontein Mine, South Africa. The cartridge was filled with crushed, sterile quartzite collected from the same mine. In-line chamber at the bottom of the cartridge contains a collection of mineral substrates for subsequent studies of microbe/mineral interactions. Cartridge was incubated in situ for two months before retrieval.

is especially applicable on large samples, which cool slowly, in effect drifting through the optimum growth temperature of every potential mesophilic contaminant as they do so. For filtered nucleic acid and polar lipid samples, ultracryopreservation ($-70\,^{\circ}C$ or colder) is best. This can be achieved for short periods in the field and during transport with dry ice or liquid nitrogen. Temporary storage at $-20\,^{\circ}C$ appears to be adequate for up to several weeks. Additional protection for nucleic acid samples can be secured at any temperature by the use of chemical preservatives such as 5-M guanidineisothiocyante buffer, (76).

CONCLUSION

Underground microbial habitats can be sampled successfully for microbiota, whether these be natural cave systems or anthropogenic excavations. Many of the technical aspects of working in these disparate habitats are similar, but there are concerns unique to each as well. Mines generally provide easier access than caves and have the benefit of the potential availability of power and heavy equipment underground. By virtue of their continual expansion into new zones, mines present additional and alternative sampling opportunities. However, contamination is always of concern. Natural cave systems offer the opportunity to study intact natural ecosystems.

Protection, Permitting, Legal, and Ethical Considerations

In the United States, all caves (including those privately owned) are legally protected (Federal Cave Resources Protection Act of 1988). In addition, caves that occur on land managed by federal agencies come under additional layers of permitting and management. The Bureau of Land Management, the USDA Forest Service, and the National Park Service are involved with cave and karst management on their lands. In other countries, similar protections are in place. Cave conservation policies have been adopted by the (U.S.) National Speleological Society, the Cave Research Foundation, the National Association for Cave Diving, and the (British) Cave Diving Group. Cave researchers should be aware of these organizations and their policies as well.

In countries lacking specific protections for their caves, ethical considerations still dictate extreme caution in undertaking any microbial sampling. Sampling programs should be designed to minimize disturbance to cave features and cave life. Caves not only house microbial communities of great value, but also contain unique higher fauna (sometimes indigenous to only one cave system) and often irreplaceable paleontological, archaeological, and historical materials. In natural caves, any excavation must be done purposefully by drilling or the chipping or breaking of material. The biogeochemical ecosystems created by cave bacteria are unusual, delicate, and easily disrupted by careless movement, thoughtless exploration (77), or overcollection (78,79). Many caves have long since ceased their active speleothem-forming period, and those geological and mineralogical specimens are truly irreplaceable. Ideally, microbiological techniques and those of auxiliary sciences will evolve toward low-impact or no-impact methods that are not consumptive for a sample.

Because mines, by definition, are extensively impacted by human activity, these sites represent very different circumstances in terms of conservation and legal issues. Permissions to sample are required as in caves, but because mines are typically private holdings, government regulations (other than safety) may not be applicable. In government operations (e.g., underground waste depositories), there are additional layers of security and regulation, which must be navigated. In either case, professional courtesy, openness, and data sharing have value in enhancing relationships with host organizations. In private mines, science is usually a secondary consideration to the economics. Mine researchers are thus well advised to minimize their impacts on the mining process. Mines often require training and heat-tolerance certification for underground workers, and guest researchers are expected to meet all legal requirements for mine workers and contractors.

Safety

All underground work has its intrinsic hazards. Safety should be the first order of business for mine or cave microbiologists. Safe terrestrial caving techniques are best learned from experienced, safety-minded, and conservation-aware cavers. The best practice for noncaving microbiologists who wish to sample caves is to work with cavers who have experience working on scientific caving expeditions (National Speleological Society website *http://www.caves.org/*). Although some caves offer walk-in access, others require extensive training and endurance. Additional insights into vertical caving that requires rope skills can be gained by consulting (80). Certain specific cave environments may present hazards out of the ordinary, for example, lethal concentrations of gases. Breathing gear ranging from simple acid gas–filtration units to carbon dioxide–removing rebreathers or complete oxygen–breathing systems may be necessary. In dry caves, the entire weight of such units must be borne without buoyant assistance from water.

Cave diving can be particularly unforgiving. Safety practices must be adhered to scrupulously at all times. Safety of divers takes precedence over all scientific objectives (81). Only experienced, "full cave" certified divers should engage in research cave diving. Research cave divers should maintain diver's accident insurance including air-ambulance coverage (26). For technical and safety details of cave diving practices, see Prosser and Grey (82), Saltsman (83), and Balcombe and coworkers (77), and older works by Exley and Young (84), Lavaur (85), Mount (86), and Lascu and Sarbu (87). For general information on research diving, consult Flemming and Max (81) and Miller (53). For specific recommendations on research cave diving, consult Balcombe and coworkers (77), Horne (51), Flemming and Max (81), Lascu and Sarbu (87), Murphey (88), Skiles (89), and Wood (90). None of these works, however, addresses methods for microbiological sampling with scuba in underwater caves or other underwater settings.

Conditions encountered in modern mines range from modern industrial settings (although deep underground) to some of the most extreme working environments. The most compelling mine sites for microbiological study tend to be deep and thus geothermal heating may be an issue. In some South African mines, virgin rock temperatures of 50 °C are not unusual and water ingressions can exceed 60 °C. Although, by law, working areas must be cooled down to industry standards, humidity and heat may challenge nonacclimatized researchers laden with samples and equipment. Heavy machinery such as locomotives and cable conveyances operating in tight quarters must be monitored. Lighting is generally provided by headlamp. Hearing protection in noisy areas is required. Accumulation of explosive gases can occur. For this reason, flame sterilization of tools can never be recommended and flash photography is generally not permitted in mines. The swabbing of tools with ethanol must suffice. As with cave sampling (or life for that matter), most dangers, however, can be minimized by adherence to common sense.

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CAVES AND OTHER LOW-LIGHT ENVIRONMENTS: AEROPHITIC PHOTOAUTOTROPHIC MICROORGANISMS

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Caves are generally considered to be aphotic habitats in which food must be imported from the surface or manufactured in situ by chemosynthetic bacteria. The

presence of photosynthetic microorganisms is, however, also frequently revealed in these environments as blue-green, brown, or gray biological patinas in areas in which sufficient irradiance is provided by natural light. Show caves equipped with lighting systems represent a particular case of cave habitats. Depending on the intensity, quality, and duration of the illumination, conditions conducive for photoautotrophic growth are created in these caves, allowing photosynthetic organisms to spread to areas in which they did not previously grow. This leads to an often luxuriant development of photoautotrophs around the sources of light; the flora that establishes under these conditions is referred to as *Lampenflora* (i.e., flora of the lamps) (1–3). Although microbial photoautotrophs are the major component of this flora, mosses and ferns (and in some cases even seed plants) are also frequently observed as soon as some soil is formed. Besides natural caves, there exist also artificial low-light environments, such as catacombs, tombs, etc., in which the illumination of frescoes, stuccoes, and marbles of historic and artistic value may lead to the development of photosynthetic organisms (4–7), often having a biodeteriorative effect on this cultural heritage.

The photoautotrophic microorganisms that thrive on the stony surfaces in low-light environments do not develop as single colonies but as components of complex biofilms. The possible role that these microbial photoautotrophs may play in the food web of these low-light habitats is still completely unknown.

In this article, the data available on the biodiversity, distribution, and biology of the aerophytic photosynthetic microorganisms living in low-light environments are presented, also focusing on the negative effects of the development of these biofilms in show caves or man-made hypogean environments of archeological interest.

HISTORICAL DEVELOPMENT

Although cave microorganisms have been studied since the eighteenth century (8), photosynthetic microorganisms were generally ignored. It is only since the second half of the Twentieth century that works devoted only to photosynthetic microorganisms have appeared. However, investigations on the taxonomy and ecology of low-light microbial photoautotrophs remain rather scarce and investigations of these microorganisms have only been conducted in about 20 different countries worldwide: Belgium, Bulgaria, France, Germany, Great Britain, Greece, Hungary, Israel, Italy, New Zealand, Romania, Russia, South Africa, Spain, Thailand, Ukraine, United States, former Yugoslavia, and Vietnam (9–14). Most data are available for limestone caves, studies from other types of substrata are scarce or absent.

THE LOW-LIGHT ENVIRONMENT

Caves can be considered as being nearly closed environments in terms of energy and mass flows. Three principal cave energy levels can be distinguished (15). High-energy caves are those that experience high-energy events such as regular winds and frequent flooding by allogenic rivers. Caves of moderate energy are influenced by events some orders of magnitude less than the previous. In low-energy caves, energy flow is minimal, consisting primarily of heat exchange between the cave atmosphere and the rock surfaces; low-energy caves are essentially closed to any impact from the outside environment. This is exacerbated in many show caves, which are completely shut off from the outside environment during the nontourist season by doors, which prevent light penetration at the entrance and atmosphere exchange with the outside (Fig. 1b).

Caves and other low-light habitats are generally hypogean environments with stable microclimates characterized by uniform temperatures throughout the year, constant and often high relative humidity, and low illumination, especially toward the deepest zones. The most important environmental factors, which govern the growth of photosynthetic microorganisms in these environments, are light, temperature, and humidity. Intensity of light is of course the governing factor for the distribution of the photoautotrophs. Fluctuations in temperature and water supply are extreme in the cave mouth (Fig. 1a), whereas light is the main controlling factor in the deepest zones. Availability of dripping water is also an important factor governing the distribution of these microorganisms in cave systems.

LOW-LIGHT MICROBIAL PHOTOAUTOTROPHS

The microbial photoautotrophs found in caves use oxygenic photosynthesis and are characterized by the presence of chlorophyll-*a*. They are phylogenetically very diverse and comprise two very distinct groups (*1*) prokaryotic cyanobacteria and (*2*) eukaryotic algae; the latter can be defined as photosynthetic eukaryotes/protists excluding land plants.

Members of the cyanobacteria have been treated both as bacteria and plants. They are also commonly referred to as *blue-green algae*, *blue-green bacteria*, *cyanophytes*, or *myxophytes*. Cyanobacteria possess some internal membranes, but lack membrane-bound nuclei, plastids, and mitochondria, and are in these respects bacteria-like. Other bacterial characteristics include their cell wall structure and chemistry and the presence of 70S ribosomes. Plant-like features include pigmentation (presence of chlorophyll-*a*) and the biochemistry of photosynthesis. Besides the liposoluble pigments chlorophyll and carotenoids, cyanobacteria also possess the hydrosoluble phycobiliproteins, which are organized as supramolecular structures, the phycobilisomes, located on the surface of the thylakoids. Several groups of cyanobacteria are capable of nitrogen fixation, often through the presence of specialized cells, called *heterocysts* (16). The classification of cyanobacteria into taxonomic categories below the level of the class has not been agreed upon. The traditional classification based on the cellular organization and the morphology of the organisms (17) is being challenged by bacteriologists (18), who point out that these criteria are, to a large extent, dependent on environmental conditions and do not reflect phylogenetic relationships upon which modern taxonomic schemes are, ideally, based.

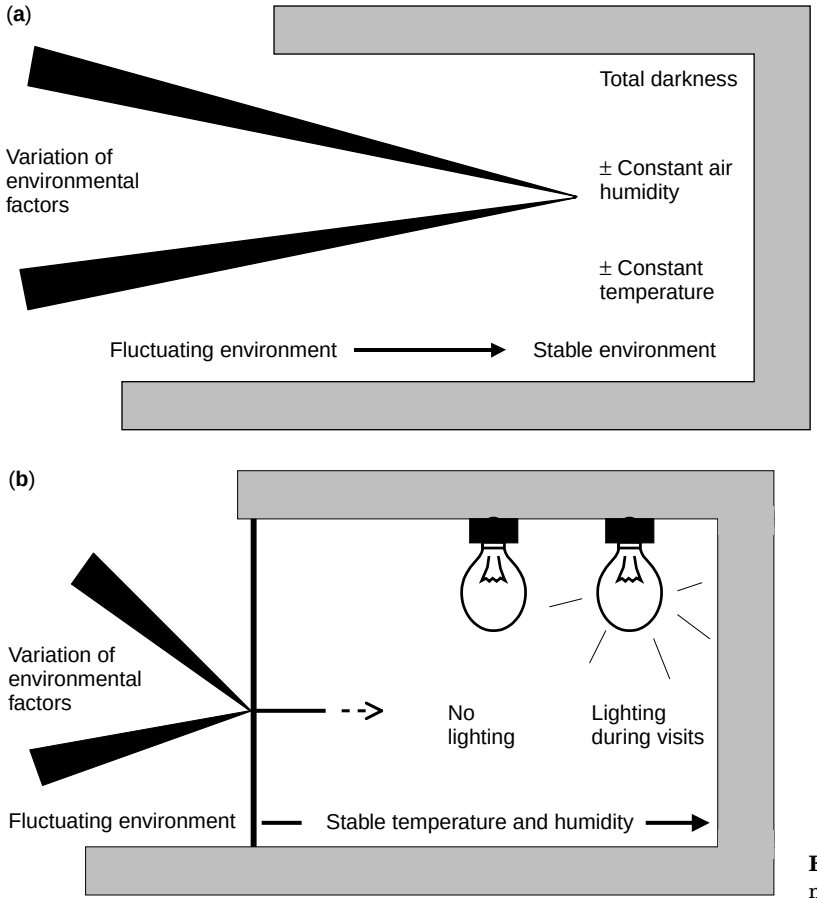

Figure 1. Variation of environmental factors in a natural cave (**a**) and a show cave closed by a door (**b**).

Eukaryotic algae are a phylogenetically heterogeneous group. The major lineages are the Chlorophyta (green algae), Rhodophyta (red algae), Glaucocystophyta, Euglenophyta (euglenoids), Chlorarachniophyta, Heterokontophyta (with the classes Phaeophyceae, Bacillariophyceae, Chrysophyceae, Xanthophyceae, etc.), Haptophyta, Cryptophyta, and the Dinophyta (19). These lineages mainly differ by their photosynthetic pigment composition, the nature and localization of their storage products, the ultrastructure of their plastids, and flagellar apparatus (Table 1).

The photoautrophic microflora of low-light environments mainly includes members of the following major groups: Cyanobacteria, Chlorophyta, Rhodophyta, and Heterokonta. Extensive growths are most frequently the result of cyanobacteria and green algae, which can also occur as lichen phycobionts but sometimes of Bacillariophyceae (diatoms), and less commonly of Xanthophyceae and Rhodophytes. Phaeophyceae, Haptophytes, Glaucocystophytes, Chlorarachniophytes, and Cryptophytes seem to be absent from terrestrial low-light environments. Under peculiar environmental conditions monospecific populations can occur, but more often different microbial photoautotrophs are associated with bacteria, fungi, mosses, and also higher plants.

The compilation of floristic lists is accomplished either by direct microscopic examination of samples scraped from the substrate in the least destructive way or by a number of indirect methods in which the presence of an alga in a sample is inferred following growth in an enriched medium. The establishment of unicyanobacterial/unialgal cultures is essential for the proper taxonomic treatment of many of the photosynthetic microorganisms. More commonly light microscopy, but also epifluorescence, scanning and transmission electron microscopy help in the identification of the taxa, as well as in the definition of the relationships among microorganisms and substrate.

About 350 taxa, of which about 58% belong to the cyanobacteria, 18% to Chlorophytes, and 18% to Heterokonts (especially diatoms) have been recorded from low-light environments, other groups are of marginal importance (10). In regard of these numbers, one might think that the photoautotrophic microflora of low-light habitats is abundant and diverse. The real diversity of the flora of low-light environments is, however, probably largely overestimated. Indeed, in many cases the floristic lists are based on the results from enrichment cultures; this often leads to the growth of organisms that only were accidentally present in the environment or as resting spores, thus falsifying the picture. The taxa recorded in the literature can be catalogued into one of the following three categories (10):

- *Troglobitic species:* these species are obligate cavernicoles and cannot survive outside of caves or other low-light environments.

Table 1. Diversity of Photoautotrophic Cave Microorganisms

Division (Common Name)	Distinguishing Features	Major Groups Present in Caves
Cyanobacteria/Cyanophyta (cyanobacteria, blue-green algae)	Prokaryotic; gram-negative cell walls; phycobilins; chlorophyll-*a*, (and -*b*); glycogen; free thylakoids with phycobilisomes	Chroococcales, Oscillatoriales, Nostocales, Stigonematales
Rhodophyta (red algae)	Chlorophyll-*a*, phycobilins; floridean starch; single thylakoids with phycobilisomes in chloroplast enveloped by two membranes, no flagella	
Chlorophyta (green algae)	Chlorophylls *a* and *b*; starch inside chloroplast; stacked thylakoids in chloroplast enveloped by two membranes	Chlorophyceae, Trebouxiophyceae, Ulvophyceae
Heterokontophyta	Chlorophylls *a* and generally *c*; chrysolaminarin; thylakoids grouped by three inside chloroplast enveloped by four membranes; heterokont	Bacillariophyceae (diatoms), Xanthophyceae (yellow-green algae), Chrysophyceae (golden algae), Eustigmatophyceae
Euglenophyta (euglenoids)	Chlorophylls *a* and *b*; paramylon; thylakoids grouped by three inside chloroplast enveloped by three membranes	Euglenophyceae
Dinophyta	Chlorophylls *a* and *c*; thylakoids grouped by three inside chloroplast enveloped by four membranes	Dinophyceae

- *Troglophilic species:* these species can grow and reproduce in low-light environments, but have their optimal development in other aerophytic habitats.
- *Trogloxenic species:* these species accidentally reach the low-light environment by air, seeping water, or other organisms; they are unable to develop in these environments and only survive as resting stages.

The last group is by far the largest group so that many of the species recorded from low-light environments are typically subaerial and ubiquitous. All Euglenophytes, Chrysophyceae, Dinoflagellates, and a large number of the recorded species of the other groups can be considered as trogloxenes (10).

Some species, such as the cyanobacteria *Loriella osteophila* Borzi, *Scytonema julianum* (Meneghini ex Frank) Richter, the red algae *Phragmonema sordidum* Zopf and *Cyanidium* cf. *caldarium* (Tilden) Geitler, and some widespread aerophytic diatom species can be considered as troglophiles.

Obligate cavernicolous species are very rare. At present only a few cyanobacteria, such as *Geitleria calcarea* Friedmann, *G. floridana* Friedmann, and *Herpyzonema pulverulentum* Hernandez-Mariné et Canals, are considered to be true troglobites. Other cyanobacteria may belong to this group, but their identification is doubtful because they are incompletely described, because they were probably misidentified (like *Atractella clavata* Serbanescu et Decu or *Ialomitzia cavernicola* Gruia initially described as cyanobacteria, but probably representing various stages of fungi) or because they were only found once (like *Symhyonema cavernicolum* Asencio, Aboal et Hoffmann, *Spelaeopogon cavarae* Borzi, *Oscillatoria rupicola* Hansgirg var. *cavernarum* Skuja, *Synechococcus sciophilus* Skuja, *Pleurochloris cavernicola* Skuja, *Chloromonas antrorum* Kostikov, *Caloneis borealis* Carter, *Cymbella diavola* Carter, *Navicula variolinea* Carter, *Navicula vula* Carter, and *Nitzschia disputata* Carter) so that it is difficult to assess their real ecological requirements.

The cave algae reach the environment as vegetative cells or spores by air (as shown by agar plate controls) and animals (insects, bats, etc.) (20). In show caves, transport by humans is of great importance, both for bringing in new spores or for the spreading of species already present. Many of the photosynthetic microorganisms recorded from caves are typical soil algae; they may be washed into the cave from the surface soil during heavy rain events. The introduced microorganisms are then subject to environmental selection, which is a product of the surrounding biological environment (species composition), climatological characters, and the geophysical nature of the substrate; only troglobitic or troglophilic taxa develop in this environment, the trogloxenes die or persist as resting stages.

SOME TROGLOPHILIC AND TROGLOBITIC PHOTOSYNTHETIC MICROORGANISMS

Cyanobacteria

***Geitleria calcarea* Friedmann.** *Geitleria calcarea* is a member of the Stigonematales lacking heterocysts. It is characterized by the presence of laterally and dichotomously branched filaments and by a characteristic calcite sheath (21–24). It is only known from limestone caves or other low-light habitats from tropical or warm temperate regions (Fig. 2) (25) in which it colonizes the deepest and least illuminated areas of the photic zone. It appears on the walls as a light gray, mold-like coat. A second species of the genus, *G. floridana* Friedmann (22), was described from caves in Florida in which it grows intermixed with filaments of *G. calcarea* and thus seems to have similar ecological requirements. The most obvious difference between the two species is size.

***Loriella* spp.** Like *Geitleria*, the genus *Loriella* Borzi (Stigonematales) is characterized by the presence of dichotomous branchings and by the presence of a calcified sheath; the two genera mainly differ by the presence (*Loriella*) or absence (*Geitleria*) of heterocysts. The genus was first recorded on various limestone substrates in low-light environments in Papua New Guinea (25) and recently rediscovered in Spanish caves (26) in which it forms almost monospecific populations or grows together with other cyanobacteria, especially *G. calcarea*.

Some other true-branching cyanobacteria were described from cave environments; these include *S. cavarae* Borzi, *H. pulverulentum* Hernandez-Mariné et Canals (27), and *Symphyonema cavernicolum* Asencio, Aboal et Hoffmann (28).

***Chroococcidiopsis kashaii* Friedmann.** *C. kashaii* (Chroococcales) forms a blue-green layer on the rock substratum, composed of microscopic clusters of cells that multiply by endospore formation. In soft substratum, it can penetrate a few hundred micrometers. Mature undivided cells are spherical, subspherical, or ellipsoid (29). It seems to tolerate substrata with high nitrate concentrations (30).

***S. julianum* (Menegini ex Frank) Richter.** *S. julianum* (Nostocales) is a heterocystous, false-branching cyanobacterium that is often observed on limestone substrates in low-light environments. It never colonizes areas of stone that are completely wetted, but high relative humidity is important for its growth. It is a calcifying cyanobacterium characterized by triradiate crystals (31–33); in dry parts the calcified sheath is often thin or even absent (7).

***Leptolyngbya* spp.** Narrow (<2 μm width) filamentous, nonheterocystous cyanobacteria of the genus *Leptolyngbya* Anagnostidis et Komárek (Oscillatoriales) often form the most abundant components of biofilms in some low-light environments. Many of these forms are very rich in phycoerythrin and are red (5,34,35).

Rhodophyta

***Cyanidium* sp.** The red alga *C. caldarium* (Tilden) Geitler is a typical representative of hot, acidic ecosystems. A morphologically similar alga has been reported, although rarely, from nonthermal, nonacidic habitats,

Figure 2. Geographic distribution of *Loriella* (dots) and *Geitleria* (squares).

especially from limestone caves (36). The cave *Cyanidium* is a bluish eukaryotic microalga, characterized by the presence of chlorophyll-*a*, phycocyanin, and allophycocyanin. It reproduces by autospores. The taxonomic relationship between the two organisms remains to be established.

***P. sordidum* Zopf.** The highly polymorphic bangioid red alga *P. sordidum* Zopf was first recorded in greenhouses (37). Its natural habitat may, however, be low-light habitats (38–40).

Bacillariophyceae (Diatoms)

Diatoms while perfectly adapted to aerophytic conditions seem to prefer humid conditions; low proportions of diatoms generally occur indeed in low-light environments of arid zones. Moisture is also the most important factor determining the distribution of diatoms in cavern systems (41,42). Although no troglobitic diatom species seems to be described so far, several diatom species are frequently observed in low-light environments; they are rarely dominant but grow generally mixed with other species in the biofilms. Among these species, *Diadesmis gallica* W. Smith, *Diadesmis contenta* (Grunow) D. G. Mann, *Orthoseira roeseana* (Rabenhorst) O'Meara, and *Hantzschia amphioxys* (Ehrenberg) Grunow can be considered as troglophilic (4,11).

Chlorophyta

Although quite a number of green algae were recorded from low-light environments, most of the recorded taxa can be considered as trogloxenes and are characteristic of other terrestrial environments. One of the exceptions is the troglophilic genus *Stichococcus* Nägeli that often forms almost monospecific thin and dry green biofilms in low-light environments.

Flora of the Lamps

A remarkable ruderal vegetation is formed by algae living in the vicinity of artificial light sources in caves (Fig. 3b). Microbial photoautotrophs generally are the pioneering organisms around lamps; with time they are followed by mosses and ferns (Fig. 4). Common atmophytic and freshwater microbial photoautotrophs often form a luxuriant vegetation here (1–3). Recorded cyanobacteria in this "flora of the lamps" are *Gloeocapsa alpina* Nägeli, *G. dermochroa* Nägeli, *G. sanguinea* (Agardh) Kützing, *Tolypothrix rupestris* Wolle, *Nostoc microscopicum* Carmichael, *Chroococcus varius* A. Braun, *Chlorogloea microcystoides* Geitler, *Aphanocapsa fuscolutea* Hansgirg, *A. castagnei* (Brébisson) Rabenhorst, and *A. saxicola* Nägeli. Common green algae are *Desmococcus olivaceus* (Pers. ex Ach.) Laundon, *Stichococcus bacillaris* Nägeli, *Gloeocystis rupestris* (Lyngbye) Rabenhorst, *G. vesiculosa* Nägeli, *Chlorella vulgaris* Beyerinck, and *Scotiella nivalis* (Shuttleworth) Fritsch. In this particular niche, cyanobacteria sometimes occur in spherical aggregates, up to 1 cm in diameter, almost exclusively composed of *G. alpina* Nägeli and *C. varius* A. Braun and in another of *A. fuscolutea* Hansgirg and *C. microcystoides* Geitler.

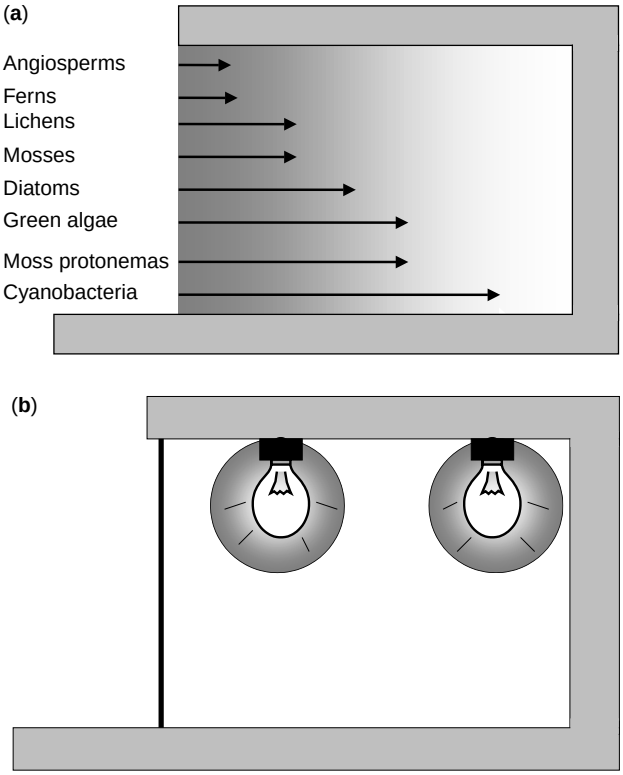

Figure 3. Distribution of phototrophs in a natural cave (**a**) and a show cave closed by a door (**b**). (The gray zone indicates photoautotrophic growth.)

DISTRIBUTION OF PHOTOAUTOTROPHS IN CAVES

Photosynthetic microorganisms living in the neighboring outside areas generally colonize the better illuminated wall surfaces near the entrance of the caves; towards the depth of the caves the microbial photoautotrophic vegetation becomes less dense and less diverse (43,44). Evidently, the depletion of species composition and of taxonomic spectrum is mainly caused by the reduction of intensity of illumination. Very few species are able to grow along the whole gradient of conditions. With decreasing light intensity, mosses and lichens generally disappear first (Fig. 3a). Extremely low-light irradiance can support the growth of photosynthetic communities mainly formed of cyanobacteria and chlorophytes (and moss protonema that can thrive at lower light intensities than the leaved gametophytes) (44,45). In most cases, the microbial photosynthetic communities at the dim end of the lighted zone are exclusively built by phycobiliprotein-rich cyanobacteria, many of which are able to regulate pigment composition.

PHYSIOLOGICAL ECOLOGY

Few species of photoautotrophs are able to withstand the low irradiance available in these environments, but data

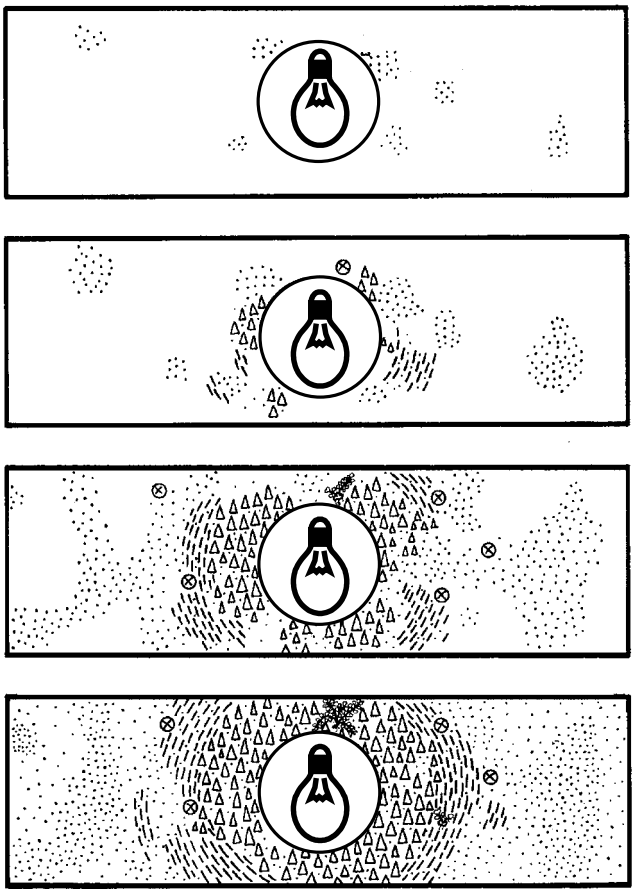

Figure 4. Ontogenesis of a "lamp vegetation" (adapted from Ref. 3). The dots indicate microbial photoautotrophs, the other symbols representing mosses and ferns.

on the biology and the ecophysiological adaptations of the microbial photoautotrophs to this particular extreme terrestrial environment are scarce. Some reports in the literature deal with photosynthetic microorganisms isolated in culture from samples taken from totally dark parts of caves. A large variety of mostly common aquatic and terrestrial cyanobacteria and eukaryotic microalgae was identified in these isolates, but no visible growth of these species was recorded in situ. Although the methodology of these studies may be beyond criticism, the presence of viable cells is not a sufficient indication of a cave vegetation. The cyanobacterium *G. calcarea*, one of the microbial cave photoautotrophs occurring in the deepest parts of the caves, lives at an irradiance that is generally below 5 $\mu mol\, m^{-2}\, s^{-1}$ (7). The photoautotrophs of low-light environments keep their color in contrast to animals, which are often discolored in cave habitats. Hypothetical adaptive characters that allow them to thrive under the extreme low photon flux densities are: (*1*) the possibility of ultrastructural changes (increase in photosynthetic membranes) as a consequence of low-light intensities (5,34,46) (this has been shown for cyanobacteria, but not for green algae); (*2*) the presence of large antenna owing to the presence of phycobilisomes in cyanobacteria, which may allow these organisms to capture the few photons available. The fact that at the lowest light intensities, eukaryotic microalgae cannot compete with cyanobacteria is generally explained by the lower maintenance requirements of the latter and their ability to adapt to low light (47–49).

The adhesion of the photoautotrophic microorganisms to stone surfaces is based on the production and secretion at the cell surface of mucilaginous substances providing the cells with a high degree of hydrophobicity. The exopolymeric substances secreted by these microorganisms vary by their consistency and thickness. They are commonly called *glycocalyx*, *capsule*, *sheath*, or *envelope*. The formation of these extracellular investments represents one of the most relevant steps in the early stage of the microbial colonization by helping to stick the cells to the substratum. Moreover, the polysaccharides secreted by the photosynthetic microorganisms contribute to the formation of firm microbial biofilms (44) supporting and stimulating the growth of other microorganisms, especially of heterotrophic bacteria and fungi (50), always present in the same microhabitats colonized by cyanobacteria and microalgae. Microbial cells attached to a surface can develop into a highly differentiated community in which photo- and heterotrophic organisms are enmeshed in a polysaccharide matrix that mediates intercellular communication as well as the interactions with the substratum. The often anionic nature of the exopolymers strongly absorbs cations and dissolves organic molecules from the underlying minerals. In these peculiar habitats, as in almost all terrestrial environments, and in contrast to aquatic systems, gram-positive bacteria appear to predominate as epiphytes (50). The release of inorganic and organic metabolites by microalgae determines interactions either with the substrate, which can be corroded, or with bacterial and fungal populations that can utilize these compounds. The production of these exocellular, highly hygroscopic, polymeric substances also ensures the survival of the colonizing organisms in adverse climatic conditions.

ECONOMIC IMPORTANCE: "GREEN SICKNESS" OF CAVES

Caves and other low-light environments are not only of interest to speleologists and scientists in general, but in many cases, they are also positively involved in the tourism and are thus of economic relevance. This is especially the case for show caves, which present interesting rock formations or are of archeological interest, as well as for many man-made low-light environments presenting various works of art. The photoautotrophic microorganisms are extremely successful in colonizing illuminated areas in these low-light environments. The reasons (52) are that these often hypogean environments are characterized by:

- A usually moist and wet atmosphere, with a relative humidity close to 100%;
- An often relatively constant illumination provided from the artificial lighting;
- Water filtering through the formations that are saturated with CO_3^{2-} and often also contains high concentrations of nutrients;

- High levels of carbon dioxide present in the atmosphere that dissolves readily into the water; and
- Uniform temperatures prevailing all over the year.

Besides the physical and chemical weathering caused by environmental pollutants, the presence of micro- and macroorganisms is a major factor for the deterioration of this cultural heritage. Indeed, once these low-light environments start to be illuminated by any natural or artificial light source, eukaryotic algae and cyanobacteria, lichens, mosses, and ferns proliferate, causing aesthetic, physical, and chemical changes. The development of photoautotrophs discolors the stalactites and stalagmites of karst formations, giving them a dirty and unsightly appearance (52). Besides stimulating the growth of the photoautotrophs, the illumination in show caves also allows the photoautotrophs to spread to areas in which they previously could not grow. Furthermore, the movement of the visitors through the caves causes a rapid spreading of the microorganisms. The concomitant increase in temperature also favors a rapid growth. An important secondary problem is carbon dioxide solution, dissolution and the impact thereof on the carbonate deposits. Microbial growth can cause the erosion of formations leading to irreversible damage. This is mainly because of their uptake of carbon dioxide and the dissolving of calcium carbonate. With increasing numbers of visitors, a degradation of the cave environs thus often becomes evident. Moderate and low-energy caves are especially vulnerable to the threats posed by tourism. Since the famous studies on the "maladie verte" (green sickness) (51) that affected the prehistoric mural paintings in the caves of Lascaux in France, the presence of photosynthetic microorganisms has been reported by a number of different authors, on walls and paintings in caves, churches, or more generally in confined low-light environments characterized by high humidity. Their conservation constitutes a problem that has nowadays assumed a worldwide interest.

Changes in the composition and luxury of the subterranean biofilms may also result from changes in the land use above the hypogean environment; this may lead for example to an increase in the nutrient concentrations in the water (owing to increased use of mineral fertilizers) or to an impact on the water cycle, decreasing or increasing the amount of dripping water.

The damaging effects on stones are produced by epi-, endo- and chasmoendolithic species. Aesthetic damage by photosynthetic microorganisms is caused by the development of colored biological patinas. Besides the production of aesthetic damage, the established microbial biofilm communities can be associated with different biodeteriorative effects.

The presence of microbial biofilms, which adhere firmly to stone surfaces, indeed increases the surface biosusceptibility to deterioration. A synergic biodeteriorative effect on stone surfaces is possibly achieved by concomitant growth of photoautotrophic and heterotrophic populations (50). Bacteria and fungi, in fact, are able to use the organic matter produced by the photoautotrophs. The metabolic activities (for example the release of acidic compounds) of the microbial consortia, and the polysaccharide matter of the biofilms contribute to chemical stone deterioration by solubilization, leaching, and chelating processes. A marked softening of the substratum and the progressive deepening of the biological growth in the layers beneath the surface is owing to the mobilization of elements and can thus cause stone surface weathering, increasing the loss of stone particles from the crystalline structure (50). The extracellular mucilages are also prone to increase the disruption damage by physical contraction and expansion of the polysaccharides.

As shown by pH microelectrode measurements in biofilms (47), both acidification owing to respiration during the dark period and alcalinization as a consequence of photosynthetic activity during lighting may occur to a sufficient extent to induce dissolution and reprecipitation of mineral compounds, especially in calcareous substrates. Intensive photosynthesis leads to a sharp increase of pH owing to the consumption of carbon dioxide and HCO_3^- and the consequent increase of CO_3^{2-}. This fact is relevant in changing the solubility of elements in the mineral substrate and thus influencing the interrelationship among resident microorganisms and lithic substrata with a subsequent increase of stone damage.

Mineral precipitation in the form of crystals has been observed in some cyanobacterial species, for example, *S. julianum* (7,31–33). The precipitation of calcium carbonate on the surface of the polysaccharide sheath of cyanobacteria in the form of calcite crystals seems to be promoted by live bacteria. Carbonate precipitation and/or solubilization are major factors in the biotransformation of calcareous substrata. After the death of calcifying microorganisms, the calcified filaments may remain on the substratum in which they can accumulate, forming a calcite layer. Deposition of successive layers, together with the processes of dissolution and precipitation of calcite, can result in the formation of whitish mineral crusts with a typical layering for stromatolithes (7).

In these low-light environments some dangerous species such as *Prototheca* sp. can sometimes occur in high concentrations among other populations of microorganisms. This genus seems to cause conjunctivitis and skin disease like protothecose for archeologists who permanently work in such environments. Furthermore, data exist that in low-light conditions, the destruction of abundant photoautotrophic biomasses can produce carcinogenic nitrosamines, compounds, which are immediately destroyed by light but which remain stable and can accumulate in low-light environments.

The eradication of the photosynthetic microorganisms living on stony substrates in low-light environments and the control of their development, necessitate a multilevel approach, involving preventative measures and the treatment of existing colonized areas (52). Nondestructive methods are especially needed in the case of valuable archeological and artistic surfaces. Preventative measures mainly concern the lighting used to illuminate the caves and how it is operated. Every effort should be taken to limit illumination of any area as much as possible.

The duration of illumination should be limited as far as possible by having alternate lamps illuminating the walkways over time, by using motion detectors and varying display illumination. Where areas need to be illuminated for longer periods of time colored lighting should be used and especially photosynthetically inactive wavelengths such as green light (white, reds, and blues should be avoided) (52). For treating colonized areas, spraying with algicides, antibiotics, hydrogen peroxide with subsequent washing with low pressure, application of gases and irradiation by UV germicide lamps have been applied (52–54).

To preserve these unique environments, it is thus necessary to determine a balance between recreational and commercial expectations versus the conservation and preservation of the cave environments. As a rule, the illumination should be reduced to a minimum and the number of visitors allowed should be such that, once the visitors leave these confined environments, parameters such as temperature and atmospheric carbon dioxide return to the same level prior to the visitors' entry (54).

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CELL VIABILITY, METHODOLOGY FOR.

See LASER SCANNING MICROSCOPY IN COMBINATION WITH FLUORESCENCE TECHNIQUES FOR BIOFILM STUDY

CHAPERONE AND CHAPERONINS.

See STRESS RESPONSE IN ARCHAEA; STRESS RESPONSE IN BACTERIA: HEAT SHOCK

CHEMICAL WEAPONS, BIODEGRADATION OF

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The U.S. Army has custody of chemical weapons (CW) containing nerve and blister (vesicant) agents located in eight different sites in the continental United States and at Johnston Island, a small island in the Pacific Ocean. The stockpile, which totals approximately 32,000 tons, consists primarily of three agents, the nerve agents GB (sarin) and VX, and the blister agent HD (sulfur mustard). The structures of these agents are shown in Figure 1. These agents are stored in one-ton bulk steel containers and in munitions such as bombs, rockets, artillery shells, and mines (Fig. 2). All the munitions and agents are between 25 and 50 years old. In addition, U.S. Army officials have identified 215 sites in 33 states that are likely to contain buried chemical weapons or to be contaminated with chemical agents (1). Besides the United States, several other nations such as the independent republics of the former Soviet Union also face cleanup tasks similar to that of the United States. The list of CW agents include organophosphorus (OP) nerve gases such as VX, GA (tabun), GB, and GD (soman); blistering agents such as HD and HT (a mixture of HD and agent T); and the organoarsenical agents Lewisite (L) and Adamsite (DA) (Fig. 1).

The Chemical Stockpile Disposal Program in the United States was initiated in 1985, when the Department of Defense (DOD) targeted to destroy at least 90% of the CW stockpile by September 30, 1994. As the program proceeded, the pace was slower than anticipated, and the target date for completion was revised several times. In 1988, Congress extended the completion date to 1997, and in 1990 to July 1999.

International events soon had an impact on this program. In 1990, the United States and the former Soviet

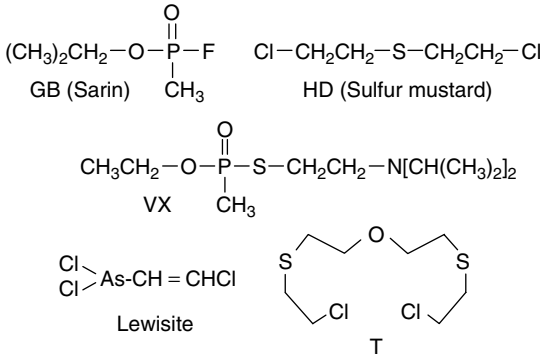

Figure 1. Primary agents in U.S. and Russian chemical weapons stockpiles.

Figure 2. Stockpile of sulfur mustard at the Aberdeen Proving Ground, Maryland.

Union signed a memorandum of agreement to cease chemical weapons production, dispose of inventories, share disposal technology, and develop inspection procedures. In addition, on September 3, 1992, the Conference on Disarmament approved the Chemical Weapons Convention (CWC), signed between January 13 and 15, 1993, by the United States and several other nations, forbidding the development, production, stockpiling, or use of chemical weapons. Upon ratification, the deadline (which supersedes previous deadlines) for stockpile destruction will be December 31, 2004, or later. In the case of the United States, the ratification of the CWC, which was on April 30, 1997, made the deadline April 30, 2007.

In the United States, the army has had an ongoing program for disposal of surplus and obsolete weapons. Before 1969, CW munitions were disposed of by open-pit burning, evaporative "atmospheric dilution," burial, and deep ocean dumping (2). The Marine Protection, Research & Sanctuaries Act of 1972, prohibited the ocean disposal of agents. In the 1970s, the principal methods employed for agent destruction were neutralization via alkaline hydrolysis for GB and incineration for HD. Because of problems with GB alkaline hydrolysis, incineration was selected as the preferred technology for destruction of obsolete components of the CW stockpile in 1982. Currently, two full-scale CW incinerator facilities are available in the Marshall Islands and in Utah.

The initial plan to use incineration for the destruction of the CW stockpiles has stalled due to considerable local and national opposition by citizens, environmental groups, and politicians. As a result, the army was directed by Congress to report on alternative technologies for CW stockpile destruction. The army requested the National Research Council (NRC) Committee on Review and Evaluation of the Army Chemical Stockpile Disposal Program to reevaluate the status of incineration. An additional NRC committee on Alternative Chemical Demilitarization Technologies was formed to examine alternatives for CW disposal. On the basis of the two NRC studies (3,4), alternative technologies were proposed. With the rapid progress in biotechnology during the last decade, one possible solution is to develop biological strategies for the large-scale decontamination of CW agents. The focus

of this article is to provide a summary of the recent developments in the area of biodegradation of chemical weapons. Direct biodegradation and the use of chemical neutralization followed by biodegradation are among the technologies under investigation.

MUSTARD GAS

Sulfur mustard, often referred to as "mustard gas," is 2,2′-dichlorodiethylsulfide (Chemical Abstract Services number 505-60-2, military designation, H or HD). Mustard is an oily liquid with a freezing point of approximately 14 °C and a boiling point of 217 °C. Because mustard is nearly insoluble in water, it is very persistent in the environment and can contaminate soils and surfaces for long periods. Mustard affects the eyes and lungs and blisters the skin. It causes severe chemical burns and painful blisters, and is lethal at high dosages, especially if inhaled. Symptoms are generally delayed for several hours after exposure (5–7). It was developed in Germany and used extensively during World War I during which it caused 80% of the gas casualties. It was also used by Italy in Ethiopia in 1936, by Japan against China in World War II, by Egypt in Yemen in the early 1960s, and by Iraq against Iran during the 1980s.

Because the blistering agent HD reacts with microbial proteins and is highly toxic to microbial cells, it is not amenable to direct biodegradation. Therefore, most studies have been focusing on combined chemical and biological degradation. The advantages of using hydrolysis as a neutralization reaction preceding biodegradation of HD include an aqueous medium, complete dechlorination, and products (primarily alcohols) that are amenable to biodegradation. Also, the hydrolysis reaction does not add any additional carbon that would require subsequent biological removal. Alkaline hydrolysis has been previously utilized for the destruction of Canadian HD stockpiles (8). Biodegradation has a widespread application in municipal and industrial wastewater treatment plants, is conducted at ambient temperatures, and offers a favorable mass balance for the process.

The mechanism of HD hydrolysis has been the subject of considerable study beginning just after World War I (9–15). It has been demonstrated that HD reacts through a series of sulfonium ion intermediates to produce thiodiglycol as shown in Figure 3. However, additional ether or thioether products can also form depending on the actual conditions of the reaction. Because HD is insoluble in water, the reaction involves hydrolysis at the water–organic interface with the products then dissolving in the water (14). Therefore, agitation during hydrolysis is a critical factor in carrying out this reaction. Both the rate of mass-transfer and the rate of hydrolysis can be accelerated at elevated temperatures.

The efficiency of conversion of HD to thiodiglycol (TDG) has been shown to be dependent on temperature and whether the base used for neutralizing the acid is added before or after the reaction (16). Elevating the temperature of alkaline hydrolysis reaction from 30 to 90 °C increased the TDG concentration from 62 to 84%. Running the hydrolysis in hot water alone (with base neutralization

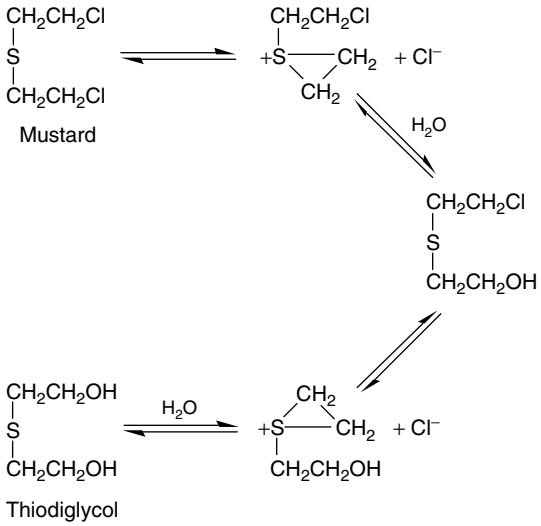

Figure 3. Hydrolysis mechanism for HD.

after cooling) further increased the TDG level to more than 95%. The hydrolysis reaction in either caustic or water was completed, with HD not being observed within a detection limit of 160 ppb.

Biodegradation of TDG

TDG, the primary hydrolysis product of HD, is a water miscible, nontoxic liquid. However, it is listed as a Schedule Two compound (chemical weapon precursor) in the CWC and therefore must be destroyed as part of any demilitarization processes. Earlier studies with *Alcaligenes* ssp. *A. xylosoxydans* demonstrated that TDG could serve as sole carbon and energy source for microbial growth (17).

The actual pathways by which TDG is metabolized have not been the subject of extensive investigation. Preliminary studies (18) with *A. xylosoxydans* indicated that this organism oxidized TDG by means of sequential NAD-dependent butanol dehydrogenase reactions to give thiodiglycolic acid (TDGA) as shown in Figure 4. NMR analysis of the cell-free dehydrogenase reactions yielded two oxidation products from TDG: hydroxyethylthioacetic acid (HETA) and TDGA. The identity of these products was confirmed by mass spectrometry analysis. Therefore, it appeared that in this organism, the biodegradation of TDG proceeds via an oxidative pathway that first yields a monoacid product and then the diacid product. Recent studies have confirmed that HETA and TDGA are intermediates in the TDG pathway (19). The fate of the TDGA has not been determined.

In previous studies with microbial consortia and in earlier work with *Pseudomonas picketii*, the transient appearance of thiodiglycol sulfoxide was occasionally observed (20). Whether the sulfoxide is an actual metabolic intermediate in the degradation of TDG or the product of a side reaction is not known. However, by whatever means the sulfoxide is produced, it is the subject of further degradation as it is not found in the final effluent.

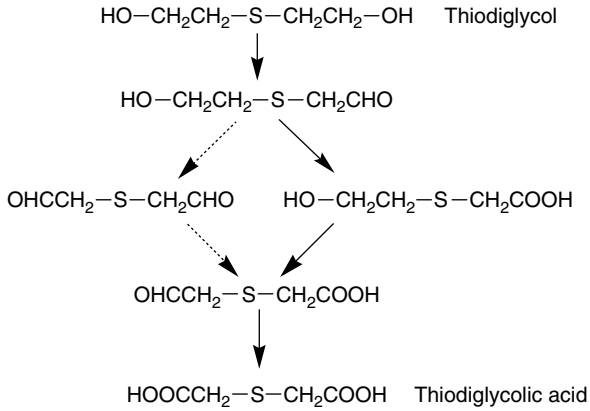

Figure 4. Thiodiglycol metabolism in *Alcaligenes* (18).

Large-Scale Biodegradation of TGD

For the biodegradation of hydrolyzed HD (21–22), the use of sequencing batch reactors (SBRs) was selected (Fig. 5). SBRs offer several important operational advantages for the treatment of chemical wastes (23). They are very efficient. The SBR is composed of a continuous flow stirred tank reactor (CFSTR) followed by a plug flow reactor, offering the ideal volumetric reactor configuration for unsteady state activated sludge systems. They operate in the batch mode that not only offers a kinetic advantage over conventional CFSTRs (therefore smaller in size) but also permit batch analysis and toxicity testing for hazardous waste treatment before discharge. They use a single tank for both treatment and settling; therefore, no secondary clarifier is required. Finally, they are robust and flexible. SBRs are intentionally operated over a rang of substrate concentrations, pH conditions, and oxygen concentrations, thereby allowing selection of a very diverse and robust population of microorganisms. Operational strategies can also be varied to accomplish carbon, nitrogen, and/or phosphorus removal.

The reactor cycle (24-hour) used in these studies normally consists of a four-hour aerated fill, an 18-hour aerated react, a 1.5-hour settle, and a 0.5 hour draw. The total organic carbon (TOC) loading in the feed was 3,400 to 3,800 mg/L. The hydraulic residence time (HRT) was 16 days and the original biomass for the SBRs was activated sludge from the Back River Waste Water Treatment Plant (Baltimore, MD). Trace metals were provided in a modified Wolin Salts Solution (24) in addition to NH_4Cl and KH_2PO_4 as nitrogen, potassium, and phosphorus sources. The hydrolyzed HD was the sole source of carbon and sulfur. The SBR was operated at a pH of between 6.5 and 8.5. Buffering was primarily accomplished through the addition of $NaHCO_3$ to the feed, although a NaOH solution was occasionally used on demand when the pH dropped below 6.5.

In SBR with caustic-hydrolyzed HD, the TDG was typically removed to greater than 99%; however, the effluent TOC showed a gradual increase until it reached approximately 1,000 mg/L. At this point, the feed was changed to the water-hydrolyzed HD. After 146 days of operation, the reactor had significantly lower levels of effluent TOC and suspended solids. MICROTOX analysis of the effluent showed it to be nontoxic (100% effluent produced no changes in the luminescence levels of the test bacteria). In addition, toxicity characteristic leachate protocol (TCLP) analysis of the effluent found none of the restricted organics and metals at levels of concern. A mass balance calculated per ton of HD is shown in Table 1

On the basis of results at the laboratory and bench scale, the neutralization-biodegradation method was selected by the army for the destruction of the HD stockpile at Aberdeen Proving Ground. A U.S. $86 million demonstration unit was approved and is in the permitting process. Site clearing for the demonstration unit and full-size facility has been initiated. The official groundbreaking ceremony for the facility was held on June 26, 1999. A U.S. $306 million contract has been awarded for the construction, operation, and dismantling of the full-size facility. The goal of the program is to meet the April 30, 2007 deadline for destruction as mandated by the CWC.

With the success obtained in the biodegradation of HD, a related material called HT was examined (25). HT is a mixture of 60 weight percentage of HD and 40 weight percentage of an analog bis-(2-(2-chloroethylthio)ethyl)ether or T. As with HD, treatment of HT with hot water hydrolyzes it into a mixture of TDG and bis-(2-(2-hydroxyethylthio)ethyl)ether (T-OH). Because the potential biodegradation of T-OH was not known, a number of studies were undertaken to determine if it could be

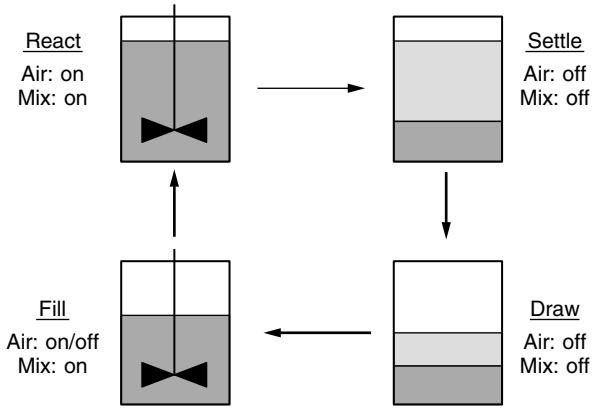

Figure 5. SBR operational cycle.

Table 1. Summary of Process SBR Mass Balance (16)

Input (tons)		Output (tons)	
HD	1.00	CO_2	0.55
NaOH	1.11	H_2O*	0.60
O_2 (air)	0.93	Biomass**	0.30
NH_4Cl	0.16	Na_2SO_4	0.88
KH_2PO_4	0.04	NaCl	0.90
		KCl	0.01
Total Input:	3.24	Total Output:	3.24

*Represents only the water actually produced from the oxidation processes.
**Dry weight of biomass.

further hydrolyzed to TDG (approx. 20% is hydrolyzed to TDG by the hot water system). It was found that a considerable portion of the remaining T-OH is converted to TDG by conducting the hydrolysis in the presence of HBr or HI. However, it was later determined that this additional hydrolysis was not necessary to achieve biodegradation. SBR studies demonstrated that the water-hydrolyzed HT (after neutralization) could be efficiently biodegraded (comparable to hydrolyzed HD) and that the effluent from the systems has little (if any) toxicity.

Immobilized Cell Bioreactors

All the studies described earlier involved the use of SBRs for the biodegradation of the hydrolyzed agents. An alternative approach is the use of biofilm systems in which the microorganisms grow attached to a solid support. AlliedSignal Environmental Systems (part of AlliedSignal, Inc.) owns and operates mobile bioreactor units for on-site neutralization of various contaminated waste streams. These reactors, commonly called immobilized cell bioreactors (ICBs), use a stationary support of polyurethane foam blocks to grow the biomass on. Cylindrical polypropylene spacers are also used to allow for more efficient aeration and liquid circulation. AlliedSignal provided laboratory-scale ICBs for evaluation with hydrolyzed HD and VX. These units had a total volume of approximately 1 liter and a working volume of 600 to 800 mL. Studies were undertaken with the ICBs and both HD and VX hydrolysates (25).

An ICB was seeded with biomass from a HD SBR and allowed to acclimate in a batch feed mode. The feed for the HD-ICB consisted of (per liter): HD hydrolysate (3.8%), 333 ml; NH_4Cl, 1.2 g; KH_2PO_4, 0.35 g; Wolin salt solution (24), 10 ml; and $NaHCO_3$, 5.0 g (for pH control). The feed was adjusted to a final pH of 7.5 with HCl prior to use. In the initial batch operations, a recirculating loop and the addition of NaOH provided additional pH control. Once sufficient biomass had been produced and continuous operations were begun, the pH was controlled entirely by adjusting the level of sodium bicarbonate in the feed. Pumping air through a frittered glass disk at the base of the ICB provided continuous aeration.

The initial working volume of the HD-ICB was 750 mL. As the biomass developed, the working volume decreased to the point that when the studies were concluded, it was down to an estimated 287 mL. The feed rate to the ICB ranged from 100 to 300 mL per day, giving a hydraulic residence time (HRT) of 1.5 to 7.5 days. Very efficient mineralization of the TDG was achieved as long as the HRT was kept at greater than three days. In the laboratory unit, higher feed rates (and lower HRT) resulted in the biomass, which nearly plugged up the reactor. This resulted in anoxic or anaerobic zones where biodegradation of the TDG ceased. In full-scale units (using larger foam blocks, spacers, higher velocity liquid flow, and aeration) this plugging would not occur. Under all conditions in this study, the biomass stayed firmly attached to the support. The total suspended solids (TSS) in the reactor effluent were generally at or below standard detection limits. Overall, once established, the HD-ICB system was very efficient and required little daily maintenance. It would therefore lend itself to pump and treat operations or continuous-flow waste streams. Further studies will be necessary to determine whether biomass buildup would reach a point where it would hinder performance, and if the same level of TDG mineralization could be maintained over long periods.

Cryoimmobilized cultures of *A. xylosoxidans* have also been investigated for the hydrolysis of TDG. More than 150 mM TDG was metabolized within 24 hours and 100% of the initial activity can be maintained after four months of continuous use (26).

OP NERVE AGENTS

German scientists first developed OP nerve agents over half a century ago while conducting research on organophosphate insecticides (27). During World War II and warfare competition afterwards, the world saw a rapid proliferation of such agents (Table 2). The use of these agents has resulted in large numbers of casualties. During the past 15 years, Iraqi troops employed nerve gas against Iranian soldiers and Iraqi Kurdish civilians and nerve gases were considered a major threat to allied personnel during the Persian Gulf War. Normally, only combat personnel would be exposed to nerve agents, although other people living close to the battle fields may get intoxicated by the soil and groundwater or drinking water. However, the use of sarin against Japanese civilians during a terrorist attack on the Tokyo subway system in 1995, resulting in more than 5,500 casualties (28), alerts us about a new and much more dangerous type of exposure (29–30).

These OP nerve agents are highly toxic and inhibit acetylcholinesterase (AchE) in the central nervous system synapses of most animals including humans (31). Exposure to organophosphates results in acetylcholine (ACh) accumulation, which interferes with muscular responses and the function of vital organs. High dosages of organophosphates can prove fatal. Chronic or prolonged exposure to sublethal doses of organophosphates may result in delayed cholinergic toxicity and neurotoxicity (32). The classic symptoms of nerve agent poisoning

Table 2. The Development and Uses of OP Nerve Agents

1854	DE: TEPP synthesized
1934	DE: fluorine atom incorporated into OPs
1937	DE: tabun and sarin synthesized
1940	DE: production capability reached 1,000 tons/month
	UK: dimethyl and diethyl phosphorofluoridate synthesized
1941	UK: DFP synthesized
1944	DE: soman synthesized
After 1945	Greater effort put in synthesizing further warfare Ops
Late 1950s	New series of nerve agents: V agents
1960–1967	USA: 4,000–5,000 tons of VX produced
1986	Iraq versus Iran: probable use of nerve agents
1994	Japan: sarin used on civilian population in Matsumoto and Tokyo

DE: Germany.

include salivation, lacrimination, urination, and defecation. Miosis and bradycardia are also common (27). In the disaster of Tokyo subway incidence, patients with severe poisoning showed greatly reduced consciousness levels, miosis, marked fasciculation, flushing, tachycardia, raised blood pressure, respiratory distress, and flaccid paralysis; whereas those with mild poisoning complained of headaches, dizziness, nausea, chest discomfort, abdominal cramps, and showed marked miosis. All are typical symptoms caused by sarin gas intoxication (29).

The progression of nerve agent toxicity, in character and speed, is not only dose-dependent but also dependent on the route of entry into the body. In the vapor phase, they are absorbed rapidly across the respiratory epithelium. They are also absorbed across the cornea where by a local effect they produce miosis. As liquids, they pass relatively rapidly across the skin and mucous membranes without damaging them. They may also be absorbed from the gut and, when carried into wounds, are rapidly distributed throughout the body. The absorption of nerve agents from air taken into the respiratory tract is almost 90% (27).

Currently, fielded decontamination solutions, such as DS2 and bleach, are quite effective in degrading OP-based CW agents (33). However, they are corrosive in nature and result in hazardous waste. Microbial- or enzyme-based decontamination systems not only provide rapid removal of CW agents but are also environmentally safe and noncorrosive in nature. The use of enzymes has considerable potential for decontamination of equipment, vehicles, large fixed sites, and cleanup operations that might result from a terrorist attack.

Enzymatic Degradation of OP CW Agents

Mazur was the first to describe the enzyme hydrolysis of the phosphorous-fluorine bond of diisopropyl fluorophosphates (DFP) by a crude preparation of rabbit tissue (34). Subsequently, several different enzymes such as paraoxonases, DFPase, and somanases from sources as diverse as hog kidney, rabbit, human, and squid have been shown to detoxify DFP or other related OP nerve agents (35–37). However, in most cases, only crude enzyme extracts were used to demonstrate the hydrolysis of these OP CW agents.

In the early 1980s, several studies were initiated to search for microorganisms that could degrade OP CW agents. The initial focus was on the microorganisms that are capable of hydrolyzing related OP insecticides. Among a range of enzymes that hydrolyze OP insecticides, only organophosphorus hydrolase (OPH) isolated from *Pseudomonas diminuta* (38) and *Flavobacterium* sp. (39) is active in degrading nerve agents, if at all (40–41). Cleavage of the P–F, P–S or P–CN bonds render the OP agents relatively nontoxic as the bond broken is that involved in the irreversible inhibition of acetylcholinesterase (42). OPH is also the only well-characterized OP-degrading enzyme, which has been shown to hydrolyze the P–S bond of OP agents (43–44).

The gene encoding OPH, *opd*, was isolated from both *P. diminuta* and *Flavobacterium* sp. (45). Because the economics of culturing the native soil bacteria is not very attractive as a result of the slow specific growth rate, the *opd* gene has been cloned into *Escherichia coli* (46), insect cell (fall armyworm) (47), *Streptomyces* (48), and soil fungus (49) to explore the possibility of expression in alternative hosts. To date, *E. coli* has been used most extensively for expression. The kinetic properties of the recombinant OPH are virtually the same as the native form (46,50).

Recombinant OPH is a homodimer with equal active sites at the C-terminus of each monomer (51–52). Each active site has a binuclear metal center, and different metal-associated forms of OPH with Zn^{2+} or Co^{2+} demonstrated significantly different hydrolytic capabilities (53); the activity of the Co-form is consistently five- to tenfolds greater (54). OPH has a broad substrate range and has the ability to hydrolyze several insecticides (55), CW analogs (41) and the actual CW agents (40,43). Catalytic specificities (k_{cat}/K_m) vary from diffusion limited for paraoxon to more than eight orders of magnitude lower for acephate (55). The rate of hydrolysis for members of OP CW agents, including VX and sarin, are slow compared to hydrolysis of insecticides such as parathion and coumaphos (56). A comparison of specificities for different substrates is shown in Table 3 (57). Practical applications of OPH-based technologies for the biodegradation of CW agents would necessitate an improvement not only in the catalytic rate but also OPH availability, shelf life, and stability.

Another interesting enzyme that is receiving more attention in recent years for the biodegradation of CW agents belongs to a class of enzymes known as organophosphorus acid anhydrolases (OPAA) (58). As shown in Table 4, the OPAA enzymes are capable of catalytically hydrolyzing a wide variety of substrates, the fluoride-containing CW agents such as soman, sarin, and GF, and also the cyanide containing agent tabun. A number of *Alteromonas* sp., containing high levels of OPAA activity, has been isolated (58–60). The genes coding for OPAA have been cloned from *Alteromonas* sp. strain JD6.5 (61) and *A. haloplanktis* (62) and functionally expressed in

Table 3. Comparison of Substrate Specificity of OPH (63)

Substrate	k_{cat}/K_m (s^{-1})
Paraoxon	1×10^8
DFP	1×10^7
Sarin	8×10^4
Soman	1×10^4
Demeton-S	7×10^2
VX	7×10^2
Acephate	2×10^1

Table 4. Activity of OPAA Against Various CW Agents and Analogs

Enzyme	k_{cat} (s^{-1})			
	DFP	Tabun	Sarin	Soman
Alteromonas sp. JD6.5	1,650	85	611	3,145
A. haloplanktis	575	113	257	1,389
A. undina	1,239	292	376	2,496

recombinant *E. coli*. Sequence and biochemical analyses of the cloned enzymes suggested that OPAA is in fact a prolidase (61–62), a type of dipeptidase cleaving dipeptide bond with a prolyl residue at the carboxyl terminus (X-Pro). OPAA is highly active in hydrolyzing two dipeptide substrates of prolidase, namely Leu-Pro and Ala-Pro, but is not active against tripeptides. Computer modeling has suggested that the hydrolytic activity of OPAA against OP CW agents may be due to the fortuitous similarity of these compounds in size, shape, and surface charges to the X-Pro dipeptides (33).

Degradation of OP Agents by OPH

On the basis of the X-ray crystal structure of OPH, several mutant OPHs with enhanced activity against CW agents or analogs were generated (57a,b,63). These mutants were rationally designed based on amino acid substitutions in the enzyme active site. In particular, because the functional catalytic role and the coordination of metal centers have been studied, the initial approach was to generate mutants with substitutions in the residues responsible for binding the divalent metal cations. Site-directed mutagenesis was used to replace the original histidine residues at positions 254 and 257 (63). Of these enzymes, three variants (H254R, H254S, and H257L) retained only one metal at the active site. However, these enzymes retained activity against paraoxon with only a small reduction in k_{cat}/K_m. More importantly, mutants H254R and H257L showed a four- to fivefold improvement in activity and specificity against both VX and its analog demeton S (57a,b,63). OPH mutant H257L catalyzed the hydrolysis of soman and its analog NPPMP about fivefold better. The activity of the double mutant H254R/257L was even better with more than 20-fold improvement against demeton S compared to the wild-type OPH.

Another rational approach to design improved OPH mutants for enhanced DFP (an analog of sarin) degradation was demonstrated by Watkins and coworkers (64). This strategy was based on engineering the hydrophobic residues (Trp131, Phe 132, Leu271, Phe 306, and Tyr 309) involved in the putative leaving group of the active site. Because DFP has a fluoride-leaving group, replacement of one side chain with a residue capable of hydrogen bond formation and proton denation (His, Tyr, or Lys) was predicted to enhance catalysis. Eleven mutants were created by site-directed mutagenesis. The double mutants, F132H/F306H and F132H/F306Y, hydrolyzed DFP up to an order of magnitude faster than the wild-type OPH. However, because the K_m values were generally higher for these mutants, the overall specificity for DFP improved only by twofold.

Because the kinetic characteristics of OPH can be altered with relatively few amino acid substitutions, there is a realistic possibility of creating OPH variants with improved activity toward other OP agents based on rational design. Unfortunately, identifying all the amino acids responsible for substrate specificity and those that might give rise to extended specificity remains an overwhelming challenge. To this end, in vitro directed evolution is perhaps the more useful way to sample this sequence flexibility in a simple and rapid fashion (65). Not only is this method considerably easier to perform, it can also test the effects of amino acid changes, insertions, and deletions in regions of the protein that do not have predictable roles in activity (66–67). Moreover, the database generated on the protein sequence space can be merged with structural information for future rational design of OPH. We have recently demonstrated the directed evolution of OPH for enhanced degradation of methyl parathion, a substrate that is degraded 50 times slower than paraoxon by the wild-type OPH (unpublished results). Mutants generated more than 30-fold faster after two rounds of screening degraded methyl parathion. More interestingly, mutations outside of the active sites were shown to be important for the improved activity. It is difficult to comment on how these mutations contribute to the improved activity against methyl parathion; however, it is clear that these positive mutations cannot be predicted "a priori" simply based on structural information. Directed evolution is powerful precisely for this ability to identify distal mutations that involve subtle, long-range interactions.

Practical applications of OPH for rapid detoxification of OP compounds have been investigated. Both native and recombinant OPHs have been immobilized onto nylon (membrane, powder, and tubing), porous glass, and silica beads to develop a reusable or recoverable biocatalyst system for the detoxification of OP compounds (68–70). In addition, OPH has been immobilized in polyurethane foams that can potentially be applied as sponges or wipes for the cleanup of spills (71–72). Dry foams stored at room temperature retained significant activity over 50 days (73). Unfortunately, the immobilization of OPH by physical adsorption or covalent binding often results in a significant reduction in operational activity and/or stability because of sensitivity to changes in pH or temperature. In addition, the enzyme kinetics parameters for these modified OPH's are often less desirable because of the inaccessibility of the enzyme's active site.

More importantly, practical applications of large-scale enzymatic degradation have so far been limited by the tedious protocol and the high cost associated with purifying OPH. Although secreted OPH from *Streptomyces lividans* can be purified from the culture medium without cell disruption (48), the level of production is relatively low and ion-exchange chromatography and gel filtration are still required. We have recently developed an economical alternative by using whole cells with OPH anchored on the surface (74). This strategy is attractive for bioreactor applications but may be difficult to implement for any in situ applications because of the use of recombinant strains. It is clear that any commercial application of OPH for a wide range of degradation necessitates the development of a reliable and economic means of purifying and immobilizing OPH.

Enzyme immobilization using an affinity tag offers the advantages of (*1*) reversible but strong immobilization under mild conditions, (*2*) proper enzyme orientation on the support material so all active sites are fully accessible to the substrate. We have recently demonstrated this possibility by generating fusion enzymes between OPH and a family III cellulose-binding domain (CBD_{clos})

from *Clostridium cellulovorans* (75), enabling purification and immobilization onto different cellulose materials in essentially a single step (76). The kinetics values of the immobilized fusion enzymes were similar to OPH with a modest increase in K_m. The pH profile of the cellulose-immobilized enzymes was also only minimally affected. The CBD-OPH fusion enzymes could be immobilized onto a variety of cellulose matrices, and retained up to 85% of their original activity for 30 days. Repeated hydrolysis of paraoxon was achieved in an immobilized enzyme reactor with 100% degradation efficiency over 45 days. These fusion proteins should prove to be invaluable tools for the development of low-cost OPH-based cellulose materials for the simultaneous absorption and degradation of stored or spilled organophosphate wastes.

Another interesting application of OPH for decontamination was recently reported by incorporating OPH into fire-fighting foam (77). This approach was motivated by the problems encountered in decontaminating surfaces because the OP agents are difficult to extract and are quite volatile. When OPH is integrated into the foam, however, volatilization is minimized and agents can be extracted.

Degradation of OP Agents by OPAA

A simple procedure for the production and purification of OPAA in recombinant *E. coli* was developed (60–61). The recombinant OPAA produced was of the same molecular weight as the native OPAA. Production in small-scale batch fermentations reached up to 1 g/L of culture. Fractionation of crude extracts with $(NH_4)_2SO_4$ resulted in OPAA enzyme that is 75% pure.

A simple and safe enzyme-based decontamination system was developed based on the use of freeze-dried enzyme (78). This approach provides long-term stability and it greatly reduced logistical burden. The lyophilized enzyme could be reconstituted with any solvent and sprayed onto the contaminated surfaces using fire-fighting equipment. Because OPAA can be fully maintained in $(NH_4)_2CO_3$-buffered water, such an innocuous and inexpensive solvent is ideal for the proposed system. Lyophilization with the disaccharide trehalose not only enhanced activity but also stabilized OPAA during prolonged storage at 37 °C with no loss in activity for 12 months (33).

A number of water-based foams and wetting agents were also investigated as potential matrices of delivery. Two such agents, "Cold Fire" and "Odor Seal" were found to enhance OPAA activity. Because both these are biosurfactants in nature, their presence in the decontamination formulation provides encapsulation of CW agents and enhance their solubility in the enzyme solution. Interestingly, OPAA also retained full catalytic activity in the commercial laundry detergent "Tide Free" and "Protectall" lotion, whose ingredients also included biosurfactants. These results again point to the stabilizing effects of biosurfactants on OPAA. Various formulations based on OPAA have already been demonstrated to be effective in degrading sarin and soman in actual testing at the Army Research Center (33).

Strategies for Degrading Bulk Agents

Destruction of CW agents must be irreversible such that even by-products must be destroyed, rendering them unusable for further military purposes. Steps are required to generate an acceptable waste stream following hydrolysis of OP agents. For example, the by-products generated by chemical hydrolysis of sarin with sodium hydroxide, sodium isopropyl methylphosphonate (IMPA), and sodium fluoride must be processed before discharge. Although no microorganism grows on IMPA as a carbon source, they can be used as the sole source of phosphorus and sulfur (79). In SBR systems in which sarin hydrolysate was supplied, IMPA concentrations decreased from 85 mg/L to less than 1 mg/L within 75 hours (1). The only drawback is the long acclimation period (six weeks) required for the consortia. Soman and VX can be similarly degraded as sarin by first treating with chemical hydrolysis followed by biodegradation. Again, the by-products of hydrolysis (PMPA and EMPA) can be used as a sole phosphorus source by microbial consortia (80). In SBR experiments, PMPA levels decreased from 164 mg/L to less than 1 mg/L within 60 hours (1).

VX-ICB Operations

The VX-ICB was also seeded from an existing SBR system. The feed for the ICB was the same as for the SBR with VX/caustic as the sole phosphorus and sulfur source. The feed rate was maintained at 50 milliliter per day for the entire study. At that time, the extra carbon source for the SBR was isopropanol. From the SBR studies, it was known that while isopropanol was readily metabolized, it did not generate much biomass. Therefore, the adaptation period for the VX-ICB was considerably longer than for the HD-ICB. At several points during this adaptation period, the feed was augmented with the addition of glycerol, glycerol and molasses, and molasses and inorganic phosphate, in an effort to increase the biomass.

Although the level of biomass production in the VX-ICB was always much lower than in the HD-ICB, the TSS was always higher. It appears that the biomass that was generated did not adhere to the support as readily. The performance of the VX-ICB for OP degradation was comparable or somewhat worse than the SBR system, the highest levels being in the 30 to 40% range. As with the SBR systems, it is presumed that increasing the auxiliary carbon source (increasing the C : P ratio) and switching to one that would give a much higher cell yield could improve this. Alternatively, a secondary stirred tank reactor could be added (with the additional carbon) to complete the OP mineralization.

On the other hand, unlike the SBR, the level of OS mineralization was surprisingly good. In general, the OS compounds were nearly always 95 to 100% degraded. There are two mysteries with regard to this performance that remain to be solved. The first is the actual metabolic pathways for the degradation of these materials. Possible metabolic intermediates were not observed in the ICB effluent. The second is the fate of the sulfur. Although the initial level of inorganic sulfate in the ICB effluent corresponded to the amount of sulfur in the feed, this

dropped considerably thereafter. This is despite the fact that the OS degradation remained at approximately 100%. Because the system was well aerated, it does not appear to be likely that it could have been converted to hydrogen sulfide or other gaseous form. Alternatively, it was thought that the activated carbon on the surface of the polyurethane foam might have preferentially adsorbed the VX-thiol (disulfide). However, if it was only adsorption without degradation, the carbon should have become saturated very early in the operation of the ICBs (which initially ran for 190 days).

More recent studies coupled the ICBs with a secondary stirred tank reactor (STR). The OS compounds continued to be totally removed in the ICB. By the addition of dextrose to the STR as a primary carbon source, the OP compounds are also fully degraded (25).

Other CW Agents

Other than mustard gas and OP agents, arsenic-containing CW agents belong to the largest group of CW agents produced (81). Arsenic is highly toxic to microorganisms; as a result, biodegradation of these mixtures is problematic. However, several Russian scientists have successfully developed laboratory-scale processes for destroying these agents (1). Following the treatment of Lewisite by chemical hydrolysis, the remaining arsenic intermediates were treated with electrolysis and electrocoagulation, yielding formate, acetate, and arsenous and arsenic acids. After precipitation of arsenic from the solution, the remaining organic acids are mineralized in a fluidized bed reactor using a natural consortium.

Large-Scale ICB Operations

The successor to The Alternative Technologies program is the Assembled Chemical Weapons Assessment (ACWA) program. Unlike the former, which only dealt with bulk agents (HD and VX), the ACWA program deals with weaponized agents and all the materials that may be involved in their destruction (explosives, propellants, etc.). As with the bulk agent program, the agent would first be chemically neutralized and the hydrolysate processed further. A number of different technologies are being evaluated, one of which is the use of ICBs followed by an oxidative polishing step. A 1,000-gallon ICB unit was set up by AlliedSignal to process hydrolyzed HD (primary carbon source) and hydrolyzed Tetrytol (a mixture of the explosives Tetryl and TNT) as primary nitrogen source. After an acclimation phase, the ICB was run at 200 gallons per day for a 40-day operational trial. The final report from this trial has not been completed. Although the biological system worked well for TDG degradation, the extent of Tetrytol degradation has not been determined. Whether this technology will be further evaluated for use in stockpile destruction is not known at this time.

CONCLUSION

Significant progress has been made on the biodegradation of CW agents during the past decade. Both microbial consortia and enzymes are now available for the destruction of various CW agents. In some cases, chemical neutralization combined with biodegradative processes can play a substantial role in destroying stockpiles of CW agents. This approach appears to be most readily adaptable to dealing with sulfur mustard (HD). Just such a method has been successfully demonstrated and is being put into operation at Aberdeen Proving Ground to destroy the 1,600 tons of the agent located there.

For dealing with the nerve agents GB and VX, the potential use of biodegradation is dependent on finding a more efficient means of dealing with the C–P bond in these organophosphonates. This would eliminate the need to add the extra carbon that greatly increases the cost and scale of the current systems. Although the use of biological systems for the destruction of the nerve agent stockpiles is still undecided, these studies have lead to the discovery of a number of very interesting enzymes. These may play an important role in agent decontamination on the battlefield, after terrorist attacks, or in dealing with spills or leaks that may occur at demilitarization facilities.

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CHEMOLITHOAUTOTROPHY. See LITHOTROPHIC MICROBIAL ECOSYSTEMS IN THE SUBSURFACE

CHOLERA

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Cholera is perhaps one of the few ancient diseases that so far has managed to prevail in the modern world, carrying a devastating power that different regions have experienced from time to time. Cholera is a diarrheal disease that has long plagued populations in tropical areas of the world and traditionally near the Ganges Delta. Cholera has also been historically a global disease. The seventh pandemic is currently in progress. In this respect, cholera is a unique and insidious waterborne disease, capable of causing massive, worldwide epidemics. Currently, more than 75 countries in the world are listed as having cases of the disease. This disease generally occurred in epidemic form only in certain countries, until recently.

A bacterium, *Vibrio cholerae*, commonly found in all aquatic environments, such as marine, estuarine, river, and pond water, is now known to cause cholera. Only in recent years has *V. cholerae* been recognized as an autochthonous member of the aquatic microbial community and an opportunistic human pathogen. Powerful and sensitive tools and techniques for detection, identification, and characterization are rapidly evolving, with revived interest within both the scientific and lay communities. This increased awareness and interest, coupled with improved methods for detection, cholera disease, and the causative agent, *V. cholerae*, are now increasingly being reported from more locales throughout the world.

HISTORY OF CHOLERA

The Disease, Cholera

Cholera is a devastating disease involving production of cholera toxin by the bacterium, causing severe diarrhea of such prodigious volume that hypotensive shock can cause death within 12 hours of the first symptom (1). It is an enteric disease and is dose dependent. Usually, the infection is caused when the pathogen, *V. cholerae*, is ingested via food or water. The minimum effective dose may vary from 10^4 to 10^6 cells, depending on the susceptibility of the individual (2). *Vibrio cholerae* is highly sensitive to acid, therefore, hypochlorohydric people, that is, individuals with low acidity in their stomach are susceptible to a lower infectious dose and are at a higher risk of contracting the disease (2–4).

There is no conclusive agreement about the origin and use of the word "cholera." In Greek, the word means "gutter" or can be derived from the Greek words "chole" (bile) and "rein" (to flow), thus meaning the flow of bile (5–7). However, there is no disagreement among cholera historians about the occurrence of cholera-like disease in Asia and Europe during ancient times. The Sanskrit word, "visuchika," believed to denote cholera, was recorded around 500 to 400 B.C. (8). Given the lack of earlier recorded evidence, cholera in India was not discovered before the sixteenth century (9).

Cholera as an Emergent and Reemergent Disease

The modern history of cholera begins after 1817, when cholera was reported in the Middle East, Asia, Europe, and the shores of America (5–7). The question remains whether the pathogen was already on the American continent as part of the natural microbial flora or, as it was widely believed until recently, physically crossed the Atlantic. Historical records show human infection and clinical descriptions that match the descriptions obtained from different parts of the world, notably India. Until 1960, the disease was rampant across the world and took tens of thousands of lives. The disease was often considered as a curse from God. One artistic rendition that has appeared in many publications depicted the disease as a "ghost." In the United States, the last recognized indigenous cases of cholera occurred in Massachusetts and New York in 1911, until an individual case appeared in Port Lavaca, Texas, in 1973 (10). Thereafter, sporadic cases of cholera in the United States have been reported, most, however, being related to foreign travel, consumption of imported food, or consumption of "bootlegged" seafood from polluted waters in the U.S. Gulf Coast.

Although in 1854 John Snow first showed the possibility of environmental water contamination as a cause of cholera, not much was done to pursue this idea because scientists were primarily interested in the clinical aspects of the disease. The first report of toxigenic *Vibrio cholerae* from an environmental water source in the United States was made in 1978 (11). Interestingly, all the toxigenic strains of *V. cholerae* isolated from environmental sources in the United States between 1973 and 1990 are identical, except for one strain, as observed by Wachsmuth and coworkers (12). These Gulf Coast strains are unique in ribotype (assessed by restriction fragment length polymorphisms (RFLP) of rRNA genes (13).

A major event in the recent history of cholera was the 1991 outbreak in South America, after a century of freedom from the disease. Since then, small outbreaks continue to be recorded in and around Central and South America (14–16). The reemergence of cholera in the Americas drew the attention of both clinicians and microbial ecologists. The next key event came in 1992, when a massive epidemic caused by a new epidemic serotype, O139, originated in the southeastern part of India, gradually moved to the north of the Bay of Bengal and proceeded to Bangladesh (17,18). Before that time, *V. cholerae* O1 was the only serotype known to cause epidemics of cholera. The question of whether serotype O139 was a "novel" serotype or merely newly recognized remains unanswered at this time. If it is due to recent origin, researchers hypothesize that it may

have been converted from other existing serotypes in the environment. One point in support of this notion is the close resemblance of *V. cholerae* O139 to the El Tor biotype of *V. cholerae* O1 (19,20).

THE PATHOGEN, *V. CHOLERAE*

Taxonomy and Classification

In 1854, Pacini first described the causative agent of cholera in Italy as *V. cholera*; it was later termed *Kommabazillen* by Koch in 1883. The name was then subsequently changed to *V. comma*, based on the shape of the bacterium as determined using the technology of the time. This name persisted in the literature for several decades, before the microorganism was finally renamed as *V. cholerae*, following Pacini's original designation.

Vibrio cholerae is a γ-proteobacterium classified under the family *Vibrionaceae*. According to *Bergey's Manual of Determinative Bacteriology*, 9th edition (21), this family encompasses the genera *Aeromonas, Enhydrobacter*, *Photobacterium*, *Plesiomonas*, and *Vibrio*. However, an earlier study based on molecular evidence suggests that *Aeromonas* and *Plesiomonas* should be placed in a new family designated *Aeromonadaceae* (22). Within the family *Vibrionaceae*, *Vibrio* is considered to be the type genus and *V. cholerae* the type species within the genus. In Bergey's Manual the genus *Vibrio* is described as, "gram-negative, straight, or curved rods, 0.5 to 0.8 μm in width and 1.4 to 2.6 μm in length. Motile by one or more polar flagella, which are enclosed in a sheath continuous with the outer membrane of the cell wall, facultatively anaerobic and chemoorganotrophic, having both a respiratory and fermentative type of metabolism." Most of the species in the genus have an absolute requirement for Na^+ and at minimum the ion stimulates growth in all species (21). Therefore, it is not surprising that these microorganisms inhabit a wide range of estuarine and marine environments.

Vibrio cholerae is conventionally distinguished from other *Vibrio* spp. by a series of biochemical tests because there is no individual phenotypic trait that can confirm the species' identity (23). As a first-order test, presumptive isolates are screened on a *Vibrio*-specific medium (e.g., thiosulfate citrate bile-salt sucrose (TCBS)). *Vibrio cholerae* is sucrose-positive and, therefore, produces yellow colonies on TCBS agar. *Vibrio cholerae* is also positive for starch hydrolysis, oxidase, catalase, nitrate reductase, tryptophanase, lecithinase, gelatinase, and lysine and ornithine decarboxylase [a complete list of biochemical reactions can be found in the *Manual of Clinical Microbiology* (24)]. Conditions for the growth of *V. cholerae* include temperatures between 15 and 42 °C, salinity up to 50‰ (NaCl concentration of 5%), and pH between 5 and 10. Although, Na^+ stimulates growth, *V. cholerae* is among the few species in this genus that can tolerate low NaCl if grown in a complex medium where sufficient Na^+ is available (23,25). *Vibrio cholerae* is also negative for arginine decarboxylase and esculin hydrolysis, and when combined with results from TCBS agar, this series of three biochemical tests plus growth at 0% NaCl in a complex medium is a fairly reliable and quick method to identify presumptive *V. cholerae* isolates (26). Inhibition of *V. cholerae* by the vibriostatic agent O/129 (2,4-diamino-6,7-diisopropylpteridine) was once considered to be a key test for identification; however, owing to increasing resistance among clinical and environmental strains, this test no longer serves the purpose (27,28).

Approximately 200 serogroups of *V. cholerae* are known to date, determined by surface antigen variations in the lipopolysaccharide (29). However, so far only two serotypes are acknowledged for their potential to cause epidemics of the disease. Serotype O1 was implicated in all large epidemics of cholera until 1992 when O139 epidemics emerged, first in India and then Bangladesh. Characterization of serotypes is traditionally accomplished by serum agglutination tests for O1 and O139; however, specific monoclonal antibodies are now available and O1 and O139 cells can be detected directly by immunofluorescent microscopy (30,31). Within the O1 serotype, isolates can be further classified into two biotypes. The classical biotype was associated with all pandemics except for the latest (seventh), pandemic, which started in 1961 and was replaced with the El Tor biotype. Differentiation of biotype is based on the presence of hemolysin in El Tor strains and susceptibility to Mukerjee's cholera type IV bacteriophage (32). In the early literature, the El Tor biotype was assigned to a species separate from *V. cholerae* O1 Classical, designated *Vibrio eltor* (6). Furthermore, any *Vibrio* spp. that did not agglutinate with O1 antiserum was designated as nonagglutinating (NAG), which is a misnomer because these strains would, in fact, agglutinate with the appropriate non-O1 antiserum (33). Additionally, clinical and environmental isolates from both endemic and nonendemic cholera regions show little variability (34–37).

Ecological Perspectives

Vibrio cholerae is a bacterium that occurs naturally in the aquatic environment. Studies over the last 20 years have clearly defined *V. cholerae* to be a part of the autochthonous bacterioplankton, found almost everywhere in the aquatic environment, if attempts are made using appropriate tools. We were able to demonstrate the seasonal fluctuations in abundance of *V. cholerae* in Chesapeake Bay throughout the year, with large numbers of the cholera bacterium present during the summer months (38–40). In addition, because cholera is now considered a re-emerging disease, the level of awareness is at an all time high among microbial ecologists, oceanographers, biologists, and the medical community at large, especially after the 1991 outbreak in Latin America, which spread to 21 countries in 24 months (41).

Several major multinational and multiinstitutional studies have been undertaken in the past five to seven years investigating the mechanisms of survival, multiplication, and persistence of *V. cholerae* in the environment, as well as its characterization and detection, using immunodiagnostic and molecular tools for monitoring the

environment. Clearly, a new era in the environmental microbiology of *V. cholerae* began during the past decade, which we believe opens avenues for multidirectional and multidisciplinary investigation.

Survival and Growth

Drinking water was first implicated in the spread of cholera almost 150 years ago by John Snow in London, England, and, subsequently confirmed by Robert Koch's isolation and description of *V. cholerae* in 1884, following the work of Pacini in 1854. Despite numerous investigations during the 1960s demonstrating the survival of *V. cholerae* in different kinds of natural water over an extended period of time (42–44), the perception remained unchanged until the late 1970s that the source of the causative agent of cholera was the patient ingesting contaminated water and/or food. Unfortunately, in studies before the early 1970s all of the diagnostic methods available for isolation and characterization of *V. cholerae* were primarily developed for clinical use (45). Most diagnostic tests have a threshold for sensitivity, which becomes critical when clinical and environmental samples are compared, solely because of concentration differences. Without other choices, researchers relied on conventional culture methods as the best-available technology. Assays for detection of viable but nonculturable cells were not available. As a result, accurate monitoring for the presence and survival of these pathogens was impaired until appropriate and sensitive methods were developed in the 1980s and 1990s (46). In addition, cells of *V. cholerae* present in natural water adapt to a variety of environmental conditions, such as low pH, variable salinity, temperature, and solar radiation, and conditions that are not optimal for growth and multiplication (47). Therefore, *V. cholerae* O1 from environmental conditions may not necessarily grow on conventional laboratory media.

Viable but Nonculturable (VBNC) State

The viable but nonculturable phenomenon has been described as a survival strategy of microorganisms, notably human pathogens in the aquatic environment (48). In the 1930s, it was noted that there were significant differences between the numbers of autotrophic and organotrophic bacterial counts of water samples (49). A few years later, another group of investigators concluded that a significant portion of a given bacterial population that did not appear on culture plates must be present in a resting stage (50). Around the same time, further investigation led to the demonstration of metabolically active bacteria, which could be detected by microscopy, but did not form colonies on microbiological media (51,52). Half a century ago, Zobel reconfirmed earlier findings that only a small percentage of bacterial cells present in a given sample of seawater will grow on agar plates (53). The evidence for viability has been crucial. Coliform bacteria not growing in culture media in the laboratory (once considered dead) were subjected to measurement of their ATP level, the results of which suggested that these *Escherichia coli* cells were alive (54). Some of the pioneering work in this area was conducted at the University at Maryland in the 1980s. The investigators examined the viable but nonculturable phenomenon in *E. coli* and *V. cholerae*. Using nalidixic acid to prevent cell division, and yeast extract to promote growth, the researchers demonstrated that cultures, which did not produce colonies on bacteriological media, were indeed metabolically active, as observed by the presence of elongating cells under microscopic examination (55,56). In these studies, for the first time, investigators were able to demonstrate induction of culturable *V. cholerae* O1 cells to a nonculturable state in laboratory microcosm experiments. These metabolically active cells do not form colonies on bacteriological culture plates, making it impossible to isolate them.

Bangladesh has been one of the major endemic areas for cholera. Without exception, there are epidemics almost every year (57). Nevertheless, it has been difficult (and in some cases impossible) to isolate *V. cholerae* from surface water in Bangladesh between cholera seasons, even with intensive monitoring and epidemiological surveillance, or from water not directly contaminated by the patient's stool (58). Yet the discovery of the viable but nonculturable phenomenon in *V. cholerae* facilitated much higher rates of direct detection in bodies of water serving as drinking water sources in Bangladeshi villages. Huq and coworkers (59) showed that these VBNC *V. cholerae* could be detected by direct immunofluorescent microscopy throughout the year in these water sources, even when the organisms could not be cultured using conventional media.

It is evident that the main obstacle in the study of *V. cholerae* in the environment is the detection of target cells when the concentration of bacteria is very low, as is often the case in environmental waters during winter months. Although water has been suspected to be the source of the cholera bacterium, as far back in time as 150 years ago, there is, even at present, no effective technique available to isolate the VBNC cells from water supplies directly. However, methods are available that allow direct detection of the pathogen in the VBNC state from environmental sources, using both immunological and molecular methods (59).

Methods for Detection

Among the problems associated with studying microbial communities in the environment, is the lack of cost-effective, reliable methods for detection. In addition to viable but nonculturable *V. cholerae* and related gram-negative bacterial pathogens, most bacteria present in seawater have never been cultured. Therefore, advances in field research rely on effective means to detect nonculturable organisms.

Traditionally, detection and identification of bacteria in environmental samples was based on conventional culture methodology. Water samples were plated onto selective or nonselective media and a battery of biochemical tests was run. While this approach has not been given up entirely, because it still can provide valuable information about those isolates that are culturable, increasingly researchers

are using molecular tools for detection and to determine viability and potential community function.

Tools for direct detection, generally speaking, can be divided into two categories, immunobased or nucleic acid–based. Immunodetection is where labeled antibodies specific to the target are added to a sample and have been used extensively for microscopic assays. Using this approach, and targeting the toxigenic serotypes O1 and O139, *V. cholerae* has been detected in a variety of source materials by both direct and indirect immunofluorescent assays (30,31). Because this approach allows detection of both culturable and viable but nonculturable cells, it has substantially improved assessment of the occurrence of *V. cholerae* in the environment. Using this approach, Ruiz and coworkers found significant numbers of *V. cholerae* in the ballast water of ships arriving in Chesapeake Bay (60).

With the advent of the polymerase chain reaction in the 1980s (61), nucleic acid–based detection gained in popularity and is now used extensively in studies of *V. cholerae* in the environment. PCR-based methods offer specificity at several levels, can target any nucleic acid sequence, and are relatively quick and easy to perform. Primers are now available for the specific detection of *V. cholerae* in environmental samples and do not cross-react with the closely related *V. mimicus* (62,63). One disadvantage of the nucleic acid–based approach is that they do not differentiate between viable and nonviable cells. Therefore, research is now beginning to focus on combining microscopic detection with nucleic acid hybridization. With fluorescent in situ hybridization (FISH) assays, fluorescent-labeled probes are added to whole fixed samples. The probe then hybridizes to the complementary sequence within the target cell and is detected by a fluorescent signal viewed under epifluorescent microscopy (64). This approach can be combined with 4′6-diamidino-2-phenylindole (DAPI) staining to determine cell viability (65).

Role of Plankton in the Ecology, *V. cholerae* and Incidence of Cholera

Most of the major outbreaks of cholera epidemics during the past 25 years originated in coastal areas (66), including the outbreak caused by the newly recognized O139 serotype of *V. cholerae* (67). As cited earlier, until recently, the prevailing view of the ecology of cholera was that *V. cholerae* was associated only with humans. In the early 1980s, Colwell and coworkers (68) showed that *V. cholerae* is an indigenous, autochthonous member of brackish and estuarine environments. Several subsequent studies provided evidence to support this hypothesis (69–71). The autochthonous nature of this organism has since proven to be an important factor in understanding the epidemiology of cholera, promoting insight leading to new knowledge and further investigation into the prevention of cholera (72).

The association of *V. cholerae* with plankton and changing environmental conditions in water, particularly changes in pH, was first addressed in the 1960s. Cockburn and Cassanos (73) demonstrated a positive correlation between the incidence of cholera and the presence of chlorophyll-bearing organisms in water. Interestingly, *V. cholerae* produces an enzyme, mucinase, which actively degrades mucin and mucinlike substances present in plant cells and in the gut of humans (74). Earlier, it was demonstrated that, with the disintegration of a large number of algae after a peak bloom, the gram-negative heterotrophic bacterial population, including *V. cholerae*, begins to rise (75). Different organic compounds and other nutrients released by the disintegrating plankton in the water served as a major source of energy for those heterotrophic bacteria. The authors further noted that the gelatinous cover of filamentous blue-green algae filaments contained high concentrations of gram-negative rod-shaped bacteria. The mucilaginous sheath of the cyanobacteria, *Anabaena variabilis*, was found to serve as a site of attachment for *V. cholerae* cells (76,77). Using direct detection methods, Islam and coworkers (78,79) demonstrated that *V. cholerae* persisted for approximately 15 months in the mucilaginous sheath of *A. variabilis*. These are convincing results to assume that the bacteria may become associated with algae as a method for prolonged survival in the environment and added evidence for the long-term survival of *V. cholerae* under varying environmental conditions. There is, however, no evidence that *V. cholerae* actually multiplies or proliferates in association with blue-green bacteria.

In addition to mucin, most vibrios, including *V. cholerae* produce a chitinase enzyme that degrades chitin and, thereby, supply nutrients needed for survival (80). The digestion of chitin appears to provide the chitinase active cells with protection from harsh acidic environments (4,81). The association of *Vibrio parahaemolyticus* with microscopic crustaceans, a major component of zooplankton, was reported by Colwell and colleagues in 1975 (81) and is considered a significant finding because of the chitin composition of the carapace of copepods. In subsequent studies, Huq and coworkers demonstrated the ecological relationship of copepods and the survival and multiplication of *V. cholerae* in the aquatic environment (81a, 81b). According to a recent study, surface proteins of *V. cholerae* play an important role in attachment by vibrios to chitin particles (82). In addition to providing a nutrient source, attachment of *V. cholerae* to chitin particles protects these organisms from the lethal actions of gastric acid (83). Such protection is perhaps an important factor for *V. cholerae* in the natural environment. *Vibrio cholerae* cells attached to live copepods are also more resistant to chlorine and, therefore, can resist water treatment (84). Chitin is also able to protect *V. cholerae* O1 against the effects of low temperature, suggesting a contribution to extended survival of cholera vibrios in the environment, especially at freezing temperatures (85).

Billions of gallons of water are transported from one part of the world to another via ballast (a device introduced in the early 1900s in ocean-going ships for balancing) and has been implicated in the rapid transmission and distribution of marine vertebrates and invertebrates globally (86). Researchers have observed several species of copepods of Asian origin in the Pacific Coast of North America within a 15-year period. Copepods are usually found in large numbers in water (86) as they are the most abundant group of zooplankton (87,88). According

to the Continuous Plankton Recorder survey in the northeast Atlantic established by Sir Alister Hardy in the 1930s and carried out each month from 1948 to the present, phytoplankton and zooplankton populations demonstrated a decline between 1948 and 1980 (89). However, since 1980 the trend has reversed (89). Gelpspan (1991) reported that algal blooms surpassing the usual spring bloom were observed at various points worldwide, such as North Carolina, California, Finland, Iceland, Tasmania, Japan, and Thailand. As phytoplankton are the "primary producers" of organic compounds (90), the size of zooplankton populations, which feed on phytoplankton, has also increased significantly. This observation coincides with the preceding period's two major modern cholera events, the reappearance of cholera in South America in 1991 and the emergence of serotype O139 in India in 1992.

Cholera is dose-dependent. Once the number of *V. cholerae* O1 cells is large enough to constitute an infectious dose (10^4 to 10^6 cell ml^{-1}), depending on the physiological state of the victim (91), the cells are ingested by humans while bathing, swimming, or drinking untreated water from ponds, rivers, and lakes of cholera-endemic countries, an epidemic can be initiated (92). The high numbers of *V. cholerae* cells attached to copepods offers a concentrated source of infection, should these organisms be present in drinking water. Furthermore, the reversion of viable but nonculturable cells to a culturable state and the association with copepods and environmental factors may have important epidemiological implications (93). These will be critical factors in predicting outbreaks.

GENETIC TRANSFER IN THE ENVIRONMENT AND EMERGENCE OF TOXIGENIC STRAINS

In general, bacteria are known for their considerable diversity, both in phenotype and genotype. Unlike other taxonomic domains, there is also a high degree of genetic variance even within a particular taxonomic group. While point mutations are a common source of variability among all forms of life, these alone cannot explain the diversity seen among the bacteria. Evidence suggests that lateral or horizontal gene transfer, whereby, pieces of genetic material are transferred from a donor to a recipient cell, is an important component of both bacterial evolution and diversification (94,95). Once a genetic element has been transferred to a new recipient species, the trait can then be passed onto progeny, along with point mutations, by vertical transfer. This type of genetic acquisition is believed to be an important mechanism by which toxigenic strains of *V. cholerae* arise from nontoxigenic progenitors, as may have been the case for the emergence of toxigenic *V. cholerae* O139.

DNA acquisition by integrons or temperate phages is a significant contributor to the genetic variability and transfer of virulence genes in *V. cholerae* (20,96,97). Evidence of significant gene reassortment in this species was also demonstrated in the recent analysis of the fully sequenced genome (95). The smaller of the two chromosomes present in *V. cholerae* contains a high percentage of genes of hypothetical function and many of these do not appear to be γ-proteobacterial in origin, which is also supported by the presence of a large integron island for gene capture (95).

In 1996, Waldor and Mekalanos first demonstrated that the *V. cholerae* CTX element was coded for by a filamentous, single-stranded DNA, temperate phage, CTXΦ, and was responsible for the production of the cholera toxin (CT) in toxigenic strains (20). This phage could be induced to form infectious particles and shared many properties of the M13 coliphage (20,96). In *V. cholerae* the main virulence genes tend to exist in clusters, or cassettes, as in the CTX element, responsible for production of the secretory enterotoxin CT (cholera toxin) and the *V. cholerae* pathogenicity island (VPI), which carries the genes for the toxin coregulated pilus (TCP) (96). The CTXΦ may be present in a host cell as a replicative form (RF, plasmid) or as a prophage integrated into the chromosome at specific attachment sites (att_{RS}). More recently, Karaolis and coworkers (97) provided evidence that the *V. cholerae* pathogenicity island, including TCP, may be encoded by a second temperate filamentous phage, VPIΦ The TCP gene in the prophage has dual role as the viral capsid protein and the receptor pilin for CTXΦ infection.

Because of the widespread clinical importance of these mobile genetic elements, research is increasingly focused on determining environmental conditions that stimulate genetic exchange via induction and subsequent infection. It has been demonstrated that CTX prophage could be induced from *V. cholerae* on exposure to sunlight (98). Interestingly, there is little evidence for prophage induction in the intestine (99), highlighting the importance of environmental factors in the propagation of viral particles. Environmental conditions may also be important in the subsequent infection and integration of *V. cholerae* phages (100).

Vibrio cholerae also show genetic rearrangements and phenotypic differentiation in other properties (101,102). Researchers have observed phage mediated biotype conversion between Inaba and Ogawa (103), and later seroconversion of non-O1 to O1 was demonstrated in laboratory microcosms (104). This evidence is especially interesting in cholera-endemic areas where O1 and O139 serotypes are far less prevalent than non-O1 in the environment. The rate of seroconversion is enhanced in seawater and by key environmental parameters, particularly warm temperatures (104).

Vibrio cholerae offers an interesting clinically and environmentally relevant model for the study of gene rearrangement and transfer. Increasingly, evidence is mounting that gene transfer among *V. cholerae* is occurring at a high rate in the environment (37,99,105–107). Research in this area is only now beginning to elucidate the patterns and mechanisms by which nontoxigenic environmental *V. cholerae* strains may give rise to new epidemic varieties.

ENVIRONMENTAL AND CLIMATE MODELING–BASED APPROACHES TO PREDICTING OUTBREAKS

During the past few years there have been studies on global climate change and its impact on ecosystems (108).

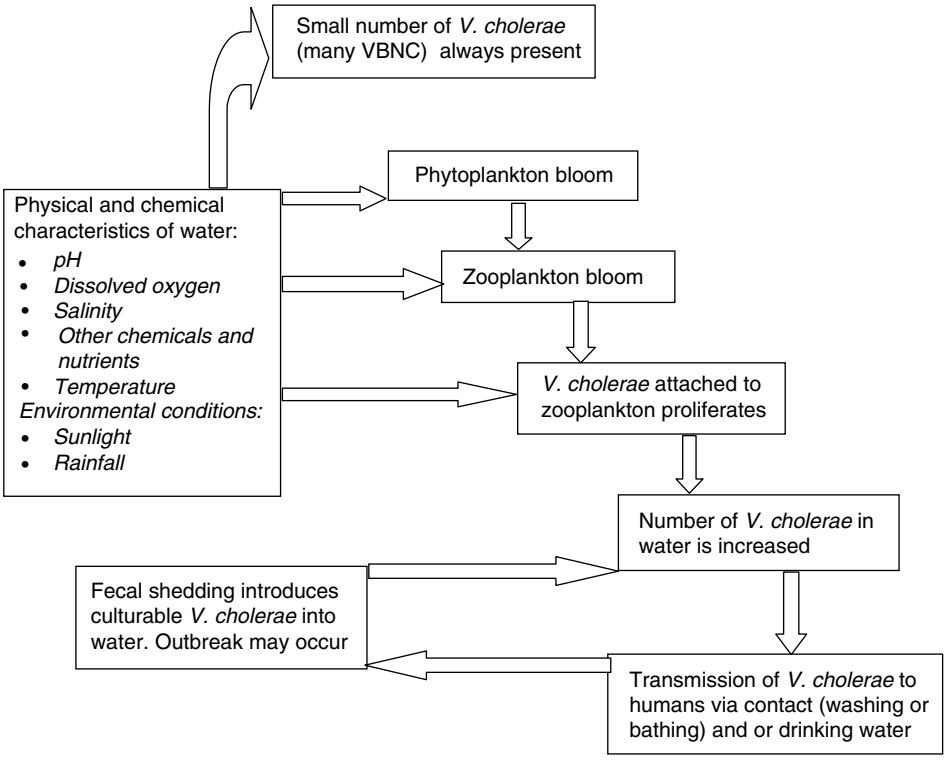

Figure 1. A model for possible survival and transmission of *V. cholerae* in the natural environment. (Modified from the original model published earlier. Permission obtained from the original: A. Huq et al., M. Yasuno and B. A. Whitton, ed., *Biological Monitoring of Environmental Pollution*, Tokai University Press, Tokyo, Japan, 1988, p. 262).

Changes in climate may directly and indirectly affect the ecology of all living creatures. Fluctuations in weather conditions have minor to major effects on the environment that may bring ecological changes to various regions, such as soil erosion, deforestation, drought, and human migrations from rural to urban areas. The direct influence of sunlight and dissolved chemical nutrients on aquatic plants and phytoplankton, resulting in increased pH and concentration of dissolved oxygen (109), provide a favorable environment for *V. cholerae*. A hypothetical model was proposed by Huq and coworkers (110) and subsequently modified by Colwell and Huq (46), suggesting a role for the various physicochemical and biological factors of the aquatic environment in the transmission of *V. cholerae*. This model is further modified by proposing the possible direct and/or indirect role of phytoplankton blooms and subsequent zooplankton blooms influenced by the physicochemical parameters that play a role in the survival, multiplication, and transmission of *V. cholerae* in the natural environment (Fig. 1). Islam and coworkers described the relationship of different environmental parameters on the formation of phytoplankton blooms and the multiplication of *V. cholerae* (79). Conditions triggering an increase in the number of *V. cholerae* in water may include increased numbers of zooplankton following blooms of phytoplankton. When untreated natural water is the only source for domestic use, copepods in the water are likely to be ingested by humans and the associated *V. cholerae*, thereby, may cause disease.

Because of the seasonality of cholera, climate is strongly considered an influencing factor in the ecology of the organisms and the epidemiology of cholera. The El Niño-Southern Oscillation (ENSO) is a major component of climate variability that appears to drive the interannual variation of the disease where temperature plays a significant role (111). Interestingly, the reappearance of cholera in South America coincides with the 1991–1992 El Niño event. Furthermore, warmer temperatures and the 1997–1998 El Niño have been implicated with increased numbers of diarrhea cases in Perú (112). Recent studies have suggested that the temporal variability of cholera in Bangladesh has an interannual component also strongly correlated with El Niño events (111). Preliminary investigations showed that cholera cases in Bangladesh closely follow sea surface temperatures (SST), which were obtained by satellite for the Bay of Bengal (113,114). Furthermore, sea surface height (SSH) can be measured remotely with the Advanced Very High Resolution Radiometer (AVHRR) satellite and such data provides insight as to the incursion of water with plankton, to more inland areas. It was interesting to note the correlation of SST and SSH to cases of cholera in Bangladesh (Fig. 2) (114). However, in 1993, the association was not observed. Although the SST were within the normal range for that year, SSH in the spring was the lowest observed during the entire study period. Therefore, less intrusion of water took place and perhaps fewer interactions between people and water suggesting SSH may also have significant implications, in addition to SST, for predicting outbreaks and epidemic of cholera.

CURRENT STATUS AND FUTURE DIRECTIONS

It has become evident during the past two decades that there is a tremendous need for further information concerning the ecology of *V. cholerae*. Specifically, we still

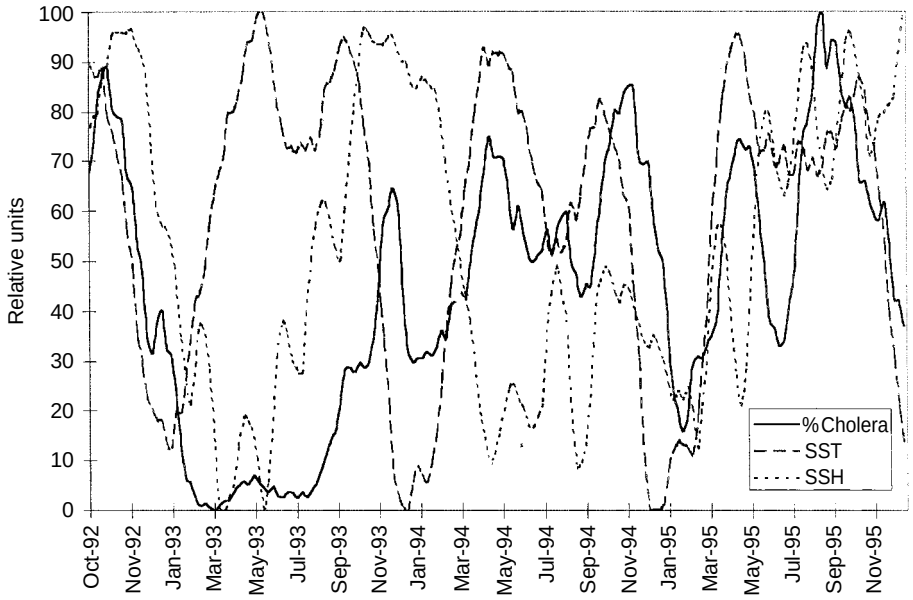

Figure 2. Cholera cases (solid line), SST (dashed line) and SST (dotted line) data from October 1992 to December 1995, cholera cases followed by the SST cycle; however in spring 1993, SST was the lowest for this period (B. Lobitz et al., *Proc. Nat. Acad. Sc.*, vol. 97, pp. 1,438–1,443, reproduced with permission).

do not understand why epidemics of cholera tend to occur only in certain parts of the world, and the causative organism can be found almost anywhere. Obviously, the level and quality of public health awareness and safety are significant factors. Accessibility to safe drinking water and reliable and effective methods for sewage treatment are critical.

Due, in part, to the lack of a successful cholera vaccine, efforts to control the disease is now relying on understanding the ecology of the causative agent and its genetic variability. Based on environmental and ecological studies on cholera, carried out during the past 25 years, one of the most interesting adaptations in the fight against cholera is the simple filtration of water using sari cloth, a domestic material available in every Bangladeshi village household, where cholera is a problem. Using four layers of sari material to filter surface water, less than or equal to 99% of the *V. cholerae* attached to copepods or other particles can be removed, significantly reducing the dose (115). A recent field trial demonstrated a reduction in cases, in the village population, using drinking water prefiltered with a sari, as compared to the population using water without such filtration (116).

The entire genome of *V. cholerae* has now been sequenced (95). This will push the field forward in understanding the ecology and pathogenicity of this important and historical pathogen. Because of the nature of this organism as an autochthonous member of the aquatic microbial community and an opportunistic human pathogen we may not be able to eradicate the disease, but certainly continued research should help reduce the numbers of cases and/or the amount of suffering.

Acknowledgments

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CHRYSOPHYTES. See PALEOLIMNOLOGY: USE OF SILICEOUS STRUCTURES OF CHRYSOPHYTES AS BIOLOGICAL INDICATORS IN FRESHWATER SYSTEMS

CILIATED PROTOZOA. See PROTOZOA IN MARINE AND ESTUARINE WATERS

CILIATES IN FRESHWATER ECOSYSTEMS. See PROTOZOAN CILIATES IN FRESHWATER ECOSYSTEMS

CISTERNS. See MICROBIAL WATER QUALITY OF RAINWATER ROOF CATCHMENT SYSTEMS

CLIMATE CHANGE, USE OF CHRYSOPHYTES AS INDICATORS OF. See PALEOLIMNOLOGY: USE OF SILICEOUS STRUCTURES OF CHRYSOPHYTES AS BIOLOGICAL INDICATORS IN FRESHWATER SYSTEMS

CLONING. See PHYLOGENETICALLY BASED METHODS IN MICROBIAL ECOLOGY

CLOSTRIDIUM

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GENERAL CONCEPTS

Bacteria from the genus *Clostridium* are gram-positive, anaerobic, generally motile, spore-forming rods. These generally obligate anaerobes are ubiquitous saprophytes common in nature and are also part of our normal flora. Many clostridia are transient or permanent members of the normal flora of the human skin and the gastrointestinal tracts of humans and animals. Unlike typical members of the human bacterial flora, most

clostridia can also be found worldwide in the soil. They induce diseases that can be acquired through contaminated foods or wounds.

The clostridia are classically anaerobic rods, but some species can become aerotolerant on subculture; a few species can grow under aerobic conditions. Most species are gram-positive, but a few species stain very weakly or are gram-negative. Also, many gram-positive species easily lose the gram reaction in culture, resulting in gram-negative cultures. Clostridia employ butyric fermentation pathways to generate energy and, as a result, often produce a foul odor.

The clostridia can vary considerably in their biochemical and physiological properties (1). Clostridia are able to ferment a wide variety of organic compounds. They produce end products such as butyric acid, acetic acid, butanol, and acetone, and large amounts of gas (CO_2 and H_2) during fermentation of sugars. A variety of foul-smelling compounds are formed during the fermentation of amino acids and fatty acids. The clostridia also produce a wide variety of extracellular enzymes to degrade large biological molecules in the environment into fermentable components. Hence, the clostridia play an important role in nature in biodegradation and the carbon cycle. In anaerobic clostridial infections, these enzymes play a role in invasion and pathology.

The clostridia produce a spherical or oval spore that results in a characteristic bulge generally located at the terminal or subterminal end, giving the bacterium the appearance of a long drumstick. The body of the bacterium is stained, whereas the spore remains unstained during a gram stain providing a visual clue to the identification that is particularly helpful when differentiating weak gram-positive or gram-negative species from true gram-negative bacteria such as Bacteroides. The spores are produced when the environment becomes unfavorable and are extremely resistant and can survive in the environment for very long periods. Poorly canned foods create an anaerobic environment under which any surviving spores can germinate and produce toxin.

Of the anaerobes that infect humans, the clostridia are the most widely studied. They are involved in a variety of human diseases, the most important of which are gas gangrene, tetanus, botulism, pseudomembranous colitis, and food poisoning. In most cases, clostridia are opportunistic pathogens; that is, one or more species establishes a point of infection in a particular site in a compromised host. All pathogenic clostridial species produce protein exotoxins (such as *botulinum* and tetanus toxins) that play an important role in pathogenesis.

Clostridium perfringens produces large oval subterminal spores (rarely seen in standard culture) and is nonmotile. This species is most often associated with feces and wound infections. *Clostridium tetani* is motile and produces terminal spores with the appearance of a squash racket. *Clostridium botulinum* produces oval subterminal spores and is motile.

Clostridium difficile produces large oval subterminal spores. Ordinarily, this species cannot compete with normal intestinal flora, but when antibiotics eliminate the normal flora *C. difficile* can flourish, producing disease.

CLOSTRIDIA IN DISEASE

Clostridia are ubiquitous pathogens that produce many toxins and hydrolytic enzymes. Because the toxin-encoding genes can be located on extrachromosomal elements or in variable regions of the chromosome, several pathovars have arisen, each of which is involved in a specific disease. The various diseases caused by clostridia are diarrhea, botulism, tetanus, blackleg, malignant edema, infectious necrotic hepatitis, enterotoxemia, and gas gangrene. They affect all types of animals (2–6).

Food-borne infections are frequent because of poor hygienic practices or incorrect food-handling practices (7–10) and have resulted in numerous outbreaks (11). These infections are the source of significant economic costs worldwide (12,13). Todd (14) reported that acute food-borne disease infections and intoxications are much more of a concern to governments and the food industry today than a few decades ago. Meaningful monitoring of increases or decreases in food-borne disease requires an effective surveillance system at the local, national, and international levels. The apparent decrease of *Staphylococcus aureus* and *C. perfringens* outbreaks in industrialized countries may be related to improved temperature control in the kitchen.

Clostridium perfringens continues to be a common cause of food-borne disease (6,9). Characteristics of this organism that contribute to its ability to cause food-borne illness include the formation of heat-resistant spores that survive normal cooking and heating temperatures, a rapid growth rate in warm food, and the production of enterotoxin in the human gut. Time and temperature abuse associated with food preparation contributes to the majority of outbreaks of *C. perfringens* food-borne disease. CPE-induced diarrhea has been reported in the absence of a defined food vehicle. These cases have been typically associated with the elderly and those following a course of antibiotic therapy. The incidence of CPE-induced diarrhea may be expected to increase with the growing population of immunocompromised (disease-, treatment-, or age-induced) individuals.

The impact of *C. difficile* has been discussed by Settle (15) and by Groschel (16). Infection with this bacterium can give rise to a wide range of symptoms, from diarrhea to fulminating colitis and toxic megacolon. Changes in the composition of natural intestinal flora, mainly due to antibiotic therapy, permit its colonization and multiplication in the colon. The disease is caused by enterotoxin A and cytotoxin B, and infection ranges from asymptomatic carrier state and mild diarrhea to pseudomembranous colitis. The clinical diagnosis is made by observing inflammatory, sometimes bloody, diarrhea and by the colonoscopic detection of epithelial necrosis, ulceration, and, in the advanced state, pseudomembrane formation.

Clostridium tetani is the causative agent of tetanus (lockjaw). The organism is found in soil, especially heavily-manured soils, and in the intestinal tracts and feces of various animals. Carrier rates in humans vary from 0 to 25%, and the organism is thought to be a transient member of the flora whose presence depends on ingestion.

The organism produces terminal spores within a swollen sporangium, giving it a distinctive drumstick appearance. Although the bacterium has a typical gram-positive cell wall, it may stain gram-negative or gram-variable, especially in older cells. Tetanus is a serious disease of humans. Mortality rates that were reported vary from 40 to 78%. The disease stems not from invasive infection but from a potent neurotoxin (tetanus toxin or tetanospasmin) that is produced when spores germinate and vegetative cells grow after gaining access to wounds. The organism multiplies locally and symptoms appear remote from the infection site. Because of the widespread use of the tetanus toxoid for prophylactic immunization, fewer than 150 cases occur annually in the United States, but the disease is a significant problem worldwide where there are more than 300,000 cases annually. Most cases in the United States occur in individuals more than 60 years of age, which is taken to mean that waning immunity is a significant risk factor. Spores are highly resistant to adverse conditions and spores of some strains resist boiling in water for up to three hours. They may resist dry heat at 160 degrees for one hour and 5% phenol for two weeks or more. In water, iodine (1%) can kill the spores within a few hours.

Clostridium botulinum is a widely distributed saprophyte occurring in soil, vegetables, fruits, leaves, silage, manure, the mud of lakes, and sea mud. Its ability to produce a potent neurotoxin in food and resistance of its spores to inactivation combine to make it a formidable pathogen of humans and a range of animals and birds. Insufficient heating in the process of preserving foods is an important factor in the causation of botulism, and great care must be taken in canning factories to ensure that adequate heating is achieved in all parts of the can contents (Reed 1994a). Botulinum toxins are among the most poisonous natural substances known that are classified into seven types (A to G), with types A, B, and E most frequently associated with human disease. Botulism is a severe, often fatal, form of food poisoning characterized by pronounced neurotoxic effects. The disease can be caused by a wide range of foods, usually preserved hams, large sausages, home-preserved meats and vegetables, canned products such as fish, liver paste, and even hazelnut puree and honey. Foods responsible for botulism may not exhibit signs of spoilage. In recent years, it has been recognized that the organism is capable of causing a distinct syndrome of illness in infants. Infantile botulism arises when spores present in food (usually honey or corn syrup) germinate in the intestinal tract and produce toxin (17,18). Typically, cases occur in the second month of life and always in infants aged one or less. The case fatality rate is about 2% compared with 15% in adult food-borne botulism.

CULTURE

Methods for the detection and enumeration of clostridia can be found in official or standardized methods (19–22). Not surprisingly, attempts to develop a single isolation medium for all species that occur in foods have not been entirely successful, and the problem is compounded by the need to recover both vegetative cells and spores, some of the latter being unable to germinate without heat activation. Most available isolation media, except some of those used in the dairy industry, include sulfite and an appropriate iron salt, so that blackening caused by sulfite reduction can serve as a differential test for clostridia. An unresolved issue is whether special precautions are needed to exclude oxygen during sample preparation and dilution, preparation of media, and in conditions used for anaerobic incubation. Although such stringency may be required for maximum recovery of sublethally damaged cells or spores, practical constraints in laboratories necessitate use of relatively simple procedures for detecting clostridia routinely. Most species will grow sufficiently well with standard anaerobic atmosphere generating systems, but for some (e.g., *C. tetani*) it is essential to ensure complete removal of oxygen for good growth.

The detection of clostridia, primarily *C. perfringens*, in water is based on the most probable number (MPN) or membrane filtration (MF) methods. Media frequently used are tryptose-sulphite-cycloserine agar (TSC) with or without egg yolk (23,24), Shahidi-Ferguson *perfringens* agar (SFPA) or oleandomycin-polymixin-sulphadiazine *perfringens* agar (OPSPA) (19), m-CP medium (25), iron milk medium (22), lactose-sulfite (LS) broth (26). The respective value of each medium is related to its particular use, and differences in efficiency have been reported (24,27).

OCCURRENCE

Habitat

The main habitat of clostridia is the soil but many species are also found in dust, sewage, rivers, lakes, seawater, milk, vegetables, fresh meat, fish, insects, and the intestinal tract. Sulfite-reducing clostridia (SRC), and in particular *C. perfringens*, are present in the feces of man and other animals (pig, chicken, dog, cow, horse, sheep, calf), although not all species are exclusively fecal in origin (4,28–32).

Occurrence of clostridia is fairly common in human feces and may be affected by diet. Geldreich (28) reported the presence of *C. perfringens* in 13 to 35% of humans and *C. tetani* in 1 to 25% of people. Numbers of SRC in human feces are typically in the region of 10^3 per gram but can rise as high as 10^{10} per gram. Vela and coworkers (32) reported carriage rates of *C. perfringens* in feces of a northern Mexican population where the organism was found in 126 of the 200 fecal samples obtained from healthy individuals. The samples had an average of 7.4×10^3 spores per gram, with the elderly population showing the highest levels.

Clostridia are found in various concentration in wastewater (typically 10^4 to 10^5 per 100 ml in raw sewage and up to 10^3 to 10^4 per 100 ml in sewage effluents) and wastewater-contaminated waters (typically 10^2 to 10^3 per 100 ml) around the world (25,33–37). The similarity of counts obtained suggests that the majority of SRC in sewage are *Clostridium perfringens*. Further evidence for this comes from a study comparing the performance of MCP agar and TSC agar for the detection of *Clostridium perfringens* and sulfite-reducing clostridia in sewage and other wastewater (38).

Food and Animal Wastes

Clostridia are frequently found in a wide range of foods (7,14–45). Anderson and coworkers (46) reviewed the problems of the food industry with spore-forming bacteria including *Clostridium*. Spore-forming bacteria are a special problem for the food industry as it is not always possible to apply sufficient heat during food processing to kill spores. *Clostridium perfringens* mainly causes food poisoning through food served in restaurants, hospitals, or homes for elderly people. The practices that lead to food poisoning are always the same: meat-containing dishes stored after cooking with insufficient cooling and reheating. Even though it should be relatively easy to control, *C. perfringens* remains one of the most common causes of food-borne diseases. Proper disinfection is necessary to control this type of food poisoning, as it is now clear that only kitchen strains of *C. perfringens* are able to produce the large amounts of enterotoxin necessary to cause food poisoning.

Fransen and coworkers (47) reported that clostridia were present in raw sludge from pig and poultry slaughterhouses at a level of 3.1 to 5.8 (in log10 N/g) (raw sludge was collected at pig ($n = 8$) and poultry ($n = 5$) slaughterhouses).

Surface Waters

In many surface waters, particularly streams and rivers, it has been shown that the principal source of SRC is municipal wastewater (30,34,35,48,49). Low counts of *C. perfringens* are generally observed in lakes and streams, draining land used for housing and grazing livestock despite relatively high levels of other indicator bacteria. Streams receiving treated wastewater contain high numbers of SRC immediately downstream of the discharge. Watercourses not receiving inputs of wastewater contain low levels of *C. perfringens* compared with fecal coliforms, whereas stream samples collected downstream of wastewater discharges contain higher levels of *C. perfringens* that decrease progressively further downstream.

A number of studies investigating the removal of indicator organisms and pathogens during the production of drinking water from river sources provide information on the levels of clostridia in surface water. Despite geographic and analytical differences (Canada, North America, and Europe), the range of counts are remarkably similar for *C. perfringens* or sulfite-reducing clostridia, ranging from less than 1 to 2,000 per 100 ml (33,35,50–52).

Bezirtzoglou and coworkers (26) determined the numbers of *C. perfringens* in river and lake waters of rural northwestern Greece. The numbers of *C. perfringens* fluctuated depending on the sampling site, ranging up to 10^2 per 200 ml in rivers with lower occurrence in lakes. It was significantly more common in the vicinity of industrial or domestic activities. Sartory (48) reported similar results for fecal clostridia (sulfite-reducing clostridia capable of growth at 44.5 °C) for an eutrophic impoundment and its feeder rivers in South Africa. While fecal clostridia levels in the river inflows ranged from 20 to 3,100 cfu per 100 ml, those in the impoundment ranged from less than 1 to 150 cfu per 100 ml. *Clostridium perfringens* made up 78% of the identified isolates.

Griffin and coworkers (53) assessed the microbial water quality in canal waters throughout the Florida Keys (United States) and determined the concentration of microbial fecal indicators and the presence of human pathogenic microorganisms. Numbers of *Clostridium* spp. ranged from less than 1 to 520 cfu per 100 ml, indicating that the canals and nearshore waters throughout the Florida Keys are being affected by human fecal material carrying human enteric viruses through current wastewater treatment strategies such as septic tanks. In the same area, effluent and waters from onshore shallow monitoring wells revealed the presence of fecal indicators in two of five nearshore wells, whereas offshore wells showed little sign of contamination.

The levels of fecal coliforms (FC), indole-positive FC (presumptive *Escherichia coli)*, fecal streptococci (FS), *Streptococcus faecalis*, and *C. perfringens* in the natural water sources used by 29 rural settlements in Sierra Leone were investigated by Wright and coworkers (54). Levels of the same indicators in human feces were also investigated. The incidence of *Salmonella* spp. in both habitats and the temperature, pH, and conductivity of water sources were also recorded. All water sources were contaminated with the indicator bacteria, with mean numbers of FC greater than those of *E. coli*, FS, *C. perfringens*, which were at a similar level, but were all in greater number than *S. faecalis*.

Clostridium difficile has been recovered from four river waters in South Wales, United Kingdom, in numbers ranging from 1 to 5 cfu per 100 ml (14 of 16 samples being positive) (55). Similar numbers, but with a lower positive sample rate (7 of 15 samples) were recorded from lakes. The majority of the isolates produced toxin A.

Marine Waters

Marine waters affected by sewage outfalls have been widely studied and clostridia provided insight on the impact of fecal pollution on beaches as well as local habitats. Davies and coworkers (56) reported their results for Australian waters. The survival of culturable fecal coliforms, fecal streptococci, and *C. perfringens* spores in freshwater and marine sediments from sites near sewage outfalls was studied. Studies using in situ membrane diffusion chambers showed that, with the exception of *C. perfringens*, die-off of the test organisms to 10% of their initial numbers occurred in both marine and freshwater sediments within 85 days. Also in Australia, a pilot study was conducted on the occurrence of sewage-derived viruses and bacteria in the beach and nearshore waters off Bondi, Sydney (57). Enteroviruses were isolated from 41% of a total of 66 sewage, seawater, grease, and sediment samples. Poliovirus vaccine strains accounted for 78% of the isolates. The number of fecal coliforms, fecal streptococci, *Aeromonas hydrophila*, and *C. perfringens* in the sewage ranged from 10^4 to 10^7 cfu per 100 mL. An initial reduction of 10^2 to 10^3 in bacterial counts was observed in the plume (the effluent's initial dilution zone) and a further reduction of 10^2 to 10^4 in counts in samples that were collected away from the plume.

In the United States, Hill and coworkers (58,59) enumerated *C. perfringens* in sediment samples collected at the Deep Water Municipal Sewage Sludge Disposal Site (also called the 106-Mile Site), off the coast of New Jersey (United States). The counts of *C. perfringens* found in sediment samples collected within and to the southwest of the 106-Mile Site were significantly elevated compared with counts of samples from reference stations of similar depth (2,400 to 2,700 m), topography, and distance from the continental shelf, indicating that the benthic environment was contaminated by sewage dumping at this site. In areas heavily impacted by sludge dumping, *C. perfringens* counts were generally highest in the top 1 cm of sediment and exceeded 9,000 cfu/g (dry weight) of sediment.

Edwards and coworkers (37) investigated the spatial distribution, movement, and impact of the untreated wastewater outfall from McMurdo Station, Antarctica, under early austral summer conditions. The benthic environment was examined to determine the distribution of *C. perfringens* in sediment cores and the intestinal contents of native invertebrates and fish along a transect of stations. These stations extended about 411 m south of the outfall. The findings revealed that the concentration of *C. perfringens* decreased with depth in the sediment and distance from the outfall. High percentages of tunicates and sea urchins were colonized with this bacterium along the transect. Coprostanol (a fecal sterol bioindicator of pollution) concentrations were also measured in sediment samples taken from each of the transect stations, and a similar trend was observed. These results are in agreement with the findings of previous studies performed with the water column and collectively provide evidence that the disposal of domestic wastes deserves special consideration in polar marine environments.

In their study of *C. difficile* occurrence, Al Saif and Brazier (55) recovered the organism from 7 of 15 seawater samples from the Bristol Channel in the United Kingdom, with counts ranging from 3 to 6 cfu per 100 ml.

Aquaculture

Pullela and coworkers (60) reported the nature and number of indicator and pathogenic microbes in fish reared using recirculating and nonrecirculating water systems. *Clostridium botulinum* was isolated from all the aquacultured fish sampled except pacu and tilapia grown in a recirculating aquaculture system, however, the counts were very low, ranging from 0.0 to 2.3 MPN/g

Hielm and coworkers (61) reported the distribution of *C. botulinum* serotypes A, B, E, and F in Finnish trout farms. *Clostridium botulinum* type E was found in 68% of the farm sediment samples, in 15% of the fish intestinal samples, and in 5% of the fish skin samples. No other serotypes were found. The spore counts determined by the most probable number method were considerably higher for the sediments than for the fish intestines and skin; the average values were 2,020, 166, and 310, *C. botulinum* type E spores per kg, respectively. The contamination rates in traditional freshwater ponds and marine net cages were high, but in concrete ponds equipped with sediment suction devices, the contamination rates were significantly lower.

Groundwater

In many parts of the world, groundwater is an important source of drinking water (wells, boreholes, etc.), and because of this the microbiological quality of water abstracted from underground sources is often monitored. Information on the presence of SRC in groundwaters is available from the scientific literature and water utility monitoring programs and databases. Surveys from Cairo (Egypt) (62) and from Teheran (Iran) (63) showed that most wells were contaminated not only by *C. perfringens* but also by other indicators of fecal contamination (total coliforms, fecal coliforms, and fecal streptococci). The presence of *C. perfringens* was influenced by the depth of the well and the extent of urbanization. Depending on the local conditions, *C. perfringens* was detected in up to 70% of sample sites within geographic areas of the city and its surroundings.

Groundwater supplying United States drinking water systems considered to be vulnerable to viral contamination, yet which were not under the direct influence of surface water nor vulnerable due to inadequate construction have been investigated (64,65). *Clostridium perfringens* was detected at one-third of the sites and was the bacterial indicator encountered least often. There was no seasonal pattern to the finding of clostridia and the relative frequency of indicator presence was not affected by the depth of the well, although the overall percentage of positive samples was. Interestingly, *C. perfringens* was not detected in any of the shallow wells (<50 ft). All indicator organisms (as above plus coliphages, F-specific RNA bacteriophages, and *Bacteroides* bacteriophages) were poorly correlated with the presence of enteric virus.

Abbaszadegan and coworkers (66) evaluated the use of the molecular methods (PCR) for detection of enteric viruses in groundwater and included clostridia as one of the potential indicator. A total of 150 samples (1,500 liters or more) were analyzed by performing cell culture assays for enteroviruses and by performing reverse transcription PCR (RT-PCR) analyses for enteroviruses, hepatitis A virus, and rotavirus. Thirteen samples (8.7%) produced cellular cytopathic effects on cell culture. By molecular methods, enteroviruses were detected in 40 of 133 samples (30.1%), hepatitis A virus in 12 of 139 samples (8.6%), and rotaviruses in 18 of 130 samples (13.8%). *Clostridium perfringens* was rarely found in this type of water (personal communication).

Clostridia were also detected as part of the microbial flora of thermal mineral water springs that are used as spas in Spain (67). The authors postulated that in the absence of *E. coli*, the presence of fecal streptococci and SRC did not represent actual fecal contamination, but rather that the organisms entered the spring from soil and hence their presence was indicative of inadequate natural filtration or insufficient protection of the spring.

In the United Kingdom, the occurrence of SRC in groundwater was reported by a water utility to be 8% over a five-year period (1990–1995) when 575 samples

were analyzed (68). Another utility investigated the quality of water drawn from a series of boreholes located in an unconfined magnesian limestone aquifer operating at depths between 70 and 200 m. Over a 20-month period sulfite-reducing clostridia were present in the absence of fecal streptococci, and fecal streptococci were isolated in the absence of sulfite-reducing clostridia. *Escherichia coli* was never isolated from what is considered to be a high-quality groundwater, with no evidence of being under the influence of surface water or other sources of contamination. Sartory (69), reviewing monitoring data from 82 groundwater sources in central England for a 27-month period reported only 2 SRC positive samples of 859.

Before the introduction of the Dutch national standard, a survey of groundwater sources used for the production of drinking water was carried out to determine the incidence of sulfite-reducing clostridia (70). During 1980–81, 585 samples were analyzed for SRC consisting of untreated groundwater ($n = 290$) and the associated final (treated) drinking water sampled at the treatment works ($n = 295$). Only one sample was positive for SRC and total coliforms were also isolated from that sample. The sample was from a plant treating groundwater that had recently introduced a new well. Clostridia were not detected in a sample taken a few days later (Havelaar, personal communication, 1996).

Bottled Waters

There is very little information on the occurrence of clostridia in bottled waters. In Europe a standard for "natural mineral waters" of absence in 50 ml has been established (71). In a survey of bottled water sold in Canada, Warburton and coworkers (72) did not detect SRC in any of the 267 samples.

Drinking Water Supplies

In Europe, SRC have been used as indicators of water quality since the beginning of the twentieth century. They were formally an intrinsic part of regulations (71), with a maximum admissible concentration, based on a most probable number analysis, of (1 per 20 ml). Monitoring was at the discretion of member states and some introduced national standards for sulfite-reducing clostridia: Netherlands (<1 per 100 ml), France (<1 per 20 ml), and Belgium (<1 per 100 ml). In the revised regulations (73), SRC have been replaced by a monitoring standard for *C. perfringens* at <1 per 100 ml, to be applied to surface water sourced supplies. In North America, neither SRC nor *C. perfringens* have been used as legal indicators of drinking water supplies.

Drinking water samples collected from several community supplies in the United States were examined by Bisson and Cabelli (25) using a newly developed medium. The source of these supplies was described as wells, springs, brooks, and surface water; many systems were not chlorinated. *Clostridium perfringens* was detected in 3.8% of chlorinated samples and 24% of samples from unchlorinated supplies. Coliforms were not recovered from any of the chlorinated samples, although enterococci were, demonstrating the relative resistance to chlorine of coliforms (least), enterococci, and clostridia (most). Although not described in the paper, it is likely that treatment was rudimentary and as a consequence, the findings are not particularly relevant to larger (and more complex) treatment systems, the most complex systems being able to efficiently remove the highest levels of bacteria.

Several studies have been undertaken to investigate the removal of pathogens and indicator organisms, including clostridia, during water treatment. Removal of clostridia ranged from less than 1 to 7 more than log removal according to the type of treatment applied (19,33,50–52,74–78).

Payment and Franco (33) proposed the use of *C. perfringens* as surrogate for the removal of pathogens and described their removal through the water treatment train of three works. When large sample volumes (100 to 1,000 liters) were used, a correlation between *C. perfringens* and human enteric viruses was observed after filtration. However, because *C. perfringens* is not present in sufficient numbers in many surface waters, their value to estimate removal is limited to sites with high fecal contamination.

As part of a national review on the occurrence of (SRC) in drinking water data derived from monitoring carried out by a number of U.K. water utilities was collated (68). Analysis of these data was complicated by differences in analytical procedures or type of samples analyzed but it does indicate that low level occurrence of SRC can be expected in normal drinking water treatment and supply (Table 1).

Distribution Systems

Few publications give information on the presence of SRC in drinking water distribution systems. Willis (79)

Table 1. Occurrence of SRC in U.K. Water Supplies

	Sample Type	*n*	SRC per 100 ml (%)	*Clostridium Perfringens* (% of SRC)
Utility 1	Treated groundwater	4,474	78(1.74)	38.5
	Treated surface water	913	30(3.29)	83.3
Utility 2	Treated surface water	65	1(1.54)	—
	Ex-service reservoirs	345	3(0.87)	—
	Distribution	870	11(1.26)	
Pooled data (3 utilities)	Treated water (unspecified)	449	12(2.67)	—

investigated a municipal water supply in England. Treatment consisted of slow sand filtration followed by chlorination. Sulfite-reducing anaerobes were detected more often in samples collected from distal parts of the distribution system than adjacent to the outlet of the treatment plants. The frequency of occurrence varied seasonally and was highest during the winter when up to 88% of samples from consumer's taps contained *C. perfringens*. Interestingly, spores were present in only 6% of these positive samples, leading the author to conclude that sulfite-reducing anaerobes were growing in the distribution system. Other studies have shown that SRC are regularly present in mains flushings (80, Meheus, personal communication 1996). SRC present in mains sediments could be the result of spores accumulating and persisting in dead ends or areas of low flow (81).

Growth of SRC in water distribution systems would be restricted to areas where amounts of oxygen are very small. Although some species require strictly anaerobic conditions, others will grow (but not produce spores) in the presence of oxygen; growth is enhanced and spores are formed only in the absence of oxygen (82). These conditions are most likely to arise within mains sediments, biofilms, and particularly corrosion tubercles. Evidence that clostridia species grow in these microenvironments within treated water distribution mains is provided in a study carried out by Emde and colleagues (83). Corrosion tubercles collected from a section of pipe removed from the distribution system contained 460 clostridia per g. Clostridia were not detected in the water drained from the same section of pipe. Organisms capable of reducing sulfate and thiosulfate were present in the same samples, indicating that clostridia may play an active role in cycling sulfur within corrosion deposits, and possibly biofilms.

REMOVAL AND DISINFECTION DURING TREATMENT

Removal

Several groups (33,50–52), have reported the removal of clostridia during the production of drinking water or during wastewater treatment. Payment and coworkers (50) reported the absence of *C. perfringens* in treated water at two water filtration plants. They later studied the elimination of human enteric viruses, coliphages, and *C. perfringens* during a conventional complete drinking-water treatment process (76). The respective concentrations (geometric mean) per 100 liters in river water were viruses, 79 mpniu, coliphages 6,565 pfu and clostridia, 11,349 cfu. After predisinfection, flocculation with alum, and settling, human enteric viruses were not detected in any of the 100 L samples (less than 4 mpniu per 100 L), but coliphages were detected in 7 of 14 samples and clostridia in 15 of 16 samples. In filtered water samples, human enteric viruses were detected in 2 of 31 samples, coliphages in 10 of 33, and clostridia in 17 of 33. Finished water was free of human enteric viruses (0 per 162 samples), but coliphages were detected in one sample (1.5 pfu/100 L) and clostridia in three, at 1.0, 4.1, and 7.0 cfu/100 L. They suggested that coliphages and clostridia, which are present in larger numbers than viruses in river water and which may have similar resistance to drinking water treatments, may be useful for estimating the level of treatment attained when large volumes of water (1,000 L or greater) are sampled and when they are present in sufficient numbers in the source water. Further studies at three water treatment plants (Payment and Franco 1993) suggested the value of *C. perfringens* as an indicator of drinking water quality and treatment efficacy with respect to chlorine-resistant pathogens such as enteric viruses and protozoan parasites.

Oxidizing Disinfectants

Venczel and coworkers (84) reported the resistance of *Cryptosporidium parvum* oocysts and *C. perfringens* spores to chlorine and other drinking water disinfectants. *Clostridium perfringens* spores have been suggested as a surrogate indicator of disinfectant activity against *Cryptosporidium parvum* and other disinfectant-resistant pathogens in water. A mixed-oxidant solution was considerably more effective than free chlorine, inactivating both microorganisms. A 5 mg/L dose of mixed oxidants produced a greater than 99.9% inactivation of *Cryptosporidium parvum* oocysts and *C. perfringens* spores in 4 hours. Free chlorine produce no measurable inactivation of *Cryptosporidium parvum* oocysts by 4 or 24 hours, although *C. perfringens* spores were inactivated by 1.4 log10 units after 4 hours.

Payment (85) reported the inactivating power of residual chlorine in a distribution system using test microorganisms (*E. coli*, *C. perfringens*, bacteriophage phi-X 170, and poliovirus type 1), which were added to drinking water samples obtained from two water treatment plants and their distribution system. Except for *E. coli*, microorganisms remained relatively unaffected in water from the distribution systems tested. When sewage was added to the water samples, indigenous thermotolerant coliforms were inactivated only when water was obtained from sites very close to the treatment plant and containing a high residual chlorine concentration. *Clostridium perfringens* was barely inactivated, suggesting that the most resistant pathogens such as *Giardia lamblia*, *Cryptosporidium parvum*, and human enteric viruses would not be inactivated. The author suggested that the maintenance of a free residual concentration in a distribution system does not provide a significant inactivation of pathogens and that it could even mask events of contamination of the distribution thus providing only a false sense of safety, with little active protection of public health.

Fujioka and colleagues (86) investigated the effectiveness of chlorine dioxide to inactivate poliovirus type 1 and indicator bacteria in sewage. They measured the fraction of organisms surviving after 10 minutes contact with a range of chlorine dioxide doses. Indicator bacteria (*C. perfringens*, total and fecal coliforms) were naturally present in sewage at sufficiently high counts. The sewage was seeded with poliovirus. Although the other indicators were inactivated by 3.2 to 3.8 log10, *C. perfringens* was inactivated by only 0.5 log10 at a concentration of 3 mg/L.

Chlorine dioxide appears more effective than free chlorine in inactivating *C. perfringens* spores. Free chlorine (dose 5 mg/L, pH 7 demand-free buffer, 25 °C)

reduces the viability of a laboratory-grown suspension by 1 log10 after one hour, rising to 1.7 log10 after 24 hours (Sobsey, personal communication, 1996).

Times taken for a two Log10 (99%) reduction in counts of pure cultures following the addition of a solution of chlorine were measured by Hirata and coworkers (87). They exposed a concentration of approximately 10^5 cells/ml suspended in chlorine-demand free phosphate buffer to different concentrations of chlorine and counted surviving cells after intervals of time. Their results are expressed as the relationship between chlorine concentration and the contact time required for a 99% inactivation. From these data it is possible to calculate the resistance relative to *E. coli*, or any of the other species tested: indigenous *C. perfringens* were 90 times more resistant, whereas a laboratory strain was 30 times more resistant.

The effectiveness of chlorine and ozone in inactivating indicator bacteria and bacteriophages in effluents from four wastewater treatment plants was also investigated by Tyrrell and coworkers (88). The results demonstrate the sensitivity of fecal indicator bacteria to chlorine compared with two types of phage. *Clostridium perfringens* was found to be relatively insensitive to inactivation by chlorine and ozone, with Log10 reductions of 0.25 and 0.125, respectively.

Ultraviolet Radiation

Nieuwstad and colleagues (89) investigated five different designs of UV reactors for disinfecting treated (secondary) wastewater measuring decimal reduction values (DR) of reoviruses and a range of indicators (bacteria and bacteriophages), including spores of SRC. SRC were barely inactivated by the treatment (less than 0.5 log10). These experimental data are borne out by findings from operational UV wastewater treatment plants in Hawaii (90) and Canada (91).

Although there are few comparative data, it is apparent that clostridia are considerably more resistant to chlorine-based biocides (hypochlorite, chlorine dioxide), ozone and UV irradiation than indicator organisms (bacteria and bacteriophages). On the basis of the available data it may be assumed that under conventional conditions of drinking water disinfection, there will be a negligible reduction in the numbers of viable spores of SRC.

CORRELATION WITH OTHER MICROORGANISMS

Clostridia, and in particular *C. perfringens*, were proposed as indicators of fecal contamination as early as 1899 (92). The spores of SSRC are capable of surviving in water and sediments for considerable periods persisting long after coliforms and other fecal indicators have disappeared. Because of their persistence, they have traditionally been regarded as indicating intermittent or remote contamination (19). In this respect, they can be valuable, for example, in assessing whether groundwater is prone to fecal pollution, especially if the water is not disinfected before use. Bisson and Cabelli (25) suggested their use as indicators of fecal pollution. More recently it has been suggested that *C. perfringens* could be used as an indicator of drinking water quality and treatment efficacy with respect to chlorine-resistant pathogens such as enteric viruses and protozoan parasites (33).

Clostridium perfringens is routinely present in the intestinal tract of humans and other animals along with *E. coli* and fecal streptococci, albeit at lower levels. Therefore, clostridia may be expected to be present in wastewater and receiving waters in association with coliforms, *E. coli*, fecal streptococci, and other fecal organisms. The extent to which indicator bacteria persist in the environment depends, among other things, on the inherent robustness of the species involved. The ability of SRC to form environmentally hardy spores enables them to persist for long periods. This is illustrated by the use of clostridia to track the movement and dispersal of sewage solids around sea disposal sites (93). Survival studies using in situ membrane microcosm chambers have demonstrated that in freshwater sediments, fecal coliforms and fecal streptococci decrease by up to five and one order of magnitude, respectively. In comparison, spores of *C. perfringens* showed no decrease in viability after 85 days (94). In laboratory microcosms, *C. perfringens* isolated from sewage was found to be three to four times more persistent in natural river water than oocysts of *Cryptosporidium parvum* (95), although the indicator bacteria, fecal coliforms, and fecal streptococci died at a rate 50-fold greater than the oocysts.

A number of investigations examining the relationships in drinking water sources between the occurrence of protozoan parasites and clostridia have been carried out (33,49,69,96). Correlation coefficients between clostridia and protozoan parasites were determined in each of these studies. There is no consistent pattern to the relationships identified in each of these studies. Two demonstrated a correlation between the presence of clostridia spores and *Cryptosporidium* and *Giardia*, although one study revealed a negative correlation with *Giardia*. The remaining studies produced no correlation. In one of these (49), significant correlation with indicators (fecal streptococci and somatic coliphages) were only evident when data from different sampling sites were grouped. When the association was analyzed for each river separately, the correlation was not significant. The authors concluded that the relationship between the density of protozoan (oo)cysts and *C. perfringens*, or any other indicator, may vary from one aquatic system to another or possibly be site-specific for a given river. The data of Sartory (69) comprised 2,503 samples from 30 river sites in central England. There was no correlation between numbers of *Cryptosporidium* and SRC, but there was an increase in frequency of detection of *Cryptosporidium* with increasing counts of SRC.

More recent data from 45 water treatment plants along the Saint Lawrence River (Canada) over a period of three years suggested that their presence in raw water provided an estimate of the probability of occurrence of *Giardia*, *Cryptosporidium*, or human enteric viruses (35). The probability estimates were derived by logistic regression analysis and suggested relationships with fecal coliforms, total coliforms, and *C. perfringens*. The described correlation in this particular study suggest that

the use of logistic regression methods might provide a better tool for analysis.

A U.K. Water Industry Research Ltd study (68) also reported that there are insufficient data from which to estimate the incidence of clostridia in samples taken from customers' taps. Clostridia may be found in drinking water distribution systems. However, these could represent spores present in the source water and which have accumulated in mains sediments. Alternatively, they may have risen due to growth of SRC in corrosion nodules, biofilms, or sediments. It is important to establish whether SRC can grow in water distribution systems, and if so, the species that are involved and to understand their ecology. If it were shown that SRC grow in distribution systems, then these organisms have no public health significance. However, there are occasions when it is desirable to confirm (or otherwise) whether contamination is fecal in nature, for example, following detection of coliforms in the absence of *E. coli* in routine samples. In these circumstances *C. perfringens* is a more appropriate indicator than the less specific SRC.

Clostridia could be used as an index of fecal pollution and indicator of pathogens in seafood. Burkhardt and coworkers (97) studied the ability of hard-shelled clams (*Mercenaria mercenaria*) to accumulate fecal coliforms and other microorganisms (*E. coli*, *C. perfringens*, and male-specific bacteriophages) over a one year period. Bacteriophages and *C. perfringens* showed significantly higher rates of accumulation than either the fecal coliform group or *E. coli*, especially during the spring. The higher incidence of human viral gastroenteritis associated with the consumption of shellfish during this period may be a result of the extraordinary concentration of certain microorganisms, including enteric viral pathogens.

Nichols and coworkers (98) reported concurrent measurement of the sewage tracer coprostanol and fecal indicator bacteria for water and sediments from coastal waters off Sydney, Australia. Good correlations were observed for both water and sediment samples between coprostanol and the two fecal indicator organisms, fecal coliforms and *C. perfringens* spores, thereby validating the use of coprostanol as a sewage signature in this environment. In a study by Sartory (48), the ratios of median SRC : faecal coliforms (FC) and SRC : faecal streptococci increased as the sampling sites became more distant from the sewage discharge. The persistence of clostridia, particularly the spores, and their resistance to disinfection means that they are not suitable indicators of the presence of labile pathogens such as *Campylobacter*, *Shigella*, or salmonellae.

It has been suggested that *C. perfringens* could be used as an indicator of drinking water quality and treatment efficacy with respect to chlorine-resistant pathogens such as enteric viruses and protozoan parasites (33). It is argued that the resistance of clostridial spores to disinfection and their small size (1 μm), may make them useful indicators for the presence of oocysts of *Cryptosporidium* or the cysts of *Giardia*. It was suggested that the organism was most valuable as an indicator of treatment effectiveness as opposed to pathogen occurrence.

Gericke and colleagues (99) examined the occurrence of *Giardia* cysts and *Cryptosporidium* oocysts in raw and treated drinking water in South Africa. Samples were collected from ten different treatment plants over a year and examined for *Giardia*, *Cryptosporidium*, total and fecal coliforms, *Candida albicans*, and *C. perfringens*. There was no correlation between the presence of *C. perfringens* and *Giardia* or *Cryptosporidium*. *Candida albicans* and *C. perfringens* both survived treatment processes better than the parasites, and the authors concluded that their suitability as indicators for the presence of parasites is questionable. Finally, in a review of routine monitoring data from 82 boreholes and springs, Sartory (68,69) reported that the range of counts was 0 to 40 per 20 ml (859 samples). For these data, there is no relationship between SRC levels and the occurrence of *Cryptosporidium* oocysts.

CONCLUSION

Of the anaerobes that infect humans, the clostridia are the most widely studied. *Clostridium* bacteria are ubiquitous microorganisms. They are involved in a variety of human diseases, the most important of which are gas gangrene, tetanus, botulism, pseudomembranous colitis, and food poisoning. In most cases, clostridia are opportunistic pathogens; that is, one or more species establishes a point of infection in a particular site in a compromised host. Of interest in environmental microbiology, is the natural resistance of the spores of these bacteria as well their occurrence in the feces of many warm-blooded animals.

Clostridium perfringens can be used as index of fecal pollution and the spores of SRC can serve as indicator of treatment process efficiency. Their resistance to environmental conditions as well as to water treatment processes are key factors that can provide both researchers and regulators with a robust parameter of water quality. Their absence, in water that also does not contain other indicator or index microorganisms, gives further reassurance that such waters are free of the most resistant pathogens.

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COAGULATION IN WATER TREATMENT PLANTS: PATHOGEN REMOVAL.

See MICROBIAL REMOVAL BY PRETREATMENT, COAGULATION AND ION EXCHANGE

COLD-ACTIVE ENZYMES.

See COLD-ADAPTED MICROORGANISMS: ADAPTATION STRATEGIES AND BIOTECHNOLOGICAL POTENTIAL

COLD-ADAPTED MICROORGANISMS: ADAPTATION STRATEGIES AND BIOTECHNOLOGICAL POTENTIAL

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Cold-adapted, psychrophilic and psychrotrophic, microorganisms are distinguished from mesophiles by their ability to grow at temperatures around 0 °C. Because a large part of the Earth's biosphere is at temperatures below 5 °C, cold-adapted microorganisms are widely distributed in nature. To survive and grow successfully in cold environments, they have evolved a series of adaptations of all their cellular constituents, including membranes, protein synthesis, energy-generating system, enzymes, and the components responsible for nutrient uptake. These adaptation strategies and the resulting considerable biotechnological potential are described in this review after a brief consideration of the terminology and the habitats of cold-adapted microorganisms.

COLD-ADAPTED MICROORGANISMS — DEFINITION

Forster (1) was the first to call attention to the growth and reproduction of bacteria at low temperatures when

he reported that organisms isolated from fish could grow well at 0 °C. The term *psychrophilic* was first used in 1902 by Schmidt-Nielsen (2) to describe microorganisms that are able to grow and multiply at 0 °C. During the following decades, many publications have dealt with these microorganisms, and during the 1950s and 1960s, in particular, there was disagreement concerning the use and meaning of the term *psychrophilic* (3). Visible growth on laboratory media within one or two weeks at 0 °C was considered as a criterion of psychrophiles (4). Some authors noted that the optimum growth temperature of many of these microorganisms was close to that of mesophiles. Morita (3), who distinguished between psychrophilic and psychrotrophic microorganisms on the basis of their cardinal growth temperatures (optimum, maximum, and minimum), acknowledged this fact. It is his definitions that are most widely accepted: Psychrophiles (cold-loving) are characterized by a maximum growth temperature of 20 °C or below, an optimum growth temperature of 15 °C or below, and a minimum growth temperature of 0 °C or below. Psychrotrophic (cold-tolerant) microorganisms are distinguished from mesophiles by their ability to grow at 0 to 5 °C, and have optimum and maximum growth temperatures greater than 15 and 20 °C, respectively. Of course, as with all definitions, there are exceptions, and the cardinal growth temperatures of some cold-adapted microorganisms span those of psychrophiles and psychrotrophs.

ECOLOGY

It is not surprising that cold-adapted microorganisms are widely distributed in nature because cold temperatures are ecologically much more common than high temperatures. Nearly three quarters of the Earth is covered by oceans, and more than 90%, by volume, of the marine environment is characterized by a temperature of 5 °C or less. Very low temperatures are also typical of the polar regions that constitute about 14% of the Earth's surface.

The ecological niche, in which psychrophiles that grow only over a restricted temperature range, can persist and compete successfully with psychrotrophs is the permanently very cold natural environment such as deep ocean waters, some polar regions and some particular terrestrial habitats (e.g., sediments in cold caves and on glaciers). Environments that are subject to periodic, diurnal, or seasonal fluctuations in temperature are favorable to psychrotrophs, which are able to grow over a wide temperature range and to restart their metabolic activity rapidly after thawing (5,6). Possible biochemical differences between psychrophiles and psychrotrophs have been discussed (7). The lowest temperature at which microbial growth is possible is assumed to be −12 °C (8) because it is at this temperature that intracellular ice formation occurs, accompanied by increases in the intracellular solute concentrations (9).

A wide diversity of cold-adapted microorganisms can be found in all cold environments, including fresh and marine waters, polar and high alpine soils, glacier ice, cold deserts, permafrost sediments, cold caves in subarctic or mountain areas, but they are also associated with plants or cold-blooded animals (5,6,10,11). Detailed information on microorganisms living in polar sea ice, freshwater ice, snow, permafrost soils, or cold deserts can be obtained from other articles in this Encyclopedia.

Cold-adapted microorganisms contribute essentially to the processes of nutrient (C, N, P, and S) cycling and biomass production in all cold ecosystems. A wide range of metabolic activities has been detected in cold habitats (5,6,10,11), including nitrogen fixation, photosynthesis, methanogenesis, and degradation of natural or xenobiotic organic compounds (e.g., proteins, carbohydrates, lignin, hydrocarbons, and aromatics). Anaerobic respiration and *in situ* nitrification have rarely been described. Cold-adapted thiobacteria and chemoorganotrophic bacteria have also been isolated. Other challenging conditions in cold environments are low nutrient contents, low water activity and high pressure.

Some cold-tolerant bacteria, including *Xanthomonas campestris*, *Pseudomonas syringae*, and *Erwinia herbicola*, are often responsible for frost injuries in plants because they are able to induce ice nucleation in crop plants under field conditions (12). This ice nucleation activity is, on the other hand, advantageous for food processing and for the energy-saving production of artificial snow or ice (see section on Application in this article). Cold-adapted (psychrotrophic) microorganisms are responsible for food spoilage in frozen or chilled food (13). Some of them (e.g., *Clostridium botulinum*, *Bacillus cereus*, *Listeria monocytogenes*) produce toxins and are therefore pathogenic.

There are psychrophilic and psychrotrophic bacteria, yeasts, higher fungi, and microalgae (5,6,10,11,13). Independent of the origin, the ability to grow well at 0 °C is possessed by a rather limited group of bacteria, mainly gram-negative, aerobic, non−spore-forming rods. Even within this restricted group, most strains belong to the genus *Pseudomonas* and to a lesser extent to the genera *Flavobacterium*, *Alcaligenes*, *Aeromonas*, *Xanthomonas*, *Vibrio*, *Serratia*, *Escherichia*, *Proteus*, *Psychrobacter*, *Shewanella*, and others. Some strains belong to the yellow-pigmented *Flexibacter-Bacteroides-Cytophaga* phylum. *Arthrobacter*, *Bacillus*, and *Micrococcus* dominate among gram-positive cold-adapted bacteria. A great number of bacterial isolates from glaciers and cold water samples were found to be pigmented, which gives them increased protection from UV radiation and free radicals.

Cold-adapted yeasts have been reported among the genera *Candida* (the most common genus), *Torulopsis*, *Cryptococcus, Rhodotorula*, and *Saccharomyces*; they were isolated from polar ice, snow, soil, and other materials, but also from refrigerated food. The preponderant genera among cold-adapted higher fungi are *Penicillium* and *Cladosporium* (14).

Snow algae are among the best cold-adapted microorganisms with a very low optimum growth temperature (usually below 10 °C) and have sometimes a narrow growth-temperature range (3). They are distributed in snow-covered habitats where they inhabit the upper 1-cm layer of snow, giving it a red, green, yellow, or grey coloration (10). Because snow algae are fixed annually in the ice accumulation area of Himalayan glaciers, snow algal biomass and community structure recorded in glacier ice

cores could be a new information source for the study of past climate change (15).

To survive and grow successfully in cold habitats, cold-adapted microorganisms have evolved a complex range of adaptations of all their cellular constituents that will be outlined in the next section of this article.

MECHANISMS OF ADAPTATION TO COLD

Growth Characteristics

When the environmental temperature of a population of microorganisms is lowered, the growth rate decreases until a point is reached when one or more critical functions proceed so slowly as to be insufficient to support cellular requirements and cell (culture) growth ceases. The effect of temperature on overall growth rate of a microbial culture is described in basic terms by the Arrhenius law relating the exponential rise of the reaction rate to the temperature increase:

$$K = \mathrm{A}\, \mathrm{e}^{-Ea/RT}$$

where A is a constant (related to steric factors and molecular collision frequency), Ea is the activation energy, R is the gas constant, and T is the absolute temperature.

A number of other plots may be used to represent the thermal dependence of growth. These are based on alternative mathematical descriptions of microbial growth in liquid culture and include the so-called "square root equation" in which the square root of the specific growth rate (μ) is plotted against temperature in degrees Kelvin (T), as developed by Ratkowsky (16). It is from such graphic representations that the cardinal growth temperatures, discussed earlier, can be calculated. The plots for mesophiles and thermophiles are shifted to the left for Arrhenius plots and to the right for "square root" plots (i.e., to higher temperatures), of course, relative to that of psychrophiles. Otherwise, the general form of the plots is the same for organisms from different parts of the viable growth range spectrum.

Whether an Arrhenius or square root plot is used, it is usually found that the slope of the curve is greater at the high temperature compared with the low-temperature end of the scale. The reason lies in the different mechanisms that are responsible for setting the upper and lower limits of growth, particularly for cold-adapted microorganisms, as explained in the following paragraphs.

The upper limit results from the (heat) denaturation of cellular proteins. In theory, it could be a single type of protein that is denatured, if that protein was pivotal to the overall growth/survival of the microorganism. Evidence for such a proposal comes from studies of mutants of *P. uratovorans* (formerly *Micrococcus cryophilus*) that demonstrated that one or a few thermolabile aminoacyl-tRNA synthetases were the cause of the upper limit of 25 °C (17).

Thus, the reason psychrophiles have upper growth limits that are at lower temperatures than those of psychrotrophs, and why both are below those of mesophiles, is because of the particular thermolability of one or more of their proteins (enzymes) that are essential for growth. The molecular basis of that thermolability is discussed later. Here, it is pertinent to note that because heat denaturation of a protein generally occurs over a narrow range of temperature, the high-temperature slope of Arrhenius plots is steep.

Another distinction between psychrophiles and psychrotrophs is that the latter often display Arrhenius plots with an extensive plateau region of optimum growth rate, which may extend for 15 centigrade degrees or more. In contrast, the optimum growth rate of psychrophiles is not only at a lower temperature but is also over a narrower range. That does not mean necessarily that the cellular enzymes are working at optimum rates over a wider range in psychrotrophs. Indeed, it is possible that none of the enzymes may be operating at $V_{\max}$ or optimum catalytic efficiency ($V_{\max}/Km$; see following section). It does mean that there is a balance of enzyme activities in psychrotrophs, which gives more or less equivalent growth rates over a wide temperature range, reflecting the more thermally adaptive lifestyle of psychrotrophs compared with psychrophiles.

The lower growth-temperature limit of psychrophiles is usually below 0 °C and it may be very difficult to determine practically because of very slow growth rates and the need to include antifreeze in the culture medium that may further reduce the growth rate. The linear nature of the square root plot makes it possible to extrapolate the regression line so as to give a theoretical minimum, which is not possible from Arrhenius plots: the theoretical minimum for psychrophiles may be as low as −26.5 °C (18). However, it must be stressed that this estimation assumes that there has been no phase change in the system. In fact, this is a real possibility because when temperature is lowered, pure water will eventually begin to freeze leaving a more concentrated salt solution outside the microbial cells that will alter the phase behavior of the external medium. The microorganisms will accumulate intracellular compatible solutes to counteract the imposed osmotic stresses and to maintain cellular enzyme function, but a point will be reached when enzymes fail and, additionally, intracellular ice may also begin to form. Thus, there are a number of potential phase changes that will make such calculated lower temperature limits an overestimate of the true biological value.

Clearly, the basis of the upper growth-temperature limit (heat denaturation of proteins) is distinct from that of the lower limit (intracellular salt concentration and/or cell water freezing). Although the phenomenon of cold denaturation of proteins is well documented (19), it generally occurs at a temperature below −15 °C (20) and so it is not likely to be the cause of the practical lower growth-temperature limit of even cold-adapted microorganisms. However, recent evidence suggests that this may not be true in all cases (see following section).

Besides the properties of proteins, the stability and functions of membranes, as reflected by the physicochemical properties of the lipid component, will also influence the growth-temperature range. The contributions of membrane fluidity and phase changes to the upper and lower thermal limits of growth are discussed later.

Cellular Adaptation

The primary sequence of cellular proteins (and therefore the three-dimensional structure that gives them cold activity; see following section) is fixed in the genomic sequence of DNA bases, so a microorganism cannot respond to low temperatures by changing the basic structure of its proteins. It can alter the pattern of proteins made through the thermal regulation of gene expression. Changes in enzyme levels will lead to alterations in the overall cellular composition of cold-adapted microorganisms in response to changes in growth temperature. The amounts of different proteins and RNA will be altered, membrane lipid composition will change, and cell wall composition may be modified. Such phenotypic changes may be constitutive or inducible, and will modify growth rate and thus the ability of the microbe to compete with other (micro)organisms, but will have little effect on the upper or lower growth-temperature limits. That would require the selection of mutations, that is, genotypic change in, for instance, the thermal characteristics of a key enzyme that was involved in setting the upper limit of growth. Genotypic changes occur over an evolutionary timescale, in comparison with phenotypic changes that occur during the lifetime of a cell or (generally for microorganisms) a population (culture).

Sudden decreases in temperature will elicit a specific alteration in gene expression known as the cold-shock response (21,22), which is distinct from the general stress response (heat shock) in terms of both the nature of the proteins induced and regulatory aspects — for example, unlike heat shock, cold shock does not involve special "cold" sigma factors. The cold-shock response involves the induction and synthesis of cold-shock proteins. Class I cold-shock proteins are expressed at very low levels at moderate temperatures and are induced at least tenfold by cold shock, whereas Class II cold-shock proteins are synthesized at reasonable levels at moderate temperatures and are less strongly induced by cold shock. While the cold-shock response is not confined to cold-adapted microorganisms, it is relevant to consider its essential characteristics because the phenomenon has a bearing on the mechanism of cold adaptation. The cellular function that is most sensitive to inhibition by cold shock is protein synthesis, particularly the initiation of translation, and the main functions of cold-shock proteins are in protein synthesis and in mRNA folding; some may act in freeze protection, although that is less well defined.

The major cold-shock protein, CspA, belongs to a family (CspA, CspB, etc.) of two to nine small proteins (~7-kDa molecular size) and is a transcriptional regulator (23,24). They share a common structural motif comprising a β-barrel with five antiparallel strands that is found in eukaryotic Y-box proteins, which are also transcription factors and RNA-masking proteins. The CspA protein is able to bind to the characteristic Y-box base sequence of ATTGG and regulate gene expression; in *Escherichia coli*, this includes the cold-induced proteins GyrA (the α-subunit of the topoisomerase DNA gyrase) and HN-S, both of which are involved in DNA supercoiling. These proteins may also be RNA chaperones, because they have several solvent-exposed phenylalanine residues that can bind RNA and so could minimize the formation of secondary folding that is favored at low temperatures. By maintaining nascent mRNA in a linear form, these chaperones would help to protect the initiation of translation from thermal inhibition.

The regulation of cold-shock protein synthesis is multifactorial, being controlled at the levels of both transcription and translation, as well as mRNA and protein stabilities. The genes of several cold-shock proteins have AT-rich upstream regulatory elements, and CspA mRNA is more stable at low temperatures because of an unusually long 5′untranslated leader region that has a specific secondary structure. Within this 5′UTR there is a highly conserved 11-base sequence called the "cold box" that has been proposed as a repressor-binding site (25). Unlike those of housekeeping genes, the mRNAs of several cold-shock proteins are still translatable after cold shock because of the presence of a downstream box within the coding region that has a complementary sequence to one close to the ribosome-binding site-decoding region in 16S rRNA. When translational capacity decreases following a cold shock, the A-site on ribosomes becomes blocked by charged species of tRNA that leads to lower levels of the regulatory guanosine nucleotides, (p)ppGpp. The induction of cold-shock proteins such as CsdA and RbfA that are ribosomal factors, restores the ability of ribosomes to recognize and translate non-cold-shock mRNAs. An important function of CsdA is the unwinding of stable secondary structures that form in mRNA at low temperatures. In this sense the ribosome acts as a thermoregulator.

A major distinction between psychrophiles/psychrotrophs and mesophiles/thermophiles is that in the cold-adapted microorganisms, the synthesis of housekeeping gene products is not inhibited by cold shock, and the number of cold-shock proteins is usually higher and increases with the severity of the cold shock. The synthesis of one set of proteins is permanently induced by continuous growth at low temperature. These are known as cold-acclimation proteins, but little is known about their cellular functions (26). At least one cold-acclimation protein is analogous to CspA (27).

Lipids and Membranes

Lipid Composition. The membranes of microorganisms, like other organisms, contain a lipid bilayer that is essential for many of the major cellular functions, including passive and active permeability, nutrient uptake, electron transport, environmental sensing, photosynthesis, and recognition processes. All of these functions demand the maintenance of membrane stability that can be considered in terms of two properties of the lipid continuum, namely, the fluidity and the phase. Although all parts of the molecules contribute, lipid fluidity is most influenced by the fatty-acyl moieties, whereas lipid phase depends more on the nature of the head-group of the membrane lipids (28). The gel to liquid-crystalline transition as well as the bilayer (lamellar) to nonbilayer phase transition are both influenced by growth temperature. However, changes in microbial culture temperature usually lead to greater

modifications in the fatty-acyl composition than the headgroup composition of membrane lipids, and so the focus of attention has been on fluidity effects. There are very few reports of lipid phase behavior specifically in relation to cold adaptation of microorganisms. One of the best documented is a study of the behavior of lipids extracted from an endolithic lichen community living beneath rock surfaces in Antarctica (29). In comparison, there is a plethora of reports on the temperature-dependence of fatty acid composition in a wide range of microorganisms.

When growth temperature is lowered, the most frequently observed change in fatty acid composition is in the extent of unsaturation. In bacteria, it is usually the ratio of saturated to monounsaturated fatty acids, whereas in eukaryotic microorganisms, there are alterations in the proportions of saturated, monounsaturated, or polyunsaturated fatty acids (28,30). An important exception among cold-adapted bacteria to this general distinction is Antarctic marine bacteria (mainly species of *Shewanella* and *Colwellia*), which contain significant proportions of polyunsaturated fatty acids, the relative proportions of which are growth temperature–dependent (31). In gram-positive bacteria, methyl-branched fatty acids are particularly common as the acyl chains of membrane lipids (32). They are iso- and anteiso-branched, and in response to a decrease in temperature the proportion of total branched fatty acids may increase and/or the ratio of anteiso- to iso-branched fatty acids may increase (30). In all microorganisms, there is the potential for temperature-dependent changes in fatty acid acyl chain length: a decrease in temperature leads to a shortening of the average acyl chain length (30). Another feature of lipid structure that influences the gel to liquid-crystalline transition temperature is the distribution of the (usually two) fatty acids between the so-called *sn*-1 and *sn*-2 positions on the (usually) glycerol backbone of acyl membrane lipids (33).

Membrane Lipid Organisation. Thus, to summarize the preceding section, a decrease in growth temperature may increase unsaturation or branching (particularly anteiso branching) or decrease chain length. All of these changes influence the way in which the membrane lipids pack within the membrane by introducing steric constraints and modifying the degrees of freedom for molecular motion, that is, they affect both the order and viscosity of the lipid bilayer, which is summarized in the combined terminology "membrane fluidity" (28). The "homeoviscous adaptation theory" (34) proposes that following a decrease in growth temperature, the lipid composition changes so as to restore the original level of fluidity, that is, it assumes that throughout the growth-temperature range, the membrane fluidity is relatively constant.

A second theory, the "homeophasic theory," emphasizes the necessity for the maintenance of the bilayer phase (35). In response to growth temperature changes, the lipid composition changes so as to adjust for thermally-triggered changes in lipid packing that are governed by such factors as the size, charge, and water-binding capacity of the lipid molecules. These factors determine whether lipid molecules associate to form a lamellar (bilayer) phase or one of a number of nonbilayer phases, and changes in lipid composition serve to maintain the overall bilayer continuum of the membrane (28). An attempt to resolve the apparent contradictions of these two models is made in the "dynamic phase behavior model" (36), which emphasizes the need for a constant balance between the gel to liquid-crystalline (fluidity) transition and the bilayer to nonbilayer (phase) transition. A number of other theories seek to explain specific aspects of membrane function, such as the maintenance of proton permeability (which is crucial for cell bioenergetics) at low temperature (37).

Membrane Lipid Biosynthesis and Thermal Regulation. When the growth temperature of bacteria is altered, it has two effects with regard to their lipid composition. The first is a direct one on the physical properties of the lipid bilayer, to increase or decrease fluidity and alter the phase behavior. The second is an indirect effect in which temperature changes modulate the activity of the enzymes involved in fatty acid and lipid biosynthesis, which leads to alterations in either the acyl chains of existing membrane lipids (fast changes) or in the pattern of fatty acid synthetase and phospholipid products for subsequent incorporation into the membrane (slow changes). The basis of fast and slow changes lies in their metabolic origins, which to some extent depend on the bacterial type (30). In all microorganisms, fluidity and phase behavior can be modified by changes in polar lipid and fatty acid compositions. Additionally, in yeasts, fungi, and algae, there may be changes in the amount and type of sterols that regulate both aspects of membrane fluidity by modulating lipid packing and acyl chain motion (28).

Fatty acids are synthesized by a fatty acid synthetase (FAS) enzyme complex in the bacterial cytoplasm (32). The primary products of FAS are saturated fatty acids that are either straight-chain or branched-chain depending on the type of primer molecule that is used. Unsaturated fatty acids are made by two mechanisms: either as secondary products of an aerobic desaturation system within the membrane, for which the substrate is probably the preformed acyl chains of membrane lipids; or as products of a modified FAS system, which is anaerobic and produces a mixture of saturated and monounsaturated fatty acids (30,32). Generally, bacteria contain only one type of system for making fatty acids, but some that live at low temperatures in sea ice or other cold pelagic habitats, and that have polyunsaturated as well as saturated and monounsaturated fatty acids, possess both biosynthetic mechanisms. The monounsaturated fatty acids are synthesized using an anaerobic system but the polyunsaturated fatty acids are made by an aerobic desaturase mechanism (38).

Temperature influences these biosynthetic processes differently: some are regulated by (de)activation of the enzyme protein(s), others by enzyme induction (30). It is the nature of the thermosensor (or "molecular thermometer") and the biochemical mechanisms of the change that together determine whether the response is fast or slow. Temperature-triggered changes that are mediated via desaturases can occur quickly, whether activation or induction is involved, because the substrates are usually

existing membrane lipids. The product of a gene that is induced by cold shock in *Bacillus subtilis* has been identified as a membrane desaturase (39). This is consistent with the rapid response time of cold shock. In contrast, alterations in unsaturation by the anaerobic pathway or of methyl branching are slower because they must be mediated by *de novo* fatty acid (and acyl lipid) synthesis, followed by integration into the membrane. Whatever the mechanism, there is tight control of membrane lipid composition by temperature, which underpins the capability of bacteria to be thermally adaptable.

Cold-Active Enzymes

Basic Aspects. Low temperatures strongly inhibit the activity of most enzymes. As described earlier, this is described by the Arrhenius equation. Accordingly, for an enzyme, drop in temperature from 37 °C to 0 °C induces a 20 to 80 times lower activity, and sometimes more, for a mesophilic enzyme. This is one of the main factors preventing the growth of mesophilic bacteria at low temperatures because the most temperature-sensitive enzyme (catalyst) in a pathway will restrict metabolic fluxes to levels incompatible with growth requirements. In order to compensate for this thermal inhibition, psychrophiles synthesize enzymes having an up to tenfold higher specific activity at low temperatures (Fig. 1). This is in fact the main physiological adaptation to cold at the enzyme level (40). Looking at the effect of temperature on the activity of psychrophilic and mesophilic enzymes in Figure 1, one can also note three other basic features of cold-active enzymes. First, the effect of temperature on psychrophilic enzymes is less pronounced. For instance, increasing the temperature by 10 centigrade degrees produces a 1.6-times higher activity for the cold-active amylase (this is the so-called Q_{10} value) compared with a 2.3 times higher reaction rate for the mesophilic enzyme. This is the consequence of smaller values of the activation energy Ea for cold-adapted enzymes, which render chemical reactions relatively less temperature-dependent (41). Secondly, the temperature for apparent maximal activity for cold-active enzymes is shifted toward low temperatures, reflecting the weak stability of these proteins and the distortion of the active site, followed by unfolding, at moderate temperatures. Finally, the adaptation to cold is not perfect. It can be seen in Figure 1 that the specific activity of the psychrophilic enzymes at low temperatures, although very high, remains generally lower than that of the mesophilic enzymes at 37 °C. These aspects will be detailed in the following sections. It is worth mentioning that these typical features of psychrophilic enzymes also apply to cold-adapted animals and, in fact, pioneering works in the field were carried out on invertebrates and fish (40–42).

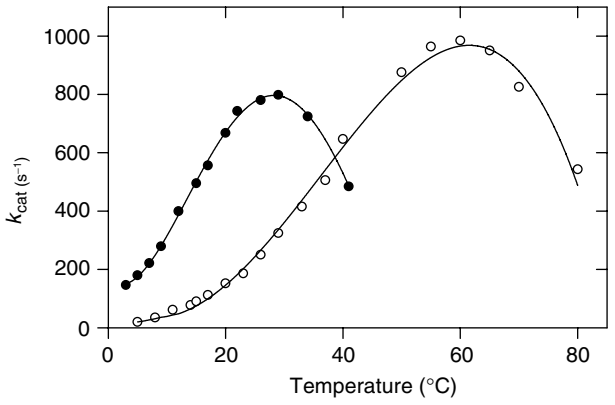

Figure 1. Effect of temperature on the activity of psychrophilic (●) and mesophilic (○) enzymes. This classical experiment, here on α-amylases from the psychrophile *Pseudoalteromonas haloplanktis* and the mesophile *Bacillus amyloliquefaciens*, illustrates the four main adaptive traits of psychrophilic enzymes. (*1*) The rate constant k_{cat} (the specific activity) is higher at low temperatures. (*2*) The Q_{10} values are lower (this is visually biased by the superior activity of the cold-active enzyme; see text for values). (*3*) The temperature for maximal activity is reduced as a result of a weaker stability. (*4*) The rate constant at the temperature of the environment nevertheless remains lower (compare k_{cat} at 3 °C for the psychrophilic enzyme and at 37 °C for the *Bacillus* enzyme). Adapted from G. Feller et al., *J. Biol. Chem.* **267**, 5,217–5,221 (1992).

Intracellular Versus Extracellular Enzymes. Extracellular enzymes secreted by bacteria and adsorbed on organic debris are thought to catalyze reactions at saturating substrate concentrations and therefore can simply increase the reaction rate constant k_{cat}. By contrast, the k_{cat}/Km ratio is the operational parameter for intracellular enzymes working at low substrate concentrations. Inspection of kinetic parameters indicates that cold-active enzymes have either increased k_{cat} or decreased Km or both, compared with mesophilic enzymes (44). It should be kept in mind that substrate binding is strongly temperature-dependent because interactions in the enzyme-substrate complex rely on weak interactions having sometimes opposite thermal dependencies: electrostatic interactions such as H-bonds, ion pairs, or Van der Waals Forces tend to be stabilized at low temperatures, whereas hydrophobic interactions are destabilized. It was noted for instance that two aromatic residues involved in substrate binding of mesophilic chitobiases are replaced by polar side chains in a cold-active enzyme, providing the possible structural basis for its 10 times lower Km value.

Structural Basis of Enzyme Adaptation to Cold. The numerous homology-based models and mainly the recently solved X-ray structures of α-amylase (45,46), citrate synthase (47), malate dehydrogenase (48), triose phosphate isomerase (49) and Ca^{2+}, Zn^{2+} protease (50) from psychrophilic bacteria have provided new insights into the molecular mechanisms of cold adaptation. The information gained from gene homology modeling has been compared with that obtained from direct structural determination from X-ray diffraction of crystals (51). The active site of psychrophilic enzymes has been subjected to careful examination, which has revealed that all amino acid residues involved in the reaction pathway, as well as all side chains pointing toward the catalytic cavity, are strictly conserved with respect to their mesophilic homologs. Therefore, point mutations are not responsible for the improved activity, contrary to Km, which should be achieved by changes

in the structure occurring distantly from the active site. The most widely accepted hypothesis accounting for the dominant adaptive traits of cold-active enzymes, that is, the high activity and the weak stability, suggests that there is a correlation among the activity, the flexibility, and the stability of the enzyme molecule (52,53). The flexible structure of psychrophilic enzymes can provide enhanced ability to undergo discrete and fast conformational changes at low temperatures imposed by the catalytic events. But the price to pay for such plasticity is, of course, the low stability of the native enzyme structure. Flexibility is a dynamic parameter, involving so-called "molecular breathing" or microunfolding, and consequently the static X-ray structures were mainly useful in revealing those structural factors responsible for the low protein stability. It was found that all structural factors currently known to stabilize the protein molecule could be attenuated in strength and number in the structure of cold-active enzymes. This involves the clustering of Gly residues (providing local mobility), the disappearance of Pro residues in loops (providing enhanced chain flexibility among secondary structures), a reduction in Arg residues that are capable of forming multiple salt bridges and H-bonds, as well as a lower number of ion pairs, aromatic interactions, or H-bonds, compared with mesophilic enzymes. The size and relative hydrophobicity of nonpolar residue clusters forming the protein core are frequently smaller, lowering the compactness of the protein interior. The N and C-caps of α-helices are also altered (weakening the charge–dipole interaction) and loose or relaxed protein extremities appear to be preferential sites for unzipping. The binding of stabilizing ions, such as calcium, can be extremely weak, with binding constants differing from mesophiles by several orders of magnitude. Finally, insertions and deletions are sometimes responsible for specific properties such as the acquisition of extra-surface charges (insertion) or the weakening of subunit interactions (deletion). Of course, all these factors are not found in every cold-active enzyme: each enzyme adopts its own strategy by using one or a combination of these altered structural factors in order to improve the local or global mobility of the protein edifice.

Recently, the surface of the molecule was also implicated in cold adaptation. Calculations of the electrostatic potential revealed an excess of negative charges at the surface of the molecule (47) and, indeed, the pI of cold-active enzymes is frequently more acidic than that of their mesophilic or thermophilic homologs. This has been related to improved interactions with the solvent, which could be of prime importance in the acquisition of flexibility near 0 °C (see below). Besides the balance of charges, the number of salt bridges covering the protein surface is also reduced. There is now a clear correlation between surface ion pairs and temperature adaptation, because these weak interactions significantly increase from psychrophiles to mesophiles, thermophiles, and hyperthermophiles, the latter showing Arg-mediated multiple ion pairs and interconnected salt bridge networks. Such an altered pattern of electrostatic interactions at the molecular surface is thought to improve the resilience or the "breathing" of the external shell of cold-active enzymes.

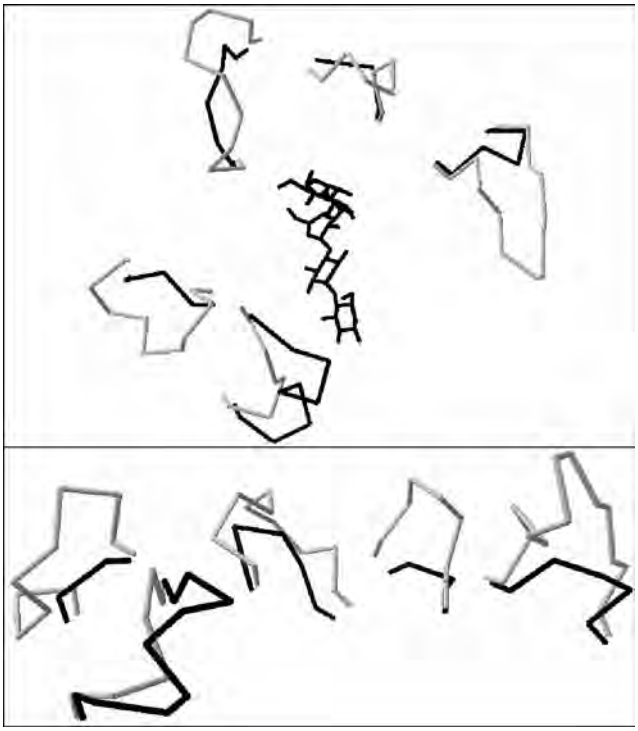

Figure 2. Active site accessibility. Upper panel: superimposition of the variable loops bordering the active site of the psychrophilic α-amylase from *Pseudoalteromonas haloplanktis* (backbone in black) and of pig pancreatic α-amylase (backbone in gray). The carbohydrate inhibitor acarbose bound to the active site is also shown (center of the figure, in black). These variable loops are markedly shorter in the cold-active enzyme. Lower panel: tangential view of the molecular surfaces facing the external medium (upper side). A carbose is removed for clarity. The loops around the catalytic cleft of the cold-active enzyme are less protruding and favor the active site accessibility. Picture generated by Swiss-PDB Viewer using data from N. Aghajari, G. Feller, C. Gerday, and R. Haser, Protein Sci. **7**, 564–572 (1998); M. Qian et al., *Biochemistry* 33, 6,284–6,294 (1994).

Another interesting feature enlightened by crystal structures arises from the calculation of the solvent-accessible area (46,47). It was noted that psychrophilic enzymes expose a higher proportion of nonpolar residues to the surrounding medium. This is an entropy-driven destabilizing factor caused by the reorganization of water molecules around exposed hydrophobic side chains (54).

The last important aspect revealed by crystallography refers to the active site that seems to be more accessible to ligands. An example of α-amylase is shown in Figure 2. This larger opening of the catalytic cleft is achieved by small deletions in loops bordering the active site or by distinct conformation of these loops, and by replacement of bulky side chains for smaller groups at the entrance. The reasons for such better accessibility are still not clearly understood but it is thought that cold-active enzymes can accommodate substrates at low-energy cost, as far as the conformational changes are concerned; it may also facilitate easier exit of products. Nevertheless, this typical feature of the active site has at least two consequences. First, nonspecific enzymes (accepting various substrates) should have a broader

specificity, because various substrates having slightly distinct conformations or sizes can fit and bind to the site. This property was in fact previously reported for a cold-active subtilisin, which is a nonspecific alkaline protease (55). As a second consequence, substrate(s) should bind less firmly in the binding site, if no point mutation(s) have occurred, giving rise to higher *Km* values. This is certainly one of the structural explanations to the observation that some cold-active enzymes, which are strongly homologous to mesophilic counterparts, have lower binding constants (44,47,52,55).

How to Reach Flexibility at Low Temperature. One can understand intuitively that low temperatures render a protein more compact or at least, less mobile. The structural factors listed earlier obviously contribute to the adaptive compensation aimed at regaining the flexibility required by catalysis at low temperatures. After a period of, say, visual inspection, the study of cold-active enzymes is now entering into a more analytical stage, which in fact converges on the general problem of protein folding. Evidently, psychrophilic proteins, as well as all extremophilic proteins, are useful models as a result of the extreme conditions imposed on the polypeptide structure and function.

A first step in this analytical approach was achieved recently by microcalorimetric studies (57). A definitive advantage of differential scanning calorimetry compared with other analytical methods is the fact that, in favorable conditions, three thermodynamic parameters related to protein unfolding are recorded directly (and not extrapolated or calculated from indirect data). The calorimetric records, or thermograms, of a psychrophilic α-amylase and of mesophilic α-amylases, including the heat-stable enzyme from *B. licheniformis* that can resist temperatures close to boiling, are illustrated in Figure 3. It can be seen that the low stability of the cold-active α-amylase is characterized by a low melting point at 44 °C (the top of the transition, corresponding to the equilibrium between the native and unfolded state) and by a low melting enthalpy (the surface below the curve, corresponding to the total amount of heat absorbed during unfolding). Deconvolution of the heat capacity function also indicates that the psychrophilic enzyme unfolds without any stable intermediate(s). This shows that all structural elements are unstable and unfold at the same time, contrary to mesophilic α-amylases that possess domains of distinct stability as illustrated for the *B. amyloliquefaciens* enzyme in Figure 3. However, in the case of phosphoglycerate kinase and chitobiase from Antarctic bacteria, it was found that the low structural stability could only affect one particular domain of the protein, giving rise to biphasic thermograms (Fig. 4). In addition, the reversibility of unfolding for these enzymes is improved because protein aggregation is not favored or does not occur at the low temperature of melting. The low hydrophobicity of the protein cores, which tend to aggregate upon solvent exposure, probably also, contributes to the reversibility of the unfolding process.

The parameters recorded by microcalorimetry were used to calculate the stability curve of the cold-active

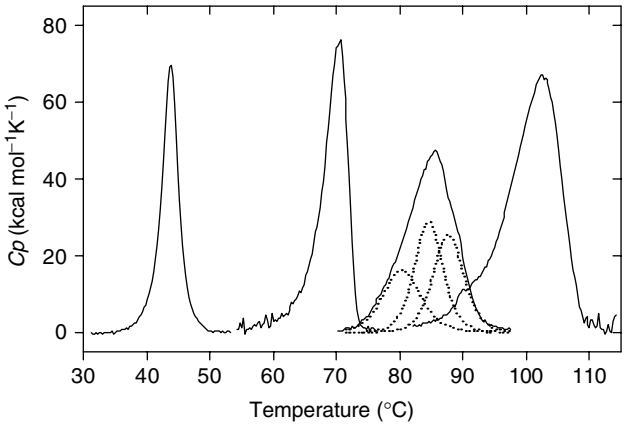

Figure 3. Thermograms of psychrophilic and mesophilic α-amylases recorded by differential scanning calorimetry. From left to right: heat-induced unfolding of α-amylases from the psychrophile *P. haloplanktis*, human salivary glands, *B. amyloliquefaciens* and *B. licheniformis*. The cold-active enzyme is characterized by a low melting point, *Tm* (temperature at the top of the transition peak) and also by a low enthalpy of unfolding, ΔH_{cal} (surface of the transition peak). Unlike the psychrophilic enzyme, all mesophilic α-amylases possess domains of distinct stability (the deconvolution into three cooperative transitions is shown as dashed lines for *B. amyloliquefaciens* α-amylase). Adapted from G. Feller, D. d'Amico, and C. Gerday, *Biochemistry* 38, 4613–4619 (1999).

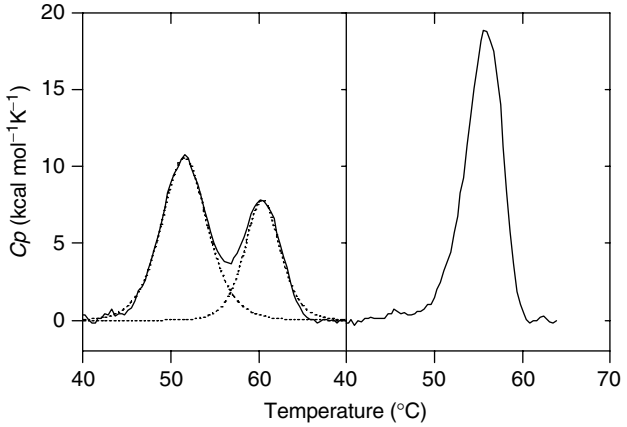

Figure 4. Thermograms of phosphoglycerate kinase. Differential scanning calorimetry thermograms of the enzymes isolated from the Antarctic psychrophile *Pseudomonas* sp. TACII18 (left) and from yeast (right). The psychrophilic enzyme is composed of two domains that unfold independently: a heat-labile domain and a heat-stable domain when compared with the yeast enzyme. Such an experiment reveals that the weak stability of psychrophilic enzymes can only affect one particular region of the protein, probably providing the required flexibility around the active site. Adapted from M. Bentahir et al., *J. Biol. Chem.* 275, 11147–11153 (2000).

α-amylase, that is, the Gibbs free energy of unfolding as a function of temperature (Fig. 5). This curve corresponds to the energy required to disrupt the protein structure at any given temperature and, by definition, is nil at the melting points. The weak stability of the cold-active

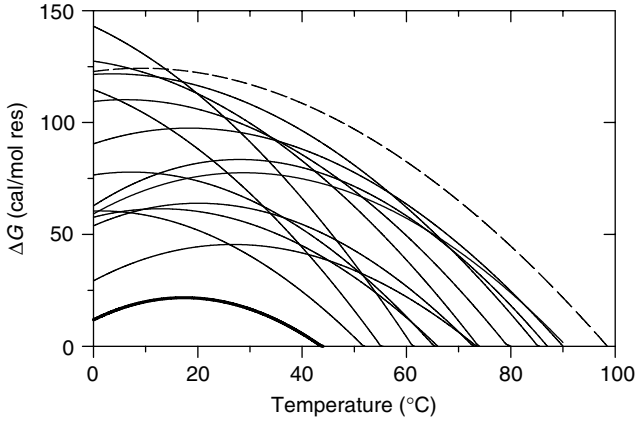

Figure 5. Stability curves of proteins from psychrophile (heavy line), mesophile (plain line) and thermophile (dashed line). Each curve displays the energy required to disrupt the protein structure (the Gibbs free energy of unfolding) as a function of the temperature. Extrapolation of the stability curve for the psychrophilic enzyme toward low temperatures indicates that cold unfolding should occur near −10 °C. Note also, that the physiological temperature for the organisms, from which the enzymes are isolated, lies on the left side of the bell-shaped curve for the psychrophilic bacteria and on the right side for both the mesophiles and the thermophiles. Adapted from G. Feller, D. d'Amico, and C. Gerday, *Biochemistry* 38, 4,613–4,619 (1999).

α-amylase is obvious in Figure 5, over the whole temperature range in which the native state prevails, that is, between both melting points. One should note that the temperature for maximal protein stability does not usually correspond to the environmental temperature of psychrophiles, mesophiles, or thermophiles. The temperature experienced by mesophiles and thermophiles lies on the right side of the bell-shaped stability curve, giving evidence that their enzymes reach the necessary balance between optimum stability (leading to a compact molecule) and flexibility (required for the catalytic function) by using the thermal dissipative force, responsible for unfolding at higher temperatures. By contrast, the environmental temperatures around 0 °C that are encountered by psychrophiles, are found far on the left side of the stability curve. We should mention at this point that the decrease in stability within this region of the curve shown in Figure 5, and ultimately leading to cold denaturation, arises from the hydration of protein groups that destabilize the protein at decreasing temperatures (54). It follows, that the origin of flexibility of psychrophilic enzymes at low temperatures involves group hydration mainly, and is therefore drastically different from mesophilic and thermophilic proteins, the latter taking advantage of the conformational entropy rise with temperature to gain in mobility. The improved interactions with the solvent of cold-active enzymes suggested by their crystal structures (see earlier section), reinforces the idea that group hydration plays an essential role in the acquisition of flexibility in the proteins of microorganisms living in permanently cold environments.

Limit of Protein Adaptation to Cold. A surprising consequence of the free energy function for the psychrophilic α-amylase shown in Figure 5 is its weak stability at low temperatures when compared with mesophilic and thermophilic proteins, whereas it was expected that cold-active proteins should also be cold-stable. This protein is in fact both heat labile and cold labile. Assuming constant properties of the solvent below 0 °C (i.e., no freezing) and the absence of protective effects from cellular components, this α-amylase should unfold at −10 °C. Incidentally, this temperature closely corresponds to the probable lower limit of bacterial growth (9) and therefore cold denaturation of some key enzymes in psychrophiles could be an additional, though unsuspected, factor contributing to the lower limit of life at low temperatures.

It was also shown that psychrophilic enzymes (or their heat-labile domains) have reached a state close to the lowest possible stability of the native state (57). Indeed, several experiments indicate that the cold-active α-amylase could not be any less stable than it is. If psychrophilic enzymes have indeed gained in flexibility at the expense of stability in the course of evolution, this implies that the actual native state precludes further adaptation toward a more mobile structure. This aspect accounts for the imperfect adaptation of the catalytic function in psychrophilic enzymes, mentioned at the beginning of this section and illustrated in Figure 1.

BIOTECHNOLOGICAL POTENTIAL

During the past decade, it has been recognized that cold-adapted microorganisms and their cellular constituents or products provide a large biotechnological potential, offering numerous economical and ecological advantages over the use of organisms and their enzymes that operate at higher temperatures (59–65).

The cultivation of microorganisms at low temperatures prevents or limits the risk of contamination with mesophilic bacteria or fungi (advantageous in continuous systems) and is energy-saving. The high activities and catalytic efficiencies of cold-adapted enzymes at low temperatures have a number of advantages: they may prevent undesired chemical transformations and the loss of volatile compounds; they can give high reaction yields from reactions involving thermosensitive components; they may shorten turn-round times for processes operated at low temperatures; they allow on-line monitoring at low temperatures; and they obviate the requirements of an increase in the enzyme concentration to compensate for the lower efficacy as is necessary when using mesophilic enzymes at low temperatures. In addition, the rapid inactivation of cold-active enzymes by mild heat treatment does not affect product quality, permits selective enzyme inactivation in a complex medium, and does not require expensive heating/cooling systems. On the other hand, low thermal stability and low reaction rates may restrict the application of cold-active enzymes.

Food Biotechnology

Food Processing. Low-temperature fermentation and ripening of food minimizes contamination by mesophilic microorganisms and provides better taste and quality of products when compared with products fermented at

higher temperatures (61,66). Psychrotrophic salt-tolerant bacteria, mainly micrococci, play an important role in flavor and taste development in the texture modification during ripening in the rind of soft cheese (67). Psychrotrophic lactic acid bacteria, mainly *Lactobacillus curvatus* and *L. sake*, have been suggested as useful starter cultures for dry sausage production (68). When ham was fermented with *Lactobacillus* sp. at 5 °C for three weeks, the desired color, cohesiveness, and consistency of the meat were obtained (66).

Cold-active enzymes are desirable for several processes in the food industry in order to improve taste, flavor, and texture, to maintain product quality and to improve preservation. Enzymatic hydrolysis of lactose, a major milk component, results in increased solubility, digestibility, and sweetness, and is conventionally carried out with ß-galactosidase enzymes obtained from mesophiles for a maximum of four hours at 30 to 40 °C or for 24 hours at 5 to 10 °C so as to limit contamination. A reduced incubation time and a minimized risk of contamination could be obtained by using cold-active ß-galactosidase (60). Such an enzyme has been isolated from psychrophilic *P. haloplanktis*; after 50 minutes at 4 °C, 33 and 12% of milk lactose was hydrolyzed by the cold-active and the commercial enzyme from *Klyveromyces lactis*, respectively (69). Commercially available thermolable microbial rennets (Marzyme II, Modilase) are useful alternatives to calf rennet in cheese manufacture, because they are rapidly heat-inactivated, and the processing of cheese does not result in residual coagulation activity when the whey is used in other dairy products (60). Transglutaminase from *Streptoverticillium* sp. that is used to improve protein texture, was shown to be useful in strengthening gels of minced fish flesh (surimi) at 10 and 25 °C (70). Biosensors (enzyme electrodes) that are functional at low temperatures could be useful for on-line monitoring and food quality control.

Ice-nucleation-active bacteria are advantageous for food processing. Nonphytopathogenic *X. campestris* has been used to freeze water at subzero temperatures above −5 °C for egg processing. High-pressure treatment at 300 Mpa and 5 °C for 5 minutes killed the cells without affecting their ice-nucleation activity. Thus, this treatment can be applied without any hygiene problems. The procedure reduced also the freezing and thawing time of egg white because of the formation of large ice crystals (71). Enhanced freeze concentration in the presence of ice-nucleation-active *X. campestris* has also been obtained for milk (72), fruit juice (73) and soy sauce (74) without loss of original flavor. Further, adding bacterial ice nucleators to food was reported to make its freeze-drying more efficient and to enhance a desirable powdering of the freeze-dried product. Antifreeze proteins (thermal hysteresis proteins) are used to improve the quality and texture of frozen food products that are eaten while in the frozen state, such as ice cream, or that are thawed before they are consumed (75).

An important aspect in food technology is preservation. According to the World Food and Agriculture Organization, hydrogen peroxide can be added to milk (0.05–0.25%) as a preservative if it is destroyed by catalase after processing (cold pasteurization). Thus, cold-active catalase is a potential enzyme for degrading the remaining hydrogen peroxide in milk at ambient temperature, which could be interesting for developing countries (76). Glucose oxidase from *Aspergillus niger* improved the preservation of shrimp at 0 to 2 °C by inhibiting psychrotrophic growth without affecting sensory factors (77).

Production of Alcoholic Beverages. A further field of application of cold-adapted microorganisms is the cost-effective production of alcoholic beverages with improved taste and aroma. Wine making at temperatures below 15 °C is not used on an industrial scale because the productivity is low. To decrease the fermentation temperature below 10 °C while retaining productivity, psychrotolerant *Saccharomyces cervisiae* strains were immobilized on solid supports, such as mineral kissiris, delignified cellulose, and gluten (78,79). The improved quality of wine produced at low temperatures can be attributed to the reduction in proportion of higher alcohols, whereas fruit esters are synthesized and retained to a greater degree, while the overall percentage of volatile compounds is increased. Further, low temperature helps the sedimentation of colloidal compounds and thus facilitates wine clarification. Beer brewed at low temperatures contains smaller amounts of higher alcohols, polyphenols, and diacetyl, with less bitterness and a lower pH than the traditional product (79).

Food Supplements. Functional mono-, di- and polysaccharides are attractive as sweeteners that cause less tooth decay. Enzymatic processes at low temperatures (5–25 °C) have been developed for the production of functional oligosaccharides, such as palatinose and cyclodextrin (80).

Long-chain polyunsaturated fatty acids (PUFA), such as docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), arachidonic acid (AA) and gamma-linolenic acid (GLA), are attracting considerable attention as dietary supplements to counteract deficiency in essential fatty acids (60,63). Currently, many PUFAs are obtained from plant seeds or fish oil but this is associated with several problems such as the undesirable fish-odor and the difficulties of large-scale purification. Some cold-adapted microorganisms have a higher proportion of unsaturated fatty acids in their lipids than mesophiles. *Mortierella alpina* was reported to produce AA in large quantities (81) and to synthesize EPA (82) at 6 to 12 °C; lowering growth temperature resulted in the increased production of EPA. Marine psychrophilic bacteria, particularly *Shewanella* spp. also produce PUFA (31). The gene cluster involved in EPA biosynthesis by a marine *Shewanella* strain has been expressed in mesophilic *E. coli* (83) and in marine cyanobacteria that can be cultured at high densities (84).

PUFA-enriched substances are also produced by microbial lipases. A psychrotrophic *Pseudomonas fluorescens* lipase (with maximum activity at 40 °C) catalyzes the selective incorporation of PUFAs in lipids at low temperature; a *Pseudomonas* sp. lipase produced a high

concentration of monoglycerides containing 40% PUFA at 10 °C ((85) and refs. therein).

Detergent Industry

A major application field of cold-active enzymes (lipases, proteases, cellulases, and amylases) is the detergent industry where the use of such enzymes allows colder washing cycles and is thus energy saving. Promising candidates for detergent additives are enzymes from cold-adapted alkaliphilic microorganisms that are not only active at low temperatures but also active and stable at alkaline pH and in presence of surfactants and oxidants.

A commercially advantageous production of a cold-active detergent lipase (Lipolase™) from the filamentous fungus *Thermomyces lanuginosus* (formerly *Humicola lanuginosa*) was achieved by cloning the lipase gene into *Aspergillus oryzae*, a suitable host for large-scale production. This enzyme has optimum activity at 35 to 40 °C and pH > 10 and is resistant toward oxidation and proteases (86).

Alkaline cellulase K from *Bacillus* sp., active at 10 °C and more active toward amorphous than to crystalline cellulose, has been added to laundry detergents as an effective component. Several microbial cold-active proteases, produced from *Bacillus*, *Paecilomyces*, and *Nocardiopsis*, and active at 10 to 20 °C, have been used in detergent manufacturing [(85) and refs. therein]. Contact lens cleaning solutions, based on an alkaline protease from a marine bacterium, were capable of cleaning and sterilization at low temperatures, in both phosphate and nonphosphate-containing detergent formulations (87).

Organic Phase Biocatalysis — Production of Fine Chemicals

Cold-active enzymes are advantageous for the enzymatic synthesis of volatile and heat-sensitive compounds. Organic phase biocatalysis (OPB) refers to the performance of enzymatic reactions in media composed wholly or partly of organic solvents. The advantages of low temperature OPB with cold-adapted enzymes include: improved biocatalysis stability at low temperatures; increased solvent choice to include polar, water-miscible, organic solvents; increased product yield; improved compatibility with heat-sensitive reactants and products; improved oxygen solubility for oxidase-catalyzed reactions. Additional advantages arise from the characteristics of cold-adapted enzymes, such as increased enzyme flexibility and activity and increased interactions with the aqueous solvent (88). Two examples of the use of cold-adapted enzymes for low temperature OPB include a lipase from psychrotrophic *Pseudomonas* sp. for ester synthesis (89) and a neutral protease from *Vibrio* sp. for peptide condensation (90).

Acrylamide is used as paper strengthener, and for the production of intermediates of pharmaceutical agents. Immobilized cold-active nitrile hydratase from *Rhodocccus* sp. and an immobilized cell system of *Pseudomonas chloraphis* have been utilized for the industrial production of acrylamide at 0 to 5 °C ((85)) and refs. therein).

Agricultural Biotechnology

Improved Symbiotic Nitrogen Fixation. Legumes constitute an important protein source; they meet most of their nitrogen (N) requirement through biological nitrogen fixation in symbiosis with rhizobia, which overcomes the high costs of nitrogen fertilizers. Cold periods during the growing season can significantly limit the establishment of this symbiosis. To improve nitrogen fixation of temperate legumes at low temperatures, the selection of cold-adapted rhizobia is a valuable tool. With Arctic rhizobia from temperate legumes *Astragalus* and *Oxytropis* it was possible to improve legume growth by 30% under controlled and field conditions, and these strains showed also better competitivity and nitrogen fixation at low temperature (91). Survival of cold-adapted rhizobia to winter freezing is advantageous for early nodulation of perennial legumes in spring. Psychrotrophic rhizobia from legumes also constitute a good genetic reservoir to transfer cold adaptation to rhizobia that are specific to legumes of agricultural importance (92).

Plant Protection. Biocontrol of plant diseases and pests is a safe alternative to chemical pesticides. Cold-adapted biocontrol agents are relevant to areas of high latitude and altitude, and to pests and diseases of winter crops, as well as postharvest diseases in cold storage. Such agents (fungi) for use in temperate soils, in which temperatures of 0 to 5 °C are common, are already commercially available: for example, "Bio-Green" (*Metarhizium anisopliae*) is used for the control of a pasture pest in southern Australia, and "Plant-Helper" (*Trichoderma atroviride*) to combat many plant diseases. Development of a cold-tolerant isolate of *Phialophora* sp. (lobed hyphopodia) for the control of the take-all disease of cereals is close to commercialization. These biocontrol agents produce antibiotics, cell wall-digestive enzymes and toxins, or induce host resistance at low temperatures (0 to 5 °C). For the efficient control of snow molds, which can attack many economical plants under a cover of snow and cause extensive damage, cold-adapted biocontrol agents (saprophytic fungi such as *Typhula phacorrhiza*, *Acremonium boreale*, *Humicola grisea*) are in development ((93) and refs. therein).

Ice-nucleation-active (Ice+) bacteria are often responsible for plant frost injuries. The introduction of indigenous or constructed *P. syringae* strains that are incapable of inducing ice nucleation can reduce the amount of Ice+ strains on crop plants (94). On the other hand, insect pests have been shown to become more vulnerable to low temperatures after the application of Ice+ bacteria (95).

Biohydrometallurgy

Biohydrometallurgical processes involve the solubilization of minerals and metals by suitable microorganisms for recovering base and precious metals. Bioleaching of metal sulfides at temperatures below 15 °C is mediated by microorganisms belonging to the genus *Acidothiobacillus* (formerly *Thiobacillus*) and particularly by cold-adapted strains of *A. ferrooxidans*. No leaching of metal sulfides occurs when these strains are not present in the

leach liquors. Case histories of low temperature biohydrometallurgical operations carried out with satisfactory bioleaching performance include the Quebrada Blanca mine in Chile (where temperatures vary from −15 to 20 °C), the El Teniente copper sulfides and oxides mine in Chile (where temperatures are below 8 °C), the San Valentino di Predoi copper mine in North Italy (the temperature is constantly 8 °C all year around), Canadian mines (constantly at 10 °C), and commercial Russian plants (temperatures below 15 °C for several months of the year) (96).

Artificial Snow and Ice

The most widespread commercial use of ice nucleators is in the energy-saving production of artificial snow. A commercial preparation of ice-nucleation-active bacteria (SNOWMAX) are added to the water used in snow-making machines to raise the critical temperature for artificial snow making by several degrees (97), or to produce ice as a construction material for installations in Arctic and Antarctic areas (98).

Low-Energy Wastewater Treatment

The temperature of wastewater treatment is an important parameter because of its effect on chemical and biological reaction rates. An effective wastewater treatment strategy with minimal energy requirement in many cold areas is the use of lagoons, the most common and economical method for treating domestic and industrial sewage in cold regions of North America (99). The temperature of typical influent wastewater during the winter months is between 0.5 and 10 °C. Aerobic, anaerobic, or facultative aerobic lagoons (ponds) consist of natural or constructed cells, whereby a major portion of organic matter entering this system is decomposed by indigenous microorganisms. Periods of ice cover change the lagoon ecology such that psychrotolerant and psychrophilic organisms are favored. Lagoons can provide significant reductions in total suspended solids, nutrients, and indicator and pathogenic microorganisms; physicochemical treatment processes can be used to enhance the final quality. An unresolved problem is the persistance of some pathogens and indicator organisms, such as *Salmonella* and fecal coliforms, because their numbers appear to be enhanced by the cold temperatures (100).

Anaerobic treatment of cold wastewaters has so far been considered as unfeasible. Recently, a cold (8–12 °C) anaerobic wastewater treatment, using two module-expanded granular-sludge bed systems, has been developed for full-scale application to low strength non- or partially-acidified wastewaters, including various industrial wastewaters and settled domestic sewage. The system provided remarkable long-term (six months) stability at low temperature, despite large variations in organic loading rate and hydraulic retention time. The enhancement of the biodegradation process can be attributed to the development of a balanced microecosystem in the sludge. The COD (chemical oxygen demand) removal efficiencies were high and comparable to those found during mesophilic or thermophilic anaerobic treatment. Implementation of these systems, combined with physicochemical resource-recovery posttreatment methods, should lead to a significant decrease in the operational costs of wastewater treatment (101).

Anaerobic digestion of municipal wastewater and animal manures at low temperatures (5–25 °C) can be used successfully to produce methane from animal manure and other organic wastes (102,103). Anaerobic digestion at 20 °C in sequencing batch reactors reduced the pollution potential of swine manure slurry by removing 85 to 95% of the soluble COD, produced important quantities of biogas, and was successful in removing odors (104).

Bioremediation of Organic Pollutants

Bioremediation of contaminated soil and water, that is, the accelerated natural biodegradation through the modification of limiting environmental factors, is an economically and sometimes logistically favorable alternative to conventional physicochemical techniques. Temperature affects the rates of biodegradation via its effect on microbial metabolism, but also through the physical nature and chemical composition of the contaminants. Cold-adapted indigenous microorganisms play a significant role in the biological decontamination of cold environments *in situ*, in which ambient temperatures often coincide with the growth-temperature range of these microorganisms.

The biodegradation of many components of petroleum hydrocarbons by indigenous cold-adapted microorganisms has been reported in a variety of terrestrial and marine cold ecosystems, including Arctic, Alpine, and Antarctic soils, Alaskan groundwater and Antarctic seawater [(105) and refs. therein]. Several oil tanker accidents, such as those of the Bahia Paraiso in Antarctica and of the Exxon Valdez in Alaska during 1989, demonstrated that temperature was not the main limiting factor for oil biodegradation, but instead the effectiveness was limited by the availability of nitrogen and phosphorus.

Comparatively little is known about biodegradation of aromatic and polycyclic aromatic compounds under cold conditions. Psychrotrophic pseudomonads have been described with the ability to degrade phenol (106), toluene (107,108) naphthalene and phenanthrene (108) at low temperatures (5 to 10 °C). Aerobic degradation of polychlorinated biphenyls with up to three chlorine substituents has been shown to occur at 4 °C in contaminated river sediment (109) and at 7 °C in Arctic soil (110). High-rate chlorophenol (tri-, tetra-, and pentachlorophenol) biodegradation in groundwater was achieved in an aerobic fluidized bed system at 5 to 7 °C, and more than 99.9% mineralization was obtained at a loading rate of 740 $mg\,l^{-1}\,day^{-1}$. The effluent quality was close to drinking water standards. This system has the advantage that it can be operated and maintained at actual groundwater temperatures and avoids the heating costs (an increase of the process temperature from 7 to 25 °C increases annual operating costs by a factor of 2.5) (111).

Di-*n*-butyl-phthalate (DBP), one of the most widely used plasticizers, is at least partially biodegradable under

anaerobic conditions at 10 °C by a psychrotrophic *P. fluorescens* (112). Simulating a Canadian subsurface environment at ambient temperature (10 °C), biotransformation of DBP was observed under aerobic, nitrate-reducing, Fe (III)-reducing and sulfate-reducing conditions (113).

Molecular Biology

There are a number of uses of cold-active enzymes in molecular biology. Alkaline phosphatase catalyzes the dephosphorylation of nucleic acids, but enzyme inactivation after the reaction is required in order not to abolish the subsequent kinase or ligase reaction. Better product retention can be obtained with a heat-labile alkaline phosphatase isolated from an Antarctic bacterium; the enzyme has an optimum temperature of 25 °C and is irreversibly inactivated after 10 min at 55 °C (114).

Uracil-DNA glycosylase (uracil *N*-glycosylase, UNG) is used to prevent carry-over contamination in the polymerase chain reaction (PCR) and must be inactivated after treatment. UNG from *E. coli* is not completely inactivated by the heat denaturation step, so a sufficient amount survives to degrade the newly-synthesized PCR product. This can be avoided by using a new heat-labile uracil-DNA glycosylase from a psychrophilic marine bacterium that is rapidly inactivated in dilute buffers at 40 °C (45 °C), with a half-life of two minutes (0.5 min) (115).

Protein expression systems for the production of cold-active proteins have recently been reviewed (63). Transfer of genetic material between mesophilic donor bacteria and psychrotrophic recipients showed that plasmid-borne genes are successfully expressed at temperatures as low as 4 °C (116,117). Cold-adapted bacteria could be exploited as cell factories for the manufacture of adventitious proteins because lowering the temperature was shown to result in an increased recovery of soluble, correctly-folded protein (118). Remaut and coworkers (119) designed an efficient expression system and introduced *E. coli*-derived expression-controlling elements into psychrotrophic hosts, using broad-host-range plasmids as vector systems. They showed that the *E. coli lacI*^{q}*-Ptrc* repressor-promoter system is functional at temperatures as low as 4 °C in two different psychrotrophic species.

CONCLUSION

A wide diversity of cold-adapted microorganisms can be found in all cold environments. To preserve membrane function and to grow at low temperatures, psychrophilic and psychrotolerant bacteria adapt their membrane lipid composition, largely through changes in the fatty-acyl components of phospholipids and glycolipids. Decreases in temperature lead to increased fatty acid unsaturation and/or methyl branching, and a shortening of acyl chain length, all of which help to maintain a relatively constant level of membrane fluidity as described by the homeoviscous theory. The alternative homeophasic theory emphasizes the need also to maintain lipid bilayer integrity at low temperatures. The changes are regulated by thermal control of cytoplasmic fatty acid synthesis enzyme systems and by membrane-bound desaturase enzymes. The tight regulation of these systems contributes to the thermal adaptability of bacteria.

Cold-active enzymes produced by psychrophilic microorganisms display a high catalytic efficiency associated with low thermal stability. These properties are beginning to become understood, and the rules governing protein adaptation to cold appear to be relatively diverse. Psychrophilic enzymes are not only of extraordinary interest at the fundamental level to understand the structure-function relationships, but also to investigate the general problem of protein folding. Increasing our knowledge of these aspects will clearly require further multidisciplinary approaches. Nevertheless, cold-adapted microorganisms have much more to give to the field of biotechnology. Indeed, the application of these extremophiles and of their enzymes offers considerable potential in the detergent and food industries, for the production of fine chemicals and in bioremediation processes.

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COLD DESERTS.

See Desert Environments — Soil Microbial Communities in Cold Deserts

COLD-INDUCED PROTEINS.

See Cold-Adapted Microorganisms: Adaptation Strategies and Biotechnological Potential

COLD SHOCK

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When an exponentially growing culture is shifted from its optimum growth temperature to a lower temperature, the organism exhibits a response called as a cold shock response. The effect of cold shock is mainly twofold: (*1*) the membrane fluidity decreases, affecting the membrane-associated functions such as active transport and protein secretion; and (*2*) the secondary structures of RNA and DNA are stabilized, which may affect the efficiency of mRNA translation and transcription (1). In addition, protein folding is too slow or inefficient, and ribosomes need to be adapted to function properly at low temperatures (2,3).

The cold shock response has been studied in detail using *Escherichia coli* and *Bacillus subtilis* as model systems (1,4–13). Enterobacteria such as *E. coli* often encounter sudden drastic temperature changes as a result of excretion from animals. Cold shock response gives this bacterium a selective advantage of quickly adapting to the new environment. The knowledge accumulated through the study of the cold shock response in *E. coli* and *B. subtilis* can be applied to other organisms.

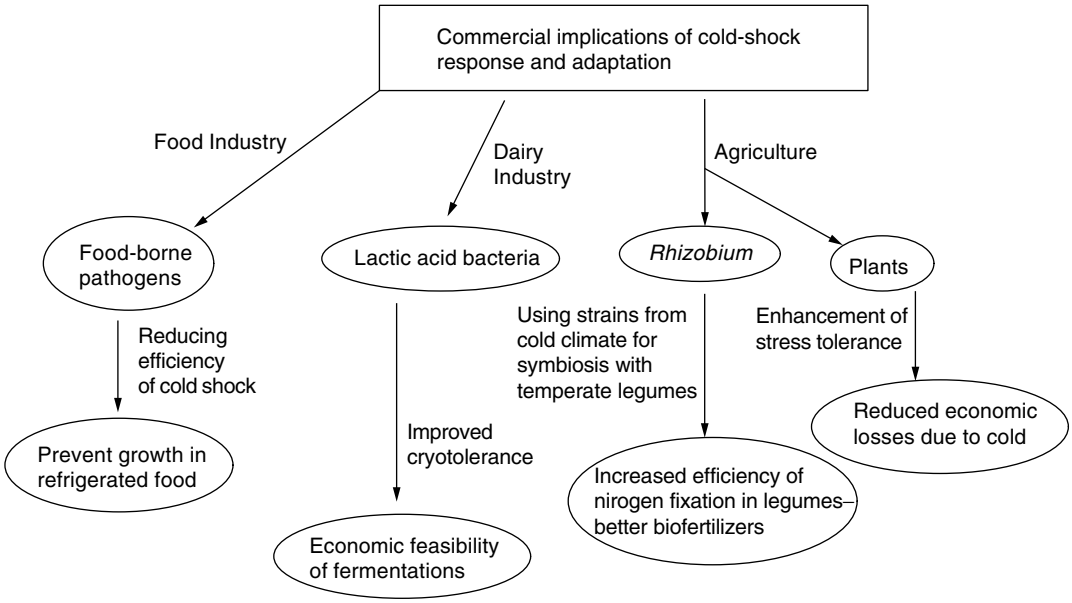

Figure 1. Commercial implications of cold shock response.

The study of cold shock response is now gaining importance because of its commercial and health implications. Figure 1 illustrates the importance of the study of cold shock response. One of the applications is in dairy industry wherein bacteria such as *Lactobacillus lactis* are used. The dairy fermentation processes involve harsh temperature changes, including lower temperatures. Therefore, it is important to use commercial lactic acid bacteria starter cultures that have been modified for better cold adaptation. This is essential to prevent losses in the activity and viability of the bacterial cultures. The economical feasibility of the fermentation process depends on this. Therefore, the study of cold shock response is important to manipulate *L. lactis* for better cold adaptation (14).

Refrigeration is a commonly used method for extending the shelf life of food. Thus, it is necessary to understand the cold shock response of food-borne pathogens. Examples of such bacteria include *Listeria* (15), *Clostridium* (16), *Pseudomonas* (17), *Enterococcus* (18), *Vibrio* (19), and *Yersinia* (20). *Listeria monocytogenes*, for example, is an opportunistic pathogen causing listeriosis, which primarily affects pregnant women, newborn babies, or immunocompromised patients, such as those suffering from AIDS (21). Thus, it is important to understand the cold shock response of these bacteria and to design means to reduce the efficiency of the cold shock response. This will prevent growth of these pathogens in the food stored at low temperatures. It is reported that if *B. subtilis* cells are cold shocked prior to freezing, the viability during the freezing is enhanced, suggesting that cold shock proteins (Csps) may directly or indirectly protect cells from the cold damage caused by freezing (22). On the basis of this report, the food-spoilage bacteria can be sensitized to damage through direct freezing. Also the viability and activity of frozen or freeze-dried commercial lactic acid bacteria starter cultures can be improved by cold shock–induced cryotolerance (14,23).

The study of cold shock response is also important in agricultural industry. *Rhizobium* is a nitrogen-fixing bacterium that lives in symbiosis with legume plants. Low temperature is one of the major limiting factors for this symbiosis in temperate climate. Thus, *Rhizobium* isolated from arctic (psychrotrophic) legumes is of considerable interest to the agricultural industry as it tries to improve the nitrogen fixation of legumes cultivated in temperate climate (24). In addition, a desaturase enzyme from a cyanobacterium, which plays an important role in maintaining the membrane fluidity after cold shock, has been shown to confer chilling resistance to tobacco plants (25). These instances imply that in the future the study of cold shock response is going to play a major role in biotechnology.

COLD SHOCK RESPONSE

The cytoplasmic membrane, ribosomes, and nucleic acids are implicated in sensing temperature changes (4). The cold shock response in *E. coli* is summarized in Figure 2 (4).

Cold Shock Response at the Level of Cytoplasmic Membrane

Bacteria exhibit a highly conserved cold shock response by adjusting the membrane lipid composition. The change in the fluidity of the membrane lipids is the first event that signals a change in temperature. The usual liquid crystalline nature of the membrane is changed to a gel-phase state upon cold shock. This affects the membrane-associated functions as the proportion of fluid (i.e., disordered lipid to ordered lipid) in the cell membrane plays an important role in membrane function. Different modes of adaptation are seen to compensate for this effect: (*1*) increasing the proportion of unsaturated fatty acids (UFAs) in the membrane lipids, (*2*) shortening of fatty

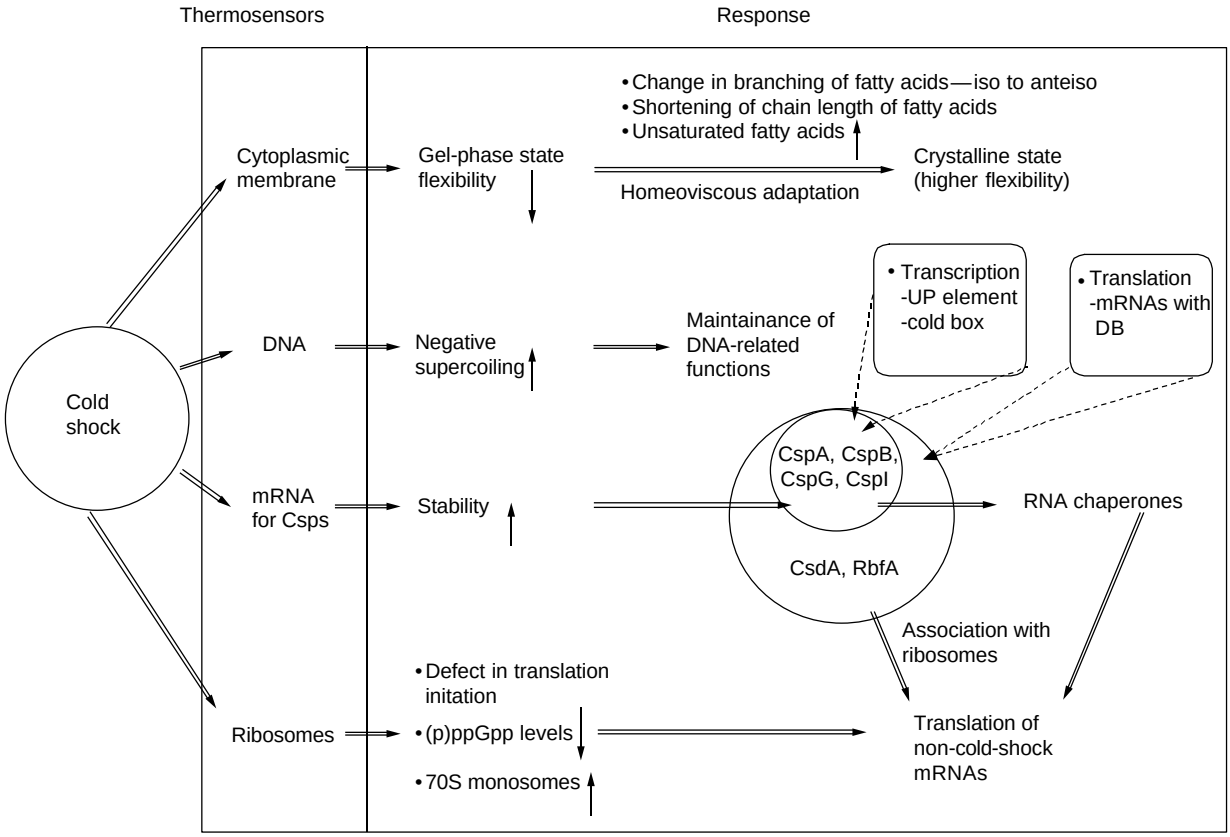

Figure 2. Model for cold shock response following the temperature downshift from 37 to 15 °C in *E. coli*. DB: downstream box and (p)ppGpp: guanosine 5′triphosphate-3′diphosphate (pppGpp) and guanosine 5′diphosphate-3′diphosphate (ppGpp) (collectively abbreviated as (p)ppGpp) (4).

acid chain length, and (*3*) alteration of fatty acid branching from *iso* to *anteiso*.

Phospholipids with UFAs have lower melting points and greater degree of flexibility than phospholipids containing saturated fatty acids. This change compensates for the negative effect of low temperature on the physical state of the lipid bilayer. This is known as *homeoviscous adaptation* (26). The increase in UFA is achieved by two mechanisms. In *E. coli*, the enzyme α-ketoacyl-acyl carrier protein synthase II converts palmitoleic acid to *cis*-vaccenic acid after the temperature downshift. Interestingly, the synthesis of this enzyme is not induced upon cold shock, but the enzyme is activated at low temperatures (27,28). On the other hand, the desaturation system in *B. subtilis* is cold-inducible. *Bacillus* (29–31) is the only nonphotosynthetic bacterium in which the presence and cold induction of desaturase is reported. The desaturase gene (*des*) transcript is barely detected at 37 °C, and following cold shock, the synthesis of a membrane *des* gene is transiently induced to 10- to 15-fold higher levels after four hours. The deletion of the *des* gene did not cause a detectable phenotype after cold shock in a rich medium (29). Recently, a two-component signal transduction system has been identified that consists of a sensor kinase DesK and a response regulator DesR responsible for the cold shock induction of the *des* gene. DesR binds to a DNA segment from the −28 to the −77 position relative to the start site of *des* gene. UFAs act as negative signaling molecules of *des* transcription. Thus, a regulatory loop composed of DesK-DesR controls the expression of the *des* gene at low temperatures (30). According to this model, DesK assumes different signaling states in response to a temperature-induced change in membrane fluidity. This is accomplished by regulating the ratio of kinase to phosphatase activity such that a phosphatase-dominant state is present at 37 °C, when membrane lipids are disordered. A kinase-dominant state predominates upon an increase in the proportion of ordered membrane lipids after a temperature downshift to 25 °C. DesK-mediated phosphorylation of DesR results in the transcriptional activation of *des*. Activation of *des* results in the synthesis of Des, which desaturates the acyl chains of membrane phospholipids. These newly synthesized UFAs inhibit *des* transcription either by favoring DesK dephosphorylation of DesR-P or by causing dissociation of DesR-P from its binding site (30).

In the case of cyanobacteria, deletion of the *des* gene caused cold sensitivity, as in these organisms, desaturation of lipids is correlated with acclimatization of photosynthetic activity at low temperatures. In these bacteria, the desaturases are bound to the thylakoid membrane. These are acyl-lipid desaturases that introduce double bonds into fatty acids that have been esterified to glycerolipids (32). They are efficient regulators of the extent of unsaturation of membrane lipids in response to temperature changes. In the case of *Synechococcus* sp., two of the desaturase genes,

desA and *desB*, are induced after temperature downshift from 38 to 22 °C. Their expression is tightly controlled by a combination of mRNA synthesis and stabilization at low temperatures (33). The introduction of the *desA* gene into the chilling-sensitive cyanobacterium *Anacystis nidulans* increased the cold resistance of this organism (34). These results suggest that desaturases are essential for the cold shock adaptation of cyanobacteria.

Using *Synechococcus* PCC 6803 strain, it was shown that the change in the fluidity of the membrane lipids is the first event that signals a change in temperature. In vivo, palladium-catalyzed hydrogenation of membrane lipids activated the transcription of *desA*, without the temperature downshift. As this catalytic technique affects only the plasma membrane, this result implies that the membrane acts as a sensor and that transcription of *desA* is enhanced in response to the altered lipid saturation (35). It was also shown that the energy produced by photosynthesis is necessary for the transcription of *desA*. Thus, the low temperature–induced desaturation of membrane lipids occurs only in the light but not in the dark. Gombos and coworkers (36,37) showed that polyunsaturated fatty acids are important for growth and for the ability to tolerate photoinhibition of photosynthesis at low temperatures. These results support the fact that the cytoplasmic membrane helps in sensing the temperature changes.

Recently, it has been shown that in *B. subtilis* the *anteiso*-branched fatty acids, and not the UFAs, are the major fraction after temperature. Using a defined minimal medium, it has been shown that cold shock adaptation of *B. subtilis* depends on the presence of isoleucine or precursors of *anteiso*-branched fatty acids. After shift to a lower temperature, the ratio of *anteiso*- to *iso*-branched fatty acids is dramatically changed. *Anteiso*-fatty acids have lower melting point than *iso* fatty acids (38). An Ile-dependent change in the fatty acid branching profile appears to be the main mechanism of cold shock adaptation of the membrane of *B. subtilis* (39). Similarly, in the case of *Listeria monocytogenes*, two modes of adaptation to lower temperature were found: (*1*) shortening of fatty acid chain length, and (*2*) alteration of braching fatty acid branching from *iso* to *anteiso*. The deficiency of *anteiso* fatty acids resulted in cold sensitivity (40).

A recent report by Weber and coworkers showed that deletion of *B. subtilis* fatty acid desaturase showed a severe cold-sensitive phenotype in the absence of isoleucine. The four UFA species of differing lengths, branching patterns, and positions of the double bond found in the wild-type strain are not synthesized in the *des* deletion mutant. Also, the mutant showed dramatically altered saturated fatty acid profile at the onset of the stationary phase in the presence of exogenous isoleucine sources. They reported that during cold shock adaptation, *des* expression could completely replace the isoleucine-dependent, long-term, fatty acid branching adaptation mechanism. They concluded that the crucial aspect in cold adaptation of the cytoplasmic membrane is not its specific molecular composition but rather its physical status in terms of its fluidity (41).

Cold Shock Response at the Level of Ribosomes

VanBogelen and Neidhardt (3) suggested that organisms sense the changes in temperature at the level of ribosomes. It was proposed that after an increase in temperature, the speed of translation is higher than that of the supply of charged tRNA; hence, the A-site of the ribosome is empty (11). On the other hand, following cold shock, translational efficiency reduced, so the A-site is blocked because of the high concentration of charged tRNA. This in turn lowers guanosine 5′triphosphate-3′diphosphate (pppGpp) and guanosine 5′diphosphate-3′diphosphate (ppGpp) (collectively abbreviated as (p)ppGpp) levels by inhibiting RelA-mediated synthesis of (p)ppGpp. Thus, the concentration of (p)ppGpp increases at high temperatures and decreases after a temperature downshift. Jones and coworkers (42) have shown that artificially induced high levels of (p)ppGpp diminish the expression of Csps, whereas low concentration increases their production. Thus, (p)ppGpp affects the magnitude of the cold shock response.

Cold Shock Response at the Level of Nucleic Acids

The extent of DNA supercoiling changes in response to various environmental factors, including exposure to chemicals and changes in osmolarity, and affects the expression of various genes (43,44). Wang and Syvanen (45) have suggested the supercoiling of DNA to be a thermosensor. To maintain DNA-related functions, such as replication, transcription, and recombination, it is important to regulate the DNA supercoiling in the cells exposed to thermal stress. DNA is usually negatively supercoiled, and in the case of *E. coli* and *B. subtilis*, negative supercoiling transiently increases after the temperature downshift (46,47). On the other hand, in *E. coli* it was seen that heat shock results in a decrease in the linking number (the number of base pairs per helical turn) (44). A change in the linking number is often due to a change in superhelical density. This change affects the normal arrangement between the −10 and −35 region of many promoters. Recognition of some σ^{70} promoters by RNA polymerase is dependent on the relative orientation of the −35 and −10 regions (45). The cold shock–inducible *E. coli recA* promoter is one such twist-sensitive promoter. It has also been shown that the change in supercoiling may be necessary to allow the DNA-dependent RNA polymerase of *B. subtilis* to start transcription from promoters of housekeeping genes after stress with unaltered efficiency (47). It has been proposed that DNA supercoiling may regulate UFA synthesis in *B. subtilis* (48).

Cold Shock Response and Csps

Microorganisms can be categorized on the basis of their temperature optima for growth. Thermophiles grow between 40 and 110 °C, whereas mesophiles grow between 10 and 50 °C. Psychrophiles and psychrotrophs can grow at or near 0 °C. The Csps are synthesized by all these three groups of organisms to counteract the deleterious effects of low temperature on RNA and DNA and consequently on transcription and translation. In the case of mesophilic

bacteria, such as *E. coli*, after the temperature downshift, there is a growth lag period during which Csps are dramatically induced, while the synthesis of most of the other proteins is arrested. The production of Csps is transient, and at the end of the growth lag period, their production is reduced to a new basal level. In the case of some mesophiles such as *B. subtilis* (22), there is no lag period after the temperature downshift and, in addition, the synthesis of non-Csps is not arrested but is reduced (49). Similarly, in the case of *L. lactis*, there is no lag period of growth after the temperature downshift (14).

As mentioned previously, *E. coli* has been extensively studied as a model system for bacterial cold shock response. In the case of this organism, proteins such as CspA (50), CspB (51), CspG (52), CspI (53), CsdA (54), RbfA (55), NusA (56), and PNP (57) are expressed at a low level at 37 °C and are dramatically induced after the temperature downshift. On the other hand, proteins such as the recombination factor RecA (58), the initiation factor IF-2 (59), the nucleoid-associated DNA-binding protein H-NS (60), the subunit of topoisomerase DNA gyrase GyrA (61), Hsc60, HscB (62), dihydrolipoamide transferase, and pyruvate dehydrogenase (63) are present at 37 °C, and their induction after cold shock is not dramatic (<10-fold). CspA, CspB, CspG, and CspI have been proposed to function as RNA and DNA chaperones; CsdA is a ribosomal associated protein with RNA unwinding activity; RbfA is a ribosomal binding factor; NusA is involved in termination and antitermination of transcription; and PNP is a ribonuclease. In addition, a trigger factor is induced after a lag of 2 to 3 hours upon cold shock, and it enhances viability at low temperatures (2). It probably helps protein synthesis and folding to continue at low temperatures. It may also help to maintain preexisting proteins in a functional form by promoting refolding of cold-damaged proteins.

Bacillus subtilis is another mesophilic bacterium, which has been studied in detail for its cold shock response. Similar to *E. coli*, *B. subtilis* also has a family of multiple CspA homologs. The three CspA homologs, CspB, CspC, and CspD, are essential for efficient growth at optimum temperature, efficient adaptation to low temperatures, and survival during stationary phase (64).

In addition to these CspA homologs, 37 other proteins were induced after temperature downshift from 37 to 15 °C in the case of *B. subtilis*. These proteins function at various levels of cellular physiology, such as chemotaxis (CheY), sugar uptake (Hpr), translation (ribosomal proteins S6 and L7/L12), protein folding (peptidyl propyl *cis*/*trans* isomerase), and general metabolism (cysteine synthase, ketol-acid reductoisomerase, glyceraldehyde dehydrogenase, and triosephosphate isomerase) (6,49). Table 1 summarizes Csps from *E. coli* and *B. subtilis*.

Table 1. Cold Shock Proteins of *E. coli* and *B. subtilis* and Their Functions

Proteins	Functions
E. coli (4)	
CspA, CspB, CspG, CspI	Proposed RNA chaperones
CsdA	RNA unwinding activity
RbfA	Ribosomal binding factor
NusA	Transcriptional termination and antitermination
PNP	Ribonuclease
RecA	Recombination factor
IF-2	Initiation factor
H-NS	Structuring chromosomal DNA
α-Subunit of DNA gyrase	DNA supercoiling
Hsc66, HscB	Cold shock molecular chaperones
Trigger factor (TF)	Maintenance and repair of proteins
Dihydrolipoamide transferase	Energy generation
Pyruvate dehydrogenase	Energy generation
B. subtilis (6,48)	
CspB, CspC, and CspD	Proposed RNA chaperones, initiation of translation
CheY	Chemotaxis
Hpr	Sugar uptake
Ribosomal proteins S6 and L7/L12	Translation
Peptidyl prolyl *cis*/*trans* isomerase	Protein folding
Cysteine synthase	Amino acid synthesis
Ketol-acid reductoisomerase	Amino acid synthesis
Glyceraldehyde dehydrogenase	Glycolysis
Triosephosphate isomerase	Glycolysis
Fructosebisphopshate aldolase	Glycolysis
Enterochelin synthase	Synthesis of enterochelin

CspA Family of Csps. The first Csp, CspA, was reported from *E. coli* (50,63) and since then its homologs, which constitute a subgroup of Csps, have been reported from a number of gram-positive and gram-negative bacteria, including psychrophilic, psychrotrophic, mesophilic, and thermophilic strains. CspA homologs have not been reported in archaea and cyanobacteria.

The CspA family of *E. coli* consists of nine homologous proteins, CspA to CspI, but among them only CspA, CspB, CspG, and CspI are cold shock–inducible. Transcription of *cspD* is dramatically induced upon stationary phase and upon glucose starvation, the induction being independent of the stationary phase sigma factor σ^s (65). CspC and CspE are produced at 37 °C and were identified originally as multicopy suppressors of a temperature-sensitive chromosomal partition mutant (66). Judging from the chromosomal location of the *csp* genes, it has been proposed that the large CspA family probably resulted from a number of gene duplications, and after subsequent adaptation, resulted in specific groups of genes that respond to different environmental stresses, for example, *cspA, cspB, cspG*, and *cspI* for cold shock, *cspD* for nutritional deprivation (1). Despite being the major Csp of *E. coli, CspA* is dispensable at both optimum and low temperatures. In a *cspA* deletion mutant, the production of CspB and CspG increased, suggesting that these proteins can compensate for CspA and probably have overlapping functions (67). Recently, it has been shown that deletion of *cspA, cspB, cspG*, and *cspE* resulted in cold sensitivity (68). CspA is differentially regulated from CspB, CspG, and CspI (53,69). The induction of CspA is observed after temperature shift from 37 to 30 °C, and high levels of CspA production are seen between 24 and 10 °C. CspB and CspG are produced only as temperature shifts below 20 °C, the maximum induction being at 15 °C. CspI is induced between 15 and 10 °C. Recently, it has been shown that CspA, CspB, and CspG are induced at low temperature under conditions that completely block protein synthesis (70). In the case of *B. subtilis*, a *cspB*/*cspC*/*cspD* triple deletion mutation was lethal, indicating that a minimum of one CspA homolog is essential for the viability of the organism, CspB being the most important of the three (64).

Regulation of CspA Family of Proteins. Expression of CspA is regulated at levels of transcription, translation, and mRNA stability. CspA is subject to transcription attenuation by virtue of the unusually long 5′ untranslated, highly conserved region (5′-UTR). It contains a unique 11-base sequence called the *cold box*, which is highly conserved in the cold shock genes. The cold box is a presumed transcriptional pausing site and is involved in the repression of cspA expression. It is proposed that CspA autoregulates its gene by attenuation of transcription (71). Immediately after the temperature downshift, RNA polymerase somehow bypasses the pausing site in the *cspA* mRNA. However, as cellular CspA concentration increases during the lag phase, CspA starts to bind its own mRNA to destabilize the elongation complex of RNA polymerase, leading to the attenuation of transcription. This is substantiated by the observation that overproduction of 5′-UTR at 15 °C resulted in the prolonged synthesis of not only CspA but also CspB and CspG (71,72). However, coproduction of CspA together with its 5′-UTR suppressed these effects.

It has also been shown that CspE negatively regulates the expression of CspA at the level of transcription (73). Using in vitro transcription assays, it was shown that CspE increases the efficiency of pausing by RNA polymerase. This is achieved by direct binding of CspE to the cold box region of *cspA*. This result is substantiated by the observation that *cspA* expression was derepressed at 37 °C in a *cspE* deletion mutant (73).

The dramatic stabilization of *cspA* mRNA (half-life more than 20 minutes) immediately following cold shock is another mechanism involved in the regulation of *cspA*. The *cspA* mRNA is extremely unstable at 37 °C (half-life less than 12 seconds). This stabilization is transient and is lost once cells are adapted to low temperatures. Deletion analysis of the *cspA* 5′-UTR showed that this region is responsible for its extreme instability at 37 °C and has positive effect on mRNA stabilization at low temperatures (74). Three base substitution mutations within the 159-base 5′-UTR caused 150-fold stabilization of *cspA* mRNA at 37 °C, which in turn resulted in the constitutive expression of *cspA* at 37 °C. This clearly indicates that the *cspA* promoter is active at 37 °C. This stabilization was found to be at least partially due to the resistance against RNase E degradation (75). It has also been suggested that *cspA* transcription is enhanced by an AT-rich sequence (upstream (UP) element) immediately upstream of the *cspA* promoter (74,76). Deletion of this region resulted in the diminished activity of the *cspA* promoter (74). In contrast to the induction of proteins by heat shock, the cold shock induction of *cspA* does not need any additional transcription factors.

Recently, significant production of CspA was detected in the early exponential phase at 37 °C. This high level of CspA expression was thought to be caused by (*1*) favorable gene dosage effect, as *cspA* maps near *oriC*; (*2*) the high concentration of its transcription activator Fis; and (*3*) higher stability of *cspA* mRNA due to lower RNase activity. With increasing cell density, the disappearance of Fis, accompanied by the accumulation of H-NS (transcriptional repressor) causes a reduction in the *cspA* transcript, and *cspA* mRNA disappears rapidly as a result of its increased rate of degradation (77). Later on, it was shown that this high level of CspA is due to the nutritional upshift and that the induction level is one-sixth of its cold shock induction level. It was also found that CspA induction can be achieved not only by the culture dilution but also by the simple addition of nutrients (78).

It has also been reported recently that polynucleotide phosphorylase selectively degrades *cspA* mRNA at 15 °C (79). It represses production of CspA homologs at the end of the lag phase. A similar phenomenon was also observed in the case of *Yersinia enterocolitica* (80). The exact mechanism of the selectivity is not known.

The *cspA* expression is also regulated at the level of translation. The *cspA* mRNA contains a sequence located 14 bases downstream of the initiation codon, designated as the downstream box (DB). The DB is also present

in CspB, CspG, CspI, CsdA, and RbfA (53,74,81,82). At 15 °C, there is a defect in the initiation of translation at low temperatures (83). It has been suggested that upon cold shock, ribosomes become nonfunctional for cellular mRNAs, except for mRNAs for Csps. DB may enhance the translation initiation by facilitating the formation of a translation preinitiation complex by binding to 16S rRNA. By having the DB, the *cspA* mRNA may be able to bypass the requirement of ribosomal factors such as RbfA and CsdA for translation initiation at low temperatures. Thus, DB increases translational efficiency for the mRNAs for Csps. After the lag period, however, cold-unadapted ribosomes are converted to cold-adapted ribosomes by acquiring cold shock ribosomal factors such as RbfA and CsdA, which are produced during the acclimation phase (84). Recently, Moll and coworkers proposed that there is no interaction between the DB and the 16S rRNA in translation initiation. However, they maintained that the DB might still enhance translation initiation in particular mRNAs. The exact mechanism of this event needs to be explored (85).

Bacillus subtilis csps, like *E. coli csps*, also have 5′-UTR regions, which are supposed to be implicated in the autoregulation of these genes. The artificial induction of CspB from *B. subtilis* in *E. coli* at 37 °C led to a marked decrease in cellular growth and a change in the pattern of protein synthesis. Hence, it was suggested that the change in the protein synthesis in response to cold shock is, at least in part, achieved by induction of Csps (86).

The CspA homologs are reported from a number of other mesophilic bacteria such as *Salmonella typhimurium* (87), *L. lactis* (14), *L. plantarum* (88), and *Enterococcus faecalis* (89). *Salmonella typhimurium* is similar to *E. coli* with respect to the regulation of the expression of CspA by mRNA stabilization and the presence of the UP element. *Lactobacillus lactis* differs from *E. coli* by lack of any lag period after the temperature downshift. Thermophilic bacteria, such as *B. caldolyticus* and *Thermotoga maritima*, also contain the CspA homologs. The CspA homologs from these bacteria are more stable than their mesophilic counterparts (90). A recent report shows that arginine3 and leucine66 confer thermostability to the CspA homolog from *B. caldolyticus* (91).

Structure and Function of CspA Homologs. CspA from *E. coli* binds RNA without apparent sequence specificity and with low binding affinity (92). It has been shown that CspA increases the translation of its own mRNA and renders the mRNA more susceptible to RNase degradation. At low temperature, secondary structures in the RNA are stabilized, and these are resistant to degradation by RNases. CspA destabilizes these structures. This is probably crucial for efficient translation at low temperature, as keeping mRNA in a linear form is an essential prerequisite for the efficient initiation of translation. Hence, CspA is proposed to act as an RNA chaperone (92). The nonspecific and weak binding of CspA to RNA and DNA is also important for the chaperone function, as this binding would not hamper ribosome movement on mRNA. CspB, CspG, and CspI are also speculated to function as RNA chaperones. *Bacillis subtilis* Csps were also proposed to act as RNA chaperones (8,64).

The three-dimensional structures of CspA from *E. coli* and CspB from *B. subtilis* have been resolved by X-ray crystallography and nuclear magnetic resonance analysis (93–97). These proteins fold in a two-state mechanism very rapidly without intermediates. They are marginally stable in solution. Two-state folding and a native-like activated state of folding seem to be inherent properties of these proteins (98,99). The protein consists of five antiparallel β-strands, β1 to β5, forming a β-barrel structure with two β-sheets. The protein has two RNA-binding motifs, RNP1 and RNP2, located on the β2 and β3 strands, respectively. The seven aromatic residues present on the surface of the protein are involved in the hydrophilic interactions between protein and nucleic acid. Mutations of three phenylalanine residues from the aromatic cluster adversely affected the DNA binding in the case of CspA from *E. coli* (100). Similarly, in the case of CspB from *B. subtilis*, the nucleic acid binding as well as the protein stability were abolished by the mutations in the two RNP sites (101,102). This surface patch has a functional role in single-stranded nucleic acid binding and it is also critical for maintaining the stability of the protein. This suggests that stability and function may have coevolved in these RNA- binding proteins. The protein has overall negative surface charge with a positively charged aromatic nucleic acid binding surface. After binding of the Csp protein to RNA, which may involve stacking of the aromatic side chains with RNA bases, the approach of other RNA for intramolecular or intermolecular base pairing will be prevented by charge repulsion. Hence, this structure is ideal for its proposed role as an RNA chaperone (8,64).

Hanna and Liu (103) demonstrated that CspE is implicated in transcription regulation. Interaction of CspE with nascent RNA during transcription elongation was detected by RNA-protein cross-linking. In addition, purified CspE was found to interfere with Q-mediated transcription antitermination. Recently, Bae and coworkers showed that CspA, CspE, and CspC decreased transcription termination at several intrinsic terminators and also decreased transcription pausing. They proposed that cold shock induction of *nusA, infB, rbfA*, and *pnp* might occur through transcription antitermination mediated by CspA and its homologs (104).

In the case of *B. subtilis*, if the levels of CspA homologs are experimentally reduced after temperature downshift, an decrease in overall protein synthesis is observed along with changes in the pattern of protein synthesis and significant reduction in the growth of the organism (64). Hence, increased levels of CspA homologs after cold shock appear to be important for compensating for higher stability of secondary structures in RNA at low temperatures.

It has been shown that CspB, CspC, and CspE can interact with RNA and single strand (ss) DNA sequence selectively. The preferred sequences for binding of these proteins are UUUUU, AGGGAGGGA residues, and AU-rich regions, respectively. This suggests that they may have additional functions than that of CspA (105). Recently, CspB from *B. subtilis* was shown to interact preferentially with polypyrimidine, especially T-based ssDNA templates (106,107). The in vivo significance of

these findings remains to be elucidated. Recently, CspC and CspE were shown to be involved in the regulation of UspA, RpoS, and subsequently RpoS-regulated stress proteins such as OsmY and Dps (108). It has also been shown that CspD forms a dimer, and with two independent β sheets interacting with ssDNA, functions as an inhibitor of DNA replication and may play a regulatory role in chromosomal replication in nutrient-depleted cells (109).

Proteins Analogous to CspA. Cyanobacteria, such as *Anabaena variabilis*, do not contain CspA homologs, but they have a family of cold-inducible RNA-binding proteins (Rbp) (110). These are similar to RNA-binding proteins from eukaryotes. These belong to the RBD (RNA-binding domain) family of proteins, which is structurally different from the CSD (cold shock domain) family of proteins. RBD and CSD seem to perform a similar function and have similar nucleic acid–binding surface, yet appear to evolve independently. This represents a very interesting case of convergent evolution in the sense that Rbp proteins could be the cyanobacterial counterparts of CspA from gram-positive and gram-negative bacteria (8,111).

In addition to Rbps, the other proteins that are induced in cyanobacteria after the temperature downshift are Clp proteins, the S21 protein in the small subunit of ribosome, and RNA helicases (112,113).

In the case of *A. variabilis*, four *rbp* genes (*rbpA1*, *rpbA2*, *rbpA3*, and *rbpC*) are regulated by cold. The level of the mRNAs for *rbp* genes increases transiently after the temperature downshift, whereas the level of corresponding proteins increases gradually and remains maximal for 24 hours after the downshift in temperature from 38 to 22 °C (112).

Transcription of the *rbpA3* gene is dependent on two different promoters, one of which is active at high temperature and whose activity is suppressed at low temperatures. The activity of the other promoter transiently increases after the temperature downshift. A putative cold-responsive *cis*-acting element in the 5′-UTR of the *rbpA1* gene has been identified. A 150-bp region between the initiation of transcription and a ribosome-binding site is absolutely necessary for its cold-induced transcription. Deletions within this region resulted in constitutive transcription at 38 and 22 °C. This suggested that transcription of the *rbpA1* gene at higher temperature might be repressed by a repressor protein. Two putative repressor proteins of about 75 and 32 kDa have been suggested to be candidates for controlling the activity of this gene. Whether these proteins are indeed the regulators of the cold shock response for this organism needs to be further explored (114).

Two genes for RNA helicases, *crhB* and *crhC*, have been identified in *Anabaena* sp. PCC 7120 (115). The *crhB* gene is expressed under a variety of stress conditions such as cold stress, salt stress, and nitrogen limitation, whereas the expression of CrhC occurs exclusively in response to cold stress. It has been suggested that CrhC has a role in the destabilization of the secondary structures of mRNAs, which allows the cells to overcome the cold-induced blockage of the initiation of translation that occurs at low temperatures (115). The cold shock induction of *crhC* is regulated at levels of transcription and translation. Similar to *cspA* of *E. coli*, *crhC* also contains an AT-rich UP element, a cold-box–like sequence, a long, highly structured 5′-UTR, and a DB.

The pattern of the cold-induced accumulation of S21 in cyanobacteria suggests that this protein may be involved in the cold acclimation of the translational apparatus, activity for which usually decreases upon exposure to cold shock (116).

Caseinolytic proteases (Clps) represent a new family of bacterial molecular chaperones that includes proteases that are expressed constitutively in some cases and induced by stress in others (117,118). ClpP1 is observed under cold stress and under UV light, and the amount of ClpP1 increases 15-fold within 24 hours of the temperature downshift (119). Deletion of its gene leads to severe impairment of growth at low temperatures.

ClpB in *Synechococcus* sp. PCC 7942 was identified initially as a heat-inducible molecular chaperone that is essential for the acquisition of thermotolerance. However, its synthesis is also strongly induced under cold stress. It has been suggested that ClpB may renature and solubilize aggregated proteins at low temperatures at which translation is repressed (120).

A low-temperature-inducible gene, *lti2*, in *A. variabilis* strain M3 was induced by temperature downshift from 38 to 22 °C, but its biological role remains to be elucidated (121).

COLD ACCLIMATION PROTEINS

Synthesis of Csps is an immediate response to cold shock, whereas cold shock acclimation proteins (Caps) are specifically synthesized during continuous growth at cold temperatures. Synthesis of Caps in mesophilic bacteria in response to continuous growth at low temperature has not been studied. *Enterococcus faecalis* is unique in the sense that despite being a mesophilic bacterium, along with 11 Csps, its cold shock response also includes 11 Caps (89).

The cold shock response of psychrophiles is well illustrated by studies on *Arthrobactor globiformis* (122,123) and *Pseudomonas fragi* (17). In the case of *A. globiformis*, four types of proteins are produced after the temperature downshift: Csps that are overexpressed only after large temperature downshift, Csps that are overexpressed after milder shocks, Caps, and late Caps. The presence of Caps is a general feature of cold-adapted microorganisms. These proteins may be involved in important metabolic function(s) at low temperature by maintaining membrane fluidity and/or by replacing cold-denatured peptides. A low-temperature-specific proteolytic system has been described for *A. globiformis* and some Caps may act as cold-specific proteases that eliminate denatured proteins whose accumulation can be detrimental to the cells. Also, unlike *E. coli*, the initiation of protein synthesis was much more resistant to a sudden decrease in temperature in the case of *P. fluorescens* (124). The CapA of *A. globiformis* is homologous to *E. coli* CspA. Posttranslational regulation has been suggested to account for the induction of the CapA mRNA. In the case of *P. fragi*, four homologs of CspA have been reported: CapA, CapB, TapA,

and TapB (temperature adaptation proteins). Persistent expression of CapA and CapB helps this organism to grow at low temperature (17).

In the case of psychrophilic and psychrotrophic bacteria, the synthesis of housekeeping proteins is never repressed following an abrupt temperature downshift, a large number of Csps are synthesized, relative level of Csps is moderate even after a severe cold shock, and synthesis of Caps is prolonged (122).

CONCLUSION

The organisms sense the temperature changes at the levels of cytoplasmic membrane, nucleic acid, and ribosomes. The content of UFAs in the membrane phospholipids increases to compensate for the reduced membrane functions at low temperatures. Shortening the fatty acid chain length and changing the branching of fatty acids from to *iso* to *anteiso* are also some of the important mechanisms for cold adaptation. Cold-inducible proteins are also important for cellular adaptation to low temperature. The cold shock response has been extensively studied in *E. coli* and *B. subtilis*. The expression of major *E. coli* Csp, CspA, is regulated at the levels of transcription, mRNA stability, and translation. Homologs of CspA are widely distributed in bacteria, and Rbps found in cyanobacteria are counterparts of CspA. Caps probably play an important role in cold acclimatization of psychrophilic bacteria.

One interesting question is why some bacteria, such as *E. coli*, have so many CspA homologs. Studies with multiple deletion strains of *cspA* homologs will help in answering this question. Csps are implicated in a number of functions, such as RNA chaperone, regulator of transcription and translation, but elaborate and extensive research is essential for thorough elucidation of their cellular roles. This is a limiting factor for the design of strategies for identifying the key regulatory elements responsible for the survival of the organism after cold shock. Understanding the expression of Csps and designing means of decreasing their accumulation can potentially reduce the efficacy of the cold shock response of food-poisoning bacteria and prevent their growth at low temperatures.

One of the major problems in expressing the recombinant proteins in *E. coli* to high levels is the formation of inclusion bodies, which are aggregates consisting mainly of the overproduced polypeptides. These proteins can refold in active conformation, but recovery yields are highly variable. Recombinant protein expression at reduced growth temperatures has the advantage of increasing the solubility of aggregation-prone recombinant proteins and limiting their degradation by heat shock proteases that are induced during the overexpression. However, efficiency of traditional promoters such as *tac* is strongly reduced at low temperatures. Hence, cold shock–inducible promoters are useful in directing the expression of recombinant proteins at low temperature. Using *cspA* promoter, β-galactosidase was efficiently expressed at low temperature (125,126).

The study of cold shock response in plants is essential for increasing stress tolerance and thus for reducing economic losses. In the case of mammals, it is gaining importance in areas such as storage of cells and organs, treatment of brain damage, and so forth. (127). These instances imply that in the future the study of the cold shock response is going to play a major role in biotechnology.

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COLD-SHOCK PROTEINS. See COLD SHOCK; COLD-ADAPTED MICROORGANISMS: ADAPTATION STRATEGIES AND BIOTECHNOLOGICAL POTENTIAL

COLIFORM BACTERIA AS INDICATORS OF WATER QUALITY

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The term *coliform bacteria* is perhaps the most common aquatic microbiological term used to define water quality in regard to fecal pollution. These bacteria are very common in the fecal tract of humans (10^7–10^9 per gram of feces), though their density is frequently exceeded by that of other fecal bacteria such as the strictly anaerobic *Bifidobacterium* (10^8–10^9 per gram) and *Bacteroides fragilis* bacteria (10^7–10^{10} per gram). Because detection of coliforms is less complex than a search for specific pathogens, these bacteria have traditionally been used as indicators of possible fecal contamination over the past century. During this time, a mass of microbiological data has been gathered from drinking water, recreational water, storm water, sewage, food-processing waste, landfill leachates, and ambient waters to demonstrate the usefulness of this indicator (1).

Coliform Bacteria: A Definition

"Coliform bacteria" by definition is an artificial grouping of convenience that encompasses a variety of common bacteria found in the intestinal tract and fecal discharges of humans and other warm-blooded animals. These organisms are gram-negative, non–spore-forming bacilli that ferment lactose (35 °C within 48 hours) with acid and gas production under facultative anaerobic conditions (2). Although this definition is useful for methodology use for multiple tube tests, the definition has been extended to the membrane filter testing approach to include detection of aldehydes. Similarly, the coliform definition was further expanded for the chemically defined test procedure to include metabolic activity for cleaving the enzyme β-D-galactosidase. Such differing characterization among coliform tests leads to a small but recognizable difference (about 5%) in the detected "coliforms" among the three different procedures.

Profile of Species. Profiling coliform species in feces reveals a number of factors that influence the makeup of the coliform population (2). The varied diet of humans and chickens appears to be a major reason there may be a large number of species and variants in strains found in the feces of these warm-blooded animals. Another factor is the exposure to antibiotics in food and medication. For example, as most humans have a predominance of *E. coli* in their intestinal tract, there may be a temporary predominance of *Klebsiella* in those individuals under medication. Coliform profiles in cows, pigs, and sheep reveal the species of coliforms to be almost exclusively *E. coli*. Fish and other cold-blooded animals have no permanent coliform flora; those present are transient in nature (lasting only a few weeks) and reflect the food and water supply in the animal's environment. Vegetation appears to have a predominance of *Klebsiella* and *Enterobacter* species that are environmental coliforms

Table 1. Enteric Bacterial Profiles in Raw Industrial Wastes

Enteric Bacteria	Occurrences (%)			
	Wood Pulp and Paper	Food Processing	Meat Processing	Municipal Sewage
Coliform species				
Escherichia coli	0.4	35.0	56.9	62.0
Klebsiella pneumoniae	92.3	55.0	21.5	18.0
Enterobacter species	6.7	3.3	13.8	14.3
Other bacterial genera				
Pectobacterium species	0.6	6.0	0.5	3.6
Salmonella species	0.008	0.7	7.3	2.1

Source: Data adapted from Herman (5).

of limited sanitary significance. This profile may change if the plants are exposed to fecal discharges of wildlife, storm water runoff, or poor-quality irrigation water. Coliforms in soil reflect contact with wildlife, agricultural use of land, or the influence of residential septic tanks and poor sewage collection systems. Thus, it is not surprising that soils gathered from remote regions of the Earth have very few fecal coliforms, whereas those in the pathway of sewage discharges may be overburdened by fecal contamination that is not reduced through natural self-purification processes in the environment. Other coliform species including *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Citrobacter freundii*, and various subspecies variants may also be detected in vegetation and soil. Furthermore, these coliforms often become the major part of the flora in a water biofilm occurrence, that is, water supply distribution systems, and in industrial processes impacted by biofouling, such as paper mill effluent and food-processing wastes, and their receiving waters downstream of discharge (1,3,4).

No single coliform species is permanently predominant in wastewaters from industrial activities, being subject to the individual processing conditions from which the organisms were introduced. However, there is a trend for certain coliform species to be found in the majority of these waters. For example, as shown in Table 1, waste in domestic sewage that does not include some industrial discharges most likely will have *E. coli* as the predominant coliform species. *Klebsiella pneumoniae* often predominates in paper mill wastes, whereas food processing discharges contain a fluctuating mix of environmental strains and some fecal contamination acquired during agricultural production.

Drinking water regulations limit the presence of coliforms in a safe water supply and when found are often the result of treatment problems or loss of distribution system integrity (Table 2). It is important to note that although a few coliforms may occasionally be tolerated in water supply (less than 5% occurrence during the month), none of these bacteria should be *E. coli*. If *E. coli* is detected, the water supply must be put on "boil water" advisory because of the concern for fecal contamination. Origin of the various non-*E. coli* strains shown in Table 2 reflects the intrusion of environmental strains from soil around a leaking or broken pipe segment or the development of a biofilm in the distribution system.

Table 2. Coliform Species Identified from 111 Public Water Distribution Systems in North America

Citrobacter	*Escherichia*
C. freundii	*E. coli*
C. diversus	*Klebsiella*
Enterobacter	*K. pneumoniae*
Enter. aerogenes	*K. rhinoscheromatis*
Enter. agglomerans	*K. oxytoca*
Enter. cloacae	*K. ozaenae*

Source: Data from water systems temporarily out of compliance in six states and Ontario Province, Canada (4).

COLIFORM SUBGROUPS AS INDICATORS

Although the majority of total coliform bacteria detected by lactose fermentation at 35 °C are found in the warm-blooded intestinal tract, the test is not specific to strains only of sanitary significance. Other coliform bacteria in the aquatic environment originate from soil and vegetation. This situation, therefore, requires careful consideration of the interpretation of total coliforms found in ambient waters and restricts their significance in treated water to that of a measure of process effectiveness.

Fecal Coliforms. In an effort to obtain a more precise measure of the coliforms associated with fecal pollution in the aquatic environment, Eijkman (6) proposed that public health authorities focus on the subgroup that produces gas from glucose at 46 °C, because coliforms that did not originate from the intestinal tract of warm-blooded animals would not survive this incubation temperature during cultivation. Unfortunately, this differential characteristic proved to be of low sensitivity. Various combinations of differential biochemical reactions (indole, methyl red, Voges-Proskauer, citrate utilization, hydrogen sulfite production, and so on) were then proposed in an effort to be more specific in this grouping of significant coliform bacteria. Again, classification was not clear-cut largely

because of the diversity of species included in the definition for all coliform bacteria (gram-negative bacilli that are non–spore-forming and facultative anaerobic, producing gas and leading to aldehyde formation from lactose fermentation within 48 hours at 35 °C).

Returning to the original concept of Eijkman, Perry and Hajna (7) redesigned the glucose medium base to include bile salts for selectivity and a buffer system as a barrier to low pH from metabolic activity in cultivation. They also reduced air temperature incubation to 45.5 °C (±0.5 °C) because even fecal coliforms die rapidly at temperatures above 46 °C. Any further reduction of temperature below 44 °C provided an opportunity for more of the environmental coliforms to survive and produce false-positive results.

Extensive studies of this concept by Geldreich and coworkers (3) on feces from a wide selection of animals, vegetation, soil, municipal waste effluents, industrial wastes, ambient freshwaters, coastal waters, and drinking water supplies led to the following conclusions:

- The most acceptable incubation temperature for differentiating the fecal group of coliforms is 44.5 °C (±0.2 °C), provided the temperature is precisely controlled in a water bath or a heat sink incubator.
- Elevated temperature incubation (44.5 °C) for positive lactose fermentation would exclude a variable small percentage of some stressed fecal coliform strains in the trade-off to eliminate those coliforms of no sanitary significance.
- Preliminary incubation for two hours at 35 °C, before selective growth at 44.5 °C, to improve recovery of any stressed fecal coliform strains may be counterproductive because some nonfecal coliforms may survive to give a false-positive result.
- Although *E. coli* is the predominant fecal coliform, there may be occasions when fecal *Klebsiella* are the organisms in dominance, such as in paper mill waste and sugar refining, but these often may be traced to combine sanitary waste introduced into plant operations.
- Provided these test conditions and environmental factors are understood, there is an excellent correlation between positive test results and those coliforms originating from the feces of warm-blooded animals.

After years of field testing and the development of specific fecal coliform procedures (A-1 medium for use in a multiple-tube procedure for testing shellfish waters and M-FC medium for the membrane filter procedure used for examining freshwater and wastewater), the fecal indicator concept has become established internationally and is used in a variety of water quality standards. The test has proven to be far superior to the total coliform concept as an indicator of sanitary significance for a variety of pollution monitoring situations.

***Escherichia coli*: A Fecal Indicator.** Efforts have been made to further narrow the detection of fecal coliforms to a specific indicator for *E. coli* (8). Rather then rely solely on fermentation in a lactose medium at 44.5 °C incubation temperature to screen out environmental strains of limited sanitary significance, detection is based on the recognition of special metabolic pathways in the test concept that is specific for most strains of *E. coli*. In this approach, *E. coli* is defined as coliform bacteria that possess the enzyme β-glucuronidase and are capable of cleaving the fluorogenic compound 4-methylumbelliferyl-β-D-glucuronide (MUG) with the corresponding release of the fluorogen when grown in EC-MUG medium at 44.5 °C within 24 hours. As is done in fecal coliform multiple-tube or membrane filter tests, this differential procedure is used as a confirmatory test, not being used directly on a water sample. Only the chromogenic chemically defined substrate medium, which incorporates the MUG reagent, can be used in a direct test of a water sample (9). In any of these procedures, fluorescence produced is observed by viewing the culture under a long wavelength ultraviolet (UV) light. The production of fluorescence by any of these methods, after appropriate culturing of the water sample, is an indication of a positive response to *E. coli* occurrence.

Unfortunately, these tests for *E. coli* and others for fecal coliforms may not detect the pathogenic *E. coli* 0157 : H7. This pathogenic *E. coli* serotype does not grow well at 44.5 °C, poorly ferments lactose, and does not produce a positive reaction in media that incorporate MUG as a reagent for differentiating *E. coli* (10). Screening for this pathogen generally is done on total coliform media incubated at 35 °C, with growth in the medium streaked onto sorbitol agar plates. Any colonial growth that produces white colonies indicates that the organism does not ferment sorbitol and may be *E. coli* 0157 : H7. These colonies are further tested for glutamate decarboxylase followed by serotyping. False-positives occasionally are encountered, one of which may be with Shigella, but because both these organisms are pathogenic, such occurrences are not considered to be a detriment to the test results.

Restricting the test to *E. coli* does result in some variable loss of recognition for other fecal coliform organisms. Approximately 30 to 40% of all warm-blooded animals, humans included, have *Klebsiella* in their intestinal flora, with individual densities ranging up to 10^8 *Klebsiella* per gram of feces (11,12). *Klebsiella* occasionally may predominate among coliforms profiled in the intestinal flora of a single individual for over a few weeks to several months. The cause often is the diet or the medical therapy. Approximately 60 to 80% of all *Klebsiella* from feces and clinical specimens are positive in the fecal coliform test. However, this trade-off in using a specific *E. coli* test also rejects the environmental strains of *Klebsiella pneumoniae* that occasionally appear when examining site-specific receiving waters downstream of nutrient waste discharges.

***Klebsiella*: A Biofilm Indicator.** The *Klebsiella* genus consists of *Klebsiella pneumoniae*, *K. oxytoca*, *K. planticola*, *K. terrigena*, *K. ozaenae*, and *K. rhinoscheromatis*. Most of these species are widely distributed in nature, occurring in soil, water, grain, fruit, vegetables, dairy products, hay, and cotton. Only those *Klebsiella* strains that are positive by the fecal coliform test (ferment lactose with gas production at 44.5 °C) are considered *K. pneumoniae* (13). This

characteristic is important because many of the commercially available differential test kits used for speciation of the coliform group do not take this parameter into account. Those coliform strains that yield biochemical profiles identical to that of *K. pneumoniae*, but negative in the fecal coliform test, may be speciated by their ability to ferment D-melizitose and grow at 10 °C. These latter strains (that should be designated as a separate species) have their origins in the aquatic environment, may be the predominant coliform in water pipe biofilm, and are of little sanitary significance.

Because environmental *K. pneumoniae* variants have been isolated from water and nutrient-conducting tissues of trees as well as from the seed embryos (14,15), it is not surprising to detect these coliforms in industrial wastes from wood pulp and paper mills. Other wastes containing significant densities of klebsiellae are textile finishing plants and sugarcane or sugar beet processing operations (16,17). Densities may range from 10^4 to 10^6 *Klebsiella* per milliliter and represent 50 to 90% of the total coliform populations in these effluents (18). As a consequence, surface water and unprotected groundwater receive *Klebsiella* from both environmental and fecal sources in storm water runoff over the water shed and effluents from municipal sewage treatment plants.

Many of the water systems reporting coliform occurrences in their distribution networks have noted that the predominant organism was a member of the *Klebsiella* genus (4,19). Most of these species have been detected in coliform-contaminated public water systems (Table 2). These organisms can be shielded by particulate materials, porous pipe sediments, biological debris, macroinvertebrates, and disinfectant demand products in the water. Furthermore, encapsulation of these coliforms can occur in hostile water environments, triggered perhaps by phosphates and depletion of iron and magnesium salts. This capsular slime coating around *Klebsiella* cells provides a measure of protection from the effects of chlorine and other disinfectants, and the organisms adjust their metabolism to survive by accumulating nutrients (assimilable organic carbon) adsorbed by pipe sediments. Once these organisms become established in the pipe environment, growth beyond meager subsistence eventually creates a biofilm that periodically sloughs cells into the flow of water traversing the pipe network.

Klebsiella colonization in the drinking water distribution system has led to much discussion and controversy about their public health significance in water supply (20,21). No evidence appears to be currently available to suggest that pathogens would similarly grow in these biofilm communities. Nevertheless, the appropriate action is to flush the entire distribution system and apply appropriate disinfection. After all, these are coliform bacteria and their presence suggests either a lack of adequate treatment at the plant or a deterioration in the cleanliness of the pipe network.

Use of a differential test for *Klebsiella* in the total coliform population of a natural water sample can provide evidence of excessive nutrients in source waters or the existence of biofilm sites in water supply distribution systems. Such a medium (M-Kleb agar) containing carbenicillin and adonitol has been used to selectively cultivate (18–24 hours at 35 °C) *Klebsiella* from other species in the total coliform population (12). Thus, parallel testing for profiling total coliform, fecal coliform, *E. coli*, and *Klebsiella* may be performed on a single water sample using appropriate membrane filter tests, and quantitative results can be achieved within 24 hours.

Opportunistic and Pathogenic Coliform Species

Although the vast majority of coliform bacteria in the warm-blooded intestinal flora are harmless, there are some serious exceptions for a few coliform strains. These opportunistic coliforms may present a public health risk to newborns, senior citizens, and immunocompromised individuals in the community. Exposure to some *E. coli* serotypes that are overt pathogens in contaminated water supply would appear to be an even more disastrous event because so many more people become ill in the community.

Opportunistic pathogens are organisms that, when found in large numbers and in the wrong place at the right time, have the potential to cause infection. Potential locations for large densities of these organisms in the water environment include dead ends and slow-flow sections of a water supply distribution pipe network, building plumbing lines that are static from minimal use, and various water line attachment devices (treatment units, humidifiers, faucet antisplash screens, and showerheads). The routes of exposure may be drinking water (ingestion), bathing or swimming (body contact), and inhalation of water vapors (air-cooling devices and fountains). For most people, these types of exposure are of no consequence because the natural defense mechanisms of the human body provide a shield to invasiveness, colonization, and infection.

Opportunistic *Klebsiella*. Among the total coliform bacteria, some *Klebsiella* strains can be opportunistic pathogens and are among the causes of illness reported in hospital records. *Klebsiella pneumoniae*, particularly antibiotic-resistant serotypes, can cause infections of the human respiratory system, genitourinary tract, nose and throat, and occasionally meningitis and septicemia (22,23). *Klebsiella*-caused infections sometimes are of apparent primary etiology, but more often are found in mixed infections or as a secondary invader (24). In the hospital environment, the nosocomial pathogen infection rate was reported to be 16.7 infections per 10,000 patients from 94 hospitals (25). *Klebsiella pneumoniae* was the cause of 1.1% of all nosocomial hospital deaths during the same period. Infections of the urinary system, lower respiratory tract, and surgical wounds were the most frequent cause of *Klebsiella*-associated illnesses or deaths.

Most of the *Klebsiella* in waterborne occurrences are not of fecal origin. In those infrequent situations in which the laboratory analyses reveal a few fecal *Klebsiella* in hospital water supply lines or their attachment devices, efforts must be made to destroy the colonization sites in an attempt to prevent amplification of this opportunistic pathogen to infective dose levels. Infective dose (ID_{50}) values for environmental and clinical isolates of *Klebsiella* have been reported to be between 3.5 and 10^1 to 7.9×10^5

cells per milliliter (26). Therefore, inhalation of moisture from vaporizers using drinking water contaminated with opportunistic fecal *Klebsiella* should be considered a risk to some individuals.

Pathogenic *E. coli.* Primary pathogens in water supply may cause illness among a large number of people in the community and frequently result in deaths among those individuals in a poor state of health (particularly individuals in extended medical care facilities), children, and senior citizens. Other factors to consider include route of exposure, application of water (ingestion, inhalation, or body contact), and dosage (density of pathogen in water). The onset of illness may begin in a few hours or may take several days after a critical exposure to contaminated water supply. The infection may result in no overt symptoms in some individuals, but in the vast majority of people infected there is pronounced illness, often involving diarrhea, bed rest, and lost of normal activity for days.

Most *E. coli* strains are harmless coliforms that live in the intestinal tract of warm-blooded animals including humans, pets (cats and dogs), farm animals (cows, pigs, sheep, and horses), and wildlife (birds, rodents, deer, and so on). However, among the bacterial, viral, and protozoan pathogens that may contaminate an unprotected or poorly treated water supply is a group of pathogenic *E. coli* that causes severe gastrointestinal illness (10). Most common victims are small children in developing countries, who have yet to develop an immunity to these exposures, and travelers to those regions. Symptoms often are watery diarrhea, abdominal pain, vomiting, and in some cases a low-grade fever and bloody stool.

Various pathogenic *E. coli* have been further characterized based on the serological and the virulence characteristics into the following subgroups: enterotoxigenic (producing toxins), enteroinvasive (colonizers of the tissues of the small intestine), or enterohemorrhagic (causing a bloody diarrhea). Incubation period for these pathogens to colonize the intestinal tract and produce symptoms is quite variable, ranging from less then 24 hours to several days, and the duration of the illness varies from three days to several weeks, depending on the virulence characteristics, health status of the person, and effectiveness of medical intervention. In every respect, these *E. coli* are primary pathogens in food and water supply contaminated by domestic sewage, livestock, or wildlife. (Details of these pathogenic strains of *E. coli* are given in E. COLI: PATHOGENIC STRAINS.)

Reassessing Coliform Interpretations

Continuing research into the science of environmental microbiology and numerous field experiences over the years have demonstrated that total coliform bacteria are not always indicators of sanitary significance (27,28). Part of the problem with erratic correlations to pathogen occurrence lies in the greater sensitivity of coliforms to disinfection agents than some waterborne agents. The other factor is that the traditional search for coliforms in water is based on the conventionally accepted protocol to test 100 ml of sample, the reason being that this amount of drinking water represents the average amount ingested at a given time. If no coliforms are found in this test volume, the assumption is that the water quality is excellent and does not provide a risk to public health. In contrast, in the search for pathogens, the sample test size is often a liter for bacterial pathogen agents and 100 liters or more for viruses and protozoan parasites. These test volume differences also apply to the microbial standards established worldwide for water quality in recreational waters, irrigation waters, and municipal wastes.

A reality check suggests that any attempt to increase the size of the test portion to one liter or more for routine coliform analysis would be impractical because increased cost for sample transport from the field to a laboratory would be significant, laboratory processing options for larger samples are limited, and possible increased interference from turbidity and other bacteria in the water sample make coliform detection difficult. Additional concerns include increased laboratory cost for additional equipment and test materials (laboratory hardware, media, and so on) and the need to upgrade operational skills for the average laboratory technician. As a side issue, many water plant operators are fearful that any change in the test baseline or decrease in the percent of coliforms permitted in total samples examined during the month might make the utility out of compliance. Similarly, changes in recreational water quality standards are often resisted by public and local government officials who are fearful that bathing beach closures will be more frequent and unreasonable. These negative concepts suggest that any primary thrust toward improved water quality through refinements in monitoring, revised standards, treatment, and compliance legislation must also include public recognition of the cost-benefit trade-off and a basic understanding of risk acceptance.

The search for a better indicator or at least a major refinement in the coliform indicator concept is in order and long overdue. Other indicator systems, such as fecal streptococci, *Clostridium*, H_2S producers, *Bacteroides*, coliphage, aerobic spore-formers, and heterotrophic bacteria, have been offered as the ultimate answer for specific situations. Each of these candidates has its champion for acceptance, but each appears to fall short of acceptance for different reasons. Considerations in the evaluation of each candidate include exclusive fecal origins, parallel resistance to that of waterborne pathogens, occurrence in sufficient densities to measure treatment process efficiency, easy speciation in the laboratory, rapid-testing capability, and cost-effectiveness in a monitoring program. These requirements are very demanding, and no known indicator system is immune to some weakness that prevents universal acceptance as a stand-alone indicator system.

Water Supply. The most subtle change over the past 20 years of public health microbiology has been the general recognition that the coliform test as used in municipal water supplies is not so much a measure of sanitary significance but more an indicator of treatment operation-effectiveness (28). It is generally agreed that properly operated water treatment systems will inactivate or

remove all coliform bacteria, whether they are of sanitary significance or are merely harmless environmental strains. There is no excuse for coliform bacteria escaping the conventional treatment barrier. Coliform data trends observed in both pilot plant research and field experience have repeatedly shown that this indicator is too coarse a measurement to be used in treatment process adjustments for better barrier protection from intrusions of protozoan agents, viruses, and opportunistic pathogens. The fact remains that many pathogenic agents may penetrate some physical and disinfectant treatment process barriers (that is, coagulation, settling, filtration, and chlorination) that normally provide a safe margin of effectiveness for total coliform removal.

More rigorous tests of treatment-effectiveness must be explored. For example, coliphage mimics many of the properties of viruses and is more cost-effective in the performance evaluation process. *Clostridium* might be another promising surrogate for testing treatment barriers, although their densities are too low in natural waters to observe the desired 6 log removals that are necessary for protection from pathogen incursions. The use of aerobic spore-formers in the evaluation of treatment performance appears to be more promising for several reasons: aerobic spore-formers are common in aquatic waters and very resistant to chlorination, do not multiply in treatment processes as a result of increased assimilable organic carbon (AOC) after ozonation, and are simple to cultivate (29). Furthermore, physical removal of aerobic spore-formers appears to parallel that of particle removals through different treatment configurations and has the added advantage of not breaking apart while passing through various unit processes. These characteristics may provide a critical parallel to the physical entrapment and removal of *Giardia* cysts and *Cryptosporidium* oocysts by various treatment processes.

Attempts have been made to improve the action response to coliform monitoring events in public water supplies. Regardless of the question concerning their poor correlation to some pathogenic agents in water supply occurrences, prompt action must be taken to verify and correct the problem. The new concept involves the use of a test for the presence or absence occurrence of coliforms per 100 ml (30,31). This qualitative approach ignores all concerns for a maximum permissible level of total coliforms in a mean value for all samples over a given month. Instead, frequency limits of 5% are placed on all coliform occurrences during the month.

For years, national regulations on drinking water quality have been based on a mean value derived from total coliform measurements gathered over each month. The limit was established at a geometric mean of one coliform per 100 ml. For many utilities experiencing coliform occurrences, the first reaction, when exceeding this limit, was to increase the number of samples taken over the next few days in an effort to drive the mean value down to below the one coliform per 100 ml limit so as to stay in compliance. Such action may have avoided a noncompliance issue but did not address why there were coliform bacteria at some location(s) in the distribution system. Now, with the opportunity to define water quality in terms of total coliform frequency (presence or absence) over a 30-day period and permitting up to 5% of coliform-positive samples during this interval, all positive samples have equal significance. In other words, a sample with only one total coliform has the same significance as another sample that may have many more coliforms per 100 ml. Unfortunately, the presence or absence procedure obscures the density in a coliform-positive sample, thereby providing no information on the magnitude of the contaminating event.

Recreational Waters. Epidemiological investigations on recreational waters have revealed poor correlations between bather illness and total coliform bacteria (32–35). These facts support the belief that this traditional indicator should be abandoned as worthless coliforms because of the predominance of environmental. More promising relationships may be found with such indicators as fecal coliform/*E. coli*, fecal streptococci/enterococci, and total staphylococci/*Candida albicans*. Much of the evidence points to the need to judge the quality of bathing waters with respect not only to fecal pollution but also to contamination introduced by bathers in close proximity. In fresh bathing waters, fecal coliform/*E. coli* and fecal streptococci/enterococci are the indicators of choice to measure the risk associated with gastrointestinal illness, whereas total staphylococci/*Candida albicans* are useful as predictors of illness associated with the ear, nose, and skin areas of bathers. These later health concerns have poor correlation with fecal contamination in bathing waters.

In marine waters, fecal coliform/*E. coli* may have an accelerated die-off that could distort correlations with pathogen occurrences. This may be the reason investigations of marine waters present some conflicting information on the use of fecal indicator bacteria (fecal coliform and fecal streptococci) as predictors of enterovirus occurrences. Important influences on the perception of safe bathing waters are the more rapid die-off of fecal indicator bacteria (fecal coliforms and *E. coli*) in marine waters, the recirculation of polluted waters near the beach, and the protection afforded to viruses in fecal cell debris.

Coliform bacteria (total or fecal coliforms/*E. coli*) are also a poor measure of body contact risks from opportunistic pathogens that occur in disinfected swimming pools, the exception being fecal contamination introduced into wading pools by small children. There is more interest in the use of enterococci and staphylococci to determine the extent of discrete pockets of epidermal cells and nasal, and saliva contaminants in the surface microlayer of water in the pool before dispersion. Additional useful information on water quality can be obtained by determining the magnitude of heterotrophic bacteria present in relation to the total number of bathers, a factor that is not possible with coliform measurements.

Nutrient Wastes. Extensive use of coliform tests worldwide has identified several disagreements in data interpretation from some types of waste discharges, namely, food-processing discharges, paper mill wastes, and animal feedlot operations (28,36,37). Several public health

concerns must be understood in these situations before drawing conclusions that the fecal coliform concept is invalid. Among these issues are the presence of thermotolerant strains of *Klebsiella*, nutrient waste loading impacts on receiving waters, depression of the natural self-purification capacity of these waters, and concern for extended survival of some bacterial pathogens downstream (38,39).

Sugar Processing Mills. Coliforms (particularly *Enterobacter* and *Klebsiella* strains) are commonly found on both sugar beets and sugarcane crops. These organisms contaminate the crop through application of poor-quality irrigation water, wildlife contact, and land application of farm animal manure if the fields are near cattle feedlots. Wildlife populations in sugar beet–growing areas include meadow mice, pack rats, Norway rats, pocket gophers, prairie dogs, and rabbits. It has been estimated that one acre of farmland may support 300 meadow mice and one square mile of similar agricultural fields can be the habitat for several thousand wild rabbits.

In separate studies of beet soil and beet surfaces from samples collected over five western states, fecal coliform results ranged from less than 2 to 230,000 per gram of material (40). Soil taken from trucks after unloading at the mill gave values of 10.9 to 700 fecal coliforms per gram. River water is used in the mill to wash beets of soil particles. Such feed water receives a substantial quantity of soil and beetroot debris. Fecal coliform levels of 120,000 to 210,000 organisms per 100 ml of spent wash water represent the effect of intermixing many portions of different field soils and beets.

Spent flume water contains a mixture of process makeup water, soil, debris washed from the incoming beets, and juices diffused from the beets in flume water. The resulting composite of pollutants is reflected by total coliform densities that can range from 43,000 to greater than 1,000,000 organisms per ml, with the fecal coliform portion being 1 to 10% of these values. Predominant total coliform species in this population (*Enterobacter* and *Klebsiella*) are particularly stimulated to explosive growth by the nutrient wastes during warm water periods. Most alarming is the protective shield provided by these nutritive wastes to some waterborne bacterial pathogens. A study of the waste from three sugar mills in Michigan during 1966 revealed a total of nine *Salmonella* serotypes, suggesting that this pathogen also can survive in fecal contamination introduced during cultivation, from processing water or sanitary waste in the plant.

Although radically different in appearance, sugar beets and sugarcanes both produce product wastes that contain significant residual sucrose, which is a biodegradable organic for many bacteria. In terms of chemical composition, sugar beet and sugarcane wastes are a complex mixture of various carbohydrates including pectin, pentosans, cellulose, and residual sugars. There are also various purines and related compounds including heteroxanthin, vicin, covicin, betanin, plus betaines, and urea in beet pulp. Vitamin analysis of 100 grams of beet pulp includes vitamin A, ascorbic acid, niacin, riboflavin, and thiamine. With a rich nutrient source such as this, it is understandable why many heterotrophic bacteria, including coliforms and bacterial pathogens, will either flourish or remain viable in the polluted aquatic environment.

These nutrient wastes and their bacterial content can best be reduced through biological activity that consumes the nutrients in waste stabilization ponds that have a long retention time (3–4 weeks). Without paying attention to the nutrient waste reduction before its release to the receiving water, there will be a disastrous collapse of the stream's capacity for self-purification over a river reach extending more than 24 hours flow time downstream (38,39). Bacterial multiplication of coliforms in this zone can exceed 1,000 times the number released from the mill and extend the persistence of fecal coliforms and *Salmonella*. Even under winter conditions, when bacteria multiplication is minimal, there can be an undesirable impact on the quality of receiving streams and lakes.

A field survey on the impact of sugar beet mill waste discharges on the Red River of the northern United States (North Dakota–Minnesota) revealed that the presence of excessive nutrient waste during winter conditions (river temperatures $<5\,^{\circ}C$) depressed stream natural self-purification in a manner such that *Salmonella* in sewage discharges along the river at Fargo, North Dakota, and Moorhead, Minnesota, were detected 73 miles (or 4 days flow time) further downstream than during off-season shutdown of the mill (40–42). In other words, sugar mill nutrient waste became a protective shield to hazardous fecal bacteria and environmental coliforms introduced into nearby receiving waters. This situation does demonstrate that the interpretation of coliform density additions in wastes from sugar mills, as being solely of fresh fecal pollution, may be misleading. Conversely, these events should be viewed as excessive nutrient releases to receiving waters that depress the benefits of a natural purification barrier to pathogenic bacteria passage downstream.

Paper and Pulp Mills. The significance of fecal indicators in discharges of wastewater from paper and pulp mill systems into receiving waters has been a sensitive point for both the industry and environmental regulators for over 40 years (27). The main issue is the quality of wastewater released into rivers and lakes and the impact these organic and sulfur complexes have on receiving water downstream. Unfortunately, both sides to the argument focus on only two aspects — release of indicator bacteria and occurrence of pathogens in the waste discharge. Little attention has been paid to reducing bacterial nutrient loading to receiving waters, which is similar to the same problem as that observed with sugar mill wastes.

Sanitary wastes are often separated from paper mill operational wastewaters, which is essential, but little attention was given to fecal contamination that originates in feed water for mill operations, rodent, and other wildlife contamination of wood chip stock stored in the plant yards, or biofilm formation in process operations. The problem is complicated by the fact that environmental strains of *Klebsiella*, *Enterobacter*, *Citrobacter*, enterococci, and other heterotrophic bacteria colonize vegetation, trees

included. This unique association begins with embryo fertilization of tree flowers. Growth continues in the water- and food-conducting tissues by various bacteria (including *Klebsiella pneumoniae*) and persists throughout the life of the tree (43). Cyclitols or wood sugars are the source of bacterial nutrients in this habitat and remain so even when the trees are reduced to wood chips (44).

Salmonella and additional coliforms are periodically introduced into the huge piles of wood chips in storage yards by rodents and other wildlife that roam the area at night as well as inputs from storm water drainage and feed water used in pulp processing. In a random survey over a nine-month period, seven *Salmonella* serotypes were isolated from effluents of three paper mills discharging into the Raisin River in Michigan (45). These samples also contained 3,700 to 460,000 total coliforms and 700 to 1,100 fecal coliforms per 100 ml. It should be pointed out that the *Salmonella* occurrences in paper mill waste were not continuous over several days of repeated sampling at each site, being unpredictable because of the status of raw material contamination and fluctuating quality of feed water.

Feedlots and Poultry Farms. Agricultural pursuits that concentrate on feeding livestock and poultry in confined feedlots can be a significant contributor of nutrient wastes (partially digested feed) and fecal organisms in the accumulating manure. During storms and subsequent runoff, streams in these water sheds often become impaired with farm animal wastes, if not continually removed by landfills and drainage from storm water runoff diverted into holding lagoons. Considering the fact that cattle feedlots often contain 1,000 to 10,000 heads of cattle over an area of about one square mile, it is reasonable to equate the magnitude of this farm waste with that of domestic waste contributed by a medium-sized city. The problem is also acerbated by the geographic location of many feedlots in regions of low rainfall and small streams. These grasslands are exposed to rainfall patterns that often are heavy cloudbursts that forcefully flush the land surface and dramatically change the stream water quality.

An illustration of this pollution problem can be found in a study of Tierra Blanca Creek and its intermittent tributary, Frio Draw (46). Tierra Blanca Creek is a small stream that has its beginning in the northwestern border area of New Mexico and the Texas Panhandle. From the New Mexico state line, it courses 66 miles toward the dam at Buffalo Lake (30 miles southwest of Amarillo, Texas). This water resource is used for irrigation and as the source water for a multiuse recreational lake that is also a wildlife refuge. The stream's major sources of pollution along its course are the sewage treatment plant effluent from Hereford, Texas, and the storm water runoff from a series of seven large cattle feedlot operations. All of these fecal and nutrient additions occurred within a 10.9-mile reach of Tierra Blanc Creek.

Rainfall for the year averages about 15 inches over the water shed and occurs largely as thundershowers in late April to early June. Following these thundershowers, the stream flow in Tierra Blanca Creek suddenly increases from about 13.7 cubic feet per second (cfs) to temporary flows ranging from 114 to 513 cfs. During the extended dry season, major pollution loading was from the poorly treated sewage plant discharge, which averaged 80,000 fecal coliforms per 100 ml. However, springtime thundershowers suddenly flushed the feedlots, and the municipal sewage treatment processes became overburdened with additional inputs. The net result was a dramatic deterioration in stream water quality, with fecal coliform densities 100 to 1,000 times those of dry weather conditions. This impact on the receiving stream created a severe loss of water quality in the delta area of the multiuse lake, some 6 to 8 miles downstream. In essence, the stream temporarily became a fecal waste conduit, and the excessive nutrient loading depressed any chance of natural self-purification within the stream.

Tropical Waters. Much of the development of coliform and fecal indicator concepts has been done in temperate aquatic environments. Applying these concepts to tropical waters has led some investigators to question their use in the tropical environment as being unrealistic (47–52). These tropical field studies have concluded that coliform and fecal coliform data from tropical streams and bathing waters do not coincide with known sources of fecal contamination. In rural New Guinea, high densities of both fecal coliforms (FC) and fecal streptococci (FS) in drinking water were associated more with domestic animal populations than with the number of people on the water shed. In fact, it was noted that when FC and FS densities were at the lowest, the human population was highest. Unfortunately, no attempt was made to confirm these results, and the implications were that animal populations on the water shed were not a public health issue for water quality. In Sierra Leone, high densities of fecal coliform (40–240,000 organisms per 100 ml) were reported, but no correlation was found between the indicator densities and the presence of *Salmonella*. In Hawaii, FC and FS were found in tropical streams that were not known to be contaminated with sewage. In Nigeria, total coliforms were stated to be quite high in areas upstream of known contaminating sources. In other studies reported from Nigeria, Hawaii, New Guinea, Puerto Rico, Sierra Leone, and the Ivory Coast, high densities of *E. coli* were reported in waters that were stated to be completely free of fecal contamination.

These are several aspects to these conflicting reports that must be understood in the selection, use, and interpretation of indicator systems in the evaluation of water quality in tropical environments:

- Tropical waters are warm waters round the year and often nutrient-laden over the entire drainage basin. These conditions stimulate the growth of many coliform species including environmental *Klebsiella*, some strains of which may be thermotolerant fecal organisms.
- Tropical soils are warm, moist, and high in organic nutrients that support the persistence of various microbes including coliform bacteria from desiccation.

- Sunlight exposures are more intense in the tropics and the UV component is an effective bactericidal agent, particularly for *E. coli*, thereby accelerating the rapid inactivation of this fecal indicator in sunlight-drenched tropical bathing waters.
- All warm-blooded animals (wildlife such as rodents, birds, monkeys, and so on as well as domestic animals including farm animals, dogs, and cats) are important contributors of bacterial pathogens and protozoan pathogens such as *Giardia*, *Cryptosporidium*, among others, and should not be ignored.
- The dense tropical vegetation supports a vast variety of wildlife that will exceed that of the human population in remote areas.
- The frequency of waterborne pathogen occurrences in water fluctuates as a reflection of the population density for infected wildlife, domestic animals, and humans living in the water shed.
- Although tropical waters and soils are host to a much larger variety of waterborne pathogens (that is, *Vibrio* chlorea, fungi, protozoans, and worms) than that encountered in temperate climates, major efforts at water quality characterization must focus on conventional indicators of fecal contamination in drinking water supplies to measure natural barrier and treatment effectiveness.
- Because the threat from recreational bathing in tropical waters and the raw consumption of agricultural produce grown in polluted waters introduce additional risks from tropical parasites, the choice of indicator systems should be expanded to include direct monitoring for endemic pathogens.

For these reasons, it is difficult to interpret the occurrences of total coliforms and fecal coliforms in ambient tropical waters without an environmental impact study or a sanitary survey of activity on the tropical water shed. Perhaps the use of only *E. coli*-testing of tropical freshwaters, augmented with fecal streptococci/enterococci analysis of coastal marine waters, is the only way to avoid the overlay of environmental organisms of no public health significance in these aquatic environments. In the meantime, the search must go on for the "perfect" indicator system that is simple to detect as well as specific to, and universally found in the warm-blooded animal fecal flora. This change in the traditional approach does not exclude the continued use of total coliform and fecal coliform tests in the monitoring of tropical drinking water supplies. In this situation, treated water should not contain any coliform bacteria.

Cisterns and untreated groundwater systems may contain a few total coliforms (<10 organisms per 100 ml) provided there are no fecal coliform/*E. coli* present in the sample. The concern with cistern supplies is poor protection from birds and rodent fecal contamination on the cachement site. Fecal contamination from surface runoff into untreated water wells continues to dictate the need to use a fecal coliform/*E. coli* test to determine the major health risk.

CONCLUSION

Coliform bacteria have long been recognized as one of the major bacterial groups in the intestinal tract of warm-blooded animals. Perhaps no other bacterial group has been studied more in the research laboratory or used more often to characterize water quality. These applications have come with some misgivings. Significant is the realization that the coliform group is an artificial collection of organisms that includes not only those bacteria common to the intestinal tract of warm-blooded animals, humans included, but also environmental strains having no sanitary significance. Most disturbing is the fact that a few coliform strains are either opportunistic bacteria or serious intestinal pathogens.

Further refinements in testing procedures have resulted in the development of a procedure for the subgroup known to be fecal coliforms. Although this subgroup has been used very successfully in a variety of waters and wastes, some nutrient discharges (paper mill, sugar processing, and farm animal feedlots) to receiving waters may distort the magnitude of pollution loading because of the growth of thermotolerant *Klebsiella* of environmental origins in these waters downstream. A specific test only for *E. coli* has also been introduced to exclude these *Klebsiella*. Unfortunately, this more restrictive test also excludes those *Klebsiella* found in the intestinal tract of approximately 30% of the people. These facts illustrate the difficulty in devising a "perfect" indicator system that is selective for all organisms of sanitary significance.

The search for better indicator systems has not yet produced the answer because of a number of conditions that must be met for the "perfect indicator:" identical responses that parallel pathogen incursions and survival in the water system; a simple, reliable, and a rapid test that is cost-effective as a monitoring tool; and occurrence of this indicator system in larger enough densities so that treatment effectiveness can be measured by six log removals. All of these requirements are challenging, and no known indicator system proposed to date is immune to some weakness that prevents acceptance for all classes of environmental waters and wastes.

Intensive investigations have shown that total coliform bacteria do not provide all of the desired characteristics for the ideal indicator. Coliforms are more sensitive to inactivation by disinfectant concentrations recommended for drinking water than are many waterborne pathogens. Most restrictive to any effective use of the coliform indicator system is the traditional requirement to test only 100-ml portions of drinking water. Such a test volume rarely correlates to the possible presence of either virus, *Giardia*, or *Cryptosporidium*, when the same water is examined for those pathogenic agents in 100-liter samples.

The most productive approach is to reassess the interpretation of coliform findings in the light of current information about their place in the ecology of aquatic environments. A review of the subject suggests the following understandings:

1. Fecal pollution concerns in the Tropics must include recognition of the contributions from wildlife and

farm animals on the water shed, not just a focus on human pollution as the sole public health concern.

2. Acknowledgment that pathogen occurrences are not constant in the aquatic environment.
3. Rejection of the acceptance of any coliform in treated drinking water supplies because proper treatment will inactivate these organisms.
4. Abandonment of the historical use of the total coliform test for bathing water quality assessments as being an unrealistic predictor of public health risk in these recreational waters.
5. Recognition that massive growth of *Klebsiella* downstream of some industrial operations is an indication of poorly treated nutrient waste that degrades natural self-purification processes in receiving waters.
6. Response to the sudden appearance of *Klebsiella*, *Citrobacter*, or *Enterobacter* strains in distribution water supply as an indication of biofilm development in the pipe environment that must be suppressed.
7. Awareness that although most coliform bacteria are harmless, there have been waterborne outbreaks caused by a few *E. coli* serotypes that are serious intestinal pathogens.

In conclusion, coliform bacteria may be viewed as a rogue group of organisms used to assess water quality, but the wealth of information on their place in the aquatic environment provides cautious reason for their continued use in lieu of the "perfect" indicator system.

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COLIFORM BACTERIA — CONTROL IN DRINKING WATER DISTRIBUTION SYSTEMS

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The occurrence of coliform bacteria is relatively common in drinking water distribution systems. Most coliform bacteria are generally not harmful by themselves. However, when they are detected, drinking water system operators and public health officials are challenged to determine their source and whether they pose an unreasonable risk to public health. Their presence may signal inadequate treatment, a breach of the distribution system, or may be the result of biofilm that comes about from chronically poor system maintenance. Systems with coliform levels that exceed government standards are in violation of the United States Environmental Protection Agency's (U.S. EPA) Total Coliform Rule (TCR). When this occurs, the water system operator is required to provide public notification, perform additional monitoring, and, in some cases, to develop a coliform bacteria control plan. These actions can be costly and highly disruptive to normal utility operations. Further, periodic or repeated public notification is likely to adversely impact consumer confidence in the local public water supply. Although there does not appear to be a completely effective means to totally eliminate coliform bacteria, practical experience shows that their impact can be minimized using a series of best management practices for treatment, disinfection, and distribution system maintenance.

COLIFORM BACTERIA IN WATER DISTRIBUTION SYSTEMS

Coliform bacteria in water distribution systems have long been problematic for the drinking water industry and regulatory community. The periodic occurrence and recovery of elevated coliforms from water distribution systems has been reported by various drinking water supply systems throughout the United States (1–9). Investigation of such occurrences frequently reveals that the source of the coliforms is not known because there is no evidence of treatment breakthrough or cross-connections in the distribution system.

In theory, one would not expect coliform bacteria to be present in drinking water that has been properly disinfected. In reality, this is not the case. Coliform bacteria are fairly common to some degree in all water distribution systems because drinking water treatment processes and distribution systems are not sterile. When coliform bacteria are detected, the system operator must retest to rule out fecal contamination and, perhaps most importantly, begin to understand and identify their source in order to take appropriate corrective action to prevent further microbial degradation in the water system.

Distribution System Water Quality Deterioration

Before undertaking a discussion of coliform bacteria occurrence in drinking water distribution systems, it is important to become familiar with the concept of water quality deterioration. In a review of the effects of the distribution system on water quality, Clark and coworkers reported that there is increasing evidence that finished water quality may undergo substantial changes as water moves through the distribution system to the consumer (10). Water quality deterioration implies an adverse change in quality between the point of distribution system entry and any point in the system where the change is detected. Although some change in water quality is expected in the distribution system, left unchecked, these changes may result in noticeable aesthetic impacts at the consumer's tap, including the appearance of objectionable tastes and odors, discoloration, and sediments or particulate matter. Adverse water quality changes in the distribution system, which generally go unnoticed by the consumer, include losses in disinfectant residual, excessive microbial growths, and internal corrosion.

Water quality deterioration can result from inadequate treatment, poor system maintenance, or long hydraulic residence times in the distribution system. Examples of inadequately treated water include insufficient disinfection that results in low residuals in the distribution system, poor pH or corrosion control, or the breakthrough of particulate matter as a result of nonoptimized filtration. Foremost among poor distribution system maintenance practices is the failure to periodically flush the system according to a prescribed plan to remove sediments, encrustations, and bacterial growths from the pipe wall. Long residence times that increase the age of water in the distribution system come about in areas where the flow or turnover of stored water is low. Low flow areas occur in dead-end portions of the system where water can stagnate because of poor circulation or in residential neighborhoods where consumer demand is low. Water can also age or become stale in systems with minimal storage tank drawdown.

Unless proper treatment and maintenance is in place, systems that use monochloramine in the distribution system for secondary disinfection are susceptible to a severe form of water quality deterioration know as nitrification. Nitrification is the biochemical conversion of reduced forms of nitrogen to nitrite and nitrate. By and large, nitrification is caused by two groups of bacteria: ammonia-oxidizing bacteria (AOB), which convert ammonia to nitrite, and nitrite-oxidizing bacteria (NOB) that oxidize nitrite to nitrate. Key factors that promote nitrification include increasing water temperature, hydraulic residence times in excess of several days, improper chlorine and ammonia ratios, and biofilm in pipes. For drinking water systems that chloraminate, nitrification can result in severe water quality deterioration in the distribution system, including the loss of chloramine residual, marked increases in heterotrophic plate count (HPC) bacteria, increased nitrite and nitrate concentrations, and possibly difficulty in complying with the TCR (11). (See also NITRIFYING BACTERIA IN DRINKING WATER, this Encyclopedia).

Significance of Coliform Bacteria as Sanitary Indicators

Most waterborne pathogens that impact human health are of fecal origin. Because waterborne pathogens are difficult to measure directly, water suppliers and public health officials rely on the measurement of indicator organisms normally associated with fecal contamination to ensure drinking water safety. One of the most widely used fecal indicator systems is based on the detection of bacteria collectively known as the total coliform group. The total coliform group bacteria include the genera *Escherichia*, *Klebsiella*, *Enterobacter*, *Citrobacter*, and some species of *Serratia*. The overall reliability of this group as an indicator of fecal pollution is somewhat questionable because most strains of coliform bacteria, except for *Escherichia coli*, occur naturally in the environment and are not of fecal origin. The total coliform group is also a poor indicator system for the presence of pathogenic protozoan cysts and some viruses. Conversely, the presence of *E. coli* in drinking water is generally regarded to be of greater public health significance because they are the only members of the total coliform group that are definitely associated with the intestinal tract of warm-blooded animals and humans.

On the basis of a limited study of communities with and without waterborne disease outbreaks, Batik and coworkers cautiously suggested that routine coliform monitoring may be of little help in preventing the transmission of waterborne disease (12). Further, the authors advised public health officials to reevaluate the importance of routine coliform monitoring for the prevention of waterborne disease. Despite this advice, the presence of total coliform bacteria in treated drinking water is unacceptable. Further, their presence without the evidence of a public health threat is indicative of deficiencies in treatment or disinfection that must be corrected.

Sources of Coliform Bacteria in Drinking Water

The presence of coliform bacteria in drinking water distribution systems without the evidence of fecal contamination or operational problems is a general indication of water quality deterioration. Because coliforms can arise from a number of sources and conditions, careful investigation is necessary to rule out fecal contamination. A partial listing of the potential sources of coliform bacteria in drinking water distribution systems is shown in Table 1. While this listing is not all-inclusive, the examples used provide a practical framework to begin the investigation of distribution system coliform occurrence. Experienced distribution system operators typically use these diagnostics to investigate coliform activity and to rule out potentially serious conditions that could pose a threat to public health.

U.S. EPA Total Coliform Rule

The U.S. EPA TCR regulates the presence of coliform bacteria in drinking water distribution systems. The rule is based on the premise that water systems should try to

Table 1. Potential Sources of Coliform Bacteria in Drinking Water Distribution Systems

Source or Cause of Coliform Bacteria	Examples
Inadequately treated water	Treatment interruptions including lapses in disinfection, loss of coagulant feed
Water treatment Deficiencies	Level of treatment not matched to source water quality, e.g., distribution of undisinfected groundwater of low or poor quality
Treatment Breakthrough	Passage of stressed or particle-associated coliforms
Distribution system breaches	Main breaks, construction and service activities including main repairs or new installations, cross-connections
Sanitary Deficiencies	Damaged or missing vent screens or hatch covers on storage tanks, inadequately protected open finished water reservoirs
Sampling and laboratory Errors	Monitoring locations of poor sanitary condition, failure to use aseptic sample collection and handling techniques, contaminated sampling bottles or lab supplies, poorly implemented analytical procedures,
Distribution system Biofilm	Colonization of pipe walls, sediments, or tuberculations in systems prone to water quality deterioration

Table 2. Percentage of US Population Served by Community Water Systems with TCR Violations During 1997

System Size	Population Range	Population Served	Population Receiving Water with TCR Violation
Small	25–3,300	25 Million	4.8%
Medium	3,301–10,000	25 Million	6.6%
Large	>10,001	202 Million	3.5%

achieve drinking water that is free of coliform bacteria because their presence indicates the possible existence of fecal and disease-causing bacteria. The TCR was published in the Federal Register on June 29, 1989 and became effective on December 31, 1990 (13). TCR compliance monitoring is based on testing throughout each month for total coliform bacteria. The number of monthly samples needed for compliance is proportional to the population served by the water system. For example, systems serving a population between 41,001 and 50,000 persons must collect a minimum of 50 samples per month. Large systems that serve a population between 450,001 and 600,000 persons are required to test at least 210 samples per month. Small systems that serve between 4,101 and 4,900 persons must test five samples per month, while systems that serve less than 1,000 persons only test one sample per month or less. Monitoring must be performed according to a state water supply agency-approved sampling plan.

The TCR maximum contaminant level (MCL) is based on monthly coliform presence-absence (PA) levels in the distribution system. Systems that are required to collect less than 40 samples per month are allowed no more than one sample per month to be coliform-positive. Larger systems collecting 40 or more samples per month can have no more than 5% coliform-positive samples in any month.

Whenever a system detects coliforms in drinking water, a set of repeat samples must be collected within 24 hours. Any routine or repeat sample that is total coliform-positive must also be analyzed for the presence of fecal coliforms or *E. coli*. Water systems that exceed allowable monthly levels for total coliform bacteria are in violation of the TCR, which must be reported to the state and the public. A positive result for *E. coli* or for fecal coliform bacteria, which grow at 44.5 °C, constitutes an acute violation of the TCR. It should be noted that testing for fecal coliforms is not always a reliable indication of fecal contamination because some environmental coliform strains of nonfecal origin are also able to grow at 44.5 °C. Conversely, because of their distinct association with the intestinal tract, the presence of *E. coli* conveys a much stronger association with fecal contamination. Any evidence of fecal contamination requires prompt state and public notification.

Systems with chronic coliform occurrence problems because of the presence of biofilm may be granted a variance from compliance with the TCR MCL if the system has no evidence of fecal contamination or waterborne disease (14). In establishing a TCR variance provision, the U.S. EPA created a set of interim criteria to be used by the states as guidelines by which systems could operate without posing an unreasonable risk to health.

Coliform Occurrence in the United States

According to U.S. EPA compliance monitoring records, distribution system coliform occurrence is relatively common among community water systems in the United States. In 1997, the U.S. EPA National Public Water Systems Compliance Report revealed that the TCR was violated by more than 5% of the nation's 54,367 community water systems (15). Further, this statistics represents the highest percentage of maximum contaminant level (MCL) violations for community systems in all size categories during 1997. Populations impacted by coliform MCL violations in 1997, according to system size, are shown in Table 2.

TC-R violations were also problematic in other U.S. EPA public water system classifications during 1997, including nontransient noncommunity and transient noncommunity systems that experienced a TCR MCL violation rate of approximately 4%. Collectively, these systems serve an additional 23 million persons annually.

A survey of 81 U.S. drinking water utilities representing 32 states conducted by Smith and coworkers for the period between 1986 and 1989 revealed that 82.7% of the responding systems reported some level of monthly distribution system coliform occurrence (16). On average, coliforms were detected in these systems six months per year. Slightly more than one-third of the systems reported monthly coliform occurrence levels above 5%.

DISTRIBUTION SYSTEM BIOFILMS (see also BIOFILMS IN NATURAL AND DRINKING WATER SYSTEMS, THIS ENCYCLOPEDIA)

Growths of microorganisms attached to pipe walls and solid surfaces in water distribution systems are commonly referred to as biofilm. Biofilm organisms adsorb nutrients from bulk water and grow as patchy mats. Abrupt hydraulic changes resulting from flow-velocity changes or changes in the chemical environment can trigger the sloughing of biofilms. Coliform-containing biofilms in drinking water distribution systems can result in violations of the TCR when disease-causing microorganisms are not present. Moreover, coliform occurrence that is the result of biofilm is not associated with breaches of the primary treatment and disinfection barriers or contamination within the distribution system. Indications of waterborne disease or evidence of fecal contamination are also absent when coliform bacteria from the distribution system are the result of biofilms. In general, water distribution systems that maintain low disinfectant residual levels throughout the system do not

practice corrosion control and those that do not regularly flush are more predisposed to biofilm formation.

Microorganisms found in biofilms can include a variety of coccoid, rod-shaped, filamentous, and appendaged bacteria, fungi, and higher organisms such as nematodes, and crustacea. While biofilms in drinking water systems are generally assumed to be innocuous, recent research has shown that viruses and parasites such as *Cryptosporidium* can be trapped in biofilms after a contamination event such as a cross-connection (17,18). Noncoliform biofilms that consist mainly of mixed heterotrophs, including iron, sulfur, and nitrogen bacteria, can cause microbially induced corrosion, nitrification in chloraminated systems, and objectionable tastes and odors.

Biofilm microorganisms are known to be associated with distribution system corrosion products and tuberculations (19–22). Tubercle surfaces possess an extremely high surface area that provides an ideal habitat for microbial growth. Unlined cast-iron pipes, present in many older drinking water systems such as the 84-year-old section of heavily tuberculated six-inch main from Milford, Connecticut, shown in Figure 1, can play host to a number of chemical and physical phenomena that favor biofilm growth and affect disinfection efficiency.

Using a scanning electron microscope (SEM), Allen and coworkers found high populations of microorganisms on or in the tubercles of pipe specimens from seven drinking water systems in the United States (23). The investigators concluded that microbial populations are able to successfully adapt to a variety of environmental conditions in a water system that is adequately treated and disinfected. Rod-shaped microorganisms on a tubercle at a magnification of 4,000X, (courtesy of M. J. Allen) are shown in Figure 2.

Microorganisms deeply enmeshed in or on a tubercle are shielded from chemical disinfection by free chlorine because of competing oxidation-reduction reactions that consume the disinfectant before a lethal concentration can penetrate the biofilm. LeChevallier and coworkers noted that biofilms grown on iron pipe surfaces exerted a disinfectant demand that was up to 10 times greater than from biofilms grown on other types of pipe materials (24). Although nutrient concentrations in most treated drinking waters are low, tubercle surfaces are also able to adsorb and store nutrients for microbial growth in an otherwise nutrient-deficient environment. Microorganisms attached to tuberculated surfaces are also protected from the shear forces of water flowing through the pipe.

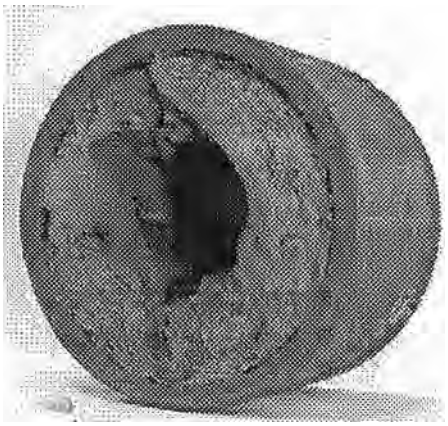

Figure 1. Tuberculated six-inch cast-iron main.

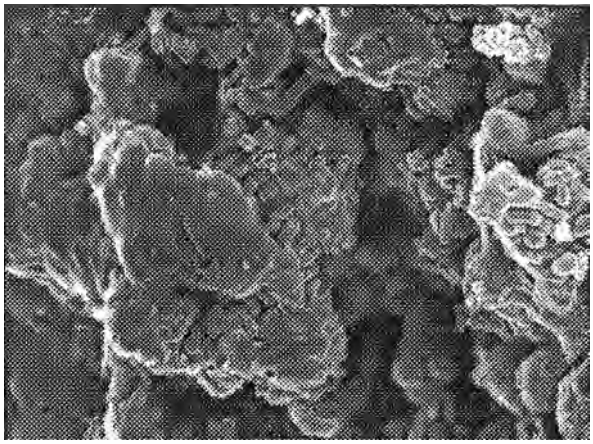

Figure 2. Microorganisms on tubercle surface (4,000 times).

Biofilm Signs and Symptoms

The most outstanding symptom of biofilm is the persistent and widespread recovery of coliforms in the presence of disinfectant residual concentrations typically found in drinking water distribution systems. In a biofilm situation, it is not uncommon for coliform presence-absence to exceed the TCR maximum contaminant level for several days to weeks. Moreover, coliform occurrences due to biofilm may last for several years or more. The highest coliform recovery rates generally occur in the summer and fall months when water temperatures are at or above 15 °C (25). An analysis of 218 coliform-positive samples in the New Haven, Connecticut, distribution system recovered between 1992 and 1998 showed that 73% of all coliform-positive samples occurred between June and November. The mean water temperature during this interval was $17.1\,^{\circ}\mathrm{C} \pm 3.5\,^{\circ}\mathrm{C}$. Between 1992 and 1998, peak coliform levels typically occurred in the month of September, when the mean water temperature was $19.9\,^{\circ}\mathrm{C} \pm 2.6\,^{\circ}\mathrm{C}$. The relationship between seasonal coliform occurrence and water temperature in the New Haven system is shown in Figure 3 for 1992 and 1993.

Traditional water quality parameters such as disinfectant residual concentration, turbidity, pH, and color are not likely to overtly reveal the presence of biofilm. However, difficulty in maintaining an acceptable disinfectant residual in conjunction with excessive HPC bacteria levels may indicate the onset of water quality deterioration that can lead to a biofilm condition.

Biofilm Factors

Biofilms are presumed to result from the complex interaction of environmental, chemical, physical, and distribution system operational factors, including the presence of growth-stimulating nutrients, seasonal changes in water

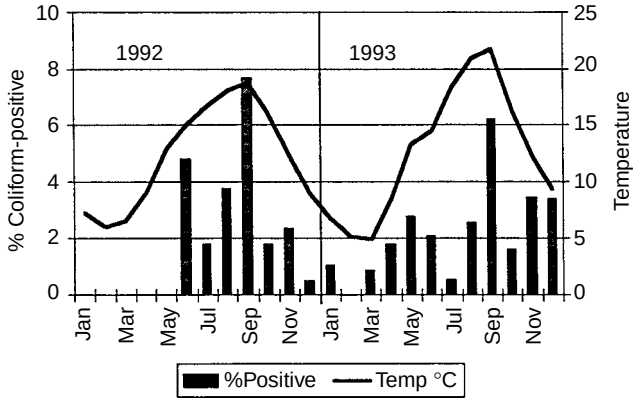

Figure 3. Seasonal distribution system coliform occurrence in New Haven, Connecticut.

temperature, distribution system hydraulics, disinfection practices, and water main corrosion. However, in an 18-month study of 31 North American drinking water systems, LeChevallier and coworkers found that no single factor could account for all observed coliform occurrences (26). Factors evaluated included filtration and disinfection practices, temperature effects, nutrient concentrations, corrosion effects, and operational characteristics. An analysis of system operational characteristics, monthly distribution system coliform occurrence, and water quality data revealed the following findings:

1. Filtration. Four systems in the study used unfiltered surface water and accounted for 64.3% of the positive coliform samples, indicating that filtration may be an important factor in preventing coliform regrowth.
2. Disinfectant Residual Type and Level. Systems that used free chlorine had more coliform bacteria than chloraminated systems. Systems with low disinfectant residuals at dead-end sites, that is, less than 0.2 mg/L free chlorine or monochloramine levels less than 0.5 mg/L, had substantially more coliform occurrences compared to systems that maintained higher dead-end disinfectant residuals.
3. Temperature. On average, the occurrence of coliform bacteria was significantly higher when water temperatures were higher than 15 °C.
4. Nutrient Levels. Systems with assimilable organic carbon (AOC) levels above 100 μg/L tended to have more coliform bacteria than systems with low AOC. Systems with high AOC levels tended to maintain higher disinfectant residuals to control coliform occurrences.
5. Corrosion. Systems using free chlorine with more than 1,000 miles of unlined cast- iron pipe appeared to have a higher incidence of coliform occurrence compared to systems with less than 200 miles of unlined cast-iron pipe. Chloraminated systems showed no apparent relationship between coliforms and the amount of unlined cast-iron pipe present in the system. Systems that used a phosphate-based corrosion inhibitor generally had lower coliform levels.
6. Operational Characteristics. Treatment process and distribution system characteristics associated with increased coliform occurrence rates included:

   - Ozonation without biological treatment
   - Large proportion of storage tanks in the distribution system
   - Lack of a systematic distribution system flushing program
   - Presence of uncovered finished water reservoirs

Biofilm Impacts

Aside from noncompliance with the TCR, distribution system biofilms can

- Interfere with disinfection
- Impact public health
- Provide a habitat for opportunistic pathogens such as *Mycobacterium avium* and *Legionella*
- Increase pumping costs
- Cause corrosion that reduces the life of pipes and other materials in the system
- Produce aesthetic problems including discolored water and objectionable tastes and odors
- Weaken consumer confidence

The South Central Connecticut Regional Water Authority reported expenditures of $875,000 in an unsuccessful attempt to control a sudden systemwide outbreak of distribution system coliforms that began in 1984 (27). The increased operating costs included $750,000 for additional chlorine and overtime labor for extensive flushing of the distribution system. The remainder of this expenditure was used to nearly triple its routine water quality monitoring program. Aside from potential public health implications and operational issues, New Haven's experience underscores the fact that biofilm coliform occurrences can be costly and disruptive to normal utility operations.

DISTRIBUTION SYSTEM COLIFORM CASE STUDIES

Utility response to distribution system coliform occurrence has been varied. Some systems substantially increased disinfectant residual levels and initiated extensive flushing programs in the distribution system. Other systems increased pH levels or changed disinfectant types (i.e., from chlorine to chloramines) or modified corrosion control programs in an attempt to control or eliminate the distribution system coliform occurrences.

To illustrate the impacts and development of various coliform control strategies associated with persistent distribution system occurrence, case studies representing four unique approaches will be presented. The four control strategies to be discussed are

1. Standardization of distribution system maintenance practices in New Haven, Connecticut

2. Year-round flushing combined with a periodic changeover from combined to free chlorine in Greenville, South Carolina
3. Implementation of a corrosion control program in Utica, New York
4. Conversion from free chlorine to chloramine in Hopewell, Virginia

New Haven, Connecticut

The Regional Water Authority, headquartered in south central Connecticut, supplies water to more than 380,000 inhabitants in the 12-town metropolitan New Haven district. The Authority has an average daily demand of 55 MGD (million gallons/day) that is provided by three surface water treatment plants and five wellfields. The distribution system is divided into 19 separate pressure zones and includes about 1,600 miles of water main ranging in size from 4 to 72 inches, 28 storage tanks and 27 pumping stations. Approximately 23% of the distribution system piping consists of heavily tuberculated unlined cast-iron main, some of which dates back to the late 1890s.

In 1984 when distribution system coliform bacteria first became problematic in New Haven, four surface water sources were in use. The surface water facilities included in-line direct, slow sand, and conventional filtration plants along with a high-quality unfiltered supply. The unfiltered surface water provided about 60% of the system's daily demand. A direct filtration plant for this supply was dedicated in 1986. The slow sand filter plant was decommissioned in 1991 after more than 80 years of service.

In the spring of 1984, the Authority unexpectedly began to recover elevated numbers of coliform bacteria from throughout the distribution system. Coliform occurrences peaked in August when the coliform average for the month was 12 times the maximum contaminant level of one colony-forming unit per 100 mL.

Coliform occurrence was systemwide, including areas of the distribution system where water from different finished water sources mixed, as well as in two hydraulically isolated systems. Routine speciation of all coliform-positive samples indicated that there was no evidence of fecal contamination in the distribution system based on the absence of *E. coli*.

After consultation with state and U.S. EPA officials, the Authority immediately increased chlorine residual levels to a maximum of 6–8 mg/L to disinfect the distribution system and insure ample disinfection of the unfiltered surface water source. At the same time, extensive flushing of the distribution system was initiated. This effort, which included all pipe sizes, was completed over an eight-week period. The combined effect of heavy flushing and increased disinfection residual levels appeared to have stimulated biofilm sloughing because the number of coliform-positive samples increased shortly after these changes were implemented. Further, many of the coliform-positive samples recovered during this period showed confluent coliform growth or were too numerous to count on m-Endo agar. Numerous consumer complaints were received through the end of 1984. The bulk of these complaints were related to chlorinous taste and odor because distribution system chlorine residuals were considerably higher than normal. Some consumers also complained of dry skin, hair damage, and the bleaching of laundry items. By the end of 1984, coliform numbers began to subside and this resulted in the gradual lowering of effluent chlorine residuals early in 1985. Chlorine concentrations leaving the treatment facilities were adjusted to maintain a target value of 1 mg/L at all distribution system extremities. To achieve this target value, effluent chlorine residuals averaged 1.9 mg/L (range 1.1 to 3.9 mg/L).

Coliform investigations following the outbreak included intense scrutiny by the State health department and various consultants of past and present water treatment and distribution practices with emphasis on disinfection at the unfiltered supply. These efforts found no major deficiencies in any of the Authority's treatment facilities and were also unable to identify any conditions or practices that could account for the coliform outbreak. Moreover, coliform occurrence rates in areas of the distribution system served by the unfiltered surface water source were found to be comparable to other parts of the system served by filtered surface water or disinfected groundwater. The investigators also found no evidence of fecal contamination or waterborne disease in the Authority's service area (28). A similarly intense review of sampling and laboratory procedures found that the Authority's water quality monitoring program produced accurate and reliable results, and that laboratory errors were not responsible for the unusually high number of distribution system coliforms. Operational and procedural changes made over the next five years following the August 1984 coliform outbreak are summarized in Table 3.

Despite these changes, significant coliform occurrence continued throughout the distribution system as shown in Figure 4. Importantly, this figure reveals that coliform bacteria were present in the distribution system before the 1984 outbreak. However, it is very likely that their significance was overlooked as an indication of biofilm. This is because throughout much of the 1970s and early 1980s, coliform density averages in the Authority's distribution system were generally below the

Table 3. Changes in Treatment and Distribution System Maintenance

Year	Operational Change
1984	Increased free chlorine residuals in all parts of the distribution system
1985	Developed and implemented a unidirectional distribution system flushing program
1985	Expanded raw, finished, and distribution system water quality monitoring
1986	Direct filtration plant online for the unfiltered surface water supply
1988	Increased zinc polyphosphate corrosion inhibitor feed rate from 1 mg/L to 2 mg/L
1990	Implemented periodic cleaning and inspection program for all distribution storage tanks and finished water reservoirs

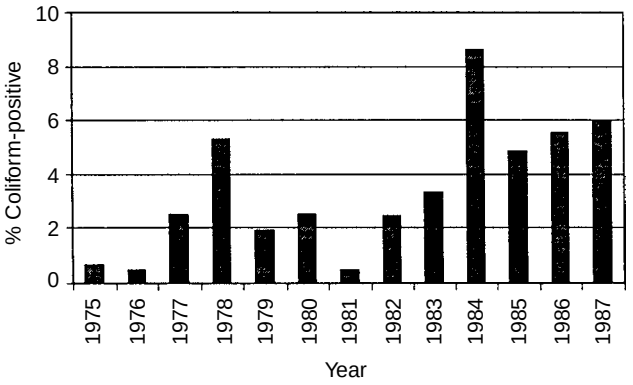

Figure 4. Annual distribution system coliform occurrence in New Haven (1975–1987).

prevailing interim maximum contaminant level, which required monthly distribution system coliform cell density, averages to be less than one colony-forming unit per 100 mL. Further, except for August and September 1984, the cell density–based coliform MCL was never exceeded between 1975 and 1983.

In an attempt to bring the coliform occurrences under control, the Authority began to examine past distribution system maintenance practices. A review of these practices from the mid-seventies through 1981 found that the Authority had no organized distribution system-flushing program and that storage tank cleaning and inspections were performed approximately every 10 years. Any flushing that was performed was limited to customer complaints and the periodic flushing of known trouble spots. In 1982, a short-lived effort was made to develop a more organized distribution system-flushing program. The goal of the program was to flush 100% of all pipes 16-inches or less in diameter on an annual basis. A true unidirectional flushing program was developed in 1985. Over the next five years, an average of 50% of the system was flushed on an annual basis. In 1989, coliform occurrence decreased by about 50% compared with the previous year. Because of this apparent success, annual system flushing averages were reduced to a target level of 25% in 1990. The Authority also adopted a three-year cycle for storage tank cleaning and inspections at this time.

From 1990 through the first half of 1992, coliform occurrence in the distribution system was at a record low since the initial outbreak in 1984. This reduction was thought to be the result of improvements to distribution system flushing, higher disinfectant residuals, corrosion control, and tank maintenance programs implemented between 1985 and 1990. The Authority's apparent success in controlling coliform occurrence was dashed in early June 1992, when coliform bacteria suddenly and unexpectedly reappeared in all parts of the distribution system, which prompted the Authority to apply for a variance from the TCR on June 8, 1992. Flushing was also halted in mid-June 1992 because it initially appeared to track spatially with the appearance of coliforms in the distribution system. A subsequent in-depth review of coliform occurrence patterns from the summer and fall months ultimately showed no relationship between flushing and the appearance of coliforms in the distribution system. Flushing was resumed in 1993 at the 25% level until 1999, when it was suspended because of a drought alert in the region.

The Authority's decision to seek a variance was strengthened by no evidence of waterborne disease in the community, no interruptions in treatment, and no evidence of fecal contamination in the distribution system. As a condition of the variance, the State Department of Public Health Water Supplies Section (DPH) issued the Authority a Consent Order in 1993. The requirements of this Order included continued maintenance of minimum target disinfectant residuals and corrosion inhibitor doses in all parts of the distribution system. Specific mandates for public notification, water quality monitoring, cross-connection control, and performance of an annual independent sanitary survey were also part of the Consent Order. The Order also required the submission of an annual variance report to DPH describing distribution system maintenance completed in the previous year and plans for the following year (29).

In 1993 and 1994, distribution system coliform occurrence continued to be sporadic, with the heaviest occurrence typically appearing in the summer and fall months. After July 1994, monthly coliform occurrence rates were generally well below 5%. Averages of annual coliform occurrence shown in Figure 5 for the period 1995 to 1999 suggest that coliform occurrence had reached what could be considered a normal "background level" for a system of the Authority's size and complexity. Because of these low coliform occurrence rates, the Authority formally petitioned DPH in January 1999 to rescind the Total Coliform Rule (TCR) variance and related Consent Order. DPH lifted the Variance and Consent Order on March 18, 1999.

The factors believed to be responsible for the reduced coliform levels in the Authority's distribution system after 1994 include the maintenance of higher disinfectant residuals throughout the system since the mid-1980s, improved corrosion control and the application of consistent distribution system maintenance practices. Importantly, the events and operational practices chronicled in the New Haven case study suggest that biofilm:

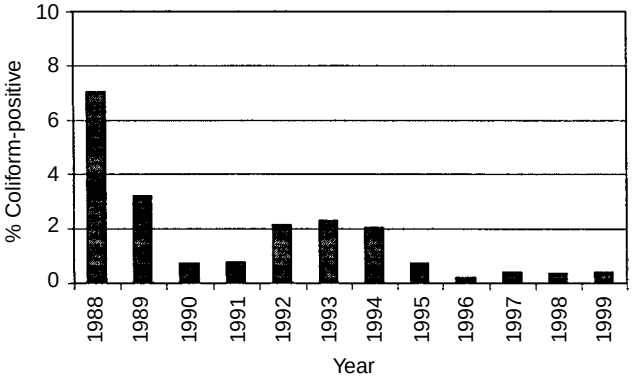

Figure 5. Annual distribution system coliform occurrence in New Haven (1988–1999).

Table 4. Coliform Control Strategy for New Haven, Connecticut

Program Element
Maintain minimum target chlorine residual of 0.3 mg/L in all parts of the distribution system
Annually clean and line 8 to 10 miles of old unlined cast-iron pipe to reduce biofilm habitat
Annually flush a minimum of 25% of the distribution system
Clean and inspect all distribution system storage tanks on a rotating three-year cycle
Apply a corrosion inhibitor to control distribution system corrosion
Maintain an effective cross-connection control program
Perform periodic sanitary surveys of source water, treatment, and distribution system facilities to insure overall system integrity

- likely to have resulted from the cumulative effects of inconsistent and/or infrequent distribution system maintenance practices in the 1970s and early 1980s
- can be held in check by the consistent and systematic application of distribution system best management practices

To ensure that distribution system coliform levels remain low, the Authority has adopted the coliform control strategy outlined in Table 4.

Greenville, South Carolina

The Greenville Water System serves a population of 310,000 residents in greater Greenville, South Carolina. Two high-quality unfiltered surface water supplies and a conventional filtration plant produce an average of 54 MGD. Treatment of the unfiltered supplies is slated to begin in the spring of 2000, when a 75 MGD dissolved air floatation plant comes on-line. The distribution system contains approximately 2,000 miles of water main and 15 storage tanks.

In 1992, the system began to experience persistent episodes of coliform contamination in the distribution system (30). Monthly total coliform bacteria MCL violations occurred three times in 1992, once in 1993, and twice in 1994. Typical of biofilm, peak coliform levels generally occurred between June and September. Distribution system flushing typically occurred only in response to consumer complaints, in areas of the distribution system with low disinfectant residuals, or where coliform bacteria were present.

In the winter of 1994, the Greenville Water System implemented a two-step biofilm control process based on year-round unidirectional flushing and a seasonal disinfectant changeover from monochloramine to free chlorine. The 30-day disinfectant changeover period typically occurs in late March or early April. Sufficient free chlorine is used throughout the system to achieve a 2 mg/L target at all distribution system extremities after flushing. Annual distribution system coliform occurrence data, summarized in Figure 6, indicate that a highly successful approach for controlling biofilm has been achieved in the Greenville Water System. Distribution system flushing crews also reported that the two-step process was responsible for shorter flushing times relative to sediment removal and higher concentrations of free and total chlorine in some areas of the distribution system compared to 1994 levels.

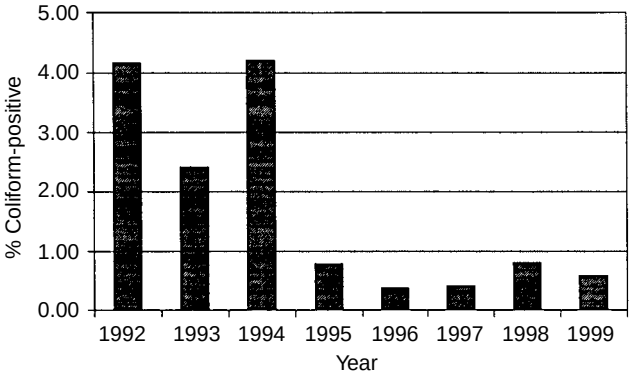

Figure 6. Annual distribution system coliform occurrence in Greenville, South Carolina (1992–1999).

Utica, New York

The Upper Mohawk Valley Regional Water Board serves a population of 126,000 in the greater Utica, New York, area. Before 1992, the system was served by unfiltered surface water from the Hinckley Reservoir. A direct-filtration plant, producing an average of 20 MGD, was dedicated in 1992. The distribution system consists of 625 miles of main and has 25 storage tanks.

Between 1991 and 1994, elevated lead levels and excessive coliform occurrences were common in the distribution system. Ninetieth percentile lead levels at consumer taps, which were the result of highly corrosive source water, were as high as 160 μg/L compared to the U.S. EPA Lead and Copper Rule action level of 15 μg/L. Coliform levels in the distribution system routinely resulted in violations of the TCR. Seasonally, up to 30% of the monthly coliform samples were positive. After the filter plant went online in 1992, distribution system coliform and lead violations continued to occur. This prompted the utility to accelerate modifications and improvements to a previously established corrosion control program, based on the adjustment of pH and alkalinity with lime and soda ash. The utility was also able to demonstrate that the coliform occurrences were biofilm-related and the utility was granted a TCR variance by the New York State Health Department, Bureau of Public Water Supply Protection (31).

Following full implementation of the corrosion control program in 1994, ninetieth percentile lead levels at consumer taps were reduced to below 10 μ/L. The effect of corrosion control on the distribution system biofilm also produced dramatic results with no TCR violations being reported after 1995 as shown in Figure 7.

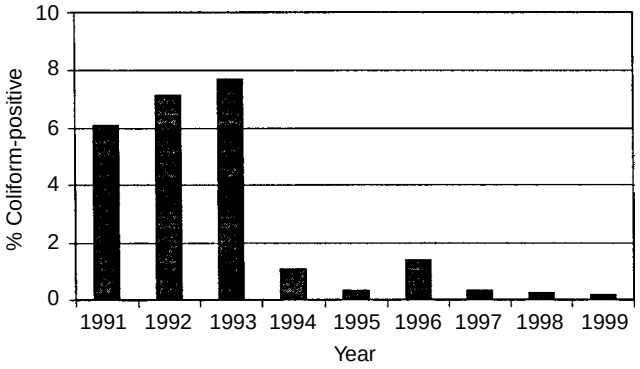

Figure 7. Annual distribution system coliform occurrence in Utica, New York (1991–1999).

Hopewell, Virginia

The Virginia American Water Company serves 15,000 customers in the City of Hopewell, Virginia. Water from the Appomattox River is treated in a rapid filtration plant that is divided into two sections to produce industrial and domestic grades of water. The conventional treatment trains employed in both sections of the plant are identical, except the domestic plant uses post-filter GAC contactors. The combined capacity of the two sections is 33 MGD. Unlike the industrial system, the domestic production is fluoridated and treated with zinc orthophosphate for corrosion control and ammonia is added to form chloramines. The separate industrial and domestic distribution systems contain a total of 162 miles of main. The industrial system contains one storage tank, while the domestic distribution system has two storage tanks.

In 1991, Hopewell began to experience persistent coliforms in the distribution system. In an attempt to control these occurrences, the average free chlorine residual in the distribution system was increased from 0.57 to 2.1 mg/L. Despite this change, the coliform occurrences continued, and total trihalomethane (THM) concentrations exceeded 100 μg/L during the summer because of the higher chlorine residuals (32). Hopewell began using mT7 agar in January 1992 to enhance the detection of injured coliforms (33). Throughout 1992, 22.3% of the distribution system samples analyzed using mT7 agar were positive for coliforms. During the first six months of 1993, 11.7% of the samples analyzed using mT7 agar were positive for coliforms. In May 1993, 5.6% of the distribution system samples were coliform-positive using m-Endo agar (24).

Because adequate distribution disinfectant residuals and pressures were being maintained and there were no coliform bacteria in the treated effluent, the regrowth of coliform bacteria in the pipe network was suspected. These conditions prompted Hopewell to change the domestic distribution system disinfectant from free chlorine to monochloramine in July 1993 (22). One week after chloramination began, all samples taken from the Hopewell distribution system were coliform-negative. Following the changeover to monochloramine, only two coliform-positive samples were detected through the end of 1994. These samples were recovered during the first week after conversion to chloramines and were isolated on mT7 agar. Subsequent coliform monitoring using mT7 agar through the end of 1998 has detected only 12 coliform-positive samples. Further, with regard to chloramines, all samples taken for compliance with the TCR have been negative for coliform bacteria through the end of 1999, and average HPC bacteria levels determined using R2A agar were below 30 CFU/mL.

Monthly THM averages throughout the distribution system did not exceed 100 μg/L during a 15-month period following the conversion to chloramines. Further, the four quarterly running averages for THMs remained below 80 μg/L. Average total haloacetic acid levels decreased slightly from 26 μg/L using free chlorine to 21 μg/L after the conversion to chloramines.

CONCLUSION AND RECOMMENDATIONS

Because some degree of water quality deterioration takes place in all distribution systems, high-quality finished water entering the system is no guarantee that the system will remain free of coliform occurrence. Further, when coliform bacteria become problematic in the distribution system, there is no completely effective means to do away with them. However, the case studies presented clearly demonstrate that the control of distribution system coliforms is possible using a series of best management practices for treatment, disinfection, and distribution system maintenance. These practices include adequate disinfection, filtration to lower turbidity and nutrient levels, corrosion control, and distribution system maintenance. Moreover, the water system operator must consider all of these practices in devising a solution to limit the impact of distribution system coliform occurrences and biofilm. In developing a strategy for coliform control, water system operators also need to be aware of all the potential causes of distribution system coliforms. Each occurrence of coliform bacteria must be evaluated in order to rule out treatment lapses and distribution system contamination before concluding that biofilm is to blame. Failure to investigate any coliform occurrence episode may create an unreasonable health risk if the coliforms are the result of operational problems.

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COLIFORM REGROWTH IN WATER DISTRIBUTION SYSTEMS.

See BIODEGRADABLE DISSOLVED ORGANIC CARBON IN DRINKING WATER

COLIPHAGE: DETECTION METHODOLOGIES.

See BACTERIOPHAGE DETECTION METHODOLOGIES

COMETABOLISM.

See PESTICIDE DEGRADATION IN SOILS

COMMERCIAL USE OF MICROORGANISMS, REGULATION OF.

See REGULATION OF THE COMMERCIAL USES OF MICROORGANISMS

COMPOST: BIODEGRADATION OF TOXIC ORGANIC COMPOUNDS

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Composting is a technique primarily applied for the stabilization of organic solid wastes, such as municipal solid waste, animal manure, biosolids, and yard cuttings. A new application of composting for bioremediation and biodetoxification is defined as compost bioremediation. Compost bioremediation comprises the remediation and restoration of contaminated soil (soil composting, biopiling), treatment of contaminated sludge and solid waste (composting), and purification of contaminated air (biofiltration). Compost bioremediation applied to landfarming and biofiltration involves the use of tailor-made mature or finished compost, which acts mainly as a source of

microorganisms and cosubstrate for the breakdown of pollutants. Compost bioremediation has proven effective in degrading and sequestering many types of toxic environmental pollutants, such as halogenated aromatics and hydrocarbons, pesticides, petroleum derivatives, explosives, solvents, heavy metals, and odors. Once applied to soil, in addition to alleviating contamination — which is the intrinsic purpose of bioremediation itself, compost has the added value of supplying nutrients and providing soil conditioning for its further restoration. The techniques of landfarming, composting, and biopiling are often collectively designated soil remediation. Landfarming consists of spreading a thin layer of the material that needs to be detoxified over the soil, which then undergoes the traditional agricultural practices of fertilization and irrigation. Mechanical plowing ensures aeration and mixing. Soil composting consists of mixing a polluted soil with compost amendments and bulking agents such as straw, wood chips, plant wastes, manures and, as required, microbial inoculum. Here again periodic turning and aeration are practiced. Biopiling consists of mixing soil with a compost structuring agent at a ratio that is lower than that used in composting. Because turning is not applied, biopiling is less homogeneous and sometimes less effective in removing pollutants than composting. This chapter focuses on the biodegradation of toxic organic compounds commonly found as soil pollutants during composting, with an emphasis on four major families of environmental pollutants: petroleum products, chlorinated compounds, explosives, and pesticides. Landfarming and biofiltration technologies, which are related to the use of compost and not to the composting process itself, are not within the scope of this chapter.

PRINCIPLES OF COMPOSTING

Composting is defined as the thermophilic aerobic decomposition of organic matter (OM) by a mixed microbial population under controlled conditions. Composting is a form of waste stabilization that requires special conditions, particularly of moisture and aeration, to yield temperatures conducive to thermophilic microorganisms (1,2). Compost is the stabilized and sanitized product of composting, which is beneficial to plant growth. The heterogeneous organic substrate has undergone an initial, rapid stage of decomposition and is in the process of humification (3).

Controlled environmental conditions distinguish composting from natural decomposition, which often occurs in open dumps, manure heaps, or field soil. The degree of control also distinguishes composting from other solid-phase bioremediation techniques, such as biopiling and soil farming. The organic wastes, comprising several components, normally have an indigenous population of microorganisms. Once the moisture content of the waste is brought to an appropriate level and the mass is aerated, microbial metabolism speeds up. Energy is obtained by biological oxidation of part of the carbon; some of this energy is used in metabolism, the rest is given off as heat. The end product (compost) is made up of the more resistant OM residues, breakdown products, dead and living microorganisms, together with products from further chemical reactions between these materials (4).

The temperature trend in most composting processes can be divided into four stages:

1. *Mesophilic:* an initial phase lasting one to two days, during which the mesophilic strains of microorganisms start to decompose the readily degradable compounds; heat is given off and the temperature rises. The pH falls slightly as organic acids are produced.
2. *Thermophilic:* the second stage, lasting two to four weeks, when, above 40 °C the thermophilic strains take over. If the temperature rises above 60 °C, the fungi become deactivated, and the reaction is continued by thermophilic bacteria. During this phase, the more readily degradable substances, such as sugars, fats, starch, and proteins, are rapidly consumed and most of the human and plant pathogens are destroyed; the pH becomes alkaline as ammonia is liberated from the proteins. The reaction rate decreases as the more resistant materials are attacked; the compost enters stage 3.
3. *Cool-down:* as the temperature falls, the thermotolerant fungi reinvade the composted material from the cooler environment. These first three stages of composting last from a few weeks to two months, depending on the composted material and technology of composting.
4. *Stabilization and maturation:* requires several months, during which little heat is generated and the final pH is normally slightly alkaline. During this phase, mesophilic microorganisms, as well as macrofauna, colonize the compost. Intense competition for food takes place between the microorganisms, involving antagonism and the formation of antibiotics (5). Humification occurs in the residual OM to produce the stable composted product (2). This process probably includes the polymerization of aromatic compounds, as well as lignin modifications. Thus, it could be important in pollutants detoxification via immobilization in the humic substances.

Thus, the composting of organic wastes is a dynamic and extremely complex ecological process in which temperature, pH, and nutrient availability are constantly in flux.

Different types of microorganisms are active at different times in the composting pile. Bacteria have the most marked effect on the decomposition process, and are the first to take hold. The numbers and species of organisms responsible also change markedly. Thermophilic bacteria, particularly *Bacillus* spp. and *B. cereus* (6), appear to dominate the early phase of high activity, but thermophilic actinomycetes predominate thereafter (7). Fungi, which compete with bacteria for food, play an important role at later stages in the process as the pile dries, as these organisms can tolerate low-moisture environments better than bacteria. Raw compost material contains about 10^6

microbial counts of mesophilic fungi per gram of raw material and 10^3 to 10^6 thermophilic fungi per gram (8). The predominant mesophilic fungi in the raw material are *Geotrichum* spp. (9) and the thermotolerant fungus *Aspergillus fumigatus* (10).

Thermophilic microorganisms prefer temperatures between 45 and 70 °C. Thermophiles generate even greater quantities of heat than mesophiles, and the temperatures reached during this stage are hot enough to kill most pathogens and weed seeds. The thermophiles continue decomposing the feedstock materials as long as nutrient and energy sources are plentiful. As these sources become depleted, thermophiles die, and the temperature of the pile drops. In mushroom compost, thermophilic fungi are responsible for the degradation of lignocellulose (11).

Thermophilic and thermotolerant fungi, which are known to possess cellulolytic and ligninolytic activities, or which have been found growing in compost, have been studied (12): for example, *Talaromyces emersonii*, *Thermoascus auranticus*, *Thermomyces lanuginosus*, and a *Coprinus* sp. (9,10). Chefetz and coworkers (13) purified phenol oxidase (laccase) from municipal solid waste compost during the thermophilic stage of composting. *Chaetomium thermophilium*, a cellulolytic fungus, was isolated from this stage and exhibited laccase activity when grown at 45 °C. The purified laccase exhibited high catalytic activity toward a wide range of phenolic substrates. These fungi may be responsible for the degradation of aromatic xenobiotics during composting.

When the temperature decreases, mesophilic and thermotolerant microorganisms recolonize the compost (8). The fungi that are dominant after peak heating are *Aspergillus* spp. (9) or *T. lanuginosus* (10). Basidiomycotina occurring in compost include *Panaeolus* spp., *Corticium coronilla*, and possibly *Mycena* spp., all of which have been isolated from compost during the cooling and maturation phases, or from mature compost (9,10).

APPLICATION OF COMPOST FOR THE DETOXIFICATION OF TOXIC ORGANIC COMPOUNDS

During composting, the organic contaminants in the raw material are exposed to the activity of microorganisms that are capable of metabolizing some of them to by-products (partial degradation or transformation) and some of them to completion (mineralization). Consequently, composting has been proposed as a means of removing such organic compounds (bioremediation) (14). Of the broad range of common toxic pollutants reported to be degraded in compost, explosives, and more specifically 2,4,6-trinitrotoluene (TNT) and hexahydro-1,3,5-trinitro-1,3,5-triazine or royal demolition explosives (RDX), have been widely studied (Table 1). Other common families of compounds intensively studied include total petroleum hydrocarbons (TPH) such as gasoline, diesel and jet fuels, oil, and grease (15); polycyclic aromatic hydrocarbons (PAH) found in wood preservation, coal gasification, and petroleum refining wastes (16), and the most distinctive family of toxic compounds studied, pesticides (i.e., insecticides and herbicides) (17,18).

In trials for examining the degradation of pesticides in cotton waste chicken manure–based compost, almost complete degradation of carbofuran (a carbamate insecticide) and 99% degradation of simazine (a herbicide of the triazine group) were found after 50 days (19). In Alaska, composting was tested as a means of clearing the soil of fuel pollutants (20). Almost complete degradation of alkylbenzene was registered after 35 days along with a drop in TPH from 1,700 to 120 mg/kg after 70 days. The ability of microorganisms to degrade many dangerous chemical compounds has been proven; among them are compounds that are known to be resistant to degradation, such as benzene, pentachlorophenol (PCP), phthalates, heavy and light fuels, phenols, PAH, mineral alcohols, and polychlorinated biphenyls (PCB) (21). Residues of organochlorines can still be found in composts because of their high recalcitrance to biodegradation.

Table 1. Examples of Organic Compounds from the Four Main Families of Environmental Pollutants Studied for Degradation During Composting

Class of Contaminant	Source	Example
Petroleum products		
TPH	Petroleum industries, vehicle repair workshops, oil-cleaning plants, oil separators: gasoline, diesel and jet fuels, oil and grease	Mainly alkanes, paraffins with 1 to 40 carbon atoms per molecule, and cycloalkanes
PAH	Incomplete burning of coal oil, wood, and garbage; coal gas production; petroleum refining; coal cooking; coal tar manufacturing; wood preservation; aluminum extraction; asphalt; cigarette smoke; fire smoke and residue	Fluorene, naphthalene, anthracene, phenanthrene, pyrene, benzo[*a*]pyrene
Pesticides	Insecticides, herbicides, fungicides	Atrazine, dicamba, diazinon, 2,4-D
Explosives	Military munitions	TNT, RDX, nitrocellulose
Nonvolatile chlorinated compounds	Wood and leather preservatives, pulp and paper manufacturing, production of synthetic polymers, and burning of chlorinated plastics	Polychlorinated dioxins (PCDD) and furans (PCDF), polychlorinated biphenyls (PCB), chlorinated phenols

The degradation of PCP, anthracene, phenanthrene, benzo[*a*]pyrene, and a mixture of benzene, toluene, ethylbenzene, and xylenes (BTEX) during mushroom composting or mushroom compost and soil landfarming was studied on a laboratory scale, using flask respirometers (10 to 100 g), bench composters (1 kg), and environmentally controlled compost bins (40 kg) (22–24). Mineralization of ^{14}C-PCP was observed in composts incubated at 30 °C for 4 weeks, and generally, neither toxic intermediates (e.g., chloroanisoles) nor polymerization products (e.g., dibenzo-*p*-dioxins) were detected. Pre-enrichment using PCP at 500 mg/kg for 3 months on a 30-kg scale induced PCP mineralization activity in composts. Further studies showed that during the thermophilic stage, compost could also be induced to degrade freshly added phenanthrene. The extent of mineralization (65% after 50 days) was greater than in noninduced compost (6%). However, the application of compost did not seem to influence the removal of aged phenanthrene from soil. In contrast to chlorophenols, PAH are strongly hydrophobic compounds that exhibit significant partitioning in the organic carbon of soil, resulting in reduced bioavailability and hence bioremediation potential.

Despite the numerous investigations performed and the wide application of composting in bioremediation, knowledge of the mechanism involved in the degradation of organic compounds during this process is relatively scarce. The following sections provide a detailed description of the four major families of pollutants studied for their degradation during composting: petroleum products (TPH and PAH), nonvolatile chlorinated compounds, pesticides, and explosives.

DEGRADATION OF PETROLEUM PRODUCTS

TPH contaminants consist of several aliphatic and aromatic fractions of crude and treated oil, ranging from simple *n*-alkanes (paraffins with 1 to 40 carbon atoms per molecule) to complex branched and cyclic alkanes, both aromatic and polyaromatic (25). The major sources of TPH discarded to the environment are wastes from petroleum industries, vehicle repair workshops, oil-cleaning plants, and oil separators (see Table 1). *n*-Alkanes, which are easily degraded by soil bacteria, are normally absent or present at low concentrations. The remaining TPH are more difficult to degrade and therefore considered recalcitrant. These include structurally complex alkanes, such as isoprenoids, steranes, hopanes, two- to six-ring PAH, cycloalkanes, asphaltenes, resins, and polar compounds. The many-branched pristane and phytane were used as endogenous tracers to estimate the biodegradability of TPH in several studies (25). TPH-contaminated soils frequently contain 5,000 to 20,000 ppm. Biopiling of soil with mature compost in laboratory-scale reactors resulted in the rapid degradation of even complex TPH within two weeks to two months, compared to the long treatment required with landfarming (up to 6 months) (26,27). The studies found degradation rates of about 375 ppm TPH/day, in contrast to the 40-ppm TPH/day reported for in situ biodegradation (28). However, despite the high extent of degradation reported, a significant amount of bound residue was found. In a field study, Bartusiak (29) reported an oil degradation rate of 110 ppm/day. Kirchmann and Ewnetu (15) studied the biodegradation of oil sludge from petrol stations and petroleum residues from a refinery through composting with horse manure. Paraffin oil was used as a reference. Oil wastes decomposed 78 to 93% within 4.5 months of composting. Mineralization accounted for 37 to 41% of the carbon degraded. Degradation was estimated by measuring total organic carbon (TOC) and total oil content (toc) based on extraction of the organic substances with trichlorotrifluoroethane. Mineralization was determined as the difference between TOC and toc. Volatilization was found insignificant. Brown and coworkers (25) found accelerated degradation of TPH in soil amended with sludge compost. Two- to three-ring PAH degraded more rapidly than others. Among the four-ring PAH, pyrenes degraded more readily than chrysenes. The more recalcitrant PAH within a homologous group were the highly methylated ones.

PAH are composed of several hundred individual compounds containing at least two condensed aromatic rings and represent the more widely studied family of TPH components. Today, anthropogenic combustion of fossil oil is by far the major source of PAH input into the environment (see Table 1) (30,31). PAH with two and three rings are often referred to as low molecular weight (LMW) PAH, whereas those with more than three rings are often referred to in the biodegradation literature as high molecular weight (HMW) PAH (32). Fluorene (two rings), naphthalene (two rings), anthracene (three rings), phenanthrene (three rings), pyrene (four rings), chrysene (four rings), and benzo[*a*]pyrene (five rings) constitute the more extensively studied congeners (Table 2). The recalcitrance of PAH is caused by their limited solubility in water, the requirement for oxygen to initiate oxidation, and their diverse and complex structure (32). In addition, some of the compounds have biocidal properties. The lighter congeners (e.g., naphthalene) are volatile; as the number of condensed benzene rings increases, so do hydrophobicity and resistance to microbial degradation (30,33).

Crawford and coworkers (33) reviewed the potential of composting for the bioremediation of soil contaminated with PAH. Windrow facilities were efficient for the degradation of PAH in compost; however, this increased the volatilization of light and semivolatile congeners. They concluded that both bench- and field-scale studies are needed to further define the variables most conducive to composting processes focused on the degradation of PAH.

Joyce and coworkers (16,40) studied the degradation of a mixture of three- and four-ring PAH during composting of municipal solid waste in a batch-type, in-vessel, laboratory-scale composter. The extent of their disappearance was monitored for 2 months. The results indicated that the major biodegradation occurred during the first 30 days (i.e., active phase). Anthracene, phenanthrene, and pyrene almost completely disappeared, whereas 80% of fluorene's disappearance was because of volatilization. Benzo[*a*]pyrene was completely recalcitrant.

McFarland and colleagues (34,41) reported higher initial rate of bound-residue formation of contaminant carbon

Table 2. Carbon Allocation During Controlled Composting of PAH-Containing Wastes on a Laboratory Scale

PAH	Percentage of Initial Carbon in the Fraction				
	Disappearance	Volatilized	Mineralized	Bound	Adsorbed
Fluorene	90–95	80	n.d.a.	n.d.a.	n.d.a.
Naphthalene	80–95	17	20–60	10–27	45
Anthracene	92–95	n.d.a.	10–30	20–70	4
Phenanthrene	80–95	n.d.a.	38	36	5
Pyrene	50–90	n.d.a.	10–45	15–45	5
Benzo[*a*]pyrene	60	n.d.a.	5–10	20–60	n.d.a.

Note: Adapted from References 34, 35–37,38,39. Data refer to ^{14}C-labeled experiments in soil and compost mixtures or sludge compost. n.d.a.: no data available.

during the first 30 days of composting by inoculating a benzo[*a*]pyrene-contaminated soil composting system with the white rot fungus *Phanerochaete chrysosporium*. Although, fungal inoculation was ineffective in significantly enhancing the extent of benzo[*a*]pyrene degradation within 95 days of composting, the extent of contaminant disappearance was around 63%. However, analysis of the volatile fraction indicated that neither mineralization nor vapor partitioning of benzo[*a*]pyrene (or its chemical intermediates) is significant during the soil composting process. Bound-residue formation was found to be the predominant transformation mechanism for benzo[*a*]pyrene in the microbially active compost systems, accounting for nearly 100% of its removal.

The effect of compost age on the degradation of the four- to six-ring PAH perylene, benzo[*a*]anthracene, and chrysene during composting of municipal solid waste was studied by Martens (42). The author concluded that microbial decay of PAH during composting occurs because no accumulation of PAH concentration in ripe compost was detected inspite of a 40 to 60% weight reduction during composting. In contrast, studies with ^{14}C-labeled PAH indicated that in fresh composts, only minor amounts of PAH are degraded.

The degradation of PAH during composting of artificially contaminated pinewood and naturally polluted pinewood (mixture of waste wood and PAH-polluted soil), mixed with animal manure, was studied at a pilot-scale level (43). PAH degradation in dewar vessels was influenced by the inoculum used. The fastest PAH degradation was achieved by compost addition, but the most intensive CO_2 evolution was measured with PAH-polluted soil as an additive. After 61 days, the PAH content of the wood was reduced from 1,000 mg/kg each to 26 mg/kg of phenanthrene and 83 mg/kg of pyrene. The relationship between microbial wood decay and PAH degradation showed that the detoxification, at least of artificially PAH-polluted wood, requires only partial wood decay. In a pilot-scale percolator, after 27 days of remediation, the portion of residual PAH was higher in the naturally polluted material. The slower degradation in the naturally polluted waste wood may be explained by the lower bioavailability of pollutants relative to artificially contaminated wood. In the former, the degradation rate of PAH depended on their degree of condensation (the higher the number of aromatic rings, the smaller the degradation rate).

Several studies have reported that adding compost to contaminated soil enhanced bioremediation because of the structure of the organic matrix. Kastner and Mahro (35) studied the degradation of naphthalene, phenanthrene, anthracene, fluoranthene, and pyrene in soil and soil and compost mixtures. To evaluate degradation and to distinguish it from sorption, they performed alkaline humic acid extraction of the samples. Kastner and coworkers (36) reported stimulated degradation of ^{14}C-labeled anthracene and hexadecane in soil amended with ripe compost. With compost, 23% of the labeled anthracene was transformed into $^{14}CO_2$ and 42% was fixed to the soil matrix irreversibly, whereas more than 88% of the original anthracene could be recovered from the nonamended control. The formation of nonextractable bound residues was markedly lower with ^{14}C-hexadecane as only 21% of the labeled carbon had become nonextractable after 103 days. Kastner and coworkers (37) also reported that anthracene decomposition in soil occurs in a two-phase process. In the first, the original compound is sequestered in the soil, as reflected by its limited extractability. In the second, PAH metabolites undergo oxidative coupling to the humic substances, forming nonhydrolyzable complexes.

Compost was also reported to enhance the oxidation of aromatic contaminants in soil to ketones and quinones, which eventually further disappear, during the composting of soils and compost mixtures artificially contaminated with coal tar PAH, including N-containing analogs (44,45). Whereas in nonamended soil only aromatics with up to three fused benzene rings were considerably degraded during the first 15 weeks of the experiment, soil supplementation with compost helped enhance elimination of all compounds monitored. After 15 weeks, substantial residues were only found for benzo[*a*]anthracene, chrysene, and benzo[*a*]pyrene. Accumulation of ketonic and quinonic degradation products, such as 9-fluorenone, anthracene-9,10-dione, 2-methyl anthracene-9,10-dione, and benzo[*a*]anthracene-7,12-dione, was observed in nonamended soil material. In mixtures with compost, short-term concentration maxima of such products correlated well with phases of enhanced contaminant elimination.

Soil composting treatment performed on field and laboratory scales was compared for the removal of PAH at mesophilic temperatures (46). PAH degradation was lower in the laboratory trial than in the field trial, and

no self-heating of the soil was demonstrated in the laboratory. The absence of microorganisms was unlikely to have limited the biodegradation of PAH in this study. Composting for the removal of PAH from soil was assessed as a treatment option at a former tar-contaminated site in Australia, alongside conventional land treatment. Soil composting was substantially more effective in removing benzo[*a*]anthracene, benzo[*b* + *k*] fluoranthene, benzo[*a*]pyrene, dibenzo[*ah*]anthracene, chrysene, indenopyrene, and benzo[*gih*]perylene, than the land treatment processes. The extent of removal of these HMW-PAH was at least 50%, over the seven-month treatment period in which composting was used, whereas degradation did not exceed 5% for each of these PAH compounds in the land treatments over the same period (47).

The relationship between biomass growth and PAH degradation in soil, and subsequent toxicity reduction, was evaluated in bench-scale compost units. Whereas all treatments resulted in significant degradation of PAH with two to four rings in their molecular structure (50 to 95%), no reduction in the concentrations of five- or six-ring PAH occurred during the 12-week study (48,49). Rates of PAH removal during the first four weeks of compost treatment correlated strongly with initial PAH concentration but not with the reactor biomass concentration. Several toxicity bioassays were used to evaluate the efficacy with which compost biomass reduces toxicity of PAH-contaminated soil. After composting, no significant genotoxicity was observed in the soil.

Degradation of PAH in aged, creosote-contaminated soil by applying spent oyster mushroom compost was studied in laboratory-scale polyethylene columns (50). Whereas degradation of three-ring compounds was similar, the four- and five-ring PAH were better degraded when soil and fungal substrate were homogenized rather than layered. Fish oil added to the spent mushroom compost (SMC) and mixed with creosote-contaminated soil gave the best degradation of PAH. This treatment, incubated for seven weeks at ambient temperature, resulted in the following removal percentages: 86% of the total 16 PAH, 89% of three-ring PAH, 87% of four-ring PAH, and 48% of five-ring PAH. Whereas numerous studies have been performed on the fate of PAH during composting, very little information is available on their volatility. The LMW congeners, with the higher vapor pressures, are expected to be more susceptible to volatilization. Thus, Silviera and Ganho (38) found 17% volatilization of ^{14}C-labeled naphthalene in laboratory reactors.

Similar to soil, PAH degradation by microorganisms in compost is limited by its availability and by the presence of microorganisms possessing suitable metabolic activities. In contrast to soil, however, composting provides temperature, pH, and redox potentials suitable for their degradation. Sorption and desorption of PAH to and from the organic matrix of compost are crucial to the bioavailability of these compounds. In a recent review on PAH in soil, Wilcke (31) concluded that PAH sorption in soil resembled its partitioning between water and an organic solvent, indicating that sorption is an entropy-driven two-phase process. The first is rapid and probably corresponds to sorption near the surface of the macromolecules. The second is slow and may be explained by diffusion of the PAH to internal binding sites. There appears to be an inverse correlation between the polarity of the organic matrix and the strength of adsorption. Similarly, studying the pattern of pyrene sorption to different types of natural organic matter (NOM), Chefetz and coworkers (51) concluded that the aliphatic moieties of NOM contribute significantly to the sorption of nonionic aromatic pollutants to complex NOM matrices.

The data presented in Table 2 summarize the distribution of the carbon fraction of the most extensively studied PAH congeners during composting under controlled laboratory conditions. As can be seen from the data summarized, covalent binding to the OM seems to be the prevalent path of disappearance, whereas adsorption seems to be a minor one, for all the congeners. Mineralization is significant for the LMW-congeners; however, it is only minor for the HMW-congeners. Volatilization seems to be an important path of disappearance for the low vapor pressure, LMW-congeners, such as fluorene and naphthalene; although, there is not enough data to make a decisive conclusion on the basis of studies performed under composting.

DEGRADATION OF HALOGENATED COMPOUNDS

The toxic halogenated organics, especially chlorinated congeners, described in this section refer to those aliphatic, aromatic, and polyaromatic compounds derived from the use of wood and leather preservatives, pulp and paper manufacturing, production of synthetic polymers, and burning of chlorinated plastics (see Table 1) (52). Volatile chloroaliphatic compounds such as dichloromethane, dichloroethene, trichloromethane, trichloroethene, tetrachloroethene, vinylchloride, and other volatile organic compounds (VOC) are normally treated by biofiltration using, among others, finished compost as a biofiltration packing matrix, rather than composting systems.

Polychlorinated dibenzo-*p*-dioxins (PCDD) and polychlorinated dibenzofurans (PCDF) are known to spread through the environment by atmospheric transport and deposition processes (53,54). PCDD and PCDF have been detected in trace amounts as unwanted by-products in final products and residues of industrial production. The sources of PCDD and PCDF can be divided into primary ones, for example the chemical industry, pulp and paper industry, etc., and combustion processes with stationary and mobile sources. The main route for the introduction of dioxins into the environment is via combustion processes, whereby the emitted dioxins may be carried by atmospheric transport to ultimate environmental sinks (55). Such processes lead to the presence of these compounds, albeit at low concentrations, in plant materials and soil, both of which are major components of the raw material used in composting. The fate of PCDD and PCDF during composting, including the more toxic congeners (2,3,7,8-substituted PCDD and PCDF), was studied by Eitzer and coworkers (54). Although somewhat variable, their studies revealed that no significant changes in concentration took place during composting. However, as the average toxic range of these compounds, measured in terms of

toxic equivalents (TEQ), was found to be below the recommended unrestricted level of 5 pg TEQ/g, the final compost was considered safe for agricultural use.

Laine and coworkers (56) applied luminescent bacteria tests to assess the fate of chlorophenols during the composting of sawmill soil and impregnated wood. The assessed toxicity decreased during composting, paralleling the chlorophenol concentrations in the compost piles. Organic chlorine compounds, which appeared in HMW sizes, were neither degraded nor remobilized during composting, although no further polymerization occurred during the composting. Large amounts of PCDD and PCDF were found in the compost piles, but their concentrations did not significantly change during the bioremediation process. As PCDD and PCDF were not degraded during bioremediation, the treatment of PCDD- and PCDF-contaminated wood chips in biopiles is not recommended. However, sampling of airborne particles during the mixing of compost windrows at a wood-preserving site contaminated with chlorophenols and PCDD and PCDF, showed concentrations of PCDD and PCDF in different particle sizes. The congener distribution of PCDD and PCDF in the collected air-particle fractions was similar to that in the compost windrows, and their level was 1,000-fold higher than the atmospheric background values reported previously (57).

Only a few PCB congeners with two or three chlorine atoms are likely to be degraded by composting or addition of finished compost to soil, whereas the higher congeners, with four or more chlorines, are present in finished compost at almost the same concentration as that added or found at the beginning of the process (58,59). The level of PCDD and PCDF and PCB in compost at different degrees of maturation from 21 Brazilian municipal solid waste composting plants was evaluated (60,61). Most PCDD and PCDF concentrations were above acceptable levels (17 ng TEQ/kg), especially for compost from metropolitan areas. For PCB, the average concentrations of six PCB congeners were 28, 52, 101, 138, 153 and 180 μg/kg, which is three times less than the value stipulated by regulations (0.2 mg/kg).

Wastewater treatment sludge and power-boiler fly ash were combined and composted in mixed and static windrows (50-m long, 4-m high, and 6-m wide) (62). The concentrations of PCB and chlorophenol in the final compost were below the levels stipulated by regulations (dioxin concentration of 41 pg/g TEQ). Applying compost to soil improved soil characteristics as measured by a number of parameters, and the dioxin concentration in the final soil and compost mixture was 3 pg/g TEQ, allowing the soil and compost mixture to be classified as agricultural soil. In contrast, Rosenbrock and coworkers (63) reported 28% mineralization of U[^{14}C]dibenzo-*p*-dioxin in soil amended with wheat straw and mushroom compost and up to 50% mineralization by mixing soil with wheat straw precolonized with several strains of white rot fungi.

Straw composting of chlorophenol-contaminated soil, performed at a pilot-scale level, was studied by Laine and Joergensen (64). More than 90% of the chlorophenols were removed during the composting period. Frequent mixing and control of the nutrient level enhanced the chlorophenol degradation activity of the indigenous microbes in the contaminated soil. In a parallel bench-scale experiment, an average 60% mineralization of [^{14}C]-PCP was obtained in four weeks, indicating that a major part of the chlorophenols had been mineralized completely.

Benoit and Barriuso (65) studied the degradation of ^{14}C-labeled 4-chlorophenol (4-CP) and 2,4-dichlorophenol (2,4-DCP) during wheat straw composting on a laboratory scale. After three weeks of composting, approximately 20% mineralization of the chlorophenols was observed, whereas approximately 40 and 70% of 2,4-DCP and 4-CP, respectively, were found bound to compost residue, and 30% 2,4-DCP and only 2% 4-CP to the water-extractable residue. Binding of chlorophenols to humic substances has been explained by polymerization via stable ester-bond formation by extracellular enzymes, accompanied by dehalogenation (66,67). Mineralization of [^{14}C]-PCP was observed in mushroom composts incubated at 30 °C for four weeks, and generally, neither toxic intermediates (e.g., chloroanisoles) nor polymerization products (e.g., dibenzo-*p*-dioxins), were detected (22).

The data presented in Table 3 summarize the distribution of the carbon fraction of the most extensively studied chlorophenols during composting under controlled laboratory conditions. As can be seen from the summarized data, the microbial transformation decreases with the degree of chlorination, and covalent binding to the OM seems to be the prevalent path of disappearance. Adsorption and mineralization are less marked for all the congeners, whereas volatilization seems to be negligible.

Table 3. Carbon Allocation During Controlled Composting of Chlorophenols-Containing Wastes on a Laboratory Scale

	Percentage of Initial Carbon in the Fraction				
Chlorophenol	Disappearance	Volatilized	Mineralized	Bound	Adsorbed
4-CP[a]	90	n.d.a.	20	70	2
2,4-DCP[b]	90	n.d.a.	20	40	30
PCP[c]	40–55	n.d.a.	4–15	n.d.a.	n.d.a.

Note: Adapted from References 22,56,64–68. Data refer to experiments with ^{14}C-labeled pollutants. n.d.a.: no data available.

[a] 4-CP: 4-chlorophenol.

[b] 2,4-DCP: 2,4-dichlorophenol.

[c] PCP: 2,3,4,5,6-pentachlorophenol.

DEGRADATION OF PESTICIDES

The use of naturally occurring microorganisms to degrade complex pesticides is not new, and composting is one of the oldest waste disposal systems known to man. Although widely used in the handling of solid wastes, such as agricultural plant residues, the potential of composting in degrading pesticide-laden wastes has only recently been gaining attention. Composting windrows may provide an accessible, low-cost mechanism in which naturally occurring microorganisms can be manipulated to achieve a rapid, cost-effective means of treating toxic wastes. A single windrow may be capable of handling a wide variety of pesticide-laden soils or rinsates simultaneously, or be acclimatized to encourage the dominance of specialized populations of microorganisms to achieve maximum degradation rates (69).

Pesticides vary largely in their chemical nature, and consequently, they vary largely in their ability to break down in compost. Older organochlorine insecticides, for example, toxaphene, break down very slowly (4% within 30 days), whereas other pesticides, for example, trifluraline, degrade more than 80% within the same time frame. Other chemicals, such as 2,4,5-*T*-atrazine, endrin, and PCP, are moderately degradable and are reduced by about 50% in 30 days. Similar variability in breakdown rates is also seen with pesticides in soils (70). In general, organophosphate and carbamate pesticides are at negligible concentrations in composting feedstock, indicating that they are well degraded in the environment. Therefore, most of the degradation studies refer to organochlorine pesticides.

The use of compost for the remediation of high concentrations of released pesticides is considered mainly for two types of applications: (*1*) bioremediation of soils contaminated with pesticides and (*2*) biodegradation of mixed pesticides in rinsates, such as those generated by the washing of sprayers and aircrafts involved with pesticide application. Much of the literature on pesticide transformation in compost refers to the effect of organic amendments, including compost, on the fate of pesticides in soils (71), or to the transformation of pesticides during composting of agricultural or yard waste (18). Neither source is readily relevant to the degradation of pesticides in contaminated soils and rinsates in which pesticides occur at much higher concentrations. Moreover, typical findings in compost-amended soil indicate increased binding and increased stabilization of the pesticides, rather than accelerated degradation (71).

For example, the transformations of eight herbicides (atrazine, simazine, terbutryn, pendimethalin, carbetamide, 2,4-D, metsulfuron-methyl, and dimefuron) in soil after compost addition were monitored during long-term laboratory incubations. Compost addition to soil generally decreased herbicide mineralization and favored the stabilization of herbicide residues. A fraction of the stabilized residues remained extractable and potentially available. However, most of them were nonextractable and formed bound residues. Sorption and desorption could be the first step of kinetically limited biodegradation, mainly for the most highly sorbed herbicides (atrazine, simazine, terbutryn, pendimethalin, and dimefuron). Compost addition had little effect on the less-sorbed herbicides (carbetamide, 2,4-D, and metsulfuron-methyl).

It is important to note that the removal of pesticides by composting is a complex mechanism involving many possible pathways. Most often, the removed pesticides are not actually degraded but their detection becomes impaired by limitations of the analytical analysis. For example, when a pesticide is strongly sorbed to the humic substances in the composting pile, it will still be present in the compost but it may become very difficult to chemically extract, thus its presence can go undetected. However, because of the irreversible nature of some covalent binding mechanisms, the risk to potential acceptors from the bound pesticides is also greatly reduced.

Obviously, complete mineralization to the pesticide's basic, nonhazardous chemical building blocks (carbon dioxide, water, and ammonia) is the best route from an environmental point of view. However, in practice, several other mechanisms normally dominate the removal of pesticides from compost. Some of these mechanisms may include: partial biotic degradation to metabolites; partial abiotic degradation by processes such as hydrolysis or photodegradation; assimilation into microorganisms, uptake by plants, complexation of parent compounds or metabolites with other similar compounds (polymerization) or with humic substances (humification); volatilization into air; leaching into groundwater, or drifting on compost dust in the wind. Volatilization of pesticides has been showed as a major mechanism of removal for a number of pesticides (see review by Buyuksomez and coworkers (17)).

A few studies have reported the disappearance mechanisms and degradation pathways of pesticides during composting using radioactive tracers. Valo and Salkeinoja-Salonen (72) conducted one of the earliest studies on using compost for the degradation of chlorophenol (fungicide) in contaminated soils. Chlorophenol was degraded after 120 days from 200 to 300 ppm to 20 to 30 ppm. Another year of composting reduced the concentration to only 15 ppm, indicating much more difficult removal of part of the aged material. The mechanism of degradation of various pesticides during composting was examined by Lemmon and Pylypiw (73) and by Bugbee and Saraceno (74). They found that 2,4-D is mineralized to carbon dioxide and water, whereas diazinon is partly degraded to water-soluble metabolites and partly to insoluble products. Other studies have shown that composting is a valuable remediation method for soils contaminated with the herbicide dicamba (75) and PCP (76). Racke and Frink (77) found approximately 5% mineralization of carbaryl, a carbamate insecticide, during composting of wastewater treatment sludge in aerated laboratory static piles. They suggested that the main mechanism of disappearance was transformation of carbaryl to 1-naphthol, which in turn binds to the humic fraction of the compost. Reddy and coworkers (78–80) studied the degradation and fate of the [^{14}C]-radiolabeled pesticides diazinon, 2,4-D, and pendimethalin during composting of yard trimmings in aerated laboratory composters. In all cases, volatilization was negligible. After approximately 50 days of composting 47% of 2,4-D and 11% of diazinon were mineralized; the

bound fraction was 49% for 2,4-D (approximately 23% of it was incorporated into humic material) and 51% for diazinon (approximately 19% of it was incorporated into humic material). Only 3% of 2,4-D was found adsorbed, whereas 36% of the diazinon was only hydrolyzed to a secondary product. Pendimethalin was found to be the most resistant to biodegradation, although 13% was mineralized. Rao and coworkers (81) reported complete disappearance and 11% mineralization of [^{14}C]-atrazine after four months of composting of pretreated wood.

The need for appropriate methods to capture and then treat pesticide rinsates generated at pesticide loading, mixing, and wash-down sites is coming to the forefront (69). Pesticide disposal methods based on the principles of bioremediation are not only safe and effective, they are also practical. Several media have been tested as adsorbents for pesticides in rinsates. SMC was tested as an adsorption medium for the removal of pesticides, including carbaryl, carbofuran, and aldicarb, at concentrations of 0 to 30 mg/L from rinsate (82,83). It was found that SMC might potentially be used for on-farm treatment of pesticide rinsate. The rapid equilibrium reached by the pesticide solution and SMC was consistent with a physical type of adsorption mechanism. The adsorption of carbamate pesticides onto SMC exhibited nonlinear, "favorable" adsorption behavior that could be well characterized by the Freundlich isotherm model. In addition, multipesticide adsorption tests displayed the characteristics of competitive behavior. The competitive ability of these three pesticides in multicomponent adsorption followed the order carbaryl > carbofuran > aldicarb, consistent with their adsorbability.

Mullins and coworkers (69,84) developed a system that utilizes lignocellulosic (organic) sorbents and subsequent chemical and microbial-mediated degradation of sorbed pesticide residue. They envisioned this system's incorporation into a pesticide loading, mixing, and wash-down pad facility. By combining or integrating bioremediation with a sorbent and wash-down pad facility, it may be possible to dramatically reduce pollution of surface and groundwater at sites in which application equipment is loaded and cleaned. Furthermore, they constructed and tested several versions of a pesticide disposal system that includes a two-step disposal process: sorption and biodegradation.

Field-scale tests on the disposal of four formulated pesticides using peat moss as the sorbent and composting of the sorbed residues were performed. Thirty-five–gallon volumes of pesticides at concentrations ranging from 1,200 to 1,500 mg/kg were treated. The pesticides mixture included: malathion (Dragon 4E), captan (Captan 50 WP), lindane (Ortho 2.5E), and diazinon (Ortho 4E). The total amount of residue remaining in the treated rinse water and composted sorbent represented less than 2% to as little as 0.003% of the initial amounts of each pesticide contained in the untreated rinse water prior to treatment (69). These results demonstrated the efficacy of using organic sorbents in a large-scale system to remove and degrade pesticides from pesticide-containing rinse water.

Field studies have also demonstrated the degradation of pesticides in a composting environment. For example, very high levels of diazinon can be degraded within a short time. Levels ranging from 4,000 to 32,000 mg/kg were effectively degraded to 1 and 61 mg/kg, respectively (after 8 weeks) and 1 and 7 mg/kg, respectively (after 18 weeks) (85). Bioaugmentation (i.e., inoculation of specialized microorganisms) is likely to enhance the remedial application of solid-state fermentation where difficult-to-degrade pesticides are concerned. More information is needed to establish composting as the method of choice for rinsate disposal. The biggest problem is still the partial degradation of pesticides in the compost pile, which in turn causes large amounts of compost to be defined as hazardous waste because of a high concentration of bound residues.

The data presented in Table 4 summarize the distribution of the carbon fraction during composting under controlled laboratory conditions of distinctive members of each of the families of pesticides commonly applied in agriculture. In contrast to other environmental pollutants, mineralization in general terms is low whereas microbial transformation, followed by binding OM, and adsorption seem to be prevalent path of disappearance. Volatilization seems to be marked in many cases.

DEGRADATION OF EXPLOSIVES

Environmentally unaware practices in areas in which explosives are produced and/or handled have resulted

Table 4. Fate of the Carbon Fraction During Controlled Composting on a Laboratory Scale of Members of the Major Types of Pesticides

	Percentage of Initial Carbon in the Fraction				
Pesticide	Disappearance	Volatilized	Mineralized	Bound	Adsorbed
2,4-D[a]	30–90	n.d.a.	48	49	3
Atrazine[a]	70–92	n.d.a.	4–11	n.d.a	40–60
Chlordane[a]	44	n.d.a.	0.2–5	n.d.a.	n.d.a.
Diazinon[b]	90–95	22	1–11	51	35
Carbaryl[c]	95	50	0.5–1	n.d.a.	n.d.a.

Note: Adapted from References 17, 18, 70, 77–81, 86. Data refer to experiments with ^{14}C-labeled pollutants. n.d.a.: no data available.
[a] Organochlorine. 2,4-D: 2,4-dichlorophenoxyacetic acid.
[b] Organophosphate.
[c] Carbamate.

in the contamination of soils and sediments with various explosives, including TNT, RDX, octahydro-1,3,5,7-tetranitro-1,3,5,7-tetraazocine (HMX), and methyl-*n*-2,4,6-tetranitroanaline (tetryl).

Until a decade ago, no biological methods were available to mineralize explosives effectively under aerobic conditions without the production of unwanted metabolites (87). As a result, very costly incineration ($300 to $500 per ton soil) was the method of choice for removing explosives from soils (88). In recent years, composting and alternating anaerobic and aerobic biological treatments have been developed and tested on full-scale operations (59,89). The centuries-old technology of composting has been refined to maximize the degradation of hazardous materials such as TNT. The advantage of using compost is associated with the use of compost microorganisms that convert TNT and related compounds into simpler, benign, organic molecules, similar to the way in which they convert organic materials into humus (59). The whole process is based on nature's own ability to decontaminate soils, but under the accelerated conditions existing in the compost pile. The most common type of composting when dealing with TNT-contaminated sites is windrow composting. During such composting, the soil is mixed with compost and spread over a field or other surface in narrow rows called windrows. To facilitate microbial growth, composting material, such as wood chips, straw, alfalfa, manure, or other agricultural products, is added (90). Windrows are kept aerated with occasional turning, and moisture content, oxygen level, and temperature are all carefully monitored. Windrow composting has been found to have a high degree of treatment success; moreover, composting costs far less than incineration and is more environmentally sound. Composting yields compost, which is a valuable material, as opposed to incineration, which yields ash that must itself be disposed of as toxic waste after it has been solidified. Composted material can be revegetated, unlike solidified material. Moreover, composting consumes only very small amounts of nonrenewable energy sources to operate the machinery, whereas incineration uses large quantities of fossil fuel with all the associated environmental consequences.

When the Umatilla Army Depot in Hermiston, Oregon, a Superfund site, composted 15,000 tons of contaminated soil instead of incinerating it, it saved approximately $2.6 million. Risk-based cleanup goals for Umatilla were established at explosive concentrations of less than 30 mg/kg for TNT and RDX. The project exceeded these expectations by achieving undetectable levels of explosives. Contaminant by-products were either destroyed or permanently bound to soil or humus (59).

It is important to note that complete mineralization is not the common biochemical mechanism that acts to decontaminate soils containing explosives. TNT-contaminated soil spiked with radiolabeled [^{14}C]-TNT and treated biologically led to complete immobilization of the reduced TNT in the soil (91). At the end of the treatment process, more than 98% of the initial radioactivity was bound to the soil. Only transformation to the solvent-extractable products, 4-amino-2,6-dinitrotoluene and 2-amino-4,6-dinitrotoluene, and conjugation to cellulosic, humin, humic acid, and fulvic acid was detected in composting of an explosives contaminated soil amended with radiolabeled TNT in a small-scale compost simulation system (92). More than half of the added radioactivity was recovered in the cellulose plus humin fractions. Microbial transformation of TNT usually begins with reduction of one of the nitro groups. Aerobic bacteria are able to reduce two of the three nitro groups of TNT; the reduction of the third nitro group requires anaerobic conditions. Many of the products of TNT biodegradation are highly reactive and covalently bind to cellular components and organic solids (in soil or compost). The latter prevents or prolongs the mineralization process, but also hinders a further spread of TNT contaminants.

Composting can be problematic because the precise fate of explosives is not easily determined. If TNT or its degradation intermediates are bound to the compost mix, these could leach back into the soil. However, extraction experiments with biologically treated soil showed that drastic desorption conditions and even strong hydrolytic agents (0.5 M HCl) could mobilize only minor amounts of the bound residue (<1%). High long-term stability of the reduced and bound radiolabeled TNT in soil was demonstrated in a leaching experiment that was carried out over a period of two years simulating 2,000 mm of rain per year (91). Nuclear magnetic resonance data further confirmed the irreversible binding of the reduced TNT in soil, indicating the safety of the biologically treated soil (91).

Although the Umatilla soil was heavily contaminated with TNT and RDX, no explosives could be detected after composting, and the soil was restored to "a better condition than before it was contaminated" (93). The composting feedstocks used at Umatilla were 30% contaminated soil, 21% cattle manure, 18% sawdust, 18% alfalfa, 10% potato waste, and 3% chicken manure. In other cases, substitutions may be made depending on the cost and availability of ingredients. Composting of explosive-laden soils has been since used in other, geographically varied regions, including the United States Naval Submarine Base in Bangor, Washington; the Navy Surface Warfare Center in Crane, Indiana; the Sierra Army Depot in Herlong, California; the Seymour Johnson Air Force Base in North Carolina; the Louisiana Army Ammunition Plant; Fort Riley in Kansas; and the Hawthorne Army Depot in Nevada.

A different composting approach was reported by Breitung and coworkers (94) and Bruns-Nagel and coworkers (95). Continually aerated compost was compared to compost aerated after an anaerobic prephase of 65 days. The continuous aeration system rapidly degraded 92% of the TNT and contaminating by-products. Although slower, the two-phase system was able to achieve almost 100% degradation of TNT and contaminating by-products (levels were undetectable). The two-phase system works by reducing TNT almost entirely to amino-dinitrotoluene (Am-DNT) during the anaerobic prephase, then using the aerobic phase to degrade the Am-DNT.

Traditional composting presents two major setbacks. First, no more than 30% of the compost pile can be made up of the contaminated soil, which results in an increase in the amount of product that must eventually be landfilled. Second, despite substantial efforts, residual toxicity of the finished compost is still a possibility (87).

To overcome these problems, Grace Bioremediation of Canada developed a proprietary solid-phase composting methodology known as DARAMEND® (89). The technology employs combinations of organic and inorganic soil amendments to modify the physical and chemical conditions in the soil to enhance the rate and extent of microbial biodegradation of organic explosive compounds. The key components of the technology, as applied to soils containing organic explosive compounds, are: proprietary organic amendments, trace quantities of multivalent metals (e.g., powdered iron), physical disruption of soil structure through vigorous tillage, and regulation of soil moisture and oxygen diffusion by irrigation. Typical application rates for the organic amendments are between 0.5 and 2% (w/w) of contaminated soil, thus virtually no bulking of the soil occurs. The number of treatment cycles required to attain the remediation goals varies with the identity of the target compounds, their initial concentrations, and the applicable remediation criteria. DARAMEND® bioremediation technology is currently being implemented full scale on soil contaminated with organic explosives and chlorinated solvents at Naval Weapons Station Yorktown in Virginia. Soil concentrations of organic explosive compounds of 11,350 mg/kg were reduced in 74 days of treatment by more than 99%. Specifically, TNT was reduced to 3 mg/kg, RDX to 4 mg/kg, HMX to below detection limits, and Am-DNT to 1.9 mg/kg (89).

Today, it is clear that the treatment of explosive-laden soils using biological alternatives can compete with the expensive and less environmentally friendly incineration methods.

THE FATE OF ORGANIC POLLUTANTS DURING COMPOSTING

One of the most complicated and controversial aspects of contaminant detoxification by composting is their real fate. Whereas disappearance seems to be very fast (a few hours to a few days, depending on the nature of the contaminants, initial availability, and concentration), the real contribution of biotic degradation or abiotic transformation is more complex and difficult to measure. The ultimate fate of the contaminants depends on the technology of composting, the size of the units, and the duration. For pilot-scale composting studies, a volume of at least 10 to 20 m^3 is required for achieving the typical temperature profiles seen in full-scale piles (17,18,59,96). Furthermore, some of these results depend directly on the chemical analysis and extraction techniques used to monitor pollutants fate. The efficiency and yield of conventional techniques, such as cold extraction and Soxhlet extraction, change during the different stages of composting, as a function of the changes in the solid matrix during humification.

Mass-balance calculations to assess the fate of toxic organic compounds during composting have been reported in lab-scale units, using ^{14}C-radiolabeled material. According to these results, at least in the short term, complete degradation (i.e., mineralization) accounts for only a small portion (10–50% of initial labeled carbon, depending on the type of compound). Biotic mechanisms such as adsorption and binding during humification, which tend to retain the contaminants and/or their metabolites within the solid matrix, appear to be a significant mechanism of transformation (25–40% of initial labeled carbon, depending on the type of compound). Volatilization is also significant; its importance depends on the physical and chemical nature of the contaminants (e.g., vapor pressure), the technique of composting (e.g., forced versus natural aeration, number of turnings), and the size of the composting plant. The schematic distribution rates of the different carbon fractions of the source pollutants of each of the main families of organic compounds reviewed in this chapter are presented in Table 5 (see also Tables 2–4).

The major difficulty in quantifying the fate of contaminants in compost is that lab-scale units (2–15 L) do not provide results similar to field-scale units, in terms of extent of degradation and time scale (59). The time allowed for composting, and especially the length of the

Table 5. Fate of Organic Carbon During Composting of the Organic Pollutants Discussed in This Chapter

		PAH		Nonvolatile		
Fraction	TPH	LMW	HMW	Chlorinated	Pesticides	Explosives
Disappearance[a]	M	H	L to M	H	M	H
Adsorption[b]	L	L to M	L	L to M	M to H	H
Binding[c]	M	M to H	M to H	H	H	M to H
Volatilization	L	M to H	L	L	M to H	L
Mineralization[d]	M	M to H	L	L	L	M

Note: Abbreviations — TPH, total petroleum hydrocarbon; PAH, polycyclic aromatic hydrocarbon; LMW, low molecular weight; HMW, high molecular weight; L: low rate; M, moderate rate; H, high rate.

[a] Disappearance of the source pollutant.

[b] Adsorption refers to the formation of an extractable residue.

[c] Binding refers to the formation of a nonextractable residue.

[d] Mineralization indicates complete degradation to carbon dioxide and water.

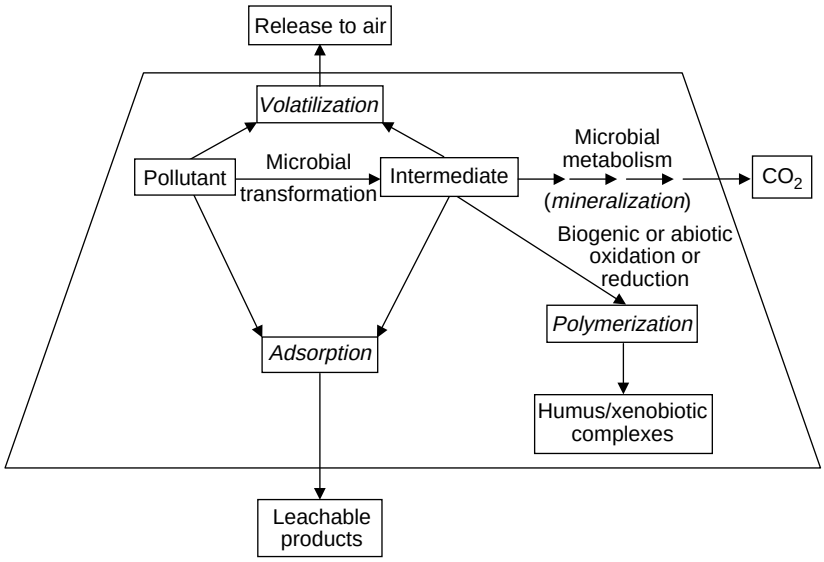

Figure 1. Proposed scheme describing the fate of organic pollutants during composting and its relation to the surroundings. The four main processes occurring are: mineralization (complete degradation), volatilization, adsorption (formation of extractable residues), binding, or polymerization (formation of nonextractable residues). Frame denotes a composting pile.

thermophilic phase, changes drastically from lab to field tests. Volume reduction during composting dramatically affects the residual concentration of the target compounds following the thermophilic phase. More difficult to degrade and less volatile compounds, in fact, concentrate rather than decrease in net reduction. In general, there are not enough data to make a definitive statement on the effect of composting on each particular compound. Moreover, the use of artificial contamination to mimic natural environments, such as soil, or sources of polluted substrates used in composting does not allow, in most cases, a useful prediction of the potential of compost to degrade organic pollutants in natural situations (96).

No remediation technology is appropriate for all contaminants and situations (59). First, the degradation rate of a specific contaminant is affected by the material being composted (97). Second, a relatively low extent of mineralization of aromatic compounds occurs in compost. In some cases, potentially toxic water-extractable intermediates are formed. Compost, as well as other bioremediation techniques, are not efficient enough to degrade highly chlorinated PCB congeners or HMW-PAH congeners (54,56,77). Third, there is the question of whether the formation of nonextractable metabolites is a satisfactory end point of remediation. In practice, loss or lack of extractability by organic solvents is suggestive of binding (39,41,43,77,98). Bound residue, especially to the humic matrix, results from the formation of reactive moieties, such as ester groups, creating low long-term stability. Ether linkages between humic materials and metabolites result in relatively long-term stabilization and decreased bioavailability (39,41,99). Hydroxylated compounds are formed during the degradation of almost all aromatic compounds during composting, which covalently couple to humic substances, resulting in long-term immobilization, but not destruction (30,100). In some cases, coupling of chlorinated compounds to humic material results in dehalogenation (66,67). On the other hand, the high level of OM within the compost matrix enhances adsorption. Adsorption sequesters chemicals temporarily, preventing their further degradation (18). However, adsorbed material eventually leaches. Dubourguier (96) claims that no research has reported on the long-term stability of these humus and xenobiotics complexes for safe use.

A proposed scheme describing the fate of organic pollutants during composting is presented in Figure 1, describing the main biogenic and abiotic processes occurring during composting: mineralization (complete degradation), volatilization, adsorption (formation of extractable residues), binding or polymerization (formation of nonextractable residues).

CONCLUSION

In this chapter have we reviewed various composting processes leading to bioremediation of PAH, chlorinated compounds, explosives, pesticides, and other toxic pollutants. Only a few studies are described on composting per se for bioremediation. However, many processes are based on the principle of composting, such as landfarming and biopiling. In these methods, either fresh OM or compost is amended at various ratios to contaminated soils. Based on the available literature, it may be difficult to conclude upon the optimum composting conditions suitable for specific decontamination treatments as in many cases, the nature and quality of the compost are not well defined. Furthermore, it is important to study bioremediation under more or better controlled environments, which can facilitate the use of tracers, such as ^{14}C-labeled compounds, and the performance of complete mass-balance calculations. It is essential to elucidate the fate of the targeted pollutants and metabolites formed, to assess the mechanism of biodegradation as well as potential residual toxicity.

It can be concluded that composting has some advantages over other in situ remediation technologies.

1. The microbial populations in compost exhibit high activity related to the degradation of organic

material, as well as high diversity of bacteria, actinomycetes, and fungi capable of metabolizing a wide array of organic compounds, including pollutants.

2. The bioavailability of the target compounds seems to be higher in the presence of labile OM, in comparison to soil containing clay minerals, and stable OM, whose interaction with the pollutant could reduce the bioavailability. Moreover, mechanical mixing occurring during composting may improve the physical contact between the different fractions present.
3. The high temperature occurring in the thermophilic phase of composting increases degradation rates although it may promote volatilization of some compounds.
4. The rate of oxidation is higher in compost than in soil because of the continuous supply of air. Furthermore, the extent of oxidation of OM in general, and toxic organic compounds in particular, is improved because of the high oxidation potential of compost compared with soil, as well as enhanced cooxidation because of the array of substrates present.

OM degradation and stabilization are accelerated in compost relative to soil. Thus, composting studies may serve as a model system to predict the potential fate of toxic organic pollutants in long-term processes in soil. Biodegradation of toxic organic compounds in compost exploits a natural process of transformation of heterogeneous OM by mixed microbial populations under controlled conditions. Thus, bioremediation through composting offers an attractive technology for rapid and effective decontamination of solid wastes.

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CONDITIONING FILMS IN AQUATIC ENVIRONMENTS

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DEFINITION

Organic and inorganic compounds present in the bulk water phase or at the water–gas interface rapidly coat substrata immersed in natural waters (freshwater, marine, or subsurface environments) producing thin films, the conditioning films. These films substitute the surface properties of original substrata by those of the combination substratum/conditioning film. Their formation precedes microbial adhesion and, therefore, influences the interaction of microorganisms with interfaces in aquatic environments. Conditioning films of interest to microbial adhesion and biofilm formation are formed by inanimate substances (colloids, molecules, and ions), of a size significantly smaller than a microbial cell, present in the bulk water phase or at the water–gas interface. These films may also serve as a source of nutrients to organisms in oligotrophic environments.

SOURCES OF ORGANIC AND INORGANIC COMPOUNDS IN ENVIRONMENTAL WATERS

Inorganic compounds in natural waters occur as dissolved cations or anions or as colloids and mineral particles. They enter natural waters by the dissolution of rock minerals, the erosion of surface soils, and atmospheric deposition. Aquatic organic carbon in streams and rivers comprise mostly recent (<30 years old) plant and soil decomposition products (1). In lakes and in the oceans, between 30% (lakes with low hydraulic residence times) and nearly 100% (eutrophic lakes fed by groundwater and oceans) of the aquatic organic carbon is autochthonous (2). Organic carbon in groundwater originates from the weathering of "old" organic matter adsorbed on clay minerals or hydrous oxides of aluminum and iron in soil, and from the release of geologic carbon (kerogen, 3). Aquatic organic carbon is divided into dissolved organic carbon (DOC, the organic carbon fraction that passes 0.45-μm filters) and particulate organic carbon (POC, organic carbon retained by 0.45-μm filters). In many natural waters, however, small particulate organic carbon fragments that pass 0.45-μm filters, such as colloidal matter and nanobacteria, the numerically dominant population of bacteria in natural waters (4), may constitute a significant proportion of DOC (5). The DOC concentrations in natural waters vary from low ppm in oligotrophic environments to tens of ppm in eutrophic waters.

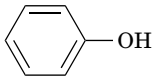
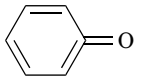
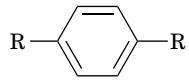
Between 50 and 75% of all aqueous dissolved compounds in natural waters are randomly assembled, high molecular weight, polymeric organic acids known as "humic substances" that form aggregates of 2 to 50 nm diameter commonly associated with clay minerals and oxides of iron or aluminum (1,6). No definite chemical structure can be assigned to these polyfunctional, polyelectrolytic macromolecules whose functionality is dominated by carboxylic acids, esters and amides, aliphatic and aromatic OH, and heterocyclic nitrogen. The generic term encompasses humic acids, fulvic acids, and the relatively insoluble fraction known as humin. Humic acids have molecular weights ranging from 2,000 to 100,000, and constitute some 10 to 15% of DOC in natural waters. They differ from fulvic acids in that they contain fewer carboxylic and hydroxyl functional groups. The remainder of the undefined DOC assigned to the humic group is a poorly understood series of macromolecules known as humin or "hydrophilic acids" with carboxylic, hydroxyl, and carbohydrate moieties (7).

Proteins and their hydrolysis products, dissolved free amino acids (DFAA) and dissolved combined amino acids (DCAA), account for between 2.5 and 5% of DOC in natural surface waters (8). This pool contains material excreted by cells, proteins leached from live or dead organisms, and proteins transformed in the water column by action of extracellular proteases or other compounds. Their size varies from 14 to 66 kDa and their profiles vary both horizontally and vertically in the water column, but their precise identity remains largely unknown. Carbohydrates comprise between 1 and 2% of the total DOC and exist both as free monomers (amino sugars, uronic acids, and aldolases) and as polysaccharides (8). Lipids represent from 10 to 25% of POC in marine surface waters (2) but are only a minor component of DOC. Dissolved lipids consist primarily of *n*-alkanes, pristane, phytane, and methyl esters, ethyl esters, and propyl esters of fatty acids, whereas triacylglycerols, free fatty acids, and phospholipids dominate the lipid pool of the particulate phase. From a mechanistic perspective, interactions between microbial cells and inanimate components of natural waters are mediated by the functional groups present in the molecules. In the case of inorganic compounds, these functional groups are predominantly free solvated ions and, in the case of colloids, oxides of silicium, iron, or aluminum. Most functional groups associated with organic matter are either charged or capable of undergoing polar type of interactions (e.g., hydrogen bonds) (Table 1). It is, however, important to note that many organic macromolecules have extensive hydrophobic domains in their structure. If this hydrophobic character predominates, then the molecule will be expelled from the bulk water phase and accumulate either at solid–liquid interfaces or at the water–air interface.

INVESTIGATING CONDITIONING FILMS

Conditioning films by their very definition are thin surface coatings. Their study therefore requires analytical techniques appropriate to the task and sample handling and manipulation methodology that avoids contamination with materials not present in the water sample or those materials from the water sample that are not part of the

Table 1. Functional Groups Present in Natural Organic Matter

Functional Group	Structure[a]	Occurrence
	Acidic Groups	
Carboxylic acid	R–CO_2H organic carbon	90% of all dissolved organic carbon
Enolic hydrogen	R–CH=CH–OH	Aquatic humic substances
Phenolic OH		Aquatic humic substances, phenols
Quinone		Aquatic humic substances, quinones
	Neutral Groups	
Alcoholic OH	R–CH_2–OH	Aquatic humic substances, sugars
Ether	R–CH_2–O–CH_2–R	Aquatic humic substances
Ketone	R–C=O (–R)	Aquatic humic substances, volatiles, ketoacids
Aldehyde	R–C=O (–H)	Sugars
Ester, lactone	R–C=O (–OR)	Aquatic humic substances, tannins, hydroxy acids
Core aromatic unit		Aquatic humic substances, hydrocarbons
Aliphatic unit	R–CH=CH–CH_2–CH_2–R	Aquatic humic substances, hydrocarbons
	Basic Groups	
Amine	R–CH_2–NH_2	Amino acids
Amide	R–C=O (–NH–R)	Peptides

Note: [a] at pH 7

conditioning film. Vessels for sample collection and storage should, whenever possible, be made of glass and have volumes sufficiently large to avoid significant alteration of the chemical bulk water composition by materials either adsorbed onto or leached from vessel surface polymers. It is also important to process water samples either by membrane filtration or by centrifugation before storage, to separate particulate biotic and inanimate matter. Again, it is important to avoid contamination of the samples with material leached from the membranes or the centrifuge tubes. In this sense, it is advisable to discard the first 100 mL of filtrate, or even better, to determine empirically at which filtration volume the amount of organic or inorganic matter contributed by the filter to the sample becomes negligible. The pore size cutoff of the membranes should be such that it allows passage of small colloids and high molecular weight organic matter but retains even the smallest bacteria. Filters with pore sizes around 0.1 μm are adequate for conditioning film studies. Samples should be stored in refrigerators but not frozen because this would cause aggregation of dissolved macromolecules into colloids during the freezing process.

It is important to ensure thorough removal of bacteria from water samples before conditioning film studies. Depending on the parameters investigated, small numbers of bacteria on substrata could significantly bias conditioning film analysis. If it is assumed that conditioning films are thin films with a thickness of approximately 80 nm (corresponding to a mature conditioning film, 9), with a density similar to that of bacteria, and that these films homogeneously coat a surface, then the material contributed by a globular microbial cell with a diameter of 1 μm (water content approximately 80%, density of organic matter approximately 1 g/cm^3) would correspond to a conditioning film of approximately 6.5 μm^2. If it is stipulated that contamination by microbial compounds should be less than 1%, then a substratum cell density of less than 1 cell per 650 μm^2 would be tolerable. Substratum cell densities observed when substrata are exposed to unfiltered surface waters rapidly reach values well above this threshold. This problem is particularly important when analyzing the chemical composition of conditioning films using extractive techniques or direct in situ analysis methods. It is of lesser importance when determining conditioning film thickness by ellipsometry, for example, because irregularities on small patches of surface do not significantly impact averaged data.

CONDITIONING FILM ANALYSIS

The comprehensive set of data required for conditioning film characterization cannot be obtained with a single analysis method. A brief assessment of the utility of the

most important methods for conditioning film analysis is given in Table 2.

Contact Angles

Contact angles formed when drops of diagnostic liquids spread on substrata are one of the few analysis techniques with good sensitivity for the very top layers of surfaces (Fig. 1). At equilibrium, the relationship between the liquid–vapor (γ_{LV}), the solid–vapor (γ_{SV}), and the solid–liquid (γ_{SL}) interfacial free energies converging at the drop edge/solid/air interface is described by the Young equation (Fig. 1, 10):

$$\gamma_{LV} \cos\theta = \gamma_{SV} - \gamma_{SL} \quad (1)$$

γ_{SV} in Equation (1) may be influenced by adsorption of liquid vapor to surfaces but the consensus in the literature indicates that for contact angles greater than 10°, the spreading pressure caused by the vapor film can be neglected. Under these circumstances, the solid–vapor interfacial free energy in Equation (1) can be replaced by the surface free energy of the solid surface, γ_S (10):

$$\gamma_S = \gamma_{LV} \cos\theta + \gamma_{SL} \quad (2)$$

Solving Equation (2) would theoretically allow one to determine the surface free energies of the substratum γ_S and of the particle γ_P, two parameters required for calculation of the solid–particle γ_{SP}, the solid–liquid γ_{SL}, and the particle–liquid γ_{PL} interfacial free energies that

Table 2. Methods Available for Conditioning Film Analysis

Method	Contact Angle	Mercury Drop Electrode	Chemical Hydrolysis
Information provided	Wettability surface free energy	Surface potential	Monomeric composition of conditioning film components
In situ capability	Yes (bubble contact angle techniques) No (all other techniques)	Yes	No
Surface resolution	Macro (mm^2)	Macro (mm^2)	Macro ($mm^2 - cm^2$)
Analysis type	Physicochemical	Physicochemical	Molecular
Characteristics	Relatively simple Fast Surface layer sensitive	Fast Simple	Relatively simple
Disadvantages	Requires air/water/solid interfaces no consensus about most appropriate thermodynamic models for calculation of surface free energy diagnostic liquids may alter constitution of conditioning films	Only one type of (toxic) substratum substratum not suitable for subsequent microbial adhesion studies	Destructive variability of the efficiency of hydrolysis of different polymers requires complex laboratory set-up for monomer analysis and identification impossible to reconstitute polymeric structure of macromolecular conditioning film components.

Table 2a. Methods Available for Conditioning Film Analysis, Continued

Method	Pyrolysis	Electrophoretic Mobility	Time-of-Flight Secondary Ion Mass Spectroscopy
Information provided	Volatilization and fragmentation pattern of conditioning film components upon heating	Surface charge	Mass spectrum of adsorbed compounds
In situ capabillity	No	Yes	No
Surface resolution	Macro (mm^2)	Macro (mm^2)	Macro (μm^2)/nano(nm^2)
Analysis type	Chemical	Physicochemical	Chemical
Characteristics	Complex experimental set-up	Simple Fast	True surface analysis technique
Disadvantages	Interpretation difficult fragmentation pattern of the same molecule may differ on different substrata	Identification of charged groups impossible no information about distribution and location of charged groups only net charge measured applicable to buoyant particles only	Ultrahigh vacuum may modify conditioning film composition reduced size of mass fragments limits applicability for macromolecule analysis poorly characterized fragmentation and ionisation process secondary ion yields vary over 6 orders of magnitude across the periodic table.

Table 2b. Methods Available for Conditioning Film Analysis, Continued

Method	X-Ray Photoelectron Spectroscopy	Matrix-Assisted Laser Desorption Ionisation Time-Of-Flight Mass Spectrometry	Electrospray Mass Spectroscopy
Information provided	Elemental composition of surfaces	Mass spectrum of adsorbed molecules	Mass spectrum
In situ capability	No	No	No
Surface resolution	Macro/nano	Macro/nano	Macro
Analysis type	Elemental	Chemical	Chemical
Characteristics	True surface analysis method quantitative determination of specific chemical bonds such as C=O, C−O, etc.	Surface capability analysis of intact polymers extensive databases available for biological macromolecules	Most accurate MS methodology for large molecules extensive databases available for biological macromolecules
Disadvantages	Ultrahigh vacuum may modify conditioning film composition difficult to relate to macromolecular structure of complex films	Not all macromolecular conditioning film components can be volatilized and ionised quantification difficult	Material needs to be solubilized and removed from surface prior to analysis mass range not high enough for many macromolecular conditioning film components.

Table 2c. Methods Available for Conditioning Film Analysis, Continued

Method	Fourier Transform Infrared Spectroscopy: Specular Reflectance (SR), Diffuse Reflectance (DR) and Attenuated Total Reflectance Modes	Surface Enhanced Raman Spectroscopy	Ellipsometry
Information provided	IR spectrum of adsorbed compounds	Raman spectrum of adsorbed compounds	Film thickness
In situ capabillity	No: SR,DR Yes: ATR	Yes	Yes
Surface resolution	Macro ($mm^2 - cm^2$	Macro (μm^2)	Macro (mm^2)
Analysis type	Chemical	Chemical	Dimensional
Characteristics	Relatively simple techniques identification of functional groups	Relatively simple technique identification of functional groups no interference from water	Relatively simple technique
Disadvantages	Similar functional groups may be associated with different macromolecules spectrum combines information from different locations on substratum water subtraction from spectra requires judgement of degree of hydration	Sensitive to fluorescence similar functional groups may be associated with different macromolecules spectrum combines information from different locations on substratum	Elimination of thickness depends on assumption about refractive index of film poor spacial resolution assumptions of refractive index are based on homogeneous films

Table 2d. Methods Available for Conditioning Film Analysis, Continued

Method	Surface Force Microscopies (SFM)	Near-Field Optical Microscopy
Information provided	Surface force profiles Surface roughness	Optical image of conditioning film
In situ capability	Yes	No
Surface resolution	Nano (nm^2)	Nano (nm^2)
Analysis type	Physicochemical/dimensional	Optical
Characteristics	Very good surface resolution Relatively simple techniques Chemically modified tips can provide Information concerning surface Chemistry and molecular recognition	Very good surface resolution Can be combined with conventional Optical techniques such as fluorescence
Disadvantages	Tip/sample interactions may distort results Flat surface (at nm scale) required to achieve good vertical resolution Nonrigid conditioning film molecules may distort from lateral tip forces	No definitive identification of conditioning film components possible Resolution currently less than SFM Small depth of field

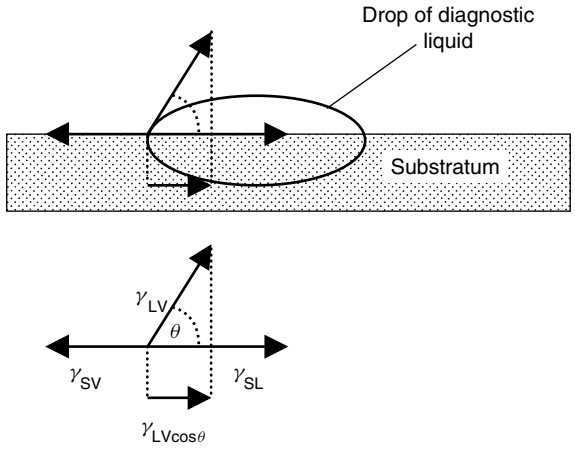


Figure 1. Surface energy equilibrium diagrams in contact angle analysis. γ_{SV} : solid–vapor interfacial tension, γ_{LV} : liquid–vapor interfacial tension, γ_{SL} : solid–liquid interfacial tension, and θ : contact angle.

determine the free energy of adhesion of a particle (or molecule) to a substratum:

$$\Delta F_{adh} = \gamma_{SP} - \gamma_{SL} - \gamma_{PL} \quad (3)$$

Of the four parameters contained in Equation (2), only θ and γ_{LV} can be determined experimentally. The remaining terms need to be estimated based on theoretical models (10). Unfortunately, there is no consensus in the literature about which of the plethora of thermodynamic models proposed to calculate the different physicochemical surface properties from contact angles produces correct estimates for the respective parameters. An evaluation of the most commonly used thermodynamic models for contact angle analysis revealed that the Lifshitz van der Waals acid-base approach with the diagnostic liquids water, diiodomethane, and formamide provided the most consistent results for conditioning film analysis (11).

Contact angles may be measured in static or dynamic mode using a variety of techniques (12). In the dynamic mode, the volume of the droplet of diagnostic liquid is continuously changed either by increasing the amount of liquid (advancing contact angles) or by retrieving liquid from the droplet (receding contact angle). Advancing contact angles wet new areas of the solid surface constantly, whereas receding angles develop over already wetted areas. Continuous desorption of conditioning film components by advancing contact angles may lead to accumulation of an organic film at the advancing edge of the drop. Receding angles on conditioned substrata certainly probe surfaces, the composition of which has been altered by the advancing angle, and thus produce results of questionable significance to the original conditioning film. Dynamic contact angle measurement should be avoided when assessing conditioned interfaces. Contact angles may also be measured in situ by placing the substratum face down in a measuring chamber filled with liquid and analyzing the contact angle of an air bubble with the coated surface (13). Care is required in the interpretation of the results obtained with this method as well. It is well known that passage of an air–water interface across a surface exerts shear forces strong enough to detach firmly bound cells (14). A similar process may occur with adsorbed macromolecules when an air bubble spreads on a conditioned surface.

Sessile contact angles need to be measured as fast as possible after deposition of the droplet on the test surface. The solvent action of many diagnostic liquids may remove adsorbed conditioning film components and thus contaminate the liquid. In addition, vapors of the diagnostic liquid may deposit at the solid–vapor interface in front of the droplet. Both processes would change the value of the contact angle. It is important to consider that macroscopic analysis with contact angles provides essentially averaged values for surface properties. For example, on a hydrophobic porous substratum, a diagnostic liquid may fill pores and create patches of liquid–liquid interfaces, whereas on a hydrophilic surface, the liquid may rest on a plane surface composed of solid and air. Averaged results such as provided by contact angles in both of these cases (or on inhomogeneous surfaces) would not be representative of the conditioned surface. The drop size of contact angles needs to be uniform because of the effects of gravity and drop cohesion on drop shape. Substratum roughness also affects contact angles, but this effect is of not much significance when the same surface is compared in the coated and in the clean state. Contact angles in air cannot be measured on fully hydrated films. It is well known from analysis of hydrated biological surfaces that contact angles change progressively from a fully wetting condition during dehydration until they reach a stable plateau value, which is claimed to be a thermodynamically significant measurement (15). However, it has recently been demonstrated by the authors of this chapter that bacterial adhesive properties of conditioning films in this partially dehydrated state are the same as those of the in situ film.

Mercury Drop Electrode

In the mercury drop electrode, the convective streaming created by the gradient in surface tension at a liquid–liquid (water sample/mercury) interface generates a measurable increase in the reduction current of mercury (II), which is manifested as a polarographic maximum (16). Dissolved organic molecules (and micelles) adsorbed at the mercury–water interface reduce the gradient in surface tension and slow down the convective streaming, which is detected as a suppression of the maximum current measured. Surface-active materials present in conditioning films can be quantified with this methodology.

Fragmentation Techniques

Conditioning films can be chemically hydrolyzed and the hydrolysis products analyzed by standard chemical analysis techniques (17). The results need to be interpreted with care because it is impossible to reconstitute the macromolecular composition of a conditioning film from analysis of the monomers. Moreover, it is important to avoid analytical bias toward more readily identifiable compounds

of biological origin such as amino acids, sugars, or lipids. Molecules from the humic group are less amenable to assessment by standard methods and are often overlooked in these studies. Conditioning film components may also be fractionated directly from the surface by pyrolysis (18), where the fragmentation pattern depends on the nature of adsorbed polymers and on the number of attachment sites of each polymer with the substratum. It is, therefore, difficult to obtain definitive data on macromolecular composition of conditioning films by this method.

Electrokinetic Techniques

Electrokinetic techniques exploit the fact that movement of charged particles in electrolytes depends on their surface charge (19). Any one of the four electrokinetic effects electrophoresis, streaming potential, electroosmosis, or sedimentation potential may be employed to determine the electrokinetic or zeta potential of a conditioned interface. In reality, the electrochemical plane of shear separating the moving particle from the solution is located at some distance from the substratum surface because of the electric double layer that forms on surfaces in electrolytes. The zeta potential measured with electrokinetic methods therefore represents an approximation of the electrostatic potential at the edge of the diffuse part of the double layer (Fig. 2). In practice, only two of the electrokinetic effects have found widespread use, electrophoretic mobility measurements on small particles in electrolytes and streaming potential, where fluid is forced through a capillary tube to generate a measurable potential at the test surface. These methods do not provide strict chemical identification of conditioning film components. Electrophoretic mobility versus pH curves of surfaces are electrophoretic titrations of ionizable surface functional groups. Thus, pK values and putative identities of acid-base functional groups in conditioning films can be investigated with carefully designed electrophoretic titrations.

Mass Spectrometry

Recent developments in mass spectrometry have greatly increased its applicability to conditioning film analysis (20). Volatilization and ionization of macromolecules can now be achieved readily with techniques such as the application of a strong electric field to the sample (field desorption), bombardment of the sample with energetic ions or atoms, the formation of ions directly from small, charged liquid droplets (thermospray ionization), electrospray ionization, and bombardment with short duration, intense pulses of laser light (laser desorption). Modern time-of-flight mass analyzers allow for true macromolecular analysis of ions with masses in excess of 100,000 Da (21). In these detectors, ions generated by pulsed beams controlled by precision timing are accelerated in a magnetic field and then allowed to drift in a field-free tube where they separate into a series of spatially discrete individual ion packets, each traveling with a velocity characteristic of its mass. Assuming that all ions have the same energy, heavier ions take comparatively longer to reach the detector. The velocity of the ions is proportional to the mass-to-charge ratio of a particular ion species.

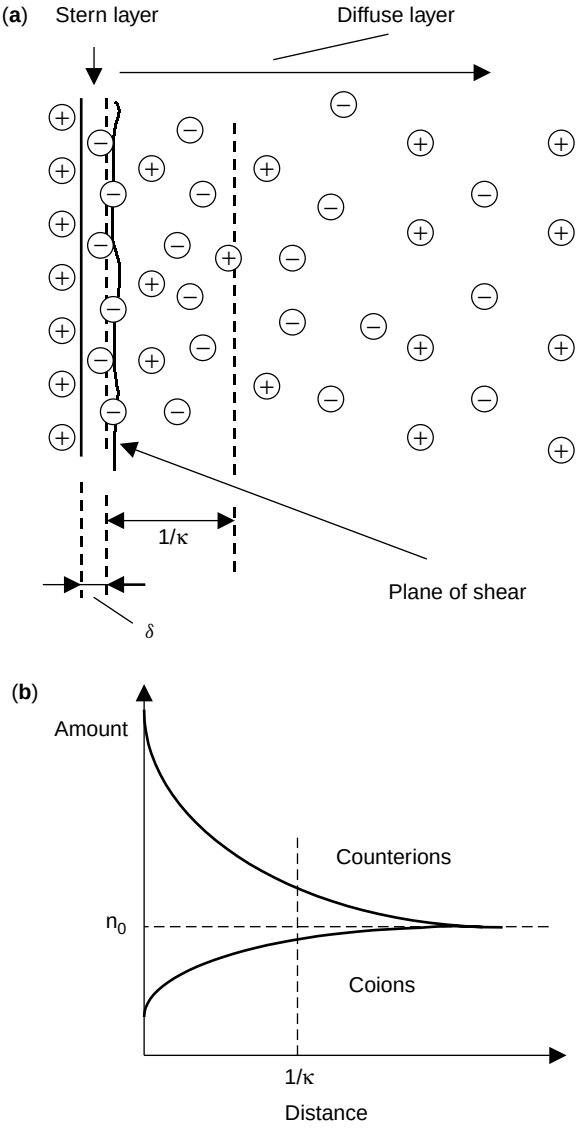


Figure 2. (**a**) Schematic representation of electric double layer. Δ : thickness of Stern layer (ions tightly bound to the substratum surface) and $1/\kappa$: thickness of electric double layer. (**b**) Concentration profile of coions and counterions in the electric double layer. n_0 : equilibrium concentration in solutions without charged surfaces.

Time-of-flight secondary ion mass spectrometry (TOF-SIMS) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) are the two truly surface-capable MS techniques (22,23). In TOF-SIMS, the sample surface is bombarded with a focused primary ion beam of O_2^+, Cs^+, or Ga^+ with an energy of 0.5–2.0 keV. The shock wave generated by the penetration of the highly energetic ions into the surface desorbs and ionizes adsorbed matter generating secondary ions that are injected into a TOF mass analyzer. The current state of the art of TOF-SIMS allows only for the generation of secondary ions of up to 10,000 Da. New developments may extend this mass range considerably in the near future (24). In MALDI-TOF MS, the sample is

mixed with an energy-absorbing matrix, irradiated with a laser, and analyzed using a time-of-flight mass analyzer. The sample probe is commonly stainless steel or silver, sometimes with a roughened sample application area to promote nucleation. Ionization and volatilization can be achieved with many different lasers, the most popular being nitrogen lasers with a wavelength of 337 nm and IR lasers with a wavelength of 10.6 μm. The matrix is believed to serve two major functions: absorption of energy from the laser light (hence, the requirement for a high molar absorbance at the wavelength of the laser) and isolation of the biopolymer molecules from each other. Low molecular weight aromatic compounds with extended electron conjugation readily meet these requirements. Typical matrix : sample ratios of $1,000:1$ to $5,000:1$ are optimum for ion production. The choice of appropriate matrix compounds is still largely empirical and one cannot predict a priori if a matrix compound will form a homogeneous solution with a given analyte molecule. Proteins and oligosaccharides can be analyzed readily by MALDI-TOF but routine analysis of humic acids and polysaccharides requires further methodology development.

Electrospray ionization (ESI) mass spectrometry is a rapid and accurate technique for the analysis of a wide range of polar compounds (25). Electrospray ionization operates by the process of emission of ions from a droplet into the gas phase. A solvent is pumped through a stainless steel capillary that carries a high potential, typically 3–5 kV. The strong electric field generated by this potential causes the solvent to be sprayed from the end of the capillary. The aerosol contains highly charged droplets. As the droplets evaporate, the ions within them also evaporate. A flow of warm nitrogen gas through the source helps the evaporation process and removal of the solvent. There is usually no fragmentation, and the spectrum contains only the pseudomolecular ion. The method is useful only for analysis of desorbed conditioning film components.

X-ray Photoelectron Spectroscopy

X-ray photoelectron spectroscopy, also known as electron spectroscopy for chemical analysis (ESCA), is a truly surface-capable method of elemental analysis (26). Irradiation of a sample with X-rays of known energy results in the emission of electrons of (lesser) binding energy. The kinetic energy of these electrons is measured by a spectrometer. The spectrum is a composite of the single-core electron (i.e., photoelectron), and an Auger electron, which is ejected following reorganization within the atom. The technique allows depth profiling of the elemental composition of conditioning films by variation of the angle of incidence of X-rays on the sample surface (27). One of the drawbacks of this method is the ultrahigh vacuum required in the sample chamber, which allows for analysis of only tightly bound, dehydrated, and nonvolatile conditioning film components.

Fourier Transform Infrared Spectroscopy

Infrared (IR) spectroscopy is one of the principal methods for identification of compounds used in chemistry. It provides extensive information about vibrational modes of atoms and functional groups in molecules. The same information can be obtained at much greater speed and sensitivity using Fourier transform IR spectroscopy (FTIR). There are many variants of FTIR (28). The ones most relevant for conditioning film analysis are specular reflectance FTIR and grazing angle FTIR, both restricted to analysis of dried films, and attenuated total reflectance FTIR that allows the study of conditioning films in situ, for example, directly in the aquatic phase (29). Specular reflectance FTIR involves the analysis of IR radiation totally reflected at mirror surfaces when the angle of reflection is equal to the angle of incidence. The amount of light specularly reflected depends on the nature of the surface and the refractive index of the substratum. In the "low reflectance" or "high absorption" variation of the technique, most of the radiation is absorbed and dissipated within the bulk of the sample. The spectrum obtained represents changes of refractive index of the sample as a function of wavelength, and the signal is usually weak. In the "high" or "absolute" reflectance mode the sample is deposited on a highly reflecting surface. Incident radiation passes through the sample, is reflected at the mirror surface, and passes through the sample a second time. The small amount of radiation reflected from the front of the sample can be used to estimate epitaxial film thickness. In grazing angle FTIR, the IR beam is directed at the specular surface at a high angle of incidence to the normal, such that the beam interacts with the coating, but not with the substratum. The grazing angle effectively increases the path length of the beam through the sample. The use of a polarizer dramatically enhances the sensitivity of the measurement because only plane-polarized light will interact with the coating. Films too thin for conventional specular reflectance (e.g., Langmuir-Blodgett films) can be analyzed by grazing angle FTIR (30).

In attenuated total reflectance FTIR, the IR beam channeled through an IR transmitting crystal of high refractive index reflects off the crystal walls many times generating an evanescent wave, which penetrates into the surrounding medium producing a spectrum of the sample in contact with the outer surface of the crystal. The depth of penetration of the evanescent wave depends on the wave number and angle of incidence of the radiation as well as on the ratio of the refractive index of the solution to the refractive index of the crystal. Wavelength scanning over a range of wave numbers produces the spectrum. Thus, crystals of different refractive index have to be used to vary depth of penetration of the evanescent wave at particular wave numbers. For example, the depth of penetration for the amide I band ($1{,}650\ \mathrm{cm}^{-1}$) is 0.14 μm for Ge or 0.17 μm for ZnSe, the most suitable materials for analysis of aquatic samples. The best performance of ATR-FTIR can be obtained with cylindrical crystals placed in a circle cell. A large number of internal reflections in the IR-transmissible crystals and a high refractive index maximize the signal from adsorbed species, minimize spectral distortions, and ensure small penetration depths into the sample that minimize interference from compounds present in the bulk solution. ATR cells have been designed to ensure

laminar flow with all areas of the surface reached by the flowing liquid. Water can be accurately computer-subtracted to obtain spectra of substances in a hydrated state and can thus analyze the composition of conditioning films deposited in situ (31).

Fourier Transform Raman Spectroscopy

Raman spectroscopy detects changes in scatter of IR radiation, rather than in absorption like FTIR spectroscopy (32). The two techniques are thus complementary. Raman spectroscopy is useful for detecting the bonds of weakly-absorbing IR species, such as C=C bonds or aromatic carbon functionalities. Raman has also proved useful in characterizing strongly IR-absorbing compounds, including amino acids and dye molecules adsorbed onto roughened metal substrata. In surface-enhanced Raman spectroscopy (SERS), the coupling of the radiation field with the oscillating, delocalized electrons of the metal substratum leads to enhanced signals from adsorbed compounds such as amino acids, peptides, and proteins (33). The principal advantages of Raman spectroscopy over FTIR are the complete lack of interference from water, and the smaller "spot-size," typically on the order of $1-3\ \mu m$ of Raman microscopes compared to $5-10\ \mu m$ for FTIR. The main disadvantage is that Raman spectroscopy is sensitive to fluorescence, a property inherent to aromatic amino acids and humic substances. For every Raman photon that is detected, about 10,000 fluorescence photons are detected.

Ellipsometry

Ellipsometry, a technique based on the principle that the state of polarization of monochromatic light incident on a specularly reflecting surface will differ following the adsorption of material onto that surface, is widely used for analysis of the thickness of adsorbed films (34). Collimated unpolarized or circularly-polarized monochromatic light is directed through a telescope that polarizes the light incident on the surface (35). The polarization state of the reflected light is subsequently analyzed. Although several variations in optics and analyzers exist, the most commonly used instrument is the "null" ellipsometer. In its most basic form, rotation of the analyzer components results in the refringent light being totally extinguished. Photoelectric detectors achieve the null condition by varying the output current or voltage. Film thickness is analyzed based on the estimation of the film's optical constants under the assumption that the film is completely transparent for the ellipsometer beam. The technique is very sensitive to surface imperfections, such as surface roughness, oxide layers, contamination, and surface damage.

Near-Field Optical Microscopy

Near-field optical microscopy overcomes the limitation of conventional optical microscopy techniques by scanning a subwavelength light source very close to the sample and building an optical image, pixel by pixel (36). In scanning near-field optical microscopy, the light source is connected to an optical aperture of 25 to 100 nm diameter fabricated into the apex of an aluminum-coated optical fiber. Using force-feedback, the tip of the probe maintains a constant separation from the sample (5 nm). As the light emanates from the tip, it only illuminates a volume of the sample of similar dimensions to the aperture. Hence, resolution is limited to the size of the aperture and not by the wavelength of the light. Near-field optics have produced the highest optical resolution to date, and can now be added to any conventional microscope. The technique is capable of generating topographical data (i.e., vertical, z-dimensions and lateral, x-y-dimensions), therefore, placing it in the same family as other scanned probe techniques. By controlling the polarization state of the laser light at the aperture and by using polarizers and filters of the collected signal, the same contrast mechanisms used in far-field imaging can be used, for example, fluorescence, absorption, reflection, or polarization.

Scanning Probe Microscopy

The variants of scanning probe microscopy (SPM) most suitable for conditioning film analysis are scanning tunneling microscopy (STM) and atomic force microscopy (AFM). The common features of both techniques are a laser-activated scanner and a sharp tip, or "probe." SPM scanners are made from piezoelectric ceramics that expand and contract proportionally to an applied voltage. A variety of probe materials are available depending on the type of measurement to be performed. STM tips are generally platinum-iridium or tungsten. AFM tips are fabricated from silicon or silicon nitride (chemically-etched or oxide-sharpened). The much stiffer silicon tips are more common for resonating probes that minimize contact-induced film damage. Cantilevers with high resonant frequencies are not suitable for solution-state work.

Scanning tunneling microscopy (37) is capable of true atomic-scale resolution. As the tip scans the sample, it encounters sample features of different heights, resulting in an exponential change in tunneling current as a function of separation distance. A feedback loop is used to maintain a constant tunneling current during scanning by vertically moving the scanner at each (x, y) data point until a "set point" current is reached. The vertical position of the scanner at each data point is recorded to form a topographical image. This technique is limited to conductive and semiconductive substrata, although the short tunneling distance allows its use in aqueous media.

Atomic force microscopy (38) uses the same general instrument layout as the STM, but the probe is a pyramidal tip attached to the end of a long, low-spring-constant cantilever. Monitoring the deflection of a laser from the top of the cantilever as it scans across the substratum produces topographical or other images. The process is therefore mechanical, and does not depend on sample conductance. There are three primary modes of AFM, which are defined according to the nature of the tip-substratum interaction. Ideally, the tip would not touch the substratum. This is the basis for "noncontact" AFM. Unfortunately, long-range van der Waals interactions are extremely weak in liquid solutions, and conditioning film images obtained with this technique are often

poor. Sometimes, experimental conditions allow for the acquisition of satisfactory imaging data of adsorbed macromolecules (39). The basic principle for "contact" mode AFM is to drag the tip across the substratum. Force curves are used to determine the minimum tip force, which can be applied without retracting the tip from the substratum, and to measure attraction, repulsion, and adhesion between the tip and sample. Force curves may also be acquired in resonating probe AFM to view changes in amplitude and phase with tip-sample separation. The probe assembly is oscillated at or near its resonant frequency such that tip-sample contact is minimal because the tip only contacts the substratum at the end of each oscillation cycle. The free-air amplitude of oscillation is reduced ("damped") by the substratum/sample, and the AFM uses this change in amplitude to map the topography. The feedback loop usually maintains the oscillation amplitude constant. Operation can take place in ambient and liquid environments. In liquid, the oscillation need not be at the cantilever resonance. When imaging in air, the typical amplitude of the oscillation allows the tip to contact the surface through the adsorbed fluid layer without being stuck.

Topographical measurements made with an SPM are a convolution of tip-sample interactions. For this reason, SPM is a mechanical, rather than an optical technique, and the tip composition and construction are the major limiting factors determining lateral resolution. The most important tip parameters are the radius of curvature (sharpness) and the angles of the tip sidewalls. An ideal tip would end in a single atom. For atomically flat substrata, this permits atomic scale resolution. Conditioning films, however, are never atomically flat. For the tip sharpness to be effective for these samples, it would need to extend vertically for the maximum height of the sample. In other words, the tip would ideally be a needle, rather than a pyramid, and even then, the accessible sample area would be still limited to 90°. When the substratum has raised or lowered areas with gradients greater than the tip angle, the trajectory of the tip over the corrugated substratum is an indication of the tip shape, rather than the true surface topography. Contamination of the tip with debris from the substratum or from adsorbed matter (in fluid) effectively dulls the tip. This does not necessarily distort the vertical resolution, but the lateral resolution can be affected drastically. Vertical resolution is determined by the resolution of the scanner movement, typically less than 0.1 nm.

PHYSICOCHEMISTRY OF CONDITIONING FILM FORMATION

The transport of conditioning film components from the bulk liquid phase to the substratum surface depends on mass-transfer processes with hydrodynamic shear as an important force field to be considered when surfaces are exposed to flowing media. At distances below 10 to 50 nm from the surface, molecular forces replace the hydrodynamic forces. At a molecular level, up to 17 different types of repulsive or attractive interactions between particles in liquid media listed in the literature can be combined into a set of five primary types of interactions (40): Brownian motion, electrodynamic or Lifshitz van der Waals forces (includes London, Debye, and Keesom forces), electrostatic forces (Coulombic forces), polar interactions (includes Lewis acid-base interactions, hydrogen donor–hydrogen acceptor interactions, hydrogen-bonding forces, and electron acceptor–electron donor interactions), and chain elasticity when layers of adsorbed polymers overlap (such as in the case where a cell attaches to a substratum surface conditioned with a macromolecular film). The terms pressure, force, and interactions, all widely used in the literature interchangeably, really mean energy and should be understood as such.

Brownian Motion

Every molecule or particle immersed in liquid medium, irrespective of its size, has a Brownian energy of approximately $1\,kT$ ($k = 1.3805 \times 10^{-23}\ \mathrm{JK}^{-1}$, T = temperature in Kelvin). This energy keeps particles in suspension, provided the energy of attraction between similar molecules or particles (substrata) is less than $1.5\,kT$ per pair. Brownian motion, therefore, plays an important role in the solution stability of small molecules.

Lifshitz van der Waals Electrodynamic Interactions

In aquatic systems, all the three types of Lifshitz van der Waals electrodynamic interactions behave similarly and decay at the same rate at distances of 5 to 10 nm from the particle or molecule surface. The decay rate for energies per unit surface is inversely proportional to the square of the distance (L^{-2}). The van der Waals-Debye interactions where a molecule with a permanent dipole induces a dipole in a neighboring molecule by polarization and the van der Waals-Keesom forces (interactions between molecules with permanent dipoles) are not subject to retardation at distances beyond 5 to 10 nm from the particle surface and are of minor importance in condensed media such as water. The London van der Waals forces (instantaneous dipoles induced by the motion of electrons in a molecule) represent more than 95% of the electrodynamic forces in liquids. They decay at a rate proportional to L^{-3} at distances beyond 5 to 10 nm from the particle or molecule surface. Lifshitz van der Waals interactions can be attractive or repulsive, depending on the properties of the interacting surfaces.

Electrostatic Interactions

Most surfaces in natural waters are negatively charged. Electrostatic interactions between them are therefore usually repulsive; their intensity depends on the electrokinetic potential of the interfaces. The interaction energy decays exponentially with distance L, as $\exp(-\kappa L)$. $(1/\kappa)$, the Debye length, is the thickness of the Gouy-Chapman electric double layer and varies with the ionic strength of the medium. The interactions between two negatively charged surfaces can be attractive when the values of their surface potentials differ significantly or when bridging compounds such as divalent cations are present in the medium.

Polar Interactions

Polar interactions between molecules or particles in aqueous phases are generally of much higher energy than Lifshitz van der Waals or electrostatic interactions. Polar interactions, on a generic level, comprise all types of electron donor–electron acceptor interactions including hydrogen-bond interactions and hydrophobic interactions. Hydrogen-bonding interactions in water between very hydrophilic materials tend to be repulsive, whereas the same interactions between more hydrophobic surfaces tend to be strongly attractive. Polar interactions decay exponentially with the distance L from the surface, as $\exp(-L/\lambda)$, where λ is the decay length characteristic of the medium (somewhere between 0.2 and 1.0 nm for water).

Chain Elasticity

Chain elasticity comprises a series of forces that apply when a polymer is compressed or stretched (41). A bacterium approaching a surface conditioned with macromolecules will initially interact with the polymers by physisorption processes. Further reduction of the bacterium-substratum distance will depend on the contraction and/or conformational alteration of the conditioning film macromolecules and of the polymers at the bacterium surface.

Other Interactions

The remaining interactions listed in the literature are disjoining pressure, hydration pressure, structural forces, depletion flocculation, depletion stabilization, entropy-driven interactions, enthalpy-driven interactions, cross-binding interactions, steric interactions, specific interactions, and osmotic pressure. All of these interactions are either part of one of the primary interaction groups identified earlier or the result of combinations of these groups (40).

Adsorption of Macromolecules

A prerequisite for adsorption of a compound to a surface is the decrease of the Gibbs free energy:

$$\Delta_{ads}G = \Delta_{ads}H - T\Delta_{ads}S \quad (4)$$

where H, S, and T are enthalpy, entropy, and absolute temperature, respectively, and Δ_{ads} indicates the change of the thermodynamic functions resulting from the adsorption process. For adsorption to be thermodynamically favorable, $\Delta_{ads}G$ has to be exothermic, for example, less than zero. With macromolecules, exothermic adsorption is often achieved at the expense of conformational alteration. The polymers most likely to be found in conditioning films from natural waters are humic compounds, polysaccharides, and proteins. Little is known about the conformational changes of humics or polysaccharides upon adsorption, but a great deal of information is available about these events in proteins, which adopt three types of structures in solution. The expanded-coil structure is highly solvated and flexible. Fibrillar proteins have a regular structure of α-helices or β-sheets. Globular proteins may contain a combination of α-helices, β-sheets, and random parts folded into a compact entity. Most proteins belong to the fibrillar or globular classes.

Norde and Lyklema (42) classify proteins as "hard" or "soft" depending on their resistance to structural modification on adsorption. "Hard" proteins are structurally stable because of their strong internal coherence. Hydrophobic groups are buried in their interior and most of the charged or polar groups are located at the protein–water interface. Internal charged groups occur as ion pairs because the reduced local dielectric permittivity inhibits dissociation. Adsorption of "hard" proteins is governed by electrostatic interactions and partial dehydration of both the substratum and protein surfaces. Calculations of the energy balance of dehydration of a polystyrene substratum (42) by a protein of 50 kDa adsorbed with a surface coverage of 2 mg/m^2 indicate a moderately exothermic reaction suggesting that dehydration of interacting interfaces alone can facilitate macromolecule adsorption. Globular proteins do not unfold significantly upon adsorption, but a modification of secondary structure into a more random configuration causes an increase in entropy sufficient for spontaneous adsorption (42). "Soft" proteins undergo structural modifications upon adsorption that result in increased entropy of the adsorbed species. The partial or even total denaturation of these proteins at interfaces leads to the establishment of many contact areas between the adsorbed macromolecule and the substratum. This considerably increases the stability of the sorptive interaction.

Electrostatic charge is another important factor that determines the rates of protein adsorption to surfaces. Adsorption of negatively charged proteins to positively charged substrata is usually rapid and diffusion-limited. Protein adsorption is maximal at pH values around the isoelectric point, where the protein has a more compact, globular, conformation. At other pH values, net positive or negative protein surface charges lead to more extended protein configurations as a result of the intramolecular electrostatic repulsion. In addition, the net charge of the protein generates an electric double layer. In most circumstances, the charge of the substratum will be of the same sign as that of the protein and the electric double layers on both of these surfaces will increase the energy barrier for adsorption.

COMPOSITION OF CONDITIONING FILMS

The predominance of glycoproteins in conditioning films from natural waters has been inferred from the presence of amide and polysaccharide adsorption bands observed in ATR-FTIR spectra obtained in some of the pioneering investigations (43). These two bands are certainly indicative of the presence of proteins and polysaccharides on the substratum surface but because unfiltered water samples were exposed for several hours or even days to substrata, it is more likely that the results obtained represent the colonization of the surfaces by microbial biofilms. Furthermore, the presence of polysaccharide and

amide adsorption bands on FTIR spectra does not imply that these compounds occur in the same macromolecules. Similar results were reported by Kristoffersen and coworkers (17), who found amino acids, sugars, myristic, palmitic, oleic, and stearic fatty acids in material hydrolyzed from surfaces exposed to raw seawater for two hours in flow cells. Electron micrographs, however, suggest the presence of a significant amount of material of microbial origin on the exposed surfaces. The patch wise distribution of lectin-binding material (polysaccharides or glycoproteins) on substrata exposed for between one and three days in seawater and its absence in areas without cells is indicative of the close association of these materials with microbial cells (44). Evidence for the presence of humic substances in conditioning films deposited from marine waters was first reported by Loeb and Neihof (9) and has been recently conclusively demonstrated by Leis and coworkers (24). The composition of conditioning films in subsurface environments is largely unknown, but it can be assumed that these films will be dominated by humic-like substances derived either from kerogen or transported into the subsurface in addition to inorganic components deposited from solution.

MODIFICATION OF SUBSTRATUM SURFACE PHYSICOCHEMISTRY BY CONDITIONING FILMS

The surface charge of particulate matter suspended in natural surface waters is always negative as a result of adsorption of organic components of conditioning films (9,45–49). Oxidation of organic matter by UV removes the ability of the coatings to modify substratum surface charge. Both large and small molecular weight compounds contribute to the surface charge of the films (50) whose major ionizable groups are carboxylic and phenolic –OH groups (48). The surface charge imparted by conditioning films on substrata appears to vary between sites, for example, the organic composition of the films seems to be specific for the sampling location (9,48). Every water sample was found to contain dissolved organic and colloidal matter that generated conditioning films, which modified surface free energy and its components (Lifshitz van der Waals, electron donor and electron acceptor) on most substrata. The effects were, however, substratum-specific and site-specific and there were no general unifying trends discernible (51,52).

KINETICS OF CONDITIONING FILM DEPOSITION

Conditioning films accrue at a rate of approximately 1 nm/s during the first few seconds of exposure, but the accumulation rate slows down rapidly till they reach their terminal thickness of between 20 and 80 nm after approximately 20 hours (53). Under optimum adsorption conditions, the surface coverages may reach 50% within the first few seconds (54). Conditioning films more than approximately 20 nm thick most probably comprise several layers of adsorbed molecules. Even after growing to a steady state thickness, the molecular composition of the films continues to change by the replacement of macromolecules with low affinity by others with higher affinity for the substratum (52,55). In analogy to the effects of conditioning films on bacterial adhesion, the first conditioning layer will influence the adsorption of the subsequent layers (56).

EFFECTS OF CONDITIONING FILMS ON MICROBIAL ADHESION

Conditioning of a surface with macromolecular organic matter should result in the formation of a coating with physicochemical properties different from those of the original surface. Such a coated surface should, therefore, retain organisms in a manner different to that of the original substratum. Studies with model compounds such as proteins have firmly established that conditioning films do influence microbial adhesion to inanimate substrata. Concentrations of dissolved organic compounds in the grams per liter range employed in these studies are well above the low parts per million (ppm) range typically encountered in most natural waters. Systematic evaluation of the effects of conditioning films from natural waters representative of freshwater, marine, and subsurface environments (52,57,58) revealed that microbial adhesion was modified on about 60% of substrata tested. The film-specific and substratum-specific effects were caused by the high molecular weight components of the films. Stripping of volatile compounds from the samples did not modify the adhesive properties of films, and films deposited from material that passed filters with a nominal pore size of less than 30,000 Da did not affect adhesion of the test organisms. Despite the low concentration of organic matter in natural waters and of its diverse nature, a few of the samples investigated produced films on four different substrata to which microorganisms attached in similar numbers, for example, the films appeared to completely mask the adhesive properties of the underlying substratum. On other occasions, however, substratum properties appeared to be somehow relayed to adhering bacteria across the conditioning film. In the case of the thin films deposited from natural waters, this may be caused by patchiness of surface coverage, for example, there may be areas of the substratum not entirely covered by macromolecular material. Substratum properties could also be relayed to bacteria if conditioning film material is removed from the bacterium–substratum interface in the early phases of attachment. Comparison of adhesion of carbon-limited and nitrogen-limited phenotypes revealed that conditioning films could modify the selectivity of substrata for microbial phenotypes (52,57,58). Adhesion of the test organisms to conditioned substrata correlated only occasionally with surface physicochemical parameters determined with contact angles (59).

The identity of the macromolecules from natural waters that affect bacterial adhesion to conditioned substrata is not known. Conditioning of a hydrophilic (stainless steel) and a hydrophobic substratum (polypropylene) with organic matter from six different fractions (hydrophobic-base, hydrophobic-neutral, and hydrophobic-acid as well as hydrophilic-base, hydrophilic-neutral, and hydrophilic-acid) of a freshwater and a marine water did not improve the consistency of the effects of the films on

microbial adhesion relative to the results obtained with the unfractionated water samples (52). Coatings from hydrophobic fractions were as effective in modifying adhesion as coatings from hydrophilic fractions. In addition, variation of the concentration of the material from the fractions in the conditioning film solutions and of the exposure time revealed that both factors influenced the adhesive properties of the conditioning films. Clearly, adsorption of the microbial adhesion-modifying macromolecules from natural waters to surfaces is a complex and dynamic phenomenon.

CONDITIONING FILMS AS SOURCES OF NUTRIENTS FOR BACTERIA

Ever since microbiologists realized that surfaces in natural environments are colonized by microorganisms, there has been speculation about the ecological advantages of the attached forms of life to microbes. Because most aquatic environments are oligotrophic, for example, low-nutrient ecosystems, one possible reason for the high affinity of bacteria for surfaces is the potential role of conditioning films as sources for nutrients (60). It is now well established that microbes have evolved a diversity of strategies to access adsorbed nutrients (60). These include reversible adhesion followed by detachment of either the mother cell or of a daughter cell once available adsorbed food sources are exhausted (61) and some sort of reversible attachment with surface movement such as crawling (62) or other forms of lateral displacement (63). Small molecular weight hydrophobic compounds such as stearic acid sorb tightly to substrata but can be accessed readily by microorganisms (62,63). It is even possible to isolate microbes targeted at the biodegradation of adsorbed small molecular weight compounds (64). The digestibility of adsorbed macromolecules by exoenzymes will depend largely on the conformation of the adsorbed species, which in turn varies depending on the surface chemistry of the substratum. Samuelsson and Kirchman (65) studied the growth of Pseudomonas S9 on adsorbed ribulose 1,5-bisphosphate carboxylase (rubisco). They found that although a larger amount of rubisco adsorbed to hydrophobic surfaces, hydrolysis of the protein was faster on hydrophilic surfaces probably because of the easier access of the exoprotease to the adsorbed protein. Growth of microorganisms was also initially faster on hydrophilic surfaces but more rapid depletion of available substrate led to higher growth rates on hydrophobic surfaces after six hours. The relevance of these results to conditioning films from natural waters depends on the following issues:

1. *The Nature of the Components of Conditioning Films from Natural Waters*. All of the studies in which bacterial growth and utilization of adsorbed organic compounds has been demonstrated employed compounds such as proteins, fatty acids, or hydrophobic pollutants, which are not likely to be significant components of conditioning films in natural waters. The compounds were used in a pure form, for example, the surface concentration of the particular substrates was very high. However, in natural waters, easily degradable organic carbon ("labile carbon") represents only between 10 and 30% of DOC (8). Furthermore, this scarce, labile fraction comprises small molecular weight compounds that either do not adsorb to surfaces or adsorb only weakly (60). The remainder of the DOC pool is constituted of recalcitrant organic matter with turnover times of up to 6,000 years (8).

2. *The Ease of Biodegradation of These Components*. The humic compounds that represent the largest fraction of natural water DOC and which have been demonstrated to be part of conditioning films, are thought to be rather recalcitrant for biodegradation by bacteria (66). Growth yields on these compounds are likely to be significantly smaller than those obtained with sugars, amino acids, or fatty acids. It is, therefore, very unlikely that the conditioning film material from natural waters present in the contact area of a microorganism with the substratum would support the production of biomass required for cell replication.

3. *The Geometry and Contact Area of the Bacterium-Conditioned Substratum Interface*. The contact area of a microorganism with a conditioned substratum is very small. Most cells are curved, and only a very small part of the curved section is in direct contact with the substratum (67). The immediate contact area of cells with conditioning films, therefore, does not represent a significant source of food for attached microbes. Rhouxhet and Mozes (68) calculated that if a disaccharide was adsorbed as a monolayer and the cell was actively respiring at rates typically encountered in growing organisms, the compounds adsorbed in an area corresponding to the surface occupied by the cell would be consumed within 10 seconds. Alternatively, one could estimate the area of conditioning film required to supply a cell with its organic carbon in sufficient amounts for the synthesis of the entire cell organic carbon. If the following assumptions are made:

- the cell is assumed to be a sphere with a diameter of 0.5 μm
- cells consist of 80% water and 20% organic matter (density of 1 g/cm^3)
- rubisco adsorption data from Samuelsson and Kirchman (65) are adopted as reference (maximum of 0.45 μg/cm^2)
- yield of biomass production with rubisco is assumed to be one (1 g of cell mass formed per gram of rubisco utilized)

then it becomes clear that the cell would need to scavenge the entire rubisco adsorbed in an area of approximately 2.9 μm^2, which is approximately 15 times the projection area of the cell diameter on the substratum surface. Clearly, organisms need to scavenge comparatively enormous areas to satisfy their food requirements if the only source of nutrients were the conditioning films, even when these films consist of easily degradable substances. Growth yields on films from natural waters are likely to be much lower because the cells would have to produce a wide range of extracellular enzymes and the substrates of the individual exoenzymes would be dispersed over a much wider

area than was calculated for rubisco. Surface displacement is the only means by which microbes would obtain sufficient nutrient sources from adsorbed films for growth.

4. *The Specific Physicochemistry of Surfaces*. Most organic compounds available to microbes in oligotrophic natural waters are macromolecules. These substances may adsorb to surfaces tenaciously forming many contact points and may even be denatured. These processes may render many of them unavailable for breakdown by exoenzymes thus restricting even more nutrient availability to microbes.

Points 1 to 4 illustrate that the content of easily degradable organic matter is low in conditioning films from natural waters. Depletion of these components from the films would facilitate their replacement by the dominant recalcitrant organic molecules in the pool. Conditioning films from natural waters are, therefore, not significant sources of organic carbon for bacteria in oligotrophic environments.

CONCLUSION

The composition of conditioning films from natural waters, the dynamic processes that occur during their formation, and their exact role in microbial adhesion and biofilm formation are still largely not known. The following list includes some of the most important issues that need to be researched to better understand the interaction of environmental conditioning films with environmental microbes:

- What are the components in conditioning films that influence microbial adhesion, either positively or negatively?
- How far into the development of microbial biofilms do conditioning films influence the process?
- Which components of conditioning films, if any, are food sources for microorganisms and what growth rates and yields can be achieved by microbes growing on these compounds?
- Does the ability to utilize conditioning film components as food source provide an advantage for microorganisms colonizing inanimate surfaces in natural environments?
- What is the exact role of conditioning films in microbial attachment?
- Do microbes attach to surfaces via the establishment of connections between their cell surface adhesive biopolymers and conditioning film components or, alternatively, do conditioning films only play a role in initial, reversible adhesion, with firm linkages between microbe and substratum established after displacement of conditioning film components from the interface between microorganism and substratum

Novel surface analysis techniques and improvements in mass spectrometry procedures that allow for analysis of entire macromolecules provide a novel set of tools for the study of conditioning films. These methods, in combination with state-of-the-art molecular biology and microbial physiology procedures, will greatly improve our understanding of the interactions of conditioning films with microbes.

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CONFOCAL LASER SCANNING MICROSCOPY (CLSM).

See IMAGE ANALYSIS OF MICROORGANISMS

CONSORTIA, MICROBIAL.

See AGGREGATES AND CONSORTIA, MICROBIAL

CONTROLLING THE MICROBIAL QUALITY OF DRINKING WATER IN DISTRIBUTION SYSTEMS

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The existence of man is dependent on water supply and it is an essential element in community life. Ancient civilizations evolved around rivers, lakes, and ground water outcroppings as springs. Catchment basins or open-dug wells were developed to catch rainwater, and open channels were created to divert water resources to village fountains, public baths, and agricultural fields. The ancient Greeks constructed clay pipes for water transport (1), and early Romans became the master engineers who created massive aqueducts for the purpose of bringing water supply from the mountains to distant cities (2). These achievements were followed by the Moorish civilization that extended water delivery systems to create marvels of water fountains in the arid lands around the Mediterranean coastal areas of Spain. In the Americans, the various centers such as the "lost Inca city" of Machu Picchu (Peru) and the Mayan cities in ancient Mexico attest to the management of water for the survival of these great centers of civilization. Today, the very existence of many villages in remote areas of the world are tied to a water source within less than two hours walking distance because water supply must be carried in buckets by women and children to their homes. From these basic discoveries of how to collect, protect, conserve, and provide water in a community, there has evolved the present day complex structure of a distribution system.

The intent of a modern distribution system is to supply water on demand to individual homes, public buildings, offices, hotels, schools, and industries for five basic purposes: safe water to drink, sanitation necessities (baths and toilets), processing food, creating manufactured goods, and fire control. These uses require a complex engineering operation that includes storage of water supply, pumping stations to move water in large mains throughout the pipe network, standpipes to afford relief from surges of water pressure in the pipelines, valves to isolate sections of the system, booster chlorination stations

and fire hydrants; all of which are connected together by miles and miles of pipes laid under the streets to reach all customers in the community. Added to this complexity are numerous water-attachment devices connected to building and home plumbing systems for a variety of uses. As a consequence, it is not surprising that water entering the distribution system from the treatment plant may be altered in quality during its passage. These quality changes are most often detected in taste, odor, and color that affect the appreciation of a pleasant drinking water. More serious are the intrusions of microbial and chemical agents that are of health significance (3). Protecting the quality and quantity of a water supply in distribution within the community is a complex engineering operation that never stops.

Basic Concepts in Distributing Microbially Safe Water

Rare has been the opportunity to design a water distribution system before a community is built. More often, its growth anticipates or follows urban sprawl in every progressive community. Water supply distribution systems in cities created over two hundred years ago were relatively simple in design because the cluster of human activities was close together. As these cities grew into urban sprawls and with increased diversity of activities, the demand for water intensified. In many cases, additional sources of water had to be found. Often the solution was met through the development of more wells or for other utilities; long transmission lines were laid to large distant surface waters in an effort to increase the availability of raw water. To keep up with the demand and to provide a water supply reserve for fire control, treated water was stored in additional reservoirs located at strategic sites further out in the pipe network. As a result of these pipe network expansions, gravity movement of the water supply over hilly terrain was found to be inadequate and pressure pumping was introduced to move the water to far reaches of the system. Today, progressive water utilities have developed strategies to keep pace with the anticipated expansion of the metropolitan area over the next decade or two. Implementing these plans must proceed carefully, however, to avoid oversizing the distribution system too early. When supply far exceeds demand, there is long retention of water supply in the pipe and in the reservoir storage tanks. The net result is that water supply stagnates and there is a rapid deterioration in quality. Whenever possible, it is desirable to keep water supply moving throughout the distribution system for short retention times and minimal quality changes.

Storage of treated water supply is mandatory in every community system to meet periods of peak demand and to provide a buffer during emergency shutdown of water treatment production. These reservoirs of drinking water supply may be held underground, at ground level, or in elevated tanks. Underground reservoirs are generally constructed as cement vaults, but older basins still in use were created with masonry or stone and are lined with concrete, asphalt, or butyl rubber to prevent surface leakage or groundwater contamination into the supply. Elevated storage tanks are constructed of steel or reinforced cement materials and sealed with an epoxy coating to deter water loss through leakage. Impounded storage of drinking water at ground level should be covered to minimize contamination from storm water runoff, birds, dust, human contact, and adjacent road spills. Over time, sediment and biofilm accumulate in these storage structures. For these reasons, it is desirable to take storage reservoirs off line every three to five years to flush out this microbial habitat, repair any structural defects, and apply a new coat of epoxy sealer.

Maintaining Distribution System Integrity

Integrity of the system is of utmost importance. This places emphasis on maintaining pipelines with minimal leaks and a positive pressure of 20 psi (pounds per square inch) to prevent backflows during cross-connections. Materials used in the manufacturing of pipe must be stable over the years of service life to resist collapse from movement in the pipe bed during ground heaving in the winter, vibrations created from road traffic, or nearby construction activity, and earthquakes. Pipe networks should be designed to minimize numerous dead-end lines by incorporating continuous loops that nullify the creation of static water areas.

The service life of pipes is not infinite. Various materials have been used for water supply pipes including cast iron, ductile iron, cast iron lined with cement, steel, reinforced concrete, asbestos combined with Portland cement, and three plastic materials: polyvinyl chloride (PVC), polyethylene, or polybutylene. Pipe material in building plumbing networks are most often copper or plastic, but occasionally, black and galvanized iron may be encountered in older homes. Pipe service life is also affected by both external and internal corrosion. External corrosion results from the intimate contact with aggressive soil environments. Some of these factors include soil resistivity, soil pH, presence of sulfate-reducing bacteria, and differences in soil type along the pipeline. Pipes laid in areas of landfill or across industrial sites may also be exposed to accelerated external corrosion. In these cases, some form of external coating or a sleeve is needed to provide substantial protection from accelerated lost of integrity.

Internal corrosion is the result of water aggressiveness in unlined metal pipe that is often aided by microbial activity of both sulfate-reducing organisms and other aerobic heterotrophic bacteria (4,5). Additional factors that cause water to become aggressive on metal pipe materials include low pH, low alkalinity and hardness, high chloride, or high sulfate in some interrelationships. These exposures lead to formation of an irregular, porous deposits (tubercles) that are suitable for microbial habitation and attack on the metal surface. Over time, the interior pipe surface becomes pitted as microbial and chemical mediated corrosion slowly reduces wall thickness and strength.

Combating corrosion is critical because deposits of rust accumulating in the pipe begin to restrict water flow, bacterial colonization of tubercles expand, and customer complaints of taste and color in the water increase (6). Strategies for pipeline salvage may vary depending on the status of the corrosion problem. Severe accumulations of

tuberculation will require mechanical scrapping of the pipe to loosen these rock-like deposits. Less severe corrosion deposition may be removed by forcing a plastic plug (termed a *pig*) through the line to sweep the material ahead and out of the pipe. Following such action, the line is flushed to remove sediments and other debris. In more serious situations, lining the pipe section with cement may be cost effective and desirable to combat biofilm development and stop loss of water through corrosion pinholes that may develop in the pipe wall.

The immediate response to a line break is to repair the fractured pipe sections or to replace. To expedite repairs at streets with high-volume traffic, pipes with longitudinal splits are often patched with metal straps. A more permanent repair approach is to cut out the broken section and replace it with new pipe. Once the repair is made and tested for leakage, on-site disinfection of the repaired section is essential to prevent the introduction of microbial contamination from drainage around the site. Generally, this procedure consists of the application of a heavy chlorine dose (50 mg/L HOCl), usually in the form of liquid chorine, calcium hypochlorite granules or tablets, or sodium hypochlorite solutions. The disinfected standing water is held in the pipe section for 24 hours after that a bacterial test for heterotrophic bacteria is made. If the bacterial density is less than 500 organisms per ml, the pipeline is accepted for return to service, if not, the disinfection procedure is again applied and the water tested for residual contamination. Any repeated contamination will require an inspection of the repair job for a cause to the loss of pipeline integrity. Frequent line breaks in the same area increase the probability of a breakthrough of microbial contamination. Repeated breaks in the vicinity should be viewed as a signal for prompt rehabilitation of that part of the pipe network.

Managing Water Quality

Maintaining the integrity of the distribution system requires a dedication to preventive maintenance of all component parts to optimize their service life and function. Cast iron and ductile iron pipe appear to have a service life of approximately 100 years; reinforced concrete, 50 years; and asbestos cement pipe, 30 years. Although thousands of miles of nonmetallic water pipes and service lines have been installed, little can be said about their service life because these materials are a relatively recent development.

Every water utility has a history of some unpredictable water loss that occurs because of slow leaks, pipeline breaks, and unmetered service to their customers. In reality, the distribution pipe network is never completely watertight but with good management, losses from slow leaks should not exceed 15%. It is important to not only minimize these water losses as a matter of water conservation and lost revenue but also to reduce possible soil contamination opportunities. A dangerous contamination pathway may be created in unchecked water loss situations and to cross-connections and back-siphonage in water attachment devices, interconnection of private and public water supplies, or lack of backflow prevention in lawn watering systems, fire control sprinklers, and car wash operations.

Back-siphonage is the movement of drainage water back against the direction of normal flow or positive water pressure. This is the reason why water supply utilities must maintain a positive pressure of at least 20 psi in all areas of the distribution pipe network. Such a policy provides significant protection from passage of storm water runoff and sewage drainage in the pipebed to a fractured pipeline, a poor pipe joint or cross-connections to various water-use devices in plumbing systems. Any large-scale reduction in water pressure is an opportunity for microbial contamination to overcome the integrity of a safe distribution system.

For those utilities that have very aggressive water, inclusion of a treatment to suppress corrosion is essential to reduce chemical and microbial mediated activity on the pipe walls (7). An effective corrosion control protocol is sometimes difficult to achieve because only certain nontoxic substances at low dosages can be employed for health reasons. With this limitation in mind, corrosion treatment has focused on raising the pH and alkalinity during water processing or adding corrosion inhibitors (carbonates, polyphosphates, blended phosphates, or sodium silicates) to form protective films on pipe walls. Unfortunately, attempts to create a passivating film of inhibitors on pipe walls may result in excessive depositions in mixing zones where the water flow shifts back and forth. The net effect is an unstable shifting between deposition and dissolution by changes in water quality and reversals of flow. This instability often results in the unpredictable release of sediments and entrapped bacteria into the bulk flow of water.

Cross-connections and back-siphonage are always a threat to water quality in the distribution system. Much of this threat is from attachment devices or poor plumbing practices that do not isolate the water supply from backflow of drainage in the device. What is also dangerous is the illegal tap-in on public water supply lines and the interconnection of a private well or cistern supply with the municipal water system. These unauthorized actions by private citizens provide opportunities for incursions of fecal bacteria, viruses, and protozoan pathogens from surface runoff around the wellhead or from bird and rodent feces on the cistern rainfall collecting surfaces.

Maintaining a disinfection residual that can inactivate pathogens associated with contaminants seeping into large volumes of high-quality water supply does provide some protection from these incursions (8). Obviously, there are limitations to this protective barrier. Chlorine residuals in distribution water often range from 0.1 to 0.3 mg/L and these concentrations will not be effective during massive intrusions of gross contamination characterized by odors, color, and milky turbidities. Fortunately, replenishment by the inflow of fresh, chlorinated water will provide significant dilution and dispersion of these contaminants to improve the potential for inactivation of microorganisms by the available disinfectant residual.

Microbial Flora of Drinking Water

Drinking water is not sterile nor does it need to be for a safe supply. The objective of water utilities is to

provide the community with a water supply that presents a minimal health risk from the invisible threats of microbiological, chemical, and radiological contaminant. Also of concern with dubious safe water is the general appearance (turbidity) and palatability (taste and odor). These conditions are often linked to the metabolic activity of microorganisms within the distribution pipe network.

Profiling the organisms found in potable water reveals a mixture of microorganisms whose origins are varied (3). They may have been introduced in the source water, survived treatment and established themselves in the distribution system, or entered during line breaks or cross-connections (9). Ecological dominance of some species is a reflection of their ability to rapidly colonize corrosion sites, low flow areas of the system, stratified water-storage tanks, and attachment devices in building plumbing networks.

Inspection of the bacterial population of microorganisms in drinking water reveals many gram-negative and gram-positive bacteria, spore-formers, acid-fast bacilli, pigmented organisms, fungi, yeast, and free-living amoeba and nematodes. Most of these organisms are ubiquitous in the aquatic environment and of no public health significance. The exceptions are those organisms in the population of innocuous organisms that may become opportunistic pathogens or transfer antibiotic resistance factors to the human intestinal flora. By contrast, fecal contamination introduces the chance occurrence of intestinal pathogens (bacterial, viral, and protozoan). The routes of exposure may not only be ingestion but also body contact with the water during bathing or inhalation exposure to indoor climate control devices. An overview of some microbial groups in potable water reveals the presence of coliforms, antibiotic resistant bacteria, opportunistic pathogens, pigmented bacteria, ultramicrobacteria, and macroinvertebrates.

Coliforms. A variety of coliforms may be found occasionally among the heterotrophic bacteria in potable water supply (4). Their occurrence fits no set profile but some species are more common that others, perhaps because they are more capable of colonizing the Spartan environment of the distribution system. These organisms include *Klebsiella pneumoniae, Enterobacter aerogenes, Enterobacter cloacae*, and *Citrobacter freundii*. Among the coliforms, *Klebsiella* occurrence must be viewed cautiously even though the vast majority of this genus are environmental coliforms of no public health significance (10,11). Approximately 30% of all human intestinal tracts contain some *Klebsiella* strains, however, *E. coli* is the predominant coliform species of the fecal flora of warm-blooded animals. Because *E. coli* does not normally persist in the potable water environment, any occurrence of this organism suggests an immediate presence of fecal contamination. However, in recent years a mutant *E. coli* strain has been found to colonize greensand (used in water-softening treatment). This contaminant is difficult to eradicate with disinfection, because the strain develops a protective mucoid characteristic. Another strain, *E. coli* 0157 : H7, has recently emerged as a serious hemorrhagic pathogen. This organism is a slow fermentor of lactose and appears to be able to form a tenacious film over the inner surface of bottled water containers with survival for more than 300 days (12).These observations suggest that *E. coli* strains are emerging with new and dangerous characteristics for survival.

Antibiotic-Resistant Bacteria. The transfer of antibiotic resistance factors among the heterotrophic bacteria in water to the flora of the intestinal tract may be a contributing factor in the declining efficacy of an increasing number of antibiotics used in medical therapy. Antibiotic resistance factors (R-factor in plasmid transfers) occurring in bacterial populations originate in surface water sources containing rural storm water runoff from animal feedlots and in upstream municipal sewage effluents. Farm animals in particular, often receive continuous doses of antibiotics in animal feed and become generators of a variety of antibiotic resistant bacteria. Wastewater and water supply process basins provide the mixing chamber for R-factor transfers and the chance for some organisms to acquire multiple resistances to different antibiotics. Many of these transformations occur in the biofilm established on process basin compartment walls, agitators, and sand or mixed media filters (3,4). Receptor organisms may include *Pseudomonas Alcaligenes, Acinetobacter, Moraxella, Staphylococcus*, and *Micrococcus*. In the water supply of Cairo Egypt, 40–70% of the heterotrophic bacteria could be expected to have antibiotic resistance factors (13). Perhaps, part of this high percentage may be a consequence of indiscriminate use and easy access to antibiotics in the metropolitan area.

Opportunistic Pathogens. Some of the microorganisms (bacteria and fungi) that survive treatment and colonize the distribution system may in turn become colonizers of the susceptible human (individuals with underdeveloped or impaired immune systems), generally, the very young and senior adults in the community as well as patients on chemotherapy, dialysis, severe burn injury repair, and individuals receiving organ transplants (14). At least 5% of new patients in hospitals acquire nosocomial infections and 1% may die as a direct result of these exposures. The source of many of these opportunistic pathogens is water and water-attachment devices; others are introduced in salads and by hospital staff or visitor contact (15).

Bacteria, common to the water environment may present a health risk to susceptible individuals if high densities (10^6 per ml) occur (14,15). Amplification of these potential opportunistic organisms in static water locations of the building plumbing system or in an attachment device is necessary for the organism to achieve infective dose levels in healthy children and adults that approach 1×10^{10} cells per exposure. Infective dose for those individuals most at risk in the community would be several magnitudes less. Although the number of cells necessary to achieve an infective dose may seem unlikely to occur often, the volume of water used to take a shower or bath can easily supply this accumulative density during a given exposure period. Some of the bacteria most often found to be the cause of nosocomial infections associated with

contaminated potable water are *Pseudomonas, Acinetobacter, Mycobacterium, Flavobacterium, Legionella*, and *Klebsiella* (16,17).

Fungi are another group of organisms in water that, when present in certain situations, may colonize the human body and become opportunistic pathogens (18). The most frequent complaint is from allergenic reactions: (sauna taker's disease), mycotoxin, and infections of the skin, nails, hair, and genitalia. Very often the contact exposure route is with some water attachment device used in the home, hospital, or health spa.

Filamentous fungi (*Penicillium, Cladosporium, Phyma, Acremonium, Sporocybe, Aspergillus*, among other) are common to the aquatic environment. Hence, it is not unusual for mycelial fragments and spores to be present in source water. Passage through coagulation, sand filtration, and disinfection will lead to some reduction in fungal densities but are quite variable in their effectiveness. Aquatic fungi find many areas (pipe joints, gaskets, joint seal compounds, and biofilm sites in areas of corrosion and sediment) of the distribution system to be a satisfactory environment for colonization (3,4).

Profiles of filamentous fungi in water supply are variable, being a reflection of seasonal water temperatures, source water quality, treatment patterns, and methods used to isolate these organisms in the laboratory. Fungi densities in potable water supplies are usually less than 10 organisms per ml (3). Any greater numbers in water supplies often result in customer complaints of poor taste and musty odors.

Yeasts are found in water supply (19). Although little is known about their profiles, frequency, density, and regrowth characteristics, one species, *Candida albicans*, is an important example of an opportunistic strain of yeast. This yeast may cause secondary infections to individuals stressed from diabetes, cancer, and immunological defects associated with AIDS, organ transplants, and the application of broad-spectrum antibiotics. Water treatment will remove 90 to 99% of yeast in raw source waters but only ozonation rather than conventional chlorination is effective in their inactivation (3). Finished water coming from the treatment plant may have very few yeast per liter but these scant occurrences may be amplified a thousand fold in old pipe sections, dead ends, and other stagnant water sites in the pipe network.

Pigmented Bacteria. Some of the bacteria found in water supply have the ability to form nonphotosynthetic and nondiffusible pigments (yellow, orange, pink, purple, brown, and black) (20). This characteristic may be observed on plate cultures if the incubation time is extended beyond three days. Such organisms (strains of *Flavobacterium, Mycobacterium, Serratia, Corynebacterium*, and *Chromobacterium*) appear in greatest abundance in waters of high quality such as public water supplies, bottled water, and product water from a variety of attachment devices such as ice machines, point-of-entry treatment packages, humidifying units, and hemodialysis equipment (3). Their origins are in source water and soil contaminants introduced in line breaks. Disinfection of water supply has little impact on pigmented bacteria (3). Of this group of organisms, nontuberculous *Mycobacterium* and *Serratia* species may be the major representatives of those pigmenters that can be opportunistic, causing skin lesions, septicemia, and postsurgery complications in the hospital.

Although pigmented bacteria entering the distribution system may be only a few organisms per liter, this density changes significantly in static pipe sections of the distribution network. Yellow-pigmented and orange-pigmented strains follow different seasonal patterns of dominance. For instance, yellow-pigmented bacteria occur in higher densities during the summer, whereas orange pigmenters are more frequent in the winter (3). Little is known about the mechanism that stimulates these cyclic growth patterns in the distribution system.

Ultramicrobacteria. This group of very small heterotrophic bacteria (less than 0.3 μm in size) is "normal"-sized aquatic bacteria that have adjusted to the harsh environment of the distribution system through gradual starvation (21). As part of their survival mechanisms, some of these ultramicrobacteria develop encapsulation and enhanced adhesion to pipe walls, tuberculations, and storage tank surfaces. As a consequence, these organisms are capable of surviving for extended periods of slow water movement during storage in static reservoirs and in bottled water stockpiled for use during natural disasters (22).

Some of these ultramicrobacteria include strains of *Klebsiella, Escherichia, Bacillus, Micrococcus, Staphylococcus*, and *Vibrio*. Pathogenic bacteria may also become minaturized in structure and metabolic activity under these circumstances and may be difficult to detect unless very dilute media are used for their cultivation with extended incubation times (3). On resuscitation in the laboratory, these organisms often return to normal size and typical metabolic functions.

Macroinvertebrates. Drinking water also contains a diverse population of free-living organisms that feed on bacteria and fungi. These are the amoebas, amphipods, copepods, and the nematodes common to surface waters (23). In water treatment, many of these organisms become entrapped in the filter media but some pass through treatment barriers, including disinfection exposure. Migration into the distribution system occurs in the search for food (bacteria and fungi) at tuberculation sites of biofilm or in areas where water flow is slow and colonized bacteria occur in the sediments. Although these organisms are not known to cause a health risk, they may become a transporter of viable coliforms, *Legionella*, and other bacteria into the distribution system (3,4). When the macroinvertebrates burst open, the engulfed bacteria are released. In some instances, excessive colonization by macroinvertebrates in old pipelines may lead to public complaints about water palatability and aesthetics because of their presence in the drinking water.

Biofilm and Water Stability

Water supply biofilm is a microbial event created by a consortium of organisms at some site within water treatment, distribution, or plumbing systems. Circumstances that

are conducive to their growth occur under challenging conditions that include adequate nutrients, a protective habitat, and favorable water temperature. Adversities to biofilm development are disinfection, unstable sediment substrate, and the shearing effects of water movement under pressure. These habitats may occur in water process basins, distribution pipes, storage tanks, stagnant plumbing lines, and attachment devices. Key requirements for successful biofilm development are the ability of organisms to adjust their metabolic activity to a limited nutrient base, form a slimy matrix of extracellular organic polymers for adhesion to pipeline surfaces and locate in an area of minimal exposure to disinfection residuals.

Upon entering the distribution system, microorganisms may follow one of several scenarios: (*1*) die in the harsh water environment for lack of suitable nutrients or from exposure to disinfectant residuals, (*2*) sustain meager survival by successfully attaching to some pipe material then take on an ultramicrobacterial existence, or (*3*) rapidly adjust to the water environment and actively colonize quiescent sites in the pipe network (24).

Biofilm development in the distribution system is a progressive process (25). Initial microbial colonization occurs among those organisms that either become embedded in the sediments or quickly produce a slimy matrix of extracellular organic polymers that adhere to the pipe surfaces. This matrix is interlaced with water channels that have been reported to constitute as much as 40 to 60% of the total biofilm volume (3).

Biofilm in Water Distribution. The adverse effect of biofilm development in the pipe network is the sudden, erratic release of coliforms and other heterotrophic bacteria into the bulk water flow that has nothing to do with a treatment barrier failure, cross-connection, or line break. The problem is most often associated with systems using surface water. These occurrences are stimulated by warm water temperature ($>15\,^{\circ}C$) in the pipe network and the accumulation of assimilable organics above a critical mass. During these episodes, coliform positive samples are detected in an almost random fashion throughout the pipe network. Coliform speciation from these positive water samples during a biofilm release period often reveals that one of several coliform species predominate (*Klebsiella pneumoniae, K. oxytoca, Enterobacter agglomerans, E. cloacae*, or *Citrobacter freundii*). Perhaps this is the reason why only *Klebsiella* and *Enterobacter* strains are most often identified as the coliforms involved in public water supply noncompliance problems (3).

Coliform releases in biofilm fragments may not be of public health significance per se but should not be ignored because of perpetual concern for a hidden fecal contaminating event (3). These biofilm releases could be masking other more serious situations that suggest (*1*) a possible partial loss in treatment barrier effectiveness, (*2*) backflow of contaminated water into a distribution line, or (*3*) accumulation of assimilable organics in slow flow pipe sections that support expanded microbial growth in localized areas and interference with disinfectant residual effectiveness.

Within the potable water distribution system, there are many safe havens for biofilm development. Foremost among these sites are areas of corrosion and slow flow where sediments accumulate and water movement is not sufficient to disrupt the development of colonization on iron pipes (9). This action is followed by tuberculation in the pipe that not only impairs water flow but also becomes a protected microbial habitat for cell attachment within the porous structure of these corrosion deposits (25–27).

Although cement pipe and PVC pipe materials are not subject to this type of corrosion attack, water characteristics may change the surface structure of asbestos/cement mains and biological activity will eventually create pitting of plastic pipe materials and splaying of cement surfaces. It is important to note that not all pipe sections may show evidence of deterioration even after 50 years or more of service: the reason being the nature of the system's water chemistry and the continuous movement of water supply through the pipe network in high-demand areas. Water storage reservoirs and standpipes may also accumulate biofilm deposits on sidewalls and in tank bottom sediments as a result of slow water exchanges and stratification. This near static situation provides opportunities for enhanced anaerobic growth of various nuisance organisms that cause taste and odors.

Growth of pioneering microorganisms in the distribution system initially proceeds slowly as the organisms adjust to the constraints of this aquatic environment (3). Eventually, other organisms traversing the bulk water movement become entrapped in this material and introduce diverse metabolic pathways and unique by-product releases. With the synthesis of more complex organics and unique by-product releases, additional organisms with specialized nutrient requirements such as *Legionella* become established in the microbial community. Thus, as the pioneering biofilm reaches maturity, the microbial community becomes a site for progressive diversity of bacteria, protozoans, nematodes, and worms.

This consortium of organisms is a dynamic entity, with profiles shifting to reflect establishment of newcomers to the habitat, impact of antagonistic organisms in the community, and changing dominance by more aggressive organisms. All of these microbial profile changes are a reflection of warm water temperatures, available organics and nitrogenous compounds, and the impact of disinfectant exposures. Portions of the biofilm may eventually slough off into the bulk flow of water, as the thickness of the biofilm is held constant by the shearing effects of water flow. Other cases of biofilm release are water pH changes that effect pipe sediment stability and mechanically scraping of pipelines to reduce tuberculations.

Although the profile of transient organisms may vary with seasonal factors in source water, the permanent flora that comprises biofilm may be expected to be more stable. Organisms such as *Pseudomonas, Flavobacterium, Proteus, Bacillus, Actinomycetes*, coliforms, fungi, and bacterial predators are often the major contributors to the permanent biofilm community (3). The bacterial pathogen *Salmonella* has also been

demonstrated in biofilm but its persistence may be tenuous because of competition from other organisms that have been estimated to be 2,400,000 total organisms per milliliter.

Drinking Water Stability. Drinking water stability can only be achieved by keeping the system clean so that there are minimal progressive changes in the microbial quality (28). Source water constituents (assimilable organics, humic substances, and turbidity), treatment configuration, poor line-flushing practices, and water stratification in storage tanks provide opportunities for the gradual accumulation of sediments and microbial presence in the distribution system. These elements provide either a source of nutrients or contribute to a protective habitat.

Essential nutritive substances for microbial subsistence and growth in this situation are nitrogen, phosphorus, trace metals and biodegradable organics. Of these substances, reduction of organics is the most cost-effective approach to bring biological stability to the water system as critical inorganic materials are ubiquitous in the aquatic environment (29). Specific treatment options include biological treatment (ozonation followed by biological removal in carbon filters), granular carbon filtration, and enhanced coagulation. These processes provide destruction of recalcitrant organics in ozone treatment, improved extraction in the filter media bed and more effective physical entrapment in the coagulation process. The net result is that less organics pass into the distribution system and become available to organisms that have become entrenched in the pipe or storage tank environment. As a consequence, microbial activity becomes limited even in the presence of warm water conditions (9,17) and biofilm expansion is held in check. As an additional benefit, chlorine demand is reduced in the pipe environment that, in turn, improves the protection provided by disinfectant residuals in the pipe network.

Biological stability in the distribution system is most often measured by determining the assimilable organic content (AOC) or the biodegradable dissolved organic carbon (BDOC) concentration. The AOC test is based on a bioassay of two test strains (*Pseudomonas fluorescens* strain P17 and *Spirillum* strain NOX) with growth in the test sample compared to calibrated growth curves established on known concentrations of standard organic compounds (acetate and oxalate) for these organisms (30,31). The BDOC procedure evaluates the reduction in the dissolved organic carbon concentrations (DOC) over time. In the procedure, an inoculum of heterotrophic bacteria in a sample taken from the filter bed is then added to the filter-sterilized test sample (32,33). Sterilization of the test sample removes any overlapping response caused by the original flora in the test portion. DOC testing of the incubated sample is done daily till the DOC measurements reach a stable value over several days. The difference between the initial and final DOC concentrations is the biodegradable organic carbon fraction of the test sample.

Using these methods, the threshold at which drinking water supply may be considered biologically stable is less than 10 μg/L of AOC or 0.15 mg/L carbon in a BDOC test. Unfortunately, low nutrient concentrations alone do not ensure coliform-free water supply because of the potential threat of a contamination breakthrough (34,35).

Monitoring Microbial Quality

Monitoring the microbial quality of drinking water supply is a regulatory requirement established by the Federal Drinking Water Act that was enacted to protect public health (36). The regulations in this legislation are enforced by the United States Environmental Protection Agency and revised periodically as new information on causes for waterborne risks are discovered, faster and more specific methods for analysis are developed and innovations in treatment are field tested to improve barriers to chemical, radiochemical, and microbial contamination. The overall goal is to reduce the health risk that might be found in public water resources, nationwide. In Europe, the European Community Directive on the Quality of Water for Human Consumption has established guidelines for drinking water quality throughout the member states but the interpretation and implementation of the directive can be different from country to country (37).

Monitoring production of water at the utility and throughout the distribution system provides managers with vital information on continued effectiveness of treatment schemes to process the varying qualities of source waters and evaluating the integrity of water supply in distribution. It must be remembered that collection and subsequent analysis of water samples only provides information on the quality of water in the distribution system and does not in itself improve the water quality. Continued monitoring without an action response to correct the problem does not solve the problem.

Pioneering development of national standards for public water quality by the U.S. Public Health Service focused on characterizing the sanitary quality of public water supplies (38). Although coliform bacteria are found in high densities in the intestinal tract and are readily detected by conventional laboratory procedures, this group of bacteria became the chosen indicator of sanitary significance. By definition, these organisms are an artificial grouping of various gram-negative bacilli. Because they generally ferment lactose sugar, this characteristic became the bases for their detection. Like all indicator systems, there are some weaknesses in this interpretation.

In the case of the total coliform indicator group, many of these organisms are dominant environmental strains in the aquatic environment. Their die-off does not closely parallel that of virus persistence in fecal cell debris or the extended survival of pathogenic protozoan cysts or oocysts. With a coliform standard set on a test volume of 100 ml (approximate capacity of a glass of water for drinking) and the search for bacterial pathogens, virus, and protozoans based at 1-, 10-, or 100-l test portions, respectively (low density occurrences in contaminated drinking water), there is little opportunity for many parallel correlations. Regardless of these shortcomings, the total coliform test has amassed an impressive record of serving as an indirect indication of a microbiologically safe water supply and providing useful information on treatment effectiveness.

Monitoring frequency for water quality in the distribution system is based on the population served by the utility (3). The rationale for this approach is based on the recognition that as population increases so does the size and complexity of the system and the potential for cross-connections and back-siphonage. Unfortunately, the one major weakness in this approach is that small water systems (serving fewer than 10,000 people) are infrequently monitored. Small water systems (that often have the most water quality problems) are only obligated to monitor water quality a few times per month in an effort to reduce the burden of laboratory costs. To counter this loss of critical information, small water utilities should be subjected to a periodic sanitary survey of all operational practices by the State Water Authority. This review would help identify faults in water quality protection. Other factors to consider in maintaining a responsive monitoring program for all water utilities are pipeline break frequency, low-water pressure problems, areas of no disinfectant residual, and historical patterns to all coliform occurrences. Every water distribution system has unique characteristics so that the frequency of monitoring and locating of sampling sites must be flexible and responsive to changing water quality conditions.

Case Studies on Pathogen Incursions

Adequate protection of the distribution system is essential to prevent the incursions of pathogens into the safe water supply. Waterborne outbreak events because of the problems in the distribution system may start from line breaks in contaminated soils, sudden loss of water pressure, and wildlife contamination around open reservoirs of finished water or bird habitation inside storage tank structures that have deteriorating covers (3). A study of distribution system deficiencies for water supplies in the United States for the period from 1971 to 1998 that have lead to waterborne outbreaks (Table 1) reveals the importance of careful management of the distribution system and an effective community plumbing ordinance to avoid the risk of drinking water contamination. Field investigation of these outbreaks has uncovered a wide range of intestinal pathogens as the causes for these community disasters (Table 2). It is important to note that there still are reports of a larger number of outbreaks for which a pathogen was never detected in the water supply. Part of this problem is because of not recognizing a contamination problem until it has passed through the water system, lack of

Table 1. Distribution System Deficiencies Causing Outbreaks (1971–1998)

Deficiency	Community Outbreaks	Systems Percent	Non-Community Outbreaks	Systems Percent
Cross-connection/Back-siphonage	45	50.6	15	62.5
Corrosion/leaching of metals	12	13.5	1	4.1
Broken/leaking water mains	10	11.2	0	—
Contamination during storage	9	10.1	6	25.0
Contamination of mains during construction/repair	5	5.6	1	4.2
Contamination of household plumbing	7	7.9	1	4.2
Inadequate separation of water main and sewer	1	1.1	0	—
Total	89	100	24	100

Source: Data from Craun and Calderon (38).

Table 2. Etiology of Outbreaks Caused by Distribution System Contamination, (1971–1998)

Etiology	Community Outbreaks	Systems Percent	Non-Community Outbreaks	Systems Percent
Chemical	35	39.3	3	12.5
Unidentified pathogen	29	32.6	11	45.8
Giardia	8	9.0	4	16.7
Salmonella	4	4.5	1	4.2
Norwalk-like virus	3	3.4	1	4.2
Shigella	3	3.4	1	4.2
Campybacter	3	3.4	1	4.2
Hepatitis A	1	1.1	1	4.1
S. typhi	1	1.1	0	—
Cyclospora	1	1.1	0	—
E. coli 015 : H7	1	1.1	0	—
V. cholerae	0	—	1	4.1
Total	89	100	24	100

Source: Data from Craun and Calderon (38).

rapid screening techniques for waterborne pathogens, and the sudden emergence of new pathogens that have not previously been found in water supply (3,4,39).

Several scenarios can be cited to illustrate a public health disaster that might have been avoided with better attention to distribution system operations. In all of these case histories, a waterborne outbreak occurred because of the presence of a specific pathogen. It is likely that other pathogens could also take the same pathways during an outbreak but may be overlooked in the search that is focused on the first pathogen detected in the water supply or found in the feces of several illness cases.

Line Breaks and Service Meter Repairs. Loss of protection in the pipe network from storm water runoff or sewage migration into the pipeline bed is always a threat whenever there are line breaks and repairs as well as service meter replacements. One example to cite is a waterborne outbreak involving the pathogen *E. coli* 0157 : H7 that occurred in the small farm community of Cabool, Missouri (population 2,090) during the period from December 15, 1989 to January 20, 1990 (39). This event resulted in four deaths, 32 hospitalizations, and a total of 243 known cases of diarrhea. The pathogenic agent was found in the bloody feces of some of the hospital patients.

Public water supply became the prime suspect after field epidemiologists ruled out contaminated raw beef and tainted milk. Further investigation revealed that persons living inside the city (using municipal water) were 18.2 times more likely to develop bloody diarrhea than those people living outside the city and using private well water supplies.

A study of the water utility records revealed that the groundwater aquifer used as untreated drinking water had a long history of meeting the bacteriological quality standard. However, several events occurred in the distribution system prior to the waterborne outbreak that may have been the major contributing factors. Weather conditions proceeding this outbreak reached record low temperatures that caused freeze blockages in 43 service meters and two major distribution line breaks that were within days of each other at two residential locations. Some meter boxes were reported to be partially submerged during replacement that could have introduced contamination. On the basis of customer recollections, the two-line breaks did not reduce water pressure systemwide although localized low pressure created opportunities for back-siphonage overnight before the breaks were repaired. In both the line breaks and service meter repair situations, line flushing with fresh water supply was done but the lines were not disinfected prior to service.

The source of contamination was though to be sewage from the municipal collection system that was undersized in pipe capacity and becoming overloaded with storm water runoff that leaked into the waste transport system. There were continual complaints from some residences of periodic overflows from nearby sewage distribution boxed into their yards, even though storm water runoff was drained by open ditches along the streets. These complaints were verified during heavy rains by observation of various paper products associated with sewage being released at several distribution boxes. Such situations provided opportunities for soil contamination and a pathway for sewage or surface runoff to intermingle with water supply at line break areas and service meter repair sites.

The pathogenic agent was not recovered in the water supply probably because a specific search for the organism in water samples was not done during the outbreak period. Additional circumstantial evidence was found in several water samples: detection of *E. hermanii*, an associate *Escherichia* strain, and several tetracycline-resistant coliforms that share characteristics with the outbreak strain *E. coli* 015 : H7. Such findings suggest that these organisms and the outbreak strain originated from a common source of contamination. At this point in the investigation, no pathogenic *E. coli* was detected in any of the samples although coliforms were present in some of the distribution samples taken at the ends of the pipe network yet there was never a report of coliforms being in the groundwater supply. The next step was to create a hydraulic mode of water flow patterns in the distribution system. The purpose was to plot a simulation for contamination movement entering the line breaks and meter repairs. The result of this study showed that by combining the main break dates with the illness start dates and resident locations, 85% of cases could be linked to the direction of first incursion of contaminated water in the distribution system. The outbreak subsided with the consumption of boiled water. Since then, the system has utilized disinfection on a permanent basis and there has been no more water associate illness reported in the community.

Water Pressure Interruptions and Illegal Tap-Ins on the Pipe Network. Water losses from perpetually leaking pipes represents not only a loss in revenue for the utility (unaccounted water losses are often between 30 and 60%) and a waste of resources but also a potential health risk from incursions of contaminates. Such a situation is often a serious problem in many third world nations. Compounding factors are problems with utility infrastructure deterioration in treatment or distribution and scheduled interruptions in pumping water throughout the system to reduce the cost of electricity. With sewage collection systems that are also in need of infrastructure repair and capacity expansion, it is not surprising to find sewage overflows linked to deteriorating water supply distribution networks. Once a waterborne outbreak begins to form in a community, defective sewage collection systems reinforce the risk and intensify the spread of contamination. This was one aspect to the rapid development of the cholera outbreak in much of South America in 1991 (40,41).

The city of Lima, Peru, obtains its water supply from a surface water source that is augmented by a series of wells in various locations throughout the city (3). Considering the gross nature of the surface source water during seasonal flash floods, it is remarkable that the water utility is capable of producing a water quality that meets international drinking water standards of less than one coliform per 100 ml. *Vibrio cholerae* was never detected in the finished water from the plant during the outbreak.

Unfortunately, the microbially safe water supply produced at the plant did deteriorate in distribution during the cholera outbreak based on a special study of 183 water samples collected from the pipe network and promptly tested. Fecal coliform bacteria were found in 18.7% of all samples. One sample had a maximum fecal coliform density of 240 organisms per 100 ml, and the fecal coliform occurrences increased with distance from the water plant. None of these samples contained cholera in the 100-ml test portions, however this pathogenic agent was detected in earlier tests from two wells supplying water to the distribution system.

There are a variety of factors that contributed to this loss of water quality. Because of a limited operational budget, sustained water pressure in the pipes was limited to restricted hours each day (4–6 hours) in different areas of the city in an effort to cut pumping cost for electricity. This action resulted in the failure of numerous building water supply tanks to fill with new water. In other situations, this loss of water pressure contributed to back-siphonage from attachment devices and encouraged some citizens to augment their water supply by interconnecting lines between private wells and the public water supply. The condition of widely fluctuating water pressures also provided many opportunities for reversals of flow and suction that mixed good quality water with contamination pathways wherever they existed in the pipe network from leaking pipes and cross-connections. Adding to the problem was a nonexisting flushing program for the distribution network because water production at the water utility barely met the demand and no funds were available to repair all of the pipe leakages in the system. These negative impacts also made it impossible to maintain a protective disinfectant residual because of significant disinfectant demand from the contaminants in most of the pipe network. As a result of the enforced low water pressure and inadequate system maintenance, integrity of the pipe network was lost to an increased health risk.

Bird Habitation in Drinking Water Storage Tanks. Wildlife habitation (sea gulls, ducks, and geese) in open, reservoirs of drinking water supply has long been recognized as a significant contributor to fecal contamination and the source of some waterborne outbreaks. The obvious solution is to enclose large, open reservoirs whenever possible, but cost considerations in engineering a cover over huge water surface areas make this approach unfeasible. Thus, it is a common practice to inject additional chlorination (booster chlorination) to water at the outlet from these open reservoirs prior to water entry into the distribution system in an effort to inactivate any bird contamination and to replace a loss in disinfectant residual from sunlight exposure.

Smaller capacity, covered water storage tanks spaced at appropriate locations within the service area are a more practical solution. Water pressure can be established more uniformly everywhere and individual tanks can be taken out of line for repair and cleaning while other tanks serve as backup. These covered storage tanks also slow the decay of chlorine residual because sunlight exposure is not a factor. Unfortunately, when preventive maintenance of the tank structure is infrequent, corrosion of the tank begins to accelerate. The most common concern is that the protective screen cover over air vents may be breached because of the action of corrosion or wind damage. This situation provides opportunities for birds to enter the tank structure to find shelter from the winter weather. Such was the case when pigeons invaded openings in water supply tanks at Gideon, Missouri (42).

The community outbreak of diarrhea, caused by *Salmonella typhimurium*, occurred during November and December 1993. It was estimated that 44% of the community (1,104 population) became ill. Two of the hospitalized patients died. A sanitary survey of the water supply revealed that water supply was derived from two wells driven 1,300 feet into an aquifer. At the time of the outbreak, the water supply was untreated because the aquifer quality met the coliform standard for a safe water supply. Water pumped from these wells was stored in two water tanks (50,000 and 100,000 gal. capacity) and satisfied customer demand in this small community. In addition, a private water storage tank of 100,000 gal. capacity (used for fire control in a cotton baling plant) was also connected to the system for filling but separated by a backflow prevention valve.

During field investigation, a large number of pigeons were observed roosting on the two 100,000 gallon tanks. Physical inspection of the water storage tanks revealed the consequences of minimal maintenance to arrest corrosion and maintain a protective barrier screen at all air vents. The tank inspector found that all tanks were deteriorating from long-term corrosion. There were holes in the top of the privately owned tank and numerous bird feathers and droppings were floating in the water supply at all the three tanks. Not surprising was the detection of *Salmonella typhimurium* in the sediment from the private water storage tank.

A combination of events set the stage for passage of the pathogenic agent into the distribution pipe network. In early November, there was a sharp climatic change in weather with air temperatures falling to record lows. This persistent and dramatic event caused destratification of the water supply in the storage tanks. The resultant mixing of stratified tank water resulted in a variety of taste and odors and an increase in complaints from customers.

Immediate water utility action was to flush the entire water supply lines over a 12-hour period. Each of the 500 hydrants was flushed for 15 minutes at a rate of 750 gal./min. This vigorous action brought much of the water out of the storage tanks thereby further mixing the contaminants and passing them into the pipe network. Not surprising was the detection of *Salmonella typhimurium* during the outbreak period at a fire hydrant nearest to one of the public water supply storage tanks.

Applying a hydraulic model simulation of water movement in the system revealed that the earliest cases of salmonellosis and the location of the fire hydrant at which this pathogenic agent was recovered, were situated primarily around the 100,000 gallon tank. This finding supports the belief that the pathogenic agent was pulled into the pipe network during the vigorous flushing program that focused in the area around the tanks.

In retrospect, the systemwide flushing program should have begun with good quality water at the wells then followed by progressive draining of system water outward to the ends of the pipe network. This strategy might have provided more protection to part of the population served; although, this was difficult to achieve because of cyclic reversals of flow in some parts of the pipe network. Lack of funding for repair and maintenance of storage tanks was a major contributing factor associated with this outbreak. The outbreak finally was brought under control with a community order to boil drinking water until tests revealed that disinfection residuals were occurring consistently throughout the entire distribution system.

CONCLUSION

Drinking water quality in the distribution system must be safe from a variety of pathogenic agents that may be present in sewage, storm water runoff, and poor quality source waters. There is a growing awareness that subsets of the population are at increased risk from exposure to opportunistic pathogens through ingestion, inhalation or body contact, whereas a greater number of consumers may be sensitive to the sudden advent of taste and odors associated with microbial activity in the pipe/tank environment. This condition need not imply that drinking water supply must be treated and delivered as a sterile product to the consumer. Rather, the solution lies in achieving a biologically stable water to minimize microbial activity.

The quality of water supply entering the distribution system will change over time as a function of system integrity, retention time in the pipe–storage tank network, and microbial response to this environment. To combat these changes in quality, every water utility must maintain a permanent program for systemwide flushing. Identify and correct water loss through leak detection, revamp the pipe network pattern to reduce dead-ends through continuous pipe loops, replace lines with a history of frequent pipe breaks, and institute triennial inspections of storage tanks that must be taken off-line for cleaning, resealing, and repair.

Each public water system is unique in some aspect, so it is important to focus on those unique features that have the potential for degrading water quality during distribution. This means that the water authority must implement an effective, routine microbiological monitoring program for the distribution system with an action response to various scenarios of quality change. Furthermore, a public awareness initiative must be in place to inform consumers about the quality of their water supply during adversities and disclose strategies for improvement.

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CORROSION. See BIOCORROSION: ROLE OF SULFATE REDUCING BACTERIA

COSMETICS, USE OF A265 BIOSURFACTANTS IN.

See BIOSURFACTANTS: TYPES, SCREENING METHODS, AND APPLICATIONS

CRUDE OIL, BIODEGRADATION OF.

See PETROLEUM AND OTHER HYDROCARBONS, BIODEGRADATION OF

CRUDE OIL SPILLS. See PETROLEUM AND OTHER HYDROCARBONS, BIODEGRADATION OF

CULTURABLE SUBSURFACE MICROBIAL COMMUNITIES.

See SUBSURFACE MICROBIAL COMMUNITIES: DIVERSITY OF CULTURABLE MICROORGANISMS

CYANOBACTERIA

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Cyanobacteria, also known as chloroxybacteria, blue-green algae (cyanophytes, blue-green plants — formerly myxophytes or "slime plants"), cyanochlorontans (blue-green organisms), and prochlorophytes, are prokaryotes with the universal plant pigment, chlorophyll a, and with "higher plant" or oxygenic (oxygen-producing) photosynthesis, wherein water is split for the electron source and oxygen is emitted. They are evolutionarily the oldest oxygenic photosynthetic organisms (1,2). Cyanobacteria were the dominant forms of life on earth for more than 1.5 billion years, and their earliest fossil record is from about 3.5 billion years ago (2). Many species have retained the ability to fix atmospheric nitrogen (dinitrogen gas) and, in fact, cyanobacteria have been estimated to fix approximately 75% of the nitrogen (N) attributed to natural sources worldwide. They were abundant in the ancient oceans and were also the earliest terrestrial autotrophs, helping to create and enrich the soil (2). In present times they remain ubiquitous, covering the air-water interface across lakes and oceans, endolithically within desert rocks and as crustose forms in the "black zone" of the high rocky intertidal worldwide; at depths down to at least 100 m in the dimly lit oceans, beneath more than 30 m of Antarctic sea ice (2), in light-free caves and tombs (3), in hot springs at temperatures up to approximately 75 °C (2), and in the lungs and digestive tract of an array of homeothermic organisms, including humans (4,5). Cyanobacteria are significant to evolution not only because of their biogeochemical importance in forming the oxygen-rich atmosphere but also because they are the likely progenitors of the chloroplasts of red algae (Rhodophyta), which are the dominant macroalgal flora of the intertidal or subtidal and of soft-water streams in various geographic regions (1). Certain species (the chlorophyll $a + b$ organisms) are also considered the progenitors of the chloroplasts of green algae (Chlorophyta) and higher plants.[1] This article provides an overview of cyanobacteria taxonomy, systematics, phylogeny, cell biology, ecology, and evolutionary significance.

TAXONOMY, SYSTEMATICS, AND PHYLOGENY

The Fossil Record

Schopf (2) wrote, "The best documented early branch of the Tree of Life is the cyanobacterial lineage, represented in the Precambrian fossil record by ensheathed solitary and colonial unicells (represented in extant taxa by the Chlorococcaceae) and cellular microscopic filaments [(mostly members of the) Oscillatoriaceae, taxonomically the most diverse cyanobacterial family]." Most Precambrian cyanobacteria-like fossils, both older and younger than 1.5 billion years (Ga), are referable to extant genera within the Chlorococcaceae and the Oscillatoriaceae. Schopf (2) described 37% (26 of 70) of coccoidal taxa in the fossil record as older than 1.5 Ga and 28% (31 of 109) of those younger than 1.5 Ga as indistinguishable from extant species. Moreover, 35% (18 of 51) of filamentous cyanobacterial taxa in the fossil record older than 1.5 Ga and 37% (56 of 152) of those younger than 1.5 Ga are virtually identical to extant species in the Oscillatoriaceae. No discontinuity has been detected in cyanobacterial community structure, biotic composition, or environments inhabited since 1.5 Ga years ago. The oldest cyanobacterial fossils known to date were found in the 3.5-Ga-old conglomeratic Apex chert (basalt), a geologic formation in northwestern Western Australia (1,2).

In that assemblage, 7 of 11 species (ca. 63% of measured specimens) are similar in cellular organization, size range, median dimensions, and pattern of size distribution to oscillatoriacean cyanobacteria, and several are indistinguishable from extant species. At that time, there were extensive shallow seas, with volcanic islands fringed by sedimentary debris (river gravels, sands), mud flats, and interspersed evaporitic lagoons. Isotopic compositions of organic and carbonate carbonate in the Apex chert indicate the occurrence of photosynthetic carbon dioxide fixation similar to that of extant cyanobacteria grown in carbon dioxide-rich environments. Other lines of evidence support these findings of 3.5-Ga-old cyanobacteria. Among prokaryotes, oxygenic photosynthesis is unique to cyanobacteria (2). The Apex sequence and coeval geologic units contain evidence of both the reactants required for oxygenic photosynthesis (H_2O, CO_2) and the products (reduced org. carbon and O_2). Evidence of carbon dioxide occurs as calcium carbonate–rich limestones, deposited as a result of the aqueous reaction between Ca^{+2} (from weathering of the land surface) and HCO_3^- (from dissolution of atmospheric CO_2).

Graham and Wilcox (1) wrote, "The rise to dominance of cyanobacteria ... has been described as the single most significant evolutionary event in the history of life on earth, for without it, subsequent origin of eukaryotic life would have been impossible." Evidence for free molecular oxygen (g) 3.5 Ga ago, and its origin predominantly from cyanobacteria, is provided through several lines of evidence. The earth's atmosphere mostly formed from volcanic outgassing of the planetary interior over geologic time (2). Unlike other components of the atmosphere (N_2, H_2O vapor, CO_2), oxygen is not released from heated rock. The presence of free oxygen is indicated by iron oxide–rich sedimentary deposits called banded iron formations (BIFs, the world's major source of iron ore), which are widespread in geologic terrains that are more than 3.5 to 1.9 to 2.0-Ga old (2). The oxygen content of the atmosphere increased dramatically between 2.2 and 1.9 Ga ago (from $\leq$ 1% to $\geq$ 15% of present levels). Nonbiological sources (e.g., thermal dissociation or UV-induced photodissociation of water vapor) would not have been sufficient to account for the massive quantity of oxygen that was added. Thus, oxygenic photosynthesis is the only quantitatively plausible source for the enormous quantities of oxygen that were sequestered in Precambrian BIFs (2).

Other evidence for ancient oxygenic photosynthesis from cyanobacteria has been obtained from 2.7 Ga-old stromatolites (layered, calcareous, mound-shaped structures) in the Tumbiana Formation of Western Australia (1,2). Stromatolites are abundant in Precambrian deposits from shallow and deeper waters worldwide, and are believed to have been produced by photosynthetic cyanobacteria that trapped sediments along with layered deposits of calcium carbonate that precipitated from the photosynthetically increased pH in the surrounding medium. The low sulfate content of many ancient stromatolites indicates that they developed in freshwater lakes as a result of oxygenic, rather than anoxygenic (anaerobic process, without oxygen production), photosynthesis. The maximum number of ancient stromatolites occurred in the late Precambrian

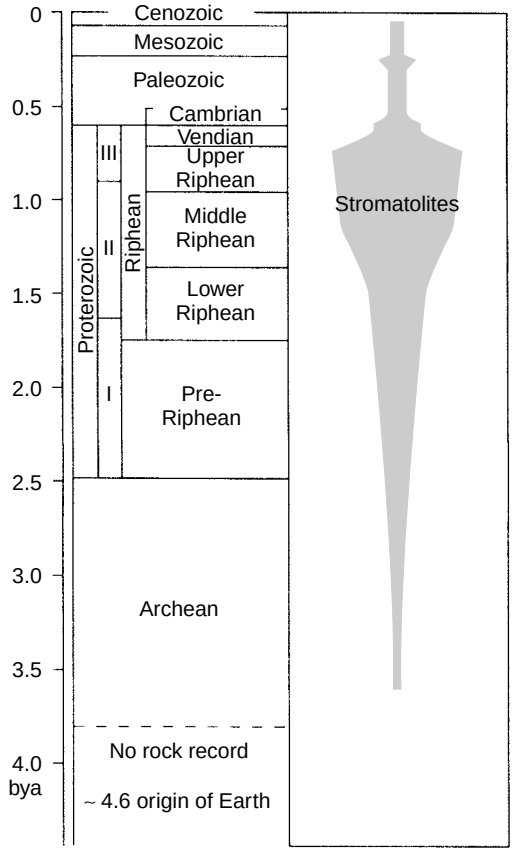

Figure 1. Stromatolite occurrence on Earth, which gradually increased throughout the Precambrian to maximum abundance approximately 800 Ma ago, after which they declined to present-day numbers. Modified from Tucker and Wright (1990), and reprinted from L. E. Graham and L. W. Wilcox, *Algae*, Prentice-Hall, Upper Saddle River, N.J., 2000, with permission from the authors and Prentice-Hall.

approximately 0.7 to 0.8 Ga ago, followed by a sharp decline during the Cambrian (Fig. 1; 1). The decrease may have been related to an increase in herbivorous gastropods that grazed on cyanobacteria-containing microbial biofilm layers or mats before they could accrue form stromatolites. Burrowing organisms that also appeared in abundance may have disrupted the growth of cyanobacterial mats, and increasingly abundant red and green calcareous seaweeds and metazoans may have competed with cyanobacteria for suitable substratum. Present-day cyanobacterial mats usually form in habitats with little or no metazoan grazing activity, such as coastal tidal sand flats, protected coastal lagoons and inland saline ponds, lakes, and seas, hot and cold deserts, caves, and geothermal springs. In habitats devoid of gastropods and other grazers, extant cyanobacteria also remain active in stromatolite formation (i.e., in hypersaline, shallow tropical habitats throughout the world, and also in permanently ice-covered Antarctic lakes) (1,2).

Blue-Green Algae, Blue-Green Bacteria, or Other Names?

The list of division-level names spanning botanical to bacterial nomenclature illustrates the major controversy

that has plagued the systematics of these organisms for nearly 30 years (6–8)—Should they be considered as primitive plantlike algae or as bacteria? They are prokaryotes with many ultrastructural and biochemical bacteria-like features; yet they share the same basic photosynthetic biochemistry of eukaryote algae and higher plants, and they share the "universal plant pigment," chlorophyll *a*, which is the pigment that participates directly in oxygenic photosynthesis and is structurally distinct from bacteriochlorophyll (1). The general view of some ecologists and phycologists can be summarized by Lewin's (6) argument in favor of blue–green algae (division cyanophyta under the International Code of Botanical Nomenclature), paraphrased as: Why combine all prokaryotes together, when they are so strikingly distinct in basic photosynthetic biology, pigments, and certain ultrastructural traits such as phycobilisomes? Should we then combine all eukaryotes together as well, for example, flowering plants in the same group as humans, because they have a nucleus? Golubec (8) similarly argued that many algae and even angiosperms show heterotrophic capacity, and that blue–green algae generally act as primary producers, not as decomposers.

Molecular and microbiological specialists, in contrast, favor placement of these organisms under the bacteriological code, in recognition of the fact that cyanobacteria represent an "undisputed distinct phylogenetic line of the eubacteria" (9). As a result, cyanobacterial publications in taxonomy and systematics have become widely scattered across bacteriological, medical, microbiological, and botanical literature. Recently, as a further complication, small prokaryotes that are green ("apple–green") in color, having chlorophyll *b* rather than blue–green algal phycobiliproteins as accessory pigments in photosynthesis, have been removed by various specialists from a previously separate algal division under the botanical code, the Prochlorophyta (10), and added to the cyanobacteria under the International Code of Nomenclature of Bacteria. Like most aspects of the taxonomy of prokaryotes with "higher plant" photosynthesis, the phylogenetic position of the chlorophyll $a + b$ prokaryotes is still in debate, and phycology texts reflect both sides (2,11). Indeed, some specialists use the terms Cyanophyta and Prochlorophyta [the latter, designated as an artificial (not phylogenetically coherent) division of "green cyanophytes"] from the botanical code, although placing these organisms within the Kingdom Eubacteria (12). The division Prochlorophyta has been described as artificial on the basis of molecular data (16S ribosomal RNA sequences) indicating that the three known genera are only distantly related (13). Other molecular data (e.g., sequences for a DNA-dependent RNA polymerase, *rpo* C; and partial sequence of the ribulose-1,5-bisphosphate carboxylase/oxygenase enzyme or Rubisco) indicate that two of the three genera (*Prochloron, Prochlorothrix*) are close relatives, whereas the third (*Prochlorococcus*) is more closely related to γ-purple bacteria (14,15) (Fig. 2; see article by Campbell in this Encyclopedia).

Operationally, species in cyanobacteria vary greatly depending on the classification scheme, and no complete comprehensive coverage of cyanobacterial species is yet available. The species concept in cyanobacteria is, in fact, uncertain and presently in a state of major flux. The difficulty stems, in part, from the fact that cyanobacteria lack gametes or sexual structures, which precludes definition by the biological species concept (i.e., two taxa belong to the same species if they interbreed and have viable offspring; 11). Cyanobacterial taxonomy began in the 1700s and the system that was developed through most of the 1900s was under the botanical code. Identification was based almost entirely on morphological traits (e.g., see Geitler (16) and the Frémy series given in (2)), but it was later realized that many of these traits are polymophic, depending on environmental conditions. For example, in the blue-green prokaryotes with oxygenic photosynthesis, hormogonia (specialized cells used to fix dinitrogen gas into inorganic N; see following text) form in low-N environments; phosphorus limitation tends to alter the degree of filament tapering and pattern of false branching; sheath color/thickness depend on the abundance of certain micronutrients and the turbulence, and so on (17,18). Less information is available on polymorphism among the "green" prokaryotes with oxygenic photosynthesis. Some morphologically distinct isolates of symbiotic coccoid organisms, *Prochloron didemni*, have been evaluated as very closely related on the basis of molecular data, implying that different physiological states of *P. didemni* in different hosts affect their morphological appearance (2).

As a proposed solution, Drouet and Daily [series given in Castenholz (9)] relied upon several simple morphological features to combine more than 2000 previously described species within more than 150 genera to 24 genera and 62 species for all known cyanobacteria. However, many of their analyses had relied on dry herbarium material that would have led to inaccurate morphological descriptions; live strains were not subjected to environmental gradients to verify polymorphic traits; and genetic information was not available. More recently, the cyanobacteria were regrouped at the family and genus level by Komárek, Anagnostidis, and Kann (2), still as blue-green algae under the botanical code. Their most recent definitive contribution of Komárek and colleagues presents a detailed, logical arrangement of the Chroococcales, considering cell shape, patterns of cell division, and cell organization within sheaths and/or mucilage (19).

Stanier and coworkers (7) and Rippka and coworkers (17,20) proposed removal of this entire group of organisms from the botanical code and placement under the bacterial code. However, at present, taxa described under either code are considered in accounts prepared under the other code (2). Descriptions at the order level (21) are similar to those in historical botanical systems. The cyanobacterial taxonomy was first to rely upon other characteristics in addition to morphology (e.g., optimum temperature for growth, fatty acid composition, motility, heterotrophic potential, DNA base pair composition, gas vacuoles, etc.). By that taxonomy, the organism formerly known as *Microcystis aeruginosa* would be called *Microcystis* strain number, followed by series of + and − signs indicating presence/absence of certain morphological, physiological,

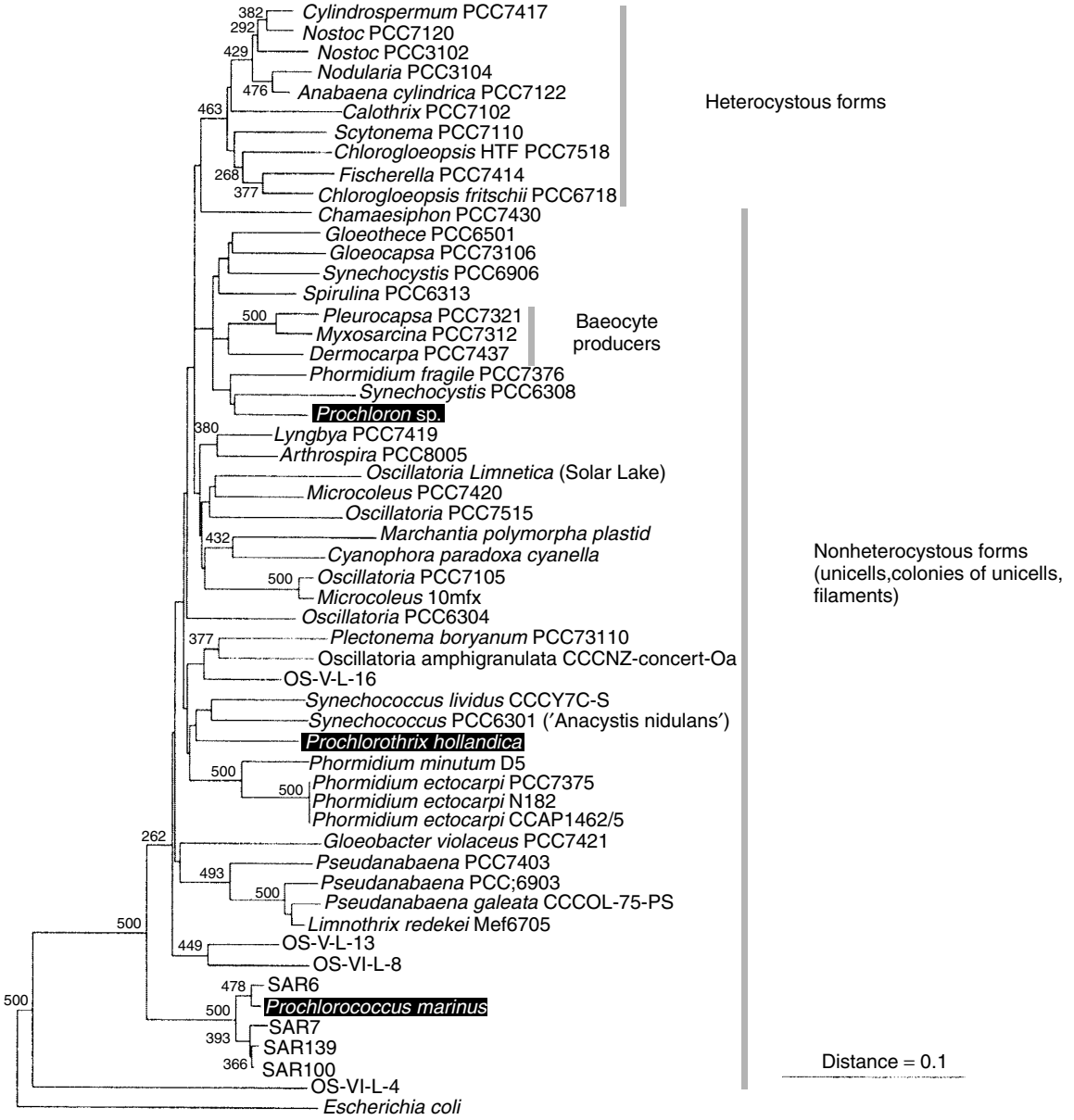

Figure 2. A phylogenetic tree of the cyanobacteria (and plastids, represented by the liverwort Marchantia and the glaucophyte Cyanophora) inferred from 16S rDNA sequences. Note that taxa with heterocysts form monophyletic groups, whereas other types of cyanobacterial morphologies appear to have arisen multiple times. Also, note the polyphyletic nature of the chlorophyll b-containing prokaryotes with oxygenic photosynthesis (Procholoron, Prochlorothrix, Prochlorococcus). Organisms listed as a series of letters and numbers are not yet named, and are known only from sequences obtained from environmental samples. The numbers on the branches represent bootstrap values (refers to a modeling procedure that estimates how closely the taxa are related evolutionarily) based on 50 resamplings of the data. After (A. Wilmotte, in D. A. Bryant, ed., *The Molecular Biology of Cyanobacteria*, Kluwer Academic Publishers, Dordrecht, The Netherlands, 1994, pp. 1–25), reprinted from (L. E. Graham and L. W. Wilcox, *Algae*, Prentice-Hall, Upper Saddle River, N.J., 2000), with permission from the authors and Prentice-Hall.

and other traits. Thus, older botanical generic names, but not species names, were retained whereas phenotypes were reordered to define genera based on clusters of characteristics. Limited attempts have been made to assess species status under the bacteriological code using additional genetic traits (e.g., molar guanine + cytosine ratios in DNA, DNA-DNA reassociation rates, thermal stability values of DNA-DNA hybrids, PCR fingerprinting with primers from randomly repeated elements, 16S rRNA sequences from variable regions V6–V8, amplified ribosomal DNA restriction analysis of the internally described spacer, and genome size) (2,9). The present review honors

the "cyanobacteria" of the bacteriological code, but treats them as primitive plantlike (algal) organisms in ecological contexts. Although (botanical) species names technically should not be used for cyanobacteria, they were retained here to facilitate comparison with other literature, because clonal cultures and strain numbers are not yet available for many previously designated species.

Molecular analyses based on various gene sequences have demonstrated that cyanobacteria are a major eubacterial clade (phylum). The basic traits shared in common between cyanobacteria and (other) eubacteria are prokaryotic ultrastructure, common presence of mucilaginous sheaths (although frequently found in eukaryote algae as well), simple binary fission, absence of the histone proteins associated with eukaryotic DNA (also missing in certain other eukaryote algae), and 70S ribosomes composed of 16S and 23S subunits.[1] Applying Stanier and coworkers' (7) scheme, and on the basis of strong molecular evidence (14,15), the "apple–green" prokaryotes that were designated as prochlorophytes under the botanical code (10) are considered as members of the cyanobacteria as mentioned (1,2). This schematic ignores the presence of both chlorophyll *a* and *b*, starchlike food storage product similar to that of higher plants (13), and stacked thylakoids in prochlorophytes but not in other cyanobacteria (1,12). These historically had been regarded as taxonomically reliable characters, especially the chlorophyll composition, in distinguishing among eukaryote algal divisions. However, among the cyanobacteria, recent findings suggest the presence of all four chlorophylls ($a - d$) (or, if the prochlorophytes were to be considered separately, then the cyanobacteria still would have three of the four chlorophylls represented) (1), creating challenges for botanical systematists and phylogeneticists that might be more consistently addressed using certain molecular traits.

Modern approaches are increasingly based almost entirely on characteristics of pure cultures and instead of a specific epithet, a strain number is commonly used (9). However, there is a serious problem inherent in use of pure cultures over time, that is, pure cultures often have aberrant physiological, morphological, and genetic (especially ploidy) traits from field material that become more pronounced over time (18). It is also important to note that in some cyanobacteria, field material with different morphologies often yields distinct cultures, indicating that data based on the morphotype of field material can be of value (22). Clonal cultures do not yet exist for many cyanobacteria. Castenholz (9) estimated that fewer than 10% of what may eventually be recognized as genera (using Stanier and coworkers' system, expanded in the "Stanier/Rippka system" to include biochemical and ultrastructural features, and limited genotypic characters) have corresponding clonal isolates in culture, and that probably far less than 5% of the "species" are known in culture. Some of the more common species from nutrient-poor (oligotrophic) waters such as various tropical *Trichodesmium* spp. have been difficult to culture (9). The prospect remains that many, if not most, of the thousands of species that were historically described and then regrouped may be ecological phenotypes (ecophenes) of relatively few genotypic clusters (9). This question can only be resolved when many more quality cyanobacterial clones become available and when aberrancies that develop over time as an artifact of culture conditions can be distinguished from traits expressed under natural conditions.

Principles of phylogenetic systematics are increasingly being used to define cyanobacterial taxa as true for eukaryotic algae, especially rRNA sequence data (Fig. 2; 9). For example, genera that previously were grouped together on the basis of more complex morphology have been divided into related clusters or branches, considering similarities in rRNA nucleotide sequence. Structurally cryptic variants distinguishable at the molecular but not the morphological level occur among the cyanobacteria. However, polyploidy (occurrence of multiple genomic DNA copies per cell; see following text), coupled with possible variation among these multiple genomes, and the occurrence of horizontal DNA transfer (uptake and genomic incorporation of foreign DNA, resulting in formation of genetic chimeras), may complicate attempts to characterize cyanobacterial species by nucleotide signatures (1,9).

Diagnostic Characters Under Light Microscopy

Basic cyanobacteria morphotypes include coccoid unicells or colonies and unbranched or branched filaments [cells of the filament, not considering the sheath, are collectively called a trichome (1); Fig. 3]. Filaments may have true or "false" branching, wherein true branches develop by continued division by a cell at an angle to the main filament axis. False branches are outgrowths of filaments adjacent to a dead cell or a specialized cell (e.g., a heterocyst, see following text). Filaments may be uniseriate or multiseriate, consisting of one or more than one row of cells, respectively. Presence or absence of reproductive cells can be helpful in identifying cyanobacteria, including baeocytes (endospores) produced by subdivision of a cell into multiple cytoplasmic units**;** exospores, which bud from the apex of a filament; and akinetes that are thick-walled, food reserve–packed resting stages of vegetative cells. Other useful structures include the presence/absence of a mucilaginous sheath, heterocysts, and hormogonia (the latter are short filaments separated from longer filaments, which can act as a form of vegetative propagation). In addition, the prokaryotic cyanobacteria have no organelles, so their pigments are not compartmentalized and cells appear uniformly pigmented in light microscopy. The cells generally are small (ca. 0.4–3.0 μm, biovolume $<1–10 \mu m^3$), but some unicellular and colonial coccoid taxa have much larger cells (maximum dimension up to 60 μm, biovolume $>10{,}000\ \mu m^3$) (1,2). As Whitton and Potts (2) pointed out in their excellent overview, the depth and detail of the information needed to identify cyanobacteria to "botanical species" have become so great that they might be most effectively accessed from a formalized, continually updated database. Such a database has been created for the 320 botanical species from the British Isles, with most of the morphological and ecological data expressed in 113 characters or character states (23). It was estimated that about 150 characters would be sufficient to encode the information for all described cyanobacterial species. In

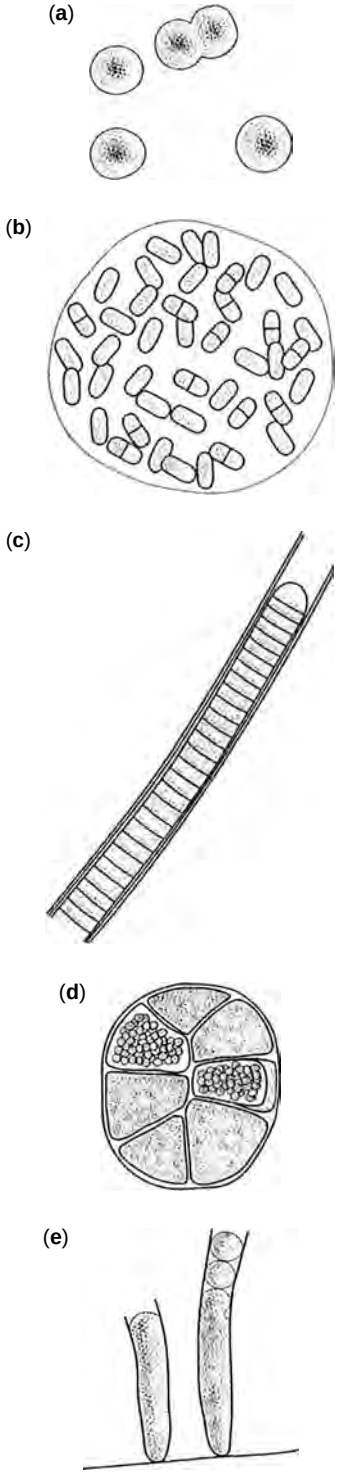

Figure 3. Representative cyanobacterial taxa and morpohologies, including (**a**) unicells such as Synechocystis; (**b**) colonies of individual cells such as Aphanothece; (**c**) unbranched filaments, here, Lyngbya; (**d**) endospore (baeocyte)-forming taxa such as Myxosarcina; (**e**) exospore-forming taxa such as Chaemo-siphon; (**f**) aggregations of multiple trichomes in a common sheath as in Microcoleus; (**g**) false-branched filamentous forms, here, Scytonema; and (**h**) true-branched filamentous forms such as Stigonema. Reprinted from (L. E. Graham and L. W. Wilcox, *Algae*, Prentice-Hall, Upper Saddle River, N.J., 2000), with permission from the authors and Prentice-Hall.

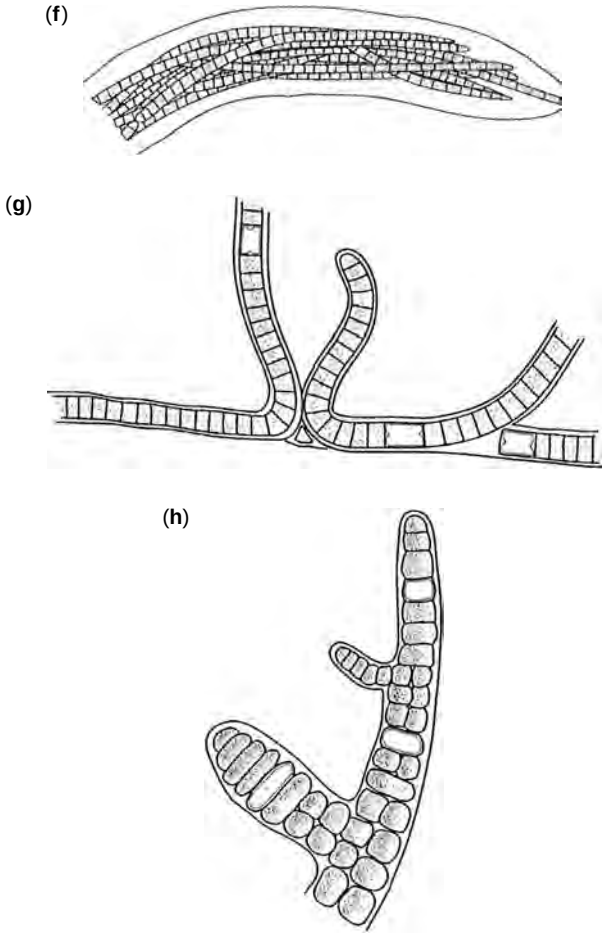

Figure 3. (*Continued*)

related work within the bacteriological nomenclature, *Anabaena* strains have been described in a detailed similarity matrix (24).

Most botanical classification schemes for the cyanobacteria are based on light microscopy. These schemes have combined unicellular and colonial cyanobacteria within the order Chroococcales, and grouped unbranched filamentous taxa lacking specialized cells within the order Oscillatoriales (14,15). However, these groupings are not consistent with recent molecular evidence, and the order Pleurocapsales is also in question (12). On the other hand, inclusion of all heterocyst-producing cyanobacteria within one order (Nostocales) is supported by molecular evidence (Table 1; 12). Molecular research is underway to clarify relationships among cyanobacteria to construct more evolutionarily meaningful classification schemes (1).

Some Genetic Features

Because rRNA sequences (especially 16S rRNA sequences) have been used to assess relationships within the cyanobacteria, and between cyanobacteria and chloroplasts, ribosome composition has become an important feature in cyanobacteria systematics (12). Cyanobacterial ribosomes consist of three types of structural RNA,

Table 1. List of Features in the Orders of Cyanobacteria. After Castenholz and Waterbury (1989), Modified from [(B. A. Whitton and M. Potts, eds., *The Ecology of Cyanobacteria—Their Diversity in Time and Space*, Kluwer Academic Publishers, Boston, Mass., (2000)]. Reprinted with permission from the authors and Kluwer Academic Publishers

NONFILAMENTOUS	
	Order Chroococcales
	Unicellular or nonfilamentous aggregates of cells held together by outer wall or gel-like matrix; binary division in one, two, or three planes, symmetric or asymmetric; or by budding. Rarely form thick-walled resting cysts (akinetes)
	Order Pleurocapsales
	Unicellular or nonfilamentous aggregates of cells held together by outer wall or gel-like matrix; reproduction by internal multiple fissions with production of offspring cells smaller than parent; or by a mixture of multiple fission and binary fission. Rarely form akinetes
FILAMENTOUS	
	Order Oscillatoriales
	Binary division in one plane giving rise to uniseriate (1 column of cells) trichomes, sometimes with false branches; do not form heterocysts. Akinetes apparently not known
	Order Nostocales
	Binary division in one plane giving rise to uniseriate trichomes, sometimes with false branches; one or more cells per trichome differentiate into a heterocyst (at least when concentration of inorganic nitrogen is low). Some also produce akinetes
	Order Stigonematales
	Binary division periodically or commonly in more than one plane, giving rise to multiseriate trichomes or trichomes with true branches or both; apparently always have the ability to form heterocysts. Some also form akinetes

each associated with a protein, including "large" 23S ribosomal RNA, "small" 16S ribosomal RNA, and "very small" 5S ribosomal RNA (ca. 2,900, 1,540, and 120 nucleotides, respectively; S = Svedberg Unit, measure of sedimentation rate when ultracentrifuged). Cyanobacterial ribosomes are smaller than eukaryote cytoplasmic ribosomes, but similar in size to ribosomes of other eubacteria and to ribosomes of eukaryote mitochondria and chloroplasts (12).

The basic morphotypes of cyanobacteria have been retained for at least 2.8 billion years, as mentioned (1,2). It is unknown whether this apparent stasis has occurred because of ecological equilibrium (unlikely, given changes on earth over geologic time) or genetic homeostasis. Many "species" or strains of cyanobacteria have apparently been selected for tolerance of widely varying environmental conditions (2). Cyanobacteria have long generation times (days to months) in comparison to many heterotrophic bacteria (9). Furthermore, genome size may be larger in the more morphologically complex (filamentous) cyanobacteria than in unicellular eubacteria and unicellular coccoid cyanobacteria (among c. 128 strains, c. 1.6×10^9 daltons in unicellular coccoid cyanobacteria, up to 8.6×10^9 daltons in filamentous taxa) (9). On the other hand, these differences in total DNA may reflect polyploidy, which is common in the relatively few cyanobacteria examined. Thus, the coccoid unicellular taxa, *Cyanothece* (formerly *Synechococcus*) and *Synechocystis*, can have up to six identical chromosomes; and in *Synechocystis* PC 7942, polyploidy can involve up to 10 identical DNA molecules (9). The entire genome of *Synechocystis* PCC 6803 has been sequenced and includes 3,573,470 base pairs and 3,168 genes (*http://www.kazusa.or.jp:8080/cyano/*, the CyanoBase database) (2). Similar projects have been undertaken for *Prochlorococcus* and *Anabaena* (2).

Genetic recombination (donation of short DNA fragments from other strains or "species") is of recognized importance in bacterial evolution, but the frequency of horizontal translocation of DNA in natural cyanobacterial populations is unknown. Transfer of short DNA fragments has been documented in some rapidly growing, unicellular culture strains (9). However, the mechanism(s) for DNA uptake has not been determined, and no confirmed example of transduction or conjugation (mating) is yet available (1,9). In addition, because of difficulties encountered in attempts to recover mutant strains, little is known about spontaneous mutation rates in cyanobacteria.

Ultrastructure and Photosynthetic Pigment Complement

The fine structure of cyanobacterial cells is consistent among phycobiliprotein-containing taxa, but somewhat distinct from that of the chlorophyll $a + b$ organisms (Fig. 4; 12). Many prokaryotic traits are shared between the two "subgroups," including absence of organelles and presence of the following inclusions: a four-layered cell wall with murein as a structural component (peptidoglycan with peptide side chains attached to linked, alternating residues of N-acetylglucosamine and N-acetylmuramic acid); thylakoids containing chlorophyll **a**; cyanophycin granules [multi-L-arginyl-poly(L-aspartic acid), nitrogen storage reserves, diameter approximately 500 nm, consisting of polymers of amino acids arginine and asparagine]; polyphosphate bodies (or volutin granules of highly polymerized phosphate, diameter 0.5–2 μm; function to some extent as P storage reserves; found in [other] bacteria, fungi, and eukaryote algae); and carboxysomes (polyhedral bodies, diameter 200–300 nm; contain the photosynthetic Rubisco enzyme system; Table 2) (1,12).

However, there are also important differences between the "blue-green" and "green" prokaryotes with oxygenic photosynthesis. The blue-green organisms have cyanophycean starch (α-1,4-linked polyglucan, similar to glycogen and to the amylopectin component of higher plant starch) as their major reserve polysaccharide. In

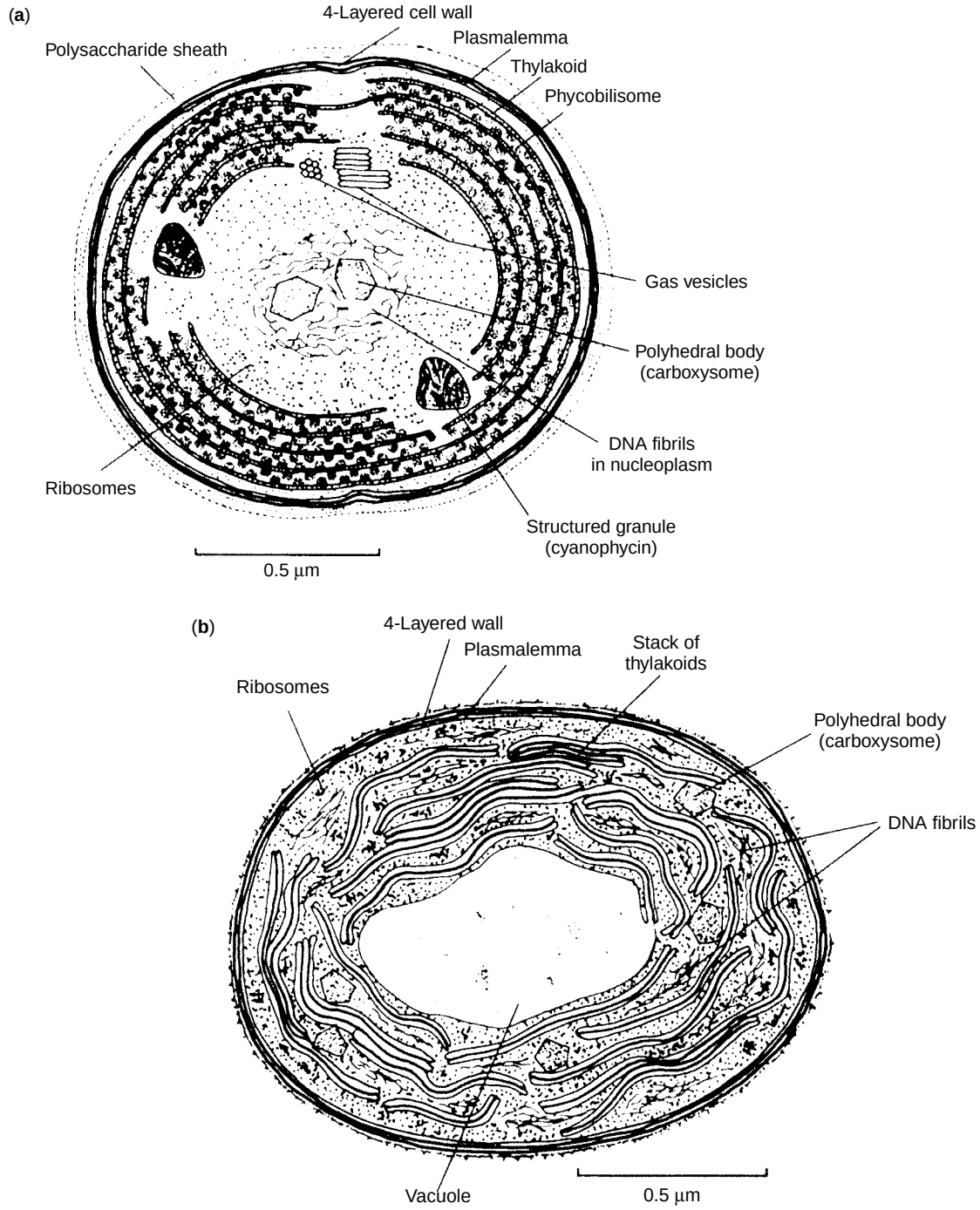

Figure 4. Diagrams comparing the ultrastructure of (**a**) the "blue-green" coccoid prokaryote, Synechocysis-tis and (**b**) the "green" coccoid prokaryote, Prochloron. Reprinted from (C. van den Hoek et al., *Algae—An Introduction to Phycology*, Cambridge University Press, Cambridge, U.K., 1998.) with permission from the authors and Cambridge University Press.

contrast, the green taxa have higher plant starchlike reserves with both amylose (β-1,6-linked polyglucan similar to amylose, and α-1,4-linked amylopectin) (12). The blue-green taxa are also strikingly distinct from the green prokaryotes in their pigment composition, especially the accessory pigments involved in photosynthesis. The blue-greens contain water-soluble phycobiliprotein pigments (the chromophores of which are called phycobilins) organized within complex, efficient light-harvesting structures called phycobilisomes [see following text; note that there is no structure of equivalent complexity known in (other) eubacteria]. The phycobiliprotein pigments are also highly significant in the ecology of these organisms (see following text). In contrast, the green prokaryotes

Table 2. Comparison of the "Blue-Green" and "Green" Prokaryotes with the Universal Plant Pigment, Chlorophyll *a*, and Oxygenic Photosynthesis. ***These organisms recently have been considered by some specialists (on the basis of molecular data, but ignoring pigment complement and certain distinct aspects of the fine structure which are regarded as major taxonomic characteristics in phycology) to be two subgroups within the cyanobacteria (1,12).***

Feature	Commonalities	Distinctions	
		"Blue-Green" Taxa	"Green" Taxa
Morphology	Coccoid, filamentous	Some colonial or with branched filaments	No colonial taxa, No filament branching
Color(s)	—	Blue, olive green,	Red bright green
Main photosynthetic pigment[a]	Chlorophyll *a* in thylakoids[b]	—	
Accessory pigments	—	Phycobiliproteins, in phycobilisomes; a few with chlorophyll *c* or *d*	Chlorophyll *b* (no phycobilisomes)
Light-harvesting proteins	Similar[c]	—	—
Major xanthophyll	Zeaxanthin	—	—
External mucilage (sheath)	Similar[d]	—	—
Cell wall[e]	4-layered, with structural murein	—	—
Gas vesicles	Present (some plankters)	—	—
Thylakoid arrangement[f]	—	Occurring singly, equidistant	Grouped (2-several per stack)
Reserve polysaccharides	—	Cyanophycean starch (polyglucan similar to glycogen)	Higher plantlike starch
Other reserves	Cyanophycin (N), polyphosphate bodies (P), lipids	Poly-β-hydroxy-butryic acid[g]	
DNA location	—	Concentrated in center of cell cytoplasm	Diffuse distribution throughout cytoplasm
Nitrogen fixation	?	Present in many	?

Note:
[a] Chlorophyll *a* is distinct from bacteriochlorophyll and chlorobium chlorophyll, the two chlorophylls found in other (nonoxygenic) photosynthetic bacteria (1).
[b] The exception is the blue-green prokaryote, *Gloeobacter violaceus* PCC 7421, which contains chlorophyll *a* but lacks thylakoids, suggesting that this taxon is relatively primitive (also supported by some molecular evidence) (1).
[c] The chlorophyll *a*/*b* binding proteins of these green prokaryotes are closely related to blue-green prokaryote light-harvesting proteins, rather than to eukaryote chlorophyll *a*/*b* proteins (1).
[d] A mucilaginous sheath is often present, composed primarily of complex polysaccharides with fibrillar appearance in electron microscopy (12).
[e] The cell wall is similar to that of gram-negative bacteria, and can be destroyed by lysozyme. The murein layers (peptidoglycan) typically is perforated by small pores with cytoplasmic extensions. The cell wall layers outside the murein layer consist mostly of lipopolysaccharides (12).
[f] Note, however, that thylakoid stacking has been documented in a genetically altered *Synechocystis* species lacking phycobilisomes, suggesting that thylakoids may stack when there is no spatial interference (here, phycobilisome structures) (25).
[g] Lipid droplets have been found in both blue-green and green prokaryotes; and several blue–green taxa have contained poly-β-hydroxybutryic acid (in vesicles ca. 200 nm in diameter; found in many [other] bacteria) (12).

have water-insoluble chlorophyll *b*, rather than phycobiliproteins, as their accessory pigment in photosynthesis.

HABITATS

General Distribution

The extant "blue-green" cyanobacteria are a diverse, ubiquitous group as mentioned. In contrast, only three monospecific genera of "green" prokaryotes with oxygenic photosynthesis are known (but note that *Prochloron* may possibly include two to three different "species") (2,15). *Prochloron didemni* was first described in the mid-1970s, and occurs as intra- and (mostly) extracellular symbionts of tropical and subtropical ascidians (sea squirts; temperature 21–31 °C). *Prochlorococcus marina* is an abundant picoplankter at 50 to 100 m depth in the open oceans, and was first detected in the early 1990s using flow cytometry (1,12). Two ecotypes have been determined (differing from one another by 2% in SSU rDNA sequence), one of which grows maximally at light levels that completely photoinhibit the other (1). *Prochlorothrix hollandica* is the only known filamentous "green" prokaryote and forms major blooms in shallow, eutrophic (nutrient-rich, highly productive) freshwater lakes of the Netherlands (12).

Cyanobacteria are widespread in neutral and alkaline waters, but do not thrive at pH less than or equal to 4. They often dominate the late summer plankton of temperature-stratified eutrophic lakes and slowly flowing lower rivers, where colonial coccoid and filamentous species such as *M. aeruginosa*, *Anabaena flos-aquae*, *Anabaena circinalis*, *Aphanizomenon* spp., *Cylindrospermum raciborskii*, and many others, form major water discoloration from noxious blooms up to 10^9 cells/mL in freshwaters worldwide (see following text) (2,5). Many of these species are capable of dinitrogen fixation, affording a significant competitive advantage over other phytoplankton. Many also produce potent toxins (see following text) that make them unpalatable to certain zooplankton grazers (2,5). The blooms commonly deplete dissolved oxygen concentrations from their high respiration during the night, causing massive fish kills (26). Filamentous and colonial coccoid cyanobacteria can also dominate the phytoplankton of shallow, polymictic, eutrophic reservoirs that sustain episodic, high sediment loading, alone or with phosphate (P) enrichment (27). Estuaries of Australia and in the Baltic Sea sustain massive blooms of the toxic cyanobacterium, *Nodularia spumigena* (28). Blooms of another filamentous, colonial cyanobacterium, *Trichodesmium thiebautii*, form "sawdust-like" patches in open-ocean blooms worldwide that were noted by Darwin and certain early explorers (29).

In the subsurface plankton of oligotrophic and mesotrophic lakes (with low and moderate productivity, respectively) and the oligotrophic oceans (to >100 m depth), cryptic, picoplanktonic cyanobacterial unicells (0.2–2.0 μm) contribute significantly to carbon production and ecosystem function (2). Their physiological ecology appears similar across oligotrophic freshwater and marine systems. In addition, picoplanktonic, coccoid unicellular cyanobacteria (*Cyanothece aeruginosa* and others) cover most lakes, slow-flowing rivers, and the oceans (2,30). And, colonial coccoid cyanobacteria (cell diameter 0.5–3.0 μm) are abundant in shallow eutrophic lakes (2) and turbid, eutrophic estuaries during summer. The abundance and major contributions of picocyanobacteria to the ecology of most of the aforementioned aquatic systems had been completely missed until the 1970s (30), and until the early 1990s their dominance of the summer plankton in turbid, eutrophic estuaries had been overlooked [e.g., Neuse Estuary, North Carolina, where this phenomenon was first reported by Fensin (31); Fig. 5; later by Paerl and coworkers (32)].

Within the benthic biofilm communities of oligotrophic lakes, pico-cyanobacteria (mostly coccoid colonial forms and unicells) can predominate throughout the growing season, contributing significantly to total community biovolume and cell number (Fig. 6; 33). These and/or larger cyanobacteria [including some toxic taxa (2)] can also become abundant in benthic habitats of eutrophic lakes and shallow, eutrophic rivers. Planktonic, filamentous cyanobacteria are stimulated to produce extracellular polysaccharides in response to episodic sediment loading, which results in coflocculation and settling to benthic habitats (34). There, they can thrive and provide an inoculum for other planktonic blooms (35;

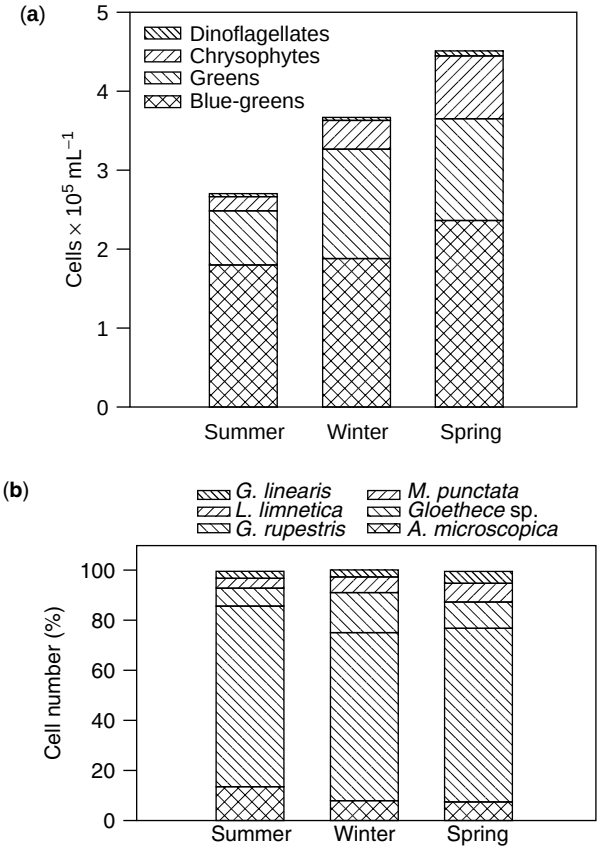

Figure 5. Cyanobacteria in the Neuse Estuary, North Carolina, United States, including (**a**) total abundance (designated as blue-greens) and (**b**) relative abundance (percentage of total phytoplankton cell number) of cyanobacterial taxa, with picoplankters including Gloeothece (G. linearis, G. rupestris, Gloeothece sp.) and Microcystis spp. (especially, M. punctata) contributing 50% or more of the total phytoplankton cell number during all seasons. From (E. E. Fensin, Population Dynamics of Pfiesteria-like *Dinoflagellates, and Environmental Controls in the Mesohaline Neuse Estuary, North Carolina, U.S.A.*, M.S. thesis, Department of Botany, North Carolina State University, Raleigh, N.C., 1997.) with permission of the author.

Fig. 7). Benthic *Phormidium* mats can be seasonally dominant or subdominant macroalgae in third-order, softwater streams of New England and the southeastern United States (36,37). Thus, cyanobacteria are important members of the flora of both open-water and benthic habitats in many aquatic ecosystems.

Success in Extreme Environments

Geothermal Springs. Hot springs, with characteristically high temperature in combination with high sulfide or acidic conditions, are scattered throughout all continents (except Antarctica, with fumaroles) and many islands, often separated by substantial distance. It has been hypothesized that there should be endemic species of thermophiles, restricted to certain hot spring clusters by geographic isolation and, perhaps, evolutionary divergence (2). Some of the major cyanobacterial taxa found in geothermal springs, such as *Cyanothece* spp., appear to

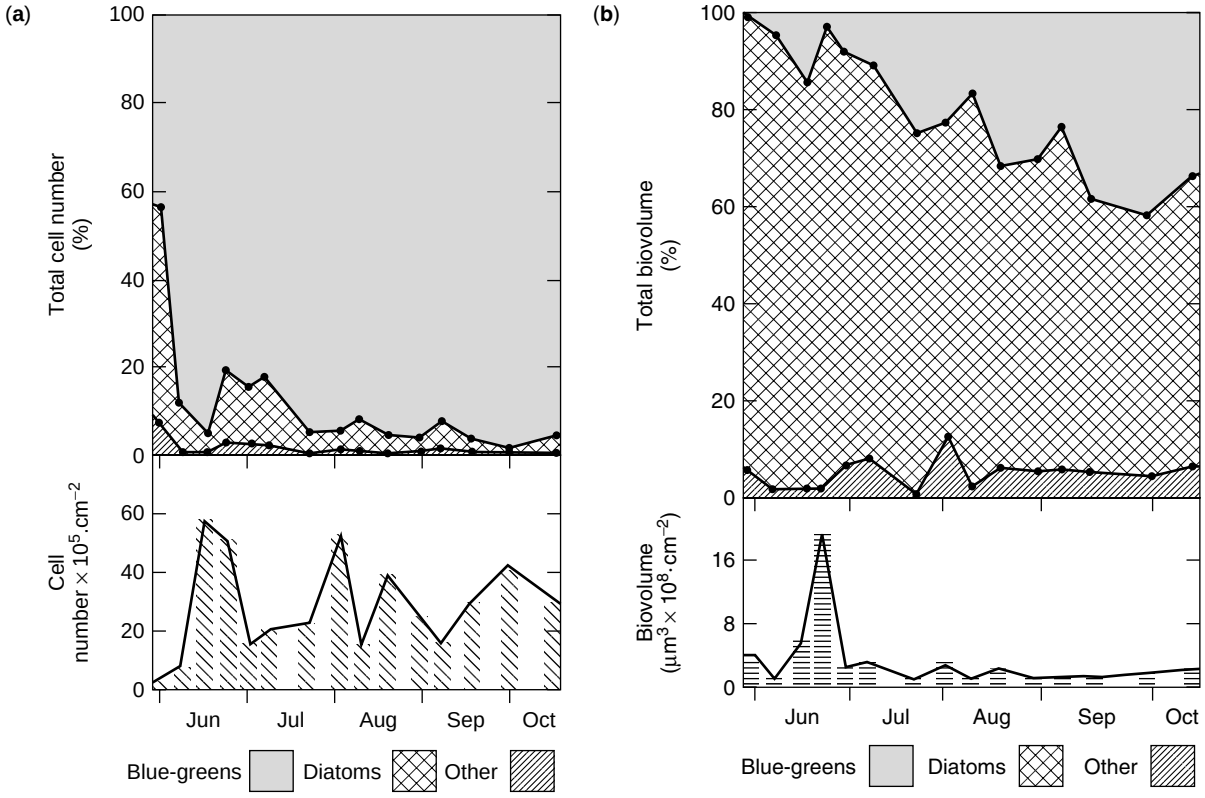

Figure 6. Epiphytic microalgae [here, including abundant pico-cyanobacteria as blue-greens; e.g., Gloeothece linearis, Cyanothece (Synechococcus) Aeruginosa, *Chroococcus minor*, *Aphanothece microspora*, *A. gelatinosa*, *A. nidulans*, and *C. minor*] on the outer leaves of *Scirpus subterminalis*, the dominant rooted, submerged plant in the littoral zone of a hardwater, P-limited, north temperate lake, including relative abundance (percentage of total phytoplankton) as (**a**) cell number and (**b**) biovolume. From (J. M. Burkholder and R. G. Wetzel, *Arch. Hydrobiol. Suppl.* **83**, 1–56 (1989).), reprinted with permission from E. Schweizerbart'sche Verlagsbuchhandlung.

be restricted by geographic distribution, whereas others such as *Mastigocladus* cf. *laminosus* and "*Chlorogloeopsis*" [taxonomy uncertain; formerly *Mastigocladus* HTF (high-temperature form)] are found in hot springs worldwide. The taxonomy of cyanobacteria from most geothermal springs is uncertain. These thermophilic cyanobacteria show distinct morphologies depending on environmental factors such as the chemical composition of the water, temperature, or irradiance (2). Within a taxon such as *Cyanothece*, there appears to be many different strains with ordered distributions along thermal and vertical gradients, suggesting an ongoing process of "speciation" (resulting from adaptive radiation of specialized ecotypes) or, at least, of development of distinct physiological races.

Temperature, pH, nitrogen supplies (especially the combined nitrogen or N_i available, usually as NH_4^+), and free sulfide are major factors controlling cyanobacterial species composition and abundance in geothermal springs (2). Hot springs taxa occur at temperatures ranging from about 50 to 74 °C, and are variable in free sulfide tolerance (range less than 0.05–3 mM). They are most common in near-neutral or alkaline conditions; species diversity becomes limited at pH below 6 and, as in other habitats, they have not been found below pH 4. For example, in areas where outflows of neutral to alkaline, nonsulfidic hot springs with combined nitrogen cool to about 74 °C, a mat of *Cyanothece* HTF may first encounter the spring water and remove most of the N_i. The downstream cyanobacteria at a temperature below approximately 58 °C may be dinitrogen fixers [e.g., *Mastigocladus* (*Fischerella*) cf. *laminosus* or HTF "*Chlorogloeopsis*" at temperature below approximately 64 °C, or *Calothrix* at temperature below approximately 50 °C). Or, a hot spring may be sufficiently nitrogen-rich to be dominated by *Cyanothece* without dinitrogen fixers, or so nitrogen-poor that only dinitrogen fixers occur. Thermophilic cyanobacteria that can grow at temperatures above 56 °C cannot tolerate more than about 10-μM sulfide, but spring water usually is depleted in sulfide once the outflow temperature reaches 52 °C. In some hot springs (e.g., Iceland), sulfide-oxidizing photosynthetic bacteria on the mat surface remove sulfide and thus allow underlying cyanobacteria to grow. Filamentous thermophilic taxa can move 5 to 6 μm/sec in response to changing light and temperature, and the mats move to avoid slow changes in stream temperature. Limited movement (0.1–0.3 μm/sec) has also been shown by coccoid unicells in positive phototaxis (2).

Thermophilic cyanobacteria have intensive localized photosynthesis and high photorespiration, with the majority of photosynthate stored as polyglucose rather

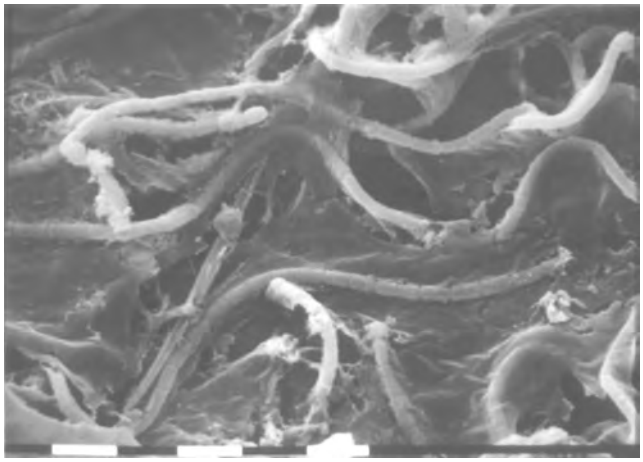

Figure 7. Scanning electron micrograph of the benthic microalgal community from shallow waters (depth 0.25–0.5 m) of a reservoir in the Piedmont region of the southeastern United States, showing abundant Anabaena filaments among a mucilaginous matrix (white bar equals 10 μm). Cyanobacteria dominated the shallow waters of experimental enclosures that had received episodic sediment loading for about 11 weeks over the summer growing season (kaolinite or montmorillonite clay, added at two-day intervals to effect an initial concentration of 25 mg suspended solids/L after additions), alone or with phosphate enrichment (mean $PO_4^{-3}P$ from 45–115 μg/L). From (J. M. Burkholder and B. E. Cuker, *J. Phycol.* **27**, 373–384 (1991).), reprinted with permission from the *Journal of Phycology*.

than as substances needed for growth. Photoexcretion of glycolate (up to 60% of the photoexcreted carbon, and 10% or more photosynthetically fixed carbon) and dark fermentation of polyglucose lead to a diel cross-feeding of most fixed carbon to heterotrophs (2). Under varying light intensities, thermophilic cyanobacteria isolated from hot springs may also excrete fructose, adonitol, oxalate, fumarate, succinate, acetate, formate, propionate, and/or β-hydroxybutryate. Volatile fatty acid products of fermentation accumulate in darkness, and are later taken up by filamentous photoheterotrophs.

Cold, Desiccation, High UV, and Other Extremes. Cyanobacteria are the predominant biota in cold environments such as ice shelves, glaciers, glacial meltwater streams, ice-capped lakes, and recently exposed glacial moraines; and they are also abundant in tundra and polar desert soils, where they are important in nitrogen and carbon cycles (2). In lower portions of polar glaciers and on alpine glaciers in temperate regions, black, mucilage-producing cyanobacteria (e.g., filamentous *Calothrix parietina*, *Phormidium frigiddum, Lyngbya martensiana, Microcoleus paludosus* var. *acuminatus, Nodularia harveyana*, and coccoid *Cyanothece aeruginosa*) can form "cryconite" (cold rock dust) by trapping dust, and the resulting dark patches absorb radiation so that the local heat balance is affected and holes, ponds, and streams are produced. The black coloration is from UVR-screening black carotenoid pigment, scytonemin, found in certain cyanobacteria of polar, high-light, and high-temperature environments. Cyanobacterial colonizers of rocks in Arctic and Antarctic streams and the surfaces of some lakes and ponds are rich in carotenoids (e.g., canthaxanthin, myxoxanthophill that neutralize reactive oxygen species) and other UV-A/B absorbing pigments such as mycosporine-like amino acids with maximal absorption at 320 to 335 nm [e.g., asterina-330, shinorine, phorphya-334, palythene; (2)].

In freshwaters and saline lakes of both polar regions, cyanobacteria can accumulate to benthic mats up to 90-cm thick, with more than 40 μg chlorophyll a/cm^2. Picocyanobacteria often dominate the autotrophic plankton of polar and subpolar freshwater and saline lakes, where they are favored because they are eurythermal (withstanding temperatures down to $-12\,^{\circ}C$ and grow from $5–35\,^{\circ}C$); tolerate desiccation, freezing, and salinity stress (e.g., a brine covering with sixfold higher salinity than seawater); have various adaptive strategies against UV radiation and high solar radiation in exposed habitats; and can shade-acclimate to allow net growth in dim-light environments (2). For example, some Antarctic mat-forming cyanobacteria were completely desiccated within five hours of being exposed to the atmosphere, but photosynthesis and respiration occurred within 10 minutes of rewetting. Thus, in many polar environments they grow slowly but gradually accumulate over many seasons, with only minor losses from biotic and abiotic removal processes (2). In polar oceans, however, cyanobacteria are rare or absent, probably because depressed growth rates at cold temperatures and low light are insufficient to balance losses from grazing, advection, and mixing (2).

Cyanobacteria synthesize compatible solutes to counteract osmotic stresses from freezing and high salinity; and they accumulate trehalose as a water replacement mechanism to maintain the functional integrity of membranes during desiccation. Freshwater cyanobacteria accumulate sucrose in response to osmotic stress; marine taxa accumulate glucosylglycerol, sometimes with trehalose and sucrose; and hypersaline taxa accumulate betaine (and, secondarily, sucrose) (2). Although typically a single, low-molecular-weight organic solute is synthesized in response to osmotic stress, many cyanobacteria produce a secondary compound (e.g., disaccharide), which may assist in rapid response. Cyanobacterial/microbial mats often evolve dimethylsulfide (DMS), produced from DMSP, which can serve as a secondary cryoprotectant.

Endolithic Habitats. Cyanobacteria penetrate porous, translucent rocks to provide the primary-production basis of endolithic communities across an extreme temperature gradient from hot to cold polar deserts. It is hypothesized that the endolithic habit enables survival under extreme osmotic, thermal, and UV or light stress; and, for cold-desert forms, cryoprotection is additionally afforded. In deserts, various translucent rock types contain cyrptoendolithic cyanobacteria (*Gloeocapsa, Chroococcus turgidus, Chroococcidiopsis*) (2). Daily rock temperatures can vary from 22 to $48\,^{\circ}C$; relative humidity above the rock decreases sharply during the day (e.g., from 47 to 24%); and rock moisture content can range from 0.05 to 1.12% (2). The spaces between the outermost rock grains are filled with mineral material ("desert varnish") that is permeable to liquids and gases, but not penetrable by

cells. The cyanobacteria attach to grains beneath this outer layer. Physiological studies on endolithic isolates, *Chroococcidiopsis* sp. and *Nostochopsis lobatus* in a hot desert in South Africa, indicated that light saturation of photosynthetic e^- transport occurred at a similar light intensity (200–350 μmol m^{-2} sec^{-1}) as for the natural community in full sunlight (20–250 μmol m^{-2} sec^{-1} in full sunlight, or 1–10% of I_o). Despite capability for substantial photosynthetic C_i fixation by endolithic cyanobacteria, carbon turnover is probably very slow. Nitrogen is mostly supplied abiotically as nitrate and as ammonia from atmospheric deposition, with little evidence for dinitrogen fixation (2).

LIGHT, PHOTOSYNTHESIS, AND ORGANIC CARBON UPTAKE

Pigments

As mentioned, the green prokaryotes have chlorophyll *b* as their main accessory pigment. Chlorophyll *b* can be formed from a chlorophyll *a* precursor by a single step that involves an oxygen-requiring enzymatic conversion of a methyl to a formyl group (1). Although this has been described as an "easy" step (1), the chlorophylls are among the most taxonomically reliable pigments, and they do not readily interconvert. On the basis of molecular evidence, it has been hypothesized that chlorophyll *b* independently evolved at least four times [represented by three "green prokaryote" or prochlorophyte lineages (see following text), and by the ancestor of green algal and higher plant plastids; 38,39]. Note, in addition, that an accessory chlorophyll *c*-like pigment has been reported in the green prokaryotes, *Prochlorococcus* (40) and *Prochloron* (41). Because these two taxa are not considered closely related on the basis of certain molecular data, it has been further hypothesized that it arose independently in both. Moreover, chlorophyll *d* (found in some red algae) was recently discovered to be the major photosynthetic pigment in the blue-green *Acaryochloris marina*, an organism that has very little chlorophyll *a* and lacks phycobiliprotein pigments (42).

Like eukaryote algae and higher plants, cyanobacteria have a suite of carotenoids such as β-carotene and zeaxanthin, which act as accessory pigments by increasing the harvest blue wavelengths of light that cannot be directly absorbed by chlorophyll *a*, for use in photosynthesis [Fig. 8; (1)]. UVB (280–320 nm) and UVA (320–400 nm) damage molecular targets directly or indirectly via production of reactive oxygen metabolites. The carotenoids partially protect cyanobacteria from UV radiation and harmful photooxidation, as indicated. Their association with chlorophyll also prevents formation of highly reactive oxygen radicals that could otherwise damage the chlorophyll(s), lipids, proteins, DNA, and other molecules (42). Carotenoids and accessory chlorophylls absorb only about one-third of light in the [bluish-]'green window' that cannot be directly absorbed by chlorophyll *a* for use in photosynthesis. That is, they fill a gap between available light energy and the absorbance capability of chlorophylls (25). But the greatest green light-harvesting capability occurs among the blue–green

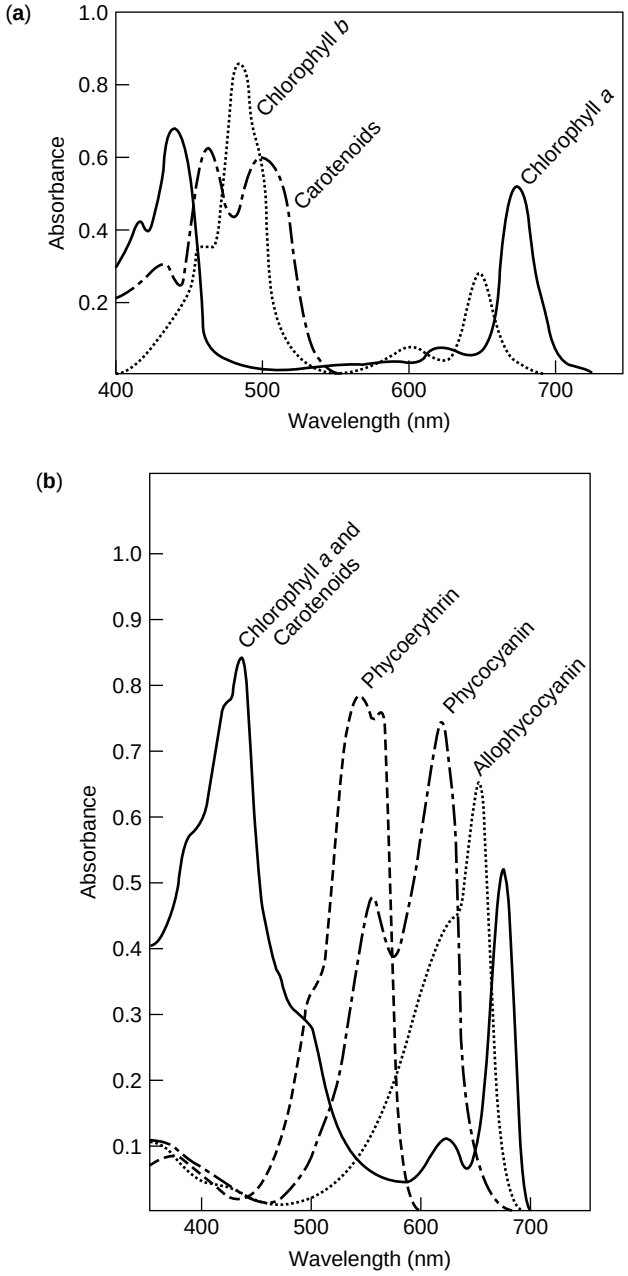

Figure 8. Absorption spectra of light-harvesting pigments in cyanobacterial photosynthesis, including (**a**) the chlorophylls a and b and carotenoids and (**b**) phycobiliproteins. Modified from Gantt (1975) by Graham and Wilcox (1); reprinted from (L. E. Graham and L. W. Wilcox, *Algae*, Prentice-Hall, Upper Saddle River, N.J., 2000) with permission from the authors and Prentice-Hall.

prokaryotes, which are afforded significant ecological advantage over most other aquatic organisms with oxygenic photosynthesis because of their phycobiliprotein pigments (Figs. 8 and 9).

Living and dead organic matter absorb red light, a low-energy photosynthetically active radiation (PAR) that is rapidly removed near the water surface and dissipated or

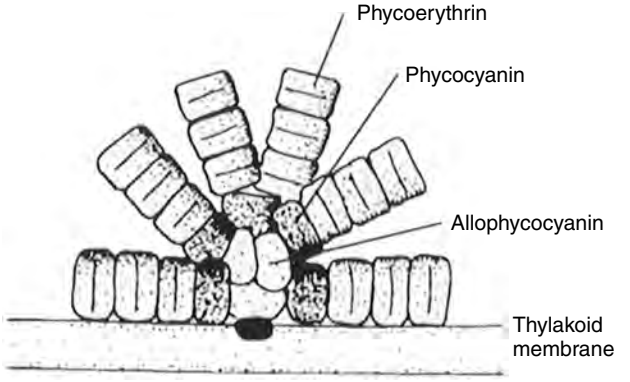

Figure 9. The ultrastructure of a hemispherical phycobilisome in cyanobacteria, showing exterior-most phycoerythrins (absorption and fluorescence maxima at approximately 545 nm and 575 nm, respectively), phycocyanin internal to the phycoerythrins (absorption and fluorescence at approximately 555–618 nm and 636 nm, respectively), allophycocyanin internal to the phycocyanin (absorption and fluorescence maxima at approximately 650 nm and 660–675 nm, respectively), and chlorophyll *a* internal to the allophycocyanin [absorption and fluorescence maxima at approximately 670 nm and 685 nm, respectively; (1)]. Hemidiscoidal phycobilisomes are similarly constructed. Note that not all blue–green prokaryotes with oxygenic photosynthesis have all three types of phycobiliprotein pigments, and that phycoerythrins do not appear to be essential for phycobilisome formation. From C. van den Hoek et al., *Algae—An Introduction to Phycology*, Cambridge University Press, Cambridge, U.K., 1998. Reprinted with permission from Cambridge University Press.

lost as heat (25), and thus effectively compete with chlorophylls. The PAR highest in energy is blue light, which is also absorbed by living and dead organic matter and by water molecules. Within the visible spectrum, especially in waters with moderate to high productivity (and moderate to high organic matter content near the surface), mid-energy wavelengths of light of greenish-yellow color penetrate to greatest depths (25). The phycobiliprotein pigments are capable, through their relatively intricate packaging in phycobilisomes (Fig. 9); of absorbing virtually the entire green window and transmitting the light energy via fluorescence to chlorophyll *a* for use in oxygenic photosynthesis. Thus, they can grow in low-light habitats with insufficient light for most aquatic plants (here, including algae). Phycobilisomes (diameter ca. 40 nm) can form up to 25% of the dry weight and 40% of the total soluble protein of cyanobacterial cells (1). Their structure maximizes light energy transfer to chlorophyll *a*, with up to approximately 90% energy-transfer efficiency (1). In addition to this remarkably efficient pigment complement, some cyanobacteria are capable of chromatic adaptation, wherein they adjust their pigment composition in response to changing light quality. They increase phycoerythrin synthesis in green light (becoming red in color) and increase phycocyanin synthesis in red light (becoming blue in color), which affords adaptive advantage in aquatic systems characterized by a changing light environment (25). The genes that regulate cyanobacterial chromatic adaptation may be controlled by sensor kinases and they encode proteins that are similar to bacterial response regulators, portions of the light-sensing phytochrome pigment of higher plants, and portions of higher plant ethylene receptor proteins (1).

Oxygenic and Anoxygenic Photosynthesis

Cyanobacteria have the typical photosystems I and II (except within heterocysts; see following text) for inorganic carbon (C_i) fixation, but their Rubisco has low affinity for carbon dioxide relative to most other oxygenic photosynthetic organisms, and they are extremely vulnerable to the effects of photorespiration [function of Rubisco as an oxygenase rather than a carboxylase under increasing oxygen; (43,44)]. Cyanobacteria are able to achieve high rates of photosynthesis in oxygen-rich environments because they can use both carbon dioxide and HCO_3^- as sources of C_i and because they have developed a highly efficient, although still poorly understood, C_i-concentrating mechanism. Cyanobacteria take up carbon dioxide, when available, by passive diffusion or active transport (enzyme as yet unknown). Experimental evidence also indicates that they can utilize HCO_3^- as a C_i source, which they take up using a membrane-bound transporter or "pump" (as yet uncharacterized). Bicarbonate is much more readily available than carbon dioxide in neutral to alkaline habitats where cyanobacteria mostly occur (2,44). Although HCO_3^- uptake would require energy, HCO_3^- would be more easily retained than carbon dioxide, which can readily diffuse back out of cells. Cyanobacteria use carbonic anhydrase (CA) to convert HCO_3^- to carbon dioxide, the substrate used by Rubisco. Both CA and Rubisco are colocalized within carboxysomes, which facilitates capture of CA-generated carbon dioxide by Rubisco before the carbon dioxide is lost from the cell by diffusion (45,46).

Although oxygenic photosynthesis predominates in cyanobacteria, some taxa can also conduct anoxygenic photosynthesis (involving only photosystem I) in anaerobic environments with sufficient light and appreciable reduced substrates such as hydrogen sulfide (H_2S), hydrogen gas (H_2), or organic compounds as electron donors. (2) Heterocysts conduct anoxygenic photosynthesis, using substrates from adjacent vegetative cells (see following text). Anoxygenic photosynthesis is also common in microbial mat cyanobacteria, mostly via use of sulfide as the electron donor: $12H_2S + 6CO_2C_6H_{12}O_6 + 12S^o + 6H_2O$. Mats formed by cyanobacteria generally have a surface layer of sand or scytonemin, then a layer of cyanobacteria, an underlying layer of oxidized iron, then a layer of purple sulfur bacteria (rarely underlain by a layer of green sulfur bacteria), and a bottom layer of FeS. The internal mat environment is characterized by steep gradients in light, oxygen, and sulfide. An alternate mode of anoxygenic photosynthesis occurs in the mat former, *Microcoleus chthonoplastes*, which undergoes oxygenic and anoxygenic photosynthesis concurrently. In the latter, it produces thiosulfate from sulfide oxidation, a process that yields twofold more electrons and, thus, appears to be twice as efficient as S^o production by other cyanobacteria (2).

Cyanobacteria have been subdivided into four general groups in degree of sulfide tolerance and potential for sulfide-dependent anoxygenic photosynthesis (2).

Group 1 cyanobacteria (sulfide-sensitive oxygenic photosynthesis only) are extremely sulfide-sensitive. These species mostly inhabit freshwater lakes and terrestrial environments low in sulfide. They cannot conduct anoxygenic photosynthesis and their oxygenic photosynthesis is inhibited at low sulfide levels (less than 0.1 mM). Group 2 cyanobacteria (sulfide-resistant oxygenic photosynthesis only) are much more resistant to sulfide, with oxygenic photosynthesis stimulated at moderate (less than 1 mM) sulfide levels characteristic of marine microbial mats with fluctuating sulfide concentrations. However, these organisms are incapable of anoxygenic photosynthesis. Group 3 cyanobacteria occur in similar habitats as group 2, but conduct sulfide-dependent anoxygenic photosynthesis concurrently with sulfide-insensitive oxygenic photosynthesis. Group 4 cyanobacteria are variably sensitive to sulfide (depending on the taxon, sulfide tolerance ranges from less than 1 to 10 mM) but conduct sulfide-dependent anoxygenic photosynthesis rather than sulfide-sensitive oxygenic photosynthesis. The freshwater bloom former, *Oscillatoria limnetica* from anaerobic H_2S-rich bottom waters of a hypersaline lake, switches off photosystem II when exposed to less than 0.1 mM sulfide, and anoxygenic photosynthesis is induced in a process requiring protein synthesis (2). This organism tolerates up to 9.5 mM sulfide, but anoxygenic photosynthesis is inhibited at more than 4 mM sulfide. The excreted elemental sulfur forms granules on the filament surface, which are believed to be of physiological use (see following text). Cyanobacteria are believed to have been anoxygenic phototrophs before they evolved oxygenic photosynthesis.

Respiration and Heterotrophy

Probably as a remnant from their evolution under anaerobic conditions, some (more likely, many) cyanobacteria use one or more fermentation pathways in which they process cyanophycean starch or other substrates in anaerobic respiration when oxygen is not available. Some fermentation pathways are energetically efficient in utilizing the organisms' accumulated osmoprotectant compounds as substrates. For example, some taxa (e.g., *O. limnetica*, several strains of symbionts, *Anabaena azollae*, *Nostoc* spp.) have homoacetate fermentation, which uses the osmoprotectant, trehalose, as substrate. When oxygen is absent, aerobic respiration apparently is limited or does not occur; many cyanobacteria die, and lysis occurs within two to three hours after transfer to dark anoxic conditions (2,47).

Many cyanobacteria, like many eukaryote algae, are capable of uptake of organic carbon (C_o) substrates and of heterotrophic growth in darkness using C_o substrates or in the light via photoheterotrophy. Substrates taken up by cyanobacteria have included D-glucose, D-fructose, D-ribose, sucrose, acetate, various amino acids, and glycerol, whereas D-galactose, maltose, cellobiose, mannitol, and D-xylose were poorly used (1,2). Where heterotrophic growth in darkness has been documented, rates were significantly lower than that for photoautotrophic growth (1,2).

Although there have been reports that cyanobacteria can contribute to the oxidation and degradation of hydrocarbons, in most cases, the cultures may not have been bacteria-free (2). Cyanobacteria immobilize oil-degrading bacteria and fungi in their outer mucilage, and have probably done so through geologic time (2). Following the largest known oil spill (by the Iraqis, into the Arabian Gulf adjacent to Kuwait, August 1990 to February 1991), visually obvious cyanobacterial mats developed over the oil layers that had drifted to cover approximately 770 km of the Saudi Arabian coast, in areas without sign of other macroscopically visible forms of life (47). Mats with strongly adhering oil layers consisted of associations of two filamentous cyanobacteria, *M. chthonoplastes* and *Phormidium corium*, with up to 10^6 oil-degrading bacteria per gram fresh weight of their sheaths. Apparently, the cyanobacteria afforded the bacteria benefit by preventing them from washing out to sea, providing oxygen, and perhaps providing fixed nitrogen as well, although their role in utilizing the oil as an organic substrate was probably indirect.

Cyanobacteria can degrade and, to varying extents, utilize other potentially toxic organic substrates as well. For example, they can mediate oxidation of naphthalene (common water-soluble pollutant) to 1-naphthol, naphthalene-1,2-oxide, and possibly other substrates, apparently via monooxygenase and dioxygenase systems (2). A monooxygenase system appears to be involved in initial oxidative attack by a cyanobacterium on phenanthrene as well. Some species oxidize 1- and 2-methyl naphthalene at the methyl group, through hydroxylation of the alkyl side chain, and oxidize biphenyl, mostly to 4-hydroxybiphenyl (2). Two autotrophically grown cyanobacteria were shown to metabolize aniline (aromatic amine used in industry for production of dyes, pesticides, and pharmaceuticals) to formanilide, acetanilide, and p-aminophenol. The cyanobacteria, *M. chthonoplastes* and *Phormidium corium*, isolated from oil-rich sediments, were incubated with crude oil or n-alkanes, and growth occurred although the alkanes were oxidized to produce increased proportions of total unsaturated fatty acids with the equivalent chain lengths in their total lipids, an effect analogous to that in hydrocarbon-utilizing bacteria and fungi. Chlorinated cyclic aliphatic hydrocarbons (e.g., lindane) can be oxidized by cyanobacteria (*Anabaena* sp., *Nostoc ellipsosporum*) (2), but the extent to which they utilize such compounds is not known.

Vertical Migration in Benthic Mats

Cyanobacteria use gliding motility to migrate downward into the lower mat or underlying sediment during periods of high light (late morning to late afternoon, summer season) and up to the surface in late afternoon or at sunset. The downward movement is mostly attributed to negative phototaxis, but in soil, may also be in response to higher nutrient concentrations at depth (2). For some species, the cue for daytime vertical migration is the intensity of solar irradiance, especially in the UVR region. The downward movement enables avoidance of high UVR radiation exposure, thereby preventing photoinhibition and photodestruction. Alternatively, some migrating species move to (low) optimum light intensities for photosynthesis. Upward movement may be controlled by chemical factors, for example, movement toward

increasing dissolved oxygen and/or movement away from increasing sulfide (but note that some cyanobacteria are incapable of movement in the dark after a short period, probably after photosynthetic energy reserves are depleted). Light probably does not control upward movement as light would be scattered so that a directional influence would be lost at depth within a microbial mat or in the soil (48).

The geothermal springs taxon, *Oscillatoria terebriformis*, exhibits different behavior. It makes a first migration downward during the high-light period and moves up to the surface in late afternoon, then makes a second migration down into the lower mat or underlying sediment after dusk and returns to the surface in the early morning (2,2). It shows positive phototaxis toward low light and negative phototaxis toward high light, controlling its movement downward during the day. In darkness, it continues random movement until it reaches the sulfide layer. Sulfide (0.7 mM or more) inhibits motility in darkness, and light reverses the inhibition (2,2). During the day, the sulfide layer is displaced downward, relieving inhibition of mobility so that the organism moves up toward the light to the surface of the mat. At high light intensity during midday, it becomes negatively phototactic and moves deeper into the sediment. At night it becomes trapped in the sulfide layer, which is essential for its survival through the dark period. In aerobic conditions, dark respiration would rapidly deplete its carbohydrate reserves, leading to death within several hours (2,2). In the anoxic sulfide layer, *O. terebriformis* undergoes the slow process of fermentation, enabling an extended period to generate sufficient energy to cover its metabolic maintenance needs.

OTHER NUTRITIONAL ECOLOGY

Nitrogen

Nitrogen, used in amino acids, nucleotides, chlorophylls, and phycobiliprotein pigments, and some energy compounds, can form up to approximately 10% of cyanobacteria cells (dry weight basis) (2). The preferred nitrogen form by most cyanobacteria for uptake is NH_3–NH_4^+ (especially at low light intensities) $> NO_3^- \gg N_2$ (49). When NH_4^+ is available (nonlimiting), cyanobacteria do not assimilate alternative sources (50). As in other organisms with oxygenic photosynthesis, nitrogen metabolism is closely connected to inorganic carbon (C_i) fixation because both processes compete for energy and reductant generated by the light reactions of photosynthesis. Assimilation of carbon dioxide to carbohydrate requires four electrons (e^-), whereas synthesis of amino-N from nitrate requires 10 e^-, and at a cellular C/N of approximately 5, up to 50% of the reductant generated by the light reactions is used for nitrogen assimilation (51,52). Nitrogen assimilation can be a carbon "drain" because substantial carbon is required for amino acid synthesis so that nitrogen assimilation influences the rate of carbon dioxide fixation, the partitioning of the fixed carbon, and the production/maintenance of carbohydrate reserves — with major effect, in turn, on cell growth, cell turgor pressure, and cell density/buoyancy (51). Inorganic nitrogen (N_i) as ammonia is taken up by passive diffusion or, as the protonated form NH_4^+, by a specific uptake system; and nitrate is taken up by another specific uptake system (involving the enzyme nitrate reductase, which includes iron and molybdenum as cofactors), and then reduced to ammonia using ferredoxin as an e-donor (thus, the process is closely linked to photosynthesis). Organic nitrogen (N_o) sources such as amino acids arginine, asparagine, and glutamine can be taken up by cyanobacteria (2). Cyanobacteria "luxury-consume" nitrogen, or take up nitrogen greatly in excess of the cell's metabolic needs, for storage as cyanophycin and later use when N_i and labile N_o substrates become depleted (e.g., in eutrophic waters late in the growing season; in many estuarine and marine waters; within the dense, compacted biomass of microbial mats and other biofilm layers, etc.).

Nitrogen gas (N_2) in air and water is much more abundant than N_i or labile N_o, forming approximately 70% of the atmosphere and approximately 95% of the nitrogen in the oceans (1). Cyanobacteria are the only diazotrophs, or N_2-fixing organisms, with oxygenic photosynthesis, affording a major advantage over other algae in usable nitrogen supplies. In nitrogen fixation, dinitrogen is converted or "fixed" into N_i (NH_3) by the following reaction, which is energy-costly and catalyzed by the enzyme, nitrogenase (Nase):

$$N_2 + 8\,e^- + 8H^+ + 16\text{MgATP} \xrightarrow{\text{Nase}} 2NH_3 + H_2(g) + 16\text{MgADP} + 16P_i$$

In cyanobacterial taxa with this capability, N_2 fixation is induced by low levels of N_i, especially NH_4^+, in the external medium. The key enzyme involved, Nase, consists of a molybdenum (Mo)-iron (Fe) protein, several Fe-sulfur clusters, a Mo-Fe-S cofactor (or, in one form of cyanobacterial Nase, vanadium) and an Fe-protein (1). Cyanobacteria actually have two Mo-dependent Nase forms, one that is operative in vegetative cells in anaerobic environments and another that is operative in heterocysts (specialized cells that reduce access of oxygen to Nase) in anaerobic or aerobic external environments.

Nitrogenases are extremely sensitive to oxygen and function only in anoxic microzones (e.g., within colonies insulated from the external aerobic environment by thick mucilage or within benthic mats and other biofilm layers) or anaerobic habitats. Rates of dinitrogen fixation are elevated in some (mostly heterocystous) cyanobacteria during the light period because of increased available (photosynthetic) energy for the process, other taxa fix dinitrogen only at night when oxygen is not evolved from photosynthesis. Other mechanisms have also been found; for example, the unicellular cyanobacterium, *Cyanothece*, downregulates photosystem II during dinitrogen fixation so that little oxygen is produced for several hours (52). The colonial marine plankter, *Trichodesmium*, is unusual among nonheterocystous taxa in its ability to conduct oxygenic photosynthesis concurrently with dinitrogen fixation during the day, which is accomplished by physical separation of dinitrogen fixation and carbon fixation (1,2,29). Similarly, some nonheterocystous taxa colonizing cave walls and rice paddy fields can conduct

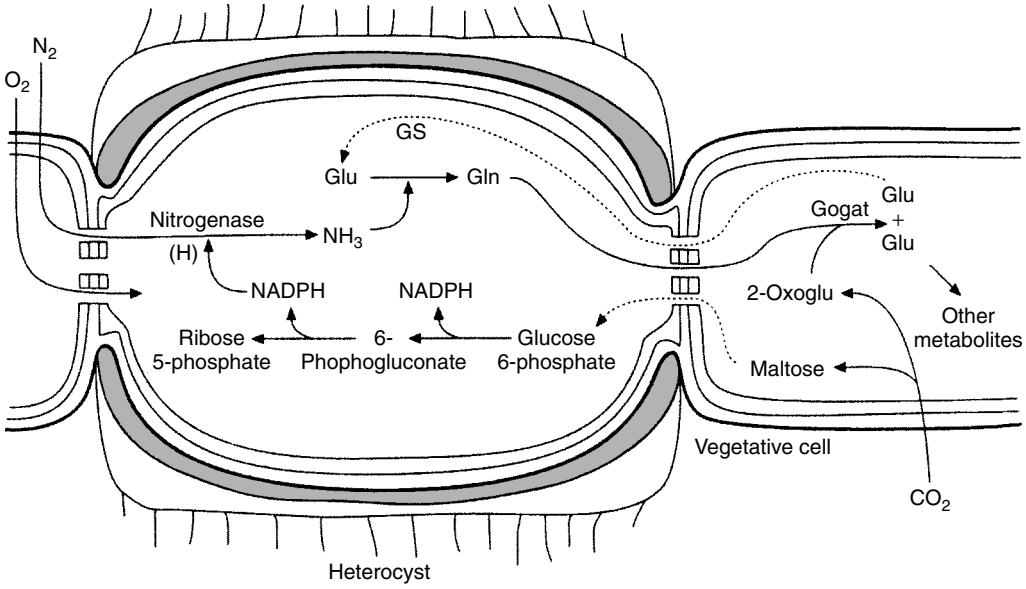

Figure 10. Diagram of a cyanobacterial heterocyst and adjacent vegetative cells, showing the relationship between photosynthetic and N_2-fixation metabolism. The thick walls of heterocysts reduce diffusion of oxygen (and N_2) inward. Thus, entry of these gases occurs via vegetative cells and small pores or channels (micro-plasmodesmata) in the cell walls connecting heterocysts and vegetative cells. These pores also allow movement of fixed nitrogen (Gln = glutamine) from heterocyts into vegetative cells. The GOGAT enzyme system (glutamate synthase (glutamine-oxoglutarate amidotransferase regenerates glutamate, which diffuses into heterocysts. Addition of NH_3 to glutamate is accomplished by glutamine synthase (GS). The NH_3 origi-nates from the activity of nitrogenase, using NADPH (reducing equivalents) generated from carbohydrates such as maltose or glucose-6-phosphate that originated, in turn, from photosynthetic carbon dioxide fixation in the vegetative cells. After Haselkorn, 1978, from (L. E. Graham and L. W. Wilcox, *Algae*, Prentice-Hall, Upper Saddle River, N.J., 2000.) with permission from the authors and Prentice-Hall.

oxygenic photosynthesis concurrently with dinitrogen fixation (2).

Heterocysts, found in certain filamentous cyanobacteria in lakes, estuaries, and the marine high-intertidal zone (but rarely elsewhere in marine habitats), enable enhanced spatial separation of dinitrogen fixation and oxygenic photosynthesis (Fig. 10; 1). These specialized cells are often covered by thick mucilage, helping to create a low-oxygen environment. The microanaerobic condition is enhanced by respiration from aerobic bacterial colonizers, attracted by amino acids that leak from the specialized, N-enriched cells. The thick heterocyst walls provide a diffusion barrier for gases and limit oxygen entry. In addition, heterocysts have photosystem I but not photosystem II; hence, carbon fixation and oxygen production do not occur in these cells. Carbohydrates are imported from adjacent vegetative cells for respiration, needed to generate ATP and reducing equivalents. Microquantities of oxygen are scavenged by intra-heterocyst respiration, and by reaction of H_2 (produced by Nase, which also functions as a hydrogenase) with oxygen to form water.

Diazotrophic cyanobacteria are capable of using dinitrogen as their sole nitrogen source for growth. These organisms contribute most of the planktonic dinitrogen fixation in freshwaters, which represents 6 to approximately 80% of the nitrogen load in some eutrophic lakes) (53); and even when dinitrogen fixation represents a small contribution of the nitrogen loading to a lake as a whole, dinitrogen fixation is of major significance to the success of bloom-forming taxa. Most marine cyanobacterial mats that have been examined also had high dinitrogen fixation (2). Cyanobacterial dinitrogen fixation directly (via leakage of ammonia or amino acid products from cells) or indirectly (after cell death, lysis, or decomposition, and release of labile N_o and N_i) contributes a significant portion of the nitrogen budget for some freshwater rivers and lakes, the high rocky intertidal, major areas of the oceans (e.g., the Sargasso Sea), benthic mat environments, and various other aquatic habitats (1,2,28,53). Through this important metabolic process, cyanobacteria are significant suppliers of nitrogen to N-limited freshwater, estuarine, and marine communities. dinitrogen fixation also is a major factor enabling certain planktonic cyanobacteria to form noxious blooms in freshwater lakes, lower rivers, and certain estuaries (1,2); see following text). Even in the open oceans, cyanobacterial dinitrogen fixation has been increasingly recognized as important to the oceanic nitrogen cycle. For example, although filamentous *Trichodesmium* spp. have been known to be important, widely distributed dinitrogen fixers in the open oceans (29), recent research has shown that unicellular cyanobacterial dinitrogen fixation (e.g., by *Synechocystis* spp. and *Cyanothece* spp.) is widespread in the subtropical North Pacific Ocean (54).

Phosphorus

Phosphate is essential to all life, required for production of genetic information (DNA), "energy currency" molecules, ATP and ADP, and phospholipids used to form membranes. About 0.6% of cyanobacterial cells (dry-mass basis) is P, excluding polyphosphates (2). Phosphate ion PO_4^{-3}, the P form readily taken up by cyanobacteria, can be liberated from insoluble materials (e.g., hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$; strengite, $FePO_4 \cdot 2H_20$) by microbial activity, including production of organic acids and chelators, dissimilatory reduction of ferric iron, and production of sulfide (2). P can occur in other oxidation states (from +5 to −3) but, unlike N and S, it is not important in redox reactions as bacteria rapidly oxidize any reduced P under both anaerobic and aerobic conditions (2). Thus, the microbial P cycle basically consists of P_i-PO_4^{-3} uptake and its release by excretion or autolysis of P_o-phosphate, which is then mineralized by acid and alkaline phosphatases. Once released into the surrounding medium, soluble reactive phosphate (SRP, or dissolved P_i) is recycled or taken up again within seconds (25), and measured concentrations are often negligible in fresh- and marine waters. Pulses of SRP enrichment (e.g., during mixing in lakes) are luxury-consumed for storage as polyphosphate granules and subsequent use.

Many cyanobacteria have high P optima for growth in comparison to [other] algae and, when N_i is limiting, low N:P supply ratios (often considered as N_i:TP in the medium) have been reported to favor their growth in lakes, reservoirs, and lower rivers (25,55; but see 2). Although microbial mats in salt marshes and coastal marine environments are often N-limited (2), mats in those habitats that are formed in part by heterocystous cyanobacteria probably become P-limited. Such mats, when fertilized with phosphate, have significantly increased dinitrogen fixation, indicating that the benthic cyanobacteria were probably P- and N-limited as a direct (because dinitrogen fixation requires phosphate) or indirect effect (by stimulating aerobic bacteria that decreased oxygen in the mat, which would, in turn, be conducive to dinitrogen fixation) (2). Similarly, dinitrogen fixation by the pelagic cyanobacteria, *Trichodesmium* spp. in the central Atlantic Ocean can be P-limited (56).

Sulfur

Sulfur is an essential constituent of amino acids cystine and methionine, nitrogenase, thylakoid lipids, CoA, and DMSP (dimethylsulfonioproprionate, used in osmoregulation or as a cryoprotectant, see following text; 1). Uptake of sulfate is energy- (ATP)-dependent. In freshwaters, sulfate can be transformed to H_2S and, thus reduce limiting levels by anaerobic bacteria. By contrast, seawater contains abundant sulfate (28 mM), and sulfate reduction is usually important in marine coastal microbial mats. Below pH 7, H_2S (g) becomes increasingly important, whereas above pH 9, the predominant form is S^{-2}. At pH 7–9, virtually all sulfide is present as HS^-. Mat cyanobacteria coexist with sulfate-reducing bacteria, which actually occur throughout the mat layers. The cyanobacteria excrete dissolved organic compounds during fermentation and photorespiration (e.g., glycolate), which can serve as substrates for the sulfate-reducing bacteria. Thus, the metabolism of the mat cyanobacteria can directly influence sulfate reduction (2). Some mat-forming cyanobacteria can reduce elemental S_o to sulfide, using electrons produced during glycogen degradation. Sulfate reduction usually does not generate biochemical energy for cyanobacteria, but allows higher acetate production. Thus, sulfur serves as an electron sink during fermentation. An exception is *Oscillatoria limnetica* that appears to be capable of true sulfur respiration as indicated (2).

In contrast to sulfate, free sulfide (H_2S, HS^-, S^{-2}) is common in anaerobic habitats. It is highly toxic to all biological organisms, reacting with iron-containing compounds such as cytochromes and hemoproteins. It can be a potent, irreversible inhibitor of photosystem II, and of anoxygenic photosynthesis as well (2) (but note that sulfide can be used as an e^- donor by some cyanobacteria in anoxygenic photosynthesis as mentioned). H_2S enters cells by passive diffusion. In high-sulfur environments (e.g., benthic mats in some geothermal springs or coastal marine habitats), sulfide can react with S_o to form polysulfides which can be tenfold more toxic than sulfide, but which may also serve as the sulfur that is transported in cells.

Trace Metals

Trace metals are extremely important in the physiology and ecology of cyanobacteria because they are required for many essential metabolic functions (1). For example, zinc is a component of the carbon-concentrating enzyme, carbonic anhydrase, and is also a necessary constituent of alcohol dehydrogenase, glutamic dehydrogenase, and (with trace metal, copper) Cu/Zn superoxide dismutase. Copper is also needed for plastocyanin and cytochrome oxidase. Manganese is required in the oxygen-evolving complex of photosystem II (1). The key enzyme in dinitrogen fixation, nitrogenase, contains molybdenum and iron; and the key enzyme in nitrate uptake, nitrate reductase, has molybdenum and iron as cofactors. Iron is essential, as well, in ferredoxins (which serve as electron donors in dinitrogen fixation, sulfur reduction, and [indirectly] carbon dioxide fixation), cytochromes, nitrite reductase, catalase, and glutamate synthetase. Recent research additionally has shown that a protein encoded by an "iron stress-induced" gene in the open ocean cyanobacterium, *Synechocystis* PCC 6803, encodes a photosystem II-like protein that associates with phototosystem I to form a complex that significantly increases the size of the PSI light-harvesting system. The "extra" antenna formed by this PSII-like protein for PSI apparently helps compensate for depressed phycobilisome and PSI levels in response to iron limitation in open-ocean waters (57).

Given their importance in major metabolic processes, it is not surprising that trace metals sometimes play an important role in controlling phytoplankton production. For example, although iron is among the most abundant elements, its biological availability is restricted by various natural binding and loss processes. In various oligotrophic areas of the oceans (mid-Atlantic, North Pacific gyre, Southern, and Antarctic Oceans), phytoplankton production remains low despite relatively high nitrogen (nitrate), P and silicate availability, apparently because of iron

limitation (58). It has been hypothesized that large surface aggregates of *Trichodesmium* trap incoming iron in dust from atmospheric sources (59). As a more widespread mechanism that actually can control levels of biologically available iron in some aquatic systems, many cyanobacteria commonly produce potent chelators (surface iron-binding substances) called siderophores (siderochromes), which have high affinity for iron and transport iron into the cells (60).

Dissimilatory iron metabolism is important in microanaerobic environments such as cyanobacterial mats in salt marshes and benthic freshwater and marine coastal habitats (2). Within the mats, both ferric (Fe^{+3}) and ferrous iron (Fe^{+2}) can act to immobilize toxic sulfide. In many microbial mats, a layer of oxidized iron often occurs (probably produced by specialized anoxygenic phototrophic purple bacteria) between the cyanobacterial layer and the underlying anoxic layers. The iron layer is believed to form a protective barrier between the aerobic and anaerobic components of the mat: Fe^{+3} would scavenge sulfide and protect cyanobacteria from its toxic effects. Fe^{+2} would react with oxygen to maintain oxygen partial pressure low enough to allow efficient cyanobacterial photosynthesis, although also protecting the underlying purple sulfur bacteria from oxygen (2). Both Fe^{+2} and Fe^{+3} can also act as an effective UV screen because they strongly absorb UV radiation at 220 to 270 nm. Some mat-forming cyanobacteria (e.g., *Microcoleus chthonoplastes*) accumulate iron at the outer sheath. The iron is bound to negatively charged polysaccharides in the sheath, and may act to protect the organisms from UV, sulfide, and high oxygen (2).

Vertical Migration in the Water Column and Nuisance Plankton Blooms

The density of phytoplankton cells (here, including cyanobacteria in an ecological context) is generally 1.01- to 1.03-fold greater than that of water (1,000 kg/m^3); thus, they tend to sink rather than to stay afloat (61). In stratified fresh- and marine waters during periods of active phytoplankton growth, dissolved N_i and P_i often are depleted in surface waters, whereas deeper waters are more nutrient-rich from decomposition of dead organisms as they settle out of the water column. Free carbon dioxide can also increase with depth from increased activity of microbial decomposers. It would be advantageous for phytoplankton to have a mechanism that enabled them to move to the lower water column to gain access to greater nutrient supplies, then to rise back up into light-replete areas for photosynthesis (62). However, in open, deeper waters phytoplankton will not survive if they sink and remain below the euphotic zone (depth in which there is sufficient light for photosynthesis).

Within a pressure-dependable water column, cyanobacteria can regulate their buoyancy in response to environmental conditions using gas vacuoles, which are unique to prokaryotes among the phytoplankton. These structures consist of groups (stacks in hexagonal arrays) of hollow, rigid, pointed cylinders (gas vesicles) with rigid proteinaceous walls that are fully permeable to gases, but with a hydrophobic inner surface that prevents influx of water and heavier cellular constituents (62). They maintain a finite volume of metabolic gases (e.g., N_2) at ambient pressure, and their density (120 kg/m^3) is much less than that of other cellular constituents (2). Gas vacuoles are used by cyanobacteria as an efficient mechanism to provide lift because gases have much lower densities than solids or liquids. For example, Reynolds and Walsby (63) reported that at 15 °C, the volume of gas needed for neutral buoyancy in *A. circinalis* is 1.1% of the total cell volume, and found that the actual volume of gas ranged from 0.7 to 2.3% of the total cell volume. Individual gas vesicles commonly withstand external pressures of 4 to 7 atm (up to 35 atm in marine *Trichodesmium*), enabling cyanobacteria to control buoyancy through a relatively deep water column. Cyanobacteria can control buoyancy in a pressure-dependable water column by regulating the number of gas vesicles, accomplished in part by controlling the internal turgor pressure, that is, the extent to which dense components such as carbohydrates and proteins (density ca. 1600 kg/m^3 and ca. 1300 kg/m^3) accumulate (2). Intracellular osmotic pressure can increase during photosynthesis to 3.5 to 5.0 atm, which is sufficient turgor pressure to eliminate weaker vesicles. Three mechanisms have been described whereby gas-vacuolate cyanobacteria regulate their buoyancy: (*1*) by changing cell density through alterations in cellular composition, mostly by accumulation of carbohydrate reserves through photosynthesis, and their reduction either through respiration or by conversion to less dense protein; (*2*) by collapse of gas vesicles from increased turgor pressure; and (*3*) by collapse of gas vesicles due to depressed gas vesicle synthesis, and dilution of remaining gas vesicles by growth (1).

Gas vesicle production is induced by low light conditions and, therefore, when light is less than optimum, there is an increase in gas vesicles relative to other cellular products. As gas vesicles accumulate, the cell becomes less dense and floats up to a more light-replete area where its photosynthesis increases. The resulting accumulation of osmotically active sugars, with uptake of ions, increases the intracellular turgor pressure which collapses a portion of the gas vesicles, causing the cell to sink. As osmotically active substances are removed by respiration and other cell processes in deeper, darker waters, gas vacuoles reform and the cycle repeats (1,2). Buoyancy tends to increase during the dark period, and to decrease over time during the light period (63). This mechanism interacts with nutrient limitation. Buoyancy tends to decrease when major nutrients such as P_i or N_i limit cell growth because carbohydrates accumulate and turgor pressure increases (2). N_i limitation leads to reduced gas vacuolation and loss of buoyancy because of reduced protein production needed for gas vesicle assembly. Sustained C_i limitation can also lead to decreased cell buoyancy because less energy is available for gas vesicle synthesis. When nutrients are abundant, buoyancy is mostly controlled by light intensity. If light is suboptimum for growth, then the energy supply will be relatively low, carbohydrate reserves are reduced, and cell buoyancy will increase. At higher light, carbohydrate production increases, turgor pressure increases and buoyancy decreases. Marine *Trichodesmium* spp. are exceptional because their extremely strong,

membrane-bound gas vesicles enable them to remain buoyant in highly variable light fields. However, their buoyancy is controlled to some extent by carbohydrate content; thus, they sometimes occur in deep waters below the euphotic zone (64).

Thus, through regulation of gas vesicles, in dependable-pressure water columns cyanobacteria have a mechanism that enables them to sink to deeper, nutrient-replete waters late in the light period and at night, and to rise to reach light-replete (but nutrient-depauperate) waters early in the light period. Additional advantage over other phytoplankton would be afforded for N_2-fixing cyanobacteria, which are favored by low-N_i environments (indicated by low water-column TN_i:TP). The movement to deeper waters would also enable avoidance of damage from photoinhibition and high UV radiation (note that Paerl and coworkers (65) reported that natural populations of *M. aeruginosa* had optimum photosynthetic rates and resistance to photoinhibition at surface irradiances, but Ibelings and Mur (66) used techniques with finer-scale resolution and provided evidence that surface populations of *M. aeruginosa* can in fact be photoinhibited (2)). Gas-vacuolate cyanobacteria are characteristic of periodically stratified eutrophic lakes and certain other locations such as the P-rich Baltic Sea and Caribbean (Fig. 11; 67). Healthy populations are distributed near the surface, then at other locations in the water column depending on the time of day, the physiological condition of the cells, the nutrient regime, and other factors. However, rapid development and persistence of noxious, thick surface blooms and surface scums at the air–water interface is a common aesthetic and economic problem in management of eutrophic lakes. Such "blooms" often include many dying or dead cells, in conditions that are highly unfavorable for growth of cyanobacteria and other aquatic life. Consider, for example, Zohary and Pais Madeiras' (26) description of *Microcystis* surface "hyperscums" several decimeters thick, covered by a crust of photooxidized cells; the bloom had formed during prolonged periods of calm weather in wind-protected sites within a hypereutrophic lake. It extended over 1 to 2 hectares and persisted for 103 days, with concentrations of 1.76×10^9 cells/mL. The environment was anoxic and aphotic with extreme, fluctuating temperatures, and low pH. How do such surface scums occur?

Cyanobacteria buoyancy regulation depends on a dependable external pressure regime that, if variable, changes sufficiently slowly to allow the cells to adjust their internal pressure (63). If the euphotic zone extends deeper than the mixing zone (depth to which wind frequently mixes the surface waters), cyanobacteria may concentrate for part of the day below the mixed layer. If the euphotic zone extends to approximately the same depth as the mixed layer, turbulence will result in distribution of the cyanobacterial cells throughout the mixed layer. Average exposure to light will permit net production little excess photosynthetic carbohydrate reserves; the cells remain neutrally buoyant and are able to adjust buoyancy in response to changing environmental conditions. However, if the euphotic zone is shallower than the mixed layer, decreasing light intensity within the mixed layer promotes increased cyanobacterial buoyancy. Cyanobacterial surface scums often develop under such conditions during calm, warm weather following deep mixing from moderate storms when cells have become overbuoyant as a result of the (previously) low light intensity (63). Because of the sudden change in hydrological conditions, the cells are unable to lose their excess buoyancy and remain at the surface long enough to encounter excessive light intensity that inhibits the photosynthesis needed to increase turgor pressure. High light intensity can also cause photooxidation of pigments and death from UV radiation. Surface tension would help to trap them at the surface, where they would dehydrate at high temperatures. Localized oxygen depletion within the developing layer would depress turgor pressure, and aggregations, or clumping of cells would promote flotation.

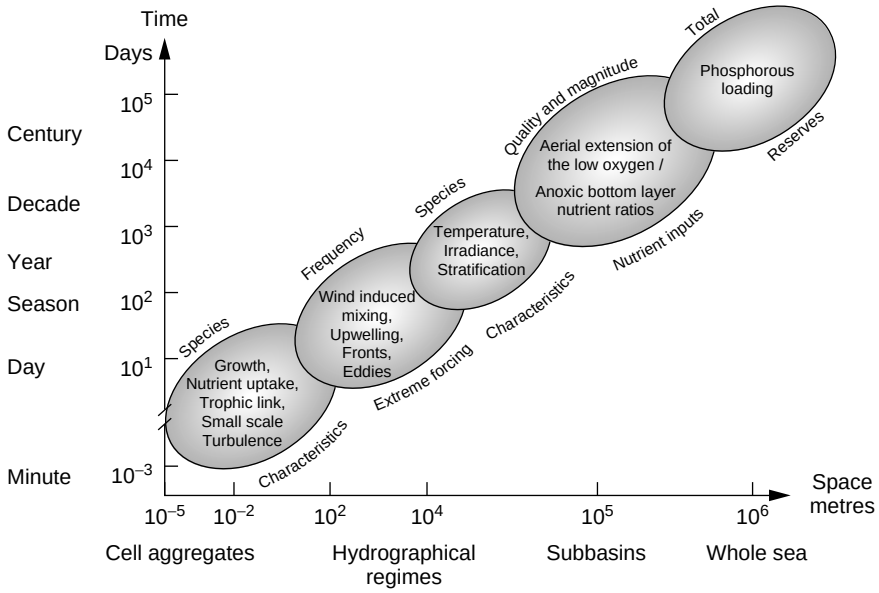

Figure 11. Depiction of the scales and time frames of processes regulating cyanobacterial blooms in the Baltic Sea. From (K. Kononen and J-. M. Leppänen, in M. Kahru and C. Brown, eds., *Monitoring Algal Blooms: New Techniques for Detecting Large-Scale Environmental Change*, Landes Bioscience, 1997, pp. 63–84) with permission from the authors and Landes Bioscience.

These factors in combination would reduce population growth and photosynthesis, and would disable buoyancy regulation. Cells at the surface that lost buoyancy would be physically impeded from sinking by the presence of the underlying, still overbuoyant population. The previously dispersed population would not have to be especially dense because it would be greatly concentrated at the surface. For example, if a population with 2×10^3 cells/mL that was dispersed through 5 m floated up to a 2-cm surface layer, its a surface density would be 5×10^5 cells/mL (2). Thus, cyanobacterial surface scums occur when conditions change too rapidly from low to high light intensity (corresponding to high turbulence, then sudden calm) so that cells cannot correct overbuoyancy; when sensescent or stressed cells cannot increase their turgor pressure; and/or when photosynthesis becomes limited by carbon dioxide diffusion through calm water (68).

BIOLOGICAL INTERACTIONS

Predators and Pathogens

Various mechanisms of grazer avoidance have been invoked in cyanobacteria including "refuge" in small size, large size, or copious mucilage; in habitat (for example, cyanobacteria that closely adhere to sand grains; or colonizers of rocks and ice); and in chemical defense (production of toxins or other bioactive antigrazer substances). All of these appear to be effective with certain grazers under some conditions; yet on a seasonal basis, grazers can significantly influence cyanobacterial populations. Grazing pressure is a major factor restricting the distribution of stromatolites and benthic cyanobacterial mats, and grazing pressure restricts cyanobacterial plankton growth in the polar oceans (2,2).

Although many microfauna and some herbivorous macroinvertebrates and fish avoid cyanobacteria, others (e.g., protozoan ciliates such as *Nassula, Frontia, Ophryoglena*; rhizopodial amoebae such as *Pelomyxa*; many rotifers) thrive in the midst of toxic blooms (e.g., *Anabaena, Aphanizomenon, Microcystis*; Fig. 12; 69) and can significantly reduce even toxic colonial cyanobacteria consisting of cells embedded in thick mucilage (2,69,70). Other zooplankton exhibit physiological and behavioral adaptations to avoid toxic cyanobacteria, including selective feeding and avoidance of depths in which toxic blooms are concentrated (also note that herbivorous carp have been reported to avoid toxic cyanobacteria (2)). Marine herbivorous copepods (e.g., *Acartia*) feed selectively, and can actively discriminate against toxic or low quality algal food. Freshwater copepods selected and consume filaments of cyanobacterium *Oscillatoria tenuis*; however, they rejected the morphologically comparable but toxic species, *Oscillatoria* (*Planktothrix*) *rubescens* (71). In some systems, calanoid copepods avoid cyanobacteria, whereas in other systems, calanoid copepods ingest cyanobacteria with wide variation in assimilation rates (72). If generalizations are possible, given the aforementioned information, reduced grazing on cyanobacteria has been associated with large size, high density, allelopathic compounds, and poor assimilability; and the extent to which grazers can control cyanobacterial blooms often depends on timing of grazing pressure during bloom development (2).

Many effects of grazing on cyanobacteria are indirect and beneficial. For example, grazing by some herbivores (e.g., the snail, *Lymnaea*, grazing benthic microalgae in an arctic lake) adds nutrient enrichment via their excreta and can favor coccoid cyanobacteria at the expense of larger eukaryote algae (73). Some colonial cyanobacteria with thick mucilage exhibit high survival following microfauna gut passage (e.g., cladoceran zooplankton, calanoid and cyclopoid copepods — hence, the cyanobacteria were referred to as "inedible"), and derive benefit from nutrient enrichment during that process. Microfaunal grazers excrete phosphatases that assist algae in phosphate acquisition, and they can also assist in dispersal (2).

Pathogens of cyanobacteria include bacteria, fungi, and viruses (cyanophages). Some aerobic gram-negative bacteria (e.g., Myxobacteriales) lyse cyanobacterial cells (*Anacystis, Coccachloris, Nostoc, Plectonema, Cyanothece*) (74). In laboratory experiments, the cyanobacteria were lysed within 2 to 10 hours (less commonly, within three days) of exposure to the pathogenic bacteria. Although little is known about the extent to which bacterial pathogens affect natural cyanobacterial populations, bacteria tested in artificial ponds were capable of eliminating a bloom of *M. aeruginosa* within two days (75). Fungi that parasitize planktonic cyanobacteria are mostly (uniflagellate) chytrids, with exception of a biflagellate (Blastocladiales) (76), and many taxa are affected including *Anabaena, Aphanizomenon, Gomphosphaeria, Lyngbya, Microcystis, Oscillatoria*, and especially *Scytonema* (2) (Fig. 12). Many of these chytrids appear to have a host range limited to a single cyanobacterial taxon; some attack a specific structure such akinetes or heterocysts (77). For example, the chytrid *Chytridium cornutus* was observed to attack only heterocysts of *Aphanizomenon*; *Blastocladiella anabaena* was observed predominantly on *A. flos-aquae* (76).

Cyanophages have been more intensively examined than other cyanobacterial pathogens because of earlier efforts (1960s–1980s), thus far unrealized, to use them as agents of biological control of noxious bloom-forming cyanobacteria. Cyanophages are lytic infectious agents that belong to three families of double-stranded DNA viruses, the genetically diverse Myoviridae, which commonly infect *Cyanothece* spp. in marine waters; and the Syloviridae and Podoiridae, which are most often isolated from freshwaters and commonly infect filamentous cyanobacteria (e.g., *Anabaena, Nostoc, Phormidium, Plectonema, Anacystis, Cyanothece* [*Synechococcus*]) (2). The myoviridaens are the most abundant cyanophages (occurring at $>10^6$/mL during warmer seasons, and at $>10^5$/gram sediment in coastal areas. For example, in a study in the Gulf of Mexico, at about 50 km from shore and about 50 m-depth there were 9.4×10^4/mL at the sediment–water interface, and 3.0×10^2/mL at 30-cm sediment depth under anaerobic conditions (2). The myoviridaen cyanophages have a turnover time ranging from hours to days in the water column, whereas in sediments they can persist for at least 100 years. In coastal

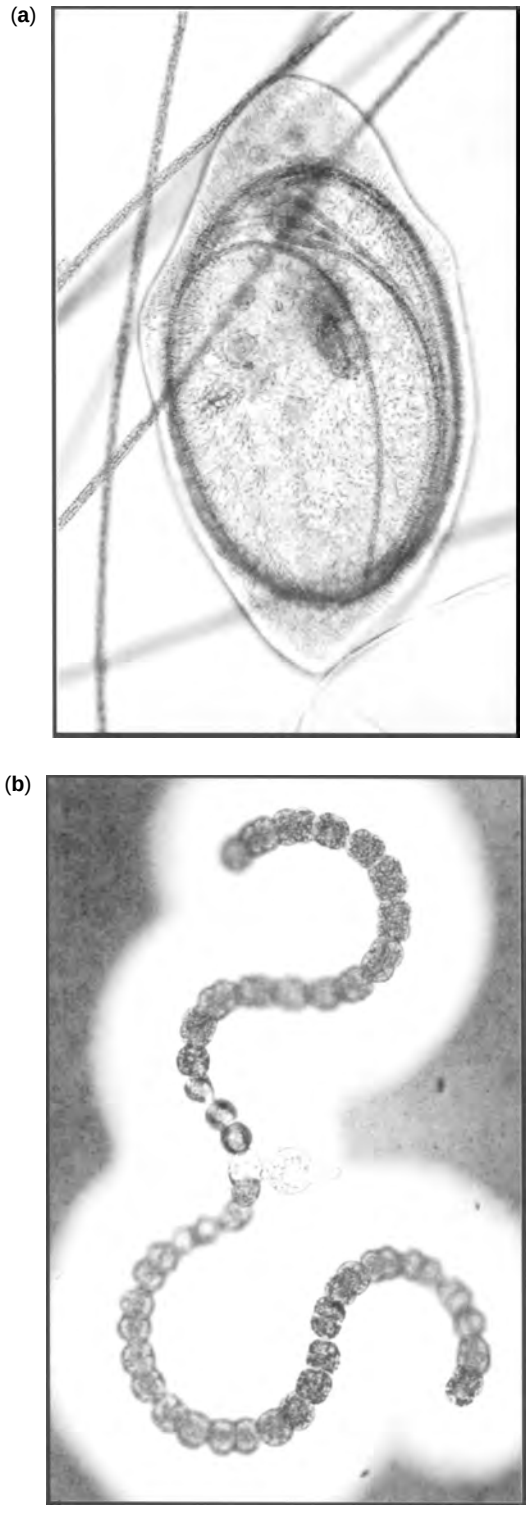

Figure 12. (**a**) The freshwater ciliate, Frontonia (420 × 234 μm), which had ingested several filaments of Oscillatoria (Planktothrix) that are coiled inside; and (**b**) The freshwater chytrid parasite, Rhizosiphon, showing its flask-shaped sporangium (24 × 12 μm) embedded in the mucilage surrounding Anabaena (white, against dark background). From (H. Canter-Lund and J. W. G. Lund, *Freshwater Algae — Their Microscopic World Explored*, Biopress, Bristol, U.K., 1995.), with permission from the authors and Biopress.

waters, high concentrations of cyanophages and *Cyanothece* result in high encounter frequencies and selection for *Cyanothece* strains that are relatively resistant to infection. Virus abundance follows that of its host, with evidence of a threshold in *Cyanothece* of about 10^3 to 10^4/mL, above which cyanophages greatly increase (2). It has been estimated that cyanophages remove approximately 3% of marine *Cyanothece* daily. By contrast, offshore *Cyanothece* populations have lower encounter frequencies and appear to have low resistance to infection. There is only one documented case thus far of a marine filamentous cyanobacterium (*Phormidium persicinum*) infected by a virus, although this phenomenon is probably more common. Freshwater cyanophages are lower in abundance (maxima > 10^3/mL), even in the most eutrophic lakes, than in productive near-shore waters.

UV-B radiation destroys cyanophages and/or depresses their infectivity, selecting for cyanobacterial communities that are more resistant to photodestruction in the summer season. Nevertheless, photoreactivation processes can restore infectivity to a major proportion of the damaged viruses (2). The physiological conditions that influence susceptibility to infection mostly remain to be examined, but there is experimental evidence that nutritional status is one important factor: All cells of P-replete marine *Synechococcus* were lysed when exposed to cyanophages, vs. only 9% lysis of P-limited *Cyanothece*. In addition to lytic infection, lysogenic associations have been shown in both unicellular and filamentous cyanobacteria, but this and other aspects of cyanophage/cyanobacterial interactions (e.g., other environmental controls on cyanophages, and physiological traits enhancing host susceptibility) remain poorly understood.

Symbiosis

Cyanobacteria form symbiotic associations with a range of eukaryote hosts including protists (microalgae, microflagellates, macroalgae), bryophytes, higher plants, fungi, sponges, ascidians (sea squirts), echuroid worms and midge larvae (2). The best understood interactions are mutualistic symbioses with terrestrial and freshwater plant hosts that are easily accessible for study. Nevertheless, cyanobacterial symbioses are widespread across terrestrial, freshwater, and marine habitats. Loose associations between cyanobacteria and other organisms are widespread and both ecologically (e.g., various N_2-fixing cyanobacterial taxa growing in close association with duckweed, *Lemna*) and economically important (e.g., epiphytic and endosymbiotic [*Azolla*-associated] N_2-fixing cyanobacteria enhancing growth and crop yield of rice (2) (Table 3). Many endosymbioses are also known from polar to tropical regions. Of these, most marine cyanobacterial symbioses occur in the plankton and benthos of nutrient-poor tropical waters. Most freshwater cyanobacterial endosymbioses are found in habitats that are enriched in some nutrients but limited by other resources that are augmented by the symbiotic interaction (78). For example, the *Azolla*/*Anabaena* association is widespread among P-rich but N-poor habitats. Similarly, unicellular cyanobionts that appear to be capable of fixing dinitrogen within the cytoplasm of certain freshwater benthic diatoms increase

Table 3. Examples of Cyanobacterial (Cyanobiont) Symbioses

Relationship/Habitat	Cyanobiont(s)	Associate or Host	N_2 Fixation
Loose Association			
Temperate freshwater epiphyte (lower epidermis, reproductive pockets)	*Nostoc, Gloeotrichia, Anabaena, Calothrix, Cylindrospermum*	Duckweed, *Lemna*	+
Temperate freshwater epiphyte	*Nostoc, Hapalosiphon, Tolypothrix*	Moss, *Sphagnum*	+
Temperate (grassland, forest)	*Nostoc*	Mosses, *Ceratodon, Funaria*	+
Tropical freshwater epiphyte (submersed roots, stems)	*Gloeotrichia pisum*	Deepwater rice, *Oryza sativa*	+
Pelagic marine epiphyte Sargasso Sea	*Calothrix*	*Sargassum*	+
Tropical/subtropical marine epizooite (cloacal cavity)	*Prochloron*	Ascidians (sea squirts), e.g., *Lissoclinum punctatum, Didemnum molle*	−
Endosymbiosis			
Temperate freshwater (soft-water lakes, marshes)	coccoid unicells	Diatoms, *Rhopalodia gibba, Denticula, Epithemia turgida*	(+); no heterocysts
Temperate freshwater (softwater, hardwater lakes and subtropical rice cultivation; dorsal leaf cavities)	*A. azollae*	Fern, *Azolla*	+
Tropical/subtropical marine pelagic	*Synochococcus Synechocystis*,	Heterotrophic dinoflagellates, *Ornithocercus magnificus, O. quadratus, Histioneis elongata, H.highleyi;*	−
Temperate marine benthic	*Oscillatoria, Spirulina, Anabaena*	Ascidians, *Pyura cancellata, P. carnea*	+ (*Anabaena*)
Temperate/subtropical marine sponges (intra- and intercellular)	*Aphanocapsa, Phormidium*, rarely *Oscillatoria*	Marine sponges (c. 40 genera)	+ (3 spp.; no heterocysts)
Pelagic north temperate/subarctic	*Richelia intracellularis*	Diatom, *Rhizosolenia*	−
Tropical/subtropical marine (in tunic cells)	*Prochloron*	Ascidian, *Lissoclinium*	
Terrestrial tropical/subtropical (mostly southern hemisphere; in coralloid roots)	*Nostoc (Calothrix)*	Cycads, e.g., *Zamia*	+
Terrestrial subtropical (in stem glands)	*Nostoc*	Angiosperm, *Gunnera*	+
Terrestrial (polar to tropics; Cyanolichens)	*Nostoc, Fischerella Calothrix, Scytonema*, various others	Fungi (usually ascomycetes), phycomycete, *Geosiphon*	+

in number in response to decreasing external N_i, but this increase does not occur under P limitation.

Although it is often unclear how the cyanobacteria in loose associations derive benefit, the other organism(s) involved typically derive nutritional advantage(s). In some loose associations (e.g., *Prochloron* in the cloacal cavity of tunicates), the cyanobacteria clearly derive nutrients from the host (2). In endosymbioses, the host may derive carbon from the cyanobionts (and N, from N_2-fixers), although the cyanobionts gain access to additional free carbon dioxide and are afforded protection from grazers, pathogens, desiccation, and other harmful external factors. The relationships probably enhance survival and growth not only of the symbionts, but of all organisms that directly or indirectly depend on them for habitat or nutrition. For example, in the tropics and subtropics, cycads containing N_2-fixing cyanobacteria in their roots can significantly contribute to the local nitrogen economy (79). The subtropical marine sponge *Chondrilla nucula,* with N_2-fixing cyanobionts, releases sufficient N_i to supply 50 to 120% of the nitrogen needed to sustain local coral reef productivity (80).

Many cyanobacterial endosymbionts are heterocystous filaments, and produce hormogonia that act as the infective agents in the hosts. These symbioses are somewhat analogous to the relationship in free-living cyanobacteria between heterocysts and adjacent vegetative cells (2). The symbionts (like heterocysts) often have negligible photosynthesis, relying on organic compounds excreted by the host for energy and growth, and thus avoiding the potential for the symbionts to inhibit dinitrogen fixation with their own oxygen evolution. All known higher plant/cyanobacterial symbioses involve heterocystous cyanobionts, and in all but one case (*Gunnera*, the only angiosperm genus with cyanobionts, found within all c. 50 spp.), the cyanobionts are extracellular (2). The cyanobionts in higher plant associations mostly are *Nostoc* spp., which especially respond to (largely unidentified)

plant chemical signals that induce hormogonia formation and attract hormogonia (with gliding motility) into the plant tissue (2). Additional signals within the plant host then repress further hormogonia formation although stimulating heterocyst development; the cyanobacteria undergo substantial morphological alterations and usually their growth rate and carbon dioxide fixation are suppressed although their dinitrogen fixation is enhanced, with much of the fixed product lost to the host (often through inhibition of the cyanobiont's glutamine synthetase activity, so that the cyanobacteria are unable to synthesize amino acids with the fixed N_i).

Cyanobionts can supply the entire nitrogen requirement of the higher plant host (2). Even in large *Gunnera* plants (leaf width, 2 m; Table 3), a *Nostoc* cyanobiont mass that is only 1% of the total plant weight can supply all of the host nitrogen needed (81). Less commonly (e.g., *Richelia intracellularis* within the diatom *Rhizosolenia*), the symbiont fixes both carbon dioxide and dinitrogen for the host (82). In symbioses where dinitrogen fixation has not been observed (e.g., in some sponges; unicellular cyanobionts or "phaeosomes" of the dinoflagellates, *Ornithocercus magnificus* and oxygen. *steinii*; *Prochloron* with the tunicate, *Didemnum molle*), the cyanobionts are photosynthetically active and transfer a portion of their fixed carbon to the host (possibly as glucose, maltose, or glycerol); or, (e.g., *Prochloron* in tunic cells of the tunicate, *Lissoclinium voeltzkowi*), the host's entire C_o requirement is met by the symbiont (2,83).

In most cases cyanobionts remain recognizable as cyanobacteria, and they can survive without the host. By contrast, freshwater cyanelles (plastids found in certain photosynthetic, unicellular, or palmelloid eukaryotes, artificial algal class Glaucocystophyceae (1)) apparently represent an extreme in cyanobacterial symbiotic relationships, wherein the symbiont has lost much of its own genetic capability and has become entirely dependent on the host (2). Cyanelles occur in genetically unrelated "Glaucocystophycean" organisms that typically are found in low density as epiphytes on filamentous algae, aquatic mosses, and submersed angiosperms. Although the endosymbionts function as chloroplasts, they still retain a peptidoglycan cell wall that is believed to be a remnant structure of free-living cyanobacterial ancestors. The cyanelles are intermediate between cyanobacteria and eukaryote chloroplasts in most features of gene organization/ sequence (79). They cannot respire because their respiratory chain lacks cytochrome carbon oxidase; they cannot fix N_2; and they contain only approximately 10% of the DNA found in free-living cyanobacteria, so that their genome is similar in size to that of chloroplasts and they have very limited genetic autonomy.

Although the cyanobacteria act as the symbionts in most of the associations, in at least one case the colonial cyanobacteria, *Nostoc parmelioides*, functions as the host and the eukaryote, the chironomid midge, *Cricotopus nostocicola*, is the endosymbiont. This mutualistic association occurs in temperate, moderately fast-flowing streams (22). The larvae inside the colony eat the *Nostoc* and are protected from predation, and *Nostoc* colonies with larvae have higher productivity than unoccupied colonies. Enhanced productivity may occur because occupied colonies grow further into the water flow, enhancing outward oxygen diffusion that would otherwise inhibit dinitrogen fixation, and promoting inward carbon dioxide diffusion for cyanobacterial growth. The larvae apparently trigger hormogonia formation, thereby promoting expansion of the colony and potentially providing habitat for later larval generations.

Toxins and Other Bioactive Substances

Toxic cyanobacterial blooms cause major economic impacts in many countries, and their lethal effects on wildlife and livestock have been reported at least since the 1870s and see the article by Falconer in this Encyclopedia. More than 40 taxa produce an array of cyanotoxins, mostly as cyclic peptides, alkaloids, and lipopolysaccharides (5) –and many other cyanobacteria probably have toxin producing (toxigenic) capability (2). Although most focus historically has been on several toxic planktonic freshwater (*A. flos-aquae*, *A. flos-aquae*, *M. aeruginosa*) and brackish (*N. spumigena*) bloom-forming taxa, recent research has shown that (*1*) many benthic and planktonic "non-bloom-forming" cyanobacteria are capable of toxin production (Table 4); (*2*) many cyanotoxin variants can be produced; and (*3*) cyanotoxins, despite their aquatic origin, generally are less toxic to aquatic biota than to terrestrial organisms including humans (2). For example, microcystin, the most potent liver carcinogen known, shows low toxicity to mussels and crayfish (2).

Most cyanotoxins appear to be secondary metabolites (2) used in defense against predation, but they may also serve other cellular functions such as nitrogen storage (1). Although little information exists on the frequency of cyanobacterial toxicity in environmental samples, the available data are compelling: for example, 44% of the cyanobacterial blooms sampled in Finnish fresh and coastal waters were potentially lethally toxic (88); and approximately 67% of the cyanobacterial blooms collected from approximately 100 freshwater locations in the United Kingdom were toxic (89). Some strains produce three or more toxins, with relative proportions influenced by environmental factors and, as true for many toxic eukaryotic algae, the factors that regulate expression of toxicity are poorly understood (90). To err on the side of caution, some specialists have advised that all cyanobacterial blooms should be regarded as potentially toxic unless shown otherwise by rigorous examination (91). Such counsel would be supported by changes in toxicity that have been documented in cyanobacteria: Toxic strains have become nontoxic (atoxigenic, apparently incapable of toxin production) but, importantly, the reverse has also occurred (2). Toxicity not only varies among strains collected from the same sample in the same bloom, but also among clones of the same isolate (2,92).

Cyanotoxins can be lethal neurotoxins or hepatotoxins, or nonlethal cytotoxins (causing damage to various types of active cells) with selective bioactivity (Table 4; 2; and see the article by Falconer in this Encyclopedia). Direct cyanotoxin poisoning of animals can occur via ingestion of cyanobacterial cells in drinking water, or indirectly via consumption of prey that had accumulated

cyanotoxins (5). Cyanotoxins can bioaccumulate in aquatic vertebrates (fish, especially the liver) and invertebrates (shellfish, especially the hepatopancreas). Cyanotoxins vary in the time required for degradation. Although some toxins degrade rapidly, microcystin and nodularin degrade slowly in living cyanobacterial cells; thus, scums that dry on lake or estuarine shores can contain high toxin concentrations for weeks to months before they are microbially degraded (5). Cyanotoxins and other bioactive substances from cyanobacterra adversely affect some

Table 4. Cyanobacteria, Their toxin(s), and Brief Description of Toxin Effects on Mammals (modified from [2-DS,5,85–88]; and see CYANOBACTERIA-TOXINS IN DRINKING WATER, this Encyclopedia)

Toxin	Taxon (Taxa)	Structure	Effects
Acutiphycin, 20,21-didehydroacutiphycin	*Oscillatoria acutissima*	Macrolides	Cytotoxic to tissue cells; but also has antitumor properties (lung carcinoma)
Anatoxin-A	*Anabaena flos-aquae, A. lemmermanni, A. planktonica, Anabaena* spp., *Cylindrospermum* sp.	Secondary amine alkaloid (MW 165) (nicotinic [cholinergic] agonist; structural analog to cocaine and neurotransmitter acetylcholamine)	Neurotoxin — neuromuscular blocking agent; binds to neuronal nicotinic acetylcholine receptors; causes an influx of Na^+, with sufficient local depolarization to open voltage-sensitive Ca^{+2}, Na^+ channels, blocking further electrical transmission; can lead to convulsions, paralysis, asphyxiation, and death by respiratory failure in mammals (e.g., livestock, wildlife, dogs). Chronic reproductive impacts shown in mice (fetus malformation).
Anatoxin-A(S)	*Anabaena flos-aquae, A. lemmermanni,*	N-hydroxy guanidine methylphosphate ester (naturally occurring organophosphate)	Neurotoxin-acetylcholinesterase inhibitor; causes similar symptoms as anatoxin-A, additionally with ataxia, diarrhea, hypersalivation, tremors.
Aphantoxin AphantoxinII	*Aphanizomenon flos-aquae*[a] *Aphanizomenon flos-aquae*[a]	Alkaloid (MW 315; neosaxitoxin) Alkaloid (MW299 — closely resembles saxitoxin)	Neurotoxins–interfere with neurotransmission (block neurone Na^+ channels across axon membrane); cause irregular breathing, twitching, loss of coordination, death by respiratory failure.
Aplysiatoxins, Debromoaplysiatoxin	*Lyngbya majuscula*,[b] *Schizothrix calcicola, Oscillatoria nigroveridis*	Alkaloids, phenols	Neurotoxins if ingested; dermatotoxins if contacted by skin; potent animal skin tumor promoters; potent tumor promoter if ingested (induces differentiation of promyelocytic leukemia cells, aggregation of lymphoblastoid cells, and stimulation of prostaglandin production and choline turnover); activate Ca^{+2}-activated, phospholipid-dependent protein kinase C to cause contraction of smooth muscle; also cause swimmer's itch (skin irritation, erythema, blisters, deep desquamation).
Ciguateratoxinc[c]	*Trichodesmium erythraeum*	Polyethers	Neurotoxins–open Na^+ channels at resting potential; prevent open channels from being inactivated during subsequent depolarization. Replace Ca^{+2} ions at sites on neuroreceptors that control sodium permeability. Can cause Ca^{+2}-dependent contraction in smooth and skeletal muscle tissues; can promote release of norepinephrine, dopamine from pheochromocytoma cells. Immunogenic after coupling to serum protein.
Cyanobacterin	*Scytonema hofmanni*	Chlorinated diaryl-lactone	Cytotoxin-cyanobacterial inhibitor
Cyanoginosins	*Microcystis aeruginosa*	Heptapeptides (MW 909-1044)	Cytotoxic hepatotoxins

Table 4. (*Continued*)

Toxin	Taxon (Taxa)	Structure	Effects
Cyanoviridin	*Microcystis* taxa of uncertain status (*M. botrys, M. viridis*)	Hepatopeptide (MW 1039)	Cytotoxic hepatotoxin.
Cylindrospermopsin	*Aphanizomenon ovalisporum, Cylindrospermopsis raciborskii, Umezakia natans*	Cyclic guanidine alkaloid (MW 415)	Cytotoxic renal and hepatotoxin; inhibits glutathion synthesis and protein synthesis; causes widespread and progressive (sometimes partially delayed) tissue injury, with cell necrosis in liver, kidneys, adrenals, lung, heart, spleen and thymus (mice). Cause of acute hepatoenteritis and renal damage among an Aboriginal population (Australia).
Demethoxy-cylindrospermopsin	*Cylindrospermopsis raciborskii*	Cyclic guanidine alkaloid	Cytotoxic renal and hepatotoxin.
Endotoxins	*Anacystis nidulans Cyanothece [Synechococcus] nägelli*	Lipopolysaccharides Galactoparanosyllipid	Cytotoxin–pyrogenic (fever-causing) agent; contact irritant, allergen and gastrointestinal irritant in humans (can be lethal to mice).
Hapalindole A	*Hapalosiphon fontinalis*	Substituted indole alkaloid	Lipophilic cytotoxin, inhibitory to algae, fungi.
Homoanatoxins	*Oscillatoria acutissima, O. formosa (Phormidium formosum)*	Secondary amine alkaloids (methylene-anatoxin-A)	Neuromuscular blocking agent, similar to anatoxin-A.
Hormothamnin A	*Hormothamnion enteromorphoides*	Cyclic undecapeptide	Cytotoxic hepatotoxin.
Laxatoxin	*Anabaena laxa*	Cyclic peptides (A, MW 1350-1400; B, MW 1150-1290)	Cytotoxic hepatotoxins.
Lyngbyatoxin	*Lyngbya majuscula*	Alkaloids	Similar activity as aplysiatoxins (e.g., Lyngbyatoxin-A).
Microcystins[d]	*Anabaena flos-aquae, A. circinalis, A. lemmermanii*, other *Anabaena* spp., *Anabaenopsis milleri, Hapalosiphon hibernicus*,[e] *Microcystis aeruginosa, Microcystis* spp. of uncertain affinity *(M. botryis, M. viridis), Nostoc rivulare, Nostoc* sp., *Oscillatoria limosa, O. aghardii/rubescens* group[e]	Heptapeptides (MW 994-1044)	Hepatotoxins (lipophilic); irreversible inhibitors of types 1 and 2A serine protein phosphatases in the liver; potent tumor promoters; cause diarrhea, pallor mucus membranes, vomiting, weakness; death from intrahepatic hemorrhage (microcystin-LR = most potent liver carcinogen known; also affects kidneys, lungs). Chronic reproductive impacts indicated (10% of young mice tested with hippocampal injury and reduced brain size); cause dose-related increase in chromosomal breakage (human lymphocytes).
Nodularin	*Nodularia spumigena*	Pentapeptide (MW 824)	Neurotoxin, hepatotoxin — promotes perchronic neurotoxicoese, gastrointestinal disturbances, cutaneous or respiratory irritation; active liver injury. Potent tumor promotor (liver, bronchogenic, abdominal carcinomas; uterine adenocarcinomas; thoracic lymphosarcomas); strong inhibitor of 1,2A protein phosphatases. Similar to microcystin in both acute and chronic effects; can be as potent as microcystins in causing liver pathology.
Saxitoxins[f]	*Anabaena circinalis, Cylindrospermum raciborskii, Lyngbya wollei, Oscillatoria* sp.	Purine alkaloids	Neurotoxins — bind specifically to the voltage-sensitive Na^+ channel; block Na^+ ion influx in neurons and action potential of cardiac muscle.

(*continued overleaf*)

Table 4. (*Continued*)

Toxin	Taxon (Taxa)	Structure	Effects
			No reports of human health effects from ingestion of drinking water containing cyanobacteria with saxitoxins. Sheep died after ingesting water laden with a bloom of saxitoxin-containing *Anabaena circinalis*.
Scytophycin	*Scytonema pseudohofmanni*	Methylformamide A (MW 821)	Lipophilic cytotoxin (e.g., against human epidermoid carcinoma, mouse fibroblasts); active against intraperitoneally implanted lymphocytic leukemia, lung carcinoma
Tubercidin	*Tolypothrix byssoidea*	Pyrrolopyrimidine	Cytotoxin.
Unidentified, volatile sulfur compounds	*Microcystis aeruginosa, M. wesenbergii*	—	Cytotoxins.
Uncharacterized neurotoxin	*Pseudanabaena catenata*	—	Causes violent convulsions when injected intraperitoneally in mice.
Polybrominated secondary metabolites (2-A)	*Oscillatoria spongelia* (in marine sponage *Dysidea herbacea*)	—	Inhibitory to a range of gram-positive and gram-negative bacteria; deter feeding by generalist fishes.
Uncharacterized cytotoxins	**Anabaena hassalii,* **A. spiroides*. var. *contracta,* **A. variabilis,* **Coelosphaerium kuetzingianum, F. muscicola* ('Fischerellin'), *Gloeotrichia echinulata,* **Gomphosphaeria lacustris,* **G. nägeliana, Nostoc linckia,* **N. paludosum, N. zetterstedtii, Scytonema mirabile*	—	Some with anti-microbial properties (e.g. lethal to protozoans, ostracods, and other potential grazers). Taxa with an asterisk (*) indicate field cases of intoxications.

[a] *Aphanizomenon flos-aquae* toxicity recently has been questioned by Carmichael (87). AphantoxinII closely resembles saxitoxin.

[b] *Lyngbya majuscula* sometimes grows epiphytically on edible algae in Indonesia and the Philippines.

[c] The information on effects of ciguatoxins is mostly from research on the ciguatoxins of dinoflagellates. Ciguatoxins have also been experimentally shown to cause peripheral nervous system damage; stimulate hormone and neurotransmitter release; activate protein kinases; and cause joint pain, miosis, erethism, cyansis, prostration, nausea, vomiting, diarrhea, low blood pressure, bradycardia (85).

[d] Also known as *Planktothrix agardhii, P. mougeotii, P. rubescens*.

[e] More than 60 variants (analogs) of microcystin have been characterized, differing mostly in the type of L-amino acid present; and in the presence/absence of a methyl group on one of the D-amino acids, or on a dehydroamino acid, or both. The basic difference between hepatotoxic and nontoxic *M. aeruginosa* (PCC7806) is the presence of one or more genes encoding for microcystin synthetases. Microcystins act similarly as another group of cyclic peptides, the hepatotoxic heptapeptides called phallotoxins (e.g., phalloidin) of the mushroom *Amanita phalloides*, the green death cap. Microcystin-LR, the best known analog, does not readily cross cell membranes and, therefore, does not enter most tissues. After oral uptake, it is transported across the ileum into the bloodstream through a bile acid-type transporter, and becomes rapidly localized in the liver via active uptake by hepatocytes. Resulting severe liver damage is characterized by disruption of liver cell structure, a loss of sinusoidal structure, increased liver weight from intrahepatic hemorrhage, hemodynamic shock, heart failure, and death. Intranasal installation of microcystin-LR in mice resulted in extensive necrosis of the epithelium of the nasal mucosa of both the olfactory and respiratory zones, progressing to destruction of large areas of tissue down to levels of deep blood vessels. Cumulative liver damage was demonstrated with repeated sublethal dosing. Microcystins and nodularin are regarded as tumor promoters because of their potent inhibition of protein phosphatase, and act similarly (and with similar potency) to other tumor promoting toxins such as okadaic acid, tautomycin, and calyculin (2-DS,85).

[f] Saxitoxins from dinoflagellates that accumulate in shellfish seafood have caused irregular breathing, twitching, and loss of coordination in humans, and death by respiratory failure. These toxins can cause peripheral and central nervous sysstem dysfunction. Eighteen saxitoxins are represented in the cyanobacteria, with neosaxitoxin considered separately as Aphantoxin (5).

microorganisms (e.g., some zooplankton), but not others. In the open oceans, *Trichodesmium* blooms have been linked to death of fish, oysters, and crabs, and tuna have shown bloom avoidance behavior (2).

Fish that are injected or force-fed cyanotoxins develop similar symptoms as laboratory mammals (Table 4), but natural routes of exposure should be considered to evaluate the potential for impacts. Immersion into toxic cyanobacterial blooms adversely affected some fish in experimental tests, but with high variability among fish species in sensitivity. Impacts have included damage to liver, heart, kidney, gills, skin, and spleen (5). Dead fish collected from senescing/dying cyanobacterial blooms had significant damage of the gills (perhaps from the high pH caused by cyanobacterial photosynthesis, and because of high ammonia from cyanobacterial decomposition), digestive tract, and liver (93). Gill damage would enhance microcystin uptake and subsequent

liver necrosis. European carp (*Cyprinus carpio*) that were exposed to microcystins under natural conditions developed atrophy of hepatocytes, gills with pinpoint necrosis, epithelial ballooning, folded lamellar tips, exfoliation of lamellar epithelium, and elevated aspartate aminotransferase activity and serum bilirubin levels –all consistent with impaired hepatocyte function (94). In coastal British Columbia and Washington state (U.S.A.), net-pen-reared Atlantic salmon smolts (*Salmo salar*) that were exposed to microcystin (probably as a feed contaminant) developed liver degeneration ("Net Pen Liver Disease"), which caused major economic loss to mariculture (5,87). Recent research has also confirmed that *M. aeruginosa* microcystin-LR can cause mass mortality of cultured catfish, the largest component of pond-based aquaculture in the United States, through feeding on planktonic diets or passive assimilation through the gills (95).

Human exposure to cyanotoxins has occurred via consumption of finfish, shellfish, or other animals that had bioaccumulated the toxins (e.g., shellfish–nodularin, saxitoxins) (5). Because the cyclic peptide and alkaloid toxins of most potential concern mostly accumulate in animal viscera, to err on the side of caution, viscera should not be consumed. More frequently, humans are exposed to toxic blooms via direct contact, ingestion, or aerosol inhalation has promoted acute, noncumulative health effects including eye and ear irritation, mouth ulcers, nausea, vomiting, diarrhea, fever, cold or flu symptoms and fever (96). These impacts, although causing discomfort, have not been associated with serious health outcomes to date. In lakes affected by cyanobacterial blooms, water-contact sports involving submersion of the head (e.g., diving) or aerosol inhalation (e.g., water skiing), inhalation and resorption through nasal and pharyngeal mucous membranes can present a high risk of exposure. A density of 20,000 *Microcystis* cells/mL (corresponding to 10 μg chlorophyll *a*/L) can contain 2 to 4 (<10) μg microcystins/L (5). This level is close to the WHO provisional drinking water guideline value of 1 μg microcystin-LR/L, intended to be safe for life-long consumption (97). Thus, serious outcomes from such exposure during water sports would be unlikely.

However, to err on the side of caution, recreationists should avoid waters with obvious discoloration potentially indicating high cyanobacterial concentrations. Cyanobacterial blooms can reach maximal densities of millions and, in extreme cases, billions of cells/mL (26). A density of 100,000 toxic *Microcystis* cells/mL (c. 50 μg chlorophyll *a*/L) was recommended (5) as a guideline for a moderate health alert in recreational waters would be expected to contain up to 50 μg microcystin/L. Twice that amount or more (up to 200–400 μg microcystin/L) would be probable if toxic *Oscillatoria agardhii* was present rather than *Microcystis* (5) (Table 4). A 15-kg child who consumed 250 mL of water during extensive play could be exposed to 10-fold more toxin than the recommended tolerable daily intake for human health protection (94). Moreover, *Microcystis* and certain other planktonic, toxic cyanobacteria commonly form thick surface deposits or scums, where densities can be up to a million-fold higher than in water-column suspensions — significantly increasing the risk of high toxin exposure. A large body of evidence exists for potentially severe health hazards from human exposure to toxic cyanobacterial scums, which can contain up to 24 mg microcystin/L (5). Given that the oral LD_{50} (dose at which half of the test mice die) of microcystin-LR in mice is 5,000–11,600 μg toxin/kg body weight, ingestion of ≤2 mg of microcystin by a 10-kg child could cause liver injury (5). Thus, ingestion of only a small amount of scum during recreational activities would be required. Fortunately, no human fatalities have been unequivocally associated with (low-volume) oral ingestion of scum, although many animals have been killed by drinking (larger quantity of) scum-laden water. Moving from lakes to marine shores, some marine beaches have been associated with incidents of severe human dermatitis from benthic-mat cyanobacteria (*L. majuscula*, also *Schizothrix, Oscillatoria* spp.) following storms that dislodged clumps of the mats into the water (2,5).

Information is generally lacking in most countries (including the United States) to evaluate the extent to which chronic human illness may occur from cyanotoxin exposure, because microcystins are not measured or considered in most guidelines for potable drinking water (see CYANOBACTERIA-TOXINS IN DRINKING WATER, in this Encyclopedia). Thus, most nations have not set limits or guidelines for cyanotoxin exposure (exceptions–Australia; in the United States, Oregon (5)). The World Health Organization [WHO] has recommended a limit of 1 μg microcystin-LR/L drinking water (as total cell-bound + extracellular microcystins) to protect public health (97). Supporting animal and human toxicity data are incomplete for most cyanotoxins with exception of microcystin-LR; thus, the WHO limit was based on information available for that toxin, as a provisional guideline to be adjusted as additional data may become available.

Recorded cases of human gastrointestinal and hepatic illness linked to cyanobacterial toxin exposure via potable water supplies have occurred during natural or copper sulfide treatment–induced dissipation of a bloom with extensive cell lysis that released cyanotoxins (e.g., United States, towns along the Ohio River, 1931; Zimbabwe, city that used a cyanobacteria-laden reservoir for potable supplies, 1966). In Australia (1979) after exposure to toxin from *Cylindrospermum raciborskii* in a water supply reservoir, 140 aboriginal children and 10 adults suffered from diarrhea, severe kidney damage and liver damage; approximately 70% of the patients required intravenous therapy and more severe cases went into hypovolaemic/acidotic shock, but all recovered with treatment (5). In China (mid-1990s), chronic liver disease in certain villages (including cancer, related to liver tumor–promoting microcystins; Table 4) was related to chronic exposure to toxic cyanobacteria (5). Epidemiological evidence suggested (but without definitive proof) that in those villages, microcystins from poor drinking water supplies acted with hepatitis B virus to promote liver cancer (see CYANOBACTERIA-TOXINS IN DRINKING WATER, in this Encyclopedia for additional information). Cyanobacterial toxins, like other toxins, most severely impact people who are innately more susceptible

such as children, the elderly, and immunocompromised people of all ages. A total of 88 deaths, mostly children, occurred during a severe gastroenteritis epidemic (Brazil, late 1980s), related to microcystin exposure in drinking water from a reservoir with a dense *Microcystis*/*Anabaena* bloom. Also, in Brazil (mid-1990s), "Caruaru syndrome" was the name given to the symptoms of severe hepatitis that were sustained by 117 patients on hemodialysis treatment, which would have represented "worst-case" or extreme toxin exposure. Of these, 100 patients developed acute liver failure and 49 died. The patients had received microcystin, concentrated through the treatment process, from a water supply containing a dense *M. aeruginosa* bloom (5).

Thus, various studies of human populations have yielded epidemiological evidence relating adverse health impacts to the presence of cyanotoxins in recreational or drinking waters. However, with rare exceptions, these studies have been retrospective and lack complete epidemiological data, especially regarding exposure (cyanobacterial taxa, densities, duration/frequency of exposure, etc. (2)). Although acute exposure can cause death from liver hemorrhage or liver failure, such incidents fortunately can be minimized by avoiding use of water with noticeable cyanobacterial blooms as potable supplies (see CYANOBACTERIA-TOXINS IN DRINKING WATER, in this Encyclopedia). In contrast, risk of long-term exposure to relatively low concentrations of cyanotoxins (especially cyclic peptides, which can promote malignant tumors) in drinking water is more difficult to avoid, because treatment procedures in many water supply plants do not remove these toxins, and also because there would be no noticeable discoloration.

Other bioactive substances produced by cyanobacteria (*Anabaena, Aphannizomenon, Lyngbya, Microcystis, Nostoc, Oscillatoria, Phormidium, Schizothrix, Symploca*) cause serious taste-and-odor problems for water supply plants (5). These compounds emit "earthy" or "musty" odors, especially geosmin (*trans*-1,10-dimethyl-*trans*-2-decalol) and 2-methyl isoborneol, which sensitive people can detect at levels as low as 10 ng/L (5).

Much has been published on adverse effects of cyanotoxins, but considerably less on their value. Cyanobacteria produce many bioactive substances with potential anticarcinogen activity and other medicinal value (85). This aspect of cyanobacterial research has, as yet, received relatively little attention but is gaining in general interest. Certain cyanobacteria (e.g., *Spirulina platensis , S. maxima*) consistently have not been found to be toxic and are mass-cultured for their high-protein content. Thus, they have been economically important to the aquaculture industry in some countries (92). *Spirulina* contains 60 to 70% protein and 50% nucleic acid (dry weight basis). In addition to its high nutritional quality; it has also been used as a diet pill; a few grams taken 30 to 60 minutes before meals causes marked reduction in appetite.

FUTURE DIRECTIONS

With many bacteria-like features, but with the universal plant pigment and higher plant photosynthetic biochemistry, these "primitive," complex organisms provide an excellent illustration of the adage, "Nature mocks at human categories." Their taxonomy has been in disarray for the past approximately 30 years, with various revisions of the historic botanically named species co-occurring with bacterial designations throughout the botanical, bacteriological, general microbiological, and medical literature. Because pure cultures and strain numbers are not yet available for many of the [former] botanically designated species (in part because of culturing difficulties), this confusing mix of taxonomic schemes will continue to characterize the field for some time. Bacteriological naming procedures depend upon such pure cultures, but from ecological, physiological, biochemical, genetic (e.g., polyploidy, gene loss), and even morphological perspectives, extreme caution is warranted because many microorganisms, including the cyanobacteria, have been shown to significantly change when cultured long-term (months to years). Long-term reference cultures frequently become highly aberrant over time in culture (9). It is recommended that cyanobacterial researchers take the necessary steps to determine whether "reference" cultures have significantly changed over time in traits of interest. Otherwise, findings and conclusions about these organisms in many important research areas will probably drift increasingly far from the realities exhibited by natural populations.

Although intensive research has been conducted on the physiology and ecology of benthic mat- and bloom-forming cyanobacteria, major questions remain to be resolved about these and other cyanobacterial communities. As examples, cyanobacteria produce many anticarcinogenic and other potentially beneficial compounds, and research is needed to improve techniques for isolating these substances and harnessing their medicinal value. In summarizing his excellent review on cyanobacterial symbioses, Adams (2) expressed that many symbiotic associations probably remain to be discovered. The identity of many host chemical signals and of the genes they regulate in cyanobionts are presently unknown, but molecular techniques are rapidly advancing insights that could yield substantial benefit — for instance, an understanding of the regulatory systems involved in establishment and maintenance of plant/cyanobacterial symbioses may enable enhancement of existing symbioses, or of establishing novel associations with agriculturally important plants to increase yield (2). Given increased recognition of the importance of cyanobacteria in the world's oceans and lakes, the significance of lysogeny in cyanophages merits additional research (2). Although the movement of cyanobacterial DNA by viruses (transduction) has not yet been demonstrated, there is evidence that lysogenic cyanophages integrate into the host genome. Lysogenic cyanophages may be important as vectors of genetic information within cyanobacterial communities.

Noxious, bloom-forming cyanobacteria in lakes and certain brackish waters produce among the most potent toxins known, adversely affect a range of aquatic organisms and terrestrial mammals including humans. Their ecological impacts will probably increase via stimulation of toxic cyanobacterial taxa by nutrient pollution,

which is increasing in many countries (84). Examples of successful management of noxious cyanobacterial blooms that have already formed are rare, and historic strategies (e.g., application of copper sulfate or other killing agents) does not eliminate the organisms because some are able to form resistant structures, representing an inoculum for the next bloom. At the same time, application of copper sulfate or other pesticides can cause lysis of the biomass, leading to higher risk for health impacts to humans and other affected organisms from the toxins that are released (5). Major questions remain as well about the toxins themselves, including synthesis pathways, environmental factors controlling toxicity, and the full range of chronic and acute effects.

Cyanobacteria have been among the most successful organisms on earth over geologic time, and present-day species are both widespread and of major ecological and economic importance. Strengthened understanding of genetic and environmental controls on their growth, survival, and bioactive substance production will continue to yield information of benefit to humans with applications ranging from environmental management, to crop yield, to improved medical treatment and protection of public health.

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CYANOBACTERIA IN SOILS.

See SOIL BACTERIA

CYANOBACTERIA-TOXINS IN DRINKING WATER

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The safety of drinking water is of paramount concern to the community. The presence of any known toxic compound in water for human consumption has to be regarded as a potential hazard to health. Although deaths of farm livestock caused by consuming toxic cyanobacteria in their drinking water were well described in 1878 (1), the implications of these organisms for the health of the human population are still being evaluated (2). Outbreaks of gastroenteritis among consumers of particular water supplies have been attributed to cyanobacterial toxins in the supply in the United States, South America, Europe, and Australia (3). The most severe cases of poisoning by cyanobacterial toxins in water occurred in dialysis patients in Brazil, who were exposed to the toxins in the dialysis fluid, as a result of which more than 50 people died (4,5).

The chemistry and toxicology of the active compounds from cyanobacteria have been investigated and the toxins include neurotoxins, hepatotoxins and widely cytotoxic compounds. As cyanobacteria are gram-negative organisms they also produce lipopolysaccharide endotoxins, about which relatively little is known. Allergic reactions to cyanobacteria are relatively common, but have only been identified from the colored photosynthetic protein phycocyanin (6). There is increasing evidence to prove that some cyanobacterial toxins can initiate or increase the growth of cancer (2).

The production of toxins occurs in a range of cyanobacterial genera, which are distributed worldwide. Drinking water supply reservoirs in many parts of the world are increasingly affected by water blooms of toxic cyanobacteria as the availability of nutrients to the organisms rises. This has resulted from intensive agriculture, population increase, urban growth, and water reuse, which are characteristic of present-day human society.

As a result of the increasing risk of injury to health from cyanobacterial toxins, the World Health Organization (WHO) has determined a safe Guideline Value (GV) in drinking water for one of the most common toxins (microcystin-LR) and is considering GV for several other toxins. The water supply industry has already evaluated treatment options for removal of these toxins, and more advanced water treatment plants can now reduce the concentrations to below the GV (3). Simple chlorination and flocculation treatment has not been effective in removing cyanobacterial toxins from drinking water (7,8).

Epidemiological studies of the effect of low concentrations of cyanobacterial toxins in drinking water supplies on human health have only recently begun (9). This area of research is vital if the evidence from animal experiments of potential human health risks from cyanobacterial toxins is to be evaluated and the data applied to the provision of safe water supplies.

THE CYANOBACTERIA

Fossil evidence suggests that cyanobacteria were among the earliest life-forms on the planet as the cyanobacterial colonies known as *stromatolites* can be found in rocks from over 3,000 million years ago to the present day (10).

These prokaryotic microorganisms possess chlorophyll-a. They carry out oxygen-liberating photosynthesis with photosystems I and II in a manner similar to eukaryotic plants (11). Their distinctive colors, which range from blue-green through green, yellow, and orange to red, are due to varying amounts of accessory photosynthetic pigments. These carotenoids, phycocyanins, and phycoerythrins assist in light trapping under the low light conditions that occur in deep or turbid water (12).

Cyanobacteria are morphologically a very diverse group, growing as single cells, chains of cells, three-dimensional arrays of cells, and single or stacked filaments, with or without a sheath surrounding the cells. Their tolerance of temperature extends from hot springs to under the Antarctic ice, with habitats from desert rocks to the sea (13). As sources of toxins in drinking water, the planktonic species that float freely in water are the most significant, although benthic species attached to sediments or submerged rocks may be equally toxic.

Cyanobacteria multiply in reservoirs, lakes, and slow-flowing rivers during the summer period of warmer surface water and temperature stratification of the water column. Stratification reduces the vertical movement of water owing to the colder, denser water remaining in the deeper levels of a lake. Due to less oxygenation these levels become anaerobic, with liberation of nutrients from the sediments. The phytoplanktons of a lake include eukaryotic and prokaryotic microorganisms. Their relative abundance will depend on competitive interactions. The toxic cyanobacteria compete most effectively through their regulation of buoyancy, which enables them to move up and down in the water, optimizing energy harvesting in the shallower layers and then sinking into the nutrient-rich deeper water to replenish phosphate reserves (14).

This capability for vertical movement is a consequence of the possession of gas vacuoles by many cyanobacterial species. The balance between sinking and rising is dependant on the carbohydrate status of the cells, counterbalancing the low density of the gas vacuoles. Thus during the day, starch will accumulate to the point that the cells sink, followed by nutrient uptake from the colder higher nutrient layers below during the night. Cell growth and division utilize the carbohydrate reserve, lowering cell density and allowing the cells to rise again (14).

Many species of cyanobacteria have heterocysts, which are specialist nitrogen-fixing cells. This enables them to compete effectively with eukaryotic green algae when water nitrate or ammonia is low (14).

Thus, during the summer when the warm surface layers of water are often depleted of nutrients, cyanobacterial growth can overtake the competing eukaryotic algae to the extent of forming water blooms. These are named from the brightly colored surface scums, which may form during the night in calm weather. Cell concentration in a water body overall may only be 20,000 cells/ml, which is just sufficient to see a greenish or reddish stain in the water. Cells rising to the surface become trapped, and cell concentration may reach greater than 1,000,000 cells/ml. Once formed, these scums accumulate in bays and shallow shorelines and may persist for weeks or months. Cyanobacterial scums are often of toxic species and present a particular risk to both domestic animals and human water users (15). This is discussed later in the article.

The public perception of cyanobacteria — blue-green algae as they are popularly called — is largely a consequence of the increasing frequency of scums in recreational waters and drinking water reservoirs. This increase in scums is a direct consequence of the increase in nutrient availability in freshwater, particularly of phosphorus and nitrate. Use of phosphatic fertilizer in crop production increases the leaching of phosphorus into rivers, and soil erosion in drier countries accentuates this process. Intensive animal production leads to large quantities of fecal material that is returned to the fields from piggeries, feedlots, dairy, and poultry units, which in wet weather increases the nutrient loads entering waterways. Added to this are nutrients from sewage processing plants

that are major components of the eutrophication of waterways. It is estimated that 53% of European lakes and 48% of North American lakes are now eutrophic, often with cyanobacterial dominance (16). In more advanced countries, removal of phosphate from sewage and remediation of lakes are increasingly carried out, coupled (in Canada, for example) with the mandatory domestic sale of phosphate-free detergents. However, reversal of the increased incidence of cyanobacterial scums is not quick because of the continued resolubilization of phosphate from sediments (14).

TOXIC SPECIES AND GENERA

The relationship between toxicity and the classification of the cyanobacteria provides a general guide to the potentially hazardous species, with many fascinating anomalies. For the purpose of water supply authorities, the frequency of toxic water blooms among the total of blooms is sufficient for all blooms to be regarded as potentially poisonous. For example, in Finnish waters, 44% of blooms were lethally toxic under the test conditions and hepatotoxic blooms were twice as common as neurotoxic blooms (17). Subsequent development of sensitive chemical and immunological tests has raised that percentage to 72% of 533 samples from Germany containing hepatotoxins alone (18).

A list of toxic cyanobacteria in fresh water that have been identified so far comprises approximately 20 species from ten genera (19). These were initially classified according to morphological criteria, with considerable success (20). More recently, molecular genetic techniques have largely confirmed the morphological taxonomy (21,22). Toxic cyanobacteria have a worldwide distribution, and the lack of records from a particular country indicates a lack of data, rather than the absence of cyanobacteria. Examples of identified toxic cyanobacteria in different countries, chosen to show diversity of location and toxins, are given in Table 1.

The most abundant toxic species with a worldwide distribution is a colonial spherical-celled organism named *Microcystis aeruginosa*. Figure 1a illustrates this species, from a sample taken from an Australian river (the Hawkesbury), used for water supply and for recreation. There are three related species in the genus that are also toxic, *M. viridis*, *M. wesenbergii*, and *M. botrys*. These cyanobacteria produce a family of cyclic peptide toxins named microcystins, which are discussed in detail later (19). The cells of this genera form approximately spherical aggregates of cells surrounded with mucilage, 2–3 mm in diameter. They can be clearly seen by the naked eye in water samples. The cells contain gas vacuoles and at night they can form floating scums under suitable warm, calm weather conditions, which collect on downwind shores. In water supply dams, scums of this and other species are frequently seen near the main dam wall, close to the off-take, during a bloom. Scums also accumulate in pools upstream of weirs, which are the site of water intakes (Fig. 2).

Table 1. Examples of the Worldwide Distribution of Toxic Cyanobacteria, the Species or Genera Involved, and Their Toxins

Location of Toxic Cyanobacteria	Genera or Species Reported	Toxins	Reference
Australia	*Anabaena circinalis*	Saxitoxins	24
	Microcystis aeruginosa	Microcystins	25
	Cylindrospermopsis raciborskii	Cylindrospermopsin	26,27
	Nodularia spumigena	Nodularin	28
Brazil	*Microcystis aeruginosa*	Microcystins	29
	Aphanocapsa cumulus	Microcystins	30
Canada	*Microcystis aeruginosa*	Microcystins	31
China	*Microcystis aeruginosa*	Microcystins	32
Denmark	*Microcystis aeruginosa*	Microcystins	33
	Planktothrix agardhii	Microcystins	33
Finland	*Nodularia spumigena*	Nodularin	34
	Nostoc spp.	Microcystins	35
Germany	*Anabaena* spp.	Anatoxin-a	36
	Aphanizomenon spp.		
	Planktothrix agardhii	Microcystins	18
	Planktothrix rubescens	Microcystins	18
Israel	*Aphanizomenon ovalisporum*	Cylindrospermopsin	37
Japan	*Microcystis aeruginosa*	Microcystins	38
	Umezakia natans	Cylindrospermopsin	39
UK	*Microcystis aeruginosa*	Microcystins	40
	Oscillatoria spp.	Anatoxin-a	41
USA	*Anabaena flos-aquae*	Anatoxin-a	42
	Anabaena flos-aquae	Anatoxin-a(S)	43
	Aphanizomenon flos-aquae	Saxitoxins	44
	Microcystis aeruginosa	Microcystins	45

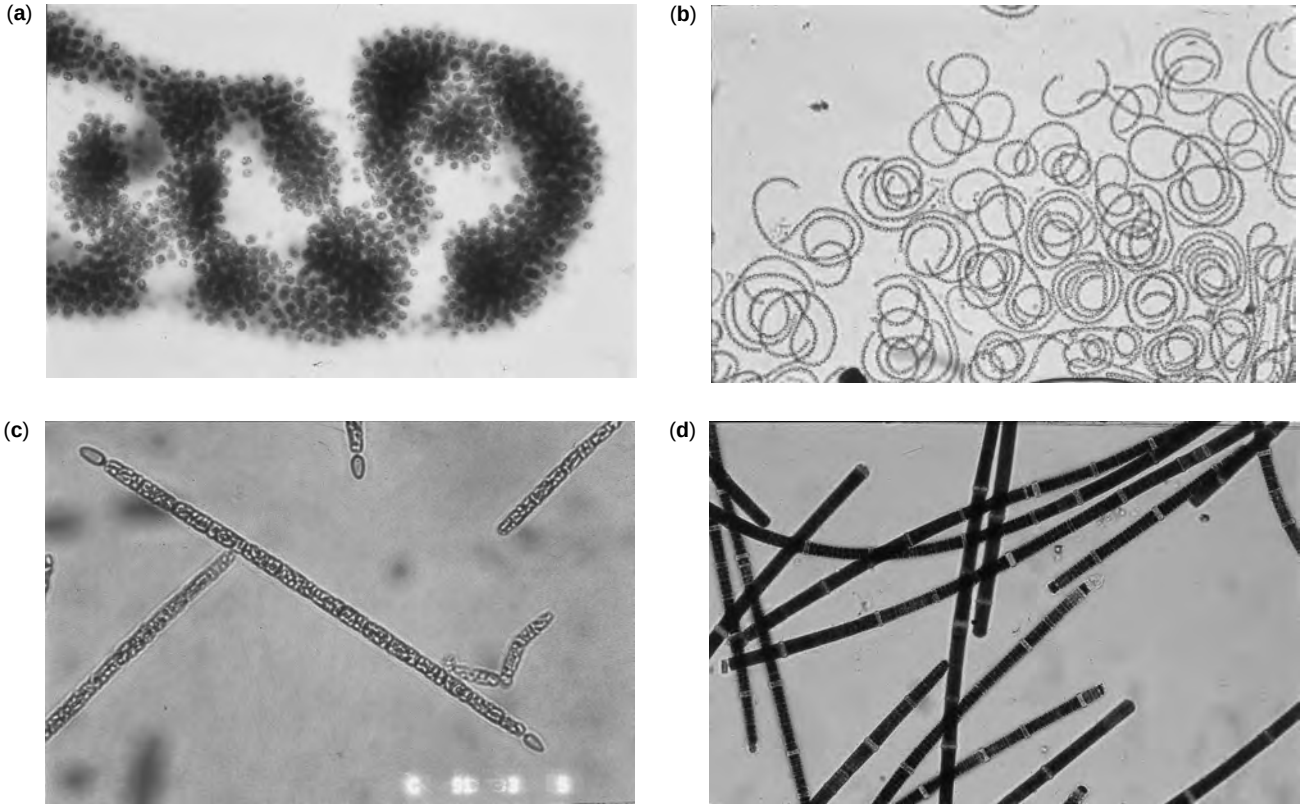

Figure 1. Toxic species of cyanobacteria. (**a**) *Microcystis aeruginosa*, (**b**) *Anabaena circinalis*, (**c**) *C. raciborskii*, (**d**) *Nodularia spumigena*. See color insert.

The next most abundant toxic genus is *Anabaena*, which has many species producing a range of toxins. The *Anabaena's* form filaments of cells, often coiled, of varying sizes (Fig. 1b). The filaments of vegetative cells also include nitrogen-fixing heterocysts and akinetes, which are large resistant spores. The cells contain gas vacuoles and blooms of *Anabaena* frequently form surface scums near dam walls (Fig. 3). The species of *Anabaena*, which have been shown to be toxic, are listed in Table 2, together with their toxins.

As can be seen from Table 2, samples of *Anabaena* from different sources possess toxins found in the *Microcystis* genus, as well as several different neurotoxins not found in *Microcystis*. This is one of the puzzling features of toxic cyanobacteria, as the same species from different locations can synthesize quite different toxins. The *Microcystis* toxins are synthesized through non-ribosomal peptide synthesis, similar to the production of cyclic peptide antibiotics. The genes have been identified as linked in a peptide synthetase operon, which contains the genetic code for epimerase activity as well as amino acid linkage and a polyketide synthetase gene (21,53). The resulting peptide contains D- and L-amino acids in a seven amino acid ring.

The same species of *Anabaena* can completely lack this peptide toxin synthesizing capacity and produce alkaloidal neurotoxins from separate genetic information. It is not clear whether the transfer of the large genetic elements required for toxin synthesis can take place through plasmid transfer between cyanobacterial species and genera or not. Within a species, strains that lack the genetic information for toxin production, therefore being nontoxic, can be isolated.

The completely different cyanobacterium *Planktothrix* (Oscillatoria) *agardhii* has isolates from different locations that contain Microcystis toxins and others with the neurotoxin anatoxin-a (18,54). This species is abundant in the cool, temperate, and subarctic regions of Europe, and is capable of surviving under ice during the winter. The red form *P. rubescens* is particularly adapted to low light intensities and cold lakes, with high concentration of *Microcystis* toxins. *Planktothrix* grows as smooth filaments that may develop as dense layers deep in clear lakes, at the boundary between the oxygenated and anaerobic layers. In the autumn, it becomes buoyant and can then form surface scum (13).

Another species that forms bands of cells well below the surface is the subtropical filamentous cyanobacterium *Cylindrospermopsis raciborskii* (Fig. 1c). The species became particularly known as a result of a major human poisoning event, which is described later (25,55). The organism has a worldwide distribution but produces different toxins from geographically separated isolates. The isolate responsible for the human poisoning was from Australia and the toxin was identified as a cytotoxic alkaloid. This alkaloid has also been found in the species in Hungary and the United States (Florida) (56,57). By contrast the same species in Brazil produces paralytic

Figure 2. Weir pool with a dense scum of toxic *Anabaena circinalis* showing the drinking water intake. See color insert.

shellfish poisons (58). *Cylindrospermopsis raciborskii* does not have gas vacuoles and does not form scums. As it forms dense layers below the surface in stratified lakes, the first warning that it is present may be the blocking of filters in drinking water treatment plants, which normally draw water from below the surface.

In brackish water estuaries and seas, another filamentous cyanobacterium has caused poisoning of livestock and pets. This is *Nodularia spumigena*, with a worldwide distribution (Fig. 1d). The largest areas of cyanobacterial

Figure 3. Dam wall showing dense scum of *Microcystis aeruginosa*. See color insert.

bloom ever observed are in the Baltic Sea, and are a result of proliferation of this organism. Ships tracking through the Baltic in summer may leave a clear wake in the scum, which can be seen from the air at 10,000 meters altitude, and the extent of the scum can be seen by satellite monitoring from space (59). Because estuarine waters are frequently used for recreation, this species has a major negative economic impact on areas where it occurs. In Western Australia, the State Government funded the cutting of a canal at a cost of approximately $40 million to successfully reduce *Nodularia* blooms in a favorite recreational area south of the State capital (60).

A range of other toxic cyanobacteria exist, and it is likely that new species will be identified as toxic in the future. A comprehensive list is provided by Sivonen and Jones (19).

CYANOBACTERIAL TOXINS

From early veterinary observations on the deaths of farm animals from the consumption of scums of toxic cyanobacteria, it was apparent that two totally different types of toxicity were being observed. In one type, typical of the consumption of scums of *Microcystis*, animals died over several hours or a few days, with postmortem evidence of massive liver damage (61). In the other type, resulting from the consumption of scums of *Anabaena flos-aquae* and many other species, the animals died rapidly, showing neurological symptoms with little postmortem evidence of injury (41).

Cyanobacterial toxins have therefore been classed as hepatotoxins (liver poisons) or neurotoxins (nerve poisons).

Table 2. Species of Anabaena That Have Been Shown to Be Toxic and Their Toxins

Toxic Species	Toxin	Location	Reference
Anabaena circinalis	Paralytic shellfish poison (saxitoxins) (neurotoxins)	Australia	24
Anabaena circinalis	Microcystins (hepatotoxins)	Finland, France	17,46,47
Anabaena flos-aquae	Microcystins (hepatotoxins)	Norway	48,49
Anabaena flos-aquae	Anatoxin-a (neurotoxin)	United States, Germany, Ireland	36,50,51
Anabaena flos-aquae	Anatoxin-a(s) (neurotoxin)	United States	52
Anabaena lemmermannii	Anatoxin-a(s) (neurotoxin)	Denmark	53

Table 3. Comparative Toxicities of Cyanobacterial Toxins From (2)

Cyanobacterial Toxin	Lethal Dose (LD_{50}) per Kg Bodyweight	Time to Death at Minimum Dose	Type of Toxicity
Microcystin-LR	60 μg/Kg	12–24 hours	Hepatotoxicity
Nodularin	70 μg/Kg	12–24 hours	Hepatotoxicity
Anatoxin-a	200 μg/Kg	10–30 min	Neurotoxicity
Anatoxin-a(s) 20 μg/Kg		10–30 min	Neurotoxicity
Paralytic Shellfish 10 μg/Kg saxitoxin		20–60 min	Neurotoxicity
Poisons	Others Higher Doses		
Cylindrospermopsin	200 μg/Kg	4–7 days	Widespread organ damage, incl. Hepatotoxicity

With increasing understanding of both the chemistry and the toxicology, the detailed mechanisms of poisoning are becoming clearer. The result is that the hepatotoxins can be separated into poisons specifically targeting the liver and those that are general cytotoxins, damaging all types of active cells. The neurotoxins can be classed as nerve channel blocking compounds, such as paralytic shellfish poisons, neuromuscular synaptic blocking or activating poisons, and anticholinesterases, similar to organophosphorus insecticides.

The cyanobacterial toxins are highly potent poisons, lethal at doses of micrograms per kg of bodyweight (Table 3). The majorities are stable chemical compounds, resistant to boiling and not removed by conventional water treatment. Consequently, there have been outbreaks of human poisoning from cyanobacterial toxins through the consumption of drinking water from chlorinated supply systems.

Microcystis toxins are predominantly hepatotoxic and form a family of related molecules called microcystins (62). The structure is a peptide ring of seven member amino acids, with only two of the normal L-form. These two vary, and within any particular isolate there may be several variants. The most common are leucine/arginine, arginine/arginine, tyrosine/arginine. About 60 different forms have so far been identified (19). The total amino acids of the cyclic peptide are D-alanine-(L-acid)-D-methyl aspartic-(L-acid)-ADDA-D-glutamate-methyl dehydroalanine, where ADDA is 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Fig. 4a). The amino acid ADDA has only been found in related compounds and is essential to the toxicity of the whole structure. The explanation for selective toxicity of microcystins to the liver is that they do not readily enter mammalian cells and are concentrated in the liver hepatocytes by a mechanism similar to the bile acid transporter (63–64). Within the hepatocytes, microcystins selectively inhibit the enzymes protein phosphatase I and IIA, with the possibility of forming a covalent complex that permanently inactivates the enzyme molecule (65–67). One consequence of inhibition of these enzymes is the gross deformation of the cell structure caused by the disaggregation of cytokeratin fibers within the cell cytoskeleton (68). This leads to the cell injury observed in acute toxicity studies of experimental animals, which is followed by death of the animal from blood loss into the liver (24). Continued or chronic consumption of microcystin in drinking water leads to active liver injury and serum enzyme changes, showing liver damage (49,50,69,70). Human liver injury by microcystins in a drinking water supply was observed following a major bloom of *Microcystis* on a reservoir that was treated with copper sulphate as an algicide (7). In these circumstances the cyanobacterial cells lyse and the toxins are liberated into the bulk water, from which they are not removed by conventional filtration techniques (discussed later). A major outbreak of gastroenteritis in the area of the Itaparica Dam in Recife, Brazil, was traced back to the drinking water supply. Of the 2,000 cases reported, 88 died. No pathogens could be found, and families which used boiled water were also affected. Toxic metals or other pollutants could not be found, but a heavy bloom of mixed cyanobacteria occurred on the dam at the time. The outbreak was attributed to the cyanobacterial toxins present in the bloom (71).

More recently in Brazil, there were at least 50 deaths of dialysis patients from liver damage that could be traced to the presence of microcystins in the dialysis fluid. The carbon filters in the dialysis unit and the patient's blood and liver tissue showed microcystins at concentrations similar to those found in experimental lethal poisoning (4,5). Poisoning during renal dialysis is, however, an extreme case, as it is equivalent to giving an intravenous injection of toxin to patients who are already ill.

There is increasing experimental evidence of tumor-promoting activity of microcystins in cell culture systems and in rat carcinogenicity studies. In cell culture, low microcystin concentrations cause hepatocyte hyperplasia, with selective cell death and an inhibition of normal apoptosis (72). In rats and mice a series of studies have shown tumor promoting activity of microcystins, with one study showing evidence of direct carcinogenicity (73–75).

In China, some regions have a frequency of primary hepatocellular carcinoma, which is among the highest in the world that is associated with the consumption of surface water from ponds and ditches. Epidemiological evidence showed a correlation of this hepatocellular carcinoma with hepatitis B virus infection and the consumption of aflatoxin B_1 from mouldy corn and other grains, both of which causes of liver cancer are widespread in these areas of China. Adjacent villages,

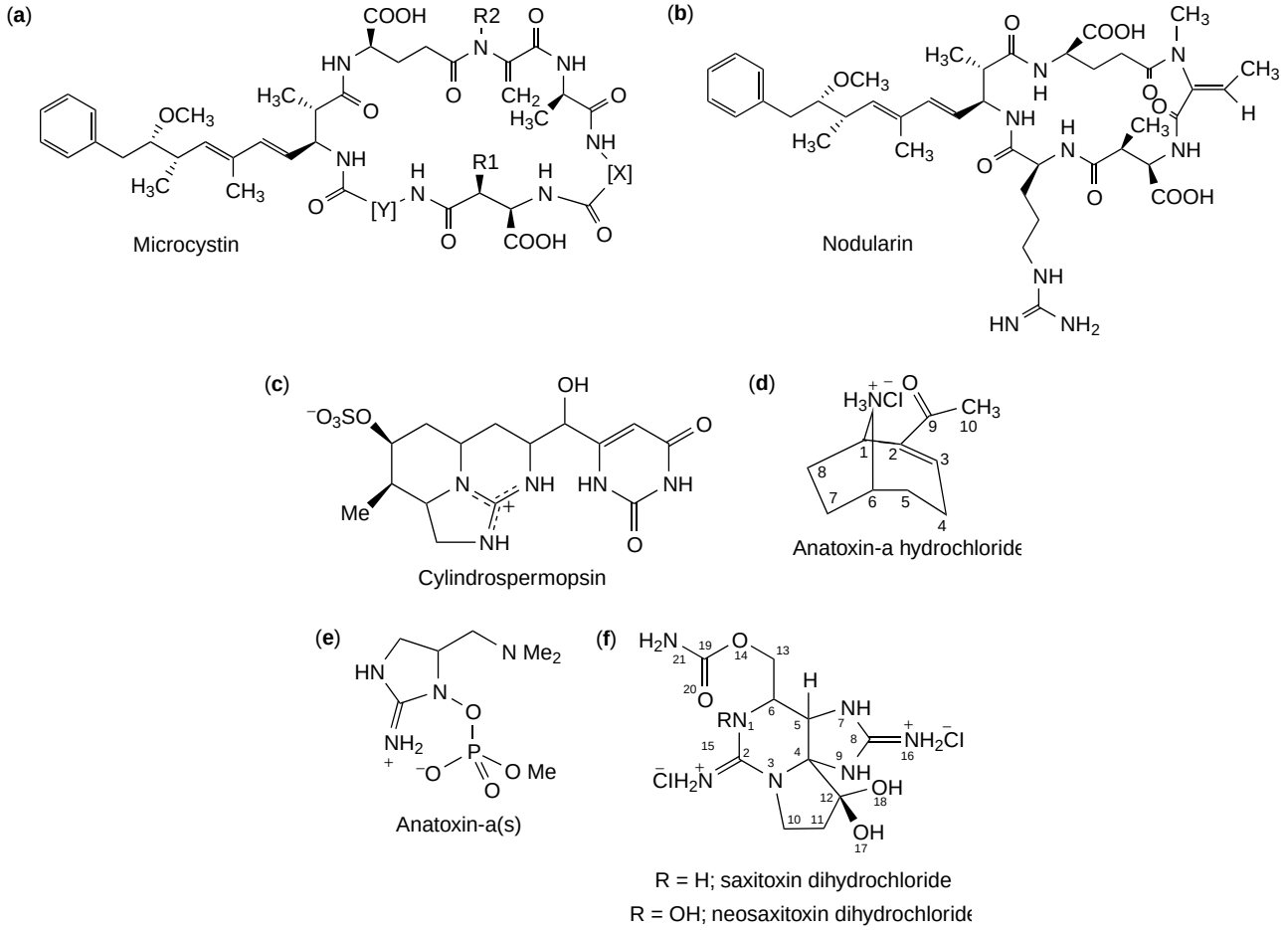

Figure 4. (**a**) Microcystin. Locations X,Y are occupied by L-amino acids. R_1, R_2 are either hydrogen or a methyl group, (**b**) Nodularin, (**c**) Cylindrospermopsin, (**d**) Anatoxin-a, (**e**) Anatoxin-a(s), (**f**) Saxitoxin.

however, had very different cancer rates, with lower rates in villages using deep well water and high rates in villages using surface water. Ponds and ditches in these areas are heavily contaminated with *Microcystis* sp. and microcystins (76,77). Although this evidence does not prove that microcystins are either carcinogens or tumor promoters in man, it is indicative that further epidemiological research is required on this issue and that a potential risk exists.

Another cyclic peptide toxin, in this case having a ring structure containing five amino acids, is produced by *N. spumigena*. The amino acids are D-methyl aspartate-L-arginine-ADDA-D-glutamate-2-(methylamino)-2-dehydrobutyric acid, giving a composition and structure very close to the microcystins (Fig. 4b). The toxin is named nodularin and has the same mechanism of action as microcystin. Human toxicity has not been reported, probably because the brackish water in which this species grows is not suitable for human consumption. Farm livestock was poisoned by drinking from a Nodularia scum in the first reported case of cyanobacterial poisoning in 1878, in Australia (1). Dogs have also been poisoned by *Nodularia* on the shores of the Baltic Sea, as a consequence of their habit of licking their coat clean after swimming (79).

The alkaloid toxin from *C. raciborskii*, named cylindrospermopsin, also damages the liver but has much wider cell toxicity (25,79). The structure is a sulfated cyclic guanidine alkaloid with a hydroxymethyl uracil group attached (Fig. 4c). The alkaloid has also been found in other cyanobacteria, to date *Aphanizomenon ovalisporum* and *Umezekia natans* (36,38). As this toxin was only recently characterized, it is likely to be found in yet more species. The mechanism of toxicity includes the inhibition of protein synthesis, which causes widespread cell injury (80). The liver shows progressive damage from a single injected dose, with mortality over five to seven days in mice. Proximal tubule damage in the kidney and death of white cells in the spleen, as well as injury in a range of other organs (25,26,81,82), have been described. The cyanobacterial species *C. raciborskii* came to attention as a consequence of a major human poisoning outbreak among Aboriginal children on Palm Island, off the tropical Queensland coast of Australia. As a result of local complaints of taste and odor in drinking water, because of the presence of a cyanobacterial bloom in the reservoir, the authorities treated the reservoir with copper sulfate. Within a week, a number of children were admitted to the local hospital with hepatoenteritis, characterized by

initial constipation, sore enlarged livers, vomiting, and headache, followed by bloody diarrhea with severe dehydration. Serum and urine analysis showed electrolyte loss, protein loss, and some cases with ketones and glucose in the urine (55). A total of 140 children and ten adults were treated, the more severe cases by relocation into a major hospital and intensive care. None died. Subsequently, the cause was identified to be the toxic cyanobacterium *C. raciborskii*, which had been lysed by copper sulfate treatment of the reservoir, liberating toxin into the drinking water.

The neurotoxins are found independent of each other, although they may be in the same species from different locations. Anatoxin-a is the most commonly observed neurotoxin in the northern hemisphere, recorded from North America and from Europe (35,83). It has a simple alkaloid structure (Fig. 4d). Cyanobacterial species containing anatoxin-a include *Anabaena flos-aquae, Aphanizomenon flos-aquae*, and *Limnothrix* (benthic Oscillatoria) (19). The toxic action has been well characterized as a depolarizing agent at the neuromuscular junction, similar in action to acetylcholine. It depolarizes both nicotinic and muscarinic receptors with a persistent action leading to a sustained blockade of impulse transmission to muscle. Death of an animal is due to respiratory failure and can occur within 10 minutes of an intraperitoneal injection of a cyanobacterial extract in the mouse (41,84). Poisoning of cattle, dogs, and bats has been attributed to this toxin in North America and Europe (40,83,85). There is no evidence of human injury through drinking water supplies, and it is unlikely that the concentrations of anatoxin-a would reach toxic levels without major prior action by the supplying authority. The predominant toxic cyanobacterial genus producing this toxin is *Anabaena*, which causes off-flavors and odors in water at cell concentrations well below those required to provide a toxicity hazard (86,87).

A further neurotoxin produced by *Anabaena flos-aquae* is the anticholinesterase toxin anatoxin-a(s). The toxin is named after the source organism, and the (s) is derived from the salivation characteristically seen with anticholinesterase neurotoxicity (51). The molecule is an *N*-hydroxyguanidine methyl phosphate ester (88) (Fig. 4e). The molecule closely resembles a synthetic organophosphorus insecticide and has a similar mode of action producing an irreversible inhibition of synaptic acetylcholine esterase. Death is due to respiratory failure resulting from hyperactivity of postsynaptic neurons as seen by salivation, lacrymation, urination, defecation, and convulsions before death (89). This toxin has caused cattle and dog deaths (90) but is unlikely to be present in drinking water at lethal concentrations. It is unstable at alkaline pH and to boiling, and is likely to be destroyed by chlorination during water treatment (89).

Paralytic shellfish poisons are the third type of neurotoxins found in cyanobacteria. These are well known hazards to human health as this type of neurotoxicity has afflicted coastal dwellers and seafarers throughout history (91). The sources of the toxins in shellfish are marine dinoflagellates. Many countries now have monitoring programs aimed at eliminating this risk from consuming shellfish (92). Paralytic shellfish poisons form a group of related molecules based on saxitoxin, and are tricyclic guanidinium alkaloids (Fig. 4f). They were first identified as cyanobacterial toxins in *Aphanizomenon flos-aquae* in New Hampshire, U.S.A. (93), when a bloom of that organism was treated with copper sulfate and resulted in a major fish kill. At that time the chemistry of the toxins was unknown, but was clarified from electrophysiological and structural studies over the next decade (94,95).

In Australia, neurotoxic *A. circinalis* blooms result in sheep deaths during most dry years (96). The chemical structure of the neurotoxin was resolved after the 1991 Darling River bloom in which more than 1,000 km of river contained a highly neurotoxic bloom associated with more than 10,000 deaths of sheep and cattle using the river as a drinking water source (97).

Studies on the physiological response of mice to the toxin indicated that paralytic shellfish poisons were likely, which was confirmed by electrophysiological experiments and structural chemical analysis (23). Since then a separate poisoning of sheep was carefully investigated and a range of paralytic shellfish poisons was identified, including neosaxitoxin, gonyautoxins, and sulfated C toxins in the intestinal contents of the animals (98). The number and diversity of paralytic shellfish poisons lead to complexity in their identification by high performance liquid chromatography (HPLC), and mouse testing is still widely used (99,100). The neuropharmacology of these toxins has been thoroughly investigated and the toxins are sodium channel blocking agents in axonal membranes, leading to interruption of nerve impulse transmission (101). This leads to relaxation of smooth and voluntary muscle and progressive inhibition of respiration, leading ultimately to death (91).

The paralytic shellfish poisons are resistant to boiling and to chlorination. The neurotoxicity attributable to them has been detected in treated drinking water during the Darling River bloom (97).

TOXIC WATER BLOOMS AND CONTROL MEASURES

Monitoring of cyanobacterial concentrations in water bodies is commonly carried out by taking water samples representative of the upper 1 to 5 meters of water, away from the shoreline. Microscopic examination of the water is used to establish the species of the cyanobacterium, and cell counting is used to obtain cell density. This is straightforward with filamentous organisms but extremely difficult with colonial spherical species such as *Microcystis*. An alternative technique used widely in Europe is the measurement of chlorophyll-a, which assesses the total of photosynthetic phytoplankton, including eukaryotic algae (102,103). When cyanobacteria are the predominant organisms, chlorophyll-a measurement provides a quick simple assessment of cell density.

The population of cyanobacteria in a water body varies greatly with the time of year, water temperature, nutrient status, thermal stratification, turbidity and turbulence, and local hydrology. As a result, each water body differs and prediction of cyanobacterial blooms requires repeated monitoring over several years to build up a pattern of cell numbers. The overall potential for

cyanobacterial bloom formation can be assessed from the total phosphorus content of the water. For example, *Microcystis* and *Anabaena* are unlikely to bloom if total phosphorus concentration can be maintained below 50 μg/liter of water (104). Adding to the complexity of bloom prediction are the effects of storms, which bring high concentrations of phosphate into rivers and lakes over a short time period and the thermal stratification of lakes, which allows solubilization of phosphate from anoxic sediments. Cell density is not uniform down the water column, and the depths at which the highest densities occur depend on the cyanobacterial species and on the mixing occurring in the lake. This has particular implications for drinking water supply as water intakes often have variable depths of water entry, which can be used to minimize cyanobacterial cell count in raw water before treatment. Scum-forming and surface-growing species of cyanobacteria, such as *Microcystis aeruginosa* and *Anabaena circinalis*, will be more obvious to plant operators through water discoloration than species such as *P. agardhii* and *C. raciborskii*, which form dense bands of cells, meters down in the water (102).

Control of cyanobacterial blooms in water supplies is becoming increasingly necessary as the number of affected lakes and rivers is increasing. In eutrophic water reservoirs in North America and in Australia, which are operated by water utilities, the use of copper sulfate as an algicide is widespread. The practice is to monitor the water body for cyanobacteria, and when cell numbers begin to increase rapidly, the lake is dosed with copper sulfate to a concentration of one to two parts per million in the upper two meters of water. This is done from aircraft, helicopters, or boats, depending on the area to be treated (105). Within hours of dosage the cyanobacterial cells lyse and any toxin present is liberated into the water. At low cell concentrations the toxin content of the water will be negligible; however, if the treatment is applied at high cell concentrations or when scums exist near the water intake, there is the possibility for human poisoning, as discussed earlier. In Australia, this practice is not permitted in rivers because of the detrimental effects of copper on the overall ecology. Notwithstanding this regulation, about 1 km of the Darling River adjacent to a township was treated with a copper complex during the 1991 *Anabaena* bloom.

More effective long-term control of cyanobacterial blooms is obtained by nutrient reduction to inflowing water sources. Phosphate stripping in sewage treatment can reduce phosphate loads to 1% of the original concentrations, removing a major source of nutrients. Use of polyphosphate-free domestic detergents not only reduces phosphate in sewage but also in septic tank output. In areas where agricultural use of superphosphate is widespread, avoiding distribution adjacent to watercourses reduces phosphate runoff during heavy rain.

In lakes with phosphate-rich sediments, remediation is difficult and costly as it involves capping the sediment to prevent solubilization of phosphorus, dredging to remove the sediment, or preventing thermal stratification. Destratification equipment has been installed in many water supply reservoirs using plumes of air bubbles to mix and oxygenate the water or propellers to mix the water layers. This technique has not been uniformly successful in preventing cyanobacterial blooms (104). In lakes and rivers used for recreation, the seasonal water blooms commonly coincide with the summer holiday period. Another strategy has been used because of the economic impact and the health hazard imposed by the presence of blooms. This is to release sufficient water from an upstream water storage to flush the bloom out of the recreational area. During January 2000, this method was used to remove a toxic *Microcystis* bloom from the Torrens Lake in the center of Adelaide, South Australia. Although this does not provide a permanent solution, this is an environmentally safe and effective method of removing a local water bloom.

SAFETY, HEALTH, AND GUIDELINE VALUES

The WHO have assessed the evidence for adverse human health impacts of cyanobacterial toxins and derived a GV for the best described and understood for these toxins. The GV is based on the determination of a tolerable daily intake (TDI) for the toxin, which is defined as the daily intake that can be consumed over a human lifetime, without detectable injury. The GV can be derived from human epidemiological data, when a dose/response curve to poisoning can be drawn from accidental human exposure. This is currently not possible for cyanobacterial toxins as measures of the amount of toxin consumed are not available in those cases in which human poisoning has occurred. The alternative method for calculation is to use animal data in which the animals have been exposed to a range of nonlethal oral doses of toxin over an extended period. This data is at present only available for microcystins, for which experiments of this type have been performed on mice (106) and pigs (70), using oral microcystin dosing. The oral toxicity of cylindrospermopsin is currently under investigation with the objective of providing data for GV determination.

To convert data from animal experimentation into safe GV for human consumption, a series of safety (uncertainty) factors are applied to the calculation. These are factors of ten for the range of sensitivity within the human population (children to aged), ten for conversion of rodent to human sensitivity and less for pig to human sensitivity as the physiology is closer between pigs and people, and ten for conversion of shorter times of exposure (subchronic) to lifetime exposure. A further modifying factor for excessive toxicity symptoms, possible tumor promotion or uncertainties in the toxicology can also be applied.

The critical experimental information is the maximum daily intake of toxin that does not cause any detectable adverse effects on the animals (No Observable Adverse Effect Level, NOAEL) and the minimum daily intake of toxin that causes an adverse effect (Lowest Observable Adverse Effect Level, LOAEL).

The TDI is thus equal to $NOAEL/10 \times 10 \times 10$ for a standard mouse experiment.

In an experiment conducted on mice for over 13 weeks, a NOAEL of 40 μg/Kg/day of microcystin-LR was found. Applying this to the formula above gives a TDI of 0.04 μg/Kg/day (106).

In an experiment using pigs, a LOAEL was found to be 280 μg/Kg/day of mixed microcystin (70), equivalent to 100 μg of microcystin-LR. In this case a safety factor of 1,500 was applied, 10×10 as before for human variability and subchronic dosage, three for differences between species and five for extrapolating a LOAEL to a NOAEL. This gave a TDI of 0.067 μg/Kg/day, close to the value from mice.

To determine the GV for drinking water, data on average human bodyweight (bw), proportion of toxin intake from drinking water (P), and average daily water consumption in liters (L) are applied to the TDI.

$$\text{Guideline Value} = \frac{\text{TDI} \times \text{bw} \times \text{P}}{L} = \frac{0.04 \times 60\ \text{Kg} \times 0.8}{2} = 0.96\ \mu\text{g/L}(1.0\ \mu\text{g/L}).$$

This GV has been provisionally adopted by the WHO (15) and is being considered by individual countries. It applies to drinking water and water used for normal domestic purposes. For particular "at risk" categories, such as use in renal dialysis or intravenous therapy, special provisions for higher quality water are required (15).

Although the GV is defined in terms of microcystin-LR, its adoption implies that other microcystins are included in the Value. Some toxic Microcystis blooms do not contain microcystin-LR at all, others only a small proportion. Mouse bioassay, using microcystin-LR as a standard, provides total toxicity equivalents that are the biologically effective measure of total toxin content. Biotests using crustaceans have also shown applicability to measure microcystin and other cyanobacterial toxins (107).

Measurement by HPLC is the standard chemical method, which gives separate peaks for each microcystin variant that has a characteristic ultraviolet (UV) spectrum with a peak or shoulder at 238 nm (108). Summation of these peak areas provides a measure of total microcystins, which does not compensate for the differences in toxicity of the variants. Enzyme-linked immunosorbent assays for microcystins are available commercially, and assays based on inhibition of protein phosphatase are under commercial development. Both these techniques have been applied with success in research laboratories (91–94,109–112). Although the operators of water treatment plants now have a standard for the microcystin content of water supply, effective monitoring of raw water sources can usefully include measurement of toxin content when species known to contain microcystins are present in cell concentrations likely to approach the GV in bulk water. The WHO has drawn up a set of Alert levels for cyanobacterial cell concentrations in drinking water reservoirs together with a flowchart for action (113). A similar approach has been taken to preserve the safety of recreational waters containing cyanobacterial blooms, with recommendations for actions at a series of cyanobacterial cell densities (15).

WATER TREATMENT

Although the prevention of cyanobacterial blooms from occurring in water storages is far better than concentrating on water treatment, the practicality of the situation is that many reservoirs and holding lakes are contaminated with cyanobacterial toxins. In a survey carried out on drinking water supplies in southwestern Manitoba in 1995 and 1996, rural municipal supplies showed microcystins in 93% of the water supplied to consumers. Seven supplies were intensively monitored in 1996, and microcystin-LR was found throughout the sampling period of June to December, sometimes at concentrations above 0.5 μg/liter (113,114). From this and similar data elsewhere it is apparent that conventional water treatment with chlorination does not remove microcystins, a conclusion that can also be reached from reports of liver injury or hepatoenteritis from chlorinated supplies drawn from reservoirs with *Microcystis* blooms.

The management of water storage reservoirs can minimize the entry of cyanobacteria into the treatment system. Water can be drawn from levels of the reservoir that carry a minimum of cyanobacteria, and alternative storages or groundwater can be used when available. If algicides such as copper are employed it is essential to withdraw the affected reservoir from supply until cyanobacterial toxin concentrations have dropped to below GV.

Cyanobacterial toxins under normal growth conditions largely remain within the cells and the toxin that leaks out will be degraded by bacteria occurring naturally in freshwater (115). The concentrations of free toxin in water are therefore low compared with the total concentration of toxin, unless algicide use or natural lysis of the bloom occurs. In water treatment it is therefore a considerable advantage if intact, live cyanobacterial cells can be removed before treatment. This may not be possible if the water drops considerably in height through pressure reduction valves or travels long distances in pipelines, between the reservoir and the treatment works (116). The common practice at water treatment plants of injecting chlorine at the flash mixer, where raw water enters the treatment plant, causes cyanobacterial cell lysis, thus immediately freeing the toxins into solution. Subsequent flocculation and sedimentation or dissolved air flotation are then largely ineffective at microcystin removal (117). When intact cyanobacterial cells are present, optimization of flocculation conditions effectively removes the cells and the toxins (118).

Alum or ferric chloride flocculation of suspended organic material does not remove appreciable amounts of soluble cyanobacterial toxins from water (117). In the case of removal of paralytic shellfish poisons from raw water in a pilot plant experiment, reduction of 20% was observed with alum flocculation (8).

Three methods are used to remove alum or ferric chloride flocculated organic matter from the water stream. The most common is sedimentation in a settling tank,

which allows heavy flocculated material to settle out. An effective technique that is particularly useful in smaller plants is the sludge blanket, in which the water with flocculant passes up through a layer of previously formed floc. This has been demonstrated to remove 95–99% of cyanobacterial cells (118). Another highly effective method is clarification by dissolved air flotation in which compressed air bubbles are injected into the bottom of the tank containing the flocculated material. The bubbles carry the floc and entrapped organic material up to the surface, where they are skimmed off. More than 80% removal of cyanobacteria was shown in eight operating dissolved air flotation plants (119).

Toxic cyanobacteria removed during flocculation and floc removal have been shown to lyse rapidly and release trapped toxins, with effectively all the toxin released within two days (120,121). It is therefore important to remove the floc prior to cell lysis, which requires frequent cleaning of sedimentation tanks and removal of sludge from blanket units during a cyanobacterial bloom. The final removal of suspended material is through rapid sand or mixed bed filtration, so that cell lysis also has implications for the frequency of filter backwashing. Backwash water is normally clarified and added into raw water intake, which can lead to recycling of toxins during a bloom.

From measurements of microcystins in treated water it is apparent that simple treatment plants do not remove the toxins (113), and considerable investigation has been undertaken to develop techniques that are effective. Use of activated carbon, either in powdered form or as granules in a filter, has been shown to be effective. The operational requirements for powdered carbon are more complex, but have been shown to remove up to 90% of microcystins (122). In practice, lower removal rates are common and are affected by the presence of alum (123). Paralytic shellfish poisons can also be removed by activated carbon (8). Granular activated carbon is installed in advanced treatment plants that recurrently have taste and odor problems with raw water or organic toxicants. It is highly effective in removing cyanobacterial toxins (8,124) as long as the adsorptive capacity has not been exceeded.

Cyanobacterial toxins can be destroyed by oxidation, which has relevance in water treatment as disinfection is carried out by oxidants. Both chlorine and ozone are in use in treatment plants and have the capacity to oxidize microcystins (122,125). The effectiveness of chlorine in water plant operation as a method of microcystin removal is reduced by the presence of other organic material (122,123) which may be particularly high when a cyanobacterial bloom occurs in the raw water supply. As noted previously, all the cases of human poisoning from cyanobacteria in drinking water occurred in chlorinated water supplies. Ozone offers more promise as an oxidant (117,126), with the ability to completely destroy microcystins, nodularin, and anatoxin-a (127). It appears likely that cylindrospermopsin and paralytic shellfish poisons can also be oxidized by ozone (127).

In advanced water treatment, ozone is used in conjunction with activated carbon filtration to remove any undesirable oxidation products. For example, at the Thames Water plant in Feltham, England, the sequence is initial low dose ozonation; flocculation followed by dissolved air flotation, rapid sand filtration, the main ozonation process, granular activated carbon filtration, and finally slow sand filtration. At the end of the treatment, chlorine is added as a pipeline disinfectant.

Recent developments in membrane filtration and reverse osmosis have demonstrated their potential for microcystin removal in fresh and salt water (128,129). Nothing is known at present about the removal of neurotoxins by membrane filtration.

CONCLUSION

Cyanobacteria are present as a normal component of ecosystems, including freshwater. Their abundance is limited by nutrients; in particular, the availability of phosphate in the water or solubilized from anoxic sediments. The majority of species that form waterblooms in drinking water sources are toxic and hence present a potential risk to consumers of that water. As eutrophication of surface waters increases the chance of cyanobacterial blooms also rises, to the extent that a majority of lakes and slow-flowing rivers have intermittent blooms in the higher populated regions of the world.

The toxins from cyanobacteria, especially microcystins and cylindrospermopsin, can reach injurious concentrations in drinking water, and have caused outbreaks of poisoning in domestic animals and people. Monitoring of surface water sources for toxic cyanobacteria is essential, especially during summer and autumn when lakes thermally stratify and the higher temperatures assist cyanobacterial growth. Water treatment technology has the capacity to remove cyanobacterial toxins but in practice may be inadequate or overloaded when a bloom occurs in a water storage, especially if the bloom is lysed with copper, liberating the toxins from the cells into the water.

The WHO GV provides a concentration of 1.0 μg microcystin-LR/liter of drinking water for safe lifetime consumption; if total microcystin toxicity can be maintained at or below this concentration, there is negligible risk to the human population. As more data are available, GV for other cyanobacterial toxins can be derived for safe drinking water. Recent research has implicated microcystin and cylindrospermopsin in experimental carcinogenesis, which may require reconsideration of GV derived from toxicity data (130,131,132).

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CYANOBACTERIAL MATS.

See BIOFILMS IN NATURAL AND DRINKING WATER SYSTEMS

CYANOBACTERIAL MATS AND NITROGENEN FIXATION.

See NITROGEN FIXATION IN THE MARINE ENVIRONMENT

CYANOPHAGES.

See CYANOBACTERIA

CYCLOSPORA: BASIC BIOLOGY, OCCURRENCE FATE AND METHODOLOGIES

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Cyclospora cayetanensis is a coccidian parasite that affects humans causing a gastrointestinal disease known as *cyclosporiasis*. Because of its biological characteristics that are somehow different from other coccidians infecting humans, its true identity took longer to unravel and it was not fully identified until 1993. *Cyclospora* has received increased attention in the past years because of reports of water or food related outbreaks in the developed world (1,2). In the United States, the first waterborne outbreak occurred in Chicago and was associated with consumption of water potentially contaminated with *Cyclospora* oocysts. The mechanisms of transmission are not completely understood; however, most of the cases of cyclosporiasis have been associated to food-borne or waterborne transmission with a case-fatality rate of 0.05% (3). Since 1995, additional outbreaks have been documented in Canada and the United States and were linked to potentially contaminated imported produce or fruits (4). These events have lead to increased awareness, improvements in the detection methods, and development of research initiatives for understanding the dynamics of cyclosporiasis better.

TAXONOMY

The genera *Cyclospora* was first observed by Eimer in 1870 and subsequently named in 1871 by Schneider (5) when *Cyclospora glomerica* was isolated from a myriapod. *Cyclospora* belongs to the *Subphylum Apicomplexa*, *family Eimeriidae*. The species name *cayetanensis* was derived from the Peruvian University where preliminary identification studies were initiated (Universidad Peruana Cayetano Heredia). The morphological characteristics relevant to this classification are anterior polar complex with apical rings, micronemes, and subpellicular microtubules. These features are common to all apicomplexan. *Cyclospora* has a complex life cycle that includes asexual multiplication (merogony), sexual multiplication (gametogony), and formation of sporozoites (sporogony). Merogony occurring within a vertebrate host is characteristic of the order Eucoccidiorida, followed by independent development of male and female gametes (suborder Eimeriorina). Upon gamete fertilization, immature oocysts are formed and excreted into the environment. The oocysts differentiate and mature having two sporocysts, each with two sporozoites, which are characteristics of the genus *Cyclospora* (Fig. 1).

To date there are fourteen additional species of *Cyclospora* that infect insectivores, rodents, and snakes. All of them are morphologically different from *C. cayetanensis*. Molecular phylogenetic studies using the 18S rDNA demonstrated that *C. cayetanensis* is more closely related to *Eimeria* species (6). Three new species of *Cyclospora* that are morphologically similar to *C. cayetanensis* have been said to infect nonhuman primates, and on the basis of phylogenetic analysis of the 18S rDNA, they are dissimilar to *C. cayetanensis* (7,8).

BASIC BIOLOGY

The first documented report attributable to *Cyclospora* infections in humans probably occurred in 1979 from patients with diarrhea in Papua, New Guinea. The

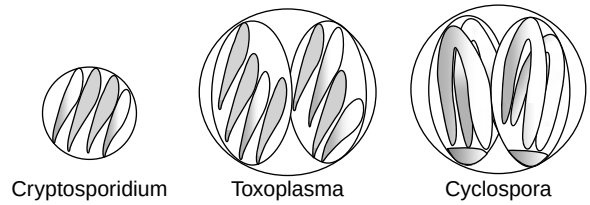

Figure 1. Fully sporulated oocysts of coccidian that cause diarrhea in humans. *Cryptosporidium* measures 4 to 6 μm in diameter, *Toxoplasma* 10 to 12 μm, and *Cyclospora* 8 to 10 μm. *Cryptosporidium* has four sporozoites and no sporocyst and is shed to the environment fully sporulated and infectious. *Toxoplasma* has two sporocysts, each with four sporozoites. *Cyclospora* also has two sporocysts, each with two sporozoites. *Toxoplasma* and *Cyclospora* are excreted unsporulated.

authors described oocysts suggesting it was a new species of *Isospora* affecting humans (9). Before this report, the genus *Cyclospora* had been associated with moles, myriapods, insectivores, and snakes, but never with humans. Previously reported in the literature as Cyanobacter-like bodies, Coccidian-like bodies (CLB), *C. cayetanensis* was recognized and described as a human coccidian parasite in the early 1990s (10). It has since been reported in multiple countries around the world, showing an endemic pattern in the developing world with isolated outbreaks or travel-linked episodes in the industrialized world.

The oocysts are spheroidal with 8.6 ± 0.6 μm diameter, a bilayered cell wall about 113 nm thick, ovoidal sporocysts 4.0 μm wide and 6.34 μm long, with sporozoites 1.2 μm wide and 9.0 μm long. Oocysts autofluoresce under UV epifluorescent microscopy (11) (Fig. 2a) and immature oocysts have internal structures with a morula type appearance (Fig. 2b). The oocysts stain variably with modified acid-fast staining. The formation of sporocyst-like structures occurs by 5 days and appearance of sporozoites within sporocysts within 7 to 13 days. Under ideal laboratory conditions, more than 40% of the oocysts collected sporulate within 7 to 13 days and become infective. In humans, which to date seems to be the only host, the proposed mechanisms of transmission is the ingestion of food or water contaminated with mature (sporulated) oocysts.

The life cycle of *C. cayetanensis* has not been fully understood. Other species of *Cyclospora*, such as *C. caryolytica* of moles, showed asexual and sexual development within the nucleus of the enterocytes (12). *Cyclospora talpae*, which also infects moles, develops asexually within the nucleus of monocytic cells in the liver, whereas sexual development was confined to the nucleus of bile duct epithelial cells (13).

Cyclospora cayetanensis has a different intracellular localization, with parasitic vacuoles containing asexual and sexual stages within the cytoplasm of jejunal enterocytes (Fig. 3); however, the exogenous sporulation closely follows that of *C. talpae* (Fig. 4).

Epidemiological studies including domestic animals in the households of individuals with cyclosporiasis did not report animals infected with *Cyclospora*. In endemic areas such as Nepal, in 1995, 196 different species of animals (14) including pigs, monkeys, dogs, cats, cows,

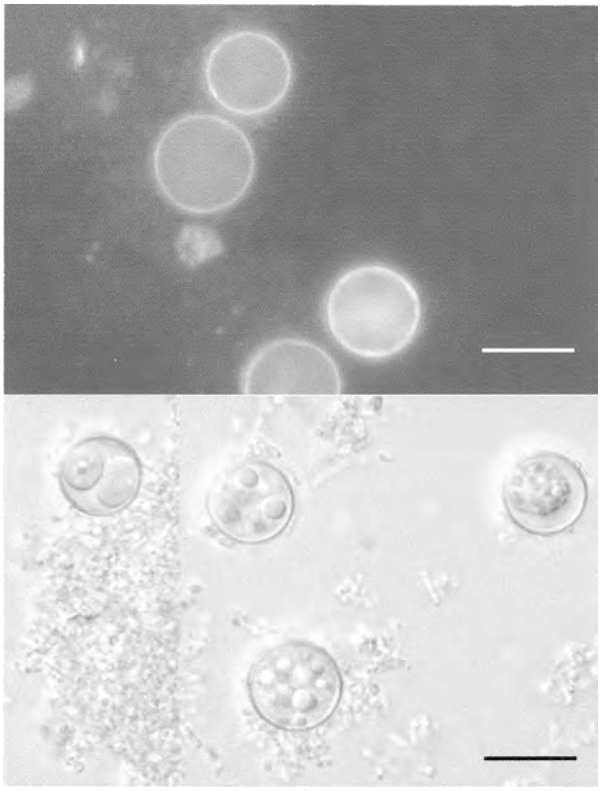

Figure 2. *Cyclospora* oocysts. (**a**) Autofluorescence. (**b**) Unsporulated and sporulated oocysts. Bar = 10 μm.

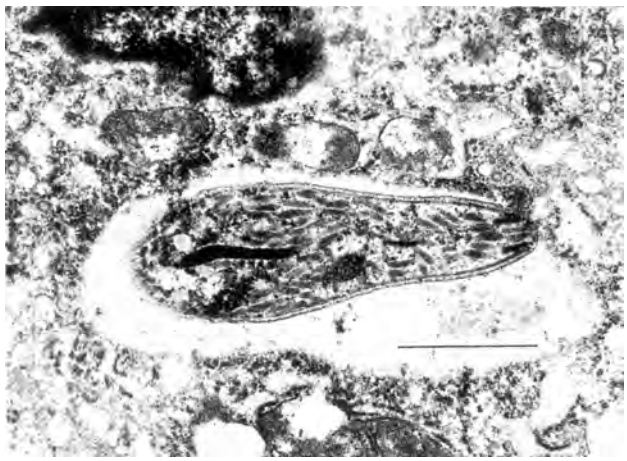

Figure 3. Merozoite in intracellular parasitic vacuole. Roptries and micronemes are characteristic to coccidians.

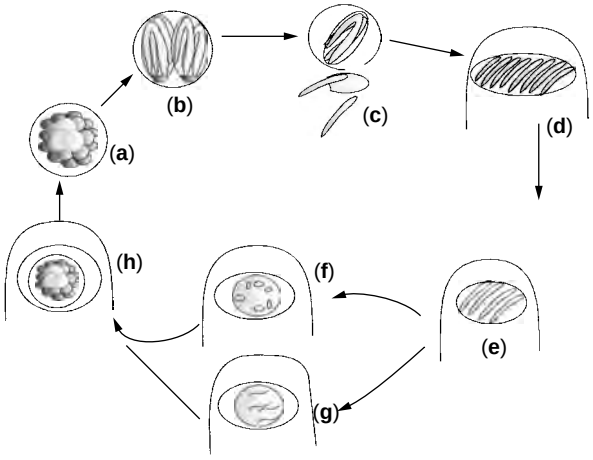

Figure 4. Life cycle of *Cyclospora*. Oocysts are excreted unsporulated (**a**). After 7 to 15 days in the environment, they sporulate and become infectious (**b**). When ingested by a susceptible host, sporozoites are released and infect epithelial cells of the small intestine (**c**). Parasites go through asexual multiplication (**d,e**) and sexual differentiation as macrogametocyte (**f**) and microgametocyte (**g**). Fecundation occurs and zygote is formed and differentiates to an immature oocyst that is excreted into the environment (**h**).

buffalo, goats, rats were surveyed and demonstrated negative to the presence of *Cyclospora*. Surveys of domestic animals in Peru also gave negative results. These surveys included commercial and home raised chickens and companion and farm animals. In 1999, 327 domestic animals from a Haitian study were surveyed and 100% found to be negative to *Cyclospora* (15).

In addition to sampling animals for natural infections, multiple controlled studies have attempted without success to study cyclosporiasis from infections of animal models such as several strains of mice including adult, neonatal immunocompetent and immunodeficient, inbred and outbred strains, and other animal species including rats, sand-rats, chickens, ducks, rabbits, gerbils, hamsters, ferrets, pigs, dogs, owl monkeys, rhesus monkeys, and cynomologus monkeys (16). These results contradict some isolated reports of *Cyclospora* in fecal samples or intestinal contents of ducks, chickens, and dogs. These communications reported parasites in fecal matter, which could be explained as being transient parasites from accidental ingestion of *Cyclospora*-infected human feces. No evidence of parasitized tissues that would demonstrate them as definite hosts for this infection was presented (17–19).

Clinical and Pathological Findings

Infection in susceptible individuals can exist as asymptomatic to severe and persistent diarrhea, mild nausea, anorexia, abdominal cramping, watery diarrhea, and weight loss (20). Vomiting is less common (21). Diarrhea alternating with constipation has been commonly reported. Infections in AIDS patients have similar symptoms but tend to last longer with reported episodes lasting up to four months (22). Onset of abrupt illness caused by *Cyclospora* has been reported in 68% of adult patients, with watery diarrhea and lasting an average of four to seven weeks. Other clinical features associated with cyclosporiasis include an impaired D-xylose and reduced vitamin B-12 absorption, and elevated-fat fecal excretion (21,23).

Cyclospora has been shown to infect epithelial cells of the duodenum and jejunum. Histopathological exam reveals a moderate to marked erythema of the distal duodenum. The lamina propria shows with mild to moderate acute inflammation. Varying degrees of jejunal villous blunting, atrophy, and crypt hyperplasia have been observed in the duodenal and jejunal portions. The intense inflammatory reaction does not correlate to the small number of parasitic vacuoles present. Gastric and colonic biopsies did not show histopathologic changes, nor the presence of parasitic vacuoles (20,24).

A description of *Cyclospora*'s life cycle has been inferred from the intracellular parasitic vacuoles observed in the cytoplasm of epithelial cells of the duodenum or jejunum (20,25). Sexual stages and two types of asexual (merogony) stages of the parasite were observed (24).

The drug of choice for treating cyclosporiasis is Trimethropim-sulfamethoxazole (TMP-SMX). The cessation of symptoms and oocyst excretion usually occurs one to three days after treatment (26). In Nepal, 94% of immunocompetent individuals with cyclosporiasis cleared the infection within seven days of treatment. In a HIV population, recurrence was about 44% in patients with cyclosporasis receiving one week therapy with TMP-SMX (27) and additional seven days of drug administration may be needed in some cases (28). Seven days of treatment using Ciprofloxacin (500 mg BID) produced a 90% cessation of diarrhea, and 64% stopped secreting oocysts in their stools (29). Although Ciprofloxacin is not as effective as TMP-SMX, it is an alternative for those patients who cannot take or are allergic to sulfa compounds. Anecdotal use of tetracycline plus folic acid showed some success (21). Norfloxacin, tinidazole, quinacrine, nalidixic acid, and diloxanide furoate were not effective to treat cyclosporiasis (30).

Diagnosis

Cyclospora can be identified by finding unsporulated oocysts in freshly collected stool samples; sporulated or nonsporulated parasites could be found in fresh vegetables or environmental samples. *Cyclospora cayetanensis* oocysts are 8 to 10 μm in diameter and bigger than those of *Cryptosporidium parvum* (4 to 6 μm), which is also infectious to humans. *Cyclospora* oocysts stain variably using a modified acid-fast technique. They stain best using the modified carbolfuchsin technique. Oocysts also can be stained using the Kinyoun, Ziel-Neelsen, and safranin methods, but do not stain well with iron hematoxylin, Grocott-Gomorri methenamine-silver nitrate, iodine, or periodic acid-schiff stains (31). Safranin uniformly stains *Cyclospora* oocysts when preheated in a microwave oven (32). Oocysts autofluoresce blue under UV epifluorescent illumination using a 365/10 DM exciter filter. To enhance the detection process, *Cyclospora* oocysts can be concentrated by sequential differential centrifugation steps or formalin ethyl acetate purification, followed by sucrose flotation in Sheather's solution.

Molecular assays have also been developed for detection of *Cyclospora*. A nested PCR amplifies 294 bp of the 18S rDNA (33). PCR inhibitors commonly found in diagnostic samples can be removed by pretreatment of the lysed preparation with a resin matrix (Instagene; Biorad, Hercules, CA) and nonfat milk.

The samples to be tested for oocysts are lysed by freeze-thaw cycles in dry ice-ethanol. Primers are those described by Yoder and coworkers. Amplification products of *Eimeria* and *Cyclospora* are of the same size. These can be differentiated by RFLP with the restriction enzyme Mnl I, and the predicted restriction fragment sizes for Cyclospora are 140, 106 and 48 bp. The detection limit of this PCR test was estimated to be 10 oocysts (34).

Other agents causing diarrhea must be considered when processing diagnostic samples. *Cyclospora* may be found in conjunction with one or more additional pathogens such as *Giardia lambia*, *Entamoeba hystolitica*, *C. parvum*, and the microsporidia (35), which also have similar transmission profiles. The confirmation process include the verification of the laboratory findings and, whenever applicable, the concordance between the reported clinical symptoms and the laboratory results. Differential diagnosis of *Cryptosporidium* is particularly

important because it also acid-fast stains, and coinfections with *Cyclospora* have been reported (36). Current immunofluorescent assays for *Cryptosporidium* rely on the use of fluoresceine isothiocyanate, and the resulting fluorescent organisms could be confused with autofluorescent *Cyclospora*. The careful measurement of the parasite and use of positive controls will demonstrate significant differences in size (*C. cayetanenesis* is 8 to 10 μm diameter versus 4 to 6 μm diameter for *Cryptosporidium*) and the intensity of the green fluorescence observed at 450 nm (very bright for FITC, weak for *Cyclospora*).

When investigating outbreaks, bacterial pathogens such as *Escherichia coli*, *Salmonella*, *Shigella*, *Campylobacter*, or viral entities such as Norwalk, adenovirus, and cytomegalovirus shall also be considered as potential causative agents. In the case of immune-compromised patients, *Mycobacterium-avium intracellulare* may also be considered as a potential etiology of diarrhea.

Epidemiology

Cyclospora has been associated to protracted diarrheal illness in humans in North, Central, and South America, the Caribbean, Africa, Bangladesh, Southeast Asia, Australia, England, and Eastern Europe. It has been reported to be endemic in the developing world, in areas of moderate to warm climate. In industrialized countries, *Cyclospora* has been detected in tourists or expatriates returning from countries where diarrheal illnesses are highly prevalent, however, there are additional reports from people with no travel history (37–39). Cyclosporiasis has also been reported in immunocompromised AIDS patients (40–45).

The first reported case in the United States in an HIV patient occurred in 1986 from a traveler returning from Mexico (40). In those days, most of the reported cases of cyclosporiasis were from travelers and expatriates returning from endemic areas (40,46,47). where *Cyclospora* is found predominantly during the warm and humid months (23). This was observed in longitudinal studies in endemic areas of Peru (10); there was an increase in the number of episodes from January to May (summer to early fall) (10,29,48,49).

Age seems to be an important factor in populations in which *Cyclospora* is endemic, with significantly greater prevalence in children older than 18 months of age and younger than 8 years (10). In Nepal, children with diarrhea under 18 months of age were not found positive to *Cyclospora*. However, children 18 months to 5 years old showed a 12% prevalence of cyclosporiasis, and 2% of children without diarrhea had *Cyclospora* (48). This age susceptibility is not fully understood and may be attributed to increased exposure to infectious *Cyclospora* after weaning. In the same area, the consumption of untreated water or unpasteurized milk diluted with water were considered risk factors for contracting cyclosporiasis for travelers and expatriates.

In a two-year prospective cohort in Peru (50), the overall prevalence of *Cyclospora* was 1.6% in children younger than eight years of age, increasing to 3 to 4% during the summer months (January to March), and 0.3% in children older than eight years. Of the infected children, 32% were symptomatic with diarrhea and some of them had concurrent infections with other pathogens. All adults that were surveyed in this studies were found negative to *Cyclospora*.

Seasonality and patterns of age susceptibility in Guatemala are very similar to those described for Peru and Nepal. June is the month with higher incidence, with rates as high as 10%. During the months of May and June, 19% of all pediatric gastroenteritis were due to *Cyclospora*, and children experienced prolonged diarrhea if untreated. Epidemiological analysis demonstrated an increased odds ratio associating cyclosporiasis with water consumption. Among the very young children, direct contact with soil seemed to be a potential source of contamination. Although not clearly understood, the presence of chickens or other fowls in the houses was also considered a risk factor.

WATERBORNE OUTBREAKS

In the industrialized countries, the epidemiology of *Cyclospora* is mainly associated to outbreaks, and in some cases *Cyclospora* has been epidemiologically linked to water consumption. The first documented outbreak attributable to *Cyclospora* occurred in 1990 and affected 20 members of the hospital staff of a Chicago hospital, where tap water was presumed to be the source of infection (37). In Utah, a man was diagnosed with cyclosporiasis after cleaning his flooded basement following heavy rains (39). In another incident, a child became ill and was diagnosed to CLBs in his feces one week after swimming in Lake Michigan. Water samples taken from the inlet of the Chicago municipal water supply system at this lake showed the presence of organisms resembling CLBs, although at that time the organism was not confirmed as *Cyclospora* (38). In Nepal, British soldiers and dependents experienced diarrhea caused by waterborne *Cyclospora*. The drinking water was a combination of river water and municipal water. Despite chlorination, oocysts were isolated from the water, suggesting that *Cyclospora* is resistant to chlorination levels recommended for water treatment (51). When assessing potential sources of infection, all factors shall be considered, including travelers or natives infected with *Cyclospora*, contaminated food, and insects as transport hosts. Further epidemiological studies will help in the assessment of these factors and in the development of control and treatment strategies for *Cyclospora*.

FOOD-BORNE OUTBREAKS

The first reported food-borne outbreak in the United States occurred in 1995 when 45 cases of cyclosporiasis were diagnosed in Florida in people without travel history. In 1996, a total of 1,465 cases from 20 states, Washington D.C., and 2 Canadian provinces were reported. Epidemiological analysis of these cases, using retrospective case-control studies of 180 people from Florida, showed that consumption of raspberries was strongly associated with cyclosporiasis (O.R. = 31.95, 95% CI 7.41–38.2). People who ate raspberries were 17.6 times

more likely to develop cyclosporiasis (RR = 17.6, 95% CI = 19.1–88.8) (52).

In the United States, these cases were related to 55 events between May 3 to June 14, 1996. Raspberries were identified as the potential source of *Cyclospora* infection and definitely served in 50 of the 55 events. In 27 of these events, there was significant association between raspberries and cyclosporiasis, p less than 0.05. The attack rate was estimated to be 56.3% for the 53 events in which information was available. Only one event had blackberries from the same country, and not raspberries. The median event-specific attack rates among people eating raspberries was 93.3% for the 29 events, with sufficient data to trace the source of the berries; all raspberries came from the same country. In sporadic cases, the consumption was 79.4% for strawberries, 57.8% for raspberries, 32.2% for blueberries, and 19.3% for blackberries (53).

The exact mode of contamination of the berries remains unclear. Studies of water used for irrigation of the raspberries showed intermittent contamination with fecal coliforms and *E. coli*, but no *Cyclospora* was detected. River water analysis showed heavy fecal contamination with *E. coli*; *Cyclospora* was found in 2 out of 30 samples with concentrations estimated to be greater than 15,000 oocysts per 10 L specimen (54). A dripping system was used to irrigate the raspberries and was intended to avoid contamination of the berries with irrigation water; however, the same water was used for spraying pesticides and fungicides on the berries themselves, and thus again was the potential vehicle for contamination. Because *Cyclospora* requires 7 to 14 days to fully sporulate and become infectious, it was considered unlikely that infectious *Cyclospora* could have been introduced into the berries during the spraying or during the preparation of dishes served on the events.

In 1997, basil was implicated with an outbreak in the Virginia/Maryland area (55). Clusters of *Cyclospora* positive patients were reported, identified, and associated with luncheons during the months of June and July. Forty eight of 54 people attending the luncheons had illness compatible with the clinical definition of cyclosporiasis. The relative risk associated to the pesto salad was determined to be 5.9, p less than 0.001. The basil used in the luncheons was traced back to a specific distribution company in which the basil (imported) was repacked and sold to the restaurant industry. Twenty five additional clusters were identified and linked to the consumption of fresh basil from the specific company in the area, and at least 20 additional possible clusters were linked to this product. The implicated company was asked to stop selling its basil products and the health departments of Virginia and Maryland issued press releases to inform the public to stop consuming fresh basil, either alone, or in products that could contain this fresh product.

Another outbreak in the Missouri area was reported and studied by retrospective questionnaires. This cohort study was conducted around during two social events and the epidemiological analysis demonstrated that in this *Cyclospora* outbreak, the most likely source of infection was basil. Chicken pasta salad and tomato basil were more strongly associated with *Cyclospora* infections. Rapid intervention allowed the analysis of specific meals linked to the presentation of cyclosporiasis. Leftover pasta salad was found positive by PCR, and one *Cyclospora* oocyst was detected by microscopy (56). Sporulated *C. cayetanensis* oocysts were isolated from the salad. It was postulated that the contamination occurred either way back at the distribution chain or at the original production site.

In a study conducted in an endemic area, *Cyclospora* was isolated from vegetables purchased from local markets (57). One hundred and ten samples were purchased from 13 markets of Pampas de San Juan, a periurban town in Lima, and analyzed for the presence of *Cyclospora*. Results indicated a 1.8% prevalence of *Cyclospora*. In a second collection from the same area, laboratory results showed that 1.6% of the samples were positive for *Cyclospora*, indicating the potential of food-borne transmission via fresh produce. *Cyclospora* oocysts have also been isolated from sewage water and water collected after washing leafy green vegetables such as cabbage, lettuce, and mustard from other endemic areas (14).

Viability can be determined by incubating the organism in potassium dichromate and looking for sporulated oocysts. An alternative method has been recently described. An electrorotation (58) method has been proposed to determine the sporulation of *Cyclospora* from water and vegetables. The lack of an in vivo or in vitro culture methodologies as a gold standard makes it difficult to evaluate the viability of *Cyclospora*.

ENVIRONMENTAL SAMPLING

Drinking water samples can be screened for the presence of *Cyclospora* oocysts. Oocysts can be isolated by filtration by using collection of 10 L water samples, using cartridges, or by flocculation methods developed for removing *C. parvum* in water (59). Oocysts were identified by examination using bright field microscopy and UV epifluorescent microscopy.

Irrigation water and wastewater samples were analyzed by collection of 1 to 5 L using the Envirocheck capsule (Gelman Sciences, Ann Arbor, Michigan). Particulate material was removed from the filter with an eluting solution consisting of phosphate buffer, SDS, Tween 80, and Antifoam A (Sigma Chemical Co, St. Louis, Missouri). The suspended particulate matter was then transferred to 250-mL centrifuge bottles and centrifuged at $1,050 \times g$ for 10 minutes. The centrifuged particulate matter was condensed as the final pellet (60).

Hannifin polypropylene cartridge filter (commercial Filters Parker Hannifin Corp. Lebanon, Indiana) was also used to recover Cyclospora oocysts. Larger volumes of water samples were filtered using these cartridges. The woven filters were then sliced to the core, and fibers washed in elution solution. The eluted suspensions were condensed and centrifuged in 100 mL aliquots at $1,050 \times g$. Oocysts were then purified by either zinc sulfate flotation or discontinuous sucrose separation.

Cyclospora was also isolated from produce. Several strategies have been examined to determine the most

efficient procedure for recoveries, such as washing of produce, sonication, and separation using lectin-coated (wheat germ agglutinin) paramagnetic beads, with recovery efficiencies in the range of 12% (57,61).

FTA filters (Fitzco, Inc., Maple Plain, Minnesota) that are impregnated with denaturants, chelating agents, and free-radical traps lyses most cells on contact, although retaining DNA within its matrix (62) has also been used to improve isolation of oocysts from produce. This methodology would circumvent the presence of inhibitors in food matrices and seems to detect as low as 30 *C. cayetanensis* oocysts per 100 g of fresh raspberries, with the potential to adapt to other food, clinical, and environmental samples.

CONTROL MEASURES

Direct and immediate person to person transmission seems to be unlikely in *Cyclospora* transmission because oocyst require 10 to 15 days from the time of excretion until they sporulate and become infective. The use of drinking water filters suitable for *C. parvum* removal could be effectively used to remove *Cyclospora* oocysts from drinking water. No significant information on effectiveness of disinfectants, temperature, or other physical inactivation or removal methods is currently available.

Washing does not completely remove pathogens from fresh produce. People can prevent infections from *Cyclospora* by limiting the opportunity of ingestion of fecally contaminated materials. Careful hand washing is useful in preventing the spread from food handlers. Travelers should follow routine recommendations and avoid drinking tap water, unpasteurized or nonboiled drinks and foods when traveling to endemic areas. Boiling or adequate filtering of water for human consumption also aid the prevention of this and other pathogens.

Internet resources such as the emerging infections program sites of FoodNet have effectively enhanced the monitoring and early detection of food-borne outbreaks. This system involves the states of California, Georgia, Minnesota, Oregon, and Connecticut.

CONCLUSION

Cyclospora is a fairly new parasite and in the last eight years, significant progress has been made in efforts to understand its biology and transmission dynamics. Important aspects of its basic biology are not yet fully understood such as its life cycle, host susceptibility, survival in environmental conditions, and ideal disinfection methodologies. The lack of laboratory methods or animal systems to propagate the parasite has proven to be the limiting factor for these research initiatives.

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D

DATA ANALYSIS AND MODELING

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Almost any attempt to monitor the presence and activity of microorganisms in the environment will be met by an abundance of data. Microorganisms are ubiquitous and so is their activity. Many mineral cycles depend on the microbiota for completion, such as nitrogen fixation or sulfate reduction, and all of them can be fully implemented by microorganisms. Another characteristic adding to their ubiquity is the metabolic plasticity that characterizes microbial communities, a response to irregular substrate availability. As a consequence, microbial communities implementing a specific process are expected to emerge whenever that process confers a selective advantage. In many cases, individual isolates will be recovered with the new functionality (such as the recent isolation of denitrifying nitrifiers), but this generalization is mostly valid at the population level, for example, no microorganism was ever isolated capable of converting glucose to methane. The combination of plasticity and ubiquity of microbes is particularly evident in the design of wastewater treatment plants where no inoculation is required. The right microbial community will emerge to make the most of the opportunities for growth provided by the design and operation of the station. In conclusion, monitoring the presence and activity of microorganisms in the environment represents a challenge in choosing and calibrating the relevant analytical technique (Fig. 1). This section focuses on data analysis and how it is ultimately dependent, first, on the choice of environmental signal and, second, on the data available describing the context of the transduced signal.

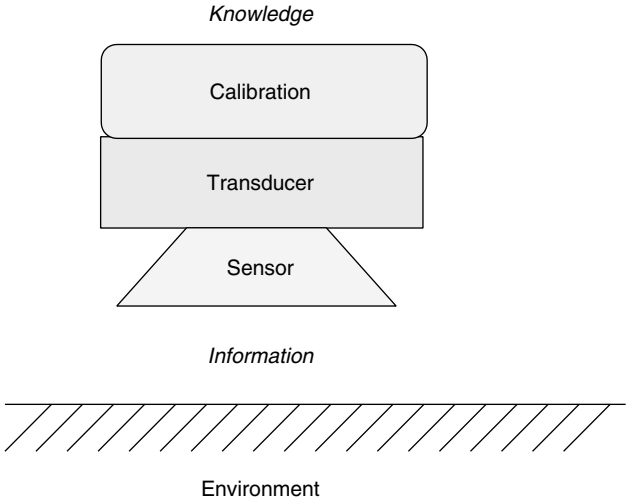

Figure 1. Analysis of environmental information as depending on the choice of the right signal, for example, microbial lipid composition; the use of the appropriate transducer, for example, gas chromatography of the hydrophobic extract; and interpretation of the results by identifying its contextuality, for example, calibration of transduced signal by comparison with database of results from other sites and other microbial communities.

DATA ANALYSIS SCHEME

The data analysis procedure depends on the objectives of the study and the type of data available. Nevertheless, the general layout invariably requires the objectives of the study to be translated in the parallel identification of objective parameters and suitable environmental signals, which are the dependent and independent variables, respectively. It is particularly important that the experimental design or field survey is closely matched by the data analysis layout to explore the possibilities for statistical analysis to the fullest. Accordingly, the scheme outlined in Figure 2 begins with the definition of objectives and proceeds to the point where a mathematical model is developed. However, in many cases, the objective of the study is purely exploratory, in which case, only the left branch of the scheme in Figure 2 applies. In some instances, data analysis is considered only after completion of data collection, and in that case, only the lower half of the scheme applies.

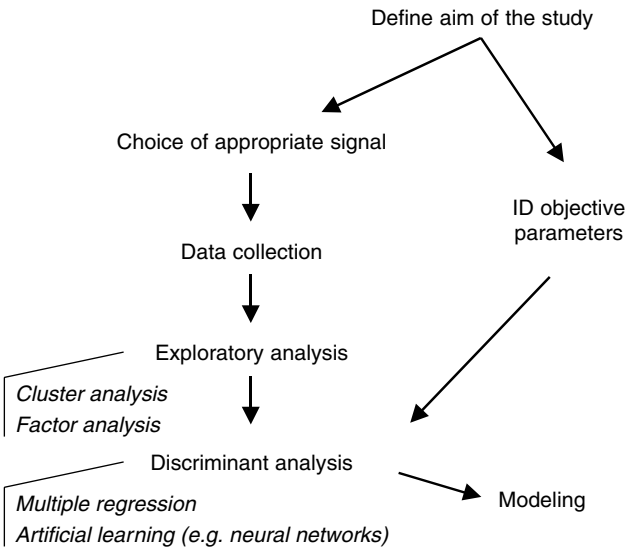

Figure 2. Data analysis scheme, from stating the objectives of the study to developing a mathematical model.

THE RIGHT KIND OF DATA

The other methodological sections overviewed a variety of techniques targeting different components of the microbial

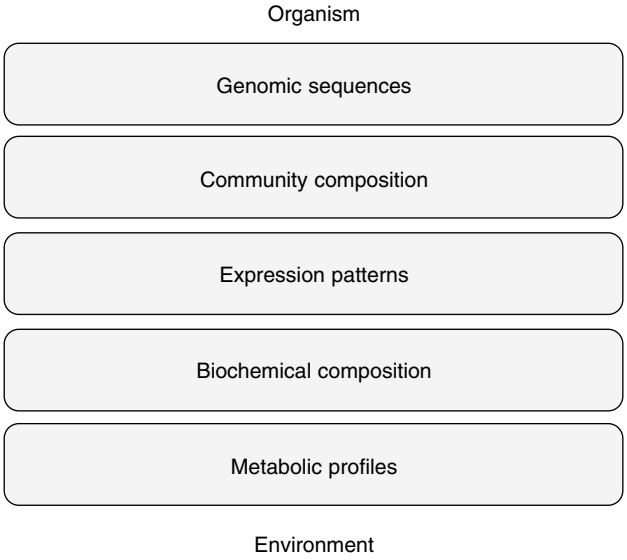

Figure 3. Microbiological signals that can be used to monitor the presence and activity of microorganisms.

community. These components range from the biochemical composition to the genetic makeup and also include expression and metabolic activity patterns (Fig. 3). The usefulness of this information is generally proportional to the mechanistic distance of the target component. For example, the physiological status of a microbial community is more apparent in the lipid composition of cell membranes than on the Restriction fragment length polymorphism (RFLP) pattern of extracted DNA. The diagram in Figure 3 purposefully ignores methods that require isolation and cultivation of microorganisms. That approach, although valid in clinical microbiology or food biotechnology, faces the insurmountable obstacle that the overwhelming majority of microorganisms in the environment would not be detected, that is, less than 1% of soil bacteria detected by direct count are culturable (1,2). Previously well-established environment safety standards based on univariate indicators, such as coliform counts, are now being downplayed in environmental protection regulations as a stand-alone indicator. Therefore, even when convenience is the only concern, multivariate biological indicators offer a better chance for the development of stable standards of environmental quality.

The data produced by these biological signals may take several formats. It may be a vector of concentrations or abundance, it can take a binary format coding for the presence or absence of indicator traits, or finally, it may be reducible to a sequence of categories. One example of each type is presented in Figure 4.

Information Content

In principle, the higher the number of variables in the multivariate signal, the better the chances of finding a predictive association with the objective parameters. However, the individual variables may be correlated and so the added measuring effort will result in an increased redundancy of the signal. The information content can be evaluated by making use of information theory tools, particularly the Shannon information index (3) (Eq. 1):

$$Sh = -\sum_{i=1}^{N} p_i \ln(p_i)$$
$$N : \text{number of components} \quad (1)$$
$$p_i : \text{likelyhood of } i\text{th component}$$

The algorithm to determine the values of p_i is detailed in Table 1, a recent example of this procedure applied to microalgae pigment profiles water samples can also be found in the work by Hofle and coworkers (4).

To compare the information content of profiles with different number of components, Sh can be normalized as an evenness index(5), E, by dividing it by the maximum Sh, which would occur if all components were equally likely ($p_i = 1/N$):

$$E = \frac{Sh}{\ln(N)} \quad (2)$$

Information content indexes are important to determine the ideal conditions to obtain a signal. Figure 5 illustrates this application, where the optimal incubation time for carbon usage tests of solid waste compost samples was found to be approximately 40 hours.

EXPLORATORY ANALYSIS

The variety of techniques available to explore multivariate data is often confusing for the beginning practitioner. The basic statistical techniques for cluster analysis, principal component analysis, correspondence analysis

Table 1. Algorithm to Calculate the Information Content of a Concentration Profile

$C_{i,j}$ = concentration of profile component j sample i. $i = 1, \ldots, n$ and $j = 1, \ldots, m$ i.e., there are m components and n samples.

1. The abundance, A_i, of a component concentration, C_i, is defined as the value relative to the maximum observed concentration. This is a simplistic approach that there may be baseline values and not all concentrations are equally likely. For increased accuracy A_i should be defined as the cumulative frequency of C_i instead.	$C_{\max_j} = \max(C_{ij}, i = 1, \ldots n)$ $A_{i,j} = \frac{C_{i,j}}{C_{\max_j}}$ or (see text) $A_{i,j} = f_{C_{i,j}}$
2. The probability value for each abundance is defined as the relative abundance with regard to the sum of abundances in the sample profile.	$p_{i,j} = \frac{A_{i,j}}{\sum_{k=1}^{m} A_{i,k}}$
3. The Shannon index, Sh, can now be calculated as described in Equation 1	

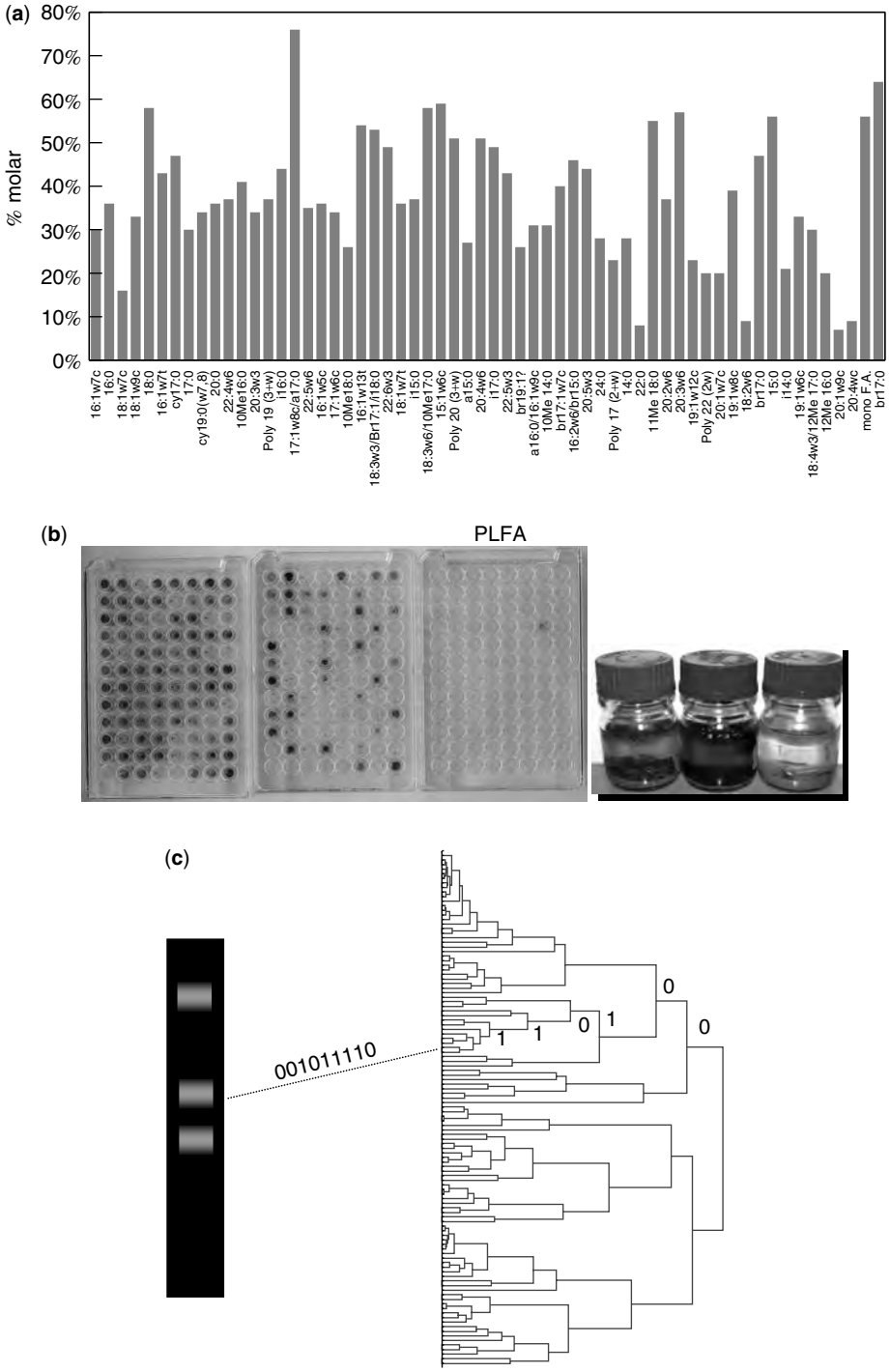

Figure 4. (**a**) Example of a phospholipid fatty acid (PLFA) signature profile. The corresponding numerical format is a vector of molar fractions. (**b**) Carbon utilization pattern obtained with BIOLOG^{TM} galleries inoculated with aqueous extracts of three different soils (flasks). (**c**) PCR amplification of rDNA fragments obtained from soil samples, followed by denaturating gel gradient electrophoresis. The fragments can be sequenced and compared with reference sequences. Its position, with regard to similarity to reference sequences (the position of the band in the gel per se is of little use), can then be translated as sequential binary code as described in the figure. See color insert.

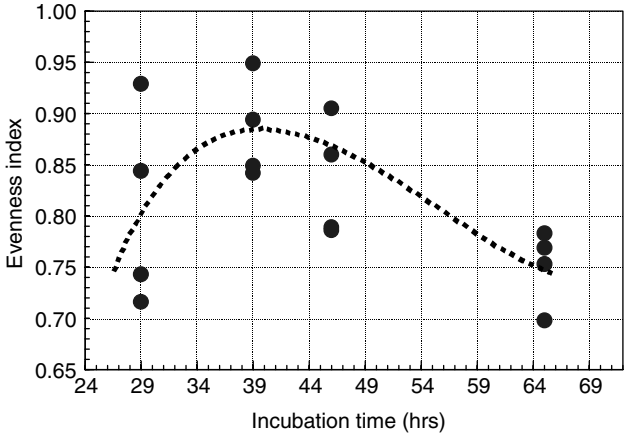

Figure 5. Evenness index (Eq. 2) applied to BIOLOG^{TM} GN carbon source usage galleries to determine the optimal incubation time.

and multidimensional scaling are introduced in this section (Fig. 6). For a more in-depth applied introduction, the reading of Everitt and Dunn Applied Multivariate Data Analysis (6) is recommended. Nonhierarchical clustering by the K-nearest means procedure and the more advanced unsupervised mapping approaches such as Kohonen Maps are also mentioned. It is important to keep in mind that all these techniques are of exploratory nature. They do not test hypotheses regarding their association with the objective parameters (Fig. 2). Instead, they analyze the relationship between the environmental signals and the contribution of their component variables.

The exploratory analysis of acquired data is based on some measure of distance (or dissimilarity) between the sampled signals that is appropriate for the system being studied. As an example, for the binary patterns generated by carbon utilization tests (Fig. 4b) or by bands in RFLP gels (Fig. 4c), it is reasonable to quantify the differences by the number of traits that differ. This is sometimes called the absolute distance or the city-block distance: $d_{i,j} = \sum_k |x_{k,i} - x_{k,j}|$. However, the comparison of concentration profiles using this measure of distance would be biased toward the furthest distances and it is more usual to adopt an euclidean distance: $d_{i,j} = (\sum_k (x_{k,i} - x_{k,j})^2)^{1/2}$. There is a wide variety of similarity indexes and a correspondingly prolific literature (7). The choice of metric is conditioned not only by the nature of the system but also by the objectives of the study. Accordingly, the measure of distance may be of statistical nature, as is usually the case in factor analysis, of geometric nature as in the two examples above, or of any metric that is deemed proportional to the perceived dissimilarity.

Bottom-Up and Top-Down Exploratory Analysis

The descriptive analysis of the multivariate signal is a very extensive field in statistics and the reader is referred to a comprehensive introductory (8) to the advanced (9) literature sources. The choice of analytical approach falls in two complementary sets of techniques, bottom-up and top-down. They both aim at representing the matrix of distances between all the sample signals, with an emphasis on the similarity between individual samples or on the overall similarity structure, respectively.

Cluster Analysis

Cluster analysis designates a technique that iteratively groups (amalgamates) the individual sample profiles by similarity. The importance of this technique for the analysis of biological field data has driven its development from the very beginning, being a statistical methodology largely developed by and for biologists (10). To use this technique, the user needs to define what measures of similarity (or the inverse, distance), and what amalgamation schemes are appropriated for the objectives and nature of the study. Just as the measures of similarity discussed in the preceding text, there is a wide variety of possibilities for amalgamation schemes,

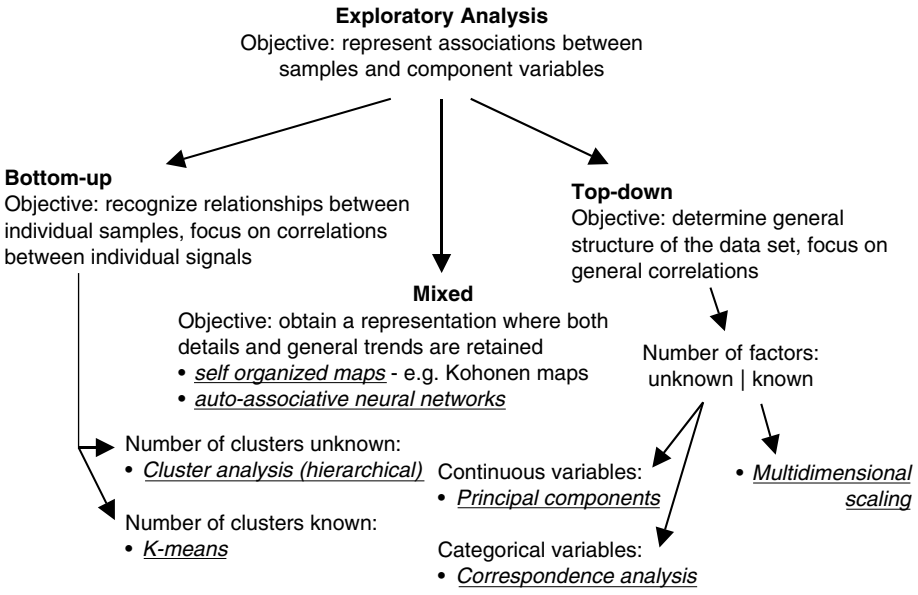

Figure 6. Decision tree to choose the appropriate exploratory data analysis technique.

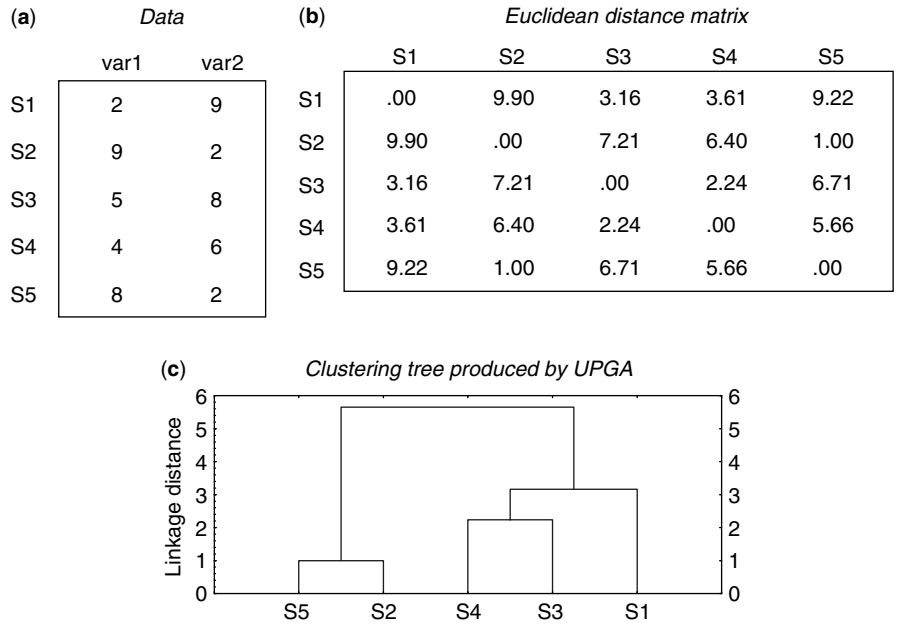

Figure 7. (**a**) Illustration of cluster analysis applied to a data set consisting of five samples characterized for two experimental measures. (**b**) The first step is to determine the distance matrix (dissimilarities, see earlier section) for all possible pairing of samples. (**c**) Then, a tree diagram is generated by following the amalgamation scheme, in this case UPGA, see text.

briefly mentioned in the following text. The linkage between a candidate member of a group can be single or complete. In the first case, the distance is measured with reference to the closest or the average group member, depending on the algorithm. Another variation consists of weighting by the size of the cluster, a useful technique when uneven cluster sizes are expected. Still another possibility is to use the analysis of variance to evaluate distance between clusters (Wards method). With regard to validation, the alternative representations obtained may be evaluated by a number of techniques. The simplest one is to compare the original distance matrix with the distance matrix built from the cluster tree (Fig. 7). The correlation coefficient between the original distance matrix and the matrix of linkage distances in the cluster tree (Figs. 7b,c) is designated as the cophenetic coefficient. More advanced validation techniques involve testing the stability of grouping by removing and reintroducing candidate sample profiles (11), which leads to branching schemes by likelihood of group membership (12). The most frequent choice of amalgamation rule is arguably the unweighted pair-group average, often referred to by the initials UPGA. In Figure 7, this amalgamation scheme is illustrated for five samples in which two variables were measured.

The example in Figure 7 illustrates a hierarchical cluster analysis procedure. As one looks deeper in a cluster, the less reliable it is to use linkage distance to predict similarity between individual samples. If the number of clusters in set beforehand, than a nonhierarchical k means that a clustering technique can also be used. This procedure will distribute the samples between k groups to minimize the profile variance within groups and maximize it between groups.

Factor Analysis

Multivariate factor analysis is the most widely used technique for top-down exploratory analysis. The purpose of factor analysis is to reduce the number of variables or axes required to represent the data set by identifying the contributions of each component of the sample profile, characterizing the data structure. The factors are identified by the linearization that represents most of the residual variance from the previous factor identification. The simple example described in Figure 8 uses the data of Figure 7 to illustrate the extraction of one principal factor from a two-dimensional data set. Factor analysis can be applied to higher dimensional sets where, for practical reasons of visualization, only two or three factors are typically identified. This approach can be applied to both continuous and categorical data. In the former case, principal component extraction is the method of choice, whereas the latter requires correspondence analysis. The resulting representations of sample profiles can be evaluated by determining how much of the total variance is explained by each of the factors extracted.

If the final number of dimensions is known beforehand, and to avoid an uneven distribution of variance by the reduced orthogonal coordinate axis, multidimensional scaling can also be used. This technique aims at representing the multivariate data in a predefined reduced dimension space such that most of the variance is represented, and is so in either reduced coordinate axis.

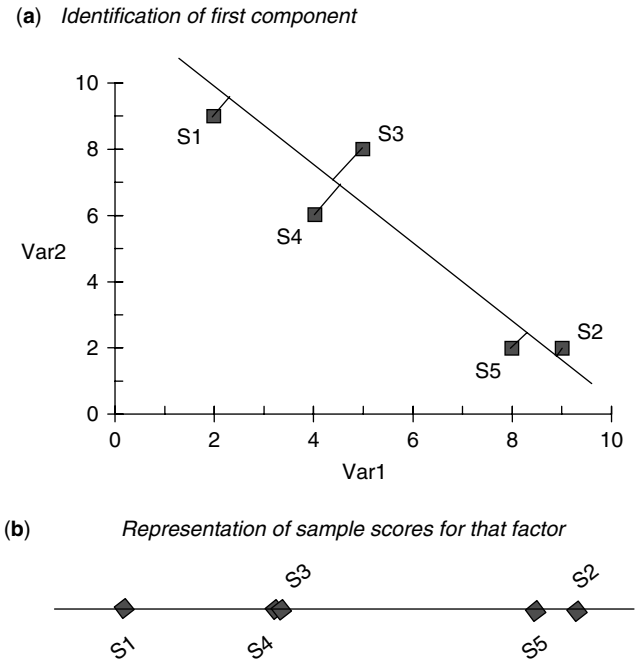

Figure 8. Illustration of extraction of first principal component for the two-dimensional data set of Figure 7. See color insert.

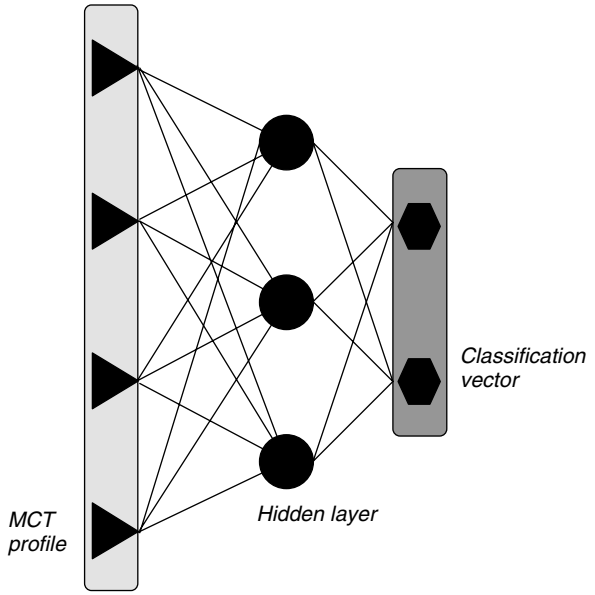

Figure 9. Schematic architecture of a three- layer feed-forward network used to associate microbial community typing (MCT) profiles with classification vectors. Symbols correspond to neuronal nodes (Eq. 8). See color insert.

Self-Organizing Maps

From an applied perspective, self-organizing maps (SOMs) represent a compromise between cluster analysis and factor analysis. The principle consists in organizing the sample in a space such that similar profiles will be close to each other. SOM achieve this by interconnecting all samples and then "straining" its lengths according to similarity between profiles. The resulting maps represent a coordinate system in which the samples can be placed such that their profiles change smoothly with position. This technique reached maturity with the landmark work of Tuevo Kohonen in early 1980s and an updated review of the methodology and application was recently published (13).

Auto-Associative Neural Networks

Feed-forward neural networks (NNs) associate input (independent) multivariate signals with one or more output (dependent) variables. The principles behind its development are discussed below under Discriminant Analysis since NN are primarily a classification technique. However, if the independent variables (Fig. 9, input) are also used as the classification or dependent criteria (Fig. 9, output), then the value obtained for the hidden nodes can be used as coordinates of a space with reduced dimensionality. The resulting map is somewhat similar to the Kohonen SOMs, except that instead of similarity, proximity represents the existence of a similar predictive model. Consequently, dissimilar profiles with component variables similarly related (for example, obeying to fixed rations) will be placed in adjacent regions. The use of auto-associative NNs as an exploratory technique has been applied alongside conventional factor analysis to map changes in soil microbial community composition signaling for bioremediation (14). This approach is sometimes compared with factor extraction by noting that its graphic representation would resemble Figure 8, except that instead of lines, one would find curves becoming a de facto principal curve extraction procedure.

DISCRIMINANT ANALYSIS

Strictly speaking, discriminant analysis is the procedure that identifies which independent variables are distinct between samples recognized as belonging to different classes (for example, subjected to different treatments, coming from different geographic locations, etc.). Out of convenience, this section also includes other modeling techniques such as multilinear regression and neural networks. The rationale is that all these procedures aim at identifying a discriminant function (Eq. 3) that can predict the value of the objective parameters (Fig. 2, right branch), which could be either continuous such as the concentration of a pollutant, or discrete, such as having been subjected to a specific treatment. The discriminant function, f_{DF}, uses the chosen signal components as independent variables (Fig. 2, left branch).

$$\text{Objective parameters} = f_{DF}(\text{signal components}) \qquad (3)$$

Regression of Explicit Functions (Multiple Regression)

The simplest multiregression technique assumes a linear relationship between dependent and independent variables. Therefore, when using multilinear regression, the discriminant function associates each of the n-objective

parameters $OP_{i=1,\ldots,n}$ to a multilinear combination of each of the m-signal components $SC_{j=1,\ldots,m}$ (Eq. 4).

$$OP_i = a_i + \sum_{j=1}^{m} (b_{i,j}\, SC_j) \tag{4}$$

The value of $b_{i,j}$ is more than a proportionality coefficient between OP_i and SC_j, it also quantifies the association between the two. However, when interpreting the associations uncovered, particular attention must be given to the level of intercorrelation between the signal components as mentioned in the section on Information Content. There are several techniques to handle redundancy, such as forward variable addition (15). Another important warning to bear in mind refers to the generality of multilinear models. If the number of components in the input signal is of the same order as the number of signals collected, not enough degrees of freedom will be left to make the model generalizable. The problem of model over-fitting is relevant for all the techniques in this section and it is further explored later in the article while discussing the optimization of artificial neural networks, the ultimate discriminant functions.

The association between environmental parameters is hardly ever linear. Therefore, if there is mechanistic evidence for a particular nonlinear formulation that should be incorporated in the formulation of the f_{DF}. A very common example is the existence of exponential relationships, such as the one that occurs between optical density and concentration. The use of hyperbolic formulations is also a widely used function such as the Michaelis-Menten equation for rate limitation by the availability of substrate (Eq. 5).

$$v = v_{max} \frac{S}{S + Km} \tag{5}$$

The latter example will now be used to demonstrate how a basic nonlinear association can be used to achieve a comprehensive mathematical description. In a waste treatment system most components can be classified as substrates, products, or microbial components. Using the reaction rates as objective parameters implies that the identification of f_{DF} corresponds to defining the dependency on each of these three components. The starting point was Equation 5, for dependency on substrate. The microbial component is the reactive element and as such defines the saturation rate, v_{max} (Eq. 6).

$$v = (X\, v_{max}) \frac{S}{S + Km} \tag{6}$$

Finally, inhibition by-product can also be formulated as a hyperbole by using the difference between the concentration for total inhibition, P_{max}, and the concentration present in the media, P (Eq. 7).

$$v = (X\, v_{max}) \frac{S}{S + Km} \frac{P_{max} - P}{P_{max} - P + Kp} \tag{7}$$

In spite of the apparent over-simplified formulation of the role of the three components in the system, a model built exactly on these principles is currently the gold standard to model microbiological wastewater treatment. Since 1987, the International Association for Water Quality (IAWQ) has been systematically compiling formulations describing the individual effects of an increasing number of microbial and chemical components playing a role in activated sludge systems. The product of this factorization exercise is the widely used ASM, Activated Sludge Model (16).

Neural Networks — A Very Quick Guide

Among the most important tools deriving from bioinformatics research is the artificial neural network (ANN) technique. An ANN is a massively parallel-distributed processor able to store experience-based knowledge and make it available for use. It resembles the human brain in two respects: (*1*) knowledge is acquired by the network through a learning process, (*2*) interneuron connection strengths known as synaptic weights are used to store knowledge (17). The ability of an ANN to identify mathematical models that closely fit data without the need for mechanistic assumptions is ideally suited for the analysis of complex data sets such as ecosystem variables and DNA microarray hybridization patterns. If properly implemented, the resulting mathematical formulation distinguishes signal from noise in the associations among an arbitrary number of parameters. ANNs have a wide range of biological applications, from modeling soil bioremediation (14), to genomic sequence analysis (18). Clustering like samples (19,20) and learning from examples without a priori knowledge of causality (21) are some of the most important uses of ANN. The reader is referred to studies by Hagan and coworkers (22) for applied design and implementation of ANNs, by Haykin (1994) (17 and Bishop (1995) (23) for the theoretical foundations, by Montagne and coworkers (1994) (24) for a review of contributions in biotechnology, and, finally, by Cheng and coworkers (1994) (25) for a discussion from a statistical perspective that approaches ANNs as universal discriminant functions. The steps involved in developing an ANN involve: (*1*) assembling the network of unit processors, (*2*) training them by changing the connection weights to minimize the predictive errors, (*3*) regularizing the solution to ensure its general nature, and finally, (*4*) repeating the previous steps for the domain of ANN topologies to find the optimum extraction of signal versus noise. This four-step procedure is inspired in the process of natural learning and is detailed in the following section for a feed-forward ANN architecture:

1. Assembling parallel processing units in fully connected layers. Each neuronal node is equated to a sigmoidal function, f, of the product of a weight matrix, w, by the input vector x:

$$y = f(wo + w\, x), f(\alpha) = \frac{1}{1 + e^{\alpha}} \tag{8}$$

2. Training ANN with cross-validation as a stop criterion, that is, part of the data are excluded from

training and are used to evaluate the generality of predictions.

3. Bootstrapping each topology evaluated. The validation subset is resampled repetitively and the ANN is retrained with cross-validation each time. The median performer is selected. This procedure avoids the possibility that the validation data set may not be representative of the whole.
4. Optimization of topology. The range of topologies is searched for the one with the best performance. The complexity of the topology selected is proportional to the complexity of the signal extracted (26).

CONCLUSION — MODELING ENVIRONMENTAL COMPLEXITY

"All models are wrong but some are useful" (27) is frequently quoted to highlight the utilitarian purpose of models. Conventionally, models are mathematical formulations of hypothesis. The validity of predictions by models is also the validity of the underlying hypothesis. Although the mathematical formalism varies widely, from simple conditional statements to multiagent systems, the convention calls for the definition of two components state variables, and state equations. The state variables are the parameters chosen to describe the dynamic behavior and the state equations describe their interdependence. The choice of variables and the formulation to describe the underlying mechanism for interaction obeys another frequent quote known as Ocham's razor: "Simplest explanation and theory is right." Ocham's razor applies not only to model formulation but also to the very choice of state parameters "No more things should be presumed to exist than are absolutely necessary" (also quoting W. Ocham). These two principles, usefulness and simplicity, command the formulation and validation of models with the goal of capturing system mechanism and the reproducing system dynamics. Modeling is the ultimate product of data analysis and that is the reason it is discussed last. Like in many other areas, the modern advances in computation are reshaping fundamental aspects of data analysis. Modeling has typically been used as a hypothesis testing tool. However, the advent of machine learning, such as ANNs, is reversing the paradigm. If, in model identification, the mechanistic description is replaced by an artificial learning component, which is allowed to regress to existing evidence, then an implicit, nonmechanistic, formulation emerges. As a consequence, the challenge is moved from model identification to the sensitivity analysis of its predictions. This role reversal turns modeling from a hypothesis testing tool into a hypothesis generating tool. The fundamental question raised by automated modeling identification through machine learning is if that solution may be more than a practical shortcut to an otherwise time-consuming problem. The modern concept of biocomplexity (28) offers some answer by claiming that real open biological systems are not wholly amenable to mechanistic reduction, for example, there is no comprehensive explicit mechanism that describes system dynamics. Accordingly, complexity is defined as a behavior that cannot be reduced to a combination of behaviors of component units. This statement reflects the observation that generation of information in complex systems resides mostly in the interaction between those component units. Since microbial ecology is inherently complex, in the sense described earlier, it is to be expected that artificial intelligence, information theory and statistical mechanics will become more prevalent as tools for data analysis in environmental microbiology.

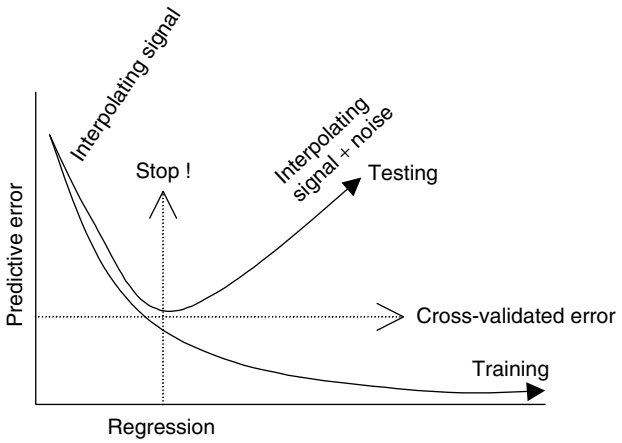

Figure 10. Description of the early stop procedure: regression is applied to the test sub-data set but the error is also calculated for the validation sub–data set. In the course of regression, the divergence between the two signals, the beginning of over-fitting and the regression is stopped.

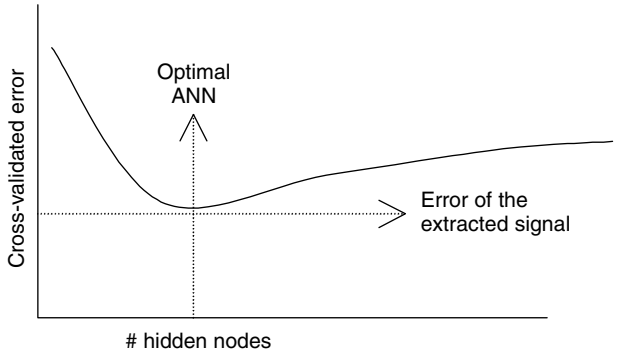

Figure 11. Optimization of topology: the procedure described in 1–3 is repeated for different number of hidden nodes, and the ANN with the smallest cross-validated error is selected.

Acknowledgments

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DEHALOGENATION OF HALOORGANICS.

See BIODEGRADATION: REDUCTIVE DEHALOGENATION AND METABOLISM OF CHLORINATED ORGANICS BY ANAEROBES

DENATURING GRADIENT GEL ELECTROPHORESIS (DGGE).

See PHYLOGENETICALLY BASED METHODS IN MICROBIAL ECOLOGY

DENITRIFICATION.

See SOIL NITROGEN CYCLE; TRACE GASES SOIL

DENITRIFICATION IN ACTIVATED SLUDGE.

See ACTIVATED SLUDGE — MICROBIOLOGY OF NITROGEN REMOVAL

DENITRIFICATION IN THE MARINE ENVIRONMENT

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DENITRIFICATION AND THE MARINE NITROGEN CYCLE

Bioavailable nitrogen, mainly inorganic and some organic species, is one of the essential building blocks of life. In marine systems, which include coastal estuaries and the open ocean, the amount of the bioavailable nitrogen influences the carbon cycle by regulating the accumulation of organic biomass, limiting the rate of primary production, and determining which major nutrient (nitrogen, phosphorus, or silica) limits primary production (1). Simply put, nitrogen cycling in marine systems influences local and global processes. An understanding of nitrogen inputs, recycling, and removal in the marine environment is required for understanding global nitrogen and carbon cycles. Biologically mediated processes govern the transformation of nitrogen among organic, dissolved inorganic, and gaseous forms (Figs. 1 and 2). Three processes serve as cornerstones for nitrogen cycling in the marine environment: nitrogen fixation, nitrification, and denitrification. These processes transform nitrogen between gaseous and dissolved inorganic forms.

The inorganic forms of nitrogen include oxidized (nitrate, NO_3, and nitrite, NO_2) and reduced (ammonium, NH_4) species. Inorganic nitrogen compounds are present at ultralow (nanomolar to low micromolar) concentrations in surface ocean waters, but concentrations increase with depth and accumulations of tens of micromoles per liter are common in deep waters. In addition

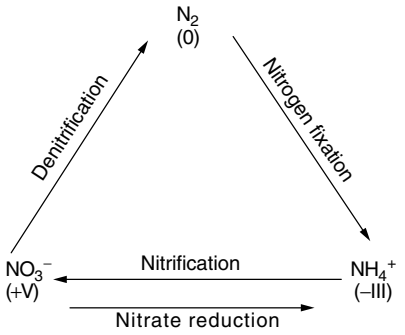

Figure 1. Linkages between the reductive and oxidative processes of nitrogen cycle, focusing on denitrification, nitrogen fixation, and nitrification. Dinitrogen gas, nitrate and ammonium (valence state in parentheses) are the major dissolved inorganic forms of nitrogen. The major reductive processes are nitrogen fixation and denitrification. Nitrogen fixation converts highly inert dinitrogen gases into ammonium, which is assimilated and used to build biological biomass. Nitrification is the oxidative component of the nitrogen cycle and is linked to denitrification, the reduction which closes the nitrogen cycle by regenerating dinitrogen gas. In some cases, nitrate is reduced to ammonium (rather than to dinitrogen) serving assimilatory (providing ammonium for incorporation into biomass) or dissimilatory (using the reduction to generate energy) functions. Conversion of organic nitrogen to ammonium recycles nitrogen to the inorganic pool and connects biological nitrogen fixation to nitrification (Fig. 2).

to dissolved inorganic forms, nitrogen is present in dissolved organic, particulate (organic and inorganic), and other gaseous forms (Fig. 2). Organic nitrogen includes particulate organic nitrogen (e.g., living organisms and detritus) and dissolved organic nitrogen (e.g., amino acids and proteins). Dissolved organic nitrogen concentrations may exceed dissolved inorganic nitrogen concentrations in marine waters. The most abundant nitrogen compound in marine waters is a dissolved gas, dinitrogen (N_2). Dissolved dinitrogen originated either from atmospheric equilibrium or from in situ production via denitrification. Nitrogen trace gases, such as nitrous oxide (N_2O), are present at low (nanomolar) concentrations. Inorganic and organic nitrogen forms are available to biological organisms (i.e., they are "bioavailable") and therefore support the production of new biomass (primary production; Fig. 2). However, dinitrogen (N_2) can serve as a nitrogen-nutrient source only to a limited number of bacteria that possess a special enzyme, nitrogenase, which reduces N_2 to NH_4 and subsequently incorporates NH_4 into the biomass (Figs. 1 and 2) (2). Small deviations in the amount of oceanic denitrification (internal loss of nitrogen) relative to nitrogen fixation (internal gain of nitrogen) can alter the uptake of atmospheric of carbon dioxide by influencing rates of primary production.

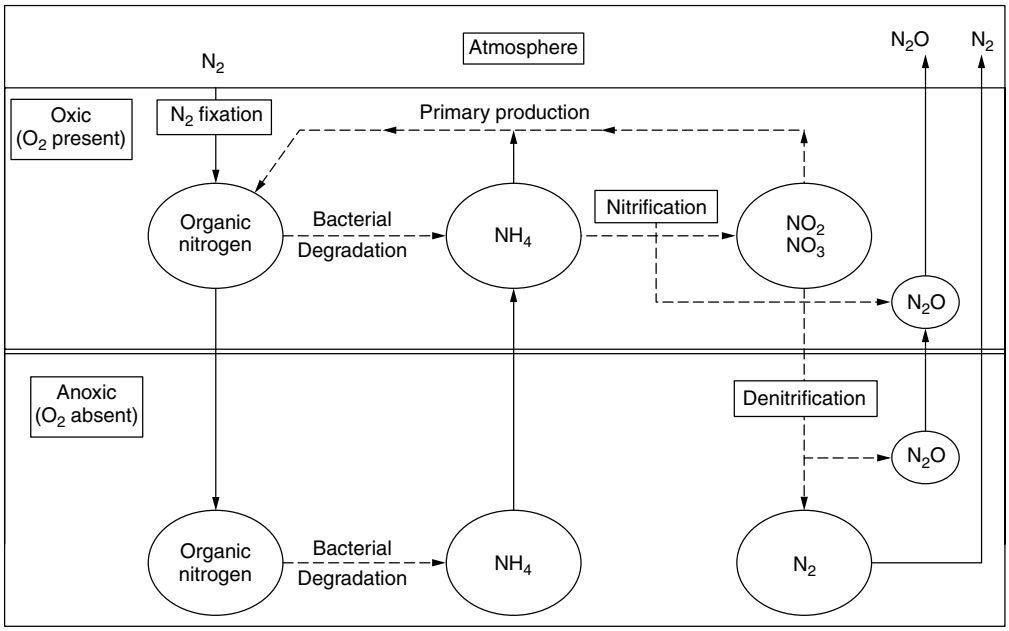

Figure 2. Denitrification in aerobic (O_2-rich) and anaerobic (O_2-poor) marine environment and the connection with the atmosphere. The dominant nitrogen species are shown in large circles, whereas the less abundant species are shown in small circles. The transition from aerobic to anaerobic conditions can occur at the water–sediment interface or within the water column in highly productive areas within a stratified water column. Generally speaking, marine waters are aerobic, whereas sediments are anaerobic. Biologically mediated processes are marked by dashed lines with arrows, whereas physical processes (settling, diffusion) are marked by solid lines with arrows. The three dominant transformations of nitrogen between pools, mainly nitrogen fixation, nitrification, and denitrification, are shown in bold. These three processes are interconnected by the processes of primary production, which produces organic nitrogen and bacterial degradation, which recycles organic nitrogen to the ammonium pool. Whereas primary production and nitrification are largely restrictive to aerobic regions of the water column, denitrification is restricted to anaerobic regions. Bacterial degradation occurs throughout the water column.

Rates of nitrogen inputs, dominant transformations, and removal vary in the different parts of the marine environment. Inputs of nitrogen to marine ecosystems include external (terrestrial or atmospheric sources) and internal (biological nitrogen fixation) terms. Rates of land-derived nitrogen loading from rivers and groundwater to the marine environment are highest near continental margins; the importance of atmospheric inputs depends on the proximity to margins and on atmospheric weather patterns (3,4). As a result, inorganic nitrogen concentrations are highest near the continents and decrease with increasing distance offshore. In stratified environments or in oxygen-limited zones, inorganic nitrogen concentrations are higher in anoxic or suboxic (bottom) waters below the photic zone than in oxic (surface) waters. Rates of biological processes, such as dinitrogen fixation or nitrification, vary as a function of bioavailable nitrogen concentration, oxygen concentration, nitrogen speciation (organic versus inorganic), and the abundance of the necessary microorganisms (5,6) (Fig. 2). Nitrogen input to marine systems is rapidly taken up by biological organisms and a single nitrogen molecule is recycled between organic and inorganic pools many times before being removed from the marine system (7). Of the nitrogen available in marine systems, some fraction is "removed" from the system on an annual basis and is thus no longer available to support biological production. Removal occurs through two mechanisms: burial or denitrification. Burial of nitrogen in sediments removes nitrogen from the active cycle by transferring nitrogen (mainly organic) into a compartment (the sediment) that cycles on timescales exceeding the residence time for nitrogen in the ocean.

The focus of this article is the process of denitrification. Denitrification is the only biological process that culminates in the production of dinitrogen gas (N_2) (8,9). As most primary producers (phytoplankton and plants) cannot use dinitrogen as a source of nutritive nitrogen (2), dinitrogen production serves to effectively regulate the amount of bioavailable nitrogen in an ecosystem. The process of denitrification, the microorganisms that mediate denitrification, and the environmental controls on denitrification will be described briefly. The methods used to quantify denitrification will be summarized, and then the distribution and rates of denitrification in different parts of the marine environment will be overviewed. Finally, important areas of future research on denitrification will he highlighted.

DENITRIFYING BACTERIA

Denitrification is the dissimilatory reduction of nitrate or nitrite to a gaseous end product (e.g., nitric oxide, NO; nitrous oxide, N_2O; or N_2) (10). Denitrification rates are influenced by a variety of microbiological and environmental factors. For denitrification to occur, microorganisms capable of mediating the process must be present. Though more denitrifiers cluster in the alpha and beta subclasses of the proteobacteria, denitrification is distributed in 40 genera of bacteria and is also present in halophilic archaea (10,11). Denitrifying microorganisms obtain energy in a variety of ways, ranging from photoautotrophy (obtaining energy from sunlight), to chemoautotrophy (obtaining energy from the oxidation of inorganic compounds), to strict heterotrophy (obtaining energy from organic matter oxidation). The majority of denitrifying bacteria are facultative anaerobes, meaning that they use NO_3^- as the terminal electron acceptor only when oxygen concentrations fall below a critical threshold concentration, commonly accepted to be around 20 μM (12). Under low oxygen conditions, and when supplied with sufficient nitrate, denitrifying bacteria couple nitrate reduction with the oxidation of organic matter (10,13):

$$4HNO_3 + 5CH_2O \rightarrow 5CO_2 + 7H_2O + 2N_2(\text{gas}) \qquad (1)$$

During this process, nitrate is sequentially reduced to dinitrogen through a series of intermediates (Fig. 3). The reduction of nitrite to nitric oxide is the first step that produces a gaseous intermediate and is thus, technically, the first step of denitrification. Respiratory nitrate reduction to nitrite, followed by nitrite excretion, does not constitute denitrification. Denitrification is catalyzed by enzymes associated with the cytoplasmic membrane, and heterotrophic denitrifying bacteria conserve energy via the generation of an electrochemical proton gradient (11) (Fig. 3). A detailed discussion of denitrification enzymology is available in Zumft (10) and will not be provided here.

At times, the process of denitrification may result in the production of N_2O, rather than N_2, as the primary product. The term "incomplete denitrification" is used to refer to such a short-circuiting in the denitrification pathway. The production of N_2O, instead of N_2, may arise from the fact that the microorganisms carrying out denitrification lack the terminal enzyme, N_2O reductase, and are thus unable to produce N_2. However, under certain environmental conditions (e.g., in the presence

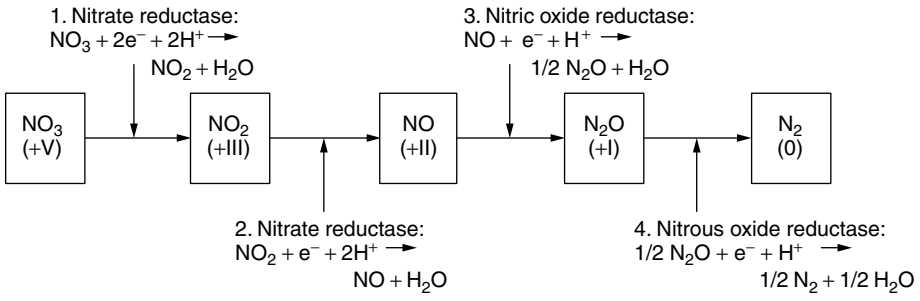

Figure 3. The four steps of denitrification process. Nitrogen species are shown in boxes with the valence state of the species given in parentheses. The enzymes mediating the sequential steps (numbered 1 through 4 on the order of occurrence) of the reaction are also shown.

of hydrogen sulfide or high concentrations of nitrate) microorganisms capable of producing N_2 may produce N_2O instead (Fig. 4; see later). High rates of N_2O production and release into the marine environment may also result from higher-than-optimal oxygen tensions in the denitrification zone. As N_2O is a radiative trace gas that contributes substantially to the greenhouse effect in the Earth's atmosphere, understanding the sources, sinks, and controls on N_2O flux from the environment is important. Denitrification is thought to be a source of N_2O in the marine environment; however, the dynamics of N_2O production and consumption are poorly understood in most marine habitats.

In addition to heterotrophic denitrification, several additional mechanisms of denitrification exist. Some autotrophic microorganisms couple nitrate reduction to the oxidation of inorganic sulfur, hydrogen, ammonia, or nitrite (10). The alternative denitrification pathways referred to as "denitrifying nitrification" (14,15) and the "anamox" reaction (16–19) have been well documented. Of these, the anamox reaction, that is, N_2 formation resulting from NO_3 reduction coupled to anaerobic NH_4 oxidation, could play an important role in the marine nitrogen cycle. However, the importance of this alternative denitrification mechanism on the global nitrogen cycle is largely unknown.

GENERAL CONTROLS ON DENITRIFICATION

The magnitude of the marine denitrification term in the global nitrogen cycle is determined by spatial and temporal variations in denitrification rates, which are influenced by a suite of environmental and physiological factors. Denitrification requires low oxygen concentration and ample supplies of nitrate and dissolved organic carbon (13). Like most enzyme-catalyzed reactions, denitrification rates vary in proportion with temperature. In a given environment, lower temperatures tend to result in lower denitrification rates and vice versa. Other environmental variables, such as salinity and pH, may also impact denitrification in some areas. In most of the marine environment, the salt content (salinity) of the water is high and stable and thus does not impact denitrification rates. However, in coastal regions where the salt content may vary significantly over short (daily, tidal) or seasonal (as a function of freshwater inflow) timescales, increased salinity may result in reduced denitrification rates (20). The pH is probably not a major control for denitrification in marine water columns but may influence denitrification rates in marine sediments. In coastal regions, where lower pH freshwaters mix with marine waters, system-scale variations in pH might affect denitrification rates. The impact of such pH variations on coastal denitrification rates has not been studied in detail. Some denitrifying bacteria use alternative sources of reductants, such as H_2 or H_2S (instead of organic carbon), and the concentrations of these chemical species may influence denitrification, particularly in low oxygen or anoxic water column and sediment environments.

The most frequently used controls for heterotrophic denitrification in the environment are oxygen, nitrate, and

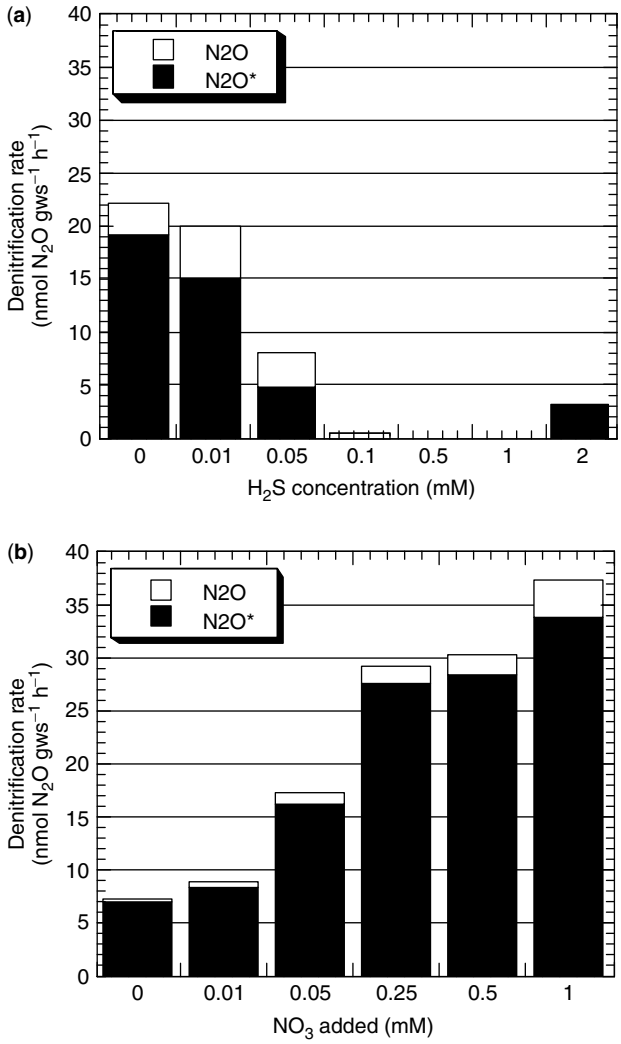

Figure 4. Potential denitrification in estuarine sediment slurries; closed bars reflect nitrous oxide (N_2O) production in the presence of acetylene and open bars reflect N_2O production without acetylene. (**a**) Rates when slurries were amended with varying hydrogen sulfide concentration in the presence of nitrate (1 mM) and glucose (0.5 mM). Denitrification rates are inhibited by low (10 to 50 μM) hydrogen sulfide; no bar infers that the rate was below the detection limit. (**b**) Denitrification rates when sediment slurries were amended with varying amounts of nitrate in the presence of glucose (1 mM) concentration. Both the denitrification rate and the acetylene-free N_2O concentration increase with increasing nitrate concentration. The acetylene-free N_2O production rate increases from less than 1% of the total N_2O production rate to over 10% of the total N_2O production rate at the highest nitrate concentration.

organic carbon concentrations. As denitrifying bacteria are facultative anaerobes, they use nitrate as the terminal electron acceptor only when oxygen concentrations are low; under high oxygen tension, oxygen is respired instead of nitrate. Once oxygen concentrations fall below a certain threshold concentration (20 μM) (12), nitrate respiration begins. However, the ultimate effect of dissolved oxygen (O_2) dynamics on denitrification can

be one of stimulation rather than inhibition. Oxygen availability can increase rates of nitrification (nitrifying bacteria are obligate aerobes). Because nitrification often supplies the nitrate to denitrifying bacteria, higher oxygen tensions could, in effect, stimulate denitrification. When nitrification supplies the nitrate that is denitrified, the process is referred to as "coupled denitrification." Processes that alter the O_2 concentration or the thickness of the oxic zone, such as primary production, aerobic respiration, and aerobic oxidation of reduced metabolites, influence the rates of nitrification and, as a result, denitrification (21).

Whereas photosynthesis may stimulate coupled denitrification by increasing nitrification and indirectly providing an NO_3 source to denitrifiers, photosynthesis may also inhibit denitrification by elevating the O_2 concentration above the critical, inhibitory threshold. High O_2 concentration often leads to a shift from denitrification to aerobic respiration (22). The ultimate effect of photosynthesis on denitrification depends on the absolute oxygen concentration, which results from the balance between respiration and photosynthesis (23). Additionally, the role of benthic photosynthesis as a stimulator or inhibitor of coupled denitrification is influenced by the nitrogen inventory of the system being studied. Stimulation of coupled denitrification by benthic photosynthesis in lake and estuarine sediments has been reported under nitrogen sufficient conditions (23,24). In contrast, inhibition of nitrification and denitrification has been inferred under dissolved inorganic nitrogen limited (i.e., low concentration) conditions (23,25). The interaction between benthic primary production and denitrification is complicated, and seasonal variation in rates of photosynthesis and in the nitrogen-nutrient status of primary producers may lead to alternating impacts (stimulation in summer and limitation in winter) (23,26) on denitrification.

Other environmental factors that alter the distribution of O_2, such as wind-induced mixing of water bodies, bioturbation (i.e., mixing of sediment or pore fluids) by infauna (27,28), or root pumping of O_2 into the sediments by benthic plants may also impact rates of nitrification and/or denitrification (29,30). Nitrification and bioturbation are often positively correlated because bioturbation stimulates nitrification by increasing O_2 availability (31,32). Spatial and temporal variations in temperature, organic carbon supply, and O_2 and H_2S concentration may affect nitrification and, thus indirectly influence denitrification (33,34) or coupling between nitrification and denitrification (35,36).

Nitrate and organic carbon concentrations also strongly influence denitrification rates (22). If either of these substrates is present in limiting amounts, then denitrification rates fall well below maximal potential values. Anoxic fine-grained, organic-rich sediments accumulate significant concentrations of labile organic carbon, such as acetate and other low molecular weight volatile fatty acids. In such environments, ample organic carbon exists to fuel denitrification; nitrate, more frequently than organic carbon, limits denitrification. Nitrate limitation arises from the fact that nitrate must diffuse from oxic environments into the anoxic sites where denitrification occurs. In sandy, organic-poor sediments, organic carbon can limit denitrification rates. Organic carbon limitation of denitrification has been inferred more frequently in tropical systems than in temperate systems. In tropical ecosystems, either nitrate and/or organic carbon may limit denitrification (37,38).

Most denitrification in the environment is dependent on nitrification, the oxidation of ammonium to nitrate (6), for its substrate supply (Fig. 2). Therefore, a close temporal and/or spatial association between nitrification and denitrification is required to deliver nitrified nitrate efficiently to the zone of denitrification. The importance of coupled denitrification in the marine environment cannot be understated. The mechanism of nitrate delivery to denitrifying zones, however, varies. Nitrate may be produced in the water column and subsequently diffuse or advect (via bioturbation) into the anoxic sediments or into an anoxic part of the water column where denitrification occurs (Fig. 2). Alternatively, nitrate may be produced in oxic sediments by nitrification and then diffuse into anoxic microsites or layers where denitrification occurs. Finally, in coastal zones, nitrate may be delivered to, or produced within, an aquifer, and then nitrate-containing groundwater may later be transported through coastal sediments where denitrification intercepts groundwater-derived nitrate (39). The term "direct denitrification" is used to describe the case where nitrate is derived from an external source, that is, a source outside of the system being studied. Though the source of this external nitrate could be nitrification, nitrification and denitrification are not explicitly linked in space and/or time. As the processes are acting independently of each other, they are uncoupled (thus the term "direct" denitrification).

As stated previously, denitrification is restricted to anoxic environments where supplies of both nitrate and organic carbon exist. Most of the marine environment do not fit these criteria; however, certain marine regions do fit the criteria and these areas support extremely high, globally significant rates of denitrification. There are three major regions of denitrification in the marine environment: in bottom sediments, within midwater oxygen minimum zones that occur beneath highly productive surface waters, and within basins where restricted water exchange leads to the development of anoxic conditions. Though denitrification has been documented in particles suspended within oxic waters in coastal environments, the importance of this phenomenon in the global nitrogen cycle is yet to be illustrated. In sediments and water columns, denitrification represents a significant sink for bioavailable nitrogen. Integrated rates of denitrification in these environments are significant compared to other fluxes in the global nitrogen cycle. Thus, denitrification influences the local, regional, and global inventories of bioavailable nitrogen, and thereby impacts patterns of nutrient limitation on short (seasonal) and long (geologic) timescales (7,9,40–42). Because quantifying in situ denitrification rates has represented an experimental challenge, the methods used to quantify denitrification will be presented before summarizing the distribution of denitrification in marine systems.

QUANTIFYING DENITRIFICATION RATES

The product of denitrification, that is, dinitrogen gas, N_2, is the dominant gas in the Earth's atmosphere (78%). Thus, marine waters in equilibrium with the atmosphere contain high dissolved N_2 concentrations ($\geq$300 μmol L^{-1}). This high background concentration makes it difficult to quantify the small changes accurately — usually less than a small percentage of the total pool — arising from in situ denitrification. As a result, many indirect methods, which do not depend on dissolved N_2 quantification, have been developed to estimate denitrification rates. The choice of method for quantifying denitrification depends primarily on whether the goal is to evaluate rates of direct and/or coupled denitrification, and on whether estimates of in situ or potential activity is desired. For simplicity, the various methods will be partitioned into three groups: direct methods, indirect methods, and potential methods. A detailed overview of the methods currently available to estimate denitrification can be found in Cornwell and coworkers (43).

Direct methods quantify the production of N_2 over time, thus providing a direct assessment of the denitrification rate. Dinitrogen concentration can be quantified via thermal conductivity gas chromatography (TCD-GC) or via membrane inlet mass spectrometry (MIMS). The TCD-GC approach is the least sensitive of the two, but this approach is also the more economical approach. Using the TCD-GC approach, samples may be degassed of N_2 and incubated for several days; then the production of N_2 over time can be used to estimate the denitrification rate (44,45). The preincubation period can be eliminated if appropriate anoxic controls are included (45). Several reports of N_2 quantification via TCD-GC without a degassing step are present in the literature (46,47); however, only high denitrification rates can be measured without initial degassing using traditional chromatographic approaches. By employing a different chromatographic column, An and Joye (23,48) were able to determine fairly low denitrification rates in a coastal sediment during an in situ benthic chamber incubation without initial degassing of N_2.

A significant step forward in the quantification of denitrification in the environment was the application of the MIMS technology to the study of denitrification (49,50). The MIMS technique makes it possible to determine denitrification rates quickly and accurately without initial removal (degassing) of dissolved N_2 or preincubation. The MIMS technique has several advantages over traditional GC techniques, including rapid analysis time (2 minutes versus 15 minutes per sample), small sample size (2 mL versus 20 to 100 mL), simultaneous determination of O_2 and argon concentration, and the absence of a gas-stripping step (which is required for the GC methods). This approach has been used to quantify denitrification in coastal sediments (49,50) and will certainly be applied to study denitrification in a variety of marine environments in the future.

There are a number of indirect methods available for estimating denitrification rates. Some indirect techniques measure the accumulation of nitrogen gases (such as N_2O), whereas others use mass balance or stoichiometric modeling approaches to estimate denitrification. The most commonly used method for estimating denitrification is the acetylene block technique (51,52). Acetylene blocks the terminal enzymatic step of denitrification, the reduction of N_2O to N_2, so the accumulation of N_2O in the presence of acetylene is assumed to reflect N_2 production. This technique works well if denitrification is dependent on water column nitrate, if nitrate is not limiting, and if the samples are not sulfidic. Because acetylene also inhibits nitrification, this technique cannot be used to estimate coupled denitrification rates. Spurious rates may be obtained with this technique if samples have low nitrate concentration or if samples are even mildly sulfidic (i.e., tens of micromolar hydrogen sulfide concentrations) (53).

Another common approach for estimating denitrification is to amend samples with ^{15}N (as $^{15}NH_4$ for assessment of coupled denitrification or $^{15}NO_3$ for assessment of direct denitrification) and to follow the production of ^{15}N-labeled intermediates or gases (54,55). An adaptation of this approach, the isotope pairing technique (56), permits the partitioning of N_2 production between direct (water column NO_3) and coupled (to nitrification) denitrification pathways. A drawback of ^{15}N techniques is that sediment nitrogen pools may not be in isotopic equilibrium with the overlying water to which the ^{15}N tracer was added (53,57), which complicates the rate calculations. Quantifying the natural abundance $^{14}N/^{15}N$ isotope ratio in nitrate and/or dinitrogen may also provide a valuable tracer of nitrogen cycling in the marine environment (58).

It is possible to estimate denitrification rates without quantifying the concentrations of nitrogen gases. Benthic flux stoichiometry is a common way to estimate denitrification rates in this manner. Using this approach, measured fluxes of oxygen or dissolved inorganic carbon or phosphorus are used to calculate the expected fluxes of dissolved inorganic nitrogen. This estimated nitrogen flux is then compared to the observed net inorganic nitrogen flux (inorganic nitrogen = ammonium + nitrate + nitrite), and any difference is assumed to reflect the net denitrification rate (59–62). This approach is typically imprecise because of the inherent difficulty in measuring benthic fluxes. Furthermore, any chemical oxygen demand may inflate O_2-based denitrification estimates and temporary storage of reduced metabolites also leads to errors in stoichiometric-obtained estimates. Fluxes of dissolved organic nitrogen that are not accounted for in benthic flux mass balances will also contribute to errors in denitrification estimates. Whole system mass balance models can be used to estimate denitrification rates (63,64), but this method can be imprecise because of the errors associated with other terms of the system nitrogen budget. Diagenetic models can be used to estimate denitrification rates (42), but such models require a great deal of input data that is not always available or easily obtained. Some models are difficult to validate, leading to large errors associated with rate estimates.

Potential denitrification rates are determined under anoxic conditions, with added nitrate and organic carbon

(typically glucose). Potential rates can be determined easily in water column or sediment (slurry) samples by quantifying the rate of nitrate depletion, by amending samples with acetylene and measuring N_2O accumulation (61), or by determining N_2 accumulation (48). Potential rates allow the experimentalist to determine the factors (O_2, NO_3, organic carbon, sulfide, etc.) that influence rates of denitrification (Fig. 4). To ensure the quality of data obtained using this adaptation of the acetylene block method, acetylene-free controls should be run to account for short-term changes in background N_2O production. Nitrous oxide production in the absence of acetylene is usually negligible at low nitrate concentration; however, background N_2O production can constitute 10% or more of acetylene-inhibited N_2O production rates at high nitrate concentration or in the presence of low concentrations of sulfide (Fig. 4) (65). We have observed increases in background N_2O production in samples amended with even moderate (100 μM) concentrations of nitrate to relieve nitrate limitation of denitrification. The rate versus concentration curves (Fig. 4b) generated in potential denitrification experiments can be used to obtain the necessary kinetic data (apparent K_m, V_{max}) for back-calculating in situ rates (61).

LOCATIONS AND PATTERNS OF DENITRIFICATION ACTIVITY IN THE MARINE ENVIRONMENT

Denitrification occurs in areas that are free of oxygen, but have sources of nitrate, from coupled nitrification, from the water column, or from groundwater. Denitrification also requires a source of labile organic carbon. Denitrification is common in sediments. The activity of denitrifying bacteria in the water column occurs only in a few select regions where low oxygen concentrations persist. Numerous studies of sediment denitrification have been conducted in a variety of habitats; however, most research has been carried out in near-shore temperate environments. In these areas, human-induced acceleration of nitrogen loading has created a need to understand the ability of these coastal ecosystems to remove excess inorganic nitrogen loading. Since denitrification can counterbalance nitrogen loading (as nitrate) to some extent, a significant research effort is under way to document the rates and patterns of denitrification in the coastal zone. Relative to near-shore coastal habitats, less information is available for continental shelf and slope sediments. Even fewer data are available for deep ocean sediments. Similarly, more data are available for temperate ecosystems, than for polar or tropical ecosystems.

Denitrification rates in temperate coastal sediments are high. Rates between 3 and 19 mmol N m^{-2} day^{-1} are common (66). Denitrification rates in temperate coastal systems tend to be related to the nitrogen loading rate of the ecosystem (63); however, it appears that the relationship between the system-wide denitrification rate and the residence time may not be as clear-cut as originally thought (67). Denitrification rates in tropical systems are markedly lower than those observed in temperate systems. Frequently, denitrification rates are offset by high rates of nitrogen fixation (31,68). Tropical environments receiving high nitrogen loading rates, via sewage discharge, for example, may exhibit substantial rates of direct denitrification (69). However, rates of coupled denitrification in tropical systems are generally low (68), suggesting that nitrifiers may be outcompeted for ammonium or that denitrifiers are outcompeted for nitrate. Chronic nitrogen limitation of other microorganisms and plants may deter denitrification in tropical environments, resulting in coupled denitrification rates that are low and insignificant. Limited supplies of labile organic carbon may also impact denitrification in tropical sediments. Polar near-shore sediments support substantial rates of denitrification (2 mmol N m^{-2} day^{-1}) (70) despite the cold temperatures; nitrate limitation of activity is common. Because the global area occupied by shallow coastal environments is small, these regions contribute only modestly to global integrated rates of denitrification (12 Tg N $year^{-1}$ removed via denitrification; 1 Tg = 10^{12} g) (8). However, denitrification is a very important process in these systems, removing, on average, between 20 and 50% of the nitrogen input to coastal systems (66).

A significant amount of marine sediment denitrification occurs in continental shelf environments (71–73); however, few direct measurements of denitrification are available for shelf regions. Using a modeling approach, the average denitrification rate for shelf sediments in the North Atlantic region was estimated to be 0.7 mmol N m^{-2} day^{-1} (74), a rate that is substantially lower than that observed in near-shore systems. Christensen (72) estimated the global denitrification rate for shelf sediments to be approximately 50 Tg N $year^{-1}$. Denitrification rates in deep ocean sediments are extremely low. Based on a limited number of actual measurements, the globally integrated rate of denitrification in deep ocean sediments was estimated to be 7 Tg N $year^{-1}$ (despite the fact that deep ocean sediments account for the largest area) (72). Since the number of actual denitrification measurements is limited, Middelburg and coworkers (42) used a modeling approach to estimate denitrification rates for marine sediments. The globally averaged sediment denitrification rate they obtained, 230 to 285 Tg N $year^{-1}$, with approximately 100 Tg N $year^{-1}$ occurring in shelf regions (42), is significantly higher than the range reported in the literature for marine sediments (12–89 Tg N $year^{-1}$) (72,75).

In certain parts of the marine environment, water column oxygen consumption rates exceed oxygen supply, thereby rates generating low (or zero) oxygen concentrations. These locations are called *oxygen minimum zones* (OMZs). Microaerophilic conditions favor both nitrification and denitrification and in these regions, rates of coupled denitrification are significant. Today there are three OMZs in the open ocean: the Arabian Sea and the North and South Eastern Tropical Pacific. Sometimes, OMZs develop near the coast. For example, coastal waters along Western continental margins, adjacent to California, Peru, and West Africa, are characterized by high rates of primary production and oxygen consumption and OMZs. Globally averaged water column denitrification rates in OMZs are about 60 to 90 Tg N $year^{-1}$. Denitrification also occurs in the water column of enclosed

or semienclosed saline water bodies, such as the Black, Baltic, and Mediterranean Seas (76–78), as well as in stratified fjords that become anoxic at depth (79). Denitrification rates in the Black Sea are believed to be approximately 1 Tg N year^{-1}. This rate is much lower than the rate in the Arabian Sea (34 Tg N year^{-1}) but is significant nonetheless. Denitrification may also occur occasionally in anoxic particles traveling through coastal water columns (80,81). The global importance of denitrification in particles or in other water column microanoxic zone (e.g., in zooplankton digestive tracts) is unknown.

In terms of the global nitrogen cycle, the available estimates of integrated denitrification rates exceed those of nitrogen inputs (Table 1). Based on the nitrogen budget presented by Middelburg and coworkers (42), the primary nitrogen sink via denitrification in the marine environment is in sediments. Whereas the highest average rates of denitrification occur in coastal sediments (average of 3.6 mmol N m^{-2} day^{-1}) (8), the total global denitrification rate in coastal sediments (12 Tg N year^{-1}) is but a small fraction of the total sediment denitrification rate (285 Tg N year^{-1}). Denitrification occurring in continental shelf sediments amounts to 101 Tg N year^{-1}, and denitrification in deep ocean sediments amounts to 172 Tg N year^{-1} (42). This upward revision in the global sediment denitrification rate, and hence in the global denitrification rate (42), is driven by significant increases in rates of denitrification in shelf and deep ocean sediments. Previous estimates of sediment denitrification in coastal, shelf, and deep ocean sediments (12 to 90 Tg N year^{-1}) (73) were similar to estimates for water column denitrification (60 to 90 Tg N year^{-1}) (75). These new globally integrated denitrification estimates suggest a large imbalance in the global nitrogen cycle. This imbalance has significant implications for nutrient controls (nitrogen versus phosphorus versus silica) on primary production and for glacial-interglacial changes in nitrogen cycling and in global primary production patterns. If these large deficits of bioavailable nitrogen in the ocean are correct, we will be forced to reevaluate the global rates of nitrogen fixation to ask whether more nitrogen fixation might be occurring in poorly studied parts of the marine environment.

Table 1. The Marine Nitrogen Budget (Tg N year^{-1})

Process	Rate
Inputs	
Riverine	25–43
Atmospheric deposition	40–50
Nitrogen fixation, accepted range	25–30
Nitrogen fixation, probable range	40–200
Total Inputs	90–293
Outputs	
Burial and export	23–43
Denitrification	
Water column	60–90
Sediments	230–285
Total outputs	313–418

Note: Adapted from Middelburg and coworkers (42).

Special Considerations and Future Research Needs

The availability and form of fixed nitrogen is a strong determinant of denitrification rates. As a result, there is a strong positive correlation between nitrogen loading rate and the integrated system denitrification rate (8,63). The relationship between nitrogen loading and denitrification is complicated, however, and is influenced additionally by physical and biological factors (see later). As discussed previously, biological processes, such as photosynthesis, can drive patterns and rates of coupled denitrification. An and Joye (23) found that photosynthesis significantly stimulated coupled denitrification in coastal sediments and that rates of photosynthetically stimulated coupled denitrification exceeded the denitrification estimated derived from the residence time in the ecosystem (based on the calculations of Ref. 63). Furthermore, the rates of denitrification that they documented were much higher than previous estimates in the same system, which also happened to agree with the residence time–based denitrification rates. They concluded that estimates of denitrification obtained during dark, rather than natural day (light) and night (dark), incubations could lead to erroneously low denitrification rates. If the pattern they observed holds for other coastal systems, then the available estimates of denitrification for near-shore environments could indeed be low. Both water column stratification (oxygen availability) and the amount of time water resides in a coastal system (i.e., the residence time) also influence the denitrification rate and the relative efficiency of nitrogen (nitrate) removal via denitrification (63,82–84). Future studies of denitrification in coastal systems need to consider the impacts of benthic photosynthesis and salinity (20) on the rates and patterns of denitrification.

Major gaps in our understanding of denitrification in the marine environment stem from the array of different methods that are used to quantify denitrification rates and from the lack of long-term data sets that include variability in both denitrification and in important environmental variables. Rigorous intercalibration experiments, including some of the old (acetylene block) and newer (MIMS) techniques, would provide us the ability to gauge the accuracy and precision of the existing data. Additional studies are needed in shelf and deep ocean ecosystems to validate global denitrification rates generated in model studies (for example, the rates put forth by Middelburg and coworkers (42)). In particular, more data are needed from polar and tropical ecosystems. Though a fair number of data sets describe denitrification in OMZ environments, the limited spatial and temporal coverage of the data, both within and between ecosystems, makes extrapolation to global scale difficult. More work is needed in these dynamic environments.

CONCLUSION

Denitrification represents a major sink for bioavailable nitrogen in the marine environment. Denitrification is

regulated by the availability of substrates (nitrate, organic carbon), oxygen concentrations, and the concentrations of inhibitors (hydrogen sulfide, salinity). Understanding the impact of photosynthesis and ecosystem residence time on denitrification warrants in depth future studies. Recent advances in the methods available to quantify denitrification, particularly the application of MIMS to study denitrification in marine habitats, promise to lead to significant advances in our understanding of the patterns and controls on denitrification in nature. Whereas denitrification rates are highest in near-shore sediments, offshore sediments dominate the global denitrification in sediments, accounting for about 96% of the total. Denitrification in the water column is approximately one-third of that present in the sediments. Understanding how global patterns of denitrification have changed over time and documenting the impact of denitrification on nitrogen recycling efficiencies in the marine environment, nitrogen to phosphorus ratios, and oceanic primary production are important research avenues that should be pursued in a variety of marine environments.

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DESERT ENVIRONMENTS.

See ENDOLITHIC MICROORGANISMS IN ARID REGIONS

DESERT ENVIRONMENTS: BIOLOGICAL SOIL CRUSTS

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Biological soil crusts are surface-bound assemblages of microorganisms that consolidate the soil into mm to cm thick crusts. They develop on a variety of environments but reach prominence in cold and hot arid lands wherever the lack of water restricts the settlement and development of higher plant cover. These largely microbial communities, variously known as cryptogamic, cryptobiotic, cyanobacterial, or simply, biological soil crusts (BSC), are initially dependent on the primary production of cyanobacteria and eukaryotic microalgae, either free-living or within lichen symbioses. In arid environments more benign with respect to water availability (such as the Northern Great Basin or the Columbia Plateau), mosses and lichens typically become dominant in mature crusts. Under regimes of strong aridity (such as in the Sonoran and Chihuahuan deserts), cyanobacteria and lichens tend to dominate mature crust communities. BSC's are typically slow growing, since periods of biological activity are restricted to events of liquid water availability linked to precipitation. Only the virtual surface and a very thin layer (1 to several mm) within these topsoil formations can be photosynthetically active. However, when wet, this layer can display very high areal instantaneous photosynthetic activity. High rates of aerobic respiration are typically found reaching down to several mm from the surface, which, if sustained, can result in local depletions of oxygen and the formation of anoxic microniches below the surface. In mature crusts, buried extracellular polysaccharide sheaths from cyanobacteria and lichen rhizoids may further extend the physically cohesive bounds of BSCs downward to about 1 cm from the soil surface. BSCs have been described from aridlands in all continents, including Antartica. In North America, for example, soil crusts cover large portions of undisturbed soils in the Colorado Plateau, the Chihuahuan, Sonoran, Vizcaino and Mojave deserts, in the Great Basin, in the Oregon, Washington, and Idaho high desert, as well as in the rangelands of Wyoming and Montana (1). The mere presence of soil crusts dramatically decreases soil erodibility by consolidating soil surfaces, affecting the hydrology of desert soils by either increasing runoff or infiltration. Their metabolic activities are large enough to contribute a large proportion of the biogeochemical cycles of carbon and nitrogen in aridlands. The ecology and biology of BSC have been recently summarized in an excellent edited volume (2) and the readers are referred to it for a more in-depth presentation.

STRUCTURE, DEVELOPMENT, COMPOSITION, AND DIVERSITY

The colonization of unconsolidated topsoils by cyanobacteria or microscopic algae represents the first step in BSC formation. The first colonizers are usually filamentous microbes that produce copious amounts of extracellular polymeric substances (exopolysaccharide) either of a diffluent nature or in the form of extracellular sheaths (Fig. 1). Filamentous cyanobacteria morphologically pertaining to the form- genera *Phormidium, Oscillatoria, Microcoleus*, and *Schizothrix* are the most commonly reported from young crusts. Little studied but important bacterial, fungal, mold, and flagellate populations may accompany the cyanobacteria at this stage. Unfortunately, very little attention has been given to microbial populations other than cyanobacteria. Because of the microbial nature of BSC's, decomposing, and mycorhizal fungi are probably not very important, but saprophitic forms seem to be able to attain significant populations making use of cyanobacterial and algal exudates. Sexual and sterile forms of saprotrophic fungi are characteristic of soil crust communities. Culturing studies by States and coworkers (3) found the pleosporaceous genera *Embellisia*, *Phoma*, *Bipolaris*, and four sterile dark-pigmented species (*mycelia sterilia*) with abundant chlamydospores most abundant in crust soils of Utah and Wyoming. Recently, culture-independent analysis of cyanobacterial community structure by means of phylogenetic reconstruction based on 16S rRNA gene sequence comparisons (4) have revealed that many of the BDC cyanobacteria form phylogenetically distinct clades, and are not closely related to cyanobacteria from other environments. If this holds true for other bacterial populations, BSC's may harbor a large inventory of untapped bacterial diversity. At the same time, such soil-specific phylogenetic clusters of cyanobacteria are deeply rooted and scattered in the overall cyanobacterial evolutionary tree. This implies that terrestrial cyanobacteria are evolutionarily old and is consistent with the view that soil crustlike communities may have been important terrestrial ecosystems of early Earth before the advent of higher plants (5). Indeed, filamentous, cyanobacteria-like microfossils have been found in terrestrial settings in the mid-to-late Precambriam (6). By far the most important and widespread cyanobacterium of soil crust is *Microcoleus vaginatus*, a filamentous type that forms bundles of filaments enclosed in a common extracellular sheath. Belnap (7) has called attention to the importance of this species in crusts of the U.S. Southwest. Morphologically and genetically indistinguishable populations of *M. vaginatus* occur in desert soils from North America, the Middle East, and Europe (4). All of these filamentous cyanobacteria seem to prefer the immediate subsurface, some 0.5 to 2 mm deep into the soil, where they encounter moderate light intensities and a refuge from UV radiation and erosional abrasion. They are motile by gliding and can make use of photosensory mechanisms to migrate up to the surface whenever incident light intensities are low (i.e., under overcast conditions). This can result in a sudden greening of desert soils under rainy, overcast conditions, making soil crusts under such conditions conspicuous to the naked eye. They return to the subsurface using hydrotactic mechanisms (8). In general, simple crusts containing only subsurface cyanobacterial populations are truly cryptic when dry and can only be distinguished by close, intentional inspection. Motility and cyanobacterial vertical migration within the crusts seems to be an important aspect in the initial stages of soil stabilization and crusting. The trails of extracellular mucopolysaccharide that filamentous cyanobacteria leave behind as they glide, glue the sedimentary particles in the soil surface together, even when the cyanobacteria are no longer present. Trapping and binding of allochtonous aeolian particles by the extracellular investments (particularly clays) may add to the initial stages of crust formation.

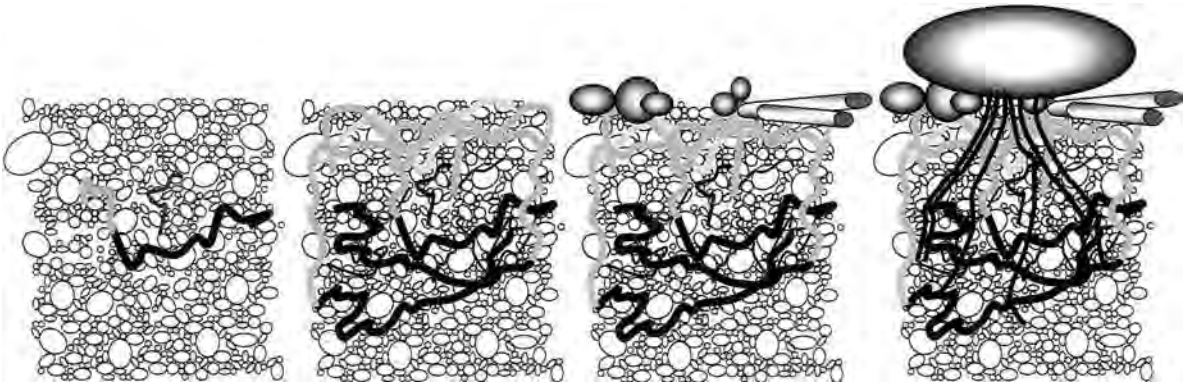

Figure 1. Abstracted view of sequential colonization steps leading to the formation of mature biological soil crusts. Left panel: initial colonization of unconsolidated soils by subsurface populations of filamentous, gliding cyanobacteria (dark lines) producing sediment-binding extracellular sheaths (gray lines). Middle left panel: vertical migrations of filamentous cyanobacteria, which leave trails of extracellular material that consolidate the soil surface. Middle right panel: sedentary, colonial cyanobacteria producing dark-pigmented envelopes colonize the crust surface imparting dark appearance to crusts. Right panel: cyanobacterial and green algal lichen colonization contributes to added physical stability.

A later stage in the formation of soil crust is the colonization of the crust's surface (Fig 1). The process of surface colonization is slow and can only occur when the substrate has been physically stabilized. But even a stable surface represents a very harsh environment for microbes, being directly exposed to full solar radiation, to the temperature extremes typical of desert environments and to the rigors of erosional forces. Only a few cyanobacteria are able to colonize the soil surface, and the process is slow. These are typically dark-pigmented, heavily ensheathed cyanobacteria of the genera *Nostoc* and *Scytonema*. Both of these genera are heterocystous (nitrogen-fixing) and, while they can differentiate specialized, motile forms for the purpose of dispersal, their vegetative cells are nonmotile. They produce copious amounts of sunscreen pigments, especially the extracellular alkaloid scytonemin, which gives their colonies a dark color and, in turn, is largely responsible for the typical black appearance of well-developed soil crusts (Fig. 2). Finally, in well consolidated crusts, given sufficient time, a large variety of soil lichens may develop, including both cyanobacterial and green algal photobionts (9). Shading of the crust by lichen cover may reduce the number of cyanobacteria in the soils, and induce shifts in the decomposer communities from bacteria and actinomycete dominated communities to those dominated by lichenicolous fungi.

FUNCTIONAL ASPECTS AND BIOGEOCHEMISTRY

The functional aspects of soil crust communities are governed by the episodic nature of water availability and by the small-scale distribution of physicochemical microenvironments. Most of the time, crust organisms are in a dehydrated state, during which only losses to the community (both in biomass and viability) occur. Eolian erosional abrasion, accumulation of photodamage, membrane and DNA damage by freeze-thaw cycles, and exposure to high temperatures contribute to this. During the short periods in which water is available, the organism hydrate, become active, and must carry out the metabolic processes for damage repair and growth that will ensure survival of the populations at least through the next drought. Indeed, microbial metabolism responds rapidly to the presence of water, and rates measured in wet crust are high. In cyanobacterial crusts, respiration starts just seconds after rewetting and initiation of photosynthesis occurs just after several minutes (10). Areal rates of photosynthesis attained in such crusts are comparable to those of mesotrophic lakes, and come close to those measured for higher plant leaves (11). Available estimates of annual carbon input in BSCs range from some 30 to 350 $kg\,C\,ha^{-1}\,y^{-1}$ (12,13). For comparison, available estimates for deserts at large, excluding BSC contributions, are about 800 $kg\,C\,ha^{-1}\,yr^{-1}$. Dinitrogen fixation by bacteria, cyanobacteria and cyanobacterial lichens seems to be the main source of nitrogen inputs to BSC's (14). Estimates for gross rates of dinitrogen fixation in BSCs range from as little as 1 to as much as 100 $kg\,N\,ha^{-1}\,yr^{-1}$ (9). The highest areal rates found in these BSCs easily surpass those measured in other terrestrial ecosystems.

Figure 2. Aspects of gypsum dune fields stabilized by biological soil crusts (Cuatro Ciénegas Valley, Coahuila, Mexico). In the foreground is an old gypsum dune field stabilized by mature crusts. The typical dark appearance is imparted by the sunscreen pigment, scytonemin, present in large amounts in surface-bound cyanobacteria and cyanobacterial lichens. The leading edge of wandering dunes of the same substrate is in the background. Note the impact of trampling on biological soil crusts visually evidenced in a seldom-used trail (arrow).

Like in other microbial habitats with high biomass accumulations within very small distances, the ecology and role of BSCs can only be completely understood by analyzing microbial activity, describing physicochemical environments, and assessing biogeochemical transformations at the microscale (μm to mm). Vertical gradients of solar radiation are caused by absorption and scattering of incident light. Vertical gradients in light intensity play an important role in defining the vertical distribution of phototrophs. Other microenvironments are the result of the metabolic activities of microorganisms and they involve localized gradients of chemical species. The formation of vertical microenvironments in the soil solution of BSC with respect to oxygen partial pressure (10,15) pH, light intensity, and light spectral composition (10) have been demonstrated (Fig. 3). When water-logged, the respiratory and photosynthetic activities of microorganisms in cyanobacterial crusts can be sufficiently high to overwhelm diffusive transport of oxygen and thus create spatially localized environments where oxygen might be depleted or supersaturated (10,15). The photosynthetically active zones (ca. the top 1 mm) reach steady states in which molecular oxygen is supersaturated. By contrast, the photosynthetically inactive, deeper zones (from 1 to 3–4 mm deep) are dominated by respiratory processes, and molecular oxygen is partially or completely depleted. In the absence of photosynthesis (wet crusts in the dark) anoxic zones can be much shallower (0.7–2 mm). The presence of internal anoxic zones has obvious implications for the mineralization of organic matter (both allochtonous and, probably primarily, autochtonous) in BSC's. Fermentation or anaerobic respiratory metabolisms may play a significant role in the mineralization of organic matter.

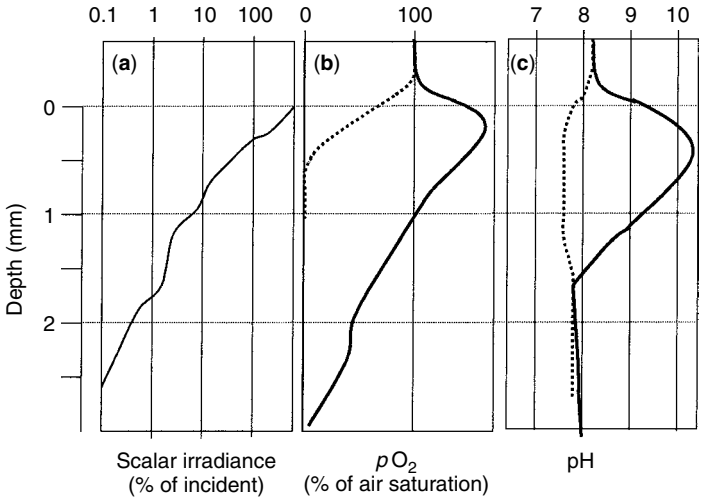

Figure 3. Some physical-chemical microenvironments present within a wet, active biological soil crust. A: extinction of incident light. B: Vertical profile of oxygen partial pressure (percent of atmospheric saturation) in illuminated (solid line) and dark (dotted line) crusts. C: vertical profile of soil solution pH in the light (dark line) and in the dark (dotted line). Redrawn from data in reference 9.

The availability of final electron acceptors other than oxygen in the crusts is likely to determine which processes may dominate. Thus, denitrification may take place in the soil crusts, largely powered by the formation of anoxic microzones. Some indirect estimates (16) point to the fact that perhaps as much as 80 % of the nitrogen assimilated by soil crusts is subsequently lost to the atmosphere during nitrification, denitrification, and volatilization, but direct evidence is still lacking. As a result of carbon dioxide uptake in photosynthesis, and its release in respiration, microscale changes in soil pH are likely to occur whenever oxygen microgradients are formed if the natural buffering capacity of the soils is low. Alkalinization of the top 1 mm of soils crusts by as much as 2 to 3 pH units in the light has been measured (Fig 3). Such depletion of protons may affect the solubilization and mobilization of silica, the establishment and germination of higher plant seeds, and/or the volatilization of ammonia (9). In the dark, acidification by 0.5 to 1 pH units in the dark, has also been measured in sandy soils, but data are lacking for other soil types.

ENVIRONMENTAL IMPACT AND CONSERVATION EFFORTS

The simple fact that aridlands cover some 35% of the total Earth's continental surface speaks for the potential significance of BSCs for global ecology. While BSC's may exert an influence on the overall fertility of soil ecosystems, research on the environmental impact of the presence, and the loss, of crust soil cover has centered on their physical influence on erosion and hydrology.

Studies on the impact of BSC cover on hydrology and water erosion has been very controversial. In some occasions, increases in runoff due to the presence of BSC have been determined (17) while, in other cases, results pointed to increases in water infiltration (18). In all probability, diametrically opposed effects are attained due to the diverse architecture of BSC's themselves. For crusts that do not present a high level of microtopography, Warren (19) has interpreted this variability in the light of known mechanistic hydrological properties of soils at large: "Where the sand content of a soil exceeds 80% and soils are not frost-heaved, the effect of biological crusts on infiltration is generally negative ... where the sand content of the soil is less than 80%, the effect of biological crusts on infiltration tends to reverse." In addition to this, when the crusts are frost-heaved (presenting a large amount of microtopography at the cm scale), BSC's tend to decrease runoff and increase infiltration by creating a multitude of microscale impoundments.

A wealth of studies has demonstrated that BSC's crusts can be a critical factor in reducing soil erosion by wind (reviewed in reference 20). Polysaccharides extruded by cyanobacteria and fungi bind soil particles together, creating soil aggregates, which, in turn, are glued together by live filaments and exudates. These larger, linked aggregates require greater wind work to displace than single grains (21). Cyanobacteria, being generally longer and larger than green algae and most bacteria, seem to impart more stability than other microorganisms (22). The passive nature of this effect is evidenced by the fact that chemically killed crusts (with intact polysaccharide glue) still protect the soil surface from erosion, at least temporarily. But BSC are highly susceptible to disturbance, as most crust organisms are brittle when dry and can be easily destroyed by mechanical forces (trampling, vehicular traffic; see arrow in Fig 2). Resistance to erosion can be significantly reduced after moderate disturbance (23,24). Sediment produced from such disturbances can "sandblast" adjacent crusts and abrade then at increased rates, thus amplifying the effects of disturbance. Because of the slow net growth rates of soil crust communities, recovery from mechanical disruption can take decades. Concerns on the environmental impact of crust degradation through cattle and human trampling on local soil erosion and suspended solid pollution in arid lands has already resulted in implementation of measures for crust protection in many rangeland management plans (25).

CONCLUSION

Because of the large global area covered by aridlands (both hot and cold) and the ubiquitous presence of soil

crusts within them, there should be no question that BSC's represent a major microbial ecosystem on Earth. Their global biomass must be gauged in the thousand millions metric tons. Their impact on global and local biogeochemical cycles has just begun to be explored, but it is likely to gain attention of the scientific community in the near future. Most of the research carried out on BSC, however, has been done at the floristic and ecological level, while virtually nothing is known about the role of bacteria (except for cyanobacteria) and associated microbial processes. The study of the microbial ecology of BSC 's represents one of the research areas where large strides will probably be made in the near future.

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DESERT ENVIRONMENTS — SOIL MICROBIAL COMMUNITIES IN COLD DESERTS

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The cold deserts of the world are characterized by relatively simple ecosystems and by physical features that approach, as nearly as is possible on this earth, the conditions of other parts of the solar system on which life may have existed. Their biota have therefore been of interest to ecologists hoping to study community structure in its least complicated form, to biochemists hoping to elucidate the molecular aspects of adaptation to cold and arid conditions, and to exobiologists as models (1) and a testing ground for the machinery of exploration (2). Cold deserts exist in polar regions (although little of the so-called Arctic polar desert is ice-free) and at high altitudes where the land lies in the precipitation shadow of mountains (for e.g., in the Atacama of Chile and Peru, which is reputed to be the driest desert on the planet, and the Xizang Plateau of Tibet). Only in the Antarctic, however, has the microbiology of a true cold desert been extensively studied.

PHYSICAL CHARACTERISTICS OF THE ANTARCTIC DESERT

The Antarctic is generally assumed to be covered with snow and ice — a view that is almost justified — as only some 280,000 km^2 that is less than 2% of the continent is ice-free. Although the terms "desert" and "cold desert" are frequently used, the ice-free areas of the Antarctic satisfy the most restrictive definition: precipitation equivalents of 100 mm or less yearly, a mean temperature of the warmest month of 2 °C or less, and a yearly radiation balance of approximately 10 $kcal/cm^2$ (3) (This definition is based on high latitude desert conditions; the radiation balance is naturally higher in nonpolar high altitude deserts). These areas generally have a mean annual air temperature of −20 °C or less and 45 mm or less of water-equivalent precipitation (4). The largest ice-free areas occur in the shadows of the Trans-Antarctic Mountains, the area known as the McMurdo Dry Valleys — because of their lack of ice cover — or the Ross Desert (Fig. 1).

While the climate varies spatially and temporally, the records from Linnaeus Terrace (Wright Valley, 1,600-m altitude) can exemplify temperature and solar radiation in the Ross Desert (5,6). The daily mean air temperature reached −5 °C in January (midsummer), the diurnal

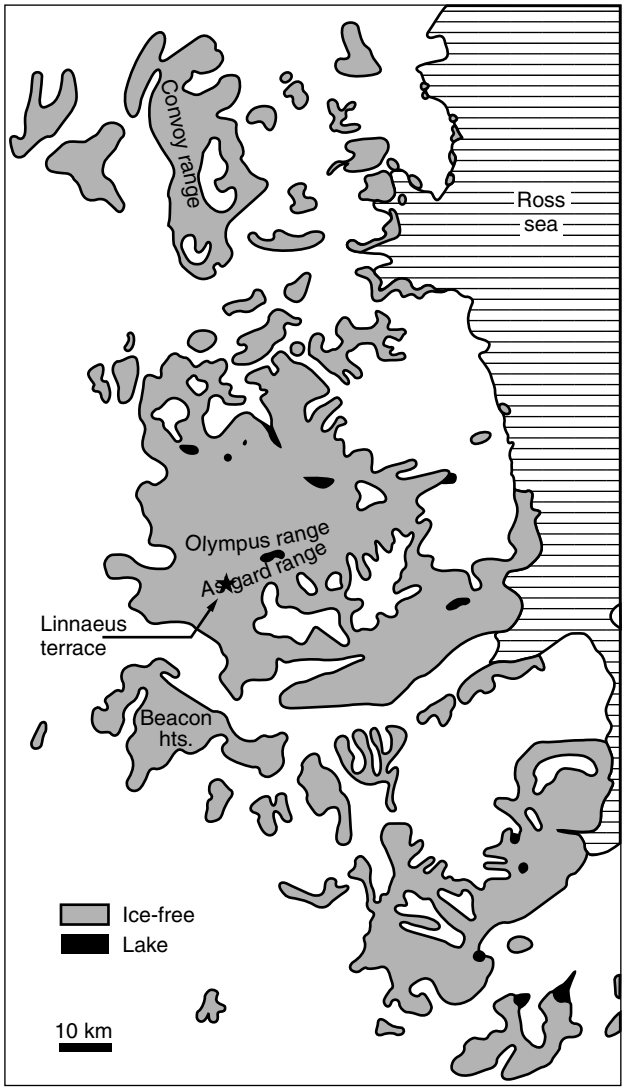

Figure 1. Ice-free areas of the McMurdo Dry Valleys (Ross Desert), southern Victoria Land, Antarctica. Reprinted with modification from J. A. Nienow and E. I. Friedmann, "Terrestrial Lithophytic (Rock) Communities" in E. I. Friedmann, ed., *Antarctic Microbiology*, Wiley & Sons, New York, 1993 by permission of Wiley-Liss, Inc., a division of John Wiley & Sons, Inc., copyright holders.

maximum in solar radiation reached 2,990–4,252 μ moles photons m^{-2} sec^{-1} (reported as 650–1,063 watts m^{-2}). The snow sensor did indicate the occurrence of snowfall but most of such snow sublimes rarely providing soil moisture. Ross Desert soils are dry and water content in the upper layers reportedly range from 0.26 to 5.1% (7).

The valleys are headed by glaciers. Temporal variation in the Antarctic climate provides additional summer moisture to sites immediately below the glaciers through the development of glacial melt streams, and also through the melting of small accumulations of snow in wind-sheltered spots. The insolation of soil and rock surfaces can warm them to temperatures well above air temperature. Heating can be quite rapid; Cameron (8) recorded a rise of 42.5 degrees Celsius (to 27.5 °C) on an insolated rock in three hours. In the Ross Desert, temperatures and the availability of water vary from valley to valley and from site to site, depending largely on exposure factors and the season. Cameron and coworkers, in the course of the first extensive investigations in the Dry Valleys, reported that Wheeler Valley had a more favorable environment than the McKelvey, Victoria, Taylor, and King David Valleys, as evidenced "by the visible presence of soil algal mats and crusts" (9).

The Soils

The soils of the Dry Valleys have been described in part by Campbell and Claridge (10) and more recently by Bockheim (4). They have been characterized as weathered surficial deposits that are barely recognizable as soils, although Bockheim pointed out that they do display horizons and do support "vegetation" (but not of agricultural interest).

Bockheim characterizes them as "skeletal-sandy," "loamy sand or sand with abundant coarse fragments," with clay and silt contents "usually less than 10% each," "unique among world soils in that they contain abundant soluble salts and permafrost." Bockheim points out that the permafrost, in some cases, lacks frost in the usual sense; that is, it is too dry to be cemented by interstitial moisture. The Dry Valley soils, away from the coast, are xerous (below the wilting point of orthodox plants every month in the year, some moisture available but with little leaching of soluble salts (10)) or, particularly in the more remote valleys (Arena Valley, Beacon Valley) and at higher elevations, ultraxerous (liquid water being rarely present). The mineral salts are derived from two sources: weathering of the parent rocks and atmospheric deposition. The parent rocks in the Trans-Antarctic mountains are variously granite and gneiss, dolerite, and sandstones. Weathering takes place largely through wind and salt erosion, coupled with the expansion and contraction resulting from insolation. The typically coarse weathered product receives marine aerosols if nearer the coast, becoming enriched in NaCl, or, if nearer the polar plateau, deposition of $NaNO_3$ through snowfall. Although salt composition varies from site to site, it typically includes chlorides (Cl), nitrates (NO_3), and sulfates (SO_4) of sodium (Na), potassium (K), calcium (Ca), and magnesium (Mg) (Campbell and Claridge, 1987). Salt accumulation, in Bockheim's samples, reached its peak well below the surface, at around 25 cm. As expected, the dominant cations in Bockheim's samples were Na, Mg or Ca, and K (in that order) and the dominant anion was Cl in subxerous soils of the ends of Wright and Taylor valleys nearest the sea ice, SO_4 or NO_3 in the xerous and ultraxerous soils of the more polar Dry Valleys. The heterogeneity of salt content in Linnaeus Terrace (Wright Valley, about 1,600 m alt.) soil samples from surface to 5-cm depths (11) presumably reflects the chance nature of liquid water availability, but should also serve as a warning against applying macroscale characteristics to microscale communities. The total μ Equivalents per gram of the major cations listed above varied from 7.13 to 60.43 in seven samples from this small area (11). The cation contents of the surface to a 7-cm layer in Bockheim's Dry Valley pedons are reported on a millimolar scale greatly

exceeding these values even in the more southerly (and less salty) samples. The organic content of Ross Desert soils is low; 0.05% organic carbon is as much or more than is present in most soils (12,13).

RESOURCES IN ANTARCTIC DESERTS

The energetic basis of community life everywhere is chemosynthesis and photosynthesis. Chemosynthetic organisms have long been sought for in vain in continental Antarctic soils. A description of communities in the Antarctic deserts must cover areas that are not strictly desert because populations at truly desert sites are obliged to import the products of photosynthesis from less arid habitats. Organic matter, although carried for long distances by winds, is very rarely carried from as far away as South America. On Signy Island (maritime Antarctica), Marshall (14) found Nothofagus pollen originating more than 1,500 kms away in South America but with a probable mean annual frequency of no more than 1.5. It has been pointed out that although others have failed to find exotic pollens on continental Antarctica, the growth of exotic "plants" around the volcanoes of continental Antarctica indicates that some immigration occurs, possibly via birds (15,16), although immigration to continental Antarctica is generally considered an unlikely event. As birds have scant motivation for flying over the desert areas of Antarctica, and wind patterns make significant imports from outside the continent improbable, organic matter for heterotrophic growth must come primarily from sources closer at hand.

Photosynthesis in Antarctica is minimized by the relatively low solar radiation and inhibited by the cold. Nevertheless, seasonal photosynthetic activity is relatively high in melt streams and ice-covered lakes and their environs, as is evidenced by cyanobacterial and algal mats visible in soil around the lakes and streams and in the ice of ice-covered waters. Ellis-Evans (17), Vincent and James (18), Simmons coworkers (19), and Vincent and coworkers (20) have written the most recent overviews of freshwater ecosystems in Antarctica. These ecosystems also contain invertebrate herbivores and predators (rotifers, nematodes, tardigrades) and also bacteria and fungi, in aquatic and terrestrial habitats.

In the Dry Valleys, Wright Valley is bisected by the Onyx River and Lake Vanda. Lake Vanda is the deepest and warmest of the Dry Valley lakes and has perhaps the greatest biodiversity; it is the only Dry Valley lake containing a moss (*Bryum algens*) (21). Deep water in Vanda can reach a temperature of 23 °C (18). The Taylor Valley lakes (Bonney, Chad, Fryxell, and Hoare) are shallower and colder, home to photosynthesizing mats in which the dominant organism is *Phormidium frigidum* Fritsch, a cyanobacterium that is "ubiquitous in southern Victoria Land, occurring in soils, lake, glacial melt streams and cryoconite holes, apparently being excluded only by high salt concentrations" (22); although exclusion from soil by aridity should be added. In the summer these lakes are bordered by temporary moats, but photosynthesis also occurs in the dim light beneath the permanent ice cover (4–6-m thick) where the maximum radiation available for photosynthesis is estimated to be on the order of 1 μ mole photons m^{-2} s^{-1}. The resulting oxygen bubbles float mat pieces up to the underside of the ice cover, where 5–10% of the floating mats freeze into the ice. The upper surface of the ice ablates bringing the mixed biota, organic matter, and captured sediments to the surface and makes them available for windborne distribution. The annual escape of organic matter was estimated at 247.4 kg for Lake Hoare and at 8,343 kg for Lake Chad (23).

To the output of these lakes may be added organic matter originating in the glacial melt streams that feed such lakes. This source has only been quantified in terms of the algal biomass represented by chlorophyll a, reported to reach (late in the summer) about 10 μg cm^{-2} for the Fryxell Stream at 0.5 km from its glacial source (24). Single collections of multiple samples from the glacial melt streams of Taylor Valley yielded much higher values at some sites; up to 514.55 μg cm^{-2} chlorophyll a for Bohner stream lower transect in Bonney Basin (25). These streams flow for only 6–10 weeks during the austral summer, then freeze and dry. Although the freeze-dried biota remains viable, as evidenced by recovery during the succeeding summers, it is friable and dispersible. The taxonomic content of these mats varies with flow rate, turbidity, and the nature of the stream bed, but is again dominated by cyanobacteria (*Gloeocapsa*, *Nostoc*, *Oscillatoria*, and *Phormidium*). Chlorophytes (*Binuclearia* and *Prasiola*) and diatoms (*Hantzschia* and *Navicula*) may be included among important genera, and nematodes, tardigrades, and rotifers may be associated with the algal mats (24,25).

At higher altitudes, the bulk of photosynthesis (15) probably occurs within Beacon sandstone, a porous, translucent rock type. The endolithic habitat provides shelter and maximizes moisture, resulting in a warmer and more moist environment (5,26). Nienow and Friedmann (5) have listed five cryptoendolithic communities: the upper zone within the rock is variously occupied by lichens in which the phycobiont is Trebouxia or Pseudotrebouxia, the alga *Hemichloris antarctica*, and various cyanobacteria (characteristically, *Gloeocapsa* spp. or *Chroococcidiopsis*). The lower zone may also contain photosynthetic algae or cyanobacteria, supporting heterotrophic bacteria and at least one yeast (*Cryptococcus friedmannii*). Photosynthesis is optimum between −2 °C and +5 °C; bicarbonate uptake occurs as low as −8 °C. On the basis of the mean annual total time (h y^{-1}) of rocks at temperatures above −10 °C, Friedmann and coworkers (27) estimated that cryptoendolithic communities were possible on Linnaeus Terrace and in the Tyrol Valley (where indeed they occur) but could not exist in some areas of Mt. Fleming or on Horseshoe Mountain. Lichen photosynthesis began at the almost incredibly low matrix water potential of −46.9 MPa (28). Extensive studies (29) of climatic conditions and the positions of rock surfaces affecting a cryptoendolithic lichen community on Linnaeus terrace (with *Trebouxia* as the phycobiont, but also

containing *Hemichloris antarctica*, cyanobacteria, and heterotrophic bacteria) allowed the estimation of the yearly gross productivity of such communities for the entire Ross Desert area at approximately $120–180 \times 10^3$ kg C. Of this only $0.6–0.9 \times 10^3$ kg C was in microbial biomass (about 0.025% of the gross productivity). These communities grow slowly and weather slowly into the surrounding environment, and have a 1,000-y carbon residence time. The great bulk of the photosynthetic product was considered more or less evenly divided between basal respiration and loss to the environment, primarily by leaching into the rock and soil. Amino sugars and amino acids have been found in the substratum of colonized rocks (30–32). Weathering of gross biological materials is visible on rock surfaces; the weathering of at least initially viable lichen biota into the soil is attested to by the isolation of the phycobiont *Trebouxia* from soil (9,33,34) and of other, less defining, inhabitants of the sandstone.

Mineral Resources

The requirements for life include more than the major elements provided by the soluble salts mentioned above, although nitrate, the major nitrogen resource of these desert soils, is thus available. Phosphate, the next highest requirement, and minor elemental requirements do not limit the photosynthetic organisms of streams (25) and rocks (5). Apparently, mineral nutrients do not limit heterotrophic life in soils (7). Indeed, nutrient limitation is not considered characteristic of communities such as these that are under great abiotic stress.

DESERT SOIL COMMUNITIES

Although trophic relationships in Lake Vanda have been diagrammed (35), the soil communities of the McMurdo Dry Valleys have only been described, somewhat incompletely, in piecemeal fashion. Areas of soil in which water is seasonally not limiting may be presumed to have relationships mirroring those in Lake Vanda, with major emphasis on microbial life. The photosynthetic base again consists of cyanobacteria, chlorophytes, and possibly diatoms (in that order) that supply heterotrophic bacteria and fungi as well as nematodes and tardigrades. These replace the rotifers and protozoa of Lake Vanda, rotifers being aquatic and terrestrial protozoa rare, although *Acanthamoeba polyphaga* and *Naegleria gruberi* have been found in lakeshore soil in Taylor Valley (36). Protozoan abundance and diversity are strongly affected by latitude and temperature (37). Tardigrades, as a group, are suctorial feeders on plant cells and other invertebrates (including other tardigrades). They are particularly suited to Antarctic life as they resist dehydration and cold temperatures by accumulating trehalose and forming a "tun" capable of withstanding short exposures to $-196\,^{\circ}C$ (38) and also longer exposures to the higher temperatures of the Antarctic winter. The dominant nematodes in the Dry Valleys are the microbivore (bacteria and yeasts) *Scottnema lindsayae* and the omnivore-predator *Eudorylaimus antarcticus* (39). These nematodes withstand drying and cold so well that they can be used as living experimental material after having been stored frozen for more than two years (40). These elements in moist soil form a simple conventional food web that includes producers, consumers, predators, and biodegraders.

Away from obvious moisture, life in Ross Desert soils is exclusively microbial and probably exclusively heterotrophic, depending on the bounty of the wind and weathering rocks. Such microbial life is concentrated in the uppermost layers of soil. The lower layers sometimes appear sterile. A series of samples representing a profile of soil taken near the junction of Victoria and McKelvey valleys contained 10^2 to 10^3 bacteria/gram in the top 2.5-cm layer but at 15 to 25 cm below the surface produced no bacterial count on a variety of media, and did not evolve significant radioactive carbon dioxide from uniformly labeled glucose (41). The apparent sterility of this and some other soil samples might be laid to the sensitivity of the radioactive assay and to failure in using appropriate culturing techniques: the 5 to 15 media used in these investigations included only the standard microbiological media of the time. Parker and coworkers (33,42) later used a relatively oligotrophic agar containing soil extract (from the site), 0.1% proteose peptone and a 0.02% yeast extract. This technique produced microbial counts up to two orders of magnitude higher than had been found by previous investigators. The varied techniques used by Vishniac and coworkers on soils from 100 to 2,200 m in the Asgard Range produced ubiquitous evidence of life (43–45). Some soils, however, appear to have been genuinely sterile. Three of Cameron's "sterile" samples from the Asgard Range inhibited microbial life when mixed with other soils, an apparent effect of their high (7–16 μg/g) boron content (46). Microbial life in Dry Valley soils is, in any case, sparser than that in more temperate and humid climes and in the Antarctic ornithogenic soils near the coast.

Although lists of the bacteria of Ross Desert soils exist (7), they are minimally useful. The bacteria reported have not often been characterized except as biomass or isolates unidentified or identified only to genus; a characterization that has been deplored as essentially irrelevant in modern taxonomy (47). Franzmann (47) commented that all of the Antarctic strains for which 16S rRNA has been sequenced to date (1996) represent new species from Antarctic lakes. New genera from Antarctic sandstone have subsequently been described with the appropriate sequences included: *Friedmanniella antarctica* (48) and *Hymenobacter roseosalivarius* (49). The identification of Ross Desert soil bacteria has so far indicated — with at least one possible exception (see following section) — that only cosmopolitan taxa had gained entry to these soils (7). Their presence need not indicate colonization as such dry and freezing conditions can prolong viability for many years, given the apparent absence of predators in the more arid soils. The fact that such bacterial taxa — lacking any obvious adaptation to life in Ross Desert soil — could greatly outnumber any indigene raised the question of whether any bacteria were active there. Evidence of activity in the absence of disturbance of environmental conditions

was provided by the presence of microcolonies on soil particles (43) and on buried glass slides (44,45). Some of the bacteria isolated (but not identified) from their soil samples may be true indigenes. The allochthonous bacteria could provide a "seed bank" able to colonize when environmental conditions are mitigated by global warming. (Global warming would not only provide more suitable temperatures for bacteria from the north but might increase the energetic basis for heterotrophic life.) The same is true of the many microfungi (50) that have been isolated from Dry Valley soils. As their mycelia typically do not survive freeze-thaw cycles, the germination of microfungal spores under current conditions would surely be a death sentence for those fungi.

Abundance does not indicate the activity of microbes in Antarctic desert soils, given the preservative effects of cold-drying. Is it possible to define the indigenous microbiota? The available clues include endemism (which may vanish on further investigation) and adaptation to local conditions. The main requisites for microbial activity in arid Dry Valley soils are the ability to survive and recover quickly from desiccation and repeated freeze-thaw cycles, psychrotolerance if not psychrophily, and an oligotrophic mode of nutrition. The rapidity of recovery from desiccation and freezing is crucial as the presence of water and the elevation of temperatures above at least $-10\,^{\circ}C$ is temporary.

The ability to withstand repeated freeze-thaw cycles seems difficult to determine experimentally as some experimental results are at odds with the experience of Wynn-Williams (51), who found that yeast biomass in Signy Island (Maritime Antarctica) peat cores increased both absolutely and in comparison to bacteria and microfungi in early spring in the field and after repeated freeze-thawing in the laboratory. The psychrotolerance required of Antarctic soil microbes is difficult to assess largely because of the absence of data. One may assume that indigenes should at least be able to metabolize and grow at the same temperatures as cryptoendoliths (microbes growing cryptically in rocks) and the photosynthetic microbes of aquatic habitats, while being able to tolerate the highest temperatures produced by surface insolation.

Psychrophily, defined as a maximum growth temperature of less than $20\,^{\circ}C$, is perhaps not to be expected in a habitat with the temperature variations of surface soil. Although some strains of Dry Valley yeast species do conform, the Antarctic microbial species that meet this definition, including both bacteria (47) and yeasts (52), have their primary habitats elsewhere than in soil.

Oligotrophy seems to be dictated by the low organic content of the arid soils and dependence on what are essentially imported substrates. Soil aridity may have a nonobvious effect on organic content: growth depends on the chance availability of liquid water but the sudden wetting of dry soil can release up to 70% of microbial biomass carbon for utilization by the survivors (53). Given the bacterial biomass in surface soils, this represents a substantial increase in available substrates.

Although Ross Desert soils have a high average salt content, halotolerance is not a prerequisite for life there. Why halotolerance is not required is hard to explain as new and apparently indigenous species of Antarctic halobacteria have been described from Antarctic lakes (47), as has a bacterium from the cryptoendolithic community that shares a recent common ancestor with the marine *Vibrio natriegens* (54). The energetic requirements of halotolerance are presumably not met in these depauperate soils.

Quick recovery from desiccation is a property of encapsulated or sheathed microbes, such as cyanobacteria and basidiomycetous yeasts. The bacteria isolated from W. V. Vishniac's collections frequently possessed extensive capsular material. Basidiomycetous yeasts are typically encapsulated. Such yeasts, a minor component of the microbiota in temperate soils, may be typified by *Cryptococcus albidus*, a phenotype commonly encountered in warmer desert soils (55). *Cryptococcus albidus* competed successfully with the natural biota of loamy sand whenever the bacteria were stressed by desiccation, an outcome resulting from the longer lag phase of the bacterial community. Encapsulated bacteria in this soil flourished only at water contents near field capacity; the dominant bacterium at 1% water content was *Nocardioides albus*, an unencapsulated actinomycete (56). Actinomycetes are reputed to include the most desiccation-resistant bacteria, although this reputation seems to be largely the result of their isolation from spores in arid soils. It is, however, possible that the tight growth habit of mycelial actinomycetes has the effect of sheathing or encapsulation. *Nocardioides albus* initially grows as a tight compact colony.

Their desiccation resistance might therefore lead one to expect basidiomycetous yeasts and actinomycetes to dominate in arid soils. This has not been demonstrated for the Ross Desert soils. Yeasts occur in arid Ross Desert soils at about one microcolony per gram of soil (7), far fewer than the recorded numbers of bacteria, although most of the latter may be inactive. Apart from the obvious ability of actinomycetes and their spores to survive in soil through the variable temperatures of temperate winters, nothing is apparent about their ability to survive repeated freeze-thaw cycles. Actinomycetes identified as Nocardia and Streptomyces are recorded only as minor (3%) elements in the bacterial population, although "coryneforms" and "coryneform-related" bacteria constitute a majority of the 568 bacterial isolates in Cameron's collection (7).

The dominant yeast in the most arid Ross Desert soils is *Cryptococcus vishniacii* (commemorating Wolf Vladimir Vishniac, who died while exploring these valleys). *Cryptococcus vishniacii* has been isolated from many sites within the Dry Valleys since the first report (57). It is an oligotrophic yeast, with a low K_s for glucose at $10\,^{\circ}C$ (between 9 and $<26\,\mu M$) (58), with strains capable of growth on a variety of amino acids (59) generated by the proteases characteristic of basidiomycetous yeasts (60) as well as sugars and organic acids, of growth at temperatures down to at least $-3\,^{\circ}C$ and up to $19–24\,^{\circ}C$ (58,61), and at water potentials down to <-4.86 MPa in liquid culture (34). However, in sand microcosms, growth was severely inhibited at -3.56 MPa

and depended on the amount of water with which additions of equal osmolarity were presented (34).

In University Valley (a small hanging valley off Beacon Valley), soil samples taken in the vicinity of a dry glacial melt stream consistently produced another indigenous species, *Cryptococcus antarcticus* (62). *Cryptococcus antarcticus* strains have maximum growth temperatures between <20 °C and 25 °C, have a higher maximum growth rate, and are slightly more halotolerant than *C. vishniacii*, although less so than *C. albidus*, suggesting that substrate availability (higher near an ephemeral melt stream) has directed the adaptation of these yeasts to their respective habitats.

At least one bacterial species possesses some features appropriate for life in these soils. *Hymenobacter roseosalivarius* (49), a taxon within the Cytophaga/Flavobacterium/Bacteroides phylogenetic line that is psychrotolerant (minimum growth temperatures between −0.5 and <5 °C), was isolated from oligotrophic media. It produces "large amounts of extracellular polymer." The 41 isolates of these bacteria were collected not only from a boulder with cryptoendolithic life on Linnaeus Terrace, but also from soil in the vicinity of the boulder, as were other microbes and some black filamentous fungi. It is possible that their presence in soil is passive; the original samples were taken with the idea that their microbial contents would be "comparable to the community in the rock" (63). The *H. roseosalivarius* isolates were not particularly halotolerant, so, if truly colonizing the soil, must live as neighbors of the *Cryptococci*. This species does have the further interesting property of lysing (unspecified) yeast cells, which speaks for its ability to find the substrate in barren soils. The actinomycete from the cryptoendolithic community, *Friedmanniella antarctica*, has not been isolated from soil.

CONCLUSION

The model presented by the McMurdo Dry Valleys leads to the expectation of physically interleaving microbial ecosystems in cold desert soils. In the McMurdo Dry Valleys, this interleaving is seen in the form of temporary glacial melt streams in the heights and more permanent streams and lakes in the valleys. Water, rather than temperature, is the most limiting factor here. Water limits biodiversity both directly and indirectly through its effect on energy production by photosynthesis. These systems are graduated, by the availability of liquid water, from the simple trophic net of lake and streamside to the depauperate population of heterotrophic yeasts and other putative heterotrophic indigenous microbes of the most arid soils. This model, although derived from the most investigated cold desert on the planet, is incompletely known. What is most needed to understand adaptation to the multiple stresses on life in cold deserts is further investigation of microbial life in the most arid soils, an area that is being neglected relative to work on lakes and their environs.

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DESICCATION BY EXPOSURE TO SPACE VACUUM OR EXTREMELY DRY DESERTS: EFFECT ON MICROORGANISMS

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Drying probably is the oldest method of preserving food. Drying of food by exposure to dry air, often in combination with sunlight, was already practiced in ancient Egypt 3,000 to 5,000 years ago. Only during the last decades, however, microbiologists, biochemists, and food scientists have begun to understand how metabolic activities including the growth of microorganisms and the decay of food components are affected as their water activity (a_w-value) is decreased. In living cells, the removal of water usually poses a serious hazard to the integrity of proteins, nucleic acids, membranes, and other molecular and supermolecular entities. Some species (including prokaryotic spores and fungal conidia), however, have developed strategies to overcome the threats presented by the loss of cellular water. Although even they cannot maintain essential metabolic activities at low water contents, they have learned to survive in a metabolically inactive ("dormant") state for months and even many years. In an extremely dry environment, however, chemically bound water also is continuously removed. This process ultimately leads to irreversible damages, especially of nucleic acids, and as such limits the survival time of dormant life-forms. The question of longevity of dormant life also has attracted public interest. This interest has focused on the question of whether "germs" arriving on Earth from outer space may have brought life to Earth almost four billion years ago (panspermia thesis).

GENERAL

The presence of liquid water within a cell is a fundamental requirement for almost any form of its biological activity. Liquid water is the solvent for all biochemical processes. The presence of liquid water also is required for maintaining the native structure of proteins, nucleic acids, membranes, and other supermolecular entities especially by means of hydrophobic bonds and/or hydration processes. Only a few biophysical events such as the absorption of radiation energy, the transfer of excited states, and some electron transport reactions do not require the presence of liquid water.

There are essentially two ways to reduce the amount of liquid cell water: by freezing or by removal. The initial phase of freezing especially is traumatic to most cells because growing ice crystals may injure cellular structures and the increasing concentration of solutes in the residual water may create osmotic stress. The process of thawing also may lead to injury of cellular components through physical stress. Liquid water ceases to exist below $-130\,^{\circ}C$. Therefore, no thermally driven reaction that requires the presence of liquid water can occur below $-130\,^{\circ}C$. Storage of biological materials at the temperature of liquid air ($-196\,^{\circ}C$) is therefore a standard method of long-term preservation, provided the damaging effects of freezing and thawing can be minimized (1). The absolute absence of liquid cell water below $-130\,^{\circ}C$ certainly represents an extremely dry environment. Primarily, however, this environment is maintained by extremely low temperatures. For this reason it is not detailed here.

In the laboratory, drying of cells, that is, the removal of cell water is typically achieved by freeze-drying under reduced pressure or by exposure to an environment of reduced water activity. The latter method allows a controlled and stepwise removal of cell water.

The water activity (a_w-value) is defined as the water vapor pressure (p_s) of a solution divided by the water vapor pressure (p) of pure water at the same temperature:

$$a_w = p_s : p \tag{1}$$

The water vapor pressure of pure water (p) is decreased by the addition of a solute. The exact correlation between the vapor pressures and the concentration of n_s moles of a solute in n moles of a solvent was first formulated by F. Raoult in 1886:

$$(p - p_s) : p = n_s : (n_s + n) \tag{2}$$

or

$$(1 - n_s) : (n_s + n) = n : (n_s + n) = p_s : p = a_w \tag{3}$$

Equation (3) shows a direct connection between Raoult's law and the definition of the water activity (a_w) as presented by Equation (1). These relationships, however, are strictly valid only for dilute solutions.

Because the temperature dependence of p_s and p is very similar the quotient p_s/p for all practical purposes shows no significant temperature dependence. It should be recalled, however, that the water vapor partial pressure (at room temperature about 20 mb) is strongly temperature-dependent. The quotient p_s/p, if multiplied by 100%, is practically identical with the relative humidity (RH). However, for certain purposes, the relative humidity is defined in terms of the weight of water vapor in a given volume. The weight of water vapor in a given volume depends on the ambient pressure and temperature. Therefore, minor corrections are required for exact comparisons. Approximate a_w-values are maintained conveniently by placing distinct solutions of inorganic salts in closed vessels at constant temperatures (2). In the presence of larger amounts of wet biological materials, however, the water activity of the solvent systems will eventually increase as the solutions absorb significant amounts of water from the biological materials. An alternative is therefore the preparation of saturated solutions of salts with excessive solid salts at the bottom (3) or the use of solid salts and related materials (e.g., silica gel) with defined hygroscopic properties.

LIMITS OF GROWTH AT LOW WATER ACTIVITIES

Minimum water activities for cell growth at normal temperatures have been reviewed earlier by Mazur (1) and Rose (2). Most eubacteria require an a_w above 0.9 and most fungi an a_w above 0.85. Even a value of 0.9 must be regarded as an extreme physiological value because it requires that the vapor pressure of liquid cell water is in equilibrium with the vapor pressure of a 3 molal sodium chloride solution. Archaea such as halobacteria may even grow at an a_w of 0.76, which is in equilibrium with a saturated (6 molal) sodium chloride solution. The most extreme adaptation to low water activities probably is represented by osmophilic yeasts (e.g., *Saccharomyces rouxii*) and a few molds (*Aspergillus echinulatus*, *Monascus bisporus*). These microorganisms grow at an a_w range of 0.65 to 0.60 (4). An a_w of about 0.6 is in equilibrium with 4.0 M $CaCl_2$. The following section shows that reduction of water activities under 0.6 causes increasing stress especially because of the removal of hydrate water from macromolecules and membranes. Under such extremely dry conditions, no terrestrial microorganism is known to exhibit any growth.

Many organisms do not need the presence of extracellular liquid water for growth. They can take up water from the atmosphere, provided the ambient water activity (relative humidity) is sufficiently high and they can provide a biochemical strategy for the import of water from the gaseous phase. Well-documented studies by Lange and coworkers (5) demonstrated that lichens in the Negev Desert in Israel may become extremely dehydrated during the day, whereas, during the night the lichens can take up some water from the atmosphere at relative humidities above 80%, even in the absence of the condensation of dew. (See the section on Survival Under Extremely Dry Desert Conditions).

According to the laws of diffusion, the uptake of liquid water from the environment is faster and more efficient than its uptake from the gaseous phase because the thermodynamic gradient between intracellular and extracellular water is more favorable for extracellular water in the liquid state.

DORMANT LIFE

The presence of free cellular water generally is thought to be required for the living state. Nevertheless, numerous organisms can survive almost complete dehydration for extended periods. The living state of the dehydrated organisms often is referred to as *anhydrobiosis*. These

organisms typically show no significant sign of metabolic activity. Their state of life is therefore often referred to as dormant. When water again becomes available, they may readily take it up and resume both metabolic activities and growth. According to Keilin (6) this phenomenon was described first in 1702 by Antony van Leeuwenhoek and has been the subject of discussion and experimentation ever since. However, the phenomenon of anhydrobiosis has been part of human experience for thousands of years, as humans have learned to store dry seeds from one year to the next in order to farm new crops of cereals or vegetables. In addition to seeds, many other anhydrobiotic organisms, such as dry yeast cells or fungal conidia, also are familiar in daily life. Less known are certain nematodes, the cysts of some crustaceans such as the brine shrimp *Artemia salina* or the huge variety of microbial spores (7). Coincidently, with the resistance to the unfavorable effects of drying, anhydrobiotic organisms are often more resistant to freezing (1,8) and heating (6,9). Many, though not all, anhydrobiotes have developed the biochemical strategy of accumulating large amounts of nonreducing sugars, especially trehalose or sucrose. The OH groups of these sugars are thought to replace the hydrate water of phospholipids or proteins in some way (10), and thus stabilizes the liquid crystalline structure of phospholipid membranes or the spatial structures of proteins. *Bacillus subtilis* spores, however, protect their integrity, especially of their DNA, by entirely different strategies (11). These include the synthesis of a special class of DNA-binding proteins for DNA protection. All dry-resistant organisms, however, have decreasing survival chances if their DNA is subjected additionally to thermal or radiation-induced lesions while being in the dormant state: in a metabolically inactive state no repair processes can operate. Thus, all damages accumulate and if (after rehydration) the general conditions for growth become favorable again, the damaged organisms fail to recover and to replicate because the number of damage exceeds their repair capacity. The molecular events induced by the removal of water will be outlined and discussed in the following section.

MOLECULAR EVENTS INDUCED BY DESICCATION

Removal of Liquid Cell Water

Fundamentally, the physical and chemical processes are the same if water is removed from any native cell by exposure to an environment of low water activity. A detailed study on the properties of cell water in successively dried *Saccharomyces cerevisiae* has been made by S. Koga and coworkers (12). These authors found that the respiration rate of dried yeast cells approaches zero if the water content was reduced to 20% by exposure to about 95% relative humidity. At this stage of drying, all free cell water that could support an organized metabolism had disappeared and about one half of the remaining cell water had obtained a gel-like structure, whereas the rest was in different states of adsorption to cellular structures. Exposure of the yeast cells to about 65% relative humidity reduced the water content to about 10%. S.Koga and coworkers demonstrated that at this stage no unbound water was left and the remaining water persisted only in various states of adsorption. This conclusion was supported especially by their measurements of the heat of vaporization (H): They observed constant values of about 10 kcal/mol for water contents above 10% and an increase to about 20 kcal/mol if the water content was reduced from 10% to 5%. The relative humidity or water activity at which all free cell water is lost certainly varies from species to species. It should be recalled, however, that so far no growth of microorganisms has been observed at water activities below 0.6. Free cell water is not only the solvent in which most metabolic reactions take place, its presence is also essential for maintaining all hydrophobic bonds (also called hydrophobic interactions) (13). Hydrophobic bonds fundamentally contribute to the stability of the bilayer structure of membranes and the spatial structure of proteins. Some essential features shall be outlined here in a simplified manner: The surface of a hydrophobic residue in contact with liquid water is covered by a netlike layer of relatively highly ordered water molecules. The ordered structure of this layer is maintained by electric dipole–dipole interactions. These interactions are much stronger here than in free water because they are influenced by the low dielectric constant of the hydrophobic residues (usually a side chain of an amino or fatty acid). It may be recalled that the force between two electric charges is reciprocal to the dielectric constant of the medium. Free water has, in contrast to hydrophobic residues, a high dielectric constant. For this reason the dipole–dipole interactions between the molecules of liquid water at room temperature are relatively weak and allow only the transient formation of ordered clusters. Thus, the state of disorder of liquid water is very high in comparison to the layer of bound water covering the hydrophobic residues. If now two or more hydrophobic residues interact with another they will finally "stick" together because they can decrease the surface area that is covered by the net of highly ordered water molecules. (Fig. 1).

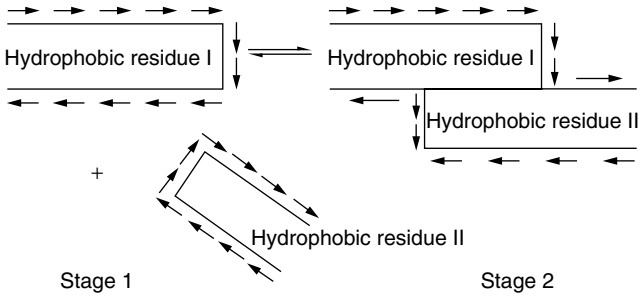

Figure 1. The arrows symbolizes the state of order of water molecules achieved because of their electric dipole properties. The highly disordered water molecules of the medium are not shown. The transition from Stage 1 to Stage 2 is especially favored because the number of ordered water molecules (number of oriented arrows) decrease from left to right and thus the number of disordered (free) water molecules increase, and so increases the entropy (S) of the system. The reaction is driven from left to right because G becomes negative because of the large increase in entropy. For more details, see the text, especially Equation (5).

The transition shown in Figure 1 can also be expressed by the following equations:

$$H = G + TS \tag{4}$$

or

$$G = H - TS \tag{5}$$

H is the change in enthalpy, S is the change in entropy (a term related to the disorder of the system), T is the absolute temperature, and G is the change in free energy. If G is negative, the reaction will be driven from left to right. This is indeed the case here because TS is much larger then H. It is a paradox of hydrophobic systems in biology that the highly ordered structures of membranes or proteins are maintained at the expense of the increasing disorder of the surrounding water molecules.

Removal of liquid water thus causes the breakdown of all structures that depend on hydrophobic interactions. This process is generally not reversible when liquid water becomes available again. Thus, most organisms are killed by dehydration. Those organisms that escape irreversible damage by dehydration have developed special strategies that allow them to regain their original activity after rehydration. Many of these organisms, including conidia of certain fungi and yeast cells, contain large amounts of nonreducing sugars, particularly trehalose (a disaccharide of glucose) (10,14). The OH groups of these sugars interact by hydrogen bonding with the phosphate groups of the lipids. These interactions appear to prevent the fusion of membranes during dehydration and they maintain the lipids in a fluid phase in the absence of water (15). In a related way, the sugars also appear to preserve the structure of some labile proteins (15). Most dry-resistant bacteria, however, do not seem to depend on trehalose, although they may apply related strategies. Many bacteria increase the production of oligo- and polysaccharides during desiccation. Concurrently they accumulate intracellular sugars (10,16), and/or they secrete various types of polysaccharides to form an extracellular coat of slimy or rigid consistency (17). The mechanism of protection by these exopolysaccharides, however, is not known. Even another protection strategy is performed during sporulation of bacilli (11). The liquid cell water of the vegetative cells is actively removed during the sporulation process. The interior of the developing spores becomes densely packed and enclosed by the very rigid sporal cortex, which may have similar protective properties as the rigid exopolysaccharide envelope of some vegetative bacteria. However, in this case also it is not exactly known how the inner membranes and intracellular proteins are protected.

Removal of Hydrate Water

The three major constituents of typical bacterial cells, for an instance of *Escherichia coli* cells, are water (about 70%), proteins (about 15%), and nucleic acids (about 7%). A significant proportion of the cell water is bound to the polar amino acids of proteins and to the bases, phosphates, and sugars of nucleic acids by means of electric interactions and hydrogen bonding. This hydrate water together with the surrounding liquid water is essential for maintaining the native structures of these macromolecules. On an average, each polar amino acid may bind about three water molecules; similarly, each nucleotide binds at least three water molecules. Jaenicke (18) estimated that "monolayer coverage" of a small protein such as lysozyme is reached at about 0.4 g water per gram protein. If these considerations are extended to all cellular proteins of *E. coli*, it can be estimated that a water content of about 6% is required to hydrate all proteins ($\sim 0.4 \cdot 15\% = \sim 6\%$, if a protein content of 15% is assumed). Actually less then 0.4 g water per gram *E. coli* protein will be bound because most *E. coli* proteins are larger than lysozyme (about 10,000 Da); therefore their surface to volume ratio is smaller. If, however, the amount of water required for the hydration of nucleic acids, sugars, metabolites, and inorganic ions is considered in addition, a value of about 10% for bound water in *E. coli* may be reached. These estimates are in agreement with data obtained by freezing of yeast or *E. coli* and related data as reviewed by Mazur (1) and with the data obtained by Koga and coworkers (12), which have been described earlier.

Dehydration is expected to destroy the native conformations of proteins (cause denaturation) because of the drastic decrease of the dielectric constant that occurs if water is removed from the hydrophilic (hydrated) pockets of proteins (18). The significance of the high dielectric constant of water in weakening electrostatic interactions has been discussed earlier. The thermodynamics of denaturation by dehydration is extremely difficult to evaluate because the relatively small change of G governing this transition results from numerous changes of stabilizing and destabilizing interactions (19), as the shielding of polar and hydrophobic groups by water disappears and electrostatic interactions become more predominant. Also, the strategies applied by anhydrobiotic organisms to achieve a renaturation of dehydrated proteins when water becomes available again, is not well understood. Evidently, two factors play a crucial role: The genetically determined design of protein folding by intramolecular bonds (18,19) and the interaction with protective components of the cytoplasm, such as sugars (20).

The structural changes of isolated DNA occurring during dehydration are, in contrast to those of most proteins, relatively well understood (21). Figure 2 illustrates the structures of B-DNA and A-DNA. B-DNA is stable above 92% relative humidity. On partial removal of hydrate water by lowering the relative humidity the B-form becomes converted into the A-form, which prevails below 75% relative humidity. At relative humidities below 44% the C-form becomes stable (not shown). Some essential data of the three DNA conformations are summarized in Table 1.

The significance of these data for cellular DNA is not clear because the conformational states of DNA in active cells are not well known. Interactions with various proteins play a crucial role. Thus, the mechanisms leading to the damages are not only species-dependent, but also dependent on the metabolic state of the cells. Moreover, many damages escape observation because of

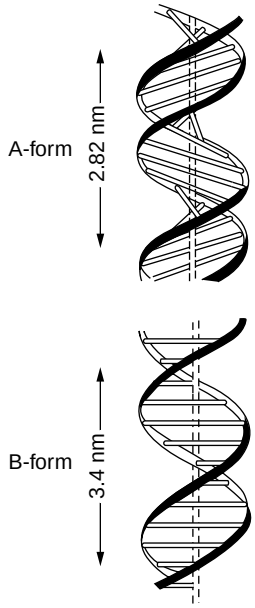

Figure 2. Two conformations of isolated DNA. The B-form is stable at relative humidities above 92%. Loss of hydrate water induces a transition to the A-form. This form becomes stable at about 75% relative humidity. If even more hydrate water is removed, another form, the C-form (not shown), becomes stable at about 44% relative humidity. Because of complexing with proteins and other components, genomal DNA may exhibit other transition states. After Dickerson (21).

Table 1. DNA-Conformations Depending on Hydration Effects

DNA Conformation	Relative Humidity	Nucleotide Pairs per Turn	Angle of Base Pairs Versus Helix Axis
A-DNA	75%	11.0	70°
B-DNA	92%	10.0	90°
C-DNA	44%	9.3	95°

Note: Data largely based on Dickerson, 1982 (21).

repair processes acting during or shortly after rehydration. Typical DNA damages that have been observed in desiccated cells after rehydration are: DNA strand breaks, both single- or double-strand breaks (22–27), formation of DNA-protein cross-links (24–26), and other damages causing mutations (28–31). It is not yet clear to what degree strand breaks are primary effects of desiccation. At least some strand breaks appear to be produced by incomplete repair of primary damages (32), whereas, others may directly result from physical stress induced by structural changes. The latter point has been corroborated by the vacuum-induced conversion of the supercoiled plasmid pBR322 into its relaxed form, which is initiated by a single-strand break (33).

The formation of DNA-protein cross-links are detailed in the next section because this reaction appears to be driven by the removal of reaction water during desiccation.

Additional Damages by Environmental Agents and Secondary Reactions

In addition to the stress caused by the removal of liquid water and hydrate water, desiccated organisms are readily damaged by a number of agents and secondary reactions. These especially include secondary interactions between cellular constituents, such as proteins and DNA, oxidations, and radiation-induced processes.

DNA cross-links with proteins are probably formed by the reaction of a protein-bound OH group with the phosphodiester group of DNA under removal of a water molecule. This reaction appears to be favored by the structural changes occurring after the loss of most hydrate water. The water released in the course of this reaction, however, is to be classified as chemically bound water. The resulting phosphotriester is a labile intermediate that will be readily hydrolyzed upon new contact with water during rehydration. The final reaction yields a DNA single-strand break, the protein being bound to the phosphate group of one of the new terminal nucleotides (25). (See Fig. 3 for more details.) In a related way also, sugars may react with DNA and cause strand breaks in addition to DNA-sugar cross-links.

Depurination reactions may also occur during long-term storage under vacuum. Greer and Zamenhof (34) have studied the heat-induced depurination of dry DNA in vacuum at temperatures between 81 °C and 131 °C. The reaction probably proceeds via the degradation of the desoxyribose. If the extrapolation of their kinetic data to about 25 °C is allowed, significant effects could be expected after several weeks of storage.

The good vacuum tolerance of *Deinococcus radiodurans* probably is related to its efficient DNA repair capacity (24,35). *Deinococcus radiodurans* repairs many of the desiccation-induced DNA damages, especially the DNA-protein cross-links, immediately after rehydration, before the first replication step (36). The same strategy appears to be used by *Bacillus subtilis* spores (11) and related organisms. In contrast to *B. subtilis* spores, however, *D. radiodurans* is inactivated strongly by exposure to relative humidities above 45% (37). The reasons for this unusual response are as yet not known (see also the section Survival under extremely dry desert conditions).

Among the biochemical constituents of cells, reducing sugars can be especially hazardous to proteins and nucleic acids during storage in the desiccated state. In metabolically active organisms, nonenzymic browning caused by the Maillard reaction is an aging process that is initiated by the reaction of the amino groups of proteins with reducing sugars (38). Evidence has been presented indicating that nucleic acids may also become modified by reaction with reducing sugars (39). Mouradian and coworkers (40) have observed that the functional integrity of freeze-dried biological membranes also is affected by glucose during long-term storage. Samples stored at 40% relative humidity showed extensive browning after 28 days of storage. Storage at 11% relative humidity, or even more efficiently in vacuum, strongly reduced the damaging reactions by glucose. Oxygen is a potentially damaging agent for lyophilized bacteria. Long-known examples are the radical production (41,42)

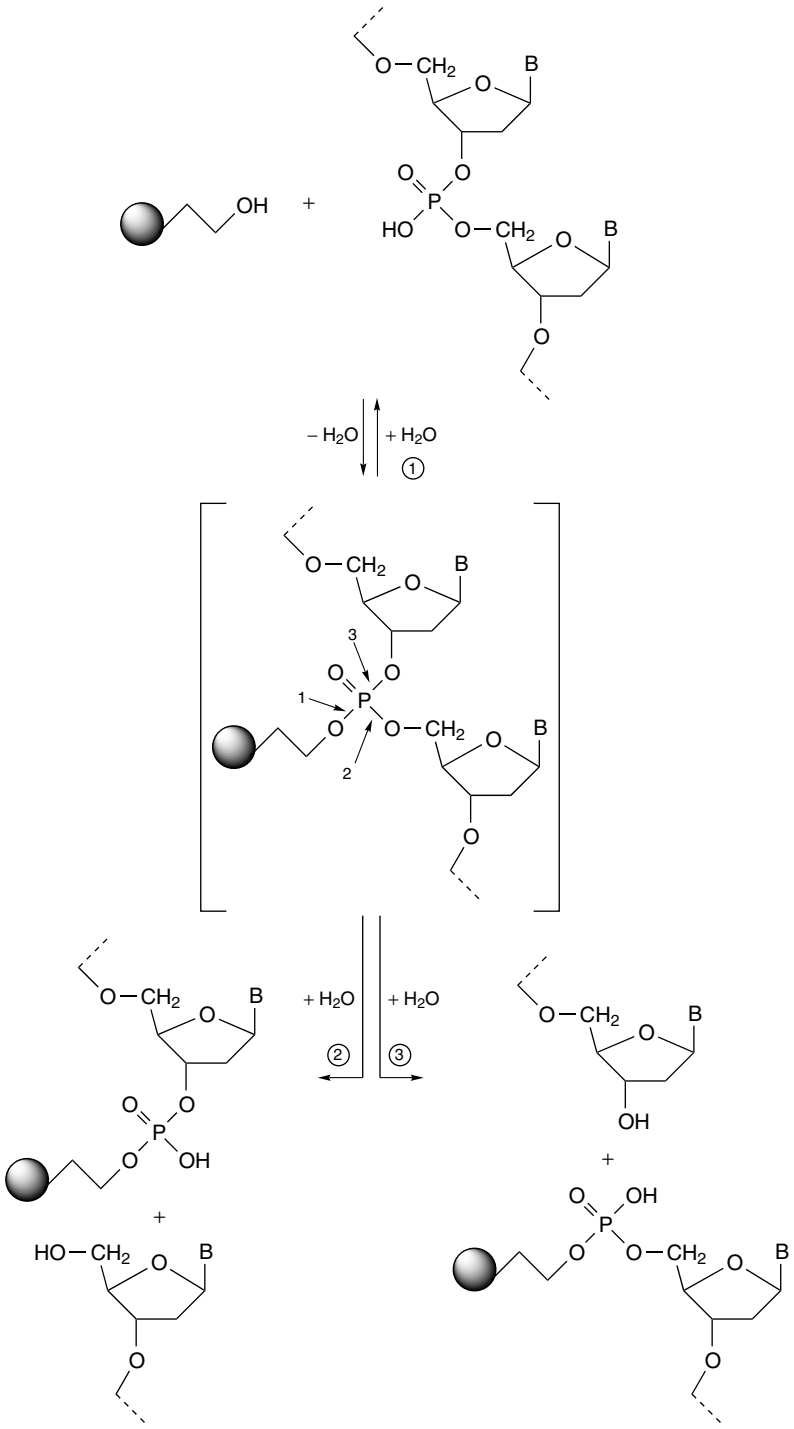

Figure 3. Mechanism for the formation of DNA-protein cross-links during dehydration and rehydration. It is suggested (25) that proteins being associated with DNA undergo a condensation reaction (for example, between a tyrosine-OH group and a phosphodiester group of DNA) yielding a reactive phosphotriester as an intermediate. The strand breaks occur during rehydration as a result of partial hydrolysis of the triester. The suggested pathway is also part of the reaction of type-I topoisomerases with DNA. These enzymes, however, again connect both DNA strands after relaxation of a supercoiled DNA structure. The protein is symbolized by the spherical structure carrying an OH group. After Dose et al., *Adv. Space Res.* **12**(4), 221–229 (1992).

and oxidation of SH groups (43). Molecular oxygen itself is a reactive diradical because its outer electron shell contains two unpaired spin-parallel electrons (a triplet state). Therefore, oxygen reacts readily with other radicals to yield even more reactive species such as superoxides (O_2), hydrogen peroxide (H_2O_2), and hydroxyl radicals (HO·). These species may contribute to DNA damage (44). A physiological source of superoxides is the reaction of oxygen with chelated Fe^{++} involving the transfer of one electron to oxygen according to:

$$Fe^{++}\ (\text{chelated}) + O_2\ Fe^{+++} + O_2 \qquad (6)$$

Therefore, storage in the absence of oxygen, preferably in nitrogen or in vacuum, is advisable (45). Cells can also be affected during long-term storage by ionizing radiation (46). However, the natural radiation environment is not expected to pose a serious hazard

unless geologic exposure times are to be considered. All cells contain a natural source of ionizing radiation: the isotope ^{40}K. Its half-life is 10^9 years. However, a single bacterial cell contains only $4 \cdot 10^3$ atoms of ^{40}K (17). Thus only storage times exceeding millions of years will yield a significant number of decays per cell.

A much more serious hazard is the exposure to solar light. About 1% of the solar light reaching the ground has wavelengths around 300 nm. This UVB fraction (UVB) is absorbed directly especially by the guanine residues of nucleic acids. The photochemistry of the resulting damages is not well known. Even in areas of moderate sunshine (50° N or S), 50% of a population of *B. subtilis* spores were killed during two hours of exposure at noon on a spring day (37).

SURVIVAL AT EXTREMELY LOW WATER ACTIVITY

In the context of this contribution, an extremely dry environment is understood as a dry environment that does not allow active growth of microorganisms. At present, a water activity under 0.6 appears to represent this limit (1–5).

Survival After Freeze-Drying

A discussion of the process of freeze-drying is beyond the scope of this contribution (see FREEZE DRYING: PRESERVATIONS OF MICROORGANISMS BY FREEZE-DRYING). Instead, only the essential damages that limit the survival of freeze-dried microorganisms are summarized here.

Survival of freeze-dried microorganisms is affected strongly by the conditions of freezing, the composition of the suspending fluids applied during freeze-drying, the gas in the storage container, the ultimate water activity, the temperature, and the time of storage. Long-term survival especially is limited by DNA damages. Freezing itself is hazardous primarily to membranes because it implicates the removal of liquid cell water (1,17). Moreover, ice crystals may cause membrane damages (1). However, the subsequent drying process in connection with the ultimate removal of bound water at extremely low relative humidities also will cause DNA damages, including single-strand breaks and mutations (28–30). It should be recalled that the native structure of DNA relies on hydrate water and that at relative humidities between 75% and 44%, the conformation of free DNA changes from the A-form to the C-form. Cells of *E. coli* show a strong increase in the number of mutations if they are desiccated at 55% relative humidity rather than at 75% (28). This effect is caused probably by conformational changes occurring during the dehydration of cellular DNA. Earlier, Clegg (10) has suggested that trehalose and other sugars may replace the water that is removed during desiccation of phospholipid membranes and proteins and thus stabilize the structures of these entities. In a related way, cellular DNA and the cellular DNA-protein complexes, respectively, could be stabilized. Moreover, gases affect the survival of dried bacteria during storage, as discussed earlier. The best survival rates are usually observed for storage in vacuum or nitrogen (45). It has been stated above that oxygen is a particular hazard (30) and that free radicals accumulate during storage in the presence of oxygen (41,42). Their damaging effects show the kinetics of thermal reactions (41): In the case of dry *Serratia marcescens*, the number of radicals increased from $-20\,^{\circ}C$ to $37\,^{\circ}C$ by a factor of 10 during six days, whereas the number of viable cells decreased by a factor of 10^{-5} (41). In view of the potential for damages to DNA occurring during drying and storage, freeze-drying is not the method of choice for preservation of microorganisms. A better way of preservation is the storage of cells at temperatures below $-80\,^{\circ}C$ (1). Not all cells, however, can survive the trauma of freezing, although there appears to exist a close relationship between resistance to freezing and dehydration. Those cells that tolerate dehydration are likely to resist freezing (8). Additionally, however, many other cell types exist, for instance, mammalian cells that are highly sensitive to dehydration although they may survive freezing in the presence of protective compounds (1).

Survival in a Dynamic Vacuum

A dynamic vacuum (pressure about 3×10^{-6} mbar and lower) is defined as an open vacuum that is maintained typically by a continuously running vacuum pump, preferentially by a turbomolecular pump. A dynamic vacuum is represented also by the vacuum of outer space. In fact, most laboratory experiments that have been conducted to study the long-term effects of a dynamic vacuum on biological materials have been designed to simulate the effects of space vacuum. During a space mission, however, the biological samples inevitably are subjected to additional stress factors that may obscure the vacuum effects (see the next section for more details). One essential feature of a dynamic vacuum is that volatile compounds are continuously removed from the sample in a nonequilibrium process. These conditions are in contrast to the storage conditions that may be applied after freeze-drying. In the latter case the sample container is sealed off under vacuum so that finally an equilibrium state is reached regarding the volatile compounds of the solid and the gas phase. Consequently, reactions that are driven by the removal of volatile by-products stop.

The early literature (before 1962) on the survival of bacterial spores or fungal conidia after exposure to a dynamic vacuum is quite controversial. Some relevant data have been reviewed by Morelli and coworkers (47) who also demonstrated that spores of *B. subtilis* var. *niger* show survival of about 50% after 35 days of exposure to an ultrahigh vacuum (10−8 mm mercury). Systematic studies on the correlation between the loss in viability and the increase in the amount of damage during long-term exposure to vacuum conditions has received increasing interest by the space research community since the mid 1980s (48,49) in connection with the planning of the long-term space mission EURECA (European Retrievable Carrier) (see next section). During the 1990s it was firmly established that the survival of microorganisms is especially limited by DNA damages. The extent of these damages increases with the time of exposure to vacuum (24,27). Their deadly impact

therefore differs crucially from that of the small number of mutations (28,30,49), strand breaks (22,50,51), and other damages that are induced during the initial period (minutes to hours) of freezing and/or drying by evacuation (see the section Molecular events induced by the removal of water). The chemistry of the primary DNA damages that are induced directly by long-term exposure to vacuum conditions is not understood fully. The formation of DNA-protein cross-links is well understood (see Fig. 3, in the section on Molecular events induced by the removal of water). This process is probably accompanied by the formation of DNA strand breaks. Additional DNA strand breaks probably are formed by incomplete excision-repair processes. These processes start immediately after rehydration, but they are blocked during the lysis of the sporal cortex or cell walls because of the breakdown of the energy metabolism by the detergents and enzymes of the lysis cocktails. Lysis is an essential step before the analysis of the genomal DNA by pulsed-field gel electrophoresis (26). The latter method allows us to determine the size of DNA fragments up to the size of a complete bacterial genome. Pulsed-field gel electrophoresis therefore is most suitable for the analysis of large genomal fragments resulting from DNA double-strand breaks.

Some typical results on the response to long-term vacuum exposure of some specific microorganisms are summarized briefly: There is a decrease in the viability of *B. subtilis* spores (Fig. 4). Exposure to a dynamic vacuum at 293 K is about twice as effective as exposure at 78 K. Exposure to an inert gas such as argon (293 K and 3% relative humidity) is also less efficient. The temperature dependence of the loss in viability supports the view that chemical reactions are responsible for the damaging effects produced by long-term exposure to vacuum. This point is substantiated by the temperature-dependent increase in the number of DNA double-strand breaks per chromosome observed after lysis of spores (see Fig. 5). About twice as many double- strand breaks are produced at 293 K than at 78 K. For comparison, the exposure to an atmosphere of dry argon (293 K and 3% relative humidity) has about the same effect as the exposure to a vacuum at 78 K. When comparing the loss in viability and the increase of the number of DNA double-strand breaks per chromosome, one has to keep in mind that only those spores that were rehydrated in a growth medium had the opportunity to repair numerous DNA damages before the first replication step. Thus, even after 25 weeks of vacuum exposure at 293 K, only 30% of the spores could repair the DNA damages so efficiently that they were able to replicate again. However, those spores that were rehydrated in a lysis buffer that actually was designed to break up the spores for DNA analysis, could only perform a very incomplete repair so that six DNA double-strand breaks per genome became analyzable. For a better understanding of these and related processes, see Figure 6.

DNA protein cross-links are not efficiently in *B. subtilis* spores during long-term exposure to vacuum (26). This effect may be due to the presence of a special class of DNA-binding proteins in these spores (11). However, in addition to other types of DNA damages a large number of DNA protein cross-links is induced in fungal conidia and even in *D. radiodurans* cells during exposure to vacuum (24,51). Figure 7 demonstrates the relatively good desiccation-resistance of *D. radiodurans* (mutant UVS_{78}). Figure 8 reveals that DNA protein cross-links are readily formed during desiccation of *D. radiodurans* (mutant UVS_{78}), although they can be repaired efficiently under growth conditions after rehydration. The same applies to other types of DNA damages such as strand breaks (24). However, despite its resistance to a huge variety of DNA-damaging agents, *D. radiodurans* has failed to survive under desert conditions (37). (See Survival under extremely dry desert conditions.)

Survival in Space

The Greek philosopher Anaximander, who lived about 610 to 546 B.C., suggested that "germs of life" may originate

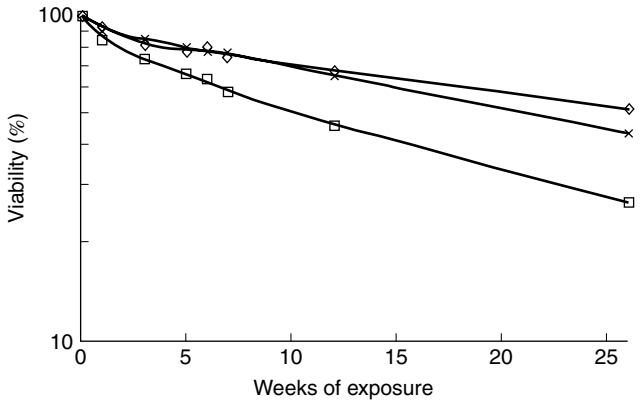

Figure 4. Loss in viability of *B. subtilis* spores (TKJ 3412) during exposure to vacuum for up to 25 weeks at 293 K (–!–) and 78 K (– –). For comparison the corresponding inactivation curve by exposure to dry argon (3% relative humidity) at 293 K is shown (– × –) (27).

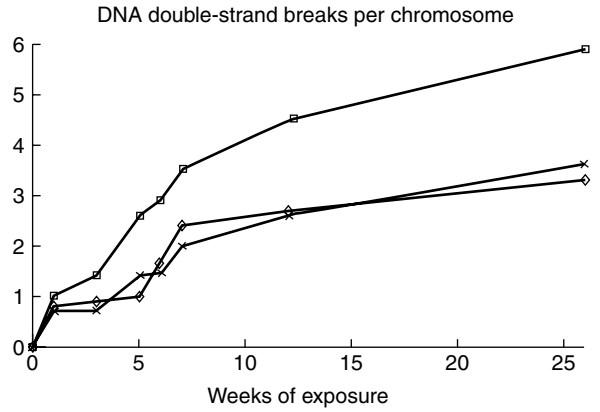

Figure 5. Formation of DNA double-strand breaks induced in *B. subtilis* spores (TKJ 3412) after exposure to vacuum for up to 25 weeks at 293 K (–!–) and 78 K (– –). For comparison the corresponding number of DNA double- strand breaks after exposure to dry argon (3% relative humidity) at 293 K is shown (– × –). It may be noted that most strand breaks occur under lysis conditions as a result of incomplete repair of other DNA damages. [Fig. 6; (27)].

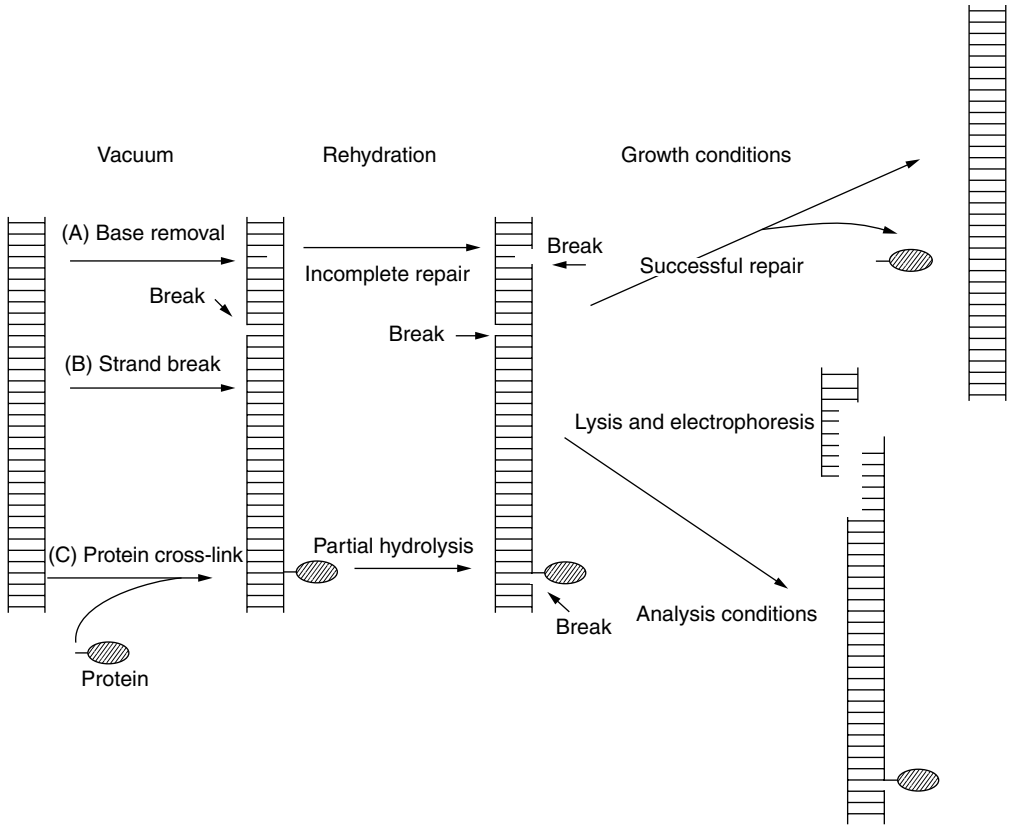

Figure 6. Possible intermediate steps leading to the formation of DNA double-strand breaks by dehydration and subsequent rehydration under lysis conditions. These conditions cause incomplete repair (lower right) especially because of the absence of ATP synthesis. For example, during excision repair the strand connecting ligases cannot function because the necessary energy resources are not available. Under growth conditions, (upper right) at least a fraction of the damaged cells may manage a successful repair. Their success, however, will depend on the number of dryness-induced DNA damages and on the efficiency of their repair systems. Even the most repair efficient strains become failure-prone and die if the number of DNA damages exceeds their repair capacity (26).

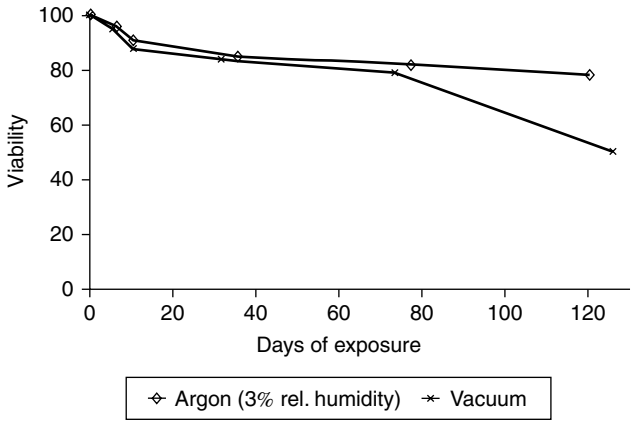

Figure 7. Survival of *D. radiodurans* (mutant UVS_{78}) after long-term exposure to vacuum and dry argon (3% relative humidity) at 293 K. Data from Dose et al., *Origins Life Evolution Biosphere* **21**, 177–187 (1991), and O. Kerz, *Dissertation*, University of Mainz, FB Chemistry and Pharmacy, 55099 Mainz, Germany, (1994).

from the "moisture" that surrounds the Earth and that life could be brought down from the sky with the rain. Related concepts have been held ever since. During the middle of the nineteenth century, it was widely accepted that airborne fungal conidia could be carried across the Alps from the Mediterranean and North Africa and that these organisms therefore could survive at high altitudes. For this reason, the German physician Richter (52) suggested in 1865 that germs of life could be picked up by passing comets or meteorites and thus be transported through space. Richter assumed that after an impact on another planet, one exhibiting favorable conditions for growth, these organisms would multiply and develop according to Darwin's theory. At the beginning of the twentieth century, Arrhenius suggested a similar hypothesis (53), which he popularized as the "panspermia" thesis. In contrast to Richter, however, he suggested that spores could be propelled through the Universe by the pressure of the light radiated from the sun or other stars. However, it was later recognized that naked spores would be inactivated within seconds, if exposed to solar UV. Spores protected by a light-absorbing coat, on the other hand, would be too massive to be transported by the pressure of light. Spores transported by meteorites or comets face numerous additional hazards. These include the heat of friction during the contact of comets or meteorites with planetary

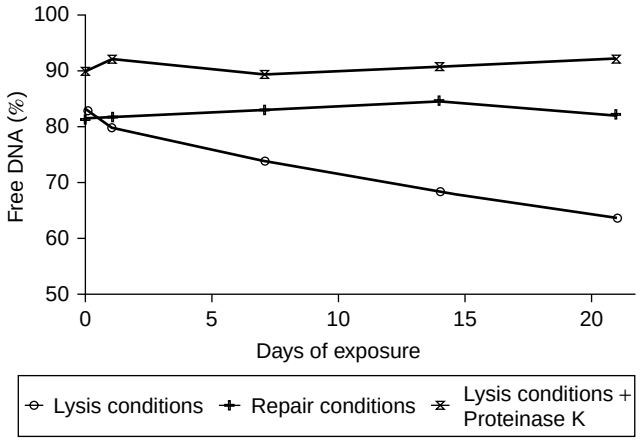

Figure 8. The desiccation-induced cross-linking of DNA to proteins causes a time-dependant decrease in "free" (extractable) DNA in *D. radiodurans* (mutant UVS_{78}) according to Dose and coworkers (24). In untreated cells about 18% of the total DNA is bound to proteins. That means only about 82% of the DNA is "free" or extractable from protoplast after lysis by detergents. The amount of "free" DNA was increased to about 90% by additional treatment with proteinase K (the protein cross-link is degraded). For repair, the cells were incubated in a growth medium for 3 hours at 30 °C.

atmospheres both during pick up and delivery of living spores and high pressures caused by shock waves during collisions (54). Other hazards include cosmic radiations and dehydration by space vacuum during travel times of geologic order (26). For these reasons Crick and Orgel (55) suggested that life may have been brought to Earth through the activity of intelligent extraterrestrial beings, either by manned spaceflight or by a programmed rocket "directed panspermia" from another planet of our Galaxy. It is true that numerous solar systems older than our own may indeed exist at a distance of 100 light-years, and that the formation of Earth-like planets in at least one of these solar systems is a possibility. It is also true that life in the Universe may have come into existence before our Earth was formed. However, space transport systems traveling at about 100 km/s (about 14 times faster than the space shuttle) would require 300,000 years to bridge a distance of 100 light-years. Because of such a long travel time, the idea of "directed panspermia" remains yet in the realm of science fiction.

With respect to the question of the origin of life on Earth, one has also to bear in mind that all hypotheses on an extraterrestrial origin of life move this problem just to another planet. To evaluate the possibility of extraterrestrial life transfer and to test for the possibility of contamination of other planets by space vehicles, especially spores of *B. subtilis*, fungal conidia, and cells of *D. radiodurans* have been exposed to the conditions of free space during various space missions (33,56,57). These missions include Apollo 16, Spacelab 1, LDEF (Long Duration Exposure Facility), the EURECA, and the D2 mission. In typical experiments, suspensions of the microorganisms are applied to small glass disks (about 2 cm in diameter) to achieve statistical monolayers or several milligrams of predried microorganisms (as a powder) are transferred to small cubic aluminum containers (side length 15 mm) that are covered by gas-permeable, but germ-impermeable silver membranes (33). Subsequently the samples are desiccated under vacuum in the laboratory and then transferred to (sealed) containers. These containers are arranged in larger exposure units that often are accommodated at the outside of the space vehicle. During the spaceflight, the exposure units are opened and closed by remote control. The samples were exposed to a space vacuum of 10^{-10}–10^{-6} mbar and at temperatures between −10 °C and 50 °C, depending on carrier and orbit. Exposure to space lasted between 1.3 hours (Apollo 16) and up to 2107 days (LDEF).

So far most space exposure experiments had at least one of the following disadvantages: The samples had to be provided several days or even weeks before the mission and they were returned to the investigator at least several days after the mission. The environmental conditions (such as temperature and relative humidity) during these periods were usually not completely monitored. In addition, the exact temperature control during the missions was often a problem. For example, during the EURECA mission some samples were heated up to 50 °C (57). Moreover, the vacuum on the surface of a space vehicle can be contaminated by gases from the propulsion system. The duplication of an experiment is often not possible because appropriate missions are not offered. For these reasons simulation experiments in the laboratory play a crucial role. The space vacuum around a space vehicle in Earth orbit can be easily be simulated for months with the help of continuously running turbomolecular pumps. Therefore, as expected, it could be confirmed that the laboratory data on the response of microorganisms to vacuum largely agree with the corresponding space data (57), especially if the undesirable effects occurring during the missions are taken into account. Noteworthy with respect to survival are the results of the LDEF mission (56). They show that *B. subtilis* spores can relatively well survive exposure to space vacuum (around 80% survival after five years of exposure), if they are exposed in thick layers with glucose as a protectant. However, if exposed in cellular monolayers and in the absence of protectants survival was reduced to about 1%. These differences are not surprising because removal and diffusion of water from a cellular monolayer proceeds faster than from a thick layer of hydrophilic biomass. Generally, the removal of chemically bound water by vacuum appears to be an extremely slow temperature-controlled reaction (27). Cellular monolayers of spores continue to become inactivated during an exposure to vacuum for more than 10 weeks (26,27).

To monitor the effects of irradiation by sunlight in space, cellular monolayers of the microorganisms are exposed on quartz disks for short periods. The evaluation of the photobiological experiments (56) has confirmed that only 5% of a population of unprotected spores will survive if exposed to the full solar spectrum for only 10 seconds. It should be noted, however, that this value was obtained by extrapolation, not by direct measurement. The UV sensitivity of spores under vacuum is about five times larger than under atmospheric conditions at about 60% relative humidity because of the synergism of dehydration

and UV effects. Action spectra of the solar light show the highest efficiency at 260 nm, the maximum DNA absorption. On the surface of the Earth, the situation is not comparable. Because of the ozone shield practically no solar UV under 300 nm reaches the surface of the Earth. On Earth, spores have to be exposed to solar light for at least 90 minutes (depending on the season and the geographic location; see the next section) to reduce their viability to about 5% (37).

The effects of solar and galactic corpuscular radiations (cosmic radiations) have been investigated during most of the above missions. However, even during the long LEDF mission (almost five years of exposure) the amount of absorbed energy was so low that no general loss in viability could be attributed to these radiations.

The present data on the inactivation of *B. subtilis* spores by long-term exposure to a dynamic vacuum reveal that the exposure time for 50% inactivation at 293 K is about nine weeks and at 80 K presumably still about 20 weeks (26,27). With respect to survival in space, the implication is that unprotected spores would be completely inactivated within a few decades both if exposed to vacuum at 293 (26) or at the extremely low temperatures found at the outskirts of the Solar System (27). The UV-sensitivity of dehydrated spores at 80 K is decreased only by a factor of ~4 in comparison to irradiation at 293 K (27). Solar radiation would therefore continue to be a lethal threat to spores at 80 K. These data seriously restrict the survival chances of unprotected spores while being transported through the Solar System and therefore further limit the validity of the panspermia thesis (53,58). In view of our data, appreciable chances of survival could only be maintained by avoiding any vacuum-induced dehydration or decomposition process. This could be achieved by embedding spores or other cells in huge icy masses at temperatures below 140 K, the critical temperature for freezing out all liquid water. Below this temperature the rate of all diffusion-controlled chemical reactions, for example, hydrolysis is practically zero (1). The size of the germ-transporting icy masses would have to be comparable to the size of the Halley's comet because evaporation processes would lead to huge losses of matter during the long journey from a remote planet. In addition, sufficient shielding from cosmic radiations would be provided by a thick cover of icy matter. The source of such a cosmic "iceberg" could be seen in the glaciers or permafrost areas of another life-bearing planet. It is well known that terrestrial glaciers provide various habitats for life. An example is the cryoconite holes investigated by Wharton and coworkers (59). Viable microorganisms have also been detected in permafrost (60). It is conceivable that a cosmic body (comet or meteorite) could have collided with the life-bearing ice cover of a remote planet several billion years ago and lifted off huge numbers of frozen masses. Some of these icy masses may have finally reached the early Earth and implanted life here. It has been realized, however, that the probability of such a life-transfer process is extremely small. Moreover, there is no significant advantage in moving the origin of life to a remote planet. The age of our Solar System is about 4,500 Myr. Contemporary forms of life may have been present on Earth about 3,700 Myr ago (61). The conditions for prebiotic evolution on Earth may have become favorable about 4,000 Myr ago. This limits the time available for the evolution of microbial life on Earth to less than 300 Myr. The available time for chemical evolution and subsequent development of primitive prokaryotes on an ancient planet could have been much larger. The upper limit for such a timescale is set by the age of the Universe (probably about 15,000 Myr), diminished by the time (probably around 5,000 Myr) required to produce an early planetary system being sufficiently rich in bioelements. Prokaryotes on an ancient planet could therefore have evolved over a period of several 1,000 Myr before appearing on Earth. This advantage, however, is counterbalanced largely by the long time required for transportation of spores through space, by the general risks of such a transport, and by its extremely low probability.

After examining all these imponderables it is realized that the data on the limited survival of spores in an actual or simulated space vacuum put further constraints on the panspermia thesis and therefore encouraged the search for the origin of life on Earth.

SURVIVAL UNDER EXTREMELY DRY DESERT CONDITIONS

The climatic environments of terrestrial deserts differ significantly. Examples are the hot Negev Desert in Israel, the cold deserts of Antarctica, and the extremely dry deserts of northern Chile (Atacama Desert) (see ENDOLITHIC MICROORGANISMS IN ARID REGIONS). Most deserts are at least occasionally subjected to precipitation, periods of high relative humidity (above 80%), and/or dew fall during the early morning hours. During these periods the growth of a variety of desiccation-resistant microorganisms becomes possible. Lange and coworkers (5) have shown that although lichens in the Negev Desert usually become extremely dry during the hot daytime, at night they are capable of absorbing enough water from the atmosphere (at about 90% relative humidity and even in the absence of dew fall) to enable a short period of photosynthetic activity during the morning. Endolithic microorganisms use a different strategy (62–64). Symbiotic colonies of a variety of microorganisms including cyanobacteria live inside rocks, usually sandstones, protected and separated from the outside by a thin translucent rock crust. Although the rock surface is abiotic, the microclimate inside the rock is comparably mild. The endolithic organisms are able to adapt their metabolic activities, including photosynthesis, in response to changes in the environment. This includes the ability to survive periods of extreme dryness in an anhydrobiotic or dormant state. In the Antarctic desert (about 162° E; 77° S) there is no sunshine from May to July, the average air temperatures range from about −30 °C (winter) to about −1 °C (summer), and the annual average of the relative humidity is about 55% (65). During the summer, solar radiation may heat the interior of the endolithic systems up to about 10 °C and some melting snow provides the necessary moisture, whereas, during the winter, the dark, dry, and cold conditions enforce a dormant state. Under these conditions a relatively diverse assemblage of prokaryotes and microeukaryotes

develop. Entirely different is the climatic environment in some areas of the extremely dry and hot Atacama Desert. Only a few areas may receive sporadic rainfall (approximately every four years) because of the recurrent climatic phenomenon called El Niño. Seasonal changes of the relative humidity and of temperature have been monitored at the location "Yungay" (24.1° S; 69.7° W). Some data are summarized in Table 2 (37).

The relative humidity reaches 70 to 80% for a few hours before sunrise on only 15 days in a summer month and about seven days in a winter month. Additional stress is imposed by the strong diurnal changes of the relative humidity. Evidently, these extremely dry conditions will not support any form of active life. Accordingly, the surface of the rocks exhibits no sign of microbial activity. These conditions, including the appearance of the landscape, appear to resemble those of early Mars after the loss of a hydrosphere about 3.5 Gyr ago (37,66).

In this context we have tested the survival chances of *B. subtilis* spores, fungal conidia, and *D. radiodurans* cells (strain R1) under the earlier- mentioned desert conditions by exposing specimens in small containers of the kind used to study survival in space. Some of the data are summarized in Table 3. The survival in the Atacama Desert is affected also by the strong diurnal changes of the relative humidity, which seems to cause additional damages to the membranes rather than to DNA. The survival of *D. radiodurans* cells (strain R1) especially is decreased. This microorganism is resistant to extreme dryness and vacuum at relative humidities under 20%, but it is inactivated readily by exposure to higher relative humidities and therefore is not adapted to survive in a terrestrial desert. Its surprising inactivation is not related to an increase in DNA damages (37). All microorganisms suffer from the daily exposure to solar light, especially UV-B. Some data for an exposure time of 90 minutes are summarized in Table 4.

The data demonstrate that the long-term survival of *B. subtilis* spores is significantly poorer under desert conditions than in a (simulated) space vacuum (26,27,37). Probably also viable spores on the surface of Mars may have soon disappeared after the planet became dry. In the extremely dry areas of the Atacama Desert, chances for microbial life appear to exist only underground. There is a flow of groundwater from the Cordillera in the east. At some places, for example, at the Oasis Pica, the groundwater rises to the surface so that agricultural activities flourish in the otherwise extremely dry and rainless desert. Similarly, on early Mars, microorganisms, if they ever existed, could only survive in an underground habitat.

Table 2. Seasonal Changes of The Relative Humidity and of The Temperature in the Atacama Desert at Yungay (24.1° S; 69.7° W)

	Summer (December)		Winter (June)	
Parameter	Maximum	Minimum	Maximum	Minimum
Temperature (°C)	33 ± 3	10 ± 2	28 ± 3	1 ± 3
Rel. Humidity (%)	60 ± 25	<10	40 ± 35	<10

Note: The data are presented as mean values ± the maximal values measured during the indicated month. Actually the values have been recorded continuously during the whole year (37).

Table 3. Survival of *Aspergillus* conidia, *B. subtilis* Spores, and *D. radiodurans* after Exposure for 13 to 15 months in the Atacama Desert (dark tray)

Sample	Laboratory (silica gel; 15 months) $(N_t/N_o) \times 100\%$	Chacabuco and Yungay (~24° S); 13–15 months $(N_t/N_o) \times 100\%$
A. niger	35 ± 5	28 ± 5
A. versicolor	25 ± 5	17 ± 5
A. ochraceus	20 ± 5	14 ± 5
B. subtilis	17 ± 3	15 ± 3
D. radiodurans	30 ± 10	0.1 ± 0.05

Note: No significant differences could be found between survival after 13 and 15 months of exposure (37).

Table 4. Inactivation of Spores and Conidia by Solar Light in the Atacama Desert

Sample	Dark Control $(N_d/N_o) \times 100\%$	Solar Light $(N_{90}/N_o) \times 100\%$
A. niger	60 ± 5	24 ± 5
A. versicolor	25 ± 5	2 ± 1
B. subtilis	29 ± 3	4 ± 1

Note: The fluence (light energy per surface area) for 90 minutes irradiation time was about 16 kJ/m^2 (wavelength range 280 nm to 320 nm). N_o is the relative number of viable cells in the fresh preparation, N_d is the relative number of viable dried cells (monolayers on glass disks) prior to irradiation in the desert, N_{90} is the relative number of viable dried cells after additional exposure to solar light for 90 minutes (37).

CONCLUSION

It is a long way from learning to store dry seeds for the next season to testing the survival of microorganisms in extremely dry environments such as Mars-like deserts and free space. Although the former is and was essential for the survival of humanity, the latter satisfies just our curiosity: Are we alone in space? Do we really originate from Earth? Neither do we have affirmative answers, nor do we know what the Earth is for and why we are here.

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DESICCATION OF MICROORGANISMS.

See FREEZE DRYING: PRESERVATIONS OF MICROORGANISMS BY FREEZE-DRYING

DESULFURIZATION OF FOSSIL FUELS

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The growth of industrial civilizations, and, in particular, the use of fossil fuels for energy, have led to the pollution of the environment. The problem with fossil fuels is that the combustion products exert a harmful influence on the worldwide environment. The carbon dioxide emission has caused global warming. The restriction of "greenhouse gas" emission (particularly carbon dioxide) is the subject of worldwide debate in 2001 although the Kyoto Protocol was adopted in 1997. All fossil fuels contain organic

Table 1. Existing and Planned Regulations for Sulfur Level in Fuels for Motor Vehicles

Year	Country	Fuel	Current Sulfur Levels (ppm)	Target Sulfur Levels (ppm)
1993	United States	On-highway diesel	2500	500
1996	Singapore	Diesel	5000	2500
	India	Diesel	8000	5000
	European Union	Diesel	3000	500
1997	Japan	Diesel	2000	500
1998	Taiwan	Diesel	5000	500
2000	South Korea	Diesel	2000	500
	Thailand	Diesel	5000	500
	European Union	Diesel	500	350
	United States	CAAA gasoline	400	50–100
	European Union	Gasoline	500	350
2005	European Union	Diesel	350	50
2006	United States	Diesel	500	15
	United States	Gasoline	300	30
2007	Japan	Diesel	500	50

sulfur compounds, and burning of the fuels releases large quantities of sulfur oxides (SO_x) into the environment, contributing significantly to air pollution and being the principal cause of acid rain. SO_x is believed to contribute a share of the ambient fine particle matter, which can cause disorders of the respiratory system. Moreover, excess amounts of SO_x reduce the ability of catalysts to remove the nitrogen oxides in the engine of motor vehicles. To avoid the production of noxious sulfoxide, sulfur must be removed from fossil fuels before, during, or after combustion.

Hydrodesulfurization (HDS) is the current method for sulfur removal, and is a treatment of the fuel with hydrogen at high temperatures (>300 °C) and high pressures (>100 atm) in the presence of a metal catalyst, mainly molybdenum- or tungsten-based catalyst. Oil refiners depend on HDS process to treat some 20 million barrels of crude oil per day (1). HDS can easily remove inorganic sulfur or simple organic sulfur compounds but not complicated polycyclic sulfur compounds. Although conventional catalysts were alumina-supported bimetallic sulfides of molybdenum or tungsten, new HDS catalysts with improved efficiencies have been investigated. For example, molybdenum phosphide (2) or cobalt molybdenum nitride (3), which differ substantially from other materials, were introduced into the catalysts, and it has been confirmed that they were more active than the conventional catalyst. However, with the depletion of low-sulfur-containing petroleum reserves and increasingly stringent regulations, adequate desulfurization by conventional HDS alone is becoming progressively more difficult to achieve.

Regulations to reduce the sulfur content of fuels for motor vehicles were introduced in the early 1990s and will be increasingly stringent after 2000 (4–6), as shown in Table 1. In the European Union, regulations reducing the sulfur levels to less than 50 ppm in 2005 have been established. The U.S. government has worked out even more stringent regulations of sulfur content in gasoline (below 30 ppm) and diesel oil (below 15 ppm). In Japan, the sulfur level in diesel oil will be decreased from 500 ppm, in 1997 to 50 ppm in 2007. Both governments and petroleum refining companies have recognized that it is difficult to meet the environmental regulations in a cost-effective manner using only conventional HDS technology because the equipment needed for such a high degree of desulfurization is extremely expensive to build and operate.

Another representative fossil fuel, coal, is used as fuel at many power stations, but not in motor vehicles. Although the fuel gas desulfurization is operated for removing SO_x released from coal at the power station, the installation is so expensive that an inexpensive sulfur removal technology has been desired.

Biological desulfurization (BDS) of polycyclic sulfur compounds in fossil fuels was investigated in the late 1970s and early 1980s, but it was not successful because the bacteria isolated at that time were not appropriate for commercial desulfurization. However, with the strict worldwide environmental regulations described earlier, BDS has again attracted attention for its application to the desulfurization of fossil fuels (7–9). BDS is expected to have the potential advantages to complement the HDS technology and to produce less greenhouse gases.

MICROBIAL DEGRADATION AND DESULFURIZATION OF DIBENZOTHIOPHENE

Microbial Degradation of Dibenzothiophene with a Cleavage of Carbon–Carbon Bonds

A variety of sulfur-containing compounds in petroleum have been characterized. Among them, various heterocyclic organic sulfur compounds remain after the current HDS treatment, and the main components are dibenzothiophene (DBT) derivatives. Therefore, for the research of BDS, DBT, which is a commercially available compound,

has generally been used as the model compound of sulfur heterocycles to be desulfurized.

The concept of microbial DBT desulfurization is not new. However, no commercially viable bioprocess for removing organic sulfur from fuels has been developed because the microbial process was not needed urgently. The metabolism of DBT by aerobic microorganisms can be generally classified into three types (Figs. 1 and 2). In type 1 metabolism, the carbon skeleton of DBT is partially oxidized and cleaved, with the carbon-sulfur bond remaining intact. This pattern of metabolism is "DBT degradation" and it should be clearly distinguished from "DBT desulfurization." All bacteria isolated for the purpose of microbial desulfurization previously had this metabolic pathway, DBT degradation. In 1968, *Pseudomonas* strains were reported to have the capacity to degrade DBT (10,11). Afterward, DBT was shown to be metabolized by hydroxylating the benzoid component of the compound to a dihydroxy-dihydro-derivative, cleaving the ring between the diol substituents, and then producing, in sequence, 4-[2-(3-hydroxy)-thionaphthenyl]-2-oxo-3-butenoic acid and 3-hydroxy-2-formylbenzothiophene (HFBT) as shown in Figure 1 (12,13). The occurrence of such a degradative pathway has been ascertained in a number of other bacterial strains (14–16). The genes for that DBT degradative pathway were proposed in *Pseudomonas* sp. C18 (17). This strain grew on naphthalene as a sole source of carbon, and the gene products for the naphthalene degradation were considered to be involved in the degradation of DBT (18). Although biodegradation of HFBT was observed in other *Pseudomonas* strains (19,20), no sulfate release was detected with those microorganisms. In the type 1

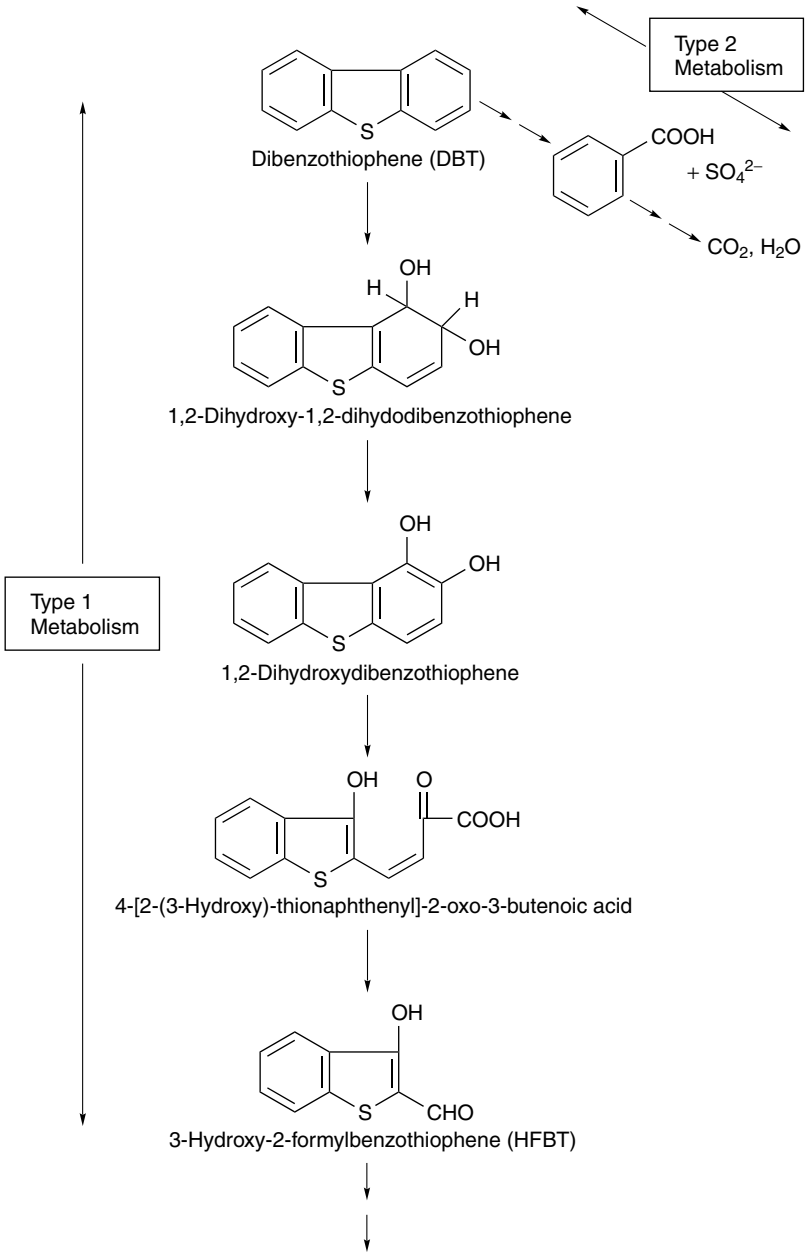

Figure 1. Microbial degradation of DBT with a cleavage of carbon–carbon bonds.

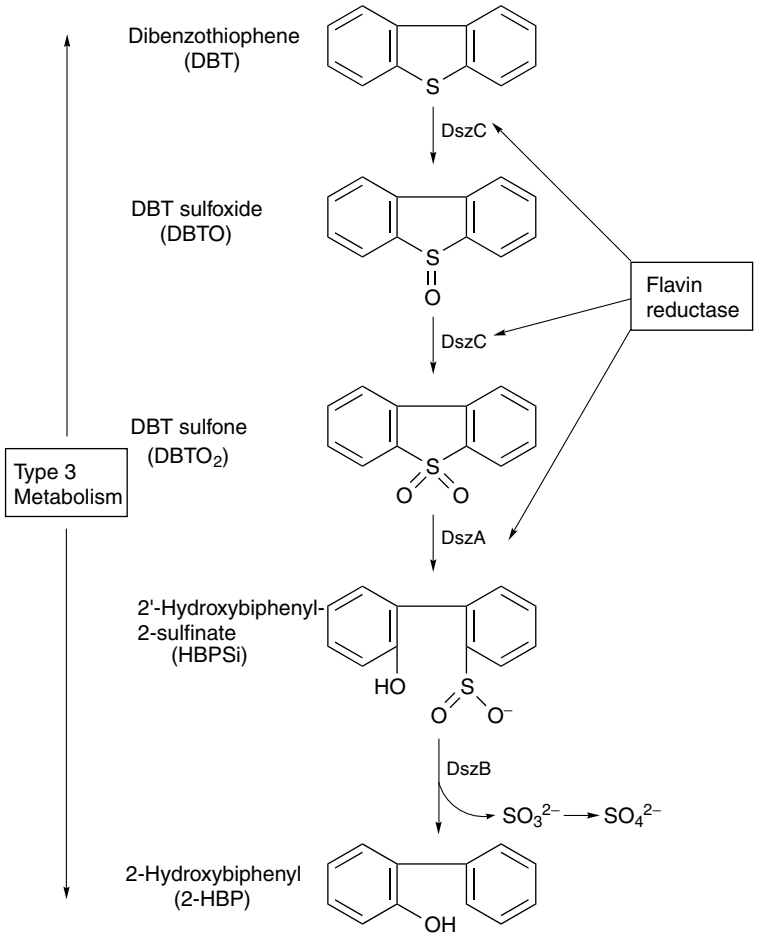

Figure 2. Sulfur specific pathway for microbial DBT desulfurization without a cleavage of carbon–carbon bonds.

metabolism described here, the bacteria assimilated the carbons of hydrocarbon leading to a loss of fuel content, but did not cleave the carbon–sulfur bond in the organic sulfur compound. That is to say, the metabolic pathway used for sulfur removal was in fact a hydrocarbon degradation pathway. The bacteria functioned in a bioremediation mode, degrading DBT to a water-soluble product, not desulfurizing DBT. Moreover, some pigments were formed in the bacterial cultures during DBT degradation, which will lead to trouble with the final oil product.

In type 2 metabolism, DBT is utilized as a sole source of carbon, sulfur, and energy. *Brevibacterium* sp. mineralized DBT, and carbon dioxide, sulfite, and water were formed as the final products (21). *Arthrobacter* DBTS2 was reported to oxidize DBT sulfoxide, but not DBT, forming sulfate and benzoate (22). Only these two microorganisms were known to have a metabolic pathway like that shown in Figure 1. They desulfurize DBT, but also break the carbon skeleton of the hydrocarbon.

It has been demonstrated that DBT is also metabolized by anaerobic microorganisms. *Desulfovibrio desulfuricans* M6, a sulfate-reducing bacterium, degraded DBT anaerobically, and biphenyl was isolated as the major degradation product (23). The degradation ratio was 42%. This bacterium removed sulfur from petroleum using electrochemically supplied electrons (24). It was shown that other sulfate-reducing bacteria also had the ability to convert DBT to biphenyl anaerobically (25,26), but the conversion ratio was very low (0.22 to 1.14%). An anaerobic process for sulfur removal would be attractive because it would not liberate sulfate as a by-product that must be disposed of. However, anaerobic microorganisms effective enough for the practical desulfurization of petroleum have not been found yet.

As described earlier, DBT metabolism, at first, was investigated using microorganisms capable of assimilating DBT as a carbon source. The modes of microbial degradation of DBT are similar to those of three DBT analogs, fluorene, dibenzofuran, and carbazole whose molecules have a carbon, oxygen, and nitrogen atom, respectively, instead of a sulfur atom of DBT molecule (18). These microorganisms seem to have some drawbacks for the microbial desulfurization of petroleum because they consume petroleum and produce unnecessary pigments.

Microbial Desulfurization of DBT with a Cleavage of Carbon-Sulfur Bonds

In type 3 metabolism, DBT serves as a sole source of sulfur, not carbon. DBT is desulfurized by the selective cleavage of the carbon-sulfur bond, resulting in the accumulation of 2-hydroxybiphenyl (2-HBP), as shown in Figure 2. The specific cleavage of the carbon–sulfur bond, rather than degradation/mineralization of the organic

sulfur compounds, is preferred for the microbial desulfurization process because sulfur is removed but the carbon and calorific values remain intact. In the past decade, several effective screening techniques have been developed and have yielded promising strains (27,28). Kilbane and coworkers (29) isolated *Rhodococcus erythropolis* IGTS8 (formerly identified as *Rhodococcus rhodochrous*), which has so far been the most extensively studied DBT-desulfurizing bacterium, and a DBT desulfurization pathway in the microorganism was proposed (30,31). According to the so-called sulfur-specific pathway shown in Figure 2, DBT is metabolized to 2-HBP via DBT 5′-sulfoxide (DBTO), DBT 5′-sulfone ($DBTO_2$), and 2′-hydroxybiphenyl 2-sulfinate (HBPSi). After *R. erythropolis* IGTS8 was found, many other bacterial strains including *Corynebacterium* sp. SY1 (32), *R. erythropolis* D-1 (33), *Nocardia globelula* (34), *Mycobacterium* sp. G3 (35), *Arthrobacter* sp. (36), and *R. erythropolis* KA2-5-1 (37) were reported to desulfurize DBT with a similar metabolic pathway. All these strains were gram-positive bacteria, and no gram-negative bacteria with a sulfur-specific pathway have been reported in the literature. as Apart from the intermediates or final products shown in Figure 2, 2,2′-dihydroxybiphenyl (DHBP) was detected in the culture of *R. erythropolis* IGTS8 (30,38), and DHBP formation was confirmed with growing cells, not with the stationary-growth-phase cells (39).

Kinetic Analyses of Microbial Desulfurization of DBT

Kinetic analyses of the microbial desulfurization of DBT were done using *R. erythropolis* N1-36 in batch, fed-batch, and continuous cultures (40,41). This strain produced more 2-HBP from DBT as a substrate than from $DBTO_2$ (40). In continuous cultures, the maximum specific growth rate was 0.235/h (41). In a fed-batch culture of *R. erythropolis* IGTS8, a high cell concentration of 33 g dry cells was obtained by keeping the concentration of acetic acid and ammonium ion below 3 g/L (42). When the cultivated cells were transferred into the synthetic medium with DBT as a sole source of sulfur, 2-HBP was detected and its production rate gradually increased with incubation time. Incubation for three to four hours was enough for the full induction of DBT-desulfurizing enzymes, and a two-phase cultivation (cell growth phase and induction phase) was proposed to obtain large numbers of cells with high enzymatic activity (42). And in a fed-batch culture of *R. erythropolis* KA2-5-1, ethanol was found to be a preferable carbon source for obtaining a high specific activity of desulfurization compared with glucose or glycerol (43).

Thermophilic Microbial Desulfurization of DBT

In petroleum refining, most of the sulfur compounds are presumed to be desulfurized by the chemical method, and the microbial process is applied to desulfurize the more recalcitrant aromatic organic sulfur compounds. Since petrochemical processing is performed at high temperatures and pressures, cooling a feedstock for bioprocess treatment is not practical. Therefore, thermophilic desulfurization bacteria would be desirable. Although the thermophilic degradation of DBT by *Sulfolobus acidocaldarius*, originally isolated from a hot spring (44), has been reported, this strain "degraded" DBT, that is, "cleaving a carbon–carbon bond." In 1997, the thermophilic desulfurization bacterium *Paenibacillus*, which can cleave carbon–sulfur bonds in DBT at temperatures up to 60 °C, was isolated (45). Maximal desulfurization by *Paenibacillus* sp. A11-2 was observed at 55 °C in the resting cells reaction, whereas that by *R. erythropolis* IGTS8 was observed at 30 °C. In addition, a moderately thermophilic bacterium *Bacillus subtilis* WU-S2B, which could desulfurize DBT at 50 °C in the similar way, was isolated (46). These thermophilic strains have potentials for the microbial desulfurization of petroleum.

GENES FOR DBT DESULFURIZATION

Identification of Genes for Desulfurization of DBT

The genes involved in the sulfur-specific pathway, DBT desulfurization, were identified and cloned with *R. erythropolis* IGTS8 in 1993 (47). Then, the DNA sequence for the genes was analyzed by two groups (48,49). The DBT-desulfurizing phenotype is conferred by a four kb gene locus on a 120-kb linear plasmid (50), organized as one operon with three genes, *dszA*, *dszB*, and *dszC*, transcribed in the same direction, coding for three proteins DszA, DszB, and DszC, respectively, under the control of a single promoter (50). DszC, a 45-kDa protein, is involved in two consecutive monooxygenation reactions, which convert DBT to $DBTO_2$. DszA, a 50-kDa protein, is a second monooxygenase, which forms HBPSi from $DBTO_2$. DszB, a 40-kDa protein, is a desulfinase that catalyzes the formation of the final products, 2-HBP and sulfite. The termination codon for *dszA* and the initiation codon for dszB overlap and there is a 13-bp gap between dszB and dszC. Although they are expressed as an operon, it was reported that there is severalfold less DszB in the cytoplasm than DszA and C (51,52). The reason for this phenomenon is not clear. By using the insertion fragments and *dsz* structural genes as probes, Southern hybridization showed the conserved nature of the *dsz* genotype among several desulfurization strains isolated from different geographic areas (50). Other genes for DBT desulfurization were cloned from the thermophilic desulfurization bacterium *Paenibacillus* sp. A11-2 (53). The DNA sequence contained an operon consisting of three open reading frames that showed an appreciable extent of homology with *dsz* genes of *R. erythropolis* IGTS8.

Regulation of Genes for DBT Desulfurization

The production of enzymes for the desulfurization of DBT is strongly repressed by sulfate or compounds containing sulfur atoms such as methionine and cysteine even in the presence of DBT (54,55). The regulatory region including the promoter was examined. Childs and coworkers cloned a fragment of the 5′ untranslated region of the *dsz* gene cluster and identified the promoter region and the transcription start site (51). The promoter region had sequence similarity with *B. subtilis* promoters (56) that use sigma factor σ^B, but not with the −35

and −10 regions of *Escherichia coli* promoters. Sulfur compounds repressing enzyme production do not inhibit the enzymatic activities (55). In contrast, accumulation of 2-HBP inhibits growth and enzymatic activities (39,55). Therefore, mutants resistant to 2-HBP may be needed for commercial applications.

ENZYMES FOR DBT DESULFURIZATION

Four Enzyme Components for Desulfurization of DBT

The enzymatic study of the microbial desulfurization of DBT was first done using *R. erythropolis* D-1. It was pointed out that NADH was the essential cofactor for the enzymatic desulfurization (57) and flavin coenzyme stimulated the enzyme activity (58) in the cell-free system. These findings have stimulated more enzymatic research. All the four enzymes involved in the desulfurization of DBT, DszA, B, C, and flavin reductase, were purified from *R. erythropolis* IGTS8 (52). The native molecular masses were 180 kDa for DszC, being a tetramer; 100 kDa for DszA, a dimer; and 40 kDa for DszB, a monomer. The subunit molecular mass of flavin reductase was 25 kDa. All proteins were colorless as isolated, indicating that no tightly associated chromophores were present. The DBT monooxygenase, DszC was also purified to homogeneity and characterized from *R. erythropolis* D-1 (59) and a *dszC*-overexpressing *E. coli* strain (60), respectively. DszC is involved in two consecutive steps of the monooxygenase reaction: DBT→DBTO→$DBTO_2$. DszC activity decreased during the purification of enzyme, and the loss in activity was due to the removal of a necessary protein component (59). Then, it was demonstrated that a flavin reductase from *Vibrio harveyi* complemented the activity of purified DszC (60,61). The second oxygenase, DBT sulfone monooxygenase, also required a flavin reductase for the catalytic activity (61). The enzyme was purified to homogeneity and characterized from *R. erythropolis* D-1 (62) and *Paenibacillus* sp. A11-2 (63), respectively. It catalyzed the oxygenolitic cleavage of the carbon–sulfur bond of DBT sulfone and gave HBPSi. Moreover, the enzyme from *R. erythropolis* D-1 also acted on dibenz[*c,e*][1,2]oxathiin 6-oxide (BPSi) and dibenz[*c,e*][1,2]oxathiin 6,6-dioxide (BPSo), and DHBP was formed from these compounds as shown in Figure 3 (38,62). Small amounts of DHBP appeared in the cultivation medium of *R. erythropolis* IGTS8 when DBT was added as a sole source of sulfur (30), and it was thought that DszA was involved in the formation of DHBP. In this case, this enzyme catalyzes the desulfurization reaction, releasing inorganic sulfur. DszA may recognize the sulfone structure moieties within the ring structures of DBT sulfone or BPSo.

HBPSi desulfinase, DszB, catalyzes the desulfination of HBPSi to form 2-HBP. This reaction is unusual because it catalyzes the cleavage of a carbon–sulfur bond of the substrate without any other proteinic component (52). The DszB reaction seems to be a rate-limiting step in the pathway since it is the slowest of the enzymatic reactions. Because of the poor activity of DszB, HBPSi might be accumulated temporarily as the only intermediate on enzymatic desulfurization by the cell-free extract of *R. erythropolis* IGTS8 (52).

Flavin reductase was purified and characterized from *R. erythropolis* D-1, and its gene was overexpressed in *E. coli* (64). The purified enzyme contained no chromogenic cofactors and was found to have a molecular mass of 86 kDa and four identical subunits with 22 kDa. It catalyzed NADH-dependent reduction of flavin mononucleotide (FMN). Flavin adenine dinucleotide was a poor substrate, and NADPH was inert. A flavin reductase was earlier identified as supplying free $FMNH_2$ to the luciferase of the bioluminescent bacterium, which catalyzed the oxidation of a long aliphatic aldehyde, resulting in the emission of light (65,66). In addition, it was reported to activate the chorismate synthetase of *B. subtilis* (67). Thereafter, a flavin reductase has been shown to be required for a number of monooxygenases (68–76).

Properties of the Monooxygenase System for DBT Desulfurization

There are some differences between the *dsz* monooxygenases involved in the microbial DBT desulfurization and the well-described monooxygenases such as methane monooxygenase. First, the known oxygenases are associated with a tightly bound flavin cofactor that can be reduced by NADH or NADPH (77), while the purified DszA and C do not contain any tightly bound flavin because they have no absorption peaks in the range of 300 to 700 nm. Second, all genes for well-known monooxygenase systems including oxygenase, electron transport protein and reductase are often present as a cluster in the plasmid of microorganisms capable of degrading the aromatic compounds. In contrast, the involvement of a flavin reductase had not been predicted by the original cloning of the *dsz* gene cluster, and the gene encoding the flavin reductase was not near the *dsz* gene cluster. Third, the *dsz* oxygenases use a flavin reductase to supply free $FMNH_2$ to them. There was no evidence for a complex between the flavin reductase and dsz oxygenase such as there was for the methane monooxygenase (78). A new class of monooxygenase, which uses a flavin reductase for activity, and includes DszA and C, as well as other enzymes described earlier, is proposed to be referred to as the two-component nonheme flavin-diffusible monooxygenase family (75).

EFFECT OF HYDROCARBON ON MICROBIAL DBT DESULFURIZATION

DBT Degradation in the Presence of Hydrocarbons

Even if the microbial desulfurization reaction proceeds in the buffer solution, it is not a meaningful and practical reaction at all because it should be done in petroleum. Therefore, effects of some hydrocarbons on the DBT degradation have been examined using several microorganisms. It was reported that degradation of DBT by *Pseudomonas* sp. was favored by *n*-dodecane or hexadecanoic acid (79). The reason for the increase in the DBT degradation rate was that the *n*-alkane formed a film around DBT that was easily attacked by the microorganisms and that the fatty acid formed a kind of micelle, which helped the DBT uptake by microorganisms (80). These experiments for DBT degradation were done with growing cells and

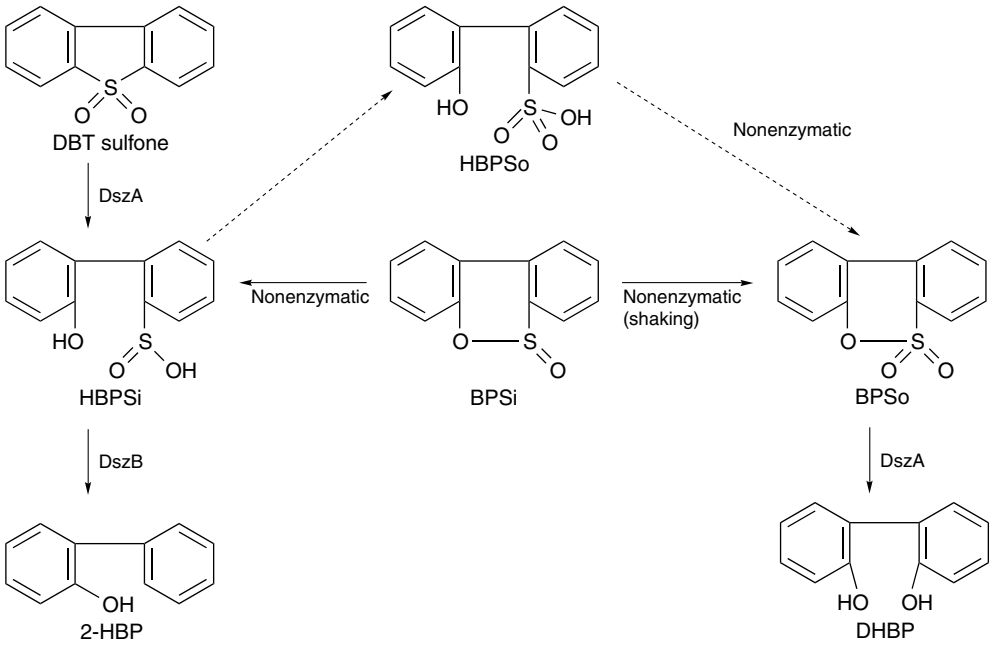

Figure 3. Proposed pathway for conversion of DBT sulfone to DHBP. Solid lines with arrowheads show the reactions verified in Reference 62; dashed lines indicate the reactions hypothesized in Reference 38.

Pseudomonas sp. strain, degraded not only DBT but also the usual aliphatic and aromatic hydrocarbons; it was not a desirable degradation. DBT was reported to be oxidized anaerobically by *D. desulfuricans* in *n*-dodecane (81) and *Desulfomicrobium escambium* in kerosene (82). However, in all the cases described here, DBT was degraded, but probably not desulfurized.

DBT Desulfurization in the Presence of Hydrocarbons

R. erythropolis H-2 with the sulfur-specific DBT desulfurization pathway utilized DBT rapidly in the presence of *n*-tetradecane and assimilated *n*-alkanes with carbon chains longer than C_8 (octane) with or without glucose (83). Because hydrocarbon was also degraded in growing cells of this strain, the reaction in resting cells was examined in the presence of hydrocarbons. Considering the practical scale of the microbial desulfurization process, a bioreactor with immobilized cells will have to be constructed; therefore, it would be essential to investigate the desulfurization by the resting cells. DBT was efficiently converted to 2-HBP by resting cells in a buffer system (33). In this strain, the addition of approximately 40% *n*-tetradecane or kerosene increased the rate of degradation of DBT (83,84). At that time, the formation of 2-HBP was observed. Moreover, the reaction in resting cells proceeded even in the presence of cyclooctane, *n*-hexane, *p*-xylene, and styrene, which at 0.5% completely inhibited the growth of this strain.

Formation of Emulsions in the Microbial Desulfurization of Petroleum

In oil-water systems such as BDS, efficient mass transfer of hydrophobic organic sulfur compounds from the oil to the water is important. Mixing is effective in creating good oil–cell water contact, and the supply of enough oxygen would enhance the reactions catalyzed by the oxygenases. However, this unfortunately leads to the formation of a tight emulsion that must be broken in order to recover the desulfurized oil and to reuse the cells. Microorganisms probably make and excrete the biosurfactant when they react with some hydrocarbon compounds. The emulsion formed by the biosurfactant was observed during desulfurization of DBT in the hydrocarbon by *R. erythropolis* H-2, and this phenomenon was reasonable for the uptake of the water-insoluble materials by the microbe. In fact, adding the surfactants, oleic diethanolamine, and Triton N101 stimulated the desulfurizing activity of *R. erythropolis* IGTS8 (85). One very effective method was the use of hydrocyclones to separate various phases (86). Passage of the emulsion through a hydrocyclone separated the clean oil from the cells and water phase.

DESULFURIZATION OF DBT DERIVATIVES OR PETROLEUM FEEDSTOCKS

Desulfurization of Substituted DBTs and Benzothiophenes

From the standpoint of the practical BDS, what should be desulfurized exists in the oil after the chemical desulfurization. Of the different classes of sulfur compounds found in petroleum, alkylated benzothiophenes, and alkylated DBTs are more resistant to HDS treatment than mercaptans and sulfides. And those with alkyl substitutions at positions four and six on the DBT ring are the most recalcitrant. *Arthrobacter* sp. assimilated 4,6-diethylDBT (4,6-DEDBT) as a sole source of sulfur (87). This strain converted 4,6-DEDBT to the sulfur-free product, 2-hydroxy-3,3′-diethylbiphenyl. This desulfurization pathway was

thought to be identical to the sulfur-specific pathway described earlier. *Rhodococcus erythropolis* H-2 desulfurized the DBT derivatives, 2,8-dimethylDBT (2,8-DMDBT), 4,6-dimethylDBT (4,6-DMDBT), and 3,4-benzoDBT by the resting cells in the presence of hydrocarbon (88). As shown in Figure 4, the initial reaction rates for 2,8-DMDBT, 4,6-DMDBT, and 3,4-benzoDBT were about 120, 60, and 20% that of DBT, and the reaction products from structurally symmetrical 2,8- and 4,6-DMDBTs were the corresponding monohydroxy dimethyl biphenyls. The reaction product from 3,4-benzoDBT was identified as α-hydroxy-β-phenylnaphthalene, indicating that the enzymes involved in the microbial desulfurization could distinguish between the two carbon-sulfur bonds of 3,4-benzoDBT. The steric hindrance caused by the naphthalene ring might lead to this specificity. Aside from *R. erythropolis* H-2, *Mycobacterium* sp. strain G3 and *R. erythropolis* KA2-5-1 were also shown to desulfurize 4,6-DMDBT (35,37).

In 1998, the benzothiophene (BT)-desulfurizing bacterium *Gordona* sp. 213E was isolated (89). This strain grew in a synthetic medium supplemented with BT as a sole source of sulfur, and 2-(2′-hydroxyphenyl)ethane 1-al, the sulfur-free compound was formed as the final product. As shown in Figure 5, the metabolic pathway of BT in this strain was analogous to that of DBT in *R. erythropolis* IGTS8 (Type 3 Metabolism in Fig. 2).

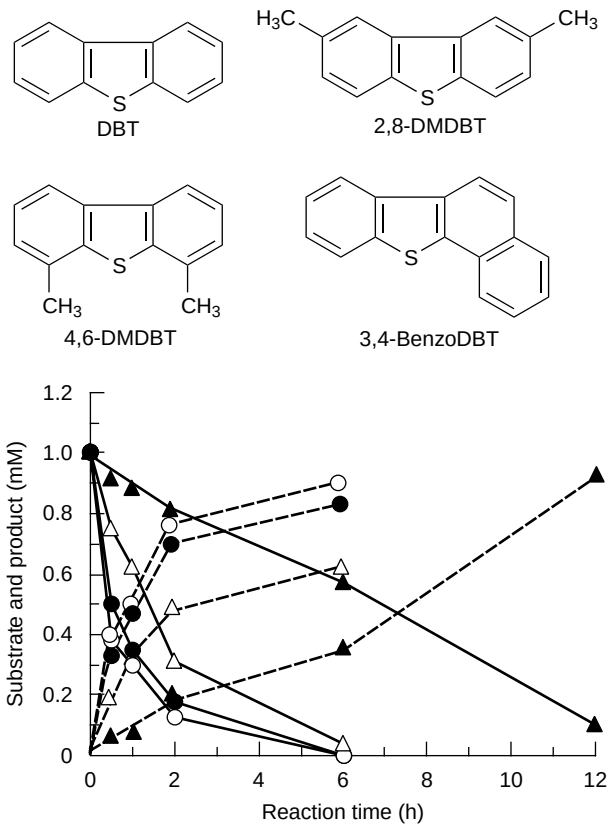

Figure 4. Desulfurization of DBT derivatives in the presence of hydrocarbon by resting cells of *R. erythropolis* H-2. DBT (closed circle), 2,8-DMDBT (open circle), 4,6-DMDBT (closed triangle), and 3,4-benzoDBT (open triangle). Solid and dashed lines indicate remaining substrates and products formed, respectively.

Another BT-desulfurizing bacterium, *Rhodococcus* sp. T09, grew with various alkylated BTs (90). Although these two strains utilized BT but not DBT, *R. erythropolis* KA2-5-1 and *Paenibacillus* sp. A11-2 isolated as DBT-desulfurizing bacteria, were also capable of utilizing BT and its analogs (37,91). However, *R. erythropolis* D-1 isolated as a DBT-desulfurizing bacterium did not utilize BT (33).

Microbial Desulfurization of Diesel Oil or Crude Oil

The possibilities for biocatalytic desulfurization have been demonstrated in diesel oil and crude oil by several researchers. Thermophilic desulfurizing bacteria *Paenibacillus* sp. strains A11-1 and A11-2 grew in the presence of light gas oil (20%) and the content of sulfur in the oil phase decreased from 800 ppm to 720 ppm after the cultivation (45). The resting cells of *Gordona* strain CYKS1 reduced the total amount of sulfur in diesel oil, the middle-distillate unit feed, from 0.15% to 0.06% after 12 hours when the diesel oil was added to the reaction mixture at 10% (92). The analysis of GC sulfur chemiluminescence demonstrated that *Rhodococcus* sp. strain ECRD-1 removed or oxidized more than two-thirds of the sulfur in a middle-distillate fraction of crude oil (93). When the crude oil was treated with *R. erythropolis* IGTS8 cells in a batch-stirred reactor, a negligible decrease in total sulfur was observed (94). However, gas chromatography-mass spectrometry analysis revealed significant desulfurization of DBT and substituted DBTs (94). This result strongly suggests that BDS should be applied to refined products such as gasoline and diesel whose predominant sulfur species are DBTs. BDS of a diesel oil by immobilized DBT-desulfurizing bacterial strains, *Gordona* sp. CYKS1 and *Nocardia* sp. CYKS2 were carried out, and the sulfur content decreased from 3,000 ppm to 2,100 ppm (95). With all the treatments described here, the sulfur content significantly decreased, but the carbon content was the same as before the treatment.

Application of Recombinant Biocatalyst to Diesel Desulfurization

DNA manipulation technology has made it possible to produce large amounts of enzyme in a desirable organism even if the original producer has little activity. Up to now, all the desulfurizing bacteria found were gram-positive. On the other hand, generally, strains belonging to *Pseudomonas* species were known to have high resistance to organic solvents. It was reported that the *dsz* genes of *R. erythropolis* IGTS8 could be expressed efficiently in two *Pseudomonas* strains and they were stably inserted into their chromosomes (96). In comparison with the wild-type *R. erythropolis* IGTS8 strain, the two recombinant biocatalysts possessed improved desulfurization ability. Moreover, the expression of the flavin reductase gene in the *Pseudomonas* strain bearing the *dszABC* gene cluster significantly enhanced the DBT desulfurization efficiency of the recombinant biocatalyst in a resting cell system (97). In addition, in *E. coli*, flavin reductase was shown to enhance the rate of DBT removal when coexpressed with the desulfurizing enzymes (98). *Rhodococcus erythropolis* I-19 containing multiple copies of *dsz* genes desulfurized

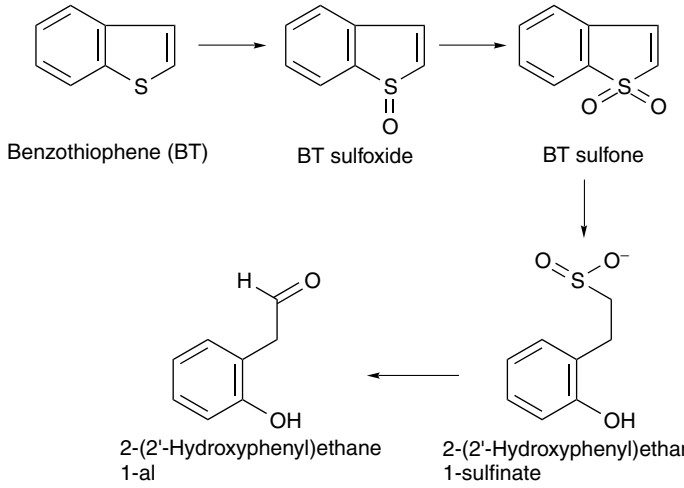

Figure 5. Benzothiophene desulfurization pathway of *Gordona* strain 213E[89].

alkylated DBTs found in a hydrodesulfurized middle-distillate petroleum. The initial desulfurization rate of this petroleum by strain I-19 was 2.5 μmol g dry cells weight^{-1} min^{-1}, more than 25-fold higher than that for wild-type bacteria (99). The recombinant *R. erythropolis* strain carrying the two *dszABC* gene clusters and one *dszD* (flavin reductase gene of the *R. erythropolis* strain with desulfurizing activity) on the vector had a DBT desulfurizing activity that was about fourfold higher than the parent strain (100). This strain also demonstrated improved desulfurizing activity for light gas oil as shown in Figure 6.

COAL DESULFURIZATION

Prior to the extensive study of microbial petroleum desulfurization, the target of fossil fuel desulfurization was coal. However, the bacteria isolated for the desulfurization did not show high levels of activity at this time (the early 1980s). The well-studied *R. erythropolis* IGTS8 was isolated a few years later. The total sulfur content of coal consists of sulfur, which is part of the coal's molecular structure (organic sulfur) and inorganic sulfidic minerals, mainly pyrite (FeS_2). Chemolithotrophic microorganisms have been used for the desulfurization of pyrite. They oxide pyritic sulfur into sulfate and ferrous ions into ferric, using the energy released by the oxidizing process for their own metabolism and growth. It was reported that the adhesion of *Thiobacillus ferrooxidans* to the pyrite surface induced the pyrite floatability by changing the pyrite's surface property from hydrophobic to hydrophilic (101) and that bacterial adhesion to pyrite occurred selectively based on the bacterial recognition of reduced iron in pyrite (102). Although research was carried out into the microbial desulfurization of organically bound sulfur in coal, the efficiency was low (103).

CONCLUSION

As described earlier, increasingly severe sulfur emission standards have forced refiners to make lower sulfur fossil fuels. Compared with the conventional chemical process,

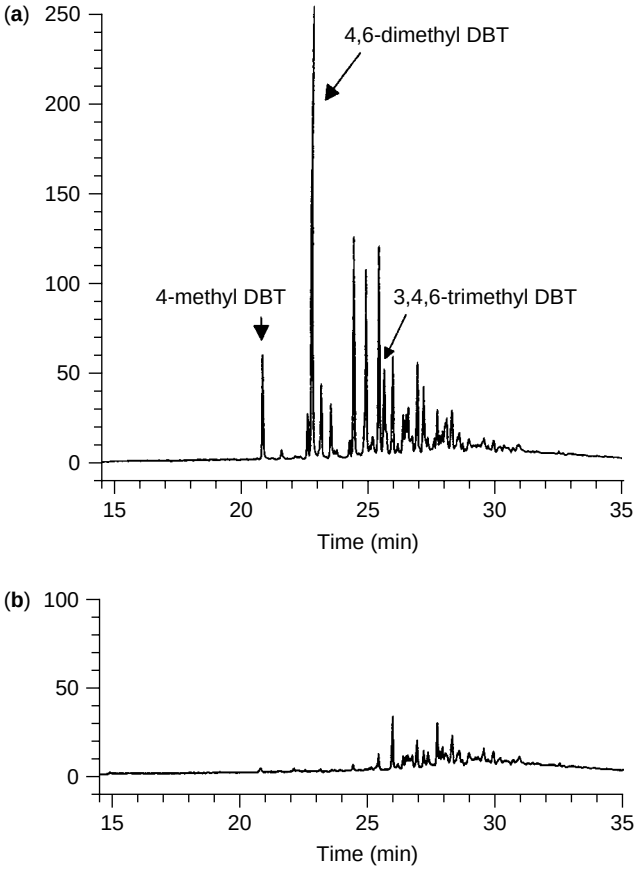

Figure 6. GC-AED sulfur detection chromatograms of light gas oil before (A) and after (B) biodesulfurization using resting cells of *R. erythropolis* KA2-5-1 harboring a plasmid with two *dszABC* clusters and one *dszD* for 23 h[100].

biocatalytic desulfurization is expected to be preferable for the purpose of the desulfurization of the most refractory sulfur-containing polyaromatic compounds because the simplicity of the biotechnology-based approach results in significantly lower capital and operating expenses. In petroleum refining, it has been postulated that BDS should be applied to desulfurize polyaromatic sulfur

compounds that remains even after the HDS treatment. The research of BDS has developed to compensate HDS process, and the promising bacterial strains including the thermophilic bacteria with the sulfur-specific pathway (type 3 metabolism in Fig. 2) were found. It has been demonstrated that these microorganisms desulfurized efficiently alkylated DBTs, which are recalcitrant to HDS. On the contrary, the drawback with biological systems is that the reaction rate is slower than for the chemical reaction. In addition, the emulsion formed during BDS will prevent the separation of oil and water. Since there are such problems to be solved yet, the genetic engineering is expected to enhance the enzymatic activity and stability for the desulfurization, and chemical engineering will bear any satisfactory separation method for the emulsion. These new technologies will make the biological refining process more attractive and cost-effective than the chemical process.

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DETECTION OF AIRBORNE MICROORGANISMS.

See Enhanced Detection of Airborne Microbial Contaminants; Bioaerosol Sampling and Analysis

DETECTION OF ENTEROVIRUSES.

See Enteroviruses in Water: Concentration and Detection

DIATOMS IN BIOFILMS

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The biofilm literature is dominated by studies on heterotrophic bacterial films. This is understandable in the medical field where biofilmed implants are a serious source of antibiotic-resistant bacterial infections, but in the environmental field, it is less easy to comprehend. Even a casual observer can appreciate that the biofilms on rocks in freshwater streams or on marine sediments in near shore areas are often brown. Further, the primary fouling layer on ships and other fabricated structures in the sea appear similarly colored. Diatoms make up the dominant biomass on all wetted and illuminated surfaces (1–3). The photosynthetic accessory pigments of diatoms are brown, which in turn lends its color to the biofilm as a whole. Figure 1 shows a typical biofilm on a stainless steel surface after exposure for 10 days in a subtropical bay. The predominance of the diatom population is obvious, but bacteria can be seen in close association with the algae. Although the interactions of bacteria and phytoplanktonic algae have been well researched (4), such cell–cell interaction in biofilms is much less well known. Diatoms are of obvious importance as attached primary producers, but they are also involved in such diverse areas as ship fouling, as indicators of chemical and physical perturbations in freshwater streams, and in the stabilization of both marine and freshwater sediments. However, generalizations are difficult to make with

Figure 1. A marine biofilm dominated by diatoms.

certainty because there are no accepted model organisms for diatom research; thus investigators have worked on a multitude of unrelated organisms. There is no organism equivalent to Escherichia coli in diatom research. Other reviews related to this subject, including information on cyanobacteria and green algae in biofilms, can be found in References 3,5–10.

SOME DIATOM MICROBIOLOGY

Cell Structure and Taxonomy

All microbiologists are familiar with the biology of bacteria, but in spite of the ubiquitous distribution of diatoms, few microbiologists are knowledgeable when it comes to this group of organisms. Therefore, some areas of diatom structure and physiology that are pertinent to their involvement in biofilms have been outlined.

Perhaps the most distinguishing feature of a diatom cell is its cell wall, which is constructed of silicon dioxide, and is highly ornate. The diatom cell wall consists of two parts that fit together like the top and bottom of a Petri dish. The two halves, or thecae, are held in place by circumferential girdle bands. In some diatoms, one or both thecae possess a slit or "raphe." This structure allows direct communication between the cell membrane and the extracellular environment. Cells vary in size from a few micrometers to several hundred micrometers. Diatoms contain chlorophylls a and c, as well as brown–yellow photosynthetic accessory pigments, which mask the green chlorophyll coloration, giving these algae their distinctive brown color. They are found in all waters in the planktonic and attached forms. In all but a few cases (e.g., *Nitzschia alba*), diatoms are photoautotrophs, but a significant number of species are facultatively heterotrophic or mixotrophic (11–13).

There are more than 10,000 species of diatoms (14). Diatoms were first noticed in the geological record in the early Jurassic deposits. They are found commonly in the upper Cretaceous period. The geological record also suggests that the radially symmetrical centric organisms gave rise to the bilaterally symmetrical pennate forms. The present classification system for diatoms is based entirely on morphology and recognizes three classes: the radially symmetrical and araphid centric forms, the araphid, bilaterally symmetrical pennate forms and the raphid, pennate organisms. This system of classification is not supported by molecular investigations based on small subunit rRNA (14). Molecular taxonomy recognizes two clades that are not related to the earlier centric–pennate split. More work with a greater selection of organisms is required before the molecular-based taxonomy can replace the traditional scheme. In any event, biofilms rarely contain organisms other than the raphid pennate kind.

Diatom Physiology as It Relates to Life in a Biofilm

Carbon Metabolism. Many phycologists will argue that the ability of diatoms to take up and metabolize small organic molecules such as glucose has little or no ecological significance. That may be true for planktonic organisms where the level of dissolved organic carbon in the water column is small and the fraction of such organisms that are not macromolecular is even smaller; however, for benthic organisms in a biofilm, facultative heterotrophy or mixotrophy may be important physiologies (13). Because the precursors of the mitochondria in eukaryotes were heterotrophic prokaryotes, it is easy to support this contention in evolutionary terms (15). It is not likely that a cell would continue to make the considerable investment in the proteins needed for heterotrophic metabolism (e.g., transporters, chemosensory receptors) if it conferred no survival advantage. Because the biofilm structure limits diffusion and mixing, it is possible that dead and lysing cells of many types within the biofilm could supply significant local concentrations of organic molecules. Self-shading and the presence of other organisms in the biofilm could reduce the available light for photosynthesis thus providing an environment where the ability to utilize already-reduced carbon would be advantageous. There is a paucity of studies on the relative contributions of organic compounds and CO_2 to the overall carbon budget of a diatom cell where both sources are available. However, Tuchman (13) reports that in a mixed species biofilm grown in an outdoor artificial stream system under low (200 μmoles/m^2/sec^1) and high light regimes (200 μmoles/m^2/sec^1), the glucose assimilated by cells at the bottom of the biofilm (under story cells) was threefold higher in relation to the total CO_2 uptake than cells at the top of the biofilm (canopy cells). The light levels impinging on the under story cells was about 85% less than the canopy cells at either light level.

Motility, Adhesion, and Chemosensing. Apart from the generation of energy and anabolic reactions in general, there are three physiological processes that are closely associated with biofilm formation in diatoms. These are adhesion processes, motility, and chemosensing.

Diatoms reach a surface by hydrodynamic means or gravitational forces (16). When they reach a surface, one of two things happens: they either stay or they are removed before they have a chance to become attached. At this point in time, there is no indication that leaving the surface is any more than a stochastic event. However, shortly after their arrival, diatom behavior is active (17), but Wang and his associates (18) believe that the events

immediately after their arrival do not require active synthetic events. The distinctions here could arise from the use of different experimental organisms by the two groups of investigators. As mentioned earlier, this is an example of a situation that makes generalizations difficult. The initial observable event after arrival on a surface is the secretion of an adhesive from the raphe slit. When the diatom falls on that part of its cell surface that bears a raphe, motility ensues immediately. When it does not fall onto a raphe-bearing surface, the diatom can be observed to turn so that a raphe is in contact with the substratum. Wetherbee and associates (3) have proposed that in the diatom Stauroneis decipiens, the strands of the secreted polymer "search" for a substratum, anchor there, and then by some contractile mechanism, move the cell onto its raphe-bearing surface. This is a far more animal-like sensory behavior than has been proposed for diatoms previously, but the fact remains that this process can be seen microscopically. Acceptance of the hypothesis requires that in this organism at least, there is a substratum-sensing mechanism in which the extracellular polymers act as transducers of substratum proximity. After moving over the substratum for some time, eventually, the diatoms secrete further polymers with different properties, and the cell is no longer motile. At this time, the cell becomes enveloped in a layer of a matrix polymer extracellular mucilage(ECM). In some species, this secondary adhesive is secreted in the form of a stalk that elevates the cell above the substratum (18,19).

The initial processes in adhesion and motility are difficult to separate and those leading to the formation of a mature biofilm are complicated. Only recently has some insight been gained at the molecular level; even so, our understanding is far from complete. The polymer responsible for the initial adhesion is synthesized in the Golgi apparatus and packaged into vesicles (20). These vesicles are transported to the raphe area of the cell where they fuse with the plasma membrane, discharging their contents into the raphe canal where they are able to interact with the substratum (for electron micrographs of vesicle fusion, see 21,22). As detected by measurements of the ability of cells to remain adhered after washing of the substratum, the adhesion process can be metabolically disrupted by inhibitors of microtubule assembly, action polymerization, transmembrane Ca^{++} flux, respiratory energy generation, and protein and glycoprotein synthesis but not by photo system-II inhibitors or darkness (3,7,21,23). Furthermore, the Ca^{++} chelator ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*,*N*′,*N*′,-tetraacetic acid (EGTA) removes the diatom *Amphora coffeaeformis* from glass, leaving behind an exact imprint of the raphes, which in this organism, are both on the ventral surface of the cell. These imprints have been referred to as footpads (23). In similar work with bacteria by Neu and Marshall (24), the term *footprints* has been used. In either case, these structures represent a cohesive break in the external polymeric adhesive induced by calcium-complexing agents. This indicates that there is an external role for Ca^{++}, most likely as a bridging agent in the ECM, as well as an internal one (23). It is significant that in the ten species examined, an external concentration of several millimolar Ca^{++} is required for successful adhesion and motility, although cellular growth needs less than 1 mM Ca^{++}. At this lower concentration, cells grow planktonically and apart from being nonmotile or adhesive, appear otherwise quite normal. Wetherbee and associates (3) have stated that diatoms may be able to detach from a surface if environmental conditions deteriorate, that is, bioadhesion in diatoms is reversible, but the mechanism is not known. In support of this, it has been observed that in axenic cultures of many diatoms in media with a high Ca^{++} concentration, cells in the stationary phase of growth are easy to detach from the surface of a glass flask, whereas in the log phase, this is not possible unless mechanical force or ultrasound is used. Detachment of the cells breaks cell-substratum bonds, but cell–cell bonds are disrupted less frequently. Thus, the detachment in this manner results in rafts of cells being produced, not a single cell suspension. The relationship between the two classes of adhesives, that is, the one involved in initial adhesion and subsequent motility and the other that becomes the enveloping ECM is unknown for most diatoms. In general, it is not even known whether these polymers are single molecular entities or collections of similar compounds, or what structural motifs explain their particular biological function(s). Some progress toward these ends has, however, been made.

Extracellular Carbohydrate-Like Polymers. A comprehensive review of the extracellular polymers of both planktonic and sessile diatoms has been prepared by Hoagland and associates (19). The review has particularly fine quality scanning electron micrographs of the extrusion of polymers from the raphe and terminal pores of the diatom frustules. In the light of modern analytical capabilities, however, the large collection of polymer chemical analyses (more than 50) is less useful. Polymers were prepared as extracts of cells or from media from which they were precipitated or lyophilized. Thus, in no case were the polymers shown to be a single molecular entity. Nevertheless, the list of the sugars participating in the potentially mixed polymer structures is rather constant. Most of them contain the sugars glucose, xylose, galactose, mannose, rhamnose, fucose, as well as glucuronic and mannuronic acids. The contentions of Allison, Sutherland, Neu and Marshall (24,25) are well worth reiterating at this juncture. They believe that the effort expended in studying extracellular polymers precipitated from media in which cells have grown may be misplaced. Their idea is that the extracellular polymers important in biofilm structure are not necessarily found in the medium. Neu and Marshall (24) consider that the "footprints" of extracellular polymer that are found underneath cells when they have been removed, either physically or chemically, are more worthy of study in the context of biofilm formation and structure. Although these workers were in fact studying bacteria, the same is probably valid for the diatoms.

The work carried on from the laboratories of Gretz, Hoagland, and Wetherbee (3,18,19,26,28,29) has gone a long way inremedying the insufficiencies mentioned by Allison, Marshall, Neu and Sutherland. Extracellular adhesives from the diatoms *Achnanthes longipes*, *Amphora coffeaformis*, *Cymbella cistula*, and *C. mexicana*

have been characterized by monosaccharide and methylation analysis, as well as lectin binding and cytochemical staining (26). Not surprisingly, polysaccharide was the main component of the adhesives formed during motility of these organisms and basal pad formation in *A. longipipes*. Components of the ECM insoluble in hot water, but soluble in $NaHCO_3$ solution, were anionic polysaccharides. For *Amphora*, these consisted mainly of galactosyl and fucosyl residues with similar sugars and the addition of glucuronyl residues in *Achnanthes*. On the other hand, adhesive polysaccharides from *Cymbella* were soluble in EDTA, contained galactosyl and xylosy residues, but lacked uronosyl residues. Fluorescently labeled sugar-specific lectin binding showed that the fucose was located in the motility polymer of both *Cymbella* and *Achnanthes*, and the adhesive pads of *Achnanthes*. Galactose was located in the capsules [ECM] of *Amphora* and throughout the shafts of the stalks of *Cymbella*. The herbicide 2,6-dichlorobenzonitrile [DCB], similar to the way in which it inhibits cellulose synthesis in higher plants and algae (27), inhibits ECM production in adhesive diatoms (26). *Achnanthes longipes* inhibited by DCB are not motile nor can they adhere, which supports the idea that motility and adhesion are metabolically linked. A similar conclusion was reached by Cooksey and Cooksey (23) from results obtained with the glycoprotein-specific inhibitor tunicamycin and the diatom *Amphora coffeaeformis*. Using yet another organism (*Stauroneis decipiens*), Lind and associates (28) were able to show that two monoclonal antibodies raised to what they named *frustule-associated components* [FACs] were able to label various extracellular parts of the diatom cell. The FACs were shown to contain four proteoglycans by staining after separation on polyacrylamide gels. One antibody bound strongly only to the exterior frustular component but not material in the raphe area, and did not inhibit motility or adhesion. On the other hand, the other antibody that bound to the frustular component, ECM trails and material in the raphe, did inhibit motility and adhesion. This work offers further evidence for the metabolic integration of motility and adhesion.

There is further work from the Gretz–Wetherbee laboratories (26,29) on the extracellular polymers that are morphologically distinct in *Achnanthes longipes*, that is, the adhesive pad, the anchoring stalk, and its collar. Chromatographic analyses of the hot water–insoluble–hot sodium bicarbonate–soluble fractions (WBS fractions) of these structures reveals that they are also chemically unique. They could also be distinguished in terms of their sugar components, amino acid content, and degree of sulfate. From their analyses, Wustman and associates (29) concluded that the outer layers of the diatom anchoring stalk were extruded from the pores in the silica cell wall adjacent to the raphe canal and contained low sulfated components of the WBS, whereas the central core of the shaft was probably secreted from the raphe canal itself. It is significant that there is chemical similarity between the central core material and the cell motility polymer. Both reach the extracellular space via the raphe canal.

McConville and associates (30) recognized another environment where biofilm formation by diatoms was visually obvious. There are extensive diatom blooms along the undersurface of the nearshore Antarctic sea ice during the austral spring. Although this environment has been investigated extensively, the extent to which the bloom is actually attached to the ice crystals as a biofilm is not known. Nevertheless, the diatom film contains a large component of extracellular polysaccharide-like materials. McConville and associates (30) investigated the carbohydrates components of *Stauroneis amphioxys* Gregory — a diatom found in the Antarctic sea ice. The carbohydrates occur as soluble EPS in the culture supernatant, as an ECM and as a part of a distinct organic layer underlying the diatom cell wall (diatotepum). During logarithmic growth, the mucilage EPS rose but fell during the stationary phase. It has often been suggested that the EPS of diatoms may contain some protein, but this has never been shown conclusively. These persons demonstrated chromatographically that the soluble EPS fraction contained about 5% protein. Staats and associates (31) also found protein in diatom EPS.

Although the molecular constituents are only currently beginning to be identified, we can make some partially substantiated efforts to explain how diatom motility operates. Figure 2 shows a schematic of a longitudinal section of a raphid pennate diatom with a series of actin filaments running parallel to the raphe. The sketch is based on work by Webster and associates (21) and Edgar (22,32). The secreted polymer molecules are anchored in the plasma membrane, either by transmembrane proteins or directly. The force generated by contractile events in the actin fibers could be transmitted via the membrane to the extracellular matrix molecules (ECM), which in turn are anchored to the substratum (at * in the figure), thus moving the cell in the opposite direction. The problem with this model is that it explains how the cell could move for only one cell length. For continuous movement, the connection of the ECM to the cell membrane and/or the substratum would have to be broken. Edgar (32) and Edgar and Pickett-Heaps (22) state that diatom trails, that is, the material left behind a cell when it is moving on a surface, are easily soluble in the aqueous milieu, and

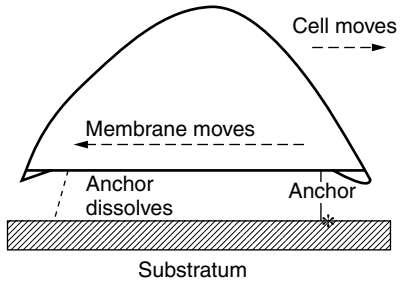

Figure 2. Representation of the interaction of a species of the diatom Amphora with a substratum leading to adhesion and subsequent motility. The cell is seen in longitudinal section. The polymer strand is anchored in the cell membrane and interacts with the surface at the place marked with an *. When, by membrane-associated activity, the strand of polymer reaches the posterior of the cell, the interaction with the membrane dissociates and the cell moves onward. For clarity, only one polymer molecule is drawn [see text for details].

that there are no trail substances evident behind diatom cells, or that the trail substance disperses rapidly. These observations suggest that the trail must detach from the membrane-associated site before it becomes soluble. Such a detachment mechanism at the posterior of the cell would allow continuous movement. However, once again, generalizations are difficult because rarely have two groups of investigators studied the same organism.

A class of Ca^{++}-binding proteins known as *frustulins* have been isolated from the cell walls of several diatoms (33,34). Initially these proteins were thought to be involved in setting the architecture of the silica cell walls. That view is no longer held (35). An alternate suggestion for their role that is in keeping with their distribution within the diatom cell that is cell wall–associated has been proposed (9). Frustulins could be the actin-associated proteins referred to by Wetherbee (3), and these molecules anchor the membrane to the cell wall. If there were no such anchors, the membrane would tend to move within the cell walls instead of transmitting the motive force to the substratum. The precedent for this idea is found in research on the slime mold *Dictyostelium* (36) where a transmembrane protein called ponticulin (for its bridging property) anchors the cytoskeleton to the inside of the cell. If the formation of such anchors is inhibited, it is likely that normal motility would also be prevented. At high concentrations, tunicamycin can inhibit diatom motility completely. At low tunicamycin concentrations, motility becomes aberrant but does not totally cease. Under these conditions, cells can perform only a shunting motion that is more than about one-cell length and are thus unable to make a complete change in position.

There are other aspects of diatom motility that are important in biofilm formation and activity. It has been shown that not only are diatoms motile, but they are capable of chemosensory motile responses. Using a diffusion chamber (37) to allow microscopy of cells in a chemical concentration gradient, Cooksey and Cooksey (38) showed that *Amphora coffeaeformis* and another species of Amphora were positively chemotactic in gradients of D-glucose, 3-*O*-methyl-D-glucose, D-glucoheptose, and D-maltose. They were negatively tactic to D-mannose and did not respond to 2-deoxy-D-glucose. Response to mannose is particularly interesting because at 2 mM, mannose is toxic to species of *Amphora* (12). Thus it seems that diatoms can avoid areas where their metabolism would be impacted negatively. By measuring the response of cells that had been pretreated with sugars and then measuring the reposes of the same cells to the same and other sugars, it was possible to postulate that Amphorae possess separate receptors for glucose and mannose, that 3-*O*-methyl-D-glucose and glucose share the same receptor, and that in this scenario, a sugar is not chemotactically active unless it possesses a hydroxyl group at position 2 of the pyranose ring. In these experiments, diatom cells suspended in minimal medium (39) were allowed to settle out and adhere to a glass surface. The result was a uniform, spatial distribution of cells on the surface. In the absence of an added chemical gradient, cells moved randomly, that is, cell–cell interactions were not detected. When cells were placed on a microscope slide so that high local concentration of cells existed (i.e., clumps), their motility was not random. Species of *Amphora*, *Navicula*, *Nitzschia*, and *Auricula* show dispersal from a clump of cells in a directed fashion (40). Wang and associates (18) have also commented on the ability of cells of *Achnanthes* to distance themselves from one another, but they did not quantify the process. Measurement of this phenomenon requires the use of a dynamic image analyzer to track cells and report the relative direction (i.e., compass bearing) of their progress from the clump. Dispersal takes place only until the cells are about 400 μm from the clump, at which point the cells resume random behavior again. This suggests that the clump is the origin of the putative signal. At this time, there is no substantiated information concerning the identity of the putative dispersal factor, but O_2, which is a photosynthetic product that causes photorespiration, has been suggested (40). Photorespiration does not result in net carbon fixation and is thus not a positive metabolic activity. It could be speculated, therefore, that environments that promote this activity should be avoided.

Both types of chemosensory behavior mentioned earlier are important in understanding how a substratum becomes colonized and a biofilm is formed. Related to this is some work by Paul reported in Reference 41. This group showed that coupons of glass and stainless steel placed in Biscayne Bay FL became colonized rapidly with diatoms. After the initial colonization period, increases in cell number took place only during the day. Because it is known that cells can adhere to surfaces in the absence of light (39), this implies that cell number increase was due to growth rather than to continued colonization from the water column. It seems that directed dispersal and growth are highly influential in the formation of a climax diatom biofilm.

Influence of Surface Energy of the Substratum on Diatom Behavior. The concept that surface energy of a substratum influences the adhesion of microorganisms, especially bacteria, to surfaces has been studied extensively (5,8,39, and 42). However, it is difficult to arrive at generalizations, although a wide variety of organisms and surfaces have been investigated. Problems arise from the fact that the physiological states of the organisms and/or the surface characteristics of the substrata were seldom well controlled. In some cases, surface chemistry was changed to derive differing surface energies on a single substratum such as glass. Although this approach produces informative data, surfaces differing supposedly only in the value of their surface energy actually differ in two ways: the intended variable, that is surface energy and the chemistry that generated it. An example will make this clear. It has been shown that the percentage of a population of the diatom *Amphora coffeaeformis* adhered to chemically modified glass surfaces in a nonlinear manner with respect to surface energy. In fact, the relationship was U-shaped with a minimum value for adhesion at about 20–25 dynes cm^{-1}. In this experiment, the cells were exposed to surfaces bearing the following, quite different, functional groups: methyl, propyl and a (fluorinated isopropoxy) propyl, as well as the physical

chemistry derived from them (5). Thus, a cell could react to either the functional group presented to it, the wettability of the surface (dictated by its physical chemistry) or both of these parameters. To overcome this problem of dual variables, Elwing and associates (43) and more recently Ruardy and group (review, 44) have described the preparation of silicious surfaces bearing spatial gradients of wettability. These gradient surfaces (about 10–40 mm in length) are hydrophilic at one end, hydrophobic at the other, with a nonlinear wettability gradient between the extremes. The use of these gradients allows only one variable at a time to be examined, in this case wettability (measured as water contact angle of the surface). This is because only the concentration of the derivatizing agent is varied, not the type of reagent. Chemical concentration gradients of the derivatizing reagent are prepared and allowed to react with, for instance, a glass plate. The presence of the gradient can be demonstrated with X-ray photoelectron spectroscopy or contact angle measurements. Using this technique, the effects of a methyl-functionalized wettability gradient on the adhesion and motility of *Amphora coffeaeformis* have been investigated (39). Figure 3 shows a steep wettability gradient on glass formed from methyl groups derived from dichlorodimethyl silane. Figure 4, curve 1, shows that response of the organism in terms of percentage motility of the population is dependent on the relative position of the cells along the gradient. By comparing Figures 3 and 4, it can be seen that cells were not motile above a substratum water contact angle of 40°. In fact, at contact angles above this value, cells actively detached. This cellular detachment can be explained in terms of extracellular interaction with the substratum as depicted in Figure 2. If the polymer strands interact weakly with the substratum, motile forces applied to the substratum from the cell membrane–associated system (the cellular "motor") via the polymer bridge will tend to cause the cell to detach from the surface, rather than cause the cell to move. Figure 4 illustrates another potential problem in measuring cellular response to substratum properties. Curves 1 and 2 were made 30 minutes apart. It seems that during the longer incubation time, the cells have made the surface more conducive to cellular motility. In other words, they may have "conditioned" the surface. This requires the secretion by the organisms of an amphipathic molecule(s) that could adsorb to the hydrophobic part of the surface and change its properties so that it becomes more hydrophilic. Furthermore, it is evident that the hydrophilic part of the gradient surface, according to this analysis, was not changed. Studies on diatom adhesion as a function of time are sometimes made in flow cells. If the ability to change the properties of the substratum with time is a general property of diatoms, conclusions derived from such studies warrant further examination. The final properties of the substratum will not be identical to those when the experiment was initiated. Although it is clear that this putative conditioning film was self-generated in situ, it is still not known from where the molecules involved in the formation of marine environmental films originate, nor is there general agreement of their role in the subsequent attachment of diatoms and other marine organisms. Discussion of the formation of conditioning films can be found in Baty and associates (45) and Characklis and Cooksey (5) and references therein, and Schneider and Leis (this volume).

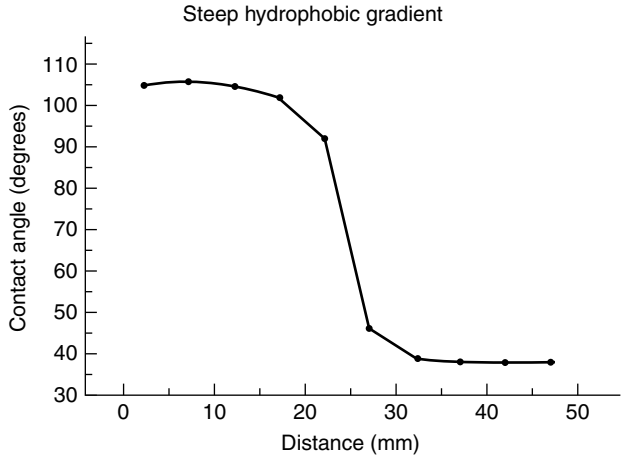

Figure 3. Wettability gradient on a glass surface represented by the advancing water contact angle. The hydrophobic side is at the left.

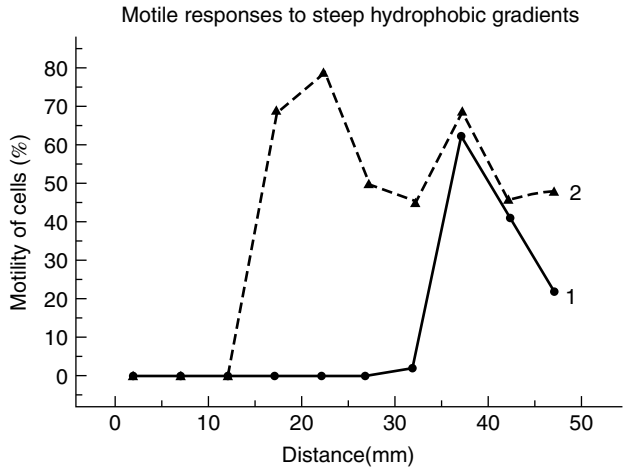

Figure 4. Motility of the diatom *Amphora coffeaeformis* on the surface depicted in Figure 3. Note that motility is not possible at water contact angles greater than 40°. Data for curves 1 and 2 were obtained 30 min apart on identical, but separate surfaces.

Cell-Cell Interactions in Biofilms. It is likely that the interactions of cells in a biofilm are mostly a consequence of their proximity to one another. In a planktonic environment, cells are many micrometers or more apart, whereas in a biofilm they are either in physical contact or only a few micrometers separates them. Furthermore, because biofilm cells are embedded in a polymer matrix, diffusion of potentially allelochemical factors is comparatively restricted. The interactions of diatoms and bacteria in the water column is well researched (e.g., 46), but there is less information on the interactions between these two types of organisms in biofilms. The planktonic environment where these interactions take place is known as the *phycosphere* (4). This term is not usually applied

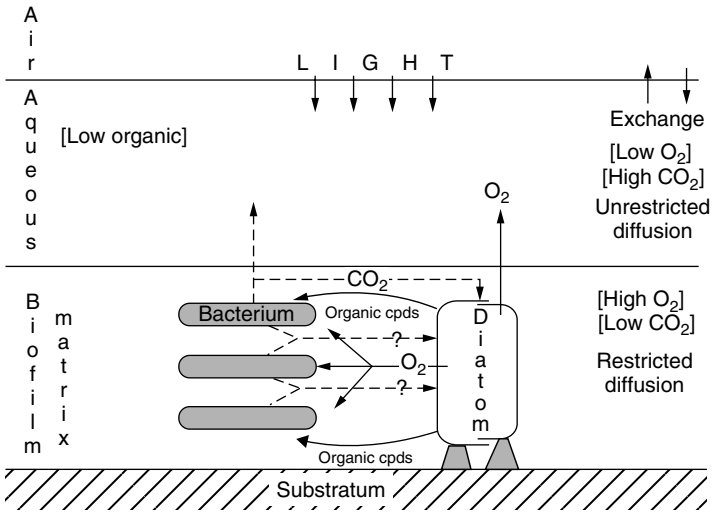

Figure 5. Potential metabolic interactions within a biofilm consisting of phototrophs (diatoms) and heterotrophs (bacteria). The possibilities of interactions at the genetic and signaling levels are not represented.

to the biofilm situation. Interactions can be mutualistic, syntrophic or toxic. Toxic interactions involving bacteria that are algicidal (47) have been described only for the water column so far. In biofilms that are illuminated, the interactions can influence either of the types of cells in the biofilm. Figure 5 shows some possible types of interaction. The secretion of organic molecules by the diatoms and their metabolism by the bacteria is an obvious interaction. An example would be glycolic acid, which is a product of photorespiratory activity of phototrophs. Because diffusion in a biofilm is reduced (48), the photosynthetic product (O_2) that stimulates photorespiration could increase in concentration, thus promoting glycolate production. On the other hand, the presence of heterotrophic bacteria that utilize O_2 as an electron acceptor would counteract this process. There is a possibility that molecules produced by one type of organism influence the metabolism of another by means other than anabolic or metabolic pathways. For instance, cell–cell signaling between bacteria in close association is known as quorum sensing (49, see also Jaeger and Wingender, this volume). Unless the so-called diatom autodispersal factor is counted, there is no such interaction documented between diatoms or between diatoms and bacteria; however, the possibility exists that such interactions can occur, particularly at the genetic level. It is reasonable to speculate that some diatom product could influence the induction of genes in bacteria that are closely associated with diatoms or vice versa. There is some evidence that there is interaction at the metabolic level. Hack and McFeters (50,51) showed that the metabolism of the bacterial component of an alpine stream biofilm that also contained diatoms was closely associated with the metabolic state of the diatoms. The experimental protocol used involved harvesting and homogenization of the attached film. This method was shown by Murray and associates (52) to cause algal cellular disruption and thus the release of intracellular components not normally available to the bacteria. A gentler method favored by Murray and associates (53) was the in situ labeling of the film by ^{3}H-thymidine. A bacterial-diatom film was constructed from *Amphora coffeaeformis* and *Vibrio proteolytica*. The film was incubated with the radioactive substrate and it was shown by autoradiography that the label was present only in the bacteria. Kinetic studies in the absence of an added organic carbon source revealed that the incorporation of ^{3}H-thymidine into the bacteria was light dependent, that is, the metabolism of the bacteria was tied to that of the diatoms. Less easily understood are the observations of Woods and Fletcher (54). On the basis of observations of sloughing from ship hulls, where presumably biofilms would also contain bacteria, axenic films of *Amphora coffeaeformis* var. *perpusilla* sloughed more easily than expected when exposed to a hydrodynamic shear. This observation could be explained in several ways. One possibility is that the axenic culture of diatoms responded to high O_2 levels that were not reduced in the absence of bacteria. The results of Jensen and Revsbech (1989), who found it difficult to form thick diatom films when the bacterial content of the culture was low, would support this idea. It is also possible that a synergistic relationship exists such that bacterial polymers and those from diatoms produce a binary adhesive, the adhesive properties of which are greater than either of the single polymers. Another possibility is that different and more adhesive–cohesive polymers are produced by either member of the consortium in the presence of the other.

Gawne and associates (55) measured the attachment of the fouling diatom *Achnanthes longipes* to various substrata as a function of the presence of several marine bacteria and their extracellular products. The diatom attached best to polystyrene (hydrophobic), but its attachment was inhibited by the presence of bacteria on the surface. Attachment to glass and a silicone elatomer (annealed and unannealed RTV 3140) was either facilitated by the marine bacterium *Pseudoalteromonas atlantica* (glass substratum) or the presence of the bacterium had no effect (RTV 3140). In other experiments, no differences were found in the adhesion of *Achnanthes* when the substrata were covered with either living bacterial cells or the extracellular polymer isolated from them. There were no conclusions to be made concerning the effects of bacteria in general. The authors made the point that the formation of the initial fouling film is highly

complex and species specific. In these experiments, one diatom was used in conjunction with five species of marine bacteria. It is likely that the picture would have been even more complex if further diatom cultures were included. Gawne and coworkers' results can be used to pose an important question concerning diatom adhesion studies: Is it more realistic to work with axenic cultures of diatoms in studies of adhesion, or is it more realistic to use natural consortia of bacteria and diatoms? Reports such as that from Velraeds and associates (56) make a valid assessment of this point even more difficult. Velraeds and coworkers showed that the initial adhesion of one bacterial culture was strongly influenced by a molecule produced by another type of bacterium. The molecule, which was proteinaceous and had the physicochemical properties of a surfactant, adsorbed to (i.e., "conditioned") the test substratum. It is certainly possible that this type of effect could have been operating in the experiments of Gawne and coworkers (55).

Fukami and associates (47) measured the influence of marine bacteria on the growth of various phytoplanktic organisms. Their main interest was in the production of the algae as food for shell fish. The growth of planktonic cultures of *Chaetoceros ceratosporum* as well as biofilms of a *Nitzschia* sp. were stimulated by indigenous marine bacteria, including species of *Alcaligenes*. On the other hand (47), growth of the dinoflagellate *Gymnodinium mikimotoi* was inhibited by a species of Flavobacterium. These differing results show the variation in the effects of bacteria on algal growth.

A potential means to investigate interactions leading to the formation of climax biofilms can be found in publications from the Canadian National Water Research Institute (57–59), where a rotating annular bioreactor was used to produce mixed species biofilms that mimic those found in nature. This type of reactor has an internal cylinder that rotates inside a second outer cylinder. The annular space between the two cylinders (6 mm) is where a biofilm is allowed to develop. The film grows predominantly on the outer wall where removable slides allow samples to be taken aseptically without disturbing the remaining biofilm. The Canadian group has used this system in conjunction with staining by conjugated lectins specific for sugars in the ECM, as well as confocal laser scanning microsopy and the measurement of other cellular parameters, to follow the development of the biofilm. Their results demonstrated that the spatial distributions and biomass of live and dead bacteria, algae and types of ECM in the biofilm were not uniform. They did not differentiate between diatom and bacterially produced ECM, but this should be possible in future work.

Given the importance of cell–cell interaction in biofilms, it is surprising that this area is not better developed; however, the discussion earlier documents the difficulty in separating the various interactions between cells.

Some Practical Considerations of Biofilms Dominated by Diatoms

Fouling. Biofouling is the accumulation of unwanted organisms and their products on surfaces of artificial objects in the aquatic environment (Flemming, this volume). It takes place in both marine and freshwater environments, although marine biofouling is more extensively studied because commercial and naval interests are predominantly marine. Biofouling refers to the accumulation of many types of organisms besides diatoms. There are reviews of the involvement of diatoms in fouling biofilms on shipping (2,5,10,60–65).

The interest in marine biofilms on synthetic surfaces, especially those of ship hulls, is related to the increase in hydrodynamic drag that these films cause compared to clean hulls. For hundreds of years it has been appreciated that fouling layers consisting of macrofoauna (largely barnacles) increase the drag on a vessel as it moves through the water, but it is only in the last 20 years or so that there has been an appreciation of the drag caused by microbes in biofilms. This is because of the fact that the tributyl tin self-polishing copolymer coatings reduced faunal fouling to the point when it was no longer a severe problem, allowing the effects of biofilm fouling — the so-called slime layer — to be more apparent. Lewthwaite and associates (66) quantified the drag caused by slime alone on a ship's hull. They found that a 1-mm thick layer caused an increase in skin friction of 80% and a reduction in speed of 15% when compared to a fouling free hull. Bohlander (64) made similar measurements on the USS Brewton, a U.S. Navy frigate, the hull of which was painted with a copper oxide–tributyl tin ablative coating. The hull showed only minor barnacle encrustation after 22 months in service, but there was a continuous biofilm over the length of the hull. In such circumstances, the biomass in the biofilm will be largely made up of diatoms. Two ship trials were made with the USS Brewton, one before and the other after hull cleaning with an automatic scrubber that removed only the biofilm. The percentage reduction in shaft horsepower for a given ship speed was 8–18% after cleaning. The engine RPM for a given speed was also reduced and the maximum attainable speed was increased by 1 knot. In terms of reduced fuel costs, it took only 20 hrs at 20 knots to repay the cost of cleaning the hull. These two papers, perhaps more than any others, emphasize the need for effective ship hull coatings that are designed to ameliorate the effects of microbial biofilms.

The so-called "fouling release" coatings must take into account the ability of the fouling diatoms to resist removal by hydrodynamic shear stress (54). There are only very few diatoms species that are common on synthetic submerged surfaces. The marine environment, on coatings consisting of self-polishing copolymers, harbor species of *Amphora*, *Amphiprora*, *Stauroneis*, *Achnanthes*, *Navicula*, and *Nitzschia*, with *Amphora* being by far the most common (65). Callow (65) made the important observation that the organisms she found on ship hulls were similar to those found on static test panels placed at various locations around the world. Species of *Amphora* are resistant to copper in antifouling coatings (65,67,68) which probably accounts for their being found frequently on the surfaces of copper-based paints. *Amphora* are able to immobilize copper in cytoplasmic vesicles thus reducing the toxicity of the metal (67), but other mechanisms are also likely (68,69,70 and 71). Some diatoms, notably *Amphora coffeaeformis*, *Achnanthes longipes*, and *Achnanthes subsessilis* are also resistant

to organotins; however, Callow (72) believes that the differences seen in the species composition of diatom slimes on organotin coatings cannot be explained solely in terms of current knowledge of relative resistances to these toxicants.

Current antifouling research and technology have moved toward fouling release coatings and away from toxicant-based materials. The influence of low wettability on the adhesion of diatoms and other organisms to surfaces is now being exploited in the design and manufacture of such antifouling coatings. All major manufacturers of marine bottom paints sell coatings based on this principle. In our opinion, information on the interaction of the adhesives produced by one organism on those fabricated by another in close proximity is needed for the design of these coatings. Because diatoms are major components of marine fouling films, they are used to assess the activity of potential components of antifouling paints (73–77).

Sediment Stabilization by Marine Diatoms. This section deals only with those aspects of the sediment biosphere that inhibit the ability of sediment particles to gain momentum and thus move. The role of microorganisms in the stabilization of sediments is not well defined. However, many of the papers dealing with sediment stabilization (SS) concern the involvement of benthic diatoms in the process. Holland and associates (78) presented the first quantitative information on the involvement of benthic diatoms in SS. They showed that the growth of several species of diatoms, as well as mixed natural populations, inhibited the movement of sediments in stirred flasks and that the effectiveness of the organisms was related to their production of extracellular mucilage. De Jonge (79) found that 13% of sand grains (>55 μm in size) taken from the Eems-Dollard estuary in the Netherlands were colonized by diatoms, but 80% of these were on the mud coating the sand grains, not on the sand grains themselves. Although this finding stresses the importance of fine particles in colonization studies, it offers no explanation why this should be so.

Grant and associates (80) described the microstructure of benthic microbial films and the means by which they influence SS. These workers point out that although there is a large body of literature concerning the means by which the mucus films produced by diatoms can inhibit sediment transport, there is little known about the mechanisms concerned (80 and references therein). Their results showed that field biomass indicators are poorly correlated with SS. SS by diatoms is related to the production of colloidal carbohydrate, which they conclude is an index of the extracellular carbohydrates produced by diatoms. Unfortunately, this view and their analyses not only underestimate diatom polymer production but also include polymers produced by other organisms, especially bacteria. Their results showed that visual estimates of the presence of organisms do not concur with sedimentary analyses. High levels of colloidal carbohydrate appear to be a better indicator of the presence of diatoms than chlorophyll. The lack of a relationship between chlorophyll and a metabolic product such as extracellular carbohydrate is not surprising because although chlorophyll levels are constant in diatoms, the products of metabolism are not. For instance, the amount and type of intracellular storage products are dependent on nutritional status. The SEM studies reported (80) here show that sand grains were colonized by many types of organism, but only the putative role of diatoms was examined. Other studies (81,82) showed the presence of mucoid materials in sediments, but their origin could not be determined microscopically. It should be pointed out that the polymers that are produced by diatoms as a consequence of their motility are soluble in seawater (21,22); so the ability of these polymers to aggregate sand particles is difficult to understand. Threadlike polymer bridges seen in SEMs are almost certainly artifacts of the preparative techniques. Madsen and associates (83) concluded that it is doubtful whether any reasonable specific quantitative information predictions about sediment stability can be made without extensive knowledge about several factors including the physiological status of the organisms. Their conclusions were based on experiments in a linear flume with natural sediments. In contrast to Grant and associates (80), they found that the only parameter which correlated with SS was the presence of epipelic diatoms sampled by exposing lens tissue to the surface of the sediment. The use of this technique has been criticized strongly by Admiraal (84). The presence of extracellular polymers in the sediment did not correlate well with their stability, although there was a qualitative relationship. It is possible indeed that there was a correlation, but it was sufficiently obscured by uncontrolled parameters in the experiment that it became statistically insignificant. Guarini and associates (85) made a multiparameter study in which they followed mud surface temperature (MST) and correlated it with microphytobenthic productivity over tidal, solar, and seasonal cycles. The fact that the populations showed midsummer photoinhibition coupled with the highest MSTs because of low tides occurring near midday indicated that there was little acclimation of this population to environmental conditions. Guarini and associates (85) emphasize that these physiological dynamics will also control sedimentary dynamics. Lack of appreciation of the involvement of variable levels of photoinhibition may explain some of the less-than-perfect correlations between the size of microphytobenthic populations(e.g., chlorophyll a) and SS seen by others. For instance, Madsen and associates (83) noted that weak correlations were obtained when the sediments contained diatoms of the genera *Amphora*, *Navicula*, and *Nitzschia* (20–40 μm).

A laboratory study by Sutherland and associates (86) using a nonaxenic culture of *Nitzschia curvilineata* showed that the bulk carbohydrate of surficial sediments was negatively correlated with erosion rate. Colloidal carbohydrates were barely detectable by the phenol–sulfuric acid method used (87). Possibly the most interesting aspect of this paper is the observation that bulk carbohydrate and erosion rate became decoupled towards the stationary phase of diatom growth. Sediments were more stable than the bulk carbohydrate content suggested. They speculated that the sugar composition (qualitative of quantitative) of carbohydrate polymer changed with time. When diatom

cultures age, the cells become less motile, the extracellular polymer becomes insoluble in the growth medium and thus easily visible with dark-phase microscope optics. There is either a change in extracellular polymer state or a different type of polymer is synthesized. Decho (88) has observed that even subtle changes in composition such as bridging, adsorption of metals and enzymic interactions within the exopolymer matrix can alter its tertiary state. The solubility of microbial extracellular polymers is highly dependent on the cationic environment and the solvent (88). It difficult to understand why polymers that are soluble in seawater (salinity c. 36 ‰) and thus will diffuse can be extracted from a sediment core quantitatively with 30 ‰ NaCl (89). This underscores some of the confusion that is found in the literature on this subject. In support of this notion, De Winder and associates (90) found that a considerable proportion of the colloidal carbohydrate produced by a diatom biofilm on a tidal sandy beach was soluble in water and therefore is likely to disappear when the sediment becomes inundated. Thus it could contribute only to a limited extent to SS.

The herbicide Diuron has been used in several studies to inhibit diatom activity and thus supposedly provide a control situation for the involvement of diatoms in SS. Diuron (DCMU) inhibits diatom photosynthesis at the level of photosystem II; however, it does not prevent motility (90). Thus, if it is postulated that the involvement of motility-derived polymers is important in SS, then DCMU-treated sediments are not a useful negative control. Daborn and coworkers (91) found that DCMU gave inconsistent results. Paterson (92) also used DCMU to manipulate diatom populations in natural sediments. He found that the SS measured was lost during the tidal cycle and concluded that this was due to the presence of diatoms and the ephemeral nature of their motility polymer. Several other points made by Paterson are relevant here. He found that the erodibility of sediments with an established diatom assemblage remained lower after (tidal) immersion than the controls, suggesting a residual effect. Paterson states that it is not possible to say whether this effect is diatom-produced or due to a stimulation of bacterial populations in the presence of diatoms. He states further that biochemical analysis of the quantity and type of mucopolysaccharides present is desirable.

Sediment populations of diatoms have been manipulated by means other than the use of DCMU. For instance, Underwood and Paterson (93) used formaldehyde spraying to reduce diatom (and other living) populations in intertidal sediments with a view to examining the influence of indigenous microflora on SS. Formaldehyde did reduce sediment chlorophyll a, but it recovered to a normal level in six days. However, grazing microfauna did not recover, and because of this, diatom populations eventually became greater in biocide-treated areas than in the untreated areas. This exemplifies very well the problems encountered in uncontrolled field situations. Underwood and Paterson also found that there was an inverse relationship between sediment water content and shear strength. This was attributed to the compaction of the sediments in the biocide-treated areas because of the absence of burrowing fauna. Freshly deposited sediments were not retained by the biocide treated sites. Colloidal carbohydrate (soluble) in sediments was correlated with chlorophyll a and presumably the number of diatoms, but total carbohydrate concentrations in sediments did not correlate with a measure of SS. It is interesting to note van Kooten and coworkers (94) found that glutaraldehyde-fixed human fibroblast cells were much harder to remove from a surface with a liquid flow than were live cells. "Fixing" the sediment and the cells in it with formaldehyde may have had a similar and undetected effect; thus the biocide may have an influence other than that proposed by Underwood and Paterson. These workers in a similar study (95) showed that there was no significant correlation between chlorophyll a concentration in sediments and bacterial density, suggesting that, given their other findings (a correlation between chlorophyll a and critical shear stress), bacteria are not involved in SS. In this study, there were no significant differences in critical shear stress between biocide-treated and untreated areas. Critical shear stress is a measure of the force necessary to erode surface particles. It is increased with an increase in microbiological biomass and a decline in water content of the sediment. As both chlorophyll a and colloidal carbohydrate were lower in very wet sediments than in drier ones, it was difficult to separate the relative importance of these variables (water content and biofilm strength) on determining critical shear strength. However, multiple regression analysis showed that a consideration of carbohydrate and water content together explained the data better. Again, this is an example of the complexities of the natural world and the interpretation thereof. The authors stress the need to use in situ methodology, particularly for measuring sediment erodibility, but also underline the problems of working with natural cohesive sediment systems, including the difficulty of separating biological and physical effects, and the problem of establishing suitable and realistic "control systems." They also suggest that future studies should be conducted in the laboratory with natural sediments. This is not acceptable. Given the varied and variable populations found in marine sediments, model controlled systems should come first.

A study that relates laboratory-based experimentation directly to the sediments is that of Smith and Underwood (96), but it only adds to the difficulty in understanding the processes that take place in natural diatom biofilms. Production of EPS (diatom motility polymer in this case) is closely related to migratory rhythms of the diatom cells. During periods of photosynthesis, assimilated carbon is stored as intracellular glucan and is used to produce EPS for motility. Diatoms are also motile in darkness and in the presence of the photosystem-II inhibitor DCMU. Under these conditions, intracellular glucan is the source of EPS carbon and presumably the energy source needed to drive motility. Smith and Underwood showed that the stimuli required to initiate the flux of carbon from glucan to EPS were dim light, darkness, and migration of the cells downward in the sediment that was linked to an internal clock driven by the tidal cycle. Such influences on sedimentary diatom metabolism complicate benthic carbon budgets as well as the design of models to explain biogenic

stabilization of sediments. Currently, there is no postulated trigger for the reallocation of glucan carbon, but it may be tied into the surface-sensing system (7). Probably a major lesson to be learned from the literature on studies on sediment stabilization by benthic diatom biofilms is that it is impossible to make generalizations. All diatoms do not appear to behave similarly. One reason for this is that the various diatom carbohydrate fractions that are extracted, analyzed and postulated subsequently to have specific roles in the stabilization process, are merely operationally defined. The fractions do not represent specific molecular entities (97). The papers of Smith and Underwood (98) and Staats and associates (99) support this. In a laboratory study with several species of diatoms, Smith and Underwood (98) found by pyrolysis-mass spectrometric analysis that the EPS changed in composition as cells moved from logarithmic to the stationary phase of growth and that EPS production increased in the dark. On the other hand, using one of the organisms (*Cylindrotheca closterrium*) investigated by Smith and Underwood (98), Staats and associates (99), showed no secretion of EPS in the dark. It is possible that the reasons for these apparent anomalies could be explained by the differing extraction techniques used. The dynamics of production of the various types of EPS should be examined further. A potential technique to accomplish this is that of Leriche and coworkers (100) who used sugar-specific lectins in an enzyme-linked lectinsorbent assay (ELLA) to quantify the components of the EPS in bacterial biofilms.

Vandevivere and Kirchman (101) demonstrated that some freshwater bacterial strains exopolymer production was greater by attached cells than their free-living counterparts. Sand-packed columns or free sand in suspension were used in this study. The lesson from this paper is that the genes controlling exopolymer synthesis are turned on by the surface. Davies and coworkers (102) have shown that an alginate gene (Alg C) activity was greater for cells of *Pseudomonas aeruginosa* on a Teflon surface than in cells free in suspension. This regulatory effect of surfaces on gene expression has obvious relevance in the colonization of sediment particles, and thus SS. There is no analogous information on diatoms, but it has been suggested that diatoms could recognize surfaces by a receptor-based process (7).

Most literature has focused on the intertidal zone. A large experimental effort is expended in this area of the ocean because it is accessible and bears the brunt of human activities. This area is also important from the point of view of its overall productivity (103). If bacteria are involved in SS, their influence will be apparent as long as there is supply of organic carbon. This condition will be met as long as there is a productive ocean above the benthos. This is likely, at least across the continental shelf, but what of phototrophs? Diatoms can metabolize autotrophically at light levels down to 0.1% of surface radiation (104). Low levels of DOC uptake by phototrophs have been measured by various workers (105–107) and mixotrophy is considered an important physiology (103,104). Cahoon and coworkers (104 and references therein) maintain that benthic microalgal abilities allow them to sustain some productivity to 0.1% photon flux density. They calculated that this light level was available at 30% of the 17,879 oceanographic stations with a depth of 200 m around the U.S. coast where light measurements were available. Diatom patches have been observed on the sediments at 70 m in the Tongue of the Ocean, Bahamas. In the samples taken by Cahoon and coworkers (104), they found mostly (97%) pennate forms of the genera *Amphora*, *Nitzschia*, and *Navicula*. Thus it seems that the proposed model system will be relevant at least to a depth of 200 m.

It is likely that the concentration of small organics in the sediment pore water is not uniform and in fact concentration gradients exist (97,108). These gradients could be a factor in the establishment of diatom films because these algae move along them toward an area of higher concentration. This has been established for two diatoms of the genus *Amphora* (38). The formation of such areas of diatom concentration in sediments will lead logically to enhanced SS. For the chemosensory perception of gradients by diatoms to fall into the former category, they must be able to derive some nutrition for the effort expended because optimal-foraging theory proposes that evolution will favor those behaviors that maximize net nutritional gains (109).

CONCLUSION

Diatoms are found in all illuminated biofilms, yet they have not been studied in detail. Most research on this subject is related to the fouling of shipping and the stabilization of marine and freshwater sediments. Work in these areas is hindered by the paucity of information available on the physiology and cell biology of the organisms concerned, as well as their interactions with other microorganisms in the biofilm. From what has been mentioned so far, it is evident that diatoms differ in some aspects of their physiology and biochemistry, such that generalizations are not wise. Because the practical and ecological importance of diatoms in biofilms are closely tied to their ability to secrete extracellular polymers, it seems reasonable to suggest that more studies of the mechanisms that control the synthesis and secretion of these molecules are needed, but research in this area is hampered by a lack of information of the molecular genetics of these organisms (110).

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DIAZOTROPHS. See NITROGEN FIXATION IN SOILS — FREE-LIVING MICROBES

DINITROGENASE. See NITROGEN FIXATION IN SOILS — FREE-LIVING MICROBES

DINOFLAGELLATES, TOXIC. See *PFIESTERIA*: THE TOXIC PFIESTERIA COMPLEX

DIOXYGENASES. See OXYGENASE ENZYMES: ROLE IN BIODEGRADATION

DISINFECTION: CHLORINE, MONOCHLORAMINE, AND CHLORINE DIOXIDE

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The disinfection process in the treatment of water is designed to insure the inactivation of microbial pathogens. These pathogens that serve as the etiological agents of waterborne disease comprise a diverse group of microorganisms, which include bacterial, viral, and protozoan species. Disinfection coupled with physical removal processes, such as clarification and filtration, comprise the basic unit processes used in water treatment. However, under certain circumstances, disinfection may serve as the sole method of treatment. Chlorine, monochloramine, and chlorine dioxide, are commonly used halogenated oxidizing agents used in the disinfection process. These chemical disinfectants are utilized in both the treatment of water and wastewater and for maintaining disinfectant residuals in water distribution systems.

The disinfection process may be effected by a variety of factors, all of which can influence biocidal activity. In terms of physical factors, two of the most important parameters in the microbial inactivation process are water pH and temperature. For halogen disinfectants, inactivation is less effective at lower water temperatures. Numerous studies have shown that disinfection rates generally increase by a factor of 2 to 3 for every 10 °C increase in temperature. Changes in pH effect individual halogen disinfectants in different ways. Water turbidity and oxidant demand are two other physical parameters that influence microbial inactivation. Particles in water and agglomerations of microorganisms themselves can serve as a means of protecting microbes from the effects of a disinfectant. This type of protection is particularly true for organisms associated with biofilms or corrosion products, which may be found in a water distribution system. The oxidant demand of a given water must be evaluated to achieve the desired level of disinfectant. The amount and the manner in which a disinfectant is applied, coupled with the requirement for adequate mixing, are important operational parameters that need to be taken into consideration. Microorganisms themselves differ widely in their resistance to inactivation by chemical disinfectants, with vegetative bacterial cells being more

readily inactivated than viruses, bacterial endospores, or encysted forms of protozoa. Prior growth conditions or metabolic status of bacterial cells may also influence their response to disinfecting agents. Lastly, the methods for determining the viability of a given microorganism after exposure to an oxidizing agent may influence the observed results. This is particularly true in the case of bioassays for determining the viability of encysted protozoans. It has also been noted that the constituents of carbohydrate-based media used for the propagation of bacteria may influence the recovery of oxidant-stressed organisms.

CT CONCEPT

As mentioned earlier, many variables can affect the inactivation process. These variables, along with basic differences in experimental designs, have contributed to the difficulties in comparing various reports on microbial inactivation. In attempts to overcome these problems, researchers have used mathematical principles to describe the inactivation kinetics of microorganisms. One of the most widely used of these mathematical procedures is known as the *CT* concept (1). *CT* values have been used by governmental agencies to provide guidance to water utilities regarding the levels of inactivation required for different microorganisms. The mathematical expression for *CT* is the product of the disinfectant concentration (C) multiplied by the time of exposure (T) required to achieve a given level of inactivation for a particular microorganism. The equation used for determining *CT* values was developed from analysis, which compared the biocidal action of a chemical disinfectant with the kinetics of chemical reactions (1). This concept is known as Chick's law

$$\log_{10} \frac{N}{N_o} = -KT$$

where $\log_{10} N/N_o$ represents the surviving microbial fraction, T represents the exposure time, and K is a proportionality constant. Watson further defined Chick's law as

$$K = C^n T$$

where C stands for the disinfectant concentration, n is a constant known as the coefficient of dilution, and T represents the exposure time required to achieve a given level of inactivation. In an analysis of inactivation studies conducted under oxidant demand-free conditions it was found that most values of n were close to 1.0 (1). The *CT* concept is essentially the Watson equation, with the implicit assumption that n is equal to 1

$$CT = K$$

where C is the disinfectant concentration (mg/L), T is time (minutes), and K is the proportionality constant. Using this equation, simple *CT* values can be calculated for different levels of inactivation for various waterborne microorganisms. Comparative *CT* data for the inactivation of several microorganisms are listed in Tables 1 to 3. Although *CT* values can be useful for comparing microbial inactivation studies and establishing compliance guidelines, it should be noted that the concept has certain

Table 1. CT Values for 99% Inactivation by Chlorine for Various Microorganisms[a]

Organism	Temperature (°C)	pH	Mean *CT* (mg min/L)
Bacteria			
Escherichia coli	5	6.0	<1.0
	5	10.0	<1.0
	15	10.0	<1.0
Virus			
Polio 1	5	6.0	2.0
	5	10.0	10.5
	15	6.0	1.0
	15	10.0	3.9
Encysted Protozoa			
Giardia lamblia	5	7.0	97
	15	7.0	32
Cryptosporidium parvum	25	7.0	7,200

[a] Adapted from Hoff, 1986 and Korich et al., 1990.

Table 2. CT Values for 99% Inactivation by Preformed Monochloramine for Various Microorganisms[a]

Organism	Temperature (°C)	pH	Mean *CT* (mg min/L)
Bacteria			
Escherichia coli	5	9.0	160
	15	9.0	66
Virus			
Polio 1	5	9.0	1,420
	15	9.0	646
Encysted Protozoa			
Cryptosporidium parvum	25	7.0	7,200[b]

[a] adapted from Hoff, 1986 and Korich et al., 1990.
[b] value for 90% inactivation.

Table 3. CT Values for 99% Inactivation by Chlorine Dioxide for Various Microorganisms[a]

Organism	Temperature (°C)	pH	Mean *CT* (mg min/L)
Bacteria			
Escherichia coli	5	6.5	<1
	10	6.5	<1
Virus			
Polio 1	5	7.0	3.6
	15	7.0	1.6
Encysted Protozoa			
Giardia muris	5	7.0	11.2
Cryptosporidium parvum	25	7.0	78[b]

[a] adapted from Hoff, 1986 and Korich et al., 1990.
[b] value for 90% inactivation.

inherent weaknesses. It has long been known that the first-order kinetics described by Chick's law is subject to various deviations that may occur for a variety of reasons, not the least of which is the failure of microorganisms to behave like the reactants in a chemical reaction. Variations in resistance among a portion of the microbial population as well as other factors such as depletion of disinfectant residual may also contribute to the observed deviations.

CHLORINE

Chlorine is the most frequently used disinfectant in water treatment. It has been successfully used in both drinking water and wastewater applications. The inactivation of microorganisms by chlorine is highly pH dependent. Chlorine disassociates in water to form hypochlorous acid and hypochlorite ion. Hypochlorous acid predominates at lower pH values and is a much more potent biocide than hypochlorite ion. Some of the earliest disinfection research centered on chlorine inactivation of enteric bacterial pathogens and sanitary indicator organisms represented by the coliform group of bacteria. More recent studies on chlorine inactivation of coliform bacteria have centered on the recovery of sublethally stressed organisms (2,3) and the protective effect from inactivation afforded from coliforms associated with naturally occurring particulate material (4). These reports have emphasized the role of different types of bacteriological media in the recovery of chlorine-stressed organisms and support the importance of water clarity in the disinfection process. Representative chlorine Ct values for various microorganisms are presented in Table 1. Using the virus polio 1 as a representative organism, it is readily seen that increasing the pH decreases the biocidal efficiency of chlorine. Similarly, when the pH level is held constant, it can be seen that increasing the temperature from 5 °C to 15 °C increases the rate of inactivation, resulting in lower Ct values.

The survival after chlorination of coliform bacteria in association with other organisms has also been reported. *Escherichia coli* and *Enterobacter cloacae* were found to be protected from chlorine inactivation when these organisms were associated with microcrustaceans such as the amphipod *Hyalella azteca* (5). Similarly, bacteria that were ingested by protozoa have been shown to exhibit an increased resistance to inactivation by chlorine. This protective effect has been demonstrated for coliform bacteria and several waterborne pathogens (*Salmonella typhimurium*, *Yersenia enterocolitica*, *Shigella sonnei*, *Legionella gormanii*, and *Campylobacter jejuni*) when ingested by free-living protozoa (6).

There have been numerous studies on the effect of chlorine on bacterial pathogens (7). Most studies have suggested that enteric bacterial pathogens are inactivated by chlorine in a manner similar to that seen for coliform bacteria. For example, studies have shown that the causative organism of hemorrhagic colitis, *E. coli* 0157 : H7, exhibited similar resistance to chlorination as that seen with nonpathogenic *E. coli* strains (8). This finding has also been shown to be true for *Helicobacter pylori* (9) and the two closely related bacterial pathogens, *C. jejuni* and *Arcobacter butzleri* (10,11). Certain nonenteric waterborne microbial pathogens such as the nontuberculosis mycobacteria, however, have been shown to be more resistant to disinfection than coliform bacteria (7).

Animal viruses and bacteriophages (bacterial viruses) are generally considered to be more resistant than bacteria to chlorination. Early chlorine disinfection studies with viruses concentrated on the inactivation of poliovirus under oxidant demand-free conditions (1). Studies have been conducted to study the inactivation of both purified preparations of single virus particles and virus particles associated with tissue culture cells to compare their resistance to chlorination. In all instances the cell-associated virus was more resistant to inactivation than were preparations of single virus particles. In studies on the inactivation of bacteriophages it was concluded that the bacteriophage, like the animal virus, is sensitive to inactivation by chlorine (1,7).

Several research studies have examined the inactivation kinetics of the waterborne protozoan parasite *Giardia* spp. (1,7). Studies using *G. lamblia* cysts derived from an animal model (mongolian gerbils) as opposed to cysts obtained from human donors have shown that the gerbil-derived cysts were somewhat more resistant than cysts obtained from human donors, suggesting that the host source of cysts may affect cyst resistance to disinfection (12). The encysted forms (oocysts) of the waterborne parasite *C. parvum* have been shown to be very resistant to chlorination (Table 1).

CHLORAMINE

The use of chloramination in water treatment has gained increasing popularity as concerns have grown regarding adverse health effects attributed to chlorine disinfection by-products. Monochloramine is considered a weak biocide in comparison with chlorine, requiring longer exposure times to achieve comparable levels of inactivation (Table 2). Chemically, chloramines are a complex group of disinfectants, however, only the monochloramine form is of major interest for drinking water disinfection. When chlorine and ammonia are mixed in equimolar proportions, nearly all free available chlorine is converted to monochloramine. This is an equilibrium reaction and is affected by the chlorine to ammonia ratio. The rate of formation also exhibits a pH dependency. In studies on the efficacy of microbial inactivation by chloramination, pH levels are generally maintained at 8 or higher to insure that monochloramine is the predominant form present. Traditionally, laboratory studies have concentrated on the use of preformed chloramine, and while these results yield conservative values for inactivation they are not representative of the disinfectant's effectiveness under field conditions. The chlorine to ammonia ratio, the pH, and the method of application are crucial parameters to consider in chloramine inactivation and must be clearly delineated to determine biocidal effectiveness under various experimental conditions (1).

As with chlorine, particle protection has also been observed with monochloramine inactivation of coliform

bacteria. Coliforms associated with smaller size particle fractions were inactivated more rapidly than the organisms associated with larger size particle fractions, thus mimicking the results observed with chlorine (4).

There has been relatively little research on the role of chloramination for the inactivation of protozoa. It has been shown that monochloramine was less effective than chlorine or chlorine dioxide for inactivating *C. parvum*. For chloramine, a *CT* of 7,200 produced only a 90% level of inactivation of *C. parvum* oocysts (Table 2).

CHLORINE DIOXIDE

Chlorine dioxide exists as an undissociated gas dissolved in water in the pH range from 6 to 9. Chlorine dioxide is a potent disinfectant and is generally considered to have a biocidal efficiency greater than chlorine or monochloramine (1). Like monochloramine, chlorine dioxide has gained interest among water utilities concerned with disinfection by-products such as trihalomethanes. Trihalomethanes are not formed when chlorine dioxide is used as a disinfectant if the oxidant is produced without excess chlorine.

The effect of prior growth conditions has been examined for the inactivation of *E. coli* and *Legionella pneumophila* by chlorine dioxide (13). Chemostat-grown bacteria were compared to batch culture bacteria in reference to their resistance to chlorine dioxide. A resistant subpopulation of each organism survived in the presence of a constant chlorine dioxide residual. The observed resistance was attributed to a phenotypic trait, which could be manipulated by altering the antecedent growth conditions in the chemostat cultures. However, in most instances chlorine dioxide is considered to be very effective for inactivating bacteria (Table 3).

Studies conducted on chlorine dioxide inactivation of viruses have shown that this oxidant has potent viruicidal properties (Table 3). Chlorine dioxide has been shown to be an effective biocide for *Giardia* (Table 3) and to be more efficacious than chlorine or monochloramine for the inactivation of *C. parvum* oocysts (14).

CONCLUSION

The disinfectants discussed in this section represent three of the most commonly used chemical oxidizing agents used in water treatment. Two important considerations that need to be addressed regarding the choice of disinfectants is biocidal efficiency and stability (15). In terms of biocidal efficiency the disinfectants may be ranked as chlorine dioxide > chlorine > chloramine. It should again be noted that temperature is an important factor to consider, with effectiveness improving with increasing temperatures within the ranges normally encountered for water samples. The effect of pH is most apparent with chlorine, with effectiveness decreasing with increasing pH levels. For chloramines the pH level needs to remain above 8 to insure that monochloramine is present. Increasing pH appears to have a beneficial effect for chlorine dioxide. However, the mechanism is not well defined. In terms of stability monchloramine is more stable than chlorine or chlorine dioxide. The biocidal effectiveness of monochloramine is less than the other two disinfectants; however, the ability to persist over a prolonged period may be beneficial, particularly in cases such as maintaining a disinfectant residual in a distribution system.

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DISINFECTION, EFFECT ON VIRUSES.

See VIRAL DISINFECTION

DISINFECTION OF LEGIONELLAE.

See *LEGIONELLA* IN THE ENVIRONMENT: PERSISTENCE, EVOLUTION, AND PATHOGENICITY

DISINFECTION OF PROTOZOAN PARASITES

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One of the challenges facing the water industry is to provide drinking water safe from disinfectant-resistant protozoan parasites and at the same time to minimize the formation of harmful disinfection by-products (DBP) in drinking water. Protozoan pathogens are unicellular

eukaryotic microorganisms that, unlike fungi, lack a cell wall. Protozoa are typically larger than prokaryotic bacteria and are distinguished from algae by their lack of photosynthetic pigments. Many protozoa are motile in aquatic environments; however, members of the group Sporozoa, to which many important human pathogens such as *Cryptosporidium* and *Toxoplasma* belong, are generally nonmotile. In aquatic environments, protozoa are found as cysts, oocysts, or spores, and all of these forms are very resistant to disinfection. Some of the protozoan parasites of concern to the drinking water industry include *Cryptosporidium parvum*, *Giardia lamblia*, *Entamoeba histolytica*, *Cyclospora cayetanensis*, *Toxoplasma gondii*, and the microsporidia. *Giardia lamblia* and *C. parvum* have especially been associated with several outbreaks of waterborne gastroenteritis and are amongst the most important etiological agents of waterborne disease (1,2). For example, *C. parvum*, which causes severe gastroenteritis that is often fatal in immunocompromised individuals, was implicated as the etiological agent in an outbreak in Milwaukee, Wisconsin, where more than 400,000 people were infected (3). More than 400 waterborne disease outbreaks were documented in the United States between 1980 and 1996, of which *Giardia lamblia* accounted for the largest fraction, 20.9%. In contrast, a highly significant 82.5% of cases of illness were attributed to *Cryptosporidium parvum* (the Milwaukee cryptosporidiosis outbreak being responsible for the elevated percentage of cases) (4). Therefore, both *Cryptospordium* and *Giardia* are of great concern to the water industry and are further described below. In addition to these two organisms, *Entamoeba hytolytica* can also be transmitted through water; however, outbreaks of disease caused by this organism appear to be limited to tropical and subtropical areas. The remaining protozoan parasites mentioned earlier are potential waterborne pathogens, but information with respect to their occurrence in water or their sensitivity to disinfection is limited.

DRINKING WATER DISINFECTION

Disinfection is the inactivation of disease-causing microorganisms, such as bacteria, viruses, and protozoa, by the addition of a chemical (a disinfectant such as chlorine) into water. Disinfectants may inhibit growth or kill target organisms. The target sites that may be affected include, depending on the type of microorganism, the bacterial peptidoglycan layer, the bacterial outer membrane, the cellular cytoplasmic membrane, structural proteins and enzymes, nucleic acids, and/or viral envelopes and capsids (5). The ideal water disinfectant should have the following properties: it should inactivate pathogens within a practical time period and over a wide range of water quality, it should be nontoxic to humans and animals at required concentrations, it should be palatable at required concentrations, it should persist in a water distribution system and maintain a residual concentration to prevent bacterial regrowth and contamination by infiltration, and ideally it should be easy and cheap to measure and use.

In a water treatment plant, disinfection is typically the last barrier in water purification, and its main purpose is to achieve a reduction in pathogen numbers (6). Disinfectants are also often used where raw water enters the water purification plant (prechlorination). Although prechlorination does achieve pathogen removal, its main purpose is typically to minimize taste and odor problems. Chlorine was first used in the beginning of the twentieth century and remains the most widespread disinfectant in the water industry (7). Other traditional disinfectants and alternative disinfection strategies include chloramination, ozonation, the use of chlorine dioxide, and the application of ultraviolet irradiation, the later method being a physical means of achieving disinfection. Each technology has some advantages under certain conditions. Because of protozoan resistance to disinfection and the concerns related to waterborne outbreaks of protozoan parasites, recent research has focused on alternative disinfection methods for the water industry; however, several investigators have noted that with the exception of ozone, the use of disinfectants alone may not be sufficient to inactivate enough protozoan parasites in water to prevent waterborne outbreaks (8,9). Disinfection technologies have to be developed along with better physical removal of organisms to ensure adequate protection of drinking water. Sequential use of disinfectants may yield synergistic inactivation and provide additional removal, but additional work is needed to confirm these results. Several of these applications are currently being studied, especially with respect to reducing the risk of *Cryptosporidium* transmission in water.

It is important to note that most studies of water disinfection of protozoan parasites have been conducted in batch-scale systems in disinfectant demand-free buffers that may not correctly simulate the operation of water-treatment plants. Although batch-scale experiments are easy to replicate and have been used to derive inactivation kinetics, they do not simulate water treatment hydraulics and do not account for other factors that are present in water treatment. Ideally, to determine the ability of a water-treatment plant to inactivate microorganisms, models of disinfection should attempt to use natural water and should simulate the flowing water process of contact between disinfectant product and target organism by making use of pilot plants.

Factors Influencing Disinfection

Many factors are known to influence disinfection (7). All microorganisms do not show the same resistance to disinfection. In general, protozoan cysts and bacterial spores are most resistant (7), whereas viruses and vegetative bacteria are much more sensitive to disinfectant action. Chemical kinetics predict that disinfection of protozoa (and other organisms) is more difficult to achieve in colder waters than in warmer waters, and this was empirically demonstrated in several studies (10,11). Other general factors include water hardness and the protection of microorganisms when they form aggregates, clumps, or biofilms in a water distribution system (12,13).

In addition to factors influencing the disinfection process itself, the analytical method for determination

of protozoan viability also affects disinfection results. All protozoan disinfection experiments must involve a measurement of viability or infectivity. Viability of protozoan parasites such as *Cryptosporidium* oocysts and *Giardia* cysts, however, has been assessed in several different ways, making interstudy comparisons very difficult. Different methods yield different results and typically, in vitro methods, such as in vitro excystation have been shown to underestimate inactivation by disinfection (14,15). Excystation of the cysts/oocysts occurs in the intestine of the host. Therefore, in vitro excystation methods simulate this condition and have been taken as an indication of survival (14,16). For *C. parvum*, the oocysts are incubated in an excystation medium made of taurocholic acid and trypsin (16,17). Numbers of oocysts, partially excysted oocysts, empty oocysts, and sporozoites are then counted by using differential interference microscopy. A correlation between in vitro excystation and infection of mice with large inocula (10^5 oocysts per mouse) of *Cryptosporidium* oocysts has been reported (18); however, the correlation with lower numbers of organisms and different susceptible species remains unclear, particularly after increased levels of inactivation (14). Therefore, consideration of some cellular injuries preventing productive infection, without necessarily preventing excystation, must be taken. The major disadvantages of in vitro excystation are the length of time (several hours) for completion of a test and the requirement of large numbers of organisms.

A second method for the measurement of protozoan viability involves the inclusion or exclusion of fluorogenic vital dyes by cysts/oocysts. For example, 4',6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) have been utilized extensively in the United Kingdom to assess the viability of *Cryptosporidium* oocysts (19). It has been shown that only cells with disrupted or broken membranes can be stained with PI (20). Motile sporozoites never incorporate PI. Correlation between inclusion of DAPI (DAPI+) and excystation yield was shown to be highly statistically significant (19). Oocysts that incorporated neither dye (DAPI- PI-), but that were not empty oocysts, could be converted to viable (DAPI+) oocysts by a trigger mechanism (for example, preincubation in acidified Hank's balanced salt solution). Such oocysts are considered viable. This method provides the advantages of speed, simplicity, and low cost. The DAPI–PI assay has been used to evaluate ozone disinfection and UV inactivation of *Cryptosporidium* oocysts (21,22). Other vital stain methods have been tested, for example, Arrowood and coworkers (23) used a modified fluorescein diacetate (FDA)–ethidium bromide (EB) staining procedure to assess the viability of *Cryptosporidium* oocysts. In this fluorogenic assay, the esterases in viable cysts convert the highly permeable nonfluorescent FDA to fluorescein that fluoresces green (24). However, the assay was not correlated with other viability assays. More recently, Belosevic and coworkers carried out an extensive study of the use of the fluorescent stain SYTO®-9 for testing the viability of *Cryptosporidium* (25). SYTO®-9 is a live or dead stain that stains dead oocysts green or yellow, leaving live oocysts either unstained or displaying a green halo. SYTO®-9 vital staining was shown to correlate with in vitro excystation and animal infectivity in CD-1 mice (26); however, other researchers (15), in a study involving inactivation by ultraviolet light and ozone, found that none of the in vitro assays tested were capable of predicting oocyst inactivation, with animal infectivity better reflecting inactivation levels in all cases. Clearly, the most effective vital stain method would be one that involves an enzyme system that is critical to the initiation of infection. Unfortunately, such an enzyme system is not known at present.

As mentioned several times in the preceding paragraphs, a third viability measurement method is the animal infectivity (8,14,27). The potential to infect neonatal mice can be used to assess the viability of oocysts. Neonatal mice are inoculated, sacrificed after several days, and parasite development stages are detected by microscopy in histological sections of the gut. As previously discussed, recent research (8,15) indicates that in vitro excystation underestimates inactivation when compared with animal infectivity. As inactivation increases, the difference between excystation and infectivity also increases. The animal infection procedure does, however, have some disadvantages, including the cost, the expertise needed, the length of the assay, and the requirement of large numbers of animals to ensure quantitative results, consequently leading to a need to maintain a large colony of mice.

In addition to the methods mentioned earlier, other viability methods for *Cryptosporidium* have recently been developed. Among the most promising ones are molecular methods in combination with cell culture (28) and a cell culture method combined with immunofluorescence and most-probable number determination (MPN-infectivity assay) (29). These methods hold great potential for future work because of their sensitivity, rapidity, and relatively low cost. Molecular methods, notably the polymerase chain reaction (PCR), have the potential to probe for the induction of synthetic processes such as mRNA synthesis that could result from an environmental trigger such as heat treatment (30). Filkorn and coworkers (31) developed a PCR assay for *Cryptosporidium* sporozoites. The assay uses excystation in combination with DNA-digestion, followed by PCR. Rochelle and coworkers (28) developed a detection procedure for *Cryptosporidium* using cell culture combined with PCR. The oocysts were inoculated onto monolayers of Caco-2 cells grown on chamber slides. The infection of the cell culture was assessed by detection of mRNA from the heat-shock protein gene by reverse transcription-polymerase chain reaction (RT-PCR). This method was tested with 10^3 oocysts. The MPN-infectivity assay consists of diluting *C. parvum* oocyst samples, plating them in triplicate onto a suitable cell culture line, incubating them for a certain period of time, and detecting infectious stages in the cell culture by immunofluorescence (29). Calculations are done using MPN tables or computer software.

None of the available viability methods is definitive. Assays based on animal infectivity cannot be extrapolated from one species to another (for example, from mice to humans). In addition, large numbers of animals are

required for statistically accurate determination of the infectious dose.

Disinfectant Concentration and Contact Time

Disinfectant effectiveness is often expressed as a *Ct* value. *C* is the disinfectant final residual concentration and *t* is the time required to kill a certain percent (or $\log_{10}$ inactivation) of the population (32). Critical *Ct* values for achieving a certain level of inactivation have been used for regulatory purposes under the Surface Water Treatment Rule (SWTR) by the U.S. Environmental Protection Agency. Tables listing *Ct* values, which provide the required *Ct* value that must be achieved to reach a certain level of inactivation, have been developed for various disinfectants under different conditions of temperature and pH.

Type of Disinfectant

Chlorine. Chlorine remains the most popular water disinfectant in North America. When chlorine gas (Cl_2) is injected into water, it hydrolyzes to form hypochlorous acid (HOCl), which dissociates in water to form hypochlorite ions (OCl^-). In general, chlorine is more efficient at low pH values (hypochlorous acid formed) than at high pH, which favors the formation of hypochlorite ions (33,34). This association between pH and chlorine has also been shown for disinfection of protozoa such as *Entamoeba hystolytica* (35) and *Giardia lamblia* (Table 1). Most studies on the mechanism of action of chlorine have been performed on bacterial cells. For example, several studies have shown that permeability disruption was the cause of chlorine damage to bacteria (32,36,37). Other studies have demonstrated that chlorine has an effect on bacterial DNA (38) and on bacterial enzymes, such as catalase, resulting in the intracellular accumulation of hydrogen peroxide (39).

Table 1 shows *Ct* values for some protozoa under various conditions. In recent years it has been recognized that the use of chlorine to provide disinfection of drinking water may not adequately inactivate all pathogens, most notably *Cryptosporidium parvum*, which is much more resistant to chlorine than *Giardia lamblia*, *Escherichia coli*, and waterborne viruses (Table 1). Quinn and Betts (40) demonstrated that preaging *Cryptosporidium* oocysts for up to seven days in tap water at room temperature did not have any significant effect on their subsequent inactivation by chlorine. Even oocysts aged in natural waters in situ for up to 26 days did not show increased susceptibility to chlorine (or monochochloramine) (16). As mentioned earlier, studies on disinfection of *Cryptosporidium* oocysts have repeatedly confirmed their relative resistance to disinfection by the levels normally used in drinking water treatment. Therefore, chlorine as a single primary disinfectant is not useful to eliminate the risk of cryptosporidiosis and other protozoan diseases in water.

Chloramines. Monochloramine is an alternative to chlorine. It has been used in some facilities for a long time. For example, in Denver and Ottawa, it was first introduced in 1917 (6). Monochloramine (NH_2Cl) is formed when hypochlorous acid (HOCl) from chlorine reacts with ammonia (NH_3). Dichloramine ($NHCl_2$) and trichloramine (NCl_3) are also formed in the process, but monochloramine is predominant at pH value greater than 8.5 (7). Chloramines are less effective as disinfectants than free chlorine; however, they appear to be very effective at controlling biofilms in distribution systems. Their use is recommended as a secondary disinfectant in systems having extensive biofilm problems (44). Monochloramine on its own is less effective than free chlorine against *Cryptosporidium* (8,10,16) and *Giardia* (45), as shown by the very high *Ct* values (Table 2), and it is not recommended as a single primary disinfectant to prevent waterborne outbreaks of protozoa (46).

Chlorine Dioxide. Another alternative disinfectant to free chlorine is chlorine dioxide (ClO_2), which may be strong enough to control *Cryptosporidium* and does not typically form as many carcinogenic disinfection by-products as chlorine. It does not form trihalomethanes or chloramines but reacts to form chlorite and chlorate, which although not carcinogenic like trihalomethanes or haloacetic acids, can be acutely toxic at high concentrations.

Chlorine dioxide can be produced by combining hydrochloric acid with sodium chlorite according to the following reaction:

$$5NaClO_2 + 4HCl \rightarrow 4ClO_2 + 5NaCl + 2H_2O$$

ClO_2does not hydrolyze in water but remains present as a dissolved gas. The pH of water has an impact on

Table 1. Inactivation of Protozoa and Other Selected Microorganisms by Chlorine

Microorganism	Temperature (°C)	pH	Inactivation ($\log_{10}$)	*Ct* value	Reference
Giardia lamblia	5	6.0	3.0	150	51
Giardia lamblia	5	7.0	3.0	300	51
Giardia lamblia	20	7.0	3.0	52–68	14
Cryptosporidium parvum	25	7.0	2.0	>7,200	16
Entamoeba hystolytica	27–30	7.0	2.0	20	52
Escherichia coli	20–29	7.0	>2.0	0.1	53
Coxsackie A2 virus	20–29	7.0	>2.0	2	53
Hepatitis A virus	20–29	6.9–7.4	>2.0	2.5	53

Table 2. Inactivation of Protozoa by Monochloramine

Microorganism	Temperature (°C)	pH	Inactivation (log_{10})	*Ct* value	Reference
Giardia muris	15	7.0	2.0	848	45
Giardia muris	15	8.0	2.0	466	45
Giardia muris	3	7.0	2.0	1,400	46
Cryptosporidium parvum	25	7.0	2.0	>7,200	8
Cryptosporidium parvum	22	8.0	>2.0	3,600	10

Table 3. Inactivation of Protozoa by Chlorine Dioxide

Microorganism	Temperature (°C)	pH	Inactivation (log_{10})	*Ct* value	Reference
Giardia muris	5	7.0	2.0	10.7	53
Giardia muris	25	7.0	2.0	5.1	54
Giardia muris	25	5.0	2.0	5.8	53
Cryptosporidium parvum	20	8.0	2.0	150	47
Cryptosporidium parvum	4	8.0	2.0	900	47
Cryptosporidium parvum	1	6.0	0.5	120	52
Cryptosporidium parvum	22	6.0	2.1	120	52
Cryptosporidium parvum	25	7.0	1.0	78	8

the formation of chlorate and chlorite; more of these DBPs being formed as the pH is increased. Inactivation rates are, however, approximately the same at pH 6 as at pH 8 (47).

The mechanism of action of chlorine dioxide on protozoa is poorly understood. In viruses, it is known to penetrate the capsid and react with nucleic acids (48,49). In bacteria, the main site of action appears to be proteins in the cell membrane and causing inhibition of protein synthesis (5,50). Few studies have been published on the chlorine dioxide inactivation of protozoan parasites in water; however, enough data have been collected so far to suggest that chlorine dioxide is a stronger oxidant than free chlorine. Accordingly, chlorine dioxide is more germicidal than free chlorine, as indicated by the much lower *Ct* values under similar conditions for a given organism (Tables 1 and 3). There is a lot of disparity in the chlorine dioxide inactivation data with *Cryptosporidium* (Table 3), and some investigators remain cautious about the possibility of using chlorine dioxide to inactivate protozoal agents (9,51). Other researchers such as Finch and Li (52) have stated that chlorine dioxide is an effective disinfectant for *Cryptosporidium*, even at low temperatures. In part, the disparity is due to different analytical methods for measuring both parasite viability and chlorine dioxide concentrations, which seems to suggest that more research is warranted to better understand the usefulness of chlorine dioxide for inactivating protozoan pathogens.

Ozone. Ozone (O_3) was first used for water disinfection in Europe in 1906 (43). Ozone forms free radicals that inactivate microorganisms. Its main effect seems to be on enzyme activity and DNA (55). Ozone has become more prevalent during the past 20 years in the United States and Canada as an alternative to chlorine for two reasons. (*1*) The recognition in the 1970s that chlorination leads to the formation of DBP and (*2*) the emergence and recognition in the past decade of waterborne cryptosporidiosis as a major health problem and the resistance of its causative agent to chlorine. Ozonation is recognized as one of the most efficient ways to inactivate protozoan parasites such as *C. parvum* during water treatment (8,14,56), as shown by the very low *Ct* values required to achieve certain levels of inactivation (Table 4). As with other disinfectants, *Cryptosporidium* is more resistant to ozone than *Giardia* (Table 4). The use of ozone for *Cryptosporidium parvum* inactivation seems to be more effective at higher temperatures and is better described by a nonlinear model such as the Hom model that accounts for ozone decay (10,14).

Only a few studies have shown that ozone in a pilot plant is efficient against *Cryptosporidium* (57). The overwhelming majority of inactivation studies have been conducted at bench-scale. It has, however, been reported that *Giardia* and *Cryptosporidium* inactivation data obtained at pilot-scale in natural waters were comparable to bench-scale results obtained in laboratory waters (57). Although such comparisons are always difficult to make, these findings seem to indicate the enormous potential of ozone for inactivating *Cryptosporidium parvum* and other protozoan parasites.

Ozonation studies have clearly demonstrated that in vitro excystation consistently underestimated *Cryptosporidium* inactivation when compared to mouse infectivity (14). Therefore, infectivity assays must be used when testing disinfectants as excystation may occur without infectivity. Comparison of past studies is also difficult because of the different viability assays utilized. Finally, comparison of various studies is especially difficult because ozone reactions are typically specific to particular water systems and vary depending on the water characteristics, suggesting that modeling must be done at each individual

Table 4. Inactivation of Protozoa by Ozone

Microorganism	Temperature (°C)	pH	Inactivation (log_{10})	*Ct* value	Reference
Giardia muris	22	5.7–8.3	3.0	0.30	14
Giardia lamblia	22	6–9	3.0	0.31	60
Cryptosporidium parvum	25	7.0	>1.0	5	8
Cryptosporidium parvum	room	—	2.0	4.6	61
Cryptosporidium parvum	7	6.9	2.0	7	14
Cryptosporidium parvum	22	6.9	2.0	3.5	14
Cryptosporidium parvum	22	6–8	2.0	1.7–2.7	62

site for proper optimization of the technology. A drawback of ozonation is that, unlike free chlorine, ozone reacts very rapidly, typically leaving no residual in the water distribution system.

Ultraviolet Light. Recently, ultraviolet light (UV) was shown to be effective at inactivating significant levels of protozoan parasites (22,27). The germicidal effect of UV irradiation involves photochemical damage to DNA within the organisms. DNA absorbs light energy in the wavelength of 240 to 280 nm, and the resulting DNA damage is often in the form of dimerization of thymine molecules, which leads to lethal mutations. Besides its germicidal action, UV has many additional advantages. For examples, it is a noncorrosive technology with no need for storage of toxic chemicals. UV irradiation also produces no known DBPs. Also, the technology is relatively low maintenance, although fouling of the lamps can be a problem. Three types of systems have been designed and evaluated for drinking purification: low-pressure UV (the more traditional technology that is employed in wastewater treatment), medium-pressure UV, and pulsed UV (15). UV is typically measured in mJ/cm^2 (that are equivalent to mW sec/cm^2). Typical UV doses in water are between 16 and 40 mJ/cm^2. One of the problems associated with UV technology is that, unlike with chemical disinfectants, no UV "residual" can be measured in water, making correct evaluation of the applied dose more difficult. Also, as for ozone, this lack of "residual" is not appropriate for biofilm control. Thus, UV technology must be used in combination with other processes such as biological filtration and/or chemical disinfection to control biofilm growth.

Using animal infectivity, bench-scale experiments have demonstrated a greater than 4.0 log_{10} inactivation of *C. parvum* with a dose of 41 mJ/cm^2. A comparable 3.9 log_{10} inactivation was achieved with a dose as low as 19 mJ/cm^2 at "demonstration-scale" at the Mannhein water treatment plant in Waterloo, Canada (15). The 111-L UV reactor tested in the later study consisted of six 1-kW medium-pressure UV lamps with an overall flow rate of 814 L/min. Therefore, the results from these recent studies suggest that UV at very low doses causes significant parasite inactivation and that this technology may become more widely used in the near future for drinking water purification. One issue that remains to be addressed, however, is the possibility of the existence of UV repair mechanisms, such as photoreactivation repair or excision repair, in protozoa similar to those known in bacteria.

Synergistic Effect of Disinfectants

Finch and coworkers demonstrated that sequential disinfection of *Cryptosporidium* with chlorine and monochloramine resulted in a synergistic effect on inactivation (63). In other words, the use of two disinfectants in sequence has an enhanced disinfection effect greater than the sum of the individual disinfectants. For example, individual applications of either chlorine (dose of 5 mg/L for 60 minutes) or monochloramine (dose of 5 mg/L for 480 minutes) resulted in less than 0.5 log_{10} inactivation of *Cryptosporidium*. When both chemicals were used sequentially at lower doses (chlorine at 1 mg/L for 60 minutes and monochloramine at 2 mg/L for 240 minutes), more than 1.5 log_{10} inactivation was measured (63). Similarly, ozone, which itself is a very good disinfectant, followed by monochloramine shows a synergistic effect (10). Synergistic effects also have been shown with chlorine dioxide (62,63). Researchers have postulated that the synergistic effect is produced when the primary oxidant affects the oocyst wall permeability, permitting the secondary disinfectant to better diffuse through the oocyst wall to damage the sporozoites (10,62).

CONCLUSION

Protozoan parasites are recognized as important etiological agents of waterborne illnesses. Historically, *Giardia lamblia* has been the most frequently cited etiology of waterborne gastroenteritis; however, in recent years, *Cryptosporidium parvum* has been implicated in several outbreaks of disease and is now recognized by water utilities as the main direct water-related threat to public health. One of the reasons for the prevalence of protozoan pathogens in drinking water is their resistance to commonly used disinfectants such as chlorine. Cysts/oocysts of these organisms are difficult to inactivate using acceptable doses of disinfectants. In the past few years, and especially in response to the 1993 waterborne outbreak of cryptosporidiosis in Milwaukee, there has been a tremendous amount of research performed to evaluate the efficacy of alternative disinfectants and disinfection procedures against *C. parvum* (and, to a lesser extent, *G. lamblia*). For use as single disinfectants, disinfectant efficacy of chemicals follows this order: ozone > chlorine dioxide > free chlorine > monochloramine; however, some

researchers have demonstrated that sequential disinfection may have a synergistic effect, thereby justifying, under certain conditions, the use of monochloramine or chlorine to inactivate protozoa. Additional research, especially at pilot-scale, is warranted to confirm these results. Some studies have also proposed that mixed oxidants may provide adequate inactivation levels (64). Finally, nonchemical means of disinfection, namely UV technology, have been shown to inactivate significant levels of *Cryptosporidium* in both bench-scale and demonstration-scale experiments and may provide an affordable alternative to chemical inactivation, at least with respect to parasite inactivation during treatment. There is no doubt that new technologies will emerge in the future to build on (or replace) current technologies, especially as concerns over toxic and carcinogenic DBPs will probably remain as long as chemical oxidants are added to drinking water. For example, membrane filtration (not a "disinfection" process) has been shown to be very efficient at removing parasites during treatment (65).

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DRINKING WATER, CYANOBACTERIA IN. See Cyanobacteria-Toxins in Drinking Water

DRINKING WATER DISTRIBUTION SYSTEMS, INVERTEBRATES AND PROTOZOA IN. See Invertebrates and Protozoa (Free-Living) in Drinking Water Distribution Systems

DRINKING WATER DISTRIBUTION SYSTEMS, PATHOGENS IN. See Pathogens in Environmental Biofilms

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DRINKING WATER, SULFUR BACTERIA IN. See Sulfur Bacteria in Drinking Water

DRINKING WATER, VIRUSES IN. See Viruses in Drinking Water and Groundwater

E

ECOLOGICAL SIGNIFICANCE OF SUBSURFACE MICROORGANISMS

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Terrestrial subsurface environments are inhabited almost exclusively by microorganisms and are in essence "aphotic" ecosystems. Photosynthesis plays only an indirect role in subsurface microbial ecology, providing reduced organic compounds that can be metabolized by aerobic or anaerobic heterotrophic bacteria. Organic compounds are introduced into the subsurface, in general, via burial of detrital organic matter or as solutes that are transported to the subsurface in the form of dissolved organic carbon (DOC) in waters that percolate downward and recharge aquifers. Microbial generation of energy in deep subsurface environments results from biochemical reactions involving the oxidation of reduced compounds and the subsequent transfer of electrons to an adjacent oxidized compound. It is these metabolic processes that have a great impact on microbial ecological interactions in the subsurface and subsequent impacts of microbial metabolism on groundwater geochemistry and geological processes such as diagenesis (1). This article will provide an overview of the sources of energy that drive microbial metabolism in the subsurface and the physical constraints on the presence and function of subsurface microorganisms. The distributions and general characteristics of microorganisms in the subsurface will be examined and critical issues with regards to sampling the subsurface and enumerating associated microorganisms will be discussed. Finally, the extent of the subsurface biosphere on the Earth will be explored along with how this concept has focused the search for life elsewhere in the solar system to the subsurface of other planetary bodies.

ENERGY SOURCES

Microbial processes involving energy generation are constrained mainly to electron transfer reactions involving the following elements: hydrogen, carbon, nitrogen, oxygen, sulfur, manganese, and iron. The scale of redox couples involving these elements and the relative energy yield of each reaction is shown in Figure 1. The electron activity, shown as ε^0 or the negative log of the electron activity in the diagram, is defined as:

$$p = -\log\{\varepsilon\} = p + 1/n \log\{\text{oxidized}\}/\{\text{reduced}\}$$

where {oxidized} and {reduced} are the activities of the oxidants and reductants, respectively. The redox couples are shown in descending order in Figure 1, from the strongest oxidants at the top to the strongest reductants at the bottom. From this diagram it can be seen that, from a thermodynamic standpoint, it is feasible to couple the oxidation of organic carbon to carbon dioxide using O_2, NO_3^-, MnO_2, FeOOH, or SO_4^{2-}. Figure 1 also provides the ΔG values for the oxidation of organic carbon coupled to various microbial electron acceptors: these values indicate that the oxidation of organic carbon should occur sequentially, beginning with the reduction of O_2, the most thermodynamically favored reaction, in descending order through methane fermentation. From microbial and geochemical standpoints, there are a number of factors that can influence the sequence and extent of these reactions, such as the bioavailability of substrates and reaction kinetics. However, at macroscopic scales in systems where microorganisms are active, such as a sediment–water interface or along an aquifer flowpath, the sequential utilization of the oxidants as

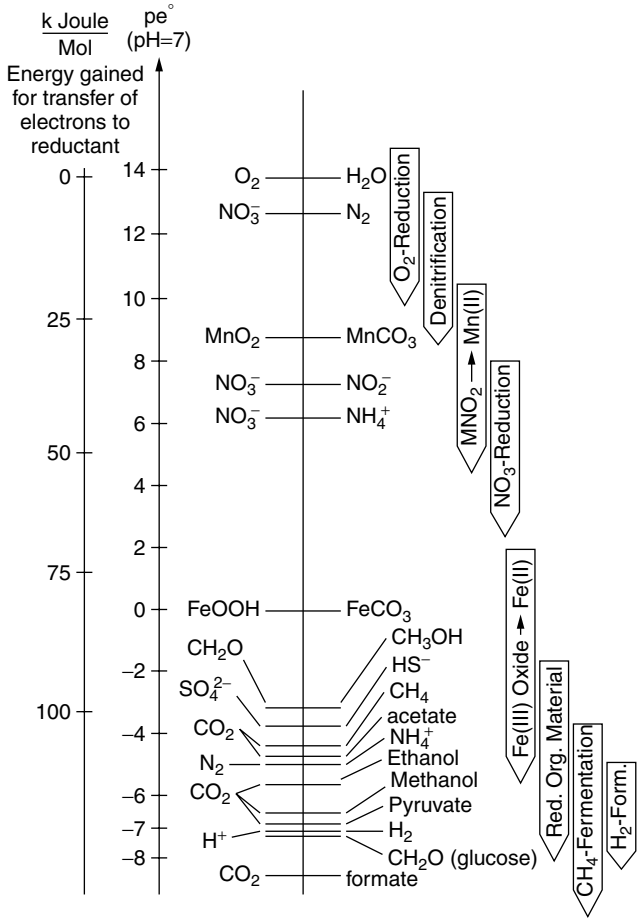

Figure 1. In a closed aqueous system, the oxidation of organic matter is achieved predominantly via microbial metabolism. Oxidation of CH_2O is coupled to the reduction of electron acceptors in order of decreasing $p\varepsilon$ or increasing thermodynamic favorability, From Reference 2, with O_2 being consumed first, followed by nitrate, Mn(IV), and so on.

electron acceptors can be observed readily. The change in relative concentration of redox active aqueous species with increasing distance along an idealized groundwater flowpath is shown in Figure 2.

One of the principal carbon and energy sources in sedimentary depositional environments is organic matter; this also holds true for many terrestrial subsurface environments. Dissolved or colloidal organic matter is likely only a significant source of reductant in shallow (<50 m) aquifers; whereas sediment-associated (detrital) organic matter of terrestrial or marine origin and soluble hydrocarbons are believed to be major sources of carbon and energy for microorganisms in deeply buried sediments and sedimentary rock. Another potentially important source of energy for deep subsurface microorganisms is methane. Although most deep terrestrial groundwaters are anoxic, methane is oxidized in anaerobic sediments (4). More recent evidence suggests that methanogens operating in reverse (oxidizing methane to hydrogen and carbon dixoide) in cooperation with sulfate-reducing bacteria may be key microorganisms in anaerobic methane oxidation in sediments (5). Given the widespread occurrence of methane in the deep subsurface, it is possible that it may be a significant source of energy for microbial metabolism, particularly in environments with significant concentrations of sulfate or Fe(III).

In addition to organic carbon, reduced inorganic elements, for example, Fe^{2+}, Mn^{2+}, S^{2-}, or H_2, can also provide reducing power for microbial metabolism. These reactions are particularly favorable when coupled to a strong oxidant such as O_2 (Fig. 1), but some microorganisms can gain energy from oxidation of some reduced inorganic species coupled to weaker oxidants such as nitrate. Microorganisms that can derive energy from the oxidation of inorganic compounds for metabolism and growth are termed *chemolithotrophs* or *chemolithoautotrophs*. Reduced inorganic species may be the dominant source of energy for microorganisms in some deep terrestrial subsurface environments, particularly those dominated by hard rocks, such as basalt (6,7) or granite (8), where organic carbon is scarce or absent. The extent to which H_2 drives deep subsurface microbial metabolism on a global scale is yet to be determined. There are a number of processes by which H_2 can be generated in deep geological strata (9) but these must be further explored and quantified before the theory of a deep biosphere driven by H_2 can be confirmed.

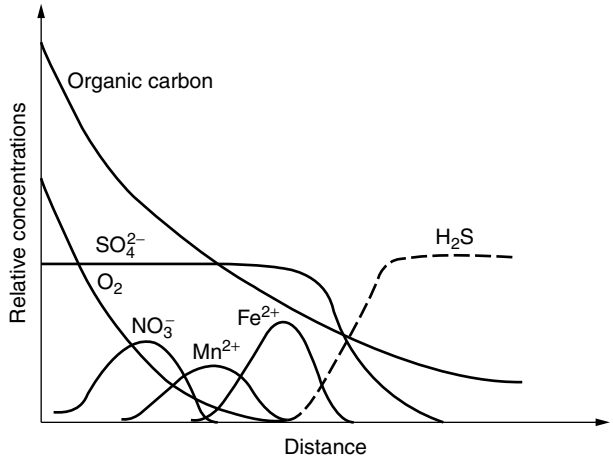

Figure 2. Changes in groundwater concentrations of ions reflecting the influence of microbial metabolism along an aquifer flowpath (3).

PHYSICAL CONSIDERATIONS

It is clear that microorganisms have evolved or adapted to occupy essentially any niche in which energy can be obtained from oxidation/reduction reactions involving organic and inorganic species, as long as those reactions have a favorable *G*. The ability of microorganisms to survive or even thrive in environments in which energy is theoretically available is influenced by a number of biological and abiotic factors. In the deep subsurface, as in other environments, the distribution, community composition, and activity of microorganisms are largely a function of the chemical and physical properties of the environment.

Assuming permissive temperatures and available energy, available pore space and water are two important requirements for microorganisms in the subsurface. Because of their small size, generally 1 mm or less, prokaryotes are more widely distributed in the subsurface than are microeukaryotes (10). Therefore, subsurface environments are microbial ecosystems that, with the exception of shallow, organic-contaminated coarse-grained aquifers (11), are generally free of microeukaryotic predators.

Spaces in rocks and sediments consist of pores of various sizes, fractures or fissures and, more rarely, macroscopic cavities. Pore size and interconnectivity are important factors controlling microbial presence and activity, particularly in sediments and sedimentary rocks. Pore size can have a direct impact on microorganisms. For example, the size of most bacteria, the dominant microbial inhabitants of deep subsurface environments, ranges from several tenths of a micron to approximately 10 μm. Therefore, bacteria are generally absent or inactive in rock or sediments where the pores average less than several tenths of a micron in diameter. For example, in deep core samples collected from a shale-sandstone sequence in northwestern New Mexico, no metabolic activities were detected in samples where pore throats were 0.2 μm or less (12). In contrast, significant activities were detected in cores with most of the porosity associated with pores from 0.2 to 15 μm. Interestingly, in some of the poorly permeable shales with small pore throats, viable sulfate-reducing bacteria were recovered and reduction of $^{35}SO_4^{2-}$ was detected (13). It has been postulated that bacteria in low-permeability subsurface sediments and sedimentary rocks may be remnants of microbial communities associated with sediments during deposition (14,15), but such hypotheses have proved difficult to test. Interestingly, the transition zones between poorly permeable but organic-rich and permeable but organic-poor strata appear to be regions of the subsurface that are relatively productive and contain a diverse microbial community that oxidizes detrital organic matter associated with the poorly permeable material (13,16,17).

Water availability (potential) and water content are also important factors impacting the ability of microorganisms to survive and function in the subsurface. Water availability and content are an important concern to microorganisms residing in the vadose zone. A detailed discussion of water availability is beyond the scope of this article and is discussed in greater detail in the articles in this section by Kieft and Brockman.

MICROBIAL DISTRIBUTIONS IN THE SUBSURFACE

The geological history of the subsurface influences the rate and age of water flowing through the system, the porosity and permeability, and the chemical composition of the rocks or sediments that ultimately influences aqueous geochemistry and nutrient availability. Although not a complete listing, Table 1 provides a number of examples of geological environments representing regional or intermediate flow systems (18) that have been sampled for microbiological analyses. Table 1 is arranged in sections beginning with sediments, followed by consolidated and indurated rock, and finally groundwaters from deep subsurface environments.

Examination of a limited data set comprising broad level measurements, such as microbial biomass and viable bacteria, does not allow the development of specific conclusions regarding ecological relationships between the microorganisms and the subsurface environment. However, there are several striking and important aspects to this summary. With the exception of the Atlantic Coastal Plain (ACP) sediments sampled on the U.S. Department of Energy's Savannah River Site (19,20), the estimates of microbial biomass associated with subsurface sediments and rocks are several, and in some cases many, orders of magnitude below the biomass in near-surface sediments and soils. Although this result might be expected, low biomass is an important feature of deep subsurface environments and impacts how scientists view and study the interactions of subsurface microorganisms with their environments.

Another important implication of the information presented in Table 1 is the relative paucity of microorganisms in consolidated and indurated rock; many of the samples investigated had microbial biomass levels near or below limits of detection. Although evidence for microorganisms being present in these environments is strong, there are equally as many or more instances of samples where microorganisms were not detected. All of these studies used extensive precautions to reduce and measure contamination during sampling. Nevertheless, it is extremely difficult to eliminate all contamination, an issue that will be discussed in greater detail in the following section. In fact, it is a testament to using rigorous procedures that microorganisms were below limits of detection in many samples. The paucity of microorganisms in these environments is expected because the lack of nutrients, water, pore space, and so on, would not be expected to support extensive microbial populations. Not surprisingly, scientists investigating these environments have had a tendency to focus on samples in which cells are observed directly or indirectly via growth in enrichments. Yet, it is also important to note environments where the populations of microorganisms are below detection using standard methods. This information provides important constraints on the types of environments that microorganisms may inhabit in the subsurface and illustrates their common patchy distribution (34).

In some contrast to rock cores, groundwaters sampled from aquifers invariably harbor bacteria at densities between 10^3 and 10^6 mL^{-1}. Valid concerns have been raised as to whether organisms associated with groundwater sampled from wells represent indigenous subsurface microorganisms, allochthonous microorganisms that colonized the well following construction, or both. Sterile solids suspended in groundwater wells were rapidly colonized by bacteria (35); the source of these organisms was believed to be indigenous aquifer bacteria. Also, recently established wells contain higher populations of bacteria than do older established wells (36), indicative of a disturbance-enhanced population. In spite of these concerns, bacteria are invariably observed in waters flowing from fissures in mines and in groundwater from established wells that have been purged to eliminate any organisms associated with stagnant water in the well bore (29), indicating they are indigenous groundwater microorganisms.

The common occurrence of microorganisms in groundwaters, almost regardless of environment, should not be surprising because bacteria are dependent upon diffusion, through water, of substrates to and of products from cells for metabolism and growth. Groundwater from wells or fissures originates from the most transmissive zones of the aquifer, and these are the zones that typically have adequate pore space and higher fluxes of nutrients to support microbial populations. Hence, deep terrestrial environments through which water is readily transmitted are more favorable habitats for microbial populations than are regions where pore spaces or fractures are small or nonconductive (12,37).

SAMPLING AND MICROBIAL ENUMERATION ISSUES

Microbiological investigations of deep subsurface environments present an exceptional challenge to scientists for several reasons. The most difficult obstacle is also the most obvious one, accessibility. Other than access via mines (26,38), caves (39), and tunnels (40), the main approach for obtaining subsurface rock and sediment samples for microbiological and geochemical analysis is via drilling and coring. Considerable effort has been directed toward adapting and modifying drilling and coring approaches for obtaining subsurface samples suitable for microbiological and geochemical analyses and for applying tracers, appropriate controls, and so on, to measure and account for contamination (41–43), and this topic is discussed in greater detail in other articles in this section. In spite of these efforts, it is extremely difficult to eliminate and measure low levels of microbiological or chemical contamination, especially when sampling environments that are deep (>300 m) and require the use

Table 1. Microbial Biomass in the Deep Terrestrial Subsurface Environment

Site Description	Geology/Sample Type	Microbial Biomass	Biomass Method	Notes	References
Atlantic Coastal Plain; Black Creek Formation	Lower delta plain sediments; lignite, Fe sulfides	10^5 to 10^6 CFU g^{-1}	Viable plate counts	Aerobic heterotrophs	19,20
		4×10^6 to 4×10^7 cells g^{-1}	Acridine orange direct counts	Total bacteria	
Atlantic Coastal Plain; Middendorf Formation	Upper delta plain sediments; fine to coarse sands	10^2 to 4×10^7 CFU g^{-1}	Viable plate counts	Aerobic heterotrophs	19,20
		8×10^5 to 7×10^7 cells g^{-1}	Acridine orange direct counts	Total bacteria	
Atlantic Coastal Plain; Magothy and Patapsco Formations	Fluvial sands, gravels, clays	10^4 to 10^5 cells g^{-1}	Acridine orange direct counts		21
		10^2 to 10^4 cells g^{-1}	Direct viable counts[a]		
South Central Washington; Ringold Formation	Lacustrine; compact silty clay	<1 to 10 MPN g^{-1}	Most probable number	Anaerobic (fermenters)	22
		$<4 \times 10^4$ to 10^5 cells mL^{-1}	Acridine orange direct counts	Total bacteria	21
		<0.5 to 45 pmol PLFA g^{-1}	Total phospholipid fatty acids[b]	Total bacterial biomass	14
South Central Washington; Ringold Formation	Paleosol; clay, silt, sand, carbonate nodules	<1 to 10 MPN g^{-1}	Most probable number	Anaerobic (fermenters)	22
		$<4 \times 10^4$ to 2×10^5 cells g^{-1}	Acridine orange direct counts	Total bacteria	22
		<0.5 to 1 pmol PLFA g^{-1}	Total phospholipid fatty acids	Total bacterial biomass	14
Nevada Test Site; Rainier Mesa	Zeolitized tuff with perched water	7×10^4 CFU g^{-1}	Viable plate counts	Aerobic heterotrophs	23
		6×10^7 cells g^{-1}	Acridine orange direct counts	Total bacteria	
Western Colorado, Piceance Basin, Wasatch Formation	Cemented sandstone; cross-bedded siltstones and shales	Occasional cell growth	Enrichments	Iron-reducers, fermenters	24
		<0.4 to 10.1 pmol PLFA g^{-1}	Total phospholipid fatty acids	Total bacteria	
Northwestern New Mexico, Cerro Negro site, Clay Mesa Formation	Shale; low permeability, high total organic carbon and total sulfur	Cell growth in one of four samples	Enrichments	Sulfate-reducing bacteria	12
		<0.4 to 4.2 pmol PLFA g^{-1}	Total phospholipid fatty acids	Total bacteria	
Northwestern New Mexico, Cerro Negro site, Cubero Formation	Sandstone; high permeability, low total organic carbon and total sulfur	No growth in five samples	Enrichments	SRB, fermenters	12
		<0.4 to 1.5 pmol PLFA g^{-1}	Total phospholipid fatty acids	Total bacteria	
Northern Virginia, Taylorsville Basin	Shale, siltstone, sandstone	Below detect to 10^5 MPN g^{-1}	Most probable number	Dissimilatory iron reducers	25
South Africa, Witwatersrand Basin	Mineralized carbon leader	3×10^7 to 7×10^7 cells g^{-1}	Acridine orange direct counts	Total bacteria	26
		<0.4 to 62 pmol PLFA g^{-1}	Total phospholipid fatty acids	Total bacterial biomass	

Table 1. (*Continued*)

Site Description	Geology/Sample Type	Microbial Biomass	Biomass Method	Notes	References
Central Sweden, Stripa mine	Groundwater from quartzbanded hematite/granite	1.6×10^3 to 2.3×10^5 cells mL^{-1}	Acridine orange direct counts	Total bacteria	27
	Glass slides contacted with groundwater for 56 to 117 days	2.2 to 5.5×10^6 cells cm^{-2}	Acridine orange direct counts	Total bacteria	
Southeastern Sweden, Äspö rock laboratory	Groundwater associated with granites	7.5×10^4 to 1.8×10^6 cells mL^{-1}	Acridine orange direct counts	Total bacteria	27
		4.1×10^2 to 3.9×10^4 CFU mL^{-1}	Viable plate counts	Anaerobic heterotrophs	
South Africa, Witwatersrand Basin	Groundwater	5×10^4 cells mL^{-1}	Acridine orange direct counts	Total bacteria	26
		<0.4 pmol PLFA g^{-1}	Total phospholipid fatty acids	Total bacterial biomass	
Gabon, Africa, Oklo natural reactors	Groundwater from wells penetrating Oklo uranium deposit	4.5×10^4 to 5.8×10^5 cells mL^{-1}	Acridine orange direct counts	Total bacteria	28
South Central Washington, Columbia River Basalts	Groundwater (artesian) from Priest Rapids aquifer	3.6×10^3 cells mL^{-1}	Acridine orange direct counts	Total bacteria	29
		$\geq 10^4$ organisms mL^{-1}	Enrichments	H_2 acetogens[c]	
South Central Washington, Columbia River Basalts	Groundwater (artesian) from Grande Ronde aquifer	7.6×10^5 cells mL^{-1}	Acridine orange direct counts	Total bacteria	29
		$\geq 10^4$ organisms mL^{-1}	Enrichments	H_2 acetogens, H_2 SRB	
North Central Montana	Groundwater from Madison Formation	0.8 to >240 MPN mL^{-1}	Most probable number	Thermophilic SRB	30
		1.0 to 1.3×10^3 mL^{-1} to 1.3×10^3 mL^{-1}	Acridine orange direct counts	Total bacteria	
Russia, Volgograd Province	Stratal waters (groundwater from artesian well)	60 to 250 cells mL^{-1}	Dilution plate counts	Methanotrophic bacteria	31

[a] Direct viable counts as determined using the method developed by Kogure and coworkers (32).
[b] A conversion factor of 5×10^5 cells μmol^{-1} for bacterial biomass in subsurface sediments has been reported (33) although this value is likely to vary by as much as one order of magnitude because of variations in cells size, degree of starvation, and so on. A value of 0.4 μmol PLFA g^{-1} is considered background.
[c] Acetogens that can grow with hydrogen and carbon dioxide.

of rotary drilling and coring equipment. This is particularly important considering that microbial populations in deep subsurface environments are typically sparse (Table 1). It is often difficult to state unequivocally that a specific microorganism or population of organisms came from a specific sample, particularly one that was obtained using rotary mud drilling and coring. The careful use of innovative drilling and coring techniques to minimize penetration of fluids or other contaminating materials into cores combined with aseptic sampling methods and the use of sensitive tracer techniques can greatly improve the quality of such samples and the confidence that they are representative.

Mines, tunnels, and caves can provide excellent opportunities for studying deep subsurface microorganisms and the processes they catalyze. However, there are a number of factors that must be considered when accessing mines for sampling to investigate indigenous microorganisms; these factors have been discussed in detail by Russell (38). A major consideration is that mines often develop microbial communities that appear in response to the development and operation of the mine and, therefore, may not be representative of indigenous endolithic microorganisms. The flow of air associated with ventilation systems can promote the development of aerobic lithoautotrophic biofilms on rock surfaces or is associated with reduced groundwater that seeps into the mine. Ventilation systems and routine mine operation can disperse allochthonous microorganisms throughout the mine. Therefore, it is important to access fresh rock surfaces, well away from any potential impacts from ventilation and wall surface colonization, when sampling for indigenous subsurface microorganisms. Tunnels and mines are particularly well suited for certain types of investigation,

such as establishing the spatial distributions of microorganisms (40,44).

Sampling of groundwater can also provide important insights into the microbial ecology and biogeochemistry of subsurface environments; in some cases it provides the only means for sampling the subsurface. For example, in low-porosity rock, microbial communities will be concentrated along fractures. Because of the size and the capacity for transmitting water, fractures are extremely susceptible to intrusion by drilling fluids that can harbor microbial contaminants (45). Sampling of groundwater may be the only means of obtaining representative microbial communities from such environments. When collecting groundwater samples for microbiological analysis, an important issue is whether the microorganisms associated with the water are representative of the in situ populations. Although it is assumed that most microorganisms in the subsurface are attached to solids, there is little information comparing groundwater-associated, or planktonic, microorganisms with those attached to solids (46). Also, groundwater, depending on the well configuration and screened interval distance, can represent mixed (42) water from several regions within the aquifer, whereas solids are usually considered "point" samples. Hence, mixed waters from different regions may not be characteristic of any particular section but rather constitute a composite sample (47). Vertical mixing of groundwater over a screened interval can result in considerable dilution of solutes and colloids, including suspended microorganisms. Because of these issues, the collection of samples from discreet zones within the subsurface is preferred. There are several approaches for conducting this type of sampling, including the use of a passive multilevel sampler (48) that has been applied to shallow subsurface environments (49).

GENERAL MICROBIOLOGICAL CHARACTERISTICS OF THE SUBSURFACE

It is estimated that more than 99% of the microorganisms existing in nature are not cultivated using standard microbiology techniques (50). Such estimates are based on comparisons of culturable microorganisms detected using dilution plating, or most probable number enumerations of total microbial populations measured by direct microscopic counts of cells, or analyses of biochemical components of microbial cells such as lipid phosphate or adenosine triphosphate (51). Although culturing methods have been used extensively by microbial ecologists, they are of limited application for studying subsurface microbial populations. Because of the requirements for sample dispersion to dislodge cells from particle surfaces, and dilution, the practical detection limit for direct microscopic counting of cells in sediment or rock samples is approximately 10^4 to 10^5 cells g^{-1}. The measurement of total microbial phospholipid fatty acids is one of the most sensitive techniques for estimating microbial biomass and has been applied extensively to subsurface samples (12,14,24,52). Even with this sensitive technique, the detection limits are in the low picomolar range (53) (per gram of sediment or rock), a value that corresponds to between 6×10^4 and 5×10^5 cells g^{-1} based on reported conversion factors (33,52). Microbial lipid measurements have an added advantage in that they can provide information on community composition and physiological state (54,55). One advantage of sampling microorganisms associated with groundwater is that the biomass can be concentrated by filtration (56), in effect improving the limits of detection.

Because of low levels of metabolic activity and the potential for the presence of dormant cells in many subsurface environments, a major challenge in interpreting biomass measurements is in establishing the extent to which cells are viable versus dead or moribund. A number of staining techniques have been developed, which have attempted to distinguish viable from nonviable cells microscopically on the basis of esterase activity as detected by the ability to cleave fluorescein diacetate (57) or membrane integrity using commercially available staining kits (58). These methods may have some utility in subsurface microbiology but they are also limited in that they do not work for all types of microorganisms and may not be sufficiently sensitive to measure low levels of metabolism. Because they are microscopy based, they are also subject to the problems described earlier. Measurements of diglyceride fatty acids (DGFAs) have been used as indicators of dead cells in deep subsurface samples (52) and in microcosm studies investigating bacterial survival (59). Where the ratio of DGFA to phospholipid ester-linked fatty acids (PLFA) has been used as a measure of dead to viable biomass in deep subsurface environments, the ratio often exceeds 1 (12,52,60).

Although phospholipids are rapidly turned over in sediments where bacteria are active (61), the turnover rate in low biomass deep subsurface sediments and rocks is unknown (12). Typically, the PLFA concentrations are higher in the clay-rich layers relative to sandy- or coarse-textured sediments. Establishing concentrations of viable biomass in deep subsurface environments is problematic because of detection limits imposed by currently available methods and because of uncertainties in distinguishing viable cells from dead cells.

The rates of in situ microbial metabolism in the deep subsurface are among the slowest in those environments where microorganisms are known to be active (15). Phelps and coworkers (62) estimated that the average carbon dioxide respired on a per cell basis in deep aquifer sands of the Atlantic coastal plain of the southeastern United States was 0.00002 pmol $year^{-1}$, which translates into an estimated doubling time of cells on the order of several centuries. Numerous other studies using either direct measurements of microbial activities associated with subsurface samples or indirect measures of in situ metabolism based on changes in concentrations of electron acceptors or donors along the groundwater flowpath within a pristine aquifer indicate that the rates of microbial metabolism are very slow compared to other environments. Direct measurements of microbial activity, such as those that utilize isotopically labeled substrates, often overestimate in situ activities based on groundwater analyses by as much as 10^6-fold (62).

In separate studies using chemoheterotrophic bacteria isolated from deep subsurface environments, Kieft and coworkers (52) and Amy and coworkers (63) found that the cells underwent typical starvation-survival responses observed in other bacteria, including reduction in cell volume and decreasing viability, based on the ability to grow on culture media, relative to the total number of cells. Microbial populations in deep subsurface environments are generally characterized as having a high proportion of miniaturized, nonculturable cells (64). The physiological state of microorganisms, termed *starvation-survival* (65,66), is probably common to many deep subsurface environments because of the low flux of nutrients and energy sources. In effect, deep terrestrial subsurface environments contain microbial cells that range from recently divided culturable to nonculturable cells in a state of starvation-survival to dead, moribund cells that are slowly decaying.

CONCLUSION: A PLANETARY PERSPECTIVE

Evidence accumulating over the past decade supports the existence of a global deep subsurface biosphere (67) which may extend to significant depths in the Earths crust. Thermophilic bacteria have been isolated from core samples collected at depths of 2.7 km (68,69). These bacteria have physiological characteristics consistent with the environment from which they were isolated. There have also been reports of thermophilic bacteria isolated from depths greater than 5 km (70) but, in this case, the bacteria were enriched from water samples collected from a borehole rather than from core samples. The current upper limit for microbial growth is 113 °C (71), and the upper temperature for survival under nongrowth conditions may be significantly higher. Onstott and coworkers (72) estimated the depth limits to be from 4 to 6 km for microorganisms, using a conservative 110 °C upper limit for growth and survival and average geothermal gradients of 26 and 19 °C/km for oceanic and continental crust, respectively. The presence of a deep biosphere has significant implications on global ecology, subsurface geological processes, the development and evolution of life on earth, and the search for life elsewhere in our solar system.

There have been several estimates of the total number of prokaryotes (25,73) in the subsurface, using a variety of assumptions and biomass values that represent a relatively small fraction of the subsurface. Although there is considerable uncertainty, the estimates indicate the total number of prokaryotes in the subsurface is immense, ranging from 10^{28} to 10^{30} cells, a value that exceeds the number inhabiting other regions of the biosphere (73). Additional scientific investigations are required to refine our understanding of the deep biosphere, to better define the limits and environmental controls, and to better understand the contributions of the deep biosphere to the overall functioning of our planet.

The subsurface represents a compartment of the biosphere that would have been largely protected from the effects of catastrophic events, such as asteroid collisions or major volcanic eruptions that were probably responsible for the extinction of many multicellular life forms over the history of the Earth. In addition, prokaryotes inhabiting the subsurface would be relatively shielded from the effects of long-term changes in the Earth's climate. Indeed, there is evidence in the form of microfossils (74,75) that prokaryotes have long inhabited the subsurface and that the ancient organisms functioned in a manner similar to modern subsurface microbial communities. The concept of a deep biosphere that functions independent of sunlight and is shielded from atmospheric and climactic changes has focused the search for life elsewhere in our solar system on the subsurface of Mars and Europa (76). Mars is a likely candidate for such a search as it has been suggested that there may remain some volcanic activity deep in the planet that could provide energy in the form of CH_4, H_2, or H_2S for chemolithotrophic microorganisms (77), such as those found in the Columbia River basaltic aquifers (6). Although conditions on the surface of Mars are unlikely to be conducive to microbial life as we know it, liquid water may be present beneath the surface of the red planet, which may provide suitable conditions for microorganisms. Although there appears to be sufficient information to suggest the subsurface of Mars and even Europa are logical targets for the search for life elsewhere in our solar system, the technical challenges of obtaining and analyzing samples while meeting contamination and planetary protection criteria (78) will be great.

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ECOLOGY OF MARINE MICROBIAL BIOFILMS

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Microorganisms attach to virtually any wetted surface submerged in aquatic environment. The cells are mostly immobilized and produce extracellular polymeric organic substances (EPS), grow and divide, thus forming the structure of an assemblage of microorganisms termed biofilm. Besides bacteria, cyanobacteria, microalgae, protozoa, fungi, and invertebrate larvae contribute to the complex structure and function of natural assemblages. The *term* biofilm is equivalent to the German word *aufwuchs* (growth on surfaces) (1). Although the latter term seems to be more appropriate because it relates to a dynamic process, it has not gained

general acceptance. To characterize natural biofilms on surfaces in aquatic environments, the adjectives epiphytic (on plants), epilithic (on rocks), and epixylic (on wood) are commonly used. The corresponding microbial communities are called epiphyton, epilithon, and epixylon, respectively.

The development of microbial biofilms is the net result of transport and exchange processes between the biofilm and the surrounding medium, and modification and decomposition processes of inorganic and organic matter within the biofilm. Biofilms consist of a mono- or multilayer of cells usually some hundred micrometers to some millimeters thick, although stabilized biofilms can grow to a thickness of some centimeters, gaining the character of mats. Biofilms and mats may be differentiated from each other by their dimensions and the stabilization, which characterize the latter. Biofilms reveal pronounced spatial and temporal heterogeneities. Besides the cells and their products, inorganic and organic material adsorb to the biofilms because of the spongy and porous structure of the complex matrix (2,3).

Although there is much information available on the attachment of bacteria to artificial surfaces of different properties as well as on the physical and chemical structure of biofilms based on pure cultures, information on the structure and function of natural microbial biofilms is much more limited. This is certainly dependent on the complex structure of natural assemblages of microorganisms which are much more difficult to analyze as compared to pure cultures. In the following review, we intend to summarize some of the information that is available on the ecology of natural marine microbial biofilms. Because virtually all available surfaces in the marine environment are covered by biofilms, the microbial colonization of sediments, plants, animals, suspended particles, and aggregates as well as of artificial (man-made) structures actually have to be considered. However, most of the information seems to be available on sedimentary biofilms. Additional information about biofilms from other habitats, and on pure culture biofilms that might be relevant for the understanding of the function of microbial biofilms in marine sediments is discussed whenever appropriate. The discussion of sedimentary biofilms, however, bears additional constraints. One is the scale of view. A biofilm already exists as an assemblage of a few cells embedded in their organic matrix on the surface of a particle, visible only by microscopic examination. Conversely, biofilms exist that are visible to the naked eye on sediment surfaces. Furthermore, it appears that almost everything that concerns the biological function of sediments relates to microbial biofilms. Dealing with sedimentary biofilms, therefore can be understood as a general discussion of the roles of microorganisms in marine sediments. This, however, is beyond the scope of the review. Instead, it focuses on the most important aspects that have specific relevance for the understanding of the structure and the function of marine sedimentary microbial biofilms.

MICROBIAL ATTACHMENT TO SURFACES

For the study of the development of microbial biofilms, various microscopic techniques have been applied, such as transmitted or incident light microscopy, and transmission or scanning electron microscopy (4). Recently, confocal laser scanning microscopy in combination with image acquisition and image analysis techniques have been used in comparison to conventional microscopic techniques (5–7). Generally, it can be concluded that confocal laser microscopy represents an invaluable tool for examinations of the spatial structure and growth of biofilms. However, species richness and relative abundance of cyanobacteria in biofilms was best enumerated by scanning electron microscopy (8).

The microbial colonization of surfaces is a matter of discussion. From the earlier work it becomes obvious that the colonization pattern generally can be divided into different phases with overlapping time sequence (9). The first step is the formation of a conditioning film of organic compounds (e.g., polysaccharides and proteins), which rapidly develops if clean surfaces come in contact with water. As a result of the decrease in the energy of the surface by the formation of the organic layer, the total free energy of the system decreases. Differences in the replication cause some doubts on the correct performance of experiments using “clean” surfaces (10). Within less than an hour after the conditioning, organisms attach to surfaces. Earlier investigations point to an obligatory order in the succession. Following bacteria, diatoms, cyanobacteria, microalgae, protozoa, fungi, and invertebrate larvae attach in the time span of hours, days, and months (4,9,11,12). Very small starvation forms of copiotrophic bacteria, adapted to growth under rich nutrient conditions, seem to be the initial colonizers. Because of the elevated nutrient concentrations on surfaces, starved bacteria may grow into rod-shaped forms (13). The subsequent appearance of oligotrophic, prosthecate bacteria, adapted to growth under low-nutrient conditions, was interpreted as a result of the exhaustion of nutrients by the initial growth of copiotrophic bacteria on the surfaces. Using a cultivation-independent genetic approach, the diversity of bacterial colonists was determined on test surfaces differing in their degrees of surface free energy. The results indicated that the succession rate of the early bacterial community may be influenced by surface physicochemical properties (14). In the literature, however, there is some doubt about the obligatory order of the sequence of colonization. Investigations imply that diatoms may colonize surfaces as rapidly as bacteria. Furthermore, problems arise from the difficulties in distinguishing between settlement on one hand, colonization and subsequent growth on the other (10).

The transport of microorganisms to surfaces is mainly governed by physical forces (4,9). Planktonic bacteria can be transported over large distances by water currents and turbulence. Fluid frictional forces slow the bacteria down in close vicinity to surfaces. Chemotactic response to a nutrient gradient around surfaces may be an additional possibility for motile bacteria to approach surfaces. After

penetrating the structured layer of water molecules surrounding all solid surfaces in water, physical forces between the cells and the surface become relevant. The cells are immobilized by antagonistic electrical repulsion and van der Waals attraction forces. During this stage the cells can be easily removed from the surface by gentle shear forces. Bacteria may overcome the electrostatic barrier by the secretion of polymers (polysaccharides, proteins, glycoproteins), which can bind the cells to the surface by various short-range forces. Therefore, the reversible attraction (adsorption) turns into an irreversible attachment (adhesion).

Results of the influence of substratum surface on the microbial attachment differ in the literature, dependent on the type of organisms and their physiological state, as well as on the experimental conditions that were not always reproducible (10). In this context, it is notable that environmental stresses (extremes of pH, high concentrations of oxygen, metals, and xenobiotics) induce formation of biofilms by an anaerobic hyperthermophilic marine bacterium (15). The attachment of bacteria to surfaces stimulates the production of extracellular polysaccharides (16). Evidence is increasing that the synthesis of extracellular polymers is the response of bacteria to (multiple) environmental signals. For a series of organisms, it could be shown that polymers used in the initial adhesive event and matrix polymers are distinct (10,17).

In a dynamic system like a biofilm, attachment and detachment occur. As a result of increased fluid shear forces, grazing, enzymatic degradation of the adhesive structures, and reproductive mechanisms, cells detach from surfaces and enter the planktonic phase (4). The extent of detachment could be derived from experiments following the rapid colonization of pure cellulose particles added to sediment suspended in particle-free interstitial water. Eventually, biofilm material is released in flocs caused by increased fluid shear forces, a process termed *sloughing*.

Equations have been described regarding attachment and growth during surface colonization (18). Growth, detachment, reattachment, attachment of foreign cells, and death of single cells were included in a mathematical model for the growth of bacterial microcolonies on marine sediments (19). By automated analysis of digitized images of acridine orange-stained culture cells, cellular RNA and DNA contents, cell volume, and frequency of dividing cells were determined as four independent growth rate–related parameters. Attached cells showed an almost constant growth rate that was independent of the dilution rate in the chemostat (20). Recently, a fluorimetric method with a high sensitivity based on the in vivo fluorescence of chlorophylls has been described to measure growth of adhering phototrophic microorganisms (21).

BIOFILM ARCHITECTURE AND FUNCTION

Biofilm systems generally consist of various compartments: substratum, base and surface biofilm, bulk liquid, and gas phase (22). The nature of the substratum plays an important role in the early development of biofilms. In the case of the colonization of wood or detritus, for example, the substratum may equally serve as a decomposable substrate. The base biofilm is relatively compact and well structured, and molecular diffusion dominates. The surface biofilm usually shows an irregular topography, and interfacial transfer processes dominate. The bulk liquid influences the transport of cells, material, and energy to the biofilm by its flow dynamics, and also facilitates the removal of end products of the microbial metabolism from the biofilm. The gas phase provides the supply of oxygen and carbon dioxide, and the removal of gaseous microbial metabolites (e.g., nitrogen, methane, hydrogen sulfide). The biofilm architecture is very complex. From confocal laser scanning microscopy (23), it is known that channel-like structures connect the film surface with deeper layers to allow the transport of nutrients and waste products in an otherwise diffusion-limited system. The composition of nutrients has a significant impact on the biofilm architecture. The cultivation of laboratory biofilms with a commercial herbicide and trichlorobenzoic acid, respectively, resulted in highly specific patterns of cellular coaggregation and growth, which did not develop when the biofilms were grown on more labile substrates (24,25).

Biofilms colonize stones in shallow water coastal inlets ("Bodden"), in the brackish water of the southern Baltic Sea (26). The communities grow to a thickness of some millimeters and consist of photoautotrophic and heterotrophic microorganisms (photoheterotrophic biofilms). The surface biofilm represents the photoautotrophic component mainly consisting of filamentous cyanobacteria, diatoms, and green algae. It reveals a loose structure and an irregular topography with filaments of cyanobacteria extending into the water. Bacteria and hyphae of actinomycetes were closely associated with cyanobacteria. With increasing depth, the biofilms became more dense. The compact base biofilm consisted of coccoid cyanobacteria and heterotrophic bacteria, embedded in an extensive slime matrix secreted by the organisms. The carbon content (C) of the photoheterotrophic biofilms varied between 50 and 70% of the dry weight. With increasing biofilm depth, the ratio between C and ATP (adenosine triphosphate, the energy storage of the cells) increased from 100 to 200, indicating less ATP per unit of carbon. This can be explained by changes in biofilm structure. From the surface to the base biofilm, the matrix became more dominating. Besides the cells, the matrix contributed to C, but not to ATP.

Until the early 1980s, the release of copious slime secretions (extracellular polymeric substances, EPS) by microbial cells was largely overlooked (27). Exopolymers are abundant as capsules closely surrounding microbial cells or as a dispersed slime in colloidal and particulate form. Microorganisms spend a large portion of their carbon and energy supply (up to about 60%) for the production of extracellular secretions, which primarily consist of polysaccharides including other components (e.g., amino acids, amino sugars, uronic acids and glycoproteins) of high molecular weight. The porous gel structure and the highly adsorptive nature of EPS are responsible for a number of biological functions, which enhance the survival and the competitive success of microbial cells.

Table 1. Functions of the Extracellular Organic Matrix for Biofilm Microorganisms and Their Environment

Functions of EPS	Literature
Attachment, detachment, movement	4,9,10,39
Concentration, localization of extracellular enzymes	2
Protection against environmental changes	4,39
Binding, storage of organic material	34,35
Binding, detoxification of pollutants	4,39
Symbiosis, syntrophic relationships	39
Genetic transfer	29
UV protection	30,31
Inhibition of adhension, motility	38
Inhibition, facilitation of settlement of larvae	37
Trophic source	36
Stability of detritus, sediments	2,131

The fibrous polymer matrix plays a key role for the organisms and their environment (Table 1). EPS contribute to the attachment of cells, although not all exopolymers secreted are involved in adhesion. Different types of extracellular polymers are secreted by microbial cells for different functions (i.e., attachment, detachment and movement on surfaces). Through the secretion of polymers around the cells, microorganisms create and maintain favorable microenvironments, which allow them to use carbon and energy efficiently. Extracellular enzymes can act in close proximity to the cells, and decomposition products of the hydrolysis of higher molecular weight material can be directly taken up by the cells. Extracellular enzymes liberated through the lysis of organisms bind to the exopolymers, which may protect the enzymes from degradation, in a manner analogous to the enzyme-humic complexes observed in soil (28). If the enzymes retain their activities, they may play a role as biocatalysts of considerable benefit for biofilm cells in their surrounding. In defined bacterial biofilms, high rates of conjugative gene transfer were observed (29). Because of its capacity to retain water, bacterial slime protects the cells against rapid changes of environmental conditions (e.g., pH, desiccation). The biofilm matrix and the deposition of ultraviolet (UV)-absorbing compounds act as a sunscreen to protect the microbial communities against UV radiation damage (30,31). Cyanobacteria apparently are able to sense UV radiation and respond by migration to limit their exposure to UV light (32).

Exopolymers possess high binding affinities for organic material through the cation exchange processes. Organic material can be concentrated and subsequently metabolized, and this is of special importance under poor nutrient conditions (33,34). Biofilm formation represents an effective mechanism by which starved cells can rapidly recover in environments of an intermittent nutrient supply (35). Besides nutrients, exopolymers bind metals and other poisons, thus presenting a general mechanism for detoxification.

The role of bacteria in symbiosis and syntrophic relationships obviously depends on secretions of polymers. Capsular EPS protect symbiotic bacteria from digestion by their host. For the colonization of heterocysts (specialized cyanobacteria cells for nitrogen fixation) by bacteria, specific polysaccharides are responsible. The slime layer reduces the diffusion of oxygen to the heterocysts, thus allowing the oxygen-sensitive process of nitrogen fixation under otherwise aerobic conditions. Although EPS can protect bacteria from grazing, the exopolymers present a potential carbon source for heterotrophic organisms, including perhaps the producers of the specific exopolymer (36). Laboratory experiments have shown inhibitory and facilitatory effects of microbial biofilms on the settlement of marine larvae (37). A glycoprotein from a gliding bacterium isolated from a biofilm inhibited the colony expansion of another bacterial strain (38). Finally, the extracellular matrix of bacteria and diatoms greatly contributes to the stability of marine sediments by increasing critical shear velocity and modifying bioroughness. For evaluating the important functions of microbial exopolymer secretions in ocean environments, the reader is referred to a comprehensive review for further details and references (39).

MARINE MICROBIAL BIOFILMS

In natural habitats, bacteria attach nonspecifically to a variety of nonbiological and biological surfaces. Specific attachment requires complementary receptors of the cell surface and the substratum. Specific polysaccharides in the EPS can bind to specific proteins (lectins) present on the attachment surface. The use of lectin probes offer a potentially useful technique for the investigation of spatial and biochemical heterogeneities of microbial biofilms (40). Information on the microbial colonization of marine surfaces is very limited. From epifluorescence and scanning electron microscopy analyses, it becomes obvious that the overwhelming portion of the microorganisms in marine sediments is attached to particles. However, the cells colonize only a very small part of the surface available on quartz sand between 0.01 and 5%, dependent on the method used (41,43). A direct comparison of these data is further restricted to the fact that cell numbers were determined applying different homogenization and staining techniques (44). Sand and silt particles are preferentially colonized, whereas clay particles are not colonized, or rarely colonized obviously because of their small size. Besides, the type and size, the roundness of the particles is important: with increasing age (roundness), the colonization decreases. Microorganisms are preferentially found in depressions and crevices of sand grains where they are protected against mechanical shear. Deep fissures of the sand grains are rarely colonized (45). The microbial occurrence in the protected areas of the sand grains is a reflection of their survival rather than their preferential colonization (43). The distribution of cyanobacteria and diatoms is the result of competition for grain sizes and temperature (46). Like many other bacteria, Bdellovibrios (bacteria that are parasites of bacteria) preferred to associate with surfaces and the numbers were significantly increased at higher salinities (47). In intertidal sand mats, the irradiance spectra were quantitatively and qualitatively correlated with the distribution of photoautotrophic microorganisms. Through their pigments, the photoautotrophs govern the penetration of light within

the mat (48). Oxygenic phototrophic microorganisms in hypersaline mats revealed a high diversity according to morphotypes, 16S rRNA genes, and carotenoids (49).

Higher organisms (plants, animals) and their biogenic structures in marine sediments are intensively colonized by microbial biofilms. Structure and function of epiphytic communities on eelgrass (50) and seagrass (51,52) reflected the nutrient availability in the water. Particular sites of marine copepods (depressed parts of the body) were colonized by bacteria of a high diversity (53,54). Infaunal burrows and tubes of worms supported elevated levels of microbial biomass and activities as compared to the bulk sediment (55–58). The stimulating effects of higher organisms and their biogenic structures on microbial communities can be understood by direct and indirect effects. Through their metabolism, plants and animals may directly provide the microbial assemblages with nutrients (for nutritional relationships between plants and microorganisms see the following section). On the other hand, through their biogenic structures (burrows, tubes), benthic animals exhibit a strong impact on sediment topography (bioroughness), thus increasing the availability of oxygen and nutrients (59).

IMPORTANCE OF GRADIENTS IN BIOFILM METABOLISM

The complex structure of microbial biofilms exhibits strong impacts on biological processes. Immobilized in their polymeric matrix, organisms metabolize in close vicinity to each other. Products released by some of the organisms can be directly taken up and metabolized by other organisms. Syntrophic interactions between microorganisms are especially pronounced in biofilms. This requires that physiological groups of organisms with complementary metabolic reactions already colonize in the vicinity. If the colonization is random, the chances of organisms with complementary metabolic reactions coming in contact with each other is low. There is no information yet known in the literature addressing this question. However, the fact that syntrophic interactions function in biofilms point to mechanism(s) of accomplishing the interactions.

From the close association of microorganisms, their high biomass, diversity, and metabolism in biofilms, pronounced gradients in biotic and abiotic parameters arise, which are the net result of various metabolic activities. It can be speculated that the gradients are the driving force for the microbial metabolism. Gradients are highly dynamic including variations in both space and time. Besides spatial and temporal fluctuations in the activity of the organisms, spatial and temporal variations in environmental conditions (e.g., light, nutrients, temperature, salinity, and currents) will greatly influence the expression of the gradients. The application of microelectrodes to biofilms turned out to be a powerful tool to follow small-scale variations in chemical parameters with a minimum of disturbance. With microelectrodes, spatial resolutions of 10 μm and less can be achieved, which characterize the direct sphere of influence of even small consortia of microorganisms. Oxygen microelectrodes were used to analyze the diffusive boundary layer as well as oxygen fluxes in sediments (60,61). Photosynthesis and respiration were calculated from the microdistribution of oxygen (62,63). Although microelectrodes are small in size, their impact on the distribution of oxygen in biological systems has to be considered (64,65). Besides electrochemical microelectrodes, fiber-optical microsensors for the measurements of oxygen, temperature, light (photosynthetically available radiation), and surface detection of biological systems have been introduced recently (66–68). Furthermore, microsensors are available for measurements of depth profiles of pH, hydrogen sulfide (69), nitrite (70), and nitrous oxide (71).

Microelectrode measurements have greatly enhanced knowledge of the impact of environmental conditions on the dynamics of biological systems. Using oxygen microsensors, it could be shown that the diffusive boundary layer covering solid surfaces in aquatic environments, is of special importance for the oxygen flux of sediment biofilms. The thickness of the layer is dependent on the flow velocity of the water and the roughness of the surface. The oxygen uptake of sediments is limited by the diffusion of oxygen through the diffusive boundary layer. Because of the restriction of the oxygen flux, sediment biofilms with high rates of oxygen consumption may become anoxic thus requiring the use of alternative electron acceptors for the oxidation of organic carbon.

ROLES OF NITROGEN, PHOSPHORUS, SULFUR, AND IRON

Limited information is available on the role of inorganic compounds (nitrogen, phosphorus, sulfur, iron) in biofilms and mats. In biofilms from nutrient-rich streams, oxygen respiration and, denitrification activity were calculated from depth microprofiles of oxygen and nitrous oxide. Denitrification occurred only in the absence or at low concentrations of oxygen, the process was determined by the penetration depth of nitrate. Most of the labile organic matter added was used by oxygen respiration in the upper layers of the biofilm (72). The availability of nitrogen and phosphorus greatly influenced the structure and nitrogen fixation of an intertidal microbial mat community. Cyanobacteria were able to circumvent the nitrogen limitation by the fixation of nitrogen, whereas diatoms were dependent on nitrogen and phosphorus in the community (73). In contrast, another study showed that the nutrient limitation of photosynthetic carbon dioxide fixation and nitrogen fixation of tidal marine microbial mats were obviously dependent on their growth rates. Mats from more productive areas showed higher frequencies of nutrient limitation, whereas slow growing tidal mats exhibited no nutrient stimulation (74). The access to phosphate supplies from the water in epiphyte communities was determined by the position of the organisms in the matrix. Loosely attached microalgae took up significantly more phosphate than did underlying adnate cells (75). Pure-culture bacterial biofilms revealed specific patterns of alkaline phosphatase expression primarily controlled by the local availability of the carbon source and the electron acceptor. The dimensions of the zone of active protein synthesis was restricted to the availability of oxygen (76,77). Significant activity

of sulfate-reducing bacteria was detected under aerobic conditions in biofilms of a hypersaline pond system. The response of sulfate-reducing bacteria to specific carbon sources in the individual layers of the mat was different, indicating different groups of sulfate-reducers and/or adaptations to different substrates (78). In aerobic trickling-filter biofilms, sulfate reduction accounted for up to 50% of the total mineralization of organic carbon. The resulting hydrogen sulfide was rapidly oxidized. Anaerobic oxidation of hydrogen sulfide with nitrate as an electron acceptor could be induced (79). The enhanced oxygen uptake of artificial cyanobacterial mats in the light reflected to a large extent the reoxidation of reduced endproducts (notably reduced sulfur) accumulated during darkness (80). In an aerobic bacterial biofilm, sulfate-reducers were present in the oxic and anoxic zones. The genetic diversity of the microbial community increased during the development of the biofilm. Sulfate-reducers detected by molecular analysis were not all sulfidogenically active in the biofilm (81). Facultative aerobic respiration, filamentous morphology, motility, diurnal migration, and aggregate formation were the most conspicuous adaptations of sulfate-reducing bacteria to the life in hypersaline cyanobacterial mats of Solar Lake (82).

Biofilms are able to concentrate minerals from extremely diluted environmental conditions (83,84). The binding of iron and manganese by surface-colonizing bacteria in a lake was accompanied by the fixation of carbon dioxide (13). In epilithic biofilms investigated in an underground research laboratory, elements were chemically complexed together to form amorphous or crystalline fine-grained minerals. The elements were thought to be concentrated by passive sorption and by energy metabolism. One of the biofilms showed both, oxidation and reduction of iron (85). The most abundant mineral associated with epilithic river biofilms was a complex iron-aluminum silicate of variable composition. It was concluded that biomineralization was a surface process associated with the anionic nature of the cell wall (86). An iron deposition associated with cyanobacteria mats was observed in hot springs. Iron was totally oxidized at the surface, whereas reduced iron was detected in greater depths of the mat. It was speculated that photoautotrophs could potentially increase the rate of iron oxidation by increasing the pH and the oxygen concentration (87).

In general, these results seem to indicate that the microbial assemblages are dependent on the uptake of nitrogen and phosphorus from the water, in their metabolism, to maintain a certain structure and/or activity. In marine environments, sulfur seems to be an important alternative electron acceptor besides oxygen for the oxidation of organic material carried out by a high diversity of sulfate-reducing bacteria adapted to various substrates. The oxidation of reduced sulfur compounds causes an enhanced oxygen demand of the community. The accumulation of iron occurs by passive sorption or by energy-yielding reactions carried out by chemoautotrophic microorganisms. The participation of both processes, however, needs further investigation.

POOLS AND SOURCES OF ORGANIC CARBON

Organic material plays a key role in aquatic environments. Particulate organic carbon (POC) represents the majority portion of organic matter in sedimentary systems, whereas dissolved organic carbon (DOC) dominates in water. The pools of DOC and POC consist of a broad variety of material, most of which is only poorly chemically identified. Because organisms contribute through the lysis of cells to the pool of organic matter, one can expect to find the whole spectrum of organic material characteristic for organisms in their habitats as well.

The microbial utilization of particulate or polymeric organic material requires processes by which the material is degraded into products that can be taken up by the microorganisms. The enzymatic decomposition of high molecular weight material is the initial and rate-limiting step in organic carbon oxidation (88). The hydrolysis processes are mediated by extracellular enzymes that are produced by the cells. The enzymes act in close vicinity to the cells, bound to the outer membrane or in the periplasm (89), which enables the cells to take up the hydrolysis products efficiently. Investigations have shown that the colonization of particles is a prerequisite for their microbial decomposition. Dead and decaying organisms also contribute to the pool of extracellular enzymes. As shown in soils, the enzymes may escape degradation by binding to humic acids bound to clay particles. They may act as "starter" enzymes for the degradation of substrates (28). For the determinations of enzymatic activities, chromogenic and fluorogenic model substrates are available. The release of the corresponding dyes serves as a measure of enzymatic response (90–92).

The DOC pool can be operationally divided into a refractory and a labile fraction. The former is more resistant to microbial attack (turnover times in the range of months and years), whereas the latter is easily available. The labile portion represents the carbon microorganisms rely on to meet their carbon and energy demands on a short-term scale. This fraction has to be continuously recycled to allow microbial metabolism. Because chemical analyses of DOC permit only a limited assessment of its bioavailability, information on the available organic carbon (ADOC) is of special interest. There have been a few attempts to determine concentrations of ADOC in waters and sediments, mostly relying on the growth of test organisms as a reflection of available carbon in filtered water or extracts of sediments (93). Recently, a biosensor for the fine-scale measurement of ADOC has been developed. This biosensor consists of an oxygen microelectrode with microorganisms attached to the tip. The oxygen consumption of the immobilized cells serves as a measure for ADOC. The response of the biosensor is sensitive and rapid. ADOC is defined with respect to the metabolic activities of the test organism chosen and the carbon source used as standard (94).

The understanding of the carbon dynamics in aquatic ecosystems has been enhanced during recent years. The adsorption of dissolved organic matter to particles (95–100) was a mechanism that supposedly "stored" labile organic carbon temporarily (101). In addition, the

sorptive preservation of labile organic carbon to mineral surfaces was shown for marine sediments (102). The concept of "storage" of labile organic carbon is supported by the observation that the adsorption of low molecular weight compounds to a hydroxyapatite surface decreased their biodegradation rates (103).

Heterotrophic microbial biofilms are ultimately dependent on the uptake of carbon from the surrounding water. On the basis of experiments with boreal river epilithon, it was discussed that slowly metabolized high molecular weight compounds from the water saturate adsorption sites on the epilithon surface. No sooner than these compounds are removed, labile low molecular weight molecules are more readily adsorbed (104). In communities consisting of photoautotrophic and heterotrophic organisms (photoheterotrophic biofilms), the autotrophs may supply the heterotrophs with carbon.

NUTRITIONAL RELATIONSHIPS IN PHOTOHETEROTROPHIC BIOFILMS

The production of photoautotrophic organisms in epilithic biofilms from shallow water coastal inlets of the southern Baltic Sea (Bodden) amounted to approximately 100 mg C/m^2 h (26). The data are close to maximum values reported for epilithic biofilms in rivers (105). Compared to the microphytobenthos and the phytoplankton in the Bodden, epilithic biofilms revealed production rates that were higher by a factor of 2 and 100, respectively. However, the productivity (taking into account chlorophyll a) of the biofilms was by 10 to 100 times lower as compared to the pelagic and benthic production, respectively (106,107).

Nutritional relationships can be expected from the close spatial associations between photoautotrophic and heterotrophic microorganisms. This is supported by a number of investigations carried out in epilithic biofilms in streams (105). It could be shown that there was a direct flux of soluble algal products to the bacterial populations, with little heterotrophic utilization of dissolved organic material from the stream water (108). Bacterial heterotrophic activities significantly decreased as the photoautotrophic community declined (109). The marked lack of response of the epilithon to the external nutrients was attributed to the nutrient supply by the photoautotrophs. Attached microbial communities grown without algae were strongly dependent on riverborne organic material (110). Bacteria associated with algae grew twice as fast in the light as in the dark (111).

Under low-nutrient conditions in the bulk water, diatom-bacteria consortia exhibited higher growth rates as compared to biofilms composed solely of bacteria (112). Marine epiphytic biofilms were closely coupled to exudation products from the primary production of eelgrass (113,114). The significant relationship between bacterial abundance and chlorophyll a in marine sediments was attributed to the release of extracellular material by the microalgae (115). A close association of heterotrophic microorganisms taking up dissolved organic matter with photoautotrophic organisms was observed in intertidal marine microbial mats (116). Photoheterotrophic microbial mats from intertidal and subtidal environments appeared to mediate coupling between autotrophic and heterotrophic processes, thus minimizing losses of fixed carbon and nitrogen from the system (74). In sedimentary biofilms of the Bodden (shallow water inlets of the southern Baltic Sea), concentrations of available organic carbon (determined with a biosensor) of up to 80% of the total dissolved organic carbon were measured. Relationships between ADOC and concentrations of chlorophyll a imply that the labile organic matter was supplied by the photoautotrophs (117). In this context it has been inferred that legionellae grew extracellularly in the absence of amoebae in tap water biofilms, obviously supplied with sufficient nutrients by the bacterial consortium (118).

Although there is no doubt about the trophic relationships between photoautotrophic and heterotrophic components in microbial biofilms, the mechanisms are poorly understood. This applies to the transport and to the chemical nature of the exudates. Microscopic observations confirmed that a number of bacteria in photoheterotrophic biofilms were closely associated with cyanobacteria and algae, thus facilitating the nutrient transfer (26). However, the majority of bacteria within the matrix must be dependent on the transport of substrates to the cells. From the response of sulfate-reducing bacteria in cyanobacterial mats to the addition of specific carbon sources, it may be concluded that glycolate was an important product released by the cyanobacteria (78).

An unknown (probably the largest) part of the organic matter supplied from the surrounding water or by primary production in biofilms is high molecular weight material and has to be decomposed by extracellular enzymes before the uptake by microorganisms. In river biofilms, the accumulation of extracellular enzymes has been demonstrated. The spectrum of hydrolytic enzymes comprised phenol oxidase, peroxidase, α- and β-D-glucosidase, *N*-acetyl-β-D-glucosaminidase, and phosphatase (105,119). In sedimentary biofilms, proteolytic enzymes contributed to the spectrum of hydrolytic enzymes as well (90,91,120,121). Deep-sea sediments are exclusively dependent on the supply of nutrients from the water via sedimentation. The association with particulate matter or with higher organisms seems to be a strategy of microorganisms to come in contact with decomposable organic material (122,123). Laboratory experiments have shown that deep-sea microbial assemblages specifically responded to the addition of nutrients (88,124). Within less than a few days, enzymatic activities increased. It is of interest that the hydrolysis products were primarily respired by the sedimentary microorganisms, yet their biomass production was delayed (121). Extracellular enzymatic activities were used to characterize the accumulation of refractory substances in marine aggregates. With increasing succession from marine snow to gel-like aggregates (increasing age), the ratio of α-D-glucosidase activity to β-D-glucosidase activity declined, which implies the accumulation of more recalcitrant β-1,4-linked polymers (125).

Determinations of enzymatic activities using chromogenic and fluorogenic model substrates contributed to our understanding of substrate decomposition in aquatic environments. However, the interpretations suffer from

a number of limitations. Because artificial (mostly soluble) substrates are applied under laboratory conditions, the data describe potential activities rather than actual degradation rates. As justification, it may be accepted that the activity of naturally occurring enzymes is measured, and that these have been produced by the organisms as a response to preceding variations in the natural food supply. Most of the measurements represent more a reflection of the spectrum of labeled substrates commercially available rather than a reflection of the spectrum of enzymes naturally occurring which is, as well as the spectrum of natural substrates, almost unknown. The "standardization" of enzyme activities based on cell numbers is more than misleading because the number of cells responsible for the enzyme activities is not known. Even if the number of active cells are known, it must be assumed that enzymes of unknown origin (not bound to individual cells) contribute to the activities as well. Further progress can only be expected if the pool of naturally occurring enzymes and their substrates, the microorganisms implicated, and the location of the enzymes are identified.

The development and activities of marine biofilms reflect the gradient of nutrient enrichment (eutrophication) in the water column. Epiphytic biomass on eelgrass increased with increasing concentrations of total nitrogen. The increase was more than tenfold higher than the increase in phytoplankton biomass (50). The effect of nutrient enrichment on epiphyte levels across a nutrient availability gradient was pronounced (52). The gradient of nutrient enrichment in coastal inlets of the southern Baltic Sea (Bodden) was clearly reflected by the contribution of primary production and heterotrophic decomposition processes (respiration, enzymatic activities) in epilithic biofilms. With increasing eutrophication, photosynthesis decreased and respiration processes became more dominant. Respiration accounted for about 20% of the photosynthesis under mesotrophic conditions, whereas under polytrophic conditions about 70% of material produced by primary production was respired (26), thus leading to anaerobic conditions below the biofilm surface. From the spectrum of hydrolytic enzymes analyzed in sedimentary biofilms in the Bodden, it may be concluded that proteolytic enzymes gain more importance with increasing eutrophication (120).

DISTURBANCE OF BIOFILMS

Disturbance of biofilms occurs by grazing and physical forces. Zooplankton and benthic animals have strong impacts on microbial biofilms and mats by grazing and physically destroying the microbial assemblages (126). However, grazing may keep the microorganisms in an active stage of growth (4). Physical disruption of hot spring cyanobacterial mats resulted in substantial structural and physiological changes of the mats (127). High sediment transport rates produced significant abrasion in the abundances of bacteria and diatoms on particles in marine sediments. Only microorganisms colonizing protected areas of the sand grains were protected against abrasion (128). This underlines the conclusion that the microbial occurrence in crevices and depressions of sand grains is more a reflection of survival rather than preferential colonization (44). Water movements caused by storm events initially reduced the diversity and activities of biofilms, however, subsequent enhanced growth led to a recovery within days (105). It is interesting to note that drifting green algae covering diatom mats exhibited no adverse effects on the biomass of benthic communities. This points to mechanisms of the diatoms to adapt to reduced light conditions (129). However, epiphytic biofilms developing on different freshwater and marine macrophytes caused a severe stress to their hosts because of shading and oxygen depletion during intensive respiration (130).

In shallow coastal environments, water movements lead to a resuspension of sediments caused by low wind speeds. The unconsolidated fluffy layer covering sediment surfaces in protected coastal areas, in particular, is easily resuspended. The effects are the release of inorganic and organic nutrients, pollutants, and the accumulation of particles in the water. Microbial biofilms colonizing particles are exposed to conditions that differ from those experienced in the much more densely packed particles in the sediments. Competition effects are reduced, and concentration gradients of nutrients and oxygen are diminished. Microbial assemblages generally respond to resuspension of sediments with enhanced activities. However, the accumulation of particles in the water may restrict the light availability for photoautotrophic microorganisms. During resuspension, planktonic microorganisms may colonize the particles, and inorganic and organic dissolved and particulate material may adsorb, thus changing the biological and chemical milieu and the composition of the particles. After transport and sedimentation of the particles, the attached microbial communities have to adapt to the life in the sedimentary environment again. Because information on the structure and function of microbial assemblages on particle surfaces in the water ("snow") has greatly increased during the recent years, this interesting subject requires separate attention.

CONCLUSION

Biofilms consisting of complex assemblages of immobilized microorganisms and their matrix represent an early evolutionary stage in the organization of organisms. Although the individual members of the biofilm community are to a certain degree independent, there exists a high microbial interspecific coordination in the transfer and use of energy and substrates. This results in pronounced gradients in biological, chemical, and physicochemical parameters, which are no longer the response of individual organisms, but the effects of microbial communities. The organization in biofilms may be regarded as a strategy for survival and competitive success of microorganisms. The high structural dynamics of biofilms is reflected by attachment and detachment occurring next to each other. Through the interactions between photoautotrophic and heterotrophic organisms, microbial biofilms present a self-sustaining miniature ecosystem.

Current knowledge on the function of natural microbial biofilms in marine environments is very limited.

Some information on the structure and the biological components of the biofilms and something about biofilm activities, mostly measured as the net result of different individual processes is known. However, within the biofilms, the interactions between the processes as well as the organisms contributing are not well understood. This becomes especially obvious from considering biofilms consisting of photoautotrophic and heterotrophic organisms. There is clear evidence for the existence of syntrophic nutritional relationships. The phototrophs provide the heterotrophs with organic substrates via exudation of material produced by primary production. The heterotrophs respond by making inorganic nutrients available via remineralisation. However, the chemical composition of the exudates and the routes of their distribution remain unclear. The problem of identification is greatly enhanced by the small scales along which material is exchanged. Adequate methods for the small-scale measurements of activity rates are almost lacking. Concentration gradients of chemical parameters are of limited value, if the processes causing the stratification are not known. It is the within-biofilm dynamics that need urgent attention.

In biofilms, the appropriate scales in time and space have to be considered. Exudates produced by photoautotrophs and the adsorption of dissolved organic material to detritus and sand grain surfaces may provide a pulse of decomposable organic matter to the biofilm, thus stimulating microbial respiration. Within a short period of time, the substrates may be used up, thus leaving the microorganisms in a stage of nutrient limitation until the next pulse occurs. If the appropriate timescale is already a problem, what are the spatial dimensions that have to be looked at in biofilms? Does the spectrum of physiological diversity measured in biofilms on an individual sand grain already reflect the overall diversity of the sedimentary biofilm? How are the activities distributed throughout the individual particles? To increase our understanding of the function of biofilms, we have to adapt our investigations to the scales in time and space relevant to marine microbial biofilms.

Marine biofilms bear a great potential to be used in marine biotechnology. With knowledge of how the biofilms function, they can be manipulated to be used as biosensors to monitor environmental parameters. The analysis of products generated by biofilm organisms as a defense mechanism against other colonizing microorganisms may help to control marine microbial biofouling.

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ECOLOGY, PATHOGENICITY, AND SYSTEMATICS OF *AEROMONAS* IN THE ENVIRONMENT

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Members of the genus *Aeromonas* are gram-negative rod-shaped bacteria that constitute a part of the natural microflora in virtually all types of freshwater ranging from sewage treatment plants to drinking water. In addition, aeromonads have also been frequently isolated from nonaquatic origins including foods, cold- and warm-blooded animals, and human clinical specimens. Except for the psychrophilic fish pathogen *Aeromonas salmonicida* subsp. salmonicida, none of the known Aeromonas taxa are considered to be primarily pathogenic in animals or humans. However, because of their emerging presence in human extraintestinal and gastrointestinal infections and their ability to produce a wide range of putative virulence factors, a number of mesophilic species are now increasingly recognized as presumptive opportunistic pathogens for humans. At present, the global research on the pathogenesis and the epidemiology of Aeromonas-associated diseases is strongly hampered by the rather complex and straightforward taxonomic structure of this genus, often leading to misinterpretation of identification results in routine analysis. The possible link between the incidence of aeromonads in aquatic environments, especially in drinking water, and the spread of waterborne diarrheal disease imply that a better understanding of the taxonomy and ecology of *Aeromonas* would benefit gastrointestinal disease control.

PATHOGENICITY ASPECTS

Aeromonas, An Emerging Human Pathogen

The first evidence for the pathogenicity of aeromonads was observed within a number of cold- and warm-blooded animals that suffered from *Aeromonas*-associated septicemic diseases. Many of these infections often had fatal consequences for the infected host (1). In fact, a group of nonmotile psychrophilic aeromonads originally known as *Bacterium salmonicida* (now known as *Aeromonas salmonicida*) was soon identified as the primary cause of fish furunculosis, a disease leading to severe economical losses in fish farming. Although the first human infection

with an Aeromonas strain was already reported in 1954, it is only during the mid-1980s that aeromonads have been increasingly recognized as opportunistic pathogens in humans. During this relatively short period, various *Aeromonas* species were recovered from a wide range of extraintestinal infection sites (1) and it could be shown that many of these isolates indeed produced a variety of putative virulence factors.

Hence, many clinical microbiologists are interested in *Aeromonas* for its role as a potential gastrointestinal pathogen for humans. Hundreds of reports, varying from single-case studies to long-term outbreak monitoring surveys, have revealed a relationship between high Aeromonas densities in drinking water or food and diarrhea in humans (2). Despite the large collection of clinical and microbiological data available, however, not a single study has so far been successful in presenting unequivocal proof for the causality of aeromonads in human intestinal disease. In this context, the developments of appropriate animal models as well as further molecular biological research on the role of disease-inducing factors are currently ongoing. Well-documented outbreaks and field studies of *Aeromonas*-associated gastroenteritis in Europe and the United States have changed the original belief that epidemic cases of this disease mainly occur in countries such as Africa, South America, and Southeast Asia. In a publication of the Institute of Medicine, National Academy of Sciences (3), members of the genus *Aeromonas* were cited as one of the emerging microbial threats to public health in the United States.

Aeromonas Infections in Animals

The pathogenicity of aeromonads was first established with cold-blooded animals that are continuously exposed to high Aeromonas densities through their natural aquatic environments (1). Among the amphibians, red-leg disease in frogs is the most frequently reported *Aeromonas* infection. This disease is mostly associated with members of the *Aeromonas hydrophila* group and typically causes hemorrhages in the leg muscles. Members of the *A. hydrophila* group have also been recovered from a wide range of freshwater fish and, to a lesser extent, from marine fish, primarily causing red-sore disease. Undoubtedly, the best-known fish pathogen in the genus *Aeromonas* is the nonmotile psychrophilic species *Aeromonas salmonicida*, which has been recognized as the etiological agent of furunculosis in salmonids. Because of its epidemic nature, furunculosis has mainly caused enormous economical losses in commercial salmon and trout farms during the past few decades (4). Although contact with diseased fish is assumed to be the main route for *A. salmonicida* to spread itself throughout the aquatic environment, survival studies have indicated that this bacterium is able to persist in fresh, brackish, and sea water as well as in natural and fish pond sediments for prolonged periods (4). Currently, two fundamental problems are encountered with the diagnosis of *Aeromonas*-associated furunculosis. First, the frequent isolation of atypical *A. salmonicida* isolates from various fish and from diverse geographical locations is leading to contradictions in identification and typing studies (5). Secondly, there is evidence that *A. salmonicida* cells can enter into a viable nonculturable (VNC) state under certain conditions of environmental stress (6). As a result, conventional medium-dependent techniques are no longer reliable for routine monitoring of furunculosis outbreaks. The majority of the studies devoted to the detection and characterization of VNC bacteria in the environment involve DNA-based enumeration techniques in combination with fluorimetric applications. The administration of antibiotics is one of the main methods to control furunculosis in fish farms next to good hygiene management including maintenance of good water quality and efficient disinfection policies. However, the generation of plasmid-mediated resistance to frequently broad-spectrum antibiotics such as oxytetracycline (OTC) in *A. salmonicida* strains as well as in the bacterial communities indigenous to salmon culture sites has seriously hampered the efficiency of the latter approach (7). Second-generation fluoroquinolones are used to overcome the problem of OTC resistance, but other methods for disease control including vaccination with genetically modified *A. salmonicida* strains and the use of probiotic Vibrio strains seem promising alternatives (4).

In warm-blooded animals, strains belonging to mesophilic *Aeromonas* species have been shown to be pathogenic for a wide range of mammals (including cats, dogs, horses, rabbits, guinea-pigs, monkeys, and mice) and avians (including chickens, turkeys, and companion birds). However, most of the reported infections occur only sporadically and are localized at the most diverse sites within the host.

Aeromonas Infections in Humans

Extraintestinal infections can be caused by a broad spectrum of Aeromonas taxa and often have severe or fatal consequences for adult individuals. Septicemia is generally considered the most life-threatening form of all systemic Aeromonas infections described so far. Although several categories of septicemic diseases are known to exist (8), 90% of all cases are typically associated with immunologically impaired adults that suffer from one or more underlying diseases. In addition to classical signs such as fever and chills, this specific form is often followed by other clinical implications such as cellulitis and liver disease, with estimated mortality rates ranging from 30 to 50% (8). Six Aeromonas taxa are known to cause septicemia in humans, that is, *Aeromonas hydrophila, Aeromonas caviae, Aeromonas veronii* biovars sobria and veronii, *Aeromonas jandaei*, and *Aeromonas schubertii*.

The second most frequently encountered site for human Aeromonas infections are the wounds next to the gastrointestinal tract. Three major types of *Aeromonas*-associated wound infections have been described so far. They are cellulitis, myonecrosis, and ecthyma gangrenosum (8), of which the latter two categories often have fatal consequences for the host. Most wound infections are probably community-acquired, involved male adults, were preceded by an injury, and resulted from environmental (i.e., freshwater or soil) contamination. The etiological agents of Aeromonas wound infections

are mostly situated in the species *A. hydrophila* and *A. veronii* (8).

Aeromonas-associated gastroenteritis seems to have a relatively narrow species spectrum and mainly occurs in infants less than five years old or adults older than 60 years old (8). No single study has been able to identify *Aeromonas* as the true causal agent of gastroenteritis (2). Single-case studies of Aeromonas diarrhea are relatively rare and mainly describe gastrointestinal infections that occurred in individuals who had traveled to developing countries or in workers who accidentally ingested aeromonads in laboratories or hospitals. In contrast, surveillance studies are believed to be far more useful in assessing the role of *Aeromonas* as an emerging enteropathogen because these reports usually contain long-time investigations on a large number of patients (2). As such, *Aeromonas*-implicated outbreaks of diarrhea have been reported in Sweden, Scotland, United States, and Australia. In these cases, drinking water and food are considered as the major sources of gastrointestinal contamination with *Aeromonas*. An overall comparison of major field studies clearly reveals that the epidemic form of *Aeromonas*-related diarrhea is most massively encountered in developing countries (2). In some of these investigations, *Aeromonas* was found as the sole bacterial enteropathogen, whereas other studies mentioned the presence of copathogens such as *Escherichia coli, Campylobacter, Shigella*, and *Salmonella*. In the majority of the cases, *A. caviae* was shown to be the major species isolated followed by *A. hydropohila* and *A. veronii* biovars sobria and veronii. In this context, it has been suggested that a pH shift in the intestine of young children may actually favor the survival and cytotoxicity of *A. caviae*, which further supports the role of this organism as a pediatric enteric pathogen (9).

Putative Virulence Factors

In recent years, intensive clinical and molecular research has resulted in the description of various structural, enzymatic, and cell-associated virulence factors. Among the structural determinants, including pili, S-layers, lipopolysaccharides, and outer membrane proteins, only the S-layer of *A. salmonicida* harboring a 49-kDa cell surface protein has been unambiguously associated with the development of a specific disease (i.e., fish furunculosis) (10). It is assumed that the highly hydrophobic S-layer may protect the bacterial cell from complement-mediated lysis and that the loss of this layer can result in autoaggregation deficiency and a reduction of virulence. The presence of S-layers has also been observed among pathogenic strains of *A. hydrophila* and *A. veronii* biovar sobria. It has been suggested that these S-layers may not be very crucial factors in the pathogenicity of these species (11).

Apart from various proteases, lipolytic enzymes, and polysaccharide-degrading enzymes, hemolysins and enterotoxins belong to the main enzymatic virulence factors described in *Aeromonas* so far (12). Hemolysins typically act as channel-forming bacterial cytolysins toward a broad range of erythrocytes and are usually produced by members of *A. hydrophila, A. caviae*, and *A. veronii*. Two major classes of hemolysins have been recognized so far. The aerolysins or β-hemolysins that produce clear hemolytic zones on blood agar and are heat-labile (56 °C, 5 min), and the α-hemolysins that exhibit incomplete hemolysis on blood agar and are not expressed at growth temperatures exceeding 30 °C. In addition to their cytotoxic activity, aerolysins also display enterotoxic effects indicating that aerolysins may have multifunctional properties and can act as cytolytic enterotoxins in human gastroenteritis (13). The cytotonic enterotoxin, on the other hand, is clearly distinct from α- and β-hemolysins and acts according to a cholera toxin-like mechanism. This toxin has been observed among environmental and clinical isolates of *A. hydrophila, A. caviae*, and *A. veronii* (14).

Recently, a number of possibly new virulence factors, either associated with the bacterial cell surface or located in the cytosol, have been described for some *Aeromonas* sp. These factors include invasins, adhesins, and plasmids. However, most of the arguments brought forward to define the precise role of these determinants in *Aeromonas* pathogenesis are often speculative and additional research in this field is necessary (13).

ISOLATION AND ENUMERATION OF *AEROMONAS*

Because of their physiological diversity and their distribution in the most diverse environmental and clinical habitats, aeromonads cannot be readily isolated using one universal medium and methodology. As such, the isolation of psychrophilic *A. salmonicida* from fish will require a very different approach compared to the recovery of mesophilic aeromonads from a human stool sample. Apart from a number of physicochemical parameters, bacteriological factors such as the density of competing microflora or the presence of injured cells are likely to vary from sample to sample and should be taken into account when choosing the most appropriate medium for isolation. In food or clinical samples, the actual isolation procedure may be preceded by an enrichment step leading to qualitative or presence–absence analysis of *Aeromonas*. The recovery of aeromonads from environmental samples, on the other hand, usually does not require preenrichment and thus allows quantitative enumeration. A concise overview will be given below of the techniques widely used for isolation and enumeration of psychrophilic and mesophilic aeromonads from diverse environments. For a full review on these methods and for literature on the isolation of aeromonads from clinical specimens, the reader is referred to reference (15).

Environmental Samples

For the isolation of mesophilic aeromonads from the environment, the standardized collection of representative samples from the aquatic or terrestrial sites of interest is a first requirement. Water samples are usually collected in sterile, screw-capped glass or plastic bottles or tubes, whereas soil and sludge samples may be collected in sterile screw-capped containers or Whirl-Pak bags. In the case of sampling raw or processed drinking water, it is

recommended that the sample recipients contain 24-mg/l sodium thiosulfate to inactivate free residual chlorine. In addition, at least 50 mg/l ethylenediamineacetic acid (EDTA) can be added for chelating free metals such as copper that are know to cause death of many bacteria in drinking water samples. In the past, the formation of biofilms has often been overlooked in bacteriological analyses of drinking water distribution systems. Regrowth of aeromonads can be determined by taking surface swab samples at technical control points in the distribution network. In general, environmental samples should be stored at 2–8 °C and processed within three to five hours after collection to minimalize the "bottle effect" on quantitative assessment of aeromonads. For routine analysis of water samples, appropriate dilutions are usually concentrated using membrane filtration before inoculation of the selection medium of choice (16).

As aeromonads are usually found together with *Enterobacteriaceae* and *Pseudomonas* spp. amongst other autochthonous bacteria in environmental samples, selective media should be used for the specific recovery of *Aeromonas* from the naturally occurring background flora. Within the range of media developed for selective isolation of *Aeromonas* spp., ampicillin-dextrin agar (ADA) (17) is most frequently used. Combining the positive aspects of mA agar and dextrin-fuchsin-sulfite agar, the ADA medium suppresses background flora and stimulates growth of *Aeromonas* by making use of the high specificity of dextrin fermentation and the addition of 10 ppm ampicillin. Except for the species *Aeromonas trota* (18), the majority of the aeromonads is believed to be ampicillin-resistant and appear as typical yellow colonies with a dark center on the blue ADA medium. For enumeration purposes, colony counting should be performed after 24 hours incubation at 28 °C as longer incubation will result in the gradual overgrowth by background flora. The addition of the vibriostatic agent 0/129 and ethanol is sometimes recommended to inhibit proliferation of vibrios (17) and *Klebsiella* spp. (16), respectively.

Fish

Fish may be analyzed for the presence of both *A. salmonicida*, the primary agent of causing furunculosis, and members of the *A. hydrophila* complex leading to septicemia. For isolation of aeromonads from fish and other cold-blooded animals, swab samples are usually taken from gills, organs, external lesions, and directly collected into an enrichment medium like tryptone soy broth. Bacteria are enriched by incubating sample suspensions at 25–26 °C for 24–48 hours. Broth cultures are then streaked on tryptic soy agar (TSA) and may be incubated at different temperatures (range 20–30 °C) to allow recovery of both mesophilic and psychrophilic aeromonads. Traditionally, *A. salmonicida* colonies can be immediately recognized on isolation on TSA by the production of a brown water-soluble pigment and through a positive oxidase spray test, result although nonpigment producing and oxidase-negative strains have been described (4).

Food

Because of the suspected association between specific cases of foodborne *Aeromonas* infections and the generation of gastrointestinal disease in humans, many attempts have been made to optimize the isolation of aeromonads from raw and processed foods. In most standard procedures, 25 g samples are collected in sterile stomacher bags to which 225 ml of 0.1% alkaline peptone water (APW) is added. The suspension is homogenized using a blender or stomacher, and appropriate dilutions are prepared for plating. Food samples need to be refrigerated during transport to the lab as some mesophilic *Aeromonas* strains are known to survive and grow at 5 °C in specific food matrices. For quantitative recovery, appropriate dilutions of the APW suspension are directly plated on the recommended isolation medium. When enrichment is desired in qualitative assessments, APW suspensions should be incubated overnight at 28 °C before direct plating and incubation at 35 °C for 18–24 hours. Currently, Bile salts irgasan brilliant green (BIBG) medium is highly preferred as the medium of choice for selective spread plate isolation of aeromonads from food products. BIBG agar provides a high selectivity for aeromonads by suppressing growth of most enterobacteria and indicates presumptive Aeromonas colonies by a positive oxidase spray test. As BIBG agar does not contain antibiotics, the medium will not inhibit recovery of ampicillin-sensitive *Aeromonas* strains as may be the case with ADA medium. Recently, modified BIBG agar (mBIBG) was evaluated to reduce the occasional inhibition of brilliant green sensitive *Aeromonas* strains on the original BIBG medium (19).

SYSTEMATICS

Identification to the Genus Level

Conventional microscopy identifies aeromonads as rods that exist singly, in pairs, or in short chains, and ranging in size from 0.3 to 1.0 μm in diameter and from 1.0 to 3.5 μm in length. Most species, except *A. salmonicida* and *A. media*, are motile by a single polar flagellum. Most aeromonads grow well and relatively fast (i.e., overnight incubation) on standard media like TSA or nutrient agar and do not require special culture conditions. To obtain optimal growth, however, it is clear that the incubation temperature should be adjusted depending on whether mesophilic (range 28–30 °C) or psychrophilic (range 22–25 °C) *Aeromonas* strains are cultivated. In general, strains can grow at a temperature range as wide as 0 to 45 °C and between pH 4.5 and 9.0. As a group, aeromonads can be easily distinguished from other gram-negative, facultatively anaerobic rod-shaped bacteria (i.e., *vibrios*, the *Enterobacteriaceae*, and *Plesiomonas*) by a few simple phenotypic tests (Table 1). In this regard, it has been noted previously that fermentation of lactose at 37 °C is an unreliable test to differentiate aeromonads from enterobacteria, and showed that the routine use of this particular test had caused false-positive counts in the determination of total coliforms in drinking water analysis. Aeromonads do not require Na^+ (sodium ion) for growth and are

Table 1. Phenotypic Differentiation of *Aeromonas* from Other Gram-Negative Rods

Feature	Reaction For			
	Aeromonas	*Vibrio*[a]	*Plesiomonas*	Enterobacteriaceae
Oxidase test	+	+	+	−
Resistance to:				
0/129 (150 mg)	+	−	−	nd
Ampicillin (10 mg)	+[b]	v	v	−

[a] Halophilic *Vibrio* spp. can be further differentiated by their inability to grow in the absence of NaCl
[b] *A. trota* is the sole ampicillin-sensitive member of the genus *Aeromonas* (8).
+, ≥ 85% positive strains; −, ≤ 15% positive strains; v, variable; nd, no data found.

resistant to the vibriostatic agent 0/129 (2,4-diamino-6,7-diiso-propylpteridine) and to most penicillin.

Taxonomy

Essentially, modern Aeromonas taxonomy has expanded from the so-called "four-species concept" of Popoff as described in the first edition of Bergey's Manual of Systematic Bacteriology (20). In the latter classification, the genus *Aeromonas* consisted of three mesophilic motile species, that is, *A. hydrophila, A. caviae*, and *A. sobria*, and one psychrophilic nonmotile species that is, *A. salmonicida*. Because the taxonomy of this genus has changed a great deal owing to the addition of newly described species and the reclassification or amended description of existing taxa. In addition, the combined use of the terms *phenospecies* (i.e., a taxon delineated on the basis of phenotypic characteristics) and *genomospecies* (i.e., a DNA hybridization group [HG] determined on the basis of DNA homology values) in the current Aeromonas taxonomy seems highly confusing to many taxonomists outside the field. At least 14 *Aeromonas* species have been validated so far, some of which are either grouped together in one single HG (e.g., *A. salmonicida* and *A. hydrophila* in HG3), are dispersed among different HGs (e.g., *A. caviae* in HG4, HG5A, and HG5B), or are further subdivided into genetically similar biovars (i.e., *A. veronii*). In addition to the detailed taxonomic review (21), Table 2 shows a list of the *Aeromonas* taxa recognized on March 2001.

Identification to the Species and DNA Hybridization Group Level

To a certain extent, the introduction of chemotaxonomic and molecular methods in bacterial systematics has addressed some of the classical problems of resolution and reproducibility typically associated with phenotypic methods. As modern *Aeromonas* taxonomy strongly relies on genotypic data, the latter methods often give inaccurate or unreliable results in the classification of unknown *Aeromonas* isolates. As a result, speciation of *Aeromonas* sp. is now increasingly becoming the responsibility of a few research units harboring specialized equipment coupled with a strong reliance on in-house expertise and up-to-date libraries. The major phenotypic, chemotaxonomic, and genotypic methods are described later that have proven to be suitable for the identification and classification of aeromonads.

At a first identification level, aeromonads can be classified in two major physiological groups including the motile, mesophilic and the nonmotile, psychrophilic aeromonads (i.e., *A. salmonicida*). However, this phenotypic division is not well defined because some nonmotile *A. media* strains are able to grow in the temperature range of 22 to 37 °C and atypical (motile, growth at 37 °C) *A. salmonicida* isolates are known to exist. Similarly, the production of a brown soluble pigment on TSA alone should not be considered typical for all psychrophilic aeromonads because only one of the four subspecies in *A. salmonicida* (i.e., subsp. salmonicida) displays this characteristic. For many years, the routine phenotypic identification of mesophilic Aeromonas isolates to the species level was based on a limited set of biochemical tests allowing discrimination among the taxa constituting the four-species concept (20). However, as increasingly new *Aeromonas* taxa were described, the classical identification scheme of Popoff (20) soon became inefficient. For this reason, Carnahan and coworkers (22) and Abbott and coworkers (23) recommended the use of 20 to 25 selected phenotypic features for the correct allocation of mesophilic *Aeromonas* strains to one of the seven major clinical species or to one of the 14 HGs (see Table 2), respectively. However, the authors conceded that it was very difficult to define reliable phenotypic markers for rare taxa such as *A. sobria* HG7 and also indicated that additional strains from diverse origins should continuously be included as a matter of postevaluation of their phenotypic schemes. To facilitate routine identification, a number of small-scale test kits have been brought on the market for the rapid speciation of unknown *Aeromonas* isolates. So far, at least 15 commercial systems including API 20E/NE, BIOLOG GN2, Vitek, B-D Crystal, and MicroScan currently have representatives of the major *Aeromonas* species included in their databases. However, the general experience is that the resolving power of these systems is often too low to deal with the high phenotypic relatedness that exists between some of the Aeromonas taxa. For instance, evaluation of the API 20E system revealed that only 52% of the *Aeromonas* strains tested were correctly identified to the genus level (24). Here, additional tests were mandatory to obtain taxonomic information beyond the genus level.

For the past 15 years, almost every chemotaxonomic technique applied in the field of bacterial

Table 2. Species and DNA Hybridization Groups Situated in the Genus *Aeromonas*

HG (= Genomospecies)[a]	Species Name (= Phenospecies)[a]	Main Source of Isolation
1	*A. hydrophila*	Humans, animals, freshwater
2	*A. bestiarum*	Animals, freshwater, humans
3[b]	*A. hydrophila*	Freshwater, foods
	A. salmonicida	
3[b]	subsp. *salmonicida*	Fish
3[b]	subsp. *achromogenes*	Fish
3[b]	subsp. *masoucida*	Fish
3[b]	subsp. *smithia*	Fish
3[b]	subsp. *pectinolytica*	Freshwater
4	*A. caviae*	Humans, animals, freshwater, foods
5A	*A. caviae*	Freshwater, foods
5B	*A. caviae*	Humans
5B	*A. media*	Freshwater
6	*A. eucrenophila*	Freshwater
7	*A. sobria*	Fish
8[c]	*A. veronii* biovar sobria	Humans, animals, freshwater, foods
9	*A. jandaei*	Humans
10[c]	*A. veronii* biovar veronii	Humans
11	*A. encheleia*	Fish, freshwater, human
12	*A. schubertii*	Humans
13	*A. trota*	Humans
14	*A. allosaccharophila*	Human, fish
ND[d]	*A. popoffii*	Freshwater

[a]Genomospecies and phenospecies represent taxa that are delineated on the basis of DNA hybridization data and phenotypic characterization, respectively (9). HG, DNA hybridization group.
[b]HG3 contains motile, mesophilic strains (*A. hydrophila*) and nonmotile, psychrophilic strains (*A. salmonicida*).
[c]HG8 and HG10 are genetically similar, but constitute two biochemically diverse groups in *A. veronii*.
[d]Not defined yet.

systematics has been evaluated for its taxonomic potential in the genus *Aeromonas*. Some of these methods were described as promising alternatives for the rather laborious methods used in phenotypic identification, whereas other techniques have not been fully evaluated (e.g., pyrolysis mass spectrometry) but seemed valuable in the characterization of aeromonads to the strain level (e.g., serotyping, phagetyping, and whole-cell protein fingerprinting). Essentially, only multilocus enzyme electrophoresis (MLEE) and gas-liquid chromatographic (GLC) analysis of cellular fatty acid methyl esters (FAMEs) have been successfully applied for the identification of *Aeromonas* isolates. In general, MLEE data show a good correlation with the DNA hybridization groups established in *Aeromonas* results, whereas a further automation of the method is required toward routine applications (25). Modern equipment for FAME analysis, on the other hand, provides many possibilities for automated strain identification and has proven to be discriminative at the phenospecies level or even at the HG level in *Aeromonas* (26). In this regard, it should be mentioned that standardization of culture media, time, and temperature of incubation are crucial to obtain reproducible and taxonomically resolving FAME patterns.

During the 1990s, a wide range of techniques based on restriction fragment length polymorphism analysis and/or on the polymerase chain reaction (PCR) was applied to identify *Aeromonas* strains. The taxonomic value of some of these DNA-based fingerprinting methods, including randomly amplified polymorphic DNA analysis, pulsed-field gel electrophoresis, and restriction endonuclease analysis (REA), is believed to be limited within the genus *Aeromonas*. For this reason, the latter techniques are more valuable when used in typing studies for individual strain differentiation. In contrast, the use of rRNA gene restriction patterns (i.e., ribotyping) and whole-genome fingerprinting using the amplified fragment length polymorphism (AFLP) technique as taxonomic tools in *Aeromonas* have been relatively successful. Visual comparison of ribotyping patterns allows discrimination among most *Aeromonas* HGs (27) and has been applied for the identification to the HG level of aeromonads from aquatic, food, and human clinical origin. Essentially, the AFLP concept combines the specificity of double REA analysis with the selective amplification of modified restriction fragments yielding a highly informative autoradiographic or fluorescent fingerprint (28). Numerical analysis of AFLP patterns show a perfect correlation with *Aeromonas* HGs and allow the construction of genotypic databases for identification of unknown or poorly characterized aeromonads. Other important developments in the molecular identification of aeromonads concern the application of the fluorometric DNA–DNA hybridizations in micro dilution wells (29) and the use of PCR assays using species-specific

PCR primers designed on the basis of 16S-rRNA sequences (30).

OCCURRENCE OF *AEROMONAS* IN THE ENVIRONMENT

Aquatic Environments

Numerous ecological investigations and microbial population studies have demonstrated that aeromonads occur in virtually all types of aquatic environments throughout the world. From the data compiled in Table 3, it is clear that every type of water displays its own specific range of *Aeromonas* counts, confirming the long-time belief that the occurrence of *Aeromonas* in specific water habitats is largely determined by physicochemical and microbial factors inherent to the site (31). The possible link between the incidence of aeromonads in drinking water and waterborne diarrheal disease implies that a better understanding of these aspects is also beneficial to workers in the field of *Aeromonas* epidemiology and gastrointestinal disease control.

In general, a limited amount of data is available on the isolation of aeromonads from naturally occurring, nonpolluted groundwater sources. In deep aerobic and anaerobic ground waters, *Aeromonas* numbers of up to 35 CFU/100 ml have been determined indicating a pronounced dominance of *A. hydrophila* over *A. caviae*. The same two species have also been found in underground waters where it was observed that the overall structure of *Aeromonas* populations autochthonous to this type of water could remain constant for long periods. Interestingly, a Belgian study indicated that free radicals spontaneously generated in oxidizing (Iron) Fe_{2+}-containing ground waters on aeration caused inactivation of *A. hydrophila* amongst other aquatic species (32).

In the past 20 years, a great deal of scientific and economical attention has been paid to the microbial quality of surface freshwaters, and this is mainly because of their importance as sources of drinking water and as habitats for aquaculture. During a two-year survey, Hazen (33) determined densities of *A. hydrophila* in a South Carolina cooling reservoir, receiving heated effluent from a nuclear reactor. The author concluded that the *A. hydrophila* densities were significantly higher in the thermally altered parts of the reservoir, and found that the mean monthly counts of this species were positively correlated with the incidence of red-sore disease in largemouth bass. If one compares studies that were devised to identify factors possibly affecting the *Aeromonas* population in surface water, different relational patterns can be observed (31). As such, high correlations are known to exist between *Aeromonas* numbers in estuaries with heterotrophic plate counts (HPC) at 35 °C and fecal coliform (FC) densities, whereas studies performed in raw surface waters used for the production of drinking water revealed that *Aeromonas* densities were positively correlated to HPC at 37 °C but negatively correlated with FC counts. Seasonal influence and the related changes in freshwater temperature, on the other hand, usually showed a highly stable correlation with *Aeromonas* densities. In temperate areas, highest *Aeromonas* counts are generally determined during summer, whereas the reverse effect is seen with surface waters located in (sub) tropical regions. Similarly, the influence of the tropic state of the water also seems to vary between studies. Schubert (34) determined that the incidence of anaerogenic aeromonads (now *A. caviae*) versus the incidence of aerogenic aeromonads (now *A. hydrophila* and *A. veronii* biovar sobria) in rivers and brooks depended upon the saprobicity of the water. In Schubert's study (34), anaerogenic *Aeromonas* strains were predominant in polysaprobic streams, but their levels declined in favor of aerogenic aeromonads when oligosaprobic sources were examined. In concordance with these data, workers have reported that *A. caviae* predominated in rivers with a high degree of organic pollution, whereas levels of *A. hydrophila* and *A. sobria* increased in less polluted waters.

Although aeromonads are not exactly typical marine organisms, the major mesophilic *Aeromonas* species autochthonous to aquatic environments have been recognized as halo-tolerant (31). In this context, most enumeration studies concentrated on coastal waters where freshwater possibly interferes with the natural marine system. As was the case for surface freshwaters, a positive correlation between *Aeromonas* densities and water temperature has been reported for marine waters. In coastal bay water, *A. hydrophila* and *A. sobria* have been shown to be predominant during summer months, whereas *A. caviae* numbers reached their peak in winter season.

Aeromonads are not only widespread in nonpolluted freshwaters and marine waters, but have also been frequently isolated from domestic and industrial wastewaters. High concentrations of fats, proteins, and polysaccharides are known to favor the selective mass growth of *Aeromonas* strains in these environments. Similarly to nonpolluted waters, studies in sewage treatment ponds have indicated that the total aeromonad densities follow a seasonal cycle, with highest numbers reported in summer (35). On the other hand, FC was dominant during winter periods. Furthermore, this study (35) also showed

Table 3. Typical Mesophilic *Aeromonas* Counts in Different Types of Water

Aquatic Environment	Typical Count (CFU/100 ml)
Sewage treatment plants	
— Influents	$10^7 - > 10^9$
— Effluents	$10^3 - 10^8$
Lagoon receiving sewage effluent	$10^4 - 10^6$
Unpolluted rivers, fresh water lakes	$10^3 - 10^4$
Marine estuary	$10^3 - 10^4$
Private unchlorinated drinking water well	$10 - 10^4$
Drinking water production/treatment plants	
— Raw groundwaters	$< 1 - 10^2$
— Raw surface waters	$10^3 - 10^5$
— Treated waters (postchlorinated)	$< 1 - 10$
— Distribution systems (unchlorinated)	$< 1 - 10$
— Distribution systems (chlorinated)	$< 1 - 10^2$
— Tap water	$< 1 - 10^2$
Bottled mineral waters	$< 1 - > 1$

Data compiled from (15).

that *A. caviae* was the dominating organism in the sewage inflow through out the year through, indicating that this species was autochthonous to the raw sewage. Conversely, the relative abundance of *A. hydrophila* and *A. sobria* is dependent highly on seasonal influence. In Italian urban wastewater purification plants, extremely high levels of *A. veronii* biovar veronii have been reported. This opportunistic human pathogen is normally associated with wounds and stool, which clearly indicates the importance of reliable *Aeromonas* speciation in cases where waste processing outflows are being used for irrigation in agriculture (31). In general, removal efficiencies of *Aeromonas* obtained in most plants is satisfactory, varying from 93% in Moroccan stabilization ponds (36) to 99.9% in a Belgian activated sludge plant (37). It should be noted that a significant number of potentially pathogenic aeromonads can be retained in dried sludge.

In essence, two factors may largely contribute to the incidence of *Aeromonas* strains in potable waters. First, aeromonads may be removed ineffectively and their numbers may even increase during the production process of drinking water. The latter phenomenon has been observed with sand filters containing significant loads of biodegradable components and with activated carbon filtration systems, which are believed to stimulate bacterial adherence and regrowth (38). In such cases, the introduction of a postchlorination step seemed absolutely necessary (38). Secondly, after growth of aeromonads has been frequently observed in several municipal distribution systems and this may be associated with biofilm formation (31). Several physicochemical parameters including water temperature and concentration of residual chlorine are known to influence the process of regrowth, whereas laboratory studies have suggested that the presence of low assimilable carbon concentrations can easily initiate growth of *A. hydrophila*. During the 1980s, a number of Dutch and Australian studies provided the first insights on the possible correlation between the high incidence of aeromonads in drinking water and an increase in *Aeromonas*-associated diarrheal cases. Other studies, although not directly linked to outbreaks of diarrhea, have shown that *Aeromonas* isolates recovered from drinking water supplies have the potential to exhibit significant cytotoxic and hemolytic activities (39). The majority of these strains were found to belong to the species *A. hydrophila* and *A. sobria* (*A. veronii* biovar sobria). As a result, health authorities in the Netherlands have defined so-called indicative maximum values for Aeromonas numbers in drinking water production, that is, 20 CFU/100 ml for water leaving the production plant and 200 CFU/100 ml for drinking water in the distribution system. According to Holmes and coworkers (31), a full understanding on the precise role of aeromonads in waterborne diarrhea requires many more outbreaks to be investigated in great detail.

Currently, only a limited number of reports are available on the isolation of aeromonads (i.e., *A. hydrophila*) from bottled mineral waters. An examination of 64 European still mineral waters did not reveal a trace of *Aeromonas* in any of the brands included. This observation could be explained by the fact that most aeromonads probably lack the potential to grow in nutrient-poor waters. Survival studies have revealed that an *A. hydrophila* strain was able to persist in filtered and filtered-autoclaved but not in unfiltered bottled water, indicating the inability of this organism to compete with the autochthonous microbial flora. On the other hand, *A. hydrophila* has been shown to survive for up to two months in double-distilled water inoculated with contaminating bacteria such as *Pseudomonas aeruginosa* and several *Enterobacteriaceae*.

Terrestrial Environments and Plants

Although it is assumed that *Aeromonas* is as widespread in terrestrial as in aquatic environments, only very few data are available on the presence of aeromonads in soil and sediments. In fact, the occurrence of mesophilic *Aeromonas* sp. has mainly been reported in metabolic studies on bioremediation of polluted factory grounds, mercury resistance adaptation in mine soils, environmental nitrogen cycles in soils and freshwater sediments, and the rhizosphere of bean plants. In addition, aeromonads have also been isolated as internally seedborne microorganisms from soybean and rice seed. Moreover, a survival study in artificially contaminated sterile or unsterilized soil has demonstrated that soil should be regarded as an important reservoir for mesophilic *Aeromonas* sp. and may play an important role in the spread of potentially pathogenic aeromonads (40).

Foods

In addition to their increased presence in potable water supplies, aeromonads have also been isolated from a wide range of foods including raw and processed meats, poultry, fish, seafood, dairy products, vegetables, and preprepared salads frequently (41). Moreover, it has been demonstrated that food isolates of *Aeromonas* can produce a wide range of potential virulence determinants such as hemolysins, cytotoxins, and exotoxins (42). Consequently, some *Aeromonas* species are now being recognized as possible foodborne pathogens, and a lot of research in this field is currently focusing on growth control and inactivation of aeromonads in foods (41). For instance, the fact that *Aeromonas* sp. are capable to grow at 4 to 5 °C has seriously questioned the purpose of refrigeration in the storage of spoilable foods. Similarly, it was demonstrated that *A. hydrophila* can survive but cannot grow on mixed vegetable salads stored in a modified atmosphere at 4 °C. Very few surveys have studied the possible sites of *Aeromonas* contamination in the line of food processing and packaging.

CONCLUSION

Over the past two decades, significant progress has been made in understanding the classification, the pathogenicity, and the ecology of aeromonads. With the aid of DNA/16S-rRNA based methods, new species have been described and better taxonomic insights were obtained in *Aeromonas* systematics. Next to the *Enterobacteriaceae*, *Aeromonas* sp. are now considered one of the major bacterial groups predominating in freshwaters worldwide.

The psychrophilic taxon *A. salmonicida* has been recognized as the primary causative agent of furunculosis, a salmonid disease that is increasingly being controlled using techniques other than antibiotic treatment including vaccination. In contrast, little is known about the true ecological relevance of mesophilic *Aeromonas* spp. in aquatic environments or their presumptive role as human opportunistic pathogens associated with sporadic cases and outbreaks of gastroenteritis. In this regard, ongoing and future research should primarily concentrate on the further optimization of virulence models and molecular subtyping methods to study the pathogenicity and epidemiology of aeromonads involved in food- or waterborne infections.

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ECTOMYCORRHIZAL FUNGI.

See MYCORRHIZAE: ECTOMYCORRHIZAL FUNGI

ELECTROPHORESIS.

See CAPILLARY ELECTROPHORESIS IN GENETIC ANALYSIS AND RIBOTYPING OF MICROBIOTA IN THE ENVIRONMENT

EMERGENCY WATER SUPPLIES.

See HETEROTROPHIC BACTERIA AS INDICATORS OF WATER QUALITY

ENDOLITHIC MICROORGANISMS IN ARID REGIONS

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Arid and semiarid regions are characterized by their limited precipitation, less than 25 cm per year in deserts, up to about 75 cm per year in adjacent steppes and shrublands. Because of the limited availability of moisture, the landscape is dominated not by the growth of vascular plants as is typical in wetter climates, but by geomorphological features such as bare bedrock cliffs, ledges and platforms, talus slopes, alluvial fans, coarse soils overlain by desert pavement, and areas of shifting sands. These geological features are inhabited by special suites of microorganisms, which often form well-defined associations. Desert soil microorganisms are treated elsewhere, as are those associated with the formation of desert varnish. Here we focus on rock-inhabiting microorganisms associated with some of the harshest environments.

Rocks and stones provide several habitats for microbial growth (1,2). The epilithic habitat consists of the surface of the rock. In milder climates it can support a rich growth of bryophytes, lichens, and subaerial algae; in the most extreme deserts, in contrast, rock surfaces are essentially abiotic. The endolithic habitat, the focus of this article, consists of the interior of the rock. Golubic and coworkers (3) identified three types of endolithic organisms. Euendolithic organisms actively bore into the surface of the rock. Euendolithic cyanobacteria are common in aquatic systems, much less so in terrestrial systems. Euendolithic lichens are common on limestone and other materials readily dissolved by weak organic acids. Chasmoendolithic organisms inhabit cracks and fissures in weathered rock. Typically, these have a direct connection with the surface. Cryptoendolithic organisms inhabit the interstitial spaces of porous rocks. Because the organisms lie below the surface of the rock, only rocks composed of light-colored or translucent grains are colonized by cryptoendoliths. The hypolithic habitat consists of the lower surface of stones embedded in the soil.

Endolithic microorganisms are widely distributed in arid regions. Examples have been reported from the Middle East (4–7), central Asia (8–9), southern Africa (2,10–13), Australia (2,10), southwestern United States and Mexico (1,10,14,15), South America (2), the Arctic (16–18), and Antarctica (19–24). It should be noted that a report of chasmoendolithic cyanobacteria from the Atacama Desert was based on a specimen from the semiarid Guanagueros Peninsula in Chile, about 200 m from the sea and at an elevation of about 10 m above sea-level, and not from the Atacama (2). Despite their global distribution and their importance in the local ecosystems, we are only now beginning to understand the ecology of these organisms.

The key for understanding the ecology of endolithic organisms often lies in the physical parameters of the immediate environment. Changes in the basic parameters (temperature, light, and moisture) can occur within millimeters. Therefore, the environment experienced by endolithic microorganisms can differ greatly from not only the large-scale macroclimate, but also the microclimate, traditionally defined as the climate in the size range of plants and animals. To emphasize this point we have proposed the term *nanoclimate* to describe the climate in the millimeter range (2,25,26). Clearly, measurements of the nanoclimate must be made with instruments operating at the appropriate scale and with the appropriate resolution (23,25–27). At the same time, because of the rigid nature of the substratum, the insertion of probes changes the environment to a degree not seen in other microbial habitats, for example, soft aquatic sediments, operating at a similar scale. One way to reduce this problem is through the development of simple computer models of the system (28,29). As will be illustrated later, these allow us to both verify the results of our measurements and extend our results to other parts of the ecosystem.

Because of the technical difficulties of making these measurements, only a few endolithic systems have been studied thoroughly. We begin our discussion with a detailed summary of the endolithic microbial communities in the Ross Desert of Antarctica.

ENDOLITHIC MICROBIAL COMMUNITIES IN THE ROSS DESERT OF ANTARCTICA

The most intensively studied terrestrial endolithic communities are those found in the higher elevations of the frigid Ross Desert in the McMurdo Dry Valleys of Northern Victoria Land, by far the largest true desert on the continent of Antarctica (21,30). The landscape is characteristic of an extreme desert: exposed bedrock and stone, soil covered by a desert pavement of small stones, no obvious vegetation (Plate 1). Air temperatures are uniformly cold, reaching a daily mean of only $-5\,^{\circ}C$ at the height of summer (mid-January), fluctuating between $-20\,^{\circ}C$ and $-40\,^{\circ}C$ during winter (25,26). All precipitation is in the form of snow, much of which sublimes away before melting (25,26,31); the absolute accumulation of snow is unknown, but probably on the order of a few tens of centimeters (30). In this environment, the only visible life consists of a few lichens growing in protected niches on rock surfaces. Endolithic organisms dominate the ecosystem.

Endolithic Organisms and Associations

Cryptoendolithic Communities. The cryptoendolithic microbial flora inhabiting porous Beacon sandstones, the prevalent rock type in the area, is surprisingly diverse. To date, five different cryptoendolithic communities have been identified, two dominated by eukaryotic algae, three by cyanobacteria (21,22,30). Their basic characteristics are summarized below.

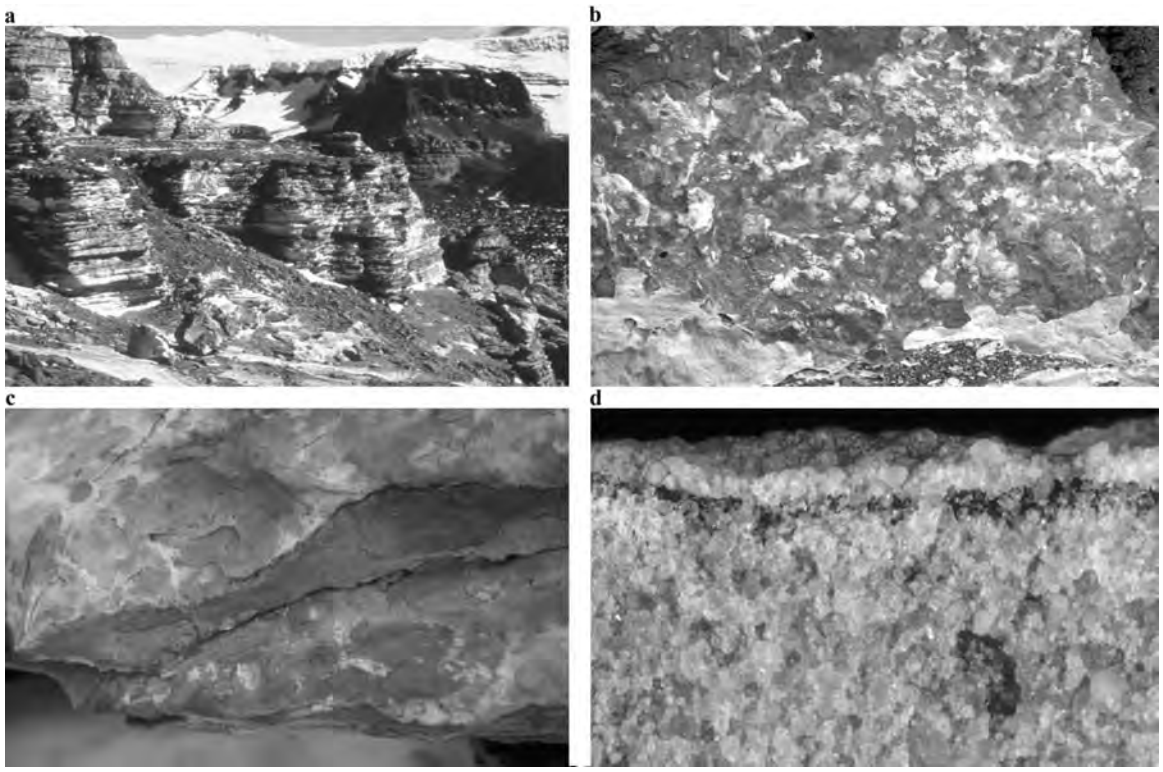

Plate 1. Endolithic communities in cold deserts. (**a**) Sandstone cliffs in University valley, Ross Desert (McMurdo Dry Valleys), Antarctica. Photo: E. I. Friedmann. (**b**) Surface of colonized sandstone with the characteristic mosaic pattern caused by exfoliative weathering from Linnaeus Terrace, Ross Desert (McMurdo Dry Valleys), Antarctica. Photo: J. A. Nienow. (**c**) Broken sandstone boulder from the same locality colonized by cryptoendolithic microorganisms. Photo: J. A. Nienow. (**d**) Colonization by the lichen-dominated cryptoendolithic community: black zone — dark-pigmented fungi, both lichenized and nonlichenized; white zone — colorless (hyaline) fungi, lichenized with *Trebouxia*, the photobiont; green zone — *Hemichloris antarctica* and *Chroococcidiopsis*. Photo: E. I. Friedmann. (Figures a and d reprinted with permission from Friedmann 1982 (*Science* **215**, 1045–1053), copyright 1982 American Association for the Advancement of Science). See color insert.

The Lichen-Dominated Community. The lichen-dominated community is the most common and best-studied in Antarctica. It appears as a conspicuous zone of parallel colored bands extending the surface crust of the rock to a depth of about 10 mm (Plate 1). Typically, there is a black upper band, extending from just below the crust to a depth of about 1.5 mm, followed by a white band of variable thickness, often extending to a depth of more than 4 mm (29). In well-developed communities, below the white band there is a green band and sometimes a blue-green band. The brown stain characteristic of Beacon sandstones in the region is absent from the colonized zone, leaving a framework of colorless quartz grains. The stain is apparently leached from the zone as a result of oxalic acid production by the fungi (23,32).

Within the black and white bands are filamentous fungi and spherical green algae, which together form a cryptoendolithic lichen. In contrast to typical lichens, including euendolithic lichens, the cryptoendolithic lichens do not have a distinctive morphology. In addition, reproductive structures are rare, so that identification of the lichen species is difficult, if not impossible. Because appressoria and haustoria are both seen in field samples (21), it is thought that at least two lichen associations are present in the community. Several strains of fungi isolated from the community are capable of forming lichen associations with lichen-algae in the laboratory (33, Friedmann and Koriem, unpublished data). Other strains of fungi, especially thick-walled and dark-pigmented black yeasts, are not. These are common in the upper black zone of the community. In some instances, they dominate the black zone, in others, the black zone is dominated by dark-pigment hyphae of the lichen-forming fungi. The white zone is inhabited by nonpigmented fungi and eukaryotic algae, which together form a lichen association. The nonpigmented fungi in this zone are almost certainly a morphotype of the dark-pigmented lichen-forming fungi from the black zone. Parasymbiontic fungi are apparently absent from this layer. The photobionts of the lichen association are species of *Trebouxia* or *Pseudotrebouxia* (21,34). Work with cultures suggests that more than one species is

present in the Ross Desert community, but that each lichen association contains only one. At least some of the cryptoendolithic lichens are morphological variants of epilithic species found in the region (35).

The green band is created by a mixture of eukaryotic algae and cyanobacteria. The dominant species in this association is the green alga *Hemichloris antarctica* (36). Other taxa include the xanthophycean alga *Heterococcus endolithicus* (37), a species from the chlorophycean genus *Stichococcus*, and a single species of each of the cyanobacterial genera *Gloeocapsa* and *Chroococcidiopsis*. *Hemichloris antarctica* and *Heterococcus endolithicus* are endemic to Antarctica. The species of *Chroococcidiopsis* is similar to forms occurring in hot deserts (2,38,39). The cyanobacteria tend to occur in the deeper layers, with *Chroococcidiopsis* deeper than *Gloeocapsa*. Occasionally, *Chroococcidiopsis* forms a separate blue-green band below the green band. Nonpigmented fungi are often present in the green band, but there is no morphological evidence of a lichen association with any of the algae.

The psychrophilic yeast *Cryptococcus friedmannii* Vishniac has been isolated from rocks containing the cryptoendolithic lichen community (40), but apparently only occurs infrequently. Heterotrophic bacteria comprise a constant, but small, portion of the community; Greenfield (41) estimated their biomass at less than 1% of the total. Included are members of the genera *Micrococcus*, *Deinococcus*, *Brevibacterium*, *Arthrobacter*, *Geodermatophilous*, *Micromonospora*, and *Streptomyces* (42–44). Siebert and coworkers (45), after a detailed study of the metabolic requirements of 41 strains of heterotrophic bacteria isolated from the endolithic habitat, characterized the community as morphologically simple but physiologically diverse.

The Hemichloris Community. The *Hemichloris* community is similar in composition to the association forming the green band in the cryptoendolithic lichen community. The dominant, sometimes sole, phototroph is *H. antarctica*. It typically colonizes the lower surface of overhanging sandstone ledges. Presumably, the light reaching these surfaces is too low to support the development of the lichen community. The *Hemichloris* community does not produce a leached zone.

The Red Gloeocapsa Community. This community is characterized by the presence of a large (3 to 12 μm in diameter) species of *Gloeocapsa* with a reddish lamellated sheath and reddish or purple cytoplasm. It forms a dark purplish red band extending from a point just below the surface crust to a depth of one to several millimeters within. Accompanying the red *Gloeocapsa* is a smaller, yellowish *Gloeocapsa*. Occasionally, other cyanobacteria are also encountered, including additional species of *Gloeocapsa*, *Eucapsis*, and *Microchaete*. This community inhabits the same types of rocks and boulders as the lichen-dominated community, but occurs much less frequently. It produces a leached zone.

The Hormathonema-Gloeocapsa Community. This complex cyanobacteria-dominated community has only been found on Battleship Promontory, where it colonizes white sandstone boulders permanently wetted by snowmelt percolating through the dolerite rubble at the base of the rocks. This community is characterized by the presence of two parallel color bands below the rock surface. The upper dark brown or blackish gray band is dominated by a species of *Hormathonema* and/or a dark gray species of *Gloeocapsa*. Members of a yellowish brown species of *Gloeocapsa* and two species of *Anabaena* also occur in this band. The lower green band is dominated by a species of *Aphanocapsa*, occasionally accompanied by several species of *Gloeocapsa*, another species of *Anabaena*, and a species of *Lyngbya*.

The Chroococcidiopsis Community. The *Chroococcidiopsis* community was the first endolithic community discovered in Antarctica (19). It is characterized by the presence of *Chroococcidiopsis* as the sole phototrophic organism. It appears as a single green or brownish band just below the surface crust. This community occurs in extreme cold and dry habitats.

Chasmoendolithic Communities. Chasmoendolithic communities occur in a variety of rocks in the Ross Desert. Cracks in weathered granites, heavily silicified sandstones, and, to a lesser extent, weathered dolerites are inhabited by rhizomorph-like extensions of epilithic lichens and associations of cyanobacteria. In granites, the chasmoendolithic lichens sometimes form masses of nonpigmented fungal hyphae interspersed with green algae of the genus *Trebouxia* (21). The cyanobacterial chasmolithic association is usually dominated by members of the coccoid genera *Chroococcidiopsis* and *Gloeocapsa* (20).

The Physical-Chemical Environment

The Substratum. The cryptoendolithic community in the Ross Desert is primarily found in porous quartz sandstones of the Beacon Supergroup. This geologic formation is common in the region since the mountain ranges are all formed of its members overlying metamorphic and intrusive rocks, mainly granites and dolerites. Both outcrops and small boulders are colonized extensively. Colonized rocks usually have a grain size between 0.2 and 0.5 mm and a porosity of 8 to 15%, but the colonization of finer-grained samples can also occur (28,41). Fe^{+3}-containing oxyhydroxides, either as part of the matrix or as stains on individual crystals, give the rocks their characteristic yellow, orange, tan, or brown colors (46,47). Other elements detected in the matrix include aluminum, calcium, lead, magnesium, manganese, phosphorus, potassium, sodium, and titanium (21,48). The relative amounts of these elements are consistent with the suggestion that the matrix material is derived from weathered dolerite (46,49).

The surfaces of colonized rocks are usually covered by thin silicified crusts less than 1 mm thick. These crusts stabilize the surface and reduce the rate of wind erosion. They develop from the accumulation of windblown dust. The dust gradually changes into a layer that is less than 100 μm, covering primary stains and framework grains. The chemical and physical processes leading to the

conversion are poorly understood but thought to be related to those leading to the formation of desert varnish (46).

The Macroclimate. Selected parameters of the macroclimate were continuously monitored through the use of automatic weather stations at Linnaeus Terrace from 1984 to 1988, and at Battleship Promontory from 1986 to 1987. The relevant data from these stations have been summarized previously (25,26,30). Briefly, the Antarctic year can be divided into four seasons on the basis of position of the sun. In winter, mid-April to September, the sun remains below the horizon 24 hours a day. Air and surface temperatures vary gradually between $-20\,^{\circ}C$ and $-45\,^{\circ}C$ in response to the movement of large air masses. Because of the cold temperatures, winter snowfalls do not contribute moisture to the system. Spring begins when the sun first appears above the horizon, sometime in early September, and lasts until the sun remains above the horizon 24 hours a day, in mid-November. During this period, air temperatures rise to about $-10\,^{\circ}C$. Surface temperatures also rise, depending on composition and orientation. Colonized horizontal surfaces remain below $-5\,^{\circ}C$, whereas north-facing slopes can reach temperatures as high as $+5\,^{\circ}C$. Snowfalls occur, but again, because of the cold temperatures, do not contribute much moisture. In summer, which lasts from mid-November to mid-February, the sun is above the horizon continuously. Even so, air temperatures remain low, rarely reaching $0\,^{\circ}C$. Surface temperatures can be much higher because of solar heating, and temperatures in excess of $+15\,^{\circ}C$ have been recorded for north-facing slopes. There can be considerable diurnal fluctuation as the sun changes its position in the sky, and the temperatures of all surfaces drop below $0\,^{\circ}C$ for some time every day. Snowfalls occur irregularly. Moisture sensors in the rocks indicate that at least some of the snow melts and enters the ecosystem. Fall begins in mid-February, when the sun drops below the horizon for an increasing portion of time every day. Temperatures drop as diurnal solar heating is reduced. By mid-April, winter conditions prevail.

The Nanoclimate

The Temperature Regime. As indicated in the previous section, the temperature of a colonized surface is not the same as the air temperature measured by weather stations. Solar heating can raise the temperature significantly, as much as $20\,^{\circ}C$ above the ambient air temperature. The amount of heating depends on both the orientation and the albedo of the surface (25,26,28,50). Calculations indicate that the diurnal maximum temperature of a north-facing surface can be as much as $13\,^{\circ}C$ higher than the diurnal maximum of a south-facing surface (28). Given the generally low temperatures in the region, this difference can account for the absence of significant colonization on south-facing slopes (21,27,28,50). Light-colored surfaces, which would be advantageous from the perspective of the light regime, may not warm sufficiently, at least under the conditions at Linnaeus Terrace, to support colonization.

Early reports (50) suggested that there was a steep temperature gradient in the colonized zone and that this gradient might influence the observed banding pattern in the cryptoendolithic lichen community. This possibility was investigated further through the use of small probes (27) and computer models (28). The combination of the two techniques demonstrated conclusively that while there is a slight temperature gradient within the colonized zone, its magnitude is too small to influence the distribution of cryptoendolithic microorganisms. The original reports of a steep gradient probably resulted from the difficulty of making measurements at the scale of the nanoclimate. Rapid changes in air temperature just above the surface of the rock may contribute to the absence of epilithic lichens in this region (27,28).

The Light Regime. The flux of light incident on the surface depends on the season of the year, the time of day, and the orientation of the surface (25,29). Seasonal data have been summarized by Friedmann and coworkers (25). During the height of summer (December and January), the average daily maximum photosynthetic photon flux (PPF) varies between 1,100 and 1,250 $\mu mol\ m^{-2}\ s^{-1}$; maxima in excess of 1,850 $\mu mol\ m^{-2}\ s^{-1}$ have been recorded. The key for the endolithic community, however, is how much of the light penetrates the rock. Measured gross extinction coefficients for colonized rocks range from 1.2 to 3.0 mm^{-1} (29), equivalent to decreases in the incident flux of 70 to 95% per millimeter. Rocks with larger framework grains and higher porosities have lower extinction coefficients; increasing the amount of matrix material or the degree of colonization increases the extinction coefficient. Because reflection at the surfaces of individual grains is a major factor in the extinction of light, changing the relative index of refraction through the addition of meltwater significantly reduces the extinction coefficient.

Both the organisms and the matrix material absorb blue light more efficiently than red light. This results in a calculated tenfold enrichment in red wavelengths at a depth of a few millimeters (29).

Although it is useful for purposes of comparison to assume that the extinction coefficient for an individual rock is constant with depth, this assumption is clearly false. First, because the quartz grains absorb relatively little of the light reaching their interiors, large grains can act as windows and allow light to reach much greater depths locally. Second, the absorbing particles, such as iron compounds, pigmented fungi, different species of algae and cyanobacteria, are not distributed uniformly. The effects of these factors on the distribution of light cannot be easily measured. Computer models (29) indicate that the steepest light gradient occurs across the crust, with up to 90% of the incident light reflected or absorbed over a distance of less than 0.5 mm. The flux of light can be reduced by an additional 90% in the black zone, with about half of the decrease attributable to absorption by members of the community. Thereafter, the rate of decrease is lower because the white zone lacks both pigmented fungi and iron compounds. At the bottom of the colonized zone, the photon flux is only about 0.005% of the incident flux, equivalent to about 0.06 $\mu mol\ m^{-2}\ s^{-1}$ (PPF) at the diurnal maximum. At this level, the effect

of water in the pore spaces is especially significant. When rocks are saturated with water, the amount of light reaching the bottom layers is increased 40-fold to about 0.2% of incident or 2.4μmol m^{-2} s^{-1} (PPF) at the diurnal maximum.

Water. Snow is the sole source of water for the community (31), but not all surfaces accumulate snow equally. On steeply sloped surfaces, snow accumulates only above the small ledges formed by exfoliative weathering (50). Even on horizontal surfaces, small depressions are more effective in accumulating snow than flat surfaces. Once the snow melts and enters the rock, it can be retained for relatively long periods, days or even weeks (25,26,50). Several factors contribute to the retention of water. First, because of the low temperatures, the potential rate of evaporation is only on the order of 1 to 2 mm m^{-2} day^{-1} (28,50). Second, the surface crust may retard evaporation by restricting gas exchange (51), although some field data suggest otherwise (50). Third, the rock matrix is capable of absorbing and retaining water at high humidity (52). The availability of matric water within the rock, in contrast to the arid conditions at the surface, seems to be the primary reason for the absence of epilithic lichens in the region.

Even during the Antarctic summer, water freezes on a daily basis, thereby reducing its availability to the community (25). Freezing occurs whenever the rock temperature drops below −5 °C; the water does not melt again until the temperature exceeds 0 °C (53). The depressed freezing point results from the physical structure of the rock. A similar phenomenon occurs in frozen soils (54).

The wetter conditions in sandstones colonized by the *Hormathonema-Gloeocapsa* community on Battleship Promontory are associated with differences in the chemistry of the rocks (22,48). Because of the lower oxidation-state of iron, colonized Beacon sandstones on Battleship Promontory are lighter than colonized sandstones from other regions. In addition, the pH is higher, ranging from 7.3 to 8.2 instead of the more typical 3.7 to 5.8. The concentrations of calcium, magnesium, and metal oxides are also higher (48).

Nutrients. Inorganic nitrogen in the form of nitrate and ammonium ions is always present in the upper few millimeters of colonized rocks (41,55). The concentration of nitrates varies from 0.1 to 25.9 μg g^{-1} rock; the concentration of ammonium ions is usually less than 2 μg g^{-1} rock. The nitrogen reaches the rock surface from the atmosphere at a rate of about 20 mg N m^{-2} yr^{-1} (30); biological nitrogen fixation is absent or very rare (41,56). The addition of inorganic nitrogen in the form of ammonia, nitrate, and nitrite does not stimulate carbon incorporation, indicating that the low levels of nitrogen are not limiting to the community (49,56). Apparently, in all endolithic microbial communities, including those in hot deserts, nitrogen demand is low because of the low metabolic rates, and can be met by the influx of fixed nitrogen from the atmosphere (55).

Interactions with the Physical Environment

Causes of the Pattern of Zonation. The most conspicuous feature of any cryptoendolithic community is the distinctive zonation pattern. Much of the structure can probably be attributed to interactions of the community with the light regime. Light gradients can result in structured communities of photosynthetic organisms through the actions of two opposing mechanisms. At the upper end of an organism's distribution, excessive light results in photooxidative damage. At the lower end, light is not sufficient for growth. This process was demonstrated qualitatively by Weber and coworkers (13) in a study of hot desert endolithic cyanobacteria. In their study, fine-grained quartz sand was inoculated at the surface with *Chroococcidiopsis*. The cultures were then incubated under different light regimes. After five months, the cyanobacteria had formed a distinct layer 3 to 7 mm below the surface of the sand. Cultures exposed to higher light intensities formed deeper bands than those exposed to lower light intensities. Qualitative evidence of these mechanisms can be seen are evident in the growth of the lichen-dominated community in the Ross Desert. The dark pigmentation of the upper layer probably provides a measure of protection against photooxidative damage (1). Fungi in the lower layers, where the photon flux is much lower, are not pigmented.

Presence of *H. antarctica* below the lichen requires a little more explanation. In culture, *Hemichloris* grows well at photon fluxes of 30 μmol m^{-2} s^{-1} (PPF), well above what it experiences in nature. Why, then, is it so deep in the rock? Several answers have been suggested (29,30), but none has been demonstrated conclusively. It is possible that *Hemichloris* is less tolerant of photooxidative damage incurred while in the desiccated state, and thus can only survive in darker habitats. It is also possible that *Hemichloris* is driven to the lower levels by competition with the lichen organisms. The competition could take the form of a simple scramble for space, with the lichen growing faster, or it could involve a more active process such as the production of antimicrobial agents by the members of the lichen. Experimental studies with cultures have demonstrated that several strains of fungi from the lichen layer are capable of producing substances active against heterotrophic bacteria and against lichenized and nonlichenized algae isolated from the parent community (57).

Biomass. The biomass of the endolithic community has been measured as chlorophyll *a* (51,58), Kjeldahl nitrogen (51,52,59), ATP (60), lipid phosphate (61), and organic matter, either by combustion (51,61) or by direct observation (41). Measured values of chlorophyll *a* range from 1.7 to 130 mg m^{-2}. The conversion of chlorophyll values to total biomass is difficult because of variability in the ratio of the photosynthetic and nonphotosynthetic components of the community. Estimates of the total organic matter based on Kjeldahl nitrogen range from 32 to 180 g m^{-2}. These are somewhat lower than estimates based on combustion (34 to 655 g m^{-2}), and significantly higher than estimates based on ATP (0.02 to 8.8 g m^{-2}) and lipid phosphate (4.4 to 7.4 g m^{-2}).

At least some of the variation among different methods results from the fact that a significant fraction of each endolithic community is composed of dead cells, as demonstrated by Greenfield (41). The proportion of dead cells in a community is variable, related, in part, to the environmental parameters at the site (41,47,62,63). The samples used by Greenfield, with only 14 to 21% of the organic matter viable, were collected from the summit of West Beacon, one of the colder sites in the Ross Desert.

Weathering. Colonization of Beacon sandstones by the lichen-dominated community is associated with a distinctive mosaiclike weathering pattern on the surface. The pattern consists of a patchwork of colors ranging from white to brown over a terraced surface (Plate 1). The microterracing results from exfoliative weathering, in which pieces of crust and subsurface material flake off the rock (Fig. 1). Exfoliating flakes are irregular in shape, up to 50 mm across and 1 to 2 mm thick. A number of factors contribute to exfoliation, including the growth of the lichen between the grains, intermittent swelling of the lichen during times of hydration, freeze expansion, and mobilization of the matrix material (47). The principal method of mobilization seems to be solubilization by oxalic acid. Oxalic acid, commonly implicated in weathering by lichens, has been identified on the basis of ion chromatography and crystal morphology in SEM (44,64,65), GC/MS (32), and FT-Raman spectroscopy (23,66). The use of FT-Raman spectroscopy allowed Russell and his colleagues to demonstrate an accumulation of oxalate just above the green layer of the community, at the depth where exfoliation occurs (23). They suggested that the dissolution of matrix silicates by oxalate may be more important than the dissolution of metal oxides or the expansion of clusters of cells. Other organic acids may also play a small role in the weathering process; 29 of 36 strains of heterotrophic bacteria tested by Siebert and coworkers (45) released some sort of organic acid into the growth medium.

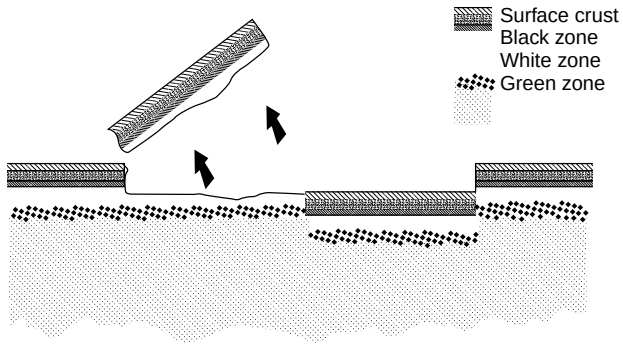

Figure 1. Diagrammatic representation of biogenous weathering in sandstones from the Ross Desert, Antarctica. From left to right: initial level of colonization, exfoliation of surface crust to biological activity, site of early exfoliation with microorganisms growing deeper into the rock substratum and the formation of a new surface crust, and a portion of the old surface crust at the initial level of microbial growth. [Reprinted with permission from Friedmann 1982 (*Science* **215**, 1,045–1,053). Copyright 1982 American Association for the Advancement of Science.]

Productivity and Growth. Gross productivity, as measured by the uptake of labeled bicarbonate in crushed rock samples, increases almost linearly with light up to a photosynthetic photon flux of about 200 μmol m^{-2} s^{-1} (56). Because the maximum light flux within the colonized zone is well below 200 μmol m^{-2} s^{-1}, the community is never light-saturated in its active state. This was confirmed by field measurements (56). Dark incorporation of labeled bicarbonate (56) or carbon dioxide (52,67) accounts for up to 10% of the total incorporation in the light. One of the lichen-forming fungi is responsible for at least a part of this incorporation. There is no evidence in support of chemoautotrophic growth (56). The rate of incorporation of bicarbonate is detectable at −8 and 30 °C, but not at −10 or 35 °C; the temperature optimum is 15 °C (56).

Net productivity, as measured by carbon dioxide gas exchange at the surface, occurs at surface photon fluxes as low as 100 μmol m^{-2} s^{-1} (PPF). The rate of uptake increases linearly up to at least 750 μmol m^{-2} s^{-1} (PPF), with no sign of light saturation (51,68). The maximum rates measured ranged from 0.22 to 0.78 mg carbon dioxide m^{-2} h^{-1} (51,68). The lower temperature limit is difficult to measure with any certainty, but also seems to be near −8 °C (51). Thus, −10 °C may be taken as the lower limit for significant metabolic activity by the community. The optimum temperature lies between −3 and 6 °C and the upper compensation point between 8 and 15 °C. The variation in these two numbers may represent the variation in the composition of the community, especially in the ratio of the photosynthetic to nonphotosynthetic components. It should be remembered that the optimum temperatures are rarely reached in nature and that the upper compensation point is only reached on north-facing slopes (25,68).

Annual gross and net productivity were estimated by coupling short-term laboratory measurements of photosynthesis with long-term field measurements of temperature and moisture conditions (68,69). Assuming that the organisms are metabolically active only at temperatures above −10 °C and only when liquid water is present, activity is restricted to an average of 700 to 800 hours per year in horizontal surfaces and 400 to 500 hours per year in north-facing slopes — the shorter period of activity in the sloped surface results from the reduced amount of snow accumulation on these surfaces. Annual net productivity in a well-colonized horizontal surface was estimated at about 600 mg C m^{-2} y^{-1} (equivalent to about 1,400 mg organic matter m^{-2} y^{-1}), which is about three times the net productivity of a sloped surface. At face value, these numbers indicate a low annual productivity in keeping with the harsh environment. The situation is actually more complicated. Johnston and Vestal (70) estimated the turnover time for organic matter in the community on the basis of the incorporation of carbon into phospholipids as 17,000 years. This is probably an overestimate of the turnover time because the temperature range below 5 °C, in which most of the net photosynthetic gain occurs, was not included in their calculations. A 10,000-year turnover time agrees with a 1,000-year residence time for carbon estimated from a radiocarbon determination (71,72). The net ecosystem productivity,

defined as the yearly accretion of organic matter, can be estimated as the quotient of biomass ($\sim$30 g C m^{-2}) and age ($\sim$10,000 years), yielding about 3 mg C m^{-2} y^{-1}. This amounts to about 0.05% of the net productivity and 0.025% of the gross productivity of the community (68). The missing material may represent the cost of remaining metabolically active in this environment, for example, the cost of compatible solutes lost during freeze-thaw cycles.

In the Ross Desert, microbial growth, a biological process, and exfoliative weathering, a geological process, seem to occur on approximately the same timescale (10^3 to 10^4 years). However, productivity measurements are typically made with mature communities. The question arises whether, after an exfoliation event with its concomitant loss of biomass, the microorganisms grow rapidly, fill the interstitial spaces in a short period of time, and then enter a period of slow or no growth, or grow slowly, but continuously, until the carrying capacity of the rock is reached. Sun and Friedmann (59) addressed this question by examining the relationship between biomass, as determined by Kjedahl nitrogen, and the color of the surface crust. Because exfoliation occurs within the leached, iron-free zone, new rock surfaces are white and the biomass of the cryptoendolithic community, having lost the dark-pigmented upper zones, is initially low. Over time, dust accumulates on the surface, resulting in an increasingly darker crust; thus the intensity of the surface provides a qualitative measure of the time since the last exfoliation. Sun and Friedmann found that biomass correlates with surface color, indicating that the community grows continuously throughout the long exfoliative cycle and that exfoliation occurs when the carrying capacity of the rock is reached.

Adaptations to the Environment. The microorganisms comprising the cryptoendolithic community do not seem to be well-adapted to the environment. Most of the strains tested are psychrotrophs, and several strains are capable of growth above 30 °C (36,37,42–45,73); true psychrophiles are present, but seem to be the exception. Two strains of the lichen-alga *Trebouxia*, the green alga *Hemichloris antarctica*, the xanthophycean alga *Heterococcus endolithicus*, the lichen fungi and some of the parasymbiontic fungi have temperature optimum between 15 and 20 °C (36,37,73), which is well above what is typically encountered during the active period. The temperature optimum for net photosynthetic activity by the community, between -2 and $+5$ °C, in contrast, is reached in nature. Apparently, the community is adapted to the environment, but the individual members are not.

This paradoxical situation can be explained if we consider the following facts. First, the temperature response curves of algae and fungi have different slopes, so the responses of the two groups do not parallel each other. Second, the temperature optimum of the fungi are usually higher than those of the algae. Third, fungal respiration continues to increase beyond the photosynthetic optimum of the algae. For a lichen community to exist in any given temperature range, the ratio of algae-to-fungi must be such that net production by the algae is greater than or equal to consumption by the fungi. In nature, the community as a whole adapts by altering the algae-to-fungi ratio to match the prevailing temperature conditions (Friedmann and Sun, unpublished data).

Extinction and Fossilization. The cryptoendolithic community exists on a tight energy budget, with no reserves to spare. The organisms living under a temperature regime far below their optimum seem to have reached the limits of their physiological potential. Maintenance of life depends on a precarious equilibrium of biological and physical processes. Even minor shifts in this equilibrium caused by a change in the orientation of the rock resulting in reduced insolation may cause extinction (47). Communities in all stages of deterioration can be found in the Ross Desert, and changes in cellular fine structure, from normal to stressed to dead cells, have been documented (23,62). Rock surfaces with extinct communities may become heavily silicified and resistant rock rinds may develop that incorporate and preserve the characteristic weathering patterns as trace fossils (47). The minimum age of such trace fossils has been estimated from quartz-rind thicknesses as ranging from about 7,000 to about 2 to 4 million years (47). It has been suggested that similar trace fossils may have been formed on the surface of Mars, preserving the last traces of a cooling and drying planet (2,47,62,74,75). Methods used to detect and analyze fossil and living endolithic communities may help in the search for life on Mars (23).

ENDOLITHIC COMMUNITIES IN THE COASTAL REGION OF ANTARCTICA

Cryptoendolithic communities are rare in coastal and maritime Antarctica, presumably because of the scarcity of suitable porous substrata. Chasmoendolithic communities are widespread and may include the major primary producers in the coastal deserts of Antarctica (76). Examples have been reported from ice-free coastal regions and nunataks in Queen Maud Land (77), MacRobertson Land (24,78), Marie Byrd Land (79,80), Princess Elizabeth Land (24), Victoria Land (20,21,78), Ross Island (81), and Signy Island (82). The most extensive studies have been made in the Vestfold Hills, Princess Elizabeth Land, and in the vicinity of Mawson Rock, MacRobertson Land.

Chasmolithic microorganisms inhabit a variety of rock types; the only requirements seem to be cracks in the stone, some light-colored mineral grains capable of transmitting light, and a supply of water. Colonized rocks types include weathered granites and anorthosites from Dufek Massif (20) and the granitic nunataks on the Edward VII Peninsula (80), weathered marbles at Gneiss Point and Marble Point (20,21), charnockite and quartz pebbles at Mawson Rock (24), and quartz-feldspathic gneisses and quartz pebbles in the Vestfold Hills (24). The growth pattern of the microorganisms is determined by the nonbiological weathering pattern of the rock. For example, vertical fissures are common in orthoquartzite, whereas granites and gneisses tend to crack parallel to the surface. In some cases, there are no obvious fissures, but just a network of small cracks. In this case, the growth pattern resembles that of the cryptoendolithic community.

The photosynthetic community in coastal Antarctica is much more diverse than that of the Ross Desert. More than 17 taxa of cyanobacteria and eukaryotic algae have been identified, although only four appear with any regularity (24,74,83). The green alga *Prasinococcus calcarius* is the most common constituent where ocean spray reaches the rock. In drier areas not reached by spray, members of the green alga genus *Desmococcus*, including a new species, *Desmococcus endolithicus* (84), are the most common taxa. Less frequently, a cyanobacterial community dominated by *Chroococcidiopsis* and an oscillatorian species develops; this community seems to have a higher moisture requirement and only develops in areas where a greater supply of meltwater is available (24). *Pseudococcomyxa simplex* was abundant as a chasmolith in an isolated boulder on the ice near the North Masson Range (85). On Signy Island, the relatively diverse chasmolithic community includes *Gloeocapsa gelatinosa*, *Merismopedia tenuissima*, *Nostoc punctiforme*, and *Plectonema battersii* (82).

The higher diversity of the coastal chasmolithic community, when compared with the Ross Desert communities, can be attributed to the milder climate. In the Vestfold Hills and on Mawson Rock, mean daily temperatures reach 2 °C in January (76,83). Signy Island is even warmer and temperatures can reach as high as 30 °C (86). In addition, winter snow accumulation is sufficient to provide a trickle of water over rock surfaces well into summer (24).

The distribution of chasmoendolithic colonization is determined primarily by wind direction; in the Vestfold Hills, the community develops almost exclusively on the leeward surfaces (24). Depressions in steep surfaces capable of capturing snowdrift enhance the development of the community.

ENDOLITHIC COMMUNITIES IN HOT ARID REGIONS

As indicated in the introduction, endolithic communities are widespread in hot arid regions. It is beyond the scope of this article to discuss each of these in detail. Instead, we will focus on three relatively well-characterized examples to illustrate the general features.

Endolithic Communities in the Middle East

The endolithic communities in the Negev Desert, southern Israel, were among the first reported from hot desert regions (1,4,5). The climate is typical of a hot desert (4,7). The yearly average temperature ranges from 21 to 35 °C in the south and east, 19 to 21 °C in the north. The annual precipitation ranges from a few millimeters in the south to a maximum of less than 20 cm in the Negev Highlands. Most of the rain is seasonal and limited to a few days during the winter. Moisture is also available in the form of fog and dew (4,7,87,88), especially in the northern parts of the desert.

Chasmoendolithic Communities. Chasmoendolithic microorganisms are found in weathered granites, dolomites, limestones, and quartz, where they can form extensive horizontal growths under surface flakes (1,4,7,89). The photosynthetic community, the only segment of the community studied in detail, is usually dominated by unicellular cyanobacteria, especially members of the genera *Chroococcus*, *Chroococcidiopsis*, and *Gloeocapsa*. However, a number of unicellular green algae and filamentous cyanobacteria occur with some regularity (4). This community is thought to be responsible for the exfoliative weathering of limestone and dolomite boulders in the region (1,7,89).

Cryptoendolithic Communities. Cryptoendolithic communities are found in porous Nubian sandstone and crystalline limestones throughout the region (5, Plate 2). They are typically dominated by a single species of cyanobacterium, almost always a member of the genus *Chroococcidiopsis*. However, other chroococcoid cyanobacteria, heterotrophic bacteria, and an occasional unicellular green alga may also be present (4,5). The community forms a green band, 0.1 to 2.5 mm thick, 0.1 to 3.0 mm below the surface of the rock. Well-developed communities frequently display a definite zonation pattern. In these cases, the upper layers are marked by the abundant growth of large colonies of cyanobacteria with pigmented sheaths and brownish cytoplasm. The pigmented sheaths probably indicate the presence of scytonemin, a compound demonstrated to provide protection against photooxidative and UV-induced damage in cyanobacteria (90,91). The upper boundary of this layer is usually sharply delimited. The lower limit is more diffuse, grading into a zone of scattered colonies with colorless sheaths and blue-green protoplasts.

Euendolithic Communities. Euendolithic lichens, especially the lichen species *Caloplaca aloiziza*, colonize the surface of limestone rocks and stones in the moister regions of the Negev (6,7,92,93). Extensive colonies give the surface of the rocks a characteristic gray color. Larger surfaces are overlain with a jigsaw-puzzle-like pattern derived from the boundaries of individual lichen colonies. The jigsaw-puzzle pattern is enhanced by the activity of terrestrial snails, which preferentially feed at the boundaries (6,94). The distribution of euendolithic lichens has been linked to the amount of rainfall, the orientation of the surface, and the size of the stone (6,7,93). The lower limit of the extensive development of the community seems to be a mean rainfall of about 30 cm y^{-1}. Wetter, cooler, north-facing surfaces are colonized by epilithic lichens, precluding their use by endolithic microorganisms. The size of the stone influences the amount of dewfall. Smaller stones cool more quickly and more completely than larger stones and, therefore, cause more dew to condense from the surrounding air (7,88). If the micro- and nanoclimates result in more than 450 hours per year of daylight imbibition time because of dew, epilithic lichens cover the surface of the stone. Lower amounts of dew and a lower cumulative daylight imbibition time (300 to 450 hours per year) favor the growth of endolithic lichens (7). Even lower cumulative daylight imbibition times seem to favor the growth of euendolithic fungi of the genus *Lichenothelia* and of communities of cryptoendolithic and chasmoendolithic

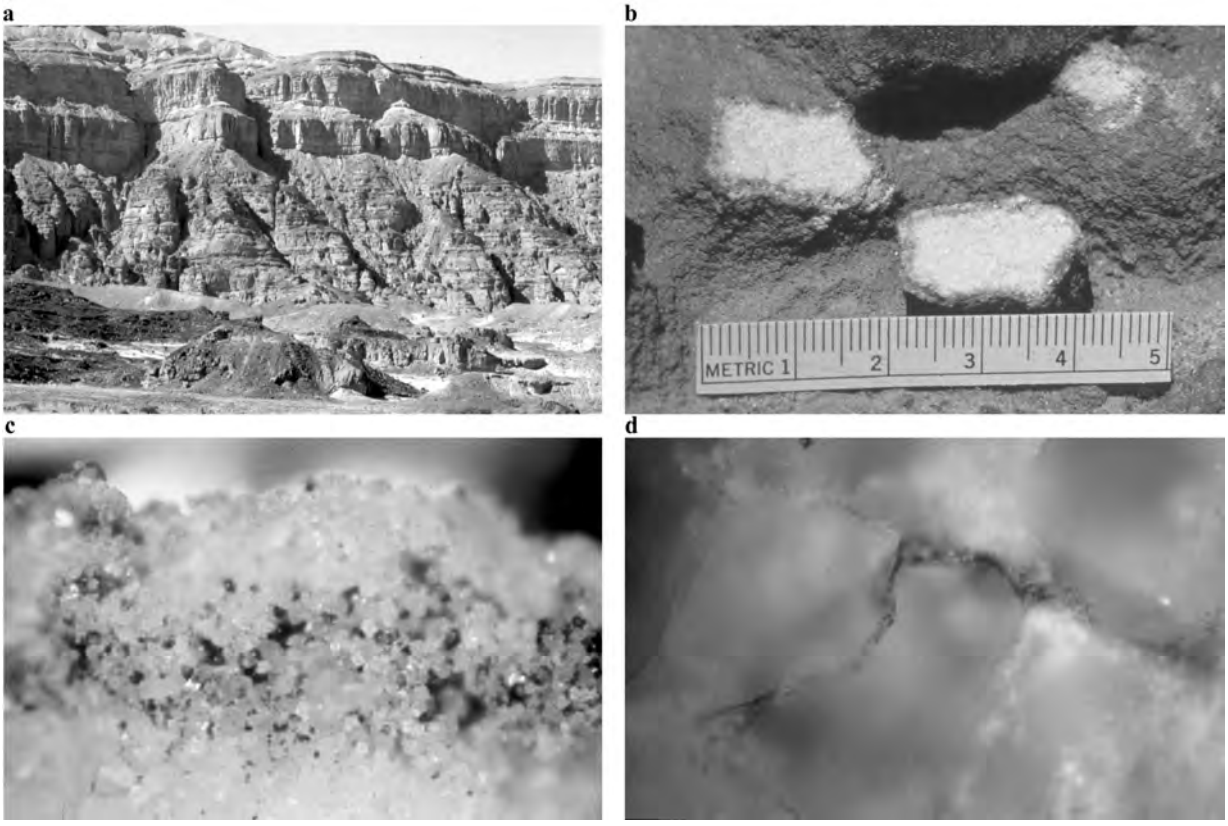

Plate 2. Endolithic communities in hot deserts. (**a**) Limestone and sandstone (darker rocks in foreground) cliffs in Timna National Park, Negev Desert, Israel. Photo: E. I. Friedmann. (**b**) Broken sandstone from the same locality colonized by cryptoendolithic microorganisms. Photo: E. I. Friedmann. (**c**) Endolithic colonization of crystalline sandstone, Mitzpeh Ramon, Negev Desert, Israel. Green zone — *Chroococcidiopsis*. Photo: E. I. Friedmann. (**d**) Section of granite colonized by chasmoendolithic microorganisms, Sonoran Desert, Mexico. Photo: E. I. Friedmann. (Figures a, b, and d reprinted with permission from Whitton and Potts 2000, *The Ecology of Cyanobacteria*, Kluwer Academic Publishers, copyright 2000 Kluwer Academic Publishers. Figure c reprinted with permission from Freidmann et al. 1967 (*Phycologia* **6**, 185–200), copyright 1967 International Phycological Society. See color insert.

cyanobacteria and eukaryotic algae. *Lichenothelia* colonies can form in limestone rocks in the southern Negev where the annual precipitation is between 3 and 5 cm y^{-1} (11).

Free-living colonies of euendolithic cyanobacteria inhabiting characteristic pits and depressions in limestone rocks in the more arid regions of the Negev Highlands have been reported (6,11,93). These reports are somewhat controversial and require reexamination. One of us (EIF) examined samples of such pitted limestone rocks from the same vicinity and found dark hyphae from *Lichenothelia* or other fungi, but no cyanobacteria. However, a euendolithic filamentous species of the stigonematalean genus *Matteia* has been isolated from the Negev (95).

Endolithic Microorganisms as Indicators of Paleoclimates. The activity of lithobiontic, especially euendolithic, microbial communities leaves distinct traces on the surfaces of colonized rocks, even when the community is destroyed by burial or fire. To the extent that these communities are associated with particular macro- and microclimatic conditions, these traces can be used to evaluate past climatic conditions. This has been attempted for sites in Israel by Danin and his colleagues (6,93). According to their observations, the co-occurrence of certain communities correlates well with current climate conditions (93). Pitting by microfungi of the genus *Lichenothelia* and by cyanophilous lichens on stones and on boulders, for example, is indicative of the driest conditions. Epilithic lichens and the jigsaw-puzzle patterns formed by endolithic lichens (genus *Caloplaca*) indicate relatively moist conditions. Using this information, Danin (93) was able to document drier conditions in Israel during the pre-Neolithic (30,000 to 12,000 BP) and wetter conditions during the Neolithic (10,000 to 9,000 BP) period by examining the weathering patterns on boulders unearthed at archeological sites. Climatic conditions have remained relatively constant in Israel for the past 6,000 years.

Endolithic Communities in the southwestern United States and Mexico

Endolithic communities have been reported from the Sonoran Desert, Mexico (14,96), and Death Valley, California (1), but the most extensively studied are those of the Colorado Plateau, northern Arizona (15,97–99). The

climate in Colorado Plateau is somewhat milder than that of the Negev Highlands; the mean annual rainfall ranges from 20 to 30 cm y^{-1} and mean temperatures range from 10 to 13 °C (97). Measurements within colonized rocks indicate that the relative humidity in the colonized zone never drops below 60% and is usually in excess of 80%; temperatures can reach as high as 47 °C (99). The predominant rock types are quartz sandstones belonging to eight geologic formations; granites, limestone, quartzite, and siltstone are also present (15,98). Chasmolithic communities are found in all rock types. These are relatively depauperate, frequently monotypic stands of *Chroococcidiopsis* or related cyanobacterial taxa (98).

The cryptoendolithic community, in contrast, is relatively rich. At least twenty species of cyanobacteria and green algae are present (15). As is typical of cryptoendolithic communities, these form a more or less well-defined band extending from just below the surface crust to a variable depth within the rock, but never more than 2 mm (15). The depth of the community depends on the grain size, the porosity, and the color of the rock, presumably through interactions with light (15). The composition of the community seems to depend on the macroclimate. Rock outcrops associated with desert scrub communities, indicating the harshest macroclimates in the region, harbor cryptoendolithic communities dominated by cyanobacteria, usually members of the genus *Chroococcidiopsis*. The same is true of outcrops associated with grasslands. In outcrops of the same type of sandstone in conifer forests, which are slightly cooler and receive more rainfall (30 cm y^{-1} vs. 20 cm y^{-1}, the community can be dominated by green algae of the genera *Chlorococcum*, *Borodinella*, *Chlorosarcinopsis*, and *Tetracystis* (98).

The biomass of the community, measured as chlorophyll *a*, ranges from 8 to 140 mg m^{-2} (15,97); these values are similar to those reported for cryptoendolithic communities in Antarctica. The biomass depends in part on the color of the rock, with light sandstones harboring a larger community than dark sandstones (15,99). Photosynthetic activity by the community, as determined by the uptake of labeled bicarbonate, ranges from 0.13 to 0.89 mg carbon oxide dm^{-2} h^{-1}. Rates of carbon fixation vary with the season and the time of the day. Interestingly, during summer, photosynthetic activity is reduced at noon, perhaps indicating some degree of photoinhibition; this reduction was not seen during other seasons (99). However, the maximum photosynthetic photon flux in the algal zone should be less than 100 μmol m^{-2} s^{-1} (99). These values are comparable to the above ground rate of photosynthesis in the region (97).

Endolithic Communities in Southern Africa

Cryptoendolithic communities have been reported from the northeastern portions of the Orange Free State and from the Northern Province of South Africa (10,13,100). Two of these communities have been studied in some detail.

The Langjan Nature Reserve Community. The cryptoendolithic community in a large brown-colored quartz sandstone has been investigated by Weber, Wessels, and Büdel (13). The climate in the Langjan Nature Reserve is typical of dry savannah grassland, with limited seasonal precipitation and high summer temperatures. The mean annual rainfall is 34 cm y^{-1}, higher than in the Negev Desert or on the Colorado Plateau, with most of the rain falling between November and March. During the dry season, which is from May to September, monthly rainfall is less than 1 cm. Air temperatures vary from 0 to 42 °C; the annual mean temperature is 23 °C. Limited measurements at the beginning of the summer season indicate that rock temperatures in excess of 50 °C are common. Observations indicate that the rock may serve as a water reservoir, but this was not investigated in detail.

The community forms a blue-green band averaging 1.1 mm in thickness starting at an average depth of 1 mm. The community is composed primarily of cyanobacteria. The most abundant organisms are members of the genus *Chroococcidiopsis*. Filamentous cyanobacteria of the genera *Nostochopsis* and *Microcoleus* are also present, as are a number of fungi imperfecti. The latter do not seem to form lichen associations with the cyanobacteria. Chlorophyll *a* ranges from 0.05 to 71.8 mg m^{-2}, with a mean of 29 mg m^{-2}, C-content from 4 to 107 g m^{-2}, and N-content from 0.7 to 4.4 g m^{-2}, in the same range as other cryptoendolithic systems. Fluorescence measurements using intact rocks demonstrated that the community is probably not light-saturated in nature. However, if the surface of the rock is removed and the community exposed, light saturation occurs at a photosynthetic photon flux between 200 and 350 μmol m^{-2} s^{-1}, and photoinhibition may occur at fluxes below 1,200 μmol m^{-2} s^{-1}.

The Golden Gate Highlands National Park. The Golden Gate Highlands are located in northeastern Orange Free State, South Africa. Aspects of the endolithic communities in the park have been investigated by Wessels and his colleagues for a number of years (100–103). The climate in the region is relatively mild. Temperatures range from 13 to 25 °C in summer and from 0 to 15 °C in winter. The mean annual rainfall, about 76 cm y^{-1}, is more than double that of the previously discussed sites.

Euendolithic lichens, primarily of the genus *Lecidea*, are common in the large sandstone outcrops characteristic of the park (103). The same species colonize sandstones in the drier Mountain Zebra National Park (12). In Golden Gate Highlands National Park, they actively grow into the sandstone by dissolving the matrix material filling in the pore spaces and cementing the grains together (101). Some of the loosened grains are removed by insects during their life cycle (102). Wessels and coworkers (103) were also able to demonstrate increased mechanical strain in the rock as the result of the imbibition of moisture from the air in response to diurnal changes in relative humidity (103). As a result of these processes, biogenic weathering of Clarens sandstone can cause the removal of about 10 mm of material per hundred years (101).

A cyanobacteria-dominated community is found in the rear wall of shallow caves eroded into a stratum of Clarens sandstone overlain by a dolerite dike. The surface of the sandstone is effectively sealed by a light brown crust of sodium carbonate, sodium sulfate, and

recrystallized siliceous material (100). This ecosystem is unique in that the rock appears to be waterlogged throughout the year. Water enters the rock at the upper surface, which is not colonized by the community. Water percolates downward, reaching the community from the inside. The total amount of water available to the microorganisms was demonstrated inadvertently by the investigators when they chipped a sample from the surface of the rock and water began to seep out. Sufficient water was still available 18 months later to support a diverse epilithic community (100). In spite of the wet conditions, the cryptoendolithic community is similar to other such communities in arid regions. It forms a blue-green zone, 1 to 2 mm thick, 0.2 to 1 mm below the surface. The dominant organism is, again, a member of the genus *Chroococcidiopsis*, and biomass (chlorophyll *a*) ranges between 44.3 and 51.8 $mg\,m^{-2}$. Strains of *Chroococcidiopsis* isolated from Clarens sandstone raise the pH of the medium to almost 10. If such an increase occurred in nature, it would increase the solubility of silica and may contribute to the weathering of the sandstone (100).

COMPARISON OF ENDOLITHIC COMMUNITIES IN HOT AND COLD ARID REGIONS

Advantages of the Endolithic Habitat in Arid Regions

Endolithic communities develop wherever suitable rock types occur, in temperate regions as well as in arid lands, as can be seen in the diverse cryptoendolithic community inhabiting dolomite cliffs in southern Canada (104–106). Why, then, are endolithic communities more important in arid regions than in regions with a milder (moister) climate? In humid regions, rock surfaces are frequently covered by dense growths of epilithic cyanobacteria, algae, lichens, and lower plants (107). Such growths preclude the development of endolithic communities by limiting the amount of light reaching the habitat. Endolithic microorganisms can only form extensive growths where external conditions are too harsh to allow the development of epilithic communities. This factor must be the primary cause of the distribution patterns of epilithic and endolithic lichens observed in the Negev Highlands. It may also be responsible for the restriction of endolithic algae to the southern walls of the limestone O. L. Basilica in northeastern Belgium (108).

Given that the external environment is too harsh to support epilithic organisms, what protection is offered by the endolithic habitat? Temperature is probably not a major factor. As discussed previously, the temperature within the colonized zone is essentially the same as the temperature at the surface. The only difference is that very short-term temperature fluctuations at the surface are damped within the first millimeter or so (28). This may be important in cold deserts, where rapid fluctuations across the freezing point have been observed, but probably only plays a small role in hot deserts and temperate climates. More important are the moisture and the light conditions. In endolithic communities, especially cryptoendolithic communities, the internal environment is moister than the external. Two factors are at work. First, the surface crust reduces the rate of evaporation or, in the extreme case of the Clarens sandstone community, the rate of outflow. Second, the physical structure of the substratum helps to condense and hold water within the rock (52,103). Because the interior is moister, the endolithic community experiences a longer period of metabolic activity after each dewfall, rainfall, or snowmelt, and a longer cumulative imbibition time than the epilithic community. However, the crucial feature is probably the reduction in the amount of light reaching the community in the desiccated state. Cryptoendolithic communities are found at depths corresponding to photon fluxes less than 5% of direct sunlight. Even with these low light levels, the organisms in the upper portions of the community produce pigments, melanin and melanin-like compounds in cryptoendolithic fungi and scytonemin in cyanobacteria, associated with protection against high light (1,90,91). Apparently, the amount of photooxidative damage accumulated at the surface during the desiccated state is too great to be repaired during the short periods of activity. The organisms require a combination of screening by the material of the rock and by their own pigments to reduce the amount of damage to a manageable level.

Cryptoendolithic Cyanobacteria in Hot Deserts

The dominance of cyanobacteria, especially members of the genus *Chroococcidiopsis*, in hot desert cryptoendolithic communities is striking. Evidently, the conditions common on rock surfaces in hot climates, namely, temperatures of 50°C or more, high levels of insolation, and extreme dryness, are less tolerated by eukaryotic algae than by cyanobacteria (2,99). This is consistent with the dominance of epilithic cyanobacteria and cyanolichens on tropical inselbergs (107), with the increased role eukaryotic algae play in the milder portions of the Colorado Plateau (98), and with the dominance of eukaryotic cryptoendolithic lichens in the milder Antarctic desert. It is also possible that *Chroococcidiopsis* and other cyanobacteria have more efficient repair mechanisms. These would allow them to recover more quickly from damaged induced by desiccation or by photooxidation, and to use more efficiently the shorter periods of activity in hot deserts. In fact, desert strains of *Chroococcidiopsis* have been shown to survive doses of X rays as high as 15 kGy, at rates of survival cited as second only to those of strains of *Deinococcus radiodurans* (109). Note that neither of these suggestions implies that *Chroococcidiopsis* is well-adapted to the endolithic environment. Instead, it seems that *Chroococcidiopsis* is the complete generalist, with an almost unparalleled ability to live under a wide range of environmental extremes (39). However, because it is a generalist, it lacks the ability to compete with more specialized species, and only succeeds where they cannot survive.

CONCLUSION

Environmental conditions on the surfaces of rocks in arid regions can be so harsh that not even lichens cannot survive; in extreme cases, the surfaces are essentially

abiotic. Endolithic microorganisms in these regions find a protected niche under the surface, in structural cavities or in fissures. These microorganisms are not adapted to the environment in the strict sense of the word because their physiological optimum lie far from the conditions they are living in. Instead, they are adapted to tolerate the environmental extremes. Often they exist near the limits of their physiological potential, in which no further adaptation is possible.

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ENDOSYMBIOSIS IN ECOLOGY AND EVOLUTION

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Almost all the forms of life present endosymbiotic organisms, that is, microorganisms that have developed the ability to live in the body or in the cells of other organisms. Such endosymbionts have originated in several lineages, both prokaryotic and eukaryotic, and include both mutualistic and parasitic lifestyles. Symbiotic associations, earlier considered as a curiosity, in reality are widespread in nature (1) and play a key role in ecology. Especially of interest are those associations in which microorganisms have chosen the intracellular habitat of another cell as an ecologic niche (2). Major examples of the ecological importance of endosymbiosis are the associations of some fungi with plant roots, forming endomycorrhizae that aid the plant's acquisition of minerals, and the endosymbioses of algae with invertebrate animals in oligotrophic tropical seas, where they may be a very important source of nutrients and may also contribute to the formation of coral reefs. Moreover, an unexpected biodiversity has been discovered near the deep-sea hydrothermal vents, where a photosynthesis-independent ecosystem exists based on sulfur-oxidizing (thiotrophic) bacterial symbionts. The evolutionary importance of endosymbiosis

is also considerable. Indeed the endosymbiosis between a unique lineage of green alga and the glomalean fungi lies at the origin of the landplants that dominate the terrestrial ecosystems. Finally, intracellular symbioses have played a key role in the origin and evolution of the eukaryotes: it is actually noteworthy that two important eukaryotic organelles, the mitochondria and the plastids, are derived from highly integrated endosymbiotic bacteria. Thus, symbiosis may be considered not simply as a mutualistic relationship but also as a way for evolutionary creativity.

DEFINITIONS

Mutualism and Parasitism

The term symbiosis originally referred to any group of differently named organisms living together, whether both organisms derive a benefit (mutualism) or the benefit for one organism derives from a damage to another one (parasitism). However, sometimes the status of an organism is difficult to identify precisely, because the mutualistic or parasitic behavior may depend on a developmental stage, or on an environmental stress, such as a contact with an unnatural partner, or simply because of our limited knowledge of the association. In addition, a mutualistic or parasitic status may change with time. The bacterial group of the rickettsiae clearly illustrates these aspects. The rickettsiae are generally nonparasitic intracellular symbionts of arthropods and protists. But some species are important human or animal pathogens transmitted by ticks or other parasitic arthropods, causing typhus, spotted fevers or ehrlichioses. Closely related nonpathogenic rickettsiae exist, and their nonparasitic status depends primarily on the fact that the arthropod host never bites a vertebrate (3). It should be noted that the mitochondrion, a fundamental organelle of almost all the eukaryotes, seems to be derived from an ancestral intracellular bacterium belonging to the rickettsial group (4). By considering both mutualists and parasites as symbionts, we follow the original definition; this approach was also adopted by Smith and Douglas (1) in their important book. In cases in which the status is not clearly defined, we adopt the term "symbiont" or "biont" and its composed forms, for the smaller partner.

Symbiont, Host, and Terminology

In most cases the size of the partners is unequal, and the terms host and symbiont are used for the larger and the smaller, respectively. The smaller size of the symbiont implies that it may be positioned in different ways with regard to the body host; some multicellular hosts have also developed specialized organs or cells to contain the symbiont. The terms *ectosymbiont* or *ectobiont* are used if the symbiont is external to the body of the host, and the terms *endosymbiont* or *endobiont* if it is located within the body of the host (Fig. 1). Furthermore, the endosymbiont may reside outside or within the cells of the host; it is then termed *extracellular endosymbiont* or *extracytobiont* and *intracellular endosymbiont* or *intracytobiont*. The intracytobiont may be free in the cytoplasm of the host cell or reside within a host cell-derived vacuole, called a *symbiosome*. The terms *parasitophorous vacuole* or *inclusion* are adopted when the intracytobiont is a parasite. The symbiotic association may be facultative or obligate for one or both the partners, if they may survive or not when the association is disrupted. However, such a distinction may be highly influenced by the laboratory techniques. For example, the term *obligate intracytobiont* is often applied to those microorganisms that could not be cultivated on available artificial media. As such situation may change with the development of more appropriate techniques, the distinction between *culturable* and *unculturable* organisms should be preferred. Finally it is experimentally possible to eliminate the symbiont by a treatment with antibiotics or by exposure to elevated temperatures. The symbiont-cured host is called *aposymbiotic*. This manipulation allows studying the effects of the symbiont on the host's phenotype.

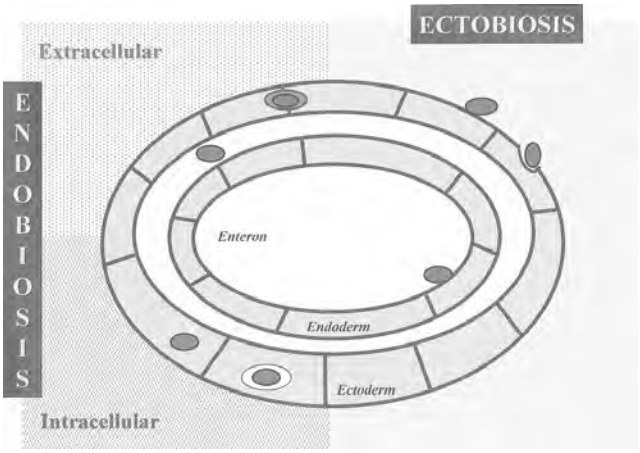

Figure 1. Terminology. Schematic drawing of a cross-section of a multicellular host to illustrate the relative position of symbionts. Ectosymbionts (at right) are positioned on the external surfaces of the host body, on the ectoderm, or on the intestinal cavity. Endosymbionts (at left) may be extracellular, or in other words, located between cells of tissues or within the host body fluids (e.g., the blood), or the endosymbionts may be harbored within the host cells, free into the cytoplasm, or enclosed in a membrane (symbiosome). See color insert.

SYMBIOTROPHIC SYSTEMS

Almost all the symbiotic associations are based on a form of *syntrophy*, that is, a nutritional situation in which two or more organisms combine their metabolic capabilities to metabolize a compound otherwise impossible to be metabolized by one alone. These are called *symbiotrophic systems*. Insects with restricted diets (i.e., plant sap- or blood-sucking insects), harbor symbiotic bacteria that provide the host with some essential nutrients, such as amino acids and/or vitamins. A very widespread symbiotrophic system is the one involving autotrophic endosymbionts, either photosynthetic (symbio-photoautotrophy) or chemoautotrophic. Finally of importance are the nitrogen-fixing bacterial symbionts of the plants.

Primary and Secondary Symbionts of Insects

Extra- and intracellular endosymbionts are commonly found in a large majority of insects, particularly in those living on nutritionally restricted diets, such as plant sap, blood, or wood. Most of these associations are obligate, the symbiont providing some essential nutrients to the host, through the synthesis of amino acids or vitamins or through the degradation of diet materials (1). The symbionts are rarely cultivated in laboratory media, and the aposymbiotic insects show several alterations, such as sterility or high mortality rates (5). These associations have been known for a long time on the basis of morphological studies (6). Recently, the development of molecular biological techniques has allowed the phylogenetic identification of most of these symbionts and the reconstruction of the evolutionary history of the symbiosis. We can distinguish two main types of insect endosymbionts: the gut symbionts, prevalently anaerobic and extracellular, and the mycetocyte symbionts, aerobic and intracellular. Additional examples of insect symbioses are those of the ants, in which both extra- and intracellular bacterial symbionts are reported, as well as complex tripartite associations involving ants and bacterial symbionts essential for the cultivation of fungal gardens (7).

The Gut Symbiosis. The gut endosymbionts are almost always extracellular, residing into the anoxic region of the insect hindgut. Such a microbiota is very heterogeneous, including both prokaryotes and protists, most being found only in this habitat. Such a microbiota is responsible for the degradation of cellulosic materials in some wood-heating insects like the lower termites (Isoptera) (the protist biota is lacking in the higher termites), but also carries out other specific anaerobic metabolic processes, such as N_2 fixation (see following section on "Nitrogen Fixation") and methanogenesis. The latter is performed exclusively by some archaebacteria, free-living in the gut or epi/endosymbiont of intestinal protists.

The Mycetocyte Symbiosis. The mycetocyte or bacteriocyte is a specialized cell harboring endosymbionts within a symbiosome; the endosymbionts are generally prokaryotes, called *Primary* (P) symbionts, and are maternally inherited. Bacteriocytes are often organized into a multicellular complex, called the *bacteriome*, generally located in the abdomen of the insect. For each P-symbiont/insect host system examined so far, the P-symbionts form independent monophyletic lineages whose phylogenies are congruent with those of the respective insect host taxon. These associations seem to be very ancient, arising once in the ancestor of the insect and evolving by cospeciation via vertical transmission of the symbiont to the successive host generations (Table 1).

The bacteriocyte symbiosis within the suborder Sternorrhyncha of the order Homoptera has been the more studied of these associations. This suborder comprises a very large group of plant sap feeding insects, partitioned into four superfamilies: Aphidoidea (aphids), Coccoidea (pseudococcids, mealybugs), Aleyrodoidea (whiteflies), and Psylloidea (psyllids). Estimates based on molecular clocks and fossils indicate a very old origin for the association between aphids and their P-symbiont *Buchnera*, a γ-proteobacterium, back to 150 to 250 million years ago (mya) (8). *Buchnera* is absent in the phylloxerids (9), and has been secondarily replaced by extracellular yeasts in a monophyletic subgroup of the family Hormaphidinae (Fig. 2; 10). The origin of the symbiosis between whiteflies and psyllids and their specific P-symbionts is estimated to about 240 mya. The P-symbiont of psyllids, for which the genus name of *Carsonella* has been proposed (11), is relatively close to the P-symbiont of whiteflies, suggesting that the two symbionts may have derived from a unique γ-proteobacterium infecting the common ancestor of the two insect lineages, about 280 mya (12). Coccoidea harbor both γ- and β-proteobacterial symbionts, phylogenetically distinct from the P-symbionts of the other superfamilies (Fig. 2).

Table 1. Examples of Cospeciation Between Insect Host Taxa and Their P-Symbionts

Insect Taxon	P-Symbiont	Bacterial Lineage[a]
Homoptera Sternorrhyncha		
Aphidoidea[b]	*Buchnera*	γ-Proteobacteria
Coccoidea[c]	P sym	β-Proteobacteria
Aleyrodoidea	P sym	γ-Proteobacteria
Psylloidea	*Carsonella*	γ-Proteobacteria
Diptera		
Glossina sp.	*Wigglesworthia*	γ-Proteobacteria
Hymenoptera		
Carpenter ants	*Blochmannia*	γ-Proteobacteria
Blattoidea		
Cockroaches	*Blattabacterium*	Flavobacteria

[a] Each γ-proteobacterial P-symbiont forms distinct lineages. However the symbionts of Aleyrodoidea and Psylloidea may have derived from an ancestral P-symbiont infecting their common ancestor.
[b] *Buchnera* is absent in the family Phylloxeridae, whereas in a monophyletic group of cerathaphidins (subfamily Hormaphidinae) it was replaced by extracellular yeasts.
[c] Both β- and γ-proteobacterial symbionts have been reported for Coccoidea.

In many insect lineages, other vertically transmitted intracellular symbionts are present, collectively called *Secondary* (S) symbionts. Whereas P-symbionts are hosted in bacteriocytes, the S-symbionts infect several organs and the hemolymph, and some may also be hosted in the bacteriome within the syncytial sheath cells and/or within a different type of bacteriocyte, called an *S-bacteriocyte*. In aphids and psyllids P- and S-symbionts are located in different cells of the bacteriome (13,14), whereas in whiteflies and pseudococcids the same bacteriocyte may harbor two different lineages of symbionts (15,16). Molecular phylogenetic analyses have demonstrated the polyphyletic origins of the S-symbionts and the complexity of the symbiotic systems, as several S-symbionts may be present in the same individual. Different species of a same insect taxon may harbor separate S-symbionts (15). Furthermore, closely related S-symbionts may be hosted by distantly related insect taxa, suggesting the possibility of horizontal transmission. For example, closely related *Spiroplasma* strains (Mollicutes) are found in *Acyrthosiphon*

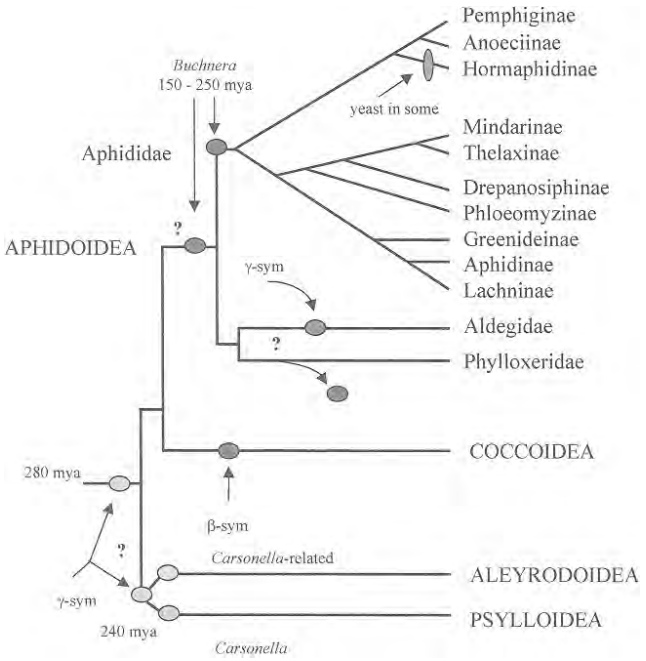

Figure 2. Phylogeny of Sternorrhyncha and origin of P-symbionts. Dendrogramm showing the evolutionary history of the four superfamilies (in capitals) of the suborder Sternorrhyncha (order Homoptera). For the superfamily Aphidoidea, the three families are indicated, and for the family Aphididae, the subfamilies are also indicated. Circles indicate the origin of the infection by the primary symbionts, with the estimated time in million of years ago (mya). Extracellular yeasts have replaced *Buchnera* in the tribe Cerataphidini within the subfamily Hormaphidinae. Members of the family Aldegidae possess a γ-symbiont, but it is not known whether it belongs to *Buchnera*, whereas the family Phylloxeridae is lacking of symbiont, perhaps lost. Coccoidea harbor both γ- and β-symbionts, but the β-symbionts seem to be the P-symbionts. They form a monophyletic clade in molecular phylogenies based on 16S rDNA analyses, whereas the γ-symbionts appear polyphyletic. Members of the superfamilies Aleyrodoidea and Psylloidea harbor closely related γ -symbionts, perhaps evolving from a unique γ -symbiont infecting a common ancestor. See color insert.

pisum (Aphididae), in *Antonina crawii* (Pseudococcidae), in ladybird beetles (Coleoptera, Coccinellidae), in a butterfly, and also in an *Ixodes* tick (15,17) (and references therein). The possibility of horizontal transmissions is suggested by the life history of both symbionts and hosts. Spiroplasmas are often maintained in nature in cycles involving the phloem of a plant and the body of a sap-sucking insect, and Coccinellidae are among the most important predators of aphids, and some species of coccinellids prey also upon coccids. Phloem sap may be a route of transmission of bacteria (18), as well as the animal blood, when the same plant or animal serves as a common source of food for different insect taxa.

More complex trophic relationships exist for parasitoid insects, which grow on an insect host. The parasitoid may be itself the host of a secondary parasitoid insect (called *hyperparasitoid*) (19). Then the parasitoid insects may be also considered as potential agents of horizontal transmissions of symbionts [(20), see following section on *Wolbachia*]. A detrimental effect caused by some S-symbionts has been reported for the insect host, but it is likely that a more complex interaction exists between the host, the S-symbiont and some environmental factors. The γ-proteobacterial genus *Arsenophonus* is a multitissue infecting S-symbiont found in both parasitoid wasps (order Hymenoptera) and triatomine bugs (order Heteroptera). Whereas *Arsenophonus triatominarum* seems to cause no effect in the triatomine host *Triatoma infestans* (21), *Arsenophonus nasoniae* has been identified as a male-killing agent in the parasitoid wasp *Nasonia vitripennis* (22). Even if the wasp is not a triatomine parasitoid, it is conceivable that an ancestor of the wasp or perhaps another parasitoid species, parasitizing the bug, has acted in the past as a vector for the horizontal transmission of the common ancestor of *Arsenophonus* sp. from one host to the other.

Symbio-Photoautotrophy

Cyanobacteria and eukaryotic algae are commonly found in symbiosis with a wide range of organisms, including several lineages of protists, invertebrate animals and plants. In the case of cyanobacteria hosted by photosynthetic organisms (plants and diatom algae), the role of the endosymbiotic cyanobacteria is almost exclusively devoted to nitrogen fixation (see following section). The symbiophototrophy seems to be very ancient, and was probably started several times since the emergence of the eukaryotes. It has been proposed that the plated forms characterizing the peculiar precambrian fauna of Ediacara may be the result of adaptations to harbor endosymbiotic algae (23). Several species of large foraminifera harbor many types of eukaryotic algae including chlorophytes, rhodophytes, dinoflagellates, chrysophytes, and diatoms, whereas planktonic ciliates are frequently found to host chlorophytes and cryptophytes. Sponges and cnidarians host cyanobacteria, *Chlorella*, and dinoflagellates; the endosymbiosis of the latter with stony corals (order Scleractinia, Anthozoa) plays a key role in the formation of tropical coral reefs, which represent a very species-rich marine habitat in mineral-poor seas. Generally, based on the type of photosynthetic pigments, the endosymbiotic algae are also called cyanellae for the blue-green cyanobacteria, chlorellae for the green algae (chlorophytes but also cyanobacterial prochlorophytes), and xanthellae for the chlorophyll *c*-containing algae (dinoflagellates and chromistan algae).

The symbiotic algae are generally intracellular, and restricted to delimited regions of the host body, for example, the gastrodermal layer of cnidaria (24), or the nondigestive zones of the regionalized cytoplasm of some protists, as the intermediary zone of the test of large discoid foraminifera (25). Some intracellular algae show intracellular displacements. For example, Foraminifera are testaceous protozoa characterized by the possession of an extra-test fine extension of the cytoplasm forming a system of anasthomized pseudopodia (*reticulopodium*), and an intra-test cytoplasm or *endoplasm*. The dinoflagellate symbionts of *Globigerinoides* are dispersed into the reticulopodium during the day to exploit a greater surface for photosynthesis, and then regrouped into the endoplasm

during the night. This type of displacement is governed by the host via cytoplasmic fluxes. In other foraminiferan-dinoflagellate symbioses, it is the alga that moves via conserved flagella into the cytoplasm of the host. A bathymetric stratification of some planktonic protist species may be also observed, in function of the different optimal values of wavelength and luminosity of the specific alga symbiont. For example, the foraminiferans *Globigerinoides ruber*, *Orbulina universalis*, and *Globigerinella siphoniphera*, which host different types of algae, live at different levels in the water column, at the surface, at a median, and at a deeper level, respectively (25).

Some cases of extracellular symbiosis exist: for example, the dinoflagellates, which are endocytobionts in several marine animals, but are hosted extracellularly in the mantle in branched ends of a tubular system originating at the stomach, of the giant clams tridacnids (26). The intracellular algae are generally located in a perialgal vacuole, a symbiosome, into the cytoplasm of the host cell. But in some associations, the alga has been observed free into the cytoplasm. In endosymbiosis, the algae show some simplification in comparison to the free-living relatives or even to the isolated organism. For example, zooxanthellae in corals and *Chlamydomonas* in foraminifera are nonflagellated in the host but become flagellated when isolated in culture. Pennate diatoms do not form frustules when harbored within foraminifera, and similarly the thecate four-flagellate green alga *Tetraselmis* lose these structures when establishing symbiosis (the intra- or extracellular localization is still disputed) with the acoel worm *Convoluta*.

In some cases some anatomical modifications of the multicellular host may have evolved. For example, some octocorals show a reduced digestive mesenteron and the loss of the nematocyts, and depend on their zooxanthellae for nutrition (e.g., some species of the family Xeniidae). However, an efficient carnivorous ability is present in other zooxanthella-harboring cnidarians, like sea anemones and stony corals; in these symbioses organic N and P and/or vitamins from the digested prey are probably utilized by the alga (24).

Several modes of transmission of the alga to the offspring host exist. Generally the algae multiply and then are distributed in each offspring host originating by asexual reproduction both in multicellular animals (e.g., fission or budding in sponges and cnidarians) and protists. When the sexual reproduction takes place, the algae may be externally associated with the eggs or may be placed within the cytoplasm of the egg prior to fertilization. For several species of cnidarians the larva must acquire the symbiont from the environment, so lineage recombinations of host and symbiont are allowed. Similarly, the algae are vertically inherited by the protist cells originating from asexual reproduction, whereas they are lost by the gametes and then the new generation originating from the sexual reproduction acquires the algae from the environment.

The basis of the endosymbiosis of algae is nutritional, the algal photosynthate being passed, in some associations up to 90%, to the host that often lives in oligotrophic waters. The release of the photosynthate seems to be controlled by a "host factor," because the alga isolated in culture does not release it. In some associations, it has been shown that the alga is also involved in recycling organic N and/or P released as wastes by the host. For some flatworm-chlorophyte associations, where the worm inhabits O_2-poor waters, there is evidence that the host utilizes the oxygen produced photosynthetically by the alga for its respiration. However, oxygen production often results in the accumulation of excess O_2 in host tissues. This leads to the production in the host tissues of elevated levels of enzymes protecting against oxygen radicals, like superoxide dismutase. Periodic digestion of the symbiont may be another source of nutrients for the host and/or a mechanism to control the symbiont population. The algal population may then be reintegrated either by the multiplication of the surviving symbionts or by acquiring new algae from the environment. In several cases in both animals and protists the plastids were digested more slowly in comparison to the rest of the alga. The host may then exploit the production of photosynthate for some days more, and then it will periodically ingest new algae to replace the no more functional plastids.

For several species, the endosymbiotic algae may be isolated in culture where they regain normal features, such as flagella or shells, and in some instances the alga does not seem to be essential to the survival of the host. However, a range of occasional-to-obligate symbiophotoautotrophies has been described. For example, the photoautotrophy in marine planktonic ciliates and foraminiferans may be based either on strictly endosymbiotic algae or on occasionally/periodically engulfed algae. In some cases only the algal plastids are retained to perform a temporary photosynthesis (25,27). Nudibranch molluscs prey upon several sessile animals (e.g., sponges, cnidarians, bryozoans), some of which harbor endosymbiotic algae. Some species of nudibranchs are able to retain zooxanthellae, either extracellularly in their gut or intracellularly in the digestive diverticula, and algae continue to multiply and to release photosynthate. Some species of nudibranchs have developed densely branched digestive diverticula to harbor intracellular zooxanthellae originating from the ingested prey. Similarly, another group of molluscs, the Saccoglossa, contains several species that live by sucking the cell contents of multicellular algae; some saccoglossans retain the plastids intracellularly in their branched digestive diverticula (1).

Another way by which the algal symbionts seem to play a key role in the life of their host, is that they contribute in some ways to the calcification of the host structures, favoring the deposition of calcium carbonate. Examples are the shell formation of foraminiferan hyaline testes (25) and the improvement of the skeletogenesis in reef-building species of corals, called *hermatypic corals*. The coral reefs are the most diverse aquatic ecosystems, and often they flourish in nutrient-poor shallow waters, where the primary production is assured by endosymbiotic zooxanthellae. Water temperature and light penetration are major limiting factors. Reef-building corals are dominated by zooxanthella-harboring stony corals (Scleractinia, Hexacorallia, Anthozoa), but important in the reef formation are also other hexacorals, octocorals, and hydrocorals (Anthomedusa, Hydrozoa).

Coral species lacking zooxanthellae can live in deep and cold waters, and generally they cannot produce reefs (*ahermatypic corals*), even if some species build significant constructions. In tropical reef corals, the rapid loss of zooxanthellae (*coral bleaching*) results often in a mass coral mortality. Coral bleaching events may be caused by several factors, the most evoked being the global warming and the increased ultraviolet radiation. Bacterial infections may also be a cause of bleaching, for example, *Vibrio shiloi* that infects intracellularly the Mediterranean Sea coral *Oculina patagonica* and produces a toxin that inhibits zooxanthella photosynthesis (28 and references therein). However, such events of bleaching may be an occasion for the coral to be repopulated by a different type of zooxanthella, perhaps more resistant. Zooxanthellae show a high diversity within corals (29), and a single coral colony may harbor several types of zooxanthellae and may also change the algal type composition in response to a bleaching event, for example, some species of the genus *Montastraea*, which dominate the Caribbean corals (30). Such multispecies dynamic communities of zooxanthellae hosted by a same coral colony may be an evolutionary adaptation to environmental stresses.

Endosymbiotic associations have also additional advantages. For example, in the *Paramecium-Chlorella* symbiosis the alga is protected against viral infections (31), and the ciliate seems to acquire a stronger resistance to microbial infections and to the attack by predatory protists (1). Several marine invertebrates (e.g., sponges, cnidarians, bryozoans, and tunicates) not only harbor endosymbiotic algae and cyanobacteria but also a high variety of heterotrophic bacteria playing a possible nutritional role [(32), for endosymbiotic bacteria of sponges]. These animals may be sources of a variety of peculiar bioactive metabolites. Such compounds may play a role in the defense of the host against predators and/or infectious microorganisms, and some of these molecules have showed to possess antitumoral, antiviral and/or immunosuppressive activities (33–35). In some cases it has been possible to ascribe the origin of some of these compounds to the endosymbionts. For example, zoocyanellae have been implicated in the chemical defense of sponges (36) and tunicates (35). Similarly, some marine invertebrates may prove toxic to a potential predator, including humans, because they may accumulate toxic algae, like dinoflagellates and diatoms (37).

Symbiotrophic Systems in Sulfur- and Methane-Rich Waters

Deep-sea hydrothermal vents on midocean ridges and other marine environments like sediments, some shallow waters, and also whale falls (38) are rich in reduced compounds like hydrogen sulfide and methane, of geothermal or biological origin, respectively. Such habitats present anoxic-to-oxygenated gradients, where a rich bacterial biota develops. Bacteria obtain energy through the oxidization of reduced sulfur and fix carbon dioxide through the Calvin-Benson pathway (thioautotrophy by sulfur-oxidizing bacteria), or by using methane as source of both energy and carbon (methanotrophs). These habitats also harbor a rich invertebrate fauna, based on symbiotrophy with such types of bacteria, hosted either as epibionts or as extra- or intracytobionts (Fig. 3). Animal groups involved in such symbiotrotrophy include Porifera, Bivalvia, Anellida, Pogonophora, Vestimentifera, Nematoda, and Crustacea. In addition, epibiont bacteria are present also on the colonial ciliate *Zoothamnium*, which lives on decaying plant material in mangrove forests (39).

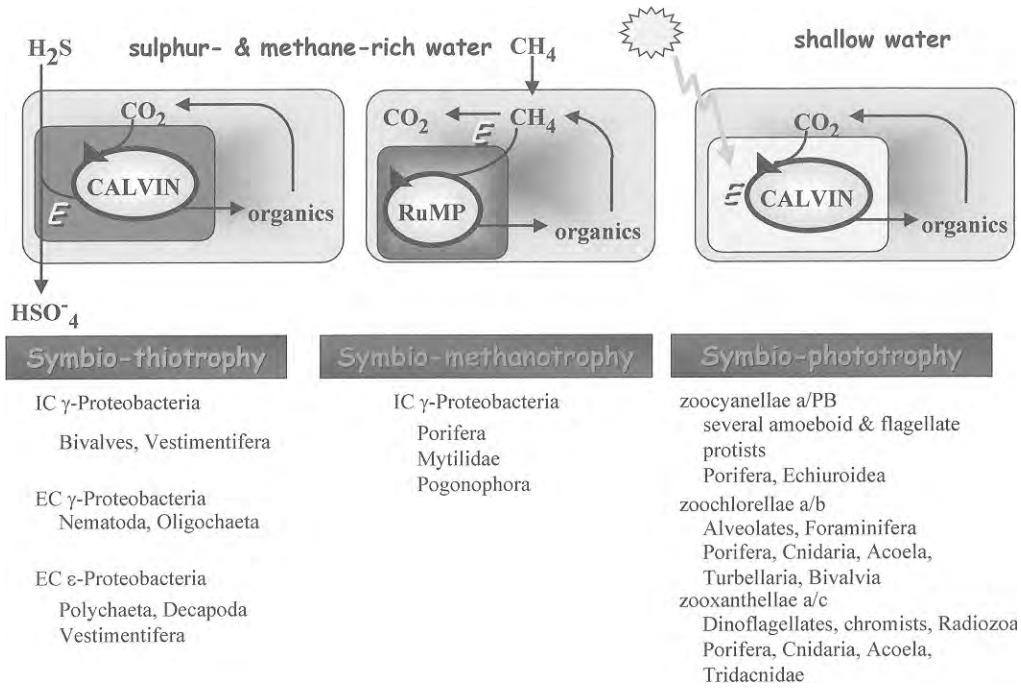

Figure 3. Symbiotrophic animals. See color insert.

Sulfur-oxidizing symbionts are mostly γ-Proteobacteria, harbored as epibionts and/or subcuticular extracytobionts by sediment-inhabiting nematodes (subfamily Stilbonematinae, e.g., *Laxus*) (40) and inter/subtidal calcareous coral reefs sands oligochaete annelids (e.g., *Inanidrilus*) (41). However epibiotic ε-Proteobacteria are also present, harbored by the hydrothermal vent-inhabiting polychaete *Alvinella* (42,43), and the shrimp *Rimicaris* (39). At least five bivalve families and the vestimentiferans (e.g., *Riftia*) harbor intracellular γ-proteobacterial thiotrophic symbionts, located within bacteriocytes in the gills or in the trophosome, respectively. Reduction or total disappearance of mouth and gut may be observed in some bivalve families and their fossils (44). The transmission of the symbiont from one generation to the next may occur via the egg if the ovary of the female is infected. The embryo becomes infected by the symbiont, and this type of transmission is called *transovarial*. A *horizontal* or *environmental* transmission of the symbiont occurs when the new generation of the host acquires the symbiont from an extracellular, free-living population of such a symbiont. Transovarial transmission has been reported for several species of bivalves, as well as an acquisition from the environment for other ones (42,45). Symbiont transmission in vestimentiferans occurs horizontally, the worm acquiring the symbiont from the environment at very earliest stages of its life when a transient digestive tract is present. Molecular analyses indicated that a significant strain-level variation between endosymbionts exists, depending on both the type of site (i.e., vent- or seep-dwelling) and on the geographic area (46).

The symbiothiotrophy seems to be very ancient. A rich fossil hydrothermal vent community has been reported from the Silurian (400 mya), including taxa, such as brachiopods and monoplacophorans, unreported in modern communities (47). In addition, the distinctive morphology of the trilobites of the family Olenidae, from the Late Cambrian to Early Ordovician (505 mya), coupled with a sulfur-rich lithology, has been interpreted as evidence for symbio-thioautotrophy (48).

Methanotrophs are a polyphyletic group of Proteobacteria able to utilize methane as the sole source of energy and carbon (49). The strains found as intracellular symbionts of invertebrates form a separate cluster closely related to the type Ia γ-Proteobacteria, within the family Methylococcaceae (50). The symbionts have been found in mytilid bivalves, a pogonophoran and a sponge. Interestingly a hydrothermal vent mytilid harbors in the same gill bacteriocyte both methanotrophic and thiotrophic endosymbionts (51).

Recently an "endosymbiotic" sulfur cycle has been described in an oligochaete worm inhabiting sediments at 6 to 8 m water depth in the Mediterranean Sea (52). In fact, a sulfur-oxidizing γ-proteobacterium, closely related to the other thioautotrophic endosymbionts, and a sulfate-reducing δ-proteobacterium, closely related to the free-living sulfate-reducing *Desulfosarcina-Desulfococcus*, occur in the immediate proximity, extracellularly, in the subcuticular space of the worm. Sulfide produced by the δ-symbiont is used by the γ-symbiont as electron donor for the autotrophic fixation of carbon dioxide. Organic carbon is then passed to the worm, which via anaerobic metabolism provides succinate and fatty acids as electron donors to the δ-symbiont.

Nitrogen Fixation

Biological nitrogen fixation is an essential process supporting life on Earth. The process is lacking in eukaryotes, but is performed by a wide range of prokaryotes, called diazotrophs or nitrogen-fixing. Several nitrogen-fixing prokaryotes are free-living, whereas others have evolved more or less strict symbiotic associations, generally with plants, such as the epiphytic *Azospirillum*, several intra- or extracellular cyanobacteria, and the endosymbiotic bacteria rhizobia and *Frankia* (Fig. 4). Nitrogen fixation is generally performed under anaerobic or microaerobic conditions, because the enzyme nitrogenase, coded by the *nifHDK* genes, is rapidly inactivated by O_2. Symbiotic diazotrophs can develop anaerobic structures, such as the heterocysts of cyanobacteria or the vesicles of *Frankia*. Nitrogenase converts N_2 into ammonia, which is generally assimilated by the host and then fixed as amino group in glutamic acid via the glutamine synthase (GS). A by-product of nitrogenase activity is H_2, consuming about 25% of the energy utilized by the enzyme. However, some diazotrophs, including also the symbiotic *Frankia* and some rhizobial strains, possess a hydrogenase ($H_2 + O_2 \rightarrow H_2O + ATP$). By oxidizing H_2 to H_2O with production of ATP, the hydrogenase recovers energy and gives additional protection to nitrogenase from the O_2.

Intestinal Symbionts of Termites. A rich bacterial flora able to fix N_2 is hosted in the intestine of termites, a group of insects living on diets of cellulose. *Citrobacter* and *Enterobacter* species are intestinal symbionts known since the 1970s. However, molecular phylogenetic analyses based on the research of the nitrogenase reductase gene (*nifH*) have revealed that termites host a richer flora of N_2-fixing bacteria, composed mainly by yet-uncultivated species belonging to several lineages like γ-Proteobacteria, *Clostridium* group, spirochetes and methanogenic archaebacteria (53,54).

Cyanobacteria. Cyanobacteria may perform N_2 fixation in the specialized heterocyst, and several eukaryotes may enter in symbiosis with them to get nitrogen compounds (1,55). Intracellular N_2-fixing cyanobacteria have been reported in some marine and freshwater diatom algae (Heterokonta), in all known species of the eudicot *Gunnera* (Angiosperms), occasionally in some species of *Sphagnum* (mossess), in the zygomycete *Geosiphon pyriforme* (Fungi), and also in a coral reef sponge. In addition, extracellular cyanobionts are present in several fungi and landplants.

In their association with fungi, cyanobacteria form bipartite (mycobiont + cyanobiont) or tripartite (mycobiont + cyanobiont + phycobiont) lichens. In *Geosiphon* and bipartite cyanolichens, the cyanobiont supplies the fungus with both ammonia and photosynthates, whereas in tripartite lichens, the fungus receives ammonia from

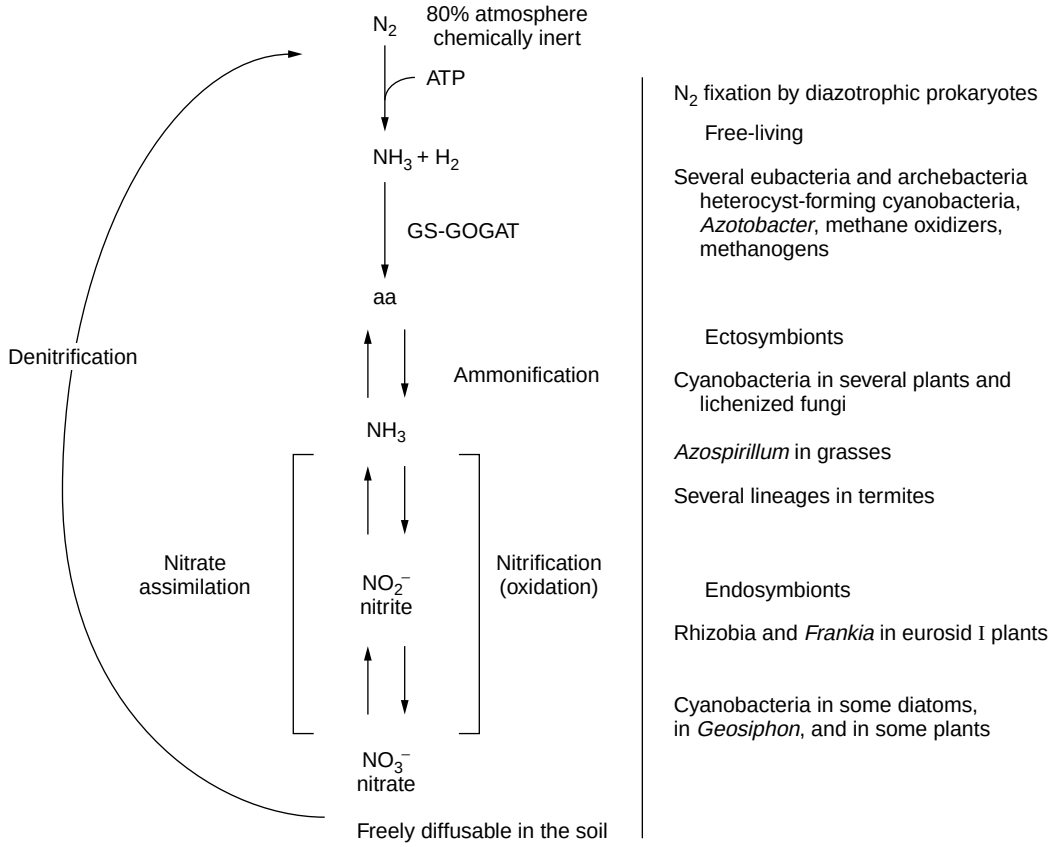

Figure 4. Nitrogen cycle. Nitrogen (N_2) constitutes 80% of the atmosphere and is a chemically inert gas. Diazotrophic prokaryotes, both free-living and symbiotic (listed at the right side of the figure) are able to fix N_2 to ammonia, which is assimilated into amino acids (aa) via the GS-GOGAT pathway. Organic nitrogen returns into the environment in the form of ammonia (ammonification), and then it is oxidized first to nitrite, and then to nitrate (nitrification) by chemoautotrophic bacteria. Nitrate is very soluble and is the main nitrogenous form available in soil for plant growth (nitrate assimilation). Under anaerobic conditions a variety of bacteria may use nitrate as a final electron acceptor in place of O_2, releasing then N_2 into the atmosphere (denitrification).

the cyanobiont and photosynthates (polyols) from the phycobiont (a green alga).

Many plant species within the landplants harbor associated cyanobionts (55): four out six genera of hornworts, but only two out the over 300 genera of liverworts, all the species of the water fern *Azolla*, and all the known cycads. In both intra- and extracellular associations with plants, the photosynthetic activity is restricted to the plant host (Table 2). Some photosynthate is provided by the plant to the symbiont, which in return supplies the host with ammonia. By contrast, in the association with the diatoms the photosynthetic activity is present in both the cyanobiont and the alga. Intracellular cyanobionts within sponges generally provide the host with the photosynthate, but the possibility of nitrogen fixation has been reported for some marine coral sponges.

Rhizobium-Legume Symbiosis and Actinorhizal Plants. Within the angiosperms, legumes and some other eudicot families have the capacity to enter in symbiosis with some Proteobacteria collectively called rhizobacteria or rhizobia, and with the actinomycete *Frankia*. Rhizobia interact with legumes (family Leguminosae or Fabaceae) and *Parasponia*, a nonleguminous genus within the family Celtidaceae, whereas *Frankia* interact with several species belonging to other eight eurosid families also called actinorhizal plants (families Betulaceae, Casuarinaceae, Coriariaceae, Datiscaceae, Elaeagnaceae, Myricacaee, Rhamnacaee, and Rosacaee) (56). The bacteria are hosted in a modified plant structure, called a nodule, where they fix N_2 and supply ammonia to the host plant. Recent molecular phylogenetic analyses have shown that such plant families are included, along other nonsymbiotic families, in a well-supported clade within the eurosid I group of the eudicot plants (56), suggesting that an unique genetic architecture has evolved within a definite lineage of plants to support symbiotic nitrogen fixation.

Nodulation genes are present in all the rhizobacteria. They code for Nod factors, that are lipochitooligosaccharides, which act as morphogenetic signals for the specific legume host to induce bacterial infection and plant nodulation (57–60).

Table 2. Cyanobacterial Symbioses

Host	Cyanobiont	N_2 Fixation	Photosynthesis		GS Activity[a]	
			Cyanobiont	Host	Cyanobiont	Host
Diatoms	IC	+	+	+	−	+
Plants						
Liverwort	EC	+	−	+	⇓	+
Hornwort	EC	+	−	+	⇓	+
Mosses (*Sphagnum*)	IC	+	−	+	⇓	+
Pteridophyte (*Azolla*)	EC	+	−	+	−	+
Cycadophyta	EC	+	−	+	+	−
Angiosperms (*Gunnera*)	IC	+	−	+	⇓	+
Fungi						
Lichens	EC	+	+	−	−	+
Geosiphon	IC	+	+	−	−	+
Animals						
Sponges	IC	(+)	+	−	?	?+

Note: Cyanobacteria may form symbioses with a wide range of eukaryotic hosts. In the table are listed the localizations of the cyanobiont: extracellular = EC, intracellular = IC. The cyanobiont performs nitrogen fixation, and the glutamine synthetase (GS) activity is performed by the host, with the exception of cycads. The cyanobiont also performs the photosynthesis, with transfer of photosynthate to the host, when the host is heterotrophic (fungi [in tripartite lichens (cyanobiont + fungus + green alga), the photosynthates derive from the phycobiont] and animals). Metabolic activities: + = present; − = absent; ⇓= decreased; (+) = activity possible but not confirmed; ? = unknown.

[a]Cycads receive amino acids synthesized via cyanobiont GS.

Rhizobacteria comprise six genera of related but distinct α-2 Proteobacteria (*Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, and *Sinorhizobium*), a unique species from the genus *Methylobacterium* (α-2 Proteobacteria), and also two members from the genus *Burkholderia* (β-Proteobacteria) (61–63). As only around 10% of the legume genera have been characterized until now, new rhizobial lineages may probably be found in the future. Each symbiotic genus has nonsymbiotic bacteria as closest relatives, as inferred from 16S rDNA-based phylogeny. Phylogenetic trees based on nodulation genes indicate a common origin of such genes, and are incongruent with 16S rDNA-based phylogenies. Assuming that the 16S rDNA is always vertically transmitted, there is strong evidence in favor of a lateral transfer of nodulation genes, often carried by large plasmids, as "symbiogenetic islands" (61,64,65).

The host ranges of rhizobia vary from very narrow, as for *Azorhirobium caulinodans*, which nodulates only legumes of the genus *Sesbania*, to very broad, as for the strain NGR234 of *Rhizobium* sp., which nodulates several species from almost all the legume tribes.

Bacteria respond to plant secreted flavonoids by producing Nod factors, which induce the curling of the root hairs. There is a local hydrolysis of the cell wall of the root cells, and bacteria penetrate within a tubelike structure called an infection thread. Such a structure grows intracellularly and penetrates several layers of the root cortical cells to reach the centrally located primordial cells. The infected primordial cells then develop into a nodule. Actinorhizal nodules have a rootlike anatomy, that is, they are constituted by several lateral structures called lobes, having a central vascular bundle and peripheral infected cells. By contrast, legume nodules are stemlike, that is, vascular bundles are peripheral and infected cells central. Specialized plant cells form a diffusion barrier into the nodule, which generates a microaerobic habitat within the tissue hosting the rhizobia. In addition, the microaerobiosis at the nodule level is also maintained by the leghaemoglobins (legume hemoglobins) that bind O_2 and release it slowly. Even if *Parasponia* nodules are infected by rhizobia and activated by Nod factors, their anatomy is similar to the actinorhizal nodule, then indicating that the plant host has a control in the nodule development. Nodulations in legumes and actinorhizal plants are related processes, yet giving different structures. Nodule-specific gene transcription has also been detected in other organs of the plant host, and interestingly some homologous genes have recently been found also in non-nodulating plant species distantly related to the eurosids. In addition, it has been demonstrated with nodulation mutant plants that a common set of host genes is essential to both nodulation with rhizobia and to endomycorrhiza formation, an endosymbiotic association with glomalean fungi present in almost all the landplants. It has been then hypothesized that the nodulation process may have originated in the eurosids by recruiting some of the apparatus evolved in landplants to form the endomycorrhiza (66).

Endomycorrhizal Burkholderia Symbiont. Another potential N_2-fixing endosymbiont is the intracellular symbiont of some endomycorrhyzal fungi. This endosymbiont belongs to the genus *Burkholderia* of the β-Proteobacteria (67) and possesses transcriptionally active *nifHDK* genes (68).

REPRODUCTIVE PARASITES

Reproductive parasites (69) form a diverse group of endosymbionts belonging to different eubacterial lineages and to the eukaryotic microsporidia, able to manipulate

the reproduction of the host, generally an arthropod. Generally these parasites cause a benign infection of the female host, and exploit the cytoplasmic transovarial passage in the next generations as a mechanism for its survival. Being then maternally transmitted, these parasites tend to promote a female-biased sex ratio of the host. Manifestations of such type of parasitism are various, ranging from the killing of the male progeny, to the feminization of the genetic male and to the induction of parthenogenesis (Table 3). Whereas the male killing phenotype has evolved several times in distantly related bacteria (70), some other reproductive disorders seem to be caused only by some specific organisms (71). *Wolbachia* is the most studied of such parasites, and may display all the manifestations of the reproductive disorders.

Wolbachia

Wolbachia belongs to the order Rickettsiales (α-Proteobacteria) characterized by an obligate intracellular life. Four wolbachial phylogenetic lineages, A to D, have been identified, infecting a wide range of arthropods (A and B lineages in several species of insects, mites and isopods) and several filarial nematodes (C and D lineages) (72,73). *Wolbachia* resides in a phagosome, mainly in cells of the reproductive organs of the host. In arthropods it is able to modify the normal chromosome kinetics, resulting in a variety of reproductive disorders. In wasps, some *Wolbachia* strains induce parthenogenesis by aborting the first mitotic phase in unfertilized eggs. The resulting diploid egg develops in a normal female, as hymenopterans have a haplodiploid system of sex determination (male is haploid, female is diploid) (thelytochous parthenogenesis). In parasitoid wasps a horizontal transfer of *Wolbachia* from infected to uninfected larvae within a same parasitized host is a possibility (20). Similarly, natural horizontal transfers of *Wolbachia* between distinct insect species have been reported, namely from the parasitized *Drosophila* host to the parasitoid wasp *Leptopilina* (74). Horizontal transfers between different parasitoid species have not been reported yet, but theoretically such events could be possible as the same food source, that is, the parasitized insect, may be shared by different species, some being occasional or obligate hyperparasitoid (19). Recently, a case of "mutualistic" symbiosis of *Wolbachia* with a parasitoid wasp has been reported, as the oogenesis of the wasp is specifically inhibited by the remotion of the bacterium (75).

In a larger range of arthropods, the most common effect of *Wolbachia* infection is cytoplasmic incompatibility (CI), that is, the production of haploid embryos from fertilized eggs by blocking the syngamy of both the paternal and maternal chromosomes, with the elimination of the paternal pronucleus. In haplodiploid insects the haploid embryo develops in normal males, and CI results then in a male-biased sex ratio. In diploid species, CI results in the death of the embryo. In haplodiploid mites the holokinetic nature of the chromosomes probably does not allow a complete elimination of the paternal chromosomes. This leads to aneuploid nuclei showing several phenotypes ranging from aborted embryos to sterile females. CI is governed by a modification/rescue (mod/res) mechanism:

Table 3. Reproductive Parasites

Parasite	Male-Killing		Feminization	Parthenogenesis	Cytoplasmic Incompatibility
	Embryo	Larva			
Rickettsiales					
Orientia				*Leptotrombidium* (Acari)	
Rickettsia	Coleoptera				
Wolbachia[a]	Coleoptera, Lepidoptera		Isopods, Lepidoptera	Hymenoptera	Insects, mites, isopods
γ-Proteobacteria					
Arsenophonus[b]	Hymenoptera				
Mollicutes					
Spiroplasma	Coleoptera, Diptera, Lepidoptera				
CFB lineage[c]	Coleoptera		*Brevipalpus phoenicis*[d] (Acari)		
Verrucomicrobia					
Xiphinematobacter				*Xiphinema* (Nematoda)	
Microsporidia[e]		Mosquitoes	Marine amphipods		

Note: Microorganisms causing a variety of reproductive disorders in their hosts. All but Microsporidia (eukaryotes) are eubacteria.
[a] *Wolbachia* causes reproductive disorders in arthropods (A and B lineages), but it is essential to the normal development of filariae (C and D lineages), and in one case of a parasitoid wasp.
[b] *Arsenophonus triatominarum* in the reduviid bug seems to be not associated to reproductive disorders.
[c] CFB, *Cytophaga-Flavobacterium-Bacteroides* lineage.
[d] Populations of this mite species are constituted entirely by haploid females. Closely related symbionts are found also in parasitoid wasps (associated to parthenogenesis) and in the tick *Ixodes scapularis*.
[e] Several species.

infected males do not transmit *Wolbachia*, but produce "modified" sperm that can be rescued only if the egg is infected by the same strain of *Wolbachia*. Several mod/res alleles exist. Other factors are involved, like a dose effect of infection density, the possibility of a double infection by different *Wolbachia* strains, the age and phenotype of the host, the temperature, etc. CI may cause reproductive isolation between different populations of a same species. If gene flow is interrupted long enough to allow the appearance of nuclear incompatibility, an event of sympatric infectious speciation may arise. Evidence in favor of the possibility of such an infectious speciation exists in the parasitoid wasps of the genus *Nasonia*. Natural populations of the three species *N. vitripennis*, *N. giraulti*, and *N. longicornis* showed bidirectional CI. Aposymbiotic interspecific crosses with *N. vitripennis* resulted in F1 hybrids, which however showed other isolation barriers, such as high lethality or abnormal behaviors of F2 hybrids. But aposymbiotic crosses between *N. giraulti* and *N. longicornis* resulted in viable and fertile F1 and F2 hybrids (76). This indicates that the hybrid incompatibility between these two latter species is mainly caused by the *Wolbachia*-induced CI, which then would precede the evolution of other isolation mechanisms.

In terrestrial isopods *Wolbachia* seem to have played a central role in the evolution of sex determination, possibly as source of a genetic element that has transformed an original male chromosome in a female one (77). Males develop because of the effects of the male hormone produced by the androgenic gland. Females emerge if the product of a "female gene" inhibits the development of the androgenic gland or the secretion of the male hormone. Sex determination in isopods is mainly heterochromosomal, that is, there are male- or female-specific chromosomes (XY/XX or ZZ/WZ, respectively). Sex chromosomes are very homologous (lack of heteromorphy), and it seems that the female chromosome is a male chromosome carrying the "female gene" (f factor). Such f factor has been hypothesized to have derived from a genetic element of *Wolbachia*.

By contrast, in filarial nematodes *Wolbachia* does not seem to be a source of reproductive disorders. The presence of the symbiont seems to be essential to the normal life of the host (78) and also it seems to participate to the inflammatory pathogenesis characterizing the filarial infection in vertebrates (79). A split in the evolution of *Wolbachia* towards the reproductive parasitism in arthropods and the mutualism in filariae, may be hypothesized. The A and B lineages comprise reproductive parasites of arthropods, without a congruency between symbiont and host phylogenies, whereas the C and D lineages are restricted to filariae, with a parallel between the phylogenies of hosts and symbionts. In addition, the filarial symbionts have a reduced genome size, about 30% smaller than those of the arthropod parasites (80), which is consistent with the genome reduction observed in several mutualistic symbionts.

Male-Killing

At present four lineages of bacteria are recognized as being responsible for the killing of the male embryos in several insect orders: the Rickettsiales with the genera *Wolbachia* and *Rickettsia*; *Arsenophonus* within the γ-Proteobacteria; unnamed species within the Flavobacteria, and some strains belonging to the genus *Spiroplasma* within the Mollicutes. The prevalence of infection in natural populations may vary greatly, from less than 1% to more than 80%, depending on several factors, such as host species bioecology (e.g., consumption of dead sibling males by sibling females), efficacy of the parasite transmission, and temperature (70). It is possible that the killer phenotype may have evolved from neutral or beneficial vertically transmitted symbionts by an event of horizontal transmission to a new host. The flavobacterial killer of ladybird beetles is specifically related to *Blattabacterium*, a beneficial symbiont of cockroaches (Dictyoptera, Blattoidea), whereas *Arsenophonus* sp. causes male-killing in wasps but not in triatomine bugs (21,22). Interestingly, all the *Rickettsia* species seem to be slightly or not pathogenic for the arthopod host when the host is a tick, but not when the host is an insect. Whereas some of these agents seem to be strictly vertically transmitted, horizontal transmission is possible and even important for others. Both *Rickettsia* and *Spiroplasma* possess lifecycles involving an animal or a plant as intermediate hosts, and horizontal transmission occurs, even if rarely, for *Wolbachia* also.

Male-killing at the larval stage has been reported for Microsporidia infecting mosquitoes, probably reflecting the ability to exploit both vertical and horizontal transmission. Indeed, whereas the parasite is vertically transmitted in the host female, the delayed death of the male at the larval stage allows it to join its aquatic habitat. The larvae then die, releasing the parasite spores for the horizontal transmission.

Induction of Parthenogenesis

The induction of parthenogenesis seems to be exclusive to *Wolbachia*. However, this mechanism is probably also used by another rickettsia, *Orientia*, the causative agent of the scrub typhus, to cause a very strong female-biased sex ratio in populations of the host, the mite *Leptotrombidium*. Recently the endosymbiotic induction of parthenogenesis has been evoked also for the longidorid nematodes of the *Xiphinema americanum* group. The populations of these worms are composed almost exclusively of females, which harbor transovarially transmitted organisms belonging to the Verrucomicrobia (81) in symbiosomes within the intestinal epitheliocytes of the juvenile and within the ovarial wall cells of the adult.

Recently a very interesting phenomenon has been reported (82) for a parthenogenetic mite species, *Brevipalpus phoenicis*, belonging to the haplodiploid superfamily Tetranychoidea. Caryological analyses surprisingly showed that females of this species are haploid, and harbor an endosymbiotic bacterium belonging to the *Cytophaga-Flavobacterium-Bacteroides* lineage. Aposymbiotic mites produce a progeny of males that are all symbiont-free. This may be a case in which a symbiont has infected the haploid male of a haplodiploid species. As such symbiont induces feminization, then a population of mite species constituted entirely of haploid females originated. Closely

related bacteria (96 to 98% 16S rDNA homology) have also been found in the tick *Ixodes scapularis* and in several species of parasitoid wasps, and in the latter they have been associated to the induction of parthenogenesis.

ENDOSYMBIONTS AND THE ORIGIN OF EMERGING PATHOGENS

As protists harbor diverse intracellular symbionts (83), they may potentially play a role as vector and/or potentiator of virulence for some pathogens as well as agents of selection for emerging parasites. Indeed protozoa may be considered as Trojan horses of the microbial world (84).

Outbreaks of pneumonia caused by *Legionella pneumophila* infection present important morbidity and mortality rates. This bacterium belongs to a rich group of intracellular parasites of several free-living and ubiquitous protozoa, including the loboseans *Acanthamoeba* and *Hartmannella*, the percolozoans *Naegleria* and *Vahlkampfia*, as well as the ciliate *Tetrahymena*. Legionellae are able to multiply within alveolar macrophages avoiding digestion by the phagocyte. Molecular and genetic analyses have shown that the infections of protozoa and macrophages are highly similar. Legionellae illustrate how a pathogen may emerge. Legionellae are naturally parasites of protozoa, in which they multiply blocking the endocytic pathway. The evolution of such a strategy of exploitation of hosts possessing tremendous digestive systems, most of these being indeed bacterivorous, has provided legionellae with the virulence traits allowing to avoid the human phagocytes (85,86). Several bacteria, among which some human pathogens, may reside into, and also multiply within environmental protozoa. New lineages of amoeba symbionts have been recently discovered, some being closely related to known human or animal pathogens. Among those are the acanthamoebal endosymbionts parachlamydiae, belonging to the order Chlamydiales, which show an increasing diversity (87), and the pararickettsiae (88), that are closely related to the rickettsiae.

Another important source of potential emerging pathogens are the arthropods. In fact it is conceivable to speculate that a new animal pathogen may arise from an endosymbiont if the bacterium is horizontally transmitted from a nonbiting arthropod species to a biting one, or if the ancestral host enters in contact with an animal. This is illustrated by the great variety existing in nature of arthropod endosymbionts closely related to well-known animal pathogen species, like *Rickettsia* and *Francisella* (89,90).

SYMBIOSES AND EVOLUTION

Obligate Symbioses

Many types of intracellular organisms exist, which are maintained in their host by either horizontal or vertical transmission. If the transmission is strictly vertical, the symbionts are constituted by small strictly clonal populations. In the absence of recombination, they then accumulate deleterious mutations that will be fixed in the genome. Such a situation results in three main features: (*1*) a higher nonsynonymous mutation rate, that is, the endosymbiont evolves faster than a free-living relative, (*2*) the reduction of the genome size, and (*3*) an increase in A + T base composition of the genome caused by a mutational bias (91). Small genomes with low G + C contents are indeed a common feature of both mutualistic and parasitic intracellular bacteria. Such a genome reduction evolution seems however to be governed to some extent by the peculiar type of intracellular association. For example, the aforementioned three main features of genome evolution have been found in P-symbionts of insects *Buchnera* and *Carsonella* (12,91). *Buchnera* possesses a highly reduced genome of 640 kbp with a G + C content of around 26% (92), but genes for the synthesis of the essential amino acids tryptophan and leucine are often amplified in multicopy plasmids. An even more drastic gene reduction is showed by *Carsonella*, the P-symbiont of psyllids, with the elimination or reduction of intergenic sequences, the reduction of the protein sizes and the modifications of 16S rRNA. In addition *Carsonella* possesses the higher A + T content found so far, that is, G + C less than 20% (93).

Endosymbiotic organisms may evolve as permanent residents of the host cell. Peculiar syntrophic consortia may provide novel metabolic processes, and the extent of host-endosymbiont integration may become highly advanced. Examples are the spirochaetes involved in the motility symbiosis with some protists, as well as the endosymbiotic algae in the symbiophototrophic hosts or the sulfur-oxidizing bacteria endosymbionts of marine invertebrates. Some ciliates possess bacterial epibionts, called *epixenosomes*, that are involved in a defensive response against predators by an extrusive apparatus (94,95). These epibionts may represent a "preorganellar state" toward the subpellicular extrusive organelles found in other protists (trychocysts) that act similarly.

The Origin of the Eukaryote Cell and Its Organelles

The main example of extreme endosymbioses is the eukaryote cell. Almost all the eukaryotic organisms are indeed characterized by harboring at least two types of genomic materials: nuclear and mitochondrial. Mitochondria are derived from highly integrated endosymbiotic bacteria, possibly a unique lineage of α-proteobacteria related to the rickettsiae. A third genome, bacterial derived, is present in photosynthetic eukaryotes, plastids being of cyanobacterial origin. Some authors have proposed a bacterial endosymbiotic origin also for the eukaryotic flagellum and the peroxisomes, a *Spirochaeta*-like and a gram-positive, respectively, but having entirely lost their genomes. Finally the ancestral eukaryote cell also is believed to have originated from an archae-eubacterial merger, several models involving different lineages of both the partners having been proposed. The integration of these bacterial endosymbionts, and perhaps of others, is hypothesized to have occurred by a stepwise manner, the so-called Serial Endosymbiosis Theory (SET). This theory was proposed in the last decades of the nineteenth century, and was reevaluated in the 1970s and it is at present widely accepted (Fig. 5).

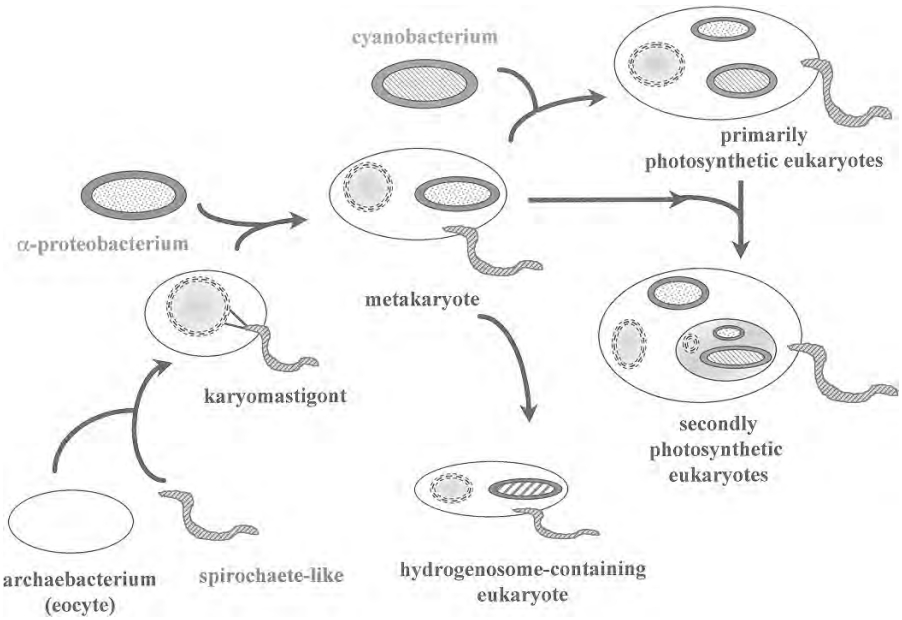

Figure 5. Serial endosymbiotic theory. The ancestral eukaryote cell is supposed to have originated from an archaebacterium (eocyte), perhaps as the result of a merger with an eubacterium (spirochaete), as a motility symbiosis, or as a failed predation. A system of internal membranes has been developed to include all the genetic materials, giving rise to the nucleus, whereas the motility symbiosis reached a high integration, giving rise to the eukaryotic flagellum. The cell at this step is called a *kariomastigont*. At one moment a novel episode of endosymbiosis with an α-proteobacterium appears, giving rise to the mitochondrion. The cell at this step is called a *metakaryote*. Some metakaryotes adapt several times and independently to anaerobic habitats changing the mitochondrion into a hydrogenosome. Endosymbiosis with a cyanobacterium has allowed the emergence of photosynthetic eukaryotes, that form at the present the kingdom of Plantae. Successive episodes of secondary symbioses between a plant and a phagotrophic metakaryote originate the secondarily photosynthetic eukaryotes (euglenoids, chlorarachniophytes, chromist algae and some alveolates). Whereas the first steps of the evolution of the eukaryotic cell are still disputed, several theories have been proposed, the origin and evolution of mitochondria and plastids are now widely accepted. See color insert.

The Karyomastigont. In the model proposed by Margulis (96,97), eukaryote cell originated from a merger between a thermoacidophil archaebacterium *Thermoplasma*-like and a heterotrophic swimmer eubacterium *Spirochaeta*-like. Both partners live in acidic environments, in which the *Thermoplasma*-like produce hydrogen sulfide that is oxidized by the *Spirochaeta* -like to ferment carbohydrates. Organic carbon is then utilized by the *Thermoplasma* -like. In addition the *Spirochaeta* -like provides motility. Such a sulfur syntrophic motility symbiosis is supported by the existence of extant *Thiodendron*, which is in reality a sulfur syntrophic consortium of spirochaetes and *Desulfobacter*. Other examples of motility symbiosis include some anaerobic flagellates, such as *Mixotricha paradoxa* (Parabasala) and *Pyrsonympha* (Metamonada), gut endosymbionts of termites, where small and large epibiontic spirochaetes are important in the motility of the protist. It should be noted that the two lineages to which these motility symbiosis flagellates belong are considered to be among the most ancestral (Archeprotista). The strict association between the epibiont spirochaete and the thermoplasmal nucleoid has originated an attachment system, called a *karyomastigont*, which assures stability to the chimera allowing the integration of the spirochetal (mastigont) DNA to the thermoplasmal genome, with the formation of the nucleus. The formation of nuclear membrane may have originated from the membrane hypertrophy induced as a defensive response to the invasive attached spirochaeta. Moreover, nucleoid membranes exist in some planctomycetes, which are peptidoglycan-less eubacteria. There are some evidences that a centriole-kinetosome DNA is still present. In Margulis's model, the karyomastigont, identifiable in some extant protists, resulted in the first microtubule-organizing center (MTOC), from which originated the 9[2] + 2 eukaryotic flagellum and the 9[3] + 0 microtubule spindle apparatus. Some extant anaerobic, mitochondria-free protists, like the Archamoeba (e.g., *Mastigamoeba*), Metamonada (e.g., *Giardia*), and Parabasalia (e.g., *Trichomonas*) are believed to be the descendants of this primitive eukaryote cell. These organisms are referred as the Archeprotista by Margulis (96), or Archezoa by Cavalier-Smith (98), or Hypochondria by Patterson and Sogin (99). Successively the endosymbiosis with an aerobe α-proteobacterium has originated the mitochondrion, and then the emerging of the aerobic, mitochondriate eukaryotes. Some of these have successively integrated a cyanobiont, which evolved in the plastid.

The Archezoa Hypothesis. In the early formulation, then, the amitochondriate Archezoa are direct descendants of the eukaryote cell, before the integration of the protomitochondrial bacterial symbiont. Phylogenetic analyses performed on the small subunit (SSU) of the

rRNA, as well as simplified cytological features (98) seem to confirm the early derivation of archezoa. But this scenario has recently been challenged by the recovery in some archezoa, namely in *Giardia* and in *Trichomonas*, of mitochondrial-derived genes. The human pathogen *Entamoeba hystolytica*, previously removed from the Archezoa owing to the presence of "advanced" features like spliceosomal introns and dictyosomes, possesses a small organelle targeted by nucleus-encoded mitochondrial-specific proteins. This organelle, termed the mitosome or crypton, contains a genome, and is considered to be a reduced mitochondrion (100). In addition, revaluations of phylogenetic affiliations based on protein-coding genes indicate that the early branching of archezoa in SSU rRNA trees may be the result of a long-branch attraction, which is the placement of highly divergent sequences toward the root of the tree because artificially affiliated to the distantly related prokaryotic ones. At present the Microsporidia are believed to be a highly derived, and then simplified, lineage of parasitic fungi, whereas trichomonads possess hydrogenosomes, which are believed to be derived from mitochondria.

The α-Proteobacteria, Rickettsiae, and Mitochondrial Origins. Members of the α-Proteobacteria are characterized by a strict association with eukaryotic cells, and several are facultative or obligate intracellular microorganisms, either symbiont or pathogens.

Rickettsia prowazekii, for which the complete genome sequence is available (101), is an aerobic obligate intracellular parasite representing at present the closest relative of the eukaryotic mitochondria. This bacterium lacks genes for the glycolysis but possesses a complete system for the utilization of the pyruvate produced by the host cell. This system is composed of three-enzyme complex for the pyruvate dehydrogenase, the tricarboxylic acid (TCA) cycle or Krebs cycle, a cytochrome-based electron transport chain, and the cytochrome oxidase, and a F_1F_0 ATPase pump. An ATP/ADP translocase allows the acquiring of ATP from the cytoplasm of the host. Molecular phylogenetic analyses of the products of all but the translocase genes showed a strong homology with those of the mitochondria. ATP/ADP translocase complex is present in mitochondria and plastids and, among prokaryotes, only in rickettsiae and chlamydiae, another group of obligate intracellular parasites. In phylogenetic analyses rickettsial ATP/ADP translocases group with the translocases of chlamydiae and of plastids, and all import ATP from the cytoplasm of the host and release ADP. The mitochondrial translocases act inversely, acquiring ADP from the cytoplasm of the cell and releasing ATP, and, in molecular analyses, form an ancient monophyletic lineage independent from the translocases of plastids, rickettsiae and chlamydiae. However, the analysis of protein-coding genome (proteome) of mitochondria reveals that of about 400 proteins only 50 are of α-proteobacteria origin. Indeed, about 200 proteins cluster exclusively as eukaryotic homologs. This suggests that the mitochondrial proteome is not the descendant of the ancestral symbiont proteome exclusively (102).

The presence of eubacterial-like sequences also in archezoan genomes has led to the proposal of an early emerging of mitochondria, perhaps contemporarily to the emergence of the same eukaryote cell. One of such models is the "hydrogen hypothesis," in which the origin of the eukaryote cell takes place on the syntrophy between an anaerobic methanogen (nucleocytoplasm) and a facultative anaerobic H_2-producing eubacterium (103). The ancestral organelle has then functioned as a modern hydrogenosome, evolving in the aerobic mitochondrion in the O_2-adapting eukaryote lineages. This hypothesis found a strong support in the extant syntrophies between methanogens (both free-living and epi/intracytobiont) and protists, which are widespread in anaerobic habitats, such as anoxic sediments and gastrointestinal tracts of animals. The protists are mitochondria-free but possess hydrogenosomes: for example, archaeamoebae inhabiting the organic-rich stagnant water sediments (sapropel) of the genera *Mastigella* and *Pelomyxa*, and several ciliates and other protists either free-living (104) or gut symbiont of ruminants (105). The H_2 produced by the hydrogenosome is then exploited by the methanogen to reduce carbon dioxide to methane. In some protists, for instance, the sapropelic ciliate *Metopus contortus*, the methanogen endosymbiont may also have morphological alterations, like partial loss of the cell wall, allowing a stricter contact with the organelle.

Another model based on methanogen syntrophy invokes a myxobacterium as the eubacterial symbiont, with a successive acquisition of the mitochondria. Anaerobic mitochondria also exist, producing ATP through pathways present in extant anaerobic bacteria but not in *Rickettsia*, an obligate aerobe. Among these anaerobic mitochondria are the denitrifying mitochondria of some fungi and ciliates, and the fumarate-reducing mitochondrion of trypanosomes and some worms. *Euglena* mitochondria utilize enzymes similar to those of hydrogenosomes. Finally, hydrogenosomes oxidize pyruvate to acetate and H_2 via pyruvate ferrodoxin oxidoreductase and hydrogenase, which are enzymes found in anaerobes. These organelles share a common ancestor with mitochondria, and at least one has been found possessing DNA (106). It is probable that the common ancestor of mitochondria and rickettsiae possessed genes for the different pathways of ATP production. As other intracytobionts, such an ancestor has undergone genome reduction with the loss of useless genes. Whereas the obligate aerobic and parasitic rickettsiae have retained only genes for aerobic metabolism, mitochondria have transferred most of their genes to the nucleus.

Whereas the genes for metabolism show eubacterial origins, the genes for replication, transcriptation and translation of DNA are archaebacterial-like. Such a situation seems to confirm an archaebacterial derivation for the nucleocytoplasm, as inferred previously from phylogenetic analyses of some protein-coding genes that showed a sister-group relationship between archaebacteria and eukaryotes. However, as sequence and sample data accumulate, a more complex situation appears. In fact eubacterial metabolic genes, and in a minor feature archeal informational genes, seem to have different origins, as showed by different roots and branching topologies on phylogenetic trees. This is consistent with the hypothesis

that lateral gene transfers (LGT) from different bacteria to the eukaryote genome have occurred repeatedly. Such LGT may have originated from bacterial endosymbionts, as it is the case for mitochondrial and plastid genes, but also from engulfed or parasite bacteria digested by the cell.

Cyanobacteria and Plastid Origins

Plastid Types and the Kingdom Plantae, the Primary Symbiosis-Derived Algae. The photosynthesis in eukaryotes is based on an oxygenic reduction of carbon dioxide performed by an intracellular organelle, called a *plastid*, which originates from an extreme endosymbiosis of a cyanobacterium (Fig. 6). All the plastids possess chlorophyll *a* (Chl. *a*) as the principal pigment, but they vary in their accessory pigments. This allows us to recognize three main types: green, containing Chl. *a* and *b* (*a*/*b*); red, containing Chl. *a* and phycobilins (*a*/PB); and yellow-brown, containing Chl. *a* and Chl. *c* (*a*/*c*). Data suggest that all the plastids are derived from a unique cyanobacterial protoplastid, which had given rise to the different types of green, red, and brown plastids by changing the composition of the pigments (107). It is probable that such a protoplastid derived from a cyanobacterium possessing all the main pigments, that is, an *a*/*b*/PB organism. Whereas the great majority of known cyanobacteria have Chl. *a* and PB (*a*/PB), some others, called *prochlorophytes*, but not representing a monophyletic lineage, have Chl. *a* and *b* (*a*/*b*). However, one prochlorophyte, *Prochlorococcus*, contains both Chl. *a* and *b* and PB. Phylogenetic analysis of the chlorophyll *a* oxygenase, the enzyme that converts Chl. *a* into Chl. *b*, shows that the enzyme sequences in the prochlorophytes and the *a*/*b* plants originated from a unique ancestor (108). It is then conceivable that a differential loss of different pigments has taken place, originating the *a*/PB cyanobacteria, the different lineages of *a*/*b* prochlorophytes within the cyanobacteria, and an *a*/*b*/PB protoplastidial cyanobacterium. The latter has changed the light-harvesting complex proteins, which in cyanobacteria and plastids belong to two different protein families. Then it has given rise to the *a*/PB plastids of the glaucophytes and the rhodophytes by losing Chl. *b*, and to the *a*/*b* plastid of the chlorophytes (green algae and lanplants) by losing PB. The evolutionary history of plastids comprises in addition several gene loss and/or transfer to the nucleus (109), leading to high integrated host-plastid systems among the different lineages of eukaryotic algae. Glaucophytes, rhodophytes, and chlorophytes form a monophyletic lineage, the kingdom Plantae (109,110). They are diagnosed (111) as having plastids enveloped by only two membranes, believed to be derived from the two membranes of the symbiotic cyanobacterium (primary symbiosis). Such an event has been ancestral to the emergence of the kingdom Plantae, the nonphotosynthetic members being so secondary.

The Secondary Symbiosis-Derived Algae. The other photosynthetic eukaryotes belong to independent lineages and possess plastids enveloped by three or four membranes (Fig. 6). These plastids are believed to have originated from secondary symbiosis between a phagotrophic protist and (*1*) an engulfed chlorophyte: the three-membraned plastid of the euglenophytes and the four-membraned plastid of the chlorarachniophytes; or (*2*) an engulfed rhodophyte: the three-membraned plastid of the pyrrophytes, or dinoflagellates, and the four-membraned plastid of the chromist yellow-brown algae. In addition a non-photosynthetic, four-membraned plastid is present in Apicomplexa, probably deriving from an independent secondary symbiosis with a green alga, but their origin by an extreme reduction of the red plastid present in a common ancestor of Apicomplexa and dinoflagellates may not be excluded. However, dinoflagellates may harbor both chlorophytes and chromist algae. In some cases the symbiosis with chromist algae seems to be highly integrated, so some dinoflagellates may represent cases of tertiary symbioses. All but the chromistan algae belong to larger protist groups probably including primitively non-photosynthetic lineages. Euglenophytes belong with the parasitic kinetoplasts (e.g., *Leishmania*, *Trypanosoma*) to the Euglenozoa, which are believed to be related to the Percolozoa (e.g., *Naegleria*), and then they are included together in the taxon Discicristata, for the discoid cristae of mitochondria. Chlorarachniophytes form an independent lineage within the Cercozoa, which also comprises monadofilosans (*Cercomonas*) and the plasmodiophorans. Dinoflagellates are included in the well-defined group of Alveolates, which comprises the ciliates and the parasitic apicomplexa (*Plasmodium*). It is interesting to note that ciliates harbor frequently endosymbiotic algae, and that Apicomplexa (e.g. *Plasmodium*) possess a relict plastid, without pigments, of uncertain derivation.

Dinoflagellates, and chromist algae contain *a*/*c* plastids, where PB have been probably substituted by Chl. *c*, but some members of both the algal groups contain *a*/*c*/PB plastids (e.g., the dinoflagellate *Amphidinium*, and the chromistan algal eustigmatophytes). Additionally, the fourth outermost periplastid membrane in the yellow-brown algae is continuous with the rough endoplasmic reticulum. Such a character has been considered as diagnostic for this group of algae, for which Cavalier-Smith has proposed the elevation to the rank of kingdom, as kingdom Chromista (111). This kingdom comprises the Cryptophyta, the Heterokonta, and the Haptophyta, and members of both cryptophytes (*Goniomonas*) and heterokonts (e.g., the oomycetes) are nonphotosynthetic, having secondarily lost the plastid.

Evidences of transitional events of primary and secondary symbioses may be found in some extant algae. Glaucophytes (*Cyanophora paradoxa*) are characterized by possessing an *a*/PB plastid that includes peptidoglycan (PG), then often called *cyanelle*. However, both the genome structure and the gene content reduction indicate that it is an organelle, not an endosymbiotic cyanobacterium. Such a plastid may represent the ancestral form of the plastid, when a genome reduction of the symbiont and a strong integration took place, but some features, such as the PG were retained. It is interesting to note that glaucophytes form the most basal lineage of the kingdom Plantae, which seems to be the unique lineage to have originated by primary symbiosis.

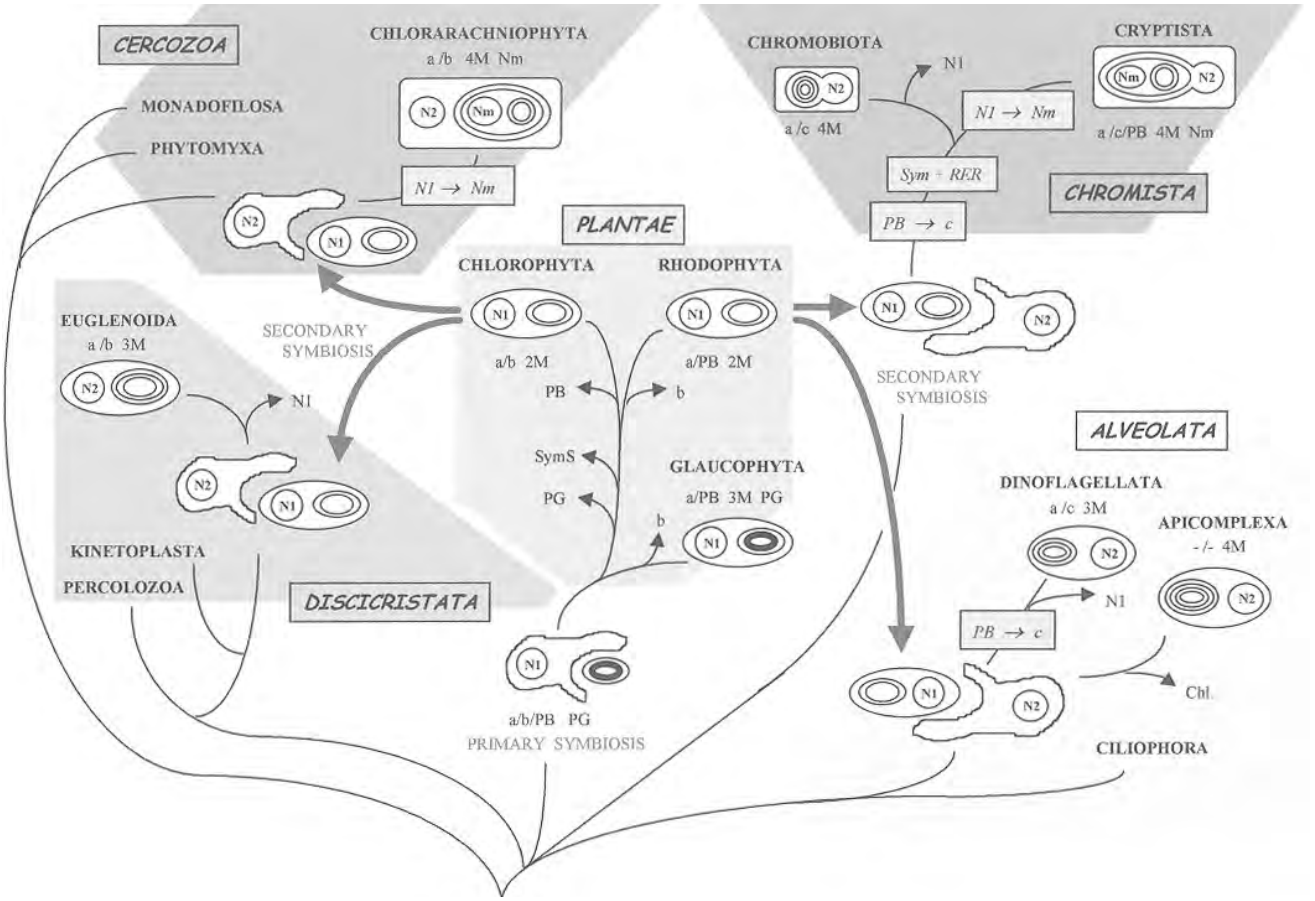

Figure 6. Origin and evolution of the eukaryotic algae. The five lineages of eukaryotes containing photosynthetic members are indicated: Alveolata, Cercozoa, Chromista, Discicristata, and Plantae (according to Cavalier-Smith 1998 (111)), with the pigment composition (a, b, c and PB, for chlorophylls *a*, *b*, *c*, and phycobilins, respectively). The number of plastid membranes is also indicated (2 M, 3 M, 4 M, for two, three, or four membranes), as well as the eventual presence of peptidoglycan (PG), and the retention of the nucleus of the primary alga as nucleomorph (Nm). N1 = nucleus of the primary alga; N2 = nucleus of the secondary alga. In squares are indicated the major transitions: substitution of phycobilins with chlorophyll *c* (PB → c), degeneration of the primary alga nucleus N1 into a nucleomorph (N1 → Nm), fusion of the symbiosome membrane with the rough endoplasmic reticulum (Sym + RER). A single event of primary symbiosis between a phagotrophic eukaryote and a cyanobacterium has led to the establishment of the kingdom of Plantae, the only primarily photosynthetic eukaryotes. Lineages within the kingdom Plantae then diverged by differential losses of plastid pigments. Episodes of secondary symbioses (large arrows in bold) between a green alga or a red alga and some phagotrophic protists, have led to the euglenophytes and chlorarachniophytes, and to the dinoflagellates and chromist algae, respectively. The nonphotosynthetic apicoplast of Apicomplexa seems to have originated from a green alga. In the Chromista, both Cryptista and Chromobiota harbor members without plastids, probably because of secondary loss. Cryptista and some Chromobiota (e.g., Eustigmatophycea) also possess phycobilins. See color insert.

Chlorarachniophytes and Cryptophytes are unrelated photosynthetic eukaryotes believed to have originated by secondary symbioses of heterotrophic protists with a chlorophyte and a rhodophyte, respectively (Fig. 6). Their plastids are enveloped by four membranes, the first two being the membranes of the plastid. The third derives from the cytoplasmic membrane of the primary host, and the outermost originates from the symbiosome membrane of the secondary host, fused with the RER in the case of the cryptophytes. These algae are very interesting because they have retained within the third membrane that is within the cytoplasm of the primary host, the nucleus of the primary host. Such nuclei, called *nucleomorphs* (Nm), contain a genome greatly reduced (three chromosomes for a total of 400 to 600 kb) with a high A + T content (60 to 70%), but they are transcriptionally active (112). The gene density as a result is very high, with that of cryptomonal Nm being the highest for an eukaryotic genome (1 gene per 0.8 kb). Spliceosomal introns are very squeezed, less then 20 bases in length in chlorarachniophyte Nm, or totally absent, in cryptophyte Nm (113). Recently the entire

551 kb genome of the nucleomorph of a cryptophyte has been sequenced (114).

CONCLUSION

Endosymbioses are widespread in nature, including both extracellular and intracellular microorganisms, prevalently prokaryotes, which have developed a mutualistic association with a larger organism, the host, that is generally an eukaryote, either unicellular or multicellular.

An endosymbiotic association is the result of a long-time adaptation process between two organisms, which may have originated from a virulent-to-avirulent evolution of an invading pathogen, or from the integration by the host of either a new metabolic process (syntrophy) or another property (e.g., chemical defense) possessed by an ingested prey. For example, the extant rhizobium-plant symbiosis may have originated from a failed plant infection by a pathogen able to infect the plant through the prevention of the hypersensitive response of the host. The localization of the genetic apparatus for both the nodulation and the nitrogen fixation on symbiogenetic plasmids has allowed its diffusion to other bacterial genera. Also, endosymbiotic algae may have originated as retained engulfed preys in many phagotrophic protists and invertebrate animals. In these types of associations some mechanisms of recognition and of regulation must have evolved. The recognition between symbiont and host is essential, for example, for the transmission of the symbiont to the next generation of the host, and for the localization of the endosymbiont in specific organs or cells of the host. The regulation of the association is essential, for example, to control the syntrophic process, and to prevent the defensive systems of the host destroying the invading endosymbiont and/or the endosymbiont causing disease to the host. The highly developed immune system of vertebrates seems to be the main cause for the extreme rarity of endosymbionts in this group of animals. In fact microbial infections in vertebrates are performed by microorganisms possessing the ability to avoid the immune response of the host at both the levels of cell and organism.

Ecological impact of endosymbiosis is particularly important. For example, the entire coral reef ecosystem in tropical, oligotrophic seas, one of the more species-rich on the Earth, is based on the endosymbiosis with zooxanthellae. There is also an endosymbiotic association between invertebrate animals and sulfur-oxidizing bacteria that characterizes the hydrothermal vent fauna, a photosynthesis-independent ecosystem. Fossil evidences indicate that such types of symbiotrophies have appeared early in the evolutionary history of invertebrate animals.

Finally, endosymbioses play also a key role in the evolution, resulting a way to create evolutionary novelties. For example, the terrestrial ecosystems dominated by the land plants from the Early Paleozoic, more than 400 mya, have originated from a lineage of green algae that acquired the ability to colonize the mainland by forming symbiosis with fungi of the order Glomales. In addition, the intracellular endosymbiosis has played a more important role in the evolution of life on the Earth, being at the origin of the eukaryotic cell and some of its organelles.

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ENDOSYMBIOSIS INVOLVING AMEBAS.

See FREE-LIVING AMEBAS PRESENT IN THE ENVIRONMENT CAN CAUSE MENINGOENCEPHALITIS IN HUMANS AND OTHER ANIMALS

ENDOSYMBIOSIS INVOLVING CYANOBACTERIA.

See CYANOBACTERIA

ENDOTOXINS IN BOTTLED WATER.

See BOTTLED WATER, MICROBIOLOGY OF

ENHANCED BIOLOGICAL PHOSPHORUS REMOVAL (EBPR) IN ACTIVATED SLUDGE.

See ACTIVATED SLUDGE — MOLECULAR TECHNIQUES FOR DETERMINING COMMUNITY COMPOSITION

ENHANCED DETECTION OF AIRBORNE MICROBIAL CONTAMINANTS

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Numerous methodologies currently are available for the detection of airborne microorganisms. Traditional detection of airborne microorganisms and microbial by-products is performed using microscopy and culture techniques (1). Microscopy generally is used to detect total microbial populations in a given sample without regard to the physiological state of the organism; both viable and nonviable organisms can be observed. Culture-based assay is limited to measurement of those organisms that proliferate under the growth conditions provided in the laboratory. Organisms that are difficult to culture because of nutrient (e.g., xerophilic fungi such as *Wallemia* spp. and *Eurotium* spp.) or temperature (e.g., thermophiles such as *Aspergillus fumigatus* and *Mycopolyspora faeni*) requirements often are not isolated. Identification of fungi to the species level based on culture and subsequent microscopy is highly subjective and difficult to perform. Direct counting methods for fungal monitoring that rely on classical microscopic features for identification cannot distinguish between the spores of some fungal genera (e.g., *Aspergillus* and *Penicillium*) or between species. Generally, the traditional detection methods of culture and microscopy are tedious and time-consuming, and generally have low sensitivity and specificity. Culture and microscopic assays also are inherently subjective making intralaboratory comparisons difficult.

Methods for enhanced detection of airborne microorganisms and by-products using bioassay, immunoassay, analytical instrumentation, and biotechnology-based technologies have been developed that increase the sensitivity and specificity of identification and reduce processing time. Traditionally, biochemical assays have been used in the microbiology laboratory to identify culturable organisms based on substrate utilization. Recently, biological and biochemical assays have been developed to detect the presence of microbial toxins and indicators of microbial biomass without the need for culture. Immunoassays involve antigen-antibody recognition between a surface antigen on the microorganism and a laboratory-prepared antibody. The use of analytical chemistry instrumentation to detect biological components in a sample and that of biotechnology-based assay methods for the detection of genetic elements unique to the microorganisms of interest are rapidly developing technologies. Categories of these detection technologies that are potentially applicable to monitoring of airborne microbial contaminants (Table 1) are discussed in the following sections, although use of the following techniques for bioaerosol detection to date has been limited.

BIOASSAYS

The limulus amebocyte lysate assay (LAL) is an in vitro biological assay used to detect endotoxin, the lipopolysaccharide in the outer membrane of gram-negative bacteria (2). The chemically distinct lipid A fraction of the lipopolysaccharide of the molecule is the toxigenic component. The 3-hydroxy fatty acids of the lipid A fraction are used as chemical markers for endotoxin in air and water samples (3). Underreporting of cell-bound endotoxin using LAL methods has been minimized by sonication of the samples, optimization of processing buffer, and extraction of samples (4,5). Commercially available kits for LAL assay are available and approved for use in the pharmaceutical industry, but they were not designed

Table 1. Listing of Selected Traditional and Enhanced Detection Technologies Applicable to Bioaerosol Monitoring

Method		Principle	Advantages	Disadvantages and Interferences
		Traditional Methods		
Culture		Growth of culturable organisms on selected media and temperatures	Simultaneous assay of many contaminants, inexpensive	Organism of interest may require special growth conditions; overcrowding on agar surface; inhibition by other organisms
Microscopy		Morphology	Rapid	Subjective; debris may obscure viewing
		Enhanced Methods		
Bioassay	LAL	Measurement of LPS as indicator of microbial presence	Used to measure endotoxin and glucans without requirement of culture	Specificity; underreporting of cell-bound endotoxin
	FISH	Fluorescent in situ hybridization and measurement of light scatter	Detection of bacterial cells	Currently high detection limits
	GC-MS-MS	Analytical instrumentation to detect muramic acid; 3-OH fatty acids; endotoxin; biopolymers	Measurement of endotoxin and glucans without requirement of culture	Reference library and fingerprints needed for identification
Biotechnology-based	PCR	Amplification of specific, unique gene sequences	Increased specificity and sensitivity	Requirement for known unique sequences; Inhibition by other nucleic acids and abiotic substances
	Microchip PCR	PCR on microscale	Decreased PCR analysis time and reagents	Currently higher detection limits than PCR; inhibition by other nucleic acids and abiotic substances
	Molecular beacons	Detection of nucleic acid	Specificity	Inhibition by other nucleic acids and abiotic substances

for use in aerobiological investigations (3). Some of the problems with use of LAL for environmental samples are because of inconsistency among the lysate preparations and storage of the samples before analysis (3). The kinetic limulus assay with resistant-parallel-line estimation (KLARE) method was developed as a kinetic LAL assay with regression analysis to measure endotoxin in environmental samples (6). The improvement in detection using the KLARE method included an ability to correct for the presence of interfering substances.

The LAL assay also has been used to detect β-(1 $\rightarrow$ 3)-D-glucans, glucose polymers that are structural components of fungal cell walls (7). Glucans have been associated with sensory irritation (7) and the inflammation-regulating capacity of airway macrophages (3). A LAL assay has been used to detect airborne β-(1 $\rightarrow$ 3)-D-glucans (7) and this assay method may be the most sensitive of the currently available methods for glucan. However, the LAL does not differentiate between β-(1 $\rightarrow$ 3)-D-glucans and β-(1 $\rightarrow$ 6)-D-glucans (8).

IMMUNOASSAYS

Enzyme Immunoassay

Enhanced detection of microbial components using immunoassays relies on the specific interaction of antigens with their respective antibodies. The enzyme-linked immunosorbent assay (ELISA) uses an enzyme to signal the presence of an antigen or antibody reaction. ELISA has been adapted to detect antigenic sites of environmental contaminants in house dust and air samples (9,10). ELISA also has been used to assay for Alternaria antigens Alt a I and GP70 using impingement air samplers (11). An inhibition enzyme immunoassay (EIA) has been developed for the detection and quantification of fungal cell wall β-(1 $\rightarrow$ 3)-D-glucans in indoor environments with a lower limit of detection in air and dust samples reported as 0.2 $\mu g/m^3$ and 0.3 $\mu g/m^3$ of air, respectively (8). The EIA also was performed on inhalable dust collected with glass fiber filters and settled dust collected with paper filters. The samples were heat-extracted and then stored at $-20\,^{\circ}C$ until analyzed in a microtiter plate format.

Electrochemiluminescence

An integration of equilibrium immunoassay with electrochemiluminescense involves a biotinylated antibody sandwich for detection of allergens. A labeled *N*-hydroxysuccinimide ester of a ruthenium (II) trisbipyridine chelate and streptavidin-coated paramagnetic beads capture the antibody-antigen-antibody sandwich complex. Detection ranges from 2.5 ng/ml to 2,000 ng/ml have been reported (12). This methodology has application for bioaerosol monitoring of allergenic constituents of microbial contaminants.

Biosensors

Detection using immunoassay in conjunction with a sensor membrane has been developed for the detection of bacteria by the food industry, medicine, biotechnology, and environmental microbiology (13). These biosensors also have been used to detect staphylococcal enterotoxin B, a matter of concern to biological warfare (14), although current detection limits are relatively high (3.0×10^5 to 6.2×10^7 cells/ml). The incorporation of a 20-MHz piezoelectric quartz crystal sensor in a flow injection system with a polyclonal antibody detected 0.1 ug/ml of staphylococcal enterotoxin B. However, inhibition was noted at concentrations greater than equal to 10 ug/ml (14).

ANALYTICAL INSTRUMENTATION ASSAYS

Mass Spectrophotometry

Gas chromatography-ion trap tandem mass spectrometry (GC-MS-MS) and conventional quadrupole GC-MS have been used to detect indicators of the presence of microbial contamination (15) and endotoxin (3). However, the GC-MS procedure for detection of endotoxin's 3-hydroxy fatty acids is approximately 1,000-fold less sensitive than the LAL assay (3). Monitoring of airborne bacteria in a stable and a dairy environment has been performed using GC-MS-MS (16). Airborne dust samples were collected with Teflon filters and analyzed for muramic acid and 3-hydroxy fatty acids as indicators of peptidoglycan in gram-positive and gram-negative bacterial cells, and endotoxin from bacterial lipopolysaccharide in gram-negative cells, respectively. Matrix-assisted laser desorption ionization (MALDI) mass spectrometry can identify gene sequences that are not readable using gel electrophoresis (17). It also has been used for the detection of quasimolecular ions of large organic molecules (up to several hundred kDa molecular mass) such as biopolymers (peptides, proteins, oligosaccharides, and nucleotides in the subpicomolar range) with an accuracy of 0.1 to 0.01% (18).

Flow Cytometery

Flow cytometry (FCM) uses simultaneous measurements of light scatter to determine cell size and structure. Incorporation of fluorescence increases the capabilities to include quantitation of cellular components, antigen detection, and estimations of cell physiology (19). Instrumentation permits the measurement of 500–5,000 objects/sec; the results are displayed in bivariate histograms. Indirect quantitation of cells is conducted using the addition of fluorescent polystyrene beads.

Staining techniques used with FCM include the use of fluorescent brighteners. An ultraviolet-excited fluorescent whitening agent (Tinopal CBS-X) with ethanol has been used as a stain for both vegetative cells and endospores in the presence of high biological background (20). FCM analysis with nucleic acid staining [e.g., 4′,6-diamidino-2 phenylindole (DAPI)] permitted the discrimination of *Pseudomonas aeruginosa* from debris and beads, and comparison with other methods of analysis (21). When comparing FCM with culture and microscopy for air samples with impingement sampling in a barn, the culture method underestimated concentrations by 2 orders of magnitude (21). The combination of FCM and fluorescent in situ hybridization (FISH) has been reported for log-phase cultures of *Escherichia coli* and *P. aeruginosa* aerosolized in exposure chambers and collected with an impingement sampling method (21). FISH also was used to detect bacteria in air samples collected in a barn. Although the FISH assay technique was reliable with the exposure chamber aerosols, difficulties were noted with the complex agricultural dust samples. Improvements to increase the permeability of the staining, to increase the sensitivity to detect cells at various stages of growth, and to enhance the detection of contaminants at lower concentrations (e.g., 10^2 cells/m^3) are in development. Advancement in detector design also will broaden the use of this technology for bioaerosol monitoring.

Immunomagnetic separation with fluorescent antibody-labeled beads and FCM is also used to detect microbial contaminants (22). This methodology has shown a detection of less than 10^3 colony-forming units for pure culture suspensions and 10^3 to 10^4 colonies of *E. coli*/ml in a mixed suspension with a one hour analysis time. Analysis can be completed in two hours and only a single antibody is required, but detection limits can be high (10^5 cells/ml) (23).

BIOTECHNOLOGY-BASED ASSAYS

Polymerase Chain Reaction Amplification

Polymerase chain reaction (PCR) amplification is an in vitro enzymatic reaction that uses amplification of specific DNA sequences for identification of a specific microorganism or group of organisms (24,25). The repetitive cycles of amplification in which specific gene sequences are copied increases the amount of DNA in the sample so that it can be detected. This technology is not dependent on the physiological state of the organism and therefore can be used to detect viable (culturable) and nonviable (nonculturable) organisms. However, the gene sequences specific to the targeted contaminant must be known. Although PCR has been used successfully to enhance the detection of microorganisms in other matrices such as clinical samples (26), food (27), water (28–30), and soil (31,32), the use of this method for monitoring airborne microorganisms is a more recent concept (33). PCR was shown to offer an increase in sensitivity over traditional culture methods for airborne bacteria (34–36) and viruses (37), and it is

possible to obtain results from air samples within hours of sample collection using PCR, compared with days or weeks for culture (35). PCR will also be useful when monitoring for airborne microbial contaminants that are difficult to culture such as *Mycobacterium tuberculosis* (38) and *Stachybotrys chartarum*.

Standard PCR involves the use of two unique primers to produce a single-amplification DNA product. To increase sensitivity when low concentrations of organisms are present in the sample, some investigators attempt reamplification (40) or the use of nested primers (41,42). Reamplification involves reassay of negative-amplification products with fresh reaction mixtures in an attempt to improve exponential amplification. When a PCR sample is amplified a second time using an inner primer set, the assay is termed *nested*. Amplification of a 208-bp fragment within a 1,247-bp product using nested PCR resulted in detection of a single plasmid copy per PCR and two *Bacillus anthracis* spores per PCR rather than the 10^3 plasmid copies and 10^4 spores detected using a standard PCR (41). Nested PCR also has been useful in detecting *B. anthracis* in tissue samples to discriminate the source of exposure (43). Multiplex PCR can be used to detect multiple contaminants by using several sets of primers to yield multiple amplification products (24). Tracking the release of fermentation products from biotechnology facilities or agricultural spraying of microbial pest control agents are potential uses for multiplex PCR in monitoring bioaerosols. Subtractive hybridization to remove homologous sequences of different strains so that unique sequences can be detected has been shown to improve PCR detection in samples containing mixed populations of similar organisms (44,45). This technique may improve the specificity of bioaerosol monitoring when a particular species within a genus is of interest. Reverse transcriptase (RT) PCR is used to detect RNA by generating a cDNA copy of the nucleic acids in a single-stranded RNA during the first cycle. The cDNA is used as the template for successive PCR cycles. RT-PCR has been shown to detect rapidly 1–10 virus particles (46).

Difficulties with the use of PCR for detection of airborne microbial contaminants includes the lack of known unique gene sequences for the organisms of interest. Selection of candidate gene sequences and the determination of likely PCR primer and probe locations using computer software have been developed. The sensitivity of the primers and probes then must be determined and the specificity tested using standard strains in laboratory experiments.

Liquid impingement and filtration sampling have been suggested for collection of air samples to be analyzed using PCR (47). Several commercially available impingement samplers and filtration devices have been used for bioaerosol collection (1), and these methods are discussed in the chapter on sampling for airborne microorganisms.

As with other environmental samples, an efficient yet simple nucleic acid extraction and purification procedure is needed before PCR can be performed on air samples. An optimized extraction and purification method will maximize yield and minimize processing time. A variety of techniques for DNA extraction have been developed, including hot detergent treatment, freeze-thaw, and bead mill homogenization. These methods have been used to detect and enumerate a variety of microorganisms including Varicella-Zoster virus (37), endospore-forming bacilli (48,49), vegetative bacterial cells (34,49), and fungal conidia (39,49–51). Six DNA-extraction methods for *Aspergillus fumigatus*, an important airborne fungal contaminant, were evaluated recently for samples from the mycelial mat of liquid fungal cultures with the highest genomic DNA yields obtained using glass bead pulverization with vortexing (52).

Detection limits using PCR for analysis of bioaerosols are affected by the physical condition and the concentration of the target DNA, and the presence and concentrations of background DNA in the reaction mixture. The presence of other airborne particles can interfere with successful detection using PCR amplification. Alvarez and coworkers (35) demonstrated interference in detection of *E. coli* with background airborne gram-negative bacilli concentrations of 10^4 CFU m^{-3}. Dilution of the sample resulted in successful amplification by diluting out the inhibitors but this procedure raises the lower limits of detection. Pretreatment of samples may be necessary to minimize interferences from biotic and abiotic material in the sample matrix and to improve detection limits. The separation of bacterial cells from PCR inhibitors by buoyant density centrifugation was tested with two vegetative gram-negative bacteria, *Shigella flexneri* and *Yersinia enterocolitica*, in food products, resulting in a detection limit of 10^4/g of blue cheese and 500/ml of milk (53), and this method may prove useful for preprocessing of air samples. Buoyant density centrifugation is performed with the sample containing target cells layered on top of Percoll media and subjected to centrifugation. Target cells are harvested from below the lighter Percoll layer while the PCR inhibitors remain in the upper layer. Sample purification using Sephadex or Chelex columns removes environmental inhibitors, and commercial kits are available. Additionally, purification of the DNA following extraction can be used to minimize interferences.

Although not yet reported for detection of bioaerosols, increasing the number of PCR samples by processing with a 864-well microwell plate has been reported. High-throughput PCR has demonstrated in 30 nine-minute cycles the analysis of more than 3,000 samples in less than 4.5 hours with simultaneous target amplification (54).

PCR does not provide information as to the viability or infectivity of the microbial contaminant. The addition of an enrichment step in sample processing can assist in discriminating infectivity of the microbial contaminant (55) or in analyzing for mRNA (24). Messenger RNA is produced before protein synthesis and has a half-life of only a few minutes. Therefore, mRNA can be used in sample analysis to estimate metabolic activity. However, mRNA is not specific to a single organism. Therefore the use of this technique for monitoring airborne viable organisms in clean–room-type environments (e.g., pharmaceutical and medical facilities) may be useful, but that in environments with mixed bioaerosol populations would be problematic.

The incubation of a sample with the appropriate host cells before extraction also can be used to establish the infectivity of viral contaminants. Integrated cell culture PCR (ICC-PCR) methodology differs from conventional PCR in that only viruses that have attached to host cells (the first step for viral infectivity) are amplified and detected, and inhibition by contaminant substances (e.g., humic acids, protease and transcriptase inhibitors) that may be present in environmental samples is minimized. Although ICC-PCR methodology has been used for detection of microbial contaminants in water (56), the application of ICC-PCR to bioaerosols has not been reported.

Until recently, post-PCR manipulation was required to detect the presence of amplified sequences, often by gel electrophoresis. The authenticity of the amplified DNA was confirmed by sequencing, restriction analysis, or nucleic acid hybridization using an oligonucleotide probe complementary to a specific internal DNA sequence within the product. Newly developed techniques have eliminated post-PCR processing with the incorporation of specific fluorescent-labeled probes that indicate amplification. The use of fluorescence correlation spectroscopy with PCR in microtiter plates combines reagents for amplification and detection in a single tube or well (57). Double-stranded target DNA is detected by two amplification primers that are 5′-tagged with two different fluorophores, resulting in a detection limit of 10–25 initial copy numbers of template. The ABI Prism 7700 Sequence Detection System (Perkin Elmer, Applied Biosystems, Foster City, California) uses TaqMan™ technology and incorporates a fluorogenic probe and the TaqMan™ PCR reagent kit (Perkin Elmer) with a 96-well microtiter format. The release of an internal fluorescent probe (cleaved during amplification) to indicate amplification provides rapid detection and quantitation of target gene sequences. The system has demonstrated reliability over several orders of magnitude and provides quantitation without the exhaustive and elaborate experimental manipulation previously described for quantitative PCR (58). Quantitative measurement of spores of a toxigenic fungus *Stachybotrys chartarum* from culture stocks (50,51) and air samples (51) has been reported using TaqMan™ technology. These investigators used reference sequences of a yeast to normalize the data for accuracy and precision of DNA extraction.

Combining PCR with immunological techniques has resulted in a rapid and efficient solution-phase hybridization of labeled targets and biotinylated capture probes. Target-probe hybrids containing biotin are captured onto streptavidin-coated microwells while other PCR components are washed away. This chemiluminescent microtiter PCR methodology uses digoxigenin-labeled amplified target detected by anti-digoxigenin-alkaline phosphatase conjugates and a chemiluminescent substrate. Although this method has not been used for bioaerosol detection, results were reported in two hours with a detection limit of 10 targets (59).

Additional PCR formats include the use of surface plasmon resonance to measure secondary structure of amplified DNA following asymmetric PCR (60) and the addition of peptide nucleic acid applied to the DNA probe with surface plasmon resonance (61). The use of rapid thermal cycling of microliter volumes inside capillary tubes containing indium-tin oxide offers additional decrease in analysis time and greater control over PCR temperature cycling (62).

Microchip-Based PCR

Integrating microchip technology and PCR can decrease the analysis time required for detection of microbial contaminants. A microchip PCR array with 10 silicon reaction chambers, thin-film heaters, and solid-state optics has been developed that provides real-time monitoring with low power requirements and no moving parts (63). Detection of *Erwinea herbicola* (vegetative cells), *Bacillus subtilis* (endospores), and MS2 (RNA virus) with a detection limit of 10^2–10^4 organisms/ml within 16 minutes has been reported (63). The simultaneous analysis of several samples can be accomplished by hybridization of fluorescent-labeled DNA on a microchip. An array of oligonucleotide probes are immobilized into gel elements fixed on a glass plate. Analysis then is performed with a two-wavelength fluorescent microscope equipped with a charge-coupled camera (64). Micro-machined silicon high efficiency reaction chambers (miniature thermal cycling chamber, MATCI) with integrated heaters and simple electronics to control temperatures provide solid-state, diode-based detection for real-time fluorescence monitoring of product DNA. The MATCI, a briefcase-sized instrument with rechargeable batteries (65), has detected single base pair substitutions in orthopoxviruses (monkeypox, cowpox, camelpox, and vaccinia viruses), human genomic DNA, and viral DNA (66). Combining cell lysis, multiplex PCR amplification, and electrophoretic sizing on a monolithic microchip has resulted in the analysis of amplified products using a sieving medium for size separation and an intercalating dye for fluorescence detection. This electrophoretic analysis has resulted in data available in less than three minutes after PCR (67). A 4.5-μl silicon microchip containing a series of 3.5-μm "weir-type" filters spanning the flow chamber was developed to minimize interference by separating the target from the background (68).

Molecular Beacons

The technology of sample analysis using nucleic acid probes that spontaneously undergo a fluorogenic conformational change when they hybridize with target fluorescent probes has been termed *molecular beacons* (69). These beacons are specific because they fluoresce only in the presence of complementary target. The reactions are carried out in a sealed tube minimizing manipulation. Application of this methodology to monitoring of bioaerosols may provide a means to screen rapidly a large number of samples with minimal analysis time.

CONCLUSION

The detection of airborne microorganisms is a difficult task confounded by the heterogeneous and highly variable composition and concentration of bioaerosols. Except

in pharmaceutical clean room-type environments, the presence of airborne microorganisms is highly variable over time and space, often fluctuating over several orders of magnitude. Enhanced methods of analysis have been reported and additional technologies are being adapted or are in development. Bioassays, immunoassays, analytical instrumentation assays, and biotechnology-based methods do not depend on viability of the organism; so culturable and nonculturable microorganisms, and cellular fragments can be detected. These techniques also demonstrate improved accuracy and precision, and provide quantitative data. Another advantage to these methods is interlaboratory comparability because the data generated are objective rather than the subjective determinations. Detection and enumeration with enhanced methods will improve the monitoring of airborne biocontaminants in indoor and outdoor environments.

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ENTAMOEBA HISTOLYTICA/ENTAMOEBA DISPAR

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Entamoeba histolytica is one of six parasitic amebas of the genus *Entamoeba* that are known to infect humans. *Entamoeba histolytica*, a protozoan parasite, is the organism responsible for the disease defined as *amebiasis*; the other amebas (*Entamoeba coli*, *Entamoeba hartmani*, *Iodamoeba butchlii*, *Endolimax nana*, and *Entamoeba polecki*) are not human disease causing organisms. Amebiasis is one of the great parasitic diseases of human kind; it predominantly affects individuals of lower socioeconomic status who live in developing countries where contaminated food and water, crowding, and inadequate hygiene are associated with disease transmission. Currently, there is a good understanding of the biology of *E. histolytica* and the mechanisms that account for its virulence and pathogenicity. Vaccines for amebiasis or reliable chemoprophylaxis, however, are not available, and the diagnosis still rests on microscopy and physician acumen in developing countries, where the disease is endemic.

HISTORY

The organism known as *E. histolytica* was first described 125 years ago by Losh (1) who named it *Amoeba coli*. Schaudinn (2) did not formally name the organism itself until 1903. After that, the taxonomy of the parasite became confused with several organisms that were morphologically similar to each other and are nonpathogenic parasites of the human intestine. These nonpathogenic organisms, as well as *E. histolytica*/*E. dispar*, have each different morphological characteristics. Differences are found in the structure of the cytoplasm, types of inclusions, motility, and size. According to the species, the nuclear peripheral chromatin is distributed in a different way or it may be absent, the karyosome may be central or eccentric and may vary in size. The morphology of the mature cyst and the number of nuclei are characteristic of the species (Tables 1 and 2). These differences can be detected if the organisms are stained, but in wet preparations, the organisms can be confused with one another.

The simple life cycle (Fig. 1) of parasitic amebas is identical in all species. The presence of nonpathogenic amebas in feces of humans is indicative of the subject having ingested contaminated foods.

In 1919, Dobell (1) published the monograph *The Ameba Living in Man*, in which he concluded that there was only one species of *Entamoeba* that produced cysts with four nuclei and the name *E. histolytica* was retained. In 1925, Brumpt (3) described *E. dispar* as an organism identical to *E. histolytica* but never causing disease in the host. Brumpt (3) based his argument on an eight-year follow-up of a patient who never developed symptoms, and on epidemiological surveys. These surveys revealed significant infection rates with what appeared to be *E. histolytica* in countries where invasive disease was almost unknown. He concluded that most infections in temperate countries were due to a distinct species, not easily differentiable from *E. histolytica*, which he named *E. dispar*. Brumpt's theory (3) was not accepted at the time, in part, because of evidence for the existence of asymptomatic carriers of ameba that were capable of causing invasive disease in others and also because these organisms could not be differentiated with the light microscope.

The name *E. dispar* disappeared from the literature for more than 50 years until 1978, when it was revealed that *E. histolytica* could be divided into two groups using isoenzyme electrophoresis. This evidence was supported by studies in several thousand isolates from all over the world and followed by a number of studies using molecular and immunological techniques (4–8). A well-documented article was published stating that *E. histolytica* was a complex of species (9), and subsequently differences between the two groups were confirmed (10). By 1993, Diamond and Clark (11) redescribed the two groups of

Table 1. Percentage of Diarrheas Related to Amebiasis

India	36–47%
Nigeria	11.2%
Calcutta	10%
Bangkok	7%
Mexico	5%
Argentina	6%
Brazil	7.5%
Colombia	33%

Source: WHO Bulletin, 1980.

Table 2. Differential Morphology of Protozoa Found in Stool Specimens of Humans: Amebae-Trophozoites

Species	Size (Length)	Motility	Nucleus			Cytoplasm	
			Number	Peripheral Chromatin	Karyosomal Chromatin	Appearance	Inclusions
Entamoeba histolytica	10–60 μm Usual range, 15–20 μm commensal form[1] More than 20-μm invasive form[2]	Progressive with hyaline, finger-like pseudopods	1 Not visible in unstained preparations	Fine granules Usually evenly distributed and uniform in size	Small, discrete Usually centrally located, but occasionally is eccentric	Finely granular	Red blood cells occasionally Noninvasive organisms may contain bacteria
Entamoeba Hartmani	5–12 μm Usual range, 8–10 μm	Usually nonprogressive but may be progressive occasionally	1 Not visible in unstained preparations	Similar to *E. histolytica*	Small, discrete, often eccentric	Finely granular	Bacteria
Entamoeba coli	15–50 μm Usual range, 20–25 μm	Sluggish, nonprogressives, with blunt pseudopods	1 Often visible in unstained preparations	Coarse granules, irregular in size and distribution	Large, discrete, usually eccentric	Coarse, often vacuolated	Bacteria, yeasts, other materials
Entamoeba polecki	10–25 μm Usual range, 15–20 μm	Usually sluggish, similar to *E. coli* Occasionally, in diarrheic specimens, motility may be progressive	1 May be slightly visible in unstained preparations Occasionally may be irregularly distorted by pressure from vacuoles in cytoplasm	Usually fine granules evenly distributed Occasionally granules may be irregularly arranged Chromatin sometimes in plaques or crescents	Small, discrete, eccentric Occasionally large, diffuse, or irregular	Coarsely, granular, may resemble *E. coli* Contains numerous vacuoles	Bacteria, yeasts

Table 2. (*Continued*)

Species	Size (Length)	Motility	Nucleus			Cytoplasm	
			Number	Peripheral Chromatin	Karyosomal Chromatin	Appearance	Inclusions
Endolimax nana	6–12 μm Usual range, 8–10 μm	Sluggish, usually nonprogressive with blunt pseudopods	1 Visible occasionally in unstained preparations	None	Large, irregularly shaped, blotlike	Granular, vacuolated	Bacteria
	8–20 μm Usual range, 12–15 μm	Sluggish, usually nonprogressive	1 Not usually visible in unstained preparations	None	Large, usually central Surrounded by refractile, achromatic granules These granules are often not distinct even in stained slides	Coarsely granular, vacuolated	Bacteria, yeasts, or other material
Dientamoeba fragilis[3]	5–15 μm Usual range, 9–12 μm	Pseudopods are angular, serrated, or broad lobed, and hyaline, almost transparent	2 (In approximately 20% of organisms only 1 nucleus is present) Nuclei invisible in unstained preparations	None	Large cluster of 4–8 granules	Finely, granular	Bacteria: occasionally red blood cells

[1]Commensal form — usually found in asymptomatic or chronic cases; may contain bacteria.
[2]Invasive form — usually found in acute cases: often remain red blood cells.
[3]Flagellate — included with amebae for diagnostic purposes.
Source: DPDX, the CDC web site for parasitology diagnosis. *www.cdc.gov/dpdx*.

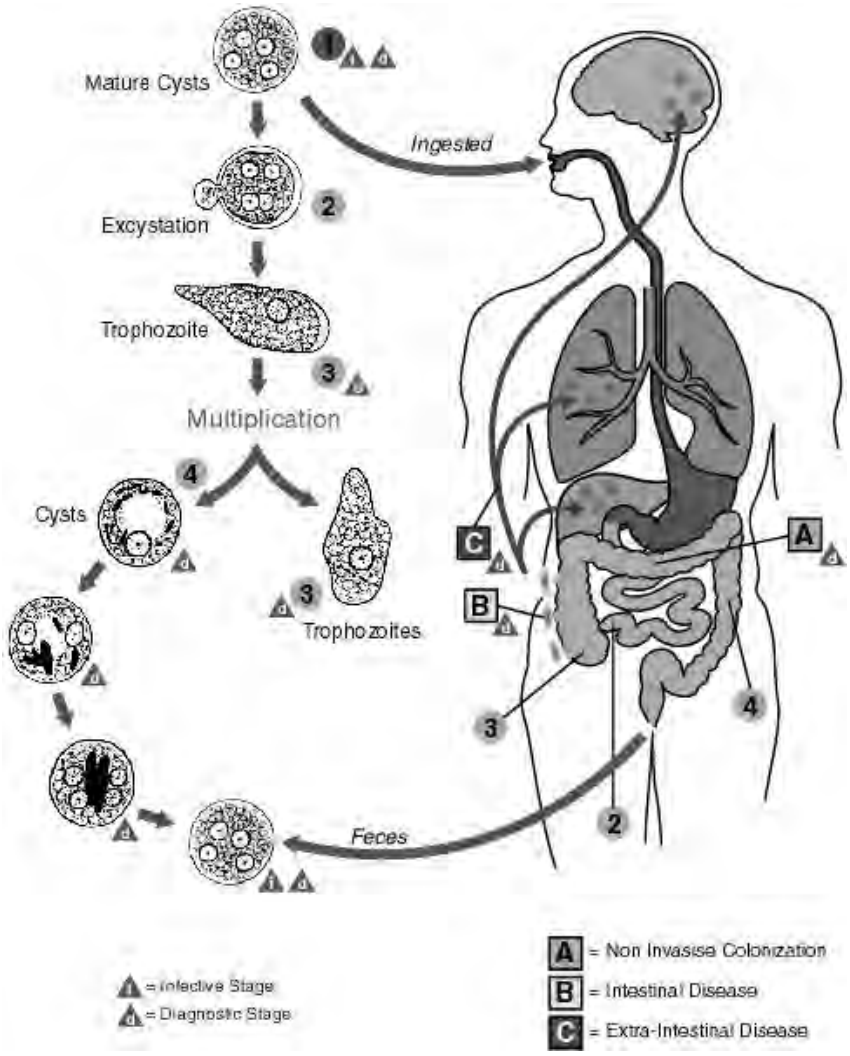

Figure 1. Life cycle of *Entamoeba histolytica*/*E. dispar*. *Source*: DPDX, the CDC web site for parasitology diagnosis. *www.cdc.gov/dpdx*. See color insert.

E. histolytica, the pathogenic and the nonpathogenic, as two different species, *E. histolytica* and *E. dispar*.

The two species present immunological differences like a 96-kDa antigen is present in pathogenic (P) strains and is absent in nonpathogenic (NP) isolates, a 30-kDa surface antigen is absent in NP isolates, a monoclonal antibody against electron-dense granules (EDG) was absent in NP isolates, finally, a monoclonal antibody detecting a 81/84 intracellular antigen was not present in NP isolates (11). Among the genetic evidence between the two species is selective hybridization of two DNA probes; cDNA encoding the 29-kDa antigen isolated from P and NP isolates showed a 4.5% difference between the two, differences in structure and expression of cysteine proteinase genes was found between P and NP isolates, the genomic organization of the actin genes appears to differ between the two forms (11).

In 1997, a World Health Organization (WHO) expert committee met in Mexico to consider the status of *E. histolytica*/*E. dispar* nomenclature, and officially recognized the existence of the two species. The growing list of differences between the two species, identical in morphology, include differences in every gene sequence examined (12–16) and differences in the cell surface scanned with light and electron microscopy (17–19). The acceptance of *E. dispar* and comparisons between the two species may provide insight into why *E. histolytica* is pathogenic (20).

LIFE CYCLE

The life cycle of *E. histolytica*/*E. dispar* was described extensively (1) and includes the description of the different stages of the parasite; trophozoite, precyst, and cyst.

Trophozoite

Trophozoites vary from 10 to 60 μm in diameter (usual range 20–30) depending on the environmental conditions. Motility and pseudopod formation is rapid and unidirectional, rarely occurring in a straight line. In wet preparations, the trophozoites have hyaline pseudopods that are fingerlike. The ectoplasm is clear and the endoplasm is finely granular and may contain bacteria and/or erythrocytes in various stages of digestion. In stained slides, the trophozoite nucleus is spherical, comprises one fifth of the trophozoite, and is difficult to see in wet preparations. The peripheral chromatin with fine granules is

usually evenly distributed in the nucleus wall. It contains a small and compact karyosome located near the center.

Precyst

In the precyst stage, the trophozoite becomes approximately the same size as the cyst. The cytoplasm is cleared of food inclusions, and occasional chromatoid bodies are present. The precystic form is uninucleated, and the enlarged nucleus contains a karyosome that is more or less central.

Cyst

A cell wall develops around the precystic form. There may be two types of inclusions within this immature cyst: a glycogen mass and highly refractile chromatoidal bars with smooth rounded edges (other species have chromatoidal bars with brushlike edges). As the cyst matures, there is nuclear division with the production of four nuclei to become the mature formed cyst. As the cyst matures, glycogen and chromatoidal material disappear. On stained slides cyst size ranges from 8.5 to 19 μm in diameter (usual range 11–14). Because of dehydration in the process of staining, cyst size may be 1 to 1.5 micrometers smaller. The mature cysts are usually spherical and contain four nuclei. The immature cysts contain one or two nuclei. The nuclear membrane is uniformly lined with peripheral chromatin. The karyosome is small, compact, and is usually located within the nucleus at the center. Cyst formation occurs only within the intestinal tract; once the stool has left the body, cyst formation does not occur. The one, two, and four nucleated cysts are infective and represent the mode of transmission from one host to another (21)

The life cycle is described in Figure 1. The infection with *E. histolytica*/*E. dispar* occurs following ingestion of a mature cyst in fecally contaminated food or water. The excystation occurs in the small intestine and trophozoites are released to migrate to the large intestine. During the process of excystation, the encysted ameba become very active, separating from the cyst wall. The ameba escapes from the cyst wall through a tiny pore and the nuclei clump together. Outside the cyst, the nuclei begin to separate from the cytoplasm and undergo division to form eight uninucleate metacystic trophozoites that continue to feed and grow, and produce cysts that are passed in the feces.

Entamoeba histolytica inhabits primarily the large intestine, where the trophozoites, or active forms, live in the intestinal lumen and on occasion may invade the mucosal crypts, where they feed upon red blood cells and form ulcers, and this gives rise to symptomatic infection with tissue invasion. The exact form of invasion is not known, but it has been suggested that amebae have enzymes that lyse host tissue, possibly from lysosomes on the surface of the ameba or from ruptured organisms. Ulceration of the intestinal wall may give rise to amebic dysentery. The majority of human infections are usually asymptomatic. Such individuals will have a negative or weak serologic response and negative stools for occult blood. They will primarily pass cysts in their stool, but if trophozoites are found, they will not show erythrocytes ingested. Isoenzyme analysis of organisms isolated from asymptomatic individuals, generally indicate that the isolates belong to nonpathogenic zymodemes (22). Asymptomatic patients infected with *E. histolytica*/*E. dispar* never become symptomatic (11). About 10% of the individuals infected have clinical symptoms presenting abdominal pain, and may eventually present dysentery. The incubation period varies from a few days to up to several months. Symptoms of amebic dysentery include diarrhea with cramping, lower abdominal pain, low-grade fever, and the presence of blood and mucus in stool. The ulcers produced by intestinal invasion of trophozoites start as superficial localized lesions that deepen into the classic flask-shaped ulcers of amebic colitis. The ulcers are separated by segments of normal tissue but can coalesce. Ameba may be found at the advancing edges of the ulcer usually not in the necrotic areas. Abdominal perforation and peritonitis are rare, but are serious complications. A more chronic presentation occurs with amebic colitis. It is characterized by intermittent diarrhea over a long period of time and can be misdiagnosed as ulcerative colitis or irritable bowel syndrome. Ameboma, a localized tumor like lesion, results from chronic ulceration and may be mistaken for malignancy. Histologically, it consists of granulomatous tissue. The ameba may find their way into capillaries, to be transported via the blood stream to the liver and other organs where abscess formation may occur. It can occur with or without previous symptomatic intestinal infection. The liver is the most common site of extraintestinal disease, followed by the lungs, pericardium, brain, and other organs. Symptoms can be acute or gradual and may include low-grade fever, right upper quadrant pain, and weight loss. Up to 5% of individuals with intestinal symptoms develop liver abscess. However, up to 50% of individuals with liver abscess have no previous history of gastrointestinal disease (22) Although the proportion of individuals infected with *E. histolytica*/*E. dispar* is small in terms of morbidity and mortality, the disease represents a major public health problem in Third World countries, where the disease is endemic.

EPIDEMIOLOGY

Entamoeba histolytica infections are well known worldwide, especially in developing countries where disease incidence is high. In 1913 (23), Walker and Sellard in their controlled experiments in the Philippines, noted that: (*1*) transmission was accomplished by cysts not trophozoites, (*2*) asymptomatic carriers were often possible reservoirs and responsible for transmission, (*3*) there are differences in the risk of infection among individuals, and (*4*) there are differences in the virulence of the strains. The acceptance of the existence of two morphologically identical species has profound implications for understanding the complex epidemiology of this parasite.

Infections by *E. histolytica*/*E. dispar* are worldwide, but are more prevalent in the tropics. It is estimated that 480 million people or 12% of the world population are infected and that the annual mortality is 40,000 to 110,000

persons (24). High rates of infection occur in the Indian subcontinent, in Southern and Western Africa, in the Far East, and in areas of South and Central America. The prevalence of the infection is related to cultural habits, age (higher among school children), level of sanitation, crowding, and socioeconomic status (25). High-risk groups include people who travel constantly, such as immigrants, and working immigrants, immunocompromised individuals, individuals in mental institutions, and homosexual men in North America (26). It is of special interest that very little invasive disease is found in patients with AIDS. The majority of strains isolated from patients positive for the immunodeficiency virus, analyzed with isoenzyme patterns, are nonpathogenic (27). Prevalence of infection varies from one area to another, as does severity of the illness from one patient to another (28). Differences in prevalence, however, are also associated with detection methods and the number of samples examined. An important limitation in epidemiological studies is that many surveys use nonrepresentative samples of the population in question (24). Approximately 10% of the people infected each year have symptoms (24). Of the 48 million with clinical symptoms, 80 to 90% have symptoms related to the intestinal mucosa (diarrhea or dysentery); however, from 2 to 20% of the population have clinical symptoms, associated with invasion through the intestinal mucosa (24).

Humans are the only reservoir of *E. histolytica*/*E. dispar* infection. Ingestion of food or drink contaminated with cysts from stool and direct oral-fecal contact are the most common ways of acquiring the infection. Transmission of *E. histolytica* by contaminated water is common in developing countries, where drinking water is not treated (24). The use of human excreta as fertilizer is also an important source of infection. Only cysts are infective, and transmission is principally from asymptomatic carriers. Depending on the environmental conditions, cysts may remain viable up to three months, in the water; however, viable cysts can be destroyed by hyperchlorination or iodization (26).

TRANSMISSION

The infective form of *E. histolytica*/*E. dispar* is the quadrinucleated mature cyst. Unlike trophozoites, cysts may remain viable for prolonged periods of time under different environmental conditions. They are resistant to gastric juice and digestive enzymes. Amebic cysts conserve their infective capacity in stools, in water and in the soil. They are resistant to chlorine in quantities normally used to purify water and thus, this procedure does not prevent epidemics caused by fecal contamination of drinking water. Cysts are very resistant to environmental conditions and can remain viable in the soil for eight days at 28 to 34 °C, 40 days at 2 to 6 °C, and 60 days at 0 °C. Cysts are destroyed by exposing them to iodine (200 ppm), 5 to 10% acetic acid, and temperatures more than 68 °C, and can be removed by filtration with sand (29).

Risk of infection is related to poor hygiene education and environmental sanitation, especially water distribution, elimination of excreta, food safety, crowding, and hygiene habits. It has been demonstrated that the frequency of cyst carriers is higher among individuals of low socioeconomic status (30). In Mexico, it was found that the frequency of cyst carriers was significantly higher in slum areas where illiteracy, the proportion of homes with dirt floors without purified drinking water or drainage was high (31). The duration of the carrier state is variable and can last from several months to two years. Carriers can heal spontaneously, which means they can stop shedding cysts in a few months (32) and they do not need to be treated.

Contamination of drinking water distribution systems with stools that contain viable cysts can cause serious epidemic outbreaks, not recorded in the literature (33), especially in developing countries where water distribution systems are deficient, and where water for human consumption is taken from rivers, wells, and other sources. Water from these sources as well as that used for irrigating crops, are frequently contaminated with fecal material (34). Contamination of food by this and other mechanisms such as unhygienic food handling and the use of feces as fertilizer are undoubtedly the main mechanisms of transmission in developing countries.

Not all individuals who ingest cysts will become carriers. This variation may be related to the size of the inoculum, characteristics of the strain of the parasite, the susceptibility of the individual, and environmental factors (33).

Because of limitations in the published literature, accurate generalizations on prevalence, incidence, morbidity, and mortality of amebiasis are difficult to make. Intestinal ameba is universal, but their geographic location marks the prevalence of the infection and the incidence of the disease. Probably the most complete information available from both serologic and stool surveys, comes from Mexico, where it is estimated that 5% of the population suffers a severe episode of invasive amebiasis annually (30).

Table 1 shows the percentage of people with diarrhea related to *E. histolytica* according to the WHO (35).

In summary, in 1981, approximately 480 million people carried this protozoan parasite in their intestinal tract, 36 million developed severe colitis, and at least 40,000 died. This infection deserves more attention and greater effort to develop control strategies, including research on better methods for prevention, diagnosis, and treatment (24).

DIAGNOSIS

The appropriate diagnosis for *E. histolytica* is accomplished by identifying the microorganisms in stools, biopsy material, liver aspirates for extraintestinal infection, and by some immunological tests. Identification of the organism can be done microscopically, but differentiation of *E. histolytica* and *E. dispar* requires other methods. The presence of red blood cells (RBC) in trophozoites found in stool samples and finding trophozoites in tissue is diagnostic of *E. histolytica* infection (36).

The collection of samples should be done in appropriate wide mouth containers, labeled with all the information about the patient, time of collection, date, and for stools,

consistency of the sample. Samples should be transported immediately to the laboratory and should be processed within half an hour after the stool is taken. Fresh samples are preferred in the laboratory to enable technicians to see moving organisms. If the sample is not processed immediately, it should be preserved in buffered formalin and polyvinyl alcohol PVA for concentration and staining respectively. It is recommended that at least three samples should be taken from the patient within 10 days, preferably every other day for a better recovery of the parasite (22,36–38).

Microscopic diagnosis should be done by trained personnel with knowledge of the morphological characteristics of the parasite (22,39) because it may be confused with white blood cells (40,41). The sample should be screened for both trophozoites and cysts in a wet mount. Permanent staining with trichrome or iron haematoxylin is mandatory to confirm diagnosis by observing morphological characteristics (22,37,39,42). Trophozoites and cysts should be differentiated from other nonpathogenic ameba (Tables 2 and 3). Biopsy specimens should be taken from the edge of ulcers. Periodic-acid-Schiff stains the parasite a magenta color, increasing the ease of detection in biopsies (43).

Microscopic techniques cannot differentiate between *E. histolytica* and *E. dispar*, other methods are needed to differentiate these species. Culture techniques have been used to cultivate the organisms and further differentiate pathogenic and nonpathogenic strains with isoenzyme electrophoresis (44). The strains were named *invasive* and *noninvasive* and three enzymes were used to differentiate them: glucose phosphate isomerase, phosphoglucomutase, and L-malate : NADP oxidoreductase (oxaloacetate decarboxylating), electrophoretic patterns were compared and analyzed by distance analysis (9). Further studies of this type and the use of an additional enzyme, hexokinase, have revealed differences, not only between strains of *E. histolytica*, but within the whole histolytica group (9,45).

Differentiation of species can be done using immunological and genetic methods. Antigen detection may be useful as a complement to microscopic diagnosis in detecting parasites and to distinguish between pathogenic and nonpathogenic strains. Antibody detection is most useful in patients with extraintestinal disease that is amebic liver abscess when organisms are not found in stool examinations. Because *E. dispar* is not pathogenic, it should not elicit a detectable antibody response and therefore patients with *E. dispar* will be negative in *E. histolytica* detection tests. Kits for antibody detection, including indirect hemagglutination (IHA), enzyme immunoassay (EIA), and immunodiffusion (ID) are commercially available in the U.S. (22,46).

The IHA was the standard for routine serodiagnosis of amebiasis for a long time. The IHA test detects antibodies specific for *E. histolytica* at titers of $>1:256$ in approximately 95% of the patients with extraintestinal amebiasis, 70% of patients with active intestinal infection, and 10% of asymptomatic persons who are passing of *E. histolytica* cysts. The EIA test has been found to be as sensitive and specific as the IHA and has now replaced the IHA in most laboratories. The immunodiffusion test is specific, but it is slightly less sensitive than the IHA and requires a minimum of 24 hours to obtain results, in contrast to two hours for the IHA and EIA tests (22,46).

Monoclonal antibodies to the galactose specific adherence lectin (GIAP) mediates amebic attachment to colonic mucins, epithelial cells, and host inflammatory cells, were screened to recognize epitopes specific to pathogenic and nonpathogenic strains (10,47). Epitope 1 and 2 Mabs bind to the lectins of both species, whereas epitope 3 to 6 Mabs recognize only the *E. histolytica* lectin. These monoclonal antibodies against GIAP of *E. histolytica* have been extensively tested (48–51). The first field test of a commercial antigen detection kit was reported in 1995 (52). Recent studies indicate improved specificity and sensitivity of fecal antigen assays. Atleast one commercial kit is available, which detects only pathogenic *E. histolytica* infections in stool, whereas several kits are available, which detect *E. histolytica* in stool but do not exclude *E. dispar* (CDC web site for parasitology diagnosis *www.cdc.gov/dpdx*).

GENITIVE EVIDENCE

Differentiation of the two species has been tested at the molecular level. DNA probes have been developed from the pathogenic and nonpathogenic strains (53,54). Amplification of the fragments differentiating the strains has been accomplished by designing specific primers for both *E. histolytica* and *E. dispar* (55,56). The primer construction was based on the pathogenic and nonpathogenic sequences of a small subunit of ribosomal RNA (rRNA) that revealed a genetic distance of 2.2% greater than that between humans and mice (5). Polymerase chain reaction (PCR) developed by Aguirre and coworkers (57), called the SHELA method uses DNA extracted directly from stool samples and allows extraction from trophozoites and cysts. PCR products are detected using a colorimetric method based on enzyme linked immunoabsorbent assay (ELISA) to read by spectrophotometry, or by eye. An improvement of the DNA extraction and changes in the primer design in the SHELA method has made it possible to analyze multiple primers within one day (58). Later in 1997 another PCR method that uses fresh stool samples concentrated in acetate-acetic acid-formalin (SAF) was developed and used digestion with proteinase K. A 0.88 kb sequence of multicopy 16S rRNA gene served as target for the amplification (59). Storage in SAF fixative before testing, resulted in decreased sensitivity after two days.

Several studies have evaluated and compared immunological and molecular methods of differentiation of the two species (60–62). The Techlab (Blacksburg, VA.) kit did not cross react with *E. dispar* antigens, but was 100 times less sensitive than PCR in detection and differentiation between the two species. The use of PCR in epidemiological studies is recommended (60). Others recommend the Techlab kit because they consider PCR too time-consuming, cumbersome, and expensive and therefore not suited for developing countries where the infection is endemic (61). Both, epidemiological studies and detection of extraintestinal amebiasis have been done at the molecular level (62–65).

Table 3. Differential Morphology of Protozoa Found in Stool Specimens of Humans: Amebae-Cysts

Species	Size (Diameter or Length)	Shape	Nucleus			Cytoplasm	
			Number	Peripheral Chromatin	Karyosomal Chromatin	Chromatoid Bodies	Glycogen
Entamoeba histolytica	10–20 μm Usual range, 12–15 μm	Usually spherical	4 in mature cyst Immature cysts with 1 or 2 occasionally seen	Peripheral chromatin present. Fine, uniform granules, evenly distributed	Small, discrete, usually centrally located	Present. Elongated bars with bluntly rounded ends	Usually diffuse. Concentrated mass often present in young cysts. Stains reddish brown with iodine
Entamoeba hartmanni	5–10 μm Usual range, 6–8 μm	Usually spherical.	4 in mature cyst Immature cysts with 1 or 2 often seen	Similar to *E. histolytica*	Similar to *E. histolytica*	Present. Elongated bars with bluntly rounded ends	Similar to *E. histolytica*
Entamoeba coli	10–35 μm Usual range, 15–25 μm	Usually spherical. Occasionally oval, triangular, or other shapes	8 in mature cyst Occasionally super-nucleated cysts with 16 or more are seen. Immature cysts with 2 or more occasionally seen	Peripheral chromatin present. Coarse granules irregular in size and distribution, but often appear more uniform than in trophozoites	Large, discrete, usually eccentric but occasionally centrally located	Present, but less frequently seen than in *E. histolytica*. Usually splinter-like with pointed ends	Usually diffuse, but, occasionally well defined mass in immature cysts. Stain reddish brown with iodine
Entamoeba polecki	9–18 μm Usual range, 11–15 μm	Spherical or oval	1 rarely 2. Occasionally visible in unstained preparations	Usually fine granules evenly distributed	Usually small and eccentric	Present. Many small bodies with angular or pointed ends, or few large ones. May be oval, rod-like, or irregular	Usually small, diffuse masses stain reddish brown with iodine. A dark area called an “inclusion mass” (possibly concentrated cytoplasm) is often also present. Mass does not stain with iodine

Table 3. (*Continued*)

Species	Size (Diameter or Length)	Shape	Nucleus			Cytoplasm	
			Number	Peripheral Chromatin	Karyosomal Chromatin	Chromatoid Bodies	Glycogen
Endolimax nana	5–10 μm Usual range, 6–8 μm	Spherical, ovoidal, or ellipsoidal	4 immature cysts Immature cysts with less than 4 rarely seen	None	Large (blotlike), usually central	Occasionally granules or small oval masses seen, but bodies as seen in *Entamoeba* spp. are not present	Usually diffuse Concentrated mass seen occasionally in young cysts. Stains reddish brown with iodine
Iodamoeba butschlii	5–20 μm Usual range, 10–12 μm	Ovoidal, ellipsoidal, triangular, or other shapes	1 in mature cyst	None	Large, usually eccentric Refractile, achromatic granules on one side of karyosome. Indistinct in iodine preparations	Occasionally granules present, but chromatoid bodies as seen in *Entamoeba* spp. are not present	Compact well-defined mass; stains dark brown with iodine

Source: DPDX, The CDC web site for parasitology diagnosis *www.cdc.gov/dpdx*

CONCLUSION

Entamoeba histolytica and *Entamoeba dispar* are now two well-defined organisms. More and more research in being done to define detailed biological, biochemical, and genetic characteristics of each organism and to specify the biological and diagnostic differences between the two organisms. Information on differences and similarities between the two organisms, however, is not conclusive (66–71). Diagnostic procedures are still being evaluated to differentiate the two organisms (72,73). Epidemiologically, the discovery or redefinition of *E. dispar* has given insight into why *E. histolytica* is pathogenic and elucidated questions about the epidemiological behavior of the species once believed to be only one organism. More research will take place until finally, all questions will be answered, or maybe more questions will be asked. This is a challenge to our capacity to decide when sufficient questions have been asked. International agencies have taken action in resolving the public health problem that diarrheal disease imposes on humanity. Especially in children, there is still much to be done.

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ENTERIC ADENOVIRUSES.

See ADENOVIRUSES

ENTEROCOCCI, FECAL.

See FECAL STREPTOCOCCI/ENTEROCOCCI IN AQUATIC ENVIRONMENTS

ENTEROHEMORRHAGIC E. COLI (EHEC).

See PATHOGENIC *ESCHERICHIA COLI*

ENTEROINVASIVE E. COLI.

See PATHOGENIC *ESCHERICHIA COLI*

ENTEROPATHOGENIC E. COLI (EPEC).

See PATHOGENIC *ESCHERICHIA COLI*

ENTEROTOXIGENIC E. COLI (ETEC).

See PATHOGENIC *ESCHERICHIA COLI*

ENTEROVIRUSES: BASIC BIOLOGY AND DISEASES

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The enteroviruses have been the most-studied waterborne enteric viruses. This was the result of their ability to be easily grown in cell culture and the occurrence of poliovirus epidemics in the first half of the twentieth century. Although almost always present in sewage and feces of infected persons, they have been seldom associated with water and food-borne outbreaks. This may be because they often cause mild or asymptomatic infections and because of lack of diagnosis. Still, they are associated with a number of common and serious infections worldwide. The development of molecular epidemiology has increased our understanding of the potential role of these viruses in heart disease and diabetes, and helps to fully understand their routes of transmission in the environment.

GENERAL PROPERTIES

Enteroviruses are a genus of the Picornaviridae family, which are among the smallest ribonucleic acid (RNA) viruses. "Picorna" means small RNA virus. The Picornaviridae family also includes the closely related rhinoviruses and hepatitis A virus (which was once classified as enterovirus type 72). Enteroviruses are subdivided into four groups, namely, polioviruses, Coxsackieviruses, echoviruses, and enteroviruses 68–71 (Table 1). Echovirus 22 and 23 have now been separated from the echoviruses as a separate genus, the parechoviruses, based on significant differences in their genome from the other echoviruses (1). They are now referred to as parechoviruses 1 and 2.

Table 1. Human Enterovirus Types

Number of Serotypes	Members
3	Polioviruses 1, 2, 3
22	Coxsackieviruses A1-22, 24 (A23 is echovirus 9)
6	Coxsackieviruses B1-6 (swine vesicular disease virus is very similar to coxsackie B5 virus)
26	Echoviruses 1–7, 9, 11–27, 29–34 echo 8 is echo 1 echo 10 is reovirus, type 1 echo 28 is human rhinovirus 1A echo 22 and 23 are now parechoviruses 1 and 2
4	Enteroviruses 68–71

Table 2. Biophysical Properties of Poliovirus

Physiochemical Characteristics of the Major Virus-Particle Population	Poliovirus Type 1
Morphology	
Diameter	28 nm
Envelope	none
Symmetry	isosahedral
Sedimentation Rate	156 S
Density (CsC1)	1.34 g/ml
Nucleic Acid	
Type	single-stranded RNA
Length	2.3 Fm
Molecular weight	2.6×10^6
Number of base pairs	7,441*
Polypetides (major)-molecular weights	24,000 25,000 34,000
Viron Mass**	8.43×10^6
% RNA (as K salt)	31.6
% Protein	68.4
Viron/mg	7.07×10^{13}

Source: F. Kee et al., *J. Public Health Med.* **16**, 145–148 (1974).
*Sabin vaccine strain. Type 2; 7,440 and type 3; 7,432
**Computed from complete nucleotide sequence

Enteroviruses are nonenveloped, icosahedral in shape, and 25 to 30 nm in diameter (Table 2). They contain a genome of single-stranded RNA of the positive sense, which acts like messenger RNA and is polyadenylated at the 3′ terminus. The RNA from all the sequenced enteroviruses are similar in length, about 7,400 base pairs. The viral capsid contains sixty subunits, each subunit containing four polypeptides. The four peptides are referred to as VP1, VP2, VP3, and VP4. In addition, one small noncapsid protein, Vpg, is covalently attached to the 5′ end of the virion RNA.

Soon after an enterovirus attaches to a cell, it is delivered into the cytoplasm as viral RNA freed from its protein shell. The single-strand genome RNA then serves as its own mRNA, which is translated to form a single large polypeptide that is subsequently cleaved to produce the various viral capsid polypeptides. Completion of encapsidation produces mature viral particles, which are then released when the cell lysis. The time required from initiation of infection to completion of virus assembly ranges from 5 to 10 hours, depending on pH, temperature, host cell, and number of particles to which the cell is exposed (2). Yields may be up to 100,000 particles per cell, but in a single passage only 1 in 1,000 or 1 in 100 may be infectious in cell culture. This has led to the assumption that most of the particles are defective, that is, lacking a complete genome or protein capsid. However, the apparent low ratio of infective to noninfective particles actually results from poor assay efficiency in cell culture. Optimizing attachment of virus to the cell and the use of different cell lines can enhance infectively of the virus in cell culture 2 to more than 10-fold (1a,1b,1c).

Enteroviruses have a buoyant density of 1.34 g/mL in cesium chloride. A characteristic of the enteroviruses is their resistance to high and low pH levels. They are stable at pH 3 to 5 for one to three hours and can tolerate pH 10 to 11 for several minutes. They are resistant to antibiotics, 70% alcohol, 5% Lysol, 1% quaternary ammonium compounds (Roccal),and insensitive to ether, deoxycholate, and most detergents. Treatment with 0.3% formaldehyde, 0.1 N HCl, or a free residual chlorine level of 0.5 mg/L will cause their rapid inactivation.

Enteroviruses are fairly stable at room temperature (days), but are rapidly inactivated at 50 °C. However, in the presence of molar magnesium chloride, only partial inactivation occurs in one hour at 50 °C (3). They are stable at 4 °C for weeks, and for years at −20 °C and −70 °C. They are rapidly inactivated by drying.

HISTORY

Poliovirus was the first enterovirus to be recognized, although it was not classified as an enterovirus until 50 years after its discovery. Poliomyelitis, the crippling disease caused by the polioviruses, has been known for centuries, but the basic studies began in 1908 when Landsteiner transmitted the disease to monkeys. In the 1930s, Paul and Trask (3) recovered the virus in feces of patients and the concept of poliomyelitis as an enteric infection became established. The first strains of what are now known as Coxsackievirus subgroup A were isolated by inoculation of infant mice with fecal material from two children during an epidemic of poliomyelitis in 1948 in Coxsackie, New York (4). Additional types of subgroup Coxsackie B were discovered shortly thereafter (5). As soon as cell culture became available in the late 1940s, other enteroviruses were isolated from the stools of both healthy and ill children. Because the viruses in the healthy children were not immediately associated with a disease they were called “orphan” viruses and later became known as ECHO (enteric cytopathogenic human orphan) viruses. As new enterovirus members were recognized, they were given sequential numbers as enterovirus 68–71.

EPIDEMIOLOGY

In the prevaccine era, epidemics of poliomyelitis occurred with great frequency among the developed countries of

the world. In the period 1951 to 1955, an average of almost 38,000 cases of poliomyelitis (approximately 21,000 of them paralytic) were reported annually in the United States. Only eight to nine cases now occur annually in the United States, and these all have been vaccine associated (i.e., conversion of the live vaccine to wild type after infection) (6). Nonpolio enteroviruses are estimated to cause 10 to 15 million symptomatic infections in the United States annually (7).

The fecal-oral route is believed to be the main route of transmission, although respiratory transmission may also be significant for some types (8). It is believed that the fecal-oral route can transmit almost all enteroviruses (except possibly enterovirus type 70, which causes eye infections). Incubation periods vary greatly with the type of virus and may be as short as 12 hours for Coxsackievirus A24 (eye infections) to as long as 35 days for poliovirus. Because of their antigenic inexperience, children are the primary targets of enterovirus infections, and thus serve as the main vehicle for their spread, which is chiefly by the fecal-oral route. These viruses are easily spread within family settings and closed institutional populations, where the rate of infection among nonimmune members may reach as high as 80%. Where unsanitary and poor socioeconomic conditions prevail, the rate of infection among infants and children may exceed 50%. As personal and community hygiene improve in a population, spread of enteroviruses becomes limited, so that increasing numbers of individuals reach later childhood, or even adulthood, without having been infected and immunized by the common serotypes. By two years of age, regardless of climate, geography, or socioeconomic conditions, most children have already experienced several asymptomatic or mildly symptomatic enterovirus infections. One set of enterovirus serotypes may be predominant for a time in some areas, or even worldwide, to be succeeded by other types.

A number of different Coxsackievirus and echovirus serotypes have been involved in sizable epidemics, and enteroviruses 70 and 71 have also been responsible for outbreaks in various parts of the world. These outbreaks have been mostly localized to one area, although one epidemic of echovirus 9 infections in the late 1950s had almost worldwide distribution, and enterovirus 70 spread explosively in Asia and Africa during 1969 to 1973. In 1981, enterovirus 70 epidemics reappeared in Asia and Africa, and also spread to the Western hemisphere. Enterovirus 71 has been involved in several outbreaks affecting large numbers of patients in several different nations, but pandemic spread has not been observed (6).

GROWTH IN CELL CULTURE

Most of the enteroviruses grow very well in human and monkey cell lines. They grow best in primary cell lines (kidney cells). Coxsackievirus A viruses do not grow in most cell lines, but can be isolated by inoculation of newborn mice. The buffalo green monkey cell (BGM) line of African green monkey kidney cells has been reported as being more sensitive than primary rhesus or green monkey kidney cells for titration of certain enteroviruses, and for recovery of plaque-forming enteric viruses from sewage and water (9,10). The BGM cell line is the cell line most commonly used today for the isolation of enteroviruses from the environment. The RD cell line, derived from a human rhabdomyosarcoma, has been shown to support the growth of the Coxsackievirus A group. A second passage in cell lines is often necessary, to obtain cytopathogenic effects (CPE) with enteroviruses isolated from environmental samples (11).

In monolayers of cultured cells, the growth of enteroviruses is generally associated with a characteristic CPE. Infected cells round up, shrink, and show marked nuclear pyknosis, become refractile, and eventually degenerate and falloff the plastic or glass surface. The time for the appearance of CPE is dependent on the concentration of virus in the inoculum, the type of virus, and if it has been repeatedly passed in the cell line. In the case of laboratory grown poliovirus, CPE can be observed usually within 48 hours.

Most enteroviruses are inhibited from propagating in cell cultures by two-(alpha-hydroxybenzyl)-benzimidazole and guanidine (12). The susceptibility of cells to a number of enteroviruses may be increased by treating with 5-iodeoxyuridine at 50 fg/mL for three days before exposing the cells to the virus (13).

IDENTIFICATION

Although isolation of enteroviruses in cell culture is fairly simple, identification of the serotype may be slow and expensive. Traditionally, specific identification tests using serum neutralization were utilized. Using specific antisera for each virus makes this a very time consuming process. However, identification is simplified with the use of antisera pools, each containing antisera to several types of enteroviruses. These pools are constituted in a pattern in such a way that, a given antiserum appears in one pool, two pools, or three pools. An unknown enterovirus may be identified by its pattern of neutralization by the pool or pools containing its homotypic antiserum (14).

Application of recombinant DNA procedures and the polymerase chain reaction (PCR) through the use of specific primers have led to the use of methods for the identification of enteroviruses in both clinical and environmental samples. These methods can be used directly on the samples, or to identify the enteroviruses after isolation in cell culture (14).

HOST RANGE

Human enteroviruses are believed to have no known natural reservoirs. They have been occasionally isolated from the feces of dogs in developing countries and rural areas (15,16). Serologically distinct enteroviruses have been found in many animals; however, humans do not usually have recognizable infections with "Animal" enteroviruses. Some laboratory animals, however, are susceptible to infection with human enteroviruses, including primates and neonatal mice. Most strains of poliovirus infect and cause paralysis in monkeys and

chimpanzees. Coxsackie A viruses, characteristically produce widespread stretching of the muscle in the skeletal muscles of new born mice, resulting in paralysis.

INFECTIVITY

The only dose-response data available are those obtained from studies conducted with vaccine strains of poliovirus or echovirus 12. Lepow and coworkers (17) conducted studies on newborn infants less than five-days old, infected with poliovirus Sabin type 1 (LSc-2ab). Minor and coworkers (18) exposed two-month-old infants, via the oral route using a syringe, to a vaccine strain of polio type 1. Plotkin and coworkers (19) and Katz and Plotkin, (20) orally exposed premature infants to an attenuated strain of poliovirus type 3 (Fox strain). Schiff and coworkers (20) studied the infectivity of echovirus 12 in adults. The virus was from stools of previously infected individuals. Teunis and coworkers assessed the probability of being infected by ingesting one virus in these studies (21). They found that the probability ranged from 7.14×10^{-4} to 1.90×10^{-1}. The range probably reflects the type of virus and method of administration.

IMMUNITY

Immunity to the enteroviruses is believed to be permanent. Immunity is generally specific to the enterovirus serotype. Virus-neutralizing antibody develops within a few days after exposure to the virus, usually before the onset of illness, and may persist for life (22). Infection of the intestinal tract results in the production of a secretory nasal and duodenal IgA antibody.

PATHOGENESIS

The portal of entry of the enteroviruses is the alimentary tract via the month. The incubation period is usually 7 to 14 days, but may range from 2 to 35 days. After initial and continuing multiplication, probably in the lymphoid tissue of the pharynx and gut, viremia may occur, and in turn lead to further growth and multiplication in other organs, namely, spinal cord, brain, meninges, heart, and skin. Usually, the virus is excreted in the stool for several weeks and is present in the throat for one to two weeks after infection in clinical or asymptomatic cases. Enteroviruses have been isolated from the feces, pharyngeal washing, cerebrospinal fluid, spinal cord, brain, heart, eye, and skin. The reported average concentration of enteroviruses in stools is about 10^6 tissue culture infectious units (23) per gram of feces.

ASYMPTOMATIC INFECTIONS

A striking future of most enterovirus infections is the large variation in asymptomatic infections seen with this group of viruses. This may vary by both the type and strain of enterovirus (24). Within each group of the major enteroviruses, a wide range of asymptomatic infection rates may be observed. For example, there is approximately a 15% asymptomatic infection rate for echovirus 9 versus 67% for echovirus 11 (25,26). As a rule of the thumb, asymptomatic infection is apparently most common for the poliovirus (90–95%), followed by the echoviruses and coxsackieviruses (about 50%). About 4 to 8% of the poliovirus infections are associated with minor illness such as upper respiratory tract infection, gastroenteritis, and influenza-like illness (8). Another 1 to 2% may develop aseptic meningitis accompanied by back pain and muscle spasms. Only 0.1 to 2% may develop paralytic poliomyelitis.

SECONDARY TRANSMISSION

Transmission within households has been well studied for the enteroviruses. Household secondary attack rates in susceptible members may be greatest for enterovirus type 70, which causes eye infections, and for the polioviruses. In some studies, the secondary attack rates may be 90% or more, although they are typically lower (24). In a study of New York families, on the transmission of viral disease, the secondary transmission rate for Coxsackievirus in susceptible persons was 76% and for echoviruses 43% (27). Enterovirus infections, often spread more frequently among children than adults, and secondary coxsackievirus infections were more frequent in mothers (78%) than in fathers (47%). The greater spread of poliovirus and Coxsackievirus may derive from longer periods of viral excretion (24). Secondary transmission rates may vary with the type and strain of enterovirus.

ILLNESSES CAUSED BY ENTEROVIRUSES

Enteroviruses cause a wide spectrum of diseases that involve almost any organ (Table 3). Disease severity can range from life-threatening with significant morbidity, to mild or subclinical. The more common syndromes include nonspecific febrile illness, aseptic meningitis, herpangina, hand-foot-mouth syndrome, and exanthems (skin infections). The clinical manifestations of infection in the neonate can be distinct and discussed separately.

Currently, there are no vaccines (except poliovirus) or treatment of enterovirus infections, except those supportive of clinical symptoms.

PARALYSIS

Before the advent of vaccination, poliovirus was a major cause of permanent paralysis in the United States. Vaccination has eliminated poliomyelitis in the United States, and no indigenous wild viruses have been detected there since 1979 (8). Most infections are asymptomatic (90–95%) with only 0.1 to 2% resulting in paralytic poliomyelitis. Most poliovirus infections occur in children under four to five years of age, but the older the age of infection, the greater the severity of the outcome. Mortality in children averages 2.5% for symptomatic infections and 30% in adults (8).

Table 3. Common Clinical Syndromes Associated with Enterovirus Infections in Children

Syndrome	Predominant Virus	Clinical Features
Nonspecific febrile illness	All types	Febrile illness (fever), with nonspecific upper respiratory and gastrointestinal tract symptoms
Aseptic meningitis	Echovirus, group B Coxsackieviruses, and polioviruses	Fever, meningeal signs with mild cerebrospinal fluid (CSF) pleocytosis, usually normal CSF glucose and protein, and absence of bacteria
Herpangina	Group A Coxsackieviruses	Fever, painful oral vesicles on tonsils and posterior pharynx
Hand-foot-mouth disease	Coxsackievirus A16	Fever, vesicles on buccal mucosa and tongue and on interdigital surfaces of hands and feet
Nonspecific exanthems	Echoviruses	Variable rash (usually rubelliform but may be petechial or vesicular), with or without fever
Pleurodynia	Coxsackievirus B3, B5	Uncommon, epidemic, fever, and severe muscle pain of chest and abdomen
Myocarditis	Group B Coxsackieviruses	Uncommon, myocarditis/pericarditis, which can present with heart failure or dysrhythmia
Acute hemorrhagic conjunctivitis	Enterovirus 70	Epidemic cause of conjunctivities with lid swelling, subconjunctival hemorrhage, and eye pain without systemic symptoms
Paralytic disease	Poliovirus, Enterovirus 71, Echoviruses, and Coxsackieviruses	Paralysis

Nonpolio enteroviruses have been associated with paralysis, but this is uncommon compared to poliovirus (28,29). Coxsackievirus A7 has been associated with outbreaks of paralytic disease (30), and outbreaks of enterovirus 71 have been involved in several outbreaks of central nervous system involvement, with most fatal cases in children (31).

PERINATAL AND NEONATAL INFECTIONS

Neonates represent a population at great risk from several enteroviral diseases. Adverse effects to the fetus are caused by enteroviral infection during pregnancy. In the prevaccine era, paralytic poliomyelitis occurred during pregnancy in apparent excess of age-adjusted expected rates, suggesting predisposition among pregnant women (32). Infections of pregnant women by the nonpoliovirus enteroviruses occur frequently. In a seroepidemiologic study, Brown and Karunas (33) found a 42% rate of infection during pregnancy, in a population evaluated prospectively over a 10-year period. In a review of Coxsackie B infections, Modlin and Rotbart (34) suggested that greater viral replication and prolonged maternal enterovirus excretion occurring in late pregnancy may well enhance the risk of infection of the newborn infant in the perinatal period.

Neonatal nonpolio enteroviral infections are common. Estimated attack rates indicate that disease in newborns and young infants is comparable or exceeds symptomatic neonatal infections caused by herpes simplex virus and cytomegalovirus (35–37). Enteroviruses were responsible for the majority (65%) of greater than three-month-old infant admissions to a hospital in one community for suspected sepsis (36–38). In another study, enteroviruses were the most frequently identified pathogens between days 8 and 29 of life, accounting for at least one-third of all cases of neonatal meningitis (36–39).

Age is the most important determinant of the outcome of enterovirus infections. Different age groups have different susceptibilities to infection, different clinical manifestations and degrees of severity, and different prognoses following enteroviral infection. Young children have higher attack rates. In one study, echovirus 9 disease attack rates in children were found to be 50 to 70% compared with 17 to 33% in adult age groups (40). Age-specific attack rates for echovirus 30 per 1,000 persons in an outbreak in the United Kingdom ranged from 19.7 (Children age 0 to 9 years) to 7.11, 4.82, 4.73, 1.5, and 0 for the succeeding 10-year age cohorts, respectively (41).

Severity of illness may also be age dependent. With poliovirus infection, adults are more likely to be severely affected, tending to acquire paralytic poliomyelitis rather than nonparalytic poliomyelitis (i.e., aseptic meningitis) or asymptomatic infection. On the other hand, Coxsackie B virus infection is clearly more severe in newborns than in older children and adults, often, causing myocarditis, encephalitis, hepatitis, and death (42,43). Coxsackievirus and echovirus encephalitis and aseptic meningitis are most frequent among those 5 to 14 years old (44,45), while myocarditis is most common in adults and neonates. In another study (46), the mean age among patients with Coxsackievirus B, meningitis was 7.7 years, pericarditis was 9.9 years, and gastroenteritis was 1.3 years.

HERPANGINA

Herpangina is characterized by a painful vesicular eruption of the oral mucosa associated with fever, sore throat, and pain on swallowing. It is seen most commonly in children ages 3 to 10 years (8). Group A coxsackieviruses are the most common etiologic agents, but group B coxsackieviruses and echoviruses also have been isolated form patients. Fever, usually mild, develops suddenly, but higher temperatures up to 41°C

(105.8 °F) can be seen, particularly in younger patients. Nonspecific early symptoms may include headache, vomiting, and myalgia. Sore throat and pain with swallowing are the most prominent symptoms and precede the characteristic exanthems (eruption of mucous membranes) by, approximately, one day. Herpangina is self-limited and symptoms resolve within one week.

ASEPTIC MENINGITIS

Nonpolio enteroviruses are the leading causes of aseptic meningitis, accounting for 70% to 90% of all cases for which an etiologic agent is identified (7,47). The most common enterovirus types associated with aseptic meningitis are Coxsackievirus B5 and echovirus 4, 6, 9, and 11. These have occurred in epidemic outbreaks, as well as sporadic cases being most common in the 5 to 15 year age group (8).

The course of enteroviral meningitis usually is self-limited and benign, but there has been an ongoing debate about the occurrence of long-term neurological, cognitive, or developmental abnormalities from this infection in older children (48). However, several investigations have documented that 10% of children younger than three months of age who have aseptic meningitis may suffer long-term sequelae, especially speech and language delay (49).

NONSPECIFIC FEBRILE ILLNESS

The most common clinical presentation of nonpolio enterovirus infection is a nonspecific febrile illness (fever). Typically, fever develops suddenly and temperatures range from 38.5 °C to 40 °C (101 °F–104 °F) and last for an average of three days. Occasionally, a biphasic pattern of symptoms can be seen, with an initial fever for one day, followed by two to three days of normal temperatures and recurrences of fever for an additional two to four days.

Studies have found that enteroviruses are the major cause of hospitalization for young infants (younger than two to three months of age) for suspected fever caused by septicemia during the summer and fall (50). A recent study of infants less than 90 days of age found that nonpolio enteroviruses were the most common cause of fever in infants requiring hospitalization (51). Greater than 25% of the infants were infected. The average length of stay was three days with average medical costs of $4,500.

EXANTHEMS (ERUPTION OF THE SKIN)

Nonpolio enteroviruses are the leading cause of exanthems in children during the summer and fall months. The most common serotype causing exanthems is echovirus 9. The classic enteroviral exanthems consist of a pink, macular, rubelliform rash. The rash may be the sole manifestation of infection, or may be present in association with febrile illness, or aseptic meningitis. Enteroviral exanthems are seen most commonly in children younger than five years of age and decrease in prevalence with age. The rash is self-limited and disappears in three to five days (7). Most infections occur in infants and young children 3 to 10 years of age (median, 4 years) (8).

The best-known enteroviral exanthem is the hand-foot-mouth disease. It is commonly associated with Coxsackievirus A16, but may also be caused by Coxsackievirus A5 and several other enteroviruses including enterovirus 71 (52). Children usually have a fever, with multiple discrete red macular lesions of about 4 mm appearing on the palms, soles, fingers, and toes. The infection is usually self-limited, lasting one to two weeks.

Complications associated with hand-foot-mouth (HFM) disease caused by enterovirus type 71 include encephalitis, meningitis, hemorrhage, acute flaccid paralysis, and myocarditis. During a large outbreak of HFM disease in Taiwan in 1990, of enterovirus type 71, 129,000 cases were estimated resulting in 405 hospitalizations and 78 deaths (53). Almost all of the severe cases and deaths were in children less than five years of age.

RESPIRATORY ILLNESS

Worldwide enteroviruses appear to account for 2% to 15% of all viruses that cause upper and lower respiratory tract diseases (54). The illness is most commonly associated with coxsackie A10, A21, A24 and B2 (8). Both children and adults are affected with infections lasting only a few days. Pneumonia associated with enteroviral infection has been reported in both outbreaks and individuals (54). Fatal pneumonia has been associated with Coxsackievirus and echovirus infections in infants and children (55–57).

ACUTE HEMORRHAGIC CONJUNCTIVITIS (AHC)

This explosive epidemic enterovirus conjunctivitis, first described in 1969 in Africa and Asia, is now found worldwide. It is common in tropical and densely populated regions. The majority of outbreaks have been caused by enterovirus serotype 70, but recently coxsackievirus A24 has been isolated during the outbreaks (8). AHC is characterized by a sudden onset of severe eye pain, photophobia, and blurred vision. Subconjunctival hemorrhages, erythema, edema of lids, and eye discharge are characteristic of infections. Recovery occurs within 7 to 10 days. Spread is by the eye-hand-fomite route in contrast to the fecal-oral route seen with most enteroviral infections. Overall, it is more common in adults, but it also does affect school-aged children. Some enterovirus outbreaks have been associated with poliomyelitis-like paralysis.

DIABETES

Insulin-dependent diabetes mellitus (IDDM) is the most common severe chronic childhood illness, affecting an estimated 123,000 children in the United States (58). More than 11,000 new cases are diagnosed annually. The disease is the leading cause of renal failure, blindness, and amputation, and a major cause of cardiovascular disease and premature death in developed countries (59). IDDM occurs most frequently at the ages of 2, 4 to 6, and 10

to 14 years, perhaps because of physiological increases in sex hormone levels and insulin resistance or because of alterations in the pattern of childhood infections. Season and latitude affect incidence, suggesting an infectious etiology (59). The infectious agents most commonly linked to IDDM have been the enteroviruses.

To date, epidemiological studies have failed to prove or disprove the association of enteroviruses with IDMM (60). This may be because the nature of the disease may involve both genetic factors of the host and environmental exposure, with the development of clinical symptoms taking years to develop. Autoimmunity, potentially induced by a preceding enterovirus infection, could play a role in human IDDM (59). Recent studies continued to support some association with enterovirus infections and IDDM (61–64).

PLEURODYNIA (BORNHOLM DISEASE)

Pleurodynia or epidemic myalgia is characterized by an acute onset of severe muscular pain in the chest and abdomen accompanied by fever. Coxsackieviruses B3 and B5 are the major causes of this epidemic disease; sporadic cases have been described with other nonpolio enteroviruses.

The muscular pain is sharp and spasmodic, with episodes typically lasting 15 to 30 minutes, although they can last up to several hours. During spasms, patients may develop signs of respiratory distress or appear shocklike with diffused sweating and pallor. Pain localized to the abdomen in young children may falsely suggest appendicitis. The illness usually lasts one to two days, but frequent recurrences are possible several weeks after the initial episode. Associated signs and symptoms include anorexia, headache, nausea, and vomiting. In contrast to many other enteroviral syndromes, pleurodynia is more common in older children and adolescents.

Cases are recognized mostly in school-aged children and adults, with the peak age being in children two to nine years old (8). However, older boys have also been reported to develop orchitis, namely, inflammation of the testes (8). It has not been established whether involvement of the ovaries occurs.

MYOCARDITIS

Coxsackievirus B infections are increasingly being recognized as a cause of primary myocardial disease in adults as well as children (2). In some studies, up to 39% of persons infected with coxsackievirus B5 developed cardiac abnormalities. Coxsackieviruses of group A and echoviruses have also been implicated, but to a lesser degree. The illness is common in neonates and adults. Older adults represent the vast majority of cases, with patients aged 40 and older, composing 69% of the cases (65). While the incidence is less in neonates, the outcome is potentially more severe with mortality among infants reported to be 30 to 50% (34). Symptoms usually begin within the tenth day of birth with fatigue, poor feeding, or mild respiratory distress.

Most children and adults recover; however, one or more recrudescences several weeks to more than a year later have been reported in approximately 20% of the cases after the initial illness (66). Persistent electrocardiographic abnormalities (10–20%), cardiomegaly (5–10%) and chronic congestive heart failure indicate that permanent heart damage occurs due to this illness.

DISEASES ASSOCIATED WITH THE IMMUNOCOMPROMISED

Enteroviruses are not prominent among the microorganisms that cause serious morbidity and mortality among the immunocompromised. In childhood, serious enterovirus infection does not appear to be particularly common in the T cell immunodeficiency syndromes (8). However, enteroviral infections pose significant risk to children who have defects in B lymphocyte functions, the most common of which is X-linked agammaglobulinemia (67). Unlike other viruses that are combated by cellular immune mechanisms, enteroviruses are eliminated from the host by humoral immune mechanisms. An intact B-cell response is believed to be necessary to block viral entry into the central nervous system. Children who have agammaglobulinemia may develop chronic enteroviral infection, most commonly meningoencephalitis. Patients experience headache, lethargy, seizures, motor dysfunction, and altered sensorium. Symptoms may wax and wane for years, but there is an overall progressive deterioration in central nervous system function. Infections are fatal in most children who are immunodeficient. Echovirus 11 has been the most common cause of chronic infection, but cases caused by other echoviruses and Coxsackieviruses have been reported (8).

Enterovirus infections in infants who have received organ transplants can result in serious complications (68). Serious life-threatening infections of both echovirus and Coxsackievirus have been documented in infants receiving both bone marrow and liver transplants. Cancer patients receiving chemotherapy may also suffer from severe illness when infected with a Coxsackievirus (69).

OTHER ILLNESSES

Enteroviruses have been associated with a number of other illnesses including juvenile rheumatoid arthritis (70) and gastroenteritis (71) (Table 4). Case reports have also linked enteroviruses to short-term mental impairment in children and other illnesses or symptoms in children (Tables 3–5). Other studies have suggested relationships between enterovirus infections and sudden infant death syndrome or SIDS (72), risk of schizophrenia from infections early in childhood (73), amyotrophic lateral sclerosis (Lou Gehrig's disease) (74), vertigo (75) and chronic fatigue syndrome (76). These studies have been limited in scope or speculation.

INCIDENCE OF ENTEROVIRUS INFECTIONS

In two studies on virus occurrence in solid waste, Peterson (85,86) isolated enteroviruses in 10% of the

Table 4. Less Common Illnesses Associated with Enterovirus Infections

Syndrome	Reference
Rheumatoid arthritis	77–79
Pancreatitis	80
Hemorrhagic syndrome	81
Gastroenteritis	82
Mental disorders	83
Alice in Wonderland syndrome	84
Schizophrenia	73
Vertigo	75

Table 5. Probability of Infection from Different Types of Poliovirus by the Oral Route in Children

Virus Type and Strain	Probability of Infection	ID_{50}*	Reference
LSc-2ab	7.14×10^{-4}	6.93×10^{4}	17
1	9.10×10^{-3}	76.2	18
3 Fox	1.90×10^{-1}	5.5	19
3 Fox	2.66×10^{-1}	5.0	20

*The number of viruses required to cause infection in 50% of the individuals exposed (77)

fecally soiled diapers that she examined. The excretion rate of enteroviruses has been found to vary by the month, with the greatest percentage from May to October in the United States. The incidence in children over the entire year ranges from 2.4% to 13.3%, with the higher excretion rate in the lower socioeconomic group (6). The most extensive work done on virus excretion was during the "Virus Watch" studies in which the incidence of virus illness and excretion was conducted in families, for many years in several locations across the United States. (6). In Seattle and New York, stools samples were collected from family members regularly (usually at monthly intervals), whether illness was present or not. Over a three-year period, the incidence of excretion of any enteric virus in children (<15 years of age) was found to range from about 10% in the winter to almost 40% during the summer. During the summer and autumn months (June through October), over one-third of the healthy children were excreting some virus in the feces, as detected by cell culture. Overall, the frequency of illness associated with echovirus infections was 44%. However, symptomatic infections were greater for children under four years of age; 78% for four-year-olds, and 12% for children, five years of age and older. The rate of symptomatic infections among the adults was 28% for both Coxsackievirus and echovirus versus 42% for children under four years of age.

ECONOMIC IMPACT

Studies on the economic cost of nonpolio enterovirus infections have only recently been attempted. Pichichero and coworkers (87) conducted a study on children older than four years to assess the economic impact of enterovirus infection. Some 380 children in two clinics, over a period of four months, in different regions of the United States, were involved in the study. The children were followed for two weeks to document absenteeism and follow-up medical care. The majority of the illnesses were mild and no hospitalizations were required. Most of the illnesses occurred in children 4 years to 12 years of age. The duration of illness in most children was prolonged, lasting 9.5 days on average. The total of direct medical care costs and indirect costs per case ranged from $132 for hand-foot-and-mouth disease to $1,193 for meningitis.

In 1991, a large outbreak of echovirus 30 meningitis occurred in New England, affecting more than 1,500 individuals (88). A cost analysis of the hospital billing for the inpatient/outpatient care of 103 patients involved in the outbreak, was performed at a hospital serving the region. The average inpatient management cost of a patient with enterovirus meningitis, in this outbreak, was $1,757 + $198 and the outpatient management costs was $477 + $63. Indirect costs were not determined.

WATER AND FOOD-BORNE OUTBREAKS

A search for instances of waterborne poliomyelitis began about 1937, and continued through much of the 1940s. The search yielded only eight likely outbreaks in which a relationship to contaminated drinking was presumed by the investigator (89). However, the epidemiological evidence was not conclusive in any of these outbreaks. Only the outbreak near Lincoln, Nebraska, has sufficient data to suggest that contaminated water was involved (90). This outbreak occurred in 1952, among families living in a cluster of temporary houses facing a number of serious difficulties with the water distribution system, fluctuations in pressure, and toilet bowl water that may have siphoned back into the water main. The most convincing outbreak linking poliomyelitis to drinking water occurred in Taiwan in 1982 (91). Having been free of the disease for seven years, 1,031 cases of poliomyelitis were reported within a five-month period. An intensive epidemiological study concluded that inadequate vaccination and receipt of a nonmunicipal water supply were significant risk factors.

Several recreational outbreaks of nonpolio enterovirus infections have been documented among children. An outbreak of Coxsackievirus B4 or B5 meningitis occurred at a boy's summer camp linked to swimming in a lake (92). Another outbreak of meningitis, this time caused by Coxsackievirus A16, occurred due to exposure to lake water (93). An epidemiological study among bathers, swimming in nondisinfected lake waters, demonstrated an association with increased risk of enterovirus infection. D'Alessio and coworkers (94) surveyed children of ages 1 to 15 at a pediatric clinic to determine where they had been swimming and the location and frequency of the swimming during the prior two weeks. Children swimming in a pool (with a chlorine residual) had a statistically significant increase in the relative risk of having an enterovirus illness. Of the 134 viruses isolated from the patients, 119 (90%) were nonpolio

enteroviruses and 33.6% were Coxsackievirus type A. Echovirus 30 transmission to children has also been associated with a community swimming pool (95). The risk of echovirus 30 was greatest among those who swallowed pool water.

A raw milk–associated outbreak of poliomyelitis in 1914 is believed to be the first recorded food-borne outbreak of a viral disease (96). Consumption of raw milk was implicated in 10 more such outbreaks in the United States and United Kingdom by 1949 (97). Combined effects of pasteurization and immunization made this a rare event after the 1950s (98). Only two outbreaks associated with foods have been reported. The first of these was in 1976 when 80 cases of aspectic meningitis were recorded among those who ate coleslaw served at a picnic; echovirus type 4 was identified as the causative agent but the actual source of the virus could not be determined (99). The other outbreak occurred in 1988 and led to 161 cases of an unspecified illness. The causative agent proved to be echovirus type 5, but the specific food and the actual source of the virus remained undetermined (100). The sole source of a food-borne outbreak due to the Coxsackievirus B subgroup comes from the former Soviet Union (101). A day care center was the site of the outbreak and Coxsackievirus types B1, B3, and B5 were reported to have been isolated from certain items of food, surfaces in food preparation areas, as well as the infected individuals.

CONCLUSION

Enteroviruses are the most studied waterborne viruses, although rarely associated with documented waterborne disease. They have been used as a model for the study of all enteric viruses in the environment. Methods are readily available for their detection in the environment and they are very stable in the environment. They are a very common cause of illness in man, causing an amazingly wide variety of illnesses, from mild to life-threatening. The impact of enteroviruses on human health is still not fully understood.

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ENTEROVIRUSES IN WATER: CONCENTRATION AND DETECTION

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Human and/or animal enteroviruses may be present in raw and treated municipal wastewater, animal manure, biosolids, groundwater, rivers, lakes, and drinking water. With the possible exception of raw sewage and sludge, the number of enteroviruses present in contaminated water is too small to be detected by methods used routinely in clinical virology (e.g., without sample concentration). To determine the presence of viruses in water, therefore, a large sample volume (100 to 1,000 L) needs to be concentrated to a smaller volume, which can then be processed by various methods for virus detection. Although progress has been made over the years for the concentration and detection of viruses from large volumes of water and wastewater (1), a "perfect" method does not exist that can concentrate and detect all types of viruses from all types of water.

The quest for methods to detect the presence of viruses in the water environment started around the 1950s, with scientists attempting to detect poliovirus in water samples (2). Since then, attention has also been focused on the concentration and detection of other enteric viruses, for example, enteroviruses, hepatitis A virus, and Norwalk (NV), and Norwalk-like viruses (NLV). Adsorption of viruses from water matrix to a solid surface and their subsequent elution in small amounts of buffer remain the basis of all concentration methods. The method is termed appropriately as *virus adsorption-elution* (Viradel) method. Surfaces that have been used for virus adsorption include microporous filters, granulated coal, organic polymers, glass powder, and polyethylene glycol (Table 1). However, methods using virus-adsorbing filters are preferred because they are simple to use and large quantities of water can be processed.

In short, a virus concentration procedure consists of the following steps: (*1*) filtration of large quantities of water or wastewater through a virus-adsorbing filter, (*2*) elution of adsorbed virus in 1 to 2 L of an eluent (usually 3% beef extract-glycine, pH 9.0 or 0.05 M glycine, pH 10.5), (*3*) further reduction in the volume of the eluate by a second-step reconcentration method (usually by organic flocculation or aluminum hydroxide-hydroextraction method), and (*4*) virus detection by inoculation in cell cultures or by molecular means. A number of excellent reviews are available that describe methods for the concentration and detection of enteric viruses from water and wastewater (1,16,17). The purpose of this review is to describe methods that have been used extensively in the field, namely, the viradel method using membrane filters.

Table 1. Media Used for Concentration of Viruses

Media	Reference
Electronegative filters	3
Electropositive filters	4
Bituminous coal sandwich	5
Glass powder, glass wool	6–8
Organic polymers	9
Erythrocyte ghost cells	10
Yeast cells	11
Inorganic salts ($CaHPO_4$)	12
Active aluminum oxide	13
Diatomaceous earth modified with metallic hydroxides	14
Animal cells growing on filters	15

ELECTRONEGATIVE FILTERS

Negatively charged pleated cartridge (25.4 cm) filters (Filterite filters) were found to readily adsorb poliovirus from water that had been adjusted to acidic pH and to which small amounts of trivalent cations (e.g., aluminum chloride) had been added (3). Adsorbed virus was eluted by treating the filter with glycine buffer at high pH. Using these epoxy-fiberglass pleated filters, approximately 70% of the poliovirus added to 400 L of estuarine water was eluted in 2 to 3 L of filter eluate, which was further concentrated by adsorbing the virus to aluminum hydroxide flocs and then recovering it in 150 mL of buffered fetal calf serum. Additional reductions in volume were achieved by hydroextraction (aluminum hydroxide hydroextraction method). By using these procedures, 60 to 80% of the virus in filter eluate could be recovered in a final volume of 10 to 40 mL. In a subsequent study, it was shown that these filters could be regenerated by autoclaving or by treating them with 0.1 N NaOH. The regenerated filters were found to retain their ability to concentrate viruses from water (18).

In another study, pleated membrane filters (Filterite filters) were used for the concentration of poliovirus from large volumes of tap water (ca. 1,000 L) by conditioning the water with small amounts of aluminum chloride to enhance virus removal by membrane filters (19). Tap water treated with 2×10^{-5} M aluminum chloride showed slight decreases in pH and turbidity and enhanced removal of poliovirus by membrane filters. The virus was quantitatively recovered by treating the filters with a basic buffer, and the eluate was reconcentrated to a small volume by adsorption to aluminum hydroxide flocs. Using these procedures, virus from 1,000 L of tap water was obtained in a final volume of 20 to 80 mL with a mean recovery of 70%. Before these studies, most of the virus concentration methods were limited to the use of flat filters that could process relatively small volumes of water.

The Viradel technique was modified for the concentration and detection of rotavirus from seawater using simian rotavirus SA-11 as a model virus for human rotavirus (20). The virus adsorbed optimally to filterite filters when seawater was adjusted to pH 3.5 and 1.0 mM aluminum chloride (Fig. 1). Adsorbed virus was eluted

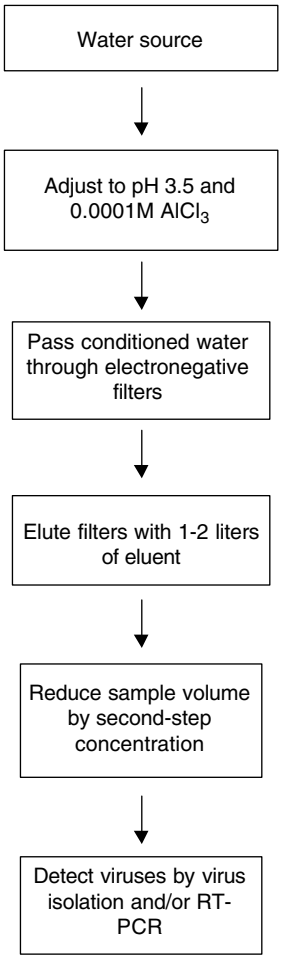

Figure 1. Schematic diagram of a virus adsorption-elution method using electronegative filters.

with 6% beef extract at pH 10.5 and was further concentrated to a smaller volume by using a modification of the organic flocculation method. When this method was used in conjunction with an indirect immunofluorescence test, rotaviruses were detected in sewage-contaminated seawater.

To enhance the virus-adsorbing capacity of electronegative microporous filters, Preston and coworkers (21) soaked epoxy-fiberglass (filterite) filters in an aqueous solution of a cationic polymer (polyethyleneimine; PEI) for two hours at room temperature and then allowed the filters to air-dry overnight on absorbent paper towels. Filters thus treated were evaluated for adsorption of coliphages (MS2, T2, ϕX174) and enteroviruses (poliovirus type 1 and coxsackievirus type B5) and were found to adsorb more viruses than untreated filters.

To determine the ability of pleated filters to adsorb bacteria, viruses, and protozoa, Payment and coworkers (22) used 3- and 1-μm wound electronegative fiberglass cartridge filters in series for the simultaneous concentration of poliovirus, coliphages, *Giardia lamblia* cysts, *Clostridium perfringens* spores, and *Legionella pneumophila* from large volumes of drinking water (Table 2). Filtration was performed at pH 3.5 in the presence of 0.001 M aluminum

Table 2. Studies on Electronegative Filters

Reference	Filter Type	Usage
3	Filterite	Concentration of viruses from estuarine water
18	Filterite	Regeneration of filters for reuse
23	Filterite	Concentration of poliovirus from tap water
20	Filterite	Concentration of rotavirus from seawater
21	Filterite	Enhanced viral adsorption by treating filter with a cationic polymer
22	Wound fiberglass depth cartridge	Simultaneous concentration of bacteria, viruses, and protozoa
24	Balston	Concentration of four different enteroviruses from water and wastewater

chloride to enhance adsorption. Elution of microorganisms entrapped or adsorbed to the filters was accomplished by a slow backwash elution with 1.5% beef extract solution containing 0.5% Tween 80 (pH 9.75). Tween 80 was shown to enhance the elution of all microorganisms tested. To further reduce the volume of the primary eluate, different second-step procedures were used. Giardia cysts were reconcentrated by low-speed centrifugation and purified by sucrose-density gradient flotation at a final recovery of 52%. *Legionella pneumophila* was reconcentrated by low-speed centrifugation at an overall recovery of 55%. *Clostridium perfringens* spores and coliphages were concentrated by a detergent—protein flotation method. Poliovirus was eluted at 93% and reconcentrated at 78% efficiency by organic flocculation.

Another negatively charged filter (grade C Balston filter, Balston, Inc., Lexington, Massachusetts) was evaluated by Guttman-Bass and Nasser (24). They used selective antibody neutralization to recover four different enteroviruses (poliovirus 1, coxsackievirus A9, coxsackievirus B1, and echovirus 7) from water and wastewater. Organic flocculation was used as second-step reconcentration procedure. The average recovery was 68% with wastewater and 97% with tap water, lake water, and seawater.

COMPARATIVE STUDIES

In subsequent years, many different investigators conducted comparative studies in which different types of filters, adsorption-elution conditions, and second-step reconcentration procedures were compared (Table 3). Beef extract (3% solution at pH 9.0) and 0.1 M glycine buffer (pH 11.5) were compared for the elution of poliovirus

Table 3. Comparative Studies on Electronegative Filters

Reference	Filters Compared
25	Fiberglass cartridge (K27); epoxy-fiberglass-asbestos (M780); Epoxy-fiberglass (filterite)
26	Johns-Manville D39, D49, and D79; Filterite 0.25-μm and 0.45-μm; Millipore AP20 and 0.45-μm; Whatman GF-D and GF-F; Gelman A-E
27	Round-robin comparison of Filterite filter in six different laboratories
28	Wound fiberglass depth cartridge filters
29	Filters from Sartorius, Millipore, Gelman, and Cuno companies
6	Macroporous glass (MPG-1000 VGKh and SO1); Nitrocellulose PNT 0.45-μm; Millipore HAWP 0.45-μm; Synpor 0.45-μm; polycapromide (Pall 0.2-μm); and FMPA 0.55-μm

adsorbed to fiberglass cartridge filters (K27), epoxy-fiberglass-asbestos filters (M780), and filterite filters (25). Poliovirus type 1 (strain LSc) was seeded into 20- to 25-gallon (ca. 75.6–95.6 L) samples of treated sewage effluent and concentrated by filter adsorption-elution technique. Adsorbed virus was eluted by using either two 600-mL portions of 3% beef extract (pH 9.0) or two 1-L portions of 0.1 M glycine, pH 11.5 (Table 4). In all experiments, beef extract elution followed by organic flocculation was found to be superior, yielding a mean viral recovery of 85% (range of recovery = 68–100%). Elution with 0.1 M glycine (pH 11.5) followed by inorganic flocculation resulted in a mean recovery efficiency of 36%. The variable range of viral recoveries with beef extract could not be improved by varying the type of beef extract or by extending the elution time to 30 minutes.

Payment and Trudel (26) compared fiberglass filters from five different sources for virus adsorption, flow rate, clogging resistance, and virus concentration efficiency. Johns-Manville D39, Filterite (0.25-μm), Filterite (0.45-μm), and Millipore (0.45-μm) filters were the most efficient for virus adsorption, retaining more than 99% of the added virus in water at pH 3.5 and 0.0005 M aluminum chloride. The Johns-Manville D79 and D49 filters retained 92 and 96% of the virus, respectively, whereas the Whatman GF-D, Whatman GF-F, Gelman A-E, and Millipore AP-20 filters retained only 28 to 78% of added virus. The best flow rate and the least clogging were seen with Johns-Manville D79 filter (or with this filter acting as a prefilter to the Johns-Manville D49, Johns-Manville D39, or Filterite 0.45-μm filters). Recovery efficiency was 87% when 20 L of tap water seeded with poliovirus was concentrated with a combination of Johns-Manville D79-Johns-Manville D39 or with Johns-Manville D79-Filterite 0.45-μm filters.

Table 4. Common Eluents Used

Eluent	Reference
Glycine buffer (0.05–0.1 M) at pH 9.5–11.5	3,23,25,27
Beef extract (3%–6%) at pH 9.5–10.5	20,25,27
Slow backwash of filters	22
4 M urea-0.05 M lysine (pH 9.0) as eluent	30
Urea-arginine-phosphate buffer as eluent	31
Inclusion of Tween 80 in eluent	22,32

To evaluate the Viradel method using Filterite filters, six laboratories actively involved in water virology research participated in a methods-evaluation study under the auspices of the American Society for Testing and Materials (27). Low (350–860 PFU) and high (1,837–4,689 PFU) doses of a vaccine strain of poliovirus type 1 were seeded in 100-L samples of dechlorinated tap water at each laboratory. The water was adjusted to pH 3.5 and 0.0005 M $AlCl_3$. The filters were eluted with 1,600 mL of 3% beef extract-glycine (pH 9.0) or 0.05 M glycine (pH 10.5) and the eluate reconcentrated to smaller volumes by either organic flocculation (33) or by aluminum hydroxide hydroextraction method (3). All test samples were assayed in a single laboratory, where titers were also determined for the virus seed. With Viradel-organic flocculation method, the average virus recovery for low- and high-input experiments were 66% and 26%, respectively. With the Viradel aluminum hydroxide-hydroextraction procedure, the average recoveries were 20% and 11%. Considerable variation was noted in virus recovery from different laboratories, which may have been due to the length of experience of the people doing the test in a particular laboratory, water quality at different locations, and quality of the eluent used in different laboratories.

Wound fiberglass depth cartridge filters with a nominal porosity of 1 μm were evaluated as an inexpensive alternative to the 0.2-μm pleated cartridge filters (28). More than 99% of experimentally seeded poliovirus was adsorbed to these filters when the water was adjusted to pH 3.5 and 0.001 M aluminum chloride. In another study, Divizia and coworkers (29) compared filters from four different manufacturers (Sartorius, Millipore, Gelman, and Cuno) for the absorption elution of a cytopathic strain of hepatitis A virus (HAV) and found that both electronegative HAWP (Millipore) and electropositive (Cuno) filters absorbed HAV with high efficiency.

Macroporous glass (MPG-1,000 VGKh from Russia and SO1 from Czechoslovakia) and membrane filters (MF) (nitrocellulose PNT 0.45 from Russia, Millipore HAWP 0.45, Synpor 0.45 from Czechoslovakia, polycapromide MF Pall 0.2 from Germany, and FMPA 0.55 from Russia) were compared for the concentration of polio and SA-11 viruses (6). Radioactively labeled poliovirus and rabbit γ-globulin were used. Nitrocellulose filters (Millipore and PNT) proved to be superior in providing 64 to 90% virus sorption and 20 to 24% protein sorption. Elution experiments were conducted using solutions of different chemicals, for example, protein solutions (tryptosophosphate broth and beef extract), amino acid mixture (glycine + arginine), and chaotropic salts (sodium

trichloroacetate + lysine). Protein solutions were found to better elute rotavirus SA-11 from nitrocellulose filters and macroporous glass (MPG). The use of nitrocellulose filters and MPG as sorbents enabled 10 to 40-fold concentration of enteroviruses depending on the eluent used.

ELECTROPOSITIVE FILTERS

The use of electronegative filters requires the conditioning of test water to low pH and $AlCl_3$. To determine if viruses can be adsorbed on electropositive filters without the necessity of conditioning the test water, Sobsey and Jones (4) evaluated Zeta Plus filters (ZP60S) composed of diatomaceous earth-cellulose-"charge-modified" resin mixtures and having a net positive charge at pH 5.0 to 6.0. These filters efficiently adsorbed poliovirus from tap water at ambient pH levels (7.0 to 7.5) without added multivalent cation salts. The adsorbed viruses were eluted with glycine-NaOH, pH 9.5 to 11.5. Similarly, electropositive asbestos-cellulose filters were able to adsorb poliovirus from tap water in the absence of salts and at pH 3.5 to 9.0. However, the adsorbed virus could be eluted with 3% beef extract (pH 9.0) but not with glycine-NaOH (pH 9.5 to 11.5). Virus recoveries with Zeta Plus filters averaged 64 and 23% for one- and two-stage concentration procedures, respectively.

In a subsequent study, Sobsey and Glass (34) described the development of an electropositive pleated cartridge filter (Virozorb 1-MDS). Virus adsorption to these filters occurred in the absence of polyvalent cations and at pH 3.5 to 7.5. Beef extract in glycine (pH 9.5) was a more efficient eluent than glycine-NaOH (pH 11.0). Although poliovirus recovery from 1,000 L of tap water was similar (ca. 30%) to either Virozorb 1-MDS or with Filterite filters (adsorption at pH 3.5 and added $MgCl_2$), the former was much simpler to use (Fig. 2). In addition, electropositive filters gave less variable virus concentration efficiencies than did Filterite filters. Clearly, electropositive filters offer distinct advantages over conventional negatively charged filters for concentrating enteric viruses from water in that they are simple to use without sacrificing viral recovery.

In another study, both electronegative and electropositive filters were compared for the recovery of indigenous bacteriophages from water samples (35). Fiberglass and diatomaceous earth filters displayed low adsorption and recovery, but when treated with cationic polymers, these filters showed approximately 99% virus adsorption. For virus elution, the eluent was passed slowly through the filter, thus increasing the contact time between the eluent and the adsorbed virus. This elution method allowed a maximum phage recovery of 71% as compared with the 47% recovery obtained with the standard elution procedure.

In most of the studies, the 1-MDS filter was found to be efficient for virus concentration, except in a study by Alonso and coworkers (32), in which coliphages were concentrated from drinking water at a recovery rate of only 2%. However, the rate of coliphage recovery could be increased by including Tween 80 in the eluent. This is in agreement with the results obtained by Payment and coworkers (28) in which Tween 80 was shown to enhance the recovery of bacteriophages, bacteria, and parasites.

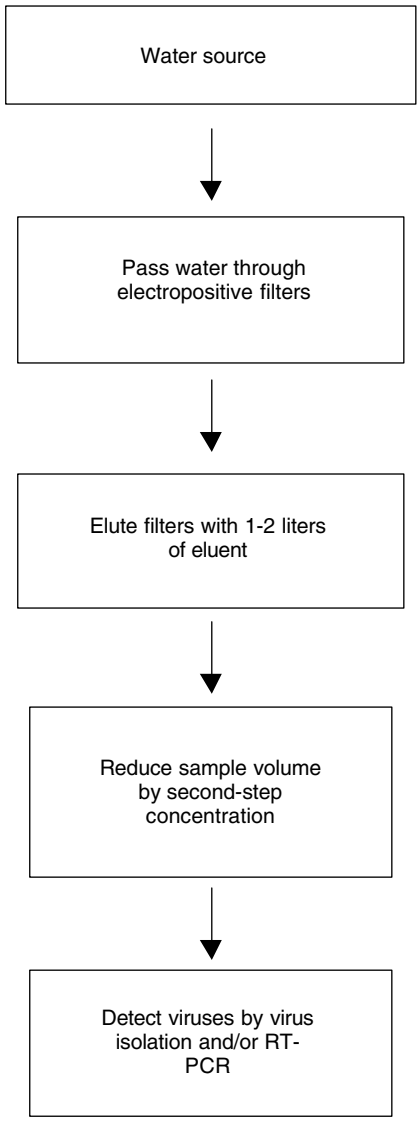

Figure 2. Schematic diagram of a virus adsorption-elution method using electropositive filters.

Another electropositively charged filter (MK filter) was evaluated against the 1-MDS filter to concentrate poliovirus type 1 (PV1) and coxsackievirus B3 (CB3) from 378-L samples of water (36). Adsorbed viruses were eluted from the filters with 3% beef extract buffered with 0.05 M glycine (pH 9.5) and reconcentrated via organic flocculation. At high virus inputs (10^6 PFU), the overall recovery of PV1 and CB3 from tap water with the MK filter was less than that achieved with the 1-MDS filter ($P < 0.05$). The average recoveries of PV1 with MK and 1-MDS filters in five trials were $73\% \pm 26\%$ and $90\% \pm 6\%$, respectively. The recoveries of CB3 in four trials were $33\% \pm 35\%$ and $96\% \pm 12\%$, respectively. Although the MK filter is less expensive than the 1-MDS filter, its use cannot be recommended because of consistently lower viral recovery.

In an effort to increase viral recovery rates, different eluents have been tested by different investigators (Table 4). Positively charged Zeta Plus filters were used to concentrate enteroviruses from 19 L of activated sludge effluent without the addition of salts and/or acidification of the effluent (30). Adsorbed viruses were eluted by treating the filters with a solution of 4 M urea buffered at pH 9 with 0.05 M lysine. The eluate was further concentrated into final volume of 1 to 2 mL by using a two-step reconcentration procedure that employed inorganic and organic flocculation. Approximately 50% of the viruses added to effluents could be recovered in the final sample. Jothikumar and Cliver (31) described the use of urea-arginine phosphate buffer (UAPB) for elution of coliphages adsorbed to positively charged filters. This method compared favorably with the beef extract elution-reconcentration procedure. The success of this method was attributed to testing the whole volume of concentrated sample, rather than partial analysis of the sample as described in standard methods for the examination of water and wastewater.

The use of Virozorb 1-MDS filters is not limited to enteroviruses alone. They have also been used for the concentration of infectious pancreatic necrosis virus from fish hatchery waters (37). The method consists of passing 100 L of hatchery water through 1-MDS filter followed by the elution of the adsorbed virus using a high pH buffer. The virus adsorbed efficiently to 1-MDS filters when the pH of the water was 5.5 and was eluted optimally with 3% beef-extract solution (pH 10). This procedure permitted the processing of 100 L of hatchery water that resulted in a 300-fold reduction in the volume of water and greater than 90% recovery of the seeded virus.

Rotaviruses were concentrated from 8 L samples of raw sewage and sewage-polluted creek water in Brazil using positively charged Zeta Plus filters (38), followed by ultracentrifugation of the filter eluate as second-step reconcentration. Rotaviruses were detected in the final sample by indirect immunofluorescence and direct immunoperoxidase assays. Using this method, rotavirus was detected in 6 of 29 sewage samples and in 19 of 55 creek-water samples. Samples examined during autumn and winter months showed a higher rate of rotavirus detection than those collected in spring and summer, corresponding to the seasonal variation of rotaviral diarrhea in the city of Sao Paulo.

Myrmel and coworkers (39) concentrated small round-structured viruses (SRSV), common cause of gastroenteritis worldwide, from 500 mL of deionized water. SRSV-containing fecal extracts were added to water and virus was recovered by filter adsorption-elution followed by organic flocculation. RNA was extracted and SRSV were detected by the use of reverse transcription-polymerase chain (RT-PCR) reaction. The sensitivity of the method corresponded to a positive SRSV detection at an estimated concentration of 0.5 to 5 virus particles per millimeter.

OTHER MEDIA

Many other media have been evaluated in an effort to find inexpensive alternatives to membrane filters (Table 1). Some of the more promising ones are pig erythrocyte membranes (erythrocyte ghost cells) (10), yeast cells (11), and inorganic salts ($CaHPO_4$) (12). Although large sample volumes cannot be processed by these methods, they may be useful as second-step reconcentration procedures. For example, erythrocyte ghost cells were used for the concentration of bacteriophages (ϕX174, MS-2, and f2) from 5 mL of saline solution (10). The adsorption and elution were carried out at pH 3.5 and 7.0, respectively. Bacteriophage adsorption on ghost cells was 93 to 100% and elution was 92 to 100%. With poliovirus, the rates of adsorption and elution were 100% and 91 to 129%, respectively.

Dahling and coworkers (5) placed a bituminous coal preparation between two prefilters and used this sandwich to recover viruses from large volumes of water. This filter was effective over a pH range of 3.0 to 7.0, and poliovirus recovery from 100 L volumes of tap water and domestic raw sewage was found to be similar to that obtained with Filterite and Millipore filters. Active aluminum oxide was used as an adsorbent for poliomyelitis virus type 3 and simian rotavirus SA-11 from sewage and drinking water (13). Optimum concentrations for effective adsorption of both rota- and polioviruses were 1.5 and 1 g/L, respectively, at ambient pH (pH 7.0 to 8.5) and at contact time of 30 minutes. Elution with 3% beef-extract solution (pH 8.5 to 9.5) was optimum for both viruses.

Farrah and coworkers (14) modified diatomaceous earth by in situ precipitation of metallic hydroxides, thereby decreasing the negative charge on the earth and increasing its ability to adsorb viruses. Filters containing diatomaceous earth modified by in situ precipitation of ferric chloride and aluminum chloride adsorbed greater than 80% of enteroviruses (poliovirus 1, echovirus 5, and coxsackievirus B5) and coliphage MS2 present in tap water at ambient pH (7.8 to 8.3), even after filtration of 100 L of tap water. Viruses adsorbed to the filters were recovered by mixing the earth with 3% beef extract containing 1 M NaCl (pH 9.0).

Papageorgiou and coworkers (15) developed a simple method in which there was no need for virus elution following adsorption to filters. They optimized conditions for the growth of Buffalo green monkey kidney (BGMK) cells on the entire surface of cellulose nitrate membrane filters in such a manner that the cells penetrated through the pores of the filter. When such conditions were used, poliovirus that had previously been adsorbed on the membranes infected the cells and replicated. In addition, this approach allowed plaque hybridization either directly on cellulose nitrate membranes or on Hybond N+ membranes after the preparations were transferred.

FACTORS INFLUENCING VIRUS CONCENTRATION

It is obvious from the preceding text that there is a high degree of variability from laboratory to laboratory when using membrane filters for the concentration and detection of enteroviruses from water and wastewater. Sobsey and Glass (40) studied the effect of various factors on virus concentration by both electropositive

and electronegative microporous filters. They seeded 1.3 L volumes of raw, finished, and activated carbon-treated waters with poliovirus type 1, echovirus type 1, reovirus type 3, and simian adenovirus SV-11. The samples were filtered through 47-mm diameter electropositive (Virosorb 1-MDS) filters at pH 7.5 or electronegative (Filterite) filters at pH 3.5 with and without 5 mM $MgCl_2$. The adsorbed viruses were eluted with 0.3% beef extract in 50 mM glycine of pH 9.5.

Prefiltration to remove particulate matter had no effect on virus recovery, but the presence of soluble organic compounds in raw and finished water was found to reduce virus adsorption to both types of filters. In the case of electronegative filters, this interference could be overcome by the addition of $MgCl_2$ to the sample being filtered. Elution of poliovirus and echovirus was much more efficient than that of reo- and adenoviruses. It was concluded that virus and water types play a significant role in recovery efficiency of any concentration scheme (40).

Lukasik and coworkers (41) studied the effects of different salts (NaCl, $MgCl_2$, and $AlCl_3$) on the adsorption of different viruses (MS2, PRD-1, ϕX174, and poliovirus 1) to commonly used microporous filters, for example, Millipore HA (nitrocellulose), Filterite (fiberglass), Whatman (cellulose), and 1-MDS filters. Both direct and indirect effects (changes in pH value of solutions following salt addition and the formation of insoluble precipitates that could adsorb viruses and be removed by filtration) were studied. The direct effects of added salts consisted of increase, decrease, or no effect on virus adsorption depending on filter type, virus type, and salt type. As reported in previous studies (3), the addition of aluminum chloride to water did enhance virus adsorption to microporous filters. However, this enhancement was due to indirect effects of salts (decrease in pH of water following the addition of aluminum chloride) rather than the direct effects (42). Increase in virus adsorption with aluminum chloride at pH 7.0 was considered to be due to flocculation of aluminum because prior removal of aluminum flocs by filtration did not enhance viral adsorption. The addition of multivalent salt and a compound that interfered with hydrophobic interactions (0.1% Tween 80 or 4 M urea) resulted in a drastic decrease in virus adsorption indicating that hydrophobic interactions are important in virus adsorption to filters. The type of water to be filtered and the possible release of toxic substances from the membrane matrix during filtration should also be considered (43) when evaluating a particular concentration method.

Table 5. Common Methods Used for Second-Step Concentration

Eluent Used	Reconcentration Method	Reference
Glycine	Aluminum hydroxide flocculation hydroextraction	3
Beef extract	Organic flocculation	33
Beef extract	Modified organic flocculation	20

SECOND-STEP CONCENTRATION

Many different methods have been evaluated for the reconcentration of filter eluates (Table 5). Kashkina and coworkers (9) described a two-phase system of water-soluble polymers for the concentration of measles virus. One of the polymers was polyethylene glycol (PEG) (MW 6,000), which was used with either dextran sulfate (DS; MW 500,000) or with dextran (D; MW 500,000). Virus concentration was found to occur in the interphase and lower phase. The concentration factors were 300 and 60 to 100 for PEG-DS and PEG-D system, respectively. Preformed magnesium hydroxide precipitate has been used for second-step concentration of viruses present in the first eluate (performed with 50 mM glycine buffer, pH 11.5)(44). The viruses were adsorbed on a preformed magnesium hydroxide precipitate. After low-speed centrifugation, the viruses were eluted (desorbed) with McIlvaine citrate-phosphate buffer. Under these conditions, 90% of the viruses present in the 300 mL of the first eluate were reconcentrated in a final volume of 40 mL. The recovery efficiency was independent of either virus concentration or water quality.

Enteroviruses added to 114 L of dechlorinated tap water were recovered in a 16 mL sample by a two-stage concentration procedure in which different types of membrane filters were used in each concentration stage. Viruses in tap water at pH 3.5 were first adsorbed to Filterite filters and then eluted with 0.2 M sodium trichloroacetate solution buffered at pH 9 with 0.2 M lysine. Viruses in this solution were adsorbed to 47-mm asbestos filters (Seitz) without pH adjustment or other modification of the solution. Viruses were recovered from the Seitz filters with 16 mL of either Casitone or fetal calf serum at pH 9. With these procedures, approximately 45% of several types of enteroviruses added to 114 L of tap water could be recovered in the final 16-mL sample (45).

A second-step concentration procedure using iron oxide was developed for the detection of rotaviruses in water (46). Samples (378-L) of estuarine water adjusted to pH 3.5 and 0.001 M $AlCl_3$ were filtered through Filterite filters of 3.0- and 0.45-μm porosity. Adsorbed virus was eluted with 1 L of 10% tryptose phosphate broth, at pH 9.5. Primary eluates were reconcentrated to a final volume of 10 to 20 mL by magnetic iron oxide adsorption and elution procedure. A 2% solution of casein at pH 8.5 effectively eluted rotavirus from iron oxide. Using this method, rotaviruses were detected in water, suspended solids, fluffy sediments, and compact sediments at different locations within the Galveston Bay, Texas.

A modification of the organic flocculation was described (47) in which bacteriophages and enteroviruses in water were adsorbed to positively charged 1-MDS filters or Seitz S filters. Adsorbed viruses were eluted with 10% beef extract, at pH 9. Organic flocculation of the beef extract at pH 3.5 permitted recovery of more than 40% of the enteroviruses but less than 15% of the bacteriophages. A method was developed that used salts at pH 7 to flocculate beef extract. Two volumes of saturated ammonium sulfate were added to beef extract, and both enteroviruses and bacteriophages were adsorbed to the flocs that formed.

More than 70% of the enteroviruses and bacteriophages were recovered by centrifuging the sample and suspending the flocs in a small volume of distilled water.

Addition of Tween 80 to a 1.5% solution of beef extract was found to enhance the elution of bacteriophages adsorbed to electronegative filters (48). When reconcentration of the eluate was attempted by ammonium sulfate precipitation, a floating layer containing most of the viruses was formed. This floating layer could be obtained with several nonionic detergents including Tween 80 and, under a salt saturation of 55%, with ammonium sulfate, potassium tartrate, and sodium phosphate. Virus recovery ranged from 91 to 103% and was obtained with several bacteriophage strains. With poliovirus type 1, coxsackievirus B-4, and rotavirus SA-11, the recoveries were 100%, 20%, and 80%, respectively, but toxicity to cell culture was encountered. After removal of the detergent by a second floating-layer method, the recovery was 32% for poliovirus. Compared with organic flocculation, this method provided improved recovery for bacteriophages.

Hollow-fiber ultrafilters have been used to remove viral contaminants from fluids. During ultrafiltration, virus concentration in the feed continues to increase because the retentate is returned to the input reservoir, although the permeate is removed to a separate vessel. Wallnerova and Simkova (12) suggested the use of the Amicon apparatus for ultrafiltration of water samples. Later, Divizia and coworkers (49) evaluated a molecular filtration system for recovering hepatitis A (HAV) and polioviruses seeded into 1 L of dechlorinated tap water. A polysulfonate membrane of 10,000 molecular weight was used. Under these conditions, HAV recovery was 100% of the input, but the percentage was reduced dramatically when the inflow pressure was increased. In contrast, poliovirus recovery was low under standard conditions, but improved when membranes were pretreated with different buffers. The best recovery was obtained using beef extract at neutral pH.

Recently, Oshima and coworkers (50) tested hollow-fiber ultrafilters with different molecular weight cutoffs to remove and detect poliovirus and phages T1 and PP7 from ultrapure water. Ultrafilters with molecular weight cutoffs of 13,000 and 6,000 were found to be very effective in removing small virus particles (25 to 30 nm) by size exclusion. In a subsequent study, they developed modified polyvinylidene fluoride (PVDF) membrane filters and tested them for the removal of different viruses from ultrapure water (51). Small volume (10 mL) filtration experiments were conducted with 47-mm disks, whereas larger volume experiments (1 L) were conducted with cartridge filters (surface area of 1.63 m2). Influenza A virus and phage T1 were removed to less than detectable limits with titer reductions of grater than 2.0×10^6 and greater than 5.8×10^8, respectively. The results of cartridge filter experiments were similar to those of 47-mm disks. Efficient virus retention suggests potential applications of this filter for second-step reconcentration of filter eluates.

A method consisting of a concentration step by tangential flow filtration (TFF) system, ultrafiltration by centrifugal concentrator, and visualization by transmission electron microscopy (TEM) has been described (52). With this method, 2 L volumes of seawater were reduced to 10 to 20 μL, which could then be dispensed on electron microscopy grids to count total viral particles. Recovery rates varied depending on both the viral morphology and flow rate. At low flow, 117% and 61% of T6 and φX174, respectively, were recovered.

Vortex flow filtration (VFF) was used to concentrate viruses and dissolved DNA from freshwater and seawater samples (53). Recoveries of T2 phage and calf thymus DNA added to artificial seawater and concentrated by VFF were 73% and 80%, respectively. Virus concentrations determined by transmission electron microscopy of VFF-concentrated samples ranged from 3.4×10^7/mL for a eutrophic Tampa Bay sample to 2.4×10^5 for an oligotrophic oceanic surface sample from the southeastern Gulf of Mexico. Viruslike particles were also observed in a sample taken from a depth of 1,500 m in the subtropical North Atlantic Ocean. Filtration of samples through Nucleopore or Durapore filters (pore size 0.2 μm) before VFF resulted in a reduction of phage counts.

MOLECULAR METHODS OF VIRUS DETECTION

Until recently, virus detection was done by virus isolation in cell cultures. The problems with this approach are that some sample concentrates are cytotoxic, not all viruses grow in cell cultures and the method is time consuming and costly. To overcome these problems, molecular diagnostic techniques have been introduced in environmental virology laboratories, the method of choice being the reverse transcription-polymerase-chain reaction (RT-PCR). Even fastidious viruses that do not grow in cell cultures, can be detected by this technology. The drawbacks of this technique are that sample concentrates may sometimes contain inhibitors of PCR reaction, PCR is unable to differentiate between infectious and noninfectious viruses, and its use is limited to sophisticated laboratories.

The development of a nucleic acid hybridization test for the detection of hepatitis A virus (HAV) in estuarine samples was described (54). The sensitivity was limited to approximately 10^4 physical particles of HAV per dot and was affected by hybridization stringency, ^{32}P energy level, probe concentration, presence of humic acid, and nucleic acid binding to filters. No cross-hybridization with other enteroviruses and unrelated nucleic acids was observed. Potential false-positive reactions between bacterial DNA in samples and residual vector DNA contamination of purified nucleotide sequences in probes were eliminated by DNase treatment of samples. Similarly, interference by organic components of virus-containing eluates was removed by proteinase K digestion, followed by phenol extraction and ethanol precipitation.

A major advance in the use of molecular techniques was made by the development of a reverse transcriptase (RT)-polymerase chain reaction (PCR)-oligoprobe (OP) or (RT-PCR-OP) method for the detection of Norwalk virus (55) that has the potential of being used for the detection of this virus in both clinical and environmental samples. Primers

were used for the amplification of RNA polymerase (260-bp product) and a putative immunogenic protein (224-bp product). The resulting DNA fragments (amplicons) were hybridized to a digoxigenin-labeled internal OP specific to each amplicon. This method was 100-fold more sensitive than radioimmunoassay (RIA). In addition, this method was specific for Norwalk virus and did not detect human and animal caliciviruses, hepatitis E virus, Snow Mountain agent, astroviruses, 16 human enteroviruses, and 5 human rotaviruses. Norwalk virus was detected in 4 of 21 coded fecal specimens that were also positive by enzyme immunoassay.

The RT-PCR technique was later used to detect hepatitis E virus (HEV) in sewage (56). HEV in sewage samples was concentrated through adsorption to membrane filters, elution with urea-arginine phosphate buffer, and subsequent reconcentration with magnesium chloride. HEV-specific cDNA was prepared by reverse transcription of the total RNA extracted from samples. Specific DNA amplification by PCR in combination with slot-blot hybridization was used to demonstrate the presence of HEV in sewage samples from the inlets and outlets of three sewage treatment plants. The assay was specific for HEV, and a 240-bp amplified product was visualized by ethidium bromide fluorescence. Sewage samples adjusted to pH 5.0 for adsorption of viruses to membrane filters were PCR positive, whereas samples adjusted to pH 3.5 were PCR negative.

Puig and coworkers (57) described the use of a nested PCR for the detection of entero- and adenoviruses and found that extraction by adsorption of nucleic acids to silica particles was the most efficient. Using this procedure, they were able to detect 24 different enteroviruses and 47 human adenovirus serotypes with a sensitivity of 1 to 10 virus particles. When 25 samples of sewage and polluted river water were analyzed, nested PCR detected more positive samples than virus isolation in cell cultures.

To determine RT-PCR compatibility of sample concentrates obtained by conventional filter adsorption-elution methods, Schwab and coworkers (58) used beef extract-glycine eluates, with or without humic acid, and seeded them with poliovirus type 1. These mock eluates were further processed by polyethylene glycol precipitation, Pro-Cipitate precipitation, a second polyethylene glycol precipitation, spin column chromatography, and ultrafiltration. The sample volumes were reduced from 1 L to 20 to 50 μL. Detection by RT-PCR was compared with cell culture infectivity procedures. As little as 3 PFU of poliovirus in an initial 1 L of mock eluate was detected by RT-PCR.

To increase the sensitivity of enteric virus detection in tap water concentrates, poliovirus 1 and coxsackievirus B3 were seeded into 378 L of tap water, concentrated with 1-MDS filters, and reconcentrated by organic flocculation (59). RT-PCR was successful in detecting viral nucleic acid following the removal of PCR inhibitory substances from water concentrate. Direct phenol-chloroform-isoamyl alcohol (PCI) extraction of viral RNA was found to be sufficient to remove inhibitory substances for RT-seminested PCR with a sensitivity of 0.2 plaque-forming units per 10 μL (0.2 PFU/L tap water).

Nucleic acid amplification by RT-PCR and confirmation by oligonucleotide probe hybridization was evaluated for the detection of poliovirus type 1 (PV1) or hepatitis A virus (HAV) in oyster extracts (60). For this purpose, seeded viruses in oyster extracts were purified by fluorocarbon extraction and concentrated by polyethylene glycol (PEG) precipitation and elution. A protein-precipitating agent, Pro-Cipitate (Affinity Technology, Brunswick, N.J.), was used in an adsorption-elution precipitation scheme to reduce sample volume to 100 μl. Virus detection by RT-PCR and cell culture propagation were comparable and correlated well with replicate samples and at different virus titers.

Schwab and coworkers (61) developed a broad-spectrum immunocapture method for indirect antibody capture (AbCap) of intact viruses, release of virion genomic RNA, RT-PCR for RNA amplification, and oligoprobe hybridization for virus detection. Enteric viruses were concentrated from large volumes of water by standard filtration-elution techniques using 1-MDS filters and 1 L of 1% beef extract-0.05 M glycine as an eluate. The eluate was concentrated and purified by polyethylene glycol (PEG) precipitation, Pro-Cipitate precipitation, and a second PEG precipitation to a volume of approximately 500 μL. Aliquots of the second PEG precipitate were further processed by RNA extraction, AbCap, or cell culture analysis for infectious viruses. The AbCap method was applied to 11 field samples of fecally contaminated surface water. Of the 11 samples, 9 were positive for enteric viruses by AbCap method, 4 were positive by direct RNA extraction of a small aliquot of the second PEG concentrate, and 4 samples were positive in cell cultures.

The PCR technique was also evaluated for the detection of enteric viruses in groundwater (62). An improved sample processing technique and a large-volume amplification protocol were used. Viruses were detected after concentration from 1,512 L of water by a filter adsorption-elution method. When 150 samples were analyzed, only 13 (8.7%) were positive in cell cultures, whereas 40 (30.1%) tested positive by RT-PCR. The authors believed that their sample-processing technique and large-volume PCR protocol (reaction volume, 300 μL) were responsible for removal or dilution of inhibitors so that more than 95% of the samples could be assayed by PCR.

Greening and coworkers (63) developed a sensitive, nonisotopic microtiter plate hybridization assay for the detection of enteroviruses in environmental samples. Following reverse transcription, viral cDNA was labeled with digoxigenin (DIG)-dUTP during the PCR amplification step. The labeled PCR products were hybridized with enterovirus-specific biotinylated oligonucleotide probe and captured in streptavidin-coated microtiter wells. Hybridized enteroviral PCR products were detected by an antidigoxigenin peroxidase conjugate using either a colorimetric or a chemiluminescent substrate and automated measurement. The chemiluminescent assay was found to be more sensitive than the colorimetric assay for the detection of poliovirus, and was specific to enteroviruses.

A multicentric study was conducted in three laboratories to evaluate the efficiency of a standardized kit for

the detection of enterovirus genome in wastewater (64). Twenty-one samples of 20 L of wastewater were analyzed before and after concentration through glass wool. Each sample was analyzed with the Amplicor kit and with techniques developed independently in each laboratory. The results showed that the Amplicor kit was well-suited for the detection of the enterovirus genome in treated wastewater and that the results were comparable to those obtained with seminested RT-PCR. In one study, Grabow and coworkers (65) compared three different cell types (L20B mouse cells, PLC/PRF/5 human liver cells and BGM cells) and RT-PCR for the detection of human enteric viruses in environmental samples. Of the 319 samples tested, 263 failed to produce cytopathic effects in either of the three cell lines but were positive by RT-PCR.

Chapron and coworkers (66) collected 29 surface-water samples using the Information Collection Rule method consisting of filter adsorption-beef extract elution and organic flocculation. Two methods were compared for virus detection in these concentrates, for example, (*1*) an integrated cell culture-RT-PCR (ICC-RT-PCR) coupled with nested PCR to detect human astroviruses, enteroviruses, and adenovirus types 40 and 41 and (*2*) total culturable virus assay-most-probable-number (TCVA-MPN) method as recommended by the U.S. Environmental Protection Agency. Twenty-seven of the 29 samples (93%) were positive by the ICC-RT-PCR method, whereas only 5 samples were positive by the TCVA-MPN method. Primers based on the helicase region of the genome of the small round-structured viruses (SRSVs) (Norwalk-like viruses) were found to be useful in detecting SRSVs in water (67).

INHIBITORS OF RT-PCR

From the foregoing account, it is clear that RT-PCR is more sensitive than virus isolation. However, it does suffer from the disadvantage that sample concentrates may sometimes contain substances that inhibit PCR amplification of target RNA. Several investigators have addressed this issue. Sephadex G-200 gel chromatography has often been used to remove PCR inhibitors (55,68). Virus concentration by adsorption onto glass powder or glass wool has been used to detect viral genomes in treated wastewater by seminested PCR (7). No enterovirus genome was detected directly in 25 samples of treated wastewater by seminested PCR but 48% and 56% of the samples concentrated by adsorption onto glass wool or glass powder, respectively, were positive for viral genome. Secondary concentration by organic flocculation of samples concentrated by glass wool resulted in a decrease in the number of RT-PCR positive samples (from 48–20%). Secondary concentration of glass wool concentrates, on the other hand, resulted in an increased recovery (from 56–72%).

Myrmel and coworkers (69) described a simple method for the preparation of Norwalk-like virus (NLV) RNA from environmental samples. They coated magnetic beads with polyclonal antibodies against a recombinant capsid protein of genogroup I NLV and used these beads for immunomagnetic separation (IMS) of NLV. Viral RNA was heat released and detected by RT-PCR. The combination of IMS and heat release was found to be more efficient than organic extraction of RNA from water contaminated with humic acids.

Lewis and coworkers (70) investigated the effects of clay, humic acid, ultraviolet light, and shellfish tissue residues on the detection of poliovirus type 2 by cell culture and RT-PCR. The latter technique was found to be 10 to 100 times more sensitive than culture in the absence of sample contaminants. The addition of 100 to 1,000 mg/L of bentonite clay and shellfish tissue residues reduced virus detection by plaque assay. Similarly, bentonite clay, humic acid (5 to 150 mg/L) and mussel tissue reduced virus detection by RT-PCR (between 1 and 8 logs), although this was mitigated in part by elution and Sephadex filtration of extracts. As expected, the exposure of virus in water to ultraviolet light reduced culturability of the virus but not detection by RT-PCR.

CONCLUSION

It is clear from this and other reviews that membrane filters are the media of choice for adsorption-elution of viruses from large quantities of water. Electropositive filters are simple and easy-to-use because preconditioning of water with acid and salt is not necessary. Molecular methods of virus detection in concentrated samples have proven useful and more progress is being made in this area. However, the problems associated with molecular methods (detection of infectious and noninfectious viruses, presence of RT-PCR inhibitors in the sample concentrates) will need to be worked out before these methods become even more popular for virus detection.

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ENTEROVIRUSES: OCCURRENCE AND PERSISTENCE IN THE ENVIRONMENT

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Evaluation of the viral contamination in the environment is subject to many variations inherent in the analysis methodology, relating particularly to the techniques used for virus concentration and to the virus material to be tested. *Enteroviruses* can be detected in the stools of patients, in environmental samples by isolating infectious virus particles on cell cultures and also by detecting the viral genome using molecular biological techniques such as the reverse transcription-polymerase chain reaction RT-PCR.

The use of RT-PCR to detect *Enterovirus* in water has several benefits over cell culture technique. It has the potential to improve detection limits as well as reducing processing times and cost. It is a sensitive and specific method but a number of outstanding problems can seriously affect its performance. For example, the enzymes used during the RT-PCR reaction can be inhibited by a number of environmental contaminants such as humic acid. Furthermore, the great sensitivity of PCR makes it susceptible to false-positive results because of contamination. Finally the detection of *Enterovirus* genome using RT-PCR is not the proof of the presence of infectious *Enterovirus*.

If attempts are made to detect infectious virus particles in cell cultures or their genome by molecular biological techniques like RT-PCR, the results for the same sample of water may differ substantially. For example, Puig and coworkers (1) found 31% of the samples to be positive when detecting infectious *Enterovirus* particles and 75% of the samples to be positive when testing for the enteroviral genome.

Viral contamination of the environment is a natural phenomenon associated with the discharge of human and animal feces, particularly into water systems. An individual infected with an *Enterovirus* whether or not he has symptoms, may excrete up to 10^6 cell culture infectious units per gram of feces. Nevertheless, the stool probably contains 10 to 100 times more viral particles (2). Given that humans produce a mean of 100 to 200 g of feces per day, approximately 10^8 *Enterovirus* particles may be released per day by an infected individual. It is logical for the enteroviral concentration to be very high in the raw wastewater that constitutes the natural receptacle for feces. However, it is important to bear in mind that the density of *Enterovirus* in wastewater varies as a function of many geographic, socioeconomic, seasonal, and, particularly, sanitary factors. Thus, the lower the level of hygiene and the larger the proportion of children in a community, the larger the number of *Enteroviruses* found in the wastewater. Indeed, children are a preferred target for these viruses. Dahling and coworkers (3) observed that the level of *Enterovirus* contamination of wastewaters was higher in Puerto Rico (a region with a very high population density and a low socioeconomic level) than in other locations in the United States, with peak concentrations of *Enterovirus* reaching more than 10^5 plaque forming units (pfu) per liter.

Similarly, the effects of other geographic and climatic factors are not negligible and it has long been known that *Enterovirus* infections show a predominance in summer and autumn in countries with temperate climates.

The diagram of the enteroviral transmission in the environment is given Figure 1.

Studies have been published concerning the level of contamination of raw wastewater (4,5). On average, 90 to 100% of raw wastewater samples contain infectious *Enterovirus*, at a mean concentration of 10^2 to 10^3 pfu/L.

In many parts of the world, these waters are discharged into the natural environment without treatment. However, in industrialized countries, they receive physicochemical or biological treatment. This treatment has two practical consequences: it improves the microbiological quality of the water discharged and reduces the amount of biologically degradable organic matter, unfortunately it also leads to the production of large quantities of residual sludge that must be disposed.

The residual sludge resulting from the treatment of wastewater contains significant numbers of *Enteroviruses* because wastewater results in their transfer to the sludge. The density of these viruses in sludge depends on the nature of the treatment process. The mean virus content of sludge is very variable, but primary sludge generally contains 10^3 to 10^4 pfu/kg of dry matter. This sludge may be used as agricultural fertilizer, discharged, or incinerated.

Viral contamination of treated wastewater has also been extensively described in the literature (6,7). Clearly, the concentration of *Enteroviruses* depends on the type of treatment applied to the raw wastewater and the initial level of contamination. The mean concentration of *Enteroviruses* following biological treatment by activated sludge is generally 0 to 10^2 pfu/L. On release from treatment plants, the number of viruses discharged into surface waters is therefore far from negligible.

In surface waters, as a result of dilution, viral concentrations rarely exceed 10 pfu/L and the frequency of samples in which infectious *Enteroviruses* may be detected is between 0 and 50%. In contrast, the enteroviral genome may be detected in as many as 30 to 100% of samples. However, in these waters, as in the environment as a whole, the presence of the *Enterovirus* genome does not constitute proof of the presence of infectious *Enteroviruses*. For example, Pallin and coworkers (8) detected the *Enterovirus* genome in 25 to 92% of seawater samples whereas no infectious *Enteroviruses* (cell culture) were isolated from the same samples.

It is also worth pointing out that most of these studies were carried out in areas likely to be polluted by waste from human activity and that these percentages therefore are not representative of surface waters as a whole. In any case, the majority of the *Enteroviruses* may be adsorbed to suspended matter and are therefore likely to sediment or to be deposited in fluvial or marine sediments.

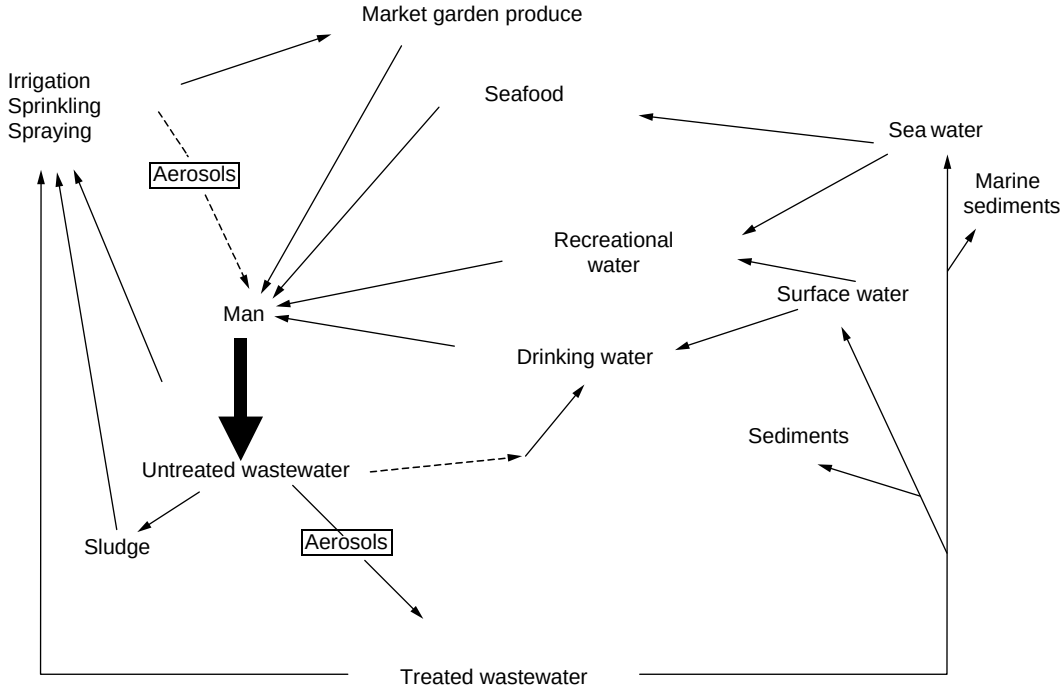

Figure 1. Diagram of the enteroviral transmission in the environment.

The superficial layer of very light readily resuspended sediments constitutes a potential source of contamination remote from the source (Fig. 2).

Viruses usually isolated in larger numbers from sediments than from the overlaying water, and values of 10^2 pfu/kg are not unusual (7,10).

If shellfish-producing areas become contaminated, the shellfish, which feed by filtering large quantities of water, may retain, and even concentrate, the virus in their digestive tract. This bioaccumulation occurs very rapidly (within a few hours) and is very efficient. Digirolamo and coworkers (11) have reported factors of concentration of up to 32 for *Poliovirus 1* in oysters. The extent of this bioaccumulation depends on the type of shellfish, its physiological state, the temperature of seawater, the type of virus, its concentration in seawater, and above all, its state, free or adsorbed. In fact, viral adsorption onto supports (feces, kaolinite, unicellular algae and so on) increases the rate of accumulation (12). In most cases, the contamination of shellfish is correlated with the discharge of raw or treated wastewater in the shellfish-producing zone. For example, infectious *Enteroviruses* and the enteroviral genome have been detected in shellfish raised in seawater into which raw wastewater, primary treated wastewater,

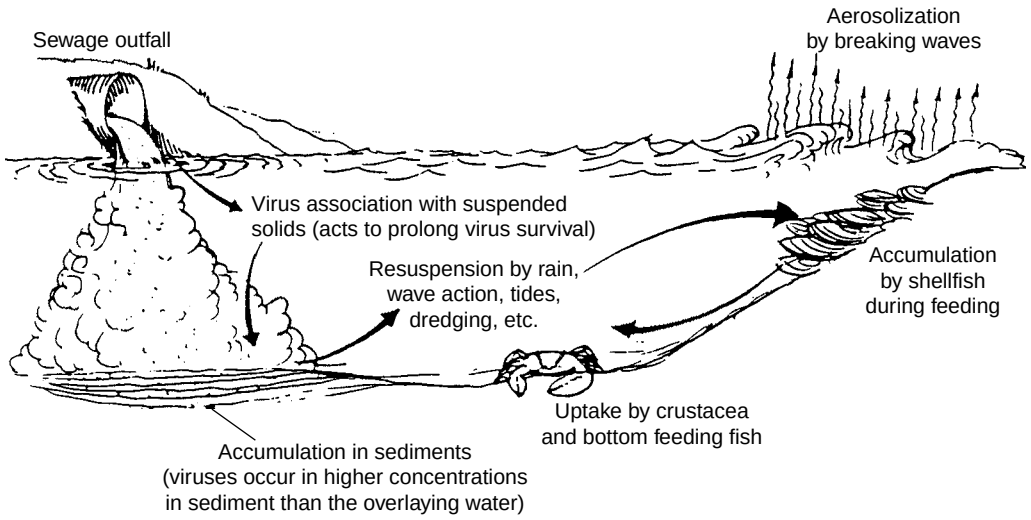

Figure 2. Fate of viruses in coastal systems (from 9).

and secondary treated wastewater have been discharged. The mean concentration in shellfish was 12 pfu, 0.3 pfu, and below the detection threshold, respectively, per 10 g of shellfish (13). But even in the last case when no infectious *Enteroviruses* were isolated, the enteroviral genome was detected in 100% of the samples. The *Enterovirus* genome has been reported to be present in molluscs on numerous occasions (7,10,14), often in the absence of infectious *Enteroviruses*. It should also be pointed out that in some cases, infectious *Enterovirus* and, more frequently, the *Enterovirus* genome, have been detected in shellfish meeting bacteriological standards for shellfish growing areas.

Wastewater (raw or treated) may be used for land irrigation and sludge spreading on fields. Infectious *Enteroviruses* and their genome have been detected in fields to which sewage sludge has been applied. Viruses have been isolated from groundwater situated vertically below fields irrigated with wastewater. Goyal and coworkers (15) isolated *Enteroviruses* in wells, at a depth of 27.5 m. In contrast, Moore and coworkers (16) did not isolate any viruses in well water while observing a site irrigated with wastewater but found *Enteroviruses* in the water of a lysimeter, at a depth of 1.37 m.

Infectious *Enterovirus* and enteroviral genome were recovered, respectively, from 3.4% and 9.2% from 303 groundwater samples (1,700 l) (17). Abbaszadegan and coworkers isolated infectious *Enteroviruses* from 8.7% of groundwater samples (1,512 l) while 30.1%, 8.6%, and 13.8% of these samples were positive for the genomes (RT-PCR) of *Enteroviruses* hepatitis A virus, and *Rotaviruses* (18), respectively.

Aerosols can form while wastewater is being treated, particularly during the activated sludge phase and when irrigation is carried out using spraying techniques. When spraying is carried out with a sprinkler, 0.05 to 1% of the liquid is transformed into aerosols. It is generally thought that 30% of the particles suspended in the aerosol could be inhaled because their size varies between 0.2 μm and 5 μm. It is therefore possible that after inhalation these particles penetrate the respiratory system and that the largest (2 μm) are evacuated by ciliary action and pass into the digestive system. If these particles contain viruses, the latter can cause infection. *Enteroviruses* have been detected in air samples taken near an activated sludge treatment plant (19). Teltsch and Katzenelson (20) found *Enteroviruses* between 40 and 100 m downwind from fields irrigated by spraying with wastewater. Similarly, Shuval and coworkers (21) have observed that 12.5% of air samples taken 730 m downwind from land irrigated by lagooned wastewater contained concentrations of *Enteroviruses* varying between 0.03 pfu and 1.94 pfu/m^3.

Finally, very little information exists on the contamination of market garden produce irrigated with wastewater. However, Bagdassaryan (22) has reported the isolation of *Enteroviruses* on irrigated vegetables but the quality of the wastewater was not indicated.

PERSISTENCE OF THE VIRUS IN THE ENVIRONMENT

Enteroviruses are obligatory intracellular parasites, and as such they can only multiply within the host cells. In the environment, the concentration of *Enteroviruses* can therefore only decrease, or at worst, remain constant. The fate of viruses therefore depends on the effects of physicochemical (temperature, adsorption onto suspended matter, salinity, pH...) and biological (inactivating substances of microbial origin...) factors in the environment. All of these factors may act separately or in conjunction and it is difficult to separate the various factors in complex environments.

Influence of Physicochemical Factors

Temperature. In the environment, viruses are inactivated by the combined effects of various environmental factors. However, many studies carried out on natural surface water (e.g., sea, river, lake) and on soils have shown that high temperatures are of primary importance in viral inactivation. An increase in temperature leads to a change in the capsid conformation and thus loses the ability to interact with the receptors of the target cell. Furthermore, it is well known that the attachment of the virus to the target cell via these receptors is the first step in viral infection and is essential for all viral infections.

Thus, increase in temperature initially affects the infectivity of the virus without causing lesions in the viral genome. This is supported by studies showing that the *Poliovirus 1* genome can be detected in the phosphate buffer after heating to 75 °C for 15 minutes, whereas infectious virus particles are no longer detected after 15 minutes at only 56 °C (23). Similarly, but at a much lower temperature (37 °C), Enriquez and coworkers (24) observed a large decrease in the infectivity of *Poliovirus 1* (5 log) after incubation in the phosphate buffer for 21 days, whereas in the same conditions a decrease of only 1.5 log was recorded for the genome after 75 days.

Adsorption. Viral adsorption also has an significant effect on the fate of viruses in the environment. Generally, viruses behave like colloidal particles with a large capacity for adsorption to surfaces of all kinds. There are many potential adsorbents in the environment including clays, suspended solids and other colloids present in the water, microorganisms, soils (in cases of wastewater irrigation), shellfish and so on. In the environment, viral adsorption depends on many factors including the nature and concentration of the salts and adsorbents present, the viral serotype, the nature and concentration of the organic matter present and so on (25,26). For example, pH plays a fundamental role. The surfaces of viruses are covered with ionizable groups, the charge of which may be modified as a function of pH. The overall charge is positive if the pH is below the pH_i (the isoelectric pH at which the overall charge of the virus is zero) and negative if the pH is above the pH_i. Most *Enteroviruses* have a pH_i between 4 and 8.2. These electrical charges, which vary with pH, enable the virus to adsorb to surfaces of opposite charge or to desorb from supports with the same charge.

The effect of salts on adsorption is also important and it has been clearly shown that both the nature and concentration of salts in solution play key roles in

determining the level of interaction between the virus and the surface. Salts may reduce the surface potential of the virus and of the adsorbent surface, thereby decreasing the repulsion between these two entities. This makes it possible for the virus particles to approach one another and to create hydrophobic and electrostatic interactions (27). It is generally accepted that when viruses come into contact with an adsorbent support, adsorption occurs rapidly and extensively. For example, 30 to 50% of *Poliovirus 1* particles adsorb to montmorillonite clay in deionized water in less than 30 minutes, but more than 99% were adsorbed in the presence of salts or in seawater (28,29). In most environmental conditions, a very large proportion of *Enteroviruses* may be adsorbed or aggregated.

This capacity for adsorption is extremely important because it enables the virus to accumulate in sediments, shellfish, and soils and may protect the *Enterovirus* against inactivating environmental factors. However, against heat, it only provides significant protection in the presence of high concentrations of suspended matter or if the temperature increase is very significant. Thus, Quignon and coworkers (29) and Gantzer and coworkers (28) reported that the presence of less than 500 mg/L of suspended matter did not affect the survival of *Poliovirus 1* at temperatures below 35 °C in sterile media. In contrast, Liew and Gerba (30) observed 1 log inactivation after 30 minutes in artificial seawater at 50 °C in the presence of marine sediments (8 g/L), whereas they observed 3 log inactivation in the absence of sediments. In fact this protection is only observed at temperature $\geq$35 °C; the temperature rarely exceeds 30°C in the environment. Protection from the inactivating of biological and other physical and chemical factors may be more important in natural waters (i.e., sunlight).

pH. The effect of pH on the survival of the virus depends largely on the type of virus. *Enteroviruses* are stable over a large range of pH, from 3 to 9, and possibly even higher, for short periods. Piirainen and coworkers (31) demonstrated that *Poliovirus* remains infectious after treatment for two hours at pH 2 at 37 °C but is inactivated after 24 hours by the same treatment. Most *Enteroviruses* are inactivated by pH greater than 11 (32).

However, in the environment, it is very difficult to demonstrate a correlation between pH and the level of inactivation because certain molecules affect both the pH and the survival of the virus. For example, ammonia, which increases pH, is toxic to *Enteroviruses* disrupting their nucleic acids (33).

Salts. The effect of salinity on the survival of viruses has been demonstrated only in very precise temperature conditions. Wetz and Kucinski (34) showed that *Poliovirus 1* was stable for one hour at 20 °C in an hypotonic buffer but was rapidly degraded at 37 °C. However, at temperatures below 34 °C, closer to those in the environment, Quignon and coworkers (35) demonstrated that neither the nature (KCl, CaCl2, or FeCl3) nor the concentration of salts affected the survival of *Poliovirus 1*. The observed effect thus results from the combined action of high temperature and low salt concentration, destabilizing the viral capsid.

Conflicting results have been obtained concerning the possible correlation between salinity or conductivity and the inactivation of the virus in the environment because of the differences in the type of salt present and because some salts may have a negative effect on the virus (virucidal), whereas others may have a positive effect (e.g., protective effect by increasing adsorption or aggregation, or by thermostabilization).

Many studies have investigated the effects of seawater salinity on the survival of *Enteroviruses* and their conclusions differ. Katzenelson (36) and Babich and Stotzky (37) found that salinity affected the inactivation of *Enteroviruses* whereas the authors found that salinity (14, 24 and 33 NaCl g/l) had no effect on the inactivation of *Poliovirus 1* in sterile water containing no suspended solids (38).

Sunlight. Since viruses are sensitive to inactivation by ultraviolet light they are more readily inactivated in the presence of sunlight. Hurst (39) reported that, in the absence of sunlight, the level of inactivation was between 0.7 and 0.8 log per day, whereas in sunlight, the level ranged from 1.3 log per day in very turbid water to 2.4 log per day in water that was only slightly turbid. Thus, sunlight inactivates viruses but this inactivation is less efficient if the water contains suspended solids as such solids both protect the adsorbed virus particles and hinder the penetration of ultraviolet light in the water column.

Influence of Biological Factors

Biological factors seem to affect the persistence of Enteroviruses in natural waters, but their nature and mode of action are not clear. Overall, it has been clearly demonstrated that more Enteroviruses survive in sterile water than in natural water. This difference in survival may be due, in particular, to the presence of biological factors that have demonstrated virucidal activity. Girones and coworkers (40) isolated marine bacteria belonging to the genus Moraxella, which had antiviral activity against *Poliovirus 1*. Similarly, Ward and coworkers. (41) isolated 27 bacterial strains with virucidal activity, notably against Enterovirus, from surface waters. Some of these virucidal biological factors are heat-sensitive. Enriquez and coworkers (24) observed a 4 log reduction in the concentration of *Poliovirus 1* in wellwater after 13 days at 37 °C, however, 19 days at this temperature were required to achieve the same level of inactivation in autoclaved water. It has also been shown that *Poliovirus* genome (RNA) is less stable in natural water than in the same water that has been autoclaved (120 °C, 20 minutes). Ward and coworkers (41) also showed that the addition of a protease inhibitor markedly decreased the virucidal activity of bacterial origin in water. This suggested that enzymes, particularly proteases (degrading the capsidal proteins) and ribonucleases (degrading the genome), may play a role in inactivating viruses in the environment. The factors that may influence persistence in soil and in water are summarized in Table 1.

Table 1. Factors That May Influence *Enterovirus* Survival in Water and Soil (from 42)

Factors	Comments
Temperature	Survival decreases with increasing temperature
Desiccation	One of the most detrimental factors; increased virus reduction in drying soils
Sunlight	May be detrimental at the soil and water surface because of ultraviolet (UV) light
pH	May indirectly affect virus survival by controlling their adsorption to soils and to suspended matter
Cations	Certain cations have a thermal stabilizing effect on viruses; may also indirectly influence virus survival by increasing their adsorption to soil (viruses appear to survive better in the sorbed state) and to suspended matter
Soil texture	Clay minerals and humic substances increase water retention by soil and thus have an impact on desiccation
Biological factors	No clear trend with regard to the effect of soil microflora on viruses; some evidence of virucidal activity by water microflora

Taking into account all these parameters it is obvious that the survival of *Enteroviruses* is largely variable in environment. Some example of T90 (time in which the initial viral concentration decreases by 90%) for infectious *Enterovirus* are given in Table 2 but it should be kept in mind that even in similar media, *Enterovirus* survival is dependant on the physicochemical and biological characteristics of each environment.

Table 2. *Enterovirus* Survival in Environment

Environmental Media	Temperature	T90 (days)	References
Raw wastewater	25 °C	30 days*	43
Primary effluent	4 °C	20 days**	
	15 °C	17 days**	44
Secondary effluent	4 °C	23 days**	
	15 °C	12 days**	
Septic tank mixed with swine manure slurry	21 °C	19 days	45
	14 °C	30 days	
Oxidation tank	18–23 °C	23 days	45
Primary sludge	23 °C	26 days	46
	2 °C	180 days	
Seawater	25 °C	6 days*	47
	15 °C	9 days*	44
	18–21 °C	6 days	48
River water	23–27 °C	1 days	49
		1–5 days***	50
Groundwater	25 °C	25 days*	43
Vegetables	4 °C	2 days	51
Soil (clay loam)	15 °C	16 days	52
	27 °C	7.5 days	

*Estimated from experimental point.
**Estimated from T99.
***Estimated from T99.9.

Influence of Wastewater Treatment

Wastewater contains large numbers of *Enteroviruses* and other pathogenic microorganisms. Therefore, in industrialized countries, they are subjected to treatment aimed at reducing their level of contamination before discharge into the environment.

Wastewater may be treated using various processes aimed at eliminating chemical (e.g., phosphate, nitrate) or biological (e.g. organic pollutants), but also pathogenic microorganisms. Some procedures involve a simple physical treatment but the most commonly used process involves four steps (Fig. 3):

The elimination of *Enteroviruses* during the treatment of wastewater clearly depends on the type of treatment. It is generally recognized that preliminary treatments have only a small impact on the concentration of viruses in wastewater, whereas the primary, secondary, and tertiary treatments have greater effects.

Primary Treatment

Primary treatment makes the reduction possible by simple sedimentation of "settleable solids," thereby also eliminating the *Enteroviruses* absorbed onto them. The reduction in virus concentration achieved by such treatments is very variable (10 to 80%), but is always less than one logarithmic unit (54). The magnitude of the decrease depends on the level of adsorption of the viruses onto settleable solids.

Biological Treatments

Biological treatments of wastewater were not originally designed specifically to eliminate pathogenic microorganisms, their original goal being the degradation of organic matter. The activated sludge process is based on stimulation of the degradation of organic matter by the bacterial biomass. Wastewater rich in nutrients and bacteria is subjected to forced aeration, which enables the bacterial population to multiply more rapidly. This bacterial population then consumes the carbon- and phosphorus-containing nutrients present in the wastewater. In a second step, bacteria flocs are eliminated from the liquid phase by sedimentation.

During this treatment, there is a transfer of viruses, from the water phase to a solid phase. The *Enteroviruses* present adsorb onto the formed flocs and sediment with them to constitute the sludge. The adsorption of viruses onto bacterial flocs is rapid and substantial. Farrah and coworkers (55) estimated that in the presence of activated sludge, between 0.7 and 1.8 log of *Enteroviruses* are adsorbed. However, *Enteroviruses* are also inactivated by substances with virucidal activity produced by the bacterial flora. It is in this environment that virucidal biological factors have most frequently been identified. The theoretical rate of inactivation of *Enteroviruses* in activated sludge is between 0 and 2 log in 48 hours (56). However, as the time spent in an activated sludge basin is on the order of 6 to 12 hours, the true level of inactivation is low.

Some studies have shown that protozoans and metazoans play a significant role in the elimination of viruses

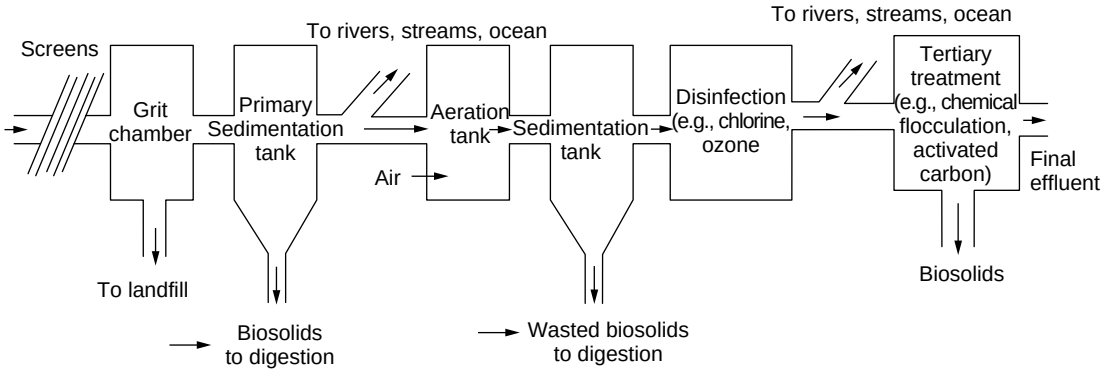

Figure 3. Flowchart of a wastewater treatment plant (from 53).

— Preliminary treatment to remove oils, sands, and large debris
— Primary treatment, generally involving simple sedimentation of suspended solids
— Secondary treatment such as activated sludge, trickling filters, oxidation ponds...
— Tertiary treatment that usually involves some type of physical and chemical treatment and disinfection.

by predation (57), but the real impact of this factor is, however, difficult to quantify.

Finally, during this type of treatment the combination of adsorption, inactivation, and predation may result in a 90 to 99% decrease in the number of *Enteroviruses* initially present in raw wastewater. The same is true for treatments involving biological filters and oxidation tanks.

The efficiency of water purification by lagooning varies from 0 to 99.9% and depends in particular on the duration of the treatment and of temperature (58).

Disinfection

In some cases, the decrease in the levels of pathogenic microorganisms achieved by biological treatment is not sufficient to satisfy current standards required for shellfish production area, bathing zones, or in case of agricultural water reuse. Then specific treatments that mostly involve disinfection are implemented (59).

Disinfection generally involves the use of chlorine (60), ozone (61), and ultraviolet irradiation (61). The inactivation of *Enteroviruses* by oxidants is a complex phenomenon, the efficiency of which depends on many factors including the aggregation or adsorption state of the virus, the nature of the oxidant, the pH, and the oxidant demands of the suspending medium. This last factor is very important because the medium itself reacts with oxidants as a result of the presence of organic matter and reduces the quantity of free disinfectant that can act on the viruses. It is therefore necessary to distinguish between the theoretical disinfecting capacity in buffered water, with no oxidant demand, and actual disinfecting capacity in wastewater.

Chlorine. Three types of chlorine compounds are generally used: free chlorine (Cl_2), chloramines (NH_2Cl), and chlorine dioxide (ClO_2).

The results obtained with buffered solutions with no oxidant demand clearly show that chlorine is an effective virucidal agent. In the presence of 1 mg/L of free chlorine, 99 to 99.9% of *Poliovirus 1* particles are inactivated within 1 minute (43,62).

In wastewater, the organic matter present reduces the virucidal capacity of chlorine because it is rapidly transformed into monochloramines (or other organic and inorganic chloramines) by reaction with ammonia and other nitrogenous compounds. In these conditions, it is not surprising that Behac and coworkers (60) obtained an inactivation of only 50% of *Poliovirus 1* in wastewater in 15 minutes, with 6.6 mg/L residual chlorine.

Chloramines have a much lower level of virucidal activity than free chlorine. In the presence of 2 mg/L of monochloramines, it requires almost three hours to reduce the level of *Poliovirus 1* to 90% in a medium with no oxidant demand (63).

Chlorine dioxide is a much more powerful oxidant. In a buffered medium, 99% of *Poliovirus 1* is inactivated in 10 minutes in the presence of 1 mg/L of chlorine dioxide. However, in effluent, it is necessary to almost double the dose to obtain the same decrease (64).

In the case of free chlorine and chloramines, inactivation is greatest at pH 6, the predominant form being HOCl. With chlorine dioxide, inactivation is maximal at high pH (around 10).

The effects of chlorine are all given with respect to infectious *Enteroviruses*. If detection of the viral genome is taken into account, significant differences are observed with respect to the results obtained by detection of infectious viruses. For example, in the presence of 1 mg/L free chlorine in water without oxidant demand, the reduction of infectious *Poliovirus 1* is between 2 and 3 log in one minute, whereas it is only 0 to 1 log for the genome (43,65). The same phenomenon is observed with chlorine dioxide and chloramines (63).

Ozone. Ozone is an excellent virucidal agent. In general, in sterile buffered media, more than 99% of Enteroviruses are inactivated in less than one minute (66,67) in the presence of 0.05 to 0.15 mg/L

residual ozone. However, as for chlorine, the presence of organic matter or suspended matter interferes with disinfection. In addition, the disinfectant action of ozone is affected by the pH of the medium. Roy and coworkers (68) showed that *Poliovirus 1* was inactivated more rapidly at pH 7.2 than at pH 4.3.

In wastewater, ozone is naturally less virucidal and thus the reduction of virus is smaller. For example, Harakeh and Butler (69) observed 80% inactivation in 10 to 15 minutes in the presence of 0.2 mg/L ozone.

Comparison of the decrease in levels of infectious *Poliovirus 1* and of its genome shows, as for chlorine, a great difference in behavior between the two entities. The infectivity of the virus is destroyed much more rapidly than the viral genome. Moore and Margolin (70) reported the total inactivation of *Poliovirus 1* in deionized water within 10 minutes of ozone treatment, whereas a significant decrease in the level of the viral genome was not detected until after 60 minutes.

Ultraviolet Irradiation. Photoinactivation is a natural phenomenon that has been known for some time to affect suspensions of Enterovirus exposed to sunlight. In phosphate buffer, Battigelli and coworkers (71) observed that a dose of 25 $mW\,s^{-1}\,cm^{-2}$ reduced the concentration of Coxsackie B5 virus by at least 3 log. In natural water samples, the efficiency of UV irradiation for disinfection depends on suspended matter content. They protect microorganisms and act as a barrier against UV irradiation.

A clear difference is observed if the fate of infectious *Poliovirus* and of their genome are compared following UV treatment. In distilled water, for a dose of 22 $mW\,s^{-1}\,cm^{-2}$, a reduction of 5 log is observed for infectious virus and only of 1 log for the viral genome (62). These results suggest that UV irradiation has an effect on the viral genome and does not prevent detection of the genome by RT-PCR.

Influence of Sludge Treatment

Wastewater treatment always produces very large quantities of sludge (i.e., biosolids), which normally undergoes physical (dewatering, heating...), chemical (lime treatment...), and microbiological (aerobic or anaerobic stabilization, composting...) treatments. In most countries, most of the treated sludge is applied to agricultural land. Therefore, to avoid any transmission to humans, it is important to understand the fate of *Enteroviruses* during sludge treatment and disposal.

Aerobic and Anaerobic Digestion. Aerobic and anaerobic digestion can be carried out at psychrophilic (low temperature), mesophilic (25 °C to 45 °C), and thermophilic (>45 °C) temperatures. The differences in viral inactivation achieved by such processes are linked essentially to differences in temperature. The level of viral reduction is greatest for thermophilic digestion, followed by mesophilic digestion and psychrophilic digestion.

Other parameters, such as the concentration of oxygen or ammonia, water content, and the presence of proteolytic enzymes may also affect viral inactivation. Psychrophilic digestion is little used and the reductions in viral contamination reported for this technique are small. For example, Martin and coworkers (72) obtained a 0.7 log reduction after 20 days of treatment at 8 °C.

Mesophilic stabilization should generally continue for at least 15 to 20 days. Nevertheless, very diverse results have been reported because it is necessary to take into account climatic data and the large variations in temperature associated with them. Reductions in contamination of between 0.5 and 3 log units have been reported for anaerobic digestion at 35 °C for 15 to 20 days (73,74,75). During aerobic digestion, Martin and coworkers (72) used a regression model to estimate that the reduction achieved in 20 days was 1 log at 18 °C, 1.1 log at 24 °C, 1.3 log at 26 °C, and greater than 3.2 log at 38 °C.

Finally, thermophilic stabilization can be considered to be an effective treatment to eliminate all *Enteroviruses* (76) provided that the sludge is continually mixed in the presence of air or oxygen, for 10 days at 55 to 60 °C. The reduction in contamination obtained is smaller if lower temperatures or shorter treatment times are used. There is generally little difference in the reduction of viral contamination obtained with aerobic and anaerobic thermophilic treatments at identical temperatures. At temperatures above 50 °C, heat is the principal inactivating factor. Generally speaking, 2 to 4 log *Enterovirus* reduction is obtained using these processes.

During anaerobic digestion at 49 °C for 20 days the reduction of *Enteroviruses* is 2.5 log (75), but if it is a two stage process (55 °C for two days and 37 °C for 10 days), the reduction is 4 log (77). In aerobic conditions, 4 log reduction is obtained at 55 °C for 28 days (78).

Composting. American legislation considers that composting as a treatment is able to eliminate *Enteroviruses* from sludge if the temperature is maintained at over 55 °C for three days with forced aeration, or at more than 55 °C for 15 days if successive turning techniques are used. The principal problem with this process is heterogeneity, particularly in terms of the temperature within the compost. This may result in the preservation of zones at a lower temperature in which the virus is able to survive. It is therefore not surprising that the results obtained are very variable (1.2 log to >4 log) despite theoretical temperatures of 60 to 70 °C for seven days (79).

Lime Treatment

Lime treatment is usually realized by addition of lime to the sludge. Such treatment increases both pH (quick and slaked lime) and temperature (quick lime). The increase in pH depends on the quantity of lime added to the sludge. The decrease of viral density is about 0.3 log at pH 8.3, 3 log at pH 10.8, and may be as much as 7 log at pH 12.5 (79,80). Finally, Hurst (58) reported reductions in contamination of 1 log in 0.47 days, four days, and seven days for the addition of 5, 3, and 1.5 kg of lime/m^3, respectively. The increase in temperature seems to be

highly variable depending on moisture and the quantity and quality of lime added.

Dehydration and Heat Treatment

Dehydration techniques are used to reduce the humidity of the sludge to various degrees and it was demonstrated there is a loss of viral infectivity proportional to the level of dehydration (81).

To conclude, heating to high temperatures (130 to 180 °C for 30 minutes) guarantees that the resulting sludge is free of *Enterovirus*.

CONCLUSION

Enteroviruses, which are excreted in large numbers in infected human stools, are currently detected in all raw wastewater.

The application of classical biological treatments significantly decreases the level of viral contamination, but, depending on the initial concentration in raw wastewater, the number of *Enteroviruses* released into the environment may be far from negligible. Therefore, in some cases, a tertiary disinfection treatment (chlorine, UV, ozone) may help to protect some environmental areas from pathogen contamination.

However, whether or not wastewater is treated, enteroviral contamination should naturally decrease from surface to seawater as a result of simple dilution. The persistence of these viruses in the environment depends on the physical and chemical characteristics of the medium.

Thus, the diversity of potential viral reservoirs in the environment, constituted not only by water (wastewater, river water, seawater, and, possibly, groundwater) but also by shellfish, which concentrate the virus in their tissues, and high levels of viral persistence, clearly result in a large potential for human contamination by *Enteroviruses*.

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ENTOMOPATHOGENS (BACTERIA, FUNGI, PROTOTOZOA, VIRUSES).

See INSECTICIDES, MICROBIAL

ENVIRONMENTAL GENOMICS.

See SOIL GENETIC ECOLOGY

ENVIRONMENTAL RELEASE OF MICROORGANISMS.

See FIELD RELEASE OF GENETICALLY ENGINEERED MICROORGANISMS (GEM)

ENZYMATIC TOXICITY TESTS.

See TOXICITY TESTING IN SOIL, USE OF MICROBIAL AND ENZYMATIC TESTS

ENZYME INHIBITION.

See SOIL ENZYMES

ENZYME KINETICS.

See SOIL ENZYMES

ENZYMES, ARCHAEAL.

See ARCHAEA IN BIOTECHNOLOGY

ENZYMES: BIOTECHNOLOGICAL APPLICATIONS

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While perhaps the most visible and profound consequence of the biotechnology revolution has been our increased knowledge of fundamental life processes and the development of novel medicines, the most pervasive use of recombinant DNA (rDNA) technology has been in the production of manufactured goods. Everyday you are using, wearing, or eating something that has been touched by industrial enzymes produced using biotechnology. For example, have you had a soft drink or a piece of candy or cake made with high-fructose corn syrup? Have you worn any denim garments today? Have you washed your clothes with laundry detergent this week? All of these products have been made possible by the advent of modern biotechnology through the use of biotechnologically derived industrial enzymes. The increasing use of enzymes to replace more traditional chemical transformation processes in these and many other industries is driven by a desire for better production economics, new product functionalities, improved workplace safety, and an increasing desire by companies to reduce the environmental impact of their operations. Often chemical transformations produce nonspecific reactions that may result in poor product yields and by-products that may prove difficult and costly to dispose. High temperatures and/or pressures needed to drive reactions lead to high energy costs and may require large volumes of cooling water downstream. Harsh and hazardous processes involving organic solvents and acidity or alkalinity at high temperatures and pressures need expensive, specially designed equipment and control systems. All of these drawbacks can potentially be eliminated using enzymes. Public, regulatory, and private industrial forces are all fueling a conversion to greener alternatives. Industrial enzymes fit this category, as enzymes do their work, become inactive, and break down into simple, nontoxic, natural components. They are increasingly being used in industrial process development and manufacturing, thereby circumventing the harsh chemical and toxic loads often put into the environment by older manufacturing methods. To meet this increasing demand for enzymes, most new enzymes are produced from genetically engineered fungal or bacterial organisms grown in large-scale fermenters. In this article, the current art of industrial enzyme production and the application of those enzymes to manufacturing processes are described.

INDUSTRIAL ENZYME PRODUCTION

Biotechnology of Enzyme Production

Historically, industrial enzymes have been made by simply fermenting an organism and recovering the fermentation broth (Fig. 1). In fact, the world's first commercialized extremophile enzyme, Esperase® (Novo Nordisk A/S), a detergent protease with pH activity range from pH 7 to 12, was developed in 1974 as a fermented *Bacillus* sp. product. Simply fermenting organisms is still done in selected cases today, as it is the mixture of enzymes in the final product that holds the desired activities. For example, a mixture of endoglucanases, cellobiohydrolases, xylanases, and hemicellulases in a product called Novozyme 342® (Novo Nordisk A/S), which is a fermented broth from *Humicola insolens*, is used in the de-inking process in paper recycling; a mixture of endo- and exoproteases in a product called Flavourzyme® (Novo Nordisk A/S), which is a fermented broth from *Aspergillus oryzae*, is used for protein hydrolysis, debittering, and flavor enhancements in vegetable and animal protein hydrolysis. In the future, this approach of "just fermenting" the organism and using the fermentation broth is expected to continue as a minor contributor to the overall source of industrial enzymes.

Today it is recombinant DNA technology that is the rule rather than the exception when it comes to producing industrial enzymes (Fig. 2). It is estimated that approximately 80% of the turnover of industrial enzymes comes from genetically modified organisms (1). Recombinant enzymes are derived primarily from extracting the gene of interest from the prokaryotic or eukaryotic source, using DNA probes based on the genetic code from primary amino acid sequence information of the purified enzyme. Alternatively, the gene encoding the desired enzyme may be chemically synthesized. In either case, the enzyme-encoding gene is then inserted into a recombinant host, such as the widely used fungal strains *Aspergillus oryzae* or *A. niger* or *Bacillus* sp. for bacterially derived enzymes (2,3). Using a wide variety of DNA expression vectors tailored for high-level expression, the active enzymes are secreted into the growth media when these organisms are fermented.

A number of techniques have been developed and are utilized to tailor enzymes for specific industrial applications. Random mutagenesis, in which the gene encoding the enzyme is mutated without regard to any structural information, results in enzyme mutants with a wide variety of amino acid substitutions, some of which may prove beneficial in a given application. When an enzyme structure is known, this information can be used to target specific amino acids using site-directed mutagenesis to improve enzyme performance in a process called protein engineering. Both approaches are typically done in an iterative manner, combining steps of mutation and screening; at each round, screening and selecting out the best protein-engineered variant for a given application and using it as a starting point for another round of mutagenesis. More recently, an improved method termed *directed evolution* has been developed that more closely resembles natural protein evolution. In this process, improved mutants selected from screening a naturally occurring or mutated pool of enzyme genes are recombined to create random chimeras that "shuffle" mutations between different enzyme mutants. This is superior to iterative mutagenesis techniques because it

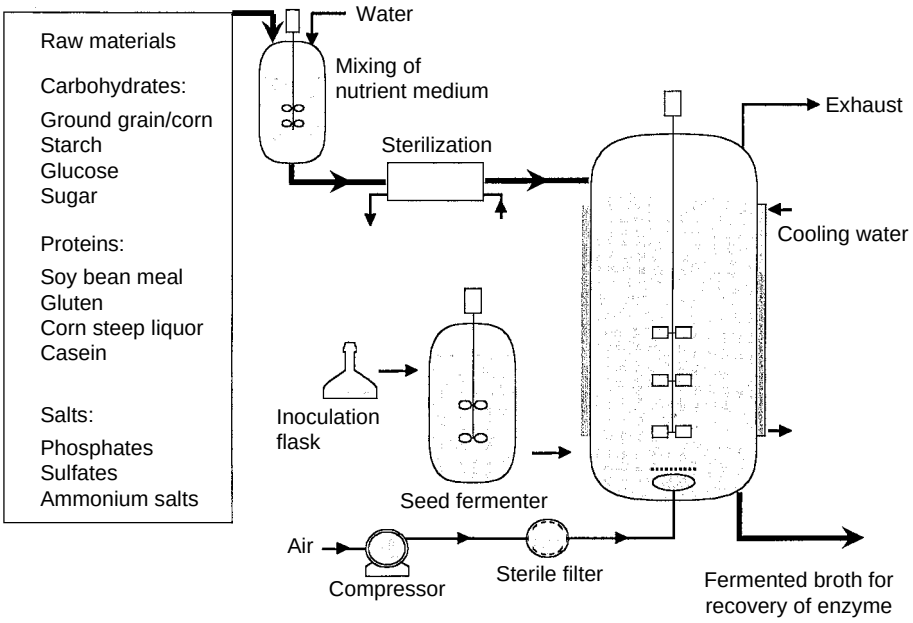

Figure 1. Schematic of large-scale industrial fermentation for enzyme production.

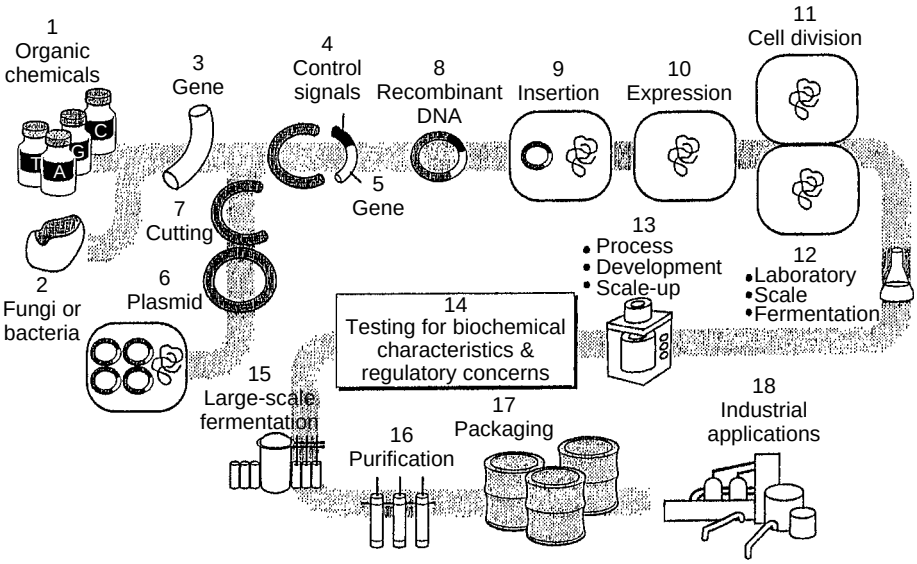

Figure 2. Flowchart of recombinant protein production showing steps in the synthesis, cloning, and expression of a heterologous gene into a production bacteria or fungus.

provides a mechanism to randomly separate beneficial and deleterious mutations (4–6). Screening is then used to identify those mutants with the optimum combination of beneficial mutations.

There are numerous examples of protein-engineered lipase, protease, amylase, glucose isomerase, cellulase, and other enzyme variants produced in small quantities by academic laboratories (7). There are a smaller, but growing number of protein-engineered and protein-evolved enzymes commercially produced (Table 1). As techniques for generating and screening mutants become more advanced, enzymes capable of catalyzing nearly any reaction under nearly any condition will be developed, the limitation being only the ability to set up a relevant screening system for the desired activity. Using such techniques, one may supplant the need for extensive environmental screening for the desired enzyme with the "proper" or "optimal" characteristics.

Enzyme Fermentation

The vast majority of industrial enzymes are produced by large-scale submerged fermentation. This involves growing a selected microorganism in closed vessles in which all the conditions critical for growth are carefully controlled: pH, temperature, feed in the form of carbon and nitrogen sources, and oxygen. The selected microorganisms are either bacteria (typically *Bacillus* sp.) or fungal (typically *Aspergillus* or *Trichoderma* sp.) that have been carefully chosen and optimized for secreted protein production. Almost all enzymes of any commercial consequence are secreted directly from these organisms into the growth medium, where they can be readily

Table 1. Commercially Produced Protein Engineered Industrial Enzymes

Enzyme	Mutagenesis Method	Advantages
Durazyme® protease	Site-specific	Enhanced storage stability in laundry detergents
Lipase Ultra®, Lipase	Site-specific	Enhanced fat-removing in laundry detergents
Guardzyme® peroxidase variants	Random, site-specific, directed evolution	Enhanced thermal and oxidative stability
Amylase	Site-specific	Reduced calcium dependence in laundry detergents

Note: The examples given are all products of Novo Nordisk A/S, Bagsvaerd, Denmark.

recovered by removal of the organism using filtration or centrifugation. As an example of the extreme productivity of this method of producing proteins, *T. reesei* has been reported to secrete some cellulase-degrading enzymes at up to 40 g/L of fermentation broth (8).

Formulation

Formulation often is a critical step in commercial production because it can stabilize the enzyme, create time-release characteristics, and allow for improved safety and ease in handling. Enzymes recovered from fermentation broth are usually concentrated by evaporation, membrane filtration, or crystallization, depending on the enzyme and its intended application. Crude enzyme can then be formulated into granulate powders or liquids, or immobilized on an inert support. As an example, enzymes used in the last step of high-fructose corn syrup production, converting glucose to fructose, are immobilized and packed in a column that can function effectively for over a year. To provide a stable, nondusting enzyme for the detergent industry, enzyme granulation is a critical step. A number of methods have been developed and patented over the years, but most granule production today is performed either in a fluidized bed or in a high-speed mixer followed by a controlled drying process. Normally, both these types of granules will also be coated by a wax or polymer to reduce dust release from the product and to protect the enzyme from other detergent components such as bleaching compounds.

THE ENZYME MARKET

The estimated worldwide industrial enzyme market in 2000 is valued at approximately $1.5 billion (1) and is traditionally divided into three segments: the largest at 73% of sales, technical enzymes include enzymes used in the detergent, starch, textile, leather, pulp and paper, and personal care industries; food enzymes, the second largest segment at 22% of the market, include dairy, brewing, wine and juice, fats and oils, and baking industries; and feed enzymes, covering enzymes used in animal feeds, contribute approximately 5% of the market (Table 2). Proteases comprise the largest selling and largest volume enzyme currently sold, primarily to the detergent industry as a stain-removing additive.

While enzymes are widely used in industry, they make up only a small percentage of the overall chemical market. This is due to a number of factors including, (*1*) the economics of the enzyme manufacture — where price competition with the competing chemical technology requires enzymes to be made in the multigram per liter amounts in the fermentation process; (*2*) the lack of understanding in many industry segments of enzymology — knowledge about basic chemical mechanisms and how enzymes might be used to solve problems is limiting in many applications-oriented fields; (*3*) the difficulties in incorporating the enzyme into many "older" manufacturing processes — capital intensive equipment and the "old way of doing things" prevents a transition to enzyme use; and, (*4*) finding the right enzyme — each industrial process has its own conditions for operation and it is important to find the best enzyme for each application. With this background, several examples are discussed where enzymes are being used, and where they might be used in the near future, to provide a more environmentally friendly solution to industrial processes.

TECHNICAL ENZYMES

Laundry Detergents

Detergents represent the single largest industry segment for the use of today's enzymes, representing about 38% of the total enzyme market. Enzymes used in detergents today include both bacterial- and fungal-derived proteases, amylases, cellulases, lipases (9,10), and the newly introduced mannanase (11); others are in the R&D pipeline. The single largest selling industrial enzymes are the detergent proteases. These bacterial, subtilisin-type proteases are one of the basic workhorses in detergents today and are considered indispensable in formulating efficient laundry detergents worldwide. As catalysts, each molecule of enzyme cuts the protein components of laundry stains thousands of times during a wash cycle, making them very space-efficient ingredients that are excellent at removing blood, grass, milk, and various foods containing proteins. In addition to the wild-type enzyme, a number of protein-engineered variants of subtilisins have entered the marketplace, with such properties as increased stability toward oxidation and increased cleaning performance.

Alpha-amylases have proven useful in catalyzing the degradation of starch stains and in improving overall soil removal by hydrolyzing the starch that binds particulate soil to the fabric. Using both proteases and amylases,

Table 2. Enzymes Sold Commercially in the Three Industry Segments, with a Short Summary of What Function the Enzyme Performs in the Process or Product

Industry Segment	Enzymes	Enzyme Function
	1. Technical Enzymes	
• Detergents	• Lipases, proteases, cellulases, amylases, mannanases	• Degradation of protein, starch, and fatty stains in laundry • Color clarification and softening of cotton laundry • Automatic dishwashing • Surfactant production • Deep cleaning
• Starch and sugar	• Amylases, pullulanases, glucose isomerase, xylanases • Cyclodextrin glucanotransferases	• Liquefaction and saccharification for the production of dextrose, fructose and special syrups for the baking, confectionery, and soft drink industries, among others • Cyclodextrin production
• Textile	• Amylases, cellulases, catalases, laccases	• Polishing of cotton fabrics • Stonewashing of denim garments • Degumming of silk • Bleach cleanup • Removal of starch from woven materials • Bleaching of denim
• Leather	• Proteases, lipases	• Soaking of hides and skins, unhairing, bating and defatting
• Pulp and paper	• Xylanases, mannanases	• Control of pitch problems caused by the use of mechanical pulps • Reduction of chlorine consumption in pulp bleaching process • Viscosity control in starch-based coatings
• Fiberboard production	• Laccases	• Free radical crosslinking of wood fibers
• Personal care	• Protease, lipases • Catalase • Lactoperoxidase, glucose oxidase, and glucoamylase • Laccases	• Removal of protein and oils from dentures, contact lenses • Removal of residual H_2O_2 from contact lenses • Oligosaccharide removal in toothpaste • Oxidation of aromatics for hair dying • Antimicrobial activity in toothpaste
• Organic chemical synthesis	• Isomerases, lipases, reductases, acylases	• Biocatalysis under mild conditions
• Alcohol	• Amylases, pullulanases, proteases, cellulases	• Degradation of starch or cellulose into sugars which are converted to alcohol through fermentation
	2. Food Enzymes	
• Baking	• Amylases, xylanases, lipases, proteases, glucose oxidases	• Reduction in bread staling • Starch, pentosan and protein modification for improvement of the baking properties. • Improvement of crust color, dough handling, stability and quality
• Dairy	• Proteases, lipases, lactases	• Cheese production, curdling of milk • Conversion of lactose in milk and whey into sweeter, more easily digestible sugars • Flavor development in specialty cheeses
• Brewing	• Amylases, beta-glucanases, alpha-acetolactate decarboxylases, proteases, pullulanases	• Degradation of starch, protein, and glucans when brewing with a combination of malt and unmalted raw materials, e.g., barley, corn, and rice • Shorten beer production time • Production of low carbohydrate beer

Table 2. (*Continued*)

Industry Segment	Enzymes	Enzyme Function
• Wine and Juice	• Pectinases, beta-glucanses, cellulases, hemi-cellulases, pullulanases, amylases, alpha-acetolactate decarboxylases, laccases	• Degradation of pectin when manufacturing fruit juices, wine, etc. • Clarification • Improved filtration • Viscosity reduction • Enzymatic peeling • Inhibition of taints in corks
• Fats and oils	• Lipases, phospholipase	• Modifications of fats and lecithins and synthesis of esters
• Food functionality	• Endoproteases, exoproteases, carbohydrases, cellulases, hemi-cellulases, phospholipases	• Improvement of nutritional and functional properties of animal and vegetable proteins • Flavor and texture improvements • Process of optimization, e.g., energy savings by lowering of viscosity • Hydrolysis of phospholipids • Reduction of starch viscosity
	3. Feed Enzymes	
• Animal feed	• Endoproteases, exoproteases, lipases, phytases, cellulases	• Degradation of feed components for improvement of feed utilization and nutrient digestion • Improved palatability

the overall performance of the detergents is increased at lower washing temperatures and there is a decrease in the detergent's overall chemical makeup.

Cellulases can be used in lieu of cationic surfactants (fabric softeners) because they improve the softness of cotton fabrics. Cellulases are also active in removal of particulate soil by removing microfibrils from the cotton fibers. As a result, there is a color-brightening effect of the cellulase treatment. Lipases catalyze the hydrolysis of triacylglycerides present in fatty stains, making them more hydrophilic and more easily removable during the wash.

Enzymes used in detergents have a positive environmental impact because they save energy by reducing washing temperatures; allow the content of undesirable chemicals in detergents to be reduced; are totally biodegradable, leaving no harmful residue; have no negative impact on sewage treatment processes; and, most importantly, do not present a risk to aquatic life. Chemicals eliminated or reduced by the use of enzymes in detergents include phosphates, surfactants, carbonates, silicates, and chlorine bleach.

Automatic Dishwashing

Automatic dishwashing detergents have traditionally contained high concentrations of silicates (sodium metasilicate) as high pH buffers, phosphate builders (sodium tripolyphosphate), and chlorine (sodium dichloroisocyanurate) as bleach. Safety, environmental factors, and performance concerns have shifted manufacturers to begin using enzymes in automatic dishwashing detergents (12). Phosphates were causing eutrophication of surface water; silicates, while being added to minimize corrosion of metals and ceramic dish surfaces, were too alkaline and posed a health threat in some European countries if ingested; and chlorine-based chemicals were reacting with organic compounds. Enzyme use, such as with proteases, lipases, and amylases, has thus lowered the requirement of these chemicals, generating "greener," safer, and higher performing automatic dishwashing detergents.

Starch

One of the most well-known applications of enzymes is in the starch processing industry for the production of high-fructose corn syrup (Fig. 3) (13). Maize starch is the most widely used raw material used, followed by wheat, tapioca (cassava), and potatoes. Starch is composed of long linear polymers of α-1,4 linked glucose with a minor number of oligoglucose branches linked α-1,6. To make the starch contained in these feedstocks available for enzymatic hydrolysis by amylase, it is pressure-cooked until a gel is formed. Liquefaction of the starch is accomplished by the addition of thermostable alpha-amylases, most of which are protein-engineered, that are added before the gelatinization. The resulting solubilized starch, composed of short glucose polymers, are then saccharified to glucose by the action glucoamylase, an exocarbohydrase specific for α-1,4 linkages, and pullulanase, an enzyme that specifically attacks α-1,6 branch points in the starch molecule. The resulting glucose syrup is then converted to the sweeter isomer fructose by the action of an enzyme called glucose isomerase.

Enzymes replaced acids and heat as processing aids in starch liquefaction and saccharification about 50 years ago. In the 1970s enzymes also replaced alkali in the isomerization of glucose to fructose. Converting starch to dextrose, in the traditional process, was achieved by acid treatment at pH 2, followed by a 140 °C heat treatment (Fig. 3).

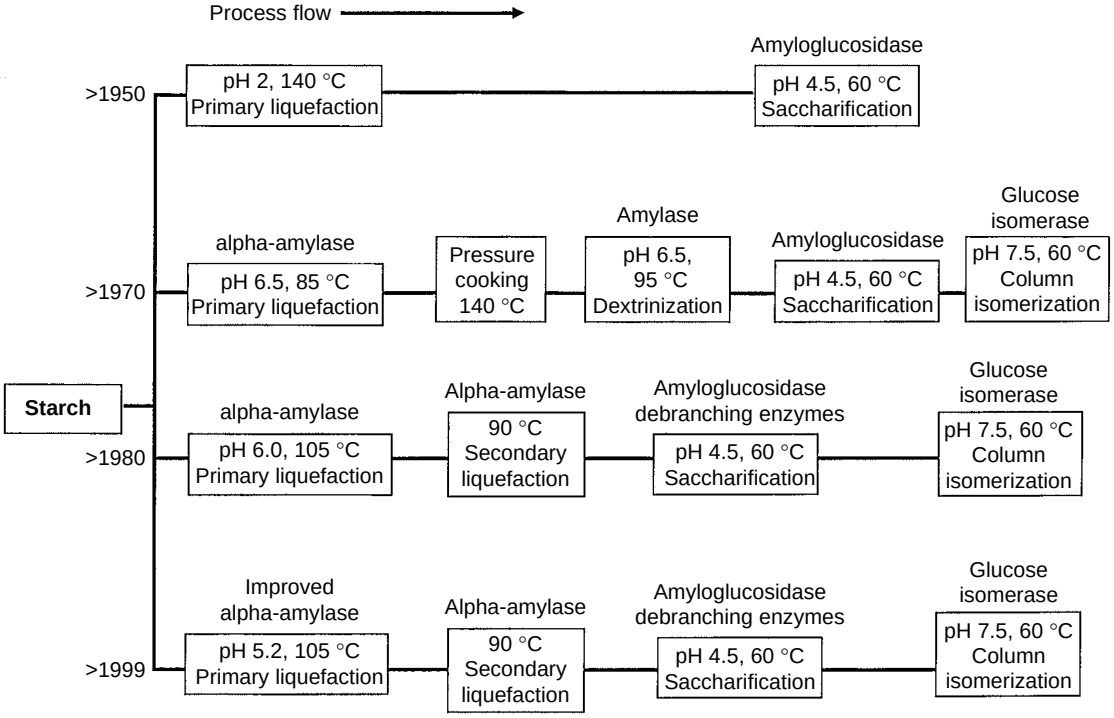

Figure 3. Enzyme-mediated improvements in the production of high-fructose corn syrup over time.

A heat-stable alpha-amylase (Novo BAN®, a bacterial amylase), operating at up to 90 °C was first used to replace the acid treatment. This enzyme, which acts randomly on α-1,4 linkages in amylose and amylopectin, required multiple dosing as the enzyme was inactivated at the higher temperatures. Later developments included the use of even more thermostable amylases (Novo Termamyl®) derived from *B. licheniformis* enabling reaction temperatures to reach 110 °C for short periods. In 1999 a Termamyl® variant called Termamyl Supra® (Novo) was introduced that allows the process to be performed at pH 5.2 rather than pH 6, dramatically reducing the cost of pH adjustment by ion exchange. Today the process may involve several different enzymes, depending on the final starch product required, including fungal and bacterial amyloglucosidases and debranching enzymes, such as pullulanase, which acts on α-1,6 linkages in the branched dextrin chain. The end result is a higher yield coupled with a more energy-efficient process and less toxic waste stream.

Textiles

Enzymes are extensively used in the textile industry for improving production methods and fabric finishing, often replacing hazardous methods with simpler, more environmentally benign alternatives. In the textile and fashion industries, product appearance is everything, so fabric dyeing must be done right. Before textile dyeing, cotton fibers are bleached to provide a uniform whiteness. The bleach must then be completely removed from the fabric or it will interfere with subsequent dyeing steps. Bleach removal is achieved either by several rinses in water or by neutralization of the bleaching chemical by a reducing agent, followed by rinsing in water. For example, in some situations approximately 40 liters of water are needed to rinse the bleach from one kilogram of fabric. When an enzyme such as catalase is used, the fabric requires no rinsing with water at all (14). The enzyme can be added and after about 15 minutes the dye can be added to the fabric. In the case of the nonenzymatic reaction, the textile manufacturer will often overdose the reducing agents, such as sodium thiosulfate or sodium bisulfate, to be sure of neutralizing the bleach. These reducing agents can also interfere with the subsequent dyeing steps and must also be rinsed away. In the traditional process, energy consumption is also required as high temperatures are needed in order for the reducing agents to react efficiently with the hydrogen peroxide. Catalase treatment therefore has a number of environmental advantages, including lower water and energy consumption, elimination of sulfate and nitrogen salt compounds in effluents, and the breakdown of hydrogen peroxide into its natural components of water and oxygen. In addition, the process is more controllable and shorter in duration.

Starch usually is coated onto cotton to give it strength in the mechanical weaving process. The starch must be removed before finishing the fabric, and this has traditionally been carried out using acid treatment. Enzymatic desizing using amylases, eliminating the acid reaction, dates back to the early 1900s. Amylases work efficiently in the desizing process and without the environmentally hazardous waste treatment problems associated with harsh chemicals such as acids, bases, and oxidizing agents.

Before dying of cotton fabric, the cotton must be "scoured" using high temperatures and highly alkaline

chemicals such as lye to remove waxes that prevent the fabric from being evently wetted. This process requires the handling of caustic chemicals, corrosion-resistant reaction vessels, and considerable inputs of energy for extensive hot water rinsing. A recently introduced alkaline pectate lyase, BioPrep™ from Novo Nordisk, is a recombinantly produced enzyme than can effectively remove these waxes under relatively mild conditions with minimal washing. The process saves energy because it can be performed at a lower temperature (50–60 °C) than the traditional process (90–100 °C) and reduces the quantity and toxicity of rinse water previously required to remove the lye from the cotton by up to twofold (Table 3). In addition, unlike the lye treatment, the specificity of the enzyme leaves the cellulose fibers in the cotton intact, resulting in increased fabric strength and weight. Enzymatic scouring of cotton therefore offers advantages in economy of production, product quality, and environmental impact over older, more traditional processes in large textile mills.

About 1.2 billion pairs of denim jeans are produced each year, as well as a significant number of other denim garments (shirts, jackets). A large percentage of these denim garments are abraded and faded, previously by such methods as stonewashing, to give a "washed out" and/or softer look to the garment. Stonewashing has traditionally involved "beating up" the garments in large washing machines in the presence of pumice stones, typically one to two kilograms of stones per pair of jeans. Bleach is often added when a lighter color is the intent. Neutral or acid cellulases have been an excellent replacement for stonewashing of denim garments, a process referred to as "biostoning" (15,16). A cupful of enzyme can replace about 100 times its weight of pumice stones, thereby preventing pumice particle dust in the manufacturing plant and preventing clogged drains by spent pumice sand.

Using cellulases as a substitute for pumice stones also prevents damage to washing machines and the garments, eliminates the need for disposal of the used stones, improves the quality of the wastewater, and eliminates the need for labor-intensive removal of dust from the finished garments. Without the use of stones, the garment load can be increased by as much as 50%, thereby increasing throughput. Neutral cellulases have an advantage in the process of biostoning because of the reduction in backstaining and its broader pH profile. Backstaining is a result of the released indigo dye from the denim being redeposited onto the surface of the fibers.

Table 3. Comparison of Enzymatic and Traditional Alkaline Cotton Scouring in a Jet Dryer Process

Measurement	Biopreparation	Alkaline Treatment
pH	8–9.5	13–14
Process Temperature	50–60 °C.	90–100 °C.
Residual Pectin	22–30%	10–15%
Weight Loss	<1.5%	3–8%
Wettability, drop test	<1 second	<1 second
Rinse water consumption	40–50%	100%
Burst Strength, % of start	95–100	90–97

Source: Bio Times **99**/03 (1999), Novo Nordisk A/S.

Laccases (1.10.3.2), also known as polyphenol oxidases, are a new entrant to the industrial enzyme marketplace. Laccase, in a product called Denilite® (Novo Nordisk A/S), was first launched in 1998 for the process of denim bleaching and abrasion. Laccases are a family of multicopper proteins with broad specificity toward inorganic and aromatic compounds, particularly phenols and anilines. These enzymes catalyze oxidations by molecular oxygen, often creating free radicals in the process. Recently several laccases were cloned, expressed (17,18), and biochemically characterized (19–21). The first laccase X-ray crystal structure has also been recently published (22).

The process of bleaching or abrading denim garments to give a "softer" or "worn-out" look involves the removal of the blue indigo dye. To remove the indigo dye from the garment, the conjugated double bonds between indigo's two carbonyl groups must be broken. The oxidative cleavage of these double bonds in the denim bleaching process typically has been carried out using hypochlorite as the bleaching agent. For enzymatic bleaching, an oxidoreductase enzyme, such as laccase, is needed. However, since the indigo dye on the garment is not accessible as a substrate to the laccase, a small organic mediator, such as *propionic acid phenothiazine* (PPT), is added. Figure 4 outlines the mechanism behind laccase denim bleaching. The technology is based on laccase using molecular oxygen as an electron acceptor being oxidized (with the concomitant reduction of oxygen to water), which then creates a mediator radical (PPT+) from the mediator itself, PPT. The PPT+ radical then reacts in a catalytic cycle in which the oxidized cation radical oxidizes solid indigo on the denim surface under simultaneous reduction back to the natural mediator. One of the advantages of this technology is that a side reaction occurs in which the PPT radical disproportionates to form an oxide (PPT-O), which depletes the catalytic cycle, thereby ending the bleaching cycle. In this way, the level of bleaching can be predefined and controlled by the amount of mediator added at the beginning of the process. In addition to the denim bleaching process, the enzyme/mediator mixture can also be used for abrasion enhancement in a totally stone-free process for obtaining highly abraded denim (24).

Other textile materials, such as wool and silk, can also use enzymes for surface modification. Selected proteases can be used to reduce pilling in wool garments by specifically hydrolyzing surface fibers. Silk normally requires a highly alkaline degumming step to remove sericin, a sticky protein that adheres tightly to the fibrin that makes up the silk fiber. Although this process is effective, it is hazardous and causes some damage to the fibrin, weakening the fiber. Certain proteolytic enzymes specifically remove the sericin without cleaving the fibrin.

A recently introduced product from Bayer, Baysolex, contains a recombinant peroxidase that effectively oxidizes residual reactive dyes on cotton after the dying process. This procedure not only reduces the amount of water and

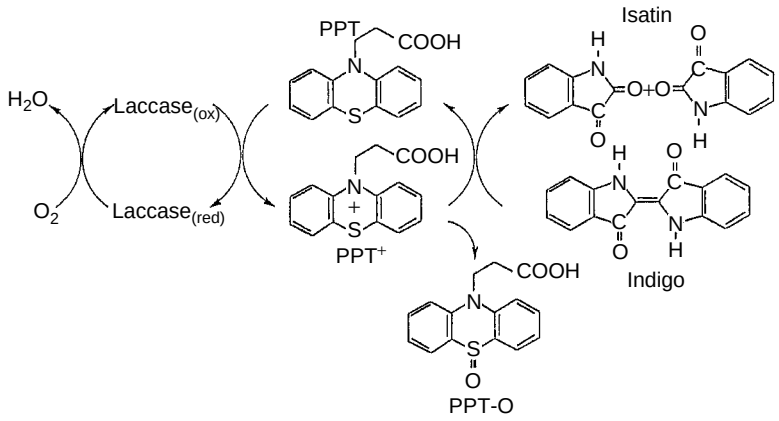

Figure 4. Outlined mechanism behind Denilite® laccase/mediator bleaching technology.

energy required for rinsing but also yields a more colorfast fabric (23).

Leather

The production of leather is one of the oldest applications of industrial enzymes. Traditionally, steps include curing, soaking, dehairing and dewooling, bating, and tanning. The process involves the use of numerous chemicals and unpleasant working environments to remove hair, fat, and unwanted protein such as elastin, keratin, albumins, and globulins, leaving the collagen fibers intact before the tanning of the hides and skins. Traditionally, dog or pigeon feces were used in the early bating steps to make the leather more pliable. The feces bating owed its softening effect to the action of proteases. In 1908 the first standardized enzymatic bating process was patented and was based on using animal pancreatic enzymes. Today bacterial and fungal proteases, mammalian trypsin (a by-product of insulin production), and lipases are used in leather processing (24). The results are a reduction in the use of sulfides, lime, nonionic surfactant compounds and organic solvents. The benefits are in a more standardized, well-defined process, with advantages with respect to the final characteristics and properties of the leather itself.

Pulp and Paper

Paper comprises wood fibers derived from pulp produced by chemical and/or mechanical means. The worldwide production of pulp raw materials is approximately 70 million tons per year, corresponding to a market value of pulp and paper chemicals of more than $5 billion. In the manufacture of white paper derived from pulp there is a strong desire to reduce (and eliminate) the use of chlorine and other chlorinated organic compounds as bleaching agents. This desire stems from intensified federal, state, and local regulatory and public environmental pressure to create fewer pollutants (25,26). A key concern in the reduction of chemical use is to provide the same high quality of brightness of the final paper product without losing paper strength. Enzymes are being used to enhance the bleachability of the chemically derived pulp.

The purpose of processing wood and bleaching the pulp is to remove lignin (delignification). Lignin is a natural resinous adhesive, which causes the yellow-brownish color. The goal is to reduce wood to cellulose fibers and to do so with as little damage as possible to the other wood constituents such as hemicellulose and, most importantly, the cellulose itself. Pulp production begins with debarking and chipping wood logs (Fig. 5). This is followed by a caustic sodium hydroxide cooking wherein the main part of the lignin is dissolved and then washed away. The result is a darkly colored pulp that has to be bleached in order to obtain white pulp for paper production. Chlorine and chlorine dioxide are the most common bleaching chemicals used in multiple stages of pulp bleaching coupled with alkaline extraction of the dissolved lignin.

Environmental pressures have been mounting over the past 15 years on the control of toxic dioxins and chlorinated organic compounds in paper mill effluents. Regulators have been pressuring industry to move away from using chlorine-based chemicals and toward other alternatives, such as the use of hydrogen peroxide, oxygen, ozone, and more recently the use of enzymes. Hydrogen peroxide, oxygen, and ozone have the disadvantages of being too expensive, requiring costly capital investments and resulting in the loss of paper strength. Considerable effort has been devoted to finding alternative bleaching agents, and alternatives have been examined to supplement the delignification stage. The application of xylanases and mannanases has turned out to be useful in removing part of the lignin after the alkaline cooking process and before the bleaching step (27,28).

In a typical xylanase procedure, a pH adjustment of the pulp from 10–11 to 7–9 is necessary. (The temperature is about 55–65 °C). After the pH adjustment, xylanase is mixed into the pulp, which is then kept in a holding tank for about two hours. Mannanases may also be added in some cases for additional bleach-boosting effects. As a result of the significant lignin removal by the enzyme treatment, much lower consumption of chlorine (10–50% reduction) is required to bleach the pulp further (depending on the type of wood and particular enzyme(s)). In addition, the enzyme treatment offers improved bleachability, reduction in the bleaching costs, less chlorinated organic compounds in the effluent, and higher brightness in the paper. The economic benefits include very simple equipment needs and savings in chlorine compounds. These benefits can easily pay for the enzyme treatment, not to mention the supplementary beneficial

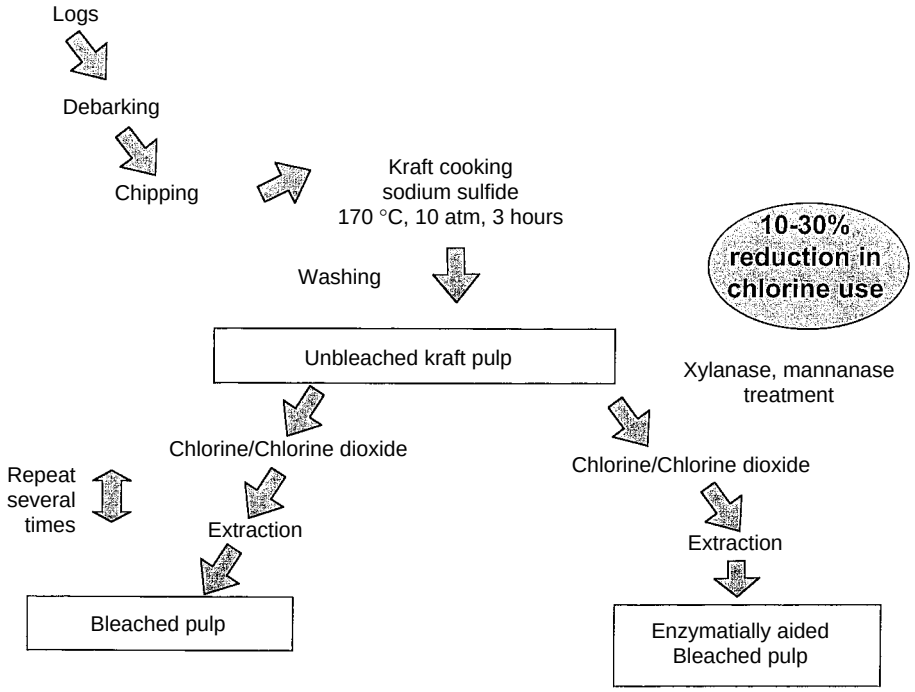

Figure 5. Enzymatic and chemical pulping flow diagram.

effect on the environment. New xylanases introduced into the market are able to withstand higher pH pulp processes without the need for neutralization and are more thermostable, further improving the attractiveness of enzymatic pulp treatment (29).

Although the mechanism of enzymatic bleach-boosting is not fully understood, it appears that xylan is solubilized during the pressurized alkaline cooking process. As the pressure, temperature, and pH drop, xylan precipitates on the wood fiber surfaces. Some lignin is chemically bound to the xylan, which may become released by the action of the xylanase. The precipitated xylan may create a physical barrier for the bleaching chemicals to get into the fibers and block the extraction of lignin and other chromophores. The paper fibers become more susceptible to bleaching after xylanases selectively remove xylan from the surface and the pores of the fibers, releasing the darkly colored chromophores.

In addition to pulp treatment, enzymes are used in the papermaking process. Residual tree resin in pulp can adhere to rollers and cause the pressed paper to be stained or tear, necessitating work stoppage and laborious cleaning. Addition of lipase before the rolling of the paper can reduce the occurrence of these resin-related problems in production. High-quality paper is often coated with starch to improve gloss, smoothness, and printing properties. The starch used in this process must be low in viscosity and was previously treated with aggressive oxidizing agents. Amylases that cleave the starch result in starch solutions with appropriately reduced viscosity and can be produced on-site at paper mills using batch or continuous processes.

Paper recycling is increasing rapidly on a worldwide basis (30), and enzymes may play a significant part in making this an economically viable alternative to new paper production. Recycled paper represents about 30% of total paper consumption and is growing yearly. Of the paper that is recycled, 60% represents old newspaper and 40% represents mixed office wastepaper and tissue paper. Since municipal waste comprises of about 35 to 40% paper, the shrinking availability of land for waste burial could be significantly alleviated with a reduction in disposed paper. Efficient ink removal is a critical requirement before paper can be effectively recycled.

Current de-inking methods involve a highly alkaline solution containing sodium hydroxide, sodium silicates, hydrogen peroxide, chelating agents, and synthetic surfactants (31). These chemicals provide fiber swelling, ink removal, and the disaggregation of the paper into fibers. As the de-inking process starts, the paper has a tendency to darken, necessitating the addition of large amounts of hydrogen peroxide to keep the pulp white. Often harsh chlorine-based bleaching chemicals are used as a final whitening step. In addition to the chemicals, the process involves a combination of thermal and mechanical energy.

Pilot plant de-inking trials using enzymes have shown that fungal cellulases are effective in de-inking newspaper (32) and, most importantly, in aiding toner removal from xerographic and laser-printed paper, largely comprising mixed office wastepaper (33,34). Toners used in office copiers are made of resilient plastic polymers that are tightly bound to the paper fibers. Their removal is difficult with the chemical process, but cellulases are able to release toners from fiber surfaces, presumably by the enzymatic removal of the tiny strands of cellulose that protrude from the surface of the paper fibers, dislodging the cellulose that holds the particle of ink to the paper. De-inking mixed office wastepaper can be achieved using low doses of alkaline cellulases without any added sodium

hydroxide. The alkaline cellulases are required because the pulp already has a high pH due to calcium carbonate used as a filler in the initial paper product. It is expected that enzyme technology will be used on a commercial scale in the near future to modify and/or replace the current de-inking methods.

Fiberboards

During the processes of making wood composites such as fiberboards, particle boards, cardboards, and the like the wood is first disintegrated into small pieces, which are afterward put together into larger boards using high energy and petrochemical adhesives such as urea-formaldehyde and phenol-formaldehyde glues. There is a growing interest in eliminating the use of such toxic and carcinogenic chemicals in the process. In addition, boards made with these adhesives give off small emissions of formaldehyde for months after manufacture.

Enzyme technology may provide a natural alternative to formaldehyde glues for board manufacturers. Lignin, a heterogeneous polyaromatic polymer, is a naturally resinous adhesive material. Nonsulfonated and sulfonated lignin can be polymerized by oxidase reaction. Enzymatic treatment of the wood particles with oxidases, such as peroxidases and laccases, leads to a rough surface of the fiber, rendering them very susceptible to interfibrous bonding. Fiberboards prepared from enzymatically treated fibers by simple pressing under normal production conditions have shown in preliminary trials to have very good technical properties (35). Both the tensile strength and the swelling properties met the required technical standards without the use of the formaldehyde-based adhesives. While a commercial process is still being developed, it should be possible to completely avoid the use of the hazardous formaldehyde-based adhesives. Enzymatic gluing of wood could significantly reduce the worldwide consumption of the approximately 300,000 tons of phenol-formaldehyde and the 2,500,000 tons of urea-formaldehyde used in the manufacture of today's fiber- and chipboards.

Personal Care

The use of enzymes in the personal care industry holds great potential, although there are only a few examples of products that contain enzymes thus far. Some brands of toothpaste and mouthwash incorporate glucoamylase, glucose oxidase, and lactoperoxidase. Although only present in the mouth for a few minutes, the solubilized enzymes attack oligosaccharides adhered to teeth and other mouth surfaces, producing hydrogen peroxide. The low levels of peroxide then effectively inhibit the formation of bacterial plaque that causes tooth decay and bad breath.

Another novel application of enzymes is a new system for hair dyeing using a laccase. The enzyme utilizes oxygen and a common hair dyeing intermediate that is oxidized, resulting in colored hair. Because of the specificity of the enzyme for the intermediate and the use of neutral pH conditions, the oxidative damage that normally occurs with hair dyeing using hydrogen peroxide and ammonia is avoided. There is also no smell of ammonia, no risk of skin irritation, with a broader precursor specificity (and a larger range of colors) (36).

Enzyme applications are also established in the field of contact lens cleaning. Contact lenses are cleaned using solutions containing proteases, lipases, or both. Methods using hydrogen peroxide as a disinfectant require complete removal of the peroxide before replacement in the eye. Catalase, which converts hydrogen peroxide to water, is used to remove residue of this oxidant before final rinsing.

Organic Chemical Synthesis

The role of enzymes in the manufacture of organic chemicals is becoming more established (37). Examples include the isomerization of glucose to fructose by glucose isomerase, beta-cyclodextrin production using cyclodextrin glucanotransferase, carnitine production using beta-ketoreductase, and aspartame production using the protease, thermolysin. The use of enzymes is particularly important when chiral molecules must be distinguished. In the production of amino acids, the conversion of racemic mixtures to the chirally pure desired product is being carried out using amidohydrolases and hydantoinases. The production processes of medically important antibiotics, penicillin and ampicillin, both benefit from enzymatically aided synthesis in lowered chemicals (inorganic and organic solvents) usage, waste, and reduction in energy. Biocatalysis offers significant advantages over equivalent chemical processes in allowing more benign pHs, temperatures, and pressures to be used. In addition, the enzymatic route of synthesis results in fewer undesirable by-products and less waste.

Oil and Gas Drilling

Oil and gas drilling requires the pressurized injection of drilling mud into the well for cooling the drilling head, transporting stone and grit up to the surface, and controlling the pressure underground. This mud often contains injected biopolymers such as guar gum to aid in stabilization and fracturing and to create a "filter cake" that prevents fluid loss. This filter cake can become impermeable, reducing field yield and well productivity. Enzymes specific for degrading the polymeric glue are currently sold to the drilling industry as replacements for strong acids and oxidants that have been traditionally used to degrade the filter cake.

More recently, enzymes have been developed that produce organic acids during the drilling process, dramatically reducing the need to interrupt drilling to degrade the filter cake. In addition, the acids produced allow the in situ generation of minerals, gels, and resins that can act to consolidate sandy drilling fractures and reduce mineral contamination in the product (38).

Fuel Alcohol

Many countries with excess agricultural capacity have a strong economic impetus to reduce their dependence on fossil fuels by production of alcohol from biomass. In a process similar to that described for the production

of high-fructose corn syrup and distilled liquor, complex starch must be converted from sugar polymer to monomer form to make the sugars fermentable by yeast. This requires liquefaction, the conversion of the solid polysaccharides into a liquid form by hydrolysis into shorter oligosaccharides, followed by a saccharification step producing fermentable monomeric sugars. The ethanol resulting from the fermentation is then recovered by distillation. In this industry, where low price is the only means of competition, reduction in capital costs, energy inputs, and efficient conversion are critical. Although the equipment and process for making fuel ethanol and distilled liquor are different, the principle of using enzymes to create fermentable sugars is exactly the same.

More recent efforts in the fuel ethanol industry are centered on converting biomass waste such as corn stover (leaves, husks, and stems), wood pulp, and sugarcane leaves and stems (bagasse) to fuel. In this case, the glucose is derived from cellulose, a β-1,4 polymer of glucose, rather than from starch. Combinations of cellulolytic enzymes capable of hydrolyzing the β-1,4 bonds such as cellobiohydrolases, endoglucanases, and cellobiases are required for this process. Research efforts aimed at reducing the cost of these enzymes are being investigated with funding from the U.S. Department of Energy.

FOOD ENZYMES

Baking

Six billion loaves of bread are baked in the United States alone each year. Approximately 600 million to over 1 billion of these loaves become unsaleable as a result of staling and mold spoilage. Research into bread staling has had a long history, with no good answers, until recent developments in the use of enzymes, such as amylases, to retard the process. Several theories exist regarding why bread stales. Bread is comprised primarily of starch (amylose and amylopectin) and a heterogeneous mixture of gluten proteins. In fresh bread, starch is mostly in an amorphous state. A modification in starch structure plays a major role in the staling of bread (39). Starch molecules tend to revert from an amorphous state to a less hydrated, crystalline state. As this happens, water is released from the starch and presumably is absorbed by the gluten proteins. Long linear amylose chains, extending through several amorphous regions of the bread, interact with the crystalline regions (which may involve as much as 15% of the starch), making it rigid, affecting the entire bread.

Many bakers employ the use of chemical emulsifiers, such as monoglycerides, to retard staling. The emulsifying agents make a softer bread and reduce the rate at which bread becomes firm (stale). Amylases can be used as natural antistaling baking ingredients because they reduce the need for chemical emulsifier additives (40). Amylases have been shown to significantly increase the freshness of bread compared with results using emulsifying agents. Bread has been shown to retain greater than 80% of its original freshness at the end of 48 hours when the dough was made using Novamyl® (Novo Nordisk A/S), a maltogenic bacterial alpha-amylase. Novamyl® is believed to act on specific regions of the starch fraction in bread that normally crystallize during the staling process (41). In fact, bread made with the Novamyl® is more likely to spoil from mold than as a result of staling. Novamyl® retards the staling process without affecting the handling of the dough, and because its action is very specific, overdosing does not reduce bread quality. Other amylases with less specific hydrolysis characteristics are also effective as antistaling agents but can more easily be over- or underdosed, resulting in sticky dough, collapsed bread, or poor resistance to staling.

A number of other enzymes are also used in the baking industry to modify dough properties for better baking results. Although the true mechanism of carbohydrases such as hemicellulase, pentosanase, or xylanase in bread making has not been clearly elucidated, it is well known that doughs containing optimized doses of these enzymes yield a more flexible, easier to handle dough with improved machinability. The strength of the protein network in dough can also be increased by the addition of lipase that partially degrades the lipid fraction of wheat flour. This ensures a more stable dough, resulting in a larger loaf volume and better bread texture. Chemical oxidants such as bromates, azocarbonamide, and ascorbic acid, widely used to increase the strength of the protein network in dough, can be replaced by the addition of an enzymatic oxidant such as glucose oxidase in some breads. Bread is comprised of starch and gluten proteins. During baking, the gluten proteins orient, align, partially unfold, and interact with one another through hydrophobic bonds and via disulfide linkages. This protein network transforms into a thin film in the bread, entrapping the carbon dioxide produced during rising and baking. The addition of oxidizing agents such as potassium bromate, azodicarbonamide, and ascorbic acid enhance the cohesive structures of hydrated gluten proteins in the bread dough, causing an increase in the toughness and elasticity of the bread. The effect is an overall increase in the volume of the bread, better crumb structure, and improved dough handling properties. Bromates and azocarbonamide have come under regulatory fire for health reasons and are restricted and/or banned in some countries. Animal feeding studies have shown potassium bromate to be carcinogenic (42). In the United Kingdom, potassium bromate as a bread improver was banned in 1990. Oxidases, such as glucose oxidase and sulfhydryl oxidase, have dough-strengthening effects and may become important players in the replacement of these oxidizing chemicals.

Dairy Products

The application of enzymes in the processing of milk has a long tradition. Since ancient times, an extract of calf stomach called rennet was used for coagulation during cheese production. We now know that the active ingredient in rennet is chymosin, a protease that cuts the milk protein casein at a specific place, rendering it insoluble. Today calf chymosin and selected fungal proteases with similar

properties are produced from recombinant hosts and sold to cheese producers to replace the traditional rennet at a fraction of the cost. While chymosin and other proteases are the major enzymes used today in cheese production, recombinant lipase cloned from the fungus *Mucor miehei* is also sold as a cheese ripening aid. As a minor component in rennet, the lipase was a component found to be important to the development of flavor in certain blue and Italian cheeses. Health fears concerning the use of animal extracts such as rennet on production of human food are avoided by the use of recombinant products made in microbial expression systems.

Another use of enzymes in the dairy industry is in the modification of milk for improved digestibility. Although all humans at birth make sufficient lactase to break down the 5% lactose that is found in milk, aging often is accompanied by reduced lactase production. Lactase (β-galactosidase) is sold as a dietary aid, as well as to improve the solubility or sweetness of various dairy products. Finally, highly purified and specific proteases are used to make infant formula from milk that is low-allergenic. The number of protein-sensitive individuals is estimated at 1 to 2% of adults and between 5 and 8% of children (43). By using proteases that cleave regions of the milk proteins that induce allergic responses, safe, highly nutritious replacements for mother's milk can be produced (44).

Brewing and Distilling

Beer is one of the oldest fermented products, resulting from the action of yeast on a cooked extract of barley malt; starchy cereal grains such as maize, sorghum, rice, or barley; and hops called sweet wort (Fig. 6). In sweet wort production, malted barley traditionally contributes an amylase activity that functions to break down the starch into smaller sugars that can be converted to alcohol and carbon dioxide by the fermenting yeast. Variability in the amylase activity present in different batches of malted barley can wreak havoc with beer making, resulting in poor filtration, incomplete conversion to alcohol, and poor flavor. Addition of thermostable alpha-amylase and other carbohydrases, such as β-glucanase, speed the fermentation process and increase the predictability of fermentation. The production of light beer, containing reduced sugar content, is accomplished by the addition of glucoamylase, which converts short sugar chains produced by other carbohydrases into glucose, which can then be easily fermented by the yeast. Finally, protease added during the production of the wort can produce increased nitrogen levels in wort made with low-nitrogen cereals. The increase in available nitrogen in turn aids in yeast growth and complete fermentations.

The production of distilled alcoholic drinks from crops rich in starch is another age-old example of fermentation. The conversion of starch to alcohol has always been an enzymatic process, but the source of enzymes before the 1960s was barley malt or koji, a rice extract containing fermenting organisms. Today the majority of distilleries use purified industrial enzymes. A few liters of carbohydrate-degrading enzymes can replace as much as 100 kg of malt, dramatically reducing the cost of raw materials and greatly simplifying the fermentation process.

Fruit, Juice, Wine, Fats, and Oils

A wide variety of consumer goods produced by processing of plant materials use industrial enzymes, including fruit juices, whole prepared fruit, wine, and oil. Traditional methods of wine and juice making begin with the crushing of the fruit to extract juice but are accompanied by the extraction of other plant cell components that may make further processing difficult. The pectin present in fruits reduces the yield of juice and clogs the filter apparatus used in clarifying juices. Pectin is a complicated polysaccharide composed primarily of linear chains of galacturonic acid with branches composed of a variety of shorter linked polysaccharides. During crushing, the addition of pectinases, capable of hydrolyzing the bonds between linear galacturonic acid chains, as well as other carbohydrase activities (rhamnogalacturonases, xylanases, galactanases, arabinases, etc.) contribute to increased juice yield and reduced filtration times. In juice for wine production, treatment also intensifies the flavor and color of the juice.

Whole citrus fruit can also be treated with a similar mix of enzymes to remove the skin ("enzymatic peeling"),

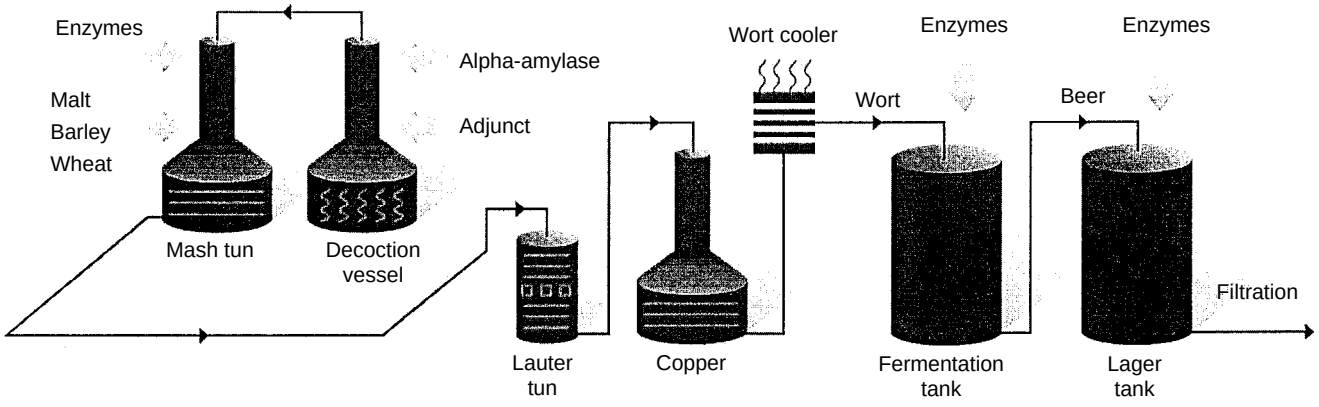

Figure 6. The brewing process.

avoiding the use of caustic soda that is traditionally used. The resulting fruit segments are fresher and no longer have a membrane surrounding them, resulting in better texture and appearance.

Wine making, beyond the production of juice for fermentation, has also been impacted by industrial enzymes. Pectinase treatment after mashing aids in wine clarification, allowing residual pectin to be degraded and reducing the viscosity of the juice.

Oil from coconut, olive, rapeseed, maize germ, sunflower seed, and palm kernels usually is crushed and extracted using an organic solvent such as hexane, an identified air pollutant. Cell wall–degrading enzymes offer a safe and environmentally responsible alternative. The degradation of the cell wall allows aqueous extraction of oil.

FEED ENZYMES

Animals have an inherent inability to digest various types and amounts of plant raw materials in feed. This can be overcome to a significant extent by the addition of enzymes in the feed. Enzymes used in animal feed act as feed enhancers. Enzymes improve the digestibility of feed raw materials, thereby increasing the efficiency of nutrient utilization. The market for animal feed enzymes has grown from about $5 million per year in 1990 to over an estimated $100 million per year today. The market potential for animal feed enzymes is much greater because today only about 6% of the manufactured animal feeds contain enzymes.

Today the primary enzymes used in animal feed are xylanases, beta-glucanases, proteases, and phytases. In addition to improving the overall digestibility of the feed raw materials and increasing the nutrient availability from the feed, there is a concomitant reduction in the production of animal waste. Animals are unable to unlock the energy in various forms of cereal- and vegetable-based non-starch polysaccharides (NSP). The addition of beta-glucanases, xylanases, and proteases will free this NSP, rendering it available to the animal. The removal of NSP also releases starch and protein, which can otherwise be masked by the cell structure in the cereal. This leads to an overall increase in metabolizable energy and in protein utilization.

Between 50 and 80% of the total phosphorus in pig and poultry feeds is present in the form of phytate, the hexaphosphate of myoinnositol, an important phosphate storage compound in plants. Phytate not only is resistant to digestion in these animals but also binds tightly to various proteins and nutritionally important metals such as calcium, zinc, and magnesium. Phytate excreted by animals also negatively impacts the environment since it is readily broken down into free phosphate by soil microorganisms and released into the soil and groundwater. Use of the enzyme phytase as an animal feed supplement therefore has two primary benefits: it increases the nutritional value of the feed by releasing phosphate and bound metals from the phytate, and it reduces the release of free phosphorus in animal waste by as much as 30% (45,46).

CONCLUSION

Enzymes are the most widespread products of biotechnology in use today. As demonstrated by the vast array of industries and applications described here, it is nearly impossible to avoid using, eating, or wearing a commercial product that has not been touched by the action of industrial enzymes. Since enzymes are responsible for the catalysis of chemical reactions in all living things, they provide a natural, biodegradable solution to numerous problems in our industrial society. Their production from fermentation of living organisms uses agricultural products as raw materials and produces only biodegradable wastes. They function specifically, are extremely efficient, and often replace chemical processes that require more energy and produce more wasteful by-products. When all the benefits of using enzymes are taken into consideration, it is not surprising that the number of enzyme applications continues to grow each year.

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ENZYMES, COLD-ACTIVE. See Use of Cold-Adapted Microorganisms in Biotechnology

ENZYMES, INDUSTRIAL. See Alkaliphiles: Alkaline Enzymes and Their Applications

ENZYMES IN SOILS. See Soil Enzymes

ENZYMES: OXYGENASES. See Oxygenase Enzymes: Role in Biodegradation

EPIFLUORESCENCE MICROSCOPY. See Image Analysis of Microorganisms

EPILITHIC MICROORGANISMS. See Subaerial Communities

EPS. See Extracellular Polymeric Substances (EPS): Structural, Ecological and Technical Aspects

EUTROPHICATION AND ALGAE

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The process of eutrophication can have wide-ranging and profound effects on algal communities in aquatic ecosystems. However, the first and by no means trivial challenge in dealing with this topic is to define the meaning of eutrophication. Despite the ubiquitous use of the term in the field of aquatic ecology, it is surprising how many different ways eutrophication is used in the literature. In keeping with the Greek origin of the word eutrophic, meaning well-fed, eutrophication is sometimes used in the context of increases in nutrient loading or concentration in aquatic ecosystems. In contrast, many limnologists and oceanographers use the term in a more reactive context, that is, referring to increases in biological productivity and/or standing crop. Obviously, these two usages are intimately related, but they are not synonymous. Increases in nutrient loading may not result in increased productivity if other factors limit the use of the nutrients. By the same token, increases in productivity may not lead to increased standing crop because of the nature and magnitude of loss functions, such as grazing, death, sedimentation, dilution, and export. For the purpose of this review, the responses of algae to eutrophication will be discussed from the point of view of both gain and loss functions.

The specific response of algae to increases in nutrient concentrations is dependent on the relative availability of the nutrient in question before the increase. If the nutrient

in question is present at growth-limiting concentrations, increases in availability should stimulate productivity, assuming the absence of other limiting factors such as light availability. Alternatively, addition of a nutrient already present at saturating levels may result in little response. The amount, rate, and manner of nutrient addition can also affect the type of algae that responds to the increase. Hence, the impacts of nutrient increases can be both qualitative and quantitative in nature. The structure and abundance of algal populations, resulting from an increase in nutrient concentrations, is further dependent on a host of physical, chemical, and biological characteristics that define particular ecosystems.

All of the aforementioned considerations manifest the need to define the impact of eutrophication on algae within the context of the fundamental spatial, temporal, and system-specific differences that exist in the structure and function of ecosystems.

NUTRIENT CONCENTRATIONS, TROPHIC STATE, AND ALGAL BIOMASS

Historically, phosphorus has been viewed as the most commonly limiting nutrient in freshwater ecosystems. This principle is in large part attributable to two important facts: (*1*) phosphorus is in low supply compared with biological demand (1), and (*2*) unlike nitrogen, which is also in low supply compared with demand, phosphorus cannot be added to an ecosystem from the air through biological fixation. The role of phosphorus in the control of algal productivity and abundance is manifested by the widely reported relationship between phosphorus concentration and chlorophyll *a* (2). For this reason, many of the most commonly used indicators of trophic state include phosphorus and chlorophyll *a* concentrations as main elements (3). A number of researchers (4–6) have proposed ranges for phosphorus and chlorophyll associated with different trophic states (e.g., Table 1). Total nitrogen concentration and Secchi disk depth are also commonly included in trophic state indices (3). Nitrogen has been shown to be a limiting nutrient for algal growth in a number of freshwater ecosystems subject to relatively high phosphorus loading (3,7). Secchi depth is a widely employed measure of light transparency, and is therefore a useful surrogate for phytoplankton abundance, except in ecosystems in which light absorption is dominated by organic color or nonalgal suspended solids (i.e., tripton).

The range of values used here to define specific trophic states must be considered rough estimates of the actual trophic state, because values observed in the field are subject to considerable spatial and temporal variation, even within well-defined ecological boundaries. As a consequence, the task of establishing an accurate measure of trophic status can require extensive sampling efforts. Adding to the complexity is the fact that in some ecosystems, most particularly those of large size and/or spatial heterogeneity, the values for key trophic state indicators can show significant regional variability (9).

More recently, the abundance of submerged aquatic vegetation (SAV) in aquatic ecosystems has been incorporated in to the evaluation of trophic state (10). This new approach has been a useful addition to defining trophic state in shallow water ecosystems in which the dominant primary producers are benthic macrophytes and algae rather than phytoplankton.

Table 1. Range of Values for Key Environmental Indicators Associated with Different Trophic States (After R. G. Wetzel, *Limnology*, 2nd ed., Saunders College Publishing, New York, 1983.). TP, Total Phosphorus ($mg\,m^{-3}$); TN, Total Nitrogen ($mg\,m^{-3}$); Chl, Chlorophyll *a* ($mg\,m^{-3}$); Peak Chl ($mg\,m^{-3}$); SDD, Secchi Disk Depth (m). Mean Values Are Shown along with Range in Parentheses

Parameter	Oligotrophic	Mesotrophic	Eutrophic	Hypereutropic
TP	8.0 (3.0–17.7)	26.7 (10.9–95.6)	84.4 (16.0–386)	— (750–1,200)
TN	661 (307–1,630)	753 (361–1,387)	1,875 (393–6,100)	— —
Chl	1.7 (0.3–4.5)	4.7 (3.0–11.0)	14.3 (3.0–78.0)	— (100–150)
Peak Chl	4.2 (1.3–10.6)	16.1 (4.9–49.5)	42.6 (9.5–275)	— —
SSD	9.9 (5.4–28.3)	4.2 (1.5–8.1)	2.45 (0.5–7.0)	— (0.4–0.5)

TROPHIC STATE AND ALGAL COMMUNITY STRUCTURE

The process of eutrophication is accompanied by profound changes in many aspects of the structure and function of aquatic ecosystems, beyond simply the abundance of algae. It is, therefore, not surprising that ecosystems of differing trophic status are often characterized by different algal assemblages (e.g., Table 2). Some of these differences are directly related to the relative ability of different algal species to compete for essential nutrients (11–13). For example, in oligotrophic lakes and rivers, algal species with a high affinity for limiting nutrients can have a selective advantage. By contrast, hypereutrophic ecosystems can favor species capable of high nutrient uptake capacity and fast growth. In another case, an ecosystem subject to pulsed nutrient loading may be characterized by species of algae capable of storing nutrients for subsequent growth among loading events. In a forth and widely important scenario, ecosystems subject to nitrogen limitation are often dominated by species of cyanobacteria capable of nitrogen fixation. The latter case is an example of how the ratio of nitrogen to phosphorus, and their concentrations, can impact community structure and biomass. Clearly, there are many possible nutrient concentration and loading scenarios. Individual aquatic ecosystems may be subject to a wide range of conditions over time and space, helping to explain the dynamic character of algal communities.

It is also important to recognize that the direct effects of phosphorus and nitrogen concentration and loading characteristics on algal biomass are only a part of the environmental mosaic that defines the impact of changing trophic status. Light availability, temperature, pH, carbon dioxide concentration, organic carbon content, and oxygen tension are just a few of the parameters affected by

Table 2. Examples of Algal Genera Containing Species Associated with Oligotrophic and Eutrophic Conditions (After R. G. Wetzel, *Limnology*, 2nd ed., Saunders College Publishing, New York, 1983, E. B. Welch, Ecological Effects of Wastewater, Cambridge University Press, Cambridge, 1980, G. E. Hutchinson, A Treatise on Limnology. II. Introduction to Lake Biology and the Limnoplankton, John Wiley & Sons, New York, 1967)

	Oligotrophic	Eutrophic
Phytoplankton	Staurastrum (desmid)	Chlorella (green)
	Tabellaria (diatom)	Scenedesmus (green)
	Dinobryon (Chrysophyte)	Cylindrospermopsis (blue-green)
	Peridinium (dinoflagellate)	Aphanizomenon (blue-green)
	Ceratium (dinoflagellate)	Anabaena (blue-green)
		Microcystis (blue-green)
		Melosira (diatom)
		Synedra (diatom)
Benthic Algae	Schizothrix (blue-green)	Cladophora (green)
	Scytonema (blue-green)	Vaucheria (green)
	Phormidium (blue-green)	Rhizoclonium (green)
	Stigeoclonium (green)	Lyngbya (blue-green)
	Ulothrix (green)	
	Epithemia (diatom)	
	Cymbella (diatom)	
	Batrachospernum (red)	

changes in trophic status. Changes in these factors can lead to a cascade of alterations in the physical and chemical structure of ecosystems that ultimately affect algal community structure. For example, high phytoplankton standing crops can enhance thermal stratification of the water column, as a result of increased absorption of solar energy. Stratification, subsequently, increases the potential for hypoxia in lower layers of the water column, increases pH in surface layers, and reduces carbon dioxide availability in the upper layers of the water column. The latter conditions favor species of algae capable of buoyancy regulation and able to use bicarbonate as a carbon source for photosynthesis. This example illustrates how changes in algal abundance can in turn shape the structure of aquatic ecosystems, which in turn precipitates further changes in community structure.

Part of the structural changes brought about by elevated trophic status in aquatic ecosystems is the appearance of algal species adapted to eutrophic and hypereutrophic conditions. The selective factors associated with elevated trophic status include; (*1*) reduced light availability in the water column (i.e., caused by light attenuation by phytoplankton), (*2*) reduced carbon dioxide concentrations (i.e., caused by photosynthetic carbon uptake and elevated pH), (*3*) reduced availability of secondary nutrients (e.g., trace metals), (*4*) increased periods of low oxygen concentrations, (*5*) elevated temperatures, and (*6*) increased frequency of nitrogen limitation. Among the various algal groups, the cyanobacteria contain a disproportionately large number of species well-suited to one or more of the aforementioned conditions. It is, therefore, not surprising that several researchers have observed that the relative importance of cyanobacteria in phytoplankton communities increases with trophic status (16,17). This relationship may, in part, be attributed to the long evolutionary history of cyanobacteria, which dates back over three billion years and includes geologic periods characterized by extreme conditions. Another important factor is the ability of many cyanobacteria to fix nitrogen, which provides them with a distinct advantage under nitrogen-limited conditions. Planktonic nitrogen-fixing genera, such as *Anabaena, Aphanizomenon*, and *Cylindrospermopsis* are found in bloom proportions in eutrophic ecosystems throughout the world. A number of non-nitrogen-fixing cyanobacteria are also prominent bloom-forming species on a global basis, including the genera *Microcystis* and *Oscillatoria*.

Despite the widespread success of cyanobacteria in eutrophic environments, there are species in other algal divisions that can dominate algal communities in eutrophic ecosystems. In the planktonic realm, these species include a number of fast-growing green algal and diatom species (Table 2). In the benthic and epiphytic domain, certain species of filamentous green algae and pennate diatoms can form extensive blooms (18,19). In shallow flowing ecosystems, filamentous green algae, frequently, dominate the algal community. The specific species of algae found in individual systems depends not only on trophic level but also on a host of other environmental parameters, such as conductivity, temperature, light, ionic balance, sediment type (20,21).

FACTORS THAT AFFECT THE RELATIONSHIP BETWEEN NUTRIENT CONCENTRATION AND ALGAL ABUNDANCE

Besides the obvious dependence of algal productivity on the availability of the key macronutrients, namely, phosphorus and nitrogen, there are other factors that can affect the specific response of algae biomass to increases in these nutrients. These factors can operate at different stages of the overall process of algal biomass accumulation, from the light reaction of photosynthesis to cell growth. The nature and magnitude of the effects also vary over wide temporal and spatial scales, from the fractions of a second associated with photosynthetic light reactions to geologic timescale shifts in climatic conditions and from subcellular differences in light absorption to latitudinal differences in seasonality. The one unifying element is that all the controlling factors can have profound impacts on the manner and degree to which changes in nutrient loading are expressed in terms of algal biomass. It is, therefore, appropriate to review some of the principal factors that are involved in determining these relationships.

Light Availability

Light is the energy source for oxygenic photosynthesis, and therefore light is the ultimate limiting factor. In the broadest sense, the varied environmental consequences of latitudinal gradients in annual incident irradiation has been shown to be a major contributor to differences in annual primary production in tropical, temperate, and boreal environments (22). Hence, given similar nutrient status, annual primary productions in ecosystems located in lower latitudes often exceed that in higher-latitudes because of differences in annual light availability. Latitudinal differences in primary production also extend to seasonal patterns of algal abundance. Algal abundance in high-latitude ecosystems frequently shows one peak coinciding with summer light maxima. Conversely, in tropical latitudes, algal abundances can exhibit multiple peaks throughout the year, reflecting the year around availability of light. In temperate ecosystems, two annual phytoplankton peaks, one in the spring and one in the fall, are common. Regional differences in weather conditions can also result in disparities in light availability. The latter considerations have led some researchers to propose that peak algal standing crop during the course of a year is a better measure of trophic status than mean annual chlorophyll concentration.

Beyond the question of variations of incident light flux, the availability of photosynthetically active radiation (PAR) in the water column is dictated by the rate and character of light extinction, depth, and vertical stability. There are a growing number of examples of aquatic ecosystems in which light, rather than nutrient availability, is the primary factor that limits primary production (23,24). As an example of one extreme case, the upper limit of phytoplankton standing crop in some hypereutrophic ecosystems can be dictated by the degree of self-shading. In a classic study of hypereutrophic lakes in equatorial Africa, Talling (25) concluded that the upper limit of phytoplankton standing crop in well-mixed shallow lakes is approximately 400 mg chlorophyll *a* m^{-2}. A similar conclusion has been reached for periphyton communities dominated by benthic cyanobacteria and diatoms (26). In contrast, benthic green algal mats in shallow flowing systems have been observed to exceed 1,200 mg chlorophyll *a* m^{-2} (18,27).

In sharp contrast to the latter scenario, there are eutrophic and hypereutrophic ecosystems in which phytoplankton standing crops only reach a small fraction of the potential represented by the concentrations of bioavailable nutrients present. In some of these systems, light absorption is dominated by the presence of high levels of nonalgal suspended solids. For example, phytoplankton standing crops in parts of Lake Okeechobee in Florida average less than 20 μg chlorophyll *a*/L, despite high concentrations of bioavailable phosphorus, because of very high levels of light-absorbing tripton (23). Similarly, high organic color levels in backwater rivers severely limit PAR light availability and can limit phytoplankton standing crops (24,28).

Besides, light extinction properties of the water column, depth, and mixing characteristics play a major role in defining the effect of light availability on algal biomass. In the case of planktonic primary producers, the movement of algal cells through the water column presents a constantly changing light environment. Therefore, primary production over the water column is an integration of photosynthesis over a range of light intensities. Historically, the relationship between mixing depth and light attenuation has been a central focus in defining the productivity of aquatic ecosystems. The two terms most commonly used to define the relationship are mixing depth and critical depth, that is, the depth at which algal respiration in the water column equals photosynthetic oxygen evolution. If mixing depth exceeds critical depth, there is a putative loss in algal standing crop over time, irrespective of the nutrient availability. This assumes that cells are circulating evenly through the mixed-layer, which is not always the case. For example, many cyanobacteria and dinoflagellates exhibit the capacity for buoyancy regulation and motility. The ability to regulate position in the water column provides these algae with a distinct advantage under conditions of light limitation in the water column. It also represents an example of how differing light environments can affect both phytoplankton community structure and abundance.

The productivity, standing crop, and composition of benthic algae are also subject to issues of light limitation, but in a somewhat different way than planktonic algae. The successful colonization of benthic environments by algae depends on the availability of appropriate substrate at depth ranges that provide sufficient light to sustain growth and reproduction. In shallow water ecosystems, benthic algae biomass can increase significantly with eutrophication. However, above a certain threshold of nutrient concentration, increased phytoplankton abundance can result in sufficient shading of benthic habitat to restrict benthic algal development. The latter phenomena can be interpreted in terms of the concept of alternative stable states (29). The latter concept refers to the presence of threshold levels for environmental conditions that once exceeded results in major shifts in community structure. Major increases in the trophic state of ecosystems can fall in this category. The establishment of high-density phytoplankton biomass can make it difficult for benthic plant communities to maintain or establish themselves without a major environmental disturbance, such as sharp reductions in water levels. Conversely, well-established benthic plant and algal communities stabilize benthic substrates and compete with phytoplankton for bioavailable nutrients, thereby reducing the potential for blooms. A major storm event, such as a hurricane, can disrupt benthic plant community structure and result in a shift to phytoplankton domination. The balance between benthic and planktonic plant/algal biomass during the process of eutrophication is a subject of major concern to managers of aquatic ecosystems. Recent decades have seen a growing effort to maintain or reestablish benthic plant/algal communities in an effort to limit the potential for phytoplankton blooms (29).

At the opposite extreme of limiting light levels is the issue of excess irradiance. High solar irradiance

can negatively impact photosynthesis and cell growth in several ways, including the production of damaging superoxides caused by exposure to excessively high amounts of PAR light and cell damage from exposure to ultraviolet light. Both of these phenomena can lead to reduced algal productivity and standing crop.

Secondary Limiting Nutrients

Although nitrogen and phosphorus are the two most frequently limiting nutrients in aquatic ecosystems, there are other elements that can limit primary production. Limitations of secondary elements, although relatively rare, can directly impact the expression of the growth potential represented by the amount of nitrogen and phosphorus present. Trace elements, such as iron, have been shown to be limiting factors in a number of systems. In some cases, the low concentrations of certain elements can have a specific effect on certain algal taxa, thereby impacting the community structure. For example, silica limitation in the Great Lakes of North America has been shown to limit the growth of diatoms resulting in major alterations in phytoplankton structure and dynamics (30). Similarly, the absence of certain vitamins can limit the growth of certain species of algae, but not others (31).

Temperature

Although light availability is the primary physical factor that limits the rate of photosynthesis, there are a number of other environmental parameters that affect photosynthetic and growth potential of algae. Principal among these is temperature that strongly influences the dark reactions of photosynthesis (e.g., Calvin cycle), and other metabolic functions of algal cells. Despite the fact that algae exhibit both genotypic (e.g., species differences in temperature optima) and phenotypic (e.g., adaptive changes in concentrations of rate-limiting enzymes) adaptations to a wide range of temperatures, short-term changes in temperature and broad geographic differences in temperature regimes (e.g., polar versus tropical or high versus low altitude lakes) have an impact on production potential. For example, low temperatures encountered in temperate and boreal ecosystems during winter can reduce the overall rate of primary production. Similarly, the extremely high temperatures associated with hot springs limit the rate of primary production and also exclude all but thermophilic species of algae.

Loss Functions

Beyond the factors that directly affect the photosynthesis and growth of algae, as previously discussed, there are also a number of key biological and physical processes that can control standing crop after growth. Among the biological processes, one of the most fundamental is grazing. Although the impact of eutrophication on algal productivity and standing crop is most often approached from a bottom-up perspective, such as nutrient limitation, standing crops of algae can also be dramatically affected by top-down processes such as grazing, associated with the food web. Grazing can affect biomass potential, and spatial and temporal patterns of abundance and composition (32). Seasonal cycles of phytoplankton standing crop are often tied to patterns of zooplankton population that vary in response to the availability of the algae biomass. Similarly, the standing crop of benthic algae can be kept down to relatively low levels by benthic grazers (i.e., invertebrates and fish), despite high rates of productivity (33).

Physical processes can also play a major role in the loss of algal biomass. One of the most fundamental physical factors is the rate of water turnover. High rates of water turnover or dilution (i.e., flushing rates) can restrict phytoplankton standing crop by limiting the time available for the accumulation of biomass. Most current models of phytoplankton dynamics include terms for dilution or residence time (34). In the case of benthic algae, high water turnover and flow rates can have the opposite effect, by increasing the flow of water across the surface of the algae (35). Flow can help increase benthic productivity and standing crop in several ways, namely, (*1*) by providing a more consistent supply of nutrients, (*2*) by breaking down the boundary layer that inhibits surface nutrient exchange, and (*3*) by enhancing gas exchange, thereby increasing carbon dioxide availability for photosynthesis and reducing inhibitory oxygen supersaturation. High flow rates can, however, also reduce benthic algal standing crop by physical scouring and erosion of mats (35).

Another physical mechanism for biomass reduction is sedimentation. Because the specific gravity of most algae is greater than water, they sink unless acted upon by physical mixing forces. In aquatic ecosystems in which depth exceeds the euphotic zone, sedimentation can significantly limit productivity and standing crop. Even in shallow water ecosystems, settling of algal cells can reduce total productivity by reducing light availability. There are processes that counteract the rate of sedimentation. The primary physical processes that reduce sedimentation are wind-, heat-, and flow-induced mixing. In shallow water ecosystems, wind mixing can also contribute to dramatic short-term temporal variation in phytoplankton standing crop. For example, highly productive shallow lakes, such as Lake Apopka in Florida, are characterized by high concentrations of meroplanktonic algae (i.e., sedimented algae located at the benthic interface) (36). Wind resuspension of the meroplanktonic layer can result in a two- to threefold increase of phytoplankton concentration in a matter of minutes. Conversely, surface scums of algae can be wind-mixed into a deeper water column, thereby reducing surface algae concentrations over short periods of time. These phenomena present added complexities to the task of determining the trophic status in ecosystems subject to vertical stratification of algal abundance.

In addition to physical factors that control the rate of sedimentation, there are biological mechanisms by which cells can adjust their position in the water column. The three primary mechanisms are buoyancy regulation, motility, and structural adaptation. Certain cyanobacterial groups, such as *Anabaena* and *Microcystis* are particularly adept at controlling buoyancy and can be found at distinct vertical layers in the water column.

Similarly, flagellated algae, such as dinoflagellates, are capable of moving within the water column in order to find optimum environmental conditions.

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EVOLUTION OF METABOLIC PATHWAYS FOR DEGRADATION OF ENVIRONMENTAL POLLUTANTS

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ADAPTATION AND ENVIRONMENTAL POLLUTION

It is undisputed that microorganisms can adapt to changes in their environment. One of the types of changes microbial communities are currently exposed to is the release of large quantities and varieties of organic and inorganic substances into the environment. These substances can act as the "triggers" for different kinds of adaptation of microbial communities. Thus, various evolutionary mechanisms at work in adapting bacterial populations can be observed. From an environmental viewpoint, adaptation of microbial communities is beneficial when microorganisms that can completely degrade polluting substances evolve (e.g., natural attenuation).

What are the ways by which environmental pollutants can act as triggers for adaptation and how might microbial communities react? In general, adaptation (in contrast to intoxication) can be discussed when the overall response of the community is "positive." Artificial organic compounds and wastes that enter the environment may represent a significant source of carbon and/or energy for microorganisms, provided their concentrations are sufficiently high. Those bacteria that can use the carbon and/or energy from the pollutant can proliferate and (perhaps) increase in population size. When this occurs, a rapid disappearance of the pollutant is usually observed. This type of reaction is what has been observed in environments polluted with oil

or oil products (1). Microorganisms degrading oil or coal components are probably ubiquitous in the environment and will immediately start to degrade those components from an oil spill, thereby increasing their population size measurably (2–4).

Other pollutants, however, might have an uncommon type of structure or side substituents, which render them nondegradable or very poorly degradable. Typical examples of such xenobiotics are halogen-substituted compounds (5). Some xenobiotics could even in principle be metabolized by existing microorganisms, but are not being degraded because they are not recognized by the pathway regulatory proteins. Therefore, the proper pathways are not expressed when the microorganisms face the compound (e.g., as for chlorobiphenyls) (6). For those poorly degradable compounds, no reaction of the community occurs and the pollutant disappears only by physicochemical and hydrological processes. However, even when nothing much might happen to a pollutant for a long time, microorganisms that can degrade it may "appear all of a sudden" (7–9). Such microorganisms often have acquired one or more genetic changes (see following section). Typical examples of compounds for which adaptation has been observed are listed in Table 1.

Biochemical Adaptation

When adaptation requires expression of a characteristic, which is present in the cell as such, one speaks of biochemical adaptation. This type of adaptation usually involves a particular signal-transduction pathway by means of signal and/or regulatory proteins that interact with the DNA and regulate gene expression. Such regulatory proteins are very common for activating the enzymes for pollutant breakdown (37,38). For example, bacteria capable of using toluene as the sole carbon and energy source will synthesize the enzymes necessary for toluene breakdown whenever they encounter larger amounts of toluene. A specific regulatory protein in the cell (XylR) is capable of recognizing toluene and activating transcription of the *xyl* genes for toluene degradation (39). Cells that suddenly face a change in carbon substrate go through a transition state (40) and adapt their metabolism to the new compound.

Table 1. Examples of Genetic Adaptation to Xenobiotic Compounds

Compound	Sole Carbon and Energy Source	Reference
Chlorobenzene	+	(10,11,12,13)
2,4-Dichlorophenoxyacetic acid	+	(14,15,16,17)
2,4,5-Trichlorophenoxyacetic acid	+	(18,19)
Hexachlorocyclohexane	+	(20)
Nitrilotriacetic acid	+	(21)
EDTA	+	(22,23)
Chlorinated alkanes	+	(24)
Nitrobenzene	+	(25)
Nitrotoluenes	+	(26,27,28)
Atrazine	+/–	(29,30,31)
Perchloroethylene (anaerobically)	+	(32)
Trichloroethylene (aerobically)	–	(e.g., 33)
Polychlorinated biphenyls	–	(e.g., 34)
TNT (trinitrotoluene)	–	(35,36)

Not necessarily complete listing or in all cases genetically "proven."

The question at what concentration a pollutant may lead to induction or selectable growth is not solved satisfactorily (41). If the concentration of the pollution is too low (below 5–10 nM), nothing much may happen. Studies on pathway induction seem to indicate that there are threshold concentrations below which no inductive effects on pathway expression are seen (42). The fact that very low background concentrations (ng/l range) of various pollutants occur in almost all environmental compartments suggests that these are too low for microbial degradation or adaptation.

Genetic Adaptation or Evolution

If the capability to recognize and degrade a pollutant is not immediately present in the cell and one or more genetic changes are required, we can speak of genetic adaptation (or evolution). This is the case when, for example, a particular mutation is needed to activate a silent gene cluster or to change the specificity of an enzyme. The capacity to produce those genetic changes (e.g., the DNA polymerase, DNA repair systems, or recombinases) resides in the cell and the basic DNA content of the cell is the source for the changes.

When the basic DNA content of the cell is not directly sufficient as source for genetic adaptation, adaptation may still occur, but involves acquisition of DNA fragments from other microorganisms (not of the same strain). Collectively, these acquisition processes are named *horizontal gene transfer*. Various ways exist by which DNA fragments can be transferred from one cell to another, can be taken up, or can be excised from the genome of a "donor" and integrated into a "recipient" organism. Some of these mechanisms are confined to a particular subclass of related microorganisms, whereas others have a broader range. The outcome, however, is that microorganisms in a community can profit to a certain extent from the DNA content of each different individual member.

The different mechanisms that can lead to changes in the DNA are described in detail. Many of these mechanisms are known from areas other than biodegradation itself, but will mostly be illustrated with examples from bacteria degrading organic pollutants.

MECHANISMS FOR GENETIC CHANGE

DNA Replication and Repair Systems

In its most elementary form, the base sequence of DNA can change through base pair substitutions, small deletions, or insertions. Some base pair changes may arise "spontaneously" as a result of chemical reactivity (e.g., deamination), whereas others might be caused by interaction with DNA-damaging chemicals or reactive oxygen species or by ultraviolet light. Some mutations, though, are the result of the activity of cellular enzymes acting on the DNA themselves (43). In growing cells, DNA polymerases, DNA repair enzymes, recombinases, and

topoisomerases are among those enzymes leading to base pair substitutions, small deletions, frameshifts, or small duplications. In nongrowing cells, DNA polymerases might not play such an important role; rather, enzymes acting on DNA supercoiling have been implicated in causing base pair mutations (44).

DNA polymerases are the main DNA-replicating enzymes in the cell, copying each DNA strand meticulously into a complementary one. Although proofreading activity is very high, DNA polymerase III makes accidental mistakes either by incorporating false bases or by incorrect exonuclease editing (45). In most cases, mispaired base pairs are corrected by the mismatch repair system of the cell (46,47). With a certain frequency, however, mispaired regions are not repaired to the original sequence but to that of the falsely incorporated bases. One or more base substitutions remain. The mean frequency for base substitutions is extremely low, but measurable, and accounts for between 5.10^{-8} and 10^{-10} per base pair per replicative round (48). The frequency of base pair mutations is not necessarily constant for all DNA templates, and the types of mutations (e.g., substitutions, deletions, frameshifts) depend on the local sequence and structure of the DNA template (44). For instance, short DNA sequence repeats can act as sites for DNA slippage (43,48–50), which may promote the occurrence of larger deletions or the formation of larger noncomplementary regions. Inverted repeats may also be more prone to base pair mutations because of the possibility of forming hairpin loops during replication or transcription. Differences between mutation rates were also found between leading and lagging strands (45).

As a result of this constant (low) rate of mutations, some cells will also suffer from mutations in certain DNA repair enzymes (such as MutS, MutH, or MutT) (47,51), or in the DNA polymerase itself (45). When these mutations cause a decrease in the proofreading activity of the DNA polymerase or of DNA repair enzymes, the cells turn into hypermutable microorganisms with a significantly higher rate of base pair mutations. On an average, hypermutable organisms seem to maintain themselves quite well, although their relative population size does not increase to much more than 2% (52). Only under specific conditions, their relative number may increase dramatically (53).

The following features of catabolic pathways have been shown to be adaptable by relatively simple base pair mutations (substitutions, small deletions, or duplications):

- kinetic properties, substrate range, inhibition characteristics, or temperature stability of catabolic enzymes
- inactivation of enzyme functions that interfere with a catabolic pathway
- effector specificities and other properties of regulatory proteins
- changes in the operator-promoter regions of catabolic pathways.

Alterations of Enzymatic Parameters

This aspect seems rather trivial in the area of in vitro genetic engineering techniques. However, the discussion here is focused on documented spontaneous changes in catabolic pathways. Single base pair changes in the *amiE* gene that encodes acetamide amidase in *Pseudomonas aeruginosa* PAC resulted in expansion of the substrate range and improved the kinetic properties of the amidase toward butyracetamide and phenylacetamide (54). In addition, different types of mutations in the *dhlA* gene for haloalkane dehalogenase of *Xanthobacter autotrophicus* could be retrieved when selecting for growth on 1-chlorohexane (55). The resulting dehalogenases had better kinetic properties than the wild-type enzyme with 1-chlorohexane as substrate (Fig. 1). Various types of mutations could also be isolated in the genes for protocatechuate 3,4-dioxygenase and vanillate demethylase of *Acinetobacter calcoaceticus*, which affected kinetic properties and thermostability (56,57).

Inactivation of Enzyme Functions

Certain enzyme activities may interfere with a catabolic pathway when they result in the formation of a toxic intermediate. In such cases, there is probably a strong selection for mutants in which the genes for these enzymes are inactivated. For example, *A. calcoaceticus* strains that lack part of the enzymes for protocatechuate metabolism produce the toxic intermediate β-carboxymuconate when grown on *p*-hydroxybenzoate. It is easy to obtain mutant strains in which the *pcaGH* genes for protocatechuate 3,4-dioxygenase, which catalyzes the formation of β-carboxymuconate, have been inactivated by base pair mutations (58). Natural isolates of bacteria with catabolic properties also display evidence for inactivation of unfavorable enzyme functions. In the *Burkholderia* sp. strain PS12, a bacterium capable of using chlorobenzenes as the sole carbon and energy substrate, the *tlpE* gene for

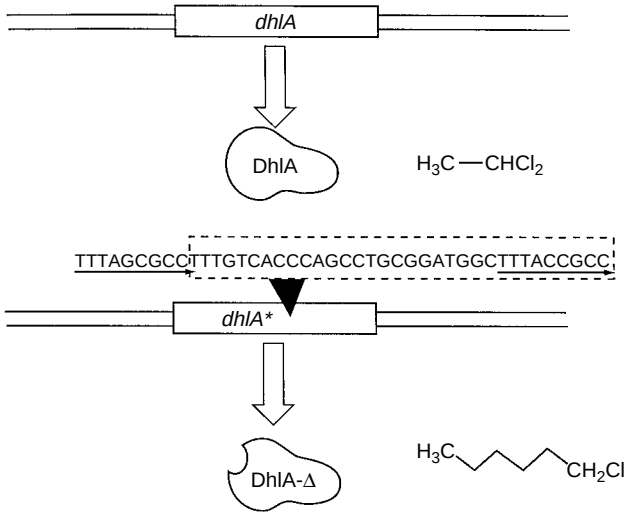

Figure 1. Spontaneous deletion occurring in the *dhlA* gene for dehaloalkane dehalogenase of *Xanthobacter autotrophicus* GJ10 that results in expansion of the substrate range to 1-chlorohexane (55).

catechol 2,3-dioxygenase is inactive, probably because this catechol 2,3-dioxygenase produces a toxic intermediate from chlorocatechols. Three base pair mutations can correct the *tlpE* gene, resulting in the formation of catechol 2,3-dioxygenase activity (10).

Regulatory Functions

Sometimes organic compounds are not used as growth substrates because they do not act as effectors for the regulatory proteins, which activate the catabolic pathway (38). Mutations in the genes for the regulatory proteins or in promoter-operator regions can then lead to pathway activation. For example, *Pseudomonas azelaica* HBP1 is a bacterium capable of growing on 2-hydroxybiphenyl (59). The three enzymes needed for 2-hydroxybiphenyl degradation are capable of converting other types of substituted phenols, such as propylphenol or butylphenol. However, the organism does not grow on these compounds as the main pathway regulatory protein, HbpR, does not recognize them as effectors (60). Mutants of *P. azelaica* can be selected by growing the organisms on isopropylphenol (61). The mutation maps in the *hbpR* gene and causes a constitutive activation of the 2-hydroxybiphenyl pathway (60) (Fig. 2). Such mutations can also be selected in various other catabolic pathways. Mutations in *dmpR* change the effector range of this regulatory protein in phenol catabolism of *Pseudomonas* sp. strain CF600 (62) or result in a constitutive phenotype (63). Similarly, mutations in XylR and XylS, the regulatory proteins of toluene and xylene degradation in *P. putida,* can result in the change of effector specificity (64,65) or in a constitutive phenotype (66,67). Spontaneous mutants of *Comamonas testosteroni* could be retrieved by selection for growth on phenol, in which a silent phenol catabolic pathway had become active. It turned out that mutations had inactivated the *aphS* gene, which normally acts as a factor to repress transcription of the phenol catabolic genes (68).

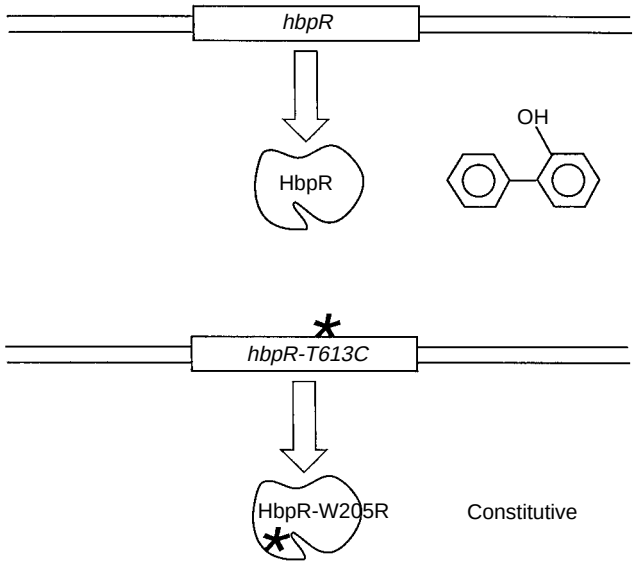

Figure 2. Spontaneous base pair substitution in the *hbpR* gene of *P. azelaica* HBP1. This mutation results in a constitutive activation of the genes for 2-hydroxybiphenyl degradation, which allows the strain to grow on 2-propylphenol (60,61).

Regulation of pathway expression can also be influenced by mutations occurring in the relevant promoter or operator regions. Illustrative in this respect are studies performed with the *pheBA* genes for phenol metabolism. When *P. putida* cells containing the promoterless phenol catabolic gene *pheBA* are incubated in a medium with phenol, mutants growing on phenol can be retrieved. Some of these genes used phenol constitutively and contained base substitutions that created a consensus $\sigma 70$ promoter (69).

Homologous Recombination

Theory. Several enzymes act on the DNA by promoting strand exchange between homologous regions, by cutting and pasting or by inserting foreign DNA. Homologous recombination by RecA and RecBCD, for example, can occur between DNA fragments with sufficient homology (i.e., around 80%) and when the length of the overlapping homologous fragment is at least 15 to 25 nucleotides (70). Because all genomic DNAs analyzed to date contain repeated sequences of this length or more (71–74), it must be assumed that homologous recombination can be important for large-scale rearrangements in genomes. In some long-term laboratory adaptation experiments, recombination between repeated sequences, which resulted in the loss of a particular gene fragment, was demonstrated (75). With a lower frequency, recombination can also occur between sequences with less than 15–25 base pair overlap. This recombination, though, is probably not RecA-dependent but involves activity of DNA gyrase (76). Studies on *A. calcoaceticus* demonstrated that even regions as small as four base pairs were sufficient for DNA recombination (58).

Effects on Catabolic Pathways. The direct effects of homologous recombination in bacteria carrying catabolic pathways are seen mostly in the occurrence of spontaneous deletions between larger homologous regions and the concurrent loss of catabolic functions (77–80). The homologous regions that promoted recombination consisted of different copies of the same insertion element (81), long sequence repeats (82), or different copies of catabolic genes themselves (83). In some cases, recombination occurred between homologous regions on plasmids and on the chromosome. This resulted in integration and excision of a plasmid into and from the chromosome, sometimes accompanied by deletions (84,85). Recombination was also assumed for the formation of hybrid genes, which are sometimes found in catabolic pathways (83,86), or for gene duplications (75,87,88). The different effects of recombinatory mechanisms have been very well studied in *A. calcoaceticus*. *Acinetobacter* can take up linear DNA fragments and the organism is very effective in incorporating such DNAs into the chromosome. It was found that RecA-dependent recombination also plays a role in mutation repair because mutations in a gene from the protocatechuate pathway (*pcaJ*) were very effectively repaired by recombination with a homologous gene (*catJ*) from the catechol pathway (89,90).

Gene Organization. It has to be assumed that recombinatory mechanisms are the general driving forces in structuring catabolic operons. The genetic organization of catabolic gene clusters very often shows blocks of genes with a similar organization, followed or interrupted by other different gene blocks (Fig. 3). For example, the gene order of the so-called *meta*-cleavage pathway cluster is conserved among bacteria using toluene (*xyl* genes), phenol (*dmp* genes), and naphthalene (*nah* genes). However, all three pathways have different upstream genes, which are part of the same operon. For the *xyl* genes they are *xylXYZL* (for toluate dioxygenase and dihydrodiol dehydrogenase), for the phenol pathway they are *dmpKLMNOP* (for a multicomponent phenol hydroxylase),

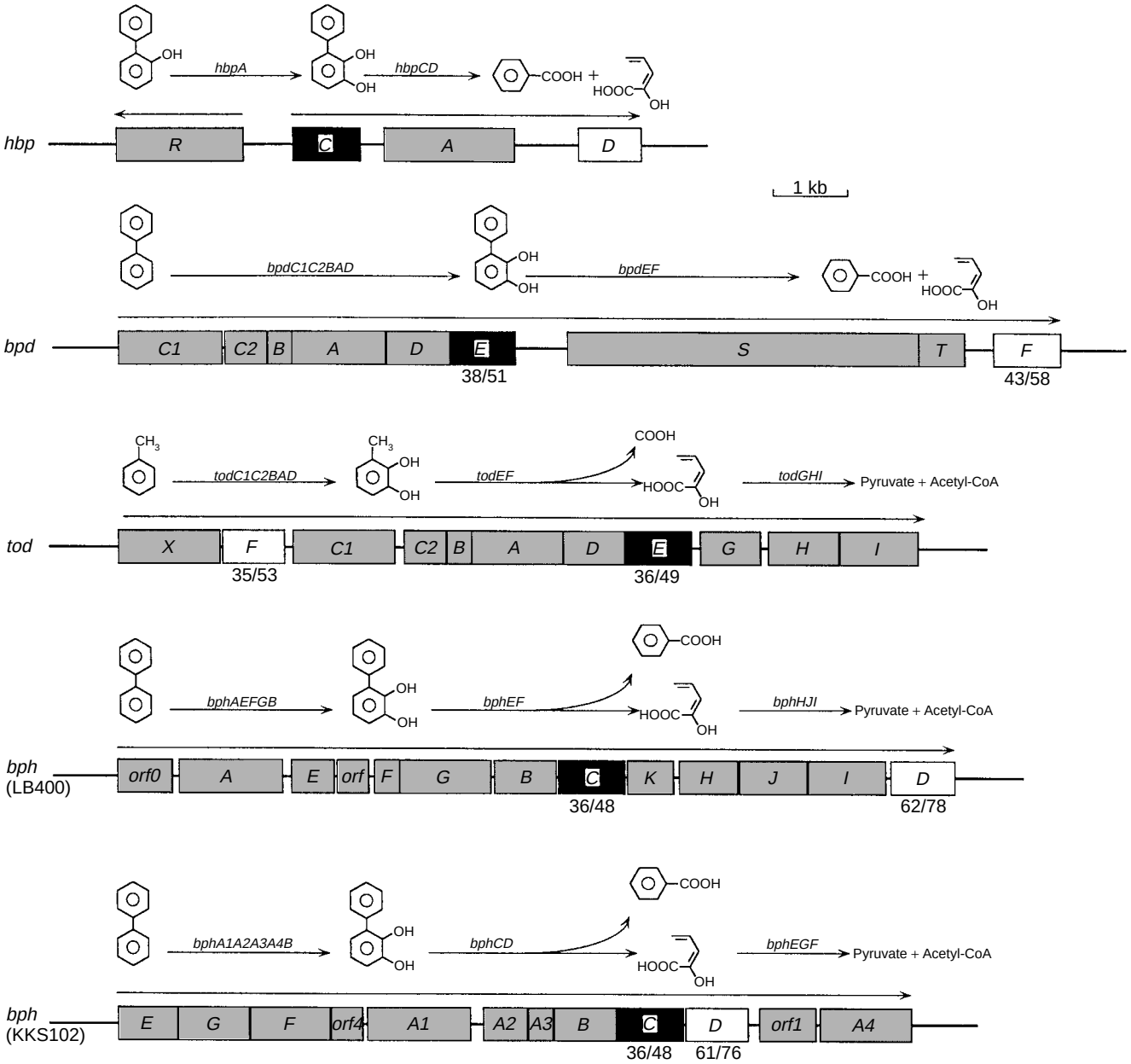

Figure 3. Examples of rearrangements in similar catabolic gene clusters. The focus is on the positions of the gene for extradiol dioxygenase (black bar) and for hydrolase (white bar). Clearly, different gene fragments are found in between those two genes in different catabolic pathways. This might point to a region, which is prone to insertions and/or deletions. Pathways and involved gene functions are summarized above the gene structures. Figures below the black and white bars indicate percentages of identity and similarity, respectively, with the corresponding genes in the *hbp* pathway. References: *hbp*, for 2-hydroxybiphenyl degradation in *P. azelaica* HBP1 (60), *bpd*, for biphenyl degradation in *Rhodococcus* sp. strain M5 (92), *tod*, for toluene degradation in *Pseudomonas* sp. strain F1 (93,94), and *bph*, for biphenyl degradation in *Pseudomonas* sp. strain LB400 (95,96) and in *P. pseudoalcaligenes* strain KKS102 (97–99). Drawing by Marco Jaspers.

and for the naphthalene pathway they are *nahG* (for salicylate hydroxylase) (37). Sometimes, only a single gene seems to have "moved" to a different position (91). Two mechanisms might be responsible for this: (*1*) genes were independently acquired into a gene cluster, but at a different position, or (*2*) gene fragments exchanged their position within a catabolic gene cluster. Interestingly, strong sequence conservation often correlates with conservation of gene organization, which could indicate that rearrangements on the DNA somehow triggered further base pair mutations (37).

Transposition and Integration

A special class of recombinatory enzymes includes transposases, integrases, invertases, or resolvases, which can promote the insertion, reversion, or deletion of discrete DNA fragments at more or less specific recognition sites. Numerous examples of these enzymes have also been found to act on the genes for catabolic pathways or have been associated with them.

Insertion Elements. Insertion elements (also called insertion sequences or IS elements) are relatively small DNA sequences (<2.5 kb) (100) that are capable of inserting themselves into target DNA molecules. The boundaries of IS elements are formed by two more or less specific inverted repeats, mostly of a length between 10 and 40 bp (100). Several hundreds of different IS elements have been described from basically all taxonomic groups of bacteria. They have been grouped in some twenty families with similar characteristics (100). Their capacity to insert into DNA molecules is dependent on a transposase enzyme, which is encoded on the insertion element itself. The transposase cuts both the DNA target and the connection of its own ends with the DNA in which it resides. The inverted repeats thereby function as binding sites for the transposase. If the transposase completely cuts its IS element out of the residing DNA, it is subsequently pasted into the new target by a cut-and-paste mechanism. In this case, no copy of the IS element remains at the original position. Other transposases only break one strand of the residing DNA and connect one strand of each IS end to the target site. The IS element is then replicated and temporarily, two copies arise (replicative mechanism). Both copies may subsequently recombine, either by the recombination system of the host cell or more effectively, by a specific resolvase enzyme (see section "Replicative Transposons").

As a result of their mode of action, IS elements can cause a wide variety of DNA rearrangements and can strongly influence gene transcription in regions near the insertion site. The following effects of insertion element activity were observed in catabolic-pathway evolution:

- gene inactivation, that is, interruption of an open reading frame (ORF)
- gene activation, for example, by deleting from an interrupted ORF
- transcription activation
- rearranging DNA, for example, by serving as targets for DNA recombination between multiple IS copies
- capture and mobilization of DNA fragments.

Gene Inactivation and Activation

Pseudomonas stutzeri strain OX1 contains about 10 copies of a 3-kb IS element (IS*Ps*1), which is involved in activation and inactivation of genes for xylene degradation (101). *Pseudomonas stutzeri* strain OX1 normally grows on *o*-xylene and toluene, but not on *p*- or *m*-xylene. The strain does have the genetic capacity to metabolize *p*- and *m*-xylene, but one of the copies of IS*Ps*1 in strain OX1 is present in front of *xylC*, thereby inhibiting transcription of the genes for *m*- and *p*-xylene degradation. Spontaneous mutants growing on *m*- and *p*-xylene but no longer on *o*-xylene could be obtained. In these bacteria, the IS element had disappeared from its location in front of *xylC*. Instead, one copy was found in the *touA* gene, which encodes *o*-xylene monooxygenase, thus inactivating synthesis of this enzyme. However, spontaneous revertants that could metabolize all xylene isomers could be isolated. In these, neither IS copy was traceable near *xylC* or *touA*.

There is also evidence for insertion inactivation of genes in natural isolates of bacteria with catabolic properties. On the plasmid pJP4 of *Ralstonia eutropha* JMP134, which contains the genes for break down of 2,4-dichlorophenoxyacetic acid, (2,4-D) one copy of an insertion element (IS*JP4*) is present in the coding region for an activator protein of the 2,4-D pathway (Fig. 4). This results in the formation of a truncated regulatory protein, which is not capable of activating *tfd* gene expression (102,103). IS*1236* is an insertion element of *A. calcoaceticus*, which inserts itself preferentially in the gene *pobR* and inactivates the pathway regulator of *p*-hydroxybenzoate degradation (104).

Transcription Activation. The ends of IS elements may contain sequences functioning as promoter-recognition sites. Among others, this was discovered when studying the expression of the *pheBA* genes of *Pseudomonas* sp. strain EST1001. The *pheBA* genes code for a short phenol-degradation pathway, that is, catechol 1,2-dioxygenase and phenol monooxygenase, respectively. When the *pheBA*

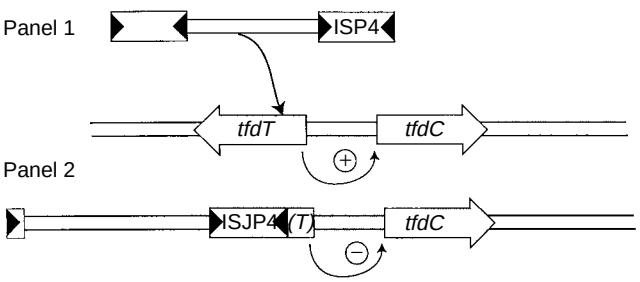

Figure 4. Insertional inactivation of the *tfdT* regulatory gene in the *tfdCDEF* chlorocatechol pathway of *Ralstonia eutropha* JMP134 (pJP4) (103). The *tfdT* gene product positively regulated the expression of *tfdC*. At some point, a transposon formed by two copies of insertion element ISJP4 inserted into the *tfdT*-coding region (panel 1), thereby interrupting the regulatory circuit. The present situation as found on plasmid pJP4 is drawn in panel 2.

genes are introduced into *P. putida* PaW85, transcription activation takes place from a promoter 1.5 kb in front of *pheB* because of cross-regulation by the chromosomally located CatR protein (105). When this region of the plasmid containing the original promoter was removed, the phenol phenotype was not expressed, but spontaneous mutants that grew on phenol could be recovered. In some of these, an IS element (IS*1411*), which is downstream of *pheA* in its original configuration, had inserted another copy of itself directly upstream of *pheB* (106) (Fig. 5). IS*1411* carries a good constitutive promoter at its 3′ end and it could be demonstrated that this promoter drives *pheBA* expression in the new situation (106). In addition, in the 1,2-dichloroethane-degrading organism *X. autotrophicus* GJ10, an insertion element can enhance gene expression. *Xanthobacter autotrophicus* normally does not grow on toxic high (5 mM) concentrations of bromoacetate, but mutants that grow on bromoacetate could be isolated after selection. These mutants could grow as a result of increased expression of the enzyme haloacetate dehalogenase. Genetic analysis demonstrated that insertion of the IS element IS*1247* had occurred at a site upstream of the *dhlB* gene, presumably driving higher constitutive expression from a promoter at the IS end (107).

DNA Rearrangements and Gene Capturing

In numerous catabolic pathways, IS elements are located in or near catabolic genes, which suggests that they have had a role in rearranging DNA fragments and perhaps in bringing new genes together. The insertion sequence IS6100 was implicated in the formation of duplications of the *nyl* genes for nylon degradation in *Flavobacterium* (87). In various natural isolates, strong evidence was found for mechanisms by which IS elements may capture gene fragments and assemble new pathway clusters. For example, two copies of an almost identical IS element (IS*1066* and IS*1067*) were discovered near the *tcbAB* genes for chlorobenzene dioxygenase and chlorobenzene dihydrodiol dehydrogenase (108,109). The structure was shown to be mobile, which made it likely that the IS elements had captured the *tcbAB* genes from another microorganism and grouped them with the genes for chlorocatechol metabolism. Recent analysis of two other chlorobenzene degrading organisms, *Ralstonia* sp. strain JS705 (11) and *Burkholderia* sp. strain PS12 (10),

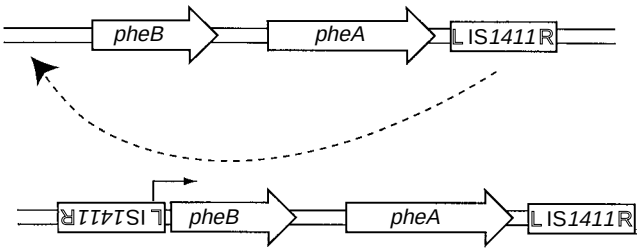

Figure 5. Events that resulted in the spontaneous formation of a constitutive promoter for the *pheBA* genes for phenol degradation (106). Inverted insertion of IS*1411* into a position upstream of *pheB* provided a proper promoter sequence, which is located at the end of the IS element.

reveals that similar events have taken place. Both strains carry gene regions for chlorocatechol degradation combined with those for an aromatic ring dioxygenase and dihydrodiol dehydrogenase, which were surrounded by (putative) IS elements or fragments of IS elements.

IS*1071*

IS*1071* is an unusual insertion element. It is larger than most other IS elements (3.2 kb) and does not produce target site duplication. It has been found in an unusual number of different bacteria as well and is very often associated with catabolic genes. For example, IS*1071* was detected in *Alcaligenes* sp. BR60 plasmid pBRC60 (3-chlorobenzoate degradation), *Achromobacter xylosoxidans* plasmid pEST4011 (2,4-D degradation), *P. pavonaceae* haloalkane dehalogenase gene region, *Ralstonia eutropha* JMP134 plasmid pJP4, *Ralstonia* sp. plasmid pTFD41 (2,4-D degradation), *P. putida* UCC22 plasmid pTDN1 (aniline degradation), *Comamonas testosteroni* T2 plasmid pTSA (toluene sulfonate degradation), and *P. pseudoalcaligenes* POB310 plasmid pPOB (4-carboxydiphenyl ether degradation) (110,111). Convincing evidence that IS*1071* had been independently involved twice in capturing the *cba* genes for 3-chlorobenzoate degradation (112) could be presented.

Composite Transposons

When two IS elements of the same kind are in the vicinity of each other, a composite transposon may be formed, which can mobilize the genetic information located in between the two IS copies. This situation is exemplified by the classic antibiotic-resistant transposons Tn*5* and Tn*10*, but also by several catabolic transposons. For example, transposon Tn*5280* contains two inverted copies of an almost identical IS element (IS*1066* and IS*1067*) and the *tcbAB* genes for chlorobenzene dioxygenase and dihydrodiol dehydrogenase in between (108,109). Both IS copies need not necessarily be intact to achieve transposition. This was shown for a transposable element on plasmid pJP4 in *R. eutropha* JMP134 that contains part of the genes for 2,4-D metabolism (14). The boundaries of the transposable element are formed by one intact and one partial copy of the IS element IS*JP4* (102). It is also not necessary for the transposition to have two copies in inverted orientation. Two copies of IS*1071*, in direct repetition, surround the *cba* genes for 3-chlorobenzoate degradation of *Alcaligenes* sp. strain BR60 and form the composite transposon Tn*5271* (81). However, the frequencies of recombination between two IS copies in direct orientation (with subsequent loss of the transposon structure) are higher than in inverse orientation (81). Not for all composite structures could transposition be demonstrated. For example, Tn*5707* and Tn*5542* are formed by two direct copies of insertion elements IS*1600* and IS1489, respectively. Both IS*1600* copies flank the *cbn* gene cluster for chlorocatechol degradation on plasmid pENH91 in *Alcaligenes eutrophus* NH9 (113), whereas the IS*1489* copies encompass the genes for benzene dioxygenase and dihydrodiol dehydrogenase in *P. putida* ML2 (111).

Replicative Transposons

Replicative transposons (alternative names: class-II transposons or Tn*3* family) do not contain two copies of an IS element. Instead, at one end, the coding regions for the transposase and, often, for a resolvase are found. The transposon boundaries are formed by two inverted repeats with a length of 25 to 100 bp (114). As described earlier (see "Insertion Elements"), the TnpA transposase in replicative transposons makes double-stranded cuts in the target DNA but single-stranded cuts in the residing copy. Only one end at each single strand is connected with the target and the complete transposon is replicated, resulting in two copies. Resolution of these two copies, after which only one transposon remains, is performed by the product of the resolvase gene (*tnpR*), which recognizes a specific resolvase binding site present on the transposon.

Some catabolic transposons belong to this type and often form complex multiple transposable elements. Classical examples are the replicative transposons of the TOL plasmids. Tn*4653* is a 79-kb large transposon that encompasses all of the *xyl* genes (115). The smaller Tn*4651* is 56 kb in size and is contained within Tn*4653*. Tn*4651* is a completely functional transposon, capable of replicative transposition and resolution of the two copies afterward. It is unusual because of the location of the resolvase coding regions and binding sites (82) and because of its transposase (116). Tn*4653* was shown to perform replicative transposition, but resolution was dependent on factors provided by Tn*4651*. A smaller structure of Tn*4651* (Tn*4652*) could arise in which the *xyl* genes had been deleted through recombination at the two direct repeat sequences of 1.4 kb that flank the *xyl* genes (82). The naphthalene plasmid NAH7 bears another (defective) replicative transposon, which contains all of the *nah* and *sal* genes (117). This 37-kb transposon structure, however, only carries a resolvase but lacks its own transposase. It can therefore only be "transposed" by other similar transposases in *trans*.

Integrons

Integrons form a special class of mobile elements, which are involved in inserting and deleting gene fragments into a predefined region contained within the integron (118,119). Integrons consist of so-called 5′- and 3′-elements and an integration site. The 5′-element contains a gene for an integrase and the 3′-element contains two ORFs of unknown function and a gene for sulphonamide resistance. A 59-bp element at the boundary of the 3′ region, or elsewhere between the 5′- and the 3′-element, seems involved in determining the integration site. The 5′- and 3′-elements are highly conserved among integrons known, whereas the 59 bp can differ, but predict a stem-loop-like structure. The recombination site itself is confined to a GTTRRRY-motif, which is common to the right end of the 5′-element and the left end of the 3′-element. Integrated genes in the integron are always transcribed from left to right, possibly from a promoter in the 5′-element. Integrated genes are further inserted very strictly, leaving very few bases upstream and downstream of the actual coding region. Genes can be inserted into integrons in a sequential manner.

Available literature does not indicate integron activity in catabolic gene clusters. In some catabolic gene clusters, however, putative integrase genes were localized near the catabolic genes. Two of these involve gene clusters for haloalkane dehalogenation in the *Mycobacterium* sp. strain GP1 (for 1,2-dibromoethane degradation), and in the *P. pavonaceae* strain 170 (1,3-dichloropropene degradation). Both strains carry an almost identical *dhaA* haloalkane dehalogenase gene, which is also conserved in a third strain *Rhodococcus rhodochrous* NCIMB13064. Comparison of the three *dhaA* gene regions showed remnants of various deletion events. Both *Mycobacterium* and *R. rhodochrous* carried a gene for an invertase upstream of the *dhaA* gene, whereas in both *Mycobacterium* and *P. pavonaceae*, (nonidentical) genes for integrases were found upstream of *dhaA*. A copy of IS*1071* was detected downstream of *dhaA* in *P. pavonaceae*. This suggests that these recombinatory elements were involved in creating the deletions and perhaps insertions (120). Another putative integrase gene was detected near catabolic genes by DNA sequencing of the plasmid pNL1 of *Sphingomonas aromaticivorans* (121).

Horizontal Gene Transfer

Although transposases and integrases promote DNA rearrangements, they do not necessarily promote transfer of genes between different bacteria. It is especially this aspect of gene transfer between different bacteria of a population or in a community between different populations that opens up the true diversity of all genes in a community. However, it is certainly not true that every piece of genetic information in a community is accessible to all others because the host ranges of the transfer mediating elements can be restricted. Generally, one considers three (or four) types of mechanisms for horizontal gene transfer: conjugation by plasmids, phage transduction, conjugative transposons, and transformation (or uptake of naked DNA).

Plasmids. Plasmids are independently replicating units of DNA in bacterial cells. Some plasmids are circularly closed, whereas others may be linear. Some plasmids contain the genetic information for their mobilization and/or transfer, which involves formation of the DNA pore or pilus and transfer of (usually) a single-stranded molecule of the plasmid into the recipient cell. Plasmids seem to be normal residents of bacterial cells and express enormous varieties of host ranges. Numerous plasmids have been described in the literature and almost all catabolic pathways have been associated with the presence of plasmids (although they are not necessarily always encoded on plasmids) (111,122). Classic examples are the TOL plasmids (for toluene degradation), the NAH plasmids (with the naphthalene pathway genes), plasmids for 2,4-D degradation, for alkane, for dichloromethane, for haloalkane, for toluene sulfonate, for chlorobenzoate, or for chlorobenzene degradation. Most of these plasmids were shown to be transmissible in the laboratory. Some plasmids were reported to occasionally give rise to a process called *retrotransfer*, which is the capture of genes

from the recipient organism by the plasmid, rather than donation from the donor to the recipient of the plasmid.

Natural Plasmid Transfer. Considerable efforts have been made to demonstrate natural plasmid transfer or plasmid transfer from introduced donors to indigenous recipient bacteria in the environment. There can hardly be any doubt left that plasmids do play an important role in the dissemination of all kinds of genetic material including catabolic genes, in microbial communities. A few recent examples may illustrate these points. Transfer of a plasmid for 2,4-D degradation to members of the indigenous soil microbial community was achieved by inoculating about 10^6 donor bacteria per gram of soil. Subsequent incubation in the presence of 100-mg/kg 2,4-D probably selected for the appearance of transconjugants, which grew up to population sizes of 10^6/g (123). Transconjugants that were retrieved from the soils were of the genera *Burkholderia* and *Ralstonia* were found to be indigenous members of the soils and contained the intact plasmid of the donor.

Another strong case for plasmid transfer in the natural environment is demonstrated in the following incident: During a major accident with contaminated mine water in 1989 in an Estonian watershed, it was decided to deliberately release phenol-degrading *P. putida* PaW85 bacteria into the river. These bacteria contained a plasmid with the *pheBA* genes for phenol degradation. A few years later, an attempt was made to retrieve the phenol-degrading bacteria from several sites in the watershed. About one-third of the bacteria growing on phenol plates were found to contain the *pheBA* genes; however, these bacteria were of a different species than the original donor strain, which apparently had not survived. It was concluded that they had been transferred from the introduced bacteria into indigenous strains (124) as the *pheBA* genes were not retrieved from any environment before.

On several occasions, circumstantial evidence was provided for gene transfer with catabolic genes in the natural environment. Mostly, this evidence consists of comparisons of isolates with the same catabolic properties from different geographic origins. Incongruent patterns between phylogenetic data obtained on the basis of 16S rDNA sequence comparisons and catabolic gene sequences (e.g., for *nahAc*: naphthalene degradation (125), for *tfdA*; 2,4-D dioxygenase (126), for *dhaA*; haloalkane dehalogenase (127), or for atrazine) (128) was taken as a proof for horizontal transfer of those catabolic genes. This can be viewed as demonstration for natural plasmid transfer as many of those markers are located on plasmids.

Plasmid Sequence Analysis. The near future will not only face a boom in complete genome sequences, but also in plasmid genome sequences. This will provide a very detailed insight into the differences between plasmid incompatibility groups, in conjugative functions, and in acquisition mechanisms. The only catabolic plasmid for which the complete sequence is available is the 184-kb plasmid pNL1 of *S. aromaticivorans* (121). About one-third of this plasmid is catabolic gene region, one-third is involved in conjugative transfer functions, and one-third is attributed to plasmid maintenance. Contrary to the classic large operons that make up single catabolic pathways in *Pseudomonas*, plasmid pNL1 contains multiple transcriptional units seemingly necessary for just one "pathway." In addition, several pathways, for example, for biphenyl and naphthalene, seem genetically intertwined. The conjugative system of pNL1 appeared mostly similar to that of *Escherichia coli's* F-plasmid. Furthermore, many hypothetical proteins were predicted with little homology to anything else known. From this and other work (129) the picture emerges that plasmids can act as collectors of junk, which might be very important for their role in horizontal gene transfer (see later section "Selection Mechanisms").

Phages. Phages are capable of transducing DNA, which means that they accidentally package DNA from the chromosome and transport this to another host cell that the phage infects. Recent genome data indicate that most genomes contain several intact phages or prophages, including a phage integrase, of which only a few phage-type proteins are left on the chromosome. Despite the fact that soils and aquatic systems contain numerous phages (130), no phages have been associated with catabolic gene transport.

Conjugative Transposons. Conjugative transposons combine both phage and plasmid characteristics. They encode the factors for conjugation and harbor a phagelike system to integrate themselves into the genome of the host. In contrast to IS elements and other transposons, conjugative transposition does not cause target-site duplication or replication (131). The integration is mediated by an integrase enzyme, which is encoded on the conjugative transposon itself. During transposition, the conjugative transposon excises and forms a circular intermediate in which both ends are connected in a heterologous hybrid. Integration into a new target proceeds from the circular form. There are a few examples of catabolic genes located on conjugative transposons or elements presumed to be such. The transposon Tn*4371* (132) from *Alcaligenes* encompasses a 60-kb region with a 13-kb region containing the *bph* genes for biphenyl degradation. The element can transpose itself into plasmid RP4, preferentially at two sites (containing the TTTTTCAT motif) and from there into other locations on the chromosome (five sites in *R. eutropha*), in other recipient bacteria or on other plasmids (pSS50). The left end of Tn*4371* locates the integrase gene, facing inward. The integrase has homology to phage P22 and Tn*1545* integrase. The right end contains a second transposon, Tn *bph*. In addition, the biphenyl and salicylate genes from *P. putida* KF715 seem to be located on a 90-kb conjugative transposon (79). The bph-sal element could be transferred from the original strain to *P. putida* AC30 and from this strain to KT2440, thereby always integrating into the chromosome. At present, it is not clear if a specific sequence is targeted by the bph-sal element and which type of integrase is responsible for integration.

The clc-element of *Pseudomonas* sp. strain B13, which carries the genes for 3-chlorobenzoate and chlorocatechol

metabolism, can also be considered as a conjugative transposon, although other features allow it to be called a "gene island" (133). It consists of a region of 105 kb that is integrated in the chromosome of its host. In *Pseudomonas* sp. strain B13, there are two sites of integration, which are formed by genes for a glycine tRNA (Fig. 6) (134). The element itself carries an identical 18-bp sequence as the glycine-tRNA gene. Preferentially, during growth on 3-chlorobenzoate, the element excises and forms a circular intermediate. The integrase of the clc-element is weakly related to that of Tn*4371* but more to phage P4–type integrases. The feature to insert site-specifically into tRNA genes is very typical for pathogenicity islands (135) and bacteriophages.

Uptake of Naked DNA. Transformation or the uptake of naked DNA molecules is also a mechanism by which bacteria can acquire new genetic material, in principle, from any source. Some bacterial strains such as *A. calcoaceticus*, *P. stutzeri*, or *Bacillus subtilis*, develop a state of natural competence (136) during which DNA is taken up preferentially. Some bacteria are also capable of producing larger quantities of extracellular DNA, which can potentially increase the possibility for natural transformation (137). Although several experiments have demonstrated that transformation of naked DNA in the environment can occur (136,138), the contribution of natural transformation to the formation and acquisition of catabolic pathways is not known.

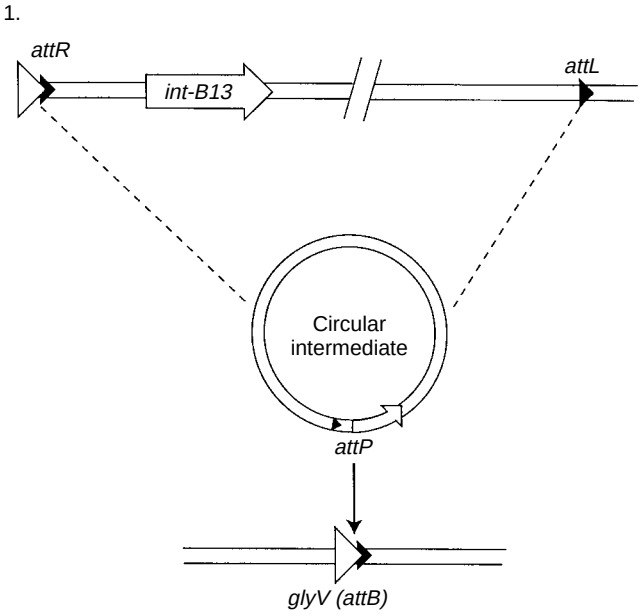

Figure 6. Mechanism of excision and integration of the conjugable clc element in *Pseudomonas* sp. strain B13 (134). The element uses a phage-type integrase (shown here as *int-B13*) to integrate site-specifically in the gene for glycine tRNA (*glyV*). An 18-bp sequence is identical between the *glyV* gene and the element (here indicated as black arrow). Upon insertion, the original *glyV* gene is restored. Attachment-site determinations (*attR*, *attL*, *attP* and *attB*) are according to phage definitions.

Trends in Catabolic Pathway Evolution

Selection of Mechanisms

What is the overall picture that seems to be emerging for the evolution of catabolic pathways in bacteria? As shown earlier, any one genetic mechanism may operate and genetic variation is the sum of all those processes. In this aspect, all the processes creating genetic variation may be considered as the "evolutionary toolbox" of the organisms (139–141). When those pathways that really seem to have been selected for by pollution of the environment with new types of chemical substances (11,18,109,127) are looked into, the most pronounced mechanism for evolution seems acquisition and recombination of sets of genes originating from different organisms, probably by insertion elements. These recombined structures disseminate in a population by conjugative elements, perhaps until the most suitable recipient organism is encountered, which can then proliferate (11). The second step, which perhaps has not yet been observed in real "novel" pathways, is the development of proper regulatory systems (see following section). Secondary mutations and deletions seem to follow the primary rearrangements if the pathway is to establish itself.

For this scenario, it has to be assumed that many of the functions that have been assigned as "typical" for xenobiotic degradation (e.g., dehalogenating enzymes) are found in a suitable form in microbial communities. It seems less likely that those "novel" genes (e.g., the chlorocatechol 1,2-dioxygenase gene) have diverged from normal catabolic genes (e.g., *catA* for catechol 1,2-dioxygenase) since the introduction of large-scale chemical synthesis. Even calculated very optimistically, such divergence times are more on the order of tens of millions of years (142). However, it is possible that the assumptions on rates of base pair substitutions are completely wrong as, for example, the hypermutable state of microorganisms may indicate.

If it is assumed that gene functions are acquired from existing organisms in the environment, then important questions remain. Bacteria with very similar (or even identical) catabolic properties can be found all over the globe, but does this indicate that they have independently picked up the same type of genes (sometimes even with the same type of mechanism)? This seems very unlikely, but might indicate that ignorance still exists on how and which genes are distributed in a microbial community, perhaps constantly, by conjugative elements. Alternatively, bacteria may be transported much more effectively throughout the globe than is thought of and thus cause distribution of bacteria that degrade toxic chemicals as well. For example, birds, wind, clouds, human beings themselves, or goods and products could be vehicles for contaminating microorganisms. Even crude chemicals such as crude oil might be contaminated with microbes degrading them.

It might be concluded that environmental pollution is not only selecting for new rearrangements and new catabolic pathways but also for particular types of genetic mechanisms. As seen earlier, several genetic processes are actually one-step evolutionary mechanisms. Transfer of a

plasmid to a new host is a one-step process, resulting in the acquisition of up to 250 kb of DNA. Activity of transposons or insertion elements is a one-step process, leading to novel DNA structures and combinations. If such genetic structures are favorable and the host carrying them can proliferate, this also means that the "evolutionary elements" themselves proliferate. In this respect, it is surprising that certain IS elements (IS*1071*) are often found near catabolic genes. Perhaps IS elements that carry strong promoters for downstream genes have an evolutionary advantage in catabolic pathway formation. Finally, some genetic adaptive mechanisms may even be directly regulated by environmental signals. It has been known for a long time that conjugation of the *tet* element in *Bacteroides* is triggered by the presence of tetracycline itself (143). In addition, the clc-element of *Pseudomonas* sp. strain B13 specifically excises when grown on 3-chlorobenzoate (144).

Regulation

If horizontal gene transfer and DNA rearrangements are considered as important mechanisms by which rapidly new functions can be acquired and assembled, then a few words should be said about the regulation of expression. Newly arranged DNA or newly incoming DNA may lack the necessary signals for optimum expression. Therefore, it can be expected that some of these DNA are not expressed properly, or are not expressed at all, or are constitutive (as discussed earlier, when insertion elements provide promoter sequences). De Lorenzo and Pérez-Martín proposed how regulatory systems might evolve in front of newly arranged catabolic operons (38). Their first premise was that regulatory genes and structural genes evolve separately. This was concluded from the observation that similar pathways have different regulators (e.g., *xylS* and *nahR*) and different pathways have similar regulators (*xylR* and *dmpR*). Secondly, novel specificities can only develop because the regulatory systems are leaky to some extent. This regulatory noise allows that regulatory proteins bind to other than their cognate operator and may have some affinity to a newly acquired gene region (e.g., activation of the *pheBA* genes by CatR in *P. putida* (105), or activation of the *tfdC*-promoter by TfdR) (103). Further fine-tuning then occurs by acquisition of secondary regulatory factors (e.g., σ^{54} or integration-host factor) or small mutations leading to a more specific effector range of the regulator.

CONCLUSION

Can it be expected that as increasingly taxonomically different bacteria will be isolated and characterized, other types of catabolic gene clusters and evolutionary mechanisms will be found? Most classical studies have been performed with "fast-growing" pseudomonads, which might not represent the true diversity of catabolic pathways in the environment or the true important pollutant-degrading organisms at a site. Some recent studies, employing more sophisticated isolation techniques (145–150) or molecular-detection techniques (4,151–153), seem to suggest that the most important species are not necessarily being looked into. In addition, recent studies on nitroaromatic degradation demonstrated the existence of completely different pathways than previously thought (26,154,155). Genetic analyses of catabolic pathways in some *Sphingomonas* strains (121,156,157) demonstrated that catabolic pathways are not expressed from nicely arranged operons. Rather, intertwined and dispersed multiple operons contribute to a particular pathway. This could indicate very different means for regulation of pathway expression. Studies on chlorocatechol degradation by *Rhodococci* indicated that rhodococcus-type chlorocatechol pathways may have evolved independently from those known in β- or γ-*Proteobacteria* (158). Finally, it may not be surprising to find very different "evolutionary mechanisms" or mechanisms with completely different ranges of activities. Only consider, for example, the very different recombination mechanisms in *Deinococcus radiodurans* (159).

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EXOBIOLOGY. See LITHOTROPHIC MICROBIAL ECOSYSTEMS IN THE SUBSURFACE

EXTRACELLULAR ENZYMES IN BIOFILMS

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The majority of microorganisms on earth live in microbial aggregates; examples are biofilms attached to solid surfaces (mostly at solid-liquid interfaces), activated sludge flocs in wastewater treatment plants, or associations of microorganisms with inorganic particles and organic detritus in natural surface water (1,2). These microbial assemblages are found ubiquitously in soil and aquatic environments; biofilms are involved in the colonization and infection of plants, animals, and man. They also occur in technical water systems and may sometimes grow on the surfaces of medical devices (3,4). A common feature of all these structures is that the microbial cells are embedded in a hydrated matrix of extracellular polymeric substances (EPS) that are produced by the biofilm organisms. Polysaccharides and proteins are characteristic components of the EPS (5). Polysaccharides have been assigned mainly structural functions in mediating the formation and mechanical stability of the three-dimensional, gel-like matrix of biofilms. In contrast, relatively little is known about the identity and functions of extracellular proteins in biofilms. Different extracellular enzyme activities have been detected in biofilms from diverse environments so that enzymes constitute at least part of the EPS proteins. Heterotrophic bacteria are common biofilm inhabitants, but fungi are also important producers of extracellular enzymes (6).

The majority of natural organic matter is composed of polymeric, high molecular weight compounds that are too large to be transported across microbial membranes into the cell. A main function of extracellular enzymes is seen in the hydrolysis of these compounds to smaller molecules that are readily taken up by the cells and used for cellular metabolism. Natural substrates of extracellular enzymes can be water-soluble polymers (many polysaccharides, proteins, nucleic acids) and water-insoluble substances (e.g., cellulose, chitin, lipids), as well as biogenic particles that are trapped in biofilms. Enzyme-mediated hydrolysis of polymers represents the rate-limiting step in the microbial utilization of dissolved and particulate organic matter in terrestrial and aquatic environments. Enzymes can also be involved in the modification and degradation of structural EPS within biofilms, promoting the detachment of bacteria from biofilms (4). Furthermore, extracellular enzymes are of importance in medical biofilms, because they can function as virulence factors in human infections (7). Some extracellular enzymes from bacteria and fungi are also of commercial interest (6) and are produced on a larger scale industrially. The activity of extracellular enzymes can be exploited in technical processes; as an example, biological wastewater treatment relies on the production of extracellular enzymes by microorganisms in activated sludge flocs and biofilms in fixed-bed reactors, which are used for the removal of organic matter. Extracellular enzymes can also be involved in biodeterioration processes, enzymatically attacking organic and inorganic materials (3). These examples underline the paramount ecological, economic, medical, and technical importance of extracellular enzymes, which strongly influence the physical, chemical, and biological properties of biofilms.

SECRETION MECHANISMS OF ENZYMES

Independent of their final localization in the biofilm, extracellular enzymes can have different sources. They are secreted by intact producer cells within the biofilm, they can be passively released from damaged or lysed biofilm organisms, or they can be trapped from the external environment.

Intact viable cells actively secrete enzymes by transport across the cytoplasmic membrane into the periplasmic space, into the multilayered cell walls of gram-positive bacteria, onto bacterial surfaces, or into the extracellular medium. These enzymes are secretory proteins that, in contrast to intracellular enzymes, only function after their passage through cellular membranes. Various distinct and specific mechanisms of enzyme secretion have been discovered in gram-negative and gram-positive bacteria. A number of recent publications have reviewed the biosynthesis and secretion of bacterial proteins, including enzymes (8–12). So far, mainly planktonic cultures of selected bacterial species were used to study the expression of extracellular enzymes, whereas relatively little work has been done to investigate the secretion of enzymes in biofilms.

Synthesis of secretory enzymes occurs inside the cell at ribosomes that are often directly bound to the cytoplasmic membrane. Secretion involves the transport of these enzyme proteins across the cytoplasmic membrane of gram-positive bacteria or across the inner and outer membranes of gram-negative bacteria. At present,

five main protein secretion pathways have been well characterized, which are designated type I to V secretion systems (Fig. 1).

The type I system is also named the *ATP-binding cassette* (ABC) pathway and involves a one-step protein export, bypassing the periplasm. An ABC exporter machinery consists of three different inner and outer membrane proteins, which form a porelike complex, directly connecting the cytoplasm with the extracellular medium (Fig. 1; 8).

The type II system or general secretory pathway, which is used by gram-negative bacteria, consists of a two-step

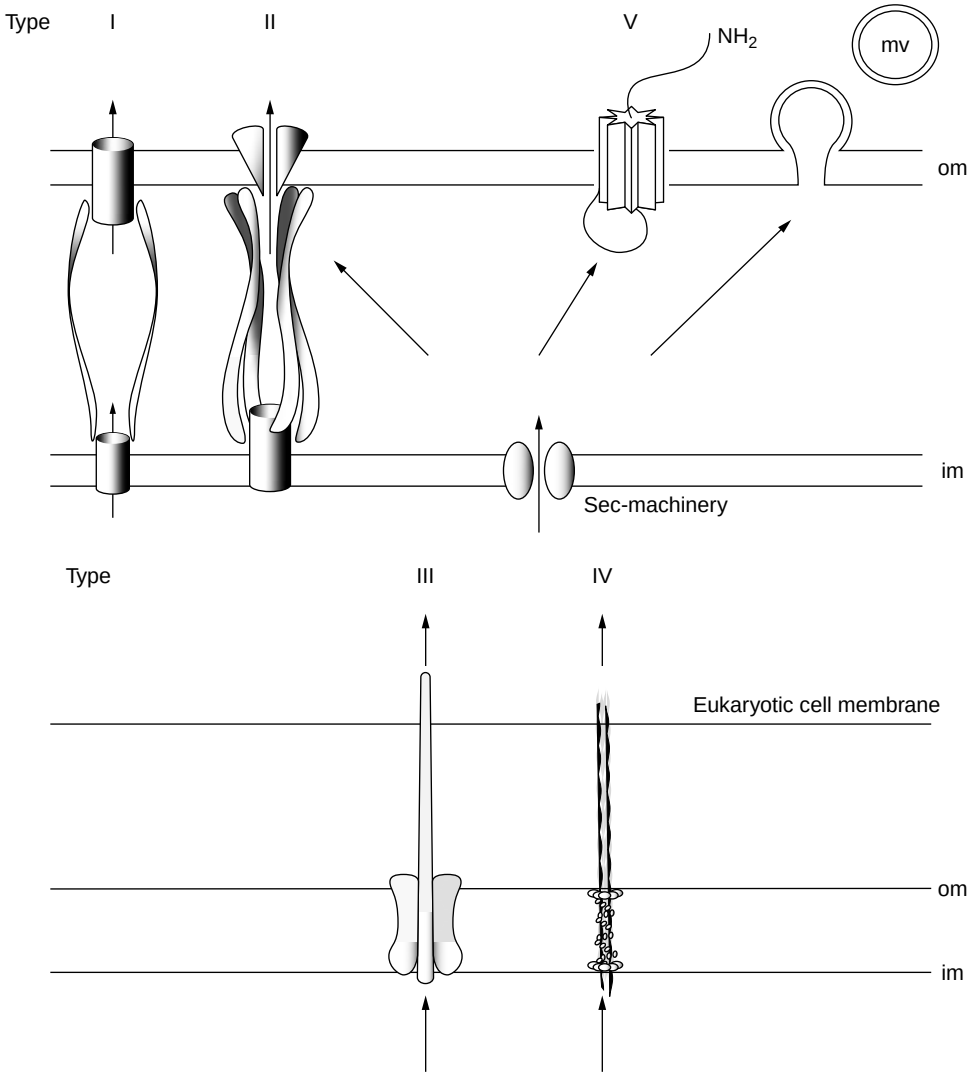

Figure 1. Pathways used by gram-negative bacteria for enzyme secretion (arrows indicate the direction of secretion). Top: The type I secretion system mediates the export of proteins in one step across both the inner membrane (im) and outer membrane (om) and is composed of a cytoplasmatic ABC-protein, a membrane-fusion protein, and a pore-forming outer membrane protein. The type II secretion system is a two-step pathway consisting of the Sec machinery that resides in the bacterial inner membrane and a complex apparatus (secreton); it is composed of 12 to 15 different proteins forming a transport channel that connects the inner and outer membrane. The type V secretion system is also named the *autotransporter pathway*. Autotransporter proteins first cross the inner membrane via the Sec machinery and subsequently insert into the outer membrane with their C-terminal domain that forms a pore through which the N-terminal domain is then secreted. An unspecific secretion pathway (top right) is characterized by the formation of membrane vesicles (mv) that are released from the bacterial cells and may contain various hydrolytic enzymes. Bottom: The type III and type IV pathways are found in many pathogenic bacteria and require contact between the secretion machinery and a eukaryotic target cell. The type III secretion system usually consists of 20 different proteins that form a large inner membrane transport channel and a needlelike structure protruding into the cytoplasm of the eukaryotic cell. The type IV secretion system is used to mobilize DNA and multisubunit proteins across bacterial membranes. About 10 proteins form a piluslike transport complex that spans both the inner and outer membrane.

process: (*1*) the initial signal peptide-mediated transport across the inner membrane and (*2*) the subsequent translocation of the transient periplasmic intermediate across the outer membrane. After translocation of the protein through the inner membrane, the N-terminal signal sequence is cleaved off by a specific signal peptidase, which recognizes a conserved cleavage site usually consisting of the amino acids alanine-x-alanine (13). Secretion proceeds through the so-called *Sec translocase*. In *Escherichia coli*, this is a multisubunit protein complex composed of the soluble dimeric SecA protein and a membrane-embedded complex formed by SecY, E, D, G, and F. The protein to be secreted is kept in a translocation-competent unfolded state by means of the chaperone SecB (14). A similar Sec translocase exists in *Bacillus* species (15). During or after translocation through the cytoplasmic membrane, some proteins additionally require the action of specific foldases to achieve their native conformation. Such foldase proteins that assist correct folding of the enzymes lipase and elastase in the periplasm have been identified in *Pseudomonas* species (16,17). Their main function seems to be helping their cognate enzymes in overcoming a kinetic barrier along their folding pathway. They may also function as temporary competitive inhibitors, preventing enzyme activity inside the bacterial cell. In addition, proteins to be secreted often contain disulfide bonds that are formed in the periplasm. In *E. coli*, a complex system consisting of Dsb proteins mediates disulfide bond formation (18). Recently, a similar system has been identified in *Pseudomonas aeruginosa* and shown to be necessary for the formation of enzymatically active extracellular enzymes (19). After folding in the periplasm, proteins to be secreted are transported through the outer membrane by means of a complex machinery called the *secreton* consisting of up to 15 different proteins forming the so-called main terminal branch of the general secretion pathway (20). In *P. aeruginosa*, lipase is secreted through such a secreton (Xcp machinery; 9,21), here encoded by 12 *xcp* genes organized in two divergently transcribed operons. The Xcp proteins are located both in the inner and the outer membrane, with XcpQ forming a multimeric pore with a diameter of 95 Å (22). Similar multicomponent secretons have been identified in *Pseudomonas putida*, *Klebsiella oxytoca*, *Aeromonas hydrophila*, *Vibrio cholerae*, *E. coli*, and a number of phytopathogenic bacteria (9,20).

The type III or contact site-dependent pathway is used by many animal and plant pathogens, which have developed a complex secretion system consisting of at least 20 different proteins (10). Most of them are located in the inner membrane; however, an outer membrane pore protein analogous to the secretins of the type II secretion machinery is also present. This pathway functions independently from the Sec translocase; the secreted proteins do not contain a signal sequence and no periplasmic intermediates can be isolated. The type III secretion system is designated to deliver bacterial proteins directly into the cytosol of a eukaryotic host cell (Fig. 1), which means that the assembly of the secretion apparatus is regulated by the contact with a eukaryotic target cell.

The type IV protein secretion systems share extensive homology to those systems that transport DNA either from bacteria to bacteria or from bacteria to eukaryotic cells. The classical example is the VirB system of *Agrobacterium tumefaciens* (23). Type IV transporters have also been found in *Bordetella pertussis*, *Legionella pneumophila*, and *Helicobacter pylori*. These consist of about 10 different proteins forming a piluslike structure that bypasses the inner membrane, the periplasm, and the outer membrane (24).

The type V secretion system is also called the *autotransporter pathway*. Autotransporter proteins are virulence factors and have been found in many pathogenic bacteria, including *P. aeruginosa* (25,26). They possess an N-terminal signal sequence and are translocated through the inner membrane via the Sec machinery. As soon as they have reached the periplasm, they mediate their own transport into the extracellular space by virtue of their C-terminal (or β)-domain, which forms a pore in the outer membrane. The N-terminal (or α)-domain is subsequently translocated through this pore to the cell surface (Fig.1).

Several secretion pathways for enzymes can be used by a single species. *Pseudomonas aeruginosa* is an example of a gram-negative bacterium, which is known to employ at least four specific pathways to secrete various enzymes (Table 1). Because this versatile organism typically forms biofilms in diverse environments such as in natural aquatic ecosystems, in technical water systems, on medical devices, and on living tissues of plants, animals, and man (4), it can be expected that extracellular enzymes may be involved in the formation, maintenance, or destruction of biofilms under widely varying conditions. Some of the extracellular enzymes of this opportunistic pathogen are known to be virulence factors; therefore, they may also contribute to the establishment of biofilm-mediated

Table 1. Enzyme Secretion by *Pseudomonas Aeruginosa*

Secretion System	Extracellular Enzyme
Specific Mechanisms	
Type I (ABC pathway)	Alkaline protease
Type II (general secretory pathway)	Elastase B (LasB)
	LasA (staphylolytic protease)
	Exotoxin A
	Alkaline phosphatase
	Phospholipase C
	Lipase
Type III (contact site-dependent pathway)	Exoenzyme S [ExoS] (ADP-ribosyltransferase)
	Exoenzyme T [ExoT] (ADP-ribosyltransferase)
	ExoY (adenylate cyclase)
Type V (autotransporter pathway)	EstA (outer membrane esterase)
Nonspecific Mechanism	
Secretion in membrane vesicles	Alkaline phosphatase
	Protease
	Phospholipase C
	Peptidoglycan hydrolase
	β-lactamase

infection processes. The activities of the *P. aeruginosa* enzymes, alkaline phosphatase, protease, and lipase have been detected in laboratory biofilms and also in the slime formed by this organism (27–30), demonstrating the expression of extracellular enzymes during the biofilm mode of growth.

Secreted enzymes have been categorized according to the final location of the functional enzyme. Here, the term *ectoenzyme* refers to any enzyme that remains associated with its producer cell (31). Examples of the localization of ectoenzymes include the periplasmic space between the cytoplasmic membrane and outer membrane of gram-negative bacteria or the cell wall of gram-positive bacteria, which consists of several peptidoglycan layers intermingled with secondary polymers (teichonic and teichuronic acids); in this wall matrix, the enzymes may be involved in cell-wall synthesis and turnover. Secreted enzymes can be bound to cell-surface structures such as lipopolysaccharide (LPS) molecules as integral parts of the outer membrane of gram-negative bacteria; this has been described for lipase LipA of *P. aeruginosa* (21,32). Enzymes can also be attached to crystalline protein surface layers (S-layers); for example, they have been shown to function as adhesion sites for an amylase of *Bacillus stearothermophilus* (33). The term *extracellular enzyme* is used for any enzyme that has lost contact with its producer cell and occurs in a cell-free form in the surrounding environment, including the EPS matrix of biofilms and/or adsorbed to other living or inert surfaces (31).

Transitions between cell-associated and cell-free states of an enzyme can occur, so that an ectoenzyme may become an extracellular enzyme; examples are alkaline phosphatase (34) and β-lactamase (6) of *Bacillus licheniformis*, amylase of *B. stearothermophilus* (33), or lipase of *P. aeruginosa* (21). The ratios between cell-bound and cell-free enzymes may depend on many factors such as growth phase, nutrient concentration, nutrient composition, association with cell-surface molecules, and accumulation of fungi and gram-positive bacteria within the cell-wall matrix.

A nonspecific mechanism of enzyme secretion, which differs from the specific pathways mentioned earlier, seems to be common among gram-negative bacteria (35,36) and has been intensively studied in *P. aeruginosa* (37,38). This nonspecific enzyme release is mediated through the formation of outer membrane–derived vesicles during normal growth and represents a process by which cellular macromolecules, including periplasmic compounds and membrane components (outer membrane proteins, enzymes, DNA, LPS, phospholipids), are shed into the extracellular space in the form of membrane vesicles (Fig. 1). The formation of membrane vesicles appears to represent an alternative route for enzyme secretion, which functions in parallel to specific secretion processes (Table 1). As an example, substantial proportions of phospholipase C, alkaline phosphatase, and protease activity of *P. aeruginosa* were found to be associated with membrane vesicles, whereas the remaining enzyme activities were localized in the extracellular, vesicle-free medium (37). Using transmission electron microscopy, membrane vesicles have been visualized in pure-culture biofilms of *P. aeruginosa* (38) and in natural freshwater biofilms (36). Membrane vesicles, into which hydrolytic enzymes are packaged (e. g., peptidoglycan hydrolases), may serve to degrade surrounding cells of foreign species in the biofilm ("predatory vesicles") (35), thereby liberating nutrients for the vesicle-forming organisms; this mechanism would be of benefit to vesicle-producing bacteria living in biofilms containing mixed microbial populations. In infection processes, membrane vesicles may facilitate the delivery of those enzymes that function as virulence factors to host tissues colonized by the biofilm bacteria (36).

DETERMINATION OF ENZYME ACTIVITIES IN BIOFILMS

According to enzyme classification, most ectoenzymes and extracellular enzymes analyzed so far in biofilms belong to the class of hydrolases, whereas enzymes belonging to other classes of enzymes (e.g., lyases and oxidoreductases) have been described rarely in biofilms. Hydrolytic biofilm enzymes include polysaccharidases, proteases, lipases, esterases, peptidases, glycosidases, and phosphatases (Table 2).

The selection of the analyzed enzymes reflects the abundance of their substrates in terrestrial and aquatic environments. Particulate and dissolved organic matter introduced into soil or water originates mostly from decaying higher plants, algae, fungi, or animals. Naturally occurring important substrates are polysaccharides (cellulose, hemicelluloses such as xylan, starch, and chitin), proteins, lipids, and lignin (64). Several different enzymes usually cooperate in the microbial degradation of biopolymers to yield successively smaller compounds. First, polymer substrates are degraded to oligomeric products; these are further cleaved to smaller oligomers or to monomeric units, which can easily be taken up and metabolized by the microbial cell. Polymer-degrading enzymes may be endoacting (endolytic), cleaving within the molecule chains and liberating relatively large fragments, or exoacting (exolytic), attacking molecule termini and thus liberating oligomeric or monomeric subunits. Polysaccharides are commonly depolymerized by polysaccharide hydrolases or polysaccharide lyases (eliminases) (65). Proteins and lipids are cleaved by hydrolytic enzymes. The biodegradation of lignin, an abundant structural plant polymer, is catalyzed by various oxidoreductase enzymes (oxidases, peroxidases) (64); the degradation products (phenols, aromatic acids, aromatic alcohols) may repolymerize, a reaction that occurs spontaneously or is catalyzed by microbial phenoloxidases, peroxidases and laccases, giving rise to humic substances (64).

The analysis of enzyme activities in biofilms and flocs is mostly restricted to representative enzymes involved in the degradation of a particular biopolymer. The activities of polymer-degrading enzymes such as cellulases, amylases, or proteases have been studied occasionally in biofilms and flocs; more frequently, oligomer-degrading enzymes involved in later steps of the decomposition of biopolymers have been assayed (Table 2). These enzyme activities are considered as indicators of the microbial

Table 2. Examples of Ectoenzymes and Extracellular Enzymes in Microbial Aggregates from Different Sources

Enzyme	Substrate	Detection Method	Source	Reference
Protein-Degrading Enzymes				
Protease	Hide powder azure	Photometric	Agar-grown biofilm (*P. aeruginosa*)	29
	Azocasein	Photometric	Activated sludge	39
	River water, casein-enriched river water	HPLC[a]	River biofilm	40
Peptidase	L-leucine-β-naphthylamide	Fluorimetric	River biofilm, drinking water biofilm	41
	L-leucine-4-methyl-7-coumarinylamide	Fluorimetric	River biofilm	40
	(L-leucine-7-amido-4-methylcoumarin)		Activated sludge	42
			Marine aggregates	43–45
			Wastewater biofilm	46
	L-alanine-4-nitroanilide	Photometric	Sewer biofilm	47,48
			Activated sludge	47,48
	L-leucine-4-nitroanilide	Photometric	Activated sludge	48
	4-MUF[b]-*p*-guanidinobenzoate	Fluorimetric	River biofilms	40,49
Carbohydrate-Degrading Enzymes				
Endocellulase	Carboxymethyl cellulose	Viscometric	River biofilm	50
Chitinase	^{3}H-chitin, ^{14}C-chitin	Scintillation counting	River and estuarine sediments	51
Alginate lyase	Native alginate in biofilm	Electrophoretic	Pure-culture biofilm (*P. aeruginosa*)	52
α-Glucosidase	*p*-nitrophenyl-α-D-glucopyranoside	Photometric	Epilithic river biofilm	50
			Sewer biofilm	47
			Activated sludge	39,47,48
	4-MUF-α-D-glucopyranoside	Fluorimetric	Stream sediment biofilm	53
			Lake sediment	54,55
			Marine aggregate	44,45
			Wastewater biofilm	46
			Activated sludge	42
β-Glucosidase	*p*-nitrophenyl-β-D-glucopyranoside	Photometric	River biofilm	50,56
			Biofilm from trickling biofilter	57
			Sewer biofilm	47
			Activated sludge	47,48
	4-MUF-β-D-glucopyranoside	Fluorimetric	stream and river biofilm	49
			Stream sediment biofilm	53
			Lake sediment	54,55
			Marine aggregate	44
			Activated sludge	42
β-Xylosidase	*p*-nitrophenyl-β-D-xylopyranoside	Photometric	Epilithic river biofilm	50
	4-MUF-β-D-xyloside	Fluorimetric	Epilithic stream and river biofilms	49,56
			Lake sediment	54
N-acetyl-β-D-glucosaminidase	*p*-nitrophenyl-N-acetyl-β-D-glucosaminide	Photometric	Epilithic river biofilm	50
	4-MUF-N-acetyl-β-D-glucosaminide	Fluorometric	Marine aggregate	44
			Activated sludge	42

(*continued overleaf*)

Table 2. (*Continued*)

Enzyme	Substrate	Detection Method	Source	Reference
	ELF 97-N-acetyl-β-D-glucosaminide	Microscopic	Pure-culture biofilm on chitin films	58,59
Chitobiosidase	4-MUF-β-D-N, N′-diacetylchitobioside	Fluorimetric	Marine aggregate	44
β-Glucuronidase	4-MUF-β-D-glucuronide	Fluorimetric	Activated sludge	42
Lipid-Degrading Enzymes				
Lipase	4-MUF-oleate	Fluorimetric	Marine aggregate	45
	4-MUF-stearate	Fluorimetric	Activated sludge	42
Esterase	4-MUF-butyrate	Fluorimetric	River biofilm	49
			Lake sediment	54
	Fluorescein diacetate	Photometric	Drinking water biofilm	60
			Sewer biofilms	47
			Activated sludge	42,47,61
			Stream sediment biofilm	53
Phosphomonoesterases				
Phosphatase	*p*-nitrophenyl phosphate	Photometric	Epilithic river biofilm	50
			Sewer biofilm	47
			Activated sludge	47,48
			Stream and river biofilms	56
	4-MUF-phosphate	Fluorimetric	Marine aggregate	44,45
			Activated sludge	62
	ELF-97 phosphate	Microscopic	Pure-culture biofilms on stainless steel	27,28
			Activated sludge	62
Oxidoreductase Enzymes				
Phenol oxidase	L-3,4-dihydroxy phenylalanine	Photometric	River biofilm	50
Peroxidase	L-3,4-dihydroxy phenylalanine, H_2O_2	Photometric	River biofilm	50
Extracellular redox activity	5-cyano-2,3-di-4-tolyl-tetrazolium chloride	Microscopic	Activated sludge	63
Extracellular redox activity	Tetrazolium salt (XTT)[c]	Photometric	Activated sludge	63

Note:
1. HPLC, high performance liquid chromatography.
2. MUF, methylumbelliferyl.
3. XTT, 3′-1-[(phenlyamino-)carbonyl]-3,4-tetrazolium-bis (4-methoxy-6-nitro)benzene-sulfonic acid hydrate.

potential of polymer degradation and metabolism in their respective environments. Thus, β-glucosidase, α-glucosidase, β-xylosidase and β-N-acetylglucosaminidase represent ectoenzymes or extracellular enzymes associated with the degradation of cellulose, starch, xylan, and chitin, respectively.

Enzymes usually act on more than one substrate. This allows for the use of synthetic substrate analogs instead of natural substrates for detection of enzyme activities. Hydrolytic activities in biofilms were mostly determined using model substrates consisting of a chromogenic or fluorescent molecule bound to a substrate monomer via ester, glycoside, or peptide (amide) bonds. Upon hydrolysis of the colorless or nonfluorescent model substrate, the chromogenic or fluorogenic groups are released; this can be monitored by an increase in absorbance or fluorescence. Examples of chromogenic or fluorogenic moieties of artificial substrates are 4-nitrophenol, 4-nitroaniline, β-naphthylamine, 4-methylumbelliferone, or fluorescein. Esterase activity is frequently measured as hydrolysis of fluorescein diacetate (FDA). Because FDA is nonspecifically cleaved by many different hydrolases (e.g., lipases, proteases, carbohydrate-degrading enzymes), this enzyme assay has been used as an indicator for general heterotrophic activity (66). Glycosidase, peptidase, and phosphatase enzyme activities are usually determined by more specific chromogenic or fluorogenic substrates (Table 2). Oxidoreductase enzyme activities have been determined photometrically by monitoring an increase in absorbance following reduction of tetrazolium salts (63,67) or L-3,4-dihydroxyphenylalanine (50). Enzyme assays are usually performed at relatively high substrate concentrations at saturation level under laboratory conditions, so that the kinetics of the reaction approach zero

order and are not dependent on substrate concentration (66); in addition, it has to be considered that the artificial substrates are analogs for naturally occurring compounds of unknown composition and concentrations, which can be expected to be limiting in certain oligotrophic environments. Thus, potential rather than actual enzyme activities are measured with the assays described earlier.

Research on enzymes in microbial aggregates has been restricted to selected environments; thus, enzyme activities have been frequently studied in river biofilms, in activated sludge flocs, in sediment biofilms, and in pure-culture biofilms grown under laboratory conditions (Table 2). Enzyme activities were measured either directly on undisturbed biofilms and suspended flocs or, alternatively, in homogenized biofilm suspensions after detaching the biofilm from solid supports by scraping and sonication techniques. The formation of undisturbed biofilms was accomplished on artificial substrata such as clay tiles glued to stream boulders in the riverbed (56), glass beads on nylon strings, or polycarbonate membranes in support holders placed in opaque pipes that were attached to the riverbed (40,49) and PVC coupons in drinking water systems (41). Alternatively, biofilms were obtained from natural substrata such as stones collected from the bottom of rivers (41,50) or sediments from streams, lakes, and oceans (53,54,68). In some studies, the biofilms were allowed to develop for several weeks before the solid supports with their attached biofilms were collected from the aqueous environment. In general, enzyme activities were determined subsequently by submerging biofilms still adherent to their substratum in solutions containing enzyme substrates or by shaking sediment samples or flocs in the respective substrate solution. After this incubation, biofilms were removed by centrifugation, before the products released enzymatically from the substrates were analyzed. Controls usually consist of formaldehyde-killed biofilms to assess abiotic substrate degradation in the absence of living biofilm organisms.

In some studies, a fractionation of biofilms and flocs was performed by methods such as centrifugation, filtration, or extraction of the EPS using ion-exchange resins (42,46,50,63). These techniques were applied to separate inorganic particulate material, cellular aggregates, and single cells from the cell-free aqueous phase in order to distinguish between particle-sorbed/cell-bound enzymes and cell-free extracellular enzymes, which were obtained by 0.2-μm membrane filtration as the final step in the fractionation procedure. In studies of freshwater and marine aggregates, greater than 3.0-μm size fractions were considered to represent particle-attached microorganisms (43,69). The separation procedures of centrifugation and/or filtration employed in many studies of biofilms and flocs do not necessarily allow discrimination between enzymes localized within the periplasm, bound to integral components of the cell surface, and those cell-free enzymes immobilized within that fraction of the EPS matrix that may not be removable but still remains associated with cells. At present, for routine extraction of EPS from many biofilms and flocs with a high yield, particularly of proteins, the best choice seems to be the use of a cation-exchange resin (Dowex), combined with stirring under defined conditions (70). The method is based on the removal of calcium ions, destabilizing the EPS structure and facilitating the separation of EPS from the cells. Because the extraction efficiency for enzymes varies depending on the type and origin of the microbial aggregate under study and on the fractionation technique used, the distinction between cell-bound enzymes, ectoenzymes, and extracellular enzymes must be considered as strictly operational.

Enzyme activities in biofilms can also be studied by analyzing the enzymatic degradation products from naturally occurring substrates or model compounds. Thus, the proteolytic activity of river biofilms was determined by monitoring the release of amino acids from river water proteins in the presence or absence of added casein using high-performance liquid chromatography (40). The size-fractionated hydrolysis products resulting from the enzymatic degradation of bovine serum albumin by wastewater biofilms were quantified with a colorimetric protein assay (71). The proteolysis of various cytokines by laboratory biofilms of *Porphyromonas gingivalis* was demonstrated using denaturing polyacrylamide gel electrophoresis, Western blotting, and antibodies recognizing both intact cytokines and their breakdown products (72). The decrease in the length of the exopolysaccharide alginate in biofilms of *P. aeruginosa*, as a result of the action of an endogenous alginate lyase, was followed by nondenaturing gel electrophoresis (52); degradation products of alginate were visualized by staining the gel with toluidine blue O. Extracellular polysaccharide lyase in a *Pseudomonas fluorescens* biofilm was detected by incubation of sample solutions in wells cut into substrate agar plates that contained biofilm-derived exopolysaccharide as the test substrate; after flooding the plates with cetylpyridinium chloride and counterstaining with congo red, enzyme-positive solutions produced a clear zone against an opaque, orange background (73).

Another approach to enzyme analysis in biofilms is the direct microscopic visualization of enzyme activity in the biofilm by the application of in situ staining procedures with fluorogenic substrates. Using this nondestructive technique, phosphatase activity in laboratory biofilms and in activated sludge flocs was detected using the water-soluble substrate ELF 97-phosphate, which yields an insoluble fluorescent precipitate upon cleavage by the enzymes; this method allowed the study of the spatial distribution of phosphatase activity in whole flocs and in vertical cross sections of biofilms (27,28,62). Extracellular redox activity in activated sludge flocs was visualized by the reduction of the tetrazolium salt 5-cyano-2,3-di-4-tolyl tetrazolium chloride (CTC) to CTC-formazan crystals, which were viewed by confocal laser scanning microscopy (63).

An indirect approach to assessing the polymer-degrading potential of microbial aggregates is the detection of extracellular enzyme-producing bacteria using substrate-containing agar media. Using this method,

amylase-, protease-, and lipase-producing bacteria from wastewater environments were quantified on agar media containing starch, gelatin, and olive oil, respectively, as the substrates (47). High colony counts of 10^9 to 10^{10} colony-forming units per gram dry weight of polymer-degrading bacteria were measured in sewer biofilms and activated sludges, indicating that these biofilms and flocs were highly active biocenoses (47).

DISTRIBUTION OF ENZYME ACTIVITIES IN BIOFILMS

Localization of Enzymes

A different situation exists for single planktonic cells in the bulk-liquid phase as compared with cells immobilized in biofilms and flocs. After secretion from single, free-living cells, extracellular enzymes can dissolve, readily diffuse away from the producing cell and be diluted in the aqueous environment. In cell aggregates, the mobility of enzymes leaving the cell surface may be restricted by diffusion limitation within the viscous gel-like EPS matrix, by immobilization through interactions with structural polymers, for example, by forming enzyme-polysaccharide complexes (74) or by binding to inorganic and/or organic particulate material incorporated in the biofilm (75,76). Thus, release of extracellular enzymes from microbial aggregates into the surrounding environment may be delayed or even prevented, resulting in a local accumulation of enzymes within biofilms and flocs. This may enhance the probability of nutrient acquisition by the whole biofilm microbial community. In the case of ectoenzymes, coupling of substrate degradation and uptake processes may even be more efficient because of the closer association of enzymes with the producer cell (34).

When enzyme activities were compared between the bulk-water phase and microbial aggregates, specific extracellular enzyme activities were usually higher in attached bacteria. This has been described for river biofilms (56) and freshwater and marine aggregates of different compositions (43–45,69). In a study of decaying marine phytoplankton, cell-specific aminopeptidase activities of detritus particle-attached bacteria were several times higher than those of free-living bacteria, and also higher than glucosidase activities (45); this could be interpreted as the potential for faster solubilization of proteins relative to polysaccharides and might reflect specializations for the hydrolysis of different polymers by free-living and attached bacteria (45). In general, higher enyzme activities of attached bacteria compared with free-living cells may reflect differences in nutrient availability and composition in the different habitats (69). Particulate matter itself can be used as a substrate, but organic matter from the bulk-water phase can also adsorb to the particulate material. These conditions provide an accumulation of nutrients, whose utilization is coupled to the synthesis and secretion of appropriate enzymes by the colonizing microbial community.

Many enzyme activities in biofilms were often found to be associated with the cells and/or other particulate material. In epilithic biofilms, phenol oxidase, peroxidase, and phosphatase activities were largely particle-associated; in contrast, the largest pool of carbohydrate-degrading enzyme activities were in the aqueous phase (50). In wastewater biofilms, most of the leucine aminopeptidase and α-glucosidase activities occurred cell-bound, whereas only a minor fraction of these hydrolytic activities (no more than 3%) was found to be located in the cell-free (<0.2 μm) fraction (46). In activated sludge, the majority of enzyme activities seem to be associated with the suspended flocs (39,48,62). Thus, 75 to 99% of phosphatase, glycosidase, and aminopeptidase activities were found to be located in the flocs (48,62). Similarly, hydrolytic enzymes (starch-hydrolyzing enzymes, α-glucosidase, protease) of activated sludge cultivated in a sequencing batch reactor were also mainly associated with the flocs (39). Using the cation-exchange technique for the recovery of EPS, approximately 85% of the total esterase activity (FDA-hydrolyzing activity) were found in the EPS extracts from activated sludge, without observing any significant cell lysis (42). This clearly indicated that esterase enzymes were part of the EPS matrix in the flocs. Similarly, a study of natural and batch-cultured stream sediment biofilms indicated that extracellular esterases (FDA-hydrolyzing enzymes) were predominantly associated with the biofilm EPS (53). In addition to the determination of hydrolytic activities, cation-exchange extraction also allowed the detection of small amounts of oxidoreductase enzymes in EPS-containing extracts from activated sludge (63).

The relationship between extracellular enzyme activity and microbial abundance in biofilms has been shown to vary depending on the enzyme studied, on the type of biofilm, on the growth phase, and the environmental nutrient conditions. In river biofilms, phenol oxidase, peroxidase, and phosphatase activities that were largely particle-bound often correlated with microbial biomass, whereas carbohydrate-degrading enzymes with their main activities in the aqueous phase of the biofilms were not correlated with biomass (50). In activated sludges, only a poor correlation of esterase activity (FDA hydrolysis) and biomass has been observed (77). Good correlations have been shown between fixed bacterial biomass and proteolytic activity (41) or esterase activity (49,60) in river and drinking water biofilms. Because these enzyme activities were considered to be near-constant properties of biofilm bacteria, the determination of protease and esterase activities measured by the hydrolysis of L-leucyl-β-naphthylamide and fluorescein diacetate, respectively, were proposed as rapid and simple methods for measuring bacterial biomass and monitoring biofilm growth on surfaces (41,60). On the basis of this principle, the measurement of extracellular proteolytic activity has been used to quantify attached bacterial biomass in various French drinking water distribution systems (78).

Association of Enzymes with the EPS Matrix

On the basis of the observation of enzyme abundance in biofilms, it has been assumed that ectoenzymes and extracellular enzymes accumulate and become stabilized in biofilms by binding at the cell surface of biofilm bacteria or by interacting with structural EPS such as polysaccharides within the biofilm matrix (49,50,75,79).

Only few reports in the literature describe the interactions between enzymes and polysaccharides, and little work has been done to study such interactions in biofilm bacteria.

The exopolysaccharide matrix may not be homogeneous, but rather may display microenvironments called *microdomains* with different physical and chemical properties at the molecular scale (80). It was proposed that extracellular enzymes could be localized in hydrophilic, exopolymer-mediated microdomains as a useful strategy to retain enzymes in close vicinity to microbial cells (80). Microdomains would provide relatively stable ionic and pH environments for enzymes to function efficiently, to prolong their half-life, and to prevent their denaturation. Because polysaccharides comprise the major structural molecules in biofilms, it can be assumed that they play a major role in localizing extracellular enzymes in the biofilm matrix, so that interactions between enzymes and polysaccharides within the extracellular matrix can be expected.

Pure-Culture Studies

In general, it is assumed that the synthesis and release of enzyme proteins and polysaccharides occur independently from each other, so that interaction between them may occur after transport across the bacterial membranes. Mucoid strains of *P. aeruginosa* provide a suitable model to study interactions between EPS, because they are characterized by the simultaneous production of extracellular polysaccharides and different enzymes (Table 1). The extracellular polysaccharide alginate, which is overproduced in mucoid variants, is supposed to be a key factor in the formation and persistence of *P. aeruginosa* biofilms on human mucosal tissues [e.g., in chronic lung infections of cystic fibrosis (CF) patients] (81). In mucoid strains of *P. aeruginosa*, the simultaneous secretion of alginate and lipase has been demonstrated (74). Mucoid strains isolated from CF patients displayed up to ninefold extracellular lipase activities (74). Enhanced de novo synthesis and release of lipase and an accumulation of enzyme activity in the EPS matrix were supposed to be the reasons for higher extracellular lipase activities in mucoid *P. aeruginosa* strains. Confirmation of an association between extracellular lipase and alginate surrounding the cells came from the observation that dissolution of the bacterial slime by the addition of a purified alginate lyase resulted in an additional release of lipase activity without killing the cells (30). From in vitro experiments using lipase preparations from *P. aeruginosa* and other microorganisms, it was concluded that alginate was able to bind lipases with a concomitant change of enzyme properties (82). As an example, lipase activity proved to be more resistant to heat inactivation at 70 °C in the presence of alginate than in its absence (82). Similarly, the extracellular polysaccharide xanthan from *Xanthomonas campestris* was also able to protect extracellular lipase activity of *P. aeruginosa* from thermal denaturation (83). The in vitro binding studies demonstrated that the interactions between alginate and lipase seemed to be based on weak binding forces (82), which are typical of interactions between EPS in bacterial biofilms (84).

The ability of alginate to interact with enzymes other than lipases has also been reported. For example, an interaction of alginate from *P. aeruginosa* with the exogenous human leukocyte elastase has been demonstrated (85). It was proposed that each elastase molecule interacted with 19 uronic acid residues of alginate, predominantly through electrostatic forces. In addition, alginate reduced the association rate between elastase and α-1-proteinase inhibitor, but increased its association rate with secretory leukoprotease inhibitor (85). It was assumed that alginate in microcolonies of mucoid *P. aeruginosa* represented an important factor in determining the local concentration of the host leukocyte elastase and in influencing the protease-antiprotease balance in the infected lungs of CF patients.

The interaction between certain polysaccharides, including alginate and lipase, with concomitant change of enzyme properties and location may be a common phenomenon in pro- and eukaryotic cells. In *P. aeruginosa*, the interaction between lipase and alginate is expected to occur after secretion. Alginate-lipase complexes may temporarily remain associated with the cell surface, before being extruded into the intercellular space within the biofilm. Detachment of lipase from surface-binding sites by alginate from the same cell may trigger novel enzyme synthesis and secretion of the enzyme (74). Thus, in biofilms of mucoid bacteria, alginate production may result in an autostimulatory effect with respect to extracellular lipase formation. In the biofilm matrix, lipase may remain noncovalently associated with alginate, resulting in increased stability and accumulation of enzyme activity in the microenvironment of the slime-embedded cells. Similarly, stabilization of other extracellular enzymes due to their noncovalent interaction with extracellular polysaccharides produced by the same bacterium has been described for an agarase from a *Cytophaga* species (86), a β-lactamase from *Bacillus cereus* (87), and a protease activity within an extracellular protein-polysaccharide-lipid complex from slime-forming *Myxococcus virescens* (88). Thus, it may be a general property of extracellular polysaccharides to function as a matrix for enzymes in biofilms with concomitant changes of enzyme properties.

In certain instances, polysaccharides already interact with enzymes during secretion. In *Micrococcus sodonensis*, the appearance of extracellular enzyme activities of alkaline phosphatase, nuclease, and protease has been shown to be dependent on the cosecretion of at least one of several polysaccharides produced by this bacterium (89). On the basis of a more detailed study of alkaline phosphatase, it was hypothesized that the polysaccharide protected the polypeptide chains of the enzyme from proteolytic degradation during a vulnerable stage of enzyme secretion (89).

Occasional reports in the article indicate that growth in a biofilm markedly influences the expression, stabilization, and accumulation of extracellular enzyme activities in the EPS matrix of the immobilized cells. Extracellular protease activity of *Staphylococcus epidermidis* studied at varying specific growth rates was generally higher in biofilms than in planktonic populations (90). In biofilms of *P. aeruginosa*, the bacteria revealed lower levels

of β-lactamase induction by β-lactam antibiotics than planktonic populations (91). One reason for this effect was supposed to be the trapping and accumulation of β-lactamase enzyme within the biofilm to such levels that afforded protection of the sessile bacteria from β-lactam antibiotics by inactivating them.

Terrestrial and Aquatic Biofilms

In studies of natural biofilms, the presence of humic substances has to be considered. Extracellular enzymes can form stable complexes with humic compounds (75), which are abundant in aquatic and terrestrial ecosystems and may be important components in the EPS matrix of soil and water biofilms. In soil biofilms, humic-enzyme complexes and enzyme complexes with clay particles were supposed to contribute significantly to total enzyme activity, giving the soil environment a persistent extracellular catalytic activity independent of the existing microflora (75). Complexes between enzymes and humic matter from soil have been reported to be extremely resistant to thermal denaturation, dehydration, and proteolysis (75). The formation of polyphenolic-enzyme complexes can partially or entirely inactivate enzymes in a reversible manner (34). It was proposed that, in these associations, enzymes can be protected from the environment, can be stored in a suppressed but chemically active state, and can be reactivated at the same or displaced sites to full enzyme activity (34). Thus, it can be assumed that potential enzyme activities in humic acid-containing biofilms may be higher than what is normally observed.

In models of river biofilms composed of autotrophic (algae, cyanobacteria) and heterotrophic microorganisms, extracellular enzymes were included as essential components of the EPS matrix. In a structural-functional model for epilithon (biofilms adherent to stone surfaces in streams and rivers), it was suggested that the polysaccharide matrix was a site for attached enzymes analogous to enzyme-humus complexes in soil (79). The biofilm matrix should retain and possibly protect extracellular enzymes, but also retain any products of extracellular enzymatic hydrolysis (79). This concept, originally developed for epilithic biofilms, was supposed to apply, with some modifications, to all biofilms on wetted surfaces (92). Biofilms submerged near water surfaces (e.g., on the upper surfaces of stones) are composed of autotrophic microorganisms (algae, cyanobacteria) and heterotrophic microorganisms, whereas biofilms within the stream bed will be predominantly heterotrophic. Thus, depending on the type of biofilm, substrates for extracellular enzymes may be exudates/photosynthates released from algae or from colonized river macrophytes and mosses, dissolved organic matter, and particulate organic matter trapped from the surrounding water (93). In biofilms on wood, products from surface and subsurface fungal hydrolysis may act as an additional nutrient source. An advantage for microorganisms in aquatic biofilms would be the availability of low molecular weight reaction products in the immediate vicinity of the immobilized cells. The long-term existence of different enzymes from the same and previous generations in the biofilm may exclude the requirement of novel enzyme synthesis and the initiation of enzyme induction, because high levels of products are maintained by the activity of the immobilized enzymes (79). The stabilization of extracellular enzymes due to their interaction with matrix polysaccharides may result in persistence of enzyme capacity within the biofilm, which is independent of the usual forms of regulation and control of enzyme synthesis and secretion. Thus, enzyme activities may be decoupled from their producer cells. This may be one reason why the activities of certain enzymes were not to be found in correlation with parameters for cell or biomass densities in biofilms (49,50). The reservoir of extracellular degradative enzymes within the polysaccharide matrix was assumed to buffer the biofilm community to sudden changes in the composition and concentration of dissolved organic matter in the bulk-water phase (49). The accumulation and stabilization of polysaccharide-bound enzymes within the biofilm matrix seem to be essential processes that have to be considered in the understanding of structure and function of biofilms in different ecosystems.

INFLUENCE OF ENVIRONMENTAL FACTORS ON ENZYME ACTIVITY IN BIOFILMS

The expression of ectoenzymes and extracellular enzymes in biofilms is dependent on various physical, chemical, and biological factors. The patterns of enzymatic activities reflect the metabolic status of microbial populations as a phenotypic adaptation to the predominating environmental conditions. Enzyme activity can be directly affected by variables such as temperature, pH, or ionic environment, because the rate of enzyme-catalyzed reactions is largely determined by these factors; enzyme activities can also be impaired when inhibitory or inactivating substances are present. An indirect mechanism of controlling the activities of secreted enzymes is the regulation of their synthesis by induction and repression processes in response to substrate availability; in addition, the expression of many extracellular enzymes is positively regulated by signal molecules, which mediate cell-to-cell communication within bacterial population (illustrated later). Observations of natural biofilms and of single-species laboratory biofilms indicate relationships between certain environmental factors and enzyme activities in biofilms.

Extracellular enzyme activities in river biofilms and in sediments have been found to be subject to seasonal variations. These differences were related to climatic characteristics (especially temperature) and to the ratio of bacterial and algal biomass, but also to the availability and composition of assimilable organic matter.

In a comparative study of the environmental conditions, biofilm composition, and extracellular enzyme activities of three Mediterranean and six Atlantic streams and rivers, the biofilms from Mediterranean streams revealed higher extracellular enzyme activities than biofilms of comparable composition from Atlantic systems (93); an explanation for this observation could be climatic differences such as lower irradiance and lower temperatures in the Atlantic-dominated streams. Water temperature has also been described to influence the production of extracellular enzymes involved in the degradation of cellulose

(β-glucosidase) in biofilter biofilms used for wastewater treatment (57). During the cold period (influent water temperature 7.5 °C to 13.5 °C), the biomass contained less extracellular β-glucosidase activity than during the warmer period. This weak hydrolytic activity, coupled with an accumulation of polysaccharides in the biofilm, was supposed to promote the rapid clogging of the biofilter observed during the colder period (57).

Ionic content of stream water seems to be another factor that can account for the variability in heterotrophic biofilm activities. In a study of three Mediterranean streams, the high concentrations of calcium and magnesium at calcareous sites were supposed to be responsible for higher extracellular enzyme activities, because both ions are known to act as activating cations in enzyme reactions (94).

In general, algal biomass in biofilms is an important biological factor stimulating heterotrophic activities. In a comparative study of stream biofilms of different geographic regions, highly heterotrophic biofilms showed lower extracellular enzymatic activities (β-glucosidase, β-xylosidase, phosphatase) than more autotrophic biofilms; maximum enzyme activity was achieved when the algal biomass was two- to threefold higher than the bacterial biomass (93). The relevance of algal biomass on the activity of heterotrophic bacteria was supposed to be related to the physical proximity between these organisms in a common biofilm matrix, facilitating the supply of algal exudates and lysis products, which are known to be major nutrient sources for heterotrophic microorganisms. A high proportion of these compounds are polymeric and can only be used by enzyme-mediated hydrolysis, so that ectoenzymes and extracellular enzymes in biofilms form the link between the presence and productivity of algae and the growth and metabolic activity of heterotrophic organisms (93).

The availability and qualitative composition of exogenous organic matter as another nutrient source in the bulk-water phase can also determine enzymatic activities of heterotrophic biofilm organisms. As an example, the enzyme activity (as β-glucosidase) of epilithic biofilms in a Mediterranean river was enhanced during higher discharge periods, which were coupled with higher dissolved organic carbon content in the stream water (56). In biofilms from different fluvial systems and grown on different substrata, the ratio of β-xylosidase to β-glucosidase activity was always similar (approximately 0.5), indicating a higher microbial utilization of cellobiosic than xylobiosic substrate molecules (93). This may reflect a higher abundance of cellulose than hemicellulose in river environments and/or a preferential degradation of polysaccharides, whose end products are energetically more favorable for bacterial growth and metabolism (β-D-glucose from cellulose vs. β-D-xylose from xylan as a major component of hemicellulose).

In littoral lake sediments, a linear positive relationship between extracellular enzyme activities (β-glucosidase, exoglucanase, endopeptidase, esterase) and organic matter content was observed (54). The patterns of enzymatic activities were different for inside and outside a reed bed; inside, polysaccharide-degrading enzymes associated with the decomposition of cellulose (β-glucosidase, exoglucanase) and xylan (β-xylosidase) and mannosidase dominated, whereas protein-degrading enzyme activity (endopeptidase) was elevated outside the bed. This pattern of enzyme expression was related to the polymeric composition of the major sources of organic matter (macrophyte-derived polysaccharides inside the bed, algal and cyanobacterial proteins outside the bed). In another study, the specific α- and β-glucosidase activities of lake sediments were strongly induced by their natural substrates (starch and cellulose, respectively) and were not inhibited by glucose, whereas proteins inhibited these activities (55). These observations suggest that an adaptation of microbial biofilm communities to the available organic matter in their environment consists of producing extracellular enzymes that are related to the composition of the predominant nutrient sources. Therefore, it was proposed to use patterns of enzymatic activities to obtain information on organic matter sources that are utilized by microbial population in natural systems (54).

Enzyme activities in activated sludge flocs also seem to be influenced by the composition of the organic matter in the wastewater feed. In full-scale plants, it was observed that addition of hydrolyzed starch resulted in high specific α-glucosidase activities, suggesting the presence of starch-utilizing populations (61). Specific alanine aminopeptidase activities were elevated in activated sludge when a higher content of proteinaceous material was in the inlet of the plant (61). When activated sludge was cultivated in sequencing batch reactors, about four times higher extracellular redox activity was measured in reactors fed with lignin compared with reactors fed with amylose. On the basis of this observation, extracellular redox enzymes were supposed to facilitate the enzymatic attack on recalcitrant molecules such as lignin (63).

The influence of environmental factors on alkaline phosphatase has been studied in pure-culture biofilms. Alkaline phosphatase was expressed in biofilms under conditions of phosphate starvation. In biofilms of *P. aeruginosa*, alkaline phosphatase was formed only in a relatively thin layer at the biofilm-medium interface, whereas in biofilms of *Klebsiella pneumoniae* it started to appear at the biofilm surface, but subsequently spread throughout the biofilm (27). In addition, alkaline phosphatase expression in *P. aeruginosa* biofilms was limited to regions of oxygen availability (28). These observations indicated a marked spatial physiological heterogeneity mediated by environmental factors such as nutrient and oxygen availability.

REGULATION OF EXTRACELLULAR ENZYME ACTIVITY IN BIOFILMS

Induction Mechanisms

Very little is known about the regulation of ectoenzymes and extracellular enzymes in biofilms. Extracellular enzymes are commonly synthesized and secreted at a low basal level. If macromolecular substrates are

present, they are degraded to small products, which are transported into the cell to induce further synthesis of the enzyme (6). Thus, the microorganisms can monitor the level of product and regulate enzyme synthesis accordingly. Enzyme synthesis may again be slowed or repressed in the presence of an excess of low molecular weight products or other carbon sources, which are easily taken up and metabolized by the cell (6). This catabolite repression allows for the efficient control of the synthesis and secretion of enzymes. Many extracellular polymer-degrading enzymes of single species in laboratory cultures have been found to be regulated in this way. However, it is unknown if these mechanisms are also employed by biofilm populations. Some observations on biofilm and floc enzymes being expressed in the presence of their natural substrates indicate that induction mechanisms may be the cause for the production of enzymes in response to the prevailing nutrients. However, changes in microbial population composition may be an alternative explanation for the dynamic expression of enzymes, occurring as an adaptation to varying concentration and composition of nutrient sources. Induction mechanisms are short-term reactions of biofilm microorganisms at the physiological level, whereas population shifts represent long-term adaptation processes. As an example, enzyme activities in starved, activated sludge were induced by the addition of lactose; within two hours, glucosidases and galactosidases reached their maximal enzyme activity (48).

Major changes in the composition of an attached bacterial community were observed during the colonization of decaying phytoplankton particles, accompanied by a pronounced increase in the cell-specific enzymatic activities of aminopeptidase, β-glucosidase, and lipase during 11 to 15 days (45). The analysis of the bacterial community composition suggested that the extensive colonization was accomplished by highly hydrolytic "particle specialist" bacteria, which belonged to the *Cytophagales* and *α-Proteobacteria* groups (45).

Regulation of Extracellular Enzyme Gene Expression by Quorum Sensing

The development and maintenance of biofilms constitute a type of community behavior among bacteria and it is now clear that these biofilm communities represent more than the sum of their individuals (95). Bacteria can obviously coordinate their activities, that is, the expression of specific genes, by producing and sending out chemical signals that are perceived by other members of the same species or even members of different species. When, with increasing cell densities, these signaling molecules reach a certain threshold concentration within a population, they bind to specific intracellular receptors, the so-called R-proteins, which can in turn activate or repress a specific set of target genes. Because the signaling molecules provide a mechanism for the bacteria to monitor their own population density, this type of cell density–dependent regulatory mechanism has been termed *quorum sensing* (96). However, because cell-to-cell signaling does not occur only at high cell densities, quorum sensing now refers to any bacterial intercellular communication by means of small diffusible molecules (97).

A prominent example of quorum sensing regulation in gram-positive bacteria is the regulation of virulence factor production in *Staphylococcus aureus* (98), in which small posttranslationally processed peptides are used as signal molecules. They interact with a membrane-bound sensor kinase named AgrC; this kinase constitutes one part of a two-component regulatory system. The corresponding response regulator AgrA is phosphorylated and subsequently activates the expression of numerous virulence genes, including those encoding lipase, protease, α-toxin, β-hemolysin, and enterotoxin.

In gram-negative bacteria, various types of signaling molecules have been detected, including N-acylhomoserine lactones (AHLs), 3-hydroxy-palmitic acid methyl ester, 2-heptyl-3-hydroxy-4-quinolone, and diketopiperazines (cyclic dipeptides) (97). Among them, AHLs are the best studied class of signaling molecules (99). Originally discovered in marine *Vibrio* species (100), these molecules have now been detected in more than 20 different bacterial species, including human pathogenic *P. aeruginosa*, *Burkholderia cepacia*, *Serratia marcescens*, several *Vibrio* species, and a wide variety of plant-associated bacteria, including *A. tumefaciens*, *Pseudomonas aureofaciens*, *Pseudomonas syringae*, *P. fluorescens*, and *Rhizobium leguminosarum* (101). These findings clearly demonstrated that signaling by low-molecular-weight compounds has emerged as a major pathway in bacteria interspecies communication.

The best studied organism with respect to gene regulation by quorum sensing is the opportunistic pathogen *P. aeruginosa*. Extracellular enzymes of this species include the proteases LasA and LasB (elastase), an alkaline protease, the ADP-ribosylating toxins (exotoxin A and exoenzyme S), the lipolytic enzymes (phospholipase C, lipases A and C), and the outer membrane esterase (EstA) (7,102). A number of these enzymes are known to be virulence factors in human infections (7). *Pseudomonas aeruginosa* possesses two quorum-sensing regulatory systems whose functions are summarized in Figure 2 (103). The Las system consists of the LasI synthetase directing the synthesis of N-(3-oxododecanoyl) homoserine lactone (3-oxo-C_{12}-HSL), which binds to the regulator LasR. The LasR-3-oxo-C_{12}-HSL complex regulates the expression of the extracellular enzyme genes *lasA*, *lasB*, *aprA*, and *toxA* as well as *lasI* itself, thereby creating a feedback regulation loop. The second quorum-sensing system, Rhl, consists of the RhlI synthetase directing the synthesis of N-butanoyl-homoserine lactone (C_4-HSL), which binds to the regulator RhlR. The RhlR-C_4-HSL complex regulates the expression of the rhamnolipid biosynthesis genes *rhlA* and *rhlB*, *lasB*, *aprA*, the stationary-phase sigma factor *rpoS* and secondary metabolites pyocyanin and cyanide. It is interesting to note that the *xcp* genes, which build up the type II secretion machinery in *P. aeruginosa*, are also subject to regulation by both the Las and the Rhl system (104). The finding that LasR positively regulates *rhlR* expression demonstrated that quorum sensing in *P. aeruginosa* operates as a hierarchical regulatory cascade with the LasR/I system being the dominant regulator.

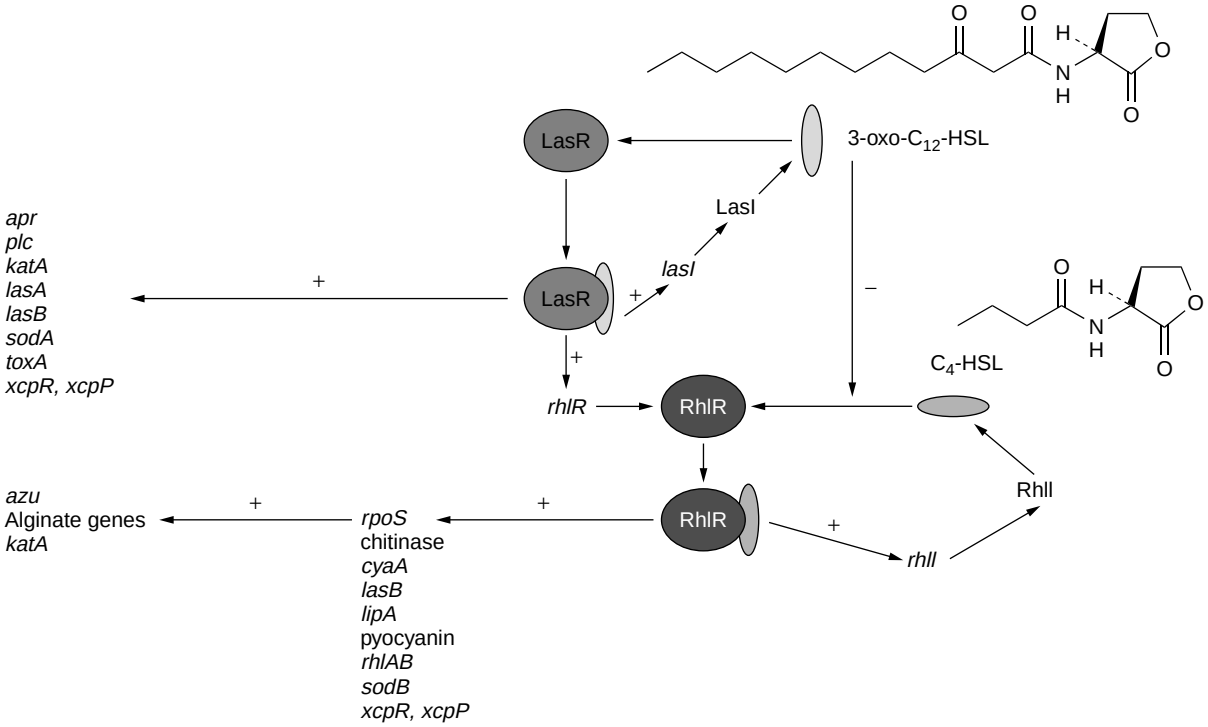

Figure 2. Quorum-sensing regulation of gene expression in *P. aeruginosa*. A plus (+) sign indicates positive regulation, a minus (−) sign indicates negative regulation. The genes encoding extracellular enzymes are printed in red. The main regulatory system consists of the regulator LasR and the autoinducer synthetase LasI, which directs the synthesis of 3-oxo-C_{12}-HSL. The LasR-3-oxo-C_{12}-HSL complex positively regulates the expression of genes *aprA* (encoding alkaline protease), *plcN* (phospholipase C), *katA* (catalase), *lasA* (staphylolytic elastase), *lasB* (elastase), *sodA* (superoxide dismutase), *toxA* (exotoxin A), *xcpR* and *xcpP* (proteins of the type II secretion system), and *rhlR* (the regulator RhlR). The RhlR-C_4-HSL complex positively regulates the expression of genes *rpoS* (stationary-phase sigma factor), a chitinase gene, *lasB* (elastase), *lipA* (lipase), *rhlAB* (rhamnosyltransferase), *sodB* (superoxide dismutase), and *xcpR* and *xcpP* (proteins of the type II secretion system). The sigma factor RpoS itself positively controls the expression of genes *azu* (azurin), *katA* (catalase), and the genes involved in the biosynthesis of alginate. See color insert.

How do the transcriptional regulators, LasR and RhlR, find their target genes? In the marine bacterium, *Vibrio fischeri*, a 20-nucleotide inverted repeat had been identified in the intergenic region between the *luxI* synthetase gene and the *luxR* regulator gene, which is required for the primary regulation of the *lux* gene expression. Such so-called *lux*-box like elements have since been identified upstream from a number of *P. aeruginosa* genes including *lasB*, *rhlI*, *rhlA*, *lasI*, *lasA*, *rhlR*, *lasR* (100), and also, more recently, *lecA* encoding the lectin PA-IL (105). *Lux*-box sequences therefore appear to be conserved regulatory elements and are likely to be targets for LasR and/or RhlR transcriptional regulators.

Biofilm formation by *P. aeruginosa* requires the coordinated expression of a specific set of genes (106). First, flagella enable the bacteria to approach a surface, attach, and form a monolayer. Subsequently, microcolonies are formed in a process that involves the so-called twitching motility mediated by type IV pili. Microcolonies then develop into a mature biofilm with its characteristic "tower and mushroom" structure (4). Finally, the biofilm cycle is closed by detachment of bacteria, which is either caused by unknown physiological events or results from mechanical forces. Recently, it was demonstrated that biofilm development by *P. aeruginosa* requires a functional Las system (107). A *lasI* mutant strain formed a thin and undifferentiated cell layer of densely packed cells. Importantly, the *lasI* mutant biofilms were susceptible to treatment with the anionic detergent SDS, whereas the wild-type biofilm was resistant. Addition of exogenous autoinducer 3-oxo-C_{12}-HSL restored the wild-type properties of the *lasI* mutant biofilm.

Pseudomonas aeruginosa plays an important role as an opportunistic pathogen, particularly in lung infections of CF patients. The bacteria are believed to exist in the form of biofilms that would render them refractile to phagocytosis, antibody and complement defense, and also to antibiotic treatment (108). Recently, it was demonstrated that sputum obtained from CF patients contained a characteristic distribution of quorum-sensing signaling molecules 3-oxo-C_{12}-HSL and C_4-HSL, with the latter compound dominating (109). The same relative abundance of acyl-HSLs was detected when *P. aeruginosa*

was grown in a laboratory biofilm model, indicating that lungs of CF patients are indeed colonized with *P. aeruginosa* biofilms.

Quorum sensing is an important regulatory circuit governing the expression of a set of genes including those that encode extracellular enzymes (Fig. 2). Also, we have described earlier that extracellular enzymes are present in biofilms. Therefore, it can be concluded that quorum-sensing regulation of extracellular enzyme gene expression will constitute a major regulatory principle when bacteria grow under biofilm conditions. However, experimental evidence for this is lacking so far. Recently, it was demonstrated that the quorum- sensing regulation under biofilm growth conditions can be fundamentally different from the one determined with planktonic bacteria (110). Future research on the role of extracellular enzymes will therefore concentrate on the identification of quorum sensing–regulated enzyme genes and the elucidation of their physiological function under biofilm growth conditions.

FUNCTIONS OF EXTRACELLULAR ENZYMES IN BIOFILMS

The primary function of extracellular enzymes is supposed to be the degradation of macromolecules to low molecular weight products, which are small enough to be transported into the cells to be available as carbon and energy sources for microbial metabolism. Thus, they are essential for providing nutrients for immobilized bacteria in biofilms by extracellular digestion of macromolecules; molecules larger than a few monomeric units cannot normally be transported across bacterial membranes. Because in many ecosystems organic material, either dissolved or particulate, is predominantly macromolecular, heterotrophic bacteria within biofilms are largely dependent on the presence of degradative extracellular enzymes in their microenvironment providing utilizable compounds for growth and maintenance of metabolic activity. In natural ecosystems, the degradation of biopolymers by microbial ectoenzymes and extracellular enzymes represents the rate-limiting step in the mineralization and cycling of particulate and dissolved organic matter. Since almost all microorganisms on earth live in microbial aggregates, polymer-degrading enzymes must predominantly originate from biofilm and floc organisms. It remains to be established what proportion of the degradation products is used by the enzyme-producing cells themselves or by other members of biofilm-mixed population, and what part of these products diffuse into the bulk-water phase to be available to planktonic organisms.

It seems that only a certain percentage of mixed microbial communities is able to produce extracellular polymer-degrading enzymes, whereas a greater number of physiological groups in a community can then metabolize the low molecular weight degradation products. The capacity of a microbial community to use dissolved and particulate organic matter is tightly coupled with the abundance and competitive success of certain species that secrete suitable polymer-degrading enzymes and provide nutrients for other members of the microbial community ("social function of extracellular hydrolysis"; 51). Thus, in a simple model of communities consisting of proteolytic and nonproteolytic *P. fluorescens* strains, the protein hydrolysates were found to be available to both strains (111). During the colonization of a chitin surface by *Pseudoalteromonas* bacteria, two physiologically different subpopulations developed: one that produced an extracellular chitinase and remained associated with the surface and another that was nonchitinase active and released daughter cells to the bulk-aqueous phase (58). It was hypothesized that the chitinase-active subpopulation supplied chitin-degradation products to the adjacent nonchitinase-active subpopulation for their cell replication and dissemination into the bulk-aqueous phase (58,112). Channels and pores in the EPS matrix near the surface were supposed to serve as conduits for transfer of soluble chitin-degradation products to nearby cells that were not producing chitinase (112). From these observations, it may be concluded that the processing of biopolymers in microbial aggregates can be mediated by cross-feeding interactions between bacteria with different enzymatic abilities.

Many extracellular enzymes of pathogenic bacteria have been shown to be virulence factors (7). The biofilm mode of growth is an important step in the establishment of human infections (4). Thus, a function of extracellular enzymes in biofilms may be the participation in the infection of eukaryotic organisms by causing tissue damage, promoting the invasion into the host organism or interfering with the defense mechanisms of the host.

The involvement in cell detachment and dispersal has been proposed as a novel function of ectoenzymes or extracellular enzymes in microbial aggregates. Certain enzymes produced by the biofilm bacteria are supposed to catalyze the partial depolymerization of structural polymers of the biofilm matrix with subsequent release of bacterial cells. Thus, enhanced expression of an endogenous alginate lyase in *P. aeruginosa* led to the degradation of the slime polysaccharide alginate and increased the detachment of cells from agar-grown biofilms, suggesting a role for this enzyme in the sloughing of immobilized bacteria from solid surfaces (52). Similarly, the action of an extracellular lyase enzyme in dense biofilms of *P. fluorescens* was specifically directed towards the biofilm exopolysaccharide (73). The enzymatic degradation of the exopolysaccharide was supposed to be responsible for the observed detachment of biofilm cells under starvation conditions during prolonged incubation (73). When exopolysaccharides isolated from biofilms of *P. fluorescens* were incubated with their corresponding spent culture media, the acetate content of the exopolysaccharide fell below detectable limits, probably because of the presence of esterase enzymes (113). These results indicate that not only depolymerization of polysaccharides, but also the enzymatic removal of noncarbohydrate substituents such as acetyl groups, may occur within the biofilm matrix. Because acetylation can provide protection against the action of polysaccharide-depolymerizing enzymes such as alginate lyases (114),

esterase enzymes may act synergistically with polysaccharide hydrolases or lyases in the destabilization of the biofilm matrix.

Methanosarcina mazei was shown to produce an extracellular disaggregatase that caused their cell aggregates to disperse into single cells (115). The enzyme appeared to be an endopolysaccharide hydrolase that degraded the capsular matrix polymer methanochondroitin to oligosaccharides, suggesting a function for this enzyme in the liberation of cells from cellular aggregates during certain stages in its growth cycle (115). Active detachment of *Streptococcus mutans* cells from biofilms on epon-hyroxylapatite surfaces coated with salivary proteins was observed (116,117); this process was mediated by the action of an endogenous surface protein–releasing enzyme.

All of these observations indicate that enzymes may cause the localized modification of the EPS matrix with concomitant changes in the physical and chemical properties of biofilms such as gelation behavior, ion binding, and mechanical stability. Enzymatic dissolution of the EPS matrix may promote the release of cells, allowing the spread of the biofilm bacteria and the colonization of new environments.

Extracellular enzymes can sometimes also function in biosynthetic processes. Extracellular polysaccharides such as dextrans or levans of *Leuconostoc mesenteroides* and *S. mutans* are polymerized from glucose and fructose, respectively, by extracellular glycosyltransferases (118). In *Azotobacter vinelandii*, extracellular calcium-dependent mannuronan C-5-epimerases catalyze the conversion of mannuronate residues to guluronate residues within homopolymeric mannuronan molecules after their polymerization and secretion to finally yield the extracellular polysaccharide alginate (119).

CONCLUSION

At present, we know that bacteria living in a biofilm mode of growth produce a number of different extracellular enzymes, which most probably also constitute a significant part of the biofilm EPS matrix. So far, our knowledge largely stems from the determination of various enzyme activities in biofilms. Future research will need more integrated experimental approaches that allow for a parallel study of (*1*) the regulation of enzyme gene expression, (*2*) mechanism(s) of enzyme secretion, (*3*) assay of enzyme activities, (*4*) identification of enzyme proteins, and (*5*) analysis of enzyme-polysaccharide interactions. Such approaches should include molecular biological techniques to construct gene fusions and defined enzyme-negative mutant strains to assess the role of extracellular enzymes for the formation, maintenance, and decay of a biofilm. Biochemical, immunological, and biophysical analysis methods, including nuclear magnetic resonance spectroscopy, analytical mass spectrometry, and atomic force microscopy, will help to identify and characterize both the extracellular enzymes themselves and their interaction with other EPS components, particularly polysaccharides. Finally, powerful in situ imaging techniques such as confocal laser scanning microscopy will allow us to understand how bacteria in a biofilm regulate the expression of extracellular enzyme genes, how the corresponding enzymes are secreted, and where they are finally localized in the intact biofilm.

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EXTRACELLULAR POLYMERIC SUBSTANCES (EPS).

See Laser Scanning Microscopy in Combination With Fluorescence Techniques for Biofilm Study

EXTRACELLULAR POLYMERIC SUBSTANCES (EPS): STRUCTURAL, ECOLOGICAL AND TECHNICAL ASPECTS

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The vast majority of microorganisms on earth live in aggregates such as films, flocs ("planktonic biofilms"), and sludges. An everyday feature of such aggregates is addressed by the term *slime*, which refers to highly, hydrated, thick biofilms. They are slippery and can have different effects. An article on diffusion in biofilms owes its existence to that property and is explicitly acknowledged by the author: "This review was written because of a biofilm that caused me to lose my grip and be carried down a small waterfall. The result was adequate to immobilize me sufficiently and for a long enough period to organize the diverse body of material presented" (1). Microbial aggregates are kept together by means of biopolymers of microbial origin, which are generally called *extracellular polymeric substances* (EPS). The abbreviation "EPS" is used as a more general and comprehensive term for different classes of macromolecules such as polysaccharides, proteins, nucleic acids, (phospho)lipids, and other polymeric compounds that have been found to occur in the intercellular space of microbial aggregates (2). They are responsible for the cohesive forces that keep these aggregates together, namely, biofilms, flocs, and sludge (3). Although somehow inexactly used, the expression "biofilm" is commonly expanded to all these forms of microbial aggregates. The EPS fill and form the space between the cells; they are responsible for the architecture and morphology of the matrix in which the cells live. Thus, the EPS can be considered as the "house" of the microorganisms.

From a technical point of view, biofilms can have both detrimental and beneficial effects. In either case, their EPS play an important role because this is the material that keeps microbial aggregates such as films, flocs, and biological sludge together and attaches biofilms to surfaces. The EPS are the prerequisite for the existence of all microbial aggregates. Although polysaccharides and proteins are the main EPS constituents, in general, only the polysaccharide moiety has been investigated further, whereas the role of proteins still remains largely unclear. To a certain extent, they may be exoenzymes, but it is possible that some of the extracellular proteins have different functions. With regard to the technical aspects, however, the focus of this article is centered mainly on the polysaccharides, for which the largest body of knowledge exists.

COMPOSITION OF EPS

The production of EPS is a general property of microorganisms in natural environments and has been shown to occur both in prokaryotic (Bacteria, Archaea) and in eukaryotic (algae, fungi) microorganisms. Biofilms containing

mixed populations of these organisms are ubiquitously distributed in natural soil and aquatic environments, on tissues of plants, animals, and man as well as in technical systems such as filters and other porous materials, reservoirs, plumbing systems, pipelines, ship hulls, heat exchangers, separation membranes, etc. EPS are mainly responsible for the structural and functional integrity of biofilms and are considered to be the key components that determine the physicochemical and biological properties of biofilms. The EPS form a three-dimensional, gel-like, highly hydrated and often charged biofilm matrix in which the microorganisms are embedded. The EPS create a microenvironment for sessile cells, which is conditioned by the nature of the EPS matrix. In general, the proportion of EPS in biofilms can vary between approximately 50 and 90% of the total organic matter (4,5). The composition of EPS as analyzed largely depends on the method used for isolation. In general, no method has been developed that reliably yields complete extraction of EPS without contamination from intracellular components. Nielsen and coworkers (6) have compared many different methods and the resulting quantities of EPS and give the most effective methods of EPS recovery for different systems. The component of EPS that is best investigated is the polysaccharide moiety (7,8). However, the matrix is composed also of other components such as proteins and nucleic acids (9,10) and lipids (11). Table 1 gives an idea about data from various natural and engineered systems.

The EPS of *Pseudomonas aeruginosa* may serve as an individual example because this is a model organism with which many biofilm investigations have been carried out and the heteropolysaccharide alginate is known to be the main EPS component. The data are summarized in Table 2.

Interestingly, even in *P. aeruginosa* biofilms, more than 45% of the overall protein mass is found in the EPS in which they represent more than 30% of the EPS mass. Using the activity of glucose-6-phosphate dehydrogenase, a strictly intracellular enzyme, as a marker for contamination of intracellular components (13), it could be shown that the cells remained intact during the entire extraction procedure. Thus, the proteins found in the EPS matrix were not of intracellular origin. Preliminary investigations revealed that they are of relatively small molecular mass, that is, between 5,000 and 150,000 Da. Strong protease activity was found, which leads to a rapid breakdown of extracellular proteins (Broekman and coworkers, unpublished results).

Considering the matrix as a whole and in nonlaboratory systems, not only polymers of microbial origin are retained in the matrix, but because of their adhesive properties, particles also can be trapped and integrated, thus influencing the microenvironment and possibly providing nutrients (14,15). Common particulate components of the matrix are debris and humic particles, clay, silt, and calcium sulfate. Substances dissolved in water can also be sorbed into the EPS molecules.

Table 1. Composition of Extracted EPS and Concentration Range of Component

Component	Content in EPS
Polysaccharides	40–95%
Protein	<1–60%
Nucleic acids	<1–10%
Lipids	<1–40%

Table 2. Composition of EPS from Agar-Grown Biofilm of *P. aeruginosa*, Data Related Per Cell

Component	Biofilm (fg/cell)	EPS[a] (fg/cell)	Proportion found in EPS
Total carbohydrates	0.99	0.76	76.0%
Uronic acids (alginate)	0.47	0.40	85.0%
Proteins	0.59	0.27	45.5%

Source: After J. Wingender and coworkers, Biofilms II, in R Doyle, J. N Abelson, and M. I Simon, eds., *Methods in Enzymology*, vol. 336, pp. 302–314 (12).

[a]The EPS components were quantitated in cell-free solutions obtained after removal of biofilm bacteria by centrifugation and membrane filtration of the supernatants.

ECOLOGICAL FUNCTION OF EPS

Adhesion and Cohesion

A major ecological advantage of the biofilm form of life is that consortia of various organisms can establish and maintain their position over a long period of time, compared with the planktonic forms of life. This applies not only to biofilms but also to flocs (16,17) and allows for the development of synergistic relationships between microorganisms. A classical example is the nitrification that takes place in biofilms and allows the spatial closeness of ammonia oxidizers to nitrite oxidizers (17). The EPS molecules, which keep the organisms together and, if they form a biofilm, are responsible for adhesion to a given surface, provide this advantage. There is literally no surface material that cannot be colonized sooner or later, but there are strong differences in the colonization kinetics. Paul and Jeffrey (18) demonstrated that one organism can adhere to hydrophobic and hydrophilic surfaces by means of different EPS components. Busscher and van der Mei (19) have emphasized the role of the forces responsible for the adhesion to surfaces because the entire biofilm can detach if these forces are exceeded either by external shear forces or by a decay of the molecules performing the binding. In some cases, attachment was found to stimulate the synthesis of EPS (20). Both adhesion and cohesion are based on weak physicochemical interactions and not on covalent bonds. Three major kinds of forces can be distinguished, namely, electrostatic interactions, hydrogen bonds, and London dispersion forces (including so-called hydrophobic interactions and van der Waals interactions) (21–23). The individual binding force of any of these interactions is relatively small compared with a covalent C–C bond. However, the total binding energies of weak interactions between EPS molecules multiply with the large number of binding sites available in the macromolecules and add up to bond values exceeding those of covalent C–C bonds. As

mentioned earlier, hydrophilic and hydrophobic properties of EPS have been demonstrated; on the basis of these results, all three types of binding forces are expected to contribute to the overall stability of floc and biofilm matrices, probably to various extents. The result is the formation of a three-dimensional, gel-like network of EPS, whose composition, structure, and properties may vary dynamically because the microorganisms respond to changes in environmental conditions. Entanglements of the macromolecules also contribute to the overall stability but cannot be addressed as binding forces in a strict sense. Their contribution will decrease with the time of stress because the molecules will glide along each other and release the tension.

The matrix network is formed by fluctuating adhesion points and the resulting matrix can behave as a gel as long as a certain shear stress is not exceeded. In this phase, the adhesion points flip back to their original arrangement. Above that point ("yield point"), new adhesion points assemble and the matrix behaves as a highly viscous fluid; fluidized biofilms could be demonstrated by Stoodley and coworkers (23). The mechanical stability of a *P. aeruginosa* biofilm was investigated by a film rheometer in which the force for compression of a flat biofilm is measured. It allows for the determination of an apparent elasticity modulus that can be taken as a parameter for biofilm stability (24). A typical compression diagram for a *P. aeruginosa* biofilm is given in Figure 1.

It could be demonstrated that in this biofilm matrix that is dominated by polysaccharides with carboxyl groups, calcium acts as an important bridging ion that increases the stability of the network significantly. This is also the case for copper and iron but not for magnesium. In such cases, surfactants will not contribute to the dissolution of biofilms. However, if other biopolymers dominate, it is possible that surfactants have a more significant effect. Hydrogen bonds are also part of the overall binding force. They can be influenced by so-called chaotropic agents, which have a high affinity for water, thus interfering with the water shell around the biopolymers, a fact known since protein biochemistry. In some cases, this type of bond dominates the binding forces. The extent to which each bond contributes to the cumulative binding force depends strongly on the nature of the EPS molecules. Because different strains can produce different EPS, the variety is considerable, suggesting that not all biofilms can be dissolved by means of only one cleaning formulation. This coincides well with observations from practice. EPS are not totally insoluble in water. A certain amount of EPS is continuously lost to the water phase. In wastewater effluents, this contributes to important measures of process parameters such as chemical oxygen demand (COD) (25).

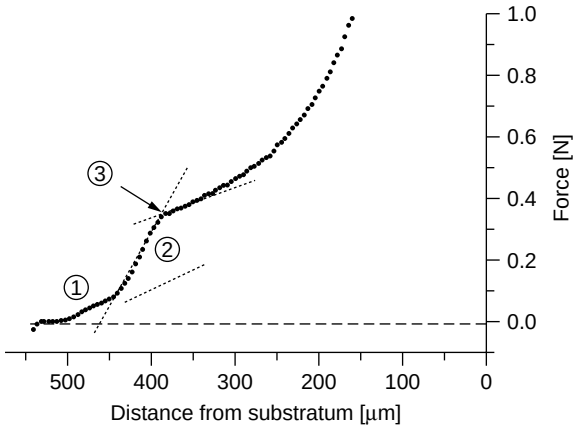

Figure 1. Compression diagram of a *P. aeruginosa* biofilm. 1: adjustment phase, 2: linear phase (apparent elasticity modulus calculated from slope), in which the matrix behaves as a gel, 3: yield point — from here on, the matrix behaves as a highly viscous fluid (after V. Körstgens, J. Wingender, H.-C. Flemming, and W. Borchard, *Water Sci. Technol.* **43**(Suppl. 6), 49–57.)

Matrix Architecture

The architecture of the EPS matrix influences the processes within biofilms profoundly. Costerton and coworkers (26) have shown that pores and channels occur in which convective transport is possible to a certain extent (27). Hoffman and Decho (28) postulated areas of different density of the matrix, which have been observed experimentally. These features result in an extremely heterogeneous structure. This structure is dynamic; Schmitt and coworkers (29) showed in a *Pseudomonas putida* biofilm, which was challenged with toluene, that a rising concentration of toluene caused the formation of more polysaccharide and, furthermore, these compounds contained more carboxyl groups. Heise and Gust (30) showed that the EPS that were formed in the exponential growth phase of a marine isolate were different from those that were formed during the plateau phase. Limitation in nitrogen and phosphorus availability is also of importance for the composition of EPS, but not investigated in depth yet.

An important question is whether the EPS matrix acts as a diffusion barrier. The major component of that matrix is water. It could be shown by NMR measurements that the self-diffusion coefficient of water within the biofilm is practically the same as in free water, and only a very small fraction, that is, less than 0.1%, displays a lower diffusion coefficient (31). There is evidence that noncharged molecules up to a molecular mass of around 10,000 Da experience practically no diffusion limitation. However, if they are consumed, as is the case with oxygen, gradients arise because oxygen consumption by aerobic organisms can occur faster than oxygen can follow the diffusion gradient. This is the manner in which anaerobic zones in biofilm arise and the reason anaerobic organisms can find suitable habitats directly below respiring-aerobic colonies. Charged molecules can interact with charged groups of the EPS, which may slow down their mobility to a certain extent. This makes perfect sense from an ecological point of view because the mobility of nutrients, exoenzymes, and other products is not restricted within the matrix, which is of great importance for cells located in the center of clusters.

Mass transport is influenced not only by the internal architecture of biofilms but also by their interface with the water phase. Some biofilms have a highly filamentous appearance, whereas others are smooth. It is obvious that

a large number of filaments will increase the surface area on which interactions with components of the water phase are possible.

Investigations on the molecular mass of *P. aeruginosa* alginate revealed a mass between 1 and 2 Mio Da [(32), Windhues and coworkers, unpublished results] that corresponds to a length of up to 5 μm of the threadlike molecule. According to a gross estimate, based on a water content of approximately 95% in the EPS matrix, a volume element of 1 μm^2 and 10-nm thickness contains around 10 molecules of alginate and 300 protein molecules with an average molecular mass of 30,000 Da. With these data, realistic size relations of alginate and proteins, such a volume element can be schematically depicted as in Figure 2.

The data given in Table 2 refer to *P. aeruginosa* as a model system. In other systems, other organisms produce other EPS molecules in other concentrations. Because biopolymers generally tend to form hydrogels, the ecological advantage of a matrix can be provided by a wide variety of different EPS. This provides a certain level of protection against enzymes specific for certain types of EPS because, subsequently, organisms producing other types of EPS can protect their matrix against such enzymes. Although alginate is a relatively stable EPS component, proteins obviously undergo a more rapid turnover. The metaphor of the EPS matrix as the "house" of biofilm organisms can be further expanded; polysaccharides may represent "walls, ceilings, and floors," whereas proteins represent the "furniture" of the "house." This system arranges the proteins in proximity to the cells and prevents washing out of exoenzymes that would be easily lost under conditions of convectional flow.

An interesting aspect of the matrix architecture is the general interaction between exoenzymes and exopolysaccharides. Wingender and coworkers (33) showed that the heat stability of an extracellular lipase of *P. aeruginosa* was significantly increased in the presence of bacterial alginate. The same was observed for pH stability. It is quite probable that there are many more interactions of this kind. Such phenomena support the view that the EPS matrix is an important component in the view of biofilms as a tissue, as suggested by Costerton and coworkers (34).

In photosynthetic systems such as microbial mats or stromatolites, the EPS play an important role in light-transmission. At the biofilm surface, the prevailing irradiance and spectral composition were found to be significantly different from the incident light. The EPS were suspected as photon traps. Multiple scattering leads to an intensity maximum for photic light (400 to 700 nm) of approximately 120% of incident quantum irradiation at the biofilm surface. At the bottom of the eutrophic zone in the biofilm, light was attenuated strongly to less than 5 to 10% of the incident surface irradiance (35).

Whether or not EPS are used as a carbon source is still an open discussion. Sutherland (36) has given an excellent overview about polysaccharide lyases, which are capable of cleaving a hexose-1-, 4-, or ß-uronic acid sequence by ß-elimination. This author assumes that EPS usually are not degraded by the organisms that have produced them (37). However, EPS must be biodegradable by other species sooner or later, or else it would persist forever, but it appears as if the polysaccharide matrix is relatively stable and not degraded in the short term, contrary to the EPS protein moiety. Again, this makes sense from an ecological point of view because many different organisms can use the "house" once the energy and carbon is spent to build it, even if this has been performed by a predecessor. Evolution has obviously not generated organisms that readily degrade EPS generally, or else such organisms would have one of the largest collective carbon sources imaginable. The generation of a strain capable of degrading any given EPS matrix in a short time would lead to an environmental disaster because it would destroy natural microconsortia, which perform a crucial role in the biogeochemical cycles of carbon, oxygen, nitrogen, sulfur, and others. It seems as if nature has prevented such a threat by the vast diversity of EPS molecules that all are capable of fulfilling the structural role required for the biofilm mode of life.

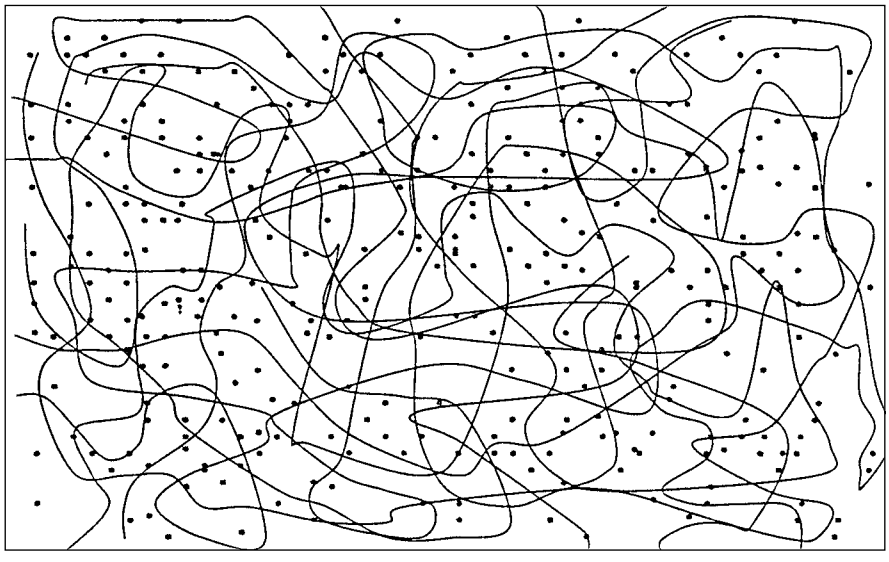

Figure 2. Size relationships of alginate and proteins in a volume element of an area of 1 μm^2 and a thickness of 1 nm, containing 10 molecules of alginate (2 Mio Da) and 300 protein molecules (30 kDa). 95% of the space is filled with water.

Enzyme preparations used for the dispersion of biofilms in biofouling cases (38) are ineffective for these reasons. This has been clearly demonstrated for papermaking processes (39).

EPS IN BIOFOULING, BIOWEATHERING, AND BIOCORROSION

EPS are involved in all kinds of biofouling (40) because they are the material that allows the existence of the microbial aggregates with biofouling properties. For example, the EPS keep biofilms on the surface of heat exchangers, ship bottoms, piping systems, medical devices such as implants, catheters, contact lenses, etc. and provide mechanical stability that is sufficient to withstand shear forces such as those occurring, for example, in pipes and on ship hulls. The damage done by biofouling is very difficult to assess, but even crude estimations lead to many millions of U.S. dollars every year in every industrialized country (41). Examples of problems caused by EPS will be mentioned here. At a first glance, some of them seem almost trivial, but they can result in severe consequences.

Mechanical Stability and Viscoelastic Properties

An important aspect of the EPS matrix is its mechanical stability (22), which has to be overcome by cleaners. Although mechanical cleaning is the most effective method, this is not always applicable, for example, when the biofouled surfaces are not accessible. Then, the cleaner has to interfere with the interactions that cause the cohesion. When biofilms develop on ship hulls or in piping systems, they will increase friction resistance because of the viscoelastic properties of the EPS matrix. This may lead to a substantial pressure drop and increase of energy consumption. Christensen and Characklis (4) assessed that a biofilm of 50-μm thickness, calculated as rigid roughness, can lead to a speed loss of 5 to 12% of a vessel. However, some EPS seem to reduce drag resistance, as is the case for organisms colonizing fast swimming fish (42). Unfortunately, this effect has not yet been transferred to engineered systems. An unexpected and significant increase of water viscosity in water as a result of the sudden increase of EPS production was attributed to phosphate limitation. This caused serious problems in a car production site where water was used for washing off excess spray paint. It could be reversed by external addition of a phosphate source (43).

Water Retention

Ophir and Gutnick (44) have investigated the role of EPS in the protection of biofilm organisms against desiccation. This is probably a very important ecological aspect of EPS because it maintains an environment in which microbial life is possible, but it also has technical relevance. When biological sludge has to be dewatered, for example, sewage or filter sludge, this water has to be removed. This is a significant economical aspect of water retention by EPS, considering the energy required for dewatering and the millions of tons of sewage sludge that have to be dewatered every year. Another more unexpected consequence of water retention is encountered when a biofilm develops on the cells of a battery, causing the battery to lose its charge (41).

Because the EPS are highly hydrated, they change the surface properties if biofilms develop on hydrophobic surfaces. When leaves fall into water, they will initially float because of their hydrophobic surface. After primary colonization, they are covered with a hydrophilic coating and sink. The hydrophobic surfaces (Teflon) of a calcium sulfate precipitation tank in the desulfurization unit of a power plant were colonized by a biofilm. This resulted in massive accumulation of calcium sulfate crystals at the wall, which had to be removed manually (unpubl. pers. obs.).

The water in the EPS dilutes the biopolymers and shields many of the potential binding sites that can bridge the macromolecules by electrostatic interactions, hydrogen bonds, and London dispersion forces (22). When a biofilm starts drying out, more of these binding sites will interact, because the water will not separate them from each other. This leads to a stronger cohesion that can be seen in many scanning electron micrographs in which the EPS appears as a filamentous structure, which is a result of the drying process required for SEM preparation and, thus, an artifact. Also, the adhesion forces to the substratum will greatly increase because they are based on the same mechanisms as in cohesion. This can be commonly observed with the naked eye on the walls of buildings when photosynthetic biofilms dry and curl up. Closer inspection reveals that, for example, on mortar, sand pebbles are removed from the mineral matrix. This is a process that increases weathering of mineral materials.

Heat Transfer

Another effect of the hydrated EPS matrix is that convectional transport of water is only possible to a very small extent when diffusive transport prevails. This is important for heat exchanger technology. Although the heat transfer coefficient for biofilms and water is practically the same (45), the performance of a heat exchanger can be significantly decreased by a biofilm because only diffusional heat transfer is possible. Convectional heat transfer as achieved by the tangential flow of water across the heat exchanger surface is hampered by the biofilm, which subsequently acts as an insulating layer for convection. This leads to significant losses in performance and increased energy demand in heat exchanger technology (41).

Biofouling and Calcium Scaling

In some cases, biofilms result not only in the unwanted accumulation of biological material on surfaces, but also promote the precipitation of minerals, especially calcium carbonate. This leads to mixed biological and nonbiological deposits (46) that are particularly difficult to remove. Calcium precipitation is an important aspect in scaling of surfaces, for example, on heat exchanger surfaces,

on separation membranes, on ship hulls, and oil rigs. This can occur preferentially in the EPS matrix, leading to deposits with remarkable mechanical stability. The same process happens in nature in a geologic scale and leads to the biogeochemical deposition of calcium carbonate (47).

Protection Against Biocides

It is well known that biofilm organisms can tolerate much higher concentrations of biocides and antibiotics than their planktonic counterparts (48,49). This can have extreme consequences. An example is given in Figure 3, which shows a SEM micrograph of a biofilm in a disinfection line constructed from copper. This biofilm developed in the presence of a quaternary ammonium compound. When isolated, the bacteria were sensitive to the biocide (50). It is a common observation that biofilm bacteria lose their biocide tolerance once they are isolated and cultivated in suspension (49).

The phenomenon is still not fully understood (51) and is associated with the EPS matrix but not with phenomena such as diffusion limitation, because diffusion of biocides is not significantly hindered by the matrix. Oxidizing biocides can be consumed by reaction with the EPS, as shown for chlorine by Wingender and coworkers (52) when comparing mucoid and nonmucoid strains of *P. aeruginosa*. However, when hydrogen peroxide was used, the mucoid strain was more susceptible than the nonmucoid strain.

Accumulation of Particles

EPS accumulate particles, which can lead to problems. An example of this is the production of paper, which is a large industry. Here, slime aggregates can develop in which cellulose fibers and organic and inorganic additives stick to surfaces and form slimy aggregates (Fig. 4). These aggregates eventually detach, leading to the formation of holes in the paper and ultimately in significant technical and economical losses (53).

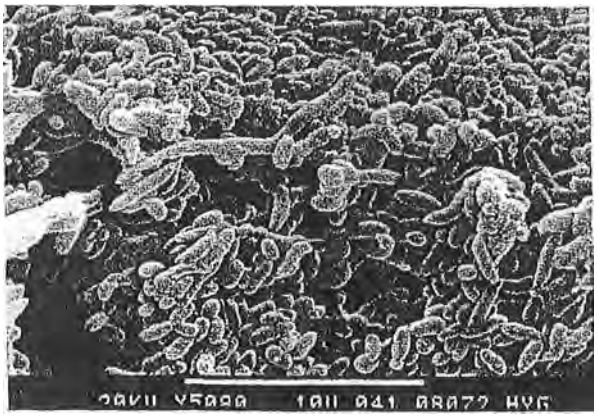

Figure 3. Scanning electron micrograph of a biofilm grown on the surface of a disinfection pipeline made of copper. The organisms have formed the biofilm in the presence of the disinfectant (quaternary ammonium compound) but were sensitive to it when isolated from the biofilm (courtesy of G. J. Tuschewitzki).

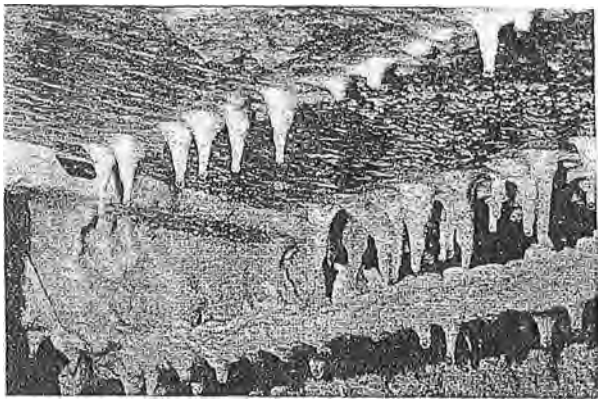

Figure 4. Aggregates of cellulose fibers caused by slime formation in a paper mill (courtesy of J. Klahre). Average length of slime aggregates: 5 to 10 cm.

EPS in Microbially Influenced Corrosion (MIC)

EPS are also involved in microbially influenced corrosion (MIC). In particular, their interaction with iron and other metals can increase the kinetics of metal corrosion as demonstrated by the EPS of sulfate-reducing bacteria (54). Little and coworkers (55) investigated the role of EPS in marine copper corrosion. They found a marine *Oceanospirillum* that produced copious amounts of copper-binding EPS when grown on copper surfaces; this may be a protective mechanism against copper toxicity. In principle, the contribution of EPS to the mechanisms of MIC is based on their binding of metal ions, either by chemical reaction, chelation, or by other ionic binding, and the subsequent dissolution of the metal. Also, EPS support the attachment of active bacteria to the biofilm. The mechanisms of MIC cases in which sulfate-reducing bacteria are not involved, however, the particular role of EPS in corrosion is sparsely investigated, although it must be suspected that they are involved in the corrosion mechanism. It is quite probable that extracellular redox enzymes may play a role in MIC.

SORPTION PROPERTIES

Dissolved substances also can be sorbed by biofilms and in particular by the EPS matrix (56). While some authors point out that the carboxylic groups of polysaccharides bind to heavy metals (57), such as copper (58), uranium (59), and cadmium (60), Wuertz and coworkers (61) could show that zinc and nickel accumulate on the cell surfaces in sewage sludge. In this case, the EPS tightly bound to the cell surface may be the major binding partners; however, this has not been investigated in detail. Decho (14) speculates that the sorption of heavy metals is part of a protection strategy against toxic effects. Not much is known about the sorption of anions but it can be expected that they are accumulated in the matrix, compared to the bulk-water phase. Although the matrix

is a hydrogel and as such hydrophilic, it must contain hydrophobic areas. This has been shown by sorption experiments with benzene, toluene, and xylene into sewage sludge. They were clearly accumulated in the EPS fraction (62). The exact sorption sites of such compounds in the EPS matrix is still unclear. Interestingly, biofilms can respond dynamically to sorption processes (63), as demonstrated earlier with the various responses of a *P. putida* biofilm to toluene (29).

Whether particle binding belongs to the aspects of sorption may be doubtful, but particles can be retained by biofilms because of the "sticky" properties of the EPS. Not only biodegradable particles, which would provide nutrients, but also sand grains can attach to biofilms because of adhesion of EPS molecules. This is an important feature in a geologic scale in diagenesis of sediments (64), mainly because of diatom EPS (65). The transport of sand in sediments is also influenced by EPS, as shown by Dade and coworkers (66) for the polysaccharides of the benthic marine bacterium, *Alteromonas atlantica*. This observation is important, for example, for modeling the transport of sediments in rivers. Also, the hydraulic conductivity of sand is influenced by EPS (67), an effect that is also known for other materials and can lead to clogging of filters (68).

EPS IN BIOTECHNOLOGY

Biotechnological applications of EPS have focused so far only on polysaccharides. Some EPS are used for increasing the viscosity of technical materials and food; they are also used as biosurfactants. Selected EPS hold a potential for medical purposes and for biologic glue. Sutherland (69) has compiled a comprehensive list of the uses of microbial exopolysaccharides, for example, in the food industry, as additives for oil-drilling emulsions and others. In most cases, EPS are incorporated to alter the rheological properties of the water present and, thus, change the texture of the product. Advantage is also taken of the ability of some mixtures of polysaccharides to exhibit synergistic gelling, which allows the application of smaller amounts of EPS. Associated with the readily measurable properties such as viscosity, others such as "mouth feel," which are more difficult to define, are of practical importance. Xanthan, a polysaccharide produced by *Xanthomonas campestris*, is used to maintain the consistency of some toothpastes. Other polysaccharides such as gellan or the synergistic mixture of xanthan-galactomannan were recommended for further use in the food industry. Polysaccharide lyases, that is, extracellular proteins, were suggested for biotechnological use, for example, to degrade the walls of marine algae in protoplast production (70). Sutherland (71) has listed established applications of microbial extracellular polysaccharides (Table 3).

EPS Used for Metal Removal

EPS were also used for use in metal removal from water. Because the EPS from *Bacillus megaterium* bind large amounts of copper, they were suggested for use in copper removal (72). A marine bacterial EPS exhibited a high affinity for cadmium (60) and holds potential for specific cadmium removal. Algal EPS are used for the same purpose in terms of natural attenuation of aqueous metal contamination by an algal mat (73). Guezennec and coworkers (74) isolated uronic-rich EPS with a potential for biotechnological usage for metal removal from deep-sea organisms. Potential and limits of this approach has been reviewed by Geesey and Jang (75).

EPS as Biosurfactants

The properties and uses of EPS as biosurfactants have been intensively investigated (76). Such organisms can be preferentially isolated from oil production

Table 3. Some Examples for Established Applications of Microbial Extracellular Polysaccharides

Properties	Use	Polymer
Biological	Antitumor agents	β-D-Glucans
	Eye and joint surgery	Hyaluronic acid
	Heparin analogs	*Escherichia coli* K 5 EPS
	Wound dressings	Bacterial cellulose
Chemical	Enzyme substrates	*E. coli* K 4 and K 5 EPS
	Oligosaccharide preparation	Curdlan, pullulan, scleroglucan
Physical		
Emulsion stabilization	Foods, thixotropic paints	Xanthan
Fiber strength	Acoustic membranes	Bacterial cellulose
Film formation	Food coatings	Pullulan
Flocculant	Water clarification, ore extraction	Various
Foam stabilization	Beer, fire-fighting fluids	Xanthan
Gelling agents	Cell and enzyme technology, foods	Gellan
Hydrating agent	Cosmetics, pharmaceuticals	Hyaluronic acid
Crystal-formation inhibitor	Frozen foods, pastilles, sugar syrups	Xanthan
Viscosity control	Oil-drilling 'muds', jet printing	Xanthan
Suspending agent	Food, paper coatings	Xanthan

Source: (After I. W. Sutherland, *Trends Biotechnol.* **16**, 41–46 (1998), modified).

sites or spills (77). Interestingly, some bacteria seem to use surface-active EPS to facilitate their transport through sandy soil (78). Some examples for surface-active EPS of technical relevance are given in Table 4 (79). These EPS may be used in tertiary oil production to mobilize residual oil from surfaces and capillaries.

OUTLOOK

It is justified to speculate that the functions of the EPS for the ecology of microbial aggregates is much more complex than it appears at a first glance. Possibly, the matrix is not just an arrangement of randomly associated biopolymers but is synthesized and degraded in a way that supports the life of the organisms in such aggregates. It is evident that the matrix is heterogeneous in composition and architecture and over time, represents a dynamic system. Possibly, an important parameter such as cell surface hydrophobicity may be regulated by surface-active EPS as suggested by Neu (76). Recent research indicates that alasan, a surface-active exocellular emulsifier produced by a strain of *Acinetobacter radioresistens* could be transferred horizontally to *A. calcoaceticus* when both strains were grown together, changing the surface properties of the latter (80). The EPS matrix and the properties that it determines represent important and complex aspects when considering biofilms as a first step to the evolution of multicellular organisms.

The use of EPS as food additives is promising but strongly restricted by legal regulations. Thus, it may be difficult to introduce new EPS because of the lengthy and costly registration procedure. However, as additives for paints and other technical materials, EPS still hold a potential that is not yet exploited. In addition, EPS may be useful as biologic glue. The role of EPS in biofouling and biocorrosion is also worth further investigating. In particular, the adhesive and cohesive forces play a fundamental role when biofilms have to be removed (22). The use of cleaners and biodispersants is still based on empirical experience and plausible considerations but few fundamental researches. Here, further progress may result in more effective approaches and concepts for prevention and removal of biofilms.

Table 4. Some Microbial Biosurfactants

Biosurfactant	Source Organism
Glycolipids	*Pseudomonas aeruginosa*
Lipopeptides	*Bacillus. licheniformis*
Fatty acids	*Candida lepus*
Emulsan	*Acinetobacter calcoaceticus*
Biodispersan	*Acinetobacter calcoaceticus*
Liposan	*Candida lipolytica*

Source: (After J. D. Desai and I. M. Banat, *Microb. Mol. Biol. Rev.* **61**, 47–64 (1997), modified).

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EXTREMOPHILES: LIFE IN EXTREME ENVIRONMENTS

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Extremophiles are organisms that can survive and grow optimally under extreme conditions. MacElroy first introduced the term *extremophile* in 1974 (1), and to define it accurately, it is necessary to define a nonextreme or normal environment. Although there are no strict definitions of a "normal" environment, there is a general view about most of the important physical and chemical factors available under these conditions. A normal environment has a temperature between 10 and 40 °C, a pH from 5.0 to 8.0, a pressure of 1 atm, and a salinity corresponding to the salinity of freshwater or of seawater (up to 30 g/l) (2). Environments in many parts of the world are considered extreme. These include geothermal environments, polar regions, acid and alkaline springs, and cold, pressurized depths of the oceans. It can be speculated that the species diversity decreases when environmental stress increases. As conditions become increasingly demanding, extreme environments become exclusively populated by microorganisms belonging to the bacterial and archaeal domains (prokaryotes). It is very likely that higher organisms are unable to survive under extreme conditions because of their cellular complexity. The realization that extreme environments harbor a different kind of prokaryotic lineage has resulted in a complete reassessment of our concept of microbial evolution and has given considerable impetus to research on extremophiles (3).

Enzymes and proteins that work under extreme conditions mediate metabolic processes and specific biological functions of these microorganisms. Consequently, constant increasing scientific interest has been aroused not only because these microorganisms grow in some of the harshest environments on Earth but also because they provide a valuable source of unique enzymes. Modern biotechnology, which provides a whole new repertoire of methods and products, still tries to mimic nature, thus demanding continuous efforts in the isolation and characterization of novel microorganisms. This article focuses on microorganisms that live under extreme conditions in terms of high and low temperature, extremes of pH, and high salinity. Such extremophiles can be found in terrestrial and marine environments, including the polar regions, solfataric fields, and soda lakes as well as in shallow and abyssal hydrothermal vent systems. Examples of microorganisms representative of each of these extreme habitats are given in the text that follows.

MICROORGANISMS LIVING AT LOW TEMPERATURE

Temperatures in many parts of the world seldom or never reach above 5 °C. This is mainly because almost 70% of the Earth is covered by deep oceans that can be considered as cold environments. Furthermore, the polar regions provide a permanently cold terrestrial habitat that is surrounded by an aquatic belt of melting ice. Numerous microorganisms, in particular bacteria, yeasts, unicellular algae, and fungi, have successfully colonized cold environments. These microorganisms, generally defined as "psychrophiles" are able to grow at temperatures close to 0 °C and have developed various adaptations in the form of finely tuned structural changes at various metabolic and cellular levels, for example, adaptations of their enzymes, enabling them to compensate for the deleterious effects of low temperature. Most of the cold-adapted microorganisms have been characterized from the Arctic and Antarctic seawaters; the latter not only are a cold habitat (around −1 °C) but also exert a high selective pressure on endemic microorganisms because the temperature of their environments is constant. Despite the harsh conditions, the density of bacterial cells in the Antarctic oceans is as high as the density reported for temperate waters (4). The microorganisms that are able to grow at or close to the freezing point of water can be divided into two main groups: psychrophiles and psychrotolerants. Pyschrophilic microorganisms are more strictly defined by an optimum temperature for growth of about 15 °C, a maximum growth temperature of about 20 °C, and a minimum temperature for growth at 0 °C or lower. In comparison, psychrotolerant organisms generally do not grow at 0 °C but do grow at 3 to 5 °C, and have optimum and maximum growth temperatures above 20 °C (4). In general, psychrophiles have significantly narrower growth temperature ranges and lower optimum/maximum growth temperatures compared with psychrotolerant microorganisms. Psychrophiles can be found in permanently cold environments such as the deep-sea, glaciers, and mountain regions, in soils, and in fresh or saline waters associated with cold-blooded animals such as fish or crustaceans. The first psychrophilic bacteria were isolated from preserved fish by Forster in 1887 (2).

Recently, a systematic investigation has been carried out to understand the rules governing the molecular adaptation of psychrophiles to low temperatures (5). These fundamental aspects are closely associated with a strong biotechnological interest aiming at the exploitation of these microorganisms and their cell components such as membranes, polysaccharides and enzymes. The specific activity of wild-type, cold-adapted enzymes and some of their recombinant forms produced by Antarctic and Arctic microorganisms have been determined for several enzymes, including alcohol dehydrogenase, α-amylase, aspartate transcarbamilase, metal-protease, citrate synthase, subtilisin, triose phosphate isomerase, and xylanase (5). In general, cold-adapted enzymes have higher specific activity at low and moderate temperatures than that of their mesophilic counterparts, and are inactivated easily by a moderate increase in temperature. These properties can be extremely useful in various applications. The possible applications include the use of enzymes (proteases, lipases, and cellulases) in detergents (cold washing), as flavor-modifying agents for food processing, such as cheese manufacture, meat tenderizing, and

lactose hydrolysis (5). Table 1 presents some representatives of psychrophilic microorganisms and their optimum growth conditions.

MICROORGANISMS LIVING AT HIGH TEMPERATURE

Microorganisms that are adapted to grow optimally at high temperatures (60 to 108 °C) have been isolated so far from high-temperature terrestrial and marine habitats (2,6). The most common biotopes are geothermally heated hydrothermal vent systems such as solfataric fields, neutral hot springs, and submarine hot vents. Submarine hydrothermal systems are situated in shallow or abyssal depths. They consist of hot fumaroles, springs, sediments, and deep-sea vents with temperatures up to 400 °C ("black smokers") (7). Shallow marine hydrothermal systems are located for instance at the beaches of Vulcano, Naples, and Ischia (Italy), Sao Miguel (Azores), and Djibouti (Africa). Examples of deep-sea hydrothermal systems are the Guaymas Basin (depth 1,500 m) (8), the East Pacific Rise (depth 2,500 m) (9), both off the coast of Mexico, the Mid-Atlantic Ridge (depth 3,700 m) (10), and the Okinawa Trough (depth 1,400 m) (11). Because of their ability to convert volcanic gases and sulfur compounds at high temperatures, hyperthermophilic communities living in such hydrothermal vents are expected to play an important role in marine ecological and geochemical processes (12). Shallow-sea and deep-sea hydrothermal systems harbor members of the genera *Igneococcales*, *Thermococcales*, *Methanococcales*, *Archaeoglobales*, and *Thermotogales*. To date, members of the genus *Methanopyrus* have been found only at greater depths, whereas *Aquifex* spp. have been isolated exclusively from shallow hydrothermal vents (7). Recently, biotopes of extreme and hyperthermophilic microorganisms were discovered in deep, geothermally heated oil reservoirs around 3,500 m below the bed of the North Sea and the permafrost soil of North Alaska (13,14).

Table 1. Microorganisms Living at Low or Elevated Temperatures

Microorganism	Optimum Growth Temperature (°C)
Psychrophilic Microorganisms	
Vibrio spp.	15–20
Micrococcus criophilus	15–20
Arthrobacter glacialis	15–20
Vibrio psychroerythreus	15–20
Aquaspirillum articum	15–20
Shivanella frigidimarina	15–20
Moderate Thermophiles (50–60 °C)	
Bacillus acidocaldarius	50
Bacillus stearothermophilus	55
Extreme Thermophiles (60–80 °C)	
Thermus aquaticus	70
Thermoanaerobacter ethanolicus	65
Clostridium thermusulfurogenes	60
Fervidobacterium pennivorans	75
Hyperthermophiles (80–110 °C)	
Thermotoga maritima	90
Aquifex pyrophilus	85
Archeoglobus fulgidus	83
Methanopyrus kandleri	88
Sulfolobus sulfataricus	88
Thermococcus aggregans	88
Pyrobaculum islandicum	100
Pyrococcus furiosus	100
Pyrodictium occultum	105
Pyrolobus fumarii	106

Microorganisms capable of growing optimally between 50 and 60 °C are designated as moderate thermophiles. Most of these microorganisms belong to many different taxonomic groups of eukaryotic and prokaryotic microorganisms such as protozoa, fungi, algae, streptomycetes, and cyanobacteria, which comprise mainly mesophilic species. It can be assumed that moderate thermophiles, which are phylogenetically closely related to mesophilic organisms, may be secondarily adapted to life in hot environments. Extreme thermophiles, which grow optimally between 60 and 80 °C, are widely distributed among the genera *Bacillus*, *Clostridium*, *Thermoanaerobacter*, *Thermus*, *Fervidobacterium*, *Thermotoga*, and *Aquifex* (Table 1). Conversely, most of the hyperthermophiles grow optimally between 80 and 108 °C (15). Interestingly, and as shown in Figure 1, the majority of the hyperthermophiles isolated to date belong to the archaeal domain of life and so far no eukaryotic organism has been found that can grow at the boiling point of water. A 16S rDNA-based universal phylogenetic tree (16,17) shows a tripartite division of the living world consisting of the domains Bacteria, Archaea, and Eukarya (Fig. 1). The archaeal domain consists of two major kingdoms: the Crenarchaeota (some genera are *Sulfolobus*, *Picrophilus*, *Pyrodictium*, *Pyrolobus*, *Pyrobaculum*, and *Thermoproteus*) and the Euryarchaeota, which include a number of hyperthermophiles (some genera are *Thermococcus* and *Pyrococcus*), methanogens (e.g., *Methanococcus*, *Methanobacterium* and *Methanosarcina*), sulfate-reducers (e.g., *Archaeoglobus*) and halophiles (including genera such as *Halobacterium* and *Halococcus*). Short phylogenetic branches indicate a rather slow evolution. Deep branching points are evidence for early separation of the two groups. The separation of the Bacteria from the Eukarya-Archaea lineage is the deepest and earliest branching point known so far (7). Hyperthermophiles are represented among all the deepest and shortest lineages, including the genera *Aquifex* and *Thermotoga* within the Bacteria, and *Pyrodictium*, *Pyrobaculum*, *Thermoproteus*, *Desulfurococcus*, *Sulfolobus*, *Methanopyrus*, *Pyrococcus*, *Thermococcus*, *Methanococcus* and *Archaeoglobus* within the Archaea (Fig. 1; Table 1).

The relative abundance of Archaea and Bacteria in high-temperature environments was, until recently, mainly studied by cultivation-based techniques. Because of the frequent isolation of Archaea from these habitats, it was assumed that Archaea dominate the high-temperature biotopes (18). Recently, the application of molecular-biological methods revealed a quite different picture. Slotbot hybridizations of rRNA-utilizing oligonucleotide probes targeting the 16S rRNA of Archaea and Bacteria revealed that Bacteria are the major component of the microbial community along a thermal gradient at a

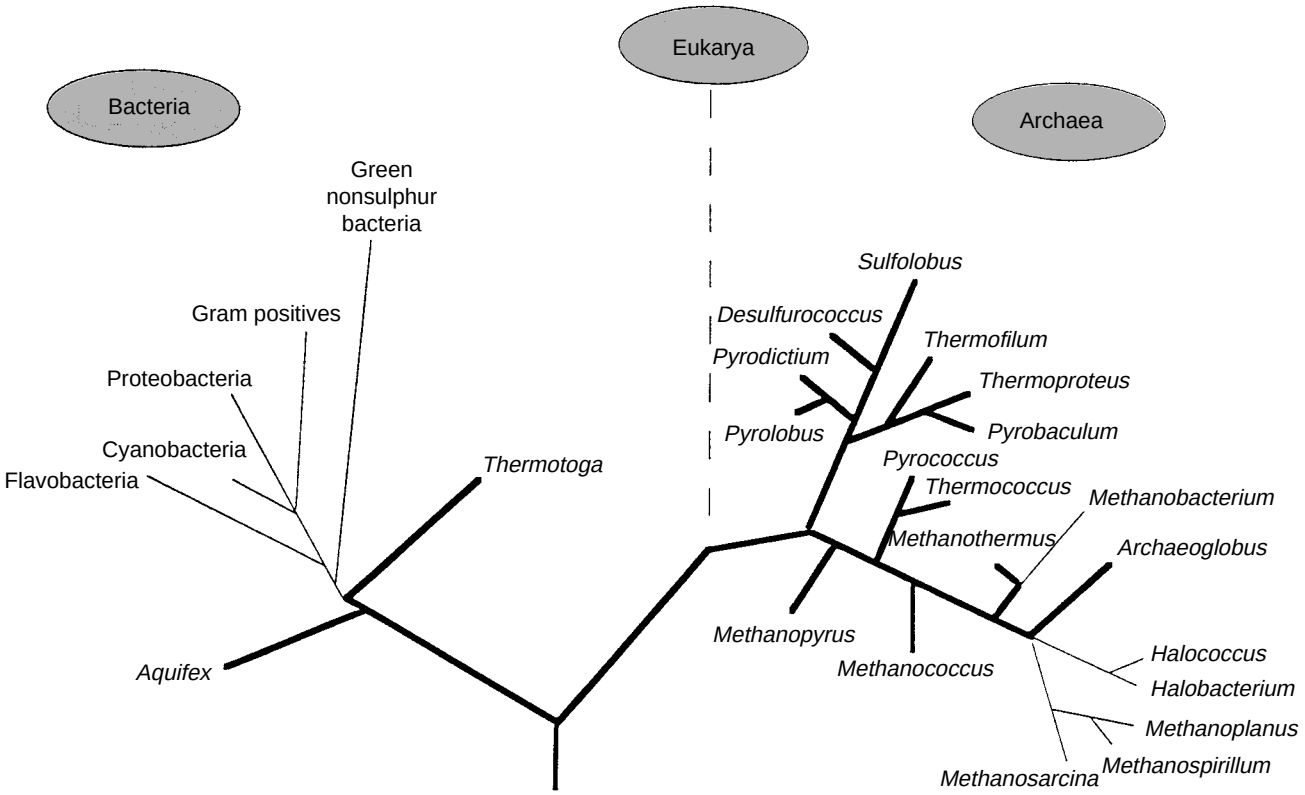

Figure 1. Phylogenetic tree of the three domains of life. Hyperthermophilic species are highlighted by bold branches. Branching order and branch lengths are based on 16S/18S rDNA sequence comparisons. The tree is a modified version of that from Blöchl and coworkers (18).

shallow submarine hydrothermal vent near Milos Island (Greece) (19). Bacteria made up at least 78% (mean 95%) of the prokaryotic rRNA. Along the steepest temperature gradient, the proportion of archaeal rRNA increased. Nevertheless, even in the hottest sediment layer, archaeal rRNA made up only around 12% of the prokaryotic rRNA. These results suggest that Archaea may generally be of lower abundance in hot environments than could be assumed from cultivation-based experiments. However, the factors that allow Bacteria to dominate in high-temperature habitats that were once believed to be the realm of Archaea remain unknown.

To date, most of the extreme thermophiles and hyperthermophiles isolated were investigated in the laboratory, and little information is available on the growth behavior in their ecosystems. In addition, a large number of hyperthermophiles remain undiscovered and continue to thrive in various ecosystems.

Although most of the hyperthermophiles appear as single cells, there have been some reports on the existence of multicellular forms during growth at elevated temperature. This has been observed in the case of *Thermococcus aggregans*, *Staphylothermus marinus*, *Pyrodictium occultum*, and *P. abysii*. The formation of large, grape-shaped cell aggregates ("giant cells") that can reach up to 1.5 mm in size, are frequently observed in *Staphylothermus marinus*. *Thermococcus aggregans*, recently isolated from a hydrothermal vent site at the Guaymas Basin was found to form aggregates consisting of up to 50 cells, particularly when cultures were grown on media containing yeast extract/peptone and elemental sulfur (7).

Of interest are the two archaeal strains *P. abysii* and *P. occultum* that grow optimally at 100 °C and build a network structure in which the cells are connected. *Pyrodictium occultum* is able to grow at temperatures up to 110 °C and can even survive one hour of autoclaving at 121 °C (18). A network of hollow tubules, therefore called cannulae, which measures about 30 nm in diameter, connects the coccoid-shaped cells of *P. occultum*. Attempts to grow *P. abyssii* in fermentors under agitation were not successful because of the instability of the network structure. It is still an open question whether these observations, that is, the network formation and aggregation, are due to specialized laboratory conditions or if they are a phenomenon that can be observed in nature (21).

Most of the microorganisms mentioned above can be found in low-salinity and submarine environments and are strict anaerobes. Terrestrial solfataric field as can be found in Italy or Iceland harbor members of the genera *Pyrobaculum*, *Thermoproteus*, *Thermofilum*, *Desulfurococcus*, and *Methanothermus*. *Pyrobaculum islandicum*, and *Thermoproteus tenax* are able to grow chemolithoautotrophically, gaining energy by anaerobic reduction of S^0 by H_2 (7,18). In contrast to these strictly anaerobic microorganisms, *Pyrobaculum aerophilum* and *Aeropyrum pernix* are able to use oxygen as final electron acceptor.

Methanothermus fervidus, is highly sensitive to oxygen and can only survive in low redox environments at temperatures between 65 and 97 °C. Some microorganisms from marine environments, such as members of the genera *Archaeoglobus*, *Methanococcus*, and *Methanopyrus* are able to grow chemolithoautotrophically, gaining energy by the reduction of SO_4^{2-} by H_2 (*Archaeoglobus lithotrophicus* and *A. fulgidus*) or by the reduction of CO_2 by H_2 (*Methanococcus jannaschii*, *Methanopyrus kandleri*). It is noteworthy that the microbial production of methane has a significant impact on the global budget of trace gases (e.g., H_2, CO_2) that are discussed to influence the world's climate. Other members of the hyperthermophilic genera *Staphylothermus*, *Pyrococcus*, *Thermococcus*, and *Pyrodictium* are adapted to marine environments. Most of them gain energy by fermentation of polysaccharides, peptides, amino acids, and sugars (22).

Among the bacterial domain of life, members of the genera *Aquifex* and *Thermotoga* represent the deepest phylogenetic branches. Cells of *Thermotoga* are rod-shaped and possess a characteristic sheath-like structure, the so-called toga, which surrounds the cells and forms terminal "balloons." Within this genus, *Thermotoga maritima* and *T. neapolitana* are the most thermophilic species, with maximal growth temperature of about 90 °C. Microscopic observations showed that depending on the nutritional conditions, few cells could grow in the same toga forming a filamentous structure (7). So far, *Aquifex pyrophilus* is the most thermophilic species among the Bacteria (T_{max} 95 °C). This strain grows strictly chemolithoautotrophically under microaerophilic conditions with hydrogen or sulfur as electron donors, forming H_2O and H_2SO_4, respectively (18).

MICROORGANISMS GROWING AT EXTREMES OF PH

Solfataric fields are the most important biotopes of microorganisms that prefer to live under both thermophilic and acidic conditions. Solfataric soils consist of two different layers that can be easily distinguished by their characteristic colors: the upper, aerobic layer has an ochre color because of the presence of ferric iron, and the layer below, which is anaerobic, appears rather blackish-blue as a result of the presence of ferrous iron. According to the chemical parameters of the two layers, different kinds of microorganisms can be isolated from these habitats. *Thermophilic acidophiles* belonging to the genera *Sulfolobus*, *Acidianus*, *Thermoplasma*, and *Picrophilus*, with growth optima between 60 and 90 °C and pH 1.0 to 5.0 are commonly found in the aerobic upper layer, whereas slightly acidophilic or neutrophilic anaerobes such as *Thermoproteus tenax* or *M. fervidus* can be isolated from the lower layer. Species of Thermoplasma (growth optima: pH 2 and 60 °C) have been found in hot springs, solfataras, and coal refuse piles (23). Their closest known phylogenetic relatives, also found in solfataras, are species of the genus *Picrophilus*, which are so far the most extreme acidophiles, with growth optima close to pH 0 (24). *Picrophilus oshimae* and *P. torridus* are both aerobic, heterotrophic Archaea that grow optimally at 60 °C and pH 0.7 and utilize various polymers such as starch and proteins as carbon source (Table 2a).

Table 2. Microorganisms Growing at Extreme pH Values

	Optimum Growth	
	(°C)	pH
a) Acidophilic microorganisms		
Sarcina ventriculi	37	4.0
Thiobacillus ferrooxidans	37	2.5
Alyciclobacillus acidocaldarius	55	2.0–6.0
Picrophilus oshimae	60	0.7
Picrophilus torridus	60	0.7
Thermoplasmaacidophilum	60	2.0
Sulfolobus acidocaldarius	75	2.5
Acidianus infernus	75	2.0
b) Alkaliphilic microorganisms		
Many cyanobacteria	20–37	6.0 to 8.0
Spirulina spp.	37	8.0 to 10.0
Chromatium spp.	37	8.5
Bacillus spp.	37	11.5
Anaerobranca gottschallki	55	9.0
Thermococcus alcaliphilus	85	9.0
Thermococcus acidoaminivorans	85	9.0

Members of the genus *Sulfolobus* are strict aerobes growing either as autotrophs, heterotrophs, or facultative heterotrophs (6,24). During autotrophic growth, S^0, S^{2-}, and H_2 are oxidized to sulfuric acid or water as end products. *Sulfolobus metallicus* and *S. brierley* are able to grow by the oxidation of sulfidic ores. A dense biofilm of these microorganisms is responsible for the microbial ore leaching process in which heavy metal ions such as Fe^{2+}, Zn^{2+}, and Cu^{2+} are solubilized. During heterotrophic growth, a range of sugars and proteinaceous substrates are utilized (25). A spore-forming sulfur-oxidizing thermoacidophile, designated *Thiobacillus caldus* (T_{max} 55 °C), was isolated by Hallberg and Lindström (26). It seems likely that *T. thiooxidans*, a sulfur-oxidizing mesophilic acidophile, would have been supplanted by the similar but more thermotolerant *T. caldus* in which the temperature exceeded 35 to 40 °C. Other hyperthermophilic acidophiles have been affiliated with the genera *Metallosphaera* (growth range: 50 to 80 °C, pH 1 to 4.5), *Acidianus* (growth range: 60 to 95 °C, pH 1.5 to 5), and *Stygioglobus* (growth range: 57 to 89 °C, pH 1 to 5.5) (3).

Alkaliphiles

Alkaliphilic microorganisms are widely distributed throughout the world. They have been found in carbonate-rich springs and alkaline soils, where the pH can be 10.0 or even higher, although the cytoplasmic pH is maintained around 8.0. In such places, several species of cyanobacteria are normally abundant and provide organic matter for diverse groups of heterotrophs, the best known of which are alkaliphilic *Bacillus* spp. Alkaliphiles require alkaline environments and sodium ions not only for growth but also for sporulation and germination. Sodium ion-dependent uptake of nutrients has been reported in alkaliphiles.

Many alkaliphiles require various nutrients for growth; few alkaliphilic *Bacillus* strains can grow in simple minimal media containing glycerol, glutamic acid, and citric acid. In general, cultivation temperature is in the range of 20 to 55 °C. Many haloalkaliphiles isolated from alkaline hypersaline lakes can grow in alkaline media containing 20% NaCl. The soda lakes in the Rift Valley of Kenya and similar lakes found in a few other places on earth are highly alkaline, with pH values between 11.0 and 12.0, and represent a typical habitat where alkaliphilic microorganisms can be isolated. Thermophilic anaerobic spore-forming alkaliphiles, such as thermoalkaliphilic *Clostridia*, have been isolated from sewage treatment plants (3). Very recently, two new, moderately thermoalkaliphic microorganisms, *Anaerobranca gottschalkii* and *Anaerobranca horikoshii*, have been isolated from Lake Bogoriae in Kenya and from Yellowstone National Park, respectively. The new isolates represent a new line within the Clostridium/Bacillus subphylum. The only archaeal thermoalkaliphiles identified to date are *Thermococcus alcaliphilus* and *Thermococcus acidoaminivorans*, both growing optimally at 85 °C and pH 9.0. (Table 2b). These alkaliphiles are unique microorganisms with great potential for biotechnological exploitation. The main industrial application of alkaliactive enzymes is in the detergent industry, where they account for approximately 30% of total worldwide enzyme production. Alkaline enzymes have also been used in the hide-dehairing process, where dehairing is carried out at pH values between 8.0 and 10.0 (27).

MICROBIAL LIFE AT ELEVATED SALT CONCENTRATIONS

The halophiles comprise Bacteria and Archaea that grow optimally in NaCl concentrations above those of seawater (>0.6 M NaCl). In general, halophilic microorganisms are classified as moderate halophiles if they can grow in salt concentrations between 0.85 and 1.7 M NaCl and as extreme halophiles if they require NaCl concentrations above 1.7 M for growth (28). Halophiles have been mainly isolated from saline lakes, such as the Great Salt Lake in Utah (salinity >2.6 M) and from evaporitic lagoons and coastal salterns with NaCl concentrations between 1 and 2.6 M (29). Saline soils have been less well explored. Bulk salinity measurements of 1.7 to 3.4 M NaCl have been reported for saltern soils (28). Saline soils constitute less stable biotopes than hypersaline waters since they are subjected to periodic and significant dilution during rainy periods. It can be assumed that microbial survival under these oscillating conditions would be even more demanding. There is no doubt that almost all hypersaline habitats harbor significant populations of specifically adapted microorganisms. However, it remains unclear what substrates for growth might be available in these biotopes. Hypersaline lakes often contain up to 1 g/L of dissolved organic carbon. In many of these lakes, primary producers such as cyanobacteria, anoxygenic phototrophic bacteria, and algae may be the main source of organic compounds (29,30). It has also been speculated that organic compatible solutes produced by many of the phototrophs as a means of counterbalancing the osmotic stress contribute significantly to the input of carbon sources. It is noteworthy that, despite the typically large surface-to-volume ratios, hypersaline environments are low in dissolved oxygen (<2 mg/L) and might be essentially anaerobic (for reviews, see 29,31).

In a study of aerobic heterotrophs in marine saltern, it has been shown that bacterial halophiles were dominant up to 2 M NaCl. Above this concentration, archaeal halophiles become predominant, almost to the exclusion of bacteria (32). Halophilic primary producers mainly belong to the cyanobacteria and anoxygenic phototrophic sulfur bacteria. The former often thrive in eutrophic salterns, in which they form large, floating mats. The latter group, on the other hand, grows either in anaerobic sediments or in the water column where they are responsible for the characteristic red color of highly saline habitats (33). The range of heterotrophic Bacteria comprises proteobacteria, actinomycetes, and gram-positive rods and cocci. Fermentative anaerobes as well as sulfur oxidisers, sulfate reducers, and nitrate reducers are also present and give rise to the assumption that all kinds of metabolic features may be found in high-salinity environments (Table 3). Halophilic bacteria do not belong to one homogeneous group, but rather fall into many taxa in which the ability to grow at high salt concentrations is a secondary adaptation.

The term "halobacteria" refers to the red-pigmented, extremely halophilic Archaea, that is, members of the family *Halobacteriaceae*, the only family in the order Halobacteriales (29,31). Most halobacteria require 1.5 M NaCl to grow and to retain the structural integrity of the cell. Halobacteria can be distinguished from halophilic bacteria by their archaeal characteristic, in particular the presence of ether-linked lipids (34). Most halobacteria are colored red or orange because of the presence of carotenoid pigments, but some species are colorless, and those with gas vesicles form opaque, white, or pink colonies. A purple hue may be seen in halobacteria that form the bacteriorhodopsin-containing purple membrane (29). Halobacteria are the most halophilic organisms known, and form the dominant microbial population when hypersaline waters approach saturation (33). Interestingly, the reddening caused by halobacterial blooms has an impact on the evaporation rates in the saltern. It is known that the carotenoid pigments of halobacteria absorb solar radiation,

Table 3. Salt Requirements of Halophilic Microorganisms

Halophilic Microorganism	Salinity (M NaCl) for Growth		
	Minimum	Optimum	Maximum
Dunaliella spp.	0.3	–	5.0
Clostridium halophilum	0.15	0.6	6.0
Haloanaerobium praevalens	0.8	2.2	4.3
Halobacterium spp.	–	2.0	5.5
Halobacterium denitrificans	1.5	2.5	4.5
Haloferax vulcanii	1.0	1.5	3.0
Methanohalobium evestigatum	–	–	4.3

thus increasing the ambient temperature and evaporation rates.

Halobacteria form a diverse group with at least nine major taxa that have all diverged from a common ancestor at about the same time (35,36). In general, soda lakes harbor a restricted range of alkaliphilic taxa characterized by a requirement for high pH and high salt levels, whereas neutral hypersaline environments harbor a wider range of neutrophilic taxa (29). It has been shown recently that neutral hypersaline waters all over the world at saturation point for NaCl harbor populations of virtually identical halobacteria belonging to the genera *Haloarcula*, *Halobacterium*, and *Halorubrum* (37). Conversely, salted foods invariably enrich for the proteolytic *Halobacterium salinarium*. Representative strains are presented in Table 3.

CONCLUSION

Extremophiles are unique microorganisms that can survive in ecological niches such as high and low temperature, extremes of pH, high salt concentration, and high pressure. These microorganisms, which belong to the archaeal and bacterial domains, produce stable biocatalysts and organic compounds that function under extreme conditions. To obtain detailed insight in the molecular properties of the enzymes and genes, further research is required. There is little doubt that extremophiles will supply novel products in the near future that will be exploited by the biotechnological industries.

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FATE AND MICROBIAL DEGRADATION OF HALOGENATED AROMATICS

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During the last 30 years, significant strides have been made in cataloguing the environmental distribution and fate of halogenated aromatic compounds in soils, freshwater, estuarine, and marine environments, and the atmosphere. Whereas the main areas of emphasis have been in qualitative aspects of their formation, photolytic degradation, and microbial mineralization, transformation, and humification reactions, the quantitative aspects of microbial contributions to mitigate aryl halide fluxes in the various environmental compartments have had limited attention. Thus, most information on the microbial degradation of this class of compounds should be considered assessments of microbial potential, rather than expressed environmental activity. Nevertheless, a fundamental understanding of the relative contributions of intrinsic chemical reactivity versus those expressed in microbial systems provides paramount guidance to applied areas of pollution prevention or green chemistry, environmental forensics, and engineering methodologies aimed at stimulating microbial activity for end-of-pipe remedial approaches. This contribution will explore aspects of the global distribution of aryl halides in light of thermodynamic and molecular properties, microbial fate mechanisms, and adaptive genetic strategies affecting the reactivity of this class of compounds

BACKGROUND

Improvement by orders of magnitude in the sensitivity of analytical instrumentation, and innovative analytical methodologies, coupled to our increased knowledge on the pathways of degradation, have allowed a better understanding and interpretation of the fate and distribution of halogenated aromatic compounds in various environmental compartments. Yet, the assessment of what constitutes a recalcitrant versus a reactive compound is largely operational and depends on a combination of analytical, chemical, and microbial expertise and insights.

Phenomenological Observations in Soils and Sediments

Halogenated aromatic compounds are ubiquitous in the various environmental compartments because of direct industrial discharge and diffuse sources such as runoff, atmospheric deposition, and biogenic formation (predominantly in marine and estuarine environments) (1 and references cited therein). Because of the halogen substitution, which confers hydrophobic properties, often in combination with reactive functional groups (e.g., carboxyl, hydroxyl, methoxy, nitro, and amino) that lend themselves to various coupling mechanisms, aryl halides tend to partition into organic-containing matrices such as biota, and soil or sediment organic matter. Hence, their ambient distribution in water is on the order of microgram or nanogram per liter in groundwater aquifers and sediment pore waters. Considering that microbial reactions are largely accepted to take place in solution, the availability of these compounds to microbial reactions may be significantly impacted (see section on matrix considerations).

Despite these characteristics, aryl halides have been demonstrated to undergo what are collectively termed "natural attenuation" reactions, which include nondestructive (e.g., volatilization, advective, and convective transport, dispersion in aqueous matrices, and sorption) and destructive (e.g., abiotic hydrolysis and microbial transformation) processes (Table 1). The combined release and attenuation processes have resulted in two main observations. First, many volatile persistent organochlorine compounds such as hexachlorobenzene (HCB), the hexachlorocyclohexane isomers (α,β,γ,δ-HCH), and p,p'-dichloro-diphenyl-trichloroethane (p,p'-DDT) and its degradation products p,p'-dichloro-diphenyl-dichloroethane (p,p'-DDD) and p,p'-dichloro-diphenyl-dichloroethene (p,p'-DDE), as well as other pesticides and nonvolatiles such as polychlorinated dibenzo-p-dioxins (PCDD) and furans (PCDF), are globally distributed because of the distillation effect, which transports organic compounds from warm source regions to areas of higher latitude. During the last few years, several global maps have been generated, which document the distribution of these compounds based on the analysis of tree bark samples (2), rural soils and isolated water bodies (3), and butter samples (4) have been generated. The second outcome has been the emergence of the field of environmental forensics, whereby the distribution and chemical characteristics of aryl halides observed in the environment are used to apportion source contributions to the contamination and to derive indicators for natural reactions. Primary emphasis has been placed on compounds, which exhibit source-specific characteristics such as congener and isomer ratios (e.g., PCDD/F, polychlorinated biphenyls — PCBs, HCH, DDT, and pesticides), and enantiomer patterns of chiral pesticides [e.g., chlorofibric acid and mecoprop 5 or recognizable degradation patterns [e.g., the accumulation of lesser chlorinated congeners and chlorobenzoic acids from commercial Aroclor mixtures, DDD, DDE; (1)]. Due to the complexity of source and sink patterns (e.g., in the case of PCDD/PCDF or PCB contamination), environmental forensics rely heavily on the use of multivariate statistical analysis to apportion the contributions of source

Table 1. Natural Attenuation Processes Affecting the Fate and Transport of Aryl Halides

Process	Description	Dependencies	Effect
Advection	Movement of solute by bulk water movement	Aquifer, sediment properties (hydraulic conductivity, porosity, hydraulic gradient) Independent of contaminant properties	Contaminant movement in the subsurface
Dispersion	Fluid mixing due to water movement and subsurface heterogeneities	Aquifer or sediment properties and scale of observation Independent of contaminant properties	Longitudinal, transverse, and vertical spreading of contamination, reduction in concentration
Diffusion	Spreading and dilution due to molecular diffusion	Contaminant properties Concentration gradients	Contaminant diffusion from high to low concentration zones
Sorption	Reaction between solid matrix (organic carbon or clays) and solute	Aquifer or sediment matrix properties (foc, clay content, bulk density, sp. surface area, porosity...) Contaminant properties (solubility, K_{ow}, substituents...)	Reduces apparent solute transport velocity Removes solutes from solution
Recharge	Movement of water across the water table or sediments into saturated layers	Aquifer matrix properties, depth to groundwater, surface water interactions,...	Dilution Changes in groundwater or pore water geochemistry
Volatilization	Contaminant transfer into vapor phase	Contaminant properties (vapor pressure, Henry's constant)	Removes contaminants from aqueous phase
Ebullition	Contaminant mobilization and transport via biogenic gases	Sediment properties (effective porosity, density,...) Microbial respiratory activity Contaminant properties	Contaminant removal from sediments to the air-water interface
Biodegradation	Microbially mediated redox reactions	Groundwater or pore water geochemistry Microbial communities Contaminant properties	Biotransformation and biodegradation Mass reduction Detoxification
Abiotic degradation	Chemical transformation reactions	Contaminant properties Groundwater or pore water geochemistry Matrix surface properties	Transformation Polymerization

contamination patterns as well as nondestructive and destructive degradation patterns. For example, the application of these techniques to dioxin-contaminated Passaic River (New Jersey) sediments, with incorporation of both point and diffusive sources, has indicated that a multiple point sources may be responsible for the majority of the contamination (8,9). However, neither sediment transport, nor environmental transformation reactions were taken into account, resulting in an incomplete assessment of the site whereby 84% of the sample variance could be explained by source contributions. The remaining 16% might then be the result of biotic or abiotic environmental transformations. The best-illustrated case that includes the contribution of microbial transformation reactions (in this case dechlorination) to the interpretation of environmental sinks, is the case of PCB-contaminated sediments (10). When sink patterns were found to deviate from known PCB sources with distinct patterns, and the potential effect of abiotic reactions was considered, the disproportionate appearance of lesser chlorinated congeners not present in the source material was attributed to natural dechlorination reactions, which were later confirmed to be of a microbial nature (11). One potentially complicating factor that may interfere with this analysis is the natural occurrence of aryl halides as the result of diagenic or biogenic processes, as is the case with dioxins and a wide range of pesticide analogs (12,13).

Hence, careful observation of aryl halide occurrence and distribution in environmental samples, and consideration of known source contributions and abiotic (nondestructive) processes, may result in an inference of environmental reactivity. This interpretation should then be confirmed and validated using environmentally relevant laboratory assessments, using the so-called "field-to-flask paradigm" or "deductive approach." This approach is counter to the much more widely used "inductive approach" in which organics are screened for their degradability or microbial degradative potentials are assessed. Whether this potential is actually expressed or can be stimulated under relevant biogeochemical conditions remains an issue of considerable contention.

Recalcitrance Versus Analytical Limitations

Because of their molecular properties, which confer extreme hydrophobicity, most aryl halides are, despite the fact that degradation pathways have been demonstrated in the laboratory, considered recalcitrant. Even the ubiquitous PCBs, which after research on their reactivity during the last two decades, have been shown to be susceptible to (even naturally occurring) dechlorination reactions, are considered refractory because of the unacceptable kinetic rates of dechlorination (one chlorine removed every 7 to 12 years). Kinetics may be a valid argument from a policymaking perspective ("to

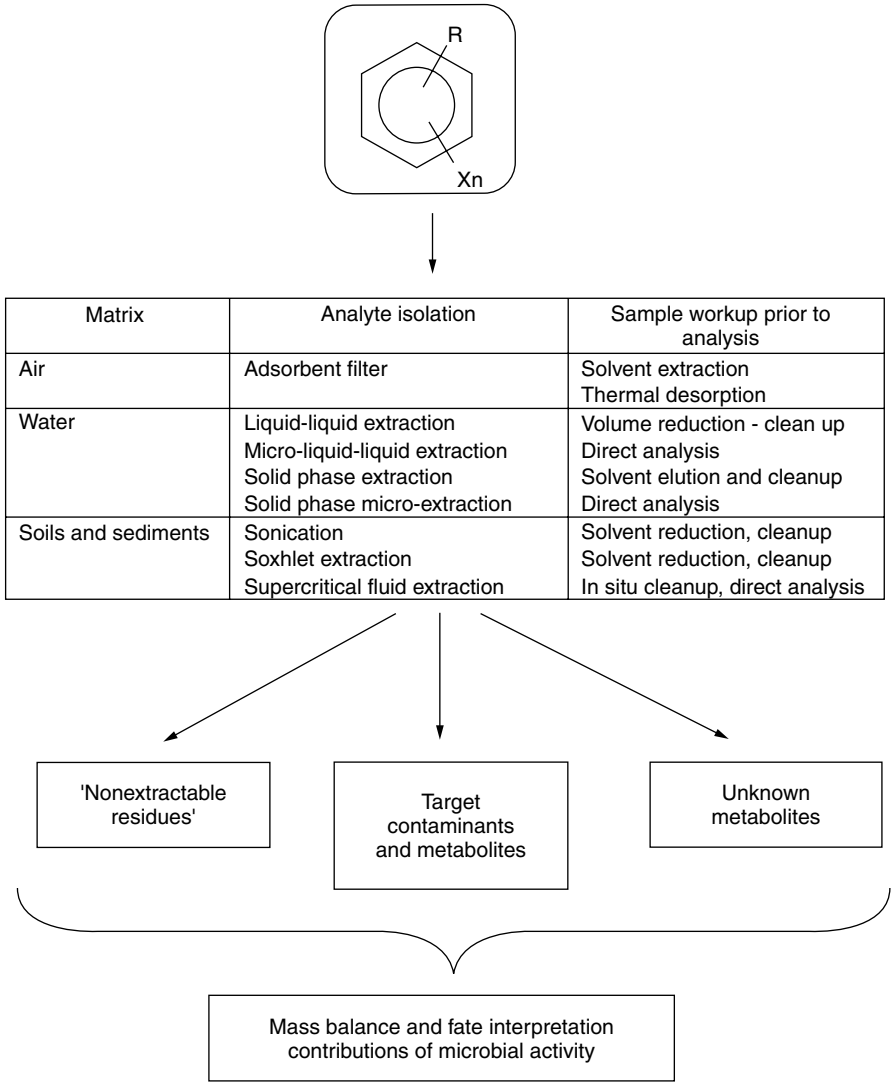


Figure 1. Operationally defined aryl halide fractions recoverable from environmental matrices.

dredge or not to dredge"), however, the operational term "recalcitrance" is often bestowed on compounds for which (*1*) no information is available on the degradation pathways, or (*2*) the availability of analytical methodology is too limiting to resolve and screen for structurally similar compounds (which may be degradation products) in the contaminated matrix (1,14). The latter is particularly the case when metabolites from aerobic degradation and hydrolysis products are considered.

Hence, the analytical limitations may be related to matrix-specific or analyte-specific considerations (1). The former refer to the implementation of proper extraction and sample work-up techniques to isolate and separate the nonpolar and often covalently bound parent compounds and metabolites from the solid-organic matrix. The latter represent challenges of analytical sensitivity and congener-, isomer-, or enantiomer-specific separation, which may impact the interpretation of fate mechanisms. These concepts are illustrated in Figure 1, which incorporates the analytical and sample work-up procedures required to assess the variety of processes affecting the fate of chlorinated aromatics in various environmental matrices. As noted on the bottom of Figure 1, environmental reactivity of this class of organics, and thus the interpretation of whether microbial processes may be responsible for these reactions are typically based on one-third of the analyte classes (target contaminant and metabolites), although most information may be hidden in the "nonextractables" (usually operationally defined) or "unknown metabolites" classes. For example, the U.S. EPA has promulgated analytical methods focused on the most toxic dioxins, that is, those with four or more chlorines and all with the lateral (2,3,7,8) positions occupied. Hence, the dechlorination of dioxins to congeners with less than four chlorines is missed, yet they may represent the dominant groups resulting from intrinsic (microbial) dechlorination (15). The story is even more poignant in the case of chlorinated pesticides (with an aromatic moiety), which often exhibit a fairly complex structure with many functional groups such as alkyl side chains (e.g, Alachlor, others). Slight modifications of the side chains would be missed in typical environmental analyses, and thus result in a conclusion that the parent compounds

are recalcitrant, although the total concentration of all potential metabolites exceeds that of the parent compound (e.g., Alachlor) by threefold (16). These and other examples indicate that there is a need for a comprehensive fundamental understanding of microbial degradation potential and intrinsic chemical reactivity to make a considered decision on whether a given halogenated aromatic compound is likely to undergo a microbially mediated transformation reaction under ambient biogeochemical conditions.

FUNDAMENTALS OF ARYL HALIDE DEGRADATION

Halogenated aromatic compounds have been distributed in the ambient environment in one form or another for millions of years. Even compounds such as dioxins appear to have been formed in million-year-old ball clays (13) by means of mechanisms that are yet known, and are by-products during natural incineration processes such as forest fires. In addition, plants, algae, and microorganisms produce and release these compounds for various physiological and ecological reasons, indicating that some latent activity for biosynthesis and thus biodegradation pathways may have been subject to substantial evolutionary processes as new aryl halides entered the various environmental compartments. Sources of halogenated aromatics have boosted fluxes immensely since the advent of the industrial revolution in the 1800s, and more significantly since heavy manufacturing was in the exponential phase in the early 1900s. Despite these relatively recent events, the microbial versatility to convert extremely stable environmental contaminants present at parts-per-trillion to parts-per-million concentrations keeps astounding us as we learn more about new enrichment techniques aimed at harnessing and exploring the microbial ecological potential (17,18). This section provides an overview of the thermodynamic, genetic, and physiological boundary conditions imposed on microbial transformations of aryl halides as we know them to date.

Thermodynamic Considerations

The propensity of organic compounds, including aryl halides, to undergo oxidation and/or reduction reactions is often expressed in terms of the energetic properties associated with their chemical structure (19–21). These properties include, but are not limited to, redox potential ($E^{o'}$, mV), Gibbs free energy of formation ($\Delta G^{o'}$, kJ/reaction), carbon-halogen bond strength, and ionization potential [often also expressed as the energy gap between the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO); in eV]. The interpretation of the former two parameters is closely related to the potential of these compounds to be used as terminal electron acceptors by microorganisms, whereby more oxidized (highly halogenated) molecules exhibit more negative redox potentials (and according to Nernst Law) and greater potential (Gibbs free) energies than lesser chlorinated compounds. Hence, the higher chlorinated (halogenated) aromatics would then compete more effectively with the terminal electron acceptors for growth. The top plot of Figure 2 illustrates that the $E^{o'}$ of aryl halides (266–478 mV) corresponds to the range for iron- and nitrate-reduction. Hence, these compounds are likely to be susceptible to reduction (resulting in dechlorination or dehalogenation — see following sections) as they reside in a range in which the addition of electrons is energetically favorable. We can further conclude that the oxidation of these already much-oxidized compounds will probably be limited to the lesser halogenated isomers because it will be difficult to release electrons. This concept is illustrated in Figure 2(a), where relative rates of oxidation and reduction are plotted as a function of level of chlorination; in practice, the literature has revealed that there may be a (chemical-dependent) zone in which oxidation and reduction reactions are equally likely. The

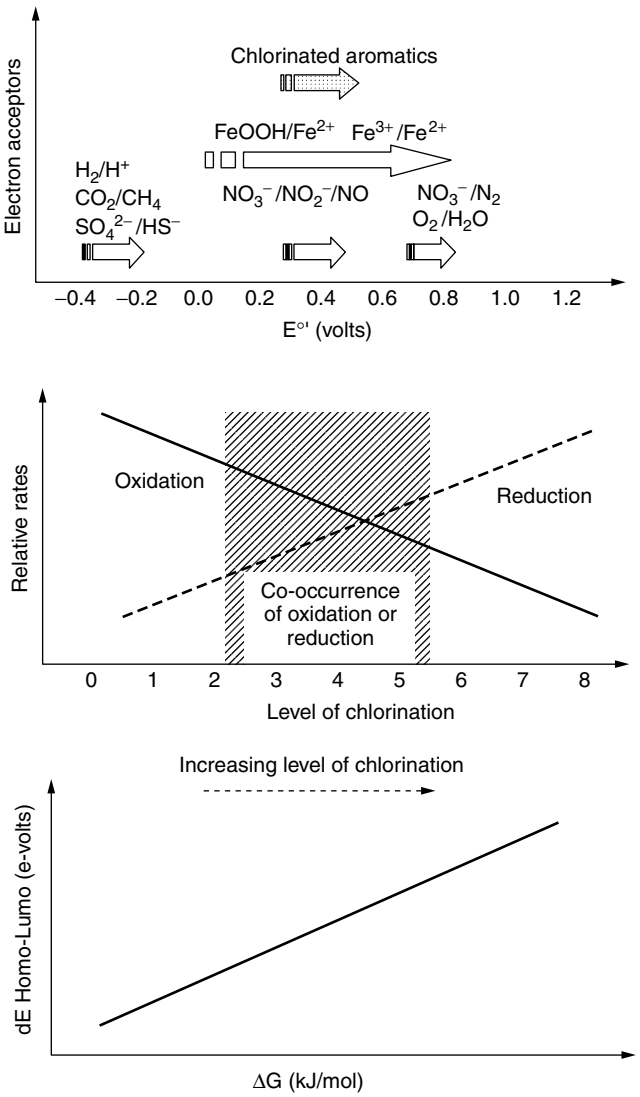


Figure 2. Thermodynamic and molecular attributed of aryl halides (**a**) comparison of redox potentials to terminal electron acceptors; (**b**) impact of chlorine level on relative occurrence of oxidation and reduction reactions; (**c**) correlation of Gibbs free energy and chlorine levels with electron potential gaps between HOMO and LUMO).

bottom plot of Figure 2 shows a trend between the Gibbs free energy and the HOMO-LUMO gap, both of which increase with increasing level of chlorination. It has been argued that the size of the gap is directly correlated to compound reactivity because it reflects the energy associated with electron release and acceptance (i.e., the ionization potential). Hence, higher chlorinated (halogenated) compounds would, according to this plot, be chemically more reactive than lesser halogenated isomers. It should be noted that there has been very limited biochemical validation of this concept, though correlations are apparent during reductive dechlorination. Other molecular descriptors describing the chemical reactivity of aryl halides, such as electronic, steric, and hydrophobic parameters, have been widely used. Their value will be assessed in the section on Structure-Reactivity Relationships.

Genetic Considerations

Considerable literature has been published on the evolutionary relationships of genetic sequences encoding, and operons facilitating the induction and expression of key metabolic enzymes required for the degradation of aryl halides (e.g., 22 to 27). The issues tend to center around the types and responsible processes for evolutionary changes, which have taken place to extend the core metabolic range of enzymes such that metabolism of halogenated aromatics can be accommodated, either through cometabolic or growth-based processes.

Under aerobic conditions, it has become widely accepted that the initial aerobic transformation steps of the aryl halides are carried out by different enzymes, but that the compounds are transformed to a limited number of intermediates such as dihydroxylated benzoic acids and substituted catechols. Further degradation of these central, dihydroxylated, metabolites proceeds then via either ortho- or meta-ring fission, which leads to intermediates of central metabolic pathways such as the tricarboxylic acid cycle. (See OXYGENASE ENZYMES: ROLE IN BIODEGRADATION, this Encyclopedia) The great diversity in the upper part of the pathway indicates that microorganisms have extended their range of biodegradative activities by evolving peripheral enzymes that "shunt" aryl halide metabolism toward central catabolic pathways. This theory is supported by amino acid analysis, and the genetic organization within operons of ring cleavage oxygenases and "upper pathway" oxygenases and dehalogenases (22,28). It was found that similarities exist in amino acid sequences of different aromatic pathway enzymes, whereas gene organization within the operon diverges strongly (22). These findings imply that microorganisms possess a range of genetic mechanisms to make evolutionary changes in existing catabolic pathways, which allows them to adapt to and grow on a wide range of aryl halides, provided sufficient selection pressure is imposed.

However, the evolution of catabolic genes does not mean they are actually expressed in environmental systems, as regulatory genes control the expression of haloaromatic catabolic pathways (Table 2). The great divergence in their evolutionary origins suggests that catabolic and regulatory genes have evolved relatively independently. Yet, despite this divergence, the general regulatory principle of activator-target DNA interaction, and transcription initiation is very similar for most known regulators (Fig. 3); regulatory specificity is then conferred by inducer molecules (e.g., pathway intermediates, target substrate). Evidence over the years has shown that catabolic promoters possess a remarkable level of nonspecificity (regulatory noise) with respect to the signals to which they respond, which allows them to evolve and to be recruited to control novel pathways. Indeed, more recently, a number of reports have been published describing the "directed evolution" of dioxygenases to accommodate chlorinated aromatic compounds through site-directed mutagenesis, in vitro DNA shuffling, and subunit or domain exchanges between dioxygenases of different bacterial origins (29). The "evolved" genes are then integrated into chromosomal operons for successful expression.

Our knowledge on the genetic evolution of anaerobic enzymes involved in microbial metabolism of aryl halides is still in its infancy, as only recently inroads have been

Table 2. Regulatory Genes of Aryl Halide Catabolic Pathways from Bacteria

Gene(s)	Microorganism (plasmid)	Function[1]	Pathway	Family[2]	Accession Number or Reference
clcR	*Pseudomonas putida* (pAC27)/R. opacus 1CP	A	Chlorocatechol	LysR	A40641/AAC38250
cbnR	*Rhodococcus eutropha* RH9	A	Chlorocatechol	LysR	BAA74529
tfdR/S	*Rhodococcus eutropha* (pJP4)	A	Dichlorophenoxyacetate	LysR	P10086
tfdR	*Pseudomonas putida* (pEST4011)	A	Dichlorophenoxyacetate	LysR	AAC78503
tcbR	*Pseudomonas* sp. P51 (pP51)	A	Trichlorobenzene	LysR	A38861
ohbR	*P. aeruginosa* 142	A	o-Chlorobenzoate	IclR	AAC69482
bpdS/bpdT	*Rhodococcus* sp. strain M5	A	Biphenyl/PCB	TCST	AAB52543/AAB52544
bphS	Rhodococcus eutropha A5	R	Biphenyl/4-chlorobiphenyl	GntR	Mouz et al., 1999

[1]The function of some gene products has been deduced from their amino acid sequence similarity with regulators of known function and, hence, requires experimental verification; A and R are transcriptional activator and repressor of the cognate catabolic promotor(s), respectively.
[2]Denotes the amino-terminal domain where the helix-turn-helix (HTH) DNA binding motif is located; TCST, two-component signal transduction.

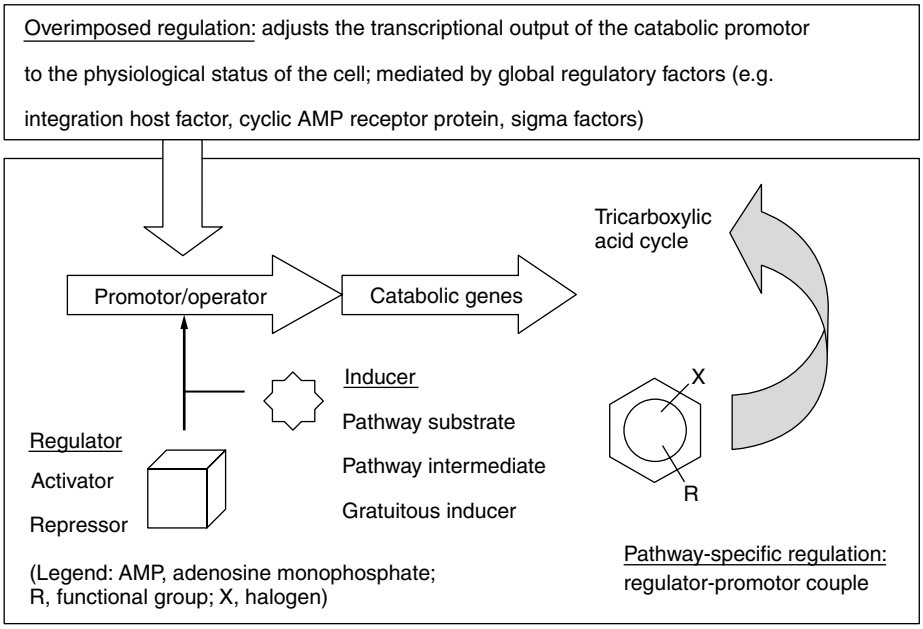


Figure 3. Levels of transcriptional regulation of aromatic catabolic pathways in bacteria (modified from E. Diaz and M. A. Prieto, *Curr. Opin. Biotechnol.* **11**, 467–224 (1998).

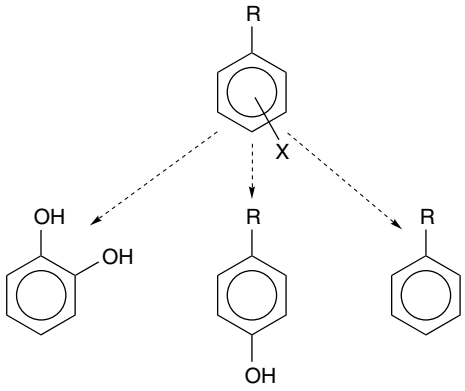
made in the functional analysis of key metabolic enzymes. Indirect evidence for the widespread, shared evolution of regulatory mechanisms has been gleaned from cross-acclimation studies. It has been proposed that derepression of enzyme induction plays a role in the acclimation period prior to dehalogenation of aryl halides, which can be triggered by amendment of the dehalogenating cultures or sediments with specific aryl halides, which may themselves serve as substrates (30,31). The possibility for cross-acclimation to induce dehalogenation, the specificity of dechlorination patterns observed (e.g., chlorobenzenes and polychlorinated biphenyls) in highly enriched cultures, and the suppression of electron transfer to the natural terminal electron acceptors during aryl dechlorination, appears to indicate that microorganisms have evolved to use aryl halides as a terminal electron acceptor (i.e., aryl halide respiration) (1,18,30–32).

Biochemical Considerations

The evolution of regulators and catabolic genes has resulted in an extraordinary metabolic versatility of microorganisms to transform or mineralize halogenated aromatic compounds, either via growth (energy-coupled) or nongrowth (cometabolic; nonenergetic) processes. The latter are often operationally defined based on specific laboratory conditions, whereas increasing evidence has come to the fore that many aryl halides are involved as fortuitous inducers controlling pathway-specific regulation (Fig. 3), although they do not serve as substrates for the catabolic enzymes (1,18). This gratuitous induction may then result in a partial transformation of the halogenated substrate, often resulting in enzyme inhibition (33). On the other hand, the cometabolic nature of dehalogenation has undergone a substantial revision during the last decade because the application of novel enrichment techniques has resulted in the discovery of a process called dehalorespiration. Dehalorespiration is an anaerobic dehalogenation processes generate energy through the promotion of a proton-motive force that allows the cells to recapture energy from the dechlorination step (18). Considering the redox potentials of aryl halides (Fig. 2a), these processes are energetically very favorable as they are at par with iron and nitrate reduction.

Adaptation of microorganisms to the presence of haloaromatics in the environment has resulted in a wide range of aerobic and anaerobic mechanisms that initiate haloaromatic degradation, including oxidative, hydrolytic, and reductive dehalogenation (Fig. 4). The former and latter processes occur predominantly under strictly aerobic and anaerobic conditions, respectively, whereas hydrolytic processes prevail in aerobic and microaerophilic conditions. It appears that complete or partial halogen removal is the predominant first step during microbial transformation of aryl halides, resulting in the accumulation of hydroxylated and/or lesser halogenated intermediates. Hydroxylated aromatics are considered "activated" as the functional group destabilizes the aromatic Π-electrons. The compounds are then rendered more susceptible to other chemical transformations, including those catalyzed by mono-or (ring fission) dioxygenases resulting in the production of catabolic intermediates that enter the tricarboxylic acid (TCA) or related ATP generating pathways. On the other hand, the extensive removal of halogens through reductive dehalogenation under anaerobic conditions allows either aerobic processes to commence or hydrogenase- and hydratase-type of anaerobic ring reduction and hydrolysis to productive catabolic intermediates.

A current list of known isolates capable of respiring chlorinated aromatics is presented in Table 3; it should be noted that the functional groups are limited to acids or hydroxyl substituents, and that ortho-dechlorination appears to be the dominant pattern. Unless experimentally proven, all other compound classes shown to undergo reductive dehalogenation should be considered cometabolic substrates. A group of compounds that may be a candidate for chlororespiration are the PCBs, as



Oxidative (dioxygenase)
R = COOH, H, NH_2
X (ortho) = F, Cl, Br, I

Hydrolytic (dehalogenase)
R = COOH, OH, NH_2
X (para) = F, Cl, Br, I

Reductive (reductase, dechlorinase)
R = COOH, H, OH, NH_2, phenyl
X = F, Cl, Br, I

Figure 4. Dechlorination mechanisms exhibited by aerobic and anaerobic bacteria.

Table 3. Anaerobic Bacteria Capable of Catabolic Reductive Dechlorination of Aryl Halides

Strain	Aryl Halide Substrate	Electron Donor	Dechlorination Pattern
Desulfomonile tiedjei	3-Chlorobenzoate; pentachlorophenol	Hydrogen, formate, pyruvate	*meta*
Isolate 2-CP1	2-Chlorophenol; 2,6-dichlorophenol	Acetate	*ortho*
Desulfitobacterium chlororespirans	2,4,6-Trichlorophenol; 3-Chloro-4-OH-phenylacetate	Hydrogen, formate, pyruvate	*ortho*
Desulfitobacterium hafniense	3-Chloro-4-OH-phenylacetetate; pentachlorophenol	Pyruvate, tryptophane	*ortho*/*meta*
Desulfitobacterium frappieri	2,4,6-Trichlorophenol; 3-Chloro-4-OH-phenylacetetate	Pyruvate	*ortho*/*meta*/*para*
Desulfitobacterium dehalogenans	2,4,6-Trichlorophenol; 3-Chloro-4-OH-phenylacetetate	Hydrogen, formate, pyruvate	*ortho*
Desulfitobacterium strain PCE1	2,4,6-Trichlorophenol; 3-Chloro-4-OH-phenylacetetate; 2-chlorophenol	Formate, pyruvate	*ortho*
Desulfitobacterium strain PCE-S	2,4,5-Trichlorophenol; pentachlorophenol	Pyruvate	*ortho*/*meta*

studies have shown that dechlorination patterns (ortho or meta+para) can be stimulated using selected PCB congeners exhibiting one pattern or another. Moreover, the recent reports that enrichment cultures have been established for ortho or meta+para dechlorination indicate that specific dechlorinases may have evolved in response to the presence of certain types of PCBs. The list of aerobic haloaromatic degraders is too extensive to be reviewed here, and the reader is referred to a number of critical reviews on the subject (e.g., 34–37).

The main observation here is that growth tends to be limited to mono- and dichlorinated compounds, with the exception of pentachlorophenol. Moreover, multiple strain–specific pathways exist for the same compound and the functional group substantially influences the reactive sites on the molecule. The effects of molecular descriptors on their reactivity is further explored in the section on quantitative structure activity relationships (QSARs). The catabolic substrate range of aerobic enzymes is evident from the number and range of cometabolic reactions that have been reported (1). As noted earlier, many aryl halides either act as gratuitous inducers for transcriptional regulators but are not actual substrates for the gene product, or serve as fortuitous substrates for the enzyme that is induced by the pathway substrate or one of its metabolites. In the latter case, the nonspecificity of the active site of the induced enzyme allows a number of "unintended" reactions to occur with either structurally similar (as in the case of biphenyl and PCBs) or dissimilar (as in the case of methane and PCBs) substrates (1,33,37). Perhaps one of the most versatile enzymes is the soluble form of the methane monooxygenase, which has the capacity to co-oxidize chlorobenzene and mono-or dichlorobiphenyls, in addition to many alkyl halides (38).

It should be noted that most of the biochemical information on microbial aryl halide degradation is based on axenic cultures isolated from a complex microbial community through selective enrichment techniques and is not necessarily representative of the original environmental system. Hence, it is imperative to place the fundamentals of aryl halide degradation within microbial ecology and ecosystem contexts to explore the impacts of system biogeochemistry and matrix effects.

ECOLOGY OF ARYL HALIDE DEGRADATION

During the last decade, substantial progress has been made to quantitatively explore the microbial community structure of contaminated environments such as groundwater, soils, and sediments. A major breakthrough has been the application of genetic tools to both phylogenetically and functionally characterize environmental samples with minimal enrichment bias, to assess the impact of chemical disturbances and matrix effects such as salinity and availability of electron acceptors. With respect to aryl halides, most of our understanding of microbial ecology and ecosystems is related to the degradation of PCBs and PCDD/F in sediments, and of chlorobenzenes and chlorophenols in groundwater environments.

Role of Microbial Communities

Considering the multitude of (bio)chemical reactions involved and the temporal and spatial variability of geochemical environments, it should not be a surprise that environmental transformation reactions in general and contaminant mineralization are the result of a complex network of microbial interactions. Hence, the selective enrichment approach aimed at stimulating and isolating target biodegradative activities are inherently artificial and biased toward a process that may actually not be relevant in the original system (39). The importance of community structure analysis and its relation to expressed microbial activities is illustrated by means of the following relevant examples.

Anaerobic Degradation of 3-Chlorobenzoate. Perhaps one of the first indications on the importance of community structure in the degradation of haloaromatics was derived from the analysis of the 3-chlorobenzoate degrading anaerobic mixed culture (reviewed in (30)). This food web consisted of a halorespirer, *D. tiedjei*, which derives energy from the dechlorination step, but requires a source of carbon and nutrients from other community members. The product of dechlorination, benzoate, is fermented to acetate and hydrogen by an acetogen strain BZ-1, which derives both carbon an energy from this process. Presumably, a feedback loop provides a fraction of the acetate to the halorespirer as a source of carbon. Both the acetate and the hydrogen are converted to methane and carbon dioxide by acetoclastic and hydrogenotrophic methanogens. Further cell leakage of amino acids and other inorganic nutrients closes the loop. The various interdependencies for sources of energy and carbon as well as the thermodynamic restrictions on the fermentative process necessitate a closely interwoven network of microbial activities to sustain 3-chlorobenzoate respiration. Similar networks presumably play a role in the physiological processes of model microorganisms listed in Table 3 and their relatives in naturally occurring microbial communities.

Anaerobic-Aerobic Degradation of PCBS. This class of compounds represents a complex mixture of congeners (biphenyls substituted with one up to ten chlorines) and isomers (different chlorine substitution patterns within each homolog group), and is often observed in parts per million (normalized per unit weight of sediment). Up to six different dechlorination patterns have been observed in sediment matrices, as a function of initial mixture and biogeochemical conditions, ultimately resulting in the accumulation of ortho- and ortho/para-substituted mono- and dichlorobiphenyls (40). As noted earlier, recent insights into the microbial ecology of PCB dechlorination have indicated that at least two types of dechlorination activity can be enriched, ortho- or meta/para- dechlorination (41,42). Furthermore, community analysis using genetic methods shows that the community structure associated with each dechlorination pattern differs markedly, indicating that at least two communities potentially exist within PCB-contaminated sediments. Sediment analysis has further indicated that chlorobenzoates and PCB ring fission products accumulate (43), suggesting that even in anaerobic and reduced sediments, some aerobic activity is prevalent, as these products require dioxygenase-mediated reactions (44).

As currently understood, PCBs do not serve as sources of carbon and energy under anaerobic or aerobic (except for some mono- and dichlorobiphenyls) conditions; thus, the cometabolizing populations need to be supplied with catabolic substrates. Some evidence exists that the PCBs or chlorinated aliphatic acid intermediates may serve as inducers for transcription of dioxygenase activity (1). Despite the fact that PCB-degradation is still operationally defined as being of a cometabolic nature, the possibility that the microorganisms reap some metabolic benefit from the reactions described earlier should be considered. For example, Kim and Rhee (45) demonstrated that the increase of PCB-dechlorinating populations can be assayed using the most probable number technique, and that different dechlorinating activities can be separated using a dilution fractionation method (46) all these reactions can be observed in single sediment core, because it follows that substantial cell-to-cell communication, chemical gradients, and sufficient labile organic carbon may be required to sustain in situ activity. Conversely, the absence of these conditions may play a role in sediments in which the PCBs have not undergone substantial alteration via dechlorination processes. For example, it has been observed that the increase in electron fluxes by amendments with simple organic substrates stimulates dechlorination (and methanogenic) activity (40). Perhaps more importantly, the amendment with brominated ortho-substituted aromatic substrates (biphenyls and benzoates) or other halogenated aromatics (benzenes, benzoates, and phenols) substantially increased dechlorination activity (40,47–50). These compounds presumably serve as an alternate electron acceptor to the dehalogenating populations (the substrate was debrominated during the process). Further, the addition of the nonchlorinated analog, biphenyl, stimulated the dechlorination activity, indicating that PCB-dechlorinators can be enriched on this substrate, whether it serves as an inducer for transcriptional activity or simply increases electron flow in anaerobic environments. Amendment strategies such as the addition of electron shuttles (e.g., vitamin B_{12}) depressed dechlorination activity for unknown reasons (51). The role

of sediment organic carbon as a potential electron acceptor or electron transfer mediator has not been explored or correlated to observed (microbial) dechlorination activity.

Anaerobic Dechlorination of Dioxins. Contrary to PCBs, dioxins represent a class of compounds, which were never produced intentionally (except for analytical standards), but result as by-products from combustion and chemical manufacturing processes, atmospheric condensation of phenols, and biogenic production (15). Their ether-linked benzene structures (Fig. 5a) and random chlorination patterns results in up to 75 congeners

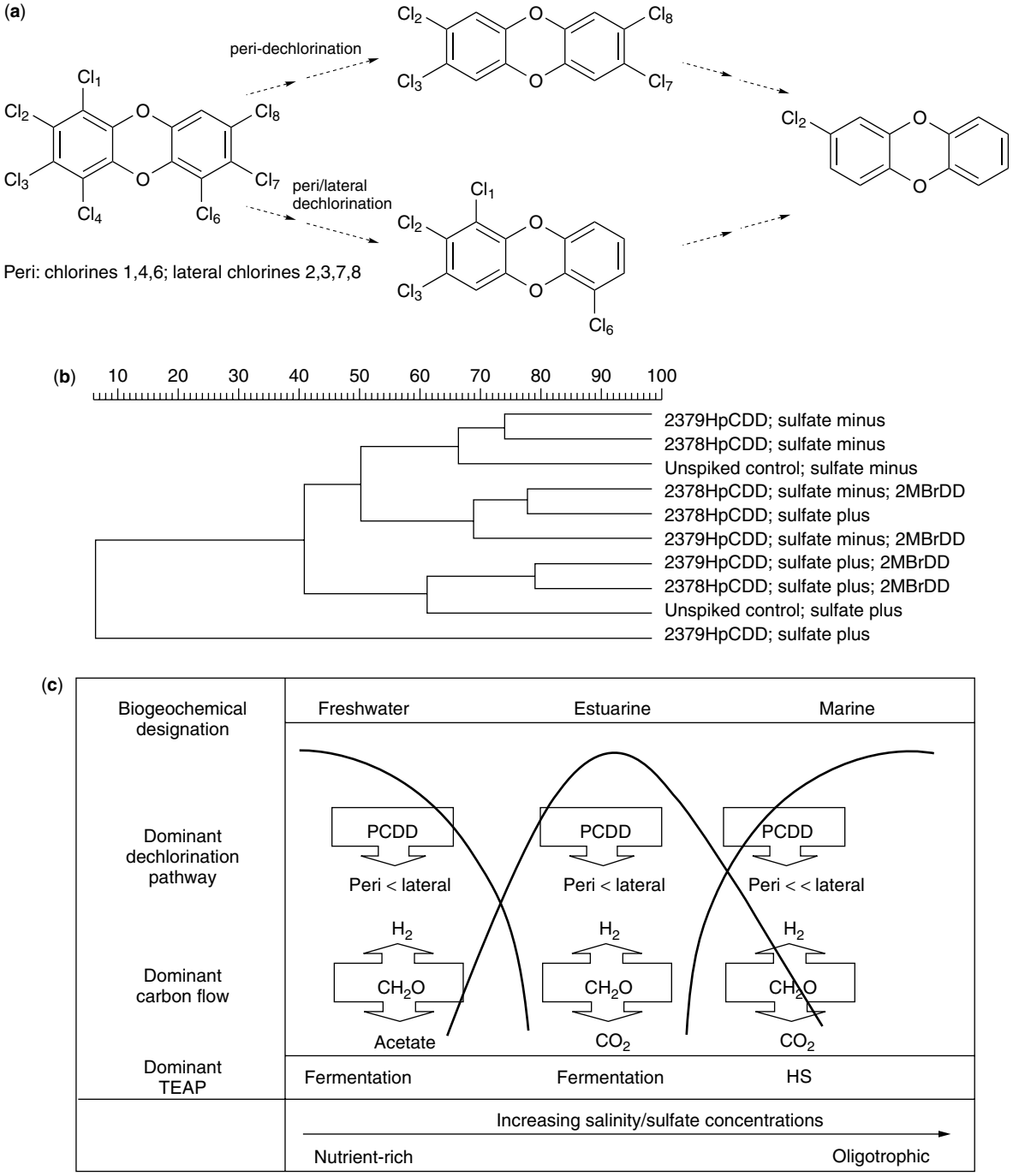


Figure 5. Effect of biogeochemical conditions on population distribution, carbon, and electron flow and PCDD dechlorination pathways (**a**) scheme of peri- and mixed peri/lateral dechlorination; (**b**) statistical analysis of community banding patterns after exposure to dioxins, methanogenic and sulfate-reducing conditions, and amendment with a priming compound; (**c**) conceptual integrative model [modified from Q. S. Fu, A. L. Barkovskii, and P. Adriaens, *Appl. Environ. Microbiol.* Submitted (2001a) and Q. S. Fu, A L. Barkovskii, and P. Adriaens, *Chemosphere* **43**, 643–648(2001b)].

with environmental patterns either dominated by the hepta- and octachlorinated congeners, or by the most toxic 2,3,7,8-tetrachlorodioxin. Ambient concentrations are on the order of parts-per-quadrillion to parts-per-billion, and thus presumably would not be able to serve as significant (extreme oligotrophs?) sources of carbon and energy. Yet, they have been shown to be subject to both microbial and abiotic (organic carbon mediated) dechlorination reactions, whereby either the peri (1,4,6,9) chlorines or a mix of the peri and lateral (2,3,7,8) chlorines are removed, ultimately resulting in the accumulation of mono- and dichlorodioxins (exemplified in Fig. 5a). Several studies have suggested that the composition of naturally occurring microbiol communities influences dechlorination processes: methanogenic and/or sulfate-reducing communities appear active on lesser chlorinated (2–4 chlorines), while fermentative activity is strongly correlated to highly substituted dioxins (4–8 chlorines). Moreover, similar to PCBs, the addition of a brominated analog, 2-monobromodibenzodioxin (2-MBrDD), substantially affected dechlorination activity by decreasing the relative ratio of peri to peri/lateral dechlorination.

This effect is likely to have been the result of a shift in the microbial community structure, as shown in Figure 5b, which uses a molecular tool for microbial community comparison (16S rRNA) to display how the community responds to external amendments. The data are presented as a similarity dendrogram comparing denaturing gradient gel electrophoresis (DGGE) banding pattern as a function of imposed geochemical conditions (high or low sulfate; plus or minus 2-MBrDD), and the resultant dechlorination pattern observed (53,55). The dominant role of the 2-MBrDD amendment on community structure is clear from Figure 5b, as the amended communities are similar regardless of other amendment. Although specific activities can be stimulated, the metabolic benefit of ppt-levels of dioxins is at best insignificant.

Effect of Biogeochemical Conditions

Our understanding of the effect of dominant electron acceptors and salinity gradients on the dehalogenation of aryl halides is predominantly derived from laboratory degradation studies of phenols, benzoates, PCBs, and dioxins under different redox conditions. The published work has emphasized the effects of alternate electron acceptors on reaction kinetics and substrate specificities, and of freshwater, estuarine, and marine conditions on dechlorination patterns and carbon flow.

Electron Acceptors. As discussed in the section on thermodynamic considerations, methanogenic and sulfate-reducing conditions effectively accommodate dehalogenation reactions because the redox potentials of these processes are well below those for halogenated aromatics. As shown in Figure 2, the range of dechlorination redox potentials coincides with those of iron and nitrate reduction. Therefore, in the case of halorespiration, competing electron acceptors (e.g., sulfate and nitrate) inhibit the dechlorination process (30,31). Indeed, most studies on (cometabolic) aryl halide dechlorination have shown strong correlations with methanogenesis and sulfate reduction, although the responsible populations have rarely been isolated. Exceptions include highly enriched sediment cultures on electron acceptors such as sulfate, iron(III), and nitrate that have been shown to support anaerobic degradation of halogenated phenols and benzoates (57 and references cited therein). In these cases, a stoichiometric correlation was observed between substrate mineralization and electron acceptor reduction, providing strong evidence for a coupled process. Moreover, inhibition of sulfate reduction and dechlorination with molybdate suggests that the sulfate-reducing bacteria were responsible for the process. The activity was found to be similar regardless of the geographic environment from which the cultures were derived, indicating that these represented ubiquitous metabolic processes.

Evidence for the direct involvement of sulfate reduction or methanogenesis during dechlorination of more complex halogenated organics such as PCBs and dioxins is more tenuous. Dechlorination of PCBs and dioxins has been observed under both methanogenic and sulfate-reducing conditions in freshwater and marine sediments, or enrichments derived thereof (15,40). Perhaps the most convincing evidence for coupled PCB dechlorination and sulfate-reducing bacteria is provided by Cutter and coworkers (42) who enriched methanogenic mixed sediment-derived marine cultures on PCB congeners selected to stimulate ortho-dechlorination activity. The inhibition of methanogenesis did not affect ortho-dechlorination activity, whereas the addition of sodium molybdate (an inhibitor of sulfate reduction) inhibited dechlorination (41). Extracted communities from estuarine sediments exposed to either sulfate or bicarbonate as the terminal electron acceptors exhibited dioxin dechlorination activity. However, insufficient evidence was available to determine whether sulfate reduction and methanogenesis, respectively, were coupled to the dechlorination activity. Electron mass balances demonstrated a strong correlation between transformation of highly chlorinated molecules and fermentative activity (54). The presence of millimolar concentrations of sulfate did not inhibit the extent of dioxin dechlorination.

Salinity Gradients. Microbial diversity in estuarine and marine environments is impacted by salinity gradients, high sulfate concentrations, and competition for electron donors. Salinity gradients between 5 and 20% reportedly exert a negative effect on aerobic heterotrophic, methanogenic, and anaerobic respiratory activities. In estuarine and marine environments, high sulfate content (20 to 30 mM) results in the dominance of sulfate reduction, but denitrification, methanogenesis, iron reduction, and fermentation have also been observed (58). Metabolic activities have been differentiated based on fatty acid turnover. Under freshwater conditions, fermentative processes were responsible for fatty acid consumption, whereas, a coupling between organic acid consumption and sulfate reduction occurred in sulfate-rich conditions (see Ref. 1 and references cited therein). The dechlorination of haloaromatics such as phenols, benzoates, catechols,

PCBs, and dioxins has been observed in estuarine sediments (1,10,59–61). On the basis of the available literature, it has been shown that sea salts affect dechlorination of PCBs in freshwater sediments, whereas a complex interrelationship exists between the effects of both sulfate and salt on dechlorination activity.

The effect of salinity on dioxin dechlorination was explored under a range of experimental conditions representative of freshwater, estuarine, and marine conditions to assess correlations between dominant terminal electron-accepting processes, carbon flow, and dechlorination patterns. As indicated in Figure 5 C, peri-dechlorination (i.e., removal of the 1,4,6,9-chlorines and concurrent transient accumulation of 2,3,7,8-tetraCDD) increased with increasing salinity of the medium (54). The shifts in dechlorination patterns are presumably due to a change in population distribution within the community. On the basis of hydrogen, the electron acceptor, and substrate analysis, the community shifted from methanogenic to sulfate-reducing via acetoclastic methanogenesis, whereas the importance of fermentation decreased with increasing salinity. No inhibitory effects of salinity on the dechlorination activity were observed.

MICROBIAL-ENVIRONMENTAL MATRIX INTERACTIONS

As noted earlier, recalcitrance of aryl halides is usually operationally defined or based on laboratory assessments in liquid culture. The former basis for recalcitrance tends to be influenced by issues of sampling heterogeneity or incomplete mass balances due to "nonextractable" fractions or unknown metabolites. For the latter, usually tight controls on the mass balance aid in the comparison of the extent and kinetics of degradation from one compound to another, yet results from these experimental systems are difficult to interpret because of sample homogeneity and selective enrichment.

Physical-Chemical and Biochemical Considerations

The physical-chemical properties of aryl halides, including generally low solubility (high distribution coefficients) and fugacity constants, and the characteristics of the dominant aryl halide sinks, generally high organic and silty sediments, affect the availability of these compounds to biochemical reactions (62,63). Additionally, because of the multitude of reactive phenolic and carboxylic functional groups in sediment organic matter and many aryl halides (or their metabolites), ample opportunity exists for cross-linking and other sequestration reactions (64).

The effect of condensation and addition reactions with humic materials on reaction stoichiometry has been clearly demonstrated for chlorinated anilines, phenoxyherbicides, and other pesticides. The fate of 3,4-dichloroaniline in an anaerobic rice paddy included the formation of operationally defined "polar material" and a condensation product of two dichloroaniline molecules (65). In the case of phenoxyherbicides, 20 to 35% of 2,4-D and 2,4,5-T were recovered from humic and fulvic acids and humin fractions, indicating the formation of polymeric humic substances (66). The fate of other aryl halide herbicides such as solan (3-chloro-2-methyl-p-valerotoluidide), propanil (N-3,4-dichlorophenyl-propionamide), and chlorodimeform (N′-4-chloro-2-methylphenyl-N,N-dimethylmethanimida mide) is similarly affected by incorporation of metabolites in soil organic matter or condensation reactions (67). Dioxin dechlorination reactions in the presence of dissolved organic matter (DOM) resulted in the production of polymeric materials and transchlorinated (transfer of the chlorine from dioxins to DOM) products (68).

In the case of aerobic degradation mechanisms, mass balances become even more complicated as the molecular structure of the contaminant changes and several oxidative coupling mechanisms have been identified. The latter result in the formation of dimers or polymers, as has been shown for chlorophenols and chloroanilines (69,70), or addition and condensation products with humic matter constituents (71), that are undetectable unless stringent extraction procedures are used. Particular problems surround the oxidative degradation of pentachlorophenol (PCP) by white rot fungi (e.g., *Phanerochaete chrysosporium*). The majority of the product was recovered as pentachloroanisole, with the remainder labeled as "unextractables" (72,73). Because peroxidases have been shown to catalyze the formation of polychlorinated dioxins and dibenzofurans from pentachlorophenol (74), this fraction should be further characterized.

Hence, it is apparent that a significant fraction of aryl halide molecules (and their microbial metabolites) will ultimately be bound to natural soil constituents. Considering the difficulty of liberating these soil-bound residues for analytical purposes, Alexander (62) and Hatzinger and Alexander (75) argue that the hazard and risk associated with toxic chemicals diminishes as they persist in soil. On the other hand, several cases have been reported in which bound residues were released and further transformed. Four possible scenarios have been recognized for soil-bound residues (64): (*1*) noncovalently bound residues are liberated via bond cleavage at the original site of attachment as has been shown for chlorophenols, and chloroanilines; (*2*) cleavage of soil constituents to monomers containing the aryl halide, as has been demonstrated for fungal oxidation of humic 3,4-dichloroaniline complexes; (*3*) bond cleavage at a site other than the original attachment, and (*4*) mineralization of the soil-bound residue. However, because the different possible binding mechanisms to high molecular weight humic material under oxic and anoxic conditions are poorly understood, predictions on the fate of these complexes are uncertain at best. A summary of possible matrix interactions is presented in Table 4.

Kinetic Considerations

Because of the factors affecting aryl halide availability mentioned earlier, it is likely that the presence of solids and organic matter (dissolved or solid) will affect not only the extent but also the rate of degradation. With respect to matrix interactions, the kinetic considerations consist of at least two components: (*1*) the differences in degradation rates between freshly spiked and historically present contaminants, and (*2*) the role of organic matter as a source of

Table 4. Biotic and Abiotic Reactions of Aryl Halides in Soil or Sediment Environments (Modified from Scheunert, 1993)

Types of Transformation	Reaction	Examples
Synthetic Processes	Conjugation	Dichlorophenols
	Reaction with natural humic substances	Chlorinated anilines, phenols chlorinated dioxins
	Interactions between aryl halides	Chlorinated anilines, phenols
	Di- and polymerization	Chlorinated anilines, phenols
	Transchlorination	Chlorinated anilines, phenols, dibenzo-*p*-dioxins
Redox reactions	Oxidative coupling	Chlorinated anilines, phenols
	Dechlorination	DDT, chlorinated dioxins, and furans hexachlorobenzene
Surface-catalyzed reactions	Dehydrochlorination	DDT, chlorinated dioxins, benzenes
	Polymerization	Chlorinated phenols, anilines

electrons during reduction reactions and as an alternative electron acceptor during oxidation reactions. The former is related to mass-transfer limitations, which because of molecular and matrix characteristics, have an impact on the extent to which sorption and sequestration take place. Hence, biodegradation in matrices in which these factors dominate are controlled by desorption and diffusion processes (76). Indeed, studies have shown that while in situ short-term aerobic degradation of PCBs was limited by the desorption kinetics from high organic sediments (77); long-term dechlorination processes may not be significantly impacted by mass-transfer limitations (51). Whereas natural in situ dechlorination reactions are estimated to result in the removal of one chlorine every seven to ten years (54), no natural aerobic field rates are available despite the occurrence of aerobic metabolism in sediments (43). In low organic environments such as groundwater systems, the first-order degradation rates of aryl halides (chlorobenzenes, chlorophenols, and chlorocresols) determined in the field (in situ microcosms) do not differ significantly from those observed in the laboratory, as based on disappearance relative to control experiments (78). Hence, attempts at increasing the availability of aryl halides from environmental matrices (e.g., via surfactant amendments) is likely to have mixed effects, depending on whether mass-transfer or biodegradation processes control the kinetics of transformation.

Organic matter (solid or dissolved can enhance degradation processes by playing a key role as electron shuttles for dechlorination, or potential electron acceptors for growth of anaerobic populations. Laboratory studies on the microbial dechlorination of dioxins by sediment-eluted mixed cultures demonstrated that dissolved organic matter was capable of enhancing the rates and extent of reaction, presumably due to electron shuttling by the phenolic functional groups (68,79). More importantly, historical dioxins in sediments dechlorinated along a pattern characteristic of abiotic rather than microbial activity (56). It is speculated that for trace levels of aryl halides, sediment organic matter may serve as the direct catalyst of dechlorination. Considering the substantial electron-accepting capacities associated with soil and sediment humic and fulvic acids (80), the contribution of quinonic moieties to the total sediment reactivity is likely to be very significant. Moreover, the potential role for fermentative microorganisms to either directly or indirectly (via hydrogen generation) mediate aryl halide dechlorination reactions (e.g., 54) would be significantly correlated to the availability of sediment organic matter.

SARs and QSARs for Aryl Halide Degradation

Structure-activity relationships (SARs) between chemical reactivity and molecular descriptors for aryl halides are valuable tools for exploring mechanistic determinants influencing biodegradation, and to predict rate constants within classes of compounds. An abundance of these statistically significant correlations based on appropriate predictor variables is available, yet very few of these yield useful quantitative expressions (QSARs). One of the limitations is the scarcity of relationships between readily available molecular descriptors and important microbial transformation reactions. Another relates to the lack of understanding of contaminant-matrix interactions, which govern the degradation process. Perhaps not surprisingly, the most robust SARs and QSARs have been generated for strictly abiotic transformation processes such as hydrolysis, direct photolysis, and oxidation. More recently, correlations have been generated describing fortuitous degradation processes (e.g., cometabolic reductive dechlorination) or for enzymes exhibiting broad substrate specificities (e.g., soluble methane monooxygenase) (20,81,82).

Most QSARs are based on the Hansch method where biological response is expressed as a linear function of hydrophobic, electronic, and steric properties (e.g., 81,83). More recently, first principle (ab initio) and semi-empirical molecular orbital calculations have been used to obtain molecular descriptors such as electron density distributions of the highest occupied π orbital (84), carbon-chlorine bond charges (82), heats of formation and ionization potentials (85), or HOMO-LUMO gaps (20,86). One or more of these parameters have been found to be strongly correlated

to the observed dechlorination pathways of dioxins and substituted benzenes (20,82), to the preferred methane monooxygenase oxidation pathways of ortho-substituted biphenyls (87), or dioxygenase-mediated oxidation of dioxins and furans (88).

A robust QSAR was developed to predict the reductive transformation constants of 45 halogenated monoaromatic hydrocarbons in anoxic sediments from four readily available molecular descriptors (83). These were the carbon-halogen bond strength, the summation of the Hammett sigma constants and inductive constants for the additional substituents, and the steric factors for these substituents. Although the electronic properties of the substituents exhibited a positive effect (increased the reaction rate), steric and bond strength factors decreased the predicted reaction rate. When the sediment organic matter content was included in the prediction, the correlations improved when the fraction of the compounds sorbed to the solid phase was considered. This indicates that, although molecular descriptors can provide a measure of reaction regiospecificity, the predicted reaction rate in more complex environmental samples is controlled by mass-transfer limitations.

CONCLUSION

As testified by the multitude of reviews over the years, the fate of halogenated aromatic compounds as affected by microbial activity is extremely difficult to summarize, both with respect to pathways of transformation and to reaction kinetics. The difficulty stems from complex interactions between diverse chemical (multiple and variable substituents) and microbial (acclimation mechanisms, enzyme specificities) processes. It is clear from this review that the transition of mechanistic observations to fate predictions in environmental matrices is complicated by the multiple attenuation processes affecting these compounds and the overall lack of mass balance due to either irreversible sequestration mechanisms or incomplete analysis for transformation products. Considering that these poorly understood issues continue to play a role in the (incomplete) decision-making framework for PCBs, (arguably the best studied group of chlorinated aromatics), research with respect to most aryl halides (except for perhaps pesticides) should still be considered in its infancy.

Despite these limitations, great strides have been made during the last decade with respect to the understanding the microbiology of aryl halide degradation. Consider, for example, the progress made in elucidating the genetic basis for microbial adaptation to structurally similar chemicals, and the requirements for transcriptional activation of key metabolic enzymes in aryl halide pathways. Dehalorespiration of chlorinated aromatic compounds, whether via direct demonstration of growth or hinted at via stimulation of specific dechlorination pathways using selected priming molecules, may be widespread in nature. This opens up a vast array of new possibilities to understand the complexity of microbial niche development. Recent advances in molecular ecological characterization methods have afforded substantial advances in this area, such as aryl halide-induced population behavior and shifts within complex communities as the result of these chemical stresses. Complementary to microbial research, the thermodynamic underpinnings of which transformations are likely to occur have aided in directing fate mechanism studies. Intrinsic chemical reactivity, as quantified using ab initio calculations have been shown to be particularly useful in radical-catalyzed oxidations and reductions. As a third line of progress, field observations have proven to be particularly useful to "calibrate" fundamental research approaches, and have in a limited number of cases demonstrated the validity of laboratory investigations for environmental fate predictions.

Ultimately, the application of each of these convergent disciplines is directed toward risk reduction from these chemicals. Key questions such as "Is the chemical transformed to a less toxic intermediate?," "Are the likely microbial reaction kinetics sufficient to prevent accumulation to levels impacting the food chain?" and "Does microbial humification result in irreversible binding to soil or sediment organic matter?" Given this state of knowledge, the regulatory community faces an enormous challenge as it is is unclear how new research information should be incorporated into risk-based decision models. Conversely, new research frontiers aimed at overcoming the limitations imposed on natural (intrinsic) microbial activity, such as mass transfer limitations, are abundant. Both regulatory and research paths will require more quantitative answers to the questions posed earlier, to allow further definition of the boundary conditions on reaction rates and pathways as a function of the relevant impacted geochemical system under consideration.

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FATE OF VIRUSES AND PROTOZOAN PARASITES IN AQUATIC SEDIMENTS

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The discharge of large quantities of domestic wastewater into coastal waters is still the most common method of disposal. Pathogens present in these discharges may eventually find their way into the sediments of natural bodies of water. Here, they may accumulate and survive for prolonged periods, to be later resuspended, resulting in a degradation of water quality. Understanding the occurrence and fate of enteric pathogens in sediments is critical in assessing their impact on water quality.

Because of their colloidal nature, free suspended viruses do not settle by themselves from the water column. However, viruses associated with large particles soon leave a water column and settle into the bottom sediments (Fig. 1). Suspended solid-associated virus that settle out of the water column accumulate as a loose, fluffy layer over the compact bottom sediment, which may contains 10 to 10,000 more viruses per unit volume than the overlaying water (1). Disturbance of these sediments by rainfall, water turbulence, or any other unnatural event may result in the resuspension of these organisms. In artificial environments, such as reservoirs for drinking water, settling tanks of drinking water or sewage treatment facilities, resuspension of sediments may result in a breakthrough, through the treatment system. In the environment, resuspended microorganisms can be transported from polluted to nonpolluted recreational water, or shellfish growing area, increasing the risk of public exposure to microbial pathogens. Giardia cysts and Cryptosporidium oocysts are very large, relative to viruses, and settle out relatively quickly from the water column. Little is known about their eventual fate in sediments.

OCCURRENCE OF VIRUSES AND PARASITES IN AQUATIC SEDIMENTS

Enteric Viruses

Recent advances in recovery methods of solid-associated viruses have made possible the studies on the concentration and distribution of enteric viruses in sediments. Studies have shown that many times more viruses exist adsorbed to sediment, than in the overlaying water on a volume basis (2), and sediments may play a role in the persistence of viruses in the marine environment (3,4) (Table 1). A 10 to 10,000-fold greater virus concentration in sediments was found, than in the overlaying water in the coastal Texas and Florida waters polluted by sewage discharge (5). In another study along Galveston Bay, Rao and coworkers (2) found a higher concentration of enterovirus in sediments compared with the overlaying water. Enteric viruses were detected 47% of the time in fluffy sediments compared to only 14% of the water samples. A greater number of enteroviruses were recovered from fluffy sediments (39–398 plaque forming units-PFU) compared with overlaying water (3–12 PFU) on a volume basis.

Protozoan Parasites

Giardia cysts and *Cryptosporidium* oocysts are known to occur in high concentration in wastewater. Sykora and coworkers (7) studied the concentration of *Giardia* cysts in raw sewage. The concentration of *Giardia* cysts in raw sewage varied from 560 to 14,000 per liter. Several other studies reported the concentration of *Giardia* cysts in the influent of constructed wetlands, which received raw sewage or secondary unchlorinated sewage. The average concentration of Giardia cysts found in the influent of a pilot scale constructed wetland receiving domestic raw sewage in Arizona was 7,444 cysts/L (8).

Rose and coworkers (9) studied Cryptosporidium oocyst concentrations in raw sewage and found an average

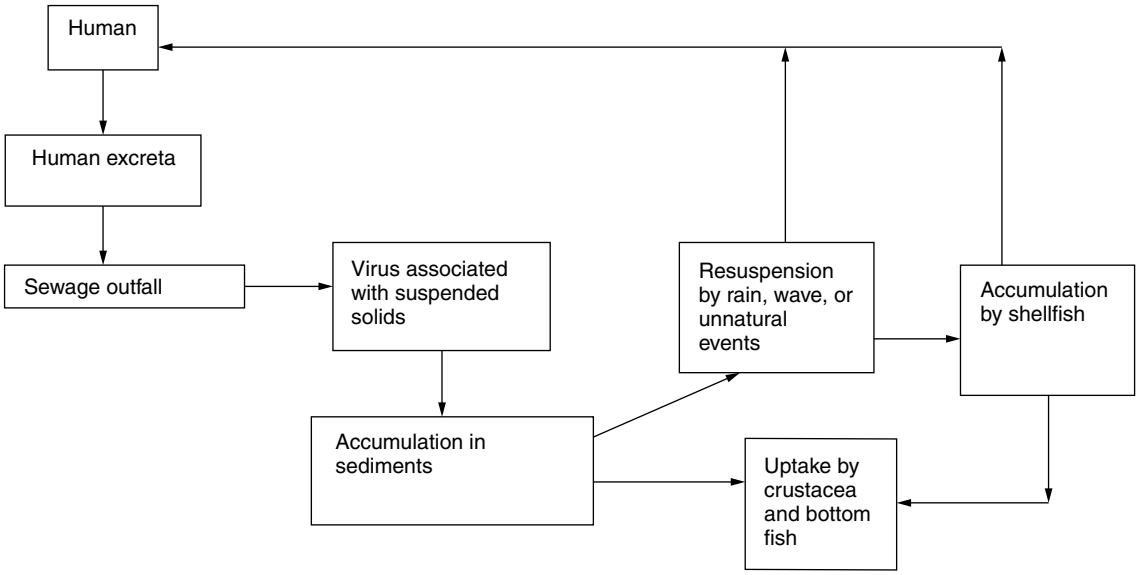

Figure 1. Transmission of viruses in coastal systems (modified from Melnick and Gerba, 1980).

Table 1. Concentration of Bacteriophage and Viruses in Sediments and Overlaying Water

Type of Sediment	Virus Type	Site Location	PFU in Overlaying Water	PFU in Sediment	Reference
Marine Sediment	Enterovirus	Hollywood	0.3[a]	3,160[a]	5
		Miami	0.5[a]	2,160[a]	
	Miami Beach	7.3[a]	9,830[a]		
Marine Sediment	Enterovirus	Galveston Bay	3–12[b]	39–398[b]	2
Wetland sediment	Coliphage	Constructed wetland (duckweed pond)	33,000[c]	14,000[c]	6
		Constructed wetland (waterhyacinth pond)	34,000[c]	8,500[c]	

[a] = Plaque Forming Unit (PFU) per 100 liters.
[b] = PFU of virus estimated for 379 liters of water.
[c] = PFU per 100 ml of water or 100 gm of wet sediment.

concentration of 5,291 oocysts per liter. The presence of *Cryptosporidium* in surface and groundwater has also been reported. *Cryptosporidium* was found to be present in 65 to 87 percent of surface water samples tested throughout the United States (10,11). One out of 18 groundwater samples tested was found to be contaminated with *Cryptosporidium* (10). The occurrence of *Cryptosporidium* in eight natural wetlands in Arizona has been studied. The concentration of *Cryptosporidium* ranged from below detection level to 0.3 oocysts/ L with an average value of 0.06 oocysts/ L (12).

Giardia and *Cryptosporidium* are larger than bacteria and viruses and would easily settle in the bottom sediment in aquatic environments. However, reports on *Giardia* and *Cryptosporidium* concentration in sediments have been very scarce. The only known study compared *Giardia* and *Cryptosporidium* concentration in overlaying water and sediment of two constructed wetlands built for the purpose of wastewater treatment. The wetlands received secondary unchlorinated wastewater. The concentration of *Giardia* in the sediment was two to three orders of magnitudes greater compared with the concentration in the overlaying water in both wetlands (Table 2). Similar to *Giardia*, larger numbers of *Cryptosporidium* were found in the sediment than in the overlaying water. Generally, from 1 to 2 $\log_{10}$ greater *Cryptosporidium* numbers were found in the sediment than in the water column. *Giardia* cysts concentration in one of the wetland (duckweed pond) waters was 130 cysts/L compared to 84,000 cysts/L in the sediment. *Cryptosporidium* oocysts concentration in water (duckweed pond) was 21.9 oocysts per liter compared with 2,300 oocysts in the sediment.

Detection of Enteric Viruses in Sediments

Detecting viruses in sediment involves a three-stage process. The first stage is the collection of samples. Sediment samples are usually collected with a grab sampler or an Ekman dredge sampler. The second stage consists of eluting the sediment-associated viruses using a beef-extract based eluant and subsequent reduction

Table 2. Concentration of *Giardia* Cysts and *Cryptosporidium* Oocysts in the Water Column and Sediment of Two Constructed Wetlands Built for the Purpose of Wastewater Treatment

Protozoan	Site	Concentration of (oo) cysts in water column/L	Concentration of (oo) cysts in sediments/L
Giardia	Duckweed pond	130	84,000
	Water hyacinth pond	95.2	14,000
Cryptosporidium	Duckweed pond	21.9	2,300
	Water hyacinth pond	47.6	3,400

Source: M. R. Karim, Survival of Indicator Microorganisms and Enteric Pathogens in Wetlands, Ph.D. Dissertation, The University of Arizona, Tucson, Ariz., 1999.

in volume by low pH organic flocculation. Viruses can be isolated from the sediments by using the following procedure (13). Briefly, viruses are eluted from sediments by suspending the sediments into three volumes of beef-extract glycine, mixed for five minutes and centrifuge at $2,510 \times g$ for 10 minutes. The pellet is discarded and the eluted virus is then concentrated using the low pH organic flocculation method. The pH of the supernatant is adjusted to 3.5, and centrifuged at $2,510 \times g$ for 5 minutes. The pellet is then resuspended into 10 ml of 0.05 glycine buffer, centrifuged at $2,510 \times g$ for 20 minutes and the supernatant passed through a 05s Zeta-plus filter to reduce toxicity. The whole process results in a known volume of concentrate containing viruses. The pellet from organic flocculation can also be processed by resuspending in 0.15 M Na_2HPO_4 (pH 9.0–9.5), as described in Information Collection Rule virus methodology (14). Several other methods for virus isolation from sediments are described in the literature (2,15)

The third stage is the assaying of viruses contained in the sample concentrate. In general, the infectivity of enteric viruses contained in the sample concentrate is examined by inoculating the sample concentrate into a mammalian cell culture. This is often done by using either a cytopathogenicity (CPE) assay or by a plaque formation assay technique. The Baffalo Green Monkey (BGM) kidney cell line is the most commonly used cell line for the detection of enteroviruses in the sediment. There is no single universal cell culture system that will detect all or even a majority of the enteric viruses present in sediments. The use of the other cell line is required to detect other groups of enteric viruses. Cytotoxicity is a problem often encountered in the assay of sample concentrate from sediments. The cell monolayer washing procedure described in the ICR virus assay method can be used for reducing cytotoxic effect. Molecular methods such as polymerase chain reaction can also be used to detect the presence of viruses in the sample concentrate. However, currently available molecular methodology cannot differentiate between infective and noninfective stages of the virus.

DETECTION OF PARASITES IN THE SEDIMENT

Sediment samples are usually collected using an Ekman dredge. Twenty milliliter of sediment is mixed with 30 ml of sterile deionized water, and after homogenization, the samples are passed through a series of two sieves of gradually finer mesh (opening two mm; 300 μm) to remove fibrous and other large materials. The sample is then centrifuged ($1,050 \times g$; 10 minutes) and the supernatant aspirated off without disrupting the pellet. The cyst and oocyst-containing pellet is washed twice with deionized water. The pellet volume is adjusted to 20 ml with an elution solution. The sample is then vortexed and clarified by using a Percoll-sucrose (sp. gr. 1.10) density gradient. The debris is pelleted to the bottom of the centrifuge tube by centrifugation ($1,050 \times g$; 10 minutes). The supernatant (20 ml) as well as 5 ml past the interface is collected with a pipette. The sample is then washed by centrifugation with the eluting solution and then aspirated down to 5 ml (plus pellet). Cysts and oocysts in the pellet are detected by immunofluorescence by filtering 0.5-ml aliquots of the final concentrate through 0.2-μm pore size cellulose acetate membrane filters. *Giardia* cysts and *Cryptosporidium* oocysts are identified on the basis of size, shape, and immunofluorescence. The results are reported as the total number of *Giardia* cysts and *Cryptosporidium* oocysts per liter of sediment or per kg of dry sediment. For those samples in which no cysts or oocysts are detected, the detection limit is calculated as follows (Eq. 1).

$$\frac{< X}{100} = \frac{[< 1(100)]}{FVR} \text{ where;} \quad (1)$$

F = volume of concentrate floated/total concentrate volume (ml)
V = volume of original sample (L)
R = volume of suspension filtered/interface volume (ml)

SURVIVAL OF VIRUSES IN SEDIMENTS

Enteric viruses are known to readily attach to particles in water and wastewater. Suspended solids-associated virus that settled out of the water column, accumulates in the bottom sediment. An assessment of the public health hazard associated with sedimented viruses depends on the survival of viruses in the sediment. During the past two decades, many reports have addressed the persistence of viruses in sediments. Virus survival was found to be prolonged in the presence of marine

sediments (4). Echovirus 1 had the longest survival time, greater than 18 days followed by coxsackievirus B3–18 days; poliovirus 1, 14 days; and coxsackievirus A9, four days. In comparison, samples containing seawater alone, coxsackievirus A9 was undetectable after two days, and coxsackievirus B3, echovirus 1, and poliovirus 1 persisted for 4, 6, and 10 days, respectively. In another study, (16) survival of poliovirus 1 and echovirus 1 in estuarine water and sediments was studied in the Galveston Bay, Texas, under field conditions. Viruses were suspended in estuarine water and sediments, and placed both in dialysis tubes and in chambers constructed with polycarbonate membrane walls. Virus adsorption to sediments greatly increased the survival time. The time required for inactivating 99% of poliovirus increased from 1.4 days in seawater alone to six days when adsorbed to sediments. Virus survival in the environment is influenced by temperature, pH, virus adsorption to soil particles, exchangeable aluminum, indigenous bacteria, and calcium hardness (17–20). However, little is known about the mechanism of how sediments prolong virus survival and infectivity. Liew and Gerba (21) studied the thermostabilization of enterovirus by estuarine sediments. Polioviruses type 1 survived longer in artificial seawater containing 12.5% (wt/vol) sediment than the water alone. In addition to prolonging virus survival time in the presence of sediments, the level of infectivity of virus in seawater containing sediments was at least one log higher than the seawater alone, suggesting that estuarine sediments protected enteroviruses against thermal inactivation. Other studies have shown that clay particles can exert some protective effect against heat inactivation of viruses (22). Particulate matter may exert a protective effect on the viruses. Gerba and Schaiberger studied the effect of particulates on virus survival in seawater (23). The presence of as little as 5mg/L clay particles greatly reduced the inactivation of virus in artificial seawater. Laboratory studies demonstrated that the sediment is also capable of protecting the poliovirus from the inactivating effects of other microorganisms as well as heat and salts, resulting in a longer survival (17). Thus, sediments could potentially serve as a reservoir of human viruses, which could be released into the water column by storms or unnatural events (i.e., dredging).

SURVIVAL OF PROTOZOAN PARASITES IN SEDIMENTS

Numerous waterborne outbreaks of giardiasis and cryptosporidiosis have been reported, yet little is known on the viability of *Giardia* and *Cryptosporidium* in different aquatic environment. DeRegnier and coworkers studied the survival of *Giardia* cysts in lakes, rivers, and tap water (24). Cysts suspended at 30 ft in lake water remained viable for up to 56 days; whereas, cysts stored at 15 ft were nonviable after 28 days. *Giardia* cysts suspended in either lake or river water were viable for 56 to 84 days compared with cysts suspended in tap water remaining viable for 14 days. Lower ($<10\,^{\circ}C$) temperature was found to be prolonging the survival of *Giardia* cysts. In a recent study, the survival of *Giardia* in the sediment and overlaying water in a constructed wetland (built for the purpose of wastewater treatment) was compared (6). *Giardia muris* cysts were placed in plastic bottles containing wastewater or wastewater plus sediment (20%). The survival of *Giardia* in water and sediment is presented in Figure 2. *Giardia* survival in the sediment was found to be opposite of what has been demonstrated for viruses. The die-off rate of *Giardia* in sediment was greater than the die-off rate in water. *Giardia* reductions in the water and sediment in five days were 0.03 $\log_{10}$ and 1.81 $\log_{10}$, respectively. The die-off rates in water and sediment were 0.02 $\log_{10}\,day^{-1}$ and 0.37 $\log_{10}\,day^{-1}$, respectively, suggesting that the wetland sediment environment is unfavorable for the survival of *Giardia*. A few survival studies suggest that temperature plays an important role in the survival of *Giardia* (24,25). Biological antagonism is thought to be a mechanism of *Cryptosporidium* oocyst die-off in natural waters (26). A plausible explanation of greater reduction of *Giardia* in the sediment could be due to biological antagonism or the presence of organic substances, which enhanced die-off of the protozoan parasites. No data exist in the literature on the survival of *Giardia* and *Cryptosporidium* in marine sediments. Future studies are necessary to study the effect of sediments on the survival of *Giardia* and *Cryptosporidium* in estuarine sediments.

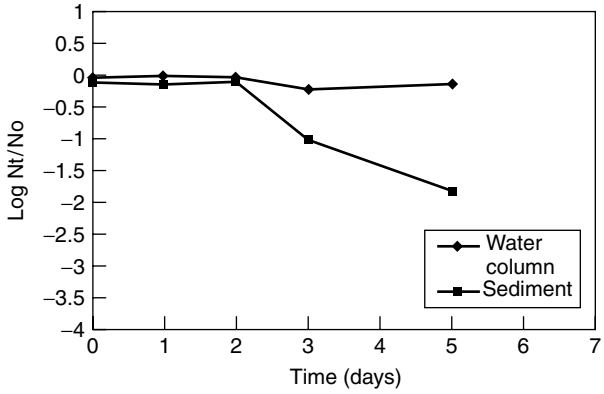

Figure 2. Survival of *Giardia muris* in sediment versus water column of a artificial wetland. M. R. Karim, *Survival of indicator microorganisms and enteric pathogens in wetlands*, Ph.D. Dissertation, The University of Arizona, Tucson, Ariz., 1999.

CONCLUSION

Sediments represent a potential reservoir of viruses contributing to the prolonged survival. In marine environments, resuspended sediment-associated viruses can be transported from polluted to nonpolluted recreational water, or shellfish growing areas, thus posing a potential public health risk. *Giardia* and *Cryptosporidium* are larger than bacteria and viruses and would easily settle in the bottom sediment in an aquatic environment. However, currently no data exist in literature on their occurrence and survival in marine and freshwater sediments. Consequently, the public health implications on sediment bound cysts and oocysts are not known.

Future research is needed to study the occurrence and survival of (oo) cysts in marine and freshwater sediments.

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FECAL CONTAMINATION, SOURCES OF

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Fecal pollution impacts many aquatic environments and it can originate from a variety of sources, including human (HS) and nonhuman (NHS) animal species. The consequences of fecal contamination include affecting the safety of surface and ground waters, as well as the economic well-being of fisheries, especially shellfish industries, where harvesting areas are strictly defined by levels of fecal contamination. Consequently, techniques for differentiating HS and NHS pollution are required to determine the sources of the fecal contamination and the appropriate level of protection for water systems, including effective remediation techniques, epidemiological information, and regulatory actions. Although much effort has been given to define better indicators of fecal contamination, such research is still in its infancy.

MICROORGANISMS USED FOR DETECTING SOURCES OF FECAL CONTAMINATION

Fecal Streptococci

Fecal streptococci have been used to indicate fecal contamination because they inhabit the intestinal tract of humans and other warm-blooded animals. The four primary species of interest are *Streptococcus faecalis*, *S. bovis*, *S. equinus*, and *S. avium*. These species show prolonged persistence in water, and do not reproduce. *Streptococcus faecalis* and *S. faecium* (enterococci) have been shown to be useful for indicating the presence of viruses, particularly in biosolids and seawater. The ratio of fecal coliforms to fecal streptococci has been used as an indicator of fecal source, whereby a ratio of 4.0 or greater was considered to indicate HS contamination, whereas that of 0.7 or less indicated NHS (1). However, after further examination, this ratio has proven unreliable for chlorinated effluents, and some investigators have questioned its overall usefulness (2).

Bacteroides. It has been suggested that bacteria of the genus *Bacteroides* might be used to distinguished HS and NHS fecal contamination. *Bacteroides* dominate the human fecal flora, and outnumber the coliform bacteria (3,4). Recently, a polymerase chain reaction (PCR) assay was developed to discriminate human and ruminant feces on the basis of host differences in *Bacteroides-Prevotella* genes encoding 16S rRNA (5,6). However, for several reasons, *Bacteroides* spp. may not be suitable for discriminatory applications. First, being obligate

anaerobes, *Bacteroides* spp. do not survive in oxygenated waters (7,8). Second, the most abundant *Bacteroides* spp. in human feces have been detected only at low levels or not at all in feces from other animals (7,9). However, the need to maintain anaerobic conditions during growth, isolation, and biochemical identification of *Bacteroides* spp. has discouraged their use as indicators. Furthermore, the survival of *Bacteroides* spp. in water is less than that of *Escherichia coli* or *S. faecalis* and other enteric pathogens. Consequently, this genus is not practical for differentiating HS and NHS fecal contamination (3,8).

Bifidobacteria

Bifidobacteria are anaerobic, non-spore-forming, gram-positive bacteria that have been proposed as indicators of fecal contamination. They are the third most common genus found in the intestines of warm-blooded animals. At the present time, the genus *Bifidobacterium* includes 28 species (10 species of human origin, 13 species of other warm-blooded animal origin, 3 species from honey bees, and 2 species from sewage) and it has been suggested that these species may help distinguish between human and animal contamination. *Bacteriodes* spp. can be differentiated on the basis of carbohydrate acidification, serological characteristics, and DNA-DNA hybridization (10,11). However, acidification tests performed with carbohydrates are not sufficient to identify bifidobacterial species. The results of other techniques that are based on electrophoretic patterns of soluble proteins (12) or transaldolase and 6-phosphogluconate dehydrogenase activities (13) do not strongly correlate with phenotypic or genomic descriptions of the species.

Clostridium perfringens. *Clostridium perfringens* is an anaerobic, gram-positive, spore-forming, rod-shaped, sulfate-reducing bacterium found in the colon of warm-blooded animals. It has been suggested as a suitable indicator for viruses and protozoan cysts because it's spores are resistant to environmental stresses and to disinfection. However, it cannot differentiate HS and NHS fecal contamination (14).

Bacteriophages

Bacteriophages are similar to enteric viruses, but are found in higher numbers than enteric viruses in wastewaters and other environments. Simple and rapid methods are available for detecting bacteriophages from various environments (15–17). Therefore, these organisms have been proposed as water quality indicators in estuaries, seawater, recreational freshwater, and potable water (18). They may also serve as indicators for assessing the process efficiency of methods to remove pathogens from water and wastewater (16). Some coliphages, particularly RNA phage f2, are more resistant to chlorination than enteroviruses, such as poliovirus type 1. However, MS2, another RNA phage, is not suitable as a surrogate for enteric viruses when determining ozone disinfection efficiency (19). Therefore, it is doubtful that bacteriophages can be used as indicators for enteric viruses in all situations (16). In other studies, investigators observed that animal and human feces contain different serotypes of RNA coliphages (20,21), which may be useful for discriminatory applications.

F-specific bacteriophage (male-specific phage) are single-stranded RNA phage (FRNA) that possess structures and sizes very similar to enteric viruses. They infect only those bacterial hosts that produce F or sex pill and were proposed as indicators of HS contamination (22). Because F-specific phage are infrequently detected in human fecal matter and show no direct relationship with fecal contamination level, they cannot be considered as indicators of HS contamination.

The potential bacteriophage of *Bacteroides* spp. to serve as indicators of viral contamination has been explored (23). Phage active against *Bacteroides fragilis* HSP 40 were detected in 10% of human fecal samples, but not in animal feces, sewage, and other polluted aquatic environments, such as river water, seawater, groundwater, sediments; the phage were absent in nonpolluted sites (23,24). In addition, bacteriophage of *Bacteroides* spp. do not replicate in the environment, they have decay rates similar to those of human enteric viruses in environmental samples (25), and they are more resistant to chlorine than bacterial indicators (26,27) but are less resistant than coliphage f2 to UV irradiation (26). Thus, these organisms may be considered as suitable indicators of human fecal contamination and might be used to differentiate HS and NHS fecal contamination. However, the primary disadvantage of this indicator is that it occurs in relatively small numbers in human feces.

Fecal Sterols

Investigators have reported a correlation between the presence of fecal sterols and fecal contamination. Fecal sterols include coprostanol, coprosterol, cholesterol, and coprostanone. The main excretory products of body cholesterol, bile acids, such as hyocholic and hyodeoxycholic acids are produced in substantial amounts by pigs as compared to humans, whereas the later produce deoxycholic and lithocholic acids, which are virtually absent in pigs. Bile acids are more resistant to degradation than coprastanol and may help distinguish between HS and NHS (pig) fecal contamination (28).

Escherichia coli. The fecal coliform, *E. coli*, has been used as an indicator of human enteric pathogens for many years (29). However, it is well established that *E. coli* also inhabits the intestines of other warm-blooded animals. Human feces potentially carry many enteric pathogens, such as *Salmonella typhi*, *Salmonella paratyphi*, *Shigella* spp., pathogenic forms of *E. coli*, hepatitis A, and Norwalk-group viruses. In contrast, animals are rarely colonized by human enteric viruses (30–33). However, before *E. coli* can be used to indicate human health risks from contact with water, it is important to define bacterial characteristics that are associated with strains representing potential source(s) of fecal contamination.

NEW APPROACHES FOR DIFFERENTIATING BACTERIAL STRAINS

Bacterial pathogens have been typed and differentiated by biochemical tests (34,35), phage susceptibility (36), outer membrane protein profiles (37), antibody reactivity (38), fimbriation (39), bacteriocin production and susceptibility, and other methods (40). However, these typing systems suffer from several disadvantages, including (*1*) the unstable nature of some phenotypic properties, (*2*) low discriminatory power at the intraspecies level, and (*3*) limited specificity (41).

Recent techniques in DNA analysis have reduced dependence on phenotypic traits. These methods, such as plasmid profile analysis, comparison of DNA restriction endonuclease digestion fragment patterns, and the use of nucleic acid probes, have been used to discriminate bacterial strains within a single species (41). Analysis of chromosomal DNA avoids the potential pitfalls associated with variable expression of phenotypes and with the biologic instability of plasmids. It has been suggested that these techniques can be used to discriminate sources of *E. coli*.

Even though one can argue that bacterial genotypic traits are more stable than phenotypic properties, the latter have been shown to provide useful levels of discrimination in certain situations, including multiple antibiotic resistance (MAR), fatty acid methyl ester (FAME), and strain serotype (31,42–44).

MAR has been used to differentiate *E. coli* from different sources by employing antibiotics commonly associated with human and animal therapy, as well as in animal feeds (31,43,45,46).

Bacterial serotyping is based on the presence or absence of somatic (O), flagellar (H), capsular or envelope (K) antigenic determinants, and their reaction with specific antisera. Several investigators have used this method for discriminating *E. coli* from different sources (42,47).

Pulsed-field gel electrophoresis (PFGE) is a measurement of restriction fragment length polymorphism (RFLP) of the whole chromosome that can highly resolve bacterial strains. Numerous investigators have described the use of PFGE in differentiating bacteria from specific sources, especially in epidemiological studies (48–51). The PFGE technique involves digesting chromosomal DNA using low-frequency restriction endonucleases to produce high molecular weight fragments. DNA fragments are then separated on a polymeric gel by alternating pulsed-electric fields.

Ribotyping, another method of "DNA fingerprinting," has provided a very powerful tool to differentiate and cluster bacterial strains (30,36,38,52–54). Ribosomal RNA (rRNA) is ubiquitous, highly conserved, and multiple copies are present on the bacterial chromosome. These factors have allowed the use of a single probe to characterize phylogenetically distant bacteria following digestion of DNA with restriction endonucleases. Generally, members of *Enterobacteriaceae* yield more restriction fragments than other bacteria, owing to a greater number of rRNA operons (55).

The purpose of this article is to discuss phenotypic and genotypic methods, which have been used to differentiate HS and NHS bacterial strains in water environments, with a focus on the historic indicator organism, *E. coli*.

MULTIPLE ANTIBIOTIC RESISTANCE (MAR)

Owing to the increasingly widespread use of antibiotics in human and animals, antibiotic resistance among bacteria is common. Because of different applications of a wide variety of antibiotics, bacterial clones can be identified via antibiotic resistance patterns that can be helpful for source tracking applications. It is important to consider that many resistance characters are transmissible, and by nature, not stable (56). However, several researchers have described patterns of antibiotic resistance in *E. coli* that are useful in determining isolate source.

Kaspar and coworkers (43) isolated a total of 202 *E. coli* from rural and urban waters and tested them with 11 antibiotics commonly associated with animal feeds and/or clinical treatments. They found that 90 and 32% of urban and rural *E. coli* isolates, respectively, were resistant to one or more antibiotics. The average MAR index for urban areas was 0.090, 3.2 times greater than that for the combined rural areas (0.028). MAR indices varied considerably among samples and geographical area.

In another study, *E. coli* isolates, which were collected from environments that were considered to pose low and high enteric disease risk to humans, were screened against 12 antibiotics (31). The MAR index for isolates from raw human sewage was greater than the index for isolates obtained by direct anal swabbing (31). It was proposed that several factors may have contributed to this difference, such as a high concentration of nutrients and exchange of plasmids in the sewage system. It was also observed that the MAR index of *E. coli* isolates from wild animals was generally low, whereas that of human and poultry isolates was relatively high (31).

Parveen and coworkers (46) reported that MAR patterns of *E. coli* may be used to differentiate HS and NHS fecal contamination. In this study, a total of 765 HS and NHS *E. coli* were collected from the Apalachicola National Estuarine Research Reserve (ANERR) in Florida and their MAR profiles were determined using 10 antibiotics. Eighty-two percent of the isolates were resistant to one or more antibiotics. *Escherichia coli* from HS showed significantly greater resistance ($P < 0.05$) to antibiotics and higher MAR indices than NHS (Tables 1 and 2). Sixty-five different resistance patterns were observed among HS isolates, compared to 32 for NHS. Examples of contrasting resistance included chlortetracycline-sulfathiazole (33.7%) and chlortetracycline-penicillin G-sulfathiazole (14.5%) for HS isolates, versus 15.4 and 1.7%, respectively, for NHS. In all 15 and 38% of HS isolates had MAR indices of less than or equal to 0.1 and greater than or equal to 0.3, respectively. Conversely, 45 and 7.6% of NHS isolates had MAR indices less than or equal to 0.1 and greater than or equal to 0.3, respectively. In addition, the average MAR index for HS isolates was 0.25 compared to 0.13 for NHS isolates.

The relationship of antibiotic resistance pattern for HS versus NHS, based on coefficients of similarity measured

Table 1. Percentage of *E. coli* Human Source (HS) and Nonhuman Source (NHS) Isolates Resistant to Antibiotics

Antibiotics	Percent Resistant Strains[a]	
	HS ($n = 407$)	NHS ($n = 358$)
Ampicillin	12.5	4.5
Chlortetracycline	88.5	61.2
Kanamycin	6.9	0.6
Nalidixic acid	2.9	0
Neomycin	3.2	0.3
Oxytetracycline	10.3	3.4
Penicillin G	38.6	37.7
Streptomycin	10.8	2.8
Sulfathiazole	66.3	21.0
Tetracycline	10.3	1.7
Total	94.6	67.6

[a]The *P*-value for HS and NHS isolates was calculated by a two-sided binomial test to determine the significant differences between HS and NHS isolates. All *P*-value for HS and NHS isolates were significant ($P < 0.05$), except penicillin G ($P > 0.05$) (46).

Table 2. Predominant Antibiotic Resistance Patterns of *E. coli* Isolated from Human Source (HS), Nonhuman Source (NHS), and Human* and Animal Feces*

Antibiotics[a]	Percentage with Each Resistant Pattern			
	HS[b]	NHS[c]	Human[d*]	Animal[e*]
C-Su	33.7	15.4	40.0	ND[f]
C-P-Su	14.5	1.7	ND	ND
C	7.9	8.4	16.7	ND
C-P	7.4	28.2	ND	6.9
Su	2.2	1.4	6.7	ND
Other resistant patterns[g]	28.9	12.5	23.3	20.7
Sensitive to all antibiotics	5.4	32.4	13.3	72.4

[a]C, chlortetracycline; P, penicillin G; Su, sulfathiazole.
[b]$n = 407$.
[c]$n = 358$.
[d]$n = 30$.
[e]$n = 29$.
[f]ND: none detected.
[g]Each of the other 59 patterns for HS, 26 patterns for NHS, 6 patterns for human*, and 3 patterns for animal feces isolates contained insignificant number of isolates are not shown.
Note: Asterisk (*) indicates isolates obtained directly from human or animal feces.

by Euclidean distance, is shown in Figures 1 and 2. At an Euclidean distance of approximately 1.8, seven clusters were formed among HS isolates, designated as P_1 to P_7. In contrast, four clusters were observed for NHS isolates N_1 to N_4. A covariance matrix of principal components clearly showed more diversity among HS isolates. In contrast, NHS isolates formed six focal groups. Clusters N_2 and N_4 were found in groups A, B, D; N_1 only in C; and N_3 only in E and F (Fig. 3). These results indicated that the MAR profile of *E. coli* was associated with source.

Discriminant analysis (DA) is a statistical method that can classify entities into groups on the basis of several classification variables (57). DA of MAR profiles was able to correctly classify 82% of HS and 68% of NHS isolates, and the average rate of correct classification (ARCC) was 75%. The ARCC is the average of the percentage of correctly classified isolates. *Escherichia coli* isolates obtained directly from human and animal feces showed that 77% of human and 79% of animal fecal isolates were correctly classified, with an ARCC of 78%. The combination of drugs that had the highest ARCC was ampicillin, chlortetracycline, penicillin G, and sulfathiazole (58).

Parveen and coworkers (59) recently found that DA of MAR patterns of *E. coli* isolates correctly classified 58% of poultry, 55% of beef, and 54% of dairy isolates. Swine isolates were poorly classified, whereby 44 and 25% of swine isolates were misclassified as poultry and dairy isolates. When beef and dairy isolates were pooled as cattle isolates, 80% of the cattle isolates were correctly classified. However, correct classification rates for swine and poultry remained similar. It was observed that omission of any of the antibiotic resistance data resulted in poorer classification of isolates. In addition, significant variations in MAR patterns were noted over the geographical areas for both beef and dairy isolates. In contrast, no significant variation was observed in MAR patterns for swine and poultry isolates, possibly because of the use of common antibiotics over a wide geographical area.

In a separate study, Harwood and coworkers (60) determined the MAR patterns for fecal streptococci and fecal coliforms and found that DA of MAR profiles of *E. coli* isolates correctly classified 54% human, 57% chicken, 54% cow, 95% dog, 73% pig, and 51% wild isolates.

Wiggins and coworkers (61) further demonstrated the utility of antibiotic resistance patterns for differentiating HS and NHS fecal contamination in water, focusing on its application for fecal streptococci. They found that 92% of HS isolates were correctly classified, with an average rate of correct classification (ARCC) of 84%. Wiggins and coworkers (62) also reported that DA of MAR profiles of fecal streptococci may be used for identifying specific animal sources of fecal contamination. They found that when isolates were classified individually, the ARCC ranged from 64 to 78%.

In similar studies, Hagedorn and coworkers (63) analyzed the MAR patterns of 7,058 fecal streptococci isolated from Montgomery County, Virginia, by DA. They found that 86% beef cow, 85% chicken, 87% dairy cow, 84% deer, and 93% human isolates were correctly classified, and the ARCC was 87%.

O-SEROGROUPING

Somatic (O)-serogrouping of bacteria is based on the presence or absence of O-antigenic determinants and their reaction with specific antisera. The O-antigen is a thermostable surface structure found in morphologically smooth strains of Enterobacteriaceae. It constitutes the O-polysaccharide of the bacterial cell wall lipopolysaccharide and is determined by chromosomal genes (64). Several investigators have used this method for discriminating *E. coli* from different sources (42,47). The distribution of various *E. coli* serotypes appears to be different for

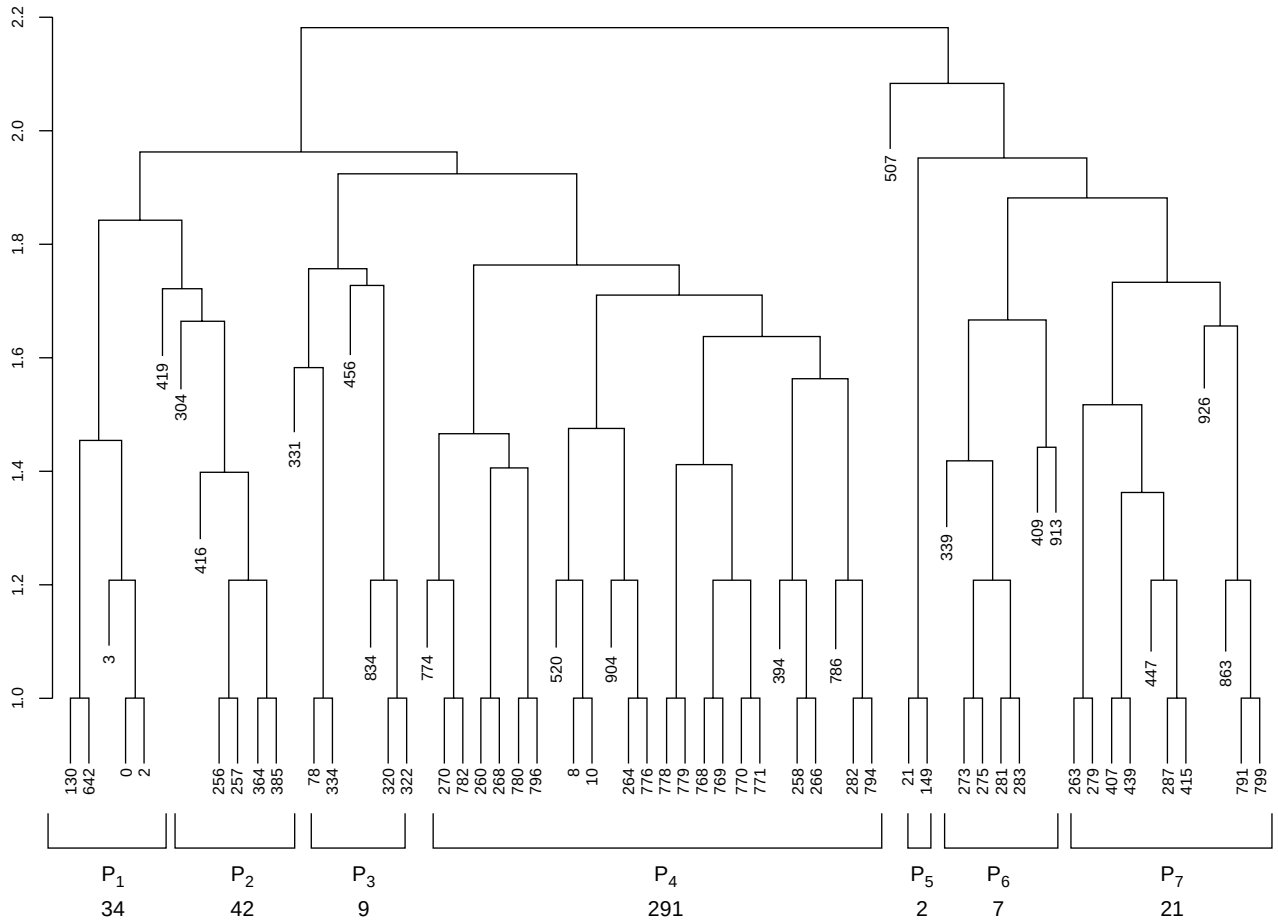

Figure 1. Dendrogram of antibiotic resistance profiles for HS *E. coli* isolates determined by Euclidean metric, average linkage analysis. Clusters were defined at a distance of 1.8. Numbers on dendrogram represent antibiotic resistant pattern, not strain numbers. Numbers at bottom indicate the number of isolates in each cluster; the Euclidean distance is indicated on the left axis (46).

humans and animal isolates (33,65,66), although many human serotypes can also be associated with other organisms (33). Parveen and coworkers (67) serogrouped a total of 100 HS and NHS isolates and found that 77% were typeable. The predominant O-serogroups are shown in Table 3. Human source isolates exhibited 19 serogroups, with 48% belonging to seven serogroups (O2, O20, O28, O79, O148, O153, O159) and the remaining isolates to 12 additional serogroups. In contrast, NHS isolates displayed 26 serogroups, with 36% in seven serogroups (O8, O11, O × 13, O19, O150, O152, O15/143), and the remaining isolates in 19 additional serogroups. Only 2 of 50 NHS isolates shared the seven most frequently detected serogroups for HS isolates ($P < 0.01$). This indicated that there was an association between O-serogroups and the isolate source. From this study, it was determined that the O-serogroups of HS isolates were rarely found in NHS isolates, similar to previously published studies (33). The majority of O-serogroups that we observed for NHS isolates were previously reported for cattle, chicken, and swine (33). These results also agree with studies by Hartley and coworkers (66) who compared the prevalent O-serogroups of diseased animals and humans, and found that most strains associated with diarrhea in pigs and humans were different, whereas those from infantile and piglet diarrhea have many common traits.

In conclusion, serogrouping may be a useful tool for differentiating HS and NHS *E. coli*. Drawbacks include the need for a large panel of antisera, many of which are not commercially available, and also that some isolates cannot be typed.

PULSED-FIELD GEL ELECTROPHORESIS (PFGE)

PFGE is a genotypic method that detects variations in nucleotide sequences of chromosomal DNA. From the literature, it is apparent that PFGE has been mainly used in epidemiological studies, especially for *E. coli* O157:H7 (68,69). Herbein and coworkers (70) reported PFGE profiles for three raccoons, one otter, one goose, three humans, and one muskrat. Within this limited sample size, they found that PFGE profiles of raccoons differed significantly from those of the otter and goose. There was no significant difference between PFGE profiles of raccoons, humans, and a muskrat. Parveen and

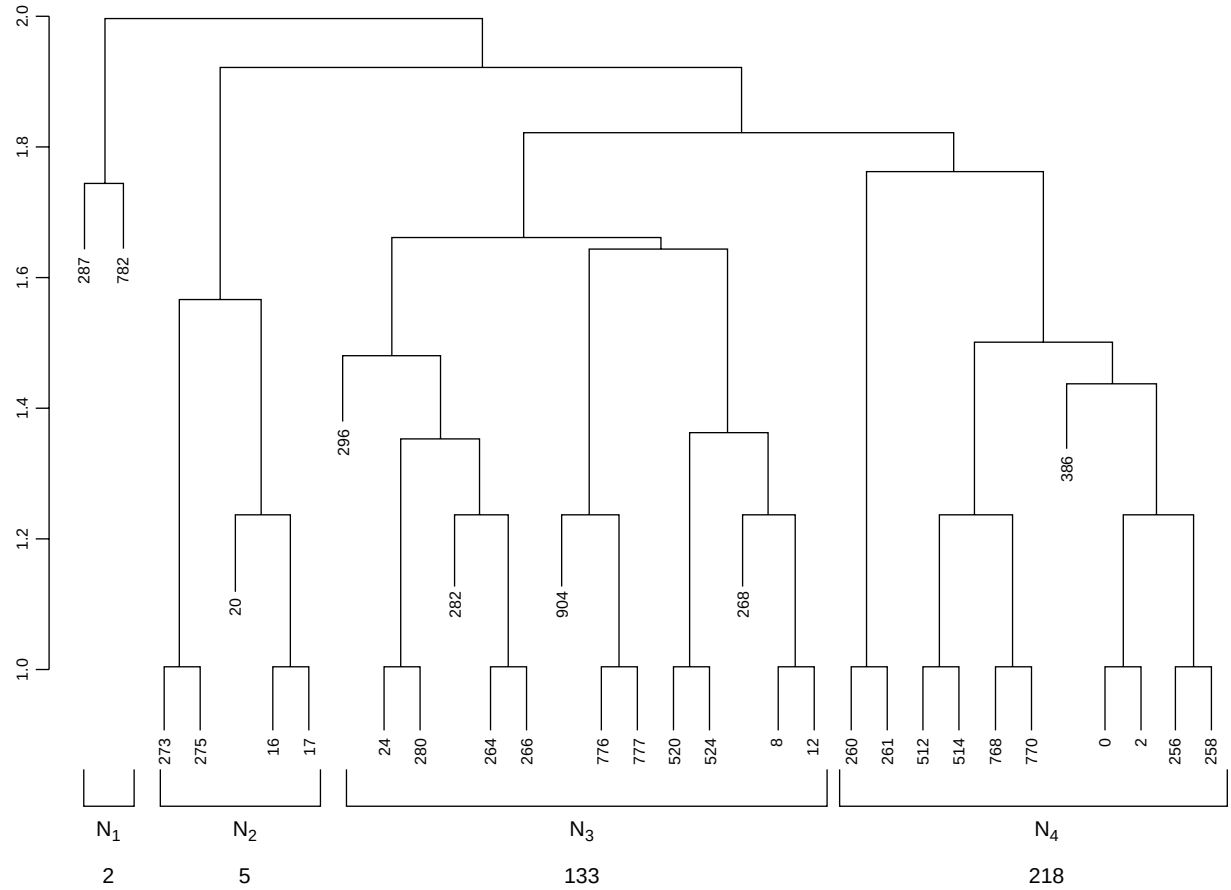

Figure 2. Dendrogram of antibiotic resistance profiles for NHS *E. coli* isolates determined by Euclidean metric, average linkage analysis. Clusters were defined at a distance of 1.8. Numbers on dendrogram represent antibiotic resistant pattern, not strain numbers. Numbers at bottom indicate the number of isolates in each cluster; the Euclidean distance is indicated on the left axis (46).

coworkers (67) analyzed a total of 32 *E. coli* isolates for PFGE profile, and found that HS and NHS isolates formed 9 and 18 PFGE profiles, respectively. There was no association between PFGE profile and isolate source. PFGE profiles of *E. coli* were not able to differentiate HS and NHS isolates. This was not surprising as PFGE detects small sequence differences that may not associate with a specific bacterial characteristic, such as host source.

RIBOTYPING

Ribotyping, a relatively recent development in DNA analysis, is the study of the distribution of the restriction sites throughout rRNA genes. It has been successfully used to characterize microorganisms belonging to different genera (55,71,72) and research has been done to differentiate *E. coli* source by ribotype (RT). Samadpour and Chechowitz (73) were able to identify 71% of HS and NHS *E. coli* isolates using ribotyping. Simmons and Herbein (74) used ribotyping for differentiating HS and NHS *E. coli* for a limited number of isolates. Hartel and coworkers (75) used an automated instrument to ribotype *E. coli* isolates from two small streams in Georgia. They were able to discriminate among ribotypes of *E. coli* from a pasture stream, a wooden stream, and cow manure, but the discrimination was insufficient within a specific site.

Parveen and coworkers (76) isolated a total of 238 *E. coli* from HS and NHS in the ANERR, and directly from human and animal feces, and tested the isolates for RT. HS and NHS isolates showed 41 and 61 RT profiles, respectively. At approximately a 50% similarity index, HS and NHS isolates demonstrated four clusters, with the majority of HS and NHS isolates located in two clusters; isolates obtained directly from human and animal feces also grouped within these clusters. DA of RT profiles showed that 97 and 67% of NHS and HS isolates, respectively, were correctly classified. The ARCC for HS and NHS isolates was 82%. DA of *E. coli* isolates obtained directly from human and animal feces showed that 67 and 100% of human and animal fecal isolates were correctly classified, respectively, and the ARCC was 84%.

In another study, a total of 287 *E. coli* strains isolated from humans, cattle, pigs, horses, chickens, turkeys, migratory geese, and dogs were analyzed by RT. It was

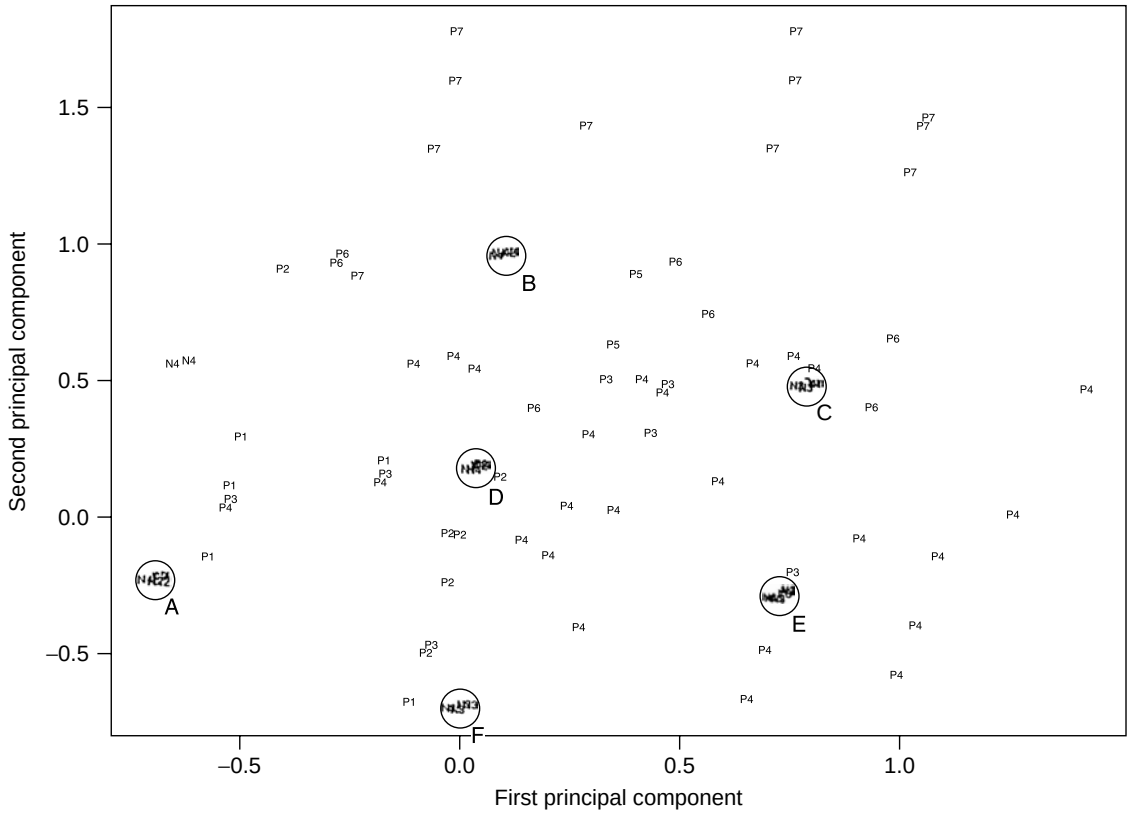

Figure 3. Two-dimensional plot of antibiotic resistance patterns by principal component analysis. Circles represent groups A to F. The specific cluster and associated number of isolates are: A, $N_4 = 117$, $N_2 = 3$; B, $N_4 = 59$, $N_2 = 1$; C, $N_1 = 2$, $N_3 = 7$; D, $N_4 = 36$, $N_2 = 1$; E, $N_3 = 113$; F, $N_3 = 13$.

Table 3. O-Serogroups of Human Source (HS) and Nonhuman Source (NHS) Isolates

	Number (%) of *E. coli* Isolates	
O-Serogroups	HS ($n = 50$)	NHS ($n = 50$)
2	3 (6)	1 (2)
8	1 (2)	3 (6)
11	1 (2)	2 (4)
x13	0	2 (4)
19	0	5 (10)
20	2 (4)	0
28	2 (4)	0
79	7 (14)	1 (2)
148	3 (6)	0
150	0	2 (4)
152	0	2 (4)
15/143	0	2 (4)
153	2 (4)	0
159	5 (10)	0
Others[a]	10 (20)	17 (34)
MR	4 (8)	0
NT	10 (20)	12 (24)

[a] 10 and 17 serogroups of HS and NHS isolates, respectively that contained only one isolate are not shown.

Note: MR, multiple reaction; NT, nontypeable; x, not accepted by World Health Organization. One NHS isolate was autoagglutinable.

found that DA of RT were correctly classified for 95% HS and 99% NHS strains. The ARCC of *E. coli* RT from human, cattle, pig, horse, dog, chicken, turkey, and goose were 93, 74, 66, 49, 55, 96, 81, and 76%, respectively. It was also observed that the classification accuracy was best when analysis was limited to three host sources (77). It can be concluded that DA of RT profiles is a useful method to identify HS and NHS fecal contamination, and potentially facilitates management practices.

Recently, Dombek and coworkers (78) reported that rep-PCR fingerprint technique can be used to differentiate human and animal sources of fecal pollution. In rep-PCR DNA fingerprinting, PCR amplification of the DNA between adjacent repetitive extragenic elements is used to obtain strain specific DNA fingerprints, which can be easily analyzed with pattern recognition computer software. The animal isolates included in this study were two types of waterfowl (geese and ducks) and common farm animals (cows, pigs, sheep, and chickens). They used two primers (BOX and REP) to generate DNA fingerprints from *E. coli* strains isolated from human and animal sources. The DNA fingerprints obtained with one primer (BOX) were more effective for grouping *E. coli* strains than the DNA fingerprints obtained with another primer (REP). A statistical analysis (Jackknife) of DNA fingerprints obtained with one primer (BOX) correctly

classified 83% human, 81% of goose, 78% of duck, 90% of sheep, 81% of pig, 100% of chicken, and 100% of the cow strains.

CONCLUSION

We have reviewed the traditional and modern methods used to detect and differentiate sources of fecal contamination in aquatic and other environments. Each method has advantages and disadvantages. For example, phenotypic methods are influenced by environmental factors, and they may vary in different geographical areas, and over time. In contrast, genotypic methods are more stable and are less influenced by environmental factors. However, the latter methods can be expensive, time-consuming, and laborious. In addition, there is the possibility of low level transfer of fecally derived organisms among different animal species.

There is probably no ideal method that is suitable for all environments. Research is in progress in many laboratories throughout the world to develop an ideal method for differentiating sources of fecal contamination. Hopefully, biotechnological advances will result in rapid, simple and reliable methods to detect sources of fecal pollution in aquatic as well as other environments.

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FECAL STREPTOCOCCI/ENTEROCOCCI IN AQUATIC ENVIRONMENTS

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INTRODUCTION: DEFINITION AND TAXONOMIC POSITION

The term *fecal streptococci* has been used to describe a group of taxonomically diverse streptococci associated with the gastrointestinal tract of humans and animals (1). Thiercelin (2) described the most common organisms isolated from human stool in 1899 as "enterococcus" to emphasize their intestinal origin. A few years, later Andrewes and Horder (3) described the species *Streptococcus faecalis* from various infections and, in 1919, Ora-Jansen (4) reported the species *S. faecium* isolated from human and animal feces. Sherman (5) indicated that the term enterococcus had been used to refer to different microorganisms ranging from the broad definition of any fecal streptococcus to a narrow definition of organisms that appeared to be identical to *S. faecalis*. Sherman proposed a classification scheme that separated streptococci into four divisions, namely, pyogenic, viridans, lactic, and enterococcus. The latter term was used for organisms that grew at both 10 °C and 45 °C, in 6.5% NaCl, at pH 9.6, reduced 0.1% methylene blue in milk, survived at 60 °C for at least 30 minutes, and were able to use esculin. Sherman's classification also correlated with the serological scheme proposed by Lancelfield (6) for the identification of streptococci. In that scheme, the enterococci reacted with group D antisera, whereas the pyogenic streptococci reacted with groups A, B, C, E, F, or G, and the viridans streptococci were ungroupable. However, streptococci classified by Sherman scheme as viridans streptococci, such as *S. bovis* and *S. equinus*, have shown to react with group D antiserum. On the other hand, some strains of enterococci were found to react not only with Lancefield's group D antiserum but often also with group Q, such as *S. avium* (7). The terms fecal streptococci, enterococci, and group D streptococci have been used indistinctively in the literature (1). Taking into account Sherman's criteria and Lancefield antigens, Clausen and coworkers (8) proposed to consider the following species as fecal streptococci: *Streptococcus faecalis*, *S. faecium*, *S. durans*, *S. avium*, *S. bovis*, *S. equinus*, *S. mitis*, and *S. salivarius*. The latter two were described as inhabitants of the nasopharyngeal tract and usually grouped with the "oral streptococci" (9). Bridge and Sneath (10) described a new group D streptococcus from chickens and it was designated *S. gallinarum*.

Kalina (11), on the basis of physiological characters, suggested that a new genus named *Enterococcus* should be proposed to include the enterococcal streptococci

including *S. faecalis* and *S. faecium* and the subspecies of these two taxons. In fact, the genus *Enterococcus*, which included *Enterococcus faecalis* and *E. faecium*, was separated from *Streptococcus* on the basis of DNA hybridization data by Schleifer and Klipper-Bäz (12). These changes were, however, not taken into account in the 1986 edition of *Bergey's Manual* (13), but they did appear in the 9th edition of *Bergey's Manual of Determinative Bacteriology* (14). The use of nucleic acid relatedness has clarified and expanded the classification scheme for enterococci. As reflected in Table 1, it has been proposed that 19 species should be included in the genus *Enterococcus* (1,15–17). In the 9th edition of *Bergey's Manual of Determinative Bacteriology* (14), only 16 species within the genus *Enterococcus* are recognized, excluding *E. columbae*, *E. flavescens*, and *E. sulfureus*.

The taxonomy of *Enterococcus* or *Streptococcus* genera has not yet been fully resolved. The terms *fecal streptococci*, *enterococci*, or *intestinal enterococci* embraces species of the genera *Enterococcus* and *Streptococcus*, although there is no complete agreement about which species should be included under these terms (Table 2; 1,8,18–22). In practice, detection and enumeration will be the conventional approach when studying these organisms in aquatic environments and therefore this disagreement may not be very important. In water samples influenced by human fecal pollution, the predominant species identified are *E. faecium*, *E. faecalis*, and *E. durans* (23–26). Therefore, the terms fecal streptococci, enterococci, intestinal enterococci, and Enterococcus group when used in the literature to refer to indicators of fecal pollution can be considered in practice synonymous. Phenotypic characterization carried out in the past may not match new taxonomic descriptions (1), and, therefore, this should be taken into account when reading the comparisons made throughout this chapter.

IDENTIFICATION

The genus *Enterococcus* consists of gram-positive (G + C content of 37 to 45 mol %), facultatively anaerobic organisms, which are ovoid in shape and may appear on smear in short chains, in pairs, or as single cells. Like streptococci, these organisms do not have cytochrome enzymes and are thus catalase negative, although some strains do produce pseudocatalase. Hydrolysis of L-pyrrolidonyl-ß-naphthylamide (PYR) and production of leucine-aminopeptides (LAP) are characteristic features coincident with group A streptococci but not with other streptococci (27). Most strains in the genus *Enterococcus* possess the characteristics of Sherman's scheme, but there are several different characteristics among enterococcal species (Table 3). All enterococci are able to hydrolyze esculin in the presence of bile salts, and so this test has been used for the rapid discrimination between *Enterococcus* and *Streptococcus* (28). However, several other gram-positive organisms can also yield a positive reaction in some of these tests, and for this reason Facklam and coworkers (29) designed a battery of tests for the complete discrimination of enterococci, including gas from glucose and vancomycin susceptibility testing (Table 4).

For routine monitoring of water samples, it will not be necessary to identify enterococci to the species level, although this may be necessary in other instances, for example, in many clinical cases, for epidemiological surveillance, or for establishing the predominance of the species in a given water environment. A number of

Table 1. Proposed Species of the Genus *Enterococcus* and Their Habitat (1,16)

Species	Previous Name in *Streptococcus*	Habitat
E. avium	*S. avium*	Avian, canine, and human GIT
E. casseliflavus	*S. casseliflavus*	Plants, soil, and chicken feces
E. cecorum	*S. cecorum*	GIT of chicken
*E. columbae**	ND	GIT of pigeon
E. dispar	ND	Human specimens and feces
E. durans	*S. durans*	Milk, dairy products
E. faecalis	*S. faecalis*	GIT of humans and animals
E. faecium	*S. faecium*	GIT of humans and animals
*E. flavescens**	ND	Human clinical specimens
E. gallinarum	*S. gallinarum*	Chicken feces
Enterococcus hirae	ND	Chicken crops and feces, GIT of cattle, pigs, canines, horses, sheep, goats, and rabbits
E. malodoratus	*S. faecalis subsp. malodoratus*	Cheese and unpasteurized milk
E. mundtii	ND	Plants, soil, and GIT of pigs, cattle, horses
E. pseudoavium	ND	Bovine mastitis
E. raffinosus	ND	Human infection
E. saccharolyticus	*S. saccharolyticus*	Cows
E. seriolicida	ND	Yellow-tail fish, subclinical mastitis
E. solitarius	ND	Rumen and clinical human samples
*E. sulfureus**	ND	Plants

*Species excluded in the *Bergey's Manual of Determinative Bacteriology* (14);
Note: New description (ND); gastrointestinal tract (GIT).

Table 2. Proposed Species to Be Included in the Group Fecal Streptococci/Enterococci

Species	Clausen (8) Sinton (19)	Devriese (18) Leclerc (1)	Dionisio and Borrego (21) Borrego and Figueras, (22)	Anon. (20)
Enterococcus				
faecalis	X	X	X	X
faecium	X	X	X	X
hirae		X	X	X
durans	X	X	X	X
cecorum		X	X	
columbae		X		
avium	X	X	X	
gallinarum		X	X	
Streptococcus				
bovis	X	X	X	
equinus	X	X	X	
suis		X	X	
alactolyticus		X	X	
hyalointestinalis		X	X	
intestinalis			X	
acidominimus			X	
mitis	X			
salivarius	X			

Table 3. Test Differentiating Between Enterococcal Species (14,15)

Test	Typical Results	Exceptions
Lancefield group D	+	*E. cecorum*, *E. seriolicida*, *E. dispar*, *E. saccharolyticus*, *E. sulfureus*, *E. columbae*, and many strains of *E. avium*
Growth at 45 °C	+	*E. dispar*, *E. malodoratus*, *E. sulfureus*
Growth at 10 °C	+	*E. cecorum*, *E. columbae*
Growth at 6.5% NaCl	+	*E. cecorum*, *E. columbae*, *E. pseudoavium*
PYR	+	*E. cecorum*, *E. columbae*, *E. saccharolyticus*
Motility	−	*E. casseliflavus*
Voges-Proskauer test	+	*E. saccharolyticus*, *E. malodoratus*, *E. avium*, and many strains of *E. solitarius*
ß-glucuronidase	−	*E. cecorum*
Alkaline phosphatase	−	*E. cecorum*, *E. columbae*
Arginine dihydrolase	+	*E. cecorum*, *E. pseudoavium*, *E. avium*, *E. saccharolyticus*, *E. sulfureus*, *E. columbae*, *E. malodoratus*, *E. raffinosus*
Hippurate test	−	*E. gallinarum*
Pyruvate test	−	*E. avium*, *E. solitarius*
Pigment production	−	*E. casseliflavus*, *E. mundtii*
Acid production from:		
Sucrose	+	*E. pseudoavium*, *E. seriolicida*, *E. durans*, and certain strains of *E. faecium*
Adonitol	−	*E. avium*, *E. pseudoavium*, *E. malodoratus*, *E. raffinosus*
Glycogen	−	*E. gallinarum*, *E. cecorum*, *E. columbae*
Lactose	+	*E. seriolicida*, *E. solitarius*, and asaccharolytic variants of *E. faecalis*
L-Rhamnose	−	*E. avium*, *E. casseliflavus*, *E. malodoratus*, *E. mundtii*, and certain strains of *E. faecalis*
D-Xylose	−	*E. gallinarum*, *E. casseliflavus*, *E. mundtii*, and certain strains of *E. malodoratus*
D-Arabitol	−	*E. avium*, *E, pseudoavium*, *E. malodoratus*, *E. raffinosus*, *E. saccharolyticus*
Inulin	−	*E. gallinarum*, *E. casseliflavus*, *E. saccharolyticus*, *E. cecorum*, *E. columbae*
Mannitol	+	*E. cecorum*, *E. columbae*, *E. dispar*, *E. durans*, *E. hirae*
L-Sorbose	−	*E. avium*, *E. pseudoavium*, *E. malodoratus*, *E. raffinosus*
Raffinose	−	*E cecorum*, *E. casseliflavus*, *E. malodoratus*, *E. mundtii*, *E. gallinarum*, *E. hirae*, *E. raffinosus*
Sorbitol	−	*E. avium*, *E. pseudoavium*, . *E. raffinosus E. mundtii*
L-Arabinose	−	*E. avium*, *E. casseliflavus*, *E. faecium*, *E. raffinosus*, *E. gallinarum*, *E. solitarius*

biochemical traits facilitate differentiation of enterococcal species, such as the ability to grow in the presence of tellurite, reduction of tetrazolium salts, acid production of several compounds (D-tagatose, sorbitol, glycerol, melibiose, and L-arabinose), motility, pigment production, hydrolysis of arginine and PYR (15,30,31). Panosian and Edberg (32) have recommended the detection of ß-D-glucosidase activity in the presence of 2.5% sodium deoxycholate, α-D-galactoside, and PYR for the rapid identification of *S. bovis*, *S. equinus*, *S. pneumoniae*,

Table 4. Tests Used to Differentiate Selected Gram-Positive Organisms (29)

Tests	% Positive					
	Enterococcus	*Lactococcus*	*Aerococcus*	*Pediococcus*	*Leuconostoc*	*Lactobacillus*
Gas from glucose	<1	0	0	0	100	50
Vancomycin resistance	<1	0	0	100	100	90
Reaction with streptococcal group D antiserum	80	0	0	95	35	25
Bile-esculine hydrolysis	99	75	60	100	90	50
Hydrolysis of PYR[a]	100	69	100	0	0	7
Growth in 6.5% NaCl	100	56	100	35	60	40
Growth at 45 °C	99	25	0	83	0	60
Growth at 10 °C	85	100	0	4	75	100

[a] PYR: Pyrrolidonyl-ß-naphthylamide.

Enterococcus spp., and the viridans streptococci. More recently, Devriese and coworkers (33) described the usefulness of a test based on acid production from methyl-α-D-glucopyranoside for differentiating *E. gallinarum* and *E. casseliflavus* from *E. faecalis* and *E. faecium* as the former species are unable to produce acid from this substrate. Manero and Blanch (34) provided a six-step biochemical key for species identification within the genus *Enterococcus*, which is easier to perform than previously proposed keys (15,35), and it allows the identification of all known species (Table 1).

Clinical laboratories generally use commercially miniaturized systems, such as the API 20 S (bioMérieux), the Vitek System GP1 card (bioMérieux), the Rapid Strep System (API), or the RapID STR System (Innovative Diagnostic Systems). Most of these test systems provide an identification in about four hours. Although Vitek and API 20S have proven useful for the identification of clinical enterococci to the species level (36), they can produce erroneous identification for environmental enterococcal strains, and the results of conventional tests and miniaturized tests do not always agree (37,38).

Commercially available chromogenic tests based on the pyrrolidonyl carboxylic acid or pyroglutamic acid conjugates of ß-naphthylamine have been evaluated comparatively (39). The chromogenic PYR tests are included in API 20 STREP kit, RapID STR system, MicroScan Conventional Gram-Positive Panels (Dade Intl.), Pasco Gram-Positive ID Panel (Difco Lab.), BBL Crystal Rapid Gram-Positive ID Panel (Becton Dickinson & Co.), and Vitek II System (bioMerieux-Vitek, Inc.).

A rapid nonradioactive DNA probe (AccuProbe Enterococcus DNA culture confirmation probe, Gen-Probe, Inc.) has been used for positive identification of enterococci because most species react positively with the probe with the exceptions of the strains of *E. cecorum*, *E. columbae*, *E. seriolicida*, *E. solitarius*, and *E. saccharolyticus* (31). However, other nonenterococcal species, such as *Vagococcus fluvialis*, have been shown to react positively with this AccuProbe genetic probe (40).

HABITAT AND CLINICAL SIGNIFICANCE

Enterococci are ubiquitous and can be found as free-living in soil, on plants, in dairy products, and as part of the normal microbiota of the gastrointestinal tract of humans, other mammals, and birds. They have been implicated in outbreaks of food-borne illnesses, and they can possess both a beneficial and detrimental role in foods (41). Enterococci are also used as probiotics to improve the microbial balance of the intestine, or as a treatment for gastroenteritis in humans and animals (41). In humans, enterococci are found in the feces of most healthy adults (more than 95%) at concentrations ranging from 10^5 to 10^7 colony forming units (CFU) per gram of feces (42,43), *E. faecalis* being more common and found in higher numbers than *E. faecium* (42).

Enterococci are less commonly found in other human sites such as vaginal tract (44) and oral cavity (45), and results are sometimes quite variable. Campbell and coworkers (46) found higher rates of enterococcal carriage among long-term hemodialysis patients and cardiac patients, *E. faecium* being outnumbered by *E. faecalis* isolates.

Several authors have thoroughly reviewed the variety of human infections in which enterococci are involved (30,47,48). These organisms most commonly infect the urinary tract, bloodstream, endocardium, biliary tract, burn wounds, indwelling devices, abdomen, and can produce enterococcal bacteremia. In addition, several infections have occasionally been reported in other organs and tissues, such as the central nervous system, lung, soft tissues, paranasal sinuses, ear, eye, and periodontal tissues.

Between 80 and 90% of clinical enterococcal isolates have been classified as *E. faecalis*, whereas 5 to 10% will correspond to *E. faecium* (31,49). Other enterococcal species, including *E. avium*, *E. casseliflavus*, *E. durans*, *E. gallinarum*, *E. hirae*, *E. malodoratus*, *E. mundtii*, *E. raffinosus*, and *E. solitarius*, are infrequent causes of human infections (50). Enterococci have become major nosocomial pathogens because they are the fourth leading cause of hospital-acquired infections and the third leading cause of bacteremia in the United States (51,52). Increases

in enterococcal infections, especially in hospitals, are associated with virulence factors (Table 5), which according to Jett and coworkers (49) are related to: (*1*) adherence to host tissues, (*2*) invasion and abscess formation, (*3*) factors potentially relevant to modulation of host inflammatory responses, and (*4*) potentially toxic secreted products.

Recent attention has focused on enterococci because of their remarkable and increasing resistance to antimicrobial agents. Antimicrobial resistance can be divided into two general types, namely, inherent and acquired. The genes for intrinsic or inherent resistance appear to reside in the chromosome, whereas acquired resistance results either from a mutation in the existing DNA or acquisition of new DNA from plasmids or transposons (30). The various intrinsic traits expressed by enterococci include resistance to semisynthetic ß-lactams, cephaloporins, low levels of aminoglycosides, and low levels of clindamycin. Examples of acquired resistance include resistance to chloramphenicol, erythromycin, tetracycline, vancomycin, and high levels of clindamycin and aminoglycosides. Resistance to high levels of penicillin and resistance to fluoroquinolones are not known to be plasmid or transposon-mediated and are presumably due to mutations, as in other microorganisms such as *Salmonella* (30,53).

Recent data from the Centers for Disease Control and Prevention indicate that up to 50% of *E. faecium* clinical strains are resistant to vancomycin (54). Vancomycin-resistant strains have disseminated throughout the United States and Europe in recent years, many of these organisms being also highly resistant to ß-lactams and aminoglycosides (55). Mortality rates for patients with vancomycin-resistant enterococcal bacteremia are extremely high (55). Vancomycin-resistant enterococci (VRE) have been isolated in stools of asymptomatic individuals who have neither been hospitalized nor received antibiotics (56,57). If asymptomatic fecal carriers of VRE are admitted to hospital, they may act as the source of hospital outbreaks (56). VRE have also been found in sewage, stool of healthy farm animals, animal products, and foodstuffs (56,57). VRE may also enter the food chain by the use of glycopeptide antibiotics in animal feed (56–58). Although enterococci have been traditionally regarded as low-grade pathogens, they now have to be seen as important opportunistic pathogens, especially when VRE are involved.

Table 5. Virulence Factors for Enterococci (49)

Factor	Observed Activities
Cytolysin	— Lytic toward gram-positive bacteria and eukaryotic cells
	— Decreased LD_{50}
	— Increase mortality in animal models
Aggregation substance	— Binding of donor and recipient cells in pheromone mating response
	— Increase the adherence to eukaryotic cells
	— Increase animal mortality in conjunction with cytolysin
Pheromones	— Chemoattractant for neutrophils
Lipoteichoic acid	— Stimulation of cytokine production
	— Binding ligand for aggregation substance in pheromone mating response
Protease (gelatinase)	— Zinc-endopeptidase
Hyaluronidase	— Mucopolysaccharidase
AS-48	— Bacteriocin with activity against gram-positive and gram-negative bacteria

FECAL POLLUTION OF NATURAL WATER ENVIRONMENTS

Concept of Indicator Organisms

Natural waters are subject to important changes in their microbial quality because of occasional discharges of wastewater as a result of human activity or stormwater runoff. Sewage effluents contain a wide range of pathogenic microorganisms that may pose a health hazard to the human population if contaminated water is used for drinking, shellfish growing, and/or recreation. The density and variety of these pathogens are related to the size of the population, the seasonal incidence of the illness, and the dissemination routes of pathogens (22,59).

The presence of enteric pathogens in drinking and recreational water is of great concern (59–61). Therefore, it is extremely important to determine the microbiological safety of these waters by analyzing them for the presence of specific pathogens of concern. However, a wide variety of microorganisms have been shown to be involved in waterborne disease outbreaks, and their presence in polluted waters is intermittent and/or in low concentrations; thus, it would be impractical to look for every pathogen potentially present in water samples (62). In addition, the methods for the detection and enumeration of pathogens are frequently complex and expensive, involving concentration processes and subsequent selective enrichment or amplification by molecular techniques. Additionally, the recovery efficiency of the procedures is low and these techniques are limited to specialized research laboratories (63). Alternative organisms that are consistently present in fecal material survive reasonably well in water compared with pathogens, and are easier to detect, and have therefore been widely used as fecal pollution "indicators."

Indicator organisms must fulfill the following requirements (64):

1. Indicators should also be present in feces, sewage, and sewage-polluted water whenever the pathogens are present, and should be absent in water without fecal pollution.
2. The levels of indicators should have some direct relationship to the density of pathogens in aquatic environments, and should be proportional to the extent of pollution.
3. The survival rate of indicators in water must be at least similar to that of pathogens. In addition, the resistance of indicators to environmental inactivation factors and disinfectants should be at least similar to that of the pathogens.

4. Indicators must be detectable by a simple, inexpensive, accurate, and rapid laboratory methodology.
5. Indicators should be nonpathogenic and applicable to all types of water samples that require a monitoring program.
6. Their characteristics must be constant.
7. Indicators must not grow and multiply in water.

Mossel (65) defined the term *model organism*, which refers to two different functions, index and indicator. "Index organisms" are related, directly or indirectly, either to the health hazards or to the pathogen presence, and they must fulfill three types of criteria, namely, similar ecology, similar resistance and persistence, and simple methodology. On the other hand, "indicator organisms" are related only to the effects of treatment processes or control of water quality. The requirements for "indicator organisms" are less restrictive than those for an index organism; their resistance to the environment must be only similar to that of pathogens and they require more simple laboratory methodologies.

The use of "index" or "indicator" organisms to assess the microbiological and sanitary quality of water is well established and has been practiced for almost a century. The most widely used indicator organisms are total coliforms, fecal coliforms (and *Escherichia coli*), and fecal streptococci (or enterococci) (66). However, it is widely acknowledged that no single indicator group or species can be expected to provide a satisfactory indication of disease risk in all situations (67), and most of them are more susceptible to adverse environmental conditions than pathogenic microorganisms are, including viruses (68).

Accordingly, there is an increasing number of investigations on alternative indicators of water quality. These include sulfite-reducing clostridia (*Clostridium perfringens*) (69,70), *Staphylococcus aureus* (71,72), *Pseudomonas aeruginosa* (73,74), bifidobacteria (75), several groups of bacteriophages (76–80), and fecal sterol biomarkers (81). The advantages and disadvantages of classical and alternative indicators have been broadly discussed in the recent literature (66,82).

Fecal Streptococci/Enterococci as Indicator Organisms

At the beginning of the twentieth century Houston (83) noted the abundance of streptococci in sewage and proposed that they might be useful for detecting contamination of water by human feces. Since then, fecal streptococci have received widespread acceptance as useful indicators of fecal pollution in natural aquatic ecosystems, on the basis of the following characteristics: (*1*) they show a high and close relationship with health hazards associated with bathing in marine and freshwater environments, mainly for gastrointestinal symptoms; (*2*) they are not as ubiquitous as coliforms are; (*3*) they are always present in feces of warm-blooded animals; (*4*) they are unable to multiply in sewage-contaminated waters; and (*5*) they die more slowly than coliforms in seawater, and their persistence patterns are similar to those of potential waterborne pathogenic bacteria or viruses (19,84–88).

To determine the usefulness of fecal streptococci as indicators of fecal pollution, it is important to ascertain whether they exist in nature only in association with feces, or whether they can persist and multiply in the environment independently of any input of fecal pollution. In animal feces, fecal streptococci generally outnumber fecal coliforms, although the overall incidence and concentration appears to differ markedly among animal species (1). In contrast, streptococcal concentrations in human feces (about 3×10^6/g) are generally lower than those for fecal coliforms, which are typically around 1.3×10^7/g (89). It appears that enterococci that dominate fecal streptococci in human effluents are present in municipal sewage in concentrations 10,100 times lower than *E. coli* (90). Although fecal streptococci are present in raw (1×10^4/mL) and treated (2×10^2/mL) wastewater at lower levels than *E. coli*, the numbers of fecal streptococci consistently exceed those of both bacteria and viral pathogens (88). The resistance of fecal streptococci to disinfection processes is about twice that exhibited by *E. coli* (88).

Numerous old studies have reported the occurrence of fecal streptococci, including enterococci, on plants and insects. Geldreich and Kenner (91) found that 46.1% of plant fecal streptococci were enterococci, 9% were *S. bovis* or *S. equinus*, and 43.9% were atypical strains. The occurrence of fecal streptococci on plants may be due to three possible causes (8): (*1*) as a result of direct contamination from warm-blooded animals and /or insects; (*2*) streptococci are temporary residents on plants, where they are capable of limited reproduction; and (*3*) they exist on plants in a truly epiphytic relationship, they spread by seeds, and are able to reproduce on the growing plant. Many of the enterococci isolated from plants have been reported to differ from strains characteristics of warm-blooded animals; thus, Geldreich and coworkers (92) detected starch hydrolysis in 37.7% of enterococci isolates from plants, whereas this characteristic was uncommon in strains recovered from humans (93).

Fecal streptococci have been recovered from a variety of insects, including *E. faecalis* and *E. faecium*, but not *S. bovis* and *S. equinus* (92,94). These authors suggested that fecal streptococci are not common residents of insects but are present as a result of chance contacts with streptococci in the environment. Clausen and coworkers (8), on the basis of similarities between plant and insect strains, suggested the existence of frequent exchange of organisms between both sources, whereas according to Leclerc and coworkers (1), the enterococci populations are most likely carried to the plants by insects. These authors indicated, however, that the studies on which these conclusions are based were performed before the genus *Enterococcus* was redefined and rearranged and that similar studies using modern taxonomic concepts are needed to validate the conclusions.

Stormwater runoff can influence the microbiological quality of surface water and, in fact, streptococcal numbers in natural water can be strongly related to their incidence in soil. Medreck and Litsky (95) found enterococci in only 2.2% of unpolluted soil samples, but Geldreich and Kenner (91), in a study of fecal streptococci species from agricultural soils, found that 35% were *S. faecalis* sub sp. *liquefaciens*, 63% were other *S. faecalis* strains, and 2%

were *S. bovis* and *S. equinus*. There are no clear data in the recent literature about the influence that these naturally occurring enterococci may have on the final concentration of fecal streptococci found in water samples.

Applications of Fecal Streptococci to Distinguish Between Human and Animal Sources

In studies of contaminated aquatic systems, it is often desirable to differentiate between humans and animals as the probable source of fecal pollution because this would assist in the overall management of microbial water quality (96). Although both fecal coliforms (FC) and fecal streptococci (FS) are present in high numbers in the feces of humans and animals, the ratio of FC to FS has been proposed as an indicator of the origin of the contamination by Geldreich and Kenner (91). These authors indicated that a ratio greater than 4 is characteristic of human fecal contamination, whereas a ratio less than 0.7 suggests animal wastes. These authors recommended that the FC to FS ratio should be considered indicative of a particular source only if the sample is collected within 24 hours of the fecal material entering the watercourse. However, this would not take into account the time between defecation and discharge, and in practice it is usually impossible to determine the age of fecal pollution in water (97). Poucher and coworkers (98) have shown that even in the first few hours after collection, the FC to FS ratio is not constant, even for samples of the same origin. Some reports have indicated that the FC to FS ratio possesses a limited value as a means of differentiating human from animal fecal pollution (66,98–100), on the basis of the differential die-off kinetics either of the two bacterial groups or of individual species within the fecal streptococcal group (101). Thus, die-off rates of fecal coliforms and fecal streptococci in natural water is *S. bovis-equinus* group greater than FC and FC greater than *E. faecium-faecalis* group (102). Feachem (103) argued that the differential rates of die-off of fecal streptococcal species, in fact, enhanced the value of the FC to FS ratio in distinguishing among pollution sources, because a predominantly human source (dominated by enterococci) should exhibit an initially high FC to FS ratio (>4), which falls during storage, whereas nonhuman sources (dominated by *S. bovis* and *S. equinus*) should exhibit an initially low FC to FS ratio (<0.7) with subsequent rises.

Wheater and coworkers (104) observed that not all animals maintain a FC to FS ratio of less than one. They determined that although FC to FS ratios in sewage effluents (human source) were invariably more than one, they did not always exceed four, and that ratios varied depending on the enumeration methodology used. Borrego and coworkers (99) concluded that FC to FS ratio does not give an absolutely trustworthy indication of the type of fecal pollution or its origin. It is more related to the pollution degree of the water sample and to the distance from the pollution source, because the index ≥ 4 can be found at the points near the pollution source, and ≤ 0.7 at more distant points.

Although many researchers who have examined fecal streptococcal population in human and animal fecal stools have commented on the implications of their findings with respect to pollution source identification, there have been relatively few studies designed to test this approach in aquatic environments. One of the difficulties associated with such field studies is the broad spectrum of streptococcal and enterococcal biotypes likely to be encountered. Thus, biochemical classification of fecal streptococci from the Nile River showed that about 78% of the isolates were distributed among atypical or unclassifiable categories (105). Bayne and coworkers (106) detected in a sewer outfall predominantly *E. casseliflavus* (formerly *S. faecium* var. *casseliflavus*), a species that is associated with plants rather than animals; and Wheater and coworkers (104) found that *S. bovis* was the predominant organism in samples from above a sewer outfall but the species was not recorded in sewage samples.

Rutkowski and Sjogren (107) characterized various pollution sources using the medium M2. In human sources (sewage treatment plants), the enterococci dominated at 61.5%, the group D viridans enterococci followed at 29.4%, and the oral streptococci ranged from 2 to 7.6%. In nonhuman sources, the group D nonenterococci constituted 93.3% of the total streptococcal population, with the remainder being enterococci. No oral streptococci (*S. mitis*, *S. salivarius*, and *S. sanguis*) were found in samples from animal sources.

The occurrence of enterococci in cattle dung water from the basins of animal farms was determined by Laukova and coworkers (108). The enterococcal predominant species were *E. faecium*, *E. faecalis*, and *E. durans* (average 36.5%) compared with *E. casseliflavus* (19.2%) and *E. avium* (1.9%). The predominance of human species should be explained by the fact that *E. faecium* and *E. faecalis* produced heat-stable bacteriocins, which exert a strong inhibitory effect on other enterococci (109,110).

Wiggins (111) used discriminant analysis of antimicrobial resistance patterns in fecal streptococci to differentiate between human and animal sources of fecal pollution in natural water. Bahirathan and coworkers (112) employed antibiotic susceptibility tests and restriction enzyme analysis (REA) of genomic DNA to characterize the relationship between sources of isolates of yellow-pigmented enterococci. More recently, patterns of antibiotic resistance in enterococci have been used to identify sources of fecal pollution in rural watershed (113) and in surface water and groundwater (114). The results obtained in these studies suggested that there are measurable and consistent differences in the antibiotic resistance patterns of enterococci isolated from various sources of fecal pollution, and, therefore, the antibiotic resistance analysis can be used to determine reliably the sources of fecal pollution (see also FECAL CONTAMINATION, SOURCES OF, this Encyclopedia).

EPIDEMIOLOGICAL STUDIES

A significant amount of epidemiological evidence has accumulated on the relationship between bathing in polluted water and illness. The majority of epidemiological studies have been conducted on marine beaches in England (87,115), France (116), Spain (117,118), Israel (119), other Mediterranean countries (120–122), Hong Kong (123,124), and the U.S. (125). Similar studies have been

carried out in fresh waters in Canada (126,127), the United States (128), France (129), England (130), and the Netherlands (131). Extensive revisions of the epidemiological studies carried out in bathing waters had been recently published (132,133). All these studies have demonstrated an excess rate of minor disease incidence among bathers when compared with nonbather control groups. The diseases observed in these epidemiological studies have been generally associated with infections of the gastrointestinal tract, ear, nose throat, and skin.

Prospective epidemiological studies of swimmers and nonswimmers at several paired (nonpolluted and barely acceptable) U.S. beaches were conducted since the 1970s (134). Two types of symptoms were defined, namely, gastroenteritis (GI) and highly credible gastroenteritis (HCGI). The principal finding was that a significant "swimming-associated gastroenteritis rate" was always observed at the more polluted beaches but not at the less polluted beaches, giving rise to a significant risk of acute gastroenteritis associated with swimming in polluted water (85,101,132,135). Skin, ear, eye, and respiratory symptoms were attributed to cross-contamination among bathers in conditions of heavy use and poor water exchange. In fact, an increasing risk of acquiring otitis externa after swimming in freshwater lakes in the Netherlands containing *Pseudomonas aeruginosa* have been demonstrated by van Asperen and coworkers (131). For marine beaches, enterococci showed the strongest relationship to gastroenteritis, and at freshwater beaches, illness was best correlated with *E. coli* densities, but the correlation with enterococci was still almost as high as that at marine beaches. On the basis of these findings, USEPA (134) established new bacteriological criteria, in which the geometric mean of the indicator bacteria densities should not exceed 126 *E. coli* per 100 mL or 33 enterococci per 100 mL for fresh water, and 35 enterococci per 100 mL for marine water. Similar conclusions have been reported by Ferley and coworkers (129) and Fleisher and coworkers (136), who indicated that enterococci were the only indicator organisms able to predict the occurrence of gastroenteritis among bathers of fresh and marine water respectively, contaminated with domestic sewage.

Although several investigations have indicated the close relationship between enterococci and the disease risks (132,137,138), other microorganisms have been reported as alternatives. Thus, staphylococci were found to be the best overall health risk indicator in several epidemiological studies, both in marine and freshwater ecosystems (119,123,126,127,139,140).

Mariño and coworkers (141) carried out an epidemiological-microbiological study on selected beaches on the Mediterranean coast of Spain to establish an association between use of the beaches and enteric and nonenteric diseases of the bathers. The results indicated that a significant correlation was established only between nonenteric symptoms of the bathers (skin infections) and the titers of *P. aeruginosa*, *Candida albicans*, and *Aeromonas hydrophila* in seawater samples. These authors also studied the applicability of World Health Organization/United Nations Environmental Program (WHO/UNEP) criteria and the European Union bathing water directive as microbial water quality guidelines (142). The conclusions of this study revealed that in coastal water with low fecal pollution level, the best indicators were fecal coliforms, *E. coli* and coliphages, whereas at high fecal pollution levels, the best indicators were enterococci and coliphages.

A preliminary epidemiological-microbiological study was conducted in Cape Town (South Africa) by von Schrinding and coworkers (143). An excess in gastrointestinal, respiratory, and skin symptoms was found among swimmers compared with nonswimmers at the polluted beach. Although not statistically significant, the results suggest a relationship between swimming-associated illness and water quality determined by analysis of enterococci, fecal coliforms, staphylococci, coliphages, and F-male specific bacteriophages.

Fleisher and coworkers (144) have identified possible dose-response relationships among bathers exposed to marine water contaminated with domestic sewage and subsequent risk of nonenteric illness in the United Kingdom. The results obtained indicated that exposure to fecal streptococci was predictive of acute febrile respiratory illness, whereas exposure to fecal coliforms was predictive of ear ailments. Estimated thresholds of effect occurred at bather exposures more than 60 fecal streptococci and 100 fecal coliforms per 100 mL of water, respectively.

The health effects of swimming in ocean water contaminated by storm-drain runoff in Santa Monica, California, was investigated using an epidemiological cohort study by Haile and coworkers (145). The higher risks for swimmers were related to the proximity to storm drains, a high level of single bacterial indicators (total and fecal coliforms, *E. coli*, and enterococci), a low ratio of total coliforms/fecal coliforms, and the presence of enteric viruses.

SURVIVAL OF FECAL STREPTOCOCCI/ENTEROCOCCI IN AQUATIC ENVIRONMENTS

Survival in Sewage, Ground water, and Fresh water

Few studies, compared with other indicators, have been performed on the survival of fecal streptococci or enterococci in aquatic environments. Some studies have shown that fecal streptococci outnumber coliforms. Thus, Cohen and Shuval (146) reported that removal of fecal streptococci (most of which were probably enterococci) from a primary settling and a biological filtration system was considerably lower than that of coliforms, and more closely resembled virus survival. In an open sewage channel receiving effluent, viruses showed the lowest die-off, followed by fecal streptococci.

However, the phenomenon of a higher survival of fecal streptococci has not been supported by other studies. For example, Hanes and coworkers (147) reported that overall die-off rates of enterococci stored in diluted sewage were similar to or greater than that of coliforms. Burman and coworkers (97) also observed die-off rates, which were similar to or greater than that of fecal coliforms in stored samples of settled sewage, diluted sewage, and river water. Granai and Sjogren (148) determined the "in situ" survival of *E. coli* and *E. faecalis* in clean and polluted lake water.

In clean water, the survival of *E. faecalis* was superior to that of *E. coli* (96 hours vs. 48 hours), whereas in polluted lake water, survival times of both microorganisms were similar (10 hours vs. 12 hours).

There are several possible explanations for the conflicting results in the literature. First, the survival rate of fecal streptococci will depend on species composition because the survival rates of different fecal streptococci species appear to differ significantly; several enterococcal species persisted significantly longer than fecal coliforms, and *S. bovis* and *S. equinus* died off rapidly in stormwater samples (91). Second, it appears that the comparative survival of coliforms and fecal streptococci in the same water sample will differ according to the available nutrients because higher organic matter concentrations are required for the growth of fecal streptococci compared with those of coliforms (86). Third, streptococcal survival is considerably influenced by water temperature, and they die off faster than *E. coli* at both high (20 to 30 °C) and low (5 to 8 °C) temperatures (97).

Data on the survival of fecal streptococci or enterococci in soil and groundwater are scarce. McFeters and coworkers (102), using survival chambers immersed in a tank through which groundwater was continually pumped, found that *E. faecium*, *E. faecalis*, and *E. durans* exhibited superior survival characteristics to coliforms, but that coliform survival exceeded that of *S. bovis* and *S. equinus*. Using a similar experimental setup, Keswick and coworkers (149) found that an unidentified streptococcus from sewage exhibited superior survival to *E. coli* and coliphage f2. Bitton and coworkers (150), using groundwater stored in a glass flask, found that *E. faecalis* survived better than *Salmonella typhimurium* and *E. coli*, and had survival characteristics similar to those of coliphage f2. Recently, Hassen and coworkers (151) concluded that fecal streptococci introduced by wastewater had a higher survival in soil and water than fecal coliforms; Ramos-Cormenzana and coworkers (152) obtained a similar persistence of fecal streptococci and fecal coliforms in groundwater.

There is very little information on the comparative die-off rates of enterococci in freshwater ecosystems, and the results are sometimes contradictory (101). McFeters and Stuart (153) obtained a T_{90} (time required for 90% of the population to die off in days) of 2.7 for *E. coli* and 4.2 for enterococci using well water. On the contrary, Hanes and Fragala (154) reported T_{90} of 4.6 for *E. coli* and 3 for enterococci in freshwater. Dutka and Kwan (155) showed that the die-off of enterococci was lower than that of fecal coliforms in a stream. In a similar study, de Vicente and coworkers (156) obtained a higher survival capability of enterococci in river water compared with total and fecal coliforms and *P. aeruginosa*.

Survival in Seawater

Slanetz and Bartley (157) compared the survival of coliforms and fecal streptococci in dialysis bags containing untreated sewage effluent and suspended in seawater. Coliform numbers in bags increased 310 times over the first two days and remained at these levels for about seven days before the onset of die-off. There was no increase in the numbers of fecal streptococci in the bags and a rapid decrease occurred within four to six days. The authors concluded that fecal streptococci would make the most accurate indicators in seawater containing organic material because they do not multiply in such water and they show appreciable die-off rates within a two- to three-day period.

Vasconcelos and Schwartz (158) investigated bacterial survival in seawater and found that *E. faecalis* survived longer than either *E. coli* or *Enterobacter aerogenes* but that all the three species survived over a period of seven days. Evison and Tosti (159), studying the survival of fecal streptococci in the Bay of Naples, found that the natural attenuation of fecal streptococci was lower than *E. coli*. In laboratory studies with seawater, these authors found that survival of both *E. coli* and fecal streptococci was inversely related to temperature, but fecal streptococci consistently survived longer than *E. coli* at all temperatures and in the dark. Lessard and Sieburth (160) reported that the decay rates for enterococci in an estuary and a salt marsh were significantly correlated with temperature. Enterococci survived longer than *E. coli* in estuary water, but showed lower survival rates in the more eutrophic salt marsh.

Borrego and coworkers (84) determined the survival capability of pathogenic and indicator microorganisms by comparing the concentrations of each at increasing distances from a marine-sewage outfall. The results obtained indicated that coliphages and fecal streptococci (predominantly enterococci) showed the lowest degree of reduction in polluted seawater (84 and 174 minutes, respectively). The other microbial parameters tested showed similar inactivation rates among them (26.4 minutes for total coliforms, 35.4 minutes for fecal coliforms, and 33 minutes for *Salmonella*). Using a similar methodology, Fattal and coworkers (161) investigated the relative dying away of bacteria and enteric viruses. They found that total and fecal coliforms disappeared more rapidly than viruses, whereas the reduction of fecal streptococcal concentrations was similar to the dying away of virus. Studies using "in situ" membrane diffusion chambers showed that the die-off of fecal coliforms and fecal streptococci to 10% of their initial numbers occurred in marine sediments near a sewage outfall within 85 days (162).

Why fecal streptococci exhibit superior survival characteristics in seawater is not completely clear. Some authors have indicated that the higher salt tolerance (163,164) or the lower susceptibility to sunlight (165,166) compared with fecal coliforms, may be some of the explanations to these findings. For this reason, controlled studies under laboratory conditions have been conducted to demonstrate the influence of several biotic and abiotic factors on fecal streptococci. de Vicente and coworkers (167) and Cornax and coworkers (68) investigated the survival of several indicator organisms and the pathogens *P. aeruginosa* and *Salmonella* spp. in the marine environment and in the laboratory under controlled conditions. The "in situ" studies showed that the die-off of enterococci in seawater was very similar to that of those pathogens, and slower than total and fecal coliforms (T_{90} of about 40 minutes for enterococci vs. 20 minutes for coliforms). The laboratory survival

experiments showed that the visible light and the nonfiltrable biotic factors produced most important inactivating effects on the microorganisms.

Similar to other allochthonous bacteria of the aquatic environments, the phenomenon of nonculturability (i.e., the inability to grow sublethally injured bacteria in culture media) has been demonstrated in *E. faecalis* in fresh and marine systems under illumination (168), in drinking water (169), and in filtered lake water (170).

ENUMERATION TECHNIQUES

The acceptance of fecal streptococci or enterococci as valid and useful fecal indicators was closely linked to the development of isolation and enumeration methods. Early attempts to quantify fecal streptococci relied on enrichment-tube procedures associated with the use of the most probable number (MPN) method, Rothe Azide Dextrose broth, followed by a confirmation in Ethyl Violet Azide (Litsky) broth being the procedure most widely accepted (171). A new modification of MPN technique to enumerate enterococci directly from Rothe broth has been presented by Donnison (172), who uses a confirmation on m-E agar and subsequent transfer to Esculin Iron (EIA) agar.

The membrane-filtration technique using the m-Enterococcus (or SB) medium was the first reported method for the enumeration of fecal streptococci or enterococci in fresh and marine water since the 1950s (173). Since then, more than 70 selective media have been proposed, such as Kenner Fecal Streptococcus (KF) agar, Pfize Selective Enterococcus (PSE) agar, and Tallous Acetate (Barnes) agar. In addition, several media have been also developed for the specific isolation and enumeration of enterococci by membrane filtration. The first and most used is m-Enterococcus medium (100). For recovery of enterococci, Standard Methods (100,174) recommends the use of m-E agar (175), followed by transfer to EIA agar, but several other media can be employed, such as Kanamycin Esculin Azide (KEA) agar (176), mSD agar (175), and mAC agar (177). The standard procedures (APHA and ISO) for the detection of fecal streptococci have recently been described by Figueras and coworkers (82).

Other media formulations and incubation procedures for fecal streptococci have been proposed for specific situations (178), increasing the membrane incubation period from 48 to 72 hours to recover stressed fecal streptococci from chlorinated effluents. Rutkowski and Sjongren (107) developed a medium that designated M2 to help in distinguishing between human and animal pollution sources. M2 supported the growth of a wider selection of streptococci than is normally associated with the fecal streptococci, but the authors reported that more than 90% of their isolates on this medium belonged to Lancefield's group D. Hernandez and coworkers (179) designed a miniaturized fluorogenic assay for enumeration of enterococci in marine water incorporating 4-methylumbelliferyl-ß-D-glucoside (MUD) into a selective medium contained in a sterile 96-well microtiter plate. This technique yields slightly lower recoveries compared with the membrane filtration technique using two selective media. This method has now become an ISO standard for the detection of intestinal enterococci from bathing water (82,180). Niemi and Ahtiainen (181), in a comparative study, reported that both the microtiter and membrane filtration methods allowed the growth of nonfecal *Enterococcus* and *Staphylococcus* species.

Several studies have been conducted to compare the selective methods used for the enumeration of fecal streptococci and/or enterococci from natural water (175,182–189); however, the results obtained were widely different and sometimes contradictory. More recently, Dionisio and Borrego (21) compared eight currently used methods to recover and enumerate fecal streptococci from natural water samples, on the basis of a few media characteristics, namely, accuracy, specificity, selectivity, precision, and recovery efficiency. The authors concluded that none of the compared methods would be considered as optimum for enumeration of fecal streptococci from water samples; however, m-Enterococcus agar using the membrane filtration technique showed the best performance characteristics of the enumeration media tested because this medium possessed the best recovery efficiency, precision, and accuracy (Table 6). Other studies have demonstrated the low selectivity and specificity of m-Enterococcus medium (26,190,191), and, for this reason, modifications of m-Enterococcus have been performed to increase its selectivity. Mates and coworkers (192) proposed the supplementation of the m-Enterococcus agar with antibiotics, whereas Figueras and coworkers (190) proposed transferring the membrane filters from m-Enterococcus to bile-esculine medium as a confirmation technique to eliminate the false positives. A variant of this last method has been adopted as an ISO method (193) for the isolation of enterococci from drinking water samples.

The most widely used media for fecal streptococci or enterococci determination by the membrane-filtration technique required a long incubation period (48 to 72 hours), and the need of a confirmatory step extend the time of the analysis to three or more days, which is a big disadvantage for their use in water analysis. The KEA agar proposed by Mossel and coworkers (176) presents two promising advantages: (*1*) the results are available in 24 hours, and (*2*) esculin hydrolysis can be observed directly on the filter. However, KEA agar presents a low capability of discrimination between typical and nontypical fecal streptococcal colonies (23), and very low specificity, allowing the growth of *Staphylococcus*, *Pseudomonas*, and *Vibrio* strains, whose colonies resemble those of fecal streptococci (21,194).

To avoid these shortcomings, Audicana and coworkers (25) modified the KEA formulation by replacement of kanamycin sulfate by oxolinic acid and increasing the sodium azide concentration to achieve the inhibition of the nonenterococcal background microbiota. This modification of KEA agar, named *oxolinic acid-azide* (OAA) agar, has been comparatively evaluated with other selective enterococcal media (25,26). The OAA agar showed the highest specificity, selectivity, and relative recovery efficiency; in

Table 6. Performance Characteristics of Several Selective Media for Enterococci Applying the Concordance Coefficient of Kendall (21)

Characteristics	m-Enterococcus	KF	PSE	KEA	Barnes	BE	MS	Rothe-Litsky
Qualitative growth								
Enterococci	3	3	3	6	3	NT	7	3
Others	1.5	1.5	5	4	6	NT	7	3
Selectivity (mean)	3	2	6	4	5	7	8	1
Specificity								
False-positive	2.5	2.5	5	6.5	2.5	2.5	6.5	NT
False-negative	2	1	4	3	5	6	7	NT
Recovery efficiency								
Typical colonies	1	4	6	5	3	NT	NT	2
Verified colonies	1	3	6	4	5	NT	NT	2
Precision								
At 0.5 limit	1	3	2	5	5	5	NT	NT
At 0.05 limit	1	3	2	4	6	5	NT	NT
Accuracy								
Seawater	1	3	2	4	7	6	8	5
Freshwater	2	1	4	3	7	5	8	6
$\Sigma R_j/N$	1.73	2.45	4.09	4.41	4.95	5.21	7.36	3.14
Ranking	1	2	4	5	6	7	8	3

Note: Not tested (NT).

addition, no confirmation of typical colonies was needed while using this medium.

Rhodes and Kator (195) modified the medium m-E by adding the chromogenic substrate indoxyl-ß-D-glucoside and reducing the concentration of TTC to recover enterococci in 24 hours. This new medium, named *mEI*, has been compared to m-E medium and showed similar results at shorter time (196).

Enterolert (IDEXX Laboratories, Inc.), a semiautomatic MPN method, has been used for enumeration of enterococci in comparison to the standard membrane filter method used for recreational natural water analysis. No statistically significant differences between the two techniques have been obtained, but Enterolert required less time for set-up (24 hours) (197–199).

Recently, a molecular biological procedure for the detection of enterococci in water samples has been designed (200). The method consists of a short enrichment of the water sample, followed by an amplification step and a hybridization reaction using 23S rDNA-targeted oligonucleotide probes. The detection limits of this molecular method is about 1 CFU per mL and the results are available within 26 hours.

CONCLUSION

The taxonomy of *Enterococcus* and *Streptococcus* general has not yet been fully resolved. The terms *fecal streptococci*, *enterococci*, or *intestinal enterococci* embrace species of the genera *Enterococcus* and *Streptococcus*, although there is no complete agreement about the species that should be included under these terms. In practice, fecal streptococci, enterococci, intestinal enterococci, and *Enterococcus* group when used in the literature to refer to indicators of fecal pollution can be considered synonymous.

Fecal streptococci and enterococci are ubiquitous and can be found free-living in soil, on plants, in dairy products, and as a part of the normal microbiota of the gastrointestinal tract of humans, other mammals, and birds. However, they have been implicated in outbreaks of food-borne illnesses, and they can possess both a beneficial and detrimental role in food. These microorganisms may be involved in a variety of human infections, named *enterococcal bacteremia*, including infections of the urinary tract, bloodstream, endocardium, biliary tract, burn wounds, and indwelling devices, *E. faecalis* being the species most frequently isolated from clinical samples.

Fecal streptococci/enterococci have received widespread acceptance as useful indicators of fecal pollution in natural aquatic environments on the basis of their close relationship with health hazards associated with bathing in aquatic environments, their constant presence in feces of warm-blooded animals, their inability to multiply in sewage-contaminated water, and their long persistence in natural water.

The survival of fecal streptococci/enterococci in aquatic environments, including sewage, groundwater, fresh water, and seawater, has also been reviewed. Several studies have demonstrated that the survival capability of these microorganisms is higher than that of coliforms, and similar to virus survival. However, the survival rate of fecal streptococci will depend on species composition because the survival rates of different fecal streptococcal species appear to differ significantly. In addition, the comparative survival of coliforms and fecal streptococci in the same water samples will differ according to the available nutrients, because higher organic matter

concentrations are required for the growth of fecal streptococci compared with those of coliforms. In addition, streptococcal survival is considerably influenced by water temperature, and they die off faster than *E. coli* at both high (20 to 30 °C) and low (5 to 8 °C) temperatures.

The acceptance of fecal streptococci/enterococci as valid and useful fecal indicators is closely linked to the development of reliable isolation and enumeration methods. For this reason, the last part of this article is dedicated to the revision of the current detection and enumeration methods for these microorganisms. Several media have been developed for the specific isolation and enumeration of enterococci/fecal streptococci by the membrane-filtration technique. However, this technique required a long incubation period and the need of a confirmatory step. To avoid these shortcomings, new media formulations, the addition of chromogenic substrates, and the application of a molecular biological procedures have recently been proposed.

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FIELD RELEASE OF GENETICALLY ENGINEERED MICROORGANISMS (GEM)

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A field release can be defined quite simply as the deliberate introduction of a bacterial strain into a natural environment. The bacterial strain being released has typically undergone some type of genetic manipulation in the laboratory to enhance its ability to perform a specific task. In bioremediation practices, for example, genetically engineered microorganisms (GEMs) are used for the cleanup of environmental pollutants in terrestrial and aquatic environments, biomining industries are attempting to use GEMs for the extraction of various natural resources, and in agriculture, GEMs can be released into crop ecosystems as biofertilizers or biocontrol agents (biopesticides, biofungicides, or bioinsecticides). In this article, the basis for why field release studies are necessary, the problems associated with introducing engineered bacterial strains into the environment, and viable methods for alleviating some of the obstacles to bacterial field releases will be explored.

THE NECESSITY FOR FIELD RELEASE TRIALS

All genetic engineering strategies begin in the laboratory, where operating conditions can be controlled and manipulated at will to thoroughly understand and predict metabolic and physiological functions of the bacterium under development. However, extrapolating laboratory-based results or scaling up laboratory-based studies to real-world environmental applications becomes all but impossible and must be approached with a great deal of caution and pessimism. While considering the vast number of biotic (i.e., competition, predation, starvation, and antagonism) and abiotic (i.e., temperature, moisture, adsorption, and pH) factors a bacterium must react to and interact within any typical environmental ecosystem, it readily becomes apparent that the environment is too complex to reproduce within laboratory confines or, for that matter, even within high-level computer modeling schemes. Therefore, true comprehension of an engineered bacterium's capabilities in bioremediation, biomining, or agricultural biotechnology requires unbiased, comprehensive exposure to the environment before adequate assessments, predictive ability, and possible benefits or hazards can be identified. However, although an abundance of GEMs have been developed for various biotechnological purposes, many of which have exhibited great potential in the laboratory, few examples of GEM field release trials actually exist (Table 1) (Fig. 1).

BARRIERS TO FIELD RELEASE STUDIES

Public Perception

The application of genetic engineering technologies has perhaps been shaped more by public fear and emotion than by an accurate understanding of the science involved and its potential risks and benefits. For example, a bacterium capable of increased metabolic degradation of crude oil constituents was met with public resistance because of the fear that these GEMs would eventually find their way into Texas oil fields and rapidly deplete U.S. oil supplies (1). Similarly, the planned release of a GEM for the prevention of frost damage in plants was met with public outcry because the "escape" of the gene involved would produce "super weeds" that would also survive frost damage (2). This "Jurassic Park" view of biotechnology has succeeded in delaying numerous field release trials and has led to what some deem an overregulated industry, in the United States as well as worldwide (3–5). Consequently, many researchers are intimidated or often outright impeded in their attempts to institute field release studies through public misperception and protest as well as through time-consuming governmental regulations and review.

Federal Regulation of Field Release Trials

Because the actual outcome of a field release cannot be reliably predicted, it becomes mandatory that oversights exist to ensure environmental and public health safety (6,7). However, the federal regulations and administrative processes required to initiate field releases of engineered microorganisms can be lengthy, complicated, and costly, and can frequently prevent GEMs from ever leaving the laboratory. Furthermore, for those GEMs that do receive field release approval, it is often the case that they are no longer state of the art, having undergone significant refinement and genetic restructuring during the regulatory waiting process. Consequently, research industries tend to avoid GEMs altogether, rather favoring methods in which environmental conditions are manipulated,

Table 1. Representative Field Release Studies Involving Genetically Engineered Microorganisms

Bacteria	Trait	Year of Release	Application	Country	Reference
Agrobacterium radiobacter K1026	Biological control agent for crown gall disease	1987	Almond seedlings	Australia	112
Pseudomonas aureofaciens 373RNL11	*lacZY*-marked	1987	Wheat plot	U.S.A.	79
Pseudomonas syringae	Ice$^-$ (biocontrol of frost)	1987	Strawberry	U.S.A.	113
Pseudomonas syringae	Ice$^-$ (biocontrol of frost)	1987	Potato	U.S.A.	114
Rhizobium leguminosarum RSM2004	Marked with plasmid-based Tn5 for determination of gene-transfer frequencies	1987	Peas, chick peas, wheat, barley	U.K.	29
Clavibacter xyli	Btk toxin (bioinsecticide)	1988	Corn	U.S.A.	115
Pseudomonas corrugata 2140	Biological control agent for take-all disease in wheat (*lacZY* marked)	1990	Wheat plot	Australia	116
Xanthomonas campestris JS414	*lux*-based marker	1990	Cabbage	U.S.A.	117
Clavibacter xyli MDR1.586 and MDR1.1413	Bioinsecticide against European corn borer	1990 1991	Corn	U.S.A.	118
Rhizobium meliloti	Biofertilizer (*nifA* and *dctABD* insertions)	1992	Alfalfa	U.S.A.	119
Xanthomonas campestris FD91L	*lux*-based marker	1992	Cabbage	U.S.A.	120, 121
Pseudomonas fluorescens SBW25EeZY-6KX	*lacZY*-marked	1993	Wheat plot	U.K.	42
Pseudomonas syringae	Tn5 insertion	1993	Bean plants	U.S.A.	122
Bacillus thuringiensis	Bioinsecticide (*cry* gene insertion)	1994	Crop plants	U.S.A.	123
Bacillus thuringiensis BtN14	Bioinsecticide	1994	Swampland soil	Sweden	124
Rhizobium leguminosarum CT0370	Marked with *Escherichia coli GUS* gene	1994	Peas	U.K.	125
Rhizobium leguminosarum Azospirillum brasilense	*lacZ*-marked	1994	Pea, broad bean, sorghum	Italy	34
Pseudomonas fluorescens R2f	*lacZ-nptII*-marked	1995	Wheat plot	Netherlands	126
Pseudomonas putida CMC4	*gef* killing gene linked to *xylS* for biological containment	1995	Soil	Spain	127, 128
Sinorhizobium meliloti RMBPC-2*	Nitrogen fixation	1995	Recombinant alfalfa	U.S.A.	71
Pseudomonas fluorescens HK44	Bioremediation of polyaromatic hydrocarbons	1996	Soil	U.S.A.	33

Note: A comprehensive list of U.S. and international field tests can be found in the Information Systems for Biotechnology database maintained by the Virginia Polytechnic Institute (*http://www.nbiap.vt.edu*).

**Sinorhizobium meliloti* RMBPC-2 was approved by the U.S. Environmental Protection Agency in 1997 for limited commercial application (500,000 pounds/year) as a microbial seed inoculant to coat alfalfa seeds before planting.

and not the microorganisms themselves, in attempts to increase indigenous microbial subpopulations to perform specific metabolic tasks. Following the Exxon Valdez oil spill in 1989, for example, fertilizers were applied to some shoreline sites to stimulate oil degradation by indigenous microbial populations (8). The exception occurs in agriculture in which GEMs are commercially available as biocontrol agents. For instance, *B. thuringiensis* strains can be genetically enhanced to produce toxins against certain crop pests. However, these engineered microbes are mass-produced in the laboratory and then killed before market release (9). Thus, only their synthesized toxins are actually environmentally applied, and not the GEMs themselves, thereby avoiding many of the regulatory overviews.

In the United States, GEMs, depending on their ultimate use, are regulated by the Environmental Protection Agency (EPA), Food and Drug Administration (FDA),

and/or the U.S. Department of Agriculture (USDA) (10). For example, GEMs destined for bioremediation, biofertilization, or biomining purposes are regulated by the EPA's Office of Pollution Prevention and Toxics (OPPT) under the Toxic Substances Control Act (TSCA) (11,12). The application process used by OPPT involves eight reviews, and several other technical documents, that detail the microorganism's genetic structure, identifies potential human and ecological risks associated with releasing the microorganism, and provides a technical plan describing the release site, the method of release, exposure risks to personnel during release, and methods to limit and monitor dissemination of the microorganism during release. Other Organization for Economic Cooperation and Development (OECD) countries require similar consents before deliberate GEM releases are allowed, and the United Nations possesses preliminary regulations in the form of a Code of Conduct and a Biosafety Protocol governing biotechnology industries worldwide.

Gene Transfer

Much of the public concern and governmental regulation concerning the release of GEMs is due to the fact that engineered genetic material can potentially be transferred from the GEM to indigenous members of the microbial consortia (13). This process occurs naturally in bacteria through conjugation (the transfer of genetic material by cell-to-cell contact), transformation (the uptake of naked DNA), and transduction (the transfer of DNA via bacteriophages). Transposition, the random "jumping" of specialized genes called *transposons*, may occur within the GEM or a recipient of a gene-transfer event (14–16). Each of these gene-transfer mechanisms occurs naturally in the environment and therefore each constitutes an inherent but poorly quantified risk factor in all field release experiments. Predicting the degree of risk is difficult because the frequency of gene transfer is dependent on the type of genetic material being transferred, the mechanism or combination of mechanisms mediating the transfer, the environmental conditions present at the time of transfer, and the perceived hazard of the gene. Studies have shown, for example, that genetic elements inserted within a plasmid can potentially be transferred at fairly high rates via conjugation (14,16). Even genes present on plasmids deficient in the transfer and mobilization elements (tra^-, mob^-) seemingly required for conjugation can still undergo gene transfer (17). It is therefore recommended that engineered genes in GEMs be chromosomally inserted. However, chromosomal DNA can still be transferred by any of the mechanisms already described, albeit at lower frequencies (14,16).

The environment into which the GEM is placed and the environmental factors impinging on the GEM will also affect gene-transfer efficiencies. Elevated transduction rates have been shown in freshwater ecosystems when particulate matter was present, probably as a result of the formation of bacteriophage–host cell microenvironments sorbed to clay particles (18). As an infected cell releases bacteriophage particles, a closely neighboring cell becomes readily infectible. Apparently for the same reason, bacteria growing in biofilms exhibit increased gene-transfer frequencies, as do microbial populations living within plant root rhizosphere systems (19,20). In soils and less so in aquatic environments, "naked" DNA released from living and dead microorganisms adsorbs to sand and clay particles in which it can exist in a transformable state for several months (21). Therefore, even after a GEM has been eradicated from its release site, the engineered DNA can still remain and be taken up by the indigenous microbial consortia. Also, because most environmental ecosystems are oligotrophic in nature, the addition of nutrients has been shown to increase gene-transfer rates simply because of corresponding increased rates of bacterial growth (22). Bacterial gene transfer has additionally been shown to occur in the digestive tract of cutworms, digestive vacuoles of protozoa, the guts of silkworms and microarthropods, and on plant leaf surfaces (23–27).

Considering the near-endless potential for gene transfer, it would seem reasonable from a critic's point of view, to ban field release studies altogether. However, much of the information available concerning gene transfer is obtained from laboratory microcosm data in which conditions were optimized to specifically enhance transfer events, thus creating a rather unrealistic "worst case scenario" portrayal of genetic exchange potentials. Considering the nonoptimized nature of the environment, it is presumed that gene transfer will occur at frequencies with lower orders of magnitude than that reported in laboratory microcosms. In fact, no direct evidence of recombinant gene transfer to indigenous microbial populations has been identified during several field release trials (28–34). This in no way signifies that gene transfer was not occurring; only that it was occurring at frequencies too low to be detected. Therefore, the risk is still present but it may be so negligible as to not constitute a significant environmental hazard. Additionally, although gene transfer is occurring in the environment continuously, the presence of identical gene sequences within distantly related bacterial species is rare. This is due to the fact that most genes obtained by a bacterium through gene transfer will be of no selective advantage and will subsequently disappear.

Persistence in the Environment

Ideally, on release into the environment, a GEM should only survive long enough to perform its specified task and then die off to ensure no further ecological risk. It is usually assumed that GEMs will gradually become extinct after release because they are unable to effectively compete with the indigenous microbial consortia. This reduced level of fitness is thought to be due to the extra energy demands mandated by the presence of the introduced genes and by the fact that GEMs are usually nurtured under optimal laboratory conditions for several years, thus making them physiologically weak and less prone to survive within harsh environmental ecosystems. Additionally, GEMs designed to survive under certain selective pressures should die off as those selective pressures are removed from their environment. For example, during bioremediation processes, GEMs maintain a selective growth advantage over the indigenous microbial

population because they are specifically designed to survive within the chemically contaminated environments into which they are placed. However, once the selective pressure of the contaminant is removed, all growth advantages cease, indigenous microbes soon flourish, and the GEMs are gradually displaced.

Numerous studies have shown GEMs to exhibit reduced fitness when direct comparisons are established against the parental strains from which they were originally derived (35–39). However, a decreased fitness level and corresponding reduction in growth rate simply implies that at some point in the future the GEM will eventually become extinct. A computer model estimated that GEMs are capable of surviving in introduced environments for approximately three years (40). In actual field release studies, a *lacZY*-marked *P. corrugata* strain was recoverable from soil four years after its introduction (41), a genetically modified *Rhizobium* strain was detectable in soil six years after its release (29), and a *P. fluorescens* strain capable of degrading polyaromatic hydrocarbons (PAHs) continues to persist in a soil ecosystem three years after its release, although the selective pressure of the PAHs has been significantly reduced (33). In contrast, a recombinant *P. fluorescens* strain was undetectable one year after its release into wheat plots (42), an engineered *P. aureofaciens* strain released by the Monsanto Corporation did not survive for more than two years (28,43), and a genetically modified *Azospirillum brasilense* strain fell to below-detection limits within one year of its field release (34). However, in other studies, GEMs that had been considered undetectable, later reappeared under suitable growth conditions (30,44). Therefore, labeling a GEM as "no longer detectable" in no way signifies that it is no longer present, only that it cannot be identified using the enumeration methods available. Enumeration techniques that rely on the isolation of visible GEM colonies are especially ineffective because microorganisms exposed to starvation conditions, as would be the norm in the environment, can enter a viable but nonculturable state, meaning that although they are still alive, they cannot be resuscitated on laboratory media (45). Molecular-based methods in which the genetic construct is identified as opposed to the microbe itself are much better indicators of GEM presence, but as will be seen later in this article, still cannot guarantee a GEM's absence. Furthermore, even if a GEM were to be completely eliminated from its environment, its engineered genetic material may still persist and contribute to gene-transfer events. Thus, it is probably correct to state that the persistence of GEMs and their genetic constructs will be impossible to predict following environmental introduction; subsequent risks must therefore be identified and minimized accordingly.

Environmental Transport

GEMs released into the environment should ideally remain within their confined application area to limit potential ecological hazards. However, preventing the dispersion and transport of GEMs in a complex environmental system is difficult. Bacteria can be transported via aerial dispersion, water flow, or plant and animal vectors. Additionally, in agricultural applications, soil tillage may affect dispersion rates. Aerial dispersion is perhaps more of a concern during the application process itself when GEMs are often applied in an aerosolized form. A model to predict aerial transport of bacteria within terrestrial environments has been developed, which suggests that few bacteria will disperse beyond a 1-m radius of the field release site (46). Actual aerial dispersion rates during soil-release experiments vary considerably, but most dispersion does indeed occur within a 1-m radius, less than 100 colony-forming units typically being detected per gram of soil (47,48). However, broad generalizations cannot be made when considering the numerous factors that can affect aerial dispersion rates (wind speed, wind direction, turbulence, humidity, and temperature), and caution during application methods is still highly warranted.

Water is perhaps the most efficient transport mechanism available to bacteria. Most water-induced transport mechanisms concerning GEMs have been studied in soil ecosystems (49) and models to predict dispersion rates have been developed (40,50,51). Water enters a field site through rainfall or agricultural irrigation where it percolates through the soil bed until it enters groundwater systems. Bacteria transferred with this water flow can potentially travel great distances. However, on distribution within groundwater flow systems, their numbers may be too low to constitute a significant environmental hazard. Also, bacteria can tightly adsorb to soil particles, making their dislodgment by percolating water difficult. The degree of bacterial adsorption to soil particles increases with time, so transport risks remain greatest directly after GEM application. In such cases a heavy rainfall could transport significant numbers of bacteria to depths approaching 2.5 m (52). In experiments in which water was not applied to the soil, introduced bacteria were detected only at depths above 40 cm (53). Adsorption is also dependent on cell surface properties, which can potentially undergo unknown alterations during the genetic engineering process making the GEM less prone to soil adhesion (54,55). Soil type and texture will also affect dispersion rates, and sandy soils exhibit higher transport frequencies than more densely textured soils. This is probably mainly due to preferential flow, the irregular flow of water through soil fissures and large pores, which has been shown to occur more readily in silty and sandy soils (56). Preferential flow effects can also be exacerbated by plant root growth or burrowing worms and insects (57). In bioremediation processes it has been demonstrated that chemically contaminated soils impede GEM transfer, probably because of an increase in soil bulk density that can limit preferential flow effects (58).

In aquatic systems, bacteria can be transported across great distances by normal water flow dynamics, but are usually so highly distributed that their low numbers in any given area pose little ecological threat. In freshwater streams, bacterial dispersion is limited by current flow and dispersion rates and is largely dependent on cycles of bacterial attachment and detachment from biofilms (59).

Bacterial transport via animal vectors during field release studies has primarily been demonstrated using

the earthworm as a model organism. Transport has been shown to occur in soil through bacterial attachment to the earthworm's surface and through ingestion and subsequent fecal deposition (60). GEMs applied to plant surfaces can undergo plant-to-plant transfer by similar ingestion and fecal deposition, as has been shown with the cutworm (61) and caterpillar (62). Similar data has been presented using grasshoppers as transport vectors (63). However, in these studies the GEMs were originally applied to the plant seeds, whereupon they subsequently colonized the roots and eventually became dispersed within aerial foliage. Grasshoppers feeding on the leaves became infested with the GEMs and transported them to previously uninoculated plants. GEM movement in this respect could substantially affect transport rates and needs to be studied in more detail before assessing the risks involved.

Transport of GEMs can also occur through bacterial taxis in which self-propulsion can be achieved through the action of a flagellum. Bacterial taxis through soil is too slow to be of any great concern but may be slightly more important in aqueous or heavily saturated environments in which flow rates are minimal (64). However, compared to the transport mechanisms already discussed, bacterial taxis should be considered an exceedingly minor form of natural dispersion.

Ecosystem Alterations

The introduction of GEMs can potentially affect the structure and balance of an ecosystem. This may occur by the displacement of an indigenous species in which the GEM outcompetes a resident microbial population. Enhanced nitrogen-fixing GEMs, for example, could overtake indigenous nitrogen-fixing bacteria in the root rhizosphere and significantly decrease or eliminate their populations. Species elimination has been shown to affect ecosystem function, but, conversely, redundancy within ecosystems may allow GEMs to simply replace an indigenous population with no adverse effects (65). Predicting what outcome will occur is extremely difficult but has been studied on a limited basis in laboratory microcosms, with conflicting results. A recombinant *P. putida* strain engineered to degrade the herbicide 2,4-dicholorophenoxyacetate (2,4-D) was found to significantly decrease the number of fungal propagules in a soil microcosm (66). Because fungi play important roles in plant growth and decomposition, thus being major contributors to soil fertility, their absence could considerably compromise a soil ecosystem. Similarly, in a study in which a recombinant *Klebsiella planticola* strain was introduced into a soil microcosm containing wheat plants, an increase in nematode populations was seen that coincided with plant death (67). However, several other microcosm studies have indicated that GEM introduction has limited influence on microbial community diversity in soil, rhizosphere, and aquatic ecosystems (68–71). The study by Doyle and coworkers (66) also demonstrated that the introduction of the GEM reduced carbon dioxide evolution, signifying that introduced GEM populations can affect ecosystem processes related to primary production and nutrient cycling, which in turn can have profound influences on global ecosystem fluxes (72). In other soil microcosm experiments, however, the introduction of recombinant microbes resulted in either no or only short-term process level changes in ecosystem fluxes (73–75).

Another ecosystem alteration more important to bioremediation field releases is the incomplete degradation of toxic chemicals by introduced GEMs. For example, microbes engineered to degrade the environmental pollutant trichloroethylene (TCE) produce as a byproduct, vinyl chloride that happens to be more toxic than the TCE itself (76). However, the degradation pathways of most bioremediative GEMs have been thoroughly studied in the laboratory before release, and risks owing to toxic intermediate production remain relatively low.

Monitoring

The ability to accurately monitor GEMs is critical to understanding their potential effects on any given environment. Considering that a bacterium is only microscopic

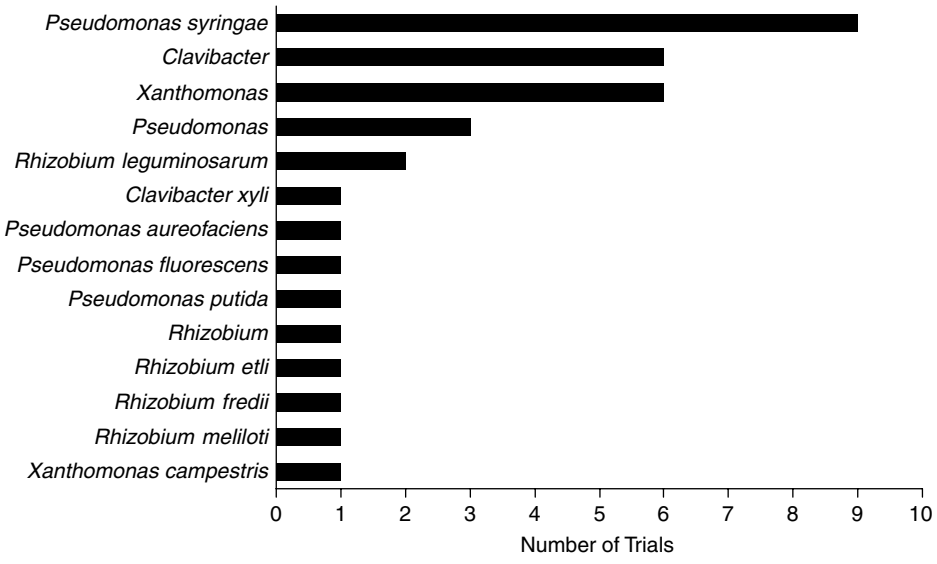

Figure 1. Number of approved field trials in the United States categorized by type of genetically engineered microorganism released.

in size, may spatially disperse within its environment, and may enter viable but nonculturable states, it readily becomes apparent that successfully tracking a particular bacterial population after introduction into a complex ecosystem will be difficult. To alleviate monitoring problems, GEMs can simply be selectively marked with an antibiotic resistance gene. In theory, plating onto media containing the antibiotic allows only the resistant GEMs to grow. However, with heterogeneous environmental samples, a background population of microbes also resistant to the antibiotic is usually present, thus making exclusive isolation of GEMs impossible. To overcome differentiation problems, antibiotic-resistant colonies can be further subjected to colony-hybridization techniques in which an additional selective marker can be identified with labeled gene probes (77). By targeting two selective markers, the GEM can first be identified phenotypically by its growth in the presence of an antibiotic and then genotypically by certifying that a specific target gene is present. However, an indigenous microbe resistant to the antibiotic that has also taken up the targeted gene sequence through a gene-transfer event will still remain indistinguishable from the GEM unless its colony morphology significantly differs from that of the GEM. Also, maintaining antibiotic resistance exerts an extra energy demand on the GEM that may affect its overall survival rate. Furthermore, because the antibiotic is not typically innate to the GEM's environment, no selective pressure persists to maintain the gene and it can subsequently be lost. Thus, although the GEM still exists in a form in which the antibiotic gene is absent, yet other engineered constructs are present, the GEM remains undetectable. The genetic transfer of antibiotic resistance genes to indigenous microbial populations is also a valid threat as has been demonstrated in medical practices in which the overprescription of antibiotics has led to numerous multidrug-resistant microbes (78). Therefore, antibiotic resistance as a genetic marker is discouraged in GEMs destined for field release.

A variety of selectable genetic markers that are less problematic than antibiotic resistance are available for integration into GEMs. The *lacZY* system, which allows GEM colonies to be distinguished by a characteristic blue color they produce on laboratory media containing the substrate X-gal, has been used in several field release studies (79). Similar in function is the *gusA* gene that cleaves the substrate X-glcA to produce blue colonies (80). Other genes include *xylE* (yellow product), *nahA* (blue product), TFD monooxygenase (red product), and *moc* (specific growth substrate requirement) (81–84). Also available are the *lux* and *luc* genes (bioluminescence) and the *gfp* gene (fluorescence) for generating "glowing colonies" as identifying markers (85,86). However, all of these marker systems rely on the growth of the tagged microorganisms; a colony existing in a viable but nonculturable state will never be initially detected on laboratory media. Additionally, increased energy demands as a result of the additional genetic load will still be prevalent and, in soil ecosystems, markers such as *lacZY*, *gusA*, and *nahA* still remain indigenous and will appear in background populations (87,88).

REDUCING RISKS DURING FIELD RELEASE TRIALS

A field release will never be risk-free. However, methods to reduce risks are available and are continually being incorporated into field release trials. These methods consist of structural and biological containment strategies that limit GEM access to the environment as well as the incorporation of better monitoring techniques following GEM release. Additionally, some risks related to the gene transfer of recombinant DNA to indigenous microbial populations can be reduced.

Physical Containment

The simplest manner in which to reduce environmental hazards associated with field introductions is to physically restrict the released microbe from freely entering the environment. This is usually accomplished by structurally enclosing the release site. For example, Figure 2 illustrates a series of subsurface soil lysimeters that served as containment structures during a bioremediation field release experiment (89). Because the GEMs never actually freely entered the open soil ecosystem, environmental hazards were significantly reduced. In agricultural field applications, containment is often achieved by confining the plants and associated GEMs to pots that are then placed outdoors. GEMs can also be applied to small plots of in-ground crop plants that are surrounded by alternating guard rows of fallow soil and plant species that are not infected (Fig. 3). In all cases, fences border release sites to prevent access by animals. With such degrees of containment, field release studies can be disputed to be nothing more than large-scale, controlled laboratory experiments performed in an artificial outdoor environment. Nonetheless, such studies represent a balance between risk assessment and environmental realism. Although always being only close approximations of natural environmental conditions, these studies will be inherently more

Figure 2. Physical containment of GEMs within a lysimeter structure. Lysimeters were 4 m deep by 2.5 m in diameter and filled with polyaromatic hydrocarbon–contaminated soil to study bioremediation-monitoring capabilities of an introduced GEM (33).

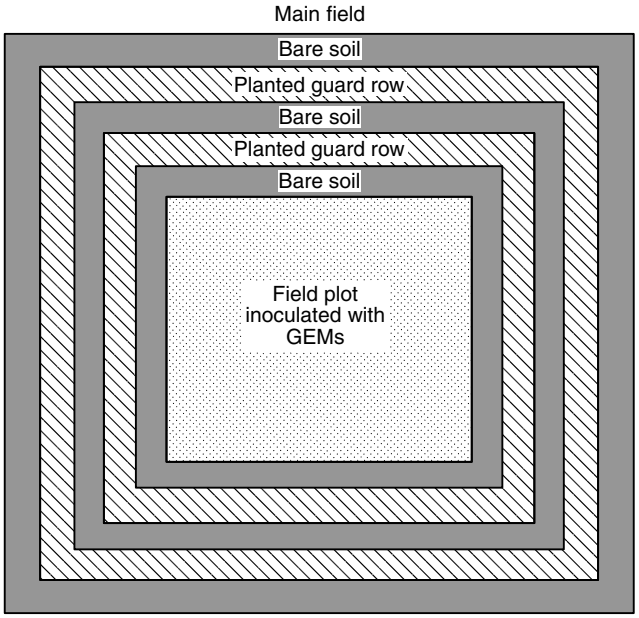

Figure 3. Typical containment design for GEMs released into agricultural crop plots.

indicative of actual field performance and ecological consequence than that achievable in conventional laboratory microcosms.

Biological Containment

One means of bypassing structural containment of GEMs is to design GEMs that are susceptible to biological containment. These GEMs are equipped with "suicide genes" that can be induced when necessary for eradication control. Induction of suicide genes such as *hok*, *gef*, and *relF* cause cell wall perturbations and subsequent death. The simplest biological containment strategies use suicide genes linked to genetic elements that are inducible in the presence of certain chemicals or substrates or by environmental factors such as temperature or nutritive status. For example, the *lac* gene is turned on in the presence of the chemical isopropyl-β-D-thiogalactoside (IPTG). Linking the *lac* gene promoter element to a suicide gene produces a genetic control mechanism in which the addition of IPTG turns on the suicide gene, thereby initiating cell death (90). In theory, IPTG can be added to the field test area and GEMs containing the *lac*–suicide gene construct will be eradicated. However, flooding a site with a control chemical is usually not environmentally or economically feasible and, because of high mutation rates, many of the cells develop suicide resistance making the degree of killing insufficient for adequate risk acceptance (91). The system can also work in reverse, where cell death by suicide genes is induced in the absence of a chemical rather than its presence (92). In bioremediation field releases such a suicide system would be advantageous because the GEMs could be designed to survive only as long as the environmental contaminant was present, after which the suicide genes would be induced to initiate self-eradication. But again, killing efficiency is unsatisfactory because of high mutation rates. Additionally, because many of the suicide vectors are plasmid-based, gene transfer risks increase. This problem has been alleviated in some systems by inserting the vector into the chromosome (93,94). Gene-transfer risks are also increased due to the large amounts of free recombinant DNA present following mass cell lysis. Attempts to overcome this obstacle have led to methods in which DNA-destroying nuclease genes are incorporated into GEMs in a fashion similar to suicide genes (95). Using this suicide scheme, GEMs are not only conditionally killed but their recombinant DNA is destroyed as well. In all of these suicide systems, however, complete eradication remains unattainable owing to the evolution of resistant mutant populations. Therefore, biological containment must be recognized as a viable method for minimizing, but never eliminating, GEM populations released into the environment, with risk factors being evaluated correspondingly.

Improved Monitoring Methods

Conventional methods for the detection and monitoring of GEMs after environmental release relied on growth and cultivation on nutrient media. Because of viable but nonculturable states of introduced populations, the need for selective markers such as antibiotic resistance, and bacterial numbers that fell below mediocre detection limits, culture methods have largely been replaced or supplemented with molecular methods. Primary among these is the polymerase chain reaction (PCR) technique that sensitively detects specific gene sequences rather than the microorganism itself (96). Thus both culturable and nonculturable microorganisms can be identified. DNA is extracted from the environmental matrix and a marker gene sequence specific to the GEM is amplified in the PCR reaction to increase detection capacity. Marker genes can consist of any unique DNA sequence that would be universally absent from the environmental introduction site. Often, markers consist of the engineered DNA sequence itself. Markers can also use gene sequences indigenously specific to the microbe, for example, 16S or 23S ribosomal RNA gene segments or the intergenic regions between these segments (97). Once amplified, PCR products are typically identified as being of GEM origin by applying DNA-hybridization techniques or, alternatively, a greater degree of sensitivity can be achieved by applying nested PCR, whereby another PCR reaction is performed to detect a specific sequence internal to the original PCR sequence (77,96). PCR was used in a soil field release experiment to detect fewer than 20 culturable Rhizobia cells per gram of soil (98) and in a soil microcosm to demonstrate that PCR was capable of detecting nonculturable microbial populations (99). New PCR techniques using fluorescently labeled probes (TaqMan, molecular probes) promise to increase detection limits even further, providing highly sensitive methods for GEM monitoring (100).

PCR can also be used to assess the effects of introduced GEMs on indigenous microbial populations. The most commonly used technique is discontinuous gradient gel electrophoresis (DGGE), which maps a microbial community

by each of its member's unique 16S ribosomal DNA fingerprint (101). The resulting pattern can be followed throughout a field release study to monitor alterations in community structure possibly attributable to GEM introduction. Various other similarly functioning PCR-based methods are also available for estimating microbial community diversity [amplified ribosomal DNA restriction analyses (ARDRA), terminal-restriction fragment length polymorphism (T-RFLP), ribosomal intergenic spacer analysis (RISA), length heterogeneity PCR (LH-PCR), repetitive extragenic palindromic PCR (REP-PCR), random amplified polymorphic DNA PCR (RAPD-PCR), arbitrarily primed PCR (AP-PCR), enterobacterial repetitive intergenic consensus PCR (ERIC-PCR)] (96,102–104). Methods that characterize bacterial populations based on energy metabolism (BIOLOG) (105) and phospholipid fatty acid composition (PLFA) are also accessible (106).

Although PCR has become the backbone of environmental monitoring methods, there still remain significant limitations to its use in field release studies. Primary among these is the presence of PCR inhibitors in most environmental samples, the commonest of which are humic acids that interfere with the Taq polymerase enzyme used in the PCR reaction (107). These contaminants can be partially removed through a series of purification steps but their complete elimination is impossible. Also problematic is the inability to completely extract and isolate total nucleic acid present within an environmental sample. Therefore, detection will always represent an underestimate of actual microbial numbers.

An alternative to PCR is to make use of immunological antibody–antigen reactions for detecting field released GEM populations (108). Monoclonal and polyclonal antibodies can be synthesized against GEM-specific antigenic determinants and used in enzyme-linked immunosorbent assays (ELISA). This technique has successfully been used to detect a recombinant *P. putida* strain in a mixed freshwater microbiota (109).

Methods for detecting and monitoring GEMs without removal from their introduced environment are also available. These methods typically use GEMs that have been engineered to bioluminescence, using either the *lux* or *luc* gene inserts. The presence of a light signal can be correlated with cell viability. Insertion of light detectors such as photon-counting modules within the field release site allows direct online visual monitoring of GEM populations (33).

Reducing Gene Transfer Risks

Early GEM constructs contained engineered gene sequences localized on plasmids, which are easily translocated through horizontal gene transfer events. Consequently, genetic techniques were developed for integrating engineered DNA sequences into the more stable host cell chromosome resulting in lowered gene transfer frequencies. Using disabled or truncated transposon vectors for inserting genetically altered DNA into the chromosome while preventing its exit further reduced gene transfer risks, as did the subsequent development of the DNA-degrading suicide genes (95,110). Further advances have been achieved using a system in which two genes, *colE3* and *immE3*, are used in concert to control gene transfer (93,111). The *colE3* gene encodes for colicin, an enzyme that inhibits protein synthesis and causes cell death. The *immE3* (immunity) gene product neutralizes colicin activity; thus in its presence, the colE3 gene product is rendered inactive and cell death does not occur. Therefore, only if both genes are present will the cell survive. In the genetic engineering strategy, the *colE3* gene is linked to the recombinant genes, whereas the *immE3* gene is placed far from the recombinant genes. If the recombinant genes undergo a gene transfer event, the *colE3* gene will follow, but the likelihood of the distant *immE3* gene being similarly transferred is remote. Thus, the recipient cell receives the recombinant genes, the *colE3* gene but not the *immE3* gene, and dies as a consequence. In test systems, gene transfer frequencies were reduced by four to five orders of magnitude. Thus, such a strategy could significantly decrease, but again not eliminate, gene transfer rates between released GEMs and indigenous microbial populations.

CONCLUSION

The future potential for GEMs in environmental applications will rely on a balanced assessment of possible adverse effects versus proposed benefits. On the basis of field release studies already performed, hazards seem remote, but owing to inherent difficulties in properly assessing GEM presence and in monitoring their diverse environmental interactions, a comprehensive and infallible evaluation of potential risks cannot, as of yet, be achieved. It is therefore necessary to exhaustively study GEMs within controlled habitats before any sort of uncontrolled or semicontrolled release to provide not a guaranteed assurance of no risk but rather a carefully derived prediction of GEM behavior on release, based on data gathered using the most sensitive techniques available. Once the ecological consequences of GEM introduction have been established, then proper regulation and management can be correspondingly applied in a manner in which some degree of uncertainty is necessarily accepted, yet overly precautionary restraint is avoided. GEMs show great promise in their abilities to bioremediate environmental pollutants and enhance and protect agricultural crops, and only through field release trials will their ultimate application be realized.

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FILAMENTOUS BACTERIA IN ACTIVATED SLUDGE: CURRENT TAXONOMIC STATUS AND ECOLOGY

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Different applications of the activated-sludge treatment process are employed worldwide for the biological treatment of wastewater. However, from the beginning of its development, sedimentation problems, in particular sludge bulking and foaming, have been observed (1). The ability of the sludge to settle is assessed by measuring the

sludge volume index (SVI), which is the volume occupied by 1 g of sludge. An SVI higher than 150 mL/g^{-} is often found in connection with one of the main problems, filamentous bulking, which is caused by the overgrowth of filamentous bacteria in the aeration basin. As a result, sludge settling and compaction are hindered by the filamentous cell structures of the organisms, ultimately leading to a decreased effluent quality (2–4; see FILAMENTOUS BULKING IN ACTIVATED SLUDGE, CONTROL OF).

Various factors such as low food to microorganism (F/M) ratios, low dissolved oxygen concentration, low nutrient conditions, or the presence of high sulfide levels may promote the growth of different types of filamentous bacteria physiologically adapted to the respective conditions. The main cause of bulking in wastewater treatment plants, however, seems to be low F/M ratios (4,5). In Table 1, a summary of factors favoring the occurrence of filamentous bacteria in activated sludge (4) is given.

It is generally accepted that basic physiological differences between filamentous and floc-forming bacteria are responsible for the mass occurrence of filamentous bacteria under certain environmental conditions that cause sludge bulking. In most cases, filamentous bacteria are able to survive better under low oxygen concentrations and relatively low nutrient conditions. In addition to sludge bulking, the occurrence of foaming in activated-sludge plants has been documented in wastewater treatment plants around the world (6; see ACTIVATED SLUDGE — FOAMING). Foaming is also caused by the overgrowth of filamentous bacteria (mainly actinomycetes) in the aeration basin often at high mean cell residence times, resulting in a brown viscous scum. Some of the most common bacteria encountered in foam are *Candidatus* "*Microthrix parvicella*," *Gordonia amarae*, *Skermania piniformis*, and sometimes *Rhodococcus* spp. or *Streptomyces* spp. The ability to produce biosurfactants and to grow on hydrophobic substrates such as hydrocarbons, their higher resistance to desiccation and ultraviolet radiation, and their ability to store polyphosphates and poly-β-hydroxybutyric acid may represent selective advantages of actinomycetes in comparison with the floc-forming microorganisms. However, more detailed physiological studies of the bacteria causing bulking and foaming have been hampered by the fact that the majority of the microscopically observable filamentous bacteria are very difficult to culture (or even not yet culturable) under laboratory conditions. Since the pioneering work of Eikelboom and coworkers (2,3), who on the basis of morphological characteristics and simple staining techniques, described seven groups containing in total 26 morphological types of filamentous organisms, several attempts have been made to develop new methods for isolation, cultivation, and also identification of these unique bacteria. However, it is now clear that morphological criteria, which are still widely used, are not reliable enough to allow exact identification of a filamentous bacterium. For example, several phylogenetically unrelated filamentous bacteria cannot be distinguished from each other morphologically (e.g., bacteria erroneously lumped together as "*Nostocoida limicola*" or Type 1863, illustrated later). On the other hand, some species, such as *Sphaerotilus natans* and *Thiothrix* sp., occur either as single cells or as filaments depending the environmental conditions (7). To overcome the limitations of the existing identification schemes, several new approaches including fluorescently labeled 16S rRNA-targeted probes (7–9) and polyclonal and monoclonal antibodies (10,11) have been developed for the in situ detection and identification of these filamentous bacteria in activated sludge. Their precise identification is an important step in finding the reasons for a particular bulking or foaming event in a wastewater treatment plant. As an example, the occurrence of "*M. parvicella*" is often associated with low F/M ratios, whereas *S. natans* can indicate low dissolved oxygen in the aeration basin (3,4). As a result of a lack of knowledge of the identity and in situ physiology of the different filamentous microorganisms, excessive overgrowth of these bacteria in wastewater treatment plants is often combated by nonspecific measures like the use of oxidants (e.g., chlorine, hydrogen peroxide) or flocculants (e.g, iron, synthetic organic polymers, lime).

The aim of this article is to give an overview of the taxonomic status of the different filamentous bacteria and their ecology, with special emphasis on comparing classical approaches with modern molecular biological methods, including their in situ detection and identification.

Table 1. Factors of the Activated Sludge Process Favoring the Occurrence of Filamentous Bacteria (4)

1. Sludge age (mean cell residence time, MCRT; or food to microorganism ratio (F/M)):
   - — Low dissolved oxygen (DO)
   - — Low F/M ratio in completely mixed and continuously fed systems
2. Aeration basin configuration (wastewater feeding regime)
   - — Nature of organic substrate (soluble versus particulate; readily biodegradable versus slowly biodegradable)
3. Presence of initially unaerated zone (anoxic or anaerobic)
   - — Septic wastewater, nutrient deficiency, low pH (<6.5), seeding from surfaces, surface trapping, and foam recycle.

METHODS FOR THE DETECTION OF FILAMENTOUS BACTERIA INVOLVED IN SLUDGE BULKING OR FOAMING

Microscopy

The classical approach to the identification of filamentous bacteria was introduced by Eikelboom (2), who investigated thousands of activated-sludge samples using the phase contrast microscope. Differentiation of the different filaments was based on cell morphological characters, such as the presence or absence of a sheath or a slime layer, true or false branching, the length and shape of the filaments, and the diameter, length, and shape of the cells. Furthermore, the presence of cell inclusions (poly-β-hydroxybutyrate, polyphosphate, or sulfur), the capability for gliding motility, and the reaction to simple staining procedures were used as diagnostic criteria to classify the organisms into 26 types of filamentous organisms grouped into 7 assemblages (2,3) (Table 2). On the

basis of these findings, simple identification keys (2–4,12) were constructed.

These, however, do not represent a phylogenetic-based taxonomy of these filamentous organisms, but the identification keys are still useful for an initial characterization of these organisms, because the microscopic methods are relatively easy to use and no special laboratory equipment is required. For an exact identification, these methods cannot be recommended, because morphology and staining reactions are insufficient criteria to differentiate between phylogenetically unrelated bacteria. For example, *Thiothrix* filaments without sulfur granules cannot be morphologically distinguished from *Leucothrix* (15). Furthermore, nonfilamentous growth forms are documented for *H. hydrossis*, *S. natans*, *Thiothrix* sp., *Leucothrix mucor*, "*M. parvicella*," and Eikelboom Type 1863 (16–18). In addition, it has been suggested that the sheath forming capacity of *L. mucor* is plasmid encoded and thus can easily be lost (15). Another problem limiting the applicability of the traditional identification schemes is that certain filamentous organisms such as "*M. parvicella*," Eikelboom Type 1863, and others display variable gram stain reactions.

Table 2. Filamentous Organisms in Activated Sludge Grouped and Listed According to Eikelboom (2)

Group I:	Sheath Forming, Gram-negative Bacteria
	1. *Sphaerotilus natans*
	2. Type 1701
	3. Type 1702
	4. *Haliscomenobacter hydrossis*
	5. Type 0321
Group II :	Sheath Forming, Gram-positive Bacteria
	6. Type 0041
	7. Type 0675
	8. Type 1851
Group III:	Sheathless, Curled, Multicellular Bacteria Resembling Blue-Green Algae
	9. Type 021N
	10. *Nostocoida limicola*
	11. Cyanophyceae
Group IV:	Slender, Coiled Bacteria
	12. *Microthrix parvicella*
	13. Type 0581
	14. Type 0192
Group V:	Straight, Multicellular, Gram-negative Bacteria
	15. Type 0803
	16. Type 1091
	17. Type 0092
	18. Type 0961
Group VI:	Filamentous Bacteria Motile by Gliding
	19. Type 0914
	20. *Beggiatoa* spp.
	21. Type 1111
	22. Type 1501
Group VII:	Additional Types
	23. Type 1863
	24. Type 0411
	25. Fungi
	26. *Nocardia* sp.

Note: For details about morphological characters see (2–4). Additional types of filamentous organisms are documented in the comprehensive manuals of Lemmer and Lind (13) and Eikelboom and Geurkink (14).

Immunological Techniques

The application of specific antibodies allows a direct detection of certain filamentous bacterial species. Immunological techniques using fluorescent antibodies were used to identify actinomycetes (19–21), *S. natans* (10), *G. amarae* (17), and *Thiothrix* spp. (11) in activated sludge. Because of their high sensitivity and speed, these methods are attractive approaches to overcome the limitations of the traditional microscopic techniques (11). However, the applicability of the immunofluorescence approach for the analysis of microbial activated-sludge communities is limited by several factors. First of all, for the development and testing of new antibodies, culturable reference organisms are necessary. Thus, this approach is currently not suitable for the majority of the described filament types. In addition, unspecific binding of the antibodies to organic particles and fungal spores can lead to high levels of background fluorescence. Furthermore, penetration problems of the relatively large antibodies through the extracellular polymeric substances of activated-sludge flocs can result in detection problem for cells within these aggregates.

Detection and Identification of Filamentous Organisms by Using Ribosomal RNA (rRNA) Targeted Oligonucleotide Probes

16S rRNA gene sequence information can easily be obtained for cultured filamentous bacteria using standard techniques (8), but can also be retrieved for not yet culturable filamentous species either after their enrichment by micromanipulation (22) or directly from activated sludge (23,24; see ACTIVATED SLUDGE — THE MICROBIAL COMMUNITY). Using these techniques, the 16S rRNA gene sequences of many filamentous bacteria have been determined (Figs. 1, 2, and 3). On the basis of this data set, for some of the filamentous species, specific 16S rRNA-targeted oligonucleotide probes were developed for their cultivation-independent detection and quantification, using either membrane hybridization (21) or fluorescence in situ hybridization (FISH) (7,8). Table 3 lists currently available probes for the detection of bacteria involved in bulking and foaming. Because very rigorous methods can be applied for total RNA extraction, membrane hybridization is well-suited also for the detection of gram-positive bacteria with rigid cell walls such as the mycolic acid-containing filamentous actinomycetes (mycolata). In contrast to membrane hybridization, FISH allows us to specifically stain and visualize the filaments within the activated-sludge biomass (Fig. 4). If combined with confocal laser scanning microscopy (24), the specifically stained filaments can be quantified using recently developed image-analysis tools (25,26). Although standard FISH protocols can be applied for the detection of all gram-negative and many gram-positive filamentous bacteria (7,27), there is a need for the development of

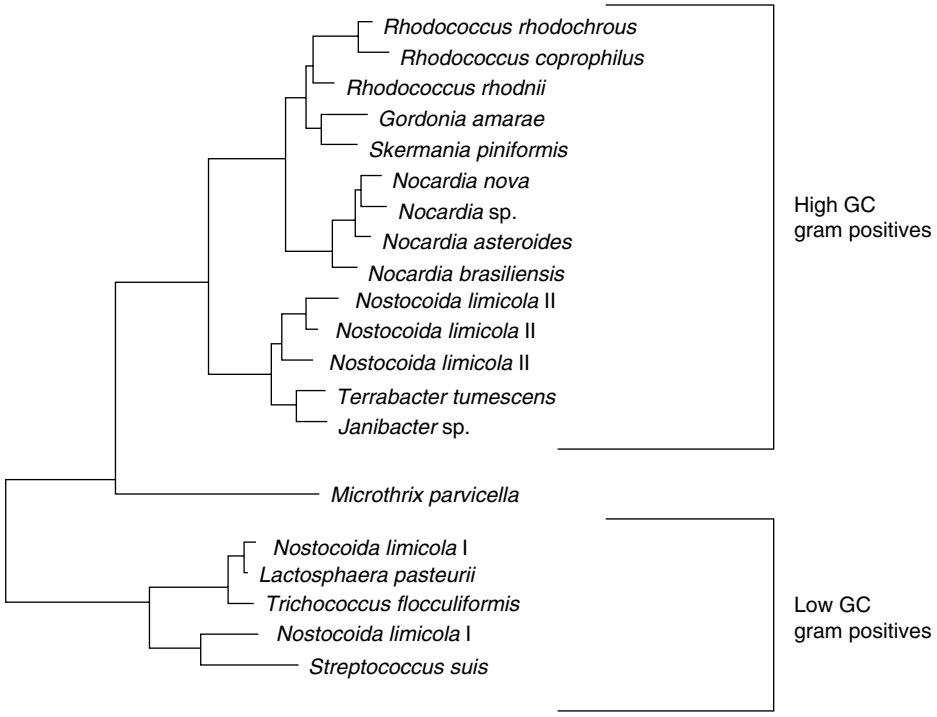

Figure 1. Phylogenetic 16S rRNA–based tree showing the affiliation of gram-positive filamentous bacteria.

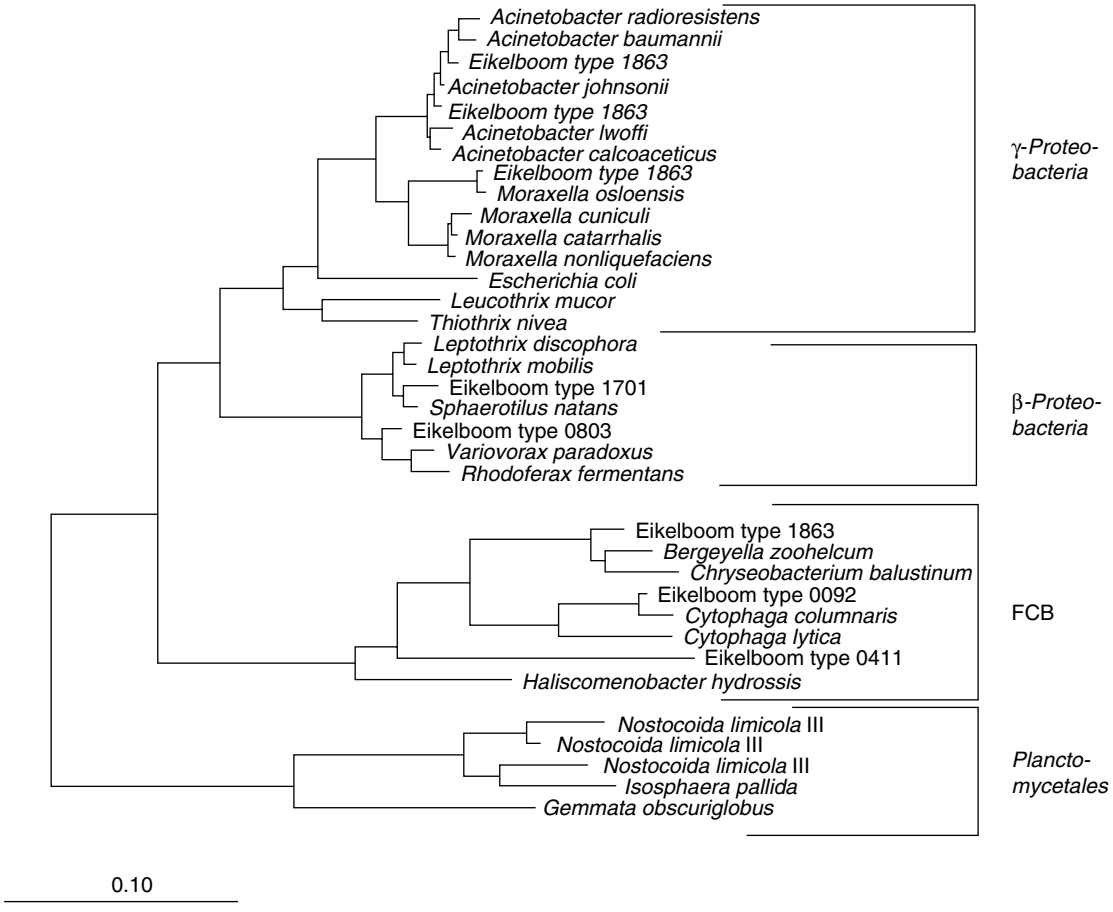

Figure 2. Phylogenetic 16S rRNA–based tree showing the affiliation of gram-negative filamentous bacteria. FCB, *Flexibacter-Cytophaga-Bacteroides* phylum.

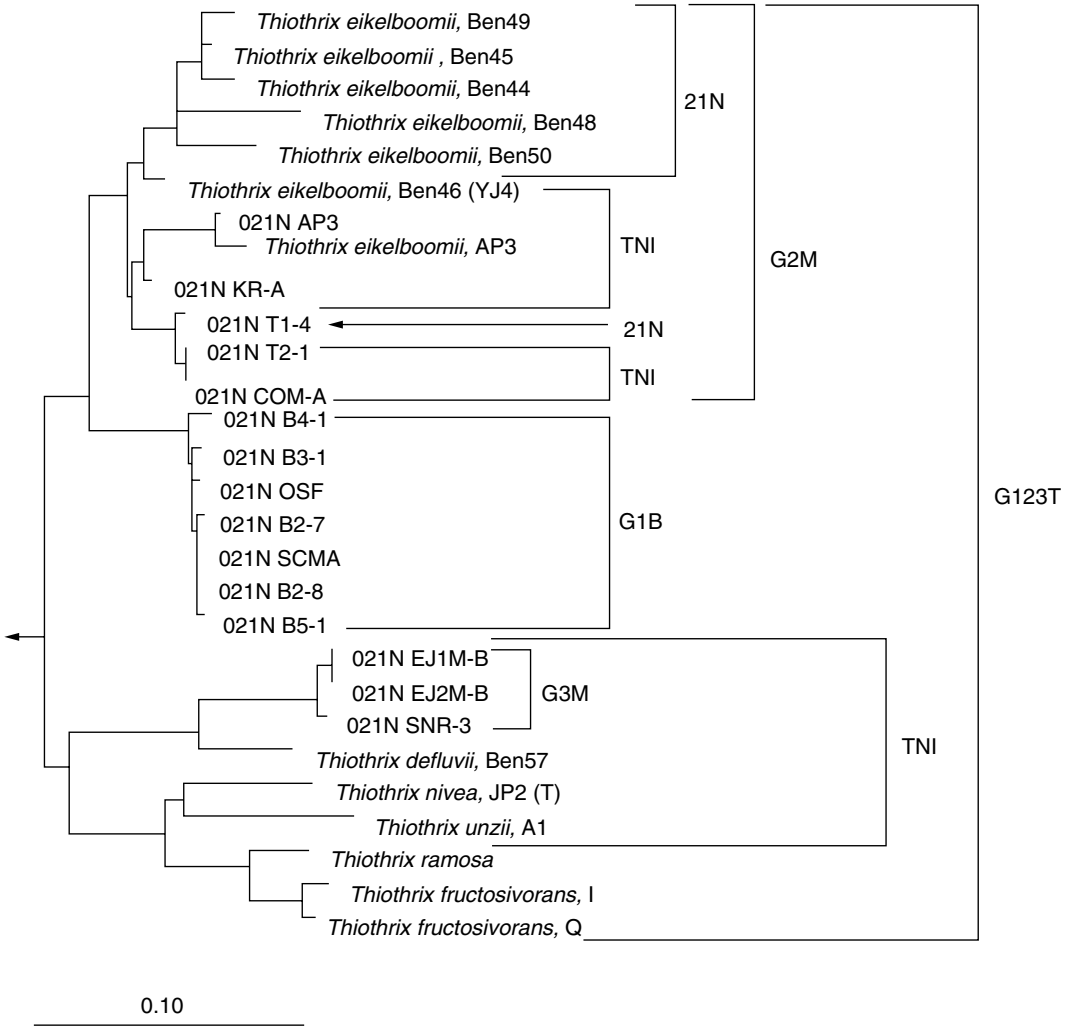

Figure 3. Phylogenetic 16S rRNA–based tree of the *Thiothrix*-Eikelboom Type 021N cluster. The specificity of the published oligonucleotide probes for these bacteria (7,8) is indicated by brackets and arrows. For probe sequences please refer to Table 3.

specific permeabilization protocols for some gram-positive organisms causing bulking and foaming (21,28,29).

ISOLATION AND CULTIVATION OF FILAMENTOUS BACTERIA INVOLVED IN SLUDGE BULKING OR FOAMING

Isolation Methods

The isolation of the majority of filamentous organisms, which is a prerequisite for the study of their nutritional requirements, is still a difficult task. Only a few exceptions to this rule that are relatively easy to isolate can be listed here: representatives of the *Sphaerotilus-Leptothrix* group, *Thiothrix* species, *G.* (formerly *Nocardia*) *amarae*, *S. piniformis* (formerly *Nocardia pinensis*), and some other nocardioform bacteria (16,35–38).

In case of successful isolation, often single isolates of various filamentous bacteria were physiologically characterized and described in detail (2,37,38). Direct dilution plating of activated sludge on different media is often unsuccessful (37–39) because the generally slow-growing filamentous organisms are overgrown by nonfilamentous rapidly growing bacteria. To overcome this problem, van Veen (40) developed a method for isolation of filaments on the basis of specific dilution procedures. An additional method was suggested by Williams and Unz (37) who used additional pretreatment procedures before plating samples on solid media. These methods were adapted and modified by Ziegler and coworkers (38). The pretreatments used in this study included washing the sludge several times after centrifugation, sonication, and subsequent dilution, gathering of filaments by using a very thin sterile pipette under a binocular and subsequent washing steps, and ultra turrax pretreatment or several minutes of whirl mixing in the presence or absence of a few small glass beads in a tube.

Another method for selective isolation of filamentous bacteria is micromanipulation with special microtools under a microscope. This technique was used, for example, for the isolation of *Gordonia* (*Skermania*) (39) and "*M. parvicella*" (41) and a modified method was applied

Table 3. Probe Sequences and Target Sites for Different Filamentous Bacteria

Target Organism	Probe Name (ODP name)[$]	Sequence	Target Site* 16S rRNA Position	Reference
"*Microthrix parvicella*"				
	MPA60	5′-GGATGGCCGCGTTCGACT-3′	60–77	106
	MPA223	5′-GCCGCGAGACCCTCCTAG-3′	223–240	106
	MPA645	5′-CCGGACTCTAGTCAGAGC-3′	645–661	106
	MPA650	5′-CCCTACCGGACTCTAGTC-3′	650–666	106
Gordonia	(S-G-Gor-0596-a-A-22)	5′-TGCAGAATTTCACAGACGACGC-3′	596–617	107
Gordonia amarae	(S-G-G.am-0192-a-A-18)	5′-CACCCACCCCCATGCAGG-3′	192–209	107
Gordonia amarae	(S-G-G.am-0205-a-A-19)	5′-CATCCCTGACCGCAAAAGC-3′	205–223	9
Gordonia amarae group 1	(S-G-G.am1-0439-a-A-19)	5′-TCGCGCTTCGTCCCTGGTG- 3′	439–457	9
Gordonia amarae group 2	(S-G-G.am2-0439-a-A-19)	3′-CGAAGCTTCGTCCCTGGCG-5′	439–456	9
Mycobacterium complex	(S-*-Myb-0736-a-A-22)	5′-CAGCGTCAGTTACTACCCAGAG- 3′	736–757	107
Mycobacterium complex	(S-*-Myb-0736-b-A-22)	5′-CAGCGTCAGTTACTxCCCAGAG-3′[&]	736–757	107
Nocardioform actinomycetes	MLP	5′-AACCCATGCAGGCCGTAGTCC-3′	182–202	27
	DLP	5′-CCACCATGCGGCAGGAGCTCA-3′	182–202	27
	GLP1	5′-ATGCAGTGGAAGGTAATATC-3′	174–193	27
	GLP2	5′-AAGGGCAGGTCATATCCGGT-3′	178–197	27
	GLP3	5′-CCAACCATGCAGTCAGAGGTC-3′	182–202	27
'*Nostocoida limicola*'–like filaments				
	AHW183	5′-CCGACACTACCCACTCGT-3′	183–200	69
	Noli-644	5′-TCCGGTCTCCAGCCACA-3′	644–660	71
	PPx3-1428	5′-TGGCCCACCGGCTTCGGG-3′	1,428–1,447	71
	MC2-649	5′-CTCTCCCGGACTCGAGCC-3′	649–667	71
Sphaerotilus natans and some other bacteria	SNA	5′-CATCCCCCTCTACCGTAC-3′	665–673	7
Leucothrix mucor	LMU	5′-CCCCTCTCCCAAACTCTA-3′	652–669	7
Leptothrix discophora and some other bacteria	LDI	5′-CTCTGCCGCACTCCAGCT-3′	649–666	7
Thiothrix spp.[§]	TNI	5′-CTCCTCTCCCACATTCTA-3′	652–669	7
someType 021N[§]	21N	5′-TCCCTCTCCCAAATTCTA-3′	652–669	7
021N group 1[§]	G1B (S-*021Ng1-1029-a-A-18)	5′-TGTGTTCGAGTTCCTTGC-3′	1,029–1,046	8
021N group II[§]	G2M(S-*021Ng2-842-a-A-18)	5'-GCACCACCGACCCCTTAG-3′	842–859	8
021N group III[§]	G3M(S-*021Ng3-996-a-A-18)	5′-CTCAGGGATTCCTGCCAT-3′	996–1,013	8
Type 021N and *Thiothrix*[§]	G123T (S-**Thioth*-697-a-A-18)	5′-CCTTCCGATCTCTATGCA-3′	697–714	8
Thiothrix fructosivorans	TFR	5′-CTCCTCTCCCACACTCTA-3′	652–669	108
Haliscomenobacter hydrossis	HHY	5′-GCCTACCTCAACCTGATT-3′	655–672	7

*_Escherichia coli_ numbering (for details, see reference 8).
[$]OPD, oligonucleotide probe database.
[§]For details refer to Figure 3.
[&]x = 5-Nitroindole.

by Hornby and Horan (42) for isolation of other filaments. Details of the methods can be obtained from the original publications. The recommended methods for isolation of the most frequently seen filamentous organisms have been summarized by Kämpfer (43).

Cultivation of Filamentous Bacteria

Selected gram-positive filamentous bacteria, such as the nocardioform organisms, *G. amarae* and *S. piniformis*, and also *Rhodococcus rhodochrous*, can be cultivated on nutrient-rich media, such as yeast glucose agar and Tryptone-yeast extract agar (35,39,44,45). *Sphaerotilus natans* can also be grown on nutrient-rich media, such as Casitone-glycerol-yeast-autolysate (CGYA) (46) and media with moderate nutrient content like R2A agar (47).

Most of the gram-negative filamentous organisms, however, prefer media with low ($<0.5\ g\,L^{-1}$ of specific and/or complex carbon sources) or moderate nutrient concentrations (0.5 to $5\ g\,L^{-1}$ of specific and/or complex carbon sources) (37,38). Recommended media include I-medium, SCY-medium (sucrose-Casitone-yeast extract, 40), GC (glucose plus sulfide), AcS (sodium acetate plus sulfide), and SS-medium (sucrose plus sulfide, 48), LT (lactate), CGY (Casitone-glycerol-yeast-autolysate), and GS medium (glucose plus sulfide, 37), GMBN (glucose mineral base medium, 49), medium of Slijkhuis (50), and R2A medium (51). Aerobic incubation at 20 °C or 25 °C

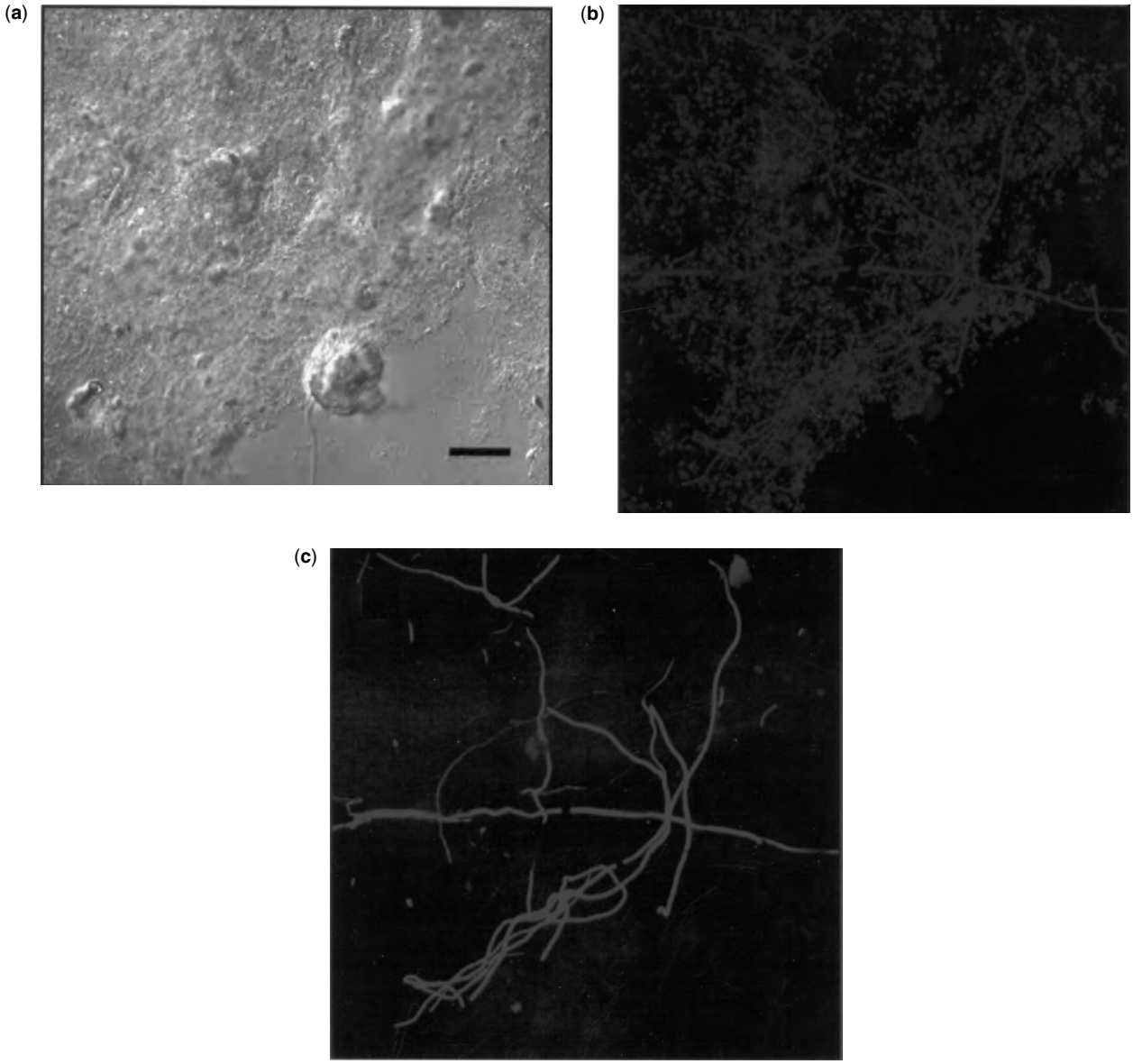

Figure 4. In situ identification of *Thiothrix*/Eikelboom Type 021N in activated-sludge flocs using confocal laser scanning microscopy. (**a**) Transmission micrograph. (**b**) FISH using probe GAM42a specific for the γ-subclass of *Proteobacteria*. (**c**) FISH with probe G123T specific for *Thiothrix*/Eikelboom Type 021N (8). Bar represents 20 μm. Micrographs were recorded by Matthias Horn. See color insert.

(sometimes up to 30 °C) is preferred (up to six weeks). Resulting growth of filamentous colonies can be followed under a binocular (40x) or a microscope (160x). A table summarizing the isolation media and conditions is given by Kämpfer (43).

TAXONOMY OF FILAMENTOUS BACTERIA CAUSING FOAMING AND BULKING PROBLEMS IN THE ACTIVATED SLUDGE PROCESS

On the basis of comparative 16S rRNA sequence analysis, the phylogeny of many filamentous microorganisms has been elucidated. Figures 1 to 3 provide an overview of the phylogenetic affiliation of the most important filamentous bacteria, which are discussed in detail in the following text.

Actinomycetales

"Microthrix parvicella." "*Microthrix parvicella*" is a gram-positive, straight filamentous bacterium, which is very difficult to cultivate and to maintain (3,52). On the basis of 16S rDNA sequencing, "*M. parvicella*" was found to be a novel deep-branching member of the actinomycetes within the gram-positive phylum of the domain bacteria (41,52) (Fig. 1).

Morphologically it can be characterized by its thin filaments (0.3 to 0.7 μm diameter), which can be hundreds of micrometers long with a characteristic winding appearance (Fig. 5; 2–4,41,50,52). Individual cells in the filaments are difficult to detect by light microscopy. On the basis of electron microscopical studies (52), filaments are clearly gram-positive, although some of the cells (presumptively empty, autolyzed cells) stain gram-negative. Cells show intracellular inclusions of polyphosphate detectable by Neisser staining. Micromanipulation is recommended for their isolation (41). In the comprehensive studies of Blackall and coworkers (41,52), a few "*M. parvicella*" isolates grew best on fresh R2A or non-Tween medium (NTM), although even on these "optimum" media very poor growth was observed. Although some phenotypic features of "*M. parvicella*" have been reported by Slijkhuss and coworkers (53), no reference strains were deposited in culture collections. Because of the lack of information of phenotypic features, the name "*Candidatus Microthrix parvicella*" was proposed (52) on the basis of the suggestion of Murray and Stackebrandt (54) that the provisional status Candidatus be used for incompletely described prokaryotes. At present, no reference strains are available from culture collections.

"*Microthrix parvicella*" is found in activated sludge plants around the world, where it is responsible for solids-separation problems both in bulking and foaming (6,52). This organism is one of the most frequently identified causative agents for solids-separation problems in wastewater treatment plants (41,52,55) and can be found in both mixed liquors and foams. According to Wanner and Grau (12), "*M. parvicella*" can occur in anaerobic and aerobic zones of activated-sludge plants and is able to accumulate storage material, which allows successful competition with floc-forming bacteria. This may explain why selectors do not always control SVI to very low levels, in cases in which "*M. parvicella*" is the causative bulking agent (4). In systems with anaerobic zones, serious bulking problems caused by "*M. parvicella*" were reported (56). "*Microthrix parvicella*" is often associated with low F/M ratios. So far, "*M. parvicella*" has never been detected outside activated-sludge plants. However, the organism seems to be also present in activated sludge plants without bulking or foaming problems (6,55). There are also a number of reports implicating "*M. parvicella*" as the main causative organisms of foaming in South Africa, Australia, and Europe (6, and references therein). By using microautoradiography on activated sludge samples (57), it was shown that from six different substrates investigated (acetate, glucose, ethanol, glycine, leucine, and oleic acid), "*M. parvicella*" consistently took up only oleic acid. The uptake was observed under aerobic and anaerobic conditions, although it is still uncertain whether "*M. parvicella*" can grow anaerobically (57). "*Microthrix parvicella*" seems to be a specialized lipid consumer, able to take up long-chain fatty acids under anaerobic conditions and subsequently use the storage material for growth when nitrate or oxygen is available as electron acceptor (58). Some information on the physiology of "*M. parvicella*" was obtained from the studies of Slijkhuis and coworkers (53). First isolated by Van Veen (40) on a mineral salts medium containing a low glucose concentration (0.15 $g\,L^{-1}$) and small amount of thiamine and biotin, in the studies of Slijkhuis and coworkers (53), the organism was found to be more readily culturable on activated sludge medium (2). Carbon sources such as glucose, fructose, lactic acid, succinic acid, and citric acid were not used, but instead "*M. parvicella*" uses oleic acid and its polyoxyethylenesorbitan ester (Tween 80) as the sole source of carbon and energy (at low concentrations <0.15 g/L) (53). Shorter-chain fatty acids were also used, but only in the presence of Tween 20. As a result, the lipid composition of "*M. parvicella*" was very high (sometimes approaching 35% of the dry weight of the organism (50)), because the organism rapidly used (five days) the oleic acid and palmitic acid moieties of Tween 80 and Tween 60 and stored these as long-chain fatty acid esters (53). Because these esters were presumed to constitute the main components of large intracellular lipid globules detected microscopically (53), the authors suggested that the ability of "*M. parvicella*" to store long-chain fatty acids in the early growth stages would favor it in competition for substrates in activated-sludge plants containing such long-chain fatty acids. Thus, extensive growth of "*M. parvicella*" in wastewater treatment plants could be controlled by decreasing the amount of fats in the influent by physical means. Also, nitrogen and sulfur requirements for this organism were demonstrated. Nitrogen is required in a reduced form, for example, as ammonium sulfate. Consequently, potassium nitrate could not be used as a nitrogen source for growth (50). Although growth was observed at 8 °C, "*M. parvicella*" grows optimally at 25 °C, but very weak or no growth is found at 35 °C (50). "*Microthrix parvicella*" was found to grow in media with initial pH 7.5, 7.7, and 8.0, but no growth was detectable when the initial pH of the medium was 7.1 or lower.

Growth of "*M. parvicella*" is suppressed under continuous oxygen supply but not with intermittent aeration (59). Two possible explanations have been suggested. "*Microthrix parvicella*" might possess a high affinity for oxygen at low concentrations and can therefore successfully compete for this electron acceptor under conditions of reduced oxygen supply (50). Alternatively, "*M. parvicella*"

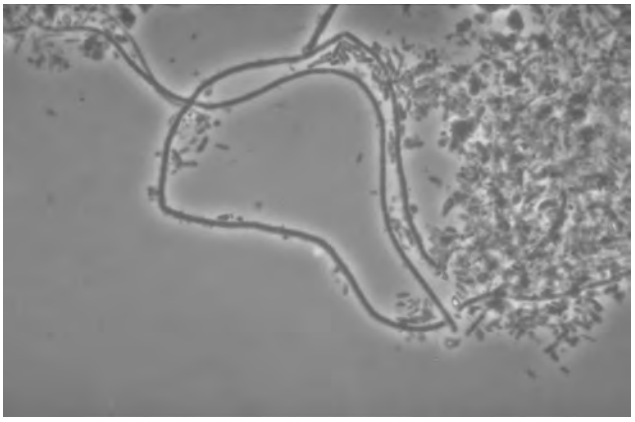

Figure 5. Filaments of "*Microthrix parvicella*" (×1250). Photo by D. Eikelboom, TNO-MEP, Apeldoorn.

might require reduced nitrogen and sulfur compounds for growth and the concentration of these in a fully aerated mixed liquor may be too low (59). It must be stressed that Blackall and coworkers (41,52), in spite of numerous attempts, could not grow "*M. parvicella*" cells according to the instructions of Slijkhuis and coworkers (53), a failure that might indicate that different organisms might have been investigated by the two research groups. In a new approach for kinetic and physiological characterization (60), two strains were studied in detail, and it could be shown that both strains were K_S-strategists with a high substrate affinity, are able to store PHA under aerobic/anoxic/anaerobic conditions, and have a resistance to long periods of anoxic/anaerobic conditions. For further understanding of the metabolism of "*M. parvicella*" and its ecological role, more pure-culture studies are urgently needed.

Other Actinomycetes

Actinomycetes have been frequently reported in connection with the formation and stabilization of biological foams in the activated-sludge process (Figure 6; 6,35,39,45,61). Although several species belonging to different genera have been detected in treatment plants all over the world, such as *Actinomadura* spp., *Micromonospora* spp., *Nocardia asteroides*, *Nocardia caviae*, *R. rhodochrous*, *Rhodococcus ruber*, *Streptomyces* spp., and some others, the most frequently found organisms are *Gordonia amarae* (former *Nocardia amarae*) and *Skermania piniformis* (former *N. pinensis*, 62). In contrast to "*M. parvicella*," most of these can be relatively easily isolated with standard spread-plate techniques, although micromanipulation can facilitate their isolation (39). The majority of them grow best at 25 to 30 °C even on relatively nutrient-rich media ($>5\ g\,L^{-1}$ of specific and/or complex carbon sources), such as yeast glucose agar and tryptone-yeast glucose agar, and can be maintained by lyophilization.

Several phylogenetic studies based on 16S rRNA sequences have been published (62–64). Further studies have clearly demonstrated the extent of actinomycete diversity in activated sludge foaming (9,65,66) and many probably still await identification (67).

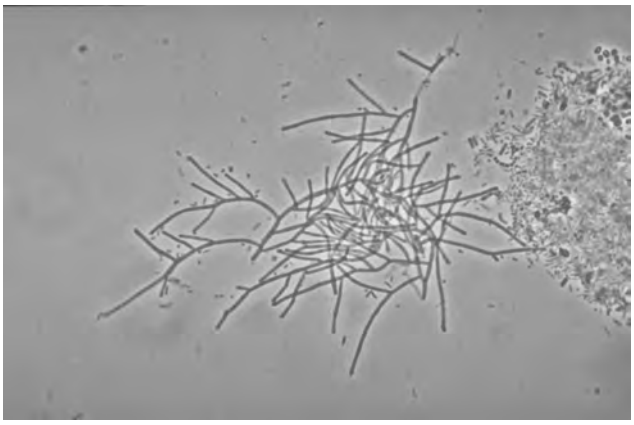

Figure 6. Filaments of actinomycetes (x 1250). Photo by D. Eikelboom, TNO-MEP, Apeldoorn.

For the identification at the genus or even species level, chemotaxonomic characterization (analyses of isoprenoide quinones, polar lipids, cell wall diamino acids, and fatty acids) is essential (62,63), in addition to morphological and physiological characterization. Cell wall types and further chemotaxonomic features differentiating actinomycetes at the genus level are shown in Table 4. Reference strains have been deposited in culture collections and should be included for control purposes in identification procedures of unknown isolates.

Gordonia amarae. *Gordonia amarae* is an aerobic gram-positive organism, producing a substrate mycelium with moderately branching vegetative hyphae that does not fragment in undisturbed cultures. Originally described as *Nocardia amarae* by Lechevalier and Lechevalier (44), it became evident from chemotaxonomic and other taxonomic studies that this species revealed note differences from the authentic *Nocardia* species with respect to the presence of MK-9(H_2) (63) as its major menaquinone, serological cross reactions, and sensitivity to nocardiophages. On the basis of 16S rRNA sequence analyses, the genus name *Gordonia* was revived, and it was subsequently shown that organisms classified as *N. amarae* belong clearly to the genus *Gordonia* (63).

Secondary branching of *G. amarae* is rare. The filaments are partially acid-fast. The substrates esculin, Tween 20, Tween 40, Tween 60, and urea are hydrolyzed, but not casein, elastin, gelatin, hypoxanthine, adenine, tyrosine, or xanthine. The organism can reduce nitrate to nitrite. A wide variety of carbon sources (sugars, alcohols, and organic acids) is used (63).

Gordonia amarae possess cell wall chemotype IV, with mesodiaminopimelic acid as the only diamino acid, and arabinose and galactose as major cell wall sugars. The predominant isoprenolog is MK-9(H_2), and polar lipids include phosphatidyl ethanolamine, diplphosphatidyl glycerol, phosphatidyl glycerol, phosphatidyl inositol, and phosphatidyl inositol mannosides. The species contains major amounts of straight-chain, saturated and unsaturated fatty acids, and tuberculostearic acid. Mycolic acids with 46 to 54 carbons and up to three double bonds are present. On pyrolysis gas chromatography of mycolates 16 : 0, 18 : 0, 16 : 1, and 18 : 1 fatty acid esters are released (Table 4). The G + C content of the DNA is 60 to 66 mol%.

Gordonia amarae was found to be the actinomycete from the most often isolated from foams from the treatment plants in the United States. In addition, *G. amarae* has been isolated several times as the predominant foam actinomycete in Australia, and also Europe (6,35,55, and references therein).

Some studies have been published on specific foam strains and it was found that, although pure cultures of *G. amarae* did not grow in sterile raw sewage without the addition of a carbon source (fructose), they did grow in sterile activated sludge. After removal of the solid components from the activated sludge, no growth was observed, suggesting that the solids supply some essential nutrients. High levels of emulsifiable fatty material (oil and grease) have also been associated with *Gordonia* and *Nocardia* growth and foaming, but until

Table 4. Chemotaxonomic Markers of *Skermania* and *Gordonia* and Other Genera of the Suborder *Corynebacterineae*

No.	DAP[c] meso	Sugar Type[a] A	Acyl Type	Menaquinones[b] 8/2	8/4	c8/4	9/0	9/2	9/4	10/0	11/0	Phospholipid Type[d]	Mycolic Acid Length[e]	Mycolic Acid Pyrolysis[f]	G + C
Skermania	+	+	G	−		+++	−	−	−	−	−	II	58–64	16–20	68
Gordonia	+	+	G	(v)	−	−	−	+++	−	−	−	II	54–66	16–18	63–69
Mycobacterium	+	+	G	(v)	−	−	−	+++	−	−	−	II	70–90	22–26	70–72
Tsukamurella	+	+	G	−	−	−	+++	−	−	−	−	II	64–78	20 : 1	67–67
Skermania	+	+	G	−		+++	−	−	−	−	−	II	58–64	16–20	68
Gordonia	+	+	G	(v)	−	−	−	+++	−	−	−	II	54–66	16–18	63–69
Williamsia	+	+	G	−	−	−	−	+++	−	−	−	II	50–56	nd	64.8
Nocardia	+	+	G	−		+++	−	−	−	−	−	II	50–62	12–18	64–72
Rhodococcus	+	+	G	+++	−	−	−	(v)	−	−	−	II	34–64	12–16	73–73
Dietzia	+	+	A	+++	−	−	−	(v)	−	−	−	I	34–38	nd	73
Corynebacterium	+	+	A	v	−	−	−	v	−	−	−	I	22–36	8–18	51–67
Corynebacterium amycolatum	+	+	nd	−	−	−	+++	−	−	−	−	nd	lacking	−	nd
Turicella	+	+	nd	−	−	−	−	−	−	++	++	nd	lacking	−	65–72

[a] Whole-organism sugar patterns of actinomycetes containing meso-(A_2pm) A, arabinose + galactose; B, madurose C, 3-O-methyl-D-galactose, D, xylose; E, rhamnose; C, no diagnostic sugars, glucose, mannose, ribose may be present.

[b] Abbreviations 8/4 = MK-8(H_4) 9/2 = MK-9(H_2).

[c] DAP (A_2pm), diaminopimelic acid.

[d] Phospholipid types with diagnostic phospholipids: II, phosphatidylethanolamine; III, phosphatidylcholine; IV phosphatidyl-N-acetylglucosamine, I diagnostic phospholipids lacking nondiagnostic phospholipids, i.e., phosphatidylglycerol, diphosphatidyl glycerol; PI, phosphatidylinositol, and PIM, phosphatidylinositol and glycolipids may be present.

[e] Number of carbon atoms in mycolic acid molecule, range of homologous series of mycolic acids.

[f] Fatty acid methyl esters released by pyrolysis of mycolic acid methyl esters.

now little experimental evidence has been presented to support a clear connection. Because of the hydrophobic cell walls of actinomycetes, hydrophobic substrates are selectively available to them, and this may lead to their successful competition for substrates in activated sludge. It should be noted that many authors have proposed that the hydrophobicity of the mycolic acid content of the actinomycete cell wall plays an important role in foaming.

Deficiencies of nitrogen and phosphorus, and also trace elements, cannot account for the excessive occurrence of nocardiae and/or gordoniae (35).

Examinations of the foaming environment have been conducted by Marshall (68), who showed that nutrients can accumulate at interfaces, leading to a relatively nutrient rich condition in an otherwise low-nutrient environment. On addition of surfactants, the growth of actinomycetes in a pilot plant increased, which suggests a role of surface-active agents, which might explain a greater substrate availability and an increased growth of *G. amarae* on the addition of hydrophobic substrates such as hexadecane, and industrial wastewater containing nonpolar hydrocarbons (35). *Gordonia amarae* grows between 23 to 37 °C, but not at 10° or 40 °C, although for some strains, growth as low as 10 °C has been reported (44).

A correlation between foaming and plant-operating temperature was suggested by Pipes (69) who observed that foaming occurred only at temperatures above 18 °C. However, Sakai and coworkers (70) detected and isolated *G. amarae* from plants operating between 14 °C and 26 °C, although in addition to temperature, growth rates also depend on the environmental pH. Optimum growth of *G. amarae* occurs at a pH of about 7 to 8, whereas only very slight growth was observed at pH 5 to 6. Oxygen supply is another important parameter, because *G. amarae* is a strict aerobic species. Blackall and coworkers (71) showed that *G. amarae* neither grows on, nor takes up, acetate under anaerobic (no nitrate, no oxygen) or anoxic (nitrate, no oxygen) conditions.

Actinomycetes found in foam and scum are generally considered as slow growers, with generation times of 4 to 13 hours under optimum conditions; however, large variations in this parameter (4 to 7 d) were described for *G. amarae* (6).

In modeling microbial growth, especially in continuous culture, the K_S value largely determines the steady state concentration of unused growth rate-limiting substrate, and thus is an important parameter showing the efficiency of conversion of substrate to biomass. Lemmer (72) proposed a model about growth strategies based on K_S values. She argued that foam-forming actinomycetes, including *G. amarae*, can switch between two strategies. The first strategy is characterized by high-substrate uptake efficiency at low substrate concentrations, low K_S values, and relatively low cell yield (K-strategy). At high-substrate concentrations, the second so-called μ_{max}- or r-strategy is used by the actinomycetes. This strategy is characterized by high biomass production and both growth rates exceeding those of K-strategists. Some other models have been proposed, and details and critical remarks on these models have been published by Soddell and Seviour (6) in their comprehensive review on activated-sludge foaming.

The bases for *G. amarae* growth and foaming in activated sludge are not well understood. In the literature, much contradictory information can be found, and many of the suggested countermeasures are based on anecdotal evidence (4). Different surveys have implicated that foam and scum problems occur in treatment plants with marked differences in sewage compositions and plant-operating systems. Ecological factors implicated with foam formation include the oxygen content of the aeration tank, the sludge age, and a high solid content of the mixed liquor, temperature, and the grease and oil content of the sewage. Details regarding these factors have been summarized by Soddell and Seviour (6). In the study of Stainsby and coworkers (67), it was shown that there is a higher diversity of mycolic acid-containing actinomycetes, including new *Gordonia* species associated with activated-sludge foam. Their role need to be elucidated in the future.

Skermania piniformis. *Skermania piniformis* was first described as *N. pinensis* by Blackall and coworkers (71) for actinomycetes responsible for extensive foaming or scumming in activated sludge sewage treatment plants in Australia. By using a combination of morphological, chemical, and physiological properties, these organisms were first assigned to the genus *Nocardia*. However, it was obvious that the organisms showed atypical features for *Nocardia*, including a relatively slow growth rate, mycolic acids, which were monounsaturated at position 2, and a different antimicrobial sensitivity pattern. The actinomycetes showing mycolic acids as distinctive chemotaxonomic features share many phenotypic properties (Table 4). On the basis of 16S rRNA sequence analyses, they form a distinct phyletic line, and are currently classified in the genera *Corynebacterium, Dietzia, Gordonia, Mycobacterium, Nocardia, Rhodococcus, Tsukamurella*, and *Williamsia*, primarily on the basis of chemical, molecular, and morphological markers. The 16S rDNA sequence of *N. pinensis* is substantially different from the corresponding sequences of the representative mycolic acid-containing actinomycetes. Almost equal levels of relatedness to the sequences of *Gordonia* strains (levels of similarity, 94.9 to 95.9%), *Nocardia* strains (94.5 to 95.9%), and *Rhodococcus* strains (94.5 to 95.9%) were observed (62). Lower levels of homology were observed with the sequences of *Mycobacterium* strains (94.0 to 94.7%), *Tsukamurella* strains (93.7 and 93.8%), a *Dietzia* strain (93.5%), and *Corynebacterium* strains (91.4 to 92.6%) (62).

Additional chemotaxonomic data confirmed a separate position for *N. pinensis*. High amounts of hexahydrogenated menaquinones with eight isoprene units, the last two of which were cyclized, were detected. In addition, the glycan moiety was rich in N-glycolated muramic acid. The organism also contains major amounts of diphosphatidyl glycerol, phosphatidyl ethanolamine, phosphatidyl inositol, and phosphatidyl inositol mannosides (41).

On the basis of phylogenetic, chemotaxonomic, and conventional phenotypic data it was evident that *N. pinensis* should be considered a member of a separate genus within

the mycolic acid-containing actinomycetes and the name *S. piniformis* was proposed by Chun and coworkers (62).

Skermania piniformis is a gram-positive, nonacid-fast, nonmotile actinomycete forming a mycelium, which does not fragment in undisturbed cultures. Secondary branching is rarely observed and aerial hyphae cannot be seen without the help of a microscope. The organism grows on tryptone yeast extract glucose (TYG) agar (71). During the early growth stages (24 hours), the light microscopic appearance of the organism resembles a pine tree. Strains isolated from activated-sludge plants often contain intracellular sudanophilic and polyphosphate inclusions.

Colonies on nutrient-rich media (TYG) are orange, opaque, macroscopically dry and friable, and microscopically moist and shiny, have a pasty texture, are difficult to emulsify or subculture, and are circular with entire edges. The minimum and maximum growth temperatures are 15 °C and 31 °C, respectively. The organisms are aerobic and catalase, oxidase, and urease positive. Growth cannot be enhanced in an atmosphere containing 5% carbon dioxide, nor does growth occur anaerobically, microacrophilically, or in a candle jar. Hydrolysis of esculin, casein, gelatin, hypoxanthine, Tween 20, Tween 40, Tween 60, and Tween 80 are negative. Nitrate is not reduced to nitrite. A large number of fluorogenic substrates are hydrolyzed (41,62,71). Because *S. piniformis* does not grow on many media, that support the growth of other actinomycetes, the exact nutritional requirements and utilized carbon sources are unknown. However, the organism grows well on media that contain glycerol as a carbon source and asparagine as a nitrogen source. The cell wall contains major amounts of mesodiaminopimelie acid, arabinose, galactose, glucose, ribose, and N-glycolated muramic acid. The peptidoglycan is of the A1-γ type. As polar lipids, diphosphatidyl glycerol, phosphatidyl ethanolamine, phosphatidyl inositol, and phosphatidyl inositol mannosides are predominant. Major fatty acids are straightchain saturated, unsaturated, and tuberculostearic fatty acids, mycolic acids with 58 to 64 carbons and monounsaturated side chains, and hexahydrogenated menaquinones with eight isoprenic units in which the last two units are cyclized. The organism has DNA that is rich in guanine and cytosine (67.5 mol%). In a numerical taxonomic study, 59 isolates showed the microscopic morphology and/or similar morphological features of *S. piniformis* clustered as two separate groups, probably representing new species (73).

Skermania piniformis was first isolated by filament micromanipulation (71). It has a relatively slow growth rate (10 to 21 d) (6) and constitutes the second most prevalent foaming organism in Australia (71) after *G. amarae*. As reported by Soddell and Seviour (6), microphotographs from activated-sludge samples outside Australia presented in earlier studies show organisms with the characteristic pine tree branching morphology (4) indicating that *S. piniformis* is not unique to Australia. Detailed knowledge on the global distribution and ecology of the organism, however, is still scarce.

Nocardia. Organisms belonging to the genus *Nocardia* are aerobic, chemoorganotrophic actinomycetes forming a rudimentary to extensively branched substrate hyphae. These hyphae often fragment in situ, or on mechanical disruption, into rod-shaped to coccoid, nonmotile elements. Aerial hyphae, most often visible only microscopically, are almost always present. *Nocardiae* have an oxidative type of metabolism. The chemotaxonomic features are listed in Table 4. Those *Nocardiae* playing a predominant role in foaming, for example, "*N. amarae*" and "*N. pinensis*," have been reclassified as *G. amarae* and "*S. piniformis*," respectively. There are only few reports on the occurrence of authentic *nocardiae* in bulking and/or foaming, although *Nocardia asteroides, Nocardia farcinica*, and *Nocardia caviae* have been isolated from foams of United States treatment plants (6,74).

Rhodococcus. The genus *Rhodococcus* contains aerobic, gram-positive, partially acid-fast, nonmotile actinomycetes-forming rods to extensively branched substrate mycelia. The growth cycle is quite complex. It starts with the coccus or short-rod stage. Different organisms then show a complex series of morphological stages: cocci may germinate only into short rods, or form filaments with side projections, or show elementary branching, or in the most differentiated forms, produce branched hyphae. Members of the genus *Rhodococcus* can grow well on most of the standard laboratory media at 30 °C although some strains require thiamine. Colonies may be rough, smooth, or mucoid, and they often show a buff, cream, yellow, orange, or red pigmentation, though colorless variants also occur. Rhodococci have an oxidative metabolism. They are catalase positive, sensitive to lysozyme, arylsulfatase negative, and produce acid from glucose oxidatively. Some important chemotaxonomic features are listed in Table 4.

Rhodococcus rhodochrous have been reported to be the main actinomycetes in foam in European treatment plants (35). However, *Rhodococcus equi, R. ruber*, *R. coprophilus*, and *R. erythropolis* have also been detected in plants from different parts of the world (6,35). They can use a wide range of different carbon sources and most of them grow between 10 and 40 °C. Although it has been speculated that rhodococci have a lower temperature optimum than *G. amarae*, and this explains the predominance of rhodococci in Europe and of *G. amarae* in the United States, some studies in Japan do not support this assumption (70). Kurane and coworkers (75) reported that *Rhodococcus* species, particularly *R. erythropolis*, which occurs in activated sludge, were able to produce chemicals that flocculate both organic and inorganic compounds, including cells of *E. coli, Saccharomyces sp.*, and *Macrocystis aeruginosa*. Therefore, it was suggested that these compounds flocculate bacteria or prevent their deflocculation in an activated sludge environment (73). In the study of Stainsby and coworkers (67), it was shown that there is a higher diversity of mycolic acid-containing actinomycetes, including new *Rhodococcus* species.

"Nostocoida limicola." Filaments of "*N. limicola*" were first characterized and isolated by van Veen (40), who characterized them as chains (0.6 to 0.9 mm long) of

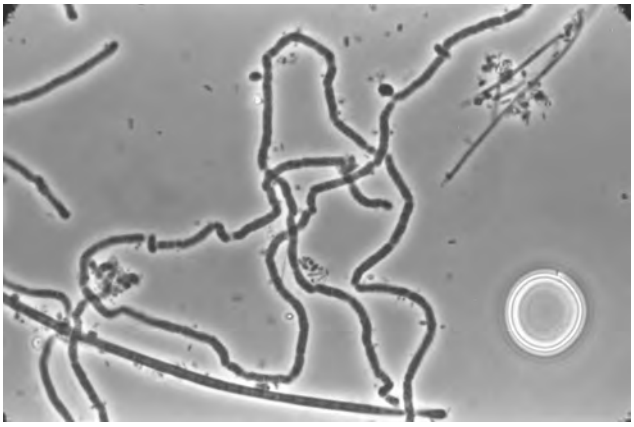

Figure 7. Filaments of "*Nostocoida limicola*." Phylogenetic group not determined (×1250). Photo by D. Eikelboom, TNO-MEP, Apeldoorn.

coccoid, gram-positive cells with spherical to ovoid appearance, 0.3 to 0.6 μm long with a diameter of 0.7 to 1.5 μm. A similar description was given by Eikelboom (2) and both authors described "*N. limicola*" as facultatively anaerobic to microaerophilic organisms. In the study of Eikelboom and Van Buijsen (3), three morphotypes were recognized and termed *N. limicola I, II*, and *III*. The incidence of "*N. limicola*" has been described for both bulking and foaming events, particularly in plants treating wastewater from industrial sources (Fig. 7). Recently, the phylogeny of all three morphotypes has been clarified (Figs. 1 and 2). "*Nostocoida limicola*" I contains at least two different low G + C gram-positive bacteria, which are very closely related to *Lactosphaera pasteurii* and *Streptococcus suis*, respectively (22). "*Nostocoida limicola*" II is affiliated with the actinomycetes (high G + C gram-positives). Representatives of the genera *Terrabacter* and *Janibacter* were shown to be the closest phylogenetic relatives of this morphotype (76). '*Nostocoida limicola*' III lumps together members of the *Planctomycetales*, which are most closely related to *Isosphaera pallida* (22). The actual diversity hidden behind the '*N. limicola*' morphotype might still be higher. Schade and coworkers (32) isolated '*N. limicola*'-like organisms, which on the basis of comparative 16S rRNA analysis were shown to belong to the green nonsulfur lineage. These results demonstrate that organisms from four different bacterial divisions possess the morphology of '*N. limicola*'. This example most drastically shows that morphological similarities between bacterial species are not indicative of a common evolutionary history. Five '*N. limicola*' filaments of the morphotype II could be isolated on R2A agar using a micromanipulator from two different activated-sludge sewage treatment plants in Italy and Australia (76). For all strains, the gram stain was extremely variable, and both gram-positive and gram-negative cells could be detected in the filaments. Neisser stain also gave positive results. The isolates were able to grow on several carbon sources, including organic acids and carbohydrates. Ammonium, nitrate, and urea were used as nitrogen source and the organisms grew in a temperature range between 15 °C and 30 °C, but not at 40 °C (76). In contrast to the description of van Veen (40) and that of Nowak and Brown (77), none of the strains could grow anaerobically or with nitrate as the terminal electron acceptor. Snaidr and coworkers (33) studied the diversity of '*N. limicola*'-like filaments from industrial wastewater treatment plants and found an additional type affiliated to the α-subclass of *Proteobacteria*. For these organism the genus name "*Alisphaera*" is proposed (33).

FILAMENTS AFFILIATED WITH THE β-SUBCLASS OF THE PROTEOBACTERIA

On the basis of 16S rRNA sequencing studies, some of the "classical" filamentous bacteria in activated sludge, such as *S. natans, Leptothrix* species, and Eikelbooms Type 0803, have been assigned to the *Rubrivivax* subdivision of the beta subclass of the *Proteobacteria* (Fig. 2). The class *Proteobacteria* has been proposed (78) as a suitable collective name for the phylogenetic relatives to the purple photosynthetic bacteria.

Sphaerotilus

Historically, organisms belonging to *S. natans* (at present the only species of the genus) have been regarded as the principal agents of filamentous bulking (37), but it is now clear that a wide variety of filamentous bacteria contribute to this problem. On the basis of 16S rRNA sequencing studies, the genus *Sphaerotilus* and the closely related genus *Leptothrix* belong to the *Rubrivivax* subdivision of the β-subclass of the *Proteobacteria* (46,79,80).

Sphaerotilus natans is a gram-negative straight rod, usually arranged in single chains with sheaths of uniform width. These sheaths may be attached by means of holdfasts to surfaces, and are not encrusted by ferric or manganese oxides. *Sphaerotilus natans* has an aerobic, chemoorganotrophic metabolism. It can grow at low concentrations of dissolved oxygen (below 1 mg/L^{-}) and has a temperature range from 10 to 37 °C (optimum between 20 and 30 °C). *Sphaerotilus natans* can be grown on a wide variety of media (43,49), with a preference for media with moderate nutrient concentrations (16,36). Reference strains are available from culture collections. Isolation can be successful by using the spread plate techniques (16), centrifugation pretreatment (37), or whirl-mix pretreatment of activated sludge (38).

Sphaerotilus natans contains ubiquinones of the type Q-8 as a major component (>90%), which is a common trait within genera belonging to the *Rubrivivax* group (81). Fatty acid analyses of all strains revealed characteristic profiles for *Sphaerotilus* with four major fatty acids, *cis*-9 hexadecanoic acid (16 : 1), hexadecanoic acid (16 : 0), *cis*-9,11 octadecanoic acid (18 : 1), and hexadecanoic acid (12 : 0). All strains contained the hydroxylated fatty acid 3-OH 10 : 0 (81). Similar to its quinone composition, the fatty acid profile closely resemble that of the genus *Leptothrix* (80). A clear differentiation at the genus level with these chemotaxonomic features is not possible. Strains of *Sphaerotilus* can grow on a wide variety of carbon sources including sugars, organic acids, and

alcohols. On the basis of carbon source utilization patterns, a differentiation between the genera *Sphaerotilus* and *Leptothrix* is possible (80,81). Because isolation and cultivation of these organisms is sometimes difficult and time consuming, the use of specific 16S rRNA-targeted oligonucleotide probes (7,46) can be very useful for in situ detection and identification of *Sphaerotilus* (43, Table 3). The mol % G + C of the DNA is approximately 70 mol%.

The original habitat of *S. natans* was slowly running fresh water heavily contaminated with sewage or wastewater from paper, potato, dairy, or other agricultural industries. Often long, slimy tassels attached to stones or plants are observed. *Sphaerotilus natans* is often present in activated sludge, but only under certain conditions does *S. natans* cause sludge bulking (2). *Sphaerotilus natans* primarily occurs in plants characterized by a low concentration of available nutrients (typically found in systems continuously fed with wastewater) and low oxygen tensions in activated sludge. The ability of *S. natans* to grow at very low pO values is an additional factor favoring the competition with floc-forming bacteria (16). However, Williams and Unz (37) isolated *S. natans* and also the morphologically very similar Type 1701 from aeration basins operated at both high- and low-bulk water dissolved oxygen conditions. They stated that certain operation parameters, such as organic loading or dissolved oxygen, should not be considered as sole reasons for bulking problems. Moreover, the interaction of coexisting factors, which are still unknown, seems to be important (37).

Although *S. natans* prefers a growth medium containing adequate amounts of easily assimilable organic nutrients, the occurrence of this organism has been reported for unpolluted water of brooklets, ditches, and ponds where unknown compounds are the substrates.

In the former habitat, the sheaths are thin and colorless; in the latter, particularly in the presence of soluble iron compounds, they may turn yellow-brown and sometimes become encrusted with ferric oxide. This characteristic can be clearly observed in a laboratory apparatus in which *S. natans* is grown in slowly running soil extract enriched with Fe (II). Under these conditions, the sheaths of *S. natans* are very similar to those of *Leptothrix ochracea*. For this reason, it has been suggested that both organisms are identical. However, in a polyphasic approach on the taxonomy of the genus *Leptothrix*, the separate standing of both genera has been clearly shown (80).

Leptothrix

Organisms belonging to the genera *Sphaerotilus* and *Leptothrix* are characterized by their ability to form sheaths. The formation of the sheaths is affected by the composition of the culture medium (16,36). *Leptothrix* is also placed within the *Rubrivivax* subdivision of the β-subclass of the *Proteobacteria* (Fig. 2). The genus includes six species, from which *L. discophora, L. cholodnii*, and *L. mobilis* (80) are available from culture collections. Strains of the remaining species, namely, *L. ochracea, L. pseudo-ochracea*, and *L. lopholea*, have never been grown in axenic culture (46). Representatives of the culturable species can be grown on nutrient-poor media, such as PTYP medium (46) and the medium described by Rouf and Stokes (82), incubated at 20 to 30 °C (optimum temperature) (16). *Leptothrix* and *Sphaerotilus* share a number of features, namely, motile cells, formation of poly-β-hydroxybutyrate (PHB), a requirement for vitamin B_{12}, but they can be clearly differentiated by the ability of *Leptothrix* to oxidize Mn^{2+} to Mn^{4+}, in addition to several other features listed by Mulder and Deinema (16), and Spring and coworkers (80). In the past, identification of both genera and the species within the genus *Leptothrix* was based largely on morphological and cytological criteria (2,16). However, for culturable representatives, several phenotypic characters have been described as well (16,37,46,55). Sheathed bacteria of the genus *Leptothrix* are often found in freshwater habitats, such as lakes, ponds, swamps, or springs. However, they have also been associated with bulking in activated sludge (2,7,36).

Eikelboom Types 1701, 1702, and 0803

Bacteria of the Type 1701 described by Eikelboom (2) share many morphological features with the *Sphaerotilus-Leptothrix* group, especially with *S. natans* (2,37,38). Eikelboom (2) stated that when grown on I-medium, colonies of Type 1701 closely resemble those of *S. natans*, but cells of Type 1701 are smaller compared with *S. natans* (3). However, detailed taxonomic studies are required to clarify the relationship of Type 1701 to *S. natans*. Phylogenetically Eikelboom Type 1701 is closely related to *S. natans* (unpublished results, Fig. 2). Type 1702 was also reported to produce long, unbranched, and slightly benched filaments. Compared with Type 1701, septa are not so clearly visible. The original description is given by Eikelboom (2). This type has never been obtained in pure culture. Type 1701 has been reported often in connection with mixed liquor suspended solids (MLSS) held under low dissolved oxygen concentrations (83,84).

On the basis of 16S rRNA sequence data, organisms of Eikelboom Type 0803 were recently shown to belong to the *Rubrivivax* subdivision of the β-subgroup of the *Proteobacteria* (85) (Fig. 2). Two strains under study showed highest sequence similarity with *Variovorax paradoxus* (94.8%), *Rhodoferax fermentans* (94.8%), and *Comamonas testosteroni* (94%) (85). Eikelboom Type 0803 bacteria form 50 to 150 μm straight, smoothly curved filaments with cells 0.6 to 0.8 × 0.9 to 3.0 μm in dimension. They are able to store volutin granules and are often found to be attached to inorganic material. The isolate studied by Bradford and coworkers (85) was able to grow on freshly prepared R2A agar, producing yellow, shiny, slightly elevated colonies. An earlier isolated strain (37) had similar properties. Morphologically, this filament may be confused with "*M. parvicella*" (37). Nutritional requirements are largely unknown, although the filament can be cultivated on fresh R2A medium (85) or SUC medium (37).

FILAMENTS AFFILIATED WITH THE γ-SUBCLASS OF PROTEOBACTERIA

The gram-negative organisms of the genera *Leucothrix, Thiothrix*, and *Beggiatoa* have all been shown to belong phylogenetically to the γ-subclass of the *Proteobacteria* (Fig. 2), and are filamentous sulfur bacteria. *Thiothrix* and Eikelboom Type 021N are frequently found in bulking sludge and their occurrences have been described in connection with specific plant operational factors (3,4,86).

Thiothrix, Leucothrix, Eikelboom Type 021N

Members of the genus *Thiothrix* produce colorless filaments, and are able to oxidize reduced sulfur compounds (87). They have striking morphological features such as their ability to produce gliding gonidia to form rosettes and to deposit intracellular sulfur granules (Fig. 8; 37,87–89).

In the past, the characterization of members of the genus *Thiothrix* was mainly based on morphological criteria, and it has been suggested that the aforementioned listed morphological features are sufficient for defining features of the genus (90). However, since the publication of Eikelboom (2) it is clear that some other filamentous bacteria like Eikelboom Type 021N (Fig. 9; 2), and also *L. mucor*, form rosettelike structures very similar to those produced by *Thiothrix* species. The production of gonidia has also been observed (37,91). As a consequence, there has been a controversial discussion regarding the value of morphological criteria for classification of these organisms, and also the relationship between Eikelboom Type 021N, *L. mucor*, and the genus *Thiothrix* (2,91,92).

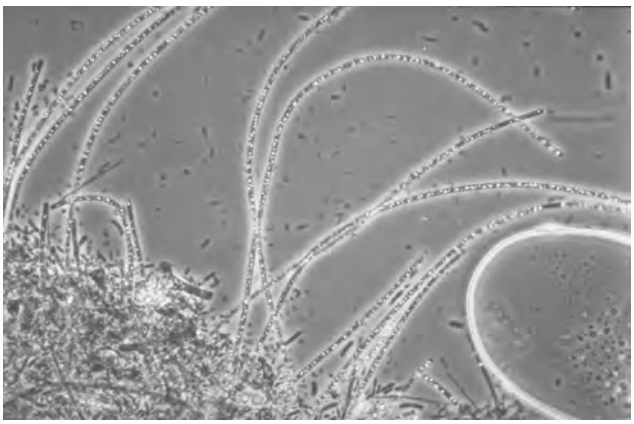

Figure 8. Filaments of *Thiothrix* sp. (×1250). Photo by D. Eikelboom, TNO-MEP, Apeldoorn.

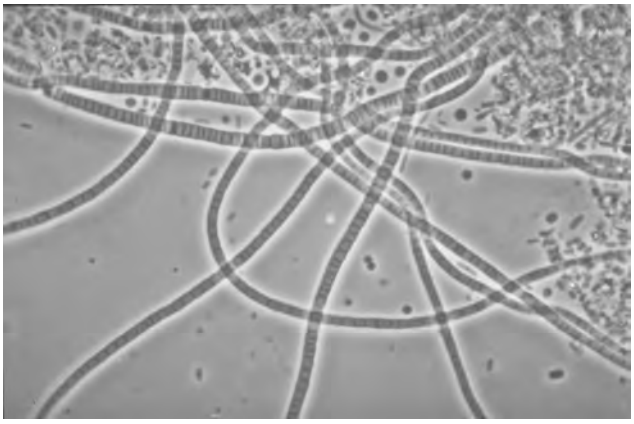

Figure 9. Filaments of Type 021N (×1250). Photo by D. Eikelboom, TNO-MEP, Apeldoorn.

Three named *Thiothrix* species, *T. nivea, 'T. ramosa'*, and *'T. arctophilia'* (excluding species incertae sedis), mainly isolated from freshwater habitats, have been studied extensively. These have been isolated in pure culture and subjected to phenotypic and genotypic characterization (90,92–95).

To clarify the phylogeny of *Thiothrix* sp. from different habitats, molecular biological approaches based on 16S rRNA sequence analysis have been conducted (90,95,96). All of the *Thiothrix* isolates and all but one of the Eikelboom Type 021N bacteria investigated by Howarth and coworkers (96) were members of the γ-subdivision of the *Proteobacteria.* This finding was similar to those of Polz and coworkers (90) and Teske and coworkers (95), who showed that *Thiothrix* formed a deeply branching lineage of the γ-*Proteobacteria* most closely related to sulfide-oxidizing symbionts of marine invertebrates and *Thiomicrospira* species. The 12 strains studied by Haworth and coworkers (96) were assigned to four new *Thiothrix* species, namely *T. unzii, T. defluvii, T. fructosivorans*, and *T. eikelboomii*, the latter species harboring the Type 021N strains. The authors who stated that the phenotypic characters frequently used to differentiate Eikelboom Type 021N bacteria from *Thiothrix* species are not totally reliable. An important result was that the Eikelboom Type 021N strains isolated independently from three different continents were phylogenetically homogeneous. However, Kanagawa and coworkers (8) recently demonstrated that additional diversity is found within this morphotype on the basis of fifteen isolates of the Eikelboom Type 021N from Japan. According to their 16S rRNA sequence, the isolates were subdivided into three distinct groups (Fig. 3). Group I is clearly separated from all currently recognized *Thiothrix* species and exclusively consists of seven isolates from Japan. Group II contains four isolates from Japan and clusters together with *T. eikelboomii*, whereas group III forms a monophyletic lineage together with *T. defluvii*. On the basis of the current perception of the diversity within the *Thiothrix*/Eikelboom Type 021N line of descent, the previously published oligonucleotide set for these microorganisms (7) was supplemented and now covers the entire group (8) (Table 2; Fig. 3).

Thiothrix unzii produces rod-shaped cell and forms multicellular filaments. Gliding gonidia have been observed. A sheath is not present, but rosettes and a holdfast are formed. Cell inclusions, such as volutin granules, sudanophilic granules, and PHB, are present within the cells. Growth is observed in the temperature range from 4 to 33 °C and pH ranges from 6.5 to 8.5, but no growth at 37 °C occurs. The organism is oxidase-positive and catalase-negative. A requirement for reduced inorganic sulfur for growth was

detected. Sulfur globules are deposited within invaginations of the cytoplasmic membrane. Hydrolysis of gelatin and casein is positive, whereas hydrolysis of starch, Tween 80, and urea does not occur. A small number of carbon sources (pyruvate, succinate, acetate, lactate, and propionate) are used if sodium thiosulfate is present. A large number of carbon sources are not used (96).

Thiothrix fructosivorans also produces rod-shaped cells and forms multicellular filaments. Similar to *T. unzii*, gliding gonidia are produced from the end of the filaments, but in contrast, a sheath is present. Rosettes and a holdfast can be detected, and volutin inclusions, sudanophilic granules, and PHB inclusions are observed within the cells. Its temperature and pH range is similar to that of *T. unzii*, but no requirements of reduced inorganic sulfur for growth are apparent. Sulfur globules are also deposited within invaginations of the cytoplasmic membrane after growth in the presence of reduced inorganic sulfur compounds. Gelatin can be hydrolyzed, but starch, casein, Tween 80, and urea are not hydrolyzed. In contrast to *T. unzii*, fructose, sucrose, and melezitose, and some organic acids are hydrolyzed.

Thiothrix eikelboomii (including some filaments morphologically similar to Eikelbooms Type 021N) also shows rod-shaped cells and forms multicellular filaments. A high degree of variablility in cell morphology is observed, including cuboidal, barrel-shaped, cylindrical, discoid, and beadlike cells (96). In addition, knots may be observed in filaments. The cells stain gram-negative or gram-variable. A sheath is not produced. Rosettes and a holdfast are not formed by all strains. As reported for *T. unzii* and *T. fructosivorans*, volutin inclusions, sudanophilic granules, and PHB inclusion within the cells are observed. The temperature range is also similar to these two species. Like *T. fructosivorans*, no requirement for reduced inorganic sulfur was detected, and sulfur globules are deposited within invaginations of the cytoplasmic membrane when cells are grown in the presence of reduced inorganic sulfur compounds. As reported for *T. fructosivorans*, gelatin is hydrolyzed and starch, casein, Tween 80, and urea are not hydrolyzed. Glucose, fructose, maltose, sucrose, trehalose, melezitose, mannitol, pyruvate, succinate, malate, acetate, lactate, and propionate can serve as sole sources of carbon.

Thiothrix defluvii shows a similar cell morphology to those reported for *T. unzii*, *T. fructosivorans*, and *T. eikelboomii*, but cell shape can be irregular. Elongated swollen cells are often present. A sheath is not produced. Rosettes, a holdfast, and gliding gonidia and knots in filaments may be formed but not by all strains. Cells can store intracellular elemental sulfur when grown in the presence of reduced inorganic sulfur compounds. In contrast to all other *Thiothrix* species, no volutin granules are present and PHB granules are not found. Because this species grows extremely slowly, its exact nutritional and biochemical properties are unknown.

Thiothrix species can be isolated from very different natural habitats, such as sulfide-containing natural water and irrigation systems, but also from activated-sludge plants (96, and references therein).

On the basis of morphology, the genera *Thiothrix* and *Leucothrix* have been grouped together in the family *Leucotrichaceae*. *Leucothrix* species can also form rosettes and gonidia, although no sheaths are produced. The capability of sulfide oxidation and accumulation of elemental sulfur are characters shared with *Thiothrix* species, and many Eikelboom Type 021N bacteria are isolated from activated sludge (37). On the basis of rRNA sequence analysis, *L. mucor* DSM 2157^T is placed as a deeply branching lineage at the base of the *Thiothrix* clade, and this may indicate that *Thiothrix* and *Leucothrix* share a common ancestry (7,96).

Eikelboom Type 1863

Filaments of Eikelboom Type 1863 are characterized by their short, *Streptococcus*-like chains of cells. They stain gram-negative and are 0.5 to 1.0 μm long and 0.8 μm wide. No sheath and slime layer is present (2). On the basis of this distinctive morphological description, Seviour and coworkers (18) isolated five filaments from activated sludge on R2A agar and identified the pure cultures using the 16S rRNA sequencing approach in connection with phenotypic features. From the results of this study, it is obvious that Eikelboom Type 1863 is not a single genetic entity. Consistent with a previous study (97), two isolates were identified as *Acinetobacter* spp., whereas another one was found to represent a *Moraxella* species (Fig. 2). Both genera belong to γ-subclass of the *Proteobacteria*. The other strains were identified as *Chyseobacterium*-like organisms belonging to the *Cytophaga* group of the *Flexibacter Cytophaga Bacteroides* phylum (18) (Fig. 2). Consequently, as discussed earlier for *N. limicola*, morphological characterization cannot be recommended for species allocation.

FLEXIBACTER CYTOPHAGA BACTEROIDES CLUSTER

The *Flexibacter Cytophaga Bacteroides* group has been described to constitute a separate phylum within the division bacteria, which contains at least three types of filaments that occur in activated sludge.

Haliscomenobacter Hydrossis, Type 0092, Type 0411

Filaments of the species *H. hydrossis* (at present the only species of the genus) are characterized by their straight, thin, needle-shaped cells (0.4 to 0.5 × 3 to 5 μm), usually in chains and enclosed by a hardly visible sheath. This organism is gram-negative and can be identified by its unique cell morphology. On the basis of 16S rRNA sequences, it was shown that the organism phylogenetically belongs to the *Saprospira* subgroup of the *Flexibacter Cytophaga Bacteroides* phylum (98; Fig. 2). Isolation can be facilitated by using a centrifugation or whirl-mix pretreatment (38). The organisms grow on I-medium (40) and GMBN medium (49,99) at 25 °C. They produce pinkish, smooth, or slightly filamentous colonies about 1 to 3 mm diameter on SCY agar. Mulder (100) described a requirement for thiamine and vitamin B_{12}. Growth occurs in a temperature range between 8 and 30 °C (optimum ~26 °C). The pH optimum is above that of

S. natans (100). The nine isolates of filamentous bacteria, obtained from activated sludge and morphologically assigned to the genus *Haliscomenobacter* in addition to the type strain of *H. hydrossis* DSM 1100^T studied by Kämpfer (99), had the MK-7-menaquinone as the major quinone, and the MK-6-menaquinone as minor component. The main components of cellular fatty acids in all strains were the 13-methyltetradecanoic acid (i15 : 0), followed by straight-chain unsaturated acids of 16 : 1 and saturated acids of 16 : 0 and 18 : 0. Hydroxylated fatty acids of 3-OH i15 : 0 and 2-OH i15 : 0 were found in higher amount in the type strain of *H. hydrossis*, whereas the isolates from activated sludge produced these acids in minor amounts or traces. Analysis of physiological properties showed phenotypic homogeneity among the tested organisms. From 240 tested organic compounds, only a few sugars (D-glucose, D-fructose, and a few others) could be used as sole carbon source (99). Instead of PHB inclusions (typically found within *Sphaerotilus* and *Leptothrix*), polysaccharide globules are detected (12). The G + C content of the DNA is 49 mol%.

If present in bulking sludge, *Haliscomenobacter* can occur in large numbers and often makes the sludge flocs voluminous. *Haliscomenobacter hydrossis* has been detected in activated-sludge plants in different continents. No clear indications concerning the causes of the predominance of *Haliscomenobacter* are available at present, however, Krul (101) found that *H. hydrossis* has a poor competition ability in heavily loaded activated-sludge plants because of the low growth rate related to the low substrate uptake rate. *Halismenobacter hydrossis* has also been detected in foams, but they are of less importance in comparison with "*M. parvicella*" and *Gordonia amarae* (55).

Eikelboom Types 0092 and 0411

The Types 0092 and 0411 also belong to the *Flexibacter Cytophaga Bacteroides* phylum (84). Type 0092 belongs to the *Cytophaga* subgroup and Type 0411 to the *Flexibacter* subgroup. Filaments of both types were isolated by micromanipulation and were grown on R2A agar (84). For Type 0092, only one earlier isolation has been described by Horan and coworkers (102).

Type 0411 has also been grown on I-medium and on SCY medium (2). In the earlier studies of Eikelboom (2) and van Veen (40), it was suggested that this type belongs to the genus *Flavobacterium* and/or *Flexibacter*. Detailed growth requirements are not known.

Eikelboom Type 1863

As mentioned earlier, Seviour and coworkers (18) isolated five filaments from activated sludge, two of which were identified as *Chyseobacterium*-like organisms belonging to the *Cytophaga*-group of the *Flexibacter Cytophaga Bacteroides* phylum.

CHLOROFLEXUS SUBDIVISION

Herpetosiphon

The occurrence of bacteria belonging to the genus *Herpetosiphon* in bulking sludge was reported first by Trick and Lingens (103) and Senghas and Lingens (104). In their study, they characterized their strains morphologically and physiologically. The organisms can be characterized by unbranched, flexible rods or filaments (0.5 to 1.5 × 5 to 150 μm) consisting of individual cells with a length of 2 to 3 μm. They stain gram-negative and are obligate aerobic organisms. Some polymeric substances can be degraded. The isolation can be performed by spread-plate techniques (104), and also by micromanipulation (85). *Herpetosiphon* belongs phylogenetically to the *Chloroflexus* subdivision of the green nonsulfur lineage (85). *Herpetosiphon* is not considered to be a prominent bulking organism.

OTHER FILAMENTOUS ORGANISMS

For several other filamentous bacteria (Types 1702, 0321, 0675, 1851, 0192, 1091, 0961, 0914, 1111, and 1501), only little information can be obtained from literature. The morphologies of Type 0041 and Type 0961 are shown in Figures 10 and 11, respectively.

The morphology of Type 0041 has been very deeply explored using electron microscopy because of specific

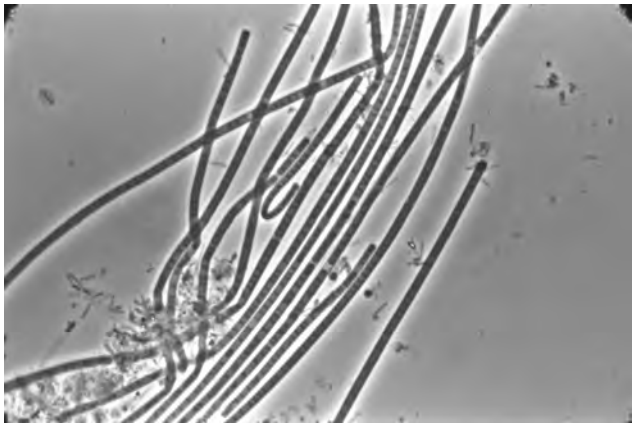

Figure 10. Filaments of Type 0041 (×1250). Photo by D. Eikelboom, TNO-MEP, Apeldoorn.

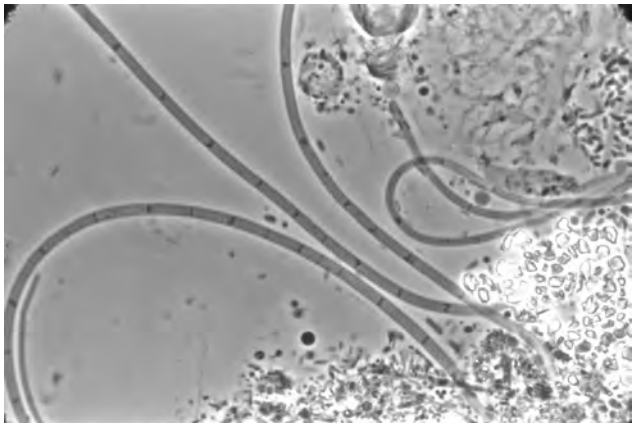

Figure 11. Filaments of Type 0961. Phylogenetic group not determined (×1250). Photo by D. Eikelboom, TNO-MEP, Apeldoorn.

interesting properties such as the presence of attached growth along the filaments of this sheathed organism (105). This organism was fortuitously studied recently when Hugenholtz and coworkers (106) designed FISH probes from environmental sequences from organisms in the recently described TM7 division. In this study, organisms morphologically described as Type 0411 bound a probe for the TM7 division and a probe for one of the TM7 subdivisions. Therefore, at least some Type 0041 filaments are phylogentically in the TM7 division.

For the other filamentous types, only their cell morphology has been studied and documented (2–4), and for some types, for example, Type 0581, successful cultivation on I- and SCY-medium has been described (2).

CONCLUSION

For the effectiveness and success of wastewater treatment (organic matter reduction, nitrogen and phosphorus removal), the complex structures and functions of the microbial communities are directly responsible. Community structure and function have also a direct effect on the ability of the activated sludge to settle and to be properly separated from the final effluent in the secondary clarifier. Filamentous bacteria have a key influence on sludge settling and consequently there is an urgent need to understand the phylogeny and ecology of these unique microorganisms. Traditionally, filamentous bacteria were classified according to morphology-based identification keys. Recent comparative 16S rRNA studies of filamentous bacteria, however, revealed that the established classification system is inconsistent with the phylogeny of these microorganisms. On the basis of morphological criteria, phylogenetically unrelated filaments were erroneously grouped together (e.g., "*N. limicola*," Type 1863), whereas closely related microorganisms were sometimes assigned to different taxonomic units (e.g., *Acinetobacter* and some filaments of the Eikelboom Type 1863). Today the 16S rDNA sequences of a large number of filaments have been determined and researchers began to develop an oligonucleotide probe set for fast, reliable, and cultivation-independent in situ detection of these bacteria directly within the activated-sludge samples. The 16S rDNA database has to be completed for the remaining filament types to establish a phylogenetically sound taxonomic system for all filamentous bacteria thriving in wastewater treatment plants. Such a system will also provide an excellent base for the design of oligonucleotide probes for all known filaments. FISH with these probes should then be used to quantify the different filament species in various wastewater treatment plants to identify process parameters, which influence their abundance. Furthermore, we need to directly investigate the physiology of the different filamentous bacteria. Keeping in mind that many filaments have not been successfully cultured or maintained in the laboratory yet, and that the metabolic activities of a bacterial species in its habitat cannot be directly inferred from pure culture experiments, methods for investigating the in situ physiology of filaments are required. Recently, a combination of FISH and microautoradiography has been developed, which allows to simultaneously identify microorganisms within their environment and to study their in situ physiology (107). This method has already been successfully used to investigate the in situ physiology of *Thiothrix* and Eikelboom Type 021N (108,109) and should in the future be applied to other filaments as well. These analyses will help to better understand the metabolic behavior and the ecology of filamentous bacteria in wastewater treatment plants with the ultimate goal of identification of causative factors for prevention of excessive filamentous growth causing bulking and foaming.

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FILAMENTOUS BULKING IN ACTIVATED SLUDGE, CONTROL OF

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The activated sludge process (see ACTIVATED SLUDGE — THE PROCESS, this Encyclopedia) is the most widespread biological wastewater treatment technique. The process is applied to both municipal and industrial wastewater treatment plants. It is used from the smallest units such as individual houses and farms to country hotels, reststops, and small settlements in rural areas, in large towns, and in metropolises or large industrial complexes. In all cases, biologically treated wastewater should be separated from the biomass of activated sludge to achieve high-quality final effluent. Although new separation processes such as membrane filtration have been tested in pilot- and full-scale units, conventional separation based on gravity sedimentation is still the only economically feasible option for most municipalities and industrial enterprises in the world. This fact stresses the importance of good settling properties of activated sludge. Activated sludge bulking caused by filamentous microorganisms is the most common operational problem associated with poor activated sludge separation. For this reason, the phenomenon of activated sludge filamentous bulking has been studied intensively since the 1960s. This article summarizes the current level of understanding of this separation problem and highlights the most common methods for control of activated sludge bulking.

WELL-SETTLING ACTIVATED SLUDGE

Requirements

One of the most characteristic features of wastewater treatment using activated sludge is that the biomass must be able to (*1*) flocculate (see ACTIVATED SLUDGE — THE FLOC, this Encyclopedia) and (*2*) settle and thicken by gravity sedimentation. With a few exceptions of small municipal plants or wastewater treatment installations in the industry, the treated wastewater is separated from the mixed culture of activated sludge in the so-called secondary clarifiers (or secondary settling tanks) in which the main driving force for separation is gravitation. Even the most sophisticated nutrient- removal activated sludge systems cannot work properly without good settling, thickening, and dewatering properties of the biomass. Poor separation and thickening of activated sludge in secondary clarifiers can lead to the following operational problems:

- The biomass of activated sludge escaping from the secondary clarifier deteriorates the quality of final effluent, not only in terms of suspended solids but also in BOD_5 (respiration of biomass), COD (organic nature of the biomass), and in N_{TOTAL} and P_{TOTAL} (the biomass contains a significant fraction of N and P compounds).
- A diluted return sludge stream does not allow the maintenance of the required biomass concentration in the aeration basin(s) and proper control over the sludge age.
- A diluted waste sludge stream results in hydraulic overloading of the sludge handling facilities.

From the point of view of the above operational problems, well settling activated sludge should exhibit the following features (1):

- It settles fast, with zone settling velocities of 3 m/h or more.
- It does not occupy an excessive volume after settling and thickening in a secondary clarifier.
- After sedimentation, it leaves a clear supernatant ($X = 15$ mg/L or less).
- It does not rise within at least a two- to three-hour period after sedimentation.

Measurement of Settling and Thickening Properties

The conditions during settling, in secondary settling tanks, can be best simulated by the measurement of zone

settling velocity. The measurement should be performed in transparent cylinders, so that the speed of movement of the sludge–supernatant interface can be noted. The higher and wider the cylinder, the more realistic the figures we obtain. A graph plotting sludge layer height versus time is called a settling curve that usually has three distinct settling phases: (*1*) reflocculation, (*2*) zone settling, and (*3*) transition and compaction.

The zone settling velocity is calculated from the slope of the curve in the phase of zone settling (II).

However, in wastewater treatment practice, the measurement of so-called sludge volume index SVI is the most common way to characterize activated sludge settleability. The standard sludge volume index is defined as follows:

$$\mathrm{SVI} = \frac{V_{30}}{X}$$

where

SVI = sludge volume index [mL/g]
V_{30} = volume of settled sludge after 30 minutes sedimentation in a 1-L cylinder
X = concentration of activated sludge (mixed liquor suspended solids) [g/L]

The numerical value of SVI is affected by many factors, especially by sludge concentration or the volume of sludge after 30 minutes sedimentation and by so-called wall effects in the cylinder. Therefore, in some countries the conditions of the settling test are standardized. Common standardization methods include:

- Stirred sludge volume index SSVI; the measuring cylinder is equipped with a slowly rotating stirring device.
- SVI at a standard concentration; the test is performed with a defined sludge concentration (for instance, 3.5 g/L $SVI_{3.5}$).
- Diluted sludge volume index DSVI; the volume of settled sludge after 30 minutes sedimentation should not exceed 200 mL/L, and if so, the test is repeated with a diluted sludge.

Activated sludge can be classified according to the zone settling velocity (ZSV) and the sludge volume index shown in Table 1.

When filamentous microorganisms cause sludge separation problems, it is useful to quantify the amount of these microorganisms in activated sludge. For that purpose, Sezgin and coworkers developed the measurement of total extended filament length (TEFL) (2), in which the total length of the filaments protruding from flocs or freely floating in the bulk liquid is measured. The TEFL values of 10^7 μm/mL or 10^4 m/g are considered to represent the boundary between nonbulking and bulking activated sludge (3).

Table 1. Types of Activated Sludge According to Its Settleability

Type of Sludge	SVI, mL/g	ZSV, m/h
Well-settling	<100	>3
"Light"	100–200	2–3
Bulking	>200	<1.2

The measurement of TEFL is rather laborious and time consuming, and so, Jenkins and coworkers developed routine examination of activated sludge, a rapid and simple method of subjective scoring of filament abundance (3). The abundance of filaments is classified according to scale from none to excessive and is given in Table 2.

In the case of increased abundance of foam-forming nocardioform actinomycetes, which are often irregular in shape and frequently branched, Pitt and Jenkins (4) recommend a modified method based on counting the number of intersections of these filaments with a hairline in the eyepiece.

Microscopic Features of Well-Settling Activated Sludge

A simple microscopic examination can be very useful for the identification of both well-settling sludge and activated sludge separation problems. Good settling sludge is composed of compact and firm flocs of quite a regular round shape. The average size of these is usually 100 μm or greater. When looking at the flocs (see ACTIVATED SLUDGE — THE FLOC, this Encyclopedia) under a microscope with higher magnification (1000×) and preferably using phase contrast, a "filamentous backbone" of the flocs can often be observed. The "filamentous backbone" can be seen better in samples stained with Gram or Neisser stain (3). This suggests that large compact flocs may be formed by bacterial growth on filaments observed inside the floc. Filaments protruding from the flocs into the bulk liquid

Table 2. Subjective Scoring of Filament Abundance According to Jenkins and Coworkers (3)

Numerical Value	Abundance	Description of Microscopic Picture
0	none	
1	few	Filaments present, but only observed in an occasional floc
2	some	Filaments commonly observed, but not present in all flocs
3	common	Filaments observed in all flocs, but at low density (e.g., 1–5 filaments per floc)
4	very common	Filaments observed in all flocs at medium density (e.g., 5–10 filaments per floc)
5	abundant	Filaments observed in all flocs at high density (e.g., >20 filaments per floc)
6	excessive	Filaments present in all flocs — appears more filaments than flocs and/or filaments growing in high abundance in bulk solution

could be one of the signs of bulking sludge (mentioned later). Then, we discuss poor floc macrostructure. In some activated sludge samples, the compact core of flocs created by bacterial biomass is missing and the flocs exhibit a loose open structure. The compact cores of activated sludge flocs may also be too small (usually 20–80 μm). Consequently, the reason for bad settling properties are seen in poor floc microstructure. These microstructure problems can be related to either insufficient production or overproduction of the glycocalyx, which is the biopolymer material binding together the cells in cores of activated sludge flocs.

ACTIVATED SLUDGE SEPARATION PROBLEMS

In wastewater treatment practice, six major problems related to microbial biomass quality can be distinguished as a consequence of three different causes (1,3).

Poor Floc Microstructure

Dispersed Growth. The activated sludge microorganisms are dispersed freely in the bulk liquid as individual cells or small clumps with a diameter of 10 to 20 μm. The sedimentation rate of these individual cells or bacterial clumps is too low for gravity sedimentation, and no zone-settling occurs in secondary settling tanks. This has two impacts on the activated sludge process:

1. The separation efficiency of the secondary settling tank is very low, and the final effluent is turbid.
2. Because of poor separation efficiency, a significant amount of biomass escapes from the system. Therefore, only low values of sludge age (Θ_X) can be maintained in the system. A system with dispersed growth resembles a chemostat more than a continuous cultivation system with biomass recycle.
3. This poor bioflocculation is caused by low production of extracellular biopolymers (the glycocalyx) creating a matrix of firm activated sludge flocs. One typical reason for the dispersed growth is a very high organic loading of biomass (high "foot to microorganisms" ratio, F/M), when bacteria do not need to produce the glycocalyx. Thus, the dispersed growth is a common problem in a start-up period of activated sludge systems. Another reason may be the toxicity of treated wastewater.

Unsettleable Microflocs. The outer symptom of this separation problem looks, superficially, very similar to the dispersed growth. The final effluent from a secondary clarifier is not clear and contains many microparticles of escaping biomass. However, the nature of the problem is different, as can be seen under the microscope. The unsettleable particles are of larger dimension (about 50–100 μm) than in the case of dispersed growth; and are approximately spherical and compact. These microflocs result from the disintegration flocs that were initially firm and sound.

During the settling test, the activated sludge rapidly separates into two parts. The larger flocs settle quickly, and when the sludge volume index is calculated on the basis of the volume of these larger flocs, its value is quite low (around 50 mL/g). However, the supernatant in the cylinder is turbid and a substantial fraction of total biomass remains in these unsettleable particles.

The reasons for this floc disintegration are:

- Insufficient production of glycocalyx or its consumption by bacteria inside the flocs due to a low organic loading of biomass (typical of high sludge age systems, extended aeration),
- Total absence of filamentous microorganisms that form the "backbone" of larger flocs (3), and
- Disintegration of flocs by shearing effects, for instance, by inappropriate mechanical aerators.

Viscous Bulking. The rather broad term, viscous bulking, describes the symptoms, but not the causes. Activated sludge contains an excessive amount of extracellular biopolymers, which impart a slimy jellylike consistency to the sludge. Because the biopolymers are hydrophilic colloids, the activated sludge becomes highly water retentive. Such a "hydrous" activated sludge exhibits low settling and compaction velocities. These biopolymers are also natural surface-active agents. When the viscous activated sludge is intensively aerated, strong foam may appear (1).

The production of biopolymers is characteristic of most floc-forming microorganisms, but under normal conditions (no toxic compounds and nutrient balanced growth) the amount of generated biopolymers is just enough for formation of firm flocs. On the other hand, zoogloeal bacteria always produce large amounts of biopolymers because the individual cells of *Zoogloea* are fixed in slimeous colonies. Thus, some authors use the term "zoogloeal bulking" for describing the settling problems caused by an excessive presence of highly hydrated zoogloeal colonies in activated sludge.

Poor Floc Macrostructure

Filamentous Bulking. Filamentous bulking is a typical problem of poor activated sludge compaction (phase III of the settling curve — mentioned earlier), which results in (1):

- Low return and wasted activated sludge solids
- Difficulties in maintaining the required activated sludge concentration in reaction basins
- Poor sludge dewaterability
- Hydraulic overloading of sludge handling facilities

Filamentous microorganisms interfere with the sedimentation and compaction of activated sludge flocs in two ways (3):

1. Some filamentous microorganisms prefer to grow inside flocs and produce flocs with a very diffuse open structure. These open flocs provide space for water retention inside them, so that although filaments protruding from the flocs do not mechanically hinder aggregation of individual flocs, excessive water remains "captured" in the settled sludge.

2. The second way that filaments can detrimentally affect the sedimentation and compaction of activated sludge flocs is much more common. Most of the filamentous microorganisms observed in activated sludges protrude from rather compact and firm flocs into the bulk liquid. The filaments, which in low numbers form a backbone of firm flocs, in large numbers, are able to prevent, mechanically, the compaction of individual flocs. This type of interference problems is called "bridging."

Foaming Caused by Filamentous Microorganisms. Biological foaming by "foam-forming" filamentous microorganisms (see ACTIVATED SLUDGE — FOAMING, this Encyclopedia) is a complex of physicochemical and biochemical interactions leading to the stabilization of a three-phase system of air-water-microbial cells. Stabilization of these biological foams results from the following features of foam-forming filaments:

- Production of extracellular materials such as lipids, lipopeptides, proteins, and carbohydrates, which have the properties of surface-active agents (biosurfactants)
- The cell walls of foam-forming microorganisms are strongly hydrophobic, contrary to other filaments and floc-formers.

The formation of stable foams in the aeration basins of activated sludge plants can create a wide range of operation problems:

1. Aesthetic problems, slippery path along the aeration basins covered by escaping foam.
2. Floating biomass in secondary clarifier deteriorating the final effluent quality.
3. Accumulation of significant amount of biomass into the foam, which is not available for treatment processes; loss of possibility of controlling activated sludge age.

Other Reasons

Rising Sludge. When this is the problem, the water surface in the secondary clarifier is covered by patches (or in worse cases at continuous cover) of floating activated sludge. If the phenomenon is observed in a glass cylinder, two phases can be distinguished:

- The activated sludge settles rapidly and a rather compact bottom layer of settled sludge and a clear supernatant are formed.
- After a certain period (at elevated temperatures even less than 30 minutes, which may raise difficulties in the SVI test), a part or a whole volume of the settled and thickened sludge starts to float and move up to the water surface.

The floating material is full of gas bubbles caused by an endogenous denitrification, which takes place in the settled and thickened layer of activated sludge. Because of a high biomass concentration, the dissolved oxygen from the previous aeration is quickly depleted and anoxic conditions are thus established, provided nitrification occurs in the system. The bubbles of nitrogen liberated during this endogenous denitrification act as sludge "carriers." Thus, the primary reason for this kind of separation problems does not lie in the activated sludge itself but in:

- Insufficient denitrification in the activated sludge system
- Poor performance of settled sludge removing equipment in secondary clarifiers, when the sludge remains too long in the bottom layer.

BASIC SELECTION MECHANISMS FOR FILAMENTOUS BULKING CONTROL

The actual microbial composition of activated sludge (see ACTIVATED SLUDGE — THE MICROBIAL COMMUNITY, this Encyclopedia) is a result of strong competition for energy and carbon sources, nutrients, and also for electrons. The competition is influenced by numerous factors, both intrinsic to the activated sludge process such as biomass retention time, actual substrate, oxygen, and nutrient concentrations in reactor or cultivation conditions (oxic, anoxic, and anaerobic) and external factors including wastewater composition, temperature, and pH. These are difficult to control, but their effect must be considered in wastewater treatment plant control strategies. The following basic mechanisms are thought to be decisive in selecting microbial species in activated sludge (1):

Kinetic Selection

This selective pressure results from differences in growth and substrate utilization rates. In the case of balanced growth (in which readily biodegradable substrate uptake and cell growth occur simultaneously), two types of competition strategy are possible, in terms of saturation kinetics.

Activated sludge floc-forming microorganisms are generally μ_{max}-strategists, whereas most of activated sludge filamentous microorganisms belong to theK_S-strategists. As shown in Figure 1, a substrate concentration gradient is necessary to support the growth of these μ_{max}-strategists. However, so-called unbalanced growth, when the phases of substrate (both soluble and particulate) or nutrient uptake and utilization are partially or fully separated, occurs in activated sludge processes with substrate concentration gradients. During competition under conditions of unbalanced growth, the microorganisms that can sequester substrate more rapidly from the bulk liquid will be favored, provided there is time enough for the regeneration of accumulation/storage capacity in these cells. In activated sludge, the accumulation or storage and regeneration selection mechanism is typical of most floc-forming microorganisms. The process of substrate accumulation or storage takes place in the contact zone of activated sludge systems (Fig. 2), which can be not only oxic but also anoxic or anaerobic. Meanwhile, endogenous metabolism (regeneration) occurs in the main aeration basin or in a

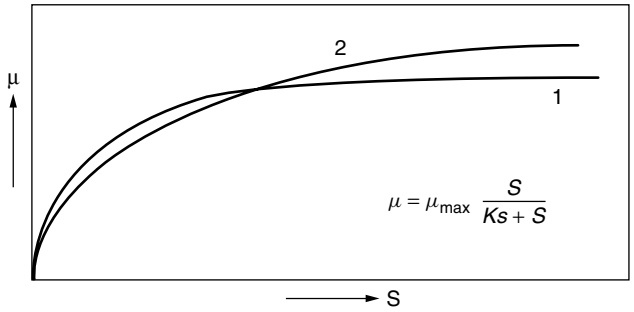

Figure 1. Schematic description of K_S-strategy (2) and μ_{max}-strategy (3) in terms of Monod kinetics. μ_{max}-strategy: maximum specific growth and substrate utilization rates at high substrate concentrations. K_S-strategy: high substrate affinity at low substrate concentrations.

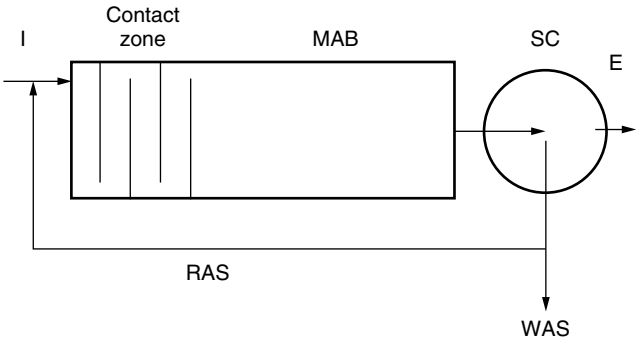

Figure 2. Activated sludge system with compartmentalized contact zone. I — influent, E — effluent, WAS — waste activated sludge, RAS — return activated sludge. Contact zone: Compartmentalized zone with substrate concentration gradient (accumulation and storage of organic substrate), MAB — main aeration basin: a part of the activated sludge system in which organic carbon removal is finished and in which the endogenous metabolism of accumulated/stored substrate takes place (regeneration). SC — secondary clarifier.

separate regeneration zone placed in the return sludge stream (Fig. 3) (1,5).

A compartmentalized contact zone of an activated sludge system is sometimes called a selector. However, for the selection of floc-forming organisms, the combination of a compartmentalized contact zone and regeneration is always necessary.

The results of competition between floc-forming and filamentous microorganisms based on kinetic selection are influenced by the following factors:

Wastewater Composition. Wastewater is a complex mixture of substrates, nutrients, and micronutrients, which support the community of activated sludge. In addition, wastewater continuously inoculates this consortium with the bacteria growing into sewers.

Readily Biodegradable Substrates. Bacterial cells can probably directly utilize low molecular weight organic compounds, that is, compounds with simple molecules. These compounds (e.g., monosaccharides, alcohols, volatile fatty acids, and amino acids) represent approximately 10

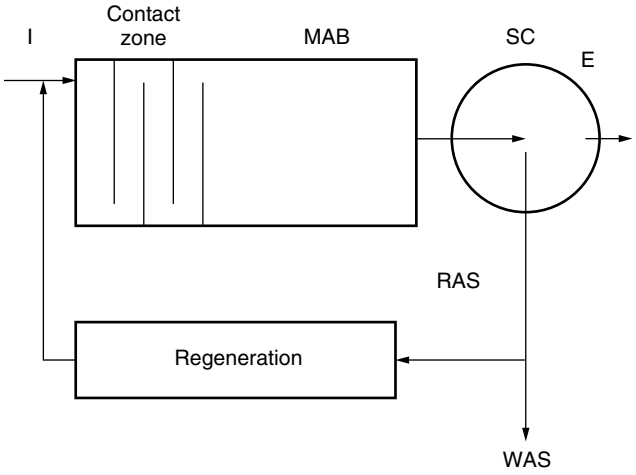

Figure 3. Activated sludge system with compartmentalized contact zone. I — influent, E — effluent, WAS — waste activated sludge, RAS — return activated sludge. Contact zone: Compartmentalized zone with substrate concentration gradient (accumulation and storage of organic substrate), MAB — main aeration basin: a part of activated sludge system in which organic carbon removal is finished, REGENERATION: a part of the activated sludge system in which the endogenous metabolism of accumulated or stored substrate takes place (regeneration), SC — secondary clarifier.

to 20% of COD of common municipal wastewaters. These substrates are thought to favor the growth of certain filamentous microorganisms, for example, Types 021N, *Sphaerotilus natans* /1,701, 0,041 and *Haliscomenobacter hydrossis*. Special attention has to be paid to the design of activated sludge systems for wastewaters containing an increased fraction of industrial wastewaters (food industry). Reduced sulfur compounds can also be readily metabolized, thus supporting the growth of sulfur-metabolizing filaments such as Type 021N/*Thiothrix* or *Beggiatoa*.

Particulate, Slowly Biodegradable Substrates. Most of the organic compounds in municipal wastewaters are present as so-called particulate substrates. These are formed by large-molecule organic compounds, which are present in the wastewater, either as colloids or as true suspended solids. In both cases, they have to be digested by extracellular enzymes before they become available for cells. The mechanism for particulate substrate disintegration is generally termed as hydrolysis, and the products of this process are very similar from a chemical point of view to the readily biodegradable substrates, originally present in wastewaters. However, the hydrolysis is a surface phenomenon and is connected with flocs. Therefore, if the main aeration basins where the hydrolysis occurs are not operated as completely mixed tanks, the readily biodegradable substrates released by hydrolysis will be more available to the floc-formers than the filamentous microorganisms.

Some specific particulate substrates can support the growth of certain filamentous microorganisms. As is seen with fats and grease, which are selectively concentrated in foams and thus, support the growth of nocardioform

actinomycetes. Long-chain fatty acids are reported as being specific substrates supporting the growth of *Microthrix parvicella* (6,7).

Inoculation of Activated Sludge Systems from Wastewaters. Sewerage network and wastewater treatment plants form one system, and processes in sewers may significantly affect the composition of the activated sludge community. Especially in extended sewer systems, readily biodegradable substrates can be taken up by bacteria growing in inner slimes. These wall growths are continuously sloughed off and they inoculate the treatment plant. In some cases, filaments such as *S. natans* can be found in that inoculum (1).

Biomass Retention Time. Biomass retention time Θ_X ("sludge age") has an impact on the distribution of individual microbial species in the consortium of activated sludge, according to their growth and decay rates. Low biomass retention time Θ_X may result in wash-out of slower-growing species whose net growth rate is slower than the dilution rate $D = 1/\Theta_X$. On the other hand, high Θ_X values favor the slow-growers. Unfortunately, the "safe" values of Θ_X, when all filamentous microorganisms are assured to be washed-out, are too low to be applied as a common bulking control measure.

Actual Substrate Concentration in Reactor. Activated sludge cultured in reactors with substrate concentration gradients acquires certain features that have been described as "selector" effect (8). They are:

1. High rates of substrate consumption,
2. High oxygen (or generally, electron acceptor) uptake rate, and
3. Enhanced growth of zoogloeal bacteria.

   The substrate concentration gradient produces a combined effect of both kinetic and metabolic selection (mentioned later). Because of floc stratification, a fourth feature can be added, that is,
4. Increased metabolic diversity of activated sludge.

Dissolved Oxygen, Nutrients, pH, and Temperature in Aeration Basins

Dissolved Oxygen (DO). Some filamentous microorganisms (e.g., *S. natans*/Type 1,701, *H. hydrossis*) exhibit a high affinity for dissolved oxygen at low concentrations because of low values of their half-saturation constant K_O (3). The boundary between "bulking" and "nonbulking" DO concentrations is not fixed because this value depends on the actual value of activated sludge loading (B_X) in the reactor. Nevertheless, DO concentrations less than 0.5 mg/L are dangerous for low DO bulking, especially when soluble substrate still remains in activated sludge mixed liquor (1,3,5).

Nutrients. As with DO, some filaments exhibit higher affinity to certain nutrients (i.e., nitrogen, phosphorus, and micronutrients). This is again the result of a K_S-strategy in certain filaments under conditions of balanced growth. Therefore, the addition of nutrients for controlling filamentous bulking is sometimes necessary. As guidelines for dosing with nutrients, the following empirical concentrations in final effluent might be used — ammonia nitrogen at least one mg/L and soluble orthophosphate phosphorus more than 0.2 mg/L.

pH. As far as is known, none of the most common filamentous microorganisms exhibits a special preference for extreme pH values. Some fungi prefer low pH values, although such a decrease in the pH in municipal wastewater treatment plants (e.g., by nitrification) leading to excessive fungal growth is improbable. Lime dosing equipment should be considered when an acid stream might be discharged into the plant.

Temperature. Temperature affects significantly the rates of all biochemical processes, and the solubility of oxygen in the mixed liquor. Thus, elevated temperatures will support the growth of filamentous microorganisms associated with low DO concentrations. The oxygenation capacity of the aeration systems should be calculated for the highest mixed liquor temperatures expected during the year in order to guarantee the recommended DO concentration is achieved in warm weather.

There are reports of significant seasonal shifts in the dominance of individual filamentous types. Typically, *M. parvicella*, which dominates the filamentous population in winters, is replaced by nocardioform actinomycetes, Type 0,041, or *Nostocoida limicola* in warm seasons. This shift is almost certainly associated with temperature changes, but an exact explanation of this phenomenon is still not available. According to practical experience, more severe *M. parvicella* foaming problems are to be expected in winter than in summers (9,10).

Metabolic Selection

In nutrient removal activated sludge systems, the decisive fraction of readily biodegradable substrates from wastewater is utilized not under oxic but under anaerobic and/or anoxic conditions. Thus, the microorganisms that are able to metabolize substrates under these conditions will be selected. This is the principle of metabolic selection.

Alternation of anaerobic and oxic cultivation conditions is a prerequisite for the enhanced biological phosphorus removal (EBPR) mechanism in nutrient removal. As the filamentous microorganisms from Groups S and C (shown later) are unable to utilize substrates under alternating anaerobic or oxic cultivation conditions at a rate comparable to that of floc-forming microbes, their suppression in nutrient removal activated sludge systems is based on metabolic principles. Although Group S and C filamentous microorganisms are quite common in conventional activated sludge plants, surveys of filaments in nutrient removal systems conducted in Europe, South Africa, U.S.A., Australia, and the Czech Republic (11) indicate that these filaments are not important in systems with anaerobic and/or anoxic zones. Elimination of Group S and C filaments in systems with anoxic zones is based on different denitrification rates between filaments and floc-formers (1,12).

Classification of Filamentous Microorganisms. The growth response of filamentous microorganisms to pressures caused by either kinetic or metabolic selection (or by the combined effect) can be used to classify the filamentous microorganisms found in activated sludge. Thus results from microscopic identification of filaments can be directly used in wastewater treatment practice in reaching proper remedial measures.

Wanner and Grau (12) summarized their own and literature data and proposed the classification of filamentous microorganisms into four groups. The groups were established on the basis of:

- Morphological similarity (sheath, trichome, and cells)
- Similar staining reactions, intracellular deposits
- Metabolic similarity, that is, the ability to utilize substrates and gain energy under the same cultivation conditions
- Occurrence in the same operational arrangements and conditions
- Similarity in problems that the filamentous microorganisms cause.

Group S: Sphaerotilus-like Oxic Zone Growers. They include sheathed filamentous microorganisms, which are able to utilize organic substrates only under oxic conditions. The presence of polyphosphates and PHB granules in cells may be observed, but the rate of their formation and exploitation is not technologically important. Their occurrence in activated sludges is connected with saccharidic and other readily biodegradable wastewaters, higher Θ_X, and low DO. Characteristic representatives are *S. natans* and Type 1701 and Types 0041 and 0675.

Group C: Cyanophycae-like Oxic Zone Growers. Group C includes filamentous microorganisms morphologically resembling colorless blue-green bacteria: Type 021N and *Thiothrix*. Type 021N is a very common filamentous microorganism in bulking activated sludges found in conventional activated sludge plants treating domestic or other wastewaters containing readily biodegradable substrates (1,3).

As opposed to oxic zone growers S, the microorganisms from group C are able to metabolize sulfur. *Thiothrix*, in particular, can take advantage of its mixotrophic way of life in systems with anaerobic zones (1,10).

Group A: All Zones Growers. The term "all zones growers" means that these microorganisms are equipped with such a diverse enzymatic apparatus that they can utilize substrates under all the conditions encountered in activated sludge plants. In addition, all zones growers accumulate low molecular substrates under both oxic and anoxic conditions, and synthesize storage products with rates comparable to those of the floc-formers. *Microthrix parvicella* and Type 0092 undoubtedly exhibit the characteristic features of the all zones growers, and *N. limicola* I and II are commonly found in full-scale plants with anaerobic zones (11). Foot (10) also added to this group Type 0803.

Group F: Foam-Forming Microorganisms. The foam-formers are microorganisms, which can produce biosurfactants enabling them to froth and create scum. The floating effect is supported by hydrophobic surfaces of cells. These hydrophobic cells stabilize the air bubbles or oil droplets entrapped in the foam. The formation of biological foams is associated with the Actinobacteria, especially the so-called norcardioform actinomycetes (*Gordona*, *Rhodococcus*) or bacteria-like *M. parvicella*. These microorganisms can be considered as primary foam-formers together with Types 0092, 0041, and *N. limicola* II. Other filamentous microorganisms commonly found in biological foams are probably not foam causers, but become associated with already established scums.

The differences among individual groups S, C, A, and F can be illustrated by the response of filamentous microorganisms to systems combining kinetic selection with metabolic selection under different cultivation conditions (Table 3).

APPLICATION OF BULKING CONTROL PRINCIPLES TO THE DESIGN AND OPERATION OF ACTIVATED SLUDGE PLANTS

Plant Configuration

The design of an activated sludge system has to combine all the factors that favor preferentially the growth of the floc-formers, that is:

- A substrate concentration gradient in the system (or at least in its head-end)
- A rapid accumulation ("biosorption") of substrate with subsequent regeneration of accumulation/storage capacity
- In the case of nutrient removal systems, utilization of most of the substrate under anaerobic and/or anoxic conditions.

There are principally four process configurations effectively supporting the growth of floc-formers. In these

Table 3. Response of Filamentous Microorganisms to Systems Combining Kinetic Selection (High Concentration Gradients) with Metabolic Selection Under Different Cultivation Conditions (13)

Group of Filamentous Microorganisms	Cultivation Conditions		
	Oxic	Anoxic	Anaerobic
S (e.g., *Sphaerotilus natans* and Type 1701)	–	–	–
C (e.g., Type 021N and *Thiothrix*)	–	–	–/+ (+ for sulfur filaments)
A (e.g., *Microthrix parvicella*, Type 0092, *Nostocoida limicola*)	0	0	0/+
F (e.g., nocardioform actinomycetes)	?	?/–	–/?

– suppression; + stimulation;
0 no effect ? effect uncertain

configurations, the basic principles of kinetic selection are employed either alone or in combination with metabolic selection:

1. Sequencing batch reactor with short filling period ("dump-fill") or with an inlet mixing zone.
2. Continuous plug-flow reactors; as even the long corridor-type reactors exhibit a high degree of longitudinal mixing, the reactor should be compartmentalized to approach the plug-flow hydraulic regime.
3. Completely mixed or compartmentalized continuous-flow reactors with a contact zone placed ahead of the main reactor, where the contact zone should, preferably, be divided into two to four compartments.
4. Activated sludge system with return sludge regeneration, in which the main reactor should be arranged in configurations two or three.

In all these configurations, the return activated sludge can be mixed with the treated wastewater either under oxic or anaerobic or anoxic conditions. An example of an activated sludge configuration combining most of the factors favoring the growth of floc-formers is given in Figure 4.

Adding the anaerobic zone in front of the denitrification zone can modify the arrangement shown in Figure 4. In this so-called R-AN-D-N process, not only can enhanced biological phosphorus removal (EBPR) be achieved, but because of much stronger metabolic selection pressures these arrangements prove to be more resistant to bulking and foaming problems (15).

Design Parameters

Oxic Contact Zone Design. The design criteria for the oxic contact zone (1,5,16) can also be applied to the design of the inlet part of compartmentalized plug-flow systems or to the design of SBRs. However, the oxic contact zone (originally patented, as the so-called selector in Czechoslovakia) can only be used in conventional activated sludge systems, and not in biological nutrient removal (BNR) plants because the readily biodegradable substrate necessary for the EBPR and denitrification is "inefficiently" consumed under oxic conditions. The following parameters should be considered in the design of oxic contact zones.

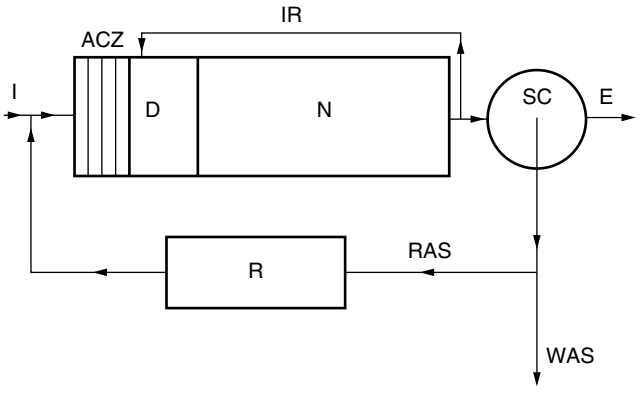

Figure 4. Schematic of the so-called R-D-N Process. ACZ — compartmentalized anoxic contact zone; D — denitrification zone; N — nitrification zone; SC — secondary clarifier; R — regeneration zone; I — influent; E — effluent; RAS — return activated sludge; WAS — waste activated sludge; IR — internal recirculation (14).

Contact Time. The contact time is based on the flow rates of mixed liquor and not only on wastewater flow rates. It is directly correlated with the size of the contact zone. When the contact time is insufficient, soluble substrate is not consumed in the contact zone, and enters the main reactor where it can support growth of filaments. On the other hand, when the contact time is too long, growth of filaments occurs right in the contact zone, especially when this is not compartmentalized. The recommended values for contact time have been summarized by Wanner (1), and lie in a range of 10 to 20 minutes.

For conventional activated sludge systems, this contact time results in the volume ratio V_{CZ}/V_{TOTAL} of about 1 : 10. This ratio is also sufficient for a full restoration of accumulation/storage capacity in the main reactor. The recirculation ratio of return activated sludge entering the contact zone should be less than one.

Activated Sludge Loading in the Contact Zone. Activated sludge loading (B_X) was selected as a design criterion instead of "floc loading." Although this parameter describes more precisely the relationship between actual substrate and biomass mass fluxes, for continuous-flow systems the activated sludge loading is more convenient for calculations. To achieve conditions for well settling sludge, the activated sludge loading B_X in the whole contact zone should be higher than three kg/kg.d (based on BOD_5 and X) (16). If the contact zone is compartmentalized, the recommended activated sludge loading B_X should be, for instance, at least 12 kg/kg.d in the first compartment of a four-compartment contact zone.

Conditions in the Main Reactor. The selection of floc-formers does not occur only in the contact zone, and the conditions in the main reactor following the contact zone are part and parcel of the selection process (in this sense the term "selector" should cover the whole configuration and not only the contact zone). The activated sludge retention time in the main aeration basin is sufficient for a complete restoration of accumulation or storage capacity, when the activated sludge loading in the whole system does not exceed 0.3 to 0.5 kg/kg.d (BOD_5, X) (5,16,17). If the main reactor is a tank with a high dispersion number, the soluble COD in the effluent from the contact zone should not exceed the final effluent soluble COD by more than 20 to 30 mg/L (1).

Design of Anaerobic and Anoxic Contact Zones of BNR Systems. The primary task of the anaerobic and anoxic contact zones in BNR (biological nutrient removal) activated sludge systems is to create conditions for nitrogen and phosphorus removal. Thus, the design of these zones has to follow principally the guidelines for BNR systems design. However, the designer should always

try to implement bulking control strategies in the design, although the conditions for bulking control can sometimes be contradictory to the requirements for BNR processes.

Anaerobic Zone. The anaerobic contact time sufficient for bulking control is about 0.5 to 1.0 hour, which is usually exceeded because of EBPR. The recommended B_X value of one kg/kg.d (BOD_5, X) in the anaerobic zone for efficient bulking control is also mostly met because of the general requirements of an EBPR design.

Anoxic Zone. A certain analogy with the design of oxic contact zones can be used because the floc-formers supported by anoxic contact zones are mostly the same microbes, which are favored by oxic contact zones. Thus, recommendations with regard to the construction and design of oxic contact zone can be applied to a certain extent, also for the design of anoxic contact zones in BNR plants.

Plant designers and operators should always be aware of the main principles of bulking control:

- In conventional plants, the majority of organic substrate available in treated wastewater should be removed in the head part of the activated sludge system, and the aeration basin should always be operated as a plug-flow reactor.
- In nutrient removal systems, the majority of the available organic substrate should be consumed in the anaerobic and/or anoxic zone.

NONSPECIFIC BULKING CONTROL METHODS

All the methods described earlier are selectively targeted at the control of excessive growth of filamentous microorganisms. However, in wastewater treatment practice, other methods of bulking control are commonly used. These methods are not aimed at the control of the growth of filaments, but at the suppression of problems resulting from the presence of filaments or at the entire elimination of the filamentous population.

Elimination of the Filamentous Population

This method is applicable when the problem of "floc bridging" is the case. The method exploits the fact that relatively thin filamentous microorganisms are exposed, directly and immediately, to concentrations of chemicals found in the bulk liquid; whereas most of the floc-forming microorganisms are exposed to lower concentrations because of diffusional resistance in the outer layer of activated sludge flocs. Therefore, the filamentous microorganisms protruding from the flocs can be selectively killed by adding toxic compounds to the return activated sludge stream, whereas most of the floc-forming organisms will survive inside the flocs. For the full-scale control of filamentous bulking, chlorine is used most often (3). However, chlorination of activated sludge is just a remedial method and should not be used as a permanent solution to bulking problems (1).

Increase of Sedimentation Velocities

As described earlier, excessive presence of filamentous bacterial populations reduces the sludge settling velocities by both mechanisms (floc bridging and "open" floc structure). One of method to compensate for this is to increase the specific weight of the activated sludge flocs. The following procedures can be used by process operators as remedial methods:

- Addition of mineral suspensions to the mixed liquor (e.g., soil suspension, primary effluent particles by reducing the capacity of primary settling tanks).
- Addition of digested sludge to the mixed liquor (this must be done very carefully to avoid any DO deficit in the aeration basins).
- Formation of heavy "cores" in the flocs by precipitation of mineral salts, the most common method of bulking control with this approach now is the addition of ferric salts to the mixed liquor (1,5,15)

Compensation of Slow-Settling Velocities

One possible solution is to construct new secondary clarifiers, which are less susceptible to poor settling properties of the activated sludge than the older constructions. The secondary clarifiers, which are more resistant to bulking activated sludge, should exhibit the following features (18):

- A specially designed zone for efficient flocculation of activated sludge.
- Deeper settling tanks providing enough "buffering" capacity.
- Reduction of any hydraulic or mechanical disturbances in the layer of settled sludge to avoid sludge resuspension.

CONCLUSION

Activated sludge process is the most common technology of biological wastewater treatment used today. Until other separation methods such as membrane filtration become economically feasible, activated sludge separation from treated wastewater by gravity sedimentation is the only real option. For this reason, good settling properties of activated sludge are still of primary importance for achieving the required quality of final effluent. Of the six different separation problems described in this article, activated sludge bulking caused by the excessive presence of filamentous microorganisms is the most serious operational problem. These filamentous microorganisms adversely affect activated settling properties by two different mechanisms, that is,

- By "bridging" firm and compact flocs
- By providing "open" and "diffuse" structure to the activated sludge flocs

The methods for activated sludge filamentous bulking control can be divided into two major groups:

1. Methods, which specifically control the growth of filamentous microorganisms on one hand and support the growth of floc-forming microorganisms on the other hand.

   The methods in this group exploit the principles of activated sludge population dynamics and support the floc-forming microorganisms in their competition with filamentous microorganisms. The selection of microbial community composition can be based either on kinetic or on metabolic principles. Kinetic selection of floc-forming microorganisms can be achieved in activated sludge systems by establishing substrate concentration gradients. For metabolic selection, the consumption of the most readily available substrates under anaerobic and/or anoxic cultivation conditions is decisive.

2. Methods eliminating the filamentous population by chemical action or compensating the negative effect of the excessive presence of filamentous microorganisms.

   From this group of methods, which do not prevent the excessive growth of filamentous microorganisms but solve the problems caused by their presence, the following are most commonly used:

   - Selective killing of filaments by chemical compounds such as chlorine.
   - Increase of floc settling velocity by addition of mineral particles or precipitants (e.g., addition of ferric salts).
   - Measures in the construction of secondary clarifiers compensating the low settling velocities of bulking activated sludge

Acknowledgments

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NOTATION

X — concentration of biomass expressed in terms of SS (suspended solids), mg/L or g/L B_X — activated sludge loading

$$B_X = \frac{S_1 \cdot Q}{V \cdot X}$$

where S_1 substrate concentration in the influent to the reactor
Q wastewater influent flow
V reactor (aeration basin) volume
X biomass concentration (MLSS) in the reactor

Θ_X — sludge age

$$\Theta X = \frac{V \cdot X + M_{SC}}{(Q - Q_W) \cdot X_2 + Q_W \cdot X_W}$$

where M_{SC} mass of biomass (activated sludge) in secondary clarifier
Q_W excess sludge flow
X_2 biomass concentration in the final effluent
X_W biomass concentration in the excess sludge stream

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FILTRATION (HIGH-RATE): REMOVAL OF PATHOGENIC MICROORGANISMS.

See Removal of Pathogenic Microbes by Granular High-Rate Filtration

FILTRATION: OCCURRENCE OF PROTOZOA IN SPENT FILTER BACKWASH WATER.

See Occurrence of Protozoa in Spent Filter Backwash Water

FINGERPRINTING TECHNIQUES. See BIOFILMS IN NATURAL AND DRINKING WATER SYSTEMS

FLAGELLATED PROTOZOA. See PROTOZOA IN MARINE AND ESTUARINE WATERS

FLOCS, MICROBIAL. See ACTIVATED SLUDGE — THE FLOC; MICROBIAL FLOCS SUSPENDED BIOFILMS

FLOODED SOILS

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Wetlands are mainly characterized by standing water, a vegetation adapted to the wetness, and unique soil conditions different from those of upland soils (1). Flooded soils are found in wetlands that are inundated for part of the year. They occur naturally in floodplains and tidal marshes or are created in wetland rice agriculture. Three major types of wetland rice exist: deepwater rice that is covered by more than 50-cm water depth, irrigated rice that is permanently flooded by artificial irrigation, and rain-fed rice that is flooded only after heavy rainfall (2). Wetlands are of great ecological importance, for instance, by constituting unique habitats for plants and animals (1); by contributing the largest source in the atmospheric methane budget, thus affecting global climate (3); and in the case of rice agriculture, by providing the food for one-third of the human population (1).

In general, wetland soils are characterized by unique biogeochemical cycles of carbon, oxygen, nitrogen, sulfur, iron, and phosphorous and unique microbial communities when compared with upland soils (1,4). Besides the various modes of organic matter input, the biogeochemistry of wetland soils is largely controlled by the restricted availability of oxygen. Flooded soils, in particular, have a microflora that is adapted to the change between submergence and drainage. In addition, biogeochemistry of rice soils also is influenced by plowing and by domination of only one plant species. In these respects, rice soils differ from marsh soils or aquatic sediments.

This article focuses on the biogeochemistry and microbiology of irrigated rice soils. In contrast to other wetland soils, irrigated rice soils are manageable and thus accessible to experiments in the field and the laboratory. Thus, they constitute a relatively simple model system. In addition, they cover an area of about 73 million hectares worldwide and annually (in 1997) produce 520 million tons of rice (5). This production must be increased in the future (by 65% until the year 2020) to cover the demand of food by the growing human population (2). On the other hand, they emit about 60 million tons of methane into the atmosphere (3). Although increasing atmospheric methane may cause severe environmental problems by global climate change, a future problem is to increase rice production for the growing human population and simultaneously mitigate methane emission (5,6). This challenge can only be met by a better understanding of the functioning of soil microbial communities.

Biogeochemical processes in flooded soils have previously been reviewed with respect to microbiology (7–9), soil chemistry (10–12), and methane emission (2,13–17). Important early studies published in Japanese have been reviewed by Kimura (9).

BIOGEOCHEMICAL CYCLING

The biogeochemical cycling in flooded soils is driven by the input of organic carbon and of oxygen and by the availability of oxidants (i.e., nitrate, Mn(IV), Fe(III), and sulfate) in the soil. If oxygen or other oxidants are available, organic carbon is oxidized to carbon dioxide. If oxygen or oxidants are not available, organic carbon is disproportionated to CO_2 and CH_4. Availability of organic carbon, O_2, and oxidants in the flooded soil varies with time, space, soil type, and management. The major habitats of microorganisms in rice soils related to availability of O_2 and organic substrates are shown in Figure 1. The major microbial catabolic reactions that are active in rice soils are shown in Table 1.

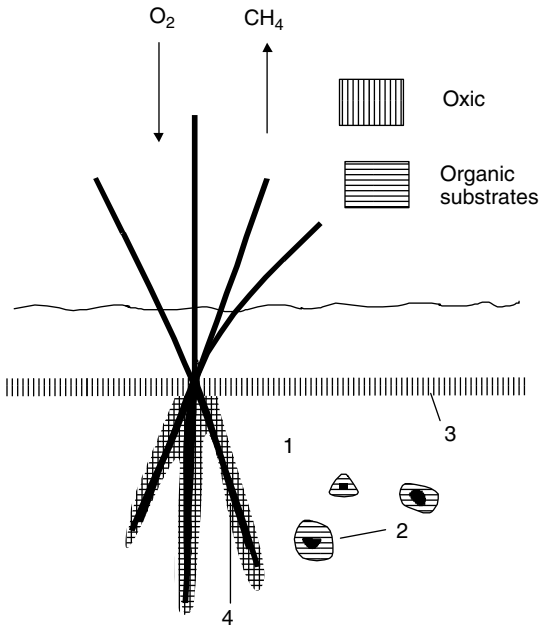

Figure 1. Scheme of a flooded rice field showing the different habitats for biogeochemically active microorganisms: (*1*) bulk soil (anoxic & substrate-limited), (*2*) decomposing straw (anoxic & increased organic substrates), (*3*) surface soil (oxic), and (*4*) rhizosphere (partially oxic & increased substrates).

Table 1. Major Biogeochemical Reactions Catalyzed by Aerobic and Anaerobic Microorganisms and the Standard Gibbs Free Energy Changes ($\Delta G^{\circ'}$) and Standard Redox Potentials ($\Delta E^{\circ'}$)[a]

(a) Aerobic microbes using O_2 as electron acceptor

No.	Phenotype	Reaction	$\Delta G^{\circ'}$ (kJ mol^{-1}) per O_2	$\Delta G^{\circ'}$ (kJ mol^{-1}) per Substrate
1	Heterotrophs	$C_6H_{12}O^b{}_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O$	−479	−2, 872
2	H_2 oxidizers	$H_2 + 0.5O_2 \rightarrow H_2O$	−474	−237
3	Methanotrophs	$CH_4 + 2O_2 \rightarrow CO_2 + 2H_2O$	−409	−818
4	Sulfide oxidizers	$HS^- + 2O_2 \rightarrow SO_4{}^{2-} + H^+$	−398	−796
5	Iron oxidizers[c]	$FeCO_3 + 0.5O_2 \rightarrow FeOOH + CO_2$	−380	−190
6	Ammonium oxidizers	$NH_3 + 1.5O_2 \rightarrow NO_2{}^- + H^+ + H_2O$	−192	−288
7	Nitrite oxidizers	$NO_2{}^- + 0.5O_2 \rightarrow NO_3{}^-$	−148	−74

(b) Anaerobic microbes using inorganic electron acceptors[d]

No.	Phenotype	Reaction	$\Delta G^{\circ'}$(kJ mol^{-1}) per e-acceptor	$\Delta G^{\circ'}$(kJ mol^{-1}) per Substrate	$\Delta E^{\circ'}$(mV)
8	DNRA bacteria[e]	$4H_2 + NO_3{}^- + 2H^+ \rightarrow NH_4{}^+ + 3H_2O$	−600	−150	+363
9	Denitrifiers	$2.5\ H_2 + NO_3{}^- + H^+ \rightarrow 0.5N_2 + 3H_2O$	−560	−224	+747
10	Sulfate-reducers	$4\ H_2 + SO_4{}^{2-} + H^+ \rightarrow HS^- + 4H_2O$	−152	−38	−217
11	Methanogens	$4\ H_2 + CO_2 \rightarrow CH_4 + 2H_2O$	−131	−33	−244
12	Iron reducers	$0.5\ H_2 + FeOOH + CO_2 \rightarrow FeCO_3 + H_2O$	−48	−95	+78
12	Homoacetogens	$4\ H_2 + 2CO_2 \rightarrow CH_3COO^- + H^+ + 2H_2O$	−48	−24	−290

(c) Fermenting microbes disproportionating organic substrates

No.	Phenotype	Reaction	$\Delta G^{\circ'}$, (kJ mol^{-1}) per Substrate
13	Homoacetogens	$C_6H_{12}O_6 \rightarrow 3CH_3COO^- + 3H^+$	−311
14	Propionate producers	$C_6H_{12}O_6 \rightarrow 4/3CH_3CH_2COO^- + 2/3CH_3COO^- + 2H^+ + 2/3CO_2 + 2/3H_2O$	−311
15	Butyrate producers	$C_6H_{12}O_6 \rightarrow 2/3CH_3CH_2CH_2COO^- + 2/3CH_3COO^- + 4/3H^+ + 2CO_2 + 8/3H_2$	−248
16	Ethanol producers	$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2$	−235
17	Lactate producers	$C_6H_{12}O_6 \rightarrow 2CH_3CHOHCOO^- + 2H^+$	−198
18	Acetoclastic methanogens	$CH_3COO^- + H^+ \rightarrow CO_2 + CH_4$	−36
19	Theoretical	$C_6H_{12}O_6 + 6H_2O \rightarrow 6CO_2 + 12H_2$	−26
(d) Syntrophs reducing protons to H_2			
20	Lactate utilizers	$CH_3CHOHCOO^- + H_2O \rightarrow CH_3COO^- + CO_2 + 2H_2$	−49
21	Ethanol utilizers	$CH_3CH_2OH \rightarrow CH_3COO^- + H^+ + 2H_2$	+10
22	Butyrate utilizers	$CH_3CH_2CH_2COO^- + 2H_2O \rightarrow 2CH_3COO^- + H^+ + 2H_2$	+48
23	Propionate utilizers	$CH_3CH_2COO^- + 2H_2O \rightarrow CH_3COO^- + CO_2 + 3H_2$	+71

Note:
[a]Calculated from the Gibbs free energies of formation taken from Thauer et al. (18), except for siderite ($FeCO_3$) and ferrihydrite (FeOOH) which were taken from Stumm and Morgan (19).
[b]$C_6H_{12}O_6$ = glucose.
[c]The Gibbs free energy strongly depends on the type of iron mineral used for formulating the reaction.
[d]The reactions are given with H_2 as electron donor. Reactions (12) and (19) can be used to replace H_2 by acetate or glucose, respectively.
[e]DNRA = dissimilatory nitrate reduction to ammonia.

Origin of Organic Carbon

The organic carbon in flooded soils is replenished by exudation and decay of roots and by incorporation of straw and other organic matter. Addition of rice straw or compost is common practice in rice agriculture and results in a strong stimulation of CH_4 production (20–22). The half-life of rice straw in flooded soils is about two years (23). Organic matter is also provided by the plant roots through exudation of part of the photoassimilated carbon, sloughed off cells, and decaying roots (24). Pulse-labeling studies showed that about 5% of the photoassimilated $^{14}CO_2$ was converted to CH_4 within a few days and reemitted into the atmosphere (25). Experiments with ^{13}C-labeled straw and carbon dioxide quantified the contribution of different carbon sources to CH_4 emission from irrigated rice (26–29). Without straw addition, plant photosynthesis accounted for more than 80% of CH_4 emission; the rest was due to decomposition of soil organic matter. Addition of straw stimulated CH_4 production, but the percentage contribution of straw carbon to total CH_4

production declined with time, so that over the entire season straw, plant photosynthesis, and soil organic matter contributed about 40%, 40%, and 20%, respectively (29).

Input of Molecular Oxygen

There are two paths of oxygen input into flooded soils: diffusion from the floodwater and transport through the plant-vascular system. In the first path, O_2 penetrates only 2 to 3 millimeters deep into the soil because of active O_2 consumption (30). Occasionally, O_2 concentrations on the soil surface can increase because of photosynthetic layers of algae, but O_2 is nevertheless depleted in a few millimeter depth (30,31). On the other hand, in the vegetated soil, O_2 can be detected in deeper soil layers because of plant-vascular gas transport (30). Aquatic plants typically transport O_2 by diffusion or pressurized ventilation from the atmosphere into the roots to allow respiration of root cells in the anoxic soil (32,33). The vascular system also allows gas transport in the opposite direction and thus mediates more than 90% of the emission of CH_4 from rice fields (34,35) and allows the emission of other trace gases (36). In rice plants, the gas transport is by molecular diffusion (37–39). Oxygen leaks from the rice roots into the surrounding soil but is so rapidly consumed that the penetration depth is very thin (<0.4 mm) (40,41). However, the impact of oxygen leakage from roots on soil biogeochemical cycling becomes significant during the season as the plants develop (42).

The rhizosphere, or more specifically the ectorhizosphere, is the volume of soil influenced by roots (43). Although the outer boundary of the ectorhizosphere may be difficult to define, the rhizoplane (root surface) and endorhizosphere (the various cell layers of the root itself) are easily distinguishable. The plant-vascular system, the aerenchyma, allows a very efficient gas exchange even by molecular diffusion alone (44). The primary function of root aeration is to support the plant tissue with O_2 for aerobic metabolism, but some O_2 will be released into the ectorhizosphere. However, O_2 release along a root is not homogenous (45–47). No O_2 is released at the root tip. Only a few millimeters from the tip aerenchyma formation starts, and the highest O_2 release rates are found along this zone. More proximally, radial O_2 release rate decreases again (40,46). This zone of low O_2 release correlates with the suberization of the exodermis and the development of a layer of sclerenchymatous fibers on the external side of the cortex, causing a barrier for O_2 diffusion (48). Temporal changes in the rhizospheric redox potential, which were recorded close to a growing rice root, agreed well with the described zonation and suggest an "oxygenation window" for the zone behind the root tip forming for about two to three days (49). Free O_2 has been found repeatedly in the rooted soil of flooded rice microcosms (30,40,50,51). However, at elevated temperatures, when O_2 consumption is accelerated, the occurrence of O_2 seems to be restricted to the very root surface (31).

Oxygen concentrations measured in the rooted soil of microcosms at 100 days after planting (dap) were lower than at 50 dap (30). This result is consistent with the observation that Fe(III) precipitates are formed around young roots (see following section) but dissolved later when the root grows older (52). Hence, the O_2 supply seems to be higher in the early season than in the late season. However, the O_2 supply also was found to be influenced in the following ways: (*1*) in vitro, O_2 release from rice roots is stimulated by the inoculation with nitrogen-fixing *Azospirillum* (53); (*2*) reducing soil conditions may favor both aerenchyma formation and O_2 release (54); (*3*) in potassium-depleted soils, the oxidizing power of rice roots is increased by K fertilization (55). To summarize, the O_2 supply in the rhizosphere is highly variable in space and time.

Sequential Reduction and Redox Gradients

Besides O_2, a number of inorganic soil constituents contribute sequentially to the oxidation of organic matter. The sequence is determined by thermodynamics as compounds with higher redox potential are reduced first, that is, $O_2 > NO_3^- > Mn(IV) > Fe(III) > SO_4^{2-} > CO_2$ (Table 1) (10,56). Note that the redox potential of natural ferric iron minerals is substantially lower (around 0 mV) than that of aqueous Fe^{3+} (770 mV) (57). After flooding of soil, O_2 generally is depleted within one day; nitrate usually is depleted within a few days; on the other hand, reduction of Fe(III) can require weeks to complete (10,56). The time required for reduction depends on the concentration of each oxidant in the soil and thus is a factor of soil type and soil management. In general, Fe(III) is by far the most quantitatively important oxidant in rice field soils and thus determines the phase during which the organic matter is oxidized to carbon dioxide (58). In iron-rich acidic sulfur soils, for example, this phase extends over practically the entire season and CH_4 production never starts. In other soils, vigorous CH_4 production generally starts when reduction of Fe(III) and sulfate is largely completed (59). This event usually (details follow) coincides with a decrease of the redox potential (measured with a platinum electrode) to <−150 mV (60). At this time, the soil redox conditions are dominated by the reduction of carbon dioxide to either CH_4 or acetate (61). The total amount of CH_4 produced during the season and the maximum rate of CH_4 production are constrained by the availability of degradable organic matter and oxidants, usually Fe(III) (58,62,63). It also depends on temperature as the time required for sequential reduction increases with decreasing temperature (64–66).

The sequential reduction of soil oxidants is also seen in the form of vertical spatial gradients in the flooded soil under quasi-steady state conditions. Nitrate is usually depleted below a few millimeter depth (51), Fe(III) below about 0.8-cm depth (67), and sulfate below about 1- to 5-cm depth (68,69). The presence of plants, however, that introduce O_2 into deeper soil layers can complicate the vertical profiles, because nitrate, Fe(III), and sulfate are regenerated by oxidation with O_2 from their reduced forms (see following).

ANAEROBIC DEGRADATION OF ORGANIC MATTER TO METHANE

The methanogenic degradation of organic matter is accomplished by a complex microbial community, the functions of which are similar in all kinds of anaerobic environments such as sediments, anaerobic digesters, rumen, or flooded soils. The functions of such a microbial community consist of (*1*) hydrolysis, (*2*) fermentation, (*3*) syntrophic degradation, (*4*) homoacetogenesis, and (*5*) methanogenesis and are illustrated in Figure 2 (70–74). Hydrolysis is the largely extracellular enzymatic cleavage of polymers to monomers, for example, polysaccharides to sugars, proteins to amino acids, and so on. Fermentation is the microbial degradation of the monomers to simpler compounds. This is basically a disproportionation of the substrate to reduced plus oxidized compounds connected to the conservation of energy. Syntrophic degradation is the oxidation of primary fermentation products, such as fatty acids, alcohols, or aromatic compounds, to acetate plus carbon dioxide with protons as oxidant, thus forming H_2 as reduced product. Syntrophic reactions are typically endergonic under thermodynamic standard conditions and thus only proceed if the concentrations of the products, especially of H_2, are kept at a very low level. This is accomplished in syntrophy with the methanogenic microorganisms. Methanogenesis also can convert H_2 plus CO_2 to CH_4 and cleaves acetate to CH_4 plus CO_2. Methanogenesis can also convert formate, methanol, trimethylamine (TMA), or dimethylsulfide (DMS) to CH_4. Syntrophic microorganisms also produce formate instead of H_2 as reduced product. Methanol, TMA, and DMS are negligible degradation products in flooded soils. Homoacetogenesis is a special type of fermentation that produces acetate as sole product (75). Sugars, for example, can be directly converted to acetate, thus bypassing the syntrophic function. Many primary fermentation products as well as H_2 plus CO_2 also can be converted by homoacetogens to acetate as sole product.

In flooded soils, anaerobic methanogenic microbial communities degrade the organic matter that is available, that is, straw and other organic amendments, root exudates and root material, and the indigenous soil organic matter. The methanogenic degradation operates preferentially when oxidants other than CO_2 and H^+ are not available (see below). The degradation process has been studied in anoxic bulk soil, on straw particles, and on the surface of rice roots.

Hydrolysis and Fermentation

Rice straw consists of cellulose (32–37%), hemicellulose (29–37%), and lignin (5–15%) and, in addition, contains silica (76,77). Soil organic matter, on the other hand, is chemically not well defined. In soils into which straw is regularly incorporated, part of the soil organic matter likely consists of partially degraded straw particles, which apparently are hydrolyzed and fermented in a similar way as fresh straw (78). Changes in the composition and quality of soil organic matter, the phenolic content in particular, were observed in intensified rice cultivation where fields are kept flooded for prolonged periods (79,80). Only

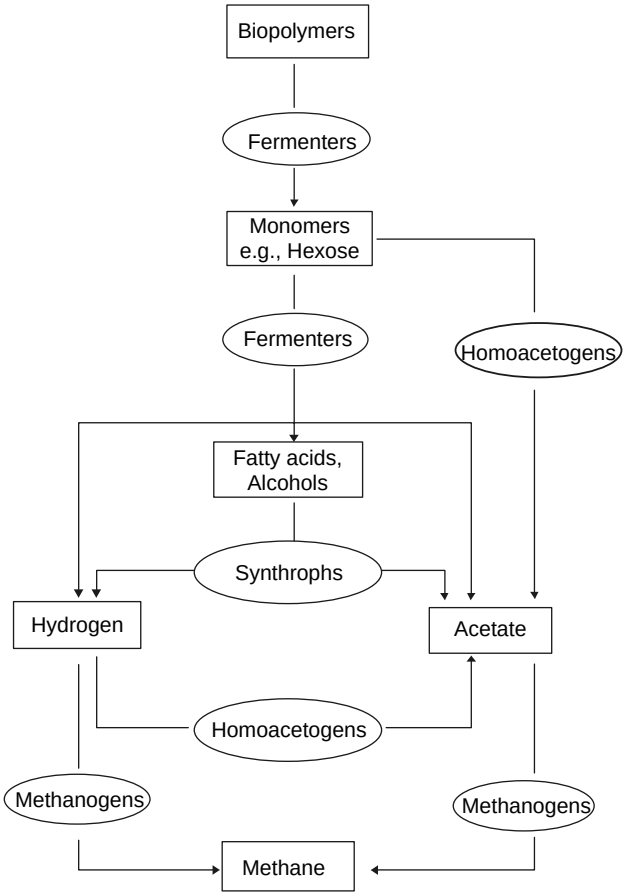

Figure 2. Pathway of anaerobic degradation of organic matter to methane [taken from Conrad 1999 (109)].

3 to 17% of the soil organic carbon is degraded to gaseous products during one season (58,76). The degradation process seems to result in changes of the oxidation state of the organic matter, which, however, are so slight that they are detected only by mass balance of oxidized and reduced compounds (81,82).

Rice straw is colonized by microorganisms, and the structure of the leaf blades and sheaths gradually disintegrates. The visible disintegration process becomes most apparent after three weeks of exposure in flooded soils (83,84). Molecular characterization of the straw-colonizing bacteria in an Italian rice field soil by targeting their ribosomal rRNA genes showed that most of the active bacteria were clostridia (55%), mainly belonging to the clostridial clusters I, III, and XIVa; others belonged to the genus *Bacillus* (1%), the *Cytophaga/Flavobacterium* cluster (5%), and the *Proteobacteria* (4%) (85). These bacterial groups also were found in flooded bulk soil, both by sequence information from clone libraries and by isolation of the dominant anaerobes (using the highest dilution steps of soil) (86–88). Saccharolytic bacteria were found to represent 50% of the total microorganisms (determined microscopically), representing about 10^8 bacteria per gram dry soil. These saccharolytic bacteria were able to grow on xylan, pectin, and various sugars, whereas bacteria able to grow on cellulose were about two orders

of magnitude less numerous. Isolates of the saccharolytic bacteria fermented sugars preferentially to acetate and propionate (87). Bulk Italian rice soil also contained bacteria belonging to the following phylogenetic groups: *Verrrucomicrobia*, *Actinobacteria*, clostridial cluster IX, and *Chlorobiaceae* (86,87). Hence, the straw-colonizing bacteria are possibly a subset of the bacterial community found in the bulk soil. The diversity of the straw-colonizing bacterial community increased immediately after exposure of the rice straw to anoxic soil but became constant after about 15 days (85). A difference in the bacterial community was also observed between dry rice soil and flooded rice soil, indicating community changes after flooding (88,89).

The fermentation pathway of straw components was found to be similar to that of the organic matter in unamended soil with acetate and carbon dioxide as major and propionate as minor fermentation products (78,90–92), that is, the same fermentation products that are observed in the dominant bacterial isolates (87). Only shortly after addition of rice straw to the flooded soil, additional fatty acids transiently accumulated to a significant extent, that is, butyrate, isobutyrate, valerate, isovalerate, caproate (78), and unidentified compounds (probably phenolic compounds) (76). Acetate and carbon dioxide were the major fermentation products of cellulose degradation (93), although small amounts of propionate were produced during fermentation of glucose, the monomer of cellulose (94). However, most of the propionate must be produced from other soil organic compounds or plant residues, possibly from xylan and pectin (95,96) or proteins (97).

Glucose is the most abundant sugar in hydrolysates of rice straw (98) and in the flooded rice soil (99). It is fermented via the Embden-Meyerhoff pathway with turnover times of more than 1 hour (94,99). Acetate is the major fermentation product, the concentration of which is effectively controlled by the equilibrium between glucose fermentation and methanogenic acetate consumption.

Rice roots are also colonized by microorganisms (100), including fermenting bacteria, but the composition of the microbial community seems to be completely different from that in the bulk soil or decomposing rice straw (101). Sequences of the 16S rRNA gene cloned from DNA extracts of rice roots showed a dominance of members from the following phyla: *Cytophaga/Flavobacterium/Bacteroides* (CFB)-phylum, the α-subgroup, β-subgroup, and δ-subgroups of the *Proteobacteria*, the *Holophaga/Acidobacterium* Phylum, and the gram-positive bacteria with low G + C content. Interesting was the detection of *Myxococcaceae* (*δ-Proteobacteria*), a bacterial group that usually lives on the lysis products of microbial and plant cells.

Little is known about the fermentation processes operating on the root surface. Pulse-labeling experiments with $^{14}CO_2$ showed that plant roots release organic material, which is converted to lactate, propionate, and acetate, probably in a sequential fermentation (25). Excised plant roots rapidly produce H_2, which subsequently is consumed by carbon dioxide reduction (102,103). Production of H_2 probably is due to fermentation processes being initiated by the exudation of soluble compounds from the roots or by the decay of the roots. Cellulose, glucose, and xylose are all rapidly fermented on excised rice roots, with acetate as the major fermentation product and propionate as the minor fermentation product (104). Hence, the fermentation pattern of excised rice roots apparently is not much different from that of rice straw and soil organic material, although the bacterial community seems to be completely different.

Syntrophy

In irrigated rice soil, propionate is the most important compound that is syntrophically degraded in the absence of oxidants other than CO_2 and H^+. Propionate accumulates in flooded soil when methanogenesis is inhibited or when high H_2 concentrations are applied, demonstrating that its turnover is controlled by thermodynamic conditions (92). However, propionate degradation proceeds at rather high Gibbs free energies (-7 to -15 kJ mol^{-1} of propionate) (105) that generally are considered as insufficient for microbial energy conservation (74). Low rates of propionate degradation were also observed under formally endergonic conditions, that led to the conclusion that propionate is degraded in soil microniches (e.g., in microbial aggregates) that are shielded from the thermodynamic conditions in the bulk soil (106). Propionate is degraded by a randomizing pathway, the succinate pathway, to one CO_2 product, one acetate product, and three H_2 as stoichiometric products (107). By producing H_2, propionate degradation can account for most of the CH_4 production from H_2 plus CO_2 (107). Propionate degradation on rice roots has not yet been studied, but the reverse process (i.e., the synthesis of propionate from H_2, CO_2, and acetate) seems to operate (103,104). The microorganisms responsible for propionate degradation in flooded soils are not known. Inhibition studies indicate the involvement of sulfate-reducers (107,108).

Homoacetogenesis

Methanogenic archaea convert the final products of fermentation and syntrophic degradation to CH_4. In the absence of homoacetogenesis, degradation of polysaccharides finally results in the production of 2 acetate, $4H_2$ plus $2CO_2$ per hexose monomer (109). Acetate is cleaved by acetotrophic methanogens to CH_4 plus CO_2. Hydrogenotrophic methanogens convert $4H_2$ plus CO_2 to CH_4. Hence, $3CH_4$ are produced from 1 hexose, two of which are produced by acetotrophic (67%) and one is produced by hydrogenotrophic methanogens (33%). In the presence of homoacetogenesis, on the other hand, more acetate instead of H_2 plus CO_2 is produced from hexose fermentation, or some of the H_2 is used to reduce carbon dioxide to acetate. In this case, more than two of the three CH_4 are produced from acetate.

Indeed, hydrogenotrophic methanogenesis frequently contributes less than 33% to total CH_4 production in anoxic rice soil, suggesting that some of the polysaccharides are fermented by homoacetogenesis (61,110–112). Homoacetogenic bacteria of the genus *Sporomusa* do occur in Italian rice field soil in numbers of up to 10^8 per gram dry soil (113). *Sporomusa* species usually are

chemolithoautotrophic, which may explain why some of the acetate (<10%) in rice soil seems to be produced by reduction of carbon dioxide (61,111). The thermodynamic conditions for the reduction of carbon dioxide to acetate usually are not favorable unless H_2 concentrations are elevated (92). Elevated H_2 concentrations and vigorous carbon dioxide reduction to acetate have, for example, been observed on excised rice roots (103,104). Also, 16S rRNA gene sequences characteristic of the homoacetogenic genus *Sporomusa* have been detected in DNA extracts from rice roots (101). In flooded soil, on the other hand, elevated H_2 concentrations and carbon dioxide reduction to acetate usually occur only if methanogenesis is inhibited (112,114). In addition, production of acetate from H_2 plus CO_2 seems to be favored over hydrogenotrophic methanogenesis when temperatures are low (e.g., 15 °C), probably because the resident homoacetogens are more psychrotolerant than the methanogens (92,115,116).

Methanogenesis

Methanogenic archaea occur in rice soils in numbers of up to 10^7 cells per gram dry soil and do not decrease significantly when the flooded soil is drained until the next season (117–119). Numbers of methanogens in rooted soil (that is assumed to receive some O_2, see earlier section) and in anoxic bulk soil are nearly the same (120). Most of the methanogenic archaea in rice soil even survive desiccation and exposure to oxygen (121). The methanogens are even able to decrease the redox potential of their medium to a compatible level (about +50 mV) if substrate is available to initiate CH_4 production (122). Obviously, the methanogens in flooded soils are not easily killed by exposure to O_2, contrary to what most textbook state, and thus are perfectly adapted to their changing environment.

So far, methanogens were isolated or enriched from rice soils belonging to the genera *Methanobacterium* (115,123–125), *Methanobrevibacter* (123,126), *Methanoculleus* (124), *Methanosarcina* (124,127,128), and *Methanosaeta* (129). Molecular techniques also demonstrated the presence of most of these genera in Italian rice field soils (129). Short fragments of the 16S rRNA genes of *Methanogenium* and *Methanoculleus* species were detected in Japanese rice soils (130). Furthermore, 16S rRNA gene sequences of novel archaea (named *Rice cluster I* and *Rice cluster II*) were detected in Italian rice soils (131,132) that fall in between the phylogenetic radiations of the *Methanosarcinales* and *Methanomicrobiales* and have a methanogenic phenotype (133,134).

Although numbers of total culturable methanogens stay fairly constant in rice soil, the individual populations change dynamically with time of anoxia and temperature. Thus, the percentage contribution of *Methanosarcinaceae* within the total methanogenic population increases during 17 to 25 days after onset of anoxic conditions in soil slurries (135,136). This relative increase may be caused by the usual increase of acetate concentrations during this period, given the fact that *Methanosarcina* species can only use acetate concentrations greater than 200 μM (137). After longer periods (90–120 days) of anoxia, acetate concentrations usually decrease, as does the percentage of *Methanosarcina* in the methanogenic population that is then dominated by *Methanosaeta*, which has a much lower threshold (about 10 μM) for utilization of acetate (116). Interestingly, *Methanosaeta* dominates in anaerobic enrichment cultures on cellulose inoculated with Italian rice soil at 15 °C, whereas *Methanosarcina* dominates at 30 °C, at conditions where acetate is nonlimiting (138). The *Methanosaeta* populations in Italian rice soil seem to be more psychrotolerant than the *Methanosarcina* populations. On the other hand, if rice soil slurries are incubated at more than 40 °C, rice cluster I is becoming the dominating methanogenic population (139).

The 16S rRNA gene sequences characteristic for Rice cluster I and Rice cluster II have been discovered on rice roots (131,133). This was surprising because rice roots had originally been considered to be largely oxic habitats (see preceding). Nevertheless, roots produce CH_4 when incubated under anoxic conditions (140) and methanogens inhabit the root surface in numbers of 10^6 cells per gram dry root and produce CH_4 predominantly from H_2 plus CO_2 (133). However, acetotrophic methanogenesis is also possible on rice roots if phosphate concentrations are lower than 20 mM (141). Interestingly, *Methanosaeta* species have so far not been detected on rice roots, although they are abundant in the soil, but acetotrophic *Methanosarcina* species are present (129,131,133). Acetate concentrations probably are much higher on roots than in the bulk soil, thus allowing *Methanosarcina* species to be active.

Clone libraries of archaeal 16S rRNA genes demonstrated the presence in rice soil and on rice roots of novel deeply branching phylogenetic lineages. Novel phylotypes were found among the *Euryarchaeota* and were named *Rice cluster III* and *Rice cluster V*. Even more novel phylotypes were found among the *Crenarchaeota* and were named *Rice cluster IV* and *Rice cluster VI* (131,132,135,136). The sequences of rice clusters III to VI are unrelated to any known bacterial isolate. Hence, it is not known which phenotypes these novel phylotypes have and which functional role they play in the flooded soils. There are also only few related sequences from clones that were obtained from other environments, for example, from lake sediments and anaerobic digestors (related to Rice cluster IV) and from other soils and lake sediment (related to Rice cluster IV) (summarized in 135,142).

MICROBIAL OXIDATION OF METHANE

In flooded soils, CH_4 and CO_2 are the end products of organic matter degradation. However, not all of the produced CH_4 is released into the atmosphere, as it is oxidized by methanotrophic bacteria to carbon dioxide. This oxidation process is possible when O_2 becomes available, that is, at the surface of soil and plant roots.

Scavenging of Methane at the Soil Surface

Vertical concentration profiles in flooded rice soil show that the CH_4 and O_2 gradients overlap at about 2- to 3-mm depth, that is, the lower end to which O_2 diffuses from the surface (31,40). At this depth, CH_4 is

oxidized, thus reducing the diffusive CH_4 flux by about $81 \pm 15\%$ (143). However, if CH_4 production rates are high and if the produced CH_4 cannot evade via the plant gas vascular system (see preceding), gas bubbles form and result in ebullition of CH_4 that is not scavenged by CH_4 oxidation (144). In planted soils, however, gas bubbles may be trapped in the rooted zone where part of the CH_4 can be oxidized. The vertical CH_4 concentration profile seems to be slightly affected by bioturbation of small animals living in the soil (144). However, the net effect of bioturbation is very small; even addition of chironomid larvae to flooded rice soil did not significantly change the flux of CH_4 across the sediment–water interface, although, methanotrophic activity in the walls of the chironomid burrows was significantly higher than in the soil (145).

The methanotrophic bacteria occur in numbers of 10^4 to 10^7 bacteria per gram dry soil (40,50,146–150). The methanotrophs possessing a soluble methane monooxygenase (*mmoX* gene) in addition to the particulate methane monooxygenase (*pmoA* gene) can be detected by naphthol utilization (147). Type I (*γ-Proteobacteria*; family *Methylococcacea*; genera *Methylobacter, Methylocaldum, Methylococcus, Methylomicrobium, Methylomonas, Methylosphaera*) and type-II (*α-Proteobacteria*; family *Methylocystaceae*; genera *Methylocystis, Methylosinus*) methanotrophs can be distinguished, among others, by the sequence of their 16S rRNA and *pmoA* genes and by phospholipid profiles (151). Both groups were detected in flooded rice field soil (152–154).

Methane Oxidation in the Rhizosphere

Methane concentrations in the rooted soil are lower than those in the bulk soil. However, the concentrations at the microsites where O_2 and CH_4 overlap are not known. From studies of CH_4-oxidizing bacteria growing in agar columns in countergradients of O_2 and CH_4, it is known that they consume their substrates to extremely low levels (155). This seems to be less plausible in the rice rhizosphere, because the equivalent CH_4 mixing ratio should not be less than that measured in the aerenchyma. At mixing ratios of 5,000 to 10,000 ppm_v in the aerenchyma, the rhizospheric CH_4 concentration corresponds to 6 to 12 μM CH_4, a concentration quite higher than the apparent K_m known from pure cultures and rice soil (156). Hence, O_2 seems to limit the activity of CH_4 oxidizers more than CH_4 does.

Methane oxidation in flooded soils usually is expressed in percentage of CH_4 production. The first reports of CH_4 oxidation in rice fields were published in 1985 (34), but the quantification later proved to be rather difficult. One method is to measure CH_4 production rates in soil samples and to compare these rates with the CH_4 emission rates in the field (34,157). The difference is taken as the oxidation rate. However, the production rate in a soil sample may be biased by the handling (e.g., cutting of roots), rendering the derived oxidation rate unreliable. For example, rice roots may be cut during the sampling and result in stimulation of CH_4 production by decaying root pieces. As an alternative approach, various inhibitors have been used to block CH_4 oxidation. With these techniques, the difference between the increased emission after application of an inhibitor compared with that before the application of an inhibitor is taken as the oxidation rate. Common methods include the elimination of O_2 (30,34,50,158) or the application of acetylene (C_2H_2) (34,159,160), a suicide inhibitor of the CH_4 monooxygenase (161). However, some authors found that CH_4 emission was reduced (162,163), possibly because C_2H_2 inhibited methanogenesis (164,165). Some authors have favored methyl fluoride (CH_3F) (166–170). However, since CH_3F also inhibits acetoclastic methanogenesis, it also may bias the results (112,140,171). Instead, difluoromethane (CH_2F_2) has recently been used as an inhibitor of CH_4 oxidation (172,173). This inhibitor did not affect methane production in vitro and was applied successfully to laboratory microcosms and flux chambers installed in the rice field (42,154). Regardless of the inherent methodological problems, most numbers published during the last years on CH_4 oxidation rates in vegetated rice soil are in the range of 10 to 50% (42,170,174,175). Compared with the oxic surface of a lake sediment with about 90% CH_4 oxidation (176) or to that of rice fields with about 80% (143), the CH_4 oxidation in the rhizosphere, therefore, seems to be quite ineffective.

The reason for this may be the competition between rice plants and rhizospheric bacteria for nutrients. In greenhouse experiments it could be shown that nitrogen deprivation limits the rhizospheric CH_4 oxidation. A treatment with ammonium-based fertilizers stimulated CH_4 oxidation (153,177), and in a field study in Italian rice field a positive correlation between potential CH_4 oxidation and the availability of ammonium was found (42). Hence, nitrogen fertilization may be an option to reduce CH_4 emission from rice fields. In a first field experiment, however, the stimulation of CH_4 oxidation after urea fertilization was counterbalanced by simultaneous stimulation of CH_4 production, resulting in zero net effect (178).

In contrast, the situation in nonflooded upland fields is quite different. Upland soils are exposed only to atmospheric CH_4 concentrations and show mostly an inhibition of CH_4 oxidation following nitrogen fertilization (179–182). However, even the reaction of upland soils is not consistent, with some published evidence for stimulating or inducing effects of ammonium fertilization on CH_4 oxidation in low CH_4 (146,183–185) and in high CH_4 environments (186,187). Interestingly, this stimulation seems to depend on the natural ammonium level because it is observed in low-ammonium but not in a high-ammonium soil (146).

Methane-oxidizing bacteria have been detected in putatively oxic rhizospheric soil, in the rhizoplane, and even in surface-sterilized rice roots and shoots (40,50,188). They were counted in relatively high numbers of 10^6 to 10^8 per gram dry soil or root biomass. The detection inside rice roots or shoots points to the ability of these bacteria to invade the rice plants, that was confirmed by study of gnotobiotic roots using confocal laser scanning microscopy (189). The number of CH_4-oxidizing bacteria in the rooted soil may or may not increase with time, but the total root-associated population increases largely in parallel to the development of the root system with ongoing season (40,50,154,188). Type II methanotrophs

seems to have a quite stable population size, but type I methanotrophs react fast upon favorable conditions, as shown by analysis of 16S rRNA genes and phospholipid fatty acids (153).

Methane oxidation was repeatedly found to occur also under anoxic conditions in marine sediments (190–194). Although CH_4 turnover in flooded rice fields can be interpreted without assuming an anaerobic CH_4 oxidation process, there are hints that it nevertheless may exist, possibly being correlated with the reduction of sulfate and/or iron (195–197).

Oxidation of Atmospheric Methane

In flooded soils, the methanotrophs act as a filter for the evading CH_4, which is supplied to the upper soil surface layer in micromolar concentrations. Nevertheless, flooded soils still act as a net source for atmospheric CH_4 (Fig. 3). In upland soils, on the other hand, the methanotrophs consume atmospheric CH_4 that is available at nanomolar concentrations. These soils act as a sink for atmospheric CH_4 (36,198,199). The diffusion of CH_4 from the atmosphere to the soil is mainly controlled by texture and moisture (200,201). The methanotrophic populations acting as CH_4 filter versus those consuming atmospheric CH_4 can be distinguished by the CH_4 oxidation kinetics, that is, their apparent K_m being in the micromolar and nanomolar ranges, respectively (36,202).

Fields cropped with upland rice and rain-fed rice (for definition, see 2) can act as sinks for atmospheric CH_4, when the soil moisture decreases to sufficiently low levels (149,203,204). Fields cropped with irrigated rice are also turned into actual upland soils when the flooded fields are drained, and they remain in this state during the fallow period until they are flooded again. Nevertheless, this type of field has so far not been observed to become a net sink for atmospheric CH_4. The residual CH_4 production activity in drained soil, although low, was always found to be higher than the CH_4 oxidation activity, thus resulting in a small net release of CH_4 (205). Hence, it is the balance between production and oxidation of CH_4 that determines the direction of CH_4 flux, which is emission in drained flooded soils but uptake in dry upland soils. The methanotrophic community resident in flooded soils was found to be more sensitive to drying than the methanogenic community was. Nevertheless, the methanotrophs were able to utilize atmospheric CH_4 with a nanomolar apparent K_m (205) and display a dramatic population dynamic in the upper soil layers upon drainage. Type-I methanotrophs (*Methylobacter*, *Methylomonas*, and *Methylococcus*), in particular, reacted with population growth, as identified by the increase of the 16S rRNA and *pmoA* gene content (206).

MICROBIAL CYCLING OF OXIDANTS

In addition to CH_4, other reduced compounds are formed in flooded soil, in particular, Fe(II) and reduced nitrogen and sulfur compounds. Analogously to CH_4, these compounds can be oxidized if O_2 is available and thus effectively regenerate nitrate, ferric iron, and sulfate in the soil (Fig. 3). These regenerated oxidants can subsequently serve as electron acceptors for reduction reactions. Hence, redox cycling of N, Fe, and S is taking place. During this process, N-containing and S-containing trace gases are produced and released into the atmosphere.

Cycling After Intermittent Drainage

Intermittent drainage is a common practice in floodwater management of irrigated rice or incidentally happens after water shortage. Drainage allows O_2 to penetrate deeper into soil, resulting in an increase of the redox potential (207). This increase in redox is brought about by oxidation of Fe(II) to Fe(III) and of sulfide to sulfate, which in turn suppress CH_4 production. Because it takes quite some time after the short-term drainage to reduce the produced Fe(III) and sulfate again, CH_4 production stays suppressed for a while (208,209). Thus, intermittent drainage is an effective option to mitigate CH_4 emission from flooded soils (207,210,211).

Availability of O_2 during intermittent drainage also allows the oxidation of ammonium and consequently the

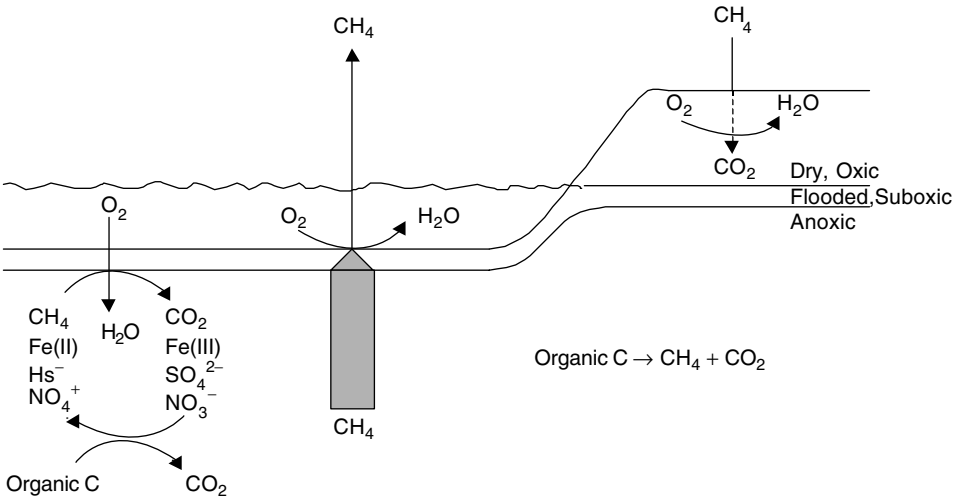

Figure 3. Scheme of redox cycling at the oxic–anoxic interface of flooded soils, of CH_4 oxidation attenuating the emission of CH_4 into the atmosphere, and of CH_4 oxidation acting as sink for atmospheric CH_4.

production of NO and N_2O during nitrification itself and during the subsequent denitrification of the produced nitrate (212). Nitrous oxide (N_2O) is an even more effective greenhouse gas than CH_4 and, in addition, results in the destruction of stratospheric ozone (3,36,213). However, nitrification and production of N_2O only operates if ammonium is available. In vegetated rice fields, the plants rapidly take up ammonium, thereby limiting soil nitrogen (51). Hence, it is not surprising that intermittent drainage results in increased N_2O emission only if the soils are well fertilized (209,214–216).

Cycling in the Rhizosphere

The cycling of matter in the rhizosphere is characterized by the relative abundance of organic matter provided by root exudation and root decay and by the availability of O_2 leaking from the plant aerenchyma system. The O_2 allows oxidation of reduced compounds such as organic substrates, ammonium, ferrous iron, and reduced sulfur, whereas the organic substrates supply electron donors for reduction processes such as the reduction of O_2, nitrate, Fe(III), and sulfate. The stimulation of both oxidation and reduction processes potentially allows for an intensive cycling of C, N, Fe, and S in the rhizosphere.

The cycling of electron donors and acceptors in the rhizosphere has a spatial and temporal component; radial gradients of the different compounds are formed around an individual root, but these gradients change with time and may even collapse at the root surface in a senescing plant. Aerobic and anaerobic processes may be mutually exclusive or may overlap to some extent, but no high-resolution studies have been undertaken as yet. Nevertheless, experimental evidence suggests that rice roots are inhabited by microbial communities that allow the conversion of nitrogen by coupled nitrification-denitrification (51,217,218), the cycling of Fe(II) and Fe(III) (67,219), and the cycling of sulfur by sulfur oxidation and sulfate reduction (69).

Nitrogen Cycling

In wetland rice agriculture, nitrogen fertilizer efficiency is rather poor, with an apparent nitrogen recovery of less than 40% (79,220). The main reasons for fertilizer inefficiency are nitrogen losses by both volatilization of NH_3 and coupled nitrification-denitrification. Nitrogen loss by volatilization of NH_3 is a physical process, caused by algal photosynthesis that increases pH in the flood water (221). Nitrogen loss by denitrification to N_2 is a microbial process that depends in a wetland soil on nitrification, the microbial formation of oxidized nitrogen species from ammonium. Once oxidized, nitrogen (mainly as nitrite and nitrate) is reduced by denitrifying bacteria. Among the processes described earlier, nitrification may compete with rice plants for N sources, but evidence is growing that nitrification and subsequent denitrification play a major role for only a limited time after fertilization. The problem is recognized in agronomic practice, and split-fertilization or deep placement of fertilizers is used to reduce the imbalance between ammonium supply and plant uptake (222). The application of wax-coated calcium carbide (a slow-release source of the nitrification inhibitor C_2H_2) has also been tested (162,163,223).

Nitrogen cycling in rice fields is characterized by spatially heterogeneous substrate concentrations that change unpredictably with time. The activities of nitrifiers and — because of the tight coupling — of denitrifiers are dependent on the presence of O_2 and ammonium. Both substrates may be limiting for nitrification. With the ongoing season, nitrogen is rapidly depleted in the rooted soil layer where the rhizosphere of different roots overlaps, ending up at nearly undetectable concentration of ammonium, nitrite, and nitrate. Nitrification and subsequent denitrification become active only if an imbalance between nitrogen supply to and nitrogen demand of the rice plant allows for increased ammonium concentrations in the rhizosphere (Fig. 4).

Nitrifying bacteria are aerobic bacteria and are assumed to be active in the same microhabitats

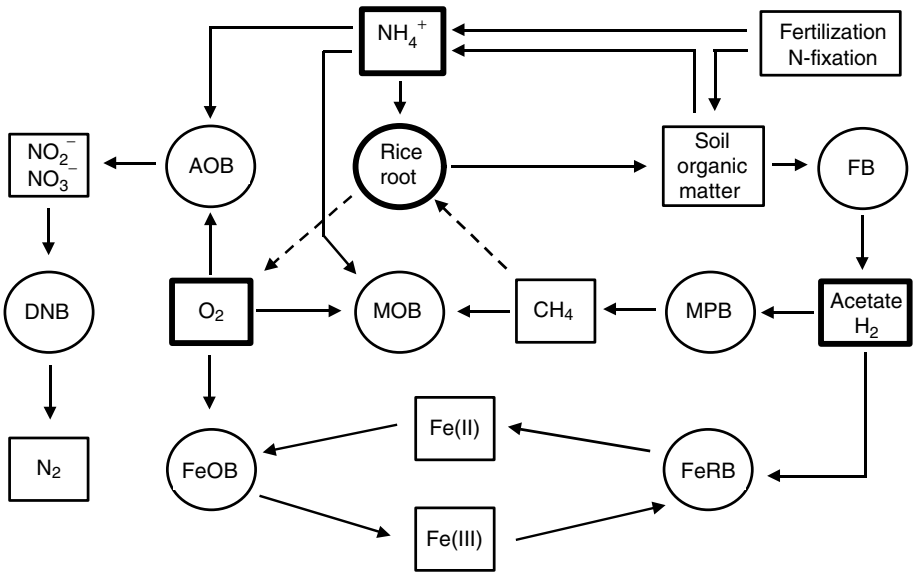

Figure 4. A simplified scheme of the interactions between microbial carbon and nitrogen cycling. Processes and/or organisms are symbolized by circles and substrate pools by boxes. Fluxes of substrates are symbolized by solid arrows with the exception of the diffusion of the gases O_2 and CH_4 into and from the aerenchyma of the root, which are marked by dashed arrows. For sake of clarity, abiotic redox processes, aerobic mineralization of soil organic matter to carbon dioxide, and dissimilatory nitrate reduction to ammonium are omitted. FB = fermenting bacteria; MPB = methane producing bacteria; MOB = methane oxidizing bacteria; AOB = ammonium oxidizing bacteria; DNB = denitrifying bacteria; FeOB = iron oxidizing bacteria; FeRB = iron reducing bacteria.

as the CH_4-oxidizing bacteria. The ammonium-oxidizing genera belong to the *β-Proteobacteria* and *γ-Proteobacteria* (224,225). Ammonium is activated by the ammonium monooxygenase, which is phylogenetically related to the particulate methane monooxygenase of methane-oxidizing bacteria (226,227), and both enzymes are inhibited by the same compounds, (e.g., C_2H_2) (226). Aerobic ammonium oxidizers can be characterized by the gene encoding for the ammonium monooxygenase, the *amoA* (228). DNA extracts from rice roots showed the presence of *amoA* sequences characteristic of the genus *Nitrosospira* (228,229). *Nitrosospira* species are also the most dominant ammonium oxidizers in the rhizosphere of the aquatic grass *Glyceria maxima* (230) and in nonflooded soils (231,232). The numbers of culturable ammonium-oxidizing bacteria in bulk soil and rhizospheric rice soil are approximately 10^4 to 10^6 per gram dry soil (177).

Methanotrophic bacteria are able to activate ammonia with the methane monooxygenase and exhibit nitrification activity (227). Indeed, it was found that CH_4-oxidizing bacteria could contribute substantially (>50%) to nitrification in ammonium-limited rice field soil (177,233). It is not known whether the recently discovered phenotype of anaerobic ammonium oxidation, represented by species of the order *Planctomycetales*, play any role in flooded soils (234–236).

Nitrification activity starts shortly after fertilization with $(NH_4)_2HPO_4$ or urea, resulting in millimolar pore water concentrations of nitrite and nitrate in the rooted soil layer (51). However, such high concentrations are locally restricted and disappear after a few days, when ammonium is again used up by the rice plant and the soil microorganisms. It is not certain whether ammonium oxidizers or methanotrophs are responsible for the nitrification activity following fertilization. However, methanotrophic bacteria are quite sensitive to nitrite (237). Ammonium oxidizers are known to preserve a high-energy charge even during ammonium deprivation (238) and to become reactivated immediately after fertilization (239). Hence, ammonium-oxidizing bacteria probably cause the rapid onset of nitrification after fertilization.

The aerobic nitrite-oxidizing genera belong to the *α-Proteobacteria* (*Nitrobacter*), *γ-Proteobacteria* (*Nitrococcus*), *δ-Proteobacteria* (*Nitrospina*) (225,240), and a deeply branching class (*Nitrospira*) within the domain *Bacteria* (241). The numbers of culturable nitrite-oxidizing bacteria in bulk soil and rhizospheric rice soil are on the order of 10^5 to 10^7 per gram dry soil (177). Phylogenetic analyses of the nitrite-oxidizing community in rice field are so far lacking.

Once nitrite or nitrates have been formed, they may diffuse from the rhizosphere into the anoxic bulk soil, where they will be reduced. Rice field soils contain populations of nitrate-reducing bacteria, denitrifying bacteria, and bacteria capable of dissimilatory nitrate reduction to ammonium (DNRA) (reviewed, see 9). The nitrate reducers (producing nitrite) and the denitrifiers (producing N_2) are mostly facultative anaerobes. The DNRA bacteria are mostly anaerobes. The phenotypes are all polyphyletic, so that reliable molecular tools based on the 16S rRNA gene are not available for environmental studies. Molecular techniques based on functional genes that are unique for the specific phenotypes are also not available yet, so that community analyses of the populations active in nitrate reduction, denitrification, and DNRA are not possible at the moment. The current picture, recently reviewed by Kimura (9), indicates that potential nitrate reducers and denitrifiers contribute up to 16 and 0.9%, respectively, of the total viable counts in rice field soil and that the rhizosphere is more densely populated than the bulk soil. Denitrification activity is obvious in rice field soil, but most studies indicate that DNRA plays no significant role. Hence, the oxidized ammonium is lost mainly by denitrification (if not taken up by the plant) and is not recycled (Fig. 4).

The coupling of nitrification with denitrification in the rice rhizosphere has been shown by balancing nitrogen addition with nitrogen recovery from plants and soil (242), by application of ^{15}N-labeled urea (217,243), and by measurements of N_2, the end product of denitrification (218). Radial profiles measured in different positions along individual rice roots have shown that net nitrification occurs closely behind the root tip at some distance from the root surface and that it was confined to the very root surface in the basal zone (51).

A simplified scheme of how nitrogen and carbon cycling in a rice field are interconnected is shown in Figure 4. The root drives microbial activity by releasing labile organic carbon (exudates, decaying cells, and so on) into the pool of soil organic matter also containing refractive material such as straw, stubble, and humic substances. Organic matter is degraded in the anoxic soil by fermenting bacteria to acetate and H_2 (for details see Fig. 2). If oxidized iron is available, acetate and H_2 are used by iron-reducing bacteria or by CH_4-producing bacteria. Organic nitrogen from the soil organic matter will end up as NH_4^+. Methane-oxidizing bacteria, ammonium-oxidizing bacteria, and iron-oxidizing bacteria compete for O_2. Iron oxidation and reduction together form an internal redox cycle (see following section). Ammonium-oxidizing bacteria produce NO_2^- and NO_3^- that will be reduced in the surrounding anoxic soil by denitrifying bacteria to N_2, making the coupled oxidation and reduction to a nitrogen sink. This loss has to be compensated for by microbial N_2 fixation and by fertilization. All organisms compete for NH_4^+, with the rice root as the top competitor, followed by methane-oxidizing bacteria. The latter may outcompete the ammonium oxidizers for ammonium. For the sake of clarity, abiotic redox processes, aerobic mineralization of soil organic matter to carbon dioxide, and dissimilatory nitrate reduction to ammonium are omitted.

Iron Cycling

Iron is a major component in soils. In fact, iron reduction dominates the redox processes in rice field soils shortly after flooding and often accounts for more than half of the electron balance over the entire rice-growing season, thus being a process that is even more important than methanogenesis (58).

Profiles of Fe(III) in unplanted rice microcosms showed a maximum in a depth of 2 to 4 mm close to the zone of highest O_2 consumption in about 2-mm

depth (67). However, iron oxidation was not an exclusively aerobic process but instead was caused in part by the reduction of nitrate (244) that was available below 2-mm depth (51,244). In slurry experiments using the same rice field soil, substantial amounts of Fe(III) were produced after addition of NO_3^- or N_2O (245). Bacteria able to couple the reduction of NO_3^- to N_2 with the oxidation of Fe(II) to Fe(III) have been found in different sediments (246–248) and are also present in rice fields in most probable numbers of about 10^6 per gram dry soil (244).

Iron-reducing bacteria are abundant in rice field soils. Ferrihydrite-reducing bacteria occur in numbers that are about 10-fold higher than those of methanogens and use H_2, acetate, and lactate as electron donors. Isolates from rice field soil were phylogenetically diverse, belonging to the α-, γ-, and δ-*Proteobacteria* (Schnell, personal, communication).

Rice roots tend to form a dense root mat within the top 3 cm (120). These roots are often covered with reddish iron plaques, making the reoxidation of Fe(II) to FeOOH quite obvious (219,249). Oxidation of Fe(II) has positive effects for the rice plant (250) because high amounts of Fe(II) are detrimental, being a major constraint for rice agronomy in Fe-rich soils (251). Iron plaques are also present in older root segments, where the measured O_2 release is comparably low. Numbers of iron-reducing bacteria are slightly higher in the rhizosphere than in bulk soil (Schnell, personal, communication). Hence, it is likely that iron is cycled on the rice roots by coupled oxidation-reduction of iron, or at the least roots stimulate iron cycling.

Iron(III) is also common in the rooted soil layer between the surface and a depth of at least 3 cm in microcosms (120) and in the field (42). CH_4 production in this layer is low and acetate is mainly oxidized to carbon dioxide (42). In vitro, methanogenesis is not totally suppressed in this soil. However, it is only after reduction of Fe(III) after 25 to 40 days that acetate concentrations reach the threshold values for methanogens and vigorous CH_4 production resumes (120). It is also during the period of iron reduction that sulfate is available in concentrations that are sufficiently high to support sulfate reduction (120), possibly caused by sulfide oxidation coupled to Fe(III) reduction (252).

Sulfur Cycling

As found for reduced nitrogen and iron, reduced sulfur compounds also can be oxidized in the rhizosphere. Indeed, sulfate concentrations are found to be highest in the rhizosphere and in the surface soil layers, indicating that oxidation of reduced sulfur compounds takes place at these oxic sites (25,68,69). Sulfur-oxidizing bacteria are present in bulk and in rhizosphere soil at numbers of 10^5 to 10^6 per gram dry soil and actively oxidize reduced sulfur species, thus regenerating sulfate (253). Isolates of sulfur-oxidizing bacteria are phylogenetically related to *Thiobacillus thioparus* (β-*Proteobacteria*) and to various genera within the α-*Proteobacteria* (253).

Sulfate reduction was studied in rice microcosms with a planar root mat (69). Rates were highest in 0- to 1.5-mm distance to the root mat. Washed excised rice roots showed sulfate reduction potential when incubated in anaerobic medium, indicating a close association of sulfate-reducing bacteria with the roots. Hydrogen-using sulfate-reducing bacteria were present at 10^7 cells per gram fresh root biomass and were related to the genus *Desulfovibrio* (254). The genus *Desulfotomaculum* (potentially utilizing acetate) accounted for 1% of the total bacterial rRNA on rice roots (255). It was suggested that the sulfate-reducers were limited by the availability of sulfate rather than electron donors, the latter would be provided by root exudation.

Traditionally, sulfate-reducing bacteria are considered to be strict anaerobes. However, they may tolerate or even respire O_2. Many of them form aggregates, resulting in a higher tolerance to oxygen exposure (256,257); fast-growing species respire with O_2 that is preferentially used when present together with other possible electron acceptors (257,258). Aerobic respiration is coupled to proton translocation (259) and ATP conservation (260), but aerobic growth in pure culture is poor or absent (261–263). Hence, sulfate-reducing bacteria are well adapted to survive in an environment like the rhizosphere that may become oxic from time to time.

In rice soils, the number of sulfate-reducers range between 10^3 and 10^7 per gram dry soil, depending on soil conditions and substrate used for cultivation (68,264). Isolates were phylogenetically affiliated with the genera *Desulfovibrio*, *Desulfobulbus*, *Desulforhabdus*, *Desulfobotulus*, and *Desulfotomaculum* (254). Dot blot hybridization showed that *Desulfotomaculum* accounted for about 0.5% of the bacterial rRNA (255). Rates of sulfate reduction were highest at about 3- to 5-mm depth. In the top layers, where O_2 was present, sulfate reduction was low but detectable; in depth below 6 to 7 mm, it was limited by sulfate. The major product of sulfate reduction was acid-soluble sulfur (i.e., H_2S and FeS), whereas chromium-reducible sulfur (i.e., S^0 and FeS_2) was a minor product (69). Hence, sulfur was mainly in a labile form. The concentration of pyrite (FeS_2) was less than about 1% of the iron content, so that sulfate reduction in rice soil contributes little to the iron cycle.

Volatile reduced sulfur compounds such as dimethyl sulfide, carbonyl sulfide, and carbon disulfide also are produced in flooded rice fields and are emitted through the rice plant into the atmosphere (265). Production of these trace gases is stimulated by addition of organic sulfur compounds, but the exact mechanism of production is not clear. The production rates of these volatile sulfur compounds are less than 0.1% of sulfate reduction rates (69).

ISOTOPE EFFECTS

Both CH_4 and its precursors show distinct isotopic signatures. In the case of CH_4 formed by methanogens, the distribution of carbon (mainly ^{12}C and ^{13}C) and hydrogen isotopes (H and D = deuterium) depends on the isotopic signatures of the source materials and on the kinetic isotope effect. Isotope data are given in δ notation ($\delta^{13}C$, δD) and expressed in per mill:

$$\delta = [(R_{\text{sample}}/R_{\text{standard}}) - 1] \cdot 10^3$$

where R is the ratio of the isotopes (e.g., $^{13}C/^{12}C$) in the sample and the standard, respectively. In short,

the molecules with the lower isotopic mass diffuse and react more rapidly than the isotopically heavier species, resulting in a kinetic effect. In the case of the carbon atom, this discrimination against ^{13}C results in its depletion in microbially produced CH_4 relative to the precursor substrates. The isotope fractionation factor ε (δ^{13}C-substrate $\rightarrow \delta^{13}CH_4$) is specific for the different methanogenic pathways and takes values from $\varepsilon = 24–27‰$ for acetoclastic (δ^{13}C-acetate $\rightarrow \delta^{13}CH_4$) and $\varepsilon = 55–58‰$ for hydrogenotrophic ($\delta^{13}CO_2 \rightarrow \delta^{13}CH_4$) methanogenesis, respectively (266). Fractionation factors in rice field soils have been investigated by Sugimoto and Wada (267,268).

Isotopic fractionation also occurs during CH_4 oxidation because methanotrophs prefer the isotopically lighter CH_4, causing an enrichment of ^{13}C and deuterium in the residual CH_4 (269–271). The isotope fractionation factor for CH_4 oxidation ($\delta^{13}CH_4 \rightarrow \delta^{13}CO_2$) is $\varepsilon = 7–31‰$ (265). Because production and oxidation have different effects, analysis of the isotopes of CH_4 may be a useful tool to quantify methanogenic pathways and the importance of CH_4 oxidation in situ. In the past, simply the isotopic signature of pore water CH_4 has been used to draw such conclusions (for review see, 266,272). Using more detailed sampling, some authors found signals in natural wetlands that were consistent with plant-associated CH_4 oxidation (273–275), whereas in other situations no rhizospheric oxidation could be detected (275).

Knowing the isotopic signature of pore water and of emitted CH_4, it should be possible to draw conclusions about the underlying microbial pathways and processes. However, it must be taken into account that the isotopically lighter CH_4 diffuses faster than its heavier counterpart. Because in rice the plant-mediated transport of CH_4 is mainly diffusive (37–39), it has a major impact on the signature of emitted CH_4. Hence, the isotopic signature of CH_4 in the aerenchyma also must be taken into account. Only a few studies have used this approach to analyze the CH_4 turnover in rice fields (276–278). These field studies have shown the potential of stable isotopes to unravel the interaction of methanogenic pathways and processes. The magnitude of CH_4 oxidation calculated from isotopic signatures agreed surprisingly well with that derived from other methods (278), but data available as yet are quite scarce. Additionally, the isotopic signatures of CH_4 from the rooted soil, the aerenchyma, and the emission seem to be difficult to interpret with respect to root-associated CH_4 production and oxidation (276–279). The interpretation becomes even more complicated if one takes into account that different groups of CH_4-oxidizing bacteria may discriminate differently against $^{13}CH_4$ and that even within one strain of *Methylococcus capsulatus* the isotope effect largely depends on the currently active soluble or particulate methane monooxygenase (280). Similarly, strain-specific or enzyme-specific discrimination may occur in methanogens. To summarize, measuring naturally occurring isotopic signatures provides a useful tool for the analysis of CH_4 turnover, but it should be applied in combination with other biogeochemical and microbiological methods.

COMPETITION AMONG MICROORGANISMS

Microorganisms require for growth and maintenance a source for energy, carbon, and nutrients. Given the limited availability of these compounds in soil, competition for these resources arises and may significantly affect biogeochemical cycling (13,14). In particular, microorganisms compete for substrates that are generated by anaerobic degradation of organic matter and for electron acceptors and nutrients.

Anaerobes

The microorganisms involved in the anaerobic degradation of organic matter typically are limited by energy. Hydrogenotrophic methanogens, for example, consume their substrate H_2 to levels that are at the thermodynamic threshold for energy conservation. The H_2 partial pressure in anaerobic environments such as flooded soils typically is in a range of only 1 to 7 Pa (58), which allows for the methanogenic reaction ($4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$) a Gibbs free energy change of only 6–7 kJ mol^{-1} H_2 or about −25 kJ per reaction (59). This free energy change is just sufficient to generate one-third ATP per reaction (74). Because H_2 also can be used by bacteria that reduce nitrate, ferric iron, or sulfate, these bacteria compete with methanogens for the available H_2. Since the reduction of nitrate, ferric iron, or sulfate is more exergonic than the reduction of carbon dioxide by methanogens, these bacteria are able to effectively decrease the H_2 partial pressure below the threshold of methanogens (Table 2). This results in the suppression of CH_4 production from H_2 plus CO_2. This suppression often (but not always) coincides with an increase of the soil redox potential that can be analyzed with a platinum electrode. The suppression basically depends on (*1*) the availability of nitrate, ferric iron, or sulfate and (*2*) the presence of active microorganisms able to reduce these oxidants.

Immediately after flooding of soil, H_2 partial pressures increase as a result of the onset of hydrolysis and fermentation. The relatively high H_2 partial pressures allow exergonic CH_4 production from H_2 plus CO_2 until nitrate reducers, iron reducers, and/or sulfate-reducers become active. These bacteria then decrease the H_2 partial pressure until methanogens become suppressed. Suppression of CH_4 production holds on until nitrate, ferric

Table 2. Standard Gibbs Free Energy Changes and Thresholds of H_2-Utilization in Anaerobic Microbes Using Different Inorganic Electron Acceptors

Microorganisms	$\Delta G^{o'}$ (kJ $mol^{-1}H_2$)	H_2 threshold (Pa)
Homoacetogens	−24	43–95
Methanogens	−33	2.5–10.0
Sulfate-reducers	−38	0.8–1.9
Iron reducers	−95	0.05
Nitrate reducers[a]	−150	0.02–0.03

Note:
[a] producing ammonium
Source: Data from Cord-Ruwisch and Coworkers (281); Klüber and Conrad (282).

iron, and sulfate are depleted. Then, H_2 partial pressures increase again and methanogenesis resumes (59,283).

A similar chain of events takes place after intermittent drainage (209) or when nitrate, ferric iron, or sulfate is added to the flooded soil (284). The bacteria-reducing nitrate, ferric iron, or sulfate can also successfully compete with methanogens for acetate and thus suppress acetotrophic methanogenesis (42,114,208,285). The suppressive effect of the oxidants is partially alleviated when degradable organic matter is in excess (284). However, nitrate reducers also compete with fermenting bacteria for sugars (285). Indeed, nitrate fertilization effectively decreased the concentration of dissolved organic carbon and eventually the concentration of dissolved CH_4 and the rate of CH_4 emission (286). During the reduction process, nitrate reducers produce nitrite, N_2O, and NO as transient intermediates, which exert a toxic effect on the methanogenic archaea in addition to competition (245,287,288).

Competition between methanogens and bacteria-reducing nitrate, ferric iron, and/or sulfate certainly also takes place in the rhizosphere of vegetated soil, where nitrate, ferric iron, and sulfate are more or less continuously recycled (Figs. 3 and 4). This recycling of oxidants explains why single additions of Fe or S to irrigated rice can result in rather strong suppression of CH_4 emission that is much larger than expected from the stoichiometry between its reduction and CH_4 production (34,63,120,289–291). Addition of Fe is especially effective because little is assimilated by the plants, lost to the floodwater, or removed by pyrite formation. Effective suppression of CH_4 production by iron has also been observed in vegetated littoral soils (292). In contrast, nitrate is regenerated from ammonium but is released as gaseous product following denitrification. These losses must be compensated for by nitrogen fixation in natural wetlands and by fertilization in wetland agriculture (Fig. 4).

Aerobes

Little is known about the competition among aerobic organisms. An important example is that ammonium oxidizers compete for ammonium with the rice plant and with heterotrophic bacteria (293,294). Furthermore, methanotrophic bacteria are able to successfully compete for ammonium, accounting for most of the detectable ammonium oxidation (177,233). Competition for O_2 is also possible among ammonium oxidizers, nitrite oxidizers, CH_4 oxidizers, ferrous iron oxidizers (Fig. 4), and all the other aerobic organisms, and even with O_2-consuming chemical reactions. Nitrate may be assimilated by the rice plant or reduced by bacteria.

Besides competition, the role of predation, parasitism, and disease in controlling populations and stabilizing the coexistence between potential competitors should not be overlooked, but little is known for flooded soils (294,295).

INTERACTION BETWEEN MICROORGANISMS AND PLANTS

Besides serving as a habitat for microorganisms, there are also more intense interactions between rice plant and microorganisms, the economically most important one being symbiotic N_2 fixation (296). Strains of *Azoarcus*, a nitrogen-fixing genus belonging to the *β-Proteobacteria*, are able to colonize rice roots endophytically (297,298). Bacteria invade the roots, colonize the cortex, and penetrate deeply into the vascular system, allowing systemic spreading into the rice shoot. *Azoarcus* cells inside roots of rice seedlings express nitrogenase, making this bacterium to a possible source of plant-available nitrogen. *Burkholderia vietnamiensis* is a root associate that has a high nitrogen-fixing potential, too. The exact interaction with the rice plant is notknown, but inoculation with a certain strain of this bacterium increased grain yield by 13 to 22% (299).

CONCLUSION

The microbiology of flooded soils is characterized by a large functional diversity that is seen in the numerous microbial phenotypes that occur in this ecosystem (Table 1). This functional diversity is mainly due to the many different ecological niches created by gradients between oxic-anoxic and substrate-rich–substrate-poor zones in the soil. These gradients are created by the limitation of the O_2 transport from the atmosphere into the soil because of slow diffusion and microbial respiration and by the deposition of organic matter into the soil by root exudation and decay of plant debris (Fig. 1). This situation results in element cycling between oxidized and reduced states within the ecosystem occurring in the soil on a microscale. The cycles of iron and sulfur are more or less closed and are driven by the reduction of O_2 and the oxidation of organic matter to CO_2 (Fig. 3). Nitrogen cycling leads to losses caused by denitrification that must be compensated for by microbial N_2 fixation or fertilization (Fig. 4). Because of O_2 limitation, part of the organic matter is disproportionated to CO_2 and CH_4, the emission of the later being of great significance for the atmospheric CH_4 budget. The relative ratio of the CH_4 : CO_2 produced and emitted matters a lot as CH_4 is a much more powerful greenhouse gas than CO_2 is. This relative ratio of CH_4 : CO_2 produced is the result of the complex microbiology of flooded soils.

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FLUORESCENCE MICROSCOPY.

See Laser Scanning Microscopy in Combination With Fluorescence Techniques for Biofilm Study

FLUORESCENT IN SITU HYBRIDIZATION (FISH).

See Fluorescent Probes for in situ Analyses of Microbial Communities

FLUORESCENT IN SITU HYBRIDIZATION (FISH): USE IN ACTIVATED SLUDGE.

See Activated Sludge — Molecular Techniques for Determining Community Composition

FLUORESCENT PROBES FOR IN SITU ANALYSES OF MICROBIAL COMMUNITIES

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The first articles describing the use of fluorescent in situ hybridization (FISH) for the analysis of bacteria were published in 1989 (1) and in the early 1990s (2–5), and since then the technique has become increasingly widely used (6,7 and references therein). Using the "mosaic" sequence structure of small and large subunit ribosomal RNA, FISH has enabled the targeting and enumeration of specific microbial populations in a enormous variety of different ecosystems, just a few examples of which are soil (8–10), hot springs (11,12), the rumen (13), aquatic environments (14,15,16), and activated sludge (17,18,19,20). Moreover, recent technological developments have included the combined use of FISH with microautotadiography (21) and with microelectrode measurements (22).

This article focuses on the practical applications of this technique ranging from the initial nucleic acid database related probe design through sample preparation, hybridization, microscopy (including conventional epiflourescence and laser-scanning confocal microscopy), and finally image analysis and interpretation. Also included is a discussion of how recent technological advances have resulted in the broadening of the methodology to many different applications. The pros and cons of the technical aspects of FISH are discussed. In doing so, we will also cover the molecular approaches required for the in situ analysis of ribosomal RNA, messenger RNA, functional genes, and proteins.

TECHNICAL BACKGROUND

Nucleic Acid Databases and Probe Design

The exponential increase in the quantity and availability of sequence information, together with the availability of supporting software on the internet, and the ability to rapidly synthesize DNA oligonucleotides has revolutionized the design and use of DNA hybridization probes. The main source of nucleic acid sequence data is the European Bioinformatics Institute (EBI) database (23), which in collaboration with the DNA Database of Japan (Mishima) and Genbank, NCBI, Bethesda, Maryland (24), maintains and distributes the EMBL Nucleotide Sequence Database (25). There are also databases that specialize in phylogenetic sequence information, such as the Ribosomal Database Project (RDP) (26) and ARB (27). These contain extensive sequence information for the small subunit (SSU) ribosomal RNA (rRNA) genes of microorganisms and to a lesser extent the large subunit (LSU) genes of microorganisms.

In a move toward an integrated center for microbial data, the Ribosomal Database Project is now hosted by the Center for Microbial Ecology at Michigan State University. This Integrated Microbial Database (IMD), which is accessible through the World Wide Web, contains up-to-date phylogeny and taxonomy, gene sequences (including genomes), biochemical, ecological and phenotypic data, and metabolic models (28).

In addition to the sequence data, some of these databases also provide supportive software from alignment editors for phylogenetic analysis. The RDP offers various software programs for handling, analyzing and displaying alignments, and trees. It is also possible to check the phylogenetic groups that putative rRNA-targeted oligonucleotide DNA probes complement. ARB

has a probe design function where rRNA-targeted oligonucleotide probes can be designed at any taxonomic level chosen within a phylogenetic tree. The oligonucleotide probe database is a resource designed to unify oligonucleotide-probe nomenclature and as a record for probe submission following publication. Many of the probe sequences available on this site have been used for fluorescent-labeling and whole-cell hybridization (29).

The procedure for developing a 16S or 23S rRNA probe for a species or genus of bacteria can be conveniently laid out in the following format:

- Comparison of target organism sequences by computer alignment
- Identification of a target sequence (usually 12–25 oligomers in length) that is unique for the taxanomic group of interest
- Chemical synthesis of complementary nucleic acid probes
- Attachment of fluorescent label to the 5′ end of oligonucleotide
- Experimental evaluation of the probe to determine practical specificity of probe. Differences in the secondary and tertiary structure of both the SSU and LSU rRNA molecule may result in steric hindrance that prevents effective probe binding. This can be even more of a problem when fluorescent labels with a large molecular weight are attached to the probe.

Fluorescent Labels and Attachment to Probe

Fluorescent dye molecules are attached to the 5′ end of an oligonucleotide via an aminohexyl linker molecule. Among the most frequently used dyes are fluorescein isothiocyanate (FITC), tetramethylrhodamine 5-isothiocyanate (TRITC), Texas Red, and the hydrophilic sulfoindocyanine dyes CY3, CY5, and CY7. Also used are bodipy complexes and coumarin-phalloidin. Absorption max, extinction coefficients, and emission wavelengths are shown in Table 1. Conveniently, oligonucleotide probes can be obtained prelabeled from the supplier, however, manufacturers will also provide "easy-to-use" labeling kits.

Such fluorescently monolabeled probes might fail to detect cells with small numbers of ribosomes. One or a combination of the following approaches can be adopted to improve fluorescence, thereby improving sensitivity:

Table 1. Spectroscopic Properties of Selected Fluorophores

Fluorophore	Absorption Max	Extinction Coeff.	Emission Max
Fluorescein	490	67	520
Tetramethyl rhodamine	554	85	573
Texas Red	596	85	620
CY 3.18 amine	554	130	568
CY 5.18 amine	652	200	672
CY 7.18 amine	755	200	778
Bodipy	500–581	80	510–591
Coumarin-phalloidin	387		470

indirect-labeling, the use of more sensitive labels, or multiple labeling. The rational behind indirect-labeling is that rather than directly attaching a fluorescent molecule to the oligonucleotide probe, a reporter molecule is attached. Following hybridization, this reporter is detected in a single step by a labeled binding protein. Reporter molecules include digoxigenin or biotin (30). Biotin-labeled rRNA-targeted oligonucleotides have been used to detect *Pneumocystis carinii* in lung sections with alkaline phosphatase-labeled streptavidin (4).

It is also possible to detect whole-cells with oligonucleotides that are covalently linked to enzymes, such as horseradish peroxidase, a more sensitive label. Compared to using FISH with probes labeled directly with fluorochrome, the use of horseradish peroxidase (HRP)-labeled probes followed by treatment with fluorescent tyramide was recently reported to increase the sensitivity of FISH by a magnitude of 10 to 20 (31). One potential restriction of the use of HRP is adequate permeabilization of bacterial cell walls because HRP is a high molecular mass enzyme with a molecular mass of 44 kDa, whereas fluorescein has a molecular mass of only 330 Da. For the HRP to enter bacteria, they must first be permeabilized with cell wall degrading enzymes, for example, lysozyme. This is particularly important for gram-positive bacteria because of their thick cell walls.

The requirement for permeabilization is considered a real problem for the characterization of complex bacterial communities most frequently encountered in environmental microbiology (31). Interestingly, the nonpermeability of bacterial cells to large HRP labeled-probes has been exploited to estimate the condition of bacterial cell walls (32). The rationale behind this approach was that the expression of intracellular, peptidoglycan-hydrolysing enzymes like autolysins would permeabilize the cell wall, making cells detectable by FISH. As a result, intact cells would not be labeled, whereas those with damaged walls would be. The effect of phage infection on the ability of the gram-positive bacterium *Lactococcus lactis* to hybridize to a general eubacterial HRP-labeled 16S rRNA-targeted probe (6) was used as a model. The results indicated that the application of FISH with HRP-labeled probes was a sensitive method for determining the state of bacterial cell walls. The relevance to the complex samples often encountered in environmental microbiology is likely to remain restricted until the problem of permeabilization is fully addressed.

There have also been several attempts to increase the sensitivity of whole-cell hybridization by directing multiple fluorescent labels to one ribosome (33). Using several monolabeled oligonucleotides to different target sites on the rRNA molecule more fluorescence is directed to the target cells. However, the application of this methodology is restricted by the limited availability of target sites with identical specificity.

Sample Preparation and Physical Sectioning for Whole-Cell Hybridization

Pure cultures, mixed cultures of bacteria grown in liquid media, or environmental samples for which the investigator does not require preservation of a three-dimensional

structure can be placed directly on a microscope slide for subsequent whole-cell hybridization. However, most environmental samples are not homogenous solutions, and preservation of structure and spatial relationships are required for a true in situ analysis. To process environmental samples, usually some pretreatment is required, the extent of which will reflect the robustness of the sample being processed.

An example of resilient structures that have been investigated using whole-cell hybridization are the sludge granules involved in the breakdown of waste organic matter (34,35). These samples are so rigid that they maintain their structure during the fixing process, with the granules washed in phosphate-buffered saline, and allowed to settle naturally before the fixation process. Less resilient samples such as feces, rumen fluid, or plant roots may first be set in an agarose gel before the embedding processes are carried out. To set the fixed mixture in agarose, the suspension is warmed to 37 °C and mixed in equal proportions with a 2% solution of agarose in phosphate-buffered saline cooled to 45 °C. The mixture is then rapidly cooled on ice. The fixation procedure is then performed as documented below. The core stages for the processes of sample preparation and physical sectioning for fluorescent in situ hybridization are: fixation, dehydration, embedding, sectioning, mounting, and washing. Each of these will be discussed in detail in the following section.

Fixation. For any successful whole cell hybridization, it is first necessary to achieve sufficient penetration of the cell membrane via a permeabilization and/or fixation step. When characterizing the microbial populations/activity in a specific environment, it is advisable to fix the samples on-site, thereby gaining a truer "snapshot" of the microbial population in situ. Fixation techniques have not changed substantially over the 10 or so years since they were first introduced for environmental applications (1,5). However, these techniques do have limitations, with the failure to permeabilize many gram-positive cells being well documented (8,20,36). As a consequence, a number of different permeabilization pretreatments have been developed including exposure to: xylene (37), lysozyme (8,38) diethyl ether (39), hydrochloric acid (36), and lipase (20). However, it should also be noted that these permeabilization techniques that may be necessary for laboratory cultured bacteria are often not required for the same organisms when analyzed in situ. Permeabilization of cultured *Streptomyces scabies* necessitated a lysozyme pretreatment, whereas the cells were easily detected via the regular 4% paraformaldehyde fixation with no pretreatment in soil (8). Similarly, a mild acid permeabilization step was required for detection of cultured actinomycetes (36), a treatment that proved unnecessary when for the successful hybridization of the same bacteria located on a root surface (9).

The fixation procedure detailed in the next section is applicable to a wide range of environments. Samples are collected and suspended in a paraformaldehyde-based fixative and left for approximately six hours at 4 °C. To prepare the fixative 33 ml H_2O of water is placed in a 150 ml flask and heated to 60 °C, one drop of 10 M sodium hydroxide solution is added, followed by 2 g of paraformaldehyde, then 16.5 ml of triple-concentrated phosphate-buffered saline is added. The pH is adjusted to 7.2, and the solution filtered through a 0.45-μm filter. This is stored on ice and should be used within 24 hours. The fixed sample is then washed in phosphate-buffered saline (PBS 0.13 M NaCl, 10 mM NaH_2PO_4, pH 7.2) to remove the paraformaldehyde that autofluoresces at many of the wavelengths used for whole-cell hybridization microscopy. At this point, samples can be stored in a 1 : 1 (v/v) mixture of phosphate buffer and ethanol at −20 °C.

Dehydration and Embedding. Where preservation of the three-dimensional structure is not required, a sample can be placed directly onto a gelatin-coated microscope slide precleaned in potassium hydroxide in 95% ethanol, then immersed (5 × 1 min) in hot distilled water containing gelatin (0.1% w/v) and $KCr(SO_4)_2$ (0.01% w/v), then dehydrated through ethanol or water baths (5 min in 50 : 50 v/v, then 80 : 20 v/v, and finally 96 : 4 v/v). The dehydrated sample is then ready for FISH. When preservation of the three-dimensional structure is required, the sample on the microscope slides is then dehydrated using ethanol and xylene baths. Subsequently, the sample is embedded in melted paraffin wax.

Sectioning. Each sample is then sectioned into a specified thickness ranging from 2 to 10 μm using a rotary microtome, the thickness of the sample depending on the subsequent microscopic approach. This is considered in the next section.

Mounting and Washing. Each section is placed on a gelatin coated microscope slide. The paraffin wax is removed by sequential washes in xylene and alcohol baths, starting with 100% xylene and ending with 100% ethanol to remove the xylene. Once they have been air-dried, the sections on the slides are ready for hybridization.

The main advantage of thin sections (1–3 μm) is the small cross section the light transverses, reducing the amount of light scattering from out-of-focus images originating from sample material above and below the plane of focus. One disadvantage of sectioning is that the three-dimensional reconstruction requires that each individual slice needs to be bought into focus. An alternative approach is to prepare thicker sections, however, this increases the amount of light scattering, although with the growing use of confocal laser scanning microscopy (CLSM), optical sectioning is rapidly becoming established as an alternative to such physical sectioning.

Hybridization Techniques

Once the sample has been physically fixed onto a microscope slide and prepared for hybridization, it is ready for the addition of the fluorescent-labeled oligonucleotide probe. The first stage in this procedure is the creation of an area around the sample on the slide that will maintain it in contact with the hybridization buffer and probes. Slides with indentations can be used for hybridization, and in this case the sample is placed in the indentation and

no further preparation is required. However, if ordinary slides are used, a wax ring can be placed around the sample. A test tube or similar object of a suitable diameter is warmed in a flame, dipped in wax, and then the wax end of the tube used to form a ring surrounding the sample on the slide. The subsequent stages in this procedure are presented below:

Hybridization. To each well is added 9 μl of hybridization solution (20–40% formamide (Table 2), 0.9 M sodium chloride, 0.1% sodium dodecyl sulfate, 100 mM Tris (pH 7.2)) and 1 μl of probe (25–50 ng μl^{-1}). This is mixed gently with a pipette tip to evenly distribute the probe taking care not to scratch the surface of the sample. An alternative means of ensuring even distribution of the probe for multiple hybridizations is to prepare a master mix of hybridization solution and probe. The concentration of formamide is varied depending on the temperature of dissociation (Td) of the probe used in the hybridization (Table 2). This is left overnight at 37 °C in a screw cap plastic tube (50 ml volume) placed horizontally in a hybridization oven with tissue paper soaked in distilled water to prevent evaporation of buffer.

Washing. The hybridization fluid is removed with a quick rinse in 25 μl double-distilled water. This is followed by a wash in wash solution I (20–40% formamide, 0.9 M sodium chloride, 0.1% sodium dodecyl sulfate, and 100 mM Tris (pH 7.2)) for 20 min at 37 °C. Wash solution I is removed with a pipette and wash solution II is added (0.9 M sodium chloride and 100 mM Tris (pH 7.2)) for 15 minutes at 37 °C. Finally, the well containing the sample is rinsed in distilled water for 10 minutes at room temperature and left to air-dry in the dark. At this point, samples can be stored dry and away from light until analysis by fluorescence microscopy.

Mounting. Before fluorescence microscopy, to retard bleaching, it is advisable to mount the sample using an antifadent. These are supplied by a number of manufacturers and include, for example, Citifluor™ (Citifluor Ltd., U.K.) and "Slowfade®" (Molecular probes, Inc, U.S.A.).

Microscopy

Conventional Epifluorescence. Fluorescence is the emission of light from a molecule in which an electronically excited state has been produced (40). Specifically, when light strikes a molecule capable of fluorescence, a quantum of light is absorbed by that molecule (in $\sim 10^{-15}$ seconds), electrons are raised to a higher excited state, and energy in the lowest vibrational state is rapidly dissipated with the lowest vibrational level of the excited singlet state obtained. The electron returns to the ground electronic state with the emission of energy (fluorescence) (41). The reemission of light is usually at a higher wavelength (Stokes fluorescence) than the initial excitation wavelength. Any fluorescent molecule will have two characteristic spectra, excitation (the relative efficiency of different wavelengths of exciting radiation that cause fluorescence) and emission (the relative intensity of radiation emitted at various wavelengths) (41). Any portion of such a spectrum where absorption occurs can produce a fluorescent light, which will be the same irrespective of the excitation wavelength; however, the fluorescence intensity will vary with the relative strength of the absorption (41).

Table 2. Concentration of Formamide Included in Hybridization Fluid in Relation to the Calculated or Experimentally Determined Td of the Oligonucleotide Probe

Formamide (% w/v)	20	25	30	40	45	50
Td of probe (◦C)	20	25	30	40	45	50
Volume of reagents	μl	μl	μl	μl	μl	μl
Sodium chloride (5 M) (μl)	180	180	180	180	180	180
Tris Cl. (1 M, pH 7.2)	100	100	100	100	100	100
Formamide	200	250	300	400	450	500
SDS (100 gl^{-1})	10	10	10	10	10	10
Distilled water	510	460	410	310	260	210

Fluorescent in situ hybridization utilizes fluorophores (fluorescent light-emitting molecules) tagged onto oligonucleotide probes. In epifluorescence microscopy, the fluorophore is illuminated by the light source, usually a mercury high-pressure lamp, the light from which passes though a excitation wavelength filter, and is then directed through the objective lens by a dichroic mirror onto the sample (Fig. 1a). Light emitted by the fluorophore is then collected by the same objective lens and picked up by the eyepiece or camera. This can be done using either upright or inverted microscopes. A principal advantage of using the inverted microscope is that it provides a stable platform for complementary physiological studies (42).

Before sample analysis using epiflourescence microscopy, it is recommended that the user locates the sample using transmitted or phase-contrast light microscopy. In this way, any excess photobleaching of the samples as a result of overexposure to fluorescent light will be avoided, and the likelihood of successful location of any probed bacteria will be maximized. Images from conventional epifluorescent microscopes can be captured on film with exposure dictated either by the user or by the fluorescence intensity. Examples of such images are presented in Figures 2 and 3. To maximize detection of fluorescence, a charge coupled device (CCD) camera can be used. A CCD is a light-sensitive silicon solid state device composed of many small pixels. The light falling on a pixel is converted into a charge pulse. The output gate of the CCD can either be connected to an analog to digital converter, to digitize the picture, or can provide a standard video signal that can be captured. The computer-reconstructed digitized images are easy to store in the computer memory before any application of image analysis software.

As a less disruptive alternative to thin sectioning, thicker sections can be used for the analysis of the in situ hybridization. When this approach is taken, submicron images of sections are reassembled to reconstruct the biological structure using image analysis software. This reconstruction is called volume rendering (43). This converts a serial collection of image sections into a 3-dimensional representation called the image stack. These stacks can be rotated or spliced to study the structure

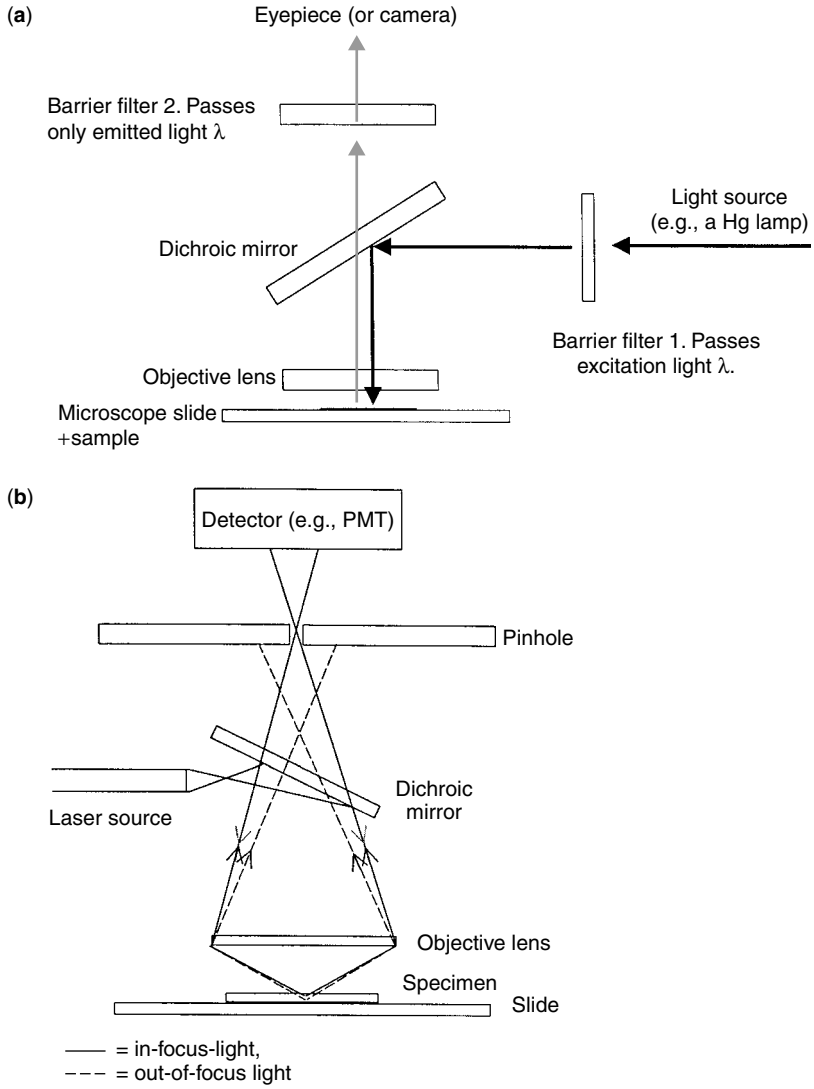

Figure 1. Schematic of (**a**) the optical system in an upright epifluorescence microscope, (**b**) the confocal laser scanning microscopy CLSM.

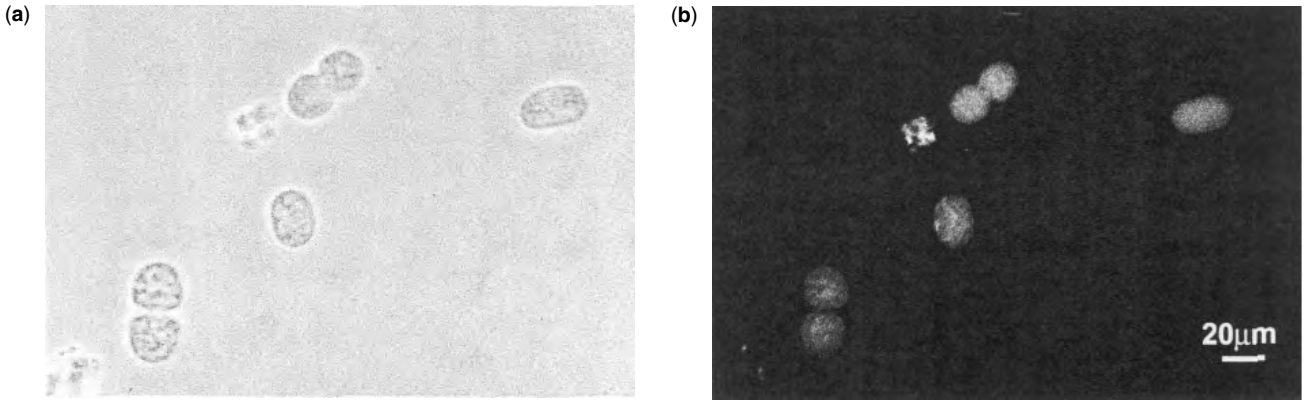

Figure 2. *Achromacia* sp. probed with fluorescently labelled 16S rRNA-targeted oligonucleotide probes visualized using conventional epifluorescence microscopy. (**a**) Probe labelled with Fluorescein and (**b**) Rhodamine. (Dr. Ian Head. The University of Newcastle Upon Tyne, U.K.). See color insert.

of the biological sample. The files for these stacks are cumbersome, however, that is less of a problem with constant advances in computer hardware. Image analysis software has been written for Macintosh, MS-DOS, UNIX workstations, and Silicon Graphics computers (SGI). A list of image analysis software available for public use is available from NIH Image (National Institute of Health, Bethesdam, MD, U.S.A.).

Confocal Microscopy

The introduction of confocal laser scanning microscopy (CLSM) has provided for significant advances in successful utilization of FISH. Increasingly widely used, CLSM has resulted in vastly improved resolution and three-dimensional image analysis. As with conventional epiflourescence microscopy, the use of an antifadant is recommended for mounting FISH samples.

With epifluorescence microscopy, the image obtained from a thick sample is blurred because of out-of-focus fluorescence. In common with conventional epifluorescence, CLSM uses reflected rather than transmitted light; moreover, sample thickness has less effect on the ability to obtain high-resolution images. CLSM enables the researcher to scan through a sample resulting in thin, nonblurry section images (44). Figure 1b shows a schematic of the basic CLSM set up. Argon or Krypton Argon lasers are most commonly used for FISH, covering as they do a broad range of emission lines. Unlike conventional epifluorescence microscopy, a single point of excitation light is scanned across the specimen in CLSM. With only a single point illuminated at any moment, the illumination intensity falls off above and below the plane of focus, thereby reducing the excitation of fluorescence of out-of-focus sample. The fluorescent light then passes back though the dichroic mirror and passes through a pinhole aperture situated in a conjugate focal plane to the sample. Any light emitting from above or below the focal plane will not pass though the pinhole, resulting in a further removal of any out-of-focus fluorescence. The light that passes though the pinhole is then detected by a photomultiplier tube (PMT). Sample scanning (in X, Y, and Z directions) is controlled by use of computers, which also control the image assembly and display functions. It is the Z-scan function (the optical sectioning function) that enables creation of three-dimensional images.

To produce a 3D image, the upper and lower limits of the sample fluorescence can be set manually, following which the section thickness and the number of sections to be scanned are decided. The depth of sections is dictated by the numerical aperture and magnification of the lens used and by the thickness of the sample. The images are then collected from each section (for e.g., on the Leica TCS-NT CLSM the galvanometer driven Z-stage is able to move at a minimum of 10-nm steps though a sample), thereby providing a set of optical sections that can be viewed either as a tableau of images or projected as a three-dimensional image (Fig. 4). Following Z sectioning, the CLSM also enables production of images through the ZX plane. A CLSM system will be coupled to a conventional epifluorescence microscope, enabling the researcher to locate and roughly focus the CLSM on the sample under investigation, before CLSM analysis.

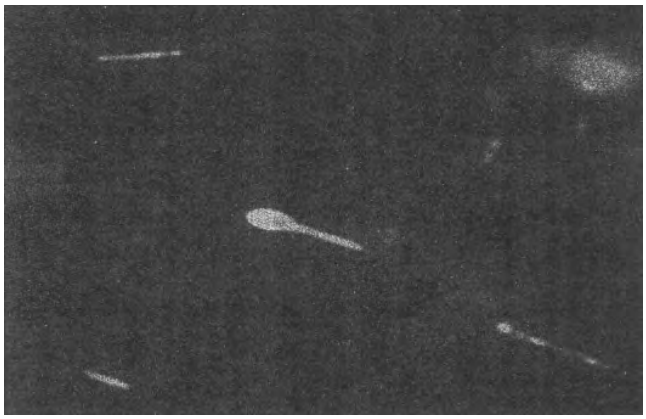

Figure 3. A fecal sample probed with a Fluorescein isothiocyanate-labelled oligonucleotide probe specific for the bifidobacteria genus (S-G-Bif-164-a-A-17) (29) visualized using conventional epifluorescence microscopy. (Dr. G. W. Welling, University of Groningen, The Netherlands). See color insert.

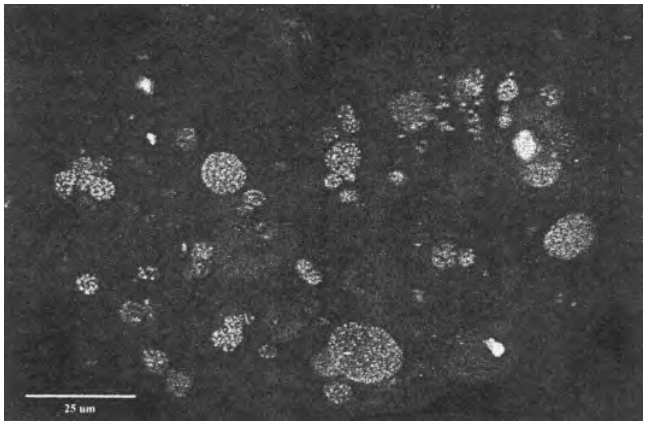

Figure 4. A reconstructed confocal image of an activated sludge floc labelled with fluorescien labelled Nso 1225 (S-*-Ntros-1225-a-A-20) (29) visualized using confocal laser-scanning microscopy (CLSM). (Dr. G. Coskunner and Dr. T. Curtis, The University of Newcastle upon Tyne). See color insert.

Pros and Cons of FISH

Problems associated with the use of FISH can be conveniently broken down into two main areas. First are those directly related to the application of the rRNA analysis in microbial ecology, and to the development of the appropriate oligonucleotide probes. Second are the methodological issues relating to the application of the technology with environmental samples. Although there are extensive databases containing the rRNA gene sequences for several thousand prokaryotes (5S, 16S, and 23S sequences), important weaknesses associated with successful application of FISH lie in this most fundamental area. The difficulties associated with the use of the polymerase chain reaction (PCR) to recover rDNA sequences from complex mixed environmental samples have been well documented (45,46,47). Among these are

chimera formation and the fact that the current database represents only a very small fraction of the extant diversity, therefore limiting primer choice. Chimeric rDNA sequences are formed when a PCR reaction combines two or more sequences derived from different bacteria into one product that is then reamplified throughout the remaining PCR. The probability of chimeric sequences forming is increased when the template DNA used for PCR is very fragmented, resulting in partial length rDNA fragments. Premature termination of elongation during PCR will also have the same effect. Using high-molecular weight, DNA as template has been found to produce chimeric clones at the rate of about 2% (2 out of 113) (45). Chimeras can be detected by performing comparative sequence analysis on different regions of the rDNA sequence. The ribosomal database project at the Center for Microbial Ecology provides an on line chimera check program (26).

Another disadvantage of the direct analysis of rDNA is that the DNA of minor members of the microbiota or of organisms that are recalcitrant to release their DNA using conventional DNA extraction techniques are unlikely to be detected by PCR using universal- or domain-specific rDNA-targeted primers. However, the retrieval of less common sequences can be facilitated using more taxonomically specific PCR primers for PCR amplification. These problems of accurate sequence retrieval for a comprehensive range of microorganisms have serious implications for the design of accurate rRNA-targeted fluorescent probes. Probe design on the basis of incomplete sequence information results in the likelihood that bacteria comprising substantial proportions of the microbial community will not be detected using FISH.

Second, in addition to the limitations to the PCR technique that can result in inaccurate sequence retrieval not reflective of the extant microbial community, there are other more specific methodological problems associated with FISH. Low-fluorescent signal intensity is frequently encountered during in situ hybridization. Such low intensity can be caused by a number of factors including: low ribosome number, low organism number, and insufficient accessibility of the target rRNA molecules.

The correlation between cellular ribosome concentration and bacterial growth rate was a key discovery of microbial physiology (48). In principle, this idea can be applied to environmental samples. Probe-conferred fluorescence should correspond to bacterial numbers and for single cells it should correspond to cell ribosome content and, by inference, to single cell activity. However, there is limited published data relating change in ribosome abundance to growth rate for environmentally isolated bacteria (49).

Problems with target accessibility are usually either related to limited cell wall probe accessibility (common in gram positive bacteria, see permeabilization section) or interference through the tertiary structure of the ribosomes. The problem of restricted accessibility because of the tertiary structure of ribosomes can be reduced by the inclusion of formamide to the hybridization buffer. Formamide is a solvent that weakens the effects of hydrogen bonds. Although the higher-order structure of ribosomes is well known, there is a great deal of variation between taxanomic groups, with some regions inaccessible in some groups, indeed shifting the target site just a few nucleotides can dramatically increase the signal (6).

MOLECULAR APPROACHES TO IN SITU ANALYSES

Ribosomal RNA-Targeted Oligonucleotide Probes

Background. The high abundance of ribosomes has ensured that rRNA is the most commonly used target for fluorescently labeled probes. Moreover, targeting of particular regions of the rRNA sequence enables phylogenetic specificity to be varied from the universal to the subspecies level (1,50,51,52). The genes that code for rRNAs (5S-, 16S-, and 23S-) are also very highly conserved. Regions of the molecule have remained sufficiently conserved through evolution to permit probe design for the three domains, whereas the more variable regions allow for discrimination between genera, species, and even individual strains. The 5S rRNA molecule (approximately 120 nucleotides) provides relatively little sequence information and is therefore of less value in the study of phylogenetic relationships and probe design. The larger 16S molecule provides more sequence information and is the most widely used for FISH. The 23S rRNA molecule (approximately 3000 nucleotides) contains twice as much information as 16S rRNA, however, it is used less frequently for probe design, but has been of value in resolving phylogenetic relationships between microorganisms that have proved to be closely related in terms of their 16S rRNA (53).

There are two different ways to use phylogenetically based oligonucleotide probes for investigating microbial populations. Firstly, quantification of a certain SSU rRNA compared with total SSU rRNA can be obtained by slot-blot hybridizations of a directly isolated mixture of RNAs (tRNA, LSU rRNA, SSU rRNA, and mRNA) with SSU rRNA-targeted oligonucleotide probes specific for the taxonomic group of interest and a universal probe. The relative abundance is calculated by dividing the amount of a specific probe bound to a given sample by the amount of hybridized universal probe. This approach was first used to determine the relative abundance of cellulose-degrading populations of the bovine rumen (54). Subsequently, this technique has been used to determine the abundance of several rumen bacterial populations (55), methanogens in bioreactors (56), microbial mat communities (57), and predominant microbial populations in human feces (58).

Second, to visualize intact organisms within their environment, whole-cell FISH is used. Analysis at this single-cell level provides a more detailed picture than does slot-blot hybridization. This microscopic identification of single cells was first performed with radioactively labeled oligonucleotide probes (59). These have been largely superseded by the use of fluorescent labels that can be instantaneously detected by epifluorescent microscopy.

Fluorescently labeled rRNA-targeted oligonucleotide probes allow the detection of individual cells that makes them a suitable tool for determinative, phylogenetic, and environmental studies in microbiology. This technique is now commonly used for analysis of a diverse

range of environmental microbial populations, such as leaf surfaces (52), sludge granules (35,60), nitrifying bioreactors (61), full-scale biofilters (62), mixed-culture biofilms (63), ruminal bacteria (64), and cricket hindguts (65).

The Use of rRNA-Targeted Probes to Identify Unculturable Organisms

An important caveat to this technology is the in situ identification of previously unidentifiable populations. The restricted number of morphological types or lack of distinct morphological features often limits in situ identification of different organisms in mixed communities. The general description rod-shaped anaerobe, potentially encompasses several thousand bacterial species. This together with the problems of isolating many prokaryotes in pure culture for identification purposes explains why FISH has become such a popular and important tool in environmental microbiology for the direct identification of organisms. As an example, members of the species *Xanthomonas* are important in environmental microbiology, in phytopathology, and in human pathogenesis. Members of this genus are difficult to identify, especially if the origin of the isolates is uncommon (62,66). In comparison to time-consuming methods based on isolation techniques, in situ hybridization has facilitated identification procedures. In a study of biofilters, the dominant bacterial populations identified in full-scale bioreactors and laboratory-scale biofilters were members of the *Xanthomonas* branch of the class *Proteobacteria*, which forms a monophyletic cluster close to the root of the γ-*Proteobacteria* (62). Previously designed probes that are specific for the γ-*Proteobacteria* did not give a positive FISH signal when hybridized with many of the *Xanthomonas* isolates found in the bioreactor samples, indeed none of the 18 tested representatives hybridized with these probes. This indicated that they were new species not previously isolated. Therefore, to allow in situ identification of these bacteria and quantification new probes had to be designed based on new SSU rDNA gene sequences of the new isolates. The new *Xanthomonas*-specific probe presented was an efficient tool for detecting species of *Xanthomonas* in subsequent bioreactor studies and for identifying potential phytopathogens.

The Use of rRNA-Targeted Probes to Study the Spatial and Temporal Relationships of Organisms

In addition to the identification of microbes in situ described in the previous section, FISH is increasingly used in environmental microbiology to study spatial and temporal relationships both within a single population and in mixed populations. In studies of spatial relationships great care must be taken when sampling to minimize disturbance of the microbial populations. This is probably one of the most critical and limiting factors to consider, rather than the microscopy of such samples where that introduction of CLSM has simplified and improved the analysis enormously. Particularly resilient structures that have been investigated in this way are the sludge granules involved in the breakdown of waste organic matter (35,60). The anaerobic degradation of organic matter leads to the intermediate formation of alcohols and fatty acids. The subsequent oxidation of these products under methanogenic conditions is coupled to proton reduction and can only proceed at low-hydrogen partial pressures and low formate concentrations. A low concentration of these metabolic intermediates is maintained by interspecies hydrogen transfer of hydrogen or formate between synthrophic consortia of bacteria. This complex metabolic relationship between different trophic levels of organisms requires a precise spatial organization of the microorganisms, usually the inner layer is composed of methanogens and the outer layer is comprised of fermentative bacteria (67). Additionally, the syntrophic bacteria are in a close juxtaposition with the methanogens.

The beginning of an understanding of how these sludge granules function to breakdown organic matter has been achieved through a combination of FISH and immunohistochemical techniques (68). However, the mosaic of microbes within the granules still requires extensive studies, particularly in relation to the location of bacteria at the genus and species level. A study on mesophilic and thermophilic sludge granules combined FISH with confocal-laser scanning microscope to elucidate the spatial distribution of particular species of methanogens and bacteria that were previously detected by whole microbial community 16S ribosomal DNA (rDNA) cloning analysis (35). Probes were designed for a cluster of bacteria closely related to *Syntrophobacter* species and for a group of unidentified green non-sulfur bacteria. Using these probes, the *Syntrophobacter* species hybridized with cells in the inner layer of the mesophilic sludge granules, whereas the unidentified green non-sulfur bacteria were found to be filamentous cells in the outermost layer of the thermophillic sludge granule sections.

Messenger RNA-Targeted Oligonucleotide Probes and Structure-Function Relationships

An important factor in understanding community level processes is the relationship between structure and function in microbial communities. Therefore, an obvious progression from the detection and visualization of organisms in their environment using fluorescently-labeled rRNA-targeted oligonucleotides described so far, is to determine the metabolic function of organisms in situ. Previous attempts to address this problem have been by a number of means, for example, the metabolic role of unculturable bacteria has been inferred from the spatial location of particular morphologies determined by in situ hybridization in the example described earlier. Another approach in environmental microbiology has been the use of microelectrodes to measure microbial processes. This approach has been of particular value in the study of sulfate-reducing bacteria in microbial mat communities (57). Here the distribution and abundance of sulfate-reducing bacteria and eukaryotes within the upper 4 mm of a cyanobacterial mat community were characterized using a combination of group-specific hybridization probes to measure ribosomal abundance and microelectrodes to determine dissolved-oxygen concentrations.

FISH has been successfully combined with microelectrode measurements to study the processes of sulfate

reduction in trickling filter biofilms (22) and nitrification in microbial flocs of a nitrifying fluidized bed reactor (69). Similarly, the use of microsensors for nitrate in combination with rRNA probes for ammonia- and nitrite-oxidizing bacteria showed that nitrification was restricted to the top layers of a trickling-filter biofilm. In these layers, ammonia and nitrite oxidizers were found in proximity to each other (69). The combination of the two methods provided reliable information relating in situ microbial activity and the occurrence of specific microorganisms in complex microbial consortia.

A more direct approach for studying the functional relationships of organisms in situ is to investigate the expression of particular genes. It is important to note that the information derived from rRNA-targeted oligonucleotide probes on the abundance of RNA for a particular population cannot be used as an indication of a specific kind of activity. Excepting members of a few highly physiologically restricted bacterial lineages, such as the ammonia oxidizers belonging to the β-proteobacteria, the apparent identification of a bacterial species in situ does not provide much information about its function in its habitat. Often, one population has the potential to catalyze different transformations, and one genotype is linked to several phenotypes. More importantly, one phenotype may be linked to several genotypes, for example, a wide range of genotypes of bacteria express cellulase enzymes. Additionally, just because a particular genotype is present in an environmental sample does not necessarily mean that it will be expressing a characteristic phenotype. Information regarding the phenotype of an organism is of more value than the actual abundance of the bacteria. A direct and sensitive means of studying the phenotype of bacteria is to study mRNA expression. Prokaryote synthesis of mRNA commits the bacteria to expression of the gene product. If there is sufficient sequence information, then probes can be designed for regions of the mRNA for particular gene products. These probes are then used to identify specific mRNAs in the pool of extracted mRNAs (70). A method for detecting RNAs in single bacterial cells by an in situ PCR has been developed (71).

It is important to note, however, that mRNA is incredibly labile and difficult to handle, particularly in environmental samples that are notoriously rich in nucleases. An alternative approach to mRNA detection is the use of genetic reporters, such as the *lux* or *lac* Z-genes. This approach has been used in a number of areas of environmental microbiology, such as the study of bacterial infection of root hairs (72), surface regulation of alginate production (73), and the monitoring of nitrogen or phosphate starvation (74).

An alternative strategy to studying structure–function relationships in bacteria is the use of radiolabeled substrates in combination with microautoradiography. This permits the analysis of the metabolic activity of prokaryotes under conditions that approach in situ conditions by direct visualization of microorganisms with active uptake systems within a complex community. To date a major limitation of microautoradiography has been an inability to correlate the activity detected with identification and detection of a responsible organism. However, the recent combination of microautoradiography with FISH performed with rRNA-targeted oligonucleotide probes permits simultaneous identification and determination of substrate uptake patterns of individual microbial cells within complex microbial consortia (21). This methodology was first evaluated by using defined artificial mixtures of *Escherichia coli* and *Herpetosiphon aurantiacus* with [^{3}H] glucose. Subsequently, this was used to visualize the uptake of organic [^{14}C] acetate and [^{14}C] butyrate) and inorganic [^{14}C] bicarbonate and [^{33}P] radiolabeled substrates in probe-defined populations from complex activated sludge microbial communities. In summary, this combined technology allows the direct analysis of the in vivo uptake of substrates by probe-identified microorganisms in environmental samples.

Fluorescent Proteins as Reporters for In Situ Gene Expression

Unfortunately reporter genes, such as the *lux* or *lac Z*-genes described earlier are not suitable for use in FISH, however, the successful cloning and heterologous expression of green fluorescent protein of *Aequorea victoria* (75) provided a suitable reporter for use with the FISH approach (63). Green fluorescent protein has become a useful tool for measuring and monitoring bacterial gene expression (76). The biggest potential application of this methodology in environmental microbiology is the monitoring of microorganisms exploited for bioremediation, biocontrol, and plant growth enhancement. This will be of particular value for genetically engineered microorganisms that potentially share their phylogeny with many existing environmental microorganisms and so cannot be differentiated with fluorescently labeled rRNA-targeted probes. An advantage of green fluorescent protein is that unlike other biomarkers, it does not require any substrate or additional cofactors in order to fluoresce. Single cells tagged with green fluorescent protein can easily be visualized by microscopy; normally a single copy integrated into the chromosome is sufficient for visualization of cells. It is the active component of a bacterial population that is the most important, because this is the fraction that exerts an effect on the environment.

Green fluorescent protein has been used in conjunction with FISH to study in situ gene expression in mixed-culture biofilms and to provide evidence of metabolic interactions between community members (63). In the biofilms, bacterial identity was determined by in situ hybridization with fluorescent labeled 16S rRNA targeted probes, while gene expression was simultaneously visualized using green fluorescent protein as a marker for the expression of enzymes involved in the catabolism of toluene. However, there are still problems with the use of green fluorescent protein. As discussed earlier not all the viable cells in a population are metabolically active and the green fluorescent protein phenotype does not indicate the metabolic status of the cells. Additionally, green fluorescent protein is a very stable protein, and therefore reflects the history of gene expression in the cell rather than expression at any one time.

An alternative marker protein for use in conjunction with FISH is β-glucuronidase. This is another promising

tool for ecological studies of biocontrol agents in soil-based environments such as the rhizosphere. It has been transformed into *Trichoderma harzianum*, a widely used biological control agent for soil-borne plant-pathogenis fungi (77). However, while there are many advantages to using this marker protein it does require an exogenous substrate that green fluorescent protein does not and some background activity of β-glucuronidase may be present in some systems.

CONCLUSION

The use of FISH with rRNA-targeted probes has evolved rapidly over the past decade from a technique to qualitatively identify microorganisms to one that provides answers concerning some of the more complex issues in environmental microbiology. With access to the appropriate microscope facilities, it is a simple and robust technique and has been and is being used in environmental microbiological laboratories the world over. Insights into the architecture of complex microbial communities are now being provided. Together with the use of marker proteins to signal particular gene expression and so relate structure to function, the opportunities for this technique in environmental microbiology are particularly valid.

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FLUOROCHROMES.

See Laser Scanning Microscopy in Combination With Fluorescence Techniques for Biofilm Study

FOAMING IN ACTIVATED SLUDGE.

See Activated Sludge — Foaming; Filamentous Bacteria in Activated Sludge: Current Taxonomic Status and Ecology

FOOD CONTAMINATION.

See Viruses and Protozoan Parasites in Food, Including Methodology

FOOD INDUSTRY.

See Biofilms in the Food Industry

FOOD PRESERVATION.

See High Hydrostatic Pressure: Microbial Inactivation and Food Preservation

FOOD PROCESSING AND BIOAEROSOLS.

See Bioaerosols in Industrial Settings

FOSSIL FUELS DESULFURIZATION.

See Desulfurization of Fossil Fuels

FREE-LIVING AMEBAS PRESENT IN THE ENVIRONMENT CAN CAUSE MENINGOENCEPHALITIS IN HUMANS AND OTHER ANIMALS

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Members of the protozoon genera *Acanthamoeba*, *Naegleria*, and *Balamuthia* are free-living, nonparasitic amebas. Some are, however, capable of causing disease as

opportunistic pathogens in humans and other animals. *Acanthamoeba* and *Naegleria* have a wide distribution in soil and water and can be readily isolated from either of these sources. It is believed that *Balamuthia* amebas also share the same habitat as *Acanthamoeba* and *Naegleria* but have yet to be isolated from environmental samples. Since their recognition as potential human pathogens, these organisms have come to occupy an increasingly important place in the fields of environmental microbiology and clinical medicine. Although they share a similar ecological niche in nature, the amebas have different phylogenetic histories and taxonomic positions. Several comprehensive reviews of these organisms and their pathogenic potential have been published over the years (1–7).

The concept that *Acanthamoeba* and other small free-living amebas can cause disease in humans and other animals was suggested by the late Dr. Culbertson in the early 1960s. Culbertson and colleagues, while growing poliomyelitis virus in monkey kidney cell cultures for vaccine purposes, noticed cleared areas or plaques in the control cell cultures. The culture supernatant, when injected into experimental animals, caused death of the animals with typical symptoms of meningoencephalitis, such as ruffled fur, aimless wandering, coma, and death. Culbertson and coworkers isolated an ameba from the culture supernatant and brains of the dead and dying animals and designated it as *Acanthamoeba* Lilly A1 strain (8). This isolate was subsequently named as *Acanthamoeba culbertsoni* (3). Culbertson and colleagues also isolated from nature several different strains of *Acanthamoeba* with varying degrees of virulence. Based on these studies, Culbertson suggested that human infections with these amebas might occur naturally (3,8). A few years later, Fowler and Carter in Australia reported meningoencephalitis in an Australian boy attributed to *Acanthamoeba*, which was later identified as *Naegleria fowleri* (3,9). Currently, it is well known that several species of *Acanthamoeba*, *Balamuthia mandrillaris*, and *N. fowleri* cause infection in humans and other animals (2,3,7).

Acanthamoeba causes a spectrum of diseases, including granulomatous amebic encephalitis (GAE), *Acanthamoeba* keratitis, skin and nasal abscesses, and bone infection. Typically, infections caused by *Acanthamoeba* occur in immunocompromised hosts, including persons with the human immunodeficiency virus and/or the acquired immune deficiency syndrome (HIV/AIDS). In contrast, *N. fowleri* causes primary amebic meningoencephalitis (PAM) in previously healthy children and young adults with a history of water-related sports. The third member of this group of amebas, *B. mandrillaris*, causes granulomatous encephalitis and skin abscesses and sinus infections in both immunocompetent and immunocompromised persons. In this review, we describe the biology and epidemiology of the amebas, their modes of infection, and mechanisms of pathogenesis, and appropriate antimicrobial therapy in the treatment of these infections, emphasizing recent advances in knowledge concerning these opportunistic pathogens (3).

Acanthamoeba spp.

Biology

The life cycle of *Acanthamoeba* includes a trophic or feeding stage, the trophozoite, and a resistant cyst stage. The trophozoite has sluggish locomotion and measures about 20 to 30 μm in diameter and is characterized by the presence of pointed pseudopodial projections, the acanthopodia. In nature, amebas feed on bacteria, a feature that makes it relatively easy to isolate and culture them in the laboratory. When food is scarce or when facing desiccation or other environmental stresses, the amebas round up and encyst. The cyst has two walls, the outer wall (ectocyst) is wrinkled and the inner wall (endocyst) is stellate, polygonal, oval, or round. At the junction of the ecto- and the endocyst are pores or ostioles and these are covered with opercula. Upon return to favorable growth conditions, the dormant ameba is activated to leave the cyst and become once again a trophic form (10).

Acanthamoeba has worldwide distribution and is commonly found in soil, dust, fresh water, sewage, swimming pools, and hot springs. It has also been isolated from tap water, salt water, ocean sediments, heating, ventilating and air conditioning (HVAC) units, hydrotherapy baths in hospitals, dental irrigation units, eye wash irrigation units, home aquaria, humidifiers, thermal and nuclear cooling tower effluents, bacterial, mycotic, and mammalian cell cultures, vegetable matter, contact lens paraphernalia, and human tissue. Given their environmental ubiquity, many opportunities exist for humans to come into contact with trophic amebas or cysts (3,4,6,11). *Acanthamoeba* is tolerant of a wide range of osmolarities, enabling it to survive in distilled water, tissue culture media, mammalian body fluids, and seawater. Strains isolated from human cases of *Acanthamoeba* infections generally grow optimally at 37 °C, although a number of clinical isolates grow better at about 30 °C. The pathogenic potential and virulence of environmental isolates can be determined by instilling amebas into the nasal passages of mice. Infected animals exhibit signs such as ruffled fur and aimless wandering, coma, and die within 1 to 4 weeks indicating the virulence and/or the pathogenic potential of the isolate. The cytopathic effect (CPE) caused by amebas on tissue cultures is another possible mode of assessing virulence (4,5) of the strain.

The taxonomy of the genus is unsettled. Until relatively recently, species were created on the basis of morphological criteria, including such features as size of trophozoites and cyst morphology. Based on such criteria, *Acanthamoeba* isolates were separated into three groups: I, II, and III. Members of group II are most frequently isolated from the environment and, perhaps because of their relative abundance in nature, are most often implicated in human infections. Increasingly, isoenzyme patterns and/or a molecular approach using 18S rRNA gene, is being investigated to improve upon the taxonomy of the genus *Acanthamoeba* and determine its phylogenetic relationships (12).

In addition to their involvement in human disease, *Acanthamoeba* spp. have also been identified as agents

of GAE in gorillas and other old-world primates, other mammals like sheep and horses, as well as in lower animals, including fish and lizards (3,4,6).

Growth and Nutrition

Acanthamoeba spp. can be readily grown and maintained in the laboratory using a variety of media. As bacteria are their major food source, they will grow in the laboratory on nonnutrient agar plates covered with bacteria such as *Escherichia coli* or *Enterobacter aerogenes*. They show a preference for bacteria that are not encapsulated, the presence of a mucoid capsule inhibiting phagocytosis by the amebas. The use of nonnutrient agar covered with a bacterial lawn is the recommended technique for isolation of amebas from both clinical specimens such as brain tissue and corneal scrapings, as well as environmental samples. Amebas feed upon the bacteria until almost all the food is gone and then encyst. Cysts remain viable for prolonged periods of time, especially if the agar plate is sealed to prevent drying and kept at a reduced temperature (4).

Acanthamoeba can also be readily established in bacteria-free or axenic culture by harvesting amebas from a bacterized culture, washing them by centrifugation in distilled water or saline to eliminate most of the bacteria, and then inoculating the washed cells to a suitable growth medium containing antibiotics. A combination of penicillin-streptomycin or gentamicin is effective in inhibiting or killing any residual bacteria present in the ameba inoculum. A medium consisting of 2% proteose peptone, 0.5% yeast extract, and 0.5% glucose will support axenic growth with yields of 10^6 amebas/mL or more. A number of the isolates from human *Acanthamoeba* infections appear to require further medium enrichment with calf serum and a standard tissue culture vitamin mixture. Also, several different species of *Acanthamoeba* have been grown in chemically defined media (4,11).

Endosymbionts

For reasons that are not known, *Acanthamoeba*, more so than other soil amebas, is often likely to harbor endosymbiotic bacteria. According to one study it is estimated that approximately 20 to 24% of clinical and environmental isolates of *Acanthamoeba* spp. harbor endosymbiotic bacteria. Many, if not most, of these bacteria cannot be cultured outside of the ameba and appear to be obligate endosymbionts. Whether endosymbiont-bearing *Acanthamoeba* serve as reservoirs for these bacteria, some of which are potential pathogens for humans, is unknown at this time.

Acanthamoeba spp. that are found in HVAC units (discussed earlier), might serve as hosts for bacteria such as *Legionella* spp., the causal agent of legionellosis and Pontiac fever. *Legionella* have complex growth requirements yet survive in nutritionally deficient environments, perhaps because of an association with amebas or other protozoa. *Legionella* are intracellular parasites of human mononuclear phagocytes and, in the course of infection, destroy their host cells. They apparently do the same to *Acanthamoeba*, proliferating within phagosomes after uptake and causing lysis of amebas. While most of this work has been based upon in vitro studies, *Acanthamoeba* infection with *Legionella*-like bacteria has been found in amebas isolated from soil samples. It is estimated that approximately 30% of the *L. pneumophila* contained in these samples were viable but nonculturable, based on DNA amplification and hybridization. *Mycobacterium avium*, another bacterium found in water supplies, was able to survive saprophytically in vitro in association with *Acanthamoeba* trophozoites. Both *M. avium* and *Legionella* have been found to survive within the ameba cyst, as has *Afipia felis*, a potential human pathogen. Further, it has been shown that *Acanthamoeba* harboring *M. avium* and *Legionella* were more virulent than noninfected amebas to mice after intranasal instillation. Likewise, in another study, increased cytopathogenicity for tissue culture monolayers by amebas harboring endosymbionts was shown when compared with those lacking endosymbionts. Other bacterial pathogens that have been shown to proliferate in vitro within *Acanthamoeba* trophozoites are: *Listeria monocytogenes*, causal agent of meningitis and septicemia in humans; *Burkholderia pseudomallei*, agent of meloidosis in humans'; and *E. coli* serotype 0157, cause of hemolytic uremic syndrome in humans. Obligate intracellular pathogens such as *Chlamydia* and *Chlamydia*-like bacteria have been found in approximately 5% of *Acanthamoeba* isolates, and *C. pneumophila*, a respiratory pathogen, can survive and grow within *Acanthamoeba*. A feature common to all the prokaryotes that can develop within amebas is that they are obligate or facultative parasites of human phagocytic cells, suggesting that pathogenesis for humans may have evolved from infection of amebas and perhaps other protozoa, and that amebas may have an important role in the etiology of several bacterial diseases (14–17).

ACANTHAMOEBA GAE

Granulomatous amebic encephalitis (GAE) caused by *Acanthamoeba* is an insidious, chronic infection of the central nervous system caused by *Acanthamoeba* spp. The precise portal of entry is not clearly known, but the wide dissemination of these amebas in the environment allows for many possible modes of infection. Trophic amebas and/or cysts of *Acanthamoeba* have been isolated from the nasal mucosa of healthy persons, suggesting a nasopharyngeal route as one means of invasion, and amebas may also enter the body through breaks in the skin or trauma or injury to the corneal epithelium because of a foreign body or use of contact lenses. Amebas were also found in a biopsy specimen from a patient's perforated gastric ulcer, suggesting that entry was via an oral route. From skin lesions, amebas can be transported by hematogenous spread to other organs and organ systems of the host. Of particular interest is that most, if not all, persons who develop GAE are immunocompromised, including AIDS and transplant patients. However, there have been a few

cases described in which there was no underlying immune suppression (2–5,18,19).

Pathology and Pathogenesis

Definitive identification of amebas usually follows upon brain biopsy and visualization of trophic or cystic amebas in hematoxylin and eosin (H&E)-stained brain tissue sections, or upon culturing amebas from clinical other samples. In formalin-fixed, paraffin-embedded and H&E-stained tissue sections, amebas can be distinguished from host cells by their prominent densely staining central nucleolus. *Acanthamoeba* is found in either the trophic or cystic stage in tissue sections. Additionally, indirect immunofluorescence testing using anti-*Acanthamoeba* serum is also helpful in the recognition of amebas in brain tissue. Characteristic cysts can be readily recognized after cultivation on agar plates (2–7).

Mechanisms of Pathogenesis

Much of the damage done by *Acanthamoeba* trophozoites in the course of corneal or brain infections is probably the result of several different pathogenic mechanisms. *Acanthamoeba* lacks the specialized feeding cups that are found in *Naegleria* amebas (discussed later), though phagocytosis of host cells is undoubtedly involved in damage to host tissues. Several studies have described enzymes produced and secreted by *Acanthamoeba* that may facilitate spread of amebas by opening avenues for invasion and providing nutrients in the form of lysed host cells (3,20,21).

Host Response

Much of the information about the host immune response to *Acanthamoeba* is from in vitro and mouse experimental studies. The alternative complement pathway and antibody formation are both important defense mechanisms that activate neutrophils to destroy invading amebas. Once stimulated, neutrophils release lysosomal enzymes and reactive oxygen intermediates, including hypochlorite and hydrogen peroxide, thus promoting destruction of amebas. Other studies also indicate that although *Acanthamoeba* activated the complement pathway, it was shown to be resistant to complement-mediated lysis. Furthermore, it has been suggested that microglial cells produce a variety of interleukins (IL-1a and b, and tumor necrosis factor) when cultured with *Acanthamoeba* and thus may be responsible for the killing of the amebas. Different conclusions reached in these studies are probably owing to a number of factors including species and strains of *Acanthamoeba* used and the growth conditions employed (22,23).

ACANTHAMOEBA KERATITIS

Acanthamoeba keratitis is the other major infection caused by these amebas. In contrast to the systemic nature of GAE, keratitis is an acute, localized infection involving the cornea. Unlike GAE, it occurs in immunocompetent persons following corneal trauma or, more commonly, as the result of poor hygiene in the care of contact lenses or contact lens cases (24,25). Wearers of contact lenses are a high-risk group for *Acanthamoeba* keratitis, with most of the cases resulting from use of nonsterile tap water in preparation of contact lens solutions, and/or failure to keep lens storage cases clean. Amebas proliferate wherever bacteria are found as a potential food source; once established in the lens storage case, amebas can transfer to the lens surface and, ultimately, to the corneal surface, in which they are difficult to eradicate with antimicrobial treatment. In the presence of an antimicrobial agent, trophic amebas encyst and can survive the course of therapy. No case of GAE has ever been reported to progress from a case of amebic keratitis, although a case of uveitis was associated with fatal GAE. Amebas isolated from keratitis infections generally have lower temperature optima than GAE isolates, consistent with their superficial location. Among the species that have been implicated as etiologic agents are *A. castellanii*, *A. polyphaga*, *A. rhysodes*, *A. culbertsoni*, and *A. hatchetti*. Symptoms of infection include severe pain, lacrimation, photosensitivity, and the appearance of ring infiltrates. Corneal transplants have been used to repair corneal damage and eliminate infection; enucleation has been performed in cases of treatment failure. This infection may be confused with viral keratitis with resultant delay in initiating appropriate therapy to eliminate the amebas. Definitive diagnosis is based on visualization of amebas upon microscopic examination of corneal scrapings or biopsies or their cultivation from affected tissue (11). Recently, confocal microscopy has been used as an aid in the diagnosis of *Acanthamoeba* keratitis (26).

ANTIMICROBIAL THERAPY

Use of antimicrobial agents against GAE has not been particularly successful, in large part owing to the spread of infection before the causal agent can be identified. Diagnosis is, more often than not, postmortem. No single drug has been effective in clearing an infection and combination drug therapy is often employed. Among the drugs used in the treatment of GAE with varying degrees of success were pentamidine isethionate, 5-fluorocytosine, itraconazole, fluconazole, and sulfadiazine (3,27,28). Other drugs that have been used include amphotericin B, rifampin, and ketoconazole. A combination of intravenous pentamidine, oral fluconazole or itraconazole, and azithromycin along with topical applications of chlorhexidine, and ketoconazole cream, were effective in treatment of skin nodule infections (3,27,28). In some cases therapy had to be discontinued because of undesirable side effects of the medications. Drugs effective against *Acanthamoeba* generally have little effect upon *Naegleria*.

Treatment of *Acanthamoeba* keratitis has been more successful than GAE. A variety of drugs have been used in treatment, including chlorhexidine, polyhexamethylene biguanide, propamidine isethionate, dibromopropamidine isethionate, neomycin, paromomycin, polymyxin B, clotrimazole, ketoconazole, miconazole, and itraconazole (3,11,29,30). Brolene, a commercially available eye

medication (in United Kingdom) containing propamidine isethionate and dibromo propamidine isethionate was found to be effective in the treatment of *Acanthamoeba* keratitis infections but may be accompanied by drug toxicity and resistance. As is the case for GAE infections, treatment of amebic keratitis often employs a combination of drugs. Currently, the drugs of choice for *Acanthamoeba* keratitis are chlorhexidine gluconate, PHMB, and propamidine isethionate (brolene).

Other compounds have been screened for efficacy against *Acanthamoeba* spp. in vitro. These include a variety of diamidine compounds, synthetic maganin compounds combined with silver nitrate, imidazole and triazole compounds, and azithromycin, phenothiazines, and povidone-iodine (31,32).

NAEGLERIA FOWLERI

Biology

The genus *Naegleria* comprises a group of amebo-flagellate protozoa. The ameboid form, the trophozoite, is the feeding and dividing stage in the life cycle, but the ameba transforms into a nondividing, nonfeeding flagellate when conditions change, for example, when its habitat is diluted by rain water. In the laboratory, transformation from the ameba to flagellate stage can be induced by washing trophic amebas in distilled water. The flagellate stage is transitory and soon reverts to the trophozoite form. A cyst stage is also found but the cysts are not as resistant to environmental stresses as those of *Acanthamoeba* (10). Although widely distributed in soil and water, *Naegleria* are not as common as *Acanthamoeba*. Both pathogenic (*N. fowleri*) and nonpathogenic isolates (*N. gruberi*, *N. lovaniensis*, and others) can be recovered from environmental samples. Another species of *Naegleria*, *N. australiensis*, isolated from environmental samples, is pathogenic to mice, but is not known to cause amebic meningoencephalitis in humans. There are, however, nonpathogenic *Naegleria* species (*N. lovaniensis*) and other ameba species that can also tolerate elevated temperatures. Pathogenic strains are more often recovered from warm water lakes, streams, spas, and thermally polluted bodies of water. These same bodies of water have often been sites for infection of humans with *N. fowleri*. *Naegleria* amebas have not been recovered from seawater, suggesting sensitivity to elevated osmolarities (4). In nature they feed upon bacteria, and can be isolated from both environmental and clinical specimens using nonnutrient agar spread with bacteria. *Naegleria* can be isolated from many of the same sites, such as swimming pools, as *Acanthamoeba* and other free-living amebas (1–7).

Growth and Nutrition

Naegleria fowleri, like *Acanthamoeba*, can be grown in the laboratory on nonnutrient agar plate coated with bacteria, such as *E. coli* or *E. aerogenes*. *Naegleria fowleri* can be identified based on its eruptive locomotion, ability of trophic amebas to transform into flagellates when suspended in distilled water, and the characteristic vesicular nucleus that remains intact throughout mitosis. *Naegleria fowleri* can also be established in axenic culture by washing amebas from a bacterized culture by centrifugation and transferring them to an antibiotic-containing medium, omitting the antibiotic once the bacteria have been eliminated. A chemically defined medium has also been developed for *N. fowleri* (4).

Endosymbionts

Naegleria fowleri, like *Acanthamoeba*, can support the growth of *Legionella pneumophila* in the laboratory. But there have been no reports in the literature of environmental or clinical isolates of *Naegleria* amebas harboring endosymbiotic bacteria. Given its somewhat more restricted distribution and a greater sensitivity to environmental stresses than *Acanthamoeba*, *Naegleria* probably plays a minor role in dissemination of bacteria in nature (4,15).

PAM Caused by *N. fowleri*

Naegleria fowleri causes an acute and fulminating primary amebic meningoencephalitis (PAM). The onset is sudden and the disease progresses rapidly and death usually occurs within a week or less. With perhaps a few exceptions, all cases of PAM reported in the literature have been fatal (3).

Pathology and Pathogenesis

As with *Acanthamoeba* encephalitis, amebas can be seen in necrotic brain tissue and within the perivascular space. Evidence points to the nasal passages as the portal of entry, and most victims of PAM have a history of swimming in freshwater lakes, streams, or pools, or washing in tap water containing the amebas. Amebas are aspirated into the nasal passages and, after attaching to the nasal mucosa, migrate across the cribriform plate to the brain via the olfactory nerves, causing extensive damage to the frontal lobes of the brain. This has been documented at the light microscopic and ultrastructural levels by infections via the intranasal route in the mouse model. Diagnosis of PAM can be made by microscopic examination of freshly collected CSF, and seeing amebas actively moving about (3).

Mechanisms of Pathogenesis

It is not clear why *N. fowleri* amebas are so highly virulent and/or invasive, causing such extensive damage in so short a period of time. Based upon, for the most part, in vitro studies, several possible explanations have been offered. In tissue culture, *Naegleria* amebas destroy the cell monolayer by causing the Cpe, and "nibbling" away at the tissue culture cells by producing suckerlike appendages. Other studies suggest the amebas produce toxins or enzymes or a cytolytic factor that causes destruction of tissue culture cells or presence within *Naegleria* amebas of a cytopathic protein that triggers the apoptosis pathway in susceptible tissue culture (3,4).

Host Response

Given the rapid onset and progression of PAM in humans, there is little opportunity for an effective humoral response to develop against the amebas. However, in one case, a 9-year-old who survived PAM, a high titered (4,096) anti-*N. fowleri* antibody of the IGM class, was found at 7, 10, and 42 days and the antibody persisted for 4 years (3,4,33). While *N. fowleri* causes a fulminating and fatal disease in some, many more humans are presumably exposed to this ameba than the small fraction that develop Pam. What distinguishes those who contract Pam from those who do not is an intriguing question, assuming that all persons are similarly exposed. That most of the victims of PAM are young and have been swimming or engaged in other aquatic activities has suggested that it might be owing to greater activity in the water, namely diving and prolonged periods of immersion with a greater opportunity of aspirating amebas into the nasal passages. Based on serologic studies carried out at different laboratories, it is believed that large numbers of humans are exposed to *Naegleria* and the antibody titer reflected the degree of environmental exposure to amebas (3,4). Domestic and wild animals, having a closer association with soil and water than humans, might be expected to show higher antibody levels. Serologic studies have shown that cattle and swine have low titered antibodies, whereas wild animals such as raccoons, rabbits, rats, and voles had high titers (34). Reports of a South American tapir and cattle dying of PAM caused by *N. fowleri* have been published (35). A recent report suggests that a number of Holstein cattle in California died of PAM probably contracted from drinking untreated river water (36). The conclusion to be drawn from these studies is that both humans and other animals are exposed to *Naegleria* amebas and develop antibodies against them. Whether these antibodies are protective remains unclear.

The laboratory mouse is an ideal animal model to study PAM. Mice can be inoculated intranasally or intracerebrally with a suspension of amebas and most die within the week. Differences in time-to-death arise because of the size of the inoculum, culture history, and virulence of the particular strain of ameba being used, and the age of the mouse. The intracerebral route is more certain than the intranasal route to establish infection, and even low virulence strains can cause PAM when so inoculated. Adult mice are less likely to become infected than young animals. Pathogenic isolates from PAM patients tend to lose their virulence with repeated subculture. Virulence, however, can be restored or enhanced by animal passage or even by culturing amebas on tissue culture monolayers (4).

Antimicrobial Treatment

Naegleria fowleri is highly sensitive to amphotericin B, and hence it is the drug of choice for treating PAM cases. Among the few cases reported to have survived PAM, only one well-documented case, that of a 9-year-old girl, was rapidly diagnosed and treated early in the course of infection with intravenous and intrathecal amphotericin B, miconazole, and oral rifampin (33). The authors suggested, based on in vitro testing, that amphotericin B and miconazole had a synergistic action, while rifampin was without effect on the amebas.

BALAMUTHIA MANDRILLARIS

Biology

Balamuthia is the third member of the pathogenic free-living amebas and the most recent of the group to be recognized as a pathogen (2,3,7,37,38). There had been reports in the literature over the years of encephalitis caused by amebas that were neither *Acanthamoeba* nor *Naegleria* by immunofluorescence staining. Ultimately, the isolation of amebas from the brain of a mandrill baboon that died in the San Diego Wildlife Park enabled infections of these non-*Acanthamoeba-Naegleria* cases to be diagnosed (37,38). The ameba, which is larger (12 to 60 μm in length) than either of the other two genera, has a cyst stage but no flagellate stage. The cyst is a triple-layered structure lacking pores. The ameba resembles in many of its features the leptomyxid amebas included in the genus *Leptomyxa* (37). Recent evidence, however, based upon sequencing of 16S-like rRNA suggests that *B. mandrillaris* may have phylogenetic affinity with the genus *Acanthamoeba* (39). With the identification of the baboon ameba, human cases were soon discovered and additional strains of the ameba were isolated (3).

Growth and Nutrition

The amebas grow well and abundantly when inoculated on to monkey kidney or rat glial cells but do not grow on bacteria as *Acanthamoeba* and *Naegleria* amebas do. Thus, attempts to isolate this organism from environmental samples (soil and/or water) using the conventional technique of nonnutrient agar and bacteria (3,37,38) do not work. This may in part be owing to (*1*) the relatively long generation times (>20 hours) of *Balamuthia* isolates in vitro and (*2*) the many other organisms (protozoa and fungi) in the environmental samples that overgrow potential *Balamuthia* isolates. Tissue culture monolayers have served as an excellent growth substrate for the amebas isolated from brain tissue samples of victims. The amebas feed on the cell monolayer, decimating it within several days. Using trophic amebas from these tissue culture monolayers, a cell-free medium has been formulated that produces approximately 5×10^5 amebas/mL. Several isolates have been adapted to grow in this medium with doubling ranging from 21 to 28 hours. In culture, the organism is sensitive to changes in osmolarity and does not readily tolerate either hypo- or hyperosmotic conditions (40).

GAE Caused by *B. mandrillaris*

The disease *B. mandrillaris* causes is similar to GAE caused by *Acanthamoeba*, and occurs in immunocompromised hosts, including AIDS patients and intravenous drug users. But it has also been reported from immunocompetent persons. In addition to the typical GAE,

B. mandrillaris may infect the sinus cavities and skin causing abscesses and lesions. The disease is chronic, developing over a period from about 2 weeks to 2 years. Humans over a wide age range from a few months to greater than 50 years have developed infections. Almost all cases of GAE caused by *B. mandrillaris* have resulted in death. Little is known about the source of these infections and *B. mandrillaris* has not been isolated from an environmental source (3,37,38).

Pathology and Pathogenesis

There is a pronounced similarity between *Balamuthia* encephalitis and that caused by *Acanthamoeba*. The amebas are found in similar locations in the brain causing hemorrhagic necrosis in the midbrain, thalamus, brainstem, and cerebellum (3). Most infections have developed in immunosuppressed or immunocompromised persons, including those suffering from AIDS, or malnourished individuals including chronic alcoholics. *Balamuthia* GAE has occurred in the very young or older persons or in patients with HIV/AIDS. Some of the *Balamuthia* GAE cases had facial skin lesions and/or rhinitis with infections of the sinus cavities or otitis media (3). Symptoms in children and adults have included headache, nausea and vomiting, fever, myalgia, weight loss, and seizures. The duration of symptoms has ranged from several days to 2 years and the period from onset of symptoms to time of death has ranged from about 1 week to greater than 2 months. Like its *Acanthamoeba* counterpart, the disease is of a chronic nature, developing slowly and insidiously. The disease has also been reported in animals, particularly those in zoological parks. Several of the cases have occurred in primates, including the original mandrill baboon, gorillas, gibbon, and monkeys as well as horses and sheep. An animal model using SCID (severe combined immunodeficient) mice has also been established (3,13,37,38).

Host Response

Limited information is available about host response to *Balamuthia*. As the infections have been of a chronic nature, it is likely that an antibody response would have occurred in the host. Recent studies to identify serum antibody response to *B. mandrillaris* in patients with or without GAE indicate that some people do mount a humoral response to these amebas (41).

Antimicrobial Therapy

Effective antimicrobial therapy is virtually nonexistent. The organism does not respond well to the drugs that are of limited efficacy in treating PAM and GAE caused by *Acanthamoeba*. Little information is available about the efficacy of antimicrobial agents in treating *Balamuthia* GAE. Most of those diagnosed as having the disease had been treated empirically with antibacterial, antifungal, or antiviral agents but these had minimal effect upon disease progression. Recently, however, a 60-year-old patient from California has survived the infection, but with severe neurologic deficits, after treatment with a combination of sulfadiazine, clarithromycin, fluconazole, and 5-fluorocytosine (Dr. Thomas Dietz, personal communication to G.S.V.). In vitro studies have shown that pentamidine and propamidine isethionate at a concentration of 1 μg/mL inhibit growth of amebas by 82 and 80%, respectively (31). The drugs, however, were amebastatic but not amebicidal. Amphotericin B, effective against *Naegleria*, had little effect upon *Balamuthia*. Among other drugs tested were macrolide antibiotics, azole compounds, gramicidin, polymyxin B, trimethoprim, sulfamethoxazole, and a combination of trimethoprim-sulfamethoxazole (3). Given the problems with diagnosis of infection and the lack of effective antimicrobial agents, the prognosis for victims is bleak.

CONCLUSION

It is estimated that more than 100 known infectious agents can cause encephalitis. Encephalitis caused by *Acanthamoeba*, *Naegleria*, and *Balamuthia* is not a major public health problem as relatively few cases have been diagnosed. Yet because of its high mortality and because children are often victims (PAM), it is of concern. Most of the amebic encephalitis cases described in the literature have been from countries in which a well-developed medical establishment is available for identifying these cases. It can be assumed that many more cases occur on a global scale, in humans as well as animals, which go undiagnosed and unreported. Amebic keratitis, caused by *Acanthamoeba*, is a very different type of infection that targets mainly the wearers of contact lenses. Although devastating in its impact, at least effective drugs for treatment are available. Areas that are in need of further investigation are (*1*) developing diagnostic procedures for early identification of the agents of PAM and GAE, (*2*) finding safe and effective antimicrobial agents for treating these infections, (*3*) developing effective molecular and/or serologic probes for the diagnosis of the infective agents, and (*4*) finding the ecologic niche of *Balamuthia* in nature.

Acknowledgments

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FREEZE-DRYING. See DESICCATION BY EXPOSURE TO SPACE VACUUM OR EXTREMELY DRY DESERTS: EFFECT ON MICROORGANISMS

FREEZE DRYING: PRESERVATIONS OF MICROORGANISMS BY FREEZE-DRYING

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Preservation of microorganisms by freeze-drying is widely practiced (1–12), but usually with an enormous loss of viability; in fact, the death rate is so severe that it is often given in log units (13–16). The literature on survival of microorganisms in the dry state is extensive (17), therefore all of it is not reviewed in this article. Instead, findings over the past decade that have provided new insights into freeze-drying technology and are likely to dramatically improve viability of microorganisms in the dry state (some of which have been published in the last few months) are discussed.

LESSONS FROM NATURE

The major sites of damage from dehydration are membranes and labile proteins (3). We have learned a great deal about how to obviate this damage by studies on organisms that normally survive dehydration. A usual feature in the biochemistry of such organisms (a phenomenon known as anhydrobiosis) is that they accumulate large amounts of disaccharides, the most common of which are sucrose and trehalose (18–28). Survival in the dry state is often, but not always (29), correlated with the presence of one of these sugars. (Some additional interesting exceptions to this observation will be described in the following text.) Over the past 20 years we and others have provided evidence that these sugars stabilize membranes (30) and proteins (31–37) in the dry state, most probably by hydrogen bonding to polar residues in the dry macromolecular assemblages (30,32,33). This direct interaction results in maintenance of dry proteins and membranes in a physical state similar to that seen in the presence of excess water (38–40). For example, when a phospholipid bilayer is dried, the liquid crystalline to gel phase transition temperature rises sharply, often by as much as 70 °C (40). Thus, the membrane will be in gel phase at physiological temperatures in the dry state (Fig. 1). This phase transition may lead to leakage across the bilayer (41–45) and to phase separation of membrane components (46), both of which may be catastrophic events for an intact cell. Disaccharides have the ability to

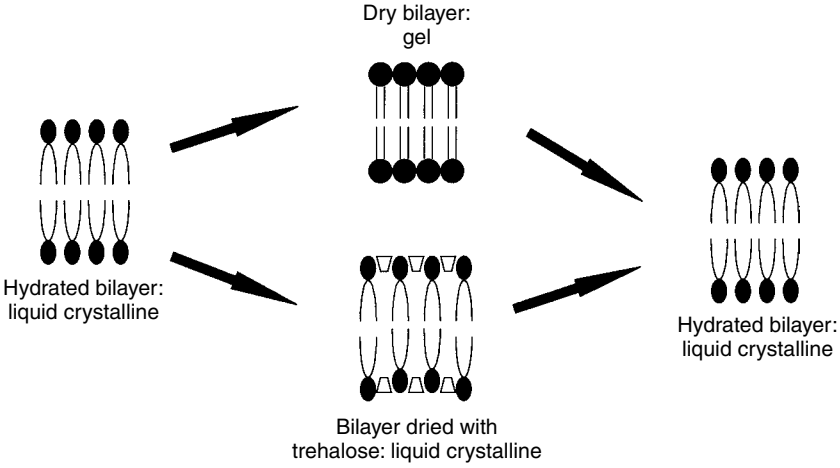

Figure 1. Diagram illustrating effects of dehydration on the gel to liquid crystalline phase transition temperature in a phospholipid dried with and without trehalose. During rehydration, the membrane dried without trehalose undergoes a phase transition, leading to temporary defects across the bilayer and to leakage of solutes (adapted from J. H. Crowe et al., in W. H. Dantzler, ed., *Handbook of Physiology*, Section 13, Comparative Physiology, vol. II, Oxford University Press, Oxford, U.K., 1997, pp. 1,445–1,477). The polar head groups are represented by the filled circles and the acyl chains by the adjacent lines. Trehalose is represented by the trapezoids between the polar headgroups in the bilayer dried with trehalose.

reduce this transition temperature (T_m) in the dry state, sometimes well below the transition temperature seen in the fully hydrated bilayer (30). We have suggested that this interaction is responsible for the stability of membranes in dry organisms (30). Because T_m is driven down below T_m in the fully hydrated bilayer, it seems clear that residual water in the sample cannot be responsible for creating this effect, but there is direct evidence as well that water cannot be involved; the dry samples contain at most 0.1 moles water/mole lipid (41). Similar observations have been made concerning the interaction between the sugars and dry proteins (32,33), with comparable arguments about the role of the stabilization of proteins in intact cells. There is some evidence in the literature that a major site of damage in freeze-dried microorganisms is the cell membrane (13,47), but evidence concerning stability of other cellular components during drying of microorganisms is lacking.

THE ROLE OF SUGAR GLASSES IN STABILIZATION

Glass Formation Is Required for Stabilization

Bruni and Leopold (51) have suggested that the glassy state may assure quiescence and stability in a living system for long periods. Indeed, it appears that maintenance of the cell in the glassy state is required for long-term stability of model systems such as liposomes (58,59) and for intact cells (55,56). The role of glasses in this regard appears to be immobilization of cellular components, thus preventing close approach of surfaces that would normally be separated by bulk water.

Properties of Glasses

A glass is a liquid of such high viscosity that it is capable of slowing chemical reactions or even, for all practical purposes, stopping them altogether (48–50). The viscous glass can, nevertheless, be readily melted by addition of water, thus restoring conditions permissive for normal metabolism. This latter point is particularly important in considerations on stability of cells in the dry state, therefore it is discussed in the following text. Excellent reviews on the properties of glasses can be found in Slade and Levine (48–50).

A glass typically is spatially homogeneous, but without any long-range lattice order (48). Glasses show temperature-dependent transitions, during which they pass from a glassy mechanical solid to a state with markedly decreased viscosity. This transition, called T_g, is a second-order transition (as opposed to a first-order transition such as a crystalline melt). It can be detected by a change in heat capacity or by direct measurement of mechanical relaxation of viscosity. Operationally, T_g is most often measured with differential scanning calorimetry (DSC), differential mechanical analysis (DMA), electron spin resonance (ESR), nuclear magnetic resonance (NMR) (48–50) or, most recently, Fourier transform infrared spectroscopy (52). The latter method is particularly useful because it permits detection of glass transitions even in single cells. With DSC, which is the most widely used method, a change in the baseline is seen at T_g, which represents the change in heat capacity.

T_g is strongly affected by the addition of plasticizers such as water. In sucrose glasses, for example, T_g falls from about 70 °C to −70 °C with the addition of water (Fig. 2). Such a diagram of the relationship between T_g and water content, known as a state diagram, has fundamental importance in the study of glasses. Construction of the state diagram for a pure solute requires some care, but it is nevertheless a straightforward procedure involving the addition of small amounts of water to the dry glass, with measurements of T_g at each increment. State diagrams for mixed solute systems are considerably more complex; a three-dimensional state diagram has recently been prepared for a ternary system, sucrose, glycine, and water (53), which yielded a three-dimensional glass transition "surface" (rather than the two- dimensional state diagram seen in a binary system). On the basis of such results, it would seem hopeless to attempt to construct state diagrams for, for example, sugar-protein mixtures, let alone sugars in intact cells. Nevertheless, it has been possible to do so possibly because the sugar contents of anhydrobiotic organisms are so high, often exceeding 20 or even 25% and sometimes as much as 50% of the dry weight (54), thus restricting the

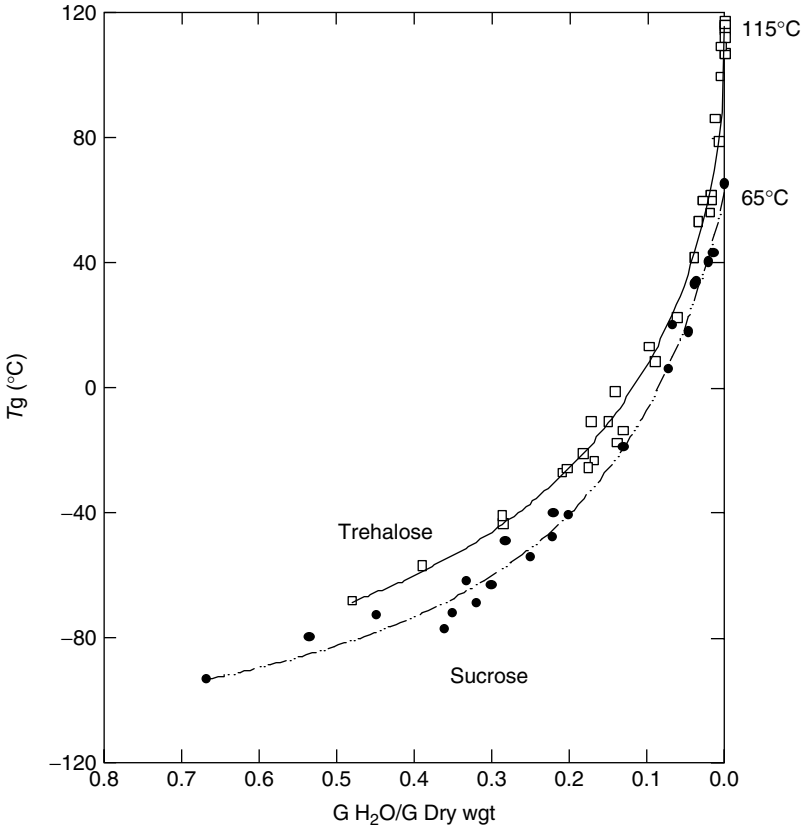

Figure 2. State diagrams for sucrose and trehalose, illustrating the large difference in glass transition temperatures (T_g) for these sugars. Data from L. M. Crowe et al., *Biophys. J.* **71**, 2,087–2,093 (1996).

participation of other compounds in the glass transition of the predominant sugar. Indeed, glasses have been detected in dry plant cells (54–56) and bacteria (57), and full state diagrams have been produced in a few cases.

To put this in practical terms, the sample temperature should not exceed the T_g line (Fig. 2) during processing for freeze-drying or during storage. It must also be noted that the damage that occurs due to devitrification has a kinetic component (60). In the case of membranes, it takes some time for the damage (fusion between adjacent membranes) to occur following devitrification. As a result, the sample may undergo short periods of devitrification without serious damage.

Are polymers useful for vitrification in cells? It would seem a priori that if maintenance of the dry cell in an anhydrobiote in the glassy state was required, an elevated T_g would be advantageous. For instance, it is clear from the state diagram for sucrose shown in Figure 2 that the addition of even modest amounts of water would result in devitrification of the sample at physiological temperatures. In seeds (61) or pollen (45), which contain large amounts of sucrose, devitrification would clearly seem to be problematic. One solution might be to add a third compound at high concentrations that would elevate T_g. Good candidates for such a role can be found among the many polymers that have elevated T_gs. T_g varies with molecular weight in a characteristic and predictable fashion; it increases nearly linearly with molecular weight of the solute, and some polymers have T_gs exceeding 200 °C (48). Thus, it would seem that such compounds ought to be found at high concentrations in anhydrobiotic organisms. Evidence is beginning to accumulate that certain polymers may play such a role in anhydrobiotes either by elevating T_g or by otherwise altering viscosity, although the mechanism is not at all clear (52,59,62,63). Interestingly, Blisett and coworkers (64) and, more recently, Bashan and Gonzalez. (65) showed good survival of microorganisms in dry polymers, but unfortunately the polymers were not characterized sufficiently to suggest a mechanism of preservation. Hill and coworkers (62) reported that *Nostoc* produces an extracellular polymeric glycan that seems to be related to its survival in the dry state. Subsequently, Hill and coworkers (63) showed that this glycan can stabilize membrane vesicles in the dry state provided a small amount of sugar is also present. About the same time, we also reported that mixtures of a large polymer, hydroxyethyl starch and glucose will stabilize membrane vesicles during drying (66). Neither component would provide stability by itself, but the two in combination worked reasonably well. It emerged that the polymer, with its high T_g, prevented fusion during drying but did not affect the lipid T_m. The glucose, by contrast, reduced T_m in the dry lipids but did not prevent fusion (Fig. 3). Thus, the two in concert satisfied both requirements. Such mechanisms may be found in the real world.

Is Vitrification Sufficient?

It is clear from the studies on glasses outlined previously that vitrification is an important aspect of preservation of cells in the dry state. But is it in itself sufficient?

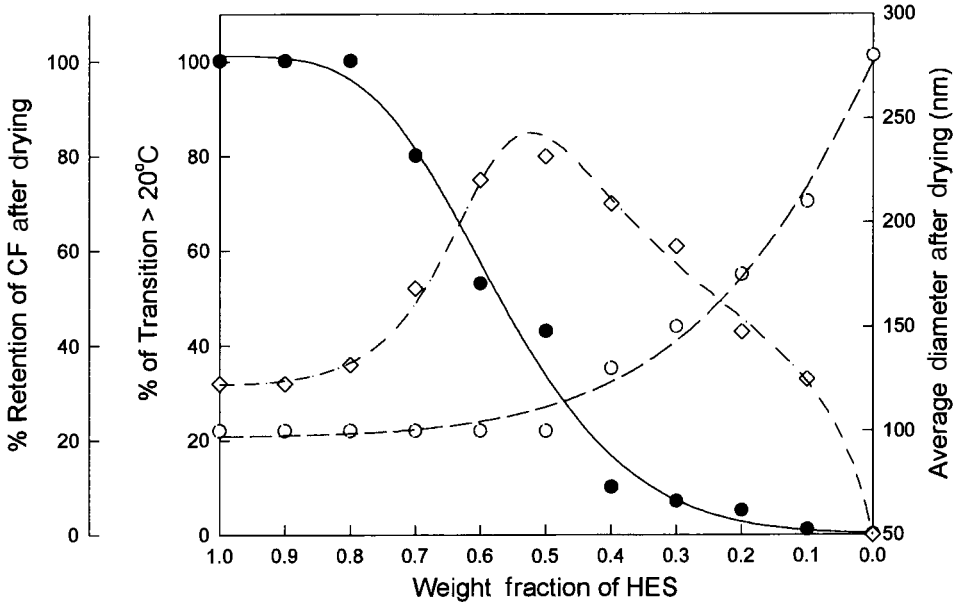

Figure 3. Effects of mixtures of hydroxyethyl starch and glucose on the stability of dry liposomes. Neither component protects the liposomes against leakage of trapped solute [carboxyfluorescein, (CF)] when used alone, but the two compounds in a mixture do so. HES inhibits fusion (because of its high T_g) but has no effect on the phase transition (T_m). Glucose has the opposite effects. Data from J. H. Crowe and et al., *Cryobiology* **35**, 20–30 (1997).

The discussion on studies with liposomes has shown that vitrification by the addition of a polymer with an elevated T_g does not preserve the dry liposomes (66). It is possible that the polymer phase separates from the liposomes (which appears to be the case with multilamellar vesicles), but that does not appear to be so with unilamellar vesicles; the polymers prevent fusion between the vesicles during drying, but have no effect on T_m in the dry lipids (66). The interpretation placed on this finding is that the polymer can prevent fusion by steric hindrance of close approach of adjacent liposomes, but does not affect T_m because the polymer is too large to interact directly with the bilayer. Thus, our own viewpoint on this matter is that although glass formation is required for long-term stability, it is not in itself sufficient (67).

Is Trehalose Special?

Trehalose has been shown by a large number of workers around the world to be remarkably (and sometimes, it is claimed, uniquely) effective in stabilizing dry or frozen biomolecules, cells, and tissues. Others have not observed any such special properties. However, trehalose has a remarkably high T_g compared with other oligosaccharides (Fig. 2). It is not anomalous in this regard because it lies at the end of a continuum of sugars with increasing T_g. Nevertheless, it is unusual in that the addition of small amounts of water does not depress T_g as in other sugars. Instead, a dihydrate crystal of trehalose forms, thus shielding the remaining glassy trehalose from effects of the added water (68). Therefore under less than ideal conditions, such as high humidity and temperature, trehalose does indeed have special properties, which may explain the stability and longevity of anhydrobiotes that contain it. Further, it makes this sugar useful in stabilization of biomolecules and intact cells of use in human welfare.

In summary, we believe that trehalose is the sugar of choice as an excipient for most investigations on freeze-drying biomolecules or intact cells. Under ideal storage conditions (low water content, low temperature, and in the presence of reduced oxygen), other sugars such as sucrose can be just as effective as trehalose. But trehalose not only has the required properties for stabilization under ideal conditions it also works well under storage conditions that are not ideal.

Other additives in combination with trehalose may be useful. Juan de Pablo and colleagues have recently discovered that the effectiveness of trehalose can be increased remarkably by adding borate ions. The addition of one borate ion per mole of trehalose raised T_g by 60 °C (69). The borate apparently accomplishes this by forming a cross-linked network between the sugars. More recently, Conrad and coworkers (70) applied this system to freeze-dry and vacuum-dry *Lactobacillus*, with excellent recovery initially. When the cells were subsequently stored at 37 °C, in air, without protection from light or oxygen, recovery remained as high as 40% even after three months under these vastly suboptimal storage conditions. Thus, de Pablo and coworkers may have discovered the best of both worlds; the trehalose-borate mixture behaves like a trehalose-polymer mixture but without the problems associated with polymers. As shown in the following section, it is possible that the trehalose gets into the cells during chilling, and this entry may be required for preservation. It is also possible that the borate may enter the cytoplasm by the same mechanism, but it is unlikely that a large polymer would do so. These promising results

may well lead to vastly improved methods for freeze drying not only for microorganisms but also other cells. As de Pablo and coworkers point out, the high T_g of this mixture is likely to be particularly useful when the samples must be stored under suboptimal conditions.

TREHALOSE HAS UNUSUAL SOLUTION PROPERTIES

Trehalose Protects Against Effects of Toxins

Several groups have reported that trehalose has unusual properties in solution (76–80). It is not at all clear just what these properties mean for stability of biological systems in the dry state, but it is interesting to note that they may be related to the observation that trehalose reduces ethanol toxicity in yeast cells undergoing fermentation (81–83). This finding may well have applications in the world of fermentation that are currently being explored. Trehalose may have similar effects for other toxic compounds (84), so this may be a generalized protective mechanism against chemical toxins.

Trehalose and Thermal Tolerance

Extensive literature has developed over the past decade suggesting that trehalose synthesis may be involved in thermal tolerance in hydrated microorganisms. Attfield (85) and Hottiger and coworkers (86) first reported that heat shocked yeast cells produce trehalose, a finding that has not been without controversy, although the vast majority of reports now seem in agreement with these original observations (20,87–89). Some workers, notably Wiemken and his colleagues, have proposed that trehalose may actually be a substitute for heat shock proteins in some cells (90–95). Recent reports showed a synergistic effect between trehalose and heat shock proteins such as HSP104 (96–98); therefore, it now seems likely that the trehalose and heat shock protein mechanisms are not mutually exclusive. In fact, the bulk of the evidence now suggests that trehalose synthesis may be a general stress response (83,95,99–104). The observation that heat shock leads to trehalose accumulation may also be useful in inducing cells to make trehalose with a view toward using that induction as a means for increasing survival during drying. In fact, some progress has already been made in that regard (86).

Relationship Between Solution Properties of Trehalose and Dehydration

It is not at all clear as to what is special about trehalose that leads to such interesting properties in solution, although some progress is being made by de Pablo and coworkers (77). However, what is clear is that the solution properties and effects of the sugar during drying do not share a common mechanism; the sugar appears to interact directly with proteins and bilayers during drying, as discussed earlier, but in solution or during freezing, the best evidence suggests that instead of a direct interaction, there is preferential exclusion of the solute from the surface of the protein or membrane (This matter is too complex to cover in detail here; see 105–106 for discussions).

TREHALOSE AND SURVIVAL FOLLOWING DEHYDRATION IN YEASTS: A CASE STUDY

Saccharomyces cerevisiae synthesizes trehalose during the stationary phase and survives drying (air-drying or freeze-drying) after trehalose synthesis is complete. In our hands, more than 40% survival can be obtained in cells dried in this fashion. By contrast, cells taken from log-phase cultures showed poor survival. Here again, survival is clearly correlated with trehalose synthesis. Studies on yeasts and the role of trehalose have yielded the following useful observations.

Membrane Lipid-Phase Transitions and Survival of Drying

It has long been known that when commercially dried yeasts are rehydrated, they must be placed in warm water, a requirement that is also shown in the data in Figure 4. This puzzling result might be explained as follows, according to Leslie and coworkers (107). Suppose the yeast cell membranes are in the gel phase at temperatures less than about 40 °C. Thus, when the cells are put in cold water they undergo a phase transition as they rehydrate, leading to leakage of solutes across the membrane. By contrast, when they are placed in warm water, the lipids would be expected to undergo the phase transition before the waterfront reached the membranes. We tested this model by measuring the leakage of cytosolic components from cells rehydrated at different temperatures and phase transitions in the dry cells. The results (Fig. 4) showed that with increasing temperature of rehydration leakage from the cells was abated in concert with the increased survival. At about the same temperature, the cell membranes were seen to undergo a gel to liquid crystalline phase transition in the dry state, suggesting that the model proposed may be correct.

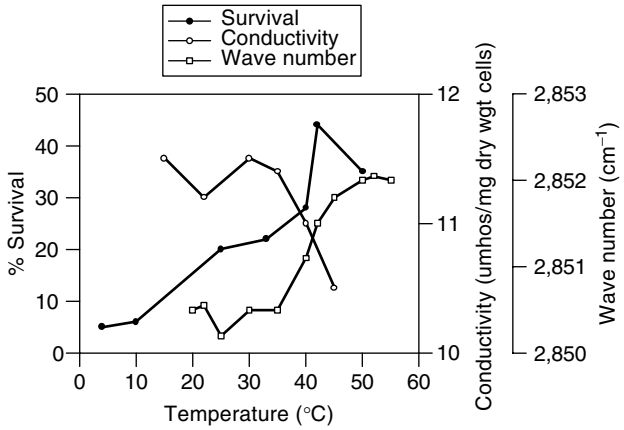

Figure 4. Survival of dry yeast cells rehydrated at the indicated temperatures. Also shown is a measure of the lipid-phase transition made with infrared spectroscopy (wave number) and leakage of solute as a function of rehydration temperature. Adapted from S. B. Leslie and coworkers, *Biochim. Biophys. Acta* **1,192**, 7–13 (1994).

Does Trehalose Depress T_m in Membranes in Dry Yeast Cells?

When membranes were isolated from yeast cells and dried with and without trehalose, the results shown in Figure 5 were obtained. In the membranes dried with the sugar, T_m was centered on about 42 °C, in good agreement with the results seen in intact cells (Fig. 4). But with the membranes dried without the sugar, T_m was seen to rise to nearly 70 °C (Fig. 5). Thus, it appears that the sugar is responsible for depressing T_m in the dry cells to a range at which it is physiologically possible to rehydrate the cells without killing them (107). If T_m were more than 50 °C (above the lethal temperature for the hydrated cells), this would clearly not be possible.

A trehalose transporter is required for yeast cells to survive drying. *S cerevisiae* has a proton-dependent trehalose transporter (108), the function of which was not at all clear until recent years. The transporter will work as a mechanism for taking up trehalose from the medium, but trehalose is rarely found in the medium surrounding these cells, so its existence was puzzling. Panek and coworkers (108,109) have provided an explanation. They produced a mutant strain in which the transporter was missing. This strain synthesizes trehalose normally, but will not survive drying. However, if trehalose is introduced outside the cells they survive. Panel and coworkers inferred from these observations that the transporter may be used to get trehalose outside the cells during the drying process. They then produced a mutant deficient in trehalose synthesis. As predicted, those cells did not survive drying unless trehalose was added outside, in which case they transported the sugar to the inside, and the cells then survived drying. These results, summarized in Figures 6–7, suggest the importance of having trehalose on both sides of the membrane, in keeping with results from liposomes showing that the sugar must be on both sides of the bilayer to achieve optimum stability (30).

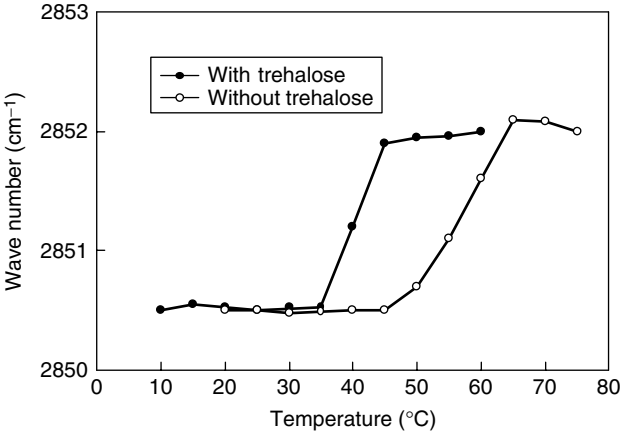

Figure 5. Lipid-phase transitions measured with infrared spectroscopy in membranes from yeasts dried with and without trehalose. The sugar reduces T_m by about 30 °C. Adapted from S. B. Leslie and coworkers, *Biochim. Biophys. Acta* **1,192**, 7–13 (1994).

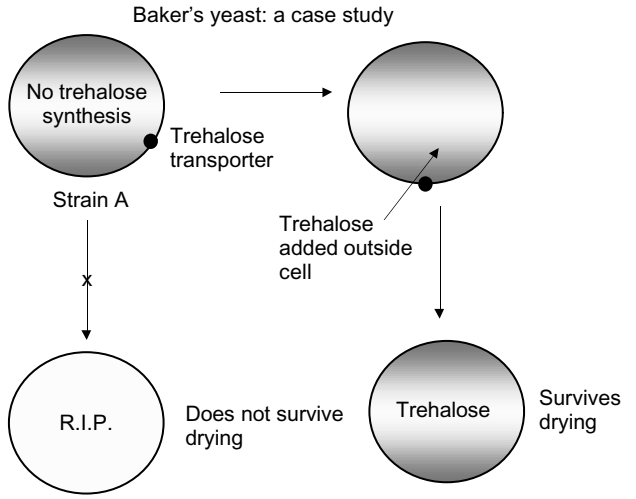

Figure 6. Diagram, illustrating a case study on the relative roles of trehalose synthesis and transport in survival of drying by yeast cells. In this strain, which lacks trehalose synthesis but has a transporter, adding trehalose outside leads to survival following dehydration. Adapted from E. C. A. Eleutherio and coworkers, *Biochim. Biophys. Acta* **1156**, 263–266 (1993).

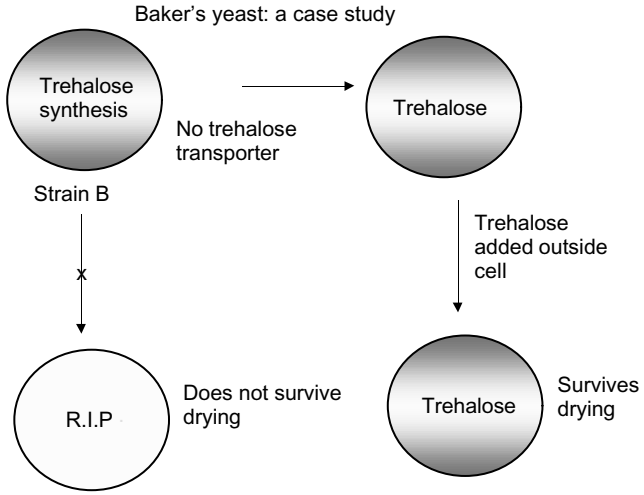

Figure 7. Diagram illustrating a case study on the relative roles of trehalose synthesis and transport in survival of drying by yeast cells. In this strain, which has trehalose synthesis but no transporter, adding trehalose outside also leads to survival following dehydration. Adapted from data of E. C. A. Eleutherio and coworkers, *Biochim. Biophys. Acta* **1,156**, 263–266 (1993).

INTRODUCING TREHALOSE INTO CELLS ARTIFICIALLY

It is clear from the earlier discussion that if a way could be found to introduce trehalose into cells that do not normally make this sugar, cellular survival following drying might be enhanced. In fact, currently it is possible to do this with a number of bacteria, with gratifying results. Several techniques are available, not all of them successful:

Chemical Permeabilization and Electroporation

Both of these widely used methods for introducing solutes across membranes may work with bacteria. We have not

tested either method with bacteria, but others and we have done so with eukaryotic cells, with generally disappointing results. The sugar can be introduced into the cytoplasm, but it appears that the membranes are so destabilized by these treatments that subsequent drying is unsuccessful.

Loading Using Lipid-Phase Transitions

Because membranes are known to become transiently leaky during lipid-phase transitions, Leslie and coworkers (110) suggested that it might be possible to use the phase transition itself to introduce sugar into the cytoplasm. The rationale here is that if one puts a high concentration of sugar outside, the main diffusion vector would be expected to be from outside to inside. Leslie and coworkers (110) reported that at least in some bacterial species, this is a practical solution to the problem. For instance, *Bacillus thuringiensis* surprisingly showed a phase transition between about 0 and 20 °C (Fig. 8). When trehalose was put in the medium around the cells, it was seen to accumulate in the cytoplasm when they were chilled below 20 °C (Fig. 8). When the cells were then freeze-dried, survival increased from less than 10% when they were dried with trehalose on the outside (but without slow cooling through the phase transition) to nearly 70% when they were chilled slowly and the trehalose was allowed to enter the cytoplasm. Lipid-phase transitions were then measured in the cells dried under optimum conditions. When the cells were dried without trehalose T_m rose by about 40 °C compared with the hydrated cells. However, in the cells dried with trehalose T_m was similar to that seen in the hydrated cells. Thus, trehalose reduces T_m in these dry cells by about 40 °C. There is some evidence that this might even work with mammalian cells; Beattie and coworkers (111) reported that mammalian islets of Langerhans were successfully preserved by freezing after trehalose was introduced into the cytoplasm by chilling the cells through a lipid-phase transition.

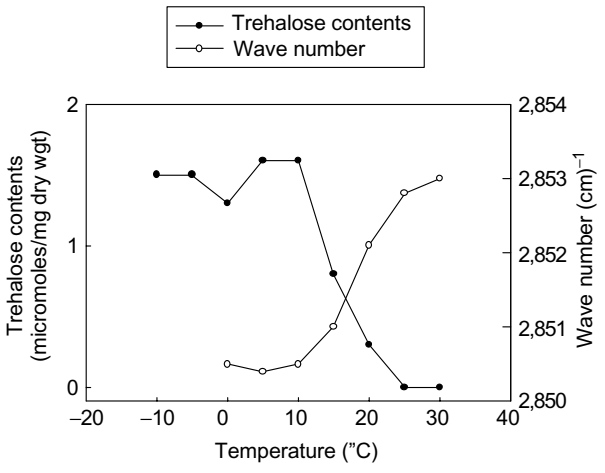

Figure 8. When *Bacillus thurengiensis* was chilled in the presence of trehalose, the sugar entered the cytoplasm between about 20 and 10 °C, coincident with a lipid-phase transition, measured with infrared spectroscopy. Data from S. B. Leslie et al., *Appl. Env. Microbiol.* **61**, 3,592–3,597 (1995).

Transfection with Genes for Biosynthesis of Sugars

The biosynthetic pathway for trehalose synthesis involves two steps (Fig. 9) and thus two enzymes. The genes for those enzymes have been cloned (102,112,113). It should be possible to transfect microorganisms with these genes with an appropriate promoter and thus induce the cells to make their own trehalose. In fact, this has been attempted with plant cells (114) with promising results, which were improved by inhibition of trehalase, which in turn led to increased accumulation of trehalose (28,115) More recently, related results have been obtained with mammalian cells (116), also with promising results. The genes were incorporated into an adenovirus vector, which was then introduced into a mammalian cell line. With multiple infections, the trehalose biosynthesis increased. The cells, which were then dried to a level at which free water was no longer detectable, retained viability for five days. It seems reasonable to suspect that the level of viability might be improved by altering the storage conditions; oxygen, for example, is known to attack dry membranes producing free radicals and free fatty acids that are extremely damaging. The first such study with bacteria was recently published by Billi and coworkers (117), who transfected *E. coli* with the gene for sucrose phosphate synthase. Presumably, the transfected cells produced sucrose phosphate, although that was not confirmed. After freeze-drying or air-drying the transfected cells showed a 10^4 increase in survival compared with controls. Furthermore, lipid-phase transition temperatures were depressed in the transfected cells, similar to the results produced by Leslie and coworkers (110).

An alternative to the pathway shown in Figure 9 that has not been explored is a novel enzyme that converts maltose to trehalose (118,119). The first suggestion of the existence of this pathway was obtained from yeasts by Panek's group (120). That work was forgotten for some years, but its independent rediscovery by two other groups (118,119), and publication of new evidence that this pathway may play a physiological role in yeasts (121) has rekindled interest in it. However, it is not clear how this pathway might be used to increase trehalose synthesis.

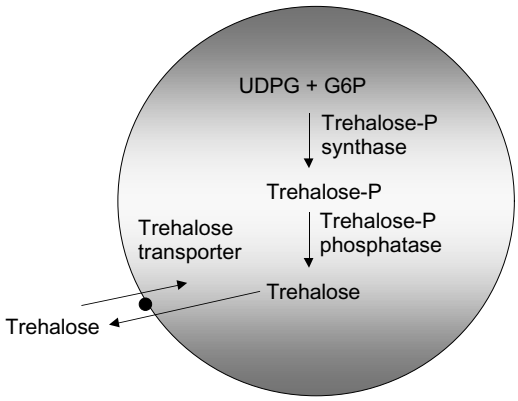

Figure 9. Cartoon, illustrating the trehalose biosynthetic pathway and the trehalose transporter. All three genes have been cloned (see text).

Transfection with the Gene for the Trehalose Transporter

The gene for the trehalose transporter from yeasts has been cloned (122), thus providing another potential pathway for the introduction of trehalose. One can imagine that transfection of cells with the biosynthetic genes and the gene for the transporter may even result in the complete mimicking of the system that occurs naturally in yeasts (Fig. 9). Several groups of investigators in the United States are attempting to do just this with mammalian cells, but so far as we know there is only limited activity in this area with microorganisms.

Use of a Pore Protein to Introduce Trehalose

Eroglu and coworkers (123) recently engineered a pore-forming hemolytic protein, α-hemolysin, so that the pore could be switched on and off. By substituting a number of residues with histidines the pore could be turned off; by adding μM quantities of Zn^{++} the pore could be closed, and by removing the Zn^{++}, the pore could be reopened. Because the pore protein spontaneously inserts into membranes, Eroglu found that they could simply incubate the cells in its presence, add trehalose in the absence of Zn^{++}, and introduce the sugar via the pore. Using this procedure, they were able to obtain very high rates of survival of two lines of mammalian cells in the frozen state. Such studies have not yet been extended to microorganisms.

THERE IS MORE THAN ONE WAY TO ACHIEVE STABILITY

We have concentrated this discussion on trehalose because it is so effective as an excipient. However, there are other routes to the same end. For example, if the rise in T_m in membrane lipids is the key damaging event during dehydration, it should be possible to prevent this damage by mechanisms other than interactions with disaccharides. This appears to be the case. Hoesktra and coworkers (43,44) showed that with certain pollen species the hydrated cells have a T_m so low that it does not rise above physiological temperature during drying. Such cells survive drying with minimal amounts of disaccharides present (43). However, to achieve this low T_m, the membrane lipids are highly unsaturated and are particularly susceptible to free radical attack (1) and to enzymatic deesterification (71–74). Such species have only a brief lifetime in the dry state. More recently, Linders and coworkers (75) showed that a species of *Lactobacillus* can be dried, although it contains only small amounts of disaccharides. When phase transitions were measured in the intact cells, T_m was seen to increase by only a small amount during drying. It emerged that the lipid composition of the membranes is probably responsible for this effect. The membranes have a high concentration of charged lipids, which presumably show electrostatic repulsion and thus a limited rise in T_m during drying. These kinds of adaptations appear to be exceptional.

CONCLUSION

Sugars can be used to freeze-dry microorganisms very effectively, provided they can be introduced into the cell. The means for accomplishing this are at hand as are described here. In fact, it may be possible by genetic engineering methods to mimic the natural system in yeasts. The most effective sugar, at least under less than optimum conditions, is trehalose. Nevertheless, even the relatively high effectiveness of this sugar can be improved by addition of polymers or borate ions, both of which elevate the glass transition and improve stability. The potential for further work in this field is enormous.

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FUEL ADDITIVES, BIODEGRADATION OF.

See MICROBIAL DEGRADATION OF FUEL OXYGENATES

FUMONISINS.

See AIRBORNE TOXIGENIC MOLDS

FUNGAL ALLERGY AND ALLERGENS

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Fungi can cause a variety of human diseases. They can act as pathogens, elicit allergic diseases, and cause other types of hypersensitivity diseases such as hypersensitivity pneumonitis, allergic bronchopulmonary mycoses, and allergic fungal sinusitis. This article focuses on the role of fungi as etiologic agents in allergic rhinitis and asthma.

Allergic diseases [e.g., allergic rhinitis ("hayfever") and allergic asthma] are classified as immediate hypersensitivity reaction, or type 1 according to the Gell and Coombs classification (1). Allergic reactions involve the production of immunoglobulin E (IgE), antibodies that are formed in response to a variety of protein or glycoprotein antigens (allergens) of which fungi are a typical example. Generation of IgE antibodies requires the central role of CD4+ T-helper lymphocytes. In the development of fungal allergy, exposure to the allergens largely occurs through inhalation of these proteins carried on spores or other particles into the nose and lung.

EXPOSURE TO FUNGAL ALLERGENS

Exposure to fungal allergens is generally considered to arise from the outdoor environment, but indoor exposure to fungal allergens can also occur. Indoor environmental exposure is a reflection of fungal spores invading the indoor environment through open windows and cracks or following amplification of fungi due to water accumulation in the indoor environment. Certain taxa such as *Penicillium* and *Aspergillus* may be recovered in higher amounts in the indoor environment compared with the outdoor environment; and, therefore, are considered to be predominantly indoor exposures (2). Homes and businesses that have been flooded or have excessive moisture problems are also sites for exposure to fungal allergens.

A number of fungi have been identified as causing allergic disease (allergenic). A listing of some of the identified allergenic fungi is found in Table 1. The best studied allergenic fungi belong to the somewhat artificial group *Fungi imperfecti* (Deuteromycetes), which are now recognized as the asexual forms of Ascomycetes. Fungi belonging to the Basidiomycotina are increasingly recognized as important fungal allergens, although they have not been as well characterized as the fungi imperfecti. Ascomycotina comprise another large group of fungi that on occasion have been shown to be allergenic. Zygomycota appear to be of lesser importance as fungal allergens.

Seasons and weather patterns influence the number of fungal spores in the outdoor air. In the northern areas of the United States, fungal spores appear in the spring as snow cover begins to recede. When the weather warms, the spores become more prevalent, particularly in the months of May and June. Peak spore counts occur in the months of late summer (July through September) with the numbers receding during October and November. In the southern areas of the United States, fungal spores are present throughout the year, although again spore counts peak during the late summer and early fall (June through October; (3). Wet and dry conditions

Table 1. Allergenic Fungi

Myxomycetes	*Coprinus*	*Cephalosporium*
Fuligo	*Dacrymyces*	*Cladosporium*
Lycogala	*Ganoderma*	*Coniosporium*
Stemonitis	*Hypholoma*	*Curvuluaria*
Oomycetes	*Inonotus*	*Dicoccum*
Plasmopara	*Merulius*	*Dreschlera*
Zygomycetes	*Naematoloma*	*Epicoccum*
Absidia	*Pleurotus*	*Epidermophyton*
Mucor	*Ustilago*	*Fusarium*
Rhizopus	*Psilocybe*	*Gliocladium*
Ascomycetes	*Stereum*	*Helminthosporium Stereum*
Chaetomium	*Gasteromycetes*	*Monilia*
Claviceps	*Calvatia*	*Nigrospora*
Daldinia	*Geastrum*	*Paecilomyces*
Erisyphe	*Lycoperdon*	*Penicillium*
Eurotium	*Pisolithus*	*Phoma*
Leptosphaeria	*Podaxis*	*Rhodotorula*
Microsphaeria	*Scleroderma*	*Spondylocladium*
Saccharomyces	*Teliomycetes*	*Sporobolomyces*
Xylaria	*Puccinia*	*Sporotrichum*
Basidiomycetes	*Tilletia*	*Stemphylium*
Hymenomycetes	*Urocyctis*	*Tilletiopsis*
Agaricus	*Ustilago*	*Torula*
Amanita	Deuteromycetes (Fungi Imperfecti)	*Trichoderma*
Armillaria	*Alternaria*	*Trichophyton*
Boletus	*Aspergillus*	*Trichothecium*
Boletinellus	*Aureobasidium*	*Wallemia*
Cantharellus	*Botrytis*	
Chlorophyllum	*Candida*	

Source: Modified from E. Levetin, Fungi. in H. A. Burge, ed., Bioaerosols, Lewis Publishers, Boca Raton, Fla., 1995, p. 108.

also affect outdoor spore recovery from the air. Spores from *Cladosporium*, *Alternaria*, *Epicoccum*, and others predominate during dry weather, especially during windy days (2). *Fusarium*, *Phoma*, Ascospores, and Basidiospores are the predominant organisms recovered during periods of wet weather (2). A seasonal relationship exists between indoor fungal recovery and outdoor fungal levels. Table 2 lists the types of fungi found in the indoor and outdoor environment during various seasons of the year in studies conducted in southern California, Finland, and England.

MECHANISM OF FUNGAL ALLERGY

The first step in the process of the development of fungal allergy consists of the production of IgE antibodies to fungal proteins or glycoproteins. This initial step is termed "*sensitization*." Fungal allergens are taken up by antigen presenting cells in the lung (4). These cells then present the allergen to T-lymphocytes. For the allergen to be recognized by the T cell, it must first be processed into small peptide fragments and presented on the surface of the antigen presenting cell in association with major histocompatibility (MHC) class 2 proteins (5; Fig. 1). Antigen presenting cells capture and internalize the protein. They migrate to draining lymph nodes where the processed peptides are presented on the surface of the cell in association with MHC class 2 molecules. Naïve T cells, through a T cell receptor that has specificity for a particular antigenic peptide, recognize the complex of the antigen and the MHC class 2 molecule. The naïve T cells become activated through a variety of costimulatory signals. The activated T cells then undergo multiple rounds of replication that are controlled by a variety of cytokines. Initially a pool of multi-potential cells (Th0) are produced. Under the influence of the local cytokine milieu these cells can differentiate into Th2 cells.

For allergic diseases, genetic predisposition is present, known as atopy. Individuals are defined as being atopic if they or close relatives have manifestations of allergic diseases such as allergic rhinitis, asthma, or eczema. The genes that control the Th2 type of response have not been discovered at present; however, clear-cut genetic influences do exist.

The production of cytokines by the Th2 cell leads to the production of specific IgE antibodies. IgE antibody has unique biological characteristics. It has the ability to bind to receptors found on mast cells and basophils. These cells contain histamine and other biochemical mediators and are found in abundance in the tissues that are the sites of allergic reactions. These cells are particularly abundant in the eyes and respiratory tract. Interaction between fungal allergens and IgE on the surface of the mast cell or basophil results in its degranulation with release of histamine and other biochemicals that can affect the tissues, which lead to the symptoms of allergy such as sneezing, coughing, and wheezing. These reactions occur rapidly within 10 to 15 minutes after exposure to the fungal allergen to which the individual is sensitive (early-phase response).

In approximately half of the allergic subjects, the so-called early-phase response is followed by a late-phase reaction that occurs three to four hours after the initial exposure to antigen. The late-phase response differs from the early response in that it involves the influx of inflammatory cells from the bloodstream, consisting largely of eosinophils and basophils. A number of cytokines produced by the lymphocytes are potent stimulatory factors for the recruitment of eosinophils. Cytokines and chemokines are important factors in recruiting eosinophils into the site of inflammation. Eosinophils can contribute to further tissue inflammation by releasing their granular proteins, cytokines, and also generate leukotrienes that have profound effects on airway function in patients with asthma.

In summary, the mechanism underlying fungal allergy involves a complex series of events. Genetic factors play an important role in governing the ability of the individual to

Table 2. Distribution of Indoor and Outdoor Allergenic Fungi

	Range-Spores/M^3						
Fungal Taxa	Indoor[a]	Indoor Summer[b]	Indoor Winter[b]	Outdoor Summer[b]	Outdoor Summer[c]	Outdoor Summer[d]	Outdoor Summer[e]
Penicillium	0–4,737	0–7,900	0–480	0–95	15,000	—	—
Cladosporium	12–4,637	0–160	0–160	11–430	600,000	1,300–42,250	200–6,000
Botrytis	0–54	—	—	—	12,000	—	—
Yeasts	0–5	0–74	0–78	0–790	10,000	—	—
Aspergillus	0–306	0–76	0–19	0–11	15,000	—	—
Alternaria	0–282	—	—	—	7,500		
Rhizopus	0–24	—	—	—	—	—	—
Nonsporulating mycelium	0–14,194	0–1,700	0–200	19–9,300	—	—	—
Epicoccum	0–155	—	—	—	—	5–130	1–150
Fusarium	0–47	—	—	—	7,500		

[a]Studies carried out in Southern California homes.
[b]Studies carried out in Finnish homes.
[c]Studies carried out in European homes.
[d]Studies carried out in Pennsylvania[63].
[e]Studies carried out in Wisconsin[64].
Modified from: Vijay HM, Thaker AJ, Banerjee B, Kurup VP. Mold allergens. In: Lockey RF, Bukantz SC, eds. Allergens and Allergen Immunotherapy, 2nd ed. Marcel Dekker, New York, 1999:113-154.

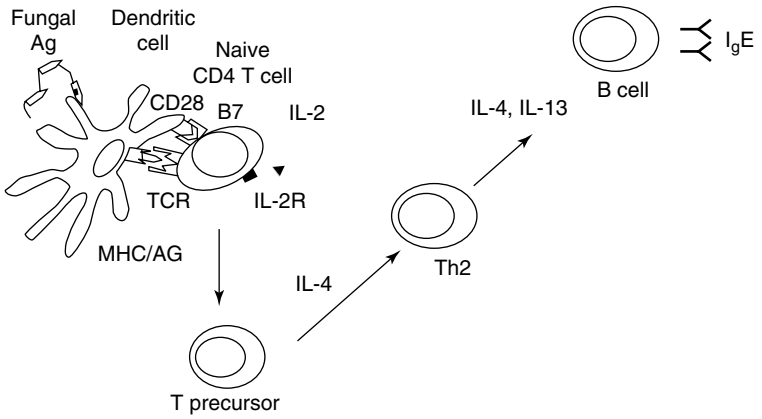

Figure 1. Antigen presentation to naïve T-cells requires recognition of antigen (Ag)/major histocompatibility (MHC) complex by T-cell receptor (TCR) and co-stimulatory signals provided through interaction of CD28 and B7. Differentiation of T-precursor cells into Th2 effector cells is influenced by the presence of interleukin (IL)-4 and IL-3. Modified from C. M. Kiekhaefer et al., In: R. K. Bush, ed., Environmental Asthma, Marcel-Dekker, New York, 2001, pp. 13–31.

develop IgE antibodies. The allergens produced by fungi, through airborne routes, lead to their uptake by antigen processing and presenting cells. These cells interact with T-lymphocytes and in the appropriate cytokine milieu lead to the generation of Th2 helper cells. The Th2 cells elaborate cytokines that are directly involved in the production of the IgE antibodies.

Circulating IgE, which is specific for fungal protein allergies, binds to the surface of mast cells and basophils. Interaction between the fungal allergen and IgE triggers the release of preformed mediators, such as histamine, and the generation of other vasoactive biochemical mediators. These mediators have direct effects on the tissue. Further, production of cytokines and chemokines result in recruitment of inflammatory cells, particularly eosinophils, into the tissue. Eosinophils, in turn, release other mediators and generate leukotrienes that result in the typical inflammation seen in the late-phase allergic reaction. Over time these events may lead to chronic problems such as asthma.

Symptoms of Fungal Allergy

The most common symptoms related to fungal allergy involve the nose, eyes, and lungs. These symptoms are known as allergic rhinitis, allergic conjunctivitis, and asthma, respectively. Nasal symptoms include nasal congestion, runny nose, and sneezing, whereas ocular symptoms include redness, itching, and watery eyes. Asthma is important because this is a potentially fatal condition. Symptoms of asthma consist of cough, wheezing, and shortness of breath. In addition to fungal allergy, individuals with asthma may experience symptoms when exposed to other allergens as well as exercise, cold air, and virus infections.

PREVALENCE OF FUNGAL SENSITIVITY

Allergy to fungi is demonstrated by skin testing with extracts prepared from fungi or by in vitro assays that use radioimmunoassays (radioallergosorbent [RAST]) or enzyme-linked immunoassays [ELISA] to detect specific IgE antibodies to fungi. Although presence of IgE antibodies does not necessarily reflect disease, it is one way of determining the frequency of sensitization and its relationship to respiratory symptoms. Unfortunately, many of the commercially available extracts for detecting fungal allergy are unstandardized materials, and, therefore, give variable results. Until such time as standardized extracts are available, estimates of the true prevalence of sensitivity to fungi will be difficult to establish. Nonetheless, studies in various parts of the world indicate that fungal sensitivity is common, particularly among asthmatics and especially in younger patients. In the general population of the United States, a large-scale epidemiologic study indicated that 3.6% of the population are sensitized to the fungus, *Alternaria alternata* (6). In reports from the United States, up to 80% of asthmatic patients demonstrate positive skin test to one or more fungi (7). In a European study in patients presenting with suspected respiratory allergy, the frequency of positive skin tests to fungi varied from country to country (8). Approximately 3% of patients in Portugal had positive skin tests to either *Alternaria* or *Cladosporium*; in Spain, 20% demonstrated positive skin tests to these allergens. In the United States, a skin reactivity study involving symptomatic patients found that 25 to 33% of these individuals reacted to one or more basidiomycete species (9). In another large-scale epidemiologic study of children with asthma residing in inner cities in the United States, the most common sensitizer was *Alternaria:* 38.3% of asthmatic children had a positive skin test to this allergen, which was comparable to their sensitivities to cockroach and house dust mites (10).

ASSOCIATION OF THE FUNGAL SENSITIVITY WITH ASTHMA

Sensitivity to house dust mites, cockroach allergens, and animal danders are known risk factors for asthma. An accumulating body of evidence suggests that sensitivity to fungi, particularly *Alternaria*, is associated with asthma. Gergen (11) found that *Alternaria* sensitivity was associated with an adjusted odds ratio (an adjusted odds ratio indicates the risk for an event, e.g., asthma is associated with a factor *Alternaria* sensitivity that is 2.3 times greater than chance alone when all other factors have been considered) for having self-reported asthma of 2.3 (95% CI, 1.5 to 3.4). Peat (12) found that children residing in the inner part of Australia who had asthma

were likely to be sensitized to *Alternaria*, which resulted in an adjusted odds ratio of 5.6 (95% CI, 3.1 to 10.1). In a study of children residing in the desert southwest United States, skin test sensitivity to *Alternaria* at age six was associated with persistent asthma and onset of new asthma but this declined by age 11 (13). This age decline in fungal sensitivity in asthmatic patients had previously been noted by Kaufmann (14).

Studies in school age children residing in North Carolina, Virginia, and New Mexico also demonstrated similar effects in that there was a link between *Alternaria* sensitivity and asthma in these populations (15,16). Although positive skin tests or the presence of IgE sensitivity to fungi by in vitro methods indicate a link between sensitization and asthma, it has been more difficult to directly correlate exposure to fungal spores and the development of asthma symptoms in sensitized individuals. Bruce (17) found that autumnal asthma symptoms were correlated with the presence of sensitivity to *Alternaria* by skin tests in approximately 50% of their subjects. In a study of children in southern California, fungal exposure was significantly associated with asthma symptoms (18). The need for inhaled bronchodilators to treat asthma symptoms and asthma symptom scores increased with increasing numbers of spores per cubic meter of air (18). These studies demonstrate the limitations in assessing exposure-response relationships. However, it does appear that fungal spores play a role in asthma symptoms.

ROLE OF FUNGAL EXPOSURE IN ACUTE ASTHMA ATTACKS

Exposure to fungal spores has been associated with emergency room visits for asthma. Nelson (19) reported that emergency room visits for asthma in children was associated with sensitivity to a variety of allergens including *A. alternata*. Epidemics of asthma have occurred at times of high spore counts in the atmosphere. These have been recognized in New Orleans at a time when there was a high basidiomycetes spore count in the air (20). Similar outbreaks have been reported in England and New Zealand during times of high fungal spore counts (21). Fungi may have also played a secondary role in the outbreaks of asthma in Barcelona, Spain, which were attributed to sensitivity to soybean hull (22).

ROLE OF FUNGAL ALLERGY IN SEVERE AND FATAL ASTHMA

Fungal sensitivity and exposure to airborne fungal spores has been associated with severe episodes of asthma. In a study of children and young adults who presented with respiratory arrest due to asthma, 10 of 11 patients were sensitive to *Alternaria* (23). Further, these patients experienced their severe episodes of asthma at the peak of the *Alternaria* season. The adjusted odds ratio was 189 ($CI = 6.5 - 5,535.8$) for a severe, potentially fatal attack of asthma that was highly correlated with *Alternaria* sensitivity (23). The risk of death from asthma has also been correlated with the presence of fungal spores in the atmosphere. In a study of death certificates from asthma in the Chicago area, the adjusted odds ratio for death on a day when the fungal spore counts exceeded 1,000 per cubic meter of air was 2.3 times as high as when the counts were less than 1,000 (24). The odds ratio increased for every increase in the fungal spore count above 1,000 per cubic meter. This observation indicates that fungal sensitivity not only carries an increased risk for the development of asthma but also plays an important role in epidemics of asthma, acute severe life threatening episodes of asthma and asthma deaths.

INDOOR FUNGAL EXPOSURE AND ALLERGIC DISEASE

Most of the studies cited above have concentrated on the relationship of fungal sensitivity to outdoor allergens and its role in allergic disease and asthma. The relationship between fungal exposure and allergic symptoms in the indoor environment is not as well characterized. Several studies have examined the relationship among indoor fungal spore counts, fungal allergen levels, and allergic symptoms. Studies conducted in Ontario, Canada, showed that allergic symptoms were higher in patients living in a damp residence (25). In Dutch children with known allergies, 63% lived in homes that had increased indoor fungal levels (26). The most commonly identified organisms recovered from homes were *Penicillium*, *Cladosporium*, *Aspergillus*, and mycelia sterilia. Peat (27) reviewed the literature for the previous 15 years regarding the relationship between dampness or fungi in the home and respiratory health. Analysis of the data from these studies (Fig. 2) indicates that there is an increased risk for children to have respiratory symptoms in a home that is damp or has visible fungal growth (odds ratio in the range of 1.5–3.5) (27). Verhoeff and Burg (28) also reviewed the association between fungi in homes and health risk. These authors came to virtually the same conclusion as Peat concerning the positive association between damp homes and respiratory morbidity of the occupants (28). In addition to these literature reviews, Beaumont (29) found that half the reported asthmatic symptoms of patients could be attributed to indoor or outdoor fungal spore peaks.

Aside from the home environment, individuals are exposed to fungal allergens in their work environment as well. Studies of Canadian office workers indicate that the office environment is also a potential source of fungal allergen exposure (30). Clearly it is important to account for exposure to fungi in the outdoor environment, home environment, and workplace.

PURIFIED FUNGAL ALLERGENS

Recent advances in molecular biology have led to a better understanding of fungal allergens and their relationship to allergic disease. Previous attempts to isolate fungal allergens by conventional protein techniques have proven cumbersome and difficult. Several major allergens have been identified from important fungal taxa (Table 3).

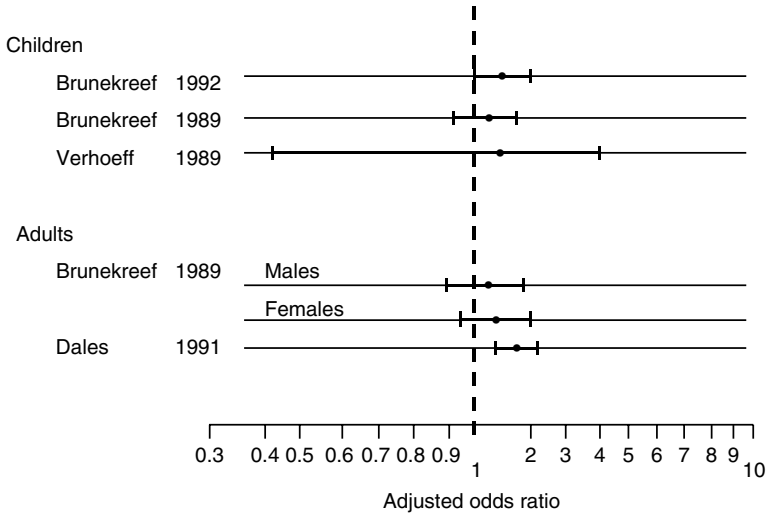

Figure 2. The association between asthma in children and adults exposed to fungal growth in the home. From: R. K. Bush, in environmental Asthma, R.K. Bush, ed, Marcel-Dekker, New York, 2001: 69–90.

Alternaria

A number of *Alternaria* allergens have been isolated. Alt a 1 is a major allergen with a molecular weight of 29 to 30 kD (21,31–33). Fragments of this protein have been molecularly cloned (34–36). The allergen is a two-chain dimer. Ninety percent of *Alternaria* allergic individuals have IgE antibodies to this protein. The biological function of Alt a 1 has not been elucidated. Alt a 2 is another major *Alternaria* allergen that has been molecularly cloned (37). It has a molecular weight of approximately 25 kD. Approximately 60% of *Alternaria* sensitive individuals have IgE antibody directed against this protein. Sequence homology has been demonstrated to a common transposable region and to mouse RNA-dependent eukaryotic initiation factor-2α-kinase. Several other minor *Alternaria* allergens have been identified as well (38). Alt a 3 is a heat shock 70 protein; Alt a 6 is a P2 ribosomal protein; Alt a 7 is homologous with YCP4 yeast protein; and Alt a 10 is an alcohol dehydrogenase. These proteins appear to be cytoplasmic housekeeping proteins that are conserved among several fungal species. GP70 has also been described, which is a 70 kD allergen from *Alternaria* (39). It accounts for 13% of the dry weight of an *Alternaria* extract and elicited positive skin tests in 87% of *Alternaria* sensitive patients tested with it.

Cladosporium

Three *Cladosporium herbarum* allergens have been purified (3). Cla h 1 and Cla h 2 are major allergens with molecular weights of 13 and 23 kD, respectively. Cla h 3 is a ribosomal P2 protein, similar to Alt a 6 (40). In addition, enolases are highly conserved major allergens found in *Cladosporium* and *Alternaria* (41). Enolases are also found to be allergens in *Saccharomyces cervisiae* and *Candida albicans*.

Aspergillus

Aspergillus fumigatus is an important indoor allergen from which several allergens have been molecularly cloned (42,43). Asp f 1 is a cytotoxin homologous with mitogillin (44). This protein is secreted into the surround media only during active growth of the organism. Asp f 2 is a major allergen in patients with allergic bronchopulmonary aspergillosis (ABPA) (45). Asp f 3 is a peroxisomal membrane protein that binds IgE from *Aspergillus* allergic asthmatic patients at a rate of approximately 72% (46). Asp f 4 binds IgE from sera with patients with ABPA (42). Asp f 5 is another secreted protein important in the allergic asthma and is a metalloprotease (42,43). Asp f 6 is a nonsecreted, manganese superoxide dismutase that is important in ABPA (42). Secreted proteins such as Asp f 1, Asp f 3, and Asp f 5 are recognized by IgE antibodies in sera of patients who have *Aspergillus* sensitivity with or without ABPA (42). Using a combination of these allergens, diagnostic sensitivity of 97% for *Aspergillus* allergy has been demonstrated (42). The nonsecreted proteins such as Asp f 4 and Asp f 6 are recognized by the IgE antibodies in patients with allergic bronchopulmonary aspergillosis (42).

Penicillium

Several allergens from *Penicillium citrinum* have been identified (47–50). A 33 to 34 kD alkaline serine proteinase has been isolated, which is cross-reactive with similar allergens from an *Aspergillus oryzase* (49). Approximately 93% of patients with sensitivity to various *Penicillium* species have IgE binding to this protein (49). A heat shock 70 protein has also been identified as an important *Penicillium* allergen (50).

Identification and purification of fungal allergen plays a critical role in improving the diagnostic capabilities for fungal allergy. Further, identification of these allergens provides the basis for development of immunoassays to quantitate allergen exposure in the environment.

ASSESSMENT OF FUNGAL ALLERGEN EXPOSURE

See Bioaerosol Sampling and Analysis, this Encyclopedia, for more information.

Table 3. Some Relevant Allergens from Fungi by Molecular Cloning

Fungal Taxa	Allergen Designation	Molecular Size (KD)	Sequence Homology/Comments
Alternaria alternata	Alt a 1	29–31	Major allergen
	Alt a 2	25	Major allergen, mouse RNA- dependent, eukaryote initiation factor 2-alpha kinase
	Alt a 3	20	hsp 70 protein
	Alt a 6	11	P_2 ribosomal protein
	Alt a 7	22	YCP4 yeast protein
	Alt a 10	53	Alcohol dehydrogenase
	Enolase	46–48	Conserved allergic protein in several fungal species
Cladosporium herbarum	Cla h 1	13	Major allergen
	Cla h 2	23	Major allergen
	Cla h 3	11.1	
	Enolase	46–48	Conserved protein in several fungi species
	hsp 70	70	hsp 70 protein
Aspergillus fumigatus	Asp f 1	18	Major allergen mitogillin
	Asp f 2	34	Important in patients with ABPA
	Asp f 3		Peroxisomal membrane protein
	Asp f 4	30	Important in patients with ABPA
	Asp f 5		Metalloprotease
	Asp f 6	26.7	Important in patients with ABPA Manganese superoxide dismutase
	Asp f 7	11.6	
	Asp f 8		
	Asp f 9	33.7	
	Asp f 10		Aspartic proteinase
	Asp 11		Peptide-prolyl isomerase
	Asp f 12	65	hsp 70 protein
Penicillium citrinum	Pen c 1	33	Alkaline serine proteinase conserved in other fungal species
	Pen c 2	70	hsp 70 protein
	Pen c 3	18	Peroxisomal membrane protein
Psilocyte cubenis	Psi c 1		
	Psi c 2	23	Cyclophilin
Coprinus comates	Cop c 1	8.9	Intermediate allergen

Traditionally, exposure to fungal allergens has been assessed by microscopically identifying and counting the number of fungal spores in the air or by semiquantitative cultures obtained from air or settled dust samples. A number of volumetric samplers are available. In general, suction devices are preferred because they are more efficient in capturing a broader range of particle sizes than impaction samplers. In addition to these methods, immunoassays to detect fungal polysaccharides (51) and markers for total fungal mass such as ergosterol, are available. There is no evidence, however, that either of these fungal products play a pathological role in fungal allergy. Lastly, the most direct way to assess fungal allergen exposure is by the measurement of actual allergen concentrations in airborne or settled dust samples. This technology is not as highly developed as it is for house dust mite, cats, or cockroach allergens, but as more fungal allergens are identified, immunoassays for their quantitation are being developed. Such assays have been developed for *A. alternata* allergens (39,52–54), *A. fumigatus* (55), and *Cladosporium herbarum* (54). Because of a variety of technical limitations of each sampling method, a combination of assays may be necessary to more accurately identify and quantitate fungal allergen exposure.

ABATEMENT MEASURES TO REDUCE FUNGAL ALLERGEN EXPOSURE

On the basis of available information, there is a correlation among fungal exposure, sensitivity to fungi, and development of allergic disease. Therefore, reductions in exposure should lead to decreased severity and incidence of illness attributed to it. This has been demonstrated for allergens such as dust mite and cat, but no controlled trials have been conducted regarding fungal exposure. The recommendations are, therefore, based on intuitive reasoning.

For outdoor fungal exposure, sensitive individuals should avoid exposure to contaminated materials such as decaying vegetation, compost piles, and farming activities.

If these activities must be performed, the use of a dust-mist mask or full respiratory protection may be necessary to reduce exposure.

Indoor fungal exposure occurs in two ways: through infiltration of spores from the outside and through the growth of fungi indoors. Successful strategies to eliminate the problem must consider both sources of contamination. Spores from the outside can be reduced in the inside environment by closing doors and windows and using air conditioning. This can reduce indoor fungal allergens at times when the outdoor levels are high [the use of window air conditioners with the vent closed has been shown to effectively exclude spore infiltration from the outdoor environment (56)]. Interestingly, indoor fungal allergen concentrations are decreased when ventilation (i.e., opening windows) is increased (57). A compromise of providing adequate filtered air and nonfiltered outdoor air ventilation is necessary to reduce spore exposure while at the same time allowing fungal metabolites to vent to the outside.

Indoor fungal growth is dependent on moisture and a carbon source. The fundamental principle for eliminating or reducing fungal growth is controlling the amount of moisture present (58). Several steps can be taken to control indoor moisture problems. (*1*) Use dehumidifiers or air conditioning to maintain indoor relative humidity no greater than 50%, (*2*) seal all leaks to prevent water accumulation, (*3*) increase the use of bathroom and kitchen ventilation by using exhaust fans, (*4*) vent clothes dryer to the outside, (*5*) reduce the number of live indoor plants that have to be watered, (*6*) heat all rooms in the winter and add heating to outside wall closets, and (*6*) use a sump pump in the basement that are prone to flooding.

Carpets, wallpaper, paneling and heating, and air conditioning systems are known to harbor fungal spores. Fungal levels are higher in the presence of old wall-to-wall carpets (57). Vacuuming can reduce fungal spore levels; however, if contamination is extensive or family members are extremely sensitive, more effective measures may be needed such as replacing carpeting with hardwood, tile, or other materials. Washable surfaces that have visible fungal growth can be treated with 5% bleach and detergent solution. However, caution should be exercised when applying this and respiratory protection should be used. Treatment can also be done with commercial antifungal agents, although not all of these products are equally effective (59).

In cases in which extensive flooding has occurred that has led to tremendous amounts of fungal growth in basements or other areas, paneling and other contaminated materials may have to be removed. If contamination is found in air ducts and filters, these may need to be cleaned or replaced to reduce fungal exposure (60).

High efficiency particulate air (HEPA) filters are the most efficient type of filter to remove spores from the air in indoor environments (61). Electronic air cleaners have a moderate effect (62). However, the effectiveness of these air filters in treating allergic disease due to indoor fungal allergy is yet to be established. Heating and air conditioning equipment in homes and the workplace should be inspected and cleaned regularly to avoid fungal contamination that can be circulated through the building.

To a person who is highly sensitive to fungal allergens, a well fitted mask that will filter particles that are less than one micrometer when engaged in activities such as cleaning that can disperse spores into the air can be of benefit. Where extensive fungal contamination exists, cleaning should be performed by individuals who are not allergic or immunocompromised.

Conclusion

Sensitivity to fungal allergens is a common clinical problem. Fungal allergen exposure is associated with the development and severity of asthma in sensitized individuals. Most of the exposure occurs from the outdoor environment, although the contribution of indoor fungal allergen exposure to allergic disease may also play a significant role. Traditional methods of exposure assessment by spore counts in quantitative cultures suggest that indoor fungal exposure indeed contributes to allergic airway disease. Methods to more directly assess fungal allergen exposure by immunoassays are still under development. Presence of fungal growth in the home or workplace suggest a problem with excessive dampness in the environment. Methods to decrease the infiltration of fungal spores from the outdoor environment, controlling indoor moisture problems, and cleaning or removing contaminated materials may be helpful in improving the health of individuals with fungal induced allergic diseases.

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FUNGAL BIOFILMS. See BIOFILMS: BACTERIAL-FUNGAL BIOFILMS

FUNGAL CONTAMINANTS

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Fungal spores are recognized allergens (1,2). Over the last 20 years, fungal amplification in the indoor environment, because of leaks and excessively high humidity, and the resulting contamination have increasingly been recognized as a major health risk to building occupants (3–7). In addition to their allergenicity, fungi are known to be capable of causing infections and capable of producing a wide variety of chemicals (3–6,8). These chemicals include irritating volatile organic compounds and highly poisonous mycotoxins. Fungi and their by-products have been suspected of causing adverse health effects ranging from irritation, hypersensitivity diseases, infections, to possibly inhalation mycotoxicosis (4–6). Some chemical by-products of fungi, such as penicillin, cephalosporin, and cyclosporin, are beneficial to humans. The growth and amplification of fungi in the indoor environment may expose building occupants to allergens, fungal glucan and volatile organic compounds (VOCs), and possibly mycotoxins over a long period of time or until the occupants and/or fungal contamination are removed from the environment. The current approach to the assessment and investigation of indoor fungal contamination includes taking notes of water damage history, visual inspection, sampling and testing for fungi in the air and from other possible sources, and, ultimately, remediation (5).

WHAT ARE FUNGI?

Fungi are ubiquitous in nature. They are eukaryotic organisms. Their cells do not contain chlorophyll and, therefore, must rely on external sources for nutrients and energy; that is, they are heterotrophic. There are approximately 70,000 species of fungi known and identified. However, it is conservatively estimated that there are more than 1.5 million species in nature (9). Fungi can form symbiosis with algae (lichens), mycorrhizal associations with plant roots, or cause diseases in plants and animals, including humans. More importantly, a majority of fungi are saprophytes, and decompose litter and organic matter to recycle nutrients. These saprophytes can be found growing in man-made environments in which they grow on many organic building materials with elevated moisture content or water activity because of the availability of excessive moisture or uncontrolled water in the indoor environment.

In the current taxonomy, true fungi may be arranged into four or five phyla (9,10). They are Chytridiomycota, Zygomycota, Ascomycota, Basidiomycota, and Deuteromycota. Many mycologists do not accept the last group as a formal taxonomic category and call them mitosporic fungi (produce spores by asexual mitotic division). Most taxa of the Deuteromycota are also called *microfungi*, or *molds*, because of their small sporocarps. Many members of the Deuteromycota have sexual states (termed teleomorphs) in either the Ascomycota or the Basidiomycota. However, a significant number of taxa in the Deuteromycota have only anamorphs (asexual state) and no known teleomorphs, and can not easily be assigned to either the Ascomycota or Basidiomycota without using molecular and ultra-structural techniques. Therefore, the anamorphic or asexual names are used in this text. Although the majority of fungi found to be associated with indoor contamination are in the Deuteromycota, a few members of the Zygomycota, Ascomycota, and Basidiomycota have also been identified growing in the indoor environment.

Fungal spores are cosmopolitan. They are in soil, in water, or in the air, and have been reported from the Arctic, the Tropics, and the Antarctic. They are known to contain allergens, and may carry mycotoxins (1,4,5,7,8). More significantly, they are propagules and will germinate and grow into colonies under suitable conditions outdoors as well as indoors. Numerous cases of fungal growth and amplification have been reported in the indoor environment (3–5). Reports of illness and adverse health symptoms have been related to mold growth and exposure to mold allergens, mycotoxins, and possibly fungal VOCs in water-damaged and moldy environments (5,11–14).

Microfungi may produce spores in dry form or in slimy masses (15). Dry spores are powdery and easily become aerosolized. Their primary dispersal mechanism is through air. Common dry-spored fungi found indoors include species of *Alternaria*, *Aspergillus*, *Cladosporium*, *Paecilomyces*, and *Penicillium*. Dry spores may be released into the air by passive means or by an active discharge mechanism. Spores of *Aspergillus*, *Cladosporium*, and *Penicillium* become airborne by air movement, whereas *Epicoccum nigrum* and *Sporobolomyces salmonicolor* are known to discharge their spores by a shooting mechanism. The slimy spores are dispersed primarily through running water, small animals or insects, and occasionally through air when they become dry and disturbed or when they are attached to other particles. Species of *Acremonium*, *Fusarium*, *Stachybotrys*, and *Trichoderma* are well-known slimy-spored fungal contaminants found indoors. Both dry- and slimy-spored fungi can be found in the same indoor environment.

FUNGI IN THE INDOOR ENVIRONMENT

Fungal growth in the indoor environment is not new. Wood microbiologists have studied wood decay by fungi for many years (16,17). *Aspergillus versicolor* and *Scopulariopsis chartarum* were reported to grow on wallpaper as far back as the 1930s (18,19). *Chaetomium* species were found damaging ammunition dumps in the Pacific during World War II. *Aspergillus* species and other molds are known to grow and damage optical instruments in the humid tropical environment. In the indoor environment, fungi have been found to grow on wood products, paper products, carpets, wallpapers (20), and insulation materials of the heating, ventilating, and air-conditioning (HVAC) system (21).

Fungi require moisture, organic nutrient, oxygen, light, and reasonable temperature to initiate and sustain growth in the indoor environment. Nutrients are usually plentiful, oxygen is normally in relative abundance, sunlight and artificial lights are required indoors for human functions, and temperature is often suitable or ideal in indoor environments. These factors usually do not pose a problem in the indoor environment. The critical factor to fungal colonization and growth indoors is moisture, particularly the moisture content of materials. Ambient relative humidity does not directly affect mold colonization and growth.

Moisture content in materials may be measured in percentage of water on oven-dry weight or in water activity. However, water content in a material does not imply the actual availability of free water to fungi. A better measurement of water availability to fungi is water activity (22), which is expressed as:

$$\mathrm{Aw} = \frac{\text{vapor pressure of water in substrate}}{\text{vapor pressure of pure water}}$$

Water activity (Aw) in a substrate can selectively allow certain fungal groups to grow. At low water activities (<0.85), xerophilic fungi, such as *Eurotium* species, some species of *Aspergillus*, and *Wallemia sebi*, are often found to dominate the fungal population. In wet substrates, hydrophilic fungi, such as species of *Acremonium*, *Stachybotrys*, *Trichoderma*, and *Ulocladium* are likely to be found. Slimy-spored species are likely to be found in a high water activity environment because of their requirement of water for spore dispersal.

Fungi can obtain their nutrients from the indoor environment. Wood and paper products, skin flakes, pollen grains, insect parts, and organic dusts are commonly available indoors (23). Wood and paper products are the

most abundant nutrients available. In fact, they are so abundant, many cellulolytic fungi, such as *Stachybotrys chartarum*, *Trichoderma species*, *Chrysosporium pannorum*, and *Chaetomium globosum*, are commonly identified from these sources in the indoor environment. Pollen is another good nutrient source for fungi. Some fungi have been found capable of attacking and using pollen for a supplementary source of nitrogen (24). Insect parts may also harbor fungi. In studies of samples collected indoors, entomophagus *Beauveria bassiana* and *Paecilomyces farinosus* are occasionally found in abundance. Insect and insect parts are very common in dust samples taken in wet and damp environments. Organic dust, including soil, compost, and mulch, is also a good source of nutrients for fungal growth.

Fungi are aerobic but may grow in reduced oxygen conditions. However, the lack of oxygen in water may actually limit fungal growth when materials are soaked deep in the water even though Aw is at 1. In the indoor environment, oxygen is highly unlikely to be a limiting factor to fungal colonization and growth.

Some fungi require light for spore production; however, light may affect some vegetative growth. Without light, some fungi, such as species of *Alternaria* and *Epicoccum*, are known to fail to sporulate. The myth that fungi grow better in total darkness was based on layman's observations. Dark places are often associated with lower temperature, higher humidity, and dampness. It is the moisture that permits fungal growth in such conditions (5). It is not uncommon to observe fungal growth on the walls of occupied spaces, although heavier fungal growth may be found in the wall cavity side of the surfaces.

Fungi may grow at temperatures below freezing to 35 °C or higher. Some of them are thermophilic (50 °C or higher) but most fungi prefer the room temperature range (20 to 30 °C). A few fungi, such as *Aspergillus fumigatus*, grow best at a temperature close to 35 °C. Several species of *Cladosporium* (*C. herbarum*, *C. cladosporioides*, and *C. sphaerospermum*) and *Penicillium corylophilum* are known to germinate and grow at relatively low temperatures (25). These fungi are commonly encountered growing in HVAC systems (21), particularly near the cooling coil where the temperature can be as low as 10 °C.

HEALTH EFFECTS

Fungi are known to cause a number of adverse health effects to humans. Four types of diseases are generally recognized: (*1*) infection in living tissues by fungi (mycosis), (*2*) allergic and hypersensitivity diseases, (*3*) mycotoxicosis owing to ingestion of foods containing toxins produced by fungi or inhalation of mycotoxin-containing spores, and (*4*) irritation caused by chemicals produced by fungi.

Fungal Infections

Of over 70,000 species of fungi, no more than 300 species have been directly implicated as causing human disease. Approximately 90% of all fungal infections are caused by fewer than a dozen fungi (26). *Aspergillus fumigatus* is the most notorious and most often encountered in fungal infections among the immune-deficient population, causing aspergillosis. This fungus can grow at a wide temperature range with the optimum near human body temperature. In addition, it produces several mycotoxins, which may facilitate invasion of the fungus. Outbreaks of aspergillosis caused by this fungus are not uncommon in hospitals and health care facilities, particularly in bone marrow or organ transplant units and in critical care units. *Aspergillus fumigatus* is not a common colonizer of the indoor environment. However, its spores are among the smallest among microfungi at approximately 2 to 3 μm (27), allowing them to easily infiltrate the air space of a building. A few other species of *Aspergillus* have also been reported to cause aspergillosis.

Dermatophytic fungi, such as species of *Trichophyton*, *Epidermophyton*, and *Microsporum*, grow on and utilize keratin in hair, skin, and nails for food. Because of their nutritional requirements, their attack rate is higher in institutions or in overcrowded conditions. Although dermatophytoses are normally not life threatening, they are the most common fungal infections in the world.

Soil-borne infectious fungi, such as *Histoplasma capsulatum, Blastomyces dermatitides*, and *Coccidioides immitis*, are usually found outdoors. However, *H. capsulatum* and *Cryptococcus neoformans* are often found in association with bird and bat droppings, or with soils enriched with bird droppings or poultry manure. Occasional outbreaks of histoplasmosis in the indoor environment have been reported because of indoor contamination by nesting birds (28). Transmission is by air. *Coccidioides immitis* causes valley fever or coccidioidomycosis. This fungus is primarily found in southwestern United States in arid, alkaline soil. Except for reports of infections in laboratory workers, there has been no report of infections in the indoor environment (26,29).

Allergic and Hypersensitivity Diseases

Fungal spores of various species have been associated with hypersensitivity diseases, such as mold allergy, rhinitis, bronchitis, asthma, and hypersensitivity pneumonitis (6). The significance of fungal spores as allergens in the indoor environment has not been fully appreciated until recently. The fungal spore is a known cause of allergic diseases (2) and has been identified as one of the major indoor allergens (1). Fungal levels comparable to outside background levels are usually well tolerated by most people. However, as atypical fungal spore levels increase because of recurrent water leaks, home dampness and high humidity, an increased frequency of respiratory problems (allergy and "sick building syndrome"-like symptoms) can be found (13,30–37). Many allergenic fungal spores are common outdoors. Because of their concentration and prevalence outdoors, these spores are the ones most likely to invade and grow in the indoor environment. Species of *Acremonium, Aspergillus, Alternaria, Chaetomium, Cladosporium, Paecilomyces, Penicillium, Phoma, Rhodotorula, Sporobolomyces, Stachybotrys, Trichoderma, Tritirachium*, and *Ulocladium*, have been

encountered and reported to grow in the indoor environment. Many of these fungi have been reported to cause allergies and hypersensitivity diseases.

The reported percentage of the human population that is allergic to fungi and molds varies from 2 to 18%. Approximately 80% of asthmatics were reported to be allergic to molds (14). The incidence and prevalence of allergic diseases are on the rise (1). In clinical allergy, patients can be specifically tested for mold allergy using skin or serological tests, and appropriate advice and treatment can then be prescribed. Patients with an atopy (a genetic trait of increased allergen sensitivity) are frequently allergic to multiple fungal species and manifest type I reactions (asthma, rhinitis, eczema, and hay fever).

All fungi may be allergenic depending on the exposure situation and dose (1). The relevant route of exposure is inhalation and the adverse effects are related to duration and intensity of fungal exposure. However, typical for allergic reactions is that once an individual develops an allergy to certain fungi, even small airborne concentrations can trigger an asthma attack or other allergic reactions. Allergy threshold levels to common mold, have been reported at 100 CFU/m^3 for *Alternaria* and at 3,000 CFU/m^3 for *Cladosporium* (2).

Hypersensitivity pneumonitis (HP), also called *extrinsic allergic alveolitis*, is a well-recognized allergenic, occupational disease, including malt worker's lung, woodworker's lung and wineworker's lung, It is characterized by symptoms of dyspnea, cough, abnormal chest X-ray, abnormal lung functions, high antibody precipitins, and possibly pulmonary fibrosis (6).

Mycotoxicosis

Many fungi produce a number of secondary toxic metabolites. These chemicals, known as *mycotoxins*, are toxic to animals and humans. Symptoms of mycotoxicosis may include skin rashes, loss of appetite, dizziness, nausea, immune-suppression, neurotoxicity, hemorrhage of the lungs and brain, birth defects, and cancer. Although mycotoxicosis can be caused by ingestion, inhalation, or skin contact of mycotoxins, the majority of our knowledge on mycotoxicosis has been learned from ingestion exposure. The significance of inhaled mycotoxins to human health is not well defined and currently is under scientific investigations. It is, however, prudent to limit exposure to such potent toxic chemicals (5), particularly when significant fungal growth and amplification is found indoors. It is conceivable that multiple forms of exposure, including ingestion, inhalation, and skin contact, are very likely when an indoor environment is infested with fungi. In addition to causing mycotoxicosis, mycotoxins may be involved in fungal infection. It was recently suggested that gliotoxin produced by *A. fumigatus* may facilitate the invasion of animal and bird lungs (38).

Organic dust toxic syndrome (ODTS), also called *toxic pneumonitis*, is a nonallergic, noninfectious form of an acute inflammatory lung reaction to fungal dust exposure (38). The differences between HP and ODTS can be difficult to distinguish. A table is available to compare differences and symptoms of HP and ODTS (6). High dosage exposures to fungal spores (possibly mycotoxins), glucans, or endotoxins are usually associated with ODTS. The significance of ODTS in occupational health is such that preventive measures have been recommended for certain occupations, such as agriculture and food processing industries, by the National Institute for Occupational Safety and Health (NIOSH). The measures include the use of industrial hygiene controls, special protective equipment, ventilation and respiratory protection (40).

Irritations

Actively growing fungi may produce VOCs with unpleasant odors. Some people consider these odors to be offensive and irritating. It has been suggested that exposure to fungal VOCs (possibly also bacterial VOCs) may be related to poor air quality (14). However, there is very little clinical data to relate exposures to fungal VOCs and symptoms of disease (5).

Strains of toxigenic *Stachybotrys chartarum* were found to induce hemolysis on blood agar incubated at 37 °C but not at 23 °C (41). Although the significance of this finding is yet to be determined, hemolysins produced by pathogenic bacteria are known virulent factors in the disease causing process. The production of hemolysin(s) by this fungus was suggested to play a role in the pathophysiology of idiopathic pulmonary hemorrhage (41).

Beneficial Fungal Chemicals

Fungi are also known to produce a number of useful chemicals and compounds. These include alcohol, organic acids, enzymes, antibiotics (such as penicillin and cephalosporin), immunosuppressive medicine (such as cyclosporin), and many anticancer agents (5,8). Some fungal chemicals also have estrogenic effects on humans (8).

ASSESSMENT AND INVESTIGATION

The most important step in the assessment and investigation of fungal contamination in a building is to visually inspect and evaluate the building structure and the HVAC system. If information regarding building water damage history is available, it may be helpful in developing an evaluation method, formulating a sampling strategy, and possibly relating water damage and fungal contamination to reported health effects. The visual inspection may include, but not be limited to, the following points. They are: (*1*) detection of moisture and leaks, (*2*) identification of sources and causes of moisture migration, (*3*) identification of structures that are conducive to moisture migration, (*4*) identification of the moisture-damaged areas and coverage, (*5*) identification of hidden moisture damage, (*6*) assessment of hygienic conditions of the HVAC system, and finally, (*7*) collection of source samples to confirm fungal growth, and to identify the types of fungi. After careful review of the building history and visual inspection of the building structures and the HVAC system, samples of various types may be collected to confirm fungal growth. This approach not only identifies the sources of fungal contamination, but also facilitates medical diagnosis and eventual remediation and control of fungal growth.

Various types of sampling may be used. Air sampling has been commonly used in the assessment of airborne fungal contaminants. However, no standard protocol is currently available. Various types of air sampling equipment are commercially available. In a medically based assessment, air sampling may help to decide whether airborne fungal spores may be the offending agents. Air sampling for culturable fungi or total spore counts is often used to determine potential human exposure (42). Air sampling for culturable fungi provides accurate identification of fungi but often underestimates airborne spore concentrations because of nonviable spores, dormant spores, or unsuitable collection media (15). Air sampling for total spore counts may provide a better estimate of airborne concentrations of fungal spores; however, identification of fungal spores is presumptive at best. A recent comparative study showed that higher fungal levels were derived from an air sampling cassette analyzed using the total spore count method when compared with samples collected with an Andersen single stage sampler and analyzed for culturable fungi. The total spore count method also had a better detection frequency of spores that have difficulty competing with fast-growing fungi on nutrient media (42).

Currently available air sampling technology and the analytical methodology are capable of sampling for a short period of time. The results are affected by the normal fluctuations of airborne fungi and usually do not reflect the temporal and spatial variations. Efforts to improve sampling efficiency and analytical capability are being pursued. Recent reports suggest that the newly developed Polymerase Chain Reaction (PCR) technology may identify species-specific DNA sequences of some important indoor fungi. The technology can be applied in the detection of specific fungi in air or in substrate, (43,44). The technology also has much better detection sensitivity than the currently used techniques.

In addition to air sampling, source sampling, by taking surface wipes, bulk material, vacuum dust samples, is used to detect and confirm the presence of fungal reservoirs and growth, and to identify the types of fungal contaminants. Because fungi do not grow in the air, source sampling and testing is necessary to locate and detect the sources of fungal growth and contamination. The samples can be processed and analyzed using conventional extraction and serial dilution techniques, or by the PCR or DNA fingerprinting technology (43,44).

To quickly confirm fungal growth, a piece of clear cellophane sticky tape can be used to remove fungal "growth" from surfaces. The tape sample is prepared and examined under a compound microscope for fungal structures. This technique provides qualitative, descriptive results. A competent mycologist may identify fungi to the genus or species level, depending on the presence of fungal structures and fungal characters. The viability of identified fungi, however, is unknown unless further tests or culturing are performed. This method has been extensively and successfully used by field personnel in building assessment and inspection for fungal contamination.

The results of various samples are used to determine and locate fungal growth and contamination, as well as the types of fungi and their levels. Properly collected data can be helpful to physicians, who make the diagnosis and relate exposure to disease. A mycologist or a microbiologist with mycology training is useful in assisting in the interpretation of results. Results of air samples require careful and systemic approach to the interpretation. There are no generally accepted numeric guidelines or standards for assessment of fungal bioaerosol exposure in the indoor environment. Comparative sampling is necessary and is the currently accepted approach (45). For practical purposes, outdoor samples and samples collected from indoor nonproblem locations are taken as a reference for comparison. Although the American Conference of Governmental Industrial Hygienists (ACGIH) had previously suggested numeric guidelines for interpretation of fungal bioaerosol data, the current 1999 publication does not support any existing numeric criteria for interpreting fungal bioaerosol data (5). In keeping with the current ACGIH recommendations (5) as well as the industry-wide approach, interpretation of possible indoor fungal exposures should focus on:

1. comparison of indoor versus outdoor concentrations,
2. comparison of species composition indoors and outdoors,
3. determination of the presence of marker species in the indoor environment, such as *Aspergillus versicolor, Penicillium aurantiogriseum, Ulocladium botrytis*, and *Stachybotrys chartarum*, which are relatively uncommon in outdoor air, and
4. determination of the origins, whether indoor or outdoors, of the identified airborne fungi based on the natural niches and habitats of the fungi.

In general, indoor fungal concentrations should be similar to or lower than outdoor concentrations. Residential buildings are usually leaky and tend to have fungal levels similar to outdoor levels. It must be kept in mind that comparison of indoor and outdoor fungal sample results requires both expert and commonsense approach. For example, in subfreezing, snow-covered weather, outdoor fungal concentrations are very likely lower than indoor levels. If significantly higher levels of total fungi as well as marker species are found indoors, this often suggests indoor fungal amplification. Furthermore, the detection of some slimy-spored fungi in the air, even at low levels, may require further evaluation. The detection of slimy-spored toxigenic fungi, such as *S. chartarum* or *Fusarium verticillioides* (syn. *F. moniliforme*), often suggests an indoor contamination source. In addition, the consistent detection of some marker fungi, such as species of *Aspergillus, Paecilomyces, Penicillium* and *Wallemia*, may indicate water damage or excessive humidity, and fungal amplification.

PREVENTION AND CONTROL

Although fungal spores have been known to be an allergenic agents for many years, their significance to human health in general and to humans in the indoor environment in particular has been mostly overlooked

and misunderstood. They produce allergens and a wide range of chemicals and compounds that can induce various health effects and responses. More importantly, in a moldy indoor environment, humans are likely to be exposed to multiple fungal types as well as a mixture of various chemicals and toxins via various exposure routes (inhalation, ingestion, and skin contact). The best treatment for the exposures is to avoid or minimize such exposures by preventing fungal growth and removing any existing fungal growth as soon as it is discovered.

Fungal growth indoors is usually the result and symptom of moisture problems. Although fungal spores are common in indoor environments, fungi normally do not grow in a dry clean building. However, they can quickly establish their presence when there is sufficient moisture. Many fungi that have been found to grow indoors are ubiquitous and fast growing. A few fungi, such as *Chaetomium globosum* and *Stachybotrys chartarum*, are found growing indoors because of their preference for cellulose-rich substrates. Any unwanted moisture may allow fungal spores to germinate and grow into colonies. It is critically important to keep water, moisture, and excessive humidity in a building under control.

In a contaminated building, moldy materials must be removed, in addition to controlling moisture and humidity and to keeping the building dry (46). Because most fungal spores are very small (mostly 2 to 10 μm), isolation and containment must be installed during remediation to avoid and minimize migration of aerosolized spores and secondary contamination. Any residual spores may last for a few years; therefore, the building environment must be kept dry to starve the spores of moisture, thus preventing their germination and growth. Biocide treatments are usually not recommended because of potential toxicity to humans and house pets, and possibly long-term residual effects. Furthermore, biocide treatments without moisture control are only a temporary solution. Fungi will grow back in a few days if moisture presence persists.

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FUNGI. See AIRBORNE TOXIGENIC MOLDS; IDENTIFICATION OF AIRBORNE FUNGI; MYCORRHIZAE: ARBUSCULAR MYCORRHIZAE; MYCORRHIZAE: ECTOMYCORRHIZAL FUNGI

FUNGI AND INDOOR AIR

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A number of fungi and microbes have been recognized to have importance in clinical medicine. These have been described to cause infections of the skin and other body organs, allergy, inflammatory or irritant effects, and toxicosis. Infections are discussed only briefly because these are commonly described in more detail in standard medical textbooks (1,2). Specifically, fungi not normally found in the interior of buildings and their chemical by-products such as (1–3)-ß-D-glucan, mycotoxins, and microbial volatile organic compounds (MVOCs) have also been implicated in conditions commonly known as "sick building problems" or building related diseases (3–5). Health problems from biological aerosolized pollutants can also be related to animal dander, dust mites, bacterial endotoxins (lipopolysaccharides or LPS) (6), viruses, *Legionella*, and protozoa, which will be discussed in other articles in greater detail.

Fungal spores and other microbial products can be deposited in the dust on various indoor surfaces (organic dust) and may be become airborne in the indoor environment and result in air contamination (bioaerosols). Such bioaerosols have been defined as microscopic organisms and biologically significant materials that are transported through the atmosphere and deposited in areas that may have "consequences for life" (7). Organic dust comprises materials of vegetable, animal, and microbial origin such as bacteria and fungi (8). Microbial materials and components in dust are made of a variety of substances including fungal spores and hyphae, actinomycetes spores, endotoxins (lipopolysaccharides from gram-negative bacterial cell walls), mycotoxins, (1–3)-ß-D-glucan, and MVOCs. Organic dust has been recognized as containing causative agents for airway disease (e.g., sinusitis, asthma), allergy and toxic reactions (7). The term *sick building syndrome* (SBS) refers to poorly defined physical conditions or nonspecific symptoms (primarily sensory complaints) that are related in time and location to a "problem building," but no definite or specific cause (exposure) can be identified in an industrial hygiene evaluation of the work area (9–12). SBS is typically a multifactoral problem that includes building factors, ventilation system problems, volatile organic compounds (from carpets, glues, office supplies, or microbes), work-organization, and job stress and dissatisfaction, among others. Common complaints and symptoms in SBS cases can vary in their clinical presentation, prevalence, and their relationship to the time the building investigation is carried out. The term *building-related disease or illness* signifies a category of clinical diagnoses that are related in time and place of occurrence to an identifiable, known hazardous exposure in a building. Such illnesses have distinct clinical findings and laboratory or imaging test abnormalities. The pathogenesis of specific building-related illnesses has been related to infectious, immunological, or toxic exposures (13–17).

Fungi (or molds) have been related to building complaints for both SBS and building related illness. There are many nutrient sources in buildings that fungi can utilize for food, such as wood, paper, paint, mineral oil, and various modern building materials. If moisture from leaks or defective ventilation intrudes into such nutrient-rich environments, fungi and bacteria can grow to levels not encountered in outdoor environments. Bacteria and fungi contribute to degradation of materials, and can lead to health problems if they become airborne and are inhaled or touched by occupants. Many fungal spores are in the respirable size of 2–20 μm and may be trapped in the human airways, including the lungs. Maximal airborne dispersal will frequently occur under dry building conditions, if fungi and bacteria and their products are not removed after moisture intrusion has been ameliorated. Fungi detected on damp and wet building materials (so-called "atypical fungi") detected in past building investigations and identified utilizing different sampling methods are listed in Table 1.

INFECTIONS

Infections caused by fungi are called mycoses, specifically, endemic mycosis and opportunistic mycosis. Opportunistic fungal pathogens have medical importance, especially in persons with immunodeficiencies, organ transplants,

Table 1. Fungi Found on Damp and Wet Building Materials (So-called "Atypical Fungi")

Alternaria alternata
Aspergillus niger
Aspergillus versicolor
Aspergillus ochraceous
Aspergillus sydowii
Aspergillus versicolor
Aureobasidium pullulans
Chaetomium globosum
Cladosporium herbarum
Eurotium herbariorum
Eurotium repens
Memnoniella echinata
Paecilomyces variotii
Penicillium aurantiogriseum
Penicillium brevicompactum
Penicillium chrysogenum
Penicillium citrinum
Penicillium commune
Penicillium corylophilum
Penicillium expansum
Penicillium spinolosum
Penicillium viridicatum
Stachybotrys chartarum (atra)
Trichoderma viride
Wallemia sebi

(*Source:* 18,19).

and patients with immunosuppressant pharmacological treatments (20). Endemic mycoses are related to the geographic distribution of certain fungal pathogens. This type of infection is caused by the inhalation of airborne spores or conidia found in regions that have a high occurrence of such fungi because of unique soils, climate, and flora or fauna. Fungi and the endemic infections that may occur through air transmission are listed in Table 2.

Opportunistic infections are secondary illnesses that occur in patients with an altered or weakened immune system. Patients at risk for fungal infections usually have major systemic diseases or health suppressed conditions such as complicated diabetes mellitus, cancer, HIV/AIDS, severe liver or kidney diseases, organ transplantation, or burn injury. These patients may be on immune-suppressive medication. These infections are not contagious and the fungi are not considered obligatory pathogens. Secondary fungal infections and medical complications related to airborne fungal contamination in hospitals and transplant units have been reported. Immune-compromised patients may be at an increased risk of contracting opportunistic infections if pathogenic fungi become airborne and a route of entry exists. Among the fungi of concern are *Aspergillus* spp., such as *A. fumigatus*, *A. flavus*, and *A. niger*. Soil, bird and bat droppings, water-damaged materials, or rich organic substrates in buildings, may be a reservoir for these fungi. Other clinically important fungal infections are candidiasis, with local mucocutaneous or disseminated systemic organ manifestations, and skin mycoses. Among fungal skin infections (dermatophytoses) are tinea corporis, capitis, curae, and barbae (named depending on the body region infected) that are caused by fungal genera such as *Microsporum*, *Epidermophyton*, and *Trichophyton* that manifest as dermatitis. Invasive fungal diseases of the paranasal sinuses may also be associated with allergic sinusitis in patients (22). Often *Aspergillus* species are involved. Noninvasive forms may be colonizing preexisting body cavities and may be asymptomatic as long as some immunological resistance can be maintained.

An increased rate of respiratory, ear, or gastrointestinal infections can be found in people who are exposed to unusually high concentrations of fungi in the indoor environment. This is possibly the result of an altered or weakened immune system caused by cytotoxic fungi. Epidemiological studies have shown that among children, especially those in daycare centers or in schools, the rate of upper respiratory infections, bronchitis, and ear infections increases with excessive mold or dampness in buildings. Among adults in such environmental situations, an increased risk of bronchitis and sinusitis has been documented.

Correct diagnosis, therapy, and prevention of opportunistic infections may be difficult for those who are not well trained and experienced in this field. Early recognition, preventive building engineering, proper hygiene, and public health intervention can reduce the incidence

Table 2. Diseases Transmitted by Airborne Fungi and Affected Tissues

Fungi	Disease	Affected Organ/Tissues
Histoplasma capsulatum	Histoplasmosis	Lung, eye, (skin & bone.)
Cryptococcus neoformans	Cryptococcosis	Lung, central nervous system, meninges, skin and viscera.
Coccidioides immitis	Coccidioidomycosis	Lung, multi organ dissemination (skin, bone, meninges, joints).
Blastomyces dermatitidis	Blastomycosis	Lung, skin & mucous membrane, bone, joints.
Aspergillus spp.	Aspergillosis	Lung, bronchial airways and sinus cavities, ear canal, eye (cornea).
Sporotrix schenkiie	Sporotrichosis	Granulomatous pneumonitis (rare), skin, joints, central nervous system, eyes
Mucorales, Zygomycetes	Mucormycosis	Nose, sinuses, eye, lung (brain & other organs), gastrointestinal system.

(*Source:* 21)

of mycoses, especially those acquired in institutional care facilities.

ALLERGY

Many fungi are known to cause an immune pathology with an exaggerated or inappropriate immune response called hypersensitivity reaction or common allergy. Fungal spores are a known cause of allergic disease and have been identified as one of the major indoor allergens (23–26). Significant methodological problems in the production of reliable allergen extract from fungi exist compared to, for example, a cat, dust mite, or cockroach allergens. Extracts that are available correspond poorly to the fungi found frequently in indoor surveys (27). Population studies have shown that prolonged indoor exposure to some fungi can result in hypersensitivity reactions and chronic allergic diseases. Most people usually tolerate mold concentrations comparable to outside levels. Normal or typical indoor molds may vary depending on climate variations and geographic regions. However, as mold levels that are atypical in the indoor environment increase because of recurrent water leaks, home dampness, and high humidity, the prevalence of allergy and respiratory problems also rises (28–30). Many epidemiological studies have shown associations between damp and moldy buildings and cough and asthma among the occupants (31). Damp building materials, particularly those containing cellulose are prone to fungal contamination. Among the most common fungal contaminants of damp and moldy buildings are *Penicillium*, *Aspergillus*, *Chaetomium*, *Ulocladium*, *Stachybotrys*, and *Cladosporium* (32).

The reported percentages of populations allergic to molds vary from 2% to 18%. The incidence and prevalence of allergic diseases is on the rise. Approximately 80% of asthmatics were reported to be allergic to molds (33). A recent study of patients diagnosed with allergic fungal sinusitis found a very high incidence of mixed fungal contamination in nasal mucus (up to 96%) together with eosinophilia, but no type I hypersensitivity (34).

In clinical allergy, patients can be tested for specific mold allergy using skin or serological tests (IgE-RAST) and specific advice and treatment can then be given. Because of the low sensitivity of some of the commercially available mold extracts, false negative test results are common. Patients with atopy (a genetic trait of increased allergen sensitivity) are frequently allergic to multiple fungal species and manifest type I reactions (e.g., asthma, rhinitis, eczema, and hay fever).

All fungi may be allergenic depending on the exposure. However, the sensitivity of clinical laboratory tests to confirm allergy or prior exposure may vary with the individual patient and his or her immune system. Atopic individuals typically have a higher rate of positive skin reactions after provocation tests and serological allergy tests that measure antibody precipitins (IgE). Some individuals with known allergy may not develop any measurable biological marker. Diseases such as allergic bronchopulmonary aspergillosis (ABPA) (35) and allergic fungal sinusitis (AFS) possibly require additional host factors that are not well documented and may be the result of a combined reaction of allergenic inflammation and the immunotoxic effect of fungal metabolites. The pertinent route of fungal exposure is inhalation. Fungal mycotoxins have been found to inhibit the motion of respiratory cilia, slowing or preventing clearance of particles including viruses and bacteria from the respiratory tract (36), which may be important in the pathology of rhino-sinusitis. Allergically sensitized individuals have a lower tolerance to airborne concentrations of fungal allergens. Such sensitivity differs categorically from an inflammatory response to fungal toxins, which has a dose-response relationship that depends on concentrations of toxins breathed in, and does not require prior sensitization. Allergy threshold levels to common molds have been reported, but variations in sampling strategies and methodological limitations make these very unreliable in practical settings (37,38). Experts agree that currently acceptable safe threshold limits for fungal indoor exposure cannot be established and it generally is recommended that unnecessary fungal exposures are avoided or minimized (39,40).

HYPERSENSITIVITY PNEUMONITIS (HP) AND ORGANIC DUST TOXIC SYNDROME (ODTS)

The clinical findings and laboratory results of allergic and inflammatory toxic reactions to airborne microbial exposure are often difficult to separate in clinical practice (41–43). Hypersensitivity pneumonitis, called extrinsic allergic alveolitis outside the United States, is a well-recognized occupational lung disease. In serious cases, it may lead to lung fibrosis (scarring of the lungs). Organic dust toxic syndrome, also called toxic pneumonitis, is a nonallergic, noninfectious form of an acute inflammatory lung reaction to high fungal or other organic dust exposure (44). The differences between HP and ODTS can be difficult to distinguish. Table 3 lists comparative features of HP and ODTS.

In the early or subclinical stages of the disease, laboratory findings, pulmonary function tests, or radiological abnormalities may not be present. In these cases, the patient's history is vital for early diagnosis. The significance of ODTS in occupational health is such that the National Institute for Occupational Safety and Health (NIOSH) has recommended preventive measures for ODTS. The measures include the use of industrial hygiene controls, special protective equipment, ventilation of the workspace, and respiratory protection (45).

Workers in several occupations are at risk of acquiring these diseases although the number of affected people may not be very high. Farming and the agricultural industry, lumber and wood industry, and waste management are included in the occupations at risk. Large-scale composting of organic waste is a new, growing technology in municipal waste management (46). Environmental monitoring suggests possible high exposure risks to several pathogenic fungi, bacteria, and viruses. Immunological

Table 3. Comparative Features of Hypersensitivity Pneumonitis and Organic Dust Toxic Syndrome

	Hypersensitivity Pneumonitis (Extrinsic Allergic Alveolitis)	Organic Dust Toxic Syndrome (Toxic Pneumonitis)
Immune responses	Type IV delayed hypersensitivity, cell mediated immune reaction	Nonallergenic, noninfectious, lack of IgG.
Affected tissue/organ	Lung alveoli, forming granulomas	Inflammatory lung reaction
Exposure levels	106–1,010 CFU/m^3 of thermophilic actinomycetes or fungi	High concentrations of fungi, >109 spores/m^3 of (1–3)-ß-D-glucan or >1–2 $\frac{1}{4}$ g/m^3 of endotoxins.
Clinical features	Dyspnea, cough, fatigue, poor appetite, weight loss, abnormal chest X-ray, abnormal pulmonary functions, high antibody precipitins; may cause pulmonary fibrosis long term.	Dyspnea, cough, headaches, fever, chills, malaise, acute inflammatory lung reaction, negative chest X-ray; may recover after exposure cessation.

blood changes (such as IgG antibody elevation) can be can observed in waste handling workers or other occupations with high fungal exposure (47).

INFLAMMATION AND IRRITATION OF THE AIRWAYS

Prolonged exposure to fungi and their products can result in inflammation of the skin and mucous membranes, and of the airways (nonallergic asthma, bronchitis) (3,48,49). Skin contact with fungal materials can cause skin irritation and lesions that are often difficult to distinguish from other chemical-contact dermatitis, unless history of exposure can be established (50). Symptoms of burning eyes, nasal irritation, sore throat, hoarseness, and irritant cough are common after repeated exposure to airborne fungi. Typically, these complaints subside once the exposure is stopped. After inflammation of the airways, patients may be more intolerant to other airborne agents or pollutants, such as tobacco smoke, perfume, diesel exhaust, and strong chemical odors. A possible pathway of the mechanism of the effects of microbial organisms and its products are shown in Figure 1. It illustrates the important systems and mediators of the lung defense.

MYCOTOXINS

Several fungi found in our environment, in household dust, and agricultural products, produce toxins termed *mycotoxins*. Most of the information on mycotoxins comes from studying ingestion-related poisoning or from animal studies (50,52–56). The control of mycotoxins in food and feed is an important public health task and has great economic importance. Food-borne diseases affect animals or cause regional outbreaks of human disease (57). Historically, mycotoxins have been known to be a problem to farmers in Eastern European and developing countries, who have problems related to wet weather and storage (58). *Claviceps purpurea*, one of the first mycotoxin producers identified, produces the substance ergot, which causes ergotism. Ergot toxins caused food poisoning outbreaks associated with dry gangrene, loss of limbs, and bizarre behaviors that may have significantly contributed to the population decline in Europe from the fourteenth to the eighteenth centuries (59). Mycotoxin poisoning may have constituted part of the plagues of Egypt in which a series of catastrophes led to scarcity of food, wet storage of grain, and fungal growth of what is believed to be *Stachybotrys*. Subsequently, this led to the death of infants (first born) and elders from ingestion of mycotoxin-contaminated food (60).

Mycotoxins have been called an agent in search of a disease (61) but may be instrumental in a number of diseases. More than 400 fungal metabolites are known to be toxic to animals or humans. Mycotoxins can act directly or as products resulting from metabolism in the body. Break-down products of mycotoxins are more frequently measured in toxicodynamic studies as the parent mycotoxins have short half-lives ranging in hours or less. Important mycotoxigenic fungal species, their mycotoxins, and associated diseases are listed in Table 4.

Mycotoxins are typically small molecules (200 to 500 mass units) that are produced by a taxonomically wide range of fungi and consist of a very diverse group of compounds including polyketides, terpenes, cyclic polypeptides, phenolic acids, and diphenyl quinones, among others. (62,63). Important toxins are aflatoxin, 12-13-epoxytrichothecenes, fumonisins, ochratoxins, and gliotoxins, among others. Examples of fungi and their secondary metabolites are listed in Table 5 (64).

The International Agency for Research on Cancer (IARC) (38) classifies aflatoxin, a toxin discovered in 1961 and produced by *Aspergillus flavus*, *A. niger*, and *A. parasiticus*, as having "sufficient evidence" for human and animal liver carcinogenicity. It may also be involved in occupational respiratory cancers among food and grain workers (65). Aflatoxin-induced disease has been well reviewed (66,67). Carcinogenicity for trichothecene toxins (T-2 toxin, *Fusarium* toxins) was listed with "limited evidence" for animals and "inadequate evidence" (no data

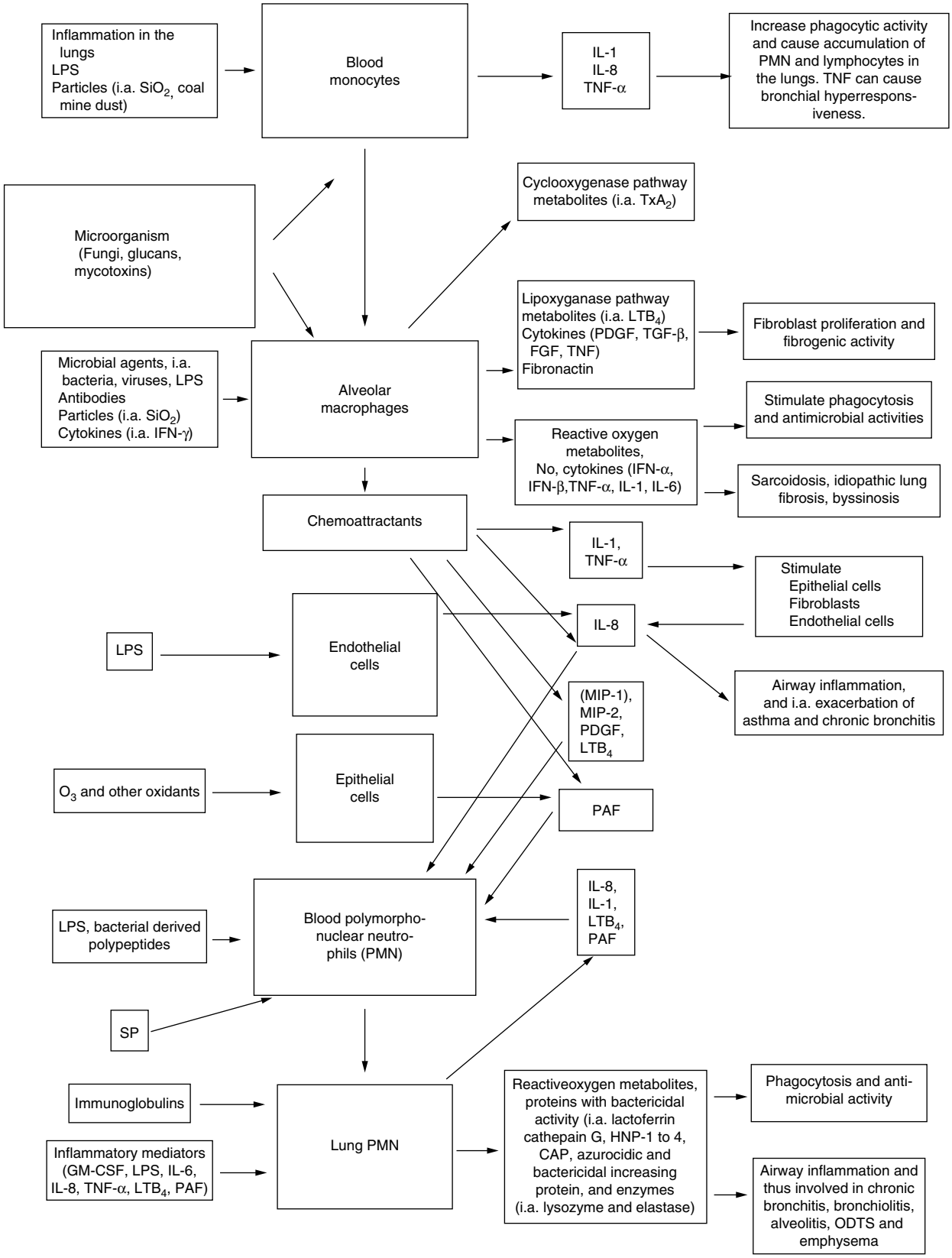

Figure 1. Possible respiratory tract effects from exposure to microbial organisms and products source: (50) G. D. Nielsen, Y. Alarie, O. M. Poulsen, B. A. Nexo. Possible mechanism for the respiratory tract effects of noncarcinogenic indoor-climate pollutants and based for their risk assessment. G. D. Nielsen, Y. Alarie, O. M. Poulsen, and B. A. Nexø, *Scan. J. Work Environ. Health* **21**, 165–178 (1995).

Table 4. Some Toxigenic Fungi, Fungal Chemical Metabolites, and Their Health Effects

Fungi	Chemical Metabolites	Health Effects
Penicillium (>150 species)	Patulin	Hemorrhage of lung, brain disease.
	Citrinin	Renal damage, vasodilatation, bronchial constriction, increased muscular tone. Nephrotoxic, hepatotoxic.
	Ochratoxin A	Neurotoxic.
	Citroviridin	Reduced cellular oxygen uptake.
	Emodin	Lung disease.
	Gliotoxin	Neurotoxic: trembling in animal.
	Verruculogen	Lung, teratogenic in rodents.
	Secalonic acid D	
Aspergillus spp.	Patulin	Hemorrhage of lung, brain disease.
A. flavus &	Aflatoxin B1	Liver cancer, respiratory cancer,
A. parasiticus	Sterigmatocyst	cytochrome-P-450-monooxygenase disorder.
A. versicolor	in	Carcinogen.
A. ochraceus	Ochratoxin A	Nephrotoxic, hepatotoxic

Table 5. Examples of Fungi and Their Secondary Metabolites

Arenaria alternata: tenuazonic acid, alternatiol, alternariol monomethylether, altertoxins
Alternaria, tenuissima: altertoxins
Aspergillus ochraceus: penicillic acid, ochratoxin A, xanthomegnin, viomellein, vioxanthin
Aspergillus flavus: kojic acid, 3-nitropropionic acid, cyclopiazonic acid, aflatoxin B1, aspergillic acid
Aspergillus fumigatus: gliotoxin, verrucologen, fumitremorgin A&B, flumitoxins, tryptoquivalins
Aspergillus niger: naphtho-pyrones, malformins
Aspergillus (Emericella) nidulans: sterigmatocystin, nidulotoxin
Aspergillus terreus: terrein, patulin, citrinin, citreoviridin
Aspergillus versicolor: sterigmatocystin, nidulotoxin
Aspergillus ustus: austamide, austdiol, austins, austocystins
Eurotium amstelodami: physcion, echinulin
Eurotium chevalieri: physcion, echinulin
Eurotium berbariorum: physcion, echinulin
Chaetomium globosum: chaetoglobosins, chetomiin
Cladosporium berbarum: epi- and fagi-cladosporic acid
Fusarium culmorum: culmorin, fusarin C, trichothecenes type B, zearalenone
Fusarium verticillioides: fumonisins, fusaric acid, fusarin C, moniliforme, naphthoquinone pigments
Fusarium solani: fusaric acid, naphthoquinone pigments
Paecilomyces variotii: patulin, viriditoxin
Penicillium aurantiogriseum: penicillic acid, verrucosidin, nephrotoxic glycopeptides
Penicillium brevicompactum: botryodiploidin, (mycophenolic acid)
Penicillium cbrysogenum: roquefortine C. meleagrin
Penicillium citrinum: citrinin
Penicillium commune: cyclopiazonic acid, rugulovasine A & B, fumigaclavine A & B
Penicillium crustosum: roquefortine C, penitrem A, terrestric acid
Penicillium expansum: roquefortine C, patulin, citrinin, communesins, chaetoglobosin C
Penicillium glabrum: citromycetin
Penicillium roqueforti: roquefortine C, isofumigaclavine A & B, PR-toxin, mycophenolic acid
Penicillium rugulosum: rugulosin
Penicillium variabile: rugulosin
Penicillium viridicatum: xanthomegnin, viomellein, vioxanthin, viridic acid, penicillic acid
Trichoderma barzianum: chrysophanol, koninginin A, trichorzianines A+B
Trichoderma viride: alamethicins, emodin, suzukacillin, trichodermin, trichotoxin A
Stachybotrys chartarum (a.k.a. atra): satratoxins, verrucarin J, roridin E and others
Wallemia sebi: walleminol A&B.

available) for humans in 1993. Macrocyclic trichothecenes, such as satratoxin H, have not been classified as carcinogenic. Epidemiological studies suggest a higher rate of upper respiratory tract and lung cancer in workers in the grain and food handling industry with high fungal product inhalation risk (65). The high rate of lung cancer among uranium miners in Silesia (so-called "Schneeberg disease") may have been related to combined effects of high radon and *Aspergillus* exposure in the underground mines (68).

Water-damaged building materials are often contaminated with fungi that produce detectable levels of mycotoxins (69–72) that may become airborne and contribute to indoor air pollution. Air sampling in buildings with fungal growth utilizing high-volume collection methods and assays sensitive to *Stachybotrys chartarum* toxins (Roridin A) showed that a very high percentage of the samples were cytotoxic if *Stachybotrys chartarum* was detected on the paper air-filters (73). Trichothecene and other mycotoxins were isolated by chemical methods (HPLC system) from extracts of samples with *Stachybotrys chartarum* and other fungal growth from buildings with water-damaged gypsum boards or other building materials (74,75). Important toxigenic fungi in damp buildings appear to be: *Penicillium* species, *Aspergillus* species, *Chaetomium*, *Fusarium* species, *S. chartarum* (syn. *S. atra*), *Paecilomyces* species, and *Trichoderma viride* (76). These fungi have been associated with adverse health effects in humans and animals, resulting in diseases that are primarily neither allergic nor infectious in nature. Inhalation of mycotoxins contained in houses appear to have stronger effects than ingestion of mycotoxins (77,78). Several mycotoxins are observed to be cytotoxic even in very low concentration and can inhibit DNA, RNA, and protein synthesis, and cause apoptosis of cells of different body organs. These toxic effects may cause a variety of short-term as well as long-term adverse health effects in animals and humans (79–82). Symptoms thought to be due to mycotoxins or toxin-containing spores (particularly those of *S. chartarum*) include dermatitis, recurring cold and flu-like symptoms, burning and sore throat, headaches and excessive fatigue, diarrhea, and impaired or altered immune function. The ability of the body to resist infectious diseases may be weakened, resulting in opportunistic infections. Certain mycotoxins, such as zearalenone, have been found to cause infertility and stillbirths in pigs and are regulated in food in Canada to prevent developmental effects in humans. Low level complex exposures from a mixture of mycotoxins encountered in typical conditions in wet and moldy buildings, may have synergistic effects. These may result in neuro-endocrine-immune changes that result in complex changes or even damage of the endocrine and nervous system (Fig. 2; 83).

The high-dose exposure to fungi and mycotoxins found in farmers and in food industries was generally considered unlikely to occur in nonfarming activities. However, many toxigenic fungi, such as *S. chartarum* and species of *Aspergillus*, *Penicillium*, and *Fusarium* have been found in high concentrations in buildings with documented indoor air problems and illnesses (84–86). An epidemiological study of adults in damp and moldy buildings suggests that inhalation exposure to mycotoxin-containing fungal spores is significant in the reported cases of building-related mycotoxicoses (87). Building and health investigations associated with toxigenic *Stachybotrys* were reported by Croft and coworkers (88), Auger (89,90). Johanning and coworkers (91) and Hodgson (92). Hemorrhagic lung disease in infants was highly associated with indoor *S. chartarum* exposure in a case cluster investigation in Cleveland (93) and in a case-home investigation in the mid-west of the U.S.A. (94). Subsequently, some consultants for the Center for Disease Control and Prevention (CDC) called for more research to prove the causal relationship of *Stachybotrys* and idiopathic pulmonary hemorrhage in infants on review of the Cleveland study. Exposure of mice to toxigenic spores of *S. chartarum*–induced bleeding from the respiratory tract (95) supporting the association found between toxic spores of this fungus and bleeding lungs in infants made in the Cleveland study. More recently, *S. atra* was isolated from bronchoalveolar lavage fluid of a child with pulmonary hemorrhage (96) and *S. atra* exposure was found in an infant that developed laryngeal spasm and hemorrhage during general anesthesia (97).

One important toxigenic fungus frequently detected in "problem buildings" is *S. chartarum*, which produces a series of potent cytotoxins such as trichothecenes (satratoxins) and spirolactones, as well as a variety of other compounds affecting the immune system (98,99). Table 6 lists chemical metabolites derived from *Stachybotrys* and reported health effects that may be related to the chemical properties.

A case study of health effects and changes in immunological laboratory values related to indoor exposure to trichothecenes and possibly other mycotoxins noted disorders of the respiratory and central nervous system, (100–102).

Trichothecenes are potent small molecule inhibitors of protein synthesis that act through inhibition of peptidyl transferase activity (103,104). These toxins can cause alveolar macrophage defects and affect phagocytosis. They have been investigated for use in cancer treatment (105) but also for use in chemical-biological warfare. The presence of fungal chemical metabolites has been reported in several cases of animal and human ingestion–related mycotoxicosis, occasionally resulting in death of animals and humans (106,107). Mycotoxins, such as Satratoxin H of the macrocyclic trichothecene group, have been shown to suppress T- or B-lymphocyte activity, suppress immunoglobulin and antibody production, reduce complement or interferon activity, and impair macrophage-effector cell function of human neutrophils (108).

Changes in laboratory measured levels of immunoglobulins (IgA, IgE, IgG, and IgM) have been reported in workers handling mycotoxin contaminated foodstuff (primarily deoxynivalenol or vomitoxin) (109). Renal failure, with deposition of IgG in the glomeruli, in a farmer who inhaled mycotoxins, primarily deoxynivalenol (vomitoxin) from *Aspergillus ochraceus*, has also been reported (110).

The treating physician may not recognize signs of mycotoxicosis because symptoms often overlap several known syndromes and exposure circumstances, and presence of certain mycotoxins are often not noted in the patient's history. Establishing exposure history and the presence of toxigenic fungi will be of importance to the differential diagnosis. Newer analytical methods involving immunoassays and cell line cytotoxicity analysis may be able to provide rapid information about the

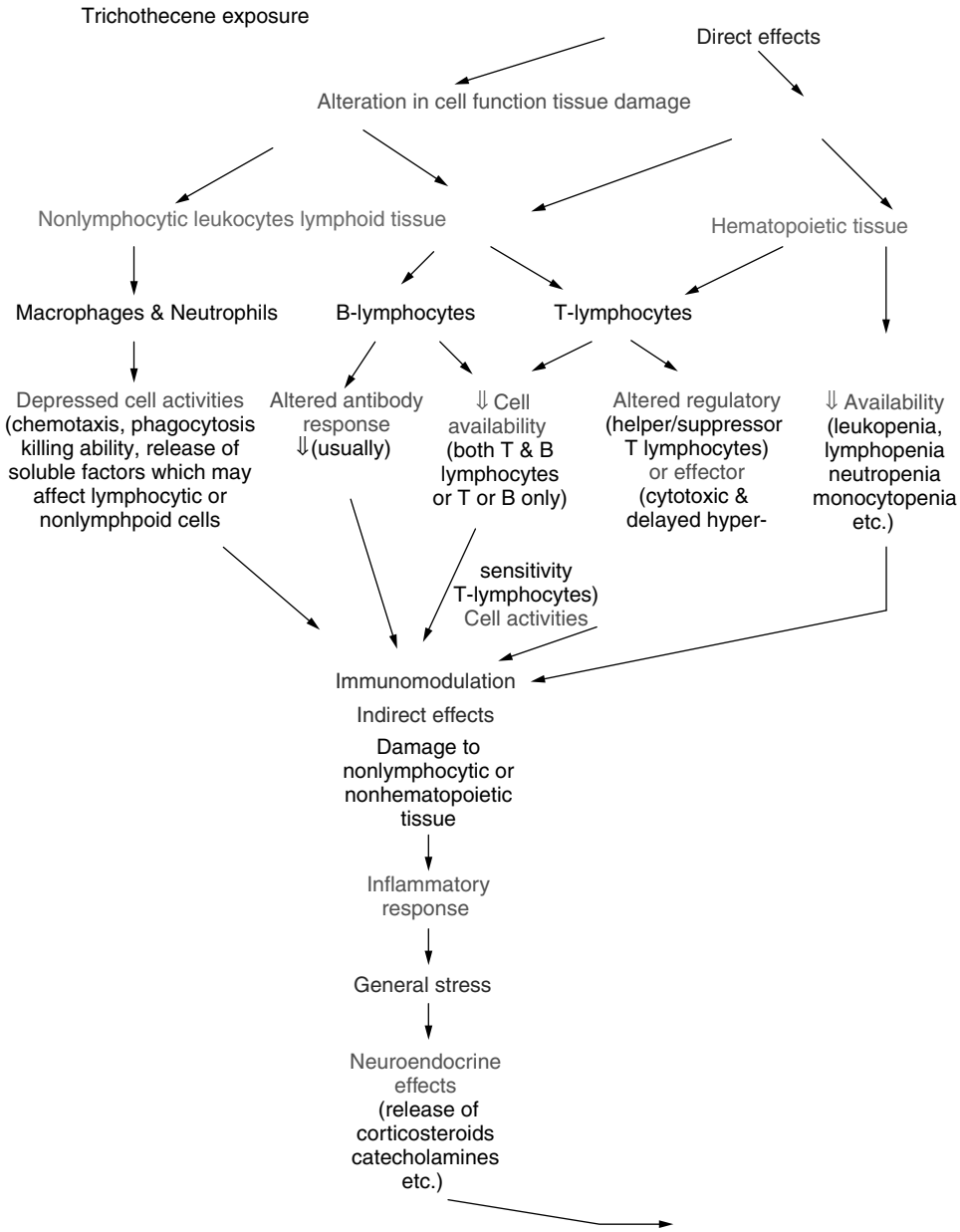

Figure 2. Immune system effects of Stachybotrys mycotoxins (trichothecenes) [*Source:* H. M. Ammann, in E. Johanning, ed., *Bioaerosols, Fungi and Mycotoxins: Health Effects Assessment, Prevention and Control*, Eastern New York Occupational and Environmental Health Center, Albany, 1999, pp. 520–521. See color insert.

presence of mycotoxins in fungus-contaminated environments (111).

MICROBIAL VOLATILE ORGANIC COMPOUNDS (MVOCS)

Fungi in active growth produce microbial volatile organic compounds (MVOC's) that are typically noticed as a mildew odor. Indoor measured MVOC levels, however, are typically low, and possible health risks are uncertain. Related mucous membrane and trigeminal nerve irritation may trigger unpleasant reactions and annoyance. Olfactorial reactions and memory formation may result in psychophysical reactions by subjects with a history of prior exposure.

Measurement of MVOC may be an indicator of excessive fungal growth indoors (112). A number of VOCs have been identified from fungi common in indoor contamination. Most of these fungal VOCs are derivatives of alcohols, ketones, hydrocarbons, and aromatic compounds. Most studies of MVOCs have been done on fungi that contribute to off-flavors and odors in food. Fungal species such as *A. niger* and *A. flavus*, isolated from indoor environments, produce the alcohols 1-octen-3-ol and 2-octen-1-ol that together account for the characteristic odor of these species (113). In a recent report, Larsen and Frisvard (114)

Table 6. Chemical Metabolites Derived from Stachybotrys and Reported Health Effects

Fungi	Chemical Metabolites	Health Effects
Stachybotrys chartarum & *Fusarium* species	Trichothecenes* (more than 50 derivatives known): T-2, nivalenol, deoxynivalenol, diacetoxyscirpenol, satratoxin H, G, other macrocyclic trichothecenes	Immune suppression and dysfunction, cytotoxic, bleeding, dermal necrosis; high dose ingestion = lethal (human case reports); low dose, chronic = potentially lethal); teratogenic, abortogenic (in animals). Hemorrhage.
		"Alimentary toxic aleukia" (ATA) reported in Russia and Siberia.
		"Staggering wheat" in Siberia.
	Spirolactone	"Red mold disease" in Japan.
	Zearalenone	Neurotoxic/nervous behavior abnormality.
		Cocarcinogen/chemotoxic.
		Anticomplement function.
		Phytoestrogen may alter immune function; stimulates growth of uterus and vulva, atrophy of ovary.
Claviceps spp.	Ergot alkaloids	Prolactin inhibitor, vascular constriction, uterus contraction promoter.

studied the in vitro production of fungal volatiles from 47 *Penicillium* taxa and detected alcohols, ketones, esters, small alkenes, monoterpenes, sesquiterpenes, and aromatics. Little is known about VOCs of other common indoor fungal contaminants.

CONCLUSION

Fungi in indoor environments may play a more important role than previously thought. Fungal contamination has been recognized as a major problem in buildings in which moisture control is poor or significant water intrusion has occurred. Synergistic inhalation effects of fungi and its by-products, such as mycotoxins in fungal spores, beta-glucans, or perhaps fungal MVOCs released into the surroundings are potentially irritating, toxic, teratogenic, carcinogenic, and immune-suppressive. Immunotoxicological effects probably depend on the exposure conditions, dose, and timing. Some immunological effects may only be transient, of short duration, and difficult to detect in routine medical tests. Medical findings often appear nonspecific and overlap several syndromes, and therefore other systemic diseases or causes need to be ruled out by the experienced clinician. Furthermore, risk assessment of human exposure to these fungi and their by-products is complex because multiple agents, hypersensitivity reactions, and different disease outcomes are involved. Several methods exist to assess and characterize exposure conditions (115). Exposure to fungi and their by-products at varying levels is ubiquitous in some indoor environments. Sampling techniques applied in building investigation should be selected on the basis of an established hypothesis, namely, on the goal and purpose of the testing. Human sensitivity and susceptibility to fungi, and microbial contaminants in general, varies from individual to individual. Some of the health implications from inhalation exposure of many fungal metabolites are not well understood, particularly at low concentrations. Little is known concerning the consequences of short- or long-term human inhalation exposures to toxic fungal metabolites. Although it appears that for most people adverse health effects are limited, reversible and not severe, there are notable exceptions that pose clinical challenges. However, based on the information available currently, it is prudent to counsel avoidance of any unnecessary exposure to infectious, allergenic potential toxic fungi. Control of moisture problems is key to preventing further growth and the potential for exposure. Because allergic and toxic properties of fungi remain even if the organisms are no longer viable, removal of molds and microbial dust from buildings is also necessary. Minimizing and avoiding indoor exposure to pathogenic fungi will generally result in resolution of any symptoms and related diseases in otherwise healthy people. A good understanding of fungal biology in the indoor environment and its impact on human and animal health will help in better diagnosis, appropriate treatment, and will contribute to the improvement of public health.

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FUNGI AND POLLUTANT BIODEGRADATION.

See FUNGI, FOR BIOTECHNOLOGY

FUNGI, CULTURE MEDIA FOR.

See IDENTIFICATION OF AIRBORNE FUNGI

FUNGI, FOR BIOTECHNOLOGY

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Biotechnology, the application of living organisms and/or their components to industrial processes and products, is expanding rapidly because of advances in genetic engineering. These advances allow for the possibility of "tailoring" organisms or their components to specific needs such as optimizing performance in the production of important metabolites, increasing stability to broaden or improve their application to a desired process, or the ability to translocate genetic material from one organism to another. Biotechnology has had, and will continue to have, a large effect on a wide variety of industries by providing the potential to lower the energy requirements and associated costs of an industrial process, and providing the means to move the process toward a base of renewable raw materials. Some of the most important organisms used in biotechnology are fungi (Table 1). The use of fungi is not new. Historically, fungi have been used as a food source or in food preparation. It is only in the past 100 to 150 years that humanity has harnessed the power of fungi and applied them to the areas of industry, medicine, agriculture, and the environment.

Evidence of the prehistorical use of fungi is limited to a few archeological relics and extrapolation from cultural practices that have survived to recent times. An example of the use of mushroom from evidence was found in the frozen, partially mummified remains of a 5,000-year-old stone age man discovered in the Tyrolean Alps. Among his possessions were fragments of three types of shelf fungi. One species was probably used as tinder, a practice that was common throughout Europe before the advent of matches. The purpose of the other two fungi is not known, but speculation suggests they may have served medicinal purposes. Another example of an ancient use of mushrooms is evident in Norse history. The Vikings ate the mushroom *Amanita muscaria* in preparation for battle. They believed that the mushroom gave them special ability to fight. In actuality, it was the psychogenic effect of the toxins in the mushroom that reduced their perception of fear and lent them their ferocity in battle. This mushroom was also used as a pest control agent in the Northern Hemisphere during the Middle Ages.

THE ROLE OF FUNGI IN BIOTECHNOLOGY

Mushroom production has a long history of importance as a food in many countries. The Romans and Greeks considered a variety of mushrooms as delicacies, which

Table 1. Selected Examples of Fungi and Their Biotechnological Uses

Organism	Use in Biotechnology
	Production of Antibiotics
Cephalosporium acremonium	Cephalosporin C
Emericellopsis sp.	Penicillin N
Fusidium coccineum	Fusidic acid
Paecilomyces varioti	Variotin
Penicillium chrysogenum	Penicillins G and V
Penicillium patulum, Khuskia oryzae	Griseofulvin
Tolypocladium inflatum	Cyclosporin
	Production of Medicinal Compounds
Claviceps purpurea	Ergotamine
Rhizopus nigracans, Phytophthora infestans	Steroid transformations
Aspergillus terreus	Lovastatin
Ashbya gossypii	Riboflavin
Blakeslea trispora, Phycomyces blakesleanus	β-Carotene
	Production of Organic Acids
Aspergillus niger	Citric acid, Gluconic acid
Aspergillus terreus	Itaconic acid
Penicillium stoloniferum	Mycophenolic acid
Rhizopus oryzae	Fumaric acid, Lactic acid
	Production of Industrial Solvents
Candida acidothermophilum	Ethanol
Pichia farinosa, Trigonopsis sp.	Glycerol
	Production of Enzymes
Aspergillus sp.	Lipase, Pentosanase, Proteases,
Aspergillus niger	β-Glucanase, Cellulase, Glucose oxidase, Lactase, Glucoamylase, Pectinase, Catalase
Aspergillus oryzae	α-Amylase
Fusarium sp.	Penicillin acylase
Penicillium sp.	Dextranase, Catalase
Trichoderma reesei	Cellulase, Hemicellulase
	Use in Food and Beverages
Saccharomyces cerevisiae	Various beers and wines, Kefir, breads
Saccharomyces bayanus	Champagne
Saccharomyces sake	Sake
Aspergillus oryzae	Sake, Miso, Shoyu
Candida holmii	Sourdough bread
Penicillium camembertii, P. roquefortii	Blue cheese
Monascus albidus	Tofu (fermented bean curd)
Candida sp., *Fusarium* sp.	Edible protein substitutes
Agaricus sp., *Morchella* sp., *Pleurotus* sp.	Edible mushroom cultivation
	Use in Agricultural Compounds and Applications
Giberella fujikuroi	Giberellin
Various species of Hyphomycetes and Zygomycetes	Bioinsecticides
Various species of Hymenomycetes and Gasteromycetes	Forestry
Puccina sp., *Phragmidium* sp., *Entyloma* sp.	Bioherbicides
Trichoderma sp.	Biofungicides

were served only to the most privileged people. The European community has a rich history of hunting the prized truffle. Eastern cultures have cultivated mushrooms for more than two thousand years. Today mushroom farming has grown to be a very large economic concern around the world. The types of fungi grown for consumption within particular regions have expanded from a single species (i.e., *Agaricus bisporus* in the United States) to include more exotic types that are popular in other countries, such as the oyster mushroom (*Pleurotus ostreatus*), which has typically been grown in the Orient. Other traditional food uses have exploited the fermentation properties of fungi to make beer, wine, and spirits, to leaven bread, to add flavors to cheeses, and to produce foods such as shoyu (soy sauce), miso, and tofu (Table 1). Modern uses of fungi in the food industry have been in the production of flavors, coloring agents, and as a protein supplement used to mimic meats.

Traditional industrial uses of fungi began early in the twentieth century with the production of citric acid from *A. niger*. Today the industrial use of fungi has increased to a variety of applications that include other organic acids, industrial solvents, and industrially important enzymes such as cellulase, amylase, and lactase (Table 1). Novel industrial uses of fungi also exist, which include coal solubilization, delignification, and bleaching of paper pulp, and removal of metal ions from solutions.

Development of fungal technology has been closely tied to the importance of fungi in medicine. In 1928, Alexander Flemming discovered penicillin, the first and most important of the medically useful antibiotics (1). With the advent of World War II, the industrial process of fungal fermentation was applied to *P. chrysogenum* to produce the penicillin required to meet the demands of the Allied Forces. Since that time, a wide variety of antibiotics and medicinally useful compounds have been isolated from fungi (Table 1). The medicinal uses of fungi are expanding because of the power of genetic engineering. Genetically engineered fungi are being used to produce, transform, or modify a variety of medicinally important compounds. Some of the proteins produced in fungi are insulin, human α-interferon, and hepatitis B surface antigen used in vaccines. Compounds that are transformed or modified by

fungi include various steroids and antibiotics. Additional compounds produced or modified by fungi are listed in Table 1.

The use of fungi in agriculture is rapidly developing. Attempts are being made to use fungi as bioinsecticides or bioherbicides and to improve crop yields (2). The advantages of using fungi as bioinsecticides and bioherbicides are related to the broad spectrum of environments that fungi can colonize and host range specificity that may decrease the possibility of undesirable secondary affects on the environment. Additionally, it is possible to permanently establish these fungi in an ecosystem, thereby sustaining long-term control over a pathogen and decreasing costs.

There are three major areas that are being investigated for the use of fungi to improve crop yields: mycorrhiza association, fungal metabolites, and genetic transformation (3). *Mycorrhiza* (fungus-root) is the term used to describe the association of fine feeder roots with root-inhabiting fungi. This association is symbiotic in nature, providing nutrients and protection from stress to both species. Current research is exploring the use of mycorrhiza-forming fungi with commercial crops to improve crop health and productivity. Many of the fungi associated with plants produce chemical compounds that are plant growth regulators. These chemicals, which include gibberellins, cytokinins, and ethylene, are currently being produced industrially for application on crops with high commercial value. These plant growth regulators have been shown to improve crop quality, increase yield, and alter harvest time of the plant product, thereby increasing a crop's economic value. Genetic manipulation is often used to increase or introduce an economically desirable trait into a plant to increase yield or resistance to stress. Traditional methods for genetic transformation have used *Agrobacterium* to introduce foreign DNA into plants. Researchers are currently investigating the use of a mycorrhizal fungus (*Olpidium brassicae*) for genetic transformation. The wide host range for this fungus, which includes grasses and many broadleaf plants, makes it a useful vector for many crops such as beans and lettuce (4).

The broad diversity of applications illustrates the important role that fungi have in biotechnology. This gives importance to understanding fungal ecology, biochemistry, physiology, and genetics to realize the full biotechnological potential of fungi. This knowledge, combined with the ability to manipulate fungal DNA, will expand the application of fungi to environmental concerns.

FUNGI IN ENVIRONMENTAL BIOTECHNOLOGY

Environmental use for fungal biotechnology has been largely devoted to the treatment of hazardous wastes and bioremediation of soils. Most significant of these applications has been the use of wood-degrading white-rot fungi. White-rot fungi are a group of ubiquitous fungi from the class Basidiomycetes that have the ability to depolymerize lignin in wood and to mineralize it to carbon dioxide, thereby making cellulose available for use as an energy source. Various species of white-rot fungi have been studied for their ability to degrade lignin and are used in a variety of biotechnological applications. Among these are *Pleurotus eryngii* (5,6), *Trametes versicolor* (7), *P. ostreatus* (8), *Bjerkandera* species (9), *Phlebia radiata* (10), *Ceriporiopsis subvermispora* (11), and *Phanerochaete chrysosporium* (12). Of these, *P. chrysosporium* has been extensively studied and shown to have the ability to degrade a variety of persistent environmental pollutants such as munitions (11,13–15) synthetic polymers (16,17), pesticides, and various organic compounds (18). This ability is related to the nonspecific, free-radical mechanism, which evolved to degrade lignin (19). The lignin-degrading enzymes can catalyze both oxidative and reductive reactions utilizing highly reactive, nonspecific redox mediators. The versatility of these enzymes to degrade a large number of diverse chemicals gives white-rot fungi great potential for application in environmental biotechnology.

Lignin

The lignin-degrading ability of white-rot fungi evolved owing to the unique nature of lignin. Unlike most biopolymers, lignin does not contain identical, readily hydrolyzable, repeating linkages that occur at regular intervals. The biosynthesis of lignin is the result of peroxidase-catalyzed polymerization of three precursor alcohols: coumaryl alcohol, coniferyl alcohol, and synapyl alcohol (20–22). Peroxidases oxidize these phenylpropanoid compounds to generate phenoxyl radicals that randomly combine to form an irregular, three-dimensional polymer that has no precise chemical structure (3,21–25). The relative concentration of each monomer, which differs with each plant species, determines the final structure of the lignin. Therefore, the final structure of lignin will vary greatly from species to species. The unusual structural features of lignin, that of being bulky, nonlinear, three-dimensional, and stereo-irregular, make its degradation a complicated process that requires a system that is extracellular, nonspecific, and nonhydrolytic.

Wood-Degrading System of White-rot Fungi

Although lignin degradation has been studied for more than 40 years, it was not until the early 1980s that the lignin-degrading system of white-rot fungi was discovered. Early work showed that ligninolytic activity was related to H_2O_2 production (26,27). The isolation of ligninase from the white-rot fungus *P. chrysosporium* soon followed (28–30). Ligninase was shown to contain a heme prosthetic group and was called lignin peroxidase (LiP) (31). Subsequent research identified that some of the ligninase activity detected in the extracellular fluid of the fungal cultures was dependent on Mn^{2+}, leading to the discovery of manganese peroxidase (MnP) (32). Ten extracellular peroxidases, six isoenzymes of LiP, and four isoenzymes of MnP have been purified from cultures of *P. chrysosporium* (33). The enzymes of the lignin degradation system are defined as the ligninases (LiP and MnP), hydrogen peroxide producing enzymes and laccases. Together with other small molecules produced by the fungus or found in wood, such as veratryl alcohol (3,4-dimethoxybenzyl alcohol, VA), oxalate, and manganese, white-rot fungi can efficiently degrade lignin. The

production of the lignin-degrading enzymes occurs during secondary metabolism of the fungus when nutrients such as carbon, nitrogen, or sulfur are limited (12,28,30). Wood is considered to be a nitrogen-poor medium (34). Other enzymes produced by white-rot fungi for wood degradation are cellulases and cellobiose dehydrogenase (CDH). These two enzymes are primarily produced when the fungi are grown on cellulose as the carbon source (35).

Peroxidases

The types of peroxidases are structurally similar. Their sequences are 48% homologous and the overall fold and organization of their secondary structure are nearly identical (36,37). The enzymes contain a penta-coordinate heme and two structural calcium ions. The heme of both LiP and MnP are coordinated by a histidine as the proximal axial ligand, whereas the distal ligand to the iron is open and comprises the hydrogen peroxide-binding site formed by a histidine and an arginine. Both LiP and MnP contain four conserved disulfide bonds, whereas MnP contains an additional disulfide bond located in the C-terminal end of the protein (37). This disulfide bond was proposed to facilitate MnP activity by providing an access channel to the substrate-binding site by moving the C-terminal tail away from the body of the protein (37).

The substrate-binding sites of LiP and MnP differ, providing the basis for the distinct activities of these two similar enzymes. Unique to MnP is a proposed Mn^{2+} substrate-binding site, formed by the carboxylate oxygen atoms of two glutamates (E35, E39) and aspartate (D179) amino acids, a heme propionate oxygen, and two water oxygen atoms (37,38). Lignin peroxidase lacks two of these carboxylic amino acids and the C-terminus tail of the protein blocks access to the gamma-meso edge of the heme (36). Experimental support for the proposed Mn^{2+}-binding site has been provided by mutation of the substrate-binding site amino acids (39,40) and modification of the heme propionates (41).

The substrate-binding site of LiP has not been well defined. Because LiP can directly oxidize a broad range of substrates, from small compounds to bulky substrates such as lignin and ferrocytochrome *c*, the substrate-binding site was proposed to be located at the protein surface and includes aromatic or nonpolar residues (42). Using the crystal structure of cytochrome *c* peroxidase as a template, homology-modeling studies identified two sites centered on tryptophan residues (W17 and W171) on the surface of LiP (42). An additional substrate-binding site was proposed in the access channel near delta-meso edge of the heme (36). Site-directed mutagenesis experiments have shown that W171 (43) and amino acids of the heme access channel (44) are important for substrate oxidation and may reflect two independent substrate-binding sites. In particular, it was shown that W171 was required for the oxidation of VA and a wide variety of other substrates (45,46).

The peroxidase catalytic cycle is shown in Figure 1. In the catalytic mechanism, hydrogen peroxide removes two electrons from the ferric enzyme, generating the enzyme intermediate compound I. One electron is taken from the ferric iron group, forming the oxoferryl [Fe(IV) = 0] species and the other electron is removed from the porphyrin, generating the porphyrin-cation radical (47,48). Compound I is converted to compound II by the one-electron oxidation of a redox mediator. This oxidation quenches the radical on the porphyrin, yielding the oxoferryl enzyme intermediate and a substrate radical. Reaction with a second redox mediator reduces compound II back to the ferric enzyme, releasing the bound oxygen as hydroxide and a second substrate radical, thus completing the catalytic cycle (49). The reduction of compound II is generally the rate-limiting step of peroxidase catalysis when hydrogen peroxide is saturating. It is usually one order of magnitude slower than compound I reduction (48).

Peroxidases can catalyze the one-electron oxidation of a variety of substrates because of the high reduction potentials of hydrogen peroxide and the catalytic intermediates of the enzymes. Hydrogen peroxide has a reduction potential of 1.8 and 1.3 V at pH 0 and 7, respectively (50,51), whereas the reduction potentials of compound I and compound II for LiP and MnP are as great as 1.4 V (52).

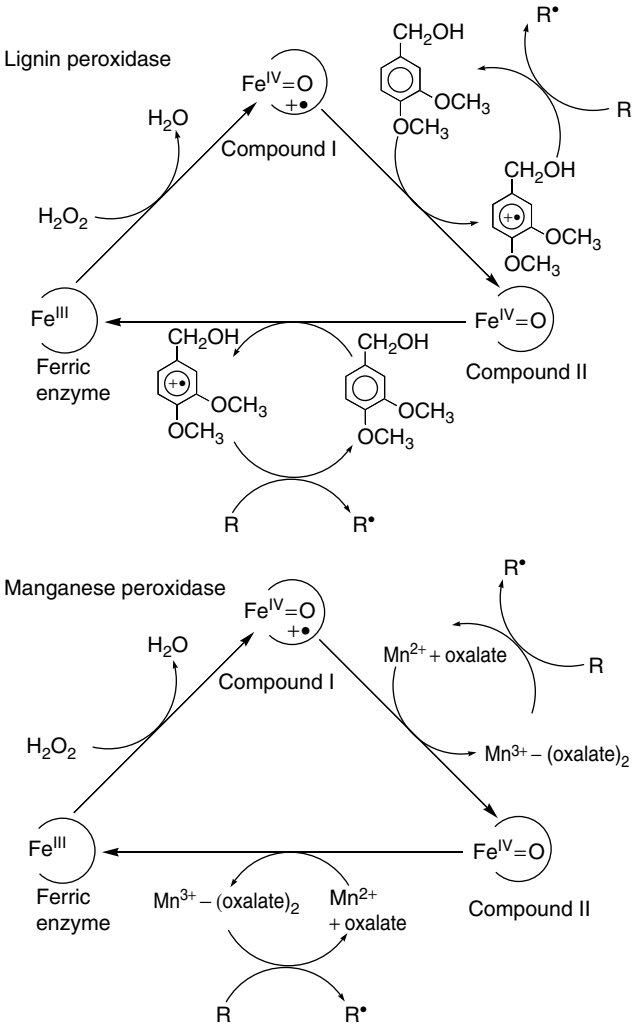

Figure 1. Mechanisms of catalysis and mediation by lignin peroxidase and manganese peroxidase from *Phanerochaete chrysosporium*.

Although LiP has been shown to directly oxidize a variety of bulky substrates (46), it is the indirect redox reactions catalyzed by these enzymes that are considered important for the degradation of lignin and environmentally persistent pollutants (53). Consequently, research has focused on identifying small redox chemicals that can mediate the oxidation of these chemicals.

The physiological substrates for LiP and MnP are VA (54,55) and Mn^{2+} (56,57). Veratryl alcohol is a by-product of lignin degradation (58) and is synthesized and secreted by the fungus during secondary metabolism (59). When VA is oxidized, it is converted to the veratryl alcohol cation radical (60) with a reduction potential of 1.4 V (52). Although it is not believed that the veratryl alcohol cation radical can diffuse away from LiP because its stability in water is believed to be very limited (61), it was shown to mediate the oxidation of other chemicals (62). Manganese occurs naturally in wood (63) and its availability increases as the wood decays (64). Manganese peroxidase oxidizes Mn^{2+} to Mn^{3+}, which is believed to act as a diffusible redox mediator to facilitate the biodegradation of lignin and pollutants (65). Unlike LiP, the catalytic cycle of MnP has a unique requirement for chelated Mn^{2+} to reduce compound II to ferric enzyme (57). Although many organic alpha-ketoacids may act as effective chelators for Mn^{2+} (62,66), it is believed that oxalate is the physiological chelator in vivo (57). Once manganese is oxidized, the chelator acts to stabilize Mn^{3+} by providing electron density to the manganese. The stabilized Mn^{3+}-oxalate complex is then able to diffuse away from the enzyme and oxidize less accessible areas of wood or other chemicals (66). The reduction potential of the Mn^{3+}-oxalate complex has been estimated to be 0.9 to 1.2 V (67,68).

Hydrogen Peroxide Generating Enzymes

Hydrogen peroxide is an oxidative substrate for the peroxidases and is required for catalysis. White-rot fungi appear to have developed a number of mechanisms to produce hydrogen peroxide. Hydrogen peroxide generation by white-rot fungi may, in part, consist of intracellular enzymes, glucose-1-oxidase, glucose-2-oxidase, and methanol oxidase (69–71), and two extracellular enzymes, glyoxal oxidase, and aryl alcohol oxidase (72–75). Glucose-1-oxidase, the first enzyme implicated in hydrogen peroxide formation in white-rot fungi, is produced during secondary metabolism (69). Glyoxal oxidase is also produced during secondary metabolism and can utilize glyoxal, methyl glyoxal, and other alpha-hydroxyl carbonyl and dicarbonyl compounds to produce hydrogen peroxide (72). The involvement of intracellular glucose-2-oxidase and methanol oxidase in extracellular hydrogen peroxide production is not known (70,71), although speculation suggests that the fungi secrete the hydrogen peroxide (69,71,73). Aryl alcohol oxidase reduces molecular oxygen to oxidize aromatic alcohols that are both secreted by the fungi and produced during lignin degradation to aldehydes to generate hydrogen peroxide (73,74). There are also some minor enzymatic and nonenzymatic pathways that may contribute to hydrogen peroxide formation. Cellobiose dehydrogenase is capable of generating hydrogen peroxide through the direct or indirect reduction of molecular oxygen (76). Cellobiose dehydrogenase can also reduce iron, which can react with molecular oxygen to form superoxide, and ultimately, hydrogen peroxide (76,77). Additionally, molecular oxygen can be reduced by carboxylate anion radical generated upon the oxidation of oxalate and glyoxylate by Mn^{3+} produced by MnP-dependent reactions (78,79). The relative contribution of each of these mechanisms to the production of hydrogen peroxide is not clear; however, it is proposed that it is the generation of hydrogen peroxide that is the rate-limiting step in biodegradation (80).

Laccase

Laccases are phenol-oxidase enzymes containing four copper ions, produced by white-rot fungi during primary metabolism (81). These proteins catalyze the one-electron oxidation of phenols to phenoxyl radicals with the concurrent reduction of molecular oxygen to water (81,82). Additionally, laccases can also oxidize nonphenolic lignin model compounds in the presence of a proper redox mediator (82,83). Laccases, or enzymes similar to laccases, are also used by higher plants to produce lignin and are used by many fungi to help in lignin depolymerization (81–83). The role of laccases in lignin degradation is not well understood. Laccases are produced at different levels by a variety of white-rot fungi. High levels of laccase are produced by *Pycnoporus cinnabarinus*, whereas *P. chrysosporium* produces only low levels of the enzyme and only under conditions in which the fungus is grown on cellulose (81). The potential of laccases in bioremediation has not been well characterized.

CDH

It has been proposed that the degradation of lignin and highly oxidized environmental pollutants may require reductive pathways (18). Cellobiose dehydrogenase is a two-domain enzyme containing FAD and heme (76). It is secreted by *P. chrysosporium* and other fungi when cellulose is used as the nutrient carbon source and it catalyzes the oxidation of cellobiose and the reduction of quinones, which are produced during lignin degradation, and other electron acceptors such as molecular oxygen, iron, and manganese dioxide (76). These redox reactions may have significant consequences on biodegradation by the formation of oxidative and reductive free radicals. Figure 2 shows the mechanism of free-radical production by CDH. Although the physiological role of CDH is not known, there are a number of proposed functions for the enzyme. Among these are cellulose depolymerization, lignin depolymerization, and diminished product inhibition of cellulases by oxidizing cellobiose. In addition, CDH can generate free radicals to promote a variety of redox reactions, which can enhance MnP activity by dissolving precipitated manganese dioxide, creating cellobionic acid to chelate Mn^{3+}, and reducing quinones (76). The potential for redox cycling between the peroxidases and CDH is alleviated by its proteolytic cleavage when the fungus enters secondary metabolism (84). The independent flavin domain retains most of the activities of

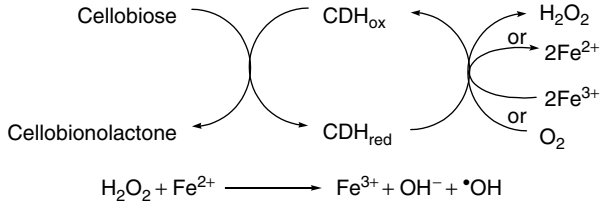

Figure 2. Proposed mechanism for hydroxyl radical production by cellobiose dehydrogenase.

CDH, but does not inhibit LiP and MnP, as intact CDH does (84).

Cellulase

Fungi produce cellulases when cellulose is used as the nutrient carbon source (85). There are several classes of cellulases that work together to depolymerize cellulose through hydrolysis reactions. Some cellulases work from the terminal ends of the cellulose and are referred to as exoglucanases. Cellulases that can hydrolyze glucose monomers located in the interior of the polymer are referred to as endoglucanases. Most endoglucanases bind to areas of imperfection in the cellulose matrix. These enzymes also differ in their ability to depolymerize carboxymethlyated cellulose with only endoglucanases capable of catalyzing its hydrolysis (86). Cellulases are receiving increased attention from government and academic researchers for use in the bioconversion of green waste to ethanol.

Methylation

White-rot fungi have a methylation system that is important in the degradation of phenolic pollutants and phenolic lignin degradation products (87,88). Methylation occurs during ligninolytic and nonligninolytic conditions. The physiological methyl donors are proposed to be S-adenosylmethionine and methyl chloride, the latter being synthesized from S-adenosylmethionine (89). The resulting methylated product can then be oxidized such that the aromatic ring is opened. This mechanism is believed to limit the ability of phenols to redox cycle, providing a mechanism for degradation instead (90). Methylation of phenol yields the corresponding anisole (18). This is of particular importance in alleviating the toxicity of some polychlorinated phenols (PCP) because some PCP are potent inhibitors of oxidative phosphorylation and are highly toxic to microorganisms.

Transmembrane Redox Potential

Fungi possess a transmembrane redox potential, and the membrane redox potential of *P. chrysosporium* has been shown to reduce a number of chemicals (Fig. 3). The transmembrane redox potential is believed to be responsible for the reduction of 2,4,6-trinitrotoluene (TNT) (91). Physiologically, the transmembrane redox potential may be important for the protection of the fungal hyphae from free radicals produced by peroxidases during lignin breakdown. It may also be involved in quinone reduction, reducing quinones in proximity

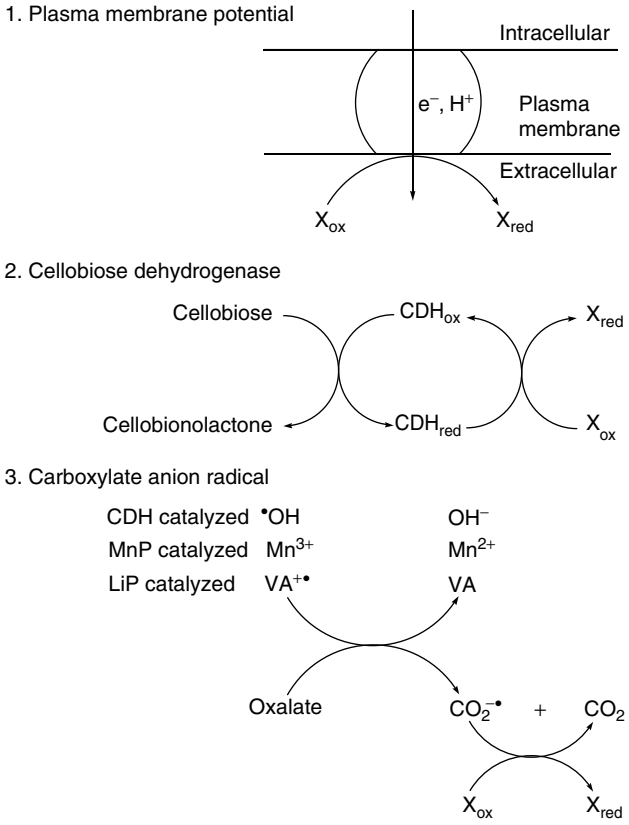

Figure 3. Reductive reactions catalyzed by the white-rot fungus *Phanerochaete chrysosporium.*

to the transmembrane methylase for methylation and subsequent degradation rather than redox cycling (92).

OTHER WOOD-DEGRADING FUNGI

Brown-Rot Fungi

There are many known species of brown-rot fungi. Brown-rot fungi are characterized by their ability to remove the cellulose, hemicellulose, and xylan from wood without the complete degradation of the lignin as seen with white-rot fungi. The remaining, partially oxidized lignin is brown. A percentage of the lignin degraded by brown-rot fungi is hydroxylated and demethylated (93,94). Brown-rotted wood has a rapid decrease in its properties of strength even before there is a significant weight loss. After brown-rot decay, there is an increase in the weight percentage of the wood that is alkali soluble. This increase is characteristic of brown-rot decay. Specific enzymes responsible for wood degradation have not been isolated but it is generally accepted that degradation involves the hydroxyl radical. It should be pointed out that most of the evidence for hydroxyl radical-mediated degradation is circumstantial (95).

Brown-rot fungi tend to maintain the extracellular pH between 1.7 and 3.5, whereas white-rot fungi typically maintain the pH between 4 and 6 (96,97). It has been proposed that the low pH allows ferri-reductases to generate hydroxyl radical without damage to the

fungi (98). The low pH decreases the affinity of organic acids for iron. This increases the reduction potential of the iron and decreases the reactivity of ferrous iron for molecular oxygen. The reduced iron diffuses away from the fungi into areas with higher pH. The iron then reduces molecular oxygen to ultimately yield hydroxyl radical at a safe distance from the fungal mycelia (98).

Soft-Rot Fungi

Soft-rot fungi are the most recently discovered group of wood-degrading fungi. Wood that has been degraded by soft-rot fungi has an increased ratio of lignin to carbohydrate when compared with undegraded wood (99). Soft-rotted wood usually has distinctive longitudinal cavities created in the wood. Some soft-rot fungi have the ability to erode lignin, but not to the extent of white-rot fungi. In contrast to white-rot fungi, wood degradation does not appear to be in response to nutrient limitation. As with brown-rot fungi, the enzymes responsible for lignin degradation have not been determined; however, many soft-rot fungi secrete CDH and some express laccase activity. These enzymes may be responsible for wood degradation (99).

Soft-rot fungi tend to degrade wood at slower rates than either white- or brown-rot fungi, but they are well adapted to adverse environments (100). Soft-rot fungi do not have peroxidases and do not cause wood to be alkali soluble, leading to the conclusion that soft-rot fungi have a different mechanism of wood decay than either white- or brown-rot fungi (99).

GENETIC MANIPULATION OF FUNGI

The application of any fungi to a specific area of concern will require a proper understanding of the genetics and physiology of the species or class of fungi. The tools of genetics have increased our knowledge greatly, and the advances in this field, from recombinant DNA techniques and microarray gene analyses to elucidating entire genome sequences, will hasten the understanding of the principles that will govern biotechnological advancements. The ability to genetically manipulate fungi has permitted researchers to improve microbial strains by alteration of the fungi's genetic code, production of proteins outside their natural host, exploration of the relationship of protein structure and function, and design of new functions into proteins. The list of fungi and the products (Table 1) that are isolated from them is evidence of the biotechnological potential of this technology.

During the late 1980s, when the traits of white-rot fungi were recognized for having great potential for degradation of environmental pollutants, researchers sought to find the best strain of fungus to degrade lignin. Early work with *P. chrysosporium* used screening to find naturally occurring mutants of the fungus that exhibited useful traits. Several strains of *P. chrysosporium* that exhibited an enhanced ability to degrade lignin or oxidize phenols were identified (3). Currently, through recombinant DNA techniques we are no longer constrained to the traditional methods for fungal strain improvement of mutation, screening, and selection. The discovery that microorganisms carry plasmids (101), which are independently inherited DNA molecules, has permitted researchers to transform microorganisms, manipulating how and where genes are expressed. The genes for the peroxidases of *P. chrysosporium* have been identified (3) and placed into plasmids for expression of recombinant proteins. The peroxidases have been expressed in prokaryotic and eukaryotic organisms. The recombinant enzymes are currently being expressed in bacteria, *Escherichia coli* (102,103), other fungi, namely, *A. oryzae* (104), and the natural host, *P. chrysosporium* (105,106).

Expression of recombinant peroxidases in prokaryotic systems has the potential to produce target protein in quantity in a short period and at low cost (107). Unfortunately, bacterial expression systems do not always produce active recombinant proteins (108,109) and thereby require additional processing steps to obtain active enzymes (109,110). Lignin and manganese peroxidases have been expressed in bacterial systems. However, as are many other plant or fungal peroxidases, these proteins are produced in an inactive form, requiring the introduction of heme and calcium to form active enzyme (102,103). Because of the limitations of bacterial systems for protein expression, eukaryotic systems remain indispensable for the production of many recombinant proteins by providing an environment that permits the production of active enzymes (111,112). Transformations of yeast and higher fungal species have provided for the recombinant expression of many useful proteins (Table 2). The use of yeast has dominated the area of heterologous protein expression as a result of the ease in which these organisms can be manipulated compared with the higher fungi. Recombinant MnP and LiP have been produced in the natural host *P. chrysosporium* by placing the genes under the control of the glyceraldehyde-3-phosphate dehydrogenase promoter, allowing peroxidase expression in nutrient-sufficient culture conditions (105,106). The reintroduction of the peroxidase genes into *P. chrysosporium* illustrates the power of genetic engineering to, hopefully, direct the improvement of fungal strains for specific characteristics. These expression systems have advantages and disadvantages for the development of an organism or enzyme for biotechnological applications.

The tools of crystallography, genetic engineering [Polymerase Chain Reaction™ (PCR) and site-directed mutagenesis], and enzyme kinetics have opened the door to understanding how enzymes function on a molecular level. From crystallographic data researchers can identify structural features that may contribute to the function of an enzyme. Site-directed mutagenesis and PCR allow researchers to make changes in the genetic code to alter a protein's structural features. Enzyme kinetics are used to evaluate the effect of the structural changes on enzyme activity. Using these tools, researchers have made significant advances in understanding the structural features that contribute to the activity of the enzymes from white-rot fungi. With this knowledge researchers can design features into proteins to obtain an enzyme with desired properties. Protein engineering has been applied to the peroxidases from *P. chrysosporium* to

Table 2. Examples of Heterologous Proteins Expressed in Fungi

Organism	Protein
Yeast	
Saccharomyces cerevisiae	Glucoamylase (*Aspergillus awamori*)
	β-endorphin
	Bacillus α-amylase
	Endoglucanase
	Granulocyte-macrophage-colony stimulating factor
	Human α-interferon
	Hepatitis B surface antigen
	Insulin
	Interlukin-2
	Leech hirudin
	Mouse α-amylase
	Mouse Immunoglobin
Pichia pastoris	D-alanine carboxypeptidase
	α-amylase
	Catalase (*Aspergillus fumigatus*)
	Kunitz protease inhibitor (APLP-2)
	Tick anticoagulant protein (TAP)
	Human CD38 (soluble portion)
	Mouse serotonin receptor
	Tetanus toxin fragment C
	HIV-1 gp120 (intracellular)
	HIV-1 gp120 (secreted)
	Human interferon (IFN)-α2b
Filamentous fungi	
A. oryzae	Manganese peroxidase (*P. chrysosporium*)
	Coprinus cinereus peroxidase
	Dye-decolorizing peroxidase
	Laccase (*C. cinereus*)
	Human lysozyme
	Human lactoferrin
Aspergillus niger	Mycocin HMK (*Williopsis mrakii*)
	Equine lysozyme
	Green fluorescent protein (S65T)
	Barley α-amylase
	Restrictocin cytotoxin
	Aldehyde dehydrogenase
Trichoderma reesi	pH 2.5 acid phosphatase (*A. niger*)

increase the thermal stability of recombinant protein (41) and to introduce veratryl alcohol oxidase activity (45,46) into MnP.

POLLUTANT DEGRADATION

The diverse and partially redundant mechanisms of the extracellular, nonspecific, free radical–based lignin-degrading system used by white-rot fungi enables the fungi to degrade a wide variety of environmental pollutants (Table 3; 18). Central to this ability are the enzymes LiP, MnP, and CDH. These enzymes can produce a number of free radicals that allow for the mediated oxidation or reduction of recalcitrant chemicals (62,113). Lignin peroxidase and MnP can use their respective substrates, veratryl alcohol and manganese, to mediate the oxidation of other chemicals (62,114). These two oxidized substrates can also oxidize oxalate to form the carboxylate anion radical that can be used for reductions (Fig. 3). The carboxylate anion radical can also be formed by CDH (76). The carboxylate anion radical can reduce molecular oxygen or iron to generate oxygen radicals, that is, superoxide (115–117). Superoxide can dismutate to form hydrogen peroxide, which in the presence of reduced iron can generate the hydroxyl radical, a potent oxidant.

The ability to form both oxidative and reductive species gives white-rot fungi the capability to degrade a variety of pollutants. Many pollutants might require reduction for effective degradation or have metabolites that must be reduced for efficient bioremediation. Three extracellular reductive mechanisms can be described for *P. chrysosporium* (Fig. 3). The reductive potential of the plasma membrane potential, or CDH, has been shown to reduce chemicals that are easily reduced. However, some highly oxidized pollutants such as carbon tetrachloride and trichloroethylene are relatively stable and cannot be reduced directly by the plasma membrane redox potential or by CDH. Also, because of the oxidation potential required to further oxidize these chemicals, they are resistant to oxidative remediation by the peroxidases. However, the highly reductive carboxylate anion radical formed by LiP, MnP, and CDH can reductively dehalogenate carbon tetrachloride and trichloroethylene (60,113,118,119), leading to their mineralization.

The various metabolites formed by the many different mechanisms of wood degradation do not inhibit the activity of the different enzymes involved. For example, the peroxidases have been shown to oxidize, either directly or indirectly, a variety of small and large compounds such as cyanide (120), polycyclic aromatic hydrocarbons, many phenols (18), iodide, ferrocyanide, and cytochrome c (46). Yet their oxidized substrates, Mn^{3+}-oxalate or the veratryl alcohol cation radical, are reduced by the reducing sugars, glucose and cellobiose, products of cellulose metabolism (17). This was unexpected, but considering the natural environment of white-rot fungi, it would be unproductive for cellobiose to act as a reductant, and therefore an inhibitor, for the peroxidases.

However, there is potential for these multiple redox reactions to compete and form futile cycles. For example, veratryl alcohol can be oxidized to quinones by LiP (121,122) that can be subsequently reduced by CDH to the hydroquinone (92). Hydroquinones are readily oxidized back to the quinone by Mn^{3+}, which results in a zero net metabolism. However, the reactions catalyzed by the fungus have evolved to work together to prevent redox cycling and promote degradation. For example, quinones can be reduced to hydroquinones and these phenolic compounds can be methylated at the cell surface (88), resulting in a compound that could be oxidized to a cation radical,

Table 3. Selected Examples of Environmental Pollutants Degraded by *Phanerochaete chrysosporium*

Biopolymers	
Cellulose	
Kraft lignin	
Lignin	
Synthetic Polymers	
Polyacrylate	
Polyacrylamide	
Nylon	
Chlorinated Aromatic Compounds	
2,4-Dichloroaniline	
2,4-Dichlorophenol	
2,4,5-Trichlorophenol	
2,4,6-Trichlorophenol	
Pentachlorophenol	
2,4,6-Trichlorobenzoic acid	
2,4,5-Trichlorophenoxyacetic acid	
Pesticides	
DDT (1,1,1-trichloro)-2,2-*bis*-(4-chlorophenyl)ethane	
Chlordane	
Lindane	
Toxaphene	
Munitions	
TNT (2,4,6-trinitrotoluene)	
DNT (2,4-dinitrotoluene)	
RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine)	
HMX (octahydro-1,3,5,7-tetranitro-1,3,5,7-tetraazocine)	
Nitroglycerine	
Polycyclic Aromatic Compounds	
Anthracene	
Benzo(*a*)pyrene	
Chrysene	
Naphthalene	
Pyrene	
Polycyclic Chlorinated Aromatic Compounds	
Aroclor 1242	
Aroclor 1254	
Dioxin	
PCB (polychlorinated biphenyls)	
Dyes	
Azo-dyes	
Azure blue	
Crystal violet	
Cresol red	
Bromophenol blue	
Others	
Aminotriazole	
Azide	
Carbon tetrachloride	
Cyanides	

which would be susceptible to further oxidation resulting in mineralization.

Transformation of insoluble and toxic chemicals can be a potential problem for bioremediation. White-rot fungi may have significantly fewer difficulties in accomplishing this than bacterial systems because of the extracellular degradation system they have evolved. The oxidative and reductive compounds produced by CDH and the peroxidases were shown to efficiently depolymerize insoluble polyacrylate polymers (16,17), transforming them to a soluble product that was easily metabolized by the fungi. Additionally, many of the extracellular reactions that lead to mineralization result in the detoxification of chemicals. In the degradation of pentachlorophenol, one of the first steps is its methylation to pentachloroanisol, which can be further metabolized (88). Highly oxidized compounds, such as TNT, can be very toxic to microorganisms. The fungus is able to detoxify these chemicals by first reducing them by a transmembrane redox potential (91). The detoxification reactions take place at the cell surface and thus are dependent on mycelial mass to effectively overcome the toxic effects of these chemicals.

The biodegradation of the munitions TNT, hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetraazocine (HMX) by white-rot fungi has generated a great deal of interest in recent years (11,13–15). Degradation of all three munitions in both soil and liquid cultures of *P. chrysosporium* have been shown. Manganese^{III}, produced by MnP, oxidizes each of these munitions (15). Additionally, TNT and RDX could be directly reduced by CDH (13). Reduction of TNT to its amino and diamino derivatives is quickly catalyzed by the transmembrane redox potential (91). TNT reduction is seen with almost all fungi, but only wood and litter-degrading fungi are able to oxidatively transform the aminodinitrotoluenes and mineralize them (91,123). Lignin peroxidase has been shown to directly catalyze the transformation of aminodinitrotoluenes (15). Cultures of *P. chrysosporium* have been shown to efficiently mineralize even high concentrations of TNT to carbon dioxide (11). In the case of RDX, nearly 100% of the carbon was oxidized to carbon dioxide (13). In soil cultures, higher concentrations of RDX caused increased rates of RDX disappearance. In liquid cultures, mineralization rates were proportional to RDX concentration until RDX became saturating (13).

The mechanisms used by white-rot fungi for the degradation of wood make it an exceptional candidate for the bioremediation of complex mixtures of pollutants. Intech180 (North Logan, Utah) has commercialized white-rot fungi technology and has overseen the application of the fungi in a number of field tests. EarthFax Engineering, Inc. (Salt Lake City, Utah) conducted a recent field

application at a wood treatment facility in southeastern U.S.A. that was predominately contaminated with PCP, dioxin, and lindane (124). Bioremediation resulted in the reduction of contaminating PCP, dioxin, and lindane to levels below EPA industrial and residential standards. Successful bioremediation should lead to the restoration of a contaminated site to use. An advantage to using white-rot fungi is that the fungal inoculum consists of fungus grown on woody by-products, and its degradation results in the formation of humus. Thus, bioremediation of soils results in the improvement of many soil factors, making it suitable to use for agricultural or landscaping purposes.

CONCLUSION

The use of fungi for some biotechnological development has one basic goal, to provide a cost-effective alternative to some specific industrial process. The development of *A. niger* to produce citric acid was driven by labor-intensive, and thus expensive, methods of isolating the acid from lemons. Many other industrial applications of fungi were developed for similar reasons; the product was less expensive to produce or a compound's structure was unknown and thus unable to be synthesized, as was the case with penicillin. Today the definition of "cost-effective" extends beyond economic concerns to include political and environmental considerations. Governments are passing increasingly restrictive legislation that controls contaminating emissions of many industrial processes. As restrictions increase, alternative processes will need to be developed to meet the current requirements for a product. A prime example is the development of "biofuels" to replace our dependency on imported oil and to stop generating the pollutants associated with their use. Biofuels are alcohols made from cellulosic biomass (green waste from agriculture and forestry). The vision of the Department of Energy National Biofuels Program is "to realize the large-scale use of environmentally sound, cost-competitive, biomass-based transportation fuels. . .." This vision clearly reflects the philosophy of a "green" biotechnology, to begin with a renewable resource to make a product by a process that is friendly to the environment.

Each of the industrial fungal processes introduced earlier may impact the environment either by reducing the level of a pollutant that is made or by removing the source of pollution. Many times the by-products of a fungal process can be used for other purposes. As in the example of bioremediation with white-rot fungi, the end product can be reintroduced into the environment for a productive use. There are many areas in which fungi can be applied: industry, agriculture, food science, medicine, and bioremediation. In all cases, the biotechnological development of fungi should, hopefully, have a positive impact upon our environment.

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FUNGI IN FRESHWATER ECOSYSTEMS.

See WATER FUNGI AS DECOMPOSERS IN FRESHWATER ECOSYSTEMS

FUNGI IN MARINE/ESTUARINE WATERS

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Eukaryotic mycelial decomposers (organoosmotrophs) of marine ecosystems were until recently all termed "fungi," but they are not all members of the kingdom Fungi (1); some belong to the kingdom Straminipila (2) (alternative spelling: Stramenopila), whose members have zoospores (biflagellate swimmers) as propagules (Table 1; 3–5). This article focuses on the marine mycelial fungi and straminipiles that are decomposers of vascular-plant material. The most clearly important of the fungi with respect to marine-ecosystem functioning are the ascomycetes (3,4,6–8) that are secondary producers using the lignocellulose of extraordinarily productive intertidal vascular plants [e.g., saltmarsh grasses (9,10)]. There are only a very few species of marine mycelial basidiomycetes [ca. 2% of all known species of marine mycelial fungi (7)]; the ecological role of marine basidiomycetes is likely to be the same as that of marine ascomycetes. The importance of the marine straminipiles, mostly species of *Halophytophthora*, is not well established, but may be analogous to that of the marshgrass ascomycetes — the principal substrates for the halophytophthoras are leaves that fall into the marine environment [e.g., mangrove leaves (4,11)]. A few species of marine fungi and straminipiles are potentially important parasites or pathogens of macroalgae and animals (4,12), including corals (13,14).

The mostly nonmycelial, unicellular marine chytrids, the only members of the kingdom Fungi that have zoospores (posteriorly uniflagellate) as propagules, also include symbionts, for example, of seagrass (16), but marine chytrids as a group are not known well enough for inclusion in this article (3). Note that the two books that still must be used for chytrid identifications date from 1960–1977 (17). The marine yeasts [unicellular ascomycetes and basidiomycetes in the kingdom Fungi (3,18)] as well as the nonmycelial straminipiles [Labyrinthulomycota: thraustochytrids and labyrinthulids (3,19,20); parasitoidal marine intertidal lagenidialean straminipiles (21–23)] and the maritime vesicular-arbuscular mycorrhizal fungi (zygomycotes; 24) are also excluded here.

THE MARINE-DECOMPOSER ASCOMYCETES AND STRAMINIPILES

Marine mycelial ascomycetes include representatives of several ascomycetous taxonomic groups (1,8,25), with the largest number belonging to the perithecial

Table 1. Contrasting the Two Major Types of Marine Mycelial Organoosmotrophs[a]

Taxon	Typical Substrate	Key Morphocharacteristics
Kingdom Fungi, Phylum Ascomata	Intertidal plant shoots, driftwood	Haploid chitinous mycelium, porous septa, complex sexual structure, nonmotile propagules (ascospores), accumulation of biomass in substrate (5–30% of total mass)
Kingdom Straminipila, Phylum Oomycota	Fallen leaves, e.g., of mangroves	Diploid nonchitinous mycelium, septal plugs, no complex sexual structure, motile propagules (zoospores), no accumulation of biomass (<1%) in substrates[b]

[a]See also references (1–3,5,7–9,15).
[b]Biomass information for oomycotes tentatively estimated, based in part on unpublished findings (transmission electron microscopy) of D. Porter, E. A. Richardson and S. Y. Newell for submerged decaying mangrove leaves.

order Halosphaeriales, many species of which produce ascospores bearing appendages (some quite elaborate) that are likely to serve as substrate-attachment devices (7,26,27). Many of the ascomycetes most important in secondary production in marine intertidal zones are pseudothecial ascomycetes [loculoascomycetes (1,28)] (Fig. 1; 7,9). Keys to the marine ascomycetes are available (6,7). Examination of rDNA base sequences has revealed that marine ascomycetes evolved from terrestrial ancestors into marine habitats (29). The fact that principally important ascomycetes of decaying saltmarsh grasses are members of the same genera common as agents of diseases of cereal grasses (e.g., *Phaeosphaeria* and *Mycosphaerella*) indicates that it is likely that at least some marine intertidal ascomycetes evolved into the sea with their substrate plants (30).

The marine mycelial straminipiles of plant-decomposition systems are members of the family Pythiaceae, and most are members of the genus *Halophytophthora* (4,5). The most common species worldwide is *Halophytophthora vesicula* (Fig. 2). A key to the species of *Halophytophthora* is available (33). The most common nonhalophytophthoran marine species is *Pythium grandisporangium* (34). The wide range of morphologies and behaviors (e.g., peculiarities of zoospore-release mechanisms) of the halophytophthoras indicate that *Halophytophthora* is a catch-all (polyphyletic) genus (4). Cooke and coworkers (35) examined rDNA base sequences for *Halophytophthora batemanensis* [probably = H. *vesicula*

Figure 1. Typical deposit of ejected ascospores of two species of loculoascomycetes from naturally decaying smooth cordgrass leaf blades of Georgia saltmarshes (9). The smaller (about 7 μm wide), hyaline, 2-celled spores are of *Mycosphaerella* sp. 2 [Kohlmeyer and Kohlmeyer (31)] (Dothideales), and the larger (about 11 μm wide), dark, 4-celled spores are of *Phaeosphaeria spartinicola* (Pleosporales). The spores were explosively expelled at a rate of hundreds per square centimeter per hour, onto a capture glass placed in front of a wetted decaying leaf, and many have germinated to hyphae. These two species are nearly always intimately mixed in the leaf-decay system, and may be mutualists (9,30,32).

(4)] and concluded that the halophytophthoras may be more closely related to the genus *Pythium* than to *Phytophthora*. However, sequencing of mtDNA (*cox2*) (D. Hudspeth, M. Hudspeth and S. Newell, unpublished) from six strains of *H. vesicula* (both "delicate" and "robust" varieties; Fig. 2) indicates that these six form a single clade that is closer to *Phytophthora* than *Pythium*.

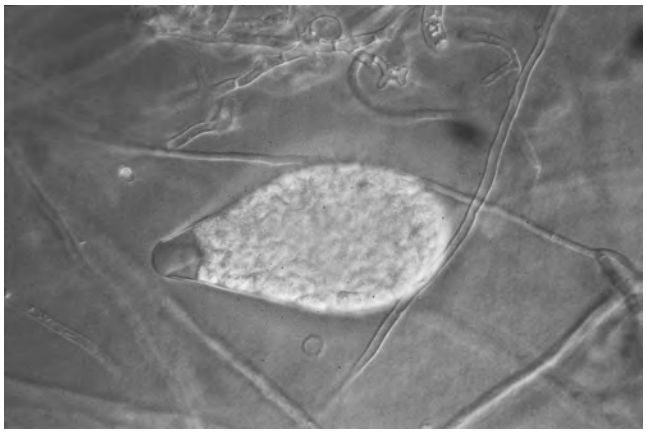

Figure 2. Zoosporangium of the cosmopolitan marine oomycote *H. vesicula* (about 40 μm wide at the widest point) (4,36). This is the "delicate" variety; see References 11 and 37 for photos of the "robust" variety. Species of *Halophytophthora* are common and rapid pervaders of fallen mangrove leaves (34,36,38,39). At full maturity, the inverted cone visible at the tip of the sporangium (left end) everts into a delicate, ephemeral release vesicle, and the biflagellate zoospores seen crammed against one another in the sporangium swim away through the vesicle.

Life histories of the marine ascomycetes and the marine straminipiles are quite different from one another (Table 1). The predominant ploidy for the ascomycetes is haploid. Haploid mycelia pervade the plant substrate, lyse and digest it, and convert it into new fungal mass [at high efficiency ($\geq$50% yield) at least in some cases (9)]. Some of the fungal digestate is used to construct ascomata (commonly spheroidal, dark-colored tissues embedded in the decaying plant material) in connection with sexual fusion of female and male hyphal or spermatial entities. Binucleate (dikaryotic) ascogenous hyphae are formed, and from these, multiple asci (usually cylindrical containers of ascospores) grow upward within the ascomata. Ascospores are formed after nuclear fusion, meiosis, and subsequent mitosis (usually eight spores per ascus). The ascospores are released, in some cases explosively expelled, from the asci, out away from the decaying plant, and some of these ascospores attach to new substrates to complete the cycle. See References 1,8,9, and 25.

The predominant ploidy for the marine straminipiles is diploid (Table 1). Haploid sexual organs are formed by meiosis, and these soon fuse in compatible pairs to form zygotes in the female partner. The zygotes mature into usually thick-walled oospores. The oospores germinate to yield new diploid hyphae that can propagate vegetatively by diploid zoospore formation. The zoospores swim and/or are transmitted through water currents to new substrates (commonly fallen leaves) to which they attach and penetrate by germination into new diploid hyphae. Some of the marine straminipiles may not undergo sexual reproduction. See References 1, 5, 36 to 38, 40 and 94.

Methods of collection and isolation of marine ascomycetes and marine straminipiles are given in reference (4) (see also 7,36,38,41). Central methods for ascomycetes involve capture and culture of ascospores expelled away from the plant material collected in the field, often as standing-decaying shoots (Fig. 1). Alternatively, ascomata can be microexcavated from decaying material, squashed with fine forceps, and dragged across agar to spread ascospores for subsequent germination and transfer. For straminipiles, samples are collected as submerged decaying leaves and small subsamples are placed on oomycote-selective medium (containing strong selective inhibitors of fungi and bacteria). When oomycotes appear, single sporangia or hyphal tips are removed to new culture plates. It is very important that investigators working with marine-mycelial decomposers keep in mind that culture of mycelial eukaryotes on common laboratory agar media, with transferring, will commonly lead to loss of natural physiological capabilities and capability for sexual reproduction (42).

MARINE ADAPTATION OF EUKARYOTIC MYCELIAL DECOMPOSERS

Neither marine mycelial ascomycetes nor straminipiles have a large presence in the plankton (11,43). Mycelial fungal biovolume concentrations are less than 0.2% of biovolumes of bacterioplankters, even in saltmarshes, where fungal volume would be expected to be maximized (9). Mycelial straminipile contribution to flagellate-plankton volume has been estimated to be only about 10^{-5} to 10^{-6} that of total flagellates, even close to the mangroves, where marine oomycotic contribution would be expected to be greatest (44). Even the single-celled fungi [yeasts (3,18)], which one might expect to be adapted to life in liquid suspension, and therefore potentially having planktonic impact (45), are present in the saltmarsh plankton at levels 10^{-8} those of bacterioplankters and at total biovolumes at most 10^{-5} those of bacteria [using generous yeast-cell volumes (*Pichia spartinae*; 18) and bacterial-cell volumes from Reference 46]. [Note that the appearances of yeasts in natural plankton samples, e.g., in direct-epifluorescence microscopic fields, are unknown; because marine-planktonic yeasts have not been direct-microscopically enumerated as have bacteria, it is possible that the above estimates of yeast cell concentrations (culture-based) are not accurate.]

Mycelial fungi are exquisitely well adapted to pervasion and lysis of solid substrates, through turgor-pressurized tip growth and lytic-enzyme release, among other capabilities (47,48). They would not be expected, therefore, to have the plankton as a major habitat, one where their relatively weak dissolved-substrate affinities ($>10^{-6}$ M) put them at a severe competitive disadvantage to the ultramicro planktobacteria, whose affinities are commonly as strong as 10^{-8} M (47,49). It is in the solid, aboveground parts of dead intertidal vascular plants (e.g., saltmarsh grass and rushes) in which marine ascomycetous decomposers have the opportunity to pervade, lyse polymeric solids, and produce new mass, with minimal competition from prokaryotes or predators (Table 1; 9,11,49,50). This does not appear to apply to parts of marine vascular plants that are permanently submerged (seagrasses) or to leaves that fall and then decay in a permanently submerged location (mangroves), unless the dead parts break away and then decay in the intertidal zone (16,51,52). Although submerged wood is known to be a substrate for many species of marine fungi (4,6,7), the extent to which submerged wood serves as a substrate for marine-fungal production is not yet clear (3).

Marine mycelial straminipiles may not build large masses in their solid substrates (fallen, submerged leaves, e.g., mangrove leaves; Table 1). They do penetrate and grow within fallen, submerged leaves [although not apparently within leaves produced within the inter- or subtidal zones (4,39,43)], but there is some evidence (see next section) for the hypothesis that they soon convert newly synthesized mass into zoospores that rapidly depart the decaying leaf system (11,53).

Marine mycelial ascomycetes are not dramatically different from their terrestrial and freshwater counterparts, perhaps in part because mycelial fungi in general are capable of countering low external water potential by production or absorption of compatible solutes such as glycerol and membrane protectants such as trehalose (54–56). These compatible solutes and membrane protectants serve equally well to provide physiological functionality in the face of desiccating conditions or salty

conditions; for this reason, it is not a simple matter to find evidence of physiological adaptations to seawater salinity using agar cultures (57,58). Therefore, the definition that has been adopted for the marine eukaryotic decomposers is "fungi that grow and sporulate exclusively in a marine or estuarine habitat" (4,31). This definition excludes species of fungi that might be capable of germination and inextensive hyphal growth, but that are capable of neither extensive substrate pervasion nor ascomatal or conidial formation in marine substrates in nature (11). It also excludes species such as *Schizophyllum commune*, which can form basidiomatal brackets on dead wood in salty, maritime environments, and exhibit resistance to salt in agar culture, but which also produce basidiomata on dead wood in nonsalty terrestrial environments (59). There are, however, subtle physiologically adaptive characteristics of at least some marine fungi, including membrane-transport enzymes that function optimally in the presence of seawater ions and the alkaline seawater carbonate-buffering system (54,60–62). It may be marine adaptations of this nature that can restrict normal propagule production to the marine environment (54,63), along with specific adaptations to high sulfate availability and to the marine substrates that are occupied and lysed (e.g., finely targeted lytic enzymes, competitive characteristics targeted to potential marine combatants, mutualisms evolved with marine invertebrate litter comminuters). Adaptations to marine substrates probably include selective adherence by seawater-borne ascospores bearing appendages (4,6,7,26,27).

Marine mycelial straminipiles generally show mycelial growth and zoospore production patterns with broad optima at salinities above that of freshwater, and it has been suggested that this, along with cuing of zoospore release by dilution and mild drying, is a sign of adaptation to intertidal zones, where salinities would be expected to be variable, along with occasional exposure of substrates to air (36,37,53,64). Straminipiles generally can respond to increasing salinity by production of compatible solutes (proline; 54). There are no reports of the characteristic marine mycelial straminipiles (halophytophthoras) from freshwater or terrestrial habitats (but see 36), so the definition given in the previous paragraph for marine mycelial ascomycetes also applies to the mycelial straminipiles (4).

BIOMASSES AND PRODUCTIVITIES

A barricade that blocked collection of data for marine mycelial biomasses and productivities was the opacity of their solid substrates. This obstacle was overcome by development of methods for chemical extraction and measurement of biochemical proxies for living fungal (membrane-containing) mass and total-fungal (cytoplasm-filled and empty hyphal) mass (especially ergosterol and glucosamine, respectively) (65–67). Subsequently, the ergosterol method was expanded such that it became possible to measure instantaneous fungal production rates (66,67). The expansion involves monitoring the flow of radiolabeled acetate into ergosterol.

There are no published methods for biochemical-proxy measurement of the biomass or productivity of marine straminipiles, primarily because the straminipiles do not synthesize ergosterol, nor do most species have glucosamine as a monomer of cell wall polymers (3,66,68). It may be that immunoassays for terrestrial, plant-pathogenic straminipiles could be adapted for measurement of mass of marine mycelial straminipiles (66).

Because marine ascomycetes are capable of high-efficiency conversion (ca. 40–60% yield) of intertidal vascular plant material, which is highly photosynthetically productive, the productivity of marine ascomycetes in saltmarshes is substantial (Fig. 1; 30,32,69,70). For smoothcordgrass (*Spartina alterniflora*) saltmarshes in Georgia, U.S.A., annual fungal productivity (3-year average) was 535 g m^{-2} of marsh. Standing dead shoot parts are essentially converted *from within* into new entities that are substantially fungal in nature, although this is not patently obvious in gross aspect (9,50). Surprisingly, fungal productivity was higher in winter and spring than in summer and autumn in Georgia (latitude 31°N) (32). Autumnal productivity of marshgrass ascomycetes at a standard temperature of 20 °C did not show decreases with increasing latitude from 29 to 43°N, and the same major species of ascomycetes were involved over the whole transect (71). Bacterial productivities on standing-decaying marshgrass leaves were approximately 1% of the fungal productivities inside the leaves (9).

High productivities of saltmarsh ascomycetes led to high content of fungal nitrogen and phosphorus in standing decaying leaves (33–88% in living fungal mass); saltmarsh-fungal production may partially drive import of nitrogen by saltmarshes (32). Bacterial nitrogen fixation in decaying marshgrass shoots may also be facilitated by fungal/bacterial consortia, and saltmarsh ascomycetes have been implicated in the lytic release of volatile organosulfur compounds from within the decaying shoots (9).

There is no published information yet for biomasses or productivities of marine mycelial straminipiles. However, three findings suggest that these oomycotes do not have a mass accumulative strategy like the marine mycelial ascomyetes (see previous section): (*1*) carbon dioxide output rates do not rise in decaying leaves containing high frequencies of marine oomycotes to the level that they do in leaves containing high concentrations of marine-ascomycete mass (39,69); (*2*) frequencies of oomycotic occupation of and rates of oomycotic zoospore release from submerged decaying mangrove leaves fall early in the decay process (53); (*3*) transmission electron microscopy has shown high densities of mycelia of marine ascomycetes in naturally decaying saltmarsh grass blades, but no clear evidence (in a preliminary study) of such densities of hyphae of oomycotes in naturally decaying mangrove leaves (50; Porter, Richardson, and Newell, unpublished).

FATES

The mass produced by marine ascomycetes (at least those of saltmarshes) has the same sort of fate as does

that produced by fungi in forest floor litter: (*1*) output to shredding invertebrates; (*2*) output as sexual propagules (ascospores); (*3*) output as remnant mycelia in small particles, breaking away from the litter decay system and moving into a sediment bacterial/meiofaunal system (9,32,72). In the saltmarsh, principal, at least partially mycophagous, shredding invertebrates are snails, amphipods, and probably crabs, but it is likely that others remain to be identified (9,72). It has been suggested that one cause of lower summer plus autumn fungal productivity in saltmarshes is higher tides that would permit greater access of mycophagous invertebrates and bacterial competitors than in winter plus spring (32). It is not known whether marine straminipiles serve as food for litter-associated invertebrates. If they do not accumulate substantial masses within decaying leaves, as hypothesized earlier, then it may be that the main output avenue for marine mycelial straminipile mass is for zoospore stages in the plankton to serve as food items for invertebrates, perhaps principally the protozoplankton.

POLLUTION AND MARINE FUNGI

There is a large literature for terrestrial ecosystems on the impacts of both: (*1*) pollutants on fungi and (*2*) fungi on pollutants (73,74). Much less is known about marine mycelial ascomycetes and straminipiles and anthropogenic contamination of the marine environment. Recent examinations of urban saltmarsh sites that had received gross pollution by toxic chemicals (Hg, Cr, PCBs, PAHs, multichlorinated insecticide) showed that saltmarsh ascomycetes in the marshgrass decay systems were not detectably impacted (75,76). Two implications of these findings are: (*1*) toxic contaminants may readily move through the fungal decay system into the coastal-marine foodweb; (*2*) because saltmarsh ascomycetes are lignocellulolytic, they may be candidates for bioremediation strategies in which polyaromatic pollutants are present (50).

FURTHER READING

For more information on mycelial ascomycetes and straminipiles pertinent to understanding of their marine ecology, additional references (77–85,15,86–94) can be consulted.

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FUNGI IN SOILS. See SOIL FUNGI: NATURE'S NUTRITIONAL NETWORK

FUNGI IN STREAMS. See STREAM MICROBIOLOGY

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G-BACTERIA IN ACTIVATED SLUDGE.

See ACTIVATED SLUDGE — THE "G-BACTERIA"

GALLIONELLA FERRUGINEA: AN IRON-OXIDIZING AND STALK-FORMING GROUNDWATER BACTERIUM

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HISTORY AND TAXONOMIC COMMENTS

The genus *Gallionella* is characterized by its chemolithotrophic growth with ferrous iron and its production of a twisted stalk consisting of a bundle of numerous fibers that makes *Gallionella* very easy to identify. *Gallionella* has been described under several different names such as *Spirophyllum ferrugineum* (1,2), *Didymohelix ferrugineum* (3), *Gloeosphera ferruginea* (4), and *Gallionella filamenta* (5). Most, if not all, of the attempts to categorize species of the genus *Gallionella* under various names arise from observed differences in the appearance of the stalk. The current phylogenetic position of the family *Gallionellaceae* is based on the only 16S-DNA sequence for *Gallionella* available in DNA databases. Additional strains must be sequenced before the diversity of other possible species or genera within this family can be determined.

Ehrenberg was the first to discover the stalks of *Gallionella* more than 160 years ago while studying ochre masses in 1836. In his description (1), Ehrenberg referred to *Gallionella* as a *fossil infusorian*, which he called *die Eisenochertierchen*, the small iron ochre animals. Haeckel (6) presented the first phylogenetic tree that included the kingdom Monera for unicellular organisms in 1866. Zopf (7) first included *Gallionella* with the bacteria in 1879. *Gallionella* has been observed and described by many more scientists from early days and there has been a continuous discussion about its morphology and physiology, but most studies have focused on the stalks. For a summary of the first 100 years of discussions about the intriguing characteristics of *Gallionella*, see Beger and Bringmann (8) and van Iterson (9). Winogradsky (10,11) proposed an autolithotrophic life of the so-called *iron bacteria* including *Gallionella* and listed *Leptothrix, Cladothrix*, and *Gallionella* as examples of lithotrophic iron bacteria. Adler (2) found only small amounts of *Gallionella* in fresh water from Karlspader, but when the water had been left in bottles for several days, the *Gallionella* reproduced profusely or, more precisely, the stalks had become elongated. Lieske (12) succeeded in cultivating *Gallionella* in carbonic water with metallic iron as the ferrous iron source. In 1924–1929, Cholodny made microscopic studies on cover slips that had been submerged in habitats of *Gallionella* (13,14). Admirably accurate sketches of cells attached to the ends of stalks were drawn, showing that the stalk was excreted by the cell and was not a living part of it. Teichmann (15), growing cultures according to the method described by Lieske (12), found a great number of bean-shaped cells in the fluid. This observation influenced Pringsheim (16) to suggest that it is "not impossible that motile cells are formed under certain conditions," a conclusion in accordance with today's knowledge of free-living cells in the exponential growth phase.

In 1953, Beger and Bringmann (8) made comparisons between earlier drawings of the stalk of *Gallionella* and their own transmission electron microscopy studies of stained whole cells and stalks. They proposed that the genus *Gallionella* consisted of five species. Vatter and Wolfe in 1956 (17) presented transmission electron microscopy images of metal-shadowed cells with stalks directly placed upon grids. One year later, Kucera and Wolfe (18) introduced an excellent growth medium containing iron sulfide as the source of ferrous iron. Wolfe could have succeeded in his work with *Gallionella* but for his premature conclusion that "these organisms (i.e., iron bacteria) are too difficult to be profitable" (19). In 1958, a thesis on *Gallionella* presented by van Iterson (9) produced excellent electron microscopy images of the organism using a methodology similar to that used by Kucera and Wolfe described earlier, and it suggested that the stalk was a living part of the organism, with sporangia in the form of membrane sacs on the stalk. Balashova (5,20–22) studied the stalk using transmission electron microscopy with chromium-stained preparations and concluded that it may have zoological forms and budding cells on its cell walls. In 1989, Hanert (23) made detailed light microscopy studies on stalk elongation using single cells and measurements of iron oxidation in both natural samples and laboratory cultures. Evidence of intracytoplasmatic membranes and autotrophic growth was presented. In 1990, Lütters-Czekalla (24) reported growth of *Gallionella* with reduced sulfur compounds instead of ferrous iron as electron donor. This observation remains to be confirmed. In 1990–1995, Hallbeck and coworkers (25–28) reported the 16S-rDNA sequence of *Gallionella*. They showed that, in the exponential growth phase, *Gallionella* has a free-living stage without a stalk. The authors demonstrated chemolithotrophic growth, with carbon dioxide as the sole carbon source, and mixotrophic metabolism. They also demonstrated an organic composition of the stalk and found that the stalk was important for survival in the environments in which *Gallionella* is found. A thesis summarizing these results was published in 1993 (29).

ENRICHMENT, ISOLATION, AND CULTIVATION PROCEDURES

Growth Medium

Various media for enrichment and cultivation of *Gallionella* have been tested. To create proper growth conditions, the medium must contain ferrous iron and carbon dioxide. Lieske (12) designed a culture medium composed of carbonic water and metallic iron. The use of iron sulfide as a source of reduced iron was first suggested by van Niel (17). In 1957, Kucera and Wolfe (18) published a growth medium composed of a salt solution and iron sulfide, which made it possible to obtain pure cultures of *Gallionella*. The salt solution was initially prepared with tap water because the medium lacked a crucial component. This component was later found to be calcium. The ferrous iron medium has since then been widely used, with some minor modifications. Wolfe's modified medium is prepared as follows. Screw-capped tubes (180 × 16 mm) are filled with 10-ml salt medium consisting of 1.0 g NH_4Cl (ammonium chloride), 0.4 g $MgSO_4$(magnesium sulfate) × 7 H_2O, 0.1 g $CaCl_2$(calcium chloride) × 2 H_2O, 0.05 g K_2HPO_4 (Potassium phosphate mono basic) and one liter double-distilled water. The salt medium is autoclaved, chilled to 5 °C and infused with sterile, filtered CO_2 (carbon dioxide) to pH 4.6 to 4.8. A ferrous sulfide or ferrous carbonate precipitate (0.5 ml) is added slowly to the bottom of the tubes with a Pasteur pipette and the tubes are left for 4 to 6 hours to allow gradient conditions to establish before inoculation. The ferrous sulfide and ferrous carbonate have to be prepared in the laboratory. Ferrous sulfide is prepared by dissolving 7.8 g $FeSO_4$ $(NH_4)_2SO_4$ (Mohr's salt) and 4.8 g sodium sulfide each in 200-ml boiling distilled water, and subsequently pouring the ferrous solution into the sulfide solution. Two 500-ml Erlenmeyer flasks are used, and mixing is done with a glass rod. The flasks are filled to the top and sealed with a rubber stopper to prevent oxidation of the ferrous iron. The iron sulfide sediment needs at least 4 hours to settle and is then washed five times with boiling water before it is centrifuged at high velocity. The iron sulfide is collected in small bottles, filled up with water, closed with an airtight lid and sterilized at 121 °C for 20 minutes. The medium is stored cool in airtight vials.

Ferrous carbonate is prepared by dissolving 3.9 g $FeSO_4(NH_4)_2SO_4$ (ferrous sulfate ammonium sulfate complex) and 1.0 g anhydrous Na_2CO_3 (sodium carbonate) each in 100-ml boiling distilled water, and subsequently pouring the carbonate solution into the ferrous solution, preferably under a nitrogen atmosphere. The precipitated ferrous carbonate is then washed five times with boiling double-distilled water, sterilized in closed vials at 121 °C for 20 minutes and stored cool in airtight vials. It is recommended that all solutions used for preparation of the medium and source of ferrous iron are filtered (using a 0.2-μm filter) to remove any cells or particles that may give a background during microscopic counts.

Isolation

Gallionella strains have been isolated successfully by serial dilution and serial transfer. The isolation procedure starts with washing one colony, if the enrichment culture contains colonies attached to the walls of the culture tubes (23). Thereafter, the colony is suspended in 10-ml fresh, sterile medium and inoculated into new test tubes with Wolfe's modified medium at serial dilutions in ten times increments. The dilution series is made up to ten times the inverse of the total number of cells per ml. One milliliter is used as inoculum from each dilution and introduced into several parallel culture tubes. Five to ten serial transfers, each starting from one colony, are necessary to achieve pure cultures in this manner. This procedure requires up to ten weeks but is quite certain to continually reduce the number of contaminants and obtain a pure culture. Purity is checked microscopically using a variety of heterotrophic and autotrophic media, such as yeast extract bouillon, nutrient agar, *Nitrosomonas* medium, and *Thiobacillus ferrooxidans* medium.

Maintenance

Pure stock cultures of *Gallionella* strains can be maintained for years using the described culture conditions with serial dilution transfers every four to eight weeks. Nunley and Krieg (30) reported preservation of *Gallionella* culture material for at least 13 weeks by freezing at −80 °C in 15% glycerol, although this procedure did not result in survival of the strain cultured by the authors.

DESCRIPTIVE CHARACTERS

Morphology

The size, shape, and ultrastructure of *Gallionella* are illustrated in Figures 1 and 2. The cell is gram-negative, bean-shaped, and usually 0.5–0.8 × 1.6–2.5 μm in size. It secretes an extracellular twisted stalk from the concave side, 0.3 to 0.5 μm in width and up to 400 μm or more in length. The stalk is composed of

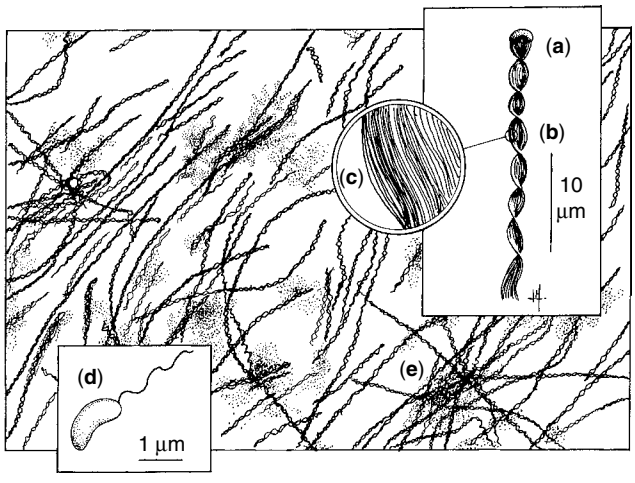

Figure 1. *Gallionella ferruginea* cell morphology, flagella position in cell suspension, and stalk arrangement. The bean-shaped cell of *G. ferruginea* (**a**) forms stalks (**b**) of a bundle of 2-nm wide fibers (**c**). Motile cells with one polar flagellum (**d**), and without a stalk, have been reported. Iron hydroxides precipitate on and around the stalks (**e**).

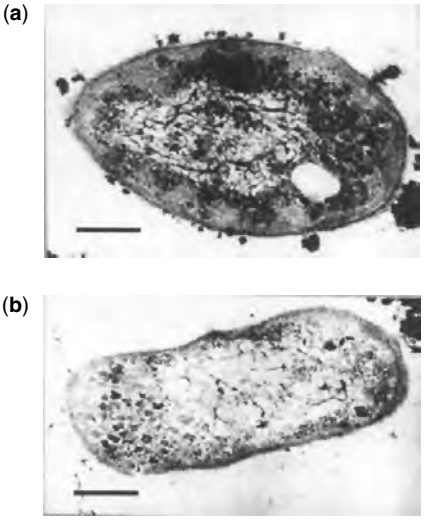

Figure 2. Ultra-thin section of *G. ferruginea* strain Johan in the stationary phase. (**a**) Cross-section of strain Johan with outer cell wall membrane. Bar = 0.2 μm. (**b**) Longitudinal section of strain Johan. Bar = 0.25 μm.

a number of 2-nm wide fibers and is produced under microaerophilic conditions when cells are in the late exponential or stationary growth phase. Cells may have a polar flagellum (17). The stalk becomes continuously encrusted with precipitated ferric iron oxides that may totally cover old stalks. The composition of the stalk has not been conclusively demonstrated, but inorganic as well as organic compositions have been suggested. Hanert (23) proposes that the stalk consist of colloidal ferric hydroxide based on observations of it being dissolved in 0.12% sodium thioglycolate. Hallbeck and Pedersen (27) reported a higher carbon-to-nitrogen ratio (C : N = 6.8) in stalk-forming cultures compared with a non-stalk-forming culture (C : N = 4.8), and concluded that the stalk is composed of an extracellular carbon skeleton, probably a carbon polymer.

Two strains of *Gallionella* have been described and published (Table 1). Strain BD from a drainpipe in Braunschweig was described by Hanert (23) and strain Johan from a 60-m deep drinking water well was described by Hallbeck and Pedersen (25–27) and Hallbeck and coworkers. (28). Strain BD was reported to have intracytoplasmatic membranes (23), whereas strain Johan does not have such membranes (Fig. 2). The isolation procedure for *Gallionella* is by serial dilution and the possibility of contaminants contained in the cultures cannot be excluded conclusively. Serial-thin sectioning should show a stalk connected to the sectioned cell to confirm it to be a cell of *Gallionella*, as in Figure 3(a–c). Inclusions that may be poly-β-hydroxybutyrate have been noted but apart from this, strain Johan does not show any specific fine intracellular structures (Fig. 2).

Growth and Physiology

Gallionella can be cultured in vitro in screw-capped test tubes using oxygen and ferrous iron concentration gradients in a salt medium, with carbon dioxide as sole carbon source (Fig. 4), as described earlier. Solid-phase ferrous iron is placed at the bottom of the tube in a fresh, autoclaved and oxygen-free salt medium. Iron sulfide or iron carbonate can be used as a source of ferrous iron. With this procedure, the concentration of dissolved ferrous iron will decrease from the bottom of the tube and upward as it dissolves and diffuses away from the solid phase. Oxygen will decrease in concentration from the top of the tube

Table 1. Differential Characteristics of *Gallionella Ferruginea* Strains Johan and BD

Character	Strain Johan[1]	Strain BD[2]
Cells bean-shaped	Yes	Yes
Diameter (μm)	0.5–0.8	0.5–0. 7
Length (μm)	1.6–2.5	0.8–1.8
Motility without stalks	Yes	Yes
Motility with stalks	No	No
Temperature range for growth (°C)	5–25	ND[a]
Optimum temperature (°C)	20	17
pH range for exponential growth	5.0–6.5	ND
Generation time in exponential growth (h)	8.3	ND
Stalks not produced in exponential growth phases	Yes	ND
Maximum cell number in in vitro culture (cells/ml)	5×10^6	ND
Length of stalks (μm)	Average 60 per cell	Up to 400 for one cell
Colony form in vitro	A ring on the tube wall	Circular colonies
Growth with carbon dioxide as sole carbon source	Yes	Yes
Growth with ferrous iron as sole energy source	Yes	Yes

[1]Strain Johan was described by Hallbeck and Pedersen (25–27), and Hallbeck and coworkers. (28).
[2]Strain BD was described by Hanert (23).
[a]Not determined.

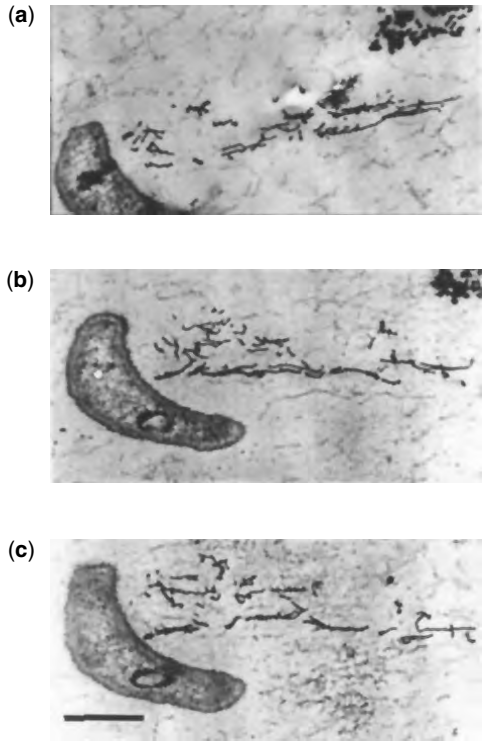

Figure 3. Serial-thin sections of a cell with an adjacent stalk. Bar = 0.5 μm.

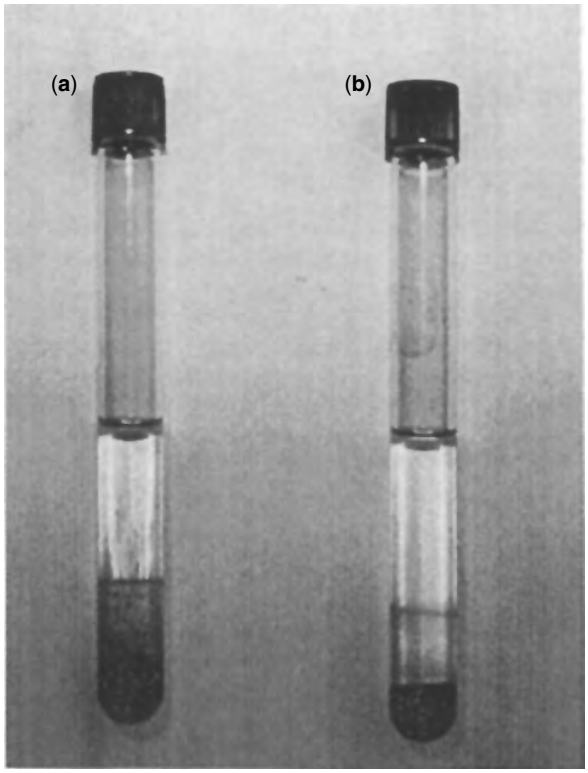

Figure 4. Screw-capped gradient culture tubes with 2-week-old growth of *G. ferruginea*. Iron sulfide as a source of ferrous iron is placed at the bottom of the tubes. (**a)** Growth of a stalk-forming strain. (**b)** Growth of a non-stalk-forming strain.

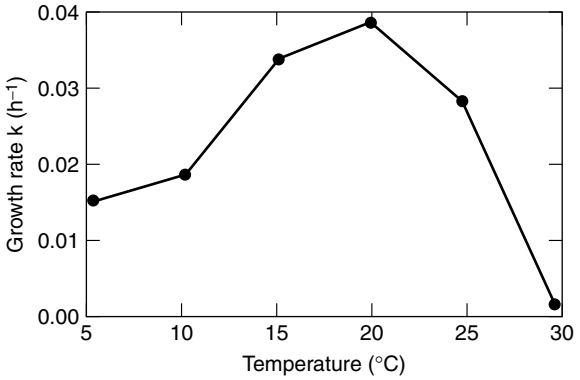

Figure 5. The growth rate (k) of *G. ferruginea* under aerobic culture conditions in the temperature range of 5–30 °C (*Source:* Hallbeck and Pedersen (25)).

downward as it diffuses into the salt medium from the air above. An optimum growth situation will develop in this gradient tube and *G. ferruginea* cells and stalks will appear in the gradient at the position most favorable to growth. Eventually, in the tube additional rings of growth may form in new places as the concentration gradient changes with aging of the culture.

The optimum growth temperature is 17–20 °C and temperatures above 25–30 °C are lethal (Fig. 5). The culture grows to a maximum of 5×10^6 cells/ml culture, which makes many traditional techniques for strain characterization impossible. Strain BD develops small, circular colonies attached to the wall of the tube within three to five days after inoculation. Strain Johan predominantly forms a ring at a specific level in the concentration gradient (Fig. 4). The cells of strain Johan colonize as new rings upward in the tube as the culture gets old, that is, ten days or older. Eventually, most of the tube will be filled with a brittle mass of stalks, iron oxides, and cells. Hallbeck and Pedersen (25) have demonstrated that *Gallionella* strain Johan is free-living in vitro in its exponential growth phase and does not produce stalks until its late exponential and stationary phases, when pH exceeds 6 (Fig. 6). Most stalk formation and iron oxidation occur at circum-neutral pH (31). The generation time at optimum temperature for strain Johan is 8 hours. Stalk production continues for many days in the stationary phase (25). Some cell division may still occur but not at the growth rate observed during the first four to five days after inoculation. The use of the term *stationary phase* implies that cell division ceases, but cell activity does not necessarily cease. Instead, the cell appears to enter a secondary phase, during which it allocates its metabolic activity to stalk production instead of cell formation. This type of metabolic switch is commonly observed among microbes producing antibiotics. The maximum mean stalk length per cell of strain Johan has been determined to be 60 μm in a 16-day-old culture (Fig. 6). Individual cells may produce a much longer stalk, with 400 μm having been reported for a single cell of strain BD (23). With prolonged sub-culturing on iron carbonate as energy source, some of the *Gallionella* strain Johan cultures have been reported to irreversibly lose their ability to form a stalk (28). Their

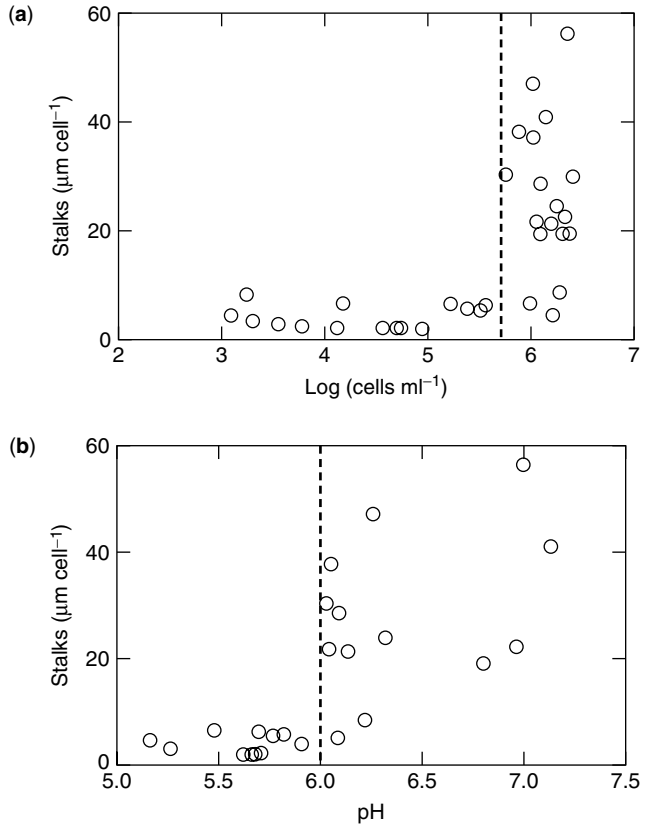

Figure 6. (**a**) Relation between total number of cells and stalk length per cell in growth experiments with *G. ferruginea* under aerobic gradient growth conditions. Data from four experiments. (**b**) Relation between pH and stalk length per cell in growth experiments with *G. ferruginea* under aerobic growth conditions. Data from three experiments (*Source:* Hallbeck and Pedersen (25)).

identity was confirmed by 16S-DNA sequencing. They still form a ring of oxidized iron, as does the stalk-forming variant, but the ring is very thin (Fig. 4).

Gallionella is chemolithotrophic, with ferrous iron as its energy source and electron donor, and with carbon dioxide as its sole carbon source. Carbon dioxide fixation by strain Johan was revealed using hydrogen [^{14}C]-carbonate (26), whereas in vivo activity of the Calvin cycle key enzyme, ribulose biphosphate-carboxylase, has been reported for strain BD (23). Mixotrophic metabolism has been shown on glucose, fructose, and sucrose although growth did not occur on these sugars without ferrous iron and carbon dioxide. Ammonium and nitrate can be used as nitrogen sources; the capacity for nitrogen fixation is not known.

Phylogeny

In 1993, the 16S-rRNA gene of *G. ferruginea* (strain Johan) was published between base numbers 47 and 1405 (*Escherichia coli* numbering) (28) Phylogenetic analysis of this sequence has placed *Gallionella* among the β-proteobacteria. The sequence was distant from other species sequences in the tree, with a 10% difference to the most closely related genus, the chemolithotroph *Nitrosospira multiformis* (32). The remote position of *G. ferruginea* in relation to other species, and its utilization of iron as its energy source and electron donor as opposed to ammonia for the closest gene cluster, *Nitrosomonadaceae*, motivates the designation of a separate family for the genus *Gallionella, Gallionellaceae*. At present, there is only one known species in this family. The capacity of chemolithotrophic iron oxidation among bacteria has previously been suggested to be evolutionarily widespread (33). This is confirmed by, for instance, the phylogenetic distance (85% in terms of its 16S-rRNA gene identity) between *Gallionella* and the iron-oxidizing genus *Thiobacillus*.

Working with *Gallionella ferruginea*

Gallionella ferruginea has a generation time of 8 hours at 20 °C and grows to at most 5×10^6 cells/ml after 80 hours. The difference between working with a slow-growing organism such as *G. ferruginea* and a fast-growing bacterium such as *Escherichia coli* is striking, as demonstrated by the following calculations. A growth experiment with *G. ferruginea* starts with 1×10^3 cells/ml and it grows at most to 5×10^6 cells/ml. An *E. coli* experiment may start at 10^6 cells/ml and within three and a half hours, it easily grows to 10^9 cells/ml. These numbers illustrate that many experiments that can be performed easily with *E. coli*, are difficult or even impossible and in all cases, very time-consuming if applied to *G. ferruginea*. For instance, biochemical analytical procedures commonly require cell amounts in the milligram to gram range. One gram of *E. coli* cells can be obtained by growing 1 l of culture left overnight. To obtain 1 g of *G. ferruginea*, 250,000 culture tubes (Fig. 4) have to be prepared and cultured for five to six days. The cells will be in 2,500 liters of medium, together with more than 50-kg ferrous iron oxide and a great number of stalks. For interest, approximately 12,000 tubes, with about 6-kg iron sulfide and 120 liters of the mineral salt solution, were used to produce the data published in Hallbeck and coworkers (25–28). The total weight of all *G. ferruginea* cells in these experiments was 0.05 g.

ECOLOGY

The environments where stalk-forming *Gallionella* can be found, commonly attached to surfaces, are in slow-flowing groundwater that is rich in ferrous iron but has a low organic carbon content and a low oxygen tension. Typical places to search for *Gallionella* are the insides of drainpipes, storage basins for groundwater from deep wells, and inside tunnels and on rock walls with seeping groundwater. A common feature of these environments is that cold (i.e., below 20 °C), reduced, anaerobic, and ferrous iron–bearing groundwater reaches an atmosphere that contains oxygen. Such environments are suitable for chemolithotrophic growth with ferrous iron as the energy source and the electron donor and with oxygen as electron acceptor. A flow appears to be an absolute prerequisite to the formation of *Gallionella*, continuously

supplying ferrous iron and possibly also carbonate from the groundwater to the attached cells. Under such conditions, the stalk may act as a holdfast and prevent the cells from being washed out to a more oxidized environment that does not contain ferrous iron.

Hallbeck and Pedersen (27) have demonstrated an additional function for the stalk. The iron oxidation that occurs in a typical *Gallionella* environment may be divided into two processes namely: (*1*) the respiratory iron oxidation performed by the cells in their energy metabolism, and (*2*) the nonmetabolic iron oxidation induced by the increasing oxygen tension as the anoxic groundwater reaches the atmosphere. Ferrous iron reacting with oxygen participates in a chain of reactions yielding highly reactive oxygen species such as perhydroxyl (HO_2), hydrogen peroxide (H_2O_2), and the hydroxyl radical (HO) (34). The survival of the stalk-forming (Sta^+) *Gallionella* strain Johan in media with a low or high potential for oxygen radical formation was compared with that of a variant of strain Johan, which irreversibly lost its ability to form a stalk (Sta^-) (27). It was found that *Gallionella* Sta^+ survived longer (nine weeks) than did Sta^- (six weeks) in cultures with a high potential for oxygen radical formation. Therefore it was suggested that the stalk of *Gallionella* protects the cells against the toxic oxygen species discussed earlier, by directing the oxidation of iron to the stalk. This phenomenon could be compared with the action of the protein ferritin, proposed to perform iron oxidation in both prokaryotic and eukaryotic cells (35). Whether the iron oxidation on the stalk is enzymatic or the stalk acts as a surface catalyst for the oxidation reaction is not known.

In conclusion, the stalk acts as a holdfast and gives *Gallionella* the unique possibility of colonizing and surviving in an ecological niche with high contents of ferrous iron and with some oxygen, a niche that is inaccessible to bacteria without a defense system against the oxygen radicals formed during inorganic oxidation of ferrous iron.

Microbial Mats and Biofilms

Systematic studies of *Gallionella* in natural and laboratory environments have been few. Emerson and Revsbech (36) reported the occurrence of *Gallionella* spp. in an iron-oxidizing microbial mat. They found *Gallionella* to predominate closer to where the groundwater emanated from the well. It was here that the water had the lowest concentrations of oxygen, confirming the microaerophilic nature of *Gallionella*. The authors continued their studies in the laboratory, constructing a chamber that mimicked the situation on the studied site (37). They concluded that the studied bacterial consortium was responsible for most of the observed iron oxidation. The other dominating species studied were *Lepthotrix ochracea* and members of the family *Siderocapsa* were also observed.

The formation of biofilms of *Gallionella* on surfaces has been reported earlier (27,38). Water from 40 to 60 m deep wells was pumped over test surfaces and in less than 48 hours, dense biofilms of stalks and cells developed. They were up to 30-μm thick, showing the growth potential of this bacterium. The Äspö Hard Rock Laboratory (HRL) tunnel, Äspö, Sweden (39), is heavily populated with mats of *Gallionella* that can reach 10 cm or more in thickness. Stones and rock surfaces in pools and ditches in the tunnel are commonly covered with moss-like biofilms of *Gallionella*, demonstrating underground tunnels, mines, and so on to be excellent sites for studies of processes related to this organism, such as metal accumulation.

Metal Accumulation on the Stalks

The stalks of *Gallionella* attract large amounts of iron hydroxides and hydroxides of other metals also. The processes governing this coating are not well established, although several intriguing theories exist. It can be anticipated that enzymatic ferrous iron oxidation by *Gallionella* in its extraction of electrons for energy production takes part in the build-up of metal oxides on the stalks. By this process, oxidized, ferric iron from this metabolic process would be expelled and adsorbed on the stalk. In addition, as suggested earlier, the stalk may act as a protective agent against the creation of toxic levels of oxygen radicals. The stalk would then have a ferrous iron oxidation capability and a sorption capacity for ferric iron hydroxides and for other metals. An interesting function of the stalk, which is in line with these assumptions, has been suggested by Banfield and coworkers. (40). This group discovered ferrihydrite nanocrystalline growth in samples containing *Gallionella* stalks and suggested that the stalks attract or flocculate 2 to 3-nm ferrihydrite particles formed by the cells' enzymatic oxidation of ferrous iron. The stalk then governs an aggregate-based growth of polycrystalline materials. Surfaces of nanocrystalline iron oxyhydroxides, for example, goethite formed from ferrihydrite are active adsorption sites for metals at neutral to alkaline pH. Consequently, *Gallionella* may have an important influence on the fate of dissolved metals in natural groundwater, as discussed later.

The transport, chemical speciation, and ultimate fate of dissolved metals in aqueous systems are largely controlled by reactions that occur on solid surfaces (34). Recognition of the importance of solid-phase reactivity in aqueous geochemistry has fostered the development of the surface complexation-precipitation theory (SCPT) as the leading model for understanding the behavior of dissolved metals in pristine and contaminated waters (34,41). This concept embraces the principles of thermodynamics and chemical equilibria to predict when solid-phase partitioning of metal ions is likely to occur in response to sorption, and to quantify subsequent surface precipitation reactions. The SCPT has thus far been applied almost exclusively to minerals, particularly to hydrous iron oxides (41); however, Warren and Ferris (42) recently demonstrated that a continuum exists between ferric iron sorption and precipitation reactions on bacterial surfaces, as anticipated with SCPT. Pedersen and Albinsson (43) reported a similar process with the iron-reducing bacterium *Shewanella putrefaciens*. This is an important step forward as bacteria are at least as widely distributed in aqueous systems and probably as reactive as are many inorganic solids. Moreover, if SCPT is to emerge

as a true guiding paradigm for aqueous geochemistry, it must be firmly established to be applicable to both organic and inorganic solids.

The behavior of bacteria as geochemically reactive solids can be inferred from extensive research documenting their performance as sorbents of dissolved metals, and nucleation templates for a wide range of authigenic minerals (43–46). This reactivity stems directly from the presence of amphoteric surface functional groups (i.e., carboxyl, phosphoryl, and amino constituents), which are associated with structural polymers in the cell walls and external sheaths, capsules, and stalks of individual cells (43). Direct interaction between these surface functional groups and dissolved metals accounts for the sorptive properties of bacteria, whereas surficially sorbed metals provide discrete sites for subsequent mineral nucleation and precipitation reactions (43).

Because of their ubiquitous distribution and reactive surface properties, hydrous iron oxides are considered to be dominant sorbents of dissolved metals in aquatic environments. This perception is somewhat tempered by work that shows that natural iron oxides often contain significant amounts of silica (e.g., siliceous ferrihydrite) and sulfate (e.g., jarosite and schwertmannite), as well as organic matter, including intact bacterial cells (44). This intermixing of compositionally variable iron oxides and organic matter produces composite multiple sorbent solids with highly variable metal retention properties, referred to as *bacteriogenic iron oxides* (BIOS).

Bacteriogenic iron oxides and groundwater samples were collected underground at the Stråssa mine in central Sweden and from the Äspö HRL tunnel. ferrous iron-oxidizing bacteria, including stalked *G. ferruginea* and filamentous *Leptothrix* sp., were prominent in the BIOS samples from Stråssa, whereas *G. ferruginea* dominated in the Äspö HRL samples. The goal of these investigations was to understand the accumulation of various metals by BIOS. Strontium, cesium, lead, and uranium were studied in the Stråssa BIOS (47), and sodium, cobalt, copper, chromium, and zink were studied in the Äspö HRL BIOS (48).

The BIOS samples were found to contain only amorphous hydrous ferric oxide, as determined by X-ray diffraction. Inductively coupled plasma mass spectroscopy revealed hydroxylamine-reducible iron and manganese oxide contents ranging from 55% to 90% on a dry weight basis. Distribution coefficients (K_d values), calculated as the ratio between the BIOS and dissolved heavy metal concentrations, revealed solid-phase enrichments of 10^0 to 10^5, depending on the metal and iron oxide content of the sample. At the same time, however, a strong inverse linear relationship was found between log K_d values and the corresponding mass fraction of reducible oxide in the samples, implying that the metal uptake was strongly influenced by the relative proportion of bacterial organic matter in the composite solids. On the basis of the metal accumulation properties of the BIOS, an important role can be inferred for intermixed iron oxides and bacterial organic matter in the transport and fate of dissolved metals in groundwater systems.

CONCLUSION

Gallionella ferruginea is a gram-negative, bean-shaped, iron-oxidizing bacterium that secretes an extracellular twisted stalk composed of numerous fibers. The stalk is produced under microaerophilic conditions when cells are in the late exponential or stationary growth phase. The phylogenetic position of *G. ferruginea*, as determined by 16S-DNA sequence comparisons, is among the β-proteobacteria. The bacterium is a member of the family of *Gallionellaceae*, with one known species, *G. ferruginea*. The most closely related species, according to 16S-rDNA sequence data, is the chemolithotroph *Nitrosospira multiformis*, which with a 16S gene similarity of 90% is only distantly related. *Gallionella ferruginea* is motile by means of a polar flagellum. Microaerophilic, chemolithotrophic growth can be obtained in vitro by using oxygen and ferrous iron concentration gradients in a salt medium, with carbon dioxide as the sole carbon source. Mixotrophic metabolism has been demonstrated with glucose, fructose and sucrose. *Gallionella ferruginea* can be found where anaerobic groundwater with ferrous iron reaches an environment that contains oxygen. Commonly, large amounts of stalk material are produced, which attract iron hydroxides and many different trace metals, giving it a brown, macroscopic appearance. The stalk and iron hydroxide masses formed may cause severe clogging of ditches, drinking water wells, and any other facilities utilizing iron-bearing, anaerobic groundwater eventually.

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GASES, TRACE. See Trace Gases Soil

GENE CHIPS. See Biochip-Based Devices and Methods in Microbial Community Ribotyping; Microarrays: Applications in Environmental Microbiology

GENE EXCHANGE IN BIOFILMS

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Microorganisms in many natural and engineered systems grow at solid–liquid, solid–gas, liquid–liquid, or liquid–gas interfaces. These microbial aggregates are called *biofilms* and contain entrapped particles of both organic and inorganic origin, extracellular polymeric substances (EPSs), and water (1). They form spatially differentiated multispecies communities. EPSs are composed of polysaccharides, proteins, nucleic acids, and other amphiphilic polymeric compounds (2); they play a pivotal role in microbial interactions with interfaces and serve in binding cells and other particulate materials together (3). Owing to the close proximity of cells, biofilms are likely candidates for multicellular communication and coordination. One such form of interaction is the exchange of genetic information, and mobile genetic elements represent an important mechanism in the adaptation of populations to environmental changes.

The principle of horizontal gene transfer, that is, a change in the genetic makeup of a cell by means of acquisition of foreign DNA, as compared to vertical gene transfer during cell division in the bacterial domain has been known since the 1940s. At that time Avery and coworkers (4) reinterpreted an earlier experiment by Griffith (5) and first demonstrated the uptake of free DNA, thereby providing evidence that DNA contained hereditary information. In biofilms, horizontal gene transfer among bacteria is beneficial because it increases metabolic capabilities and helps the biofilm community adapt to changing environmental conditions. In natural systems, conditions are frequently oligotrophic and bacterial growth rates are low. Because bacterial cells divide by binary fission, thus enabling vertical gene transfer, with only a handful of daughter cells receiving chromosomal mutation, the evolutionary significance of horizontal gene transfer is considered to be substantial. In open engineered systems, such as wastewater treatment plants, cell densities and growth rates are much higher. Here, the impact of horizontal gene transfer may be compounded by a rapid division of transconjugants passing on their newly acquired genetic information to the next generation.

The role of genetic exchange processes in the environment has only begun to be investigated fairly recently (6), mostly in relation to the biosafety aspects concerned with the release of genetically modified microorganisms. In many ways, the use of bioengineered organisms in applied technologies involving less than absolute control of the employed biota has been hampered by uncertainties regarding the fate of genetic information in the environment. Until quite recently, available information did not

allow an exact estimate of the likelihood of gene transfer in various habitats. This has led to numerous risk assessment scenarios and the use of bacterial plasmids as vehicles for foreign DNA, which are restricted in their host replication range and their ability to be transferred. Horizontal gene transfer in biofilms can be considered highly likely because of high cell densities, the long retention time of cells, their relative immobility, and the fact that DNA can adsorb to surfaces. However, very little research on gene transfer in biofilms has been carried out to date despite the obvious importance of gene flux for microbial taxonomy and ecology. In fact, the existence of a fixed biomass or biofilm has often been ignored in the characterization of environmental samples. In this article, the focus is mainly on the role of gene transfer in natural and engineered systems in which the establishment and role of biofilms has been experimentally verified. Further, a distinction will be made between those studies dealing with gene exchange in biofilms based on average transfer frequencies and rates and recent reports on the extent of transfer as measured microscopically, in situ, at the single-cell level in undisturbed biofilms.

MECHANISMS OF GENE TRANSFER

There are three major mechanisms by which gene exchange in the environment can occur: (*1*) conjugation, in which DNA is transferred from a donor to a recipient cell by cell-to-cell contact; (*2*) transformation, in which extracellular DNA is taken up by competent cells; and (*3*) transduction, in which bacteriophages act as vectors for transfer of bacterial DNA to a new host.

Conjugation

During conjugation, plasmids are transferred from a donor to a recipient cell. This form of gene transfer requires physical contact and places the greatest demands on donor and recipient cells. The information to be transferred must reside on a plasmid or transposon, and both cell types must be metabolically active. Cell contact is established via conjugative pili, which are encoded by the tra genes (Fig. 1). Some smaller, naturally occurring plasmids do not contain a complete tra system. They can be transferred by the process of mobilization (7), provided they carry their own *oriT* site as well as genes required for conjugative DNA processing (mob region) (Fig. 2). Plasmids can also integrate into the host chromosome via recombination. If the plasmid later becomes excised, it may carry chromosomal genes, which will be transferred to a recipient during conjugation. Conjugative transfer can be inhibited by DNA restriction, surface exclusion (8), incompatibility of plasmids, or fertility inhibition (9). It is a relatively nonspecific process, which can occur between distantly related bacteria (10). Certain plasmids belonging to the incompatibility groups IncQ and IncP have been transferred from *Escherichia coli* to bacteria as distant as the Chlorobiaceae, that is, green sulfur bacteria. Conjugation systems in gram-negative bacteria are frequently but not universally repressed, and can be activated by stress factors including temperature, UV-irradiation, or the antibiotic erythromycin.

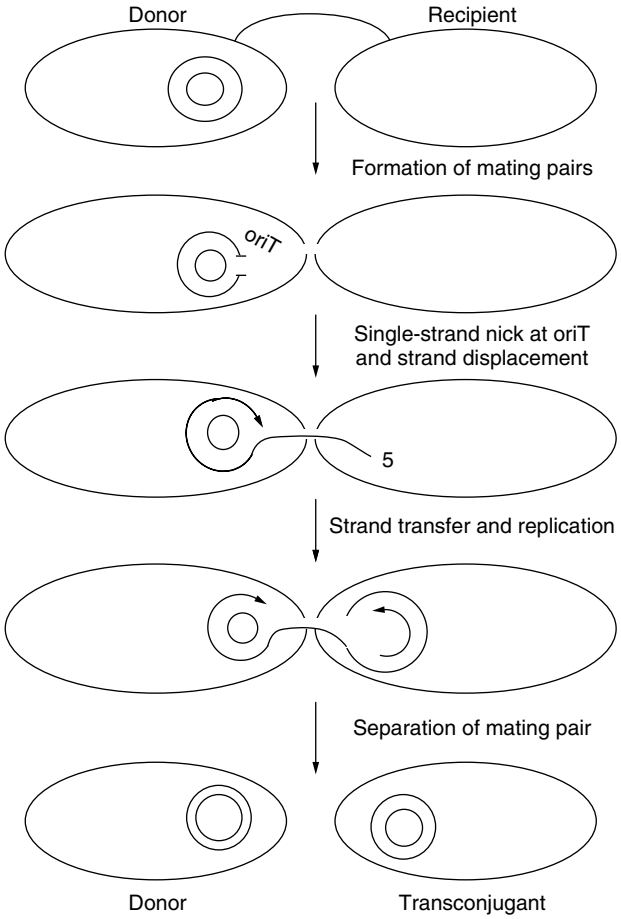

Figure 1. Mechanism of DNA transfer during conjugation. The donor cell produces a pilus, which is encoded by the plasmid and contacts a potential recipient cell that does not contain the plasmid. Retraction of the pilus brings the cells into close contact and a pore forms in the adjoining cell membranes. Formation of the mating pair signals the plasmid to begin transfer from a single-stranded nick at oriT. The nick is made by plasmid-encoded tra functions. The 5′ end of a single strand of the plasmid is transferred to the recipient through the pore. During transfer, the plasmid in the donor is replicated, its DNA synthesis being primed by the 3′ OH of the *oriT* nick. Replication of the single strand in the recipient proceeds by a different mechanism with RNA primers. Both cells now contain double-stranded plasmids, and the mating pair separates. *Source:* redrawn after Ref. 11 with permission from the American Society for Microbiology, © 1997.

Gram-positive organisms including *Streptococcus*, *Staphylococcus*, *Enterococcus*, *Bacillus*, *Streptomyces*, *Nocardia*, and *Clostridium* spp. are known to carry transmissible plasmids, but apparently conjugation does not involve the formation of pili (9). One conjugation system specific for enterococci is inducible by signal peptides ("clumping inducing agents") that are excreted by recipients. These molecules then subsequently entice the plasmid-carrying donor cell to synthesize surface proteins involved in cell clumping. For *Enterococcus faecalis*, clumping is not observed below temperatures of ca. 12 °C. In streptococci and staphylococci, plasmid transfer by conjugation in the laboratory requires a solid surface, but the mechanisms of transfer have not been elucidated.

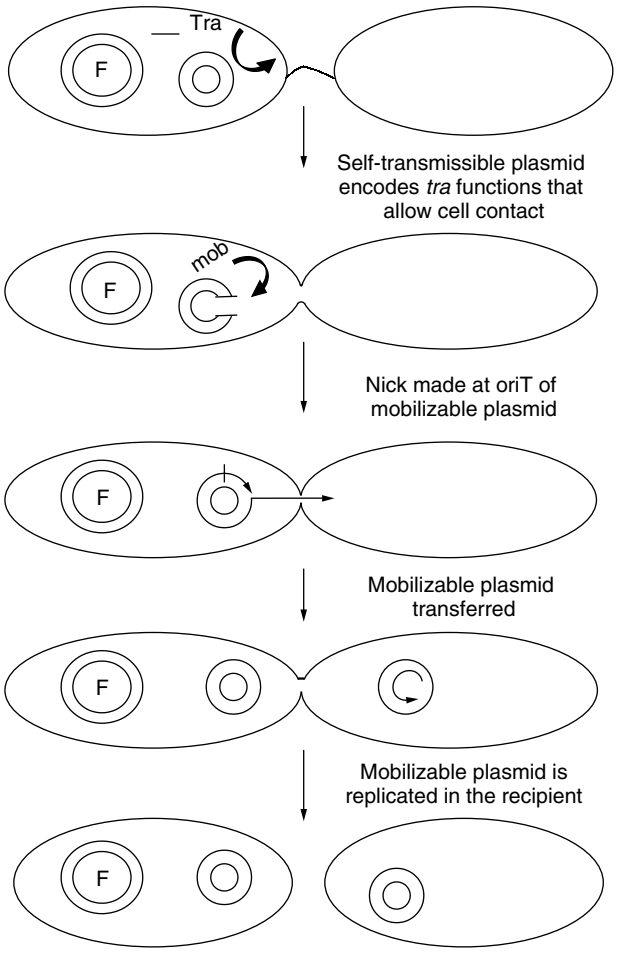

Figure 2. Mechanism of plasmid mobilization. The donor cell carries two plasmids, a self-transmissible plasmid, F, which encodes the tra functions that promote cell contact and plasmid transfer, and a mobilizable plasmid (blue). The mob functions encoded by the mobilizable plasmid make a single-stranded nick at oriT in the mob region. Transfer and replication of the mobilizable plasmid then occur. The self-transmissible plasmid may also transfer. *Source:* redrawn after Ref. 11 with permission from the American Society for Microbiology, © 1997.

Conjugation has been shown to occur in aquatic systems (12), including wastewater and sludge (13–15), in soil (16), on plant leaves (17), and in each of the intestinal, urogenital, and respiratory tracts (18,19); it may be the most important gene transfer mechanism in the environment because many plasmids are known to have a broad host range and the ability to transfer plasmids is widespread among bacteria.

Conjugative transfer of plasmids in biofilms under environmental conditions has been demonstrated with an elegant method called *exogenous isolation* (20). The procedure calls for the addition of a genetically characterized recipient bacterial strain to a mixed natural bacterial community present in the epilithon, the biofilm present on river stones. The method uses suspended cells rather than intact biofilms (Fig. 3), and the new plasmid to be transferred into the recipient is obtained by applying a direct selection for markers, such as Hg resistance (21). Another version of this method involves tripartite exogenous isolation of plasmids (Fig. 3) (22). Here, known recipient and donor strains are mixed with the suspended epilithic community and incubated together. The donor contains a small, mobilizable plasmid, which cannot transfer itself (mob^+tra^-). During incubation with the epilithic community, another plasmid, which is tra^+, must enter the donor strain and then mobilize the tra^- replicon into the plasmid-free recipient. Importantly, this procedure does not involve selective pressure on the tra^+ plasmid, and hence a variety of plasmids are obtained without any predetermined phenotype. This method has also been used successfully under environmental conditions, that is, in a river (23).

To approximate conditions as they occur in biofilms, another method was devised in which donor and recipient bacteria are placed onto membrane filters to be incubated on stones (membrane method, Fig. 4). Alternatively, bacteria have been introduced directly into the epilithic bacterial community on separate stones (stone method, Fig. 4). Next, the two stones are brought into contact in the river. Plasmid transfer is confirmed by plating presumptive transconjugants on selective agar plates. Hence, conjugative plasmid transfer can occur in natural biofilms in an environmental setting such as river stones. The transfer frequencies in various microcosms and in rivers have been assessed using the stone and filter methods. They varied from 10^{-2} to 10^{-8} transconjugants per recipient and were greatly affected by temperature (Table 1). Frequencies were lower or higher in situ than on agar plates in the laboratory depending on the microorganisms tested, indicating that additional species-specific factors influence conjugation in environmental biofilms. Exogenous isolation has also been used on disrupted epilithon and air–water interface samples, which had been filtered onto membranes and floated on artificial seawater (25). Frequencies were in the order of 10^{-8} and 10^{-9} recipients per filter for the biofilm and the air–water interface samples, respectively.

In the terrestrial environment, the rhizosphere (root microenvironment), phylloplane (leaf surface microenvironment), and, to a lesser extent, soil particles may represent favorable conditions for biofilm formation. On the phylloplane, conjugation is stimulated by leaf exudates, such as carbohydrates, amino acids, and organic acids, and takes place primarily in junctions between epidermal cells and in substomatal cavities (17). Similarly, gene transfer is stimulated by root surfaces (32). A minimum water content or relative humidity is important for cell survival, but above this level they do not have a direct effect on conjugation frequencies. Clustering of cells into interstices found on the phylloplane can lead to transfer frequencies that are higher than those found on filters placed on agarose (17). The metabolic activity of bacteria is not rate limiting in specialized niches like the phylloplane and the rhizosphere (33), as long as a certain threshold activity is maintained.

An example of a specialized and selective environment for biofilms is the mammalian intestine (19). Plasmid transfer takes place in the viscous mucus layer covering the epithelial cells. On the basis of selective enumeration of transconjugants in the feces of streptomycin-treated

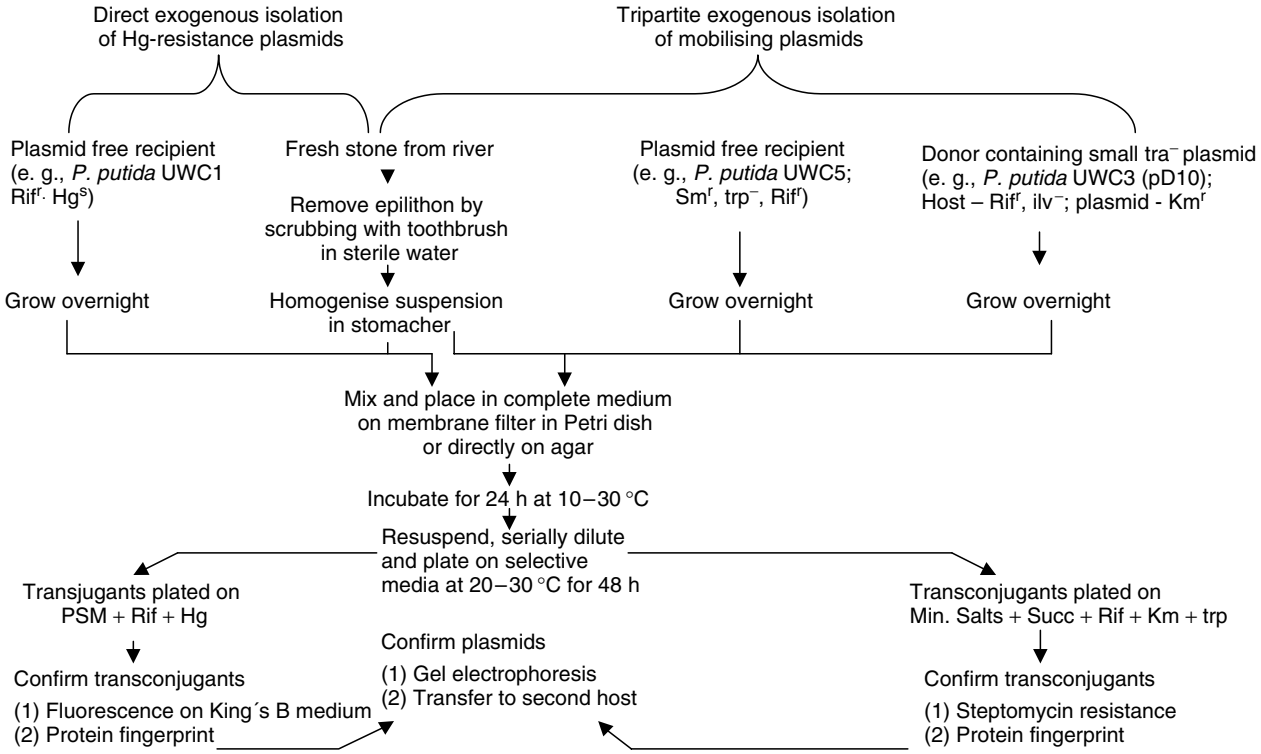

Figure 3. Schematic diagram for the exogenous isolation of plasmids from river epilithon by the direct (24) and tripartite (22) approaches. Rif^r, rifampicin resistant; Strep^r, streptomycin resistant; trp$^-$, tryptophane deficiency; ilv$^-$, isoleucine, leucine, and valine deficiency; Succ, succinate; Hg^s, sensitive to mercury; PSM, *Pseudomonas* selective medium. *Source:* redrawn after Ref. 21 with permission from BioLine, © 1994.

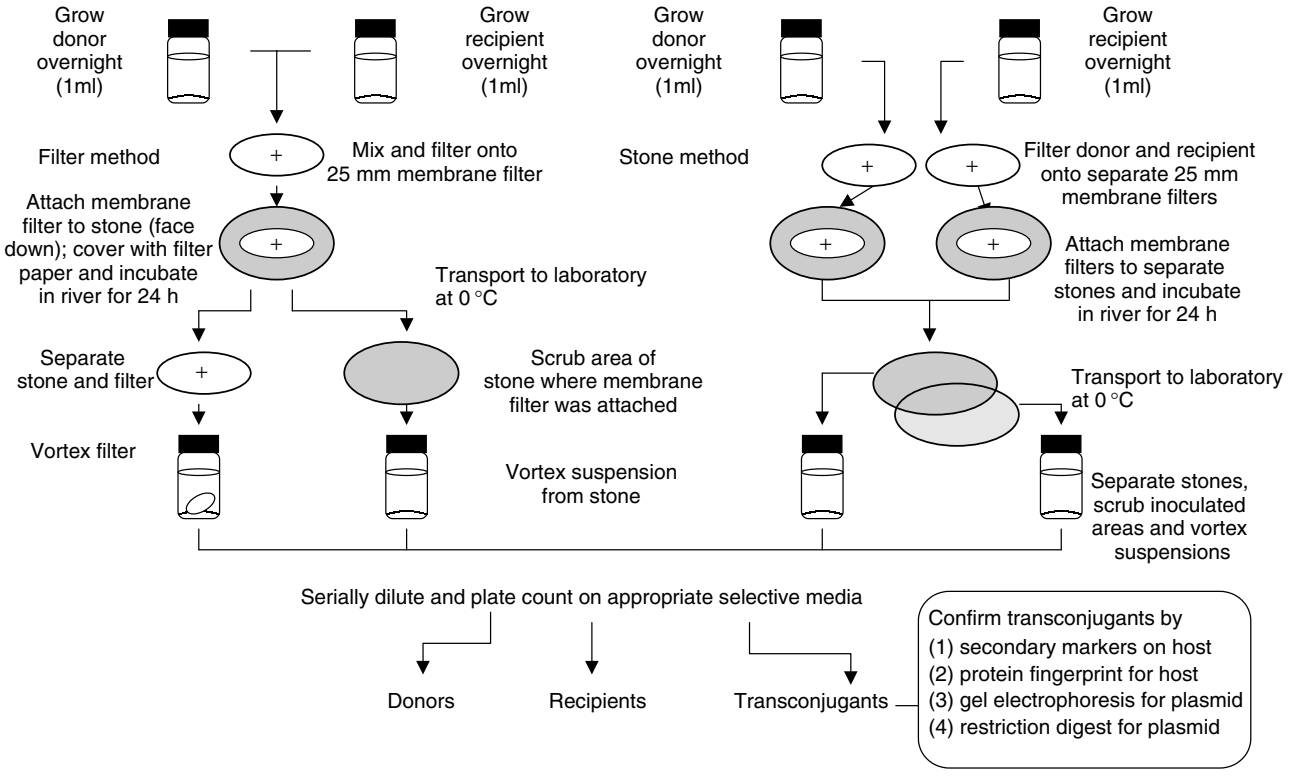

Figure 4. Schematic diagram for estimating plasmid transfer between donor and recipient bacteria with the filter (23) and stone (26) methods. *Source:* redrawn after Ref. 21 with permission from BioLine, © 1994.

Table 1. Maximum Plasmid Transfer Frequencies Between Bacteria in River Biofilms with Indigenous Bacteria Present

Bacteria[a] (Plasmid)	Experiment Type	Transfer Frequency	Reference
Conjugation			
P. Aeruginosa (pQM1)	Filer method in beaker microcosm	2×10^{-3}	23
	Filter method in stream microcosm	1×10^{-1}	27
	Filter method, in situ	1×10^{-2}	26
	Stone method, in situ	2×10^{-1}	26
P. putida (pQM1)	Filter method, in situ	3×10^{-3}	27
	Stone method, in situ	3×10^{-3}	28
P. putida (indigenous plasmids)	Filter method, in microcosm	2×10^{-6}	29
	Filter method, in situ (no donor used)	2×10^{-6}	29
P. fluorescens (pQM1)	Filter method, in situ	1×10^{-2}	27
	Stone method, in situ	3×10^{-5}	28
P. aeruginosa (pQM1) × *P. Putida*	Filter method, in rotating disk microcosm	3×10^{-4}	30
P. fluorescens (pQM1) × *P. Putida*	Filter method, in rotating disk microcosm	5×10^{-5}	30
Transformation			
A. calcoaceticus (pQM17)	Filter method, in situ	3×10^{-7}	31

[a] Donor and recipient were the same species unless defined separately.
Note: P. = *Pseudomonas*; *A.* = *Acinetobacter*.
Source: after Ref. 21. Reprinted with permission from BioLine, © 1994.

mice and in the effluent of flow cells with biofilms developing on the glass surfaces, it was reported that conjugative gene transfer occurs rapidly after the addition of donor cells. After this initial burst of activity, no further transfer takes place. Thus, only a fraction of the biofilm microbial population obtains the plasmid, and primary transconjugants do not act as donors for neighboring recipient cells. There is some evidence in support of this hypothesis in the way of microscopic observations based on the detection of the green fluorescent protein (GFP) of transconjugants forming a thin layer on top of recipient microcolonies (34). This bears implications for the relevance of conjugative gene transfer in biofilms, such as those found in the intestine, in which the transient influx of high numbers of plasmid-bearing cells may lead to the transfer of antibiotic resistance and other plasmid-encoded genes to the intestinal flora. If bacterial movement in the mucus layer was restricted by biofilm-like conditions, then the further dissemination of newly acquired genes would be slowed considerably, but yet other environmental factors may play a role in determining secondary transfer. In a biofilm reactor study, the rate of plasmid transfer (expressed as cells/cm^2/hour) initiated by transconjugants was found to be highly dependent on the ambient nutrient levels (35). At the highest substrate concentration used, the rate of secondary transfer surpassed the rate of transfer initiated by the recipients by an order of magnitude. Hence, it is necessary to consider the type of habitat when assessing gene transfer scenarios.

Laboratory-grown biofilms in reactors or flow cells operated under continuous flow conditions have been useful for the study of gene transfer as the environmental conditions can be controlled. In biofilms, there is no homogenous mixing of cells, and contact as required for conjugation occurs only between individual layers of cells. Hence, plasmid transfer cannot be described by the mass action model employed for conjugation events between cells suspended in liquid media (36). Rather, the spatial biofilm structure, that is, architecture, accounts for the difference in transfer frequencies. This is apparent from looking at the effect of nutrient concentrations in a controlled rotating annular biofilm reactor (35). Higher substrate concentrations can dramatically suppress the occurrence of transconjugants over time. As conjugation is an energy-requiring process, there appears to be no physiological reason for this observation. It may be a result of interruption of mating pairs owing to rapidly dividing cells or caused by greatly varying biofilm architecture. Shear stress has a considerable effect on biofilm architecture, according to measurements of biofilm thickness and areal and volumetric density. As a rule of thumb, low shear (0.07 N/m^2) biofilms tend to be thicker and less volumetrically but more areally dense than high shear (0.99 N/m^2) biofilms (37). It also affects the efficiency of conjugative transfer of a plasmid, such as RP4, which tra genes code for rigid pili. In a rotating annular biofilm reactor, conjugation was only observed below a shear of 0.085 N/m^2, which corresponds to a laminar flow regime (38). Besides temperature and

nutrient concentration, the ratio of donors and recipients is important for initial conjugation events, with an ideal ratio of 1 : 1.

Although gene transfer in biofilms is most frequently expressed in terms of frequencies of transconjugants (T) per recipient (R) or donor (D), this does not reflect relative differences in the localization of donor cells or the kinetics of transfer. The total number of transconjugants may be high owing to the growth and division of cells or transconjugant-initiated secondary gene transfer, despite relatively low frequencies of initial horizontal gene transfer. To more accurately account for the role of the donor, the transfer frequency, F, can be expressed as the number of transconjugants divided by the product of recipients and donors (39),

$$F = T/R\,D$$

Despite the fact that there are more chances of cell collisions in suspended systems, there have been reports of higher transfer frequencies in biofilms. For example, a biofilm, which had formed on the surface of glass beads in a reactor, supported much higher frequencies of plasmid transfer under no-flow conditions than did the planktonic population in the liquid phase (40).

Retrotransfer. In the mid-1980s, Max Mergeay and his colleagues in Mol, Belgium, reported a phenomenon occurring in the opposite direction of conjugation as it is commonly defined: the resident plasmid or chromosomal genes of a recipient are transferred to the donor of a conjugative plasmid (41). Hence, the donor cell, which has received DNA from the intended recipient, becomes a *retrotransconjugant*. Retrotransfer has been demonstrated for several plasmids belonging to various incompatibility groups (42). It proceeds unidirectionally in two steps and is de facto mechanistically identical to conjugation. The recipient cell is first converted to a donor cell by receiving a plasmid capable of retrotransfer. The tra genes must be expressed in the recipient before retrotransfer can occur. In a second round of conjugation, a mobilizable plasmid (tra^-mob^+) is transferred back to the original strain that donated the tra^+ plasmid. Even nonmobilizable plasmids (as judged by the lack of conjugation), as well as chromosomal markers, can be retromobilized (43,44). For this reason, retrotransfer is important from the point of view of release of genetically modified microorganisms. The presence of plasmids that can both mobilize and retromobilize other plasmids was confirmed in soil and aqueous systems (45). Until now, there have been few studies to assess the relevance of retrotransfer in biofilms. In a rotating annular biofilm reactor containing a pure culture of *Bacillus azotoformans* with the conjugative plasmid RK2, a donor *Pseudomonas putida* PB2440 harboring the mobilizable plasmid pDLB101 was capable of retromobilizing this plasmid and transferring it to *B. azotoformans* after having first received plasmid RK2 (35). The initial rate (expressed as transconjugant cells/cm^2/hour) was slower than for mobilization, confirming the need for RK2 to be transferred and replicated in strain PB2440 before retrotransfer is possible. After 2 hours, this rate increased considerably and even exceeded direct mobilization rates. When the calculated growth rates of retrotransconjugants were taken into account, the increase in cell numbers of *B. azotoformans* containing both plasmids was because of the growth of retrotransconjugants and not because of the continuous retrotransfer events. As retrotransfer involving plasmids from different incompatibility groups occurs at different frequencies, these studies provide only a glimpse of the potential role of gene transfer in natural biofilms.

Transformation

The uptake of free DNA from the extracellular environment is referred to as *transformation*. Over 40 bacterial species, including representatives of the genera *Acinetobacter*, *Pseudomonas*, *Bacillus*, *Staphylococcus*, *Streptococcus*, *Neisseria*, and *Haemophilus*, are known to be competent of taking up DNA during their natural lifecycle; this phenomenon is referred to as *natural transformation* (10). Other species, such as *E. coli*, can be rendered competent by physicochemical manipulations (e.g., electroporation). The latter form of competence is unlikely to be of importance in the environment. The process of natural transformation (hereinafter referred to as "transformation") in biofilms is sensitive to inhibition by extracellular nucleases and involves the following steps (Figs. 5–7):

1. Release of DNA from dead or viable cells
2. Dispersal of released DNA in biofilms
3. Persistence of the released DNA in biofilms
4. Development of competence in bacterial cells
5. Binding of DNA to potential recipient cells

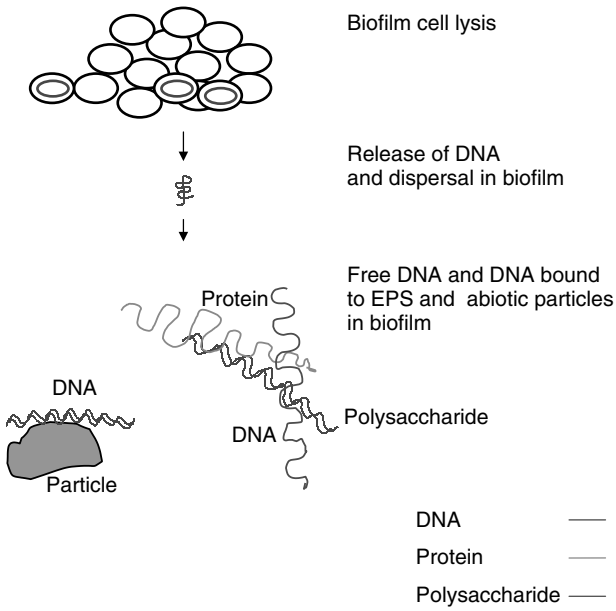

Figure 5. Conceptual view of the fate of DNA after cell lysis in a biofilm, depicting the first three steps in natural transformation (see text for details). The released DNA is either bound to biofilm constituents such as EPSs, cells, and abiotic particles or present as free DNA. See Figures 6 and 7 for uptake of DNA and internal processing by cells. See color insert.

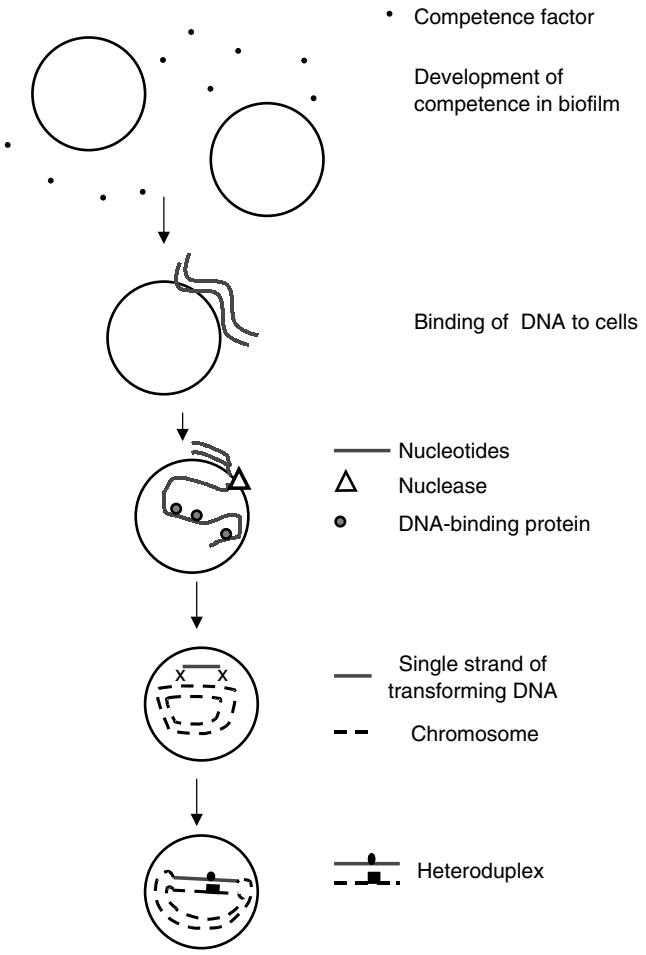

Figure 6. Uptake by and integration of transforming DNA into the chromosome of *Streptococcus pneumoniae*. Competence factors accumulate as the cells reach a high density. Double-stranded DNA binds to the cell and one strand is degraded. The remaining single strand replaces the strand of the same sequence in the chromosome, creating a "heteroduplex," in which one strand comes from the donor and one comes from the recipient. *Source:* redrawn after Ref. 11 with permission from the American Society for Microbiology, © 1997. See color insert.

6. Uptake of exogenous DNA and internal processing by cells
7. Integration of transforming DNA into the chromosome (Fig. 6) or reassembly of a transforming plasmid molecule (Fig. 7)

Transformation is known to be dependent on cellular functions that are distributed on the chromosome and are expressed under certain environmental conditions (46). The state of competency, when nucleic acids are adsorbed at the cell surface and can be taken up by the cell, is transient in liquid culture in most of the organisms studied. Its development and duration are variable among different species. In gram-positive bacteria, it is cell density dependent and occurs as a result of the accumulation of a competence factor in the growth medium of the culture. It is thought to be an inducible state of existing cells and can occur as an immediate response (e.g., *Streptococcus*) or a gradual event (*Bacillus subtilis*). One exception is *Neisseria gonorrhoea*, in which competence is constitutively expressed (47). Important for an assessment of transformation in the environment is the question of accessibility of free DNA because of the widespread occurrence of DNases. In gram-negative bacteria such as *Haemophilus*, *Azotobacter*, *Acinetobacter*, and *Pseudomonas* spp., no competence factor is produced, and the development of competence is controlled internally, for example, by an environmental stimulus leading to unbalanced growth (48). It is probable that the state of unbalanced growth is a fact of life for bacterial cells in the environment and hence, gram-negative cells should be continually competent for transformation. This was shown for *Acinetobacter calcoaceticus* grown in flow cells in the laboratory both under stress and no-stress conditions (Hendrickx, Hausner, and Wuertz, unpublished information). Likewise, the accumulation of a competence factor in biofilms should allow gram-positive species requiring the factor to also reach the state of competency. This may explain why the dental plaque organism *Streptococcus mutans* transformed 10- to 600-fold more efficiently when growing in biofilms than when growing in liquid culture (49).

It is known that DNA adsorbs to mineral surfaces, a phenomenon that protects it from degradation. Bacteria with natural competency, such as *Pseudomonas stutzeri*, may take up this particle-associated DNA by an unknown mechanism (50). For example, the efficiency of DNA uptake by *B. subtilis* was 3,200 times higher at an interface formed by sand particles and water than when the cells occurred in a planktonic state (51). However, two other competent species, *P. stutzeri* and *A. calcoaceticus*, did not display this trait (51). Likewise, *Vibrio* sp. WJT-1C took up more free plasmid DNA in water than in the sediment (52). Overall, it seems that particulate plasmid DNA is less accessible than dissolved plasmid DNA, although chromosomal DNA can increase the rate of transfer when present at the same time (51). In laboratory studies, plasmid DNA was transferred intergenerically from *E. coli* to *B. subtilis* (53). and to a *Vibrio* sp. (54). Interestingly, some forms of transformation require cell contact, but unlike conjugation, the donor cells can be metabolically inactive or dead. However, as the transfer involving live donor cells was inhibited by nalidixic acid or rifampicin, it was hypothesized by Paul (54). that some form of active transport of plasmids was involved in this type of transformation. The process is DNase sensitive and any type of plasmid, including nonconjugative plasmids, can be transferred. Its relevance to transformation in the environment remains to be shown. Clearly, these results are important for the release of genetically modified microorganisms. Little information is available on the rates of uptake in biofilms; this may be because of the lack of knowledge concerning the distribution and availability of extrachromosomal DNA in complex systems (51).

The only direct evidence of biofilm-associated transformation in a natural environment comes from river epilithon (20). Using *A. calcoaceticus*, a genus common in aquatic environments, as the recipient and either lysed

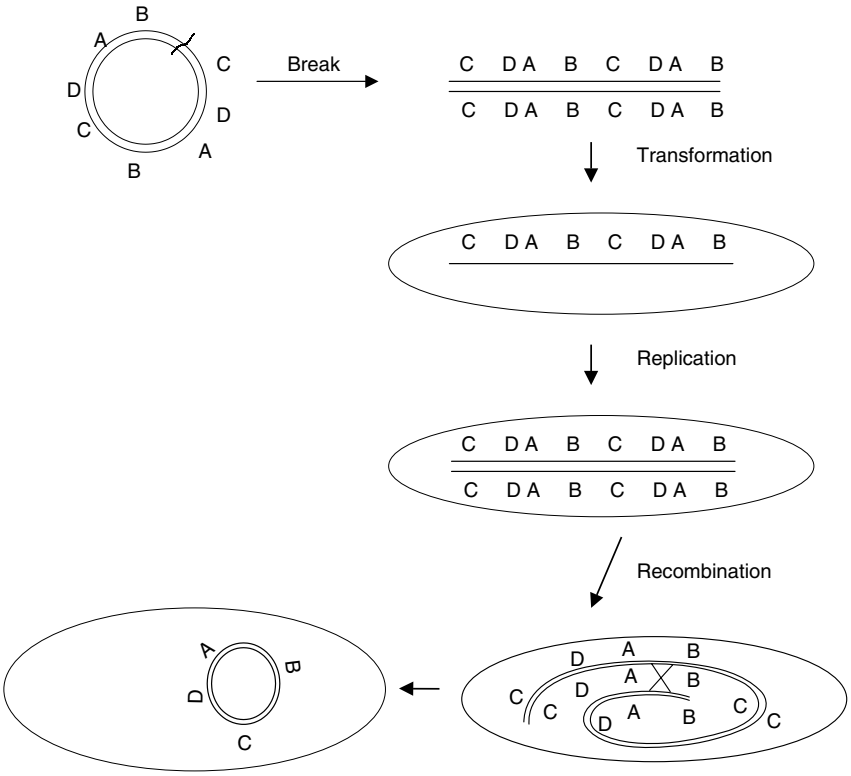

Figure 7. Natural transformation of bacterial cells by dimeric plasmids. After the single-stranded dimeric plasmid DNA is taken up, it can serve as a template to make the double-stranded DNA. The repeated ends can recombine with each other to form a circular plasmid. *Source:* redrawn after Ref. 11 with permission from the American Society for Microbiology, © 1998.

cells or live cells of the same species as the donor, transformation frequencies of 4.0×10^{-4} and 1.7×10^{-5}, respectively, were reported (55). Here, transformation can be considered to have occurred truly in situ. The experimental setup involved placing two nitrocellulose filters, one containing the source of DNA and one the recipient, in contact with each other on the surface of a sterile stone. The stone was placed in a nylon mesh bag and left on the bed of a river for 24 hours before selecting for transformants. The method is similar to the one described earlier for conjugation (Fig. 1) and *A. calcoaceticus* was transformed both by free chromosomal DNA and live donor cells via cell-contact transformation (56). These investigations, although not strictly biofilm studies owing to the lack of EPS, reveal the potential for natural transformation under in situ environmental conditions when high cell numbers are present. It has been reported that high DNase activity in wastewater treatment plants prevents uptake of free DNA. On the other hand, the presence of EPS in biofilms may help bind and protect extracellular DNA (57). In the light of reports of adsorption of DNA to some surfaces such as clay minerals (51), the potential for transformation in biofilms in a variety of environmental settings, including soil, epilithon, phylloplane, rhizosphere, and wastewater reactors, is considerable.

Transduction

Transducing bacteriophages can act as carriers of foreign DNA pieces, which they inadvertently insert into their own DNA structure during lysis of their respective bacterial hosts. During the next infection cycle, a phage can pass on this information to its new host (Fig. 8). This process may be *generalized* transduction, in which a phage can contain any piece of donor chromosomal or extrachromosomal DNA as a result of general cell lysis, or *specialized* transduction, in which a lysogenic bacteriophage, after having integrated into the host chromosome, may transduce genes that are adjacent to its insertion site upon the onset of the lytic cycle. Transduction is specific, as bacteriophages have a very limited host range (10). Hence, this form of gene transfer is only possible between closely related species (58). The fact that the number of viruses in the aquatic environment is much higher than previously thought, that is, up to 10^8 particles per milliliter in marine waters (59), gave rise to speculation by investigators that about one-third of the bacterial population can be attacked by bacteriophages within 24 hours. T4 bacteriophages can infect *E. coli* cells in laboratory-grown biofilms (60), and similar scenarios are conceivable for natural biofilms. The spread of genes by transducing bacteriophages in the aquatic environment has been shown in environmental containment chambers submerged on site both in the absence and presence of the natural microbial community (58). Virus-mediated gene transfer has also been shown on the phylloplane (61). It should be noted that bacteriophages can transfer not only chromosomal DNA but also plasmid DNA, and are therefore candidates for the spread of any foreign gene in the environment or in bioreactors. Native, lysogenized bacteriophages — phages which are replicated along with the host chromosome and are induced to become virulent by environmental conditions — were identified as important agents of horizontal gene dissemination in freshwater microbial populations (58). Even lytic

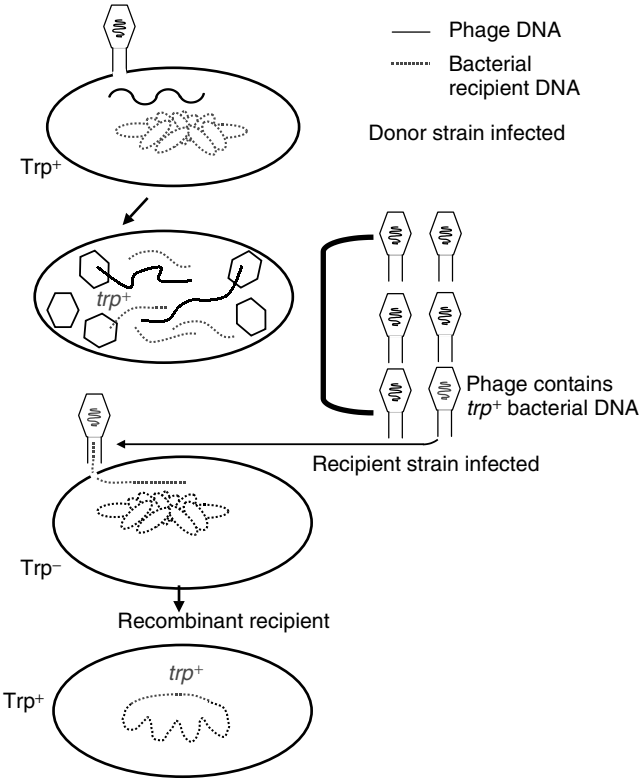

Figure 8. An example of generalized transduction. A phage infects a Trp^+ bacterium, and in the course of packaging DNA heads, the phage mistakenly packages some bacterial DNA containing the trp region instead of its own DNA into a head. In the next infection, this transducing phage infects the Trp^+ bacterial DNA instead of phage DNA into the Trp^- bacterium. If the incoming DNA recombines with the chromosome, a Trp^+ recombinant transductant may arise. Only one strand of the DNA is shown. *Source:* Redrawn after Ref. 11 with permission from the American Society for Microbiology, © 1997. See color insert.

phages establish a pseudolysogenic relationship with their bacterial hosts under low nutrient conditions (62). There are few reports of transduction in wastewater treatment facilities (63), and the importance of transduction as a means of gene transfer in biofilms in general remains to be established.

IN SITU FREQUENCIES OF GENE EXCHANGE

Transfer to Unknown Organisms

Until quite recently, gene transfer frequencies in biofilms have been evaluated exclusively by plating transconjugants, transformants, or transductants on selective agar media. Detection of transfer is thus dependent on the culturability of recipients under selective pressure. Obviously, such an approach ignores the vast potential of gene transfer to that part of the natural population that is not amenable to present cultivation techniques. It is estimated that only 0.1 to 10% of bacterial species in the environment can be readily cultured in the laboratory. The remainder has physiological requirements, which preclude their easy isolation in axenic culture. Furthermore, bacterial cells, which would normally grow under laboratory conditions, can enter the state known as *viable but nonculturable* (64).

The discovery of the GFP, which provides a label for living bacteria and can be used as a reporter molecule for the investigation of gene expression or protein localization (65), enabled the in situ microscopic detection of gene transfer events on a single cell level. This approach has been used to study conjugal gene transfer in situ in biofilms (17), activated sludge microcosms (66), and seawater bacteria filtered onto membranes (67). For activated sludge, transconjugants that had received the GFP-labeled RP4 plasmid were characterized microscopically by fluorescent in situ hybridization (FISH) with oligonucleotide probes for various subclasses of Proteobacteria as well as aeromonads and pseudomonads. This allowed the assignment of a phylogenetic affiliation of transconjugants by colocalization of fluorescent signals based on GFP and FISH without selective plating on solid media. The study revealed that more than 95% of transconjugants hybridized with the GAM42a probe, which targets bacteria belonging to the γ subclass of Proteobacteria, and using a more specific FISH probe, transconjugants were shown to belong predominantly to the genus *Aeromonas* (66). This information is important in so far as the plasmid RP4 is known to have a broad host range, yet in this experimental activated sludge system, its transfer was obviously limited to a well-defined group of organisms.

Quantitative Analysis of In Situ Gene Transfer

Recently, a quantitative method has been developed based on the concurrent use of GFP-labeled plasmids, automated confocal laser scanning microscopy (CLSM), and semiautomated quantitative image processing (68,69) to determine conjugation (70). and transformation (71) frequencies in biofilms. Using this technique, large volumes of biofilms can be scanned and analyzed in a systematic way. In addition, this approach delivers information concerning the localization and distribution of transformants and transconjugants within the biofilm (Fig. 9). Gene transfer frequencies detected in situ in biofilms using CLSM are higher than those quantified on selective agar media. For example, the conjugative transfer of the mobilizable (mob^+tra^-) plasmid pRK415, which belongs to the incompatibility group IncP and into which the gene *gfp* had been cloned, from *E. coli* to *Ralstonia metallidurans* in biofilms occurred at rates 1,000-fold higher than those determined using standard plating methods for the selection of transconjugants (70). It follows that the majority of cells that had received the plasmid were either compromised in their ability to divide on agar plates in the presence of selected pressure or did not pass the plasmid on to their progeny. The cells were not generally impaired in their ability to grow on agar because the total recipient cfu count on nonselective media approximated the total microscopic cell count. The quantitative determination of in situ horizontal gene exchange has also been applied to biofilms grown in flow cells (Fig. 10). This approach allows the careful control of environmental parameters such as the Reynolds number, *Re*, which is characterized by the ratio of inert forces, to frictional forces and describes the type of hydraulic

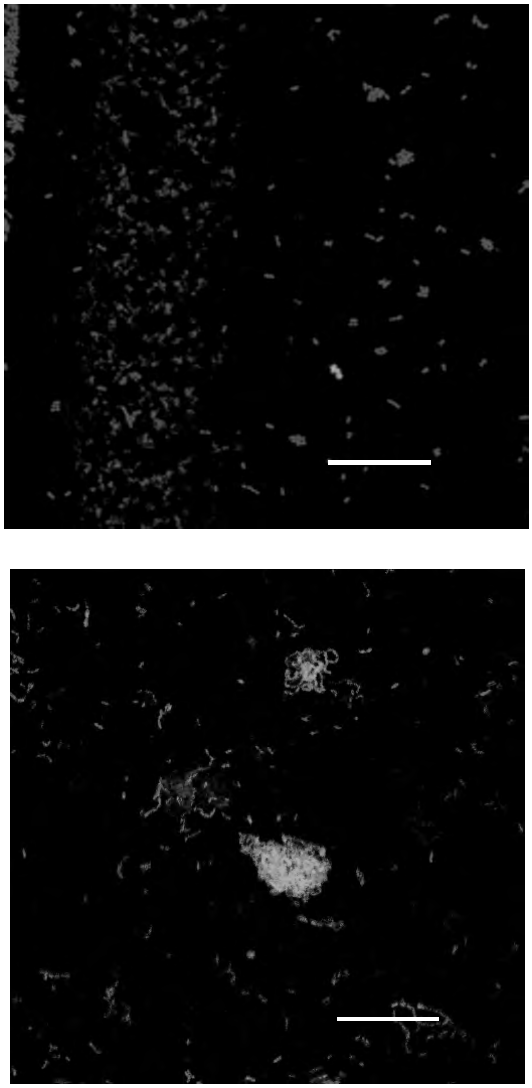

Figure 9. Transconjugant cells in a biofilm of *R. metallidurans* AE104 cultivated under nutrient-rich (**a**) and nutrient-poor (**b**) conditions. Recipient cells were grown as a confluent biofilm on microscope slides and exposed to high densities of donor and helper cells to allow transfer of the mob^+ plasmid, which carried the gene for the GFP. CLSM signals were collected consecutively and stored as gray scale images. AE104 cells were detected by FISH with a TRITC-labeled rRNA-directed oligonucleotide probe specific to the β-proteobacteria subgroup. With computer-assisted coloring, recipient AE104 cells were assigned the color red. Donor *E. coli* strain GM16 cells were detected based on the fluorescence emitted by GFP and are depicted in green. Transconjugants emitted both green light owing to GFP and red light owing to hybridization with the TRITC-labeled rRNA-directed oligonucleotide probe and are shown in yellow. Bar, 25 μm. *Source:* after Ref. 70;. reprinted with permission from the American Society for Microbiology, © 1998. See color insert.

conditions in a flow cell (72). Hence, gene transfer can be estimated in a defined hydrodynamic environment in the absence of a true measure of events as they occur in aqueous ecosystems.

The spatial structure or architecture of the biofilm plays a decisive role in gene transfer. While studying conjugation in flow cells, donor cells were shown to penetrate biofilms to varying depths depending on the distribution of microbial cell clusters and EPSs (73). The latter can act either as an obstacle preventing transport of cells or as an aid in facilitating cell-to-cell contact by the stabilization of donor cells. Recipient cell clusters of a certain size occurring deeper in the biofilm and close to the substratum (the glass surface upon which the biofilm was grown to enable microscopic visualization) supported the highest transfer frequencies. These recipient cell clusters could be divided into outer and inner groups using 3-D image analysis techniques. Interestingly, more transconjugant cells were detected in the inner portion of cell clusters (73). Taken together, these studies indicate considerable movement of donor cells inside biofilms and even within cell clusters.

Similarly, natural transformation has been quantified in situ in *Acinetobacter* sp. BD413 biofilms based on the expression of a variant GFP, the yellow fluorescent protein (YFP) (Fig. 11). It was shown that as little as 1 pg free plasmid DNA per milliliter resulted in detectable transformation (74). The plasmid DNA taken up had no known homology to any part of the host genome. Hence the plasmid was either recircularized via homologous recombination of two plasmid molecules or transcription and synthesis of YFP proceeded without the plasmid being present in a replicative form in the cell. As in the case of conjugation, in situ transformation frequencies are several orders of magnitude higher than those determined by plating transformants on selective media (74). Recent studies have also revealed that transformants become established deep inside biofilms. Even genes that exert an adverse effect on the biofilm, such as overexpression of certain proteins, may be taken up via transformation (74) and become established long enough to allow further transfer via conjugation to other bacterial species in which they have no negative effect. These results demonstrate that biofilms represent a habitat allowing the induction of competence in gram-negative bacterial species. The relevance of transformation in the environment is closely linked to the presence of biofilms.

CONCLUSION

Gene transfer in biofilms is an important mechanism of information exchange and adaptation of microbial communities. All three main routes of horizontal transfer, namely, conjugation (including retrotransfer), transformation, and transduction, have been demonstrated in laboratory-grown biofilms. Conjugation and transformation were also detected in the epilithic environment of rivers by introducing marker strains into the community and placing stones together so that inoculated areas may contact. The use of flow cells and CLSM in conjunction with fluorescently tagged plasmids has advanced our understanding of the dynamics, both spatial and temporal, of gene transfer by facilitating in situ investigations

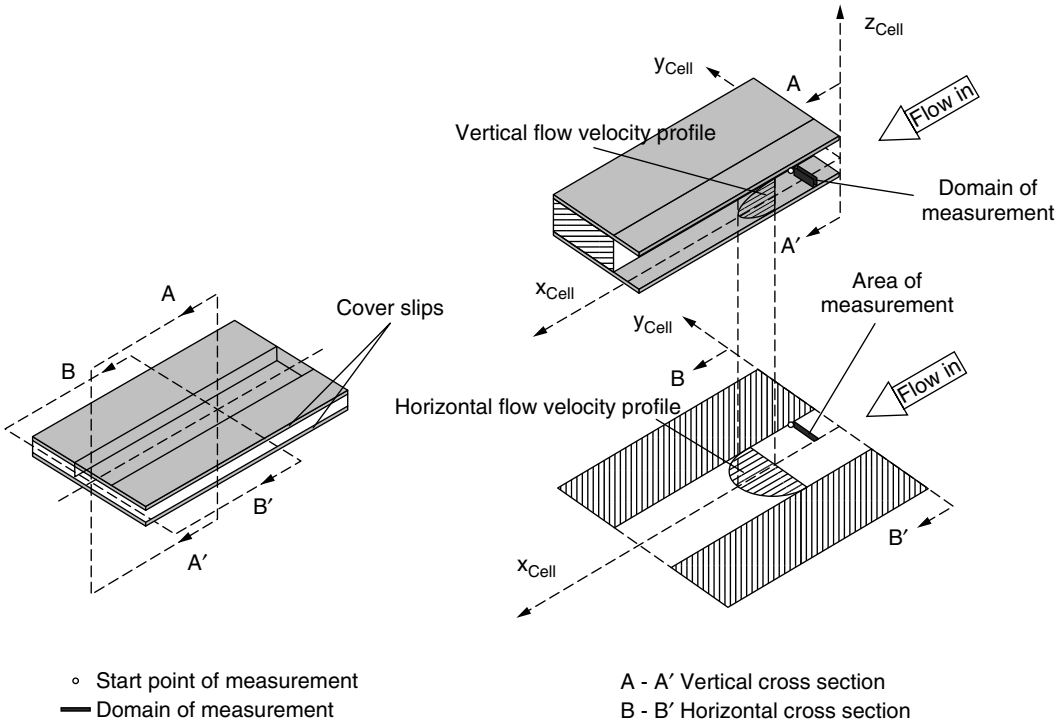

Figure 10. Schematic view of the flow cell indicating fluid velocity profiles and domain of measurement typically used to investigate gene transfer events. *Source:* after Ref. 68; reprinted with permission from the American Society for Microbiology, © 1998. See color insert.

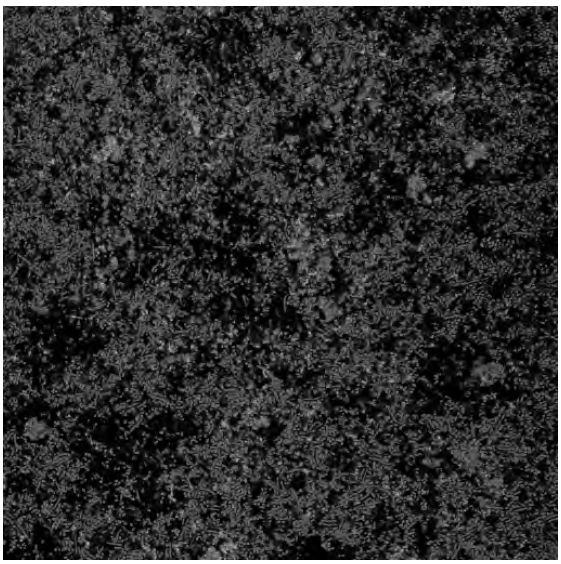

Figure 11. Transformant cells in an 18-hour biofilm of *Acinetobacter* sp. BD413 grown in a flow cell and exposed to 1 μg plasmid DNA/mL for 20 minutes. Cells were visualized with the general nucleic acid stain Syto 60 (Molecular Probes, Eugene, Oregon) and were detected with an LSM410 confocal laser scanning microscope (Zeiss, Jena, Germany). Transformants were detected by the emission of light owing to the GFP encoded by the plasmid, pGAR1. See color insert.

no longer solely reliant on the isolation of transconjugants, transformants, or transduced cells on selective growth media. Depending on the architecture of a biofilm and environmental factors such as nutrient supply, gene transfer may be limited to the top layer at the interface with the passing fluid, or it may occur deep within the biofilm. The very high frequencies of gene exchange reported in biofilms may be responsible for the dissemination of antibiotic resistance genes and the acquisition of multiple resistances by pathogenic strains. Cells that have received a plasmid may or may not divide in a biofilm. Vertical transfer after cell division must occur for the long-term establishment of genetic traits. Transformation in biofilms can account for the dispersal of DNA released by lysed cells. Plasmids thus transferred can be passed on to other species in the biofilm by conjugation. It is thus likely that conjugation is the most important route for the establishment of new genes. However, the importance of transduction in biofilms in the environment needs to be determined in view of the long underestimated relevance of viruses in planktonic populations.

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GENE PROBES. See Laser Scanning Microscopy in Combination With Fluorescence Techniques for Biofilm Study

GENE TRANSFER IN BIOFILMS. See Biofilms in Natural and Drinking Water Systems

GENETIC ECOLOGY OF SOIL. See Soil Genetic Ecology

GENETIC MANIPULATION OF FUNGI. See Fungi, for Biotechnology

GENETICALLY ENGINEERED MICROORGANISMS (GEMS). See Field Release of Genetically Engineered Microorganisms (GEM)

GENETICALLY ENGINEERED MICROORGANISMS FOR BIODEGRADATION OF RECALCITRANT COMPOUNDS

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The mineralization of organic molecules by microbes is essential for the carbon cycle to operate. The massive mobilization of compounds stored in natural resources or the introduction of xenobiotics into the biosphere produces unidirectional fluxes that result in the persistence of many chemicals in the biosphere, where they are a source of pollution. Molecular biology offers the tools to optimize the biodegradative capacities of microorganisms, accelerate the evolution of new metabolic activities, and construct totally new pathways through the assemblage of catabolic segments from different microbes. The number of genetically engineered microbes (GEMs) for potential use in biodegradation has increased significantly in the last few years and a number of catabolic modules for the specific evolution of new pathways are being constructed so that a number of new recombinant microorganisms will soon be available. The behavior and functioning of some of the already available recombinant microbes have been tested in microcosms in which the survival and fate of recombinant microbes in different niches under laboratory conditions have been found to be similar to those of unmodified parental strains. Recombinant DNA (rDNA), both on plasmids and on the host chromosome, is usually stably inherited by GEMs. The potential lateral transfer of rDNA from GEMs to other microbes is significantly diminished, although not totally inhibited, when rDNA is incorporated on the host chromosome.

KEY CONCEPTS

A large proportion of organic compounds of biological and chemical origin are ultimately mineralized (degraded to carbon dioxide, water, and other inorganic compounds), predominantly by microorganisms, as part of the continuous cycling of carbon between inorganic and organic states. The mineralization of a compound involves its structural alteration and the formation of metabolic intermediates that serve either as carbon skeletons for cellular constituents or as fuels for energy generation. Mineralization of organic compounds is a central feature of the carbon cycle and is a process critical to the maintenance of life on this planet.

Most naturally occurring molecules are easily mineralized, as are industrial chemicals with structures similar to organic compounds of biological origin. However, many xenobiotics (compounds that exhibit structural elements not found in natural ones) are not readily mineralized, and persist in the biosphere. This is because the structural elements of such compounds are chemically very stable, have novel substituents that are not generally found in organic molecules of biological origin, are toxic for microorganisms, or inhibit degradative enzymatic attack. Some organic compounds in nature undergo partial degradation; however, this is not necessarily beneficial: microbes may not gain energy for growth, and more recalcitrant toxic compounds or highly reactive products, which subsequently undergo chemical changes such as polymerization, may be formed.

Xenobiotics introduced in natural resources are not readily integrated into the natural nutrient cycles.

As a consequence of the progressive accumulation of pollutants in the biosphere, environments hostile to biological systems, that is, those polluted by aliphatic and aromatic hydrocarbons, chlorinated compounds, nitrotoluenes, dyes, explosives, organic solvents, and others, are appearing with increasing frequency (1).

Effective steps should be taken to protect environments from pollution. A variety of alternative treatments, including physical, chemical, and biological approaches, have been developed to reduce or eliminate contamination by hazardous compounds (1–3). Physical treatments comprise, among other methods, adsorption to activated carbon, filtration, and incineration. Chemical approaches involve solvent extraction or surfactant precipitation. These treatments are expensive, and may in some cases generate unwanted products. The biological approach, including continuous and batch treatments of liquid wastes, composting in situ and on-site soil treatments, and so on is usually cheaper and involves less risk to human health or the environment (3–8). The most promising approach is to optimize the biodegradative capacities of microorganisms, accelerate the evolution of new activities, and exploit them to eliminate these pollutants (3,8).

The introduction of some chemicals into the environment exerts selective pressure for the evolution of the corresponding catabolic activities. For example, the herbicide 2,4-dichlorophenoxyacetic acid, insecticides such as DDT and parathion, and explosives such as nitrate ester derivatives can be mineralized by single microbes or communities of microbes (7–15). However, other chemicals such as certain organic solvents, polychlorinated aromatic compounds, dioxins, and dibenzofurans are highly recalcitrant. In such cases, the evolution of new activities in the laboratory may be helpful because the frequency and types of genetic events needed (mutation, alteration of gene expression, gene dosage, gene transfer, and so on) may be carefully controlled under selective conditions (3,8,16–18). The experimental evolution of catabolic pathways offers considerable potential for accelerating the evolution of bacteria that are able to degrade toxic industrial chemicals, and this may be useful for reducing environmental pollution. Several strategies have been successfully applied to construct bacteria that are able to eliminate a wide range of organic solvents such as toluene derivatives, a number of chlorinated aromatic compounds arising from human industrial activities (chlorobenzoates, chlorosalicylates, and chlorophenols), and the more recalcitrant polychlorinated biphenyls (PCBs) and explosives such as 2,4,6-trinitrotoluene (TNT) (3,8,17–23).

Many of these bacteria belong to the genus *Pseudomonas*, a group of microorganisms that exhibit a wide range of metabolic activities against natural and xenobiotic compounds. The so-called fluorescent *Pseudomonas* group includes strains whose biochemical, physiological, and genetic characteristics have been well characterized (24,25). A number of genetic tools — wide host range cloning plasmids and cosmids, transposons and mini transposons, gene markers and probes, reverse genetics, and so on — have made it possible to design recombinant derivatives of this group of bacteria with increased biodegradative properties (26–31). Some of these constructions are summarized in the following chapter. Furthermore, some of these recombinant bacteria and their parental wild-type strains have been selectively introduced in polluted and nonpolluted environments, and their survival, performance, and ability to transfer recombinant DNA have been monitored.

HYBRID PATHWAYS FOR METABOLISM OF CHLOROORGANIC COMPOUNDS

Hybrid Pathways for Chlorobenzoates and Chlorotoluenes

Pseudomonas sp. B13 exhibits two ortho-cleavage pathways, one for the metabolism of benzoate and the other for the catabolism of *m*-chlorobenzoate (23). These pathways do not allow the mineralization of other chlorobenzoates or any alkylbenzoates (23,32). The chromosomal ortho-cleavage pathway for benzoate seems to be similar to other catabolic pathways for the metabolism of benzoate by Pseudomonads. The *m*-chlorobenzoate pathway is partially chromosome-encoded and partially plasmid-encoded. In this pathway, *m*-chlorobenzoate is first oxidized to *m*-chlorocatechol by chromosome-encoded genes; then *m*-chlorocatechol undergoes ring cleavage and chlorine elimination, a process involving four plasmid-encoded enzymes — catechol 1,2-dioxygenase, chloromuconate cycloisomerase, dienelactone hydrolase, and maleylacetate reductase. The products resulting from the action of the plasmid enzymes are further metabolized by chromosomally encoded enzymes. In *Pseudomonas* strains growing on *m*-chlorobenzoate, the plasmid-encoded genes involved in the degradation of this compound were duplicated; this duplication ensured an increase in protein dosage (33,34). *Pseudomonas* sp. B13 cannot grow on *p*-chlorobenzoate or mixtures of alkylbenzoates and halobenzoates. In contrast, *Pseudomonas* sp. FR1 (pFRC20P), a derivative of B13 that was constructed through the recruitment of genes from different microorganisms, is able to grow on *p*-chlorobenzoate and mixtures of alkylaromatics and chloroaromatics (17). Its construction involved chromosomal integration of the genes for the metabolism of *p*-chlorobenzoate and alkylbenzoates to *p*-chlorocatechol and alkylcatechols, respectively. The construct was completed by introducing a mobilizable plasmid (pFRC20P) carrying a key gene for the metabolism of alkyllactones. The genes encoding the TOL plasmid pWW0 toluate dioxygenase (*xylXYZ*) and the next enzyme in the pathway (a dehydrogenase encoded by *xylL*), together with the positive regulator *xylS*, were cloned into Tn5 and delivered into the host chromosome (Fig. 1). This allowed the recombinant bacteria to grow on *p*-chlorobenzoate and also allowed the metabolism of *p*-methylbenzoate to γ-methyl-lactone (Fig. 1). The resulting bacterium was called *Pseudomonas sp. FR1*. Plasmid pFRC20P carries the gene that encodes for an isomerase from *Alcaligenes* that allowed the conversion of γ-methyl-lactone into β-methyl-lactone (17) and subsequently allowed mineralization of the alkylbenzoate. Furthermore, this isolate grew and simultaneously assimilated mixtures of alkylbenzoates and halobenzoates.

Hybrid strains that mineralized *p*-chlorobenzoate (17,22), although able to transform 3,5-dichlorobenzoate,

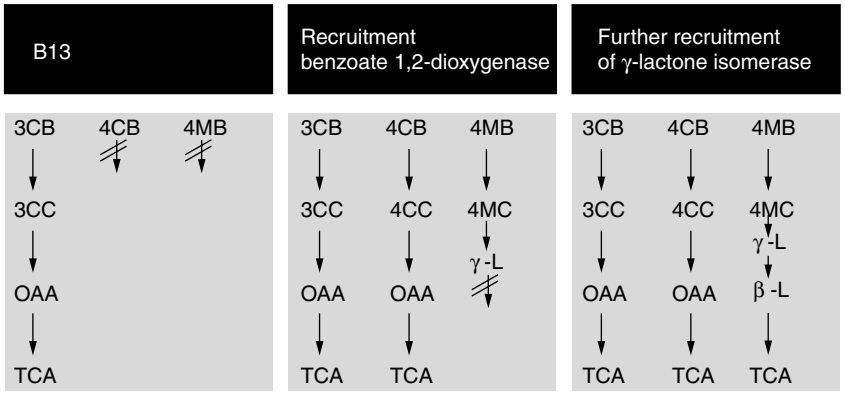

Figure 1. Expansion of the range of chloroaromatic compounds degraded by *Pseudomonas* sp. B13. Details of the pathway expansion strategies are as described in the text.

did not grow on this compound because the XylS regulator was not activatable by 3,5-dichlorobenzoate. By recruiting a XylS mutant regulator that is activated by this compound, a derivative mineralizing 3,5-dichlorobenzoate was constructed.

Recruitment into *Pseudomonas* sp. B13 of the upper pathway enzymes (xylene monooxygenase, together with benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase) encoded by the TOL plasmid pWW0 (34–36) resulted in strains that are able to mineralize *m*-chlorotoluene (19,37,38).

Haro and de Lorenzo have investigated the degradation of *o*-chlorotoluene, a compound for which microbes with biodegradative activities have not been found. They took advantage of the relaxed substrate activity of toluene dioxygenase of *P. putida* F1, which functions as a monooxygenase with 2-chlorotoluene and converts this into *o*-chlorobenzyl alcohol (39). It was suggested that further metabolism of the latter to *o*-chlorobenzoate may be mediated by the TOL pathway benzyl alcohol dehydrogenase (XylB) and benzaldehyde dehydrogenase (XylC) (39). A gene cassette has been engineered so that the chromosomal *todABC* genes encoding toluene dioxygenase and the *xylBC* are expressed from the Pu promoter for the upper pathway of the TOL plasmid. This construct is now available for the transformation of *o*-chlorotoluene to *o*-chlorobenzoate (39). Once this module is transferred to an *o*-chlorobenzoate degrading strain, mineralization of *o*-chlorotoluene will be achieved.

Self-Inhibiting Metabolic Routes: The Case of Chlorobiphenyl Metabolism

A number of biphenyl-degrading microorganisms that have been isolated cometabolize a variety of polychlorinated biphenyls (PCBs) (39–44). The degradation of biphenyl and chlorinated analogs is initiated by dioxygenation at the 2,3-position. The 2,3-dihydro-2,3-dihydroxybiphenyl formed is dehydrogenated to 2,3-dihydroxybiphenyl, which then undergoes *meta* cleavage. Benzoates are produced by hydrolysis of the ring cleavage product. In most cases, biphenyl-degrading organisms are not able to mineralize the chlorobenzoates formed from chlorobiphenyls, although some strains cometabolize chlorobenzoates. An inhibitory effect of chlorobenzoates or their metabolic products has been observed in the *Pseudomonas testosteroni* strain B356, with *m*-chlorobenzoate being the most effective inhibitor (45). In the case of 3-chlorobiphenyl, the rapid formation of *m*-chlorocatechol from *m*-chlorobenzoate led to toxicity, manifested as a decrease in viable cells during substrate utilization (46). This toxicity was because of *meta* cleavage of *m*-chlorocatechol, which might produce a reactive acyl chloride intermediate. A similar mechanism for the interference of *m*-chlorocatechol with the utilization of biphenyl and monochlorobiphenyl is inactivation by *m*-chlorocatechol of 2,3-dihydroxybiphenyl dioxygenase, which is necessary for biphenyl metabolism. Two approaches have been tested to overcome chlorobenzoate-mediated inhibition of PCB degradation: the use of mixed cultures consisting of PCB and chlorobenzoate degraders and the in vivo and in vitro combination of PCB and chlorobenzoate pathways (46–51).

The *Burkholderia cepacia* strain JHR222 is a hybrid strain that was able to mineralize: 2-, 3-, and 4-chlorobiphenyl and *o*-, *m*-, *p*-chloro-, and 3,5-dichlorobenzoate, but not other isomers such as 2,3-, 2,5-, 2,6-, and 3,4-dichlorobenzoate. A problem related to the mineralization of complex mixtures of chlorobiphenyls by this strain is that certain dichlorobenzoates inhibited the metabolism of monochloro-substituted biphenyls (53). Further developments in this area are foreseen via the use of sequential anaerobic treatments to achieve dechlorination of PCBs and further aerobic metabolism of low chloro-substituted biphenyls (54).

A Hybrid Enzyme for Trichloroethylene Cometabolism

Trichloroethylene (TCE) has been recognized as one of the most significant environmental pollutants in soil and groundwater (1). This and related compounds persist in the environment and are suspected carcinogens. Wackett and Gibson (55) showed that toluene dioxygenase plays a role in the cometabolic elimination of TCE. Later, Suyama and coworkers (56) constructed a hybrid strain in which the *bphA1* gene coding for biphenyl dioxygenase was replaced by the *todC1* gene, which codes for toluene dioxygenase of *P. putida* F1 within the chromosomal biphenyl-catabolic *bph* gene cluster. This hybrid strain efficiently removed trichloroethylene and *cis* 1,2-dichloroethylene.

MODIFIED PATHWAYS FOR ALKYLAROMATICS

Expansion of the Catabolic Potential of the TOL Plasmid Catabolic Pathways for Metabolism of Alkylaromatics

Pseudomonas putida KT2440, harboring the self-transmissible TOL plasmid pWW0, grows on a variety of aromatic hydrocarbons used as solvents, including toluene, *m*- and *p*-xylene, and *m*-ethyltoluene (36). The lateral alkyl chain of these aromatics is oxidized to yield alkylbenzoates, which are further metabolized to Krebs cycle intermediates via catechol and alkylcatechols. In this strain, the metabolism of *p*-ethylbenzoate is blocked (21). By introducing a series of mutations in the TOL plasmid, the recombinant TOL plasmid pWW0-EB62 that allows the host microbe to grow on *p*-ethylbenzoate while maintaining its ability to grow on benzoate and *m*-methylbenzoate and *p*-methylbenzoate (21) was constructed. The strategy involved the isolation of several mutants, which are detailed later. XylSArg45Thr is a mutant protein that allows the induction of the meta-cleavage pathway in response to *p*-ethylbenzoate as an effector. When the mutant regulator was introduced in *Pseudomonas* bearing the TOL plasmid, *p*-ethylbenzoate was oxidized to *p*-ethylcatechol, which inactivated the wild-type *xylE*-encoded catechol 2,3-dioxygenase. Ramos and coworkers (21) then isolated a mutant *xylE* gene that encoded an enzyme resistant to inactivation by its substrate *p*-ethylcatechol. This mutant catechol, 2,3-dioxygenase, exhibited a single amino acid substitution (threonine 253 isoleucine) (57). The *xylS* and *xylE* mutations were recombined in the TOL plasmid pWW0-EB62 so that bacteria grew on *p*-ethylbenzoate as the sole C-source. This microbe has been extensively studied in microcosms (see following text).

de Lorenzo and coworkers (58) used the upper TOL operon of plasmid pWW0, together with its regulator *xylR*, to construct a cassette that would allow the bioconversion of toluene and a number of derivatives to the corresponding benzoates. This cassette can be transferred to microorganisms that use the housekeeping orthocleavage pathway of catechol for the metabolism of toluene and its derivatives.

Mineralization of Mixtures of Several Aromatics

The construction of a hybrid strain that is able to mineralize components of a benzene, toluene, and *p*-xylene mixture was achieved by redesigning two metabolic pathways of *P. putida* for toluene metabolism. Genetic and biochemical analyses of the *tod* and the *tol* pathways revealed that dihydrodiols formed from benzene, toluene, and *p*-xylene by toluene dioxygenase in the *tod* pathway could be channeled into the *tol* pathway by the action of *cis*-*p*-toluate-dihydrodiol dehydrogenase, leading to complete mineralization of a benzene, toluene, and *p*-xylene mixture (59). A hybrid strain was constructed by cloning the *todC1C2BA* gene encoding toluene dioxygenase on plasmid pRSF1010 and introducing the resulting plasmid into *P. putida* bearing the TOL plasmid pWW0. The hybrid strain, called *P. putida TB105*, was able to mineralize a benzene, toluene, and *p*-xylene mixture without accumulating any metabolic intermediates.

HYBRID PATHWAYS FOR NITROAROMATICS

Mineralization of Mononitrotoluenes

Apparently, the mononitrotoluene that can more easily be mineralized by microbes is *p*-nitrotoluene (60–62), because *o*-nitrotoluene and *m*-nitrotoluene are more recalcitrant, although a *Pseudomonas* strain that is able to metabolize *o*-nitrotoluene has been described (62). To date no microbe that is able to deal with *m*-nitrotoluene has been described. Two independent reports originally documented the mineralization of *p*-nitrotoluene by bacteria belonging to the genus *Pseudomonas* (10,60).

Nitrotoluenes may be regarded as structural analogs of toluenes and xylenes, the original substrates of the TOL plasmid-encoded pathway. In fact, the degradative pathway found in *p*-nitrotoluene-mineralizing microbes involves stepwise oxidation of the methyl substituent with *p*-nitrobenzoate as the intermediate (10,60). Further metabolism of *p*-nitrobenzoate occurred via *p*-hydroxylaminobenzoate, which was then transformed into protocatechuate, a central intermediate of the metabolism of aromatic compounds (61).

In contrast to *p*-nitrotoluene, *o*-nitrotoluene and *m*-nitrotoluene are more recalcitrant, and until recently only one *Pseudomonas* strain had been isolated because of its capability of metabolizing *o*-nitrotoluene (62). Delgado and coworkers (63) examined the ability of nitro-substituted compounds to serve as substrates for the TOL pathway enzymes toluene monooxygenase, benzyl alcohol dehydrogenase, and benzaldehyde dehydrogenase. All three enzymes were able to transform the substituted nitroaromatic substrates, when the nitro group was located at the meta or para position of the aromatic ring, revealing that enzymes of the TOL catabolic pathway can metabolize *m*-nitrotoluene and *p*-nitrotoluene to the corresponding *m*-nitrobenzoate and *p*-nitrobenzoate. The XylR regulator controls transcription of the genes of these enzymes from the Pu promoter. Its effector specificity is such that it recognized *o*-nitrotoluene and *p*-nitrotoluene as effectors, but was not activated by *m*-nitrotoluene (64). Therefore, the potential of the TOL upper pathway seems to be limited to the degradation of *p*-nitrotoluene as it is the only compound that acts as both effector and substrate. Transfer of the TOL upper pathway module to a *p*-nitrobenzoate-degrading *Pseudomonas* sp. strain resulted in the expansion of its catabolic potential to include *p*-nitrotoluene (65; Fig. 2).

A mutant XylR regulator that gained the ability to recognize *m*-nitrotoluene without losing its ability to recognize the other effectors was selected (64). This mutant regulator, together with the TOL upper catabolic pathway, provides the potential for the biotransformation of *m*-nitrotoluene to *m*-nitrobenzoate. This module can be transferred to a Pseudomonas strain that is able to mineralize *m*-nitrobenzoate (66). This was achieved after cloning the *xylR7* mutant allele and the upper genes enzymes into a mini Tn5. The mini Tn5 was transferred to strain JS51, and mineralization of *m*-nitrotoluene was achieved (67).

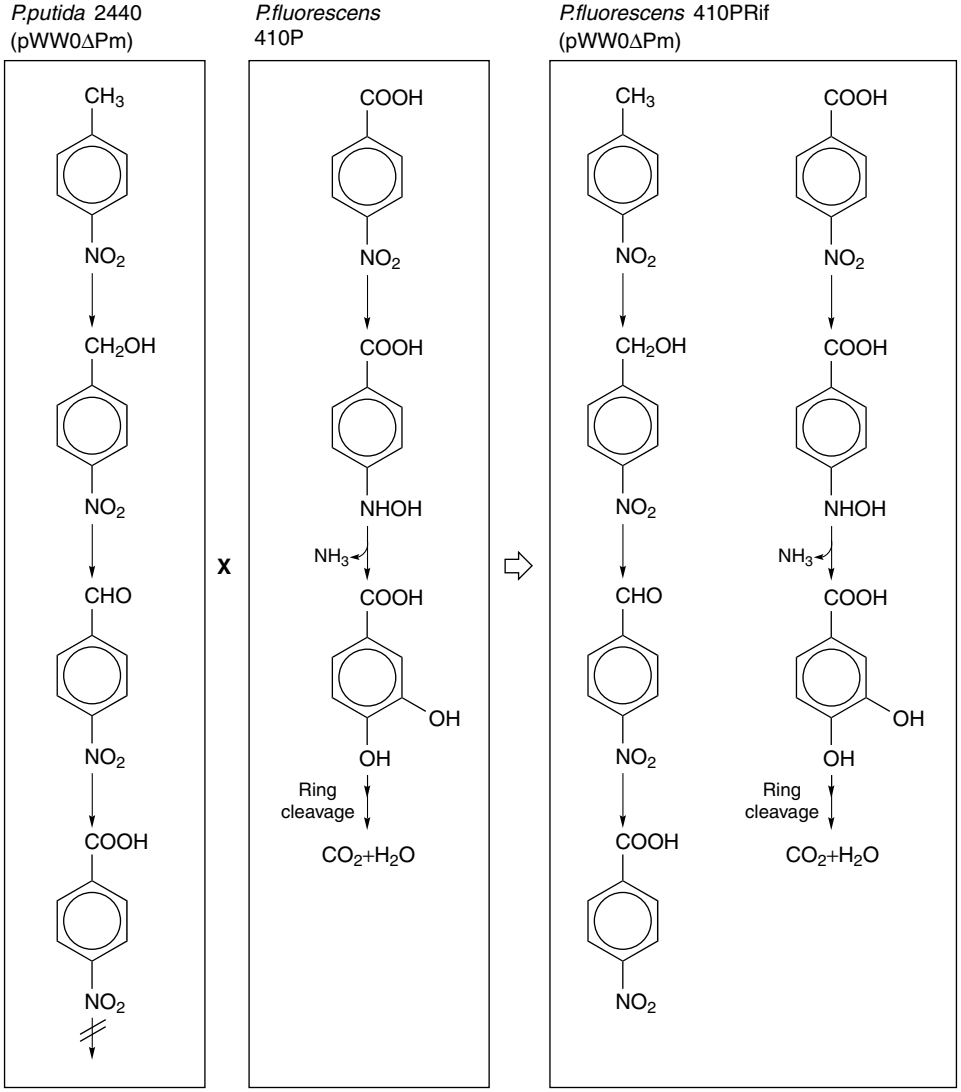

Figure 2. A catabolic pathway for the metabolism of 4-nitrotoluene. The hybrid pathway was as constructed by in vivo mating between strains with different catabolic potentials (35).

REMOVAL OF ORGANOMERCURIALS

Mercury (Hg) is a toxic metal that has been released into the environment in substantial quantities. Its toxicity results from the capacity of Hg(II) to bind sulfhydryl, thioether, and imidazole groups and thereby inactivate enzymes (68). Organic species of mercury (both alkyl and aromatic derivatives) accumulate in tissues of higher organisms, causing serious health problems (69).

The *merTPABD* genes of transposon Tn501, which encodes the enzymes involved in organomercury resistance (69), were cloned into a mini Tn5 that was used to insert the genes into the genome of *P. putida*. Transconjugants that constitutively expressed the *mer* genes (high resistance to phenylmercury) were selected (69). This allowed the engineered bacteria to cleave Hg from an organic moiety and reduce the Hg(II) released to Hg^0. These properties were also combined with benzene and toluene catabolism of several *Pseudomonas* strains, allowing the degradation of the aromatic moiety of the organomercurial as well as the detoxification of the metal component.

IMPROVED REMOVAL OF POLLUTANTS IN THE PLANT RHIZOSPHERE

The Bioremediation of sites polluted by compounds at very low concentrations may be favored by microbes that are able to grow in plant rhizospheres, in which the nutrient supplied by the plant may help to colonize soil sites that are poor in available substrates and ultimately enhance the elimination of pollutants (70–76). This process is known as *plant-assisted microbial bioremediation* or *rhizoremediation*. *Pseudomonas fluorescens* sp. F113 is an isolate from the sugar beet rhizosphere, which is an excellent root colonizer and shows potential for serving as an instrument of biological control (75). This microbe has been subjected to pathway expansion to include biphenyl and certain monochlorinated biphenyls (20). The *bph* genes

were cloned into a mini-Tn5 operon encoding resistance to a herbicide in the suicide plasmid pDDPCB (20) and then delivered into the host chromosome through this vector system. *Pseudomonas* sp. F113-PCB is a derivative that carries the *bphABCD* genes for the conversion of biphenyl into benzoate.

Plant compounds that induced biphenyl metabolism by bacteria have been identified. For example, *l*-carvone induced *Arthrobacter* sp. strain B1B to cometabolize Aroclor 1242 (a mixture of PCBs), resulting in significant degradation of 26 isomers in the mixture. Several compounds structurally related to *l*-carvone, including limonene, *p*-cymene, and isoprene, also induced cometabolism of PCBs by certain bacteria. These results suggest that in the plant rhizosphere gratuitous inducers may facilitate the activation of catabolic pathways and the removal of xenobiotic compounds.

In the plant rhizosphere, the metabolism of alkylbenzoates was stimulated so that degradation was enhanced and the remaining levels of these aromatics were lower than those found in soil without plants (76). A similar observation was seen for removal of TNT in the plant rhizosphere (A. Esteve-Núñez and J. L. Ramos, unpublished).

TRACKING GEMS IN MICROCOSMS AND BEHAVIOR OF RECOMBINANT MICROBES

Tools for Tracking GEMs in the Environment

Most of the recombinant bacteria constructed for biodegradation bear antibiotic-resistance markers. Although these markers are extremely useful in molecular genetics and microbial ecology, their use in uncontained applications is not very important (77). To avoid the use of antibiotic-resistance selection markers in GEMs designed for environmental applications, a series of nonantibiotic-resistance markers were developed. These markers include spontaneous resistance to phosphinothricin, bialaphos, heavy metal ions such as mercury, arsenate, or tellurite (28,29,77–79), and nutritional markers such as the genes that enable the organism to grow on lactose as the sole C-source (80). Some of these markers were introduced into the host microorganisms via mini-Tn5 derivatives (28).

Another approach, which may ultimately solve the problem of the presence of selectable markers, is the use of a selectable marker, that is, kanamycin resistance, flanked by two tandem *res* (resolution) sites of plasmid RP4, which can be provided as a cassette within a mini transposon (81). The *res* site is a short sequence that, once recognized by the resolvase of the ParA system, undergoes site-specific recombination that results in the deletion of the intervening DNA sequences (82). ParA can be provided in a suicide replicon.

An alternative is the use of genes such as *lux* and *luc*, which encode for light emission (83). This system now has been used to mark and track *Pseudomonas* strains that are able to colonize a number of plant roots (71,73).

An alternative to the introduction of a selectable marker in the target strain is to use tools that specifically recognize the target microbe. Monoclonal antibodies (mAbs) are powerful tools for tracking microorganisms because they can recognize epitopes on the surface of bacteria and thus serve as in situ identifiers. A series of mAbs were produced against whole cells of *Pseudomonas putida* KT2440 (84). One was shown to recognize the *O*-antigen of *P. putida* LPS. In the laboratory, this mAb specifically recognized the strain when grown in different culture media and at different growth stages. The mAb was used to track the strain after its release in a mesocosm established in Plussee Lake in northern Germany (85).

Another approach used in our laboratory has been to introduce new epitopes on bacterial surface proteins. For example, a mammalian coronavirus epitope (86) has been cloned into the OprL protein, a surface protein of *P. putida*, and is now being used as a reporter to track bacteria bearing rDNA (87).

The use of direct gene probes and PCR is a powerful approach to detect microorganisms without prior cultivation (88,89). For example, amplification of chromosomal genes from a highly specialized subpopulation of the total microbial community from the top layer sediment of the Elbe River in Germany made it possible to identify aerobic microbes that are able to degrade biphenyl (90).

Survival, Propagation, and Stability of GEMs in Their Target Ecosystems and Their Effects on Indigenous Microorganisms

A series of features that are crucial for the safe and effective functioning of some of the earlier recombinant and wild-type bacteria have been examined in soil microcosms with and without plants and in sewage microcosms. Microcosms offer a suitable approach to evaluate the survival and functioning of GEMs; however, it should be recalled that the samples are taken from nature and introduced into the laboratory. This imposes some limitations as a number of parameters are closely controlled, that is, incubation temperature, light/dark cycles, and so on. The survival of some of the recombinant microbes described earlier was assayed by introducing both parental and recombinant strains in the edaphic and the sewage water microcosms. These recombinant bacteria were designed to eliminate pollutants and were added to polluted soils and aquatic microcosms in relatively high numbers, for example, 10^6–10^8 colony forming units (CFU) per gram of soil or milliliter of sewage water.

Most studies have focused on the behavior of these microbes during the initial period after introduction into the microcosms, usually between four and eight weeks. It was generally observed that both the recombinant microbes and their parental strains were able to establish in soil, rhizosphere, and aquatic microcosms. In each ecosystem, the number of bacteria tended to reach the microcosm's carrying capacity, so that in microcosms rich in organic matter, the number of microbes was usually higher than that in microcosms poor in nutrients (72–74,88–96). Recombinant bacteria survived better than parental strains only when the former were introduced in soil or aquatic microcosms where up to 0.1% (wt/wt or wt/vol) pollutant had been added, that is, under conditions strongly favorable to the GEM. For example, the introduction of *P. putida* EEZ15 (pWW0-EB62) or *Pseudomonas* sp. FR1 (pFRC20) in soils supplemented with 0.1% (wt/wt) *p*-ethylbenzoate

and *p*-methylbenzoate allowed the strain to become established at at least one order of magnitude higher than in soils without the supplement (63,78,93). A similar finding was obtained when these bacteria were introduced into microcosms consisting of wastewater from a sewage treatment plant (91,92). Although survival of parental and recombinant bacteria approached 100% in short-term experiments, in the long term, a steady decline was observed in CFU per gram of soil until numbers in some cases fell below the detection limits (93).

Recombinant and parental bacteria survive in soils, and in certain cases, the total number of CFU increased. For example, when a low number (10^4 CFU per gram of soil) of *P. putida* (pWW0-EB62) was introduced in nonsterile soils, the strain multiplied to a density of 10^6–10^7 CFU per gram of soil (78). The stability of the genetic information introduced into *Pseudomonas* sp. FR1 (pFRC20P), *Pseudomonas* sp. F113-PCB, and *P. putida* EEZ15 (pWW0-EB62) has been studied (63,75,78). These strains can be considered genetically stable bacteria as the phenotype acquired through genetic manipulation was maintained under laboratory culture conditions in the absence of selective pressure. Furthermore, some natural properties of these strains that are not related to the recombinant phenotype, that is, biocontrol traits, growth rates, and pigment production, remained unaltered. The recombinant trait did not affect the competitive ability of the *Pseudomonas* sp. F113-PCB and *P. putida* strains in colonization assays in nonsterile soil microcosms on sugar beet, corn, tomato, and spinach-seedling roots (72–75). Furthermore, these strains have been shown to be stable in soil; 100% of the bacteria recovered after prolonged incubation in soil retained the ability to use the aromatic compounds that they were designed to deal with.

The effect of the introduction of wild-type or recombinant microbes into the indigenous microbiota has been studied by estimating the "total" number of culturable microbes in relatively rich medium (i.e., peptone agar) or by counting the indigenous population that is able to use a certain compound as the sole C-source, (i.e., *p*-hydroxyphenylacetic acid degraders), the denitrifying bacteria, or the "heterotrophic" population (74,89–95). Neither the wild-type nor the recombinant microbe affected indigenous microbiota, which suggests that natural environments have a certain buffering capacity against the introduced microbes.

The earlier cases suggest that the possible risks from the use of recombinant microbes in bioremediation are similar to those posed by the parental nonmodified strains. It also seems that the physicochemical and biological parameters of the microcosms affect the parental and recombinant microbes equally.

Functionality of Recombinant Microbes in Target Microcosms

The introduction of bacterial strains into the environment for in situ bioremediation will usually require that microorganisms be able to survive in high numbers and express the desired catabolic phenotype. For pollutants to be efficiently mineralized by natural and genetically modified microbes, the degrading microbe must not only become established in the polluted sites but also must express catabolic genes in response to the pollutant, even in the presence of other compounds. Soils, river sediments, and sewage treatment plants are complex environments where gene expression can be inhibited or stimulated. *Pseudomonas putida* harboring either the wild-type pWW0 or the recombinant TOL plasmid were able to mineralize ^{14}C-labelled substrates (*p*-methyl-^{14}C-benzoate) for at least a month (95,96). In soils, mineralization was monitored as the evolution of $^{14}CO_2$, whereas in aquatic microcosms the metabolism of alkylaromatics and chloroaromatics was monitored chromatographically by measuring the disappearance of the target chemical from the polluted site (92). It was shown that *Pseudomonas* sp. FR1 (pFRC20P) enhanced the rate of degradation of a mixture of *m*-chlorobenzoate and *p*-methylbenzoate that had been added to the water column of sediment cores made of intact-layered sediments from the Plussee Lake and the Rhine River (92).

Pseudomonas sp. FR1 bearing the *bph* genes inserted on the chromosome was able to remove up to 100 ppm of *p*-chlorobiphenyl per gram of sediment slurry in five days (20). In these assays, recombinant bacteria established at close to 10^8 CFU per gram of sediment.

Transfer of Recombinant DNA from *Pseudomonas* to Other Microorganisms

Gene transfer from recombinant microbes has been analyzed under optimum laboratory conditions and in soil and aquatic microcosms.

The genetic information introduced into *Pseudomonas* sp. F113-PCB was inserted into the host chromosome via a mini Tn5 lacking the transposase gene. In *Pseudomonas* sp. FR1 (pFRC20P), the information was located partially on the bacterial chromosome (Tn5:*xylXYZ*, *xylS*) and partially on a mobilizable broad host-range plasmid (pFRC20P). In *P. putida* (pWW0-EB62), the recombinant information was on a self-transmissible TOL plasmid. The recombinant DNA in these three strains was stably maintained under laboratory growth conditions and in bacteria introduced into soil and aquatic microcosms. In the laboratory, there was no transfer of the recombinant DNA from the chromosome of *Pseudomonas* sp. F113-PCB or *Pseudomonas* sp. FR1 (pFRC20P) to other *Pseudomonas* or to indigenous bacteria in soil and aquatic microcosms (63,75,91). In contrast, plasmid pFRC20P was transferred to other microbes, but only if a helper plasmid was supplied in the mating mixture (93). In these cases, transfer of the plasmid led to about 10^{-5} transconjugants per recipient. Transfer of the wild-type TOL plasmid or the recombinant pWW0-EB62 was restricted to microorganisms belonging to members of the pseudomonad rRNA group I (*P. putida*, *P. fluorescens*, *P. stutzeri*, and so on) and some enterobacteriaceae. In matings on plates, the rate of TOL transfer was on the order of 1 to 10^{-2} transconjugants per recipient, and the rate of intergeneric transfer ranged from 10^{-4} to 10^{-8} transconjugants per recipient (97,98).

In soils, the TOL plasmid was transferred from *P. putida* to other strains of *Pseudomonas* only at high cell densities (higher than 10^6 CFU/g soil). Transfer

was influenced by the type of soil used, the incubation temperature, the initial inoculum size, and the presence of chemicals that affected the survival of donor or recipient bacteria. Maximal transfer was observed in soils incubated at 15–17 °C, when the donor and recipient loads were about 10^8 CFU per gram of soil (97).

In conclusion, recombinant DNA can be transferred between microorganisms. Transfer is limited by the vector used to introduce the recombinant DNA into the host microbe and by the nature of the recipient microorganism. Chromosomal information is less likely to be transferred than is information on mobilizable plasmids. The latter is less likely to be transferred than recombinant DNA on self-transmissible plasmids.

Not all locations on the chromosome are similar in terms of expression, physical structure, or mobilization. This is particularly true in *Pseudomonas*. Ramos-González and coworkers (98) labeled the chromosome of *P. putida* randomly at 34 independent positions with the same marker, a mini-Tn5-Km. They found that the TOL plasmid was able to mobilize these insertions on the host chromosome at a rate between 10^{-4} and 10^{-8} transconjugants per recipient. Furthermore, mobilization of the host chromosome by the TOL plasmid occurred when the TOL plasmid and the marked chromosome were located in the two independent microbes, a phenomenon called *retrotransfer* (98–100). Retrotransfer involves the movement of a self-transmissible replicon to a recipient organism, the capture of DNA, and its return to the original donor (100). It follows that although chromosomal insertions are less likely to be mobilized than rDNA on plasmids, they can nonetheless be transferred potentially.

The deliberate release of GEMs in the environment raises a series of scientific and public concerns, and an active biological containment can provide a means to increase the predictability of the behavior and fate of recombinant microbes (101). Such biologically active containment systems have been shown to control the survival of GEMs and to inhibit lateral gene transfer (95,101,102) (see FIELD RELEASE OF GENETICALLY ENGINEERED MICROORGANISMS (GEM), this Encyclopedia).

CONCLUSION

Bringing about in vitro evolution of the catabolic pathways usually requires thorough knowledge of the biochemistry of the reactions involved in the degradation of a compound and of the genetics of the pathway. More specifically, intimate awareness of the organization of the genes that encode the enzymes and of the operation of the regulatory circuits that govern the activation of gene expression, including alteration of substrate and effector specificity by DNA shuffling (18,102,103), is required. These regulators can be useful for the design of new biosensors (104). The judicious combination of segments from different metabolic pathways in the appropriate bacteria can provide complete catabolic routes for recalcitrant xenobiotic compounds, and can circumvent certain problems related to substrate incompatibilities, and to the formation and accumulation of toxic intermediates. An example of this is the newly identified catechol 2,3-dioxygenase that is able to deal with chlorosubstituted substrates (105–107). This, in turn, can increase the catalytic potential and efficiency of microorganisms for the degradation of xenobiotics and can enhance their survival in environmental settings. Specifically designed pathways can also be introduced into microorganisms derived from contaminated sites of interest and hence, adapted to prevailing environmental conditions. As information accumulates on the genetic determinants of the characteristics that are important for treatment processes, such as tolerance or resistance to the toxic effects of solvents and other pollutants, surfactant production, and so on, our ability to generate more effective biocatalysts will increase (108–117). In recent years, a number of new catabolic pathways for toxic and recalcitrant compounds have been elucidated. These include pathways for the metabolism of dibenzodioxins and new pathways for chloroaromatics and polar and apolar nitroaromatics such as nitrobenzoates, monosubstituted, and polysubstituted nitrotoluenes (6,10,12,65,118–130). Equally important is the fact that many enzymes are extremely relaxed in their substrate specificity and are thus able to deal with multiple substrates. Some dioxygenases for one-ring aromatic compounds function also as monoxygenases and attack compounds with two or three aromatic rings and heterocycles (131–134). It is therefore expected that new catabolic pathways will be constructed on the basis of these catabolic modules, and as a consequence, the number of microbes able to mineralize halo-substituted and nitro-substituted aliphatic and aromatics, organic solvents, insecticides, and other xenobiotics should increase steadily.

Bioremediation research in simulated environments does not accurately represent real environmental conditions, and therefore, such experiments cannot test how prospective bioremediation schemes respond to the changes in the weather and to the movements of materials that may typify polluted sites. For these reasons, and to more fully address concerns regarding the safety and reliability of the approaches that are now being developed, carefully controlled field studies for bioremediation purposes need to be performed (see FIELD RELEASE OF GENETICALLY ENGINEERED MICROORGANISMS (GEM), this Encyclopedia).

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GENETICALLY MODIFIED MICROORGANISMS (GMM) IN SOIL ENVIRONMENTS

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The early 1970s witnessed a major breakthrough in molecular biology with the discovery of restriction enzymes. From that moment on, it has been possible to modify the genetic constitution of all organisms, and a new era in human history started. In the early years of genetic modification, the perspectives of these approaches were tremendous. It was, for instance, thought that it would not take long before one would be capable of modifying organisms in such a way that they could easily cleanup spills of waste or polluting materials or successfully control pathogens. Also, in

agricultural practice, expectations were high, for instance, considering the transfer of nitrogen fixation genes from bacterial hosts into plant genomes. Application of such plants would enable developing countries to decrease their expenditures on chemical fertilizers. Also, genetic modifications would make it possible, at least in theory, to reduce crop diseases, improve nutrient availability and, thus, drastically increase agricultural yields.

However, these expectations have been tempered ever since and have even led to an upheaval because of two reasons. Firstly, genetically modified organisms might not be as well adapted to their natural environments as their unmodified ancestors and thus perform poorly after release. Secondly, public concern was raised about the infringement on nature by genetic modification (often called genetic "manipulation" by persistent opponents to the application of these techniques). Maybe the first backlash was a result of the realization that genetically modified microorganisms (GMMs) would often indeed be outcompeted in their natural environments. In addition, the horizontal spread of recombinant genes was a second major concern because of the possible occurrence of unpredictable events due to lack of (ecological) knowledge about the effects of these new gene combinations on indigenous species.

However, it is currently well accepted that the application of GMMs to the environment needs a thorough understanding of the host strains carrying the genetic modification as well as the heterologous genes, with special emphasis on their regulating systems. Also, it is essential to gain an understanding of the spread and potential dormancy of modified bacterial cells in the environment, in order to assess the potential risks involved in their use in nature.

In this article, we focus on the requirements for the application of GMM strains in natural, unconstrained environments such as agricultural fields, and discuss approaches for tracking released GMM strains as well as limiting their spread by the construction of so called self-containing mechanisms.

CONSTRUCTION OF GMMs AND CONTAINMENT

For the construction of a genetically modified bacterial strain, developed to perform under field conditions, two considerations are important; (*1*) optimum survival and activity and (*2*) spatial and temporal containment. First, survival and activity of the introduced GMM often must be optimum; that is, metabolically active cells of the GMM strain should remain present in sufficient numbers for the required period of application (1). On the other hand, the strain must ideally remain restricted only to the location and period for which heterologous gene activity is desired (1). Both considerations may be contradictory when optimum survival is necessary on the one hand, while on the other hand the GMM has to ultimately be eliminated in order to prevent its persistence or escape from the site of application. However, sufficient survival as well as containment may be combined when different regulatory genes responding to intrinsic cellular or environmental signals are used. Regulatory genes necessary for controlling the heterologous genes inserted can be triggered under different conditions as genes necessary for containment of the GMM strain. In this section, special attention is given to the regulation of the gene inserts, both to improve survival and to contain the introduced strains with respect to time and location.

In general, GMM strains are ecologically less competent than their (unmodified) parent strains, as demonstrated by competition experiments in soil (2). Because of the expression of the heterologous gene, the cellular energy expenditure is increased, and hence GMM cells may be deprived faster of their energy sources. Therefore, uncontrolled expression of inserted genes can be harmful to the released GMM; controlled gene regulation at the appropriate time and site of application would be preferable. The use of regulatory genes specifically responding to cellular physiological status (e.g., exponential versus stationary phase) or specific environmental signals may help overcome cellular energy depletion. On the other hand, the signal used for beneficial gene expression can also be applied to regulate containment of gene expression via a "negative loop" construction (3). A repressor gene can be placed under the control of a promoter regulated by the environmental signal, whereas the intended containment gene is constructed downstream of a promoter/operator region controlled by the repressor protein (4).

The choice of different signals and their responding promoter sequences, with the aim to control heterologous gene expression, is an important first step. Subsequently, a screening of appropriate promoter sequences should be carried out and these promoter constructs should be used for further application. In this section, a study is included in which two different promoters of *Pseudomonas fluorescens* that respond to signals from the soil environment were characterized.

Promoter probe reporter systems are excellent tools for investigating the expression of genes involved in the response of bacteria to soil conditions. Plant-induced reporter gene expression has been determined with transcriptional fusions using reporter genes such as *lac*Z [ß-galactosidase] (5,6), *gus*A [ß-glucuronidase] (7,8), *lux* genes (light emitting genes from Vibrio fisheri) (9,10), *xyl*E (xylene degradative gene) (11), and *ina*Z (ice nucleation gene) (12). For microscopic detection of *in situ* induced reporter gene activity, specific fluorescence markers such as the green fluorescent protein (GFP) gene (*gfp*), which has been cloned from jellyfish (*Aequorea victoria*) (13), can be used. Using promoterless *gfp* as a promoter probe reporter, specifically induced expression can be observed by epifluorescence UV microscopy in which intracellular GFP will emit a green light.

Although testing of induction of reporter genes in situ is relatively unexplored, progress has been made recently. Bacterial strains with reporters inserted in genes that show expression upon exposure to stress conditions such as phosphorus limitation (14) have been studied *in vitro* and in soil, and the genes

identified were shown to function under soil conditions. Similarly, Kragelund and coworkers (15) showed the expression of N and C responsive genes under soil related conditions. Responsiveness to root exudates and carbon limitation was investigated in soil, soil supplemented with nutrients, and rhizosphere soil (16–18). Reporter gene expression upon carbon limitation was observed in unamended bulk soil, indicating that the carbon limitation induced promoter can be used as a regulatory element to control gene expression in GMM strains intended for application in soil (16). In situ root exudate-controlled reporter gene activity under natural conditions has been reported by Vande Broek and coworkers (8) and Brennerova and Crowley (9). Furthermore, *in situ* root colonization studies under gnotobiotic conditions have been performed with various different markers and bacterial species. For example, de Weger and coworkers (10) used naphthalene-inducible *lux*CDABE and constitutively induced *lux*CDABE and *lux*AB gene constructs for *in situ* root colonization studies with *P. fluorescens*. Reporter gene activity was detectable in the rhizosphere with the naphthalene-inducible *lux*CDABE and constitutively induced *lux*AB genes at a colonization level between 10^3 and 10^4 CFU/cm root.

Studies on the induction of reporter genes by soil or plant stimuli in natural soils are technically feasible (10,16–18). The promoter/reporter gene combinations can be used in the natural environment, for example, as biosensor systems or to determine the expression of genes of interest. Reporter gene expression was observed near wheat roots in microcosms as well as in the fields (16); the promoter identified proved to be an excellent regulatory gene of the expression of recombinant genes in a new generation of GMM constructs.

BIOTIC AND ABIOTIC FACTORS INFLUENCING BACTERIAL SURVIVAL IN SOIL

For decades, bacteria have been isolated from soil and characterized in the laboratory with the aim to study and improve their performance as biopesticides (19), biofertilizers (20,21), or bioremediation agents (22,23). Symbiotic nitrogen-fixing bacteria such as rhizobia (24), free-living nitrogen-fixing bacteria such as *Azospirillum* (25), as well as plant growth-promoting rhizobacterial (PGPR) gram-negative species such as *Pseudomonas* (26–28) and *Flavobacterium* (29) or gram-positive species such as the *Bacillus* species (28,30,31) are among the most frequently used soil inoculants. However, many species do not survive well in soil, either because they are not indigenous to soil or because they survive well only in specific soil types (25). It should be noted that, in addition to the specific intrinsic properties, such bacteria have often been selected on the basis of the fast growth of colonies or broth cultures under laboratory conditions and their suitability for genetic modification.

Much effort has been spent on the isolation and identification of ecologically adapted bacteria from specific sites in soil, such as the rhizosphere (32,33), under the assumption that these organisms possess traits that helped them adapt to the conditions prevailing at these sites. Copiotrophic gram-negative species such as those of the genus *Pseudomonas* (32,33) are abundant in the rhizosphere and have been suggested to be the best soil inoculants because of their fast growth and alleged optimum survival under prevailing conditions in the rhizosphere as well as their genetic accessibility. However, upon introduction, bacterial cells are often subjected to the harsh conditions present in the rhizosphere and bulk soils, which can limit their survival. How the bacteria cope with these conditions depends on their genetic makeup and their physiological condition at the time of introduction into the soil (34). For practical applications, bacterial cells should aptly colonize soil sites; optimum survival and activity is only ensured after successful occupation of such an ecological niche. It is clear that the site (i.e., rhizosphere or bulk soil) in which inoculant bacteria reside in soil greatly determines their physiological and metabolic status, which in turn determines the persistence and activity in soil.

Introduced Soil Populations

The main goals of field tests that have been conducted to date have been to monitor the fate of genetically marked bacteria as models for subsequent GMM releases with practical applications and to study the ecological effects of these inoculations (17,35–37). Most of these studies assessed the putative risks involved in the release of GMMs into soil by measuring parameters such as survival, spread, and gene transfer, but not the fundamental properties of the introduced population, such as its physiological status or its response to environmental stresses. The latter items are important as they determine the efficacy of the application.

Knowledge of the behavior and fate of indigenous soil bacteria upon their introduction into soil is of importance in all cases in which these bacteria are applied to practical purposes such as biological control or bioremediation. Survival of the introduced populations in soil has been studied for a wide range of organisms, and the generally observed progressive decline of bacterial numbers following introduction into soil has often hampered the effectiveness of bacterial inoculants [reviewed by Van Veen and coworkers (1)]. For instance, comparison of the population dynamics of fluorescent pseudomonas in different soils showed that all introduced populations declined to low numbers within time spans of several weeks to months. Overall linear decay rates varied from approximately log 0.2 to log 1.1 colony forming units (CFU) per 10 days, and depended on the strain and the soil used. Similar responses have been found in other introduced bacteria such as *Salmonella typhimurium* and *Klebsiella pneumoniae* (38) and *Flavobacterium* spp. and *Alcaligenes* spp. (29,33). Such responses might be characteristic of copiotrophic organisms exposed to soil conditions. Oligotrophic bacteria introduced into soil may also show progressive decrease in CFU numbers, albeit slower than copiotrophic bacteria (33,39). The slower decline of oligotrophic bacteria in bulk soil is possibly related to a better adaptation to the carbon-limited conditions of soil.

It is difficult to pinpoint a single dominating reason for the decline of introduced bacterial populations in

soil. However, introduced bacteria, particularly shortly after release, are likely to be affected by the same adverse soil conditions that affect indigenous bacteria, such as limitation of a range of nutrient sources. Upon introduction in soil, increasingly higher numbers of cells of an introduced *P. fluorescens* strain were detectable via specific immunofluorescence than via selective plating (28). This suggested a possible conversion of part of the introduced population into nonculturable forms that, however, might be still viable. Such viable but nonculturable (VBNC) cells were also found in other studies (40); recently it was found that their presence does not enhance the persistence of introduced *Pseudomonas* populations (41). The occurrence of nonculturable cells, in the indigenous as well as introduced bacterial populations, indicates that one or several major factors controlling the status of bacterial cells in soil are common to bacteria in soil. Moreover, given the relative immobility of bacterial cells in soil, the localization of introduced bacteria following their introduction is likely to affect their ultimate fate (42), that is, localization determines whether inoculant cells are successful in colonizing soil and developing a cellular form resistant to soil stress or whether they will die out.

PHYSIOLOGICAL ASPECTS THAT AFFECT THE FATE OF BACTERIA INTRODUCED INTO SOIL

The metabolic activity and physiological state of bacterial populations will greatly affect their fate and efficacy after introduction into soil. Moreover, it is important to determine whether GMM strains are impaired in this adaptation due to the presence or expression of heterologous genes, resulting in an increased metabolic load.

Different approaches have been taken to assess the metabolic and physiological conditions of cells in soil. Traditionally, the detection of specific strains in a natural environment has been based on CFU counts on selective plates or on immunofluorescence (IF) cell counts (43,44). Using both techniques in combination, information on the presence of nonculturable cells can be obtained (17). However, these approaches provide little information about the activity of inoculated cells in soil. To assess the risks involved in the release of GMMs in soil, it is important to determine whether such nonculturable cells are alive (viable) and metabolizing, alive but in a metabolically arrested state, or dead (45,46). To detect the presence of heterologous genes in bacterial cells in soil, including nonculturable ones, direct molecular detection techniques have been developed (47). Discrimination between viable and nonviable cells has often been performed using the direct viable count method (DVC, based on cell elongation by addition of nutrients in the presence of a cell division inhibiting compound) (48) or redox dyes such as 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT) (49) or 5-cyano-2,2-ditolyl tetrazolium chloride (CTC) (50). The viable cell numbers, determined by either or both techniques, often appeared to be intermediate between the total (IF) cell and selective CFU counts in different habitats such as plant surfaces (51) and soil (52,53). However, it remains to be determined whether viable but nonculturable populations are responsive to environmental stimuli, or if they are metabolically arrested. At least in one study it was concluded that the viable but nonculturable state of introduced *P. fluorescens* cells does not represent a physiological stage in which cells are optimal adapted to the harsh conditions as present in soil (41).

Another criterion by which bacterial adaptation can be measured is their responsiveness to different, often stressful conditions that prevail in soil, such as carbon deprivation that limits growth (4). In general, bacterial growth-limiting conditions provoke cellular responses, leading to an increased, overall, generalized, resistance. For instance, *Escherichia coli* cells that were limited in their growth after depletion of carbon sources showed an enhanced resistance to stress conditions such as high temperature, osmotic stress, and the presence of oxidative or noxious compounds (54,55). A similar response was observed in a typical soil bacterium, *Pseudomonas putida*, and adaptation coincided with *de novo* synthesis of proteins upon growth limitation (56). Later, enhanced stress resistance was also demonstrated for *P. fluorescens* cells introduced into two texturally different soils (57). Although the rates of cell decline upon introduction of this strain in the two soils were different, the time spans needed for complete adaptation (1–5 days) were the same. This led to the conclusion that differences in survival time as a result of residence in different soil types are not related to differences in adaptation, but rather to other factors such as protection against predation. To address questions about bacterial responses that serve as signals for the onset of adaptation to soil, host cells carrying environmentally controlled reporter genes may help identify triggers to which these organisms will respond upon their release (16).

TRACKING GENETICALLY MODIFIED STRAINS IN SOIL ENVIRONMENTS

Sampling

Bacterial cells released into soil will not always remain at the site of application but may spread after their release. Such spread can be lateral (spurred by wind, rain, insects, or human activity) or vertical (induced by rain or burrowing worms). Also, cells may become nonculturable and die, whereas other cells become active and are mobilized. The relative density of introduced strains may thus fluctuate in soil. In order to obtain a reliable overview of the presence of released GMM strains in the field, it is necessary to use an appropriate sampling strategy (58).

Dispersal of a genetically modified *P. fluorescens* strain was monitored during a field release study in the Netherlands (17). From the ninth day onward, detectable GMM CFUs were observed just outside the inoculated plot, with a maximal distance of about 2 m. However, CFU numbers in soil never exceeded 10^3 per g of dry soil, and the released GMM strain outside the plot could no longer be detected after 3 months, whereas in the inoculated part in the plot, the introduced strain survived for more than one year. This example illustrates that the number and

location of the samples drawn should carefully be taken into account. Thus, the sampling strategy chosen will depend on: (*1*) the mode of introduction, (*2*) the time span after release, and (*3*) factors influencing the mobility of inoculant cells such as plant growth, soil water flow, and spread by wind, animals, or human treatments. Obviously, all considerations about the statistics of sampling play a role, as outlined by Van Elsas and Smalla (58).

The mode of application of (GMM) strains will determine the location where cells reside. Cells can be applied either directly to soil in suspension via injection or spraying, or indirectly by immobilization in carriers using coatings (seed coating or root dipping) or slow-release carriers (e.g., in alginate or κ-carrageenan beads) (59). Cells applied directly to soil will colonize the top soil layer, whereas immobilized cells tend to remain at the site of application. The way bacteria are introduced into the field (e.g., spraying versus application as immobilized cells) will influence the sampling strategy. Shortly after its release the effect of introduction is clearest, whereas, later, these effects may fade away because of dispersal in soil. Also, it should be taken into account that the introduced strain may spread heterogeneously over the field. To establish statistically significant differences between treatments related to the release of GMM strains, enhanced numbers of replicates will be needed to overcome increasing standard deviations.

Vertical movement of introduced bacterial cells in soils has been observed under controlled conditions (60–62) as well as in fields (63). Bacterial migration to deeper soil layers is mainly the result of water flow by heavy rainfall. However, in the same field experiment performed with a genetically modified *P. fluorescens* strain, vertical movement occurred without heavy precipitation (17). Supposedly, bacterial cells migrated in substantial numbers to deeper layers by other means, for example, through soil cracks or by activity of soil animals such as earthworms (64).

Plant growth is a major factor affecting the spread and activity of introduced bacterial cells. Bacterial growth near plant roots may result in increased ecological competence and thus competition with indigenous species residing near plant roots. Therefore, sampling of the rhizosphere of wild plant species near or in the test field is a prerequisite to determining the fate of the introduced GMM strain (65).

Methods for Sample Analysis

GMM strains in natural environments can be tracked with methods based on (*1*) their growth in selective media (cultivation-based methods), (*2*) intrinsic properties of the host (using antisera or probes targeting specific epitopes or nucleic acid sequences), and (*3*) the recombinant DNA (using probes or primers).

In most cases, GMM strains, when released into soils, are monitored over time using cultivation-based methods. Plating techniques are most convenient to use because of the selectable markers commonly present in GMM strains, as well as the possibility for quantification. Additionally, isolated colonies can be tested for phenotypic traits by carbon utilization testing [e.g., by using commercially available test systems such as API (Montalieu-Vercieu, France) or BIOLOG™ (Hayward, CA)] and molecular fingerprinting [e.g., by using Repetitive Extragenic Palindromic (REP)-sequences, Enterobacterial Repetitive Intergenic Consensus (ERIC-) or BOX-PCR] (66), and presence of the genetic insert can be tested by using molecular probes (47). However, bacterial growth is a prerequisite step for plate-counting methods and, thus, only the culturable fraction of the populations is assessed. Additional measurements by direct, cultivation-independent methods, such as selective cell counts, will be necessary to determine the total number of GMM cells present in soil. The nonculturable fraction can then be deduced by subtracting the CFU numbers from the cell numbers. The determination of the total GMM cell numbers can be accomplished by the use of fluorescent dyes conjugated to molecules targeted against host-specific properties such as epitopes (using antibodies) (44) or nucleic acids, generally 16S rRNA (using molecular probes) (67). Cells extracted from soil can be stained directly by using specific antibodies conjugated to a fluorescent dye such as fluorescein isothiocyanate (FITC) (immunofluorescent cell staining). However, when using molecular probes, cells must be fixed and permeabilized in order to allow entrance of the conjugated probes (whole-cell hybridization). Treated cells are visualized by their fluorescence under UV light (wavelength depends on the dye used) at about 1,000 times magnification. In both cases, the targeted molecules present in bacterial hosts (epitopes for antisera and nucleic acids for molecular probes) should be sufficiently available, even under conditions of cell starvation. Therefore, polyclonal antisera as well as probes directed against ribosomal 16S or 23S RNA are preferred, as the targets (multiple epitopes and ribosomal RNA) are expected to be most stable, albeit not constant.

The inserted genes in the released GMM strain can be detected independent of cell growth. For that purpose, DNA extracted from soil can be amplified by PCR using primers directed against the heterologous gene. Protocols for DNA recovery from soil are routinely applied in many research laboratories (68); they generally allow the detection of target molecules on the order of about 10^2 to 10^4 per g of soil. Quantification can be accomplished either by quantitative (Q)-PCR (quantification is based on the relationship between the number of target molecules initially present and the number of amplified products during several PCR cycles), competitive PCR (competition between amplification of target molecules and added target sequences) (69), and most probable number (MPN) PCR (end point dilution of target DNA followed by PCR). Moreover, PCR amplification in extracted and fixed cells (whole-cell PCR or *in situ* PCR) is a promising new tool for detection and quantification of released GMM strains (70).

The side-by-side use of a range of different techniques for tracking GMM strains in soil is recommended in all field releases. Only such a polyphasic approach allows the establishment of the possible occurrence of nonculturable cells, the number of cells that lost their heterologous DNA, as well as the occurrence of heterologous DNA without the presence of the original host cells. Concerning the last point, heterologous DNA may be present in soil as 'naked'

DNA (from lysed cells) or located in other hosts as a result of gene transfer (see later). Therefore, only a polyphasic detection strategy will allow an appropriate judgment of the presence and fate of GMM strains and their DNA in environmental samples.

ASSESSING THE EFFECTS OF GMMs ON POPULATIONS INDIGENOUS TO SOIL

Perhaps the most troublesome public concern about the release of GMM strains is the potential for concurrent undesirable effects on the indigenous microbial community upon introduction into the open field. Hence, most national governments currently require extensive data on such possible effects, prior to granting permission for field release of any GMM. Preceding every field release, a protocol must be set up that includes measurements of expected effects and, if possible, consequences that may not be directly related to the application. These considerations can either be directly related to an effect of the inserted (heterologous) gene on the native organisms or related to changes in the occupation of different (micro) habitats of natural species caused by the application of inoculant strains. Furthermore, an important potential consequence of the introduction of GMM strains into the environment is the transfer of recombinant DNA to indigenous species. The effects of GMM releases on natural populations can thus be considered by using two criteria; (*1*) an ecological and (*2*) a genetic criterion.

Ecological Effect of a GMM Release

In considering the impact of GMM releases, we deal only with the effects brought about by the novel genetic combination, thus disregarding effects that would have been caused by wild-type strains. Effects on natural populations can be expected from the expression of the inserted heterologous genes whose products are intended to target particular organisms, but which may, unintentionally, also affect nontarget, organisms. For instance, constructs consisting of genes whose products are aimed at a reduction of plant damage caused by soil-borne pathogens may also affect populations of nonpathogens. Examples are specific *Bacillus thuringiensis* crystal protein (*cry*) products targeted against larvae of dipteran insects (2) and genes encoding antifungal compounds from *P. fluorescens* such as phenazines (71). Both genes are not specific to just one particular pathogen, but can affect a broader range of organisms, although there is considerable specificity for limited insect groups among the various *cry* gene products.

Assessment of effects that cannot directly be rationalized from the function of the heterologous gene is more complicated. In fact, all possible targets should theoretically be included in the risk assessment protocol, which is a truly daunting task. Hence, we propose that, first, a reasonable assessment should be made as to which organisms might be possibly affected. Then, an appropriate technique that is capable of surveying the effects on all organisms of the selected groups must be chosen.

Recently, possible shifts in fungal community structures in field soil were studied upon release of a *P. putida* strain genetically modified by insertion of a gene responsible for the production of the antifungal compound phenazine-1-carboxylic acid, with the intention of a later application as a biocontrol agent (71). Fungal isolates were obtained from the rhizosphere of crop plants (*Triticum aestivum*) in different field plots treated with modified or parent strains and analyzed by Amplified Ribosomal DNA Restriction Analysis (ARDRA). A transient change in fungal community structure was observed in wheat rhizospheres from plots inoculated with either strains, and the effect on fungal populations in rhizosphere soil with the GMM strains was different from that containing the unmodified strain.

Exposure of soil microbial populations to different carbon sources in BIOLOG^{TM} microtiter plates is a commonly used approach to study differences or shifts in soil microbial compositions (72). Community Level Physiological Profiling (CLPP) using the 95 different carbon sources present in the Biolog microtiter plate (73) helped determine the effect of a soil-inoculated GMM strain on indigenous populations. As bacterial growth is not required, this approach can be considered as a measure of total activity of all bacterial population residing in the soil.

Studying microbial populations by molecular techniques excludes biases caused by growth steps prior to analysis, whereas large numbers of species can be investigated in a single profile. Nucleic acid extraction is a prerequisite, and methods for extraction and purification of DNA and RNA from soils are nowadays commonly available in many text books (68,74). In general, molecular fingerprinting of microbial communities is based on PCR amplification of hypervariable regions present on 16S rDNA genes using primers that target conserved regions directly adjacent to these (for a schematic overview, see Fig. 1). Ribosomal genes are "par excellence" suitable for molecular fingerprinting and different primers targeting 16S or 23S ribosomal genes have been developed. Analyses of the amplified products are performed by Temperature or Denaturing Gradient Gel Electrophoresis (TGGE and DGGE) (75) or Single Strand Conformational Polymorphism (SSCP) (65) (Table 1). Both methods have successfully been applied by different laboratories to study microbial community changes in different habitats. A fingerprint consisting of different bands, each representing individual organisms, reveals the complexity of the microbial populations in the environment, whereas, their activity can be determined by amplification from RNA (76). Individual bands can be sliced out from gels, cloned into vectors, and analyzed for DNA sequencing. Database comparisons of the sequences, for instance, by BLAST searches on the Internet, helps establish the potential affiliation of the selected bands.

Schwieger and Tebbe (65) described a field release study in which molecular fingerprinting of microbial communities was applied. Two *Sinorhizobium meliloti* strains chromosomally tagged with the luciferase gene (inserted for convenient recovery; one strain was mutated in a gene responsible for DNA repair after damage, i.e., *rec*A) were introduced into field plots planted with alfalfa (*Medicago sativa*, the natural host for *S. meliloti*). Three months after

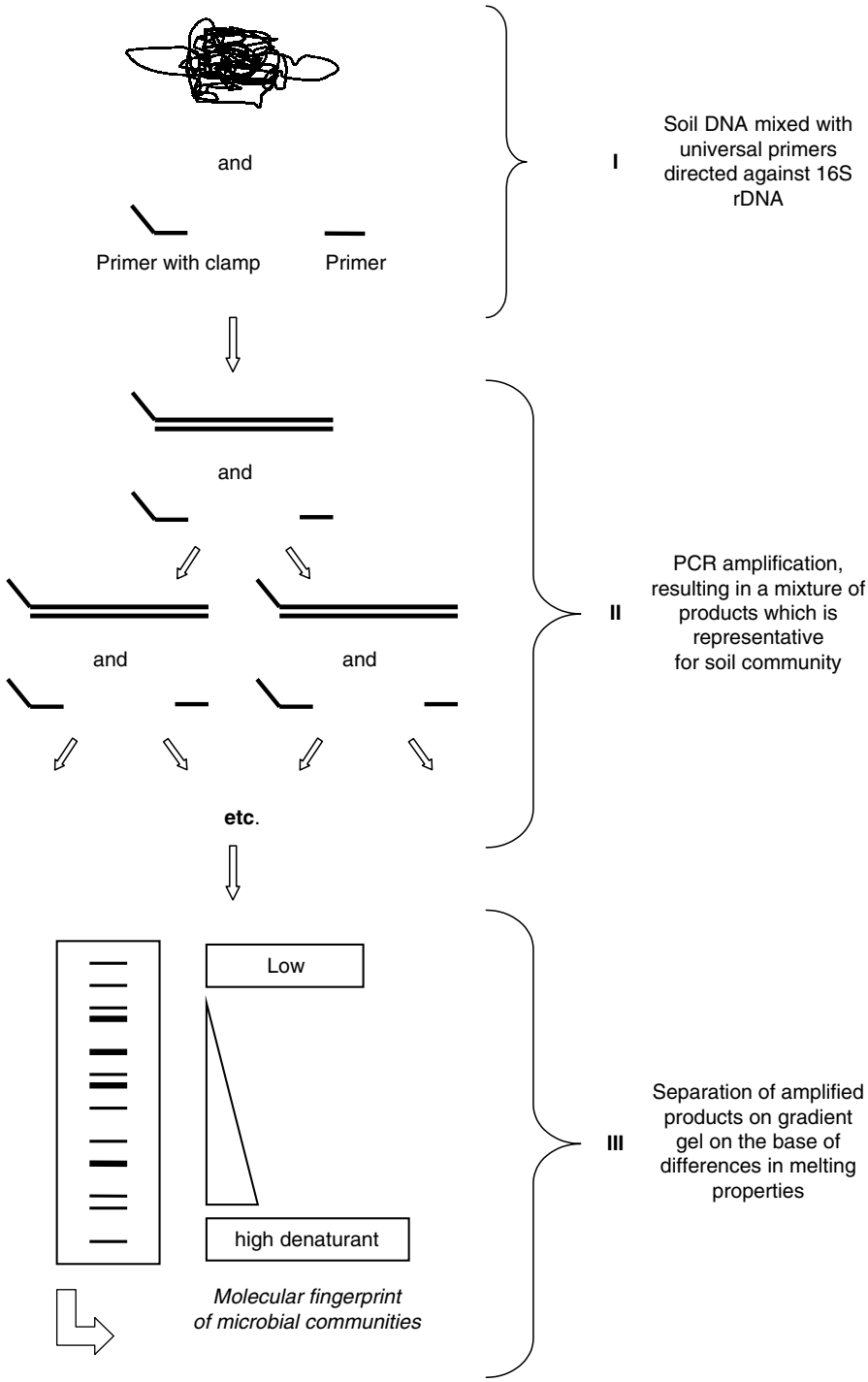

Figure 1. Schematic representation of the procedure for DNA fingerprinting of soil habitats. Molecular analysis of soil communities is independent of preceding culturing and consists of the following steps: (*1*) extraction of soil DNA, (*2*) PCR amplification of 16S ribosomal DNA using a primer set with GC clamp (necessary for analysis of the products on denaturing gradient gel), and (*3*) separation of the PCR products on a gradient gel.

introduction of both populations, the community structures near the alfalfa roots were clearly affected by the release, as demonstrated via SSCP. The dominant *Pseudomonas* population was reduced in the rhizosphere of alfalfa plants treated with the modified strains. However, the rhizosphere community structure near a weed commonly observed in the field plots (*Chenopodium album*) was not affected by the introduced strains.

Although the data obtained by molecular fingerprinting of inoculated soils show a great deal of detail, a major drawback of the method is the use of highly conserved ribosomal primers. Only the most dominant species (over 0.1% of the total) can be visualized on gel. Therefore, development of primers for selected groups of organisms, including prokaryotes and archeal and fungal species, are in progress. Such systems would allow the in-depth study of specific groups of organisms.

In conclusion, the development of molecular techniques, either or not in combination with classic techniques such as culturing or enrichment steps, allows the study

Table 1. Methods Used for Studying GMM Impact on Microbial Populations Indigenous to Soil Ecosystems

Method		Remarks	References
Growth-based	Plating/colony formation on:	Only culturable populations assessed. Tedious.	77,78
	—General media		47,79–81
	—Selective media		
	Biolog Physiological Community Level Profiling	Measurement of potential microbial community activity.	53,72,73,82–84
Microbial biomass assessments	Extraction of microbial compounds	Compounds related to specific groups of organisms such as muramic acid (bacteria) and phospholipid fatty acid (fungi).	85,86
	Microscopy	Direct biomass assessment.	87,88
	Soil enzymes	Potential activity assessed.	85,89
Fingerprinting of PCR-amplified products from extracted nucleic acids	PCR/DGGE* or TGGE* and CSPD*	Profiling based on differential melting of 16S PCR products obtained from community DNA.	65,75,90,91
	PCR/ARDRA*	Profiling by restriction of 16S PCR products obtained from community DNA.	92,93
	PCR/T-RFLP*	Profiling by restriction of fluorescent end-labeled 16S PCR products obtained from community DNA.	94

*DGGE: Denaturing Gradient Gel Electrophoresis.
TGGE: Temperature Gradient Gel Electrophoresis.
SSCP: Single Strand Conformational Polymorphism.
ARDRA: Amplified Ribosomal DNA Restriction Analysis.
RFLP: Restriction Fragment Length Polymorphism.

of changes in natural populations as a result of the introduction of GMM strains. So far, different effects on natural populations caused either by the introduced strain or by expression of the inserted gene(s) have been shown (17,35–37,65,71). None of the effects shown are thought to pose a threat to ecosystem functioning. It is prudent to allow the testing of a wealth of novel microbial gene products on a case-by-case basis, building on the experience of no apparent risk gained so far.

Genetic Impact of GMM Releases

Transfer of genetic inserts from released GMM strains into ecologically competent species that reside in soil may have consequences for the persistence and expression of these genes in the environment. Therefore, special care has to be taken during construction of GMM strains with respect to the possibility of transfer of heterologous genes to natural species. Most commonly, genes are inserted into the chromosome either by genetic crossing-over using DNA sequences homologous to the target site for insertion or by transposon mutagenesis. The advantage of chromosomally inserted constructs is: (*1*) the inserts are present in the cells as single copies, (*2*) the mutants are genetically stable, and (*3*) transfer is limited and only possible via interactions with mobilizing elements such as plasmids, conjugative transposons, insertion elements, and/or phages. Such elements would first have to enter the inoculant cell from the natural microflora, a low-frequency occurrence in soil (95). A drawback of chromosomal insertion, however, may be the low copy number of the heterologous genes, possibly resulting in restrictions on expression. Therefore, nonmobilizable plasmids carrying these constructs are favored in some occasions.

Movement of genes between organisms in soil is possible via the prokaryotic gene transfer mechanisms: transduction, transformation, and conjugation. Transduction, a phage-mediated DNA transfer, has been only incidentally described for soil environments but it cannot be ignored that it plays an important role in the movement of genes between bacterial species. Transformation, the transfer of extracellular DNA by competent bacterial cells, and mobilization, for example, plasmid-mediated transfer of DNA, are better known processes in soil environments. Transformation between introduced strains in natural soils has been conclusively demonstrated by Lee and Stotzky (96). Nielsen and coworkers (97) extended this work, showing that cells of the transformable bacterium *Acinetobacter* sp. BD413 can become naturally competent in soil and

then effectively capture available DNA (97). However, most information about bacterial gene transfer in soils has been obtained for conjugation using self-transmissible plasmids such as RP4 and pIPO2 and mobilizable plasmids (IncP and IncQ plasmids) such as RSF1010 and pIE723 (98–100). Although transmissible plasmids are not recommended for use in GMMs intended for release, these studies have conclusively demonstrated that gene transfer does take place in natural soils. The use of mobilizable but non–self-transmissible plasmids (IncQ plasmids) may be a safer option, although the isolation of a cryptic mobilizable and self-transmissible plasmid from soil (100) may imply that IncQ plasmids can be transferred into indigenous soil bacteria by retromobilization. As indicated, chromosomally inserted genes are transferable to other species via excision and insertion into incoming plasmids or conjugative transposons from the natural microflora, although the incidence of such an occurrence is likely to be very small.

In general, gene transfer is an unwelcome event when releasing GMMs to the environment. However, transfer of heterologous genes to indigenous species may sometimes be very advantageous as it may result in stable integration of these genes into species that are well adapted to the habitat and are, thus, ecologically more competent than the inoculant (host) strain. This strategy has been applied under contained conditions in bioreactors, in which the xenobiotic compounds 3-chlorobenzoate (101) and 3-chloro-aniline (102) were degraded. Introduced GMM strains carrying the respective catabolic genes on mobilizable plasmids were transferred to indigenous strains and the transconjugants that appeared were successful in the degradation of noxious compounds. Although, up to now, this application is restricted to bioreactors, it may be extended to soils, in particular, in the degradation of xenobiotics in contaminated soils.

PROSPECTS FOR THE USE OF ENVIRONMENTALLY-INDUCED PROMOTERS IN SOIL

Environmentally controlled promoter regions can certainly be applied to regulate the expression of biological control (control of plant diseases) or biological containment (containment of GMMs) of genes in soil inoculant strains. To achieve this goal, the reporter gene that enables the detection of the regulatory regions should be replaced by an appropriate biocontrol or biocontainment gene [using a negative loop (3)]. Suitable candidates for the construction of environmentally regulated biocontrol agents are bacterial genes involved in the synthesis of antifungal antibiotics, such as phenazine-1-carboxylic acid (103,104), and 2,4-diacetylphloroglucinol (105) from fluorescent *Pseudomonas* spp. or genes toxic to soilborne insect larvae, such as the *B. thuringiensis cry*IVB gene (106). Genes of rhizosphere bacteria involved in antagonism toward plant-pathogenic fungi are of special interest and may be used as targets for cloning experiments. When these genes are brought under the control of a rhizosphere-induced promoter, the timing and extent of gene expression can be controlled depending on the promoter of choice. This new strategy in the utilization of GMMs as biocontrol agents is promising because it can enhance and concentrate the beneficial action of the control agent to the site and time that it is most needed, for example, at the onset or peak of pathogen activity. However, in addition to the production of antibiotics (1), bacterial antagonism toward phytopathogenic microorganisms in the rhizosphere may also be affected by competition for nutrients (107), or it can be based on a combination of both actions (108).

Genes proposed for biological containment are the *E. coli gef*/*hok* (host killing) and *rel*F genes (109) and the *Serratia marcescens nuc* (nuclease) gene (110). Overexpression of *gef*, *hok*, and *rel*F genes causes a collapse of the membrane potential, whereas *nuc* gene expression results in intracellular degradation of DNA. The result of both actions is severe damage to the host cell, resulting in cell death. These genes can be brought under the control of a carbon starvation inducible promoter or a rhizosphere induced promoter region connected to the "negative loop" construction, as discussed before. Induction of the host killing genes by carbon limitation in bulk soil can result in cell death, thus confining the active GMM cells to the rhizosphere, which is the site where expression of biocontrol genes is desired. Thus, a GMM strain can be constructed that expresses its biocontrol gene in the rhizosphere whereas, after escape from this site, expression of the biocontrol gene is switched off and the host strain will be killed by expression of the biocontainment gene.

CONCLUSION

The success of the application of GMM strains in soil environments depends on the selection of the bacterial host that should be ecologically competent in the selected habitat. Survival of the inoculant strain can be optimized by using appropriate carrier materials, whereas its activity can be regulated by selection of appropriate regulatory sequences used for the control of expression of the heterologous gene(s). From a biotechnological point of view, there are no limitations in the optimization of suitable inoculants. However, more information is needed about the adaptation and survival of released GMMs, upon introduction into the target environment. Up to now, we lack fundamental knowledge of a full understanding of the adaptational processes with respect to the metabolic activity and dormancy of inoculant cells (111) (for overview, see Fig. 2). Unraveling these mechanisms will potentially allow an interference in the cellular properties and, thus, optimize survival under selected conditions.

Restricting GMM strains to the site of application may decrease the putative risks involved in the release of these inoculants into the environment. The development of optimized modes of introduction, aimed to restrict released GMM strains to specific sites, as well as the tools necessary to track these strains and to determine fate, activity, and effect on natural microflora, is needed on a case-by-case basis. Techniques for impact assessments now make it possible to pinpoint drastic effects on microbial fluctuations, but more development is necessary to enable detection of subtle changes in microbial community structure upon releases of GMMs into the environment.

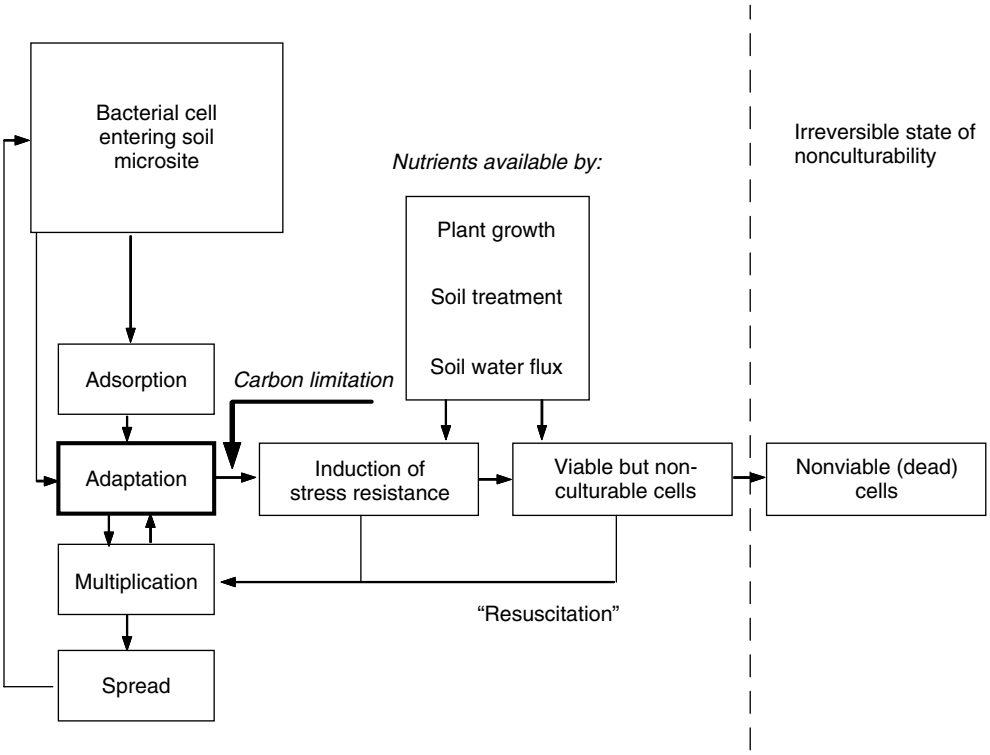

Figure 2. Concept of the sequence of events following the introduction of bacterial cells into soil and the specific factors influencing the physiological status of bacterial cells in soil. (Data taken from L. S. van Overbeek, in Responses of Bacterial Inoculants to Soil Conditions, Ph.D. Thesis, Leiden, The Netherlands, 1998.)

Nevertheless, mankind should not lose its feeling of optimism about the great potential offered by the prospect of GMM releases in bioremediation and biocontrol. Compared with the prospects available only 10 years ago, major steps forward have been taken. It is very feasible that genetically modified biocontrol strains will replace chemical pesticides, or be used in an integrated fashion, to control soil-borne diseases in some of the key crop plants that feed the world.

Acknowledgments

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GENOMICS, ENVIRONMENTAL

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In 1995, the complete research of genome sequence of the first free-living organism, *Haemophilus influenzae* (1), was completed. This accomplishment opened the door to the genomic era in microbiology. From this point, until recently, material on 28 microbial research genomes including *Archaea, Eubacteria*, and *Saccharomyces cerevisiae* have been published and are available (*http://www.tigr.org/tdb/mdb/mdb.html*) (Fig. 1). Several hundred additional microbial genomes are expected

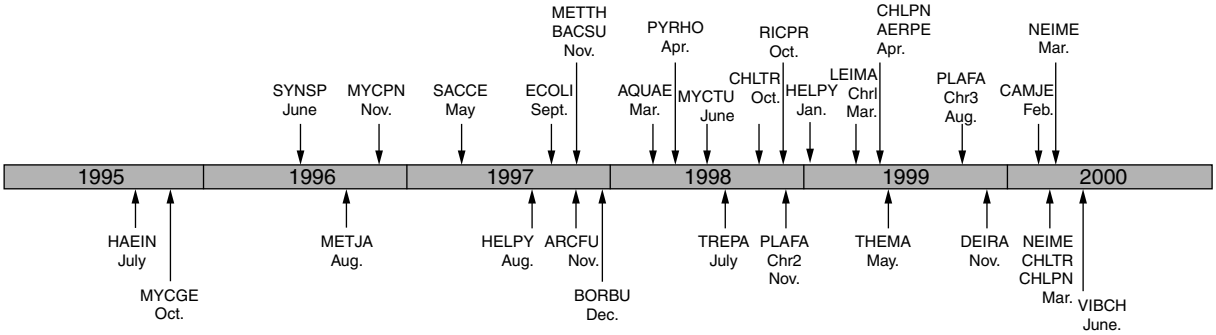

Figure 1. Sequencing timeline of complete microbial genomes. The arrows indicate the dates of publication for each of the completed genomes. The organism abbreviations are as follows: HAEIN, *Haemophilus influenzae*; MYCGE, *Mycoplasma genitalium*; SYNSP, *Synechocystis* PCC6803; METJA, *Methanococcus jannaschii*; MYCPN, *Mycoplasma pneumoniae*; SACCE, *Saccharomyces cerevisiae*; HELPY, *Helicobacter pylori*; ECOLI, *Escherichia coli*; METTH, *Methanobacterium thermoautotrophicum*; BACSU, *Bacillus subtilis*; ARCFU, *Archaeoglobus fulgidus*; BORBU, *Borrelia burgdorferii*; AQUAE, *Aquifex aeolicus*; PYRHO, *Pyrococcus horikoshii*; MYCTU, *Mycobacterium tuberculosis*; TREPA, *Treponema pallidum*; CHLTR, *Chlamydia trachomatis*; RICPR, *Rickettsia prowazekii*; PLAFA, *Plasmodium falciparum*; LEIMA, *Leishmania major*; CHLPN, *Chlamydia pneumoniae*; AERPE, *Aeropyrum pernix*; THEMA, *Thermotoga maritima*; DEIRA, *Deinococcus radiodurans*; CAMJE, *Campylobacter jejuni*; NEIMA, *Neisseria menigiditis*; VICHO, *Vibrio cholerae*. See color insert.

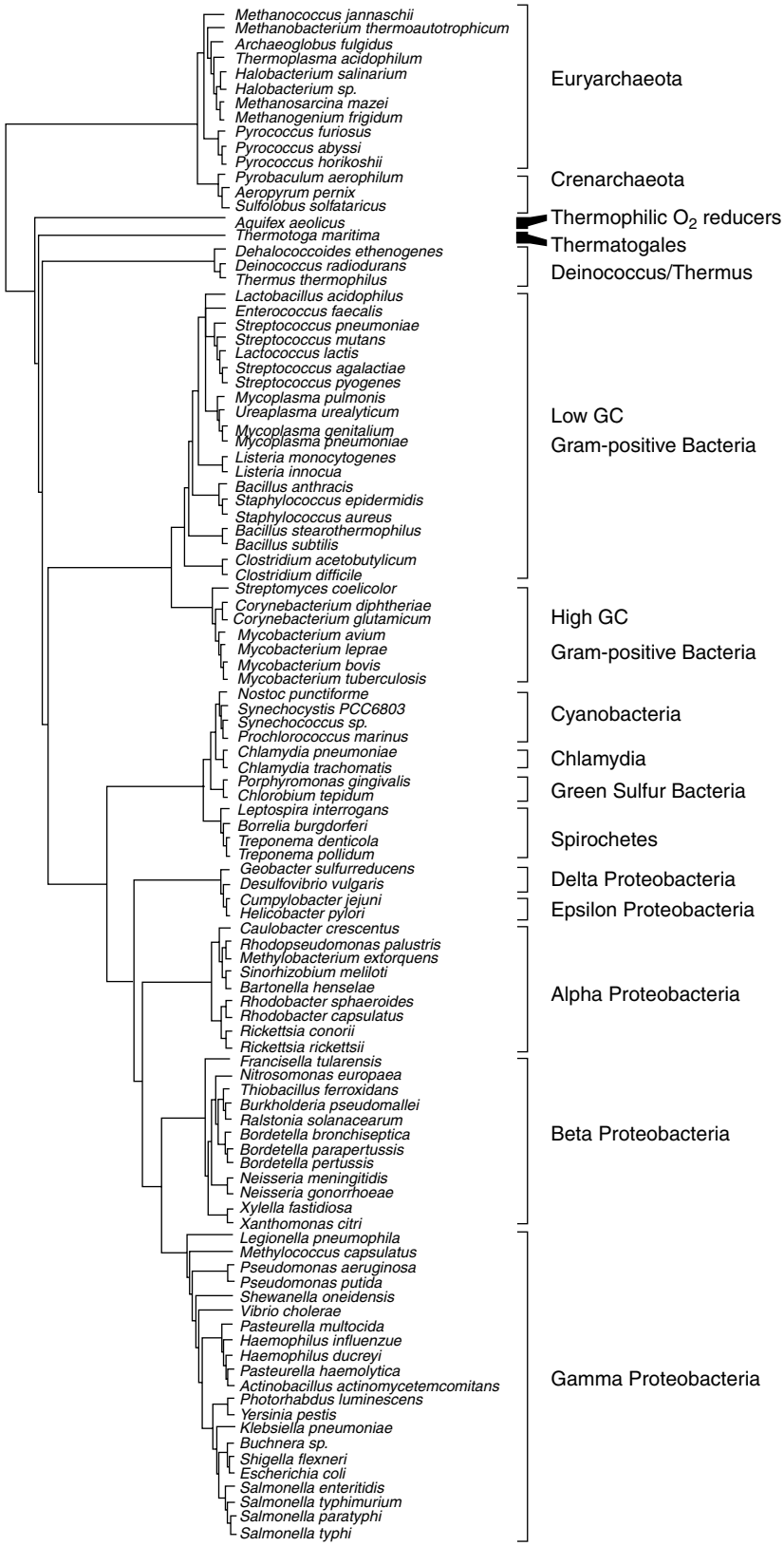

Figure 2. Phylogenetic tree based on 16S-rRNA sequences for each of the prokaryotic organisms whose complete genome sequence has been published (highlighted in green) and other organisms for which genome sequencing projects are underway (*http://www.tigr.org/tdb/mdb/mdb.html*). The major prokaryotic phylogenetic groupings are indicated to the right of the tree. The *archaea* are indicated in red and the *eubacteria* are indicated in blue. The phylogenetic tree was derived from sequences from the Ribosomal Database project (RDP) (*http://www.cme.msu.edu/RDP/html/index.html*) using Phylip (52) for tree construction. In some instances where the rRNA sequence of the particular strain was not available, the rRNA of a close relative was substituted. See color insert.

to be completed in the next 5 to 10 years. Additionally, chromosomes and genomes from eukaryotic model systems such as *Caenorhabditis elegans, Drosophila melanogaster*, and *Arabidopsis thaliana* have been published (2–4).

The current range of genome-sequencing projects includes representatives from all three domains of life. These genome projects provide good coverage of most of the major groupings within the *archaea* and *eubacteria*, as can be seen from a 16S-rRNA phylogenetic tree (Fig. 2). There is a relative concentration of sequencing projects on well-studied groups such as the γ-Proteobacteria and the low (GC) gram-positive bacteria. Other groupings such as the crenarchaeota are underrepresented. Another way to consider the type of microorganism being sequenced is by their ecological role. Considered in this manner, pathogenic organisms and organisms from extreme environments are well represented in current genome sequencing efforts. However, organisms of agricultural significance and difficult to culture organisms are currently relatively poorly represented. With the growing rate of genome sequencing, it is anticipated that these deficiencies will only be temporary.

The diversity in the representative organisms allows for comparative studies of individual organisms, as well as for comparative studies of genome composition, and gene organization within and across the domains. Insight has also been gained into how genes are acquired and shared between organisms (5,6), and the ability of bacteria to change their genome composition rapidly by capturing and maintaining mega plasmids (6,7). The later events have been suggested to increase the competitive nature of *Vibrio cholerae* in the aquatic ecosystem, which may be responsible for the dominance of this genus in such environments.

With each newly sequenced, annotated, and publicly released genome there is typically increased research activity surrounding the organism, and the genome data has resulted in some major achievements for the field of environmental microbiology. Examples of unusual bacteria with completed genome sequences that will continue to allow significant advances for microbial ecology include the only known aerobic hyperthermophilic archaeon, *Aeropyrum pernix* (8), the hyperthermophilic bacteria *Thermotoga maritima* (5) and *Aquifex aeolicus* (9), and the radiation-resistant bacterium *Deinococcus radiodurans* (7).

In these early days of genomics, a major challenge to the scientific community is both keeping up to date with the remarkable amount of genomic data being released, digesting away the "wheat from the chafe" for study, and determining how to best apply these data to science. This chapter will review the current status of the field of microbial genomics, discuss hypotheses being addressed in environmental microbiology by the use of genomic data, and give an overview of where the field is going.

MICROBIAL GENOME SEQUENCING AND ANNOTATION

For the complete sequencing of microbial genomes, the most efficient and cost-effective strategy has proven to be the random shotgun sequencing method (Fig. 3; 10). This approach has successfully been used to completely sequence microorganisms with varying genomic characteristics including variations in genome size (560 kb–6.2 Mb), base composition (19%–67% G + C), presence of various repeat elements, insertion sequence (IS) elements, and multiple chromosomal molecules and plasmids (6,7,11,12). In the random shotgun method, total DNA of the organism of choice is isolated, randomly sheared, size selected, cloned into a plasmid, and the ends of the clones are sequenced to give a predetermined level of coverage that represents the entire genome.

The theory for shotgun sequencing follows from the Lander-Waterman application of the equation for the Poisson distribution (13). On the basis of the Lander-Waterman model, and assuming an average sequence read length of 500 base pairs (bp) for a 2.0 Mbp genome, it would be necessary to sequence approximately 32,000 sequencing reactions to obtain eight-fold sequence coverage of the genome (Table 1). This approach, with an ideal library of

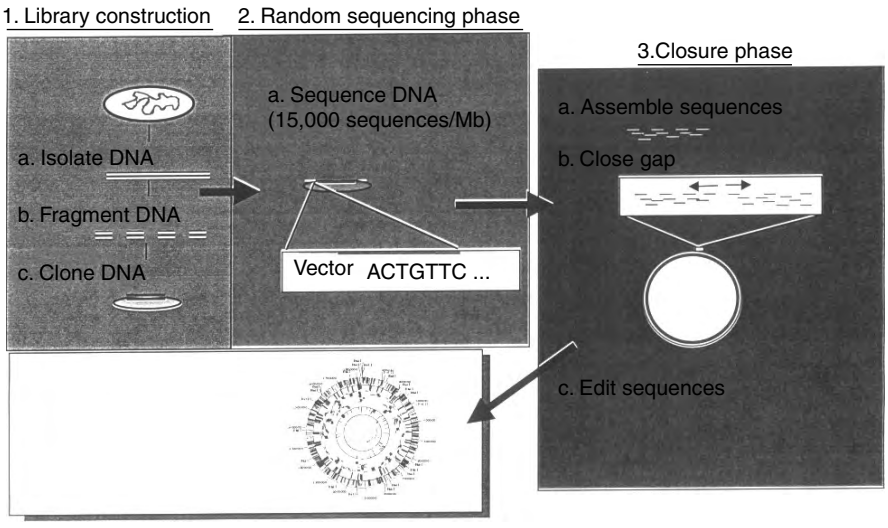

Figure 3. Strategy for random shotgun sequencing of microbial genomes. The first phase involves construction of small and large insert libraries with DNA from the organism of interest. The second phase involves the sequencing of random clones from the libraries to a predetermined level of coverage. The third phase involves assembling the random sequences into contiguous segments and subsequent gap closure. Finally, the completed sequence is annotated and published. See color insert.

Table 1. Computer Simulation of Random Sequencing where the Genome Size is 2.0 Mbp and Assuming an Average Sequence Read Length of 500 bp

Clones (n)	% Unsequenced	bp Unsequenced	Gaps	Av.Gap Length (bp)	Fold Coverage
5,000	28.65	573,010	1,433	400	1.25
10,000	8.20	164,170	821	200	2.5
15,000	2.35	47,035	353	133	3.75
20,000	0.67	13,476	135	100	5.0
25,000	0.19	3,861	48	80	6.25
30,000	0.06	1,106	17	67	7.5
32,000	0.03	671	11	63	8.0

random inserts and random clone selection, should result in approximately 11 gaps (regions of the genome with no sequence coverage) with a total length of about 700 bp of unsequenced DNA after the sequencing reactions have been assembled (Table 1). Note that in practice, more gaps are likely to occur because of repeat areas, secondary structures, and unclonable regions in the genome.

The successful construction of random libraries, with few no-insert and chimeric clones, is the most critical step for the generation of good representation of the entire genome during the random-sequencing phase. Once a sufficient number of sequences are generated, the sequences are retrieved to be assembled into contigs. Current systems for managing microbial sequencing projects are designed to automate data flow as much as possible, thereby reducing user error. These databases store and correlate information collected during the entire sequencing operation from library construction and template preparation to final analysis of the sequence.

After assembling the contigs, any remaining unsequenced regions of the DNA are closed by a combination of methods. Contigs that are linked by forward and reverse pairs become groups, and these clone-linked contigs can usually be closed by sequencing off a spanning clone, or by sequencing a polymerase chain reaction (PCR) product generated from primers designed at the ends of the contigs. Gaps for which there is no linking information are termed *physical gaps*, and are ordered by multiplex or combinatorial PCR (14), or optical maps. Spanning clones from large insert libraries that point off the ends of these contigs can also be used as linking information. These gaps are closed by sequencing the PCR products generated from the large insert clones, or from chromosomal DNA. Direct walking on bacterial DNA can also be used to close these gaps. All repetitive sequence regions including IS elements, ribosomal RNA (rRNA) regions or transposons are confirmed by walking spanning clones across the repetitive regions.

Completion of the sequencing phase does not represent the end of the project. Bioinformatic analysis of the completed genome is essential for interpreting and understanding the sequence data. This involves identification of all the genes and other features (tRNA, rRNA, repeated sequences, etc.) in the genome and subsequent analyses of these features. Gene prediction programs using Hidden Markov models (HMMs) or Interpolated Markov models (e.g., GLIMMER (15)), have proven effective at identifying microbial genes in an automated fashion. Biological names and functions are assigned where possible; assignments are being made by a combination of computer programs and human annotation/curation. Functional predictions are based both on traditional methods such as BLAST or FASTA searches against sequence databases, as well as approaches based on homologous families of proteins, such as HMMs, Pfams and COGs (16,17). In addition to the identification of all open reading frames (ORFs), annotation also involves the identification of intergenic regions, and novel features on the genome including nucleotide biases, origins of replication, and putative regions of horizontal gene transfer, repeat structures, insertion elements, and plasmids. More detailed analyses of the genomic sequence can allow for a reconstruction or complete description of the biology of the organism. See for example a recent reconstruction of the physiology and transport abilities of *V. cholerae* (Fig. 4).

A major problem encountered with the dissemination of genome data is cascading gene nomenclature error also known as transitive catastrophe error. This occurs when an overly ambitious gene name and biological function is assigned to an ORF with no experimental evidence. This incorrect gene assignment can then in turn be passed onto the next genome during annotation, and so on. This type of transitive error can be reduced in several ways; the first is by careful and consistent reannotation of genomes and consulting new computational models and phylogenomic methods for gene naming. It is critical that future ORF assignments take into account this potential problem.

THE ENVIRONMENTAL APPLICATIONS OF GENOMICS

In mid-2000, there are 28 published complete microbial genomes. These include those from five *Archaea* and 22 *Eubacteria* and yeast (see *http://www.tigr.org/tdb/mdb/mdb.html*; Figs. 1 and 2). Currently, sequencing efforts exist worldwide for at least 90 additional microbial genomes. This availability of genomic data from a diverse assemblage of organisms allows scientists to address numerous specific hypotheses. However, and perhaps more importantly, these data increase the ability to consider the larger picture of the mechanisms and regulation involved with a microorganism and how it interacts with its environment. Also, in combination with functional genomics, the genome sequence and annotation information allows for better modeling of an organism's global response to changes in its ecosystem. As a result,

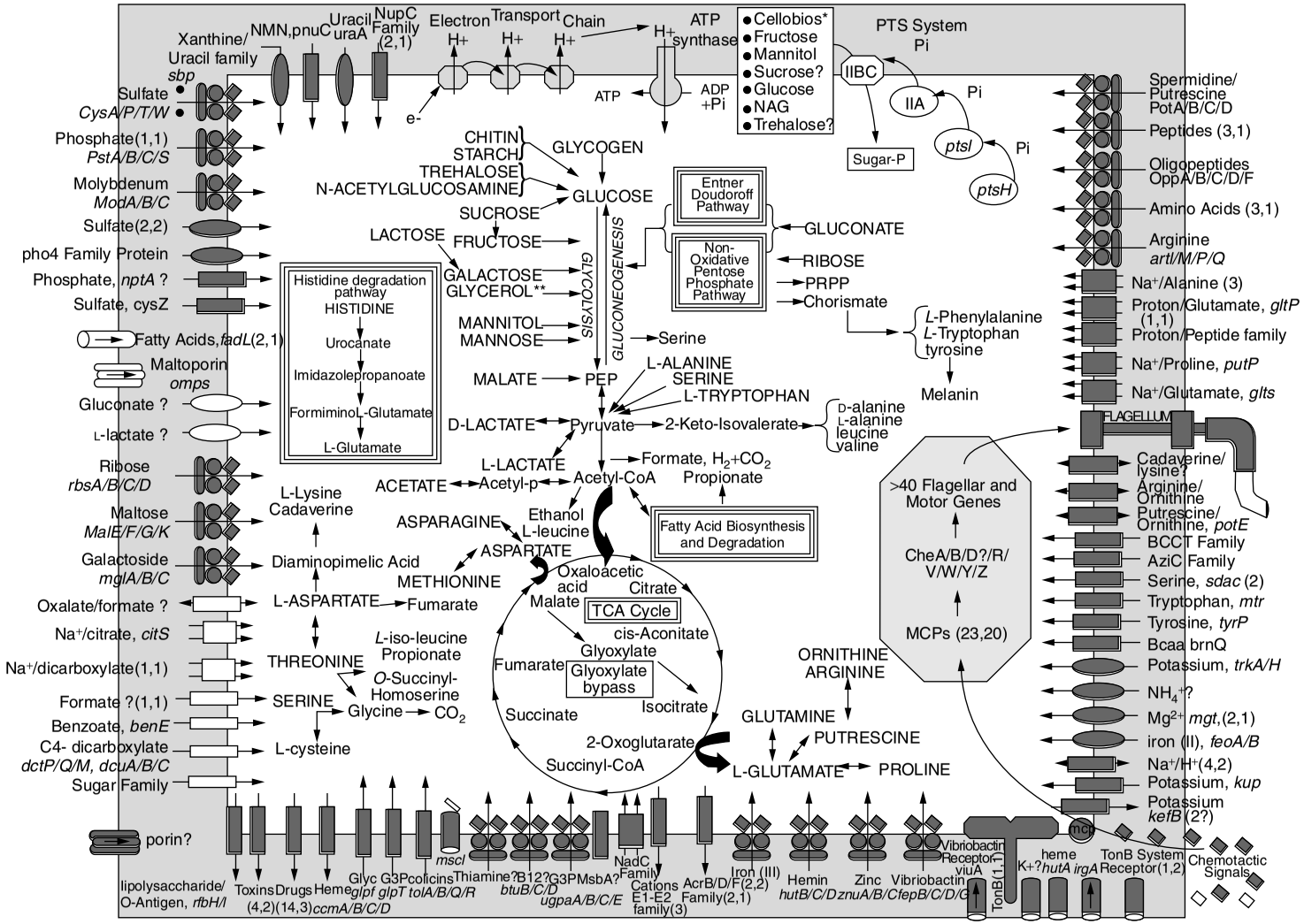

Figure 4. (*Continued*)

Table 2. Current and Past Genome-Sequencing Projects for Environmentally Significant Organisms

Archaea	Size (Mbp)	Completion	Significance*
Aeropyrum pernix	1.67	1999	Aerobic hyperthermophile, biotech.
Archaeoglobus fulgidus	2.18	1997	Hyperthermophile, sulfate reducer, oil well problem, biotech.
Halobacterium sp.	2.50	—	Halophile, biotech.
Halobacterium salinarium	4.00	—	Halophile, biotech.
Methanococcus jannaschii	1.66	1996	Hyperthermophile, methanogen, biogas production, biotech.
Methanococcus maripaludis	?	—	Mesophile, methanogen, biogas production, biotech.
M. thermoautotrophicum	1.75	1997	Hyperthermophile, methanogen, waste digestion, biotech.
Methanogenium frigidum	?	—	Psychrophile, methanogen.
Methanosarcinia mazei	2.80	1999	Hyperthermophile, methylotroph, biogas production, biotech.
Pyrobaculum aerophilum	2.22	2000	Hyperthermophile, sewage digestion, biogas production, biotech.
Pyrococcus abyssi	1.80	1999	Hyperthermophile, biotech.
Pyrococcus horikoshii	1.80	1998	Hyperthermophile, biotech.
Pyrococcus furiosus	2.10	—	Hyperthermophile, biotech.
Sulfolobus solfataricus	3.05	—	Hyperthermophile, sulfur-reducer, biotech.
Thermoplasma acidophilum	1.70	—	Thermophile, sulfur-oxidizer, biotech.
Bacteria			
Aquifex aeolicus	1.50	1998	Hyperthermophile, biotech, evolution, chemolithoautotroph.
Bacillus halodurans	4.25	1999	Alkaliphilic, deep-sea adaptations, industrial applications.
Bacillus subtilis	4.20	1997	Industrial applications.
B. stearothermophilus	?	—	Extracellular xylanases.
Caulobacter crescentus	3.80	2000	Cell cycle regulation.
Chlorobium tepidum	2.10	2000	Photosynthetic, evolutionary implications.
Clostridium acetobutylicum	4.10	—	Solvent production.
Clostridium thermocellum	?	—	Degradation of plant polysaccharides.
C. glutamicum	3.10	—	Amino acid biosynthesis.
Deinococcus radiodurans	3.20	1999	Radiation resistance, environmental clean up.
D. ethenogenes	1.50	2000	Tetrachloroethene degradation.
Desulfovibrio vulgaris	1.70	2000	Microbial transformation of sulfites.
Geobacter sulfurreducens	2.50	2000	Iron reduction.
Nitrosomas europaea	2.20	—	Nitrite removal.
Photorhabdus luminescens	5.50	2000	Biological insecticides.
Pseudomonas putida	6.20	2000	Pollution control, bioremediation.
Rhodobacter capsulatus	3.70	2000	Photosynthetic.
Rhodobacter sphaeroides	4.34	—	Photosynthetic.
Shewanella oneidensis	4.50	2000	Metal reduction.
Streptomyces coelicolor	8.00	2000	Makes 66% of all natural antibiotics.
Synechocystis sp.	3.57	1996	Photosynthetic.
Thermotoga maritima	1.86	1999	Hyperthermophile, evolution, bioremediation, biotech.
Thermus thermophilus	1.82	2000	Thermophile, biotech.
Thiobacillus ferrooxidans	2.90	2000	Iron reduction.

Note: Hyperthermophiles grow at or above 90 °C. Psychrophiles grow below 18 °C. Abbreviations: *B. stearothermophilus, Bacillus stearothermophilus; C. glutamicum, Corynebacterium glutamicum; D. ethenogenes, Dehalococcoides ethenogenes; M. thermoautotrophicum, Methanobacterium thermoautotrophicum*.

Figure 4. (*continued*) Reconstruction of transport and metabolism of *V. cholerae* on the basis of the annotated genome sequence. Pathways for energy production and the metabolism of organic compounds, acids and aldehydes are shown. Transporters are grouped by substrate specificity: cations (green), anions (red), carbohydrates (yellow), nucleosides, purines, and pyrimidines (purple), amino acids/peptides/amines (dark blue), and other (light blue). Question marks associated with transporters indicate a putative gene, uncertainty in substrate specificity, or direction of transport. Permeases are represented as ovals, ABC transporters are shown as composite figures of ovals, diamonds, and circles, porins are represented as three ovals, the large-conductance mechanosensitive channel is shown as a gated cylinder, other cylinders represent outer membrane transporters or receptors; all other transporters are drawn as rectangles. Export or import of solutes is designated by the direction of the arrow through the transporter. If a precise substrate could not be determined for a transporter, no gene name was assigned and a more general common name reflecting the type of substrate being transported was used. Gene location on the two chromosomes, for both transporters and metabolic steps, is indicated by arrow color: all genes located on the large chromosome (black), all genes located on the small chromosome (blue), all genes needed for the complete pathway on one chromosome, but a duplicate copy of one or more genes on the other chromosome** (purple), required genes on both chromosomes (red), complete pathway on both chromosomes (green). Gene numbers on the two chromosomes are in parenthesis and follow the color scheme for gene location. Substrates underlined and capitalized can be used as energy sources. Abbreviations: PRPP, phosphoribosyl-pyrophosphate; PEP, phosphoenolpyruvate; PTS, phosphoenolpyruvate-dependant phosphotransferase system; ATP, adenosine triphosphate; ADP, adenosine diphosphate; MCP, methyl-accepting chemotaxis protein; NAG, N-acetylglucosamine; G3P, glycerol-3-phosphate; glyc, glycerol; NMN, nicotinamide mononucleotide. *Because *V. cholerae* does not use cellobiose, we expect this PTS system to be involved in chitobiose transport. **Complete pathways, except for glycerol, are found on the large chromosome. See color insert.

genomic data mining is likely to become one of the most powerful molecular tools available. The following sections look at various aspects of environmental biology and describe some of the relevant genome-sequencing projects that are expected to impact on these areas (Table 2).

UNDERSTANDING PHYLOGENY AND EVOLUTION

Horizontal Gene Transfer and Acquired Genes

Initial analyses of genomic data across the sequenced microorganisms suggest that single universal phylogenetic trees (e.g., Fig. 2) may not be the best way to depict relationships among organisms (18). Instead a net-like pattern that reflects the frequency and significance of horizontal lateral gene transfer has been proposed (18). Complete genome sequences of a number of environmental and pathogenic microorganisms, including closely related *Pyrococcus* sp. and *Chlamydia* sp. have shown significant differences in genome structure, organization, and composition (19,20). A very recent example of lateral gene transfer has been shown from the genome sequences of the two hyperthermophilic archaea *Pyrococcus furiosus* and *Thermococcus litoralis* (21). Both organisms share a 16-kb region on their genomes that contains only 173 nucleotide differences from one to the other; the 16-kb insert in *P. furiosus* is flanked by insertion elements with inverted and direct repeats. These transposable elements were proposed to be the mechanism for the exchange of this 16-kb segment between these two archaea (21).

Analysis of the *T. maritima* genome sequence suggested that almost one quarter of the genome was acquired by lateral gene transfer with the thermophilic archaea. There was also extensive conservation of gene order between *T. maritima* and the archaeon that it shared the highest level of similarity with (5). These acquired genes probably convey some selective advantage to these thermophiles, or alternatively may not be detrimental to the organism and have subsequently ameliorated into the genome. Lawrence and Ochman (22) have attempted to calculate time frames for lateral gene transfer in *Escherichia coli*. They have concluded that subsequent to the divergence of *E. coli* and *Salmonella* (100 million years ago), 10% of the *E. coli* genome was acquired in more than 200 events of lateral gene transfer. Their data also suggest that a significant percentage of *E. coli* might have been acquired recently, at an average rate of 16 kb per million years (22).

Clearly, the comparisons of various genome sequences in parallel with new methods for analyzing this data, has and will continue to provide fundamental insight into evolution. This is particularly significant for the archaea that inhabit the extremes of temperature and anaerobic conditions for life.

Captured Megaplasmids

Another mechanism for an environmental bacterium to rapidly change its overall genomic content is by capturing a megaplasmid and all the associated genes. Required genes can be moved from the chromosome to the megaplasmid, thus making the captured megaplasmid essential for the survival of the cell, and thereby stabilizing this new replicon. This model was proposed for the small chromosome of *V. cholerae* (6). In this case, the genome sequence analysis suggests that the smaller replicon was captured by an ancestral *Vibrio* subsequent to travelling through a broad range of hosts. The capture of this megaplasmid, which presumably contained genes that gave the ancestral *Vibrio* a competitive advantage in its ecosystem, resulted in it being stabilized by the transfer of essential genes to this replicon.

In addition to containing genes that make the cell more competitive, second chromosomes (and megaplasmids) may increase survivability and speed recovery from hostile environmental conditions. Such situations have been suggested from analysis of the small chromosomes from *D. radiodurans* and *V. cholerae*. For *D. radiodurans*, it appears that the small chromosome may have genes involved in de novo synthesis and importing precursors (7). For *V. cholerae*, the small chromosome has been suggested to help cells survive in biofilms and as a suggested model to help explain the VBNC (viable but nonculturable) state.

BIOREMEDIATION

Hope for Mixed Waste Sites Exposed to Radiation

The potential remediation of radioactive waste sites has driven much of the focus on the recently sequenced *D. radiodurans* as a model organism. *D. radiodurans* is the most radiation-resistant organism known, capable of surviving exposure to ionizing radiation doses of 15,000 Gy. The completed *D. radiodurans* genome sequence, however, gave little insight into the extreme ionizing radiation resistance of this organism. In addition to the earlier-mentioned radiation resistance, the bacterium reduces Fe(III)-nitrilotriacetic acid coupled to the oxidation of lactate to CO_2 (carbon dioxide) and acetate. *Deinococcus radiodurans* can also reduce uranium and technetium in the presence of humic acids or synthetic electron shuttle agents (23).

Mutant strains of *D. radiodurans* expressing the cloned mercury resistance gene (merA) from *E. coli* have recently been developed (24). These mutant strains reduce Hg(mercury) (II) to volatile elemental mercury, which is less toxic. The strains also grow in the presence of both radiation and ionic mercury at concentrations well above those found in radioactive waste sites. Recombinant *D. radiodurans* expressing toluene dioxygenase have also been constructed (25), the recombinant bacterium able to oxidize toluene, chlorobenzene, 3,4-dichloro-1-butene, and indole. The bacterium is capable of producing the toluene dioxygenase in a highly irradiating environment. The above studies support the potential use and practical applications of engineered *D. radiodurans* for bioremediation of mixed wastes exposed to radiation.

A Better Understanding of Metal Oxidation/Reduction

Shewanella oneidensis is a model organism for a comprehensive understanding of bioremediation of metal-contaminated sites. This bacterium grows both aerobically and anaerobically, acting as a metal reducer in its anaerobic phase. Iron oxides are specific substrates, so

competition for the electron acceptor is minimal and in a stratified aquatic environment, reduced iron will diffuse upward into the overlying oxic zone and be oxidized, and then return to the anoxic zone through gravity, thus acting as a "pump" for oxidizing equivalents.

Geobacter sulfurreducens is a dissimilatory metal-reducing microorganism and sulfur-reducing microorganism isolated from surface sediments (26). This bacterium grows in a defined medium with acetate as an electron donor and ferric PPi, ferric oxyhydroxide, ferric citrate, elemental sulfur, Co(cobalt) (III)-EDTA (ethylenediaminetetraacetic acid), fumarate, or malate as the sole electron acceptor. It also couples the oxidation of hydrogen to the reduction of Fe(iron) (III). The genome sequence will undoubtedly increase our understanding of the mechanisms that this organism utilizes to reduce metals and give insight into other novel pathways that it may have.

Biodegradation of Halogenated Aliphatic Compounds, PCBs, and Ground Water Pollutants

Many sites of environmental concern contain groundwater contaminated with nonaqueous phase liquids, dry cleaning, and degreasing solvents. Tetrachloroethene, for example, is a groundwater contaminant often persisting in subsurface environments. It is recalcitrant under aerobic conditions and is not readily susceptible to oxidation. *Dehalococcoides ethenogenes* strain 195, which is currently being sequenced, is the only organism so far isolated that dechlorinates tetrachloroethene all the way down to ethylene (27,28). This bacterium is therefore capable of degrading dry-cleaning, and degreasing solvents that pollute ground waters.

Nylon-6 is another man-made polymer used in the manufacture of car tires, fabrics and automobile parts (29). The unreacted monomer used in its synthesis (epsilon-caprolactam) can be found in wastewater generated during production of nylon-6. Epsilon-caprolactam can, however, be degraded by *Pseudomonas aeruginosa* (MCM B-407), an organism that was first isolated from activated sludge used to treat waste from a factory producing nylon-6. Other *P. aeruginosa* strains have demonstrated abilities to degrade 2,4,6-Trinitrotoluene (TNT) (30) that can accumulate in the environment as a result of the manufacture of explosives. *P. aeruginosa* strains that can utilize gasoline as the sole carbon source have been isolated from gasoline-contaminated aquifers (31). *Pseudomonas putida* can mineralize numerous organic wastes and pollutants including many ring-based compounds. Chromate, a widespread environmental contaminant can be converted to the insoluble and less toxic Cr(III) by the novel chromate reductase from *P. putida*. The genome sequence is expected to reveal additional previously uncharacterized pathways for the degradation of environmental contaminants.

In wastewater systems, the microbial removal of ammonia commences with ammonia oxidation to nitrite, and the resulting nitrate being reduced to molecular nitrogen. The individual steps are carried out by nitrifying and denitrifying bacteria, respectively. *Nitrosomonas europaea* has been shown to efficiently remove nitrogen from various wastewaters. An additional benefit is that this organism can conduct ammonia-stimulated aerobic transformation of halogenated aliphatic compounds including dichloromethane, dibromomethane, trichloromethane, bromoethane, ethylene dibromide, vinyl chloride, trichloroethylene, and 1,2,3-trichloropropane (32).

INDUSTRIAL APPLICATIONS

New Enzymes for Degradation of Refractory Carbohydrates

The complex plant polymers cellulose and xylan are recalcitrant to degradation, but a diversity of organisms and enzymes have been identified, which are capable of degrading these polymers that are based on glucose and xylose, respectively. The recently completed *T. maritima* is known to have an extensive ability to degrade both simple and complex plant polymers. On the basis of the genome sequence, numerous other proteins likely involved in plant polymer degradation were identified. Similarly, *Clostridium thermocellum* contains a cellulosome, a large multienzyme complex that allows for adherence to plant surfaces and initiates plant cell wall degradation. The complete sequence will no doubt reveal other mechanisms associated with the efficient degradation of plant cell walls by aerobic bacteria. *Bacillus stearothermophilus* produces an extracellular xylanase that is thermostable and alkaline tolerant that can bleach pulp optimally at pH 9 and 65 °C. This enzyme has successfully been used in large-scale biobleaching mill trials.

Chitin is a carbohydrate polymer that consists of alternating β-1, 4-linked *N*-acetylglucosamine residues. Estimates are that more than 10^{11} metric tons of chitin are produced annually (33), making chitin the second most abundant organic compound in nature. *Vibrio cholerae* is an autochthonous aquatic organism that can grow with chitin serving as the sole carbon and nitrogen source. *Bacillus halodurans* is an alkaliphilic bacterium that produces keratin-decomposing enzymes, which devolve keratinous proteins including hair, nail, and feathers.

Natural Production of Solvents, Drugs, and Insecticides

Clostridial acetone-butanol fermentation by *Clostridium acetobutylicum* is one of the largest biotechnological processes known, and there is a significant potential for the use of cheap agricultural wastes as novel substrates. Many continuous culture methods have been successfully established that would allow for various substrates to be used to generate solvents produced by microorganisms.

Streptomycetes are used to produce the majority of antibiotics applied in human and veterinary medicine and agriculture. They also have an interesting mycelial, sporulating life cycle, which involves complex regulation of gene expression in space and time. The genetic control of antibiotic and enzyme production is both temporally and causally related to the morphological differentiation (34). The Sanger Centre (*http://www.sanger.ac.uk/*) is currently sequencing the model streptomycete *Streptomyces coelicolor* A3 (2). Elucidation of the *S. coelicolor* sequence should help clarify the complex genetic and metabolic

regulation of antibiotic production. Comparative genomic analyses of the streptomycetes may assist in genome engineering of *Streptomyces* sp. to make polyketide antibiotics more efficiently and to create novel secondary metabolites.

Novel biological insecticides have been cloned from *Photorhabdus luminescens*, a gram-negative bacterium belonging to the *Enterobacteriaceae*. This bacterium is mutualistic with certain nematodes and can kill the insect by a combination of toxin action and direct infection (35). Initial studies have highlighted that this bacterium can be used to produce insecticides, and present an alternative mechanism to concerns arising from the use of transgenic crops that express toxin genes from *Bacillus thuringiensis*.

Improving Oil Well Productivity

Archaeoglobus fulgidus (36) is a sulfate reducer that has been associated with the contamination of oil wells. The organism serves as a model for understanding corrosion and the souring of hydrocarbon reserves. Inhibition of certain essential biochemical pathways can disrupt its normal metabolism, and therefore limit its detrimental effects in the environment. The genome sequence revealed numerous pathways, which could serve as candidates for initial inhibitory studies.

UNDERSTANDING ENVIRONMENTAL PATHOGENESIS

Several environmental bacteria have also acquired the capacity to cause serious human disease. Notable bacteria that have had their genome sequenced that fall into this category include *V. cholerae* and *P. aeruginosa*. The genome sequence of these environmental pathogens allows for a more complete understanding of how environmental bacteria emerge to become significant human pathogens. For example, although *V. cholerae* is the etiological agent of cholera, it is also an autochthonous aquatic organism. This bacterium seems to have achieved its human pathogenicity in several different mechanisms. These include the chromosomal integration of a filamentous phage (CTXϕ) containing the cholera toxin genes, and other recently acquired regions of DNA (the VPI or vibrio pathogenicity island). Both of these reside on the large chromosome and have a trinucleotide composition that is significantly different from the rest of the *V. cholerae* genome. Also, the small chromosome (apparently a captured megaplasmid) contains an integron island to allow additional gene capture and several of the ORFs in this region maybe important to pathogenicity (e.g., drug resistance genes and virulence factors).

Additionally, genome analysis offers evidence to evaluate genes implicated in human pathogenicity. The maltose-sensitive hemagluttinin (MSHA) was originally implicated in intestinal colonization; however, recently several investigators have reported that MSHA is not required for intestinal colonization, but instead is important in biofilm formation. Therefore, MSHA maybe more important in the "environmental fitness" of *V. cholerae* rather than pathogenic potential. Interestingly, this gene cluster does not appear to be recently acquired (i.e., there are no integrase, transposase, or phage homologs that might suggest an origin other than *V. cholerae*), and the trinucleotide composition is similar to the rest of the chromosome. These genome analysis tools lend evidence to these genes being with *V. cholerae* longer than the other pathogenicity genes, and thereby suggest their greater importance in the environmental aspects of this bacterium rather than the pathogenic.

AS ENVIRONMENTAL MODEL SYSTEMS

Although the many applications from the genomics sequence are evident, the environmental organism also acts as a model organism for understanding how many systems function. For example, *Caulobacter crescentus* currently being sequenced at The Institute for Genomic Research (TIGR), stands out as a model organism for increasing our understanding of life cycles that include an asymmetric cell division and an obligate cell differentiation. With each cell division, a motile but replication-inert swarmer cell and a sessile replication-competent stalked cell are generated. Although the stalked cell immediately reinitiates DNA replication and cell division, the swarmer cell remains motile and chemotactically active for a period of the cell cycle before it differentiates into a stalked cell. Motility is lost by ejecting the flagellum, a stalk is synthesized, and the swarmer eventually initiates chromosome replication and cell division. Development phases in this bacterium appear to be controlled in part by localized cues that control morphological changes and cell cycle progression (37). Additionally, Caulobacters are biofilm-forming members of the natural flora of soil and aquatic environments, which exhibit several characteristics that make them attractive for development of high surface area microbial bioreactors or biosensors. Other microorganisms recently or currently being sequenced, which stand to give insight into particular metabolic processes include *Bacillus subtilis*: a model system for understanding sporulation, *Chlorobium tepidum*: for understanding the origin of photosynthesis; *Desulfovibrio vulgaris*: for understanding sulfate respiration; and *Methanobacterium thermoautotrophicum*: for understanding methane production and single carbon metabolism.

WHAT THE FUTURE HOLDS IN STORE

Functional Genomics

The elucidation of complete genome sequences has led to the development of new fields of research in the area of functional genomics. The term *functional genomics* is widely used but somewhat ill defined. One possible definition is that functional genomics uses high-throughput or large-scale approaches that investigate the roles of large numbers of genes or proteins in a systematic fashion rather than more traditional approaches that investigate the role of a single gene or protein. This definition includes areas such as microarray expression analysis, large-scale gene knockouts and high throughput phenotypic screening, structural genomics and proteomics.

DNA array technology, more colloquially known as *gene chips*, can be used to measure expression patterns

of thousands of genes in parallel. This technology involves the immobilization of large numbers of PCR fragments or oligonucleotides, typically corresponding to all of the genes from a particular organism, onto a support matrix (glass slide, nylon membrane, or silica chip). This can then be probed with fluorescently labeled mRNA isolated from cells grown under different conditions to examine gene expression or with DNA from different strains or isolates to look at environmental variability. The use of arrays reduces a dependence on extensive biochemical analyses to identify the functions of unknown genes. Although comparisons of genome sequences allows for a prediction of enzyme activity, arrays can provide a more reliable identification of genes associated with particular pathways. By increasing our understanding of gene function, arrays ultimately allow for new and more accurate classification schemes.

A complementary approach to that of microarray expression are proteomic studies using two-dimensional gel electrophoresis to examine protein production and localization. Traditional two-dimensional gel electrophoresis methods used Edman degradation methods of peptide sequencing for identification of protein spots on gels. A more sensitive and automated approach, MALDI TOF (Matrix Assisted Desorption/Ionization-Time of Flight) Mass Spectrometry, enables high throughput screening of protein samples derived from two-dimensional gel electrophoresis (38). Using such an approach it starts to become practical to undertake whole proteome analysis studies for completely sequenced microbial organisms.

Complete genome sequences also enable large-scale gene knockout studies either through use of saturation transposon mutagenesis and identification of the transposon insert sites by sequencing, or by making targeted gene knockouts (39,40). The construction of such knockouts enables the presumptive identification of essential genes, and enables the generation of large banks of gene mutants, whose phenotypes can be examined using high throughput screening processes such as Biolog plates (41). One example of a large-scale gene knockout study was the transposon mutagenesis of *Mycoplasma genitalium* and *Mycoplasma pneumoniae*, to identify nonessential genes under laboratory growth conditions (40). As *M. genitalium* has the smallest known microbial genome with only 480 protein-encoding genes, this approach indicated that only approximately 265 to 330 of these genes were essential for growth under the conditions examined, thus providing an estimate of the minimal genome required for life.

The area of structural genomics deals with high throughput protein structure determination, involving gene overexpression, protein purification, and subsequent structural determination using X-ray crystallography or NMR (nuclear magnetic resonance) spectroscopy (42). This approach works on the assumption of being able to deduce information on the function of unknown proteins based on their structure. Current structural genomic projects are focussing on determining the structures of representative members of different protein families whose members are not predicted to conform to any currently known structures with the aim of identifying all of the possible protein folds in nature. Currently, such studies are only in preliminary or formative phases, and this approach is problematic for membrane proteins that are typically recalcitrant to crystallization and frequently too large for NMR-based approaches.

GENOMIC ENGINEERING

The ability to release genetically modified microorganisms for environmental remediation is still under debate. However, numerous examples of successfully engineered microorganisms exist and will continue to increase as we elucidate new pathways from completely sequenced or newly identified organisms. The genetically engineered *E. coli* cells manipulated to express mercury transport systems (43) effectively accumulated the toxin and were thought to have excellent properties for bioremediation of mercury-contaminated environments. The gene for toluene dioxygenase has recently been inserted into the chromosome of *D. radiodurans* (25), and the recombinant bacterium degrades toluene and other organic compounds in high-radiation environments. A synthetic acetone operon derived from *C. acetobutylicum* genes was constructed and introduced into *E. coli*. Ace4 was expressed in *E. coli* and demonstrated the ability to use clostridial genes in nonclostridial hosts for solvent production.

Improvements in our ability to engineer microorganisms to synthesize molecules, to have desired biodegradative pathways, and to increase and optimize enzyme activity will continue to reveal the potential applications from genomics for environmental microbiology.

BIOINFORMATICS APPLICATIONS

Databases

As more genomes are discovered, the amount of data being generated is enormous. To make reasonable use of this data, researchers should be able to easily compare data among these organisms. To do this, databases that can be openly accessed by the research community and that can be easily queried must be available. A variety of second generation biological databases have been developed, which address particular demands resulting from the mountain of genomic data. An increasingly important feature of databases such as the Omnione and Ecocyc is that they incorporate detailed manual curation of the data in addition to sophisticated automated analysis. Examples of such bioinformatic databases are described later.

TIGR's comprehensive microbial resource (CMR) (*http://www.tigr.org/*) enables intergenomic and intragenomic comparisons of microbial genomes. The CMR contains a variety of tools for querying the Omnione database, which incorporates the detailed curated genome dataset from each of TIGR's microbial genome projects as well as the original annotation and further automated annotation by TIGR for all of the non-TIGR microbial genome-sequencing projects. Thus, the CMR allows comparison between genomes based on role categories, protein families, best matches, and other criteria, and enables complex queries based on a variety of features.

Interpro (*http://www.ebi.ac.uk/interpro/*) presents an integrated database based on SwissProt, TrEMBL, Pfam, PRINTS, and PROSITE, and thus includes data on proteins, protein families, and domains/motifs, thereby proving useful for proteome analyses of completed genomes and for predicting protein structure and function.

The Ecocyc database (*http://ecocyc.PangeaSystems.com/ecocyc/*) is an *E. coli*–specific database that combines functional and bioinformatic information describing the metabolic pathways, signal transduction, membrane transport, and gene regulation of *E. coli* (44). Each of the proteins and enzymes in *E. coli* is annotated in detail, including references to the original literature. Thus, Ecocyc acts both as an online review article and a qualitative model of the *E. coli* biochemical machinery. The Pathway Tools graphical user interface provides a wide variety of query operations and visualization tools. The related MetaCyc database is a more generalized metabolic-pathway database that describes pathways and enzymes of many different organisms (44). Other metabolic databases include WIT (*http://wit.mcs.anl.gov/WIT2*) (45) and KEGG (*http://kegg.genome.ad.jp/kegg/*) (46). Such metabolic databases are valuable in metabolic reconstruction of pathways in newly sequenced genomes.

Orthologs are genes in different organisms that evolved from a common ancestral gene by speciation, and paralogs are homologous genes that are diverged by gene duplication (47) and hence may have diverged in function. Accurate functional predictions of protein function are increasingly dependent on methods that take into account assignment of orthologs and paralogs within families of homologous proteins. Phylogenetic approaches are particularly valuable for determining orthology. Other family-based approaches for identifying orthologs and paralogs include the COG database (*http://www.ncbi.nlm.nih.gov/COG*) (17) and Pfam database (*http://www.sanger.ac.uk/Software/Pfam/*) of protein family sequence alignments and Hidden Markov models (16).

COMPARATIVE GENOMICS

Comparison of the Transport Capabilities of Microorganisms

The availability of complete genome data enables systematic genomewide comparisons to provide insight into the overall physiology of each organism. Comparison of 76 families of cytoplasmic membrane transport systems in each completely sequenced microbial genome (48–50) provides one example of such a comparative genomic approach. The overall numbers of membrane transporters was found to vary over 15-fold between organisms, but was generally proportional to the genome size of the organism. The transport bioenergetics matched the overall mode of energy generation in each organism. For example, organisms such as the mycoplasmas and spirochetes, which lack a TCA cycle and an electron transfer chain and hence can only generate a proton motive force by substrate-level phosphorylation, were highly dependent on ATP-dependent rather than proton-dependent transporters, whereas the converse was true of more metabolically versatile organisms such as *E. coli*.

The overall transport substrate specificities were found to correlate with the organisms lifestyles, that is, with the concentration and diversity of nutrients in their particular ecological niche. For instance, organisms from deep marine environments were found to possess only a limited array of transporters for organic nutrients but a preponderance of transporters for inorganic cations and anions, which presumably reflects the availability of these substrates in this environment. Similarly, intracellular parasites, such as the chlamydias and *Rickettsia prowazekii*, have an extensive set of transporters for amino acids and nucleotides, but little ability to transport free sugars, which almost certainly reflects the relative accessibility of these compounds in an intracellular environment.

Thus comparative analysis of transporters appears to provide insights into both the physiology of the organism and the environment in which it dwells. It is anticipated that such comparative genomic studies will become increasingly pertinent as complete genome data becomes available for representative organisms from different phylogenetic lineages and different lifestyles or environments.

Unknowns and Conserved Hypotheticals

It is important to note that for each completed genome sequence project, an average of 40 to 50% of the ORFs in the genome is either shared with other organisms but has not been previously characterized (conserved hypothetical), or is completely unknown (unique) (Table 3). One major challenge remains in the elucidation of these unknowns and conserved hypotheticals. Hopefully the use of microarrays, in combination with proteomic studies and other functional genomic approaches (see earlier) will accelerate the rate at which we can begin to characterize these ORFs. Undoubtedly, many of these unknown genes will prove to have interesting or important functions relevant to environmental microbiology.

EXPLORING MICROBIAL DIVERSITY ON EARTH

Nonculturable Microorganisms and Genomic Potential

One of the most exciting future steps for genomics is the analysis of the huge population of uncultured microorganisms. To date, studies on unculturable bacteria have been primarily limited to phylogenetic analysis based on 16S-rRNA sequence and enumeration of specific 16S-rRNA containing cells. These methods have greatly increased our knowledge of the phylogenetic diversity of many ecosystems, but they do not allow accurate determination of the functional niche these microorganisms occupy. On the basis of 16S-rRNA sequence, the only way that biogeochemical function can be assigned to an uncharacterized organism is by relatedness to cultured bacteria. However, environmental genomics confers the ability to examine both the biogeochemical capabilities of uncultured bacteria (genomic potential) and what specific genes and metabolic pathways are being

Table 3. Comparison of Total Number of Predicted Open Reading Frames (ORFs), ORFs of Unknown Function, and ORFS Unique to Each Organism for Each of the Completed Genomes

Organism	ORFS	Unknowns	Uniques
Aeropyrum pernix K1	2694	523(19%)	1538(57%)
Archaeoglobus fulgidus DSM4304	2437	1315(54%)	641(26%)
M. thermoautotrophicum Δ H	1855	1010(54%)	496(27%)
Methanococcus jannaschii DSM2661	1749	1076(62%)	525(30%)
Pyrococcus horikoshii OT3	2061	859(42%)	453(22%)
Aquifex aeolicus VF5	1521	663(44%)	407(27%)
Bacillus subtilis 168	4100	1722(42%)	1053(26%)
Borrelia burgdorferii B31	1751	1132(65%)	682(39%)
Campylobacter jejuni NCTC 11168	1654	223(14%)	144(9%)
Chlamydia trachomatis serovar D	894	35(4%)	255(28%)
Chlamydia pneumoniae CWL029	1073	251(23%)	186(17%)
Deinococcus radiodurans R1	3187	692(22%)	1002(31%)
Escherichia coli K-12	4288	1632(38%)	1114(26%)
Haemophilus influenzae KW20	1692	592(35%)	237(14%)
Helicobacter pylori 26695	1657	744(45%)	539(33%)
Mycobacterium tuberculosis H37Rv	3924	1521(39%)	606(15%)
Mycoplasma genitalium G-37	470	173(37%)	7(2%)
Mycoplasma pneumoniae M129	677	248(37%)	67(10%)
Neisseria meningitidis MC58	2158	345(16%)	532(25%)
Rickettsia prowazekii Madrid E	834	104(13%)	208(25%)
Synechocystis sp. PCC 6803	3168	2384(75%)	1426(45%)
Treponema pallidum Nichols	1040	461(44%)	28(27%)
Thermotoga maritima MSB8	1877	863(46%)	373(20%)
TOTAL	46,761	18,568(40%)	12,519(27%)

expressed in response to changes in the environment (functional genomics, see earlier).

To determine the genomic potential of an environment, the DNA from the environment is isolated, cloned into large (100 Kbp) bacterial artificial chromosomes (BAC), and these BACs are sequenced to closure. These BACs can then be annotated similarly to an entire microbial genome (i.e., genes found and roles assigned, RNAs found, etc.). Although this does not necessarily give the genome of any single uncultured bacterium, the gene content of the BACs can give an idea of what important biogeochemical processes may be going on in an environment. From end-sequencing and annotation of 450 BACs derived from Monterey Bay surface water DNA extraction, several interesting results can be seen (DeLong and Heidelberg, in preparation). Most of the Monterey Bay BACs have greatest amino acid sequence similarity to *R. prowazekii*, and α-Proteobacteria, the same group that comprises the greatest percentage of the 16S-rRNA sequences in this ecosystem (Fig. 5). Also, by comparing the functional roles of genes identified from the Monterey Bay BAC sequences to the complete gene complement of *V. cholerae*, it can be seen that the representative set of BACs tend to have a greater percentage of genes involved in amino acid biosynthesis, purines, pyrimidines, nucleosides, and nucleotides, and protein synthesis (likely a result of smaller genome size), whereas *V. cholerae* has a greater percentage involved in transport and binding, regulatory functions, and cellular processes proteins, possibly because *V. cholerae* needs to be competitive in a greater diversity of environments that the microorganisms inhabiting the Monterey Bay surface water (Fig. 6). Such methodologies may also prove valuable commercially because of the large potential for new gene discovery. This also has the advantage of providing a mechanism for expressing genes from unculturable organisms in alternative hosts such as *E. coli*.

Use of Subtractive Hybridization Methods to Identify Differences Among Environmental Strains

Despite the indisputable value associated with whole genome sequencing, it is often cost-prohibitive to obtain sequence data for multiple strains of the same microbe, or for related species. Alternatively, gene diversity and genome plasticity can be examined in related microbes through the use of suppressive subtractive hybridization. Suppressive subtractive hybridization was developed by CLONTECH and kits have been optimized for use with genomic DNA and cDNA. For genomic differences, genomic DNA from the strain of interest (tester) and the strain to subtract with (driver) is fully digested using a frequent cutter. The fragments that are obtained average between 250 and 2,000 bp. Following a series of reactions, complexes of unique DNA's containing different adaptors at either end allow for logarithmic amplification. A nested PCR is run using a small amount of the primary PCR product as template, to give a final product that is highly enriched for unique DNA's. Unique regions can be further characterized to identify the unique metabolic characteristics of individual strains or species. This method has been used to identify unique regions in the closely related species of *Helicobacter pylori* (51) and *P. putida*. The differences are likely a reflection of the environmental niche that individual

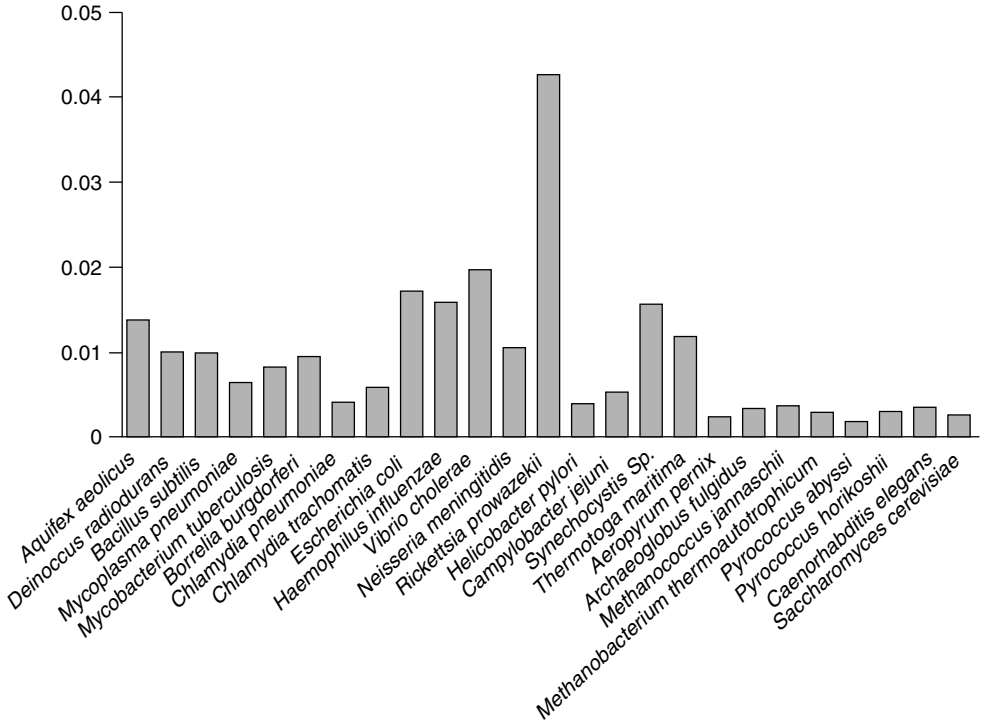

Figure 5. Comparison of the Monterey Bay BAC library environmental sequences to ORFs of other completed genomes. The vertical axis shows the total fraction of best hits for the BAC genes to each currently sequenced genome.

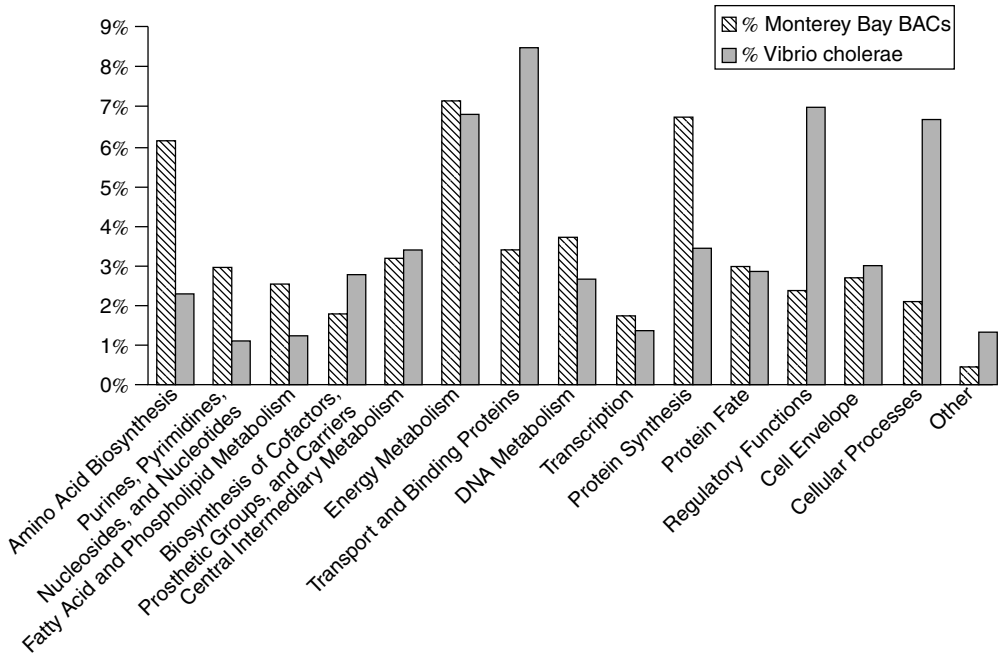

Figure 6. Functional role categories of the genes identified from the Monterey Bay BAC library end sequences compared to the gene content of the *Vibrio cholerae* genome.

species occupy. Having at least one completely sequenced genome of the organisms of choice is invaluable to the successful interpretation of the data generated from this procedure.

CONCLUSION

Complete genome sequences have increased abilities to address biological questions. The field of genomics

continues to expand, and each new sequence gives insights into genome organization, gene regulation, gene content, novel genes, and gene families, and the many biochemical pathways that reside in these organisms. Although many unknown and conserved hypothetical proteins remain to be characterized, techniques for meeting these challenges are being continuously developed. Sequence information and functional genomic studies continue to lead the identification of enzymes with unique properties, physiological adaptations, evolution of genes and gene families, polypeptide structure–function relations, and metabolic regulation. The development of new and improved activities in these organisms will be possible using current gene technology.

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GEOCHEMICAL AND GEOLOGICAL SIGNIFICANCE OF SUBSURFACE MICROBIOLOGY

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The concentration and isotopic composition of the inorganic ions and gases dissolved in groundwater and the mineralogy, organic geochemistry, and isotopic chemistry of the associated rock strata can be used to delineate the impact of microbial processes on (*1*) the geochemistry of groundwater, (*2*) the diagenesis of sediments, and (*3*) the cycling of elements at redox gradients in the subsurface. This article deals with the current state of knowledge with regard to the isotopic and mineralogical signatures of microbiological activity reported in the laboratory and compares them with those identified in the subsurface.

The discussion will focus on deep terrestrial environments where resident microbial populations must rely on endogenous energy sources rather than those replenished by transport from the surface. Microbial populations in this case are insensitive to 10-kyr changes in the surface climate and vegetation. Their activity, instead, relies on their ability to access energy from more recalcitrant organic substrates or reduced inorganic mineral surfaces.

These surfaces may quickly become depleted of bioavailable nutrients and/or coated with the by-products of microbial and geochemical reactions. As a result, the overall activity of "deep"-subsurface microbial communities will reflect changes in fluid flow and mineral precipitation induced by long-term volcanic or tectonic episodes (1) or changes in sea level. In the deep subsurface, the anaerobic microbial processes that are occurring today may also be very similar to those that dominated the earth's surface in the Archean. Clues to the formation of isotopic signals or inorganic "biomarkers" in Archean rocks may exist in modern, subsurface microbial ecosystems.

BACKGROUND

The extent to which microbial communities affect subsurface geochemistry was first explored in the 1920s, when a geologist at the University of Chicago, Edson Bastin, examined the source of hydrogen sulfide and bicarbonate in water from deeply buried oil fields in Illinois. In an experiment reported in Science in 1926 (2) Edson Bastin and Frank Greer, a microbiologist also from the University of Chicago, cultured sulfate-reducing bacteria (SRB) from groundwater samples collected from oil fields at depths of 150 to 600 m. Their results suggested that microorganisms were responsible for the in situ reduction of sulfate to sulfide. See Geochemical Aspects of Subsurface Microbiology, Esotopic Fractionation, Subsurface Microbiology

The presence of microorganisms in groundwater is not surprising because bacteria are dependent on diffusion, through water, of substrates to and products from cells for metabolism and growth. Groundwater sampled from wells typically originates from the most transmissive zones of the aquifer, and these zones usually have sufficient pore spaces and higher nutrient fluxes to support microbial populations. For this reason, groundwater, almost regardless of the environment, invariably harbors bacteria at densities between 10^3 and 10^6 cells per milliliter, and evidence of their activity is present in the concentrations and isotopic values of the dissolved inorganic or organic species and gases. The isolation and analysis of the microorganisms whose physiology is consistent with the inferred in situ geochemical process (3–5) also provides evidence that the microorganisms are active in that environment. Deep terrestrial environments through which water is readily transmitted are more favorable habitats for microbial populations than are regions where pore spaces or fractures are small or nonconductive (6,7).

In contrast to groundwater, a relative paucity of microorganisms occurs in the consolidated, indurated rock of the deep terrestrial subsurface. Many of the samples examined till date have had microbial biomass levels below limits of detection. Although evidence of microorganisms existing in these environments is strong, for many rock samples, microorganisms were not detected by either molecular or enrichment approaches. Progress in the detection in rock cores of diagenetic mineral phases produced by modern, subsurface microorganisms has been slow because of the low biomass of deep subsurface samples with some notable exceptions (8).

MICROBIAL FLUID INTERACTIONS

Microbially Catalyzed Oxidation-Reduction Reactions

Microorganisms have evolved or adapted to occupy essentially any niche where energy can be obtained from oxidation-reduction reactions involving organic and inorganic aqueous species, as long as those reactions have a favorable ΔG. The ability of microorganisms to survive or even thrive in environments in which energy is theoretically available is influenced by temperature, low abiotic reaction rates, and available pore space.

Deep subsurface environments inhabited by microorganisms can essentially be considered 'aphotic' systems. Photosynthesis plays only an indirect role in subsurface microbial metabolism, providing reduced organic compounds by burial and metamorphism that can be metabolized by heterotrophs. Microbial generation of energy in deep subsurface environments, therefore, is derived from biochemical reactions involving the oxidation of reduced compounds and the subsequent transfer of electrons to an adjacent oxidized compound of the following elements: Hydrogen, nitrogen, oxygen, sulfur, manganese, and iron.

From a thermodynamic perspective, the oxidation of organic carbon to carbon dioxide using O_2, NO_3^-, MnO_2, FeOOH, or SO_4^{2-} as oxidants or electron acceptors is exothermic, though kinetically frustrated. The ΔG for the oxidation of organic carbon also diminishes in order from O_2 to SO_4^{2-}, indicating that the oxidation of organic carbon should occur sequentially, beginning with the reduction of O_2, the most thermodynamically favored reaction, in descending order through methane-fermentation. From microbial and geochemical standpoints, the bioavailability of the electron acceptor, the reactive transport of the products, and the reaction kinetics will influence the sequence and the extent of these reactions. At macroscopic scales in systems in which microorganisms are active, such as a sediment–water interface or along an aquifer flow path, the sequential utilization of the oxidants as electron acceptors can be readily observed.

One of the principal reductants in sedimentary depositional environments is organic matter; this also holds true for many subsurface environments. Dissolved or colloidal organic matter is probably the only significant source of reductant in shallow (<50 m) aquifers, whereas sediment-associated (detrital) organic matter of terrestrial or marine origin and soluble hydrocarbons are a major source of reductant in deeply buried sediments.

In addition to organic carbon, reduced inorganic species, for example, Fe^{2+}, Mn^{2+}, S^{2-}, NH_4^+, CH_4, or H_2, can also provide reducing power for microbial metabolism. These reactions are particularly favorable when coupled to a strong oxidant such as oxygen, but some microorganisms can gain energy from oxidation of some reduced inorganic species coupled to weaker oxidants (such as nitrate or even carbon dioxide). Microorganisms that can derive energy from the oxidation of inorganic compounds for metabolism and for growth are termed

chemolithotrophs or *chemolithoautotrophs*, respectively. Reduced inorganic species may be the dominant source of energy for microorganisms in some deep terrestrial subsurface environments, particularly those dominated by hard rocks such as basalt (9) or granite (8) in which organic carbon is scarce or absent.

For confined aquifers where the DIC is not equilibrating with soil carbon dioxide (referred to as a *closed* system for carbon cycling), the DIC represents a balance between subsurface microbial and inorganic processes. These processes include: (*1*) the flux of soil carbon dioxide from the recharge zone, (*2*) the depletion of carbon dioxide during dissolution of calcite and dolomite, (*3*) the liberation of carbon dioxide during calcite or siderite precipitation, (*4*) the microbial oxidation or fermentation of either dissolved organic matter (DOM) or sedimentary organic matter (SOM), (*5*) the disproportionation of acetate during methanogenesis (10), (*6*) the depletion of carbon dioxide during methanogenesis by carbon dioxide reduction, (*7*) oxidation of methane (i.e., methanotrophy, 11,12) (*8*) the fixation of carbon dioxide in the microbial biomass, and (*9*) the thermal disproportionation of acetate. For deep subsurface environments at temperatures greater than 100 °C, the abundance of DIC may reflect metastable equilibrium reactions among DIC, carbonate, feldspar, and clay, and is inversely proportional to the Ca^{2+} concentration (13–15). In this case, as the temperature and depth increase, the pH will decrease, pCO_2 will increase, and both will be insensitive to microbial activity.

Similarly, subsurface organic acid concentrations represent the balance between production by either microbial fermentation or acetogenesis and by hydrous pyrolysis of kerogen (Carothers, 1978 #658) or petroleum (Helgeson, 1993 #551), consumption by microbial respiration (16), and, at higher temperatures, metastable equilibrium with DIC (15).

Dissolved oxygen generally diminishes with distance along the flow path in shallow aquifers as a result of aerobic bacterial respiration (either heterotrophic or chemolithoautotrophic). Dissolved nitrogen varies in concentrations and is composed of numerous species. Dissolved NO_3^- tends to decline with distance along the flow path in shallow aquifers as a result of NO_3^- reduction to N_2 by bacterial activity, producing a wide variety of intermediate products, such as NO_2^-, NO, N_2O, or NH_4^+. In deep subsurface environments, the dominant nitrogen species is either N_2 or NH_4^+, the latter produced by deamination of organic matter or perhaps from other abiotic reactions. Sinks for NH_4^+ are oxidation to NO_3^- by ammonia-oxidizing bacteria or fixation of NH_4^+ in the microbial biomass.

Dissolved Fe(II) reflects the production of Fe(II) by reduction of Fe(III)-bearing mineral phases by iron-reducing bacteria or by dissolved sulfide. Sinks for Fe(II) include the sorption of Fe(II) to mineral surfaces, the precipitation of Fe(II) bearing minerals such as green rust, vivianite, FeS, $FeCO_3$, and Fe_3O_4, and the oxidation of Fe(II) to Fe(III) oxide phases abiotically or by iron-oxidizing chemolithoautotrophic bacteria using either O_2 or NO_3^- as an electron acceptor.

Groundwater SO_4^{2-} concentrations typically reflect a balance between SO_4^{2-} production by gypsum dissolution, biotic or abiotic pyrite, or sulfide oxidation and bacterial SO_4^{2-} reduction and barite precipitation. Dissolved HS^- or S^{2-} concentrations reflect a balance between production by bacterial SO_4^{2-} reduction and FeS precipitation, S fixation into the microbial biomass and, possibly, sulfur or thiosulfate disproportionation reactions. Intermediate sulfur species, such as S_2O_3 and SO_3^- tend to be ephemeral in groundwater systems.

Because the enzymatic electron acceptor processes have varying affinities to hydrogen, the concentration of hydrogen can reflect the dominant electron-acceptor reaction (17). In subsurface environments that are electron-donor limited, the hydrogen concentration appears to be thermodynamically controlled by the principal, terminal electron-acceptor reaction so as to maintain a constant ΔG (18) under varying temperatures and pH. The segregation of the dominant electron-acceptor processes along the flow paths of many aquifers also reflects this competition for acetate and hydrogen (19). In deeper, hydrocarbon-bearing, sedimentary formations at temperatures of 100 to 150 °C, hydrogen concentrations may primarily reflect abiotic, irreversible, hydrolytic disproportionation, hydrolysis of light paraffins, and metastable equilibrium among organic acids (20). The groundwater hydrogen concentrations reflect the production of hydrogen by water disproportionation reactions (9,21), pore water radiolysis (22), or microbial fermentation of organic matter, and the microbial consumption of hydrogen linked to various terminal electron-accepting processes (17).

Finally, in natural gas reservoirs, the concentration of dissolved hydrogen, methane, and organic acids will be controlled by DIC concentration, the partitioning factor between the aqueous and gas phase and the total pressure (23).

Geochemical Evidence of Subsurface Microbial Activity: Case Studies

Hydrogeologists and geochemists have commonly observed systematic decreases in the E_h of groundwater as it migrates from elevated recharge areas to low lying discharge areas under conditions of confined flow, and have attributed the changes to microbial metabolic processes (24). The spatial scale for the transition from one redox zone to the other in aquifers may extend over kilometers in contrast to the centimeters typical for modern sediments. The redox status of the groundwater at any specific geographic position will represent the integrated effect of a heterogeneous mixture of redox couples varying over scales from centimeters to millimeters. Finally, accurate knowledge of groundwater flow path and detailed delineation of the redox gradients are far more difficult to obtain in the deep subsurface than in most surface sediments, because of the spatial heterogeneity of redox properties in combination with aquifer inaccessibility and limitations associated with sampling water from wells.

E_h declines along the groundwater flow path for the Middendorf formation of the Atlantic coastal plain, southeastern United States (25). Redox zonation has also been

delineated by changes in the concentrations of chemical species involved in redox reactions, such as sulfate, and when linked to the microbially catalyzed oxidation of organic carbon to carbon dioxide in the extensive Madison aquifer of Montana, Wyoming, and South Dakota (26). Observations reporting similar zonation of redox processes have been reported for other regional groundwater systems including the Fox Hills-Basal Hell Creek aquifer of North Dakota and South Dakota (27), but not the chalk aquifers of the United Kingdom (28) (including the Lincolnshire Limestone).

One of the first studies that closely linked microbial analyses of sediment core samples to carbon dioxide increases in groundwater of deeply buried coastal plain sediments of the eastern United States was conducted by Chapelle and coworkers (29). In this study, the presence of bacterial cells, including methanogens and sulfate-reducing bacteria, was unequivocally identified in deep sediment cores, indicating that the source of carbon dioxide was microbial oxidation of organic matter (possibly sedimentary lignite). Later work by Murphy and coworkers (25) identified geochemical changes along a groundwater flow path in the Middendorf aquifer of South Carolina that were consistent with the physiology of microorganisms cultured from Middendorf core samples, including aerobic chemoheterotrophs and iron-reducing and sulfate-reducing bacteria. They suggested that lignite inclusions in the aquifer sediments were the principal source of organic carbon for microbial fermentation and respiratory processes. Redox processes within the Middendorf were heterogeneous and probably linked to lignite distribution in the sediments. Subsequent research confirmed the presence of active bacteria in core samples by measuring the oxidation of ^{14}C-labeled glucose or acetate (30), incorporation of ^{14}C-labeled acetate into lipids and ^{3}H-thymidine into DNA of bacterial cells (7). Jones and coworkers (31) found that some subsurface sediment slurries accumulated acetate in the absence of amendment with any organic compounds, indicating the presence of endogenous fermentable carbon compounds. Later, McMahon and Chapelle (16) identified that microbial fermentation of organic matter was occurring in fine-grained aquitard sediments, and was probably responsible for driving bacterial respiration in adjacent course-grained aquifers. McMahon and Chapelle (32) also demonstrated the biological generation of carbon dioxide from endogenous carbon in ACP sediments incubated with O_2 ,NO_3^-, or SO_4^{2-} as the electron acceptor, providing additional evidence of sedimentary organic carbon as the principal electron donor driving microbial metabolism in deeply buried Atlantic coastal plain sediments.

In oil-rich environments, microbial degradation of petroleum manifests itself by the progressive depletion of n-alkanes, followed by branched alkanes and alkyl aromatics, leaving a residue enriched with napthlenes and polynuclear aromatic hydrocarbons, resins, and asphaltenes (33). These features have been reported for subsurface petroleum reservoirs with maximum formation temperatures of 88 °C, and are normally associated with aerobic (oxygen respiring) conditions (33). More recently, however, anaerobic oxidation of n-alkanes and alkylbenzenes to carbon dioxide has been reported for mesophilic and thermophilic SO_4^{2-} reducing bacteria (34,35). Hyperthermophilic SO_4^{2-}-reducing archaea and thermophilic SO_4^{2-} reducing bacteria have been reported from oil fields worldwide (36–39) to depths as great as three kilometers beneath the seafloor. Such cultures were shown to grow at temperatures as high as 102 °C (36).

In deep igneous rock environments where solid phase–associated organic carbon is sparse (40,41), low E_h (< -200 mV) groundwater is typical with relatively high concentrations of Fe(II) and HS^- (40,42). Interestingly, dissolved hydrogen concentrations as high as 60 μM in the Columbia River basalt aquifer of south-central Washington and 70 μM in the granite aquifer of southeastern Sweden have been reported (9). Hydrogen has also been found to comprise up to 30% of the natural gas discharged freely from deep subsurface fractures (43). These values are several orders of magnitude greater than the sediments in which microbial fermentation is occurring (17) and the origin of the hydrogen remains controversial. Many of the bacteria that reside in these environments can grow autotrophically with hydrogen as the sole electron donor and carbon dioxide as the carbon source, including acetogenic bacteria and autotrophic methanogens (9). The acetogenes represent a potential dissolved carbon source for heterotrophic bacteria. Alternatively, the CH_4 and C_{2+} compounds found in these environments may originate from hydrous pyrolysis organic matter disseminated nearby or the degassing of the underlying mantle. Recently, abiogenic Fischer-Tropsch synthesis has been proposed as a source mechanism for CH_4 and C_{2+} compounds from the subsurface of the plutonic rock-hosted aquifers of the Canadian and Fennoscandian Precambrian shields on the basis of their δ^{13}C and δD values (44,45).

To determine the extent to which a subsurface relies on indigenous energy sources requires estimates of the rates of various microbial respiration processes and the flux of electron donors and acceptors from the surface. In modern depositional settings where microbial activity measurements are based on geochemical and radiolabeled substrate, incubations are compatible with each other and with the rate of sediment burial. Geochemical estimates of microbial respiration rates within aquifers are on the order of nmoles $L^{-1}yr^{-1}$, which are a factor of 10^3 less than those derived from modern lakes or marine sediments (46,47). Microbial activity measurements derived from laboratory incubations with radiolabeled substrates using homogenized sediment from the same aquifer (46) yield rates that are 10^6 times more than those derived from geochemical mass balance, and are unrealistically high. Geochemical estimates, however, have relied on assumptions of mass balance that are difficult to establish in deeply seated aquifers. Stable isotope analyses partly alleviate the need for this assumption and can provide more reliable estimate of in situ microbial respiration rates.

The Impact of Subsurface Microbial Activity on Isotopic Composition

Elements commonly involved in biological redox reactions contain more than one isotope and the reaction rate

varies slightly with the atomic weight. Reactions that break bonds preferentially break those between the lighter isotopes of an element, ^{12}C versus ^{13}C, for example. This is true regardless of whether the reaction is biologically driven or in thermodynamic equilibrium, but the degree of preference for the lighter isotope differs between the enzymatically driven microbial reactions and thermodynamic equilibrium between products and reactants.

The degree to which the kinetic (first-order) reaction rate for one isotope, k_1, is greater than that of another, k_2, for the same element is reflected in the value of ε, where

$$\varepsilon = [k_1/k_2 - 1] \times 10^3 \quad (1)$$

Determinations of the ε values for electron donor/acceptor reactions of different bacterial strains have been mostly based on batch experiments where the product of the reaction cannot equilibrate with the reactant. The isotopic compositions of the reactant and/or the product, expressed as δ values, are determined for multiple experiments terminated at varying degrees of F, the fraction of reactant remaining. Under these conditions, which are defined as isotopically "closed" conditions in Table 1, ε is derived from either of the following Rayleigh distillation relationships,

$$\varepsilon = [\delta_{rf} - \delta_{ri}]/\ln(F) \quad (2)$$

$$\varepsilon = [\delta_{ri} - \delta_{pf}]/[F \times \ln(F)/(1-F)] \quad (3)$$

where δ_{rf} and δ_{ri} are the final and initial isotopic compositions of the reactant and δ_{pf} is the final isotopic composition of the product and δ is defined as,

$$\delta = [(R_{sample} - R_{standard})/R_{standard}] \times 10^3 \quad (4)$$

where R_{sample} and $R_{standard}$ are the isotopic ratios of the sample and a standard. Isotopic determination of both the reactant and product phases provides two independent measurements of ε. If these two measurements are not in agreement, then a significant portion of the reactant is being converted to some other phase, such as fixation in a growing biomass, or partial equilibration between the reactant and product is occurring.

In some instances isotopic fractionation experiments are performed under steady state conditions, defined as isotopically "open" conditions in Table 1, in which the reactant and product concentrations are kept constant during the course of the experiment. In this case, the value of ε is given simply by,

$$\varepsilon = \delta_p - \delta_r \quad (5)$$

The kinetic fractionation values are quite distinct from equilibrium fractionation values (Table 1) given by,

$$\delta = 10^{13} \times \ln(R_{product}/R_{reatant}) = \delta_p - \delta_r \quad (6)$$

where $R_{product}$ and $R_{reactant}$ are the isotopic ratios of the product and reactant. Typically, these quantities are determined at high temperatures where reaction rates are enhanced. To compare the δ values with the ε values derived from the lower temperature microbial experiments, Δ values are extrapolated using the following relationship,

$$\Delta = a(10^6/T_K^2) + b(10^3/T_K) + c \quad (7)$$

where a, b, and c are experimentally derived constants (81).

To illustrate the differences between Δ and ε, consider the SO_4^{2-}-reduction reaction. Sulfate and dissolved hydrogen will react to form S^{2-} at 300 °C relatively quickly, and the reaction is reversible (87). The lighter ^{32}S preferentially composes the S^{2-} at equilibrium; whereas the ^{34}S tends to remain in the residual sulfate. The $\delta^{34}S$ of the S^{2-}, δ_{pf}, and SO_4^{2-}, δ_{rf}, depends on the initial $\delta^{34}S$ of the SO_4^{2-}, δ_{ri}, and the fractional amount of reaction progress and the $\Delta_{S^{2-}-SO_4^{2-}}$, δ, according to the following relationships,

$$\delta_{rf} = \delta_{ri} + \Delta \times (F - 1) \quad (8)$$

$$\delta_{pf} = \delta_{ri} + \Delta \times (F) \quad (9)$$

where $\delta S^{2-} - SO_4^{2-} = -20‰$ at 300 °C (or −73‰ at 25 °C, Table 1). If pyrite (or greigite) is produced or the reaction is performed by SRB at low temperatures, however, then the reversibility of the reaction is eliminated and a Rayleigh distillation enrichment in the residual SO_4^{2-} results. A plot of the $\delta^{34}SO_4^{2-}$ as a function of InF typically yields linear relationships. In the case in which SO_4^{2-} is continuously fed to a culture of SRB (steady state experiment or "open" system in Table 1), the $\delta^{34}SO_4^{2-}$ and $\delta^{34}S^{2-}$ are given by the expressions. Because S^{2-} is isotopically lighter than the SO_4^{2-} by 20‰, the more sulfur that is S^{2-}, the more enriched in ^{34}S the S^{2-} and SO_4^{2-} become, but the $\delta^{34}S$ of the total S still has to match that of the inlet SO_4^{2-}.

In groundwater, the $\delta^{34}S$ of the source SO_4^{2-} may not be known, in which case it is derived from the aforementioned mass balance relationship by assuming a value for Δ and by using the measured concentrations of S^{2-} and SO_4^{2-} (i.e., F) and values of $\delta^{34}SO_4^{2-}$ and $\delta^{34}S^{2-}$ (26). For groundwater in which the S^{2-} concentrations are too low for accurate determination of either S^{2-} or $\delta^{34}S^{2-}$, the amount of SO_4^{2-} reduction, F, is obtained by inferring the $\delta^{34}S$ of the source SO_4^{2-} (e.g., modern marine systems) or deriving it from sediment measurements, by assuming the $\Delta_{S^{2-}-SO_4^{2-}}$ and by measuring the $\delta^{34}S$ of the SO_4^{2-} (47). The isotopic geochemical estimates of microbial SO_4^{2-}-reduction, therefore, are dependent on which mass balance law is selected, Rayleigh distillation versus steady state mass balance, and on the value of $\delta_{S^{2-}-SO_4^{2-}}$ or $\varepsilon_{S^{2-}-SO_4^{2-}}$.

Rayleigh Versus "Open" System Isotope Behavior

Plots of $\delta^{34}S$ of SO_4^{2-} for bacterial cultures and for marine sediments conform to Rayleigh distillation trends,

Table 1. Isotopic Fractionation Factors for Catabolic and Anabolic Reactions

Electron Acceptor/Taxon	Product/Substrate	T (°C)	ε_{p-s} (‰)	ε_{b-s} (‰)	Δ_{p-s} (‰)	Ref.
	Substrate → Product					
O_2 reduction: $2C_3H_4O_3 + 3H_2O + 3O_2 \rightarrow CH_3COO^- + 5HCO_3^- + 6H^+$						
Escherichia coli K12(c.)	$HCO_3^-/C_6H_{12}O_6$	37	−3.4	−0.3–0.6		48, 49
	$CH_3COO^-/C_6H_{12}O_6$	37	12.3	n.a		
Escherichia coli (c.)	HCO_3^-/O_2	?	-18.2 ± 0.9	n.a.		50
Pseudomonas halodurans (c.)	HCO_3^-/O_2	20	-18.6 ± 0.6	n.a.	−24.2	51
O_2 reduction: $CH_3CH_2OH + O_2 \rightarrow CH_3COOH + H_2O$						
Acetobacter suboxydans (c.)	CH_3COOH/CH_3CH_2OH	30	−5.1	n.a.	n.a.	52
	$\mathbf{C}H_3COOH/\mathbf{C}H_3CH_2OH^*$	30	−11	n.a.	n.a.	52
	$CH_3\mathbf{C}OOH/CH_3\mathbf{C}H_2OH^*$	30	−0.8	n.a.	n.a.	52
O_2 reduction: $CH_4 + 2O_2 \rightarrow HCO_3^- + H_2O + H^+$						
mixed cultures (o.)	HCO_3^-/CH_4	23	−5–31	n.a.	69	53
Unidentified strain A(c.)	HCO_3^-/CH_4	26	−25	n.a.	68	54
Unidentified strain B(c.)	HCO_3^-/CH_4	12	−13	n.a.	73	54
Unidentified strain B(c.)	HCO_3^-/CH_4	26	−16, −24	n.a.	68	54
Unidentified strain A(c.)	H_2O/CH_4	26	−281	n.a.	98	54
Unidentified strain B(c.)	H_2O/CH_4	12	−98	n.a.	106	54
Unidentified strain B(c.)	H_2O/CH_4	26	−260	n.a.	98	54
Methylococcus capsulatus (o.)	HCO_3^-/CH_4	25?	n.a.	−30		55
Methylococcus capsulatus (o.)	HCO_3^-/CH_3OH	25?	n.a.	−10		55
Methylobacte. Organophilum (o.)	HCO_3^-/CH_3OH	25?	n.a.	−4		55
Methylomonas methanica (o.)	HCO_3^-/CH_4	25?	n.a.	−30		55
Methylomonas methanica (c.)	HCO_3^-/CH_4	25?	−30	n.a.	68	56
Methylosinus trichosporium (o.)	HCO_3^-/CH_4	25?	n.a.	−6		55
Methylosinus trichosporium (c.)	HCO_3^-/CH_4	25?	−30	n.a.	68	56
O_2 reduction: $2H_2 + O_2 \rightarrow 2H_2O$						
Alkaligenes eutrophus (c.)	$Cell/CO_2$	24	n.a.	−26.1		57
O_2 reduction: $HS^- + 2O_2 \rightarrow SO_4^{2-} + H^+$						
Thiobacillus thiooxidans	SO_4^{2-}/HS^-	?	−20	n.a.		58
O_2 reduction: $2NH_4^+ + 2O_2 \rightarrow 3H_2O + N_2O + 2H^+$						
?	NH_4^+/N_2O	?	−30	n.a.		59
?	NH_4^+/N_2O	?	−65	n.a.		60
O_2 reduction: $S_2O_3^{2-} + 2.5O_2 \rightarrow 2SO_4^{2-}$						
Thiobacillus neapolitanus (c.)	$Cell/CO_2$	21	n.a.	−25	n.a.	61
Thiomicrospira sp. L-12(c.)	$Cell/CO_2$	21	n.a.	−25	n.a.	61
NO_3^- reduction: $2CH_3COO^- + 3NO_3^- + e^- \rightarrow ?4HCO_3^- + 1.5N_2 + H_2O$						
Paracoccus denitrificans (o.)	NO_3^-/N_2	30	−28.6	n.a.	90	62
Pseudomonas denitrificans (c.)	NO_3^-/N_2	30?	−13–20	n.a.		63
Pseudomonas stutzeri	NO_3^-/N_2	30?	−20–30	n.a.		64
Nitromonas europaea	NO_3^-/N_2	30?	−35–36	n.a.		60
NO_3^- reduction: $CH_3COO^- + N_2O + 3H_2O \rightarrow ?N_2 + 2HCO_3^- + 3H_2 + H^+$						
Paracoccus denitrificans (o.)	N_2O/N_2	30	−28.6	n.a.	10	62
NO_3^- reduction: $2NO_3^- + S^0 + H_2O \rightarrow ?N_2 + SO_4^{2-} + H_2$						
Thiobacillus denitrificans (c.)	SO_4^{2-}/S^0	25?	0	n.a.		65
S^o reduction: $S^0 + H_2 \rightarrow ?HS^- + H^+$						
Chromatium vinosum (c.)	H_2S/S^0	33	0	n.a.		66
Thermoproteus neutrophilus (c.)	$Cell/CO_2$	85	n.a.	−8.2		57
SO_3^{2-} reduction: $C_6H_{12}O_6 + 4SO_3^{2-} \rightarrow 6HCO_3^- + 4HS^- + 2H^+$						
Clostridium pasteurianum (c.)	H_2S/SO_3^{2-}	37	−15–20	1	−58	(67,68)
	$HCO_3^-/C_6H_{12}O_6$	37	−11	0		
	$Cell/N_2$	37	n.a.	0		67

(*Continued*)

Table 1. (*Continued*)

Electron Acceptor/Taxon	Product/Substrate	T (°C)	ε_{p-s} (‰)	ε_{b-s} (‰)	Δ_{p-s} (‰)	Ref.
SO_3^{2-} reduction: $3CH_3CH_2COO^- + 2SO_3^{2-} \rightarrow 3CH_3COO^- + 3CO_2 + 2H_2S + 2H^+$						
Desulfovibrio desulfuricans (c.)	S^{2-}/SO_3^{2-}	20–40	−25.5	n.a.	−64–58	(68,69)
Desulfovibrio desulfuricans (c.)	HS^-/SO_3^{2-}	30	0–3	n.a.	−60	70
Desulfofotomaculum nigrifs (c.)	HS^-/SO_3^{2-}	55	−5–13	−2.5	−53	70
Desulfovibrio desulfuricans (c.)	$CO_2/CH_3CH_2COO^-$	5	−6.9–6	n.a.	−72	71
Desulfovibrio desulfuricans (c.)	$CO_2/CH_3CH_2COO^-$	15	−9.1–6	n.a.	−66	71
Desulfovibrio desulfuricans (c.)	$CO_2/CH_3CH_2COO^-$	25	−10.8–6	n.a.	−62	71
Desulfovibrio desulfuricans (c.)	$CO_2/CH_3CH_2COO^-$	35	−12.8–6	n.a.	−59	71
SO_3^{2-} reduction: $SO_3^{2-} + 4H_2 \rightarrow H_2S + 3H_2O + 2e^-$						
Desulfovibrio desulfuricans (c.)	H_2S/SO_4^{2-}	20–35	−25	n.a.	−64–59	(68,69)
SO_4^{2-} reduction: $2CH_3CH_2COO^- + SO_4^{2-} \rightarrow 2CH_3COO^- + 2CO_2 + H_2S + 2OH^-$						
Desulfovibrio desulfuricans (c.)	H_2S/SO_4^{2-}	11–40	−23.7	n.a.	−64–75	(68,69)
Desulfovibrio desulfuricans (c.)	HS^-/SO_4^{2-}	19–25	−5–12	n.a.	−72–75	70
Desulfovibrio vulgaris (c.)	HS^-/SO_4^{2-}	30	−8–10	n.a.	−70	70
Desulfovibrio salexigens (c.)	HS^-/SO_4^{2-}	30	−9–11	n.a.	−70	70
Desulfofotomaculum orientis (c.)	HS^-/SO_4^{2-}	30	−4–6	n.a.	−70	70
Desulfofotomaculum nigrifs. (c.)	HS^-/SO_4^{2-}	55	−5–13	−2.5	−62	70
Desulfovibrio desulfuricans (o.)	H_2S/SO_4^{2-}	23–40	−15–35	−2.4	−65–75	72
Desulfovibrio africans (c.)	HS^-/SO_4^{2-}	35	-14.6 ± 4.1	n.a.	−69	73
Desulfovibrio desulfuricans (c.)	HS^-/SO_4^{2-}	35	-14.6 ± 4.1	n.a.	−69	73
Desulfovibrio gigas (c.)	HS^-/SO_4^{2-}	35	-14.6 ± 4.1	n.a.	−69	73
Desulfofotomaculum salexns. (c.)	HS^-/SO_4^{2-}	35	-14.6 ± 4.1	n.a.	−69	73
Desulfovibrio vulgaris (c.)	HS^-/SO_4^{2-}	35	-14.6 ± 4.1	n.a.	−69	73
Desulfofotomaculum nigrifs. (c.)	HS^-/SO_4^{2-}	55	-14.6 ± 4.1	n.a.	−62	73
Desulfovibrio desulfuricans (c.)	$CO_2/CH_3CH_2COO^-$	15	−8.8	−6	−74	71
Desulfovibrio desulfuricans (c.)	$CO_2/CH_3CH_2COO^-$	35	−7.4	−6	−66	71
Unknown strain	H_2O/SO_4^{2-}	25–30	1.2–25**	n.a.	−30	(74,75)
Desulfobacter hydrogens.(c.)	$Cell/HCO_3^-$	24	n.a.	−8–13		57
SO_4^{2-} reduction: $SO_4^{2-} + 5H_2 \rightarrow H_2S + 4H_2O + 2e^-$						
Desulfovibrio desulfuricans (c.)	S^{2-}/SO_4^{2-}	20	−5–11	n.a.	−72	69
Desulfobacterium autotroph. (c.)	$Cell/HCO_3^-$	24	n.a.	−36		57
SO_4^{2-} reduction: $CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O$						
Sediment	C	2	-8.8 ± 1.3	n.a.		11
	H	2	-157 ± 23	n.a.		11
CO_2 reduction: $8H_2 + CO_2 \rightarrow CH_4 + 2H_2O$						
Methanosarcina barkeri (o.)	CH_4/CO_2	40	−43	−12	−63	76
Methanosarcina barkeri (c.)	CH_4/CO_2	36	−46	n.a.	−65	77
Methanobacterium M.o.H (o.)	CH_4/CO_2	40	−59	9	−63	76
Methanobacterium sp. (o.)	CH_4/CO_2	37	−35	−24	−64	78
Methanobacterium sp.(o.)	CH_4/CO_2	46	−34	−19–24	−62	78
Methanobacterium formicicum (c.)	CH_4/CO_2	34	−48	−5–30	−65	79
	CH_4/H_2***	34	300–420	630–720	1,169	79
Methanobacterium thermo.c.)	CH_4/CO_2	65	−25	−13	−56	76
Methanobacterium thermo.(o.)	CH_4/CO_2	65	−34	−24	−56	80,81
	CH_4/H_2	65	n.d.	686*	1,019	80,81
CO_2 reduction: $2H_2 + 2HCO_3^- \rightarrow CH_3COO^- + H_2O$						
Acetobacterium woodii (c.)	$CH_3\mathbf{C}OO^-/\mathbf{C}O_2$*	30	−58.6	n.a.		82
	$\mathbf{C}H_3COO^-/\mathbf{C}O_2$*	30	−58.6	n.a.		82
Acetobacterium woodii (c.)	CH_3COO^-/CO_2	24	−40	−15–27		57
Disproportionation: $CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$						
Methanosarcina barkeri (c.)	$\mathbf{C}H_4/\mathbf{C}H_3COO^{-}$*	37	−21	n.a.		83
	$\mathbf{C}H_4/CH_3\mathbf{C}OO^{-}$*	37	−21	n.a.		83
Methanosarcina barkeri (c.)	CH_4/CH_3COO^-	37	−21.2	n.a.		77
Disproportionation: $CH_3OH + H_2O + 2e^- \rightarrow CH_4 + 2OH^-$						
Methanosarcina barkeri (cl.)	CH_4/CH_3OH	37	−72.5–74.8	n.a.		77
Disproportionation: $3S^0 + 2Fe(OH)_3 \rightarrow 2FeS + SO_4^{2-} + 2H_2O + 2H^+$						

(*Continued*)

Table 1. (*Continued*)

Electron Acceptor/Taxon	Product/Substrate	T (°C)	ε_{p-s} (‰)	ε_{b-s} (‰)	Δ_{p-s} (‰)	Ref.
ÅB_1(c.)	FeS/S^0	24	−8.6	n.a.		84
	SO_4^{2-}/S^0	24	15.3	n.a.		84
ÅB_2(c.)	FeS/S^0	24	−7.3	n.a.		84
	SO_4^{2-}/S^0	24	12.6	n.a.		84
Phototrophy: $H_2S \rightarrow hv \rightarrow S^0 + H_2$						
Chromatium vinosum (c.)	S^0/H_2S	33	2.2	n.a.		66
Phototrophy: $S^0 + 4H_2O \rightarrow hv \rightarrow SO_4^{2-} + 3H_2 + 2H^+$						
Chromatium vinosum (c.)	SO_4^{2-}/S^0	33	0	n.a.		66
Phototrophy: $S_2O_3^{2-} + 5H_2O \rightarrow hv \rightarrow 2SO_4^{2-} + 4H_2 + 2H^+$						
Chromatium vinosum (c.)	Cell/CO_2	30	n.a.	−19.6		85
	Cell/H_2O	30	n.a.	−170		
Chromatium strain D (o.)	Cell/CO_2	20	n.a.	−23		86
Chlorobium lim. thiosulf. (o.)	Cell/CO_2	20	n.a.	−12		86
Chlorobium vibrio. Thiosulf. (c.)	Cell/CO_2	30	n.a.	−4		85
	Cell/H_2O	30	n.a.	−160		85
Chlorobium phaeovibrioides (c.)	Cell/CO_2	30	n.a.	−2−3		85
	Cell/H_2O	30	n.a.	−146		85
Chlorobium lim. thiosulf. ((c.)	Cell/CO_2	30	n.a.	−6		85
	Cell/H_2O	30	n.a.	−163		85
Chlorobium vibrioforme (c.)	Cell/CO_2	30	n.a.	−4		85
	Cell/H_2O	30	n.a.	−148		85
Phototrophy: $H_2 + HCO_3^- \rightarrow hv \rightarrow$ *Cell mass*						
Rhodspirillum rubrum (o.)	Cell/CO_2	20	n.a.	−20.5		86
Rhodspirillum rubrum (c.)	Cell/CO_2	20?	n.a.	−12		85
	Cell/H_2O	20?	n.a.	−71		85
Rhodopseudomonas caps. (c.)	Cell/CO_2	20?	n.a.	−11		85
	Cell/H_2O	20?	n.a.	−101		85

Note: $\varepsilon_{p-s} = \delta_p - \delta_s$ and $\varepsilon_{b-s} = \delta_b - \delta_s$, where p = product, s = substrate, b = biomass, $\delta = [(R_p - R_{\text{standard}})/R_{\text{standard}}] \times 10^3$, R is the isotopic ratio and $\delta_{p-s} = a(10^6/T_k^2) + b(10^3/T_k) + c$.n.a. = not analyzed.

*Bold letters in chemical formula specify the specific molecular site for which the isotopic fractionation was determined.

**See discussion in text. The $\delta^{18}O$ of SO_4^{2-} was found to depend on the $\delta^{18}O$ of H_2O.

***See discussion in text. The δD of H_2, CH_4 and the cells were found to depend on the δD of H_2O.

but those for aquifers typically do not. In subsurface environments, the definition of Rayleigh or "closed" systems versus "open" isotopic systems depends on the exchange rate of the fractionated substrate between an elemental volume of water and its surroundings relative to the rate of microbial SO_4^{2-}-reduction. Following Jorgensen (88), the degree of isotopic "openness" for SO_4^{2-} reduction is determined by the following steady state relationship,

$$\begin{aligned} SO_4^{2-}\text{-reduction rate} = {} & \text{pore water diffusion supply rate} \\ & + \text{groundwater advection} \\ & \text{supply rate} \end{aligned} \quad (10)$$

If the diffusion term dominates, the system is considered "open," whereas if the groundwater advection term dominates, then the system is "closed." Instead of having one pair of equations defining the biogeochemical mass balance, we have two pairs, one for each isotope of sulfur, ^{32}S:

$$\partial[^{32}SO_4^{2-}]/\partial t = D^{32}SO_4^{2-}\partial^2[^{32}SO_4^{2-}]/\partial x^2 + V\partial[^{32}SO_4^{2-}]/\partial x - R^{32}SO_4^{2-}(x) \quad (11)$$

where

$$R^{32}SO_4^{2-}(x) = [^{32}SO_4^{2-}]/[SO_4^{2-}] \times (1 + 0.5 \times \varepsilon^{32}_{SO_4^{2-}-S^{2-}}/10^3) \times R(x) \quad (12)$$

and

$$\partial[^{32}HS^-]/\partial t = D^{32}HS^-\partial^2[^{32}HS^-]/\partial x^2 + V\partial[^{32}HS^-]/\partial x + R^{32}SO_4^{2-}(x) \quad (13)$$

An identical set of relationships exist for ^{34}S.

In depositional environments, plots of $\delta^{34}S$ versus $^{32}SO_4^{2-}$ mimic Rayleigh fractionation trends, but because of their partially "open" system behavior (88), the $\varepsilon_{S^{2-}-SO_4^{2-}}$ is less than that in "closed" systems and depends on the rate of SO_4^{2-} reduction. Because microbial SO_4^{2-} reduction preferentially depletes the SO_4^{2-} of ^{32}S, the diffusive flux of $^{32}SO_4^{2-}$ is greater than that of $^{34}SO_4^{2-}$ toward the zone of SO_4^{2-} reduction, although $D^{32}SO_4^{2-}$ is indistinguishable from $D^{34}SO_4^{2-}$. Simultaneously, the diffusive counterflux of $^{32}S^{2-}$ is greater than that of $^{34}S^{2-}$ away from the SO_4^{2-} reduction zone. With increasing

burial depth, the SO_4^{2-} reduction rate decreases, the sulfur isotope system becomes more "closed',' and the $\varepsilon_{S^{2-}-SO_4^{2-}}$ increases, but the Rayleigh enrichment trend is still observed.

In the low porosity, low permeability environment typical of the deep subsurface, dissolved electron donor/acceptor fluxes are largely controlled by diffusive rather than advective transport of the spatial scale directly relevant to microbial activity. The length scales, L, for observed redox or activity gradients in aquifer microsites (25,89) are on the order of millimeters. Soluble substrates will diffuse toward the pore that contains the bacteria, because of the microbiologically induced concentration gradient. The same argument can be made for nonmotile cells metabolizing in liquid or solid media (90). The mass-transfer to the cell's surface is governed by the expression:

$$\partial C/\partial t = D\{\partial^2 C/\partial r^2 + 2/r\partial C/\partial r\} \quad (14)$$

where c is the concentration of the soluble chemical substrate and D is its diffusivity. The transient solution to this equation assuming constant bulk-phase, C_0, and cell surface, C_1, concentrations is:

$$C - C_0/C_1 - C_0 = R/r \text{ erfc } \{(r - R)/2\text{vDt}\} \quad (15)$$

where R is the radius of the cell assuming a spherical shape. The steady state solution is given by Crank (1975):

$$\text{C} = \text{R/r } \text{C}_0/\text{C}_1 + \text{C}_0 \quad (16)$$

The net substrate flux, in units of atoms per cell-time, is equal to:

$$\Phi = 4\pi\ \text{r}^2\text{J} = -\text{D}\partial\text{C}/\partial\text{r} = 4\pi\ \text{D}(\text{C}_1 - \text{C}_0)\text{R} \quad (17)$$

The substrate diffusion rate within the pore in which the cell resides is governed in some cases by the Stokes-Einstein relationship,

$$\text{D} = \text{k}_\text{B}\text{T}/6\pi\mu\text{R}_\text{o} \quad (18)$$

where k_B is Boltzman's constant, μ is the media viscosity, and R_o is the substrate radius. However, in deep subsurface environments, which are undersaturated, natural gas deposits, for example, diffusion of gaseous substrates are modeled by the Chapman-Enskog theory (91):

$$\text{D} = \{0.00186\text{T}^{3/2}\text{v}(1/\text{M}_1 + 1/\text{M}_2)\}/\{\text{p}\sigma_{12}^2\Omega\} \quad (19)$$

where T is temperature °K, p is the pressure in atmospheres, M_1 and M_2 represent the molecular weight of the diffusing substrate and the average molecular weight of the gas medium, and σ_{12}^2 and Ω are molecular properties derived from viscosity.

The substrate diffusion rate toward the pore, D_{eff}, will be somewhat more restricted by porosity, θ, and toruosity, τ(91),

$$\text{D}_\text{eff} = \theta\text{D}/\tau^2 \quad (20)$$

and by viscous drag as modeled by the Rankin relationship (91),

$$\text{D}_\text{eff} = \text{D}\{1 + 9/8\lambda \ln(\lambda) - 1.54\lambda\} \quad (21)$$

where λ is $2\text{R}_\text{o}/\text{d}$ and d is the pore diameter.

Microbial Isotopic Fractionation

Expressions 1 through 20 can be used to constrain the rates of microbial activity if the total isotopic fractionation factor, ε, is known for the specific redox reaction. ε can vary, however, as a function of reaction rate. This relationship has been established for SO_4^{2-} reduction. The total isotopic fractionation, $\varepsilon_{S^{2-}-SO_4^{2-}}$, associated with the reaction sequence for SRB is the sum of several enzymatic steps, each with a distinct ε_i, linked in series to produce a net $\varepsilon = \sum_i \varepsilon_i$. By examining *D. desulfuricans* cells for various growth rates, temperatures, electron donors, and for cell-free enzyme extracts, Kemp and Thode (69) deduced that dissimilatory SO_4^{2-} reduction proceeds by three reactions linked in series: (*1*) SO_4^{2-} diffusion through the membrane and reaction with ATP sulfurylase to adenosine-phosphosulfate, (*2*) conversion of SO_4^{2-} to SO_3^{2-} and production of adenosinemonophosphate, and (*3*) reduction of SO_3^{2-} to S^{2-}. The ε value for the first step is only 1 to 3‰, whereas those of the second and third reactions are both approximately −20 to −25‰ (72). The total $\varepsilon_{SO_4^{2-}-S^{2-}}$ value depends on the reaction rates and concentrations for each step. When SO_4^{2-} concentrations are greater than 10^{-3} M, the $\varepsilon_{S^{2-}-SO_4^{2-}}$ varies inversely as a function of reduction rates from a maximum of −45‰for less than 10^{-17} moles/cell-hr to a minimum of −6‰for greater than 5×10^{-16} moles/cell-hr for organic electron donors (Fig. 1; 92). When hydrogen is the electron donor, the $\varepsilon_{S^{2-}-SO_4^{2-}}$ varies from −10 to −20% and may exhibit a slight positive correlation with reduction rates. At SO_4^{2-} concentrations less than 10^{-3} M, the $\varepsilon_{S^{2-}-SO_4^{2-}}$ decreases, approaching 3‰as diffusion of SO_4^{2-} into the cell becomes the rate-limiting step (93). The $\varepsilon_{S^{2-}-SO_4^{2-}}$ was the same, −14‰, for *Desulfofotomaculum nigrificans* cultures grown at 55 °C as that for *Desulfofotomaculum salexigens* grown at 35 °C (73). Unlike the $\Delta_{S^{2-}-SO_4^{2-}}$ values for equilibrium systems, the $\varepsilon_{S^{2-}-SO_4^{2-}}$ does not inversely correlate with temperature.

A parallel assimilation reaction competes for intracellular S^{2-} and can significantly perturb the isotopic composition of S^{2-} during the log-growth phase, because the S isotopic fractionation during assimilation, $\varepsilon_{biomass-SO_4^{2-}}$, is −2.4‰ (70). *Clostridium* growing on SO_3^{2-} (67) produces S^{2-}, which is isotopically enriched instead of depleted. The effect of biomass incorporation would be to reduce the $\varepsilon_{S^{2-}-SO_4^{2-}}$.

For Other Microbial Reactions

Microbial reduction of O_2, NO_3^-, and CO_2 produce large isotopic fractionation trends in $\delta^{18}O$, $\delta^{15}N$, and $\delta^{13}C$ of the residual electron acceptor (Table 1). In the case of aerobic respiring bacteria, the $\varepsilon_{HCO_3^- - O_2}$ is −18% for pure cultures (Table 1). Isotopic enrichment of residual NO_3^- during bacterial reduction in steady-state laboratory cultures (62)

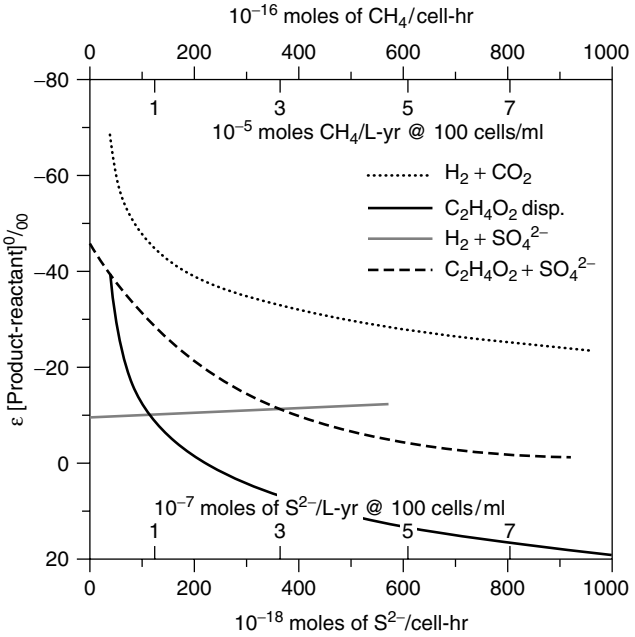

Figure 1. The $\varepsilon_{product-reactant}$ in parts per trillion versus reaction rate for four reactions: (*1*) $4H_2 + CO_2 = CH_4 + 2H_2O$ (gray dashed)(56); (*2*) $CH_3COOH = CH_4 + CO_2$ (solid black)(56); (*3*) $4H_2 + SO_4^{2-} = HS^- + 3H_2O + OH^-$ (solid gray)(92); (*4*) $CH_3COOH + SO_4^{2-} = HS^- + 2CO_2 + H_2O + OH^-$ (black dashed)(92); reaction rate units are in moles of product per cell-hr and in moles of product per L-yr assuming 100 cells/ml.

yields an $\varepsilon_{NO_3^- - N_2}$ of 28.6%. This represents the maximum fractionation because $\varepsilon_{NO_3^- - N_2}$ decreases with increasing denitrification rates. When NO_2^- concentrations become limiting (<2.5 mM), the $\varepsilon_{NO_3^- - N_2}$ is less.

The greatest isotopic enrichment occurs during reduction of carbon dioxide via methanogenesis. Batch laboratory experiments conform to Rayliegh distillation trends and yield $\varepsilon_{CH_4-CO_2}$ values ranging from −25 to −59% (Table 1). Zyakun (94) reported $\varepsilon_{CH_4-CO_2}$ values as high as −80% and decreasing to −10% as CH_4 production rates increased from 10^{-15} to 10^{-12} moles per cell-hr (Fig. 1). The enrichment of residual carbon dioxide by acetogens is equally as great as in methanogens with $\varepsilon_{C_2H_4O_2-CO_2}$ values ranging from −40 to −59% (Table 1). As in the case of SO_4^{2-} and NO_3^- reduction, when carbon dioxide is less than a critical value concentration, the $\varepsilon_{CH_4-CO_2}$ decreases (94), but this critical value is probably well below the inorganic carbon concentrations that typify the deep subsurface.

The $\delta^{18}O$ of the residual SO_4^{2-}, NO_3^- and CO_2 are also enriched during bacterial reduction. The time required for oxygen in water and carbon dioxide to reach thermodynamic equilibrium is essentially instantaneous, however, on the timescale of microbial respiration in laboratory incubations, and so no effect is observed. The time required for the oxygen in water and SO_4^{2-} to equilibrate, however, is on the order of thousands of years (75). During the SO_4^{2-} reduction process, however, the oxygen of the SO_4^{2-} bound to the enzyme complex can isotopically equilibrate with that of water far more rapidly than isolated SO_4^{2-} (74,95). As a consequence, the more enriched the $\delta^{34}SO_4^{2-}$ of residual SO_4^{2-}, the closer to equilibrium the $\delta^{18}O$ of the SO_4^{2-} is with water (95). Because the $\varepsilon_{S^{2-}-SO_4^{2-}}$ is larger when the reduction rate is lower, the slower the SO_4^{2-} reduction rate, the closer to equilibrium the $\delta^{18}O$ of the SO_4^{2-} becomes with water (95). Unlike SO_4^{2-} reduction, the oxygen in the NO_3^- reduction process does not appear to isotopically equilibrate with the oxygen in water. Böttcher and coworkers (96) reported isotopic enrichment in the oxygen of the residual NO_3^- consistent with Rayleigh fractionation with an $\varepsilon_{NO_3^- - H_2O}$ of −8.0‰.

Oxidation of CH_4, NH_3^+, H_2, and HS^- yields enrichment trends in the $\delta^{13}C$, $\delta^{15}N$, δD, and $\delta^{34}S$ of these electron donors. Batch experiments on methanotrophs follow Rayleigh distillation trends (97) and yield $\varepsilon_{CO_2-CH_4}$ of −5 to −31‰ (53,54,56,94). The cause of the variability in the $\varepsilon_{CH_4-CO_2}$ has not been established, but Zyakun (94) reported that the $\varepsilon_{CO_2-CH_4}$ decreased from 30‰to −10‰ when CH_4 became limiting. The effect of the competing assimilation reaction can also be significant as methanotrophs can fix up to 50% of the carbon from CH_4 and the fractionation associated with assimilation of the carbon can be as high as −30‰ and −11‰ for particulate MMO and soluble MMO, respectively (55). The carbon isotopic fractionation has been found to be associated primarily with the first step of methanotrophy, the conversion of CH_4 to CH_3OH. The subsequent conversion of CH_3OH to cell biomass yielded $\varepsilon_{biomass-CH_3OH}$ values of −3.0 to −10‰ (55). The isotopic enrichment of the *D* in the residual CH_4 is more profound with $\varepsilon_{H_2O-CH_4}$ as high as 325‰ (54). Similar enrichment factors for carbon and hydrogen are observed in anaerobic CH_4 oxidation experiments, but neither the specific mechanisms nor the active microbial communities are known (11).

The $\delta^{15}N$ of the residual NH_3 becomes heavier upon progressive oxidation by nitrifying bacteria yielding $\varepsilon_{NH_3-N_2O}$ of −65‰ (60). The concomitant enrichment of the δD of the residual NH_3 is probably equally as great as observed for CH_4 during methanotrophy, but has not been reported.

During oxidation of hydrogen by methanogens the δD of hydrogen, CH_4 and the cells were found to correlate with the δD of the water and not with the rate of hydrogen consumption (79,80). The δD values of the hydrogen were close to those predicted by equilibrium fractionation, whereas the δD values of the CH_4 were still far removed from complete isotopic equilibration with water. The H isotopic exchange rates between water vapor and hydrogen and methane (98) are rapid relative to the geologic timescale, but they are too slow to account for these laboratory effects. Balabane and coworkers (79) proposed that the enzymatic reduction of carbon dioxide to produce methane involved an intracellular water-reduction step in the pathway. This would imply that not all of the H in the CH_4 is derived from extracellular, dissolved hydrogen. Whether this is true for other microbial reactions in which hydrogen is the electron donor is not known. This partial isotopic equilibration of the H in CH_4 with that of water makes the δD_{CH4} formed by carbon dioxide reduction heavier (−150 to −250‰ SMOW) than that formed during

aceticlastic methanogenesis (−250 to −400‰ SMOW), in which some of the H originates from the methyl group within the acetate (99).

Kaplan and Rittenburg (58) reported $\varepsilon_{HS^- - SO_4^{2-}}$ of −20‰ for the oxidation of HS^- to SO_4^{2-} by pure cultures of *Thiobacillus concretivorus*. Bacterially mediated oxidation of base metal sulfides produces SO_4^{2-} with $\varepsilon_{S^{2-} - SO_4^{2-}}$ of −2 to −5.5‰ (100). The oxidation or reduction of solid-phase S^0, however, produces no measurable fractionation of the $\delta^{34}S$ (Table 1).

Oxidation of C_{2+} organic compounds to carbon dioxide generally results in a respired carbon dioxide that is isotopically lighter by 3 to 12‰ (Table 1), with the exception of hydrocarbons for which little fractionation has been observed. During fermentation reactions, the $\delta^{13}C$ of acetate, on the other hand, may be either isotopically enriched or depleted in ^{13}C with respect to the organic substrate depending on the proportion of carbon oxidized to carbon dioxide. Aceticlastic methanogenesis enriches the ^{13}C of the residual acetate with an $\varepsilon_{CH_4 - C_2H_4O_2}$ of −21‰ (Table 1), but this fractionation value may also depend on the reaction rate (94). At high reaction rates (Fig. 1), the $\delta^{13}C$ of methane becomes heavier than that of the source acetate, whereas the $\delta^{13}C$ of carbon dioxide becomes lighter.

Subsurface Isotope System Behavior: Case Studies

To deconvolve the multiple sources and sinks for carbon and SO_4^{2-}, geochemical estimates of the microbial carbon dioxide production and SO_4^{2-}-reduction rates in aquifers have used stable isotope analyses of the DIC, the SO_4^{2-}, and S^{2-} (25,26,101). Isotopic analyses of other electron acceptors, such as O_2 and NO_3^-, have been used to delineate biogeochemical processes in marine environments, but their application to subsurface environments has been limited to contaminated shallow aquifers (96).

In the Cubero sandstone of northwestern New Mexico, enrichment in the ^{34}S of the groundwater SO_4^{2-} is related to the activity of SRB (6,102), and the overall trend approaches a Rayleigh distillation trend with an $\varepsilon_{S^{2-} - SO_4^{2-}}$ of −5‰. On the basis of the measured temperature, porosity, and the average pore diameter determined from Hg porosimetry, the D_{eff} for SO_4^{2-} using Equations (18) and (21) was approximately 6×10^{-6} cm^2/s. Microautoradiography of the Cubero sandstone revealed that SO_4^{2-} reduction was concentrated in microsites with radii of several millimeters (89). Radiotracer measurements also indicated that the minimum SO_4^{2-} concentration that promoted SO_4^{2-} reduction was less than 6×10^{-5} M and the groundwater SO_4^{2-} concentration in the Cubero sandstone was 8×10^{-4} M (6). These measurements suggest that the concentration gradient surrounding a SO_4^{2-} reducing microsite is approximately 10^2 M/m^2 and that the diffusive SO_4^{2-} flux would be 5×10^{-10} moles/m^2-s using Equation (17). The groundwater velocity in the Cubero sandstone estimated from ^{14}C model ages and hydrogeological modeling is 0.2 m/y (102). This yields an advective flux of 5×10^{-9} moles/m^2-s, and according to Equation (10) the isotopic system is predominantly "open" at the pore scale. This suggests that the observed $\varepsilon_{S^{2-} - SO_4^{2-}}$ will be less than the actual $\varepsilon_{S^{2-} - SO_4^{2-}}$ at the microsite. Furthermore, the $\varepsilon_{S^{2-} - SO_4^{2-}}$ will vary as a function of the size and distribution of microsites. For the horizontal scale greater than 100 m sampled at Cerro Negro studies, diffusive mixing is insignificant compared with advective transport, and isotopic variations should behave according to a Rayleigh distillation trend, unless pore scale S cycling or groundwater mixing from multiple reservoirs is occurring.

The $\varepsilon_{S^{2-} - SO_4^{2-}}$ at a microsite in the Cerro Negro aquifer should be small because the concentrations often fall below the 10^{-3} M level (102). For the case of the Cubero Sandstone at Cerro Negro, the substrate flux expression, SO_4^{2-} concentrations and diffusivity used in the preceding text indicate that the SO_4^{2-} flux to a cell that is 0.5 μm in diameter is approximately 10^8 atoms per cells. The limited isotopic fractionation implies that the SO_4^{2-} flux is the rate-limiting step and that most of the intracellular SO_4^{2-} is being converted to S^{2-}. Such a conversion rate would correspond to 10^{-8} moles per cell-yr of S^{2-}. On the basis of data presented by Walvoord and coworkers (102), the SO_4^{2-} reduction rate along the flow path over a time interval of 20,000 years is approximately 10^{-6} moles per L-yr. These two rate estimates are compatible only if about 10^2 cells/L of metabolically active SRB exist in the Cubero Sandstone. MPNs from microbial enrichments, however, suggest much higher cell densities (6). Sulfur isotope data on the dissolved sulfide is required to determine whether the low $\varepsilon_{S^{2-} - SO_4^{2-}}$ implied by the sulfate data is representative and that additional sulfate is not being supplied to the groundwater by gypsum dissolution.

For the Madison aquifer in which isotope data exist for both sulfate and sulfide, the data are too complex to be represented by a simple Rayleigh distillation trend. Plummer and coworkers (26) suggested that microbial SO_4^{2-} reduction is occurring in the Madison aquifer at depths of 2.2 km and temperatures of 87 °C, based on the S isotopic differences between SO_4^{2-} and HS^-. The isotopic enrichment of the residual SO_4^{2-} implies an $\varepsilon_{S^{2-} - SO_4^{2-}}$ of less than −7‰; whereas the $\delta_{S^{2-} - SO_4^{2-}}$ reported ranges from −20 to −38‰ (26). With such fractionation values, the isotopic enrichment of the residual SO_4^{2-} would be very large if additional SO_4^{2-} was not being supplied by the dissolution of gypsum. Measurement of the $\Delta_{S^{2-} - SO_4^{2-}}$ is, therefore, critical to determining both the rate of microbial SO_4^{2-} reduction and the source of the sulfate. The observed $\Delta_{S^{2-} - SO_4^{2-}}$ values correspond to laboratory reduction rates ranging from 10 to 100 × 10^{-18} moles/cell-hr (Fig. 1). The population density of SRB in the Madison aquifer is approximately 100 MPN/mL (103). Combination of these two estimates yield a net SO_4^{2-} reduction rate of 10^{-7} to 10^{-8} moles/L-yr, which is comparable to the SO_4^{2-}-reduction rate estimated by geochemical modeling.

In principle, the actual $\varepsilon_{S^{2-} SO_4^{2-}}$ values observed in enrichments of SRB from an aquifer under varying SO_4^{2-}-reduction rates could be compared with the observed $\Delta_{S^{2-} SO_4^{2-}}$ values to model the rate of in situ microbial SO_4^{2-}-reduction rate. Comparisons of the SO_4^{2-}-reduction rates determined by $^{35}SO_4^{2-}$ radiotracer experiments with the rates derived by stable isotope modeling, however, are very limited (89). The published values of $\Delta_{S^{2-} - SO_4^{2-}}$ for aquifers range from −9 to −65‰ and appear to be

an inverse function of temperature (26), similar to that observed for high-temperature equilibrium systems. If SO_4^{2-} reduction is controlled by microbial activity, the inverse relationship between $\delta_{S^{2-}-SO_4^{2-}}$ and temperature observed by Plummer and coworkers (26) and Figure 1 imply that the specific SO_4^{2-}-reduction activity increases with depth or with temperature.

Some $\Delta_{S^{2-}-SO_4^{2-}}$ values, such as those reported for the Floridian aquifer (104), are too large to simply attribute to low SO_4^{2-}-reduction rates based on current laboratory observations. Disproportionation of an intermediate product of sulfide oxidation, thiosulfate, can increase $\Delta_{S^{2-}-SO_4^{2-}}$ values, because the isotopic difference between oxidized and reduced sulfur constituents of thiosulfate are such that the resulting sulfate is enriched with respect to ^{34}S and the sulfide is depleted in ^{34}S (105). Although this mechanism has been proposed to explain the extreme isotopic differences between solid-phase sulfide and sulfate, Wortmann and coworkers (106) have recently reported $\Delta_{S^{2-}-SO_4^{2-}}$ values of up to 70‰ for deep coastal marine sediments. Disproportionation reaction of thiosulfate can be used by facultative anaerobes, for example, *Shewanella putrefaciens* (107) and SRB's (108) facilitating the rapid turnover of thiosulfate in anoxic depositional settings. The spatial juxtaposition of a relatively permeable and microaerobic sandy aquifer and an organic and sulfide-rich clay aquitard could also provide the requisite components for indigenous sulfur cycling by sulfide oxidation using oxygen or Fe(III) as an oxidant, thiosulfate disproportionation, SO_4^{2-} reduction, and the fractionation of sulfur isotopes. If such environments occur in the subsurface, they could provide a potent source of chemical energy for subsurface microbial ecosystems.

Unlike the sulfur system, the $\Delta_{CH_4-CO_2}$ observed in natural environments is frequently consistent with the $\varepsilon_{CH_4-CO_2}$ observed in laboratory cultures. Jenden and Kaplan (109) documented the $\delta^{13}C$ of carbon dioxide and methane in deep-sea sediments from 28 to 336 meters below the sediment-water interface. The $\delta^{13}C$ of the diminishing carbon dioxide conformed to a Rayliegh relationship with a $\varepsilon_{CH_4-CO_2}$ that decreased from −84‰to −63‰ with increasing depth. The methane production rate must be extremely high in these organic-rich sediments for the carbon dioxide-reduction rate to exceed the rate of carbon dioxide production by anaerobic oxidation of organic matter, acetate dissimilation, and thermal decarboxylation. In sandstone aquifers of the Gulf Coast, Carothers and Kharaka (110) reported evidence for aceticlastic methanogenesis at temperatures as high as 80 °C, on the basis of the $\delta^{13}C$ of DIC.

A much lower abundance of organic carbon characterizes the CRB aquifer where Stevens and McKinley (9) reported evidence for in situ microbial carbon dioxide reduction to methane. In this case, the deeper the water, the lower the DIC and the heavier the $\delta^{13}C$ of carbon dioxide. The data followed a Rayleigh distillation relationship in which the $\varepsilon_{CH_4-CO_2}$ was −60‰. Because groundwater ages in the CRB are younger than 35,000 years, the methane-production rate is greater than approximately 10^{-8} moles/L-year. The specific rate derived from $\varepsilon_{CH_4-CO_2}$ and Figure 1 is 5×10^{-15} moles CH_4/cell-hr. These two rate estimates are compatible only if three metabolizing methanogens are present per milliliter of water. The microbial enumeration of the groundwater samples suggested larger population densities, up to 10^4 cells/mL (9). The use of $\varepsilon_{CH_4-CO_2}$ and Zyakun's (94) results as a measure of in situ respiration, if reliable, implies that either the groundwater ages are much younger or that methane cycling is occurring by methanotrophy. Isotopic analyses of both CH_4 and DIC for the CRB aquifer are required in order to verify that the $\varepsilon_{CH_4-CO_2}$ derived from the DIC is representative.

The fixation of carbon dioxide into microbial biomass will increase $\delta^{13}C$ of the residual carbon dioxide. The $\varepsilon_{cell-CO_2}$, however, varies from −3 to −36 (Table 1) making it difficult to incorporate into geochemical models. In the case of carbon dioxide methanogenesis, the increase $\delta^{13}C$ of the residual carbon dioxide is more likely to be caused by catabolic versus anabolic conversion of the carbon dioxide.

MICROBIAL–MINERAL INTERACTIONS

With increasing depth or distance along a flow path, groundwater tends to become supersaturated with respect to carbonates, sulfides, or sulfates, silica, and clay (26,32,102). Although the formation of these phases is thermodynamically favored, abiotic precipitation may be kinetically inhibited. In this case, subsurface microbial participation in these solid/liquid reactions could play a significant role in diagenesis. Microbially mediated changes in the isotopic composition of the DIC and HS^- are usually reflected in the isotopic composition of precipitated carbonates and iron sulfides, but whether the mineral type or form is also specific to biotic precipitation processes, as is the case for aragonite precipitation in marine systems, has not been determined.

In vitro experiments indicate that a variety of extracellular iron sulfides (111) and dolomite (112,113) are formed during microbial SO_4^{2-} reduction. Bacterial Fe(III)-reduction experiments have yielded extracellular formation of siderite (114), magnetite (4,115), vivianite (because of phosphate in the growth media), and green rust (116) (because of chlorine in the growth media). Slobodkin and Wiegel (117) demonstrated that extracellular magnetite and siderite could be produced by Fe(III)-reducing and hydrogen-oxidizing bacteria growing at temperatures as high as 87 °C. Bacteria have also been implicated in the direct or indirect nucleation and precipitation of silica and various carbonate and sulfate minerals in the laboratory (118,119) and in natural surface and aqueous environments (120–124). These studies indicate that the types of minerals, their crystal symmetry, size, and morphologies are a complex function of biotic and abiotic factors including solution and cell surface chemical properties such as pH, Eh, ion composition, temperature, and type of microbial metabolism.

Because many of these experiments are performed under artificial conditions in the laboratory, it is difficult to determine the extent to which microbial–mineral precipitation reactions occur in the subsurface relative to abiotic processes. It is also unclear whether all

microbial biominerals are distinguishable from those produced as a result of abiotic reactions, but recently Thomas-Keprta and coworkers (125) used a combination of morphological, textural, and compositional properties to distinguish magnetite formed by magnetotactic bacteria from other forms of biotic and abiotic magnetite and applied this approach to the analyses of magnetite from Martian meteorites. Experiments using subsurface bacteria, media with compositions similar to groundwater and seed mineral or rock substrates, are becoming more common (126). Dong and coworkers (127) demonstrated that a subsurface bacterial strain, *S. putrefaciens* CN32, could reduce the Fe(III) in fine-grained magnetite. Kostka and coworkers (128) reported bacterial reduction of Fe(III) within smectite and later determined that this process had a dramatic effect on the chemical properties of the clay (129). Both phases are more recalcitrant and representative of deep subsurface environments than amorphous or microcrystalline forms of Fe(III). Detailed investigation of bacterial dissolution and precipitation of minerals under subsurface environmental conditions, however, is still in its infancy.

An alternative approach to examining subsurface bacterial mineralization is to study recently contaminated aquifers where microbial activities are elevated above background levels by the pollutant. In a hydrocarbon-contaminated sandy aquifer, Bennet and coworkers (130) found, by using submersed in situ mineral microcosms, that groundwater-inhabiting bacteria enhanced the weathering of silicates and the precipitation of clay minerals. Because contaminants impose geochemical conditions within aquifers that are distinct from those that prevailed throughout the aquifers' geological history, diagenetic phases formed during the contamination history are often distinguishable from those precipitated over geologic time. Tobin and coworkers (131) used this approach to detect traces of pore-filling ferroan calcite in a TCE-contaminated basaltic aquifer, where a normally aerobic environment had been replaced by anaerobic conditions, resulting in Fe(III) reduction.

Pore or fracture-filling diagenetic minerals, therefore, may provide constraints on the type and magnitude of subsurface microbial activity. Diagenetic carbonate and sulfide assemblages are particularly informative, because the relative contributions of microbial Mn(IV), Fe(III), and SO_4^{2-} reduction and methanogenesis can be delineated by determining the iron and maganese content, the $\delta^{13}C$ of the carbonate, the abundance, and $\delta^{34}S$ of the sulfide, and by calculating the mass balance as constrained by the redox equations (123). High positive values of $\delta^{13}C$ (ca. 10 to 20‰ PDB for the carbonate reflect methanogenesis by carbon dioxide reduction. Inferences with respect to aceticlastic methanogenesis are undermined by the potential variation in the $\varepsilon_{CO_2-C_2H_2O_2}$ with activity (Fig. 1) and the potential variation in the $\delta^{13}C$ of the acetate. Intermediate negative values of $\delta^{13}C$ (ca. −15 to −30‰ PDB) for carbonate reflect the combined effects of fermentation and microbial oxidation of formate or acetate coupled to microbial Mn(IV), Fe(III), and SO_4^{2-} reduction. Finally, very light $\delta^{13}C$ values in carbonate (ca. < -35‰ PDB) are indicative of methanotrophy (8,132).

Measurement of ^{13}C values in carbonate has been successfully used to model the organic oxidation and inorganic mineralization in low-temperature, near-surface marine sediments (133–135), and the formation of carbonate concretions in shallow nonmarine settings (123,136) where microbial processes dominate most redox reactions. Mineralogical evidence of low level, deep subsurface, and microbial activity can be inferred by determining whether diagenetic cement was formed at depth or near a surface or shallow subsurface sediment-water interface in the remote past. Several approaches exist for deriving the age of diagenetic carbonate/sulfide formation, relative to a rock unit's geologic history. The active precipitation of diagenetic carbonate in an aquifer can be verified by comparing the $\delta^{18}O$ and trace metal concentration, $^{87}Sr/^{86}Sr$, of the carbonate to that of the groundwater for the present-day formation temperature (137). The timing of pore-filling carbonate formation can also be related to a basin's burial history by measuring the detrital porosity, because differential burial compaction of partly cemented sediments produces a heterogeneous porosity distribution. If the burial and thermal histories of a rock unit are known, determining the temperature of diagenetic carbonate formation can constrain its age. The $\delta^{18}O$ can be used to surmise the temperature of carbonate precipitation, if the $\delta^{18}O$ of the paleogroundwater can be constrained. Analyses of primary aqueous fluid inclusions in the carbonate not only can establish the formation temperature, but analyses of secondary inclusions can determine whether the temperature of the rock has exceeded approximately 120 °C, which is the generally accepted upper limit for microbial life (138). Finally, in carbonate that contains hydrocarbon inclusions and/or is associated with sulfide, U(IV) can substitute for Ca(II) (139). Consequently, the secondary carbonate formed in anoxic environments is amenable to radiometric dating by either the U-series disequilibrium method, as in the case of the 500 kyr Devil's Hole vein calcite (140), or the U-Pb approach, as in the case of secondary calcite in Paleozoic limestone (141,142).

McMahon and Chapelle (32) related the diagenetic mineral assemblages present in a 50 to 80 m deep confined aquifer to microbial activity. The volumetric abundance, isotopic composition of calcite and pyrite cement, and the distribution of secondary porosity of 70 Ma. marine ACP sediments were used to derive the rates of microbial fermentation and SO_4^{2-} reduction. The sparry calcite tended to have light ^{18}O and ^{13}C signatures, characteristic of precipitation in present-day meteoric water, whereas the micritic calcite preserved a marine origin. When averaged over 70 myr, the volumetric abundances yielded rates for carbon dioxide production and SO_4^{2-} reduction of 10^{-6} and 10^{-8} moles $L^{-1}yr^{-1}$ rates, respectively. These estimates are similar to those derived from present-day pore water fluxes of acetate and SO_4^{2-} from the clays into the sandy aquifer (32). Furthermore, the cement and secondary porosity was concentrated near the interfaces between the clay aquitards and sandy aquifers, consistent with observations that present-day fermentation in the clay layers supplies organic acids to the sandy layers, where they are oxidized by SO_4^{2-}-respiring bacteria.

Because the small pore sizes in the clays restrict access of bacteria to organic matter and because the organic matter that is accessible is consumed and/or becomes more recalcitrant with continued biodegradation, the subsurface microbial respiration rate should decrease with time. Consequently, much of the cement may have formed early in the ACP burial history and respiration rates derived from the diagenetic cements only provide maximum estimates of the present-day subsurface microbial activity.

Evidence of the involvement of subsurface and hyperthermophilic SO_4^{2-}-reducing archaea in the formation of diagenetic carbonate has been reported for a North Sea chalk oil reservoir (143). These investigators reported the presence of biodegraded hydrocarbon inclusions within fracture-filling calcite comprised of light $\delta^{13}C$ values, associated with pyrite depleted in ^{34}S. The aqueous inclusion data and $\delta^{18}O$ of the carbonate indicate that the biodegraded oil was trapped at temperatures between 95 and 130 °C, sometime during the late Miocene to the present. These results suggest that hyperthermophilic SO_4^{2-}-reducing bacteria have been active in the subsurface for an extended period.

Grossman (144) documented sulfur cycling in a shallow subsurface environment using a combination of microbial assays and stable sulfur isotope analyses. In this case, however, the sulfur isotopic signature of the solid-phase sulfide in the aquifer sediments clearly indicated that it had not formed because of the modern sulfur cycling.

Analyses of many carbonate concretions in sandstone, which can attain diameters of several meters, indicate they grew at depths sufficient to prevent the diffusive transport of solute from the sediment-water interface and precipitate from meteoric groundwater (123,145). Textural features preserved in some of these carbonates suggest bacterial involvement in the precipitation process (146), and may reflect subsurface microbial activity.

Reduction halos are centimeter-sized, bleached, carbonate-cemented spheroids, with heavy metal and/or organic-rich cores that typically occur in terrestrial red beds, but are also reported in igneous rocks (22). Hoffman (22) has hypothesized that many reduction halos form as a result of microbial reduction of Fe(III) minerals in the host rock and precipitation of reduced metals in the core of the reduction halo. This supposition is based in part on Th-Pb ages of 100 Ma for reduction halos in Permian red beds, indicating a formation depth of at least 1,000 m (147), fluid inclusion homogenization temperatures of 40 to 70 °C, suggesting formation at depth, and sulfur isotope composition of sulfide in the core that suggest significant fractionation.

CONCLUSION

Microbial populations in the deep subsurface are generally characterized as being present at relatively low-population densities, heterogeneously distributed, and having very low metabolic activities. Their activity is constrained primarily by the availability of energy sources, pore space, and water. They obtain energy for growth and maintenance from oxidation of sediment or rock associated organic matter (heterotrophy), or from reduced inorganic compounds such as H_2, CH_4, or S^{2-} (lithoautotrophy). These metabolic reactions include, for example, oxidation of organic matter to carbon dioxide, reduction of Fe(III), to Fe(II) Mn(IV) oxides to Mn(II), and SO_4^{2-} to S^{2-}. Although their metabolic rates are glacial, they persist over eons and thus impart major changes in the geochemistry of the aqueous and solid phases of the subsurface. These changes are most easily recognized by progressive decreases in the $p\varepsilon$ of groundwater as it moves from the point of recharge to distal points in a confined aquifer. Microbial processes are also evident in groundwater and diagenetic minerals from the isotopic composition of elements such as carbon and sulfur that are fractionated during enzymatic catalysis.

In spite of recent advances in our scientific understanding of the microbiology of the deep terrestrial subsurface and of the biogeochemical processes microorganisms catalyze in this environment, many unanswered questions remain. Although evidence mounts that lithoautotrophic microbial communities may be common in the deep subsurface, we know little about their distribution and ecology or the origin of their primary energy source(s). There has been considerable speculation, but little actual scientific evidence that such microbial communities may support subsurface heterotrophic communities and that the entire ecosystem may make significant contributions to carbonaceous deposits in the earth's crust. Future scientific investigations should focus on addressing these questions, by applying developing technologies for deep drilling and sampling, by using conventional and molecular analyses for characterizing microbial communities and the enzymes they express, and by adapting isotope geochemistry to constrain in situ rates of respiration, growth, and mineralization. Exploration of the present-day deep subsurface ecosystem may provide insights to unraveling the surface biogeochemistry of the Archaen and guide the search for extraterrestrial life in our solar system.

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GEOCHEMICAL ASPECTS OF SUBSURFACE MICROBIOLOGY.

See GEOCHEMICAL AND GEOLOGICAL SIGNIFICANCE OF SUBSURFACE MICROBIOLOGY

GEOSTATISTICS FOR DETERMINING THE DISTRIBUTION OF SOIL MICROORGANISMS.

See SOIL DISTRIBUTION OF MICROORGANISMS

GIARDIA: BASIC BIOLOGY, GENETICS AND EPIDEMIOLOGY

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Giardia duodenalis, also known as *G. lamblia* and *G. intestinalis*, is the most commonly detected flagellated protozoa in the intestinal tract (1). The parasite is regarded as zoonotic by the World Health Organization (WHO), although there is evidence that host range can be restricted (2,3), and there is a lack of evidence for the extent of zoonosis (4) such that animal reservoirs of human outbreaks have not been unequivocally identified, although several likely sources have been reported (5,6). It has been estimated that 280 million people are infected each year with *Giardia*, with the incidence very high in children in developing countries, decreased to 2 to 7% in some industrialized nations (1,7), and varying significantly with locale (8,9). More recently, the WHO has estimated that three billion people live in unsewered environments in developing countries. In these people, the rates of giardiasis approach 30%, suggesting closer to one billion cases of giardiasis at any one time and contributing to 2.5 million deaths annually from diarrheal diseases (10).

HISTORY

Giardia duodenalis was the first protozoan parasite of man to be discovered when in 1681 the Dutch lens maker, Antonie van Leeuwenhoek, observed the parasite in his own stools using a simple self-constructed microscope (11,12). Van Leeuwenhoek's chronic diarrhea, which he described as "a looseness of bowels.... So I went to stool some twice, thrice, or four times a day... especially when I took hot smoke beef that was a bit fat, or bacon..." is an old complaint. Giardia cysts having been found in fossilized human feces several thousands of years old (13). His clear description of what he saw, "...very prettily moving animalcules..." and "...although they made rapid movement with their feet... they made but slow progress" is likely to be the first description of Giardia. In 1859, Vilem Lambl, from whom Giardia obtained one of its names, rediscovered the parasite in children's stools, but it was not until the twentieth century that convincing evidence was presented that the clinical syndrome of persistent diarrhea, colicky abdominal pain, abdominal distension, a feeling of bloating often associated with nausea, vomiting, and loss of appetite were attributed to Giardia parasites (12).

BIOLOGY

Organisms of the genus *Giardia* belong to the class Zoomastigophorea (zooflagellates), order Diplomonadida (cells with bilateral symmetry) and family Hexamitidae (6–8 flagella), and representatives of the genus have been reported to inhabit the intestinal tract of a wide variety of vertebrate hosts. The soft-bodied trophozoite, the feeding stage of the parasite, attaches to the small intestinal epithelium of its host by means of a large ventral disk. It reproduces by binary fission until, in some cases, the gut lining is completely covered with parasites. The trophozoite, 11 to 16 μm in length, is pear shaped, flattened like a leaf, with two nuclei dorsal to the ventral disk, four pairs of flagella, and complex microtubular structures including a claw hammer–shaped median body (Fig. 1) (14). A flange that overhangs the disk is also a distinguishing feature of *G. duodenalis* (15). Most of the information regarding Giardia biology and giardiasis, the disease, is derived from studies of the trophozoite stage.

The parasite has a simple life cycle (Fig. 2). Passage of the trophozoite through the gut induces encystation, whereby the binucleate trophozoite becomes a binucleate cyst and subsequently the nuclei undergo division within the cyst to form a quadrinucleate cyst (14,16). Only the cyst will survive in the environment and stomach acid, allowing it to reinfect another host. In fact it is the stomach acid that initiates excystation and which is completed in the small intestine (16,17). The cyst is responsible for transmission directly via the fecal/oral route or via fecal contamination of food and water. It is this form of the parasite that concerns water treatment authorities.

No sexual stage has yet been observed in the life cycle of *Giardia*. However, other organisms such as the ciliates have very complex mating systems, so presently we cannot fully discount the sexuality of *Giardia*. The observed

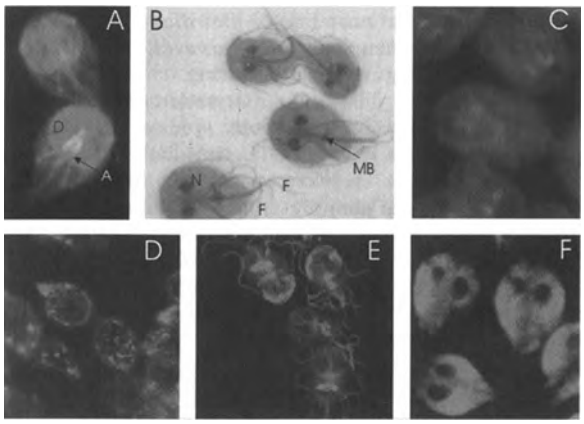

Figure 1. (**a**) Immunofluorescence of Giardia trophozoites reacted with mouse polyclonal sera raised against proteins of around 32 kDa (including giardins) extracted from a polyacrylamide gel. The disk and axonemes are clearly visible. (**b**) Giemsa-stained trophozoites showing nuclei, median body, and flagella, but not the disk. One trophozoite is undergoing division, which is almost complete. (**c**) Trophozoites stained with Hoechst showing nuclear staining. (**d**) Live trophozoites (hence, some are not in the focal plane) exposed to the fluorescent drug quinacrine overnight. The drug is apparently accumulated in dorsal vesicles. (**e**) Trophozoites reacted with a fluorescein-labeled antitubulin antibody. All of the microtubule bearing structures are visible; disk, flagella, and median body. (**f**) IFA of trophozoites reacted with a monoclonal antibody against a Giardia cytosolic protein. Trophozoites are 12–15 μM × 6–8 μM (15).

Figure 2. Life cycle of *Giardia*. Trophozoites colonize the small intestine, particularly the duodenum and jejunum. When trophozoites move through the digestive tract, encystation commences, the nuclei divide, and the protective cyst wall is synthesized. Cysts are passed in the feces and the length of time of viability depends on environmental conditions. A new host is infected via fecal/oral, food-borne, waterborne, or sexual transmission. Ingested cysts excyst under the influence of stomach acid and recolonize the gut.

polyploidy and aneuploidy (18), however, is consistent with the lack of regular meiotic pairing.

Much of the following information described in this article was obtained from studies on *Giardia* parasites grown in vitro (19). The invaluable techniques of excysting cysts in vitro, passaging cysts through a rodent model to amplify trophozoite numbers prior to committing them to continuous culture (20) and the medium with which to sustain trophozoites in culture (19) represent vital contributions to our understanding of this organism.

Speciation

Morphology-Based. Apart from *G. duodenalis*, which infects mammals, several other species belonging to this genus have been described. On the basis of the shape of the median body and body size and form, Filice (21) proposed that the bewildering list of *Giardia* species, generally named after their host species, be reduced to just three, *G. duodenalis*, *G. muris* (confined to rodents), and *G. agilis* (found in reptiles). More recently, two additional species have been proposed — *G. ardeae*, found in blue herons (22) and *G. psittaci*, first reported in budgerigars (23). The distinguishing features of *G. ardeae* are claimed to be a rudimentary caudal flagellum and a distinctly different chromosome profile to the *duodenalis* species (22). An incomplete ventral lateral flange and a rough, deeply pitted dorsal surface appears to be characteristic of the *G. psittaci* trophozoite (23).

Sequence- and Isozyme-Based. Although the above mentioned proposals are morphologically satisfying, molecular biology and biochemical studies point to further divisions in the *duodenalis* group. Analysis of many isolates established in culture in several laboratories around the world have revealed at least two demes, assemblages, or subspecies within the *G. duodenalis* sp. on the basis of differences in the rDNA repeat unit (24–26), differences in isoenzyme electrophoretic mobility patterns (27–28), sequence variation in housekeeping enzymes (29–30), surface antigen variability (31), DNA fingerprinting (24), and electrophoretic karyotype or chromosome profiles (6,24,32–35).

Over many years, many isolates obtained from children admitted to the Royal Children's Hospital in Brisbane with chronic gastrointestinal problems have been analyzed. Forty-one isolates collected over 12 years were divided on the basis of rDNA, electrophoretic karyotypes, and restriction fragment length polymorphism into two distinct demes (33). Since this study was completed, a third group has been defined by an isolate obtained from a calf (34), but more recently, an isolate of *G. duodenalis* was established in culture from a bird which died of an overwhelming infection of the parasite (36). The latter isolate, which chronically infects mice, mimicking the failure-to-thrive syndrome seen in children, is by all criteria a human isolate. This exemplifies the lack of practical significance of current *duodenalis* systematics. There are presently no reliable genetic markers or classification systems to distinguish pathogenic, drug-resistant, or potentially zoonotic strains derived from either clinical or environmental samples, apart from the extensive molecular biology data accumulated to date.

Giardia's Place in the Animal Kingdom

Evolution. *Giardia* shares many characteristics with both eukaryote and anaerobic prokaryote. It is on the basis of primitive characteristics that Sogin and coworkers (37) and Cavalier-Smith (38) proposed that the Metamonada, which includes the diplomonad *Giardia*, are primitive and "that they represent a surviving relic of a very early stage in eukaryotic evolution before the three organelles mitochondria, peroxisomes, and dictyosomes evolved" (39). However, malate dehydrogenase (40), valyl-tRNA (41), cpn60 and triose phosphate isomerase (42), which are classically of eubacterial origin, are present in *Giardia,* suggesting that the *Giardia* ancestor may have carried a symbiont related to those that went on to generate mitochondria. To complicate matters further, horizontal gene transfer is now believed to have had a major impact on species diversity and evolution (43), and consequently it is unlikely to be definitively resolved

whether *Giardia* ever carried, transiently or otherwise, a symbiont. An alternative and more parsimonious interpretation is that similar cell fusions generated the first eukaryotes, and *Giardia* retained predominantly the anaerobic gene repertoire, whereas others pursued the aerobic, mitochondrial evolutionary route (44).

Cell Structure. The highly developed cytoskeleton and membrane-bound nuclei (two) of Giardia trophozoites are clearly eukaryotic. Microtubules are typical features of eukaryotes and *Giardia* has several types of microtubule structures; the ventral disc, the flagella and axonemes, funis, and the median body (15) (Fig. 1). The ventral disc is the most distinctive structure of the trophozoites. It is composed of a single layer of uniformly spaced coiled microtubules adjacent to the ventral plasma membrane (15). Microribbons extend from the microtubules into the cytoplasm and are cross-bridged by electron-dense structures connecting adjacent microribbons (45). Novel *Giardia* proteins, the giardins, a heterogeneous group of some 23 proteins, are major components of the cross-bridges and the microribbons, but their organization in these structures is not well understood (45). Giardins are confined to the ventral disk, and the flagella and axonemes are the typical $9 + 2$ microtubules common to most eukaryotes (15). The median body, which is a separate and compact bundle of microtubules lying across the top of the funis (15,46) is not always present and has no defined function. However, it has been suggested that median body tubules, which are associated with giardins and the median body-specific protein, nucleate giardin core assembly (47). It is not surprising, considering this highly developed cytoskeleton, that the anthelmintic drugs, the benzimidazoles, specifically albendazole, are potent antigiardials. The benzimidazoles are known to interact with tubulin, preventing microtubule polymerisation or dissociating mature microtubules in helminths (48). They are not, however, effective against mammals. Conversely, colchicine, which binds to mammalian tubulin (48), does not affect *Giardia*. Consistent with the helminth data, albendazole appears to act via disruption of the Giardia cytoskeleton (49) and albendazole-resistant parasites induced under laboratory conditions have an altered cytoskeleton with enlarged median body (50). Together with the microtubular structures, *Giardia* has a collection of microtubule-associated proteins including those mentioned above — actin, median body protein, a head-stalk protein with homology to motor proteins, and dynein as would be expected for a highly motile, flagellated eukaryote (18).

Giardia cysts are elliptical and range in size from 6 to 10 μm. The thick cyst wall has an outer filamentous portion, which apparently forms from successive layers of cyst wall materials (51), and an inner membranous portion. It is tightly associated with the surface of the organism. In the periphery of the cyst cytoplasm, an extensive network of vacuoles and tubules exists just beneath the cell membrane (15). The cytoplasm of the cyst contains an apparently disorganized array of all of the highly ordered structures seen in the trophozoite (15). On excystation, the cyst ruptures at the pole opposite the nuclei and the tetranucleate organism within the cyst is liberated apparently with assistance from the flagella. Several minutes after excystation, the organism undergoes cytokinesis (division) and the typical morphology of the trophozoite is evident (15).

Trophozoite Metabolism. *Giardia* is amitochondrial and microaerotolerant (52). Its glycolytic and fermentative metabolism generates ATP by substrate level phosphorylation (53). The key ATP-generating step following the glycolytic pathway, the decarboxylation of the oxoacid, pyruvate, is executed by the anaerobic bacterial homolog pyruvate:ferredoxin oxidoreductase (PFOR) (54,55, Genbank L27221). In aerobic organisms, the mitochondrial multienzyme complex pyruvate dehydrogenase (PDH) performs this function (56). PFOR uses ferredoxin as an electron acceptor, whereas PDH uses NAD^+ and four cofactors. Acetyl CoA, a product of the decarboxylation reaction can in turn be used to generate ATP. But anaerobes, including the microaerophiles and microaerotolerant organisms, have many alternative energy producing pathways including alternative oxoacid oxidoreductase (54,57,58) and amino acid catabolism (59).

Cyst Formation. In suckling mice the jejunum is a major site of encystation and one suggestion is that primary bile salts trigger encystation (60). An alternative proposal relates to the inability of Giardia trophozoites to synthesize cholesterol de novo (61). The absorption of lipids in humans occurs almost completely in the jejunum, so trophozoites travelling through the intestine will encounter a lipid-free environment that initiates encystation (62).

Once initiated, encystation proceeds with the synthesis, processing, and transport of unique cyst wall constituents and their assembly into a protective cyst wall. Genes encoding two of these components, *CWP1* and *CWP2*, predict acidic and leucine-rich proteins, respectively, targeted to the secretory pathway by amino-terminal signal peptides. Both proteins share many features including induction kinetics, sequence similarity, and colocalization in encystation-specific vesicles (62).

Giardia Genome. *Giardia* trophozoites have two apparently identical, transcriptionally active nuclei. Unlike the bacterial nature of its metabolism, Giardia chromosomes are eukaryotic; linear and capped with typical telomere repeats $(TAGGG)_n$ (63). Each nucleus carries several copies of 5 to 8 chromosomes ranging in size from approximately 1 to 7 Mb. There are also a number of variable "minor" or "accessory" chromosomes (in representation), which are partial duplications of most or all of the major chromosomes and are associated with drug resistance (44). The relatively small size and the ease with which the chromosomes, once stripped of their proteins, can enter and migrate through an agarose gel matrix has allowed detailed study of the Giardia genome (44).

Giardia is not clearly diploid or haploid. Apart from its two nuclei and partial duplications, many chromosomes are present in greater copy number than others and chromosome representation varies from isolate to isolate.

Giardia is therefore polyploid and aneuploid but has survived successfully as an asexual organism. It has also been proposed that polyploidy may be the asexual means to replace and repair defective genes and mutations in three-way genetic crosses (44).

GIARDIASIS: THE DISEASE

Epidemiology

Giardia ranks in the list of "top ten" human parasites. It is more prevalent, with more severe symptoms, in children than adults (64). *Giardia* infections are common in institutions, particularly day care centers, where infected children may be asymptomatic. Giardia infection rates typically range from 100% of children infected at some stage during their first three years in rural Guatemala (65), to 2 to 6% of children under 11 in suburban Brisbane, Queensland, who were infected at the time of study (66). A recently reported longitudinal study of well-nourished children, four years and under, in day care centers in Havana indicated that 9% of children appeared to be predisposed to Giardia infections (they were always infected), 51% were never infected, and the remainder were infected once or twice (67). Reinfection after cure is very common, with one study among the Australian Aboriginal population reporting a 37% prevalence of *Giardia* infection before treatment, 12% soon after, and 28% within a month of treatment (68). Giardiasis is the most common cause of chronic diarrhea in travellers (7,69–70), with travel to St. Petersburg notoriously associated with the disease and cyst-contaminated food and water well documented sources (65,71).

Symptoms

The incubation period for giardiasis is one to two weeks duration after ingestion of viable cysts, following which various grades of symptoms including nausea, stomach cramps, diarrhea, and vomiting ensue. The acute stage can last from 3 to 4 days, but can persist for much longer. In children, a failure-to-thrive syndrome may occur (7), and in the developing world giardiasis is an important cause of morbidity. Persistent infection and diarrhea may occur in immunocompromized individuals, for example, IgA deficiency, but is less obvious in cases of acquired immunodeficiency syndrome (HIV/AIDS) (5,7,65). In a study of 200 patients with diarrhoea in a two-week window in a drought rehabilitation camp in Korem, Ethiopia, 98% of apyrexial patients carrying trophozoites of *Giardia* or *Entamoeba* had dysentery (72).

Infestation by the organism causes decreased small intestinal brush border surface area, microvillus and villus atrophy, enterocyte immaturity, disaccharidase and luminal enzyme deficiencies, and malabsorption of electrolytes, a multifactorial pathogenesis which is not well understood (73). A number of other symptoms have been associated with giardiasis (5), but these have not been routinely or consistently found. Similarly, granulomatous hepatitis and cholangitis have been associated with chronic diarrhoea attributed to giardiasis (74). A more recent survey of 25 patients with giardiasis and raised liver enzymes demonstrated return to normal function after antiparasite therapy (75). This may be consistent with poor fatty acid uptake and steatorrhoea in giardiasis (7); previous anecdotal observations may therefore have a stronger foundation, but vary with patient (e.g., immunological status), parasite burden, or strain.

Mechanisms of Pathogenicity

It is not known why symptoms range from a carrier state to failure-to-thrive syndrome although a Giardia gene was recently reported, which has 57% homology with the sarafotoxins, which cause stomach cramps, nausea, diarrhoea, and vomiting (76). It has long been postulated that toxins were the reason for symptoms, but the Giardia toxin homolog was the first solid data. It is only present in some isolates, and subject to the silencing of telomeric position effects, where expression of the toxin-like protein can also be induced to very high levels (77). The gene is a member of a family of genes that vary via a cassette-like mechanism (44,78), and is not present in all isolates (77). Such complex expression patterns of genes encoding diverse toxin and cell-signaling molecules may explain the variability in symptoms.

An alternative mechanism of conferring virulence appears to be via a *Giardia* plasmid. Such a plasmid was harbored by parasites obtained from a child admitted to hospital with hematemesis, and who had lost sufficient blood to warrant a transfusion. Endoscopy, biopsy, and extensive workup failed to find any aetiologic agent other than *Giardia* (18). In microbiological terms, the presence of a plasmid is usually associated with drug resistance or increased virulence, conferring upon the host cell the ability to adapt to a more hostile environment. However, the metabolic burden of carrying such elements is not conducive to their continued maintenance in the entire population unless the prevailing conditions require their presence. In fact, the *Giardia* plasmid decreases in copy number upon serial in vitro culture. This is suggestive that the plasmid conferred an advantage only on the parasite in vivo. The plasmid has since been identified in some other isolates.

A number of reports have documented a range of pathogenicity among Giardia isolates. These included a report of three isolates which differed in their ability to cause functional mucosal damage (79); only one of nine strains that was able to infect adult mice of a particular mouse strain (80), and *G. duodenalis* isolated from a bird that died together with its cage mates from an overwhelming infection of the parasite. This highly pathogenic strain established chronic infections in mice that were associated with weight-gain impairment. Before the description of this strain in 1997 (36), it was believed that avian species of *Giardia* could not be transmitted to mammals. Mice infected with the avian strain carry about a fourfold higher parasite load compared to other strains tested, and this may be responsible in part for the severe symptoms. Other molecules from the avian isolate associated with cytotoxicity are currently under investigation.

Table 1. Antigiardial Drugs: Doses and Side Effects

Drug	Recommended Dose	Side Effects
Metronidazole (Flagyl)	250 mg (adults) or 5 mg/kg (children) thrice daily, 10 d	Nausea, malaise, headache, dizziness
Tinidazole (Fasigyn)	2 g single dose or, 150 mg twice daily, 7 d	Nausea, fatigue, malaise, headache
Furazolidone (Furoxone)	100 mg (adults) four times daily, 10 d; 2–3 mg/kg (children) thrice daily, 7–10 d	Nausea, malaise, headache, hemolysis in G-6-PD[a] deficiency
Quinacrine (Atebrine or Mepacrine)	100 mg thrice daily, 7 d	Nausea, malaise, headache, hemolysis in G-6-PD[a] deficiency, neurotoxicity (including psychosis)
Albendazole (Zantel)	200 mg twice daily, 7 d	
Dual therapy (treatment failures)	500 mg thrice daily, 10 d metronidazole + 400 mg twice daily, 7 d albendazole	

[a] G-6-PD, glucose-6-phosphate dehydrogenase

Persistence of giardiasis in patients with nodular lymphoid hyperplasia almost certainly reflects inability to mount a secretory IgA-response against *G. duodenalis*. Other antibody deficiency syndromes that also predispose individuals to severe and prolonged giardiasis are further evidence for the immune involvement in parasite clearance (81). Under normal circumstances, in immunocompetent individuals, it is likely that IgA-coated trophozoites are unable to adhere to the intestinal epithelium and expulsion of the parasite is mediated by peristalsis (81). Children with a severe T-cell deficiency and $CD4^+$-deficient AIDS patients are not more susceptible to giardiasis than immunocompetent individuals, and thus the role of cell-mediated immunity to *Giardia* in humans is uncertain (82).

Finally, symptoms are also very subjective, and similar symptoms may be disregarded by one community but considered important by another.

Diagnosis

Diagnosis of giardiasis currently depends on microscopic identification of the parasite either as the cyst in stools or as the trophozoite in the small intestine. Trophozoites can be occasionally seen in stools of patients with diarrhea. Because cysts are not continuously excreted, it is recommended that three successive samples are examined (64). Some chronically infected patients who continually test negative for cysts may require duodenal biopsies and juice sample for microscopic and histological examination and for mucosal enzyme assessment (64). Other methods including commercially available enzyme-linked immunosorbent assay kits that, detect among other proteins, CWP1 (83,84), and PCR detection of DNA from Giardia cysts (85) are viable alternatives for diagnosis.

Chemotherapy

Current treatment for giardiasis includes tinidazole, metronidazole, furazolidone, and quinacrine with success rates less than 90%, followed by albendazole (along with paromomycin) with success rates of 10 to 95% (5,86–89) [Table 1 (52,64–65,86–87,93,100)].

Quinacrine was the first effective antigiardial (90) until it was supplanted by the nitroimidazole, metronidazole (91). It is a substituted acridine and was introduced as an antimalarial in the mid-1930s. Quinacrine is not recommended in Australia because of side effects, although it is still used in Europe and has been used effectively in cases of nitroimidazole treatment failure (92).

Metronidazole is regarded as the drug of choice, with the derivative tinidazole being an alternative, except in the United States where it is not available (93). Single high-dose regimens, or three, five, and seven-day regimes with lower doses have variously been recommended (Table 1) with the single, high doses of metronidazole or tinidazole being an advantage for compliance. Clinical resistance prevalence levels as high as 20% have been reported (7,94), with recurrence rates as high as 90% (95). There is a large range in susceptibility of strains to metronidazole, and resistant organisms have been isolated from patients refractory to the drug (7,52,91,96–98). Metronidazole is activated to its toxic nitroso and other radicals in *Giardia* via PFOR/ferredoxin reduction, and consistent with this mechanism of action of metronidazole, both PFOR and ferredoxin levels are downregulated in metronidazole-resistant *Giardia* (52,54,91,99).

The nitrofuran, furazolidone, where available, is recommended for children because of its availability as a suspension (93). Like metronidazole there is a range of susceptibilities of strains to furazolidone, and the development of resistance has been demonstrated in patients (97).

Albendazole was first introduced in 1982 as an anthelmintic. The first published (and successful) report of albendazole against *Giardia* was in 1986 with other benzimadazoles also being tested prior to 1990, and having mixed success (93). The first large-scale human study was in Bangladesh where the average efficacy of albendazole was 62 to 95% compared with 97% for metronidazole (100). Higher levels of efficacy with albendazole require multiple doses. Albendazole is proving very useful as an alternative,

or in combination, particularly in cases of metronidazole treatment failure (Table 1). Consistent with binding of albendazole to tubulin as its mechanism of action, albendazole-resistant *Giardia* have altered cytoskeletons with particularly enlarged median bodies (50,52).

Animal Models of Giardiasis

The ability of axenically cultured *G. duodenalis* to infect and complete its life cycle in suckling mice is well documented (101). The model has been used to assess the pathogenicity of strains by monitoring villous atrophy and associated deficiencies (102–103), drug susceptibilities in vivo (104), and to establish cultures in vitro (24). In most cases the infections spontaneously resolve after about three weeks (101). Gerbil and suckling-rat models are also used for giardiasis, the former has the advantage of using adult animals (105). However, specific pathogen-free (SPF) gerbil colonies are not as readily available as SPF mice, and in Australia gerbils are not available.

CONTROL AND IMPLICATIONS OF ENVIRONMENTAL CYST CONTAMINATION

Giardia duodenalis continues to be the most frequently identified etiologic agent in waterborne diseases outbreaks in the United States (1). Of all 22 outbreaks of waterborne disease, both chemical and microbial in origin in the United States between 1995 and 1996, giardiasis involved the largest number of cases in an outbreak with 1,449 ill people (106). In all other cold climates, where cysts remain viable for many weeks in water at 8 °C (107) (viability decreases rapidly with increased temperature), waterborne outbreaks have been attributed to *Giardia* (65,71). However, in Australia, where only sparsely populated regions are likely to have reservoir and recreational water this cold, despite some anecdotal evidence, no proven case of waterborne outbreaks has been documented. Wherever outbreaks have been investigated, fecal-oral transmission was the most likely route of infection (108). Nevertheless, waterborne contamination with *Giardia* cysts is an issue for all travelers and backpackers in cold and mountain climates worldwide, and drinking raw water should be avoided in these areas.

Water Purification and Detection of Cysts

The most efficient method of disinfecting water contaminated with *Giardia* cysts is by boiling, whereby cysts are immediately killed (107). Steam cleaning of animal enclosures is effective. For large bodies of water, chlorination is of some use, but coagulation and filtration are recommended in addition to disinfection (71,107). A variety of disinfection methods have been described, including ultraviolet radiation and chemical agents such as chlorine, iodine, and ozone (107,109). Optimal filtration of water through diatomaceous earth or sand can effect a 99.9% (3-log) reduction in *Giardia* cyst numbers (110).

The U.S. EPA requires that public water systems associated with surface water be treated to comply with at least a 3-log reduction of cysts or cyst viability (111) and an action level of 3 to 5 *Giardia* cysts per 100 L based on monitoring outbreaks has been proposed (112).

During the 1998 Sydney water contamination events, as many as 4,000 cysts per 100 L were detected in the eastern central business district water supply at one sampling (113). In comparison, a Canadian study reported a maximum of 2,000 in raw water in a longitudinal study of 86 sites in British Columbia (114).

In order to detect cysts in water they must be concentrated. This can be carried out using wound fiberglass depth filter cartridges (Diamond filters) (115), membrane and particulate filter methods, or using yarn wound cartridge filters (116). Detection of cysts after concentration employs essentially the same methods as used for diagnosis using fluorescent anticyst monoclonal antibodies (114), although the whole process may be automated using flow cytometry in water-treatment plants (117).

The severity of an outbreak will depend on a number of factors including viability and numbers of cysts, although as few as 10 can establish an infection (16), and previous exposure and herd immunity of the infected population (118).

Source of Cyst Contamination — Zoonosis

The role of animals in the transmission of giardiasis to humans is unclear, but there is considerable evidence that some *Giardia* found in humans is the same as that found in some animal species (6). In a Canadian waterborne outbreak, electrophoretic karyotyping of Giardia isolates from a giardiasis epidemic correlated with the karyotype obtained from a beaver believed to be responsible (6), and one carefully executed study reported that a human volunteer infected with Giardia cysts of animal origin shed cysts and developed symptoms consistent with giardiasis (119). The establishment of avian *G. duodenalis* in culture and chronic infection of mice with this isolate suggests that birds, previously thought not to be associated with Giardia contamination of watersheds, are indeed a potential source of contamination (3). On the other hand, it appears that *Giardia* from dogs is unlikely to be involved in transmission to humans (120). A further issue to be considered is that cyst-shedding in young animals is significantly greater than in older animals (121), and thus watersheds are likely to be heavily contaminated with animal Giardia cysts during spring (122,123). This may be controlled if young animals are located outside major water-catchment areas.

Whether contaminating cysts are infectious to humans has only been looked at carefully where beavers have been implicated (6). However, it is important to identify the source of a contamination where drinking water supplies are concerned, to stem an outbreak or prevent future similar problems. In some cases, associated coliform contamination data may indicate the likely host source of contamination, thus human fecal contamination may have arisen from sewage contamination, but Giardia cyst contamination has been reported in the absence of bacterial contamination (71). Ideally, stock and humans should not have access to water- catchment areas, but this is not always possible.

Viability of Contaminating Cysts

The contamination of Sydney's water supply in 1998 with Giardia cysts and Cryptosporidium oocysts (113) at levels more than 10 times higher than those of 13 oocysts per 100 L measured during the infamous Milwaukee outbreak (124) was not accompanied by an increase in giardiasis or cryptosporidiosis. In spite of the lack of clinical cases, boiled water alerts were posted for the entire city. This costly exercise may have been entirely avoided if the viability of the organisms had been assessed. Although this may not be an easy task, several stains have been used to measure cyst viability. SYTO-9 and Live/Dead BacLight available from Molecular Probes (Eugene, OR, U.S.A.), for example, are effective in distinguishing dead and viable cysts and staining correlates with animal infectivity (109). Live cysts fluoresce dark green and nonviable cysts light green to orange-yellow when stained with Live/Dead BacLight. Other stains including eosin (107), and propidium iodide (114), together with DAPI (125), have been successful. PCR is claimed to be useful assaying viability (115), and the neonatal mouse model (104), gerbils (126) or severe combined immunodeficient (SCID) mice (114) can be infected with cysts, although the results of viability would not be known for several days following animal infection — until either the animals begin shedding cysts or sufficient trophozoites have colonized the animal's small intestine to be detected after gut dissection.

Well-established methods for excystation in vitro have also been used to assess cyst viability (127). These involve exposure of the cysts to hydrochloric acid, salts, cysteine, and sodium bicarbonate followed by incubation and exposure to excystation medium containing trypsin. Reported excystation rates determined microscopically have been as high as 90%, but depend on the source and age of the cyst preparation (127).

FUTURE CONSIDERATIONS

Human *Giardia* infections are unlikely to be ever eradicated, and thus, chemotherapy and other methods of control of the disease will always be required. The drugs available cannot be replaced in the short term, so every effort should be made to ensure their long-term efficacy. The search for new drugs will inevitably continue, and successful drugs may well be based on inhibition or inactivation of *Giardia*-specific molecules. Such molecules will continue to be exposed by future research efforts such as those involving genome projects (128), but may also involve combinatorial chemistry (129).

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GIARDIA: DETECTION AND OCCURRENCE OF IN THE ENVIRONMENT

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Giardia duodenalis is a protozoan parasite that causes symptoms ranging from mild diarrhea, flatulence, abdominal cramps, and epigastric tenderness to steatorrhea and full-blown maladsorption syndrome in individuals who become infected. Asymptomatic infections also occur. Giardiasis is one of the most commonly reported enteric diseases, and according to some sources *G. duodenalis* is the most frequently isolated intestinal pathogen in humans. Estimates of the annual incidence of giardiasis in the United States range from 260,000 to 2.5 million cases and the overall incidence of *Giardia* carriage may be as high as 2% (1,2). The organism can be transmitted via person-to-person contact and food (3) but the waterborne route is considered by many to be the main source of giardiasis. It has been estimated that up to 60% of all *Giardia* infections are caused by drinking contaminated water (4). However, other authors stated that waterborne transmission is the least common of the regular routes of infection with person-to-person contact accounting for the majority of cases (5). *Giardia* cysts are very common in environmental waters and *G. duodenalis* has historically been the most frequently identified cause of waterborne disease outbreaks (6). The organism is also frequently a cause of diarrheal illness among children in day care centers and backpackers who drink untreated river water.

Table 1. Reported Animal Hosts of *Giardia duodenalis*

Alpaca	Cockatoo	Guinea pig	Pig
Baboon	Cow	Hare	Raccoon
Bear	Coyote	Horse	Rat
Beaver	Dog	Human	Rhesus monkey
Cat	Elk	Moose	Sheep
Chimpanzee	Gerbil	Mule deer	Slow loris
Chinchilla	Goat	Muskrat	Water vole

The highest rate of giardiasis occurs among children below 5 years and the overall incidence peaks during late summer and early autumn (2). *Giardia lamblia* was originally considered to be a human-specific pathogen but it is now recognized by most authorities to be the same species as that found in many other mammals, *G. duodenalis*. The organism has also been called *G. intestinalis* by some authors with claims that this name has a precedence in standard nomenclature (7). Therefore, *G. duodenalis*, *G. lamblia*, and *G. intestinalis* are synonymous. Historically the taxonomy of *Giardia* spp. has been somewhat confused with many host-specific species being named. In a 1952 paper, morphological criteria were used to define three species types: *G. duodenalis* from mammals; *G. muris*, which infects rodents, birds, and reptiles; and *G. agilis*, which infects amphibians (8). A species more recently isolated from the intestine of a gray heron was named *G. ardeae* (9) and *G. psittaci* from budgerigars has also been described. Some isolates from cats, dogs, and rats have been named *G. cati*, *G. canii*, and *G. simoni*, respectively, but the wide host range of *G. duodenalis* (Table 1) and the demonstration of its occurrence in all of these animals raises questions about the use of species designations based on animal hosts. The focus of this review will be *G. duodenalis* since it is the species known to infect humans. However, many studies of *Giardia* in the environment do not use species-specific detection methods, so much of the data on occurrence in natural environments and prevalence in animals is on "*Giardia*" rather than on specifically *G. duodenalis*.

BIOLOGY AND GENETICS

Giardia duodenalis is a single-celled, multinuclear, amitochondrial, anaerobic (but aerotolerant), flagellated protozoan. It has a simple life cycle involving an infective cyst and a vegetative trophozoite (Fig. 1). Following ingestion of a cyst by a suitable host animal, excystation in the small intestine leads to the formation of two trophozoites. The trophozoites replicate by binary fission resulting in giardiasis in the host. Most cases of giardiasis respond well to treatment, with metranidazole (Flagyl) being the most commonly prescribed drug. Albendazole and mebendazole are also effective. Some of the trophozoites encyst, resulting in the formation of new cysts that are excreted in the feces. The mechanism of encystation is not fully understood, but it has been suggested that trophozoites are triggered to encyst by a decrease in host cholesterol concentration as they move down the intestine (10). The cyst is relatively resistant

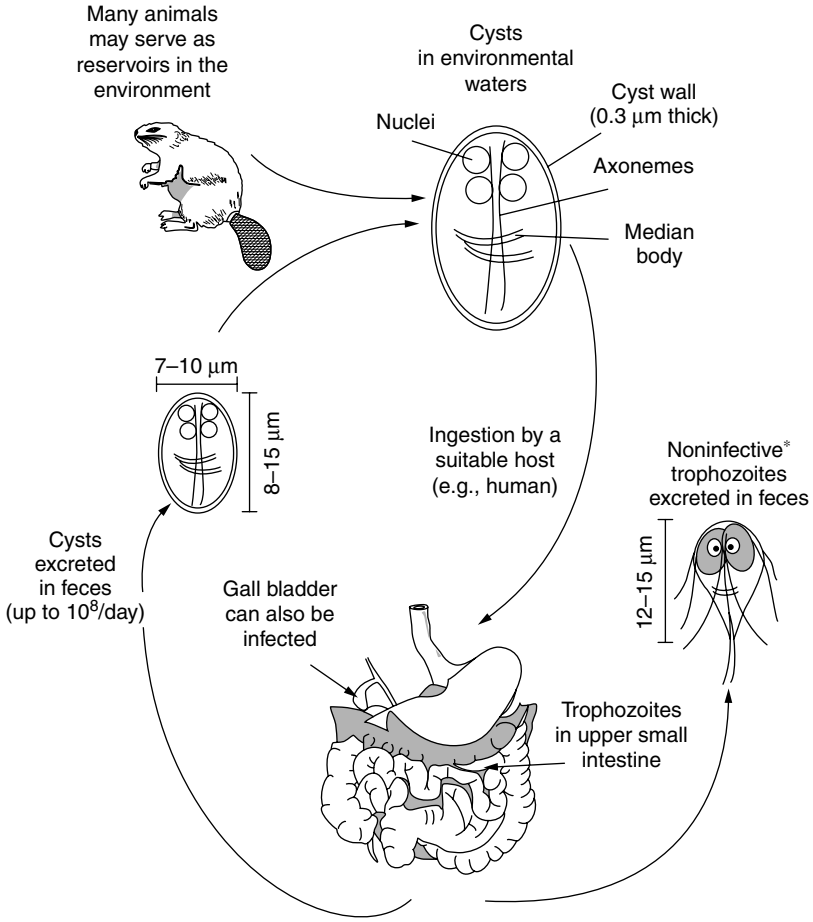

Figure 1. Diagrammatic representation of the life cycle of *G. duodenalis*. Following ingestion of cysts the incubation period is usually one week to the onset of illness. Without treatment, in otherwise healthy individuals, the average duration of illness is approximately six weeks. *, Trophozoites have been shown to initiate infection experimentally (38) but cysts are by far the most common cause of infection. Trophozoites do not survive well outside of the host organism.

to physical and chemical stresses and is the form found in the environment. Cysts are ovoid and measure 8 to 15 μm by 7 to 10 μm. The internal structures of the cyst include 2 to 4 nuclei, 2 to 4 median bodies, and axonemes of the flagella. The newly formed cysts have two nuclei but the older ones that are excreted usually have four. The median bodies are *Giardia*-specific structures that may be functionally related to the ventral disk, which acts as a suction cup in attachment of the trophozoite stage to the intestine (11). These internal structures can be observed by light microscopy using Nomarski differential interference contrast optics but the nuclei are more easily visualized by staining with nonspecific fluorescent compounds such as 4′,6-diamidino-2-phenylindole (DAPI) and epifluorescence microscopy. Other internal structures such as lysosomal vacuoles, ribosomal granules, and fragments of the ventral disk are generally only visible by electron microscopy. A variety of bacterial and virus-like endosymbionts are also present (11). The number of chromosomes and overall genome size is unresolved.

Phylogenetic analysis of the small subunit rRNA gene places *Giardia* as a very early branch of eukaryotes with no close relatives yet identified (Fig. 2a). It is considered to be one of the most primitive extant eukaryotes. The small subunit (SSU) rRNA gene of *G. duodenalis* is only approximately 1,450 bp compared with at least 1,750 bp for most other eukaryotes. It has long been recognized that there is a high level of genetic heterogeneity between isolates of *G. duodenalis* (Fig. 2b). Using both phenotypic and genetic criteria, it has been established that most isolates recovered from humans and many other mammal species can be classified into two primary groups. Just as there is still confusion over naming the *duodenalis* species, there are also naming inconsistencies within the species. The names of the two groups of isolates differ according to continent, but they appear to be synonymous. Group 1/2 isolates (North American name) are generally called the Polish group in Europe and Assemblage A in Australia. They have been detected in humans, livestock, cats, dogs, beavers, guinea pigs, and slow loris (12). The North American Group 3 is referred to as the Belgian group and Assemblage B in Europe and Australia, respectively, and have also been recovered from a wide range of animals including humans, chinchillas, dogs, beavers, and rats. The genetic distance between these two groups is much greater than the distance separating many species of higher organisms. Recent analysis has demonstrated that each of the two groups can be further subdivided. Within Group 1/2, one cluster contains isolates from humans and other animals. Isolates in a second cluster have so far only been recovered from humans (12). In addition, it has been reported that some isolates from dogs, cats, livestock (alpaca, cattle, goats, sheep, pigs),

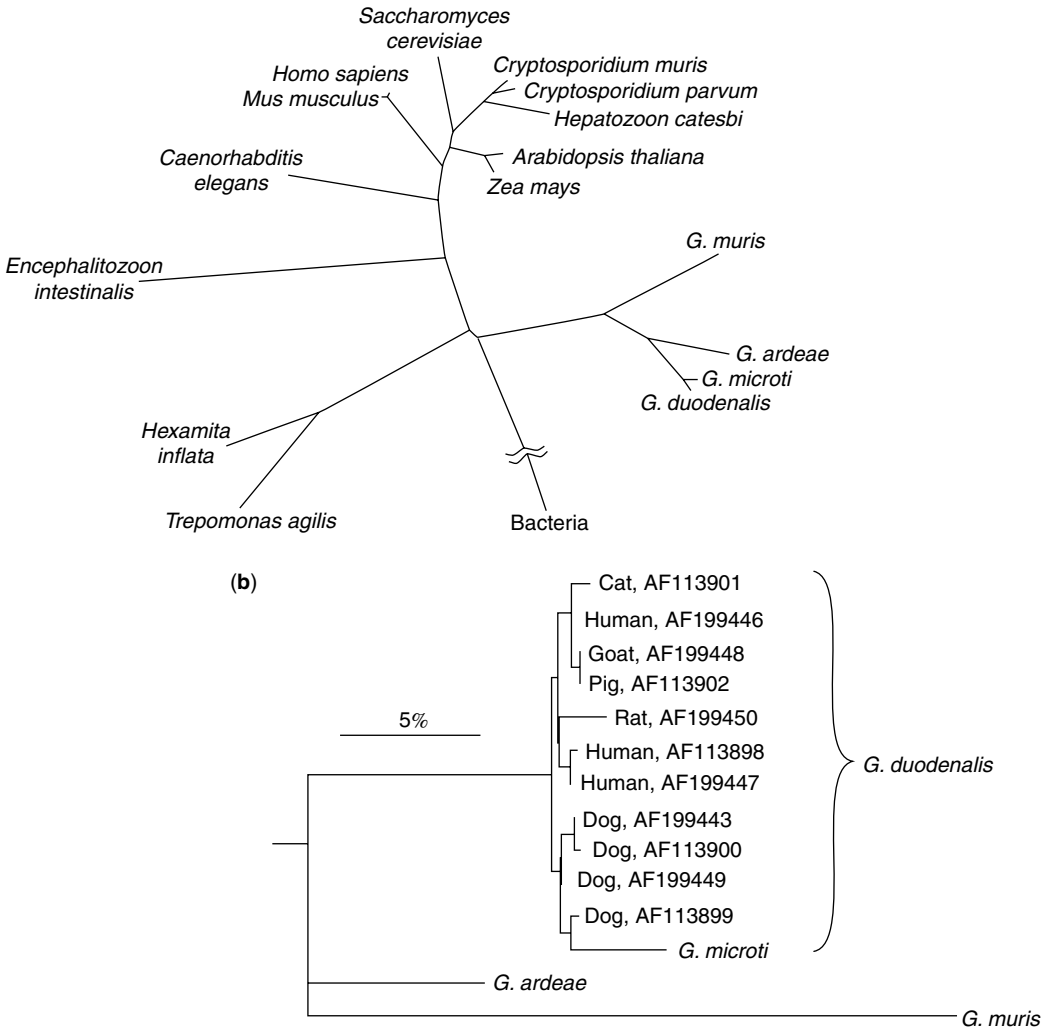

Figure 2. Phylogenetic analysis of a portion of the *G.duodenalis* small subunit rRNA gene in relationship to other major groups of eukaryotic organisms (**a**) and within the species *G. duodenalis* (**b**). The analyses in A and B were based on 780 and 424 aligned bases, respectively, and the dendrograms were constructed using the PHYLIP package of programs (Phylogeny Inference Package, ver. 3.57c). All sequences were obtained from the GenBank; the alphanumeric codes for each organism in B are GenBank accession numbers.

rats, and muskrats form separate lineages distinct from Groups 1/2 and 3. These various lineages have been called Assemblage C (dog isolates), D (dog), E (hoofed livestock), F (cat), and G (rats) (7). As has occurred recently with *Cryptosporidium parvum*, the taxonomy of *Giardia*, and specifically *G. duodenalis*, is undergoing revision. A novel livestock genotype within Group 1/2 was reported to have an exclusive association with cloven-hoofed animals (13). In addition, a group of morphologically distinct isolates from water voles and muskrats identified as *G. microti* (14) falls within the *G. duodenalis* group, as found in an analysis of the SSU rRNA gene. Genetic variation within the *G. duodenalis* group differs depending on the gene used for analysis. In a comparison of four genes, the greatest interisolate variation was displayed by the triose phosphate isomerase gene, whereas a gene encoding an elongation factor demonstrated the least variation (7). The heterogeneity seen within the species *G. duodenalis* suggests that although multiple synonymous names have been used historically, new species names may eventually be assigned to the various distinct clusters. There is currently very little information regarding the relative risk to human health posed by the various genotypes of *G. duodenalis* or whether some types are more dominant in the environment than others. However, it is likely that genotypes with restricted animal hosts will be less common in environmental water samples.

DETECTION METHODS FOR *GIARDIA* CYSTS IN WATER

Although *Giardia* is biologically and genetically very different from *Cryptosporidium* (Fig. 2a), both organisms are pathogenic protozoa and so there is a general tendency, at least within the water industry, to think of the two

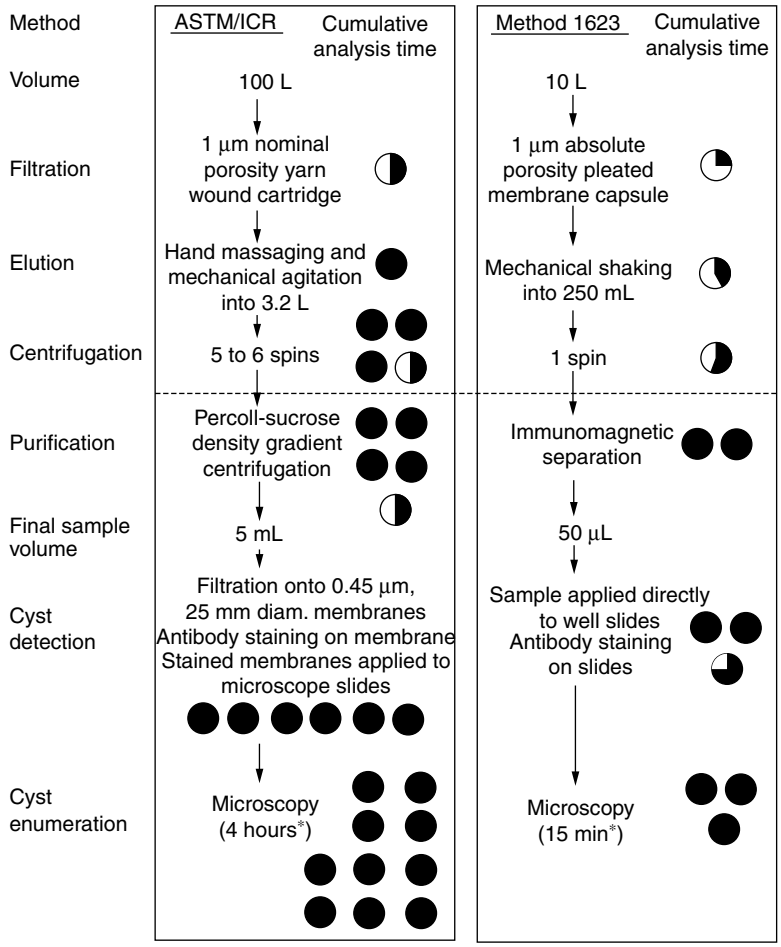

Figure 3. Comparison of ASTM/ICR and Method 1623 procedures for recovery and detection of *Giardia* cysts in water. These methods were developed for the concurrent recovery and detection of *Giardia* cysts and *Cryptosporidium* oocysts. *, Approximate microscopy time to analyze the equivalent of a 10-L sample. DNA-based detection methods can be substituted at any position below the dashed line.

organisms together. Consequently, methods have been developed for detecting both organisms simultaneously in environmental samples and the majority of environmental surveys have assessed the occurrence of both organisms.

Microscopy-Based Methods

The only methods currently approved by the United States Environmental Protection Agency (U.S. EPA) for detecting *Giardia* cysts (and Cryptosporidium oocysts) in water are based on recovery of cysts by filtration, steps to concentrate and purify the cysts, and finally detection by an immunofluorescence microscopy assay (Fig. 3). Until recently, the most widely used method was that described in a proposed standard detection procedure (15) later modified for incorporation into the U.S. EPA Information Collection Rule (ICR; 16). This method requires filtration of large volumes of water (up to 600 L for source water and more than 1,000 L for treated drinking water) through yarn wound cartridge filters. Samples are usually collected on site using a gasoline-powered vacuum pump. Filters are cut open and the fibers teased apart, and cysts are removed from the filter by a combination of manual and mechanical agitation. Cysts are concentrated and purified from the eluate by multiple centrifugation steps including Percoll-sucrose density centrifugation (specific gravity of 1.1). Cysts are stained using an indirect method with a primary unlabeled anti-Giardia antibody followed by a secondary fluorescein-labeled antiprimary antibody. Cysts are detected by fluorescence microscopy (excitation wavelength 450–490 nm, emission 525 nm) based on the color (apple green) and intensity of fluorescence, size, and shape of fluorescent objects. Cysts presumptively identified by immunofluorescence assay (IFA) are supposed to be confirmed using differential interference contrast optics to identify internal structures such as the nuclei, axonemes, and median bodies.

Studies with seeded samples have demonstrated that this method can grossly underestimate the numbers of *Giardia* cysts (and *Cryptosporidium* oocysts) in environmental water samples because of poor recovery efficiencies. Recovery efficiencies can vary greatly between laboratories and between analysts within a laboratory. Both false-positives and false-negatives are quite common. An evaluation of 12 commercial laboratories offering *Giardia* and *Cryptosporidium* detection services reported an average recovery efficiency of only 9% (range = 0 to 140%) and a high rate (36%) of false-negatives (17) although a standardized methodology was not used in the study, possibly leading to poor performance. Average cyst recovery efficiencies of 57%, 63%, and 80% were reported for just the centrifugation and Percoll-sucrose flotation steps for each of three analysts, with varying levels of experience, in the same laboratory (18).

Because of the low and variable recovery efficiencies of the ASTM/ICR method, a number of investigators have evaluated the various steps of the procedure to determine sources of loss and variation and to develop method improvements or alternatives. LeChevallier and coworkers (19) reported losses of 1 to 17% of 7-μm-diameter particles during filtration through a variety of 1-μm nominal porosity yarn-wound filters and although they recommended cotton filters, there were no significant differences between recoveries with cotton, rayon, or nylon filter materials. These authors also recommended increasing centrifugation RCF to 7,000 to 10,000 X g and increasing the specific gravity of Percoll-sucrose gradients to 1.15 to improve recovery efficiencies. Using an optimized procedure, they reported average recovery efficiencies of $50 \pm 14\%$ ($n = 6$) for samples seeded with 96,400 cysts. Nieminski and coworkers (20) reported a 12% recovery of cysts for the ASTM method compared to the 49% for an alternative recovery procedure involving filtration through 293-mm-diameter, 2-μm porosity flat polycarbonate membranes and purification by centrifugation through a two-step Percoll-Percoll gradient (specific gravity 1.09/1.05). However, high cyst losses occurred during the density gradient flotation steps of both methods. Modifications to the cyst elution procedure following filtration (essentially involving more vigorous agitation) and elimination of the density gradient centrifugation step resulted in recovery efficiencies of 60 to 80% for water samples with turbidities of 0.6 to 160 NTU (21). In this latter study, a portable battery-powered filtration device that had the capacity for introducing defined cyst spikes during sample collection for more accurate and representative determination of recovery efficiencies was used.

Typically the volume of the sample concentrate resulting from filtration, elution, and centrifugation of greater then or equal to 100 L of some water can be in excess of 5 mL, depending on sample turbidity. Since the volume of concentrate that can be applied to the 25-mm-diameter membrane prior to antibody staining ranges from 100 μL to 1 mL, depending on sample turbidity, a total of 5 to 50 membrane filters or more would need to be examined microscopically to analyze the entire sample. However, the labor costs involved in such analyses frequently requires that less than the entire sample is analyzed. In a study of backcountry waters in the Adirondack mountains, analyses were frequently limited to less than 3% of the original sample volume (22). This "subsampling" of water concentrates does not present problems so long as cysts are homogeneously distributed throughout the sample concentrate. However, in reality, the coefficients of variation (CV) for subsamples of environmental water concentrates ranged from 23 to 200% (22). Unless the entire original sample was analyzed using multiple slides, the reported cyst concentrations could have varied by as much as nine-fold.

Recognition of the limitations of the ASTM/ICR procedure led to the development of a new method (Method 1623, Fig. 3) for detecting *Giardia* cysts (and *Cryptosporidium* oocysts) in water (23). This method involves filtration of 10-L volumes of water through a 1-μm absolute porosity capsule filter, recovery of cysts by agitation in a laureth-12 based elution buffer, concentration by centrifugation, and purification of cysts by immunomagnetic separation (IMS). The resulting concentrate (50 μL) is relatively free of contaminating organisms and debris and is applied directly to a well-slide for direct fluorescent staining. Interlaboratory evaluations of this method demonstrated average *Giardia* cyst recovery efficiencies of 9.9 to 76.7% with a CV of 8.4 to 69.1% for reagent water samples seeded with 129 cysts (24). Seeded surface water samples resulted in mean recovery efficiencies of 8.9 to 73% and CVs of 7.1 to 130.4%.

Although Method 1623 has been validated using Envirochek filtration capsules (Pall Gelman) and one particular IMS kit (Dynal), alternative filtration, IMS, and fluorescent antibody staining kits can be used provided that equivalency with the approved method can be demonstrated. Alternative filtration procedures may include flat membrane disk filters, vortex flow filtration, and different types of capsule filters. Continuous centrifugation is a potential alternative to filtration. In the United Kingdom, the current routine method for sample collection uses a filter composed of compressed foam disks (Genera Technologies). Cysts (and oocysts) are eluted by repeated decompression/compression of the foam in a detergent-based solution. This method has recently been approved by the U.S.EPA for use with Method 1623. *Giardia* and *Cryptosporidium*-specific IMS kits are available from a few suppliers (Dynal, Clearwater Diagnostics) and "homemade" kits can be put together using commercially available antibodies and paramagnetic beads from various suppliers. Fluorescently labeled antibodies are also available from many suppliers (Meridian Diagnostics, Waterborne, Cel Labs). However, all alternative recovery and detection methods should be developed and evaluated within a framework that allows robust statistical analysis of results and comparison with approved, standardized methods.

Method 1623 only allows analysis of 10 L of water compared with the ASTM/ICR method, which allows 100 L or greater. With volumes above 10 L, cyst recovery efficiencies may decrease due to inefficient removal of cysts from the filter matrix. Therefore, a simple analysis would suggest that routine recovery efficiencies would have to be at least five times higher with Method 1623 compared to the ASTM/ICR procedure for the method to demonstrate equivalent sensitivity. However, a great advantage of Method 1623 is that because of the IMS purification step, the entire sample can be analyzed on a single microscope slide. Subsampling is frequently necessary with ASTM/ICR-generated concentrate, resulting in much less than the original 100 L being analyzed. Also, the rate of sample processing is much higher with Method 1623, allowing for multiple samples to be collected either temporally or spatially, thus increasing the effective volume sampled. In addition, a modification of Method 1623 using alternative capsule filters is currently being evaluated for large volume (>100 L) samples.

The advantage of microscopy-based assays is the ability to easily enumerate cysts. However, the accuracy of enumeration depends not only on the recovery efficiency of the various methods used but also on the quality

of the antibodies used for final detection. Variations in antibody specificity, avidity, and fluorescence intensity can affect enumeration accuracy. False-positives are often due to binding of the antibody or autofluorescence by non-*Giardia* organisms. Some algal cells are approximately the same size as *Giardia* cysts and may be mistakenly enumerated if they have green fluorescence. If cysts are not lost in the concentration and purification procedures, false-negatives may still be recorded if the background fluorescence and debris mask the fluorescing cysts. These problems are far less serious with Method 1623 because the additional antibody-based procedure of IMS removes most (but not all) of the background debris and nonspecific organisms. However, acknowledged problems with the specificity of fluorescent antibodies provided the impetus for the development of molecular-based detection assays.

Molecular Detection Methods

DNA-based methods are often mistakenly referred to as an alternative to the ASTM/ICR procedure for detection of protozoa in water. In fact, DNA-based methods are only an alternative to the final antibody-based detection step (IFA) of these methods. Cysts still have to be recovered and concentrated from water using filtration and centrifugation steps such as those used in the ASTM/ICR and Method 1623 procedures. DNA can be extracted at any of the procedural steps below the dotted line in Figure 3. There are numerous published methods for extracting DNA from environmental samples including water concentrates obtained with the ASTM/ICR procedure. The advantage of molecular detection techniques based on DNA extracted from such concentrates is that once extracted, the "total community" DNA can be used for detection of multiple pathogens, not just a single organism such as *Giardia*. However, the disadvantage is that DNA extracted from crude environmental samples such as water concentrates often contains coextracted materials, which are inhibitory to subsequent molecular detection methods such as the polymerase chain reaction (PCR). Consequently, DNA extraction after cyst purification by density gradient centrifugation or IMS may be preferable. However, such purification steps are semi- to very selective, and so narrow the range of organisms that can be targeted.

The first application of nucleic acid–based technology for the detection of *Giardia* in water was a radioactively labeled 265-bp probe that hybridized to a region of the small subunit rRNA gene (25). The reported sensitivity was 1 to 5 cysts in 1 mL of environmental water concentrate and there was complete agreement between the probe assay and IFA. However, the increased specificity and sensitivity possible with PCR led to the abandonment of unamplified hybridization assays. A variety of PCR primer pairs have been developed for *Giardia*, some of which are genus-specific, whereas others are specific to just *G. duodenalis* (Table 2). While some of these primers were developed for genotyping *Giardia* cysts recovered from environmental and fecal samples, others have been used for direct detection in environmental

Table 2. PCR Primers and Probes Used for Detection of *Giardia*

Primer/Probe	Sequence (5′-3′)	Target Gene	Amplicon Size (bp)	Specificity[a]	Reference
JW1	GCGCACCAGGAATGTCTTGT	Small subunit rRNA	183	GD, GM[b], GA[b]	26
JW2	TCACCTACGGATACCTTGTT				
RDR34*	AGGGACGCGTCCGGCG				
GGL405	CATAACGACGCCATCGCGGCTCTCAGGAA	Giardin	218	GD	27
GGR592	TTTGTGAGCGCTTCTGTCGTGGCAGCGCTAA				
GGP510*	AGCTCAACGAGAAGGTCGCAGAGGGCTT				
GGL639	AAGTGCGTCAACGAGCAGCT	Giardin	171	GD, GM, GA	27
GGR789	TTAGTGCTTTGTGACCATCGA				
GGP751*	TCGAGGACGTCGTCTCGAAGATCCAG				
F	AGGGCTCCGGCATAACTTTCC	Heat shock protein	163	GD, GM	28
R	GTATCTGTGACCCGTCCGAG				
Probe	GTGCAGCACAGAGGCGCTGCTG				
4131	ATGCCTGCTCGTCGCCCCTTC	Triose phosphate isomerase	683	GD	29
4130	CACTGGCCAAGCTTCTCGCAG				
GLONGF	GCTCSTTCAAGTACGCGTGG	Elongation factor 1α	650	GD, GM, GA	7
GLONGR	GCATCTCGACGGATTCSACC				
F (group1/2)	GGTGGATCCTGCCGGAGCG[c]	Upstream of SSU rRNA	296	GD	30
F (group 3)	GGTGGATCCTGCCGGAATC[c]				
R (both groups)	GCTCTCCGGAGTCGAAC				
Group 1/2 probe*	UCGCGCTCCGGCAGGAT[c]				
Group 3 probe*	UCGGATTCCGGCAGGAT[c]				

[a] GD, *Giardia duodenalis*; GM, *Giardia muris*; GA, *Giardia ardeae*.
[b] Amplicons larger than 183 bp which did not hybridize with the RDR34 probe.
[c] Underlined bases denote sequence regions that differentiate between the two phylogenetic groups of *G. duodenalis*.
* Internal probe sequences.

water concentrates. Generally, DNA has been extracted following centrifugation of filtration eluants. Mahbubani and coworkers (27) developed two sets of primers targeting a giardin gene, one genus-specific pair and a second pair amplifying a 218-bp fragment from just *G. duodenalis*. The giardins are *Giardia*-specific proteins located only in the ventral disk and at least 23 distinct giardin proteins have been identified (11). A single cyst (recovered using a micromanipulator) was detected with both primer pairs under ideal conditions. Detection sensitivities in seeded Ohio river water samples were less than 1 cyst/mL for 100-mL samples. PCR did not detect 10^5 cysts seeded into concentrates of 400-L samples. However, a sensitivity of 3 cysts/mL in highly turbid water samples was achieved with the giardin genus-specific primers if cysts were first purified by immunomagnetic separation (31).

Primers targeting a heat shock protein (HSP) gene successfully detected a single cyst seeded into an amplification reaction (28) and a comparison of giardin and HSP primers demonstrated maximum sensitivities of 1 to 10 purified cysts per amplification reaction and 5 to 10 cysts/100 L for cysts seeded into environmental water concentrates of greater than 600-L samples (32). Also, these authors reported the successful detection by PCR of *Giardia* in two unspiked water samples containing less than 1.3 and 7 cysts/100 L (cyst concentrations determined by IFA). Unfortunately, PCR-based detection assays for water samples have not been evaluated in a thorough manner and there has been little effort to standardize methods. Water samples still have to be concentrated before DNA extraction and PCR, and these methods also concentrate inhibitory compounds. In a comparison of six DNA extraction and purification methods, including hexadecyltrimethylammonium bromide (CTAB) precipitation, Chelex-100 and Sephadex resins, and activated charcoal, the authors reported that the inhibitory effects of coextracted humic substances on PCR amplification of *Giardia* DNA in environmental water samples could not be completely eliminated (33). Increasing $MgCl_2$ concentrations in the amplification reaction and diluting the DNA alleviated some of the inhibition but dilution reduces detection sensitivity and so is not really a suitable solution to inhibition.

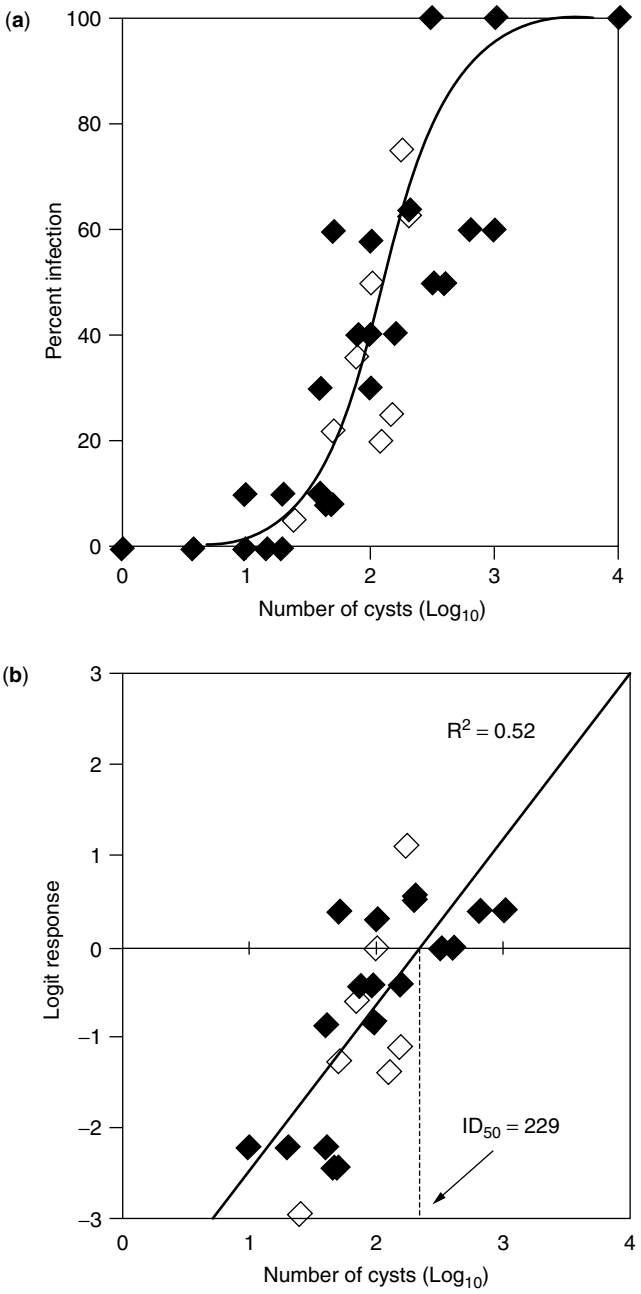

Figure 4. Dose response curve for *G. duodenalis* in artificially infected gerbils. Data was compiled from various publications (30,35–37). Open symbols indicate the values for cysts that were recovered from environmental water samples (30). Data was plotted as the percentage of animals that became infected at each dose of cysts (**a**) and as the logit response (**b**), which linearizes the dose response curve.

METHODS FOR ASSESSING VIABILITY

The most informative method for determining viability of parasites is an in vivo infectivity assay, with the gerbil being the animal of choice for *G. duodenalis*. Such methods address not only viability but also the capacity of the parasite to initiate an infection. Human volunteer studies have been conducted indicating that the minimum infectious dose is 10 cysts or less (34) but such studies are not practical for routine purposes and so they are rarely performed. Some authors have reported establishing infections in gerbils with isolates recovered from water while others have been unsuccessful. A compilation of data from published reports gives an ID_{50} of 229 for various isolates of *G. duodenalis* in gerbils (Fig. 4). The ID_{50} for *G. muris* in CD-1 mice has been reported as 36 cysts (38) and 100% infectivity in gerbils has been reported with only five *G. duodenalis* cysts (5). However, animal infectivity is a highly specialized technique limited to a few laboratories. Consequently, alternative methods of determining viability have been developed. *Giardia duodenalis* infections have been established by inoculating trophozoites onto Caco-2 cell cultures (39) but the methods

have not been applied widely. There have been no reports of initiating infection in cell culture with cysts directly. *Giardia duodenalis* trophozoites can be cultured in axenic media typically containing bovine serum, bile salts, casein digest, yeast extract, ascorbic acid, and cysteine (11). For application to environmental samples, which contain the cyst form of the organism, both cell culture and axenic culture would require in vitro stimulation of excystation as a prerequisite. In vitro excystation methods have been developed (40) and could be used as a measure of viability but they require purified cysts and lack the sensitivity and robustness necessary for use with environmental samples.

All of the viability methods described in the preceding text involve observing the actual biological functioning of *Giardia*. While these methods may give the most accurate assessment of viability, they are not practical for routine purposes. Therefore, viability surrogates that detect indications of viability but not direct biological functions have been developed. A variety of staining techniques have been developed, which allow microscopic determination of cyst viability. A combination of the fluorescent dyes fluorescein diacetate (FDA) and propidium iodide (PI) was originally used, but more recently, FDA has been replaced by DAPI (41). The assay is based on inclusion of DAPI and exclusion of PI by viable cells and staining of dead cells by both compounds. Thiriat and coworkers (42) used the method to demonstrate that up to 21% of cysts in human stool samples were viable but a maximum of 3% and less than 1% were viable in primary sewage sludge and digested sludge, respectively. However, stain-based viability assays can be inconsistent, difficult to interpret, and give variable results in different laboratories. DAPI/PI staining was included in the initial stages of developing Method 1623 but was dropped before publication of the final method. Alternative vital dyes including hexidium, SYTO-9, and SYTO-59 were all reported to provide good discrimination between live and dead *G. muris* cysts (43).

Molecular methods for assessing viability generally rely on the presence of naturally labile RNA in live cells and its absence in dead ones. RT-PCR-based detection of mRNA transcribed from the giardin gene was used to distinguish between viable and dead cysts, but positive amplification depended on the method of inactivation (44). Primers targeting the giardin gene were also used by Kaucner and Stinear (45) to detect viable cysts in river water, treated sewage discharge, and finished drinking water. The RT-PCR method detected viable *Giardia* cysts in 69% of samples ($n = 29$) compared to 24% using IFA with DAPI and PI staining. The reported sensitivity of an RT-PCR assay targeting a *G. duodenalis* heat shock protein gene mRNA transcript was 10 viable cysts in a 100-μL amplification reaction (28) but other authors have reported inconsistent viability determination using these primers (45). Dead cysts were not detected by this assay but its performance with environmental water samples was not evaluated. However, difficulties obtaining reproducible RT-PCR results, the presence of inhibitors in environmental samples, and questions on the stability of some forms of RNA have resulted in this technique being used only in a limited fashion.

PREVALENCE IN ANIMALS

Giardia duodenalis (or *G. duodenalis*-like organisms) have been detected in many animals (Table 1) and domestic or companion animals are often implicated as the source of human *Giardia* infections. The mean prevalence of *Giardia*-positive animal fecal samples (assumed to be dog feces) in seven public parks in Scotland was 11% ($n = 100$; 46). In New Zealand, cysts were detected in the feces of 24% of possums ($n = 76$), 42% of rats ($n = 19$), 31% of mice ($n = 46$), 33% of hedgehogs ($n = 6$), 1 of 3 ferrets, 20% of rabbits ($n = 5$), 35% of blackbirds ($n = 20$), 50% of thrushes ($n = 14$), 15% of sparrows ($n = 104$), and 60% of chaffinches ($n = 10$; 47). The cysts detected in birds may not have been *G. duodenalis* since some birds appear to carry distinct host-adapted species (*G. ardeae* in herons and *G. psittaci* in budgerigars). However, an extremely virulent isolate confirmed as *G. duodenalis* was recently isolated from a sulfur-crested cockatoo (48). Acute giardiasis resulted in the death of the cockatoo and several other caged birds and led to severe infection in experimentally inoculated mice. *Giardia* cysts have also been identified in fecal droppings of migratory geese (49). Consequently, contrary to earlier assumptions, birds need to be considered as potential sources of *Giardia* contamination in water. Atwill and coworkers (50) concluded that given their propensity for activity in riparian areas and the detection of *Giardia* cysts in 7.6% ($n = 221$) of the animals studied, feral pigs should be considered as a potential source of surface water contamination. Farming activities also serve as potential sources of contamination. A broad survey of farm animals across Canada detected cysts in 29% of cattle ($n = 104$), 38% of sheep ($n = 89$), 9% of pigs ($n = 236$), and 20% ($n = 35$) of horses (51). Among dairy calves 2 to 10 weeks of age in Western Canada and Western Australia, 57% ($n = 28$) and 58% ($n = 36$), respectively, were positive for *Giardia* with geometric mean cyst concentrations of 839 to 3,475/g of feces (52).

OCCURRENCE IN THE ENVIRONMENT

Considering the widespread prevalence of *Giardia* in both domesticated and wild animals, it is not surprising that cysts are found to be very common in natural bodies of water. Numerous surveys have reported prevalence figures for *Giardia* cysts ranging from 9 to 100% in surface waters (lakes, rivers, and streams), with concentrations as high as 16,666 per 100 L (Table 3). Surveys by Rose and coworkers (53) and LeChevallier and coworkers (54) demonstrated that higher cyst numbers were associated with source waters that were polluted by industrial or sewage effluents. However, it has also been reported that there was no difference in cyst prevalence between pristine and unprotected source waters (5). Pristine watersheds can of course have large populations of wild animals, which may contribute significant quantities of cysts to the water. A survey of 22 remote pristine raw water samples in Yukon, Canada, reported 32% positive for *Giardia* (65). Six percent of groundwater samples were

Table 3. Surveys of *Giardia* Occurrence in Environmental Waters

Location	Sample Type	Prevalence (%)	n^a	Cyst Conc.[b]	Reference
Southern California					
	Lake Castaic watershed	16	50	3 to 16,666	55
	Silverwood Lake watershed	55	116	2 to 5,953	
Wisconsin					
	Statewide rivers	31	210	2,610	56
Canada					
	Source waters	21	1,173	230	30
Pennsylvania					
	Allegheny River	63	24	421	57
	Youghiogheny River	54	24	526	
	Stream through dairy farm	55	22	13 to 1,527	
British Columbia					
	Source waters	98.5	140	7 to 2,215	58
New Zealand					
	Countrywide surface waters	22.5	112	375	59
British Columbia					
	Province-wide raw water	68	153	0.02 to 16.3	60
	Single community supply	100	70	0.3 to 100	
Pennsylvania					
	Urban stream in dry weather	92	12	13 to 6,579	61
U.S. multistate survey					
	Surface waters (combined)	16	257	<1 to 625	53
	Pristine rivers and lakes	9	93	12	
	Polluted rivers and lakes	29	62	625	
U.S.					
	Groundwaters	6	199	0.1 to 120	62
U.S. and Canada multistate survey					
	Surface waters	81	85	4 to 6,600	54
U.S. and Canada multistate survey					
	Surface waters	45	118	2 to 4,380	63
Pennsylvania					
	Delaware and Schuylkill rivers	34.5	27	58	64
Australia					
	River water	84[c]	19	–[d]	45

[a] Number.
[b] Cyst concentrations are given either as the maximum or as the range detected per 100 L.
[c] Based on detection by RT-PCR.
[d] Information not available in report.

also positive, with up to 120 cysts/100 L (62). *Giardia* cysts also appear to be very common in treated drinking water, with 4.6 to 77% of samples testing positive (Table 4). Cyst concentrations in these samples ranged from 7.7 to 107 per 100 L, with an average maximum of 33 cysts/100 L for the surveys included in Table 4. Most of the occurrence data in these surveys was based on the ASTM/ICR methodology described earlier, so the actual incidence and concentrations of cysts in the environment are probably higher than has been reported. It is likely that most of the cysts detected in the surveys of treated drinking water were either dead or damaged due to a combination of environmental stresses and treatment processes. However, cysts can survive for prolonged periods in surface water, depending on the temperature of the water and other conditions. Outbreaks do occur, indicating that some cysts retain their infectivity. For example, cysts recovered from 2.2% ($n = 223$) of untreated source waters established infections in gerbils (30). These samples were collected during an outbreak of giardiasis, with an attack rate of 30% in a population of 1,000. Wallis and coworkers (30) also reported that 7.6% of treated drinking water samples ($n = 79$) resulted in positive infection. Most of these samples were collected from a single treated water supply but no outbreak of giardiasis was reported in the community served by the supply. In a second study, covering much of British Columbia, 45% of cyst-positive raw water samples ($n = 91$) inoculated into gerbils were infectious (60). This study also detected infectious cysts in 10% of treated drinking water samples ($n = 42$). On the basis of a two-year cyst survey of two rural communities drinking chlorinated surface water and an IgM seropositivity of 7%, the authors suggested that there was a low level of endemic waterborne transmission in the communities. Using microscopically observed morphological criteria as a measure of viability, it was reported that 12.8 to 14.6% of cysts detected in various surface waters were viable (54,63) but most cysts detected in treated drinking water were dead (67).

Table 4. Surveys of *Giardia* Cysts in Drinking Water

Location	Prevalence (%)	n^a	Cyst Conc.[b]	Reference
New Jersey[c]	14	120	0.7 to 107	66
U.S. and Canada	4.6	262	0.98 to 9	63
U.S. and Canada	17	83	0.3 to 64	67
British Columbia[d]	59	91	0.04 to 7.8	60
British Columbia[e]	77	77	0.2 to 7.7	60
U.S. Virgin Islands[f]	26	45	<1 to 3.8	68
Canada	18	423	–[g]	30
U.S. multistate	11	2,372	–[g]	5

[a]Number.
[b]Cyst concentrations are given as the range detected per 100 L.
[c]Open finished water reservoirs.
[d]Province-wide survey.
[e]A single community water supply.
[f]Water collected in cisterns.
[g]Information not available in original report.

It is generally accepted that cysts enter surface waters on a periodic basis, but there is little information on the travel characteristics or distribution of cysts once they are in the water. Weekly sampling of a river at three times on each sampling day for a period of two months demonstrated that cysts were homogeneously distributed with an approximate concentration of 700 to 1,000 cysts per 100 L (18).

Cyst concentrations in surface waters increase during wet weather, particularly in areas with dry summers where the first rains of the wet season wash many months of accumulated animal feces into watersheds. During watershed sampling in Southern California, 45% of wet season samples ($n = 31$) were *Giardia*-positive compared with 15% ($n = 72$) during the dry season. The maximum cyst concentration of 16,666/100 L was obtained with a first-flush sample immediately following a rainstorm (55). Similarly, an extensive survey of surface waters in Wisconsin detected cysts in 47.5% of winter samples compared with 25 to 28.8% in other seasons (56). However, other authors have reported no seasonal variation in cyst occurrence or concentration (60).

Specific PCR primers are not yet available for all of the distinct genotypes of *G. duodenalis*, so it is not known whether isolates in water represent all genetic lineages. However, both the main genetic lineages of *G. duodenalis* (Groups 1/2 and 3) have been recovered from water, with no apparent difference in geographical distribution (30).

OUTBREAKS OF GIARDIASIS

The first outbreak of giardiasis in the United States, which was indisputably linked to drinking water by detection of infectious *Giardia* cysts in the water supply during the outbreak, occurred in New York state in 1974–1975, with 350 confirmed cases (69). Since this time there have been many waterborne outbreaks in many states of the United States and in many countries around the world. In many of these outbreaks involving small community systems, the number of infected individuals was low. However, in some incidents, more than 700 people have become sick. From 1971 to 1985, 92 waterborne disease outbreaks were attributed to *Giardia*, with a total of approximately 24,000 infected individuals, and between 1976 and 1994, *Giardia* was the most frequently identified etiologic agent in outbreaks caused by drinking water (70). During 1993 and 1994, *G. duodenalis* was the causative agent in 9 out of the 39 (23%) waterborne disease outbreaks in the United States, where the etiological agent was identified (70). These outbreaks were associated with both surface water ($n = 3$) and groundwater ($n = 2$) intended for drinking and two of these were linked to unfiltered, chlorinated drinking water. Four outbreaks were associated with recreational activity (swimming in pools, rivers, and lakes). One of the largest waterborne outbreaks of giardiasis with 1,400 confirmed cases (and an estimate of at least 3,000 cases) was caused by an overflow of sewage into the drinking water system at a Swedish ski resort (71). There were four waterborne outbreaks in the United States, totaling 159 confirmed cases during 1997–1998, all associated with drinking unfiltered surface water or untreated groundwater (72). During one of these outbreaks with 50 confirmed cases, tap water samples were found to contain up to 200 cysts/100 L.

Considering the widespread occurrence of *Giardia* in animals, tracing the source of contamination in outbreak situations is difficult. However, beavers have frequently been referenced as a potential source. Karyotype analysis by pulse-field gel electrophoresis demonstrated that an infected beaver found at a drinking water intake had an identical profile to human isolates recovered during an outbreak of giardiasis in Creston, British Columbia, thus implicating the beaver as the original source of contamination (73). In another study of isolates recovered during an outbreak of giardiasis, 20 *Giardia*-positive water samples were collected (cyst status determined by IFA) but DNA extracted from the samples was not successfully amplified by primers targeting the triose phosphate isomerase genes (74). However, restriction enzyme digest patterns following PCR were identical to DNA extracted from beaver fecal samples and human isolates collected during the outbreak, again implicating the beaver. In some areas of Colorado, cysts were detected in up to 45% of beavers and 100% of muskrats, with some animals shedding 1×10^8 cysts per day (5).

Cyst Removal During Water and Wastewater Treatment

Giardia cysts can survive in river water for prolonged periods, depending on water temperature. Their sedimentation velocities are low (1.4 μm/sec) so they will remain suspended in the water column unless attached to larger particles (75). They are relatively resistant to disinfection so correctly operating filtration systems are essential for removal of cysts from drinking water. The majority of waterborne outbreaks have been attributed to drinking unfiltered water or failures of filtration during treatment of surface waters. The predicted chlorine CT (contact time × concentration) value for 99.99% inactivation of cysts is approximately 245 for temperatures of 0.5

Table 5. Distribution of *Giardia* Cysts During Sewage Treatment

Sample Type	Prevalence (%)	n^a	Cyst Conc.[b]	Reference
Raw sewage	73	164	88,000	30
	100	43	26 to 3,022	65
	100	42	1,100 to 52,500[c]	78
	100	121	21 to 9,760[d]	78
	100	–	25,000	79
	100	29	130 to 7,900	80
Combined sewer	100	5	37.5 to 1,140	57
overflows	100	11	90 to 2,830	61
Effluent from sewage	83	24	1 to 461	57
treatment plant	96	28	5 to 940	78
	86[e]	35	2 to 310	80
	82[f]	33	4 to 130	80
	100	5	2 to 3,511	65
	92.5[g]	40	0 to 3	79
	67[h]	6	–	45

[a] Number.
[b] Cyst concentrations are given as either the maximum or the range detected per liter.
[c] Samples concentrated by centrifugation with no purification.
[d] Concentrated samples purified by sucrose density gradient centrifugation.
[e] Secondary effluent.
[f] Final effluent from a water reclamation treatment plant.
[g] Final effluent.
[h] Cysts detected by RT-PCR.

to 5 °C (5), and an ozone CT of 4.2 was required for 99.99% inactivation of *G. duodenalis* cysts (35).

Although apparently ubiquitous in surface waters, a large proportion of *Giardia* cysts are effectively removed by proper operating of drinking water treatment plants. Studies of a full-scale treatment plant demonstrated an average of 99.88% removal of cysts (76). In addition, the configuration of treatment plant filters (sand, anthracite/sand, deep anthracite/sand) did not affect cyst removal efficiencies, with 99.5 to >99.99% removal occurring regardless of filtration media (77). States and coworkers (57) reported that 63% of raw surface waters were cyst-positive, with an average concentration of 34 cysts/100 L, but no positive treatment plant effluents were detected. Nevertheless, *Giardia* is frequently detected in treated drinking water (Table 4).

The data in Table 5 demonstrate that *Giardia* cysts are detected in an average of 95.5% of raw sewage samples, 88% of samples following secondary sewage treatment, and far less frequently in final effluents. Inoculation of 22% of samples recovered from raw sewage ($n = 135$) resulted in infection in gerbils (30). In general, sewage treatment reduces the concentration of cysts by 90 to 99.99%. In one study it was demonstrated that the majority of wastewater treatment plant effluents contained cysts but cyst concentrations were reduced by at least 99.8% (79). With this removal efficiency, effluents contained an estimated average of 60 cysts/L but 97% of these cysts were determined to be dead based on morphology and staining with propidium iodide. Thiriat and coworkers (42) used vital dyes to demonstrate that only 3% of cysts in primary sewage sludge and less than 1% of cysts in digested sludge were viable. However, because cyst concentrations can be very high in raw sewage, treated effluents may still contribute significant cyst loads to receiving waters. On the basis of the reported occurrence and removal data, a 100 million gallons per day sewage treatment plant may be introducing 5×10^8 potentially viable cysts per day into receiving waters.

CONCLUSION

Giardia duodenalis is ubiquitous in aquatic environments and has a high prevalence in a broad diversity of animals. It is globally distributed and is the most frequently isolated intestinal pathogen in humans. The organism has multiple potential routes of transmission including consumption of water, recreational use of water, food, and contact with animals, but the true significance of zoonotic transmission has not yet been determined. Although giardiasis is not considered a fatal disease and it is not as devastating on a worldwide scale as diseases such as malaria, there are very large health and economic burdens resulting from widespread infection by the parasite. Consequently, giardiasis continues to represent a significant threat to public health. Continued efforts to improve recovery and detection methods for environmental samples and further molecular characterization of isolates will help elucidate the epidemiology of the disease and may lead to the development of tools that can be used to definitively identify the sources of contamination in environmental waters.

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GRANULAR ACTIVATED CARBON, BACTERIOLOGY OF

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The goal of drinking water treatment has always been to provide microbiologically safe water to the consumer. To accomplish this, especially when surface water is being treated, gravity filters containing sand and/or anthracite are typically used to remove particulate matter. When filtration is followed by disinfection, pathogens should be removed and the microbial load of the water reduced to an acceptable level. As water quality regulations have become more restrictive, granular activated carbon (GAC) has been used to a greater extent as a filter medium because of its ability to adsorb regulated synthetic organic carbon compounds, materials that contribute to taste and odor in finished water, and organic matter that leads to the formation of disinfection by-products. GAC is known to become colonized with bacteria, and this trend has been exploited in treatment by using the organisms on biologically active carbon (BAC) to metabolize a fraction of the organic carbon found in the water. However, there has been concern that indicator organisms and bacteria of public health interests may colonize and proliferate on GAC and BAC filters. For these bacteria to be found in finished water they must successfully colonize and compete with the indigenous organisms (or be retained in large numbers), be released from the filter and penetrate the disinfection barrier. This review describes these issues and the beneficial aspects of BAC filtration in water treatment.

PROPERTIES OF GAC

Granular activated carbon (GAC) has the beneficial property of adsorbing compounds from water. Consequently, its use in drinking water treatment has increased substantially over the past few decades due in large measure to increasingly stringent regulations on finished water quality. Of rising importance are regulations related to the requirement to remove organic compounds involved in the formation of disinfection by-products. GAC can also adsorb gasoline constituents, pesticides, and other regulated synthetic organic compounds. The aesthetic properties of potable water can also be improved because GAC can remove a variety of organic compounds that cause tastes, odors, and color.

The adsorptive properties of GAC are related in part to its surface area. GAC particles are characterized by very high surface area ranging from a few hundred to 1,500 m^2/g, most of which is provided by their porous nature (1). Pore size distributions are in the range of $1–10^4$ Å, with most pores being smaller than the average bacterium ($1–2$ μm or $1–2 \times 10^4$ Å in length). Although the majority of the pores is small, the porosity of GAC appears to be an important factor that enhances the ability for bacteria to colonize GAC particles in water treatment systems.

GAC can also react with oxidizing disinfectants such as free chlorine (1,2), combined chlorine (3) and chlorine dioxide (4). This property may also be important in providing a hospitable environment for bacteria attached to GAC in filters subjected to chlorinated backwash water.

COLONIZATION OF GAC

Virtually any surface immersed in water will eventually become colonized with microorganisms, and granular activated carbon is no exception. Indirect evidence for colonization was noted by treatment system operators who observed that microbial counts emanating from GAC filters frequently exceeded those found in the influent. This was also observed in early pilot scale work with a GAC filter, and the increase in counts was attributed to the growth and subsequent release of the organisms from biofilms on the filter media into the effluent water (6). This phenomenon has led to the belief in some industries that GAC columns act as "bug farms" and are therefore undesirable in the treatment train (7). Biological activity on activated carbon in the drinking water industry was initially investigated in the 1970s (8–10), with a subsequent flurry of research on the properties of GAC that make it conducive to bacterial colonization.

One such property is the strong affinity between GAC and bacterial cells. In experiments where the initial attachment of *Escherichia coli* to fresh powdered activated carbon was followed, nearly 100% of the initially suspended bacteria attached within 30 minutes (11). In similar experiments, approximately 90% attachment was seen for *Klebsiella oxytoca* (12) and *E. coli*, (13) on fresh GAC. Rollinger and Dott (14) demonstrated nearly 100% attachment of *E. coli*, *Klebsiella pneumoniae*, *Streptococcus faecalis*, and *Pseudomonas aeruginosa* within six hours, whereas *Pseudomonas putida* required four days.

Another property that may enhance bacterial colonization is the adsorption of organics in the pores of the carbon. These organics may then act as a growth substrate for the attached bacteria. Davies and McFeters (12) reported that the growth rate of attached *Klebsiella oxytoca* was 10 times higher than suspended cells in a low nutrient medium containing glutamate as the carbon source. The GAC had been exposed to the glutamate before colonization by the bacteria. The attached organisms were also more active, as determined by tritiated thymidine and uridine uptake. Compared with the suspended cells, the attached bacteria did not exhibit a reduction in cell size, that is typical for organisms entering into a starvation

state. Others have noted that organic carbon adsorbed on GAC can desorb, be transported out of the micropores, and then be degraded by bacteria attached to GAC (15,16). This mechanism may enhance the attachment of bacteria and subsequent growth of biofilms on carbon particles.

Regardless of the mechanisms, it is common knowledge that bacteria populate GAC in water-treatment systems. The actual numbers will vary depending on location, time of year, and the enumeration technique used. At one location, Parsons (17) found values ranging from 1.5×10^4 to 1.8×10^8 colony-forming units (CFU) per gram wet weight. The high end of this range was comparable to the average value of 3.6×10^8 CFU/g wet weight carbon at a New Jersey pilot plant (18). In another pilot system, culturable cell counts varied from 3×10^5 to 6×10^7 per gram (dry weight) of carbon (6). These same authors used ATP to estimate cell concentrations, and obtained levels of 5 to 490 ng ATP per gram dry weight of GAC. Using C^{14} glucose uptake to assess biomass concentrations on carbon, Prevost et al. (19) reported a range of approximately 15 μg C/cm^3 to 7.5 μg C/cm^3, depending on the location within the filter. If the biomass is assessed using a phospholipid assay, concentrations varied in an experimental carbon filter system from 500 to 20 nmols lipid-P/g (20).

Scanning electron microscopy images of GAC particles from drinking water filters have shown that the surfaces are colonized by a diverse suite of morphological types, including filaments, rods, cocci, fungi, and protozoans (21–23). A recently taken image of a particle from a laboratory biologically active carbon column is shown in Figure 1. These organisms are often associated with the macropores and cracks on the surface of the carbon (13) where they are most likely protected from the mechanical effects of shear and scraping from backwashing of the filters. Images of these particles also indicated that the organisms had produced extracellular polymeric substances (EPS, see Fig. 2), suggesting that the organisms were actively attaching and growing on the surfaces.

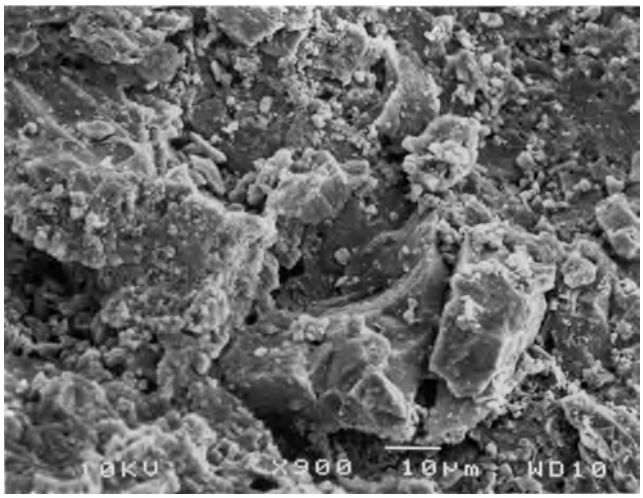

Figure 1. Scanning electron micrograph of microorganisms and detritus associated with the surface of granular activated carbon.

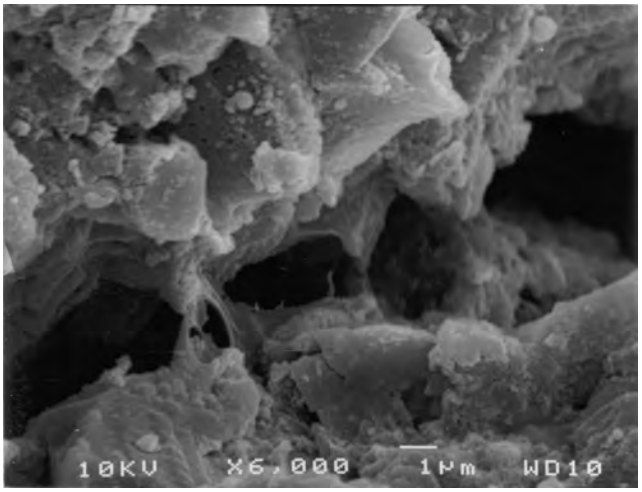

Figure 2. Scanning electron micrograph illustrating the presence of extracellular polymeric substances (EPS) in the crack of a granular activated carbon particle.

The bacteria colonizing and proliferating on the surface of GAC are likely to be nonpathogenic heterotrophs and autotrophs because the carbon and energy sources present in water support the growth of these types of organisms. Bacteria typically identified in GAC filters and their effluents are common soil and water organisms. Using culturing techniques and metabolic characteristics for classification, researchers have identified *Achromobacter*, *Arthrobacter*, *Alcaligenes*, *Acinetobacter*, *Bacillus*, *Flavobacterium*, *Micrococcus*, *Corynebacterium*, *Pseudomonas*, and *Moraxella* (14,17,22,24). At one plant, members of the genera *Pseudomonas*, *Acinetobacter*, *Bacillus*, and *Corynebacterium* were found on the filter media, with effluent heterotrophic plate counts ranging from 0 to 600 CFU/ml (25). Wilcox and associates (6) detected *Achromobacter*, *Acinetobacter*, *Aeromonas*, *Alcaligenes*, *Bacillus*, *Chromobacterium*, *Corynebacterium*, *Micrococcus*, *Microcyclus*, *Paracoccus*, and *Pseudomonas* in water and on GAC particles from a pilot facility. At other locations, *Acinetobacter*, *Alcaligenes*, *Bacillus*, *Pseudomonas*, *Micrococcus*, *Flavobacterium*, *Aeromonas*, *Achromobacter*, *Chromobacterium*, *Corynebacterium*, *Arthrobacter*, *Caulobacter*, *Nitrosomonas*, *Nitrobacter*, and actinomycetes were isolated from GAC filter media (26,27). Using fatty acid analysis for bacterial identification, Norton and LeChevallier (28) found 19 gram-negative and 5 gram-positive genera in biofilms on GAC. Gram-negative bacteria predominated, with the highest number of isolates belonging to the genera *Acidovorax*, *Hydrogenophaga*, *Pseudomonas*, *Xanthomonas*, and *Sphingomonas*. The majority of these isolates matched the organisms identified in the filter effluent.

It is also possible for coliforms to grow on GAC filter media. The proliferation and subsequent release of these organisms may cause a violation of the total coliform rule (TCR), even though there is no immediate fecal contamination. The TCR uses the presence of coliforms to indicate the potential presence of enteric bacterial pathogens in drinking water. Requirements state that

fewer than 5% of water samples collected on a monthly basis from the distribution system can be positive for total coliforms. The potential for positive tests resulting from detached biofilm coliforms is obviously of great concern to utility operators. There are several cases where coliforms (*Escherichia*, *Enterobacter*, *Klebsiella*, *Citrobacter*, *Serratia*, *Hafnia*, *Aeromonas*) have been found in filter effluent when they were absent in the influent water (6,24,29,30,31), suggesting that the organisms were accumulating and replicating in the filter. This is supported by the observation that coliforms such as *Citrobacter freundii*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, and *Escherichia coli* have been detected in filter media samples (13,32). The growth of coliforms in filter media may also contribute to the observations of Bucklin and coworkers (33) who reported elevated coliform levels in filter effluent the first few hours after backwash. It is important to note that coliforms are not typically isolated from GAC filters, even if detailed analyses are performed (14,22,28), indicating that colonization may be a site-specific issue.

BIOLOGICAL FILTRATION

Biological filtration uses filter media, usually GAC, which has been optimized for the growth of indigenous heterotrophic bacteria that degrade the available fraction of organic carbon in the water. The filter media used in the process is termed biologically active carbon (BAC). Benefits of biological filtration include improved total organic carbon (TOC) removal and reduced disinfectant demand (35–38), improved aesthetic properties (38), and the potential for reducing microbial growth in distribution systems (39).

The process was first implemented in western European countries nearly 20 years ago (10,40,41). In the most traditional form, conventional treatment including filtration to remove particles takes place upstream from the biological filters, and ozonation is often included in the treatment train. The downstream biological filters are operated with exhausted carbon, that is, the chemisorptive capacity of the GAC has been exceeded. The surfaces of the filter media act as a support for microbial attachment and growth, resulting in a biofilm adapted to using the organic matter found in that particular water. TOC removals in these filters range from 5 to 75% (42). Conversion of organic carbon to biomass can eventually lead to a complex ecosystem containing higher life forms such as oligochetes and rotifers (43). Controlling the amount of biomass by periodic backwashing is necessary so that these organisms are not released into the filtered water (44).

One of the first observations in the full-scale use of biological filtration was that the type of filter media had an influence on the net removal of organic carbon from the water. For instance, although rapid sand filters do have the capacity to biologically remove carbon (45–48), GAC filters have superior performance [(49); comparison between DeWaters and DiGiano, (50) and Hozalski and coworkers, (51)]. LeChevallier and coworkers (49) demonstrated that there were 37 times more bacteria per unit surface area on GAC than sand, and that the TOC removal rates were 51% (vs) 26%. Greater biomass levels on GAC (vs) anthracite were also correlated with better organic carbon removals (52). Although correlations between biomass and filter performance were established in the above cases, Urfer and coworkers (53) contend that as long as the biomass level exceeds a minimum concentration, cell numbers do not limit filter performance. The ability to retain this biomass may be material-related, however, because GAC can remove potentially inhibitory compounds (8), sequester organic molecules for growth (12,54) and may protect bacteria from removal by abrasion because of its microporous structure. Another practical advantage of GAC over other media is that the attached microbial population is less prone to shock from changes in water quality, down time, or accidental application of disinfectant (55,56).

Ozonation before biological filtration is often practiced because it frequently improves the biological removal of the smaller molecular weight organic compounds created during oxidation of the natural organic matter (46,57). Preozonation can also result in increased biomass within the filters, most likely because of a higher concentration of usable organic matter (53,58). This effect is not universal, however, because there are cases where ozone does not enhance the metabolism of the organic carbon (59). It has also been observed that populations developing in biological filters fed ozonated water are different from those given nonozonated water. Microbial communities established on nonozonated water used a wider variety of carbon sources at all depths in the filters. In contrast, there was a greater segregation in substrate utilization specificity with depth in filters that were exposed to ozonated water (60).

Because ozonation tends to produce a variety of easily utilized organic compounds, microbial communities in biological filters generally exhibit greater activity at the top of the filters (58,61). The stratification is due to the rapid utilization of the easily degradable organic matter in the top of the filter, with the subsequent dependence on degradation of more recalcitrant compounds as depth and hydraulic residence time increases. This activity may be characterized by both a decrease in the number of carbon substrates used and a change in population with increasing filter depth as determined using phospholipid fatty acid profiles. In the same experimental system, arbitrarily primed PCR sequencing also identified a higher diversity of organisms in the tops of the filters (60). The biomass profile through a filter has been described as first order, and if the filter is sufficiently deep, a portion of it will not be contributing to the biological removal of organic carbon constituents (61).

The potential for a wide range of metabolic functions of the microbial communities in biological filters is further exemplified by the multiplicity of compounds that can be removed from the water. Micropollutants such as phenol can be metabolized when fed simultaneously with ozonated natural organic matter (50). This effect also occurs in simulated wastewater situations, where a biofilm on GAC degraded a mixture of phenols, *N*-heterocyclic compounds and aromatic amines (62). It is also possible to reduce bromate to bromide in biological filters, providing

that the competitive effects of nitrate and oxygen as alternative electron acceptors are controlled (63).

THE PERSISTENCE OF PATHOGENS AND INDICATOR ORGANISMS ON GAC

There has been apprehension expressed by the drinking water industry over the potential proliferation and/or concentration of organisms of public health concern on granular and biologically active carbon filters. As noted earlier, coliforms can become a component of the microflora on filter media. Because biological filtration encourages the growth of bacteria on the filters, it is important to know whether indicator organism and pathogen growth would be enhanced along with the general heterotrophic population.

To address this concern, the potential for coliforms to persist on laboratory columns with virgin GAC, GAC that had been in operation in a filter for a few months, and biologically activated carbon (BAC) from a full-scale plant, was evaluated. The columns were challenged with a suite of five coliforms that had been originally isolated from drinking water distribution systems. The release of the coliforms was tracked by enumerating the organisms in the column effluent. The loss of coliforms in the water for fresh GAC was 0.12 to 0.16 log/day and 0.12 to 0.25 log/day for BAC. The coliforms were detected in the column effluent for approximately one month, although up to 100 mL was filtered to obtain a positive result. Overall, the loss rate from the biologically active carbon was the most rapid of the three media tested. At the end of the experiments, carbon samples were processed and the numbers of coliforms per gram dry mass GAC were determined. Levels were highest on the fresh GAC, intermediate for the precolonized GAC and lowest for the BAC. Conversely, the BAC had the highest concentration of heterotrophic bacteria (38). These results suggest that there is competitive exclusion of the coliform bacteria on the BAC and that the presence of initially attached heterotrophs may decrease the potential for the long-term persistence of undesirable organisms.

In studies performed using BAC filters inoculated with *Pseudomonas aeruginosa*, *P. putida* and *Escherichia coli*, Rollinger and Dott (14) showed that all of these organisms were eliminated from the column between ten and sixteen days. In the same experiments, fresh GAC was colonized with *E. coli*, *K. pneumoniae*, *S. faecalis*, *P. aeruginosa* and *P. putida*. The two pseudomonads and *E. coli* colonized the filter media, whereas the other two organisms were eliminated. These data suggested that perhaps antagonistic compounds were produced by the autochthonous bacteria in the BAC, but less than 1% of the tested strains inhibited the test organisms. Even though the mechanism was not discovered, the observation that colonized BAC reduces the potential for long-term persistence of organisms of public health was confirmed in this study.

The potential growth of coliforms on filter media is of regulatory concern, but has little direct public health significance. However, because coliforms can colonize and persist on GAC filters, there has been the concern that various pathogens may act in a similar manner. The drinking water industry is interested in the survival on GAC and BAC filters of both opportunistic (causing disease only in the immunocompromised individual) and primary pathogens (causing disease in the entire population providing that the infective dose is sufficiently large).

A review of opportunistic pathogens in drinking water has been prepared by Geldreich (64). This review lists a wide variety of organisms that are known to be components of the general heterotrophic population. As such, the presence of these bacteria in drinking water treatment and distribution systems can be expected. Two examples are *Klebsiella* spp. and *Aeromonas* spp. Both of these organisms are capable of expressing virulence factors for human infections that are not waterborne (65–67), and both have been found in granular activated carbon filters (13,68). Others that belong to this group are the *Mycobacterium avium* complex (MAC) and *Legionella* spp. Both are known to grow in biofilms in distribution systems (28,69–73), but there have been no documented reports of their presence in filter media.

In contrast to the opportunistic pathogens, the primary pathogens are not natural components of environmental systems; they are more likely to be found in the gastrointestinal system of a warm-blooded host. To colonize and persist on filter media, these pathogens must be able to successfully compete with the heterotrophic bacterial populations. As with the coliform bacteria, competition with the existing microflora may be a key parameter in preventing proliferation of these bacteria. In a study where the ability of primary pathogens to persist on granular activated carbon was assessed, three enteric pathogens (*Yersinia enterocolitica*, *Salmonella typhimurium*, enterotoxigenic *Escherichia coli*) were added to: (*1*) fresh GAC as pure cultures, (*2*) fresh GAC in the presence of unsterilized river water containing naturally occurring bacteria, and (*3*) GAC previously colonized with river water bacteria (74). Using cultural methods, the greatest level of persistence of the pathogens was on the fresh GAC with sterile river water. Survival decreased when the natural organisms were present, and was the lowest on the previously colonized GAC. Pathogens could be detected in the column effluent by culturing for more than 20 to 24 days when introduced to fresh GAC but were eliminated between 8 and 14 days if the support medium was precolonized GAC. The rate of elimination on the precolonized GAC was 0.11 to 0.7 log per day. Other laboratory studies (14) with several pathogens on GAC reported similar results. The pathogens persisted when introduced to sterile GAC and fed sterile water, but were eliminated from the medium when they were subsequently challenged by autochthonous bacteria in tap water. These data demonstrated that colonized GAC was likely to be beneficial in preventing the proliferation of pathogens.

In the earlier experiments, detection of the pathogens was limited to culturing techniques, which are known to underestimate the numbers of organisms present. Subsequent work has used cultural media combined with direct microscopy and polymerase chain reaction (PCR) methods to follow the fate of bacterial pathogens (*E. coli* O157 : H7 and *S. typhimurium*) in laboratory biologically

active filter media columns. Suspensions of the pathogens were circulated through the columns for 24 hours followed by a continuous flow of dechlorinated tap water with and without relevant concentrations of ozonation by-products. The ozone by-products (aldehydes, etc.) were supplied to determine whether the potential for pathogen survival increased if more degradable low molecular weight compounds were present in the water. Column effluent was sampled routinely, and filter media cores were taken at the end of the experiment. Semi-quantitative PCR showed that there was a 90% reduction in pathogens in the recycled column inoculum before initiation of the tap water feed. These results were mirrored by fluorescent antibody direct counts for *E. coli* O157 : H7. One may assume the reduction is due to attachment to the filter media, because the control system (batch system, no exposure to filter media) showed no decrease in cell numbers over the same period. When throughflow was resumed, no culturable pathogens were detected, and PCR and direct counts using fluorescent antibodies indicated a decline of cells in effluents over the 2.5-week duration of the experiment. After this time, filter media were directly examined for the presence of pathogens. PCR amplification from DNA extracted from the filter media were positive for both *S. typhimurium* and *E. coli* O157 : H7 in one replicate experiment and only for *E. coli* O157 : H7 in the second run, although the signal intensity was quite low. Columns that were supplied additional organic carbon in the form of ozonation by-products did not have enhanced pathogen bacterial persistence or proliferation (68).

RELEASE OF BACTERIA AND COLONIZED PARTICLES FROM GAC FILTERS

As water passes through GAC filters, particularly those that are optimized for biological activity, a significant number of bacteria can be released. The increase in the numbers of organisms from filter influent to effluent can be two orders of magnitude (75). These organisms may be released as single cells, aggregates, or attached to filter media particles (filter fines) that escape through the underdrain of the filters.

In field studies, Camper and coworkers (76) collected released filter fines from GAC filters by passing water from the underdrain during an entire filter run through a gauze filter in a 47 mm Swinnex. The average volume sampled was 18,600 liters. The number of released carbon fines in 201 samples was determined using image analysis. Recognizing that small fines may not have been efficiently retained in the gauze, the average number of particles in a sample was 2,333 ranging in size from 1.0 to $3.5 \times 10^3 \mu m$ in diameter. The bacteria were desorbed from the surface (24) and the number of heterotrophs and coliforms determined by plate count. The results indicated that 17% of the filter runs released carbon fines that contained coliform bacteria, and 28% of these coliforms exhibited the fecal biotype. It should be noted, however, that none of these utilities experienced elevated coliform numbers in their distribution systems. In a similar study, Stewart and coworkers (77) used a modified Swinnex with a polycarbonate filter to trap carbon particles released from a pilot GAC filter. An average of 36 particles per liter were detected with sizes ranging from 2 to greater than 40 micrometers. Using the homogenization technique it was determined that 200 to 7,000 viable bacteria could be recovered from 1,000 particles. Numbers of coliforms were low, with one reported fecal coliform isolated from the released filter fines.

The type of filter media and filter operations may influence the release of colonized filter particles. In a comparison between bacterial counts on released particles from biologically activated sand, anthracite, and biologically activated carbon, the BAC particles contained significantly higher numbers of heterotrophs (49). When the release of particles from full-scale anthracite and sand filters was evaluated (76), particle counts were similar to those from GAC filters. In the same study, where particles released from GAC filters throughout a filter run were enumerated, higher filtration rates, deeper GAC filter beds, and higher applied water turbidity resulted in higher particle release. The age of the carbon was not an important factor. In a similar study, Stringfellow and coworkers (78) demonstrated that the number of particles released from sand or GAC contactors were similar. They also observed that these particles were frequently colonized by heterotrophs, although no coliforms were detected.

Amirtharajah and Westein (79) showed that an elevated number of particles were released both immediately before backwash and shortly after the filter was put back in operation if proper filter-to-waste procedures were not followed. It is probable that colonized filter fines or detached bacteria were also released during these two times, as illustrated by an elevated number of coliforms detected in the filtrate immediately after backwash (33). More recently, Moran and coworkers (80) showed that there can be breakthrough of turbidity due to detachment of particles from deep laboratory filters at the end of a filter run, and these particle sizes were comparable to the range associated with *Cryptosporidium* oocysts.

DISINFECTION OF ATTACHED BACTERIA

The release of bacteria from GAC filters, especially those attached to carbon particles, is important because cells in aggregates or attached to filter media are less susceptible to disinfection than single cells (13,17,24,77). The level of protection appears to be dependent on the size of the fine (78), with bacteria associated with larger particles less susceptible to disinfection than those attached to particles of approximately the same size as the bacterium. The lack of disinfection efficacy against particle-associated bacteria may be the result of biofilm formation on the surfaces. It has been demonstrated that biofilms in general are far less susceptible to disinfectants than single suspended cells (81). In the case where oxidizing disinfectants are used, the phenomenon has been explained as a reaction-diffusion issue. The oxidizing partially disinfectant is consumed in the outer layers of the biofilm, resulting in levels insufficient to kill cells at the base of the biofilm (82,83). However, this phenomenon is inadequate

to explain the apparent lack of disinfection efficacy against very thin biofilms seen on GAC particles. Work by Srinivasan and coworkers (84) showed that thin biofilms were resistant to monochloramine, and suggest that physiological changes in these cells is responsible for the decreased sensitivity.

Regardless of the mechanism, the magnitude of this reduced sensitivity has been demonstrated in many experiments. LeChevallier and coworkers (13) showed that GAC-associated bacteria were not affected by exposure to 2-mg/L free chlorine for one hour. In similar experiments, free chlorine or monochloramine at concentrations of 1.5 mg/L for a 40-minute exposure time did not decrease the plate counts of GAC attached cells (77). Camper and coworkers (24) treated filter fines from operating GAC filters with 2-mg/L free chlorine for 30 minutes, and still recovered culturable cells from the GAC particles. In experiments where free chlorine and monochloramine were compared, monochloramine provided better log reduction of GAC attached cells only if the concentrations of both disinfectants exceeded 2 mg/L (38).

Even though particle-associated bacteria have been shown to be less susceptible to disinfectants, the quality of water produced by GAC filters has not been shown to be compromised. In a study where GAC pilot system effluent samples containing coliforms in the range of <1 to 300 CFU/100 ml were exposed to 2.5-mg/L free chlorine for 30 minutes, no coliforms were detected in 62 of 64 samples. For the two positive samples, the maximum detected number was 7/100 ml. In the same study, heterotrophic plate counts were reduced from an average of 58 CFU/ml to $<1-4$/ml (85). Similarly, the efficacy of disinfection downstream from biological filters in reducing microbial loads in finished water has been documented in both research and utility settings (35,36,38,47,49,55).

IMPLICATIONS FOR DISTRIBUTION SYSTEMS

It is known that heterotrophic bacteria carried by particles can penetrate the disinfection barrier, attach to the pipe surface, grow, and develop into a biofilm; this becomes critical if pathogens are involved (86,87). With the evidence mentioned earlier that particle associated bacteria are less susceptible to disinfection in mind, utilities have concerns about the possible transport of organisms on filter fines through the disinfection barrier to the distribution system and subsequent regrowth of undesirable bacteria.

To determine if particles can be transported to biofilms, mixed population drinking water biofilm reactors were challenged with slug dose inoculations of carbon particles in the size range of either 1.2 to 8μm or 1.2 to 50μm in diameter. The retention of the carbon fines in the biofilms over time in the presence and absence of disinfection was followed using microscopy techniques. A low percentage (20%) of the small fines initially attached to the biofilm. In contrast, nearly 70% of the larger fines were retained, resulting in a 10-fold increase in carbon mass in the biofilm over that seen with the smaller carbon fines. The larger fines were retained more effectively in the biofilm, but a reduction of nearly 80% of the particles was observed over the three-week experiment. In addition to specific information on the carbon fines, general observations on the response of biofilms to monochloramine (1 mg/L influent concentration) and free chlorine (0.5 mg/L influent concentration) were obtained. Although it took considerably longer, monochloramine ultimately decreased the number of culturable biofilm cells to essentially the same level as free chlorine. The reduction of culturability by approximately three logs was not reflected in loss of viability as assessed by staining techniques; this decrease was only one log. Neither disinfectant caused significant loss nor detachment of biofilm cells from the surface as documented by total cell counts. As determined using activity stains, the biofilm organisms in proximity of the carbon particles did not exhibit reduced susceptibility to the disinfectants, nor was there an accumulation of bacteria around the fines. These results suggest that large particles can be retained in biofilms, but that their effect on the biofilm ecology may be limited (88).

These studies were then extended to investigate the potential for previously colonized carbon particles to act as a mechanism for transporting bacteria to an established biofilm. The larger fines were incubated in the presence of *K. pneumoniae* and then added as a slug dose to biofilm reactor systems. The coliform was monitored in the biofilm using a monoclonal antibody and epifluorescence microscopy. The particles did transport the bacteria to the biofilm surface, resulting in colonization. Over the three-week period, the coliform did not appear to replicate, nor were there cells detected in areas other than the proximity of the carbon particles. If free chlorine was added, there was preferential removal of the coliforms and carbon fines over that of the general heterotrophic population, but 10% of the initially introduced organisms and 20% of the carbon fines remained in the biofilm. Because the detection method was a monoclonal antibody, there is no information available about the culturability or viability of the remaining target bacteria (89).

These findings answer some of the concerns that utilities have had over the release of colonized filter fines from GAC filters into distribution systems. Even if the fines are colonized and attach to the biofilm in numbers comparable to the numbers of culturable heterotrophs present, there is no apparent long-term impact on the biofilm. Disinfection preferentially removed the carbon particles, and there was no dramatic proliferation of an environmental coliform in the biofilm.

CONCLUSION

With growing demands on water resources and the need to respond to increasingly stringent water quality regulations, the use of GAC and BAC will become more widespread in drinking water treatment. GAC will become colonized with organisms, and this is encouraged when BAC treatment is utilized. For biological treatment, BAC appears to be the support medium of choice in most cases because of higher biomass and greater resiliency

against shocks to the system. Colonization of GAC with indigenous organisms appears to reduce the potential for long-term persistence by pathogenic bacteria, although indicator organisms and opportunistic pathogens may become a component of the ecosystem within the filter. Even though disinfectant-resistant particle-associated bacteria are released from properly operated filters and these particles may associate with distribution system biofilms, typical disinfectant regimes have controlled these potentially deleterious effects. On the basis of the results of research and practical experience, it is apparent that the overall benefits of granular activated carbon filtration and biological filtration far outweigh the concerns that may be expressed about deleterious microbiological impacts on potable water.

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GRANULAR HIGH-RATE FILTRATION: REMOVAL OF PATHOGENIC MICROORGANISMS.

See REMOVAL OF PATHOGENIC MICROBES BY GRANULAR HIGH-RATE FILTRATION

GRANULAR SLUDGE.

See ANAEROBIC GRANULES AND GRANULATION PROCESSES

GREEN FLUORESCENT PROTEIN (GFP)

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A powerful tool of molecular microbiology was created with the description of GFP. First described in its natural

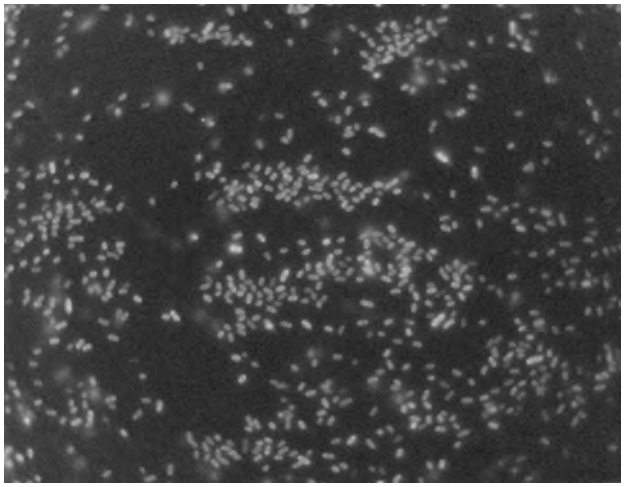

Figure 1. GFP-labeled bacteria as seen under the epifluorescent microscope. Magnification 1000X. Because the bacteria do not need to be fixed before staining, they can be viewed as a wet mount, allowing the observer to see them interact in real time. See color insert.

host, the jellyfish *Aequorea victoria*, this protein emits a bright green fluorescence when excited by ultraviolet light (UV). The utility of this protein for molecular analysis was shown after the gene for GFP was cloned and introduced into other species, both prokaryotic and eukaryotic (Fig. 1). When expressed, the gene faithfully produces GFP, which then folds into the fluorescent configuration and becomes very noticeable. Applications involving the expression of this gene became very popular, and the number of users of this technology is steadily increasing.

Information on GFP is also contained in the encyclopedia entry on BIOREPORTERS: LUCIFERASE AND GFP, and the interested reader is advised to refer to that entry for a detailed analysis of the structure of GFP. This article discusses the science and technology of GFP use, mostly in prokaryotic organisms. This is not an exhaustive analysis, but instead demonstrates the versatility of the system while acknowledging the limitations. Variations of the GFP have also been produced and these are discussed.

GFP and Its Relatives

GFP was studied extensively as a purified protein from the jellyfish before it was used as a marker by Chalfie and coworkers (1). This work opened the door for further experiments in marking cells of all description with the *GFP* gene. All the bioreporter genes discussed in this article are commercially available. Convenient cloning vectors are offered, which enable rapid construction of gene fusions. The leader in this area is acknowledged to be Clontech (*http://www.clontech.com/*), which offers a full range of plasmid vectors for both prokaryotic and eukaryotic work. In addition, some of these genes are available from investigators in transposon vectors that facilitate mutagenesis in many microbial strains (2,3).

By mutagenesis of the GFP some very useful variants have been isolated. These include alternative emission colors (e.g., cyan, yellow) and mutations that are more easily expressed in prokaryotic organisms. A particularly valuable variant of GFP has been described by Andersen and coworkers (4). They attached a short peptide to the carboxy terminus of GFP, which makes it far more labile to proteases than the wild-type version. This allows GFP to be used as a bioreporter of dynamic expression signals, and not merely as a plus or minus response.

The latest bioreporter gene to be introduced is the red fluorescent protein (RFP). The gene for RFP was isolated from *Discosoma*, a type of coral from the Indo-Pacific ocean (5). It has an excitation maximum at 558 nm and an emission maximum at 583 nm, making it a very attractive choice for tracking microorganisms. The red emission wavelength is rarely found in nature, and so the background will almost certainly be negligible. It is anticipated that many researchers will take full advantage of RFP. A description of the fluorescent characteristics (6) and the detection of RFP in a biological system (7) have recently been published.

MOLECULAR CHARACTERIZATION

The *GFP* gene has been sequenced, and descriptions of the protein are available (8,9). The protein is not especially fluorescent until it forms a fluorescent center, defined by three amino acids near the center of the protein. The reaction is shown in Figure 2. After this center is formed, the protein becomes extremely fluorescent, and the ring structure is very difficult to disrupt. The reaction comes about through an autocatalytic reaction, that is, the GFP protein catalyzes its own change. This is very convenient because only a single gene is needed for the synthesis of the bioreporter. Cofactors are not needed for the reaction (another great advantage), however,

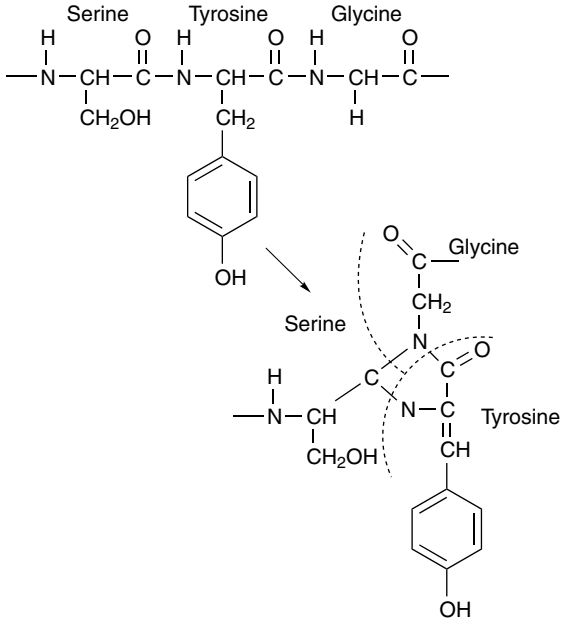

Figure 2. The folding of GFP into a fluorescent chromophore require the three internal amino acids shown.

it does require molecular oxygen, although in small concentrations. This requirement effectively restricts the use of GFP in obligate anaerobic microorganisms.

APPLICATIONS IN ENVIRONMENTAL MICROBIOLOGY

The most obvious use of GFP in bacteria is, as a readily identifiable marker (i.e., a tagant), so that the cells can be located and enumerated. This is true even in mixed culture conditions because the GFP-tagged cells will be easily identified. "Mixed cultures" include virtually every environment outside of controlled laboratory conditions, and therefore the use of GFP has lead to an explosion of research in this area. Before fluorescent biomarkers were available, the cells could be labeled with fluorescent antibodies, or the cells could be enumerated on selective agars, but these methods had substantial problems. With GFP, the cells can be examined under natural conditions and without destruction of sample material.

Fluorescent cells are easiest to see under the microscope. The UV excitation may cause other cells and particles to fluoresce, but they will appear dull in comparison to GFP-tagged cells. The variety of environments in which this technique has been attempted is quite impressive, and demonstrates the utility of fluorescent markers. An excellent review is available (10). A few examples can be cited here:

- Biofilms present unique challenges in examination because they are difficult to analyze in real time. Aspiras and coworkers (11) introduced GFP into *Streptococcus gordonii* to track the cells in a simulated oral biofilm community. This work has obvious implications for the study of the cause of dental caries, which is promoted by complex microbial communities on the surfaces of the teeth. Christensen and coworkers (12) studied gene exchange in environmental biofilms by detecting the passage of a plasmid tagged with the GFP gene. They were able to show that plasmid transfer was a rare event, which is useful information for planning other environmental release experiments.

- Soil communities may have many thousands of species in a single gram of material. Differentiating between your strain of interest and all others is a difficult task. Boon and coworkers (13) studied an activated sludge community containing a GFP-labeled *Comamonas testosteroni* strain. They were able to show survival of the strain over long periods of time. Tresse and coworkers (14) used a GFP-labeled *Moraxella* strain to follow survival in soil while it was catabolizing nitrophenol. Survival of an introduced microorganism is essential if it is to perform a bioremediation task to completion.

- Both freshwater and marine environments contain high concentrations of microorganisms. Enumeration of GFP-labeled *Escherichia coli* cells in a freshwater stream microcosm was performed by Leff and Leff (15). In an experiment analogous to the work mentioned earlier (12), Dahlberg and coworkers (16) examined plasmid transfer between species in a marine environment. They found much higher rates of plasmid transfer, suggesting that conjugation may be far more frequent in marine environments.

The preceding examples rely on constitutive expression of GFP to mark the cells, but it can also be used to detect gene expression in a time-dependent manner. In this regard it's use is closest to the work performed with eukaryotes, in which GFP is used to detect when a particular gene is expressed, for instance, in a developing embryo. This comparison is evident in the work of Kataoka and coworkers (17) in their study of *Streptomyces lividans* colony development. They found a gene that was expressed primarily during colony differentiation, and followed the activity of the gene, both temporally and spatially as the colony developed. Joyner and Lindow (18) isolated a gene that encodes a membrane receptor for a siderophore and fused it to the *GFP* gene. This creates a sensitive sensor for ferric iron in the environment, which they used to evaluate iron bioavailability on plant tissues. Hansen and Sorensen (19) produced a sensitive bioreporter of mercury by fusing the promoter of the *mer* operon to GFP. This proved to be as sensitive as comparable bioreporter constructs, utilizing the bioluminescent *lux* genes. The wild-type version of GFP was largely unsuitable for such assays because the GFP protein is so unusually stable. The protein persists for hours to days inside most bacterial cells, and therefore dynamic responses of genes over time cannot be studied. However, the new unstable versions of GFP described by Andersen and coworkers (4) have opened up this possibility.

An unusual use of GFP was described by Fischer and coworkers (20). They isolated a strain of *Pseudomonas* that responded to the explosive trinitrotoluene (TNT), and fused the TNT-responsive gene to GFP. When applied in the field, the bacteria responded to trace amounts of TNT and began to fluoresce. By finding the fluorescent bacteria one can find the source of the explosive, such as a landmine or other ordnance. To obtain clear images in the field, a laser-induced fluorescence imager (LIFI) system was employed. A computer-enhanced scan of one of these images is shown in Figure 3. During the field test, the bacteria pooled on the vegetation at the site, which was possible because of the waxy layer found on the blades of grass. TNT conducted through the plants then triggered the *GFP* gene and a concentrated signal was seen. In the figure, the strongest fluorescent image is seen in the middle of the blades of grass, which are easily discernible against the nonfluorescent background. This result demonstrated that vegetation was no impediment to the field application of the GFP-based technique, but was in fact, enhanced by it. In this figure, the actual location of the TNT was about 0.5 m from the brightest fluorescent spot, suggesting that root trails conduct the TNT efficiently.

One of the most popular uses of GFP is in the study of plant–microbe interactions, particularly with respect to the root zone of plants. It has long been recognized that microbial activity around the roots is important in the health of the plant, but the interactions are very

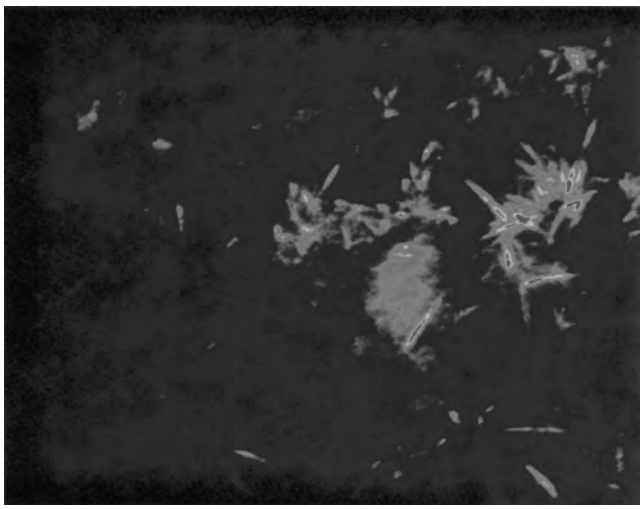

Figure 3. TNT-sensitive bacteria containing GFP were sprayed on a field containing buried landmines. Trace amounts of TNT reach the surface through plants. Shown here are plants (blades of grass) that have been sprayed with the bacteria and which are fluorescing because of GFP. A false color image shows red as being the most intense fluorescence and blue as the least intensity. Length of the figure is approximately one meter. See color insert.

difficult to examine because of the complex and dynamic nature of the microbial communities, the effects of soil particles, the presence of plant exudates, and other factors (hydrology, mineralogy, and temperature). Observing the survival and activity of cells in situ is a big improvement over the previous method of enumeration by plate counting of strains that had been given an antibiotic resistance marker.

Three recent reports have described the interaction of *Pseudomonas* species in the rhizosphere of barley (21–23). Typically, these experiments involved the use of fluorescence microscopy to visualize the bacteria on the root zone, and confocal laser scanning microscopy to visualize the three-dimensional structure of the bacteria on the developing roots. To investigate a plant–microbe symbiotic relationship, Gage and coworkers (24) used a GFP-labeled *Rhizobium* strain and watched the progress of infection threads in the rhizosphere.

FUTURE DEVELOPMENTS

The examples cited earlier show the range of projects that can be aided by employing GFP. Fluorescent proteins are both valuable and versatile, however, there are certain critical areas where improvements could be made. New colors (emission spectra) have been the trend in recent years. With the addition of useful bioreporter genes in cyan, blue, green, yellow, and red, all on convenient cloning vectors, the number of possibilities increase. However, it should also be stressed that changes in the excitation spectrum of a bioreporter are just as useful. This was the basis for the first successful alternative GFP molecule, the red-shifted GFP (RS-GFP) (25). In this molecule, the emission maximum was nearly unchanged from the wild-type molecule, but the excitation maximum was shifted to a higher wavelength ("red-shifted"). In addition, it may be possible to identify molecules that fluoresce outside of the visible spectrum, especially in the infrared region.

An area of special concern is the development of bioreporters for obligate anaerobic microorganisms. The fluorescent proteins require small amounts of oxygen to form their chromophore, and therefore completely anaerobic conditions are incompatible with bioreporter function. A fluorescent molecule that is independent of oxygen is therefore highly desirable.

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See also LUCIFERASE AND GREEN FLUORESCENT PROTEIN AS BIOREPORTERS IN MICROBIAL SYSTEMS.

GREENHOUSE EFFECT, ROLE OF MICROORGANISMS

See Weathering: Mineral Weathering and Microbial Metabolism

GROUNDWATER, PROTISTAN COMMUNITIES IN

See Protistan Communities in Groundwater

GROUNDWATER SAMPLING

See Igneous Rock Aquifers Microbial Communities
See Sampling Techniques for Environmental Microbiology

GROUNDWATER, SULFUR BACTERIA IN

See Sulfur Bacteria in Drinking Water

GROUNDWATER, VIRUSES IN

See Viruses in Drinking Water and Groundwater

GROWTH, DIAUXIC

See Metabolism of Mixtures of Organic Substrates

GROWTH, ON MIXED SUBSTRATES

See Petroleum and Other Hydrocarbons, Biodegradation of

H

HABER-BOSCH PROCESS. See NITROGEN FIXATION IN SOILS — FREE-LIVING MICROBES

HALOGENATED COMPOUNDS, BIOREMEDIATION OF. See BIOREMEDIATION: AN OVERVIEW OF HOW MICROBIOLOGICAL PROCESSES CAN BE APPLIED TO THE CLEANUP OF ORGANIC AND INORGANIC ENVIRONMENTAL POLLUTANTS

HALOGENATED HYDROCARBON (GASES). See TRACE GASES SOIL

HALOORGANICS DEHALOGENATION. See BIODEGRADATION: REDUCTIVE DEHALOGENATION AND METABOLISM OF CHLORINATED ORGANICS BY ANAEROBES

HALOPHILES. See EXTREMOPHILES: LIFE IN EXTREME ENVIRONMENTS; SALINITY EFFECTS ON THE PHYSIOLOGY OF SOIL MICROORGANISMS

HALOPHILES: AEROBIC HALOPHILIC MICROORGANISMS

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This article will focus on the moderately and extremely halophilic microorganisms isolated from "extreme environments "as defined by Horikoshi and Grant: "those in which a restricted range of organisms can grow; the majority of organisms are excluded" (1). Typical niches for halophilic microorganisms are hypersaline environments such as salt lakes (e.g., the Dead Sea, the Great Salt Lake, alkaline soda lakes), salterns distributed all over the world, etc. (2). Despite what may seem like rather hostile conditions, salt lakes can be highly productive ecosystems. Archaeal and bacterial halophiles are not the only microorganisms found. Eukaryal algae are the major phototrophs in most salt lakes. Moderately halophilic microorganisms were also isolated from seawater and estuarine environments. Anaerobic halophiles, including extremely halophilic methanogenic Archaea, have been isolated from hypersaline environments, but they are not dealt with in this article.

DEFINITION OF HALOPHILIC MICROORGANISMS

Kushner (3) and Kushner and Kamekura (4) defined various categories of halophiles, halophilic (salt-loving) microorganisms. Slight halophiles grow best in media containing 0.2 to 0.5 M NaCl (1.2 to 2.9%, w/v), moderate halophiles are defined as microorganisms growing optimally in the presence of 0.5 to 2.5 M NaCl (2.9 to 14.6%), and extreme halophiles grow best in media containing 2.5 to 5.2 M (saturated) NaCl.

On the other hand, halotolerant microorganisms grow best in media without added salt as well as in the presence of relatively high concentrations of NaCl (e.g., 1 M). Extremely halotolerant microorganisms extend their growth range to more than 2.5 M NaCl. It should be pointed out that the salt requirements of halophiles depend on the growth conditions such as temperature, concentrations of other minor salts, particularly magnesium ions, presence of growth factors, and so forth. Furthermore, some halophiles are able to grow in the presence of high concentrations of salts other than NaCl, such as $MgCl_2$, KCl, RbCl, or CsCl (4).

Living organisms are classified into three domains: Bacteria (Eubacteria), Archaea (Archaebacteria), and Eukarya (Eukaryotes) (5,6). Most of the moderate halophiles belong to bacteria, whereas most of the extreme halophiles are members of the domain Archaea. Sometimes the term *extreme halophiles* is used as a synonym of the group of members of the family Halobacteriaceae of the domain Archaea, but strictly speaking, this is not correct. Some of the members of Halobacteriaceae are moderate halophiles, and there are a few extremely halophilic bacteria.

"Approved Lists of Bacterial Names" published in 1980 (7) included a few species of extreme halophiles: *Halobacterium cutirubrum*, *Halobacterium halobium*, *Halococcus morrhuae*, etc. isolated from the Dead Sea, solar salts, salted codfish, or hides. Also included were a few species of moderate halophiles, *Flavobacterium halmophilum* (now *Halomonas halmophila*), *Micrococcus halobius* (now *Nesterenkonia halobia*), *Paracoccus halodenitrificans* (now *Halomonas halodenitrificans*), *Planococcus halophilus* (now *Marinococcus halophilus*), *Pseudomonas beijerinckii*, *Vibrio costicola* (now *Salinivibrio costicola*), etc. Besides these strains, there are some isolates that have been used for physiological and biochemical studies (8).

During the last two decades, numerous halophiles have been isolated from extreme environments. Because they were assigned to many taxa mainly according to their phenotypic characteristics, their taxonomy was in a flux. The situation has been solved gradually by

chemotaxonomic methods as well as by phylogenetic analyses of their 16S rRNA gene sequences, and generic and specific names of some isolates have been altered recently. In this article, the updated situation of the halophiles will be presented.

HYPERSALINE ENVIRONMENTS

Most of the hypersaline habitats such as the Dead Sea, soda lakes, and many solar salterns are located in hot and dry areas in the world (2). Individual salt lakes vary considerably in their ionic compositions because the nature of surrounding rocks, geology, and general climatic conditions determine the predominant ions (9). In addition to these environments exposed to the surface of the earth, there are subterranean hypersaline environments, some of which are accessible as rock salt mines or alternatively as materials indirectly accessible through solution mining. These environments once presumed to be devoid of any living organisms are in fact habitats of a variety of microorganisms. Heavily salted cod or hide for preservation and food for which high concentrations of salts are used during preparation, for example, fish sauce or soy sauce, are also hypersaline environments.

In the following sections, halophilic microorganisms are dealt with in the order of halophilic Archaea, halophilic bacteria, and halophilic Eukarya.

HALOPHILIC ARCHAEA

Isolation of Halobacteria

Members of aerobic halophilic archaea are classified within the single family Halobacteriaceae in the order Halobacteriales (10). They are also known by the trivial names *halobacteria* and *haloarchaea*. Most of the halobacteria are pigmented pink to red because of the presence of carotenoid pigments. Microorganisms of the domain Archaea are known to lack murein, the peptidoglycan polymer consisting of N-acetylmuramic acid and N-acetylglucosamine, and are thus insensitive to beta-lactam antibiotics such as penicillins. Cytoplasmic membranes of noncoccoid species of halophilic Archaea are surrounded by S-layers of glycoproteins, whereas coccoid species (genus *Halococcus* and *Natronococcus*, discussed later) possess rigid cell wall made of polymers of heteropolysaccharides (11). Most of the strains grow best at concentrations of 3.5 to 4.5 M NaCl. To compensate for the high salt concentrations in the environments, the organisms accumulate mainly KCl, up to 5 M.

The cell envelopes of the coccoid halobacteria are stable in the absence of salts. Those of noncoccoid isolates maintain their integrity only in the presence of high concentrations of NaCl or KCl. Upon gradual dilution of the culture with water, the cells change their shape, through irregular forms, to spheres that finally undergo lysis. A similar phenomenon may happen in the natural hypersaline environments on heavy rainfall.

Historically, studies on the extreme halophiles were initiated to isolate microbes that caused spoilage of salted fish, meat, or hides (12). Thus, most of the media for extreme halophiles are based on hydrolyzates of proteinaceous materials, such as casamino acids, tryptone, yeast extract, etc. Maximum recovery rates of halophilic Archaea were reported to be obtained on media containing natural brines and a whole-cell extract of *Hbt. cutirubrum* (13).

Bacto-Peptone from Difco should not be used in the cultivation of extreme halophiles because it contains high concentrations of bile acids that cause lysis of cells. Analyses of various peptones showed that the Bacto-Peptone contains 2.9 mg/g glycocholic acid and 3.7 mg/g taurocholic acid, glycine and taurine conjugate of cholic acid, respectively as the major bile acids. Lysis in 25% NaCl solution containing 1% Bacto-Peptone is specific for noncoccoid halobacteria. No lysis was observed with *Halococcus* and *Natronococcus* cells, methanogenic archaea, or halophilic eubacteria. Peptone Bacteriological Technical from Difco and Oxoid Bacteriological Peptone are recommended for use in media because they contain practically no cholic acid (14). In some of the past ecological studies of saline environments, Bacto-Peptone was used as a component of media, thus, probably having caused underestimation of halobacterial community.

Salts required to be added are high concentrations of NaCl, depending on the strains to be isolated (see description of each genus), enough amounts of KCl (e.g., 0.2%), and relatively high concentrations of magnesium as $MgSO_4$ or $MgCl_2$. To isolate alkaliphilic haloarchaea, sodium bicarbonate is added or pH is adjusted from 9 to 11 by adding NaOH, and the magnesium concentration is reduced to less than 0.1%; otherwise, precipitates of magnesium hydroxide disturb the clarity of media. The pH is adjusted from 5.0 to 5.5 to isolate strains that are able to grow at acidic condition. In order to isolate strains of halophilic Archaea from environments in which the halophilic bacteria are dominant, penicillin G is added to the media to inhibit growth of contaminant bacterial halophiles.

Media, liquid or agar plates, to which samples were inoculated, are incubated at temperatures depending on the microorganisms to be isolated. All halophiles known so far are able to grow at 30 to 37 °C, but a few strains are able to grow at low and high temperatures; *Halorubrum lacusprofundi* grows well at 4 °C, while *Haloterrigena thermotolerans* is able to grow up to 60 °C. When cultivated on agar plates, Petri dishes should be sealed by vinyl tape or placed in a plastic container to prevent drying of the agar surface. Some strains show very slow growth on agar plates compared with that in liquid media.

Members of the 15 Genera of Halobacteria

The family Halobacteriaceae contains 15 valid genera. Readers are recommended to consult chapters on halobacteria, Vol. 1 of the 2nd edition of the Bergey's Manual of Systematic Bacteriology (15), for the relevant references. In this article, the generic names will be abbreviated according to the recent proposal (16).

Halobacterium. *Halobacterium* is the type genus of the family Halobacteriaceae. *Halobacterium salinarum* is the only species of this genus, with strain NRC 34002 as the

type strain. *Halobacterium cutirubrum* and *Hbt. halobium* are now included in *Hbt. salinarum* as belonging to the same species (17). *Halobacterium salinarum* contains two major membrane glycolipids, sulfated triglycosyl diether-1 and sulfated tetraglycosyl diether, structures of which are depicted in reviews (18,19). These two glycolipids are signatures of the strains of this genus because they have not been detected in any members of other genera. Strains of *Hbt. salinarum* require at least 3 M NaCl. Most strains grow best at 3.5 to 4.5 M and growth occurs even at saturated NaCl. Thus, they are extremely halophilic sensu stricto. They also require relatively high Mg^{2+} concentration (0.05 to 0.1 M) and a pH range of 5.5 to 8.0. Optimum temperature is 50 °C. Many strains are pigmented a deep red. They require several amino acids for growth. Strains of *Hbt. salinarum* were isolated from such environments as the Dead Sea, solar salts, salted codfish, or hides. However, analysis of natural communities of halophilic Archaea, such that occur in hypersaline lakes, has shown that *Halobacterium* is not dominant and is outnumbered by members of other genera. This was shown by characterization of strains isolated as colonies on agar plates (20), by analysis of glycolipids extracted directly from the communities (21,22), and by analysis of sequences of cloned 16S rDNA amplified from DNA extracted from the communities (23).

A procedure for the specific enrichment and isolation of strains of the genus *Halobacterium* was designed on the basis of their ability to grow anaerobically by fermentation of L-arginine (24). Anaerobic enrichment cultures were set up by inoculating 10-mL portions of brine in 150-mL screw-capped glass bottles completely filled with growth media with added 5 g/L L-arginine, and inoculating the bottles in dark. After 7 to 10 days incubation at 35 to 38 °C, a growth of motile long, rod-shaped cells was detected. Thin layer chromatography of glycolipids of the cultured cells showed that they all are members of the genus *Halobacterium*; they had the two characteristic glycolipids.

Haloarcula. Seven species of this genus have been isolated from various hypersaline environments. They are listed in the following text with their optimum NaCl concentrations for growth in parentheses. *Haloarcula vallismortis* (formerly *Halobacterium vallismortis*) (4.3 M) from salt pools of the Death Valley, California; *Har. argentinensis* (2.5 to 3.0 M) and *Har. mukohataei* (3.0 to 3.5 M) isolated from saltern soil in Argentina; *Har. hispanica* (3.5 to 4.2 M) from marine salterns in Spain; *Har. japonica* (3.5 M) isolated from saltern soil in Japan; *Har. marismortui* (3.4 to 3.9 M) from the Dead Sea; and *Har. quadrata* (3.4 to 4.3 M) isolated from a brine pool in the Sinai Peninsula, Egypt. The following unvalidated strains have also been known but they are not well characterized. *Haloarcula sinaiiensis* (2.5 to 3.5 M) from a Red Sea sabkha (discussed later); "*Har. californiae*" (3 to 4 M) isolated from a Californian saltern; and "*Har. aidinensis*" (3.4 M) isolated from Aidin salt lake in China. Thus, all members of this genus are extremely halophilic. Minimal Mg^{2+} requirement for growth is 0.005 to 0.05 M. They do not require amino acids for growth.

The major membrane glycolipid of these species is triglycosyl diether-2, except for *Har. mukohataei*, which has the sulfated diglycosyl diether-1. Details on the difference of this species from other members of *Haloarcula* were discussed elsewhere (25).

Strictly square-shaped, gas-vacuolated, flat halophilic Archaea were first found in the Gavish Sabkha, a coastal brine pool in the Sinai Peninsula, Egypt. Their presence has since been documented in a variety of hypersaline environments, but no pure culture is available. A recent fluorescence in situ hybridization (FISH) has suggested a probable positioning of the Sinai square halophile in the genus *Haloarcula*. A. Oren's group noticed that a saltern crystallizer pond in Eilat, Israel was dominated, more than 55%, by a similar kind of square halobacteria. They analyzed the glycolipids of cells collected from 10 L brine and found they contained the glycolipid sulfated diglycosyl diether-1.

Halobaculum. Enrichment cultures in which Dead Sea water or sediment was used as inocula have yielded three species of extreme halophiles, *Haloferax volcanii* (discussed later), *Har. marismortui*, and *Halorubrum sodomense* (discussed later). A mass bloom of halophilic Archaea developed in the Dead Sea in the summer of 1992. The glycolipid present in the biomass was the sulfated diglycosyl diether-1, suggesting that the dominant organisms were relatives of the genus *Haloferax* (discussed later). One isolate from the biomass, a pleomorphic rod-shaped organism, was further analyzed and named as *Halobaculum gomorrense*. This isolate requires a relatively low level of NaCl for growth; a combination of 1 M NaCl and 0.8 M $MgCl_2$ supported growth. Optimum growth occurs in the presence of 1.5 to 2.5 M NaCl and 0.6 to 1 M $MgCl_2$.

Halococcus. Many strains of *Hcc. morrhuae* have been isolated from sources such as offshore seawater, saline lakes, salterns, salted products etc. *Halococcus saccharolyticus* was isolated from ponds of a Spanish saltern, and *Hcc. salifodinae* was isolated from rock salt obtained from an Austrian salt mine. All strains of the three species require at least 2.5 M NaCl for growth and 3.5 to 4.5 M for the best growth.

The cells of *Halococcus* species do not lyse in distilled water, not even with N-lauroylsarcosine, a detergent that causes complete lysis of noncoccoid halobacteria (26). The *Halococcus* cells are also resistant to repeated freezing and thawing. They have a thick, rigid cell wall of sulfated heteropolysaccharides (10). They contain the sulfated diglycosyl diether-1 as the major membrane glycolipid.

Haloferax. This genus now contains four species. *Haloferax volcanii* was originally isolated from the Dead Sea by enrichment in a complex medium containing 0.2 M Mg^{2+} and 4 M NaCl, but best growth occurs in media containing 1.5 to 2.5 M NaCl; thus, *Hfx. volcanii* is a moderate halophile. *Haloferax denitrificans* isolated from salterns in San Francisco, California, grows in media containing 1.5 to 4.5 M NaCl. *Haloferax gibbonsii* was isolated from a saltern in Spain; best growth occurs in the presence of 3 to 4 M NaCl and 0.2 M Mg^{2+} at 40 °C, but 2 to 3 M NaCl at 30 °C. *Haloferax mediterranei* isolated from a

saltern in Spain grows in the presence of 1 M to saturated NaCl. Optimum temperature is 43 to 54 °C depending on the growth medium. A similar strain "*Hfx. alicantei*" was also isolated from the Spanish saltern. Strain D1227 isolated from soil contaminated with highly saline oil brine near Grand Rapids, Michigan, was also shown to belong to the genus *Haloferax*. Growth occurs over the range of 0.86 to 5.2 M NaCl, and the optimum concentration is 1.7 to 2.6 M. The strain is extraordinary in its ability to use a variety of aromatic compounds (benzoic acid, cinnamic acid, 3-phenylpropionic acid, 3-hydroxybenzoic acid, etc.) as sole carbon and energy sources. All members of this genus contain the sulfated diglycosyl diether-1 as the major membrane glycolipids.

Halogeometricum. Halophilic archaeal strains were isolated from solar salterns of Cabo Rojo, Puerto Rico. Three strains were similar in morphological and physiological properties and lipid compositions, and they were designated as *Halogeometricum borinquense*. The cells are very pleomorphic and motile. They require at least 8% NaCl for growth, with an optimum at 3.5 to 4 M NaCl and 0.04 to 0.08 M Mg^{2+} at 40 °C, pH 7. The structure of major glycolipid of this species is not known.

Halorhabdus. *Halorhabdus utahensis* was recently isolated from a sediment sample collected from the southern arm of the Great Salt Lake, Utah. Growth was possible from 10 to 30% NaCl, with an optimum at 27%, at pH 5.5 to 8.5.

Halorubrum. *Halorubrum saccharovorum* was isolated from a saltern in San Francisco. The cells grow optimally at 3.5 to 4.5 M NaCl. *Halorubrum coriense* isolated from a saltern along Corio Bay near Geelong, Australia, grows optimally at 2.2 to 2.7 M NaCl. *Halorubrum distributum* was isolated from saline soils in Russia. The cells grow best in media containing 2.5 to 4.3 M NaCl. *Halorubrum lacusprofundi* isolated from Deep Lake, Antarctica is exceptional for halobacteria in that the cells are able to grow at as low as 4 °C. The optimum condition is 2.5 to 3.5 M NaCl and 31 to 36 °C. *Halorubrum sodomense* was isolated from the surface water sample of the Dead Sea. The cells grow in media containing between 0.5 M (in the presence of 1.5 to 2 M Mg^{2+}) and 4.3 M NaCl, and optimum growth is achieved at 1.7 to 2.5 M in the presence of 0.6 to 1.2 M Mg^{2+}, a condition close to that for *H. gomorrense* isolated also from the Dead Sea. *Halorubrum trapanicum* was originally isolated from solar salt in Trapani, Sicily. The cells grow from 2.5 to 5.2 M NaCl. Some strains of this species with apparently different morphologies have been circulated for many years, and finally a neotype strain was deposited as NCIMB 13488.

Halorubrum vacuolatum isolated from the alkaline soda lake, Lake Magadi, Kenya, is the only alkaliphilic species in this genus. It grows optimally at 3.5 M NaCl, pH 9.5. No glycolipids have been detected, whereas most other species of the genus *Halorubrum* are characterized by the presence of glycolipid, sulfated diglycosyl diether-3, but an exception has been reported: sulfated diglycosyl diether-5 was found in *Hrr. trapanicum*.

Haloterrigena. *Haloterrigena turkmenica* was proposed to accommodate the following three strains. *Halococcus turkmenicus* isolated from saline soils collected in Turkmen (Turkmenistan) grows optimally in media containing 15 to 25%. NaCl. A strain designated as GSL-11 was isolated from the Great Salt Lake.

Recently, another species *Htg. thermotolerans* was isolated from a solar saltern in Puerto Rico. This is the most thermotolerant extreme halophile known so far. The optimum temperature is 50 °C, and growth is possible up to 60 °C. Optimum NaCl concentration for growth is 3.0 to 3.5 M.

Natrialba. *Natrialba asiatica* was isolated from beach sands with granular salts attached in Japan and *Nab. taiwanensis* was isolated from solar salts produced in Taiwan. *Natrialba asiatica* is able to grow at 2.0 to 5.3 M NaCl, but requirement for NaCl for growth is partially replaceable by KCl. Growth is obtained in a medium containing 1 M NaCl and 1.6 M KCl, but no growth occurs in 2.6 M KCl without added NaCl. Both *Nab. asiatica* and *Nab. taiwanensis* are exceptional in the family Halobacteriaceae in that they are not pigmented. The contents of bacterioruberins were less than 0.1% of that of *Hbt. salinarum*. Cultivation under illumination does not cause pigmentation of cells on agar slopes. They have a glycolipid disulfated diglycosyl diether-1.

Natronobacterium magadii, an alkaliphilic halobacterium isolated from Lake Magadi, Kenya, was transferred to the genus *Natrialba* as *Nab. magadii*. Growth occurs in media containing 2 to 5.2 M NaCl, with an optimum at 3.5 M at pH 9.5. No glycolipid has been detected in *Nab. magadii*, but the *Natrialba* strain SSL1 isolated from the saline and alkaline brine (7.2 g salts/l, pH 9.0) of Sambhar Salt Lake, India has a minor amount of glycolipid diglycosyl diether-4. Recently, a variety of extreme halophiles were isolated from hypersaline ponds in Northern China: "*Natronobacterium chahanensis*", "*Nbt. chahannaoensis*", "*Nbt. innermongoliae*", and "*Nbt. wudunaoensis*." These strains may belong to the genus *Natrialba* as judged from their 16S rRNA gene sequences.

Natrinema. The members of this recently established genus were isolated many years ago from salted hide and salted codfish; thus, they probably originated from solar salts used for the salting. They have long been assigned to several species of the genus *Halobacterium*, but a recent detailed study has established the new genus *Natrinema*, which includes *Natrinema pellirubrum* and *Nnm. pallidum*. These strains grow best in media containing 3.4 to 4.3 M NaCl. The third species *Nnm. versiforme* was isolated recently from Aibi salt lake in China. It also shows optimum growth at 3.4 to 4.3 M NaCl in the presence of 0.15 M $MgCl_2$. All these species have several unidentified glycolipids.

Natronobacterium. The alkaliphilic members of Halobacteriaceae form a distinct physiological group, because they require not only high NaCl concentrations, but also high pH and low Mg^{2+} concentrations for growth. They have been isolated from a variety of alkaline,

hypersaline lakes and soils. Microscopically, the isolates consist of rods and cocci and were accordingly separated into two genera, *Natronobacterium* and *Natronococcus*. The genus *Natronobacterium* used to contain four species as described earlier, but after phylogenetic analyses *Nbt. gregoryi* turned out the only species of this genus. *Natronobacterium gregoryi* was isolated from the solar salt pans at Lake Magadi, Kenya and grows best in media containing 3.5 M NaCl at pH 9.5 to 10.0. The cells lack major amounts of glycolipids in their membranes. Recently, the group of W.D. Grant has isolated a variety of alkaliphilic Archaea from several soda lakes in the Kenyan section of the East African Rift Valley (27,28).

Natronococcus. *Natronococcus occultus* isolated from the solar salt pans at Lake Magadi, Kenya grows optimally at 3.5 to 3.6 M NaCl, pH 9.5. *Natronococcus amylolyticus* isolated from the same lake grows optimally at 2.5 to 3.4 M NaCl, pH 9.0. The cells of these species retain the coccoid form even in distilled water, as do the cells of the strains of genus *Halococcus*.

Natronomonas. Alkaliphilic strains were isolated from highly saline and alkaline lakes of Wadi Natrun, Egypt, located in a desert depression west of the Nile delta. All lakes showed pH values of approximately 11 and a total salt content of generally more than 30%. Main brine components were chloride, carbonate, sulfate, sodium, and a minor amount of potassium. The concentrations of Mg^{2+} and Ca^{2+} were below detection level. An isolate *Natronomonas pharaonis* grows in media containing 2.2 to 5 M NaCl at pH 7.7 to 9.3, with 3.5 M as an optimum.

Natronorubrum. Two alkaliphilic Archaea were isolated from sediment of the Bange Soda Lake (pH 10, 18 °C) in Tibet. They required at least 12% NaCl and pH between 8 and 11 for growth. Optimum NaCl concentrations were 22.5 and 20%, respectively. They were designated as *Natronorubrum bangense* and *Natronorubrum tibetense*.

Physiological Characteristics of Halobacteria

Archaeal halophiles have a number of unique features not found in bacterial and eukaryal halophiles. Some of the features are described later (29).

Bacteriorhodopsins. Many halobacteria have retinal-based pigments capable of the light-mediated translocation of ions across the cell membrane (30). The best known of these is the bacteriorhodopsin (bR). When *Hbt. salinarum* is grown under low oxygen tension and illumination, purple membranes, hexagonal crystalline patches containing bR as the only proteins, are formed in the cell membrane. The bR with one molecule of retinal absorbs light and catalyzes an outwardly directed transfer of protons across the membrane. ATP is generated as the protons pass back into the cell through a membrane-bound ATPase. Some strains of the genera *Haloarcula* and *Halorubrum* are known to produce similar bR proteins.

Other retinal pigments are halorhodopsin (inwardly directed Cl^- pump), sensoryrhodopsin, and phoborhodopsin. The last two are responsible to phototactic responses of halobacteria. Because halobacteria grows perfectly well in the dark, the systems are not essential for their growth, but there is no doubt that the possession of bacteriorhodopsin confers survival value on cells under sunlight exposure in natural hypersaline environments.

Carbon Dioxide Fixation. Cells of halobacteria have been shown to fix carbon oxide. Thus, halobacteria might contribute significantly to the light dependent fixation of carbon dioxide in the hypersaline environments. The primary reactions involved have been suggested to be reductive carboxylation of propionate, glycine synthase reaction, or formation of pyruvate (22). The enzyme was of Calvin cycle, ribulose-1, 5-bisphosphate-carboxylase/oxygenase, was purified from *Hfx. mediterranei* grown heterotrophically, but further extensive studies are required for the elucidation of mechanism of carbon dioxide fixation in nature.

Gas Vesicles. Some halobacterial strains, *Hbt. salinarum* and *Hfx. mediterranei*, produce buoyant, intracellular gas-filled organelles called *gas vacuoles* consisting of aggregates of individual gas vesicles. It is believed that the accumulation of gas vesicles allows the halobacterial cells in natural hypersaline niches to float at the surface, thus increasing the availability of both oxygen for respiration and light for the photophosphorylation using bacteriorhodopsin in the case of *Hbt. salinarum*.

Halocins. Halocins are bacteriocidal proteins produced by halobacteria. Halocin producers make growth inhibition zones around colonies grown on lawns of other halobacterial cells. It is now believed that the production of halocin is a practically universal feature of noncoccoid halobacteria. Well-characterized halocins include Halocin H4 produced by *Hfx. mediterranei* R4, Halocin H6 from *Hfx. gibbonsii*, Halocin Hal R1 produced by a *Halobacterium* sp. GN1, and Halocin S8 produced by an unidentified halobacterium strain S8a isolated from salt crystals from the Great Salt Lake (31). A recent investigation of 12 brine samples obtained from Israel, California, and Spain, however, failed to detect any halocin activities. Thus, the contribution of halocins in the competition between different halobacteria in hypersaline environments might not be so strong (32).

HALOPHILIC BACTERIA

To isolate bacterial halophiles from hypersaline environments, Bacto-peptone may be used as a component of media to inhibit growth of archaeal halophiles, or penicillin may be added. Because it is not possible to cite all of the original references in the following descriptions, only a relevant review (33) and representative papers are cited.

Bacillus and Related Genera

There have been some reports on halophilic strains of *Bacillus*. A strictly aerobic, gram-positive, rod-shaped, spore-forming, moderate halophile was isolated from a piece of rotting wood on the seashore of Nauru Island in

the Central Pacific Ocean. A taxonomic study included the strain into the genus *Bacillus* as *Bacillus halophilus*.

A group of 91 moderately halophilic, gram-positive, rod-shaped strains were isolated by a Spanish research group from enrichments prepared from water samples of the Dead Sea collected 57 years ago by B. Volcani. All strains formed endospores and were motile, strict aerobes. They grow in media containing 5 to 25% (w/v) total salts, (a mixture of salts of the same composition of seawater) and showed optimum growth at 10% salts. Eighteen strains chosen as representatives from physiological and biochemical characterizations were designated as *Bacillus marismortui*.

Strains of another moderately halophilic species *Bacillus salexigens* were isolated from salterns and hypersaline soils located in different geographic areas of Spain.

Two moderate halophiles isolated from hypersaline sediments of the Great Salt Lake were designated as representatives of a new genus *Halobacillus* as *H. litoralis* and *H. trueperi*. This genus now comprises *Halobacillus halophilus*.

Low G + C Cocci

Marinococcus albus is a gram-positive motile moderately halophilic coccus isolated from solar salterns. Strains of the species are nonpigmented, able to grow over a wide range of NaCl concentrations with optimum growth at 1 to 2.5 M NaCl. *Marinococcus halophilus* is also able to grow optimally at 1 to 2.5 M NaCl. The cells are pigmented a yellow-orange and many strains have been isolated from salterns, saline soils, sea sands, or salted mackerel.

Salinicoccus roseus is a pink-red-pigmented moderately halophilic coccus isolated from soils and ponds of a solar saltern in Alicante, Spain. Optimum growth occurred at 10% NaCl. *Salinicoccus hispanicus* isolated from the same location is another species of this genus. They are pigmented a reddish orange and grow optimally at 10% salts.

Tetragenococcus halophilus is a moderately halophilic lactic acid bacterium easily isolated from salted fermented foods containing more than 25% NaCl, for example, salted anchovies. The cells are tetrad cocci and grow in the presence of wide range of NaCl concentrations, 1 to 25%, with optimum growth at 7 to 10% NaCl. *Tetragenococcus muriaticus* is another species isolated from fermented squid liver sauce.

High G + C Cocci

Nesterenkonia halobia is a moderate halophile isolated in Japan from an imported solar salt. G + C content is 70 to 71.5 mole%. The strain grows best in Sehgal and Gibbons complex medium or Nutrient Broth with 1 to 2 M NaCl or KCl. Recently, six coccoid moderate halophiles isolated from ponds and saltern in Huelva, Spain were shown to belong to this species. Another *Micrococcus* sp., "*Micrococcus varians* subsp. *halophilus*" strain 41 to 3 isolated in Japan from a mash containing 18% imported solar salt for soy sauce fermentation has been used for physiological characterization and studies on extracellular enzyme production (8). This strain is unique in its ability to grow abundantly in media containing high concentrations of salts other than NaCl, such as 1.5 M LiCl, 3.0 M KCl, 2.0 M RbCl, 2.5 M CsCl, etc. (4).

Actinomycetes

The extremely halophilic actinomycete *Actinopolyspora halophila* was isolated in a laboratory in Ottawa as a contaminant on unsterilized plates of complex medium containing casamino acids, yeast extract, and salts including 25% NaCl. The strain is extraordinary in that it requires a minimum salt concentration of 12% for growth in liquid media, and best growth occurred in liquid medium between 15 and 20% NaCl. Thus, this is a bacterial extreme halophile, sensu stricto. Another species of this genus, *A. mortivallis* was isolated from a soil sample obtained from Death Valley, California. It is moderately halophilic, with optimum growth around 10 to 15% NaCl. *Actinopolyspora iraquiensis* isolated from a saline soil sample obtained in Iraq grows optimally at 10 to 15% NaCl.

Nocardiopsis halophila is another moderate halophile isolated from a saline soil sample obtained in Iraq. This organism grows in media containing NaCl up to 20%.

Flavobacterium-Bacteroides

Moderately halophilic orange and yellow pigmented, rod-shaped bacteria are readily cultured from Organic Lake, a hypersaline meromictic lake in the Vestfold Hills region of the Australian Antarctic Territory. Two new halophilic species *Flavobacterium gondwanense* and *Flavobacterium salegens* were proposed. They grow up to 20 and 15% NaCl, respectively, with 5% as optimum.

Proteobacteria

Members of the genus *Rhodospirillum* grow preferably photoheterotrophically under anaerobic conditions in the light, but they also grow under microaerobic to aerobic condition in the dark. A moderately halophilic species *Rhodospirillum salinarum* grows faster under aerobic conditions in the dark at 6 to 15% NaCl. Under anaerobic conditions in the light, the cells are more halophilic requiring 15 to 21% NaCl for optimum growth. This strain was isolated from terminal crystallization ponds of solar salt production plants in Portugal. *Rhodospirillum salexigenes*, another moderately halophilic species isolated from seawater, grows between 5 and 20%, with the optimum at 6 to 8% NaCl.

Many halophilic strains belonging to the gamma subclass have been isolated from various extreme environments. Recently, many of them have been reclassified into the family Halomonadaceae, which comprises three genera, *Halomonas*, *Chromohalobacter*, and *Zymobacter* (34). *Zymobacter palmae*, the only species of the genus, has not been reported to be halophilic. The genus *Halomonas* was created to accommodate a group of moderately halophilic, gram-negative, rod-shaped strains isolated from a solar salt facility in the Netherlands by direct surface spread plating on agar plates containing

8% solar salt produced at the study site. The strains were able to grow over a wide range of salt, from 0 to 32% salt at temperatures of 23 to 37 °C, and from 3.5 to 20% salt at 15 °C and 45 °C. Optimum growth was obtained in complex media containing 3.5 to 8% solar salt at 30 to 37 °C. These typical moderate halophiles were designated as *Halomonas elongata*. Many species of the genus *Halomonas* have been isolated from salterns, The Dead Sea, Antarctic saline lakes, etc. The following are concise descriptions of some species.

Halomonas pantelleriense, with an optimum at 10% NaCl, was isolated from the hard sand of the volcanic lake of Venere in the island Pantelleria in the south of Sicily, Italy by enrichment in a medium with 10% NaCl, pH 9.0. *Halomonas salina* isolated from various saline habitats in Spain grows optimally at 5% salts at 32 °C and 7.5% salt at 42 °C. *Halomonas eurihalina*, which shows optimum growth in media containing 15% salt at 42 °C, are isolated from various hypersaline habitats, as well as seawater. *Halomonas variabilis* is one of the many moderate halophiles isolated from the hypersaline surface water of the north arm of Great Salt Lake. The cells grow well between 1.2 M and 4.9 M NaCl with optimum growth at 1.6 M. *Halomonas halophila* isolated from hypersaline soils of Alicante, Spain is a moderate halophile that grows best at 7.5% salt at 32 °C. *Halomonas halodenitrificans* isolated from meat-curing brines requires at least 3% NaCl and grows optimally at 4.4 to 8.8%. The following species are slightly halophilic or halotolerant: *Halomonas aquamarina*, *H. cupida*, *H. desiderata*, *H. halmophila*, *H. halodurans*, *H. magadii*, *H. marina*, *H. meridiana*, *H. pacifica*, *H. subglaciescola*, and *H. venusta*.

Chromohalobacter marismortui was isolated from the Dead Sea. The cells are yellow or violet blue pigments and grow optimally at about 10% salts. *Chromohalobacter canadensis* NRC 42112 was isolated as a contaminant on a 25% NaCl-agar plate in a laboratory in Ottawa. The cells grow optimally at 8% NaCl at 30 °C, but at 45 °C they require at least 8% NaCl for growth. *Chromohalobacter israelensis* Ba1 was isolated from unrefined solar salt obtained from the Dead Sea. Optimum growth occurred at 8% NaCl and 30 °C.

Pseudomonas beijerinckii was isolated from salted beans and remains a valid species of the genus *Pseudomonas*, but 16S rRNA gene sequence suggested strongly that this species is a member of the family Halomonadaceae.

Other Halophilic Bacteria

Arhodomonas aquaeolei was isolated from a petroleum reservoir production fluid derived from subterranean brine. The cells grow between 6 and 20% NaCl, and optimum growth occurs in the presence of 15% NaCl.

Several strains of moderately halophilic bacteria were isolated from the head of an oil-producing well on an offshore platform in southern Vietnam. One strain that grows at 0 to 3.5 M NaCl with an optimum of 0.85 M (5%) NaCl could degrade n-hexadecane, pristine, and some crude oil components, and was designated as *Marinobacter aquaeolei*.

Two moderately halophilic strains were isolated from kusaya gravy, a traditional Japanese fermented brine. They grow in media containing 0.5 to 9.0% NaCl (w/v) with an optimum of 3%, and were designated as *Marinospirillum megaterium*. *Marinospirillum minutulum*, was isolated from putrid infusions of marine mussels.

Salinivibrio costicola, formerly *V. costicola*, is a moderate halophile originally isolated from rib bone in a sample of Australian bacon, but similar strains have been isolated from various hypersaline environments. In a survey of microbial populations in a multipond saltern, vibriolike strains were shown to be dominant organisms in saltern ponds that had intermediate salt concentrations of 10 to 15%.

Cyanobacteria

Cyanobacteria are widely distributed and a diverse collection of unicellular to multicellular oxygenic photosynthetic bacteria. It is believed that they are common inhabitants of extremely saline habitats. Species commonly found in hypersaline habitats are *Aphanothece halophytica*, *Aphanocapca marina*, *Chroococcus minor*, *Entophysalis granulosa*, *Microcoleus chthonoplastes*, *Schizothrix arenaria*, *Spirulina subsalsa*, etc. Only *A. halophytica* and *S. arenaria* are found in salinities of 25 to 33%. Sometimes cyanobacterial cells form mats, from which halobacterial strains are also isolated (2). The presence of microbial mats on the bottom of ponds of salterns is effective to seal the bottom and prevents the leakage of brines.

HALOPHILIC EUKARYA

Dunaliella is the best known and possibly the most ubiquitous Eukarya in hypersaline environments. It is a unicellular green alga that often assumes an orange to red coloration because of the synthesis of β-carotene in strong brine. It is an obligatory phototrophic, oxygenic, aerobic organism. As primary producers, *Dunaliella* may introduce new organic matter into brine up to NaCl saturation. The cells achieve osmotic balance by intracellular accumulation of glycerol compatible with protein and enzyme functions (2,35,36). Another green algae encountered in hypersaline environments is *Asteromonas gracilis* that grows at NaCl concentrations of up to 4.5 M.

Several *Dunaliella* species are able to proliferate over nearly the entire range of salt concentrations while maintaining a low internal salinity. *Dunaliella salina*, *D. viridis*, *D. parva*, *D. tertiolecta*, and *D. minuta*. The first two species *D. salina* and *D. viridis* have been reported from numerous hypersaline thalassic (marine) and athalassic environments. *Dunaliella parva* is the only species found in the Dead Sea.

Dunaliella salina grows best in 12% salinity and tolerates up to 35%, whereas *D. viridis* grows optimally in 6 to 9% salinity and tolerates up to 23% salinity. Thus, *D. salina* was dominant in the northern arm of the Great Salt Lake (discussed later) and in the southern arm *D. viridis* is a dominant species.

ECOLOGICAL ASPECTS OF HYPERSALINE ENVIRONMENTS

Enumeration of Archaeal and Bacterial Halophiles

Enumerations of halophilic microorganisms in natural brines are difficult to conduct. In addition to the general drawbacks of direct microscopic counting (difficulty in distinguishing between living and nonliving cells), the pleomorphic nature of some halobacteria makes it difficult to distinguish them from inorganic particles of similar size. A. Oren of the Hebrew University of Jerusalem designed a method to estimate cell numbers of noncoccoid halobacteria in natural brines by using bile acids as specific lysis-reagents of noncoccoid halobacteria as described earlier (see Isolation of Halobacteria) (37,38). A 10 mL aliquot of brine was centrifuged. To another aliquot of the same brine was added 0.1 mL of 0.3% sodium deoxycholate, incubated for five minutes, then centrifuged. Supernatants were removed, the remaining brines stirred to make even cell suspension, and cell densities were counted under phase contrast microscope. Sodium deoxycholate has been shown to lyse cell of halobacteria examined so far except for members of the genera *Halococcus* and *Natronococcus*, which possess rigid cell walls as described earlier. Cells of eubacterial halophiles are also resistant to cholate. Thus, the difference in cell counts between with and without sodium cholate treatment represents the cell numbers of noncoccoid members of the Halobacteriaceae.

Another approach is the use of antibiotics specific for Archaea and bacteria. Addition of Archaea-specific inhibitors, for example, anisomycin, to the Dead Sea brines virtually abolished the incorporation of amino acids, whereas the addition of bacterial inhibitors, for example, cycloheximide, had little effect. This is an evidence to support the view that halobacteria make up the overwhelmingly dominant population in environments with salt concentrations of more than 25% (39). Although generation times of isolated halobacteria are several hours in general in organic complex media, generation times of halobacterial population in natural brines were measured as 54 to 120 hours.

16S rRNA Sequences Recovered from Hyper Saline Environments

In recent years, diversity of archaeal and bacterial halophiles in hypersaline environments has been studied by analysis of 16S ribosomal RNA genes amplified by PCR from DNA extracted directly from samples using archaeal, bacterial, or universal primers. Molecular phylogenetic studies indicate a much greater phylogenetic and probably physiological diversity of halophiles than was assumed previously. For example, several archaeal sequences obtained from sample of crystallization pond in Alicante, Spain were found to be different from all other 16S rRNA gene sequences of isolated species (40). Novel bacterial sequences were recovered from samples of crystallizer ponds with salinity from 30 to 37% (41).

The same approach was taken for sediment samples from the Colne Point Salt Marsh located in northeast Essex, United Kingdom (42). Although the salinity of the sample was only about 0.8 M NaCl, a variety of sequences were detected in gene libraries related to different halobacteria. Some sequences were closely related to those from genera *Natrialba* and *Haloferax*. This is an unexpected result because cultivated halobacteria, except for coccoid forms, are known to lyse in hypotonic solution, such as 0.8 M. It has been speculated that within the soil granules there might be microniches with sufficiently high NaCl concentration to maintain the life of halobacteria. The author of this article has succeeded in isolating rod-shaped, pigmented halobacteria from the sands of an Indonesian saltern, which had been washed repeatedly with seawater.

Solar Salterns

Solar salterns are artificial, extremely thalassohaline (seawater derived) environments consisting of multiponds of a discontinuous salinity gradient, which are commercially operated for producing NaCl. In the course of the evaporation of seawater by the solar irradiation and wind blow, a sequential precipitation of calcium carbonate, calcium sulfate (gypsum), and sodium chloride (halite) occurs, but magnesium chloride remains soluble.

Extensive microbiological studies have shown that these ponds can be divided into three categories. In the first step, seawater is concentrated to about three times its original salinity. The microflora is similar to that of seawater, with great species diversity and a low cell number of individual species. At the second step, salinity is increased from three to seven times of seawater, and brines become dark owing to the growth of *Dunaliella* spp. and many species of moderate halophiles. The third step is characterized by hypersalinity, up to saturation, and often, a dense microbial community of halobacteria and red *Dunaliella* spp. causes bright red color. After crystallization of halite, bitterns remain, which are rich in Mg^{2+} and apparently devoid of life. This has been believed to be due to extremely high Mg^{2+} concentrations, but it is also shown that halobacteria are entrapped within the fluid inclusions of salt crystals. Cells of halobacteria are commonly visible within fluid inclusions in salt crystals formed in salterns and salt lakes (43,44) (see Salt Deposits/ Evaporites/ Rock Salts). Those entrapped halophiles in solar salt (10^5 to 10^6 cfu/g salt) are able to survive at least for several years. Thus, when the solar salt was used for the curing of hides or preservation of harvested cod fish, herring, etc., the halophiles caused spoilage of those products by forming “pink eye” on salted fish or “red heat” on cured hides.

In a study of salterns in Alicante, Spain (45), scores of colonies according to the pigmentation was used as an indicator of bacterial and archaeal halophiles; red to pink colonies were scored as halobacteria. The nonpigmented organisms were shown to be more common in ponds containing up to 6 to 14% salt, whereas red-pigmented colonies began to appear at about 18% salt and was dominant at about 27 to 30% salts. Isolated nonpigmented strains grew in 2 to 10% salt, and predominant genera were *Salinivibrio*, *Flavobacterium*, *Alcaligenes*, and *Chromohalobacter*. On the other hand, the red-pigmented strains required at least 20% salt. The bile acid method as described earlier was applied

successfully to estimate the contribution of halobacteria to the bacterial mass and activity in solar salterns (38).

Many other ecological studies have been conducted on other salterns such as a major salt-producing plant in Mexico, Western Salt Company near San Diego, California, a solar salt facility located in Bonaire, Netherlands Antilles, etc. (46).

Halobacteria are the most halophilic organisms as described earlier, and they form the dominant microbial population when hypersaline waters approach saturation, frequently imparting a red or pink coloration to the brines. The carotenoid pigments of halobacteria trap solar radiation, increasing the ambient temperature and evaporation rates in salterns, thus promoting rapid precipitation of salts. Neutral salt lakes and solar salterns may contain 10^7 to 10^8 cells/mL as judged from microscopic examination (37). Salt yields may be diminished in nutrient-poor pond systems, in which fertilizers may be added to stimulate the growth of halophiles.

Solar Lake, Sabkhas, Pools, and Lagoons of Sinai

Sea-marginal environments subject to hypersalinity include a variety of tidal flats and depressions, salt marshes, lagoons etc. The Red Sea coast of the Sinai Peninsula is noted for several hypersaline environments. Solar Lake is a small (50×140 m) sea-marginal pond. Salinities at mixing periods in summer are 15 to 18% and in the stratification period, September to July, a salinity gradient occurs from 6% at the surface to 16 to 18% at the bottom. Microbiological studies have shown that the predominant microbes are cyanobacteria (*A. halophytica*, *A. littoralis*, *Oscillatoria salina*, etc.), diatoms, and heterotrophic bacteria (2).

Sabkhas are smooth, flat plains or salt flats that gradually grade upward from a tropical sea, or they are separated from the sea by a low or permeable barrier. Sometimes hypersaline brines are formed within the sediments and occasionally on top of them. Many halophilic cyanobacteria (*A. halophytica*, *S. arenaria*, etc.) and halobacteria of the genus *Haloarcula* have been isolated.

The Dead Sea

The Dead Sea is an athalassohaline (not seawater derived), terminal lake in the Syrian-African rift valley, 410 m below sea level, the lowest point on the earth's terrestrial surface. The inflow comes mainly from the Jordan River, and the only outlet of water is evaporation. The level is reported to be lowering at a rate of 0.5 m annually owing to extensive exploitation of the Jordan River and the extreme aridity of the region (annual precipitation is about 60 mm). The chemical composition of surface water is 1.8 M Mg^{2+}, 1.7 M Na^{+}, 0.4 M Ca^{2+}, with a total salinity of 33% (wt/vol). The Dead Sea has been used as an economic resource for more than 10,000 years for obtaining salts.

The Dead Sea had long been considered a hypersaline hostile environment devoid of any living organisms. The first microbiological studies of the Dead Sea were conducted by Elazari-Volcanii in the 1930s. The microorganisms observed included many halobacteria and a phytoflagellate of the genus *Chlamydomonas* or *Dunaliella*. So far many extreme and moderate halophiles have been isolated from the Dead Sea; *Har. marismortui*, *Hfx. volcanii*, *Hrr. sodomense*, *H. gomorrense*, *C. marismortui*, *C. israelensis* Ba1, etc. Brine shrimp *Artemia*, densely populated in the Great Salt Lake, is absent. Recently, many halobacterial strains were isolated from samples of the Dead Sea water collected 57 years ago. Most of them were shown to belong to the genera, *Haloferax*, *Halobacterium*, and *Haloarcula* (47).

A. Oren conducted quantitative studies on the microbiology of the Dead Sea (48). In the winter of 1979 to 1980, massive rain floods diluted the upper water layer. In the summer of 1980, mass development of the green unicellular alga *D. parva* (8,800 cells/mL) and red Archaea (2×10^7 cells/mL) was observed. The dense community of red halobacteria imparted a reddish color to the lake. Growth of halobacteria depends on organic matter produced by *D. parva*, the only primary producer in the lake. The *Dunaliella* community declined rapidly at the end of the year 1980, but a stable community of halobacteria of about 5×10^6 cells/mL subsisted in the upper water layer for more than two years. This bloom disappeared at the end of 1982 as a result of complete mixing of the water column. During the period of 1983 to 1991, the lake was holomictic and no *Dunaliella* cells were observed and viable bacterial count was very low. Unprecedented heavy rain floods during the winter of 1991 to 1992 caused a rise of water level by almost 2 m. Thus, the upper water column was diluted down to 70% of the ordinary salinity. In May 1992, *Dunaliella* appeared again (up to 3×10^4 cells/mL) and a bloom of red halobacteria (3×10^7 cells/mL) imparted a red coloration of the lake. A. Oren intensively demonstrated the role of glycerol in the nutrition of microorganisms of the Dead Sea (49).

In connection with the relatively rapid decline of the bloom, Oren and coworkers observed electronmicroscopically the presence of high numbers of viruslike particles in water samples from the Dead Sea in October 1994, when the halobacterial number was declining. The viruslike particles, 0.9 to 7.3×10^7 /mL, outnumbered the halobacteria. In November 1995 to January 1996, the number of viruslike particles declined to less than 10^4/mL (50).

The Great Salt Lake

This lake is located in the arid Great Basin deprived of rainfall by the Sierra Nevada. The Great Salt Lake is the remnant of a prehistoric freshwater lake, Lake Bonneville. Geologic evidence from the area surrounding the lake basin indicates at least ten cyclings within the last 100,000 years from freshwater Lake Bonneville to the highly saline Great Salt Lake passing through a marinelike salinity stage twice during each cycle. The present day Great Salt Lake is merely the latest low water point in the most recent cycle (51). In 1959, a semipermeable rock railroad causeway was completed across the lake. Because there is a considerable evaporation of water, as a result of the arid climate, and 95% of the water entering the lake flows into the south end, the causeway has created practically two lakes at

different elevations. The water chemistry of the Great Salt Lake is in marked contrast to that of the Dead Sea. The ion composition of water from the northern arm of the Great Salt Lake is essentially the same of concentrated seawater, approximately 10 times, whereas the Dead Sea is principally a magnesium chloride lake. The southern arm is less saline.

One more important physical difference between the Great Salt Lake and the Dead Sea is temperature. The water temperature of the Dead Sea is generally between 21 and 36 °C, whereas that of the Great Salt Lake ranges from −5to + 35 °C. The air temperature over the lake ranges from −30to + 45 °C.

Microbiological study of Great Salt Lake began as early as 1930s by Zobell and coworkers. The northern arm community consists primarily of, on the order of biomass, halobacteria, two algae *D. salina* and *D. viridis* as the primary producer, *Artemia salina*, a brine shrimp, and brine fly. Peak densities of *D. salina* reported were 3 to 10×10^3/mL.

Moderate halophilic bacteria such as *Pseudomonas halophila*, *H. variabilis*, *H. litoralis*, *H. trueperi* predominate in surface water of the south arm, whereas halophilic Archaea such as *Halobacterium* spp., *Halococcus* spp., *Haloterrigena* strain GSL11, and *Hrd. utahensis* make up a predominant population in the north arm. The number of bacterial and archaeal halophiles range from 4 to 10×10^7 cfu/mL. In spite of the high numbers present, rates of breakdown of organic compounds, for instance, hydrocarbons, are reported very low.

Ammonia is the key nutrient controlling biological process in the Great Salt Lake. Little or no ammonia is detected in winter, and *Dunaliella* survives in a dormant state; in summer bacteria and Archaea excrete ammonia, on which the algae multiply, then the brine shrimp population continues to excrete ammonia, supporting a dense algal and archaeal bloom (36).

The picture described in the preceding text may have lost some of its validity because it is reported that the salinity of the north arm has decreased recently as a result of massive rainfall.

Alkaline Saline Habitats

The stable, naturally occurring alkaline hypersaline environments are the results of geologic, geographic, and climatic conditions. Because of the high level of carbonate minerals from the surrounding rocks, the pH is quite high, 10 to 12; thus, contents of soluble magnesium and calcium ions are very low (52). Lake Magadi of Kenya, Wadi Natrun lakes in Egypt, Sambhar Salt Lake in India, and Bange Soda Lake in Tibet are sites that have been studied geochemically and microbiologically. Total salinity may vary, from 70 to 394 g/L.

These lakes are densely populated by halophilic and alkaliphilic Archaea as well as alkaliphilic halophilic bacteria. The blooms of alkaliphilic halobacteria are observed sometimes. The following strains have been isolated: *Natrononococcus* spp., *N. pharaonis*, from Wadi Natrun lake, *Nrr. bangense* and *Nrr. tibetense* from Bange Soda Lake in Tibet. A bacterial halophile, *H. pantelleriense*, was isolated from Venere Lake, Pantelleria island of Italy, *Bacillus haloalkaliphilus* from Lake Gabara in the Wadi Natrun. From Lake Magadi, Kenya, many halophiles have been isolated: *Hrr. vacuolatum*, *Ncc. occultus*, *Ncc. amylolyticus*, *H. magadii*, etc.

An extensive microbiological study was conducted on many soda lakes in the East African Rift Valley (53). The salinities of these lakes range from 5% total salts to saturation. The microflora was astonishingly diverse: many gram-negative alkaliphiles related to the family Halomonadaceae, whereas gram-positive alkaliphiles were found in both high and low G + C divisions, including the genus *Bacillus*. On the other hand, alkaliphilic archaeal isolates were closely related to *Natronobacterium* and *Natronococcus*.

Antarctic Hypersaline Lakes

There are a few cold saline lakes in the Antarctic, such as Lake Vanda, Lake Bonney, Don Juan Pond, Deep Lake, etc. (2). Don Juan Pond is an extremely hypersaline, ice free body of water in the Wright Valley, with an average depth of 11 cm. It is essentially $CaCl_2$ brine. According to a report the total ion content was 339 g/L, but it is also reported that there is a seasonal dramatic variation in the composition. There are controversies over the possible life in the presence of high Ca^{2+} content of this pond.

Deep Lake is located in the Vestfold Hills. It is 36 m deep, ice free, and hypersaline throughout (ca 28% salinity); the salinity is believed to be derived from evaporated seawater. *Halorubrum lacusprofundi* is a halobacterium isolated from this lake.

Salt Deposits/Evaporites/Rock Salts

Salt deposits are the relics of ancient hypersaline evaporitic environments now buried, crystallized from brines. Theoretically, the complete evaporation of a column of seawater 300 m deep would precipitate about 4.8 m of salts. There have been many reports on the isolation of halophilic bacteria from subsurface salt deposits and underground brines (43). Norton and coworkers performed typical explorations (54). They isolated halophilic bacteria from two rock salt mines: Winsford salt mine in Cheshire, and Boulby potash mine in Cleveland, England. Both are estimated to be formed around 225 million years ago, and the mining operations were at depth of 170 m and 1,200 m, respectively. Spreads of brines and moist salts that were diluted or dissolved in media produced diverse populations after incubation for two to three weeks. Eighty to 90% of colonies were white- to cream-colored. Efflorescences and moist salts from Winsford had approximately equal numbers of white/creamy type and red bacterioruberin type colonies. In contrast, all Boulby samples yielded only red-pigmented halobacteria.

To recover microorganisms from the inside of rock salts, small fragments (2 to 5 g) of rock salt newly exposed by blasting were selected and dropped into vials of absolute alcohol for surface decontamination. After several hours, the salt fragments were transferred to sterile enrichment media, and incubated at 37 °C. By this procedure, positive enrichment was obtained from approximately 500 g of rock salt inoculum. The efficacy of the surface

sterilization procedure was established by the following control experiments. Orange-pigmented halobacteria were incorporated into halite crystals, and the crystals were placed into a suspension of pink-pigmented halobacteria for four hours, and then transferred to vials of absolute alcohol. Following the immersion for two hours, the crystals were transferred back to media, which yielded only orange-pigmented halobacteria.

Eight rock salt isolates, seven from Winsford and one from Boulby, together with randomly selected 46 red isolates from brine and effloresence were analyzed for their polar lipid patterns. Most of them were assigned to the genera of *Halorubrum*, *Haloarcula*, *Halobacterium*, and *Halococcus*.

The group of Vreeland in the United States did another survey of halophiles in rock salts (55,56). The Waste Isolation Pilot Plant is an underground repository built by the U.S. Department of Energy approximately 650 m below ground level near Carlsbad, New Mexico. The repository is constructed on Permian age Salado salt formation for the safe, permanent, underground disposal of defense-related radioactive wastes. This site has been shown to experience little geologic disturbance since its original deposition. Vreeland' group conducted a survey of the bacterial populations existing within the facility. Bacterial populations heterogeneously distributed throughout the mine, and populations in some mine areas reached as high as 1×10^4 cfu/g NaCl. A biochemical comparison showed the presence of extreme halophiles with wide biochemical diversity. In a most recent survey, they selected a 250 million-year-old salt crystal from the Salado salt formation; delicate crystal structures and sedimentary features indicated the salt has not recrystallized since its formation. After careful sterilization of the surface with strong alkali and acid, they succeeded in isolating a spore-forming bacterial halophile, *Salibacillus marismortui*, from a brine inclusion (57). This finding indicates that spores can effectively be immortal, and has raised fascinating questions. What is the biochemistry that allows the spores to survive for so long? Where else on the earth and to what depth might ancient life be lurking?

The Salar de Atacama located in northern Chile is a large salt deposit (approximately 300 km^2) at 2,700 m above sea level. Samples were taken from water, soils, and sediments of various salt concentrations, 3 to 10% total salts, and many moderate halophiles were isolated, including strains belonging to the genera *Marinococcus*, *Acinetobacter*, etc.

Other Saline Extreme Environments

Solar salts are produced in salterns distributed all over the world, exported and imported, and used in the production of many salted foods. As described earlier, halobacteria are known to be incorporated into the NaCl crystals. The solar salts are also contaminated with halophilic bacteria. Thus, halobacteria are easily isolated from foods containing a high content of solar salts. Many strains of *Hbt. salinarum* and *Halococcus* spp. are isolated from salted foods such as bachalao (dried salted codfish), curing of anchovies, Thai fish sauce containing 25% NaCl. Bacterial halophiles are isolated from salted foods, and some of them are able to grow in complex media containing either 1 to 4 M NaCl or 1 to 4 M KCl (58).

Leaves of a desert plant *Atriplex halimus* are known to excrete salts, predominantly NaCl, to salt bladders through salt glands. The dominant organisms isolated from the salt-coated leaf surfaces were orange-pigmented *Pseudomonas* spp. growing at 0.05 to 20% NaCl.

CONCLUSION

Aerobic halophilic microorganisms in hypersaline environments have been surveyed. It has been a sort of surprise to find so many different halophiles distributed throughout the saline niches. Although strictly anaerobic, extremely halophilic eubacteria (e.g., *Haloanaerobium lacusroseus* isolated from the sediment of hypersaline Retba Lake near Dakar, Senegal) and halophilic methanogenic archaea are known, no obligately anaerobic members have been found in the family Halobacteriaceae. Also, extremely thermophilic halobacteria able to grow at 70 or 80 °Care still to be isolated.

There are many more hypersaline extreme environments not referred to in text that await further geochemical and microbiological studies, for example, Lake Assal of French Somaliland, Zuni Salt Lake in New Mexico, Hot Lake in Washington, hypersaline lakes in Saskatchewan, Canada, Lake Eyre, and other hypersaline lakes in Australia, deepsea hypersaline basins, etc. (2). Novel halophiles are waiting to be isolated from these saline habitats.

The readers are recommended to consult excellent reviews (59,60) on the biotechnological applications of the halophiles.

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HALOPHILES: ANAEROBIC PROKARYOTES FROM HYPERSALINE ENVIRONMENTS

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Numerous aerobic and anaerobic extremophilic microorganisms have been isolated in recent years, but most attention has focused on thermophilic and hyperthermophilic microbes from domain *Bacteria* such as the members of the orders *Aquificales* (e.g., *Aquifex* species) and *Thermotogales* (e.g., *Thermotoga* species), and the family *Thermanaerobiaceae* and from domain *Archaea* such as the members of the orders *Thermoproteales* (e.g., *Thermoproteus* and *Pyrobaculum* species) and *Thermococcales* (e.g., *Thermococcus* and *Pyrococcus* species) (1–4). There are a number of reasons for the interest in such extremophilic microbes: (*1*) The analysis of central metabolism may reveal adaptations to thermal environments, (*2*) modifications in their pathways may lead researchers to identify novel/modified biomolecules, (*3*) the

potential usefulness of extremophilic enzymes in industrial processes due to improved stability of enzymes under a range of adverse conditions, and finally (*4*) the search for new and more ancient organisms after the recognition of archaeal life by Woese and coworkers (5). These studies have provided us with essential information about primary biochemical pathways operating in these organisms, which may unravel the puzzles of early cellular evolution.

Halophiles, a group of extremophilic microorganisms, have also created an interest among researchers. Halophiles have an obligate requirement of NaCl for growth and can thus be differentiated from halotolerant microbes that can grow either in presence or absence of NaCl. Three groups of halophiles are currently distinguished on the basis of NaCl growth requirements (6): (*1*) The slight halophiles, which grow most rapidly in the presence of NaCl concentrations between 2 and 5% (0.34 to 0.85 M), (*2*) the moderate halophiles, which grow most rapidly at NaCl concentrations between 5 and 20% (0.85 to 3.4 M), and (*3*) the extreme halophiles, also called hyperhalophiles, which grow most rapidly at NaCl concentrations ranging between 20 and 30% (3.4 to 5.1 M). The moderate and the extreme halophiles possess a large and diverse array of intracytoplasmic osmotic solutes that enable them to adapt and inhabit terrestrial and subterrestrial ecosystems with high salt concentrations (7). Research on these solutes has provided an insight into the various biochemical strategies employed by these microbes for maintaining and regulating osmotic balance essential for their survival and growth in such harsh environments (8).

We present a review on the characteristics of the strictly anaerobic halophiles that include carbohydrate fermenters, sulfate-reducers, methanogens, and phototrophs, and discuss their ecology and their involvement in the oxidation of organic carbon as an energy source.

THE ENVIRONMENTS AND PHYSICOCHEMICAL CHARACTERISTICS

Saline and hypersaline environments contain between 4% and saturated (34 to 35%) NaCl. The most saline environments observed on this planet include the inland terrestrial lakes (e.g., Dead Sea, and Great Salt Lake) and subterrestrial ecosystems (e.g., oil reservoirs). Solar salterns produced as a result of human activities, estuaries, and shoreline rockpools exposed to intensive evaporation also become extremely saline with NaCl concentrations reaching saturation.

Saline and hypersaline terrestrial ecosystems vary and fluctuate dramatically in ionic composition, total salt concentrations, and pH. As a result of this, a highly versatile and adaptative microflora that can cope with such environmental fluctuations develops. Most saline lakes possess close to neutral pH (e.g., the Great Salt Lake, the Dead Sea, and the Orca Basin in the Gulf of Mexico) but some are alkaline with pH ranging from 9 to 10 (e.g., the Big Soda Lake, the Mono Lake, and the Soap Lake of the Great Basin of the Western United States) (9). Sodium and/or Chlorine ions are the predominant ions in neutral pH environments (e.g., the Great Salt Lake, Lake Assal, and marine salterns), but Mg^{++} and Ca^{++} are also important components (10,11), especially in alkaline brines. Large variations in the sulfate concentrations of saline and hypersaline environments occur with up to 21.22 g/L recorded for the Soap Lake (9). The presence of sulfate is important because sulfate-reducing bacteria play a crucial role in the terminal stages of organic matter decomposition in these environments.

Both salinity and pH are compatible with bacterial activity in oil reservoirs and various halophilic anaerobes have been recovered from these ecosystems (12). The waters in oil formation vary from fresh to salt-saturated, with pH between 5 and 8. Microbes have never been cultured from oil reservoirs whose in situ temperature exceeds 130 to 150 °C, indicating that the principal determinant in limiting microbial life in oil reservoirs is temperature.

Organic Matter and Its Availability in Hypersaline Environments

Most of the organic matter found in hypersaline ecosystems is because of aerobic and/or anaerobic microbes, but some eukaryotes (e.g., the brine shrimp *Artemia salina*, brine flies, and the algae *Dunaliella salina*) also contribute to organic matter availability in these environments. Algae and plants growing nearby may also contribute organic matter into the hypersaline environments (13). Therefore, a large number of biopolymers including cellulose, hemicelluloses, pectine, chitin, and proteins and also monomers such as glycerol or betaine may contribute to the overall carbon cycle within hypersaline environments. The extremely halophilic members of domain *Archaea* contain high concentrations of salts in their cytoplasm, but the halophilic and halotolerant members of domain *Bacteria* have only low levels of salts in their cytoplasm that are regulated by synthesizing or accumulating a large number of different organic osmolytes, also known as compatible solutes (14,15). These osmolytes contribute to the overall carbon cycle in saline and hypersaline ecosystems and are available as growth substrates.

Organic osmotic solutes accumulate under increasing salt concentrations (13,16,17) and include polyols (glycerol and arabitol), sugars and sugar derivatives (sucrose, trehalose, and glucosylglycerol), and amino acids, their derivatives, and quaternary amines (glycine betaine, and ectoines). Glycine betaine has been reported to be present in members of the genus *Halorhodospira* (formerly *Ectothiorhodospira*) (18,19), *Methanohalophilus* (20–23), and *Cyanobacteria* (24). In *Cyanobacteria*, sucrose and trehalose accumulate in osmotically stressed cells (24), whereas glycerol is the major compound that enables growth of the unicellular green alga, *Dunaliella*, in saline habitats (25). A novel class of compatible solutes called ectoines (cyclic N-acetyl diamino acids) have been identified and isolated from *Halorhodospira*, an extremely halophilic archaeon (16,26). Various organic osmotic solutes including β-glutamine, β-glutamate, N-acetyl- β-Lysine, and glycine betaine (20–23,27) have been detected in halophilic methanogens. The strategy of accumulating osmotic solutes to regulate salt concentrations is energetically expensive (8) with synthesis of small

molecules (glycerol, glycine betaine, and ectoines) being less energy consuming than that of the larger molecules (e.g., disaccharides). The synthesis of glycerol, so far identified only in eukaryotic halophiles, the most inexpensive to produce in terms of energy (8). Caldas and coworkers (28) have reported that the osmoprotectant glycine betaine, which accumulates in eukaryotic and bacterial cells, also acts as thermoprotectant in *Escherichia coli*. Recently, Kuntikov and Gorlenko (29) have demonstrated that the interrelation between halotolerance and thermotolerance, which exists in some halophilic green sulfur phototrophic bacteria, cannot be explained based entirely on osmolytes and therefore other factors may also play a role.

Organic Matter Decomposition

Under normal conditions, acetate, a key intermediate in anaerobic degradation of organic matter, is oxidized by sulfate-reducing bacteria and methanogens, thereby preventing its accumulation in nature. However, an abnormal accumulation of volatile fatty acids (VFA) including acetate and hydrogen occurs in sediments with salinities above 15% (30,31) suggesting that the acetoclastic process but also hydrogen oxidation in the presence of electron acceptors such as sulfate and/or carbon dioxide is very limited (31). Skyring (32) reported that acetate oxidation occurred in moderately saline sediments via sulfate reduction. Brandt and Ingvorsen (33) isolated pure cultures of sulfate-reducers capable of acetate oxidation at salinities below 13% but enrichment cultures with greater than 20% NaCl were never obtained. In addition, methanogens isolated to date from saline sediments are specialized in utilizing methylated compounds (e.g., methylamines) (34) rather than acetate.

Hydrogen also accumulates in hypersaline sediments with up to 200 μM dissolved hydrogen being measured in sediments of the Great Salt Lake (35). This seems to indicate that the catabolism of reduced compounds such as butyrate or propionate hardly occurs via interspecies hydrogen transfer. Accordingly, hydrogen and formate but not propionate, acetate, or lactate stimulate the reduction of sulfate from Dead Sea sediments (31). Further proof of this comes from enrichment studies initiated with sediments of a hypersaline lake (340 g/L salts) in Senegal. Acetate, propionate, and butyrate were not metabolized even after two months of incubation (36). A number of hydrogenotrophic moderately halophilic sulfate-reducers such as *Desulfovibrio halophilus*, *Desulfohalobium retbaense*, and *Desulfovibrio oxyclinae* have been reported to grow at NaCl concentrations greater than 15% (8,34). The most halotolerant (12% NaCl) hydrogenotrophic methanogen reported to date was isolated from an oil reservoir (37).

It is evident from the data presented above that even if oxidation of acetate and hydrogen was possible, the process would be slower than that of carbohydrate fermentation and would result in the accumulation of acetate and other VFA in saline and hypersaline sediments. It has been suggested that the energy requirement for the cells to adapt their growth at high salinities is too expensive and this may therefore preclude the existence of some trophic groups of microorganisms usually important for establishing complete mineralization of organic matter (e.g., hydrogenotrophic and/or acetotrophic methanogens and the dissimilatory complete oxidizing sulfate-reducing bacteria) (8). Although, the absence of acetotrophic methanogens in hypersaline environment might be the result of little energy available from the substrate and also the need for these microorganisms to synthesize organic osmoprotectants considered as energetically expensive molecules (8), the thermodynamic parameters of the methanogenic reaction from hydrogen cannot completely preclude the possible existence of hydrogenotrophic methanogens (8). In addition, it is possible that such hypersaline environments may be inhabited by other hydrogenotrophic homoacetogenesis performing anaerobes such as *Acetohalobium arabaticum* (38) and *Natrionella acetigena* (39).

Microbial Ecology

Although there is a marked difference between the microbial community structures of terrestrial hypersaline sediments with NaCl concentration greater than 15% and the slightly or moderately saline ones, photosynthetic bacteria represent the major primary producers in all these environments (40). And although aerobic and anaerobic microbes have been successfully recovered from all terrestrial hypersaline environments, anaerobes have always been considered to be the dominant microorganisms of subsurface terrestrial hypersaline environments such as saline oil fields (12). However, recently, this dogma has been challenged because microbiological studies on subterranean salt deposits have demonstrated that they can also inhabited by the aerobic haloarchaea (41). Interestingly, the authors reported that no difference was observed between the 16S rDNA sequence of surface and subsurface haloarchaea populations. Because the subterranean salt deposits studied are considered to be ancient evaporites of hypersaline environments, the authors speculated that halobacteria may be able to survive for long periods in deep subterranean environments (41). The marine salterns, on the other hand, develop a variety of saline gradients because of the salt extraction process, and not surprisingly, possess the greatest diversity of halophilic and halotolerant bacteria observed in saline and hypersaline environments. Slight halophiles are recovered from the primary ponds of salterns, whereas the intermediary ponds in which the seawater is concentrated to salinities of around 10 to 20% NaCl, contain a larger number of moderate halophiles. The ponds representing the final stages of NaCl crystallization processes are inhabited mainly by the extremely halophilic organisms including aerobic members of the domain *Archaea* (35) and to a lesser extent by members of domains *Bacteria* and *Eucarya*.

The number of extremely halophilic anaerobic members of domain *Bacteria* that have been isolated from anoxic hypersaline environments is far less than the extremely halophilic aerobic archaeal counterpart. The anaerobic extremely halophilic members of domain *Bacteria* include *Halanaerobium lacusrosei*, which grows optimally in the

presence of 20% of NaCl (42) and the phototrophic sulfur-oxidizing *Halorhodospira halochloris* that grows optimally in the presence of 21% NaCl (19,43). Phototrophic sulfur-oxidizers are widespread in a narrow zone of anoxic sediments of sulfide-rich hypersaline environments that receive light. They use various substrates as carbon sources and use sulfide as an electron donor for photosynthesis. Diverse populations of phototrophic sulfur-oxidizing bacteria are encountered throughout the different salt concentrations of marine salterns. The primary ponds of marine salterns (8 to 10% NaCl) contain the most numbers of phototrophs, with moderate and/or extreme halophiles inhabiting the more saturated salt ponds (44). The extremophilic methylotrophic anaerobic archaeon *Methanohalobium evestigatum*, which is unable to oxidize hydrogen and acetate, produces the highest concentration of methane in the presence of 25% NaCl (45).

The sulfide from the anoxic sediments of hypersaline ecosystems is produced mainly by sulfate reduction. Because sulfate is one of the major inorganic components of seawater (25 mM), its concentration increases to saturation in the salterns and hence precipitates in the form of calcium sulfate (gypsum). As a consequence, sulfate is never a limiting factor for sulfate reduction in salterns and in other hypersaline environments and is used by sulfate-reducing bacteria as a final electron acceptor for oxidizing hydrogen and various low molecular weight organic compounds (e.g., lactate, ethanol, etc...). It is generally accepted that both the phototrophs and sulfate-reducers contribute significantly to the turnover of the sulfur cycle in the anoxic zones of hypersaline environments.

Halophilic mesophilic and thermophilic anaerobes have also been isolated from the saline subsurface environments (e.g., oil field environment). Rengpipat and coworkers (46,47) reported their isolation from oil-water injection filters and Gevertz and coworkers (48) reported the first isolation of a halanaerobe, which grew optimally at 2.5% NaCl but did not above 10%, from oil brines. Five moderately halophiles were also isolated from the hypersaline brines of the Southeast Vassar Vertz Sand Unit in United States. These isolates produced acids, solvents, and gases from carbohydrates (49,50). Moreover, these microorganisms were capable of forming ultramicrobacteria under particular physiological conditions, thereby making them suitable candidates for microbial enhanced oil recovery (12).

CHARACTERISTICS, TAXONOMY, AND PHYLOGENY

Halophilic anaerobes have a varied physiology and this is reflected in their phylogenetic distribution. The physiological groups include mesophilic or thermophilic carbohydrate/peptide fermenters, sulfate-reducers, and methanogens. Phylogenetically, they are distributed in domain *Bacteria* as members of the order *Thermotogales*, the green sulfur bacteria, *Deferribacter* assemblage, order *Spirochaetales*, the *Proteobacteria* (α and γ divisions) and orders *Clostridiales* and *Halanaerobiales* of the low G+C DNA containing gram-positive group, and in domain *Archaea* as members of the kingdoms *Crenarchaeota* and *Euryarchaeota*. The scheme for phylogeny described in this section has been modified from Collins and coworkers (51) and takes into account the proposed changes to be published in the new edition of the Bergey's *Manual of Determinative Bacteriology*.

Anaerobic Halophilic Members of Domain *Bacteria*

Order *Thermotogales*. Members of the order *Thermotogales* are exclusively thermophilic anaerobes and represent the second deepest branch in domain *Bacteria* (52). Within this order, the slight halophiles are represented in the genera *Geotoga*, *Petrotoga*, *Thermotoga*, *Thermosipho*, and *Marinitoga* (53) (Fig. 1; Table 1). All utilize carbohydrates with acetate being the major fermentation end-product formed. *Thermotoga maritima* is a hyperthermophile (growth temperature optimum of 80 °C) (54) that uses elemental sulfur and thiosulfate as a terminal electron acceptor.

***Deferribacter* Assemblage.** *Deferribacter thermophilus*, a gram-negative slightly halophilic (optimum growth in 2% NaCl) thermophilic (optimum growth temperature of 60 °C) anaerobe was isolated from the production water of Beatrice oil field in the North Sea (U.K) (55). It is unique among halophiles because it obtains its energy from the reduction of manganese (IV), iron (III), and nitrate in the presence of yeast extract, peptone, casamino acids, tryptone, hydrogen, malate, acetate, citrate, pyruvate, lactate, succinate, and valerate. Phylogenetic analysis of the 16S rRNA gene indicates that it forms an independent line of descent with the moderate thermophile "*Flexistipes sinusarabici*" as its closest relative (Table 1; Fig. 1).

The Green Sulfur Bacteria. The halophilic members of green sulfur bacteria are also phototrophic and seven species belonging to four genera have so far been described (Table 1; Fig. 1). They grow optimally at salinities between 2 and 5% NaCl and are therefore regarded as slight halophiles. They have been frequently isolated from marine environments although they are also present in abundance in the first ponds of marine salterns (6 to 8% NaCl concentration) connected to the sea (34). Green sulfur bacteria have also been observed in hypersaline environments but none have yet been isolated (56).

The halophilic members of the green sulfur bacteria, like their phototrophic counterparts represented in the Alpha division of *Proteobacteria* (see following text), accumulate sugars (trehalose or sucrose), and N-acetylated compounds such as N-acetyl-glutaminyl glutamic acid (34) for osmoregulation.

Order *Spirochaetales*. Four validated moderately halophilic species of the genus *Spirochaeta* have been described (Fig. 2). Of these, *Spirochaeta smaragdinae* isolated from a deep subsurface Congolese oil field habitat is the only subsurface spirochaetal strain described to date (57). Three other species, namely, *Spirochaeta africanus*, *Spirochaeta alkalica*, and *Spirochaeta asiatica* are haloalkalophiles and produce acetate, ethanol, lactate, and hydrogen from carbohydrate fermentation (58).

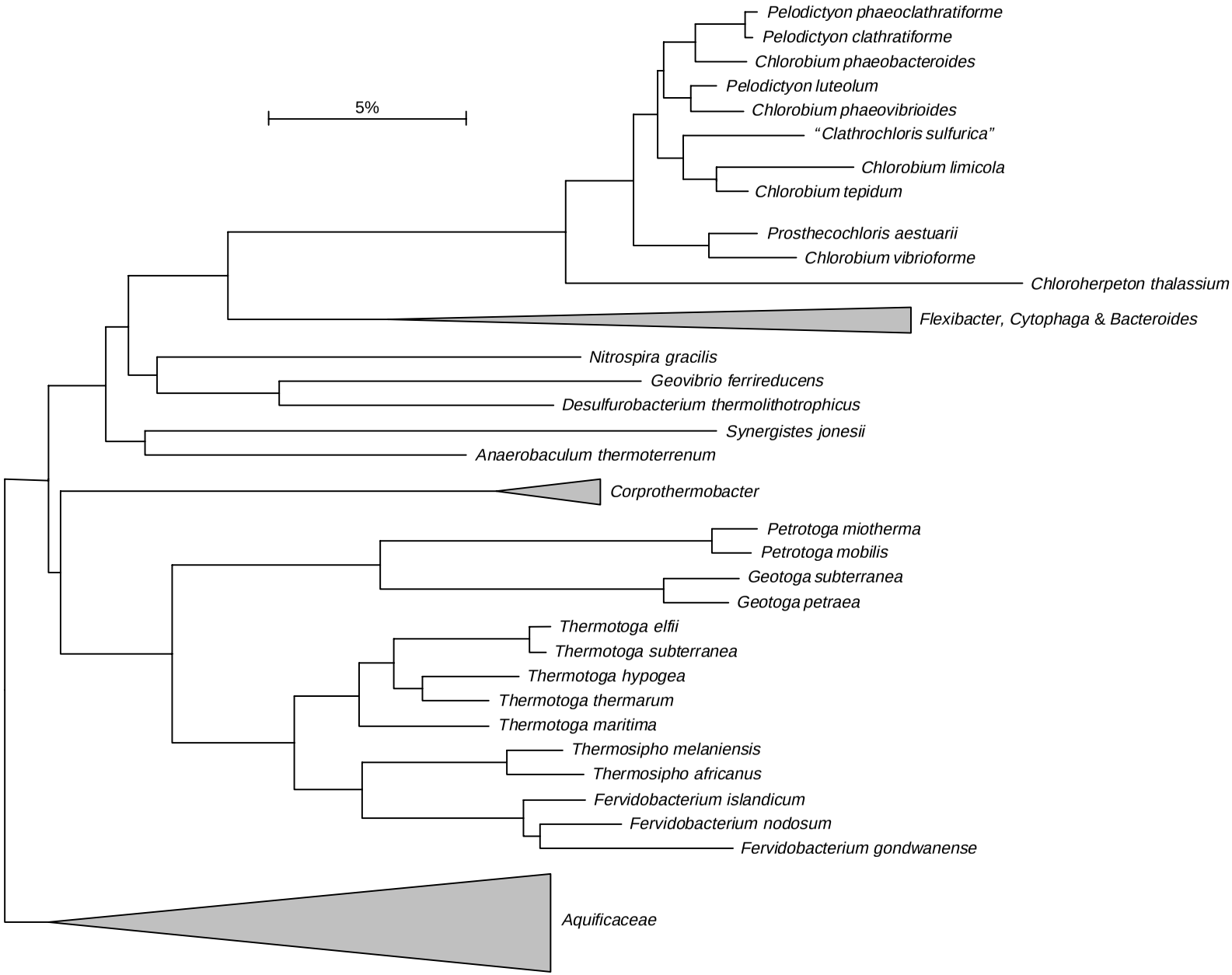

Figure 1. Dendrogram showing the position of members of the order *Thermotogales* and the green sulfur bacteria in domain *Bacteria*. Bar indicates evolutionary distance.

Table 1. Halophilic Bacteria of the Order Thermotogales, the Green Sulfur Bacteria, and Deferribacter Assemblage (see Fig. 1 for Phylogenetic Representation)

Species	Salinity Range (% NaCl)	Optimum Salinity (% NaCl)	Optimum Temperature °C*
Order *Thermotogales*			
Slight halophiles			
Geotoga petraea	0.5–10	3	50
G. subterranea	0.5–0	4	45
Petrotoga miotherma	0.5–10	2	55
mobilis	0.5–9	3–4	58–60
Thermosipho melanesiensis	0.5–6	3	70
Thermotoga maritima	0.25–3.75	2.7	80
Marinitoga camini	1–4.5	2	55
Green sulfur bacteria			
Slight halophiles			
Chlorobium chlorovibrioides	1.25–7.5	2–3	
C. phaeovibrioides	nd	1	
C. vibrioforme	nd	1	
Prostecochloris aestuarii	1–8	2–5	
P. phaeoasteroidea	0.2–7	0.5–2	
Pelodictyon phaeum	2–4.75	nd	
Chloroherpeton thalassium	1.5–3.75	nd	
Deferribacter assemblage			
Slight halophiles			
Deferribacter thermophilus	0–5	2	60

*optimum temperature only given for thermophilic microorganisms.

The *Proteobacteria.* The phylum *Proteobacteria* is currently divided into five divisions, namely, α, β, γ, δ, and ε. Slight, moderate, and extreme halophiles are represented in only two out of the five divisions, namely, the α and δ divisions. The phototrophic halophiles are housed in the α division, whereas the gram-negative sulfate-reducers and the non-sulfate-reducer, *Hippea maritima*, are housed in the δ division.

The *α Proteobacteria.* The α-division of phylum *Proteobacteria* comprises a number of phototrophic halophiles (Table 2; Fig. 2) that require H_2S and/or hydrogen, and organic compounds for anaerobic growth and active radiation for photosynthesis (59–65). Slightly photosynthetic halophiles are usually isolated from marine environments, whereas the moderate to extreme photosynthetic halophiles have been successfully isolated from hypersaline habitats. Moderately phototrophic halophiles isolated from hypersaline ponds of marine salterns grow optimally with salinities between 6 and 11% NaCl (Table 2). They belong to the genera *Rhodovibrio*, *Roseospira*, *Rhodothalassium* (formerly *Rhodospirillum*), *Halochromatium* (formerly *Chromatium*), *Thiohalocapsa* (formerly *Thiocapsa*), *Ectothiorhodospira*, and *Rhodovulum* (Table 2). The most halophilic phototrophic isolated so far are *Halochromatium salexigens* (66), *Thiohalocapsa halophila* (67), and *Rhodovibrio salinarum* (68).

Extremely phototrophic halophiles have most commonly been isolated from alkaline brines in athalassohaline (nonsaline marine water) environments such as desert lakes (43,69). They require about 20 to 25% NaCl for optimum growth and are members of the newly created genus *Halorhodospira* (formerly *Ectothiorhodospira*). The three species of this genus that have been isolated and described include *Halorhodospira abdelmalekii* (69), *Halorhodospira halophila* (70), and *Halorhodospira halochloris* (43) (Table 2).

Phototrophic halophiles use various reduced sulfur compounds to produce corresponding oxidized sulfur compounds with sulfate as the final product. They also actively participate in anaerobic degradation of cellulose and organic compounds in anaerobic halophilic communities (80). They are therefore regarded as one of the major trophic groups responsible for cycling of carbon and sulfur living in hypersaline environments.

The phototrophic halophiles control their osmoregulation by synthesis or uptake of organic solutes that accumulate in their cytoplasm, for example, glycine betaine (81–83). Glycine betaine biosynthesis by *Halorhodospira* species operates via methylation of glycine with S-adenosylmethionine being used as a methyl donor (82). Some members also accumulate sugars (trehalose or sucrose), whereas some accumulate N-acetylated compounds such as N-acetyl-glutaminyl glutamic acid (34). Some compatible solutes also serve as an energy reserve (8) as shown for *H. halochloris*. In this case, trehalose is produced as a minor component for osmoregulation but is degraded under mild stress (84).

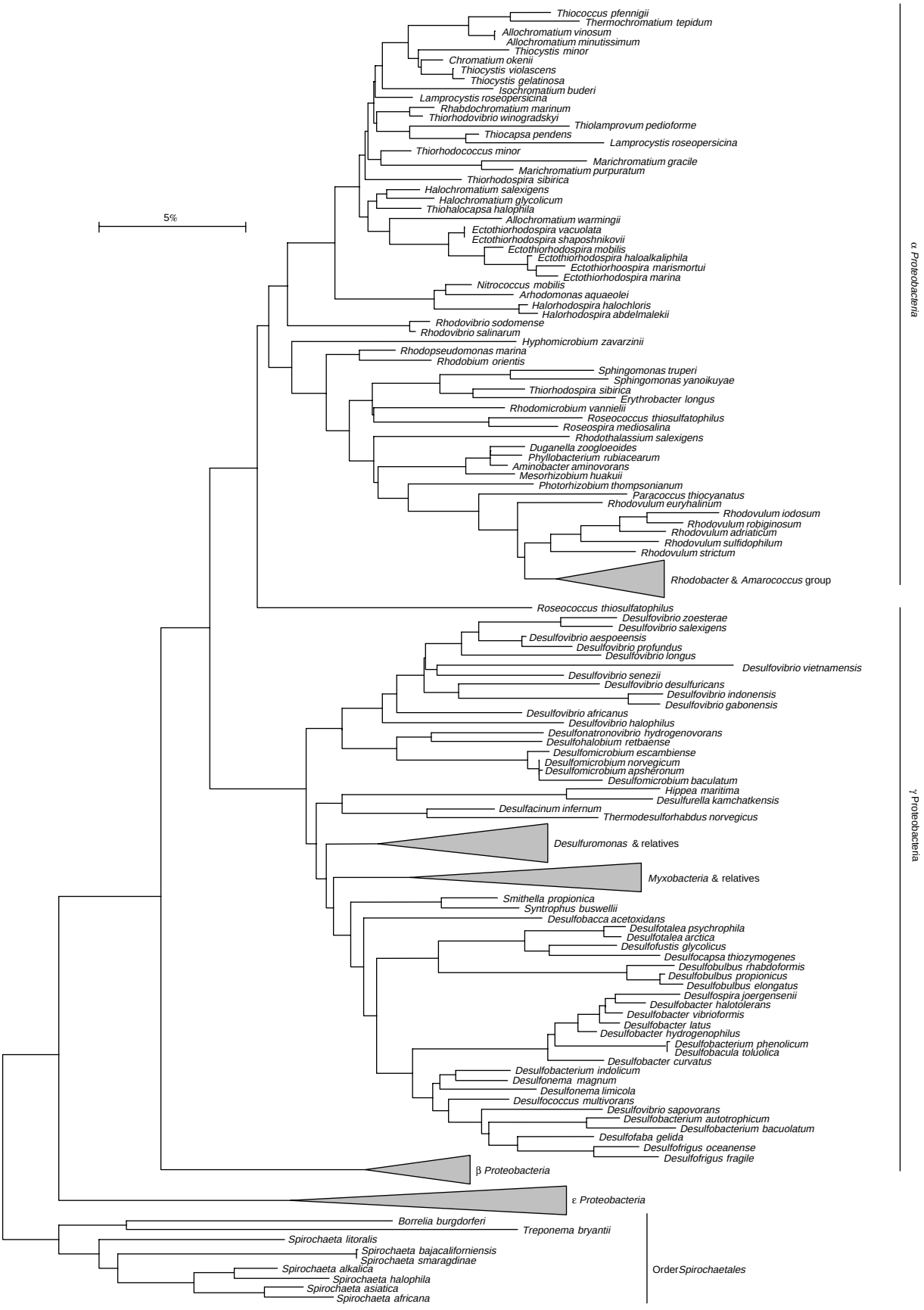

Figure 2. Dendrogram showing the position of members of order Spirochaetales and α and β *Proteobacteria* in domain *Bacteria*. Bar indicates evolutionary distance.

Table 2. Halophilic Phototrophs of the Alpha Division of *Proteobacteria* (see Fig. 2 for Phylogenetic Representation) (Data from 20,66,67,71,72,73–78,79)

Species	Salinity Range (% NaCl)	Salinity Optimum (% NaCl)
Slight halophiles		
Allochromatium vinosum	0.5–8	2.5–4.5
Isochromatium buderi	1–4	1–3
Marichromatium gracile	0.5–8	2–3
purpuratum	2–7	5.8
Rhabdochromatium marinum	1.5–5	nd
Lamprobacter modestohalophilus	1–4	1–2
Ectothiorhodospira mobilis	2–10	2
shaposhnikovii	1–7	nd
vacuolata	1.5–7.5	nd
Rhodovulum adriaticus	2.5–7.5	nd
sulfidophilus	1–6	2
Rhodopseudomonas marina	1–5	nd
Thiococcus pfennigii	nd	1–2
Thiorhodococcus minor	nd	2
Thiorhodovibrio winogradskyi	0.5–7	2–3
Roseospira trueperi	0.5–5	2
Thioalkalicoccus limnaeus	0–7	5
Moderate halophiles		
Halochromatium salexigens	5–20	7
H. glycolicum	2–20	5–6
Thiohalocapsa halophila	4.5–20	5–7
Ectothiorhodospira haloalkaliphila	2.5–15	5
E. marina	0.5–10	2–6
E. marismortui	3–20	7
Roseospira mediosalina	0.5–15	4–7
Rhodothalassium salexigens	5–20	7
Rhodovibrio salinarum	3–24	4
R. sodomensis	6–20	12
Rhodovulum euryhalinum	0.5–20	0.5–12
Extreme halophiles		
Halorhodospira abdelmalekii	12–18	14
H. halophila	11–32	18.5
H. halochloris	14–27	21

The phototrophic halophiles of the α division of *Proteobacteria* can be readily distinguished from other phototrophic halophiles represented in the phylum green sulfur bacteria on the basis of their light-harvesting pigments, bacteriochlorophylls (BChls) and carotenoids used for photosynthesis (the green sulfur bacteria, discussed earlier).

The γ *Proteobacteria.* Slight to moderate gram-negative halophilic sulfate-reducing bacteria and a non-sulfate-reducing bacterium, *H. maritima*, described as slightly halophile, are included in the γ-division (Fig. 2).

Halotolerant and slightly halophilic sulfate-reducers (SRB) belonging to 11 genera and 26 species are listed in Table 3. In marine environments such SRB mainly inhabit the anoxic sediment and the bottom anoxic waters of stratified lagoons. They are also common inhabitants of subterrestrial ecosystems (e.g., the oil field environment), and their activities are responsible for serious and costly problems in the offshore oil industry (12). In the oil field ecosystem, they develop over a wide range of salinities and temperatures and can therefore be considered as ubiquitous inhabitants. Identification of SRB in oil fields by reverse sample genome probing indicated the presence of two distinct communities that could be discriminated on the basis of the salt concentration of the production waters (85). Cord-Ruwisch and coworkers (86) also reported that salt concentrations had a strong effect on the growth of oil field reservoirs SRB populations.

The subterrestrial isolates include members of the genus *Desulfovibrio* such as *D. vietnamensis* (87) and *D. gabonensis* (88). Both are mesophilic, considered to be moderate halophiles with an upper limit for growth in the presence of NaCl between 0 and 10% and between 1 and 17%, respectively, use a limited range of substrates including hydrogen and pyruvate, and incompletely oxidize lactate. *Desulfomicrobium apsheronum*, isolated from stratal waters of the Apsheron peninsula (Russia), is also a mesophile that tolerates up to 8% NaCl for growth (89), incompletely oxidizes lactate to acetate, and differs physiologically from the *Desulfovibrio* spp. described earlier by its ability to grow autotrophically. Members of the genus *Desulfobacter* have been identified by oligonucleotide probes in oil field environments (90) and *Desulfobacter vibrioformis* has been isolated only recently from a water-oil separation system (91). *Desulfobacter vibrioformis* grows optimally with 1% NaCl and uses acetate as the only carbon and energy source while dissimilating sulfate to sulfide.

The alkaliphilic slightly halophilic SRB *Desulfonatronovibrio hydrogenovorans* (92) and *Desulfonatronum palustre* (93) use a limited range of substrates that include hydrogen and formate, and ethanol, respectively.

The eight species belonging to the four genera of moderately halophilic sulfate-reducers (*Desulfovibrio*, *Desulfohalobium*, *Desulfotomaculum*, and *Desulfocella*) were isolated from various hypersaline terrestrial or subterrestrial environments and with the exception of *Desulfocella halophila* and *D. vietnamensis*, are hydrogenotrophic. Seven out of the eight moderately halophilic SRB also

Table 3. Halophilic Sulfate-Reducing Bacteria and *H. Maritima* of Gamma Division of Proteobacteria (see Fig. 2 for Phylogenetic Representation)

Species	Salinity Range (% NaCl)	Salinity Optimum (% NaCl)
Sulfate-reducers		
Slight halophiles		
Desulfovibrio desulfuricans subsp. *aestuarii*	0.5–6	3
D. giganteus	0.2–5	0.2–2.5
D. indonensis	0–10	nd
D. longus	0–8	1–2
D. salexigens	0.5–12	2–4
D. senezii	0–12.5	2.5
Desulfomicrobium apsheronum	0–8	1
D. baculatum	0–6	1
Desulfobacter halotoleranss	0.5–13	1–2
D. hydrogenophilus	nd	2
D. latus	nd	2
D. vibrioformis	1–5	1
Desulfococcus multivorans	nd	0.7–2
Desulfobacterium autotrophicum	nd	2
D. indolicum	nd	2
D. phenolicum	nd	2
D. vacuolatum	nd	2
Desulfonema limicola	nd	1.2
D. magnum	nd	2
Desulfonatronovibrio hydrogenovorans	1–12	3
Desulfospira joergensenii	0.6–4	1.2–2
Desulfofrigus oceanense	nd	1.5–2.5
D. fragile	nd	1–2.5
Desulfofaba gelida	nd	1.4–2.5
Desulfotalea psychrophila	nd	1
D. arctica	nd	1.9–2.5
Moderate halophiles		
Desulfovibrio gabonensis	1–17	5–6
D. halophilus	3–18	6–7
D. oxyclinae	2.5–22	5–10
D. profundus	0.2–10	0.6–8
D. vietnamensis	0–10	5
D. retbaense	0–24	10
Desulfocella halophila	2–19	4–5
Others		
Hippea maritima	nd	2.5–3

use thiosulfate or sulfite as electron acceptors in addition to sulfate, and a further four species can reduce elemental sulfur to sulfide. Trüper (94) cultured a SRB from hot brines in the Red Sea that tolerated up to 17% NaCl and looked similar to *D. halophilus*, a moderate halophilic SRB isolated by Caumette and coworkers (71) from the hypersaline Solar Lake in the Sinai Peninsula. *Desulfocella halophila* oxidizes butyrate and higher fatty acids (from C_3 to C_{16}) incompletely (95). *Desulfohalobium retbaense* presents the upper limit of NaCl concentration (24%) for growth of all the moderately halophilic SRB described so far (96).

Although SRB are generally regarded as strict anaerobes, metabolic activity in the presence of oxygen has been reported for the slight halophile *Desulfovibrio salexigens* and the moderate halophile *D. oxyclinae* (97–101).

Sulfate-reducing bacteria play a crucial role at the terminal stage of organic matter oxidation in anoxic environments such as sulfide-rich soda lakes, marine, and terrestrial/subterrestrial hypersaline environments (31,72,102–106). However, only a limited range of substrates including H_2, formate, and lactate are involved in this process (35,36,96,104). In general, estimates of poor sulfate reduction in presence of acetate were noted in salinities greater than 15%. Although in situ studies of moderately hypersaline sediments has shown that a major part of acetate oxidation is performed by SRB (32), only *Desulfobacter halotolerans*, a slight halophile has shown this characteristic among pure cultures (33). This isolate from the sediments of the Great Salt Lake (Utah, U.S.), grows optimally on acetate in the presence of 1 to 2% NaCl and tolerates up to 13% NaCl. Studies on the sediments of the same lake have demonstrated that acetate oxidation occurs, albeit very slowly, at 20% NaCl, thus suggesting that acetate oxidation by moderately and/or extremely halophilic SRB, if they exist at all, may be insignificant (33). These observations indicate that acetate oxidation in hypersaline environments can occur at only low rates, thus resulting in its accumulation in sediments (31).

Low $G + C$ Dna Containing Gram-Positive Group. The members of this group include the fermentative bacteria of the order *Halanaerobiales*, Clusters XI and XII of the order *Clostridiales*, and two sulfate-reducing bacteria of the genus *Desulfotomaculum* (Table 4; Fig. 3). All members are moderate to extreme halophiles with the exception of *Desulfotomaculum geothermicum*, which is a slight halophile.

Order *Halanaerobiales*. The members of order *Halanaerobiales* are the most widely studied moderate halophiles consisting of 10 genera and 22 species. They are divided into two families, namely, *Halanaerobiaceae* and *Halobacteroidaceae*. The family *Halanaerobiaceae*, which contains 3 genera and 11 species (34,107–110), ferments carbohydrates to acetate, hydrogen, carbon dioxide, and various other metabolites such as ethanol, lactate, and VFA higher than acetate. *Halocella cellulosilytica* is the only cellulose degrader included in this family (111). However, cellulase activity has been demonstrated in a hypersaline African lake but no isolates were cultured (36), indicating that other cellulolytic halophilic bacteria may exist in nature. The family *Halobacteroidaceae* houses the remaining 12 species belonging to seven genera (112,113) all of which, with the exception *A. arabaticum*, ferment carbohydrates. The later degrades amino acids and betaine to acetate and trimethylamine (38). Five spore-forming species belonging to three different genera are also members of this family.

Table 4. Halophilic Low *G + C* DNA Containing Gram-Positive Bacteria (see Fig. 3 for Phylogeny)

Species	Salinity Range (% NaCl)	Salinity Optimum (% NaCl)
Sulfate-reducers		
Slight halophiles		
Desulfotomaculum geothermicum	0.2–5	2.4–3.4
halophilum	1–14	4–6
Order *Clostridiales*		
Moderate halophiles		
Clostridium halophilum (cluster XI)	1.5–10	6
Natronoincola histidinovorans (cl. XI)	4–16	8–10
Thermohalobacter berrensis (cl. XII)	2–15	5
Order *Halanaerobiales*		
Moderate halophiles		
Halanaerobium acetoethylicum	6–20	10
H. alcaliphilum	2.5–25	10
H. congolense	4–24	10
H. fermentans	7–25	10
H. kushneri	9–18	12
H. praevalens	2–30	12.5
H. saccharolyticum	3–30	7.5–12.5
H. salsuginis	6–24	9
Halothermothrix orenii	4–20	5–10
Halocella cellulosilytica	5–20	15
Halobacteroides elegans	10–30	10–15
halobius	8–30	9–15
Halanaerobacter salinarius	5–30	14–15
Sporohalobacter lortetii	4–15	8–9
Orenia marismortui	3–18	3–12
O. salinaria	2–25	5–10
O. sivashensis	5–25	7–10
Natrioniella acetigena	10–26	12–15
Extreme halophiles		
Halanaerobium lacusrosei	6–34	20
Acetohalobium arabaticum	10–25	15–18
Halanaerobacter chitinovorans	3–30	12–18
Halobacteroides lacunaris	5–30	15–18
Selenihalanaerobacter shriftii	10–24	21

Members of the order *Halanaerobiales* have their origins in saline lakes but the moderate halophile *Halanaerobium salsugo* (114), *H. congolense* (115), and *H. acetoethylicum* (formerly *Halobacteroides acetoethylicus*) (46) have all been isolated from oil field brines.

All members of the order *Halanaerobiales*, with the exception of three species of the genus *Halanaerobium*, are motile by peritrichous flagellation. Most species in the order should be considered as moderate halophiles because most rapid growth occurs with 7.5 to 18% NaCl with the exception of the extreme halophile, *H. lacusrosei*, which grows optimally with 20% NaCl, with an upper limit of growth at 34% NaCl (42). A recently described new species of a new genus, *Selenihalanaerobacter shriftii*, isolated from the Dead Sea, grows optimally with 21% NaCl, with an upper limit of growth at 24% NaCl (116). It oxidized glycerol or glucose with concomitant reduction of selenate to selenite plus elemental selenium. *Halanaerobium saccharolyticum* (117,118) exhibits a homoacetogenic pathway for the metabolism of glucose and the nonsaccharolytic *A. arabaticum* (38) performs homoacetogenesis from $H_2 + CO_2$. *Halothermothrix orenii* order *Halanaerobiales* (119) and *Thermohalobacter berrensis* order *Clostridiales* (120) represent the only true thermophilic anaerobic moderately halophiles (growing up to 68 to 70 °C, with most rapid growth at 60 to 65 °C) described to date.

Alkalitolerant heterotrophs belonging to the genera *Halanaerobium* and *Acetohalobium* have been isolated from neutrophilic salt lakes (38,121). Recent microbiological investigations of soda lakes (Lake Magadi, Kenya) revealed the presence of an alkaliphilic saccharolytic anaerobe (strain 7,986) representing a new taxon in the order *Halanaerobiales* (122). *Natrionella acetigena*, isolated from soda lakes grows optimally at pH 9.7 to 10 and ferments a limited range of substrates such as lactate and ethanol to acetate (39). Other haloalkaliphilic anaerobes have also been isolated from the sediments of the same lake (40) and are phylogenetic relatives of clostridial clusters VIII and XI and will most probably represent a new genus of anaerobic haloalkaliphiles (40).

It is noteworthy that in contrast to other members of domain *Bacteria*, halophiles of the order *Halanaerobiales* are unique in using the salt-in strategy for growing at high salinities (8). They balance the osmotic pressure of the environment with high intracellular KCl concentrations and generally do not produce organic osmotic solutes similar to the aerobic members of the order *Halobacteriales*, domain *Archaea* (8).

Members of Cluster XI and XII. *Clostridium halophilum* is a moderate halophile and grows optimally with 6% NaCl (range 1.5 to 10%) (123). Strains of the haloalkaliphile *Natronoincola histidinovorans* have been isolated from soda deposits in Lake Magadi, Kenya (124). The strains grow optimally in the presence of 8 to10% NaCl (range 4 to 16%) at pH 9.4 using substrates such as histidine and glutamate. The end-products of amino acid fermentation are acetate and ammonium. Analysis of 16S rDNA sequence of both species indicates that they belong to cluster XI of the low G+C DNA containing gram-positive bacteria. *Thermohalobacter berrensis*, a member of cluster XII, is a motile gram-negative nonsporulating rod-shaped bacterium isolated from the sediment of a French solar saltern feeding canal (120). The optimum temperature for growth is 65 °C (range 45 to 70 °C) and the optimum NaCl concentration for growth is 5%

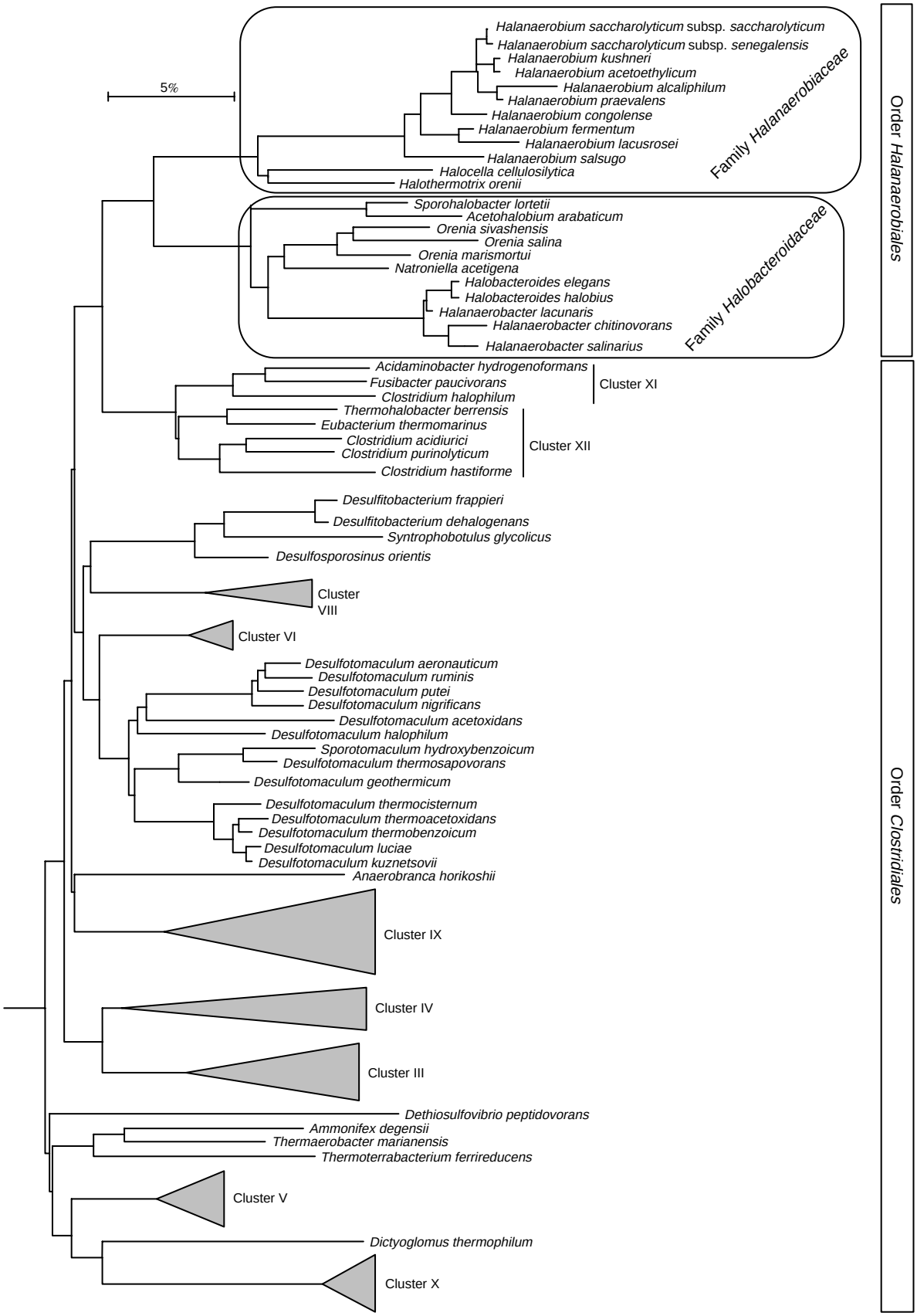

Figure 3. Dendrogram showing the position of low G+C DNA containing gram-positive. Members of orders *Haloanaerobiales* and *Clostridiales* and genus *Desulfotomacum* in domain *Bacteria*. Bar indicates evolutionary distance.

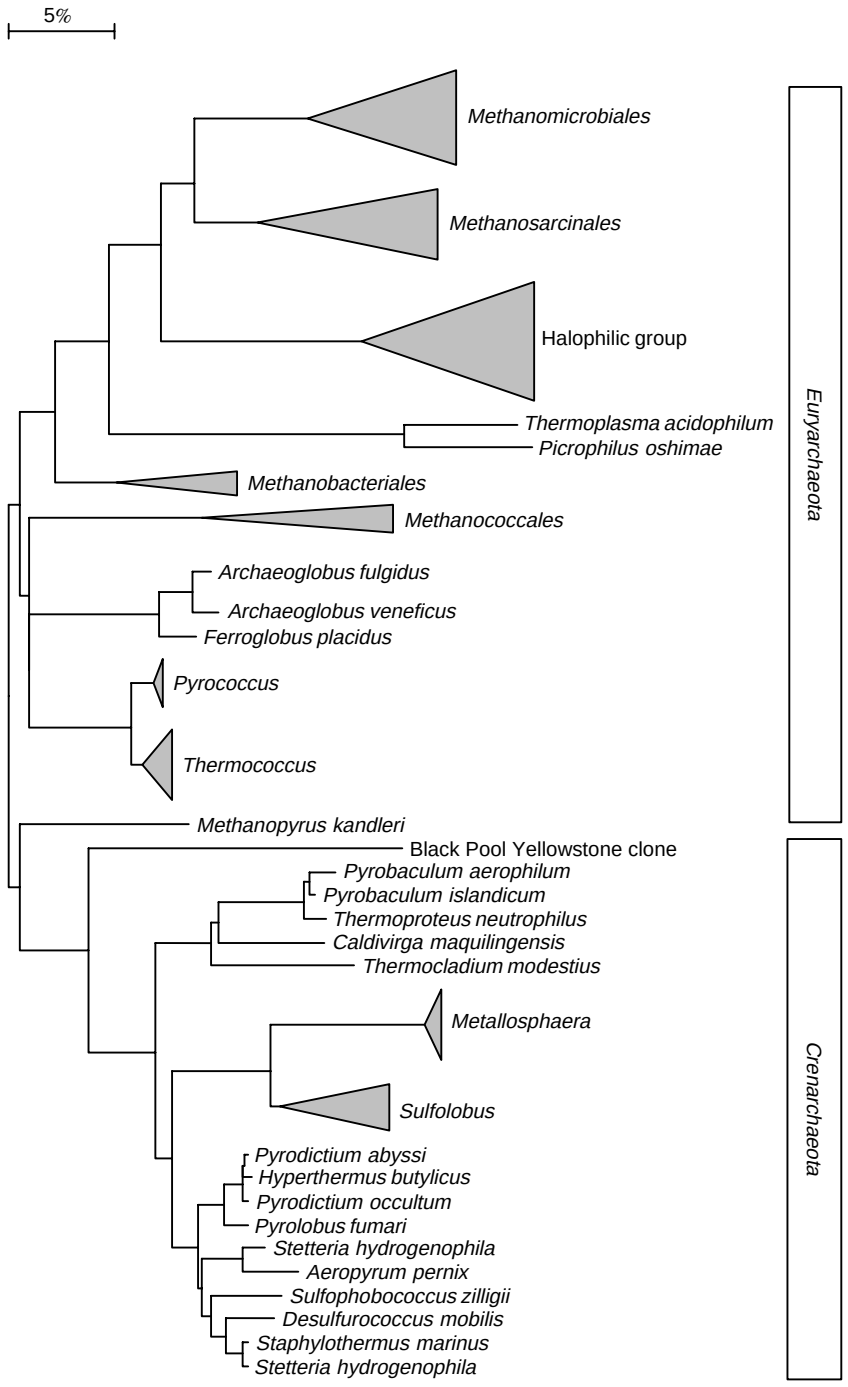

Figure 4. Dendrogram showing the position of methanogens and fermentative archaeons in domain *Archaea*. Bar indicates evolutionary distance.

(range 2 to 15%). It ferments carbohydrates, pyruvate, and biotrypticase. Acetate, ethanol, hydrogen, and carbon dioxide are produced from glucose fermentation.

Genus *Desulfotomaculum.* Only two species of the genus *Desulfotomaculum* family *Peptococcaceae*, which originate from subterrestrial environments, have been described as halophiles. *Desulfotomaculum geothermicum*, a slightly halophilic thermophilic sulfate-reducer, isolated from geothermal groundwater, completely oxidizes its substrates (125), whereas *D. halophilum*, isolated from oil field reservoir, is a moderately halophilic mesophilic sulfate-reducer that oxidizes incompletely a limited range of substrates such as lactate and ethanol to acetate (126).

Anaerobic Halophilic Members of Domain Archaea

The Methanogens. Seventeen species of slightly halophilic or halotolerant methanogens belonging to orders *Methanococcales*, *Methanomicrobiales*, *Methanosarcinales*, and *Methanopyrales* (Fig. 4) have been mainly isolated from marine sediments or subsurface environments such as hydrothermal vents. They show a salinity range from 0 to 12% NaCl and most

Table 5. Halophilic Methanogenic *Archaea* (Euryarchaeota, see Fig. 4 for Phylogeny)

Species	Salinity Range (% NaCl)	Optimum Salinity (% NaCl)	Optimum (temperature ° C*)	Substrates
Slight halophiles				
Methanococcus deltae	2–7	4		H2-CO2
M. fervens	0.5–5	3	85	H2-CO2
M. maripaludis	0.5–2	1–2		H2-CO2
M. voltae	nd	2–5		H2-CO2
M. vulcanius	0.5–6	2.5	80	H2-CO2
Methanofollis liminatans	0–5	0–4		H2-CO2
M. tationis	0–7	1		H2-CO2
Methanosarcina frisia	1–5	2		methyl compounds
M. siciliae	nd	2.4–3.6		methyl compounds
Methanococcoides burtonii	1–3	1		methyl compounds
M. methylutens	0.5–7	1.5–4		methyl compounds
Methanolobus bombayensis	1–12	3		methyl compounds
M. oregonensis	0–10	3		methyl compounds
M. taylori	1–7	3		methyl compounds
M. tindarius	0.5–8	3		methyl compounds
M. vulcani	0.5–7	4		methyl compounds
Methanopyrus kandleri	0.2–5	1.5	98	H2-CO2
Moderate halophiles				
Methanocalculus halotolerans	0–12	5		H2-CO2
Methanohalophilus euhalobius	1–14	6		methyl compounds
M. halophilus	2–21	4–15		methyl compounds
M. mahii	3–21	12		methyl compounds
M. portucalensis	7–18	12		methyl compounds
Methanosalsum zhilinae	1–13	4		methyl compounds
Extreme halophiles				
Methanohalobium evestigatum	15–30	25		methyl compounds

*only for thermophiles.

rapid growth from 1.2 to 4.8% NaCl and include hydrogenotrophic (e.g., members of the genus *Methanococcus*) and methylotrophic microorganisms (e.g., members of genera *Methanococcoides* and *Methanolobus*). Extreme to hyperthermophiles (e.g., *Methanocaldococcus jannaschii*, *Methanoignis igneus*, and *Methanopyrus kandleri*) have been reported among the slightly halophiles. Moderately to extreme halophilic methylotrophic methanogens belonging to order *Methanosarcinales* (Fig. 4) comprise three genera and six species (Table 5). Most of them are obligate moderately halophilic cocci, isolated mainly from sediments of hypersaline lakes or lagoons, with growth ranging from 1 to 21% NaCl and most rapid growth from 3.6 to 15% NaCl, with the exception of the extreme halophile *M. evestigatum* (45) with growth ranging from 15 to 30% NaCl and having most rapid growth at 25% NaCl. Marked differences in optimum pH and temperature for growth were observed. One species, *Methanosalsus zhilinae* (127,128), is alkaliphile; the others are neutrophiles. *Methanosalsum zhilinae* and *M. evestigatum* are moderately thermophilic bacteria with a temperature optimum of 45 °C and 50 °C, respectively. Methylotrophic methanogens such as *M. zhilinae* (127) and *Methanolobus oregonensis* (129), which grow optimally around pH 9.0, were isolated from alkaline lakes.

A halotolerant hydrogenotrophic methanogenic irregular coccus belonging to order *Methanomicrobiales* (Fig. 4), and growing at up to 12.5% NaCl with most rapid growth at 5% NaCl, *Methanocalculus halotolerans*, was recently isolated from an oil well (37); it uses $H_2 + CO_2$ only in the presence of acetate as carbon source (Table 5). It represents so far the hydrogen-oxidizing methanogen growing in the widest range of NaCl concentration (from 0 to 12%). It has been suggested that the ability of *M. halotolerans* to grow in a wide NaCl range and optimally at NaCl concentration close to that of the oil well from which it was isolated might be indicative of its indigenous origin (37). Identically, *Methanohalophilus euhalobius* (formerly *Methanococcoides euhalobius*) (130) isolated from stratal fluid of the Bondyuzhskoe oil field was shown to be dependent on calcium for growth. This physiological characteristic was attributed to development of this microorganism in these stratal brines having a high calcium content. Accordingly this feature might have been indicative of indigenicity of this methanogen to the oil field environment (131). Beside these two microorganisms isolated from the subterrestrial environment, isolation of other methanogens has been successful from slightly saline to saline oil well waters in the mesophilic range of temperature (12). It has been demonstrated in studying the Bondyuzhskoe oil field (131) that the methanogenic activity decreased with an increase of salinity. In addition, the role of methylamine as the methanogenic substrate increased with the mineralization of stratal waters,

suggesting that methylotrophic methanogens are also representative of subterrestrial saline ecosystems as already described for terrestrial saline to hypersaline environments (12,34). *Methanosarcina siciliae* H1350, isolated from an oil well at High Island in the Gulf of Mexico, was described as a slightly halophilic methylotrophic methanogen unable to use acetate as the energy source (132,133).

Hydrogen may be an important energy source for methanogens in oil field environments (12) and may explain the presence of microorganisms similar to *M. halotolerans* in saline oil reservoirs. In addition, the specific presence of methylotrophic methanogens in saline to hypersaline oil field waters is also probably indicative of the availability of methylated compounds such as methylamines in the oil field waters as already reported for terrestrial hypersaline environments in which these compounds may originated from the breakdown of osmoregulatory amines (34). It is noteworthy that methanogenesis but also sulfate-reducing activity from acetate have not been reported so far in moderately to hypersaline oil reservoirs, thus suggesting that the process of acetate oxidation is most hardly to occur at high salinity similarly to what has been generally observed in terrestrial hypersaline environments (8,34).

Methanogenesis has been reported from various saline and hypersaline ecosystems including a submarine brine pool in the gulf of Mexico (134), an alkaline lake (135,136), the Great Salt Lake (35,137), and the lake Retba in Senegal, West Africa (36). In most of these environments, a strictly methylotrophic activity related to methanogens has been observed and an absence of H_2 and acetate oxidation via methanogenesis has been noted. Several methanogens using methylated compounds (methylamines, trimethylamine, methanol, and dimethysulfide), but not acetate or hydrogen have been isolated from such ecosystems (31,129,138–141). Halophilic hydrogenotrophic and acetotrophic methanogens were also found to be absent in the Solar Lake sediments (Sinai) with salinity around 7% (142) though methanogenesis occurred at 8.8% salt concentration with $H_2 + CO_2$ in the terrestrial Californian Mono Lake (9) that is the highest salt concentration at which methanogenesis from $H_2 + CO_2$ has been demonstrated to occur in nature in a terrestrial ecosystem. The upper NaCl concentration so far reported for methanogens using hydrogen or formate is 8.3% (73). This indicates that methylotrophic substrates is the most important energy source for methanogenesis followed by hydrogen but not acetate in these environments.

In slightly saline ecosystems such as marine sediments in which sulfate is not limiting, sulfate-reducing bacteria are known to outcompete methanogens in particular for hydrogen and acetate (135). In moderately to hypersaline ecosystems that contain larger amounts of sulfate than marine ecosystems, the major pathway for hydrogen and acetate oxidation is via sulfate reduction. The existence of methanogens in hypersaline environments is therefore related to the presence of noncompetitive substrates such as methylamines, which mainly originate from the breakdown of osmoregulatory amines (e.g., glycine betaine). Consequently, it is generally admitted that methanogenesis does not contribute to the mineralization of carbohydrates because they do not use acetate and/or hydrogen at NaCl concentration higher than 15%. Above this concentration, sulfate reduction is probably the preferred way for oxidizing hydrogen and in a minor extent acetate and occupies a crucial function in the final steps of carbohydrate degradation. However, this function decreases when salt concentration increases in hypersaline environments.

The Fermenters. Numerous thermophilic and slightly halophilic members of domain *Archaea* that have been isolated from thermal environments and in particular from deep-sea volcanic habitats (Table 6), require between 1.5 and 4% NaCl for optimum growth. Most of these slightly halophilic archaea belong to the genera *Thermococcus* and *Pyrococcus* (Kingdom *Euryarchaeota*) and represent one of the deepest branch within the domain *Archaea* (Fig. 4). Most of them ferment proteinaceous compounds and reduce elemental sulfur to sulfide and can grow optimally at temperature above 80 °C (1,4). There are only three representatives of slightly halophiles within Kingdom *Crenarchaeota* (Table 6; Fig. 4). They belong to genera *Pyrodictium* and *Stetteria*. *Pyrodictium brockii* and *P. occultum* grow optimally at 105 °C, whereas *Stetteria hydrogenophila* grows optimally at 95 °C; they are therefore considered as hyperthermophiles.

CONCLUSION

Halanaerobes including fermenters, sulfate-reducers, phototrophs, and methanogens have been recovered from both the terrestrial and the subterrestrial ecosystems. They belong to various phylogenetic groups and comprise the low G+C DNA containing gram-positive group, the *Proteobacteria*, deep-branching members of the order *Thermotogales*, the green sulfur bacteria, and order *Spirochaetales* within domain *Bacteria*, and the Kingdoms *Euryarchaeota* and *Crenarchaeota* within domain *Archaea*. Fermentative bacteria grow on a wide range of substrates, including carbohydrate and/or proteins, and produce a variety of volatile fatty acids with acetate being the common end-product formed. The most intensively studied halanaerobes are the mesophilic thermotolerant fermenters that belong to the order *Halanaerobiales*. The members of the order *Halanaerobiales* are divided into two families, namely, *Halanaerobiaceae* and the *Halobacteroidaceae*. Although *H. cellulosilytica* is the only cellulolytic microorganism so far described in order *Halanaerobiales* (111), *A. arabaticum* grows on betaine and trimethylamine (38) and is the only halanaerobe that performs homoacetogenesis from $H_2 + CO_2$. The existence of thermophilic moderately halophilic anaerobes is limited so far to *H. orenii*, a member of the order *Halanaerobiales* (119) and *T. berrensis*, a member of the order *Clostridiales* (120). The nature of metabolites produced by fermentative bacteria leads to the development of microbial communities in which SRB appear to play an important role. For example, ethanol and hydrogen produced by the halanaerobic fermenters are known to be common growth substrates for halophilic sulfate-reducers. The accumulation

Table 6. Slightly Halophilic Fermentative Members of Domain *Archaea* (see Fig. 4 for Phylogenetic Representation)

Species	Salinity Range (% NaCl)	Optimum Salinity (% NaCl)	Optimum Temperature °C
Euryarchaeota			
Caldococcus litoralis	1–6	2.5	88
Ferroglobus placidus	0.5–4.5	1.8–2	85
Pyrococcus abyssi	0.7–5	3	96
P. furiosus	0.5–5	2	100
P. horikoshii	1–5	2.4	98
P. woesei	nd	3	100–103
Paleococcus ferrophilus	2–7.3	4.3	80
Thermococcus acidaminovorans	1–6	2–3	85
T. alcaliphilus	1–6	2–3	85
T. barophilus	1–4	2–3	85
T. celer	nd	3.8–4	88
T. chitinophagus	0.8–8	2	75
T. fumicolans	0.6–4	1.3–2.5	85–90
T. gorgonarius	1–5	2–3.5	80–88
T. guaymasensis	1–5	3	88
T. hydrothermalis	1.5–5	2–2.5	80–90
T. litoralis	1.8-6.5	nd	85
T. pacificus	1–6	2–3.5	80–88
T. peptonophilus	1–5	3	85
T. profundus	1–6	2–4	80
T. siculi	1–4	2	85
Crenarchaeota			
Stetteria hydrogenophila	0.5–6	2–3.5	95
Pyrodictium brockii	0.2–12	1.5	105
P. occultum	0.2–12	1.5	105

of acetate, produced as one of the end-product of carbohydrate fermentation by halanaerobes and/or incomplete oxidation of lactate and ethanol by SRB, at salinities higher than 15%, indicates that the rate of acetate oxidation via sulfate reduction or methanogenesis is most probably slow or nonexistent (31,34). This hypothesis is strengthened by the isolation of only moderately halophilic hydrogenotrophic sulfate-reducing bacteria that incompletely oxidize lactate to acetate in hypersaline sediments. Activity of these hydrogenotrophs is thought to be responsible for most of the biological sulfate reduction, considered as an important process in hypersaline ecosystems (104). No extremely halophilic sulfate-reducer has so far been isolated, and *D. retbaense*, a hydrogenotroph with a growth upper limit in the presence of 25% NaCl, remains the most halophilic sulfate-reducing bacterium (96). It will therefore be challenge for researchers to isolate new SRB from hypersaline ecosystems that grow at higher salinities than that reported for *D. retbaense*. A second alternative would be to add suitable compounds (e.g., osmolytes) in the growth media that would allow the currently isolated strains to cope with higher salinities. Saline and hypersaline ecosystems, in contrast to most other ecosystems, offer no competition between hydrogen- and/or acetate-utilizing SRB and methanogens because the latter use methylated compounds such as methanol, methylamines, trimethylamine, or dimethylsulfide that do not serve as energy sources for halophilic SRB (31). In addition, the most important source of methylamines for methanogens is probably glycine betaine decomposition (38), a widespread osmoprotectant found in eukaryotes and prokaryotes living in hypersaline ecosystems. The presence of methanol in hypersaline sediments is thought to be a result of a pectinolytic activity (74), which has been demonstrated in *Halanaerobium praevalens*.

As recently reported by Oren (8), life at high salt concentrations is energetically costly. Although members of the *Halanaerobiales* balance the osmotic pressure of the medium with high intracellular KCl concentrations, methanoarchaea, phototrophs, and sulfate-reducers may balance it by synthesizing organic solutes (8). Consequently, the absence of metabolic pathways such as the acetoclastic reaction pathway in halophilic methanoarchaea may have resulted from bioenergetical constraints necessary to cope with life at high salt concentrations (8). In this respect, aspects on physiological investigations in hypersaline terrestrial or subterrestrial microbes is a promising research area. Besides, experiments to be undertaken to improve our knowledge in understanding the different ways that microorganisms use to adapt their growth in hypersaline conditions, the challenge of successfully isolating new types of halophilic anaerobes is still open. In addition, studies of these halophiles may also provide biochemists with organisms from which new polymeric substances, enzymes, or osmolytes of industrial interest could be harvested.

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HALOPHILIC ARCHAEA. See ARCHAEA IN SOIL HABITATS

HALOPHILIC MICROORGANISMS. See SALT PRODUCTION

HALOTOLERANT BACTERIA. See SALINITY EFFECTS ON THE PHYSIOLOGY OF SOIL MICROORGANISMS

HARMFUL ALGAL BLOOMS (HAB). See RED TIDES AND OTHER HARMFUL ALGAL BLOOMS

HEAT-SHOCK GENES. See STRESS RESPONSE IN BACTERIA: HEAT SHOCK

HEAT-SHOCK PROTEINS. See STRESS RESPONSE IN BACTERIA: HEAT SHOCK

HEAVY METAL TOXICITY. See METALS: MICROBIAL PROCESSES AFFECTING METALS

HELICOBACTER PYLORI

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The bacterial pathogen *Helicobacter pylori* is an etiological agent of both acute and chronic gastritis. The organism is found in the human stomach and upper gastrointestinal tract. It has been associated with peptic and duodenal ulcer disease and the development of gastric carcinoma (1,2). *Heliobacter pylori* is a spiral-shaped bacterium equipped at one end of the cell with multiple, powerful flagella that readily enable it to move in the viscous fluids found in the gastric mucosa. The organism produces large amounts of urease enzyme which assists in neutralizing acids in the microecological niche of the gastric mucus–producing epithelial cells. It has been shown that *H. pylori* can convert from the spiral shape to a coccoid form. The significance of the coccoid form is currently unclear with regards to its role both in the disease state and in the transmission of the organism (3,4).

Heliobacter pylori is a fastidious organism and thus very difficult to culture from environmental sources. Most studies on occurrence in the aquatic environment have been limited to the use of molecular methods using polymerase chain reaction (PCR) amplification. Recovery and survival studies in water have been conducted with seeded organisms using cultural methods, autoradiography, and PCR analysis (5–7). Results from these studies suggest that the organism may be capable of surviving in aquatic environments.

The mode of transmission for *H. pylori* is not fully understood. Person to person contact has been proposed as the most likely method. The oral-oral route, the gastro-oral route, and the fecal-oral route have all been proposed as possible means of dissemination. Epidemiological studies have shown an increased prevalence among lower socioeconomic groups dwelling under crowded living conditions (8), suggesting that sanitation practices may play an important role in transmission of the organism. *Heliobacter pylori* has been reported in the feces of children from an area of endemic infection (9). Studies employing PCR amplification techniques have suggested the presence of *H. pylori* nucleic acids in sewage and drinking water. Other studies using microscopic immunological procedures have reported the occurrence of respiring cells in surface and ground water sources (10). Epidemiological findings have cited increased risk factors for infection associated with drinking water (11) and the consumption of uncooked vegetables irrigated with untreated sewage water (12). Pure culture inactivation studies have shown that the organism is sensitive to disinfection and is readily inactivated by chlorine (13). Further research regarding occurrence and mode of transmission should help elucidate the potential risk of this organism as a waterborne pathogen.

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HEPATITIS VIRUSES (HAV-HEV)

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Hepatitis A virus (HAV) and hepatitis E virus (HEV) are the two known viral hepatitis etiologies associated with enterically transmitted hepatitis disease. Worldwide occurrence of both HAV and HEV is both sporadic and epidemic in nature and is predominantly featured in the developing world; poor sanitation through fecal–oral routing characterizes the environmental dimension predisposing transmission (1–4). Both HAV and HEV have been implicated as causative in most outbreak episodes involving hepatitis (5). The preponderance of epidemic enteric hepatitis cases can be attributed to HEV in that most children have already been exposed to HAV by the time of adolescence, particularly in developing areas (Table 1). Moreover, HEV epidemics have generally been found to impact sizeable populations, often overwhelming immunologically and environmentally susceptible populations: some examples include 10,000 cases in Kathmandu (1973–74), 20,000 in Kashmir (1978), 11,000 in Kashmir (1981–82), 15,000 in Kashmir (1981–82), 79,000 in Kanpur (1991), and 20,000 in Mandalay (1976–77) (5). Epidemic HEV occurrence on the Indian subcontinent, first recognized from the 1955 outbreak (affecting 30,000 persons) in New Delhi, has since been identified as a significant public health concern throughout South Asia and as a major epidemic and sporadic disease entity, whereas HAV is probably the leading cause of sporadic, clinically recognized viral hepatitis (6). While HEV is molecularly distinct from HAV, it is also proving to be epidemiologically distinguishable (Fig. 1). Notably, HEV is uniquely synonymous with high case fatality rates (CFRs) in pregnant women, approximating 10–24% in affected populations, whereas CFRs associated with acute HAV infections are generally less than 1/1,000 persons, although rates as high as 1.5/1,000 and 27/1,000 among groups aged less than 5 years and more than 50 years, respectively, have been reported (7). As with HEV, most HAV infections in young children do not usually translate into acute, clinical disease episodes (8–10). Finally, the sporadic or epidemic forms of HEV, unlike HAV, are not ubiquitous in occurrence relative to geography: sporadic cases of HEV predominate in places such as North and East Africa (the Horn), in contrast to more outbreak-related recognition in Southeast Asia (10–14).

HEPATITIS A

Biology

HAV is a nonenveloped RNA virus of 27–28 nm diameter (Fig. 2) with icosahedral symmetry, belonging to the *Picornaviridae* family. There are four recognized genotypes based on primary sequence variability, but only one known serotype (15). Many strains of HAV have been described on the basis of different growth characteristics, nucleotide sequence, or geographic origin (16).

STABILITY

Stability of HAV is determined under both optimized laboratory and natural field conditions. HAV is extremely heat stable and temperatures above 60 °C are necessary to destroy infectivity (17–19). HAV is highly stable at low pH; the purified HAV remains infectious for up to eight hours of exposure to pH 1 at room temperature (20). It is resistant to many detergents such as Nonidet P40 and deoxycholate as well as to organic solvents such as ether, chloroform, and trichlorotrifluoroethane (19,21). HAV can resist higher levels of chlorine than other enteroviruses (22) and its resistance to inactivation is more remarkable in its natural state in feces, water, or wastewater (23). Exposure of 30 minutes to a free residual of 2.0- to 2.5-mg chlorine per liter is required to completely inactivate HAV (22). A lower level of 0.2 mg/L is required for complete inactivation (23) when chlorine dioxide is used because it is more microbiocidal. HAV is extremely resistant to chloramines, and treatment with 10 mg of residual chloramines per liter for nearly two hours is necessary for inactivation (24). HAV can be reliably inactivated by autoclaving (121 °C for 30 minutes), treatment with a 1 : 4, 000 dilution of formaldehyde at 37 °C for 72 hours (25), or exposure to ultraviolet irradiation (25). At 20 °C, 0.25–0.38 mg/L of ozone is required for complete inactivation of HAV (26), however, under different laboratory conditions and using a different strain of HAV, an increased concentration was required, at 1 mg/L (27). Quaternary ammonium formulations containing 23% HCl (toilet bowl cleaner), 2% gluteraldehyde, or sodium hypochlorite with free chlorine in excess of 5,000 ppm are recommended for effective surface disinfection (28) as several commercially available disinfectant preparations act poorly against the virus (29).

The survival of enteric viruses in water depends on a large variety of physicochemical and biological factors, the most important being temperature (30). The survival time increases considerably as the water temperature decreases. The T99 (the time required to inactivate 99% of microorganisms) is reported to be 19–20 days at 25 °C for HAV (31). HAV can survive in experimentally contaminated freshwater, seawater, and wastewater for

Table 1. Main Epidemiologic Differences Between Hepatitis Viruses A and E

	Hepatitis A	Hepatitis E
Mode of transmission	Fecal–oral: Person-to-person (major) Food-borne (common) Waterborne (minor) Blood transfusion (rare)	Fecal–oral: Waterborne (major) Person-to-person (minor) Food-borne (minor)
Geographic distribution	Worldwide prevalence Infection is universal in developing countries	Central and Southeast Asia, Indian subcontinent, Middle East, Northern and Western Africa, Mexico
Seasonal pattern	Fall, winter in temperate zones Rainy season in tropical countries	During or after rainy season
Age preference	All age groups Children in developing countries	Young to middle-aged adults
Sex predilection	Equal	Higher in males
Mortality in pregnancy	Not increased	10–24%
High-risk groups	Institutions for the intellectually handicapped, daycare centers, homosexual men, intravenous drug users, travelers to endemic areas	Travelers to endemic areas

Note: This table was produced by Dr. Khin Saw Aye Myint.

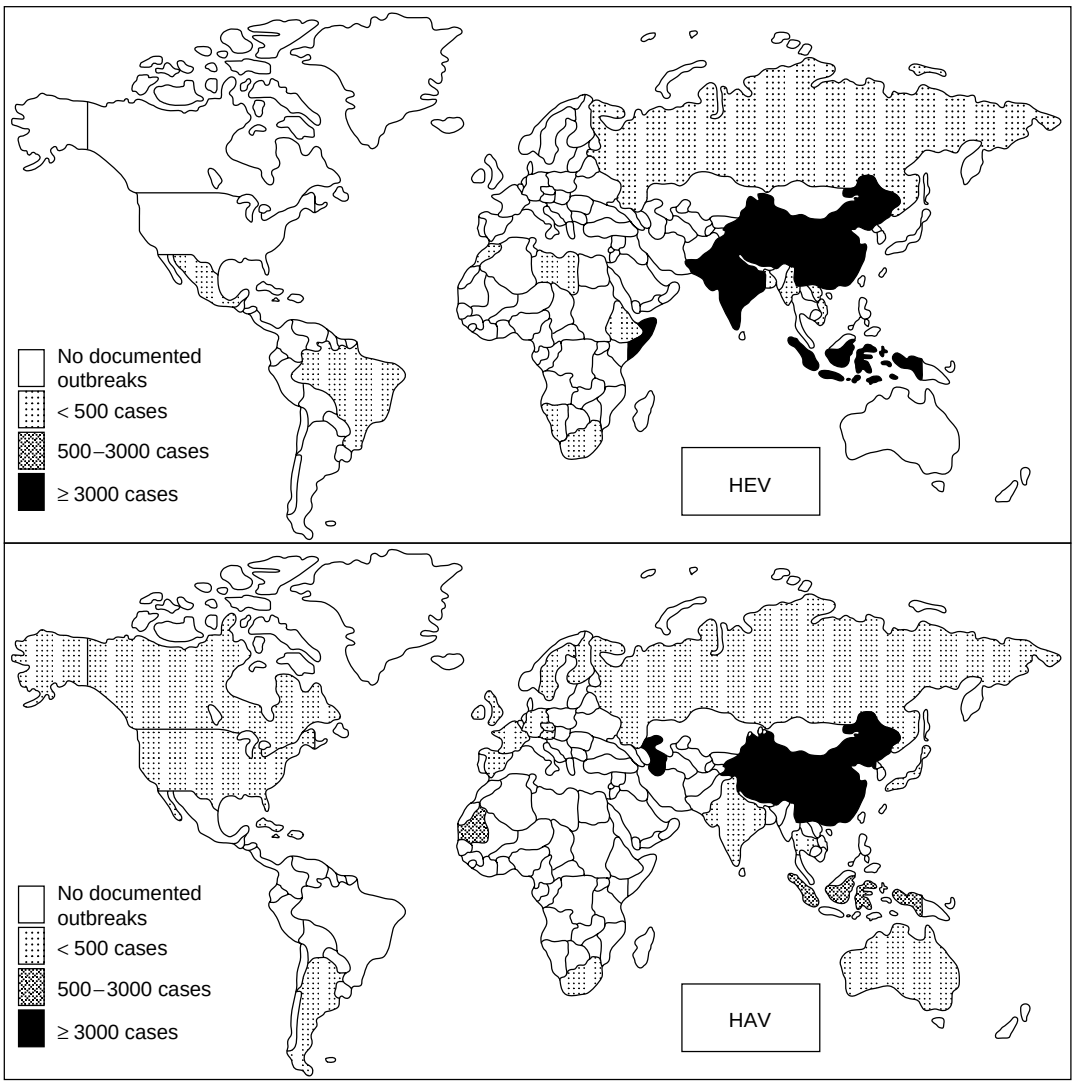

Figure 1. Worldwide outbreaks of hepatitis E virus (HEV) and hepatitis A virus (HAV), 1987–2000. Reflects largest instance of documented epidemic occurrence by country. [This graphic mapping presentation was produced by Kanti Laras, Emerging Disease Program U.S. NAMRU-2, (e-mail address: Kanti@namru2.med.navy.mil) based on an exhaustive review of the literature and compilation of 49 publications].

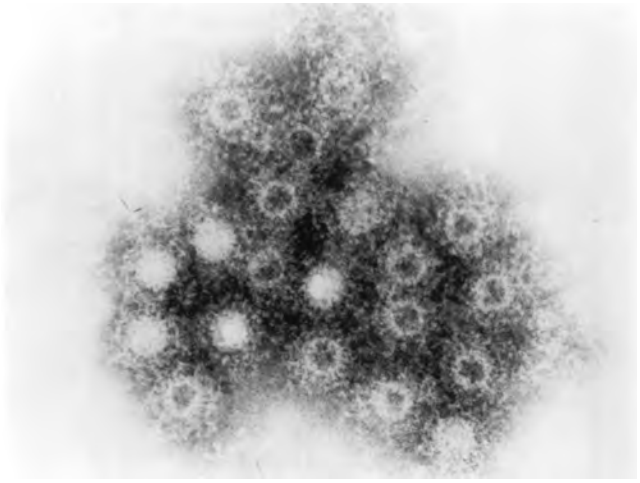

Figure 2. Immunoelectron microscopy of 27-nm HAV particles from the bile of an experimentally infected owl monkey. The virus particles are heavily coated with reagent antibody to HAV from the convalescent-phase serum of another owl monkey. (Photograph kindly supplied by Dr. John Ticehurst, U.S. Food and Drug Administration and Johns Hopkins Medical Institutions.)

as long as three months (32), and it is reported to last for nearly eight months in artificial sterile seawater (33) and at least six months in sampled groundwater after an outbreak (34). The survival of HAV in mineral water is longer at 4 °C compared to room temperature and is still infective after 300 days at room temperature (35).

It is shown that an increase in fat content contributes to the heat stability of HAV as higher exposure times are needed to achieve a 1 log reduction in virus titer in cream, when compared to skim and homogenized milk (36).

The life span of enteric viruses in immersed shellfish varies according to the microbiological quality and temperature of the water. HAV does not replicate but rapidly accumulates, and has persisted for about seven days in mussels. Conventional depuration does not provide adequate protection against the transmission of HAV (37,38). An internal temperature of 85 to 90 °C is necessary for complete inactivation of HAV in shellfish (39,40).

HAV can survive for a long time on several types of routinely used materials (41). Stability of HAV on environmental surfaces depends on relative humidity (RH), temperature, and type of surface contaminated (41,42). HAV survival is enhanced at high RH and low temperatures, especially on nonporous environmental surfaces (41).

ANIMAL RESERVOIR

The presence of antibodies in some species of nonhuman primates (great apes, Old World and New World monkeys) at the time of capture indicates that a reservoir of infection might be present in their natural habitat. There are several reports of isolation of HAV-related viruses from monkeys, several of which have significant sequence variation and minor antigenic divergence from human HAV (43,44).

VIRUS EXCRETION

Duration of virus excretion is based largely on studies in volunteers as well as epidemiologic observations and studies in experimentally infected animals. Large numbers of virions are excreted in the feces during the late incubation period and early acute phase of the disease (45–47). Fecal shedding of HAV can be studied by immunoelectron microscopy, radio- or enzyme-immunoassay, molecular hybridization and Reverse Transcriptase-Polymerase Chain Reaction assays. Recent studies using highly sensitive PCR have indicated that infected persons can excrete HAV for three months or longer (48,49).

VIRUS TRANSMISSION (TABLE 1)

Person to Person. The most predominant mode of transmission is from person to person via the fecal–oral route. Transmission is generally limited to close contacts, especially those within families. Infection rate is highest in young children, who are frequently involved in the spread of infection in households (50). Highest virus concentrations are found in feces (10^7 to 10^9 infectious doses per ml), blood (10^3 to 10^5 infectious doses per ml), and in saliva (10^0 to 10^5 infectious doses per ml) (51).

Food- and Waterborne-Transmission. About 0.4% of the documented hepatitis A in the U.S.A. is food-borne (52). In many countries, an important cause of outbreaks appears to be consumption of raw or undercooked shellfish (53). Shellfish feed by filtering large quantities of water, as a result they accumulate and retain virus in their digestive tract and tissues (54). HAV may survive in shellfish for months. Shellfish are often eaten raw or steamed, and these preparations are inadequate for virus inactivation. Food may also be contaminated during restaurant preparation or at the supplier's. Other vehicles that have been involved in outbreaks of hepatitis A include cold foods typically eaten raw or uncooked, such as milk and dairy products, fruits, meat, pastries, lettuce, and caviar. HAV is transmitted not only by raw food but also by food heated at temperatures insufficient for the virus inactivation. Although waterborne epidemics of hepatitis A have been well documented (55), waterborne transmission plays a minor role in developed countries and accounts for less than 1% of all cases seen in the United States (56). Transmission of HAV by sewage-polluted recreational water has been reported in many developing countries (57), however, there are no data indicating that a significant number of cases occur in this way (58). Nevertheless, recreational HAV exposures principally associated with swimming and bathing, for example, recreational lake water and jacuzzi, respectively, are frequently reported, temporarily and spatially focused, outbreak phenomena (57,58).

Blood Transmission. Although HAV is present in the blood for a significant period during the incubation period and early acute phase of the disease, blood-transmitted hepatitis A appears to be a rare event and only a few cases of posttransfusion hepatitis have been reported (59).

METHODS OF HAV DETECTION

Detection in Clinical Specimens

Specific diagnosis for Hepatitis A is readily made by an enzyme-linked immunosorbent assay (ELISA) or radioimmunoassay test for specific IgM (60,61). IgM anti-HAV can be detected in 95 to 100% of patients with acute hepatitis A at their first clinical presentation and usually persists for three to six months. IgG anti-HAV may also be detected at the onset of disease. The titer increases during the first weeks after onset of illness and reaches a maximum after 6 to 12 months. Immunological methods, molecular hybridization, or PCR commonly used for environmental and research studies are not practical for general clinical use. Similarly, virus isolation is impractical for diagnosis because it is insensitive, inconsistent for clinical isolates, cumbersome and time-consuming.

Detection in Environmental Samples

Environmental detection includes recovery of virus concentration and assay. A number of concentration methods have been developed; most suffer from limitations and there is no single method that can be used to detect HAV in all types of samples. Most concentration methods have achieved adequate virus recoveries with samples that have been experimentally inoculated with known quantities of HAV (62–70). Some methods have been used successfully under field conditions to recover the virus in naturally contaminated environmental samples (64,71–75). The concentration method should be customized depending on the volume of water needed to be concentrated, the probable virus concentration, and the presence of interfering substances. To monitor virus recovery efficiency, seeded samples should be included as internal controls whenever field samples are processed (76). Different techniques that have been successfully used for HAV concentration from environmental samples are described later.

HAV Concentration by Adsorption-Elution Methods

HAV HAV can be concentrated by adsorption to microporous filters and recovered by elution (77–80). The eluent can be a high-pH 0.05 M glycine buffer, a 1–3% beef extract solution at pH 9.5 to 10, or a combination of 1 to 3% beef extract-0.05 M glycine (62,68,81). The elution process is generally a single step in which the eluent is forced under pressure through the filters. HAV is efficiently adsorbed by both negatively or positively charged microporous filters (62,82,83). Efficient virus adsorption with electronegative filters requires preacidifying (usually pH 3.5) the water and the addition of multivalent cationic salts. Electropositive filters adsorb viruses efficiently over a wide range of pH without the need for chemical modifications of water. The efficiency of these filters is influenced by the amount of humic acid and other organic compounds present in the water (84,85).

For small sample volumes (86) the adsorbed virus can be eluted with a small volume of fluid. But environmental water samples are usually collected in large volumes to increase the chance of detecting low quantities of HAV. For such large volume samples, the adsorbed viruses are eluted with approximately one liter of eluent and the eluates are further concentrated to final test-volume samples suitable for virus assay (75,81). There are several techniques used for secondary reconcentration, including organic flocculation (87), ammonium sulfate flocculation (88), modified polyethylene glycol (PEG) precipitation (64), ultrafiltration (89,90) and readsorption to, and elution from, smaller diameter microporous filters (91). HAV in nonproteinaceous eluates (glycine buffer) originating from finished tap water or other waters containing low concentrations of organic matter can be reconcentrated by adsorption–elution with smaller filters (92,93).

HAV Concentration by Organic Flocculation

Organic flocculation involves precipitating viruses by acidifying eluates (beef extract or beef extract-glycine) to pH 3.5, recovering the viruses adsorbed to the flocs by centrifugation, and resuspending the pellet in small volumes of phosphate buffer for virus assay (87). Beef extract, casein, or powdered skimmed milk are usually used for flocculation. The efficiency of HAV recovery may be increased by using powder instead of paste beef extract (62). HAV can be concentrated 100- to 10,000-fold onto microporous filters and an additional 10- to 100-fold by organic flocculation (79,94).

HAV Concentration by Ammonium Sulfate Flocculation

Ammonium sulfate flocculation is an efficient method to reconcentrate HAV from beef extract eluates at neutral pH (65). This method is based on the ability of saturated ammonium sulfate to flocculate beef extract at neutral pH (88). HAV is adsorbed to the flocs and the virus-containing flocs are recovered by centrifugation. It is suitable for small volumes of all types of water but detoxification is necessary before the virus can be assayed in cell culture. It can be used as a primary or a reconcentration procedure.

HAV Concentration by Polyethylene Glycol (PEG) Precipitation

PEG, a chemically inert, nontoxic, water-soluble synthetic polymer known to precipitate a number of proteins, can be used either as a primary or secondary method for HAV concentration. For reconcentration, the eluate is adjusted to a neutral pH, supplemented, and incubated with PEG at a final concentration of 8% (wt/vol.). The precipitated HAV is centrifuged and the pellet is resuspended in 0.15 M phosphate buffer (pH 9) and recentrifuged to collect the supernate for virus assays.

PEG precipitation can be used with a variety of water samples and is unaffected by high concentrations of humic acid (64,68). It can also efficiently reconcentrate HAV in eluates from wastewater, sediments, and shellfish samples (64,69,70). PEG precipitation is superior to the organic flocculation method for HAV recovery under both laboratory and field conditions, and can be used without extensive pH treatment (64). Double precipitation with

PEG results in small volume concentrates suitable for sensitive HAV detection (68,95).

HAV Concentration by Ultrafiltration

Ultrafiltration is a process that separates particles according to their molecular weight. The membranes for ultrafiltration are defined according to the molecular weight cutoff (MWC), which is the molecular weight above which most microorganisms are retained by the membrane. HAV can be concentrated by several commercially available ultrafiltration units, depending on the volume of sample to be treated (68,72,96–98). The ultrafiltration method has several advantages: pH adjustment or additional multivalent cationic salts are not required (89,90) and organic compounds or proteinaceous solutions do not interfere with the virus recovery. HAV is successfully concentrated by ultrafiltration from a variety of environmental samples (72,83,97,99). Ultrafiltration can either be used as primary or secondary concentration.

HAV Concentration from Sludge

HAV is recovered from sludge through two steps, virus extraction and concentration to a small volume (72,100). The extraction method varies depending on different kinds of sludge. The sample is first centrifuged and the sludge pellet is suspended in an eluent (3–10% beef extract solution or glycine) with an alkaline pH, followed by homogenization. This can be done by mechanical agitation (101), magnetic agitation (102), or sonication (103). The homogenate is then recentrifuged to recover the supernate, which is neutralized to pH 7.2. Concentration of the supernate is generally done by organic flocculation, PEG precipitation, membrane adsorption—elution, or ultrafiltration for efficient HAV recovery. Freon treatment can be added before recovering the supernate (66).

HAV Concentration from Shellfish

The processing method involves homogenizing the mollusk tissues in their intervalvular fluid and extracting the viruses from the homogenate by a modified adsorption–elution–precipitation method (104). The extract is further purified and concentrated by a procedure involving fluorocarbon (Freon) extraction and PEG precipitation (64,69,70,105,106). The PEG precipitation method can obtain a high level of recovery (>90%) of HAV from shellfish (64,105). Clam tissues are more acidic than oyster tissues and constant readjustment of pH during the concentration procedure is important for effective recovery of HAV (92). Other commonly used concentration procedures for shellfish are organic flocculation (69) and ultrafiltration (107).

HAV Concentration from Food

The procedure involves washing food samples with a guanidinium phenol–based reagent (TRIzol solution), clarifying the wash retentates by centrifugation followed by extraction of the viral RNA-containing aqueous layer with chloroform, and precipitating in isopropanol (108). The equivalent of 10^2 viral genome copies of HAV seeded onto delicatessen meat could be detected by this method (108).

Identification of HAV in Sample Concentrates

Cell Culture Assays. Cell culture is the classic method by which the infectivity of HAV can be tested. Since most HAVs lack cytopathic effects (CPE), virus growth can be demonstrated by inoculation into the human hepatoma cell line PLC/PRF/5 and detection by radioimmunofocus assay (RIFA). An alternative method is to test RNA extracts from cell cultures by hybridization. However, conventional cell culture techniques have limited sensitivity, especially for the wild-type strains from field samples (109).

Molecular Biology Techniques. The most rapidly growing area of detection is based on the specific identification of viral nucleic acids by hybridization and PCR. Although dot blot hybridization is reported to be useful for the rapid detection of naturally occurring HAV (63,105,110,111), improvement of test sensitivity will be necessary for the detection of low level of viruses in many field samples (105). PCR can be used to enzymatically amplify to detectable levels nucleic acid sequences that are present in low copy numbers in field samples. The sensitivity of the PCR technique and confirmation of PCR amplification should be evaluated by techniques such as oligonucleotide probe hybridization and nested PCR assays. Nested PCR increases both sensitivity and specificity enabling the detection of low concentrations of HAV (112). The drawback of PCR is that it cannot be used for quantification or determining the infectivity of the virus. RT-PCR-based detection of HAV uses primers focused on HAV target sequences from areas in the 5′ noncoding region and the regions coding for the VP3-VP1 capsid protein or the 3D RNA polymerase (113,114). Natural strains of HAV share a high degree of nucleotide identity and can be expected to react specifically with these target sequences.

Modified versions such as antigen-capture PCR (AC-PCR) (115) and immunomagnetic capture (RT)-PCR (IC-PCR) (70,73,116) have been employed for environmental detection. AC-PCR is based on the capture of virus particles by binding to a HAV-specific monoclonal antibody, followed by PCR, and IC-PCR uses magnetic beads coated with anti-HAV antibodies against surface epitopes of HAV. In addition to avoiding the concentration of inhibitory substances (73), these methods have the advantage of indicating the presence of intact viral particles, suggesting possible infectivity (116). This is of great public health significance as there is no reliable confirmation method to determine infectivity of natural HAV strains. IC-PCR is 100- to 1,000-fold more sensitive than RT-PCR (116). Multiplex-PCR, a rapid and cost-effective method for simultaneous detection of HAV and other enteric viruses with a single amplification procedure, has recently been developed (98).

Removal of PCR Inhibitors. PCR cannot be performed with most concentrated water samples unless reaction inhibitors are removed before RT and/or the amplification reaction (PCR). Humic acid and other organic materials

that are concentrated along with viruses in environmental samples may interfere with nucleic acid polymerase function (117). Various methods to remove PCR inhibitors from environmental samples have been described (118–120). Beef extract, which is commonly used for eluting adsorbed viruses, also contains high concentration of proteins, salts, and other solutes (121) that can interfere with molecular detection methods. Sample concentrates can be purified by removing inhibitory substances prior to PCR by chemical means such as phenol-chloroform extraction (75,110), guanidinium isothiocyanate (GIT) extraction (70,111), or by physical means such as sephadex gel chromatography (68,74,119,120). A proprietary protein-precipitating agent, Pro-Cipitate, can also be used to further purify viruses in sample concentrates for RT-PCR (68,69,106). A combination of purification measures can be used to efficiently remove PCR inhibitors (68). Removal of shellfish polysaccharides from nucleic acid is also necessary before viral RNA can be detected. In addition to the above methods, the cationic detergent cetyltrimethylammonium bromide (CTAB) is also effective for removal of shellfish polysaccharides (105). CTAB can selectively precipitate nucleic acids while leaving proteins and polysaccharides in solutions (122).

Indicators of HAV Contamination in Aquatic Environments

Monitoring for hepatitis A in aquatic environments is not in the best interest for public health protection because the virus is present in very small concentrations in the environment; methods are not yet practical for the purpose of routine monitoring.

Bacterial Indicators. At present, viral contamination of water and wastewater is based mostly on bacteriological quality indices. Monitoring the traditional bacterial indicators (*Escherichia coli* fecal streptococci and coliforms) is rapid, easy, and inexpensive, but HAV is more resistant to environmental conditions, sewage or water treatment processes than current bacterial indicators (123). Studies have showed that HAV contamination is not associated with *E. coli* (124) and fecal coliforms (110,125). Outbreaks of HAV from water and food considered to be safe based on bacteriological standards (126,127) indicate that more reliable microbial indicator systems are needed.

Bacteriophages. (see BACTERIOPHAGE AS INDICATORS) Bacteriophages are viruses that arise from host bacteria that become infected and subsequently lyzed. Three basic groups of phages have been proposed as candidate indicators for enteric viruses: somatic coliphages (128), F-specific (F^+) bacteriophages (129), and phage-infecting *Bacteroides fragilis* (130). B40-8 phage infects *B. fragilis* HSP40, which is considered of human origin (130,131). As a result of their inability to multiply in the environment (131,132) and resist water treatment better than other phages (133), B40-8 are potentially useful as indicators of the virological quality of water. However, *B. fragilis* phages are found in low concentrations in groundwater, and the techniques for detection are more complicated than those used for other phages (134). There is no association of bacteriophage with hepatitis A virus recovery (124,135).

Other Viral Indicators. It has been suggested that enterovirus could be used as an indicator of HAV presence (117,136). However, there is little correlation between enterovirus and HAV occurrence; river water samples that were negative for enterovirus have been found positive for HAV (137), and conversely, river water and tap water samples positive for enterovirus were negative for HAV (112). More recently, it has been suggested that the detection of adenoviruses by PCR could be a better indicator of enteric viruses than enteroviruses as they are more stable in various environments and are more resistant to water treatment (138). However, HAV is more resistant to disinfection than human adenovirus (134).

PREVENTION

General

Providing populations with clean water, proper waste disposal, and improved living conditions rapidly reduces the incidence of hepatitis A. General hygienic measures are also most important in limiting person-to-person transmission. Transmission by foodhandlers can be reduced by training on proper hygiene and foodhandling practices. Proper handwashing during all phases of food preparation should be emphasized. The public needs to be educated that HAV is heat sensitive and that infection can be avoided by adequate heat treatment. Shellfish should be heated for 1.5 minutes at 85 to 90 °C for consumption (39). Travelers to developing countries should be advised to eat only properly cooked food and be careful with uncooked vegetables and shellfish.

HAV Passive Immunization

Until recently, the mainstay of hepatitis A immunoprophylaxis available has been passive immunization by means of pooled immunoglobulin (IG). If administered before exposure, passive immunization can reduce the incidence of hepatitis A by up to 90% (139). Postexposure prophylaxis with IG is also effective if administered within two weeks of exposure and will usually prevent or reduce the severity of disease (140).

HAV Active Immunization

Inactivated and live-attenuated vaccines against hepatitis A have now been developed as a result of progress in hepatitis A research. A safe and highly effective formalin-inactivated whole virus vaccine of high efficacy has been licensed throughout the world (141–143). In addition, a live-attenuated vaccine has been developed (131) and a novel vaccine antigen delivery system, termed *virosomes*, has been utilized to construct hepatitis A vaccines (145,146).

HEPATITIS E

Biology

Hepatitis E (HEV) is an icosahedral, nonenveloped, positive sense single-stranded RNA virus of 27 to 34 nm

(Fig. 3). The virus remains unclassified, although its genomic organization most closely resembles that of caliciviruses and is substantially different from that of picornaviruses such as HAV (147). The first complete nucleotide sequence for HEV was published by Tam and coworkers in 1991 (148). To date, four different strains (Burma, Mexico, Pakistan, and China) have been completely sequenced (149). A recent phylogenetic analysis of 26 HEV strains, based on nucleotide sequence, revealed three principal genotypes: Asia Africa (I), United States (II), and Mexico (III) (150). The HEV genome has three overlapping open reading frames (ORFs), with the nonstructural proteins located at the 5′ end of the 7.5 kb genome and the structural protein(s) located at the 3′ end. Although most of the structural proteins are coded within ORF2, all three ORFs contribute to the morphology of HEV (151). Studies showed that physicochemical properties of HEV regarding size, morphology, sedimentation coefficient, and buoyant density closely resemble those of caliciviruses (148,152). Subsequent analysis of nonstructural genes has shown similarity to the alpha-viruses, furoviruses and rubella viruses (153). To date, HEV is known to exist as a single serotype, that is, HEV isolates from different geographic regions share the same antigenic pattern. Accurate characterization of HEV has been hampered because of a lack of a tissue culture system, and the biology of HEV is poorly understood.

STABILITY

HEV is reportedly more labile than HAV. HEV does not survive exposure to high concentrations of salt, including cesium chloride, or freeze-thawing (154), but it is unaltered by exposure to trifluorotrichloroethane (155). The viral particles tend to lose their integrity after routine laboratory procedures, whereas in the environment they are more stable and survive exposure to extreme conditions (156). Epidemiological data suggest that boiling water may inactivate HEV (157,158). However, no data are available regarding the efficacy of water and wastewater disinfectants, including chlorination, in inactivating HEV.

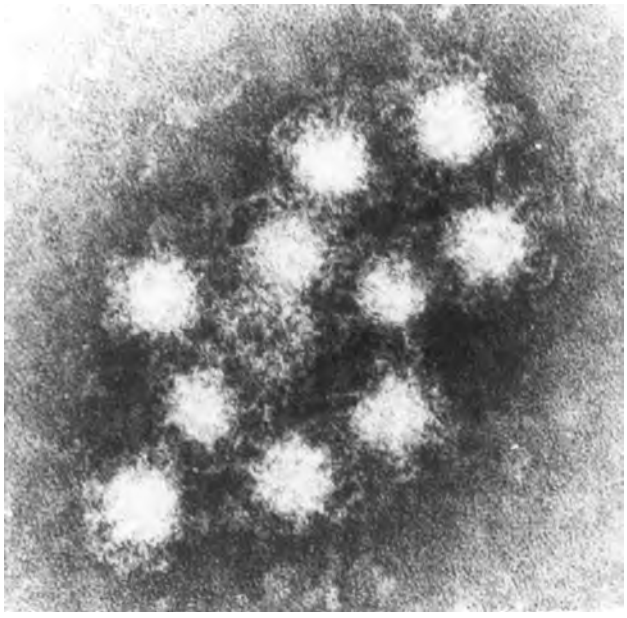

Figure 3. Immunoelectron microscopy of 29-nm HEV particles from the bile of an experimentally infected cynomolgus monkey. The virus particles are heavily coated with reagent antibody to HEV from the acute-phase serum of another cynomolgus monkey. (Photograph kindly supplied by Dr. John Ticehurst, U.S. Food and Drug Administration and Johns Hopkins Medical Institutions.)

ANIMAL RESERVOIR

Various species of nonhuman primates are known to be susceptible to HEV infection, at least under experimental conditions (155,159). It has been shown that some of these species can be infected in their natural habitat (3). In an endemic area of Nepal, naturally acquired HEV specific antibodies were detected in chickens, pigs and rats (160). Recent evidence suggests that swine may be an important reservoir of infection in the United States (161) and Taiwan (162,163). The possible ramifications of this zoonotic reservoir give caution to future xenotransplantation into humans (164). HEV was detected by immunoelectron microscopy in the blood of wild rats and mice trapped near an outbreak of hepatitis E in Kirgizia (165). Recently, it was reported that anti-HEV was prevalent in rodents throughout the United States, providing evidence that rodents might be a natural reservoir of HEV (166). These findings suggest that HEV may be a zoonotic virus, however, there is no evidence supporting the transmission of HEV from animals to humans.

VIRUS EXCRETION

Fecal excretion of HEV is in low titers compared to HAV and occurs predominantly during the first week of jaundice (167), but may persist for 14 days after the onset of clinical illness (168). The viral particles are fragile in vitro and little is known about their viability in the environment.

VIRUS TRANSMISSION

Fecal-oral transmission (Table 1) is well documented for HEV. Most hepatitis E outbreaks are caused by fecally contaminated water. Use of river water for drinking and cooking, personal washing, and human excreta disposal were all significantly associated with high prevalence of HEV infection in Southeast Asia (10). Epidemic situations often arise during the rainy season or floods when sewage waters gain access to open water reservoirs (169,170). Unlike HAV, which has a 10 to 20% secondary attack rate among household contacts, there is low incidence of person-to-person transmission, around 2% (157,171,172). The low titer of infectious virions shed in the feces during infection may account for the decreased secondary spread of hepatitis E (173). No chronic or carrier state has been reported following infection with hepatitis E (174). Only a few cases of food-borne transmission have been

reported (175,176), and food-borne hepatitis E is regarded as of small significance. In these cases, shellfish was identified as the vehicle for transmission.

METHODS OF VIRUS DETECTION

Detection in Clinical Specimens

Molecular cloning of the HEV viral genome has led to the development of sensitive and reproducible diagnostic tests, including enzyme immunoassays for antibody by immobilization on recombinant proteins or synthetic peptides (159,177–181). An excellent, comprehensive review of HEV detection, collection, and storage techniques was published recently (182). IgM anti-HEV appears early during clinical illness but disappears rapidly over a few months. IgG anti-HEV appears a few days later and persists for at least a few years (183). Commercially available ELISA helps detect antibodies of both IgM and IgG class and is useful in diagnosing acute and past HEV infection. Commercially available serological tests using recombinant proteins are more sensitive, reportedly detecting anti-HEV in 80 to 90% of patients with acute hepatitis during outbreaks of hepatitis E (178,184). Different methods to detect viral-specific nucleic acid by RT-PCR have been developed and evaluated for their efficiency in detecting viral-specific sequences (185). Recently a rapid RT-PCR procedure using universal oligonucleotide primers was developed for detection of HEV-RNA in serum (186). While HEV detection in serum or stools has high sensitivity and specificity, the process is labor-intensive, and when the method is used in less-experienced laboratories, problems of nonspecificity, primarily due to specimen contamination, might complicate the interpretation of results (187). Sensitivities for the various HEV assays have been reported to vary between 17 and 100% in nonendemic areas, suggesting caution in interpreting results from low-prevalence populations (188). Moreover, these tests may perform differently in endemic and nonendemic settings (189). Immunoelectron microscopy is not practical due to low levels of virus excretion (190).

Detection in Environmental Samples

Environmental detection of HEV has been reported (191,192), but more data are needed on applicability to various field samples. Environmental detection of these viruses requires three general steps: collecting a representative sample, concentrating the virus in the sample, and identifying the concentrated virus.

HEV Concentration by Adsorption-Elution Method

HEV from environmental samples can be adsorbed by either granular activated carbon (GAC) column (192) or membrane filters (191), and eluted with urea-arginine phosphate buffer at pH 9.0. Further reconcentration of the primary eluate is done by adding magnesium chloride, and the precipitate formed is recovered by centrifugation for virus assay.

Identification of HEV in Sample Concentrates

At present there is a lack of efficient cell culture methods for HEV, and the few reports of HEV detection in field samples is based mainly on RT-PCR with confirmation by hybridization (191,192).

PREVENTION

Although variation in pathogenicity and infectivity of HEV in one area may be due to different strains, environmental factors are a major contributor to the endemic and epidemic spread of HEV. Management of a community outbreak involves identification and correction of the environmental factors promoting fecal contamination of drinking water. Seasonal monsoons can lead to floods that can overwhelm water treatment facilities, leading to contaminated drinking water. Water treatment facilities should be upgraded, and improved hygiene standards and general sanitation could reduce transmission. Travelers to HEV-endemic regions should avoid drinking water (and beverages with ice) of unknown purity and eating uncooked shellfish, fruit, or vegetables. In an HEV epidemic, drinking water should be boiled as chlorination alone may be inadequate (158).

HEV Passive Immunization

Based on recent studies (184), immunoglobulin may be beneficial, but there have been no controlled clinical studies. Passive immunization of nonhuman primates with HEV antibodies protects them from disease after HEV challenge (193), and it is possible that prophylactic treatment, by passive immunization, may have an impact on the natural course of infection during pregnancy. This is important because pregnant woman, particularly in the third trimester, can suffer fatality rates as high as 32% (194).

HEV Active Immunization

Currently no commercial vaccine is available. The development of an inactivated or live attenuated virus vaccine has been hampered because HEV does not replicate efficiently in cell culture. Recombinant vaccines have given good results with primates against experimentally induced hepatitis E (195,196) and they may be particularly useful for travelers to disease-endemic areas and for pregnant women (183). Phase I safety and immunogenicity evaluation of a new recombinant HEV vaccine have been successfully completed in Nepalese volunteers by the U.S. Army laboratory based in Bangkok, Thailand.

In conclusion, HAV and HEV share more epidemiological attributes associated with mode of transmission, as compared with their respective, and very much distinctive, environmental morphological characteristics. Fecal–oral mechanisms contributing to the spread of both viral agents make them both highly communicable from an outbreak perspective, particularly in areas where water sanitation and hygiene are compromised. At the same time, the water-related nature of transmission allows for reasonably effective measures in preventing exposure type opportunities. Finally, surveillance attention

should be paid to changes in disease virulence against a background of increasing environmental pressures, including induced protection afforded by vaccination and even global warming.

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HETEROTROPHIC BACTERIA AS INDICATORS OF WATER QUALITY

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The heterotrophic bacteria in water comprise many diverse groups of organisms. Some of these organisms are ubiquitous to all aquatic systems, whereas others represent some contact with varying inputs of stormwater runoff, waste discharges, wildlife populations, soil, vegetation, and airborne dust particles. Their continued survival or potential for colonization in the aquatic environment are qualified by availability of nutrients, favorable water temperatures and pH, and the status of dissolved oxygen concentration. Counter influences in the aquatic environment include microbial predation by amoebae and nematodes, antagonism from competing organisms, sunlight exposure, and entrapment in sedimentation that limits the extent of

population growth. Seasonal changes in water temperature and pollution spills into the aquatic environment are other powerful influences that distort bacterial profiles and shift the dominance of these organisms at any given time (1).

It has been estimated that the community of heterotrophic bacteria may comprise up to 90% of all aquatic bacteria detected in surface waters (2) and largely are responsible for the wide fluctuations in bacterial populations noted in surface waters. Because groundwaters generally are protected from the intrusion of contaminants by a soil barrier, species diversity and density of heterotrophic bacteria are very limited and show little change.

Predominant genera of heterotrophic bacteria include *Flavobacterium, Cytophaga, Achromobacter, Acinetobacter, Alcaligenes, Moraxella, Vibrio, Aeromonas, Pseudomonas*, environmental coliforms (*Klebsiella, Aerobacter*, and *Citrobacter*), and fecal coliforms (*Escherichia* and fecal *Klebsiella*) (3). Among this wide spectrum of organisms are some species that have the ability to form brightly colored nonphotosynthetic, nondiffusible pigments (yellow, orange, pink, purple, brown, and black). This unique property is often not observed in the routine processing of water samples unless the incubation time for heterotrophic bacteria is extended beyond three days. These pigmented bacteria appear in greatest abundance in high-quality water (water supply, bottled water, and water attachment devices) and often are found to be *Flavobacterium* and some species of *Mycobacterium, Serratia, Corynebacterium*, and *Chromobacterium*. For example, the proportion of pigmented bacteria in public water supplies may represent 10–62% of the heterotrophic bacterial density. Water treatment processes, particularly granular activated carbon (GAC) filtration and disinfection may be selective factors for the occurrence of pigmented bacteria in some water supply microfloras. At the Ohio River intake, yellow-pigmented bacteria often were predominant (26% of the heterotrophic population) during the summer, all other color groups (orange, pink, purple, brown, and black) accounting for less than 6% of the total percentage of pigmented bacteria in any season (4). Why this pattern of occurrence is common is not known. Public health significance of these pigmented organisms is also not known; however, some of them are known to be plant pathogens.

Other unusual heterotrophic bacteria are known to occur in various aquatic environments such as marine waters, various waste discharges, mine drainage, heat-exchange water systems, and irrigation-return waters (5). Density magnitudes expected for these organisms may vary widely, depending on the aquatic environment and the introduced contaminants. Typical examples of heterotrophic bacterial densities in rivers and raw sewage are given in Table 1 to illustrate the impact that wastes may have on defining the state of a surface water resource.

Although heterotrophic bacterial measurements often are ignored in the characterization of safe drinking water, their general presence in water should not be ignored because this population may contain some undetected agent of pubic health concern. Any chance multiplication in drinking water systems to more than 1,000 organisms per milliliter should not be tolerated because of the concern that some chance occurrence of a pathogenic agent may reach an infective dose level. Furthermore, this situation may lead to an upgrade in density for some opportunistic pathogen presence in the water that impacts on some susceptible individuals, increases the potential for coliform colonization in a biofilm, and elevates the density for bacteria that create taste and odors. Table 2 illustrates several of these concerns with different kinds of drinking water supplies available to the public. Disinfecting water supply does suppress heterotrophic bacterial growth and dramatically reduces coliform occurrences. Although rain water generally is of excellent microbial quality on release from the atmosphere, catchment and storage provide opportunities for the greatest deterioration of its quality because there is little effort to keep the catchment area free of dust, debris, and bird fecal droppings. Many of these cisterns receive very infrequent clean-out of sediment accumulations and infrequent disinfection, and the water supply is prone to stratify rather than circulate. Such environments often lead to water-quality deterioration induced by microbial contaminants, biodegradable sediments, and poor sanitation practices. (See MICROBIAL WATER QUALITY OF RAINWATER ROOF CATCHMENT SYSTEMS).

Table 1. Heterotrophic Bacterial Densities in Various Rivers and Sewage

Water Quality	Heterotrophic Bacterial Density per mL
River Characteristic	
Remote streams	<500
Relatively clean rivers	5,000–10,000
Moderately polluted river water	25,000–50,000
Polluted river water	≤100,000
Grossly polluted rivers	≤1,000,000
Municipal Raw Sewage	
Worcester S.A.	11,100,000
Pietermaritzburg, S.A.	13,700,000

Source: Data adapted from I. Daubner, *Microbiologie des Wassers*, Inst. f. Limnologie, Slowakischen Academic der Wissenschaften, Bratislava, Slovakia, 53–104, 1972 (6); and W. O. K. Grabow and E. M. Nupen, *Water Res.* **6**, 1557–1563 (1972) (7).

DEFINITION

The heterotrophic group of bacteria has differences in morphology, gram-staining response, and biochemical reactions but all have an essential need for energy-source compounds and trace elements that contain carbon, nitrogen, sulfur, and inorganic ions necessary for the synthesis of living cell materials. The metabolic requirements of individual species in this diverse group of bacteria vary tremendously as to the essential compounds necessary to stimulate growth (17,18). For example, some of these bacteria (autotrophic organisms) have simple nutritional requirements that include only water,

Table 2. Heterotrophic Bacterial Densities in Different Water Supply Sources

Water Class	No. Samples	Heterotrophic Density Range per mL	Total Coliform % Occurrence	Reference
		Public Water		
System A[a]	525	<1–310	1.7	Emde et al. (8)
System B[b]	808	<1–600	1.6	Emde et al. (8)
		Rural Water		
Wells, springs	42	10–8,400	50.0	Lamka et al. (9)
Wells	20	10–19,000	25.0	LeChevallier and Seidler (10)
		Packaged Supplies		
Bottled water	164	<10–390,000	5.5	Geldreich et al. (11)
	189	<10–>2,000	0.0	Toronto Rpt. (12)
		Home Treatment		
Point-of-use[c]	48	4,900–170,000	10.4[d]	Geldreich et al. (13)
	25	<10–>2,000	44.0[d]	Toronto Report (12)
		Rainwater Catchment		
Tropical Cisterns	47	<10–56,000,000	56.1	Crabtree et al. (14)
Tropical Cisterns	240	270–23,000,000	90.0	Ruskin and Callender (15)
Temperate Cisterns	30	2,000–220,000,000	30.0	Lye (16)

[a]Total chlorine residual 1.77–2.24 mg/L; Canadian utility
[b]Total chlorine residual 0.58–2.40 mg/L; American utility
[c]Carbon filter device
[d]Several tap water inputs to point-of-use devices had adventitious coliforms passing through the distribution system.

carbon dioxide (CO_2), and appropriate inorganic salts; the conversions to energy being derived from oxidation or the driving force of a photosynthetic process not unlike the metabolic activity common to plant life. *Nitrobacter* that assimilates CO_2 and obtains energy from the oxidation of nitrite to nitrate are often found in groundwaters. Sulfur and iron bacteria that synthesize basic inorganics are other examples of autotrophic bacteria that may colonize some well water supplies (19). At the other end of the spectrum there are heterotrophic bacteria that are very fastidious in their nutrient requirements, requiring a variety of very complex organic compounds such as vitamins and amino acids (growth factors) that they are not capable of synthesizing. For this reason, organisms such as *Legionella*, which have fastidious nutrient requirements, depend on the presence of by-products released by the metabolic activity of different bacteria in the aquatic flora to survive.

CULTIVATION

Maximum recovery of the heterotrophic bacterial population in water is driven by cultivation methodology. Consideration must be given to four interrelated factors: medium formulation, cultivation temperature, incubation time, and in situ placement of captured organisms on a nutrient surface for growth (20–22). Two different approaches to medium development have evolved in an attempt to stimulate optimum resuscitation of stressed organisms and bacteria with special nutritional needs for cultivation.

- Dilute Medium

  The medium must provide not only a nutrient base that mimics the dilute chemical constituent levels found in various water environments (water supply and marine water) but also be inclusive of essential salts and organics necessary for resuming the rapid cell-growth rates. Most challenging is the need to be responsive to the recovery of stressed organisms whose cell membranes are functioning poorly through dysfunctional membrane osmotic gradients and to metabolic pathways disrupted by damaged enzyme systems or starvation. Such is the status of organisms known as ultramicrobacteria. These are organisms that have become very small through gradual starvation and thereby are capable of surviving for extremely long periods of time in low nutrient waters. In this state some of these organisms develop a mucoid mutant that becomes more predominant in the population over time, whereas others may go into a resting spore stage. Detection of these organisms may be difficult if attempts are made to cultivate them immediately on nutrient-rich media. Therefore, their occurrence is often overlooked unless a very dilute medium, such as R-2A, is used with extended

cultivation time (five or seven days) at an incubation temperature of 20 to 28 °C (23).

- Enriched Medium

  For organisms that are in a vigorous state of health (e.g., fecal bacteria found in raw municipal sewage or food-processing wastes), enriched medium formulations will result in rapid bacterial growth and early visualization. Standard plate count (SPC) agar (tryptone glucose yeast extract agar) and m-HPC medium (formerly known as m-SPC medium) are examples of enriched medium formulations with cultivation established at two days in an environment of 35 °C (24).

- Incubation Temperature/Time

  Incubation temperature controls not only the spectrum of organisms that may be cultured but also the rate of growth into colonies. The traditional incubation temperature of 35 °C is not realistic for many of these organisms whose origins are water shed soils, vegetation, and nonfecal waste discharges or who are stressed by self-purification forces and have become adjusted to the environment. As a consequence, many of these bacteria respond better to a cultivation temperature that has been established between 20 and 28 °C. Incubation time is interrelated with selection of temperature for the visualization of colonies. Organisms in general grow much slower at 20 than at 35 °C so that although two or three days may be sufficient for adequate visualization of colony development at 35 °C, five to seven days are necessary to see more developing colonies on media incubated at 20–28 °C. For a few organisms, seven days of incubation is not enough time to visualize those very slow growing organisms. As a consequence, incubation for 28 to 30 days has been suggested to achieve a near "total count" of all viable heterotrophic bacteria present in a drinking water sample (25). However, this length of incubation time is impractical for routine laboratory analysis of water samples in the distribution system and provides little additional significant information about water quality. There also is the serious problem of moisture loss in the culture medium and the potential for air contamination during the extended incubation time that will impact cultivation adversely unless strict requirements for moist, sterile air in the incubator are met. As a practical consideration, the incubation time for optimizing the recovery of heterotrophic bacteria in drinking water has been set at five or seven days to fit into the normal operational schedule of the laboratory.

- Laboratory Cultivation

  The placement of organisms in contact with the medium also may influence the recovery in several ways. Selection of the appropriate laboratory procedure is critical for recovery of some organisms or the visualization of others. For example, better development of pigmentation is possible when the organisms are not imbedded in agar medium.

Pour-Plate Technique. This procedure has been a traditional method for the enumeration of bacteria in a water sample (20,21). One milliliter or less of the water sample is added to liquid agar medium that has been tempered between 43 and 46 °C to prevent heat-kill of the organisms. The appropriate medium had been previously formulated and prepared to contain selected nutrients so as to promote growth of many of the heterotrophic bacteria in water. By adding the sample portion and mixing it into the medium, viable organisms are spread throughout the culture dish that, upon cultivation, will develop into discrete colonies. Problems to consider are the choice of medium formulation, temperature of the liquid agar during sample addition, and dispersion of bacteria throughout the medium. Deep agar pour cultures may restrict growth of some strictly aerobic bacteria, whereas characteristic spreading surface growth of other bacteria on the surface may suppress development or mask visualization of neighboring colonies. Various aspects of these problems can be avoided by adequate media selection, good sample dispersion within the culture dish, and avoiding formation of water droplets from condensation of hot liquid medium during the pour plate procedure. This latter problem also can contribute to confluent surface growth that obscures accurate counting of discrete colonies.

Spread Plate Technique. The spread plate method circumvents problems associated with the pour plate technique because the inoculum is spread over the surface of a solidified agar plate (20,21). Spread plates give higher counts although precision of replicates sometimes is erratic. Much of this discrepancy occurs in the spreading technique. Although the sterile glass rod spreads the water sample over the agar surface to achieve uniform dispersion, some organisms remain attached to the spreading device and are lost in the transfer process. Because this is not a uniform loss, some variation in replicate spread plates is unavoidable.

Membrane Filter Procedure. Membrane filter techniques provide another approach to surface cultivation of organisms (20,21). The principle of membrane filtration is passage of a water sample through porous membranes (pore size typically at 0.45 microns or less) with entrapment of microorganisms on the surface of the filter. The filter is then placed over a nutrient substrate that provides passage of the nutrients to the filter surface where viable cells are nourished during cultivation in a high-humidity incubator at a prescribed temperature and time. The unique advantage of this technique is that it permits the analysis of larger sample volumes of high-quality water than either the pour plate or the spread plate agar cultivation procedures.

The only limitations to size of the sample analyzed are turbidity, bacterial density, and spreading colony formation on the filter surface. Fine sand particles are not as serious a problem as are colloidal clay or debris entrapment. These latter particulates plug the membrane filter pores and interfere with discrete colony development. Because the diameter of a standard membrane filter is 47 millimeter and the effective filtration surface somewhat less, the density limit for discrete colony development will be less than 200 colony-forming units (c.f.u.).

ORIGINS OF THE HETEROTROPHIC BACTERIAL FLORA

All natural water environments (i.e., surface waters, groundwater, springs, and marine waters) have a signature profile of bacteria that are specific to that aquatic source. In addition, there are various contributions of transient organisms from life's many controlled and uncontrolled uses of water in nature and by anthropological activities (22,26).

- Indigenous Bacteria

  These are the naturally occurring bacteria found in surface waters that are remote from wildlife and human activities or are protected from the influences of surface contamination in deep aquifiers. They largely are ubiquitous, saprophytic organisms, some of which belong to the genera *Micrococcus, Pseudomonas, Chromobacterium, Serratia, Flavobacterium, Acinetobacter*, and *Alcaligenes*. Origin of these organisms may be from particulates traveling in rain or dust storms and from interfaces with contacting soil and vegetation. Some indigenous bacteria are difficult to detect using conventional laboratory techniques because of their slow growth or fastidiousness. In recent years there has been a growing interest in these organisms because of the part they may play in the development of biofilm, particularly in groundwater, water pipe networks, cooling towers, and industrial processes (3,18).

- Transient Bacteria

  Perhaps the greatest public heath concern with natural waters is contact with organisms that are introduced in sewage discharges, food-processing wastes, urban and rural storm water runoff, animal feedlot operations, and wildlife (27,28). The microbial flora of sewage is predominantly derived from fecal wastes, bath water, food preparations in the kitchen, and urban storm water. Thus it should not be surprising to expect that a variety of pathogens shed by ill people, pets, and wildlife in the community will find their way into water resources via wastewater. Many of these pathogenic bacteria, such as *Salmonella, Shigella, Campylobacter, Yersinia*, and *Escherichia coli* 0157 : H7, are heterotrophic organisms. Fortunately, within the complex sewage flora there are many beneficial organisms that are responsible for much of the biological breakdown of degradable wastes. Some of these beneficial bacteria also have mutated to better degrade unique organic complexes released in domestic or industrial liquid wastes.

Sugar processing from beets or sugarcane, cannery food processing, and paper mill wastes also have a drastic impact on water quality. Elevated concentrations of nutrient materials released in those wastes often lead to sudden, dramatic growth of heterotrophic bacteria by depressing natural self-purification processes in receiving waters. Urban and rural storm water runoff over the water shed flush out entrapped heterotrophic bacteria from soil and vegetation as rainfall washes over ground and cement surfaces. In the rural environment, storm water runoff can be a serious vehicle of bacterial transport from animal feedlot operations (e.g., cattle, pigs, and poultry) (29,30). These agricultural activities create a massive animal waste–loading problem that may exceed that of municipal sewage in small towns nearby. If the animal wastes are not diverted to a lagoon or landfill, the storm water runoff will add extremely high levels of heterotrophic bacteria, including intestinal pathogens, to the drainage basin and receiving waters. Seepage from landfills of solid wastes in domestic garbage (Table 3) and

Table 3. Microbial Characterization of Solid Wastes from Various Cities

Waste Collection Site[a]	No. Samples	Organisms g (wet wt)[b]		
		Heterotrophic Bacteria	Spores	Total Coliforms
A	4	110,000,000	270,000	3,000,000
B	3	450,000,000	110,000	6,700,000
C	6	78,000,000	38,000	1,600,000
D	3	480,000,000	31,000	1,100,000
E	4	680,000,000	1,900,000	51,000,000
F	2	54,000,000	35,000	13,000,000
G	2	4,000,000	25,000	340,000
H	3	300,000,000	160,000	8,600,000

Data from M. L. Peterson, *Pathogens Associated with Solid Waste Processing: a Progress Report*, SW-49r, U.S. Environmental Protection Agency, Cincinnati, Ohio, 1971 (31).

[a] Waste collection sites in Cincinnati, Chicago, Memphis, Atlanta, and New Orleans.

[b] Average moisture content 41%.

sludges from water processing and sewage treatment may penetrate the soil barrier and migrate into groundwater, thereby introducing microbial pollution into the nearby aquifer that otherwise would be populated sparsely with organisms.

HEALTH CONCERNS WITH AMPLIFICATION IN WATER SUPPLY

The heterotrophic bacterial population in drinking water includes many organisms that never become established in the distribution system, whereas others are more adaptive, being capable of surviving on minimal nutrients, of attachment to pipe sediments, and of actively participating in a developing biofilm. Some of these adventitious organisms may cause taste and odors; others cause in situ pipe corrosion, interfere with coliform detection, or are transferable antibiotic-resistant organisms. Microbial populations in water leaving the treatment plant generally are below 100 organisms per milliliter but rapidly increase to densities that often are 1 or 2 logs higher at static water locations in dead ends of the distribution system and in building plumbing systems. Two factors play a part in these situations: no available disinfectant residuals to suppress growth and the presence of warm water conditions in building pipe networks. Such microbial population expansions in drinking water may be of no health risk to most people in the community, however, some of the bacteria may present a opportunistic risk to some individuals under severe health stress.

- Opportunistic Pathogens

  Opportunistic pathogens generally are considered to be organisms that exist as part of the normal body microflora but under certain situations can cause disease in a compromised host. In general, these are the organisms that when found in large enough numbers and under optimum conditions, may have the potential to cause an infection in susceptible individuals (3). Such organisms particularly become invasive in some individuals [e.g., the elderly, newborns, acquired immune deficiency syndrome (AIDS) victims, cancer patients receiving chemotherapy, burn cases, dialysis patients, trauma patients, and individuals receiving organ transplants].

  Infective dose levels for a 50% attack rate among healthy children and adults may range up to 10^{10} cells per dose. Although the number of cells required to achieve an infective dose by ingestion may seem unlikely, the volume of water used to take a shower or bath easily can supply this density during a given exposure period. By contrast, the density of such opportunistic organisms needed to establish infection in newborn babies, postoperative or immunosuppressed patients, and the elderly with infirmities are much lower than that in healthy individuals. The route of exposure may be ingestion, inhalation, or body contact with water supply. A large part of the problem is due to colonization of these organisms in water attachment devices used in the home, hospitals, and clinics (3,22). Acinetobacter infections have been associated with the use of ventilator spirometers, room humidifiers, and moisturized Wright respirometers. Other organisms such as *Pseudomonas* have been the agent in a waterborne outbreak in a newborn nursery, provide a major threat to recovery from serious burns, and can be the source of infections from dental equipment; *Legionella* has been a respiratory agent in indoor air climate–control devices; and nontuberculous *Mycobacterium* has been involved in the spread of infections from aerosolized whirlpool therapy. Several studies have suggested that water supply organisms are part of the problem but not necessarily the major source of nosocomial infections in the hospital environment.

- Antibiotic R-Factor Transfers

  Heterotrophic bacteria in water supply, recreational waters, and whirlpools that are resistant to one or more antibiotics may pose a health threat if these strains are opportunistic pathogens or serve as donors of the resistant factor to other bacteria that could be pathogens (3,22). Antibiotic-resistant (R-factor) bacteria may originate in fecal wastes of ill people taking antibiotics or farm animals being fed antibiotics. On entering waste-treatment systems, these bacteria come in contact with receptive organisms and may pass on their resistance factors through plasmid transfers while in the process basin. In so doing, some of these bacteria acquire multiple antibiotic resistance. Treatment does not destroy all of these antibiotic-resistant organisms and those that survive pass into the wastewater effluent released to receiving waters. Much the same scenario of multiplication and transfer contact occurs with farm animal wastes as it is spread by stormwater runoff over the drainage basin and into streams and lakes. Downstream, these waters often are the source of raw water used in water supply treatment. Although treatment processes inactivate or remove many introduced antibiotic-resistant organisms (e.g., *Aeromonas, Hafnia*, and *Enterobacter*) from the source water, a shift of this transmissible factor to other organisms (e.g., *Pseudomonas* or *Alcaligenes* group, *Acinetobacter, Moraxella, Staphylococcus*, and *Micrococcus*) may occur (32). In the process some of the surviving organisms that acquire multiple resistance to different antibiotics also are resistant to disinfectant exposure. Many of these transformations occur in the established biofilm found in activated carbon or mixed media filtration basins. The reason for the common occurrence of streptomycin resistance among bacteria that survive chlorination is not known. In a typical population of 100 heterotrophic bacteria that may be present in a glass of drinking water, 40 to 70 of these organisms could be expected to have some antibiotic-resistance factors (32). What health risk this presents when the heterotrophic bacterial population in water supply ranges above 1,000 organisms per milliliter is not clearly understood yet.

COLONIZATION IN WATER SUPPLY

Heterotrophic bacteria are a major nuisance in water supply (e.g., biofilm creation in well casings, treatment basins, distribution systems, biologically mediated corrosion of pipe materials, and producers of unpleasant tastes and odors in static water areas) (3,18). Biofilms of iron and sulfur bacteria in well casings eventually may overwhelm efforts to extract water from the aquifer even when high concentrations of disinfectant are applied to inactivate the organisms. Treatment process management that includes routine flushing of basin walls, connecting flumes and coagulant agitators with high-pressure water jets can be very effective in suppressing the development of biofilms.

Water quality produced at the plant begins to change soon after it enters the distribution system and arrives at the home, office, or hospital tap. It has long been recognized that water must be keep moving in the distribution system to maintain the best microbial quality. Once it reaches a static zone, water quality begins to change because of microbial colonization that may or may not be of public health significance. Sites for colonization of heterotrophic bacteria will be found in slow-flow areas of the pipe network where water demand is low, in tanks with stratified water storage, in areas of changing pipe diameters, and in pipe network dead ends. These are the locations where the disinfectant residual is diminished and the sediment often accumulates and becomes the nutrient substrate for bacterial accumulation and colonization. Beyond the service meter, bacterial colonization of various water attachment devices found in homes, hospitals, and clinics are also sites for potential colonization.

Water supply systems in larger housing projects, high-rise office buildings, hotels, hospitals, and large public buildings exacerbate the problem of deteriorating water quality as a consequence of static water storage and of infrequent water demands created in variable occupancy rates and type of activity (3). Cold water storage in high-rise buildings must be covered to prevent introduction of contamination from nesting birds and atmospheric dust and to prevent the growth of airborne algae. Various heterotrophic bacteria also may enter via airborne contamination and eventually colonize the accumulating bottom sediments. Static water in building plumbing networks is often at warm water temperatures that stimulate bacterial growth year-round. Even new plumbing networks may present a problem of deteriorating water quality as a result of construction practices that introduce dust, dirt, and excessive solder flux in the lines during pipe assembly. Solder flux can be a nutrient source and an attachment site for numerous heterotrophic bacteria.

Building water supply attachment devices have a significant impact on the microbial quality of water. Long stagnation of public water supply in the warm environment of a building water system encourages various heterotrophic bacteria, including *Legionella*, to colonize pipe joint packing materials, valve stem seals, vacuum breakers (used in backflow prevention), and faucet aerators (33). Hot water tanks in homes and hospitals should not be overlooked as a cause of water-quality deterioration. If thermostats on hot water tanks are set below 55 °C as an energy-conservation measure or to prevent scalding of patients, growth of *Legionella* may occur in the hot water tank. In such situations, legionellae densities may reach infective dose concentrations either in the hot water tank or in an attached showerhead (34,35). Most residential hot water tanks are heated from the bottom, near the cold water–entrance pipe so that the water supply can be quickly heated to above 55 °C; however, after years of use, sediments may accumulate at the bottom of the tank and provide a heat-buffered environment for Legionella colonization. Water in large institutions is often heated by internal steam coils located at middepth in the tank; thus the cooler water in the bottom may not be heated sufficiently to kill *Legionella* (See *LEGIONELLAE*).

HETEROTROPHIC BACTERIA: AN OPERATIONAL INDICATOR OF MICROBIAL QUALITY

There are a variety of bacteria to be found in all natural aquatic environments and their kind and magnitude have often been used as a criterion of water quality (Table 1). Such arbitrary classifications based on consensus opinion are only a gross indication of water quality and have little scientific correlation to health risk, appearance, taste, odor, and intended use of the water. The problem with such a broad characterization is that the composition of the heterotrophic bacterial profile is so variable and sensitive to the ecology of the water as to make any interpretation of sanitary significance very difficult. Nevertheless, the use of a heterotrophic bacterial measurement continues to be applied as a broad indication of treated water supply quality. More definitive information can be found through periodic monitoring for shifts in the profile of dominant species in the population or by daily monitoring to detect a magnitude of changes in population densities. Perhaps the development of a rapid test (<4 h) for this general group of bacteria might prove useful as an alert (early warning system) to water-quality changes (waste spills and sewage bypasses) over the water shed, the buildup of assimilable organics in water treatment process basins, or the sudden emergence of cross-connections at sensitive locations on the pipe network.

PUBLIC WATER SUPPLIES: TREATMENT, DISTRIBUTION, AND STORAGE

Heterotrophic bacterial densities are not used as an enforceable criterion and standard in public water supplies; however, their occurrence is used often to evaluate treatment performance, as an alert to clean pipe networks before restoration of a protective disinfectant residual, and as an integral part of the condition-acceptance agreement with contractors before acceptance of new pipe lines (36). These applications should be dependent on use of a dilute media such as R-2A medium and incubation at 28 °C for five days or more to achieve optimum recovery data.

Water plant operators need to be alert to the possible colonization and amplification of heterotrophic bacteria

in process basins. An example of the magnitude of heterotrophic bacteria to be found in various water treatment processes and the effectiveness of treatment barriers may be seen in Table 4. Because of the fractured nature of GAC and the organic characteristics of the process water, these particles can be a more attractive substrate than sand for bacterial colonization. Colonization of sand and mixed media filtration beds is often accelerated when the plant must recycle wash water from the backwashing of these process basins. Without adequate treatment of backwash water, more heterotrophic bacteria and biodegradable nutrients will be inserted into the process water (Table 5). The concern is that spiraling elevation in densities and selective dominance of some undesirable opportunistic pathogens may break through the treatment train and enter the finished water supply.

Using aerobic spore-forming heterotrophic bacteria as a measure of disinfectant effectiveness is a more critical indication of inactivation for some pathogens in process water than the conventional practice of using chlorine-sensitive coliforms. These spore-forming organisms are more resistant to conventional disinfection practices and their physical removal appears to parallel that

Table 4. Heterotrophic Bacterial Densities in Water Treatment Processes

Treatment Process	HPC[a]/ml	Total Coliform/100 ml	Turbidity NTU	Free Cl_2 (mg/l)	Total Cl_2 (mg/l)
Cincinnati WTP (before Modification)					
Source: Ohio River	—	9,600	32	—	—
Stored source (48 h)	—	200	1.0	—	—
Coagulation and settled	500	<1	1.2	1.8	2.0
Filtered	<1	<1	0.1	1.6	1.8
Finish	5	<1	0.1	1.5	1.6
Cincinnati WTP (after Modification)					
Source: Ohio River	—	84,000	14	—	—
Stored source (48 h)	—	2,400	0.80	—	—
Coagulation and settled	5,500	1,400	1.1	0	0
Filtered	15	<1	0.07	1.8	2.0
Finish	<1	<1	0.06	1.4	1.5
Western Penn. WTP (before Modification)					
Source: Monongahela River	—	21,000	51	—	—
Plant intake	490	4	38	0.4	0.8
Coagulation	200	1	5.7	<0.1	0.4
Settling	50	1	8.5	<0.1	0.3
GAC filtered	150	8	0.6	<0.2	0.2
Finished	3	<1	0.2	<0.6	0.8
Western Penn. WTP (after Modification)					
Source: Monongahela River	—	14,000	6.8	—	—
Plant intake	29,000	4,200	5.2	<0.1	<0.1
Coagulation	4,790	100	6.3	<0.1	<0.1
Settling	7,100	43	2.3	<0.1	<0.1
GAC filtered	850	44	0.3	0.1	<0.1
Finished	1	<1	0.2	<0.4	<0.1

Source: Data from E. E. Geldreich, in J. M. Symons et al., eds., *Treatment Techniques for Controlling Trihalomethanes in Drinking Water*, Report E.P.A. 600/2-81 156, U.S. Environmental Protection Agency, Cincannati, Ohio, 1981 (37).
[a]HPC = heterotrophic plate count (R-2A medium, 28 °C., five days incubation).

Table 5. Impact of Recycled Backwash Supernatant to Treatment Train

Characteristic	Raw Source Water	Backwash Supernatant (Waste Clarifier) to Rapid Mix	Plant Effluent (Chloramination) 3.0 mg/L
AOC	117 μg/L	217 μg/L	542 μg/L
TOC	4.6 mg/L	8.1 mg/L	4.0 mg/L
HPC	20,000 per mL	2,100,000 per mL	300 per mL

Data from E. E. Geldreich, in R. A. Denninger, P. Literathy, and J. Rartam, eds., *Security of Public Water Supplies*, Kluwer, Academic Publishers, Dordrecht, Netherlands, 2000 (38).
AOC = Assimilable organic carbon
TOC = Total organic carbon
HPC = Heterotrophic plate count; R2A agar (25 °C for 5 days).

of physical entrapment and removal of *Giardia* cysts and *Cryptosporidium* oocysts through different treatment configurations (39).

The United States Environmental Protection Agency does not have a specified standard for heterotrophic bacterial densities in finished water entering the distribution system. Most water treatment operations using disinfection have a finished water quality ranging from <25 to 500 heterotrophic bacteria per one milliliter. This range is subject to some variation depending on the medium and procedure used in the heterotrophic bacterial determination.

Heterotrophic bacteria are being used in a variety of ways to characterize changes in the microbial quality of water supply in distribution systems (3,36). The most common use of heterotrophic bacterial data from the distribution system is to determine when lines need to be flushed to remove sediments and restore disinfectant residual. Although the heterotrophic bacterial count of water entering the distribution system generally will be below 100 organisms per milliliter, this density will often rise to several thousand organisms per milliliter at the ends of the lines and in areas of slow flow. Flushing the pipe network not only removes much of the sediment accumulation but also the organisms colonizing these sites. The net result is cleaner water and restored disinfectant residual at the ends of the distribution system. Water authorities using chloramination carefully monitor the heterotrophic bacterial density for significant increases as an indication to flush lines and possibly return to free chlorine residuals for a few weeks. The purpose is to disrupt the buildup of organisms that cause taste and odors in their utilization of free ammonia available from the conversion of free chlorine to chloramines.

For many years, many state and local water authorities have required contractors of new water lines to demonstrate that before acceptance, pipe sections not only were coliform-free but also had less than 500 heterotrophic bacteria per milliliter. The intent is to demonstrate that the line is clear of soil contamination that might have been introduced during construction. When the test is done on successive days, the data may provide some evidence of regrowth potential from deposited sediments. Another approach is to hold a sample from a new line at room temperature for several days, then examine it for elevated densities of the heterotrophic bacterial population. If the heterotrophic bacterial density increases to 1,000 or more organisms per milliliter, some protective sediments still are present in the pipe and the new pipe work needs another line-flushing and a test for bacterial regrowth. This repeat protocol is important because the practice of line-flushing followed by heavy doses of free chlorine for 24 hours often result in a negative coliform result but not necessarily an indication that the lines are free of soil and other debris.

Long-term storage of water supply (4 weeks or more) in enclosed reservoirs is not desirable. During these quiescent water periods, persisting heterotrophic bacteria begin to grow in the sediments, biofilm expands over the interior wall surfaces, and the water stratifies. These actions cause a further degradation in the microbial quality of the drinking water supply. For these reasons, the utility often selects a site downstream of the storage tank to monitor general bacterial quality of the static stored water supply. The evaluation may demonstrate that stored water has deteriorated when heterotrophic bacterial counts rise from below 100 organisms per milliliter to several thousand organisms per milliliter. Any evidence of coliform occurrences or substantial growth of the heterotrophic bacterial population should signal the need to drain the tank, to inspect tank interior and air vents for corrosion, to remove sediments, and to refill with fresh chlorinated water supply. The net result is cleaner water and restored disinfectant residuals.

RURAL WATER SUPPLIES

Rural water supplies may originate from surface water (farm ponds and creeks), naturally occurring outcroppings of the water table (springs), cisterns to store water from roof or hillside catchments, dug wells of less than 75 meters depth to catch surface water runoff, and drilled wells with casing installed that may reach more than 1,000 meters into an aquifer. The heterotrophic bacterial flora of these supplies reflect the status of barrier protection from surface drainage, septic system leaching fields and wildlife activity over the catchment area. Many of these bacteria have a strong aggressiveness that can initiate vigorous biofilm growth in the static water situations such as in well casing, water pipe, water-storage tanks or cisterns, and attachment devices. Mixed populations of indigenous organisms and transient contaminants provide opportunities for cooperative adjustments within the microbial community for survival and possible growth in an otherwise austere water environment. Some of the aquatic bacterial genera (e.g., *Pseudomonas, Aeromonas, Acinetobacter*, and *Alcaligenes*) become so adjusted to their ecological environments that soon they dominate the heterotrophic bacterial flora in specific situations. Selective dominance of iron and sulfur bacterial biofilms may clog the well casing so tightly that extraction is impaired or taste and odors become so offensive that the well must be abandoned (19). In waters containing significant concentrations of nitrates, numerous nitrate or nitrite bacteria become dominant and create unpalatable tastes and odors. Many of these situations are acerbated by nitrate-nitrogen concentrations around barnyards and corrals (40).

The most important, unanswered question concerning heterotrophic microbial populations in rural drinking water supplies is their health effect. There is growing evidence that substantial densities of some heterotrophic bacteria in these supplies may provide a significant health risk to some people. Some of these organisms may be multiple antibiotic–resistant (41). Also of growing concern are the reports of large densities of some opportunistic pathogens (*Flavobacterium, Legionella, Mycobacterium* avium complex, *Pseudomonas, Serratia, Staphlycoccus*, and several fungi) that may be present in these untreated water supplies (9). Sensitive subpopulations at risk to many untreated rural water supplies are the elderly, infants, pregnant women, AIDS victims, and individuals with diabetes.

HETEROTROPHIC BACTERIA IN ALTERNATIVE WATER SUPPLIES

Further treatment of the public water supply by the consumer to remove taste of chlorine, enhance the chemical quality, or provide a barrier to protozoan pathogens are available to the public. These alternative drinking waters include bottled water, stored public water supplies, and point-of-use devices. Such choices place responsibility for acceptance of water quality on the consumer who foregoes the protection afforded by public water authority oversight except during emergency situations caused by disasters, outbreaks, and noncompliance with safe water regulations. In both alternatives there may be microbial degradation of the product in terms of heterotrophic bacterial buildup that goes unseen and not understood by the consumer.

- Bottled Water (see BOTTLED WATER, MICROBIOLOGY OF)

  Storage of a safe drinking water supply in containers does circumvent potential exposure to contamination problems associated with distribution systems; however, storage in a container in itself introduces a different environment that may lead to microbial deterioration. Water packaged in a plastic bottle or in larger containers for use as a portable or emergency supply of drinking water is in a static environment. The rate of change in bacterial density is related to a variety of factors including assimilable organic nutrients in the water, organisms competing for dominance in the microbial flora, water pH characteristics, and shelf life under ambient temperatures (42). High-quality bottled waters undergo slow rates of change because generation of the indigenous organisms may take hours, not minutes and the available nutrients are in trace amounts. Waters bottled from sources that have fluctuating water quality and perhaps an occasional coliform quickly deteriorate. Emergency bulk supplies of drinking water are generally derived from some municipal water source and over time in storage will follow similar patterns of microbial quality change.

  The source of bottled water is often groundwater or protected springs but may be reprocessed public water supply. It can be argued that these special water sources will contain few organisms and those present are indigenous to the source and not of any public health concern. Yet, there have been several waterborne outbreaks that have been caused by the use of bottled water. Between April and November 1994, Portugal had a cholera epidemic that caused 2,467 confirmed cases and 48 deaths (43). Most of the country was affected and the source of the outbreak was found to be in the consumption of a particular brand of commercially bottled water and poorly cooked shellfish. The source water may have been contaminated from cholera-infected individuals living on the water shed or involved in the bottling operation. This case clearly illustrates the importance of water shed protection for groundwaters and springs that may have a poor soil barrier to safeguard quality from surface-runoff contamination.

  Source protection remains of highest priority as do sanitation practices in the bottling plant to avoid fecal contamination. What remains as a concern is the storage history of the product before and after the consumer purchases the product. During this time interval, the heterotrophic bacterial population will undergo a significance increase in density and a possible shift in dominant species. Bottled-water refrigeration in the supermarket will slow the progressive deterioration in microbial quality. As might be anticipated, the microbial quality of freshly bottled water (within 48 h of bottling) is excellent in most cases. In a study of 129 freshly bottled water collected directly from 25 different bottlers, only 10% had an initial heterotrophic plate-count density greater than 500 bacteria per milliliter (11). Total coliforms were detected in six samples; one sample reported also to contain fecal coliform bacteria. Another coliform-positive sample also contained 42 *Pseudomonas aeruginosa* per 100 milliliters.

  Bottled water with high densities of heterotrophic bacteria always are of concern because these increased densities may make it more difficult for the laboratory to detect fecal pollution and pathogens that also might be present. Excessive densities of heterotrophic organisms (more than 1,000 organisms per milliliter) in a water sample can desensitize both the multiple-tube test (including the P-A test) and the membrane filter total coliform procedure. Organisms such as *Pseudomonas* species and *Flavobacterium* strains can suppress the growth of total coliforms, preventing detection of gas production in lactose fermentation broth and spreading growth over the surface of the membrane filter that interferes with coliform sheen development on M-Endo media. These interference problems may be avoided with the use of chemically defined coliform media although high densities of *Aeromonas* and *Flavobacterium* may give occasional false readings in the total coliform and E. coli tests, respectively.

  The other concern with high-density populations in bottled water is the possible emergence of significant densities of some opportunistic pathogen that may be of health risk to some consumer with health problems. How much a concern this really is continues to be debated because the research evidence still is inconclusive for a variety of situations. There is no doubt that for individuals debilitated by some illness or treatment that undermine the natural body–immune system exposure to bottled water with *Pseudomonas*, *Flavobacterium*, or *Acinetobacter* may acquire a secondary infection from such opportunistic pathogens (42).

  Studies on the various genera and species of heterotrophic bacteria in bottled water have revealed that *Pseudomonas* species (many being multiresistant to antibiotics) often are a dominant part of this bacterial population as storage time increases. *P. stutzeri, P. diminuta, P. maltophilia, P. putida,*

P. fluorescens, and *P. acidovorus* have been reported in densities of 10^3 to 10^5 organisms per milliliter (44), largely because these organisms can slow down their metabolic activity to survive on minimal nutrient concentrations available in protected aquifers. *P. aeruginosa*, most prevalent *Pseudomonas* in human disease, is rarely found in high-quality bottled waters. When introduced through contamination of the source or in bottling operations, this organism will maintain a stable density and then decline to nondetectable densities after six to seven months (42,45).

- Stored Emergency Water Supplies

  The requirements for stored emergency water supplies set aside for use in cases of shipwreck, at times of natural disasters, or during wartime devastations are unusual. These stored supplies may be used for drinking water among the stressed population that also could include those injured in the disaster (1,9). Examination of the bacterial populations in emergency supplies of water stored after approximately five years revealed that 22.8% of 167 samples contained species of *Flavobacterium* and 16.2% contained species of *Pseudomonas*. Flavobacteria were frequently dominant and the counts ranged from 10 to 26,000 per milliliter. This information suggests that after years of long-term storage, nutrients are reduced and organism subsistence is in balance until the containers are contaminated during opening for inspection and sampling.

  For this reason, stored water must be of good bacteriological quality that goes beyond monitoring for fecal contamination and recognizes the concerns with opportunistic pathogens. Ensuring the continued availability of a high-quality emergency water supply over extended storage periods is difficult. Initially a high-quality source water must be selected and care taken when filling and sealing storage containers so as to prevent contamination with bacteria and dust particulates. Because the quality must be maintained indefinitely, all emergency water supplies should be given supplemental disinfection during annual inspections and before use.

- Point-of-Use Treatment Devices (see Home Treatment Devices — Microbiology of Point of Use and Point of Entry Devices)

  Home water supply treatment devices have a significant impact on the microbial quality of public water supplies. Although treatment devices may be very effective initially in providing microbial or chemical improvement in the public water supply, their effectiveness may diminish because of unpredictable service life of the filter, ambient water temperatures, frequency of static water conditions, poor maintenance, and unit design that create sumps where bacteria can grow in the moist ambient environment. The inclusion of a carbon filter in some of these devices to remove organics, taste, and odor will also provide an ideal habitat for microbial colonizations with subsequent releases of additional heterotrophic bacteria into product water.

Adventitious organisms passing through the distribution system may become transient colonizers in a carbon filter for varying periods of time (46). A study on occurrence and persistence of various heterotrophic bacteria passing through several different point-of-use devices from a dechlorinated tap water revealed that coliform bacteria were released from one or more carbon filters during periods ranging from 1 to 10 months, although the treatment devices being evaluated in a pilot study were not challenged with any coliforms during a 12-month test run. Three coliforms species were detected in single samples at various times rather than for prolonged periods: *Enterobacter aerogenes* (4 times), *E. cloacae* (4 times), and *Klebsiella pneumoniae* (once). These observations suggest that occasional coliform occurrences in public water supply may be more readily detected by monitoring the passage of tap water over time through a carbon filter device than from scheduled grab samples taken periodically from distribution-sampling sites. Because the point-of-use devices had been disinfected before the beginning of this study period, the organisms must have been present in the dechlorinated distribution water passing through the test system and were able to survive and multiply to some extent in the individual treatment device filter cartridge. Other organisms detected in the dechlorinated tap water and the number of times isolated during the 12-month study were *Alcaligenes* spp. (twice), *Serratia marcescens* (4 times), S. *rubidaea* (twice), and on different one-day occasions, *Pseudomonas cepacia*, *P. fluorescens*, and *P. maltophila*. Although the densities initially increased 10–100 times more than those of dechlorinated tap water, these densities tended to stabilize after several months.

In a pilot study to challenge carbon filter treatment devices with heterotrophic bacteria that would be anticipated in cross-connections, line breaks, or back siphonage, it was revealed that carbon filter units provide an ideal environment for colonization and opportunities for bacterial release into the product water (47). This could be of particular concern if there is infrequent filter replacement by the user. Among the organisms released in product water were *Klebsiella pneumoniae*, *Aeromonas hydrophila*, and *Legionella pneumophila*. Other heterotrophic bacteria (*E. coli and Salmonella*) did not pass through the carbon filters under test. This was thought to be due to the aggressive competition of other organisms in the high-density heterotrophic population colonizing the water treatment filters.

Static water conditions overnight or for longer intervals provide opportunity for continued growth of organisms colonizing carbon filters (13). In fact the first draw of water in morning samples had higher bacterial densities than at any other time during the day or evening (Table 6). In winter the carbon filter on the nonchlorinated source water produced water with lowest bacterial densities. These results suggest that warm water and ambient air temperatures are important factors that stimulate the higher densities on all filters during summer months. This phenomenon was even more pronounced with third faucet units that had a 1 log higher bacterial density

Table 6. Microbial Quality Deterioration Related to Static Periods Without Use

Average HPC per ml	Nonchlorinated Source				Chlorinated Tap Water Carbon # 3 (Control)
	Acrylic Fiber Wound Carbon	Carbon-Impregnated Paper	Carbon # 1	Carbon # 2	
			Winter		
A.M.	280	4,700	1,300	13,000	380
P.M.	160	5,800	770	12,000	100
			Summer		
A.M.	8,200	200,000	7,300	7,500	8,500
P.M.	6,200	340,000	3,800	11,000	5,000
Average HPC count per ml	29	25,000	260	2,600	11
6-week static test	693,000	1,100,000	103,000	30,000	29,000
Cyclic flushing resumed	130	11,000	500	5,100	190

Data revised from E. E. Geldreich and D. J. Reasoner, in G. A. McFeters, ed., Drinking Water Microbiology: Progress and Recent Developments, Springer-Verlag, New York, 1990 (13).

in the morning than from a full service unit located under the sink, probably because water demand was limited to drinking use only, thereby providing more static water contact time in the carbon bed. Whether individuals who consume water from a biologically active filter suffer adverse health effects is dependent on the bacteria involved, the number of organisms ingested, and the individual's general health and resistance to the particular organism(s).

Although the heterotrophic bacterial flora that colonize carbon filters often may reach 10^2 to 10^5 organisms per milliliter, there appears to be no apparent health risk to most people based on conclusions from recent epidemiological studies of carbon filters in home use. Perhaps these established microbial populations are a significant factor in preventing many overt pathogenic strains of bacteria from successfully colonizing and persisting in carbon filter treatment devices. The trade-off may be the chance colonization and release of some opportunistic pathogen that successfully competes within the bacterial flora and becomes predominant, thereby representing a health risk to some susceptible individuals using these devices.

CONCLUSION

Heterotrophic bacteria comprise a diverse population of bacteria in the aquatic environment. Many of these bacteria are ubiquitous and of no sanitary significance. Their origins are a reflection of water contact with soil, vegetation, airborne dust particles, and the fecal wastes of all animals. Their survival is a reflection of the status of natural self-purification forces including water chemistry, water pH, aeration of receiving waters, sunlight exposure, microbial antagonisms, and predation by bacterial predators. Counteracting forces that depress the natural die-off of heterotrophic bacterial populations in water include discharges of poorly treated waste and stormwater runoff over the water shed.

Among the heterotrophic bacteria of concern are those that are pathogenic, opportunistic, or carriers of R-factor transfers. Contact with these bacteria may come from exposure to water supply, recreational waters, whirlpools, and aerosols in climate-cooling devices. Unfortunately, differentiation of these organisms of concern is not practical in routine monitoring, so the approach has been to limit the "total" density of all heterotrophic bacteria in a water contact situation, thereby limiting the number of such organisms of concern to below a risk level.

No single examination of a water sample will detect all of the heterotrophic bacteria to be found in a water sample because of varying nutrient requirements, unique incubation temperatures, and extended incubation-time requirements. Regardless of these limitations to their complete detection in a single test, the concept of relating density of heterotrophic bacterial populations to water quality continues to be used for a measure of water treatment effectiveness and indication of microbial colonization in water supply distribution pipe networks.

Areas to be explored are the use of a rapid test (<4 h) for detecting heterotrophic bacteria in an early warning monitoring system that might be used to detect a change in water quality over a water shed designated for drinking water source use and the development of a heterotrophic bacterial profile technique (fingerprinting) in water samples to identify origin of pollution.

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HIGH HYDROSTATIC PRESSURE: MICROBIAL INACTIVATION AND FOOD PRESERVATION

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The metabolic and physiological activities associated with the growth and multiplication of most microbial cells are well regulated and occur within a narrow range of hydrostatic pressure (as well as with other physical

parameters). There are some barophilic microorganisms, which are grouped together with the extremophiles, that need higher pressure for growth and survival. Generally, the barophiles need a hydrostatic pressure for these functions well below 100 MPa (1). As the hydrostatic pressure is increased to 100 MPa and above, microbial cells start to lose viability, which under a given set of conditions is directly proportional to the pressure level. Destruction of bacterial spores requires pressure of 700 MPa or more, although they can be induced to germinate and outgrow at a pressure of about 300 MPa (2).

Because high hydrostatic pressure (HHP) in the range of 100 to 800 MPa can destroy microbial cells and inactivate some bacterial spores, there is an interest in using this as a potential nonthermal technique to destroy food-borne spoilage and pathogenic microorganisms to enhance the shelf-life and safety of food. The concept of preserving food by destroying microorganisms by HHP was originally applied by Hite in 1899 (3). He showed that the shelf-life of raw milk, following pressurization at 600 MPa for 1 hour at 25 °C, could be extended for 4 days. He and his coworkers also pressurized several fruits at about 800 MPa to achieve commercial sterility, and the products remained unspoiled even after five years (4). For the next 70 years there was no report available on the application of HHP in food, although sporadic reports were published on the viability loss of microbial cells, bacterial spores, and viruses in broth or buffer suspensions by HHP as well as by the combination of pressure, pressurization time, and pressurization temperature. Since the 1980s and more particularly in the 1990s, a large number of studies have been conducted in many countries to determine the viability loss of food-borne microbial cells and spores at different pressures in combination with different pressurization times and temperatures and in the presence of several antimicrobial compounds during pressurization (2,5,6). The success of these studies is evidenced by the commercial marketing of several pressure processed foods in Japan by 1991 and afterward in a few European countries and the United States (2,5–9). This is probably the shortest time in the history of commercial food processing that it has taken between studying a novel food processing technique and successfully marketing of a food that has a high rate of consumer acceptance. Initial success was with low pH foods pressurized to kill aciduric bacterial cells, molds and yeasts, but not bacterial spores (since low pH prevents spore germination). At present, high pH foods are also being studied and these will require destruction of the bacterial spores to reach commercial sterility (as in canning).

The HHP (also called high pressure processing, HPP and ultra high pressure processing, UHP) technique, as opposed to most prevalent thermal treatments, is a uniquely three-dimensional process comprising pressure, pressurization time, and pressurization temperature. If one or more effective antimicrobial compounds is incorporated during pressurization, the process can become four-dimensional with a very high microbial destruction capability. The process involves placing a packaged-food inside a closed steel chamber containing water (or a mixture of water and a little oil to prevent water from freezing at a high pressure) followed by closing the chamber hermetically and pumping more liquid inside the chamber (Fig. 1). The pressure increase is instantaneous and transmitted uniformly throughout the liquid as well as in the food mass, relatively independent of shape, size, viscosity, and composition of the food and packaging material. Once the desired pressure is reached, pumping of more liquid is stopped and the valve in the opening is closed holding the pressure in the liquid constant. At the end of the pressurization period, the valve opens automatically and the excess liquid flows out of the chamber by gravity during decompression. Depending on the pressure unit, the come-up time could be one to two minutes and the come-down time could be less than 30 seconds. During pressurization, the temperature of the food rises about 3 °C per 100 MPa due to adiabatic heating, but then reverts to the original food temperature following decompression. Also, during pressurization, the volume of a food mass is reduced by about 10 to 15% between the range of 300 to 700 MPa, but then reverts to original volume following decompression. The liquid in the chamber, if necessary, can be heated to a desired temperature by installing a heating coil around the wall of the unit. For a food to reach a temperature of the liquid during pressurization, it is necessary to heat it prior to

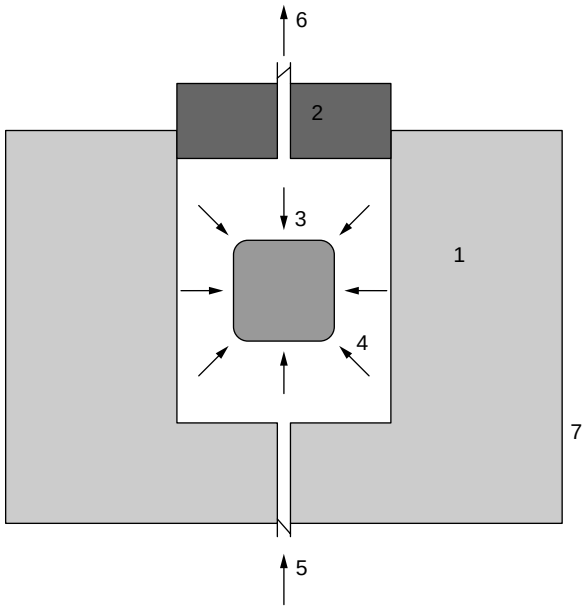

Figure 1. Schematic diagram of a high hydrostatic pressure vessel (7). The food package (4) is put in liquid (3) inside the chamber (1), which is closed at the top with a movable cylinder (2) with an opening and a valve (6) to remove air and excess liquid prior to pressurization. Once the chamber is closed, excess liquid is pumped through the inlet tube (5) that has a valve which closes when the desired pressure level is reached. The pressure is maintained constant during pressurization time, at the end of which the inlet valve (5) opens and the excess liquid is removed from the chamber by gravity. The liquid in the chamber can be heated by installing a heating coil around the vessel (7). The process is clean, energy efficient, and requires relatively less space compared with other food-processing methods. See color insert.

putting the food inside the chamber. Pressurization does not have any detrimental effect on the covalent bonds of food components. Thus there is no loss of vitamins and minerals as well as no change in color and flavor. However, pressurization breaks noncovalent bonds such as ionic, hydrophobic interaction, and H-bonds. As a result, macromolecules (of food and microorganisms) undergo unfolding during pressurization and may not correctly refold during decompression, leading to an alteration in the original structure. This, in addition to killing microorganisms, has opened the possibility of developing novel products especially when the food has a high protein content (2,5,6,10).

In addition to preserving foods by destroying microorganisms, HPP has been found to have many other novel applications in food processing. Some of these are listed in Table 1. Because of its versatile applications in food, HPP has currently generated worldwide interest and is being considered to be a major technology to be used to produce food in the twenty-first century. Among the different applications, this article covers only the destruction of microorganisms by HHP (6,10).

INACTIVATION OF BACTERIAL CELLS

Sublethal Injury and Viability Loss

Studies with pure cultures of several food-borne gram-positive and gram-negative bacterial suspensions, either in buffers or in bacteriological media, have revealed that following pressurization, a population generally has three distinct groups of cells, namely, the uninjured or normal, the sublethally injured, and the dead (11). The three groups are differentiated arbitrarily on the basis of physiological and metabolic characteristics (12–14). While the normal subpopulation of cells retains the characteristics of the original strain and the dead cells permanently lose the ability to multiply, the sublethally injured cells develop several distinct but transient characteristics. These cells become sensitive to many chemical compounds to which the normal cells are resistant, and temporarily lose the ability to multiply and some metabolic activities. These altered characteristics are associated with the structural and functional changes or "injury" to the cell wall, cell membrane, some enzymes, ribosomes and DNA. In this subpopulation, the injured cells differ greatly in the degree or extent of injury; some being highly injured while some others have very little injury as observed from their rate of repair (Fig. 2). Once they are exposed to a favorable environment, these injuries are repaired by the cells themselves through metabolic activities. Following repair, the cells regain the normal characteristics of the strain including the ability to multiply. Before repair, if they are exposed to some adverse chemical or physical environment to which the normal cells are resistant, the injured cells lose viability. This fact has been exploited to increase the viability loss of a bacterial strain by pressurizing it in the presence of some other physical or chemical parameter(s) and is discussed later.

The usual procedure to differentiate the injured population from the normal population, among the survivors, following exposure to hydrostatic pressure is to enumerate the colony forming units. The cell suspensions before and after pressurization are enumerated simultaneously in two agar media, one of which is selective and the other nonselective (Fig. 2). The two media are formulated in such a way that the bacterial strain forms equal numbers

Table 1. Some Potential Applications of HHP in Food

Antimicrobial applications
To destroy microbial cells at lower pressure range
To destroy bacterial spores at higher pressure range
To germinate bacterial spores at lower pressure range
To prevent microbial growth in unfrozen food
Novel product development
To produce fruit and vegetable products with natural taste
To improve color of fruit products and egg yolk
To facilitate gelation of proteins without heat
Improved food processing
To tenderize meat
To enhance cheese ripening
To open valves of shellfish
To inactivate undesirable food enzymes
To thaw frozen food rapidly
To store food unfrozen at −20 °C
To inactivate allergens and toxins

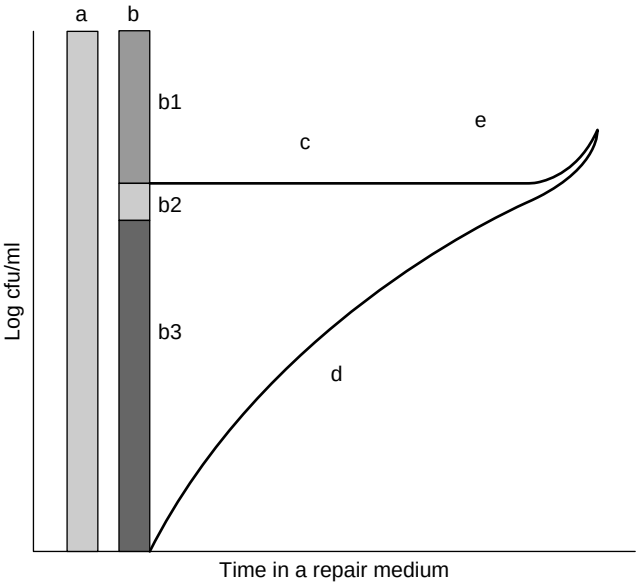

Figure 2. Schematic presentation of the effect of pressurization of a normal bacterial cell suspension (**a**) leading to production of three subpopulations (**b**), namely, dead cells (**b1**), normal cells (**b2**) and sublethally injured cells (**b3**). During subsequent incubation of the cells in a favorable environment for a period, the total survivor counts (b1+ = b2 = c) in a nonselective agar medium remain unchanged, suggesting no cell multiplication. However, the counts on selective agar medium increases rapidly (**d**) due to repair of injury and regain in resistance to selective environment; the rate of repair differs due to differences in the extent of injury among different cells. Cell multiplication starts following repair of injury as indicated by simultaneous increase in counts in both media (**e**). See color insert.

of colony forming units (cfu)/ml in both media before pressurization. Generally, one or more antimicrobial agents (for e.g., bile salts, NaCl, etc.) are included in the nonselective medium to convert it to a selective medium. The compound is used at a concentration that will inhibit growth of the injured cells, but not of the normal cells. Following pressurization and plating, only the normal survivors will form cfu on the selective medium, whereas all the survivors will form colonies in the nonselective medium (since the injured cells can repair and multiply in it). The fraction of injured cells is then calculated from the differences in cfu in the two media (11–13).

In general, at a lower pressure range (about 100 to 200 MPa) the fractions of both dead and sublethally injured cells in a bacterial suspension is low. As the pressure increased to between 300 and 400 MPa proportionately, there will be more cells with sublethal injury and also more dead cells. Above 500 MPa, the number of dead cells becomes much higher than the number of sublethally injured or normal cells. Other characteristic effects of pressure treatment on bacterial cells include a higher sensitivity of gram-negative than gram-positive bacteria, and a greater sensitivity of exponential-phase cells than stationary phase cells to a particular pressure; the latter difference is more pronounced between 300 and 600 MPa. In addition, bacterial species and strains within a species differ greatly with respect to the damaging effects of hydrostatic pressure, which again is more evidenced at a low pressure than at a high pressure range (2,5,6).

Sublethal Injury. Metrick and coworkers (15) reported that following pressurization of two serovars of *Salmonella* at 230 MPa for 30 minutes at 25 °C, there was a 3-log difference in cfu between the nonselective tryptic soy agar (TSA) medium and selective eosin methylene blue agar medium supplemented with 2% NaCl (EMBSA medium). The injured cells could not form colonies due to their developed sensitivity to 2% NaCl, to which normal cells were not sensitive. They also reported that a subsequent incubation of the pressurized cells for 2.5 hours at 37 °C in a chicken broth, but not in a phosphate buffer, enabled the injured cells to repair their transient sensitivity to NaCl and regain the ability to multiply and form colonies in EMBSA. The sensitivity of the injured cells to NaCl suggests that pressure most likely produced reversible injury to the inner membrane of the cells. Kalchayanand and coworkers (12,16–18; unpublished data) observed sublethal injury among the survivors of pressure-treated *Listeria monocytogenes, Escherichia coli* O157:H7, *Salmonella* Typhimurium, *Staphylococcus aureus, Leuconostoc mesenteroides, Lactobacillus sake, Pseudomonas fluorescens*, and *Serratia liquefaciens* strains. Depending on the species, the cells showed sensitivity to bile salts [suggesting cell wall or outer membrane (OM) injury; (12,14)], and NaCl (suggesting cytoplasmic membrane or inner membrane injury) both of which could be repaired in appropriate environments. The number of cells in the injured population greatly varied with bacterial species and strains and pressurization conditions (Table 2). Similar observations were also made by Alps and coworkers (18). Ludwig and coworkers (19) found that pressure-injured cells of *E. coli* have reduced the ability to form colonies on violet red bile agar medium due to their sensitivity to bile salts and crystal violet. They also became susceptible to the lethal effects of lysozyme, nisin, and EDTA at concentrations that were ineffective against normal cells. Susceptibility of cells to these three compounds was observed only during pressurization but not after pressurization. Since the lipopolysaccharide (LPS) molecules in the OM of *E. coli* provide protection against the antimicrobial effects of the above three compounds, it could be that during pressurization, the physical state and permeability barrier imposed by LPS molecules changes but after decompression is restored to the original state. A similar situation has been reported in freeze-injured *E. coli* cells (14).

Viability Loss. The inability to multiply permanently and form cfu by the bacterial cells in a nonselective agar medium, following a stress such as pressurization, has usually been used to determine viability loss or death. Major studies have been conducted with several food-borne pathogens to determine the rate and level of viability loss, that is, the D-values (time in minutes to reduce viability by 1-log cycle of a bacterial strain under a given condition) as well as pressurization parameters that could reduce viability of a strain by 6-log cycles to ensure safety to the consumer. Many of these studies have been discussed in several recent reports (2,5,6,19,20). The predominant pathogens studied include strains of *Salmonella* serovars (16–18,21), *Listeria monocytogenes* (16–18,20–28), *Escherichia coli* O157:H7 (16–21,29,30) *Vibrio parahaemolyticus* (22,24), *Yersinia enterocolitica* (21,31), *Staphylococcus aureus* (10–16,21,29), and *Campylobacter jejuni* (32). Some of the indicator, spoilage, and lactic acid bacteria studied were *Escherichia coli* (14,32), *Serratia liquefaciens* (16),

Table 2. Pressure-Induced Sublethal Injury Among Foodborne Pathogens

Pathogens	Unpressurized (log_{10} cfu/ml)	Pressurized (log_{10} cfu/ml)		
		Dead	Injured	Normal
Listeria monocytogenes Scott A	11.8	6.8	2.1	2.9
Staphylococcus aureus 581	9.8	5.7	4.1	<0.1
Salmonella typhimurium M1	10.2	5.0	5.2	<0.1
Escherichia coli O157:H7 #932	10.1	8.7	0.9	0.5

The cell suspensions in 0.1% peptone solution were pressurized at 345 MPa for 5 minutes at 25 °C. The nonselective medium was tryptic soy agar supplemented with 0.6% yeast extract (TSY-agar). The selective media used were: TSY-agar with 7.5% NaCl for *Staphylococcus*, modified Oxford (MOX) agar for *Listeria*, xylose lysine deoxycholate (XLD) agar for *Salmonella* and violet red bile (VRB) agar for *Escherichia* strains.

Pseudomonas species (16), *Citrobacter* species (33), *Streptococcus* species (32), *Leuconostoc* species (16), and *Lactobacillus* species (16,34). Most studies were conducted in phosphate buffer, saline or peptone broth. However, some studies were conducted in food systems, namely, milk, sea food, chicken, pork, beef, liquid egg, cheese, salsa, and others. The results of these studies can be summarized as follows (2,6):

- Gram-negative cells are relatively more sensitive to HHP than gram-positive cells. A higher sensitivity is also observed among rods than cocci and among exponential growth phase cells than stationary phase cells.
- There is a considerable variation in pressure-resistance among different bacterial species and among strains of the same species.
- Bacterial cells are more resistant to pressure in food systems than in a bacteriological broth or in a buffer. Resistance increases with the increase in food density and lipid content.
- A low pH, high A_w or the presence of an antimicrobial compound during pressurization enhances viability loss.
- Viability loss also increases with the increase in pressure, pressurization time and pressurization temperature (especially above 35 °C; see next section).
- Pressure-induced sublethally injured cells, when exposed to low pH, mild heat, antimicrobial agents, or repressurization, lose their ability to repair and grow.

Interaction of Several Parameters

The bactericidal efficiency of hydrostatic pressure processing is dependent on three parameters, namely, the pressure level, pressurization temperature, and pressurization time. Other parameters can be adjusted, namely, the environment of the material (food) being pressurized, in the form of low pH (below 6.5) and/or antimicrobial compounds (such as preservatives), to enhance bactericidal efficiency. Depending on the food system, all four parameters can be combined to achieve a desired level of destruction of pathogenic and spoilage bacteria, even under a moderate level of pressurization.

In one study, cells of four pathogenic and four food spoilage bacterial strains from the early stationary growth phase, were suspended in 0.1% peptone solution and exposed to a combined effect of hydrostatic pressure between 107 MPa and 483 MPa, pressurization temperature between 25° and 60 °C, and pressurization time between 5 and 30 minutes (16,17). The viability losses of the strains were determined from the differences in cfu/ml before and after a given treatment. In general, the results showed that the viability losses of the eight strains ranged between <0.1 and 1.5 log cycles at 138 MPa in 5 minutes at 25 °C. By raising the pressure to 345 MPa under the same conditions of pressurization, the viability losses (or death) increased to between 0.9 and ≥8 log cycles. By raising the pressurization temperature to 50 °C even an exposure of 107 MPa for 5 minutes raised the viability losses of the strains from 0.2- and 2.9 log cycles at 25 °C to 1.8- and ≥7.8 log cycles at 50 °C. However, increasing the pressurization time from 5 minutes to 30 minutes did not increase the viability loss, which remained around 1 log cycle. These results indicated that pressure per se', even at mild temperatures above 35 °C, has a much more detrimental effect on bacterial cells than pressurization time above 5 minutes. Similarly, viability loss increased proportionately with the increase in pressure at any pressurization time and temperature combination. Some of these results are presented in Table 3 (16,17). In this study, cell suspensions (about 10^8 cfu/ml) in 2-ml cryovials in duplicate were subjected to combinations of hydrostatic pressure, temperature, and time, and the viability losses were determined from the differences in cfu/ml on tryptic soy agar plates before and after pressurization. These results also revealed that a much greater viability loss was achieved in all four strains both at a higher pressure and a higher pressurization temperature as compared with a longer pressurization time. Studies by Kalchayanand and coworkers (16,17) also have shown that an 8-log cycle viability loss of most food-borne bacteria could be achieved by a combination of pressurization at 345 MPa for 5 minutes at 50 °C, a combination that gave a D-value of ≤0.6 minutes. This combination of medium pressure and temperature could be of advantage over using a very high pressure (≥700 MPa) at 25 °C. Other studies have reported that a higher pressurization temperature is more detrimental to bacterial cells (18,22,29,35) than a longer pressurization time (18,22).

Several other parameters, incorporated either in a cell suspension or in a food product, during pressurization were found to enhance viability loss of bacterial cells. As mentioned earlier, pressurization not only kills bacterial cells but also causes sublethal injury to many survivors. The injury is manifested by an increase in permeability and an increase in sensitivity to many antimicrobial compounds, especially those that are cell wall and cell membrane active. In the presence of one or more of these compounds during pressurization, the injured cells also lose viability. Depending on the effectiveness of a compound, and the bacterial strain, the viability loss could be increased by an additional 2- to 3-log cycles. As the injury is reversible, the compound is most effective when it is present during pressurization.

Pressure Resistance

Bacterial strains from different genera and species differ considerably in sensitivity to high hydrostatic pressure, as they do toward many other physical and chemical parameters. As shown in Table 3 among the four species used in the study, *Leuconostoc mesenteroides* and *Listeria monocytogenes* were more resistant to pressurization at 345 MPa for 5 minutes at 25 °C than *Serratia liquefaciens* and *Salmonella* Typhimurium. Limited recent studies have shown that strains of species of food-borne bacteria also differ in sensitivity to hydrostatic pressure, especially when pressurization is done at an ambient temperature. This is of special importance in developing pressurization parameters for the destruction of food-borne pathogens. For this purpose, it is important that the strains of a

Table 3. Viability Loss of Food-Borne Bacteria by the Combined Effect of Hydrostatic Pressure and Pressurization Temperature and Time

	Log_{10} Viability Loss as a Function of					
	Pressure (MPa)[a]		Temperature (°C)[b]		Time (min)[c]	
Bacterial Strains	138	345	25	50	5	15
Listeria monocytogenes Scott A	0.1	3.0	0.8	6.9	0.8	0.9
Salmonella Typhimurium ATCC 14028	0.2	>8.0	0.7	>7.5	0.7	0.9
Leuconostoc mesenteroides Ly	0.6	2.7	0.9	5.8	0.9	2.2
Serratia liquefaciens FM1	0.1	>8.0	0.3	7.0	0.3	1.0

[a]Pressurized for 5 minutes at 25 °C
[b]Pressurized at 207 MPa for 5 minutes
[c]Pressurized at 207 MPa and 25 °C.

pathogen that are highly pressure resistant should be used in modeling studies.

Earlier studies reported that cells of two serovars of *Salmonella* and two strains of *L. monocytogenes*, when pressurized in phosphate buffer at 22 °C between 138 and 340 MPa differed greatly in viability loss (15,22). Variations in pressure sensitivity among three *L. monocytogenes* strains at 375 MPa and three *E. coli* O157:H7 strains at 600 MPa were also reported by Pattersen and coworkers (21). In this case, stationary phase cell suspensions in phosphate buffered saline (pH 7.0) and pressurization temperature of 20 °C for 5 to 30 minutes were studied. Benito and coworkers (35) observed that viability loss of stationary phase cells in phosphate buffer of six *E. coli* O157:H7 strains following pressurization at 500 MPa and 30 °C for 5 minutes ranged between 0.2- and 6.1-log cycles.

The pressure-resistant stationary phase cells were also relatively resistant to mild heat treatment (57 °C for 10 min), exposure to pH 2.5 (adjusted with either HCl or acetic acid) and high osmotic environment (20% NaCl in broth). However, the differences in pressure resistance disappeared when exponentially grown cells were pressurized even at 200 MPa. Exponentially grown cells, as compared to stationary phase cells, also became sensitive to acetic acid but not to HCl, possibly due to a bactericidal effect of undissociated acetic acid molecules. The researchers suggested that the difference in pressure sensitivity of cells in the two growth phases was due to a relatively greater degree of membrane damage of the pressure-sensitive strains as opposed to the pressure-resistant strains.

Alpas and coworkers (36) compared the pressure resistance of cells (suspended in 1% peptone) from late exponential growth phase of nine *L. monocytogenes* strains, seven *S. aureus* strains, six *E. coli* O157:H7 strains, and six *Salmonella* serovars following pressurization at 345 MPa for 5 minutes at 25 °C. Viability loss ranged between 0.9- and 3.5-log cycles among *L. monocytogenes* strains, 0.7- and 7.8-log cycles among *S. aureus* strains, 2.8- and 5.6-log cycles among *E. coli* O157:H7 strains, and 5.5- and 9.3-log cycles among *Salmonella* serovars. However, when one of the pressure-resistant and one of the pressure-sensitive strains of each species, taken from the late exponential growth phase were pressurized at 345 MPa for 5 minutes at 50 °C instead of 25 °C the viability losses were very high and the differences in pressure sensitivity between strains in a species disappeared (Table 4). D-values following pressurization at 50 °C were ≤0.6 in all strains except for the pressure-resistant *S. aureus* strain. The pressure-resistant strains were also relatively resistant to mild thermal treatment. In a recent study, Alpas and coworkers (18) observed that the differences in pressure resistance among these strains was most pronounced at 25 °C; as pressurization temperature was raised to 35 °C or above variation in resistance among strains started to diminish. It was suggested that the differences in pressure resistance at lower temperature could be dependent on the relative ability of the strains to resist phase transition of membrane lipids (see next section); the ability to resist a phase transition is gradually eliminated with the rise of pressurization temperature perhaps due to the increase in lipid fluidity. These results emphasize the advantage of using a moderately high temperature to eliminate the effects of pressure resistance among bacterial strains especially when pressurization is conducted ≤500 MPa.

Mechanisms of Cell Death

A bacterial cell exposed to high hydrostatic pressure (>100 MPa) is subjected instantaneously and uniformly to two major effects, the adiabatic heating (approximately 3 °C per 100 MPa) and a volume reduction (about 10 to 15% between 300 to 700 MPa at 22 °C). These effects are produced during pressurization, and on decompression, both the temperature and volume return to the original levels. Among these two parameters, the effect of volume reduction is more detrimental to cells as it can alter the structure of macromolecules and their associations through breaking of ionic bonds, H-bonds, and hydrophobic bonds. Following decompression, macromolecules may not go back to original conformations. Since many vital biochemical and physiological processes are dependent on the specific macromolecular structure, any alteration in conformation can adversely affect the ability of the cell to carry on normal metabolic activity and growth. In addition to changes in molecular configuration, a decrease in cell

Table 4. Viability Loss and D-Values After Five Minutes Pressurization at 345 MPa and Thermal Treatment at 50 °C up to Five Minutes for One Pressure-Resistant and One Pressure-Sensitive Strain from Four Species

	Viability Loss (log_{10} cfu/ml) After		Estimated D-value After	
Bacterial strains	Pressurization at 25 °C	Pressurization at 50 °C	Pressurization at 50 °C	Thermal treatment at 50 °C[b]
L. monocytogenes				
CA	0.9	>8.1[a]	≤0.6	11.7
Ohio 2	2.8	>8.0	≤40.6	11.2
S. aureus				
485	0.7	5.4	2.6	21.1
765	7.8	>8.1	<0.6	14.6
E. coli O157:H7				
933	2.8	>8.3	<0.6	9.9
931	3.3	>8.2	<0.6	8.8
Salmonella enteritidis				
FDA	5.5	>8.2	≤0.16	9.9
Salmonella typhimurium				
E21274	5.7	>8.4	≤0.6	7.9

[a]No cfu was detected following plating of 2-ml cell suspensions in TSY agar plates indicating all the cells have been killed, except for *S. aureus* strain 485.
[b]Viability loss due to thermal treatment for 5 minutes at 50 °C without pressurization was ≤1.0-log cycle.

volume by pressure can also cause phase transitions for some biomolecules such as lipids and enhance reactions among cellular enzymes and other chemicals. These also can adversely affect the normal functions of a cell following decompression (2,5,6).

Both the sublethal injury and lethal injury (or viability loss) caused by pressurization, as discussed before, are the manifestations of these changes; they are enhanced as pressurization temperature is increased above the ambient temperature. The conformational alteration of the lipopolysaccharide (LPS) molecules on the surface of the outer membrane has been implicated in developed sensitivity of pressure-induced sublethally injured gram-negative bacteria to surface active agents (like SDS), lysozyme, and bacteriocins (11–14). In the normal cells, these macromolecules are stabilized by divalent cations through ionic bonds, and act as a barrier to many antimicrobial compounds and provide resistance against them. Pressurization breaks the ionic bonds and impairs the barrier function of LPS molecules, making the cells sensitive to many chemicals. Since pressurization does not disturb covalent bonds, the molecules remain intact and following decompression reform ionic bonds with divalent cations, but likely in a different way than occurred originally. This may allow the cells to regain partial resistance against some antibacterial compounds, such as lysozyme and nisin (37). For this reason, to achieve a maximum viability loss, the antibacterial compounds should be present during pressurization of the cells (or foods). Pressurization of gram-positive bacterial cells also can bring about changes in the cell wall, probably through alteration of the normal functions of surface layer proteins (16,17).

The cell membrane of gram-positive cells and the inner membrane of gram-negative cells are considered to be greatly affected by pressurization. These structures are composed of lipid bilayers in which many types of large protein molecules (with specific conformations) are embedded by hydrophobic bonds. Many of these proteins have vital functions in the transport of nutrients, synthesis of ATP and enzymatic functions for cell growth and survival. Pressurization by destroying the hydrophobic, H-, and ionic bonds can alter the conformations of these proteins and affect their functions. Although following decompression proteins try to restore their native conformations, they may end up being different from the original structures. In addition, the lipid bilayer at high pressure undergoes a phase transition from the natural liquid crystalline phase to an initially reversible gel phase, then finally to an irreversible integrated phase with reduced thickness (38). These changes, both in the protein molecules and in the lipid bilayer, impair many functions of the membrane, namely the permeability barrier, transport of nutrients, the transmembrane proton gradient and proton motive force, and the ability to synthesize ATP. The pressurized cells become sensitive to NaCl, undissociated organic acids and bacteriocins (12,16,17). Pressurization also can cause dissociation of proteins from intracellular macromolecular assemblies such as ribosomes, and alter functions of cytoplasmic enzymes. Both viability loss and sublethal injury of pressurized cells result from the combination of the changes in the cell wall (or outer membrane), cell membrane (or inner membrane) and cytoplasmic macromolecules; however, the injured cells differ in the extent or level of these changes.

An exposure to high hydrostatic pressure probably does not grossly alter the morphology, disrupt or lyse bacterial cells. However, very little information is available in this area. Recently we pressurized at 345 MPa for 5 minutes at 25 °C a suspension of the food spoilage bacterium *Leuconostoc mesenteroides* Ly and then incubated the cell suspension at 25 °C. Immediately after pressurization, the OD of the suspension (original OD: 100%, and cfu:

2×10^8/ml) increased to 142% and viability decreased to 4×10^1 cfu/ml (a loss of more than 6-log cycles). After 60-minute incubation at 25 °C, the OD reduced to 34% (a loss of 66% of original value) and viability dropped to 2×10^1 cfu/ml. Scanning electron micrographs of the cells at different times showed that immediately after pressurization, the cells remained intact, but showed slight swelling; however, after 30-minute incubation many of the cells were lysed (Fig. 3). The results showed that lysis occurred after pressurization. Further studies showed that cell lysis was due to the action of cell wall–lytic enzyme(s), since pressurization of heated (70 °C for 10 min) cells did not show such lysis. It was suggested that pressurization initially caused alterations in the cell wall and cell membrane and permealization of the membrane. The membrane damage resulted in a loss in proton and electrochemical gradients across the membrane and a loss of the ability of cells to synthesize ATP. Finally, specific lytic enzymes in the cells were activated resulting in the degradation of the cell wall. Similar observations have been made with cells stressed by other treatments that cause cell wall and cell membrane damage (39).

INACTIVATION OF BACTERIAL ENDOSPORES

Hydrostatic pressure processing was initially used for the preservation of low pH (≤4.5) food products such as fruit juices, jams, and jellies. These products were generally pressurized around 600 to 700 MPa, at 22 to 25 °C for 5 to 30 minutes. The main objectives were to destroy the aciduric microorganisms, namely, yeasts, molds, and some bacteria (such as lactic acid bacteria) that can grow in these products, as well as the vegetative cells of food-borne pathogens. The objectives here are quite similar to those for pasteurization of thermally processed foods and are not aimed at destroying thermoduric bacteria and bacterial endospores. To destroy bacterial spores, foods are given commercial heat sterilization treatment to ensure destruction of the most heat-resistant spores of the pathogen *Clostridium botulinum*. Before high pH (>4.5) foods can be produced by pressurization, it will be important to develop methods either for destroying spores of *C. botulinum* to achieve commercial sterility or preventing bacterial spores from germination, and subsequent growth now is achieved in low heat–processed meat products by incorporating nitrite and refrigerated storage. Currently, there is an interest in determining the pressurization parameters necessary to destroy spores because nitrite is considered detrimental to health due to the possibility of it changing into carcinogenic nitrosamine.

Many reports are available in the literature that describe the effectiveness of high hydrostatic pressure in combination with other parameters for the destruction of spores of several *Bacillus* and *Clostridium* species and strains (40–51). These findings can be summarized as follows (2,5,6):

- A pressure of 1000 MPa or more is required for a substantial inactivation of spores of food-borne bacteria.

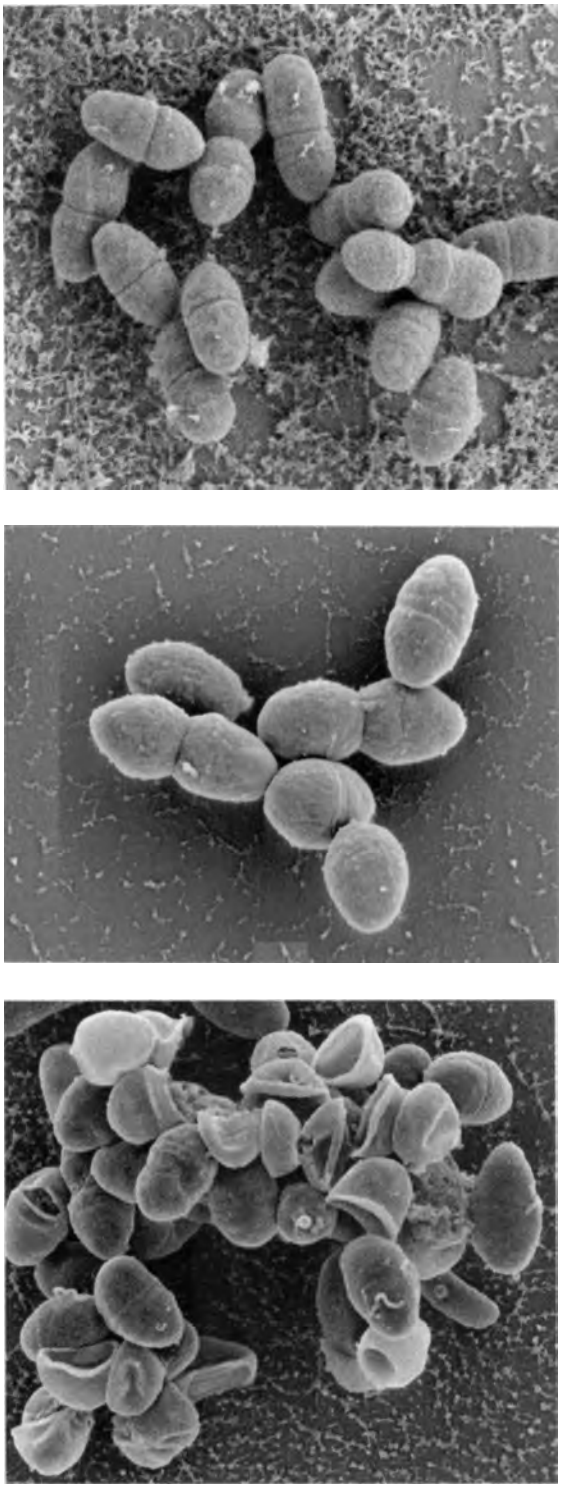

Figure 3. Scanning electron micrographs of *Leuconostoc mesenteroides* Ly cells (25,000 X). (**a**) Unpressurized control. The cells are lenticular, about 1×1.5 mμ in size diplococcal stage with distinct constriction between the two cells. (**b**) Immediately after pressurization at 345 MPa for 5 minutes at 25 °C. Pressurization caused more than 99% cell death, but the cells did not show a major visible change on the surface or on gross morphology. (**c**) Pressurized cells after 30 minute incubation at 25 °C after pressurization. Many cells were lysed (with the separation of cell wall between the two cells). See color insert.

- In general, *Bacillus* spores are relatively more sensitive than *Clostridium* spores.
- The level of destruction is dependent on the species and strain and is directly proportional to the pressure.
- An environment with high solute concentrations, low A_w, and relatively high pH provides protection against pressure inactivation of spores.
- High temperature, low pH (<5.0), and chemicals such as EDTA during pressurization enhance spore destruction.
- Many spores, depending on the species and strain and pressurization parameters, can be induced to germinate and outgrow at a pressure range around 100 to 300 MPa followed by incubation for 1 hour or more.
- Pressure-induced spores become sensitive to repressurization and other physical and chemical antibacterial treatments.

Several recent studies have been conducted to determine pressure inactivation of spores of several *Bacillus* and *Clostridium* species associated with food spoilage and food-borne diseases. A 5-log cycle reduction was achieved by pressurizing spores of a strain of *B. stearothermophilus* at 400 MPa and 90 °C (40,41). It was necessary to pressurize at greater than and equal to 700 MPa and 60 °C to obtain similar levels of inactivation of spores of strains of *B. subtilis, B. coagulans, B. cereus*, and *B. licheniformis* (42–44,51). An initial pressurization at 150 to 200 MPa followed by repressurization at 400 MPa increased spore inactivation of several *Bacillus* spp. This can be attributed to induction of germination by the initial pressure treatment followed by destruction of germinated spores by repressurization (45,46). Pressure-induced germination could be achieved by pressurizing spores in suspensions containing some salts, amino acids, and glucose (47) and incubating subsequently at 37 °C for at least 1 hour (2,5). The physicochemical events induced by hydrostatic pressure appeared to be similar to those induced by heat treatment as the spores lose dipicolinic acid and other spore components (2,5,6,44).

Several pressurization studies have been conducted with spores of *C. sporogenes* PA 3679, which is a nonpathogenic strain used as a surrogate for *C. botulinum* spores in thermal inactivation studies. The results of one study showed that a 5-log cycle destruction of PA 3679 spores required 1400 MPa at 54 °C for 5 minutes; when the temperature was increased to 75 °C, similar inactivation was achieved at 500 MPa. Also, a D-value of 0.7 was achieved by pressurizing the spores at 800 MPa and 108 °C (48,49). One study reported that very little inactivation of *C. botulinum* spores was achieved by pressurizing at 827 MPa and 75 °C (50).

Pressurization in the presence of antimicrobial compounds such as bacteriocins, lysozyme, EDTA, and other compounds has been reported to increase inactivation of both *Bacillus* and *Clostridium* spores (2,5). Since these compounds do not have antibacterial activity against the spores per sé, it is believed that they act on pressure-induced germinated and outgrowing spores. The use of these and other antibacterial compounds, such as sucrose laurate and monolaurin, in the agar media used for enumeration of spores after pressurization seemed to have a bactericidal effect against pressure-induced spores of several *Bacillus* and *Clostridium* species and strains (2,51).

We have studied pressure-induced germination of several species of *Bacillus* and *Clostridium* strains by exposing spore suspensions to pressures between 138 and 483 MPa at 60 °C for 5 minutes (Table 5). The results showed that the amount of germination induced varied widely with the species and strains. The induction

Table 5. Pressure-Induced Germination of *Bacillus* and *Clostridium* Species After 1 Hour and 24-Hour Incubation at 4 °C Following Pressurization

Spore of	Incubation (h)[a] at 4 °C	Log Cycle Germination-Induction Following Pressurization at the Indicated Levels (MPa)				
		138	276	345	414	483
B. cereus	1 h	0.2	0.4	0.9	1.4	1.5
(ATCC 10876)	24 h	5.0	5.0	5.3	5.5	5.8
B. stearothermophilus	1 h	0.1	0.1	0.2	0.2	0.2
(ATCC 12890)	24 h	3.2	3.1	2.9	2.5	2.5
C. sporogenes	1 h	0.3	1.3	1.3	1.2	1.3
(PA 3679)	24 h	0.9	2.7	2.7	2.6	2.6
C. perfringens	1 h	0.2	0.4	0.9	1.0	1.0
(ATCC 1027)	24 h	0.2	0.6	1.1	1.1	1.2
C. tertium	1 h	0.1	0.2	0.2	0.2	0.2
(FM 1)	24 h	0.6	1.1	1.1	1.1	1.2
C. laramie	1 h	0.2	0.2	0.2	0.3	0.3
(ATCC 52154)	24 h	0.2	0.2	0.2	0.3	0.3

[a]Spore suspensions in 0.1% peptone solution were pressurized at 60 °C for 5 minutes at the indicated pressures. Immediately after pressurization, the samples (duplicate tubes) were kept at 4 °C for 1 hour and for 24 hour. The pressurized and control spore suspensions were then heated at 80 °C for up to 30 min and enumerated for cfu/ml by recommended methods. The difference between control and pressurized samples were used to calculate pressure-induced population. Heating at 80 °C did not cause any spore inactivation.

was much less within 1 hour but increased after 24-hour incubation at 4 °C (incubation at 25 °C in place of 4 °C, did not increase the germination amount; data not presented). At a higher pressure range of 276 to 345 MPa, the amount of germination generally did not increase; in fact, in some strains it decreased. In another study, when pressurized spores were suspended in a mixture of two bacteriocins, pediocin AcH and nisin, at 5,000 activity units/ml with or without lysozyme, all of the induced spores were killed (data not presented). This suggests that although spores are insensitive to these antibacterial compounds, they are killed following pressure induction. Thus, these biopreservatives could be incorporated into food prior to pressurization at a medium range of hydrostatic pressure (300 to 400 MPa) and at a moderate pressurization temperature (60 °C) for 5 minutes to achieve better spore destruction. During storage of the foods (at 4 °C or 25 °C), the germinated spores will be killed by the biopreservatives, preventing their subsequent growth and associated spoilage or food-borne diseases.

INACTIVATION OF OTHER MICROORGANISMS

Viruses

Many food-borne and waterborne pathogenic viruses have been implicated in causing diseases in humans. No studies have yet been reported on the effectiveness of HHP on their destruction. One reason could be, that except for Hepatitis A virus, techniques for quantitative and qualitative detection of food-borne viruses are not currently available. Since there is an interest now in using HPP for destruction of pathogenic *Vibrio vulnificus* in raw oysters and other shellfish, it could be important to see if the same treatment can also destroy Hepatitis A virus in raw oysters. In addition, in countries such as France where raw milk is used to make cheese, it would be interesting to see if bacteriophages of starter culture lactic acid bacteria, which often are present in raw milk and associated with the destruction of starter cultures and fermentation failure, could be destroyed (along with other pathogenic bacteria) by HPP. This would provide an effective means to treat raw milk prior to cheese manufacture.

Several studies have been conducted to determine the effectiveness of high hydrostatic pressure on the destruction of viruses associated with human immunodeficiency diseases. These results showed that pressurization at 400 MPa for 10 minutes destroyed over 5-log cycles of these viruses (52,53). A recent report indicated that at 300 MPa for 1 hour, HIV particles were inactivated, and the pressure-treated viral particles showed an enhanced immunogenicity (54). It could be assumed that at 400 MPa and higher, at which the foods are processed, most viruses (and bacteriophages) probably will also be destroyed.

Yeasts and Molds

The destruction of food-borne yeasts, molds, and their spores is of major interest in the commercialization of pressure-processed fruit juices, jams, and jellies. Because of their large size, most studies showed that vegetative cells of yeasts and molds are destroyed within a few minutes following exposure at 300 MPa at 25 °C. However, spores of some molds required much higher pressure and some even needed 800 MPa at 70 °C for 10 minutes for a 6-log cycle reduction. A lower pH, mild heat, presence of nisin, and repressurization for several cycles also enhanced the level and rate of their destruction (7,55–60). Electron microscopic observations have revealed that pressurization of yeast cells above 200 MPa for 10 minutes produced damage to the cell wall and altered subcellular structures, especially in the nucleus and mitochondria. For example, the nuclear membrane had pores and the mitochondrial cristae were damaged. In addition, electron dense materials occurred in the cytoplasm (61) as a result of protein degradation.

Protozoa and Parasites

Several pathogenic protozoa, such as species of *Cryptosporidium, Cyclospora, Entamoeba*, and *Giardia*, are important because of their ability to cause food-borne (and waterborne) diseases in humans. Increased consumption of raw fruits, juices, and vegetables has been associated with many outbreaks, and pressurization could be a means to reduce the incidence. A recent study has reported that pressurization of oocysts of *Cryptosporidium purvum* at 500 MPa for 1 minute in fruit juices resulted in a 5-log or more reduction in viability and infectivity. Microscopic observations revealed a crumpled appearance of the oocysts (62). Another study has also reported that the round worm *Trichinella spiralis* in meat could be killed at 200 MPa in 1 minute (63). Although there are few studies on pressure inactivation of food-borne protozoa and parasites, the general assumption is that these organisms could be effectively destroyed by hydrostatic pressure in the range of 400 to 500 MPa and low pH. With mild heating, a lower pressure range could still cause their destruction.

CONCLUSION

The results referred to in this review describe studies mostly conducted with pure microbial cultures in buffer and bacteriological media and with several food systems. In recent years, more studies are being conducted in many different food systems that include both low pH and high pH food products from dairy, meat, and fruit and vegetable (64–74) groups. In a current study, we observed that several food-borne pathogens, namely, *L. monocytogenes* and *E. coli* O157:H7 strains and *Salmonella* serovars could be destroyed completely in contaminated frankfurters by a combination of moderate hydrostatic pressure and medium heat along with a bacteriocin-based biopreservative (Table 6). These and other studies have generated enough interest worldwide among consumers and food processors to start the marketing of some products in several countries. The indications are quite favorable and there is every reason to believe that hydrostatic pressure processing will be a major method not only for the microbiological destruction and preservation of foods but also for many other

Table 6. Complete Destruction of Food-borne Pathogens by a Combination of Pressurization Treatments

	cfu/g							
	Roast Beef			Summer Sausage			Frankfurters	
Pathogens/ treatments[a]	1 d	7 d (at 25 °C)	84 d	1 d	7 d (at 25 °C)	84 d	1 d	7 d (at 37 °C)
L. monocytogenes								
Control	6×10^3	1×10^{10}	NT[b]	4×10^3	1×10^1	<10[c]	4×10^2	5×10^4
HP	<10	<10	<10	<10	<10	<10	2×10^1	5×10^2
HP + BP	<10	<10	<10	<10	<10	<10	<10	<10
E. coli O157 : H7								
Control	5×10^2	2×0^{10}	NT	9×10^2	4×10^1	<10	2×10^3	NT
HP	<10	<10	<10	<10	<10	<10	<10	<10
HP + BP	<10	<10	<10	<10	<10	<10	<10	<10
Salmonella serovars								
Control	2×10^4	4×10^{10}	NT	7×10^2	2×10^2	<10	1×10^3	NT
HP	<10	<10	<10	<10	<10	<10	<10	<10
HP + BP	<10	<10	<10	<10	<10	<10	<10	<10

[a] Pathogens were inoculated in the meat products with or without biopreservatives (BP; a 1 : 1 mixture of pediocin AcH and nisin A at 5000 AU/g), vacuum-packaged and pressurized (HP) at 345 MPa for 5 min at 50 °C. The samples (5 per test period) were stored either at 25 °C or 37 °C and examined for the pathogens at different storage intervals.

[b] NT: Not tested due to positive results in the previous study.

[c] <10: Testing by both enumeration and isolation procedures failed to detect any colonies. Enumeration for each sample involved testing of 0.4 g product. Isolation involved adding 10 to 25 ml of enrichment broth in each sample bag followed by 24 h incubation at 37 °C, streaking of broth culture on a selective agar medium plate, incubation of plates at 37 °C up to 2 d and examination for the absence of colonies.

novel applications in food processing. HHP should also be beneficial for production of foods that otherwise are not possible to develop by existing processing methods.

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HOME TREATMENT DEVICES — MICROBIOLOGY OF POINT OF USE AND POINT OF ENTRY DEVICES

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Consumer concerns over the quality of drinking water have increased steadily over the past 15 to 20 years. This is primarily because of the perception of consumers, significantly influenced by news media reports that contaminants (chemical and microbiological) in municipal drinking water make it unsafe for consumption. The occurrence of low levels of disinfection by-products resulting from the reaction of chlorine or other water disinfectants with organic compounds in the water to form compounds that may be carcinogenic, mutagenic, or toxic, has been demonstrated. Waterborne disease outbreaks caused by pathogenic bacteria, viruses, and protozoa have occurred due to inadequate treatment, treatment process failures, and posttreatment contamination of potable water. In addition, the aesthetic quality of tap water varies due to source water characteristics (hardness, odor, taste, or appearance) and residual disinfectant level. Many consumers turn to bottled water or to commercially available water treatment devices that can be installed in their homes because they do not like the taste, odor, or appearance of the tap water delivered to their homes. Thus, as a result of the lack of confidence of consumers in the quality of municipally supplied tap water and aesthetic concerns related to tastes, odors, and appearance, there is a rapid growth in point of use (POU) and point of entry (POE) treatment unit sales.

According to the Water Quality Association (1), in 1999, consumer use of home water treatment systems has increased by 19% since 1997 and by 28% since 1995. Thirty-eight percent of 1,007 adults over the age of 18 surveyed in 1999 used some type of home water treatment device (1).

Home treatment POU units generally fit one of four categories: (*1*) Pour-through devices that stand alone on the counter top, (*2*) counter top units that attach to the kitchen faucet by means of a diverter valve that allows the flow from the faucet to be directed through the unit, (*3*) under the counter units that are tapped into the cold-water line and supply a separate faucet, or units that treat all the cold water going to the faucet, and (*4*) small faucet attached devices through which the tap water can be diverted (Figs. 1,2). Pour-through granular activated carbon (GAC) filter units (Fig. 2) have

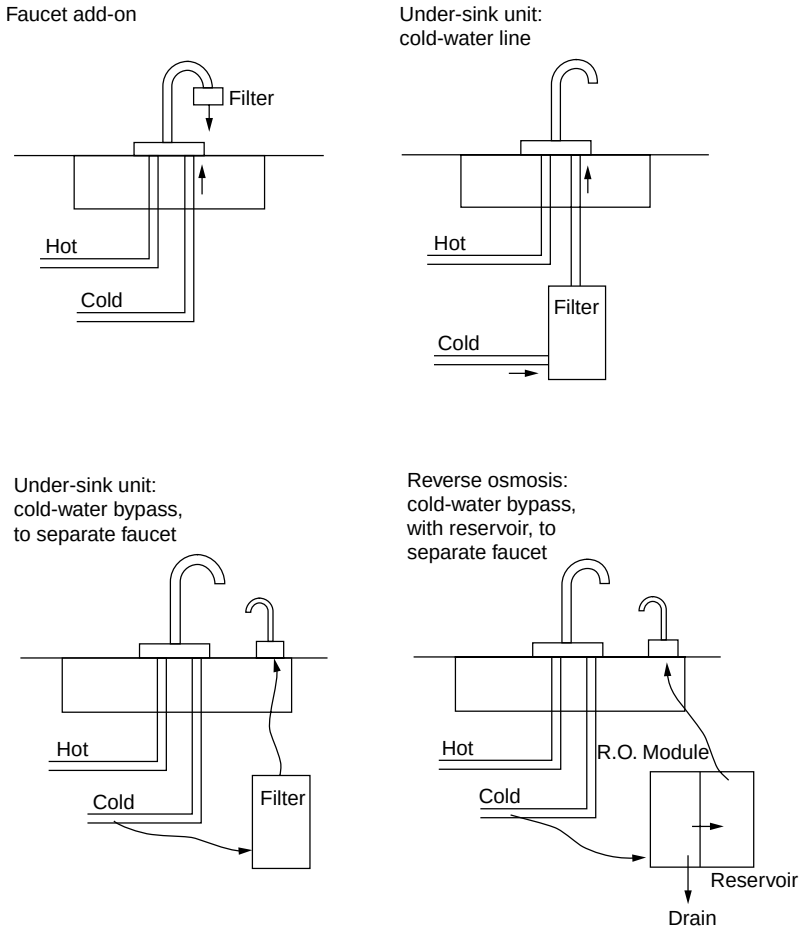

Figure 1. Basic applications of point of use units. (Modified from Fig. 2, *J. AWWA*, 75(1): 42–50 (1983), by permission. Copyright, 1983, American Water Works Association).

become extremely popular in the United States (e.g., Brita®). However, there have been very few studies of the microbiological characteristics of pour-through filter devices (see Bacteriological quality of water from POU and POE treatment devices).

Microbiological quality concerns related to POU/POE treatment generally involve the control of heterotrophic bacteria levels, measured by the heterotrophic plate count (HPC) method in treated water. The bulk of these concerns have been directed at POU/POE treatment devices that contain GAC or powdered activated carbon (PAC) that are sold as filters to improve the aesthetic quality of water, including removal of any residual disinfectant (usually chlorine) from influent water. Additional claims may include removal of particulate contaminants, including parasitic protozoan cysts and oocysts, asbestos fibers, ordinary sediment, rust and turbidity, chlorine disinfection by-products (trihalomethanes), insecticides and pesticides, and other organic compounds. Some treatment devices contain special adsorbents or ion exchange resin(s) for removal of inorganics such as lead and arsenic. These treatment devices should only be used to treat water that is "microbiologically safe" to begin with, and such a statement is generally included in the manufacturers' product literature.

In addition to the use of POU-GAC filters in home applications, such filters are also used as in-line filters to treat water going to refrigerator cold-water dispensers and icemakers, commercial icemakers, and soft drink dispensing machines. Studies of microbiological problems of these types of filters in commercial equipment applications were not found. However, based on the information from studies of similar types of GAC filters used in home treatment POU devices, unless such in-line filters incorporate a final filtration step using an absolute filter of less than or equal to 1.0 μm to remove bacteria, it is probable that high heterotrophic plate count (HPC) levels would also be found in the effluent from these filters.

GAC filters, including those that contain silver as a bacteriostatic agent, have been shown to promote heterotrophic bacterial growth. Additionally, high bacterial levels have been found in product water storage reservoirs of treatment devices such as reverse osmosis (R.O.) units. The key concern has been the possibility that ingestion of high levels of heterotrophic bacteria in drinking water may cause illness, particularly for the very young, the elderly, and for immunocompromised individuals.

POU VERSUS POE

Home water treatment devices that are installed to treat only the water at a faucet location (usually the kitchen tap), or at multiple faucets, are called point of use (POU)

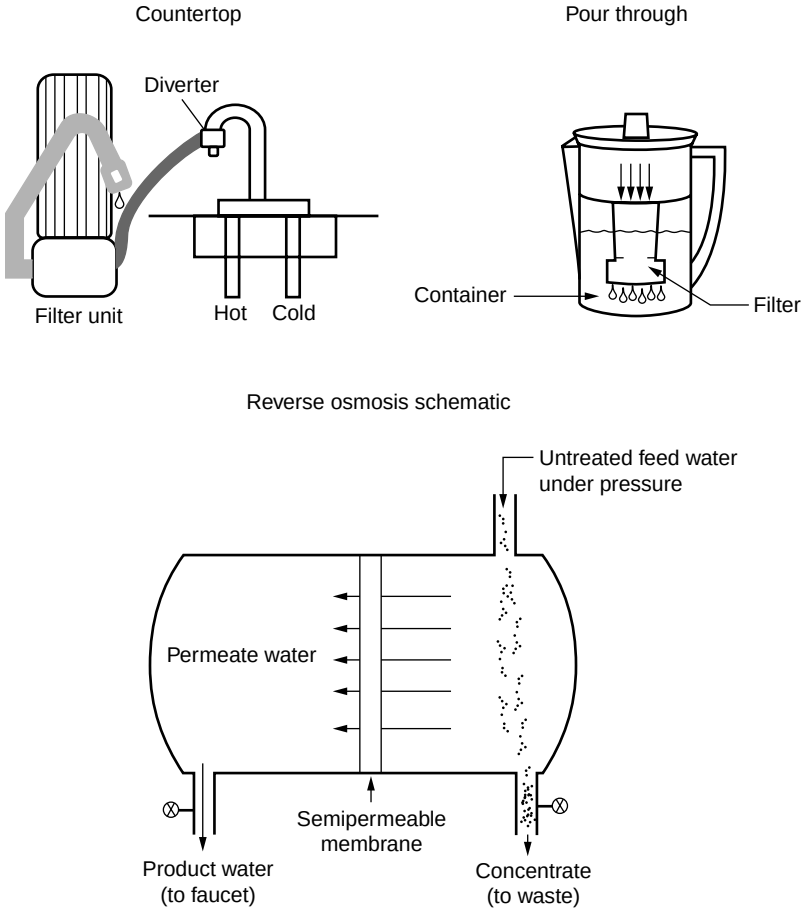

Figure 2. Countertop and pour-through point of use devices (upper) and a schematic of how reverse osmosis works.

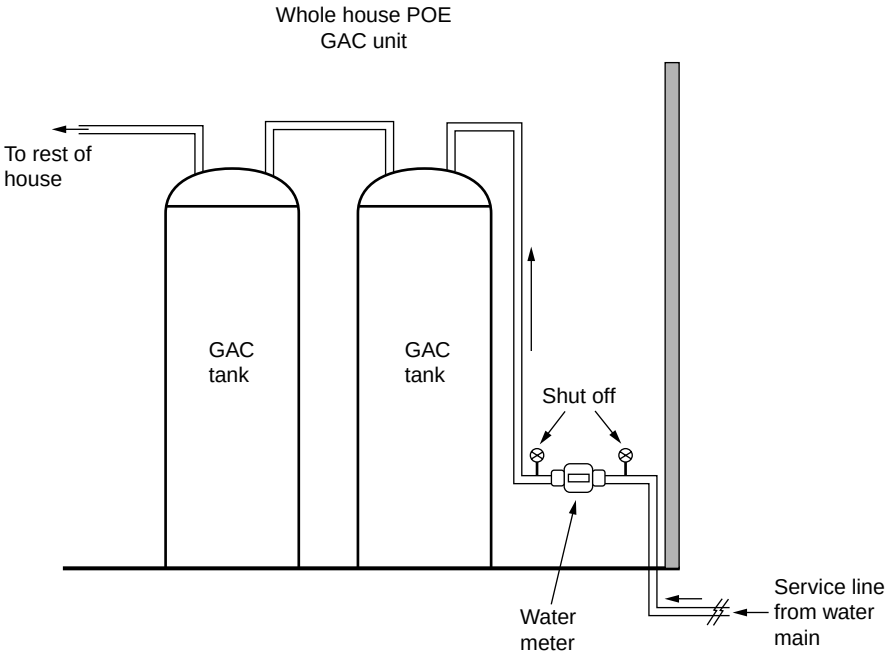

Figure 3. Example diagram of a whole-house point of entry GAC water treatment unit. Other configurations that use a combination of treatment technologies such as GAC + UV treatment, ion exchange + UV treatment, or particulate filter + GAC + UV treatment, etc., are available.

devices. Most POU drinking water systems are installed to treat water used only for drinking and cooking. Home water treatment devices that are installed to treat all the water entering the home are called point of entry (POE) devices (Fig. 3). A variety of both types of water treatment units are commercially available to consumers worldwide. This article addresses (*1*) general microbiological concerns associated with the use of some types of these treatment devices and does not address specific brands or models and (*2*) microbiological studies of POU water purifiers. It does

not address removal of chemical compounds, inorganic or organic, by POU and POE treatment devices.

TYPES OF HOME TREATMENT UNITS

Home treatment devices or appliances may use a variety of basic processes to treat water, including filtration, adsorption, ion exchange, reverse osmosis, distillation, and disinfection (2). Table 1 summarizes the basic processes that may be used by POU/POE devices and the contaminants they can remove. In some cases, combinations of two or more of these processes may be used in a single treatment device to achieve multiple treatment goals (e.g., filtration for removal of particulates and adsorption to remove chlorine, organic substances, and odors). A brief description of each of these treatment processes, their effectiveness, and some of their limitations for home POU/POE treatment is provided.

Adsorption

GAC or PAC is used to remove tastes and odors associated with chlorine or other disinfectant residual, chemical compounds produced by microorganisms such as actinomycetes, fungi, algae, iron and sulfur bacteria, and other organic chemicals. Activated carbon is obtained by destructive distillation of wood, nut or coconut shells, bones or other carbonaceous material, and activation by heating to 800–900 °C with steam or carbon dioxide. This creates a microporous structure with a very high surface area that can adsorb organic compounds. The activated charcoal is ground, sized, sieved for uniform grain size, and used to pack filtration cartridges sized according to the type of treatment unit desired. GAC filtration cartridges vary in GAC content from as little as 30 grams in a faucet mounted filter to several kilograms in large undersink devices or even more in POE filters. Some GAC POU devices use a bed of GAC, others use a pressed cake or block of activated carbon, and still others use a GAC bed followed by PAC or a diatomaceous earth precoat on the influent side of a fabric filter barrier.

GAC filters have a limited effective life because the carbon can become saturated with adsorbed organic chemical compounds, and some of these may begin to be released back into the water as sorptive sites on the GAC become filled. In addition, GAC provides an ideal environment for bacterial growth because of the tremendous surface area and the availability of adsorbed nutrients. Some manufacturers use GAC treated with silver as a bacteriostatic agent to attempt to control growth of bacteria in the filter cartridge or treatment unit.

Regular filter cartridge replacement (if so equipped) and maintenance of the filtration device in accordance with the manufacturer's specifications must be done to assure acceptable performance of the treatment device. In general, GAC treatment devices should only be used to treat drinking water that is microbiologically safe to begin with because the devices are not designed for removing and/or inactivating pathogenic bacteria, viruses, and protozoan cysts/oocysts, that is, they are not water purifiers.

Table 1. Point of Use and Point of Entry Water Treatment Processes

Treatment Process	Contaminant(s) Removed
Adsorption (activated carbon)	Chlorine, organic chemicals, tastes, and odors
Mechanical filtration	Particulates, turbidity, color, asbestos, cysts/oocysts
Reverse osmosis	Total dissolved solids, metals, nitrates, bacteria, cysts/oocysts
Water softeners, cationic	Calcium, magnesium, iron, manganese, barium, radium
Anionic	Sulfates, nitrates, bicarbonates, chlorides, arsenic
Distillation	Inorganics, dissolved solids, nonvolatile organics, volatile organics (?)
Disinfection, chemical	Bacteria, viruses
Disinfection (UV)	Bacteria, viruses, *Cryptosporidium* oocysts
Water purifiers	Bacteria, viruses, protozoan cysts/oocysts

Mechanical Filtration

Mechanical filters physically remove particles such as sediment and rust by straining. These filters may be made of porous ceramic or plastic, diatomaceous earth, or synthetic fibrous polymer materials. Fibrous filters include spirally wound acrylic or other polymeric filaments, pleated paper filters, and woven or nonwoven fabric filters. Except for R.O. membranes, POU/POE manufacturers do not use biodegradable materials in filters. The effective pore size of the filter will determine the size of particulate materials that it can remove from water. Fine filters with an effective pore size of 0.2–0.4 μm diameter can be used to remove most bacteria and protozoan cysts/oocysts from water. However, the smaller the pore size, the slower the passage of water through the filter and the more quickly the filter may become plugged if the water contains a high level of small particulates. Also, the smaller the pore size, the greater the pressure that will be needed to push the water through the filter material.

Reverse Osmosis (R.O.)

Reverse osmosis is a process by which almost all foreign materials in water, including microorganisms, minerals, and most organic materials, are separated from water using pressure to force the water through a semipermeable membrane (0.0005 μm pore diameter) into a storage tank (Fig. 2c). Production of treated water by R.O. is slow (about 1 L/h.) and about 75% of the influent water is wasted. Therefore, a storage tank is needed to collect the permeate and to provide a ready supply of treated water.

Reverse osmosis membranes may be made of cellulose acetate, cellulose triacetate, or a thin film composite membrane made of polyamid resin or other synthetic material. The thin film composite membranes are stronger than the cellulose acetate and triacetate membranes, but they cannot tolerate chlorine and a dechlorinating prefilter

is needed to protect them from chlorine in the influent water. The cellulose acetate and triacetate membranes are subject to degradation by bacteria over time and are less desirable for this reason. They can, however, tolerate chlorine and have been used in situations in which a chlorine residual was needed in the product water. As long as the R.O. membrane is intact, high quality water is produced by the R.O. device. However, once the membrane integrity has been compromised, treatment effectiveness is lost, the quality of the product water is degraded, and the storage reservoir and any other downstream components then become colonized by bacteria.

Ion Exchangers. Cation and anion exchange units are the two kinds of ion exchangers. Cation exchangers, often called softeners, exchange positively charged sodium or potassium ions for positively charged calcium and magnesium (hard water), lead, radium, and iron and manganese ions. Anion exchangers exchange negatively charged hydroxyl or chloride ions for ions such as sulfates, nitrates, and bicarbonate.

Distillation. Distillers heat water to boiling in one vessel, condense the vapor, and collect the condensate in another vessel. Dissolved solids, metals, minerals, nonvolatile organic compounds, and particles remain in the boiler vessel, and the heat kills any microorganisms. Some volatile organic chemicals (VOCs) may be carried over into the condensate vessel if heating is not carefully controlled. There are distillers that vent volatile organics, but their performance in reducing organics in the distilled product water is in question. There appear to be no microbial problems with distillers other than potential bacterial contamination of the condensate-receiving vessel.

Disinfection. Disinfection treatment units include chlorinators, iodinators, ozonators, and ultraviolet (UV) irradiation devices. Chlorinators dose a chlorine-releasing chemical such as calcium hypochlorite or sodium hypochlorite (bleach) into the water. Devices that contain iodine as the disinfectant generally use an iodinated resin that releases iodine into the water as it flows through the device. Ozonation involves the injection of ozone into the water to destroy some organic compounds and to inactivate bacteria, viruses, and unencysted protozoa. Ozone must be generated on site by the treatment unit and although it is a powerful oxidant, it does not leave a lasting residual in the water. Ultraviolet disinfection devices house a UV lamp that emits UV light at a germicidal wavelength (254 nm) that inactivates viruses and bacteria and possibly oocysts of Cryptosporidium. None of the devices in this category is able, by itself, to eliminate or inactivate all pathogenic microbes from the processed water. Protozoan cysts or oocysts generally must be removed physically, which means coupling a fine filter ($<4.0\ \mu m$) with the disinfection process, although recent studies indicate that UV exposure renders oocysts of Cryptosporidium noninfectious to a mammalian host (3,4).

Water Purifiers

Water purifiers are a special category of POU/POE treatment units that are designed to treat raw source water. POU or POE devices that are claimed to be water purifiers and are used to treat raw water must provide treatment that would assure complete removal or inactivation of all pathogenic microorganisms (viruses, bacteria, and protozoa). Such devices generally will incorporate two or more treatment processes to achieve the goal of eliminating pathogens, for example, filtration and chemical disinfection or filtration and UV disinfection. Water purifiers must be carefully evaluated for their capability to remove or inactivate bacteria, viruses, and cysts/oocysts of protozoa in test waters that are representative of worst case conditions under which the device might be used. The U.S. EPA developed a Guide Standard and Protocol for Testing Microbiological Water Purifiers (5) that specifies the testing levels and organisms to be used in testing water purifiers. The protocol is meant to be flexible to accommodate efficacy testing of all types of POU water purifiers. Manufacturers can obtain certification of drinking water treatment devices (DWTDs) through an independent testing organization such as National Sanitation Foundation (NSF) International (formerly NSF Ann Arbor, Michigan), Underwriters Laboratories (UL, Northbrook, Illinois), or Canadian Standards Association (CSA) International (formerly CSA Toronto and Ontario, Canada). Certification testing involves laboratory testing of a DWTD to verify the manufacturer's claims of contaminant reduction, a toxicological review, health effects evaluation, and performance and pressure tests to be sure that the treatment devices themselves do not contaminate the drinking water. Depending on the specific claims and the product type, certification testing of the POU treatment device for compliance with existing standards [American National Standards Institute/NSF standards (ANSI/NSF)] can be accomplished for a fee.

BACTERIOLOGICAL QUALITY OF WATER FROM POU AND POE TREATMENT DEVICES

The bacteriological quality of water delivered by the municipal water system may be significantly modified by POU/POE filters that utilize only GAC for treatment. The bacteriological quality is generally assessed by the HPC procedure and expressed as colony forming units (CFU)/mL (6). Depending on various factors, the bacterial count in public drinking water (tap water) may range from less than one colony forming unit (CFU)/mL to more than 10^3 CFU/mL, whereas the water from some POU-GAC treatment devices may range from 10^1 CFU/mL to more than 10^6 CFU/mL (Table 2). In general, in typical GAC filters, the bacterial level increases in treated product water is relative to the bacterial level in influent tap water. The magnitude of the increase may vary considerably but generally is in the range of 10- to 1,000-fold and is due to the growth of bacteria on the GAC in the filter cartridge. For a GAC-precoat combination filter, however, the density of bacteria in the product water may be lower than that of

Table 2. Heterotrophic Plate Counts (CFU/mL) in Influent Tap Water vs. POU/POE Product Water

Filter Type	Influent Mean (Range)	Product Water Mean (Range)	Reference
GAC#4–Run 1	23 (2–120)	180 (16–600)	7
GAC#4–Run 2	25 (3–100)	610 (35–8,000)	
GAC A–0 sec.[a]	9,500 (ng)[b]	84,000 (84,000–85,000)	8
30 sec.	780 (ng)	11,000 (7,900–14,000)	
4 h	160 (ng)	3,500 (3,500–3,600)	
GAC B–0 sec.	9,500 (ng)	2,800 (1,600–40,000)	8
30 sec.	780 (ng)	3,500 (2,600–4,400)	
4 h	160 (ng)	920 (830–1,000)	
GAC C–0 sec.	9,500 (ng)	109,000 (57,000–162,000)	8
30 sec.	780 (ng)	17,000 (13,000–21,000)	
4 h	160 (ng)	6,100 (4,100–8,000)	
GAC#1 (N = 95)	2,800 (100–11,000)	2,300 (130–10,000)	9
GAC#2 (N = 95)	2,800 (100–11,000)	71,000 (3,400–340,000)	8
GAC#3 (N = 95)	2,800 (100–11,000)	2,700 (290–15,000)	8
GAC#4 (N = 95)	2,800 (100–11,000)	19,000 (570–89,000)	8

Note: [a]Bacterial count after running the tap water for the time indicated.
[b]ng = not given

the influent water because of the pore size of the membrane filter in the device (10).

Bacterial growth in GAC filters depends on a number of factors including the length of time, the unit that has been in service, seasonal changes in water temperature, type of GAC, influent water quality, length of time since previous use, species of bacteria present, ambient temperature at which the filter unit is installed or kept, design of the unit, and presence or absence of a disinfectant residual.

A tremendous surface area (nominally, 1,000 nm^2/g GAC) is the factor that makes GAC so effective at adsorbing organic chemical compounds from water, it is also the factor that provides an ideal growth habitat for many of the heterotrophic bacteria found in water (12,13). The GAC surface becomes colonized by the bacteria, which along with adsorbed and extracellular material released by the bacteria, form a biofilm. The bacteria found in biofilms derive at least two benefits from attachment to the surface: (*1*) a ready source of nutrients that have concentrated at the surface added to a constant or intermittent replenishment of nutrients by water flow through the carbon and (*2*) physical protection from toxic or inhibitory chemicals. During periods when the POU device is not used, such as nighttime or vacation periods, water contained in the device stagnates and the bacteria on the GAC proliferate. The data in Table 3 show that the bacterial density in the water increases during a period of nonuse (stagnation). For the extreme case of a six-week nonuse period, the data showed that bacterial count changes were significant and the increase in CFU/ml ranged from 1-$\log_{10}$ to 4-$\log_{10}$ during the six-week stagnation period, and following resumption of use, dropped from 1- to 3-$\log_{10}$, depending on the unit (9). For nonuse periods of 20–92 hours, a granular or precoat filter showed significantly increased bacterial levels in the product water relative to samples collected before the shutdown. Interestingly, the bacterial levels in the influent water were much higher after the shutdown than they were before the shutdown, but the product water bacterial levels were much less than the influent bacterial levels, indicating that bacterial removal by the precoat filter was about 2- to 3-$\log_{10}$ (10).

Smaller time of day changes in bacterial levels in the product water were observed for four different GAC units when bacterial levels were determined in the morning following an overnight no-flow period versus bacterial levels determined in the afternoon. This was attributed to flushing during several flow periods that occurred during the day. The bacterial counts in the afternoon samples were generally, but not always, less than the morning bacterial counts.

In a study of bacterial contamination of POU filters, Daschner and coworkers (11) found that for 64% of 48 households that used water treatment devices, the bacterial counts of the product water were higher than for tap water of the same households. They also found differences in the bacterial levels as a result of storage of the product water and/or the treatment unit at 4 °C or 22 °C. For the type of pour-through filters that they evaluated (Aquafine, Brita®), bacterial levels ranged as follows: fresh filtrate (filter system stored at 22°C), HPC = 1 to >10, 000 CFU/mL versus fresh filtrate (filter system stored at 4 °C), HPC = 3 to 9,000 CFU/mL; 24 to 72-hour-old filtrate (filter system stored at 22 °C), HPC = 0 to >10, 000 CFU/mL versus 24 to 72-hour-old filtrate (filter stored at 4 °C), and HPC = 0 to 183 CFU/mL. Clearly, storage of both the filter unit and the filtered water at 4 °C was beneficial in holding down the bacterial density of the product water. After only one week of use, however, the bacterial density of freshly filtered product water was greater than that of tap water, indicating significant colonization of the GAC filter.

Daschner and coworkers (11) examined 13 newly purchased, unused filters for microbial contamination and found that the contamination at the bottom of the filter in 10 of the 13 filters ranged from 2 to 300 CFU/16 cm^2

Table 3. Effect of Non-Use Periods on Bacterial Counts in Product Water from POU Treatment Devices

Filter Type	Mean CFU/mL Before Non Use	During Non Use	After Non Use	Reference
GAC				
#1	25[a]	690,000	130	9
#2	25,000	1,100,000	11,000	9
#3	260	100,000	500	9
#4	2,600	30,000	5,100	9
Dechlor. Tap Water	11	29,000	190	9
Granular Precoat[b]				
Influent	280[c]	130,000	NG[d]	10
Effluent-1	490	22,000	NG	10
Effluent-2	540	19,000	NG	10

Note: [a]Non use period of 6 weeks; HPC numbers in all columns for this reference are mean values for a six-week period before, during and after shut-down, respectively.
[b]Powdered activated carbon on influent side of the barrier portion of the filter.
[c]HPC numbers are mean values calculated from data in cited reference for shut-down periods ranging from 20–92 hours
[d]NG = not given.

(Rodac plates). When the GAC filter material was checked for bacterial contamination, 5 of the 13 filters (38%) yielded bacterial counts that ranged from 4 to 99 CFU/g. Thus the fact that filters may come from the manufacturer already contaminated with bacteria must be recognized. Identification of bacteria isolated during the study yielded *Aeromonas hydrophila*, *Pseudomonas cepacia*, *P. fluorescens*, *P. putida*, *Sphingomonas paucimobilis*, *Acinetobacter lwoffi*, and coagulase-negative staphylococci.

Bacteria Isolated from Pou-GAC Filters

Bacteria isolated and identified from POU water treatment devices are listed in Table 4. This list includes both coliform bacteria and noncoliform bacteria found in specific studies. It is unlikely that these organisms represent all the bacteria that may be found in such treatment units, but these are representative of bacterial isolates from treated drinking water in general (9). The list includes some bacteria that are known opportunistic human pathogens (e.g., *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Aeromonas hydrophila*, etc.). The occurrence of coliform bacteria on this list is not surprising because no water treatment plant is 100% efficient in removing or inactivating all microorganisms, and low levels of bacteria do survive and colonize the pipe walls of water distribution systems. Some coliform bacteria that escape disinfection are able to survive and grow in distribution system biofilms and are shed back into the water under some conditions (15).

New carbon filters become colonized by bacteria very rapidly, and the HPC/mL levels tend to spike and then drop marginally. The HPC/mL of the product water becomes relatively stable, often at a fairly high level, for filter cartridges that have been in service for more than two weeks. This is illustrated in Figure 4 to show the bacterial levels in dechlorinated tap water influent versus bacterial levels in product water for three third-faucet type GAC treatment units used in the study (16). Within two weeks of being in service, all three units showed high levels of heterotrophic bacteria in the product water, and for two of the three test units the bacterial load in the product water was the most during the first six weeks of use. The curve marked TAP in Figure 4 represents water

Table 4. Bacteria Isolated from Product Water from POU Treatment Devices

Genus and Species	Filter Type	Reference
Coliform Group		
Citrobacter freundii	GAC	9
Enterobacter aerogenes	GAC	9
Ent. cloacae	GAC	9
Klebsiella pneumoniae	GAC	9
Non coliform		
Acinetobacter calcoaceticus	R.O.	14
Acinetobacter lwoffii	GAC	11
Aeromonas hydrophila	GAC	11
Alcaligenes sp.	GAC	9
Alcaligenes sp.	R.O.	14
Chromobacterium sp.	R.O.	14
Flavobacterium sp.	GAC	8
Flavobacterium sp.	R.O.	14
Moraxella sp.	R.O.	14
Pseudomonas aeruginosa	GAC	8
Ps. cepacia	GAC	9,11
Ps. fluorescens	GAC	9,11
Ps. maltophilia	GAC	9
Ps. paucimobilis	R.O.	14
Ps. putida	GAC	11,14
Serratia liquifaciens	GAC	9
Ser. rubidaea	GAC	9
Sphingomonas paucimobilis	GAC	11
Coagulase negative staphylococci	GAC	11

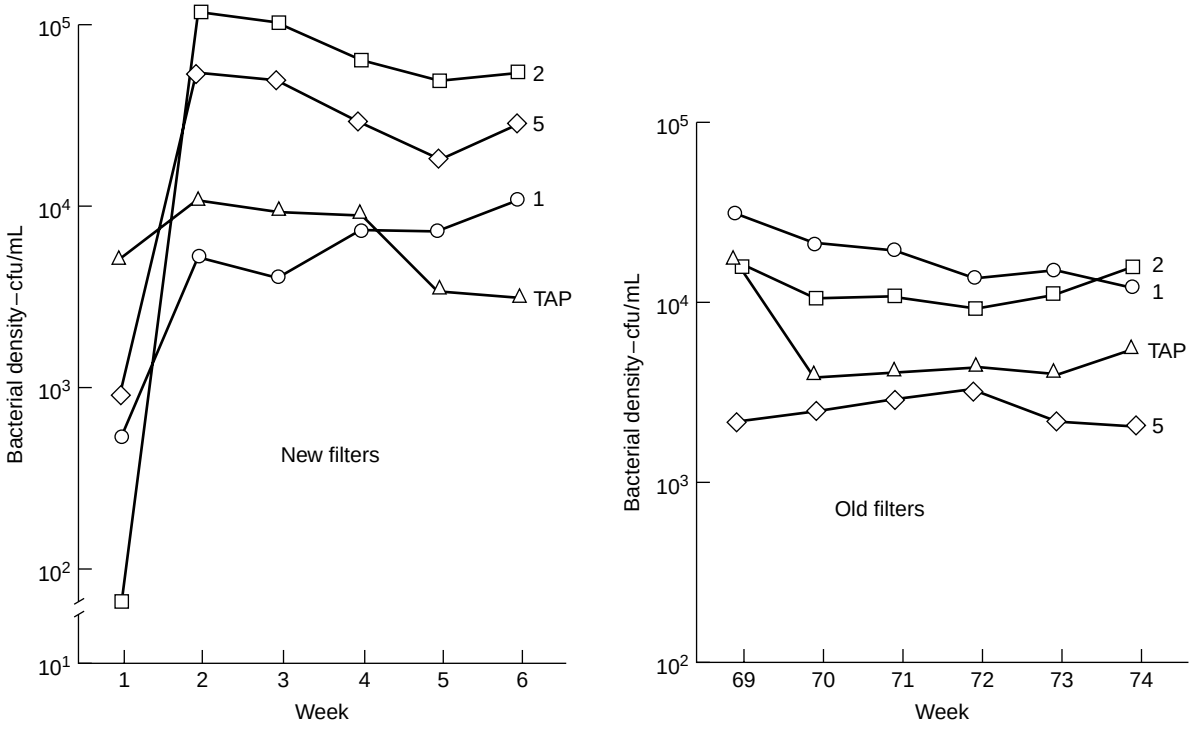

Figure 4. Comparison of bacterial densities in product water from new and old filter cartridges. The number at the end of each curve (1, 2 and 5) represents different filter units tested; TAP indicates dechlorinated tap water (modified from Fig. 12, *J. AWWA* **79** (10): 60–66 (1987), by permission. Copyright, 1987, American Water Works Association).

used as the influent to the treatment units. This feed water was municipal tap water that was dechlorinated by passage through a GAC filter. The graph of the bacterial levels for the TAP samples for both the new and the old filters (Fig. 4) showed relatively stable bacterial levels that were generally lower than the bacterial levels associated with the product water from the treatment units. This pattern was similar to that reported by Tobin and coworkers (8).

High levels of heterotrophic bacteria can develop in GAC cartridge filters and are shed into the product water, and it is not possible for the consumer to determine when a cartridge has ceased to function well in the removal of organic chemical compounds from the water. Consequently, it is important for the filter unit or cartridge to be maintained and changed according to the manufacturer's recommendations. The only clues a consumer may have that the GAC filter cartridge may need to be replaced are changes in the flow rate or taste and/or odor of the product water. However, such a change could occur before the manufacturer's recommended filter cartridge use limit is reached. If this were to happen, the GAC cartridge or the complete unit (depending on the type of unit), should be replaced. If it is economically feasible, the consumer may wish to replace the filter more frequently than the manufacturer recommends because the consumer cannot easily determine when exactly the carbon filter is no longer functional, and the bacterial concentration in the product water may achieve a very high, relatively stable level.

Bacteriostatic Filters

Some GAC treatment devices contain silver as a bacteriostatic agent to control growth of HPC bacteria. However, silver is only effective as a growth-inhibiting agent for some bacteria, not all. In addition, the effectiveness of silver may become attenuated as particulate loading and organic compound adsorption increase, and bacterial biofilm builds up on the GAC during use. Reduced effectiveness of silver may also result from loss of silver caused by leaching.

Reasoner and coworkers (16) found that two of the three GAC filters that contained silver as a bacteriostatic agent shed bacteria in the product water that were at levels as high as for GAC filter units that did not contain silver. Qualitatively, there appeared to be fewer bacterial colony types in the HPC population from silver-containing GAC filters than there were from plain GAC filters.

Silver is moderately effective as a bacteriostatic agent for coliform bacteria. Tobin and coworkers (8) spiked tap water with coliforms in settled sewage and compared counts of coliform bacteria in water filtered through a GAC-silver POU filter with coliform counts in water filtered through a plain GAC filter of the same manufacturer. Water samples were collected from the GAC-silver unit immediately after adding the coliform spike to the influent. The samples were quickly neutralized and analyzed, and the results showed that some coliforms could still be enumerated. After a contact time of 30 minutes before sample neutralization, no coliform could be detected. In the same samples, there was very little

effect on the total heterotrophic bacterial population. However, water samples collected from the plain GAC filter and handled in a similar fashion showed coliform counts nearly as high as the initial coliform spike level (8). In another study, an *Enterobacter aerogenes* challenge was reduced from 8,200/100 mL to 830/100 mL (1-$\log_{10}$ reduction) in 30 seconds and to less than 1/100 mL (>3-$\log_{10}$ reduction) in 17 hours; the reduction of *E. aerogenes* from 52,000/100 mL to less than 1/100 mL (>4-$\log_{10}$ reduction) occurred in 68 hours (18). In the same study, GAC-Copper yielded a similar reduction in 68 hours. However, the authors concluded in that study that the effect of silver or copper on the general heterotrophic bacterial population was negligible. The use of a precoat filter in conjunction with GAC was also effective in reducing coliform bacteria challenge levels, yielding log reductions ranging from 99% to 99.999% (2- to 5-$\log_{10}$) (18).

Although silver in GAC filters has been shown to be bacteriostatic or even bactericidal for some bacteria, including some coliforms, other bacteria have been shown to be resistant to silver. A pour-through GAC-silver treatment unit was tested to see whether the three species of nontuberculous mycobacteria (*M. avium*, *M. fortuitum*, and *M. mucogenicum*) would colonize and grow or survive (19). The results showed that nontuberculous *M. avium* colonized and grew in the device, but the other two species were never detected in the filtered water during an eight-week experiment. The three *Mycobacterium* species were further tested for susceptibility to silver by disk diffusion assay; *M. avium* grew in the presence of 1,000 μg/mL silver, whereas *M. fortuitum* and *M. mucogenicum* were inhibited by 50 μg/mL silver. When Rodgers and coworkers (19) surveyed 45 strains of nontuberculous mycobacteria (11 species) isolated from drinking water for sensitivity to silver using the disk diffusion assay, 26 isolates (57%) were resistant to 1,000 μg/mL silver, including 20 *M. avium* isolates. These researchers concluded that the presence of nontuberculous mycobacteria in drinking water treated by a filtration device that incorporates silver as a bacteriocidal or bacteriostatic agent could pose a health risk for immunocompromised consumers.

Included in the general concern over bacterial growth in POU-GAC filters is the possibility that frank or opportunistic pathogens that reach the filters may become established, multiply, and pose a health threat to the consumers. Because of this concern, various studies have challenged POU-GAC treatment units by dosing pure culture suspensions of various bacteria to determine if the bacteria would simply pass through, be retained by the filter units and survive at low levels, or colonize the filter units and multiply to higher levels than the challenge dose. Table 5 lists bacteria that were used to challenge POU treatment units in various studies and the length of time the organisms survived and were detected in the product water. In the studies reported by Geldreich and coworkers (9), Reasoner and coworkers (16), and Geldreich and Reasoner (20), there was considerable variation in the size of the challenge doses used. The doses reflect the total number of challenge cells delivered to each unit, not CFU/mL; doses varied from a low of 200 to 300 CFU to a maximum of 3.72×10^9 CFU. The higher doses were usually associated with bacteria that were more difficult to recover in attempts to establish that they could or could not survive or colonize. Some bacteria (*E. coli*, *Salmonella typhimurium*, and *Enterobacter cloacae*) appeared to pass through some of the filter units and were not recovered even a few hours (shown as <0.5 d in Table 5) after the small challenge dose. Others apparently colonized the treatment units and were detected in the product water for periods that varied from 0.5 days to as high as 150 days. Most challenge bacteria, when detected in the product water, were present at low levels ranging in mean values from 0.2 CFU/100mL to 110 CFU/100mL (9). Several bacteria that were dosed at low levels (200–300 CFU/unit) were retained by the filters, apparently colonized, and were released over periods ranging from six days (*Citrobacter freundii*) to as long as 150 days (*Pseudomonas aeruginosa* and *Serratia marcescens*). Other bacteria used to challenge the test units, although dosed at very high levels, were unable to survive or colonize the GAC treatment units for more than a few hours at best (*Campylobacter jejuni*, *Salmonella typhimurium*, and *Yersinia enterocolitica*). Finally, of three species of Mycobacterium seeded into water and filtered through a pour-through GAC-silver water treatment device, only *M. avium* was found to colonize the GAC and remain recoverable in the product water throughout the eight-week testing period (19). The authors concluded that because such an opportunistic pathogen could survive or colonize in such a unit, water treated by a POU device that relies on the bactericidal effect of silver could pose a health risk to immunocompromised consumers. This concern could be extended to some other opportunistic pathogens listed in Table 5.

A general concern raised here is whether the benefit of using POU devices to remove cysts and oocysts, heavy metals, trihalomethanes (THMs), and other contaminants outweighs the risk of infection for AIDS patients or other immunocompromised persons, posed by nontuberculous mycobacteria such as *M. avium* or other opportunistic pathogens such as *Pseudomonas aeruginosa* (cystic fibrosis) that may be found in the general heterotrophic bacterial population in nonpurified water. By and large, this concern narrows down to an issue of individual choice, but that choice needs to be based on education and an awareness of the potential risks involved in the use of nonpurified drinking water. Studies that specifically expanded on this particular issue were not found.

Based on the information presented in Tables 2 to 4, it is apparent that GAC and GAC-silver POU treatment units that do not incorporate some form of additional treatment to reduce or inactivate bacteria have generally been found to yield product water containing high numbers of bacteria. In many cases, the numbers of bacteria in the product water were several orders of magnitude greater than the bacterial levels in the water influent to the treatment units. Thus, from a bacteriological quality viewpoint, some types of GAC and GAC-silver POU water treatment units degrade the quality of the water treated although there may be aesthetic improvement based on taste and odor of the water or removal and reduction of other contaminants

Table 5. Persistence (Survival/Colonization) of Bacteria Used to Challenge POU GAC Treatment Devices

Organism	Challenge Dose CFU/Unit	Persistence Time	Reference
Aeromonas hydrophila	100–46,000	0–15 d	16
Campylobacter jejuni	1.17×10^6–3.72×10^9	0.5 d	16
Citrobacter fruendii	200–300	6–10 d	9,20
Enterobacter aerogenes	200–300	30–50 d	9,20
Ent. cloacae	200–300	<0.5 d[a]	9,20
Escherichia coli	200–300	<0.5 d	9,20
Escherichia coli	4,000,000	>17 d	21
Klebsiella pneumoniae	1,000	0–63 d	16
Legionella pneumophila	14,000,000	0.1–5.5 d	16
Mycobacterium avium	290,000	56 d	19
M. mucogenicum	48,000	<1 d	19
M. fortuitum	18,000	<1 d	19
Pseudomonas aeruginosa	200–300	150 d	9,16
Pseudomonas aeruginosa	4,000,000	>17 d	20
Salmonella typhimurium	200–300	<0.5 d	16
Serratia marcescens	200–300	30–150 d	20
Yersinia enterocolitica	Variable	0–0.5 d	16

Note: [a] <0.5 d indicates the organism was undetected in the first sample following the spike dose.

such as cysts or oocysts, inorganics (lead, arsenic, radium), or chlorinated organic disinfection by-products (THMs).

However, an observation in contrast to the negative perception of high bacterial levels in POU-GAC filters is the suggestion that high bacterial levels may be beneficial in preventing colonization of GAC filters by frank or opportunistic pathogens. This may be due to what has been termed *competitive inhibition* or *biocompetitive exclusion* caused either by inhibitory materials produced by the biofilm bacteria that act against pathogens, or simply the inability of the pathogens to compete for nutrients in the presence of a heterotrophic bacterial biofilm population, or both. The exclusion or inhibition of pathogens by resident biofilm bacteria has been shown or suggested by the results from several studies in which some pathogen challenges did not result in pathogen colonization of the filters or GAC that already had a biofilm population (Table 5) (9,16,21,22). The results from these studies and the lack of documented evidence of human illness caused by high HPC levels in drinking water form the basis for an argument that has been posed that high HPC levels in POU and POE treatment devices are beneficial in preventing colonization by pathogenic bacteria (23,24).

POU Water Purifiers

POU water purifiers pose a different concern and challenge than POU-GAC filter units because by definition, water purifiers are designed to be used to treat nonpotable water and must remove or inactivate all pathogens that might be present in the source water. Therefore, the microbiological concern focuses specifically on the performance of these treatment units for removing and inactivating microbial pathogens. Performance evaluation of water purifiers involves using a testing protocol such as that developed by the U.S. EPA (5) to test production models of a given water purifier over the manufacturer's claimed lifetime for the unit. The intention is to verify or establish that the purifier is capable of performing as claimed by the manufacturer for removal or inactivation of bacteria, viruses, and protozoan pathogens. The EPA protocol requires microbial removal or inactivation of: bacteria, *Klebsiella terrigena*, 99.9999% (6-$\log_{10}$); viruses, Poliovirus 1 (LSc) (ATCC-VR-59) and Rotavirus (Wa or SA-11) (ATCC-VR-899 or VR-2018), 99.99% (4-$\log_{10}$); and protozoa cysts, *Giardia muris* or *G. lamblia*, 99.9% (3-$\log_{10}$), or optionally for treatment units that use occlusion filtration, particles, or microspheres of 4–6 μm diameter. Because the protozoan parasite *Cryptosporidium parvum* has been shown to be a major agent of waterborne disease in the past few years, the testing protocol must be modified to test for removal of oocysts of this organism and a filtration device must have an effective pore size of 2 μm or less to remove the oocysts.

The effectiveness of several POU water purifiers for removing or inactivating bacteria, viruses, and protozoa is summarized in Table 6. All of the purifiers evaluated incorporated two or more treatment processes such as GAC pressed carbon block filtration and UV irradiation, GAC filtration and heating, candle filtration and GAC filtration and UV irradiation, or membrane filtration and iodine disinfection. Purifier testing for the studies cited in Table 6 was largely based on the U.S. EPA protocol (5).

Overall, removal or inactivation of microorganisms by the water purifiers was generally excellent when seeded water of qualities specified by the U.S. EPA protocol was used in the testing and met the protocol goals. Removal and inactivation results ranged as follows: viruses — from >99.99% to >99.99998%; bacteria — from 99.4% (heterotrophic bacteria) to >99.99999%; bacteriophages or coliphages — from >99.997% to >99.9999999%; cysts or oocysts — from 99.95% to >99.998%; yeast — from >99.9997%; fungal spores — >99.9994%; and algae — 99.99993%. Some of

the studies tested removal or inactivation of a variety of microorganisms other than those required by the U.S. EPA protocol, hence the values for heterotrophic bacteria, yeast, fungal spores, algae, and bacteriphage other than coliphage. Conversely, not all the studies tested for bacterial removal or inactivation using *Klebsiella terrigena*, the bacterial challenge organism specified in the U.S. EPA protocol. In the study by Grabow and coworkers (25) the purifier was also challenged with untreated river water and treated wastewater after the unit had been operated with seeded tap water (Table 6). With raw river water, although good removal or inactivation of heterotrophic bacteria (99.9998%) occurred, it would not have met the U.S. EPA protocol requirement for bacteria. Removal of seeded Poliovirus 1 (>99.9998%) would have met the U.S. EPA protocol requirement for virus removal or inactivation, but removal or inactivation of coliforms and somatic coliphages were only 99.97% and 99.92%, respectively. For treated wastewater, removal or inactivation of heterotrophic bacteria (99.99998%) and seeded Poliovirus 1 (99.9998%) would have met the U.S. EPA protocol requirements for bacteria and viruses, respectively, whereas total coliform removal or inactivation (>99.9998%) would not have met the U.S. EPA protocol requirement for bacteria. All the studies cited in Table 6, except those by Grabow and coworkers (25,28), tested the purifier units for 100% or greater, of the manufacturer's stated lifetime for the treatment unit. As a result, it would appear that the water purifiers functioned as claimed and produced water of potable quality throughout and beyond their ascribed lifetimes.

HEALTH EFFECTS RELATED TO HOME TREATMENT UNITS

As indicated earlier, concerns about high bacterial levels in the treated water from many POU home treatment devices are related to the potential for adverse health effects caused by ingestion of the bacteria, especially for those consumers whose immunological health status may be compromised. This includes the very young, the elderly, persons who are immunosuppressed by medical treatment because of organ transplant or cancer chemotherapy, and others who are immunocompromised because of diseases such as AIDS. Perhaps the more specific concern is whether high numbers of one or more specific opportunistic bacterial pathogens could colonize and dominate the bacterial population of a POU-GAC treatment unit so that the consumer would be exposed to a potentially infectious dose and subsequent illness. This concern prompted the experiments reported by Geldreich and coworkers (9), Reasoner and coworkers (16), and Rodgers and coworkers (19) in which POU-GAC or GAC-silver filters were challenged with specific bacterial strains to observe if they could colonize the treatment units. In fact, very few of the organisms were able to colonize and persist for any extended period (Table 5), and for those that did persist, the numbers recovered were generally low. However, the experiments did demonstrate that some of the challenge strains were able to colonize and persist in low numbers for substantial periods.

Very few studies have been conducted with the specific goal of attempting to demonstrate adverse health effects caused by ingestion of high numbers of heterotrophic bacteria in water from POU/POE home water treatment units. Two epidemiological studies conducted by Yale University researchers attempted to establish a relationship between consumption of high numbers of heterotrophic bacteria in water from POU GAC and POE filters and waterborne illness in consumers (32,33). Neither study was able to establish a direct link between ingestion of high numbers of HPC bacteria in drinking water and human illness. However, the Yale studies did not have large enough study populations to be definitive. Also, the study populations were composed primarily of "healthy" individuals or families and lacked proportional representation of consumers who may be at the greatest risk — the very young, the elderly, and other immunocompromised persons.

Results of epidemiological studies of waterborne illness conducted in Canada indicated that there was a significant level of endemic waterborne illness in consumers of municipal water that met current Safe Drinking Water Standards (34,35) of the United States and Canada. In the Payment and coworkers (34) study in a community that received tap water treated by a conventional water treatment plant, 300 RO water filtration units were installed in randomly chosen households. The rate of gastrointestinal illness (G.I.I.) in the households that had R.O. units was compared to the G.I.I. rate in a similar group that did not have R.O. water treatment. The results indicated that the households that had consumed R.O. treated tap water had a G.I.I. rate that was lower by about 35% than that of those who drank municipal tap water. Thus, the R.O. treatment units appeared to prevent a significant level of waterborne illnesses in all population subgroups within the study population.

Analysis of the microbiological data collected on the R.O. units showed that HPC levels in water from most of the units ranged from 1,000 to 100,000 CFU/ml, and weekly monitoring showed that the numbers were relatively stable (36). Further analysis showed significant univariate correlations between the HPC levels at both 20 °C and 35 °C and the G.I.I. rate, suggesting that bacteria growing in the R.O. units were responsible for an increase in the G.I.I. symptoms. However, there was no correlation between the G.I.I. rate and the amount of water consumed, which suggested that the statistical correlation with bacterial density could be spurious. A correlation was also found between the length of an illness episode and HPC counts at 35 °C, but not with HPC counts at 20 °C, suggesting that illness symptoms were more severe as the 35 °C HPC counts increased. Because there was no correlation between the amount of water consumed without further treatment (heated or used for cooking) and the HPC counts at 20 or 35 °C, the implication is that there were no negative aesthetic effects that caused a possible decrease in consumption by the study participants. The authors concluded that their data suggested that heterotrophic bacteria (35 °C plate count) that grew in R.O. treated drinking water were associated with a low incidence of gastrointestinal disease. It must be

Table 6. Effectiveness of POU Water Purifiers for Removal or Inactivation of Microorganisms

Treatment Unit[a] (Test Water)[b]	Test Organism	Unit Lifetime Gal/h/cycles(%)[c]	Influent Conc.	Effluent Conc.	% Removal	Reference
GAC Block/UV	Poliovirus 1	475gal(150)	24,000/mL	<1.10/mL	>99.995	26
(Seeded tap)	Hepatitis A virus	475gal(150)	36,000/mL	<1.10/mL	>99.997	
	Rotavirus	475gal(150)	22,000/mL	<1.10/mL	>99.995	
	Coliphage MS-2	475gal(150)	280,000,000/mL	<1.10/mL	>99.99999	
	Vibrio cholerae	475gal(150)	94,000/mL	0.1/mL	99.99989	
	Salmonella typhi	475gal(150)	320,000/mL	0.11/mL	99.99997	
	E. coli (EHEC)	475gal(150)	200,000/mL	<0.1/mL	>99.99995	
	Shigella dysenteriae	475gal(150)	570,000/mL	0.1/mL	99.99998	
	Heterotrophic bacteria	475gal(150)	17,000/mL	100/mL	99.4	
	Giardia lamblia cysts	475gal(150)	9,700/mL	<1.10/mL	>99.989	
	parvum oocysts	475gal(150)	20,000/mL	<1.10/mL	>99.995	
	Saccharomyces cerevisae	475gal(150)	37,000/mL	<0.1/mL	>99.9997	
	Aspergillus niger spores	”	18,000/mL	<0.1/mL	>99.9994	
	Synechococcus sp.	”	19,000/mL	.013/mL	99.99993	
GAC/Heat Cycle	Polio 1	240 (?)	12,500,000/L	<111	>99.998	27
(Seeded tap)	Rotavirus SA-11	240 (?)	10,300,000/L	<111	>99.999	
	Coliphage MS-2	240 (?)	11,900,000/L	<333	>99.997	
	Giardia muris	240 (?)	76,400/L	<20	>99.97	
FC/GAC/UV	*E. coli*	23 gal (?)	23,000/100 mL	5/100 mL	>99.99783	28
(Seeded tap)	*S. faecalis*	23 gal (?)	4,400,000/100 mL	16/100 mL	>99.99996	
	C. perfringens	23 gal (?)	100,000/100 mL	0	>99.999	
	Coliphage MS-2	23 gal (?)	16,000/100 mL	1/100 mL	99.99375	
	Coliphage V1	23 gal (?)	400,000/100 mL	0	>99.999	
	Poliovirus 1	23 gal (?)	5,000,000/100 mL	47/100 mL	99.99906	
	Hepatitis A virus	23 gal (?)	1,600,000/100 mL	0	>99.999	
	Adenovirus 40	23 gal (?)	250,000/100 mL	0	>99.999	
	Adenovirus 41	23 gal (?)	560,000/100 mL	0	>99.999	
	Rotavirus SA-11	23 gal (?)	170,000/100 mL	0	>99.999	
	Rotavirus HRV-3	23 gal (?)	27,000,000/100 mL	5/100 mL	99.99998	
GAC/UV	Poliovirus 1	750gal(150)	46,000pfu/mL	<1	>99.997	29
(Seeded tap)	Rotavirus SA-11	750gal(150)	14,000pfu/mL	<1	>99.992	
	Hepatitis A virus	750gal(150)	14,000pfu/mL	<1	>99.992	
	Coliphage MS-2	750gal(150)	170,000,000pfu/mL	<1	>99.9999999	
	Giardia lamblia cysts	750gal(150)	99,000/mL	<1	>99.990	
	parvum oocysts	750gal(150)	28,000/mL	<1/mL	>99.997	
	E. coli 0157 : H7	”	19,000/mL	<0.1/mL	>99.99994	
	Salmonella typhi	”	220,000,000/mL	<0.1/mL	99.99994	
	Vibrio cholerae	”	46,000/mL	0.1/mL	99.9997	
	Shigella dysenteriae	”	50,000/mL	<0.1/mL	>99.999998	
R.O./Iodine	Polio virus 1	500h(100)	8,300,000/L	<111	>99.997	30
Resin	Rotavirus SA-11	500 h (100)	8,300,000/L	<111	>99.997	
(Seeded)	*parvum* oocysts	500 h (100)	2,600,000/L	<33	>99.998	
	Klebsiella terrigena	500 h (100)	1,400,000,000/L	<3.3	>99.9999	
MF/Iodine	Polio virus 1	100gal (100)	36,700,000/L	175	>99.99	31
Resin	Rotavirus SA-11	100gal (100)	36,700,000/L	175	>99.99	
	>99.9998					
	fragilis phage B40-8	33.3gal(?)	40,000/100 mL	0	>99.998	
	Polio virus 1	33.3gal(?)	600,000/100/mL	0	>99.9998	
(Raw River Water)						
	Heterotrophic bacteria	3.7gal(?)	400,000/100 mL	1	99.9998	
	Total coliforms	3.7gal(?)	1,500/100 mL	0	99.97	
	Somatic coliphages	3.7gal(?)	1,300/100 mL	0	99.92	
	Poliovirus 1 (seeded)	3.7gal(?)	600,00/100 mL	0	>99.9998	25
(Treated Wastewater)						
	Heterotrophic bacteria	3.7gal(?)	4,000,000/100 mL	2	99.99998	
	Total coliforms	3.7gal(?)	470,000/100 mL	0	>99.9998	
	Somatic coliphages	3.7gal(?)	24,000/100 mL	0	>99.996	
	Poliovirus 1 (seeded)	3.7gal(?)	500,000/100 mL	0	>99.9998	

Note: [a]Type of treatment unit tested: GAC Block/UV = pressed activated carbon block filter followed by ultraviolet treatment; GAC/Heat Cycle = carbon filter plus heating to 88–94 °C; FC/GAC/UV = polypropylene candle prefilter followed by a GAC-silver filter followed by ultraviolet treatment; RO/Iodine = reverse osmosis membrane filtration followed by disinfection passage through an iodine (I_5) resin filter cartridge; MC/Iodine Resin = microfiltration followed by disinfection passage through an iodine resin bed filter.

[b]Seeded tap is used to indicate that the “Guide Standard and Protocol for Testing Microbiological Water Purifiers (U.S. EPA, 1987), or a modification of it, was used as the test procedure.

[c]Manufacturer’s stated lifetime of the treatment unit, or subunit, in gallons/liters, hours of use, or cycles, and () indicate the percentage of the lifetime the units were actually tested; (?) indicates lifetime not given in citation.

pointed out that there were no instances of illness in which G.I.I. was shown to be directly attributable to ingestion of high concentrations of HPC bacteria in the filtered water from an R.O. treatment unit. Additionally, when a redesigned epidemiological study was conducted (36) the correlation between HPC levels in R.O. treated water was not confirmed.

Concerns over ingestion of high bacterial levels in drinking water, either from municipal tap water or from home POU treatment devices, will probably continue although there have been no studies that could directly prove that the HPC bacteria do or do not cause illness in the consumer. Much of the concern is related to the perception of bacterial quality. Specifically, despite adverse news media reports concerning drinking water quality, people generally take it for granted that their drinking water is free of harmful impurities (including bacteria), whether it comes from the municipal supply or from a home treatment device that treats municipal tap water. They do not expect that there will be any bacteria in the water at all, and if they find out that there are bacteria in the water, albeit apparently harmless bacteria, they are likely to be perturbed. Their perception is that all bacteria are bad and should not be consumed. The likelihood is that for healthy, immunologically competent people, ingestion or other exposure to high numbers of heterotrophic bacteria may not pose a significant health risk. However, for immunologically compromised persons, exposure to high bacterial levels in drinking water may carry a significant risk of infection and disease. The size of the sensitive population (i.e., immunologically compromised population) in the United States is growing rapidly, and exposure to opportunistic pathogens in drinking water is becoming a matter of increasing concern. For this reason, POU home treatment units should be designed to minimize the potential for high bacterial levels in the product water.

DISCLAIMER/NOTICE

The views expressed in this article are those of the author and do not necessarily reflect the views and policies of the U.S. EPA. It has been subjected to the Agency's peer and administrative review and has been approved for publication as an EPA document.

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HOT DESERT SOIL MICROBIAL COMMUNITIES

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Microorganisms of hot desert soils carry out essential nutrient cycling and soil-building functions of soil communities despite significant environmental challenges, foremost among them being extreme desiccation. Deserts are variously described as extremely arid and arid environments that receive less than 60 to 100 mm and from 60 to 100 to 150 to 250 mm annual precipitation, respectively (1,2). Semiarid lands receive from 150 to 250 mm to 250 to 500 mm precipitation and may be subject to desertification, that is, loss of vegetative cover and productivity usually as a result of human activities (1,3). Moisture availability is generally considered to be the controlling factor for biological activities in desert systems, with the moisture input occurring as infrequent, discreet, unpredictable events (2). The majority of arid and semiarid environments occur between the latitudes of 25 and 35° (1) where high temperatures occur during at least part of the year, and thus the majority of deserts can be termed hot deserts (as contrasted with cold deserts such as the Antarctic dry valleys). Maximum surface soil temperatures of hot deserts may be as high as 80°C (4). Low available moisture as well as extreme heat limit primary production and restrict the plant communities to scattered "islands of fertility." This in turn limits the availability of plant-derived organic carbon for microbial metabolism. Unique features of desert soil communities that contrast with those of more mesic systems include generally low biomass, low diversity, spatially and temporally heterogeneous patterns of biomass and activity, and adaptations for survival of adverse environmental conditions that can include low-moisture availability, extreme heat, low nutrient availability, high pH, and high salinity. Though not necessarily extremophiles, desert soil microbes do exhibit extreme tolerance of adverse environmental conditions. Microorganisms are essential to ecosystem processes in deserts. They may also be important in reclamation of disturbed, desertified systems. This article deals with hot desert conditions that influence soil microorganisms, microbial abundance and diversity in desert soils, microbial adaptations to the extremes of desert soils, and practical applications of desert soil microbiology. Previous reviews of the topic include those of Skujins (5,6) and Kieft (7).

SOIL WATER POTENTIAL

Water is usually considered to be the single overriding controlling factor for biological activities in desert ecosystems (2,4–6,8) However, nitrogen is often the limiting nutrient for plant productivity during the brief periods when water is readily available (9,10). Water potential is a measure of free energy of water in a system, for example, soil, relative to a pure water reference (11). Water potential (ψ) is measured in pressure units and is related to water activity (a_w) of a system and to the relative humidity (rh) of an atmosphere in equilibrium with the water in a system according to the following:

$$\psi = \mathrm{RT}(V_w)^{-1} \ln a_w = RT(V_w)^{-1} \ln\left(\frac{\mathrm{rh}}{100}\right)$$

where

ψ = water potential (MPa)
R = gas constant (8.31×10^{-4} m^3 MPa mol^{-1} K^{-1})
T = temperature (K)
V_w = partial molal volume of water (1.8×10^{-5} m^3 mol^{-1})
a_w = water activity, and rh = relative humidity (%).

Matric water potential (ψ_m) and solute water potential (ψ_s) are the most significant components of total water potential in soil: $\psi_{total} = \psi_m + \psi_s$. Pure water has a water activity of 1 and a water potential of 0. As a porous medium such as soil loses water, ψ_m becomes increasingly negative; as solutes such as NaCl increase in concentration, ψ_s also becomes more negative. Matric and solute water potentials impose related but different stresses on microbial cells. Cytoplasmic water potentials must approximate extracellular water potentials to avoid excessive turgor pressure, plasmolysis, or plasmoptysis (cell explosion); however, some positive turgor pressure is required for cellular expansion during growth. Microbes respond to solute water potentials by accumulating intracellular compatible solutes, a response that has been well characterized in many different microorganisms (12–15). Responses to matric water stress are less well understood: cells may accumulate intracellular compatible solutes and/or passively lose water to the surrounding medium. Matric water potentials in desert soils can be as low as −150 MPa (16). In extremely saline soils, the solute water potential can approach −41 MPa as the NaCl concentration approaches saturation. Arid and semiarid soils with poor drainage are often salt affected.

Low water potential in a porous medium has the added effect of decreasing nutrient availability. As a soil loses water, the water films on the surfaces of soil particles become thinner and also discontinuous. This limits solute diffusion and also impedes microbial motility. Solute diffusion is reduced by a factor of approximately 2 in a typical soil as the matric water potential drops to −0.1 MPa (11). Bacterial motility is essentially nil at matric water potentials below approximately −0.1 MPa (17). Filamentous organisms (fungi, algae, cyanobacteria, and actinomycetes) may be able to overcome this limitation by extending filaments through air voids in a partially desiccated soil. Matric water potential affects nutrient limitation in nearly any unsaturated soil; however, the effects are especially severe in desert soils, where the duration of desiccation stress can be months or even years. In addition to the stresses imposed by extremely low absolute water potentials, rapid changes in water potential can induce extreme imbalances in water potential between the cytoplasm and the surrounding environment. Rapid increases in water potentials as desiccated soils are wetted can cause

loss of intracellular solutes and/or plasmoptysis (18). These rapid osmotic challenges may be attenuated by extracellular polysaccharide matrices that retard water loss (19). See the section on "Low water potential: salinity and desiccation" for a more complete discussion of microbial adaptations to water potential stress.

DESERT SOIL MICROBIAL ABUNDANCE

The numbers and activities of microorganisms in desert soils are generally lower than in soils of more mesic environments. Plate counts of aerobic, heterotrophic bacteria in various hot desert soils have been measured in a range of approximately 10^4 to 10^7 cfu g^{-1} (6,7). This is at least two orders of magnitude lower than what one would expect to culture from agricultural or forest soils. Bamforth (20) counted 1 to 7×10^8 bacterial cells per gram by direct microscopy in a Sonoran Desert soil in Arizona. This total count of bacteria is also considerably lower than what one would typically find in more mesic soils. As with any soil, the total number of bacteria exceeds the number of culturable heterotrophs by a factor of at least 100. This difference likely reflects the number of dead and dying cells and the presence of viable cells with metabolisms that are not suited to the plate-count medium. As with bacteria, the biomass of fungi in desert soils is generally less than in more mesic soils (6). Typical numbers of fungi range from 10^3 to 10^4 fungal propagules per gram of soil (7). Hyphal lengths, which may be a better estimate of fungal abundance, range from approximately 10 to 200 m g^{-1} soil (7). The ratios of fungal to bacterial biomass are typically greater in deserts than in nondesert soils. Soil fungi may be favored in low-moisture environments because of their desiccation-resistant spores and the ability of filamentous fungi to grow through air-filled soil pores. Actinomycetes are similarly favored by dry conditions. Numbers of protozoa can range from less than 100 cells g^{-1} in extremely dry deserts to approximately 10^4 cells g^{-1} in semiarid soils (21–23). Parker and coworkers (23) counted 2.5×10^4 amoebae, 4.9×10^3 flagellates, and 7×10^2 g^{-1} ciliates in a Chihuahuan Desert (New Mexico) soil. The most extreme, arid terrestrial environments may have even lower numbers of microorganisms. The Atacama Desert, which may be the driest area on Earth, has been estimated to have fewer than 10 culturable bacteria g^{-1} and may be devoid of eukaryotic microbes (24).

SPATIAL AND TEMPORAL DISTRIBUTIONS

The spatial distribution of microbes within desert soils tends not to be uniform, instead, microorganisms are concentrated within islands of fertility that immediately surround desert shrubs (5,16,25–31) and also areas of concentrated animal activity (32). The transition from grassland to desert shrubland is accompanied by an increase in spatial heterogeneity of soil resources as mineral nutrients are concentrated into patches that favor the establishment of desert shrubs (33,34). The soil beneath the canopies of these shrubs is then enriched in organic carbon from root exudates, material sloughed off from roots, and accumulated plant litter, resulting in higher microbial biomass and activities. Total organic carbon (TOC), microbial biomass carbon, basal respiration rates, and soil dehydrogenase activities were all greater in soil beneath creosotebush canopies than in surrounding bare soil at a Chihuahuan desert site (29). The ratios of biomass carbon to TOC and of respiration to biomass carbon (metabolic quotient) were also higher beneath shrub canopies than in bare soil, indicating a higher proportion of readily metabolizable carbon in the canopy soils. Similar patterns were observed in an *Atriplex* shrubland and a semiarid grassland site (16). The effects of desert plants on microbial abundance are especially pronounced in the rhizosphere. The ratio of the number of rhizosphere microbes to those in the surrounding bulk soil (R/S ratio) can be 700 or more in a desert, 100 times greater than in a nondesert soil (6,7). The localization of nutrients in the immediate vicinity of mammal mounds (35,36) can also stimulate microbial activities. Ayarbe and Kieft (32) measured greater TOC, microbial biomass carbon, basal respiration, biomass carbon/TOC ratios, and metabolic quotients in the soil of banner-tailed kangaroo rat mounds than in the surrounding soil. Dhillion and Zak (37) reported higher numbers of bacteria and greater fungal hyphal lengths in the soil of western cottontail burrows than in the surrounding soil at a site in western Texas.

Temporal variations in biomass and activity in deserts are driven primarily by moisture patterns. Temporal variations in plant primary productivity in deserts have been described as following a "pulse and reserve" model in which plant metabolism and growth are stimulated by precipitation. Later, as the soil dries, a portion of the plant biomass dies, but a reserve of viable biomass retains sufficient energy and nutrient reserves to become active when moisture again becomes available. The majority of desert soil microbial activity is heterotrophy based on plant-produced organic carbon, and so microbial patterns of activity are similar to those of the primary producers. Blooms of activity follow immediately after precipitation events; these are followed by periods of slow decline in activity and biomass as the soil dries (38,39). Microbial reserve biomass includes intracellular storage compounds (e.g., poly-β-hydroxybutyrate and glycogen in bacteria), compatible solutes (e.g., trehalose and sucrose; 15), and extracellular polysaccharides (EPS). As with spatial patterns, temporal variability in biomass and activity has also been found to be greater in a desert shrubland than in an adjacent grassland (29). Temporal variances in TOC, biomass carbon, basal respiration, nitrogen mineralization potential, and dehydrogenase activity were higher in a Chihuahuan desert creosotebush shrubland than in an adjacent grassland (29).

DIVERSITY OF DESERT SOIL MICROBES

Environments in which physical and chemical parameters exert a greater influence than biological interactions on biological processes are often considered to have lower species diversity (40–42). This generalization is

likely valid for deserts, especially if soil microbial diversity reflects plant diversity, as has been suggested for broad-scale comparisons among ecosystems (43). Nonetheless, desert soil microbial communities include a very large number of microbial types, as evidenced by traditional culture-based studies (44,45), culture-independent molecular approaches (44–46,62), metabolic activity studies (47–49), and membrane phospholipid fatty acid (PLFA) profiling (50,51). Microorganisms that are typically cultured from arid and semiarid soils are not especially different from those in more mesic environments; however, the frequency distributions may be more skewed toward desiccation-tolerant forms, such as spore-formers and other gram-positive bacteria along with fungi.

Bacteria

The most commonly cultured bacteria from desert soils are members of the genus *Arthrobacter* (52). Other commonly cultivated bacterial genera include *Corynebacterium*, *Pseudomonas*, *Acinetobacter*, *Bacillus*, *Proteus*, and *Micrococcus* (6,53). Endospore-forming bacteria, especially *Bacillus* spp., but also *Clostridium* spp., can comprise a significant proportion of culturable bacteria, approaching 50% in extremely arid soils (6). Actinomycetes are important constituents of the soil community, with actinomycete propagules (mainly spores) comprising as much as 50% of bacterial communities in desert soils (6). Common actinomycete genera include *Streptomyces*, *Micromonospora*, and *Thermoactinomyces* (6). Isolates belonging to the genus *Geodermatophilus* have most often been cultivated from the surfaces of desert rocks and soils (54). *Geodermatophilus obscuras* are dark-pigmented filaments and/or coccoid cells in aggregates; their phylogenetic association is in the Frankiaceae (54). *Geodermatophilus* has been found in abundance in decaying plant litter (55) and in rhizospheres (56) in deserts. Myxobacteria, which form desiccation-tolerant myxospores and microcysts, have also been cultured from arid soils. Examples include *Archianium*, *Cystobacter*, *Stigmatella*, *Polyangium*, and *Chondromyces* (57,58).

As in most natural environments, the majority of bacteria in desert soils are not culturable by current methods. When 16S rDNA-based molecular methods for characterizing communities are applied to desert soils, the microbial diversity is revealed to be far greater than indicated by methods that rely on culturing alone (44). In comparing bacteria diversity in arid soils using culture-based and culture-independent 16S rDNA sequencing, Dunbar and coworkers (44) cultured 34 phylotypes representing three bacterial divisions, whereas the culture-independent approach yielded 498 different phylotypes representing at least seven bacterial divisions. Some of the previously overlooked, uncultivated bacteria represent novel lineages and may carry out metabolic functions not previously known in desert soils (Table 1). Kuske and coworkers (45) found a variety of bacterial 16S rDNA sequences belonging to a monophyletic but diverse group of bacteria related to the genus *Acidobacterium* in two arid Arizona soils. In a further investigation, members of the *Acidobacterium* group were found to be widespread in a variety of terrestrial environments (59). Holmes and coworkers (2000) used 16S rDNA methods to identify another taxonomically distinct group of common arid soil bacteria that are rarely, if ever, cultivated. These are high G + C gram-positive bacteria related to the genus *Rubrobacter*, and they were found to make up 2.6 to 10.2% of bacterial clone libraries derived from arid Australian soil samples. Many more novel bacterial taxa will likely be unearthed as molecular, culture-independent approaches are applied more widely to hot desert soils.

Table 1. Novel Groups of Uncultured Desert Soil Microbes Evidenced by 16S rDNA Sequence Analysis

Microbial Group	Environment	Reference
	Archaea	
Soil Crenarchaeota, closely related to sequences reported by Bintrim and coworkers (60) and Jurgens and coworkers (61)	Chihuahuan Desert, New Mexico	Kieft, unpublished data
	Bacteria	
Acidobacterium group	Sunset Crater National Monument, Arizona; Dugway, Utah	45,59
High G + C gram-positive bacteria related to *Rubrobacter*	Sturt National Park, New South Wales, Australia	Holmes and coworkers (62)

Archaea

Archaea are also present in hot desert environments; however, desert Archaea have received relatively little study. Methanogens are present, but restricted as in other soils to strictly anoxic sites, such as the centers of organic-rich soil aggregates. Extraction, amplification, cloning, and sequencing of 16S rDNA using universal archaea primers has demonstrated the presence of as yet uncultured Crenarchaeota in soils (60,61). These soil Crenarchaeota form a phylogenetically distinct group, but their metabolic function is currently unknown. We have recently found 16S rDNA sequences with close homology to other soil Crenarchaeota in a Chihuahuan Desert soil in New Mexico (Kieft, unpublished data). We also have evidence that these desert soil Crenarchaeota can form cells small enough to pass through a 0.4-μm filter (Kieft, unpublished data). Efforts are under way to cultivate soil Chrenarchaeota. Increased knowledge of the biodiversity of Archaea in desert soils is needed.

Fungi

Fungi in desert soils are generally of the same genera as those of soils in moister climates. Commonly isolated fungi

from desert soils include *Aspergillus* spp., *Penicillium* spp., *Cladosporium* spp., *Alternaria* spp., *Chaetomium* spp., *Fusarium* spp., *Mucor* spp., *Helminthosporium* spp., *Curvularia* spp., and *Stemphylium* spp. (63). Fungal activity has been characterized as being limited to temporal and spatial "windows of opportunity" that allow favorable moisture conditions (10). At the soil surface and in litter layers, these windows of opportunity occur following precipitation events. Spatially, these windows of opportunity occur in the vicinity of plants and in burrows of desert animals. The spatial and temporal heterogeneity has been credited with generating an unusually high fungal diversity in desert systems (10). Much of the characterization of fungal biodiversity in desert soils has focused on habitats associated with plants and with animal activities.

The litter layers beneath the canopies of desert shrubs provide a more favorable habitat for fungi than the open soil between plants. Zak and coworkers (10) identified species of fungi in decaying creosote wood placed beneath the canopies of shrubs at Chihuahuan and Sonoran Desert sites. Dominant fungi were *Alternaria alternata*, *Coleophoma* sp., and *Fusarium acuminatum* at the Chihuahuan Desert site; the Sonoran site was dominated by the same three species plus *Phoma* sp. and two unidentified species. Arbuscular mycorrhizal (AM) fungi are important symbionts of many desert plants, and plants infected with mycorrhizae exhibit greater drought tolerance (37). These are mostly members of the Glomaceae, with the most common species being *Glomus fasciculatum*, *G. intraradices*, and *G. mosseae* (64). Dhillion and coworkers (65) surveyed plants in a coastal desert in Chile and found that 98% of the 38 plant species present formed AM associations. In a survey of North American and Namibian desert AM fungal communities, Stutz and coworkers (64) found 21 different species of AM fungi, with similarity in species composition between the North American and Namibian sites ranging from 54 to 79%. Mycorrhizal fungi can be critically important in restoration of disturbed desert systems (66,67) (see section on Plant-Microbe Symbioses in Reclamation of Disturbed Arid Ecosystems).

The caches of seeds in the mounds of kangaroo rats have been shown to provide a favorable habitat for growth of diverse microfungi (68–70). Seed caches may be liters in volume and conditions are generally moist (68,69). Herrera (70) identified 273 fungal species belonging to 37 genera in kangaroo rat food stores at a Chihuahuan Desert site. The most common genera included *Aspergillus*, *Fusarium*, *Mucor*, and *Penicillium*. It has been suggested that the kangaroo rats have a mutualistic relationship with the fungi, in which the mammals' behavior serves to inoculate stored seeds and to maintain favorable conditions in return for unknown benefit, for example, nutritional improvement of the seeds or protection from bacterial degradation (Hawkins, 1999). The burrows of small mammals also serve as stores of mycorrhizal spores (37).

Heterotrophic Protists (protozoa)

The majority of protozoa in desert soils are types that can form desiccation-resistant cysts, and they remain encysted except for brief periods of moisture availability (23). Flagellates and amoebae are favored in arid and semiarid soils; however, ciliates can also be found (20,22). Species richness of ciliates is generally less than 10 in desert soils, whereas species richness of ciliates in mesic soils is typically in the 10 to 50 range (22). Rodriguez-Zaragoza (71) found 40 species of naked amoebae belonging to 19 genera at a desert site in Mexico; *Acanthamoeba* and *Vahlkampfia* accounted for 12.5 and 15% of the species, respectively. Arid soil protozoa have been shown to increase following the increase in bacterial prey populations that generally occurs after rainfall (22,23,71).

Algae and Cyanobacteria

Desert phototrophs can contribute significantly to primary production, and in the case of the cyanobacteria, to nitrogen fixation. They can occur as desert crusts, as lichen symbionts, and within protective endolithic, chasmolithic, or sublithic habitats (72). As with other components of desert biota, the abundance and diversity of phototrophic microorganisms is dependent on moisture availability. Lichens generally occur in higher numbers in semiarid environments than in arid deserts; the hottest, driest deserts may be entirely devoid of lichens. Cyanobacterial crusts flourish during brief periods of moisture availability and survive intervening dry periods in a severely desiccated state. *Microcoleus vaginatus* is the most common of cyanobacterial crust species in the arid western United States; other common cyanobacteria include *Lyngbya*, *Phormidium*, *Nostoc*, *Oscillatoria*, and *Scytonema* (72). Desert *Chroococcidiopsis*, which inhabits rock environments, can be the sole photosynthetic organisms in extremely arid environments (73) and may be the most desiccation-tolerant of all the cyanobacteria (73–75). Cyanobacterial crusts are important in soil aggregation and stabilization; they are also extremely susceptible to physical disturbance. (See Desert Environments: Biological Soil Crusts, this Encyclopedia.)

PLFA Profiles for Microbial Diversity

Membrane PLFA profiles serve as microbial signatures for quantifying broad groups of organisms in a community (76,77). Steinberger and coworkers (50) quantified diversity over time at arid and semiarid sites in the Judean Desert (Israel) by monitoring changes in membrane phospholipid fatty acid profiles. Both biomass (measured by the total PLFAs) and diversity (measured by the number of different PLFAs and their concentrations) were greatest in the site with the highest average precipitation. Biomass was greatest at this site following precipitation; during the dry season, biomass declined, with gram-negative bacteria (evidenced by monounsaturated and hydroxy-substituted PLFAs) declining sharply relative to other microbial groups. At the more arid sites, there was either no significant change in biomass and PLFA profiles, or a slight increase in eukaryotes (shown by polyunsaturated PLFAs) during the dry period. The PLFA approach of monitoring and comparing microbial diversity could be put to more widespread use in desert soils.

Functional Diversity

Functional diversity of microbial communities has been quantified and compared in arid soils using the community-level physiological profile (CLPP) approach (47–49), which was pioneered by Garland and Mills (78,79). Functional diversity in this case is defined by the numbers of substrates that can be utilized by a microbial community and the rates at which each of these substrates is utilized (10). This has most often been quantified using BIOLOG gram-negative and gram-positive microtiter plates, each of which contains 95 different substrates plus iodonitrotetrazolium (INT) as a redox-sensitive dye indicator. The INT dye is initially colorless but is reduced to a purple-colored formazan when a substrate is oxidized. The number of different substrates used by the community is scored and the absorbance of the dye in each well is compared to the absorbance of a control well containing dye, but no substrate. Liu and coworkers (49) assessed functional diversity along an elevational gradient in the Chihuahuan Desert (New Mexico) as part of an experiment to test the effects of grazing, seasonal drought, and fire. Functional diversity was generally lowest in the summer drought and fire treatments.

Dobranic and Zak (48) adapted the CLPP approach for use with fungal communities by using a tetrazolium dye that can be reduced by fungal electron transport systems. They named the method FungiLog and applied it to fungal communities in decaying lechugilla leaves in Big Bend National Park (Texas). The method detected significant differences in fungal functional diversity along an elevational gradient. Fungal communities in the lower elevation sites utilized the fewest substrates, that is, they had the lowest substrate richness. Sites with the highest fungal functional diversity also had the highest rates of litter decomposition.

ADAPTATIONS TO DESERT SOIL CONDITIONS

Low Water Potential: Salinity and Desiccation

As discussed earlier, water is the major factor controlling biological activities in desert soils, and the best measure of water in soils is water potential, which includes both solute and matric water potential. Solute and matric water potentials can be measured on the same scales, but the effects of these two forms of water loss on microbial cells are fundamentally different (74,80). This is evidenced by the tolerance limits for growth and activities of microorganisms. Extremely halophilic microbes can grow in saturated salt solutions at solute water potentials of −41 MPa. Some fungi can grow in concentrated sugar solutions with solute water potentials as low as −70 MPa. In contrast, the lower matric water potential limits for growth and activity of even the most desiccation-tolerant microorganisms are much higher. *Arthrobacter*, which is extremely desiccation tolerant (81), has been reported to grow at matric water potentials as low as −17 MPa; the spore former *Bacillus subtilis* has a reported lower limit of −10 Mpa; the minimum for *Pseudomonas* is reported as −5.6 MPa, and other bacteria are reported to cease respiration at −5 MPa matric water potential (74 and references cited therein). Although growth is inhibited at −5 to −17 MPa, many desert soil microbes can survive much lower matric water potentials. Surface soils in deserts can dry to −100 to −150 MPa matric water potential (16) and still contain viable microorganisms. The duration of this severe matric water potential stress can be months or even years in the most arid deserts. The amount of water that can be lost from a microbial cell under matric stress is greater than the loss from a cell in the presence of a concentrated solute (74). Enzymatic activity within the cell is thought to cease when all of the free water is lost, leaving only water bound to cell constituents (74). Further air-drying can cause the remaining cell water to be insufficient to cover cytoplasmic macromolecules with even a monolayer of water molecules (74). Air-drying has the further effect of destabilizing macromolecules, exposing them to increased oxidative stress (74). Survival of low water potential and related stress requires multiple adaptations (Table 2).

Microbial adaptations to low solute water potentials have been well documented, whereas microbial adaptations to low matric water potentials, that is, desiccation, have received much less study and are not nearly as well understood (Table 2). The best-characterized microbial response to low water potential stress is the accumulation of intracellular compatible solutes, also known as osmolytes, to match cytoplasmic water potential to that of the surrounding medium (12–15,74,94). These compatible solutes are accumulated intracellularly through transport from the external environment or they can be synthesized de novo by the cells. Sodium ions are toxic to many intracellular processes, even those of the most extreme halophiles, and so all halophilic and halotolerant organisms exclude sodium from their cells and instead accumulate solutes that are compatible with cellular function. In bacteria, these compatible solutes include relatively soluble amino acids such as proline and glutamate, sugars such as trehalose, and also KCl. Cyanobacteria and other photosynthetic prokaryotes accumulate betaines, particularly glycine betaine (15,95). Many other microbes that are unable to synthesize glycine betaine are able to take it up from the environment and use it as a compatible solute (15). Extremely halophilic Archaea exclude Na^+ and accumulate K^+ (with Cl^- as the counterion) as the sole osmolyte, even in saturated salt solutions. Many eukaryotes tend to accumulate polyols such as glycerol. The organic compatible solutes are low molecular weight solutes that stabilize proteins by enabling them to be preferentially hydrated as the cell undergoes dehydration (74). The details of microbial production and accumulation of intracellular compatible solutes in response to solute stress have been elucidated for a large number of microorganisms, and these adaptations are of major importance to microorganisms in salt-affected soils (88). However, it is not clear to what extent intracellular compatible solutes play an essential role in microbial response to low matric water potentials in natural environments. Some desiccation-tolerant bacteria can accumulate large amounts of compatible solutes such as trehalose or sucrose (20% or more by weight, 15,74,96). It has also been demonstrated that if microorganisms have been induced

Table 2. Selected Microbial Adaptations to Desert Soils

Adaptation	Reference
Adaptations to low solute water potential (saline soils)	
Accumulation of intracellular compatible solutes, for example, proline, glutamate, glycine betaine, trehalose, KCl	12,13
Adaptations to low matric water potential (desiccation)	
Accumulation of intracellular compatible solutes, e.g., trehalose, sucrose	12,13,14,74
Protein and lipid stabilization by trehalose or sucrose	74
Membrane lipid modifications: for example, conversion of monoenoic fatty acids to cyclopropyl fatty acids and *cis*-to-*trans* conversion of monoenoic fatty acids	74,82,83
EPS capsules	84
Efficient DNA repair, evidenced by gamma radiation resistance	85,86
Scavenging enzymes, for example, catalase and superoxide dismutase, to detoxify reactive oxygen species	74
Specialized dormant structures, for example, endospores, akinetes, cysts, actinomycete spores, fungal spores, and so on	87
Filamentous growth forms for bridging air gaps in unsaturated soil	88
Adaptations to low nutrient availability	
Oligotrophy (high-affinity transport enzymes, metabolic versatility, carbon and energy storage products)	89,52
Slow rates of endogenous metabolism	90
Small size	91
Starvation proteins	92
Autochthony: metabolism of ubiquitous but recalcitrant substrates	93
Specialized dormant structures, for example, endospores, akinetes, cysts, actinomycete spores, fungal spores, and so on	87

to synthesize compatible solutes, especially trehalose, or when such solutes are made available extracellularly, then a higher proportion of these cells will survive desiccation (97–100). However, rapid dehydration may preclude compatible solute accumulation. As a porous medium, such as soil, becomes desiccated, the ability of microorganisms to acquire exogenous carbon and energy sources diminishes rapidly. In desert soils, the concentrations of organic substrates in the soil solution are generally quite low, anyway. Without access to new carbon and energy, cells in a drying soil may be unable to take up or to synthesize significant quantities of compatible solutes. The alternative is passive water loss, that is, plasmolysis, and this may be the normal state of vegetative microbial cells in a dry desert soil. Bacterial cells dried to 40 and 30% rh have been found to contain about 10 and 3% water by weight, respectively (74). Specialized, thick-walled, desiccation-tolerant cells, such as endospores, cysts, and cyanobacterial akinetes, have higher amounts of cytoplasmic water per gram dry weight (74). However, it should be noted that differentiation into these specialized cell types occurs during times of high water availability and in response to other environmental cues, such as nutrient depletion.

The stage of growth may also be a factor in desiccation tolerance of microorganisms. Stationary-phase bacteria and yeast have been shown to be more desiccation tolerant than exponential-phase cells (74,97,100). Several morphological and physiological changes that occur as a cell enters stationary phase may be responsible for enhanced desiccation survival. Smaller, coccoid cells may be more structurally stable. Changes in membrane phospholipid fatty acids, for example, conversion of unsaturated fatty acids to cyclopropyl forms, may be important in maintaining membrane integrity in the face of extended desiccation and also during the stress imposed by rapid rehydration (74,82). Desiccation of membrane lipids lowers the melting temperature, causing a transition from the liquid-crystalline phase to the gel phase (74,101). Synthesis of trehalose during onset of stationary phase in some cells may also be instrumental in survival of desiccation (15). A strong case has been made for trehalose as a nearly universal protectant against desiccation damage as well as other environmental stresses (15). Trehalose, and to a lesser extent sucrose, have been shown to stabilize proteins and membrane phospholipids during dehydration (15,74,102,103). One mechanism for this stabilization may be the tendency for trehalose to form an inert glass (74,101). (See also FREEZE DRYING: PRESERVATIONS OF MICROORGANISMS BY FREEZE-DRYING, this Encyclopedia.) Trehalose is produced by a variety of organisms, including heterotrophic bacteria, fungi, and cyanobacteria; it is synthesized in response to starvation, low oxygen conditions, heat shock, and various chemical stresses; and it is known to be produced during sporulation of fungi, actinomycetes, and slime molds (15,74,104). It has even been proposed that desiccation tolerance may be engineered into desiccation-sensitive microbes by genetic manipulation to introduce or to enhance production of trehalose or other compatible solutes (74). Billi and coworkers (86) significantly increased the desiccation tolerance of *Escherichia coli* by introducing the sucrose-6-phosphate synthase gene, thereby conferring the ability to synthesize sucrose. This approach or a similar one may be useful in enhancing the desiccation tolerance of environmentally useful microorganisms, for example, pollutant-degrading bacteria; however, accumulation of trehalose or sucrose alone may not be sufficient to confer the extreme desiccation tolerance required for survival in the driest deserts (100). The reverse approach, enhancing the pollutant-degradation potential of naturally desiccation-tolerant bacteria, is also under way (105) (see section on Pollutant Degradation in Deserts)

Microbes in unsaturated porous media, that is, soils and underlying vadose zones, have been shown to accumulate large amounts of EPS (80,84). The role of this EPS in desiccation tolerance is still not well understood. The capsule may act as a sponge to retain water, thereby slowing down or attenuating water loss from

the cell (19,106–108). As an encapsulated cell begins to dry, the EPS may even contain more water than the cytoplasm (74). By maintaining a higher matric water potential in the environment immediately surrounding the cell, the EPS may also prolong nutrient availability during soil desiccation by allowing greater solute diffusion (84). The EPS may act to retain compatible solutes that are lost from a cell during rapid rehydration; these can then be taken up and used again as the soil dries. The polysaccharides of the capsule may also serve as carbon and energy storage products and may protect cell walls from damage induced by shrinking and swelling (80).

It has been suggested that the well-characterized gamma irradiation resistance of the bacterium *Deinococcus radiodurans* is actually an adaptive response to desiccation in soils rather than to high radiation doses (85). The radiation resistance of *D. radiodurans* is attributable to its unique DNA repair mechanism. Each cell contains multiple copies of its genome and can recombine fragments of these copies into an intact, repaired version of the genome following damage induced by gamma irradiation (109,110). These extraordinary bacteria are able to survive much higher gamma radiation fluxes than occur naturally anywhere in the biosphere, and so presumably the unusual repair mechanism of this organism is an adaptation to a different environmental stress. *Deinococcus* has been isolated from soil, as well as other environments that are frequently dry (110,111). Desiccation has been used a means of selecting for radiation-resistant *Deinococcus* (112). Moreover, dehydration induces the same double-stranded breaks in DNA that are caused by gamma irradiation. In support of the hypothesis that radiation resistance in *Deinococcus* is an adaptation to desiccation, Mattimore and Battista (85) demonstrated that radiation-sensitive strains of *D. radiodurans* showed significantly lower survival of severe dehydration than a wild-type, radiation-resistant strain. Presumably, DNA repair of desiccation-induced damage occurs upon rehydration of the cell. Strains of extremely desiccation-tolerant cyanobacteria of the genus *Chroococcidiopsis* have also been shown to be relatively resistant to ionizing radiation (35 to 80% survival of 2.5 kGy gamma irradiation); the DNA repair mechanism that enables this radiation resistance is also likely an adaptation to extreme desiccation (86). Desiccation tolerance in other desert soil bacteria may prove to be mediated in part by efficient DNA repair mechanisms as well.

Energy Availability

Nearly all soils are considered to be low-nutrient environments in which energy availability, especially organic carbon for heterotrophs, is severely limiting to soil microorganisms (113,114). Even though organic carbon can be a significant component of the soil mass, much of it is in a stable, recalcitrant form. Soil organic carbon is often patchily distributed and, as discussed earlier, it becomes increasingly difficult for microorganisms to gain access to it as the soil dries. For these reasons, the soil environment selects for highly efficient organisms that are able to glean organic carbon from low nutrient sources (i.e., oligotrophic microbes), microbes that can survive for extended periods without exogenous energy sources, and autochthonous microbes that are specialized for utilization of ubiquitous but recalcitrant compounds. (See Oligotrophic Bacteria, this Encyclopedia.) Essentially, this describes three different (but overlapping) styles of *K*-selected microbes, that is, ones that have evolved to allocate a significant proportion of their resources to survival rather than to reproduction (115). In hot deserts where primary production is low and soil organic carbon is concomitantly low, the selective pressures favoring these types of organisms should be especially robust; and in fact, all three of these styles of *K*-selected microbes are well represented in desert soils.

Arthrobacter spp. have been described as the archetypical soil oligotrophs in that they have relatively low maximum specific growth rates, they are able to use a wide spectrum of organic substrates, they accumulate large amounts of reserve organic carbon during times of plenty, they form small coccoid cells as nutrients are depleted, and they decrease their rates of endogenous metabolism for long-term survival of nutrient deprivation (52,89). These traits undoubtedly contribute to the dominant role that Arthrobacters play in many arid soils and are likely shared by many other microbes in hot deserts.

The diminutive size of most soil bacteria, including those in desert environments, is consistent with the generally low availability of soil organic carbon (91). Using microscopic studies Bae and coworkers (115a) found as much as 72% of soil bacteria to be dwarfs, that is, coccoid cells with diameters less than 0.3 μm. The lower limit for cell size has not been firmly established, but it has been estimated at approximately 0.2 μm diameter (91). Bamforth (20) reported that the majority of bacteria in Sonoran Desert soils are cocci, and studies at a Chihuahuan Desert site have shown that a significant proportion of soil bacteria can pass through a 0.4-μm pore-size filter (Kieft and coworkers, unpublished data). Many of these small cells are undoubtedly dwarf forms of larger bacteria that have changed morphologically in response to nutrient deprivation. Dwarfing and also fragmentation (division without growth) have been well documented in laboratory-grown cultures that have been starved (91,116–118). Others appear to be intrinsically small cells, that is, ones that do not develop into larger forms in response to nutrient amendment (119–122). Dwarf bacteria have a high surface-to-volume ratio, which favors efficient uptake of nutrients when they become available along with minimal energetic cost for maintenance of cellular functions during starvation. Initial responses to starvation usually involve a brief increase in metabolic rate to provide energy for synthesis of starvation-specific proteins (123,124). After this initial burst, microbes that successfully survive nutrient starvation for extended periods are able to diminish their metabolism to glacially slow rates, thus using endogenous substrates in frugal manner. *Arthrobacter crystallopoietes* has been shown to decrease its rate of endogenous metabolism 80-fold during the first two days of starvation (90). In an experiment in which *A. crystallopoietes* was subjected to

the combined stresses of starvation and desiccation, 50% of cells remained viable after six months and the rate of endogenous metabolism was so slow that it could be predicted that 50% of cellular carbon would remain after 12 years (81).

Autochthony is another approach to low availability of organic carbon in desert soils, and it is exemplified in the actinomycetes and fungi, which are known for their abilities to degrade ubiquitous but relatively recalcitrant soil compounds such as cellulose, hemicellulose, and lignin. The lignin-degrading white-rot fungi are a good example. Other desert microbes are also adapted for metabolizing less palatable substrates. For example, in addition to degrading a range of sugars, organic acids, and amino acids, some strains of *Arthrobacter* have been found to degrade relatively recalcitrant substrates such as lignin, petroleum hydrocarbons, and 2,4-dichlorophenoxyacetic acid (52).

Temperature Stress

Temperatures at or near the surface of desert soils or on the surface of desert rocks may be severely challenging to the survival of microorganisms. Temperatures as high as 81°C have been measured at the surface of the Saharan Desert and surface temperatures in the Sonoran Desert in Arizona can exceed 70°C (4). Fortunately for the microbes, this extreme heat normally occurs only when cells are desiccated, a condition that generally increases heat tolerance. Desiccation stress may increase the heat stability of microorganisms and their macromolecules by inducing production of stress compounds such as trehalose (15). Many different types of organisms are known to respond to a rapid upshift in temperature by producing heat shock proteins (101). The heat shock response is also known to overlap with responses to other environmental challenges. For example, cellular adaptations for starvation survival may also confer heat tolerance (124a–124c). The heat-shock protein and molecular chaperone DnaK is involved in the regulation of expression of starvation genes in *E. coli* (124c). Similar mechanisms may function in heat tolerance of desert soil microbes. High soil temperatures inhibit legume–*Rhizobium* nitrogen-fixing associations (125). Thermophiles are rare in hot desert soils and are probably present only as incidentally transported from environments that feature hot water, for example, thermal springs.

PRACTICAL APPLICATIONS

Plant-Microbe Symbioses in Reclamation of Disturbed Arid Ecosystems

Arid ecosystems are especially susceptible to disturbances such as overgrazing, mining, and intensive agriculture. Disturbance effects are often augmented by drought conditions. Efforts to restore heavily disturbed, desertified sites to native vegetation can benefit from a consideration of plant–microbe interactions (3,37). As stated earlier, the majority of desert plants are mycorrhizal. Intense grazing and soil cultivation have both been shown to reduce the abundance and diversity of mycorrhizae (126). Strip-mining activities completely remove the plant community; physical and chemical disturbances of the soil can greatly diminish AM. The weedy annual plants that typically colonize these disturbed areas as the first stage of secondary succession are less likely to have mycorrhizal associations (37,66,126a,127,128). Progression of secondary succession beyond the weedy annual plant stage can be facilitated by inoculation with propagules of mycorrhizae that can associate with plants of the native, climax community. Inoculation with nitrogen-fixing *Rhizobium* along with mycorrhizae has potential benefit in establishment of desirable legumes (67,129–131). Mycorrhizal infection has been shown to improve nitrogen fixation by legume–*Rhizobium* associations under drought stress (125,131). Further understanding of plant–microbe interactions in desert environments may facilitate more rapid and successful reclamation of disturbed arid lands.

Pollutant Biodegradation in Deserts

As with other terrestrial environments, deserts have been subjected to contamination by hydrocarbons and other pollutants. Hydrocarbons have been introduced into desert soils through spills from petroleum production, refining, and storage sites. In the aftermath of the 1991 Persian Gulf War, nearly 50 km^2 of the Kuwaiti desert was contaminated with crude oil to depths of 50 cm to 2.5 m (132,133). Bioremediation offers the most feasible technology for cleaning up the soil. Hydrocarbons are also applied to soils in landfarming operations for biodegradation of waste oil and for bioremediation of soil that is heavily contaminated with hydrocarbons; these are often sited in arid and semiarid sites. Water, inorganic nutrient availability, and oxygen are the usual limiting factors in soil hydrocarbon degradation. Biostimulation (nutrient amendment) is the most widely applied bioremediation technique. Radwan and coworkers (132) reported that addition of water and nitrate was effective in increasing rates of biodegradation of *n*-alkanes in oil-contaminated Kuwaiti soil. The numbers of hydrocarbon-degrading bacteria in their experiment reached 10^{10} to 10^{11} hydrocarbon-degrading bacteria per gram of soil. Dominant genera were *Bacillus*, *Pseudomonas*, *Rhodococcus*, and *Streptomyces*. Bioaugmentation (inoculating with pollutant-transforming microbes) has rarely been shown to be effective in accelerating bioremediation of a natural environment. In a simulated oil spill experiment using Kuwaiti desert soil, Radwan and coworkers (133) found that a single strain of *Arthrobacter* dominated the hydrocarbon-degrading community. Inoculation with other strains of hydrocarbon-degrading *Arthrobacter* isolates had no effect on hydrocarbon degradation and, in some cases, caused a decrease in the native *Arthrobacter* population. Longer-term remediation of the polluted Kuwaiti desert has led to the reappearance of native plants, and the rhizospheres of these plants have been shown to contain abundant hydrocarbon-degrading bacteria, including *Cellulomonas flavigena*, *Rhodococcus erythropolis*, and an *Arthrobacter* sp. (134).

In landfarming operations, water and nutrients are added and the soil is tilled at intervals for aeration and

mixing. In a landfarming experiment using Kuwaiti soil contaminated with 2 to 3% and 6 to 8% hydrocarbons, 80 and 60% of hydrocarbons were eliminated, respectively, during a 15-month experiment (135). Treatment with nitrogen, phosphorus, and potassium fertilizers significantly increased the amounts of hydrocarbon degraded; bioaugmentation with hydrocarbon-degrading microbes increased the initial rates of hydrocarbon disappearance, but did not change the final percentage of hydrocarbon removal. Despite frequent irrigation, rates of degradation slowed during the hottest, months and increased during the winter rainy season. Residual hydrocarbons were high molecular weight aliphatic and aromatic compounds that are relatively resistant to biodegradation. This study illustrates the potential and also the limitations of landfarming in the desert. Indigenous soil microbes have the potential to degrade enormous amounts of petroleum hydrocarbons if sufficient water, oxygen, and nutrients are applied. However, the rates of degradation decline as readily degraded hydrocarbons are eliminated, leaving residual recalcitrant hydrocarbons that will likely remain for decades or longer. Landfarming can also contribute to air and groundwater pollution.

Bioaugmentation may be useful for remediation of soils contaminated with organic pollutants that are especially refractory to biodegradation. Weekers and coworkers (105) introduced genes encoding biodegradation of recalcitrant compounds (isopropylenebenzene, trichloroethene, 3-chlorobenzoate, 4-chlorobiphenyl, and biphenyl) into naturally desiccation-tolerant, hydrocarbon-degrading strains of *Rhodococcus erythropolis*. The resulting transconjugants retained the desiccation tolerance of the recipient strains and were also shown to express the new catabolic genes and grow on the recalcitrant carbon sources. This approach may be useful for generating biodegradative strains that combine native abilities to survive under the stressful conditions of a natural environment such as a hot, desert soil with catabolic capabilities that are tailored to a particular contaminant or suite of contaminants.

CONCLUSION

The microbial communities of hot desert soils remain poorly characterized relative to more mesic environments that are more economically important, such as agricultural and forest soils. This is unfortunate when one considers that 43% of the Earth's land surface is arid or semiarid and that more than 15% of the human population lives in desert environments (3). Much has been learned in the last decade about the diversity of microorganisms in desert soils, especially because of the availability of molecular-based, culture-independent tools such as 16S rDNA amplification and sequencing. Further application of these techniques to hot desert soils can greatly expand our understanding of biodiversity of extreme environments, especially if the metabolic functions of these previously unknown organisms are determined. Plant- and animal-associated habitats are likely sources of novel microbes. Improved understanding of the functional diversity should promote understanding of desert ecosystem function and may have practical applications for restoration of disturbed, desertified arid lands. Extreme environments continue to fascinate biologists. The means by which desert microorganisms survive the multiple abuses of dehydration, rehydration, heat, and starvation are beginning to be understood, especially for a few extraordinarily tolerant organisms such as *Arthrobacter*, *Deinococcus*, and *Chroococcidiopsis*. Lessons learned from these hardy organisms may be applicable to other desert microbes and may also engender genetic manipulations to generate environmentally useful, desiccation-tolerant microorganisms.

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HOT TUBS MICROBIOLOGY.

See SPA AND HOT TUB MICROBIOLOGY

HUMAN CALICIVIRUSES: BASIC VIROLOGY AND EPIDEMIOLOGY

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Diarrheal illness has undoubtedly afflicted the human species for centuries, and viruses are responsible for an appreciable percentage of diarrheal illness (1,2). Norwalk-like viruses (NLVs) are probably the most common etiologic agents of acute viral gastroenteritis in humans, and these viruses comprise a distinct genus within the family Caliciviridae. NLVs have a worldwide distribution and have been implicated in numerous common source (food or waterborne) outbreaks of gastroenteritis.

HISTORICAL BACKGROUND

Epidemic gastroenteritis with short-term, self-limited vomiting and diarrhea, and a high secondary attack rate was first described in the southern United States as a clinical syndrome in the late 1920s. The syndrome was referred to as winter vomiting disease or "hyperemesis hiemis" (3). Soon after, this syndrome was also described in the United Kingdom and elsewhere (4–6). Studies conducted from the 1940s to the 1970s repeatedly demonstrated the transmissibility of an infectious agent obtained from several outbreaks of epidemic nonbacterial gastroenteritis by serially feeding bacterial-free filtrates of outbreak stools to volunteers (7–15). However, a definitive agent was never identified for these outbreaks of nonbacterial gastroenteritis.

In October 1968, an outbreak of acute nonbacterial gastroenteritis struck an elementary school in Norwalk, Ohio. Over the course of two days, 50% of the students and teachers in the school developed classic symptoms

of epidemic vomiting disease. As in similar outbreaks, laboratory studies on outbreak specimens failed to identify a known etiologic agent. The unknown agent was shown to be transmissible to volunteers by feeding a bacterial-free filtrate from a rectal swab of a secondary case of gastroenteritis (14). Several years later, researchers discovered viruslike particles in stool material from the Norwalk outbreak using immune electron microscopy (IEM). The spherical particles appeared fuzzy with a diameter of 27 to 32 nm (16). The IEM technique was used to show a serologic response on the basis of immune aggregation of the 27 nm particles by paired sera from both volunteers, from a human volunteer feeding study and individuals who became ill during the original outbreak. This serological evidence and the virion shedding pattern suggested that the viral particles were the etiologic agents responsible for the Norwalk outbreak (16). The particles were initially considered to be parvovirus-like, on the basis of size, indistinct morphology, buoyant density, and stability in acid, heat, and ether (17,18). However, subsequent detection of a 59-kDa viral protein prompted reclassification of the Norwalk agent as a calicivirus (19). Recent genetic (nucleotide sequence and genomic organization) information supports this reclassification (20).

Subsequently, several other related or antigenically distinct 20 to 30 nm viruslike particles were identified as etiologic agents in various outbreaks of nonbacterial gastroenteritis and were named after the location of the outbreaks, including the Hawaii agent, Montgomery County agent, Ditchling agent, Marin County agent, and Snow Mountain agent, among others (21). Collectively, these agents were referred to as small round viruses, or 27-nm viruses, which led to the development of an interim classification scheme based on comparative morphology and physicochemical properties (22). This scheme recognized that small round viruses (SRVs) could be divided into two groups: the "featureless" SRVs without resolvable surface structure, including the parvo- and picorna-viruses, and a second group with distinct surface morphology, including astroviruses, classic caliciviruses, and particles with a "ragged" or "fuzzy" edge (referred to as SRSVs or small round structured viruses) exemplified by Norwalk virus. Figure 1 is a negatively stained direct electron micrograph of a NLVs. This scheme of classification was considered a temporary measure until more definitive criteria were developed. After several years of surveillance using IEM and later immunoassays, the SRSVs were found to be the most important cause of epidemic nonbacterial gastroenteritis (23–25).

GENERAL CHARACTERIZATION OF NLVs AND SAPPORO-LIKE VIRUSES (SLVs)

NLVs are nonenveloped, 27 nm by 32 nm spherical particles, which appear to have a ragged edge when viewed by electron microscopy. These particles comprise a single-stranded, positive-sense RNA genome surrounded by a protein capsid. NLVs have a buoyant density between 1.33 and 1.41 g/cm^3 (17,18). To date, no human NLVs have been effectively propagated in cell culture. Lack of a cell culture system has limited the study of the basic production and processing of viral proteins. However, the cloning of the NV genome has allowed the gene coding for the virion capsid to be cloned into various expression vectors that produced the capsid protein in culture (26–29). Capsid proteins produced using a baculovirus expression system self-assemble into viruslike particles (VLPs) that resemble empty virions and are slightly larger than actual viral particles (30–32).

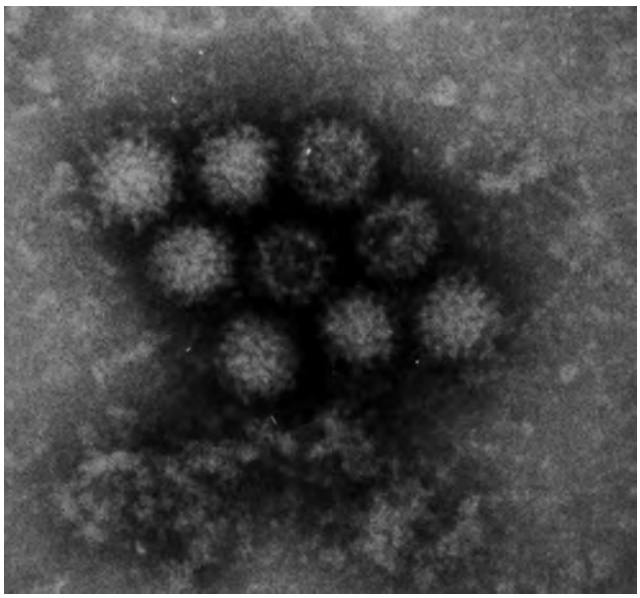

Figure 1. Negatively-stained, direct electron micrograph of a NLVs. (From Molecular Detection and Epidemiology of Human Caliciviruses by Jan Vinje, 1999.)

Other SRSVs with somewhat different surface morphology and later shown to be both serologically and genetically distinct from the NLVs were discovered. These viruses, referred to as Sapporo-like viruses or SLVs have a classical calicivirus morphology. When viewed under EM along their threefold axis, a "Star of David" pattern can be observed. The viral particles comprise a single-stranded, positive-sense RNA genome within a protein capsid. This type of virus was first observed in the feces using negative-staining EM techniques (33). SLVs have a diameter of 31 to 38 nm and their buoyant density is 1.37 to 1.38 g/cm^3 (34).

GENOMIC ORGANIZATION

The first cloned sequence of the NV genome was published in 1990, leading to rapid progress in genomic analysis of NLVs. The genome was shown to be positive-sense, single-stranded RNA approximately 7.5 kb in length, excluding 3′ terminal polyadenylation. Figure 2 illustrates the genomic organization of NLVs and SLVs. The genetic clones contained a consensus amino acid motif characteristic of RNA-dependent RNA polymerases (35). Since the original cloning of the NV genome, several other NLV sequences have been determined, including at least three full-length sequences (Norwalk, Southampton,

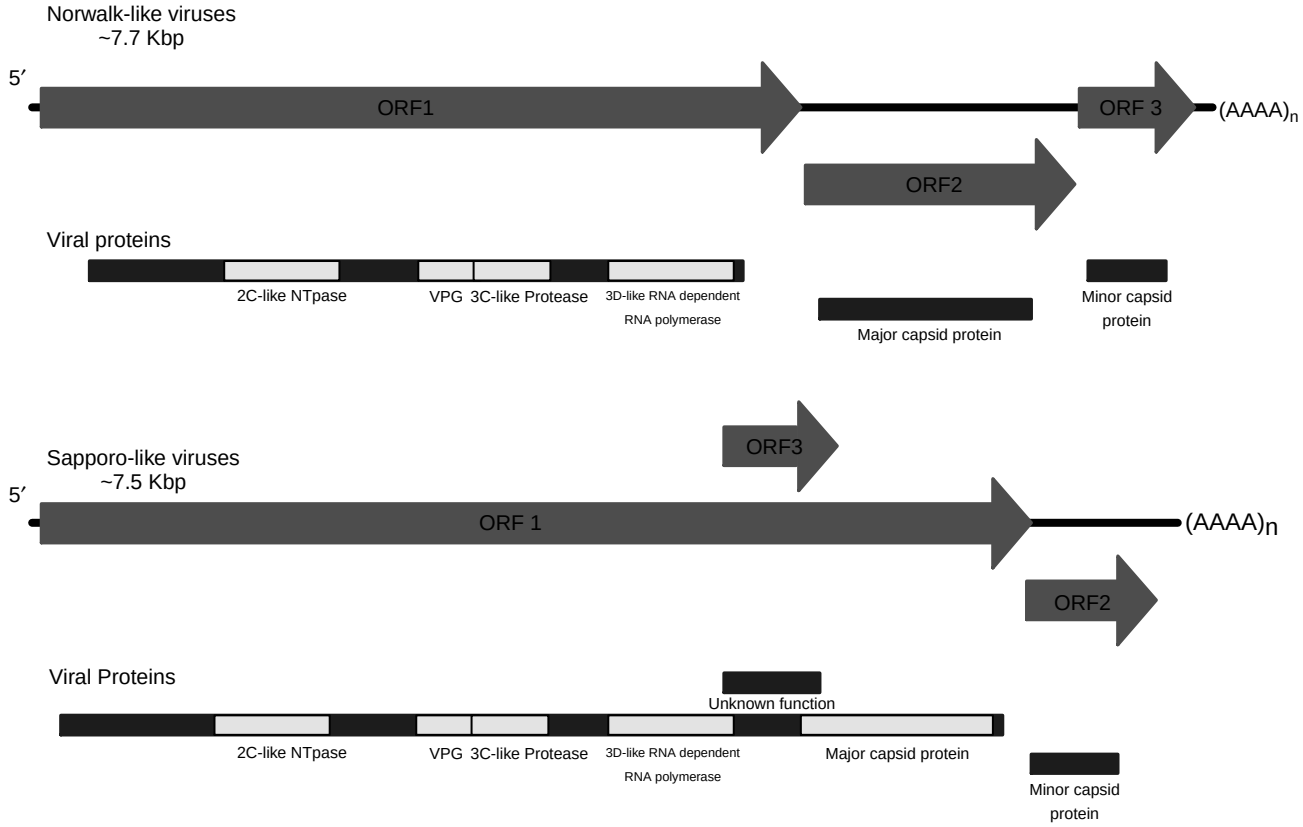

Figure 2. Comparison of the genomic organization of NLVs and SLVs. Open reading frames are indicated by red arrows, corresponding viral proteins are indicated in blue. Yellow highlighted regions represent individual proteins, presumably posttranslationally cleaved from the ORF1 polyprotein. See color insert.

and Lordsdale viruses) and numerous partial sequences, usually of the RNA polymerase region. At least two distinct genogroups have been described for NLVs (36). Genogroup I genomes (7.7 kb) appear to be slightly larger than those of genogroup II (7.5 kb). NLVs characteristically have three open reading frames (ORFs), and predicted ORFs are similar for both genogroups. ORF1 is the largest and codes for a large nonstructural polyprotein of approximately 1,700 amino acids that contains motifs characteristic of a picornaviral 2C putative helicase, 3C protease, and 3D RNA-dependent RNA polymerase. ORF2 codes for the major capsid structural protein (20). The small 3′ ORF3 codes for a basic protein recently shown to be a minor capsid protein (37). Differences in size between the viruses in the different genogroups is due to a smaller ORF1 in genogroup II. The 5′ region of ORF1 is highly variable and thought to reflect differences in secondary structure or regulatory signals between the two genogroups (20,30).

There is a frameshift between ORF1 and ORF2 in the NLV genome, so that the N-terminal amino acids of the capsid coding region overlaps the C-terminus of the RNA-dependent RNA polymerase region. Differences in the reading frame overlap between genogroups have been reported (38). Group I is reported to have a 17-nt overlap, whereas group II is reported to have a 20-nt overlap. ORF3 is also frameshifted in relation to ORF2, the first residues of the initiator codon for ORF3 overlap the terminator codon for ORF2 (38).

Production of a 3′ co-terminal polyadenylated subgenomic RNA containing ORF2 and ORF3 has been documented in animal caliciviruses (rabbit hemorrhagic disease virus, RHDV) and is thought to provide additional message for production of the capsid protein (39). Characteristically, the 5′ end of the animal calicivirus genomic RNA and subgenomic RNA are highly conserved (i.e., the 5′ terminal sequence of ORF1 is repeated at the 5′ end of ORF2) (40). Similar conserved sequences in the genomic termini of both genogroups of NLVs and the detection of RNA over 2 kb in the stool of an NV-infected volunteer by Northern hybridization suggest that the NLVs also produce a subgenomic RNA (20,41,42). However, the evidence for this remains tentative because of the lack of a cell culture system for viral replication or an in vitro translation system in which to study NLV replication events.

The genomic organization of SLVs differs from the NLVs and is more similar to animal caliciviruses. The single-stranded RNA genome is approximately 7.5 kb with 3 predicted ORFs and a polyadenylated 3′ tail. ORF1 occupies more than 90% of the genome and encodes the nonstructural proteins (2C putative helicase, 3C protease, and RNA-dependent RNA polymerase), and the major 62-kDa capsid protein (43). ORF2 is a small 3′ terminal ORF,

which is predicted to encode a 165 amino acid protein of unknown function. ORF2 in the SLVs has an analog in all other sequenced caliciviruses and corresponds to ORF3 in NLVs that has been recently shown to code for a minor capsid protein (37). Another small ORF in the SLVs is predicted to overlap the capsid coding region and encode a protein of 161 amino acids. ORF3 in the SLVs has no corresponding ORF in the NLVs (43,44). In other caliciviruses conserved sequences on the 5′ genomic terminus and the 5′ end of the capsid coding region are observed, whereas in the SLVs the level of similarity is not as great as in the NLVs or animal caliciviruses. In the animal caliciviruses these conserved regions are thought to be important in the regulation of subgenomic RNAs (43). As with the NLVs, no cell culture system is currently available for the SLVs. Consequently, production of subgenomic RNAs in SLVs cannot be confirmed. Comparison of the genomic organization of the NLVs and SLVs is illustrated in Figure 1.

Viral Proteins

Capsid Protein. The 59-kDa structural protein of NV was identified using radioimmunoprecipitation of CsCl gradient purified virions with paired (acute and convalescent) sera from infected individuals (19). The Snow Mountain (genotype II) capsid protein was found to be slightly larger, 62 kDa (45). The major capsid protein of Sapporo virus was also found to be 62 kDa (34). Computer analysis of ORF2 of several other strains is consistent with production of a single protein of approximately 60 kDa. In addition to the single capsid protein, a smaller 30-kDa protein is detectable in the feces of infected individuals (19). In baculovirus systems expressing NV capsid proteins, both 58-kDa and 34-kDa proteins were observed in the cell supernatants (27). Trypsin digest studies of baculovirus-expressed NV capsid proteins resulted in a trypsin-specific cleavage at the amino acid residue 227. The resulting cleaved protein was demonstrated to have the same size and amino acid sequence as the smaller soluble protein isolated from cell supernatants. It was also demonstrated that only the soluble 58-kDa protein and not the self-assembled recombinant Norwalk virus particles were subject to trypsin cleavage. Further, it was demonstrated that the 30-kDa protein had an identical N-terminal amino acid sequence as the 32 kDa trypsin cleavage product, thus suggesting that the soluble NV capsid proteins are subject to proteolytic cleavage in vivo (46). In another study, Japanese scientists detected NLVs in 14 related fecal isolates. In 11 of the isolates a structural protein of 63 kDa was isolated and in the other 3, a 33-kDa protein was isolated. Proteolytic and denaturing experiments failed to establish that the smaller protein was a subunit or cleavage product of the 63-kDa protein. However, the two proteins did appear to be antigenically related (47).

Studies have shown that baculovirus recombinant expression of either ORF2 alone, or ORF2 and ORF3 together, may form recombinant virus like particles (rVLPs), thus ORF3 is not necessary for formation of empty virus particles (27,48–50). However, recent studies have indicated that multiple forms of the protein expressed by ORF3, in particular, 23-kDa and 35-kDa forms, are detectable in both native NV virions and in baculovirus expression systems (37). These studies have also established through association with the formation of recombinant virus like particles (rVLPs) that the 23-kDa form of the ORF3 protein is a minor structural protein. Further, the 35-kDa form of the protein has been shown to be phosphorylated, unlike the 23-kDa form. These findings are consistent with studies on RHDV, which indicate that the equivalent protein in animal caliciviruses is a minor structural protein (51). The ORF3 protein is hypothesized to be located in the interior of the virion and to function in the encapsidation of the viral RNA (37).

Using the baculovirus-expressed capsid proteins that self-assemble into virus like particles (VLPs) resembling empty virions, the empty virion structure has been determined to a resolution of 2.2 nm by cryoelectron microscopy and computer image analysis (52). The capsids exhibit $T = 3$ icosahedral symmetry with a average diameter of 38 nm. The capsid is composed of 90 arch like capsomeres that are dimers of a single capsid protein. Deep depressions at the three- and fivefold axes result in 32 hollows surrounded by protruding arches on the surface of the capsid. Twelve of the surface hollows exhibit a small hump in the center and are located on the fivefold axis. The remaining 20 surface hollows are located on the threefold axis and appear flat. A similar architecture has also been described for a primate calicivirus (53). Further, the amino acid sequence of the capsid protein may be organized into distinct domains: (*1*) an N-terminal of 250 residues forming an 8-strand barrel in the lower shell and (*2*) C-terminal forming protruding domains. The C-terminal domains exhibit significant variation in sequence between NLVs (53).

Classic calicivirus particles, as determined by electron cryomicroscopy and computer imaging techniques on a primate calicivirus, have $T = 3$ icosahedral symmetry with 32 surface depressions at the fivefold and threefold axes. Like the NLVs, the surface depressions are surrounded by 90 archlike dimers of capsid proteins at the twofold axis (53).

Nonstructural Proteins. ORF1 of the NLVs encodes a large nonstructural polyprotein. Computer analysis of ORF1 sequence data has identified conserved amino acid motifs typical of a picornaviral 2C putative helicase, 3C protease, and 3D RNA-dependent RNA polymerase, as previously noted. Presumably the NLV polyprotein is cleaved by proteolytic enzymes analogous to cleavages of the nonstructural polyproteins of the *Picornaviridae*. Several recent studies have identified proteolytic cleavage sites in NLVs (54–56). Activity of the 3C-like protease has been demonstrated in bacterial expression studies (54,56). RNA-dependent RNA polymerase activity has been demonstrated in caliciviruses using bacterially expressed 3D-like polymerase from RHDV (57). A recent study expressing polypeptide p41, containing the motifs similar to the picornaviral 2C helicase, found no detectable helicase activity. Polypeptide p41 was demonstrated to have nucleoside triphosphate binding and hydrolysis

activities similar to the 2C protein in Poliovirus (58). There is evidence that a protein similar to the picornaviral Vpg protein is also encoded in ORF1 (55). Some animal caliciviruses have also been shown to have a Vpg-like protein associated with their genomic and subgenomic RNAs, which may or may not be required for RNA infectivity (51,59–62).

Original NV sequence data indicated that translation of ORF1 began at one of seven internal, in-frame initiator codons, three of which were favorable (20). The observed size of the ORF1 polyprotein translated from synthetic, full-length RNAs was consistent with an internal initiation site for translation at the fifth AUG. However, sequence analysis did not indicate a oligopyrimidine tract analogous to the internal ribosomal entry site of the enteroviruses, and comparison of group I and II sequences revealed extreme diversity on the 5′ proximal end of the genomes. Subsequently, an additional 12 nucleotides were found to be missing from the 5′ end of the NV and Southampton virus genomes (41,63). Within these 12 nucleotides are three in-frame initiation codons, the third of which is favored under Kozak's rules (63,64). Further, alignment of 26 bases at the 5′ end of the genome shares 88% homology with sequences at the start of the capsid coding sequence of ORF2 (63). The conserved sequences at the beginning of ORF1 and ORF2, including the presence of a favored initiation codon, suggests that NLVs begin translation nearer the 5′ terminus rather than internally.

TAXONOMY OF HUMAN AND OTHER CALICIVIRIDAE

Although originally described as parvovirus-like, on the basis of biophysical and biochemical properties, NLVs are now more appropriately classified as members of the family *Caliciviridae*. On the basis of phylogenetic analysis of nucleotide sequence data, the family *Caliciviridae* is currently composed of four genera: Lagoviruses, Vesiviruses, SLVs, and NLVs. The genus names Sapporovirus and Norwalkvirus were rejected by the International Committee on the Taxonomy of Viruses (ICTV) as it was inconsistent with ICTV nomenclature rules. Thus SLVs and NLVs were selected as provisional names until more appropriate names can be agreed upon (65). Phylogenetic analysis of the RNA-dependent RNA polymerase of the Caliciviridae indicates that SLVs are most closely related to Vesiviruses and that SLVs and NLVs are more distantly related to Lagoviruses.

Currently only two species of human calicivirus are recognized by ICTV, Norwalk virus in the NLVs genus and Sapporo Virus in the SLVs genus, although a recently isolated Vesivirus (SMSV-5) has been demonstrated to cause pathogenic lesions in humans (65,66). Both NLVs and SLVs cause gastrointestinal illness in humans. NLVs are typically associated with illness in older children and adults, whereas SLVs generally cause pediatric illness.

Historically, the term *SRSV* was routinely used interchangeably with NLVs, and the convention for naming NLVs was based on the geographic location of the outbreak. The sheer number of NLV strains, along with antigenic and genetic diversity among strains, made communication between researchers confusing. Recently, a nomenclature framework has been suggested, on the basis of the most recent decisions of the international committee on taxonomy of viruses (ICTV) (65). Under an earlier version of this framework, it was suggested that candidate human caliciviruses identified by EM should be classified as SRSVs until the virus was genetically shown to be a calicivirus, at which time it would be reclassified as a Human Calicivirus (HuCV) (67). The current cryptogram designation for a particular virus would appear as follows: Host/Genus/Species/Virus Name/Year Isolated/Country Isolated (65). For example, the framework nomenclature for Norwalk virus (NV) is Hu/NLV/NV/68/US. It has also been suggested that the cryptogram should evolve to reflect future decisions by ICTV and consensus decisions by calicivirus researchers, for example, subspecies designations and genogroupings (65).

Genogrouping and Antigenic Typing

Phylogenetic analysis of the genetic sequences of NLV strains supports their division into distinct genogroups (36,68,69). Within a genogroup, viruses share 92 to 98% amino acid homology in the polymerase region, and 77 to 98% homology in the capsid region. Nucleotide homology in the polymerase region is 72 to 88% (67). Originally two genogroups were described for the NLVs, and the SLVs were considered to comprise a third genogroup (36,70). Between genogroups I and II, nucleotide homology of the polymerase region is approximately 60%, and amino acid homology is 76 to78% (67). More recent phylogenetic analysis of NLVs and SLVs defines specific genetic clusters within the genogroups. According to a recent study analyzing the three ORFs of 31 strains of NLVs, at least 12 clusters of NLV were identifiable within two distinct genogroups, including 7 clusters in genogroup I and 5 clusters in genogroup II. A thirteenth cluster was suggested by the analysis of the ORF2 sequences (71). Another recent study analyzing the genetic sequence of ORF2 of over 100 strains found five clusters within genogroup I and 10 within genogroup II (72). Prototype strains of the genetic clusters within genogroup I include Norwalk, Southampton, Desert Storm, and Cruise Ship viruses. Genogroup II prototype strains include Snow Mountain, Bristol, Hawaii, Toronto, White River, and Gwynedd viruses. With the formal designation of NLVs and SLVs as distinct genera, it is no longer appropriate to refer to the SLVs as genogroup III. Further, it has been suggested that the SLVs should be divided into three genogroups. Genogroup I of the SLVs includes the prototype Sapporo virus and the Manchester virus. Genogroup II includes London 92 strain, and genogroup III includes the Parkville virus (73).

Until recently, the only viruses described for the NLV and SLV genera were human pathogens. However, animal viruses have recently been isolated that are closely genetically related to each genera (74–78). Recent sequence data from porcine enteric viruses have identified strains closely related to the genogroup II NLVs and the SLVs (74,77,78). Similarly, bovine enteric caliciviruses

have been isolated that are related to the genogroup I of the NLVs (Newbury agents and Jena virus) (75,76). However, phylogenetic analysis suggests that the Jena and Newbury viruses may comprise a distinct genogroup within the NLVs (72).

Early cross-challenge infectivity experiments in human volunteers and comparative serological response studies to different NLVs demonstrated that these viruses are antigenically diverse (18,79,80). As many as 14 distinct antigenic types have been suggested. Although several attempts have been made to separate the NLVs into antigenic types, there is currently no universally accepted scheme for antigenic typing of human caliciviruses. In the United Kingdom, at least four distinct antigenic types are recognized by IEM or solid-phase IEM (SPIEM) (81–83). At least six serotypes, including the four previously recognized in the United Kingdom, have been recognized in the United States (84). Nine antigenic types have been described in Japan by IEM (85). In contrast, a recent study using a panel of monoclonal antibodies identified a possible common epitope among genogroup I NLVs, but not genogroup II NLVs (86). The epitope was found to be present in the soluble 32-kDa cleavage product and was mapped to the C-terminal of the capsid protein (86,87).

METHODS OF DETECTION

Cell Culture and Animal Models

Although several animal caliciviruses have been propagated in cell cultures, human caliciviruses (NLVs and SLVs) to this point have not been propagated in this way (31,88–91). Aside from a single 1984 report of abortive replication of a candidate human calicivirus (SLV) in human embryonic kidney (HEK) cells using a trypsin containing medium, all attempts at growth in cell culture have failed (67,92). Similar unsuccessful results have been reported for studies attempting to identify a suitable animal model for NLVs. Studies attempting to induce illness in several animal types have largely been unsuccessful (21). Animals found to be inadequate models include: mice, guinea pigs, pigs, rabbits, kittens, calves, and various primates. However, studies using chimpanzees have demonstrated a serologic response and virus antigen excretion, although no apparent illness was observed (93).

Electron Microscopy

Immune electron microscopy (IEM) uses convalescent sera from an infected individual to aggregate viral particles, thus demonstrating a specific antigen-antibody reaction and also improving the detection of low concentration of virus particles. Additionally, this technique may employ paired (acute phase and convalescent) sera to identify which antigenic particles have generated an immune response (16). Despite the development of ultrasensitive molecular detection methods, EM is still the gold standard of most clinical diagnostic virology laboratories for detection of NLVs in stool specimens. Shortcomings of EM include availability of immune reagents, high detection limit, and the variable morphology of the viruses depending on preparation methods, orientation, and structural integrity.

Immunological Methods

Several immunological methods have been developed for the detection of NLVs and SLVs and diagnosis of their infections, including radio-immunoassays (RIA), enzyme immunoassays (EIA), enzyme-linked immune serum assays (also called enzyme-linked immunosorbent assay or ELISA), and immune adherence assays (IAHA) (32,94,95). IAHA is impractical for the detection of NLVs because it requires a greater quantity of purified antigen than other immunologically based methods.

First-generation immunologic detection methods were reliant on the convalescent sera of infected individuals, and therefore not practical for widespread study of NLV epidemiology. However, recent expression of rVLPs using a baculovirus expression system has allowed the production of a ready supply of hyperimmune sera to antigenically diverse NLVs. This, in turn, has led to the development of sensitive enzyme immunoassays and ELISAs (50,96–99). The sensitivity of the assays may be as little as 25 pg of capsid protein. These sensitive detection techniques have been invaluable in determining the distribution and prevalence of NLVs (96).

Molecular Methods

In the early 1990s, sensitive molecular methods based on reverse-transcription (RT) and the polymerase chain reaction (PCR) were first developed for the prototype strain, Norwalk virus (100,101). Since then, many advances have been made in RNA extraction techniques and optimization of RT-PCR protocols. These methods will be reviewed in detail elsewhere in this Encyclopedia. Briefly, improved primers have been developed to detect a wider range of NLVs, and RNA extraction techniques have been found to increase sensitivity and eliminate RT-PCR inhibition (102,103). However, a study also reported that heat release of viral nucleic acid before RT-PCR was as effective as chemical extraction procedures for sensitive RT-PCR detection (104). An internal RNA standard for RT-PCR coamplification has been proven to be effective in detecting inhibitors present in samples (104).

CLINICAL SYMPTOMS AND DIAGNOSIS

Clinical Presentation

The clinical characteristics of NLV infection have been established from volunteer studies and epidemiologic data (105). Symptoms of illness for different NLVs (Norwalk, Hawaii, Montgomery, and Snow Mountain) used in volunteer feeding studies were clinically indistinguishable (80,105,106). The incubation period of the disease is dose dependent and ranges from 18 to 48 hours, with an average time of 24 hours (38,105). Onset of symptoms is acute. The most common symptoms include nausea, vomiting, and diarrhea. Other symptoms may include headache, malaise, chills, cramping, and abdominal pain. Stools typically do not contain blood or mucous. Vomiting may be

projectile and may be a result of delayed gastric emptying (107). As many as one-third of the infections may be asymptomatic (98).

NLV infection is self-limited and rarely requires hospitalization. The common duration of illness is from one to three days. However, severe cases of dehydration resulting from NLV infection and requiring intravenous fluid replacement have been documented. Mortality from NLV is extremely rare. Deaths, although rare, are usually attributable to a predisposed condition or concurrent illness.

SLV infection is characterized by an incubation period of one to three days, followed by expression of symptoms for an average of four days (108). Symptoms of diarrhea and vomiting are common, with fever and respiratory symptoms being possible, but less likely. Subclinical infection appears to be common. In contrast to NLVs, SLVs primarily affect young children, but outbreaks in adults have been described (109). Transmission is primarily through person to person spread by the fecal-oral route, although contaminated food and water have been implicated in outbreaks (70).

Diagnosis

Presumptive diagnosis of NLV infection may be made if four criteria are met: a bacterial or parasitic agent is not identifiable, vomiting occurs in approximately half the cases, the average duration of illness is 12 to 60 hours, and the incubation period is one to two days (110). Definitive diagnosis requires isolation of NLV particles from stool samples and identifiable serologic response. However, more recent epidemiological studies of common source outbreaks, such as water- or food-borne, have relied on virus detection by RT-PCR in ill persons and in the incriminated vehicle of exposure.

Shedding

NLVs are shed typically at an approximately 10^6 virus particles per gram of feces, although shedding of up to 10^9 particles per gram of feces has been reported. Virus shedding may begin as soon as 15 hours after inoculation, although peak shedding occurs between 25 and 72 hours, corresponding with the period of clinical illness (98). Further viral shedding may persist once symptoms have ceased, up to seven days after inoculation (98,111). One account documents NV shedding two weeks post challenge (112). Shedding will also occur during asymptomatic infection (98).

PATHOGENESIS

Volunteer feeding studies have determined that the NLV dose necessary to initiate infection in an individual may be as low as 10 to 100 particles, perhaps lower (21,113). One study examining Norwalk virus dose-response in humans found 70% of individuals developed infection, when exposed to a dose of 6×10^3 RT-PCR detectable units. A dose of 6 RT-PCR detectable units was demonstrated to cause infection in 20% of individuals exposed (113). Although not detected in the cells, viral replication is presumed to occur in the mucosal epithelial cells. Biopsies of the proximal small intestine (jejunum) from infected volunteers show intact mucosa with blunting and shortening of the villi (106,114,115). Other characteristic pathology seen in individuals infected with Norwalk virus include disorganization of the epithelial cells, vacuolization of the cytoplasm, and mononuclear infiltration of the lamina propria. Hyperplasia of crypt cells was also common. Dilation of both the smooth and rough endoplasmic reticulum and swollen mitochondria have also been documented at the ultrastructural level (106,114,115). Additionally, alkaline phosphatase and trehalase activity was found to be decreased. Transient maladsorption of D-xylose, fat, and lactose was also observed during acute infection (114). The characteristic histological lesions and biochemical alterations associated with NLV infection are reversible. Normal histology and biochemical activity returns to baseline levels within two weeks postillness (106,114).

IMMUNITY

Despite numerous volunteer studies and epidemiologic investigations, immunity to NLVs is not well understood. Currently available assays do not directly detect neutralizing antibody, thus the protective effect of serum antibodies remains unclear (67,116). Early multiple challenge studies did describe a serotype-specific short-term protective effect (6 to 14 weeks) by serum antibodies against reinfection. However, the protective effect was not long term (80,117). Long-term immunity to NLV infection, on the other hand, is somewhat paradoxical. In volunteer studies, individuals that developed illness on initial challenge also developed illness on rechallenge, 2 to 4 years later (117). Further, those that were resistant to initial infection were also resistant on rechallenge. The resistant individuals also had a lower prechallenge serum antibody level than those that became ill, and they did not develop a significant serologic response postchallenge. Individuals that did become ill showed a serologic response after each challenge. Interferon does not play a significant role in immunity to NV (118). More recent studies have indicated similar rates of infection for individuals with or without preexisting serum antibodies (98). Another recent study has suggested that several challenges must occur to generate immunity (119). In this study, all subjects with a high prechallenge titer of serum antibodies became ill upon challenge, whereas approximately 60% of those with a low titer of serum antibodies became ill. On rechallenge six months later, only four individuals became ill. All of these individuals had low prechallenge titers before the initial challenge, but three had high titer serum antibodies before the second challenge. Upon a third challenge after an additional six months, no individual became ill.

High concentrations of maternal antibody can be detected in the sera of neonates (97,120). Recent studies using sensitive enzyme immunoassays (EIAs) have documented maternal antibody in 96% of neonates during their first month of life (97). Studies show that maternal antibodies to NLV may be detected at 6 to 8 months of age (97,121).

The immune response to recombinant Norwalk-like virus particles (rNLV) was studied in mice and human volunteers (122,123). In mice the rNV were immunogenic with or without adjuvant. Higher levels of antigen were necessary to generate an intestinal IgA response than was necessary to generate a serum IgG response (123). IgG responses were dose dependent and observed earlier than IgA. Tolerance was not induced with oral immunization. IgG and IgA response were noted after each oral dose (123). Similar results were observed in humans. IgG responses were dose dependent. Most individuals showed a serologic response of greater than fourfold increase after one dose, but no increase in IgG titer after a second dose three weeks later (122). Both rNVLPs and NLVs stimulate dominant IgG1 subclass and serum IgA antibodies. However, significantly higher elevations in antibody titer were observed in volunteers infected with live virus (122).

CONTROL AND PREVENTION

Treatment

NLVs typically cause a mild, self-limited illness that resolves without complications. Oral rehydration therapy is usually sufficient treatment. Occasionally, parenteral rehydration may be necessary in cases of severe dehydration (21). Administration of bismuth subsalicylate as a treatment for NLV infection has been shown to help mitigate symptoms of cramping and shorten the duration of the gastrointestinal symptoms (124,125). However, no effect was demonstrated on diarrhea and viral shedding.

Vaccines

Vaccine studies using recombinant Norwalk virus particles (rNV-VLP) are in the early stages. A systemic and mucosal antibody response is induced by the oral administration of rNV-VLP to mice or humans (122,123). An immunogenic response is observed with or without coadministration of adjuvant (126). However, it has not yet been determined whether rNV-VLPs protect against infection or illness when delivered orally.

EPIDEMIOLOGY

Transmission

Volunteer studies and outbreak investigations have indicated that transmission of human caliciviruses (NLVs and SLVs) is by the fecal-oral route (13,18,80,98,119). There is a high rate of secondary spread associated with the NLVs. Fecally contaminated food and water may serve as vehicles of transmission (127–132). Transmission by fomites is also possible. Person-to-person spread is common (24,133). Respiratory transmission is unlikely, although transmission by aerosolization of vomitus has been suggested for several outbreaks in hospitals, aboard ships, and from food (134–137).

A high secondary attack rate is a characteristic of NLV outbreaks. NLV outbreaks have been associated with a number of relatively confined settings, where presumably person-to-person transmission is likely. Norwalk virus was first isolated from a community wide outbreak beginning in an elementary school (14). Outbreaks have occurred at camps (Snow Mountain virus), in hospitals (Tauton virus), in hotels, and within families (Hawaii and Montgomery County viruses) (137–141). NLV outbreaks have also been described in nursing homes, on cruise ships, and at universities or colleges (142–146).

NLV outbreaks are often explosive and linked to a common source of contamination. Common or point source outbreaks may affect thousands of people (147). Water and food are commonly incriminated. Municipal water supplies, private wells, and commercially produced ice are all linked to common source outbreaks of NLVs (132,148–151). Recreational contact with water has also been associated with NLV outbreaks. Several outbreaks have resulted from primary contact (swimming) in contaminated lakes or swimming pool water (152–154). Even secondary recreational contact with contaminated water, such as canoeing, has been linked to NLV outbreaks, although capsizing and eating before changing clothing was associated with a greater risk than canoeing (155,156).

NLVs are estimated to be the leading cause of food-borne illness attributable to a known agent (24,157). The number of estimated annual cases of NLV gastroenteritis in the United States is 23,000,000 (out of an estimated 38,629,641 total cases of illness from known food-borne pathogens), 40% of which are food-borne (158). It is estimated that 50,000 hospitalizations will be caused by NLVs, 20,000 of which will be attributed to food. Additionally, researchers estimate 310 deaths will result from NLV illness, of which 124 will be food-related (158). However, it is acknowledged that these morbidity and mortality figures were estimated from hospital and community studies and are difficult to verify (158). The most common contributing factors to food-borne NLV outbreaks were poor personal hygiene, food from an unsafe source, and inadequate cooking (159).

Numerous food-borne outbreaks of NLVs are well described and a wide range of foods have been implicated as the contaminated vehicle. Raw and minimally processed fruits and vegetables are frequently implicated in NLV outbreaks (143,160–163). Additionally, sandwiches and pre-cooked meats have been implicated (111,164,165). Even more complex food items, such as bakery items (wedding cake with frosting), pasta, spring rolls, potato salads, coleslaw, and raspberry mousse, have been linked to outbreaks (166–172). Perhaps the most common food types associated with epidemic NLV infection are shellfish, in particular, bivalve mollusks. Each year numerous outbreaks of shellfish associated viral gastroenteritis are reported worldwide (173–176). Shellfish are typically filter feeders that retain and accumulate particles from the large volumes of water they pump through their bodies. Hence, sewage contamination of shellfish beds may lead to contamination of the shellfish meat as viruses are taken up, retained, and concentrated by the animals.

Generally, contamination of food may be distinguished into two groups; contamination by an infected food handler

and contamination by exposure to fecally contaminated water. Vegetables and fruits may be contaminated both by infected food handlers or washing or irrigating with fecally contaminated water (177). More complex or heated food items are typically contaminated by infected food handlers having poor personal hygiene and sanitation (178–180), although a community level outbreak associated with consumption of custard slices may have been caused by reconstitution of a dried custard mix with contaminated water (181). Outbreaks have been traced to pre-and post-symptomatic food handlers, and those that were sick on the job (166,178,182).

Incidence and Prevalence

Worldwide, NLVs are the most important causes of epidemic nonbacterial gastroenteritis (110,183). In the late 1970's, only a remote Equadorian Indian tribe, of those populations screened, did not have antibodies to NV (184). In the United Kingdom in 1995 and 1996, NLVs were responsible for more outbreaks of infectious gastroenteritis (42%) than Salmonella (15%) (24). In 1992 and 1993, Salmonella were responsible for slightly more outbreaks of infectious gastroenteritis (32%) than were NLVs (27%) (133). Person-to-person transmission and food-borne outbreaks were the primary mode of transmission (24,133). Between 1990 and 1994, the majority of reported cases, with known age, fell into two age groups: children from age 1 month to 4 years and adults over 65. However, cases were reported in all age groups (38). From 1990 to 1995, there is an apparent increasing trend in the number of reported cases of viral gastroenteritis associated with NLVs. Additionally, there appears to be a winter seasonality to the reported cases (38).

Molecular evidence indicates that the predominant strains of NLVs now in circulation are genogroup II viruses. Further, a naturally occurring recombinant virus between two distinct clusters of genogroup II has been identified (185). Although genogroup I viruses are still detected, they are considerably more rare (186–188). Mixed genogroup infections have been documented (156).

Early seroprevalence studies of NVs in the United States suggested that antibodies are acquired gradually through childhood. However, more recent evidence suggests that antibody acquisition to HuCVs varies with several factors, including age, geographic location, and development status of the country or region. The rate at which antibodies to Norwalk virus are acquired appears related to how developed a country may be. In lesser developed countries, antibodies are acquired very early (67). Reported seroprevalence data is summarized in Table 1.

At least four distinct antigenic types of SLVs have been described, although all share a common antigen detectable by radio-immunoassay (RIA) (189). SLVs have worldwide distribution with a seroprevalence of 30% at six months, increasing with age to greater than 90% for older children (>5 years old) and adults (190–192). In the United Kingdom, SLVs are responsible for approximately 1% of cases of viral diarrhea, with 90% of those detected occurring in young children (193).

Reservoirs

Humans are thought to be the only reservoir of HuCVs. However, recent studies identifying NLVs genetically (by nucleotide sequence analysis) in farm animals suggest that animal reservoirs cannot be completely ignored (75–78).

Age, Sex, Race, and Occupation

Traditionally, NLVs are thought to affect primarily older children and adults, particularly affecting the elderly, and only rarely the neonates and young children. However, recent advances in methods of detection (RT-PCR and sensitive enzyme immunoassays based on recombinant virus like particles) have indicated that NLVs are more widespread than previously thought, even infecting infants and young children (194–197). Geriatric facilities, adult-care facilities, and nursing homes seem particularly susceptible to NLV outbreaks (142,198–201). This may be related to the relatively confined setting of these institutions, the waning immunity, and the declining health of their resident populations. No significant differences in susceptibility have been documented between the sexes. Sexual preference apparently also does not have an effect on seroprevalence to NV because male and female homosexuals have comparable antibody levels as other adults (21,184). Studies determining the effects of race and occupation on NLV epidemiology are lacking.

Seasonality

As the term "winter vomiting disease" implies, NLVs were originally thought to have a winter seasonality. Later studies indicated that in developed countries, NLV outbreaks occur year-round (110,202). Despite year-round occurrence, a recent analysis of eight surveillance studies and unpublished data from four researchers confirm that peak incidence of cases of NLVs occurs in winter (203).

Other Risk Factors for Hucv Infection and Illness

Several studies have investigated the prevalence of diarrheal viruses, including NV, in individuals with HIV-infection (204–208). The prevalence of NV in the stools of HIV-infected children with chronic diarrhea in Tanzania was significantly greater than in HIV-negative children, also with chronic diarrhea (204). In a Venezuelan study of HIV-positive individuals with or without diarrhea, NV was not observed in stool samples (205). Another study in the United States documented a slightly elevated prevalence of caliciviruses in stool samples (6–1%) of HIV patients with diarrhea versus those without diarrhea. However, no seroconversion to NV was observed (207).

Investigations indicate that NV is rarely associated with exacerbation of Crohn's disease and ulcerative colitis (209). Other studies found no difference in antibody prevalence or titer between Crohn's patients and controls (210,211). Antibody levels to NV were also similar in individuals with cystic fibrosis and controls (212).

Table 1. Reported Seroprevalence Data for NLVs

Population Examined	Location	Antigen	Seroprevalence	Citation
Infants (under 6 months)	Bangladesh	NV	7%	Black, et al. 1982
Infants (6–11 month)	England	rNV	24.60%	Gray, et al. 1993
Infants (7–11 months)	China	rNV	41%	Jing, et al. 2000
Infants (7–11 months)	China	rMX	36%	Jing, et al. 2000
Children (age 1)	China	rNV	65%	Jing, et al. 2000
Children (age 1)	China	rMX	70%	Jing, et al. 2000
Children (under age 2)	England	NV	little	Parker, et al. 1994
Children (under age 2)	Finland	rNV	49%	Lew, et al. 1994
Children (by age 2)	England	rNV	12%	Parker, et al. 1995
Children (by age 2)	England	rMX	70%	Parker, et al. 1995
Children (by age 2)	Mexico	rNV	85%	Jiang, et al. 1995
Children (age 3)	China	rNV	85%	Jing, et al. 2000
Children (age 3)	China	rMX	90%	Jing, et al. 2000
Children (2–5)	Bangladesh	NV	80%	Black, et al. 1982
Children (by age 5)	Venezuela	rNV	50%	Pujol, et al. 1998
Children (under age 5)	Sweden	rNV	50%	Hinkula, et al. 1995
Children (under age 5)	USA	NV	20%	Kapikian, et al. 1978
Nursery School	Japan	rMX	50%	Honma, et al. 1998
Children (ages 8–9)	China	rNV	100%	Jing, et al. 2000
Children (ages 8–9)	China	rMX	98%	Jing, et al. 2000
Children	Australia	NV	Up to 90%	Parker, et al. 1994
Older Children (11–16)	England	NV	70%	Parker, et al. 1994
Adolescents	Japan	rMX	80%	Honma, et al. 1998
Individuals (9–19)	Canada	NV	55%	Payment, et al. 1994
Children and Adults (over age 10)	Sweden	rNV	80%	Hinkula, et al. 1995
Young Adults (18–35)	USA	NV	45%	Kapikian, et al. 1978
Individuals (20–39)	Canada	NV	79%	Payment, et al. 1994
Military Recruits	Norway	NV	29.50%	Myrmel, et al. 1996
Adults	Chile	NV	67–83%	O'Ryan, et al. 1998
Adults	Chile	MX	90–91%	O'Ryan, et al. 1998
Adults	Japan/Southeast Asia	rMX	82–88%	Honma, et al. 1998
Adults	Kenya	rNV	60%	Nakata, et al. 1998
Adults	Kenya	rMX	80–90%	Nakata, et al. 1998
Adults	Kuwait	rNV	98%	Dimitrov, et al. 1997
Adults	Kuwait	rMX	96%	Dimitrov, et al. 1997
Individuals (40–49)	Canada	NV	87%	Payment, et al. 1994
Individuals (50–59)	Canada	NV	84%	Payment, et al. 1994
Individuals (over age 60)	Canada	NV	100%	Payment, et al. 1994
Older Adults (45–65)	USA	NV	Up to 60%	Kapikian, et al. 1978
Older Adults (over age 60)	England	rNV	89.70%	Gray, et al. 1993
Individuals (9–79)	Canada	rNV	53–100%	Cubitt, et al. 1998
Individuals (9–79)	Canada	rHV	65–100%	Cubitt, et al. 1998
Amerindians Average	Amazon	rNV	40–100%	Gabbay, et al. 1994
Average	Canada	NV	79%	Payment, et al. 1994
Average	China	rNV	89%	Jing, et al. 2000
Average	China	rMX	91%	Jing, et al. 2000
Average	England	rNV	73.30%	Gray, et al. 1993
Average	Italy	rSH	51%	Pelosi, et al. 1999
Average	Italy	rLD	91%	Pelosi, et al. 1999
Average	Southern Africa	rNV	>90%	Smit, et al. 1999
Average	Southern Africa	rMX	>95%	Smit, et al. 1999
Urban Avg	Venezuela	rNV	47–53%	Pujol, et al. 1998
Rural Avg	Venezuela	rNV	80%	Pujol, et al. 1998
Amerindians Average	Venezuela	rNV	73–93%	Pujol, et al. 1998

CONCLUSION

Human caliciviruses, including the NLVs and SLVs, are among the most important and widespread causes of viral gastroenteritis in the world. Illness is characterized by a short incubation period, acute onset, and short duration, with typical symptoms of diarrhea, vomiting, nausea, and abdominal pain. NLVs cause illness in all age groups, whereas SLVs infect mostly infants and young children. Immunity to HuCVs is short-lived and repeated infections probably occur throughout life, with increasing severity in elderly populations. HuCVs are transmitted

by the fecal-oral route, including direct and indirect contact, fecally contaminated water, food and fomites, and possibly by aerosolized vomitus. The infectious dose of HuCVs has been demonstrated to be low for those that have studied, and based on epidemiological investigations suggest that it is low for the others. HuCVs have not been propagated in cell cultures or other animals, and this has hampered progress in understanding their biology and in developing prevention and control measures. However, the cloning and molecular characterization of HuCVs, including the expression of HuCV proteins, has led to the availability of nucleic acid detection and serologic methods that have greatly facilitated characterization of these viruses, the understanding of their epidemiology, including their detection in infected people, and various vehicles of exposure.

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HUMIC SUBSTANCES IN AQUATIC ENVIRONMENTS.

See Activity and Carbon Transformations in Biofilms

HYDROCARBON BIODEGRADATION IN COLD ENVIRONMENTS.

See Use of Cold-Adapted Microorganisms in Biotechnology

HYDROCARBONS, BIODEGRADATION OF.

See Petroleum and Other Hydrocarbons, Biodegradation of

HYDROCARBONS, BIOREMEDIATION OF.

See BIOREMEDIATION: AN OVERVIEW OF HOW MICROBIOLOGICAL PROCESSES CAN BE APPLIED TO THE CLEANUP OF ORGANIC AND INORGANIC ENVIRONMENTAL POLLUTANTS

HYDRODESULFURIZATION.

See DESULFURIZATION OF FOSSIL FUELS

HYDROPHOBICITY OF MICROORGANISMS: METHODOLOGY

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Hydrophobicity is by definition and meaning of the word the degree of dislike of a surface for water (1), but since the pioneering work of Rosenberg and coworkers (2), it was invariably associated with the ability of microorganisms to adhere to hydrocarbons in an aqueous suspension. Adhesion, however, whether to hydrocarbons in aqueous suspension or any other surface, is always an interplay of all factors involved in the process (3) including not only hydrophobicity but also electrostatic charge interactions (4) and influences of structural cell surface properties (5,6). Table 1 lists currently used assays to determine microbial cell surface hydrophobicities.

In the past, there has been extensive debate in the literature about the extent to which the results of different hydrophobicity assays correspond (23–25). The general consensus seems to be at present that the results of different hydrophobicity assays do not correspond (26–27) unless confined to a collection of closely related strains (28–29). Strictly speaking, hydrophobicity is an acid–base phenomenon (30) that can only be measured by water droplets on carefully prepared and dried microbial lawns (8,31). If water molecules have a greater preference to surround each other than to contact a microbial cell surface, the surface appears as hydrophobic (see Fig. 1) and water droplets do not spread, whereas if water molecules favor a microbial cell surface rather than each other, the surface appears hydrophilic. Contact angles with liquids on microbial lawns can be measured without an intervening influence of electrostatic charge, but it must be realized that drying of the lawns is a critical step as it determines the degree of collapse of microbial surface appendages and therewith the contact angle (8,31–32). Usually, contact angles measured on microbial lawns increase as a function of drying time until a so-called plateau is reached (Fig. 2). Note that the duration of the plateau state may also vary. As long as the lawns are dried to the plateau for water contact angles, relevant microbial contact angles can be measured. Theoretically, cell surface hydrophobicity cannot be measured solely by water contact angles, as this does not allow accounting for acid–base interactions as occurring between water molecules and between water molecules and the microbial cell surface. Full surface thermodynamic analysis of the cell surface hydrophobicity requires contact angle measurements, with at least three different liquids with varying acid–base properties (33).

The formal surface thermodynamic analysis of contact angles with liquids on microbial lawns yields one value for the so-called thermodynamic cell surface hydrophobicity of microbial cell surfaces, ΔG_{mwm}, whereas the term intrinsic cell surface–hydrophobicity is used for the contact angle (θ)–based hydrophobicity. Microbial adhesion to hydrocarbons (MATH) is, however, still the most widely used assay for cell surface hydrophobicity and, for that reason, will be described here in its so-called kinetic mode forwarded by Lichtenberg and coworkers (34) as a more quantitative and reproducible variant of the

Table 1. Summary of Different Assays to Measure Microbial Cell Surface Hydrophobicity

Description of the Assay	Comments	References
Contact angle measurements	Quantitative, intrinsic property, but overall property	7–9
Microbial adhesion to hydrocarbon (MATH)	Qualitative, interplay of more than one property	2,10–12
Microbial adhesion to solvents (MATS)	Qualitative, interplay of more than one property	13
Salt aggregation test	Indirect	14,15
Hydrophobic interaction chromatography	Interplay of more than one property	16
Two-phase partitioning system	Qualitative, interplay of more than one property	17
Direction of spreading	Qualitative, but contact angle based	18
Hydrophobic microsphere attachment	Mostly used for yeast cells, interplay of more than one property	19
Adhesion to polystyrene	Interplay of more than one property	20
Adsorption of nonionic and anionic surfactants	Qualitative, interplay of more than one property	21
Image analyses technique	Interplay of more than one property	22

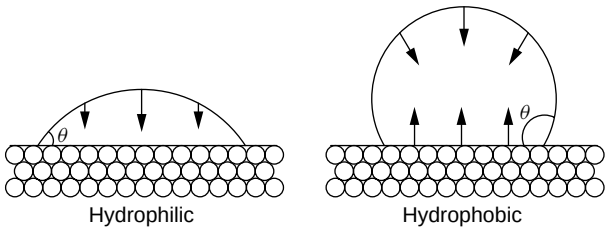

Figure 1. The contact angle equilibrium for a hydrophobic and a hydrophilic microbial cell surface, with the strongest interaction indicated by arrows in each diagram.

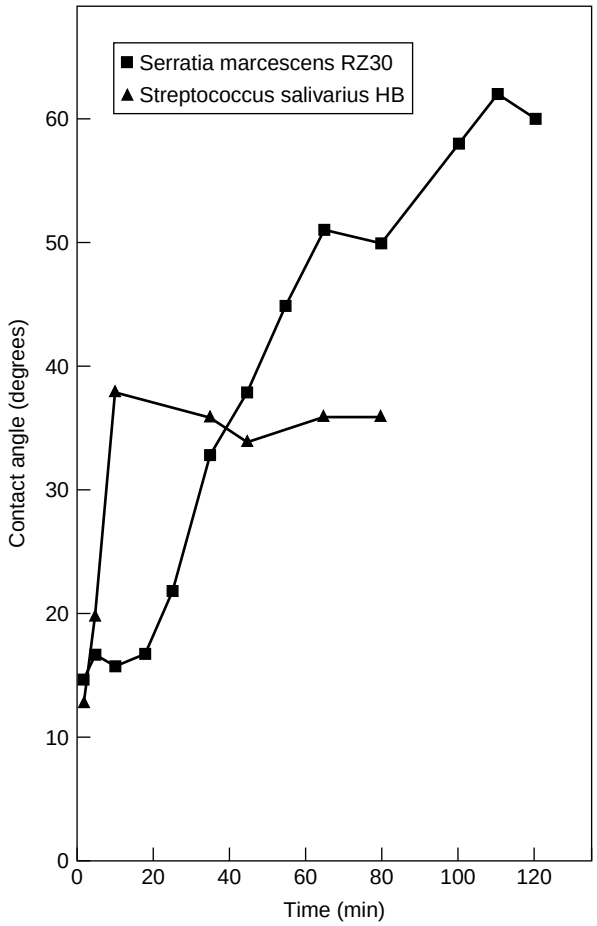

Figure 2. The water contact angle on different microbial lawns as a function of the drying time of the lawn. Note that the plateau angle arises after different drying times for different strains.

assay as originally described by Rosenberg and coworkers (2). Because microbial adhesion to hydrocarbons does not involve acid–base interactions between the hydrocarbon and the microbial cell surface, Bellon-Fontaine and coworkers (13) have suggested replacing the hydrocarbon phase by an organic solvent capable of direct acid–base interactions and proposed the acronym microbial adhesion to solvents (MATS) for this assay. MATS is included in this chapter, as a comparison of MATH and MATS results give an estimate of the acid–base character of a cell surface. Finally, it will be demonstrated that MATH and MATS disqualify as measures for cell surface hydrophobicity, predominantly because of the intervening effect of electrostatic charge interactions.

MICROBIAL CELL SURFACE HYDROPHOBICITY FROM MEASURED CONTACT ANGLES AND SURFACE THERMODYNAMIC ANALYSIS

Microbial lawns for contact angle measurements with liquids can be prepared by slowly depositing approximately 50 layers of bacteria suspended in water, on a membrane filter (pore size 0.45 μm) placed on a fritted glass support while applying negative pressure. These wet filters with bacteria can then be attached on sample holder plates with double-sided adhesive tape and dried at room temperature till the so-called plateau contact angles can be measured. The plateau is indicated by stable water contact angles in time, as can be seen in Figure 2 (31). To determine whether the cells are homogeneously deposited, scanning electron micrographs can be made. Contact angles can be measured with a variety of liquids. For a surface thermodynamical analysis that accounts for acid–base interactions contact angles should be measured with water, formamide, methyleneiodide, and/or α-bromonaphthalene. These liquids have varying Lifshitz-Van der Waals (LW) and acid–base (AB) surface free energy components, whereas in addition, their electron-donating and electron-accepting surface free energy parameters differ (Table 2). The Young equation describes the contact angle equilibrium (Eq. 1) as

$$\gamma_{\mathrm{lv}} \cos\theta = \gamma_{\mathrm{sv}} - \gamma_{\mathrm{sl}} \tag{1}$$

where θ is the contact angle and γ_{ij} is the surface free energy of the liquid (l)-, solid (s)- or the vapor (v)-interface. In a surface thermodynamical analysis, the measured contact angles with the different liquids are inserted in Equation 1, while using

$$(\cos\theta + 1)\gamma_{\mathrm{l}} = 2(\gamma_{\mathrm{mv}}^{\mathrm{LW}}\gamma_{\mathrm{l}}^{\mathrm{LW}})^{1/2} + 2(\gamma_{\mathrm{mv}}^{+}\gamma_{\mathrm{l}}^{-})^{1/2} + 2(\gamma_{\mathrm{mv}}^{-}\gamma_{\mathrm{l}}^{+})^{1/2} \tag{2}$$

to eliminate γ_{sl} from the equation. Details about this mathematical procedure have been given in several papers (30,33,36).

The surface free energy data of the microbial cell surface thus derived G can be employed to calculate a free energy balance ΔG_{mwm}

$$\Delta G_{\mathrm{mwm}} = -4\left(\sqrt{\gamma_{\mathrm{mv}}^{\mathrm{LW}}} - \sqrt{\gamma_{\mathrm{wv}}^{\mathrm{LW}}}\right)^2 - 4\left(\sqrt{\gamma_{\mathrm{mv}}^{+}\gamma_{\mathrm{mv}}^{-}} + \sqrt{\gamma_{\mathrm{wv}}^{+}\gamma_{\mathrm{wv}}^{-}} - \sqrt{\gamma_{\mathrm{mv}}^{+}\gamma_{\mathrm{wv}}^{-}} - \sqrt{\gamma_{\mathrm{mv}}^{-}\gamma_{\mathrm{wv}}^{+}}\right) \tag{3}$$

in which γ^{LW} is the Lifshitz-Van der Waals surface free energy component, γ^{+} and γ^{-} are the electron-accepting and electron-donating surface free energy parameters, and ΔG_{mwm} is a thermodynamic measure for the cell surface hydrophobicity that accounts for acid–base interactions and is negative for hydrophobic organisms and positive for hydrophilic organisms. Table 3 present some examples of microbial contact angles and thermodynamic cell

Table 2. Total Surface Tension (TOTAL), Lifshitz-Van der Waals (LW) and Acid–Base (AB) Components and Electron-Donating (−) and Electron-Accepting (+) Parameters for Water, Formamide, Methyleneiodide, α-Bromonaphthalene, and Different Hydrocarbons and Organic Solvents used in MATH and MATS, Respectively (35)

Liquid	γ^{Total}	γ^{LW}	γ^{AB}	γ^-	γ^+
Water	72.8	21.8	51.0	25.5	25.5
Formamide	58.0	39.0	19.0	47.0	1.92
Methyleneiodide	50.8	50.8	0	0	0
α-bromonaphthalene	44.4	44.4	0	0	0
n-octane	21.6	21.6	0	0	0
n-hexadecane	27.5	27.5	0	0	0
p-xylene	28.4	28.4	0	1.8	0
Toluene	28.5	28.5	0	2.3	0
Diethylether	16.7	16.7	0	16.4	0
Chloroform	27.2	27.2	0	0	3.8

Note: All data in mJ/m^2.

Table 3. Contact Angles (degrees) for Water (W), Formamide (F), Methyleneiodide (M) and α-bromonaphthalene (α-b) on Different Microorganisms, together with the Thermodynamically Derived Interfacial Free Energy of Adhesion ΔG_{mwm} (mJ/m^2)

Strain	θ_W	θ_F	θ_M	$\theta_{\alpha-b}$	ΔG_{mwm}
Leuconostoc mesenteroides NCDO523	17	39	52	36	56.8
Enterococcus faecalis 1131	23	30	42	27	34.5
Staphylococcus epidermidis 169	37	31	58	45	15.8
Acinetobacter calcoaceticus RAG1	48	49	43	38	18.9
Serratia marcescens RZ30	54	55	88	91	3.8
Actinomyces naeslundii T14-J1	64	37	43	29	−34.9
Lactobacillus plantarum RC20	79	43	45	30	−60.7
Staphylococcus epidermidis 3081	87	50	49	32	−72.4
Streptococcus mitis T9	91	47	55	36	−64.6
Lactobacillus acidophilus T13	102	47	55	38	−36.6

Note: Note that for hydrophobic organisms $\Delta G_{mwm} < 0$, whereas for hydrophilic organisms $\Delta G_{mwm} > 0$.

surface hydrophobicities, taken from a recently published reference guide (37).

The cell surface hydrophobicity as derived here from a surface thermodynamic analysis of contact angles describes an overall surface property, whereas the hydrophobicity of surface appendages, such as fibrils or fimbriae may be substantially different. Handley and coworkers (5), for instance, demonstrated that hydrophobic, colloidal gold particles only adsorbed to the tip of *Streptococcus sanguis* CR311 and PSH and concluded that the cell surface hydrophobicity was confined to the ends of the fibrils. Contact angles measured with liquid droplets on a microbial lawn are essentially representative of a fuzzy coat of cell surface material collapsed into a lawn. Therewith these results are useful to interpret the long-range interactions between an organism and a substratum surface, but not necessarily the short-range interaction, which may be dominated by structural and chemical cell surface heterogeneities (6).

MATH AND MATS ASSAYS

The hydrocarbons employed in MATH are essentially nonpolar, that is, they have a zero acid–base surface free energy, as can be seen from Table 2. By replacing the hydrocarbons by an organic solvent, acid–base interactions between the microorganisms and the solvent droplets in the assay can be introduced because these solvents have a nonzero acid–base surface free energy (Table 2). MATH and MATS are experimentally identical.

MATH and MATS can be carried out as originally proposed by Rosenberg and coworkers (2), but Rosenberg himself criticized the assay five years after its first publication as not being sufficiently quantitative (34). The preferred kinetic mode of the assay is still not widely used, as it is slightly more time-consuming. However, because the quality of the data superior as compared with the original assay has been considered, the kinetic assay will be described here.

Microorganisms are suspended in 3 ml of a buffer solution to an OD_{600} of between 0.4 and 0.6, generally referred to as OD_0. Subsequently, 150-μl hexadecane or another solvent is added to the suspension. The suspensions are vortexed for 10 s and left to settle for 10 min to allow separation of the two-phase system, when the water phase of the OD_{600} is again measured and referred to as OD_t. Vortexing and measuring are repeated until the total vortexing time is 60 s. Log $(OD_t/OD_0 \times 100)$

is plotted against the vortexing time, in which the negative slope yields the initial removal rate (R_0) as a measure of the adhesion of the bacterial cells to hexadecane or the other solvent.

Figure 3 illustrates the determination of the initial removal coefficient for *Streptococcus salivarius* HB adhering to hexadecane in 10-mM potassium phosphate with pH 7. Sometimes MATH (or MATS) is performed at various pH values to determine the degree of involvement of electrostatic interactions in the adhesion process (38,39) and interestingly, the initial removal rates by hexadecane are dependent upon pH, as can be seen in Figure 4. Although not all strains show a pH-dependent removal by hexadecane, the occurrence of a maximum removal coefficient at a certain pH value is common for many strains. Initial removal rates by the electron-accepting solvent chloroform (Table 2) are also pH-dependent and generally greater than the removal rates by hexadecane (Fig. 5) when the organism has a strongly electron-donating character. In contrast, Bellon-Fontaine and coworkers (13) showed that strains with electron-accepting character adhered less to chloroform than to hexadecane, but preferred diethylether above hexadecane. Comparison of MATH and MATS data can thus be useful to obtain an impression of the acidity or the basicity of the cell surface.

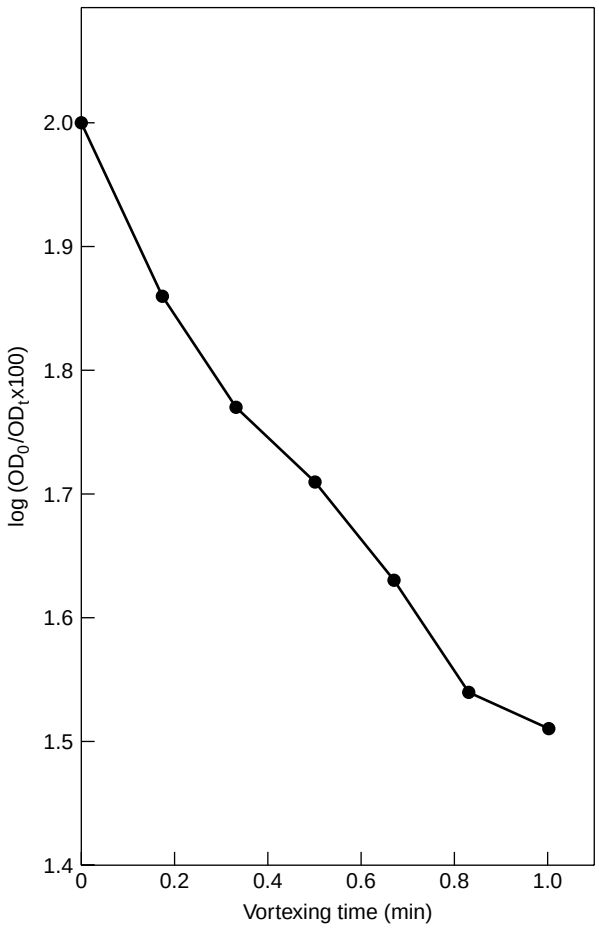

Figure 3. Illustration of the determination of an initial removal coefficient in the kinetic MATH assay for *S. salivarius* HB in 10 mM potassium phosphate at ph 7.

Mechanism of MATH and MATS

Adhesion-based hydrophobicity assays measured an interplay of all factors involved in microbial adhesion (28–29,38–39), which is actually confirmed by the pH-dependence of MATH results (see Fig. 4). The suspension pH dictates the protonation of ionizable groups on the cell surface and the degree of collapse of surface appendages (40) and a pH-dependence of adhesion results may point to electrostatic charge interactions. Several years ago, hydrocarbon droplets in aqueous suspensions have been found to be negatively charged (41). More recently, ζ-potentials of the different hydrocarbons in aqueous solutions used in MATH have been measured as a function of pH and were found to be highly negative above a pH of 3, whereas being slightly positively charged below a pH of 3 (38–39,41). The electrostatic interaction between hydrocarbons and microorganisms arising from this charge can be calculated in a DLVO (Derjaguin-Landau-Verweij-Overbeek) approach (4)

$$\pi\varepsilon a(\zeta_1^2+\zeta_2^2)\left[\frac{2\zeta_1\zeta_2}{\zeta_1^2+\zeta_2^2}\ln\frac{1+\exp(-\kappa d)}{1-\exp(-\kappa d)}+\ln\{1-\exp(-2\kappa d)\}\right] \quad (4)$$

in which ε denotes the dielectric constant of the medium, a is the radius of the microorganism, ζ, ζ-potential, κ^{-1}, double layer thickness and d, the separation distance between the interacting surfaces. If calculated for a separation distance of 1.57 Å, i.e. the distance of closest approach between two surfaces (42,43), the electrostatic contribution per adhering organism, ΔG_{el}, can be directly compared with the thermodynamic cell surface hydrophobicity per unit area, ΔG_{mwm}, although closest approach does not necessarily occur, and both measures have different units.

Figure 6 presents MATH (hexadecane) data for 23 different strains and species as a function of ΔG_{el} and ΔG_{mwm}, as determined in potassium phosphate solutions with varying pH. From Figure 6 it becomes apparent, that microorganisms adhere to hexadecane in MATH only in the absence of strong electrostatic repulsion, that is, ΔG_{el} needs to be negative, and when ΔG_{mwm} is negative too, that is, the organisms need to be hydrophobic (see also Table 3).

Synthesis: Thermodynamic Hydrophobicity, Electrostatic Interactions, and MATH(S)

Table 4 summarizes the observations from Figure 6. Hydrophilic organisms will always prefer the aqueous phase over adhesion to hydrocarbons and organic solvents, regardless of electrostatic repulsion. For hydrophobic organisms, whether adhesion to the hydrocarbons and organic solvents will occur depends on the electrostatic interaction. Hydrophobic organisms clearly adhere only in the absence of electrostatic repulsion. Close to the isoelectric points of the organisms, adhesion in MATH or MATS assays is consequently maximal. Therewith, MATH and MATS disqualify as assays for microbial

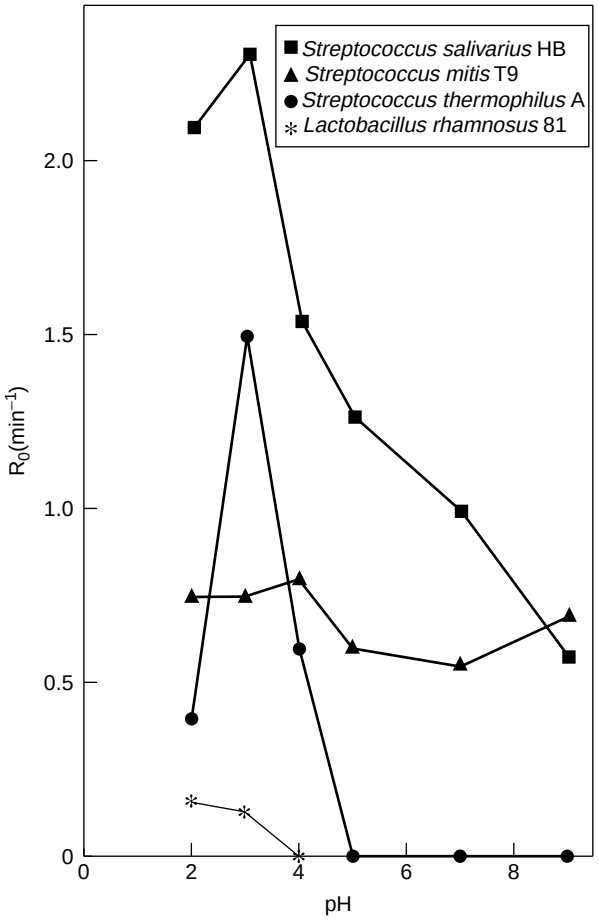

Figure 4. pH-Dependence of the removal by hexadecane in MATH for a variety of different strains.

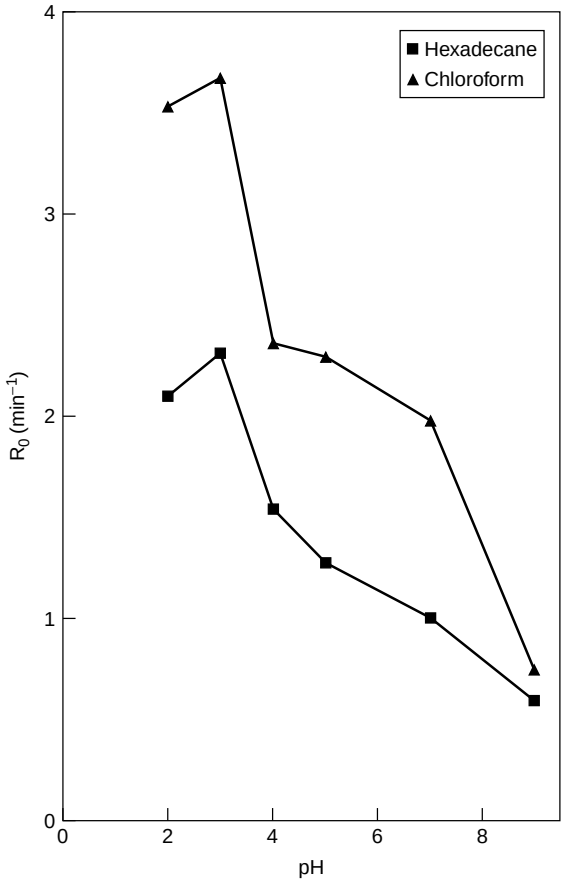

Figure 5. pH Dependent removal by hexadecane and chloroform for *S. salivarius* HB in MATH and MATS, respectively.

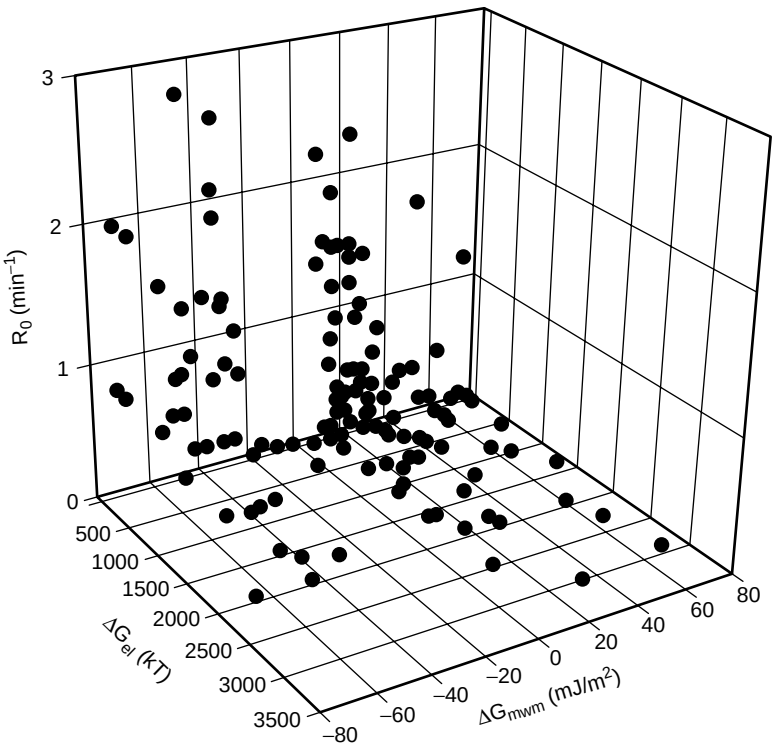

Figure 6. Removal by hexadecane of 23 strains as a function of ΔG_{mwm}, derived by thermodynamic analysis of measured contact angles, and the electrostatic interaction energy at closest approach. Note that adhesion of hydrophobic organisms to hexadecane only occurs in the absence of strong electrostatic repulsion.

Table 4. Schematic Summary of the Relations Between the Hydrophobicity of Microbial Cell Surfaces by Contact Angles or Surface Thermodynamic Analysis, Electrostatic Surface Charge and Adhesion to Hydrophobic Surfaces in Aqueous Suspensions, like in MATH

Cell Surface Hydrophobicity	Microbial Zeta Potential	Adhesion in MATH
Hydrophilic	Highly negative	Low
Hydrophilic	Approaching zero	Low
Hydrophobic	Highly negative	Low
Hydrophobic	Approaching zero	High

cell surface hydrophobicity. However, these assays provide excellent tools for studying microbial adhesion to smooth and homogeneous model interfaces in aqueous suspensions. Microbial cell surface hydrophobicity must be inferred intrinsically from (water) contact angles and can be further evaluated thermodynamically to include acid–base interactions.

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HYDROTHERMAL VENTS: BIODIVERSITY IN DEEP-SEA HYDROTHERMAL VENTS

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The discovery of deep-sea hydrothermal vents along the East Pacific Rise (EPR) has provided an expanded view of the deep sea, which was long believed to be a cold, dark, high-pressure, and nutrient-poor environment (1,2). Unexpectedly, a dense, abundant, and diverse population

of animals was found in and around the venting of geothermally superheated water, and it soon became apparent that these macrofaunal populations were strongly dependent on the primary production of symbiotic and free-living microorganisms in the ecosystems (3–5). Since the first discovery, a number of deep-sea hydrothermal vents have been explored in the Pacific and Atlantic Oceans and, very recently, in the Indian Ocean (Fig. 1; 6).

In the microbial ecosystems of the deep-sea hydrothermal vent environments, at least three generic microbial habitats and communities have been identified: (*1*) free-living microbial population associated with the discharged vent fluids and presumably growing and reproducing within the subvent system, (*2*) free-living microbial population associated with the geochemical and physical gradients formed in the range between the discharged vent fluids and the ambient seawater and containing the greatest physiological and genetic diversity, and (*3*) endo- and exosymbiotic microbial population associated with vent fauna.

For a long time, studies of the microbial diversity in the deep-sea hydrothermal vent environments have been performed by microscopic observation of samples and isolation of a restricted number of mesophiles. Because microbial habitats falling within the temperature range for mesophiles (15–50 °C) are probably abundant, mesophilic microorganisms may have the greatest impact on the microbial communities in the deep-sea hydrothermal vent environments. Nevertheless, a greater number of thermophilic microorganisms have been isolated from these environments because of the increasing interest of microbiologists in the evolutionary traits, novel biochemistry, and industrial potential of such extremophiles (7). It is now generally accepted that conventional laboratory cultivation and isolation have substantial limitations for understanding the naturally occurring microbial communities, including most culture-resistant microorganisms (8,9). Yet, conventional, culture-dependent surveys of microorganisms inhabiting deep-sea hydrothermal vents have revealed their great phylogenetic and physiological diversity in these extraordinary environments.

In addition, recent culture-independent, molecular, phylogenetic approaches have demonstrated much greater phylogenetic diversity of microorganisms in the deep-sea hydrothermal vent environments than previously indicated by culture-dependent approaches (8,10–17). Using molecular techniques, the localization of invertebrate-associated endo- and ectosymbionts, the quantification of uncultivated dominant phylotypes in low-temperature communities, and the distribution of thermophilic microorganisms in the vent fluids and chimneys have been determined. The combined use of culture-dependent and independent techniques may allow us to elucidate the occurrence and distribution of microbial communities in deep-sea hydrothermal vent environments and their significance for the circulation of substances and the geochemical processes associated with hydrothermal activities. In this review, microbial diversity is summarized focusing on the invertebrate-associated population, the uncultivated low-temperature population, and the thermophilic population. The possible existence of a subvent

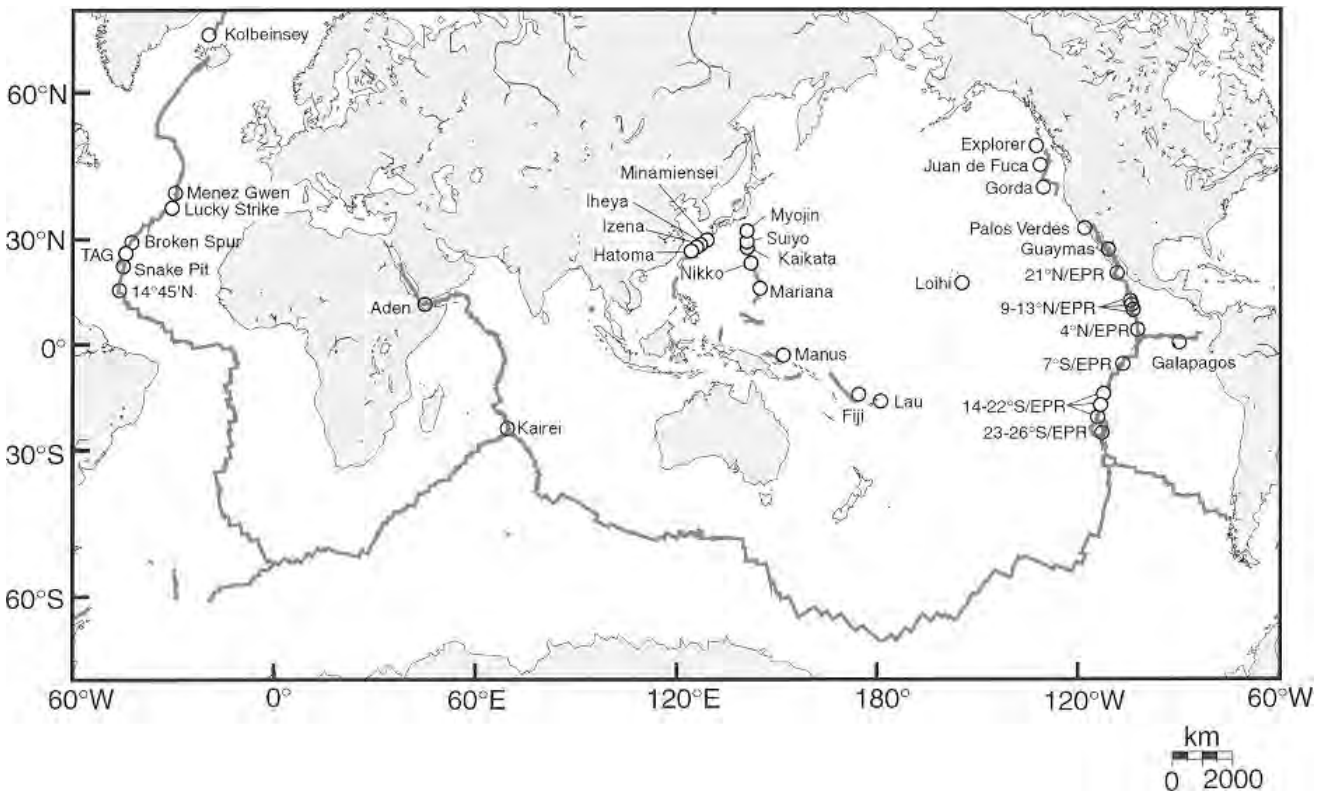

Figure 1. Location map of deep-sea hydrothermal vent sites found so far.

biosphere populated with extremophilic microorganisms and extensive distribution of deep-sea hydrothermal vent microorganisms in global subseafloor environments are also discussed.

INVERTEBRATE-ASSOCIATED MICROBIAL POPULATIONS

Chemoautotrophic and Methanotrophic Endosymbionts

The first conclusive discovery of endosymbiosis between chemoautotrophic bacteria and deep-sea hydrothermal vent invertebrates was in the trophosome of the vestimentiferan tubeworm, *Riftia pachyptila* (18,19). Many other symbiotic associations have since been reported to occur in hydrothermal vents and cold seeps around the world (Table 1). The diversity of invertebrate taxa harboring chemotrophic bacterial symbionts is quite broad (20,21). Chemotrophic symbioses are abundant in five families of bivalves, two of which (Vesicomyidae and Mytilidae) are endemic to hydrothermal vents and cold seeps. Lucinacean bivalves (families Lucinidae and Thyasiridae) are widely distributed in a variety of habitats with access to reduced sulfur compounds, including many seeps and some vents. Symbiosis with chemoautotrophic bacteria is also characteristic of bivalves in the family Solemyidae, and these bivalves are found in a wide variety of reducing habitats. Two hydrothermal vent gastropods of the family Provannidae are also known to harbor chemotrophic symbionts. Similar symbionts are also found in association with several phyla of worms including annelids, nematodes, and platyhelminthes. All currently examined members of the phylum Pogonophora, which includes vestimentiferan tubeworms, harbor chemoautotrophic symbionts. In some of the associations outlined earlier, the symbionts are extracellular, but even in those cases the associations appear to be very specific. Most chemotrophic symbionts are thioautotrophs. However, the first documented methanotrophic (methane-oxidizing) symbiosis in a deep-sea mussel was reported in 1986 (22).

Characterization of endosymbionts has been difficult because of their resistance to cultivation when they are removed from their hosts. As a result, the chemoautotrophic endosymbioses were initially confirmed by electron microscopic observation, stable isotope analysis, lipid analysis, and enzymatic characterization. These techniques revealed the physiological aspects of the symbioses and suggested the energy conversion and carbon assimilation systems of symbionts. They were classified into two types: one using sulfide, oxygen, and carbon dioxide as an electron donor, acceptor, and carbon source (thioautotrophy); and another using methane as an energy and carbon source (methanotrophy). However, the taxonomic and phylogenetic identification and the host specificity of the symbionts was unclear. Molecular phylogenetic analyses based on the 16S rDNA sequences have revealed that most host species contain a single phylotype of endosymbionts belonging either to thioautotrophic or methanotrophic bacteria, and a few have both (Table 1).

Phylogenetic analyses for 16S rDNA of symbionts demonstrated that most symbiotic bacteria in vent/seep species belonged to a limited domain of the γ-subdivision of *Proteobacteria*, despite the wide taxonomic range of their hosts (Fig. 2). All previously reported endosymbionts of vesicomyid clams (representative genera: *Calyptogena*, *Vesicomya*, and *Ectenagena*) are thioautotrophs and are located in host gills. Phylogenetic analyses of 16S rDNA sequences of the symbionts showed that these sequences formed a monophyletic clade that is closely related to the thioautotrophic symbionts of deep-sea mussels (Fig. 2). Deep-sea mussels (representative genus: *Bathymodiolus*) harbor thioautotrophs and/or methanotrophs as endosymbionts in their gills. *Bathymodiolus thermophilus* (East Pacific Rise) and *B. septemdierum* (western Pacific) have thioautotrophic endosymbiotic bacteria, *B. japonicus* (western Pacific) and *B. platifrons* (western Pacific) have methanotrophic bacteria, and *B. puteoserpentis* (Mid-Atlantic Ridge) has both (Table 1). Each trophic type of bacteria forms an independent monophyletic clade (Fig. 2). Methanotrophic symbionts of deep-sea mussels are more closely related to many free-living methanotrophs than to thioautotrophic symbionts in hydrothermal vent hosts (Fig. 2).

Endosymbionts of vestimentiferan tubeworms (representative genera: *Riftia*, *Escarpia*, and *Lamellibrachia*) are also thioautotrophs, located in trophosomes. Unlike the endosymbionts of vesicomyid clams and deep-sea mussels, the symbionts of the tubeworms form a paraphyletic group with thioautotrophic symbionts of solemyid clams (representative genus: *Solemya*) and clams belonging to the superfamily Lucinacea (representative genera: *Codakia*, *Lucinoma*, *Thyasira*, and *Anodontia*) (Fig. 2).

The causes of these differences in phyletic characteristics are still unclear. One possible explanation is a difference in the symbiont transmission mechanism. Direct evidence of symbiont transmission is rare, but three possible paths of transmission are recognized for chemotrophic symbiont-harboring species from deep-sea vents and seeps (23). These include vertical transmission from parent to progeny, horizontal transmission involving the spread of symbionts between contemporary hosts, and environmental reinfection of each generation of hosts from an environmental stock of microorganisms. The operational distinction between the last two is that a free-living form of the symbiont is required for the latter mechanism.

Vesicomyid clams and deep-sea mussels are reported to have a horizontal transmission mechanism. The vesicomyid clam *C. soyoae* contained bacteria within primary oocytes and follicle cells (24). Symbiont 16S ribosomal RNA genes (16S rDNA) were consistently amplified from the ovarial tissue of three species of vesicomyid clams, namely, *C. magnifica*, *C. phaseoliformis*, and *C. pacifica* (25). In situ hybridization showed that the symbionts were located in follicle cells surrounding the primary oocytes (25). Hybridization within oocytes was not observed, but several sections showed follicle cells invaginating and developing oocytes, thereby indicating a possible infection mechanism. The authors also mentioned a similar transovarial transmission mechanism in the symbiont of the deep-sea mussel *B. thermophilus* (25). The symbiont genealogy, based on bacterial 16S rDNA sequences, had a strong affinity with the host genealogy, which was based on clam mitochondrial cytochrome

Table 1. Symbiont-Harboring Invertebrates

Species	Location	Habitat	Depth (m)	Symbiont Symbiont Type	16S rDNA Accession No. of Symbionts
Bivalvia Solemyidae					
Solemya occidentalis	—	—	—	T	U41049
Solemya pusilla	Sagami Bay, Jpn.	Eelgrass bed	Intertidal	T	U62130
Solemya reidi	Santa Monica Bay, CA	Sewage outfall	150	T	L25709
Solemya terraeregina	Lizard Island, Austral	Coral reef sediments	—	T	U62131
Solemya velum	Wood Hole, MA	Eelgrass bed	Intertidal	T	M90415
Lucinacea					
Lucinidae					
Anodontia phillipiana	Ferry Reach, Bermuda	Mangrove Swamp	Intertidal	T	L25711
Codakia costata	Whalebone Bay, Bermuda	Eelgrass bed	Intertidal	T	L25712
Codakia orbicularis	Guadeloupe, Caribbean area	Sea-grass bed	Intertidal	T	X84979
Lucina floridana	St. Joseph Bay, FL	Eelgrass bed	Intertidal	T	L25707
Lucina nassula	Guadeloupe, Caribbean area	Sea-grass bed	Intertidal	T	X95229
Lucina pectinata	Guadeloupe, Caribbean area	Mangrove Swamp	Intertidal	T	X84980
Lucinoma aequizonata	Santa Barbara Basin, CA	Seep	500	T	M99448
Thyasiridae					
Maorithyas hadalis	Japan Trench	Seep	7,326–7,434	T&?	AB042413 AB042414
Thyasira flexuosa	Jennycliff Bay, UK	Reducing sediment	15	T	L01575
Vesicomyidae					
Calyptogena elongata	Santa Barbara Channel, CA	Seep	500	T	AF035719
Calyptogena fossajaponica	Japan Trench	Seep	6,329–6,809	T	AB044744
Calyptogena kilmeri	British Columbia, Canada to N. CA	Seep	549–1,464	T	AF035720
Calyptogena magnifica	East Pacific Rise, Galapagos Rift, Oregon subduction zone	Vent, Seep	2,036–2,700	T	AF035721
Calyptogena pacifica	Southern CA to Alaska	Seep	55–2,200	T	AF035723
Calyptogena phaseoliformis	Japan Trench, Kurile Trench	Seep	5,300–6,500	T	AF035724
Calyptogena soyoae	Sagami Bay	Vent, Seep	830–1,455	T	S79668
Calyptogena sp.	Oregon subduction zone	Seep	2,000	T	L25710
Calyptogena sp.	Monterey Bay, CA	Seep	3,400	T	L25708
Calyptogena sp.	Florida escarpment	Seep	3,313	T	AF035722
Ectenagena extenta	Northeast Pacific	Vent, Seep	~3, 000	T	AF035725
Vesicomya chordata	Gulf of Mexico	Seep	600	T	L25713
Vesicomya gigas	Northeast Pacific	Vent, Seep	1,000–2,700	T	AF035726
Vesicomya lepta					
Mytilidae					
Bathymodiolus japonicus	Sagami Bay, Okinawa Trough	Vent, Seep	700–1,400	M	AB036711
Bathymodiolus platifrons	Sagami Bay, Okinawa Trough	Vent, Seep	1,100–1,400	M	AB036710
Bathymodiolus puteoserpentis	Mid-Atlantic Ridge	Vent	3,476	T&M	U29163 U29164
Bathymodiolus septemdierum	Myojin Knoll, Suiyo Knoll, Mokuyo Knoll, Jpn	Vent	1,200–1,400	T	AB036709
Bathymodiolus thermophilus	East Pacific Rise, Galapagos Rift	Vent	2,500–2,600	T	M99445
Vestimentifera					
Escarpia spicata	Santa Catalina Basin, Guaymas Basin	Vent, Seep, Whale fall	1,240–2,016	T	U77482 AF165908 AF165909
Lamellibrachia columna	Lau Basin	Vent	1,859	T	U77481
Lamellibrachia sp.	Green Canyon, Gulf of Mexico	Seep	700	T	U77479
Ridgeia piscesae	Gorda Ridge	Vent	2,847	T	U77480
Riftia pachyptila	East Pacific Rise, Galapagos Rift, Guaymas Basin	Vent	2,016–2,637	T	U77478

Note: T: thioautotroph; M: methanotroph.

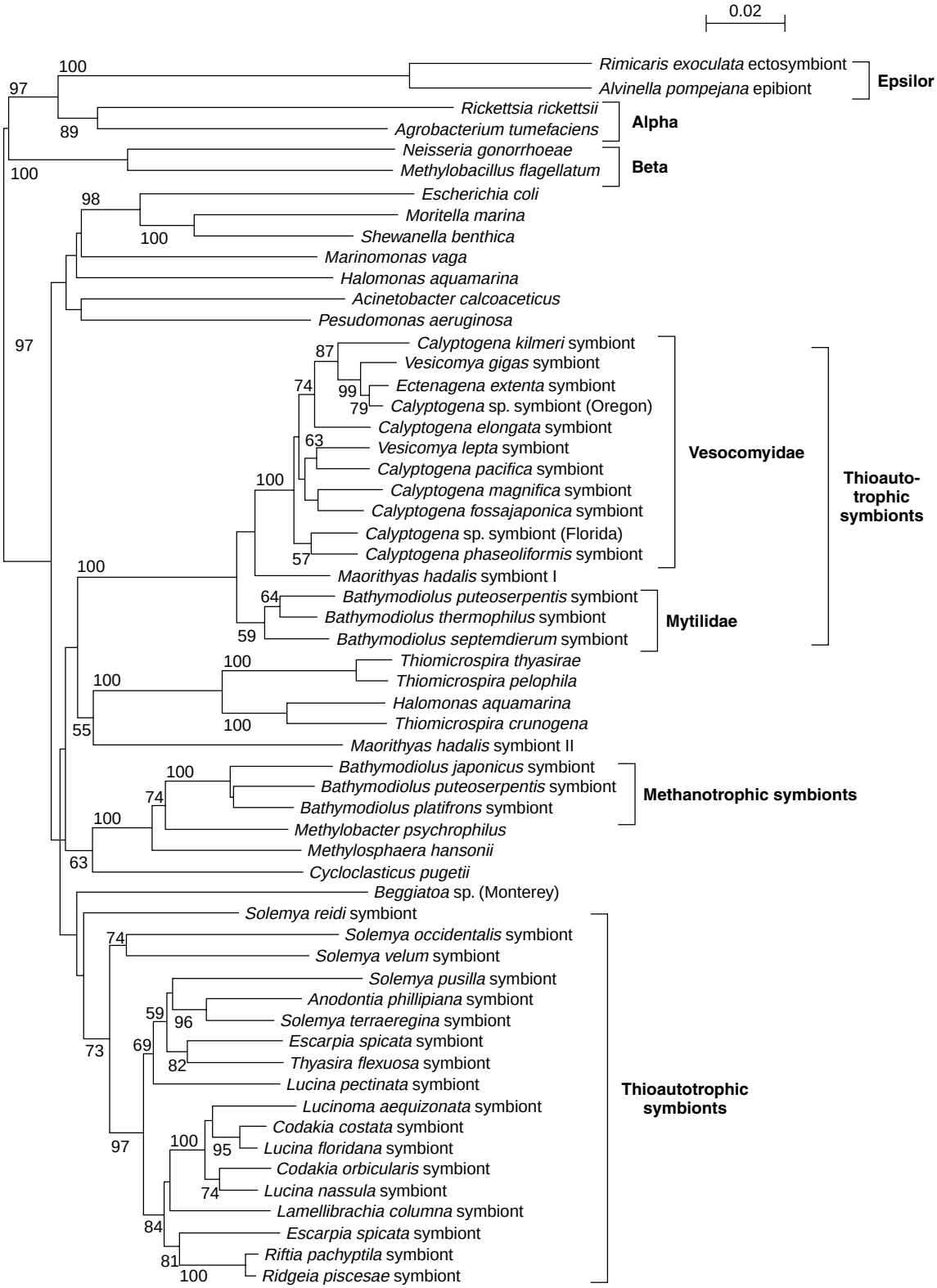

Figure 2. Phylogenetic tree based on small subunit rDNA sequences including various endosymbionts. Neighbor joining (NJ) tree within the members of the χ-subclass of Proteobacteria with the α-, β-, and ε-subclasses of Proteobacteria as an out-group is shown (1,357 homologous positions analyzed). Scale bar represents 0.02 nucleotide substitutions per sequence position. The percentage of 1,000 bootstrap resamplings is indicated. Bootstrap values greater than 50% are shown.

oxidase subunit I and 16S rDNA sequences. This also indicated nearly complete vertical transmission over long-term associations (26).

Evidence is accumulating to suggest that horizontal transmission or environmental reinfection is the mode of transmission in vestimentiferans and lucinid clams. An analysis of restriction fragment length polymorphism (RFLP) was employed on three vestimentiferan symbionts using three symbiont-specific genes, namely, eubacterial 16S rRNA, RuBPC/O Form II, and ATP sulfurylase (27). The results indicated that all of the symbionts from the three different hosts were conspecific and that the *Riftia* and *Tevnia* symbionts were indistinguishable in hosts separated over an 1,800-km range. These results strongly suggest that the symbionts are acquired de novo by each generation of juvenile tubeworms from a common source in the surrounding seawater (27). Feldman and coworkers (28) examined host-symbiont coevolution by comparing phylogenetic trees from symbiont 16S rDNA and host mitochondrial *COI* genes. The endosymbionts comprised two distinct clades, one associated with tubeworms from basaltic vent habitats and the other associated with tubeworms from sedimented seeplike environments. Within each symbiont clade, 16S rDNA sequences were nearly identical, suggesting that vent vestimentiferans share a single endosymbiont species that is distinct from the seep endosymbiont species. In the case of the lucinid clam *C. orbicularis*, amplification of bacterial 16S rDNA sequences using specific primers was unsuccessful in ovaries, eggs, veligers, and metamorphosed juveniles cultivated in sterile sand. Successful amplifications were obtained from gill tissues of adult specimens and from metamorphosed juveniles cultivated in sand from an unsterilized sea-grass bed (29). Hybridization using specific probes and observation of endosymbiotic bacteria in the gills of numerous juveniles cultivated in this sand demonstrated that the sulfur-oxidizing endosymbionts of *C. orbicularis* are environmentally transmitted to the new generation after larval metamorphosis.

In the case of solemyid clams, several lines of evidence seem to be contradictory. Krueger and Cavanaugh (30) showed that the symbiotic associations of solemyid clams appear to have multiple evolutionary origins on the basis of comparative sequence analysis of 16S rDNA. However, Cary and Giovannoni (25) reported a transovarial transmission mechanism in the symbiont of *S. reidi*. Cary (31) suggested that the *S. reidi* symbionts are vertically transmitted with the egg according to his molecular and histochemical analyses. Therefore, it is still unclear how such a paraphyletic group might arise.

The influence of bacterial endosymbionts on host distributions seems to be strong in hydrothermal-vent ecosystems. Fujiwara and coworkers (32) reported deep-sea mussels containing only methanotrophs (lacking thioautotrophs) from hydrothermal vents in the Okinawa Trough. Comparison of methane and hydrogen sulfide concentrations in end-member fluids from deep-sea hydrothermal vents indicated that methane concentrations were much higher at habitats containing *Bathymodiolus* that harbored only methanotrophs than at other hydrothermal-vent mussel habitats. The known distribution of other mussels containing only methanotrophs has till now been limited to cold-seep environments with high methane concentrations from the interstitial water. Therefore, the authors concluded that the distribution of methanotrophic symbioses between deep-sea mussels and methanotrophs is strongly influenced by the methane or hydrocarbon concentrations provided by hydrothermal-vent and cold-seep activities or that methanotrophic symbionts are required for the survival of host mussels in methane-rich environments. Selection of host habitats as related to symbionts might be an important process in the diversification of both hosts and symbionts.

Epibionts

The most extensively studied epibiotic microbial populations are of a tube-dwelling annelid polychaete *Alvinella pompejana* and of a highly motile alvinocaridid shrimp *Rimicaris exoculata*, which are endemic in the deep-sea hydrothermal vent fields of the East Pacific Rise and the Mid-Atlantic Ridge, respectively. On the basis of microscopic observations, it has been concluded that morphologically diverse microbial populations are present in the surface and subsurface of the epidermal organs of these animals. In the case of *A. pompejana*, dorsal epidermal expansions are externally covered by filamentous morphotypes that dominate the worm-bacteria association. Rod-shaped, prosthecate, spiral-curved, and filamentous sheathed or unsheathed bacteria are scattered on the surface of the worm integument, whereas clumplike assemblages of rod-shaped, coccoid, and filamentous bacteria are associated with cuticular protrusions in the intersegmental spaces (33). The extremely dense ectobacterial population consisting of rod-shaped and filamentous morphotypes with varying thickness is also noted on the anterior appendages, especially the hypertrophied gill-bailer, of the hydrothermal vent shrimp *R. exoculata* (34,35). Although culture-dependent approaches have been attempted to characterize the epibiotic microorganisms of these polychaete and shrimp, several nonfilamentous heterotrophic mesophiles have been successfully cultured from *A. pompejana*'s dorsal integument alone (36–40). Most of these heterotrophic strains are probably substantial components of epibiotic microbial communities. However, the most dominant filamentous epibionts have eluded all attempts at culturing, and so molecular phylogenetic techniques based on 16S rRNA sequences were applied to characterize the epibionts of these animals (41–43).

Several molecular phylogenetic analyses have revealed that most of the phylotypes recovered from rDNA assemblages of the epibiotic microbial populations are within the ε-subclass of *Proteobacteria*. This group of bacteria encompasses the genera *Arcobacter*, *Campylobacter*, *Helicobacter*, *Sulfurospirillum*, *Thiovulum*, and *Wolinella*, and the majority of these genera are known to be involved in multiple metabolic pathways regarding oxidation and/or reduction of sulfur compounds under microaerophilic and/or anaerobic conditions (44,45). In phylogenetic trees of the ε-subclass of *Proteobacteria*, the epibiotic phylotypes obtained from *A. pompejana* and *R. exoculata* are closely

related to each other, indicating certain evolutionary biases for the selection of epibionts. In situ hybridization and epifluorescence microscopy demonstrated that these phylotypes might represent the predominant morphotypes found on the surface and subsurface organs of the animals. In addition, epibiotic phylotypes and their close relatives were detected in considerable proportions in the total microbial rRNA assemblage, not only on the exterior surfaces of other invertebrate genera (*Paralvinella* sp. and *Riftia* sp.), but also on the surfaces of the rocks and sulfide blocks around the habitats of the hosts.

The epibionts within the ε-subclass of *Proteobacteria* may be sulfur-oxidizing chemoautotrophs contributing the trophic and sulfide-detoxifying roles in the gradients formed in the mixing zone of the reduced and oxidized waters. A combined use of culture-dependent and culture-independent techniques will shed light on the exact function and role of the epibionts for their hosts and surrounding microbial ecosystems.

MICROBIAL COMMUNITIES IN LOW-TEMPERATURE HABITATS

In low-temperature habitats that are probably formed in the steep geochemical and physical gradients between the discharged vent fluids and the ambient seawater, the availability of dissolved free oxygen renders aerobic or microaerobic oxidation of the reduced sulfur compounds, methane, and hydrogen the predominant energy-conversion systems. Sulfur-oxidizing bacteria colonizing rocks, chimneys, sediments, and animal surfaces exposed to vent emissions have been studied most extensively. With the exception of endosymbiotic chemolithoautotrophs, the sulfur-oxidizing microbial population identified in deep-sea hydrothermal vent environments include (*1*) uncultivated large filaments, probably consisting of *Beggiatoa* and *Thiothrix* in dense bacterial mats (46,47), (*2*) numerous base-producing facultative chemolithotrophs and heterotrophs (48,49), (*3*) rather few acid-producing obligately chemolithotrophic bacteria limited to two fully described species, *Thiobacillus hydrothermalis* (50) and *Thiomicrospira crunogena* (51–53), and (*4*) filamentous, sulfur-excreting species of the ε-subclass of *Proteobacteria* (54). As often observed in the endosymbiotic microbial population, methanotrophs are also probably dominant microorganisms in the mixing zone of the reduced vent water and the oxidized seawater. However, very few cultures have been obtained from the free-living methanotrophic population in the deep-sea, hydrothermal-vent environments. Hydrogen-oxidizing chemolithoautotrophs are very often cultivated from terrestrial hot springs and shallow marine hydrothermal vents. Although it was not fully described, the first hydrogen-oxidizing thermophilic bacterial strain EX-H1 was cultivated from chimney fractions in the East Pacific Rise (55).

On the basis of culture-independent molecular techniques, the bacterial rDNA community structures have been determined in several deep-sea, hydrothermal-vent environments. Muyzer and coworkers (56) found that the members of *Thiomicrospira* and *Desulfovibrio* belonging to the α- and δ-subclass *Proteobacteria*, respectively, were the possible dominant bacterial components in the microbial rDNA assemblages of two independent chimney samples using a denaturing gel gradient electrophoresis (DGGE) technique. This result indicated that not only sulfur-oxidizers, but also sulfate-reducing bacteria (SRB), predominantly occurred in the deep-sea, hydrothermal-vent environments, which was consistent with the evidence of sulfate-reduction activity in the sediments at the Guaymas Basin (57,58). In addition, the isolation and dominant occurrence of SRB within the δ-subclass *Proteobacteria* have been reported from different hydrothermal-vent sites [(59,60), Takai and coworkers, unpublished data].

Enrichment cultures and molecular signatures of typical deep-sea aerobic heterotrophs (61) belonging to the genera of *Alteromonas*, *Pseudoalteromonas*, *Vibrio*, *Pseudomonas*, *Halomonas*, *Marinobacter*, *Shewanella*, and *Colwellia* are often retrieved from deep-sea, hydrothermal-vent environments. Most of these genera of bacteria are probably derived from the ambient seawater. However, the high primary production and relatively high concentration of organic compounds in the seawater around the deep-sea, hydrothermal-vent emissions might harbor such ubiquitous deep-sea heterotrophs at much higher community density than in the typical deep-sea water and provide suitable habitats for certain populations of heterotrophs that specifically adapt to the deep-sea, hydrothermal vent environments.

The dominant occurrence and remarkable genetic diversity of the members of the *ε-Proteobacteria* have been demonstrated by a number of molecular phylogenetic surveys. Moyer and coworkers (14,62) reported that approximately 60% of the total bacterial clones constructed from a bacterial mat at a deep-sea hydrothermal vent in the Loihi Seamount was closely affiliated with *Thiovulum* sp. within the *ε-Proteobacteria*. As described in the previous section, the members of the *ε-Proteobacteria* represent major components in the epibiotic habitats and the surface area of the deep-sea, hydrothermal-vent animals.

Similarly, the deep-sea, hydrothermal-vent fluids and plumes may be niches for various members of the *ε-Proteobacteria*. To analyze the bacterial and archaeal diversity over time in the habitats just adjacent to vent emissions, an in situ titanium growth chamber (vent cap) was designed and deployed over several vents at the Snake Pit site in the Mid-Atlantic Ridge. During the deployment of the vent cap, the temperature was observed to decrease to approximately 20 °C (16). The rDNA clone analysis indicated that the most abundant bacterial rDNA sequences were of the *ε-Proteobacteria*, with a great phylogenetic diversity. These experiments did not necessarily demonstrate the community structures in the vent fluids, although the lower temperatures of vent fluids and plumes mixing with ambient seawater are able to provide suitable environmental settings for growth of the *ε-Proteobacteria*. In fact, a number of rDNA clones phylogenetically associated with the *ε-Proteobacteria* were found in a water sample directly collected from black smoker vent water at a temperature greater than 300 ° in the Okinawa Trough (Takai and coworkers unpublished data). The high abundance of rDNA sequences of the

ε-Proteobacteria was also observed in the surface layer (3 mm inside from the surface) of a black smoker chimney emitting vent fluid at a temperature greater than 300° in the Suiyo Seamount, Japan (Takai and coworkers unpublished data).

Archaea, the other domain of the prokaryotes, are substantial components even in low-temperature microbial communities occurring in deep-sea, hydrothermal-vent environments. As described in the next section, a great number of archaeal thermophiles have been isolated from the vent fluids, chimneys, rocks, sediments, and animals. Considering the hydrogen and carbon dioxide–rich environmental settings and the presence of mesophilic sulfate-reducing bacteria, it seems very likely that a certain population of psychrophilic or mesophilic methanogens occur in the anoxic zones of low-temperature habitats. However, no mesophilic or psychrophilic archaea have been cultivated from deep-sea, hydrothermal-vent samples. On the basis of recent molecular phylogenetic analyses, a great phylogenetic diversity of archaea containing possible psychrophilic and mesophilic entities have been identified in deep-sea, hydrothermal-vent environments (15–17,63). Most often, recovered archaeal phylotypes are classified into marine crenarchaeotic group I (MGI) (Fig. 3a). This group of phylotypes is an uncultivated, cosmopolitan archaeal group that is found in the global ocean environment (64–66). Cultivation of MGI is probably one of the greatest challenges for marine microbial ecologists because of their largest biomass potential within the domain of Archaea in the global ocean environment. Very limited but significant information on their physiology has been obtained from the vertical and seasonal distribution profiles in the coastal and oceanic waters (67,68), the molecular traits of some proteins restored from their DNA fragments (69), and the nutritional signatures using radioisotope-labeled substrates (70). At present, it is widely accepted that MGI might be in a category between psychrophiles to mesophiles, microaerobes to aerobes, and oligotrophs to heterotrophs. These predictions may lead to a simple answer that the ubiquity of MGI in deep-sea, hydrothermal-vent environments results from contamination from the ambient seawater; nevertheless, the formation of certain environmental optimums for their growth in the mixing zone of the vent emissions and the deep-sea water is not completely excluded.

Putative nonthermophilic archaeal phylotypes are noted within the euryarchaeotic kingdom as well (Fig. 3b). Marine euryarchaeotic group (MGII in Fig. 3b) and marine benthic group (MGIII in Fig. 3b) are also cosmopolitan nonthermophilic archaea widely distributed in the global ocean environments (64–66,74). Highly divergent uncultivated euryarchaeotic phylotypes [deep-sea euryarchaeotic group (DSEG) and miscellaneous euryarchaeotic group (MEG) in Fig. 3b] were obtained from sediments of a simmering point in the Okinawa Trough (17). The deep-sea, hydrothermal-vent systems located along the spreading Backarc axis are attributed to methane- and hydrocarbon-rich vent fluids by means of several geochemical surveys (89–91). Several archaeal phylotypes (pISA14 and pISA16 in Fig. 3b) recovered from the hydrothermal-vent systems are relatively abundant in the archaeal rDNA population (17) and had close phylogenetic relationship with the archaeal phylotypes found in subseafloor methane hydrate samples and methane-seep sediments (84,92,93). It is suggested that these archaeal phylotypes are derived from possible methanogenic archaea probably involved in anoxic methane oxidation syntrophically associated with sulfate-reducing counterparts (84,92,93). The anoxic oxidation of methane and hydrocarbon forms may play an important role in the primary production and energy conversion of the deep-sea, hydrothermal-vent ecosystems.

MICROBIAL COMMUNITIES IN HIGH-TEMPERATURE HABITATS

Cultivation and Isolation

Thermophiles have been isolated from various deep-sea, hydrothermal-vent sites since immediately after the discovery of deep-sea hydrothermal vents (94). These thermophiles are physiologically and phylogenetically diverse groups of microorganisms and include members of both the bacterial and archaeal domains (7,95–113). Among them, the members of *Thermococcales* are the most frequently isolated thermophiles, and these organisms are regarded as the major decomposers of organic matter within marine hot water ecosystems (99,100,104–107,109–117). They are strictly anaerobic, obligately heterotrophic microorganisms capable of using complex substrates for growth. Their growth is also strongly associated with the reduction of elemental sulfur (S^0). Within the order *Thermococcales*, three genera are known. The members of *Thermococcus* most predominantly occur as a culturable thermophilic population in the global deep-sea, hydrothermal-vent environments and are also obtained from oceanic and terrestrial oil reservoirs (118–120). Only two species of *Pyrococcus*, (*Pyrococcus horikoshii* and *Pyrococcus abyssi*), and one species of *Palaeococcus* have been cultured from the deep-sea vents and described (110,113).

The other thermophilic archaeal isolates are the sulfate-reducer *Archaeoglobus profundus*, the sulfite-reducer *A. veneficus* (101,121) and chemolithoautotrophic methanogens including the genera *Methanopyrus* (98,122) and *Methanococcus* [(95,123–126), Takai and coworkers unpublished data] within the Euryarchaeota. The thermophilic species of genera within the Crenarchaeota, such as *Desulfurococcus* (97), *Pyrodictium* (103), and *Staphylothermus* (96), are also listed in the culture collection of deep-sea, hydrothermal-vent archaea. A facultatively aerobic, obligate chemolithoautotroph *Pyrolobus fumarii* (108) is a deep-sea, hydrothermal-vent crenarchaeotic hyperthermophile currently representing the life-form with the highest temperature limit (113 °C) for growth.

Although relatively few of the fully described thermophilic isolates are classified as the domain Bacteria, the presence of various thermophilic genera is evident in deep-sea hydrothermal ecosystems. The occurrence of aerobic, heterotrophic thermophiles represented by *Bacillus*, *Thermus*, and *Thermaerobacter* has been reported till now (111,127,128). The bacterial chemolithoautotrophic thermophiles, consisting of

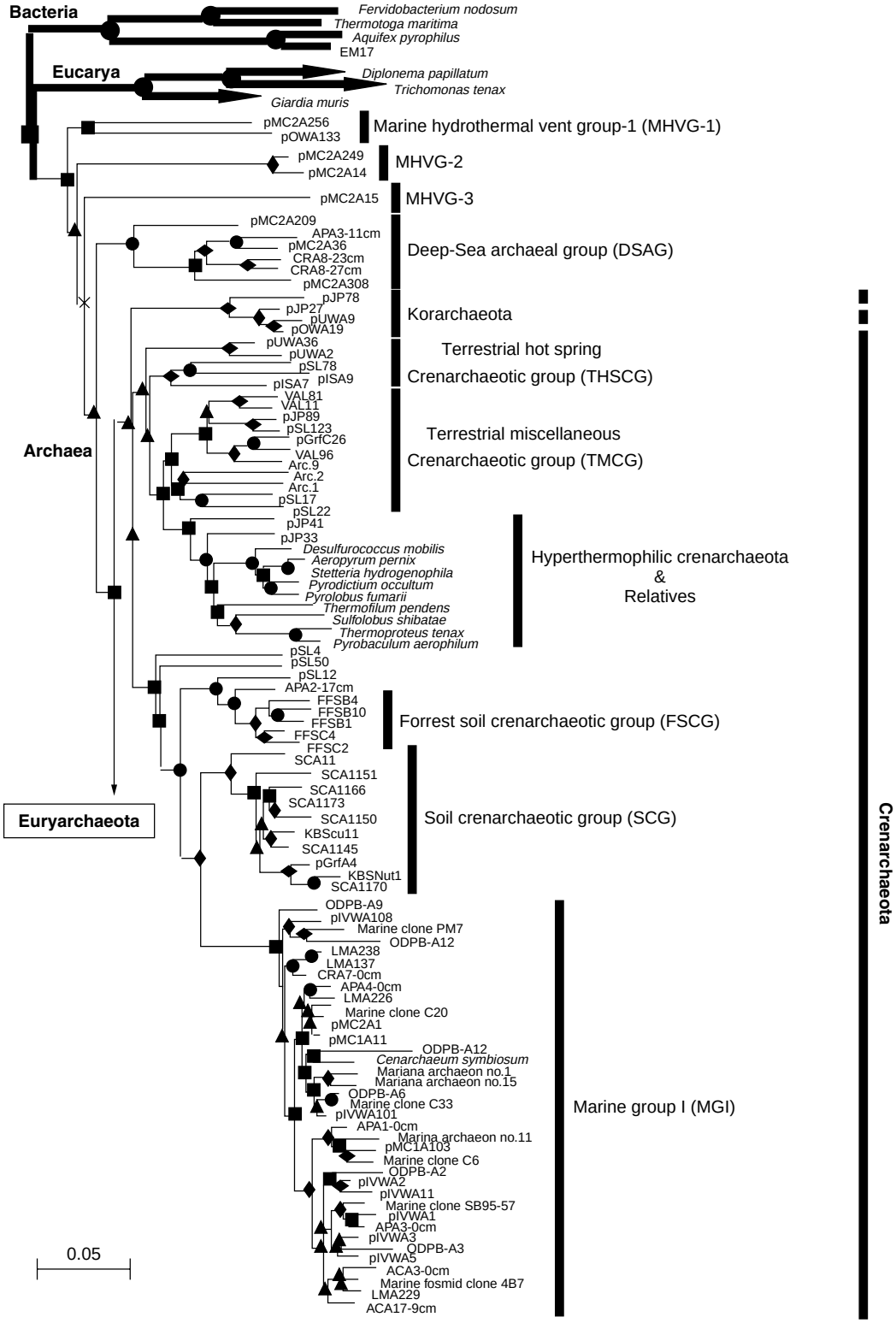

Figure 3. Phylogenetic tree based on small subunit rDNA sequences including various rDNA clones obtained from deep-sea, hydrothermal-vent environments. The tree was inferred by neighbor-joining analysis. (**a**) The tree indicates the phylogenetic relationship among the domains of Bacteria and Eukarya, the deep branches of uncultivated Archaea and the crenarchaeotic kingdom. (**b**) The tree indicates the phylogenetic organization within the euryarchaeotic kingdom. The marks on the branches represent the bootstrap confidence values: (●) greater than 80%, (■) 60 to 80%, (▲) 40 to 60%,(×) less than 40%. The scale bars indicate the expected changes per sequence position. (*Figure legend continues to next page*)

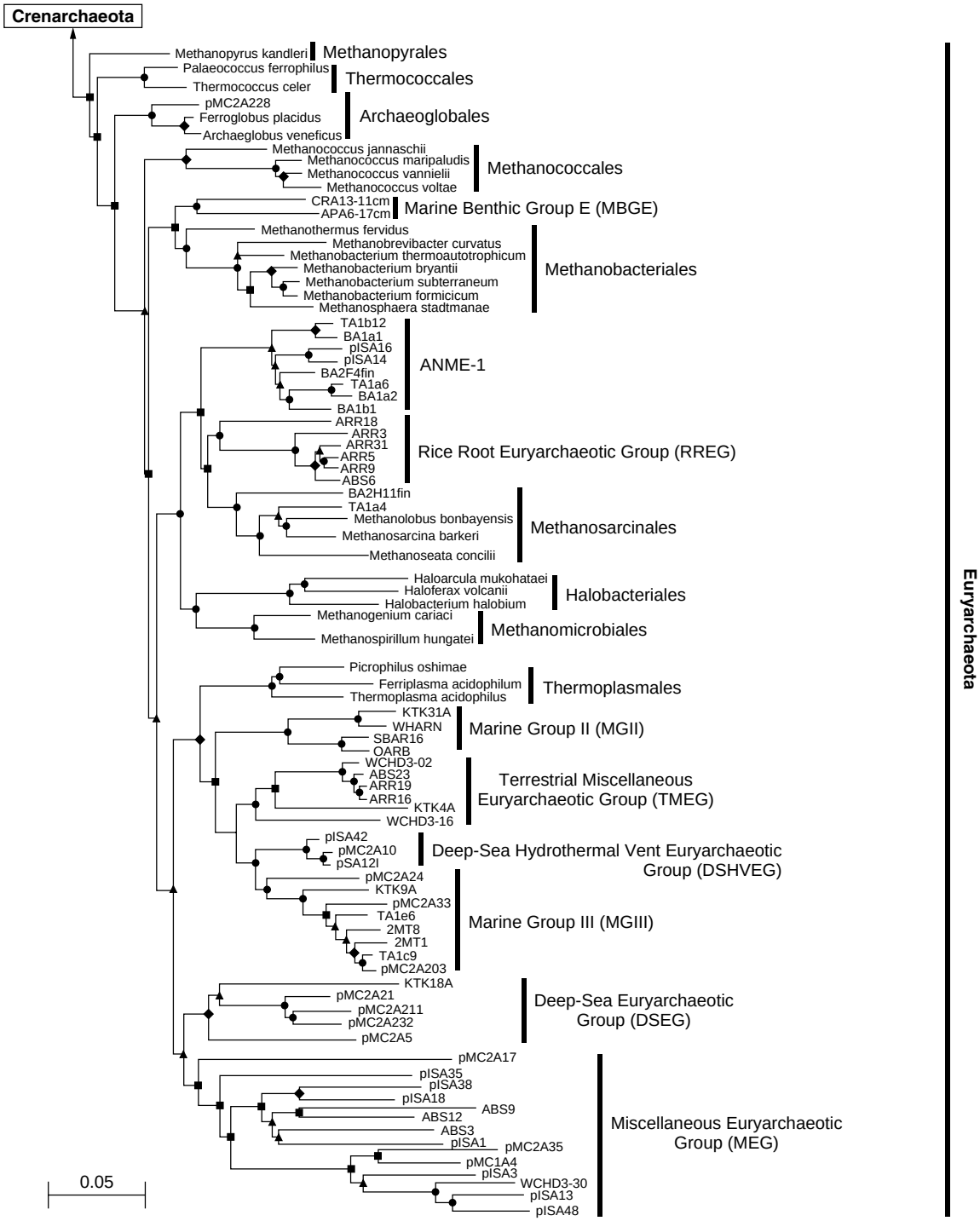

Figure 3. (*Figure legend continued from previous page)* Abbreviations indicate rDNA clones corresponding to uncultivated organisms derived from the following environments: bold pMC1A, pMC2A, pISA, and pIVWA from deep-sea, hydrothermal-vent environments (17); pOWA and pUWA from shallow-marine, hydrothermal-vent water and terrestrial acidic, hot spring water, respectively (71); pJP and pSL from sediments in Yellowstone National Park hot springs (72,73); CRA, APA, ACA, and LMA from deep-sea sediments (74); VAL from boreal forest lake water (75); soil clone SCA from agricultural soil (76); KBScul and KBSnat from soils (77); pGrifA and LMA from freshwater lake sediments (78,79); Arc from a subsurface paleosol (80); marine clones C and PM from ocean water samples (81); ODPB-A from subseafloor sediments and water (82); Mariana archaeon from sediments in Mariana Trench (83); TA and BA from marine anoxic methane-oxidizing layers (84); ARR and ABS from rice roots and paddy soils (85); KTK from deep-sea brine sediments (86); SBAR, OARB, and WHARN from coastal water (64); 2MT from salt marsh sediment (87); and WCHD from a subsurface soil (88).

microaerobic hydrogen-oxidizers of deeply branched *Aquificales* [(55), Sako pers. comm.] and anaerobic sulfur-reducing hydrogen oxidizers of *Desulfurobacterium* [(129), Sako pers. comm.], might partially sustain the deep-sea, hydrothermal-vent microbial communities as primary producers with thermophilic methanogens and hydrogen-oxidizing archaea. The facultatively sulfur-reducing, fermentative strains of *Thermosipho* (107,112) are isolated from various deep-sea, hydrothermal-vent samples. *Thermosipho* species are physiologically similar to *Thermococcales* members despite lower temperature ranges for growth of *Thermosipho*, and are often cocultivated in early enrichment cultures from the same inocula (112). It seems likely, therefore, that the thermophilic strains of the orders *Thermococcales* and *Thermotogales* share almost identical microhabitats in the deep-sea, hydrothermal-vent environments. Recently, a strain of dissimilatory iron (III)-reducing thermophiles has been successfully isolated from a black smoker chimney in the Suiyo Seamount (Takai and coworkers unpublished result).

Molecular Phylogenetic Approach

Compared with the number of culture-dependent approaches, only a few investigations have been made using molecular phylogenetic techniques for high-temperature microbial communities in deep-sea, hydrothermal-vent environments. The genetic diversity of thermophilic bacteria and archaea have been characterized using rDNA clone analyses in the microbial rDNA assemblages obtained from the vent cap system deployed in the Mid-Atlantic Ridge (16) and from vent fluids, chimneys, and sediments around Japan (17). In the vent cap experiments, most of the archaeal and bacterial phylotypes had strong phylogenetic associations with known cultivated groups of thermophiles such as *Thermococcales*, *Archaeoglobales*, *Aquificales*, and the *Desulfurobacterium* group, although several novel phylotypes having no apparent phylogenetic similarity with any other rDNA sequence were also recovered (16). Considering the deep branching and the short branch length of these novel phylotypes, they may represent uncultivated thermophilic populations in deep-sea hydrothermal vents. However, further phylogenetic analysis and its use in combination with enrichment cultivation will be required to determine their thermophily and physiological properties. The detailed analysis of archaeal diversity in the vent chimneys has provided greater genetic diversity of archaea in high-temperature microbial communities (17). It was demonstrated that several, at least division- or order-level novel, ancient lineages of archaeal phylotypes [marine hydrothermal vent group I (MHVG-1), MHVG-2, MHVG-3, and deep-sea archaeal group (DSAG) in Fig. 3a] were obtained from the distinct deep-sea, hydrothermal-vent chimneys in the Izu-Bonin Arc. The relatively high G + C content of the rDNA sequence and their limited occurrence in marine hydrothermal-vent systems strongly suggested that these ancient lineages of archaea were thermophiles. The occurrence of putative thermophilic, deep branches of archaea was partially supported by in situ whole-cell hybridization (FISH) (17). The occurrence of some of these phylotypes was detected from another deep-sea, hydrothermal-vent system in the Manus Basin (130).

The distribution of discrete thermophilic microbial communities in the deep-sea, hydrothermal-vent environment was reported by Harmsen and coworkers (131), by applying enrichment culture and whole-cell fluorescence in situ hybridization (FISH) techniques (132) to analysis of black smoker chimneys obtained from the Mid-Atlantic Ridge. The microbial-community density varied in subsamples obtained from different parts of a chimney structure, with the top part having the highest density (131). In addition, the increased density of the total archaeal population in the top part of the chimney was observed by the FISH technique. The preferred colonization patterns of potential *Thermococcales* members in the surface area and of potential *Igneococcales* members in the top parts and the vent area were also indicated by enrichment-culture techniques (131). A similar distribution pattern of microbial communities was also observed in the analysis of archaeal community structures in different microhabitats of a black smoker chimney from the Manus Basin, which is geologically and geographically distinct from those in the Mid-Atlantic Ridge (130). These results suggest that fine and detailed analyses of microhabitats dispersed a few centimeters apart, by using a combination of culture-dependent and culture-independent techniques, is necessary for future investigations on the diversity and function of microbial communities in the deep-sea, hydrothermal vent environments.

Subvent Biosphere

On the basis of several microbiological, geochemical, and geophysical observations, Deming and Baross (133) proposed the possible existence of a subvent biosphere, a viable microbial ecosystem populated with hyperthermophilic microorganisms beneath the active hydrothermal seafloor. Positive enrichments of hyperthermophiles from cold plume water shortly after an eruption event (134,135) also support the hypothesis for the subvent biosphere. This biosphere is now recognized as one of the most important research targets for elucidating the global terrestrial and oceanic subsurface biosphere. A direct approach to the subvent biosphere using a submarine drilling system, boring machine system (BMS), is now being undertaken to obtain samples from the subvent environment of active deep-sea, hydrothermal-vent sites in the Suiyo Seamount by a Japanese research team (136). This project will involve a multidisciplinary study of the subvent biosphere and may give direct evidence of its existence. However, an effective approach indirectly accessing the biosphere can also give great insight.

A deep-sea. hydrothermal-vent chimney is formed by chemical interaction between cold seawater and hot vent water. In the chimney structures and the underlying sulfide mounds, steep geochemical and physical gradients can be formed by equilibration between the vent water and the seawater, and similar environmental gradients are evidently formed in the subvent environments. Microbial rDNA showing substantial diversity was recovered from black smoker vent water in the Okinawa Trough, at a temperature greater than 300 °C which is far above the

upper temperature limit for growth of even the most hyperthermophilic archaeon, *P. fumarii* (113 °C) (17). The microbial rDNA may serve as a genetic signature indicative of the microorganisms thriving in the subvent habitats, conveyed there by vent water. It is probable, therefore, that a hydrothermal vent chimney is an environment analogous to the subvent biosphere, and elucidation of its microbial community structure will serve to characterize the features of the subvent microbial ecosystem.

The microbial population probably occurring in the subvent biosphere is believed to share similar habitats in the vent fluids and the interface between the vent fluids and the inside structures of chimneys. In these microhabitats, it was demonstrated that hyperthermophilic and extremely halophilic archaeal phylotypes were present (130). The hyperthermophilic rDNA population of the interface between the vent fluid and the chimney consisted largely of members of *Ignicoccales*, including the facultatively chemolithoautotrophic members with the highest temperature range for growth. This might indicate that these hyperthermophilic archaea thrive lithotrophically, using the reduced chemicals provided by hydrothermal activity in the extraordinary high-temperature environment. The discovery of extremely halophilic archaeal rDNA and the subsequent cultivation of halophilic microorganisms revealed the hypersaline microhabitats in the subvent biosphere. In fact, the formation of brines or salt deposits in deep-sea hydrothermal systems has been proposed (137–139). These brines or salt deposits provide suitable microhabitats for the extremely halophilic archaea. The global distribution of extremely halophilic archaea and hyperthermophilic *Thermococcales* members has been observed in nonhydrothermal pelagic subseafloor sediments (140) and in subseafloor methane hydrate core samples (Komatsu and coworkers, unpublished data). The widespread distribution patterns of *Thermococcales* in the terrestrial and oceanic subsurface oil reservoirs are other examples (118–120). The distribution of archaeal components found in the deep-sea hydrothermal or the subvent environments in global subsurface microbial communities may shed light on the origin of subsurface microorganisms and the biogeographical propagation of hyperthermophiles and extreme halophiles.

CONCLUSION

Since the first discovery of deep-sea hydrothermal vents, the investigations on deep-sea hydrothermal vents, with the dense, abundant, and diverse populations of animals and microorganisms, have fascinated numerous ocean biologists and microbiologists. With the recent development of technology and machinery for approaching deep-sea hydrothermal vents, a number of microorganisms composed primarily of thermophiles and hyperthermophiles have been isolated by cultivation techniques. In addition, advanced, culture-independent, molecular techniques have extended our view to deep-sea, hydrothermal vent microbial communities, with a great physiological and phylogenetic diversity. The microbial populations found in deep-sea hydrothermal vents were initially considered to be very limited, unusual, and specific microbial ecosystems. However, it has become apparent that microbial activities associated with these vent systems have great impact on the global microbial ecology, and on the geologic and geochemical processes of the earth, past and present. We believe in the future, because the discovery of deep-sea hydrothermal vents in the Mid Ocean Ridge revolutionized the oceanography in the twentieth century with the establishment of active plate tectonics; this knowledge of deep-sea, hydrothermal-vent ecosystems led to the elucidation of the origin of life and the exploration of extraterrestrial life in the 21st century.

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HYDROTHERMAL VENTS: PROKARYOTES IN DEEP-SEA HYDROTHERMAL VENTS

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The exploration of the deep-sea, from a microbiology point of view, started with the expedition of the "Travailleur" and the "Talisman" between 1882 and 1883, which demonstrated the occurrence of living bacteria in marine sediments at a depth of 5,000 m (1). After this pioneer work, deep-sea microbiology interested only a rather small part of the marine microbiologist community, although the deep sea (oceanic waters >1,000 m deep) represents 75% of the total volume of the oceans and 62% of the Earth's biosphere (2). The cost of deep-sea technologies may probably partially explain this situation.

The deep sea is mostly characterized by low temperature, low nutrient concentration, and elevated hydrostatic pressure. For these reasons, deep-sea microbiologists focused on psychrophilic bacteria, the degradation of organic matter under in situ conditions, and responses to hydrostatic pressure (3). Barophiles and obligate barophiles, in particular, have been isolated and studied for their physiology and molecular adaptations (4). Despite several very fascinating discoveries, the deep sea remained, for many scientists, a cold, dark, almost desert-like remote environment where all processes are very slow.

This view of the deep oceans was totally modified by the discovery of the first deep hydrothermal vent fields in the Galapagos Rift (5). In February 1977, the pilot and passengers of the manned submersible Alvin were faced with totally unexpected luxuriant animal communities at a depth of 2,500 m. Two years later, at 21°N on the East Pacific Rise, another Alvin dive led to the discovery of the now famous "black smokers," sulfide chimneys from which

hot fluids vented at temperatures up to 350 °C (6). This was a new departure for deep-sea biology and microbiology, which heralded further developments in the fields of biochemistry, molecular biology, biotechnology, or even astrobiology.

PRESENTATION OF HYDROTHERMAL VENTS

Hydrothermal Processes and Fluid Composition

Physiochemical processes that occur at deep-sea hydrothermal vents have been well documented by several authors (7,8). Briefly, in areas exposed to tectonic activity, seawater penetrates through cracks of the oceanic crust, to a depth of several kilometers below the sea floor, where it interacts with hot basaltic rocks. The seawater becomes an acidic solution enriched with minerals and metallic elements. This superheated solution (kept liquid because of high hydrostatic pressure) rises up through the ocean floor. The mixing of this solution with cold oxygenated seawater at discharging points results in the precipitation of calcium sulfate and polymetallic sulfides (the black smoke) and subsequent construction of the sulfide chimneys from which fluids vent at temperatures up to 350 °C. However, it is also possible that the hot fluid may mix with cold water before reaching the sea bottom. In this case, fluids vent out at rather low temperatures (10 to 30 °C) and the surrounding areas may be colonized by invertebrate communities.

Because of rock leaching by heated seawater, vent fluids have a chemical composition different from seawater and are characterized by low pH and high concentrations of dissolved gases (hydrogen sulfide, methane, carbon monoxide, carbon dioxide, hydrogen) and minerals (silicon, iron, manganese, zinc, etc.). However, the chemical composition and the temperature of the fluid, and, consequently, smoker wall minerals, may vary according to the vent site location. Also, speed of emission may vary from several centimeters per second to 2 m/second. Recent analysis for a Mid-Atlantic Ridge hydrothermal vent (9) reported concentrations of 3 to 12 mM kg^{-1} for H_2S, 25 μM kg^{-1} for CH_4, 750 to 6,500 μM kg^{-1} for iron, or 360 to 1,140 μM kg^{-1} for manganese, all these not being detected in seawater. Also, lead, copper, and zinc had concentrations of 10 to 360 nM kg^{-1}, 10 to 40 μM kg^{-1}, and 40 to 100 μM kg^{-1}, for an enrichment factor, compared to seawater, of 1,000, 1,500, and 4,000, respectively. On the contrary, magnesium and sulfate ions are absent from vent fluids.

Distribution of Vent Fields

Hydrothermal vent fields have been discovered and explored in many parts of the oceans, but are all located along oceanic ridges or in back-arc basins. About 40 active sites have been listed (10), from which about 30 are located in the Pacific Ocean (Guaymas Basin, East Pacific Rise at 21°, 17°, 13°, 11°, 9.5°N and 13°, 20°, 23°, 26°S and the Galapagos Rift for the eastern side; Marianna Basin, Lau Basin, North Fiji Basin, and Okinawa Basin for the western side). In the Atlantic, eight sites have discovered on the Mid-Atlantic Ridge between 14°45′ and 37°51′ N. The less and more deep sites known are located in the Atlantic at depths of 800 and 3,650 m, respectively.

Deep-sea hydrothermal vents represent very complex ecosystems inhabited by complex microbial communities. However, it is usual to distinguish three main categories: nonsymbiotic mesophiles, invertebrate-associated microorganisms, and thermophilic prokaryotes.

NONSYMBIOTIC MESOPHILES

Abundance and Activity in Surrounding Waters

As is common in aquatic microbial ecology, the abundance of bacteria in the waters surrounding vent biotopes has been estimated by several methods. Direct counts indicate numbers ranging from 6×10^4 to 10^9 cells/mL, whereas viable counts are in the range of 10 to 2×10^6 cells/mL for autotrophs and 10^2 to 2×10^7 cells/mL for heterotrophs (3). Both autotrophic and heterotrophic bacterial activities were also measured, but data obtained indicated a great variability between vents and even between samples from the same vent. However, all data indicate that the rate of bacterial production in seawater was too low to support the invertebrate biomass present. Several studies also considered the bacteria present in vent plumes (some plumes are detectable several kilometers away from the vent), and particularly manganese-metabolizing bacteria (11,12). Manganese oxidizers are rather abundant in vent waters but some plume bacteria can also bind manganese in their capsular polysaccharides.

Inert Surfaces and Bacterial Mats

Early reports from dives at hydrothermal vents mentioned heavy bacterial colonization of the sea bottom and the existence of spectacular bacterial mats, particularly at the Guaymas Basin, where they may be several centimeters thick. Scanning and transmission electron microscopic observations revealed not only a diversity of morphotypes but also the presence of some metabolic types, such as nitrifying and methane-oxidizing bacteria, identified thanks to their intracellular membranes (3). Most of the morphotypes observed were filaments with or without sulfur granules. Those sulfur granules were assigned (from morphological observations only) to either *Beggiatoa* or *Thiotrix* (13,14).

The exposure of artificial surfaces (glass, stainless steel) at vent sites indicated that the rate of bacterial colonization was similar to the rate observed in coastal waters. More recently, the use of vent caps (in situ growth chambers) exposed for several days at the Mid-Atlantic Ridge has allowed us to analyze the molecular diversity of the surface microbial communities from low-temperature vents (about 20 °C). In one instance, after a 5-day exposure, 47 phylotypes were determined by restriction fragment length polymorphism on a 87 clone library, all belonging exclusively to the epsilon subclass of Proteobacteria (15). In another study of a second vent at the same Atlantic site, Epsilon Proteobacteria were found, but also phylotypes corresponding to thermophilic bacteria and Archaea (16).

Cultivated Species

Among mesophilic bacteria isolated from hydrothermal vents, some were obtained from water samples and from various surfaces or pieces of invertebrate tissues when attempting to isolate symbionts (3). It is not possible to fully describe mesophilic microbial communities from the data found in the literature. However, all reports agree that there is a wide metabolic diversity (autotrophs, heterotrophs, sulfur-oxidizers, methane-oxidizers, nitrifiers, denitrifiers, sulfate reducers, etc.) of these communities. This is not surprising if one considers the density and complexity of the animal assemblages. It is worthy of noting that mesophilic halotolerant (16 to 27% NaCl) bacteria have been found in different vent plume samples collected at the Juan de Fuca Ridge, where they may form up to 28% of the total microbial community, as estimated by means of most probable number viable counts. Several isolates have been assigned to the genus *Halomonas* and *Marinobacter*, but these have not been fully identified. These organisms may have originated from sub–sea floor brine environments (17).

From all the strains isolated, only a small number have been fully characterized and identified (Table 1).

These strains do not reflect the real biodiversity of free-living mesophiles but were obtained from experiments dedicated to autotrophy and sulfur oxidation (18,19), surface colonization (20), or specific enrichment and screening for exopolysaccharide production (21–23).

INVERTEBRATE-ASSOCIATED BACTERIA

Vestimentifera Symbionts

The large tube worm *Riftia pachyptila* is probably the most fascinating invertebrate living in the East Pacific Rise deep-sea vents, but other species of Vestimentifera have also been described, such as *Tevnia jerichonana* and *Ridgea piscesae*. The lack of a digestive tract is a characteristic they all have in common. They obtain their nourishment, instead, through a special organ called the *trophosome*, which is full of autotrophic sulfur oxidizers. Despite several attempts at cultivating them, these symbionts have so far only been characterized by their 5S and 16S ribosomal RNA (rRNA) sequences. It was demonstrated that a single endosymbiont, belonging to the gamma subclass of Proteobacteria, inhabited each host. Further experiments using DNA/DNA hybridization indicated that the symbiont did not vary in different geographical areas, but was also identical for *R. pachyptila* and *T. jerichonana*. (24). Specific nucleotidic probes have been constructed to elucidate the mode of transmission of the symbionts. No signal was detected through the polymerase chain reaction amplification of DNA from gonad or oocytes, suggesting that the bacteria might be acquired from the environment after metamorphosis and settlement of the larvae (25).

Table 1. Mesophilic Bacterial Species Isolated from Deep-Sea Hydrothermal Vents

Genus	*Species*	Metabolism	Reference
Thiomicrospira	*crunogena*	Sulfur-oxidizer	18
Thiobacillus	*hydrothermalis*	Sulfur-oxidizer	19
Hyphomicrobium	*hirschiana*	Heterotroph	20
Hyphomicrobium	*jannaschiana*	Heterotroph	20
Alteromonas	*macleodii/jijiensis*	Heterotroph	21
Alteromonas	*infernos*	Heterotroph	22
Vibrio	*diabolicus*	Heterotroph	23

Mollusc Symbionts

Independent of the functionality (*Bathymodiolus thermophilus*) or nonfunctionality (*Calyptogena magnifica*) of their digestive tracts (3), bivalve molluscs harbor monospecific assemblages of endosymbionts (gamma subclass of the Proteobacteria) within their gill filaments. Whereas sulfur-oxidizing endosymbionts are found in most cases, it appears that some Mytilid populations can also harbor methanotrophic endosymbionts (26). It has also been demonstrated for Mytilids from the Snake Pit vent site on the Mid-Atlantic Ridge, that both metabolic types can coexist within the bacteriocytes in the gill filaments of the same mussel (27). Symbiont transmission was investigated for *C. magnifica* and *B. thermophilus* using specific 16S rRNA probes. Symbionts were detected in the follicle cells surrounding the oocytes, providing strong evidence for the vertical transmission between parents and progeny (28).

Crustaceans

Dense clouds of shrimps swarming close to the active chimneys and beehive-like structures are typical of the Mid-Atlantic Ridge vent sites. *Rimicaris exoculata*, one of these shrimps, has been shown to harbor dense epibiotic bacterial populations composed of rods and different-sized filaments on their hypertrophied mouth appendices (29). Again, attempts at cultivation were not successful, but the epibionts were characterized from their 16S rRNA. In fact, the different morphotypes appeared to correspond to a single phylotype belonging to the epsilon subclass of the Proteobacteria (30). Data obtained from sequence analysis were confirmed by in situ hybridization, which indicated that the epibionts could correspond to either a single pleomorphic species or very similar species with similar 16S rRNA. Interestingly, it was found that this phylotype was also abundant on the sulfide rocks within the same vent area. Although the relationship between the shrimps and their epibionts has not been completely elucidated, the most probable significance of this association is nutritional (31).

Polychaetes

Whereas Vestimentifera and molluscs live in warm (10 to 20 °C) waters and the shrimps swarm around active chimneys, Polychaetous annelids (*Alvinella pompejana*) build their tubes onto the wall of active smokers and are exposed to rather elevated temperatures and high concentrations of toxic compounds (32). Measurements of temperatures for the worm are not so easy, and temperatures ranges of 25 to 40 °C and 40 to 50 °C or

even 70 to 80 °C have been reported for the opening of the tubes and within the worm colonies, respectively. A single individual of *A. pompejana* has even been observed twisted on the temperature probe of the submersible Nautile, whereas the probe indicated a temperature of 100 °C. Although the true optimal temperature for the life cycle of this organism has not yet been determined, it is probably the most eurythermal or thermotolerant metazoan species presently known (33).

Zoologists who first described this novel species, using scanning electron microscopy for fine observation, were surprised by the abundance of epibiotic microorganisms living on the dorsal parts of the worm integuments. Rod-shaped, prosthecated, spiral-curved, sheathed, and unsheathed filaments were scattered on the worm surface, whereas more specific filamentous epibionts were found associated with cuticular extensions in the intersegmental spaces (34). Following these morphological studies, culture methods revealed a very metabolically diversified community, particularly adapted to elevated heavy metal concentrations, and displaying resistance to arsenate, cadmium, silver, zinc, or copper (35). About 20% of the isolates (*Acinetobacter, Alteromonas, Pseudomonas*, or *Vibrio*) harbored one to five plasmids of sizes ranging from 4.6 to 157 kb. Hybridization experiments indicated that plasmids of identical sizes existed within different genera, indicating a probable transfer of genetic elements (36,37). However, the dominant filamentous morphotypes again escaped attempts of being cultured and so are only characterized by their 16S rRNA. Four dominant clone families (32) represented 65% of the clone library. These were placed into two different clades within the epsilon subclass of Proteobacteria (38). Only few genera are known for this subclass. These are mostly adapted to low oxygen concentration environments, and are involved in several types of sulfur compounds' metabolisms, under both microaerophilic and anaerobic conditions. The two dominant phylotypes were detected from worm specimens collected in different geographical areas. In situ hybridizations indicated that they represented the dominant forms living on the dorsal integument of the worm. These two phylotypes were also detected on *Paralvinella* sp. integument, *R. pachyptila* tube and rock surfaces, and on *Alvinella caudata* integuments, indicating that these epibionts are not obligate companions of *A. pompejana* (39). The role and significance of this association are yet to be fully elucidated.

THERMOPHILIC PROKARYOTES

Abundance and Distribution

As it will be shown in what follows, hyperthermophilic Prokaryotes have been isolated mostly from debris or pieces of active smokers or hot sediments. Although the real habitat of these organisms is not totally known (see the section on Hydrostatic pressure), it is well documented that thermophilic and hyperthermophilic organisms are present within the mineral walls of the active black smokers. Their abundance has been evaluated using various methods, including lipid analysis, direct counts, cultures, and molecular probes.

Membrane lipids are often used as biomarkers to estimate bacterial abundance, or even to detect specific taxa. Lipids have been detected in all sections of a flange structure collected from the Juan de Fuca Ridge (40). Because Archaea typically possess lipids with ether bonds, the ether/ester lipid ratio is an indication of Archaea versus Bacteria abundance. In this example, archaeal lipids were present in all layers of the flange, with an exception of the external layers; the highest concentration was found in the middle parts. The ratio of diether to tetraether lipids increased from the coolest to the hottest sections analyzed. From the lipid concentrations measured, the abundance of prokaryotic cells within the flange was estimated at 10^6 to 10^8 cells per gram (41). Another set of measures indicated bacterial densities above 10^5 and archaeal densities in the range of 10^7 to 10^9 cells per gram of smoker wall (dry weight) (42). All these data are consistent with a distribution of bacteria mostly in the outer parts (cooler) of the smoker walls and archaea in the inner (warmer), with a decrease in the central parts (too hot).

Other estimations were based on direct cell counts using fluorochromes such as acridine orange or DAPI. Cell densities of about 10^9 to 10^{10} per gram (d.w.) were obtained for the outer parts of the walls, but only 10^5 per gram within the chimney wall; densities decreased below the level of detection (10^3 to 10^4) in the very inner parts of the samples (43).

Viable counts for anaerobic heterotrophic sulfur-reducing cocci were also performed and gave densities up to 3×10^6 cells per gram (d.w.) for heterotrophic sulfur-reducing cocci (44). These results were consistent with those obtained by the same research team using whole-cell hybridization with domain- and kingdom-specific oligonucleotide probes. The upper and outer parts of the sample appeared to be the most heavily colonized. Bacteria and Archaea cell numbers were almost similar and Crenarchaeota, when detectable, remained at a rather low level (maximum 19% of Archaea for the inner outer part of the sample).

Probes specific to the genus *Bacillus*, different *Thermus* species, the genus *Thermotoga*, and the order Aquificales were also used on the same samples (45). Although the two first genera had been detected by culture methods, no positive signals were noted using their specific probes, and only one sample appeared to contain cells hybridizing with the *Thermotoga* probe (also confirmed by enrichment cultures). The combination of the Aquificales-specific probe and a Bacteria-specific probe (that does not hybridize with the described species of Aquificales) made it possible to estimate that cells responding to these two probes (including cells of the genus *Desulfurobacterium* (46), see subsequently) may represent up to 40% of the bacterial population.

The Bacteria Domain

As has been reported earlier, the temperature gradient at deep-sea hydrothermal vents is particularly steep, between 350 °C within the active smokers to 2 °C, some decimeters away, for the cold deep-sea water. It is also estimated that the boundary between the oxic and

anoxic zones occurs at about 30 °C, which eliminates the possibility of a niche for aerobic (or facultative) thermophiles. However, certain active smokers (beehives), particularly at the Mid-Atlantic Ridge, have a porous structure that allows some entry of cold oxygenated water, and consequently the existence of some warm (60 to 70°C) oxygenated habitats within the beehives. These structures allowed us to isolate the first aerobic thermophiles from deep-sea vent habitats.

***Bacillus* sp.** Spore-forming facultative aerobic bacilli have been isolated from Mid-Atlantic Ridge smoker samples, from sediments collected in the Guaymas Basin, and from smoker samples from the Lau Basin (47). Although not completely characterized, these organisms were assigned to the genus *Bacillus*. They appear to differ from reference strains of thermophilic bacilli (with the exception of several strains from the Lau Basin) when compared by means of numerical taxonomy. Although it is always difficult to come to a conclusion regarding the real origin of spore-forming bacteria, particularly from aquatic habitats, the results of the numerical taxonomy study lead us to conclude the existence of novel and distinct thermophilic spore-forming communities at least in the Mid-Atlantic Ridge and Guaymas samples.

***Thermus* sp.** Bacteria belonging to the genus *Thermus* have been isolated from all thermal environments of the world, including man-made systems. For reasons reported earlier, aerobic thermophiles were not expected in the black smoker habitats until the beehive structures with potential habitats for such organisms were described. Non–spore-forming bacteria, growing aerobically and heterotrophically with a maximum temperature range from 70 to 80 °C, were isolated from the Mid-Atlantic Ridge beehives and Guaymas Basin sediments (48). From a numerical taxonomy study, several strains were assigned to the genus *Thermus*; one of them was later identified by means of DNA/DNA hybridizations as *Thermus thermophilus* (49), extending to the deep sea the already ubiquitous distribution of this organism.

***Thermotoga* sp.** Several species of Thermotoga (50) have been described from coastal hot springs and from other hot habitats such as oil reservoirs. This genus has rarely been reported from deep-sea vent habitats, and the strains, enriched or isolated, never identified.

Thermosipho melaniensis. *Thermosipho melaniensis* 5(51) has been isolated from the gills of a vent mussel *Bathymodiolus brevior* collected in the Lau Basin (1,887 m). This organism cannot be considered as a commensal of the mussel, rather it is an organism trapped within the particles collected by the filtration organs of this bivalve. The cells are gram-negative rods (0.4 to 0.6/1 to 3.5 μm), surrounded by a sheathlike structure and ballooning over the ends. They occur singly, in pairs, or in short chains of up to five cells. They are strict anaerobes and grow heterotrophically on complex organic substrates such as yeast extract or brain-heart infusion. Growth can be also supported by malt extract, tryptone, or carbohydrates, if supplemented with yeast extract. Hydrogen is a growth inhibitor, but inhibition is overcome by elemental sulfur respiration, and consequently hydrogen sulfide production. Growth occurs between 45 and 80 °C, with an optimum at 70 °C (doubling time 10 minutes). Optimum pH and salt concentration are 6.5 to 7.5 and 3%, respectively. DNA base composition is 30.5 mol% G + C. Type strain BI429 has been deposited at the Collection of Institut Pasteur (CIP 104,789).

Thermosipho japonicus. *Thermosipho japonicus* (52) was isolated from deep vents located in the Okinawa area (972 m). Cells are slightly curved nonmotile rods (0.5/3 to 4 μm), surrounded by a sheathlike structure. This organism is a chemoorganotroph and uses yeast extract, peptone, tryptone, or casein as carbon and energy sources. In addition to these complex substrates, this organism also uses maltose, glucose, galactose, starch, and so on. Growth is enhanced in the presence of sulfur or thiosulfate and is inhibited by hydrogen. The temperature range for growth is 45 to 80 °C with an optimum at 72 °C. The optimum pH is 7 to 7.5, and the optimum salt concentration is 4%. The DNA base composition is 31.4 mol% G + C. Type strain IHB1 has been deposited at the Japan Collection of microorganisms (JCM 10,495).

Desulfurobacterium thermolithotrophicum. This novel genus (46) was isolated from a smoker sample collected at the Mid-Atlantic Ridge (3,500 m). Cells are gram-negative and occur singly or in pairs as small highly motile rods (0.4 to 0.5/1 to 2 μm). They are obligate chemolithoautotrophs that use hydrogen as electron donor and sulfur, thiosulfate, or sulfite as electron acceptors. Growth occurs between 40 and 75 °C (optimum 70 °C, doubling time 135 minutes). The optimum pH is about 6.25, and the optimum salt concentration is 3.5%. The DNA base composition is 35 mol% G + C. Type strain BSA has been deposited at the German collection of microorganisms (DSM 11,699).

Marinitoga carminata. *Marinitoga* (53) represents a novel genus within the Thermotogales. It was obtained from a chimney sample collected at the Mid-Atlantic Ridge (980 m). Cells are rod-shaped with a sheathlike structure (0.5 to 1/1 to 3 μm). They are obligate anaerobes and chemoorganotrophs, fermenting gluten, peptone, brain-heart infusion, tryptone and saccharose, glucose, fructose, maltose, and cellobiose in the presence of yeast extract. Carbon dioxide, hydrogen, acetate, isovalerate, and isobutyrate are formed during glucose fermentation. Growth occurs between 25 and 65 °C with an optimum at 55 °C. The optimum pH is 7, and the NaCl concentration 2%. The DNA base composition is 29 mol% G + C. Type strain MV 1,075 has been deposited at the Collection of Institut Pasteur (CNCM I-2,413) and the German collection of microorganisms (DSM 13,578).

Caloranaerobacterazorensis. This novel genus (54) was obtained from a Mid-Atlantic Ridge chimney (1,650 m). Cells appear as motile rods (0.3 to 0.5/0.5 to 2 μm) by means of peritrichous flagella. They stain gram positive, but heat-resistant spores were never observed,

and cultures do not survive to heat exposure. 16S ribosomal DNA (rDNA) sequence analysis placed this organism within cluster XII of the genus *Clostridium*. This organism is strictly anaerobic and chemo-organotrophic, and ferments gluten, brain-heart infusion, glucose, starch, xylan, and pyruvate. Acetate, isovalerate, butyrate, and propionate are formed during glucose fermentation. The temperature range for growth is 40 to 70 °C (optimum 65 °C). The optimum pH is 7, and the optimum salt concentration is 2% NaCl. The DNA base composition is 27 mol% G + C, one of the lowest for a thermophilic anaerobic bacterium. Type strain MV 1,087 has been deposited at American Type Culture Collection (ATCC) and the German collection of microorganisms (DSM).

The Archaea Domain

Because of the very elevated temperatures of hot fluids recorded at deep-sea hydrothermal vents, many microbiologists focused on hyperthermophilic organisms and were rather successful with the isolation of novel genera and species of Archaea.

Methanopyrus kandleri. *Methanopyrus kandleri* (55) was isolated from sediment samples collected in the Guaymas Basin (2,000 m) and from a shallow marine hydrothermal system at Kolbeinsey Ridge (106 m, Iceland). Cells are rod-shaped with a gram-positive structure, 0.5 μm in diameter, and 2- to 14-mm long (average 8 to 10) occurring singly or in chains of up to 70 cells. Polar tufts of flagella make the cells motile. They are strictly anaerobic and form methane from hydrogen and carbon dioxide. Growth is stimulated by yeast extract and bacterial cell homogenates. H_2S is formed in the presence of sulfur, but cells tend to lyse. 16S rRNA sequence analysis indicated that this organism was not related to any of the three methanogenic lineages already known. Growth occurs between 84 and 110 °C with an optimum at 98 °C (doubling time: 50 minutes). The optimum pH and salt concentration are 6.5 and 2%, respectively. The DNA base composition is 60 mol% G + C. The type strain AV 19 has been deposited at the German collection of Microorganisms (DSM 6,324).

Methanococcus jannaschii. *Methanococcus jannaschii* (56) was obtained from a smoker collected on the East Pacific Rise at 20°50′ (2,600 m). Cells are irregular cocci, motile, with a complex flagella system. They are obligate anaerobes that produce methane from hydrogen and carbon dioxide. No organic compound is required, and do not stimulate growth. But selenium stimulates growth significantly, and sulfide is required. The temperature range for growth is 50 to 86 °C with an optimum at 85 °C for a doubling time of 25 minutes. The optimum pH is 6.0, and the optimum salt concentration is 0.5 M. The DNA base composition is 31 mol% G + C. The type strain JAL-1 has been deposited at the German collection of microorganisms (DSM 2,661).

Methanococcus infernus. *Methanococcus infernus* (57) is an autotrophic methanogen isolated from a hydrothermal chimney collected at the Mid-Atlantic Ridge (14°45′N, 3,000 m). Cells are coccoids (1 to 3 μm), single or in pair, and highly motile, with tufts of flagella. This organism is strictly anaerobic, and uses hydrogen and carbon dioxide as energy and carbon sources to produce methane. Growth is stimulated by selenate, tungstate, and yeast extract; sulfur is reduced to H_2S in the presence of carbon dioxide and hydrogen. Growth occurs between 55 and 91 °C with an optimum around 85 °C and a doubling time of 35 to 40 minutes. The optimum pH and salt concentration are 6.5 and 2.5%, respectively. The DNA base composition of the type strain is 33 mol% G + C. The type strain has been deposited at the German collection of microorganisms (DSM 11,812).

Methanococcus vulcanius. *Methanococcus vulcanius* (58) has been isolated from an active smoker collected on the East Pacific Rise (13°N, 2,600 m). It is an autotrophic methanogen, showing coccoid cells (1 to 3 μm), motile by means of three tufts of flagella. It is a strict anaerobe that produces methane from hydrogen and carbon dioxide as energy and carbon sources. Growth is stimulated by arsenate, tungstate, and yeast extract; hydrogen sulfide is produced from sulfur in the presence of hydrogen and carbon dioxide. Growth occurs between 49 and 89 °C, with an optimum at 80 °C (doubling time 45 minutes). The optimum pH and salt concentration are 6.5 and 2.5%, respectively. The DNA base composition of the type strain is 31 mol% G + C. The type strain has been deposited at the German collection of microorganisms (DSM 12,094).

Methanococcus fervens. This species (58) was first described as strain AG86, and was isolated from hot sediments collected in the Guaymas Basin (2,003 m). It was assigned to a novel species when chosen as a reference strain for the description of *M. vulcanius*. Cells are regular or irregular motile cocci. They use hydrogen and carbon dioxide to produce methane, and growth is stimulated by yeast extract, casamino acids, trypticase, selenite, and tungstate. Growth occurs between 48 and 92 °C, with an optimum at 85 °C (doubling time 20 to 30 minutes). The optimum pH and salt concentration are 6.5 and 3%, respectively. The DNA base composition of the type strain is 33 mol% G + C. Strain AG 86 is deposited at the German collection of microorganisms (DSM 4,213).

With the description of *Archaeoglobus fulgidus*, a novel lineage within the Euryarchaeota was reported, and was presented as a tentative missing link between the methanogens and the sulfur-metabolizing Archaea. Two novel species have been isolated from deep-sea vents.

Archaeoglobus profundus. *Archaeoglobus profundus* (59) was isolated from smokers and hot sediments collected at the Guaymas Basin (2,000 m). Similarly to *A. fulgidus, A. profundus* is a sulfate reducer which shows a blue-green fluorescence at 420 nm. Cells are nonmotile irregular cocci (about 1.3 μm). This species is mixotrophic: H_2 is required as an electron donor, but acetate, lactate, pyruvate, yeast extract, meat extract, or peptone can be used as organic carbon sources. Thiosulftate and sulfite may also serve as electron acceptors. Elemental sulfur is reduced, although it inhibits growth. The temperature range for growth is

65 to 90 °C with an optimum around 82 °C. The optimum pH and salt concentration are 6.0 and 1.8%, respectively. The DNA base composition is 41 mol% G + C. The type strain, AV18 has been deposited at the German collection of microorganisms (DSM 5,631).

Archaeoglobus veneficus. *Archaeoglobus profundus* (60) was isolated from black smoker samples collected at the Mid-Atlantic Ridge (3,500 m). Cells are very irregular cocci to triangular plate–shaped lobes (0.5 to 1.2 μm), and are motile with polar flagella. They show a blue-green fluorescence at 436 nm. They grow chemolithoautotrophically with molecular hydrogen as the electron donor and sulfite or thiosulfate as the electron acceptors. They show organotrophic respiration with sulfite as the electron acceptor, and formate, acetate, pyruvate, isopropanol, ethanol, fumarate, and glucose as the electron donors and carbon sources. They never use sulfate, nitrate, or nitrite as electron acceptors; elemental sulfur inhibits growth. End products are hydrogen sulfide and, to a lesser extent, methane. Growth occurs between 65 and 85 °C (optimum 80 °C), and optimally at pH 7 and 2% NaCl. The DNA base composition is 45 mol% G + C. The type strain SNP6 has been deposited at the German collection of microorganisms (DSM 11,195).

Among all thermophilic and hyperthermophilic organisms isolated so far, Thermococcales are represented by a large number of species. Several reasons may be suggested to explain this situation. Thermococcales are probably abundant within geothermally heated systems where they show a great specific diversity; they are relatively easy to handle in the laboratory (although strict anaerobes, they resist to exposure to oxygen at temperatures below temperatures allowing growth), they are organotrophic, grow rather quickly and represent a great potential for thermophilic enzymes, and consequently have been enriched intensively by microbiologists. They are distributed within three genera: *Pyrococcus, Thermococcus*, and *Palaeococcus*.

Pyrococcus abyssi. *Pyrococcus abyssi* (61) was isolated from a fluid sample (more probably a chimney particle contained in the sample) collected in the North Fiji Basin (2,000 m). Cells are slightly irregular cocci (0.8 to 2 μm), motile by means of a polar tuft of flagella. They are obligate anaerobes, fermenting organic compounds (tryptone, gelatin, amino acids). H_2 produced during fermentation inhibits growth, but addition of elemental sulfur, cystine, or polysulfides in the medium prevents inhibition. In that case, H_2S and mercaptans are produced. Growth occurs between 67 and 102 °C (optimum 96 °C, 33 minutes doubling time). The optimum pH and salt concentration are 6.8 and 3%, respectively. The DNA base composition is 45 mol% G + C. Type strain GE5 has been deposited at the Collection of Institut Pasteur (France) (CNCM I-1,302).

Pyrococcus horikoshii. *Pyrococcus horikoshii* (62) was isolated from hydrothermal fluids collected at the Okinawa Trough vents at 1,395 m. Cell morphology, motility, and metabolism are rather similar to *P. abyssi*. However, *P. horikoshii* requires tryptophan for growth. Growth occurs between 80 and 102 °C, with an optimum at 98 °C. The optimum pH and salt concentration are 7 and 2.4%, respectively. Elemental sulfur is not essential, but greatly stimulates growth. The DNA base composition is 44 mol% G + C. The type strain has been deposited at the Japanese collection (JCM 9,974).

Pyrococcus glycovorans. This species is very similar to the two other deep-sea *Pyrococcus* with respect to its morphology and metabolism. However, in addition to proteinaceous compounds, *Pyrococcus glycovorans* (63) ferments carbohydrates, particularly glucose. Growth occurs between 75 and 104°C, with an optimum at 95 °C. The optimum pH and salt concentration are 7.5 and 3%, respectively. The DNA base composition is 47 mol% G + C. Type strain AL 585 has been deposited in the Institut Pasteur collection with the number CNCM I-2,120.

Thermococcus profundus. This strain (64) was isolated from a vent system at the middle Okinawa Trough (1,395 m). Morphologically this organism is very close to the earlier-mentioned species of *Pyrococcus* (coccoid motile cells of 1 to 2 μm). It is also an anaerobic chemorganotroph that ferments organic compounds (tryptone, peptone, casein, gelatin, yeast extract or pyruvate, maltose, starch). Growth was observed between 50 and 90 °C, with an optimum at 80 °C. The optimum pH and salt concentration are 7.5 and 2 to 4%, respectively. The DNA base composition is 52.5 mol% G + C. Type strain is numbered DT 5,432.

Thermococcus chitonophagus. This species (65) was obtained from a sample collected at 20°50′N on the East Pacific Rise, containing smoker material and vestmentifera tube pieces, inoculated into a chitin-supplemented medium. In addition to features common to the Thermococcales described earlier, *Th. chitonophagus* ferments chitin with hydrogen, carbon dioxide, NH_3, acetate, and formate as end products, plus hydrogen sulfide when grown in the presence of sulfur. Growth occurs between 60 and 93 °C (optimum at 85 °C). The optimum pH and salt concentration are 6.7 and 2%, respectively. The DNA base composition is 46.5 mol% G + C. The type strain GC74 has been deposited at the German collection (DSM 10,152).

Thermococcus peptonophilus. *Thermococcus peptonophilus* (66) was isolated from hydrothermal systems in the western Pacific Ocean (Izu-Bonin and South Mariana Trough Areas). The species is distinct from other *Thermococcus* as it only grows on complex protein compounds and peptides, and cannot use amino acids, organic acids, or carbohydrates. Temperature range for growth is 60 to 100 °C (optimum 85 °C). The optimum pH and salt concentration are 6 and 3%, respectively. The DNA base composition is 52 mol% G + C. Type strain OG-1 is registered at the Japanese collection of microorganisms (JCM 9,653).

Thermococcus fumicolans. *Thermococcus fumicolans* (67) was isolated from the North Fiji Basin (2,000 m).

Although rather similar by certain features to the *Thermococcus* species described earlier, *Th. fumicolans* exhibits some specific physiological features: temperature range for growth is 73 to 103 °C (optimum 85 °C); the optimum pH is 8, and the optimum salt concentration varies from 1.3 to 2.6% NaCl. The DNA base composition is 54 to 55 mol% G + C. Type strain ST557 has been deposited at the Collection of Institut Pasteur (CIP 104,680).

Thermococcus hydrothermalis. *Thermococcus hydrothermalis* (68) was obtained from a smoker sample collected at 21°N on the East Pacific Rise. The strain was selected among other Thermococcales for the presence of a nicotinamide adenine dinucleotide phosphate–dependent alcohol dehydrogenase activity. Although close to other known *Thermococcus* species, *Th. hydrothermalis* was assigned to a novel species that grows between 55 and 100 °C (optimum 85 °C); the optimum pH is 6, and the optimum salt concentration is 3 to 4%. The DNA base composition is 58 mol% G + C. Type strain AL662 is registered at the Insitut Pasteur Collection (CNCM 11,319).

Thermococcus guaymasensis. This species (69) has been isolated from hot marine sediments at the Guaymas Basin hydrothermal vent site (2,000 m depth) in the Gulf of California. Cells are coccoids (1.0 to 3.0 μm), nonmotile, and occur mainly as single cells or as diploid forms. They are strict anaerobes and use proteinaceous substrates (yeast extract, and trypticase, but also casein, dextrose, maltose, and starch) in the presence of elemental sulfur. With yeast extract, trypticase, and sulfur, the end products are acetate, propionate, isobutyrate, isovalerate, carbon dioxide, and H_2S, whereas with the other substrates, only acetate, carbon dioxide, and H_2S are formed. Growth occurs from 56 to 90 °C, with an optimum at 88 °C. The optimum pH and salt concentration are 7.2 and 3.0%, respectively. The DNA base composition is 46.0 mol% G + C. The type strain has been deposited at both the German and the Japanese collections of microorganisms (DSM 11,113 and JCM 10,136, respectively).

Thermococus aggregans. *Thermococcus aggregans* (49) was isolated from a hot sediment sample collected in the Guaymas Basin (2,000 m). Cells are coccoid (1.0 to 1.5 μm), nonmotile, occur as single cells or as diploid forms, but may produce aggregates of up to 50 cells. Substrates and end products are similar to those used and produced by *Th. guaymasensis*. Growth occurs from 60 to 94 °C with an optimum at 88 °C. The optimum pH and salt concentration are 7.0 and 2.5%, respectively. The DNA base composition is 42.0 mol% G + C. The type strain has been deposited at the German and the Japanese collections of microorganisms (DSM 10,597 and JCM 10,137, respectively).

Thermococcus barossii. *Thermococcus barossii* (70) was obtained from rock fragments of a hydrothermal vent flange formation located at the Juan de Fuca Ridge. Cells have the typical coccoid morphology of Thermococcales but are apparently nonmotile. For this species elemental sulfur is required whereas it only stimulates growth of other deep-sea *Thermococcus* species. Temperature range for growth is 60 to 92 °C, with an optimum at 82.5 °C. The optimum pH varies from 6.5 to 7.5, and cell density was not affected by salt concentrations between 1 and 4% NaCl. The DNA base composition is 60 mol% G + C.

Thermococcus siculi. *Thermococcus siculi* (71) was obtained from the Mid-Okinawa Trough hydrothermal vents (1,394 m). Although it may resemble several already described species of *Thermococcus* in some criteria, *Th. siculi* features do not totally match with those species, and this has been confirmed by DNA/DNA hybridizations with close species. Growth only occurs on proteinaceous substrates in the temperature range 50 to 93 °C, with an optimum at 85 °C. The optimum pH is 7.0, and salt concentrations between 1 and 4% NaCl are required (lysis occurs for concentrations below 1%). The DNA base composition is 55.8 mol% G + C. The type strain has been deposited at the German collection (DSM 12,349).

Thermococcus barophilus. *Thermococcus barophilus* (72) has been isolated from a smoker sample collected at the Mid-Atlantic Ridge (3,500 m). Morphologically and metabolically it is a very classic *Thermococcus* species. However, it has been purified after enrichment and subcultures, all performed under elevated hydrostatic pressure (40 MPa). Although it also grows under atmospheric pressure in a temperature range of 48 to 95 °C, with an optimum at 85 °C, its growth rate under hydrostatic pressure is twofold higher, for the same optimum at 85 °C. Hydrostatic pressure is required for growth between 95 and 100 °C. The DNA base composition is 37.1 mol% G + C. Type strain MP has been deposited at the Collection of Institut Pasteur (CNCM I-1,946).

Palaeococcus ferrophilus. This strain (73) representing a novel genus within the Thermococcales was isolated from a smoker collected in Ogasawara-Bonin Arc (1,388 m). Although it closely resembles to other Thermococcales, it is physiologically different by its requirement for sulfur, especially ferric iron. In the latter case, H_2 is produced during fermentation of proteinaceous substrates. It is noticeable that contrary to other microorganisms, including several hyperthermophilic Archaea that utilize ferrous iron as an electron donor or ferric iron as an electron acceptor, *Palaeococcus ferrophilus* does not use iron in such a way. Although the role of iron has not been totally elucidated, it has been suggested that "relatively high concentrations of ferrous iron might be required for proper operation of the iron(II)-regulated fermentation pathways of *P. ferrophilus*." Temperature range for growth of this species is 60 to 88 °C, with the optimum being 83 °C. The optimum pH and salt concentration are 6.0 and 4.3%, respectively. The DNA base composition is 53.5 mol% G + C. Type strain DMJ has been deposited at the Japan Collection of microorganisms (JCM 10,246).

***Desulfurococcus* sp.** Four species of *Desulfurococcus* (74) have been isolated from nonabyssal hot environments, but deep-sea strains assigned to this genus have never been identified fully. Cells are irregular cocci and

may or may not be motile. They have been reported from various vent sites in the Atlantic and Pacific oceans. They are strictly anaerobes, chemoorganotrophs, and only ferment complex proteinaceous substrates, preferentially but not obligately in the presence of sulfur. They grow in the range of 50 to 95 °C with an optimum at 85 or 90 °C.

Staphylothermus marinus. *Staphylothermus marinus* (75) has been isolated from coastal geothermal areas, but also from the East Pacific Rise at 11°N (2,500 m). Cells are slightly irregular cocci, nonmotile, occurring singly, in pairs or in chains and aggregates up to 100 cells. Cell size may vary from 0.5 to 15 μm according to culture conditions. They are strictly anaerobes and sulfur dependent. They grow heterotrophically on complex proteinaceous substrates, forming carbon dioxide, acetate, isovalerate, and H_2S. Growth occurs between 65 and 98 °C with an optimum at 92 °C and a doubling time of 270 minutes. The optimum pH is 6.5, and the optimum salt concentration is 1.5%. The DNA base composition is 35 mol% G + C. Type strains F1 and Al2 have been deposited at the German collection of microorganisms (DSM 3,639 and DSM 3,666, respectively).

Pyrodictium abyssi. This novel species of *Pyrodictium* (76) was isolated from hot sediments and smokers collected in the Guaymas Basin (2,000 m) but also from hot sediments collected at the Kolbeinsey Ridge (Iceland, 103 to 106 m). Cells are polymorphous disk-shaped (1 to 2 μm) with flat protrusions. This species is strictly heterotrophic and ferments proteins, carbohydrates, cell extracts, acetate, and formate, growth being stimulated by H_2. Fermentation products from yeast and meat extracts and H_2 are isovalerate, isobutyrate, butanol, and carbon dioxide. In the presence of H_2, H_2S is formed from S^0 or $S_2O_3^{2-}$. Growth occurs between 80 and 110 °C (optimum 97 °C); the optimum pH is 5.5, and the optimum salt concentration is 2% NaCl. The DNA base composition is 60 mol% G + C. Type strain AV2, isolated from the Guaymas Basin, has been deposited at the German collection of microorganisms (DSM 6,158).

Pyrolobus fumarii. *Pyrobolus fumarii* (77) was isolated from a chimney sample collected at the Mid-Atlantic Ridge (3,650 m). Cells are nonmotile, irregular coccoid-shaped (0.7 to 2.5 μm). This novel genus is a strict chemolithoautotroph using H_2 as the electron donor under microaerophilic conditions or anaerobic conditions with NO_3^- or $S_2O_3^{2-}$ as the electron acceptors. Growth is not stimulated by organic compounds and is even inhibited by acetate, pyruvate, glucose, starch, or elemental sulfur. Growth occurs between 90 and 113 °C with an optimum at 106 °C (doubling time 60 minutes). The optimum pH is 5.5, and the optimum salt concentration is 1.7%. *Pyrobolus fumarii* tolerates a pressure of 25 Mpa, and exponential cultures survived 1 hour autoclaving at 121 °C. The DNA base composition is 53 mol% G + C. Type strain 1A has been deposited at the German collection of microorganisms (DSM 11,204).

Molecular Diversity

It is a well-known fact that despite the best efforts and imagination of microbiologists, culture methods do not permit us to obtain a pure culture of all of the living microbial species within a single sample. The list of organisms reported earlier does not reflect the total and true diversity of hydrothermal vent microbial communities (78). The ability to extract total DNA from a sample, and then to amplify 16S rDNA genes is considered as an alternative method for the estimation of microbial diversity.

This approach has been used in the case of smoker samples, but not very successfully until recently. The main problem encountered was the extraction of DNA from smoker samples, which failed in part because of the patchy distribution of the microbial communities within the mineral structures, but also because of the strong attachment of cells to minerals and/or chemical interactions (78). However, it was recently reported that some authors were successful in extracting and measuring up to 80 μg of DNA per gram of chimney (42), and in detecting novel methanogens using amplification of the *mcrA* gene from DNA extracted from freshly collected samples of an active smoker (79). Takai and Horikoshi were able to obtain DNA from hydrothermal water and chimney samples collected in the Ogasawara and Okinawa areas and conduct a molecular phylogenetic analysis of the archaeal communities (80). Novel phylotypes distinct from other cultivated archaea or environmental clones were obtained. These clones were mostly placed in the Euryarchaeota, but a few were placed in the Crenarchaeota. Interestingly, several clones were placed close to *Picrohilus* and *Thermoplasma*, two acidophilic thermophiles.

An alternative to DNA extraction from chimney samples involved the deployment of the "vent caps," in situ growth chambers, on the top of active smokers with intermediate temperatures (81,15). This approach allows the investigator to extract DNA from coupons exposed within the growth chamber and colonized by microorganisms. Again unique novel rRNA sequences were obtained. These corresponded to members of the Thermococcales or Archaeoglobales, well known from the vent environments, and also to members of the Thermoplasmales, confirming that thermoacidophiles may be present in the vent ecosystems, although they have so far escaped all attempts at being cultured.

Genetic Elements

The first genetic elements found in deep-sea hyperthermophiles were plasmids from the methanogen *Methanococcus jannaschii* and a relative strain AG-86, with sizes of 64, 18, and 20 kb (82). Plasmids of sizes ranging from 3.5 to 24 kb were also found when screening a collection of 57 strains of Thermococcales (83). The plasmid pGT5 from *P. abyssi* has been studied more intensively (84). As it was the first discovered within a phylogenetic group studied intensively for basic and biotechnological research, this plasmid presently serves as a base for the construction of shuttle vectors. It is a

3.5-kb cryptic plasmid that has been sequenced fully. Two open reading frames were detected, one of them coding for a protein involved in the replication through a rolling circle mechanism (85). This plasmid has also been used for DNA topology experiments (84) where it was shown to be in the relaxed state at physiological temperatures despite the presence in this archaeon of a reverse gyrase that was expected to favor a positively supercoiled DNA. Further experiments showed that the plasmid topology hyperthermophilic archaea may vary from a relaxed to a positively supercoiled state (all mesophiles, including Archaea, have their DNA in a negatively supercoiled state), depending on growth phase and temperature variations (86). However, the plasmid pGS5 from *A. profundus* (87) was found negatively supercoiled, probably because of the coexistence of histones, reverse gyrase, and gyrase in this organism.

Responses to Hydrostatic Pressure

Deep-sea hydrothermal vents have been documented at depths ranging from 800 to 3,500 m (10). At each of these vents, hydrostatic pressure is 80 to 350 bars or 8 to 35 MPa. The responses of some continental mesophiles (such as *Escherichia coli*) or deep-sea psychrophiles to elevated hydrostatic pressure have been well documented. However, very little is known about the response of thermophiles and even deep-sea thermophiles to this parameter. Different types of responses have been observed by several authors (88). For instance, it was shown that elevated pressure increased the maximum temperatures for growth of several Thermoccoccales strains, including *Pyrococcus abyssi*, from 1 to 4 °C. The optimum temperature for growth was also shifted up from 2 to 4 °C; the growth rate increased as well (61). Similar experiments were carried out for a few methanogenic strains, with the added difficulty that these organisms require a CO_2/H_2 headspace that prevents the efficient pressurization of the liquid medium. (A common technique for pressure studies consists in culturing the cells within a glass syringe, without head space above the liquid medium. The syringe is immersed into a high-pressure reactor, filled up with water, and connected to a pump. Pressurization of water within the reactor transmits the pressure to the culture through the piston of the syringe.) It was noted that a pressure of 200 bars decreased the growth rate of deep-sea thermophilic methanogens strains CS1 and FS (89). However, pressure of up to 750 bars was shown to increase growth rate and methanogenesis of *M. jannaschii* at 86 and 90 °C, when helium was used as the pressurizing gas (90). Using argon or H_2/CO_2 as the pressurizing gas, methanogenesis did not occur above 86 °C. After an examination of the studies dealing with responses of deep-sea hyperthermophiles to hydrostatic pressure, Deming and Baross (91) distinguished four different behaviors. For barosensitive strains, high pressure reduces growth rate; for barotolerant organisms, growth rate is unaffected by pressure; for barophilic strains, growth rate is stimulated by high pressure. The fourth category, obligate barophiles, corresponds to organisms that can grow only under elevated pressure. For instance, *P. abyssi* is an obligate barophile between 102 and 106 °C, as it cannot grow above 102 °C under atmospheric pressure; its maximum temperature for growth is shifted up under elevated pressure, and growth for this supraoptimal temperature is only possible when elevated hydrostatic pressure is applied.

It must be noted that all deep-sea hyperthermophiles exposed to elevated hydrostatic pressures had been isolated previously under atmospheric pressure (or under a slight gas pressure that prevents boiling of the culture medium). Recently, *Th. barophilus* was isolated after enrichment and subcultures were all carried out under elevated pressure (40 MPa) (72). This organism, which can grow also under atmospheric pressure, showed an interesting behavior. The maximum temperature (but not the optimum) for growth was enhanced by pressure, and, in particular, the growth rate was increased twofold. The total protein profiles of the cells grown either under atmospheric or under hydrostatic pressures were studied and compared (92). Under pressure, a 60-kDa protein was expressed, which is still unknown. But under atmospheric pressure, a 35-kDa protein was expressed, which appeared to be close to a heat shock protein reported previously for the hyperthermophilic archaeon *Desulfurococcus* strain SY. This would indicate that this organism is in a stress situation when it grows under atmospheric pressure. Deming and Baross (91) also noted that for most of the strains showing a barophilic behavior, the optimum pressure for growth was above the pressure existing where the sample from which the strains had been isolated were collected. For instance, *P. abyssi* was obtained from a North Fiji Basin vent site at 2,000 m (pressure: 200 bars), but its optimum pressure for growth was 400 bars. This is in contrast to the situation with deep-sea psychrophiles, where it has been shown that the optimum pressure for growth was always below the pressure existing at the sampling depth (93). From these observations, it was suggested that black smokers could represent "windows to a deep biosphere." If such a subterranean biosphere really exists, its inhabitants would be exposed to an elevated pressure (hydrostatic and lithostatic), higher than the pressure existing at the sea floor. Arguments for the existence of this novel biotope were given in the report of thermophilic and hyperthermophilic microorganisms in moderate temperature hydrothermal fluids (3 to 30 °C) following a deep-sea volcanic eruption (94,95).

FUTURE RESEARCH

Thirty years ago, deep-sea hydrothermal vents were unknown. Since then, 40 sites have been discovered and explored, which is a lot, but also very few if one is aware of the fact that oceanic ridges have a total length of 60,000 km. Although many of these sites, but not all, have been studied by microbiologists, a significant level of research effort is still needed to fully understand these fascinating ecosystems. It is obvious that low and moderate temperature habitats have been studied very little. About the microbiological aspects of symbiosis, several points must be clarified, including symbiont transmission, ecology and physiology of free-living states of the symbionts, cell recognition

mechanisms, or prokaryotic cell division control by the host.

Apparently much more is known about thermophilic prokaryotes. But despite a rather long list of novel species, the first attempts at a molecular approach to thermophilic microbial diversity indicate that many novel phylotypes exist, whose whole organisms are yet to be cultured (for instance thermoacidophiles). Novel culture techniques (with imaginative sets of electron donors, acceptors, and carbon sources) must be developed (continuous cultures, mixed cultures, nutrient gradients, immobilized cells, etc.).

An exploration effort also must be encouraged to investigate other sites, particularly deeper sites that certainly exist, where more barophilic organisms may exist. The hypothesis of a deep sub–sea floor biotope also will require many efforts to be supported. Deep drilling in areas surrounding active vents should be organized in the future. Though the active exploration of the solar system is presently scheduled for the twenty-first century, the exploration of microbial diversity of planet Earth is not yet completed, and must go on.

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HYPERTHERMOPHILES

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Over the past few decades, hyperthermophilic microorganisms (those with optimum growth temperatures of 80 °C and above) have been isolated from a number of geothermally heated habitats, including shallow terrestrial hot springs, deep wells, sediment from volcanic islands, and deep-sea hydrothermal vents (Table 1; 1–5). A significant number of isolates, representing a broad spectrum of growth physiologies (e.g., methanogens, fermentative anaerobes, sulfur-reducers, sulfate-reducers, microaerophiles), have successfully been propagated in laboratory pure culture (6). This capability has facilitated metabolic studies and efforts to examine scientific and technological issues associated with the intrinsically thermostable and thermoactive proteins produced by these unique microorganisms (4,7,8). Because of their small genome sizes (typically ~2 Mb), interest in their evolutionary significance (9) and biotechnological potential (3,10,11), a relatively large amount of genome sequence information currently exists for these organisms (see Table 2). In fact, efforts are underway to further expand these databases (12). This situation presents some important opportunities. Although genetic systems for these organisms are currently unavailable in forms that would be readily useful for physiological studies, other approaches, including DNA microarrays and proteomics (13) coupled with genome sequence data, could be used to help decipher novel features underlying high-temperature microbial lifestyles (14). No doubt such efforts will also lead to the identification of interesting biocatalysts whose function in extreme environments is an important technological attribute (7). Paramount to such efforts to utilize genomic data is the capability of culturing hyperthermophiles in experimentally meaningful ways. This is particularly important because nearly half of the ORFs in hyperthermophilic genomes sequenced to date cannot be linked to a particular function (Table 2; 12).

HYPERTHERMOPHILIC ENVIRONMENTS

The natural habitats from which hyperthermophiles have been isolated often contain steep thermal gradients and have distinctive chemical and physical characteristics. Biotopes that have provided hyperthermophiles may be shallow or deep, terrestrial or marine (Table 1; 5). Shallow terrestrial environments, including hot springs, mud holes and solfaterras, are usually located in proximity to regions of significant geothermal activity and are widespread geographically. Such biotopes can be found in, for example, Iceland, New Zealand, Russia, Japan, Italy and the United States. An interesting feature of some hot, shallow terrestrial biotopes is the variation in pH encountered. For example, in Yellowstone National Park in the northwestern United States, hyperthermophiles can be isolated from sites with moderately alkaline pH (up to approximately pH 9) and from extremely acidic pH (down to approximately pH 1) (88). Hyperthermophiles have also been found in the deep, terrestrial subsurface while drilling oil reservoirs in places such as Alaska (89). Many hyperthermophiles have been isolated from readily accessible, shallow marine springs in locations such as Vulcano Island, Italy (62), and the Azores. Probably the most intriguing source of hyperthermophiles has been deep-sea hydrothermal vent environments that give rise to temperatures ranging from cold seawater at 2 °C to superheated hydrothermal fluids at more than 400 °C. Within this temperature gradient, a complex, nonphotosynthetic ecosystem operates that not only includes hyperthermophiles but a variety of microscopic and macroscopic life forms. Although new high-temperature habitats continue to be discovered, both marine and terrestrial, a relationship between geographic diversity and biodiversity has yet to be established.

ISOLATION AND CULTIVATION OF HYPERTHERMOPHILES

Discovering new hyperthermophiles and obtaining them in pure cultures for further study continues to be a central focus in this field. Although it is clear from phylogenetic fingerprinting that the biodiversity of hyperthermophiles in samples collected from natural environments is extensive (90), extracting selected species from these samples for further microbiological study can be an elusive undertaking. Whether many such members of the hyperthermophilic communities are viable but not culturable remains to be seen (91).

Table 1. Hyperthermophilic Archaea and Bacteria

Order	Genus	Species	Ref	Sample Site	Morphology	T_{opt} (°C)	pH Range (opt.)	Aerobic/ Anaerobic	G/C Content
Bacteria									
Thermotogales	*Thermotoga*	*T. maritima*	15	Marine mud	Rods with sheath	80	5.5–9 (7)	An	46
		T. neapolitana	16	Submarine hot springs	Rods with sheath	80	5.5–9 (7)	An	41
Aquificiales	*Aquifex*	*A. pyrophilus*	17	Hot marine sediments	Rods	85	5.4–7.5 (6.8)	Ae	40
		A. aeolicus	17	Hot marine sediments	Rods	85	N/A	Ae	43.4
	Thermocrinis	*T. ruber*	18	Pink filaments of Octopus Spring	Rods	80	7–8.5	Ae	47.5
Archaea									
Sulfolobales	*Sulfolobus*	*S. acidocaldarius*	19	Acid hot springs	Lobed cocci	75	1–5.9 (2)	Ae	37
		S. solfataricus	20	Volcanic hot spring	Lobed cocci	70	3.2–5.0 (4.5)	Ae	36
		S. shibatae	21	Geothermal pools and mud pots	Cocci	81	(3)	Ae	35
		S. yangmingensis	22	Acidic, muddy hot spring	Lobed cocci	80	2.0–6.0 (4.0)	Ae	42
	Acidianus	*A. infernus*	23	Geothermal spring	Lobed cocci	88	1.0–5.5 (2)	Ae/An	31
		A. ambivalens	24,25	Solfataric field	Lobed cocci	80	1–3.5 (2.5)	Ae/An	32.7
	Stygiolobus	*S. azoricus*	26	Hot springs	Lobed cocci	80	1–5.5 (5.5–3.0)	An	38
	Sulfurisphaera	*S. ohwakuensis*	27	Acidic hot springs	Cocci	85	1.0–5.0 (2.0)	An	33
Thermoproteales	*Thermoproteus*	*T. tenax*	28	Acidic hot springs	Regular rods	88	1.7–6.5 (5)	An	55.5
		T. neutrophilus	29	Hot springs	Rods	88	5.5–7.5 (6.5)	An	56
		T. uzoneinsis	30	Hot springs	Rods	90	4.6–6.8 (5.6)	An	56.5
	Pyrobaculum	*P. islandicum*	31	Solfataric field in Iceland	Rods	100	5–7 (6)	An	62
		P. organotrophum	31	Solfataric field in Iceland	Rods	100	5–7 (6)	An	51
		P. aerophilum	32	Boiling marine water	Rods	100	5.8–9 (7)	Ae/An	52
		P. oguniense	33	Terrestrial hot springs	Rods	90–94	5.4–7.5 (6.3–7.0)	Ae/An	48
		P. arsenaticum	34	Terrestrial hot springs	Rods	95	(6)	An	58.3
	Thermofilum	*T. pendens*	35	Solfataric hot springs	Slender with regular rods	88	4–6.5 (5.2)	An	57.4
		T. librum	29	Hot springs (neutral pH)	Filamentous rod	N/A	N/A	An	57
Desulfurococcales	*Desulfurococcus*	*D. mobilis*	36	Solfatraric hot springs	Cocci with flagellae	85	2.2–6.5 (6)	An	50.8
		D. mucosus	36	Solfatraric hot springs	Cocci	85	2.2–6.5 (6)	An	51.3
		D. saccharovorans	29	Solfatraric hot springs	Cocci	85	4.5–7 (6)	An	50
		D. amylolyticus	37	Hot volcanic vents	Cocci	90–92	5.7–7.5 (6.4)	An	42.1
	Staphylothermus	*S. marinus*	38	Shallow marine sediments	Cocci in aggregates	92	4.5–8.5 (6.5)	An	35
		S. hellenicus	39	Shallow hydrothermal vents	Irregular cocci with aggregates	85	4.5–7.0 (6)	An	38
	Sulfophobococcus	*S. zilligii*	40	Hot alkaline spring	Irregular cocci	85	6.5–8.5 (7.5)	An	54.7
	Stetteria	*S. hydrogenophila*	41	Sediment of hydrothermal system	Irregular cocci	95	4.5–7.0 (6.0)	An	65
	Aeropyrum	*A. pernix*	42	Coastal solfataric vent	Irregular cocci	90–95	5–9 (7)	Ae	67
	Igneococcus	*I. islandicus*	43	Submarine hydrothermal vents	Irregular cocci	90	3.8–6.5 (5.8)	An	41
		I. pacificus	43	Submarine hydrothermal vents	Irregular cocci	90	4.5–7.0 (6.0)	An	45
	Thermosphaera	*T. aggregans*	44	Mud volcano area in Yellow stone	Regular cocci	85	5.0–7.0 (6.5)	An	46
	Thermodiscus	*T. maritimus*	29	Submarine solfataric field	Irregular dish, discs	90	5–7 (5.5)	An	49

Table 1. (*Continued*)

Order	Genus	Species	Ref	Sample Site	Morphology	T_{opt}(°C)	pH Range (opt.)	Aerobic/ Anaerobic	G/C Content
	Pyrodictium	*P. occultum*	45	Submarine solfataric field	Discs with fibers	105	4.5–7.2 (5.5)	An	62
		P. brockii	45	Submarine solfataric field	Discs with fibers	105	4.5–7.2 (5.5)	An	62
		P. abysii	46	Hydrothermal vent	Polymorphous disk-shaped	97	4.7–7.1 (5.5)	An	60
	Hyperthermus	*H. butylicus*	47	Sea floor	Irregular cocci with pili	95–106	(7)	An	56.5
	Pyrolobus	*P. fumarii*	48	Deep-sea hydrothermal vent	Lobed cocci	106	4.0–6.5 (5.5)	An	53
Thermococcales	*Thermococcus*	*T. celer*	49	Solfataric marine water	Cocci	87	4–7 (5.8)	An	57
		T. litoralis	50	Shallow marine sediments	Cocci	88	4.0–8.0 (6.0)	An	38
		T. profundus	51	Deep-sea hydrothermal vent	Cocci	80	4.4–8.5 (7.5)	An	52.2
		T. alcaliphilus	52	Shallow hydrothermal system	Cocci	85	6.5–10.5 (9.0)	An	43
		T. chitonophagus	53	Deep hydrothermal vent	Irregular cocci	85	3.5–9 (6.7)	An	46.5
		T. fumicolans	54	Deep-sea hydrothermal vent	Cocci	85	4.5–9.5 (8.0)	An	54
		T. peptonophilus	55	Deep-sea hydrothermal vent	Cocci	85	4.0–8.0 (6.0)	An	52
		T. acidaminovorans	56	Shallow marine sediments	Coccoid-shaped	85	5.0–9.5 (9)	An	49
		T. aegaeicus	39	Shallow hydrothermal vents	Irregular cocci	88–90	4.5–7.5 (6)	An	45
		T. aggregans	57	Hydrothermal vent	Irregular cocci	88	5.6–7.9 (7.0)	An	42
		T. barophilus	58	Hydrothermal vent	Irregular cocci	85	4.5–9.5 (7.0)	An	37.1
		T. barossii	59	Hydrothermal vent	Irregular cocci	82.5	4–9 (6.5–7.5)	An	60.0
		T. gorgonarius	60	Geothermal vent	Irregular cocci	80–88	(6.5–7.2)	An	50.6
		T. siculi	61	Deep hydrothermal vent	Cocci	85	5.0–9.0 (7.0)	An	55.8
	Pyrococcoccus	*P. furiosus*	62	Heated marine sediments	Cocci	100	5–9 (7)	An	38
		P. woesei	63	Heated marine sediments	Elongated cocci	100–103	(6–6.5)	An	37.5
		P. abysii	64	Hydrothermal vent	Irregular cocci	96	4.0–8.5 (7.0)	An	45
		P. glycovorans	65	Hydrothermal vent	Cocci	95	6.5–8.5 (7.0)	An	47
		P. horikoshii	66	Hydrothermal vent	Irregular cocci	98	5–8 (7.0)	An	44
Archaeoglobales	*Archaeoglobus*	*A. fulgidus*	67	Heated sea floor	Irregular cocci	83	5.5–7.5	An	46
		A. profundus	68	Deep-sea hydrothermal vent	Irregular cocci	82	4.5–7.5 (6.0)	An	41
		A. veneficus	69	Deep-sea hydrothermal vent	Irregular cocci	75–80	6.5–7.0 (6.5)	An	45
	Ferroglobus	*F. placidus*	70	Hydrothermal vent	Irregular cocci	85	6.0–8.5 (7.0)	An	43
Methanobacteriales	*Methanothermus*	*M. fervidus*	71	Hot solfataric spring	Rods	83	¡7 (6.5)	An	33
		M. sociabilis	72	Solfataric mud hole	Rods in clusers	88	5.5–7.5 (6.5)	An	33
Methanococcales	*Methanococcus*	*M. thermolithotrophicus*	73	Heated-sea sediments	Cocci	65	6.0–8.0 (6.5–7.5)	An	31.3
		M. jannaschii	74	Submarine hydrothermal vent	Irregular cocci	80–85	5.2–7.0 (6.2)	An	31
		M. igneus	75	Marine hydrothermal system	Irregular cocci	88	5–7.5 (5.7)	An	31
		M. fervens	76	Deep hydrothermal vent	Regular-irregular cocci	85	5.5–7.6 (6.5)	An	33
		M. vulcanius	76	Deep hydrothermal vent	Cocci	80	5.25–7 (6.5)	An	31
		M. infernus	77	Deep hydrothermal vent	Cocci	85	5.25–7 (6.5)	An	33
Methanopyrales	*Methanopyrus*	*M. kandleri*	78	Heated deap sea sediments	Rods in chains	98	5.5–7.0 (6.5)	An	60

Table 2. Hyperthermophiles for Which Genome Sequences Have Been Reported

Organism	Genome (Mb)	G+C (%)	T_{max} (°C)	Habitat	Initial Isolation	Physiological Genotype	% ORFs with unknown function (12)	Ref
Archaea								
Pyrococcus horikoshii	1.80	41.9	102	Deep marine hydrothermal vent	Okinawa trough, Japan	Fermentative Obl. Anaerobe Fac. S° reducer	43	(79,80)
Pyrococcus furiosus	1.91	38	103	Shallow marine volcanic vents	Vulcano, Italy	Fermentative Obl. Anaerobe Fac. S° reducer	N/A	(81)
Pyrococcus abyssi	1.76	45	102	Hydrothermal vent	North Fiji basin	Fermentative, Obl. Anerobe,	45	Direct sub. To NCBI
Archaeglobus fulgidus	2.18	48.5	92	Deep-sea volcanic vents	Vulcano, Italy	Respiratory Obl. Anaerobe Obl. Sulfate reducer	53	(82)
Methanococcus jannaschii	1.66	31	86	Deep-sea volcanic vents	East Pacific Rise	Obl. $H_2 + CO_2$ to CH_4	62	(83)
Aeropyrum pernix	1.67	67	100	Deep-sea volcanic vents	Kodakara, Japan	Respiratory Obl. Aerobe	57	(84)
Pyrobaculum aerophilum	1.7	52	104	Boiling marine water	Ischia, Italy	Respiratory, Fac. Aerobe, O_2, nitrite, nitrate reducer	N/A	(85)
Sulfolobus solfataricus	2.99	36	87	Volcanic hot spring	Yellow Stone National Park, U.S.A.	Respiratory, Fac. Aerobe, O_2 reducer	30	(86)
Bacteria								
Thermotoga maritima	1.86	46	90	Shallow marine volcanic vents	Vulcano, Italy	Fermentative Obl. Anaerobe, Fac. S° reducer	46	(87)
Aquifex aeolicus	1.55	43.4	95	Shallow marine volcanic vents	Kolbeinsey Ridge near Iceland	Respiratory Microaerophilic	44	

Isolation

Given the difficulty in accessing certain high-temperature habitats, isolation of hyperthermophiles from natural settings into pure culture presents a number of challenges (5). Not only are plating techniques problematic because of the scarcity of solid media that function at hyperthermophilic temperatures (92), but the obligate anaerobicity and sulfur requirement of many of these organisms are additional complications. There has been some success reported using solid media for hyperthermophile cultivation (93), but the method of choice has been serial dilution in liquid media (94). Despite potential problems with ensuring the presence of a pure culture, serial dilution techniques have been used to isolate most of the hyperthermophiles listed in Table 1. However, although initially the serial dilution approach led to the isolation of a host of hyperthermophiles with unique physiological and phylogenetic features, the pace of discovery of new organisms with distinctive characteristics has slowed considerably in recent years. High-temperature marine enrichment cultures at neutral pH consistently yield rapidly growing heterotrophic hyperthermophiles, such as those from the orders *Thermococcales* and *Thermotogales* (29). New isolation methods are needed to tap the extensive biodiversity in geothermal habitats inferred from the morphological variability under the microscope and from molecular phylogenetic analysis (Fig. 1). Stetter and colleagues have recently reported using an optical tweezers trap (95,96) for single cell isolation from mixed cultures (5,6). This method used in conjunction with specific whole-cell hybridization using 16S rRNA phylogenetic probes is especially promising for isolating yet to be isolated hyperthermophiles into pure culture from natural enrichments (90,91).

Cultivation

Before the availability of genome sequence information for selected hyperthermophiles, efforts to examine their physiology and enzymology relied to a great extent on the ability to grow these microorganisms in pure culture in laboratory and pilot plant settings (97). Many different reactor systems have been developed in this regard (4,98). Until efforts to clone and express genes encoding hyperthermophilic enzymes in mesophilic hosts, such as *Escherichia coli* (10), were successful, hyperthermophilic enzymes studied in purified form were obtained from hyperthermophilic biomass. Cultivation of hyperthermophiles on a scale sufficient to provide biomass for protein purification efforts presents many challenges (97,99). In short, lack of knowledge of microbial physiology, low biomass yields, generation of copious amounts of hydrogen sulfide, explosive substrate and product gases, salt-laden media, anaerobic conditions and cell sensitivity to shearing are all potential obstacles. A few heterotrophic hyperthermophiles, e.g., *P. furiosus* (62), *T. litoralis* (50) and *T. maritima* (15), can be cultivated without sulfur (albeit, anaerobically) and have, thus, been the primary focus of direct purification efforts. These organisms can be grown to biomass yields of 1 kg (wet) or higher in 600-liter fermentation systems (100) and can also be grown for long periods of time in

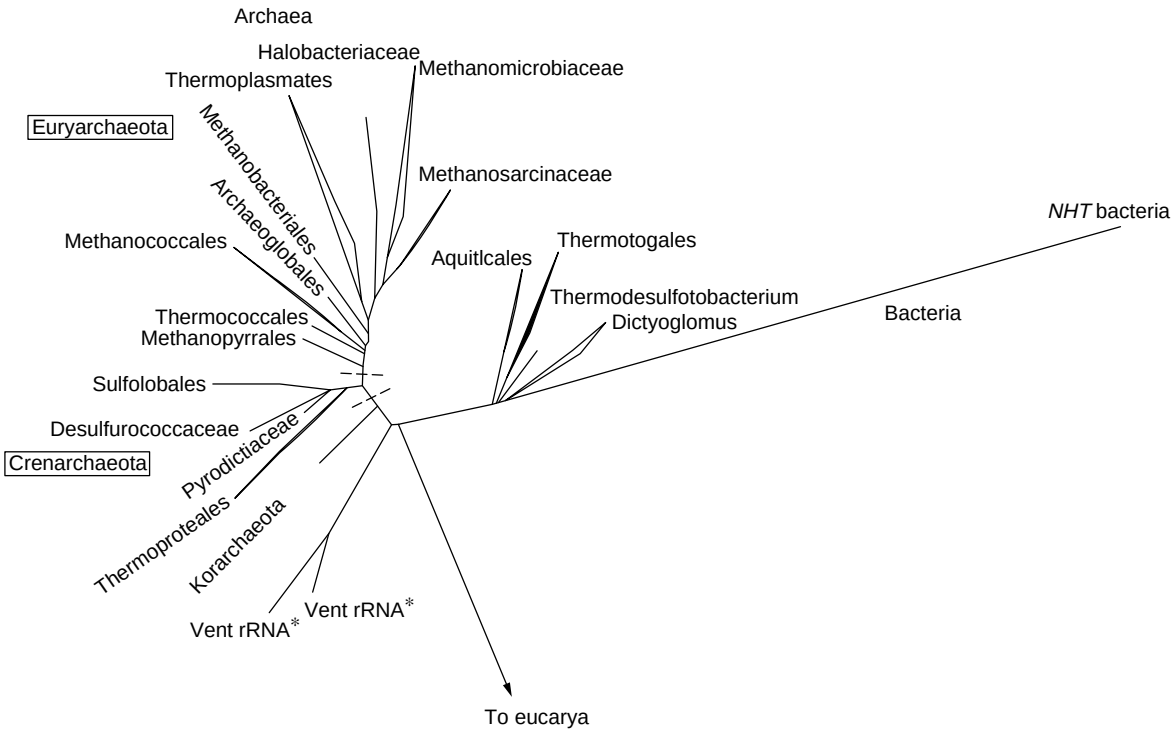

Figure 1. Families and orders of hyperthermophilic Archaea and Bacteria (9). Hyperthermophilic groups are indicated in bold text. Korarchaeota represents rRNA sequences cloned from hot springs. Vent rRNA represents cloned rRNA sequences from submarine hydrothermal vents. NHT, nonhyperthermophilic bacteria.

continuous culture (101,102). However, although sulfur is unnecessary for the growth of these organisms, their cultivation still presents significant challenges (101). The advent of genome-sequence data overcame an important barrier to the study of specific hyperthermophilic proteins. Using amino acid sequence homology, genes encoding candidate proteins could be identified, cloned and expressed in mesophilic heterologous hosts. The fact that a heat treatment step could be used to facilitate purification is an additional advantage of this approach. Of course not all genes from hyperthermophiles can be converted into actively expressed proteins in mesophilic hosts, thereby still necessitating direct purification methods be pursued. This is important given the significant fraction of genes in hyperthermophilic genomes with unidentified function (Table 2).

Unraveling the functional genomics of hyperthermophiles will need to be pursued through a combination of approaches, namely, examining the physiological response of genes to changes in growth environment (e.g., media formulation, temperature, pH, etc.) and relating these to variations in bioenergetic parameters, differential expression of genes, and protein production patterns and metabolic products. The combination of classical methods for studying microbial physiology with molecular techniques can be an advantage when used to determine the relationship of the genome to the proteome to the physiome. However, it is critical that the response of a particular organism to environmental changes be interpreted in an unequivocal way so that cause and effect relationships can be established. A particularly valuable tool for this is continuous culture, which can be used to provide biomass resulting from steady-state operation or transients in cultivation conditions. This approach works particularly well for hyperthermophilic microorganisms, given the reduced chances for contamination at elevated temperatures and, thus, the prospect of long periods of stable cultivation (101,103,104). For example, Han and coworkers (104) showed that continuous culture in conjunction with two-dimensional gel electrophoresis was useful for studying heat shock in the extreme thermoacidophile *Metallosphaera sedula*. Transcriptional (i.e., differential gene expression) and translational (i.e., two-dimensional gel electrophoresis in conjunction with mass spectrometry) responses of the organism to changes in growth environment can be related to specific genes and proteins, thereby contributing to elucidation of physiological and metabolic patterns. This information, taken together with insights into cellular bioenergetics arising from classical approaches developed for continuous culture, can be used to further the knowledge of biological function in extreme environments.

METABOLISM OF HYPERTHERMOPHILES

Hyperthermophiles as a group represent a diverse set of metabolic features reflecting to some extent the various environments from which they are isolated (5,105–112). Lithotrophic metabolism, utilized by most anaerobic or microaerophilic hyperthermophiles, involves oxidation of H_2 or S, coupled to the reduction of S, SO_4^{2-}, CO_2 and NO_3^-. Autotrophic hyperthermophiles use CO_2 as the sole carbon source via reductive tricarboxylic acid (TCA) cycle or reductive acetyl CoA/carbon monoxide dehydrogenase pathway, whereas heterotrophic hyperthermophiles metabolize carbohydrates or peptides, by either oxidation to CO_2 or fermentation to free acids such as acetate and other metabolic products. In sugar catabolism, glucose is converted to pyruvate through either a modified Embden-Meyerhof (EM) or a nonphosphorylated Entner-Doudoroff (ED) pathway, although members of the genus *Thermotoga* appear to use the typical EM and phosphorylated ED pathways. Some hyperthermophiles can utilize complex peptides as a source of carbon and energy, with S, $S_2O_3^{2-}$, SO_4^{2-}, O_2 or NO_3^- as possible electron acceptors, producing H_2S or CO_2 by respiration or acetate and other free acids, such as isobutyrate and isovalerate, by fermentation. Hyperthermophiles with completed genome sequences (Table 2) represent the metabolic variety found in this group of microorganisms, aspects of which are discussed later for particular species. Two hyperthermophilic bacteria, *A. aeolicus* and *T. maritima*, represent among the deepest lineages of Bacteria in the 16S phylogenetic tree, and the early divergence indicates that they are closely related to the universal common ancestor (Fig. 1). Their metabolic functions may represent those of early life on earth (87,113). The other eight organisms are from the domain Archaea. *Aeropyrum pernix* and *Pyrobaculum aerophilum* are aerobic hyperthermophiles with optimum temperatures above 95 °C, and their metabolic functions provide insight into the origin of oxygen respiration on earth (84,85). *Pyrobaculum aerophilum* can also carry out denitrification, a metabolic function that is unique in genus *Pyrobaculum* (32). *Archaeglobus fulgidus* is the first sulfur-metabolizing microorganism with its genome information available, and its sulfate-reducing pathway can help us understand the global sulfur cycle (82). *Methanococcus jannaschii*'s genome is the first sequenced archaeal genome. As a methanogenic autotroph, its metabolic function is to synthesize all cellular materials from CO_2, and form methane (83). Three closely related *Pyrococcus* species, *P. horikoshii* (79), *P. furiosus* (81) and *P. abysii* (direction submission to NCBI), have also been sequenced recently, and their different metabolic functions are reflected in their genome sequences. Along with *P. furiosus*, *Sulfolobus solfataricus* is one of the most studied hyperthermophiles. Originally isolated from high temperature (75 °C–90 °C) solfataric field, it is an aerobic acidophile, and its sugar metabolism enables it to utilize a wide range of carbohydrates from the environment (86,114).

Aquifex aeolicus

Aquifex aeolicus is a hydrogen-oxidizing, microaerophilic, chemolithoautophic bacterium, with an optimum growth temperature of 85°C (113). Incapable of growing on organic substrates, it uses CO_2 as the sole carbon source for biosynthesis by reductive tricarboxylic acid (reverse TCA) cycle, using two CO_2 molecules to form acetyl coenzyme A (Acetyl-CoA) and other intermediates for biosynthesis. Different from the conventional TCA cycle in aerobic bacteria, two important enzymes are required for the

process: a ferredoxin-dependent 2-oxoglutarate synthase, which catalyzes reductive carboxylation of succinyl-CoA to 2-oxoglutarate, and an ATP citrate lyase, which cleaves citrate to oxaloacetate and acetyl CoA (115). In *A. pyrophilus*, a close relative of *A. aeolicus*, both enzyme activities were detected in the cell extracts using H_2, CO_2, O_2 and thiosulfate; other enzymes involved in the reverse TCA cycle were also detected (115). In *A. aeolicus*, ferredoxin oxidoreductase, which can catalyze the reversible carboxylation/decarboxylation of 2-oxoglutarate, pyruvate, or 2-isoketovalerate with different specificities, is found in the reverse TCA cycle. Two distinct sets of ferredoxin oxidoreductase gene clusters, encoding two enzymes with 4 subunits each, were identified in its genome (113). The citrate synthase gene (*gltA*), along with other required genes encoding various enzymes to complete the reverse TCA cycle, have also been identified in the genome. From the genome sequence, it is clear that the reverse TCA cycle is essential for *A. aeolicus* metabolism. A number of key enzymes in the pathway have two copies of genes, resembling similar functions, but with varied specificities, such as two ferredoxin oxidoreductase gene clusters, two fumate hydratase genes, and two succinate-CoA ligase genes (113). Also, there is an absence of any other enzymes essential for other CO_2 fixation pathways, such as would be found in the Calvin cycle or reductive acetyl CoA pathway. *Aquifex pyrophilus* contains neither ribulose1,5-bisphosphate carboxylase, an essential enzyme for Calvin cycle, nor two enzymes, CO dehydrogenase and formate dehydrogenase, which are key enzymes in reductive acetyl CoA pathway (115). The energy metabolism of *A. aeolicus* is based on O_2 respiration, gaining energy by Knallgas reaction with H_2 as electron donor. Although the solubility of O_2 is low at high temperatures, *A. aeolicus* can grow at oxygen levels as low as 7.5 ppm (17,113). Its highly developed oxygen-respiration system is similar to those found in less thermophilic bacteria, causing speculation that aerobic respiration had either originated before the phylogenetic lineage including members of the genus *Aquifex*, or been laterally transferred, and acquired by *Aquifex* later on (113). When grown under strictly anaerobic conditions, *A. pyrophilus* was able to reduce nitrate to N_2 as an end product (nitrate respiration) (17,32). However, despite the presence of nitrate reductase (*nasA*) and nitrate transporter (*narB*) genes in the genome of *A. aeolicus*, nitrate respiration has not been observed, leading to the speculation that the two genes may be involved in nitrogen catabolism rather than respiration (113).

Aeropyrum pernix

Aeropyrum pernix, an archaeon with an optimum growth temperature of 95 °C, is a strictly aerobic hyperthermophile (42). It metabolizes peptides and gains energy by aerobic respiration, completely oxidizing peptides to CO_2. Although not essential for growth, thiosulfate stimulates the growth of *A. pernix* eightfold, without producing H_2S. Other hyperthermophiles, such as *P. aerophilum* and *T. tenax*, can also oxidize peptides to CO_2, with sulfur or thiosulfate as electron acceptor, either aerobically or anaerobically (28,31). The *A. pernix* genome contains all genes involved in TCA cycle, with the genes coding for a dimeric 2-oxoacid:ferredoxin oxidoredactase replacing alpha-ketoglutarate dehydrogenase found in bacterial TCA cycle (84). A homologous 2-oxoacid:ferredoxin oxidoredactase was previously purified from a *Sulfolobus* sp., with broad substrate specificity toward 2-oxoglutarate, 2-oxobutyrate, and pyruvate (116). *Aeropyrum pernix* cannot survive under any anaeorobic condition (42), yet three genes in its genome encode a nitrate reductase enzyme complex typically involved in nitrate respiration. Phylogenetic analysis showed that the nitrate reductase subunit A was closely related to several bacterial oxidoredutase genes, but not archaeal or eukaryotic genes, indicating that the genes are probably laterally transferred between Archaea and Bacteria (117).

Archaeoglobus fulgidus

Archaeoglobus fulgidus, a strict anaerobe, can grow both chemolithoautophically in the presence of H_2, CO_2, and thiosulfate, and chemoorganotrophically on various organic carbon sources, such as lactate, glucose, formate, starch, peptone, with sulfate, sulfite, and thiosulfate, but not sulfur, as electron acceptors (67). Because of its anaerobic natural environment, *A. fulgidus* carries out sulfate respiration as its energy metabolism (109,118). Similar to the pathway found in mesophilic sulfate-reducing bacteria, sulfate is first activated to adenylylsulfate (adenosine-5′-phosphosulfate; APS) by ATP sulfurylase, followed by reduction of APS to sulfite by adenylylsulfate reductase, before final conversion to sulfide by sulfite reductase (109,119). All the enzymes in the pathway have been identified in *A. fulgidus* (118,120–122). Reduction of adenylylsulfate to sulfite is an essential step of this metabolic pathway. The adenylylsulfate reductase from *A. fulgidus* is composed of two subunits, encoded by two genes separated by 17 base pairs in the genome, and arranged in order of *agrB-agrA*. Subunit A, a 73-kDa protein, is closely related to subunits of several bacterial succinate dehydrogenases and fumarate reductases. Subunit B, with M_r of about 17 kDa, is an iron-sulfur protein, and its seven cysteine residues form two clusters, assisting in the formation of an ion-sulfur center with eight ions and six sulfide atoms in the protein (118). Chemoautotrophically, *A. fulgidus* can fix CO_2 via a reductive acetyl CoA/carbon monoxide dehydrogenase pathway (123). When grown organotrophically, the organism utilizes various organic carbon sources, such as formate, lactate, and methanol, oxidizing them to CO_2 (67). The presence of 57 β-oxidation enzyme genes and five types of ferredoxin-dependent oxidoreductase genes in its genome further confirmed *A. fulgidus*' ability to use organic carbon sources (82). In a proposed lactate catabolism, a membrane bound lactate dehydrogenase and pyruvate:ferrodoxin oxidoredutase oxidized lactate to Acetyl CoA and CO_2. The acetyl CoA from both chemoautotrophic and organoheterotrophic pathways undergoes modified acetyl CoA/carbon monoxide dehydrogenase pathway by acetyl CoA decarboxylate/synthase (ACDS) complex (119,123). The acetyl CoA decarboxylate/synthase complex (ACDS) catalyzes reversible conversion of CO_2 and Acetyl CoA, carrying out acetyl CoA synthesis and

CO-dependent of methyl-tetrahydromethanopterin (CH_3-H_4MPT) reduction simultaneously. It is composed of five subunits with total M_r of 2.0 MDa, and resembles an ACDS complex from *Methanosarcina thermophila* (124), not only in overall and α-, γ-, ε-subunit molecular weights, but also immunologically (125). Two genes, clustered as *cdhA2B2* under one operon in the genome, encode acetyl CoA synthases, which are α- and ε- subunits of the complex, and are homologous to *M. thermophila*'s ACDS α- and ε- subunits genes (125). It is still unclear whether the gene sequences encode β-, γ-, δ-subunits of the ACDS complex, although two carbon monoxide dehydrogenase genes, *CooF* and *CooS*, are identified in the genome (82). The genome sequence has also resulted in some conflicting results about other metabolic functions of *A. fulgidus*. Although glucose was listed as a carbon source (67), no glucose uptake- or transport-genes, or its route of catabolism can be identified in the genome. On other hand, there are multiple clusters of acetyl CoA synthase genes, *cdhE-cdhD-cdhC*, *cdhA1-cdhB1*, *cdhA2-cdhB2*, although *A. fulgidus* cannot grow on acetate (82).

Methanococcus jannaschii

Methanococcus jannaschii is a strictly anaerobic, obligate autotrophic methanogen, utilizing H_2 and CO_2 as food and energy sources (74). Its energy metabolism is methanogenesis, involving the stepwise reduction of CO_2 to CH_4 (see recent reviews for detail mechanism discussion (109,112,126). As a critical enzyme in methanogenesis, formylmethanofuran (formyl-MF) dehydrogenase reduces carbon dioxide to formylmethanofuran with H_2. Formyl-MF dehydrogenase in *M. jannaschii* has seven subunits, A, B, C, D, E, F, G, encoded by a gene cluster *fwdEFGDAXC*, in which *fwdX* encodes a hypothetical protein with no homology to any function-known enzymes. All subunits contain only tungsten as a cofactor (83,112). Formyl-MF dehydrogenase from a thermophilic methanogen, *Methanobacterium thermoautotrophicum*, has four subunits, all of which have both tungsten and molybdenum as cofactors (127). By comparing with other mesophilic and hyperthermophilic methanogens, it was believed that the use of tungsten cofactor in this enzyme is unique to hyperthermophiles (112). Formylmethanofuran:tetrahydromethanopterin formyltransferase exogenetically catalyzes the transfer of formyl group from formyl-MF to H_4MPT, which is encoded by one gene cluster, *mtrEDCBAFGH*, in *M. jannaschii* genome, compared to two copies of such gene are present in *M. thermoautotrophicum* (128). Methyl-coenzyme M reductase catalyzes the release of methane from coenzyme M. In *M. jannaschii*genome, there are two clusters of genes, *mrtBGA* and*mcrBDCGA*, encoding for two three-subunits methyl-CoM reductases. Two homologous methyl-CoM reductases, MCRI and MCRII, are characterized in *M. thermoautotrophicum*; both enzymes consist of three subunits, with native M_rS of about 300 kDa, but differ in kinetic properties and pH optima (129,130). Fixation of CO_2 in *M. jannaschii* is through the reductive Acetyl-CoA-carbon monoxide dehydrogenase pathway (83), similar to that of *A. fulgidus* (see earlier discussion). Gluconeogenesis and glucose metabolism are not present in *M. jannaschii*, yet there are two genes encoding polysaccharides-degrading enzymes in the genome, an α-amylase and a glucoamylase. These genes are clustered around a portion of the genome in which the G + C content is less than 95% of rest of the genome, suggesting they were laterally transferred from other organisms (83).

Pyrobaculum aerophilum

Pyrobaculum aerophilum is capable of utilizing a variety of metabolic pathways (32). It grows aerobically on organics, such as peptides or acetate, or inorganic compounds, such as CO_2, H_2, and thiosulfate. It carries out denitrification as its energy metabolism under strict anaerobic conditions, a mechanism that has not been identified in its close relatives *P. islandicum* (31), *P. organotrophum* (31), *P. oguniense* (33), or *P. arsenaticum* (34). *Aquifex pyrophilus* is the only other hyperthermophile capable of denitrification (17). When grown under anaerobic conditions with complex carbon sources, *P. aerophilum* requires tungstate, but not molybdate, and nitrate reductase, nitrite reductase, and nitric oxide reductase activities were detected associated with cellular membranes (131). Although nitrite reductase and nitric oxide reductase were not affected, nitrate reductase activity was inhibited by high level of tungstate concentrations, decreasing fourfold from tungstate concentration of 0.1 μM to 5.0 μM and indicating that tungstate is involved in a complex metabolic pathway of *P. aerophilum* (131). In the partially (95%) completed genome sequence, a nitrate transporter gene and three genes (*narZ*, *narY*, *narG*), encoding for α-, β-, γ-subunit of nitrate reductase, are cotranscribed under one operon; a ferredoxin-nitrite reductase was also identified (85). Heterotrophically, *P. aerophilum* completely oxidized complex peptides to CO_2, similar to *A. pernix*. The presence of genes encoding pyruvate-flavodoxin oxidoreductase and malate oxidoreductase indicate that TCA cycle is involved. Recently, it was shown that under anaerobic conditions, *P. aerophilum* grew organotrophically in the presence of arsenate (34). As a close relative *P. arsenaticum* grows chemolithoautotrophically on CO_2, H_2, and arsenate, or heterotrophically on complex peptides and arsenate, both anaerobically (34). An enzyme system for the arsenate respiration has yet to be characterized.

Pyrococcus sp.: *P. abyssi, P. horikoshii,* and *P. furiosus*

The three *Pyrococcus* species have similar growth physiologies in that they have comparable optimum growth temperatures, are all strict anaerobes, grow fermentatively on peptides, and reduce sulfur. *Pyrococcus furiosus*, isolated from shallow marine sediments, can grow on a number of carbohydrates, such as starch, maltose, cellobiose and barley glucan, without sulfur, and on complex peptides with sulfur (62,110,132). *Pyrococcus abysii* and *P. horikoshii*, both isolated from deep hydrothermal vent environments, grow on complex peptides with sulfur, but not carbohydrates (64,66). *Pyrococcus furiosus* possess a wide range

of glycosyl hydrolases, many of which have been identified and characterized: a β-glucosidase (*celB* gene) (133,134), two α-amylases (135–137), a β-mannosidase (138), a pullulanase (139), a laminarinase (140), an endoglucanase (141), and two chitinases (Gao et al., unpublished results). These endo- and exo-acting enzymes synergistically hydrolyze complex carbohydrates into glucose, providing *P. furiosus* with carbon and energy sources (142). In the *P. abysii* genome, six putative glycosyl hydrolases can be identified (*http://www.genoscope.cns.fr/cgi-bin/Pab.cgi*). Although at least 12 putative glycosyl hydrolases are found in *P. horikoshii* genome, only two are endo-acting enzymes. The difference in glycosyl hydrolases among the three *Pyrococcus* species might be indicative of their natural habitats. *Pyrococcus furiosus* was isolated from shallow marine sediments, which contain high carbohydrate but low protein content, whereas *P. horikoshii* and *P. abysii* may have more limited access to carbohydrates in deep hydrothermal vent environments (81). *Pyrococcus furiosus* uses a modified EM pathway for glucose degradation to pyruvate, using ADP-dependent hexokinase and ADP-dependent 6-phosphofructokinase (109,143), which might be related to the higher stability of ADP over ATP at or near 100 °C (110). The ADP-dependent hexokinase has been purified and exists as a dimer, exhibits high substrate specificities toward glucose and ADP, which cannot be replaced by fructose or ATP/GTP molecules; K_m values for ADP and glucose are 0.033 mM and 0.73 mM, respectively (144). The ADP-dependent phosphofructokinase, a tetramer, exhibited lower specificities to its substrates with K_m values of 0.11 mM for ADP and 2.3 mM for fructose-6-phosphate; ADP can be replaced by ATP, GP, or GDP (145). *Pyrococcus horikoshii* and *P. abysii* probably obtain pyruvate through degradation of peptides, although the metabolic pathways responsible are still unclear. In *P. horikoshii*, many seemingly essential metabolic enzymes are still unidentified (80,81). Pyruvate has two possible metabolic fates in *P. furiosus*. It can be further oxidized to acetyl CoA by pyruvate:ferredoxin oxidoreductase (POR) and subsequently converted to acetate and ATP via substrate level phosporylation by the acetyl CoA synthase (109). In *P. furiosus*, POR is a 4-subunits protein with thiamine pyrophosphate and two ferredoxin-like clusters; unlike the same enzyme from *T. maritima* (146), it does not contain a copper ion, resulting in different catalytic mechanism (147). The homologous genes encoding the pyruvate:ferredoxin oxidoreductase are identified in the other two *Pyrococcus* species. The second metabolic fate of pyruvate is to serve as an alternative electron sink by its conversion to alanine through the coordinated action of the glutamate dehydrogenase and alanine aminotransferase (148,149). *Pyrococcus furiosus* cannot use a TCA cycle to further oxidize acetyl CoA to CO_2, due to absence of two important enzymes for the TCA cycle, succinyl-CoA hydrolase and 2-oxoglutarate synthase. Instead, acetyl CoA is fermentated to acetate, via acetyl CoA synthase, coupled with phosphorylation of ADP, a mechanism present only in Archaea (150,151). An entire operon, containing three enzymes of TCA cycle, is missing in *P. horikoshii* (81).

Sulfolobus solfataricus

Sulfolobus solfataricus is a microaerophilic heterotroph, capable of utilizing a number of simple carbohydrates, including glucose, xylose, maltose, sucrose, as carbon and energy source (20,114). With the availability of its genome sequence, other complex carbohydrates, such as starch, cellulose, and xylans, can also be listed as potential growth substrates for the microorganism (86). Two genes, encoding a xylanase and a cellulase homologous to glycosyl hydrolase family 11 and 12, have been identified; the presence of signal peptide sequences indicate that both genes are secreted in the media, degrading polysaccharides into oligosaccharides. These two genes, along with a putative β-xylosidase gene encoding an intracellular xylosidase, are cotranscribed under one operon. Another gene cluster identified in the genome involves an α-amylase and a pullulanase gene, presumably degrading α-linked polysaccharides into oligo-, disaccharides. After being carried into the cytoplasmic membrane by a number of transporters, the oligo- and disaccharides are degraded into glucose and xylose by intracellular glucosidase and xylosidase (86). A family 1 β-glucosidase, encoded by *lacS* gene, has been studied extensively and its crystal structure has been solved (152–154). Recently, an α-xylosidase (XlyS) was characterized (155). The enzyme was exo-acting, and exhibited high hydrolytic activity on the xylose-containing disaccharides, releasing xylose from oxyloglucan oligosaccharies at optimum temperature of 90 °C. It acted synergistically with the β-glucosidase (LacS) on the xyloglucan oligosaccharides, releasing glucose and xylose shortly after incubation. No glucose and only trace amounts of xylose were observed when the substrate was incubated with LacS or XlyS alone. These genes, along with a major facilitator superfamily of transporter gene, and a gene with an unknown function, are cotranscribed as a polycistronic message (155). *Sulfolobus solfataricus* then degrades glucose to pyruvate via non-phosphorylated ED pathway (86,109,114). Glucose dehydrogenase ($NAD(P)^+$-dependent) oxidizes glucose to gluconate, which is then oxidized to 2-keto-3-deoxygluconate (KDG) by gluconate dehydratase. KDG aldolase then degrades KDG to pyruvate and glyceraldehyde, which is further oxidized to glycerate by glyceraldehyde dehydrogenase. Finally, a kinase phosphorylates glycerate to 2-phosphoglycerate, which is then converted to pyruvate by enolase and pyruvate kinase. A number of genes encoding these glycolytic enzymes are clustered in the genome, such as a cluster containing phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes, and a cluster with NADP-dependent glyceraldehyde-3-phosphate dehydrogenase, fructokinase, 2-keto-3-deoxy-gluconate aldolase genes (86). Pyruvate that is generated by sugar metabolism is utilized by *S. solfataricus* via the TCA cycle involved a 2-oxoglutarate:ferredoxin oxidoreductase (105).

Thermotoga maritima

Thermotoga maritima is a strictly anaerobic bacterium, growing fermentatively on peptides and many simple and complex carbohydrates, including glucose,

sucrose, starch, cellulose and xylan (15). A number of glycosyl hydrolases involved in sugar catabolism have been characterized, including amylases (156), xylose isomerase (157), two β-glucosidases (158,159), a β-galactosidase (158), two xylanases (160), two cellulases (161,162), and a α-galactosidase (163). Other glycosyl hydrolases genes, encoding for a pullanase, a laminarinase, four α-glucosidases, two α-mannosidases and two β-mannosidase, have also been identified in its genome (87). Many of these genes are under the same operon with ATP-binding cassette (ABC) transporter genes, indicating that metabolizing and transporting carbohydrates are coregulated in *T. maritima*. Overall, 7% of the total function-identified genes are involved in sugar metabolism, significantly more than for any other genomes sequenced so far (87). There are several unique features about *T. maritima*'s metabolic functions. Unlike other anaerobic hyperthermophiles, *T. maritima* produced two moles of acetate, CO_2, four moles of H_2 and 2 moles of ATP per mole of glucose, without other minor metabolic products, such as lactate, ethanol (164). Its metabolism, like those of other anaerobic bacteria, involves an EM pathway with ATP-dependent hexokinase and ATP-dependent-6-phosphofructokinase, whereas other hyperthermophiles, such as *Pyrococcus* and *Thermococcus* species, use ADP-dependent hexokinase/phosphofructokinase (109,165). *Thermotoga maritima* also uses a phosphorylated ED pathway in addition to the EM pathway. With ^{13}C-labeling experiments, it was found that two pathways operated simultaneously, with the majority (85%) of glucose undergoing the EM pathway, and the rest undergoing the ED pathway. Two key enzymes for the phosphorylated ED pathway, glucose 6-phosphate dehydrogenase and 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase (but not KDG aldolase), were detected in the cell extract (165). *Thermotoga maritima* was also found to be able to utilize Fe^{+3} as a terminal electron acceptor, thus avoiding the production of toxic sulfide end-products and H_2 (166). Additionally, a flavoprotein, consisting of α- and β-subunits, and multiple ion-sulfur identified in the genome as potential electron transporter (87). The last step of metabolism in *T. maritima* involves the conversion of acetyl CoA to acetate ATP by the coordinated action of the phosphate acetyltransferase and acetate kinase, in contrast to the single enzyme system of acetyl CoA synthase found in *P. furiosus* (109).

GENETICS OF HYPERTHERMOPHILES

The hyperthermophiles represent a diverse group of microorganisms that display a range of metabolic and genetic properties and span both the bacterial and archaeal domains. Accordingly, the genetics, transcription, translational, and genetic elements, of the hyperthermophiles are equally as diverse. From an evolutionary standpoint the hyperthermophiles cluster at the base of the 'tree of life' and many of their branches are short. Also, hyperthermophiles may represent the closest relatives to our last universal common ancestor(s), and by studying the genetics and molecular biology of these unique microorganisms we may gain insight into early evolution. Furthermore, a solid understanding of the genetics will be necessary to develop genetic systems for the hyperthermophiles.

Transcription and Translation

The transcriptional and translational apparatus of the hyperthermophilic bacteria *Thermotoga* and *Aquifex* is similar to that of *E. coli* and for this reason this review will only briefly address the hyperthermophilic bacteria. The three subunits (α, β, β') of the core RNA polymerase (RNAP) are present in the genome sequences of both *T. maritima* (87) and *A. aeolicus* (113). In addition, four sigma (σ) factors that determine promoter specificity, have also been identified in both organisms. The promoter elements consist of -35 and -10 regions as found in *E. coli* and in *T. maritima* have the consensus TTGAC and TATAAT (167). Similar to *E. coli* and *Bacillus subtilis*, the AUG triplet is used as the translational start codon. However, to a lower extent both the GUG and UUG triplets are also used as the translational start codon.

The hyperthermophilic archaea, however, display a chimeric transcriptional and translational apparatus with features from both eukaryotic and bacterial organisms (for recent reviews see 168–172). Similar to transcription in bacteria, archaeal genes are often located on the chromosome in clusters and can be transcribed as polycistronic messages. Further, there does not appear to be significant posttranscriptional modification of the mRNA such as poly-A tailing or a 5′ cap. However, the archaeal DNA is complexed with histones into nucleosome-related structures similar to eukarya (173). The archaeal RNAP also resembles the eukaryotic RNAP II in its complexity possessing at least 10 subunits as opposed to the four-subunit bacterial enzyme (174). This complexity was also observed in the genome sequences of *A. fulgidus*, *M. jannaschii*, *A. pernix*, and *P. horikoshii* with the identification of the individual subunits and subsequent comparison to their eukaryal homologs (80,82–84). The archaeal RNAP is unable to recognize promoters unaided and requires two additional *trans*-acting factors: the TATA-binding protein (TBP) and transcription factor B (TFB), which are homologs of the eukaryal TBP and transcription factor IIB. These three elements have been found to be sufficient to drive transcription in vitro (175,176). However, it cannot be ruled out that in vivo there are other factors yet to be identified that are also involved. The archaeal promoter element consists of two *cis*-acting elements, which include the TATA Box, and the TFIIB-responsive element (BRE). The TATA box is centered around $-25/-26$ relative to the transcription initiation site, and the BRE immediately precedes the TATA box with a AA doublet found at approximately -33 and -34 (177–180). A comparison of mapped promoters from *P. furiosus* is given in Figure 2. An analysis of the transcriptional start sites reveals a slight bias for a purine base. The TATA box is readily identified and is centered around -25 relative to the transcription start site and is preceded by an A-rich stretch of bases that make up the BRE.

Figure 2. Promoters from the hyperthermophilic archaeon *P. furiosus* Mapped promoter of *P. furiosus*. *celB* and *adhA* (181), *gdh* (175), *aat* (Ward et al., unpublished results), *lrp* (182), *aroQ* and *tyrB* (Ward et al., manuscript submitted), *nox1* (Ward et al., manuscript submitted), *gor* (183), *pfpI* (184), *pls* (185), *pol* (186), *pdk* (187), *hydB* (188), and *argF* (189).

Archaeal transcription is initiated by binding of the TBP to the 8-base pair TATA box, which is followed by the binding of the TFB to the BRE element. It has been shown that it is the BRE/TFB complex that determines the polarity of transcription in Archaea (190). The crystal structure of the archaeal preinitiation complex containing TBP, TFB, and the promoter region, including the TATA box and BRE has also been determined, which identified the sequence specific interactions between the C-terminal helix-turn-helix domain of the TFB and the BRE (191). It is this final ternary complex that is required for recruitment of the RNAP. Similar to eukaryotes, the N-terminal domain of TFB contains a zinc-finger, and is essential for recruitment of the archaeal RNAP to the promoter and subsequent promoter clearance (192,193). Although there has been significant focus on transcription initiation in the Archaea, this is not the case so far for transcription elongation and termination, and little is known about the factors involved. Posttranscriptional modification of messenger RNA (mRNA) does not appear to be as significant in the Archaea and 5′-capping of mRNA has never been observed. Transcription termination appears to resemble the bacterial Rho-independent mechanism, and probably involves an inverted repeat followed by a stretch of T-residues between four to thirty nucleotides in length.

Efforts to elucidate the mechanisms behind transcriptional regulation have focused on the identification and characterization of regulators identified through genome sequences. Analysis of archaeal genomes identified a large number of transcriptional regulatory proteins that were more closely related to bacterial sources than eukaryal (170). Three regulators from hyperthermophilic archaeons have been characterized in detail: the LrpA from *P. furiosus* (182), Lrs14 from *S. solfataricus* (194), and MDR1 from *A. fulgidus* (195). Both LrpA and Lrs14 belong to the Lrp/AsnC family of transcriptional regulators and were found to bind to their own promoters and repress transcription in vitro. The Lrs14 was found to bind the promoter region including the TATA and BRE sequences and it was proposed that inhibition may be via direct competition between the TBP and Lrs14 (194). The LrpA from *P. furiosus* binds from position −22 to +24 of its promoter and although it may not exclude binding of TBP it is possible its mechanism of repression is through the inhibition of the RNAP recruitment (182). The addition of possible effector molecules such as leucine did not have an effect on the binding of either LrpA or Lrs14. The crystal structure of the LrpA from *P. furiosus* was recently elucidated and could provide a great deal of insight into the how Lrp binds DNA thereby effecting transcription (196). Despite the similarity of these two regulators to the global regulator Lrp from *E. coli*, their roles as activators or global regulators is still purely speculation, and the actual genes, besides themselves, that they regulate is currently unknown. The LrpA is found just downstream of the gene encoding the glutamate dehydrogenase (*gdh*) and was originally thought to possibly play a role in its regulation. However, binding of the LrpA to the *gdh* promoter region has never been observed in vitro even in the presense of various potential effectors. The MDR1 (metal-dependent repressor 1) from *A. fulgidus* is located within a gene cluster that also encodes a metal-importing ABC transporter and is expressed as a polycistronic message (195). Transcription was found to respond to the availability of metal ions in the growth medium and MDR1 bound in a metal-dependent manner to repress transcription. It was also shown that binding of MDR1 to the promoter region did not inhibit TBP and TFB binding, but did block recruitment of the RNAP. The majority of regulators identified in the genomic sequences appear to be of a bacterial origin and are repressors. A transcriptional activator (GvpE) has been identified in a halophilic archaeon that activates genes involved in the formation of gas vesicles (197). The GvpE resembles the eukaryal basic leucine zipper proteins, but the precise mechanism of activation remains to be elucidated.

Very little is known about translational initiation in Archaea other than that the mRNAs can be found as either mono- or polycistronic messages, are usually preceded by Shine-Delgarno (SD) sequence, and the initiation usually begins with a AUG codon, although

GUG and UUG can also serve as the initiation codons. Analysis of genomic sequences reveals a combination of bacterial and archaeal features (for reviews see 168,169). Recently, it has been shown that two mechanisms exist for translation initiation in *S. solfataricus* (198). It was found that the SD-like ribosome binding motif was essential for efficient initiation and that disruption of the SD resulted completely blocked translational initiation. However, this could be overcome by the removal of the entire 5′ untranslated region. This leaderless mRNA was correctly translated, suggesting that a secondary mechanism for translational initiation exists in the hyperthermophilic archaea. The translation of leaderless mRNA's has now been observed in *Pyrobaculum* (199). It was found that genes that were expressed as a monocistronic message or were the first gene in an operon utilized a leaderless mRNA for translation. However, genes that were internally located within operons required the presense of a SD-like sequence for translation.

Genetic Elements

In recent years genetic elements have been found throughout the hyperthermophilic archaea and bacteria, including plasmids, viruses, and IS elements (for recent reviews see 200–202). These plasmids and viruses can serve as excellent models to study DNA replication, gene expression, and have great potential to serve as templates to build genetic systems for the hyperthermophiles. Furthermore, the study of genetic diversity could provide insight into the genetic variation, biodiversity, and evolution of these ancient organisms. With the completion of a number of genomes from hyperthermophiles horizontal transfer of genes has been shown to play a role in the genetic diversity of prokaryotes. The transfer of genes are often linked to genetic elements such as parts of prophages, transposases, integrases, and recombinases (203,204).

Mobile elements such as insertion sequence (IS) elements are integral parts of the bacterial genome and are also found in eukaryotic organisms (205). These elements are generally less than 2.5 kb and only contain the elements necessary for transposition, which usually involves a set of terminal inverted repeats (IR) flanking the element and a gene encoding a recombinase/transposase. The activity of these mobile elements can result in numerous genomic rearrangements such as deletions, inversions, and gene fusions. IS elements were found to be responsible for the spontaneous generation of β-galactosidase-deficient mutants (ISC1217), and uracil-auxotrophic mutants (ISC1058, ISC1217, ISC1359, and ISC1439) in *S. solfataricus* (206). The activity of these mobile elements probably plays a significant role in genomic rearrangements in this organism. IS elements have also been found in the genomes *P. furiosus* (*http://comb5–156.umbi.umd.edu/genemate/*), *P. abyssi* (*http://www-archbac.u-psud.fr/Projects/Pab_r/Pab_r Genome.html*), *T. maritima* (87), and *A. fulgidus* (82). In *P. furiosus*, a 16 kb region of the genome was found to be identical to 16 kb of the genome of *T. litoralis* with exception of 153 bp (207). This 16 kb region in *P. furiosus* is flanked by two IS elements and, on the basis of this, was given to be evidence of a recent lateral transfer between these two organisms.

Many hyperthermophilic bacteria and archaea have been found to harbor endogenous plasmids varying in size from 0.8 to 41 kb (Table 3). The presence of multiple plasmids within an organism is also common, as it has been observed in both the Euryarchaeota and Crenarchaeota. Most of the reported plasmids appear to be cryptic, despite significant effort to link the plasmids to phenotypic traits. Only one plasmid has been identified and characterized in detail in the hyperthermophilic bacteria: the 846-bp cryptic plasmid pRQ7 from *Thermotoga* sp. strain RQ7, which is one of the smallest replicons described in any organism (208). The plasmid has been sequenced and a single open reading frame (ORF) was identified that encodes a protein of 25.4 kDa and probably encodes the Rep protein necessary for plasmid replication. Sequence analysis of the Rep protein and putative origin of replication suggested that the plasmid replicated via a rolling-circle mechanism and single-stranded DNA intermediates of pRQ7 were identified confirming that the plasmid replicates via this mechanism (209). This plasmid has been found in three other strains of *Thermotoga* isolated from distantly located hydrothermal vent systems suggesting that the plasmid is both highly conserved and widely distributed among the *Thermotogales* (210). An extrachromosomal element (ECE) of 39.4 kb was identified in *A. aeolicus* and is present at approximately twice the copy number of the chromosome (113). The ECE encoded 32 coding regions of which only one could be assigned a function, a transposase. One coding region was also present on the chromosome in two identical copies suggesting that there has been transfer of genetic material between the ECE and the chromosome.

In the hyperthermophilic archaea, a large number of genetic elements have been identified, sequenced, and characterized in detail. The two model organisms of study are the aerobic, acidophilic Crenarchaeote *Sulfolobus* and the strictly anaerobic, heterotrophic Euryarchaeotes *Pyrococcus* and *Thermococcus*. A study was carried out to determine the distribution of plasmids in the *Thermococcales*, and of the 57 strains tested, 11 harbored plasmids that ranged in size from 3 to 24 kb suggesting that plasmids are diverse and widely distributed among the *Thermococcales* (212). Three of the strains isolated contained two plasmids, one of 3 kb and a second larger plasmid of 24 kb. On the basis of the relatedness of these three strains and that the 3 kb plasmids cross-hybridized, as did the 24 kb plasmids, they are probably closely related. To date, only the small cryptic plasmid pGT5 from *P. abyssi* has been characterized in detail (211). The plasmid is 3.4 kb and contains 2 ORFs with the larger ORF encoding the Rep protein necessary for plasmid replication, and was also found to replicate via the rolling-circle mechanism. The function of the second smaller ORF is unknown, but may play a role in copy number control. A second plasmid has been isolated recently and sequenced from *Pyrococcus* sp. strain JT1 and was found to be distinct from pGT5. The 3.4 kb plasmid (pRT1) was sequenced and encoded two proteins, both of which are

Table 3. Plasmids and Viruses from the Hyperthermophilic Microorganisms

Genetic Elements	Size (kb)	Sequence	Copy Number	Host Organism	Function	Ref.
Plasmids						
pGT5	3.4	U49503	35	*Pyrococcus abyssi* GE5	cryptic	(211)
pRT1	3.4	AF393813	high*	*Pyrococcus* sp. strain JT1	cryptic	
pGN23	16.8	nd	nd	*Pyrococcus abyssi* GE23	nd	(212)
pGN31	5.2	nd	nd	*Thermococcus* sp. strain GE31	nd	(212)
pSN559	3.0	nd	nd	*Thermococcus* sp. strain I559	nd	(212)
pLN559	24.0	nd	nd	*Thermococcus* sp. strain I559	nd	(212)
pRN1	5.0	NC001771	20	*Sulfolobus islandicus*	cryptic	(213)
pRN2	6.9	NC002101	35	*Sulfolobus islandicus*	cryptic	(214)
pHEN7	7.8	AJ294536		*Sulfolobus islandicus*	cryptic	(215)
pSSVx	5.7	AJ243537	high	*Sulfolobus islandicus*	SSV2 satellite	(216)
pDL10	7.6	AJ225333	high	*Acidianus ambivalens*	cryptic	(217)
pGS5	2.8	nd	high	*Archaeoglobus profundus*	cryptic	(218)
pRQ7	0.8	L19928	200	*Thermotoga* sp strain RQ7	cryptic	(208)
Conjugative plasmids						
pING1	24.5	AF233440	low	*Sulfolobus* sp.	nd	(219)
pING4	24.7	AF233440	low	*Sulfolobus* sp.	nd	(219)
pING6	26.6	AF233440	low	*Sulfolobus* sp.	nd	(219)
pNOB8	41.2	AJ010405	low	*Sulfolobus* sp.	nd	(220)
pNOB8–33	33.0	AJ010405	high	*Sulfolobus* sp	del. variant	(220)
pSOG2/4	28.4	nd	nd	*Sulfolobus* sp.	nd	(202)
Viruses						
SSV1	15.5	NC001338		*Sulfolobus shibatae*		(221)
SIFV	~ 41	nd		*Sulfolobus islandicus*		(222)
SNDV	20	nd		*Sulfolobus neozealandicus*		(223)
SIRV1	32.2	nd		*Sulfolobus* sp.		(224)
TTV1	16	X14855		*Thermoproteus tenax*		(225)

nd- not determined.
*greater than 30 copies per chromosome (c.c.).

expressed. Similar to pGT5, the plasmid replicated using the rolling-circle mechanism (Ward et al., manuscript in preparation).

The distribution and diversity of genetic elements has been best characterized in *Sulfolobus*, and this is largely due to the work of Zillig and coworkers. They have now discovered more than 25 plasmids and 30 novel viruses (202). The plasmids have been divided into two groups; conjugative and nonconjugative. The nonconjugative plasmids range in size from 4.7 to 35 kb and similar to the plasmids described in the Thermococcales appear to be cryptic (202). A number of these plasmids have been sequenced and five of them, pRN1, pRN2, pHEN7, pDL10, and pSSVx, are closely related and are grouped together as the pRN family of plasmids (216). These plasmids exhibited significant sequence identity in three ORFs and in two noncoding regions. Two of the ORFs appear to be involved in plasmid replication and its control, as one ORF encodes the putative Rep protein involved in plasmid replication and the other appears to be a CopG homolog and has been proposed to be involved in copy number control. It has been suggested that the pRN family of plasmids replicate via the rolling-circle mechanism. The ORF encoding the CopG homolog of pRN1 (ORF56) has been expressed and the recombinant protein binds its own promoter (226). In all 5 plasmids, the gene encoding the CopG homolog lies immediately upstream of the gene encoding the Rep protein and it is possible that these two genes are cotranscribed and binding of the CopG to its own promoter could affect expression of the Rep protein and control the copy number of the plasmid (226). The plasmid pSSVx of this family is especially intriguing in that it appears to be a cross between a plasmid and a virus (216). The plasmid was isolated from *S. islandicus* strain REY15/4, which also contains a novel virus SSV2. The plasmid could be spread to *S. solfataricus* but only in combination with SSV2 either during infection or with purified virus. Despite the similarity of pRN1 and pRN2 with pSSVx, neither plasmid could be spread, even the presence of the viruses SSV1 or SSV2.

Conjugation has been found to be a more frequent event in the Crenarchaeota than previously thought. From 300 novel strains of *Sulfolobus* 11, conjugative plasmids were identified and range in size from 24 to >40 kb (227). These plasmids were found to efficiently spread from donor to recipient cells by direct contact of the cells. The transfer of DNA was not mediated by pili

as is found in some bacterial conjugation systems, but instead contacts involving large sections of the cell surface were observed (227). The authors believe that the high frequency of these plasmids in isolates could in part be attributed to the self-spreading nature of these plasmids. The copy number of these plasmids is generally low in the host cell, but on transfer into the recipient strain, the copy number increased to at least 35 c.c., which resulted in inhibition of growth (220,227). Another characteristic of these plasmids is significant genetic variation that results after prolonged growth of the recipient cells. This was most pronounced with pNOB8, where a 10-kb deletion event occurs resulting in formation of pNOB8–33 (220). The complete nucleotide sequence of the conjugative plasmids pNOB8, pING1, pING4, and pING6 have been determined and the sequence of pSOG2/4 is in progress (219,228). The pING, pNOB, and pSOG plasmids were found to contain regions of extensive identity across the three subfamilies. These regions included ORFs that may be involved in plasmid replication, maintenance, and conjugative transfer, and noncoding regions (219,228). IS elements were also identified in both pNOB8 and the pING plasmids, which appear to play a significant role in the genetic variation of these plasmids and could possibly be involved with the transfer of genetic material between the plasmids and the host chromosome.

Approximately 35 viruses have been found in the archaea and in the hyperthermophiles they so far have only been isolated in the Crenarchaeotes *Thermoproteus* and *Sulfolobus* (reviewed in (202,229). These viruses have been found to be morphologically unique and resulted in the formation of three novel virus families. All the genomes have been found to be dsDNA and were either linear (SIFV, SIRV1, and TTV1) or circular (SSV1, SSV2, and SNDV) and the genomes of SSV1 and TTV1 have been completed. All of these viruses, with the exception of SNDV1, integrate into the genome of its host. The spreading and subsequent integration of these viruses could be a significant source of gene disruption or replacement, and a mechanism for lateral gene transfer.

MICROBIAL ECOLOGY OF HYPERTHERMOPHILES

On the basis of the information gained from 16S rRNA phylogenetic probes (90) and from visual inspection of samples from geothermal sites using electron microscopy, it is clear that only a small fraction of the hyperthermophile world has been captured in laboratory pure culture (5). One of the current challenges is to relate information gained from phylogenetic analysis to physiological and ecological phenomena. The fact that hyperthermophiles participate in a complex ecosystem in high temperature environments is beginning to be examined more carefully. Stetter and coworkers, for example, have tried to relate phylogenetic and physiological information gained from samples taken from regions within the Pisciarelli Solfatara near Naples, Italy (5). Consistent with what is known about hyperthermophile physiology, they noted that the distribution of previously studied hyperthermophilic species was consistent with their expected pH growth ranges as determined in laboratory pure culture. At the same time, phylogenetic analysis and microscopic examination revealed the presence of numerous organisms unrelated to known hyperthermophiles.

Significance of Polysaccharides in Hyperthermophile Microbial Ecology

Over the past decade, the enzymology and physiology of carbohydrate utilization by hyperthermophiles has been examined (110,111,230–234). These efforts, in conjunction with genome sequence data, have revealed that hyperthermophiles produce a wide array of glycosyl hydrolases (GH), representing a number of GH families (235). Genome sequence information has been enlightening in view of the expanded list of carbohydrates that may be substrates for actual and putative GHs in a given organism. For example, *P. furiosus*, once thought to only grow on starch and cellobiose, has in fact the enzymatic inventory to utilize a wide array of complex β-linked carbohydrates, including lamanarin, barley glucan and chitin (236). Whereas *P. furiosus* that normally grows best near 100 °C, can grow on simple sugars such as glucose, maltose and cellobiose, polysaccharide utilization by this hyperthermophile and others offer the advantage given the apparent stabilization of sugar moieties conferred by the polymeric structure (142).

The use of complex carbohydrates by heterotrophic hyperthermophiles raises questions about the sources of these compounds in their natural environments. Very little has been done to this point to address this issue, although it is clear that polysaccharides are found in extreme environments. For example, *S. acidocaldarius*, an extreme thermoacidophilic aerobe, produces a sulfated, exocellular, glucose-, mannose-, and galactose-containing polysaccharide (237), whereas the archaeon *Haloferax mediterranei*, a mesophilic halophilic aerobe, produces a sulfated, exocellular polysaccharide (238). Anaerobic archaea have been less studied in this regard, but the moderately thermophilic *M. thermophila* produces a heteropolysaccharide outer layer (239) that may aid in colonization (240), and hyperthermophilic *Thermococcus* species typically produce glycogen, apparently as an intracellular storage polymer (241). Unusual extracellular polysaccharides have also been identified in hydrothermal vent mesophilic bacteria (242–244). Complex carbohydrates typically associated with higher plants are good growth substrates for certain hyperthermophiles. *Thermotoga maritima*, for example, grows extremely well on carboxymethylcellulose and guar galactomannan; synergistic enzyme systems in this organism are indeed present to hydrolyze such material for transport and utilization (Chhabra et al., submitted). However, except for shallow marine and terrestrial geothermal vents, hyperthermophiles are usually found in the deep-sea hydrothermal vent settings, which lack any photosynthetic life. The question arises as to how these compounds are made available to hyperthermophiles and how have these organisms acquired the enzymatic machinery to use them.

The answer to the aforementioned question may lie, at least in part, in the recently discovered capability

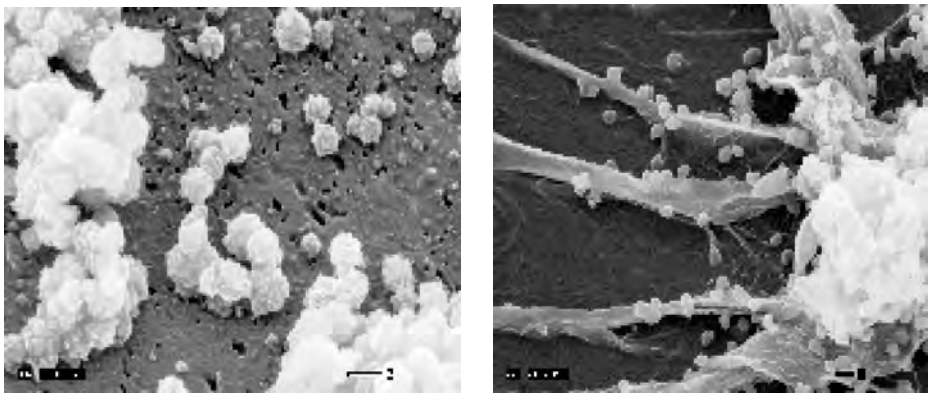

Figure 3. *Pyrococcus furiosus* grown on cellobiose (left) and maltose (right) in batch culture at 98 °C. Note formation of capsular polysaccharide (left) and exopolysaccharide (right) (Pysz and Kelly, unpublished data).

of hyperthermophiles to produce exocellular and capsular polysaccharides. It has been reported, for example, that the hyperthermophile *M. jannaschii* forms a biofilm based on exopolysaccharides it produces (245). Rinker and Kelly (99) showed that *T. litoralis* was able to produce large amounts of a mannan-based exopolysaccharide under certain growth conditions. Furthermore, this organism was also able to form biofilms with this material. Rinker and Kelly (246) have shown that *T. litoralis* forms copious amounts of polysaccharide in chemostat culture, as does *T. maritima*. It is possible that stress induces polysaccharide formation; Rinker and Kelly (246) revealed that exopolysaccharide formation by *T. litoralis* was induced at elevated NH_4Cl concentrations, which at the same time served to inhibit growth. Indeed, Laplagia and Hartzell (245) reported that stress induced polysaccharide formation by the hyperthermophile *A. fulgidus*. We have recently been able to establish growth conditions that induce exocellular and capsular polysaccharide formation by the hyperthermophile *P. furiosus* (Pysz and Kelly, unpublished data) (see Fig. 3). These two types of polysaccharides may play different roles in microorganisms (247). The genes and enzymes regulated in the formation of polysaccharides in hyperthermophiles are currently being investigated.

Interspecies Interactions at High Temperatures

The focus so far in the study of hyperthermophiles has been on isolating organisms in pure liquid culture for subsequent characterization. However, in natural settings, microorganisms are typically not planktonic but rather associated with surfaces and biofilms that offer higher concentrations of nutrients and protection from predators and toxic agents (248). It has been proposed that the concept of communities existing and interacting within microbial biofilms represents a more unifying theory of ecology and that evolution may have been more of a proliferative and associative process than competitive and selective (249). Certainly, geothermal systems are now known to harbor a diverse set of microorganisms, which may be difficult to separate into pure cultures (90,250). Although much can be learned from the analysis of electron flux from donor to acceptor in pure culture, such processes in natural settings take place within a web of other organisms and may be much different. The difficulties typically encountered in establishing and maintaining certain hyperthermophiles in pure culture probably stem from the elimination of cell to cell interactions that occur in natural consortia. Hence, there are likely to be synergistic relationships within hyperthermophilic consortia, which may be an important factor in establishing stable ecosystems containing these microorganisms. For example, it has been shown that many heterotrophic hyperthermophiles are positively affected in coculture with hyperthermophilic methanogens (251,252). Muralidharan and coworkers have shown that *T. maritima* and *M. jannaschii* in hyperthermophilic coculture results in a 10-fold increase in cell densities of the heterotroph as a result of removing H_2 inhibition through methanogenesis (253). Also, although H_2 levels in the headspace in these cocultures are negligible, *M. jannaschii* was able to grow to in excess of 2×10^7 cells/ml on the CO_2 and H_2 generated by the fermentative anaerobe. Cell sorting and electron microscopy revealed that these organisms form very tight interactions in the coculture. Using a mathematical model to describe coculture interactions, it was shown that the high growth rates of hyperthermophilic methanogens relative to mesophilic methanogens led to an increased dependence on H_2 availability, hence the close physical interaction observed experimentally (Fig. 4).

CONCLUSION

Much has been learned about the enzymology and physiology of hyperthermophilic microorganisms over the past two decades. In many respects, not many new rules have been uncovered that underly the biological mechanisms enabling life to thrive at such biologically extreme temperatures. On the other hand, genome sequences for these organisms have only been partly diciphered and thus may include information and insights not fully appreciated at this point. In any case, hyperthermophiles have expanded the scope of the biosphere and presented the intriguing possibility that life, extant or extinct, may be associated with other solar bodies that are just beginning to be explored.

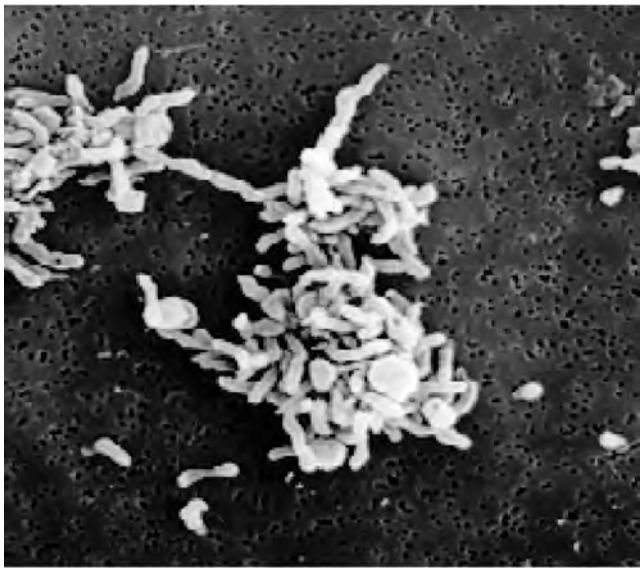

Figure 4. *Thermotoga maritima* (rods) and *Methanococcus jannaschii* (cocci) coculture (252).Interactions within hyperthermophilic consortia may also help explain the presence of a diverse set of glycosyl hydrolases produced by these organisms. For example, *T. neapolitana* and *T. maritima* both produce a set of hemicellulases that act together to hydrolyze galactomannans (254). One of these hemicellulases, β-mannanase, is produced extracellularly, and is effective in hydrolyzing the mannan-based exopolysaccharide produced by *T. litoralis* (99). Thus, one can envision a consortium. The importance of biofilm-based consortia in hydrothermal environments cannot be overlooked and such structures may in fact be the defining framework of any ecosystems that exist in these niches. However, at present, little is known about mixed culture dynamics at high temperatures, especially with respect to intra- and inter-consortial processes. The central importance of polysaccharide-forming and–hydrolyzing enzyme systems cannot be overlooked in this regard.

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I

ICE ENVIRONMENTS. See SNOW AND ICE ENVIRONMENTS

ICE MICROBIAL COMMUNITIES. See SEA ICE MICROORGANISMS

IDENTIFICATION OF AIRBORNE FUNGI

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The fungal kingdom consists of macroscopic and microscopic organisms that can be parasitic, symbiotic, or saprophytic. A fungus made of filaments forming a colony is defined as a *mold*. Mold colonies are generally visible to the naked eye; however, the individual filaments (hyphae) and spores forming the colony are visible only with the aid of a microscope. Mold spores can become airborne, because many fungi use air currents for dispersing their spores. In addition, their small size and weight makes transportation through the air possible. Fungi are known to occupy natural and artificial habitats in both indoor and outdoor environments. Dead or living plants, decaying or freshly cut wood, food, grains, soil, and building materials such as paint, wallpaper, and cellulose products can be colonized and damaged by fungi, especially under humid or wet conditions. Certain molds growing on environmental surfaces can produce toxins that can cause disease when inhaled, ingested, or on direct contact with skin. Therefore, it is important to sample and accurately identify fungi to properly remediate or treat the contamination. Fungi are identified by observing the colony morphology and the color, shape, and arrangement of the spores they produce. Difficulties surrounding fungal identification include the lack of appropriate references and trained personnel, the inability of certain fungi to produce classical structures in the laboratory, and variations in the colony morphology that is observed, depending on the culture medium used. Fungal identification is achieved after a sample is collected, processed, and/or cultured. Types of samples that can be analyzed for fungal identification are tape samples of visible colonies on building materials or surfaces, microscope slides coated with an adhesive film used for air sampling, and colonies growing on fungal media in the laboratory. This chapter summarizes the criteria and techniques used for identifying environmental fungi and the characteristics of the fungal genera that are most often found in environmental samples.

CLASSIFICATION OF FUNGI

Molds and yeasts are eukaryotic organisms that belong to the kingdom Fungi. Approximately 60,000 species of fungi have been described (1). They are characterized by being nonmotile, having a cell wall, lacking chlorophyll, and developing by means of spores (2). Like any other kingdom, fungi are classified into divisions, classes, orders, and families (3). Classification of microscopic fungi is based on their reproduction and on the type and characteristics of the spores they produce. Fungi can reproduce sexually or asexually. Organisms showing both types of reproduction (at different times in their life cycles) are given two names [e.g., *Aspergillus* (asexual form) and *Emericella* or *Eurotium* (sexual forms)]. Asexual fungi are also referred to as "microfungi" or "Fungi Imperfecti" and belong to the phylum Deuteromycota (4). Sexual fungi that are common in indoor environments are classified into three phyla: Zygomycota, Ascomycota, and Basidiomycota (5).

TERMINOLOGY AND DEFINITIONS

Microscopic fungi can be either yeastlike or filamentous in nature. Yeastlike microscopic fungi are referred to as *yeasts*, whereas filamentous microscopic fungi are referred to as *molds*. Some fungi, referred to as "dimorphic," present both as a yeast or a mold at different stages in their life cycles. Colonies vary in texture. They may be cottony, powdery, woolly, velvety, leathery, or granular. In addition, their color may be white, brown, black, green, yellow, or other colors (5,6). Molds forming black dots on clothing, books, or damp materials are referred to as "mildew"; however, true mildews are parasitic fungi growing on plants (7).

Spores are reproductive fungal structures that can be produced either sexually or asexually. Depending on the type of reproduction or function, spores are referred to with names such as *arthroconidia*, *ascospores*, *basidiospores*, *sporangiospores*, *blastoconidia*, or *chlamydoconidia*. However, the name most commonly used is "spore" or "conidium" (pl. conidia). In addition, when a fungus produces both large and small conidia, these are referred to as "macroconidia" and "microconidia," respectively. Spores generally form on the side or at the end of hyphae (long chains of cells comprising the mold) or conidiophores. Conidiophores are specialized hyphae that serve as a structure that bears conidia. The size, color, shape, and arrangement of spores are generally constant for each genus, thus making them a very useful tool for identification (8). The combined characteristics of the conidiophores and spores are unique to the genus. Several characteristics such as the arrangement of spores around the conidiophore, spore pigmentation, roughness or smoothness of the conidiophore and spores, and length

of the conidiophore need to be considered in the speciation of fungi.

Fungi that are capable of reproduction are said to be viable. Fungi that grow under laboratory conditions are said to be culturable and can de detected by culture analysis of air or bulk samples. Certain fungi that are viable may not grow in culture under laboratory conditions for various reasons such as sampling stress or lack of proper nutrients. These fungi are referred to as viable but nonculturable and can be detected by genetic amplification or microscopic analysis of air samples such as those collected onto an adhesive-coated slide.

FUNGAL HABITATS

Molds are ubiquitous in nature and are essential in nutrient cycling, the decomposition of dead or dying matter. However, molds have specialized nutritional requirements (see the following text). Fungi can live in a symbiotic relationship with other organisms, can be parasitic, saprophytic, or both (5). The habitat that a mold occupies depends on several factors such as the kind and availability of nutrients, competition, and spore dispersal (8). Fungi occupy natural and artificial habitats in both indoor and outdoor environments (9). Among the habitats molds occupy are dead or living plants, decaying or freshly cut wood, food, grains, water, and soil. In addition, artificial products such as paint, wallpaper, and cellulose products (e.g., paper, cardboard, and wood derivatives) can be colonized and damaged by fungi especially under humid or wet conditions. In the process, certain molds can produce toxins (mycotoxins) that can cause disease on direct contact with skin, inhalation, or ingestion (5).

NUTRIENTS

Fungi need sources of energy (i.e., organic carbon), nitrogen, minerals, and occasionally vitamins. Many fungi can be grown in the laboratory; however, some may not produce spores (or toxins) when cultured away from their natural habitat (10,11). In the laboratory, molds and yeasts are grown in a substance called a *medium* (pl. media) that can be semisolid or liquid. Semisolid media are generally preferred for identification purposes (8). Growth media can be made by diluting all the necessary nutrients and a solidifying agent (i.e., agar) in water. Otherwise, growth media in dehydrated form or poured into ready-to-use plates are available from commercial sources. Regardless of the source, media should be inspected for the appropriate appearance, pH, sterility, and performance (12).

CULTURE MEDIA AND INCUBATION CONDITIONS

There are several publications that provide recommendations on microbiological media for the isolation of fungi (5,13). A list of common fungal media and their formulations is presented in Table 1. For the primary isolation of fungi, a general culture medium such as malt-extract agar (MEA) should be used. In samples in which a large number of bacteria are anticipated, a bactericidal agent such as chloramphenicol (100 μg/ml final concentration) should be used (5). Dichloran glycerol-18 (DG-18) (14) is useful for the isolation of xerophilic fungi (13). In addition, DG-18 and 2% MEA without glucose or peptone have been recommended for the inhibition of fast-growing fungi (e.g., *Rhizopus* and *Trichoderma*) (5). Cellulose agar (CA) contains rose bengal that retards growth of fast-growing fungi and allows slower-growing organisms to be detected. Because rose bengal is light-sensitive, this medium should be protected from light (5). Cellulose agar is useful for the isolation of cellulolytic fungi such as *Stachybotrys* species. Water agar (20 g agar/L of water) overlaid with sterile filter paper has also been suggested for the isolation of *Stachybotrys* from environmental samples (5). For promoting sporulation, potato dextrose agar (PDA) is recommended. Czapek solution agar (CZA) has proven useful for the identification of certain *Aspergillus* species (3).

Light is not required for fungal growth; however, periods of light and darkness necessary for normal sporulation are usually provided by the normal handling of the cultures during incubation (2). Environmental fungi are generally incubated at 23 to 25 °C for up to 10 days. Preliminary identification of fast-growing organisms can usually be performed after three days of incubation by using a stereoscope and the methods discussed in the following text. Plates should be reincubated and checked periodically to observe the organism at the proper stage of sporulation. Higher incubation temperatures should be used if special interest in thermophilic fungi exists. Although incubation at 45 °C (5,13) is not necessary for the isolation of *Aspergillus fumigatus*, it is useful for speciation. For the detection of *Stachybotrys* on MEA plates, samples should be checked periodically and incubated for 10 days. However, if fast-growing or numerous other fungi are present, detection of this organism on MEA will be difficult if not impossible. Therefore, it is recommended that for the isolation of *Stachybotrys* species, CA plates be used in conjunction with a primary isolation medium. Care must be taken in handling the plates because spores can be dislodged from the original colonies, forming satellite colonies that produce an overestimation of the quantity of colonies on the plate. Plates with numerous colonies, toxigenic fungi, or fast-growing fungi such as *Rhizopus* should be taped or sealed to prevent accidental exposure to the organism(s).

SPORE DISPERSAL

Mold spores can become airborne, because many of the fungi use air currents for dispersing spores. In addition, their small size and weight makes transportation through the air possible (5). Several mechanisms exist for spore dispersal by air currents, such as exposure of spores to the air, shooting spores into the air, and droplet adhesion (8,15). Fungal spores are subjected to both seasonal variations and daily fluctuations that are influenced by several factors including wind velocity and rain (1,16,17).

Table 1. Summary of Commonly Used Fungal Growth Media and Stain Formulations

Malt-Extract Agar (MEA)[a]

1.275% maltose
0.275% dextrin
0.235% glycerol
0.078% peptone
1.5% agar
Final pH = 4.7

Potato Dextrose Agar (PDA)[a]

20% potato infusion
2% dextrose
1.5% agar
Final pH = 5.6

Sabouraud Dextrose Agar (SDA)[a]

1% neopeptone
4% dextrose
1.5% agar
Final pH = 5.6

Cellulose Agar (CA)[b]

0.2% sodium nitrate
0.1% potassium phosphate, dibasic
0.05 % magnesium sulfate
0.05 % potassium chloride
2 % cellulose powder[d]
0.005 % rose bengal
1.5 % agar
Final pH = 8.0

Lactophenol Cotton Blue[e]

20% concentrated phenol
20% lactic acid
40% glycerol
20% distilled water
0.05% cotton blue

Corn Meal Agar (CMA)[a]

5% corn meal infusion
1.5% agar
Final pH = 6.0

Czapek Solution Agar (CZA)[a]

3% saccharose
0.2% sodium nitrate
0.1% potassium phosphate, dibasic
0.05% magnesium sulfate
0.05% potassium chloride
0.001% ferrous sulfate
1.5% agar
Final pH = 7.3

Rose Bengal Agar (RBA)[a]

0.5% soytone
1% dextrose
0.1% potassium phosphate, monobasic
0.05% magnesium sulfate
0.005% rose bengal
1.5% agar
Final pH = 7.2

Dichloran Glycerol-18 (DG-18)[c]

1.0% glucose
0.5% peptone
0.1 % potassium phosphate, monobasic
0.05% magnesium sulfate
1.5% agar
18% (wt./wt.) glycerol
0.0002% dichloran (in ethanol)
0.0005% chlortetracycline (in water)
Final pH = 5.6

[a] MEAC can be prepared by adding chloramphenicol (100 μg/ml final concentration) to the MEA formulation [chloramphenicol is added to the cooled media (51°C) after autoclaving]; formulation obtained from Difco Manual, 10th ed., Difco Laboratories, Detroit, 1985.
[b] Formulation courtesy of W. Sorenson, NIOSH, Morgantown, West Virginia, and B. Jarvis, University of Maryland, Maryland.
[c] Filter-sterilized chlortetracycline is added to the cooled media after autoclaving; formulation obtained from A. D. Hocking and J. I. Pitt, *Appl. Environ. Microbiol.* **39** (3), 488–492 (1980).
[d] Sigmacell type, Cat. No. S-6790, Sigma Chemical Co., St. Louis, Missouri.
[e] Formulation obtained from D. H. Larone, *Medically Important Fungi. A Guide to Identification*, 3rd ed., ASM Press, Washington, 1995.

METHODS OF IDENTIFICATION

Air sampling is the traditional method used for the collection of spores for identification of airborne fungi. Two approaches exist for air sampling: collection of viable spores onto growth media for the culture of colonies and collection of total spores (viable and nonviable) onto an adhesive-coated glass slide for the microscopic examination of fungal spores (8). Chemical analyses for ergosterol and glucans have been developed as a means to measure fungal biomass. In addition, immunological analyses that can detect certain fungal allergens and mycotoxins have also been developed. However, both chemical and immunological assays have relatively low sensitivity of detection (5). Of increasing interest is the detection of environmental fungi using molecular techniques such as polymerase chain reaction (PCR). However, the limited number of published unique nucleic acid sequences and the similarities between closely related genera have made this approach difficult. A more detailed discussion on alternative methods of sampling and analysis of airborne microorganisms can be found elsewhere in this Encyclopedia.

IDENTIFICATION CRITERIA

Fungal (mold) identification is achieved by the observation of temperature requirements in combination with both macroscopic and microscopic characteristics. Macroscopic

characteristics include colony appearance and growth rate. Colony appearance and growth rate can vary, depending on the culture medium that is used and the age of the colony. Therefore, it is important to note the culture medium being used by the reference text of choice, especially if the manual includes color pictures of the fungal morphology. The colony appearance refers to the color of the surface and the reverse of the colony, the texture, configuration, margin, and elevation (18). The growth rate refers to the diameter of the colony and can be recorded periodically for identification purposes. Most reference books for fungal identification give emphasis to the surface and reverse colors of the colony, indicating growth rate in a subjective manner rather than by measurements. Microscopic characteristics include septation of hyphae, size, color, shape and arrangement of spores, and specialized structures. Microscopic characteristics can vary, depending on the age of the colony. Hyphae may be septate, with cross walls, or aseptate (coenocytic) with no cross walls. They can appear with or without color; however, colorless hyphae will tend to absorb the stain being used for mounting (i.e., structures appear blue when stained with lactophenol cotton blue). The size, shape, arrangement, and color of the spores are the most important determinants of fungal identification. The presence of specialized structures should also be noted because it may be important for the identification of some genera such as *Bipolaris* sp. and in the identification of *Aspergillus* to the species level. Taxonomic, dichotomous, and illustrated keys are generally used for the identification of fungi (3,6,12) and the the easiest one is probably that of the dichotomous or illustrated key, in which a flowchart approach guides the user in the identification process (6). Beginning with a fungal colony and a microscopic slide preparation of the organism of interest, this approach focuses initially on the macroscopic morphology (especially surface and reverse colors) before guiding the user to microscopic drawings of the possible matches and to more detailed information on each possible match. This approach narrows down the possibilities until a match is found for the organism of interest.

PROCESSING AND IDENTIFICATION OF SAMPLES

Fungal identification is achieved following four steps: (*1*) sample collection, (*2*) sample processing, (*3*) culture and incubation, and (*4*) analysis. A detailed discussion on sampling for airborne microorganisms can be found elsewhere in this Encyclopedia.

Analysis of Air Samples

The approach suggested for the identification of culturable fungi present in air samples is summarized in Figure 1. Following collection and incubation of an air sample, observation of the colonies through a stereoscope often reveals the presence of spores. With the interchangeable use of the top and bottom lighting on the stereoscope, it is often possible to visualize both the spores and their arrangement. With experience, fungi such as *Alternaria*, *Cladosporium*, *Penicillium*, *Epicoccum*, *Aspergillus*, *Paecilomyces*, *Chaetomium*, and *Rhizopus* can be identified to the genus level without the need for further microscopy work. Care must be taken to avoid misidentification of organisms with similar spore shape or arrangement (e.g., *Aspergillus* and *Penicillium*; *Bipolaris* and *Curvularia*; *Alternaria* and *Ulocladium*). If observation of the colony through a stereoscope does not reveal the presence of spores or if the spores observed cannot be identified with absolute certainty, it is necessary to prepare a mount of the colony (see the following text) for observation through a light microscope. The fungal mount is stained with a dye and observed through a light microscope at low magnification (100 X total magnification). Although several dyes are available for the staining of fungal structures, lactophenol cotton blue (Medical Chemical Corporation., Santa Monica, California) (Table 1) is the one that is most often used. This dye is both a staining and a mounting agent that preserves at the same time that it kills the fungal elements present in the sample (6). Scanning the mount at low magnification will reveal the areas containing spores. Certain areas may be too dense with spores impeding proper visualization, whereas others may be ideal for closer observation. Once fungal spores are located, increasing the total magnification to 400 X will reveal details of the spore shape and arrangement that will aid in identification. With the aid of dichotomous or taxonomic keys present on fungal manuals, it is possible to identify the fungus to the genus (and sometimes species level) by combining the colony morphology with the microscopic observations. Also, the use of selective culture media and incubation temperature will aid in the speciation of certain fungi such as *A. fumigatus*. In cases in which the spores cannot be identified because they are not present in any of the fungal manuals available, the organism is called an unknown. If no spores can be

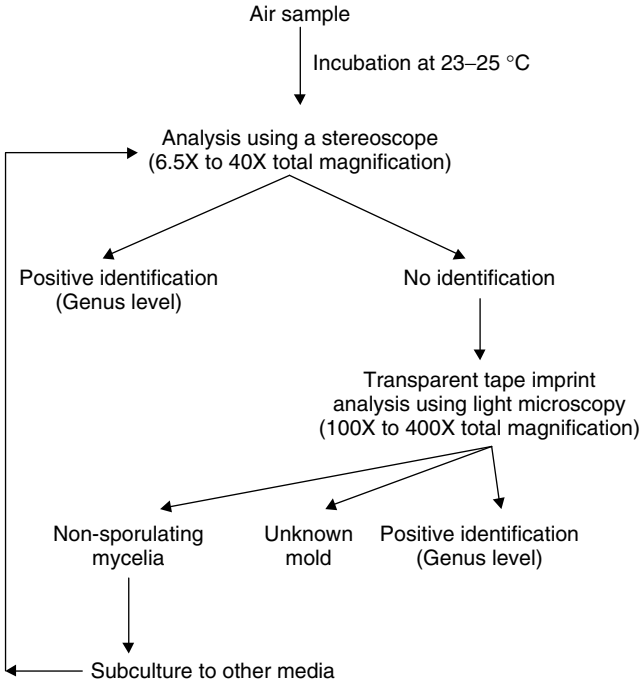

Figure 1. Flowchart summarizing the approach suggested for the identification of culturable fungi in air samples.

found, the organism should be subcultured onto other microbiological culture media such as PDA or CZA. Both the original culture and the subcultures should be incubated and examined periodically for up to 10 days. If the organism does not produce spores after these attempts, the organism is identified as nonsporulating mycelia or mycelia sterilia.

Impaction sampling onto an adhesive-coated surface [e.g., Burkard personal sampler (Burkard Manufacturing Co. Ltd., Rickmansworth, England), Air-O-Cell air sampling cassette (Zefon Analytical Accessories, St. Petersburg, Florida)] is often used for the detection of airborne fungi. It is particularly useful in the detection of fungi that would otherwise be missed by culturable sampling, either because the fungi are nonculturable or because they are dormant or slow growers that do not compete well with fast-growing fungi. Slides are mounted by placing a drop of lactophenol cotton blue on top of the adhesive portion of the sample and examined using a microscope. Similar to tape sampling (discussed in the following text), spores often have to be differentiated from debris. It is usually impossible to differentiate between organisms with similar spore shape. Also, because the spores observed cannot be cultured, there is usually only one opportunity to identify the fungi present. In addition, once the slide is stained, it must be analyzed within a few hours because air bubbles will form in the slit area under the cover slip, making analysis and quantitation difficult. This effect is of special concern with heavy samples showing a wide slit. In contrast to tape sampling, Burkard and Air-O-Cell sampling are volumetric methods and therefore quantitative.

Analysis of Surface Samples

Airborne fungi can settle on surfaces that can promote growth and amplification when sufficient water and nutrients are available. Therefore, identification of fungi present on surfaces is included in this review. The approach suggested for the identification of culturable and nonculturable fungi present in surface samples is summarized in Figure 2. Agar contact plates are incubated and analyzed in the same manner as culturable air samples (described earlier). However, they must be checked often because of the chance for overgrowth in the reduced area available on the sample surface of small agar plates. Swab, bulk (e.g., ceiling tile, wallboard, carpet, and air filter), and dust or vacuum samples require processing followed by incubation before identification. Fungal identification of the processed samples is achieved in the same manner as culturable air samples. Transparent tape imprints are often used as a sampling method for suspect fungal growth, especially of water-stained or visibly moldy building materials. Identification of fungi present in tape imprints involves mounting the tape on a drop of lactophenol cotton blue and examining with a light microscope. Examination of this type of sample is often lengthy because the spores and fungal elements must be discriminated from the debris present in this type of sample. Unless spore arrangement and critical identification structures are present in the sample, it will be difficult to differentiate between organisms with similar spores. Therefore, spores of the genus *Aspergillus* that resemble *Penicillium* spores are identified as belonging to the *Aspergillus or Penicillium* group; the same applies for *Bipolaris or Dreschlera* and *Alternaria or Ulocladium*. This method is only qualitative and there is usually only one opportunity to identify the fungi present because the spores observed cannot be cultured. In addition, once the tape is stained, it must be analyzed within a couple of hours because the adhesive in the tape will begin to dissolve with prolonged contact with the dye.

SAFETY PRECAUTIONS

It is important that safety precautions be used when handling fungal colonies and fungal contaminated material. A biosafety cabinet is necessary whenever fungi-containing samples are examined. Surfaces on which samples have been placed should be decontaminated often with a 10% bleach solution or other biocide such as 70 to 75% isopropanol solution. Discards should be properly wrapped and autoclaved before disposal or washing. In addition, gloves should be worn and hands should be washed thoroughly with soap and water after handling fungal plates and slides. Under no circumstance should fungal colonies or fungal contaminated materials be smelled. For a more comprehensive list of safety precautions, mycology books (especially medical mycology books) should be consulted (3,6).

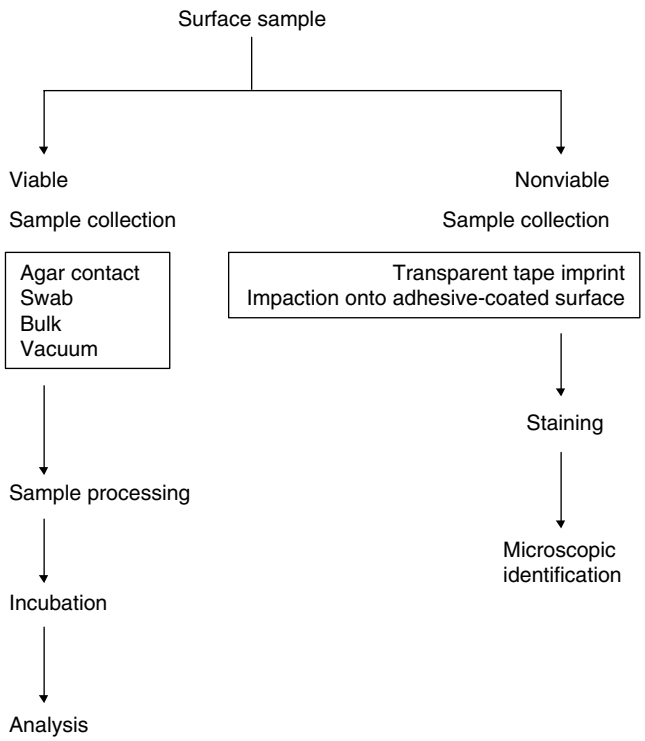

Figure 2. Flowchart summarizing the approach suggested for the identification of culturable and nonculturable fungi in surface samples.

TOOLS OF IDENTIFICATION

Several methods are available for the preparation of fungal mounts: transparent tape imprint, cover slip culture, and slide culture (6).

Transparent Tape Imprint

This is the fastest and easiest way to prepare a microscopic mount for fungal identification. Imprints are generally made of colonies growing on fungal media in the laboratory but they can also be used as a field-sampling method (as described earlier). It is important to use transparent rather than frosted cellophane tape to obtain a clear mount. It is a matter of personal choice, whether the $\frac{1}{2}$" or $\frac{3}{4}$" tape width is used; however, the wider tape is easier to handle. Following the safety precautions already mentioned, a piece of tape approximately 1.5"long is cut and looped with the adhesive toward the outside. Care must be taken to handle the tape only by the edges. The adhesive part of the tape is pressed lightly against the surface of the colony. If required, a finger or a disposable swab can be used to press down on the tape. Fungal colonies growing on agar are circular, growing from a central point toward the outside; both the center and edge of the colony should be sampled because these areas will contain old and new fungal growth, respectively (2). When pressing the tape, one must avoid pressing too hard because this will produce a sample that is too dense and difficult to analyze; when in doubt, a second (less dense) sample should be prepared. Touching adjacent colonies should be avoided because this can lead to misidentification of fungal colonies. When sampling very small colonies, the region of the tape containing the sample should be noted to make analysis easier. The tape is unlooped and mounted on a drop of lactophenol cotton blue on a glass slide, avoiding air bubbles or wrinkles in the mount. Using too much lactophenol should be avoided or cleaned up immediately. Dye on top of the tape will make visualization impossible, and any dye on top or sides of the tape can damage the microscope objectives. Once the tape is stained, it must be analyzed within a couple of hours because the adhesive in the tape will begin to dissolve with prolonged contact with the dye. Observation through a microscope using 100 X total magnification will reveal the areas with spores that are not too dense for observation at higher magnification. Slides should be discarded (e.g., inside empty slide boxes and taped when full) and autoclaved before disposing. When using this method for field sampling, the sample is collected and mounted onto a glass slide without the dye. At this stage, the sample can be stored indefinitely until it can be stained and analyzed. Therefore, it is important to label the slide properly and to store it in a cool, dry place. In addition, it is best to store each slide individually on collection either in a plastic slide holder or inside a Whirlpack bag (Nasco, Fort Atkinson, Wisconsin) to prevent cross-contamination. Staining and analysis is performed in the same manner as for imprints taken off a colony in the laboratory.

Cover Slip Culture

This is a method used for the identification of fungi on subculture and/or for the preparation of microscope mounts that can be used for taking pictures when a microscope with a camera setup is available. This method is a modification of the traditional slide culture method, in which hyphae and spores grow on the surface of a cover slip. The colony of interest is subcultured on the media of choice using a three-point inoculation. A swab usually works better than an inoculating loop for this purpose. Using sterile forceps, sterile cover slips are positioned over the inoculum at a 45° angle and inserted halfway into the agar. Up to three cover slips can be inserted on a plate; however, it is best to do only two while the third colony is left untouched to observe macroscopic fungal characteristics better and to prepare a tape imprint in case it is necessary. The plate is incubated with the agar side down and examined periodically until sporulation begins; this examination can generally be done by using a stereoscope. Hyphae and spores will grow on the glass surface of the cover slip, producing an intact arrangement of the spores. The cover slip is pulled gently using forceps and mounted on a drop of lactophenol cotton blue on top of a glass slide. Very often, fungal growth will be present on both sides of the cover slip. Therefore, the side of the cover slip away from the dye should be cleaned with an alcohol-moistened swab (70% isopropanol or 70% ethanol). This is difficult to achieve because the cover slip is already mounted on the dye. Alternatively, a second drop of lactophenol is placed on top of the cover slip that has already been mounted and a second cover slip is applied on top; this traps the fungal growth in between two layers of dye. This approach will produce a mount that although sharp and clear needs to be visualized in two planes of focus. The mount is observed under a microscope at low and high magnification and analyzed as indicated previously. If no spores are visible, the plate can be reincubated and a second cover slip can be removed and mounted later. Cover-slip preparations can be sealed around the cover slip edges with clear nail polish and stored flat in a slide holder. However, with time, the seal may start to break and the dye can start leaking out or the mount will start drying out. A commercially available dye that contains lactophenol cotton blue with an adhesive (Mycoperm, Scientific Device Laboratory, Inc., Glenview, Illinois) can be used for the preparation of permanent slides.

Slide Culture

Slide culture has been used traditionally for the preparation of fungal microscope mounts, especially for those organisms with fragile spore chains (8). It provides an undisturbed arrangement of the fungal colony of interest; however, the macroscopic morphology cannot be appreciated with this method. For this technique, a sterile glass petri dish is preferred because of the depth they provide over the disposable type. The petri dish is lined with a sterile piece of filter paper and sterile water (~2 mL) is added to the bottom of the plate. A U-shaped glass tube is placed on top of the filter paper and a glass slide is positioned on top of the glass tube. An agar square of approximately 1 cm × 1 cm is aseptically cut and removed from an agar plate (poured 4-mm deep with the media of choice) and transferred to the center of the glass slide

inside the petri dish. The agar block is then inoculated with the organism of interest by using an inoculating loop to touch the sides of the square. A cover slip is placed on top of the inoculated agar block and slight pressure is applied. The petri dish is incubated and checked periodically for signs of sporulation. It is important that water be added as required to the bottom of the petri dish to prevent the agar block from drying out. Once ready to analyze, the cover slip is removed gently with forceps and mounted on a drop of dye on a glass slide. The mount is observed using a microscope at low and high magnification and analyzed as indicated previously. If no spores are visible, a clean cover slip should be placed on top of the inoculated agar block and the plate should be reincubated until sporulation occurs. It is also possible to prepare an additional mount by removing the agar block from the top of the glass slide and disposing it in a biohazard bag. The slide should be stained by placing a drop of dye followed by a cover slip. Like cover slip culture, this type of preparation can also be sealed around the cover slip edges with clear nail polish and stored flat.

COMMON ENVIRONMENTAL FUNGI

Although mold populations are seasonal and vary from one region to another, certain environmental fungi are present throughout the world (2). Generally identified to the genus level because of the difficulties discussed previously, the fungi included in this section comprise the majority of the organisms that are generally encountered in environmental samples. A description of the general macroscopic and microscopic characteristics of each genus has been summarized from several mycology books (6,12,19). These descriptions are based on morphologies observed on Sabouraud dextrose agar. The following genera (arranged in alphabetical order) are discussed: *Alternaria, Aspergillus, Bipolaris, Chaetomium, Cladosporium, Curvularia, Epicoccum, Fusarium, Paecilomyces, Penicillium, Rhizopus, Stachybotrys*, and *Trichoderma*. A brief description on yeasts is also included.

Alternaria

There are approximately 50 known species with some having spores measuring up to 200 μm in length. Generally, spores are 23 to 34 μm in length by 7–10 μm in diameter at the widest part. Sporulation occurs in approximately five days. Macroscopic morphology is characterized by a woolly colony with a surface that starts as pale gray and becomes olive/black or brown and with a black reverse. Microscopic characteristics (Fig. 3a) include (*1*) brown (dematiaceous) hyphae and conidia; (*2*) muriform (longitudinally and transversely septate) conidia; (*3*) chains of elongated conidia; and (*4*) a beaklike apical cell. The presence of elongated conidia in chains is the characteristic that distinguishes *Alternaria* from the genus *Ulocladium* (Fig. 3b). *Ulocladium* spores also have septate conidia; however, these are usually smaller, rounder, and their arrangement is in an alternate fashion rather than in chains.

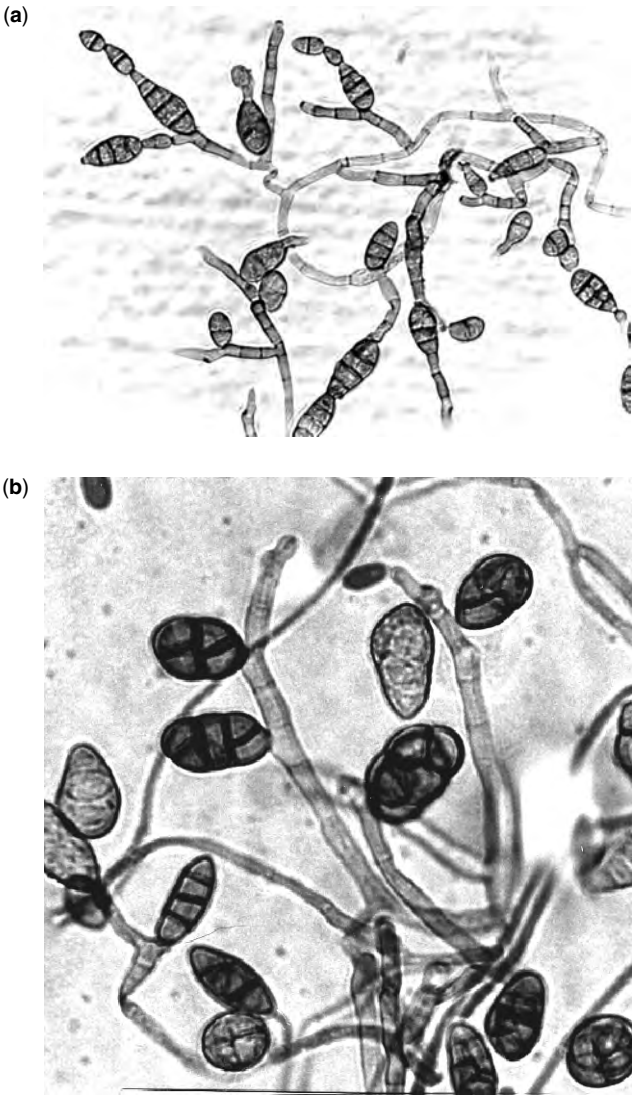

Figure 3. (**a**) Alternaria spores in chains. (**b**) Ulocladium spores and hyphae.

Aspergillus

There are approximately 185 known species, with spores generally measuring 2 to 5 μm in diameter. Most species are rapid growers, showing sporulation in approximately three days. Some species such as *A. niger* have conidiophores completely covered with spores that in culture are visible with the naked eye. Macroscopic characteristics include a velvety, powdery, or cottony colony with a surface color that starts as white and becomes yellow, green, brown, or black and a reverse that can be white, gold, or brown. Microscopic morphology is characterized by (*1*) foot cells (merging of hypha with the base of the conidiophore forming an inverted letter T) (Fig. 4a); (*2*) vesicles (swollen structures at the end of a conidiophore) that are partially or completely covered by

phialides (cells that produce conidia) and conidia (Fig. 4b); (*3*) the presence of round or oval conidia in chains; and (*4*) conidia with smooth or rough walls. Single spores of *Aspergillus*, such as those encountered in impaction sampling onto an adhesive-coated surface, cannot be distinguished from those of the genus *Penicillium*. The arrangement of spores around the vesicle, its size, and shape are the main characteristics used in the speciation of *Aspergillus*.

Many of the *Aspergillus* species produce both an asexual and sexual (teleomorph) stage. The species is generally identified by the asexual name because of the difficulty in obtaining the sexual stage in the laboratory (12). However, if the sexual stage is observed, many mycologists identify the species by the sexual name (e.g., *A. glaucus* and *A. nidulans* are the asexual forms and *Eurotium* and *Emericella* are the sexual forms, respectively). There are several publications that aid in the speciation of *Aspergillus* (20,21).

Bipolaris

There are approximately 52 known species. Generally, spores measure 16 to 35 μm in length by 6 to 12 μm in diameter. Sporulation occurs in approximately five days. Macroscopic morphology is characterized by a downy colony with a gray or brown surface becoming black, and a black reverse. Microscopic characteristics (Fig. 5a) include (*1*) brown hyphae; (*2*) knobby conidiophores, curved at the end bearing conidia; (*3*) spores born in an alternate fashion on the conidiophore; (*4*) black scars on the spores at points of attachment to the conidiophore; (*5*) brown, thick-walled conidia; and (*6*) oblong to cylindrical conidia containing four or more cells. The absence of curved conidia and a darker central cell distinguishes *Bipolaris* from the genus *Curvularia* (described in the following text). Two other genera (*Drechslera* and *Exserohilum*) also resemble *Bipolaris*. *Drechslera* produces germ tubes (germination of hyphae from a spore) emerging from any cell of the conidium, perpendicular to the long axis, whereas the germ tubes of *Bipolaris* are polar and in the direction of the long axis of the conidium. In addition, *Drechslera* isolates tend to become sterile in the laboratory. Therefore, when unable to differentiate between these two organisms, they should be reported as *Bipolaris* or *Drechslera*. Conidia produced by the genus *Exserohilum* are longer (14 by 80 μm in length), have a protuberant hilum (scar remaining at point of attachment), and the end cells of some species are delimited by dark septa (Fig. 5b). The hilum of *Bipolaris* is scarcely protuberant.

Chaetomium

There are approximately 81 known species. Spores (ascospores) generally measuring 6 to 11 by 3.5 to 8.5 μm in diameter are contained within asci (cells producing and containing ascospores) in a saclike structure called a perithecium (pl. perithecia) that measures approximately 100 to 150 by 110 to 225 μm in diameter. Perithecia are often visible in a culture with the naked eye. Sporulation occurs in approximately five days. Macroscopic characteristics include a cottony colony with a surface, which starts as white and becomes tan/gray or gray/olive with age, and an orange/tan

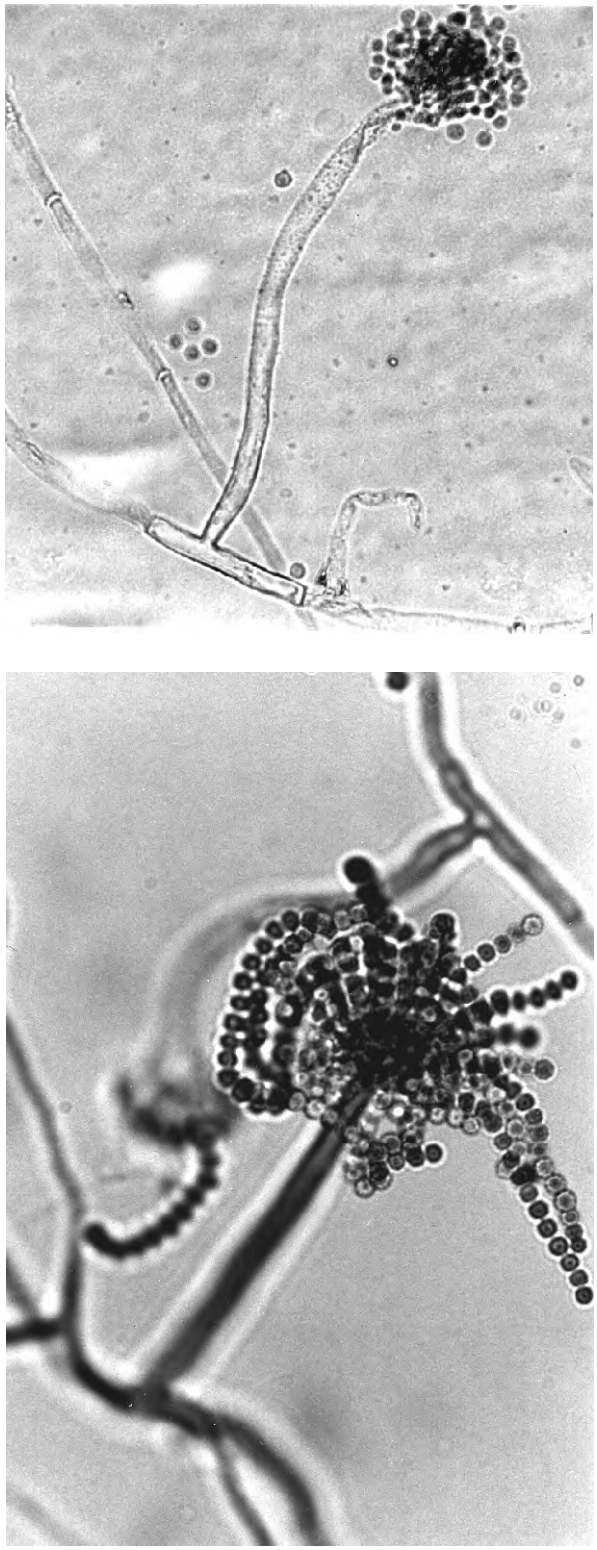

Figure 4. (**a**) Aspergillus conidiophore with visible foot cell. (**b**) Aspergillus vesicle covered with spores.

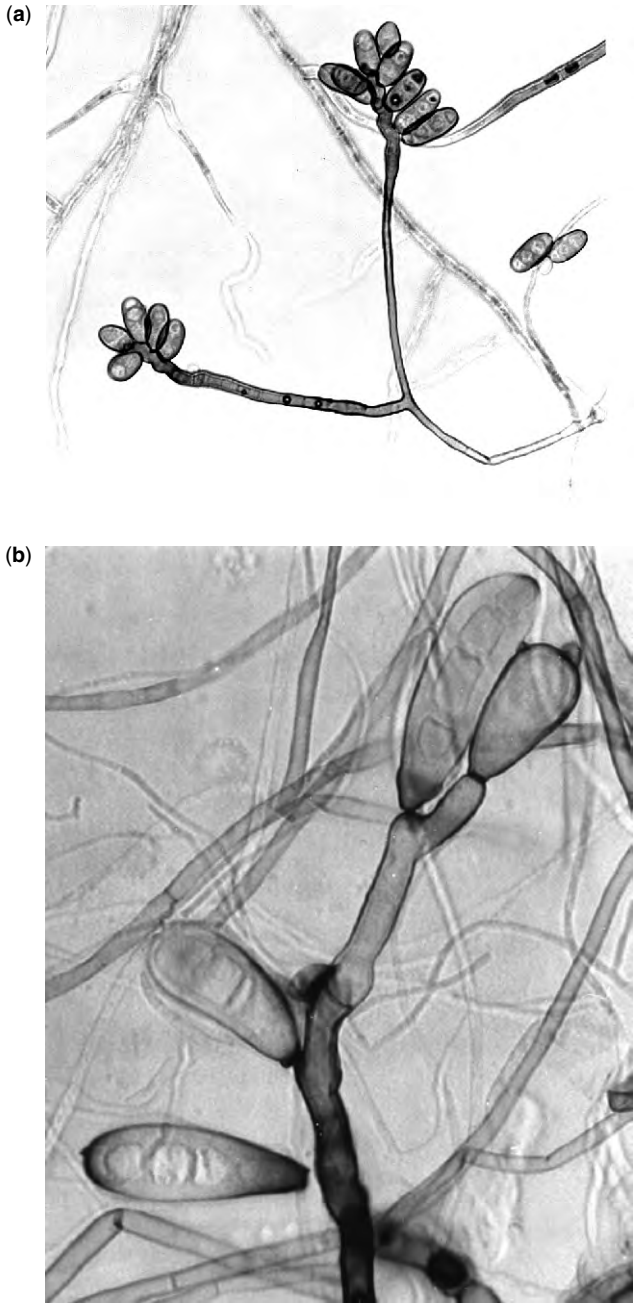

Figure 5. (**a**) Bipolaris conidiophores. (**b**) Exserohilum conidiophore.

or brown to black reverse. Microscopic morphology is characterized by (*1*) round to ovoid, olive to brown perithecia; (*2*) perithecia surrounded by spinelike hairs (Fig. 6a); (*3*) long, undulant, helical and/or erect hairs; (*4*) asci containing four to eight ascospores that readily dissolve on release from the perithecia; and (*5*) brown, unicellular, oval to lemon-shaped ascospores (Fig. 6b). One feature that distinguishes *Chaetomium* from the genus *Phoma* is the presence of spinelike hairs (Fig. 6c). In addition, *Phoma* produces large fruiting bodies (pycnidia) that have an opening (ostiole) from which spores are released. The ostiole produced by *Phoma* is usually more readily visible than that produced by *Chaetomium*. Also, the spores produced by *Phoma* tend to be slimy, smaller (3 to 5 by 2 to 3 μm in diameter), are oval to round, and are lighter in color than those of *Chaetomium*. In cases in which the pycnidia produced by *Phoma* cannot be distinguished from the perithecia produced by *Chaetomium*, it is recommended that the microscopic mount be pressed (e.g., with the eraser of a pencil) to break open these structures and visualize its contents. *Arthrinium* also produces spores that resemble those produced by *Chaetomium*. Single ascospores of *Chaetomium* such as those encountered in impaction sampling onto an adhesive-coated surface can be confused with those of the genus *Arthrinium*. Characterized by caramel-colored hyphae with swollen cells, *Arthrinium* produces conidia with an equatorial germ slit (Fig. 6d).

Cladosporium

There are approximately 50 known species. Generally, spores measure 4 to 12 μm in length by 3 to 6 μm in diameter. Sporulation occurs in approximately seven days. Macroscopic morphology is characterized by a velvety colony with very short hyphae. The surface color can be olive/brown or black and the reverse is black. Microscopic characteristics (Fig. 7) include (*1*) brown hyphae; (*2*) brown spores resembling very pointy rice grains with black scars at points of attachment; (*3*) very fragile branching treelike spore chains; and (*4*) shield-shaped cells at the base of the chains.

Curvularia

There are approximately 33 known species, with spores generally measuring 8 to 14 μm in length by 21 to 35 μm in diameter. Sporulation occurs in approximately five days. Macroscopic characteristics include a pink-gray woolly colony with an olive-green, brown, or black surface and a dark reverse. Microscopic morphology (Fig. 8) is characterized by (*1*) brown hyphae; (*2*) curved conidia usually with 3 to 4 cells; and (*3*) central cell that is typically expanded and is darker than the other cells. The spores of *Curvularia* can be confused with those of *Bipolaris* (Fig. 5a). However, the curved spores and the darker central cell distinguish *Curvularia* from the genus *Bipolaris*. In addition, the spores of *Curvularia* are septate, whereas those of *Bipolaris* are distoseptate (cells contained within sacs, lacking conventional septations).

Epicoccum

There are two known species. Generally, spores measure 15 to 30 μm in diameter. Sporulation occurs in approximately seven days. Macroscopic morphology is characterized by a cottony colony that starts with a yellow to orange surface becoming brown to black. The reverse color can be red, with some species producing a yellow, brown, orange, or red pigment that diffuses into the medium. Microscopic characteristics (Fig. 9a,b) include (*1*) hyaline (clear) to brownish hyphae;

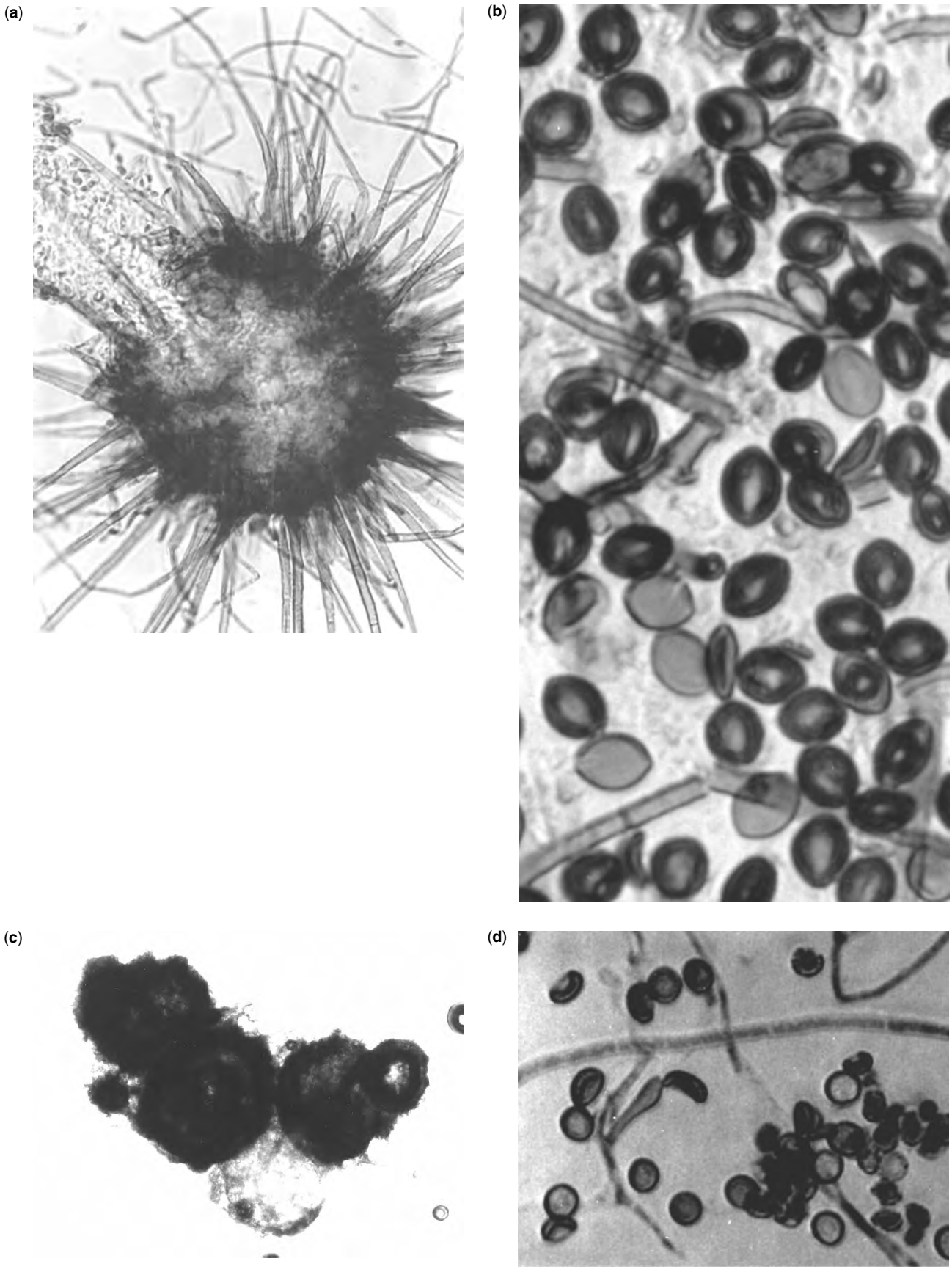

Figure 6. (**a**) Chaetomium perithecium. (**b**) Chaetomium ascospores observed at high magnification. (**c**) Phoma pycnidia. (**d**) Arthrinium spores and conidiophore.

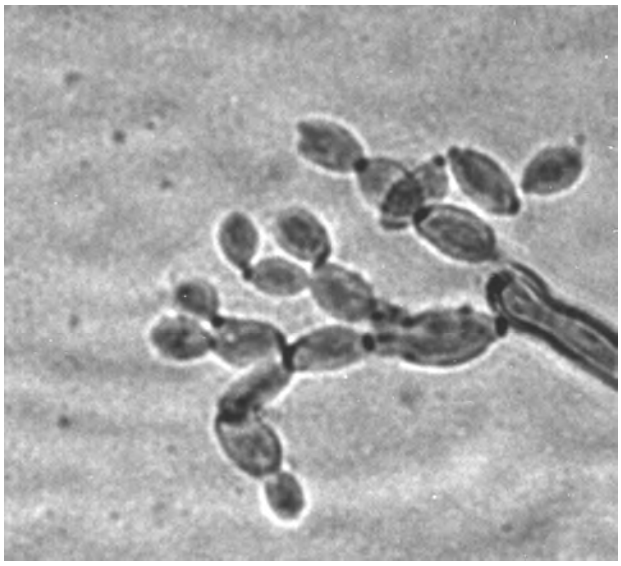

Figure 7. Cladosporium conidiophore.

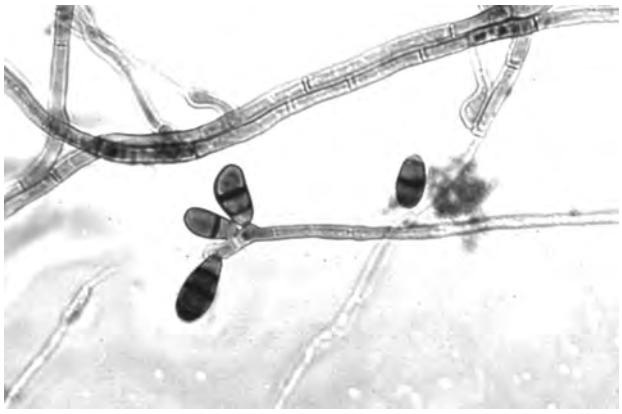

Figure 8. Curvularia conidiophore and hyphae.

(*2*) short conidiophores bearing more or less round conidia; (*3*) brown-black mature conidia with rough walls; (*4*) conidia with both transverse and longitudinal septations; and (*5*) conidia grouped in clusters. Mature clusters of spores are often visible in a culture with the naked eye. For isolates suspected of being *Epicoccum* that do not produce spores in the laboratory, sporulation may be stimulated by incubation under ultraviolet (UV) light alone or in combination with growth on 2% water agar (12).

Fusarium

There are approximately 50 known species; in which large (macroconaidia) and small (microconidia) spores are produced. Macroconidia generally measure 11 to 70 μm in length by 3 to 8 μm in diameter. Microconidia measure approximately 4 to 8 μm in length by 2 to 4 μm in diameter. Sporulation occurs in approximately four days. Macroscopic characteristics include a cottony, sometimes mucoid, colony with a surface that

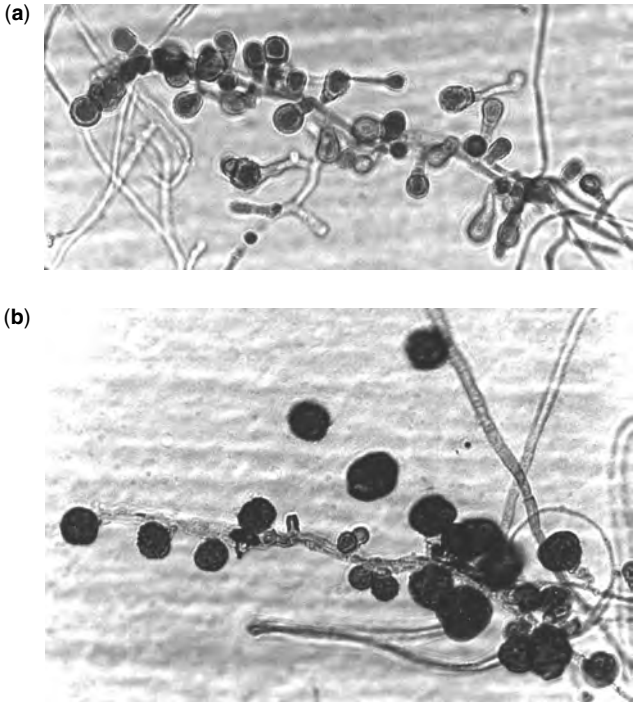

Figure 9. Epicoccum young (**a**) and mature (**b**) spores.

starts as white and becomes pink or violet in the center and with a light reverse. Microscopic morphology is characterized by (*1*) hyaline hyphae; (*2*) macroconidia that are curved and multiseptate; (*3*) foot cells present at the base of the conidiophores; and (*4*) microconidia that are unicellular, sometimes bicellular, ovoid to ellipsoid in slimy heads or in chains (Fig. 10a,b). The microconidia of *Fusarium* can be confused with the spores of *Acremonium* (Fig. 10c). However, the presence of macroconidia and foot cells distinguishes *Fusarium* from the genus *Acremonium*. The genus *Cylindrocarpon* resembles *Fusarium* by producing macroconidia and microconidia; however, the macroconidia produced by *Cylindrocarpon* have rounded ends, whereas those produced by *Fusarium* have pointed ends. In addition, the absence of foot cells distinguishes *Cylindrocarpon* from *Fusarium*.

Paecilomyces

There are approximately 41 known species. Generally, spores measure 2 × 3.5 μm in diameter. Sporulation occurs in approximately three days. Macroscopic morphology is characterized by a powdery or velvety colony with a surface that is yellowish-brown, lilac, or another color (except green), and an off-white to brown reverse. Microscopic characteristics (Fig. 11a,b) include (*1*) hyaline hyphae; (*2*) brushlike clustered phialides elongated at the tips; (*3*) conidia oval to fusoid in long chains; and (*4*) phialides/conidial chains that bend away from the axis of the conidiophore. The brushlike appearance of the conidiophores of *Paecilomyces* may be confused with that of the genus *Penicillium* (Fig. 12a). However, the phialides

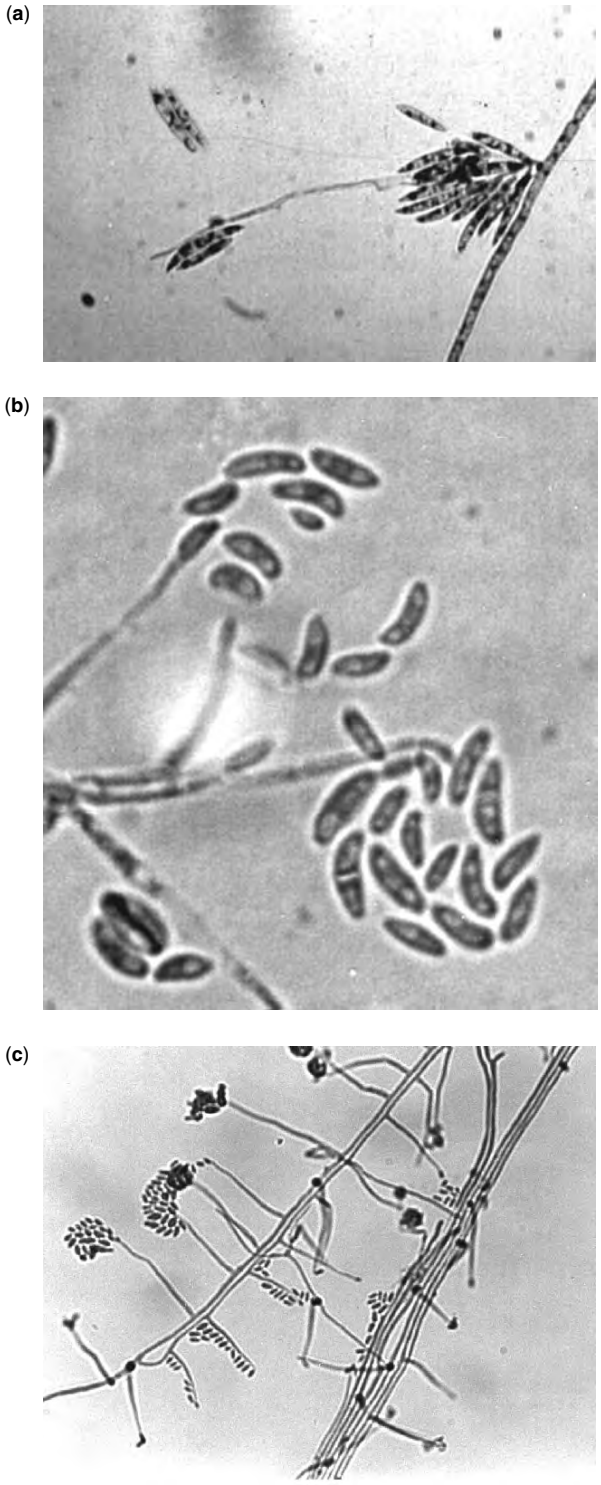

Figure 10. (**a,b**) Fusarium macroconidia. (**c**) Acremonium conidiophores and hyphae.

of *Paecilomyces* are swollen at the base and slender at the top that bears the conidia. In addition, *Paecilomyces*

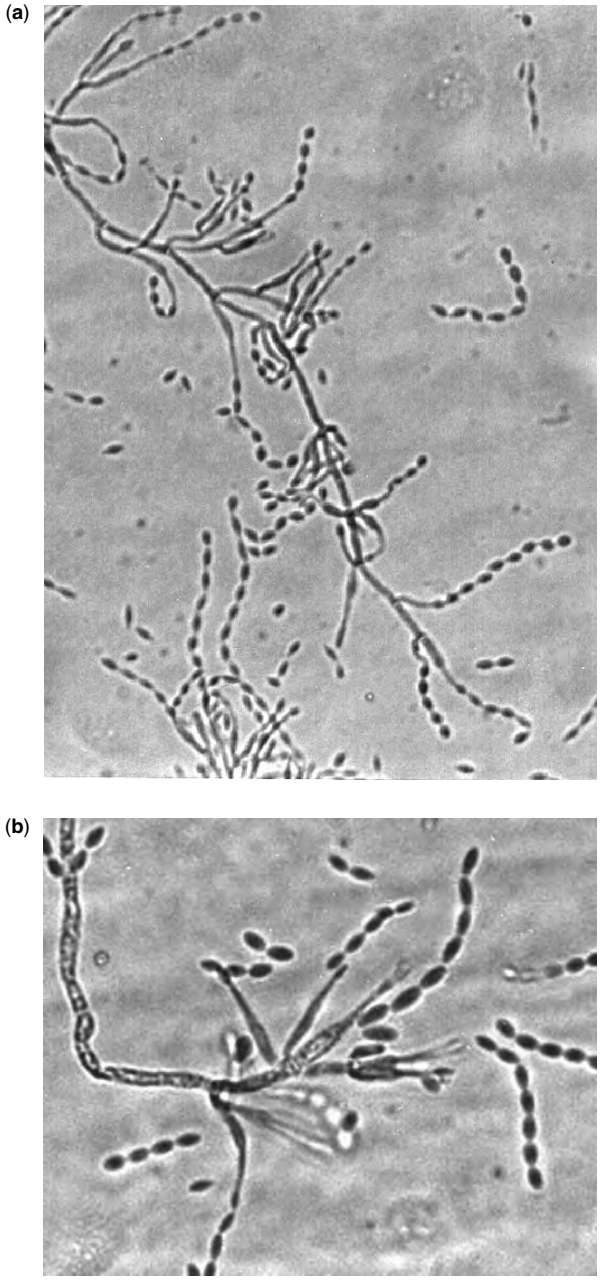

Figure 11. (**a**) Paecilomyces spores and hyphae. (**b**) Detail of Paecilomyces phialides.

produces spores that are oval as opposed to those produced by *Penicillium* that are round to oval.

Penicillium

There are approximately 223 known species. Conidia generally measure 2.5 to 5 μm in diameter. Sporulation occurs in approximately four days. Macroscopic characteristics include a powdery colony with a surface that starts as

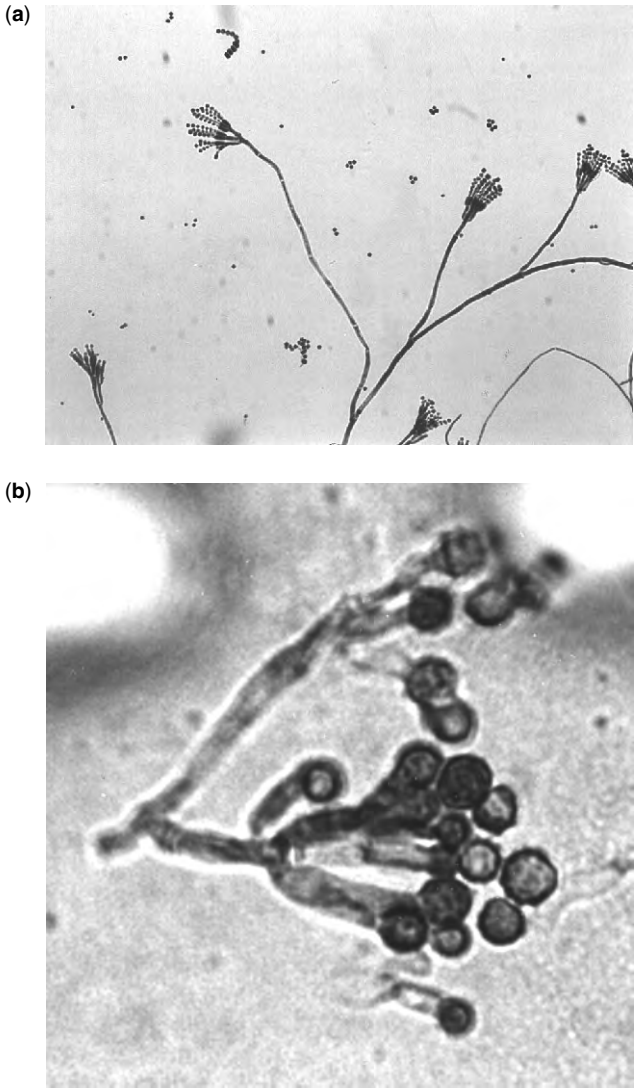

Figure 12. (**a**) Penicillium conidiophore and spores. (**b**) Scopulariopsis conidiophore.

white and becomes blue green with a white margin. However, other surface colors and textures have been observed in less common species. The colony reverse is white, sometimes red or brown. Microscopic morphology (Fig. 12a) is characterized by (*1*) hyaline hyphae; (*2*) brushlike conidiophores; (*3*) round to oval spores in chains; (*4*) hyaline or pigmented spores; and (*5*) spores with smooth or rough walls. The spores of *Penicillium* can be confused with those of at least four genera, namely, *Paecilomyces* (described in the previous section) (Fig. 11a,b), *Trichoderma* (described in the following text) (Fig. 15), *Scopulariopsis* (Fig. 12b), and *Gliocladium*. Conidia produced by *Scopulariopsis* are round to lemon-shaped, thicker-walled, larger (4 to 9 μm in diameter), and cut off at the base (truncate base). In addition, their mature spores often have spiny walls and their conidiophores generally are shorter than those of *Penicillium*. *Gliocladium* produces brushlike conidiophores containing masses of sticky conidia at their tips that (unlike *Penicillium*) tend to clump.

Some *Penicillium* species produce both an asexual and sexual stage. The sexual stage is characterized by the presence of sexual fruiting bodies. Many mycologists identify these species by their sexual name (i.e., *Eupenicillium* or *Talaromyces*) (23). Several publications exist that aid in the speciation of *Penicillium* (4,24).

Rhizopus

There are approximately 10 known species. Spores (sporangiospores) generally measuring 4 to 11 μm in diameter are contained within a saclike structure called a *sporangium* (pl. sporangia) that measures approximately 40 to 350 μm in diameter. Mature sporangia are visible in a culture with the naked eye. Sporulation occurs in approximately four days. Macroscopic morphology is characterized by a cottony colony that grows very rapidly. The surface of the colony starts as white and becomes gray or yellow/brown and the reverse is white. Microscopic characteristics include (*1*) hyaline hyphae; (*2*) wide hyphae with few or no septations; (*3*) long sporangiophores (the equivalent of a conidiophore, approximately 4-mm long); (*4*) dark, round sporangia; (*5*) ovoid spores; (*6*) hyaline or brown spores; and (*7*) rhizoids (rootlike hyphae opposite the sporangiophore that are submerged in the growth medium) (6) and stolons (horizontal hyphae often connecting sporangiophores and bearing rhizoids) are present (Fig. 13). The microscopic morphology of *Rhizopus* can be confused with that of three genera, namely, *Mucor*, *Absidia*, and *Rhizomucor*, *Mucor* lacks rhizoids and stolons and produces branched and unbranched sporangiophores that are mostly hyaline. *Rhizopus* produces sporangiophores that are mostly brown. In addition, it is reported that *Mucor* has a maximum growth temperature below 37 °C, whereas that of *Rhizopus* is approximately 45 °C (6). *Absidia* produces indistinct rhizoids that form between stolons and not directly opposite to a rhizoid. It is often useful to use a stereoscope to visualize the rhizoids formed by *Absidia* isolates. *Absidia* also produces pear-shaped sporangia and finely branched sporangiophores that are almost hyaline. Its maximum growth temperature is the same as that of *Rhizopus* (6). *Rhizomucor* produces branched, dark brown sporangiophores. In

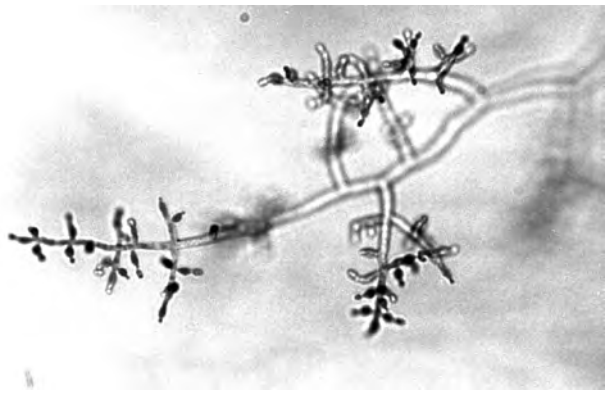

Figure 13. Trichoderma conidiophores (young).

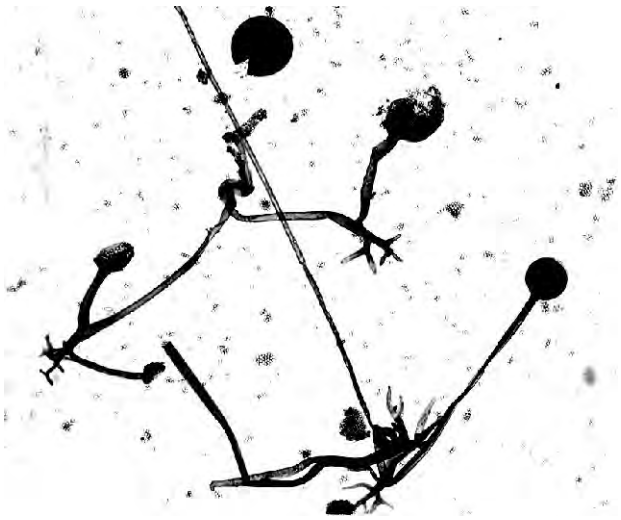

Figure 14. Rhizopus conidiophores with visible sporangiospores and rhizoids.

addition, short rhizoids forming between stolons are produced. Its maximum growth temperature is approximately 54 °C (6).

Stachybotrys

There are approximately 15 recognized species of *Stachybotrys* (22). Conidia generally measure 4.5 to 9 μm in diameter. Sporulation in laboratory media occurs in approximately four days in pure culture. However, up to 10 days may be required when other competing fungi are present. Macroscopic characteristics include a yeast-like colony surface that starts as white to beige. As the colony matures, cottony mycelia develop that become covered with black, oily droplets. The reverse of the colony starts with a light color that becomes dark. Microscopic morphology (Fig. 14a,b) is characterized by (*1*) hyaline to dark hyphae; (*2*) hyaline or pigmented conidiophores; (*3*) hyaline or pigmented phialides; (*4*) ellipsoidal phialides in groups of 3 to 10; (*5*) dark unicellular conidia; (*6*) oval conidia forming slimy masses; and (*7*) conidia with smooth to rough walls. The conidiophores of *Stachybotrys* can be confused with those of the genus *Memnoniella* (Fig. 14c). However, the spores produced by *Memnoniella* are smaller, round, and are produced in chains rather than in clumps.

Trichoderma

There are approximately nine known species. Spores generally measure 3 μm in diameter. Sporulation occurs in approximately five days. Macroscopic morphology is characterized by a rapidly spreading white fluff becoming woolly. As sporulation occurs, the surface of the colony is covered by scattered green patches often forming concentric rings. The reverse of the colony can be pale or light orange/tan to yellow. Microscopic characteristics (Fig. 15) include (*1*) hyaline hyphae; (*2*) branched conidiophores; (*3*) conidiophores bearing phialides attached at right angles; (*4*) phialides swollen at the base; and (*5*) round or oval conidia forming clusters at tips of phialides.

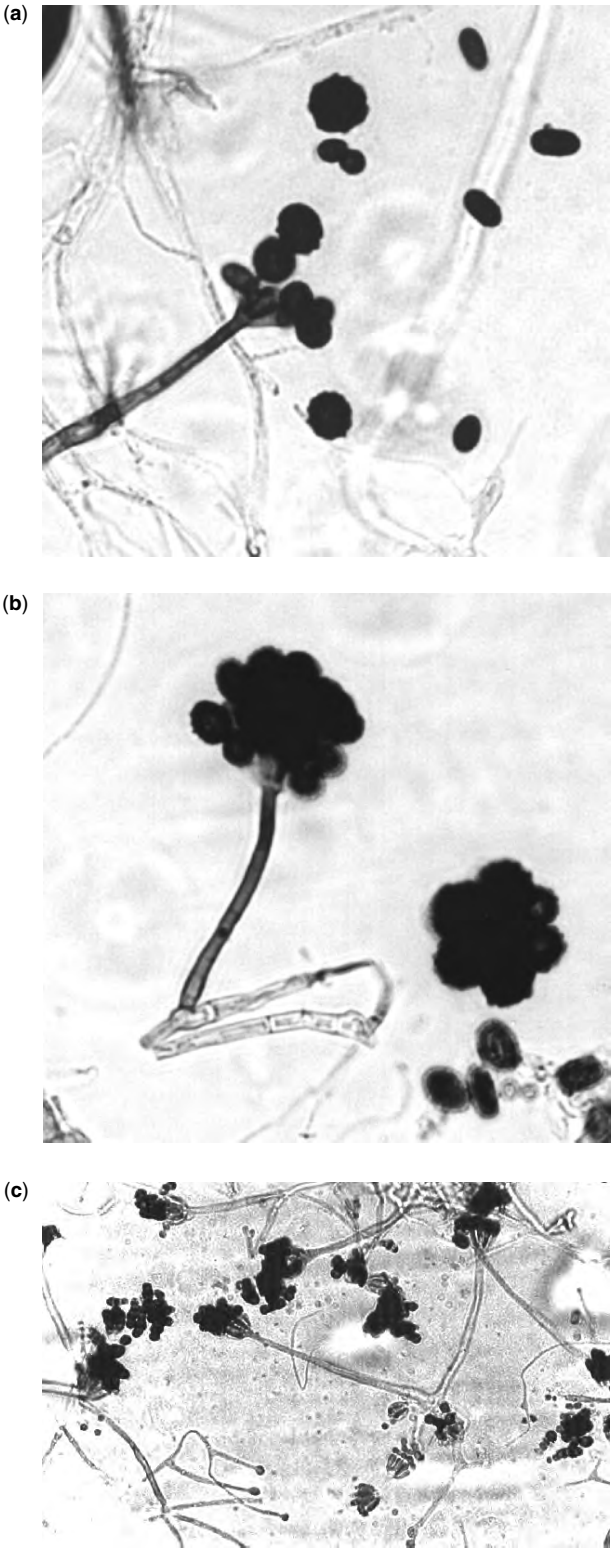

Figure 15. (**a**) Stachybotrys conidiophore and different shaped spores. (**b**) Stachybotrys spores covering conidiophore. (**c**) Memnoniella conidiophores and spores.

Yeasts

Macroscopic morphology is characterized by smooth, moist, or mucoid colonies that resemble bacterial colonies. Colony color is usually white to cream but can also be orange, pink, or tan. Reproduction occurs by budding or binary fission, a type of reproduction in which parent cells divide to produce two identical cells. Yeasts are characterized by the presence of buds, small outcroppings present in parent cells. Microscopically, yeasts are characterized by (*1*) single cells; (*2*) oval or round cells; (*3*) budding cells; (*4*) large cells observable at low magnification (100 X total magnification); and (*5*) sometimes pseudohyphae or elongated buds (2,12). Some organisms can produce true hyphae or no hyphae at all, and few can produce ascospores. Because it is necessary to perform biochemical and physiological tests for their classification into genus and species, yeasts generally are not identified but reported as the number of yeasts per sample (5). Yeast-identification procedures include determination of the presence of pseudohyphae or true hyphae, the germ tube test, carbohydrate assimilation or fermentation, the Dalmau test, capsule production, cycloheximide resistance, and temperature studies. There are several manuals (6,24) that can provide the reader with a more detailed description of yeast-identification methods.

CONCLUSION

With an increased awareness of the health effects associated with exposure to environmental fungi, there is also an increased interest in the identification of these microorganisms. Traditionally, mycology has emphasized on the study of pathogenic or opportunistic fungi and treated environmental (saprophytic) fungi as contaminants that can be encountered during routine analysis of medical specimens. It is the purpose of this review to give an overview of the culture media and methods that can be useful in environmental mycology and to present the reader with the basic methodology for identification of the genus level of several of the most common environmental fungi. Although identification of environmental fungi has its difficulties and requires practice, building a large reference library and acquiring and maintaining stock cultures can make identification easier and more accurate.

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IGNEOUS ROCK AQUIFERS MICROBIAL COMMUNITIES

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Independent scientific work has unambiguously demonstrated life to be present in most deep geological formations investigated, down to depths of several kilometers (1,2). Sub-seafloor environments (3,4), sedimentary rocks (5), and igneous rocks (6) all harbor life. The distribution of

underground life is conceptually restricted only by temperature. The temperature limit for life is, as far as is known, 113 °C (7). This temperature is reached at very different depths around the earth, from the seafloor surface at marine hot springs to 10 km or deeper in massive sedimentary rock formations. In the Fennoscandian Shield, the temperature typically increases between 1 and 2 °C per 100 m, implying that at igneous rock sites in Scandinavia, microbial life may extend as far as 10 km underground. Such rocks are penetrated by humans for a variety of purposes such as mining for metals, drilling boreholes for the extraction of groundwater and rock heat, and building tunnels and vaults for communication, transport, defense, storage of hazardous waste, and radioactive waste disposal. Microbial processes should, if possible, become an integral part of preinvestigations of such sites. This review presents research record from the last decade of microbiological research performed on microbial communities in deep igneous, Fennoscandian rock aquifers, the ultimate aim being to contribute to a safe disposal of high-level nuclear waste (8).

EXPLORATION METHODOLOGY

Eleven subterranean sites in Fennoscandian igneous rocks have been explored down to a depth of 1,700 m for diversity and activity of microbial communities (Table 1). A total of 75 specific borehole positions in 53 different boreholes have been investigated for geology, chemistry, total numbers of viable microorganisms, and microbial diversity and activity.

Drilling and Sampling of Rocks and Groundwater

All sampling of igneous hard rock aquifer material and groundwater require penetration of the rock to reach the target aquifers. There is only one main way of achieving this goal and that is via the drilling of holes (Fig. 1). The detailed procedure may be modified in many ways, but there are two main drilling approaches, namely, from the ground surface and from an underground tunnel. Drilling in hard rock is done either with percussion-boring or with core-drilling. The percussion drill does not recover any rock material and may introduce air into intersected aquifers. The debris, created during drilling, and groundwater, once an open fracture has been intersected, are forced to the surface by air. This type of drilling depends on the use of compressed air with a pressure higher than groundwater pressure. This sets a limit of about 150 to 200 m, at which depth the pressure needed will be too high for normal percussion drilling machines. Deeper boreholes must instead be drilled by core-drilling producing a core, which can be used for mapping the geological strata penetrated. The retrieval of rock aquifer material during drilling in igneous hard rock always requires core-drilling. Triple-tube drilling (Fig. 2) is the best available method

Table 1. Site Information Comprising All Boreholes in Fennoscandian Igneous Rock Investigated for Microbiology Since 1987

SITE	Year	Borehole	Geological Situation	Depths (m)	Levels
Hålö (S)	1992–1996	HBH01, HBH02	Porphyritic monzo-granite	10–45	(2 levels)
Hästholmen (F)	1997–1998	HH-KR1, H-KR2, HH-KR3, H-KR4, HH-KR5, HH-KR6	Rapakivi-type granite	65–943	(6 levels)
Kivetty (F)	1997–1998	KI-KR5, KI-KR13	Granodiorite and granite	497–721	(2 levels)
Laxemar (S)	1988–2000	KLX01, KLX02	Småland granites	100–1700	(7 levels)
Olkiluoto (F)	1998–1999	OL-KR3, OL-KR4, OL-KR8, OL-KR9, OL-KR-10	Gneisses, schists, grandiorite, and granites	248–863	(7 levels)
Palmottu (F)	1998–1999	R302, R337, R387	Supracrustal volcanic and sedimentary rock	32–309	(5 levels)
Romuvaara (F)	1998–1999	RO-KR10, RO-KR11	Gneisses, granodiorite, and metadiabase dykes	543–564	(2 levels)
Stripa (S)	1987–1991	V1, V2	Granite	799–1,240	(4 levels)
Ävrö (S)	1987	KAV01	Porphyritic granite-grandiorite	420–924	(4 levels)
Äspö (S)	1988–1996	KAS02, KAS03, KAS04	Porphyritic granite-grandiorite	129–1,002	(11 levels)
Äspö Hard Rock Laboratory (HRL) tunnel (S)	1992–2001	KR0012, KR0013, KR0015, SA813B, SA923A, SA1062A, HA1327B, SA1420A, KA2511A, KA2512A, KA2858A, KA2862A, KA3005A, KA3010A, KA3067A, KA3105A, KA3110A, HD0025A, KA3385A, KA3539 G, KA3548A01, KA3600F, KJ0050F01, KJ0052F02, KJ0052F03	Porphyritic granite-grandiorite	68–450	(25 levels)
11 sites	1987–2001	53 boreholes		10–1,700	(75 levels)

Detailed information about the boreholes and the rock formation studied can be obtained from Reference 9.
F = boreholes in Finland; S = boreholes in Sweden.

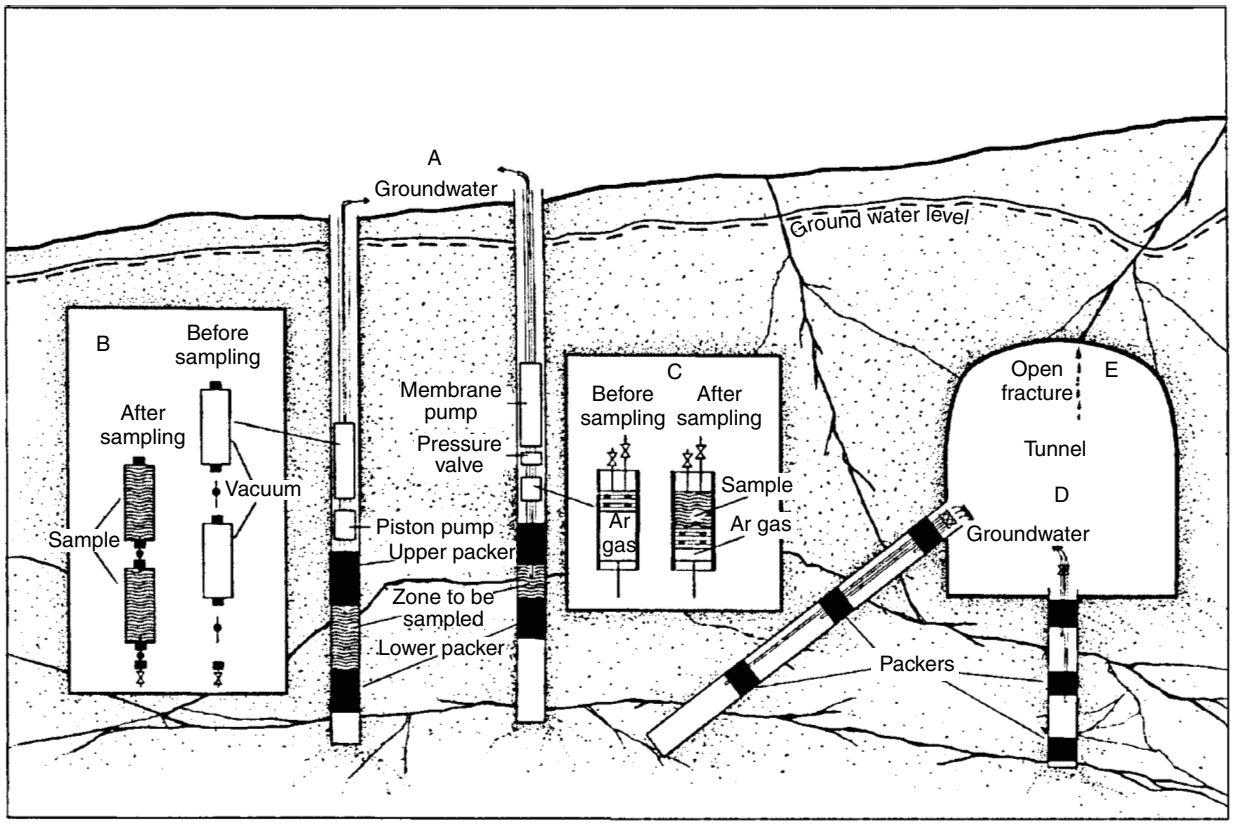

Figure 1. Access to aquifer material and groundwater occurs via the drilling of boreholes from the ground surface or tunnels. After retrieval of drill core material, the boreholes are packed off in one or several sections, each of which isolates one or more specific aquifers. A. Down-hole pumps of various types force groundwater from the aquifer to the ground surface for subsampling. B. Borehole BAT sampler, which can be opened and closed from the surface and is designed for gas-sampling (10). C. The PAVE borehole sampler, which can be opened and closed from the surface, is designed for gas and microbiological sampling (11), and allows one or more sample vessels to be used simultaneously. D. Tunnel boreholes do not require pumps when the tunnel is below the groundwater table. Aquifers can be packed off and connected to sampling devices in the tunnel with pressure-resistant tubes. It is important, however, to understand the potential danger and technical problems connected with the high hydrostatic pressure, which occurs at depth and increases by approximately 1 atmosphere per 10-m depth penetration. E. Open fractures in tunnels can be sampled directly and represent groundwater with minimal disturbance except for the pressure decrease caused by the tunnel.

for obtaining cores with the smallest possible disturbance to the rock environment.

Evaluation of the Contamination Risk During Drilling and Excavation

The risk of contamination of an intersected aquifer during drilling is obvious. Drill water may penetrate aquifers. Therefore, control of drill water contamination is done by the addition of one or more tracers to the drill water. Fluorescent stains may be employed on a routine basis for drilling of the boreholes. The stains used for the boreholes listed in Table 1 were fluorescein and uranin (Aldrich, Germany). Drilling in tunnels reduces the risk of drill water contamination of aquifers when drilling at tunnel depths more than about 60 to 70 m. The risk decreases with the depth because of an increasing aquifer pressure that is higher than the pressure used for the drill water, at least in water-conducting fractures. Pumping or flushing of boreholes to measure the maximum hydraulic water capacity will concurrently clean aquifers and the borehole from drill water, mud, and cuttings, provided there is enough water inflow for intense pumping.

An investigation of the potential risk of microbial contamination of hard rock aquifers has been performed during drilling of boreholes in a hard rock tunnel, using 16/18S ribosomal DNA (rDNA) sequencing and culturing methods (12). It was found that the tubing used for drill water supply constituted a source of bacterial contamination to the aquifers via the drilling equipment. The sequencing results showed that although large numbers of contaminating bacteria were introduced into the boreholes during drilling, they did not establish at detectable levels in the aquifers. The number of microorganisms in drill water introduced into the boreholes varied from 10^5 to 10^6 cells ml^{-1}. Drill water contamination of the studied boreholes was below 1 part

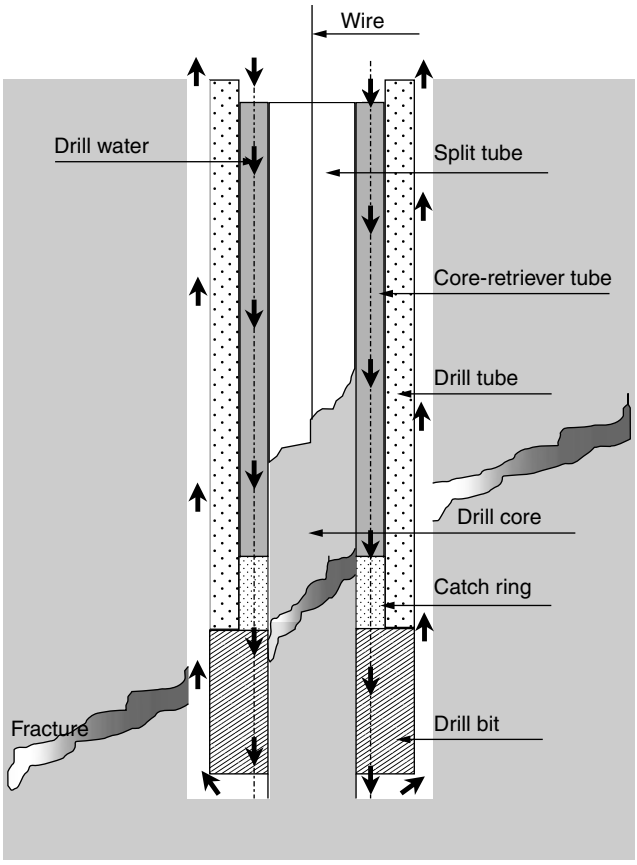

Figure 2. The triple-tube drilling principle. The use of a core retriever minimizes the exposure of the core to drill water and delivers the core intact to the surface, even when multifractured rock is penetrated. The drill tube protects the drill core from contact with aquifer systems intersected during drilling. This is of great importance when layers with contaminants, such as hydrocarbons, are penetrated. The split tube keeps intersected fractures intact with small pieces of rock in their original place.

per 1,000, so that fewer than 10^3 cells ml^{-1} in the aquifers of the drilled boreholes could be expected to originate from contamination during the drilling operation. This number was more than one order of magnitude less than that found directly after drilling and could, therefore, not explain the origin of the observed total cell numbers in the new boreholes. Thus, microorganisms appear to have been present in the aquifers before the drilling operation was undertaken.

Groundwater-Sampling

Surface boreholes generally require pumping for the retrieval of groundwater, whereas tunneling below the groundwater level offers access to artesian water that can be sampled without pumps, thereby reducing the risk of contamination. It is easy to collect samples with a retained in situ pressure. Most tunnels used for igneous rock microbiology research are found in mines at various stages of mining. Some, such as the Stripa mine in Sweden (Table 1) and the Kamaishi mine (13) and Tono uranium mine (14) in Japan, have been closed for mining and are only used for research, whereas others, such as the Witwatersrand gold mines in South Africa (15), are being researched while mining is still going on. Mines generally penetrate large bodies of rock, depending on the age of the mine and the rock formation of interest. This fact, together with the extensive pumping needed to keep the mines dry, will after some period of operation alter the surrounding groundwater system in a way that makes it difficult to assure that the observations made reflect a pristine groundwater. These disturbances can be decreased significantly when underground facilities are built specifically for research. A few tunnels, such as the Äspö Hard Rock Laboratory (HRL) tunnel (6), Grimsel in the Swiss Alps (16), and the Underground Research Laboratory in Pinawa, Canada (17), have been built for the purpose of research only. Data on changes in the hydrodynamic situation and the biogeochemical record during construction are obtained and can be used as a baseline when interpreting information obtained at a later stage.

Pumping from the surface and all withdrawal of groundwater via tubes may introduce unwanted effects. These include degassing because of a pressure decrease and the possibility of microbial biofilm formation on tube walls during prolonged pumping over periods of several weeks. Such long pumping times are commonly needed before the geochemical situation, especially redox potential, becomes stable in the groundwater. Most of these effects can be avoided by the application of down-hole samplers. The suitability of two types of down-hole samplers has previously been tested, in addition to that of sampling via pumping. The samplers used were the BAT (10) and the PAVE (11) samplers (Figs. 1b and c, respectively). The BAT sampler was constructed with gas-sampling as a major aim and consists of two sterile cylindrical tubes or one larger tube made of stainless steel. The tubes are supplied with nitril rubber stoppers and evacuated. In the study testing the BAT sampler the tubes were opened and closed at sampling depths by penetration of the stoppers with hypodermic needles by means of a mechanical device controlled from the ground level. For several reasons, however, this sampler was not a reliable microbiology-sampling tool. The piston pump, which was very difficult to clean, was placed before the sample containers and the sudden decrease in pressure, when the sample containers opened, ripped biofilms from the pump into the sample container. Also, the pressure difference between the evacuated sample cylinders and the groundwater at depth (up to 100 atmospheres) may have caused a "French press" effect on cells in the groundwater, disrupting some of the cells by a sudden drop in pressure when they passed out of the narrow hypodermic needle orifice, penetrating the sampling containers. The BAT sampler (Fig. 1b) never came into routine use for microbiology-sampling. The boreholes at ground level at Hålö, Laxemar, Palmottu, Ävrö, and Äspö (Table 1) were sampled by the borehole pump technique (Fig. 1a). A mobile chemistry laboratory was used at ground level for sample retrieval and preparations (18).

The PAVE system was designed with both gas and microbiological sampling as major objectives. The system consists of a rubber membrane pump placed above a

sample container with two sterile, evacuated, and closed pressure vessels filled with argon gas so that the piston moves to the top of the pressure vessel (Fig. 1c). The argon pressure is set just below the hydrostatic pressure at the sample depth, which makes the pressure-drop during sampling negligible. In experiments conducted by Haveman and coworkers (11) the complete PAVE system was disinfected before sampling by rinsing for 30 minutes. with a 10-mg l^{-1} chlorine dioxide water solution (Freebact, Wecantech AB, Märsta, Sweden), then flushed with sterile water for 10 minutes. Control samples were analyzed to ensure the efficiency of sterilization, and growth could not be detected (11). The section of the borehole to be sampled with PAVE was packed off with inflatable rubber packers, as with the BAT system (Fig. 1b). Groundwater was pumped from the packed-off zone, past the closed-pressure vessels, and out of the borehole. Until they stabilized, groundwater parameters (pH, E_h, conductivity, and temperature) were monitored in N_2-shielded flow through cells in the field laboratory at the surface. At this point, samples for field and laboratory analysis for hydrogeochemical characterization were collected. Thereafter, the pressure valve of the PAVE was opened. Groundwater pressure pushed down the piston in the sampler to fill the sampler with groundwater. The valve was left open for several hours to allow water to pump through the sampler, and then the PAVE sampler was closed again and raised out of the borehole. This system was employed to sample the Hästholmen, Kivetty, Olkiluoto, and Romuvaara sites in Finland (Table 1).

Sampling from tunnels below the groundwater table (Fig. 1d) significantly reduces sampling difficulties. Boreholes become artesian, and packers and pressure-resistant tubes with valves are all that are needed for successful sampling. The high pressure encountered as the tunnel gains depth requires very robust anchoring and packer systems but such equipment is readily available. This sampling method was used for all the Äspö HRL tunnel boreholes (Table 1).

A final possibility is to sample open fractures that enter tunnels underground (Fig. 1e). These are free from drilling effects but may reflect some disturbance encountered during the blasting operations used for tunnel construction. Parts of the Äspö HRL tunnel have been drilled with a tunnel-boring machine (diameter of drill = 5.5 m) and the disturbance on the surrounding rock mass is minimal in those (lower) parts of the tunnel.

The Groundwater Environment

The groundwater environment in igneous rock aquifers is generally anaerobic, normally with a pH of between 7 and 8. The pH is seldom below 7 but may approach 10 in some water (19). The presence of ferrous iron and sulfide attests to reduced and anoxic conditions (Fig. 3). The content of organic carbon is generally low, being in the range of 0.1 to 10 mM. The content of dissolved solids is usually dominated by chloride, sodium, and calcium (Fig. 3), but these vary in concentration by four orders of magnitude. Such variability in groundwater composition makes it difficult to interpret chemical information of the type presented in Figure 3. Multivariate-mixing and mass balance calculations, so-called "M3 modeling", can be used to trace the effect from present and past groundwater flow with fair accuracy (20). This technique has identified five end-members, so-called "reference waters", that mix to form most types of groundwaters found in the Fennoscandian Shield aquifers. These waters have meteoric (i.e., through precipitation), glacial, marine (from the Baltic Sea), altered marine, or deep brine characteristics.

Attempts to correlate cultivable and total numbers directly with groundwater characteristics have not been very informative. Except for some correlation between total numbers and DOC, no correlation could be found. However, when all groundwater characters were taken into consideration in an M3 plot, it was found that, as the water tends toward brine, sulfate-reducing bacteria (SRB) and iron-reducing bacteria (IRB) can no longer be cultured. Other physiological types, such as heterotrophic acetogens, could be cultured from almost any groundwater type in the M3 plot. The complexity in deep groundwater

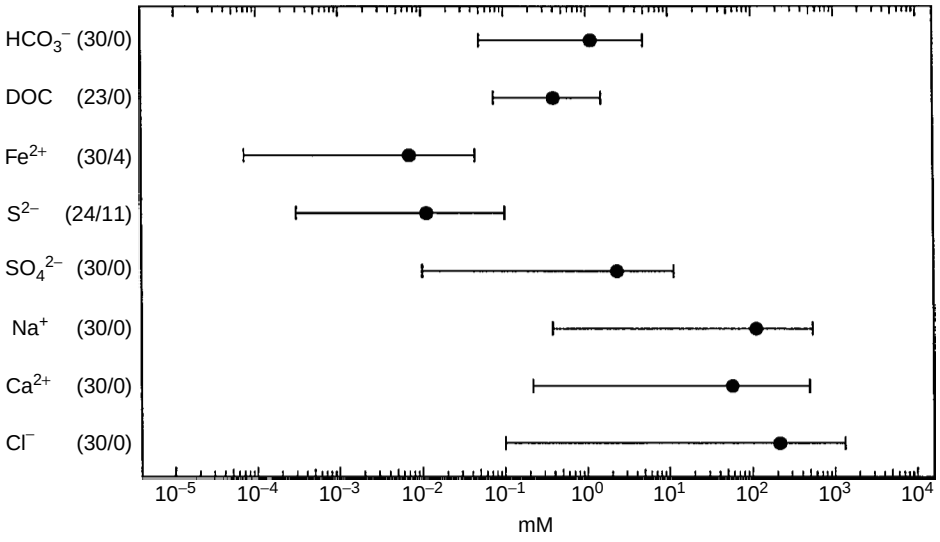

Figure 3. Chemical data observed at investigated sites. The figure shows the average concentration of the chemical species measured; the bar gives the range of data used to calculate the average. The numbers in parentheses following the site name give the sum of observations for the site/sum of observations below the detection limits for the respective analytical procedure. DOC = dissolved organic carbon.

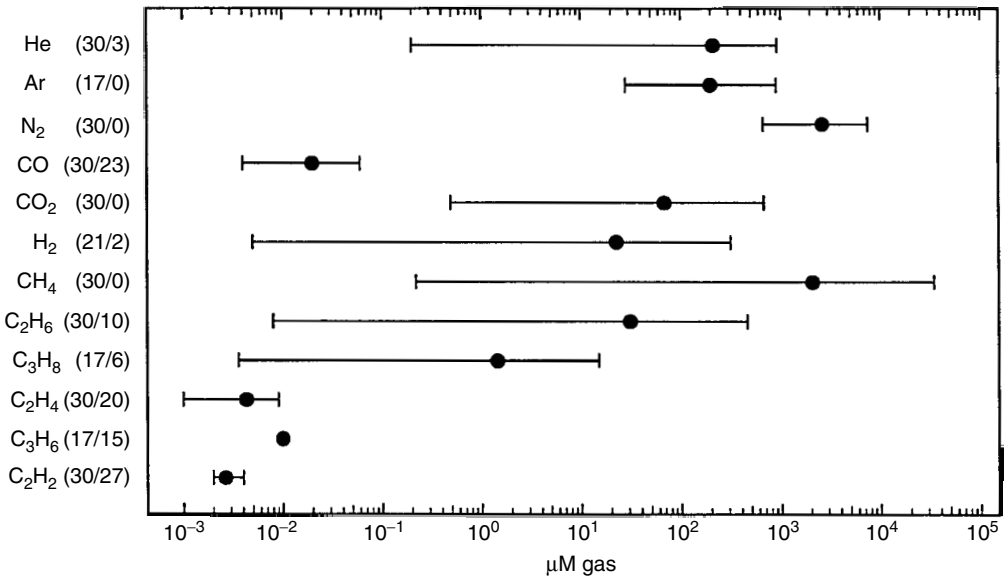

Figure 4. The composition of the gas observed at investigated sites. The data have been extracted from Haveman and coworkers (11) and Pedersen (6). The figure shows the average amount of the gas measured; the bar gives the range of data used to calculate the average. The numbers in parentheses following the site name give the sum of observations for the site/sum of observations below the detection limits for the respective gas.

hydrogeochemistry suggests that many different ecological niches may exist because each point in an M3 plot represents a more or less unique mix of different groundwaters. If this is correct, then a significant microbiological diversity (see "Diversity and Phylogeny of Aquifer Communities" in the following text) with many different species should be expected, each adapted to one or several of the observed hydrogeochemical conditions.

Several gases occur in the studied groundwaters (Fig. 4). Nitrogen generally dominates, followed by helium and argon. Traces of carbon monoxide are found, whereas carbon dioxide occurs in much higher concentrations. Hydrogen is almost always present, although it varies significantly in concentration. Methane is always found and, in some of the deep boreholes at Olkiluoto in Finland, it is the dominant gas (11). Ethane and propane occur, suggesting that some of the methane is of mantle origin, as discussed further in the following text (see "Stable Isotope Signatures").

MICROBIAL NUMBERS IN DEEP GROUNDWATER

Total Number of Microorganisms

Total numbers of subsurface microorganisms vary notably, depending on the site studied. Values in the range of 10^3 to 10^8 ml^{-1} groundwater or gram sediment have been reported for deep environments (1,5,21,22). The total number of microorganisms in igneous rock groundwater samples has been examined ever since the first boreholes listed in Table 1 were visited, back in 1987. Unattached microorganisms have been counted with epifluorescent microscopy after filtration on 0.2-μm filters and staining with acridine orange (AO) and/or 4′6-diamidino-2-phenylindole (DAPI) (23,24). Generally, the

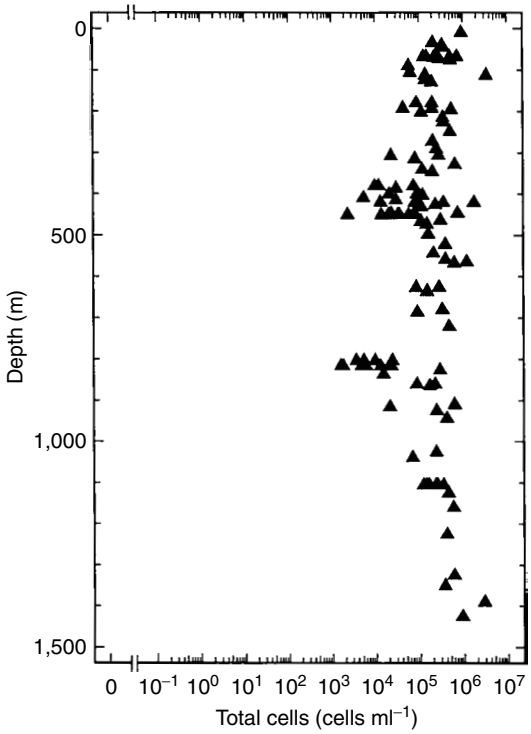

Figure 5. Total number of cells observed at the sites listed in Table 1. At least one determination was done on each level in each borehole. The number of observations depicted in the figure is 112.

average total number of cells commonly registered in the Fennoscandian igneous rock aquifers is within the interval of 10^5–10^6 cells ml^{-1} (Fig. 5), although the range of single observations is from 1×10^3 to 5×10^6 cells ml^{-1}. A large

set of boreholes examined at a site results in a larger range of total cell numbers than does a small set of boreholes. This correlation can be expected if there are large local variations between the aquifers examined at one site, which indeed seems to be the case for Laxemar, Stripa, and Äspö HRL, showing ranges in total cell numbers of almost three orders of magnitude. The total number of cells in specific boreholes was studied extensively at Stripa and Äspö and the variability was found to be remarkably small. The Stripa borehole V2 delivered reproducible and nonvariable numbers for the whole period studied, which was four years (19,25). Four new drilled boreholes in the Äspö HRL tunnel were revisited three times during one year and showed matching total numbers over this period (12), as did seven other boreholes at Äspö HRL revisited three times over a period of six months (26). The variability in total numbers between boreholes and the nonvariability in total numbers within specific boreholes is indicative of stable environments with little or no change in the conditions for microbial life. These conditions may, however, vary considerably between sites and boreholes intersecting the Fennoscandian Shield igneous rock aquifers. This observation compares well with data on the groundwater chemistry in boreholes, which may vary significantly between boreholes, depths, and sites, but is nonvariable within specific boreholes over time (27).

Viable Counts of Microorganisms in Igneous Rock Groundwater

The plate-count technique has been employed for the determination of the number of colony-forming units (CFUs) in deep igneous rock groundwater. The percentage of total numbers that could be cultured from Äspö borehole groundwater with this method ranged from less than 0.1 to 10%, with an average of 1.7% (28). The media used were of general purpose types for heterotrophic bacteria, and further characterization was required for information about the kinds of CFU obtained. *Pseudomonas* and *Shewanella* were typically found. More recent use of this method for Äspö HRL tunnel borehole groundwater resulted in very low CFU percentages of less than 0.1% of the total numbers (12). These low viable count numbers and the inability of the plate-count method to reveal information about the metabolic diversity of the investigated microorganisms motivated the adaptation of more selective media for different predominant physiological groups of microorganisms. A range of anaerobic-culturing media for physiological microbial groups were, therefore, developed and applied to Äspö HRL tunnel groundwater and at the investigated five groundwater sites in Finland. Anaerobic Hungate tubes and serum bottles with aluminum crimp–sealed butyl rubber stoppers (Bellco, U.S.A) were used for most probable number (MPN) determinations with a set of various media (11,29). Figure 6 summarizes the MPN results of IRB and SRB, heterotrophic and autotrophic acetogens, and heterotrophic and autotrophic methanogens.

IRB and SRB in the range of 10^0–10^4 cells ml^{-1} were detected at many of the depths investigated (Figs. 6a–b). An inverse relation between IRB and SRB correlating with the predominating types of fracture minerals at the sites became evident when the Finnish Hästholmen and Olkiluoto sites were compared (11). Olkiluoto has a relatively high average number of SRB and a low number of IRB, whereas the opposite is true for Hästholmen. Hästholmen's groundwaters were found to be rich in iron, containing up to two orders of magnitude more total iron than what is found at Olkiluoto. Fracture minerals at Hästholmen include iron hydroxides but pyrites are only sporadically present, whereas Olkiluoto fracture minerals have pyrite as one of the major components. This indicates that the presence or absence of pyrite as a fracture mineral correlates well with the presence or absence of SRB at the compared sites. Pyrite formation on fractures in these cold aquifers may reflect long-term SRB activity, which is not apparent from groundwater chemistry data. Interestingly, no correlation could be found between sulfate or sulfide concentrations and MPN of SRB. Some of the largest numbers of SRB were found in boreholes with both very low and very high sulfate and generally low sulfide concentrations. This example demonstrates that it is important to identify indicators of microbial activity that are both conservative and insensitive to transport processes because under steady state conditions, the time frames of various processes in hard rock may span millions of years. Even the slowest transport rate may replenish sulfate to SRB within this time frame, resulting in a steady state concentration of sulfate and a buildup of pyrite precipitates. Long-term and very slow processes should be in focus when searching for evidence of subterranean microbial activity. Signatures in fracture minerals seem to be reliable indicators of past and present microbial activity, especially if stable isotope ratios are added to the analysis protocol (30,31).

In most hydrothermally oxidized fractures, IRB have access to an almost unlimited source of ferric iron, provided they can reach it. Humic and fulvic acids are common in most deep groundwaters and these complex compounds have been demonstrated to act as electron shuttles between ferric iron sources and IRB (32). The molecular size of these compounds is small enough to allow penetration of the rock matrix, which then enables iron reduction in parts of the rock that are not directly accessible to the IRB. Attempts to correlate numbers of IRB with amounts of ferric and ferrous iron have not been successful because much more than the sulfur redox couples, the iron redox couple is sensitive to inorganic processes, at least where reduction is concerned. Therefore it is not possible to discriminate between biological and chemical iron redox reactions. Adaptation of mixing models has been demonstrated to be more fruitful (33). The effect of IRB on carbon dioxide and ferrous iron production was demonstrated for a shallow groundwater-intrusion system at the Äspö HRL tunnel. Organic carbon in the groundwater that reached the studied fracture zone was oxidized with ferric iron as the electron acceptor, and this process rapidly reached a steady state that has been sustainable over the measuring period of nine years. A similar approach was taken at the Äspö HRL for the

determination of sulfate reduction along the tunnel. It was found that the MPN of SRB correlated well with geological, hydrological, and groundwater isotope data, indicative of ongoing sulfate reduction (6,34).

Pure cultures and 16S rDNA sequences of acetogenic bacteria from Äspö HRL groundwater indicate that this physiological group of bacteria is important in the subterranean environment (26). Later applications

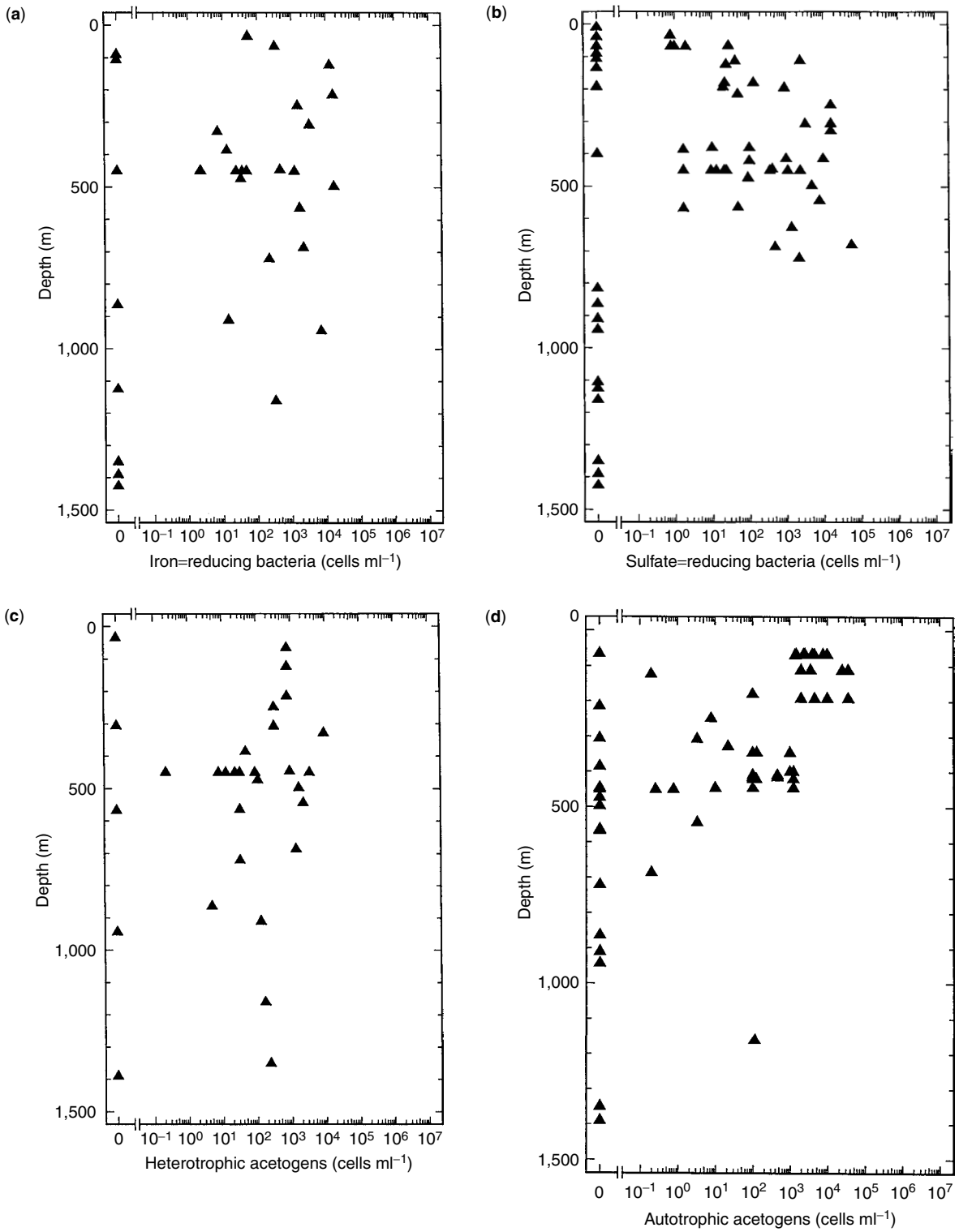

Figure 6(a-f). Most probable numbers of different physiological groups of microorganisms observed at the 11 sites investigated (Table 1). Each observation consists of an MPN determination with three or five parallel tubes in the dilution series.

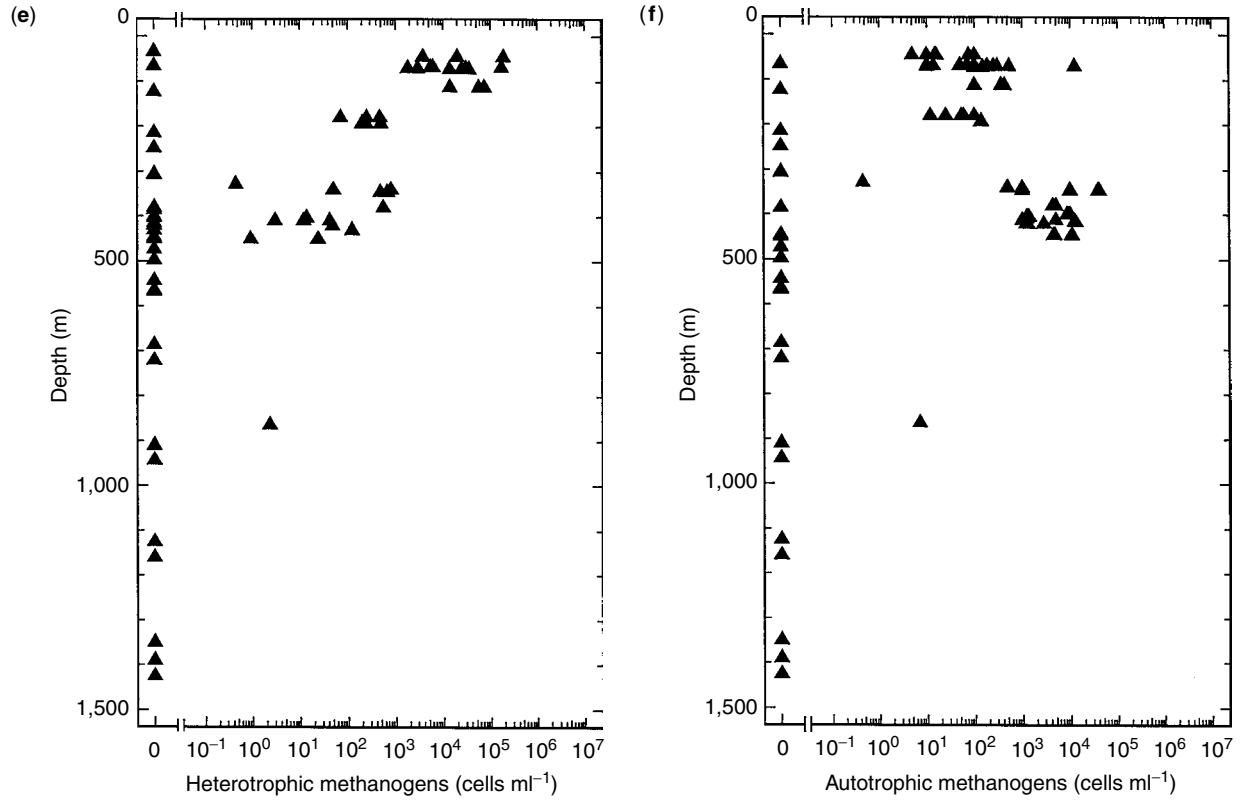

Figure 6(a-f). (*continued*)

of MPN media for heterotrophic and autotrophic acetogens lend support to this hypothesis. Autotrophic acetogens form acetate from hydrogen and carbon dioxide and thereafter the carbon may be further transformed to methane by the acetoclastic methanogens. Heterotrophic acetogens have, with five exceptions, been found at all depths studied (Fig. 6c) and autotrophic acetogens have also been shown to occur frequently (Fig. 6d). The numbers of autotrophic acetate–producing bacteria (Fig. 6d) correlate well with the numbers of heterotrophic methanogens (Fig. 6e) including acetoclastic ones (29).

The presence of hydrogen and carbon dioxide in most deep groundwater examined (Fig. 4) indicates that autotrophic methanogenesis should be possible and the MPN analyses indeed report significant numbers of organisms responsible for this process at Hålö, Äspö HRL, and Olkiluoto (not shown; 11,29). There is no simple explanation for the lack of positive indications of methanogens in most samples from Finland, other than the media used, which were developed and adjusted at Äspö during many repeated sampling occasions in the tunnel. All Finnish samples from boreholes were collected from the ground surface with the PAVE method (Fig. 1), which offers one sample of 300-ml groundwater per level. It is generally not possible economically to repeat such sampling campaigns. However, it cannot yet be excluded that the MPN determinations were accurate and that there were no, or very few, methanogens at most studied Finnish sites.

CARBON-TRANSFORMATION ACTIVITIES

Isotope Techniques

Radioactive compounds for the estimation of microbial activity have been used in microbial ecology for several decades (35). With this technique, samples are incubated with the radiotracer of interest and then examined. Cells, or products, can be separated and examined for radioactivity using standard liquid-scintillation techniques. The method gives average activity results for the whole sample. The activity of individual cells can be examined using a microautoradiography (MARG) technique (36,37). Both liquid-scintillation and the MARG technique have been applied on microorganisms from the Laxemar, Stripa, and Äspö HRL sites (Table 1) with different radiotracers and incubation times. A strong advantage of the MARG technique is that individual cells can be examined and the method can be successfully combined with nucleic acid–probing, offering specific information on selected metabolic activities (38). However, the MARG method is less applicable at very low metabolic rates than is liquid scintillation. In an aquatic sample supplemented with a radiolabeled substrate for a short period an individual cell must have a minimum uptake rate to become sufficiently labeled to produce a positive microradiogram. The lowest radioactivity that resulted in cells sufficiently radioactive to expose the film faster than the background radiation was found to be 10^{-3} disintegrations per minute (19). This level corresponds to $0.1–1 \times 10^{-16}$ mole ^{14}C per bacterium and 2.1×10^{-19} mole ^{3}H per bacterium for the method

used to generate the MARG data. A prolonged incubation for more than a few hours will lower the detection limits but will concurrently increase the background and also allow growth on the added substrates.

Both the Laxemar and the Stripa populations transformed all added compounds, although at varying rates. Carbon dioxide was assimilated at relatively low rates, as were formate, acetate, and glucose. The fastest uptake was reported for lactate. Generally ten times more lactate than other ^{14}C-labeled compounds was transformed, and up to 83% of the deepest population at Laxemar was active in lactate transformation. Lactate appeared to be the preferred carbon source and can be used by SRB and heterotrophic acetogens under anaerobic conditions prevailing in deep groundwater. Figures 6b and 6d shows that these two physiological groups of bacteria are indeed common. The incorporation of acetate was not high but, as ^{3}H was used, the sensitivity of the MARG with this isotope was higher than when ^{14}C was used. The MARG technique revealed that a large proportion of the cells did take up this compound (for a detailed discussion on method sensitivity, see 19). Acetate is used by many SRB and heterotrophic methanogens and both these groups are frequently represented in the MPN data (Figs. 6b,e). Acetoclastic methanogens have also been found to be active in many of the studied Äspö HRL boreholes. Uptake of carbon dioxide by the Stripa and Laxemar populations suggests that autotrophic organisms were present. Later studies (29) confirmed this conclusion and autotrophic acetogens and methanogens have since been enriched, enumerated (Figs. 6d,f), and isolated at many of the studied sites. Autotrophic methane production was common at Äspö HRL, as shown by using the radiotrace- scintillation technique.

A relatively large data set describing carbon transformations has therefore been obtained over a 12-year investigation period. When these data are compared with MPN data, it appears that most of the observed radiolabeled carbon transformations can be correlated with the detected physiological groups of microorganisms that are able to accomplish the observed transformations.

Significant methane and acetate formation rates were obtained in vitro with Äspö groundwater at a temperature (17 °C) close to the in situ temperature of 10 to 17 °C. The general trends for heterotrophic methane formation and acetate formation followed the trends observed with MPN and enrichments (29). The highest activity was found in shallow boreholes (i.e., at 45–68 m), which also had the highest numbers of heterotrophic methanogens and homoacetogens. Autotrophic methane formation did not, however, follow the cultivability trend. This may have occurred because with increasing depth it is more difficult to mimic in situ conditions in vitro for parameters such as pressure and dissolved gases. With this exception, three independent methods, MPN (Fig. 6), enrichments (29), and radiotracer assays, all established the presence of active heterotrophic methanogens and homoacetogens in the examined groundwater.

Stable Isotope Signatures

Microorganisms express a kinetic isotope effect by favoring lighter isotopes over heavier isotopes. Isotope fractionation may, therefore, reflect past and present activity of microorganisms in deep rock environments. The presence of hydrogen, carbon dioxide, and methane in most igneous rock groundwaters examined suggests that methanotrophic microorganisms are present. As discussed earlier, culturing and activity data support this conclusion. Methane of biogenic origin commonly has a $\delta^{13}C$ value that is lower than −50% PDB (Peedee belemnite) (39). Microbial gas is also depleted in higher alkanes so that $C_2H_6 + C_3H_8$ is less than 1% of the amount of methane. A plot of the $\delta^{13}CH_4$ values versus the quotient of methane over alkanes shows biogenic and thermogenic sources of the methane found in Fennoscandian Shield groundwater (Fig. 7). The five values that plot in the biogenic box were obtained with Äspö HRL groundwater, whereas all other data were obtained from Finnish groundwater. Several values plot between the two possible sources, suggesting that a mix of them was observed. The main component seems to be thermogenic for Finnish groundwater and biogenic for Äspö HRL groundwater. This is in line

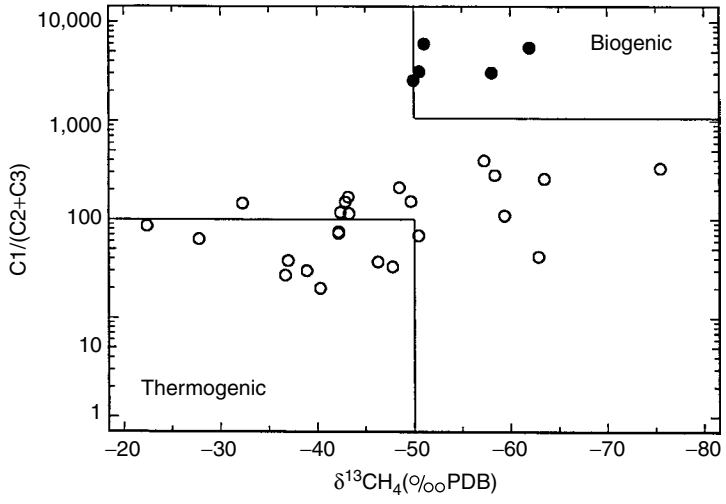

Figure 7. Stable isotope values of ^{13}C in methane dissolved in deep Fennoscandian groundwater versus the quotient of methane gas over the sum of ethane and propane (Fig. 4). Biogenic and thermogenic boxes indicate typical values for methane produced by microorganisms and deep mantle reactions, respectively. Filled circles represent samples from the Äspö hard rock laboratory, Sweden; open circles represent data from boreholes in Finland.

with culturing data, suggesting that methanogens are more active around the Äspö HRL than in the Finnish groundwater examined. Cultivability of methanogens has been significantly higher for Äspö HRL groundwater than for groundwater collected from Finnish igneous rock aquifers.

DIVERSITY AND PHYLOGENY OF AQUIFER COMMUNITIES

Molecular Investigations

The MPN assays and activity measurements described earlier have supplied extensive information about present and viable physiological groups in the examined deep aquifers but these methods do not reveal species diversity and phylogeny. Classical microbiology comprises characterization and species affiliation based on large sets of phenotypic and genotypic data, which is a very time-consuming procedure not suitable as a species diversity–screening technique for environmental samples. The concept of microbial diversity has been changed by the increase in available sequence data on ribosomal 16/18S rDNA. The cloning and sequencing of DNA from microbes living in their natural environments have revealed a genetic diversity way beyond the dreams of past researchers whose tools were limited to culturing and microscopy (40). An early strategy in the study of Fennoscandian rock aquifers was to add 16/18S rDNA sequencing of environmental DNA to the investigations. The DNA was amplified with polymerase chain reaction (PCR) methodology using so-called universal primers. The first site to be thus examined was the Stripa borehole V2 (41). Attached microorganisms were studied and all sequences found among the 72 clones investigated belonged to the Proteobacteria. Two of the major groups of clones fell into the β-group, and the third group fell into the γ-group. Because specific archeal primers were not used in this study, the probability to detect organisms belonging to the domain *Archaea* was strongly reduced.

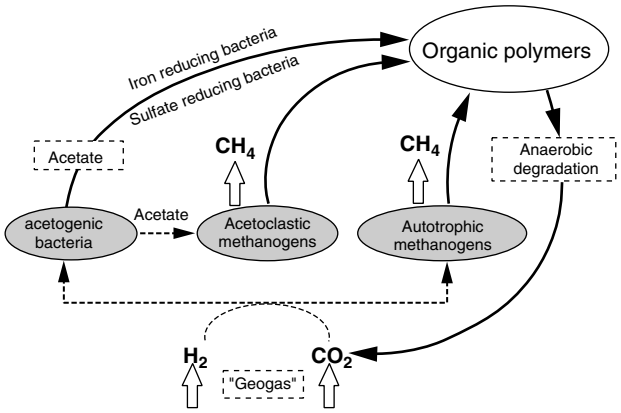

Figure 8. The deep hydrogen-driven biosphere hypothesis illustrated by the carbon cycle. At relevant temperature and water-availability conditions, subterranean microorganisms are theoretically capable of performing a life cycle that is independent of sun-driven ecosystems. Hydrogen and carbon dioxide from the deep crust of the earth or organic carbon from sedimentary deposits can be used as energy and carbon sources. Phosphorus is available in minerals such as apatite. Nitrogen, nucleic acids, and so on can be synthesized via nitrogen fixation, and nitrogen gas is the predominating dissolved gas in most groundwaters (Fig. 4).

The next site to be investigated with 16/18S rDNA studies was the Äspö HRL tunnel. In the first campaign, 155 clones of unattached and attached bacteria from 9 boreholes were sequenced (26). A comparison of predominating 16S rRNA gene sequences with international sequence databases revealed three clone groups that could be identified as bacteria on the genus level, the *Bacillus*, *Desulfovibrio*, and *Acinetobacter* genera. One of the clone groups could be identified as belonging to the fungi. A second campaign was executed during a contamination-control investigation while drilling boreholes in the Äspö HRL tunnel (12) and 158 clones were sequenced. Several clones showed close similarity to 16S rRNA genes from known and sequenced bacteria such as *Bacillus, Desulfovibrio*, *Desulfomicrobium*, *Methylophilus*, *Acinetobacter*, *Shewanella*, and the yeast *Candida*. Archeal primers were not used for those studies.

The diversity size of the bacterial community detected in the Äspö HRL groundwaters is not large compared with the numbers expected in surface soils, for example, 4,000 species in 1 gram of soil (42). Of the total of 385 sequenced clones from Stripa and Äspö HRL, 122 constituted unique sequences, each representing a possible species not reported to the database at the time the comparison was done. On an average, approximately one-third of the sequenced clones represented unique species. In other investigations similar to the Äspö HRL study, a matching molecular biodiversity per total number of sequenced clones was observed. For example, 44 specific clone groups out of 130 sequenced clones from 5 boreholes were observed at the natural nuclear reactor in Bangombé, Gabon, Africa (43), as well as 20 specific clone groups out of 67 sequenced clones from nuclear waste buffer material (44), and 23 specific clone groups out of 87 sequenced clones from alkaline spring water in Maqarin, Jordan (45). These investigations and the Äspö HRL and Stripa studies have clearly not exhausted the sequences to be found because new sequences have been found in nearly every additional sample repetition. The 16/18S rDNA sequence data–collection effort therefore clearly needs to be scaled up significantly for the study of most groundwater sites and furthermore requires automated procedures. Bacteria are very likely to be the first group of organisms for which such automated biodiversity assessment will be available, because as a major constituent of the microbial community, they deserve assessment in their own right in addition to their value as indicators (46).

Characterization and Description of New Species from Deep Igneous Rock Aquifers

The molecular work already described has given a fair insight into the phylogenetic diversity of igneous rock aquifer microorganisms but it does not reveal species-specific information. None of the 122 specific sequences mentioned earlier had 100% identities with

described species. Even if a 100% identity is obtained, there may yet be strain-specific differences in some characters and such differences are not revealed by the 16S rDNA information (47). If species information is required, time-consuming methods in systematic microbiology must be applied to a pure culture, including DNA–DNA reassociation studies. Known genera or species can be identified with these methods. Several isolates from the Laxemar, Äspö, and Äspö HRL sites have been identified as *Shewanella putrefaciens, Pseudomonas vesicularis*, and *Pseudomonas fluorescens* (26,28).

If the target isolate does not match a known genus or species, then the opportunity of describing a new species is provided. Three new subterranean species from deep igneous rock aquifers have been described and published. The isolate Aspo-2 has been characterized in detail and described as a new species, *Desulfovibrio aespoeensis* (48). It is a mesophilic species with growth characteristics that appear well adapted to a life in the aquifers from where it was isolated. Similarly, a methanogenic isolate, A8p, has been studied in detail and placed with the genus of *Methanobacterium*. Phenotypic and phylogenetic characters indicate that the alkaliphilic, halo-tolerant strain A8p represents a new species and the name *Methanobacterium subterraneum* was proposed. Finally, several oxygen-dependent methanotrophic isolates have been obtained during the investigations of microbial methane oxidation in the Äspö HRL tunnel, and a first isolate, SR5, has been successfully described (49). On the basis of phenotypic and genotypic characteristics, the strain SR5 has been proposed as the new species *Methylomonas scandinavica*.

The prospect of anaerobic methane oxidation is an intriguing possibility that has been approached in different environments (50), but absolute evidence in the form of a laboratory culture of an anaerobic methane–consuming species or a consortium is still lacking.

HYDROGEN DEPENDENCY OF DEEP MICROBIAL COMMUNITIES

The repeated observations of autotrophic, hydrogen-dependent microorganisms in the deep aquifers studied (Figs. 6d and f) imply that hydrogen is an important electron and energy source and carbon dioxide is an important carbon source for the subsurface biosphere. Hydrogen and carbon dioxide have been found in μM concentrations at all sites investigated for these gases (Fig. 4) together with methane, which is a major product of autotrophic methanogens and has been shown to be very active in vitro at Äspö HRL. Therefore a model of a hydrogen-driven biosphere in deep Fennoscandian Shield igneous rock aquifers has been suggested with various modifications (6,8,29,51,52). The organism base for this biosphere has been suggested to be composed of autotrophic acetogens that have the capability of reacting hydrogen with carbon dioxide to produce acetate, and autotrophic methanogens that yield methane from hydrogen and carbon dioxide or from acetate produced by autotrophic acetogens (acetoclastic methanogens) (Fig. 8). All components needed for the life cycle in Figure 8 have been shown to be present in deep igneous rock aquifers and the microbial activities expected have been demonstrated at significant rates in vitro. The model has, consequently, convincing support from the qualitative data obtained. It remains to examine in situ rates, a process that will require meticulous experimental conditions because of the expected very slow metabolic rates at nondisturbed conditions. The central question to address during such an experimental endeavor is whether hydrogen-driven microbial chemolithotrophic in situ activities at depth are in balance with renewal rates of hydrogen. An indisputable affirmative answer to this question is crucial for the unequivocal confirmation of a deep hydrogen-driven biosphere in the deep igneous rock aquifers of the Fennoscandian Shield.

The theory of a deep biosphere driven by hydrogen generated in deep geological strata (Fig. 8) requires more research. There are at least six possible processes in which crustal hydrogen is generated: (*1*) reaction between dissolved gases in the carbon-hydrogen-oxygen-sulfur system in magmas, especially in those with basaltic affinities; (*2*) decomposition of methane to carbon (graphite) and hydrogen at temperatures above 600 °C; (*3*) reaction between carbon dioxide, water, and methane at elevated temperatures in vapors; (*4*) radiolysis of water by radioactive isotopes of uranium, thorium and their decay daughters, and potassium; (*5*) cataclasis of silicates under stress in the presence of water; and (*6*) hydrolysis by ferrous minerals in mafic and ultramafic rocks (53). It is important to explore the scale of these processes and the rates at which the produced hydrogen is becoming available for deep microbial ecosystems.

CONCLUSION

Extensive investigations of deep aquifers in Fennoscandian igneous rocks have revealed active microbial life. As the rock is too hot to host any life when formed, this life must have migrated via groundwater-conducting fractures into cooled rock mass and adapted to the environmental conditions prevailing there. The life found represents a large physiological and genetic diversity, reflecting the variability in groundwater chemistry and rock composition of the Fennoscandian shield. These deep microbial communities may be independent of solar energy. Instead, a range of independent results have demonstrated a hydrogen-dependency of the populations, which implies that a deep hydrogen-driven biosphere may exist in the depth of Fennoscandian shield rock.

Jules Verne's famous book *Journey to the Center of the Earth* reports how Prof. Lidenbrock and his nephew, Axel, found advanced life in natural caves and tunnels deep under Europe. At that time the story was genuine science fiction. Now, 135 years later, it is obvious that Jules Verne in a sense was correct because deep under the terrestrial ground and deep under the seafloor, life is very abundant, albeit mostly in smaller cavities than suggested by Verne (2). Possibly, parts of this deep biosphere are self-sustained, without the need of

a surface biosphere. The discovery of a deep biosphere that may be independent of solar energy is of great importance to how we should search for life on other planets and to our theories about the origin of life. The predominant idea that life originated on the surface of the earth, strongly dependent on a hypothetical primordial soup, has recently come up against strong competition. Subsurface environments were relatively stable, calm, and rich in chemical and physical gradients compared to the surface environments that experienced meteoritic impacts, volcanic eruptions, and space radiation. A subsurface place of birth for life, probably very hot, is likely.

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IMAGE ANALYSIS OF MICROORGANISMS

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Microbiologists have exploited various forms of light microscopy to directly observe bacteria in pure cultures, waters, soils, sediments, biofilms, and bioaggregates. Microscopy is an important tool in environmental microbiology, providing the only direct approach to the microbial environment. Combining microscopy with an increasing range of reporter molecules such as phylogenetic probes allows observation of which bacteria are where, what they are doing, and what the environment is like at that scale. Various studies suggest that it is critical to study bacteria at this scale through direct observation (1,2). The application of computer-based image analysis to microscope images provides a powerful method to extract quantitative information. Although this technology has been used in a number of studies in applied and environmental microbiology, its potential has not been fully exploited. This review is intended as a point of departure for those interested in applying analytical imaging methods in environmental microbiology.

OBTAINING AN IMAGE

Basic System Requirements

Imaging of bacteria requires the application of various microscope techniques described below, a camera or digital imaging device, suitable connectors and cables, a monitor and computer. Useful image analysis and image processing may be carried out with as little as an Intel 80486 processor, 16-MB RAM, Windows 95 operating system, display of 256 colors, and 800X600 pixel resolution. Manz and associates (3) used a 200-MHz PC running under Microsoft Windows 95 to accomplish deconvolution of image stacks, although operating times were long. In general, a user can never have enough processor speed, RAM, storage memory, or resolution. It is also recommended that image storage systems such as CDs and archiving be considered before venturing into image processing and analyses. There are also many output options, in terms of black and white and color printers, resolution, cost, and so on. Basic manuals such as: 4, 5, 6, 7, 8, and 9 all contain valuable general information, and details with respect to microscopy, digital imaging, and analyses.

Conventional Light Microscopy

Performing effective image analyses of microorganisms requires a high-quality initial or primary image. It is always best to improve the quality of the primary image through sample-preparation steps and selection of the most suitable imaging approach. In addition, regardless of the microscopy technique selected, alignment and cleanliness are important considerations.

The small size of bacteria, often less than 1 micron, presents a challenge to the resolving power of even the highest quality light microscope. As a result, it is important to be aware of the principles of light microscopy and the microscopy options available. In general, all light microscopy techniques can be used with digital imaging methods. These techniques include phase contrast, epifluorescence, dark-field, bright-field, interference contrast, and other less commonly applied approaches (9). Each of these microscope methods serves to magnify the object of interest while enhancing the contrast between the object and its background, allowing small, low contrast objects such as bacteria to be seen. Increasingly, technology-based methods such as confocal laser scanning microscopy (CLSM) and 2-photon laser microscopy (2-PLSM) are also used to obtain high-information-content images of microbes. Basic procedures for each of these microscope methods have been detailed in other publications (9,10), and hence will be briefly discussed in the next section. Furthermore, other imaging techniques, including electron microscopy, acoustic microscopy, and scanning probe microscopy methods can be used to create images of bacteria that are amenable to image processing and analyses.

Light is a wave and therefore a perfect point cannot be imaged. Rather, resolution in a microscope is a function of the wavelength of the light used, the numerical aperture of the objective lens used for observation, and the medium between the lens and the specimen. Objective lenses have their best resolution in the *xy* plane and relatively poor resolution in the *xz* dimension. As a consequence of poor resolution in the *xz*, out-of-focus information recorded by most imaging devices degrades image quality. This *xz* error is a significant problem for *xz* imaging, 3D imaging and reconstruction, and image analysis particularly for thicker biological materials. However, the error can be modeled as a point spread function and mathematically corrected through a process referred to as deconvolution (see discussion next section).

Phase Contrast Microscopy

Phase contrast microscopy remains extremely useful because it provides for high magnification, excellent resolving power, and may be found in many laboratories. It is also a relatively simple and convenient method because it avoids the need for drying, fixation and special staining, it is nondestructive, and slides can be returned to a test system if necessary. It also avoids the problems of under- and overestimation of cell size inherent in bright-field or dark-field and epifluorescence microscopy. This approach can readily be used for making

continuous observations (11–13). Liu and associates (14) used phase contrast microscopy to image suspended bacterial populations. Cells were prepared and mounted on agarose-coated slides (15), a technique that provides a high quality phase contrast image of bacterial cells within a single focal plane. It is, however, limited to use with optically transparent substrata; that is not the case for some other approaches such as epifluorescence or laser microscopy.

Dark-Field–Bright-Field Microscopy

Dark-field microscopy achieves contrast between organisms and their surroundings by scattering light from a point source (10). Relatively inexpensive dark-field condensers and objective lenses are required, but can be used with standard microscopy equipment. Because of the increased contrast between the object and the background, dark-field images are advantageous for analyses where accurate size representation of the objects is not critical. For example, dark-field image analysis has been used to quantify rates of bacterial deposition, quantify the growth and development of bacteria and bacterial microcolonies, and monitor the motile behavior of both bacteria and surface-associated protozoans (1,16,17). In bright-field microscopy, the most common illumination on light microscopes, the object contrasts with the background if they differ in their light absorption. For bacteria to appear in bright-field images they must be stained and are frequently fixed, so continuous observation of bacterial cultures is not possible.

Differential Interference Contrast (DIC)

Another method of obtaining images of bacteria is differential interference contrast, wherein two beams are produced optically; one carrying the image and the second, a reference beam causing interference patterns (10,18). This method exploits the differences in indices of refraction of the specimen. Furthermore, the interference contrast images contain interference colors that are useful for studies of microstructure. Nomarski DIC has been used in a reflected mode to observe bacteria (19).

Hoffman Modulation Contrast

Dark-field, phase contrast, and DIC are all useful for observing unstained bacteria in suspension or on surfaces. Another approach is Hoffman modulation contrast microscopy (20). This method is also useful, although unlike phase contrast and DIC it may produce halos around objects being observed, and so can create problems for subsequent image collection, processing, and analyses.

Epifluorescence Microscopy

Typical laboratory grade microscopes are often equipped for epifluorescence microscopy. Epifluorescence microscopy requires an appropriate light source and filter sets to observe specially stained (see next section) bacterial cells. Epifluorescence microscopy focuses incident light through the objective lens that functions as both a condenser and an objective. The light source may either be a quartz-halogen-tungsten filament lamp, a mercury vapor arc or a xenon arc lamp, each of which provide particular wavelengths of light to excite special fluorescent stains. Epifluorescence approaches have the advantage of allowing analyses to be conducted on solid or opaque surfaces, and when linked with probes specific for certain bacteria, can provide detailed information from within complex microbiological systems. Rost (21,22) has published an excellent two-volume review on fluorescence microscopy that may be referred to for additional details.

Other Light Microscopy Options

Images of bacteria have been obtained using various other techniques, including transmitted, incident, or polarizing light microscopy techniques (23). The use of color infrared photography for microscopic visualization of nonstained microorganisms, modified metallurgical reflected light, continuously variable phase and amplitude contrast system microscope, interval scanning microscopy, polarizing microscopy and interference reflectance microscopy have recently been reviewed by Lawrence and associates (9).

Confocal and 2-Photon Laser Scanning Microscopy

Confocal laser scanning microscopy (CLSM) combines traditional light microscope hardware with a laser light source, photomultiplier detectors, and computerized digital imaging. Two-photon laser-scanning microscopy (2-PLSM) employs a high peak power infrared laser with an extremely short mode–locked pulse in the femto- or picosecond range to produce a high photon density. Laser microscopy is an advanced digital form of fluorescence microscopy that allows optical sectioning of living fully hydrated samples with enhanced xy and xz resolution and quality. During excitation of a fluor-stained specimen with the laser, a confocal pinhole allows only those fluorescence signals that arise from the focused xy plane to be detected by a photomultiplier tube (PMT). Typically, the selection of the fluorescence colors to be detected is accomplished using a beam splitter and filter sets. Other commercial systems allow selection of excitation emission without the need for optical lenses through the application of a filter-free prism-spectrophotometer-based system. The PMT converts the signals to a digital image. With the advent of Krypton-Argon, commercial UV and recently two-photon and multiphoton laser microscopy systems, multiple parameter imaging of samples is practical, allowing the collection of multiple quantitative data sets at a single location (24,25). Laser microscopy provides a digital data base that is highly amenable to image processing and analysis. So the user may obtain quantitative information on a wide variety of parameters including cell numbers, cell area, and object parameters such as minimum–maximum dimensions, orientation, average gray value, and so on. These techniques have been described in detail (9,26,27). In addition, the Handbook of Biological Confocal Laser Microscopy is also an excellent source of information on nearly all aspects of confocal microscopy (5).

FLUORESCENT LABELS FOR EPIFLUORESCENCE, CLSM AND 2-PLSM

Epifluorescence and laser microscopy require that the specimen be stained with special fluorochromes such as acridine orange (AO), 4'6-diamidino-2-phenylindole (DAPI), fluorescein isothiocyanate (FITC), 5-(4,6-dichloroztriazin-2-yl), aminofluorescein (DTAF), tetramethyl rhodamine isothiocyanate (TRITC), other rhodamine stains, and Texas red. Many fluorochromes (fluorescein (FLUOS, FITC) or rhodamine (TRITC) derivatives are used, however, cyanins (CY3, CY5), the blue-fluorescing aminomethylcoumarin (AMCA), or ALEXA dyes, phycoerythrine (PE)) can also be conjugated to molecules that recognize specific structures of the target microorganisms. These fluorescent probe-conjugates include lectins, poly- and monoclonal antibodies, and deoxyoligonucleotides used to detect specific sugars, antigens, or complementary nucleic acid sequences, respectively. See Haugland (28) for a comprehensive listing of commercially available fluorescent probes. Additional technical information is also available in Wang and Taylor (29).

Green fluorescent protein genetic constructs (30,31) may be used in conjunction with laser microscopy and epifluorescence microscopy to monitor gene expression in single cells. Applications of green fluorescent protein have been reviewed by Errampali and associates (32) (see GREEN FLUORESCENT PROTEIN (GFP), this Encyclopedia).

It should be noted that further research and development are required before specific recommendations can be made regarding fluorescent labels for application in 2-Photon microscopy in which excitation and emission spectra for specific fluors may be very different from that seen in traditional epifluorescence or CLSM (33).

In some instances, the application of fade retardants can be useful or necessary to extend the signal life in epifluorescence and laser microscopy. Commercial fade retardants such as Citifluor or those provided by Molecular Probes (Eugene, OR) will reduce bleaching of the fluor and extend working times for fluor-labeled samples.

VIDEO/DIGITAL CAMERA SELECTION

Although photographic film provides a suitable image for subsequent digitization, the video camera (a charge coupled device (CCD)) is the most common instrument used to obtain an analog signal of a microscopic or macroscopic image. This image may be digitized directly or fed to a frame grabber to be digitized and stored in an appropriate format. The fidelity of the primary image is again crucial, as all downstream image processing and analysis steps will vary in difficulty relative to the quality of the primary image. Video cameras vary in terms of their horizontal resolution, light sensitivity, blooming characteristics, geometric distortion, and spectral response. The camera system should be matched with the intended end application in terms of the sensitivity, resolution, and linearity of response. For example, epifluorescence microscopy images often require greater sensitivity and intrascene dynamic range to record detailed images. Manual adjustment of the camera gain and voltage control is preferable, as self-adjusting cameras alter their light sensitivity in response to changes in inter- or intrascene brightness, limiting the quantitative use of the images. In general, the low- to mid-sensitivity CCD (10^{-5} to 10^{-1} lux) cameras, are cost effective, less subject to electronic noise and geometric distortion, have relatively broad intrascene dynamic range and low blooming tendencies. These traits make them applicable for phase contrast, bright field, DIC, and most epifluorescence microscopy. Peltier cooled CCDs also offer RGB color imaging (Optronics Engineering, Goleta, CA.), providing very quiet, or noise-free imaging as a result of extremely low dark current while performing on-chip integration (summation of image information) over extended exposure times (up to 4 minutes). CCD systems, such as these also have a broad exposure range (from 0.0002 to 13,000 lux) and onboard digital contrast control, so weakly fluorescent and bright-field images can be digitized using the same basic system. Highly sensitive, cooled (−40 to −70 °C) CCD cameras having a wider range of gray-level intensities (e.g., 12–16 bit, 4096–65,686 gray-levels) may also be obtained (Photometrics Equipment, Roper Scientific, Tucson, AZ; Astromed Ltd., Cambridge, England; Micro Luminetics, Inc., Los Angeles, CA). Cameras with photon-counting capabilities (limiting low level illuminance, 10^{-11} lux) are suitable only for highly specialized applications (e.g., bio- or chemiluminescence, *lux* gene expression). These systems require specialized support equipment, darkrooms, and are not generally applicable for routine lab use (34).

The selection of peripheral equipment may also influence the quality of primary images. These include the quality of coaxial cables and connectors, random camera noise, and incorrect termination. Analog to digital conversion (ADC) circuitry and the high-frequency clock used for digital time base control may further contribute to signal degradation. Currently, completely digital CCD cameras are available that eliminate the need for ADC circuitry and associated problems.

IMAGE ANALYSIS/PROCESSING SYSTEMS

Many commercial and freeware systems for image analysis and processing are available. It has been found that no single software package offers all the features required by a specific user, so selection of software must be made with care. It is important to note that software will vary in terms of grey level resolution, programming language, capacity for software modification, design of macros or plug-ins, and system memory configuration. Equally important are details that may affect suitable end uses of the system, including their capacity to perform operations on serial image stacks produced in confocal microscopy or to perform object-based image analysis. Commercial systems available include the Quantimet System offered by Leica (Heidelburg, Germany) that has been used to analyze CLSM images of bacteria (35). PC-based three-dimensional imaging/image analysis systems include, MicroVoxel (Indec Systems, Sunnyvale, CA), VoxelView (Vital Images, Fairfield,

Iowa), or VoxBlast (VayTek, Inc., Fairfield, Iowa). The silicon graphics–based software offered by Molecular Dynamics features the 3D image analysis of CLSM image stacks. Additional commercial software suppliers are listed in Lawrence and associates (9) or on the Internet.

There are also excellent software in the published literature or available from web sites (36,37,38). Möller and associates (38) used Cellstat, a program for UNIX workstations (see *http://www.lm.dtu.dk/cellstat/index.html*). There are two excellent freeware programs that may be downloaded over the Internet. NIH Image is a versatile freeware analysis package developed for Apple platforms (*http://rsb.info.nih.gov/nih-image/*) and also compiled for Windows-based systems (ScionImagePC at www.scioncorp.com). This package was specifically designed to deal with confocal images and image stacks. NIH Image 1.62 performs well, is user friendly, and provides a wide range of analysis/enhancement and user-programing functions. ImageTool is a PC-based freeware image analysis package available at *http://ddsdx.uthscsa.edu/dig/itdesc.htlm*. This software offers many excellent object recognition and analysis features. Liu and associates (14) provide an excellent example of the application of ImageTool for studying microbial populations. In this study the authors demonstrated recognition and enumeration of 11 bacterial morphotypes. Image Tool plug-ins for studies of cell morphology are available through the Internet. Neural network systems have also been proposed for analysis of fluorescence images (39).

Specialized software is also available for the removal of out-of-focus haze by mathematical deconvolution (3,40,41). A UNIX based package for this is available over the internet that may be applied to CLSM or other fluorescent images (see *http://ibc.wustl.edu/bcl/xcosm/xcosm.html*). Deconvolution software such as EPR is available from Scanalytics (Billerica, M.A.) and was demonstrated by Manz and associates (3). The Huygens system (Scientific Volume Imaging, B.V., Hilversum, The Netherlands) runs on silicon graphics computers and includes many image-processing features, including deconvolution.

THE BASICS OF IMAGE ACQUISITION

The Primary Image

After the sample is ready to be observed, images may be collected directly (single scan), or the signal to noise (S : N) ratio of the image may be enhanced through application of various mathematical filters during collection. Typical filters include the running average, exponential, or Kalman type mathematical filter. Kalman filtration averages sequentially collected images, reducing the noise level of the image while maintaining edge features. The use of these filters may cause bleaching as a result of high frequency of scanning, and are not recommended during collection of images for applications such as ratiometric imaging.

Image-averaging techniques are best used when light is not limiting and the image is stationary. Averaging functions to eliminate nonimage interference such as pixel dropout and electronic noise. The improvement of image quality (S : N ratio) is proportional to the square root of the number of images averaged. Image-averaging may also be utilized to eliminate objects that are not stationary such as protozoans or motile bacteria (see next section) (17,42).

Another option during image collection is to use image integration, wherein the imaging device integrates images over time, allowing intensification of very faint signals. Other collection methods are available that permit image enhancement through summation of signals or collection to preset maximum signal levels. Image integration is preferred under low-light conditions, most typically during epifluorescence microscopy applications when the fluorescent signal is very weak. An example of image collection using a summation input versus a running average is shown in Figure 1a,b. These functions may be applied during image acquisition through software, or performed "on-chip" in some video cameras. The number of gray levels (e.g., 12 bit or 16 bit cameras) available for on-chip integration also enables the accumulation of low light information over a extended range of measurable brightness without saturating image pixels, a feature especially valuable during low light fluorescence microscopy applications. Subsequent application of functions such as local contrast enhancement, median filters, linear smoothing filters, and gradient filters (5,6) may serve to reduce noise levels in images, smooth the data set, and enhance the edges of objects within the image (see next section).

PRESEGMENTATION PROCESSING STEPS

As noted by Wilkinson (43) there are two types of images; those that are easy where objects stand out from a background with little detail in it and difficult images where the background is confusing or complex. Figure 2 provides an example of easy and difficult images, image A has reasonable contrast and an absence of debris, in contrast B contains problematic non-microbial objects. While it should be emphasized that sample preparation and quality of the imaging system are key factors in the optimization of the primary image, additional processing may be required in order to facilitate the identification of objects to be measured. Processing steps that may be applied before the actual segmentation of object pixels (thresholding) to improve image quality and define the edges of the objects are outlined in the next section. The result of this image-processing is an image that is gray scale, binary, or color that may be subjected to image analyses.

Shading Corrections

Optical imperfections and microscope misalignment frequently result in the uneven illumination of the primary image, often causing difficulties during subsequent image-processing steps (44). So primary images should be corrected for uneven illumination before image-processing. Peters and associates (45) used shading corrections to eliminate uneven lighting and to remove particulate debris

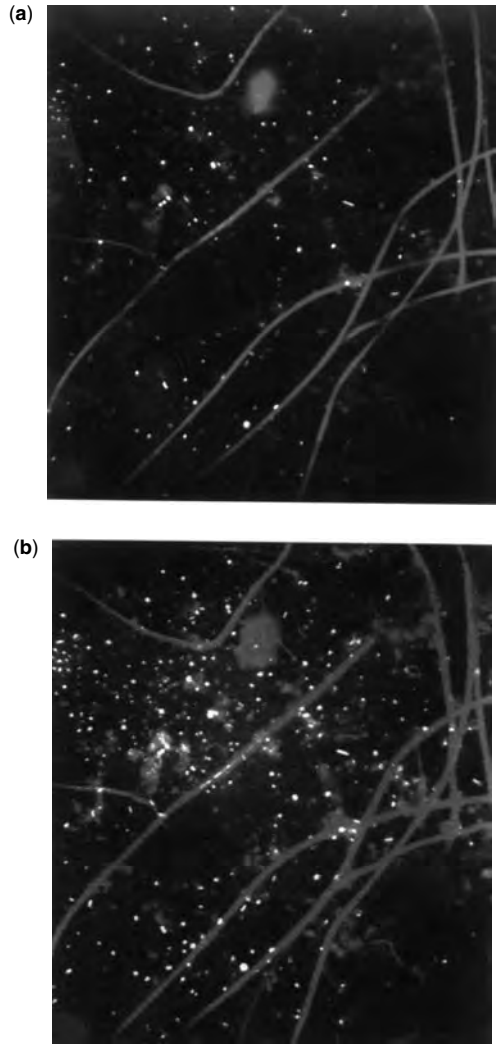

Figure 1. Confocal laser micrographs illustrating the effect of collection method on images of the same location in a river biofilm, (**a**) Kalman or line averaging mode (**b**), and image integration to increase contrast and detect faint objects in the image.

present within the optical system. This is also important for measurements because uneven lighting will result in differential sizing of objects in the image. Increasing contrast from the center to the edge of the image will result in an apparent increase in size of the same objects with distance from the center. Generally, shading corrections involve the digitization and storage of the study surface illuminated under study conditions before the sample is introduced (a reference image). If the system is aligned and no debris is present, the gray level shading correction will have a zero gray level value. If uneven illumination exists, the gray level of the shading correction will be subtracted from the study image, thereby eliminating localized bright spots or surface imperfections but not the cells introduced after collection of the reference image. Shading corrections may also be performed in the event

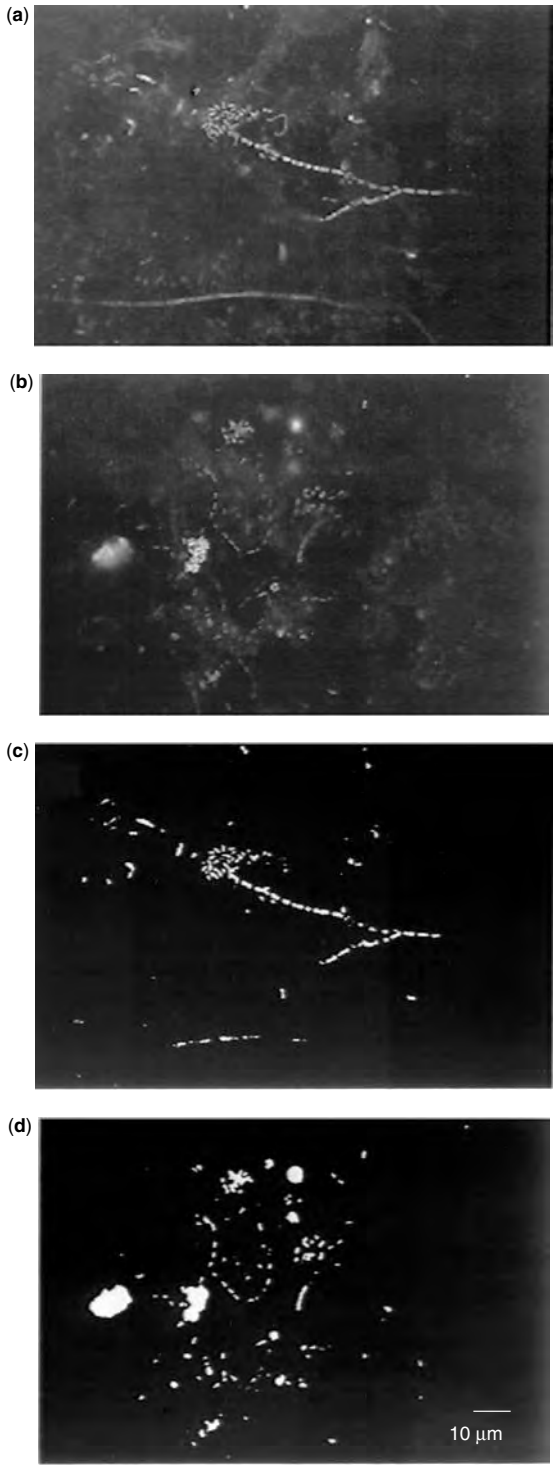

Figure 2. Two gray scale images of hybridized cells showing an "easy image" with minimal debris and nonspecific staining (**a**) and a "complex image" with noncellular debris and nonspecific fluorescence signals (**b**), the images in (**c**) and (**d**) are the respective binary images. In this instance, size selective cutoffs might reduce the error, or manual editing to remove nonobject material could be required. (Reprinted from Manz and associates, 1998).

that a reference or blank image has not been obtained. In this case, image-processing is used to remove the objects from the image leaving only the background to be used as a shading correction (6). Figure 3 illustrates the effect of applying a shading correction to improve image quality before analysis.

Mathematical Filtration

A number of mathematical filtration operations have been used to facilitate the analysis of microbiological systems, including smoothing, (Gaussian, median, lowpass), sharpening, (highpass, Laplacian, and Sobel), and so on.

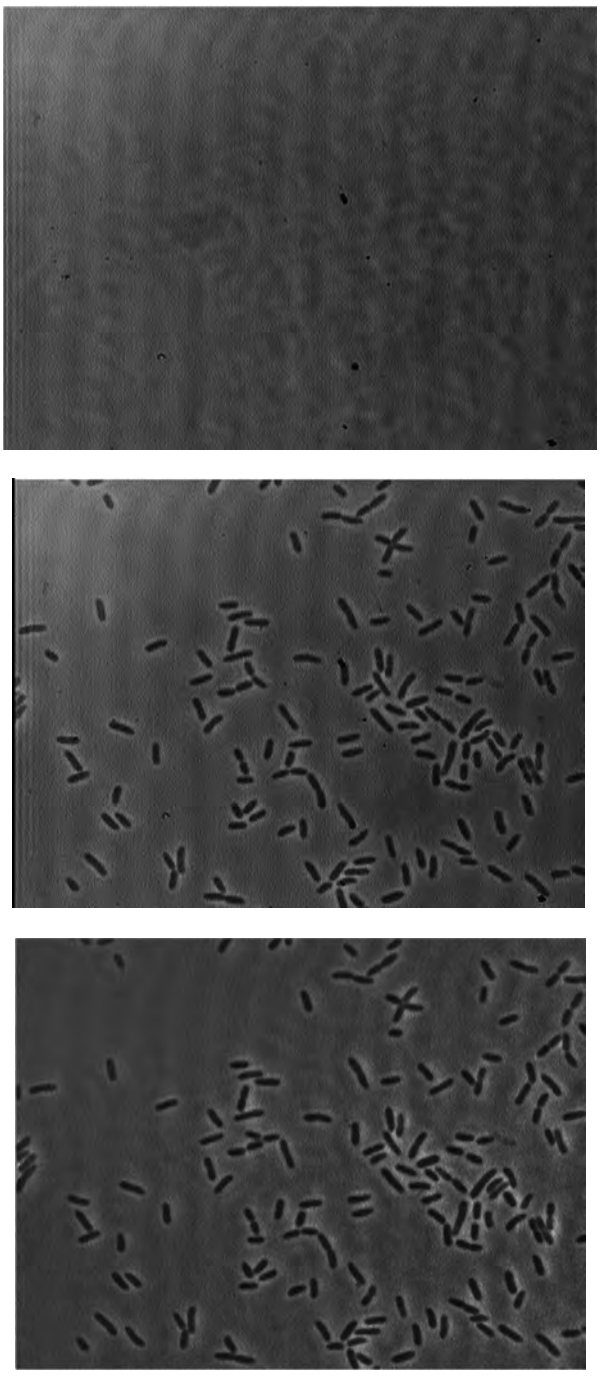

Figure 3. Effect of a shading correction (**a**) on an unevenly illuminated image (**b**) and the resultant image shown in (**c**).

Smoothing filters are commonly used to reduce image noise, and generally rely on the reassignment of the gray level value of a central pixel with the average value of itself and a matrix of surrounding pixels (may be referred to as a kernel operation). The size of the matrix is user-defined, and when applied, noise pixels are replaced with reasonable values resulting in an improved image. However, excessive application of smoothing filters can result in the loss of small objects, a loss of lateral resolution, and a decrease in the number of object pixels. Neighborhood-ranking filters such as the median filter may similarly be applied to remove noise, however, in this case a median pixel brightness value is assigned to the central pixel. An advantage of median filtration is that it does not shift boundaries as may occur in averaging or smoothing operations. There are many variations on the theme of image noise reduction (6). An example showing the impact of various strengths of median filter on noise, image quality, and object boundaries is shown in Figure 4.

High-pass filters are used to sharpen images, particularly the location of object edges. Laplacian filtration acts to increase image contrast at boundaries, thereby making edges more visible or sharper. Similar effects may be achieved using other common high pass filters such as the Roberts and the Sobel.

In some instances objects of interest may be clouded or obscured. Unsharp masking is a photographic process that may be performed digitally, where an out-of-focus image is used to mask bright regions allowing detail in these regions to be observed (see Figure 5).

Contrast Expansion and Histogram Equalization

Image analysis systems only detect object pixels having gray levels different from those of neighboring pixels. Ideally, the distribution of gray levels contained in an image will span the entire available range (i.e., 0–255 for 8-bit system; 0–4096 for 12-bit), however, this is seldom the case. Contrast expansion reassigns the image gray level values to encompass the full available range of gray levels but does not create any additional gray level information. So the image has a greater overall contrast (facilitating segmentation), but the same number of gray level values are missing. By averaging a number of contrast-expanded images that have been collected sequentially, a high-contrast image with a greater number of gray level values represented may be obtained. Histogram equalization is the selective spreading and compression of the displayed gray values so that the same number of pixels in the image show each of the possible brightness levels. Equalization may be used to bring out subtle detail in low contrast regions of an image.

Deconvolution

Deconvolution refers to the sharpening, or deblurring of a microscope image by removal of out-of-focus information, thereby mathematically producing an optical thin section. It may be used to improve both fluorescence and

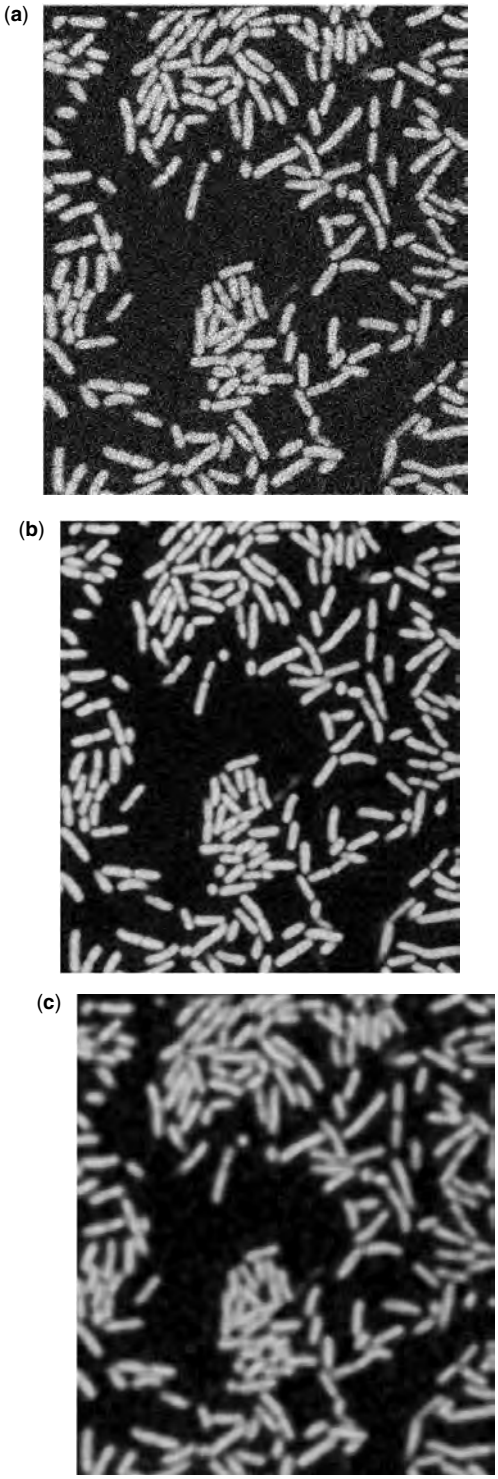

Figure 4. Presegmentation processing of a gray scale image of bacterial cells (**a**); to improve signal to noise ratio, and eliminate small debris through median filtration (**b**). The same image with a greater strength median filter showing degradation of cell edge features (**c**).

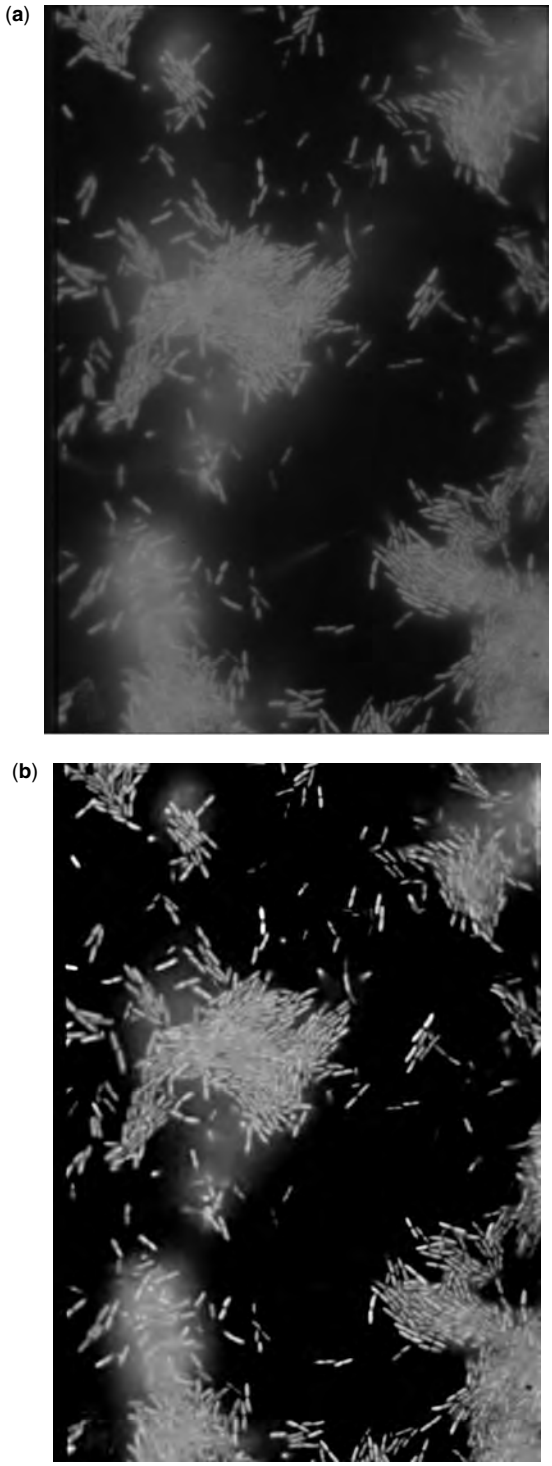

Figure 5. Application of an unsharp mask filter to an over-exposed or too bright image (**a**); to reduce haze and increase information content (**b**).

CLSM images. An ordered series or stack of high-quality primary images with perfect registration are required. A computer then calculates that information which results from objects located in other focal planes, and repositions these object pixels within the image stack. The most common algorithm is based on nearest-neighbor calculations; however, it is also the least accurate and the most artificial. Other options such as Blind Deconvolution, Iterative Maximum Likelihood

Estimation, and Iterative Constrained Tekhonov-Miller are included in certain software packages. Deconvolution methods that are based on the use of the point spread function are the most accurate for reconstruction, as it incorporates the distortion caused by the non-ideal optics of the microscope and out-of-focus pixels are placed back where they came from (photo reassignment processing) (3,46). These methods can be applied to images from the microscopy techniques described earlier, provided an image stack has been obtained.

Although mathematical approaches for deblurring microscopic images are less expensive than hardware-based optical-sectioning methods (CLSM, 2-PLSM), there are limitations to the number of depths that may be used during the calculations, and the complexity of the image. For example, a structurally simple, 3-slice image stack may be deconvolved rapidly and with excellent results. Manz and associates (3) were able to process 21-image stacks using a 200-MHz PC running under Microsoft Windows 95. Verveer and associates (47) applied various algorithms for the deconvolution of images obtained using fluorescence microscopy and CLSM. Both Agard (40) and Gorby (41) discuss the benefits of applying deconvolution. See also Squire and Bastiaens (48) for more information. Manz and associates (3) provide an excellent illustration of the application of deconvolution with EPR-deconvolution software (Scanalytics, Billerica, MA). They used a practical determination of the point spread functions using fluorescent beads (Fluospheres, Molecular Probes Inc., Eugene, OR) that had a broad excitation emission spectra. Deconvolution software such as Huygens (Scientific Volume Imaging, B.V., Hilversum, The Netherlands), AutoDeblur (Autoquant, Inc. Waterfliet, New York, U.S.A.), Micro-Tome^{TM}, HazeBuster (VayTek, Inc., Fairfield, IA) are available either through commercial sources or in the open literature. For examples of the application of deconvolution to serial epifluorescence images of hybridized bacteria including color illustrations, the readers are referred to the paper by Manz and associates (3).

SEGMENTATION/THRESHOLDING

Images must be segmented or thresholded to tell the computer which objects are to be measured during the analysis. Figure 6 shows a zoomed image of a bacterial cell illustrating the nature of the pixels forming the image and the boundary zone between what is cell (object) and background (nonobject pixels). The effect of selecting two different gray values for boundary values is shown. The resulting binary (black and white) image is then used to extract geometric data, on size, shape, number of objects, and so on. Most segmentation involves determining the boundary or the edge of the object to be measured, based on brightness or on textures. In the case of microorganisms, brightness or gray value is usually the basis for determining the object. In contrast, objects may be defined directly by grouping pixels that share certain properties and are connected, or only on their properties without regard to connectivity (43). There are also contrasting approaches concerning the degree of user interaction. Thresholding may be performed

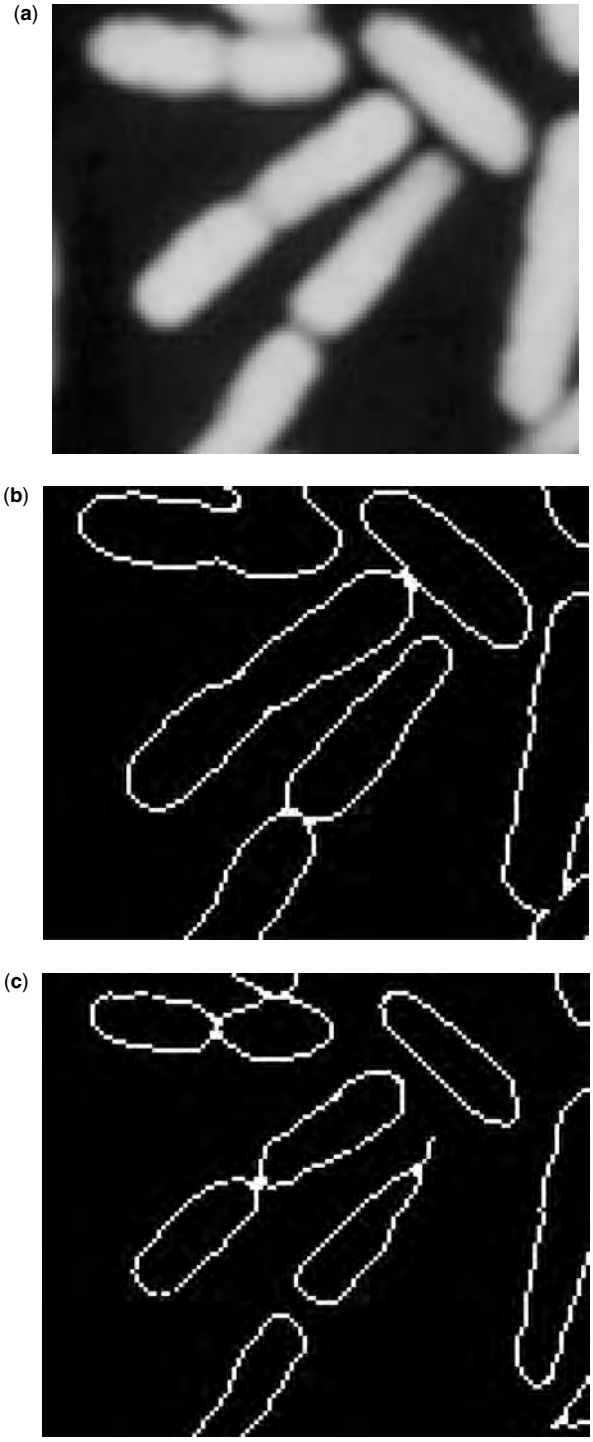

Figure 6. (**a**) Magnified boundary of the digitized image of a bacterial cell illustrating the pixelated nature of the image and how image processing software "sees" a bacterial cell. The challenge in segmentation is to correctly interpret which gray-level boundary represents the boundary of the cell for the purposes of defining and measuring the object. Once the decision is made, the software will reliably if not accurately place that boundary for all measurements. (**b**) Boundary is defined at a gray-level cutoff of 130 where black is 255 and white is 0. (**c**) Boundary of the cells is defined at a gray-level cutoff of 80. Note that cell area is reduced when the lower the gray level is used to define the object.

subjectively (with the user manually defining the gray-level cutoff value) or using automatic thresholding functions (e.g., using a probabilistic model) (49). During manual thresholding, pixels with a gray value below a user-set level (between 0–255) are considered background (gray value reset to 0), whereas pixels with gray values above this level are redefined as objects (gray level reset to 255), creating a binary (black and white) image. Manual methods are relatively easy to use, routinely available, and allow segmentation of any image that the user can interpret correctly. Liu and associates (14) used interactive discrimination to establish the cell boundary in phase contrast images, and also used a manual pencil tool technique to eliminate pixels and separate touching cells. Detection and placement of the cell boundary may be done with reasonable accuracy using phase contrast or bright-field illumination; however, accurate boundary or object detection in fluorescence images is confounded by a number of factors. These include the length of exposure to light (long times result in bleaching and an apparent reduction in object size), the "halo" effect (which makes the fluorescent object seem larger), and user subjectivity. Some authors have concluded, for example, that it may be impossible to find the true edge of fluorescently stained bacteria. In this instance, there may be a need or advantage to the use of automated methods. Additional considerations in manual versus automated segmentation include the need to exclude bias, the need to manually edit the image to remove nonobject material, and the number of images to be handled (the tedium factor). When there are large image sets to handle, it may be worthwhile to spend time developing algorithims for semiautomated or fullyautomated analysis. Automated segmentation functions should not be universally applied to all image analysis situations, as automatic settings do not necessarily agree with each other or with the manual settings of an experienced operator.

There are a wide variety of automated, edge-seeking algorithms. Sieracki and associates (50) evaluated nine different automatic thresholding methods, using fluorescent microspheres with known diameters as controls, to position the boundary of fluorescent objects. They also found that user threshold selection usually resulted in an overestimation of the object size. They reported that the gray-level histogram analysis of individual cells, present within images containing a number of cells could be used to construct a circular cell profile. This profile was then analyzed to determine the maximum in the second derivative, and used to calculate the "best" thresholding level for the entire image. Viles and Sieracki (51) subsequently found this approach, although suitable for nanoplankton analyses, did not function well with smaller picoplankton and so used a combination of edge detection and adaptive edge strengthening. Wilkinson (43) provides an excellent overview of the various methods used for determination of object boundaries in fluorescent images including Marr-Hildreth (a combination of a Gaussian smoothing function and a Laplace filter for edge amplification) and edge strength methods.

Møller and associates (38) used isointensity-thresholded images of fluorescently stained cells with the cell boundary positioned at a point equivalent to 20% of the smoothed, cell intensity maximum gray-level value. Blackburn and associates (39) found that applying Marr-Hildreth filtration in conjunction with thresholding to a constant value provided acceptable edge-detection results. Adiga and Chaudhuri (52) also deal with segmentation and counting of CLSM images of fluorescence in situ hybridization.

It should be noted that the approaches mentioned earlier should only be required where accurate details on the area of the cell or average gray value are required, and would not be necessary for simple object enumeration. The user can consider two schools of thought: (*1*) attempting to accurately size objects for determination of, for example, biomass, versus (*2*) simple counting or obtaining reproducible comparable data to assess treatment effects, and so on. The former may represent the "holy grail" of image analysis of bacteria, whereas the other represents the practical efficient application of the available technology.

Attempts to count single bacterial cells illustrates several additional problems associated with object recognition. Problems include distinguishing between specifically stained microbial cells and other fluorescent objects (e.g., soil particles), and whether the cell number is determined accurately despite the presence of microcolonies, clumps, and different cell morphologies. Simple solutions have been applied, such as the exclusion of cell clumps from the analysis (38) or dividing the number of objects by two in the case of analyzing a diplococci monoculture (53). Exclusion of circular objects allowed determination of hyphal length and biomass of fungi (37). Pattern recognition systems have allowed the extraction of single cells from small clumps in a mixed culture of methanogenic *Archaea* based on cell shape (36). These also represent situations where user intervention or manual editing may be required to achieve realistic analysis as is illustrated by Liu and associates (14) or Manz and associates (54) (Fig. 2).

Double- or multiple-thresholding may be applied when difficult to threshold objects are of interest. In this case, the user initially segments an object but finds that the objects are poorly represented. A second segmentation operation is then performed where more pixels are recognized as objects, showing all the object boundaries but including more image background noise. A Boolean feature-AND (6) then combines the two binary images, and a skeletonization step erodes the boundaries to a defined width resulting in accurately placed object boundaries.

ENHANCEMENT OF BINARY IMAGES

Following segmentation and formation of a binary image, the user may then choose to interactively edit the image to remove minor imperfections, or measure the desired parameters directly.

Erosion and dilation are commonly applied in combination to remove nonobject debris not in the same size class as objects of interest. Essentially, objects are eroded by a set number of iterations at a user-defined intensity, eliminating pixels from a binary image that should not have been present (e.g., resulted from noise). This erodes the

outer layer of pixels for image features by a user-defined amount. The image is then dilated by the same user-defined amount, returning all remaining objects to their original size. In Figure 7, an example of postsegmentation processing shows the application of an erode function to remove noise and noncell debris, and a dilate function to restore the cells to their original size and fill in missing pixels in the objects.

Close and open functions involve erosion and dilation operations but are in general used to connect or disconnect closely positioned features in binary images. Note that the open function acts as a combined erosion and dilation, where an erosion step removes edge-touching pixels (disconnecting closely positioned objects), and a dilation step restores the size of the objects while not reconnecting the object boundaries. The steps are reversed in closing, in which a dilation step is performed, connecting boundaries or joining fractures in the binary image, followed by an erosion to bring the object boundaries to their original size.

Binary images may also be eroded into skeletons that represent connected lines and indicate boundaries between objects. During the process of skeletonization, essentially all connected pixels are retained during erosion steps. This procedure may result in improved feature recognition, extraction, and measurement. This type of operation has proven useful in analysis of fungal hyphae (55). Furthermore, Boolean operators (AND, OR, NOT) may be exploited to improve images where pixels have improperly been classified as an object or background, or where the user wishes to selectively combine binary information. Korber and associates (56) used the Boolean operator OR to logically sum eight images of motile diatoms, allowing the construction of a motility track. This data was subsequently used to determine the chemotactic responses of *Amphora coffeaeformis* in mannose gradients (56).

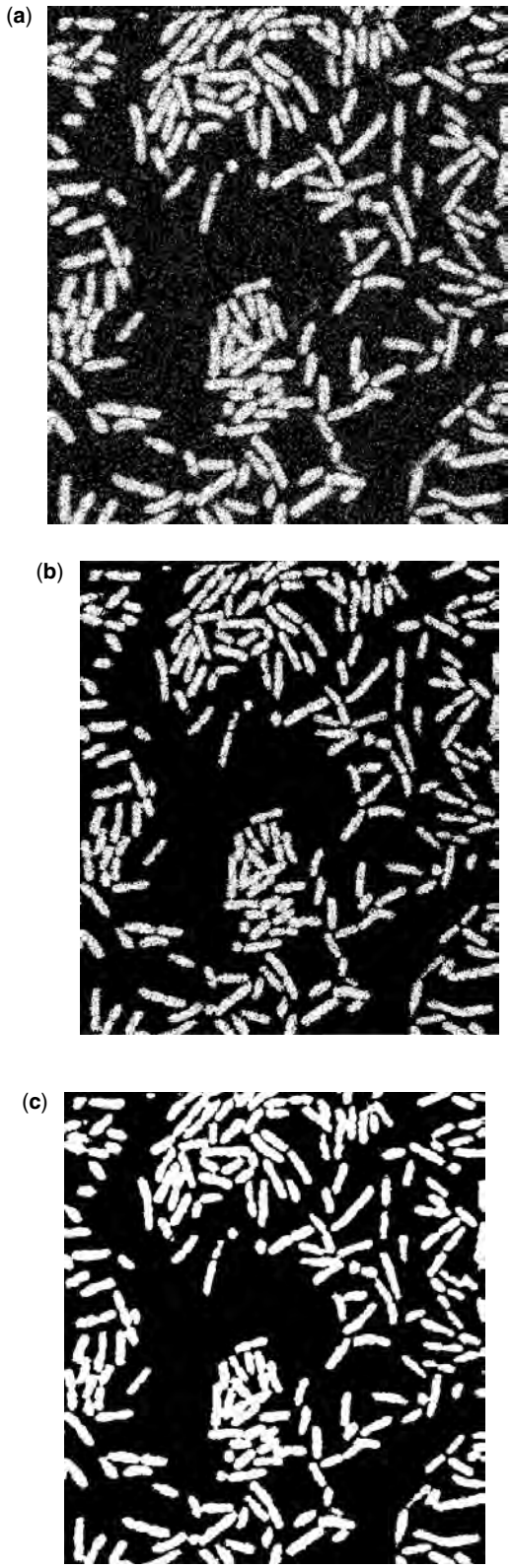

Figure 7. Postsegmentation processing showing the impact of erode and dilate operators on S/N in a binary image. The erode function was applied to eliminate the noise and small noncell objects, whereas dilate functions were applied to restore the bacterial cells to their original size, and connect pixels inside the objects.

DIFFERENCE IMAGERY

Difference imagery (the digitization of two sequential images and subtraction of their respective gray values) may be used to detect changes within the field of analysis over discrete time intervals, showing the difference between the two images as black objects on a gray background (44). During microbiological analyses, difference imagery may be used to detect the number of moving objects within the field of analysis, or may identify those cells or microcolonies that exhibit growth over a defined interval. Caldwell and Germida (57) used difference imagery to evaluate the growth kinetics of *Ensifer adherens* in agar slide culture, evaluating the potential for the separation of growth events from nonbiological debris. Difference imagery has also been used to enumerate the number of motile cells present within the hydrodynamic boundary layer of continuous-flow slide culture chambers during the recolonization phase of surface colonizing bacteria (42,58). Difference imagery may also be used to detect changes in images obtained from different focal planes within biological material. This may prove useful during the comparison of optical thin sections obtained from different regions of

biofilms using laser microscopy. An example of difference imagery showing change in location of cells with time is shown in Figure 8.

Alternately, image summation may also be performed. Lawrence and associates (59) used image summation and Helmert transformation (60) to digitally increase the depth of field. Phase micrographs were obtained from different focal planes, enhanced, aligned, and summed to create composite 2D images for discrimination and quantitative analysis of *Pedomicrobium* sp. growth and development. Using summed images, growth rates were determined for mother and daughter cells that were not developing within the same focal plane. Thus, this technique increased the effective resolution and operating depth of field of the light microscope.

FIELD AND OBJECT PARAMETERS

Parameters measured may be field-specific, meaning that all object pixels in the image are presented as a single value, or object-specific, meaning that each object in the image is identified and analyzed independently. Values obtained may be either direct (i.e., total number of objects in the field), or derived (calculated) (i.e., the percentage of the field occupied by objects).

Typical field and object-specific parameters include, field area, object area, or object number, whereas a derived parameter would be percentage field or object area. A program such as ImageTool can calculate 19 different object-specific attributes including object size, Feret diameter, major and minor axis, shape (roundness, elongation, compactness), orientation, gray scale density, integrated density, and so on and spatial position (x, y centriodes). Liu and associates (14) have developed plug-ins that expand these to include maximum curvature of the object, length, width, width/length, length/width, area bounding box area, eight Fourier descriptors, and aspect ratio. Addition of these features allows calculation of all the parameters required to use the CMEIAS Morphotype classifier facilitating identification and enumeration of 11 bacterial morphotypes. Other commercial systems may allow the user to derive as many as 250 parameters per object. Thus, the user must select the program and the parameters that are required to achieve quantitative image analysis and data required to test their hypotheses.

CALIBRATION

Image analysis is based on determination of the number of pixels that are associated with the defined objects, however, it is usually necessary to convert these to absolute values. This may be complex as in the case of fluorescence images or relatively straightforward as in phase contrast. Calibration may be achieved using a stage micrometer to check microscope objectives and magnification. Fluorescent or nonfluorescent beads (Molecular Probes Inc., Eugene, OR) are also useful, they may be purchased with specific sizes and volumes and applied to calibrate microscope images in terms of

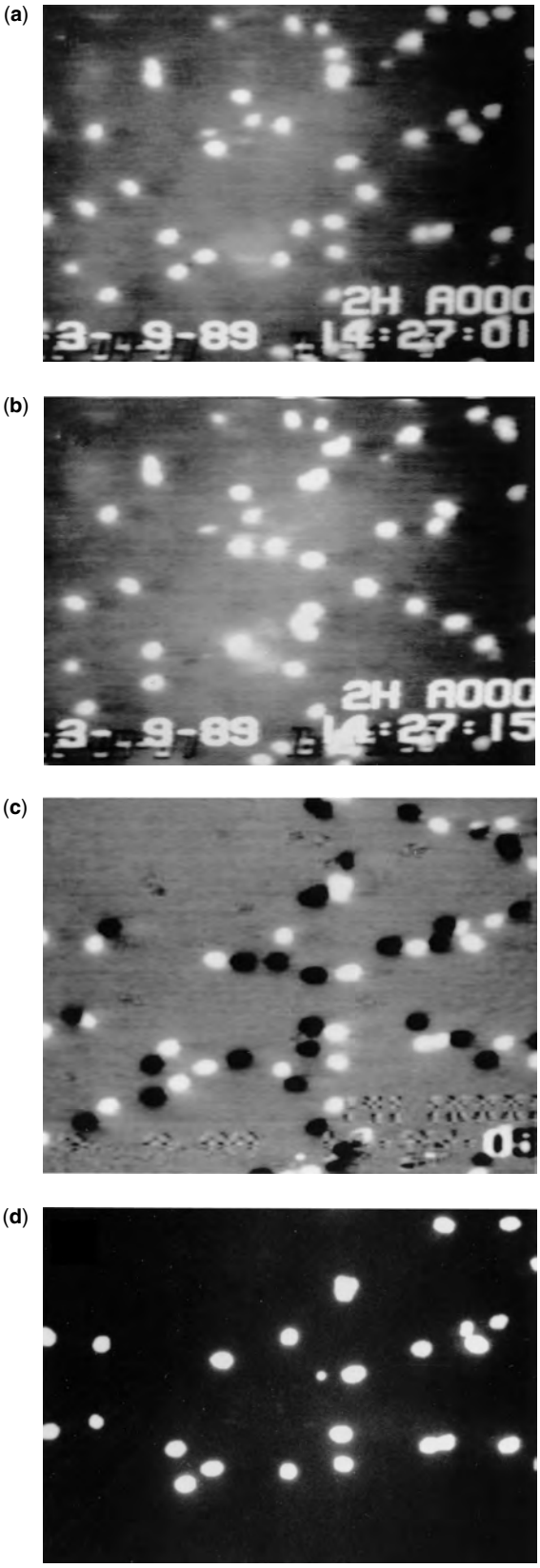

Figure 8. Images illustrating the result of application of difference imagery, (**a**) time 1, (**b**) time 2 (**c**) the resultant difference image showing movement of the diatoms, and (**d**) selection and segmentation of moving objects before enumeration.

magnification and point spread functions (3). Massana and associates (61) used Polysciences Fluoresbrite latex beads that were 0.51, 0.71 and 2.44 microns in diameter. They reported that they found 3–8% over estimation of bead area and under estimation of bead volumes by 4–13%. Similar values have been reported by other authors (35).

REPLICATION AND SAMPLING INTENSITY

The application of image analysis in microbiology is relatively new and efforts have focused on improved observation and analysis of the microbes growing in a variety of habitats. An aspect that has not received enough consideration in many studies is statistical representation. Similar to other areas of scientific research, image analysis of microbes requires representative and statistically reproducible sampling. Usually this can be achieved only if the sample size or number of replicates is large enough. Factors that may have an effect on the representative value of data were discussed by Kuehn and associates (62). These include the species under investigation, the absence or presence of growth inhibitors or stimulators, microbial behavioral factors, and experimental design. Furthermore, the distribution and type of extracellular polymeric substances, the presence of large cells such as algae and protozoa in the case of mixed species biofilms or debris may also have a significant effect.

Although image analysis offers the potential for analysis of extensive numbers of images with greater facility, the level of effort involved is still an important consideration. A number of studies have addressed the question of sample size and reproducibility. In general, the more cells, samples or area examined, the better the estimate. However, beyond a specific point, increasing effort results in only minimal improvement in the estimate. Massana and associates (61) considered the number of cells and images to be examined to obtain accurate estimates of bacterial volume. They concluded that 200 to 250 cells in 4 to 6 images resulted in a good estimate with minimal variation for the least effort. Korber and associates (63) used a computer-controlled microscope stage and CLSM to construct montages of contiguous biofilms and analyzed elements of these montages to define the area required for representative analysis. Using this approach, Korber and associates (63) demonstrated that biofilms formed by a nonmotile *Pseudomonas fluorescens* were more variable than the motile variant of this species and therefore required a larger area of analysis for statistically valid comparisons. Moller and associates (64) used CLSM and examined contiguous biofilm areas in the same size order ($>2.8 \times 10^5 \mu m^2$) during a study of the architecture of biofilms formed by a degradative microbial community. Dandurand and associates (65) also applied geostatistical analysis using covariance to compare colonization patterns of the rhizoplane, whereas Gibbs and Bishop (66) applied these approaches to the analysis of microbial biofilm surface roughness. Fractal analysis has also proven useful for the analysis of biofilm structures (67).

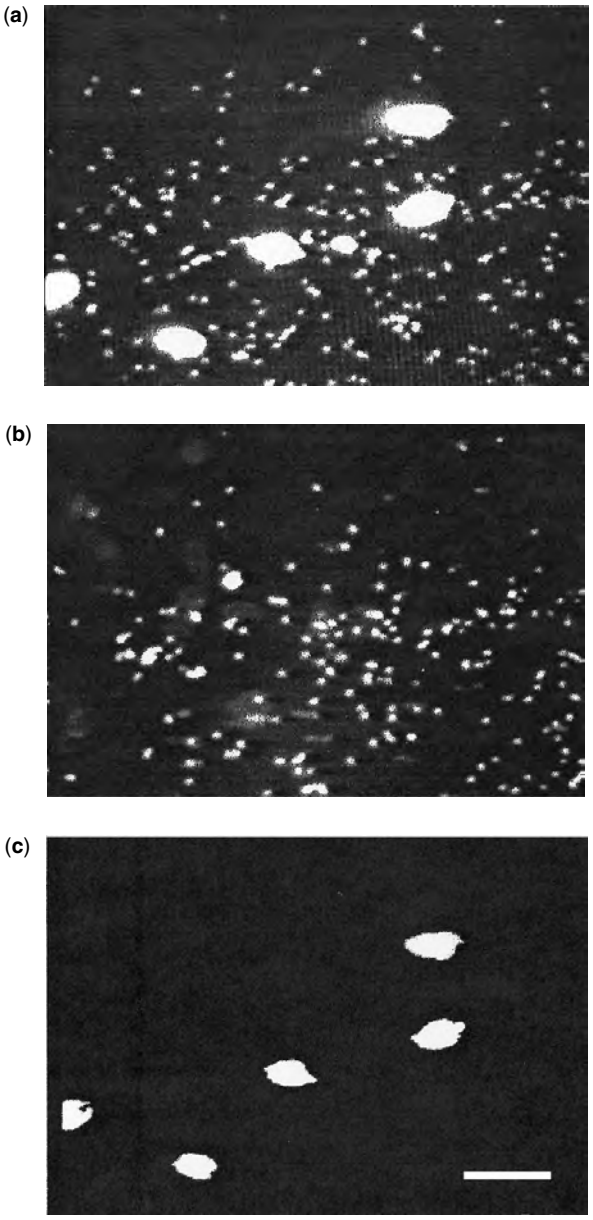

Figure 9. Digitized images showing the presence of both ciliates (*Euplotes* sp.) and bacteria (**a**), an averaged image showing the ghosting of the moving ciliates and their elimination from the image (**b**), and application of size-selection based on maximum dimension to a segmented image to eliminate the bacteria and enumerate the ciliates (**c**) (the reverse procedure allows enumeration of the bacteria). From Lawrence and Snyder (1998).

APPLICATIONS OF IMAGE PROCESSING AND ANALYSES

The use of digital image analysis is becoming increasingly widespread in environmental microbiology. The number of published studies and range of applications is extensive. The following publications all show good examples of multiple step image analysis applied to studies in environmental microbiology (3,8,24–27,35,38,39,42,50,56,59,63,68,69).

Image analyses have been used for quantification of microbial cell numbers and/or biovolume, in aquatic samples (51,61) on surfaces (38,53) or in soil samples (35). Manz and associates (54) used rRNA probes and image analyses to determine population dynamics of bacterial groups in river biofilms. Species specific counts of *Methanosaeta* and *Methanosarcina* in anaerobic granules have been made using fluor conjugated rRNA probes and digital image analysis (70). An example of determining the change in bacterial cell area with time and as impacted by protozoan grazing is shown in Figure 9. In this case, attached cells were imaged using phase contrast microscopy, images digitized, individual cells segmented, binarized, and cell area measured. Combining various input modes with size selective cutoffs and segmentation can be used to determine the rate of change in the number of bacteria and ciliates within an observation area (17,42). Figure 10 shows typical results of applying this type of processing and analysis scheme.

Increasingly, more sophisticated use of object-derived parameters to determine shape of objects are being applied to differentiate and enumerate morphological types (14) and to study filamentous organisms such as fungi (55,71).

In addition to the analysis of binary images, gray scale images may be used to determine dynamic processes associated with bacterial metabolism and activity. A number of papers describe the quantification of signal intensity after hybridization of bacteria with rRNA-targeted probes to determine the ribosome content and thus estimate the actual metabolic activity or growth rate in pure cultures or biofilms (38,72–75). Laser microscopy and digital image analysis were used to follow the influx of

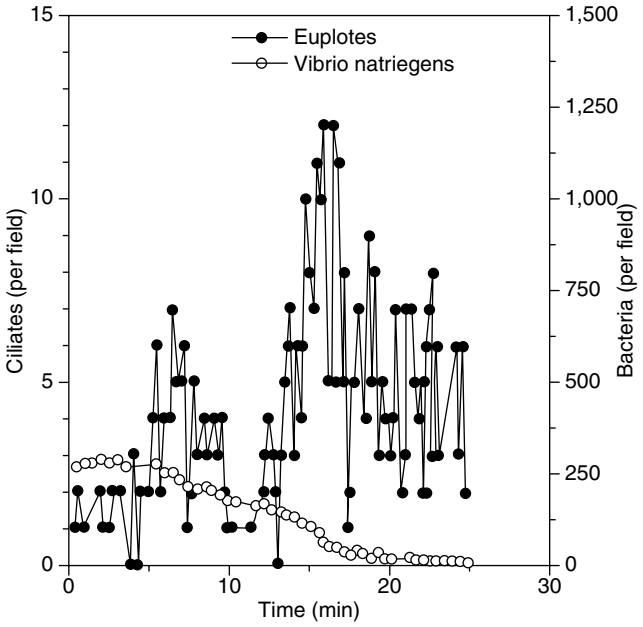

Figure 10. Time course data illustrating the removal of attached *Vibrio natrigens* cells by grazing *Euplotes* sp. Data derived using the approach shown in Figure 9. From Lawrence and Snyder (1998).

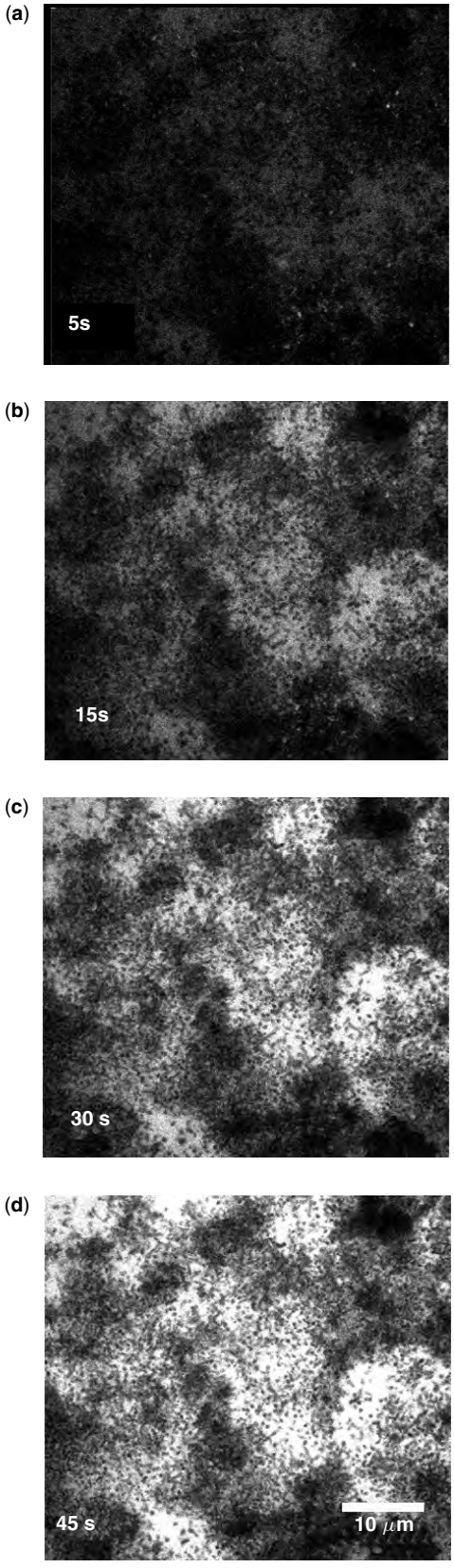

Figure 11. Confocal laser micrographs showing the appearance of a fluor-conjugated dextran at a monitoring site in a microbial biofilm. Gray scale images (**a**, **b**, **c**, **d**) show increasing brightness with time from 5 through 45 seconds.

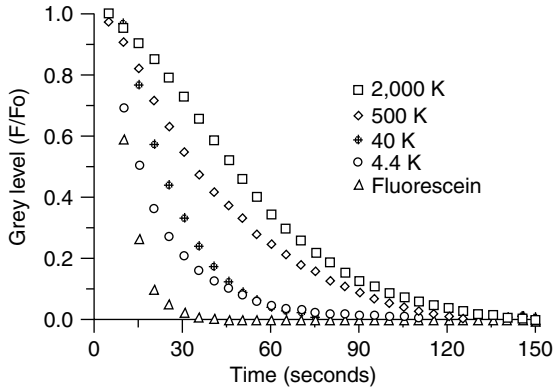

Figure 12. Results of image analysis of several time series of images (see Figure 11 **a**–**d**) showing the change in gray value and pattern of diffusion for a range of size fractionated fluorescent probes.

ethidium bromide into individual Bacillus spores during germination (76).

Lawrence and associates (77) used analysis of the rate of change in gray level to determine the rate of diffusion of various fluorescent size fractionated probes and subsequently calculate diffusion coefficients for mixed species biofilms. De Beer and associates (78) used a similar approach to determine diffusion rates of molecules in biofilm matrices. Figure 11 shows a time series of CLSM images showing the diffusion of size-fractionated fluor labeled dextrans in a microbial biofilm. The results of image analysis of a number of these time series following the diffusion of a range of fluorescent probes are shown in Figure 12. A more extensive discussion and presentation of this approach is presented in Lawrence and associates (77).

CONCLUSION

Digital image processing and analysis are powerful tools that can be and are being applied in environmental microbiology. This combined with the evolution of fluorescent probe technologies, for example, those specific for phylogeny, gene expression, activity, and environmental chemistry, permits truly comprehensive ecological studies of events occurring at the microscale. For example, using phylogenetic probes one can enumerate specific groups while autofluorescence may be used to detect phototrophs, general nucleic acid stains to enumerate bacteria, and lectins to detect and quantify exopolymers. Furthermore, the existence of relatively inexpensive and user friendly hardware and software opens the application of these approaches to any researcher.

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IMAGING.

See IMAGE ANALYSIS OF MICROORGANISMS

IMMOBILIZATION OF MICROORGANISMS ON SOLID SUBSTRATA.

See ACTIVITY AND CARBON TRANSFORMATIONS IN BIOFILMS; ADHESION, IMMOBILIZATION AND RETENTION OF MICROORGANISMS ON SOLID SUBSTRATA

INDICATORS OF WATER QUALITY: AEROBIC SPORES.

See AEROBIC ENDOSPORES

INDICATORS OF WATER QUALITY: BACTERIOPHAGE.

See BACTERIOPHAGE OF ENTERIC BACTERIA: OCCURRENCE AND PERSISTANCE IN THE ENVIRONMENT

INDICATORS OF WATER QUALITY: CLOSTRIDIUM. See *Clostridium*

INDICATORS OF WATER QUALITY: COLIFORMS. See Coliform Bacteria as Indicators of Water Quality

INDICATORS OF WATER QUALITY: FECAL STREPTOCOCCI/ENTEROCOCCI. See Fecal Streptococci/Enterococci in Aquatic Environments

INDICATORS OF WATER QUALITY: SOURCE WATER PROTECTION. See Source Water Protection: Microbiology of Source Water

INDOOR EXPOSURE TO FUNGI. See Fungal Allergy and Allergens

INDOOR HEALTH. See Fungi and Indoor Air

INDUSTRIAL ENZYMES. See Alkaliphiles: Alkaline Enzymes and Their Applications

INDUSTRIAL SETTINGS, BIOAEROSOLS IN. See Bioaerosols in Industrial Settings

INFECTIOUS AIRBORNE BACTERIA

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Infectious airborne environmental bacteria consist of those bacteria naturally present in the environment that cause human disease as a result of transmission through the air. Airborne transmission occurs by dissemination of either dust particles containing the infectious agent or airborne droplet nuclei from evaporated droplets of water containing microorganisms. Infectious bacteria are those that cause disease through the multiplication of organisms within the body, thus excluding those diseases caused by hypersensitivity or the direct inhalation of toxins. Despite their abundance in soil and water, few environmental bacteria are responsible for airborne diseases. Bacteria that thrive in soil are generally saprophytes (ones that live and feed on dead organic matter) of low pathogenicity to humans. Although outdoor and indoor air contains many bacteria and particles containing bacteria for people to inhale, few of these bacteria ever colonize or infect the respiratory tract, the principal effects of airborne exposure. Fewer bacterial species multiply in water rather than soil, and aerosolization of water is usually associated with artificial sources that use water treated to eliminate most pathogens, which reduces the risk of disease transmission. The principal pathogen in water aerosols, *Legionella* species (spp.), is reviewed in Legionellae, this Encyclopedia.

For many of the agents discussed here, the role of the bacteria that are airborne relative to other routes of exposure is uncertain, even for pulmonary infections. Ingestion and inoculation followed by hematogenous spread to the lungs or ingestion followed by aspiration are considered alternative, if not more important, routes of exposure for *Mycobacterium avium* complex (MAC) and *Burkholderia pseudomallei*. Cystic fibrosis patients colonized with *B. cepacia* can transmit the agent to noncolonized fellow patients in health care and social settings (person-to-person airborne transmission), but infection also may be acquired by inhalation of dust from soil that contains the organism. The relative importance of these two exposure routes for these patients is uncertain. Some organisms that are present in outdoor air, originating from soil and water, (e.g., species of the aerobic gram-negative bacilli *Pseudomonas, Klebsiella*, and *Enterobacter*) also are found in hospital environments in discharges from colonized patients. These bacteria are primarily nosocomial pathogens (ones that affect persons in hospitals and other health care settings), but an agent may have been acquired from the environment and have colonized the respiratory and gastrointestinal tracts of a patient before the manifestation of the disease in that person while hospitalized. Characteristics that favor survival and growth in the external environment would confer these same advantages to bacteria in hospitals and other health care settings.

Airborne bacterial pathogens for which the principal reservoir is humans, [e.g., *Mycobacterium tuberculosis* (tuberculosis)] and those for which the principal reservoir is animals [including *Bacillus anthracis* (anthrax), *Brucella* spp. (brucellosis), *Coxiella burnetii* (Q fever), and *Chlamydia psittaci* (psittacosis)] exist in the environment to little or no extent or have limited impact on human health as a result of their environmental presence. Although *B. anthracis* spores are commonly found in the soil where infected animals died, few if any cases of human illness have been reported as a result. Bacterial pathogens that affect humans in agricultural and outdoor settings are

discussed in Bioaerosols in Agricultural/Outdoor Settings, this Encyclopedia. Airborne endotoxin is an important cause of human disease in occupational settings, but this cell wall component of gram-negative bacteria does not cause infection and is covered separately in Endotoxins, this Encyclopedia. The environmental bacteria that do infect humans as a result of airborne transmission, although few in number and of low pathogenicity, are of increasing importance because of the rising population of immunosuppressed and otherwise compromised people. The human immunodeficiency virus (HIV) epidemic, in particular, has transformed previously obscure pathogens such as MAC into major worldwide causes of morbidity and mortality. The global movements of humans, animals, soil, and water can increase the geographic distribution of these agents. The widespread distribution of bacteria in soil and water makes control of environmental exposure extremely difficult, if not infeasible. Thus, the impact of environmental airborne bacteria on human health and our appreciation and understanding of resulting infectious diseases will continue to increase for the foreseeable future.

ENVIRONMENTAL RESERVOIRS AND SOURCES

Bacteria are found in a wide range of ecological sites in the environment, primarily natural water, groundwater, wastewater, soils, and in association with plants. However, bacteria often go unrecognized because of their small size and frequent absence of warning properties (such as odor or taste) to alert people of their presence. The bacterial agents discussed in this article are saprobes (saprophytes) because they use nonliving organic material (e.g., dead or decaying plant or animal debris) as nutrient sources. Along with fungi and protozoa, saprobic bacteria are responsible for the decay of organic materials and the recycling of nutrients.

A reservoir of a bacterial agent is any person, animal, arthropod, plant, soil, substance, or combination of these in which the bacterium normally lives and multiplies, on which it depends for survival, and where it reproduces and from where it can be transmitted to a susceptible host. A person or other living animal is a host to a bacterial agent if the human or animal provides subsistence to the bacterium under natural conditions. A person, animal, object, or substance becomes a source of a bacterial agent when it is the immediate location from where the bacterium passes to a host. For instance, an outbreak of melioidosis occurred when the source, a potable water supply, became contaminated and people drank, bathed in, and inhaled aerosols of the water. However, the reservoir for the etiologic agent, *B. pseudomallei*, was the soil surrounding the water-treatment facility where the contamination occurred. Identifying the reservoirs of infectious agents (not just their immediate sources) often is important for understanding their transmission and achieving long-term prevention (1).

AIRBORNE TRANSMISSION

Transmission describes the physical means by which an agent spreads from a source or reservoir to a susceptible host. Infectious agents are transmitted by five routes, namely, contact, droplets, air (droplet nuclei, dust, and spores), common vehicles, and vectors (invertebrate animals such as ticks and mosquitoes). Before people understood the causes of infectious diseases, diseases were called *contagious* if they were known to be transmitted through contact. However, this term has lost much of its significance and these diseases now are considered *communicable* diseases. Diseases referred to as communicable are transmitted from an infected person, animal, or inanimate reservoir to a susceptible host. This transmission may be direct or indirect, the latter involving an intermediate plant or animal host, a vector, or the inanimate environment. Airborne transmission is generally thought to be directly from a source to a host, although infectious material may be resuspended from surfaces.

Some of the bacteria in this article have multiple transmission routes, which may result in the same or distinct diseases. For example, pathogenic actinomycetes such as *Nocardia* spp. cause pulmonary disease when inhaled and cutaneous or ocular infections when directly inoculated. Some of the nontuberculous mycobacteria (NTM), notably MAC, and *B. pseudomallei* may cause pulmonary disease when inhaled but are believed to do so primarily as the result of septicemia following ingestion or direct contact.

True airborne transmission results from the inhalation of bacterial spores, dust particles carrying bacteria, or droplet nuclei containing bacterial cells. Droplet nuclei are the solid residues of dried droplets, usually less than or equal to 5 μm. The diameter of infectious particles containing vegetative bacterial cells or spores is determined not only by the physical size of a single organism or cluster of cells, but also by the presence of salts, proteins, and debris in varying states of hydration that accompany airborne bacteria (2).

Small particles can remain suspended in air for minutes to hours. Thus, people very close to a source of a bacterial aerosol readily are exposed, but people at some distance also may become infected if sufficient numbers of bacteria were generated and enough survived until they reached a susceptible host. Inhaled particles with diameters larger than 3 μm most often deposit in the upper respiratory tract (the nose, pharynx, and larynx). Deposition in the lower respiratory tract (the trachea, bronchi, and pulmonary or alveolar regions of the lungs) is greatest for particles with diameters in the 2 to 5 μm-range. Only infectious agents to which the upper respiratory tract is relatively resistant but the alveoli are vulnerable are considered truly airborne because few particles greater than 5-μm reach the alveolar region in appreciable numbers.

INFECTIOUS DOSE

Successful transmission of a bacterial disease requires not only that the agent reach a host but also that the bacterium arrives in sufficient numbers to resist defense mechanisms and multiply. Outdoor air typically contains 10 to 10,000 live bacteria per cubic meter of air and activities such as turning dry compost or

working with soil can expose people to even higher concentrations. Indoor air may be cleaner or contain more bacteria depending on the location. Efforts are made to keep the number of airborne microorganisms very low in hospitals and microbiology laboratories, but the air in animal confinement buildings and indoor waste-processing facilities may have several million airborne microorganisms per cubic meter. Thus, at a typical breathing rate of 1 cubic meter per hour, an adult inhales 24 cubic meters of air per day. Along with this air, we inhale hundreds to billions of living microorganisms, of which we capture a variable percentage somewhere in our respiratory tracts.

The infectious dose of a pathogen is the number of cells or amount of toxin required to produce infection or disease. Epidemiologists and researchers often express infectious dose as ID_{50} (the dose that produces infection in half of exposed hosts). The infectious dose is not known for most human infections and probably varies greatly depending on agent virulence, host resistance, and other factors. The ID_{50} has been determined for few airborne bacterial infections. While animal experiments suggest that deposition of a single cell of *M. tuberculosis* in a susceptible lung may lead to tuberculosis (3), the relevance of this information for human risk is uncertain.

AIRBORNE BACTERIAL PATHOGENS

Nontuberculous Mycobacteria

Introduction. The NTM are the *Mycobacterium* spp. other than the *M. tuberculosis* complex (which includes *M. tuberculosis*, *Mycobacterium bovis*, *Mycobacterium microti*, and *Mycobacterium africanum*). They are bacilli that are considered gram-positive and are acid-fast. First isolated in 1885, three years after Koch's discovery of *M. tuberculosis*, they were not recognized as important human pathogens until the discovery of *Mycobacterium ulcerans* in 1948. Between 1949 and 1954, cases of infection were reported caused by *Mycobacterium kansasii*, *M. avium*, *Mycobacterium marinum*, *Mycobacterium chelonae*, and *Mycobacterium intracellulare* (4). Before 1979, these organisms were referred to as *atypical mycobacteria*, but since then they have been treated as separate disease entities (5). The acquired immunodeficiency syndrome (AIDS) epidemic dramatically changed the epidemiology of nontuberculous mycobacterial disease. Before the epidemic, nontuberculous mycobacterial disease was predominately pulmonary (caused throughout the world by *M. kansasii*, *M. avium*, and *M. intracellulare*) in elderly males with underlying lung disease or heavy occupational exposure to dust, such as in farming (5). Exposure to NTM was presumed to be from environmental aerosols. *Mycobacterium malmoense*, identified in 1977, presented a similar picture but was confined primarily to northern Europe and Great Britain. Additionally, *M. marinum* caused skin infections following cuts and abrasions and exposure to water. The epidemiology of *M. ulcerans* infection, which results in skin ulcers, has remained unchanged since its first description in 1948. Today, as a consequence of AIDS, nontuberculous mycobacterial disease is most commonly caused by MAC; 20 to 40% of AIDS patients (depending on the stage of disease) in the United States and Europe have disseminated infections of MAC. There has been a concomitant increase in both pulmonary and disseminated infections caused by the other NTM that cause pulmonary disease in immunocompetent patients, that is, *M. kansasii*, *Mycobacterium xenopi*, and *Mycobacterium simiae*. While isolation of *M. malmoense* has been increasing, in contrast to the preceding NTM, it is only occasionally isolated from AIDS patients (6).

The NTM are generally considered opportunistic pathogens, primarily causing disease in individuals with immunodeficiency, underlying disease, or following trauma or surgery. However, their presence in the environment provides them an opportunity to enter and colonize the human respiratory tract and also to contaminate clinical specimens. It is often difficult, particularly in immunodeficient patients, to distinguish among infection, colonization, and specimen-contamination. Recently discovered NTM that occasionally cause pulmonary disease in immunocompetent individuals with underlying pulmonary disease or disseminated disease in immunosuppressed patients include *Mycobacterium celatum*, *Mycobacterium genevense, Mycobacterium conspicuum*, *Mycobacterium lentiflavum*, *Mycobacterium haemophilum*, *Mycobacterium heidelbergense*, and *Mycobacterium szulgai* (4). *Mycobacterium gordonae* is ubiquitous in the environment and is commonly isolated from water and soil (4). Because it is found in hospital water, it can contaminate clinical and bronchoscopy specimens during collection and processing, often resulting in pseudooutbreaks. *Mycobacterium gordonae* occasionally causes pulmonary and disseminated infections similar to those caused by the other pathogenic NTM (4). The following NTM species have been isolated from human or environmental sources but have not yet been associated with any disease: *Mycobacterium alvei*, *Mycobacterium bohemicum*, *Mycobacterium brumae*, *Mycobacterium cookii*, *Mycobacterium gadium*, *Mycobacterium gastri*, *Mycobacterium hibernae*, *Mycobacterium confluentis*, *Mycobacterium hasiacum*, *Mycobacterium valientiale*, *Mycobacterium madagascarence*, *Mycobacterium mageritense*, *Mycobacterium murale*, *Mycobacterium novastrense*, and *Mycobacterium triplex* (4).

All of the aforementioned organisms grow slowly on standard mycobacterial media. The rapidly growing mycobacteria, *Mycobacterium fortuitum*, *M. chelonae*, and *Mycobacterium abscessus*, most often cause skin and soft tissue (including wound) infections and disseminated disease, associated with direct contact with contaminated potable water. They also have been reported to cause pulmonary tract colonization and occasionally pulmonary disease, presumably as a result of inhalation of water aerosols.

Mycobacterium spp. in water-based metalworking fluids also may cause hypersensitivity pneumonitis in machine workers. Outbreaks of hypersensitivity pneumonitis in association with NTM in metalworking fluids have been reported in which the NTM identified

in one case was *M. chelonae* (7) and in another was a newly described *Mycobacterium* sp. (proposed name being *Mycobacterium immunogen*) (8).

Natural Occurrence. Mycobacteria may be obligate parasites, saprophytes, or opportunistic pathogens. Most of the latter two groups are free-living and are isolated readily from water, soil, plants, house dust, and other environmental sources (9). Clinically important NTM have been linked with potable water systems as sources. A number of species (*M. kansasii*, *M. xenopi*, and *M. simiae*) that are isolated only rarely from natural waters or soils have been isolated from tap water near people with clinical disease or frequently positive specimens (9). *Mycobacterium malmoense* has been isolated from water and soil mainly in northern Europe and Great Britain, the same regions where infections have been identified. The MAC consists of serovars of two distinct species, *M. avium* and *M. intracellulare*. Isolates have been cultured from natural water (fresh, brackish, and ocean), public drinking water systems, soil, and food. The physiologic characteristics of MAC organisms together with their relative chlorine resistance favor their presence in municipal drinking-water systems and their concentration in hot water distribution systems in hospitals (5). In San Francisco, sampling of the home environment of 290 HIV-infected people revealed that MAC was isolated more often from potted plant soil (55%) than from water (0.76%) (10). Although MAC can be found in water in diverse areas of the world, culture of 91 water samples found higher isolation rates in the United States and Finland (18 of 52, 35%) than in Zaire and Kenya (4 of 39, 10%) (11). MAC was isolated from hospital water in the United States and Finland, but not in Zaire or Kenya. The rapidly growing mycobacteria have been found in numerous natural water sources, potable water systems, and hospital water sources, and soil.

Routes of Exposure. There is no evidence of person-to-person transmission for any of the NTM, although *M. simiae* can be transmitted among monkeys (5). For those organisms causing primarily pulmonary disease in immunocompetent individuals (*M. kansasii*, MAC, *M. xenopi*, *M. simiae*, and *M. malmoense*), exposure is thought to be by inhalation of soil dust or water aerosols. Exposure to MAC is presumed to be from the lungs by inhalation of water aerosols, from the gastrointestinal tract by ingestion, or by ingestion and aspiration; whether one or more of these exposure routes are predominant is uncertain (12). One study was able to specifically link MAC isolates in potable water in hospitals to those in patients, but exposure could have been by inhalation or ingestion (13).

Incidence. Approximately 25 to 50% of patients with AIDS in the United States and Europe are infected with NTM, mostly MAC. Disseminated MAC infection has an annual incidence of 20 to 35% after the occurrence of a first event defining the presence of AIDS, reaching 40% of patients with advanced AIDS (CD4 counts <10 cells per cubic millimeter) in the United States (14). The population incidence in the United States is unknown because MAC infection (along with other nontuberculous mycobacterial diseases) is not a reportable condition. In the Houston and Atlanta metropolitan areas, where CDC conducts active surveillance, the incidence is approximately 1 per 100,000 per year (15). This is consistent with studies in the United States, Japan, and Switzerland, showing incidences of 1.3, 1.3, and 0.9 cases per 100,000 per year, respectively (7). Incidence is decreasing among HIV-infected patients as a result of new treatment modalities, for example, combination therapy with nucleoside reverse transcriptase inhibitors and protease inhibitors, and antimycobacterial prophylaxis. However, antimicrobial resistance may be increasing.

Nontuberculous mycobacterial disease is much more common in the developed rather than the developing world, despite the high incidence of AIDS in the latter. The reason for the low incidence of disseminated MAC in AIDS patients in Africa ($<1\%$) is unclear. Decreased exposure (e.g., lower likelihood of MAC in water), disease-related factors (such as death of AIDS patients from other causes before CD4 cell counts become extremely low), and protection by prior exposure to *M. tuberculosis* have been suggested as possible explanations (14).

The most common nontuberculous mycobacterial disease in immunocompetent individuals is either *M. kansasii* or MAC, depending on geographic location (5). Surveillance for NTM is not conducted by any country, and so incidence data are unavailable. The rate of isolation of *M. malmoense* has been increasing in the United Kingdom, Scotland, and Scandinavia (5), but this may be due in part to improved laboratory methods and increased awareness of mycobacteria as potential pathogens (6).

Disease in Humans. Pulmonary disease caused by the NTM is similar to tuberculosis. Patients may have localized infiltrates, cavitation, or solitary or multiple pulmonary nodules (14). Fever is usually present, whereas weight loss depends on the duration of the untreated disease. The causative agent usually can be found in sputum by culture if not by smear. Among people with MAC pulmonary disease, isolates of the *M. avium* group are similar in number to those of the *M. intracellulare* group (14). MAC have been identified in the sputum of up to 14% of the patients with cystic fibrosis, a high percentage of whom have skin tests that are positive to *M. avium* sensitin, indicating infection and a possible role of MAC in the pathogenesis of this disease (16).

MAC, *M. kansasii*, and *M. malmoense* are known to cause lymphadenitis, primarily in the cervical lymph nodes in children less than five years of age. Infected children are immunocompetent and have no identifiable risk factors. Ingestion of milk may be the route of transmission for these infections, at least for MAC, which frequently is cultured from milk (14).

MAC is by far the most common cause of disseminated disease, almost entirely in AIDS patients. Infection usually begins in either the respiratory or gastrointestinal tract, suggesting acquisition through either route (14). Disease rapidly becomes systemic, involving fever, night sweats, anemia, weight loss, and hepatosplenomegaly.

Diagnosis is made by blood culture. *Mycobacterium kansasii* is the second most common NTM causing disseminated disease in HIV-infected patients (17). However, disease in these patients often resembles that from *M. tuberculosis*, more often causing pulmonary rather than disseminated disease. In one series of 49 HIV-infected patients with *M. kansasii* infections, 32 (65%) had pulmonary isolates only, whereas 17 (35%) had disseminated disease documented (17). Coinfection with another NTM, usually MAC, was present in about one-third of the patients. This behavior of *M. kansasii* may be the result of it being antigenically the NTM most closely related to *M. tuberculosis* (17). Disseminated disease in AIDS patients occurs less frequently from *M. xenopi*, *M. simiae*, *M. malmoense*, and *M. haemophilum* (5).

Actinomycetes

The term *aerobic actinomycetes* is an informal designation for bacteria belonging to the order *Actinomycetales* (18). Most stain as gram-positive, branched, filamentous rods with various degrees of acid-fastness. Their branched filaments often are incorrectly referred to as *hyphae*. Actinomycetes have many microbiological characteristics in common with the genera *Corynebacterium* and *Mycobacterium*. *Mycobacterium* spp. are considered to belong to the order *Actinomycetales* but, because of their clinical significance and inclusion of *M. tuberculosis* (whose principal reservoir is humans and not the environment), the mycobacteria usually are discussed separately, as they are here. The remainder of the order has been adopted into descriptive groups rather than formal taxonomic families (18).

Aerobic actinomycetes are ubiquitous in the environment, found worldwide in soil and organic matter. *Nocardia* spp. and related bacteria are considered saprophytic soil organisms, primarily responsible for the decomposition of organic plant material (18). They also have been found in fresh and marine water and in wastewater. Although nocardia are encountered in clinical practice infrequently, they can be important causes of human infections. The clinical manifestations, severity of disease, and prognosis vary with the route of infection and state of the host's immune function. Species from two genera, *Nocardia* and *Rhodococcus*, cause pulmonary disease in humans, primarily immunocompromised individuals, as a result of airborne exposure. Species from the genus *Gordona* (removed from *Rhodococcus* in 1988) occasionally are recognized as causing pulmonary infection, catheter-associated sepsis, or nosocomial wound infections (18). The pathogenic actinomycetes, most notably *Nocardia* spp., also cause cutaneous and ocular infections as a result of local, usually traumatic, inoculation. Members of the genus *Actinomadura*, notably *Actinomadura madurae*, cause infections of the skin, usually of the lower extremity ("madura foot"), as a result of soil contamination of a penetrating wound. *Dermatophilus congolensis* causes dermatitis in animals and humans, usually as a result of contact with infected animals or contaminated animal products (18). Thermophilic actinomycetes cause pulmonary disease that is immunologically mediated, and not infectious.

Nocardia

Introduction. *Nocardia* is a genus of the family Nocardiaceae. The misperception of their filaments as hyphae led to their being misclassified as fungi for many years. Nocardia can cause pulmonary disease as a result of airborne exposure, cutaneous, subcutaneous, and lymphocutaneous disease as a result of percutaneous introduction, and systemic and extrapulmonary disease either from the pulmonary or percutaneous exposure. Infections most often are caused by *Nocardia asteroides* and less frequently by *Nocardia brasiliensis*, *Nocardia farcina*, *Nocardia nova*, *Nocardia otitidiscaviarum*, and *Nocardia transvalensis*. *Nocardia asteroides* is a heterogeneous taxon from which *N. farcina* and *N. nova* now have been separated (18).

Natural Occurrence. Pathogenic species of *Nocardia* have been found in soil, house dust, beach sand, tap water, and swimming pools (18). In an outbreak in a renal transplant unit, the same strain isolated from patients was cultured from dust and air in the unit (19). There is limited information available on the geographic distribution of *Nocardia* spp. *Nocardia asteroides* appears to be widespread. *Nocardia brasiliensis* (the second most frequently isolated aerobic actinomycete in the clinical laboratory) is responsible for mycetoma in Mexico and South America and is most often identified in the United States in the southeast and southwest (especially Texas, North Carolina, California, Oklahoma, and Florida) (18). *Nocardia farcina* infections have been reported from Asia, Europe, and North America, emerging as the predominant nocardial pathogen in Germany (19).

Routes of Exposure. Pulmonary infection occurs as the result of inhalation of bacteria or bacteria-containing dust, which becomes airborne from soil, water, or vegetable matter. Rarely, pulmonary infection occurs from other nonairborne routes such as ingestion of contaminated food or following dental procedures (19).

Incidence. Nocardiosis is not a reportable condition, and so population estimates are unavailable. Infection occurs in people of all ages and in men three times more than in women. It is chiefly an opportunistic disease, with estimates of the percentage of cases in otherwise healthy individuals ranging from 10 to 40% (19). There are many conditions that increase the risk of infection. Among these are lymphoreticular and other neoplasms, systemic immunosuppression [particularly transplantation (accounting for approximately 4% of infections in renal transplant patients) and also steroid therapy, HIV infection, alcoholism, diabetes, intravenous drug use, and collagen, vascular, and immune dysfunction diseases], and preexisting pulmonary disease (19).

Disease in Humans. Pulmonary nocardiosis can be an acute, subacute, or chronic infection (19). Immunocompromised patients often suffer from a brief acute pneumonia. Cavitation may be present, with or without pneumonia. Remissions and exacerbations often occur. Anorexia and weight loss commonly are present along with pulmonary symptoms. In immunocompromised patients, soft tissue

swellings, abscesses, or central nervous system manifestations may occur in conjunction with evidence of pulmonary involvement. The presence of lesions in two or more organs indicates dissemination. Common sites include the brain, skin, subcutaneous tissue, eyes, kidneys, joints, bones, and heart. The initial pulmonary infection may have cleared spontaneously before the diagnosis is established. *Nocardia farcina* may be more pathogenic than other species, with more than 50% of patients having disseminated disease (19). Treatment of pulmonary infections caused by strains susceptible to first-line antibiotics is usually successful, particularly if therapy is prolonged.

Rhodococcus

Introduction. The genus *Rhodococcus* is part of the phylogenetic group nocardioform actinomycetes, which includes the genera *Mycobacterium* and *Nocardia*. *Rhodococcus* spp. are aerobic, gram-positive, and partially acid-fast. The principal human pathogen, *R. equi*, previously was assigned to the *Corynebacterium* and *Mycobacterium* genera. *Rhodococcus equi* originally was isolated from pulmonary lesions of foals in 1923 and has since become a well-recognized veterinary pathogen, particularly in foals (18,20). Since the first reports of disease from *R. equi* in a patient receiving corticosteroid therapy in 1967 and in an HIV-infected patient in 1986, it has been identified with increasing frequency as a cause of pneumonia in immunocompromised patients (18,20).

Natural Occurrence. *Rhodococcus* spp. are widely distributed in the environment, particularly in soil. *Rhodococcus equi* is found in soil and herbivorous dung and multiplies in greatest numbers in the presence of herbivorous manure (18).

Routes of Exposure. Exposure is assumed to occur as a result of inhalation of contaminated dust. Although *R. equi*–infected patients often are reported to have a history of contact with farm animals, soil, or both (18), in HIV-infected patients, such exposure history frequently is lacking (21).

Incidence. Population estimates are unavailable, but infection with rhodococci is uncommon and primarily occurs in HIV-infected patients; it also is often misdiagnosed or undiagnosed. In addition to HIV-infected people, pulmonary and disseminated disease have been reported in patients receiving chemotherapy for hematologic and other malignancies, renal transplant recipients, patients receiving corticosteroids, and chronic alcoholics (18).

Disease in Humans. Infection with *R. equi* in HIV-infected patients usually is manifest as pneumonia, with fever, cough, dyspnea, and chest pain (20). Pulmonary abscess formation and cavitation frequently occur. The case mortality rate is 25 to 50% despite administration of antibiotics and surgical intervention (21). Delay in diagnosis, which often requires biopsy, contributes to the severity of the illness. *Rhodococcus equi* may be misidentified or undetected because the organisms often appear as nonspecific coccoid forms in macrophages. Cavitary lesions, detection of acid-fast bacilli in sputum, and an interim response to antituberculous drugs may result in a misdiagnosis of mycobacterial infection (18). Infection in immunocompetent patients has been reported in only 19 cases (22). In contrast to HIV-infected patients, in immunocompetent patients, localized infections are more common than pulmonary and the mortality rate is 11% (22).

Aerobic Gram-Negative Bacteria

Aerobic gram-negative bacteria include numerous pathogenic species that are widely distributed in soil and water and on plants. Many of these same species (but not necessarily the same strains) also can colonize human and animal intestinal, respiratory, and urinary tracts and can cause infections in those sites, including pneumonia. However, even if the strains that cause human disease were to occur in the environment, there is little evidence linking airborne or any other environmental exposure to human disease. The major exception is *B. pseudomallei*, the etiologic agent of melioidosis, which is discussed separately later. There also is evidence that airborne transmission of *Acinetobacter* spp. has a role in nosocomial transmission, as discussed later. However, it is possible that sporadic cases of pneumonia are caused by airborne aerobic gram-negative bacteria but, because of their sporadic nature, cannot be linked to an exposure. It is only in the context of outbreaks or endemic nosocomial occurrence that environmental exposures are likely to be suspected or sought.

Some pathogenic aerobic gram-negative bacilli (including *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *S. marcescens*, and *Enterobacter* and *Citrobacter* spp.) are common causes of nosocomial infections. In the hospital setting, the pathogenic reservoirs are the gastrointestinal tract and hands of health care personnel. Aqueous reservoirs (including medications, irrigation fluids, disinfectants, soaps, and dialysis fluids) have been responsible for point-source outbreaks, presumably as the result of contamination by human, not environmental, sources, and through direct inoculation, rather than airborne transmission. Contamination of nebulizers and other respiratory therapy equipment is a mechanism for entry of bacteria into the respiratory tract. These organisms may be found in any equipment using water (including sinks, aerators, shower heads, and whirlpool and hydrotherapy baths) but may have been deposited by patients or health care workers rather than an environmental source of exposure.

Burkholderia cepacia, *S. maltophila*, *Ralstonia pickettii*, and *Acinetobacter* spp. also may be present in the environment and increasingly are recognized as opportunistic nosocomial pathogens. *Burkholderia cepacia* is an important cause of respiratory tract colonization and disease in patients with cystic fibrosis and chronic granulomatous disease; the manner in which it is acquired, possibly from environmental sources, is uncertain. Evidence of person-to-person transmission of *B. cepacia* has led to the institution of surveillance programs and separation of colonized from noncolonized patients. Continued acquisition of the organism despite these precautions has

led to the speculation that the environment may be an important source. An environmental survey of 55 sites in a botanical complex yielded only 12 *B. cepacia* isolates, none of which displayed the phenotypic properties of a multiresistant epidemic strain associated with pulmonary colonization in patients with cystic fibrosis (23). In another study, 916 samples from homes of patients with cystic fibrosis, control homes, salad bars, and food markets were analyzed for *B. cepacia* (24). The agent was isolated from 5 (18%) out of 27 homes, and from 20 (4%) out of 509 cultures collected outside homes. These studies suggest that the environment is a relatively low-risk source of *B. cepacia* for these patients. However, the organism has been developed as a biological pesticide for protecting crops from fungal diseases and has potential as a bioremediation agent for breaking down recalcitrant herbicides and pesticides. This has lead to concern about increased human exposure through environmental contamination, and the possibility of acquisition of increased virulence through horizontal gene transfer between the strains applied and existing soil organisms, particularly *B. pseudomallei* (25).

Acinetobacter spp., primarily *Acinetobacter baumannii*, have been recognized as a nosocomial pathogen of increasing importance (26). *Acinetobacter* spp. can be found in soil and water and grow well in water (27). Compared with other genera of gram-negative bacilli, they survive better on dry environmental surfaces, probably contributing to their propensity to cause prolonged outbreaks of nosocomial infection (26,28,29). However, there is no evidence that outbreak strains have enhanced environmental survival capacity compared with nonoutbreak strains (28,29). *Acinetobacter* spp. have been isolated from the air of hospitals, vents of nursery air conditioners, and aerosols in association with nosocomial infections (30). Although transmission may have occurred through airborne contamination of equipment, medication, or skin in some cases (in particular, in outbreaks of bloodstream infections), direct transmission to the respiratory tracts of patients is evident in others. Nosocomial *Acinetobacter* spp. infections in the United States have been observed to demonstrate seasonal variation, with significantly higher rates during July to October than during November to June (30). This may be mediated through changes in moisture within the hospital environment as a result of changes in outdoor humidity or increased use of air conditioners that emit contaminated aerosols.

Acinetobacter spp. rarely cause community-acquired infections, even in the presence of underlying disease. However, an outbreak of acinetobacter pneumonia in foundry workers has been reported (31). Foundry and other metal workers are at increased risk of pneumonia because of chronic exposure to metal particles; an *Acinetobacter* sp. was found in the air in the foundry where the outbreak occurred, but a source was not identified. Nontuberculous mycobacteria and Legionellae have been cultured from water-based metalworking fluids and have been associated with hypersensitivity pneumonitis, as described earlier, or Pontiac fever.

Most pathogenic aerobic gram-negative bacteria have intrinsic resistance to multiple antibiotics and have demonstrated increasing acquired-antibiotic resistance, particularly as a result of acquisition of extended-spectrum β-lactamases (32). Thus, selective antibiotic pressure in hospitals (particularly in settings such as intensive care and transplant units, where use of antibiotics and invasive devices is particularly common) contributes to the increasing incidence of nosocomial infections from aerobic gram-negative bacteria.

Burkholderia pseudomallei (Melioidosis)

Introduction. Melioidosis is a disease with a wide and variable spectrum of clinical manifestations, including acute, localized suppurative infection, acute pulmonary infection, acute septicemic infections, and chronic suppurative infection. It is recognized primarily in the tropics, particularly in Southeast Asia and northern Australia, but has occurred throughout the world between 20 degrees north and south latitudes (33). The etiologic agent, *B. pseudomallei*, is a gram-negative aerobic bacillus that, with *B. cepacia*, recently was moved from *Pseudomonas* to its own genus on the basis of RNA homology. The bacterium first was isolated in 1911 from a patient with pneumonia, and the disease was named in 1921. The term is derived from the Greek "melis" meaning "a distemper of asses." Another common name for infection with *B. pseudomallei* is pseudoglanders because the disease resembles glanders, which is predominantly a pulmonary disease in asses caused by *Pseudomonas mallei* (33).

Natural Occurrence. Melioidosis initially was thought to be a zoonotic disease with a reservoir in rodents. However, *B. pseudomallei* now is known to be a widely distributed environmental saprophyte that can be isolated from soil, surface water (stagnant streams, ponds, and rice paddies), and market produce in endemic areas (34). The disease is endemic in Southeast Asia, with the greatest concentration of cases in Thailand, Malaysia, Cambodia, Myanmar (Burma), Laos, and northern Australia (35). Indigenously acquired cases also have been reported in Indonesia, southern China, the Philippines, Guam, Sri Lanka, the Indian subcontinent, Africa (Kenya and Central West Africa), Borneo, Iran, Turkey, and Central and South America. In Thailand, the percentage of positive soil and rice paddy water cultures and disease incidence are highest in the northeast section of the country (36). *Burkholderia pseudomallei* is able to survive in water for prolonged periods without any form of nutrient and is tolerant to a range of adverse environmental conditions such as low pH. However, little is known about the climatic, physical, chemical, and biological factors that control the proliferation and survival of *Burkholderia* spp. in the environment, although epidemiological studies show space-time clustering of melioidosis (34).

Routes of Exposure. It is assumed that most human and animal melioidosis arises from exposure to contaminated soil or muddy water, although only 6% of human cases have a clear history of inoculation and a further 0.5% of cases follow near-drowning (34). Occupational or recreational exposure to soil or muddy water is thought to increase the risk of infection. Laboratory animals have

been infected by inhalation, but evidence of infection acquired naturally by this route remains anecdotal. Infection of American helicopter personnel in the Vietnam War has been viewed as evidence of airborne exposure. Sporadic cases have resulted from laboratory accidents and person-to-person or animal-to-person spread. An outbreak in western Australia was linked to contamination of the potable water supply, but the route of exposure could not be determined (37).

Incidence. Melioidosis is reportable only in Thailand, Hong Kong, and Singapore. Clinically apparent disease is common in Thailand but rare elsewhere. The infection rate in patients attending government hospitals in 1997 in northeastern Thailand (137.9 per 100,000 inpatients) was significantly higher than that in the northern (18 per 100,000 inpatients), central (13.4 per 100,000 inpatients), and southern (14.4 per 100,000 inpatients) regions and accounted for 19% of the admissions in that region (36). Serological surveys indicate that clinically inapparent infection is common in other endemic areas. Most of the clinical cases in the United States have occurred in people who have traveled or lived in endemic areas, with only one or two cases of unequivocal transmission from native soil to a patient.

Disease in Humans. Infection ranges from rapidly fatal septicemia, with or without pneumonia, to more chronic soft tissue involvement of almost any part of the body. The most common form of melioidosis is pulmonary, which may be a primary pneumonitis or hematogenous pneumonitis as a result of septicemia. Illness can be acute, subacute, or chronic. A notable feature of melioidosis is recurrence months or even years after the initial acute infection, due to the ability of *B. pseudomallei* to survive inside phagocytic cells. Cases have been reported in which acute infection occurred after a disease-free interval of decades following presumed environmental exposure. The severity of disease is higher in people with diabetes or chronic renal failure.

Infection is diagnosed by culture of *B. pseudomallei* from blood, sputum, or other focal site (as indicated by the clinical presentation), or by rising antibody titer. A rapid immunodiagnostic test is available. Confirming the identity of a suspected *B. pseudomallei* culture isolate can be difficult. Some commercial culture identification systems are prone to misidentify *B. pseudomallei* as other genera or species such as *Chromobacter violaceum*, *B. cepacia*, or *P. aeruginosa*.

PREVENTION AND CONTROL MEASURES

Elimination or Reduction of Reservoirs and Sources

All the airborne bacterial infections discussed in this article are transmitted in the workplace and other settings. Occupational exposures often can be anticipated, on the basis of locations and activities of the workers. Consequently, practices that potentially put workers at risk of exposure to bacterial agents can be identified and modified. However, given the ubiquitous nature of most of these organisms in the environment and their diffuse distribution when they are present, elimination or reduction of reservoirs generally is not feasible. When bacterial presence in a health care facility poses a risk of nosocomial infection to patients, modification of the identified environmental reservoir may be considered. The possibility of control of MAC in hospital potable water, through methods similar to those used for *Legionella*, has been proposed (12). Identification and elimination of gram-negative bacilli in environmental reservoirs often is necessary to control nosocomial outbreaks. A comprehensive set of recommendations for environmental management in health care settings is under preparation by The Centers for Disease Control and Prevention and will be available in 2001.

CONCLUSION

Many species of bacteria are present in soil and water and hence become airborne, often in particles that can be inhaled. Yet, only a small proportion of these bacteria cause infectious diseases in humans, because many environmental bacteria are opportunistic pathogens, primarily affecting people with immune dysfunction or other underlying diseases. Currently, adverse health effects related to airborne bacteria account for a relatively small burden of human disease, often of limited geographic distribution. However, that burden is increasing and their geographic range is expanding; as the global population of immunocompromised individuals increases, the movement of humans, animals, equipment, and soil expands, and travel for business and pleasure increases. Thus, infectious airborne bacteria are expected to be of increasing importance in the future, as the population at risk increases and new pathogens and diseases are recognized.

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INORGANIC NUTRIENT USE BY MARINE MICROORGANISMS

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Microbes participate in reactions involving virtually every element found in the periodic chart, but of the 90 natural elements only those with atomic numbers less than 53 are considered biogenic (1), and only a few of these are essential for microbial growth. Some of the biogenic elements occur in compounds used as electron acceptors or donors in redox reactions mediated by bacteria (e.g., the reduction of SO_4^{2-} and SeO_4^{2-} by sulfate-reducing bacteria) and are not assimilated into biomass; that is, they are not nutrients. Compounds containing the most abundant biogenic elements, C, H, and O, are also usually not considered nutrients, although they make up nearly all of microbial biomass. This article focuses on the remaining elements (Table 1) found in nutrients that supply either major biogenic (e.g., N, P, and S) or trace (e.g., Zn and Fe) elements, all of which are necessary for microbial life. All marine microbes are considered, but heterotrophic bacteria are emphasized.

A fundamental problem in examining the ecophysiology of marine microbes is that few can be isolated and studied in pure culture. This problem is especially acute for heterotrophic bacteria. It has been known since ZoBell (2) that less than 0.1% of the bacteria observed by direct microscopy can be grown and isolated on agar plates. One consequence of this inability to culture most marine bacteria is that it is necessary to infer the physiology of uncultured microbes from field studies of mixed assemblages and from the physiology of nonmarine bacteria and those marine microbes that can be cultured. Molecular approaches are beginning to provide some insights into nutrient uptake by uncultured marine microbial assemblages (3). In addition, inorganic nutrient use by cultured bacteria appears to represent what marine bacteria do in the oceans, with some notable exceptions.

It is difficult, however, for laboratory experiments to reproduce the biogeochemical environment that marine microbes must contend with. Foremost, concentrations of all nutrients are usually very low in marine systems compared with standard laboratory cultures and other

Table 1. Summary of Elements Found in Inorganic Nutrients Used by Marine Microbes

Element	Form in Seawater[a]	Location or Use in Cells
Major Biogenic Elements		
N	N_2, NO_3^-	Protein, nucleic acids
P	PO_4^{3-}	Nucleic acids, phospholipids
S	SO_4^{2-}	Protein
Si	SiO_4^{4-}	Diatom frustrales
Trace Biogenic Elements		
V	$H_2VO_4^-$	Nitrogenases
Mn	Mn^{2+}	Superoxide dismutase
Fe	Fe^{3+} organic	Electron transfer system (see Table 3 for others)
Co	Co^{2+} organic	Vitamin B_{12}
Ni	Ni^{2+} organic	Urease; hydrogenase
Cu	Cu^{2+} organic	Electron transfer system, superoxide dismutase
Zn	Zn^{2+} organic	Carbonic anhydrase, protease
Se	SeO_4^{2-}	Formate dehydrogenase
Mo	MoO_4^{2-}	N_2 fixation
Cd	Cd^{2+} organic	Carbonic anhydrase
I	IO_3^-	Electron acceptor (anaerobic respiration)[b]
W	WO_4^{2-}	Hyperthermophilic enzymes

[a] (Taken from W. Stumm and J. J. Morgan, *Aquatic Chemistry*, John Wiley & Sons, New York, 1981). Those metals with "organic" occur mainly in organic complexes.
[b] See reference (118), for example. The text cites references for the other metals or also see reference (119).

environments. Little is known about the consequences of these extremely low concentrations on the physiology of marine microbes because few studies have examined the physiological processes of bacteria growing on low concentrations of both organic and inorganic compounds (4). In addition, marine microbes live in a complex chemical milieu consisting of many inorganic and organic compounds, a concoction that is difficult to reproduce in a laboratory experiment. Because of the differences between the ocean and the laboratory and between uncultured and cultured bacteria, it is important to consider field studies to help us understand inorganic nutrient use by marine bacteria.

THE MAJOR NUTRIENTS

Nitrogen

Basic Pathways and Enzymes. Nitrogen is found mainly in protein, which makes up about 60% of the microbial cell (Table 1). Heterotrophic marine bacteria obtain much of their nitrogen from dissolved organic nitrogen, mostly in the form of amino acids, but two inorganic forms, ammonium (NH_4^+) and nitrate (NO_3^-), are also major nitrogen sources for heterotrophic and autotrophic marine bacteria and autotrophic eukaryotic algae. Nitrite (NO_2^-) can be ignored in considering assimilatory nitrogen uptake as nitrite concentrations are usually negligible in marine environments. Nitrite is produced and consumed during nitrification and denitrification, but the nitrogen compounds involved in these redox processes are not considered as nutrients.

Figure 1 summarizes the uptake mechanisms for nitrogen acquisition by marine heterotrophic bacteria. A schematic diagram for autotrophic microbes would be similar, but would not have pathways for use of free and combined amino acids, although some phytoplankton may have cell-associated amino acid oxidases (5). Much of Figure 1 is based on work on nonmarine bacteria, because few of these pathways have been examined explicitly in marine microbes. Fortunately, the basic mechanisms are likely to be the same in all microbes. The kinetic parameters and regulation of nitrogen acquisition may differ.

Three important enzymes mediating assimilation of inorganic nitrogen have been found in marine bacteria and in natural assemblages of bacterioplankton. Nitrate assimilation depends on assimilatory nitrate reductase, which catalyzes the reduction of nitrate to nitrite. Genes for this enzyme have been found in coastal waters (3). The two other enzymes are glutamine synthetase (GS) and glutamate dehydrogenase (GDH), which are involved in ammonium assimilation. As observed in nonmarine bacteria, GS is found in the marine bacterium *Vibrio harveyi* growing on low ammonium concentrations (<1 mM, which is actually quite high for natural environments), whereas GDH mediates ammonium assimilation at high ammonium concentrations (Fig. 2) (6).

Glutamate is the end-product of both pathways, but the energetic costs differ. The low affinity GDH system expends only one nicotinamide adenine dinucleotide

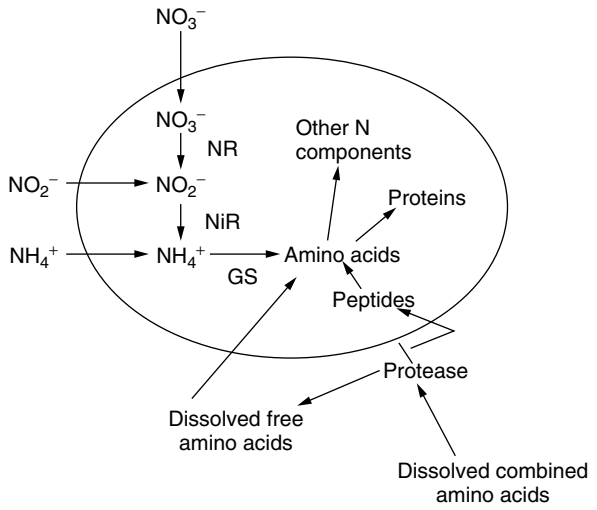

Figure 1. Summary of nitrogen acquisition by heterotrophic bacteria. GS = glutamine synthetase; NR = nitrate reductase; Nir = nitrite reductase. "Dissolved combined amino acids" is the term used by organic geochemists to refer to the amino acids released by acid hydrolysis from proteins, other polymers, complexes or adsorbed to other material. (Taken from D. L. Kirchman, in D. L. Kirchman, ed., *Microbial Ecology of the Oceans*, Wiley-Liss, New York, 2000, pp. 261–288).

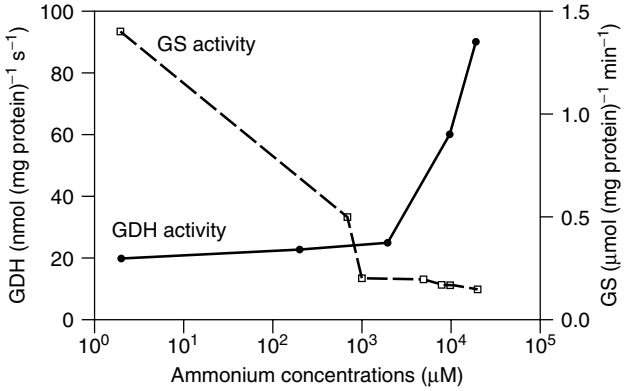

Figure 2. Activity of glutamate dehydrogenase (GDH) and glutamine synthetase (GS) in the marine bacterium *Vibrio harveyi* as a function of ammonium concentrations. (Data taken from M. P. Hoch, M. L. Fogel, and D. L. Kirchman, *Limnol. Oceanogr.* **37**, 1,447–1,459 (1992)).

(phosphate) (NAD(P)) in assimilating ammonium in a reaction catalyzed by a single enzyme, GDH:

$$NH_3 + \alpha\text{-keto-glutarate} + NADH = \text{glutamate} + NAD^+ \quad (1)$$

whereas the high affinity system requires two enzymes and expands one adenosine 5′-triphosphate (ATP) in addition to the one NAD(P)H. First, ammonium is assimilated to form glutamine, a reaction catalyzed by GS:

$$NH_3 + \text{glutamate} + ATP = \text{glutamine} + ADP + Pi \quad (2)$$

To regenerate glutamate, GS must be accompanied by glutamine α-ketoglutarate aminotransferase (GOGAT), which catalyzes the following transamination reaction:

$$\text{glutamine} + \alpha\text{-keto-gluturate} + \text{NADH} = 2\ \text{glutamates} + \text{NAD}^+ \quad (3)$$

Glutamate produced by the GDH and GS-GOGAT systems is a nitrogen donor in transamination reactions with appropriate α-ketoacids to form the other amino acids and nitrogenous compounds needed by microbial cells for growth.

Ammonium concentrations are never high enough in marine waters for the GDH system to be effective and thus ammonium must be assimilated by the GS-GOGAT system. However, concentrations may be sufficiently high in sediments for GDH to be active. Highest concentrations excepted (6), ammonium transport across the cell membrane is probably an active, ATP-requiring process, because the charged species, ammonium (not ammonia) predominates at seawater pH (ca. 8.2); the ammonium/ammonia reaction has a pK_a of 9.5.

Regulation of Inorganic Nitrogen Uptake. Extensive work with nonmarine bacteria has shown that ammonium transport and assimilation are inhibited, and genes synthesizing enzymes mediating these processes are repressed, by glutamate and glutamine. Analogous studies with natural marine bacterioplankton suggest that similar mechanisms may be operating with uncultured bacteria in the ocean (7,8). Presumably, use of amino acids avoids the energetic costs associated with transporting and assimilating ammonium. These feedback mechanisms, however, do not appear to prevent use of ammonium in the presence of amino acids when the organic carbon supply is adequate, as demonstrated in both lab (9) and field experiments (10).

The energetics of nitrogen use lead to the prediction that growth rates of marine bacteria using amino acids will be higher than growth rates of bacteria using ammonium and a carbon source. Indeed, there is some evidence for this hypothesis (11), but other studies have failed to find any difference in growth rates (12). It has been argued that the transport costs of using amino acids cancel the energetic advantage of avoiding ammonium assimilation (12,13), thus explaining the similarity in growth on amino acids and ammonium. The impact of concentrations on energetic costs has not been examined.

The energetic cost of using nitrate seems clearer, although no study has examined this issue specifically for marine bacteria. In probably all microbes, reduction of nitrate eventually to ammonium requires at least five NADH, four more than ammonium assimilation using GDH (14). For this reason, it was not surprising to learn that nitrate is the least preferred nitrogen source for marine bacteria (15) and eukaryotic phytoplankton (16), although concentrations of nitrate are much higher than other nitrogenous compounds, except dissolved organic nitrogen. Energetic considerations may be particularly relevant in understanding growth of marine bacteria in seawater with very low concentrations of organic compounds. Often the growth rate of natural bacterioplankton is limited by organic carbon (17,18), which supplies both the carbon and energy needed by heterotrophic bacteria.

Ecological and Biogeochemical Considerations. Ammonium and nitrate are the two largest nitrogen sources supporting growth of marine primary producers (cyanobacteria and eukaryotic phytoplankton), but these compounds also supply much nitrogen for heterotrophic growth. Heterotrophic bacteria can account for as much as 50% of the total uptake of both ammonium and nitrate in the oceans (15). In addition to helping us understand biological production in marine systems, the uptake of ammonium and nitrate by heterotrophic bacteria has several important ecological and biogeochemical implications.

First, uptake of these nitrogen sources by bacteria and other small microbes helps to shape the size structure of the phytoplankton community in the oceans. The large surface area to volume ratio of bacteria and other microbes gives them a competitive advantage in using low concentrations of dissolved compounds. This competitive advantage explains the abundance of small cells in oligotrophic oceans and their success in using ammonium (19), forcing large phytoplankton to use an energetically less favorable nitrogen source, nitrate. Heterotrophic bacteria cannot truly compete with phytoplankton for inorganic nitrogen because phytoplankton provides the organic carbon needed by these heterotrophs. Because low availability of nitrogen often limits primary production and phytoplankton growth in many oceanic regimes, a superior heterotrophic competitor would eventually lead to a decrease in primary production and ultimately less organic carbon. The decrease in organic carbon would inhibit growth of the heterotrophic bacterium and lower its use of inorganic nitrogen.

Nitrate and ammonium fluxes are used in carbon and nitrogen budgets and for examining fluxes of both elements out of the upper layer of oceanic regimes. Uptake of nitrate is used to examine the influx of new nitrogen and the export of both carbon and nitrogen from the surface oceans (20), as illustrated in Figure 3. Nitrate uptake is used to estimate "new production" (21) because, if other external nitrogen sources (e.g., N_2 fixation and terrestrial runoff) are ignored, nitrate is the only nitrogen compound imported into the upper oceanic layer and thus is "new." Because ammonium, on the other hand, comes only from grazing activity and bacterial mineralization, uptake of it represents "regenerated production," if nitrification in the euphotic zone is negligible. Uptake of either nitrate or ammonium by heterotrophic bacteria may not necessarily change estimates of new or regenerated production, but it certainly complicates these concepts (15).

Phosphorus

Nitrogen is generally thought to be the limiting element in the oceans, but there are now several reports of phosphorus limitation, and the North Pacific gyre may be switching from nitrogen to phosphorus limitation (22). Because evidence of phosphorus limitation in the oceans is quite new, phosphate uptake has not been examined

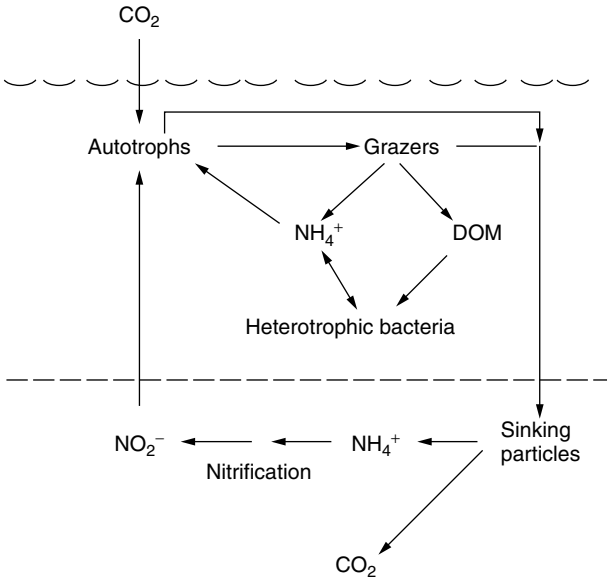

Figure 3. A schematic diagram to illustrate "new production" (nitrate uptake) and its link to carbon dioxide drawn down from the atmosphere and export of carbon below the euphotic zone (represented here by the dashed line, about 100 m deep).

by marine microbial ecologists as extensively as pathways of the nitrogen cycle. Furthermore, most of the work on phosphorus use by bacteria has focused on the uptake of organic phosphorus compounds, although orthophosphate uptake may account for much of the phosphorus needs of heterotrophic bacteria in the oceans (23). One indication of the importance of phosphate uptake by heterotrophic bacteria is that these microbes appear to account for over 50% of total phosphate uptake, on average, in marine systems (24). Phosphate undoubtedly supplies much phosphorus for both heterotrophic and autotrophic bacteria in the oceans.

The impact of phosphate on organic phosphorus use may be unusual in marine bacteria. Alkaline phosphatase, which releases phosphate from organic phosphorus compounds, is usually inhibited by the presence of phosphate in bacterial cultures and in freshwater. Natural marine bacterioplankton apparently have another phosphatase, a 5′-nucleotidase that is not inhibited by phosphate (25). With this enzyme, bacteria could continue to use organic phosphate even in the presence of orthophosphate. This potentially novel regulation may be an adaptation to low phosphorus concentrations of the marine environments. Even if phosphorus does not limit biological activity in the oceans, concentrations of orthophosphate and labile organic phosphorus compounds are very low, often undetectable in surface waters. There may be no need for regulation of phosphatases by phosphate if concentrations are never high.

Phosphorus Content of Marine Bacteria. In addition to the high uptake of phosphate by bacteria, another ecologically-relevant observation is that cellular phosphorus is higher in bacteria than in eukaryotic phytoplankton larger than 1 μm. The measure of phosphorus content commonly used by freshwater ecologists and oceanographers is the C : P ratio. This ratio for phytoplankton is classically set at the Redfield ratio; that is, C : P is equal to 106. In contrast, bacteria have much more phosphorus and consequently, the C : P ratio is much lower, as low as 50 (15). Cell size explains the difference in C : P ratios.

The diameter of an average heterotrophic bacterium in the oceans is about 0.5 μm, significantly smaller than phytoplankton, including cyanobacteria, which are usually 0.8 μm or bigger. Consequently, the surface area to volume ratio is much larger and thus the amount of membrane per cytoplasmic material is higher for heterotrophic bacteria than for phytoplankton. Membranes are phosphorus-rich because of phospholipids, whereas proteins with little phosphorus dominate the cytoplasm; some enzymes are regulated by phosphorylation. The net result is a higher P : C ratio for heterotrophic bacteria than for phytoplankton.

It is also important to note that the C : P ratio for bacteria varies greatly, by as much as 60-fold, compared with a fourfold variation in C : N ratios (9,15). This difference is probably due to changes in the biochemical composition of bacteria as a function of growth rates (15) as illustrated in Table 2. The amount of phosphorus-rich ribosomal ribonucleic acid (RNA) increases with growth rate in both marine and nonmarine bacteria (26). Growth rates also affect cell size and thus the relative amounts of phosphorus-rich membranes. Likewise, the amount of DNA per cellular carbon varies with growth-related changes in cell size, even if genome size (DNA per cell) is constant (Table 2). The net result is large changes in the phosphorus content of bacteria with different growth conditions. In contrast, the carbon and nitrogen content of bacterial cells is largely set by the largest cellular constituent, protein, which is an approximately constant 60% of bacterial dry weight, regardless of growth rate.

Bacteria are often said to be more nitrogen-rich than phytoplankton, but the difference is relatively small compared with the large difference in C : P ratios. The Redfield ratio for phytoplankton is 6.6, whereas the most recent estimates of C : N ratios for natural marine bacterioplankton average 6.8 ± 1.2 and 5.8 ± 1.1 for coastal and oceanic samples, respectively (27). Lab studies have found similar ratios (28), although a C : N ratio as low as 4.5 for a bacterial culture has been reported (12). Recently, Goldman and Dennett (9) found C : N ratios as high as 13 for slowly growing marine bacteria in nitrogen-limited continuous cultures. If these laboratory experiments can be applied to marine bacterial assemblages, field studies showing that bacteria have C : N ratios of Redfield or lower argue against nitrogen limitation and are consistent with the hypothesis that growth of these microbes is carbon-limited.

Sulfur

The high concentrations of sulfate in seawater ensure that sulfur never limits the growth of bacteria or phytoplankton in marine systems, with the potential exception of anoxic habitats where sulfate reduction can deplete sulfate to low levels. Even when sulfate concentrations are high, the energetic cost of assimilatory sulfate reduction presumably exacerbates the difficulties of heterotrophic

Table 2. Biochemical Composition of a Bacterium Growing Fast (Generation Times of One Hour or Less) and Slow (Day Timescale)

	% of Dry Weight		Source of Data for
	Fast[a]	Slow	Slow-Growing Cells
Protein	55.0	55	Simon and Azam (120)
RNA	20.0	13.7	Calculated by difference[b]
Lipids	9.0	12.0	Same lipid/SA ratio[c]
Lipopolysaccharides (LPS)	3.4	3.3	Watson and coworkers (121)
Cell wall (peptidoglycan)	2.5	4.1	Same PG/LPS ratio
C Storage (glycogen)	2.5	0.0	Assume C limitation
DNA	3.0	10.0	Fuhrman and Azam (23)
Monomers	4.0	2.1	Set arbitrarily

[a] Cell composition for a bacterium growing rapidly in the lab (122).
[b] Amount of RNA was calculated by subtracting the sum of all other amounts from the total estimated dry weight for a slow-growing natural bacterium.
[c] I assumed that the ratio of lipid or peptidoglycan (PG) per surface area (SA) measured for fast-growing bacteria in the lab can be applied to slow-growing natural bacteria. We do not know lipid or peptidoglycan amounts of natural bacteria.
Note: The dry weight of a slow-growing cell was estimated from the carbon content (12 Fgc/Cell) measured by Fukuda and coworkers (27) and assuming that carbon is 50% of dry weight.
Source: Taken from D. L. Kirchman, in D. L. Kirchman, ed., *Microbial Ecology of the Oceans*, Wiley-Liss, New York, 2000, pp. 261–288.

bacteria living on low concentrations of organic compounds in seawater. Indeed, marine bacteria prefer to use sulfur amino acids rather than sulfate, because sulfur amino acids inhibit sulfate uptake (29,30). Recently, Kiene and coworkers (31) suggested that marine heterotrophic bacteria obtained much of their sulfur from two nonamino acid sulfur compounds, dimethylsulfoniopropionate (DMSP) and methanethiol. DMSP may be particularly important in providing sulfur and some carbon for heterotrophic bacterial growth (32), which has consequences beyond understanding marine bacterial ecology, because dimethylsulfide (DMS) is released during DMSP utilization (33). Oceanic DMS is a major source of reduced sulfur in the atmosphere and acts as cloud nucleation sites (34).

Silicon

Silicon occurs as silicate (SiO_4^{4-}) in the oceans and is considered a major nutrient because fairly high concentrations of it are required for growth by selected eukaryotic phytoplankton and protozoa. Diatoms, which are algae and the most abundant and ecologically important silicon-containing organisms, require approximately equal amounts of silicon and nitrogen on a mole basis. Low silicon concentrations can limit diatom growth, especially following spring plankton blooms, which diatoms often dominate. A few rarer eukaryotes (e.g., the protozoa Radiolarians and silicoflagellates) in the plankton require silicate, but it is not required by other phytoplankton, nor by any prokaryotic group, including cyanobacteria. Heterotrophic bacteria are involved in mineralization of silicate during degradation of diatoms (35).

TRACE ELEMENTS AS ENZYME COFACTORS

Several key enzymes in microbial metabolism require metallic cofactors. None of these trace metals are needed in high concentrations, and it is common to grow marine bacteria in simple media without the addition of these metals because they are present as contaminants in other media constituents. Although high amounts are not needed, dissolved concentrations of these trace metals are very low in the oceans, low enough to affect (if not limit) the metabolism of all marine microbes. Marine microbiologists and oceanographers have examined the impact of Fe the most. Growth of autotrophic cyanobacteria (*Synechococcus* and *Prochlorococcus*) and eukaryotic phytoplankton can be limited by iron, but heterotrophic bacteria are generally not thought to be limited directly by these elements, as discussed in the following paragraphs.

Iron

Marine bacteria live in some of the most iron-poor environments in the biosphere, the open oceans in which iron concentrations in the surface layer are often below detection limits, that is, less than 50 pM (10^{-12} M). In general, bacteria do not require high amounts of iron, and in fact, a few nonmarine bacteria appear not to have any nutritional need for iron; examples include the soil bacterium *Lactobacillus plantarum* (36) and the Lyme disease pathogen *Borrelia burgdorferi* (37). However, iron has been shown to be essential for the growth of all marine bacteria isolated so far into pure culture. In fact, lab and field data indicate that marine cyanobacteria and heterotrophic bacteria contain as much iron, if not more, as other microbes (38,39).

Cellular Requirements for Iron. Table 3 summarizes the dominant iron-containing enzymes found in heterotrophic bacteria. Most of the iron in marine bacteria and other microbes is found in the electron transfer chains of photophosphorylation (phototrophic

Table 3. Common Microbial Enzymes with Iron

Pathway	Enzyme	Fe per Cell (10^{-20} mol)	% of Total Cellular Fe
Electron transfer chain	NADH dehydrogenase	89.4	57.8
	Cytochrome b_1	5.2	3.4
	Cytochrome oxidase	3.8	2.5
Kreb's cycle	Aconitase	1.1	0.7
	Succinate dehydrogenase	46.7	30.2
	Total[a]	155	100
		Fe per enzyme	
Others	Superoxide dismutase	1	5.3
	Catalase	1	0.1
	Nitrogenase[b]	30	
	Nitrate reductase[c]	4	
	Nitrite reductase[c]	1	
	Hydrogenases[d]	2 or 6	

[a]Includes superoxide dismutase and catalase with 8.2 and 0.2×10^{-20} mol iron per cell, respectively.
[b]Component I of nitrogenase in *Azotobacter vinelandi* (71). Component II has only four iron.
[c]Assimilatory nitrate and nitrite reductases (123).
[d]Catalytic sites of iron-only hydrogenases have six iron, whereas NiFe-hydrogenases have a binuclear NiFe center. See papers cited in reference (124).
Note: The percentages were forced to add to 100% and the data cannot rule out the presence of other iron-containing components.
Source: Data from P. D. Tortell, M. T. Maldonado, J. Granger, and N. M. Price, *FEMS Microbiol. Ecol.* **29**, 1–11, (1999) unless indicated otherwise.

cyanobacteria and eukaryotic algae) and oxidative phosphorylation (heterotrophic bacteria) (40). Other iron-containing enzymes include assimilatory nitrate reductase and nitrogenase. Low iron is one reason that nitrate builds up in some oceanic regimes (discussed in the following sections). Heterotrophic bacteria probably use nitrate only as a last resort because they are limited by organic carbon, not iron, but nitrate use by phytoplankton is thought to be limited by iron because of the requirement of nitrate reductase for iron.

Low iron concentrations in marine habitats may select microbes that can decrease their requirement for iron by switching to enzymes that do not require iron. Examples include the switch from ferrodoxin to flavodoxin in phototrophs, which has been demonstrated in both lab and field experiments (41). Ferrodoxin is an iron-sulfur protein in the electron transfer chain of photophosphorylation. An example from heterotrophic bacteria is superoxide dismutase, a ubiquitous enzyme in oxic environments. The nonmarine bacterium, *Streptococcus sui*, has managed to replace iron in superoxide dismutase with manganese (42).

Iron concentrations are probably too low, even in waters where production is not limited by iron, to enable marine bacteria to store iron (43); but iron storage by marine microbes has not been examined.

Iron Acquisition by Marine Bacteria. Iron is present mainly as Fe (III) in most natural environments, including seawater. Although inorganic Fe (III) is quite insoluble, the oceans do contain very low concentrations of dissolved iron (the iron passes a 0.2 μm-pore-size filter) that is complexed with soluble organic chelators (44,45). How bacteria in the oceans acquire this complexed iron is not completely understood. Extensive work on nonmarine bacteria has demonstrated that bacteria can acquire iron by excreting siderophores, which are low molecular weight organic compounds (often peptides) that bind to Fe (III) with a high affinity. Eukaryotic algae do not synthesize siderophores, although they can acquire iron complexed to siderophores (46). Siderophores may be excreted and reacquired by attached bacteria because bacterial densities can be high on organic detritus and other surfaces, and the distance between sibling cells is likely to be small. Under these conditions, excreting siderophores probably benefits, if not the original cell that synthesized the siderophore, a daughter cell or another cell of the same species. But attached bacteria are not representative of all marine bacteria. Unattached bacteria, which usually dominate marine waters, live in a very different environment and may use a different mechanism for acquiring iron.

Microbial ecologists are faced with a dilemma in trying to understand iron acquisition by free-living bacteria. On the one hand, several pieces of evidence indicate that marine bacteria do acquire iron by excreting siderophores. On the other hand, this strategy does not make ecological sense. Unlike detritus-associated bacteria, unattached bacteria live in a nutritionally dilute, but species-rich environment. The number of different bacterial "species" in the oceans is not known, but may be as high as 1,000, each separated by about 60 μm, if the abundance of free-living bacteria is about 10^6 mL^{-1}. In this dilute but diverse world, a siderophore (or an extracellular enzyme) released by a bacterium is likely to be encountered and used by another microbe from a different phylogenetic group rather than the original bacterium or its siblings. Not surprisingly, marine bacteria are able to use iron complexed to siderophores produced by other species (47). In addition to iron piracy, siderophores may be used as

carbon and nitrogen sources by other microbes. Thus, excretion of siderophore by free-living microbes seems futile.

But siderophore-like compounds have been found in the dissolved organic matter pool in seawater (48), as if these compounds had been excreted by bacteria. In addition to structural similarities, dissolved iron organic ligands in seawater have stability constants similar to bona fide siderophores (49). Further, both cultured bacteria and natural microbial assemblages rapidly use siderophore-complexed iron (50,51); the existence of an adaptation to use this complexed iron is one argument for the existence of excreted siderophores. At least for those microbes cultured and studied in the laboratory, excretion of siderophores is the only iron acquisition mechanism known for marine bacteria (51–55). Finally, our ecological intuition about the futility of siderophore excretion may be faulty. Völker and Wolf-Gladrow (56) used a theoretical model to argue that the benefits of siderophore excretion can outweigh the costs under some conditions. These data and model results indicate that free-living bacteria do acquire iron by excreting siderophores.

There are counterarguments, however. First, the current model of Völker and Wolf-Gladrow (56) may not be totally applicable to real microbial assemblages. One problem is that heterotrophic bacteria were not examined explicitly by Völker and Wolf-Gladrow. The diversity, size, and abundance levels of various bacterial groups differ from that of the phytoplankton considered by Völker and Wolf-Gladrow. Also, the cost of siderophore excretion is probably higher for heterotrophic bacteria, because these microbes are often limited by organic carbon, whereas cyanobacteria are not. In their analysis of a problem analogous to siderophore excretion, Vetter and coworkers (57) pointed out that excretion of enzymes to hydrolyze biopolymers was energetically favorable only for attached bacteria.

Questions on the applicability of laboratory experiments to real oceans can also be raised. Perhaps the iron acquisition system used by cultured bacteria is not representative of bacteria found in the oceans. It is well known that cultured heterotrophic bacteria are quite different from those found in the oceans by culture-independent approaches (58). Several of the dominant cyanobacteria of the oceans have been brought into culture, so it is difficult to dismiss laboratory studies demonstrating siderophore excretion by these microbes (59). Incidently, siderophore production has not been found for a few marine bacteria (51), leaving open the possibility of alternative iron-acquisition systems for even cultured bacteria.

Cell-associated siderophores would seem more effective for free-living bacteria than excretion of siderophores, again analogous to cell-associated enzymes. Siderophores could become "dissolved" by viral lysis or by grazers egesting partially-digested bacteria, similar to the pathway for many other components of the dissolved organic pool (60). To date, a cell-associated siderophore-like complex has not been demonstrated for any bacterium, but one siderophore from a marine bacterium has some properties that are likely to be found in a cell-associated iron chelator. Martinez and coworkers (61) presented the structure of two classes of siderophores produced by two isolated marine bacteria, *Halomonas aquamarina* and *Marinobacter* sp., both members of the gamma subdivision of proteobacteria. What makes these siderophores interesting is their fatty acid moieties (Fig. 4), which potentially could anchor the siderophore into a cell membrane. The siderophores found by Martinez and coworkers, however, are excreted. The few siderophores from the other marine bacteria examined so far (54,55) are not like marinobactin and aquachelin (61).

Oceanographic and Ecological Importance of Iron. Several oceanic regimes have relatively high concentrations of the major nutrients (nitrate, phosphate, and silicate), yet algal biomass is low as measured by chlorophyll concentrations. These high nutrient, low chlorophyll (HNLC) oceans include the subarctic Pacific, the equatorial Pacific and the Southern Ocean. The Southern Ocean is especially important in global change models and in attempts to understand climate in the geologic past. John Martin (62) originally hypothesized that increases in iron fluxes to the Southern Ocean led to higher primary production and lower atmospheric CO_2 during the last glacial maximum. This hypothesis has generally been supported by other data, but a recent study questions it (63). Upwelling regions, such as off the coast of California, are also iron-limited at times (64).

Bottle and in situ fertilization experiments have shown that growth and standing stocks of large phytoplankton, most notably diatoms, are greatly enhanced by the addition of iron (65). Growth rates of autotrophic cyanobacteria

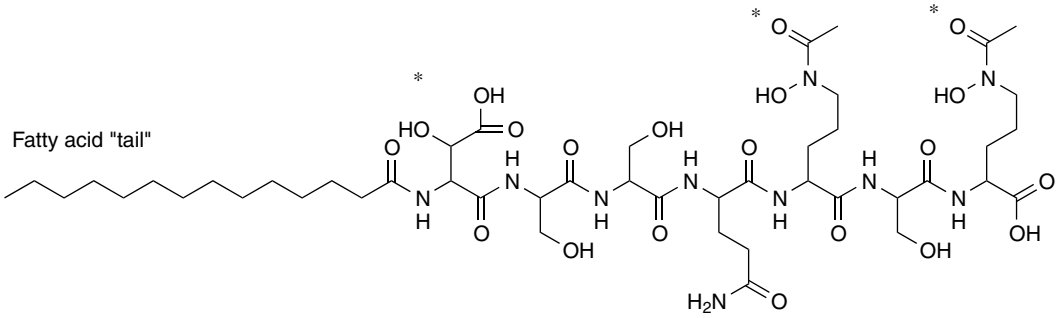

Figure 4. Structure of aquachelin D, a siderophore from the marine bacterium, Halomonas aquamarina. The asterisk indicates the probable iron-binding sites. (Taken from J. S. Martinez et al., *Science* **287**, 1,245–1,247 (2000)).

(*Synechococcus* and *Prochlorococcus*) are also limited by iron (66) but biomass levels do not change greatly following iron additions (67,68), unlike the large phytoplankton response (65). The lack of change in cyanobacteria and other picoplankton is because grazing rates by protist grazers and zooplankton increase and thus prevent large changes in biomass levels of their prey.

Nitrogen fixation, a process carried out exclusively by prokaryotes, specifically cyanobacteria in the oceans (69), is another process that may be limited by iron (70). Component I of nitrogenase, the key enzyme in dinitrogen fixation, alone requires 30 moles of iron (71). Raven (72) suggested that nitrogen-fixing cyanobacteria require orders of magnitude more iron for photosynthesis than phytoplankton, relying on ammonium. However, Sanudo-Wilhelmy and coworkers (73) found a mathematical error in those calculations. Their recalculations suggest much lower iron requirements for nitrogen-fixing cyanobacteria than originally reported by Raven, although the theoretical iron efficiency of photosynthesis based on nitrogen fixation is still 2.5- to 5-fold lower than ammonium-based photosynthesis. More importantly, the experimental evidence of Sanudo-Wilhelmy and coworkers (73) indicates that nitrogen fixation in the Sargasso Sea is limited by low concentrations of phosphate, not iron. Regardless, rates of oceanic nitrogen fixation are currently being revised and may be higher than previously believed.

In contrast to the impact of iron on phytoplankton growth, marine heterotrophic bacteria do not appear limited by iron (17,74), although this may not always be the case (75). In the HNLC oceans and in the California upwelling, growth rates of heterotrophic bacteria are limited by organic carbon (17,74,76). Perhaps heterotrophic bacteria are iron-stressed (Fig. 5), because addition of organic carbon along with iron occasionally stimulated growth more than just organic carbon additions alone (17,74). Heterotrophic bacteria appear to compete successfully for iron and can account for a large fraction of iron uptake (38,77). In contrast to the numerous studies on phytoplankton, few studies have examined iron-heterotroph interactions.

Other Trace Elements

Many trace elements in addition to iron have "nutrient-like" depth profiles in the oceans (Fig. 6), consisting of relatively high concentrations below the euphotic zone and low concentrations in the euphotic zone, where biological activity is highest because of light-driven primary production. Also, similar to iron, the concentrations of these other trace elements in surface waters are extremely low (Table 4), and many of these elements are found in organic complexes that impact their availability to the biota (Table 1). Microbes and other marine organisms may still require an element not having a nutrient-like depth profile. One example is manganese (discussed later).

Molybdenum

Although the regulation of nitrogen fixation may differ among organisms, the nitrogen fixation apparatus itself

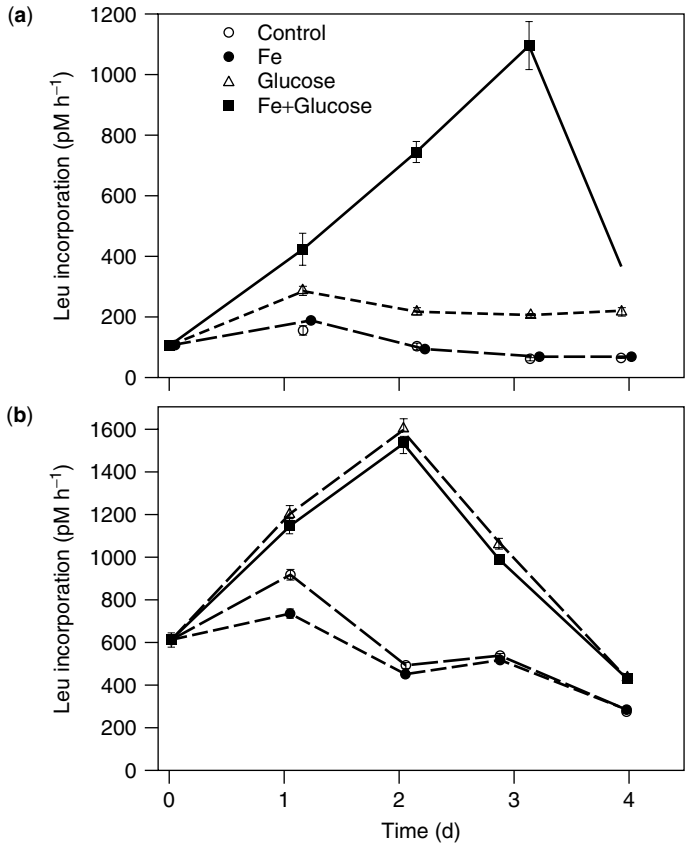

Figure 5. Examples of the effect of organic carbon and iron on bacterial growth as measured by leucine incorporation. Experiment conducted at a station with high dissolved iron concentrations (**a**); experiment conducted at a station with low dissolved iron concentrations (**b**). (Data taken from D. L. Kirchman et al., *Limnol. Oceanogr.* **45**, 1,681–1,688 (2000)).

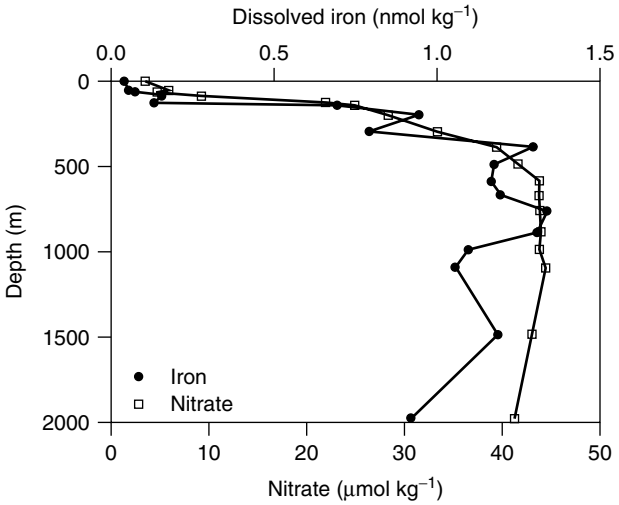

Figure 6. Depth profile of iron and nitrate in the northeast Pacific. The concentration units of μmoles and nmoles per kg are approximately equivalent to μM and nM, respectively. (Data taken from J. H. Martin and R. M. Gordon, *Deep-Sea Res. I* **35**, 177–196 (1988)).

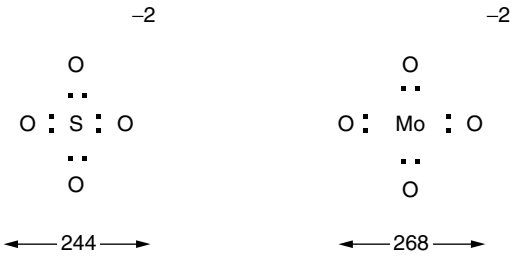

Figure 7. Lewis dot diagram illustrating the similarity of SO_4^{2-} and MoO_4^{2-}. The other six outer shell electrons for the oxygens were omitted for clarity. The units for the molecular dimensions are 10^{-12} m (George Luther, unpublished data).

is quite similar among all nitrogen fixers, ranging from *Trichodesmium* spp. in oligotrophic oceans to the symbiotic bacterium, *Rhizobium* sp., in organic-rich soils. However, marine nitrogen fixers face the unique problem of obtaining molybdenum, which component I of nitrogenase requires, in the presence of high sulfate concentrations. Molybdenum occurs as molybdate (MoO_4^{2-}) in surface oceanic waters, which is quite similar in charge and size to sulfate (Fig. 7).

Howarth and coworkers (78) first suggested that high sulfate concentrations sterically hinder molybdenum uptake and cause molybdenum limitation of dinitrogen fixation, although cyanobacteria require only low levels of molybdenum. Subsequent work demonstrated that Molybdenum transport was indeed inhibited by sulfate (79) and that statistical models for explaining cyanobacterial abundance in saline lakes were improved by including data on molybdenum concentrations (80). But additional experiments have failed to show any evidence of molybdenum limitation in the oceans (81,82). One reason for marine nitrogen fixation not being limited by molybdenum is the prevalence of "alternative nitrogenases" that have vanadium (V) instead of molybdenum (83,69).

Zinc

Depth profiles of zinc in the oceans are similar to the major nutrients because zinc is used as a cofactor by several enzymes in planktonic organisms. Of the many enzymes that use zinc, two are especially relevant for a review of marine microbes.

Morel and colleagues have been examining the impact of zinc and other metals on carbonic anhydrase in marine phytoplankton (84–86). Carbonic anhydrase is a widespread enzyme that catalyzes the reaction

$$H^+ + HCO_3^- = CO_2 + H_2O \qquad (4)$$

This reaction does occur spontaneously but at rates too slow for many cellular functions. The role of carbonic anhydrase in carbon fixation by terrestrial C4 plants is well known, but it is also important in marine phytoplankton. Because the dominant form of inorganic carbon is bicarbonate (HCO_3^-) at the pH of seawater, phytoplankton have evolved transport

Table 4. Concentrations of Some Trace Metals in Surface and Deep Waters of the Subtropical Oceanic Gyres (Units, nM)

Element	North Atlantic Gyre		North Pacific Gyre	
	Surface Water	Deep Water	Surface Water	Deep Water
"Nutrient-like"				
Cd	0.0020	0.29	0.0015	0.87
Cu	1–1.5	2.0	0.5	4.0
Ni	2.3	6.0	2.1	10
Zn	0.06	1.7	0.07	8.5
Conservative				
Pb	0.17	0.025	0.075	0.005
Mn	2.4	0.6	1.0	0.2

Note: See figure 6 for an example of "nutrient-like" depth profiles of elements in the oceans. Conservative elements are those with concentrations that covary with NaCl and are not appreciable used by microbes.

Source: Data from R. Chester, *Marine Geochemistry*, Unwin Hyman, London, U.K., 1990.

mechanisms of bicarbonate (HCO_3^-) (87), necessitating intracellular conversion of it by carbonic anhydrase to carbon dioxide, the substrate for the carbon dioxide fixing enzyme, ribulose bisphosphate carboxylase. Low zinc concentrations may lead to changes in carbonic anhydrase activity and thus carbon dioxide fixation patterns that impact fractionation of ^{12}C versus ^{13}C (84) and perhaps even the success of various phytoplankton groups (88). Data on $^{12}C/^{13}C$ ratios are useful in food web studies, oceanography, and paleoceanography.

Recently, Lane and Morel (86) showed that when grown under zinc limitation and low carbon dioxide partial pressure, the marine diatom *Thalassiosira weissflogii* produces another form of carbonic anhydrase that does not have zinc, but requires cadmium instead. Previously, it was thought that cadmium did not have any biological roles, although it was known that cadmium concentrations follow a nutrient-like depth profile.

It is not as well known that carbonic anhydrase is also found in heterotrophic bacteria (89). In *Escherichia coli*, carbonic anhydrase is part of the cyn operon that encodes the enzyme cyanase and catalyzes the following reaction (90):

$$NCO^- + 3H^+ + HCO_3^- = 2CO_2 + NH_4^+ \qquad (5)$$

Carbonic anhydrase facilitates degradation of cyanate (NCO^-) by maintaining high intracellular concentrations of HCO_3^- via the reverse of Reaction 4 given in the preceding section. Carbonic anhydrase also appears to have a role in regulating intracellular pH in a heterotrophic marine bacterium in which the enzyme is induced by high carbon dioxide partial pressure (91). The impact of low zinc concentrations on carbonic anhydrase in heterotrophic bacteria has not been examined.

The other zinc enzyme of interest to marine microbiologists is the ectoenzyme leucine aminopeptidase. Fukuda and coworkers (92) compared the activity of leucine aminopeptidase with the activity of another ectoenzyme, β-glucosidase, which does not require zinc. They found that the activity of leucine aminopeptidase, relative to β-glucosidase, correlated with zinc concentrations in depth profiles and along an east-west gradient in the North Pacific Ocean (92). Although overall growth of heterotrophic bacteria may not be controlled by zinc or other trace elements (see section on iron), the study of Fukuda and coworkers raises the possibility that degradation of organic matter may be impacted by trace elements such as zinc.

Nickel

Five microbial enzymes are known to require nickel (93): urease, hydrogenase, methyl coenzyme M reductase, carbon monoxide dehydrogenase, and one class of superoxide dismutase. Of these, urease is perhaps best known and most important to marine microbes in the surface ocean. Urea is commonly excreted by zooplankton and sometimes bacteria (94,95), and it can be an important nitrogen source for phytoplankton growth (96,97). In laboratory cultures, nickel limitation prevents urea from being used by phytoplankton as a nitrogen source (98). Palenik and coworkers (99) showed that use of another nitrogen source (hypoxanthine) by eukaryotic phytoplankton was also affected by nickel limitation, suggesting a role for urease in degrading this nitrogenous compound, although perhaps some other unknown enzyme required for hypoxanthine degradation has a nickel cofactor.

Copper

Copper is a necessary metal for several enzymes found in microbes but it can also be toxic in high concentrations. The bacterial enzymes known to require copper include superoxide dismutase (100), methane monooxygenase (101) that participates in methane oxidation, dissimilatory nitrite reductase (102), and amine oxidases (103). A more common use of copper, however, is in the oxidative phosphorylation electron transport chain of heterotrophic bacteria and other cells. The requirement for copper by these enzymes has not been examined in marine microbes, except for a superoxide dismutase in a marine yeast (104).

More work has been done on copper toxicity of both heterotrophic (105) and autotrophic microbes (106). Moffett and coworkers (107) suggested that the relatively high copper concentrations characteristic of estuarine and coastal water explain why the marine cyanobacterium *Synechococcus* is more abundant in open oceanic regimes.

Tungsten and Other Metals in Marine Hyperthermophiles

Some of the most unique environments in the biosphere are hydrothermal vents that are found at tectonic spreading zones in the ocean floor. Hydrothermal vents release many reduced metallic and other inorganic compounds, including sulfides used by chemolithotrophic bacteria, the primary producers in these environments. Chemolithotrophic production supports diverse communities of bacteria, archaea, and eukaryae in and near hydrothermal vents. Some of the most interesting organisms are the hyperthermophiles, those bacteria and archaea able to live in exceptionally high temperatures (>60 °C). These organisms are interesting for several reasons, one being their role in understanding the evolution of early life.

A hyperthermophile may have been among the first life-forms on the planet. Some of the oldest microbial fossils are from a Precambrian submarine thermal spring system (108), and thermophilic bacteria and archaea are the most deeply branched groups in 16S rRNA phylogenetic trees (109), implying an ancient origin. The metals found in selected enzymes from these microbes are unusual, and the enzymes as well as the bacteria provide several interesting test cases about the evolution of early life.

Adams and colleagues have been examining tungsten (W)-containing enzymes in marine hyperthermophilic archaea (*Pyrococcus furiosus*) originally isolated from hydrothermal vents (110,111). Some examples of enzymes with tungsten include formate dehydrogenase, formyl methanofuran dehydrogenase, and acetylene hydratase (112). These tungsten-dependent enzymes may model ancestral forms of the now more common molybdate-containing enzymes. Tungsten and molybdenum share several chemical properties because both

are in the chromium group (Group 6) of transition elements. Tungsten is also used by moderate thermophiles; but when grown without tungsten, these organisms produce analogous molybdoenzymes (113). Tungsten may be more readily stabilized than molybdenum because of the higher first ionization energy of tungsten (114). This stability is necessary for enzymes operating in the high temperatures of hydrothermal vents (113). As life moved away from hydrothermal vents and ready sources of tungsten, molybdenum may have taken its place during evolution in low temperature environments.

SODIUM, CHLORINE, POTASSIUM, AND CALCIUM: OSMOTIC BALANCE AND SALT BRIDGES

In concluding this review, it is appropriate to comment on some of the most common elements required by marine microbes, although they are not nutrients in the same sense as the compounds discussed earlier. The following elements include the most abundant ones in seawater: Na^+, Cl^-, K^+ and the divalent cations, Ca^{+2} and Mg^{+2}. These are not trace elements, of course, as the concentration of NaCl, for example, is 0.6 M in seawater. They are neither used as enzyme cofactors, nor in the synthesis of macromolecules. Magnesium (Mg^{2+}) is needed for the proper function of several enzymes (e.g., DNA polymerases) and Ca^{+2} helps to stabilize polymers, but these roles are important in all organisms. Calcium is used, however, in a unique way that is restricted to one group of marine microbes. A group of eukaryotic marine phytoplankton, coccolithophorids, uses Ca^{+2} and CO_3^{-2} to build unusual cell wall structures (coccoliths). Formation and subsequent sinking of these coccoliths contribute to the biological pump and the export of carbon from the upper layer to the deep oceans, one mechanism by which the oceans influence atmospheric carbon dioxide concentrations and climate change. Once preserved in sediments, coccoliths become a marker for deciphering primary production in the geologic past. Other than coccolithophorids, however, marine and nonmarine microbes have very similar uses for the common ions found in seawater.

But a defining feature of marine microbes is their capacity to grow in saltwater. Although bacteria had been isolated from seawater since before Zobell (1946), their great similarity to nonmarine strains raised doubts of whether there were any bacteria unique to oceanic environments (115). The growth of a microbe in seawater, per se, is insufficient evidence to conclude that it is truly marine; even *E. coli* grows in otherwise sterile seawater (116). Unlike other microbes, however, marine bacteria lyse when salt concentrations are dropped below seawater concentrations, although some strains can adapt and grow slowly in as low as one-tenth oceanic concentrations (115). In some strains, NaCl is required and cannot be replaced by Li^+ or Br^+ (115). In any case, the requirement for these salts was used to demonstrate that truly "marine" bacteria existed and that bacteria found in the oceans had not just washed in from land or freshwater.

CONCLUSION

The similarities in the inorganic nutrient needs of marine and nonmarine microbes are more striking than the differences. Both groups of microbes require and are composed of the same elements. On the basis of the few studies with relevant data, the mechanisms of uptake are similar for all microbes. What may differ are the kinetic parameters and regulation of the acquisition mechanisms. Unlike laboratory cultures and many nonmarine environments, concentrations of nearly all inorganic nutrients and organic compounds are very low in the oceans. Even the most productive regions have pico- to micromolar concentrations of key inorganic nutrients and organic substrates. These low concentrations and the complexity of the oceanic chemical environment may select for suites of regulatory mechanisms that differ from those observed to date in laboratory-reared microbes. More studies of marine bacteria growing on the vanishing low concentrations found in the open oceans may reveal new uptake mechanisms and regulatory pathways not seen in microbes from other environments.

Marine microbes may also teach us more about the evolution, biochemistry, and physiology of trace elements in biological systems. The extremely low concentrations of elements such as iron and molybdenum probably select microbes with enzymes that replace scarce metals with more abundant ones, for example, manganese for iron in the case of superoxide dismutase. The biochemical role of various metals in enzymatic reactions is also illuminated by comparing tungsten and molybdenum-containing enzymes, for example. Nature has already conducted the experiments to examine the impact of these switches, but now more work is needed to decipher the results.

The study of marine microbes provides more than just new models of regulation pathways or novel insights into biochemical reactions. The various inorganic nutrients discussed here are essential for microbial growth in the oceans. Understanding the acquisition and fate of these nutrients will also help us understand the ecology and biogeochemistry of the basis of oceanic life, the microbes.

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INSECTICIDES, MICROBIAL

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Simply defined, microbial insecticides consist of a single microorganism or several microorganisms or metabolic end product(s) as the active ingredient. Microbial insecticides control a variety of insect pests, although each separate microorganism or by-product is relatively specific for its target pest(s). They may be used to control insects by inducing diseases in target insects, or to suppress the population of insects directly or in combination with chemical insecticides. All types

of microorganisms are represented among the potential microbial control agents.

There are more than 2,000 naturally occurring microorganisms or their products, which hold promise for the control of major insect pests. Microorganisms that affect insects are termed *entomopathogens*. More than 100 species of bacteria and more than 1,000 viruses have been isolated from arthropods and more are being discovered every year. All classes of fungi are represented among the 750 known entomopathogenic fungi. Protozoa are also likely candidates as microbial control agents, because many insects not attacked by other entomopathogens are susceptible to at least one of the 300 known species of entomophilic protozoa.

Among the known microbial species with insecticidal action, the most widely used and appreciated microorganism is the ubiquitous soil bacterium *B. thuringiensis*. In fact, use of microorganisms as biological pest control began with the recognition of *B. thuringiensis* as an entomopathogen (1). *Bacillus thuringiensis* has been used for more than 40 years as a microbial insecticide to control numerous pests and vector insects, which have importance in agriculture, forestry, and medicine, and it is proven to be one of the most effective, safe, and intensely used biological pesticides. There are more than 30 species of microorganisms registered by the U.S. Environmental Protection Agency (EPA) and most of them are subspecies of *B. thuringiensis* carrying different Cry toxin genes. Extensive information regarding the scientific, economic, and regulatory issues relating to *B. thuringiensis* and its toxins is available. There are reviews emphasizing the development of *B. thuringiensis*–based biopesticides and the utility of *B. thuringiensis* toxins in transgenic plants for field crop protection against pest insects (see Additional Reading).

The aim of the this article is to summarize (*1*) the importance of microbes and their toxins as insecticides, with particular emphasis on Cry toxins of *B. thuringiensis* as insecticides, (*2*) recent and emerging insights into understanding Cry toxin receptors and insect responses to toxins, (*3*) mechanisms and effects of resistance to Cry toxins, and (*4*) future trends.

PRODUCTION TECHNOLOGY

Entomopathogens are produced in living insects, in cell/tissue cultures and by fermentation methods. Fermentation technology is used for some bacteria and fungi, whereas, living insects are used for the production of obligatory parasitic viruses and protozoa. Both processes have been successfully used to produce commercial entomopathogenic products. Submerged fermentation, generally, is used for the commercial production of *B. thuringiensis* and *B. sphaericus* and the fungi *Beauveria bassiana* and *Entomophthora virulenta*. Surface fermentation is employed to produce pathogenic fungi such as *Nomuraea rileyi* and *Metarrhizium anisopiliae*, and a combination of both surface and submerged techniques can be used for *B. bassiana* and *Hirsutella thompsonii*. Living insects are used almost exclusively as substrates for the production of the respective nucleopolyhedrosis viruses of *Heliothis zea*, *Porthetria dispar*, and *Hemerocampa pseudosugata*. Recent developments in cell and tissue culture methods have made their use more practicable and efficient for mass production of a number of entomogenous viruses. A book on the formulation of microbial pesticides has been written (2).

SAFETY AND SPECIFICITY

Entomopathogens are infectious, replicating living organisms that are a natural part of our environment. Evidence that microbial insecticides pose little human or environmental hazard has been demonstrated by laboratory animal testing data developed to support federal pesticide registration. Nevertheless, safety cannot be absolutely guaranteed for all entomopathogens in every living system, and it is important that potential hazards for new entomopathogens be known before their use. The EPA has listed several advantages of microbial insecticides that include the following: (*1*) usually, inherently less harmful than conventional pesticides, (*2*) generally, affect only the target pest and closely related organisms, in contrast to broad spectrum, conventional pesticides that may affect organisms as different as birds and mammals, (*3*) often, are effective in very small quantities and often decompose quickly, thereby resulting in lower exposures and largely avoiding the pollution problems caused by conventional pesticides, and (*4*) when used as a component of Integrated Pest Management programs, they can greatly decrease the use of conventional pesticides, whereas crop yields remain high.

No deleterious effects at normal field-use rates have been reported in tests with a number of bacteria such as the gram-positive sporeformers *B. thuringiensis*, *B. sphaericus*, *Bacillus subtilis*, *Bacillus popilliae* and *Bacillus lentimorbus*, and the gram-negative nonsporeformers *Agrobacterium radiobacter*, *Streptomyces griseoviridis*, and several species of *Pseudomonas*. Baculoviruses do not appear to replicate in vertebrate embryos or in cell lines derived from birds, fishes, amphibians, and mammals. Although allergens are encountered among the fungi, including *Beauveria*, *Entomophthora*, *Hirsutella*, *Metarrhizium*, and *Nomeraea* species, these fungi are not toxic or infectious to vertebrates. Entomopathogenic protozoa infectious to grasshoppers, for example, *Nosema locustae*, to mosquitoes, for example, *Nosema algera*, and to beetles, for example, *Mattesia trogodermae*, are not infectious to nontarget organisms, including vertebrates.

EFFICACY

Entomopathogens are effective variably against a number of diverse insects including mites, ticks, mosquitoes, black flies, sand flies, grasshoppers, beetles, and moths, among others. Microbial insecticides, like chemical insecticides, usually are sprayed or dusted on agricultural crops, stored products, forest trees, ornamental trees, and shrubs, and in aquatic habitats for mosquito control. Entomopathogens may also be successfully introduced and established in an ecosystem by other application methods to provide

long-term control of pest populations. Examples are (*1*) insects themselves that can be used to disseminate the entomopathogens, (*2*) transgenic insect-resistant plants, and (*3*) microbial materials harboring genes that encode toxic end products, such as the insecticidal proteins of *B. thuringiensis*.

Virus or fungus epizootics might be induced in an insect population before crop-damaging proliferation takes place. It is possible in some instances to manipulate the environment to create conditions in which naturally occurring pathogens exert their greatest effect. Some of the approaches may provide levels of control equal to or better than those currently obtained with chemical insecticides.

The development and use of entomopathogens or their by-products as microbial control agents are rapidly increasing in popularity worldwide among environmentalists, industrialists, scientists, users and consumers, and government policy makers. Unfortunately, their commercial development and use is underexploited.

VIRUSES

More than 1,000 viruses have been isolated from insects. Most of these viruses (>85%) have been isolated from moth and butterfly larvae, which represent some of the most serious economic pests. Insect viruses are grouped into five major categories: (*1*) nucleopolyhedrosis viruses (NPV), (*2*) cytoplasmic polyhedrosis viruses (CPV), (*3*) granulosis viruses (GV), (*4*) entomopox viruses (EPV), and (*5*) noninclusion viruses (NIV). The most promising candidates are the NPV and GV because of their specificity, safety, virulence, and stability. No representative of the CPV, EPV, and NIV have been registered for commercial use by the EPA.

Insect viruses are strict parasites and must be mass-produced in living hosts or cell/tissue cultures. The standard protocol entails rearing the host insect and producing the virus by artificial infection, harvesting the virus, and formulating an effective insecticidal preparation. Obviously, such a process is labor-intensive and costly. However, some recent advances in tissue/cell culture and recombinant DNA technology may allow for the efficient in vitro production of insect viruses, thus reducing the manufacturing costs normally associated with in vivo production.

FUNGI

As is true for the viruses, there are more than 1,000 fungal species, representing at least 100 genera, which have been reported to infect insects. Nearly all major fungal groups are represented, as is virtually every kind of insect. The EPA for commercial use has authorized approximately 15 species. The production of fungi varies from growing them in laboratory media to harvesting the organisms from their natural substrates. In short, fungi can be produced in virtually any locale with technology geared to available facilities and personnel. Because fungi are slow acting, requiring up to 10 days to kill their hosts, attention has been directed toward identifying and purifying metabolites and by-products, which have insecticidal activity. Often, these compounds are quick acting and can be formulated in much the same way as chemical insecticides. Safety considerations, however, are of the utmost concern because many fungal metabolites are carcinogenic and allergenic.

PROTOZOA

Although the majority of protozoa are free-living, many are intimately associated with insects, in relationships ranging from compatible to harmful. Most of the approximately 300 described species of entomophilic protozoa are included in the orders Microsporidia (Subphylum Cnidospora) and Neogregarinida (Subphylum Sporozoa). Microsporidia, particularly common in insects, generally possess the greatest potential for use as control agents. Among the microsporidia, the genus *Nosema* contains most of the promising entomopathogens. Some species are pathogenic to grasshoppers, crickets, mosquitoes, and various caterpillars.

The biggest obstacle to using protozoa as insecticides is that they must be produced in living hosts. The primary hosts are generally used to produce spores, although alternate hosts sometimes can be used. Most protozoa are relatively host-specific, although some have a fairly expansive host range. For example, more than 60 species of grasshoppers and crickets are susceptible to *N. locustae*. Plants, human beings, and animals seem to be resistant to infection by insect protozoa.

Few field studies of protozoa as insecticides have been conducted. Most protozoa are considered more suitable for long-term suppression programs. The slower, debilitative effects of chronic infections, such as reduced fertility and shortened life span, generally preclude their widespread use as quick-acting agents. A comprehensive review of entomogenous protozoa and fungi has been written (3).

BACTERIA

Many bacteria are associated with insects, most of which belong to the families Pseudomonadaceae, Enterobacteriaceae, Lactobacillaceae, Micrococcaceae, and Bacillaceae. Members of these families may be obligate or opportunistic entomopathogens, depending on their host association in nature. Obligate entomopathogens, generally, are fastidious and are restricted to growth in a living host insect. The occasional or opportunistic pathogens are free-living in nature, although they commonly may be found associated with one or more hosts. About 200 bacteria have been reported as entomopathogens, but only a few, including *B. thuringiensis*, *B. popilliae*, *B. lentimorbus*, and *B. sphaericus*, have been closely examined as insect-control agents. These species are sporeformers, the first two produce, in addition to the spore, discrete parasporal crystalline inclusions within the sporulating cell, the last two do not.

Fermentation processes are employed in the production of *B. thuringiensis* and *B. sphaericus* spores, whereas *B. popilliae* and *B. lentimorbus* are produced exclusively in living insects. Two types of fermentation methods are used

for the production of *B. thuringiensis* and *B. sphaericus*, namely, surface or semisolid and submerged. For the in vivo production of *B. popilliae* and *B. lentimorbus*, third instar Japanese beetle (*Popillia japonica*) larvae are infected with spores and incubated in soil seeded with rye to feed the larvae. The infected larvae are harvested 1,621 days after infection. Living insects must be used to propagate these bacteria because artificial culture methods, which will support cost-effective, large-scale sporulation of *B. popilliae* and *B. lentimorbus*, have not yet been developed.

Bacillus thuringiensis, *B. popilliae*, *B. lentimorbus*, and *B. sphaericus* have been subjected to many safety tests, which had no harmful effects on animals or human beings. Furthermore, commercial applications of various formulations of these four bacillus species have been used for insect control worldwide for the past 15 to 30 years without any documented report of adverse effect on humans and animals, nontarget organisms, or the environment. In other words, these particular microbial insecticides have withstood the tests of time and utility.

BACILLUS THURINGIENSIS

Bacillus thuringiensis is a ubiquitous bacterium and has been isolated from a variety of ecological niches including soil, stored grain, insect cadavers, and plant surfaces. It is a spore-forming bacterium characterized by the production of parasporal protein crystals along with the spore. It is likely that production of the crystal proteins during sporulation provides a means for the bacterium to replace water during spore formation and afford a survival advantage by exerting lethal action on target insects that, in turn, can provide sufficient nutrients to allow germination of the dormant spores and their return to vegetative growth. The toxic crystalline proteins are referred to as "Cry toxins" (4) and are of great agronomic importance and scientific interest. Mixtures of *B. thuringiensis* spores and parasporal crystal are used in a variety of formulations as biological insecticides. Furthermore, Cry toxin genes have been expressed in transgenic plants to render crops resistant to insect pests. Transgenic maize, cotton, and potato carrying certain *cry* genes have been introduced for large-scale cultivation, and, since 1996, both the U.S. and the worldwide market shares of these and other transgenic crops has increased.

CRY TOXINS

To date, 89 different genes encoding crystal proteins have been cloned from *B. thuringiensis* and two other species. A given strain may produce single or multiple Cry toxins in various crystal formations. For example, a new subspecies of *B. thuringiensis* (Fig. 1) has been isolated in Egypt, which contains at least 15 *cry* genes. The genes for Cry toxins can be found both on plasmids and on chromosomal DNA. Individual Cry toxins have highly specific insecticidal activity, usually restricted to a few species within one particular order of insects. To date, toxins for insect species in the orders Lepidoptera (butterflies and moths), Diptera (flies and mosquitoes), Coleoptera (beetles and weevils), and Hymenoptera (wasps and bees) have been identified. A few toxins have an activity spectrum that spans two or three insect orders. The most recent Cry toxin nomenclature (5) is based on comparative amino acid sequence identity, molecular mass, and toxicity.

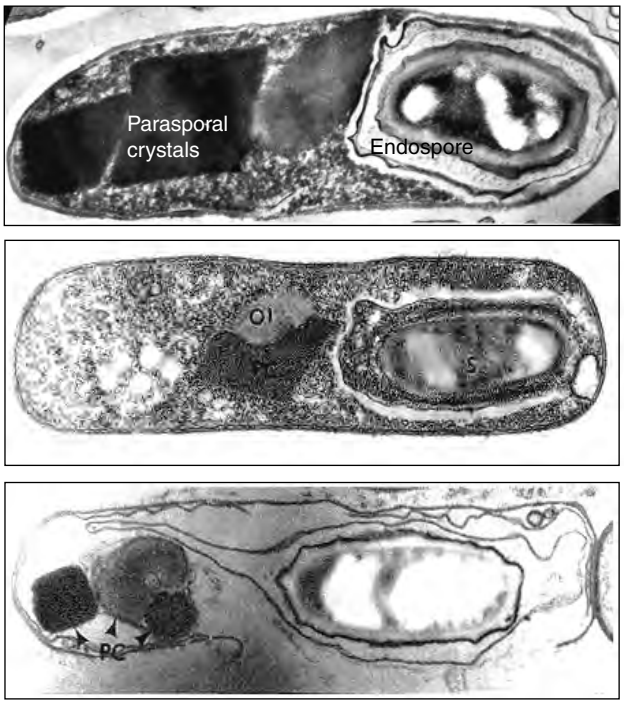

Figure 1. *Bacillus thuringiensis* spores and parasporal crystals. The bacterium has a life cycle, which includes vegetative growth and sporulation. The spore is the dormant stage of the organism and can withstand adverse environmental conditions, including extreme heat and low humidity. The transmission electron micrographs of sporulating *B. thuringiensis* subspecies (from top): *aegypti*, *berliner*, and *israelensis* reveal parasporal crystals (PC) lying adjacent to the endospore (S) of all three subspecies. The crystals vary in size, shape, and number and are composed of protoxin proteins that are expressed by *cry* genes. The outer coat of the spores also contains protoxin protein, which is insecticidal. Subspecies *aegypti* harbors 15 different *cry* genes, rendering entomopathogenic activity against three different orders of insects, Diptera, Coleoptera, and Lepidoptera. Subspecies *berliner* and *israelensis* produce cry toxins effective against moths and beetles, respectively.

The parasporal crystals of the different subspecies of *B. thuringiensis* all contain protoxins (ca. 70,140 kDa) that are single repeating subunits, which are converted, on activation, to lower molecular weight (ca. 6,067 kDa) toxins. Parasporal crystals associated with endospores of three different species of *B. thuringiensis* with different host insect range specificities are provided in Figure 1. For *B. thuringiensis* subsp. *tenebrionus*, the protoxin is a 72-kDa protein, which is converted to a 67-kDa toxin, whereas the protoxin of *B. thuringensis* subsp. *berliner* is a 130-kDa protein that, on activation, produces two molecules of about 65 kDa. One of these conversion products is protease-resistant and is toxic, whereas the other protein

is protease-sensitive and is not toxic (Fig. 3). The tertiary structures of Cry 3A, Cry1Aa, Cry2Aa, and Cry3Bb1 toxins have been elucidated (6–9). The Cry3A toxin of subspecies *tenebrionis*, the Cry3Bb1 toxin of subspecies *tolworthi*, and the Cry1Aa and Cry2Aa toxins of subspecies *kurstaki* bind to specific receptors located in the epithelial cells that line the midgut of a number of susceptible insects, including beetles by Cry3A and Cry3Bb1, moths by Cry1Aa, and both moths and mosquitoes by Cry2Aa.

CRY TOXIN RECEPTORS

Although most of the Cry toxins described in the literature exert highly specific activity toward insects in the orders Lepidoptera, Diptera, and Coleoptera, there also are reports of other isolates of *B. thuringiensis* active against other insect orders, including Hymenoptera, Homoptera, Orthoptera, and Mallophaga, and against nematodes, mites, and protozoa. Apparently, they are not effective against other nontarget organisms, including humans and animals. The toxicity and specificity of *B. thuringiensis* Cry toxins correlates directly with the binding of toxins to the epithelial cells that line the midgut of susceptible insects. The interaction of toxins with the midgut surface brings about distortion, enlargement, and detachment of the epithelial cells (Fig. 2), leading to devastation of the midgut tissue (Fig. 3) and death of the insect (Fig. 4).

Specific high-affinity receptor molecules that line the midgut epithelium of susceptible insect larvae mediate the lethal action of the *B. thuringiensis* Cry toxins. For example, the tobacco hornworm *M. sexta* contains a high-affinity receptor, BT-R_1, for the Cry1A toxins of *B. thuringiensis* (10). Both the cloned and natural BT-R_1 have high affinity ($\sim$ 1 nM) and specificity for the Cry1A family of toxins, all of which are extremely toxic to the tobacco hornworm. Expression of BT-R_1 is tissue-specific and only the epithelial cells of *M. sexta* midgut tissue harbor BT-R_1 mRNA and its translated protein. On the basis of the deduced amino acid sequence and predicted molecular structure, BT-R_1 is a 210-kDa membrane glycoprotein (Fig. 5) composed of four domains, ectodomain (EC), membrane-proximal extracellular domain (MPED), transmembrane domain (TM), and cytoplasmic domain (CYTO). The ectodomain consists of 12 cadherin repeats composed of β-sheets that are structured as ectodomain modules (EC1 through EC12).

The expression profile and structural features, along with the protein-domain architecture of BT-R_1, indicate that the protein is important in specific cell-to-cell adhesion events in the insect midgut. Most probably, the molecule has heterophilic interaction with other cell adhesion molecules. Toxin binding involves protein-to-protein interactions and appears to concern multiple structural determinants on both toxin and receptor, possibly intervening with the function of the receptor and disrupting cell-to-cell attachment and structural integrity of insect midgut epithelium. Indeed, the histpathological changes associated with *M. sexta* midgut epithelial cells exposed to Cry1Ab toxin support the notion that the pathological symptoms of Cry toxin exposure involves adverse effects brought about by dysfunctional cell adhesion (Fig. 2). Recruitment of cadherin receptors by *B. thuringiensis*

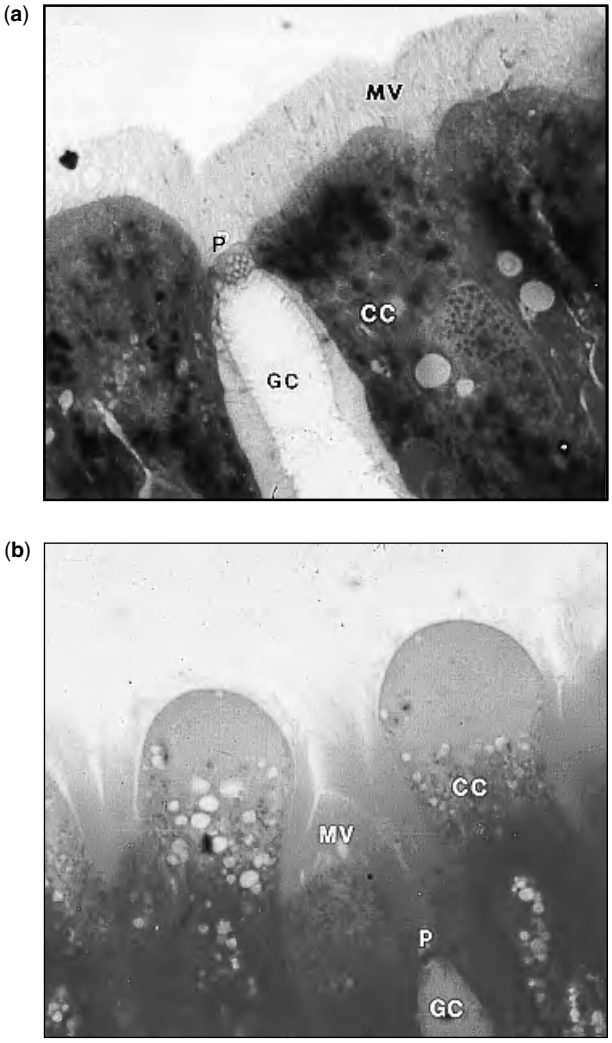

Figure 2. Effects of Cry1Ab toxin on *Manduca sexta* midgut epithelium. The midgut of insect larvae is a tube lined with columnar epithelial cells (CC). The apical ends of the midgut epithelial cells contain microvilli (MV) and this surface appearance is referred to as a *brush border*. Another type of cell that is present in the midgut is the goblet cell (GC). Goblet cells are intercalated with the columnar epithelial cells. Goblet cells contain a pore (P) at their apical ends through which enzymes and ions are secreted into the midgut. All cells that are facing the inner cavity of the midgut are attached to a basal membrane, which surrounds the tissue. The normal morphological features of the midgut epithelium change dramatically in about 1,530 minutes when a susceptible insect ingests Cry toxin. Micrographs of longitudinal sections of healthy (Panel A) and intoxicated (Panel B) midgut tissue from fourth instar *M. sexta* larvae show the morphological changes in the epithelial cells. On exposure to Cry1Ab toxin, the columnar cells swell and lose their microvillar structure, whereas the goblet cells do not appear to be affected, suggesting that toxin receptors are specifically expressed on the epithelial cells. Apical cell membrane distortion and total cell disruption occurs within one hour of intoxication, leading to devastation of the midgut tissue and death of the insect. See color insert.

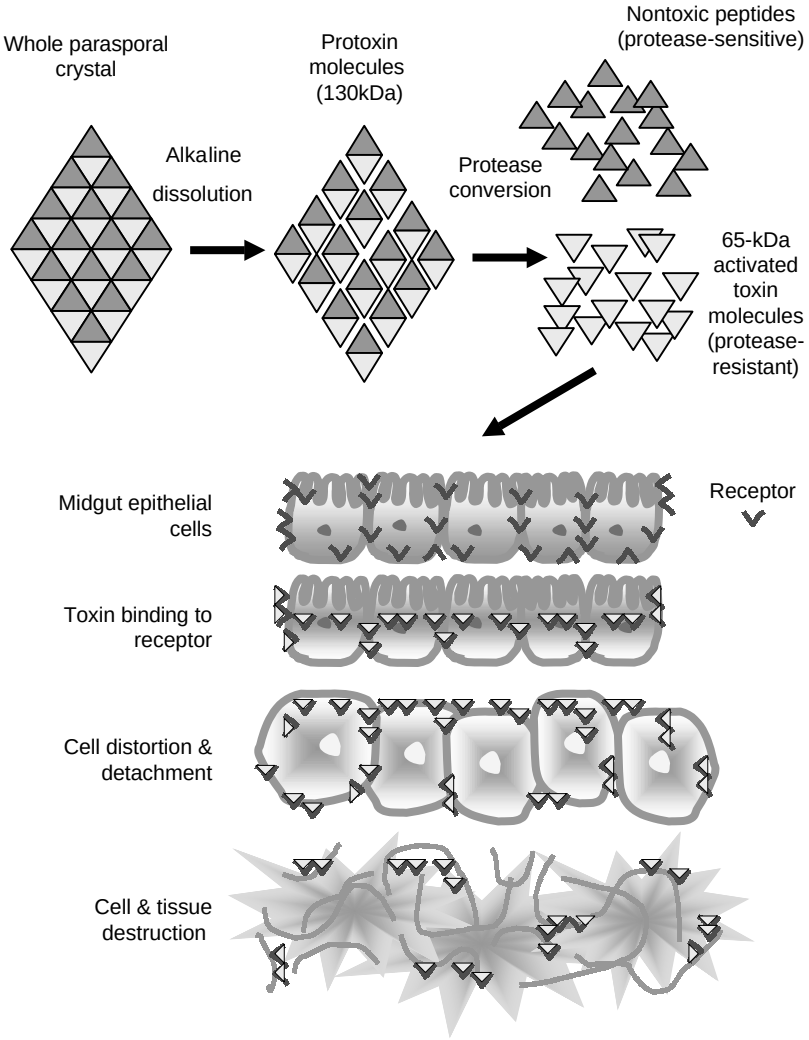

Figure 3. Activation of parasporal crystals and receptor mediated toxicity of Cry proteins. The entomopathogenic activity of *B. thuringiensis* mainly resides in the parasporal crystals that are composed of protoxin protein molecules. Following ingestion by a target insect, the protoxin is solubilized and converted to active toxin that binds to specific receptors on the surface of midgut epithelial cells. Both the specific physiological environment and the activity of proteolytic enzymes in the midgut of susceptible insects are involved in protoxin activation. Protoxin activation generates two polypeptide fragments, one that is hydrolyzed by midgut proteases and one (activated toxin) that is quite resistant to proteolytic attack in the target insect. Toxin binding to specific receptors on the epithelial cells brings about distortion, enlargement, and detachment of the cells, leading to devastation of the midgut tissue and death of the insect. Specific receptor-toxin interactions also determine the range of insect species killed by individual subspecies of *B. thuringiensis*. See color insert.

Cry toxins appears to be similar to that observed for certain other bacteria, such as *Clostridium perfringens* and *Listeria monocytogenes*, in which these pathogens target cell-to-cell and cell–matrix interactions of their host cells. It is intriguing that the Cry1A toxins of *B. thuringiensis* recruit a molecule related to cell adhesion, which is essential for a diverse range of functional properties including differentiation, growth, cell migration, and development. Certainly, *B. thuringiensis* Cry toxin receptors are pivotal to understanding insect responses to the entomopathogen and to developing new biorational insecticides.

BT-R_1 of the tobacco hornworm *M. sexta* is the first receptor cloned and characterized for the Cry1A toxins of *B. thuringiensis* (11). Since then, homologous cadherin receptors have been identified and studied in other insects. In addition to cadherin receptors for Cry toxins, several 120-kDa Cry toxin-binding proteins also have been identified in some lepidopteran insects. These proteins, in fact, exist as multiple forms of a ubiquitous gut protease, aminopeptidase N (APN). Although APN interacts with Cry toxins and is an important component of the insect midgut surface, APN's do not serve as functional receptors to mediate toxicity of Cry proteins. On the other hand, BT-R_1 and related homologous cadherin receptors for the Cry proteins are linked directly to toxicity exerted by the Cry toxins. Indeed, heterologous expression of BT-R_1 on the surface of both insect and mammalian cells mediates the toxicity of the Cry proteins, providing strong support to the idea that cadherin receptors are involved in cytotoxicity and insecticidal action of Cry toxins.

INSECT RESISTANCE TO MICROBIAL INSECTICIDES

One of the most desired goals for the utilization of *B. thuringiensis*–based insecticides is to replace most chemical pesticides. Because the binding of *B. thuringiensis* toxins to high-affinity receptors in insect midgut is critical to insecticidal activity of the toxins, the means for developing new and improved *B. thuringiensis*–based insect-control agents relies on understanding the mode of action of the toxins and how insect receptor molecules for the toxins mediate toxicity. Therefore, identification of Cry toxin receptors and understanding the mechanism of cytotoxicity will facilitate the design of novel

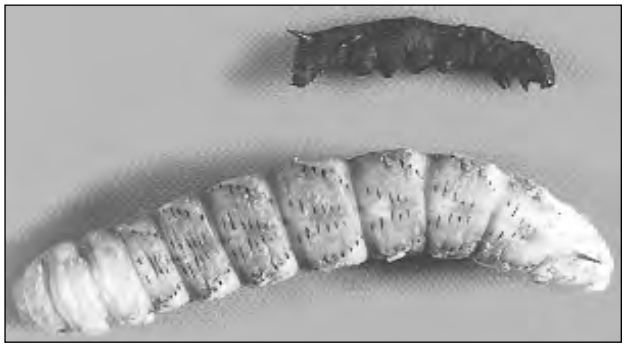

Figure 4. Effect of Cry1Ab toxin of *B. thuringiensis* on *M. sexta* larva. *Bacillus thuringiensis*–based insecticides are applied much like synthetic insecticides. Such insecticides are inactivated within a few days in most outdoor situations compared with the long-lasting presence of many chemical insecticides. Soon after ingesting whole cells of *B. thuringiensis* or activated toxin, susceptible insect larvae are poisoned and they stop feeding. If the appropriate amount of material is applied at the right time, the immediate and highly selective effect of *B. thuringiensis* is realized. *Manduca sexta* larvae feeding on a normal diet are large and green in color. Larvae that have consumed Cry1Ab toxin turn black and usually die immediately or within a couple of days after sepsis. Vegetative cells of *B. thuringiensis* may multiply in the infected host, however, without producing abundant spores or parasporal crystals. The end result is that no infective units are released into the environment when a poisoned insect dies. Until the early 1980s, commercial *B. thuringiensis* products were effective only against caterpillars. However, strains that kill not only caterpillars but also several other types of pests have been identified and developed for commercial use. New isolates of *B. thuringiensis* with new Cry toxin variants will increase the spectrum of pests that can be controlled. See color insert.

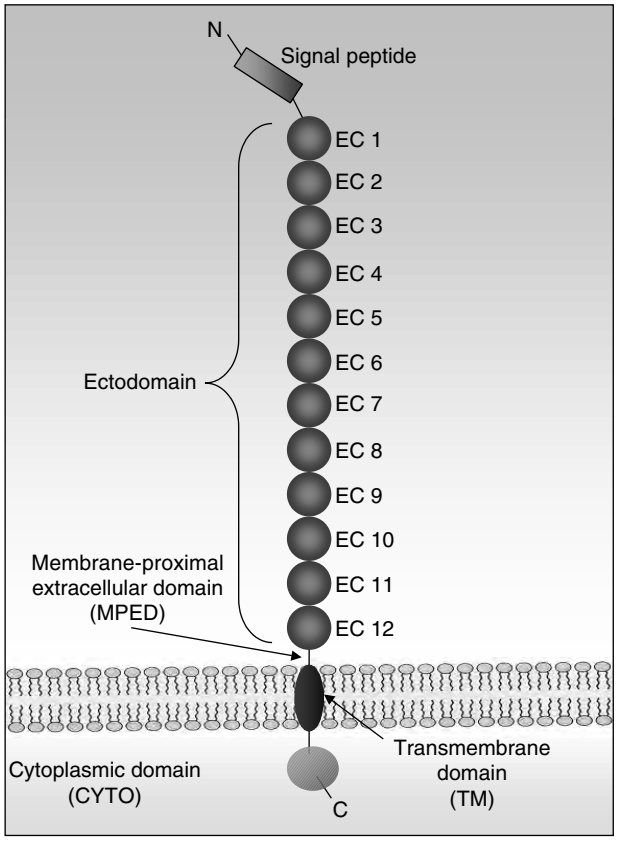

Figure 5. BT-R_1, the cadherin receptor of *M. sexta* for Cry1A toxins. On the basis of the predicted amino acid sequence, the BT-R_1 receptor (210 kDa) is composed of four domains: (*1*) ectodomain (EC), (*2*) membrane-proximal extracellular domain (MPED), (*3*) transmembrane domain (TM), and (*4*) cytoplasmic domain (CYTO). Downstream of a membrane signal sequence, the ectodomain consists of 12 cadherin repeats composed of β-sheets that are structured as ectodomain modules (EC1 through EC12). The ectodomain harbors putative molecular adhesion sequences. The structural features along with the protein-domain architecture indicate that BT-R_1 is a new type of heterophilic cadherin with a close relationship to protocadherins in the cadherin superfamily. The Cry1A toxins of *B. thuringiensis* bind to BT-R_1 with high affinity and specificity. Most probably, toxin binding adversely affects the structure and function of BT-R_1, consequently, compromising the integrity of the midgut epithelial cells that express the receptor molecule. See color insert.

insecticidal agents with higher affinity to receptor(s) and with the desired specificity against targeted insects. Furthermore, insects exhibiting resistance to particular Cry toxins have reduced or no binding capacity for the toxins, underscoring the importance of identifying additional toxin receptors and advancing our knowledge of toxin-receptor interactions. Such knowledge most assuredly will aid our understanding of why insects become resistant to *B. thuringiensis* and how this problem can be circumvented for the continued usage of this entomopathogen as an effective and safe biological control agent. Unfortunately, the widespread and intensive use of Cry toxins commercially to control agriculturally and industrially important insects have increased the possibility of the development of insect resistance to *B. thuringiensis*. In fact, there is a growing list of field-selected and laboratory-selected insect species, including the diamondback moth, the Indianmeal moth, the Colorado potato beetle, the cotton leafworm, the beet armyworm, the tobacco budworm, and the European corn borer, all of which exhibit varying degrees of decreased susceptibility to the Cry toxins.

One contributing mechanism of resistance to Cry toxins is the decrease in the receptor binding to toxin in the insect midgut. Epithelial cells in the midgut tissue of resistant insect strains retain their structural integrity on exposure to Cry toxin action, whereas the toxin devastates the midgut tissue of susceptible insects. Also, changes in physiological and proteolytic activities in the midgut of susceptible species are implicated in resistance to Cry toxins. Overall decreased receptor binding and alterations in the composition and activity of midgut proteases are correlated with the resistant state in the midgut epithelial tissue, implicating the involvement of adaptive responses in the resistance phenomenon.

Microorganisms share common strategies to overcome their hosts during pathogenesis. One common strategy is to make effector molecules that interact with specific receptor molecules in the respective host to mimic, suppress, or modulate host pathways and to enhance pathogenicity, thus rendering survival advantage to the

pathogen. However, the interactions of pathogens trigger innate defense reactions in the host cells. These innate cellular defenses consist of nonspecific responses and are found in organisms ranging from plants to humans. In particular, insects, which do not produce antibodies and T cells to combat microbial challenges, rely mostly on innate cellular responses to defend themselves against invading microbes and their toxins. Constant interaction with microorganisms and molecules has necessitated the evolution of innate defense mechanisms to guard the insect against invasion by pathogens while tolerating normal microbial flora and a plethora of potential antigens present in food. In fact, the interactions with pathogenic microorganisms generate evolutionary forces aiming to destroy an endless race among species to find new ways to become refractory to microbial challenges (Fig. 6). In this race, innate responses are significant, because they not only determine the outcome of microbial challenges at the first site of pathogen contact or attack, but they also are involved in information relays inherent in adaptive immunity as well. The innate defenses against unwelcome microbes and antigens include rapid-acting responses such as secretion of small antimicrobial peptides and adaptive changes that modulate cell surface and activity. The innate responses triggered on the surface of midgut epithelial cells, in turn, promote special physiological adaptations on the cell surface that somehow protect the cell against foreign invaders. Most often, these reactions sustain cellular adaptation on prolonged exposure to microbial activity or other stress conditions and, eventually, lead to resistance, or, in some instances, cell death.

There appears to be two levels of adaptive responses in insects that bring protection against the lethal action of Cry toxins. The first level involves changes in the profile of midgut proteases that physiologically position the tissue for response to toxin stress. The second level involves adaptive responses that can be genetically transmitted to progeny insects for future survival advantage. The innate responses and adaptive changes in the insect midgut epithelium and their functional involvement in rendering immunity to *B. thuringiensis* toxins is fundamentally important in explaining resistance. Indeed, elucidating the mechanism(s) of how midgut epithelium cells in resistant insects avoid toxin binding is critical in understanding insect resistance.

To date, the control of pest insects by microbial insecticides represents the most successful biological control strategy. However, the emergence of insect populations resistant to biological insecticides raises significant concerns. Like global warming, the resistance phenomenon may be part of the reason for the recent increase in insect infestations worldwide. These factors necessitate a fundamental understanding of insect responses to microorganisms. Certainly, the continued and improved use of microbial insecticides depends on progress in several areas, including identification of insect receptors of microbial toxins, elucidating their mode of action in mediating toxicity, and understanding how microorganisms design entomopathogenic actions. Diverse, natural microbial products and genetically engineered materials should

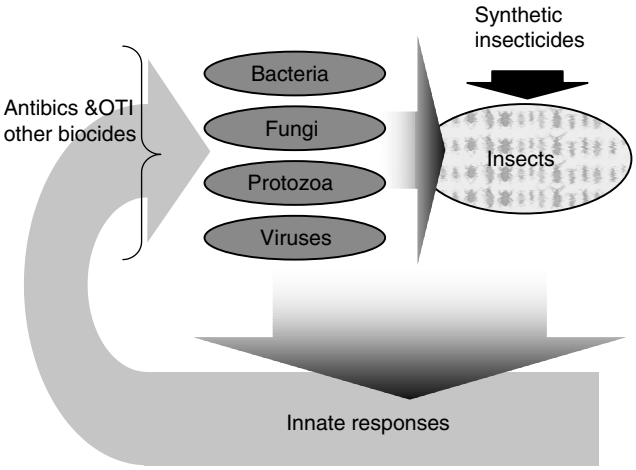

Figure 6. The relationship between innate responses and resistance to microbial insecticides. Innate cellular defense involves nonspecific responses found in organisms ranging from plants to humans. Invertebrates, including insects, do not produce antibodies and T cells to combat microbial challenges, as do vertebrate animals and humans. Instead, they rely primarily on innate responses to defend themselves against biological challenges. Constant contact of epithelial cells in the mucosal linings of animals harboring various microbes, and a large number of diverse antigens, has brought about the evolution of innate defense mechanisms. Innate responses are first-line cellular defenses and are pivotal to protecting an organism from potential invasion by various pathogens, while tolerating an indigenous microbial flora and a plethora of antigens commonly present in different food sources. Furthermore, information relays between innate defense reactions and adaptive protective devices now are appreciated as ancient evolutionary conserved immune responses. In fact, since the discovery of antimicrobial activities in the cabbage looper *Hyalophora cecropia*, studies of the fruit fly *Drosophila melanogaster* and mosquitoes have revealed notable conservation of innate defense mechanisms in both insects and mammals, especially in mucosal epithelia. Some innate defense mechanisms that guard against unwelcome microbes and antigens respond rapidly and promote adaptive functional changes, including the expression of those molecules that modulate various cell surface activities. The responses are triggered on the surface of epithelial cells on recognition of particular molecular motifs by certain surface receptors. Communication with signaling pathways promotes special physiological adaptations to the cell surface that somehow protect the cell against foreign invaders. Most often, these reactions sustain cellular adaptation on prolonged exposure to microbial activity or other stress conditions and eventually lead to resistance. However, the quick evolution of microbial pathogens is a potential for overcoming such resistance, generating natural competition involving continual challenges and responses that lead to resistance development among species. For instance, widespread commercial use of *B. thuringiensis* Cry toxins to control pest insects has produced insects that are resistant to this entomopathogen. Moreover, chemical insecticides stress insect populations forcing many individuals to acquire an enhanced detoxification capacity and increased state of resistance, raising the potential for insects to become refractory to multiple insect-control agents. Therefore, it is crucial to implicate integrated pest management strategies with microbial insecticides and chemical-based pesticides to gain the greatest possible benefits. See color insert.

provide sufficient biological resources to safely and effectively control pest insects now and in the future. However, new discoveries and development efforts are needed to meet the complex challenges in agriculture, medicine, and industry because insects have the uncanny ability to cope with newly designed insecticides regardless of their origin or creation.

SUSTAINABLE AGRICULTURE, HEALTH, AND ENVIRONMENT

Agriculture is fundamental to human society and it significantly contributes to the advancement of other industrial technologies. Sustainable agriculture refers to the means used to maintain yields and quality of crops while reducing potential threats to public health and the environment. One overriding concern related to sustainable agriculture is the use of chemical pesticides. The comprehensive knowledge gained by the research of soil, water, genetics, biochemistry, and population dynamics indicates that synthetic chemicals pose a major threat to both human health and the environment. Obviously, reducing our dependence on chemicals by introducing new, improved biological control agents and strategies is critical to sustain agriculture.

Microorganisms are essential for the makeup of the earth's ecosystems. Microbes can be pathogenic, parasitic, mutualistic, or existent in a variety of other symbiotic relationships. A number of microorganisms and their ecological relationships have been studied and the knowledge gained from studying these associations have contributed to our understanding of the importance of beneficial microorganisms in agriculture and medicine. Applications of this knowledge include disease and pest control, proper recycling of nutrients, and environmental remediation. For example, biological agents derived from certain microorganisms for the control of pests, pathogens, and weeds to reduce dependence on chemical treatments are important for human health, management of biological diversity, and habitat conservation. There is an arsenal of disease-and pest-resistant genes and useful microbes, which can be used for agricultural, biomedical, and industrial purposes. Thus, it is critical to understand and intelligently manage the diversity and complexity of microbes involved in helping maintain all sustainable systems. More reliance on natural systems and less on synthetic products for pest and disease control, to sustain suitable crop yields, is the watchword for the future health and well-being of human kind. Certainly, biotechnological advances hold promise in delivering appropriate, rational solutions to manage sustainable ecosystems. The technological scenario includes microorganisms for the creation of new products and processes, which are safe to users, consumers, and the environment.

Continued expansion of the use of *B. thuringiensis* and other microbial-based pesticides depends on progress made in a variety of research and development activities. In the case of *B. thuringiensis*, the search for new toxins continues to progress, and, so far, the discoveries have provided sufficient diversity to meet the challenge of field resistance. However, new scientific discoveries are equally important in developing new and improved insecticidal products. Also, insights into the basis of insect resistance to Cry toxins are essential as well. All in all, the continued use of microorganisms such as *B. thuringiensis* will require a combination of the discovery of new strains and new toxins, identification of target receptor molecules, and a fundamental understanding of the structural and functional relationships between toxins and their receptor molecules.

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INVERTEBRATE-ASSOCIATED MICROORGANISMS IN DEEP-SEA HYDROTHERMAL VENTS.

See HYDROTHERMAL VENTS: BIODIVERSITY IN DEEP-SEA HYDROTHERMAL VENTS; HYDROTHERMAL VENTS: PROKARYOTES IN DEEP-SEA HYDROTHERMAL VENTS

INVERTEBRATES AND PROTOZOA (FREE-LIVING) IN DRINKING WATER DISTRIBUTION SYSTEMS

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"Just a glass of water! You see a glass in front of you, filled with pure water from a water supply. It is without taste and odor; it is clear; there is nothing remarkable in sight. Nonetheless, this glass contains a world of wonders (...). Although your eye presumably sees nothing but pure water, it is the residence of a large number of animals that are invisible to the naked eye, to which the glass is a large pool, a vast lake, a sea (...). However, drink it without concern: the little creatures are completely harmless and will, taken in by your body, soon have lost their lives."

These translated parts of a chapter of the *Reading Book for Primary Schools*, published in 1899 in The Netherlands (1), present a view on the significance of free-living invertebrates and protozoans in drinking water on health, that most likely is still valid for drinking water in developed countries in temperate zones. It, however, also emphasizes the unease that was and still is stirred in most drinking water consumers when confronted with the knowledge that "animals," although microscopically small, are emerging from their taps. When invertebrates emerge in a clearly visible form, it is not surprising that these animals usually evoke downright disgust. Man has learned to associate the presence of worms, insect larvae, and crustaceans in food and water with a diminished wholesomeness and, since the discovery of microorganisms as possible origins of diseases, with diminished hygiene. In many domestic situations, this association is justified and the presence of protozoans and invertebrates in drinking water in developing countries may be far from harmless.

The presence of invertebrates, being present in all natural freshwater habitats, is to be expected in most, if not all drinking water distribution systems in the world. Drinking water and the distribution systems by which it is supplied are not sterile, nor should they be. Adequate source water treatment and hygiene during operations will prevent contamination with pathogens and will limit multiplication of (micro)organisms in distribution systems. Free-living invertebrates should however be considered a natural part of the biocoenosis (i.e., an association of organisms forming a community) in drinking water supply systems. This contribution will discuss the significance of the presence of invertebrates and, briefly, free-living protozoans in drinking water and water supply systems, preceded by a summary of the significance of their parasitic relatives. The fact that monitoring of invertebrates is apparently not required by law anywhere is the probable cause of the relative scarcity of knowledge available on the subject. An overview of the most important taxa is presented, followed by an evaluation of ecological processes (immigration, survival, feeding, growth, and reproduction) and an impression of the abundance of invertebrates found in drinking water and mains. Finally, control strategies and benchmark values are presented.

SIGNIFICANCE

Parasitic Invertebrates and Protozoans

The WHO (2) assumes that pathogenic invertebrates only may be present in drinking water in Asia, Africa, and South America and only when contaminated surface water or groundwater is not treated prior to (distribution and) consumption. *Dracunculus medinensis* (Nematoda) and *Schistosoma* spp. (Platyhelminthes) are mentioned as the most important pathogens; other worms are considered of lesser or very little importance for drinking water. An infection by the guineaworm *D. medinensis* is considered the only disease transmitted exclusively by drinking water. The larvae reach the stomach of many millions of people via their intermediate hosts, some cyclopoid Copepoda [mostly *Mesocyclops* spp. (3)], and 5 to 10 million people are taken ill each year. The infection usually is not lethal, but the adult female can reach lengths of more than 50 cm in limbs (mostly the legs) and causes painful ulcers (from within). Cercaria (i.e., one of the larval forms) of *Schistosoma* spp. in most cases enter the human body via the skin during bathing or washing of clothes in contaminated water, snails being the intermediate hosts. Although infections may occur by the distribution of untreated surface water, most infections occur in wells and ponds. An estimated 200 million people are infected yearly by schistosomiasis and some die from this intestinal or kidney infection (2,4). In temperate zones as well, invertebrate parasites are known to be (possibly) waterborne, such as *Ascaris lumbricoides* (Nematoda) and nonhuman parasitic Schistosomatidae (Platyhelminthes). Outbreaks via drinking water have not been reported (5). However, the presence of parasitic Nematoda and other invertebrates in drinking water from surface water supplies may be a transmission route for plant diseases (6).

As for parasitic protozoans, the possibility of contamination of finished water with pathogenic protozoans such as *Entamoeba histolytica* has long been acknowledged, as well as the multiplication in drinking water distribution systems of free-living protozoans such as *Naegleria fowleri* and *Acanthamoeba* spp. that may be pathogenic under certain conditions (5,7). For a large part of the twentieth century, water supply utilities in most developed countries relied on the capacity of disinfectants — in most cases

chlorine, chlorine derivatives, or ozone — to inactivate pathogens in surface water treatment. The importance of multibarrier treatment to remove chlorine-resistant (oö)cysts of the pathogenic protozoans *Cryptosporidium* spp. and *Giardia* spp. from surface water and their high resistance to disinfectants became painfully clear in both small and large epidemics caused by their presence in drinking water at the end of the twentieth century in the United States and the United Kingdom (5,8–11). Other pathogenic protozoans (*Toxoplasma*, *Cyclospora*, *Microsporidium*) are possibly important for drinking water hygiene as well and are presently under scrutiny (5,11).

Invertebrate and Protozoan Hosts for Pathogens

As invertebrates may harbor pathogenic viruses, bacteria, and protozoans in and on their body, mostly in their digestive tracks, invertebrates resistant to disinfection may take these pathogens with them while penetrating drinking water distribution systems through treatment plants. Nematoda, in particular, have been under scrutiny and are still suspect, although their role in pathogen entry into drinking water has never been fully established. It has been suggested that small crustaceans and fly larvae could also affect microbiological safety by harboring pathogenic microorganisms in or on their body (12). No pathogenic bacteria or fecal coliforms could however be found associated with individuals isolated from drinking water (13), although midge fly larvae (Tipulidae) have recently been associated with the number of total coliforms in finished water of a groundwater treatment plant in Germany (14). Some protozoans have been shown to protect pathogenic bacteria from chlorination (15). *Legionella* bacteria may multiply in free-living amebae in distribution systems and also be protect by them from chlorination (16,17); *Acanthamoeba* spp. have been known to harbor *Mycobacterium* spp. and viruses, accentuating the public health importance of these organisms (5).

The significance of intermediate hosts in distribution systems as possible vectors for invertebrate parasites in temperate zones also has been suggested (18). Theoretically, the penetration of parasitic worms or their intermediate hosts into the distribution system cannot be excluded, for example, in the case of mains bursts and other situations in which the integrity of the system is impaired. The intermediate hosts may be infected with parasites and may survive in the distribution system. A small percentage of surface water Asellidae (Crustacea, Isopoda), for example, can be infected with the larvae of Acanthocephala, a group of parasitic worms that infect the digestive tract of vertebrates (19,20). Freshwater snails can be intermediate hosts of blood flukes (Schistosomatidae, Platyhelminthes) causing "swimmer's itch," and Copepoda may be intermediate hosts of parasites such as *Gnathostoma spinigerum* (Nematoda) and *Spirometra mansonoides* (Platyhelminthes) (5). Although finished water of treatment plants is microbiologically safe, chances that an infected intermediate host (*1*) penetrates into the distribution systems, (*2*) survives, (*3*) emerges from a consumer tap, and (*4*) can pass on the parasite to a human will have to be considered negligible.

Aesthetic Significance and Consumer Complaints

An anonymous pamphlet distributed in London in 1827 pointed out the presence of a large variety and quantity of invertebrates, protozoans, and algae in the Thames river water distributed for domestic use. The pamphlet drew public attention to the inlet being situated within three yards of one of the largest city sewer outlets (21). After the start of slow sand filtration in London in 1829 (21), an introduction of this technique throughout Europe followed, although many cities (partly) continued to be supplied with untreated surface water long after. The quality of the water in distribution systems subsequently improved greatly in Europe and the rest of the industrialized world in the nineteenth and twentieth century, causing a distinct reduction of drinking water-related outbreaks of microbiological diseases. Simultaneously, major changes took place in the invertebrate communities in distribution systems. However, at the same time, consumer expectations regarding water quality rose. The sudden infestations of invertebrates emerging from taps and the resulting consumer complaints have driven water utilities to despair. Presently, consumer complaints concerning invertebrates under normal conditions probably are not abundant in most distribution systems in developed countries, but there is little or no literature to corroborate this assumption. In The Netherlands (population 16 million), consumer complaints regarding invertebrates currently are estimated to be limited to an average of 10 each year, although there is no comprehensive overview available. In Antwerp, Belgium, complaints about Asellidae and Gastropoda emerging from taps were approximately 25 to 30 each year before major air-scouring mains started in 1975, reducing complaints to maximally one per year since 1985 (22). During a symposium on consumer complaints in 1965, the London Metropolitan Water Board disclosed that 160 (3%) of the complaints in the period between 1957 and 1964 concerned "crustaceans, worms, insects and so forth" (23). The literature shows that most problems elsewhere are also caused by these three groups of invertebrates.

Invertebrate taxa that also have caused complaints are Gastropoda (snails) (22,24–27), Cladocera (water fleas) (27), Copepoda (copepods) (26,27), Hirudinea (leeches) (27,28) and Gammaridae (freshwater shrimps) (27,29,30). Not just invertebrates but their fecal pellets (31), body parts, and degradation products as well can lead to consumer complaints. The death of large numbers of invertebrates used to cause taste and odor problems in the early days of water supply, especially when oxygen concentrations dropped. Large numbers of invertebrates present in distribution systems normally are noticed because individuals are likely to emerge from taps, but sessile invertebrates such as Bivalvia (mussels) (28), Ectoprocta (bryozoans) (32) and Porifera (sponges) (32,33) were only detected when they caused taste and odor problems or clogged pipes. Their presence is usually limited to untreated surface water because these sessile filter feeders depend on relatively high densities of POM (particulate organic matter) in the water. Mussel larvae (Bivalvia), for

instance, may penetrate surface water treatments, but levels of POM in drinking water are usually too low to sustain mussel growth in present-day distribution systems (34).

The other effects of large numbers of invertebrates can be the clogging of service lines and water meters. Clogging by Asellidae has been reported in The Netherlands. Furthermore, freshwater snails and mussels will easily cause clogging owing to their hard shells (22,24–26,35). Especially in the early years of distribution systems, when water treatment was absent or rudimentary, mains constrictions and clogging were more common, for example, by Bivalvia (36), Ectoprocta (32), and Porifera (33).

Invertebrates not originating from the distribution system can be a nuisance for water utilities as well: spiders and adult insects seeking refuge in the neck of taps will emerge when water is used. Particles may also be mistaken for invertebrates. Convincing consumers of the excellent hygienic quality of their drinking water in these cases usually has been proven difficult.

Microbiological Deterioration of Water Quality

A detectable transmission of pathogenic microorganisms into drinking water by invertebrates has never been reported. However, protozoans and bacteria may multiply on the remains of invertebrates [e.g., ciliates (37,38).] and in or on their fecal pellets [*Aeromonas* bacteria in pellets of midge fly larvae (39)]. These substrates presumably contribute to heterotrophic plate counts in sediments [as high as 6.10^8/g DW (40) and 5.10^9/g DW (41)] and plate counts of *Aeromonas* spp. (30 °C) in sediments [as high as 5.10^9/g DW (41)]. Many heterotrophic bacteria will grow on easily assimilable low-molecular compounds from the water, forming a biofilm on the sediment particles, much in the same way as it is formed on all water-exposed surfaces in distribution systems. *Aeromonas* bacteria however are also able to use high-molecular substrates present in dead (micro)organisms such as proteins, fats, and complex carbohydrates (42), for example, chitin from the exoskeletons of insects (unpublished personal observations by D. van der Kooij). As the bacteria were hardly found in the fresh biofilms (43), it is likely that multiplication of *Aeromonas* in distribution systems in The Netherlands predominantly occurs in sediments, using dead microorganisms and invertebrates as part of their food supply. Total coliforms have been found in sediments from distribution systems as well, with averages ranging from 10 to 1.10^5/g DW. Samples were collected via hydrants and were not handled aseptically and therefore contamination of the samples could not be ruled out (41). In conclusion, it is likely that invertebrates lower the overall density of bacteria and protozoans in distribution systems by feeding on them, therefore increasing the conversion rate of organic carbon to carbon dioxide and hence limiting the total food supply for microorganisms. Invertebrates however also change the composition of the microorganism community in distribution systems by their presence (stimulating symbiotic microorganisms) and by converting their food into fecal pellets and eventually in molting cuticles and dead bodies, changing the substrates available for microorganisms. It is likely that these changes benefit coliforms and (opportunistic) pathogens.

DRINKING WATER FAUNA

Figure 1 shows pictures of a protozoan and invertebrate taxa commonly found in drinking water distribution systems, giving an impression of their morphology. Protozoans, Rotifera, and Tardigrada are very small and are not known to have caused consumer complaints or specific problems for water utilities. Hydrachnellae have been encountered in distribution systems in Japan, probably contributing to the colonization of swimming pools (44), but have not been known to be a nuisance. Ostracoda normally are not ubiquitous and have a high density owing to their hard shell and will not be flushed out easily via consumer taps, probably explaining the lack of consumer complaints about these organisms. Collembola (springtails, 0.5 to 5 mm, not shown in the figure) are insects sometimes found in samples of sediment flushed out of mains via hydrants. Although these organisms most likely originate from the dry part of hydrants, contaminating the samples during sampling, they are sometimes quantified in samples as possible members of drinking water fauna.

Protozoans

A wide variety of free-living protozoans are present in surface water and groundwater. It is very likely that many species that are able to feed on bacteria from biofilms also multiply in water supply systems. Many species have been found in treatment works (47), finished water (reservoirs) (48,49) and drinking water distribution systems (37,45,46). In finished water reservoirs and distribution systems they have been found to multiply on the cadavers of invertebrates (37,38). Free-living species of the Mastigophora (flagellates) (37,49), Sarcodina (amebae) (37,49), and Ciliophora (ciliates) (37,38,47) have been reported.

Nematoda

Nematoda (nematodes or roundworms) are eel-like worms (Fig. 1) that usually range in length from 0.5 to 2.5 mm. Although able to swim, nematoda usually glide through biofilms. Free-living species prey on microorganisms and small invertebrates, generally reproducing sexually via eggs. Upon investigating tap water for the presence of amebae in Ohio (U.S.A.) in 1955, Chang and coworkers (50) detected the nematoda (*Diplogaster nudicapitatus*[1]) in their samples. A few years earlier, the presence of the nematoda (*Trilobus gracilis*) was also established in finished water and tap water in Norwich (U.K.) (51), and even long before that, nematoda were reported to be common in drinking water (52). Although the abundance of the organisms in Norwich led to investigations on control methods, the (free-living) organisms were not considered a possible health threat (51). Chang and collaborators however suggested the possibility of drinking water contamination by pathogenic bacteria, ingested by

[1] It should be noted that in many cases the names of species and genera of organisms referred to in literature have been changed since publication. In this contribution, the names originally used have been presented.

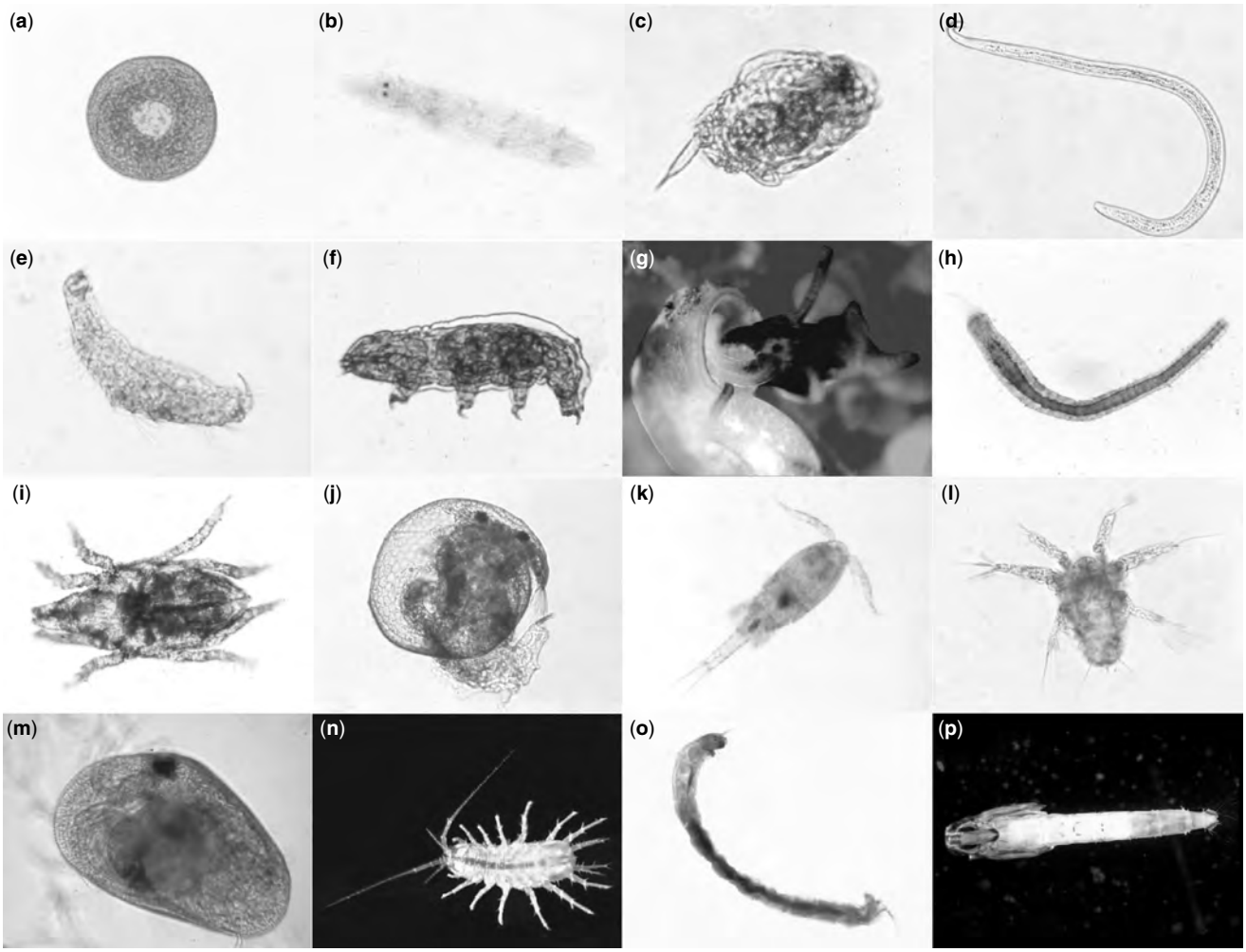

Figure 1. Examples of a protozoan and invertebrate taxa commonly found in samples from drinking water distribution systems (**a**) Testacea (shelled amoebae, c. 25–400 μm); (**b**) Turbellaria (flatworms, c. 0.5–20 mm); (**c**) Rotifera (0.1 to 1 mm); (**d**) Nematoda (roundworms, 0.5 to 2.5 mm); (**e**) Gastrotricha (0.1–1 mm); (**f**) Tardigrada (0.1–1 mm); (**h**) Oligochaeta (common worms, 0.5–100 mm); (**g**) Gastropoda (snails, 1 to 10 mm); (**i**) Hydrachnellae (water mites, 0.3–1 mm); (**j**) Cladocera (water fleas, 0.5–2 mm); (**m**) Ostracoda (0.5 –2 mm); (**k**) Copepoda (0.5–3 mm); (**l**) larvae of Copepoda (nauplius larvae, 0.2–0.5 mm), (**n**) Asellidae (aquatic sow bugs, 1 to 12 mm), (**o**) larva of Chironomidae (midge flies, 4 to 30 mm); (**p**) pharate adult of Chironomidae (c. 4 mm). Dimensions are common diameters or lengths in distribution systems, excluding extremities (from J. H. M. van Lieverloo, *J. Am. Water Works Assoc.*, (submitted).

nematoda and thus protected against chlorination during surface water treatment. They found many species during a survey of nematoda in the water supplies in United States (53). Pathogens (*Shigella*, *Salmonella*, and Coxsackie virus) were found to be able to survive several days in some chlorine-resistant species (*Cheilobus quadrilabiatus* and *D. nudicapitatus*) (54). The latter results were later corroborated by research on *Salmonella* survival in *Pristionchus lheritieri* (55). Chang and collaborators however could not prove the infectiveness of nematoda on mice (56) and concluded that under normal conditions the chances of pathogens actually being transmitted into drinking water distribution systems by nematoda must be considered very remote (57). This conclusion however did not lessen interest and ever since, investigations on nematoda removal in treatment plants and presence in drinking water have been reported predominantly in the United States (58,59,60), but also in Canada (61,62), Germany (63), India (64), Italy (65), and Poland (66). In Italy, no pathogenic bacteria could be isolated from nematoda collected in both raw and finished water of a surface water treatment plant (67). In the Netherlands, many water utilities monitor finished water of surface water treatment plants for nematoda, among other invertebrates (68). Numbers of more than 1,000 per m^3 are considered very high for finished water of surface water treatment plants. For groundwater treatment plants, numbers of 100 per m^3 or more are very high (45).

Oligochaeta (Annelida)

Oligochaeta (oligochaete worms) form a group of segmented freshwater worms (see Fig. 1) and terrestrial worms (e.g., the earthworm), also known as the common worms, generally feeding by ingesting detritus and sediments, digesting the organic matter. Although some species may grow to be much longer, the length of Oligochaeta usually ranges from 1 to 100 mm. They reproduce by dividing or budding, but the organisms are hermaphrodite and can reproduce sexually as well, provided they are sexually mature and densities are high enough to find a mate. The organisms usually burrow through these sediments; many species however are able to swim as well. Common genera such as *Nais*, *Tubifex* (Aristotle already noticed worms resembling *Tubifex* in large quantities in polluted water wells (21)), *Aeolosoma*, *Chaetogaster* or *Stylaria* probably are part of the biocoenosis in all drinking water treatment plants and distribution systems. Generally, Oligochaeta may be abundant in rapid sand filters and GAC filters (69–72). A freshwater family of Oligochaeta that may infest filter beds, namely, the Lumbriculidae, resemble earthworms and provide a less attractive sight during backwashing or when the filter beds are at rest (personal observation). Removal of Oligochaeta from filter beds is possible by backwashing with 5% NaCl (70) or with 0.01 M NaOH. The presence of biofilm as food supply will however provide for the reproduction of the few remaining Oligochaeta, their eggs or new immigrants. The numbers may also be high in the distribution system, even when numbers in the filter beds are low. It is likely that the organic matter in treatment plant filter beds and the sediments in distribution systems provide the food to sustain the Oligochaeta populations in water supplies. In the Netherlands, major Oligochaeta infestations have occurred in water supplies in 1939 (73) and 1964 (74), causing many complaints and much negative publicity. Little is known about the causes and control of the first infestation, other than that it instigated the installation of the Biological Research Commission, leading to many decades of (micro)biological research since. Limiting detritus in distribution systems to cut food supply was considered the most effective long-term control measure. The second Oligochaeta infestation was the result of overloading the 90-year old river water treatment plant in Rotterdam, which was only relieved when in 1966 the new plant increased the city's water supply capacity. Meanwhile, chlorine concentrations were increased by dosing chloramine, starting at 2 mg/L Cl_2, causing disintegration of the worms. After the infestation in the distribution system subsided, numbers were controlled with chloramine doses of 1 mg/L Cl_2. Similar problems and solutions have been reported by utilities in the United Kingdom and the United States (52,75). Maintaining concentrations of 0.2 mg/L free chlorine is considered a long-term control measure for Oligochaeta (27). Short-term control measures include high chlorine concentrations and mechanical cleaning methods, for example, flushing. The long-term effects of periodic control methods are limited (76) because of reproduction of worms that survive in dead ends (e.g., mains couplings) or those that cling to mains surfaces (e.g., *Aeolosoma* and *Nais* for this purpose use their mouthsuckers and their tails respectively).

Asellidae (Crustacea, Isopoda)

Asellidae, commonly known as water lice or aquatic sow bugs, are crustaceans that feed on dead organic matter in surface water (in Europe e.g., *Asellus aquaticus* (see Fig. 1), *Proasellus meridianus* and *P. coxalis*) and groundwater (in Europe e.g., *P. hermallensis* and *P. cavaticus)*.

Figure 2 shows the mouth parts of *A. aquaticus*, enabling the organism to scrape the surface of (organic) matter, preferably in decaying condition. Reproduction is sexual, limiting chances to establish a population from low initial densities. Locomotion is limited to walking on surfaces. Their well-developed ability to cling to these surfaces enables them to migrate upstream and to pass vertical obstacles. The Asellidae which, on a regular basis, emerged from taps in Rotterdam, the Netherlands, at the end of the 19th century were thought to be reproducing in abandoned uncovered slow sand filters in the river water treatment plant and were thought to enter the finished water through cracks in the finished water canals (29). Some 30 years later in Amsterdam it was assumed that the organisms, causing complaints in this city as well, were reproducing in the distribution system itself, using biofilm as a food source (77,78). A recent survey in the Netherlands showed that Asellidae were found in 35 of 36 investigated distribution systems (45,76,87). Most consumer complaints about invertebrates in the Netherlands presently concern Asellidae and the clogging of water meters by these organisms has been reported. The number of complaints in the United Kingdom are higher in the summer because many adults die after reproduction and are swept more easily from the mains (27).

Since 1930, dozens of publications and notes described attempts to remove Asellidae from water supplies in Germany (28,79–81), the United Kingdom (30,82–86), Belgium (22), the Netherlands (78), and France (79). The most effective method to remove the organisms, albeit temporarily, proved to be flushing of the distribution system after treatment with the botanical insecticide

Figure 2. Scanning electron micrograph of the ventral view of the head showing the mouthparts of *Asellus aquaticus* (Crustacea: Isopoda), scale line = 1 mm; from (45).

pyrethrum (78,80,82) or the synthetic variant, permethrin (pyrethroids). This latter method is still applied in the United Kingdom (27) although under strict conditions to prevent consumer exposure to the risk of poisoning for kidney patients that apply extracorporeal dialysis at home and also commercial and domestic fish collections. The use of chlorine or other disinfectants to control Asellidae is very difficult (27) because the organisms are very resistant and it is difficult to maintain a high disinfectant residual in all parts of the distribution systems. In Antwerp (Belgium) and Hamburg (Germany), periodical air scouring of the distribution system proved to be very effective (22,81). Air scouring is advised in the United Kingdom as well (as is swabbing), especially because sediments and other invertebrates are removed better than during flushing with permethrin, preventing an increase of other species numbers that frequently follows a pyrethroids dosing exercise (18,27). Experiences in the Netherlands indicate that flushing the distribution system may be more cost effective than intensive methods such as swabbing and air scouring (76,88). Limiting biofilm formation and sediment accumulation in mains is thought to be the most effective long-term control measure.

Chironomidae (Insecta, Diptera)

Of the insects, the larvae of the nonbiting Chironomidae midge flies (Fig. 1) are most commonly found in distribution systems. Midge fly larvae usually range in length between 4 and 30 mm. Most likely, the species that live in drinking water distribution systems feed on organic material in sediments, which is also used by some species to build a case glued to surfaces. Outside this sediment case, they can swim in an undulating manner, much like the nonburrowing members of the same family. Especially in the first part of the twentieth century and also more recently (89,90), utilities in the United States and elsewhere were confronted with the infiltration of distribution systems by midge fly larvae via uncovered finished water reservoirs or poorly screened air inlets in treatment plants and reservoirs. In some cases, these infestations led to larvae emerging from consumer taps (6,89,91,92). The covering of reservoirs and the screening of ventilation ducts in most cases proved an effective remedy, but other control methods were used as well, including the method of adding DDT into the reservoir that is currently strongly discouraged (93). Because most insects can only reproduce sexually and adults depend on the presence of air for their oxygen supply, preventing immigration usually is effective to control the presence of insect larvae in distribution systems.

Some midge fly larvae however may procreate in the distribution system itself. This was discovered in 1937 in Germany (94) and in 1973 in Essex (England) (95). The water supplies were not able to control the organisms by closing all entrances for adult midge flies. The organisms eventually were identified as *Paratanytarsus* sp., a parthenogenetic genus that can reproduce eggs asexually in the so-called pharate adult stage before the adult stage (Fig. 3). Flushing after treatment with the insecticides pyrethrin or permethrin has proven to be an effective method (27,94,96), as it is for controlling

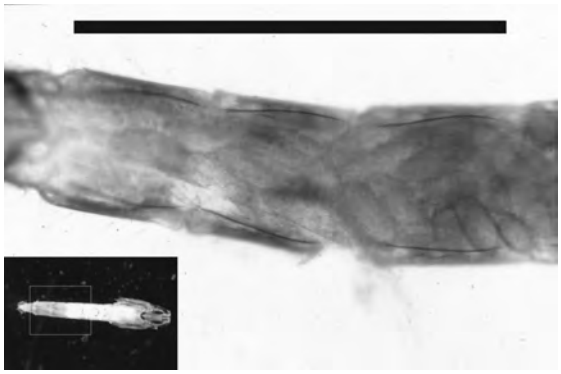

Figure 3. Pupal cuticle, containing discharged eggs, at the abdominal end of a pharate adult female of an unidentified parthenogenetic midge fly (Diptera: Chironomidae) collected from a drinking water mains in the Netherlands. Insert shows the habitus of the female, scale line = 1 mm; from J. H. M. van Lieverloo, et al., *J. — Am. Water Works Assoc.* (submitted).

Asellidae. Recently, a food-grade coagulant has been found effective in controlling the parthenogenetic species *Paratanytarsus grimmii* (97) as was shock chloramination for normal midge fly larvae (90).

Vertebrates

Eels and sticklebacks were common in early distribution systems supplying untreated surface water (98,99). Still, however, incidents of vertebrates such as a small eel clogging a water meter (in 1983) and a frog clogging a mainscock (early 1990s) have been reported in the Netherlands. These organisms must have entered the mains at one point, possibly after a mains break. Although it is not likely that these organisms survive in the mains for a long period, owing to the scarcity of food, a whole colony of eels has recently been pigged out of a potable water mains in the United States (100). Evidently, public reports on these incidents are rare.

ECOLOGY

Immigration from Groundwater

Although less conspicuous than in surface water, groundwater biocoenoses may harbor an abundance of invertebrates that are visible to the naked eye. The composition of the biocoenosis highly depends on soil structure and hydrology, because evidently the biocoenosis is interstitial (i.e., between the grains). Coarse sediments and cracks in rocky bottoms will facilitate the passage of surface water into groundwater supplies, especially via shallow wells. Soils with finer grains and coarse deep layers that are covered by fine-grained layers only harbor invertebrate species that are confined to groundwater and caves. Large sand formations usually are relatively poor in organic matter, resulting in the presence of oxygen. Even at concentrations of 0.2 mg/O_2L, invertebrates may be ubiquitously present, although it is not known whether the organisms are concentrated in pockets with higher

oxygen concentrations (101). Straining sometimes is necessary to remove relatively large groundwater species such as *Niphargus* sp. (Crustacea, Amphipoda) and *Proasellus* sp. (Crustacea, Asellidae) from groundwater obtained from coarse sediment layers (unpublished observations in groundwater from horizontal wells at 18-m depth, by the author and J. Notenboom). Finer sediments operate as giant slow sand filters and therefore allow only low numbers of small invertebrates such as Copepoda, Hydrachnellae, and Nematoda to pass into the water supply. Marshes, peats, and other formations rich in organic matter result in quick oxygen depletion, and therefore few or no invertebrates are present (101).

Immigration from Surface Water

Earlier, the abundance and diversity of the organisms in distribution systems carrying untreated surface water depended largely on the biocoenosis in the source system. Many species however cannot survive in distribution systems because of physical conditions, such as the lack of light and air, and the colonization of the system is limited by biological conditions, such as predation or unsuitable food. The constant supply of invertebrates from the source however will result in the presence of a large variety of surface water species, as was the case in many large cities in the nineteenth century and the start of the twentieth century, for example, in Breslau, Poland (102), London, United Kingdom (103); Boston, United States (33); Hamburg, Germany (98,99); and Paris, France (36). The microbiological quality of the water, although underestimated for a long time, was however of more concern than the zoological quality. Regrettably, this is still the case in large parts of developing countries. From the start of central water supply in the Netherlands (e.g., in 1853 in Amsterdam and in 1873 in Rotterdam), slow sand filters were used for surface water treatment (104). Presently, in most surface water treatment plants, protozoan and invertebrate removal is part of algae removal and is usually considered of minor importance. Microstrainers probably form the only treatment process applied specifically to remove invertebrates, especially those straining the filtrates of rapid gravity filters and GAC-filters. However, small motile invertebrates such as nematoda (59,60,62,64,65) and Rotifera (38,105) are hardly removed by some surface water treatment plants using coagulation and sedimentation followed by rapid gravity filtration, with or without chlorination. Nematoda are known to be resistant to high chlorine concentrations (106), but chlorination or ozonation before rapid gravity filtration improves the removal of invertebrates (27,38,107). Planktonic species however are better removed in treatment than benthic species (107). Furthermore, slow sand filters are known to remove invertebrates better than rapid gravity filters (27,107,108), and the backwash rate of rapid gravity filters controls invertebrate removal (109). In a survey of 17 finished water reservoirs in Germany, sediments in reservoirs of surface water supplies were found to harbor high numbers and a wide variety of planktonic species next to some benthic species, whereas the sediments in reservoirs of groundwater supplies contained only low numbers of Rotifera and nematoda. Furthermore, the numbers of Rotifera and larvae of Copepoda in one of the supplies dramatically decreased with distance from the treatment plant (110,111), probably owing to diminished food supply. Similar results were obtained in Switzerland (112). All these results indicate that penetration of invertebrates through surface water treatment plants is rather common. In finished water of at least 64% of 55 surface water treatment plants in the United States, invertebrates (predominantly nematoda) and free-living protozoans were found. Not all of these however were also found in the influent, indicating that part of the organisms may have originated from multiplication in the treatment plant itself (48).

Multiplication in Treatment Plants and Finished Water Reservoirs

Protozoans (47,49) and invertebrates may enter distribution systems by elution from water treatment systems and finished water reservoirs in which they multiply. Before slow sand filters and finished water reservoirs were covered, contamination of these systems led to infestation of the distribution systems supplied. Unscreened or poorly screened ventilation ducts still can be important immigration routes, especially for flying insects such as midge flies and for crawling invertebrates. Contamination may also occur via cracks in walls and roofs, although coliform counts will rise as a result of contaminated water seeping through, instigating inspection of structural integrity. For Asellidae, even backwash water outlets are possible routes to enter treatment plants from backwash water reservoirs or surface waters (79). Nowadays, in most cases, penetration into a treatment plant predominantly occurs from the water source, and the filter biocoenosis would seem most likely to resemble the benthic interstitial biocoenosis in (oxygenous) surface water sediments or groundwater systems. In surface water treatment plants, filter beds are expected to be harboring surface water species (21). The biocoenosis of the slow sand filters in Bremen (Germany), predominantly harboring groundwater species, resembles that of the banks of the supplying river (113). In Zürich (Switzerland), the surface water species *Canthocamptus* sp. (Copepoda, Harpacticoida) dominates the slow sand filters and the finished water reservoirs. Although the organism is not regularly found in the raw water, it is present in the sediments of the source water lake (114). However, in some plants in Germany, a limited variety of taxa have been found in the slow sand filters, predominantly consisting of groundwater species of Nematoda, Oligochaeta, and harpacticoid Copepoda, although Turbellaria, cyclopoid Copepoda, Cladocera, Asellidae, and Gammaridae have been found as well. For the slow sand filters in the water supplies using water from the river Ruhr (Germany), a colonization with groundwater species from below is likely because the filters are used to supplement the groundwater that is collected for drinking water production (115–117). GAC-filters have also been found harboring invertebrates (63,71,72,118). Filter grain size and backwashing procedures prove to be very crucial for filter colonization and invertebrate numbers in

filtrates. Removal of invertebrates from filtrates by straining with, for example, 30-μm mesh microstrainers is often necessary (68).

Immigration by Intrusion into Distribution Systems

When distribution system integrity fails (e.g., during mains breaks), organisms may inadvertently enter the mains, even when the water is still running (e.g., Asellidae). Rough surfaces, dead ends, and blind spaces in mains junctions prevent the invertebrates from being flushed out after repair. Holland (83) ascribed the presence of Asellidae in the Coventry (United Kingdom) distribution system, first detected in a water meter in 1942, to contamination of the system after many mains were broken during heavy bombardments in 1940 and 1941. Other possible immigration routes are as follows:

- Back-siphonage of wastewater or surface water via unprotected connections;
- Cross connections (18);
- Contamination during mains construction and repair (18).
- Locking-in of soil and dirt by unprofessional use of underground hydrants (i.e., opening the hydrant valve before opening the top-piece).

Chances of colonization due to (temporarily) diminished integrity of the distribution systems probably are much lower than the chances of immigration via surface water and groundwater treatment plants, but research data to corroborate this assumption are not available.

Survival and Settlement

Survival and settlement of invertebrates that penetrated into a distribution system depends on physical, chemical, and biological factors. Although air pockets may remain in mains after air scouring, normally air is absent and adult insects are bereft of oxygen (their larvae breathe through their skin or use gills). Most planktonic organisms will not withstand the current in distribution systems, although velocities are usually very low, and will be flushed out via consumer connections. Most Monogononta (Rotifera), for instance, are planktonic surface water species and their presence in distribution systems will depend entirely on their ability to pass surface water treatment. As far as chemical composition is concerned, normal concentrations of chlorine and chloramine in water leaving treatment plants are not very effective against most invertebrates found in distribution systems (27), but do limit the survival of some invertebrates. For instance, 1 to 2 mg/L of free chlorine will limit settlement of freely swimming mussel larvae (Bivalvia) (27). Considering drinking water standards in most countries, toxicity of other compounds will probably not be a determinative factor. Salinity, acidity, or alkalinity may however be too low to support some freshwater organisms. Facultative parasites, pathogenic microorganisms, and especially predators may have a large impact on chances for colonization of the distribution system by an invertebrate species. Competition for food however is most likely to determine long-term chances of successful colonization.

Feeding, Growth, and Reproduction

Availability of sufficient and suitable food is most likely to determine the abundance of invertebrates in distribution systems. Planktonic species (most Rotifera, Cladocera, and Copepoda) and sessile filter feeders [Porifera (sponges), Coelenterata (polyps), Bivalvia (mussels), and Ectoprocta (bryozoans)] depend on relatively high densities of algae or POM (particulate organic matter) in the water. Removal from surface water by coagulation and sedimentation, especially if followed by filtration, usually suffices to limit growth of these taxa in distribution systems. A study of invertebrates in sediments in finished water reservoirs in Germany revealed a significant decrease of Rotifera and larvae of Copepoda during distribution. The numbers of two planktonic surface water species of Rotifera (*Notholca caudata* and *Triarthra longiseta*) clearly decreased, whereas the numbers of benthic Rotifera slightly increased (111).

In most distribution systems, concentrations of dissolved compounds present in finished water from treatment plants are most likely to determine the abundance of bacteria, protozoans, and invertebrates. On all water-exposed surfaces in the distribution systems, benthic bacteria form the basis of a biofilm, a slime layer colored yellow, brown, or black by suspended particles that attach to it. Probably most of the bacteria are heterotrophic, using organic matter both as an energy source and as a source of base materials. Additionally, incomplete removal of ammonium, nitrite, and methane in treatment plants using anoxygenous groundwater will lead to growth of nitrifying bacteria and methane-oxidizing bacteria in distribution system biofilms as well. In these biofilms, the bacteria form the living food for protozoans and invertebrates (Nematoda, benthic Rotifera, Gastrotricha, Tardigrada, Turbellaria, Gastropoda, Ostracoda, benthic Copepoda, benthic Cladocera, juvenile Hydrachnellae, and Chironomidae larvae) that graze on these bacteria and protozoans. Large invertebrate species will mainly be found feeding on the biofilms at the bottom of mains and the biofilm-covered sediment particles accumulating there. These sediments are formed by (*1*) precipitation of particles suspended in finished water, (*2*) insoluble compounds formed in mixed water types, (*3*) parts of mains materials, and (*4*) sloughed-off biofilms, increasing the surface area available for bacteria and protozoans to grow on. Oligochaeta burrowing in these sediments ingest whole sediment particles and Asellidae scrape off biofilm from surfaces, meanwhile ingesting rust, bitumen, and asbestos fibers, as shown by the composition of fecal pellets of Asellidae ubiquitously found in sediments collected from mains (41). Small invertebrates such as Rotifera and small nematoda are attached to the biofilm while feeding and are likely to be ingested by biofilm grazers and sediment feeders. Planktonic Copepoda are found whirling up particles to feed on while skimming the sediment surface. Predators of many taxa may thrive on other invertebrates, provided the abundance of prey is sufficient and predators are not visually orientated. Figure 4 schematically summarizes the hypothesis

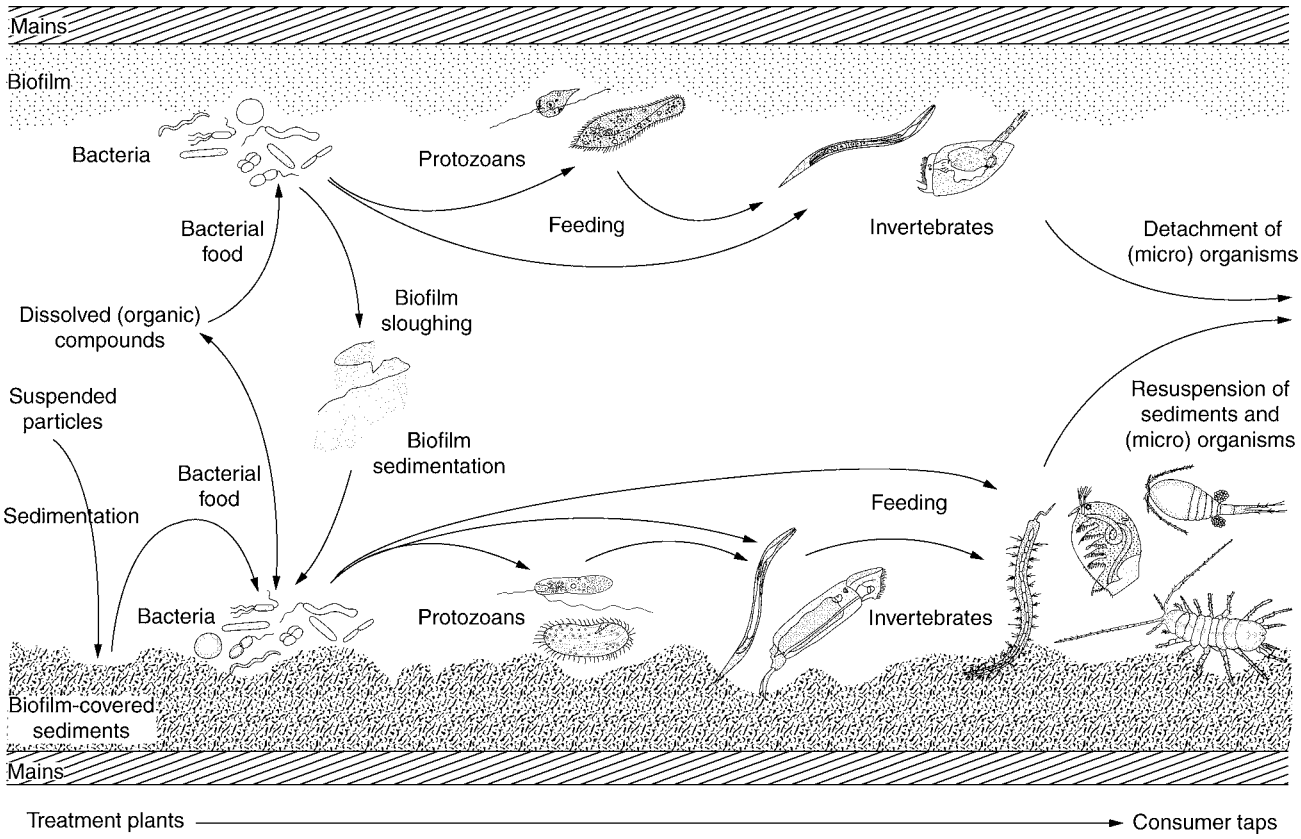

Figure 4. Schematic depiction of the hypothesis on food supply and feeding in drinking water distribution system biocoenoses; from J. H. M. van Lieverloo et al., *J.—Am. Water Works Assoc.* (submitted).

of food supply and feeding processes in the distribution systems (45).

Those species that are successfully competing for food in distribution systems will also depend on reproduction for establishing and maintaining a population. Although protozoans and most drinking water invertebrates can reproduce asexually, some taxa, for example, Copepoda and Asellidae, can only reproduce sexually, causing their chances of colonizing a distribution systems to depend on the initial population density, that is, the chances of mating. The abundance of relatively large benthic invertebrates in distribution systems such as Asellidae and Oligochaeta, compared with the low abundance of most of these organisms in finished water from treatment plants, could be seen as an indication that reproduction in the distribution system is largely determining abundance for these taxa. It is however very difficult to quantify immigration of benthic invertebrates walking and sliding into distribution systems, considering the size of trunk mains carrying the finished water of treatment plants.

ABUNDANCE

Methods to Assess Invertebrate Abundance

Freshwater zooplankton is usually collected by filtering water through plankton nets. Benthic invertebrates usually are scooped from the bottom or are filtered from sediment samples. Owing to the closed character of distribution systems, filtering of water emerging from the system is the most practical and cost-effective sampling technique. Planktonic invertebrates and suspended benthic invertebrates are collected by filtering finished water from treatment plants and by filtering drinking water from consumer taps, using plankton nets or filters, usually ranging between 10- and 45-μm mesh. In the Netherlands, 10-μm mesh filters are most commonly used, for it is known that large percentages, especially of protozoans, Nematoda, and Rotifera will pass even these filters. Benthos is collected by flushing or air scouring the mains via hydrants and filtering the flushing water with plankton nets, usually ranging between 30- and 500-μm mesh. Figure 5 shows a special sampling top-piece that was developed in the Netherlands over a period of 50 years for collecting invertebrates from mains. Of most taxa, an average of 50% or more is flushed from the mains; Oligochaeta and Chironomidae are flushed out less effectively however. On an average, only of Nematoda, Turbellaria, and larvae of Copepoda, less than 50% of the individuals is retained in the 100-μm mesh filters. Presently, extra 30-μm mesh filters are used to improve sampling efficacy (119). The invertebrates in the samples are identified and enumerated using an inverted microscope (drinking water samples) or a stereomicroscope (flushing-water samples).

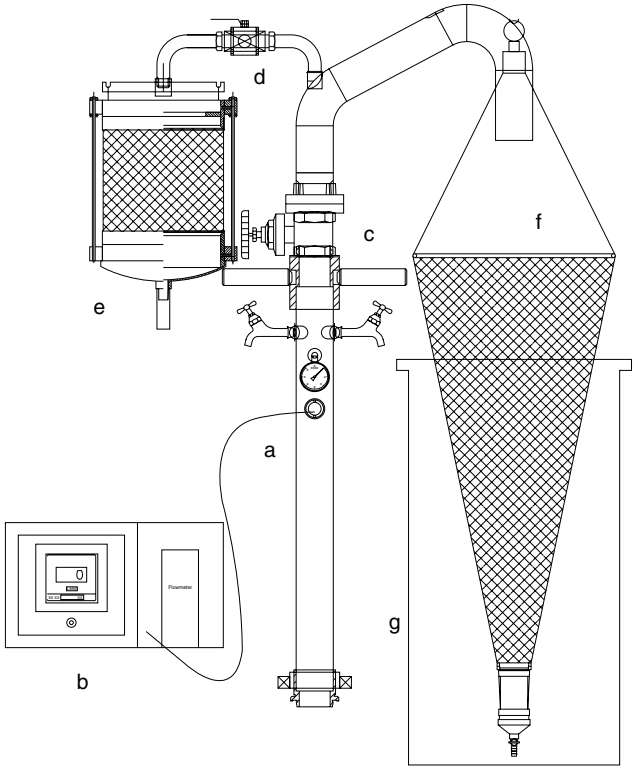

Figure 5. Drawing of the device for sampling flushing water, consisting of a top-piece for underground hydrants and filters a = Georg Fischer MK515 paddle wheel flow sensor in an aluminum plug; *b* = Georg Fischer F86 flow and volume indicator; *c* = slide valve; *d* = ball valve; *e* = 100-μm mesh plankton gauze filter containing a removable 500 μm mesh plankton gauze filter; *f* = 500-μm mesh plankton net; *g* = filtrate vessel with overflow. Not shown is the 30-μm mesh plankton gauze filter presently used to filter the filtrate of the 100-μm mesh filter. (From J. H. M. van Lieverloo et al., *Water Res.* (submitted).

Invertebrate and Protozoan Abundance in Drinking Water

Although water utilities may be aware of the presence of invertebrates in their distribution systems, not many periodically assess the size and composition of the community, most likely because it is not obligatory. If they do so, this information usually is not published (120). Results of some surveys, mostly targeting Nematoda, have been published (53,61,111,121,122) along with results of periodical monitoring by water utilities (22,58,68,114,116,123). In Figure 6, mean numbers of invertebrate taxa in finished water from treatment plants, drinking water from consumer taps and flushing water from mains are presented. The figure shows that data from literature (period 1948–1996), data from six utilities in the Netherlands using surface water (period 1964–1995), and those from a recent survey of 36 distribution systems in the Netherlands (period 1993–1995) (45,76,124) match surprisingly well for most of the invertebrate taxa.

Cladocera and Copepoda dominate the mains biocoenosis in numbers, mean numbers mounting to several hundreds per cubic meter of flushing water, whereas these and other visible invertebrates are hardly found in finished water from treatment plants and in drinking water from consumer taps. Most organisms under normal flow conditions apparently are not easily whirled up from the bottom of the mains. Owing to their size, Asellidae and Oligochaeta dominate the mains biocoenosis in biomass, forming approximately 86% and 12% respectively of total invertebrate biomass in the Netherlands (based on estimated biomass per individual organism) (45). Numbers in finished water from treatment plants and drinking water from consumer taps are dominated by protozoans, Rotifera, Nematoda, and Copepoda. Numbers of protozoans, Rotifera and Nematoda probably also are very abundant in the mains, but on account of their limited size, their numbers usually are not accurately (Nematoda) determined in water flushed from mains.

CONTROL

In 1964, the Biological Research Commission in the Netherlands, consisting of biologists and operators of research institutes and water utilities, formulated the following recommendations for invertebrate control: (*1*) limit assimilable organic matter in drinking water by intensive water treatment, (*2*) prevent mains contamination during mains construction and repair (and caused by cross connections) and (*3*) systematically flush distribution systems (18). In 1982, this view was still endorsed by the industry, adding the obvious necessity to adequately remove invertebrates in treatment plants (68). The balance of the intensity of all these control measures should depend on cost-benefit evaluations that include benefits for other variables (e.g., microbiology, color), environmental costs (e.g., wasting flushing water), and inconvenience to communities (e.g., pressure drops and colored water during flushing).

Limitation of Immigration

When finished water is microbiologically safe, immigration of invertebrates is not likely to play a major role in the abundance of invertebrates in distribution systems and drinking water. The increased awareness of possible contamination of finished water with the very small (oo)cysts of *Giardia* spp. and *Cryptosporidium* spp. will enhance the quality of many treatment plants throughout the world, probably diminishing the possibilities of a contamination by invertebrates even further. Furthermore, awareness of the risks of intrusion of microorganisms into distribution systems is growing (127), which could lead to the limitation of intrusion of invertebrates as well.

Periodical Removal from Distribution Systems

Flushing of distribution systems, either only with water or by injecting air (air scouring) or by introducing plastic swabs (swabbing), is only effective temporarily because the organisms that were not removed will be able to reestablish the population, using sediments and biofilms as food. If distribution systems are flushed periodically, abundance and species diversity will diminish and some species will be flushed out entirely. Many benthic species such as Asellidae however are used to periodic increases of water flows (128) and will be able to withstand high water

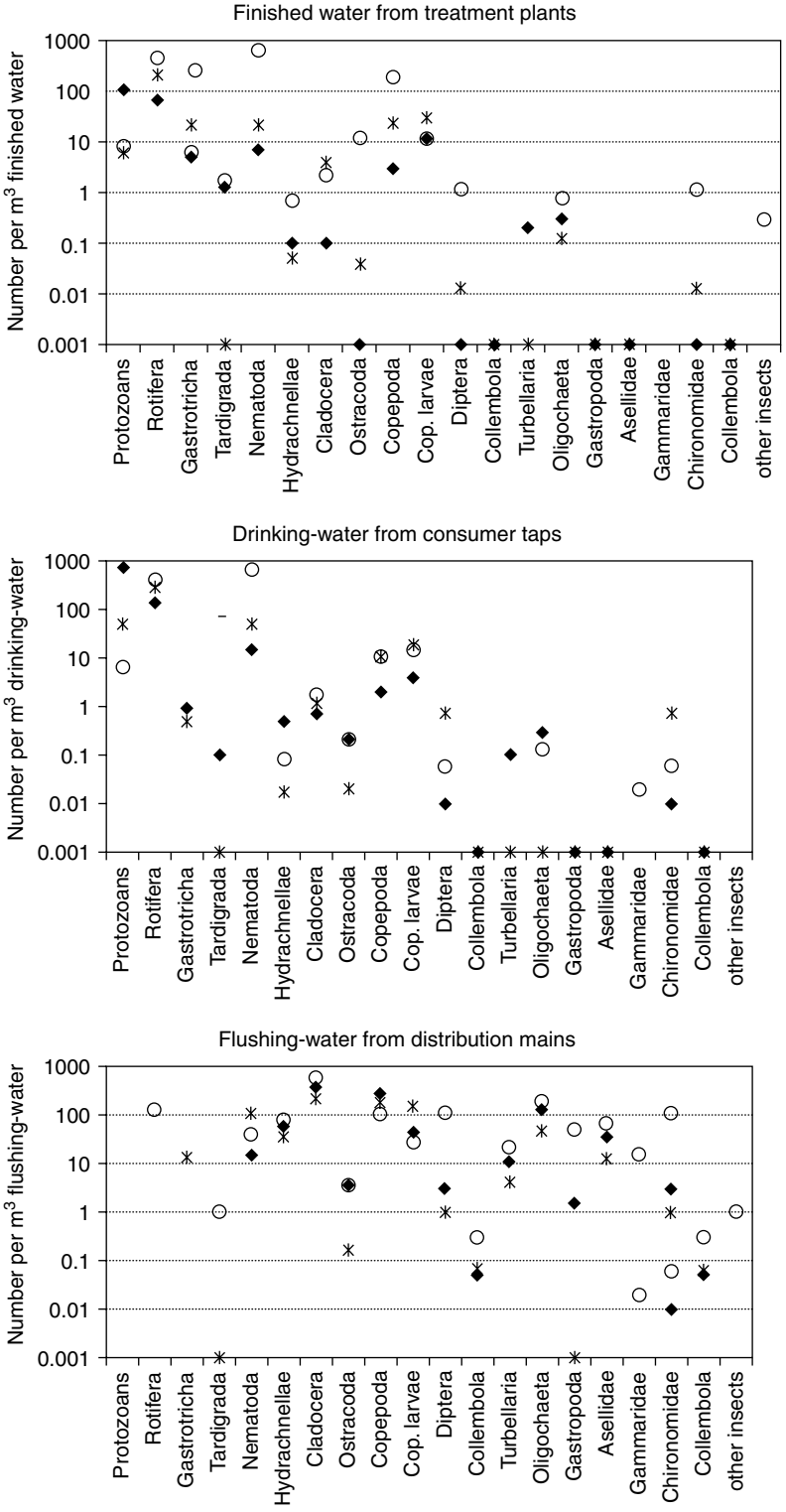

Figure 6. Abundance of protozoans and invertebrates in water supplies. ○ Literature data: arithmetic mean of means provided, calculated, or estimated per reference (1948–1996) (22,50,58,59,61,65, 68,78,95,105,107,111,114,116,121–123,125,126). ∗ Data from six utilities in the Netherlands predominantly using surface water, one report per utility (1964–1995): mean of means provided per report. ♦ Averages from a survey in distribution systems of 36 treatment plants in the Netherlands (1993–1995) (45,76,124). Means of invertebrates taxa that were looked for, but were not found, are presented as 0.001 per 1,000 L.

velocities (83). In seven experiments studying sampling accuracy in the Netherlands, only 42% (SD 15%) of living Asellidae and 83% (SD 29%) of dead Asellidae were sampled from mains at 1,0 m/s (119). Another study in the Netherlands showed that levels of invertebrates (predominantly Asellidae and Oligochaeta), sediments, and bacteria in flushing water from hydrants were back to previous levels within nine months after systematically cleaning parts of distribution systems. Only fine sediment volumes (retained in 30- to 100-μm mesh filters) remained significantly lower for more than 15 months. Although the immediate effects of intensive techniques such as air scouring or pigging were larger than the effects of flushing, there were no long-term differences, probably

because of reaccumulation of sediments from trunk mains (not cleaned) and from the treatment plants (76,88,124). In Antwerp, Belgium, good results were reported in removing Asellidae and Gastropoda only after five years of annually air scouring all mains generally distributing water at low flows, that is, dead ends, mains with drastically changing diameters and shuttle zones. Consumer complaints about invertebrates dropped from 25 per year (1975) to below 1 per year (since 1985) (22). In Hamburg, Germany, and in the United Kingdom, air scouring is used successfully as well (27,81). The cleaning frequency required is likely to depend on the rate of sediment accumulation and biofilm formation in the distribution system. If only mechanical methods are used, multiplication of invertebrates is usually only limited by cleaning very frequently. Although the organisms under these conditions may not get very large, the reproduction rate of species such as Asellidae and Oligochaeta probably is enough to keep the population stable albeit small. Low-frequency application of mechanical cleaning methods is less effective because important parts of distribution systems cannot be reached by mechanical methods, that is, dead ends, mains couplings, and, in case of corroding cast iron mains, encrustations. Chemical methods are more effective in killing these invertebrates, when used in combination with systematic flushing, although diffusion rates into dead spaces do limit the efficacy of these methods as well. Asellidae, Gammaridae, and Insecta are very sensitive to pyrethrins or permethrin, pesticides that are used in the United Kingdom for controlling these organisms under strict conditions (27). Oligochaeta can be controlled by maintaining 0.5 to 1.0 mg/L free chlorine residuals for weeks in combination with systematic cleaning of the mains (27).

Prevention of Multiplication of Invertebrates and Microorganisms

Multiplication of invertebrates in distribution systems probably depends on the presence of food sources, namely, biofilms and (biofilm-covered) sediments. Biofilm formation may be limited by maintaining a disinfectant residual in the distribution system and it may have a limiting effect on some invertebrates as well. An infestation with Oligochaeta can be prevented with residual concentrations of 0.2 mg/L free chlorine (27). Normal residuals of chlorine and chloramine in water leaving treatment works however are not very effective against most of the invertebrates found in distribution systems (27). Secondly, it is difficult to maintain a disinfectant residual throughout the distribution system. In the Netherlands, 65% of drinking water is produced from groundwater supplies, treated and distributed without a disinfectant, while surface water is treated in multiple barrier plants including chemical disinfection. Only 22% of all drinking water is chlorinated after treatment and in only 10% of all drinking water, a disinfectant residual is detectable, most of it barely (0.05 mg/L). Multiplication of microorganisms and invertebrates in distribution systems is limited by a far-reaching removal of biodegradable compounds in water treatment, aiming at achieving water with a high degree of biological stability. Biological stability of water can be assessed by measuring concentrations of easily assimilable organic carbon (AOC) (129,130), concentrations of biodegradable dissolved organic carbon (BDOC) (131,132), or by assessing the biofilm formation rate (BFR) (133).

Furthermore, maintaining biological stability in distribution systems also implies the use of biostable materials, for example, plastic materials that do not release biodegradable compounds into the water (134).

Benchmark Values

Guideline values for invertebrate numbers in distribution systems should be the result of balancing costs for invertebrate control and consumer perception of water quality. A quantitative relation between invertebrate numbers in distribution systems and consumer perception however is not yet available, partly because of the low number of consumer complaints. Benchmark values, based on percentiles of numbers found in distribution systems, can be used by water utilities for comparing their chances of consumer complaints.

If monitoring shows high numbers compared with benchmark values, chances of consumer complaints are higher as well, increasing the incentive for control. In the Netherlands, national benchmark values have been collectively set in 1993 for surface water supplies (136), which recently have been extended and amended to apply for all water supplies (Table 1). Finished water from surface water treatment plants is sampled monthly, independent of abundance. Other sampling frequencies depend on invertebrate numbers in previous samples. Samples are collected every five years if numbers previously found were low to normal, every three years when numbers were elevated, every year when numbers were high, and every six months when numbers were very high. One sample per series is collected from finished water of a (groundwater) treatment plant, and for each 5.10^9 L/yr drinking water distributed, one flushing-water sample is collected in the distribution system (but at least three flushing-water samples are collected for each treatment plant) (45,135). Benchmark values proposed in East-Flanders (a province of Belgium) for individual samples are less discriminating: 0.4/1000 L (elevated), >= 2/1000 L (high), and >= 20/1000 L (very high) for all large invertebrates (generally the same taxa as in the Netherlands, benchmarks valid per taxon, values per square meter converted to values per 1000 L, assuming an inner mains diameter of 100 mm). Values for small invertebrates are 1,000 times higher, values for very small organisms are approximately 2,000 times higher than values for large invertebrates (137). In the city of Antwerp, Belgium, guideline values for Asellidae and Gastropoda (snails) are below 10 per 100 m^2, that is, 4/1000 L (assuming 100 mm inner diameter) based on a relationship between complaints and abundance (22). The benchmark value for median numbers of Asellidae in the Netherlands is strikingly higher ($\geq 30/m^3$ is considered high), considering the limited numbers of complaints reported by the water utilities in the Netherlands (estimated 10 per year) (45).

Table 1. Benchmark Values in the Netherlands for Protozoans and Invertebrate Taxa in Finished Water from Treatment Plants and Water Flushed from Mains (Numbers/1000 L) [From J. H. M. Van Lieverloo, *J. Am. Water Works Assoc.*, (submitted) (45).]

	Finished Water From Treatment Plants[a]							Water Flushed from Distribution Mains[b] (both surface water and groundwater)			
	Elevated numbers[c]		High numbers		Very high numbers						
	Surface water	Groundwater	Surface water	Groundwater	Surface water		Ground water	Elevated	High	Very high numbers	
Numbers/1000 L	Median ≥	Sample ≥	Median ≥	Sample ≥	Median ≥	Sample ≥	Sample ≥	Median ≥	Median ≥	Median ≥	Sample ≥
Very small (<0.5 mm)											
Protozoa	10	30	30	100	500	3,000	1,000	Not determined			
— Ciliophora	5	nd[d]	10	nd	500	1,000	nd	These very small organisms are not collected quantitatively by filtering through 30-μm mesh plankton filters. Furthermore, these organisms are difficult to identify and quantify between the sediment particles in samples.			
— Gymnamoeba	5	nd	5	nd	5	100	nd				
— Testacea	5	nd	10	nd	30	500	nd				
Rotifera	300	30	1,000	100	5,000	10,000	500				
Gastrotricha	5	5	5	10	300	1,000	50				
Tardigrada	5	5	5	5	10	30	30				
Small (0.5–2 mm)											
Nematoda	30	5	80	10	100	1,000	100	50	300	1,000	10,000
Hydrachnellae	5	5	5	5	5	5	5	10	50	100	1,000
Cladocera	5	5	5	5	5	30	10	50	300	1,000	10,000
Ostracoda	5	5	5	5	5	5	5	10	10	10	100
Copepoda	5	5	80	10	100	1,000	50	30	300	1,000	5,000
Copepoda larvae	10	5	300	30	1,000	5,000	100	10	100	300	5,000
Large (>2 mm)											
Turbellaria	5	5	5	5	5	10	10	10	30	100	1,000
Oligochaeta	5	5	5	5	5	30	10	30	100	300	10,000
Gastropoda	5	5	5	5	5	5	5	1	1	5	50
Asellidae	5	5	5	5	5	5	5	1	30	100	100
Chironomidae	5	5	5	5	5	5	5	1	1	3	50

[a] 200 L from a finished water tap is filtered through a 10-μm mesh filter.

[b] 1,000 L is flushed out of mains via a hydrant at a flow increase (in mains) of 1.0 m/s and filtered through 500-μm mesh plankton filters. Simultaneously, approximately 100 L of the volume is also filtered through a 30-μm mesh filter (119).

[c] Median numbers per system (treatment plant, distribution system) or numbers in individual samples are classified 'low to normal numbers' when lower than the benchmark value for 'elevated numbers'. Numbers are 'elevated' when higher than or equal to the benchmark value for 'elevated numbers' but lower than the benchmark value for 'high numbers' etc. Benchmark values can be equal for different classifications due to detection limits. Detection limits are 5/1000 L for finished water from treatment plants and 10/1000 L for water flushed from mains.

[d] nd: not distinguished. Protozoan taxa are not distinguished separately in groundwater supplies.

CONCLUSION

The presence of invertebrates in drinking water can be a nuisance to consumers and therefore to water utilities, causing complaints about visible organisms, their body parts, or fecal pellets emerging from taps. The blocking of water supply, especially in water meters, and effects on taste, odor, and microbiological quality are known to occur as well. According to the World Health Organization, the presence of invertebrates in finished water from surface water supplies however is no health risk in developed countries (2). This view is not generally endorsed because of risks attributed to surface water invertebrates protecting pathogenic microorganisms from disinfectants in treatment plants (13,138). Protozoans are known to protect (micro-)organisms from disinfectants, and in some species *Legionella* bacteria may multiply. Therefore, protozoans and invertebrates, especially nematoda, are still under scrutiny from a health point of view (5,139,140).

Invertebrates and protozoans present in source water may immigrate into distribution systems by passing treatment systems, others may intrude via openings in the structure of treatment plants, finished water reservoirs, and distribution systems. In these systems, the organisms may multiply, provided they can adapt to the conditions and they can find enough and suitable food. Adult insects, for instance, are not able to survive in distribution systems because of the absence of air. Planktonic species and filter feeders will perish in most systems owing to a lack of suspended food particles. Benthic grazers, scrapers, and sediment feeders are most likely to flourish, especially when biofilm formation rate on water-exposed surfaces, including sediments, is high. Normal disinfectant residuals will only partly limit biofilm formation, invertebrate survival, and invertebrate multiplication, whereas periodical removal with pesticides, high disinfectant concentrations, or mechanical methods will only temporarily control invertebrate numbers. Because invertebrate populations are able to reestablish quickly, using biofilm and newly accumulated sediments, water utilities will be forced either to frequently repeat reactive control measures or to proactively limit biofilm formation rates and sediment accumulation rates. Considerations about increasing disinfectant residuals to control invertebrates would have to include a balance between consumers opinion about rarely detecting visible invertebrates and a continuous presence of a taste of disinfectants. The limitation of consumer complaints and microbiological deterioration of drinking water during distribution is likely to be attained most efficiently by limiting the availability of food sources for bacteria, protozoans, and invertebrates in distribution systems. An integral cost-benefit evaluation, including the costs of flushing and the use of bottled water as well as the benefits on other quality aspects such as microbiology, toxicology, and discoloration, is however necessary to substantiate further investments in treatment plants.

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ION EXHANGE IN WATER TREATMENT PLANTS: PATHOGEN REMOVAL.

See MICROBIAL REMOVAL BY PRETREATMENT, COAGULATION AND ION EXCHANGE

IONIZING RADIATION, EFFECT ON MICROORGANISMS.

See SPACE MICROBIOLOGY: EFFECTS OF IONIZING RADIATION ON MICROORGANISMS IN SPACE

IRON CYCLING.

See METAL (U,Fe,Mn,Hg) CYCLING

IRON OXIDATION.

See BIOMINERALIZATION BY BACTERIA; *GALLIONELLA FERRUGINEA*: AN IRON-OXIDIZING AND STALK-FORMING GROUNDWATER BACTERIUM

IRON-REDUCING MICROBES IN PETROLEUM RESERVOIRS. See PETROLEUM RESERVOIRS, INFLUENCE, ACTIVITY AND GROWTH OF SUBSURFACE MICROFLORA IN

ISOSPORA

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Isospora species are coccidial parasites of man and domestic animals. The intestinal disease caused by infection by these intracellular parasites is called *coccidiosis*. Human cases of *Isospora belli* coccidiosis are often found in underdeveloped countries of the world where sanitation is poor. Detection of the parasite in feces is the best way to confirm infection.

CLASSIFICATION

Isospora species are in the Kingdom Protista, Phylum Apicomplexa, Class Sporozoasida, Subclass Coccidiasina, Order Euccocidiorida, Suborder Eimeriorina, Family Sarcocystiidae, Genus *Isospora* (1,2). They are in the group of organisms referred to as *coccidia*. They are a group of parasites that must live inside the cell of a host animal. Only their resistant cyst stage (the oocyst) can survive outside of a host cell in the environment (Fig. 1). Coccidia are economically important pathogens of poultry, domestic farm animals, and humans. Coccidia have extremely complex life cycles. Members of the genus *Isospora* can complete their entire life cycle in a single host. However, several have evolved the ability to use paratenic hosts (transport hosts) in their developmental cycles. *I. belli* is the only member of the genus *Isospora* that infects humans, whereas other *Isospora* species are parasites of cats, dogs, pigs, and monkeys. The present discussion primarily considers *Isospora belli*.

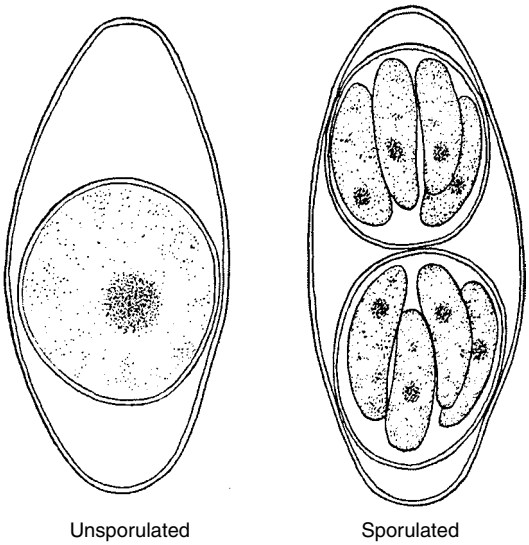

Figure 1. Line drawings of unsporulated and sporulated oocysts of *Isospora belli* from humans.

STRUCTURE AND DEVELOPMENT

Isospora species are eukaryotic protozoan parasites. Their developmental stages are inside of a host cell within a host cell membrane–derived vacuole. Only the environmentally resistant oocyst stage can live outside of a host cell. The Isospora life cycles are complex (Fig. 2). The structure of a specific stage varies with its place in the developmental cycle. The asexual stages are structurally different from each other and anisogametes are produced. Different organelles are present in asexual and sexual stages, but the cell membrane, nucleus, ribosomes, endoplasmic reticulum, golgi bodies, and mitochondria are present in all Isospora developmental stages. The motile asexual stages possess an additional compilation of organelles that form the apical complex of the parasite. The coccidian apical complex is composed of polar rings, conoid, rhoptries, micronemes, and subpellicular microtubules (3). The apical complex is probably involved in host cell penetration. The body of the sporozoite or merozoite is enclosed by a trimembranous pellicle.

The infective Isospora oocyst consists of an oocyst wall that surrounds two sporocysts. Each sporocyst consists of a sporocyst wall that encloses four sporozoites. The wall may contain a pluglike Stieda body in some species or the sporocyst wall may be composed of four plates in other species. The size and shape of an oocyst and sporocyst are characteristic of a particular *Isospora* species.

Genetics

Little is known about the genetics of the *Isospora* species. Phylogenetic analysis of small subunit ribosomal RNA gene sequences from the two major types of *Isospora* species — those with a Stieda body in their sporocysts and those without a Stieda body in their sporocyst (i.e., *I. belli*)–indicates that the two belong to different families (2,4). Based on genetic analysis there is agreement that members of the genus *Isospora* that lack Stieda bodies in their sporocysts should be removed from the family Eimeriidae (2,4,5) and placed into the family Sarcocystidae within the suborder Eimeriina (4).

Life cycle

Coccidial life cycles are complex (Fig. 2). Exogenous (outside the host) and endogenous (inside the host) cycles are present. Paratenic (transport) hosts may also be present.

Exogenous cycle. Sporogony occurs outside the host and is the exogenous phase of the coccidial life cycle. Sporogony (sporulation) is the developmental process that leads to production of infective sporozoites within sporocysts inside the oocyst. Sporogony is dependent on moisture, temperature, and adequate oxygen. Temperatures greater than 40 °C or less than 20 °C inhibit sporulation of Isospora oocysts. Rapid sporulation (<16–24 hours) of

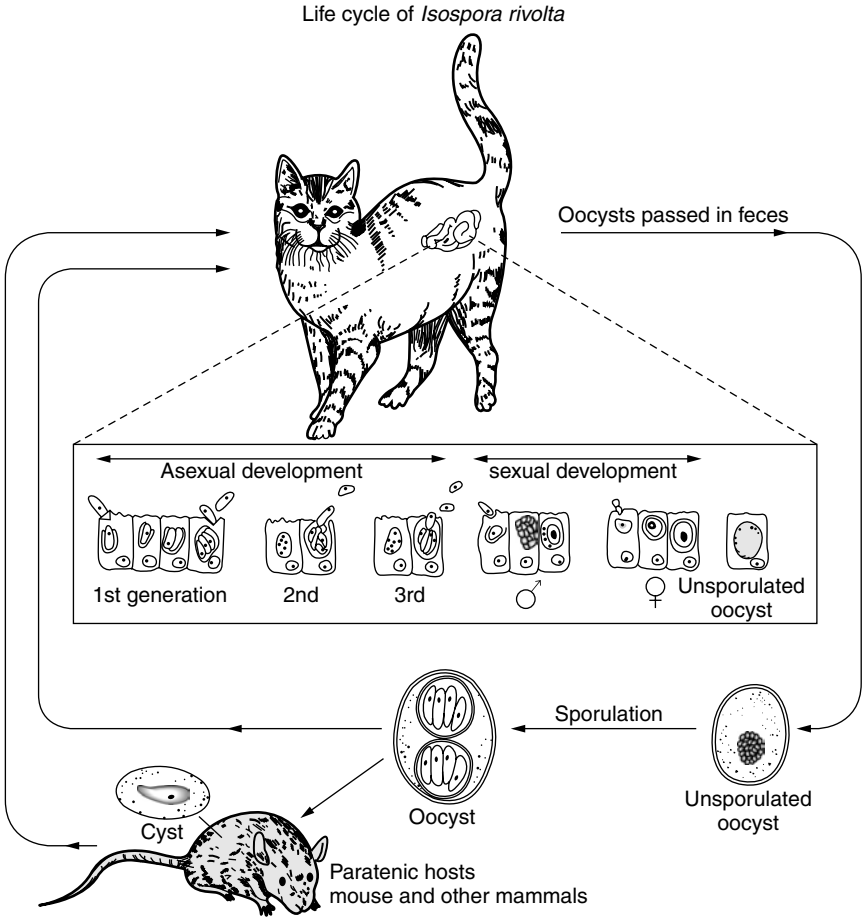

Figure 2. Life cycle of *Isospora rivolta.*

oocysts occurs at 30 or 37 °C. Structural events that occur during sporogony are similar in all *Isospora* species of mammals. Oocysts are excreted in the feces and they usually have a contracted sporont. A few oocysts that are in the sporoblast stage (two-celled stage) are excreted. As the nucleus of the sporont divides, a clear nuclear streak is formed; nuclear division occurs and the sporont divides to form two uninucleate sporoblasts. Nuclear division occurs again and the nuclei are visible as clear areas at the poles of the sporoblast. Nuclear pyramids may be seen at the poles of the sporoblasts. The sporoblasts become elongated and form the sporocyst stage. Nuclear division occurs again and the outline of developing sporozoites soon becomes visible. When the sporozoites are fully visible the oocyst is considered sporulated.

Excystation. Excystation is the process by which sporozoites are released from the sporocysts and oocysts. The process is basically the same for all *Isospora* species of mammals (6). Sporozoites become motile within the sporocysts and tumble or glide around one another. This movement is not continuous, but is interrupted by periods of inactivity. Eventually, the sporocyst wall opens along four platelike junctions and the sporozoites exit through the openings that are formed. Sporozoites excyst from oocysts through indentations or fractures that form in one or both ends of the oocyst wall.

Endogenous Development. Sporozoites enter cells in the intestine but usually do not form rounded uninucleate trophozoites. Some sporozoites and/or merozoites leave the intestine and form dormant cyst stages in extraintestinal tissues. Intestinal sporozoites may retain their elongate sporozoite shape, become binucleate, and divide by endodyogeny to form two daughter merozoites. These daughter merozoites divide by endodyogeny an indefinite number of times. For this reason, the number of sequential asexual merogenous cycles cannot be determined and developmental stages are referred to as structural types instead of generations (6). Eventually, multinucleate meronts are formed. These meronts are elongate and retain their merozoite shape. Several meronts may occur in the same host cell. Eventually, sexual stages are formed. Macrogamonts are uninucleate and are the female gamete, whereas microgamonts are multinucleate and produce biflagellated microgametes (sperm). Microgamonts and macrogamonts may occur in the same host cell. After fertilization, a resistant oocyst wall is laid down around the zygote. The endogenous life cycles in animals that ingest oocysts and in those that ingest paratenic hosts are similar (7). The prepatent period may be shortened in these infections.

Extraintestinal Stages. Extraintestinal stages occur in the tissues of the definitive host in canine and feline *Isospora* species (7–9) and *I. belli* of humans (10). Instead

of undergoing the normal developmental cycle in the intestinal tract some sporozoites or merozoites leave and invade extraintestinal sites in the host. Mesenteric lymph nodes are most often infected; other tissues such as the liver, spleen, and tracheobronchial and mediastinal lymph nodes can also be infected. Infected host cells are probably macrophages.

Mice, rats, hamsters, dogs, cats, cattle, sheep, and camels have been shown to be paratenic hosts for several *Isospora* species. Sporozoites exit from oocysts and invade extraintestinal tissues. Mesenteric lymph nodes are most often infected; other tissues such as spleen, liver, and skeletal muscles are sometimes parasitized. Parasites are most often found as single organisms: division has not been confirmed. For this reason it is more accurate to refer to the host as a paratenic, rather than an intermediate host.

CLINICAL DISEASE AND EPIDEMIOLOGY

Clinical Symptoms

Isospora belli causes serious and sometimes fatal disease in immunocompetent humans. As in all coccidial infections, infants and young children suffer more serious diseases than do adults. Symptoms of *I. belli* infection include diarrhea, steatorrhea (fatty diarrhea), headache, fever, malaise, abdominal pain, vomiting, dehydration, and weight loss (11,12). The cause of the headache is not known. Blood is not usually present in the feces, helping to differentiate *I. belli* diarrhea from other intestinal protozoa (i.e. *Entamoeba histolytic* and *Balantidium coli*). The disease is often chronic, and parasites are present in the feces or small intestinal biopsies from several months to several years. Recurrences are common because of extraintestinal stages in lymph nodes (10).

Isospora belli infection produces diarrhea in AIDS patients. The diarrhea is often very fluid and secretory like, and leads to dehydration necessitating hospitalization. Fever and weight losses are also common findings. Other opportunistic pathogens are also common copathogens in these patients. *I. belli* induces intestinal lesions and responses to chemotherapy are usually similar to those observed in immunocompetent patients.

Isospora belli infections are essentially cosmopolitan in distribution but are more common in tropical and subtropical regions, especially Haiti, Mexico, Brazil, El Salvador, tropical Africa, Middle East, and Southeast Asia. Poor sanitation is a major contributor to *I. belli* transmission.

Modes of Transmission

The only known mode of transmission of *I. belli* is by ingestion of sporulated oocysts in contaminated food or water. Transmission studies indicate that humans are the only true definitive hosts following administration of *I. belli* oocysts. The use of human feces as fertilizer can lead to the contamination of food that is eaten uncooked. Transport hosts such as flies and cockroaches may mechanically carry *I. belli* oocysts on or in their bodies and spread the oocysts from one location to another.

Reservoirs

It is not known if animals may serve as paratenic hosts for *I. belli*. The role of paratenic hosts in the transmission of *I. belli* needs to be investigated to establish if a possible mode of transmission other than by ingestion of oocysts into contaminated food or water exists.

Persistence in the Environment

Coccidial oocysts differ in their ability to survive in the environment, depending on their being sporulated or unsporulated. Unsporulated oocysts are more sensitive to environmental conditions than are sporulated oocysts because sporogony is an active aerobic process. Environmental temperature and availability of oxygen and moisture influence sporogony and oocyst survivability. High temperatures ($>35\,^{\circ}C$) and low temperatures ($<10\,^{\circ}C$) adversely affect the sporulation of coccidial oocysts. Unsporulated oocysts may survive freezing to $-7\,^{\circ}C$ for nearly two months but are usually inactivated within 24 hours at $40\,^{\circ}C$. They do not survive desiccation.

High environmental temperatures are more detrimental to the survival of sporulated oocysts than are low temperatures. Sporulated oocysts are unlikely to survive freezing for more than a few days, or temperatures of $>60\,^{\circ}C$ for more than a few hours. Sporulated oocysts will probably survive for a year or more in the environment under appropriate conditions. Sporulated oocysts cannot survive desiccation.

Methods of Detection

Isospora belli oocysts are 23–36 μm in length and 12–17 μm in width, making them the largest of the coccidial species present in human feces (13) and readily visible unstained with light microscopy. Many methods have been used to detect *I. belli* in human feces. Stained fecal smears made from concentrated samples may aid in the detection of *I. belli* oocysts (13). The modified acid-fast stain produces pink-staining oocysts that contain bright red sporonts or sporoblasts. Additionally, oocysts stained with carbol-fuchsin fluoresce bright red under green light (546 nm). Oocysts stained with the auramine–rhodamine procedure fluoresce bright yellow. When the Giemsa stain is used both oocysts and sporoblasts stain pale blue. The heated safranin-methylene blue technique produces oocysts that are orange red. The trichrome stain, routinely used for other intestinal protozoa, is of little diagnostic value.

Sheather's sugar (specific gravity 1.33) flotation method is an excellent concentration method for recovering oocysts of *I. belli* in fecal samples (14). Oocysts will be in a slightly higher plane of focus than other parasite cysts or ova. Flotation methods are superior to direct fecal smears for recovering *I. belli* oocysts in fecal samples. Other flotation media such as zinc sulfate (specific gravity 1.18) can also be used to concentrate oocysts for recovery from fecal samples.

No work has been published on methods for recovering or concentrating *I. belli* oocysts from environmental samples. Methods used to recover and concentrate

Cryptosporidium parvum oocysts from environmental samples should be effective for *I. belli* oocysts.

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ISOTOPIC FRACTIONATION. See GEOCHEMICAL AND GEOLOGICAL SIGNIFICANCE OF SUBSURFACE MICROBIOLOGY

K

KINETICS (MICROBIAL): THEORY AND APPLICATIONS

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Growth kinetics define the dynamics of microbial processes such as the rates of nutrient exchange with the environment and the rates of population change. In natural aquatic environments growth constitutes the synthetic portion of a quasi steady state process in which the organisms produced are consumed by others, resources are used, and waste products are liberated. The uses of growth kinetics include (*1*) estimating the ambient rates of microbial growth, productivity, and metabolic product input such as recyclable minerals; (*2*) measuring the efficiency of nutrient capture for growth and energy as compared to those nutrients that are bioconverted, leaked, or lost, (*3*) evaluating the rate of nutrient acquisition by whole cells as they may be affected by environmental parameters such as alternative nutrients, temperature, and, in particular, the concentration of nutrients, (*4*) determining how the ambient levels of nutrients in the environment are set through kinetic control of in situ microflora, (*5*) assessing the significance of toxins and allelopathic chemicals to the ability of particular species to compete for substrates, (*6*) explaining how particular microbial species in a multinutrient environment may successfully compete with other species, and (*7*) evaluating aspects of cytoarchitecture such as how permeases are distributed on the surface of the cell for maximal capture efficiency with minimal investment in permease and cytoplasmic protein investment.

BASIC CONCEPTS

In kinetic terms microbial growth is an autocatalytic process in which the increase in microbial mass X (Table 1) depends on the amount of mass present and a pseudo first-order rate constant defined by Monod (1) as the specific growth rate μ

$$\frac{dX}{dt} = \mu X \tag{1}$$

The rate constant is actually second-order; both biomass and substrate concentration S control the rate of growth when S is small, as described later. The population attained after a period of growth depends on the initial population and the duration of growth to give a geometric increase in population over time t

$$X = X_0 e^{\mu t} \tag{2}$$

In excess substrate the growth rate constant μ assumes a maximal value μ_{max} that is characteristic of the organism and conditions. In predominantly pelagic systems such as the oceans, μ is typically much smaller than for commonly grown cultures such as *Escherichia coli* due to the evolutionary pressure of limited substrate availability and the energy necessary to transform this substrate into macromoleacules. Then the rate of growth depends on the rate (historically velocity v) of substrate acquisition and the efficiency or yield Y of cells produced from substrate acquired

$$\mu = vY \tag{3}$$

This conversions of substrate S to cell material is at rate μ and observed doubling time of $t_d = \ln 2/\mu$, so that the rate of growth depends principally on the rate of substrate acquisition and yield.

Steady State

The kinetics of growth are often taken from the kinetics of enzyme reaction in vitro. One distinction is the idea of steady state. In enzyme kinetics, steady state means that there is equilibrium between the rates of formation and the rates of release of product from the enzyme surface, with little change in the amounts of enzyme-substrate present as compared to the large changes in the concentrations of free substrate and product over the course of the reaction. Microbial kinetics differ from those used to describe catalysis by isolated enzymes. As used in the laboratory when producing cells or in the fermentation industry, where organisms or their products such as ethanol are a short-term objective, there is also depletion of substrate and an increase in the concentration product over time. But nutrient flux on a per organism basis remains constant and maximal. Often a growth curve with three phases is observed. These are (*1*) lag phase in which adjustment to the new environment occurs — there may be some nutrient uptake with an increase in cell size without formation of new cells, (*2*) exponential growth in which there is a geometric change in population and the exponent in Equation 2 is constant, (*3*) a stationary phase in which growth is terminated due to exhaustion of nutrient or some other reason. Quorum sensors (2) that control populations before exhaustion of nutrient may be involved. Otherwise, the logarithmic phase breaks sharply into the stationary phase as limiting nutrient is exhausted. The transition between exponential and stationary phases is rapid at usual concentrations of limiting nutrients, particularly for aquatic organisms because affinity constants are very small; on the order of 10^{-8} M for organics. When substrate limitation is the prevailing force, such a culture inoculated at 10^{-2} M substrate would grow at its maximal growth rate over nearly its first millionfold reduction in concentration, and (*4*) a decline phase in which the population count decreases due to factors such as starvation, apoptosis, and phage attack.

The steady state for aquatic organisms is somewhat more complete than that for isolated enzymes or for batch

Table 1. Symbols and Units

Term	Definition	Dimensions
a°_S	specific affinity for substrate S; base or unsaturated value	l mg-cells^{-1} h^{-1}
a°_{max}	specific affinity of a perfectly absorbing sphere	l mg-cells^{-1} h^{-1}
a_s	specific affinity for substrate S as reduced by saturation	l mg-cells^{-1} h^{-1}
c	collection constant	5 D M r_S^2 (2 r_X^4)$^{-1}$ l cell (g-cells site h)$^{-1}$
D	molecular diffusion constant	cm^2 s^{-1}
k_{cat}	catalytic constant	moles substrate transformed mole-protein^{-1} s^{-1}
K	substrate concentration at half maximum membrane potential	g l^{-1} or molar
K_a	affinity constant, S at $a^\circ_S/2$	g liter^{-1}
K_μ	Monod, S at $\mu_{max}/2$	g liter^{-1}
K_p	Per particle rate constant	liter particle^{-1} time^{-1}
K_m	Michaelis-Menten constant: concentration of S at $V_m/2$	g liter^{-1}
L	translation coefficient	h^{-1}
M	molecular weight	Daltons
N	number of molecules of a particular permease	molecules cell^{-1}
ψ	membrane potential	volts
R_X	radius of a spherical cell	cm
r_S	effective radius of a permease site	cm
R	ratio of cytoplasm to total organism mass	dimensionless
S	intracellular concentration of substrate; S_i, inside substrate concentration; S_n natural substrate, S_a, added substrate.	g l^{-1} or molar
t_d	time for population to double	time^{-1}
μ	specific rate of growth, μ_{max}, maximal rate	time^{-1}
V	rate of substrate uptake by a cell (v_X) or population of cells	g S l^{-1} h^{-1}, g S cell^{-1} h^{-1}, or g S g-cells h^{-1}
V	maximal rate of substrate moved through a particular enzyme at a given enzyme concentration, V_m, maximal rate	g-substrate accumulated g-cells^{-1} h^{-1}
X	Biomass	g-cells (wet weight) l^{-1}
Y	cell yield	g-cells produced g-substrate-consumed^{-1}

cultures undergoing exponential population increase. Where both organisms and their substrate have a supply and a sink, changes over time are smaller. Over short periods, such as hours, they can be taken as constant. Such systems are at steady state in a different sense. For example, phytoplankton blooms may release amino acids through leakage and sloppy feeding by predators. These nutrients are accumulated in aquatic bacteria largely by active transport in order to attain sufficient cytoplasmic concentrations for efficient distribution to the many enzymes that harvest energy and produce macromolecules. The bacteria reproduce and would also grow in number but are also removed by bacterivores and viral attack. All processes are regulated by negative feedback. If the bacterial removal terms are small, the bacterial population increases, their nutrient consumption rate increases, the concentration of nutrients decreases, and their rate of growth then slows due to nutrient depravation. This is similar to the process in continuous culture in which the rate of growth is controlled by the rate of dilution with fresh medium and the biomass is separately controlled by the amount of limiting nutrient in the fresh medium. The continuous culture steady state process was formulated and elegantly discussed by Herbert and coworkers (3). The amount of limiting nutrient around the cell is controlled by the organism's ability to sequester it, a characteristic of the cytoarchitecture of the species involved. In a well-behaved continuous culture, the concentration of the limiting nutrient can remain constant for days to months, and until upset by an external force. This steady state is formulated as

$$X = Y(S_0 - S) \tag{4}$$

where X is biomass, Y is cell yield, S_0 is substrate concentration in the influent medium, and S is the concentration of substrate surrounding the cells. Under any particular set of conditions, all parameters are constant. However, a change in any parameter such as the flow rate of influent medium, changes all parameters. An increase in the rate of fresh medium supply would lead to a larger S, and therefore a faster rate of growth. The faster rate of growth leads to induction of more enzymes, a change in the distribution of chromosome numbers as given by cell cycle theory (4), larger cells, and

a change in the yield constant Y (5). The yield may either increase due to a decrease in endogenous requirements for enzyme repair and the maintenance of membrane potential relative to consumption by growth processes or decrease due to decreased efficiency and increased loss of metabolic products. The biomass would increase with the added substrate as modulated by changes in yield Y. So at each set of conditions there is a different steady state comprising organisms of different composition and with different properties. Formulations of the kinetics of microbial growth seek to relate rates to nutrient concentration. But these relationships, unlike those for enzymes, span a set of organisms that, even of common species, have different biochemical properties such as DNA content (chromosome number), cell size, and enzyme content as described.

Kinetic Constants as a Measure of S

Growth-rate limiting concentrations of S are too small to easily measure and there have been many attempts to evaluate them by use of kinetics. Droop (6) formulated the rate of growth as a function of the concentration of limiting nutrient by relating changes in cell yield to both μ and S. This was done with a factor called the cell quota Q. The cell quota is the amount of limiting nutrient per cell rather than the amount of cell material produced per limiting nutrient consumed. The difference depends on the amount of limiting nutrient leaked or discarded after harvesting some of its free energy and discarding the waste products in the case of microheterotrophs. For autotrophs the amount of limiting nutrient per cell represents the accumulation of reserves. Q was seen as varying between a minimum value at low rates of growth to a large one when growth rate was maximal. Steady states at different conditions involve organisms of different composition, a change that should be taken into account in kinetic expressions because the nature of the catalyst (the organisms) has changed. But there was too much difference in the way various nutrients were allocated to cell material and the system was too species-dependent to be of general use.

Another technique was to evaluate the change in the rate of change of incorporation of added radiolabeled substrate. The concentration intercept of a Woolf plot, S/v versus S, is the negative of the ambient substrate concentration plus the Michaelis constant, and the ambient concentration of substrate alone when K_m is small (7). But as shown in Figure 1, isotope dilution gives hyperbolic-looking Monod plots (Fig. 1b) even when the kinetics are zero-order (Fig. 1a). Hyperbolic kinetics, like linear kinetics, give linear Woolf plots (Fig. 1c), which in this case, extrapolate to the ambient concentration of substrate since K_m is zero. When the kinetics are mixed as may occur in single-species systems, and certainly in mixed cultures, the S intercept reflects the kinetics of the system with the smaller K_m. In all cases of K_m and S_n the ambient concentration of substrate is ambiguous. Values obtained for carbon sources such as glucose tend to be near analytical values but not for phosphate. This agreement between ambient substrate concentrations and Michaelis constants may simply

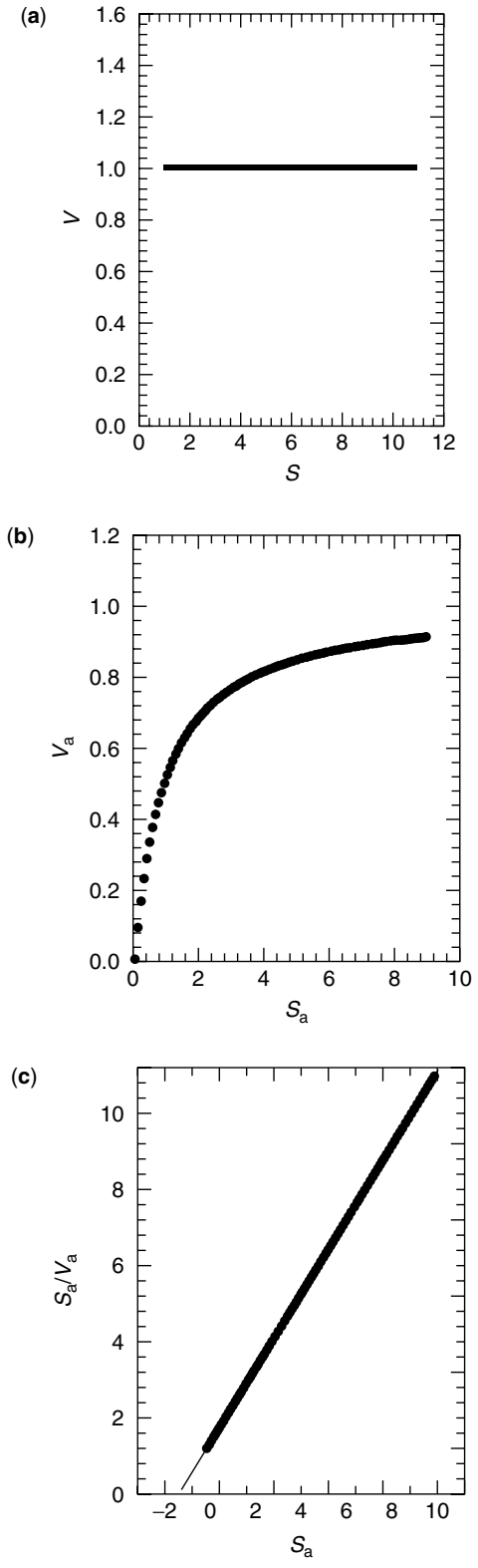

Figure 1. Isotope dilution. (**a**) Michaelis Menten plot of zero-order kinetics with no change in rate with concentration. (**b**) Michaelis Menten plot of apparent kinetics due to dilution of the added isotope S_a with natural substrate S_n calculated from the equation $v_a = va\, S_a/(S_a + S_n)$. (**c**) Woolf plot of the apparent rates showing the same linear transformation as obtained from truly hyperbolic kinetics.

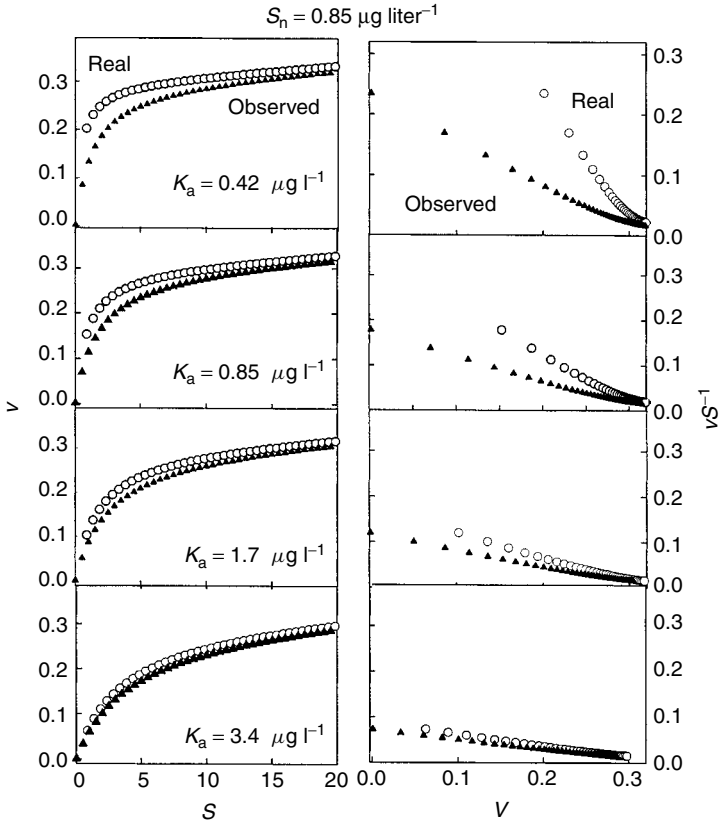

Figure 2. Effect of background substrate S_n on real and observed kinetics on a system having a constant affinity constant K_a. Left, Michaelis Menten plots; right, affinity plots.

reflect the competitive advantage of building only enough cytoplasmic enzymes to use transported substrate at the rate it is supplied from ambient concentrations by the permeases. Truncation of the maximal velocity V_m, for example, by a limited quantity of cytoplasmic enzymes consistent with unneeded rapid rates in low-substrate systems, leads to a proportionate reduction in K_m. This appears to be the main reason that ambient substrate concentrations are reflected by the Michaelis constants of the indigenous microflora.

The effect of background substrate on simulated kinetics from *Marinobacter arcticus* leucine-uptake kinetics is shown in Figure 2. The rate constant that specifies uptake when multiplied by the concentration S is called the affinity a_s (see Equation 18). As background increases, data at small concentrations are eliminated and real specific affinities (ordinate intercepts) of the Scatchard or affinity plots on the right increase. Note that apparent specific affinities decrease with the concentration of naturally occurring substrate. One may calculate the apparent affinity constant by determining v_a/S_a at the half-maximal value, $0.088/0.155 = 0.57$ μg leucine l^{-1}. The real value may be calculated the same way if S_n is known. The effect of the affinity constant on the real and observed kinetics are shown in Figure 3. Notice that the effect of background substrate on kinetics is minimized with increasing K_a. Also, the vertical positioning of the data in the affinity plots remains constant, values are only shifted with respect to rate. This means that the specific affinity of a culture or population a_S may be determined irrespective of the concentration of background substrate. The rate will be that at ambient substrate concentration and calculated from measures of S_b. Even when unknown, values are an accurate comparative determination of the activity of the population toward the added substrate that may be reported in the absolute units of specific affinity.

KINETIC FORMULATIONS FROM COLLISION FREQUENCY

A General Rate Constant

Other factors being constant, the rate of substrate acquisition depends on the concentration of the substrate. For the special case of a single substrate such as phosphate or glucose limiting the rate of substrate collection by a reasonably large cell ($r_x \approx\geq 0.5$ μm), uptake rate depends on the frequency of successful collisions of substrate with the cell (8). For a perfectly absorbing particle the rate is defined by the second-order rate constant k_p. considering a single cell, the population term is unity and

$$v_p = k_p S \tag{5}$$

The rate constant k_p is a composite constant that may be influenced by (*1*) the diffusive-resistance of the medium, (*2*) the abundance, kinetic characteristics, and energization of the permeases, (*3*) the disposition of transported substrate among resources for cytoarchitectural and maintenance requirements, and (*4*) potential downpathway limitations to the flow of material such as few cytoplasmic enzymes. It has been the implicit subject of several reviews (5,9,10). The simplest and most popular

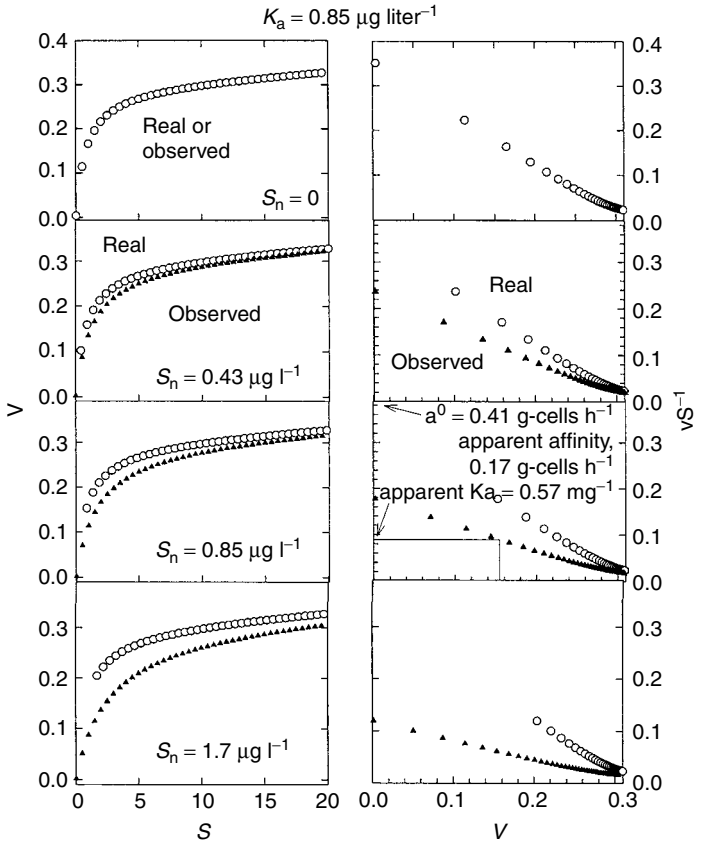

Figure 3. Same as Figure 2, showing the effect of the affinity constant K_a on system kinetics when background substrate S_n is constant.

formulation derives from the hyperbolic Michaelis Menten relationship for an enzymatic reaction (11).

$$v = \frac{V_m S}{K_m + S} \tag{6}$$

It is written for the rate of transport by a whole cell by taking V_m as the maximal rate of substrate transport by the cell. The basis of the formulation is that V_m is a characteristic of the organism set by the rate of substrate release from the substrate-permease complex or related rate-limiting step. At low concentrations of substrate, saturation is alleviated and the rate constant will increase due to a larger portion of the substrate-permease collisions being amenable for collection. When the substrate concentration is lowered sufficiently to reduce the transport rate by half, the Michaelis constant K_m is defined by that concentration. K_m is affected both by the decrease in transport due to the smaller concentration of substrate and by the increase in the rate constant for transport because the effects of saturation are less significant. It is commonly taken as a property of the permease that reflects increasing affinity with decreasing values. However, increasing numbers of permeases increase the ability of the cell to accumulate substrate without directly affecting K_m due to a corresponding increase in V_m. K_m can therefore be eliminated from Equation 6 by dividing through by V_m/K_m. When V_m/K_m is defined as the maximal value of the rate constant for uptake called the specific affinity a°_S is V_m/K_M (12). It obtains from the rate constant portion of Equation 6, $V_m/(K_m + S)$ at $S = 0$, and

$$v = \frac{V_m a^\circ_S S}{V_m + a^\circ_S S} \tag{7}$$

Specific Affinity

Specific affinity can be taken as a measure of the ability of cells to accumulate substrate. Values maximize at decreasing concentrations of substrate so long as nutrition is sufficient to maintain activation of the permeases. The associated Michaelis constant is taken as the concentration at which transport rate is reduced by saturation to half the unsaturated value in cells that have abundant cytoplasmic enzymes sufficient to process the product, intracellular substrate, as it is pumped in. This may apply to many easily cultivated large bacteria such as *E. coli*. For abundant but difficult-to-isolate aquatic bacteria it might better be taken as a measure of the ratio of cytoplasmic enzymes to permeases. These organisms require abundant permeases to collect substrate but few cytoplasmic enzymes to process them because substrate concentrations are always low. Then as substrate concentrations increase this capacity is overloaded, saturation appears, and K_m decreases. In either case the specific affinity should remain unchanged in its ability to reflect the capacity of the cells to accumulate substrate at concentrations that are small. It may be determined in the presence of background substrate if the background is small with respect to K_m,

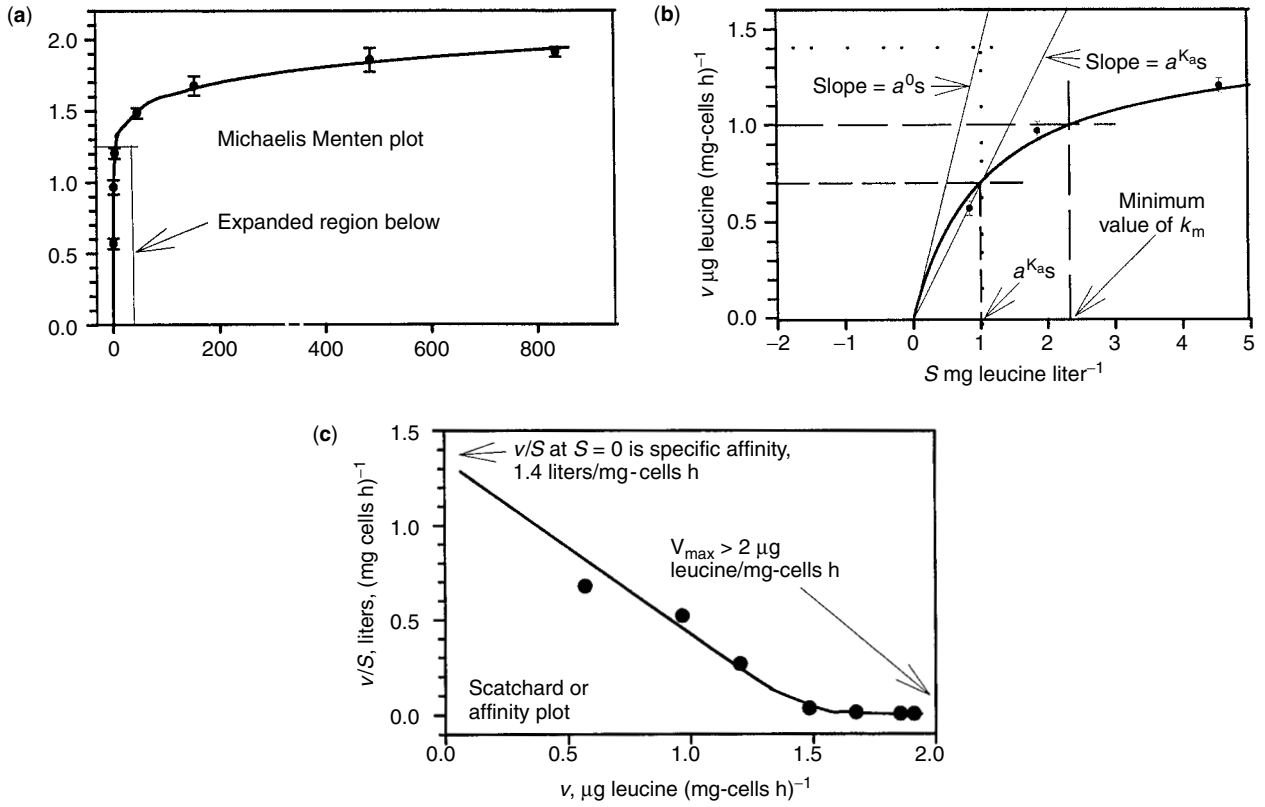

Figure 4. (**a**) Michaelis Menten plot of radiolabeled leucine uptake by toluene grown *M. arcticus* over time. (**b**) Expanded low-concentration data. (**c**) Scatchard or affinity plot of all data. Ordinate intercept gives the specific affinity and abscissa intercept gives the maximal velocity, which in this case is indeterminant. Kinetic constants are identified in (**b**) showing the specific affinity as the initial slope of the kinetic curve, the affinity constant as the concentration when the specific affinity is reduced by half, and the Michaelis constant as the substrate concentration when the maximal velocity is reduced by half.

or more accurately K_a, because a°_S is a measure of the second-order rate constant and both the uptake rate and concentration of radiolabeled substrate are diluted with background substrate by equal amounts. The various kinetic constants are represented diagrammatically in Figure 4. The specific affinity is a rate constant that measures affinity while K_a is a capacity term that measures how large S must be before saturation becomes significant. The other capacity term V_m is a measure of maximal capacity. When on a biomass basis it is like the specific affinity, and unlike K_a and K_m, which depend on V_m, in that it can be independently specified.

Monod Growth Kinetics

The common derivative of the Michaelis Menten equation for substrate transport is the Monod equation for growth (1) that is obtained from Equation (6) by replacing V_m with the maximum rate of growth μ_{max}, and K_m with K_μ the substrate concentration at $\mu_{max}/2$ so that $\mu = vY$ (Equation 1) and

$$\mu = \mu_{max}\frac{S}{K_\mu + S} \tag{8}$$

Limiting concentrations of substrate are generally below those which may be accurately analyzed so departures of experimental data from the theory are generally insufficient to distinguish among various models (10) and the Monod formulation, like its parent Michaelis Menten relationship from enzyme kinetics, and substrate transport discussed above remains a popular way of expressing the relationship between nutrient concentrations and growth. In aquatic systems additional factors such as endogenous metabolism and membrane potential attain dominant significance as affectors reducing μ when S is small. In this case it is useful to consider the underlying theory.

Molecular Collision Frequency and Nutrient Accumulation

The theoretical upper limit for the rate of substrate transport at concentration S depends on the radius of the cell, r_X, and the molecular diffusion constant D of the substrate (13).

$$v = \left(\frac{4\pi r_X D}{1,000}\right)S \tag{9}$$

This rate, written in terms of the second-order biomass-based rate constant for uptake or specific affinity gives

the theoretical maximal rate constant $a^\circ_{\max}$ and assumes complete coverage of the cell surface with permease. The real value a°_S is smaller according to the area (radius) of the active site r_S of each *active* permease, their number N, and the radius of the organism to give the base (unsaturated) value of the specific affinity a°_S

$$a^\circ_S = a^\circ_{\max}\left(\frac{N\pi r_S^2}{4\pi r_X^2}\right) \tag{10}$$

At substrate molecular weight M, the number of permease molecules per organism, as specified by the specific affinity according to the active surface area based on the diffusivity of the substrate D is

$$N = \frac{2a^\circ_S r_X^4}{5DMr_S^2} \tag{11}$$

For the case of hyperbolic saturation, usual in nonsteady state systems in which the organism composition is constant, incubation times are short, and all permeases are active, then $a^\circ_S = V_m/K_m$ and uptake rate in terms of the specific affinity, maximal rate, and substrate concentration is approximated by Equation 7. V_m defines the reduction in rate of substrate import due to saturation because it specifies the time τ that the relevant protein is occupied by substrate according to $\tau = NV_m^{-1}$ in the case of limitation by permease alone. If the activity of a particular enzyme is taken as reflective of maximal rate through the pathway, then the maximal rate V_m is $k_{cat}N$ where k_{cat} is the catalytic constant for that representative protein. The concentration-dependent specific affinity a_S becomes

$$a_S = a^\circ_S - \frac{va^\circ_S}{k_{cat}N} \tag{12}$$

or in terms of residence time in the permease

$$a_S = a^\circ_S - \frac{va^\circ_S\tau}{N} \tag{13}$$

If $5\ D\ Mr_S^2/2r_X^4$, which has the units liters cell (g-cells site h)$^{-1}$, is set equal to a constant c, the maximal value of the specific affinity $a^\circ_S = Nc$. Since rate is related to substrate concentration by the saturation dependent affinity a_S and $v = a_S S$, Equation 14 becomes a more compact formulation of the specific affinity

$$a_S = \frac{Nc}{1 + Sc\tau} \tag{14}$$

which gives the permease-dependent rate constant for substrate uptake in molecular terms.

Janusian Kinetics

Oligobacterial cytoarchitecture is thought to favor high permease content over large amounts of cytoplasmic enzyme to facilitate nutrient collection without the overhead of a large or densely packed cytoplasm (14). Flux control may then shift from transport to metabolism. Conventional treatments of coupled enzyme reactions are generally designed to explicitly treat a changing concentration of substrate and become complex. However, the kinetics can also be treated with Janusian kinetics, so named because the central rates respond or "look" in both directions, forward and back like the Roman god Janus (15).

We may formulate transport as a series of steady state processes with rates and rate constants that differ at each concentration. Solution is obtained by writing a material balance around the concentration of the cytoplasmic substrate (Fig. 5) to arrive at a different concentration of transported substrate at each rate of transport, where R is the ratio of cytoplasm to total organism mass.

Change in cytoplasmic concentration of S_C = Transport of S into cytoplasm − output of S_C from cytoplasm

$$R\frac{dS_C}{dt} = \frac{NcS}{1 + Sc\tau} - \frac{VS_C}{K_m + S_C}$$

The relationship between the concentration of substrate in the cytoplasm and the rate of transport of substrate to the cytoplasm can be specified by a translation coefficient L. Based on experimental evidence for the transport of phosphate (16) this relationship was taken as linear and first-order in substrate. When rate is translated into concentration with L, the overall rate of transport $v = LXRS_C$, where X is biomass and R is the ratio of cytoplasm mass to cell mass. At steady state $dS_c/dt = 0$, and the second two terms are equal. Solving for S_C and substituting into Equation 7 gives the rate of transport in molecular terms

$$v = \frac{VNcS}{K_mRL(1 + S_c\tau) + NcS} \tag{16}$$

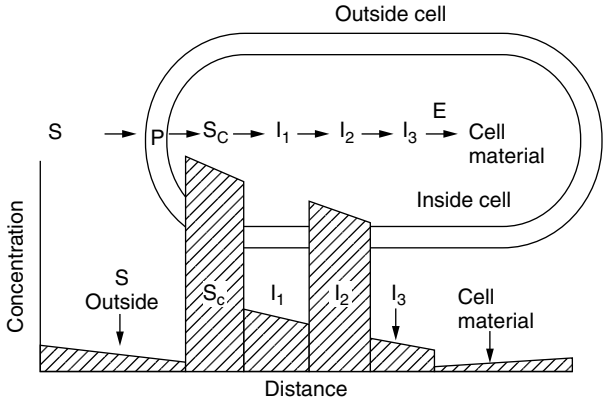

Figure 5. Model for solving Janusian kinetics in which the system flux is taken as flow to and from an intracellular pool S_C. These are in equilibrium with a series of intermediates I at various concentrations (histogram) depending on their relative amounts and kinetic characteristics. One enzyme labeled E catalyzes a particular difficult step that controls rate when S is large. Otherwise that rate is controlled by the number of permeases P and the concentration of S. Due to changes in S_C and the various intermediates, some control of rate is shared by all steps.

Specific affinity is the initial slope of Equation 15, that is, the rate constant for uptake at any external concentration of substrate when all the permeases are activated but concentrations are sufficiently low to avoid significant saturation by substrate. Differentiating as described (15) we obtain the rate of transport in terms of kinetic constants as

$$a^{\circ}_{S} = \frac{NVc}{KRL} \tag{17}$$

SOME PROBLEMS THAT MAY BE SOLVED BY USING MICROBIAL KINETICS

Theoretical Analysis of Cytoarchitectural Requirements

Equation 18 shows the value of increasing both number of permeases and cytoplasmic enzymes for an increased specific affinity. However, an increased cytoplasmic enzyme content at constant cell volume to mass ratio leads to larger cells, which decreases the collection constant c. So specific affinity and permease content are directly related, counter to the common paradigm taken from enzyme kinetics (17) in which affinity is a property of the quality of an enzyme or microbial species. Cytoplasmic enzyme concentration, when modeled as an increase in V_m, increases the ability of cells to collect substrate at concentrations that are small due to S_c removal, but the effect on rate at large substrate concentrations is greater. However, maximal velocity may not be constant over a change in the steady state concentration of substrate and the rate is likely to be a complex function of S. For example, when substrate concentration is low for extended periods, the membrane potential $\Delta\Psi$ may collapse and the effective number of permeases is decreased until sufficient substrate concentrations are experienced to empower them. In this case N should be replaced with the number of energized or active permease molecules N_a where $N_a = f\ (N,\ \Delta\psi)$. Thus, kinetics may be used to resolve the physiological composition of microbial populations.

Nutrient Acquisition Ability

For purposes of producing many cells or much product, μ_{max} is the most appropriate measure. Organisms require many cytoplasmic enzymes and much RNA for this purpose. For growing in dilute environments specific affinity is the best measure. From it the rate of growth can be calculated from the ambient concentration of nutrients. If a particular nutrient is used by an independent pathway, the specific affinity of that pathway adds to that of others to give a net effective value. If different nutrients are transported through the same permease and the permease is limiting, then inhibition transport of one nutrient competes with another. If limiting is by independent cytoplasmic enzymes with specificity for particular nutrients, then inhibition is absent.

Rates of Growth

Most direct measures of growth rate are imprecise, but values can be calculated from specific affinities and ambient concentrations of nutrients. These concentrations are difficult to measure as well, but precision is possibly greater than the uncertainty in the rates of growth. Kinetic constants for a number of systems are shown in Table 2. Taking a large springtime value for the uptake of leucine by the heterotrophic bacteria in Resurrection Bay as 830 liters/mg-cells h, and the concentration of leucine as 1 μg/liter the rate of growth from leucine may be calculated. Using the per particle rate constant from Equation 5 as the specific affinity before saturation becomes significant,

$$v = a^{\circ}_{A}A \tag{18}$$

where A is the single substrate leucine from a mixture. Marine bacteria are thought to be very dilute, that is, low in cytoplasmic enzyme content, to maximize surface to mass ratios and give ample room for their genomes, below 20% in dry mass (14). Taking the yield constant as unity for wet mass produced from substrate consumed

$$\mu = vY \tag{19}$$

Combining Equations 2 and 19

$$\mu = \left(\frac{830\ \text{liter}}{\text{g - cells h}}\right)\left(\frac{1\times10^{-6}\ \text{g leucine}}{\text{liter}}\right)\left(\frac{1\ \text{g cells}}{\text{g leucine}}\right) = \frac{8\times10^{-4}}{\text{h}} \tag{20}$$

The calculated doubling time is $\ln 2/\mu = 860$ h, a large value for an active system. Assumptions are that all the bacteria used to measure the specific affinity are alive and able to use leucine. Other substrates could contribute to the substrate flux and shorten the doubling time accordingly.

The ambient value for growth rate may also be determined by diluting out natural populations with filtered raw fresh or seawater. Since predation is a second-order process, their effects are minimized and the rate of growth is approximated by Equation 2 and the apparent rate should approach the true rate as X approaches 0 (40). These dilution-culture based measurements of growth rate are complicated by nutrients released from larger organisms during filtration and the fact that dilution inhibits growth and metabolism for reasons unknown. Again values should agree with values computed from nutrient flux or other methods.

Leakage Rates

Unidirectional fluxes and associated rate constants may be computed from a pulse of radiolabeled substrate. The best experimental platform for conducting these relaxation experiments is a continuous culture system in which the radioactivity of nutrient feed can be controlled and the system remains at a chemical steady state. Easily derived, the equations are somewhat bulky and the reader is referred to detailed treatments (16,41).

Ambient Concentrations of Nutrients

These can be accurately determined in continuous culture by extrapolation of Equation 4 to zero-added substrate concentration while changing the rate of growth by altering the rate of dilution. As the chemical composition

Table 2. Kinetic Constants from the Recent Literature[a]

Culture	Substrate	Specific Affinity, a°S Liters mg-cells h^{-1}[c]	Maximal Velocity, V_m, mg S mg-cells^{-1} h^{-1}	K_M, $\mu g\ l^{-1}$	K_A, $\mu g\ l^{-1}$	Reference
Cycloclasticus oligotrophus	toluene	20	1.2	10	1.3	In prep. this laboratory
Escherichia coli	glucose	7.3	4.8	50	at least 50	18
Pseudomonas P-15	phenanthrene	1.2	0.06	>400	400	19
Seawater, Resurrection Bay[b]	leucine	0.83	0.006	>2.6	2.6	Unpub. this laboratory
M. arcticus	toluene	0.32	0.14	44	44	20
Rumen isolate SR	arginine	0.30	0.41	13,900	1,044	21
Escherichia coli	leucine	0.21	0.010	1,570	~260	22
Sphingomonas RB 2256	glucose	0.2	–	–	–	23
Corynebacterium glutamicum	glycine betaine	0.11	0.25	1,015	–	24
Sphingomonas RB 2,256	alanine	0.06	0.029	440	440	23
Mixed methanotroph culture	trichloroethylene	0.06	0.51	7,990	–	25
Desulfovibrio G-11 and syntropic benzoate isolate	benzoate	0.025	0.045	1,732	–	26
Corynebacterium glutamicum	tyrosine	0.02	0.011	543	–	27
Escherichia coli	gluconate	0.017	0.08	4,900	–	28
Pseudomonas sp. strain B13 (chemostat)	3-chloro- benzoate	0.016	0.032	6,768	–	29
Peter Lake	leucine	0.016	–	–	–	30
Escherichia coli	leucine (repressed)	0.008	0.009	7,000	500	22
Saccharomyces cerevisiae	glucose	0.0016	0.54	5.6×10^6	1.1×10^5	28
Cycloclastic oligotrophus	acetate	0.0016	0.13	20,000	20,000	In prep. this laboratory
Lactobacillus brevis 367	trimethyl β-galactoside	0.0010	0.14	1.1×10^5	–	31
Escherichia coli	*p*-nitrophenyl phosphate	0.0072	0.65	44,630	149,000	32
Penicillium chrysogenum	phenylacetic acid	3.3×10^{-4}	>6.1	$>5 \times 10^6$	–	33
Vibrio parahaemolyticus	glucose	2.6×10^{-4}	0.027	1.0×10^5	–	34
Halobacterium saccharovorum	glucose	2.8×10^{-4}	0.07	1.4×10^5	28,000	35
Seawater; Resurrection Bay[c]	toluene	0.85×10^{-6}	–	–	–	36
Escherichia coli K12	glycine	1×10^{-6}	2×10^{-5}	2.0×10^5	–	37

[a]Conversion factors used: 400 mg/l (wet wt.) OD^{-1} (38), 3 mg cells wet wt. (mg dry wt)$^{-1}$, 1.9 mg cells (dry wt) mg protein^{-1}.
[b]Biomass taken as 53% of the total population which used leucine according to autoradiography and corrected for the 0.8 $\mu g\ l^{-1}$ ambient leucine present according to (39).
[c]Biomass taken as the total bacterial population which was 7% toluene oxidizers. The specific affinity was corrected for saturation according to the kinetic constants shown; constants obtained from *C. oligotrophus*.

of the feed is known, it can be isotopically labeled, the intercellular radioactivity can be separated into substrate and products if any, and concentrations and kinetics can be determined. If the organisms used are typical of the system, the ambient concentration can be inferred.

For natural systems one can follow indirect measures of growth such as the ratio of two to one chromosome cells, membrane potential, RNA content, and others. The first two (unpublished, this laboratory) and possibly the third (42) can be done by flow cytometry. Nutrient combinations are then added until a positive response is attained and the ambient concentrations are inferred from the additions required to elaborate the response. If the organisms are also labeled with species-specific probes, the response can be related to particular species. One problem is to isolate changes in the concentration of the probed parameter from interspecies differences in permeability to the probe. Another is toxicity of the probes. These methods remain to be developed.

CONCLUSION

Microbial kinetics, the quantitative description of how nutrient concentrations control microbial dynamics, are an integral component aquatic system behavior. Formerly based on chemical catalysis by isolated enzymes,

formulations may now be based on first principles that specify aspects of organism cytoarchitecture. Continued advances depend in part on improved quantitative analyses of both the molecular biological components of microorganisms such as the molecule count of particular enzymes and the concentration of identified nutrients in aquatic systems. Key kinetic constants are the specific affinities of the organisms because they give the rate of nutrient uptake from the ambient concentrations of nutrients and also give an absolute comparative basis of the ability of species or populations to sequester nutrients, and maximal velocity, which gives the rate of nutrient uptake when nutrient concentrations are large and a measure of the nutrient uptake capacity of the system. These completely define the kinetics when the kinetics are Michaelian. When maximal velocities increase with substrate, saturation can also be specified with an affinity constant that identifies half-saturation concentrations by comparison with the maximum value of the specific affinity. These kinetic constants define a quasi-steady state that largely sets the total biomass in the oceans and the rate that both the microflora and the concentrations of nutrients turn over because populations have maximized when nutrients become too dilute to support growth. The nutrient concentration just sufficient to support growth is set by the nutrient sequestering ability or specific affinity of the organisms. That is, in turn, a biochemical property of the organisms that is set by organism cytoarchitecture. So organism kinetics set the nutrient concentration of the oceans as well. Specific affinities can be obtained by measuring the rate of acquisition of added radiolabeled nutrients together with organism biomass present but the nutrient concentrations that they act upon are best determined by chemical analysis.

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KINETICS OF MICROBIAL PROCESSES AND POPULATION GROWTH IN SOIL

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The kinetics of microbial growth and substrate utilization in liquid culture have been worked out in great detail (1,2). However, it has become acknowledged that kinetics in soil are greatly affected by the complex physical environment of soils. Much progress has been made towards incorporating soil physical effects into the current understanding of the kinetics of microbial processes in soils. It is also becoming recognized that significant microbiological activity occurs in soils under extreme environmental conditions. For example, recent work shows significant microbial activity in frozen soils. Further integration of soil physics and microbiology is needed to address this issue. This review synthesizes new and classic work on the physical and biological factors that affect kinetics in soil. Physical properties of soils that affect movement and availability of chemicals, properties of soil microbial populations and communities that affect growth and uptake kinetics, and environmental factors that modulate the activities of soil microbes will be discussed.

SOIL PHYSICAL FACTORS

Important factors that can limit the rate of microbial processes in soil are properties of the soil that limit the movement of chemicals to microbes. The effect of abiotic processes on the bioavailability of organic molecules has received much recent attention by ecotoxicologists and soil microbiologists (3). In addition, many empirical studies have demonstrated the rapid decrease in availability of chemicals after addition to soil (e.g., 4,5). One study (4) showed that cinnamic acid (0.1 mg/g) could not be extracted from sterile soil after only two days of incubation. This decreased bioavailability of chemicals in soil can be because of several factors including: (*1*) slow rates of chemical diffusion in soil, (*2*) sorption of chemicals to soil particles, (*3*) processes such as sequestration into soil organic matter or micropores, and (*4*) interactions between microbial enzymes and the soil matrix. More details of how each of these physical factors affects the kinetics of microbial processes are given in the following sections.

Diffusion of Solutes Through Soil

Slowed diffusion of chemicals in soil relative to aquatic environments can be explained in terms of the complexity and tortuosity that chemicals must transcend as they move through the soil matrix (Fig. 1). In order for a compound to get from point A to point B, it not only has to diffuse through the soil solution but it also has to diffuse around and through the labyrinth that is the three-dimensional structure of soil. This tortuosity greatly slows the movement of chemicals from their point of release to organisms capable of transforming the chemical. The size, structure, charge, and polarity of the chemical can also affect this rate of chemical movement in soil. Another consequence of the tortuosity of soil is that chemicals interact with many surfaces and micropores as they move through soil. This increases the chances of the chemical being sorbed or sequestered as it moves through the soil.

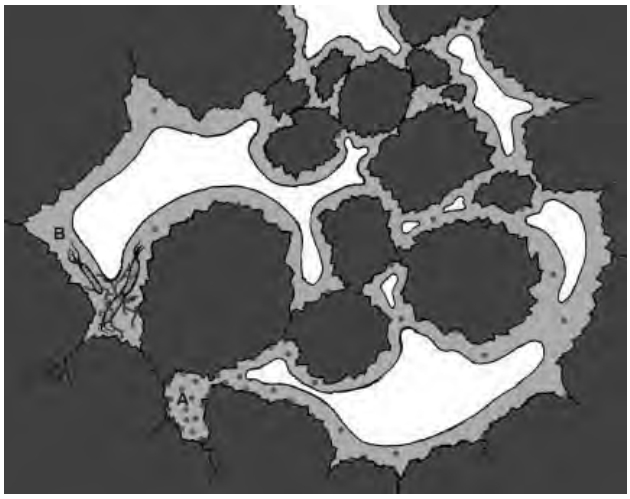

Figure 1. Diagram showing substrate molecules (red dots) diffusing down a concentration gradient from micropore A to bacterial colony B. The solid phase is shown in brown, the liquid phase in blue, and the gas phase in white. Note the relatively long distance that molecules must travel despite the short linear distance between A and B, because of the tortuosity of the water films. The substrate molecules are also shown adsorbing to soil surfaces, further slowing their movement. See color insert.

The diffusion rate of molecules down a concentration gradient through soil is directly proportional to the Nye diffusivity parameter, D_e (units of $m^2\ s^{-1}$), which is defined by the equation:

$$D_e = \frac{\theta f D_L}{\beta_e} \tag{1}$$

where θ is the volumetric soil water content (mL H_2O cm^{-3} soil, unitless), f is the impedance or tortuosity factor (unitless), D_L is the diffusion coefficient in liquid ($m^2\ s^{-1}$), and β_e is the buffering capacity of the soil (unitless). The characteristics of the soil affect most of these parameters. The volumetric soil water content is, of course, subject to the water holding capacity of the soil, which is determined by texture and organic matter content. The impedance factor is a function of water content, bulk density, and texture. For silt loams and lighter-textured soils, workers (6) found that:

$$f = 1.6\theta - 0.172 \tag{2}$$

In a different study (7), it was found that f was the highest in silt loams at a bulk density of 1.3, and decreased above or below that value. Values of f are much lower (i.e., higher tortuosity) for finer-textured soils.

The liquid diffusion coefficient is described by the Stokes-Einstein equation:

$$D_L = \frac{K_B T}{6\pi r_i \eta} \tag{3}$$

in which K_B is the Boltzmann constant (1.3807×10^{-23} J/K), T is the absolute temperature (K), r_i is the ionic radius (m), and η is the viscosity of the liquid (kg m^{-1} s^{-1}). Because of the temperature term in the equation, and because the viscosity of water decreases with increasing temperature, the diffusion rate is sensitive to temperature.

The buffering capacity, β_e, describes the tendency for a specific compound to bind to soil surfaces. The general effect of a high β_e on kinetics is to lower the effective concentration of the substrate in the soil solution. This results in higher apparent half-saturation constants (K_s) of microbes in soil compared to liquid culture (8,9). For example, one study (9) found that the amount of dinitrophenol needed to induce the half-maximal growth rate (μ_{max}) was 20 to 130 times higher in soil than in liquid culture for two different microbial populations. The buffering capacity is defined as:

$$\beta_e = \frac{\delta C_S}{\delta C_L} \tag{4}$$

where C_S is the bulk soil concentration of the compound and C_L is the soil solution concentration (both in units of kg m^{-3}). This parameter is related to the slope of the adsorption isotherm (10). The adsorption isotherm shows equilibrium values of partitioning between the solid and liquid phases for different initial concentrations. In most cases, the isotherm asymptotically approaches a maximum as the solid phase becomes saturated at high concentrations. This curved type of adsorption isotherm is known as the "L (Langmuir) type." Unless the adsorption isotherm is of the linear type, or the soil concentration is sufficiently low as to lie on the quasilinear part of the curve, β_e varies with substrate concentration. The buffering capacity is also affected by soil pH and the presence of high salt concentrations. Nonlinear adsorption can complicate kinetics; one study (11) found that biodegradation of glutamate diffusing out of artificial aggregates did not always fit a model that included constant diffusion and sorption terms.

The buffering capacity (β_e) is a measure of solutions at equilibrium with the solid phase. The use of β_e in Equation (1) therefore assumes that equilibration of the diffusing molecules is rapid relative to their motion through the soil. This is not necessarily the case. One researcher (12) pointed out that experimentally derived adsorption isotherms rely on artificially high solution:soil ratios, which could affect substrate-soil interactions, and presented a method for calculating D_e from a measured diffusion profile in a soil. Some studies have used two-compartment kinetic models to describe release of substrates from fast- and slow-desorbing soil fractions (13,14). In the latter study, the slow-desorbing fraction was interpreted as constituting a limited number of binding sites with high affinity for the substrate. In the former study, the slow-desorbing fraction was interpreted as soil aggregates or organic matter particles, from which diffusion was slow. The presence of aggregates can affect degradation rates in soils by controlling diffusion of oxygen and substrates. Researchers (15) combined two models to simulate degradation of contaminants within aggregates and in macropores between aggregates. It was found that biodegradation rates were lower in soil with large aggregates (>1 cm) compared to homogenized soil. Experiments and model simulations of biodegradation in artificial aggregates showed that biodegradation was slower in soil with larger aggregates, and that the effects of diffusion and sorption were required to model this process (11,16).

Diffusion of Gases Through Soil

Unlike the positive relationship between soil moisture content and the diffusion of solutes through soil solution discussed in the preceding section, soil moisture content strongly limits the rate of gas diffusion in soil because gas diffuses much more slowly through water-filled pore spaces than through air-filled pore spaces (17). This fact is illustrated by the one-dimensional equation for diffusion of gas through soil derived from Fick's law:

$$F = \tau \theta_A D \left[\frac{\delta C}{\delta z}\right] \tag{5}$$

where F is the flux rate (mol m^{-2} s^{-1}), τ is the tortuosity of the air-filled pore space (unitless), θ_A is the fraction of pore space filled with air (unitless), D is the diffusion constant for the gas in free air (m^2 s^{-1}), and $\delta C/\delta z$ is the concentration gradient (mol m^{-4}). This tortuosity factor (τ) (not to be confused with f mentioned earlier in terms of water films) has been modeled (18) as:

$$\tau = \theta_A^{(1/3)} \tag{6}$$

$\tau = 1$ in free air. Both, the tortuosity and the pore space decrease with increasing water content, in turn slowing the diffusion of the gas through soil.

The availability of oxygen (O_2) can be the limiting factor for aerobic microbial activity, and can determine the extent of anaerobic activities such as denitrification or methane production. Therefore, CH_4 and N_2O production are frequently related to soil water content (19–23). However, Sierra and Renault (23) found that immediately after heavy rainfall events, O_2 can be trapped in soil pore water and that an equilibrium between soil water and atmosphere is not reached for 12 to 24 hours in these cases.

Sorption

The kinetics of microbial processes in soil is greatly complicated by the mineral and organic matter fractions of soil. These solid phases bind substrate both reversibly and irreversibly. Reversible sorption leads to slowed

rates of movement and lower substrate concentrations in the soil solution, whereas irreversible sorption leads to overall decreases in the quantity of substrate available to microbes. Soils with high levels of organic matter or high clay contents generally retain compounds more than sandy soils (4,24). The types of clay and aluminum and iron hydroxides in soil can influence the ability of soil to adsorb molecules (25). Because many phenolic acids have a relatively low pK_a (<5), they are ionized at the pH values of all but the most acidic soils (4) and as such are more likely to interact with positively charged sites on clay minerals.

Sorption usually involves reversible interactions with exchange sites on soil surfaces, but irreversible complexation of chemicals can also occur. Although the exact mechanisms of these reactions are not completely understood, they do seem to be of importance in certain soils. In general, such reactions lead to strong (probably covalent) linkages with the humic fraction of soil rendering the chemical unavailable to microorganisms (3,26). Humus can fix ammonium and amino acids to a state where these compounds become resistant to acid hydrolysis (27), and a wide variety of organic compounds are associated with mineral fractions of soils (28). In one study, binding of surfactants to humics prior to incubation in soil slowed biodegradation of the surfactants, whereas preincubation with illite or montmorillonite reduced the overall extent of biodegradation (29). In another study, the addition of moderate amounts of clay minerals to protein slowed degradation by bacteria without affecting the overall yield, whereas higher clay to protein ratios protected some of the protein from hydrolysis, thus lowering the yield (30). Clearly, the extent of irreversible sorption depends on many factors, including the concentration and type of substrate and the types of organic and mineral soil colloids present.

Extracellular Enzymes

Another novel aspect of soils that affects the kinetics of microbial processes is the widespread presence of extracellular enzymes in soil. These enzymes are especially important in the degradation of large and complex molecules, such as protein, cellulose, and lignin. Extracellular enzyme activity is generally correlated with microbial biomass levels (31–33), however certain enzymes can persist in soils long after the death of the microbes that released them. Stable enzyme-clay and enzyme-humic complexes have been shown to function in soils (34,35). Interactions between enzymes and soil components have a variety of effects on enzyme activity, affinity and stability. For example, urease forms a stable complex with hydroxyapatite in alkaline soils. Relative to the free enzyme, the complexed form shows an increased pH optimum and stability to storage and proteolysis, whereas the half-saturation constant (K_s) is unchanged and the maximum velocity (V_{max}) decreases only slightly (36). The adsorption of enzymes onto clays frequently increases their pH optima, probably because of conformational changes in the enzyme owing to charge interactions with the clay surfaces (37). Clay and humus colloids can increase enzyme resistance to thermal stresses, such as heating and freeze-thaw cycles (38). In the case of acid phosphatase, enzyme-clay complexes have higher K_s values and lower stability to storage and proteolytic attack relative to the free enzyme (39). Whereas montmorillonite binds and inhibits acid phosphatase, it was found that the addition of aluminum hydrous oxide to the enzyme-clay mixture increased both the binding of the enzyme and its residual activity (40). Manganese oxides aid in the formation of phenolic-enzyme complexes, which may occur in the formation of humus (41).

Microbial enzymes can act in concert with soil abiotic factors in immobilizing chemicals. Soil microorganisms can transform chemicals into more recalcitrant and less toxic forms, which contribute to the humic fraction of soils. A number of microorganisms have been shown to polymerize phenolic compounds using enzymes, such as polyphenoloxidases and laccases (42,43). Polymers produced by these fungi are usually dark colored and can appear rapidly in response to additions to soil of suitable substrates such as ferulic acid (44).

Catalytic Properties of Abiotic Soil Components

Some abiotic components of soils are capable of catalyzing reactions in soils. For example, clays can catalyze the polymerization of amino acids to form peptides (45). Amino acids can polymerize or be mineralized by reactions with phenolics in soil (41). Manganese oxides, such as birnessite can enhance the abiotic mineralization of carbon and nitrogen from amino acids (46). The coenzyme, pyridoxal-5′-phosphate, can deaminate amino acids in the absence of enzyme using metal salts and clays as catalytic surfaces (47).

BIOLOGICAL FACTORS

Many processes in soil are microbially mediated, and so the properties of the microbial populations present in soil have fundamental importance for the kinetics of these processes. The most obvious controlling factor is the size of the functional group or population that can carry out a specific process. However, the growth and uptake kinetics of these organisms are equally important, as is their requirements for limiting nutrients and electron acceptors. Each of these factors will be discussed in turn.

Population Size

In many cases, the zero-order rate of a process is directly related to the size of the functional group responsible for carrying it out. This relationship is so obvious that the rate of a given process is often used as a way to estimate the size of the functional group carrying out the process. For example, substrate induced respiration (SIR) (48), the substrate-induced growth response (SIGR) method (49–51), and arginine ammonification (52) all correlate with various other more direct measures of microbial biomass. In one study on the rate of amino acid degradation in soil, differences in rates of glycine and glutamate degradation were related to the size of the group of microbes able to metabolize each substrate (53). In the extreme case, a process will not occur at all unless there

Table 1. Differential Forms of Equations for Substrate Disappearance and the Conditions Under Which They Theoretically Appear (S is the Substrate Concentration, X is the Biomass, K_s is the Half Saturation Constant, and μ_{max} Is the Exponential Maximum Growth Rate)

	Growth ($S_0 \gg X_0$)	Nongrowth ($S_0 \ll X_0$)
$S_0 \ll K_s$	Logistic $-dS/dt = dS(S_0 + X_0 - S)$	First order $dS/dt = \mu_{max}(X_0/K_s)S$
$S_0 > K_s$	Monod $-dS/dt = \mu_{max}S(S_0 + X_0 - S)/(K_s + S)$	Michaelis-Menten $-dS/dt = \mu_{max}X_0S/(K_s + S)$
$S_0 \gg K_s$	Logarithmic $-dS/dt = \mu_{max}(S_0 + X_0 - S)$	Zero order $-dS/dt = \mu_{max}X_0$

is a population capable of carrying out the process, as in many bioremediation studies in which specially selected organisms are added to contaminated soil (e.g., 54).

The organisms that are collectively responsible for carrying out a specific observable process are referred to as a functional group. The size of a functional group affects not only the overall rate, but also the type of kinetics observed. If a group of organisms is utilizing an organic substrate, the size of the functional group relative to the substrate concentration determines whether growth or nongrowth kinetics will result (see Table 1, and discussion in the section titled The Use of Respiration Data in Studies of Kinetics in Soils). For example, workers attempting to model mineralization kinetics for low concentrations of pesticides in surface and subsurface soil found that growth models best fit the subsurface soil, whereas nongrowth models best fit the surface soil (55). This is easily explained by assuming a smaller microbial population in the subsurface soil that was stimulated by low pesticide inputs that were insufficient to support growth of the larger surface population. On the other extreme, if a small population is confronted with excessive amounts of substrate, long lags can occur before measurable degradation occurs, or degradation of the substrate can halt altogether. In a study of the degradation of various concentrations of mecoprop in surface and subsurface soil, the maximum concentration that allowed growth was lower in the subsurface soil, again indicating a smaller subsurface microbial population (56).

Growth and Uptake Parameters of Soil Microbial Populations

The ability of microbial populations to absorb and assimilate a particular substrate directly affects its fate in soil. The kinetics of microbial population dynamics in soil can be described by a number of mathematical models, most of which were conceived for microbes growing in liquid media, for example, Michaelis-Menten or Monod kinetics (1,50). As one example, the Monod equation describes the essential properties of a microbial population with respect to the disappearance of a growth-limiting substrate:

$$-dS/dt = \mu_{max}\frac{S(S_0 + X_0 - S)}{(K_s + S)} \quad (7)$$

where S is the substrate concentration at time t ($mol\,m^{-3}$), S_0 is the initial substrate concentration ($mol\,m^{-3}$), X_0 is the initial biomass ($g\,m^{-3}$), μ_{max} is the maximum specific growth rate (s^{-1}), and K_s is the half-saturation constant ($mol\,m^{-3}$). Because biomass appears in this equation in units of substrate required to create the biomass, the growth yield is another parameter that is implicitly contained in this equation. Put more simply, the rate determining factors for a given population size using a growth-limiting substrate are the growth rate, the affinity for the substrate, and the efficiency with which the substrate is assimilated into biomass. The Monod equation is a good heuristic tool for understanding the relationships between population characteristics and the utilization of substrate, but it seldom accurately describes microbial processes in soil. The reasons for this include soil physical factors that limit the movement of chemicals to microbial cells (see preceding section titled Diffusion of Solutes Through Soil), and nutrient limitation and other biological factors, discussed in the following sections.

Limiting Factors and the Effect of Second Substrates

Often the substrate of interest is not the growth-limiting factor. In these cases, other characteristics of the microbes become important, such as requirements for a limiting nutrient (e.g. 57) or terminal electron acceptors (58,59). Nutrient limitation completely changes the kinetics of biodegradation (60,61). Degradation under such conditions is determined by the supply rate of the limiting nutrient, which can cause logistic or linear growth curves (62,63). Nutrient limitation can also enhance degradation; some nitrogen- or phosphorous-containing organic compounds will only be utilized if the microbial community is nutrient limited (64). Another important characteristic of a given functional group is the range of substrates it can utilize. In one study (65), workers induced growth kinetics in the degradation of low levels of *para*-nitrophenol by adding larger levels of phenol to the soil. Thus, the rate of transformation of a compound in soil is affected by the presence of similar compounds that the microbial population can utilize.

Effects of Microbial Community Composition

Soil microbial communities are complex, containing thousands of bacterial types (66). A study of the kinetics of DNA reassociation showed soil microbial communities to be orders of magnitude more complex than aquatic microbial communities (67). The composition of the microbial

community can cause deviations from standard growth models and has a profound influence on kinetics in soil. Filamentous microbes, such as fungi and actinomycetes, grow linearly over time on agar, and in liquid culture the cube root of the mass increases linearly over time (68). The predominance of filamentous microbes in a community could lead to nonexponential growth rates in soil. There is evidence that first-order kinetics does not always apply to fungal cultures in soil under conditions in which first-order kinetics would be expected with bacterial populations (63). The fungal-to-bacterial ratio in soil can also affect the apparent growth yield of functional groups in soil (69). Changes in growth yield by microbial biomass have been observed in response to fertilization (70,71). In both studies, microbial biomass was not affected by inputs of mineral fertilizer, but rates of respiration were affected. Long-term inputs of nitrogen can also cause shifts in the relative sizes of functional groups. Nitrogen limitation during decomposition of wheat straw caused a reduction in fungi but bacteria were unaffected (72). Nitrogen-fertilized alpine soils showed significantly reduced phenol degradation but degradation of glutamate and glucose were relatively unchanged (Schmidt and Lipson, unpublished data). Community shifts can also occur in response to seasonal changes, causing shifts in substrate utilization (73), and temperature response (74). Competition experiments using bacteria with different temperature optima and substrate affinities show that different organisms would dominate in different seasons (75), and so kinetic properties of the community could change as well.

Microbial community composition can also affect the kinetics of microbial processes in cases in which multiple populations are utilizing the same substrate. For example, it has been observed (9,76) that the kinetics of 2,4-dinitrophenol mineralization in soil indicated that two physiologically distinct populations were responsible for the process. One population functioned at high dinitrophenol concentrations (high K_s population) whereas the other was dominantly active at much lower dinitrophenol concentrations (low K_s population). Such "multiphasic" kinetics has been observed in several other aquatic and soil studies (77,78).

RESPONSES OF MICROBIAL ACTIVITY TO ENVIRONMENTAL FACTORS

From what has been stated in the preceding text, it is clear that the rate of a microbially mediated process in soil at any given moment is affected by a large number of variables. The picture is further complicated by the ever-changing soil environment. Microbial processes are subject to nonlinear effects from the dynamics of temperature, soil moisture, and predation. These are discussed in the following section.

Temperature

The rate of most chemical reactions increase exponentially with higher temperature according to the Arrhenius equation:

$$k = A \exp\left(\frac{-E_a}{RT}\right) \tag{8}$$

where k is the first-order rate constant (s^{-1}), A is a constant called the frequency factor (s^{-1}), E_a is the activation energy ($J\,mol^{-1}$), R is the ideal gas constant (8.3145 $J\,mol^{-1}\,K^{-1}$), and T is the absolute temperature (K). However, the effect of temperature on biological processes is harder to predict. Biologically mediated reactions are usually the result of several processes working in concert, each with its own response to temperature. Furthermore, enzymes and membranes can only function within a certain temperature range and so biological processes peak at some optimum temperature close to normal environmental conditions. Near the freezing point of water, physiological and diffusional effects further complicate rates of biological processes. Despite these factors, a narrow range usually exists where process rates obey the expected exponential relationship with temperature. This is especially true for enzymatic activity measured in isolation from other biological activities. One study (79) found that several soil exoenzymes obeyed Arrhenius kinetics within the range of 2 to 30 °C. They reported Q_{10}s (defined as the proportional increase in rate with a 10 °C increase in temperature) ranging from 1.3 to 4.1, with most falling close to 2.0. Workers (80) reported Q_{10}s for proteolysis and microbial uptake of amino acids as 1.98 and 2.57, respectively. As an example of how temperature responses of complex biological reactions can lead to subtle results, it was noted in the previous study that the higher temperature sensitivity of amino acid uptake compared to production could lead to higher availability of amino acids at lower temperatures. In an experiment with laboratory-incubated forest soils, researchers (81) reported that net nitrogen mineralization increased with temperature over the range of 5 to 25 °C, but that respiration showed a relatively flat response to temperature and peaked at 10 °C. The authors reasoned that microbial respiration became substrate limited at higher temperatures. In one study (82) it was observed that more labeled carbon and nitrogen from added substrate was retained in microbial biomass at lower temperatures, indicating that microbial turnover increased with temperature. This illustrates that mineralization is a balance between uptake and microbial turnover and that these processes have different temperature responses. A wide variety of positive, neutral, or negative relationships between microbial biomass and temperature have been reported (83).

Temperature affects not only the growth rate of microorganisms, but their growth yield and substrate affinity, as well. Lower growth yields are associated with higher growth rates (84), and hence, with higher temperatures. On the other hand, very slow growth rates can lead to low growth yields as well, as the energy required for maintenance of cells becomes significant relative to the carbon being assimilated into biomass (60,85). Substrate affinity sometimes decreases near 0 °C (75), although it has also been reported to increase with decreasing temperature (86).

Microbial and enzymatic activity can occur below the freezing point of water, and biological activities in soils at subzero temperatures have been reported (51,87–89). Significant mass loss of litter during winter has been observed in several ecosystems, although physical effects

could also be involved (90,91). The limit to biological activity in cold soils is not temperature, itself, but the availability of liquid water. There is always some liquid water present in frozen soils, but the width of the liquid film decreases, more or less sharply depending on mineral type, as temperature drops from 0 to −5 °C; liquid films of 50 Å or wider exist at 0 °C, and of about 6 Å at −5 °C or below (92). This creates a tortuous and discontinuous distribution of water in frozen soils, just as in very dry soils, that limits diffusion of molecules to microorganisms. Thus, diffusion coefficients of ions decrease with temperature in the same way the liquid water film width does (93).

Another important effect of freezing on the kinetics of soil processes is the disruptive effect of freeze-thaw events on soil aggregates and microbial cells. Freeze-thaw events tend to stimulate microbial activity in the short term (94–96) by releasing nutrients occluded in soil aggregates (97) or from lysis of cells (98,99). Researchers (95) have argued that repeated freeze-thaw cycles can have a long-term inhibitory effect on microbial activity by reducing the microbial population size. The rate of freeze determines the severity of effect on the soil microbial biomass. In an alpine soil, microbial biomass was not affected by freeze-thaw events designed to simulate spring and fall conditions (74,100).

Soil Water Content

In addition to the direct effects of soil water content on diffusion mentioned above, kinetics in soils are also affected indirectly by physical effects of fluctuating water content and by biological effects of osmotic stress on microbes. In dry soils, microbial activity can be limited both by osmotic stress and by diffusional limitation (101). Dry-rewet cycles can physically disrupt soil aggregates and microbial biomass much like freeze-thaw cycles do, as mentioned in the preceding text. This effect can enhance microbial activity by freeing substrate occluded in aggregates and by releasing nutrients from microbial biomass (102,103), but can reduce activity by lowering population sizes (104).

As discussed for temperature in the preceding section, the effect of soil water content on a given process depends on a complex set of factors. For most processes there is an optimum moisture content that balances both O_2 and water availability. In a field study of several alpine communities, CH_4 oxidation rates were stimulated by rainfall in drier soils, whereas the oxidation rates were negatively correlated with soil moisture in more moist soils (105). The stimulation of CH_4 oxidation by rainfall in dry soil may not have been purely because of release from water limitation of the methanotroph population, but may have been caused indirectly by creating anaerobic microsites in which methanogenic activity provided substrate for the growth of the methanotroph population (22,106). This again illustrates the complex interdependency among processes and environmental factors in soil.

Soil Texture and Carrying Capacity of Soil

Microbial growth in soil frequently follows a logistic pattern in which growth approaches a finite limit because of some environmental factors, such as substrate limitation (63). The carrying capacity can be also determined by some extrinsic factor such as the number of ideal sites for an organism. The abundance of colonizable surfaces and pores depends on soil texture; finer textured soils have smaller pores and more surface area. Bacteria living in small pores would be protected from predation by protozoa, whereas those in larger pores could be readily eaten. Various modeling studies (e.g., 107,108) predicted the turnover of microbial biomass according to the clay and silt content of soil.

MODELS OF MICROBIAL GROWTH AND SUBSTRATE USE

Many approaches have been used to model kinetics in soils. These include empirical models and models based on biological and/or physical theory. The shape of substrate disappearance curves depends on all the factors mentioned herein: the soil physical factors that affect substrate movement and availability, the initial substrate and biomass levels, the kinetic properties of the microbial community, and the many potential environmental factors, such as temperature and the availability of nutrients and water. In the following section we briefly review models of microbial activity, and discuss the practical matter of how to analyze respiration data from soils. For thorough reviews of mathematical models of microbial growth and biodegradation, see references (63,109).

The Use of Respiration Data in Studies of Kinetics in Soils

Carbon dioxide evolution from soil is commonly used to monitor degradation of added substrates (e.g. 48,49,110). Special caution should be exercised when analyzing carbon dioxide evolution data from soil incubations. Several research groups (e.g. 49,110,111) have stressed that analyzing accumulated CO_2 data leads to a number of statistical problems including nonrandom residuals, autocorrelated parameters and residuals, and underestimation of experimental error (Fig. 2). It is therefore preferable to use nonaccumulated rate data and the differential form of kinetic models to analyze soil respiration data. Examples of this approach to analyzing soil respiration data can be found in the literature (49,78). Most commercially available statistics packages contain algorithms for performing nonlinear regression and several reviews of this approach are available (109,112,113).

Variations on the Monod Equation

In liquid culture growth of bacteria occurs when initial substrate concentration (S_0) is high compared to initial biomass (X_0). Exponential growth occurs if S_0 is much higher than the half-saturation constant for the microbial population ($S_0 \gg K_s$), Monod kinetics occurs when S_0 is slightly higher than K_s ($S_0 > K_s$) and logistic growth occurs

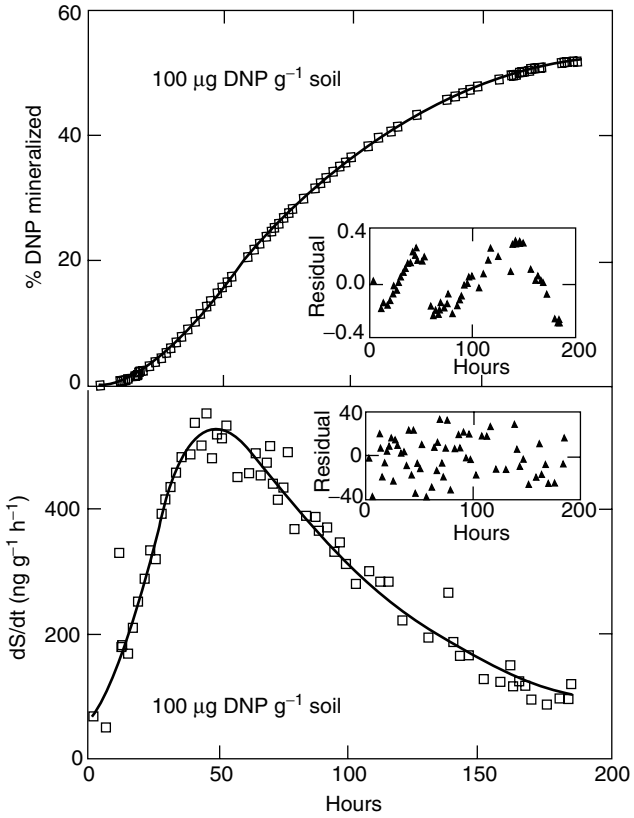

Figure 2. Respiration of ^{14}C-labeled dinitrophenol in soil graphed as cumulative CO_2 produced vs. time (A) and as CO_2 evolution rate vs. time (B). The residuals are shown in the inset of each panel, and show that fitting of accumulated data leads to nonrandom residuals. (Modified from Hess and Schmidt (110)).

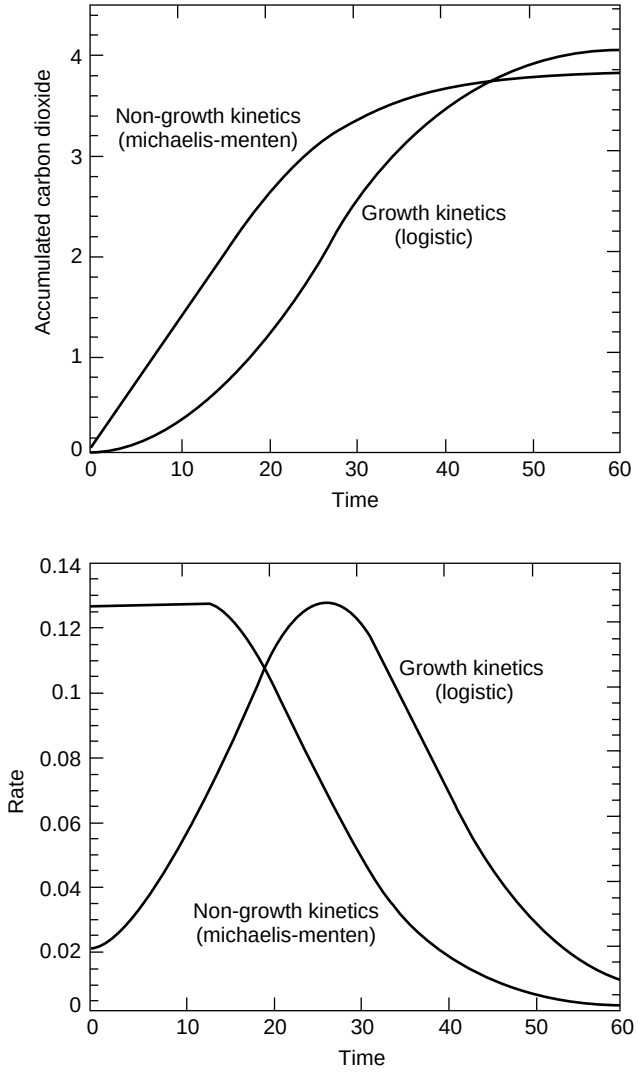

Figure 3. Shapes of typical growth (logistic) and nongrowth (Michaelis-Menten) kinetic curves: (A) graphed as accumulated product vs. time, and (B) as rate of product formation or substrate disappearance vs. time.

when $S_0 \ll K_s$. If S_0 is low relative to X_0, nongrowth kinetics occur, and conditions when $S_0 \gg K_s$, $S_0 > K_s$, and $S_0 \ll K_s$, result in zero-order, Michaelis-Menten, and first-order kinetics, respectively. The forms of these equations are all derived from the Monod equation, and are shown in Table 1. Examples of some of these types of curves are shown in Figure 3.

The Monod equation has been modified to include various biological effects (109). For example, several studies (e.g., 114) have modeled the biodegradation of inhibitory substrates using the variations of the Haldane equation:

$$\mu = \mu_{\max} \frac{S}{\left[(K_s + S)\left(1 + \frac{S}{K_i}\right)\right]} \tag{9}$$

where K_i is the inhibition constant, defined as the highest concentration of S at which $\mu = \mu_{\max}/2$. The Monod equation has also been modified to include the effect of metabolic maintenance requirements when microbial populations are subsisting on very low concentrations of substrate. The modified equation takes the form:

$$\mu = \mu_{\max} \frac{S}{(K_s + S) - a} \tag{10}$$

where a is the specific maintenance rate associated with nongrowth metabolic processes. This modification improved the modeling of degradation of low levels of pentachlorophenol and dinitrophenol in soils (85). The maintenance term, and other zero-order terms added to other models discussed in the following text, seem to be most important when very slow growth is occurring. This is when theory predicts growth yields would be low and maintenance requirements would be high (60).

Models of Microbial Activity in Soil

In soil, the effects of diffusion and sorption cause further deviations from the Monod model. Theoretically, observed kinetics in soils are best dealt with using two-compartment models that take into account the slow and fast release of substrate from soil aggregates and surfaces (e.g., 13–16). These models can be quite complex but represent a fairly realistic view of kinetics in soil. Data can also be modeled

effectively with an empirical approach, most commonly using 3/2 power kinetics (e.g., 29,56,62). The linear and exponential growth versions of the 3/2-order model are represented, respectively, as:

$$P = S_0 \left\{1 - \exp\left[-k_1 t - \frac{(k_2 t^2)}{2}\right]\right\} + k_0 t \quad (11)$$

$$P = S_0 \left\{1 - \exp\left[-k_1 t - \left(\frac{E_0}{\mu}\right)(\exp(\mu t) - 1)\right]\right\} + k_0 t \quad (12)$$

where P is product concentration, S_0 is the initial substrate concentration, and the k_n are rate constants. In Equation (11), k_2 is the linear growth rate, and in Equation (12), μ is the exponential growth rate. E_0 is related to the initial biomass by the relation, initial biomass $= E_0/a$, in which the constant, a, describes the dependence of the first-order substrate disappearance rate on biomass. It was found (62) that the linear model generally fit the data better except when microbial populations were first greatly reduced by gamma irradiation. The authors attributed this effect to the slow diffusion of substrates through thick layers of cells coating soil surfaces. The 3/2-order model is generally most effective under conditions in which little or no growth occurs, and has the advantage of fitting an initial acclimation phase and a late slow mineralization phase (62,63,115). Empirical models have the disadvantage of providing little heuristic information about the mechanisms affecting the observed kinetics. The parameters in the above 3/2 models are hard to interpret in a biologically or physically meaningful way. Some workers have interpreted the zero-order rate constant, k_0, to represent the "indigenous" slow, steady turnover of carbon in soil (62), others as the slow release of substrate from the soil matrix (29), and yet others as the metabolic energy expended by the microbes to maintain their biomass during long periods of slow growth (85).

Nutrient limitation or microbial community effects can cause growth to be linear or biphasic under conditions where exponential growth would otherwise be expected. The presence of stable soil colloid-extracellular enzyme complexes can further decouple kinetics from existing microbial populations. One study (29) found no correlation between the mineralization of contaminants and total microbial biomass or activity, but rather found major effects of the abiotic soil fractions present. In short, when observing microbial processes in soil, anything can happen. Fortunately, a variety of models have been developed, and existing models can be modified for most any given system.

CONCLUSION

Soil is a labyrinth of mineral and organic fractions with ever-changing microclimatic conditions and a diverse community of poorly understood microorganisms. To realistically include all potentially important factors in predicting a process rate would result in an excessively complicated and unworkable model. But despite the seemingly intractable complexity of the soil ecosystem, researchers have made great progress in understanding and predicting the kinetics of microbially mediated processes in soils. If one is flexible in one's model selection and allows for the myriad of possibilities that could arise, the situation is not hopeless. However, gaps in our knowledge still exist. More research is particularly needed to understand microbial community-level effects on processes in soil. Although we know almost nothing about the characteristics of most of the thousands of microbial species in soils, how they interact with each other, or their spatial and temporal distributions in soil microenvironments, this type of information is becoming increasingly available by way of powerful molecular techniques (116). It would be fruitful to integrate the current rapid advances in microbial ecology with the existing knowledge of microbial kinetics in soils.

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LABELLING OF MICROBIAL CELLS (ENZYMATIC, FLUORESCENT, IMMUNOLOGICAL, PHYLOGENETIC, AND PHYSIOLOGICAL LABELS).

See METHODS FOR FLOW CYTOMETRY AND CELL SORTING

LAKES, PERIPHYTON IN.

See PERIPHYTON

LANDFILLING OF MUNICIPAL SOLID WASTES: MICROBIOLOGICAL PROCESSES AND ENVIRONMENTAL IMPACTS

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Waste treatment and disposal are increasingly assessed against the hierarchy of waste management (1), in which disposal is viewed as the ultimate option for those wastes whose management cannot be addressed by recovery (e.g., recycling, composting, and energy recovery), reuse, or minimization/reduction. With municipal solid waste (MSW), some waste minimization can be achieved through reduced packaging or by use of longer-lasting products. Materials recycling is well established but may not be appropriate in all cases, for example, in which the cost of reprocessing is greater than virgin production, in which there are abundant raw materials, or in which there is no market for the recycled product (1). In the United Kingdom, the landfill tax was introduced in 1996, as a nonregulatory instrument to encourage recycling and reduce the volumes of waste going to landfills. However, the effectiveness of this strategy in terms of MSW, in which disposal costs are passed on to local taxpayers, is not yet evident. What is clear is that landfilling is still the major route for MSW disposal (Fig. 1) and is likely to remain so for many years. Even in countries where geography, economics, and legislation have stimulated other management options (Fig. 1), landfill is still a major disposal route.

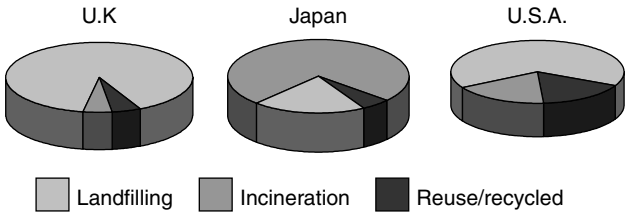


Figure 1. Waste disposal methods in the United Kingdom, Japan, and the United States.

MSW DECOMPOSITION AND STABILIZATION

The composition of MSW (1) shows clear geographic variations (Table 1), reflecting, for example, diet and the extent of domestic food preparation, solid fuel for domestic heating (ash content), and use of packaging materials (paper/card and plastics). Despite this variability, the decomposition and resultant stabilization of MSW follows a common pattern. Initial aerobic activity, whereby aerobic bacteria use soluble substrates such as sugars, as electron donors, is constrained by rapid depletion of oxygen (O_2). Within successive, layered, refuse depositions, conditions rapidly become anaerobic and a succession of chemical and microbial interactions mediates biodegradation of

Table 1. Composition (as % Wet Weight) of MSW

		Country					
		U.K.	Greece	Spain	Bulgaria	Hong Kong	U.S.
Ref.		2	2	2	2	3	4
Year		1992	1990	1992	1990	1983	1988
		Component					
Paper/board	Potentially	34.8	22.2	20.0	8.6	33.1	40
Food/garden	biodegradable	19.8	42.5	49.0	36.7	26.4	25
Plastics		11.3	10.5	7.0	6.9	23.1	8
Glass	Effectively	9.1	3.5	8.0	3.8	4.2	7
Metal	biologically	7.3	4.2	4.0	4.8	4.7	8.5
Textiles	inert	2.2	Ns	1.6	Ns	8.1	Ns
Other (including ash)		15.5	11.3	10.4	39.2	0.4	11.6

Note: Ns denotes not specified.

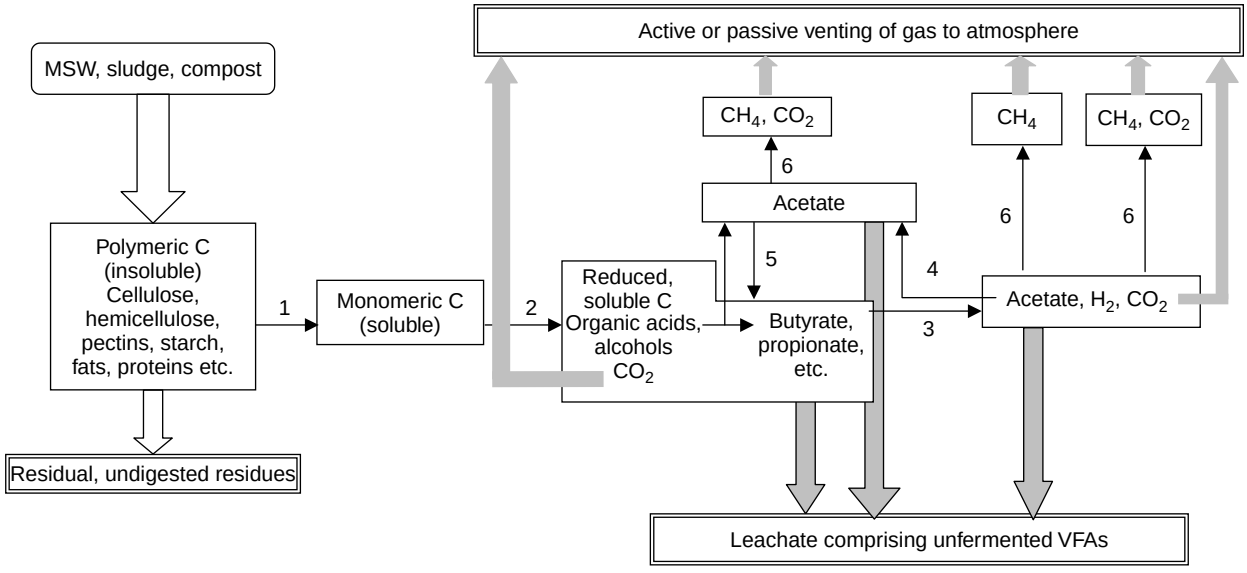
organic polymers. The transient aerobic phase, similar to a composting process, is, nonetheless, important, because it is only the waste metabolic heat, which is retained within the site owing to the insulating properties of refuse layers, that permits long-term refuse temperatures greater than 30 °C.

Landfill sites may be considered as multimillion cubic meter anaerobic digesters, in which a heterogeneous, mainly carbohydrate, feedstock is digested under suboptimum environmental conditions, with minimal intervention, by a diverse microbial population (Fig. 2). MSW typically comprises 40 to 50% cellulose, 12% hemicellulose and 10 to 15% lignin on a dry weight basis (5), of which only cellulose and hemicellulose are potentially amenable to anaerobic biodegradation. Not only is lignin highly recalcitrant under anaerobic conditions, but its integration into cellulosic tissues may also limit access of hydrolytic enzymes to cellulose (6). The predominantly carbohydrate nature of MSW is reflected in the estimation that more than 90% of the methane potential of refuse can be attributed to the cellulose-hemicellulose fraction (5).

Microbial degradation of insoluble polymers requires extracellular, hydrolytic enzyme activity, and a range of studies has demonstrated the presence in MSW of proteolytic, cellulolytic, and amylolytic organisms (7,8). Reports either describe total numbers (7) or attempt species identification (8). For example, 10 mesophilic cellulolytic Clostridia isolated from an MSW anaerobic digester showed similarity to, but were distinct from, *Clostridium cellulolyticum* and other previously isolated mesophilic Clostridia (8). It is generally accepted that there is in landfill sites a diverse hydrolytic population, whose composition reflects specific site conditions, and that hydrolysis is not limited by the metabolic capabilities of the genetic pool but rather by mass-transfer and kinetic properties.

In close association, fermentative bacteria reduce these soluble products to volatile fatty acids (VFAs), alcohols, ammonia, and carbon dioxide (CO_2). The fermentation, in turn, of these VFAs and alcohols to acetate, CO_2, and hydrogen (H_2), by acetogenic bacteria (see BIOSOLIDS: ANAEROBIC DIGESTION OF, this Encyclopedia) requires low partial pressures of hydrogen. For example, the acetogenic fermentation of propionate is only energetically favorable ($\Delta G' = -5.3$ kJ) at pH_2 less than 10^{-4} atm (9). Despite transient H_2 concentrations less than or equal to 20% by volume that have been recorded in landfill gas (10), suitably low H_2 concentrations are common and presumed to be due to the hydrogenotrophic activities of methanogenic, sulfate-reducing (SRB), or homoacetogenic bacteria. The significance of homoacetogenesis in MSW has not been assessed, although it was suggested that methanogens would outcompete homoacetogens (11).

The low sulfate and sulfide concentrations in most landfills, as evident from leachate analyses (Table 2), suggest that sulfate reduction is not a significant hydrogen sink. Wang and coworkers (12) measured the biochemical methane potential (BMP) of refuse samples and calculated that even if all sulfate removal from the cultures was at the expense of methane production, the corrected BMP was still at least 96% of the uncorrected, theoretical value. Moreover, although it is generally accepted that SRB can theoretically outcompete methanogens, because of their enhanced substrate affinities for H_2 (13), it has been demonstrated



1. Extracellular, hydrolytic enzymes
2. Fermentative enzymes
3. Syntrophic, acetogenic fermentations
4. Homoacetogenesis
5. C-1 elongation reactions
6. Methanogenesis

Figure 2. Stages in the anaerobic microbial decomposition of the organic fraction of MSW.

Table 2. Key Constituents of Landfill Leachates

	Montreal	Hong Kong		Greece		Taiwan	Turkey	U.K.	
Ref.	16	17		18		19	20	21	
Leachate age/ site maturity	Ns	10–11 y/ "closed" site	<3 y/ "active" site	[a]Ns/ acetogenic	[b]Ns/ stabilized	2y	3.5y	<2y	>20y
pH	6.8	7.0–8.3	5.6–7.3	5.97	7.5	6.3	7.3–7.8	6.21	7.45
BOD ($mg\,l^{-1}$)	450	7,300	1,600	36,500	72	15,710	10,875	11,900	260
COD ($mg\,l^{-1}$)	860	17,000	6,610	69,400	1,140	22,750	18,100	23,800	1,160
VFAs ($mg\,l^{-1}$)	[c]Ns	Ns	Ns	Ns	Ns	13,740	8,600	5,688	<5
NH_4-N ($mg\,l^{-1}$)	20	3,000	1,500	1,260	261	171	1,810	790	370
PO_4^{2-} ($mg\,l^{-1}$)	Ns	10	10	15	0.1	Ns	64	0.7	1.4
SO_4^{2-} ($mg\,l^{-1}$)	Ns	1,000	39	Ns	Ns	Ns	350	Ns	Ns
Cl^- ($mg\,l^{-1}$)	190	12,000	3,400	3,800	3,450	Ns	Ns	1,315	2,080

[a]drainage well.
[b]leachate pond.
[c]not specified.

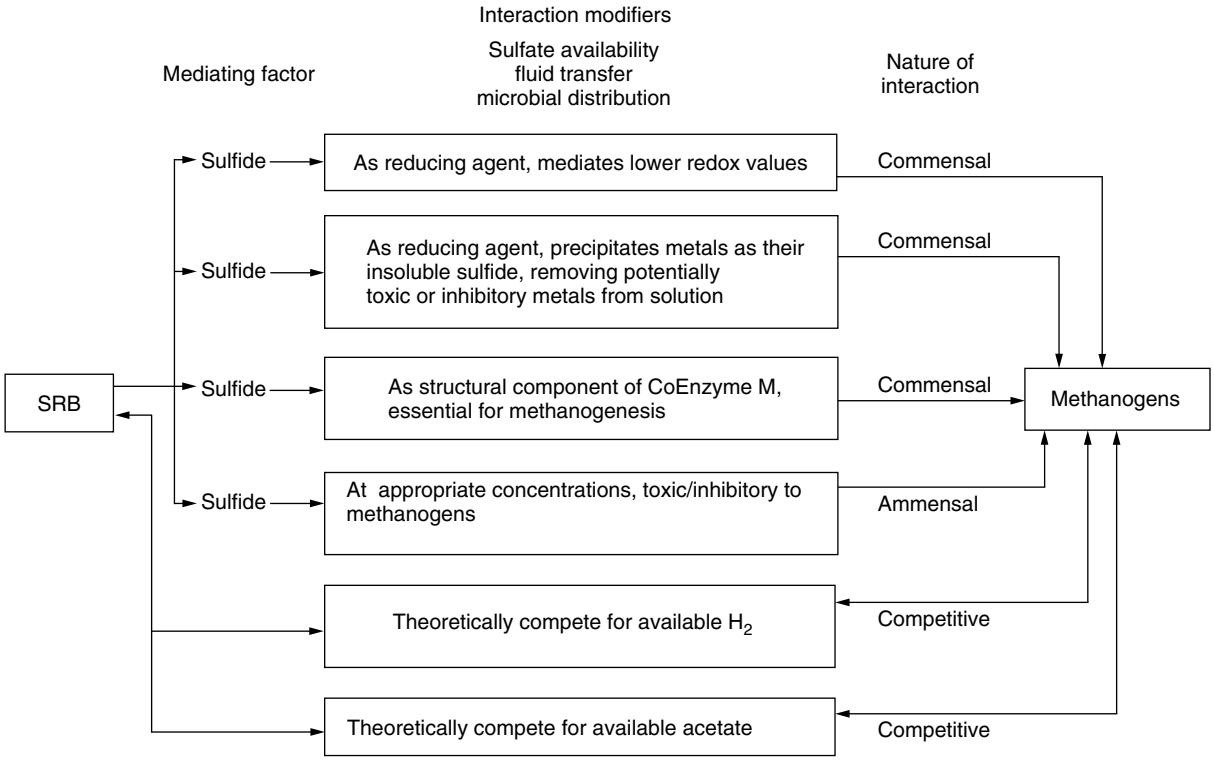


Figure 3. Potential interactions between sulfate-reducing and methanogenic bacteria in landfill sites.

that under specific circumstances, for example, in which growth is predominantly attached, electron flow may be preferentially directed to methanogenesis (14). However, although of little significance in terms of overall electron flow, SRB may play an important role in the complex web of refuse-microbial interactions (Fig. 3). For example, as a reducing agent, sulfide may contribute to the low redox conditions (often <-300 mV) required for the growth of methanogens (15).

In actively methanogenic landfills, most electron flow is clearly directed toward methanogenesis. The transition of a site from the acetogenic to the methanogenic phase is signaled by concentration decreases in both H_2 and acetate. The initiation of significant methanogenesis may occur only after many months of acetogenesis, due presumably to a range of factors such as a requirement for redox potentials <-300 mV and low methanogenic growth rates. The significant decreases in soluble C subsequent to methanogenesis are commonly used to support the hypothesis that polymer hydrolysis and not syntrophic H_2 transfer ultimately limits methane release. The methanogenic phase may persist for several decades, depending on the quantities and characteristics of the landfilled refuse, environmental conditions, and site-management practices. Even under optimum conditions, complete degradation of the organic fraction of MSW

cannot be expected, owing to the biological recalcitrance of materials such as plastic, rubber, and leather, and the anaerobic persistence of lignin and lignocellulosic materials. Thus, landfills represent a net carbon (C) sink, whose significance in the global C-cycle was estimated (6) to be at least 119 million tonnes per year, or 5 to 10% of the 1–2 Gt C y^{-1} of "missing" C previously identified.

It is clear, however, that in situ C releases, as methane, from landfill sites fall far short of the theoretical yields. Variability in measurement techniques and waste type limits the usefulness of comparing yields. Theoretical yields have been reported to vary from 229–372 $m^3\,t^{-1}$ wet material (2) to 152 $m^3\,t^{-1}$ dry material (5), whereas estimated average yields vary from 100 to 240 $m^3\,t^{-1}$ wet material (2).

FACTORS INFLUENCING GAS YIELD AND COLLECTION

The factors that influence methane production in landfills have been extensively investigated and reviewed (4,22).

Moisture

There is general agreement that moisture in many sites is the single most influential modifier, although the optimum moisture levels reported have ranged from 60% to 80% (22), significantly in excess of fresh refuse values (15–45%). It is thus not surprising that leachate recycle has been shown to enhance the onset and rate of methanogenesis (23), given that this will increase moisture content and substrate availability, and provide a degree of mixing. A dry site cannot, therefore, be assumed to pose less of an environmental hazard, because future moisture infiltration could elicit a rapid microbiological response.

Temperature

In landfill sites, in which temperatures are defined by the length and activity of the aerobic phase and by site-management practices (for example, leachate recycle and refuse compaction), temperatures generally are mesophilic (22) and often below the optimum 35–40 °C. All methanogens so far identified are either mesophilic or thermophilic (15) (see BIOSOLIDS: ANAEROBIC DIGESTION OF), and although methanogenesis has been recorded environmentally at temperatures as low as 4 (24) and 6 °C (25), the laboratory samples in all cases exhibited optimum temperatures around 35 °C. Where methanogenesis has been recorded in sites at temperatures greater than 50 °C (26) it is not clear whether this reflects a truly thermophilic population.

Refuse pH

Significant methanogenesis can occur in refuse samples at pH 5.7 (27) and has been recorded in sites in which the pH ranged from 5.5 to 8.3 (28), although most methanogens are considered to have optima near neutrality (15). It is probable that this reflects refuse heterogeneity, by which the pH in refuse microniches may be greater than that in pore water. Nonetheless, a pH value less than or equal to 5.5 is still considered detrimental to the initiation of methanogenesis in situ (28). Whether the inhibition is due to general pH effects or to specific inhibition by individual VFAs was investigated by use of multistage continuous culture systems, in which the pH values were poised and which were supplemented with acetate, propionate, or butyrate (100 mmol l^{-1}) (29). Sulfate-reducing bacterial activity in a butyrate-catabolizing association was completely inhibited by acetate and propionate, although methanogenesis was stimulated, particularly by butyrate supplementation. It is more likely that elevated VFA concentrations in "young" leachate reflect the prolonged lag period before methanogenesis rather than inhibition of methanogens.

There are difficulties, often observed in laboratory cultures (27) and probably underreported, of consistently inducing methanogenesis in homogenized refuse samples, despite the ease with which temperature and moisture content can be controlled, and in which acidic leachate pH values can be neutralized. It is thus perhaps pertinent to ask whether the induction of methanogenesis in situ is in fact encouraged by the presence of suboptimum conditions. For example, reduced rates of acidogenesis may permit concurrent growth of the slower-growing methanogens, in the absence of acids accumulation and reduced pH values. Refuse heterogeneity and reduced fluid flow may provide protective niches for methanogens. The limited degree of intervention that is possible in situ will certainly not permit operators to achieve optimum rates of methanogenesis but may, paradoxically, allow the transition of sites from the acetogenic to the methanogenic phase.

BIODEGRADATION AND INHIBITION BY XENOBIOTIC CONSTITUENTS

Although most landfill sites are not licensed to accept hazardous or toxic wastes, it has been estimated that each household contributes greater than or equal to 4.5 liters of hazardous waste per annum to municipal refuse (30). These result in the presence, in leachate, of a wide range of organic xenobiotics (Table 3). The impacts of these organic compounds on refuse-microbial processes and their biodegradative potentials are poorly understood. Although, analyses indicate (Table 3) that most are present in leachate at low and presumably noninhibitory concentrations (<0.5 mg l^{-1}), the spectrum of compounds presents the possibilities of substrate interactions. For example, bacteria may use the aggregate of many compounds to gain electrons and energy, although the contribution of each specific compound is negligibly small (31). Where degradation requires enzyme induction, concentrations may be below the threshold value. However, the presence of a closely related molecule may induce the appropriate enzyme (31). Although, individually, concentrations are below the respective toxicity thresholds, aggregative effects may be significant, such that the total toxicity is either additive (the sum of the individual toxic values) (32) or synergistic (greater

Table 3. Selected Xenobiotic (Organic) Constituents of MSW Landfill Leachates

Compound	Concentration ($mg\,l^{-1}$)	Ref.
1,1,1-trichloroethane	0.086	2
1,2-dichloroethane	0.015–0.36	33,34
Benzene	0.02–3.8	2,33,34
Chlorobenzene	0.007	2
Dichlorobenzene	0.31	33
m-cresol	0.6	34
Methylene chloride	17.0	33
m-ethylphenol	0.2	34
Naphthalene	0.29	33
PCBs	0.00073	2
p-cresol	1.5	34
Pentachlorophenol	0.045	2
Phenol	0.38–1.2	2,34
Tetrachloroethylene	0.073	33
Tetrahydrofuran	0.002–0.33	34
Toluene	0.41–41.0	2,33
Trichloroethene	0.043	2
Trichloroethylene	0.36	33
Vinyl chloride	0.04	2

than the sum of the individual toxic values). Data are, however, lacking for toxic values of MSW.

Most studies on anaerobic biodegradability use digested sludge as inoculum, and relatively few have examined the potential for biodegradation in MSW samples. Both refuse-column (with and without leachate recycle) and batch-bottle studies were used (35–37) to investigate the anaerobic biodegradation of phenolics. With leachate recycle, 100% removal of phenol ($188\,mg\,l^{-1}$) was recorded with concomitant enhanced rates of methane release. The degradation of *o*-cresol was also reported (38), although its relative recalcitrance is emphasized by other MSW studies (39) in which no *o*-cresol mineralization was noted. Conversely, the degradation of *p*-cresol and m-cresol has been consistently identified in anaerobic MSW, although with a longer lag period than that recorded with phenol (39). Radiolabeling was used (39) to confirm the degradation of toluene in refuse-inoculated bottles, although other BTEX compounds (benzene, ethylbenzene, and xylene) were not degraded. Laboratory reactors have also been used, and the anaerobic degradation observed of chlorinated aliphatic compounds (1,1,1-trichloroethane, tetrachloromethane, and tetrachloroethylene) (40), trichlorofluoromethane, 1,1-dichloroethylene, and 2,4,6-trichlorophenol (41). In the latter study, no transformation of 3-chlorobenzoate, 2,4,6-trichlorobenzoate, or chlorotrifluoromethane was observed. The anaerobic biodegradation of other compounds such as ethylene glycol (42), 2-chlorophenol (43), 2,4,5-trichlorophenoxyacetic acid (44), and alkylbenzenes (45), albeit with sludge (42,43) as the inoculum or in groundwater samples (44,45), is also encouraging. It may be, however, that a combination of concentrations below enzyme-induction levels, poorly mixed conditions, low moisture and temperature levels, and limited in situ fluid flow prevent realization of the potential of landfill sites for xenobiotic treatment and removal.

INHIBITION BY INORGANIC MSW CONSTITUENTS AND GENERATED COMPOUNDS

The presence of potentially toxic or inhibitory metals in leachate (Table 4) has prompted speculation that these may inhibit initiation of methanogenesis (46,47). However, this seems unlikely at the concentrations typically recorded in leachate. In an MSW lysimeter amended with metal-plating sludge at a loading rate of $3{,}897\,mg\,Zn\,kg^{-1}$ MSW, leachate concentrations were not significantly different from the control. This was attributed to ligand formation and precipitation of the metal as its insoluble sulfide (48). On supplementation of anaerobic MSW leachate with zinc (Zn), lead (Pb), copper (Cu), and iron (Fe) at concentrations of 100, 10, 5, and $1{,}000\,mg\,l^{-1}$, respectively, inhibition was observed only with Zn (46). The importance of sulfide alleviation is supported by experiments in which Enterobacter and Aeromonas, isolated from landfilled refuse (47) but incubated aerobically, showed lower minimum inhibitory concentrations for Zn, Pb, and Cu, of 5,5, and $20\,mg\,l^{-1}$, respectively. In anaerobic digesters inoculated with sewage sludge the recorded toxicity scale was different, such that Cu > Zn > Pb (49). The importance of hydrogen sulfide on the alleviation of heavy metal inhibition (Fig. 3) was demonstrated (50) in a digester in which complete

Table 4. Selected Xenobiotic (Inorganic) Constituents of Leachates

Heavy-Metal Concentration ($mg\,l^{-1}$)	Germany	Greece	U.K.	Canada	Finland	Hong Kong	
						Closed Site	Active Site
Reference	2	18	21	16	51	17	
Al	2.4	5.5–18.3	Ns	Ns	<0.14	Ns	Ns
As	0.014	<0.4	Ns	Ns	Ns	Ns	Ns
Cd	0.014	<0.07	<0.005	Ns	<0.08	<0.01	<0.01
Cu	0.054	0.03–4.8	0,08	1.7	<0.02	0.5	0.02
Pb	0.063	<0.5	0.11	1.0	<0.2	1.0	0.04
Hg	0.0006	<0.5	Ns	Ns	Ns	Ns	Ns
Ni	0.17	0.23–0.82	0.18	Ns	0.056	0.5	0.1
Zn	0.68	0.5–8.56	17.6	2.5	0.57	1.0	1.0

Note: Ns denotes not specified.

inhibition was recorded with 20 mg Cu l^{-1} at a Cu^{2+}/VSS of 0.015. However, with sulfide addition, before spiking with Cu, recovery was rapid although the Cu^{2+}/VSS ranged from 0.054 to 0.058.

POPULATION STUDIES

Bacterial Populations: Conventional Techniques

Quantification and characterization of the microbial population of landfill sites was until recently performed by use of conventional enumeration techniques, based on culture on selective media and most probable number (MPN) counts. For example, the addition of ball-milled cellulose to mineral medium was used for the enumeration of cellulolytic bacteria (7), which were identified by clear zones around colonies. Similar clearing techniques were used for the identification of starch-degrading and proteolytic bacteria. By use of these techniques, from samples of refuse taken from depths greater than 3 meters, relatively high densities of both aerobic and anaerobic bacteria were recovered. Anaerobic counts ranged from 0.2 to 32.8×10^6 colony forming units (cfu) (g dry wt)$^{-1}$ at 22 °C; lowest numbers were found in the oldest refuse (24–27y). Interestingly, cellulolytic organisms were not recovered from any of the three site samples, although cellulolysis was recorded in other refuse samples (52), by use of similar techniques. As the refuse became methanogenic (Fig. 4), increases in cellulolytic, acetogenic, and methanogenic counts were recorded. Before this, total anaerobic counts increased, by 2 orders of magnitude, to exceed the methanogenic and acetogenic populations by a factor of 10^6. This increase, concomitant with accumulation of short-chain fatty acids, was presumed (52) to reflect population increases in fermentative bacteria.

Conventional techniques such as these have recognized limitations. The counts on solid media undoubtedly were conservative (7), because only some bacteria would be culturable on the medium provided. Moreover, isolation of viable organisms does not necessarily reflect in situ activity. Where fermentation is dependent on interspecies interactions, as in highly anaerobic environments, enumeration of fermentative bacteria will be underestimated if incubation conditions are not sufficiently rigorous to support the growth of hydrogen sink bacteria such as methanogens. Biomass measurements based on ATP or ester-linked phospholipid fatty acid concentrations have been applied to leachate-contaminated aquifers (53) but are likely to be of limited use in the heterogeneous refuse matrix. Direct examination of acridine orange-stained samples (7,53) may be an overestimation of viable bacteria.

Bacterial Populations: Molecular Methods

Increasingly, molecular methods are being applied to environmental samples (see ACTIVATED SLUDGE — MOLECULAR TECHNIQUES FOR DETERMINING COMMUNITY COMPOSITION) and are demonstrating the presence of as-yet uncharacterized and unidentified species. One of the earliest refuse studies (54) used conventional enrichment methods to isolate methanogenic bacteria from seven U.K. landfill sites before antigenic fingerprinting. Although two isolates were

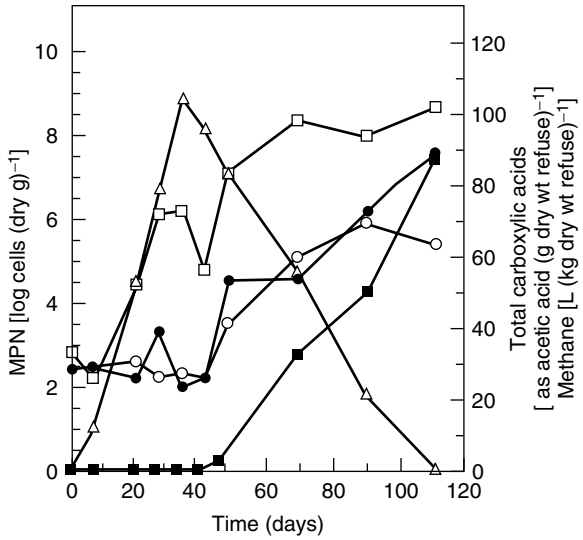


Figure 4. Changes in cellulolytic (○), acetogenic (●), and methanogenic (❑) populations, methane concentrations (■), and total carboxylic acid concentrations (△) in refuse samples [adapted from M. A. Barlaz et al., *Appl. Environ. Microbiol.* **55**, 55–65 (1989)].

closely related to *Methanobacterium formicicum* MF and *Methanosarcina barkeri* MS, a coccoid isolate was unrelated to any of the reference methanogens. Most research has, however, focused on the methanogenic subpopulations in other environments such as peat bogs (55), marine marshes (56), or anaerobic bioreactors (57). A polymerase chain reaction (PCR) method was used (58) to amplify 16s RNA sequences from digester contents, and 27 sequences were obtained that could be only partially matched with the sequences of bacteria in a general database. A major weakness of molecular methods so far is the requirement for characterized reference strains: a low correlation score may indicate either a generic match, or, conversely, a close match to a reference strain that has not been extensively characterized.

Similar techniques have been used to characterize other subpopulations such as syntrophic (57) and cellulolytic bacteria. In one study 10 cellulolytic mesophilic Clostridia were isolated from an MSW digester and differentiated by hybridization experiments with three cloned fragments carrying cellulase genes of *C. cellulolyticum* (8). From the results of these and other techniques (e.g., base composition of DNA, electrophoretic separation of soluble proteins, and CMCase activity) the authors concluded that all the strains were different from *C. cellulolyticum* and from other Clostridia described in the literature.

Both conventional microscopic or cultural examinations and amplification of nucleic acids have indicated that a huge diversity exists in a range of anaerobic environments. It is also clear that significant shifts in subpopulation development, in response to changes in environmental conditions, are characteristic of landfill sites (7,52). In such a temporally and spatially heterogeneous environment the high diversity represents a reservoir from which minority species may proliferate as conditions change.

Protozoa

Protozoa are widely distributed in anaerobic habitats, where they are the predominant and often the only consumers of other microorganisms (59). The presence of anaerobic protozoa in landfilled MSW was first reported in 1991, with the isolation of eight species (59), of which the most ubiquitous was the ciliate *Metopus palaeformis*. Each ciliate contained up to 500 methanogens, probably *Methanobacterium formicicum*, which were retained and transmitted in the cysts. It is likely that protozoa in landfills are commonly encysted (60), especially in drier (<40% water) sites, and the significance of protozoan activity in landfill sites is as yet not clear. In laboratory studies, methanogenesis was closely coupled to host metabolism and growth and although maximum methane-release rates of 0.35 pmol per ciliate per hour were recorded (61), it is difficult to extrapolate from this to likely rates in situ. There is, however, evidence that in anaerobic cultures, enhanced bacterial activity was positively correlated with the number of ciliates present (62). Ciliates with methanogens also produced methane under microaerophilic conditions, in which pO_2 was as high as 0.63 kPa (3% atm sat). In the rumen, another anaerobic environment characterized by the degradation of cellulosic substrate, anaerobic fungi as well as bacteria and protozoa are part of the autochthonous microflora. However, research has failed to enrich and isolate anaerobic fungi from landfilled MSW samples (63).

ENVIRONMENTAL IMPACTS OF MSW LANDFILLS

Health

Health and environmental concerns that arise from landfilling practices are diverse and include air quality and odors, leachate escape, and disease vectors, whether animal (seagulls, flies, rats, etc.), aquatic, or airborne. Studies on the health hazards associated with landfilling have included epidemiological studies (64,65), application of a public health assessment process (66), and direct examination of the microbiological identity of MSW (67), dust, and aerosols (68,69).

Epidemiological Studies. Epidemiological studies initially focused on sites that accepted hazardous wastes. For example, Paigen and coworkers (70) studied the health problems in children living near Love Canal, New York. Compared to the control sample, Love Canal children significantly were more likely to suffer from conditions such as seizures, hyperactivity, skin rashes, and incontinence. Intensity of exposure, measured in terms of either distance from the site or proximity to possible paths of chemical migration (i.e., wet homes) also demonstrated a dose response. Similar results were reported from a later study by Berry and Bove (64) who examined the effects on birth weight of residence near a hazardous waste site in which disposal ceased in 1971. Examination of birth-certificate information (1961–1985) indicated that proximity to the landfill had a significantly adverse effect on both average birth weight and proportion of low–birth weight babies, of a magnitude comparable to that associated with smoking in pregnancy. Although studies such as these may identify pathways for community exposure, such as inhalation of volatilized chemicals emitted from the landfill, the exposure of individuals to specific chemicals is almost impossible to measure. However, the presence of volatile chemicals such as benzene, toluene, methylene chloride, 1,2-dichloroethane, phenol, and formaldehyde was confirmed.

Potentially toxic volatile organic compounds (VOCs) have also been identified in the gas emitted from municipal waste–disposal sites not licensed to accept hazardous wastes. In a study on seven U.K. landfill sites, more than 140 VOCs were identified, of which chloroethene (>1–$87\ mg\,m^{-3}$) was the most abundant toxic component (71). At two of the sites studied, levels were in excess of the U.K. maximum occupational exposure limits ($18\ mg\,m^{-3}$) by factors of 5 and 3. In a comparable study on four Finnish landfills (72) the main chemical of concern was tetrachloromethane, which at concentrations from 1.7 to $34.3\ mg\,m^{-3}$ was occasionally present at levels above occupational air–quality norms ($14\ mg\,m^{-3}$). Although the dilution of vented gases in the atmosphere rapidly reduces VOC concentrations, there remain some concerns about possible effects on site employees. For example, at Fresh Kills landfill sites (N.Y.), which with a daily input of 14,000 tons of solid waste is one of the world's largest, there was a higher prevalence among site employees of a range of symptoms (dermatologic, neurologic, hearing, and respiratory) than among off-site employees (65). However, because of the exposure of employees to a number of potentially harmful substances and conditions including fungi, bacteria, diesel exhausts, dusts, medical wastes, heavy metals, noise, and temperature extremes, it was not possible to categorize employee exposure, and hence difficult to hypothesize specific health outcomes (65).

Microbial Pathogens. The actual health risks posed by the presence of microbial pathogens in landfilled refuse are also difficult to assess. Several studies have addressed the disposal of clinical wastes. Although most is incinerated, there is no doubt that some is still landfilled, legally or otherwise (67). In addition, the increasing tendency toward early hospital discharge and care at home generates clinical waste in a domestic setting (73); attendant disposal routes are dependent on local practices. Certain categories of clinical wastes such as incontinence pads, stoma bags, and urine containers can be landfilled, depending on site licences. Clinical wastes are not the only source of bloodstained or fecal materials, and large numbers of used disposable diapers and sanitary pads or tampons are discharged, with domestic refuse, to landfill sites. In the United Kingdom, the National Household Waste Analysis Project (74) reported that 4.3% of MSW comprised disposable diapers, of which approximately one-third may be soiled with feces (75). Other potential sources of pathogens include soiled cat litter and municipally collected dog feces. Undoubtedly, therefore, landfill represents a sink for pathogenic and

opportunistically pathogenic microorganisms. Collins and Kennedy (67) reviewed the pathogenic species found in both municipal and clinical waste (Table 5) but noted that in situ conditions at controlled landfill sites do not favor the growth or even survival of many pathogens.

Enteric viruses are common causes of juvenile gastroenteritis and thus likely to be deposited in landfills, in soiled disposable diapers. Although it was reported (76) that, in general, 10% of fresh soiled diapers were positive for poliovirus type 1 and echoviruses, few studies have addressed the persistence of enteric viruses in MSW. Of 110 soiled diapers recovered from landfill sites, and aged from 2 to 10 years, three were positive for poliovirus but negative for rotavirus and hepatitis A virus (77). This suggests that viruses were not viable after two years or more in the landfill environment. This conclusion is supported by earlier work on viral seeded lysimeters in which none were recovered after four months (78), due presumably to either inactivation at the temperatures generated in situ or their adsorption to MSW in the presence of high concentrations of dissolved salts. More than 90% inactivation of poliovirus type 1 was recorded within days at 37 °C, compared to 70% after 27 days at 4 °C (78).

Reports on the elution, in leachate, of enteric bacteria and fungi have focused largely on lysimeter and test-cell studies, in which MSW was artificially seeded. Reviewing the results, Gerba (76) attributed the variability of the results to variations in reactor configuration and operation, which render it difficult to assess accurately the risks posed by leaching of pathogenic species. The studies that have examined the microbial composition of leachate-contaminated aquifers (53) have not addressed possible pathogen transport.

Few studies have addressed the microbiological qualities of the air at landfill sites. In the working air of two large Finnish landfills, mesophilic bacteria and fungi exceeded 10^5 and 10^4 c.f.u. m^{-3}, respectively (68). In 67% of the samples, the concentrations of gram-negative bacteria exceeded 10^3 c.f.u. m^{-3}, although endotoxin levels were all below $0.1 \mu g\, m^{-3}$. The most commonly isolated bacteria were Pseudomonas, Enterobacter, and Bacillus spp., and could be considered opportunistic pathogens. Although inhalation of gram-negative bacteria may induce symptoms such as fever and chest tightness, and although such symptoms were shown to be associated with refuse handling (69), the effects of such exposure on health undoubtedly are difficult to evaluate, in the context of simultaneous exposure to other risk factors on site (65).

Table 5. Pathogens and Some Other Organisms in Both Municipal and Clinical Waste

Pathogens	Other Organisms
Aspergillus spp.	*Alcaligenes* spp.
Bacteroides melanogenicum	*Bacillus* spp.
Candida spp.	*Clostridium* spp.
Escherichia coli	*Citrobacter* spp.
Klebsiella pneumoniae	Coliforms
Listeria monocytogenes	*Enterobacter* spp.
Pseudomonas aeruginosa	*Enterococcus* spp.
Pseudomonas maltophila	*Halfnia* spp.
Salmonella serotypes	*Staphylococcus* spp.
Serratia marcescens	Yeasts and microfungi
Staphylococcus aureus	
Streptococcus pyogenes	
Yersinia enterocolitica	

Gas Emissions

Concerns about future climate change rest to a significant degree on the increasing concentrations of a number of long-lived radiatively active gases, of which CO_2 and CH_4 are two. The present atmospheric CH_4 concentrations of 1,700 ppbv have increased from approximately 650 ppbv preindustrial levels, which links the increase to anthropogenic emissions (79). Estimates for total anthropogenic methane range from 277 to 477 $Tg\, y^{-1}$ (80). Methane, although responsible for only a small percentage of the direct greenhouse warming, has a great indirect influence, because any increase in temperature leads to increases in water vapor and clouds, which collectively are responsible for more than 90% of the natural greenhouse effect and keep the earth about 33 °C warmer than would otherwise be the case (81). From an actively methanogenic landfill site the gas released comprises, typically, 50–70% methane with the balance predominantly carbon dioxide. Trace constituents include H_2, hydrogen sulfide, and potentially toxic volatile compounds such as benzene, chloroethene, and toluene (71). Worldwide, estimates of the venting of landfill methane to atmosphere range from 10–20 $Tg\, y^{-1}$ (82) to 19–40 $Tg\, y^{-1}$ (80), although such data are difficult to equate with the suggested U.S. emission rates from landfill sites of 35 $Tg\, y^{-1}$ (83). The estimate of 2 $Tg\, y^{-1}$ (79) for U.K. landfills appears more realistic.

Passive venting of landfill gas may have more immediate effects, ranging from the relatively trivial (e.g., plant die-off) to the catastrophic. In 1986 a house in Derbyshire, United Kingdom was destroyed by a methane explosion, the cause of which was traced to uncontrolled subsurface migration of landfill gas from an adjacent closed site (84).

Alternatives for the management of landfill gas emissions include

1. encouraging the natural microbial process of methane oxidation in landfill-cover soils through which the gas is passively vented,
2. through a series of perforated pipes and sunk wells, directing the gas to flares, where it is converted to CO_2 and H_2O, or
3. recovering and exploiting the energy potential of the gas.

Methane Oxidation. Annually, soils consume about 40 $Tg\, CH_4\, y^{-1}$ from the atmosphere and contribute significantly to the atmospheric methane budget (85). In most soils, methane oxidation is constrained by the diffusive transport of methane in the aqueous phase, and thus rates of methanotrophy in soils from environments

as diverse as tundra, savannah, tropical forests, and prairie are comparable, typically falling into a range of 0.5–2 mg $CH_4\,m^{-2}\,d^{-1}$ (85). Landfill-cover soils present an environment in which subsurface biogenesis permits the diffusive transport of elevated methane concentrations in the gaseous phase, which was considered (83) to account for the high rates (45 g $CH_4\,m^{-2}\,d^{-1}$) recorded in 12-cm soil cores from a closed landfill site in California. Soil with an open structure is optimum for enhanced methanotrophy. Kightley and coworkers (86) considered that a 50-cm layer of porous coarse sand might prevent the emission of methane from up to 13-m depth of landfill. However, a coarse sandy soil will be particularly sensitive to moisture changes, and Whalen and coworkers (83) considered that oxidation rates were moisture- rather than methane-dependent. A relationship between methane oxidation and moisture content was reported in cores excavated from a German landfill site (87), where oxidation was recorded from depths of 55 cm, although rates were 18.4% of those recorded at 10-cm depth. In one of the few field studies, no net methane emissions were measured from a midlatitude landfill (Illinois, U.S.A.) (88), a warning not to predict emissions from estimated generation rates.

Energy Recovery from Landfill Gas. The European Commission's White Paper on Renewables proposed a target of doubling, by 2010, from 6 to 12%, the contribution of renewables to Europe's primary energy requirements (89). In the United Kingdom, where renewable energies already meet more than 2% of the electricity needs, energy recovery from landfill gas is an integral component of renewables technology. Under the NonFossil Fuels Obligation (NFFO) in England and Wales, and Scottish Renewables Orders (SRO) in Scotland, more than 288 projects had been contracted by November 1999 to provide 30.61 MW DNC (declared net capacity) (89).

The diminishing gap between costs and market unit price reflect the development of larger, highly engineered sites with purpose-built extraction systems. However, projections of the amount of gas generated throughout the lifetime of a site are highly variable, with estimates from 39 to 500 $m^3\,t^{-1}$ (1), at rates that typically range from 6 to 8 $m^3\,t^{-1}\,y^{-1}$. With these site variations it is clear that in most sites methane release may continue for many years, and that release rates may be significantly below optimum values. Much recent research has focused either on optimizing gas release [e.g., through manipulation of the moisture regime and microbial decomposition rates (23,90) or by improved understanding of fluid flow in landfills (91)] or on the development of more sophisticated and accurate models for prediction of the generation and release of methane (92,93).

Leachate Emissions

Leachate will be generated in any landfill site in which moisture content, as a result of inherent moisture content on emplacement and subsequent infiltration, exceeds field capacity. Leachate composition will thus reflect the climatic conditions, infiltration rate, and refuse constituents, density and absorbency. Infiltration rate is heavily influenced by preferential channeling, a movement mechanism not as yet well understood (94). The composition of leachate (Table 2) is also heavily influenced by site age; "young" leachates are characterized by low pH values, high biochemical oxygen demand (BOD) and chemical oxygen demand (COD) levels, and intermediate ammonia concentrations. In older leachates the higher pH values are associated with lower BOD values, and the BOD : COD and COD : TOC (total organic carbon) ratios have been used as indicators of leachate (and hence site) maturity (95). In a study on Hong Kong landfills, the COD : TOC ratios decreased from 4.1 in a young fill to 2.7 for an old fill (17). The BOD and ammonia concentrations of leachate represent an environmental hazard if leachate is allowed to discharge untreated to watercourses, whereas the other constituents, which include heavy metals and potentially toxic organic compounds (Tables 3,4), pose ecotoxicological challenges. Although temporal fluxes are commonly observed with leached BOD and COD, inorganic ions show less variation with time. However, as leachate pH rises, metal ions are less soluble and concentrations tend to decrease (1). The continuous elution of inorganic ions has proved useful as a tracer of groundwater contamination by leachate (96). Although leakage of leachate from older sites continues to present a threat, in particular to groundwater aquifers (53), the collection and extraction of leachates from lined landfills permit its treatment before discharge.

Landfill Leachate Toxicity. A range of bioassays has been used to evaluate the ecotoxicology of landfill leachates. For example, when the results of Daphnia acute-toxicity tests were compared with those of fish (sockeye salmon) bioassays (97), comparable $LC_{50;96-h}$ values were recorded for the two tests. Daphnia were, however, less sensitive to pH changes, and a pH decrease from 7.0 to 5.0 increased toxicity by only one order of magnitude, compared to 2 orders of magnitude with the salmon. Correlation of observed toxicity to leachate composition indicated that zinc was likely to be the most significant determinant. In one of the few studies of leachate genotoxicity (98), three standard bioassays (Tradescantia micronucleus, Tradescantia stamen hair mutations, and Allium root anaphase aberrations) were used, of which the Tradescantia micronucleus assay was the most sensitive. Micronucleus frequency increased by 258 (dry season) and 153% (wet season) on exposure to leachate, demonstrating the effects of leachate dilution on toxicity. The criteria used to evaluate toxicity vary: for example, duckweed (*Spirodela polyrhiza*) was used to derive leachate $EC_{50;96-h}$ values, that is, the concentration of leachate that inhibited growth by 50% compared to a control (99). The authors concluded that the toxicity probably was due to the un-ionized ammonia content (NH_4–N 1,300 $mg\,l^{-1}$; pH 8.3), although no evidence was presented. One of the few studies on anaerobic leachate toxicity (100) reported that in acetate–propionate supplemented cultures, the addition of less than or equal to 10% leachate had no adverse effect, as measured by methane release. The inhibitory effects at higher leachate concentrations were not attributed to specific physiological groups.

Ferrari and coworkers (101) drew attention to the range of reports that have described bioassays for the measurement of acute, chronic, or genotoxicity by use of diverse species (for example, *Vibrio fischeri*, *Salmonella typhimurium*, *Lemna minor*, *Daphnia magna*, and *Lactuca sativa*), and to the difficulties inherent in comparing these disparate studies. In an extensive program, vascular plants (oats, lettuce, and Chinese cabbage), bacteria (*V. fischeri*), algae (*Pseudokirchneriella subcapitata*), and microinvertebrates (*D. magna* and *Ceriodaphnia dubia*) were challenged by six different leachates. The results indicated that the alga was more sensitive than the vascular plants, in contrast to other studies that reported the vascular plant *Spirodela polyrhiza* was more sensitive than an alga (99), suggesting that algal tests cannot be used as a surrogate for vascular plant bioassays and vice versa. Bioassays, whichever are applied, ideally should form one thread of hazard evaluation (102) and be used in conjunction with physico-chemical analyses to develop strategies for treatment and discharge.

Leachate Treatment

"Dilute-and-Disperse" Sites. In older, so-called "dilute-and-disperse" sites, the absence of a site liner or containment matrix such as clay permits the emission of leachate. Although an array (103) of physicochemical mechanisms and biological activity in the subsurface may achieve a degree of leachate treatment, the degree of attenuation is often such that groundwaters or surface waters are polluted. The significance of specific attenuation mechanisms depends on the soil characteristics. For example, selective retention of K^+, Ca^{2+}, and Mg^{2+} over Na^+ was recorded in clay soils (16) and attributed to cation exchange, electrostatic adsorption, and chemisorption. The same study observed that Cl^- attenuation was relatively low; Cl^- is often used as an inorganic tracer or "pollution marker" to monitor leachate plume subsurface migration (96). It has been suggested that other elements may also be useful markers, either of landfill-influenced groundwaters [e.g., boron (B), bromine (Br), barium (Ba), nickel (Ni), Cu, cobalt (Co), titanium (Ti), and vanadium (V)] or of the presence of hazardous or industrial wastes in the refuse [e.g., arsenic (As), silver (Ag), cesium (Cs), mercury (Hg), and tungsten (W)] (96). A high dissolved organic carbon (DOC) content is also characteristic of groundwater contaminated with landfill leachate. Although there exists a limited understanding of the composition of the DOC, it largely comprises high-molecular-weight compounds, the fulvic acid component accounting for approximately 60% of the total DOC content (104). The humic and fulvic acid fractions may be important attenuating mechanisms (104) through their abilities to form complexes with heavy metals and to bind hydrophobic organic contaminants. However, as a result of doubts on the applicability of laboratory studies and the difficulties inherent in field studies (105), the extent of leachate attenuation in the subsurface is equivocal. For example, the persistence of *o*-cresol in an anaerobic leachate plume was observed (105), although the anaerobic degradation of *o*-cresol in refuse samples has been clearly demonstrated (38). Clearly, reliable and predictable treatment of leachates from dilute-and-disperse sites is not possible.

Containment Sites. Controlled methods for treating leachate have largely been drawn from the wastewater-treatment industry. Physico-chemical processes such as coagulation or precipitation, adsorption on to granular activated carbon, and reverse osmosis have been applied to the removal of specific leachate components such as heavy metals and oils (106). Such methods are most suitable for treating leachate before a biological process or as a final polishing stage. Although biological processes are widely implemented, the range of configurations or operational practices emphasizes that treatment solutions, because of the diversity of leachate composition, are largely site- and age-specific (107).

Perhaps because aerobic processes have the advantages of economy, simplicity, speed, and ease of control (106), they are more common than anaerobic processes. Aerobic lagoons, characterized by low maintenance and control measures, are popular, although because no intervention such as aeration or biomass retention is practiced, their size must be larger than activated processes. Aerobic-sequencing batch reactors are effective for the treatment of high-ammonia, methanogenic leachate, in terms of both COD removal and ammonia-N removal (17).

Anaerobic processes are suitable for "young" high-strength leachates, and have the added advantage of energy recovery, as methane (19). Configurations used have included anaerobic filters (108), two-phase anaerobic digesters (19), and anaerobic-sequencing batch reactors (20). Effective COD reductions can be achieved. For example, anaerobic-sequencing batch reactors achieved 64–85% COD removal at a volumetric loading rate of 0.4–9.4 $g\,COD\,l^{-1}\,d^{-1}$ from a high-strength leachate (16–20 $g\,COD\,l^{-1}$) (20). Similar values (92.6% COD removal and loading rate 0.51–3.4 $g\,COD\,l^{-1}\,d^{-1}$) were obtained with an anaerobic filter (19).

The additional heat required for the efficient operation of anaerobic digesters, at 35 °C (19,20,108), can be supplied by utilization of methane generated. Low-strength leachates (COD $\leq 1,000\ mg\,l^{-1}$) generate insufficient methane, and, hence, anaerobic digestion of low-strength leachates is only viable if it can be operated at ambient temperature. At these temperatures, reactor configurations that retain, and hence increase, biomass are required to reconcile the necessity for efficient COD removal at low hydraulic-retention times (HRTs) with the low anaerobic microbial growth rates. Laboratory-scale anaerobic-sequencing batch reactors, with an operating regimen of feed, react, settle, and decant, achieved 80–90% soluble COD (400–800 $mg\,l^{-1}$) removal, at temperatures and HRTs that ranged from 15 to 35 °C and from 12 to 48 h (109). Encouraging results were also reported from a pilot-scale upflow anaerobic sludge blanket (UASB) reactor (see WASTEWATER TREATMENT MICROBIOLOGY) treating leachate (COD 1.5–3.2 $g\,l^{-1}$) at temperatures from 13 to 23 °C (51). Despite changes in leachate quality, 65–75% COD removal was constantly achieved at temperatures down to 18 °C.

An alternative strategy for the treatment of leachate at temperatures higher than ambient, which eliminates the requirement for an external heat supply, is leachate recirculation through the refuse mass. Recirculation may serve other ends. One means of increasing the sustainability of landfilling is to promote rapid refuse stabilization, and because moisture content is generally considered to be the single most important parameter limiting refuse decomposition and methane production, leachate recycle may address this. In addition, moisture additions accelerate long-term site settlement, facilitating final site closure and after-use. It has been suggested (90) that the combination of enhanced biodegradation and settlement could shorten by 3–10 years the period required for the completion of stabilization. On a pragmatic basis, recirculation in temperate climates may be used as a management tool for the in situ storage of leachate, particularly in winter months when the capacities of treatment facilities are reduced.

Evidence of leachate treatment can be measured in terms of changes in the BOD : COD ratio. For example, the analysis of samples from both leachate drainage wells ("fresh" leachate) and sampling wells in the older waste through which the "fresh" leachate was recycled indicated that the BOD : COD ratio decreased from 0.53 to 0.1, whereas the pH increased from 6 to 8.3, reflecting the use of VFAs (18). Interestingly, population studies have failed to account for this apparent enhancement. In one study on actively methanogenic refuse samples the total anaerobic population and subpopulations of cellulolytic, acetogenic, and methanogenic bacteria were the same whether or not leachate recycle was imposed (110). Although enhancement may be due to more uniform nutrient distribution in the presence of water flux, this has not been demonstrated.

Recycle will not address the problems of leachate nitrogen content, because the absence of an anaerobic-transformation route for NH_4^+ implies that concentrations will increase with recycle through the refuse mass. In one study (18) a 30% increase between "fresh" and recycled leachate was recorded. A recent survey of U.K. landfill leachates (111) reported that acetogenic and methanogenic leachates contained from 194 to 3,610 and from 283 to 2,040 mg $NH_{3/4}^+$-N l^{-1}, respectively. It has been argued that to recycle such leachates risks the inhibition of decomposition processes. Because it is un-ionized ammonia that is toxic, the toxicity of ammonia is both pH- and temperature-dependent. At 20 °C, 50% of ammonia is in the form of NH_3 at pH 9.245 precisely, and for one unit decrease in pH, NH_3 concentrations decrease by a factor of 10. Moreover, toxicity increases with temperature, because at 15 and 35 °C the pKa values are 9.564 and 8.947, respectively. It is thus not possible to assess leachate ammonia toxicity without presentation of pH and temperature data, which often are lacking. Prediction is further confounded by likely concentration differences between the refuse matrix itself, interstitial water, and leachate. Adaptation of the methanogenic associations in anaerobic digesters may explain their tolerance to high concentrations ($\leq$5,000 mg $NH_{3/4}^+$-N l^{-1}) (112), which are equivalent to the total amount of nitrogen present in MSW. In one of the few studies to investigate differential effects (113), acetoclastic methanogens were shown to be more sensitive than propionigenic bacteria or SRB with threshold inhibitory levels for NH_3 of 77,105, and 123 mg l^{-1}, respectively.

Conservative elements such as Cl^- and heavy metals also accumulate. In addition, care should be taken that the additional hydraulic load does not cause structural stability problems (30). Ultimately, recirculation can be only a temporary measure for the management of leachate, which must at some point be abstracted and, if required, treated before discharge.

SUSTAINABLE WASTE MANAGEMENT

As traditionally practised, landfilling is not environmentally sustainable, in that landfills are significant sinks for a range of materials (such as glass, paper, textiles, metals, and plastics) whose manufacture and production rely on finite sources of raw materials. Moreover, the environmental impacts of landfill sites may persist for many decades. For landfills to be more environmentally sustainable, legislators and operators must reduce energy consumption, pollution of land, air, and water, and loss of amenity (2). One approach, of much recent interest, attempts the accelerated stabilization of biodegradable wastes, with concomitant benefits in increased resource (methane) generation. This could, potentially, be achieved by continuously recirculating water and/or leachate through the site. In such a "flushing bioreactor" a leachate collection and recirculation installation is required, capable of uniform leachate distribution through the refuse mass. Significant acceleration of stabilization, however, require infiltration rates (3,000–10,000 mm y^{-1}) greatly in excess of those generally recorded (50–250 mm y^{-1}), which require hydraulic conductivities greater than 10^{-7} m s^{-1}. To achieve such high conductivities require changes in operational practices, such as shredding or pulverization to produce a more homogeneous matrix, reduced compaction, and maximum site depths 20–30m (1). Although accelerated stabilization and increased gas production have been recorded at most sites where leachate recirculation has been implemented (90), leachate volumes are greater and require storage, control, and treatment measures (30). Although, overall, leachate quality does not appear to be adversely affected, without some form of aerobic-treatment stage, ammonia concentrations will accumulate, as will those of conservative species such as Cl^{-1}. Nitrification of leachate in aeration lagoons or bioreactors, and the return of nitrate to the landfill will permit denitrification to gaseous nitrogen, leading to a net loss of nitrogen and a reduction in the degree of treatment required before discharge (114). In this way the refuse mass itself is used to provide the electron donors required for denitrification, which may need to be added to a conventional denitrification system. Whether the "flushing bioreactor" can mobilize and transform ammonia concentrations to achieve final discharge levels of 5 mg l^{-1} within 30 to 50 years, as required

by the concept of sustainability, remains to be confirmed.

Although retrofitting of sites fully to accommodate accelerated "flushing" may be difficult, it may be that this is where the technology will achieve more recognition. In Europe, at least, future legislative changes aim to reduce the volume, and alter the composition, of MSW going to landfills. Given the requirement in the European Waste Landfill Directive for member states to reduce landfilling of biodegradable solid waste, by 2010, to 25% of the baseline 1993 level (115), the importance of recycling initiatives clearly will increase. Composting (aerobic) and biogasification (anaerobic) processes may be considered not only as treatment but also as recycling options, in that compost or compost/biogas, respectively, are recovered from the organic and paper fractions of MSW. Although composting technologies (1,2) are well developed, in the United Kingdom currently less than 0.5% of MSW is thus processed. Although compost can be used as a soil conditioner, fertilizer, mulch, or peat replacement, the market is so far constrained, because of the limited acceptance of waste-derived composts. Anaerobic digestion of MSW offers several advantages over composting (116), in cost (26–42% less expensive), production of biogas (CH_4/CO_2), and a stable end-product that contains fewer pathogens and weed seeds. In addition, the potential to collect biologically generated hydrogen, for use as a clean fuel, has attracted interest (117). Full-scale reactors, of various designs (batch, continuous, or two-stage continuous) and operating conditions (mesophilic or thermophilic), are in extensive use in Europe (116) despite current limitations related to the low demand for the humuslike end-product. However, the possibility of increasing biogas production, and hence profitability, by codigestion with agricultural and food-processing wastes (118) is promising, as is the drying of the end-product, in developing countries, for use as firewood substitute (116).

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LASER SCANNING MICROSCOPY IN COMBINATION WITH FLUORESCENCE TECHNIQUES FOR BIOFILM STUDY

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Facts such as the "great plate count anomaly" in microbiology (1,2) suggest that the investigation of complex environmental communities may be best carried out using methods not involving cultivation of bacteria. These integral methods include among others, biomarkers, molecular probes, activity measurements, flow cytometry, microelectrodes, and microscopy. Comparisons of all the different microscopic methods indicate that laser scanning microscopy is best suited for the investigation of fully hydrated samples and allows the four-dimensional visualization and quantification of digitized signals of the whole microbial community (3–5).

Natural microbial communities are mostly associated with interfaces and usually referred to as biofilm systems. The definition of biofilm includes bacterial cells and their extracellular products. In addition, other cells and compounds from the environment will be associated with an interfacial microbial community (Table 1). The complexity of the biofilm architecture is dependent on physical, chemical, and biological parameters present in a specific habitat. Consequently, an environmental biofilm system will show a very different structure compared to pure culture or defined mixed culture biofilms, which have been used in many biofilm studies to date.

Table 1. Cellular and Matrix Constituents of Environmental Biofilm Systems

Cells in Biofilms	Biofilm Matrix
Prokaryotes	Particulate material
— bacteria	— geogenic and biogenic minerals
— archaea	— organic debris
	— fecal pellets
Eukaryotes	— phages, viruses
— algae	— lysed cells
— fungi	— precipitates
— protozoa	
— micrometazoa	
	Polymeric material
	— polysaccharides
	— structural proteins
	— enzymes
	— S-layers
	— nucleic acids
	— polymeric surface-active substances
	— Lipopolysaccharides, Lipoteichoic acids
	— lipids
	— chelators
	— polyphenols
	— humic substances

As a result of studying numerous environmental biofilm samples with very different properties by using laser scanning microscopy it became clear that some features are very obvious and can be easily imaged and analyzed. However, in a lot of situations the structure of interest is not visible e.g. the presence and distribution of extracellular polymeric substances (EPS) in biofilm systems. Thus it is in the imagination and skill of the scientist who has to develop a sense of "WYPIWYS" ("What You Probe Is What You See"). Consequently, the investigator has to test a variety of probes to search for the characteristic bio-physico-chemical properties of the unknown biofilm structure.

Usually, the stepwise analysis of a microbial community follows a cascade of questions. The key questions in approaching an unknown environmental sample are: (*1*) what is there, (*2*) who is there, (*3*) who is active, and (*4*) what type of activity are they doing (1,6). The first question can be answered using the intrinsic properties of the sample and general stains for typical constituents within the microbial community. The second question can be answered by employing rRNA-targeted oligonucleotide probes for in situ hybridization. In addition, new approaches with mRNA-targeted probes at the gene level may help to identify certain bacterial groups and their activities. The third question can be answered in a variety of ways. There are methods for direct activity measurements at the cell level, the determination of enzyme activity may be used as another measure, and there are several methods in which the activity of a certain gene is visualized using molecular biology techniques. The fourth question can be answered by analyzing the production or disappearance of various compounds. The tools for analysis may include enzyme assays, chemical analysis, and environmentally sensitive fluorescent probes and microelectrodes. Finally, information regarding the identity of the organisms, derived from in situ hybridization, maybe used to infer what the microorganisms may be doing.

In this entry, the sequential steps of approaching to an unknown environmental biofilm sample are discussed. Figure 1 provides an overview of the various levels of the approach and the anticipated results. The emphasis is on visualization and analysis using laser scanning microscopy (1-photon and 2-photon) as this technique allows the investigation of natural, hydrated, living samples in space and time by collecting multiple lines of information in multiple channels.

LASER SCANNING MICROSCOPY (LSM)

The potential of confocal laser scanning microscopy (CLSM) for investigations of microbial biofilms was first demonstrated in 1991 (7). In the meantime, numerous LSM applications in environmental microbiology have been published and discussed in various review articles (5,8–10).

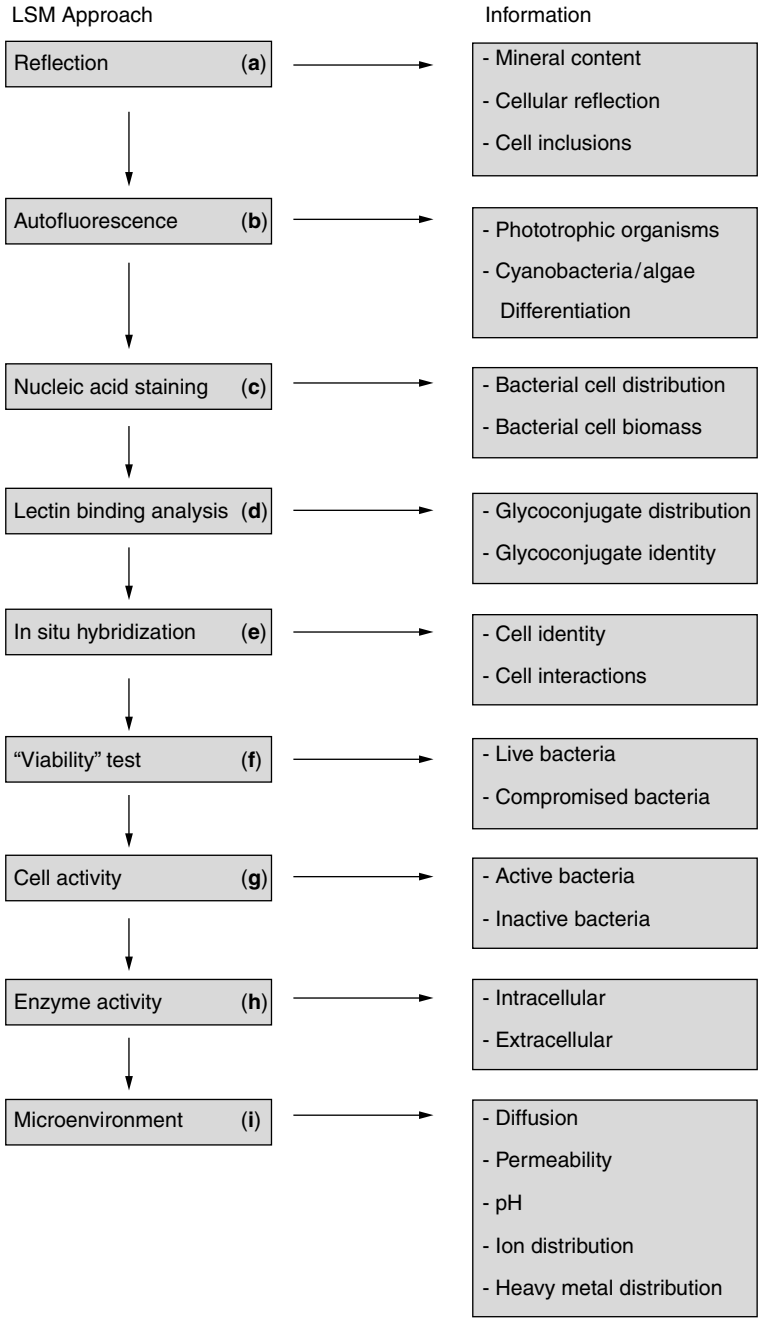

Figure 1. Laser scanning microscopy strategy to characterize an unknown biofilm sample. In the left column of the flowchart the major approaches are indicated. The letters A-I refer to the section in the text. In the right column the information gathered is listed.

The application of laser scanning microscopy allows the examination of thick biological samples in four dimensions (x, y, z, time) with the assessment of multiple parameters. Conventional confocal laser scanning microscopy uses 1-photon excitation, whereas new LSM systems may have the option of 2-photon laser scanning microscopy (2-PLSM), where 2-photons are used simultaneously for excitation of a reporter molecule. The advantages of both LSM systems are summarized in Table 2 (1-photon) and Table 3 (2-photon). Currently, most applications of LSM in microbiology use conventional 1-photon CLSM.

2-PLSM has been used in only a few studies. One group investigated defined mixed culture biofilms, which were grown in a constant depth film reactor producing very dense oral biofilms (11,12). The results confirmed previous findings from cell biological studies (13) and showed that in comparison with CLSM, the infrared laser could penetrate four times deeper into the dense biofilm structure. In another report, marine stromatolite sediments were studied after embedding in nanoplast resin (14). The study showed 2-photon imaging of DAPI-labeled bacteria and 1-photon imaging of lectin-labeled EPS compounds in different regions of the sample. A direct comparison of 1-photon and 2-photon imaging of the same biofilm location has been demonstrated very recently. Using various types of biofilm systems it could be demonstrated that 2-PLSM produced superior results

Table 2. Advantages of Confocal Laser Scanning Microscopy (CLSM)

1.	examination of fully hydrated, living, microbial communities up to several 100 μm thick
2.	noninvasive optical sectioning of samples with virtually no scattered light from out-of-focus regions
3.	sectioning in horizontal (xy), vertical (xz), and temporal (xt) dimension
4.	application of fluorescent (fluorescent mode) and nonfluorescent (reflection mode) probes
5.	simultaneous or subsequent application of multiple probes and multichannel recording of digital enhanced signals (4-fluorescence channels plus transmission and reflection)
6.	quantitative static and dynamic analyses of three-dimensional organization of polymeric aggregates, single cells, and microcolonies and complex microbial communities
7.	three-dimensional presentation and animation, for example, xyz slicer, movie, gallery view, two-dimension projection, intensity histograms, rotation, various types of stereoscopic imaging, different three-dimension projections, surface rendering

Table 3. Advantages of 2-Photon Laser Scanning Microscopy (2-PLSM)

1.	excitation only in the focal region, excitation volume is extremely small (femtoliter)
2.	caged fluorochromes are excited in extremely localized spots
3.	as a consequence, no out-of-focus bleaching and cell damage
4.	background fluorescence is also not a problem
5.	compared to 1-photon excitation, infrared has a higher depth of laser penetration (0.5–1 mm)
6.	consequently there is an inherent depth resolution
7.	less scattering and less filter problems
8.	no pinholes necessary and thus no pinhole throughput loss
9.	no UV laser necessary, no UV photo damage possible, no problems with UV optics

in terms of imaging and resolution of deep biofilm regions (Fig. 4) (Neu and coworkers, submitted). Nevertheless, 2-PLSM requires further evaluation of fluorochromes and other methodological development in order to employ this approach as a routine technique in environmental microbiology.

Direct LSM in the Reflection Mode

The laser scanning microscope (LSM) can be used in the reflection or fluorescence mode (Fig. 1, level A). Generally, in microbiological studies, the LSM is used in the fluorescence mode after applying specific fluorescent stains or other fluorescent reporter molecules. In this mode most conventional CLSMs typically equipped with an Argon/Krypton laser producing three distinct visible laser lines (488 nm, 567 nm, 647 nm) can excite three different fluorochromes. The emission signals of these fluorochromes may then be collected in the green, red, and far red part of the spectrum. This allows for either simultaneous or sequential imaging of samples using a variety of fluors. Nevertheless, the reflection signals from an unstained biofilm sample may be an additional source of information. The reflection mode can be used separately or simultaneously with other fluorescent channels. The reflection mode allows the characterization of several biofilm features. Firstly, the reflective mineral constituents of a biofilm can be recorded. The minerals adsorbed or embedded in the polymeric matrix may be of geogenic or biogenic origin (15,16). This mineral content is an important part, especially in environmental biofilm systems, and is usually not found in artificial pure or defined mixed culture biofilms. Secondly, the reflection signal of biofilm microorganisms can be imaged. Bacteria may contain reflective inclusions such as sulfur granules (17), sulfur and calcite (18) poly-beta-hydroxy butyric acid, polyphosphate, gas vesicles, and so on. In addition, it is generally known that spore-forming bacteria show a strong reflection signal. Certain bacteria may also precipitate ions on their cell surface, which may result in a reflection signal (19,20). Furthermore, environmental biofilms may contain a variety of higher organisms such as fungi or algae, also showing a reflective signal. Some of these eukaryotic biofilm organisms may have a very specific reflection signal as for example, the silicate frustules of diatoms. Several examples of reflection signals are presented in Figure 2. Thirdly, the reflective mode may be used to characterize the surface to which the biofilm is attached. By this means the surface topography and texture of the substratum can be imaged.

Direct LSM in the Fluorescence Mode

As already indicated phototrophic organisms are frequently part of environmental biofilms. The autofluorescence signal of their pigments can be valuable in the characterization of natural biofilm systems (Fig. 1, level B). In algae, the size and shape of the chloroplast can be used for taxonomic description and quantification of autotrophic organisms. The presence of two types of pigments in cyanobacteria allows the separation of their signals from green algae (Fig. 3). Nevertheless, in some cases the strong autofluorescence of algae or cyanobacteria interferes with the emission signal of fluorescent stains. If there are green algae present, the chlorophyll signal in the far red channel may preclude the use of, for example, CY5 labeled compounds, whereas the presence of cyanobacteria results in problems in the red and in the far red channel. Consequently, the detection of fluorochrome emission signals in the red (e.g., TRITC or CY3) and in the far red (e.g., CY5) channel becomes difficult as separation from pigment signals is not straightforward. Apart from these aerobic phototrophs, anaerobic phototrophic bacteria have a variety of pigments, which may be used for imaging, including bacteriochlorophylls a, b, c, d, e, and several types of carotenoids. Fluorescence of chlorophyll was used by Lawrence and coworkers to identify and localize algae in microbial mats and river biofilms (15,21). Similarly, Wiggli and coworkers used autofluorescence and CLSM to examine the distribution of photosynthetic bacteria in complex mat communities (16).

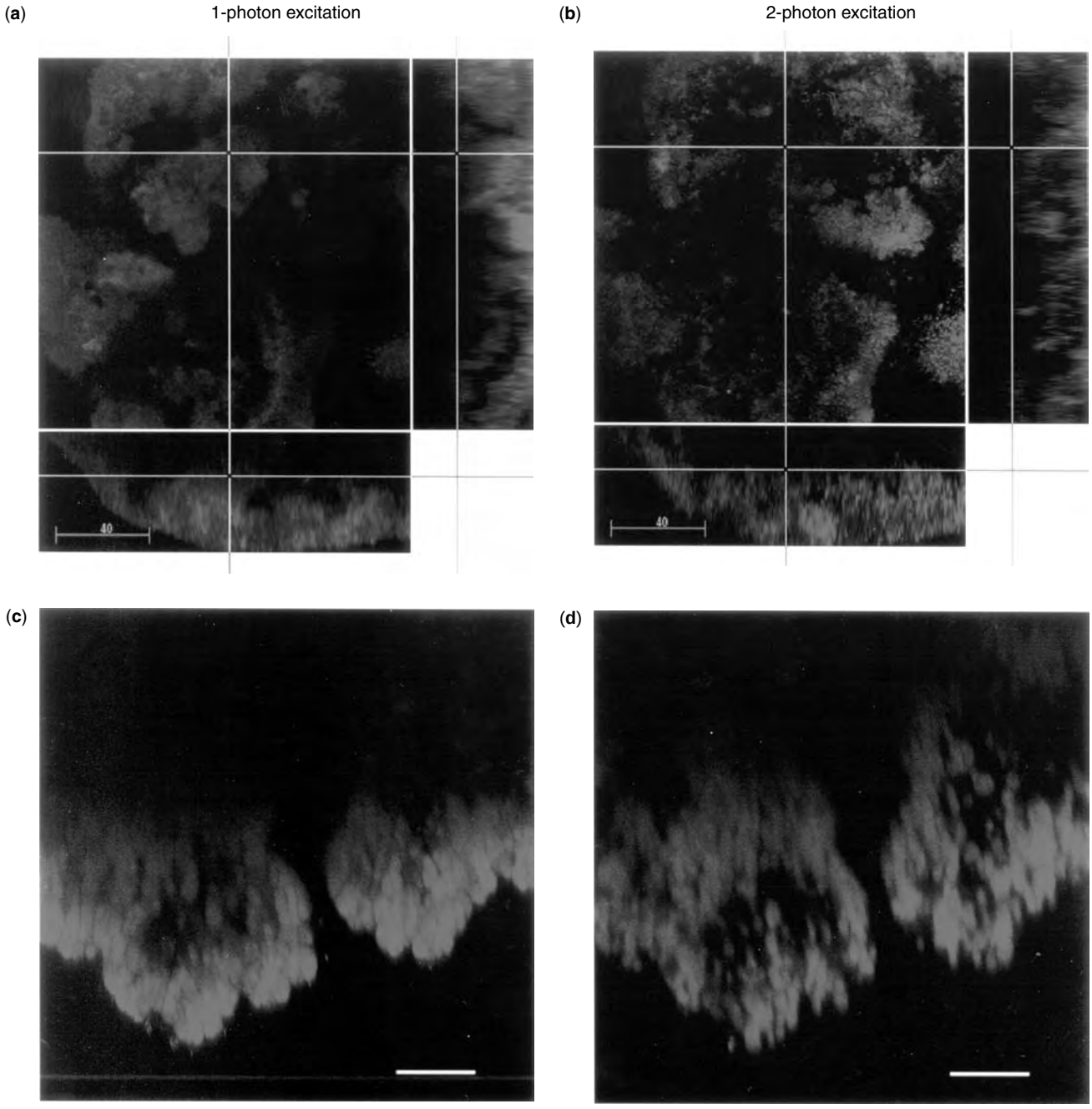

Figure 2. 2-photon (plate with 2/4 images 1p/2p). See color insert.

In addition to the phototrophic procaryotic and eucaryotic biofilm organisms certain bacteria may show an autofluorescence caused by other cellular compounds. For example, in methanogens, the autofluorescence is due to high levels of coenzyme F420, which can be used to image methanogenic aggregates (Fig. 4). Several other coenzymes are also known to show an autofluorescence signal. In cell biological studies the signal of NADP has been used as a cellular marker (22). Eubacteria may fluoresce for a variety of reasons, some cellular constituents may be fluorescent; for example, H4MPT is a coenzyme found in eubacteria, that is, a yellow fluorescent compound (23). Bacteria, in which an autofluorescence has been reported, include *Legionella*, *Vibrio parahemolyticus* (J. R. Lawrence, personal observation), *Archaeoglobus* (24), and *Thermoplasma* (25). In conclusion, depending on the habitat to be studied, and there may be many more that have not yet been examined, the autofluorescence of specific bacteria may be used for imaging. It is also necessary to assess the presence and nature of autofluorescence signals as part of the interpretation of signals resulting from application of fluorescent probes.

CLSM systems may also be equipped with nonconfocal transmitted light detection systems. The detector may

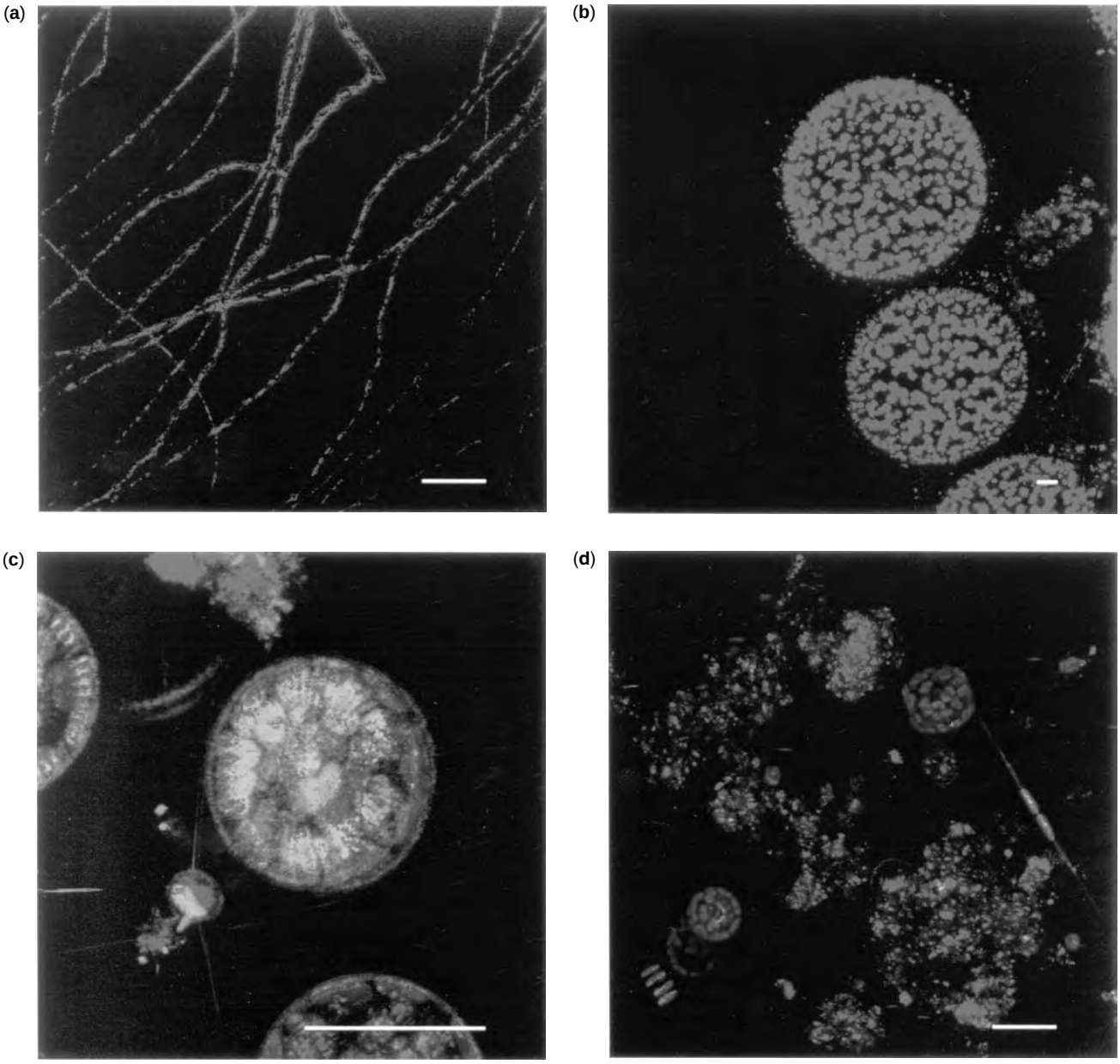

Figure 3. Reflection of diatoms, fungi, microbial mat, thiomargarita (plate with four images). See color insert.

be a photodiode or photomultiplier with a fiber-optic connection below the condenser of the host microscope. If the microscope is equipped for dark-field, phase contrast, or differential interference contrast (DIC), these images may also be digitized. Thus, confocal multichannel imaging can be combined with conventional microscopy techniques to allow correlative light microscopy of specimens under study (3). Most current LSM systems are capable of obtaining at least four images resulting from different applications of fluorescent, reflective, and transmitted light, although as many as 8+ may be obtained with additional laser options such as He Cd 442 nm (1-photon exitation), ultraviolet (1-photon excitation), or infrared (2-photon excitation).

Probes for Cell Distribution

A major application of LSM in biofilm studies is the measurement of cell distribution (Fig. 1, level C) after application of nucleic acid specific stains. Traditional stains include acridine orange (AO) and 4′6-diamidino-2-phenylindole (DAPI). However, neither is preferably employed for LSM. AO has a strong signal across the entire emission range in the green, red, and far red channels. Consequently it cannot be used in experiments with multilabeling and subsequent detection of the various signals in separate channels. In contrast, DAPI requires excitation with an expensive UV laser source, which is not readily available on most confocal laser scanning microscopes. In addition, the emission signal

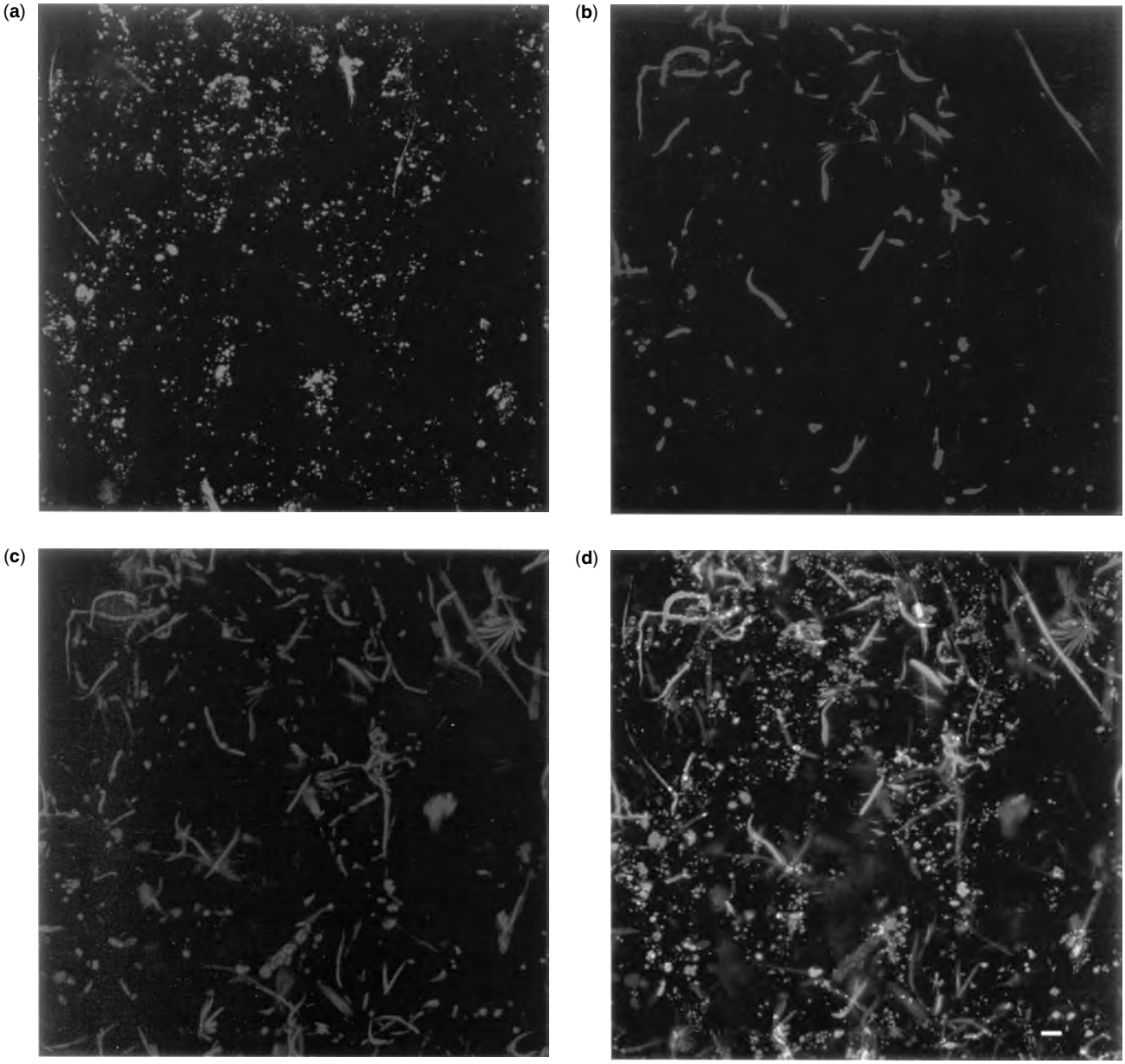

Figure 4. Autofluorescence with differentiation algae-cyanobacteria (plate with four images green-red-blue-overlay). See color insert.

of DAPI is also very broad and may interfere with emission signals in the green and even the red channel. Nevertheless, both stains have been used in numerous studies as "proven nucleic acid stains for bacterial cell counts" and their compared application has been critically reviewed (26).

In the meantime, several other options for nucleic acid specific cell labeling are available. The so-called SYTO stains (27) are now offered in all colors, including emissions in the blue, green, orange, red, and dark red. Depending on the sample properties and the other staining procedures applied, the nucleic acid stain with the appropriate emission signal can thus be selected. The labeled bacterial cells can then be easily located and quantified within the heterogenic structure of an environmental biofilm (Fig. 6). This is, however, an ever-expanding field — additional more sensitive stains such as PicoGreen or SybrGreen are available and have been employed in several studies (see references from Table 4).

Another option for staining of bacteria in biofilms, aggregates or at surfaces, is so called negative staining. This approach has been used for optical sectioning of biofilms (45). Negative stains include fluorescein, resazurin, and fluorochrome-dextran conjugates. If these are applied at appropriate concentrations the bacteria are imaged as dark objects against a bright background (7,46,47). The major advantages of this approach are lack of fading and maintenance of contrast. However, there is also significant quenching of excitation and emission signals by the high fluor concentration. Thus, the

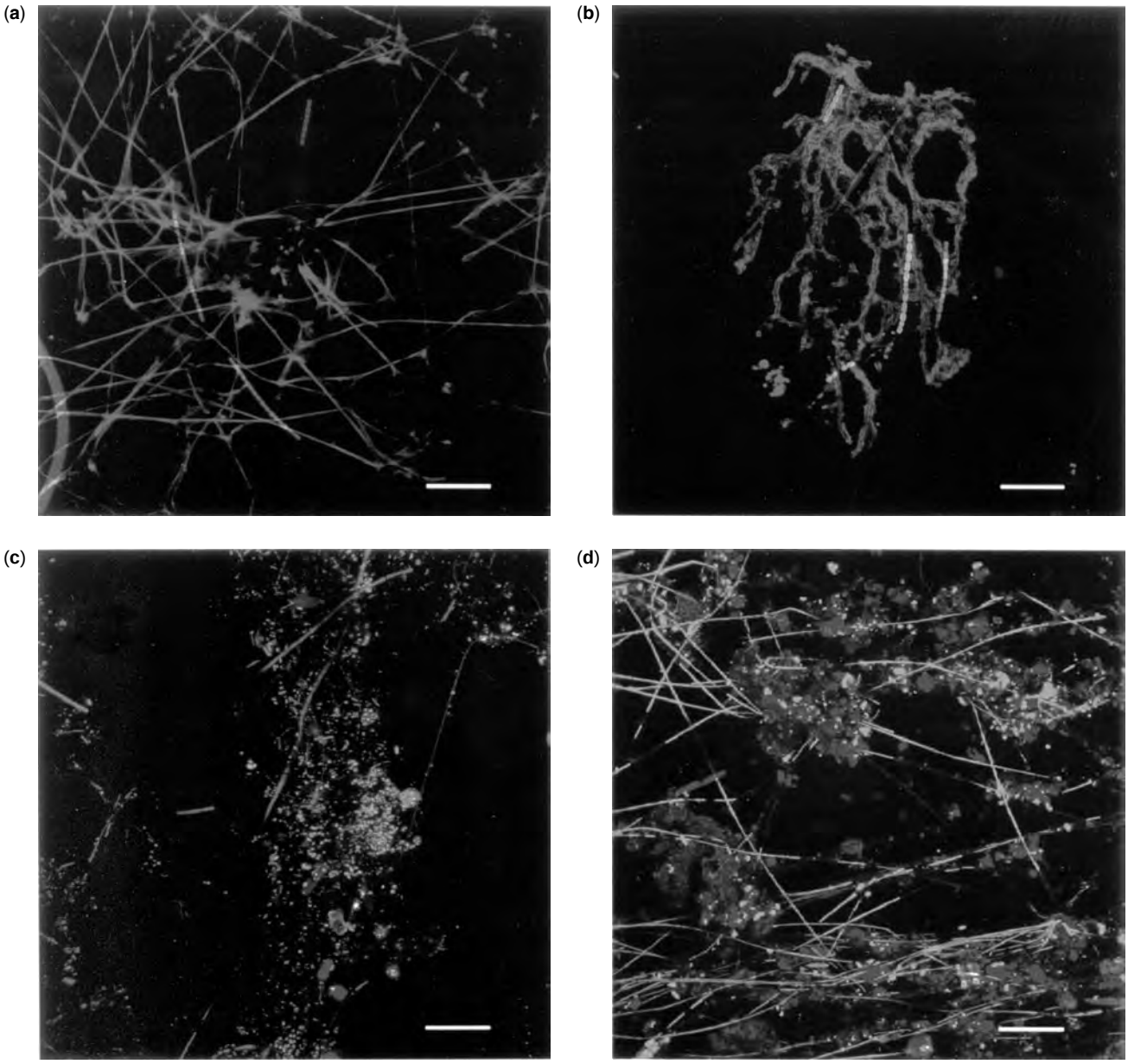

Figure 5. Bacteria and lectins 3/4 channels (plate with four images). See color insert.

microscopist has a wide range of options to consider when staining bacteria.

Probes for EPS

The term EPS (extracellular polymeric substances) stands for chemically different compounds such as polysaccharides, proteins, nucleic acids, and amphiphilic polymers. The in situ detection of these polymers remains a significant challenge (Fig. 1, level D; 48–50). In principle, several fluor conjugated probes with a specificity for each of the different polymers have to be used. Some of the stains may not only give a signal from the EPS but also result in a signal originating from the bacterial cell surface. Thus, the differentiation between cellular and extracellular signals has to be based either on the different geometric appearance of the signals or on colocalization after multiple staining and detection in different channels.

For polysaccharides there is no universal stain that will detect all types of polymers. An example of a fluorescent stain for polysaccharides is Calcofluor White M2R, specific for $(1 \rightarrow 4)$- and $(1 \rightarrow 3)$-β-D-glucan polysaccharides (51). If this type of polymer is present, it will give a very clear signal but only after excitation with a UV laser source. However, the experience to date with environmental biofilms indicates that these polymers are rarely detected. Another stain, Congo Red, is specific for similar types of polysaccharides and has an emission at 625 nm. Both stains were employed in a few microbiological studies [see (3) for literature].

Polysaccharides in environmental biofilms have been characterized by a new in situ approach employing lectin-binding-analysis. By this means, the lectin-specific glycoconjugates in the EPS can be imaged and subsequently quantified (52). A prerequisite for this staining technique is the evaluation of different types of lectins with a given biofilm sample in order to select the most appropriate lectin or most probably a panel of several lectins. This approach has been successfully used to probe for lectin-specific EPS compounds in a variety of environmental biofilm systems (Table 5). These studies have shown that lectins can be effectively used to localize EPS types not only in pure culture but also in complex biofilm systems (43,53). However, thorough evaluation of this approach is required in order to facilitate the interpretation of lectin specificity and binding patterns in biofilms (44,54). Examples for lectin-stained glycoconjugates in complex biofilms are shown in Figure 6.

Specific stains are available for proteins, some of which may be useful for proteinacious EPS compounds. Most of the protein stains have been developed for measuring proteins in solution or for detecting proteins in electrophoresis gels. Nevertheless, they may be employed to stain different kinds of proteins in biofilm systems. For example, Hoechst 2,495 has been used to label bacterial footprints on surfaces (57,58). A further option may be SYPRO protein staining, which has been employed to quantify the protein content of fixed bacterial cells by flow cytometry (59). Very recently, NanoOrange has been used as a fluorescent marker for bacterial flagella and consequently would be another stain for proteinaceous EPS compounds (60).

The presence of extracellular nucleic acids has been also demonstrated after extraction and chemical analysis of EPS compounds, which showed large amounts of nucleic acids not necessarily having their origin in cell

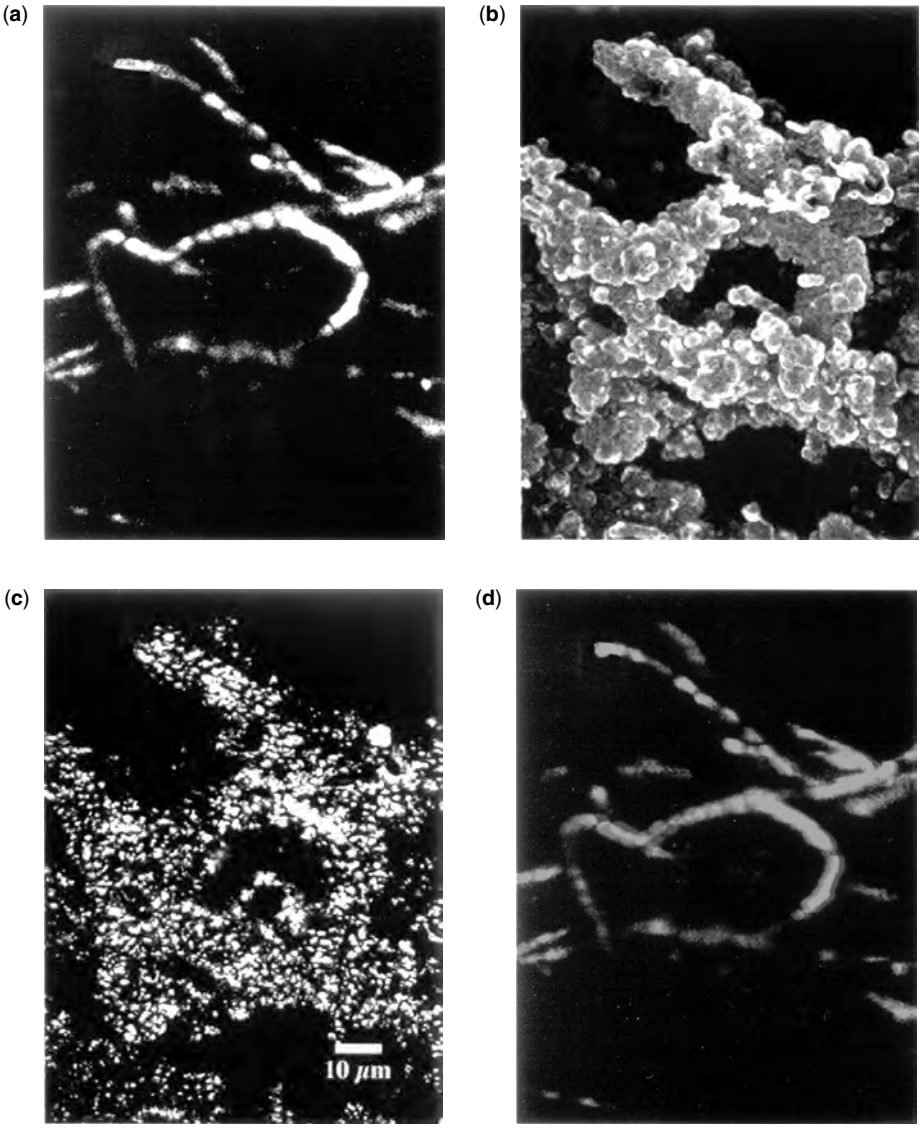

Figure 6. Stereo images (plate with red/green anaglyph and stereo pair). See color insert.

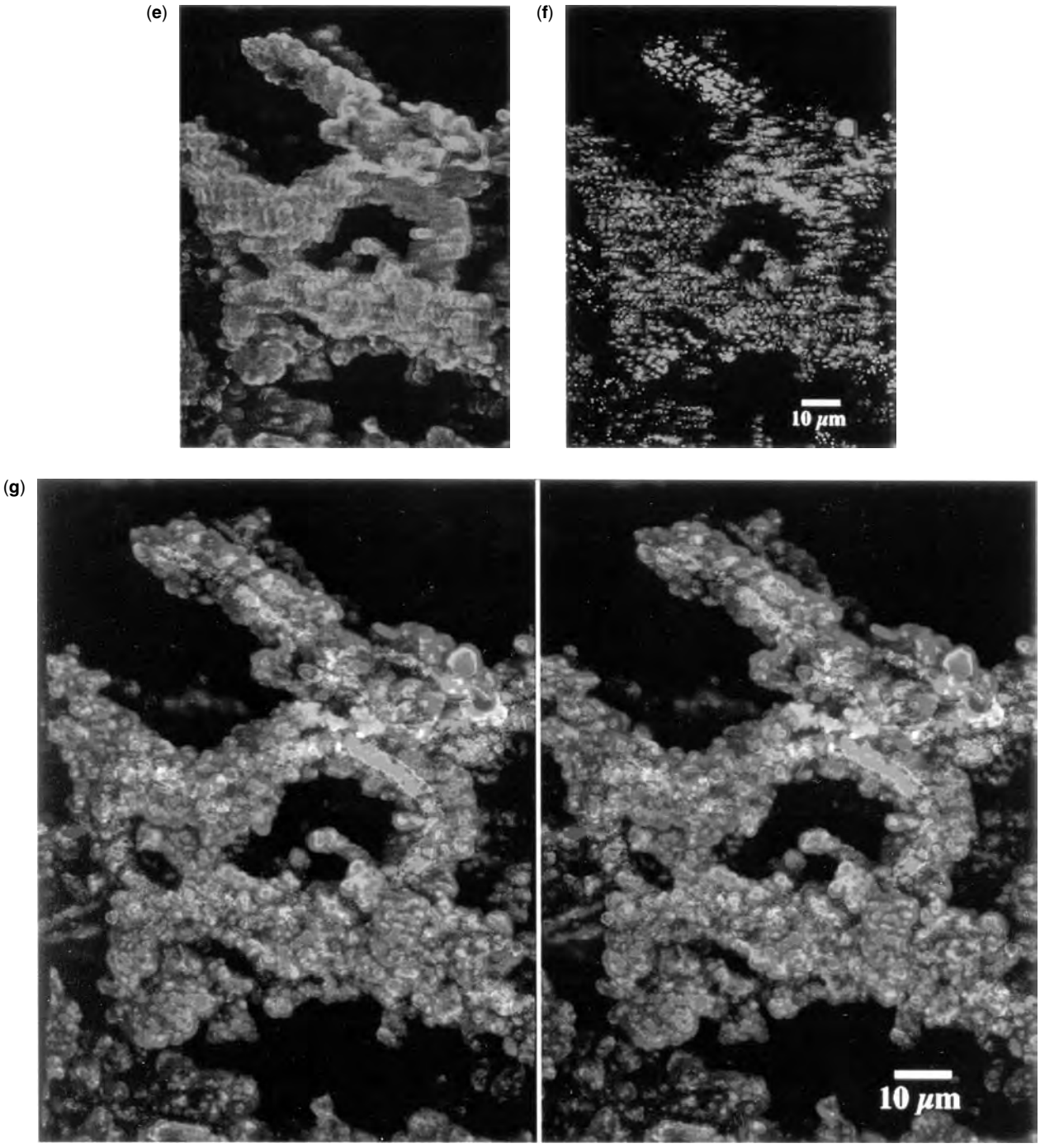

Figure 6. (*Continued*)

lysis (61). These nucleic acids, as pointed out in the preceding text, might be localized by using one of several options for fluorescent staining. A study on marine snow employed these for staining the nucleic acids associated with specific EPS compounds (62). However, the presence and lifetime of extracellular nucleic acids is still under discussion.

The charged nature of biofilm components may also be used as a target for fluorescent probes via charged (i.e., neutral anionic, polyanionic, and cationic) dextran molecules conjugated to fluorescein or other fluorochromes. These probes have proven useful for localization of charge in biofilms (53). Hydrophobicity and hydrophilicity may also be monitored through localization of the binding of fluors such as Nile Red (53) or hydrophobic/philic beads (63), whereas porosity and flow in biofilms may be evaluated through observation of the movement of fluorescent beads using confocal microscopy (64). New size

fractionated, that is, 20 nm, 40 nm, surface-modified (i.e., carboxylated, sulfonated) beads (MOLECULAR PROBES, Eugene, Oregon, http://www.probes.com) may also be used to assess the permeability and chemical nature of EPS.

An example of combining reflection, autofluorescene and fluor conjugated lectin staining is shown in Figure 6. The figure also illustrates the use of red/green anaglyphs and red/green/blue (RGB) stereo projection to simultaneously show the 3-dimensional impression of the data set(15).

Probes for Cell Identity

Antibodies. Immunologic (fluor-conjugated poly- or monoclonal antibodies) fluorescent probes can be used to assess the diversity contained in microbiological systems (Fig. 1, level E). These methods have been adapted to allow their application in various ecological studies (65). Details on preparation of antibodies are provided in various sources (66). In each system analyzed, there is always a need for optimization of technique, including blocking to prevent nonspecific binding, assessment of specificity, and so on. In studies in which there is extensive EPS such as biofilms, concerns may arise from lack of penetration of the antibody as well as cross reactivity with polymer and unknown bacteria in the biofilm. In Table 6, several excellent examples of using antibodies to track bacteria in biofilms are given. Good general reviews and evaluation of antibody applications in microbial ecology may be also found in Reference 67. Although antibodies are usually targeted to bind to specific cell surface structures, they have also been used to identify specific intracellular enzymes (see later in the text).

Table 4. Nucleic Acid Specific Stains and their Application

Nucleic Acid Stain	Applications (References)
AO/DAPI	numerous studies, for references see (26)
Hoechst 33,342	attached and planktonic bacteria (28) flow cytometry (29)
TOTO-1/TO-PRO-1	planktonic bacteria (30) marine prokaryotes (31)
YOYO-1/YO-PRO-1	marine prokaryotes (31,32)
POPO-3	bacteria in geologic samples (33)
PicoGreen	marine prokaryotes (32) bacteria in lake and seawater (34)
SYBR-Green I/II	marine picoplankton (35) marine viruses and bacteria (36) soil and sediment bacteria (37)
SYTO blue	2-photon LSM of biofilms (Neu and Lawrence submitted)
SYTO green	river biofilms (38) lake plankton (31,39) Mycobacterium (40) bacteria in water samples (41) bacteria in seawater (42)
SYTO red	river snow (43,44)

Note: SYTO stains are listed due to their emission color.

rRNA Targeted. One of the most effective means for direct assessment of the biodiversity in natural microbial populations and complex systems is based on phylogenetic data, allowing the identification of specific bacteria (1,79,80). The technique is based on the conserved nature of 16S or 23S prokaryotic ribosomal RNA sequences and the synthesis of short, labeled, complementary, oligonucleotides to these conserved RNA regions (80). A major advantage of this approach is that it does not require per se the isolation and cultivation of specific bacteria, allowing studies of unculturable and hard-to-culture bacteria or those of unknown ecological significance (1,81). The application of fluor-conjugated probes is referred to as fluorescent in situ hybridization or FISH. This approach has been used extensively with CLSM and epifluorescence microscopy to document microbial diversity in a range of habitats. These include flocs, sludge granules (82,83), biofilms (84–86), and the rhizosphere (87). Amann and coworkers provide excellent reviews of FISH for the analysis of microbiological systems (1,88). FISH may also be combined with microautoradiography, allowing the assessment of metabolic activity of specific microbial groups (89–91).

mRNA Targeted. Specific bacteria and their activities can be visualized in situ by the detection of target mRNA sequences. The technique utilizes the ability of PCR to amplify either mRNA or genes of interest within bacterial cells. The mRNA of interest is amplified by in situ PCR and then visualized by labeled probes complementary to the amplified DNA (92,93). Prokaryotic in situ PCR (PI-PCR) or in situ reverse transcription in combination with PI-PCR may be used to image or visualize specific functional genes and their expression products (mRNAs) (94). The technique has been applied to study the diversity of sulfate-reducing bacteria in microbial mats (95). In general, these are potentially powerful techniques although they remain technically challenging in their execution.

Green Fluorescent Protein. An additional approach recently developed for tracking and enumerating specific bacterial cells in natural or complex systems is the use of bacteria labeled with green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* (96). This technique is suitable for the simple enumeration of cells previously transformed with plasmids or transposons containing the *gfp* gene (97). GFP is also used extensively as a reporter system. For example, a GFP reporter system was used to detect metabolic interactions during the degradation of benzyl alcohol in mixed culture biofilms (98). The application of GFP for environmental applications has been reviewed by Errampali and coworkers (99) (See also "Green Fluorescent Protein," this Encyclopedia).

Others. Specific types of bacteria may also be detected using various fluorescent systems for Gram staining (100). These approaches have been based on differential staining such as that observed with the combination hexidium iodide and SYTO 13 staining. Karthikeyan and coworkers used the BacLight Gram stain to quantify gram-positive

Table 5. Lectins Used to Characterize Glycoconjugates in Complex and Defined Environmental Microbial Communities

Type of Lectin	Specificity (Major Only)	Applications (References)
Canavalia ensiformis	methyl α-D-mannopyranoside	
	D (+) mannose	
Helix pomatia	*N*-acetyl galactosamine	
Limulus polyphemus	*N*-acetyl neuraminic acid	Marine biofilms (55)
Arachis hypogaea	D (+) galactose	
Canavalia ensiformis	methyl α-D-mannopyranoside	
	D (+) mannose	
Erythrina cristagalli	*N*-acetyl-D-galactosamine	
	D-galactose	
Ulex europaeus	L (−) fucose	Latic biofilms (56)
Triticum vulgaris	NN′-diacetylchitobiose	
	NN′N″-triacetylchitotriose	Grazing of biofilms (21)
Arachis hypogaea	D (+) galactose	
Erythrina cristagalli	*N*-acetyl galactosamine	
	D (+) galactose	
Canavalia ensiformis	methyl α-D-mannopyranoside	
	D (+) mannose	
Glycine max	*N*-acetyl galactosamine	
	D (+) galactose	
Limulus polyphemus	*N*-acetyl neuraminic acid	
Lycopersicum esculentum	NN′-diacetylchitobiose	
	N′N″-triacetylchitotriose	
Ulex europaeus	L (−) fucose	
Vicia faba	D (+) mannose	
	D (+) glucose	Contaminants in biofilms (53)
Arachis hypogaea	D (+) galactose	
Canavalia ensiformis	methyl α-D-mannopyranoside	
	D (+) mannose	
Glycine max	*N*-acetyl galactosamine	
	D (+) galactose	
Limulus polyphemus	*N*-acetyl neuraminic acid	
Tetragonolobus purpureas	L (−) fucose	
	N-acetyl-D-glucosamine	
Triticum vulgaris	NN′-diacetylchitobiose	
	NN′N″-triacetylchitotriose	Methodology (52)
Abrus precatorius	D (+) galactose	
Arachis hypogaea	D (+) galactose	
Bandeiraea simplifolia	D (+) galactose	
	1-*O*-methyl α-D-galactopyranoside	
Canavalia ensiformis	methyl α-D-mannopyranoside	
	D (+) mannose	
Lens culinaris	α-methyl-D-mannopyranoside	
	D (+) mannose	
Limulus polyphemus	*N*-acetylneuraminic acid	
Ricinus communis	D (+) galactose	
	N-acetyl galactosamine	
Tetragonolobus purpureas	L (−) fucose	
	N-acetyl-D-glucosamine	
Triticum vulgaris	NN′-diacetylchitobiose	
	NN′N″-triacetylchitotriose	
Ulex europaeus	L (−) fucose	River snow (43)
Arachis hypogaea	D (+) galactose	
Canavalia ensiformis	methyl α-D-mannopyranoside	
	D (+) mannose	
Limulus polyphemus	N-acetylneuraminic acid	
Tetragonolobus purpureas	L (−) fucose	
	N-acetyl-D-glucosamine	
Triticum vulgaris	NN′-diacetylchitobiose	
	NN′N″-triacetylchitotriose	
Ulex europaeus	L (−) fucose	assessment (33)

and gram-negative bacteria in a complex microbial community grown on various carbon sources (101). Similarly, Wolfaardt and coworkers used a fluorescent Gram stain to identify the presence of a *Bacillus* sp. in a degradative microbial community (53). Commercial fluors for the identification of fungi such as Fun-1 (MOLECULAR PROBES) or Fungalase (ANOMERIC, U.S.A.) may also be used to provide broad indications of system biodiversity. However, as many of these probes have been developed and tested using pure batch cultures, the user must apply them with appropriate caution in assessing natural communities.

Probes for Cell Viability

In general, microbiologists define a viable cell as one capable of division, giving rise to a viable daughter cell. However, detecting and determining viability has proven to be a complex task (Fig. 1, level F). The methods tend to focus on membrane integrity, evidence of cell division, or metabolic activity. Methods such as difference imaging (102) direct determination and quantitation of cell growth (103,104) or physical viability assays [i.e., plasmolysis (103,104)] may be applied. Kogure and coworkers developed the direct viable count (DVC) method, in which bacterial samples are incubated with appropriate nutrients in the presence of an antibiotic preventing DNA-synthesis. Since other cellular functions are not affected, viable bacteria elongate and can be detected using microscopy (105). However, one step assays are becoming increasingly popular. One method gaining acceptance is the commercial two component LIVE/DEAD *Bac* Light™ viability kit (MOLECULAR PROBES). In this case there are two stains — one is a membrane permeable green fluorescing DNA-labeling probe (SYTO-9) and the other is a membrane impermeant red fluorescing DNA-labeling probe (hexidium iodide). When observed with epifluorescence or CLSM, cells with an intact membrane (living cells) appear green whereas "dead" cells fluoresce red. A number of studies have applied and assessed this staining system and others to define the numbers of viable cells in biofilms and flocs (Table 7). In most cases the LIVE/DEAD system must be checked or "ground truthed" using other methods in combination and it is clear that in complex systems interpretation may be difficult. Stains such as the SYTOX fluorescent stains (MOLECULAR PROBES), which penetrate cells that have damaged or fixed cell membranes, also offer the potential of detecting "nonviable cells" in biofilm and bioaggregate systems (106,107).

Probes for Cell Activity

5-Cyano-2,3-Ditolyl Tetrazolium Chloride. Various approaches have also been applied to assess the relative activity of a bacterial cell's electron transport system (ETS) (Fig. 1, level G). Initially the compound 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT) was applied and may be reduced by bacteria, leading to the deposition of opaque formazan crystals that are easily seen in cells (120). Alternatively, another compound, 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), may be employed. CTC is soluble and nonfluorescent when oxidized but when reduced by an active bacterial respiratory system, it accumulates inside the cell as an insoluble fluorescent CTC-formazan. In the meantime, several studies have confirmed the utility of this approach in biofilm communities (121,122). The suitability of the technique has been critically evaluated recently by Sherr and coworkers (123–125). Application of these types of tetrazolium salt indicators may be complicated by factors such as their concentration, their toxicity, the temperature, microenvironment (pH and Eh), and growth phase of the cells (126). In addition, the presence of anaerobic microenvironments in biofilms may result in chemical reduction of the CTC, creating spurious fluorescent crystals.

Table 6. Immunofluorescence Techniques Employed for the Detection of Bacteria with the Cell Surface as Target

Specificity	Applications (References)
Pseudomonas	
Achromobacter	
Comamonas	
Vibrio	marine fouling (68)
Legionella	aquatic biofilm (69)
Azospirillum	wheat rhizosphere (70)
Desulfovibrio	marine sediment (71)
Pseudomonas	barley roots (72)
Cytophaga	
Comamonas	
Aeromonas	freshwater environment (73)
Campylobacter	water microcosm (74)
Degradative bacteria	groundwater bacteria (75)
Nitrobacter	nitrite oxidoreductase (76)
Pseudomonas	sugar beet root surfaces (77)
Pseudomonas	atrazin-utilizing biofilms
Ammonia oxidizers	ammonia monooxygenace

Reporter Genes. Molecular approaches may also be used to assess cell viability and growth rates. The concentration of rRNA as detected by quantitative hybridization with ribosomal probes may be used as an indicator of cellular activity (127–129). Both epifluorescence and CLSM images may be used in conjunction with digital image analyses to determine growth rates and activity levels. As with most methods, concerns exist and these include ribosome stability, steady state growth conditions, poorly characterized organisms, and the relationship between growth rate and ribosome concentration (130).

Gene expression may be monitored in situ using a number of genetically constructed expression vectors. Early studies applied fluorogenic substrates, such as methylumbelliferylgalactoside (MUG), carboxymethylfluorescein digalactoside, fluorescein β-D-galactopyranoside (FDG), or the lipophilic C_{12}FDG. GFP-based expression vector systems have been developed with stable and modifiable fluorescent characteristics, extending the potential for monitoring in situ gene expression. Sternberg and coworkers used a GFP-based gene reporter system to monitor cell activity, showing that individual *Pseudomonas putida* cells in a toluene-degrading system had different levels of activity that changed over time (130). The use of

Table 7. Combinations of Fluorescent Stains Evaluated for Viability of Bacteria Monitored by Flow Cytometry or Confocal Laser Scanning Microscopy

Fluorescent Stain/s Tested	Applications (References)
propidium iodide ethidium monoazide carboxyfluorescein diacetates rhodamine 123	
$DiBAC_4$ (3) rhodamine 123 propidium iodide oxonol	pure cultures (108)
$DiBAC_4$ (3) oxonol	survival of bacteria in (109)
calcofluor white ethidium bromide propidium iodide carboxyfluorescein diacetates rhodamine 123	pure culture study (110)
bis-oxonol	mixed bacterial populations (111)
$diOC_6$ (3)	(112)
LIVE/DEAD BacLight kit	marine planktonic bacteria (113)
LIVE/DEAD BacLight kit	(104)
LIVE/DEAD BacLight kit carboxyfluorescein diacetate chemchrome B BCECF-AM rhodamine 123	lotic biofilms (56)
LIVE/DEAD BacLight kit propidium iodide	Listeria (114)
SYTOX Green	pure cultures (106)
LIVE/DEAD BacLight kit propidium iodide DAPI 16 S rRNA probes AO CTC	pure cultures (115) (116)
LIVE/DEAD BacLight kit	bacteria in drinking water (117)
LIVE/DEAD BacLight kit SYBR-II CTC CSE	(118)
ChemChrome V6	bacteria in water samples (119)
LIVE/DEAD BacLight kit	oral biofilms (152)

unstable GFP forms can permit real-time analysis of gene expression (131). Thus, these reporter systems provide the opportunity to assess bacterial viability, activity, and specific gene expression in complex biofilm environments.

mRNA. It is also possible to monitor bacterial activity through detection of the expression products, the mRNAs. See in the preceding paragraph "Probes for cell identity, mRNA-targeted" on in situ PCR (PI-PCR) and in situ reverse transcription in combination with PI-PCR.

Probes for Enzyme Activity

Intracellular. The presence of specific enzymes in bacterial cells may be employed as a target to identify and localize certain bacterial groups (Fig. 1, level H). For example, immunolocalization of nitrogenase in cyanobacteria microcolonies was demonstrated using an AMCA-labeled secondary antibody. It could be shown that only a fraction of the *Trichodesmium* cells within a colony has the potential to fix nitrogen (132). Monoclonal antibodies have been used to identify nitrite oxidoreductase of nitrite-oxidizing bacteria. Comparison of the immunofluorescence technique with in situ hybridization confirmed the specificity for pure cultures as well as new environmental isolates (76).

Extracellular. A starting point and excellent overview of extracellular microbial enzyme activity may be found in a collection of manuscripts addressing a variety of aquatic environments. These include rivers, lakes, sediments, estuarine, and marine habitats (133). In addition, there are several research papers on exoenzyme activity, which are listed in Table 8. The techniques to measure enzyme activity include radioactive labeled compounds and fluorogenic substrates. The enzymatic measurements may also be combined with other staining techniques to colocalize several features simultaneously. Modeling the costs and benefits of extracellular enzyme activity showed that this is a powerful bacterial feeding mechanism in high-surface-areas such as biofilm systems (134). Furthermore, Unanue and coworkers gave evidence that hydrolytic exoenzymes may be described by a biphasic kinetic model (135).

Probes for the Microenvironment

Diffusion is a critical parameter in biofilm systems and several approaches have been used to assess permeability and porosity in biofilms (Fig. 1, level I). Fluor-conjugated, size-fractionated dextrans and other fluor conjugates have been used to examine diffusion and permeability of biofilms (146,147). In these cases the rate of change in fluorescence as determined using digital image analyses is used to calculate D_e, the effective diffusion coefficient for the EPS matrix. In addition, techniques such as fluorescence recovery after photobleaching (FRAP) may be used to assess diffusion and migration of a range of fluors and fluor conjugates in biofilms (11).

Evidence for the existence of environmental gradients within biofilms has been shown using CLSM techniques in combination with pH sensitive probes such as 5,6 carboxyfluorescein (45). Recently, Vroom and coworkers demonstrated the presence of pH gradients in biofilms using laser microscopy (11). Additional fluorescent probes such as NEWPORT GREEN may be used to assess the local distribution of heavy metals such as cadmium and nickel bound in bacterial biofilms (148).

Other Factors to Consider

Although these laser-based microscopy techniques have many advantages, there are some limitations and concerns. These may be divided into three categories: (1) sample properties such as absorption, scattering, background fluorescence, or bleaching, (*2*) fluorochrome/probe characteristics such as specificity, penetration, toxicity, and bleaching, and (*3*) instrumental parameters such as

Table 8. Measurement of Extracellular Enzyme Activity in Environmental Samples

Enzyme/s Tested	Community/Habitat (References)
phenol oxidize	
phosphatase	
α-1,4-glucosidase	
β-1,4,-*N*-acetylglucosamidase	
β-1,4-glucosidase	
other enzymes	lotic epilithon (136)
aminopeptidase	river, beach (137)
β-1,4-glucosidase	
β-1,4-endoglucanase	
cellobiohydrolase	
β-xylosidase	
β-*N*-acetylglucosaminidase	
phenol oxidase	
peroxidase	surface sediment (138)
leucine aminopeptidase	
phosphatase	
β-*D*-glucosidase	
β-*D*-galactosidase	
β-*D*-xylosidase	rivers (139)
β-glucosidase	
α-glucosidase	
α-glucanase	
β-xylosidase	
mannosidase	
endopeptidase	
Esterase	sediments (140)
α-glucosidase	
β-glucosidase	model aggregates (141)
aminopeptidase	
β-glucosidase	marine sediments (142)
Alkaline phosphatase	biofilms (143)
phosphatase	activated sludge flocs (118)
β-*D*-glucosidase	
β-*D*-xylosidase	
phosphatase	river biofilms (144)
phosphatase	
aminopeptidase	limnetic biofilms (145)

lateral and axial resolution, laser penetration, and sampling. Some of these issues are discussed in the following section.

Resolution. The resolution of an LSM is comparable to the normal light microscope and dictated by the wavelength of light involved in imaging. Consequently, if resolution is an important factor, other microscopic techniques such as transmission or scanning electron microscopy have to be used. Furthermore, the digitized image has also a limited resolution in the form of pixels. With most LSM systems the usual pixel resolution for routine work is still 512×512, applicable to four channels. New instruments may have a pixel resolution of 2.048×2.048 or even more but this option is usually restricted to fewer channels due to memory limitations. In this high-resolution mode the LSM operates slowly and produces extremely large image files, which have to be handled by the host computer or subsequent image analyzing systems. Therefore, there is always a balance among the image size, image resolution and the end use of the image being collected.

Nyquist Sampling Frequency. For high-resolution three-dimensions imaging it is necessary to sample at least twice the spatial resolution of the optical system. In practical terms this means x and y sampling at 70 nm intervals while in the z axis, which has far poorer resolution and an interval of 150 nm is necessary. Consequently the sample will be extremely bleached and conventional CLSM may become impossible. In this case 2-photon laser scanning microscopy may offer a solution as excitation and bleaching will occur in the focal plane only (Table 3). Nevertheless, if sampling is carried out at these intervals then the data set is sufficiently continuous especially to allow correct image restoration (deconvolution). In addition, quantification of correct volumes becomes possible and subsequent processing may allow exact three-dimensional presentation of the data (149). Clearly, for large data sets such as those created by LSM imaging, extensive RAM, storage, and archiving are required, essentially there is never too much of any of the above computer components.

Restoration. Another drawback of all light microscopic systems is poor axial resolution. So far, this can only be compensated by computing intensive restoration procedures called deconvolution (150). Deconvolution has been applied in wide field microscopy (151) and especially in LSM (152) of microbiological communities. Several commercial software packages at different levels of sophistication are available for deconvolution of three-dimensional LSM data, for example, AUTO-DEBLUR (http://www.aqi.com), HAZE-BUSTER, MICROTOME (both http://www.vaytek.com), HUYGENS (http://www.svi.nl). Some of the packages calculate with an estimated point spread function, whereas others require the measurement of the point spread function before applying a deconvolution algorithm. The complex issue of confocal image restoration for subsequent quantification has been discussed in detail (153,154).

Laser Excitation/Emission. Penetration of the laser into biological samples is a function of wavelength of light, opacity of the sample, adsorption by the sample, scattering, the presence of inhomogeneities as well as the numerical aperture and working distance of the objective lens. Light wavelength is a major factor, many biological materials are more transparent to infrared (IR) wavelengths, and as indicated by Vroom and coworkers, penetration of biofilms using 2-photon IR lasers allowed four times greater penetration over conventional CLSM (11). The nature of the staining procedure and fluorochromes are also factors. For example, negative staining will result in reduced penetration due to quenching of both the excitation and emission wavelengths by the high fluorochromes concentration in the environment relative to typical positive stains.

Bleaching of Sample. Sample bleaching and loss of signal is often a problem in both epifluorescence and laser scanning microscopy. Minimizing observation and scan time, choosing the correct combination of zoom, pinhole, PMT setting, and laser intensity is essential. However, the use of antifade reagents is recommended

for many fluor applications such as FISH. One can only achieve the optimum image and performance from a LSM system in the absence of bleaching or fading. The reader is referred to the following paper dealing with these reagents (155). These reagents are highly toxic, must be used with caution, and may be ordered pre-mixed from MOLECULAR PROBES and CITIFLUOR Ltd. (London, U.K.).

Toxicity of Fluorochromes. Toxicity of the various fluorochromes and other reagents is a concern to both the user and the sample materials. This is particularly true in cases in which time course observations are being made on fully hydrated living biofilm samples. Although in many cases there is no immediate observable effect, negative impacts may be seen with some nucleic acid stains applied over extended time periods. Some fluorochromes such as CTC (1 to 20 mM) have demonstrated toxicity for microbial samples (124,126). In contrast, other fluors such as fluorescein (45) appear to have no detectable negative impacts when used on microbial biofilms. In a recent study, CELL TRACKER (MOLECULAR PROBES) has been used to stain intracellular bacteria in living host cells. It could be shown that the fluorochrome had no negative effect on the bacteria and the host cells (156).

CONCLUSION

The approach suggested will allow the assessment of major biofilm features by using confocal laser scanning microscopy in combination with a panel of fluorescent probes and reporter molecules. Nevertheless, new technical developments such as 2-photon excitation and time-resolved fluorescence are emerging techniques with significant advantages in fluorescence sensing and microscopy. Another necessity is the design of new fluorescent probes that can be excited with a variety of light sources and are highly specific in complex real-world microenvironments such as interfacial microbial communities.

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LEAF DECOMPOSITION BY FUNGI IN FRESHWATER ECOSYSTEMS.

See WATER FUNGI AS DECOMPOSERS IN FRESHWATER ECOSYSTEMS

LEGIONELLAE

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Legionellae are gram-negative bacteria that are common to freshwater environments. These bacteria are human pathogens and cause a substantial number of cases of pneumonia in developed countries. Disease occurs when a susceptible human host inhales aerosolized water that contains the bacteria. Legionellae cause two forms of respiratory illness that are collectively referred to as *legionellosis* (1,2). The more severe form is named *Legionnaires' disease* in reference to the infamous 1976 outbreak that occurred during an American Legion convention in Philadelphia. The milder, flulike illness caused by these bacteria is named *Pontiac fever* after the first documented outbreak, which occurred at a health department in Pontiac, Michigan (1). Currently, there are 43 species comprising 65 distinct serogroups in the genus *Legionella* (3,4). Species identification and differentiation are performed serologically or by DNA hybridization (5). One species of *Legionella*, *L. pneumophila*, causes approximately 90% of all documented cases of legionellosis (6). Although there are now 15 serogroups of *L. pneumophila*, 82% of all legionellosis cases are caused by *L. pneumophila* serogroup 1. Approximately one-half (i.e., 20) of the 43 species of Legionellae have been associated with human disease. It is likely that most of the legionellae can cause human disease under the appropriate conditions; however, these infections are infrequently reported because they are rare and because of the lack of diagnostic reagents.

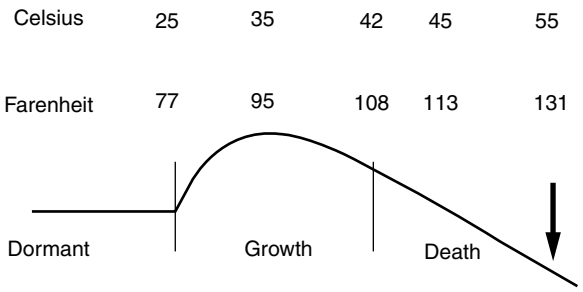

Figure 1. Temperature range of legionellae.

Incidence studies of community-based pneumonia have estimated that between 10,000 and 15,000 cases of legionellosis occur annually in the United States (7). The disease is a major concern of public health professionals and individuals involved with maintaining building water systems. Legionellosis is generally considered a preventable illness because controlling or eliminating the bacterium in certain water reservoirs will prevent cases of the disease. This concept of preventable illness has resulted in a number of guidelines aimed at reducing the risk of legionellosis in building water systems and in a growing industry based upon the litigation of Legionnaires' diseases cases. Although the disease can be effectively treated with appropriate antimicrobial agents, controlling the bacteria in freshwater environments has proven much more difficult. The factors that lead to outbreaks or cases of Legionnaires' disease are not completely understood, but certain events are considered prerequisites for infection. These include the presence of the bacterium in an aquatic environment, amplification of the bacterium to an unknown infectious dose, and transmission of the bacteria via aerosol to a human host that is susceptible to infection (8).

Water is the major reservoir for legionellae, and the bacteria are found in freshwater environments worldwide (9). Legionellae have been detected in as many as 40% of freshwater environments by culture and in up to 80% of freshwater sites tested by polymerase chain reaction (PCR) (10,11). Natural freshwater environments do not usually act as reservoirs for outbreaks of legionellosis. *Legionella pneumophila* multiplies at temperatures between 25 °C and 42 °C with an optimal growth temperature of 35 °C (Fig. 1). Most cases of legionellosis can be traced to artificial aquatic environments where the water temperature is higher than the ambient temperature. Legionellae probably have existed in freshwater environments for a very long time, whereas only in the past century has industrial technology created environments that result in increased transmission to humans. As stated in the 1992 Institute of Medicine report (12), "Emerging Infections: Microbial Threats to Health in the United States"; "technology and industry can cause, or at least contribute to, the emergence of infectious disease." This is precisely the case with Legionnaires' disease. Legionnaires' disease may be regarded as a consequence of altering the environment for human benefit. Some outbreaks of legionellosis have been associated with construction, and it was originally believed that the bacteria could survive and be transmitted to humans via soil. However, legionellae do not survive in dry environments, and these outbreaks are more likely the result of massive descalement of plumbing systems because of the changes in water pressure during construction (13,14).

Presence of the bacteria in an aquatic environment and the water temperature are the two factors that can increase the risk of Legionnaires' disease. However, these bacteria require a distinct combination of nutrients to amplify in an environment. Initially, the unusual nutritional requirement of legionellae appeared to contradict the pervasiveness of the bacteria in aquatic environments. The levels of nutrients that legionellae require would rarely be found in freshwater and, if present, would serve only to amplify faster-growing bacteria that would compete with the legionellae. However, these nutrients represent an intracellular environment, not soluble nutrients commonly found in fresh water. Legionellae survive in aquatic and in some moist soil environments as intracellular parasites of free-living protozoa (15,16). Legionellae have been labeled *protozoonotic*, a term that appears to suit their natural history (17). Legionellae have been reported to multiply in 14 species of amoebae, 2 species of ciliated protozoa, and one species of slime mold, whereas growth of legionellae in the absence of protozoa has been documented only on laboratory media (15,18,19). Protozoa naturally present in environments implicated as sources of Legionnaires' disease can support intracellular growth of legionellae in vitro (20). *Legionella* can infect and multiply intracellularly in both protozoa and human phagocytic cells (15,21). It appears that protozoa are the natural hosts of legionellae, whereas human phagocytic cells occasionally become ill-fated surrogates. Understanding the crucial role of protozoa in the ecology of legionellae is critical to the development of successful prevention strategies. The ecology of legionellae is not limited to the bacteria's interaction with protozoa. Legionellae are known to exist within biofilms found in building water systems and these biofilm-associated legionellae are more easily detected than their planktonic counterparts (22). A true understanding of the ecology of legionellae will require studies of these organisms as they interact with complete microbial communities, not as independent inhabitants of freshwater environments.

Inhalation of legionellae in aerosolized droplets is the primary means of transmission for legionellosis (23) and these aerosolized droplets must be of a respirable size (1–5 m). No person-to-person transmission of Legionnaires' disease has been documented. A number of devices have been implicated as sources of aerosol transmission of legionellae. These sources are of two general types: those producing aerosols of contaminated potable water including showers, tap water faucets, and respiratory therapy equipment; and those from nonpotable water such as cooling towers and evaporative condensers, whirlpool spas, decorative fountains, ultrasonic mist machines, and humidifiers (23). Meaningful identification of sources of transmission requires a multidisciplinary approach including epidemiology, molecular epidemiology, and microbiologic techniques including water and, occasionally, air sampling.

DETECTION OF LEGIONELLAE IN THE ENVIRONMENT

Rationale for Testing Environmental Samples

Environmental isolates of legionellae can be obtained from water or air samples, although water is the primary source of the organism (11). These bacteria can be found only in air samples after a contaminated water source has been aerosolized. Air sampling is of limited value because legionellae do not survive drying and these procedures are technically demanding. Most water samples tested for legionellae are collected either to identify a reservoir associated with cases of legionellosis or as part of a strategy to prevent cases of legionellosis (24). There is considerable controversy concerning the public health benefits of monitoring certain environments for legionellae. These viewpoints are best summarized by guidelines for healthcare facilities where there is a more susceptible population and greater need for control measures. There are at least two general strategies for preventing healthcare-associated legionellosis, especially in facilities where no cases or only sporadic cases of the illness have been detected. The first is an environmental surveillance approach, with periodic culturing of water samples from the hospital's potable water system for the purpose of detecting *Legionella* spp. (25,26). If any sample is culture-positive, diagnostic testing is recommended for all patients with healthcare-associated pneumonia. In-house testing is recommended in particular for facilities with transplant programs. When $\geq$30% of the samples obtained are culture-positive for *Legionella* spp., the healthcare facility's potable water system is decontaminated (27). The basic premise for this approach is that no cases of healthcare-associated legionellosis can occur if *Legionella* spp. are not present in the potable water system, and, conversely, if *Legionella* spp. are cultured from the water, cases of healthcare-associated legionellosis could potentially occur. Additionally, when physicians are informed that the potable water system of the hospital is culture-positive for *Legionella* spp., they are more inclined to order the necessary tests for legionellosis. A potential advantage of using this approach in these situations is that periodically culturing a limited number of water samples is less costly than routinely performing laboratory diagnostic testing for all patients who have healthcare-associated pneumonia. There may be an even greater advantage for healthcare facilities that house organ transplant patients because of the great susceptibility of these patients.

The main argument against this approach is that, in the absence of cases, the relationship between the results of water cultures and the risk for legionellosis remains undefined (24). *Legionella* spp. are sometimes present in water systems of buildings, often without being associated with known cases of disease. In a study of 84 hospitals in Québec, 68% of the water systems were found to be colonized with *Legionella* spp. and 26% were colonized at greater than 30% of sites sampled; cases of Legionnaires' disease, however, were not frequently reported from these hospitals (27).

Interpretation of the results of periodically culturing of water might be confounded by differing results among the sites sampled within a single water system and by fluctuations in the concentration of *Legionella* spp. at the same site. In addition, the risk for illness after exposure to a given source might be influenced by a number of factors other than the presence or concentration of organisms. These factors include (*1*) the degree to which contaminated water is aerosolized into respirable droplets, (*2*) the proximity of the infectious aerosol to the potential host, (*3*) the susceptibility of the host, and (*4*) the virulence properties of the contaminating strain (8). Thus, data are insufficient to assign a level of risk for disease even on the basis of the number of colony-forming units detected in samples from the healthcare environment in immunocompetent patient-care areas. Conducting environmental surveillance would obligate hospital administrators to initiate water-decontamination programs if *Legionella* spp. are identified. Because of these problems, periodic monitoring of water from the hospital's potable water system and from aerosol-producing devices is not widely recommended in general patient-care areas (28).

Collection and Culture of Water Samples

The number and types of sites that should be tested to detect legionellae must be determined on an individual basis. This is because of the diversity of plumbing and heating, ventilation, and air-conditioning (HVAC) systems in a variety of institutions that may be sampled. These institutions can include industrial facilities, hotels, hospitals, retirement homes, public facilities, and domestic environments. An environmental sampling protocol addressing selection of the appropriate sites to sample within a hospital was published in 1987 (29). An amended version of this protocol is listed in Table 1. This protocol can serve as a prototype for identifying sites that should be sampled in a variety of institutions. Generally, any water source that may be aerosolized should be considered as a potential source for the transmission of legionellae. The bacteria are rarely found in municipal water supplies and tend to colonize plumbing systems and point-of-use devices. To colonize a system, the bacteria must multiply and this

Table 1. Abbreviated Protocol for Sampling Environmental Sites for Legionellae

Category	Examples
Potable water	Incoming water line Water softener/demineralizer Water heaters Holding tanks
Potable water final outlets	Showers Sinks Whirlpools (including filter) Humidifiers Decorative fountains Misters
HVAC system	Cooling towers Evaporative condensers
Hospital associated	Water used for respiratory therapy Ice makers

requires temperatures above 25 °C (5). Therefore, legionellae are most commonly found in hot water systems. The bacteria do not survive drying (13), and so condensate from air-conditioning equipment, which frequently evaporates, is not a likely source.

Two primary sample types should be collected when sampling for legionellae: water samples and swabs of point-of-use devices or system surfaces (11,30). Collection of at least 1 liter of water allows concentration of the sample if necessary. If the water source has recently been treated with chlorine or bromine, 0.5 ml of 0.1 N sodium thiosulfate may be added to each 1-liter sample to neutralize the disinfectant.

Swabs allow sampling of biofilms, which frequently contain legionellae. These can be taken from various points within plumbing systems or from surfaces of basins of cooling towers or spas. Swabs of faucet aerators and shower heads should be taken in conjunction with water samples from these sites and should be taken with the aerator or the showerhead removed. The swabs can be streaked directly onto an agar plate or submerged in a small volume of water taken at the same time to prevent drying during transportation to the laboratory.

All samples should be transported to the laboratory in insulated coolers as protection against extreme heat or cold. Samples that will not be processed within 24 hours from the time of collection should be refrigerated.

Pretreatment of Water Samples

A schematic representation of methods for processing water samples for culture is shown in Figure 2. The procedure chosen depends on the expected degree of total bacterial contamination in a particular sample. Water samples are either concentrated and processed directly or subjected to a selective procedure such as acid or heat treatment. Potable waters generally have low bacterial concentrations and are either cultured directly or concentrated to detect legionellae. Nonpotable waters, such as those from cooling towers, generally do not require concentration because of their high bacterial concentrations.

Samples may be concentrated 10-fold or more by using either filtration or centrifugation. Filtration is used more frequently, although either procedure can be used successfully (11,30,31). Water should be filter-concentrated in a biological safety cabinet, using 0.2-μm-pore-size polycarbonate filters. Polycarbonate membranes allow suspended particles to collect on the filter surface without being trapped as with matrix-type filters. The filter membrane is then resuspended into a volume of the sterile water and vortexed for 30s. Samples may be concentrated by centrifugation at $1000 \times g$ for 10 min, removing all but 10 ml of the supernatant, and vortexing (31).

A selective procedure is required to reduce the number of non-*Legionella* bacteria before culturing some water samples with high total bacterial concentrations. Non-*legionellae* bacteria can be selectively killed by either acid pretreatment or incubation at 50 °C for 30 minutes (32,33). Legionellae are more resistant to lower pH and brief exposures to higher temperatures than many other freshwater bacteria. For acid pretreatment, the sample is mixed and incubated with an acid buffer (pH 2.2) for 3 to 30 minutes (32). The sample is neutralized by the buffer within buffered charcoal yeast extract (BCYE) agar

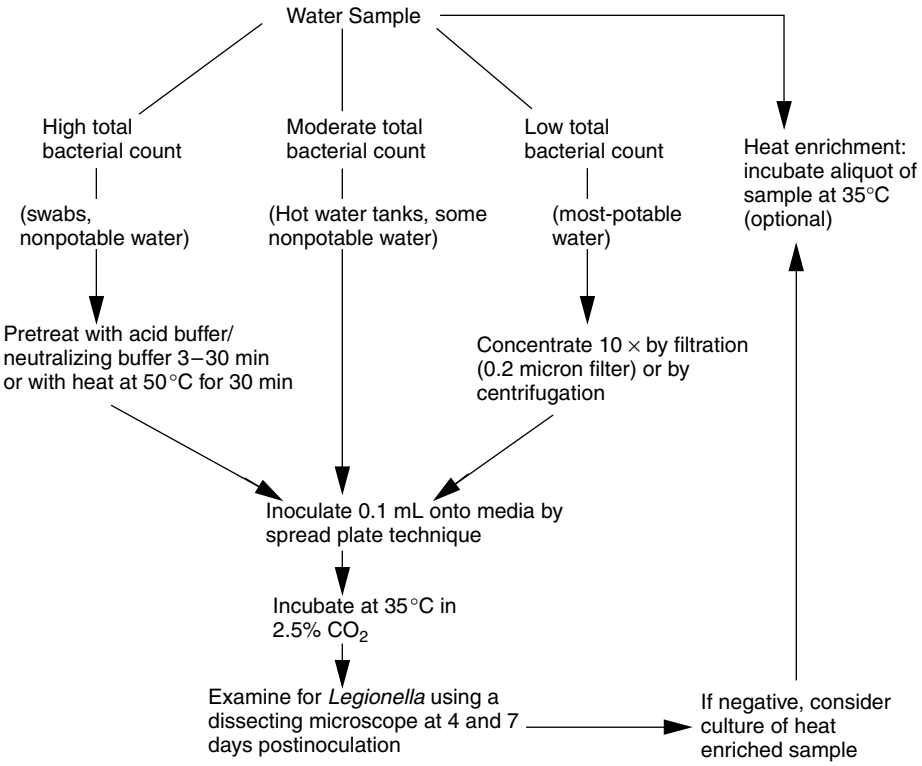

Figure 2. Overview of procedures for the culture of water samples to detect legionellae.

and therefore must be spread on the agar plate at the end of the period of incubation with the acid buffer. Heat pretreatment is accomplished by incubating 10 ml of sample in a 50 °C waterbath for 30 minutes (33).

Heat enrichment or incubation of specimens at 35 °C can improve recovery of legionellae by up to 30% (34). However, this procedure requires a considerable length of time before results can be obtained and may not be practical in many situations. Heat enrichment relies on autochthonous protozoa to amplify undetectable levels of legionellae. Aliquots of samples are incubated at 35 °C. Incubated samples can be cultured after 2 to 6 weeks.

Culture Media

Legionellae were first isolated on bacteriological agar, using Mueller-Hinton agar supplemented with hemoglobin and Iso Vitale X (MH-IH) (35). The essential component in hemoglobin was found to be a soluble form of iron, and L-cysteine is the essential amino acid provided by the Iso Vitale X. These refinements lead to the development of Feeley-Gorman agar, which provides better recovery of the organism from tissue (34). Later, starch was replaced with charcoal to detoxify the medium and the amino acid source was changed to yeast extract, resulting in charcoal yeast extract agar (36). Charcoal yeast extract agar is the base form for most media used for growing legionellae. The medium used for the culture of legionallae has been improved several times, eventually resulting in the medium currently used, BCYE (36,37,38). The most widely used form of BYCE agar is supplemented with α-ketoglutarate (37,38). Table 2 lists the primary components of BCYE agar and the supplements added for various purposes (36,39,40,41).

Table 2. Components and Supplements of BCYE Agar for Culturing Legionellae from the Environment

Component	Concentration	Purpose
Charcoal	2.0 g/liter	Base component
Yeast extract	10.0 g/liter	Base component
ACES[a] buffer	10.0 g/liter	Base component
Ferric pyrophosphate	0.25 g/liter	Base component
L-cysteine	0.4 g/liter	Base component
Potassium α-ketoglutarate	1.0 g/liter	Base component
Agar	17.0 g/liter	Base component
Glycine	3.0 g/liter	Selective agent
Polymyxin B	50–100 U/ml	Selective agent (gram negative)
Vancomycin or cefamandole	1–5 g or 4 mg/liter	Selective agent (gram positive)
Anisomycin or cycloheximide	80 μg/ml (for either)	Selective agent (fungal)
Bromocresol blue	10 mg/liter	Indicator dye
Bromocresol purple	10 mg/liter	Indicator dye
Bovine serum albumin	10 g/liter	Supplement for some fasitidious legionellae

Note: [a]N-(2-Acetamido)-2-aminothanesulfonic acid.

Culture of environmental samples requires the use of selective and nonselective media in conjunction with previously described selection procedures (11). Most laboratories use multiple plates for each sample, including a BCYE agar plate, a BCYE agar plate containing three antimicrobial agents, and a BCYE agar plate containing the three antimicrobial agents plus glycine (Table 2). These media can be prepared with or without the indicator dyes, which impart a color specific for certain species of *Legionella* (40). Although the majority of *Legionella* spp. grows readily on BCYE agar, some require supplementation with bovine serum albumin to enhance growth. *L. micdadei* and several strains of *Legionella bozemanii* show a preference for BCYE with 1.0% albumin (39). All agar plates are inoculated with 0.1 ml of sample by the spread plate technique and incubated at 35 °C in a humidified 2.5% CO_2 atmosphere or candle extinction jar.

Identification of Legionellae

Colonies of legionellae require approximately 72 hours to appear on BCYE agar and may require 7 days or longer. Ideally, plates should be examined after 4 days of incubation and a second time after 7 and 10 days before discarding incubation. Plates should be examined with a dissecting microscope and a light source to detect bacterial colonies resembling legionellae. After approximately 4 days of incubation, these colonies are 2 to 4 mm in diameter, convex, and round with entire edges. The center of the colony is usually a bright white with a textured appearance that has been described as *cut-glass-like* or *speckled*. The white center of the colony is often bordered with blue, purple, green, or red iridescence. Some species of legionellae produce colonies that exhibit blue–white or red autofluorescence (33). The primary isolation plates can be examined with long-wave UV light to detect these autofluorescent colonies.

Colonies resembling legionellae can be presumptively identified on the basis of their requirement for L-cysteine by subculture on blood agar or BCYE agar without L-cysteine. Subcultured colonies that grow on BCYE agar, but not on blood agar or BCYE without L-cysteine, are presumed to be legionellae. Legionellae are relatively inert in many biochemical test media, hence, these tests are of limited value in identification of these bacteria. Definitive identification is usually accomplished by using a direct fluorescent antibody (DFA) or slide agglutination test with specific antisera (30). Identification can also be accomplished using fatty acid analysis and DNA hybridization (5).

Air Sampling

Examination of water samples is the most efficient microbiological method for identifying sources of legionellae. Air sampling is an insensitive means of detecting these bacteria and therefore, it is of limited value in environmental sampling for legionellae. In certain instances, it may be beneficial to demonstrate the presence of legionellae in aerosol droplets associated with suspected reservoirs of the bacterium. Air sampling has been used to better define

the roles of certain devices such as showers, faucets, and evaporative condensers in disease transmission (42). It is usually used to establish the presence of the legionellae in aerosol droplets and occasionally to quantitate or determine the size of particles containing legionellae. Information regarding particle size and numbers of viable bacteria can be calculated using these procedures, but this approach requires much more stringent controls and calibration (30). Samplers should be placed in locations representative of human exposure, and investigators should wear an Occupational Safety and Health Administration-approved respirator if sampling involves exposure to potentially infectious aerosols.

Methods that have been used to sample air for legionellae include impingement in liquid by using an all-glass impinger (AGI), impaction of solid medium by using Andersen samplers, and the use of settle plates (42). Except for settle plates, these methods require a vacuum source and a means of controlling airflow. Several configurations of air sampling equipment can be used; they usually incorporate a device for controlling airflow (flowmeter-manometer) connected in the vacuum line between the sampler and vacuum source.

AGIs with the stem 30 mm from the bottom of the flask have been used successfully to sample for legionellae (42). These samplers use the principle of impingement and washing of air, in which organisms are entrapped in a liquid medium. Because of the velocity at which samples are collected, clumps tend to be fragmented, leading to a more accurate count of bacteria present in the air. The disadvantages of this method are that this velocity tends to destroy some vegetative cells, it does not differentiate particle sizes, and AGIs are easily broken in the field. Yeast extract broth (0.25%) is the recommended liquid medium for AGI sampling of legionellae (30). Once the sample has been collected, the yeast extract broth may be processed by methods used for the culture of water samples.

Andersen samplers are viable particle samplers in which particles pass through jet orifices of decreasing size in cascade fashion until they hit an agar surface (43). The agar plates are then removed and incubated to culture any legionellae present. The stage distribution of the legionellae should indicate the extent to which the bacteria would have penetrated the respiratory system. The advantages of this sampling method are that the equipment is more durable, the sampler can determine the number and size of droplets containing legionellae, and agar plates can be placed directly in an incubator with no further manipulations. Both selective and nonselective BCYE agar can be used in an Andersen sampler. If the samples must be shipped to a laboratory, they should be packed and shipped without refrigeration as soon as possible.

Detection of Legionellae by DFA and PCR

Nonculture methods offer the potential of greatly increased sensitivity, however, culture remains the method of choice for detecting legionellae, primarily because nonculture methods cannot provide information regarding the viability of the bacteria. These nonculture methods include detection of the organisms with specific antisera by DFA staining and procedures to detect nucleic acids of legionellae by using PCR.

The use of DFA to detect legionellae is limited by the number of specific antisera that can be used. There are no antisera that specifically react with all *Legionella* species, a different antiserum must be used for each species or serogroup. Reports on the sensitivity and specificity of DFA testing of environmental specimens vary greatly, with most studies indicating that the test is relatively insensitive and nonspecific (44).

The use of PCR for detecting nucleic acids of legionellae in the environment has proved to be valuable in some investigations of outbreaks of legionellosis (45). A number of Legionella genes, including 5S rRNA, 16S rRNA, and *mip* genes have been used as targets for PCR (46,47). Use of PCR to detect legionellae in the environment has suggested that up to 80% of freshwaters are positive, whereas only 20 to 40% are positive by culture (10,48). This discrepancy could be because of the presence of nonviable or injured organisms, viable but nonculturable legionellae, a nonspecific reaction with unrelated organisms, or the presence of new species of legionellae (49).

Most investigations of epidemic legionellosis have used culture to detect legionellae in the environment. As a result, most of our epidemiologically relevant information concerning legionellosis is based on direct culture data. Until we possess a better understanding of the diversity and distribution of the legionellae, results from nonculture-based methods should be interpreted cautiously.

Molecular Typing Techniques

Associating an environmental isolate of *Legionella* with a clinical isolate from a patient with legionellosis usually requires a molecular subtyping procedure. *L. pneumophila* serogroup 1 (Lp1) accounts for most of the cases of legionellosis (23). However, Lp1 can be divided into a number of subtypes by using various techniques, indicating that this is a fairly heterogeneous serogroup (50). Identification of the bacterium, even to the serogroup level, is not sufficient to implicate an environmental isolate as the source of disease.

Initially, legionellae were identified to the serogroup level during investigations of legionellosis. This form of serologic subtyping uses polyvalent or monoclonal antisera and may be adequate for identifying reservoirs of some of the uncommon legionellae causing disease. The variety of strains and distribution of Lp1 necessitate more elaborate subtyping procedures to discriminate within these bacteria. Several groups of monoclonal antibodies have been developed for this purpose (51). An international panel of seven monoclonal antibodies was proposed in 1986 (51,52). Although much information has been gained through the use of this panel, several of the cell lines have been lost and most of these reagents are no longer available. Use of these monoclonal antibodies has identified 10 type strains within Lp1. The ability to differentiate Lp1 into 10 subtypes has greatly improved our ability to correctly identify sources of disease. For example, several outbreak investigations have found Lp1 in both the potable water system and cooling towers

of a particular institution (42,53). Differentiation with monoclonal antibodies indicated that the epidemic strain had colonized only one of these systems (either cooling tower or potable systems) indicating that the presence of Lp1 in the other system was not causing disease at that site. Without this level of subtyping, it would be much more difficult to confidently confirm the source of transmission.

Recent investigations have shown that the use of monoclonal antibodies may not be sufficient for discriminating between disease-causing strains and other environmental isolates of Lp1 (54). Molecular techniques such as pulsed-field gel electrophoresis (PFGE), arbitrarily primed PCR (AP-PCR), and amplified fragment length polymorphism (AFLP) are able to discriminate within monoclonal subtypes of Lp1 and identify sources of disease-causing strains (50,55). These techniques appear to complement the use of monoclonal antibodies and are the most recent of several techniques that separate strains based on DNA polymorphism (50). Other techniques used to discriminate between isolates of legionellae include restriction fragment length polymorphism analysis, plasmid analyses, electrophoretic alloenzyme typing, and RNA/DNA probing of DNA digests (56,57). PFGE, AP-PCR, and AFLP offer better discrimination and are less labor-intensive than these other techniques.

PREVENTION OF LEGIONELLOSIS

It is unlikely that legionellae can be eradicated from aquatic environments, even on a limited scale, because they are integral members of the aquatic microbial community. The bacteria are ubiquitous in freshwater environments, and they are present in relatively low concentrations in most water supplies. They are rarely isolated from water treatment facilities. The difficulty in detecting legionellae in water treatment plants and municipal water supplies is probably because of the lower temperatures of these waters. Higher concentrations of legionellae are more frequently present in warm and thermally altered environments where they have increased potential contact with humans (58). To minimize the number of cases of legionellosis, many countries have produced guidelines or codes of practice relating to the control of legionellae. However, research to substantiate these practices is scarce, and the prevailing rationale for these recommendations is primarily empirical (59). There is no single established protocol to prevent legionellosis. The two primary approaches for controlling this disease are (*1*) maintaining building waters systems as to limit colonization and growth of the bacteria and (*2*) intervention measures if cases of legionellosis are associated with a particular environment. Table 3 list guidelines for the control and prevention of legionellosis from U.S. Federal agencies, State and Local government agencies, and nonprofit professional organizations.

Traditional means of eliminating legionellae from a particular reservoir include use of oxidizing biocides, such as chlorine and bromine and heat eradication. Additional alternative measures include UV irradiation, copper–silver ionization, monochloramine, and ozonation (60–63). Two of these procedures, copper–silver ionization and use of monochloramine have received favorable attention recently. Kool and coworkers published a case-control study that compared disinfection methods for drinking water supplied to 32 hospitals that had outbreaks of Legionnaires' disease with the disinfection method used in 48 control hospitals (64). Hospitals supplied with free chlorine were 10 times more likely to have reported outbreaks of Legionnaires' disease than those using monochloramine as a disinfectant. The authors suggest that 90% of nosocomial outbreaks may be prevented by the use of monochloramine for residual disinfection. Copper–silver ionization was documented to control legionellae at several

Table 3. Guidelines for the Control and Prevention of Legionellois from U.S. Federal Agencies, State and Local Government Agencies, and Nonprofit Professional Organizations

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hospitals, however, long-term efficacy of this procedure has not been documented (65). Further studies are needed to determine the efficiency of both of these procedures.

As previously mentioned, temperature is a critical factor in the ability of legionellae to colonize reservoirs in which they are amplified. Other microorganisms and factors critical to the growth of legionellae are almost universally present in freshwater environments, and it is temperature that governs the numbers of these bacteria. An Australian study of cooling towers found that legionellae colonized or multiplied in towers with basin temperatures above 16 °C and multiplication that became explosive at temperatures above 23 °C (59). Conversely, legionellae are killed at temperatures between 44 and 55 °C, and it has been suggested that potable hot water systems be maintained at temperatures between 55 and 60 °C, to prevent growth of the bacteria (63). This may be impractical for many institutions, especially hospitals, where the potential for scalding of patients exists or where state regulations are prohibitive.

Effective prevention strategies will require a multidisciplined approach to identify more effective decontamination techniques and approaches to prevent amplification of these bacteria in reservoirs.

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LEGIONELLA IN THE ENVIRONMENT: PERSISTENCE, EVOLUTION, AND PATHOGENICITY

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The environment has always been at the crux of the evolutionary process at both the macro and micro levels. Organisms are constantly faced with the challenge of adapting to environmental changes and to other inhabitants of their ecosystems. One outcome of such adaptation appears to have been the rise of pathogenic microbes that are a major source of morbidity and mortality to humans and animals. A common theme for many of those bacterial pathogens is their ability to infect and survive within free-living amoebae (1,2). In addition to allowing bacteria to replicate and increase in numbers, amoebae provide a protective barrier between such microbes and adverse extracellular conditions (3). Important human and animal pathogens such as *Mycobacterium avium* (4,5), *Chlamydia pneumonia* (6,7), and *Listeria monocytogenes* (8) are capable of infecting and surviving within protozoa. Moreover, a link may exist between the ability of these pathogens to infect amoebae and their ability to infect mammalian cells (2,9). An example of this comes from studies that showed that an avirulent strain of *Mycobacteria*, *Mycobacterium smegmatis*, cannot infect the same amoebae that the virulent strain, *M. avium*, can (4). Studies investigating members of the *Legionella* species and their interaction with the environment and amoebae have been most revealing (2,10–15). Over the past two decades a clear link between legionellae and their environments, and their ability to cause disease in humans has been established. This article discusses the important aspects of the critical role played by the environment in the evolution, persistence, and pathogenicity of legionellae. In addition, the molecular ecology of legionellae within amoebae is highlighted as a model for understanding the pathogenicity of legionellae to mammalian cells.

LEGIONELLAE; HISTORICAL PERSPECTIVE AND PUBLIC HEALTH ISSUES

Historical Perspective

Legionellae became known following a notorious outbreak of a mysterious respiratory illness in the summer of 1976 that affected 182 people in Philadelphia, Pennsylvania, of whom 29 died (16,17). Because the disease mostly affected attendees of an American Legion convention, it was called Legionnaires' disease. Several months following this outbreak, the causative agent of Legionnaires' disease was isolated and determined to be a rod-shaped facultative intracellular gram-negative bacterium (16). This bacterium was subsequently classified as a new species (*Legionella pneumophila*) and placed in a new genus, *Legionella* (18). Interestingly, it was subsequently determined that several outbreaks and sporadic cases of pneumonia with unknown etiologies dating back to as early as 1947 were indeed caused by legionellae and the bacterium was isolated as early as 1943 (19–22). Most of the Legionellaceae are motile by means of polar or lateral flagella (23–25). *Legionella* possess some unique features that may be useful in their identification, such as their utilization of amino acids as a sole carbon source (26) and their requirement for L-cysteine for growth on artificial media (27).

Since the initial description of *L. pneumophila*, 42 species of legionellae have been discovered (19,28–30). Many of the Legionellaceae such as *Legionella longbeachae*, *Legionella bozemanii*, and *Legionella micdadei* cause disease in humans (31–35). In addition, several *Legionella*-like amoebal pathogens that can also cause disease in humans have been described (19,31). A link between the environment and the ability of legionellae to cause disease became apparent soon after the 1976 outbreak of Legionnaires' disease when the source of infection

was determined to be a water-chilled air-conditioning system (17). This was followed by the discovery that legionellae are ubiquitous within both natural (water and soil) and "unnatural" (artificial aquatic devices) environmental settings (17,36). Finally, the complexity of the relationship between the habitat of legionellae and its pathogenicity began to emerge following the discovery that legionellae are able to survive within water and soil protozoa (37).

Public Health Issues

Clinically, Legionnaires' disease is characterized as an acute pneumonia that may be associated with high fever, dry cough, chills, diarrhea, and pleuritic pain and can lead to severe multisystem manifestations if left untreated (20,38–40). Unfortunately, the initial clinical symptoms are often indistinguishable from pneumonia caused by other etiologic agents making it often difficult to dispense appropriate treatments early during infection (38,41,42). Furthermore, many hospitals often fail to detect legionellae in culture specimens or simply do not attempt to identify this bacterium (19,43). Out of all the pathogenic species of *Legionella*, *L. pneumophila* accounts for up to 80% of the cases of Legionnaires' disease (11,13,19). Furthermore, among the 15 serologically distinct groups of *L. pneumophila*, serogroup 1 accounts for up to 82% of Legionnaires' disease caused by *L. pneumophila* (44).

It is now recognized that Legionnaires' disease and a milder nonfatal form of the disease, Pontiac fever, occur sporadically or in outbreaks in both community and nosocomial settings (11,40,41). It is estimated that 10,000 to 25,000 cases of Legionnaires' disease occur annually in the United States accounting for approximately 10% of cases of community-acquired pneumonia (11,13,19,41,45). Increased risk of contracting Legionnaires' disease has been found to occur among the elderly, smokers, and immunocompromised individuals (11,40,44,46).

The number of reported cases of infection caused by legionellae is believed to be an underestimate because of the difficulty in isolating the bacteria and the similarity of the initial clinical symptoms of Legionnaires' disease to pneumonia caused by other agents (11,31,42). Furthermore, sporadic cases of Pontiac fever with its flulike symptoms or asymptomatic legionellae infections are likely to occur (47). This is accentuated by a study in which one-third of surveyed adults in South Australia showed evidence of previous silent infection by *L. pneumophila* (48). In addition, a high percentage of outpatients (36%) and dental personnel (up to 50%) exhibited elevated antibody titers to different species of *legionella* (33,49).

THE TRANSMISSION AND ECOLOGY OF LEGIONELLAE

Transmission of Legionellae

Since the 1980s, there have been hundreds of documented outbreaks of Legionnaires' disease and Pontiac fever. Transmission of legionellae occurs via inhalation of aerosolized water droplets or soil particles containing the bacterium (11,32,50–53). In most cases of legionellae infection, the source of transmission has been attributed to aerosol generating artificial devices such as cooling towers, showerheads, grocery store mist machines and whirlpools (54–58). However, mechanical aerosolization of soil particles during gardening has also been described as a mode for transmission of legionellae (32,59,60).

The nature of the aerosolized infectious particle is not known, but several possibilities exist (Fig. 1; 61). First, it is possible that free extracellular legionellae could be inhaled following aerosolization. This is supported by the fact that animals can be experimentally infected with *L. pneumophila* (62,63). In addition, legionellae can escape from its protozoan hosts (see molecular ecology of legionellae in the following text) and can survive extracellularly for prolonged periods (64). Second, it is possible that amoebae harboring legionella may constitute the infectious particle (3,65,66). Amoebae are small enough to be inhaled and provide a sanctuary for legionellae (67,68). In addition, *L. pneumophila* grown within amoebae exhibits enhanced virulence in the A/J mouse model of Legionnaires' disease (66). Third, amoebae infected with legionellae are capable of excreting vesicles containing viable bacteria. Such vesicles are able to protect legionellae from the action of biocides and can be inhaled; thus, making them likely infectious particle candidates (69,70). The later two possibilities may help explain the "infective dose paradox" (70). This paradox is based on the fact that only low concentrations of legionellae are generally present in aerosols (believed to be insufficient to cause disease) (52,62) and that transmission may occur at some distance from the aerosol-generating source (71). However, aerosolized amoebae or vesicles containing legionellae may contain several million bacteria (69,70,72).

Clearly a link exists between the appearance of Legionnaires' disease and the industrialization of our society. However, the fact that simple mechanical aerosolization of soil particles harboring the bacteria can result in infection may be a sign that transmission of legionellae to humans may be as old as gardening itself. Person-to-person transmission of Legionnaires' disease has never been documented. Furthermore, natural infection with legionellae is believed to occur only in humans, although one case of naturally occurring pneumonia in animals due to legionellae has been reported (73).

Natural Ecology of Legionellae

Since the initial discovery of *Legionella*, members of this genus have been detected in natural aquatic (marine and fresh water) and soil environments (Fig. 2). Legionellae have been isolated from diverse aquatic sources such as rivers, lakes, and hot springs (36,64,74–76). In a study that investigated the prevalence of *Legionella* spp. in Puerto Rican waters, a high incidence of several pathogenic strains of legionellae including *L. pneumophila*, *L. bozemanii*, *L. longbeachae*, and *L. micdadei* was observed (77). *Legionella pneumophila* was found most commonly and all existed in diverse environments from rainwater collected from epiphytes

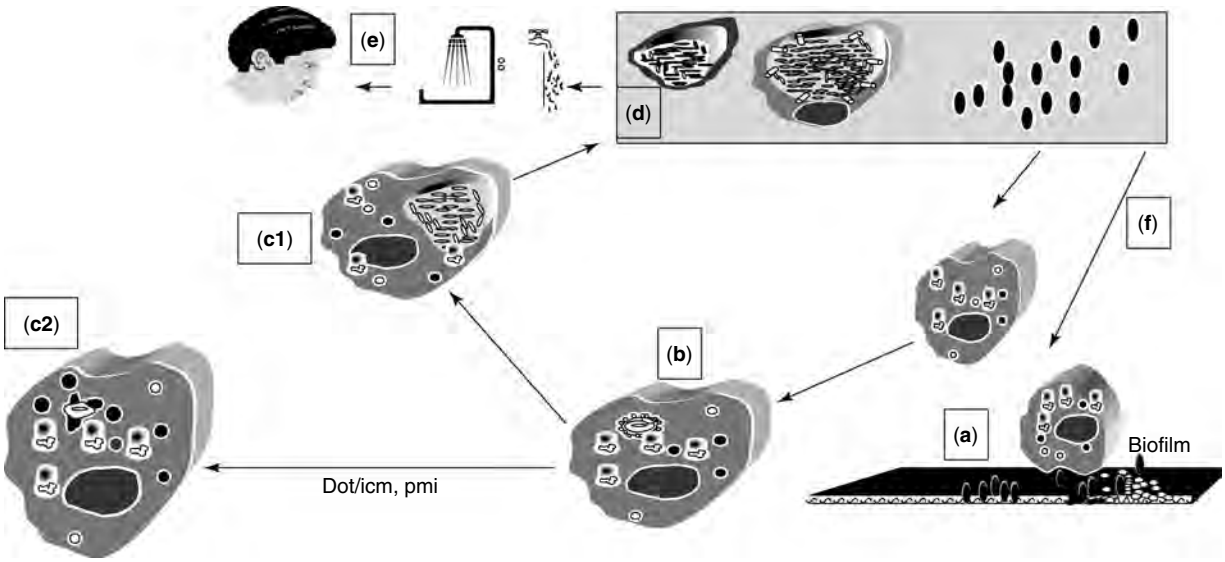

Figure 1. The environmental life cycle of *L. pneumophila* within protozoa. (**a**) *Legionella pneumophila* from biofilms with other bacteria or in suspension infecting protozoa. (**b**) Following entry, *L. pneumophila* resides in a membrane bound vacuole that recruits host cell organelles such as the mitochondria and the rough endoplasmic reticulum, and does not fuse with lysosomes. (**c1**) *Legionella pneumophila* replicates within this specialized vacuole and reaches large numbers. (**c2**) *Dot/icm* and *pmi* mutants fuse to lysosomes. (**d**) The infectious particle is not known but may include excreted legionellae-filled vesicles, intact legionellae-filled amoebae, or free legionellae that have lysed their host cell. (**e**) Transmission to humans occurs via mechanical means, such as unclear faucets and showerheads. Infection in humans occurs by inhalation of the infectious bit and establishment of infection in the lungs. (**f**) Legionellae that have escaped their host cell may survive in suspension for long periods, reinfect other protozoa, or recolonize biofilms.

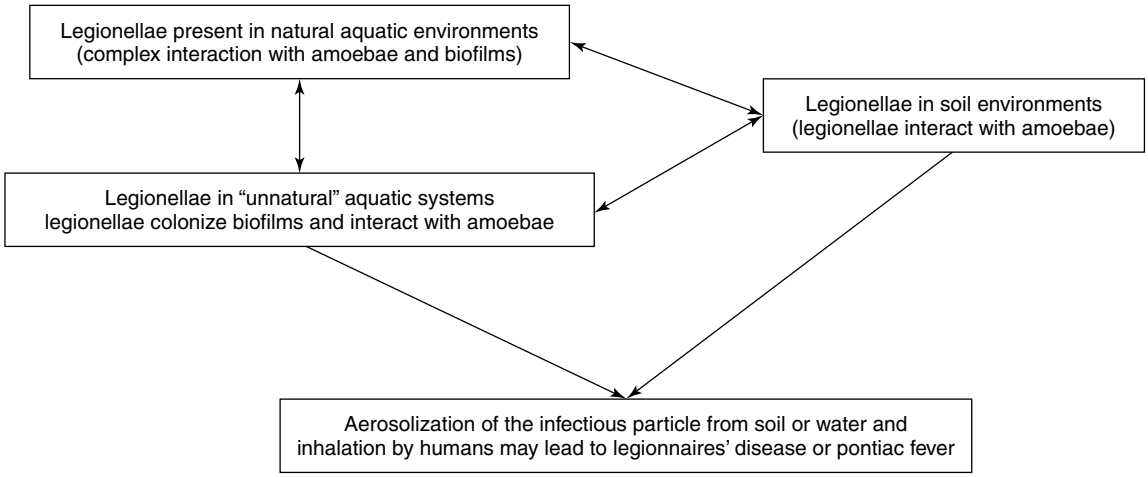

Figure 2. A diagram depicting the complex relationship of legionellae with their environments. Notice arrows between legionellae in the soil, natural aquatic environments, and "unnatural" aquatic environments are bidirectional indicating the possibility of movement of legionellae between its diverse environments. Transmission of legionellae to humans occurs from soil or artificial aquatic systems.

to sewage-contaminated coastal marine water (77). Interestingly, an average of 10^4 bacteria per milliliter of *L. pneumophila* was detected — a number sufficiently close to the estimated number of bacteria required for establishment of infection in humans (70,77).

Legionella pneumophila is able to survive high concentrations of salt (up to 3%) and no loss in viability is observed in natural seawater (78). This is interesting in light of the fact that repeated culture of *L. pneumophila* on synthetic media containing 0.65% sodium chloride allows for the isolation of salt-resistant and avirulent strains of this bacterium (79,80). Whether natural environmental exposure of legionellae to salt results in development of mutant bacterial strains is not known. However, this can

be viewed as a potential environmental factor that may contribute to the genetic diversity of legionellae.

Soil, is emerging as a potentially important reservoir for pathogenic species of this bacterium. In several studies investigating sporadic cases of Legionnaires' disease particularly in Japan and Australia and most recently in the United States, *L. longbeachae, L. pneumophila*, and *L. micdadei* were detected in potting soil (59,60,81–83). A study of potting soil in South Western Australia revealed a high rate of *Legionella* contamination (43% of sampled potting soil mixes) in particular *L. longbeachae* (59). Additionally, it is becoming clear that direct transmission of *Legionella* from soil occurs via the aerosolization of infectious particles during gardening (82–85). This is interesting in light of the common conception that transmission of *Legionella* requires mechanical aerosolization through artificial devices such as air-conditioning units or mist machines (see following text). Thus, transmission of *Legionella* to humans may not have been completely dependent on the industrialization of society.

The "Unnatural" Ecology of Legionellae

One outcome of the industrialization of humans has been the development of new contained aquatic ecosystems that act as reservoirs for diverse species of microorganisms including protozoa, bacteria, and viruses. Such aquatic systems may provide ideal conditions for the amplification of microorganisms because the environments of such systems remain relatively stable for prolonged periods. Because species of *Legionella* are ubiquitous in nature, it is not surprising that most artificial water reservoirs are contaminated with this bacterium. In a study of the occurrence of *L. pneumophila* in hot water (up to 60 °C) obtained from nosocomial, domestic, and community sources, it was observed that more than 20% of the samples were contaminated with this bacterium (86). Several other studies investigating the prevalence of legionellae in domestic potable-water systems have revealed a high incidence of this bacterium and the capacity of such systems to generate aerosolized infectious particles (52,87–89). This raises the possibility that such water systems may be sources of sporadic infection by legionellae, although incidences of such cases are relatively low (89,90).

Recurring outbreaks of Legionnaires' disease are prevalent in nosocomial settings. In a survey of 192 hospitals in the United States, more than 60% reported at least two outbreaks of Legionnaires' disease over a six-year period (91). The source of infection in nosocomial settings is often traced back to both cooling towers and potable-water systems (91). Contamination of cooling towers may be more common than previously thought as evident from a survey of hospital cooling towers in Japan, in which 45 out of 49 samples were contaminated with various species of *Legionella* (92). Furthermore, legionellae may persist in these systems for prolonged periods following decontamination treatment (93). In a study of the water system in the University of Iowa Hospitals and Clinics, *L. pneumophila* was detected over a period of 13 years and was implicated in nosocomial outbreaks of Legionnaires' disease during the same period (94).

The widespread presence of legionellae in water supplies can be viewed in certain instances as an occupational hazard. Indeed, this may be the case for dental clinicians who are at a higher risk of being exposed to legionellae. In a study of the prevalence of legionellae-specific antibodies among dental workers, 20% tested positive compared to only 8% of a nonclinical population (95). Antibodies to *L. pneumophila* were found most commonly, followed by antibodies to *L. micdadei* and *L. bozemanii* (95). In addition, the same study revealed a strong correlation between the length of clinic exposure time and the likelihood of testing positive. The reason for this high rate of exposure to legionellae among dental workers can be traced back to the dental equipment (96,97). Such equipment contains tubing, which permits water stagnation that in turn allows for the development of biofilms where microbes can thrive (See following text). Added to this is the aerosol-generating potential of various water-cooled dental devices (97). A survey of dental waters in several areas of the United States revealed that 68% of dental units were contaminated with legionellae of which 8% contained detectable levels of *L. pneumophila* (98). This survey also indicated that 61% of potable water collected for comparison was contaminated with *Legionella* species and only 4% of those contained *L. pneumophila* (98). In addition, 20% of the contaminated dental units contained upward of 10,000 legionellae organism per milliliter, a level that was never observed in the potable water control samples (98).

The Molecular Ecology of Legionellae

The molecular ecology of legionellae can be addressed by examining the interaction of this organism with its natural microecosystem that is composed of biofilms and its natural host, free-living amoebae (2). Biofilms are complex ecological niches within which microbes reside in close proximity and communication with each other (99,100). Biofilms can form virtually anywhere and sufficient nutrients are available both on natural and synthetic surfaces upon which microbes can attach (99,100). The complexity of biofilm structure and the intricate nature of inter- and intraspecies communication that occurs within such ecosystems makes them resemble a multicellular organism (101). This feature is likely to have contributed to the diversification and coevolution of biofilm inhabitants. Two lines of evidence support this idea. First, microbes within biofilms undergo high rates of conjugation of genetic material (102,103). This phenomenon may be instrumental in bacterial evolution and diversification. *Legionella pneumophila* is naturally competent for the uptake of DNA, a fact that may have influenced the evolution of this bacterium (104). Second, protozoal grazing on bacteria within biofilms is likely to have contributed to the evolution of diverse protective bacterial responses. Several examples of bacterial adaptation to their protozoan predators exist (2). These include increased bacterial productivity to ensure survival (105), production of toxic compounds against protozoa (105,106), and the development of endosymbiotic relationships with protozoa (107–109). The latter is likely

to have contributed to the evolution of intracellular bacterial pathogens like legionellae (2).

Legionella are commonly thought to exist as intracellular parasites of amoebae in nature. However, biofilms have been shown to support the growth of *L. pneumophila* and provide a protective environment for this bacterium (110,111). Microflora within biofilms are generally more resistant to the effect of biocides, chlorination, temperature, and UV irradiation (111–114). Thus, legionellae growing on biofilms in aquatic water systems such as dental water units are likely to exhibit an increased resistance to disinfection methods (see following text). In a study by Rogers and colleagues, the growth of *L. pneumophila* in biofilms was influenced by temperature and the type of plumbing material utilized (111). *Legionella pneumophila* was most abundantly found on polybutylene surfaces at 40 °C (111). Interestingly, amoebae that support the growth of legionellae were not present at this temperature, indicating that *L. pneumophila* was able to increase in numbers in the absence of its natural amoebal hosts (111). The ability of legionellae to grow extracellularly may be attributed to nutritional support provided by other microorganisms present in the biofilm (111). For example, *L. pneumophila* is able to grow extracellularly in association with environmental isolates of *Flavobacterium breve* on media lacking the essential legionellae nutrient, L-cysteine (115). In addition, *L. pneumophila* can grow extracellularly in association with blue-green algae at 45 °C (116). Thus, legionellae are protected by biofilms, and inhabitants of these ecosystems may provide nutritional support for extracellular growth of this organism.

Since the initial discovery that legionellae can survive within free-living amoebae (37), more than 13 species of amoebae have been discovered to support their growth. (2,10,11). These include common water and soil protozoa such as species of *Acanthamoeba* and *Naegleria*, respectively (37). Interestingly, different species of *Legionella* exhibit preferential growth within certain species of protozoa (14,117). In addition, a correlation may exist between the pathogenicity of a *Legionella* species to humans and the number and types of protozoa they infect (118,119). For example, *L. pneumophila* in particular serogroup 1, which causes most cases of Legionnaires' disease, can grow in the largest variety and numbers of amoebal species (10). In comparison, *L. micdadei*, which is the second most common cause of Legionnaires' disease, can grow in *Hartmannella vermiformis*, but not in *Acanthamoeba polyphaga* (120). Another species of *Legionella*, *L. longbeachae*, which is primarily associated with soil transmission of Legionnaires' disease, does not grow within *Hartmannella vermiformis* (117), and Acanthameoba castellani (119), but grows within the ciliated protozoa *Tetrahymena pyriformis* (121).

Amoebae in the environment play an important role in the persistence of legionellae and influence their virulence to mammalian cells (2). First, Legionellae existing within protozoa are highly resistant to the action of several adverse extracellular conditions, such as elevated temperatures or toxic chemicals (122,123). *Legionella pneumophila* residing within cysts of *A. polyphaga* are resistant to the action of free chlorine, explaining why legionellae often are not eradicated following disinfection of water systems or following sewage treatment (124,125). Second, amoebae are likely to play a critical role in the amplification of legionellae in the environment. Legionellae can reach substantially large numbers within a relatively short period during intracellular growth within amoebae (126). Third, intracellular growth of legionellae within amoebae increases their virulence severalfolds toward mammalian cells (127,128). This may be a result of the fact that several virulence genes that are required by legionellae to infect amoebae are also required for the infection of mammalian cells (see following text) (2). Interestingly, following intracellular replication within amoebae *L. pneumophila* exhibits a dramatic (1,000-fold) increase in resistance to the action of antimicrobial compounds (129,130).

THE LIFE CYCLE OF LEGIONELLAE WITHIN THEIR PROTOZOAN HOST CELLS

Attachment, Entry, and Intracellular Survival

Species of *Legionella* have evolved diverse mechanisms for attachment and entry into their host cells. Attachment of *L. pneumophila* and *L. micdadei* to the amoebae *H. vermiformis* is mediated through a protozoan Gal/GalNAc lectin receptor (131,132). This event triggers a dramatic tyrosine dephosphorylation of a number of protozoan proteins including the receptor itself and several cytoskeletal proteins that include paxillin, vinculin, and focal adhesion kinase (132,133). The tyrosine dephosphorylation of these proteins is likely to be mediated through a tyrosine phosphatase and results in the disruption of the cytoskeleton (11,134). This in turn may facilitate entry of *L. pneumophila* into *H. vermiformis* through a cytoskeleton-independent receptor-mediated endocytosis (133,135). Interestingly, the ability of *L. pneumophila* to manifest the entry events requires specific gene expression by *H. vermiformis* because the inhibition of protein expression of *H. vermiformis* prevents bacterial entry (136). Analysis of *L. pneumophila* entry into another protozoan, *A. polyphaga*, revealed it to be partly mediated through a similar Gal/GalNAc lectin receptor (137). Furthermore, the pattern of tyrosine-dephosphorylated *A. polyphaga* proteins was markedly different from the observed pattern in *H. vermiformis* with only mild dephosphorylation of the putative receptor molecule (137). Thus, *L. pneumophila* may have adapted different mechanisms to invade different protozoan hosts.

The bacterial ligands utilized by legionellae to initiate interaction with protozoa have not been clearly established, although several possibilities exist. The type IV pili of *L. pneumophila* are involved in the attachment process because mutants that do not express these pili fail to adhere to *A. polyphaga* (Fig. 3; 138). Other candidate ligands include a heat-shock protein (Hsp60) and the major outer membrane protein of *L. pneumophila*, both of which are involved in the attachment of this bacterium to mammalian cells (139–141). Also, *enh* loci involved in entry into mammalian monocytes and epithelial cells may also be involved in entry into protozoa (142). Several mutants

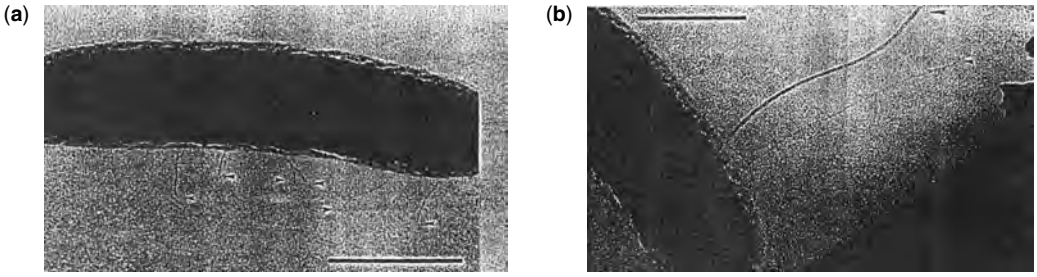

Figure 3. Electron micrographs of *L. pneumophila* expressing short pili (**a**) the long CAP pili (competence and adherence associated pili) (**b**). The two pili are expressed by different cells within the same culture, and no cells that express both pili have been detected. (Adapted from B. J. Stone and Y. Abu Kwaik, *J. Bacteriol.* **181**, 1,395–1,402 (1999)).

of *L. pneumophila* that are defective in attachment to protozoa have been recently described and should prove useful in the identification of other bacterial factors involved in the invasion process (133,137,143).

Uptake of *L. pneumophila* by its protozoan host is mediated through conventional or coiling phagocytosis (Fig. 4; 144,145). In addition, a study utilizing a flow cytometric technique revealed that *L. pneumophila* enters its host cell within five minutes of coculture with *Acanthamoeba palestinensis* (146). Following entry, *L. pneumophila* resides in a unique organelle that is surrounded by mitochondria and other vesicles (144,145,147,148). Within four hours of entry, the *L. pneumophila* phagosome becomes surrounded by the rough endoplasmic reticulum (RER) (144,145). In addition, the *L. pneumophila* phagosome is inhibited from maturation into a lysosome (11,145). The initial 4-hour period after entry precedes the replication of *L. pneumophila* and is presumed to be an adaptation period required by this bacterium to adapt to its new intracellular environment (Fig. 4; 11). The identification of several *L. pneumophila* mutants that are defective for intracellular survival within amoebae should prove useful in understanding the mechanisms used by this pathogen to parasitize its host cells (2,143). The phenotypic characteristics of the *L. micdadei* phagosome within amoebae are surprisingly different from those of the *L. pneumophila* phagosome. Studies investigating the *L. micdadei* phagosome in *H. vermiformis* have revealed that it never becomes surrounded by the RER (120,132). The importance of the phenotypic difference in the pathogenicity of legionellae is not clearly understood although it suggests heterogeneous mechanisms of intracellular parasitism by different species of legionellae (2). This is supported by the findings that *L. micdadei* does not infect another species of amoebae, *A. polyphaga*, does not exhibit pore-forming activity (see following text), and is avirulent in the A/J mouse model of Legionnaires' disease (120). In addition, *L. micdadei* and *L. pneumophila* do not share the same virulence loci (118), indicating a divergence in virulence mechanisms between these two species of legionellae.

Killing of the Host Cell

Following 24–72 hours of replication, *L. pneumophila* kills its protozoan host by a necrotic mechanism followed by release of the intracellular bacteria (149). The killing of *A. polyphaga* by *L. pneumophila* has been described to be a monophasic event in which necrosis is the only mode of bacterial-induced host cell killing (Fig. 5; 149). This is in contrast to the killing of mammalian cells by *L. pneumophila* that occurs by two mechanisms; rapid apoptosis soon after bacterial entry and necrosis before the release of the intracellular bacteria (150–153). Several studies have shown that *L. pneumophila* does not induce apoptosis in amoebae (149,154). The necrotic killing of amoebae by *L. pneumophila* is mediated through a pore formation (149). The same pore-forming activity of *L. pneumophila* is required for the killing of mammalian cells (155,156). In addition, mutants of *L. pneumophila* that are defective in pore formation cannot escape from the host cell despite reaching large (149). Expression of the pore-forming activity is triggered during

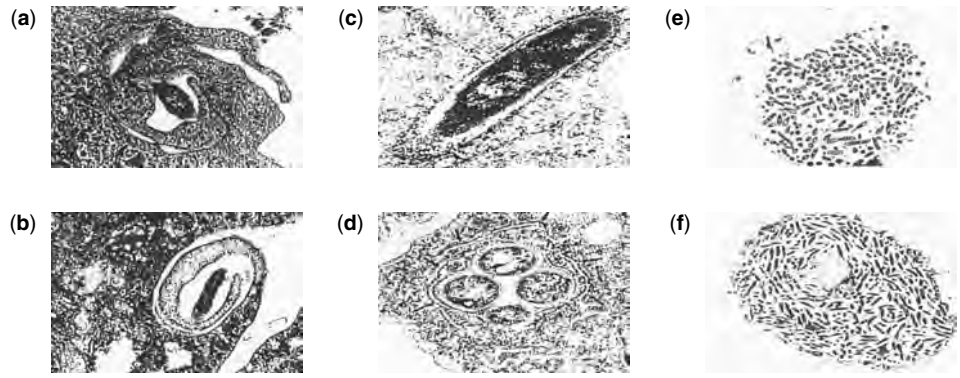

Figure 4. Electron micrographs of the infection of U937 macrophages (top panels) and *A. polyphaga* by *L. pneumophila* (lower panels). Coiling phagocytosis (**a** and **b**); formation of the RER-surrounded phagosome (**c** and **d**); and late stages of the infection (**e** and **f**). Note that in (**e**) and (**f**) there is no intact phagosomal membrane and in some cases the bacteria are making contact with the plasma membrane. (Adapted from O. S. Harb and Y. Abu Kwaik, ASM News, 66, 609–616 (2000)).

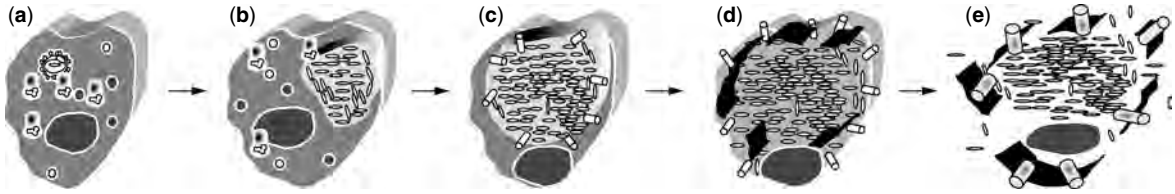

Figure 5. A model of growth phase-dependent cytolysis of *A. polyphaga* by *L. pneumophila* upon termination of intracellular bacterial replication to egress the spent host cell. During the early stages of formation of the mitochondria and RER-surrounded phagosome (**a**) and during exponential intracellular replication (**b**), expression of the pore-forming activity is turned off. This is essential to maintain integrity of the host cell, which is fundamental for intracellular bacterial proliferation. Upon transition to the postexponential phase of growth, expression of the pore-forming activity is triggered, which results in insertions of pores in the phagosomal membrane first (**c**), leading to its disruption (**d**). This is followed by insertions of the pores in the plasma membrane (**e**), leading to osmotic lysis of the cell, and release of the intracellular bacteria, which are not cytotoxic to *A. polyphaga* from an extracellular location. (Adapted from O. A. Alli and colleagues, *Infect. Immun.* **68**, 6,431–6,440 (2000)).

postexponential growth of *L. pneumophila* (149). Indeed several virulence traits of *L. pneumophila* have been described to be linked to the bacterial growth phase (157). Only postexponential phase *L. pneumophila*, in vitro, express the following traits associated with virulence: sodium sensitivity, cytotoxicity, resistance to osmotic lysis, evasion of phagosome-lysosome fusion, infectivity, and motility (flagellated) (157). Furthermore, flagellation and sodium-resistance are expressed upon macrophage lysis by intracellular *L. pneumophila* (157). Gao and colleagues, have shown that pore formation is growth phase-regulated both in vitro and in vivo (149). Interestingly, the trigger for this dramatic phenotypic change by *L. pneumophila* is thought to be nutrient starvation. In particular, depletion of five amino acids (serine, tyrosine, asparagine, proline, and threonine) appears to be crucial for the expression of these virulence traits (157). Little is known about what mediates the response to amino acid starvation by *L. pneumophila*, although a (p)ppGpp synthetase (RelA) may be involved (13,158). Thus, it is thought that *L. pneumophila* is able to repress virulence traits that are required for the invasion of and escape from its host cells during intracellular replication in the nutrient-rich intracellular environment. However, upon depletion of nutrients and entry of the bacteria into the postexponential growth phase within its host cells, the virulence traits are activated to mediate release and subsequent reinfections (13,149,157). It is likely that *L. pneumophila* may possess several effector molecules that are involved in mediating this dramatic phenotypic transformation.

Genetic Factors Required by Legionellae for Intracellular Survival Within Amoebae

Several techniques have been utilized for the identification of genetic factors necessary for the infection process of eukaryotic cells by legionellae and other intracellular bacterial pathogens (159). Such genetic analysis has allowed for a further understanding of the nature of the intracellular environment that legionellae are exposed to. Several genes involved in biosynthetic pathways are required for intracellular survival indicating that the legionellae phagosome may be deficient in or inaccessible to certain nutrients. For example, mutants of *L. pneumophila* defective in the aspartate-β-semialdehyde gene are auxotrophs for diaminopimelic acid (DAP), and can survive normally when a supplement of this compound is present on synthetic media (160). However, such mutants are defective for intracellular survival even in the presence of DAP supplement, indicating that the *L. pneumophila* phagosome is inaccessible to this compound (160). Other examples of this include the *prp* locus involved in citrate biosynthesis (161), and the Rep helicase required for survival within mammalian macrophages but not in amoebae (162). In addition, several macrophage-specific infectivity loci required for infection of mammalian cells but not protozoa have been described (163–165). The existence of these genes indicates that legionellae have evolved unique heterogeneous mechanisms for intracellular survival within different cell types.

Several genetic loci of legionellae are involved in intracellular survival of within both mammalian cells and protozoa. These include the macrophage infectivity potentiator (*mip*) gene (166), the *dot* (defect in organelle trafficking) and *icm* (intracellular multiplication) loci (167–169), the protozoan and macrophage infectivity (*pmi*) loci (143), and the *pilBCD* pilin genes (138,170). The Mip protein is a bacterial surface protein involved in the early stages of mammalian cells and protozoa infection (166) and belongs to the peptidyl-prolyl *cis*/*trans* isomerase (PPIase) family of proteins (171). However, the exact function of Mip is not known because specific mutagenesis of its PPIase activity indicated that this activity is not involved in the virulence of *L. pneumophila* (172). The *dot*/*icm* loci comprise a cluster of 23 genes that are required for the assembly of a type IV like secretion apparatus in *L. pneumophila* required for infection of both mammalian cells and protozoa (143,167,169,173,174). This secretion apparatus is involved in the proper maturation of the *L. pneumophila* phagosome (175,176), induction of apoptosis in mammalian cells (151), and assembly of a pore-forming toxin (156,169,174). The *pmi* genes consist of

89 insertion mutants of *L. pneumophila* that are all defective for intracellular survival within mammalian cells and protozoa (143). Characterization of these loci should prove useful in further understanding the virulence mechanisms of legionellae. The *pilBCD* genes of *L. pneumophila* are required for the biogenesis of type IV pili (138) and type II secretion (170). Further, *L. pneumophila* mutants of *pilBCD* are defective for intracellular survival within mammalian cells and protozoa (170,177,178).

DETECTION AND DISINFECTION OF LEGIONELLAE

Detection of Legionellae in the Environment

Several methods have been developed over the years for the detection of different species of legionellae from environmental sources. The standard method utilized for detection has been cultivation of viable legionellae on selective media (97). However, this method has several drawbacks including contamination with other bacterial species, and limited detection sensitivity and specificity. Water from environmental samples needs to be concentrated severalfolds before the detection of small numbers of bacteria is possible. In addition, supplementation of media with inhibitory compounds is often necessary to eliminate contamination (98). Perhaps, the single most important shortfall of this method is its inability to detect viable but nonculturable species of legionellae. Amplification of environmental legionellae before cultivation can be achieved via a growth cycle in amoebae (179). Steinert and colleagues were able to resuscitate a strain of *L. pneumophila* to cultivatable levels following amplification in *A. castellanii* (180).

Several immunological methods for the detection of legionellae in the environment have been developed. These include direct fluorescence detection (36,181), and the use of flow cytometry techniques (182). However, immunological detection requires the use of legionellae specific antibodies that are generally species-specific and thus is limited to the detection of only a small number of *Legionella* species. In a comparison study of culture and immunofluorescence methods for the detection of *L. pneumophila* serogroups 1 to 6 in domestic hot water supplies, Alary and colleagues showed that the indirect immunofluorescence method detected *L. pneumophila* more frequently (183). However, considerable variation existed between both methods in determining the serotype of *L. pneumophila* (183).

The most sensitive and specific methods for the detection of environmental legionellae are those that are based on the amplification of DNA fragments present in all species of legionellae. Most of these methods are based on amplification of common regions of the rRNA genes or the *mip* genes of all legionellae species using the polymerase chain reaction (PCR) technique (184–186). These methods have proven useful in the detection of legionellae from various environmental locations such as cooling towers (92), reclaimed water sources, and air (187). A drawback of the DNA amplification method is that positive results are not necessarily indicative of viable bacteria (187). Thus, the culture and imunofluorescence methods should still be utilized when the number of viable legionellae is desired (97). PCR may also lose its sensitivity in the presence of contaminants in the samples tested. However, this method is amenable to slight modifications to enhance sensitivity (92).

Disinfection of Legionellae

Eradication of legionellae from water systems is essential for controlling the occurrence of Legionnaires' disease and Pontiac fever. This is particularly important in nosocomial settings where large outbreaks of Legionellae associated diseases can occur. Several methods of disinfection have been utilized with varying degrees of success (188). First, thermal eradication was the first method utilized for the eradication of legionellae. This method involves increasing hot water tank temperatures to 60 to 70 °C followed by flushing all water outlets including faucets and showerheads for a minimum of 30 minutes. A variation of this method uses instantaneous heating systems that heat water to temperatures greater than 88 °C followed by mixing with cold water before distribution to the rest of the system (188). Thermal eradication is very useful for rapid elimination of legionellae during an outbreak and does not require any special equipment. The major disadvantage of this method is that legionellae can recolonize the water system within weeks following disinfection. In addition, it is difficult to maintain an elevated temperature in the entire water system, which may allow legionellae to survive in locations that were not sufficiently heated. Second, another method for the elimination of legionellae is by introducing copper and silver ions in the water system. Copper and silver ions are bactericidal to legionellae (188). This method is effective in the elimination of legionellae (188) and recolonization is retarded possibly because of residual ions that remain in the water system following treatment (189). The disadvantages of this method are that the concentration of copper and silver ions required to eliminate contamination may be from levels that make water unsuitable for human consumption (190). In addition, legionellae may develop resistance to the biocidal effects of copper and silver (191). Third, hyperchlorination can be used to eradicate legionellae in water systems. This method involves increasing chlorine concentrations in a water system to approximately 50 ppm (188). Although chlorine can eliminate legionellae in suspension, legionellae residing within biofilms or within amoebae will be protected. Furthermore, areas of stagnation or low circulation in the water system will not be affected by hyperchlorination (188).

All the methods utilized for disinfection are incomplete and do not provide adequate eradication of legionellae and prevention of recolonization (188). A combination of these methods and continuous surveillance of water systems for the presence of species of *Legionella* is necessary (188). Alternative means of disinfection may be achieved by further understanding the microenvironment within which legionellae survive.

CONCLUSION

The ability of legionellae to survive within a wide range of environments is indicative of its remarkable evolution. Furthermore, a clear link exists between the relationship of legionellae with its environment and it pathogenicity to humans. This, in addition to the amenability of legionellae to experimental manipulation, provides a unique opportunity for the dissection of the evolutionary process that has taken legionellae from the environment through protozoa to human cells. Indeed, some pieces of the puzzle are beginning to come together. Within biofilms, legionellae reside in a multicellular environment reminiscent of its state during the infection process of humans. Similar principles that govern the interaction of legionellae with its surroundings in biofilms may exist during the infection process in humans.

Protozoa can be viewed as an evolutionary bridge linking legionellae from the environment to humans. The interaction of legionellae with protozoa is likely to have provided the necessary conditions for the evolution of mechanisms of invasion and intracellular survival. This in turn may have been essential for its ability to invade and survive within mammalian cells. Several molecular and phenotypic similarities exist between the infection process of protozoa and mammalian cells by legionellae. Furthermore, a relationship appears to exist between the virulence of different species of *Legionella* to mammalian cells and protozoa. This may be utilized as an indicator for the potential pathogenicity of environmental microbes to humans.

The existence of novel bacterial pathogens in the environment is almost certain. Such novel pathogens may yet have to evolve mechanisms of eukaryotic parasitism or are simply waiting for the appropriate conditions for transmission to humans and animals. Understanding the intricate interactions that occur between legionellae and its environment may be essential for predicting the appearance and combating new human pathogens.

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LEGUME INOCULATION. See NITROGEN FIXATION IN SOILS (SYMBIOTIC)

LEPTOSPIROSIS

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Leptospirosis is a zoonosis associated with a bacterium that is emitted in the urine of infected vertebrates. Leptospires may survive in environmental freshwater and

contaminated hosts either by mucosal ways or through cutaneous lesions.

At the beginning, leptospirosis was confused with other infectious diseases including hepatitis, malaria, and yellow fever. The first undoubtful descriptions were in 1886 from both a French:Mathieu (1) and German:Weil (2) physicians who independently published the association of the prominent symptoms and characteristics of this disease.

Leptospirosis is clinically quite polymorphic and diverse. A large diversity of the isolates corresponds to the polymorphism of the disease at different levels, namely, epidemiologically, antigenically, and genetically.

There are some correlations between a given infective serotype and the level of gravity of the generated disease. For instance, serovars icterohaemorrhagiae or australis often lead to serious disease. However, this association is very theoretical, and finally, any serotype may drive any clinical presentation.

The first observation of the bacterium is attributable to Stimson who, in 1907, described spirochetes in the kidneys from a patient in New Orleans suffering from yellow fever (3). Probably the diagnosis was wrong and the patient had in fact contracted leptospirosis.

The first isolations of leptospires have been published in 1916 by Inada and coworkers but had been performed some years earlier (4). These authors have cultured leptospires, showed that they were responsible for experimental disease and that rats were a prominent reservoir of this bacterium.

Soon after, in Europe, German researchers (5), and also English and French ones, identified leptospires in ill soldiers in trenches in the 1st World War front, usually in France or in Belgium. The first strain (RGA) isolated by Uhlenhuth, which for years has been the reference strain of the *Leptospira* genus, was isolated near Paris (5).

In 1918, Martin and Pettit (6) described the agglutination-lysis reaction, which after being renamed micro-agglutination Test (MAT) by Babudieri in 1961 (7), remains 80 years later, the most powerful serological test in leptospirosis. This test also allowed the description of new leptospiral serotypes. Indeed, when a leptospire was isolated from a patient whose serum did not react with the already known serotypes, it suggested the infecting serotype to be a new one.

The basic taxon of serotypes is the serovar. There are about 220 pathogenic serovars. The serovars are clustered in serogroups according to their serological affinities. There are 23 pathogenic serogroups. Arbitrary criteria allow us to define each of these taxa.

More recently, genomic and genetic methods have been applied to bacteria including leptospires. Therefore, besides the aforementioned serological classification, a DNA-based taxonomy that recognizes among leptospires several complexes of species has been developed.

BACTERIOLOGY

Taxonomy

Bacteria from *Leptospira* genus belong to the *Leptospiraceae* family, which together with the *Spirochaetaceae* family, is within the Spirochaetales order (8). This group of bacteria, initially defined on morphological characters (9), is equivalent to the Spirochetes phylum as defined by Woese (10) in the phylogenetic studies of *rrs* genes coding for 16S ribosomal ARN (11).

Two nonpathogenic genera with a single species each, namely, *Leptonema illini* and *Turneria parva*, in addition to *Leptospira*, are comprised in the *Leptospiraceae* family (12).

The taxonomy within genus *Leptospira* is confusing, because of the coexistence of two classifications that do not quite correlate but are both useful (13) — a traditional classification that delineates (on fastidious and hazardous criteria of culture inhibition) two species, *Leptospira biflexa* (saprophytic) and *L. interrogans* (pathogenic), but this character may be lost when subcultured and no satisfying animal model is available.

Within the species, the leptospires are identified as serovars on serological criteria. There are currently 225 serovars identified in *L. interrogans* (more than 300 are individualized) (14). The definition of the serovar is based on an arbitrary cutoff level of antibodies in a procedure of cross-adsorption using MAT test (15). The serovars are further clustered in serogroups according to their antigenic proximity. *Leptospira interrogans* comprises 23 serogroups (14,15).

This traditional classification is hampered by many disadvantages, such as it is time-consuming, costly, and difficult to interpret (15). Such an identification is restricted to reference laboratories that need several months to confirm the identification. The interpretation itself is partially subjective and requires a complete collection of the reference serovars and corresponding experimental immune sera (one rabbit at least per strain). It is, however, often used because it is closely related to the serology diagnosis method, the MAT, which, even now, is the method allowing most of the biological diagnosis of leptospirosis. Furthermore, several serovars are associated with epidemiologically (elective host) or clinically (a given symptomatology) relevant characters.

Leptospires as other bacteria have been characterized by whole DNA/DNA hybridization (16), resulting in seven potentially pathogenic species and an unknown number of nonpathogenic species (17). Phylogenetic analysis of sequences from *rrs* genes coding for 16S ribosomal product shows that three distinct clusters of *Leptospira* species are obvious (18,19).

The first cluster comprises three validly described species (*L. biflexa*, *L. meyeri*, *L. wolbachii*) and several genomic groups of saprophytic leptospires. The second cluster comprises the seven undoubtfully pathogenic species (*L. interrogans* sensu stricto, *L. kirschneri*, *L. borgpetersenii*, *L. weilii*, *L. alexanderi*, *L. noguchii*, and *L. santarosai*). Finally, the third cluster comprises two species (*L. inadai* and *L. fainei*) (19) and probably other genomic groups. This group is diversely considered as regards to its pathogenicity.

This modern and scientific taxonomy established by standardized and universal methods has not been easily accepted by leptospirologists. However, it is also based

on the 300 delineated serovars that were distributed in the corresponding species. It is also correlated with PCR-based identification methods (20,21). Finally, it also allowed a molecular support to the serovar concept. Currently, the serovars may be identified more quickly at lower cost and more accurately by objective methods, such as ribotyping (22), pulsed field gel electrophoresis (23,24), and arbitrarily-primed PCR (25), rather than by serological cross-adsorptions. These molecular methods allow the identification of species or serovars or both (26). Only the serogroup loses its distinction because serovars belonging to the same serogroups are dispersed among several species and a species comprises serovars from distinct serogroups.

Morphology (27)

The morphology of spirochetes is typical (9) that is thin (frequining dark-field or phase contrast microscopic observation), but long bacterium. They exhibit an intense motility with a combination of translation, contraction, torsion and screw like movements.

Spirochetes are helicoidal and motile bacteria with flagella localized between the outer and inner (cytoplasmic) membranes instead of being free outside the cell. Leptospires have only one flagellum per extremity that does not overlap at the center of the cell. The cell is flexible and its motility is quite specific of spirochetes. Leptospires are also characterized by their narrow diameter of 0.1 μm, which allows them to pass through 0.45- and even 0.22 μm filters, thus complicating filtration based decontamination methods.

Leptospires are approximately 10 to 15 μm in length and the spires are very tight. Often, leptospires present a hook at each extremity. The outer membrane is three-layered and characterized by its fluidity. The cytoplasmic inner membrane is also three-layered.

- The fragile outer membrane is made of proteins, lipids, and the nontoxic lipopolysaccharide (LPS). Some immunogenic proteins of this membrane have recently been characterized (28).
- Under the periplasmic compartment is a flexible cell wall constituted by the peptidoglycan that contains muramic acid and diaminopimelate as amino acid (29).
- The two flagella inserted in the peptidoglycan comprise an axis and elements that constitute the insertion in the cell wall. A *flaB* gene coding for a 32-kD protein has been cloned (30). It forms the core of the flagellum, although a *flaA* gene codes for the sheath.

Lipids are important in proportion (15 to 25%) and some are unusual. Their composition partially reflects the medium one. Polysaccharids are essential constituents, probably playing a role in immunity (LPS) and virulence (peptidoglycan) (27).

Genome

The genome of leptospires has been studied by pulsed field gel electrophoresis. It comprises a large (5,000 kb) chromosome and another smaller mini chromosome (31,32).

Three genetic maps have been drawn concerning three serovars belonging to *L. interrogans*. They show that

- Ribosomal genes are not organized in operons and are not in equal numbers (1 *rrf* coding for 5S RNA, 2 *rrs* coding for 16S RNA, and 2 *rrl* coding for 23S RNA) (32–34).
- Physical maps differ according to each serovar (32,33,35).
- Several insertion sequences (IS) are present. Their identities differ according to the species and their number according to the serovars (35–37).
- The order of genes are quite different even for serovars close to one another (32,33,35).

These points lead to the hypothesis that the formation of a new serovar is associated with a rearrangement of chromosome fragments from a previous serovar because of the mobility of IS: deletion, insertion, inversion. Only the combinations allowing the best adaptation to a given ecological context (host serovar association) should be maintained.

In saprophytic leptospires, a phage isolated from serovar patoc (38) allows to build a shuttle vector (39). It would therefore be possible to realize genetic transfer in vitro to study the actual role of candidate-virulence genes.

Finally, in Shangai, China, a team is sequencing the whole genome of *L. interrogans* serovar lai. The complete sequence will be published in 2001.

PHYSIOLOGY — METABOLISM

Culture (27)

Optimum growth of leptospires occurs at 30 °C; however, they have been observed to grow within the range of 13 to 37 °C. Saprophytic leptospires grow at 13 °C. Hypertonicity is an adverse condition for leptospires. In optimum conditions, the doubling time is six to eight hours and the maximum density is about 10^9/ml. Mobility of leptospires increases with the medium viscosity. Culture on solid media is very slow. Growth of pathogenic leptospires require approximately 20 days before colonies can be detected.

Energy and Respiration (27)

Leptospires are aerobic bacteria with both catalase and oxidase. Their carbon source are long-chain fatty acids (and not glucides) that are used by β-oxidation. In culture, free fatty acids are toxic for leptospires and must be supplied as tweens and detoxified by Bovine Serum Albumin. Ammonium salts must be supplied or are obtained by desamination of amino acids. Saprophytes synthesize both purines and pyrimidines and normally grow in the presence of a purine analog (8-azaguanine), which, in opposition, is inhibitor for pathogenic leptospires. Glycerol and pyruvate increase growth of leptospires.

Vitamins B1 and B12, and chlorides, phosphates, calcium, magnesium, and Fe^{+++} are needed. In practice, zinc, manganese, and sulfate also are added.

Resistance to Chemical and Physical Agents (27)

Leptospires are quite sensitive to desiccation and hypertonicity. If halophile leptospires do exist, pathogenic ones are grown in 1/10 NaCl conditions as compared to physiological values. Copper Sulfate at concentrations exceeding 10 mg/L has been shown to be especially toxic to pathogenic leptospires.

Leptospires can be maintained at $-70\,^{\circ}C$ ($-20\,^{\circ}C$ for histological pieces). In culture, they are killed above $42\,^{\circ}C$ and by pH below 6.8. However, they may survive alkalinization up to 7.8. They are killed by the usual disinfectants, detergents, and most antibiotics (with the exception of rifampicin, phosphomycin, neomycin, kanamycin, and sulfamides).

Antigenic Structure

The immunodominant antigen is closer to the surface; it is the LPS carrier of the serovar specificity. It elicits protective antibodies but their activity is restricted to a given serovar (40).

Another polysacharidic antigen, close to the surface, is what is called *antigen of group* (42). This antigen, abundant in patoc serovar, serologically cross-reacts with an equivalent found in most pathogenic serovars (41). Sometimes called *TR* for "thermoresistant," this antigen is used in several diagnosis tests (TR (42), ELISA (43), Dipstick (44), etc.). Many proteins are observed in SDS PAGE electrophoresis, but few of them have been characterized or even identified (46). Recently, however, several were described and studied. A heat shock protein, Gro EL (45), and, DnaK of 72 kD has been identified (46). The endoflagellum comprises several proteins of 34 to 36 kD, which exists in the core and sheath of the endoflagellum and other pieces of endoflagellum insertion (47). These antigens exhibit cross-reactions with their homologs from other bacteria.

Finally, genes coding for *Leptospira*-specific proteins such as LipL32, LipL36, and LipL41 were cloned, sequenced, and expressed (27,48). All are lipoproteins associated with the outer or inner membrane. LipL41 is a protective antigen, mainly if injected simultaneously to OmpL1 a porin (49).

EPIDEMIOLOGY

Geographic Distribution (27)

Leptospirosis is a zoonosis, the animal spectrum of which is very large. It is therefore spread all over the world with almost no exceptions, and is found even in deserts and arctic areas. In arctic areas, they have been found in foxes and seals, and in deserts, in kangaroo-rats and gerbils.

However, its frequency differs according to the climate. Incidence is prominent in equatorial and tropical regions and decreases from temperate to boreal climates.

Another heterogeneity is observed concerning geographic distribution of leptospiral species. Indeed, the serovars associated with animal species, such as icterohaemorrhagiae serovar in rats, canicola in dogs, or hardjo and some other ones in cattle, can be found worldwide. But it has been noticed that most of the *L. interrogans* sensu stricto serovars and all those from *L. weilii* and *L. alexanderi* have been isolated in Asia (17).

Similarly, *L. borgpetersenii* is endemic in both Europe and Asia and *L. kirschneri* in Africa and Europe. In the American continent, *L. santarosai* predominates in South and Central America, although *L. noguchii* is mainly found in North America (17).

The disease usually occurs as isolated cases or in a sporadic way, although outbreaks of grouped cases (called *anademia*) may happen after rainy periods. This is the case in rural areas (Guatemala, Nicaragua), and in urban areas (Rio de Janeiro, Sao Paulo).

Seasonal Distribution of Leptospirosis (27)

Human contact with contaminated surface water from infected animals is the primary cause of leptospirosis.

Leptospires may survive for long periods (several months in freshwaters if their pH is neutral or slightly alkaline and their temperature high enough). Leptospirosis is a prominent disease in hot and damp areas such as tropical areas.

Usually, in the Northern Hemisphere, a majority of leptospirosis cases occur during the second part of the year. In contrast, in the Southern Hemisphere, the first six months of the year exhibit a recrudescence of cases. The rhythmicity of natural phenomenon such as rain, or wild animal behaviors (births, searching for a sexual partner) and sociooccupational human activities (harvests, cattle breeding, water activities during the holidays) explains this seasonality. In the tropics, transmission of infection tends to be less seasonal and can occur throughout the year. Usually, in the rainy season, temperature increases. Under these conditions, humidity rises above 70 to 80%. The subsurface soil is fully saturated, allowing leptospires to survive particularly well, and most cases occur during the most humid part of the year. However, in the driest times of the year, leptospires may not be present in the same way, but cooler nights delay the drying out process. Additionally, dry periods often are the ones when rice is irrigated and sugarcane is harvested, and workers become infected at this time and not typically in the rainy season. However, from one year to the next, large fluctuations in leptospirosis incidence happen. Meteorologic phenomena and their consequences on animal populations (mainly rodents) play a major role in these fluctuations. Catastrophic events, such as hurricanes in tropical areas, may lead to large outbreaks with hundred of cases.

RISK FACTORS (27)

Recreational activities in freshwaters such as canoeing, windsurfing, rafting, swimming, and other related activities are important risk factors in human leptospirosis.

Other more traditional risk factors, such as fishing, and hunting, also play a determining role. Occupational leptospirosis is associated with many different professions such as breeding, agriculture, fish-farming, slaughtering, veterinary, people involved in sewage, road repairs, and also in food storage and maintenance or building activities, plumbing, etc. The combination of climatic features and exposure to risk exposition may lead to high frequency of the disease. For instance, in Asia, rice culture in damp conditions increases both rodent population and water transmission, which explains that, in spite of being underestimated, leptospirosis in some areas is actually a public health problem (50). In Vietnam, it has been shown that 20% of the population presents serological evidence of a past contact with leptospires, although 2.9% probably contracted the disease per year (51). Similarly, in New Caledonia, for instance, existence of extensive cattle breeding and tropical environment contributes to a high-endemicity rate of about one per thousand (52).

In addition, these incidence rates only represent the detected cases. In La Reunion, it has been shown that the actual prevalence of the human infection was about ten times higher than the diagnosed disease (53). Although the disease was prominent in men, because of occupational activities and peridomestic environment and association with serovar icterohaemorrhagiae and serovar canicola, the antibodies prevalence was higher in women (53). Usually, the reported cases show a male predominance (60 to 90%) and a mean age of 35 to 45.

In the United States, the number of cases detected per year is rather low as compared with European countries. From 1955 to 1996, the number of human cases has ranged from 50 to 100 every year with a maximum in 1965 of 142 cases (54). Icterohaemorrhagiae (35%) is the main serogroup, followed by canicola and autumnalis. Comparatively, from 250 to 400 cases are detected every year in continental France (55).

Finally, a high risk factor for people from developed countries (where leptospirosis is usually drastically decreasing) is traveling in tropical countries. Sport or exploration trips in tropical areas, mainly in southeastern countries, represent a major risk.

The following are some examples from cases with a common source to large outbreaks resembling epidemics:

- In France, in 1950, 77 soldiers contracted Grippotyphosa leptospirosis after swimming in the Loing River (56).
- In China, in 1958, an "epidemics" of anicteric leptospirosis occurred in Wenjiang District of Sichuan Province. A careful study was focused on 852 patients. It revealed that they were mainly rice growers. Respiratory symptoms were prominent; most of the 21 patients succumbed as a result of asphyxia associated with long hemorrhages. Most of the cases were due to serovar lai belonging to the Icterohaemorrhagiae serogroup (57).
- In Washington state, in 1965, 61 people contracted leptospirosis after swimming in a river. Antibodies against pomona and autumnalis were present only among swimmers.
- In Lisboa, in 1967, a flood occurred during the night of the 25th and 26th November 1967. Many patients were later on hospitalized with typical leptospirosis symptoms (58).
- In Italy, in 1984, 33 confirmed cases of leptospirosis were recorded in Pietra Cuta, a small town in Central Italy. An inquiry revealed that a water fountain was used for drinking by most of the patients. A dead hedgehog was found in a reservoir of the fountain (59).
- In 1995, after a flood in Nicaragua, an epidemic of "hemorragic fever," which turned out to be leptospirosis, broke out mainly because of the Canicola serogroup. In the studied area 2,259 nonmalarial febrile patients were recorded (60).
- In 1996, 9 out of 26 rafters in Costa Rica suffered from leptospirosis (61).
- In the United States, in 1998, 1,194 athletes who attended one or both triathlons in Illinois and Wisconsin were under surveillance; 84 (11%) of the participants in Illinois, 20 (5%) of those in Wisconsin, and 6 (7%) of those having participated in both the events became ill with leptospirosis (62).
- In Brazil, Sao Paulo, Rio de Janeiro, and probably in other Brazilian cities, after rainfall large urban outbreaks of leptospirosis occur involving hundreds of cases with a fatality rate of 5 to 15%. Icterohaemorrhagiae serogroup is largely prominent and Canicola is also observed (63).

TRANSMISSION MECHANISMS (27)

Origins of leptospirosis transmission include contact with infected urine, contaminated freshwater and mud by infected urine or animal tissues. Pathogenic leptospires may survive several months in water, at neutral or slightly alkaline pH. Similarly, herbivorous or omnivorous animals may remain infective for years by the presence of leptospirosis in their urine. Penetration of leptospires in the host organism takes place through intact mucosa such as conjunctiva, nasopharyngeal or pulmonary mucosa in case of water inhalation. Alternatively, skin erosions or excoriations, even minute ones, represent means of access for leptospires. In several animal species, mainly domestic ones such as cattle, a congenital transmission occurs. More rarely (in pigs), venereal transmission may happen.

Interhuman transmission is exceptional, such that leptospirosis does not give birth to epidemics but rather to sporadic or clustered cases. Interhuman direct transmission cases that have been published consist of urinary, sexual, transplacentary, or even milk suckling cases.

Host Reservoirs (27)

The primary reservoir of pathogenic leptospires are animals but is extended to the environment.

The infected animals include both domestic and wild animals. Their bodies or remains may also be infective

during a limited extent of time. Infected animals excrete in their urine huge quantities of leptospires for extensive periods (years for rodents). Leptospires are able to survive for weeks or months in freshwater or muddy soil mainly when protected from light (mines, sewers, and so on.).

The contamination risk is high in damp areas where animal urine frequently contaminates the soil or surface water. Indirect transmission (by soil or water) is the preeminent way (rather than urine) for human contamination. Animal spectrum of leptospires is an extensive one but the epidemiological importance of a given species depends on several factors such as:

- receptivity of the species,
- density of animals,
- density of human population,
- way of life of both animal species and humans (occupational and/or recreational practices of humans and practices of animals).

Sometimes, a wild species has few opportunities for contact with humans but domestic animals may be used as an efficient intermediaries. Among wild mammals, rodents (rats, mice, voles, muskrats, etc.), insectivores (hedgehogs, shrews, etc.), marsupials (opossums bandicoots, spiny rats, etc.), and also bats, cervidae, and carnivores, are usual reservoirs. Rodents and insectivores are undoubtedly the natural reservoir hosts of many leptospiral serovars, particularly those in serogroups Icterohaemorrhagiae, Ballum, Grippotyphosa, and Hebdomadis.

Territory-marking carnivores are probably the second most efficient in transmission in mammals. When these wild animals are in close proximity to humans and their domestic animals, the extent of transmission depends on the intensity of farming and animal husbandry. Intraspecific transmission within livestock and dog population follows, and there can be cycles of both intra- and interspecific transmission with occasional shifts taking place as new serovars are introduced into domestic populations.

Unusual hosts, such as birds or lizards, have been recorded (64). A particular case is the one of batrachians, in the Caribbean (Trinidad, Grenada, and Barbados) in which both toads (*Bufo marinus*) and frogs harbor in their kidneys several serovars belonging mainly to Australis and Autumnalis serogroups that can also infect humans (65). These animals could be the primary hosts for endemic serovars (bim in Autumnalis serogroup (66) and bajan in Australis serogroup) (65).

Among domestic species, dogs, and also cattle, horses, and pigs play a major role.

Dogs. Dogs generally live in and around man's dwelling places, and therefore their ability to carry and transmit leptospirosis is of major public health significance. The risk of human infection from dogs is particularly important in developing countries or subtropical environments where the animals are frequently not restrained. Usually, canicola and icterohaemorrhagiae (both serovars constitute the dog vaccine valences) are the most common serovars prevalent among dogs. However, in tropical areas, the spectrum may be more diverse. Similarly, in developed countries where vaccination of dogs is common, it seems that new serovars could become infective for vaccinated dogs.

Livestock (27)

Cattle. In North America, Australia, New Zealand, and a part of Western Europe, as in many other parts of the world, interest in bovine leptospirosis is focused on serovar hardjo. It plays a major role in abortion and is harbored in the genitals and urinary tracts of cows.

Pigs. Just as serovars sejroe-hebdomadis (mainly hardjo) are particularly adapted to cattle, pomona and tarassovi serovars have a well-known association with pigs and can cause severe economical loss.

Sheep and Goats. Leptospirosis has been reported in sheep and goats from most parts of the world. Commonly reported serovars are pomona, grippotyphosa, and hardjo.

Horses and Other Equines. Interest in equine leptospirosis has developed from two main standpoints, namely, uveitis/periodic ophthalmia and abortion. Equine abortion, stillbirths, and premature live births have been diagnosed serologically, clinically, and by circumstantial evidence. Serogroups *Australis*, *Pomona*, *Hebdomadis*, and *Icterohaemorrhagiae* were involved.

VIRULENCE AND PATHOGENESIS (27)

When studying pathogenesis and virulence of *Leptospira*, the difficulty in quantifying the virulence and maintaining it stable along subcultures is a primary limitation. Such a maintenance is usually done by animal passages (guinea pigs under 150 g or hamsters under 70 g) sensitive to a lethal infection by Icterohaemorrhagiae serogroup. Other serogroups, sometimes lethal for humans (Canicola, Australis, Bataviae, Pomona, and so on), may be studied on either hamsters or *Meriones unguiculeatus*. However, both the infection (inoculation) and doses are far from those occurring in nature. In fact, the Saïmiri monkey (*Saïmiri sciureus*), naturally infected by Icterohaemorrhagiae serogroup, presents a severe disease resembling the human one and could constitute a satisfying animal model (67,68).

The first stage of the infection is the transcutaneous passage of leptospires, which remains unknown at the molecular or cellular level. Some authors consider that leptospires are able to cross healthy skin, although a cutaneous discontinuity probably facilitates the penetration (69). Mucosa surfaces are, however, considered to be usually penetrated by leptospires. Virulent leptospires escape phagocytosis and invade blood, lymphatic vessels, and then mainly the host tissues where they multiply with a generation time of about eight hours (70,71).

In BalbC mice (nonlethal model infection), specific antibodies are detected in the second day (72) (the eighth in humans) (70). This humoral immunity is transferable

to another mouse by transfer of either serum or splenic lymphocytes (73). These protective antibodies are opsonic and facilitate phagocytosis by macrophages and by neutrophiles (74). Avirulent leptospires also are killed by the complementary/antibodies system in opposition to virulent ones (75). The efficiency of human antibodies to clean leptospires is responsible for leptospirosis to constitute in humans an acute disease (76). However, in some occasions, the persistence of leptospires has been demonstrated (77–79).

At the cellular level, the mechanisms of interactions between leptospires and host cells have been first studied in the 80s using endothelial or epithelial cells. Adhesion of virulent leptospires (and not saprophytic ones) to host cells has been noticed (80). Extracellular matrix and mainly fibronectin has been shown to play a major role in adhesion (80,81). Recently, a leptospiral protein of 36 kD has been shown to be responsible for interaction with the gelatin domain of fibronectin, which could be the first step in adhesion phenomenon (82).

Virulent leptospires also induce apoptosis of macrophages, which could help them to escape to professional phagocytes at the initial step of the infection (82). In vivo (guinea pigs) apoptosis has also been evidenced in hepatocytes but not in other tissues (84). Other approaches have been directed toward identification of toxins. Preliminary experiments were not highly demonstrative and have not been reproduced (85).

The presence of several genes coding a phospholipase C (sphingomyelinase) (activity responsible for hemolysis), is a potential virulence factor (85). These genes have been cloned, sequenced, and expressed in *Escherichia coli* (86,87). They are present in the pathogenic leptospires but neither in saprophytic ones nor in *L. inadai*.

THE DISEASE

Human Leptospirosis (88–90)

Leptospirosis is a highly polysystemic and polymorphic disease. It comprises several syndromes that are diversely associated:

- Infectious syndrome, which is constant. It is characterized by a high fever reaching 40 °C in a few hours. Typically, the fever is accompanied by vasodilation of skin and mucosa. There is sometimes a brief relapse after 10 to 12 days. When untreated, the fever remains high for 7 to 10 days, and then decreases.
- Algic syndrome, which is quite frequent and characterized by myalgia, arthralgia. The intensity of pain may be severe (90,91).
- Meningeal syndrome characterized by neck stiffness, cephalalgias, and vomiting. Photophobia, hyperesthesia, and obnubilation may also be present. The CSF shows elevation of albumin and hypercytosis (89,90).
- Renal syndrome characterized by nephritis with hematury, albuminury, hyperazotemia, and oligury or anury. The duration of oligoanury may lead to renal failure and require dialysis (89,90).
- Hepatic syndrome, either clinical with icterus on skin and mucosa or subclinical. The association of icterus and vasodilation leads to a particular orange colour of the skin. Biologically, bilirubin is increased, although transaminases are subnormal (89,90).
- Hemorrhagic syndrome, which may be present on different organs such as purpura, epistaxis, digestive hemorrhages, hemoptisis, hematury and so on (92). Hemorrhages are in fact infrequent but thrombocytopenia is usual (89,90).

Other more rare symptoms may be present such as digestive pains, pulmonary syndrome (93), cutaneous rash at the end of the fever period, conjunctivitis, and infrequently cardiovascular syndrome with low blood pressure and trouble of the cardiac rhythm, which may lead to this situation.

Rarely, the disease begins with an encephalitic syndrome with coma and polysystemic failure, which is quickly lethal (94).

Evolution (89,90). At the beginning, only infectious syndromes, which are algic, and (sometimes) meningeal, are present.

After four to five days the signs of distinct organic dysfunctions appear in the kidney, and liver. They persist for five to seven days and decrease simultaneously with fever. Later on, after 12 to 15 days, a fever relapse may occur, usually isolated, but sometimes accompanied by algia or even icterus, oligury, and so on. Convalescence is long; the patient recovers slowly but usually without sequelae. Sometimes, several weeks later, ocular lesions such as uveitis or iridocyclitis may occur. All serovars from the seven pathogenic species, namely *L. borgpetersenii*, *L. interrogans*, *L. kirschneri*, *L. noguchii*, *L. santarosai*, *L. weilii*, and *L. alexanderi* are able to infect humans.

Although some serovars are known to lead to a particular clinical expression, this relationship is not absolute. The severity of the human disease also depends on the inoculum and the sensitivity of a given patient. From the influenza-like disease (grippotyphosa serovar) to the lethal multivisceral form with hemorrhagic suffusions (icterohaemorrhagiae serovar), there are many different clinical expressions. The death rate varies from 1 to 2% in developed countries and from 20 to 30% in developing countries without intensive care units. The causes of death may be encephalitis, renal failure, respiratory failure, or cardiac failure. The only sequelae that may happen are ocular such as uveitis, iridocyclitis, keratitis, and hemorrhagic retinitis, which, in rare occasions, leads to cecity (88,89).

Diagnosis

Nonspecific Biological Signs (15,27). A hyperleukocytosis with polymorphonuclear cells is usual (sometimes lymphocytosis) but not systematic. At the beginning of the disease, during the preserological phase, the following biological signs are of value and the association of two or more of them is quite significant:

- Thrombocytopenia ($<1, 50000/mm^3$)
- Increase of creatinin phosphokinase (CPK >130 UI/1) with transaminases level normal or moderately increased.
- Triglycerides elevated (>1.70 g/L) (95).
- Decrease of HDL cholesterol (<0.85 mmol/L) (95).

Specific Biological Signs (15,27). Hemoculture must be done during the first 10 days (day 0 is marked with fever onset) before antibiotherapy. Leptospires may be isolated from the CSF during the second week and from urine later on.

Direct examination. It has no value.

Culture. Current cultural methods use the medium of Ellinghausen, McCullough, modified by Johnson and Harris (EMJH), also called *tween albumin medium* (96,97). It has been commercialized and may become selective by addition of 5-Fluorouracile (98) or other antibiotics such as Fosfomycin, Rifampicin, Inoculation must be done as quickly as possible. The inoculum should represent 10% of the medium volume.

Hemoculture. It is necessary to perform several hemocultures (four to five dilutions at 10%) to decrease a possible inhibitory effect of the blood.

Urine. Filtration through 0.45 μm and 0.22 μm filters could decrease the risk of contaminating the medium by usual bacteria. Dilutions as for blood are useful and a selective medium is to be preferred.

Tubes are incubated at 30° and examined each week. A systematic subculture after 15 days is favorable. Tubes must be kept two months before concluding they are negative.

Identification (15,27). Morphological characters (dimension, terminal hooks, etc.), motility and growth on EMJH allow to confirm *Leptospira* genus. Further identification at the species level is obtained by molecular methods. The serogrouping is performed with 23 rabbit-immune sera. Finally, the serovar identification is obtained either by pulsed field gel electrophoresis or in reference centers by a complex procedure of cross-adsorption of rabbit-immune sera by reference strains.

Serological diagnosis (15,27). Many tests have been used in the past, such as complement fixation test (CFT), MAT on slides.

At present, in addition to the MAT, which is the confirmative test, the main presumptive tests still in use are ELISA (99), Dipstick (100), hemagglutination, or latex test. They are all more or less based on the same antigenic compound, that is, a secreted nonproteic determinant (thermoresistant) that is produced by most pathogenic leptospires, and also by a saprophytic serovar, Patoc strain.

The ELISA test may be revealed by anti-IgM antibodies. In these conditions, it becomes positive earlier than MAT (sixth to eighth day) and is very sensitive and specific. However, there are some caveats in the spectrum of serogroups this test is able to detect.

The MAT (15) or confirmative test consists of evaluating with the dark-field microscope the agglutination rate of suspensions of different leptospires by the serum of the patient. The panel of leptospires is representative of the main serogroups. For a reference laboratory, this panel comprises about 20 strains. The MAT becomes positive from the 8th to 10th day of the disease. Antibodies decrease from the third to sixth month but may persist at low titers for years. Therefore, it is necessary to get several samples to assess kinetics.

PCR diagnosis (27,78,79,101). Both delay and low sensitivity in culture diagnosis, and late appearance of specific antibodies, explain the reason for leptospirosis being more effectively directed by PCR-based technology.

It allows to detect about 10 leptospires. Pathogenic leptospires may be detected by PCR within 48 hours. Any tissue, including blood, urine, CSF, aqueous ocular humor, allows a quick detection of leptospires. The sooner the sampling is done, the more the PCR is positive.

In conclusion, PCR, culture, and serology are not alternative methods but should be considered as distinct and complementary methods, which must be used sequentially to allow an optimum diagnosis.

Leptospirosis in Animals (15,27)

In Dogs. Three main clinical expressions are observed:
- Febrile gastroenteritis or typhic disease.
- Nephritic leptospirosis with high fever.
- Icteric leptospirosis.

In Horses. Many different either acute or chronic clinical forms are observed:

- Hepatonephritis
- Encephalitis
- Genital: abortion
- Iridocyclitis, with relapses after long intervals

In Cattle. Again, symptoms are quite polymorphic:

- Fever, weight loss, lameness
- Orange color of the skin and mucosa
- Bloody and dark urine
- Photosensibilization (due to hepatic failure)
- Reddish colour of the milk

Usually, it occurs as a mild, slightly febrile illness with inappetence, drop in milk yield, and sometimes symptoms of mastitis (with a thicker and yellower milk tinged with bloody flecks). Abortion may take place several weeks later, most commonly around the seventh month of pregnancy.

CONTROL AND TREATMENT OF LEPTOSPIROSIS (15,27)

Prevention

Leptospires survive in freshwater or mud (102). The control of leptospirosis may depend on many different available measures, only a few of which are relevant to a given epidemiological situation. The measures enumerated later represent a large panel of possibilities. Among them, only the most specific ones, according to the locally dominant transmission route, would be efficient.

A careful study of the epidemiological conditions is required:

- to evaluate the actual prevalence frequently underestimated because of clinical polymorphism,
- to determine the predominant transmission route,
- to define the most cost-effective control measures to be implemented.

Control of Infection in Man

Rodent Control.

1. In food production or storage areas omnivorous anthropophile rodents that live in a wide range of habitats such as *Rattus* sp. and *Mus musculus*, are prevalent. This control is related to economical aspects (spoiling prevention) and other public health interests.

a. The best and safest way is preventing the entry of rats in building by reinforced doors that are kept closed, resistant ducts, and pipes.

Basic hygiene procedures are also recommended, for instance, resistant storage containers or food packaging, cleanliness of areas, food being inaccessible.

b. Rodent poisoning is difficult to employ. Rodenticides are usable but present environmental problems and human danger. In dry environment, water poisoning (anticoagulants with delayed activity) is efficient. Fumigation with carbon dioxide presents less hazards but requires air-proof premises and specialized equipment. Biological weapons, such as bacteria lethal for rats (*Salmonella typhi murium*, *Pasteurella multocida*, etc.), have been proposed and used but are dangerous for many species including man.

c. Finally, trapping is the safest method.

2. Rodent control in domestic environment Methods are similar but difficult to apply and more hazardous. Trapping is again the best compromise between efficiency and safety.

Control of Exposure in a Potentially Contaminated Environment. This requires draining for safety.

Occupational Hygiene

1. To avoid penetration of leptospires through skin and mucous membranes (conjunctiva), two measures must be used simultaneously:
   - Reducing the number of leptospires in the environment. Humid premises must be drained and ventilated.
   - Protecting the skin with the help of protective clothing, waterproof footwear, gloves, and even spectacles in highly infective conditions. Such methods are difficult to impose in warm and humid areas.

Wounds must be immediately treated and covered. Smoking, drinking, and eating must be prohibited during exposed work and in risk areas.

2. Immunization In high-risk occupational situations (sewer workers, garbage workers), vaccination could be useful, because usually a single serogroup (Icterohaemorrhagiae) is responsible.

In France, for instance, such a monovalent vaccine is recommended for sewage workers. In Paris, before vaccination of 600 to 700 people involved in sewage plants from 1951 to 1973, 29 leptospirosis cases with 3 deaths have been recorded (incidence: 180/100,000 inhabitants). From 1974 to 1981, during vaccine trials and implementation methods, seven cases (no death) occurred (rate: 40/100,000). From 1981 to 2000 (generalized vaccination), two cases due to Grippotyphosa and probably acquired elsewhere than in sewage plants, occurred (103).

A monovalent vaccine (serovar lai) is also used in China among farmers working in irrigated areas. Multivalent vaccines have been tried among rice farmers in Italy, in Spain, in Japan, and in Korea, but are not used any more. In Russia, a quadrivalent vaccine is still commercialized for people involved in agricultural and breeding activities. A human vaccine in leptospirosis is hampered by the necessity of frequent injections and mainly the narrow spectrum of immunity in terms of serogroups. Therefore, its indications are very exceptional and must be restricted to a long-lasting, high, and exposure to a unique serogroup. In case of temporary, high-risk exposure, chemoprophylaxis with doxycyclin, 200 mg taken once or twice a week, could be efficient (104).

Control of Infection in Animals: Herd Management

Rodent Control in Livestock Environment. Radient control in livestock environment is similar to that in food production areas with a supplementary dimension, which is reduction of field mice population.

Prophylaxis and Chemotherapy for Domestic Animals. The disease is eliminated by antibiotics, mainly penicillin, streptomycin, or tetracyclin. The latter two could be used as chemoprophylaxis of the carrier state. But the consequences of drug resistance on usual bacteria must be evaluated. Such practices are now discouraged.

Immunization of Domestic Animals

- Dogs should be vaccinated (usually they carry mainly canicola or icterohaemorrhagiae serovars).

- Immunization of pigs and cattle is a controversial procedure.

However, it has been practiced successfully, for instance, with pomona among cattle in the United States and a trivalent vaccine in New Zealand where it has been reported that vaccination of cattle drastically decreased infections not only in cattle but also among humans involved in breeding activities.

Isolation of Infected or Potentially Infected Animals. Newly introduced animals must be isolated. Possibly infected animals, serologically detected, must be separated from healthy ones and treated. Floors, soils, and tools must be disinfected.

Contaminated Products from Infected Domestic Animals. Animals slaughtered for food should be checked. Infected animals must be eliminated. Aborted fetuses and membranes must be destroyed. Milk is usually safe on account of its leptospiricidal action. Urine must be safely drained; waterproof floors and distant draining of effluents must be introduced. Storage areas of effluents must be inaccessible to humans and animals and treated preferentially by physical or biological procedures.

Wildlife. Ecological control of feral population is an unsolvable problem. Natural predator populations are usually decreasing; they are either actively destroyed because they are considered as noxious or intoxicated by pesticides (predatory birds).

General Control Measures in Agriculture and Farming

1. Some high-risk culture practices present unsolvable difficulties. The risk factors in particularly exposed occupations, such as sugarcane cutting (warm climate, few clothes and protection lead to many skin abrasions by leaves) may be improved, not only by mechanization, but also by prior cane burning.
2. Wet farming presents a dispersion of the risk at many steps. When possible, mainly in temperate climates, dry farming should be preferred. Animals are stabled indoors and potentially infecting contacts are reduced.

Finally, to prevent this zoonosis, the roles of occupational medicine and veterinary medicine, which can promote hygiene education, are of major importance.

Treatment (27,88,90,105)

- Sensitivity to antibiotics

Like other bacteria growing slowly on highly specific media, there are no standardized methods to study the in vitro sensitivity. Several studies have been done in quite different conditions. As other spirochetes, leptospires are resistant to sulfamides, kanamycin, neomycin, rifampicin and metronidazole. Some of these drugs are used in selective media to isolate leptospires. Other antibiotics are usually active. The concentration minimal inhibitors are, for β-lactamin, ampicillin (0.025 mg/L), cefotaxime (0.05 mg/L), ceftizoxine (0.05 mg/L), moxalactam (0.2 mg/L), and penicillin (0.39 mg/L). Tetracyclin (3.13 mg/L), and ciprofloxazin (0.2 mg/L), are less active.

An experimental study on hamsters allowed to distinguish two groups of antibiotics:

- Those curing the disease but not the urinary carrier state, such as piperacilline, doxycyclin chlortetracyclin.
- Those that quite eradicate leptospires, such as ampicillin, bacampicillin, mezlocilline, cefotaxine, and mexalactam.

In man, it has been considered that antibiotics were less efficient if administered four to five days after the beginning of the disease.

It has been shown recently by randomized studies that doxycyclin (200 mg/day for one week) (106) and penicillin (6 M intravenous/day for one week) (107) were both active on the duration of the symptoms and leptospiruria, even when initiated lately. Penicillin seemed to be more active than doxycyclin.

In conclusion, as a zoonosis, leptospirosis cannot be eradicated. But the incidence of the disease can be decreased by prophylactic measures. Its severity is decreased by early treatment and when needed hospitalization in intensive care unit.

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LIPID BIOMARKERS IN ENVIRONMENTAL MICROBIOLOGY

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Microbial communities in natural environments are complex assemblages of microorganisms composed of a variety of different species of bacteria, archaea, and microeukaryotes including fungi, algae, and protozoa. Adding to this diversity is a large temporal and spatial variation in growth and activity, that is, at a given time and place only a fraction of the microbial community might be metabolically active. It is well accepted among microbial ecologists that the classical methods including isolation and culturing of microorganisms are not suitable for analyzing the diversity and activity of such communities. In most environments, less than 1% of the total number of microorganisms can be cultured. The introduction of various molecular methods have opened up new possibilities to assay the diversity and activity of microbial communities including the large fraction of the unculturable microorganisms. With the new methods, the microbial communities are assayed by extracting and analyzing specific components of the microbial cells, without the need for isolating and culturing the microorganisms. Depending on the components analyzed, they can be used as biomarkers for biomass, species diversity, and activity of microbial communities. An important group of biomarkers that have been widely used during the last few years are nucleic acids, particularly ribosomal RNA (rRNA). Analyses of rRNA has made it possible to analyze the phylogenetic and taxonomic diversity of microbial communities in natural environments (1). However, rRNA analyses provide very limited information on the phenotype and the activity of the microorganisms in the environment. Such information can be obtained by analyzing lipid biomarkers, including membrane and storage lipids of microorganisms. The use of lipids as biomarkers for microorganisms in environmental microbiology was introduced by David C. White and coworkers in the late 70s and has then been used in numerous studies and applications (2). The objective of this article is to give an overview of the lipid biomarkers used to characterize microbial communities, present the used procedures, discuss the different types of information that can be obtained, and provide some examples from environmental studies.

THE DIVERSITY OF MICROBIAL LIPIDS

The term *lipid* covers an extremely diverse range of molecular species; thus, unlike proteins, nucleic acids, and carbohydrates, it is not possible to provide a single chemical definition. Usually, lipids are defined as substances that are sparingly soluble in water but readily soluble in organic solvents such as chloroform. Lipids may be very simple molecules like fatty acids and hydrocarbons, but most microbial lipids are complex molecules containing sugars, amino acids, and phospho- or sulphogroups. A number of different systems for classifying lipids have been published, but a practical way of classifying, which reflects the procedures used for isolating lipids from environmental samples, is the division into neutral lipids (NL), glycolipids (GL), and polar lipids (PL) (Table 1; Fig. 1). A certain group of polar lipids are the lipoconjugates (LC), in which the hydrophilic part of the molecule has the dominant influence on soluble characteristics (3).

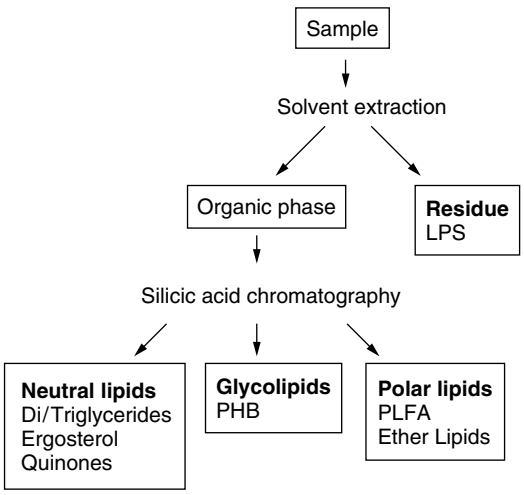

Figure 1. Scheme for analyzing lipid biomarkers in environmental samples.

Table 1. Examples of Lipid Biomarkers used in Environmental Microbiology

Lipid	Lipid Class[a]	Cellular Function	Taxonomic Distribution	References
Triglycerides	NL	Storage material	Eukaryotes	4,5
Ergosterol	NL	Membranes	Fungi	6–8
Respiratory quinones	NL	Respiratory chain/ electron transport	All cells	9,10
Hopanoids	NL	Membrane	Prokaryotes	11
Poly-β-hydroxybutyrate (PHB)	GL	Storage material	Prokaryotes	12–14
Phospholipids	PL	Membranes	All cells	15–17
Phospholipid fatty acids (PLFA)	PL	Membranes		18–20
18 : 2ω6[b]			Fungi	21,22
16 : 1ω5			AM fungi	5,23
i15 : 0, i15 : 0, i16 : 0, i17 : 0, a17 : 0			Gram-positive bacteria	19,20
10Me18 : 0			Actinomycetes	24
10Me16 : 0			*Desulfobacter*	25
15 : 1ω6, 17 : 1ω6			*Desulfobulbos*	26
i17 : 1ω7c			*Desulfovibrio*,	27
16 : 1ω8c, 16 : 1ω6c			Type I methanotrophs	28,29
18 : 1ω8			Type II methanotrophs	28,29
Phytanyl-ether lipids	PL	Membranes	*Archaea*	30,31
Lipopolysaccharides (LPS)	LC	Cell envelope	Gram-negative bacteria	32,33

[a]NL, neutral lipids; GL, glycolipids; PL, polar lipids; LC, lipoconjugate.
[b]Fatty acids are designated as total number of carbon atoms: number of double bond closest to the aliphatic end (ω) of the molecule is indicated with the geometry "*c*" for *cis* and "*t*" for *trans*. The prefixes "i" and "a" refer to iso- and anteiso branching. Other methyl-branching is indicated from the carboxylic acid end. Cyclopropal fatty acids are designated using the prefix "cy."

The diversity in the structure of microbial lipids is also reflected in the diverse cellular function of lipids. some lipids such as the triglycerides (triacylglycerols) present in eukaryotic cells and poly-β-hydroxybutyrate (PHB) found in certain prokaryotes are storage materials. A number of lipids are responsible for the structure of membranes including the phospholipids and sterols. Other lipids, such as the lipopolysaccharides (LPS) in gram-negative bacteria, participate in the organization of the cell envelope. Other lipids which are electron carriers in the respiratory chains and electron transport systems, such as the terpenoid quinones, have more special functions.

Some lipids are found in all microorganisms, such as the membrane phospholipids. Other lipids are associated with certain taxonomic groups of microorganisms (Table 1). Thus, lipids can be used as biomarkers or "signatures" for a specific group of microorganisms. More detailed information on the taxonomic distribution of microbial lipids that can provide support for the designation of specific biomarkers are found in the books by Ratledge and Wilkinson (34,35).

LIPIDS AS BIOMARKERS

There are four sorts of information that can be obtained from the analysis of lipid biomarkers in environmental samples: (*1*) microbial biomass, (*2*) community composition, (*3*) nutritional status, and (*4*) metabolic activity.

Biomass

When determining biomass by measuring the concentration of a cellular component, several requirements have to be considered: (*1*) the measured component must only occur in living microorganisms and not exist in dead cells and (*2*) the component should exist in fairly uniform concentrations in the cell (36). In environmental microbiology, particularly membrane lipids, such as phospholipids and ergosterol, have been used as markers for biomass. Phospholipids are found in the membranes of all living cells, they are not found in storage polymers, they have a relatively rapid turnover rate in aquatic sediments (15,37), and they are degraded when added to soils (38); thus, assays of phospholipids give a measure of the viable cellular biomass. The phospholipid content of microorganisms varies to some extent among different species and in response to growth conditions, which can make it difficult to convert the values of phospholipid content to cell numbers (19). However, several studies (17,33,39) have reported a good correlation between the total amount of phospholipids and the microbial biomass determined by other methods such as ATP measurements and microscopic counts.

Ergosterol is the predominant sterol in most fungi (40). It can be used as a biomarker for living fungi because it is rapidly degraded in the environment (41). Like phospholipids, the ergosterol content in fungi varies depending on species and growth conditions, and a number of different conversion factors relating ergosterol concentrations

to fungal biomass have been presented (7,8,42). Linoleic acid (18 : 2ω6) has also been proposed as a signature for fungi (21), and the soil concentration of this PLFA correlated well with the concentration of ergosterol (22). However, 18 : 2ω6 can be present in large concentrations not only in fungi but also in plants (43). Thus, 18 : 2ω6 is a good indicator for fungal biomass when plant cells are not present in the system.

Community Composition

As pointed out earlier, many lipids are associated with specific taxonomic or functional groups of microorganisms and can thus provide insights into the types of microorganisms that are present in an environmental sample. Particularly, analyses of the ester-linked fatty acids in phospholipids (PLFA) have been shown to give insights to the structure of microbial communities (18–20) (Table 1). The use of an individual PLFA as a biomarker for a specific group of microorganisms assumes in the best of cases that all members of that group contain that lipid and members of other groups of organisms do not. For example, the fatty acid 16 : 1ω5 has been used as a signature for arbuscular mycorrhizal fungi (5,23). Terminally branched fatty acids (*i* and *a*) are largely found in gram-positive bacteria, whereas cyclopropyl fatty acids are common in some gram-negative bacteria (34,35). Methyl-branching on the tenth carbon atom (such as 10Me 18 : 0) is specific for actinomycetes (24). Signature PLFA have also been identified for the sulfate-reducing bacteria (25–27) and methanogens (28,29). More extensive lists of signature PLFAs used in environmental microbiology are found in refs. (19,20).

A limitation of the PLFA method is that the occurrence of individual markers can only be derived from the analysis of isolated pure cultures. Furthermore, in most cases, there are extensive overlaps in the patterns of PLFA present in various groups of microorganisms and it is not possible to identify unique or signature PLFA. Another problem associated with the PLFA analysis is that the composition of PLFA can to a great extent depend on the growth conditions of the microorganisms. Therefore, many investigators use the "complete" PLFA profile (typically including 50 to 100 fatty acids) as a "fingerprint" of the microbial community. Using this approach, multivariate statistics such as principal component analysis (PCA) is used to simplify data and reveal patterns (44,45). For example, fingerprinted data can be used to determine the PLFAs that are most strongly correlated with an environmental variable. If the influential lipids are taxonomic biomarkers or physiological indicators (illustrated later), these changes can be interpreted as taxonomic or physiological variations.

The community composition can be further characterized by analyzing groups of lipids other than the PLFAs (Table 1). For example, methylhopanoids can be used as biomarkers for cyanobacteria (11). The composition of respiratory quinones, including the naphtoquinones and benzoquinones, vary among different groups of microorganisms and have been used as biomarkers for bacteria in environmental samples including aquatic systems, soil, and composts (9,10). Gram-negative bacteria contain unique hydroxy fatty acids in the lipid portion (lipid A) of the lipopolysaccharide (LPS) in the cell wall (46). Particularly β-OH fatty acids have been used as indicators for gram-negative bacteria in environmental samples (32,33). *Archaea* are characterized by their unique biphytanyl (archaeol) and di-biphytanyl ether lipids that are not found in other organisms (47). These lipids have been used as biomarkers for *Archaea* in sediments, hot spring mats, and soils (30,31,48,49).

Nutritional/Physiological Status

The composition and content of cellular lipids reflects the growth conditions and physiological status of an organism. Many microorganisms accumulate lipid storage polymers during conditions of unbalanced growth when sufficient levels of carbon and energy resources are present but some essential nutrient (e.g., nitrogen, phosphorus) needed for growth and division is lacking (12,50). The bacterial PHB [or PHA, poly-β-hydroxyalkanoic acid, ref. (12)] and eukaryotic triglycerides are endogenous storage polymers (Table 1), and the relative amounts of these compounds required to biomass can be used as a measure of the nutritional status of a microbial community (12–14). The diglyceride fatty acids (DGFA) are degradation products of phospholipids and have been used as indicators of dead cells (51,52). Specific patterns of PLFA can also indicate physiological stress. Exposure to toxic environments can induce the conversion of *cis* to *trans* PLFA (53), and some bacteria form a larger proportion of cyclopropane PLFA relative to their precursor (monoenoic) fatty acid in response to nutrient depletion or stress (54).

Because of a large interspecific variation in the level and metabolism of various lipids, there are potential problems in interpreting changes in patterns of PLFA to changes in nutritional and physiological status in complex assemblages of microorganisms. For example, PHB production varies widely among different species and some appear to be incapable of producing it. Likewise, the ratio of trans/cis fatty acids and cyclopropyl fatty acids to monoenoic precursors vary considerably among different bacteria (19). In spite of these difficulties, valuable information on the physiological status of microorganisms in complex environmental samples have been obtained, particularly when the information can be linked to changes in specific chemical parameters that may reflect or influence microbial growth conditions. Such an example includes the analyses of the nutritional status of microbial communities in contaminated soils (55,56).

Metabolic Activity

Lipid biomarkers can also be used to analyze the metabolic activity of specific microbial population. One strategy is to add a ^{14}C- or ^{13}C- labeled substrate to the environmental sample and to determine the degree of incorporation of the labeled substrate into the cellular lipids. The incorporation rates of ^{14}C-acetate into phospholipids can be used to measure the activity of the total microbiota (57). Labeling experiments with ^{14}C-acetate can also be used to measure the rate of synthesis of PHB and PLFA (58). Roslev and coworkers (59) developed a method for analyzing the

incorporation of various ^{14}C-labeled substrates (e.g., ^{14}C-methane and ^{14}C-phenanthrene) into PLFA biomarkers, thus making it possible to fingerprint the metabolically active part of the microbial community (59). The metabolic activity of fungi has been assayed by measuring the incorporation of ^{14}C-acetate into ergosterol (60).

There are some advantages in using ^{13}C- labeled substrates over those labeled with ^{14}C in that, stable isotopes have higher specific activities and they can be detected with high sensitivity and precision using mass spectrometry. ^{13}C-acetate and ^{13}C- methane have been used to identify metabolically active bacteria in sulfate reduction and methane oxidation in freshwater sediment (61) and to detect atmospheric methane–oxidizing bacteria in soil (62).

An alternative approach for assaying the substrate usage of various microorganisms is to measure the stable carbon isotope abundance in signature lipids using isotope ratio mass spectrometry. Stable isotope ratios have been used extensively by ecologists to study carbon usage in various organisms but have only recently been introduced for analyzing lipid biomarkers (63–65). One problem associated with this method is that δ^{13}C pattern is not uniform for different fatty acids and lipid fractions and also varies with the type of growth substrate (63). However, by δ^{13}C analysis of archaeol and 2-hydroxyarchaeol, it has been possible to detect methane consuming *Archaea* in marine sediments (65).

PROCEDURES

Generally, chemical analyses of lipid biomarkers involve the extraction of the sample with organic solvents, followed by isolation and separation with various chromatographic techniques. The procedure outlined in Figure 1 was introduced by David C. White and colleagues 20 years ago and is in large still used by most workers (15). The analyses are based on the efficient one-phase Bligh and Dyer extraction (chloroform : methanol : buffer) of the sample (66). The one-phase solvent system is then divided into two phases with the addition of one portion of chloroform and one portion of buffer. The lipids are recovered in the lipid phase and the residue can be used for analysis of more polar lipid conjugates such as LPS. The extracted lipids are separated on silicic acid into three different fractions that contain NL, GL, and PL. After extraction and isolation of the various lipid classes, they are analyzed by gas chromatography (GC) or high-performance liquid chromatography (HPLC) (Fig. 1).

Brinch-Iversen and King (67) proposed to use dichloromethane as an alternative to the more hazardous chloroform in the Bligh and Dyer extraction medium, and this procedure has been used by numerous investigators for extracting PLFA from soils. It has also been shown that the amount of PL recovered from soils depends on the buffer used in the extraction medium (17,68). Notably, the lipid extraction procedure can also be used for extracting nucleic acids from environmental samples. Nucleic acids can be recovered from the lipid-extracted residue and used for enzymatic amplification and gene probing (69). One drawback of the Bligh and Dyer extraction procedure is that it is relatively labor-intensive and slow, taking up to 24 hours for the initial extraction. Macnaughton and coworkers (70) showed that the extraction step could be significantly accelerated by using a pressurized hot solvent extractor. Another way of accelerating the analyses of lipid biomarkers is to use solid-phase extraction (SPE) columns to fractionate the lipids (71).

Ergosterol can be extracted with the Bligh and Dyer mixture and recovered in the NL fraction (72). More often, it is directly extracted by methanol, saponified, and analyzed by HPLC using a UV detector (6,7,41). More recently, Montgomery and coworkers (8) introduced a microwave-assisted extraction method to isolate ergosterol from soils. Respiratory quinones can be recovered in the NL fraction and analyzed by HPLC (9). Alternatively, they can be directly extracted from environmental samples (soil, sediment, activated sludge) using a mixture of hexane : water (10).

The ester-linked fatty acids in PLFA and di/triglycerides are usually transesterified to methyl esters by mild alkaline methanolysis, and the obtained fatty acid methyl esters (FAME) are analyzed by capillary gas chromatography using a flame ionization (FID) detector. Environmental samples usually contain a complex mixture of PLFA, for example, 30 to 50 fatty acids are typically identified in samples from soils and sediments. FAME can at least initially be identified by comparing the GC retention times with those of standard fatty acids; however, in most cases mass spectrometry (MS) is needed for identification of the FAME (36). Methyl esters of fatty acids have electron impact (EI) spectra that, in most cases, give information on the carbon chain length, presence of methyl branches, hydroxy groups, unsaturations, and cyclopropyl groups. The position of double bonds in monoenoic fatty acids can be conveniently determined in FAME by analyzing their dimethyl disulfide adducts with GC/MS (73). For most applications, the sensitivity of the aforementioned GC/FID procedure is appropriate. However, the sensitivity in the analysis of PLFA can be significantly improved by using the mass spectrometer as a detector (74).

Zelles have introduced a more extended scheme for analyzing PLFA in soils (20,75). According to this method, the saponified products obtained after the mild alkaline methanolysis of the PL fraction are separated into six different fractions using various SPE columns. These fractions include both ester-linked and amide-linked fatty acids. Using this procedure, it has been possible to identify from 190 to 360 different PLFA in various soil samples (20,75).

The biphytanyl (archaeol) and di-biphytanyl ether lipids of *Archaea* can be analyzed in environmental samples by HPLC (76). Methods have also been developed that analyze archaeols using GC-FID (31,49).

APPLICATIONS IN ENVIRONMENTAL MICROBIOLOGY

The use of lipid biomarkers in environmental microbiology has increased significantly during the last few years. PLFA analysis has become a standard method to characterize microbial communities in both soils and

aquatic environments. The following section will provide some recent examples on the application of lipid biomarker analysis in environmental microbiology.

Soils

Analysis of lipid biomarkers have been used to identify the effects on various cultivation and management practices on microbial communities in agricultural soils. Bardgett and coworkers used PLFA analysis to show that the cessation of fertilizer input to temperate grassland soils can induce a rapid shift from bacterial to fungal dominance in the soil microbial community (77). Further analysis of grassland soils along a fertility gradient verified that the fungal to bacterial ratio was highest in the unfertilized and lowest in the fertilized soils (78). This investigation also showed seasonal differences in the PLFA pattern that were related to soil mineral-N and soil moisture content. PLFA analysis have also been used to compare the microbial community compositions between organic and conventional farming systems (79).

The effects of fertility gradients on the structure and biomass of the microbial community have also been examined in boreal coniferous forest stands (80). Although the total microbial biomass and respiration rate were unchanged along the fertility gradient, shifts in the PLFA patterns indicated gradual changes in the structure of the microbial community. The relative abundance of fungi decreased and that of bacteria increased with increasing fertility. In addition, the structure of the bacterial community changed along the gradient. In another study, the PLFA method was used to demonstrate that the spatial pattern of the microbial community structure in a forest stand were related to the position of trees (81). Spruce trees had a much stronger influence on PLFA patterns than birch trees. PLFA analysis was recently used to examine microbial community changes during primary succession on a glacier forefront (82). The lipid data indicated that the biomass increased over successional time and that the microbial community shifted from bacterial-dominated to fungal-dominated.

A number of studies have characterized methane-oxidizing bacteria in soils using PLFA biomarkers (cf. Table 1). Sundh and coworkers (83) found a good correlation between the concentration of the PLFAs specific for type I and type II methanotrophic bacteria and the potential methane-oxidizing bacteria in boreal peatland soils. Such a correlation was not found in a podzolic soil profile (84). Bull and coworkers (62) added $^{13}CH_4$ to a forest soil system and found by PLFA analysis that a new methanotroph, similar at the PLFA level to known type II methanotrophs, was the predominant soil microorganism responsible for atmospheric methane oxidation.

Analysis of soil PLFA patterns has been used to detect heavy-metal effects on forest soils with low pH and high organic content, and in agricultural soils with neutral pH and low organic matter content, both in the laboratory experiments (85,86) and in field studies (87–89). In all these cases, the analysis of the PLFA pattern detected metal effects at similar or sometimes lower contamination levels compared with measurements of microbial biomass and activity. Thus, the fingerprinting PLFA analysis proved to be a sensitive measurement of heavy-metal effects irrespective of soil type and type of metal pollution (44).

Aquifers

Lipid-based methods have proven to be a valuable tool for characterizing the structure and activity of microbial communities in contaminated and pristine aquifers (19). PLFA biomass analysis has indicated trends in the distribution of microorganisms in aquifers. Several investigations have shown that the viable microbial biomass decreases with depth from the soil surface, because of the decrease in available carbon with depth (90–92). The biomass at contaminated sites is usually higher than at pristine sites, and a correlation between biomass and contaminant concentrations has been observed in several sites (55,56).

The ratio of PHB to PLFA has been analyzed in a number of aquifer studies. In groundwater from a granite aquifer, a high ratio was interpreted as a sign of nutrient limitations (93). Other studies have indicated that pristine, oligotrophic aquifers have higher PHB/PLFA ratios than contaminated and more nutrient-rich groundwater (19). The physiological status of the microbial communities in aquifers have also been examined by analyzing the *trans*/*cis* and cyclopropyl/monoenoic ratios of PLFA. Ludvigsen and coworkers (55) observed lower values of these ratios near a landfill indicating that microbial growth was enhanced by contact with the leachate. PLFA analysis of aquifer samples suggested relationships among the structure of the microbial communities, sediment types, and sample locations (55,90,92). Methanotrophs and sulfate-reducing bacteria tend to be more abundant near the contamination source (55,56,92).

Aquatic Environments

A significant part of the early applications of lipid biomarkers was done on microbial communities in aquatic environments and sediments (2,4,12,15,18,21,25,26,57,58,72). Lipid biomarkers have been used in studies examining the role of archaea in anaerobic methane utilization. Hinrichs and coworkers (65) identified isotopically depleted lipid biomarkers and archaeal 16S rRNA genes occurring together in cold seep sediments in which anaerobic oxidation of methane (AOM) is supposed to occur. Further studies of this and other sediments confirmed the presence of extremely depleted archaeal lipids, in addition to identifying isotopically depleted bacterial fatty acids and glycerol ethers, most probably originating from the AOM syntrophic partners (94). One of these partners has been identified as sulfate-reducing bacteria (95).

Isotope-labeled substrates (^{13}C) in combination with PLFA analysis were used to identify populations involved in acetate and propionate consumption in anoxic brackish sediments (96). The data showed that acetate and propionate were consumed by different specialized groups of sulfate-reducing bacteria. The acetate-labeling pattern was similar to known species of sulfate-reducing bacteria but that of propionate did not resemble any known strain.

Pollution and Bioremediation

PLFA analyses have been used to indicate microorganisms involved in the methylation of mercury in mercury-polluted lake sediments (97,98). In both investigations, the microbial community composition was strongly related to mercury methylation potential. A high correlation was found between the methylation potential and the abundance of 10Me16 : 0, which is a biomarker for *Desulfobacter* spp. (25).

Several groups have recently used isotope-labeled substrates and PLFA analysis to characterize the part of the microbial community metabolizing various pollutants. Roslev and coworkers added ^{14}C-labeled phthalate (PA) and di-(2-ethylhexyl) phthalate (DEHP) to sludge-amended soils and followed the metabolism using PLFA fingerprinting techniques (99). The data showed that organisms with different PLFA composition metabolized the two phthalates. Furthermore, it was concluded that DEHP is mineralized by indigenous microorganisms in sludge rather than by indigenous microorganisms in soil. Hanson and coworkers (100) added ^{13}C-labeled toluene to soils. After incubation, a significant part (85%) of the incorporated ^{13}C was detected in PLFAs contained in a toluene-metabolizing bacteria isolated from the experimental soil.

The effects of polycyclic aromatic hydrocarbon (PAH) contamination on freshwater sediments were determined using both PLFA biomarkers and nucleic acid probes of PAH–degradative genes (101). Principal component analysis of PLFA profiles indicated that moderate to high PAH concentrations altered the microbial community composition and that seasonal fluctuations were comparable in magnitude to the effects of PAH pollution. PAH–degradative genes were detected in all sites, but their frequencies were typically higher at contaminated sites. Similar methods were used to follow the degradation of PAH in a bioslurry treatment of a PAH–contaminated sediment (102). PLFA analysis revealed a threefold increase in microbial biomass and a dynamic microbial community composition. Nucleic acid analyses showed that the copy number of genes encoding PAH–degrading enzymes increased several orders of magnitude, and this shift correlated well with shifts in specific subsets of the microbial community (102).

Macnaughton and coworkers used PLFA analysis in combination with 16S rDNA denaturing gradient gel electrophoresis (DGGE) to identify the bacterial community members responsible for the bioremediation of an experimental oil spill (103). The PLFA analysis indicated a community shift from primarily eukaryotic biomass to gram-negative biomass with time. Data obtained from the DGGE and DNA sequence analysis verified the dramatic shift to a community dominated by gram-negative bacteria.

Composts and Waste Treatment

Analyses of lipid biomarkers have been used to follow microbial community dynamics during composting and anaerobic digestion of waste material. Herrman and Shann showed that PLFA profiles changed in a consistent and predictable way during composting of municipal solid waste. The PLFA profiles were characteristic of specific stages of the composting and it was suggested that such profiles could be used for evaluating the progress of material processing and product development (104). Hellman and coworkers related changes in the microbial community composition to the emission of trace gases during open-windrow composting of solid waste material (105). The emission of carbon dioxide, methane, and nitrous oxide (N_2O) increased successively during compost maturation. The microbial biomass (total amount of PLFA) showed a discontinuous decrease during the composting process. The level of ether lipids used as biomarkers for archaean methanogens increased at elevated methane concentrations. The period of enhanced nitrous oxide emission corresponded with an increase of PLFA, which is indicative of gram-positive bacteria and actinomycetes. Oude Elferink and coworkers used both 16S rRNA probing and PLFA analyses to identify sulfate reducers and *Syntrophobacter* sp. in anaerobic bioreactors while treating different wastewater (106). Although the PLFA method was regarded as a useful method for fingerprinting the microbial community, it was not suitable for an accurate characterization and identification of the sulfate-reducing bacteria in the sludge.

CONCLUSION

Analysis of lipid biomarkers provides a rapid and, in most cases, an inexpensive way for describing the biomass, structure, and physiological activity of microbial communities in environmental samples. The lipid biomarker methods and the methods analyzing nucleic acids such as rRNA have different strengths and weaknesses and thus complement one another (107). One advantage of the lipid biomarker method is that they can be quantitatively extracted from complex environmental samples and analyzed without the requirement for an amplification step like the PCR needed for most rRNA analysis. The PCR method can introduce artifacts and biases in the amplification processes leading to misinterpretation of the original sample. A disadvantage of the lipid biomarker method is that they cannot give the taxonomic resolution at a level that is obtained using the rRNA methods.

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LIPIDS, ARCHAEAL.

See ARCHAEA IN BIOTECHNOLOGY

LITHOTROPHIC MICROBIAL ECOSYSTEMS IN THE SUBSURFACE

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The study of microorganisms in or from the terrestrial subsurface has waxed and waned several times since the very roots of microbiology, when Leeuwenhoek first observed bacteria in well water [reviewed in reference (1)]. As subsurface resources were exploited during the industrial revolution, it became apparent that microorganisms were active in coal beds and oil fields. Nearly a century later, the ability of subsurface microorganisms to degrade or immobilize anthropogenic contaminants came under intense investigation. Most recently, with expanding exploration of the solar system, subsurface ecosystems are often regarded as the last feasible models for how living organisms could potentially exist on other Solar planets (2–5). It is now widely accepted that microorganisms persist and metabolize below the Earth's surface to depths of more than 3 km. The lower limit to the biosphere likely varies with geographic location and is probably controlled by the depth at which the geothermal gradient causes temperatures to exceed the habitable range, as well as the flux of nutrients mediated by groundwater flow and the composition of the surrounding rock.

The surface of the Earth is universally and profoundly impacted by biological oxygenic photosynthesis. Effects range from the approximately 21% O_2 content of the atmosphere to the incorporation of organic matter into the crust. Subsurface microorganisms have generally been thought to subsist by slow recycling of buried photosynthate. In fact, most studies of subsurface microorganisms have investigated their effects on fossil carbon sources. However, if primary production could occur in situ, then subsurface ecosystems might exist that are independent of biological processes at the surface. This is probably the only remaining prospect for extant living organisms on other solar system bodies, as the surface of all known extraterrestrial planets are probably devoid of liquid water, unprotected from extremes of radiation, temperature, and pressure, and hence inimical to life as we know it (6). Furthermore, the surface of the Earth may have been uninhabitable at or near the time of the origin of life because of the fainter luminosity of the young sun (7) or planet-sterilizing impact events during late coalescence of planetary materials (8). If primary production takes place underground without dependence on surface-based photosynthesis, it is possible that the earliest continuously habitable portion of the biosphere may have been the deep subsurface (4).

CHEMOLITHOAUTOTROPHY

Chemolithoautotrophic organisms obtain energy from the reaction of inorganic electron donors and electron acceptors, and produce biomass from inorganic carbon. It is at least conceivable that any of these organisms could carry out primary production in subsurface ecosystems, given an appropriate environment. Several sites where this may occur have been identified (reviewed in Ref. 5). Many chemolithoautotrophs, such as nitrifying bacteria, iron-oxidizing bacteria, and sulfur-oxidizing microorganisms, are ultimately dependent upon photosynthetic organisms to produce the O_2 needed as an electron acceptor. Others, such as methanogenic archaea and homoacetogenic bacteria, are able to grow using resources available from purely mineral sources. This makes them of particular interest as the potential foundation for pre-photosynthetic ecosystems. Until recently, anaerobic chemolithoautotrophs were not known to act as primary producers — they were discovered in the context of organic

matter degradation. In anaerobic food webs, H_2-consuming microorganisms frequently mediate the terminal electron-accepting process in the degradation of organic detritus. Their role there is the conservation of energy from organic chemical bonds, rather than primary production. Clearly, it is possible that these organisms could carry out primary production, albeit to a lesser extent than photosynthesizers, given an appropriate environment (Fig. 1). However, because the surface biosphere, and much of the subsurface, is so saturated with the products of photosynthesis, it has been difficult to identify such niches.

Energy is available to microorganisms in niches where a chemical disequilibrium exists. Most frequently, this occurs when materials in a reduced state are juxtaposed with relatively oxidized materials. In the most general terms, this occurs because the Earth is a differentiated planet in which slow mixing between the disparate components occurs at various timescales through the combined

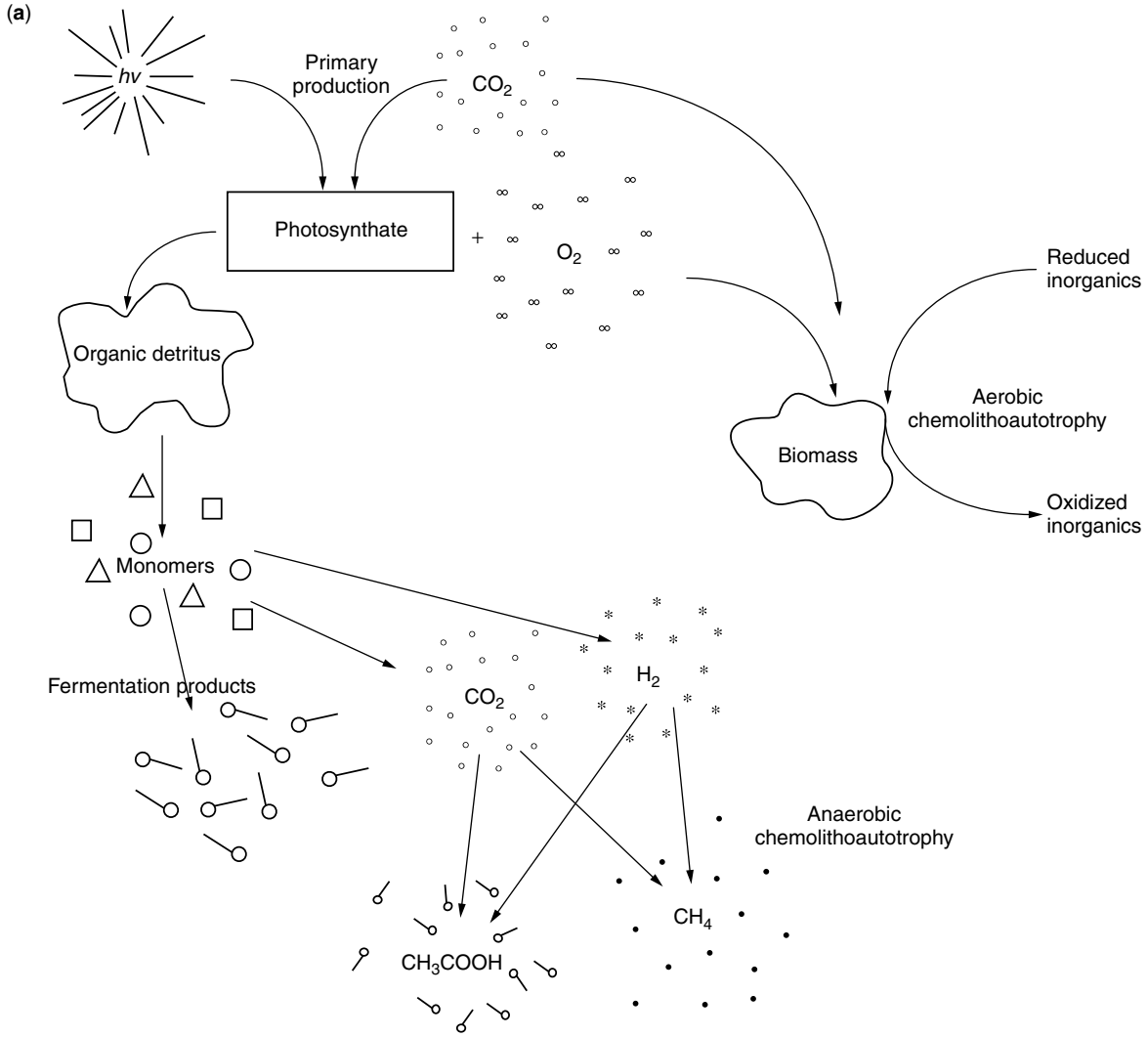

Figure 1. Ecological niches for chemolithoautotrophic microorganisms. Metabolic processes carried out by various organisms are shown as arrows. (**a**) Common niches of lithotrophs in ecosystems based on photosynthesis. Photosynthetic organisms carry out primary production and create a chemical disequilibrium, producing reduced organic matter and oxidized O_2 (from water, not shown.) Anaerobic chemolithoautotrophs conserve energy from chemical bonds of the reduced photosynthate. By consuming products of fermentation, they also improve the thermodynamic efficiency of detrital food webs. Aerobic chemolithoautotrophs depend on the other product of photosynthesis, conserving energy from reactions of photosynthetically produced O_2 with reduced inorganic compounds. (**b**) A simplified food web that might arise in environments not impacted by photosynthesis. Anaerobic chemolithoautotrophs now carry out primary production using H_2 produced as a by-product of abiotic rock-weathering reactions. Methanogenesis and acetogenesis are shown. Note that if other oxidized electron acceptors were available (e.g., Fe(III), SO_4^{2-}) other processes and complex food webs could occur. Aerobic chemolithoautotrophs (not shown) might also carry out primary production using O_2 produced by abiotic photooxidation reactions in certain subaerial environments.

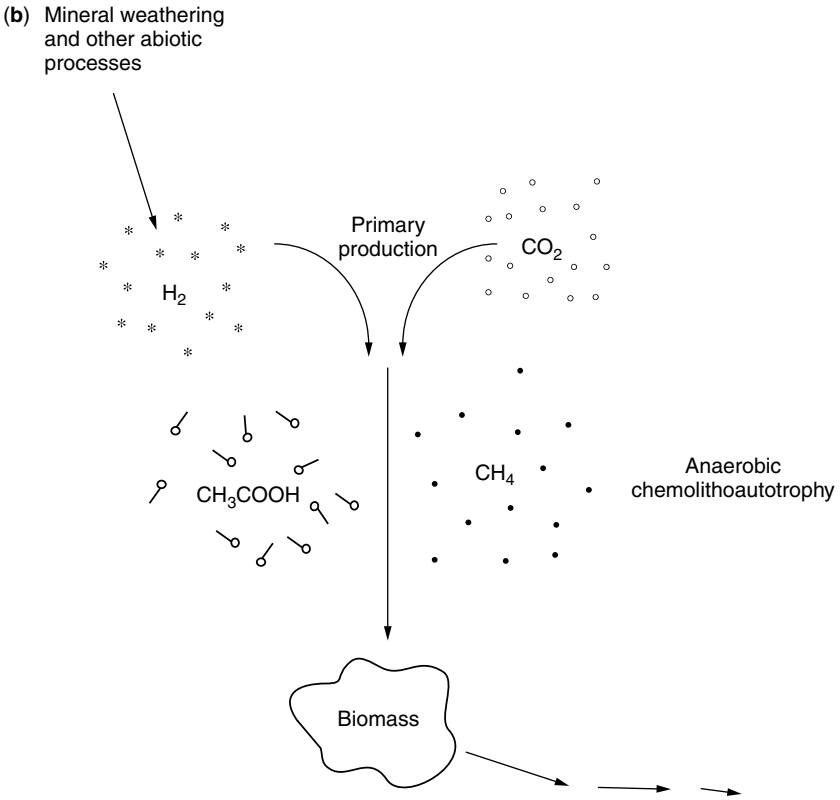

Figure 1. (*Continued*)

actions of the rock cycle (plate tectonics, erosion, volcanism), the hydraulic cycle (precipitation, runoff, groundwater flow), and subaerial processes (irradiation, lightening, aeolean flux). Reactions between the mixed materials may be thermodynamically feasible, but kinetically hindered (for example, by mineral dissolution rates) so that they remain in a state of metastability. By catalyzing these reactions at faster rates, microorganisms may be able to tap these sources of potential energy. The greatest concentrations of such exploitable energy are probably located at mixing zones where high-flux processes transport material between contrasting environments. Examples include terrestrial hot springs and mid-ocean spreading centers (9).

Igneous rocks are composed of crystalline minerals that have intruded as fluids from Earth's mantle into the crust and surface. They can be classified along a continuum from rocks containing mostly felsic (silica-rich) minerals, such as anorthosites and granites to those containing more mafic (magnesium and iron-rich) minerals, such as basalts and peridotites. These rocks are out of equilibrium with the surface environment and undergo slow reaction processes known as weathering. The more mafic minerals, which contain ferrous iron at very low redox potentials are particularly reactive.

However, as these crystalline rocks have very low solubility, weathering proceeds very slowly in most environments, and igneous rocks persist throughout the Earth's crust and over large areas of the surface. Although the weathering of igneous rocks is very slow, they are so widespread that they may constitute a very large potential energy source for microbial metabolism on a global scale. Energy flux from this source would be much more diffuse than that at high-flux mixing zones, but potentially larger in the global balance (1). However, as most igneous rocks at or near the surface are juxtaposed with the rich photosynthetically based biosphere, contributions from microorganisms growing at the expense of rock weathering may be difficult to sort out.

A MICROBIAL ECOSYSTEM IN THE COLUMBIA RIVER BASALT GROUP

A potential model system for studying microbial interactions with mafic rocks is found in the Columbia River Basalt group (CRB) in western North America. The CRB is the youngest of 12 known "flood basalt" provinces on Earth [see reference (10)]. Flood basalts were formed when so-far unknown events caused enormous volumes of magma to erupt and rapidly spread across the surface in flows that covered tens or even hundreds of thousands of square kilometers. (Many of these events have been linked to global mass extinctions in the fossil record.) In the CRB, these catastrophic flows recurred periodically over the course of a few million years and resulted in deep sequences of layered flows. These are essentially enormous volumes of almost entirely mafic rocks. The CRB covers more than 160,000 km^2 and lies up to 3 km deep, with an average thickness of 1 km. The individual flows are largely impermeable and prevent vertical groundwater flow, except through fracture zones. Zones of rubble between the flows, known as *flow tops*, are much more permeable and result in a series of aquifers with slow horizontal movement of groundwater. The time for water to flow from recharge

zones to deep aquifers in the central CRB has been estimated at greater than 30,000 years. Reaction with ferrous minerals removes oxygen from the groundwater relatively rapidly, and the deep old waters are anoxic, reducing, and mostly somewhat alkaline.

The CRB groundwaters also contain relatively large populations of microorganisms (11,12) and water pumped from the CRB frequently contains more than 10^5 entrained cells per milliliter. Most of these aquifers fall into one of three types. Shallow aerobic aquifers, generally less than 500 m deep, have not yet become strongly reducing. Deep carbonate-dominated aquifers contain relatively high concentrations of dissolved methane (up to 100 mM) and hydrogen gas (frequently around 1 μM). Deep chloride-dominated aquifers contain relatively high concentrations of sulfate (up to 2 mM) and sulfide. Higher numbers of methanogenic archaea can be recovered from the methane-containing aquifers, whereas higher numbers of sulfate-reducing bacteria can be recovered from the sulfide-containing aquifers (11). Interestingly, homoacetogenic organisms can be recovered equally well from both types (12). Lower numbers of anaerobic heterotrophic organisms could also be recovered from both sites.

As a cautionary matter, it should be noted that microbiological samples from CRB aquifers have been obtained only by sampling water produced from wells. More reliable observations of the in situ communities would require aseptically obtained core samples, which can require considerable resources and may not yet be technologically feasible in deep igneous rocks (13–15). However, observation of secondary minerals and microfossils in ancient fracture-fill material (from nonaseptically obtained cores) suggests that anaerobic microbial ecosystems similar to those present today have been present in the CRB throughout its history (16).

In fact, the methane and sulfide in CRB groundwaters appear to be products of microbial metabolism, based on trends in concentration versus depth (depth is assumed to be a proxy for time) and stable isotope values (12). Most work to date has focused on the methane-rich aquifers. The ratios of the stable isotopes of carbon can yield important clues about the metabolic functions of gas-producing organisms (17). As CRB groundwaters age, concentrations of dissolved inorganic carbon (DIC) decline, whereas concentrations of methane increase. Simultaneously, the residual DIC becomes enriched in ^{13}C, indicating that its depletion is caused by a process with strong isotope discrimination, such as microbial metabolism. In fact, if one assumes that autotrophic methanogenesis is the sole process affecting DIC, then the predicted stable isotope values match the measured values from the aquifers very closely (12). This suggests that microorganisms within these aquifers are carrying out primary production in situ. If the microbial community was producing methane from the degradation of detrital organic matter, then increases in methane concentration would be accompanied by increases in DIC from fermentation, rather than decreases, as observed (18). Furthermore, a rather wild coincidence would be required to achieve the close match between predicted and observed isotope values. Thus, field data from the CRB suggest that autotrophic methanogens carry out primary production through methanogenesis, and associated biomass production, from hydrogen and carbon dioxide.

The frequent observation of micromolar H_2 concentrations in the CRB suggests the ready availability of this electron donor, but provides few clues as to its origin. Unlike the case with methane, there does not appear to be a trend in H_2 concentration versus depth. The geochemical literature contains reports of widespread occurrences of significant H_2 in subsurface fluids [reviewed in references (12,19)]. A number of mechanisms for H_2 formation potentially exist in the subsurface [reviewed in reference (19)]. The possibility that reduced minerals in basalt react with water to produce H_2 was investigated in a series of laboratory experiments (12,19). (Such reactions are well known for ultramafic rocks, which are richer in ferrous silicate minerals than is basalt, and that produce H_2 during conversion to serpentinite during the weathering process(es) known as *serpentinization* (20).) In fact, reaction of CRB-basalt with water in the laboratory produced H_2 gas under anaerobic conditions. Experiments with different minerals present in basalt showed that the reactive components were the ferrous silicate minerals pyroxene and olivine. The production of H_2 was controlled by temperature, pH, and dissolved ferrous iron, and was inhibited in the presence of oxygen. Interestingly, H_2 production was also kinetically inhibited by product accumulation and could be enhanced by processes that remove H_2, such as microbial metabolism. Another group (21) reported inability to repeat these results at alkaline pH values, such as are found in CRB aquifers, using undescribed basalt samples from outside the CRB. This discrepancy may be caused by some compositional difference of the basalt samples, or perhaps more likely, failure to remove traces of O_2 from the experimental vessels.

The ability of microorganisms to take advantage of this reaction has been tested in vitro in a series of laboratory microcosms (T. O. Stevens and N. Landau submitted). Microbial growth, dissimilatory metal reduction, acetogenesis, and methanogenesis have been demonstrated in microcosms that contained only inorganic carbon and ground basalt as a source of energy. Consistent with the kinetic experiments with H_2 production, microcosms containing methanogens produced between 2- and 30-fold (depending on experimental conditions) more reduced gas than abiotic control microcosms.

In summary, the evidence suggests that the slow weathering of basalt minerals in deep confined aquifers results in the production of H_2 gas. Autotrophic microorganisms use the H_2 as an electron donor for reduction of carbon dioxide. In so doing, these organisms appear to carry out primary production in the deep subsurface with no link to biotic processes at the surface, and they may support the existence of subsurface ecosystems. These hypotheses might be tested by continued synergistic field studies and laboratory experiments.

At this time, relevant field data is rather sparse, because few measurements have been made specifically to test the hypotheses. Some tentative extrapolations can be made to compare laboratory results with existing field data, though a number of questionable assumptions are

required. If one assumes a single source of methane and steady-state production, then the rate of H_2 production observed in laboratory microcosms, when normalized for surface area, agrees remarkably well with the rate required to produce the highest methane concentrations observed in the CRB aquifers (19). However, transport of atmospheric carbon dioxide through groundwater recharge does not appear to provide enough DIC to support the observed methane. Dissolution of carbonates or volcanic outgassing (both present in the region, but not quantified) could potentially provide this missing DIC source. In fact, the putative CRB ecosystem may well be DIC-limited, rather than H_2-limited, which may explain the persistence of scattered but widespread micromolar concentrations of dissolved H_2. Extrapolating from the very few data available to estimate the wetted surface area in the CRB, and assuming probably unreasonable homogeneity through time and space, one can estimate that the complete reaction of ferrous minerals in a given volume of basalt would require between 10^8 and 10^{10} years. Although a more reliable estimate would require significant new data, this appears to be consistent with the accumulation of 5 to 10% alteration products over the 10^7 year age of the CRB.

SPECIAL CASE OR WIDESPREAD PHENOMENON?

If subsurface lithotrophic microbial ecosystems constitute a quantitatively significant phenomena in Earth's biosphere, it should not be confined to a special case or location, such as the CRB. From the present data, one might predict that this sort of primary production should occur in any environment that contains sufficient ferrous silicate minerals and isolation from atmospheric oxygen [reviewed in reference (1)]. An obvious environment to look for such phenomena is seafloor basalt formations, which cover 70% of the earth. Where these rocks are hot, near seafloor spreading centers, the process of serpentinization produces voluminous hydrogen and methane abiotically (22). As the rocks cool and move away from spreading centers, there appears to be great potential for microbial reactions similar to those observed in the CRB, however, very few observations have been made in these environments, other than weathering patterns that suggest the presence of subsurface microorganisms (23,24). Peridotites are assemblages of ultramafic rocks formed by eruption of mantle materials at spreading centers. They are richer in ferrous silicate minerals than basalts, and one might predict that they provide a much greater potential for microbial activity. However, we know of no investigations to test this hypothesis. Conversely, granites are much more silica-rich and lower in ferrous minerals than are basalts, and one might predict that microbial activity would be lower in such environments. However, biotic methane and significant concentrations of autotrophic microorganisms have been observed in some deep granitic environments (25,26). It may be that other mechanisms of H_2 production are active in those environments, or that H_2 is transported by groundwater flow from adjacent and intercalated ultramafic rocks that are often associated with granites. Many soils and sediments also contain ferrous silicate minerals, derived from mechanical weathering of igneous parent material. If these soils are anoxic, the weathering of these minerals should contribute H_2 to any microbial community that is present, though it may be masked by contributions from organic matter decomposition. Such a phenomenon might be difficult to measure, though some suggestive data can be found in the geochemical literature. For example, some strata of marine sediments in the Astoria Fan at the mouth of the Columbia River have been observed to contain anomalously isotopically heavy DIC, suggesting that autotrophic methanogenesis is prevalent (27). These strata are composed largely of detrital CRB basalt deposited during cataclysmic pliestocene-age floods (28). It seems likely that the same processes hypothesized for the CRB are occurring in these sediments.

SPECULATIONS ON A BROADER SIGNIFICANCE

The importance of potential subsurface primary production can be exemplified in the following rather speculative discussions.

Though Earth's current biosphere is largely supported by photosynthetic primary production, it seems possible that primary production linked to mineral weathering may have been proportionally more important early in Earth's history [reviewed in reference (4)]. Near the time of the origin of life, more than 3.8 billion years ago, the atmosphere is thought to have contained very little oxygen, and the crust would have been dominated by igneous rocks. Thus, ecosystems similar to extant ones in the CRB and other deep subsurface environments may have been active nearer to the surface, before the evolution of photosynthesis. However, ancient subsurface ecosystems may have also been important to the evolution of the biosphere. The early earth was subjected to repeated surface-sterilizing cosmic impacts (8), and subsurface ecosystems may have provided refugia from which the surface was periodically recolonized. Similarly, phenomena known as *snowball earth* events are thought to have periodically rendered the surface uninhabitable, and may have been more frequent early in Earth's history, when the luminosity of the sun was much lower. The ability of microorganisms to persist in the subsurface over geological time may potentially have been of great importance to the survival and evolution of the biosphere.

It has been suggested that chemolithoautotrophic microorganisms carrying out primary production in the subsurface may be the best prospect for potential life elsewhere in the solar system (2). Surface conditions on all of the other planets in our system are probably inimical to life. A commonly invoked litmus test for the ability of an environment to harbor life is the presence of liquid water. Only on Earth can liquid water exist at the surface. Thus, ecoystems based on photosynthetic primary production, which can only occur at the surface, are unlikely in extraterrestrial settings. However, in the subsurface, geothermal gradients and lithostatic pressures ensure that if water is present, at some depth it will be in a liquid state. Subsurface water is thought to be likely on Mars, Europa, and perhaps other icy moons.

Given a potential habitat, a source of energy would be required to sustain microorganisms in such a setting. Ferrous silicate minerals like those in terrestrial basalt aquifers are ubiquitous in the solar system. They are abundant on any planet with a rocky crust. It seems almost certain that there are extraterrestrial environments where ferrous silicate minerals are bathed in liquid water. Thus, it seems possible that if microorganisms ever existed on other planets, they could feasibly persist today in habitats analogous to the CRB aquifers. However, the mere presence of basalt and water are probably not enough to sustain subsurface ecosystems. Some mechanism for circulation and atmospheric exchange is probably necessary. In the absence of circulation, the system would come to equilibrium, and no energy would be available for microorganisms. Mechanisms for large-scale subsurface circulation on Mars have been proposed (29) but few data are currently available with which to evaluate them. Perhaps the subsurface of Europa is more conducive to energy flux, as suggested by current data. A possible ocean of subsurface water would certainly allow large-scale circulation, and evident circulation of surface ices could provide a steady flow of oxidants from the surface (30).

To evaluate these possibilities, much more information is needed about both extraterrestrial environments, and about the functioning of putative analogs on Earth.

CONCLUSION

Evidence from a number of locations suggests that anaerobic chemolithoautotrophic microorganisms play major roles in some deep subsurface ecosystems. The ability of these organisms to function as primary producers of organic matter depends on the availability of abiotically derived electron donors. Weathering reactions of igneous rocks potentially could provide such a source, through a variety of mechanisms. The large extent of the CRB and the relative isolation of aquifers within it from the surface biosphere make it an appropriate environment to study such interactions. In fact, microbiological and geochemical field observations, as well as laboratory tests of these hypotheses, have provided evidence that subsurface microorganisms may carry out primary production using energy from rock weathering. Further testing of the hypotheses may require an iterative sequence of field and laboratory investigations. If these hypotheses are correct, the phenomenon may be important in understanding the origin and evolution of Earth's biosphere, and potential biospheres elsewhere.

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LOW LIGHT ENVIRONMENTS. See CAVES AND OTHER LOW-LIGHT ENVIRONMENTS: AEROPHITIC PHOTOAUTOTROPHIC MICROORGANISMS

LUCIFERASE AND GREEN FLUORESCENT PROTEIN AS BIOREPORTERS IN MICROBIAL SYSTEMS

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A reporter signals to the investigator that a biological event of interest is taking place. Most typically, the event is a change in gene expression and the change is ordinarily an upregulation (increase in gene expression vs. control). Thus within a population or at the single-cell level, an increase in transcriptional rate can be discerned and factors governing the expression of specific genes can be investigated. Depending on the persistence of the signal, it is possible to assess temporal changes in gene expression rather than simply an on or off state. The perfect microbial reporter system should fulfill the following criteria:

1. detectable without expensive or complicated equipment,
2. rapid linear response to gene expression,
3. directly coupled to gene expression rather than coupled through intermediate compounds/events, and
4. stable on the order of minutes but unstable on the order of hours, thereby allowing downregulation (disappearance of signal) to be detected.

Many kinds of microbial reporter systems, other than the two described here, exist (1). Some, such as ice-nucleation genes, are specific to bacteria. Others, such as the enzymatic systems β-galactosidase (*lacZ*) and β-glucuronidase (*gus*), have had broad application to eukaryotes and to bacteria. This contribution will not discuss reporters as used in eukaryotic cell culture; it will focus instead on luciferase and green fluorescent protein (GFP) as reporters in bacteria and, to a lesser extent, in microeukaryotes (e.g., yeasts). Finally, biomedical literature has not been cited because this work targets readers from environmental microbiological fields.

LUCIFERASES

Luciferases are enzymes that produce light (bioluminescence) in eukaryotes and in prokaryotes. The ecological significance of bioluminescence is very high; light production results from a suite of diverse biochemical reactions. The luminescent organism must devote large amounts of metabolic resources (ATP, reducing power, and protein synthesis) to the production of light, and the parallel development of bioluminescence (frequently through unique metabolic pathways) in many different genera supports the notion that strong evolutionary selection for bioluminescence exists. The reader is referred to the review by Wilson and Hastings (2) as a starting point in this interesting saga of the unity of nature. In the present entry the difference between bioluminescence reactions will be emphasized.

In eukaryotes, bioluminescence involves what have been historically termed *luciferin-luciferase* complexes. Luciferases are enzymes directly responsible for light production; bacterial luciferases are analogous, but not homologous, to eukaryotic luciferases. Luciferins are specialized substrates upon which luciferases act. In prokaryotic systems, fatty aldehyde intermediates from natural biosynthetic pathways are equivalent to luciferins; in naturally bioluminescent bacteria, enzymes for the regeneration of these aldehydes are synthesized as part of the luciferase operon. In prokaryotes, the luciferase gene(s) are called *lux*; in eukaryotes, they are referred to as *luc*.

Eukaryotic Luciferases

Dinoflagellates. Bioluminescence of dinoflagellates, such as *Gonyaulax*, is not well understood. The luciferin, a linear tetrapyrrole (3), is very different from other eukaryotic luciferins and it is not clear how this molecule is oxidized by the 137-kDa luciferase, both of which are contained in a special organelle, the scintillon (4). This compartment may be necessary to keep the luciferin, which is quite easily chemooxidized, in a state enzymatically compatible with the luciferase. These luciferin-luciferase complexes are thus not easily employed as bioreporters.

Coelenterates. The sea pansy *Renilla* is the organism in which coelenterate bioluminescence has been most extensively described (5). Here, and in other coelenterates, the luciferin is named *coelenterazine* (6). This polycyclic imidazole is, in the absence of calcium, bound to the luciferin-binding protein (LBP). When the concentration of free intracellular Ca^{++} rises, coelenterazine is released from the LBP and is oxidized by the *Renilla* luciferase (a 35-kDa protein) through an intermediate to coelenteramide. This oxidized (excited) molecule can emit a blue photon (480 nm) in vitro. However, in vivo, the photon is immediately captured by an accessory protein (one of several GFPs), which in turn emits green light (509 nm). The photoprotein aequorin is a stable complex of the luciferase and an oxidized coelenterazine that, in the presence of Ca^{++}, finishes the bioluminescent reaction to yield the photon. Because the reaction is Ca^{++}-dependent, aequorin has been used as a Ca^{++} reporter. In prokaryotic systems, it has been used as a probe for free Ca^{++} (7–9), to correlate putative Ca-binding proteins with Ca^{++} levels in cells (10) and to investigate Ca^{++} transients in chemotaxis (11). These authors, for the most part, recognize that their population-based measurements do not preclude the possibility that very different levels of Ca^{++} exist between

individual cells (see "Signal Detection" in the following text).

The *Renilla* luciferase component alone has been expressed as a reporter in yeast (12), and the luciferase has been expressed (but not used as a reporter) in bacteria (13). It should also be emphasized at this juncture that without the sometimes esoteric research on coelenterate bioluminescence, modern biology would not have its most exciting bioreporter, GFP. This photoprotein, best known from the bioluminescent jellyfish *Aequorea victoria*, emits green light when excited by blue light and is discussed in the latter part of this contribution.

Insects. As in coelenterates, bioluminescence in insects follows a general scheme but species-dependent variation exists, primarily in the wavelength of emitted light. Insect luciferins are assumed to be quite similar to the model molecule known from fireflies because all insect luciferases will function using firefly luciferin as a substrate (14). The luciferin, which is strikingly different from that evolved in coelenterates, is acted upon by a 62-kDa luciferase which, in turn, is very similar in organisms in the same family. The luciferase reacts with ATP and luciferin in an Mg^{++}-dependent reaction to yield the excited (oxidized) luciferin that then decays to produce a photon. The structure of the catalytic site in the luciferase is thought to regulate the wavelength of emitted light (species-dependent, from green to red) (15).

Insect *luc* genes from various sources have been expressed and produce light in many different bacteria, Gram-positive and Gram-negative. The general strategy is to have the bacterium express the *luc* gene and to supply luciferin, ATP, and Mg^{++} exogenously. *Bacillus subtilis* was used to express functional firefly and click beetle luciferases from a *lacZ* promoter (16) and click beetle *luc* was functionally expressed in *Streptococcus mutans* to yield a strain that was used in a rapid assay of antibiotic sensitivity (17). *Pseudomonas putida*, *Rhizobacterium meliloti*, *Agrobacterium tumefaciens*, and *Alcaligenes faecalis* have also been used to express functional click beetle *luc* from isopropyl β-D-thiogalactoside (IPTG)-inducible plasmid constructs (18). The ability to differentiate colonies that expressed in different wavelengths has been shown (19), and such work demonstrates the potential for environmentally or physiologically relevant use in a wide variety of microbial systems. Especially intriguing is the possibility of spectrally distinct sensing of multiple reporter systems.

Prokaryotic Luciferases

Heterodimers of ~40 kDa and ~35 kDa (α- and β-subunits, respectively; the products of the *luxA* and *luxB* genes) (20) bacterial luciferases catalyze the oxidation of a long-chain (fatty) aldehyde and reduced flavin mononucleotide ($FMNH_2$) through several intermediates (some known, some postulated) to the eventual release of a photon of blue light (about 490 nm). When comparing this reaction to the analogous situation in eukaryotes, the fatty aldehyde is analogous to luciferin.

Two general strategies exist for the use of *lux* products as bioreporters. The most common approach is to link the synthesis of a *luxAB* cassette to the promoter of interest. In this case, a fatty aldehyde (normally *n*-decanal) is usually provided externally to eliminate any possibility that the bioluminescence reaction is limited by fatty acid turnover in the cells. The second strategy is to link the entire *luxCDABE* cassette to the promoter. The reporting organism then synthesizes not only the luciferase enzyme subunits (products of *luxA* and *luxB*) but also enzymes necessary for the recycling of the fatty acid substrate (a reductase, a transferase, and a synthetase; products of *luxC*, *luxD* and *luxE*, respectively; the components of the fatty acid reductase complex) (20). Thus this approach relieves problems associated with limitation by intracellular fatty acid pools.

Signal Detection

For work with microorganisms, one must distinguish between population-based measurements (an average of signals measured from many cells simultaneously) or single-cell measurements (signal from individual cells within a population). Population measurements are generally incorporated into experiments as the single variable being measured. In contrast, single-cell measurements are most frequently done through a microscope with a camera and thus lend themselves to the simultaneous application of different microscope-based approaches (correlative microscopy) (21,22).

Light can be detected in myriad ways that distinguish themselves primarily by sensitivity. Film (photographic or X-ray) can be used; this approach is not very rapid and requires a relatively large photon flux such as obtained from a population of highly expressing cells. Spatial resolution depends on the proximity of the film to the emission source and is not high enough to detect light from single microbial cells.

Luminometers (similar in design to scintillation counters but less sensitive and therefore less costly) are the most commonly used measurement tool in luciferase detection. These devices can be quite sensitive, are inherently quantitative, and are becoming very flexible in design (23), but they lack spatial resolution on the scale necessary for single-cell microbial work.

Enhanced charge-coupled device (CCD) cameras are a good compromise between sensitivity and the ability to resolve the origin of light in two dimensions. Under proper conditions, they are as sensitive as luminometers, and they produce an image showing the origination points of the light. Quantification of the amount of light emitted is more difficult, primarily because of the potential range of emission that must be measured (from essentially zero through several orders of magnitude). This problem has been addressed by the development of "photon-counting" cameras: devices more sensitive than luminometers that use computer-assisted approaches to determine relative photon fluxes at various light origins within the image (24). Figures 1 and 2 demonstrate major advantages over most commonly used reporters afforded by the use of luciferase and a photon-counting camera: spatial discrimination in monitoring (Fig. 1 and Fig. 2a) and the ability to monitor downregulation of a gene (Fig. 2b).

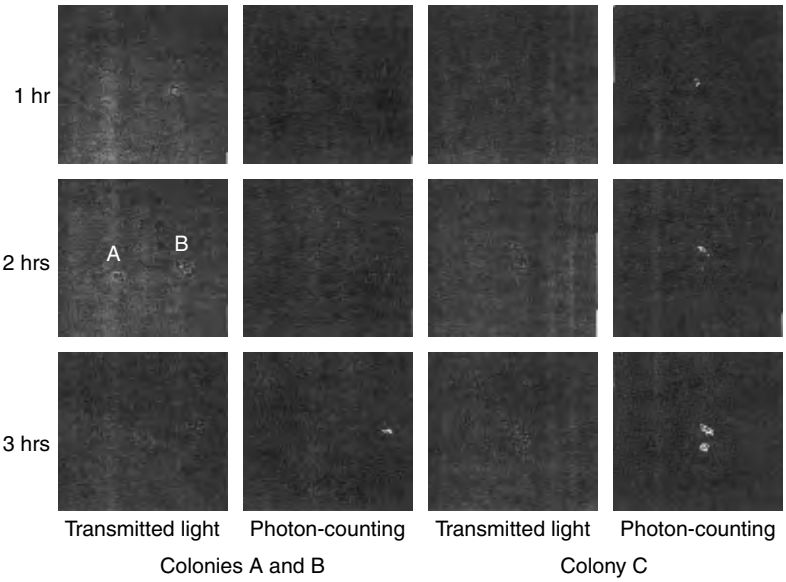

Figure 1. Spatiotemporally resolved monitoring of toluene metabolism in genetically engineered *Psuedomonas putida* cells growing in a biofilm. Transmitted light images and photon-counting images (light production) of colonies in which a chromosomally integrated fusion between the toluene-degradation gene and luciferase (*tod-lux*) responds to the presence of toluene introduced at time zero. Three colonies were monitored over the course of three hours. The columns at the left show limited growth in two colonies (transmitted light images) and no induction (light production) from colony A, whereas colony B shows increased light emission over the three-hour monitoring period. The two right columns show marked growth and high light production from a third colony in the same experiment. This colony shows two distinct centers of light production (photon-counting image at three hours). The pseudocolor scale in the photon-counting images shows low light production in black and blue, with high light production in green through red/yellow.

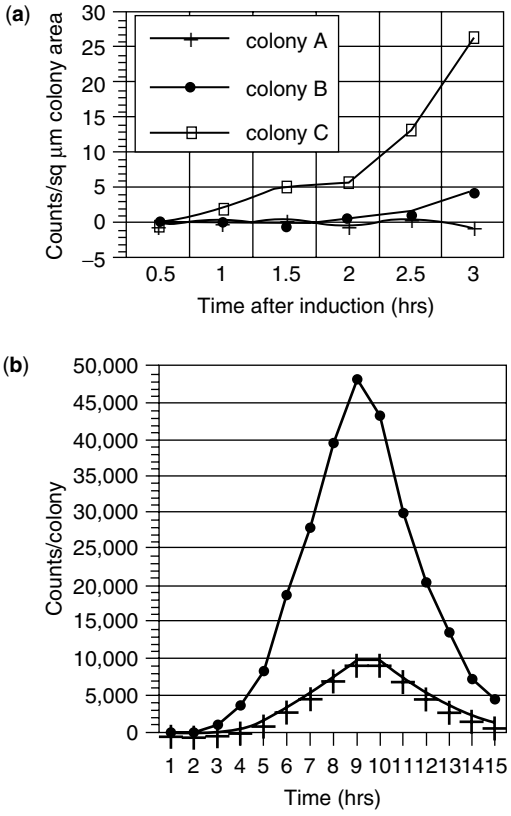

Figure 2. Quantitative interpretation of photon-counting images. (**a**) Light emission normalized to colony area for the three colonies shown in Fig. 1. (**b**) Real-time, nondestructive monitoring of up- and downregulation in two mircrocolonies of the same *tod*/*lux* reporter strain shown in Fig. 1. Light production increases from time zero (introduction of toluene). Toluene was removed after six hours of induction; light production continued to increase until nine hours, and dropped over the following six hours to preinduction levels. These data demonstrate a lag of about three hours in the response of this reporter system and show the utility of luciferase-based reporter strains as real-time biosensors.

The most sensitive detection is afforded by scintillation counters but microbiologically relevant spatial discrimination is not possible. As a result of the increasing application of these instruments for high-throughput luminescence detection, designs that incorporate the ability to read light production from individual wells within standard-format microtiter plates are available; approaches to the elimination of cross-talk between wells distinguish the instruments from one another.

Luciferase Reporters in Practice

In addition to the applications already noted, reporter systems have been applied to environmentally relevant situations, particularly to the sensing of toxins and pollutants. From a methodological standpoint, some controversy exists in the literature about the "proper" use of bioluminescence systems. Firstly, there is little consensus on the potential effects of naturally occurring variation in the myriad environmental factors known to alter bioluminescence (e.g., iron availability and oxygen concentration), on bioavailability of certain target molecules such as heavy metals, and even in the manner in which reagents are introduced in an assay (25). However, such issues seem more critical to the detailed interpretation of results than to the ability of the reporter to function when the target molecule is present. Secondly, reporters frequently respond to a suite of closely related chemicals resulting in synergistic effects when several of these effector molecules are present (26); however, in certain applications this may be advantageous. Thirdly, some assays are clearly not relevant to in situ environmental applications because they require cell lysis (27) and therefore are not capable of providing repeated measurements.

Despite these very real problems, the application of luminescence-based biosensors is on the horizon. A vast majority of work has been conducted under laboratory conditions; successful field applications of these systems remain sparse and are limited to the demonstration

of efficacy in online monitoring and process-control applications (28) and for use in mechanistic studies of bacterial contamination (29). It is also clear that reporter systems are very valuable in laboratory-based toxicity assays (30). *Luc*- and *lux*-based systems have both demonstrated potential as environmental sensors. Firefly luciferase has been expressed from a promoter of mercury resistance in bacteria to detect Hg and arsenate in environmental samples ex situ (31). The *luxAB* system and the *luxCDABE* system have been used to detect alkanes and benzene, toluene, ethylbenzene, xylene (BTEX) in ex situ samples (32,33). One example of true in situ monitoring using *lux*-based reporters is that of polyaromatic hydrocarbon (PAH) degradation in soil (34). Large (4-m deep × 2.5-m diameter) pipes were filled with PAH-contaminated soil that contained the reporter strain (*Pseudomonas fluorescens* HK44–harboring plasmid with the *luxCDABE* cassette linked to the naphthalene degradation pathway). Over a two-year period, light production of the reporter strain on soil particles was directly monitored (using a photomultiplier tube inserted into tubes in the soil), and the reduction in vapor-phase PAH levels in the soil was monitored using biosensors (the same reporter strain encapsulated in alginate) lowered into the tubes. This project not only demonstrates the utility of reporters for in situ work but also points out some of the difficulties experienced when moving from the laboratory to the field. One offshoot of this long-term field-release experiment was the development of a *lux*-based MPN assay (35) that demonstrated the presence of the plasmid-containing reporter strain over a significant background of endogenous antibiotic-resistant bacteria; the only alternative to the MPN determination was a more complicated hybridization–plating assay (34). Much effort in in situ detection studies has revolved around the development of a probe that would house immobilized whole-cell bacterial biosensors. Initially, fiber-optic-based designs were prevalent (34,36), but with the rapid development in miniaturized optoelectronics, bacterial cells and all electronic circuitry necessary to perform light measurement can be housed on a 4-mm^2 chip (37).

GREEN FLUORESCENT PROTEIN

Green fluorescent protein (GFP) is a nontoxic 238-amino acid peptide synthesized by the jellyfish *Aequorea victoria* (38,39) that emits green light at 509 nm when excited with light of 385 to 480 nm. After synthesis the protein folds and, via an autocatalytic process, three amino acid residues are modified to form intramolecular covalent cross-links (40). The protein requires only a low concentration of oxygen for maturation, and no other external factor, making it an excellent candidate as a marker in environmental microbiology (41,42). GFP was isolated in the early sixties (38) and purified a decade later (43) but was not used as a molecular marker until Chalfie and collaborators cloned it in 1994 (44). Since then, GFP has become the marker of choice for almost any biological application.

GFP Properties and Variants

Wild-type GFP has been extensively studied and characterized since Shimomura and coworkers first isolated the protein from crude jellyfish protein extracts (38). The minute amount of purified protein limited the investigation, and the detailed information available today is derived mainly from recombinant protein studies. GFP is a monomeric protein of 238 amino acids (27 kDa) with dual excitation peaks, the major peak at 395 nm and a minor peak at 470 nm. Emission is maximum at 509 nm, with a shoulder at 540 nm (38,45). This means that long-wave ultra violet (UV) light is required for efficient excitation, although blue light does excite the protein to a lesser extent. The wild-type protein has a maturation time of several hours, which makes it less interesting for gene-expression studies (41). Soon after the cloned gene was publicly available, the hunt for optimized variants commenced. One of the first useful variants described was the S65T mutant (41); the name indicates the nature of the mutation, which is a single amino acid replacement of serine 65 to a threonine residue. S65T is "red-shifted": the excitation peak is shifted toward the red end of the spectrum. S65T is still excited by blue light (488 nm) but not by long-wave UV light, whereas the emission is largely unaltered at 507 nm. Excitation with 488-nm blue light is relatively harmless for living cells when compared with the UV light required for efficient wild-type GFP emission. Furthermore, S65T has a somewhat shorter maturation time compared to that of the wild-type protein. Further refinements to the protein structure have been carried out. One of the most useful enhancements was made by Cormack and coworkers (46) who used flow cytometer–assisted cell sorting (FACS) for selection of mutants. They produced the mut1–3 mutants that are red-shifted and have significantly shorter maturation times compared to the wild type. Although the wild type takes two hours for folding of 50% of a protein pool, mut1–3 mutants have T1/2 of less than 30 minutes. Furthermore, mut1–3 mutants are brighter, that is, they have a higher quantum yield than wild-type GFP. A number of mutants with altered emission spectra have also been engineered, notably the BFP (47,48), CFP (49) and YFP (50), which emit blue, cyan, and yellow-green light, respectively. The BFP requires UV for excitation (380 nm) and emits blue fluorescence at 440 nm, whereas CFP is excited by 433 nm and 453-nm light and fluoresces at 475 and 501 nm. The YFP is excited by green light (513 nm) and emits light at 527 nm. Other physico-chemical properties of these variants are typically a higher quantum yield and shorter maturation times. For use in eukaryotic systems, codon-optimized variants that enhance translation efficiency have been engineered (Clontech, Palo Alto), but for prokaryotic systems the jellyfish codon usage works quite well. Codon-optimized mutants have been designed (e.g., for use in *Escherichia coli*), but the effects of these enhancements appear marginal (F. G. Hansen, personal communication). One last class of mutants, destabilized GFPs, deserves mention. The wild-type protein is very stable after maturation and remains intact in the cells for several days after synthesis. This obviously makes the

protein less useful for dynamic studies. Two types of destabilized proteins have been designed for use in eukaryotic or prokaryotic cells. The eukaryotic system is constructed by fusing a part of the mouse ornithine decarboxylase to the C-terminal end of the GFP protein. The tag contains a so-called PEST domain that is the target for indigenous protein degradation in higher organisms (51). In the prokaryotic setting the protein is destabilized by adding a short oligopeptide (from 3 to 10 amino acids) containing a target sequence for the ClpX protease (52). In both systems the unstable GFP-variant protein is degraded relatively rapidly, making dynamic investigations possible.

Tools for Investigating Cells Transformed with GFP

The main advantage of GFP is its visual nature, which makes it ideal for microscopic observation. Quantitative measurements of the protein are still somewhat complicated but have been achieved in a few cases. The following applications have especially benefited from the marker: epifluorescence microscopy, FACS/flow cytometry, confocal microscopy and, to a lesser extent, fluorometry.

Fluorescence microscopy of red-shifted GFP variants uses filters and light sources very similar to those needed for the observation of fluorescein-labeled specimens, but for other color variants different combinations are required. Wild-type GFP can be observed using fluorescein filter sets but fluorescence is not optimal because only the secondary excitation peak is used. Similarly, fluorescein filters can be used for flow cytometry or FACS. Using FACS machines it is possible to select mutants with higher GFP signals, for example, for selecting regulatory mutants in shotgun cloning experiments. As already mentioned, FACS was used to select some of the most frequently used GFP variants, the mut1–3 (46). These mutants were selected for higher intensity compared to the wild-type GFP. Although mercury lamps have been used as light sources in flow cytometry, a more useful configuration includes a laser light source, for example, the Argon laser line at 488 nm, which nearly coincides with the secondary excitation peak of wild-type GFP and the excitation peak of most red-shifted mutants.

The confocal microscope has been the tool of choice for investigating GFP-tagged cells. The confocal scanning laser microscope (CSLM) enables the researcher to make three-dimensional representations of thick specimens. A thorough description of the confocal microscope falls outside the scope of this chapter; details can be found in the Handbook of Biological Confocal Microscopy (53). In short, confocal microscopy is based on a conventional epifluorescence microscope, with a modification in the light path. A small pinhole is located at the focal point of a defined image plane, which allows only light from that single plane to pass. Light from planes above or below this image plane will have focal points accordingly in front of or behind the pinhole, with the result that most of the signal from these planes will not pass the aperture. In practice this means that it is possible to virtually slice the sample simply by moving the sample stage up or down and record images from the different positions. Subsequently, the slices can be combined in a computer, generating three-dimensional representations of the sample. This technique is only useful for samples of a certain size as the physical constraints on optical microscopy also apply to confocal microscopy. Single bacteria are so small that they will mainly be detected in single planes, which means that a standard microscope in many cases would be just as useful. However, for intracellular localization in large cells (eukaryotic systems) or in aggregates of cells (e.g., bacterial biofilms) or host interactions (e.g., bacterial infections), the confocal microscope is invaluable in determining the precise location of fluorescent objects. Quantitative measurements of GFP have been done using fluorometers, but several problems with this approach exist. Protein folding is not immediate or always complete, and the protein tends to precipitate to some extent. It is consequently difficult to make absolute quantitative measurements; however, it is possible to measure relative responses.

Applications of GFP

The first report on recombinant GFP expression in microorganisms was that of Chalfie and coworkers (44). They cloned the gene from the jellyfish and expressed it in *E. coli* and in the nematode *Caenorhabditis elegans*. In the bacterial system they inserted the wild-type *gfp* gene after the T7 phage promoter, and achieved green fluorescent cells. There were no other additional components than those required for expression from the T7 promoter. The excitation and emission spectra were virtually identical to those derived from the purified jellyfish protein. This work caused a revolution in the molecular biology community: since 1994, more than 3,300 scientific papers have been published that employ GFP (Science Citation Index). In the spring of 1995 the first international conference devoted to this protein was held in Palo Alto, California, and the proceedings reported initial applications of GFP in biology other than just making cells green (54). GFP is expressed efficiently in almost any bacterium, Gram-positive or Gram-negative. In higher organisms, wild-type GFP has been expressed but variants with improved codon usage which give higher efficiency of expression have been constructed.

E. coli was the first bacterium to be transformed with *gfp*, and since Chalfie's report in 1994 (44), several papers which focus on *E. coli* physiology investigated using GFP tools have been published. Numerous areas have been investigated, for example, physiological state, gene transfer, intercellular localization, identity/identification in multispecies communities, among many others. Most often, the *gfp* gene is fused to an inducible promoter such as the *lac* promoter or the T7 promoter. These promoters are often chosen for convenience of construction and because they can result in high amounts of protein. Several other prokaryotes have been used in addition to *E. coli*. The species used range from Gram-negative *Salmonella* spp. through pseudomonads to Gram-positive lactobacilli and *Bacillus* spp. Here only a few examples of the use of GFP in bacteria in environmental microbiology will be given. More comprehensive lists can be found in studies conducted by Valdivia and coworkers (55) and Errampalli and coworkers (56).

Examples of GFP as a Molecular Marker in Environmental Microbiology

Burlage and coworkers (57) were the first to use GFP in bacteria for environmental monitoring. They followed the transport of GFP-tagged *Pseudomonas* cells in a sand column. They used a Tn5 transposon to label the cells by the insertion of an expression cassette into the host chromosome. Christensen and coworkers (58,59) also used *Pseudomonas* cells to demonstrate gene-transfer events. In these experiments, a conjugative plasmid, the TOL plasmid, was labeled with the *gfp* gene fused to a repressible promoter. The donor cells also expressed the repressor protein; only when the plasmid entered repressor-free recipient cells was GFP synthesized. Tombolini and coworkers (60) labeled *Pseudomonas putida* with a Tn5 cassette containing the *gfp* gene fused to a constitutive *psb* promoter. Using this strain, GFP signal was detectable in the root hairs of *Lotus japonicus* seedlings inoculated with the *Pseudomonas* strain. *Pseudomonas* cells transformed with a stabilized plasmid-encoding GFP was used for experimental environmental monitoring in a mixed population together with unlabeled *Burkholderia* cells (61). This way it was possible to detect the presence and relative position of labeled cells in binary community. The same *Pseudomonas* strain was used for the inoculation of tomato seedlings and subsequent tracking of the cells on developing roots (61). Webb and coworkers demonstrated the utility of GFP expression in the Gram-positive *Bacillus subtilis* (62). They fused *gfp* to a spore coat protein gene, *cotE*, and demonstrated that it was possible to precisely determine the intracellular localization of the compound protein. It was also possible to show fore-spore- or mother cell–specific gene expression using promoters under the control of the sigma factor σF, a sporulation-specific transcription factor, driving GFP expression. Other Gram-positive bacteria successfully transformed with *gfp* include *Lactococcus lactis*, *Streptococcus gordonii*, and *Streptococcus bovis* (42,63). The marine bacteria *Vibrio* spp. and *Psychrobacter* spp. were tagged with GFP expressed from either a *lac* or an *npt-2* promoter by Stretton and coworkers (64). A third marine species, *Pseudoalteromonas* S91, did not show any green fluorescence when using either promoter. To achieve a fluorescence signal from this strain, the group used a promoter-probe transposon with a promoter-less *gfp* gene. This way a number of green fluorescent mutants were isolated and it was possible to identify a chitinase-negative mutant, in which the *chi* promoter was fused to the *gfp* gene, which was inducible by *N*′-acteylglucosamine (64). When the mutant cells were grown in biofilms it was found that GFP expression in mutant cells was associated with the proximal presence of a natural chitin substratum. This may be one of the first reports of determination of localized gene expression in biofilms.

Biofilm research constitutes a separate field in which GFP has proven very useful. Initially, experiments attempted simply to demonstrate the feasibility of using GFP in a living biofilm. Simple tagging to follow one species in a complex environment is still one of the prominent uses of GFP. Eberl and coworkers (65) showed an example of cell-tracking in a simple activated sewage sludge model system. They combined GFP-labeling of one particular species (*Pseudomonas putida* KT2,442) with analysis using oligonucleotide chromosomal rRNA-targeted probing. The use of GFP facilitated online tracking of a strain inoculated in a complex community, whereas subsequent fixation and probing with rDNA oligonucleotide probes made it possible to map the entire sample to establish the spatial relationship (community structure) between species. Similar tagging was used in a study of cell adhesion to activated sludge particles (66). Skillmann and coworkers (67,68) tracked tagged individual species (*Enterobacter agglomerans*) in mixed species biofilms. In these studies GFP fluorescence measurements were additionally used to quantify adhesion efficiency in an in vitro assay.

Møller and coworkers (69) used promoter fusions in model biofilm experiments to demonstrate conditional expression of catabolic pathway genes in a binary model biofilm, consisting of *Pseudomonas putida* R1 and an *Acinetobacter* sp., both capable of using benzyl alcohol as sole carbon source. They showed that commensal behavior occurs and can be measured in an intact microenvironment. When the *Pseudomonas* strain was colonizing a substratum alone the Pm promoter (responding to the presence of benzoate) was not induced on a benzyl alcohol substrate, probably because the amounts of benzoate were rapidly degraded by the strain and consequently kept at very low concentrations. Benzyl alcohol is degraded to catechol via a degradation pathway that has benzoate as an intermediate. When both strains cocolonized the substratum, Pm activity could be detected (as green fluorescence derived from the Pm-GFP cassette). The explanation offered was that *Acinetobacter* was leaking benzoate to the environment owing to a less-efficient degradation of benzoate, and *P. putida* R1 was able to use this (indicated by Pm activity). In a recent study the general physiological status of individual biofilm cells was assessed using an unstable variant of GFP (70). Using a fusion between the ribosomal RNA synthesis promoter P1 from the *E. coli* rrnB operon fused to a variant *gfp* gene, encoding a GFP protein with a half-life of 30 minutes (in batch growth experiments (52)), GFP fluorescence indicated active ribosome synthesis. The P1 promoter is growth phase–regulated, and synthesis of ribosomes occurs only in actively growing cells. Immediately before entering the stationary phase, ribosome synthesis stops. Because the GFP variant has a relatively short half-life, that is, persists only briefly after synthesis has ceased, green fluorescence could be correlated directly to cell proliferation. In the study by Sternberg and coworkers (70) it was demonstrated that cells in biofilms after a period of establishment will stop to proliferate, or do so at a relatively low rate. The same expression cassette was used by Ramos and coworkers (71) to demonstrate spatial and temporal heterogeneity of physiological activities of individual *P. putida* cells colonizing barley seedlings at the single-cell or community level.

RED FLUORESCENT PROTEIN

Recently, the red fluorescent protein (RFP) from the coral *Discosoma* sp. has been cloned (72) and made

commercially available (Clontech, Palo Alto, California). The protein has very different spectral properties than does GFP : RFP has an excitation maximum of 558 nm and an emission maximum of 583 nm. GFP variants have the drawback of spectral overlap; multiple labels based on GFP variants are difficult to differentiate. RFP, on the other hand, has a spectrum that is sufficiently different from GFP to allow easy differentiation of GFP and RFP labels in the same sample. Also, Fradkov and coworkers recently reported a gene-shuffling experiment of RFP from two different subspecies of the Discosoma coral, resulting in a red-shifted RFP with an emission maximum of 616 nm (73) which suggests that the development of spectral variants of RFP may provide a suite of different proteins just as was the case for GFP. However, RFP currently has some serious drawbacks when compared to GFP. A long maturation time and a relatively low fluorescence yield are two major factors that seem to have prevented RFP from having the same immediate application as did GFP. In *Pseudomonas aeruginosa* cells, RFP fluorescence seems to be inversely correlated to growth rate, and fluorescence is easily seen only in cells in late log or stationary phase (74). This appears to reduce the usefulness of RFP in studies of changing gene expression. The problem of low fluorescence (an unfortunate combination of low extinction coefficient and low quantum yield) can be somewhat compensated by enhancing expression efficiency (e.g., through the use of an efficient promoter). Despite these problems, RFP (alone or used in combination with other labels) is well suited as a positional marker for in vivo labeling of cells in a complex community. For example, double labels were used to show that two isogenic bacterial strains (carrying either RFP or GFP) mix very little during biofilm formation (Fig. 3) (74). Similarly, RFP-labeled cells were visualized together with GFP- and GFP-variant-labeled cells during rhizosphere colonization of tomato seedlings (75); three different populations of bacteria were differentiated.

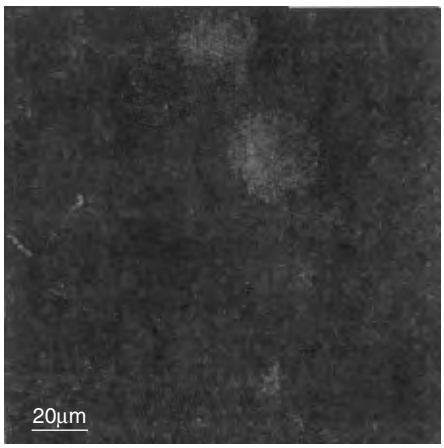

Figure 3. *Pseudomonas* sp. B13 was transformed with either GFP or RFP (chromosomal integration of the fluorescent protein gene under control of a strong promoter), and the fluorescently labeled cells were inoculated simultaneously into an in vitro biofilm growth system. This confocal micrograph shows the biofilm after five days of development. Note that the two bacterial types present in the inoculum, although isogenic except for the respective fluorescent protein gene, tend not to mix within the colonies.

CONCLUSION

Luciferases have a longer history of use than do fluorescent proteins and, although they are not simple to use, the potential rewards (real-time nondestructive data of up- and downregulation) of careful luciferase usage are great. Many problems exist in adapting luciferase-based laboratory assays for use "in the field," but the problems are no greater than those associated with any other switch from the laboratory bench to the factory. The future for luciferases is bright; the development and application of spectral variants will lead to the ability to monitor several genes simultaneously, and the push toward more sensitive detection and ultramicro (single-cell, single-chip) biosensors will assure a role for luciferases in real-world applications.

Fluorescent proteins are relatively new and extremely valuable components in the molecular microbiologist's toolbox. As with luciferases, GFP labels make nondestructive real-time monitoring of bacterial processes possible, but fluorescent protein synthesis and signal production seem to be less affected by cellular metabolic processes or extracellular conditions than for luciferases. Previous approaches to gene-expression monitoring normally required destructive (or at least system-disturbing) procedures. Currently, through the application of luciferases and GFP, the intricate three-dimensional structures of complex microbial communities can be preserved while changes in gene expression can be monitored at the level of the single cell. Although some of these proteins are suitable for only very general studies, others have been highly optimized and customized to create specialized and more informative tools. GFP has been modified with respect to spectral range, stability and maturation times to accommodate a very wide range of applications, and the recently cloned RFP is similarly under intense investigation with the aim of creating a suite of proteins similar to GFP derivatives. The number of research programs in which fluorescent proteins and luciferases play a crucial role underscores the vast impact of these small molecules on biology in general and on microbiology in particular. The impact will probably increase, and these proteins will come to be recognized as the "β-galactosidases of the 90s" and beyond.

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LYME BORRELIOSIS

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DEFINITION

Lyme borreliosis is a zoonosis transmitted by ticks belonging to the *Ixodes* genus (1). The infection is usually subclinical in wild animals. If accidentally transmitted to man, it leads to a two-step disease (2). The first step, an enlarging cutaneous reddish spot, *Erythema migrans* (EM), usually disappears after treatment or even spontaneously. The second step, which may occur alone, is a polymorphic and polysystemic chronic disease (2).

The disease is spread over temperature areas of the four continents in the Northern hemisphere. Although some symptoms have been known from the beginning of the twentieth century, the complete disease has been described after the discovery and first isolation in the United States in 1982 of its causal agent, a spirochete called *Borrelia burgdorferi* (3).

HISTORY

The first cutaneous symptom to have been described, now associated with Lyme borreliosis, is Acrodermatitis Chronicum Atrophicans (ACA), also called *Pick Herxheimer disease*, an extensive skin atrophy that predominates in the extremities (4). In 1910, the Swedish physician, Arvid Afzelius, published the description of the disease hallmark *Erythema migrans*, which followed a tick bite (5). Finally a third symptom has been described named *Lymphadenosis Benigna Cutis* (LBC) or pseudo lymphoma, an inflammatory nodule consecutive to a tick bite (6). Neurological symptoms were shown to accompany EM (7), LBC (8), and ACA (9). Human-to-human experimental transmission has been described by German physicians considering EM (10), ACA (11), and LBC (12). Penicillin was shown to be active on ACA (13), LBC (14), and ECM (15). In the 1950s, it was known that these three cutaneous symptoms were due to the transmission of a bacterium by a tick. Their unification in a single disease was discussed. However, decisive progress was made in the United States when Steere first described Lyme arthritis following EM cutaneous lesion (16). This evolutionary form has only been recently and infrequently reported in Europe, considering ACA (17) and EM (18). The second and definitive arguments were the association of a spirochete to Lyme disease and its isolation (19) from a tick. Such a spirochetal etiology had only been hypothesized in Europe (7,20) before its confirmation (21).

Following the isolation of the bacterium, serologic tests were made possible (1) and allowed Steere to describe in 1982 (22) the whole disease, which was further shown to include LBC and ACA (21). The spirochete responsible for Lyme borreliosis has been called *B. burgdorferi* (3).

BACTERIOLOGY

Taxonomy

An initial study by whole DNA/DNA hybridization, the golden standard in bacterial taxonomy (23), showed that three isolates:

1. B31 from a North American *Ixodes dammini* (now *scapularis*)
2. 297 in a patient's cerebrospinal fluid, and
3. IRS, a European *Ixodes ricinus*, belonged to a unique species (3). This species was unrelated to *Leptospira* genus, loosely associated with *Treponema*, but closely associated with *Borrelia* responsible for relapsing fever, although they constitute a separate and new species called *B. burgdorferi* (*B.b.*) (3).

A second study confirmed the uniqueness of Lyme disease–associated *Borrelia*. However, 9 out of the 10 isolates included in this further study were from North America and the European one was IRS isolate (24).

When considering electrophoretic profiles of many isolates from different origins, it was clear that a large polymorphism among European isolates contrasted with a relative monomorphism of North American ones (25). When considering human isolates, a certain level of homogeneity among European isolates was restored when these isolates were classified according to the tissue from which they had been isolated (25).

Similarly, when isolates from different origins were characterized by distinct molecular typing methods, they usually led to three main groups and several minor ones. It was the case for ribotyping (26,27), *RFLP* Restriction Fragments Length Polymorphism (28), *APPCR* Arbitrarily Primed PCR (29), *PFGE* Pulsed Field Gel Electrophoresis (30), and conformational polymorphic PCR (31).

Further studies by whole DNA/DNA hybridization, including isolates from the main three groups and several minor ones (32–42), led to the delineation and official naming of 10 species. Three of these, *B. burgdorferi* sensu stricto (*B.b.s.s.*), *B. garinii* (*B.g.*), and *B. afzelii* (*B.a.*), include all the isolates that have been isolated from patients (36,42). The other minor species were therefore considered nonpathogenic for humans.

It was later shown that pathogenic isolates from a given species lead to a particular evolutionary clinical presentation. Serology (43,44) and PCR identification (45) lead to these correlations between the species and the clinical presentation, although some authors disagree on the elective role of *B.b.s.s.* in arthritis (46). Indeed, the geographic predominance of a given *Borrelia* species could be correlated with the corresponding preferential organotropism locally prominent. The whole group of *Borrelia* transmitted by hard ticks (*Ixodes*) is called *B. burgdorferi* sensu lato (*B.b.s.l.*).

Morphology

Borrelia have all the features characteristic of the spirochetes (47). Compared with leptospires, *Borrelia* helicoidal cells are larger (10–20 μm) and thicker (0.2 μm). Their spires also are larger and looser. However, they also can pass through 0.45 and 0.22-μm filters.

The mobility of *Borrelia*, due to endoflagella localized in the periplasmic compartment, is a combination of translation, traction, torsion, and screwlike movements. The flexibility of the cell and the fluidity of the outer membrane are spirochetal characteristics.

Frequently, the envelope separates from the protoplasmic cylinder, leading to blebs. This process occurs in adverse conditions and is common to spirochetes. It may even lead to spherical corpuscles of small size, formed by the envelope in which the spirochete body is tightly coiled. The adverse conditions differ among spirochetes — hypertonic conditions for *Leptospira* and hypotonic ones for both *Borrelia* and *Treponema*.

Chemical Composition

Structure and chemical composition are quite homogeneous among the three pathogenic genera of spirochetes. However, there are some striking differences:

- The outer membrane is as usual composed with protein and lipids but, in opposition to *Leptospira*, *Treponema*, and *Borrelia*, do not possess lipopolysaccharides (48). Unlike *Treponema*, *Borrelia* exhibit many proteins and mainly lipoproteins at the surface of their outer membrane. Many of these have been extensively studied and will be detailed later.
- The cell wall (separated from the outer membrane by the periplasmic compartment) is constituted by the peptidoglycan that contains muramic acid and ornithine as amino acid in both *Borrelia* and *Treponema*.
- The flagella (between 6 and 10 per cell extremities) overlap at the center of the cell. As usual, they are constituted by a 41-kd flagellin core (encoded by *fla* gene), although a sheath is more external.
- Lipids are important. Fatty acids are required for the growth of *Borrelia* and are incorporated without modification into cellular lipids. It has been shown that *Borrelia* is unable to either elongate fatty acid chains to β-oxidize long-chain fatty acids.

Characteristics of the Genome of *Borrelia*

The genome of *Borrelia* is original by several features:

- The presence of a chromosome of small size (usual among parasitic bacteria) and, more unexpected, linear (49,50), which is typical of eucaryotic organisms, GC % of the genome is 27 to 33 (34–43 for *Leptospira*).
- The presence of many plasmids (up to 22), some circular and most linear (51), which are called mini chromosomes. Together, these plasmids represent in size more than one-third of the whole genome (52).
- The atypical organization of the genes coding for the ribosomal RNAs. Usually, in bacteria, these genes are encoded in an operon in a canonic order (*rrs, rrl*, and *rrf* coding respectively for 16S, 23S, and 5S rRNAs). Such operons are repeated from 1 to 10 times. In *Borrelia*, a single *rrs* gene is distantly localized from *rrl* and *rrf* genes tandemly repeated (53–55). This unique organization of ribosomal genes among bacteria allowed the design of a specific PCR, although the primers were within highly conserved genes (36).
- It has been shown that many potential or identified pseudogens (and very short open reading frames) are present in most plasmids (56). Such structures, interrupted by stop codons, reflect the fact that plasmidic genes are involved in active duplications and transformations. The observation of large families of paralogous genes (filiation of these genes is due to numerous duplications) on these plasmids reinforces the interpretation of plasticity assigned to plasmidic genes (57). Even a family of highly homologous plasmids with seven members in strain B31 is observed. They are circular plasmids of 32 kb (cp32). This cp32 family also shares homology with a small circular plasmid, cp9, and a large linear one, lp56 (56). This leads to the suggestion that linear plasmids originated from circular DNA. It has also been shown that there are homologies among the telomeric (terminal ends) sequences of some plasmids and even of the linear chromosomes (58).
- It appears that there are recombinations between the different replicons of *B. burgdorferi*, including the chromosome extremities. In conclusion, plasmidic structures are highly plastic ones involved in recombinations, genes duplications, and inactivations, revealing a fast-evolving behavior. Interestingly, the nine circular plasmids plus lp 28-2, lp54, and cp32-like portion of lp56, have been considered as 12 "ameliorated plasmids" although the 10 other linear plasmids should be "rapidly evolving" ones (59). Finally, it has been shown that cp32 has a prophage structure and could be used experimentally for DNA transfers (60).
- The discovery of the linearity and the small size of the *Borrelia* chromosome was a mystifying feature (61). The circularity of the bacterial chromosome was considered as a major difference with the linearity of eukaryotic ones. The homology of sequences in the terminal loop (hairpinlike) of the *Borrelia* chromosome and those from some linear plasmids with one telomere from African Swine Fever virus (ASFV) was surprising. ASFV is an eukaryotic virus transmitted by soft ticks that are also vectors for African relapsing fever *Borrelia* closely related with Lyme disease *Borrelia*. This suggested that a trans-kingdom genetic transfer had occurred (62).
- The genetic maps of one representative from each of the pathogenic species, *B. burgdorferi* sensu stricto, *B. afzelii*, and *B. garinii*, revealed a high level of gene order conservation (55). It could be concluded from this observation that few, if any, genetic

rearrangements occurred among and within these chromosomes.

A further study of phylogenetic trees drawn with sequence data from three genes: two chromosomal ones, *fla* and *bmp* (P39), and a plasmidic one, *ospA*, confirmed the clonal evolutionary mode of the *Borrelia* chromosome that seems to slowly diverge mainly by punctual mutations (63). Only plasmidic genes occasionally showed evidence of genetic transfers.

- In 1997, the genome of *B. burgdorferi* (stain B31) has been sequenced [by the Institute for Genome Research, TIGR (48)]. Among the expected discoveries was the identification of genes potentially associated with the virulence of *Borrelia*. Many pathogenic mechanisms have been evoked concerning *Borrelia*, but few have been confirmed. Therefore, bacteriologists expected that genes homologous to genes already known to confer virulence to other bacteria would be identified.

They were disappointed in this waiting; no clear homology with major genes previously associated with virulence was evidenced. However, many other surprising features were discovered or confirmed. Some of them are described in the paragraph on plasmids. Others are:

- The absence of many metabolic ways or steps, which is indeed expected in strictly parasitic organisms because they reduce their genomes that allow for discretion when invading a host (48). The associated loss of genes as fundamental as those responsible for amino acid synthesis is made possible by the presence of achieved products in ticks and in vertebrates. In correlation with this lack of metabolic enzymes, a high level of membrane transport systems is noticed (64).
- The large number of genes is associated with chimiotactism or motility, which accounts for more than 6% of the chromosome (48,65,66). The particular locomotion apparatus of *Borrelia* localized below the outer membrane should allow a maximal velocity in viscous environments in which external flagella of usual bacteria are inefficient.
- Also, a significant percentage of the coding genome (8%) is dedicated to lipoprotein coding (48). Most of the genes coding for lipoproteins are localized on the plasmids. Some of these lipoproteins have been carefully studied because their expression on levels are regulated according to the different steps of the transmission cycle (67). Indeed, at least two of these are associated with the colonization, either of the tick or the vertebrate host.

PHYSIOLOGY-METABOLISM

Culture

In nature, *Borrelia* live both in arthropods at ambient temperature and in vertebrates that maintain a 37 to 40 °C temperature. Therefore, they are usually grown from 25° to 37 °C; 37 °C is the optimum and growth stops at 40 °C. Due to its microaerophily, it grows best in partially filled (2/3) tubes and growth is prominent one centimeter below the surface. In culture, doubling time is 10 to 12 hours, and a maximum density of 10^9/ml may be achieved. Hypotonicity is for them an adverse condition that leads to blebs and conversion to spherical forms (47,51).

Energy and Respiration

Borrelia are microaerophilic bacteria that do not possess catalase or oxydase. Their carbon sources are carbohydrates and mainly glucose. *Borrelia* incorporates fatty acids that are required for their growth, but do not alter them.

Nitrogen Metabolism

Borrelia are not able to use ammonium salts or urea. They require a supply with amino acids to synthesize proteins. This is also why they are cultured in a medium on the basis of eukaryotic cell requirements.

Other Nutritional Requirements

The BSK II medium that is used to grow *Borrelia* is a complex and very rich medium (51,68). To the basic CMRL that is used in eukaryotic cells, culturing elements are added:

- Hepes as buffer
- Bovine serum albumine
- Normal rabbit serum
- Gelatin (providing a certain level of viscosity in the medium)
- Yeastolate
- N-acetyl-glucosamin, which is a component of chitin that composes the exoskeleton of arthropods.

Resistance to Chemical and Physical Agents

Borrelia are quite sensitive to droughness or hypotony. Similarly, they survive only in a narrow range of pH close to neutral (6.5–8).

They can stay alive in medium at 4 °C for months. Usually, they are kept in deep freezers (10% glycerol or DMSO). In culture, they are killed above 40 °C.

They are sensitive to usual disinfectants, detergents, and most antibiotics (with the exception of rifampicin, phosphomycin, neomycin, kanamycin, sulfamides, and metronidazole).

Antigenic Structure

Antigenic structure (51,69) of *B.b*.s.1. has been extensively studied with two main objectives:

- to optimize the serologic diagnosis potential, which up to now remains rather low in several conditions,
- to define protective antigens that could lead to an efficient vaccine.

The immunodominant antigens are the most outer ones — they are called Osp (Outer Surface Protein) — OspA, OspB, ..., OspF are characterized. They are encoded by plasmidic genes and are lipoproteins. Their potential roles and regulations are described in the Virulence and Pathogeny section. OspA (34–35 kd) and OspC (20–23 kd) are protective antigens (70). *dbpA* and *dbpB* are two genes present on the Lp54 plasmid (harboring also operon OspA B) encoding decorin binding proteins, which are expressed on the surface outer membrane and are protective in a syringe model but not in naturally tick-transmitted infection (71). OspE and OspF encoded by *erp* genes exhibit a certain level of protective immunity.

Many other proteins encoded by chromosomal genes are localized on the surface: P39, whose protective effect conferred by antibodies is controversial (72), and encoded by *bmp* genes or *osm28* and *p66* which are porins, the latter being inaccessible by antibodies when OspA is expressed (73,74).

In the periplasm, another immunodominant protein present is the flagellin (41 kd), encoded by the chromosomal *fla* gene (75). It is not protective.

Others that are deeper in the cell structure, such as the glycosylated p83, are less characterized (76).

EPIDEMIOLOGY AND ECOLOGY

Life Cycle of *Ixodes* Ticks (77)

Ixodes of the *ricinus* group (this *ricinus* group comprises most of the vectors of *B.b*.s.1.: *I. scapularis* and *pacificus* in the United States, *I. ricinus* in Europe, and *I. persulcatus* in Asia) are quite sensitive to droughness. They require the shadow and hygrometry maintained by forests. They cannot survive at altitudes superior to 1,200 m. Their seasonal activity is from February to March and October to November, with sometimes a decrease in summer when it is hot and dry, which determines the infection period and EM occurrence.

Their lifestyle exhibits three stages (larva, nymph, and adult) during a lifetime of 1 to 4-a maximum of 4 to 5 years. Each passage at the next stage by moult (although the third "passage" is egg formation) requires a blood meal. Thus, three (or two in males) blood meals are necessary for a complete cycle.

Larvae feed preferentially on small animals: micromammals or passerine birds (*Peromyscus leucopus* is, for instance, the main host for *I. scapularis* larvae). Nymphs feed usually on larger animals (small carnivores, horses, squirrels, hedgehogs, passerines, etc). Finally, *Ixodes* females require large mammals such as deer, wild boars, or even sheep or dogs. This point is quite important: in the United States, deer are quite necessary to an optimum success of the *I. scapularis* cycle. It explains why a Lyme borreliosis focus exists only in areas where a forest is colonized by many games, including big game.

However, not all animals are competent reservoirs, which means they are not able to harbor for a long term and transmit *Borrelia* to an uninfected tick. This ability depends on both *Borrelia* species and host species (rather than tick species, which have been experimentally shown to harbor and transmit several *Borrelia* species; see following text). Sometimes, vertebrates that are nonpermissive, for a given *Borrelia* species have a zooprophylactic effect on this particular species. Even some hosts that are nonpermissive for any *Borrelia* could play a role in actively decreasing the infection rates in ticks. This is the case for lizards in California (78).

Geographic Distribution of *Borrelia* Species

The geographic distribution of *B.b*.s.1. seems to be associated with vectors (42). Dry areas and high altitude ($>1,200$ m) are not favorable to ticks and are therefore conditions are not favorable for Lyme disease.

B.a. and *B.g*. are present in Eurasia (79), where either *I. ricinus* or *I. persulcatus* do exist (additionally in Korea *B.a*. is transmitted by *I. nipponensis* and *I. granulatus*). *B.b*.s.s. in the United States is mainly associated with either *I. pacificus* in California of *I. scapularis* in the Eastern part of the North American continent (80). In Europe, *B.b*.s.s. seems to be clearly restricted to the *I. ricinus* area; none has been isolated from *I. persulcatus* and none has been isolated from areas where *I. persulcatus* exists alone (79).

However, it has been shown experimentally that any of these vectors could harbor any of these three species, although that does not mean they are efficient vectors for the species they are not usually associated with (81). Another point, which complicates the situation, is that each *Borrelia* species may be isolated from many secondary different vectors: ticks: *Dermacentor variabilis*, *Amblyoma americanum*, *I. uriae*, *I. hexagonus*, *I. trianguliceps*, *Haemophysalis leporipalustris*, and fleas: *Ctenocephalis*, *Ctenophtalmus argyptes*, or even mosquitoes: *Aedes vexans*, and so on (82).

The association between *I. uriae*, a tick parasiting oceanic birds, and *B.g*. has been considered as being able to play a major role in disseminating *B.g*.. Indeed, *B.g*. has been isolated form *I. uriae* in colonies of transoceanic migratory birds all over the world, even in the Southern hemisphere where Lyme disease has not been clearly and unequivocally demonstrated (83–85).

In the same way, the existence of *B.b*.s.s. on two noncontiguous continents was unexpected. Surprisingly, whatever the typing method used, North American and European isolates of *B.b*.s.s. were closely related. This suggested that these two populations were evolving the same way, which supposes that exchanges are frequent. It has been shown that European population of *B.b*.s.s., as compared to North American one, reflects what is called in population genetics a "founder event." It could be inferred from sequences of *ospC*, a highly variable gene that the European population derived recently by importation from the North American one, probably by human transportation (86).

The nonpathogenic species of *B.b*.s.1. are less well-known: in the United States, *B. andersoni* is spread in the area where *I. dentatus* (vector) and the cottontail rabbit (host) are endemic (East part of the United States) (37).

B. bissettii is spread in the Southern states, from California to Florida (39). Although one isolate, 25015,

has been isolated from a *I. scapularis* in New York, the usual vector of most *B. bissettii* are *I. neotomae* and its host is *Neotoma fuscipes*. *B. bissettii* has been reported to be present and pathogenic in Europe (87), but these data are controversial and were not confirmed by other authors.

In Europe (and Asia), *B. lusitaniae* has been rarely isolated from *Ixodes* in Southern areas (Portugal, Moravia, Tunisia, etc.) (40). Potential hosts remain unknown.

B. valaisiana also is spread in both Asia (Korea, China, Japan when it has been found respectively in *I. nipponensis*, *I. granulatus* and *I. columnae*) and Europe (41,42). However, in Europe its distribution is very patchy because it is rarely isolated and its demonstration by PCR has been reported with frequency only in Ireland, Switzerland, and the Netherlands.

Borrelia and Ticks

Some parasitology features are important to appreciate ticks and *Borrelia* associations:

Ticks, when feeding, in opposition to mosquitoes, ingest not only blood but also skin cellular component and intercellular liquid. It has been shown for some viruses that infection of ticks is more frequent by co-feeding through the skin than by blood (88).

Ticks by themselves are unable to move at significant distances — less than 1 meter diameter (50 cm for larvae) (77). Therefore, after egg laying, there are patches of high density of larvae separated by large safe areas. Repartition of nymphs and adults are less known, but it is considered they also are aggregated.

Transtadial transmission of *Borrelia* in ticks is rare (1%) but considering transovarial transmission when it occurs, 50 to 97% of larvae are infected (77). It leads to a mosaic of patches, few of which are highly infecting but most often safe.

In most ticks, *Borrelia* remain in the midgut laying on the mucosa. However, in some of the ticks, a generalized infection occurs and *Borrelia* disseminate in the whole body, including salivary glands (89).

Although larvae are more abundant than nymphs, it is thought that nymphs, more frequently infected (20 times more), play a prominent role in transmission (90).

- In Europe, little is known about *Borrelia* species distribution in the main vector, *I. ricinus*. It has been suggested that *B. afzelii* predominates in nymphs and *B. garinii* and *B.b.*s.s. in adults and therefore in larvae (90). A factor that contributes to further complicate vector-host *Borrelia* relationship is that several strains, which could belong to distinct species, may be present in both hosts and ticks (79,42).
- In terms of temporal distribution, it may vary according to the vector and the climate. Questing ticks are encountered from February to November.
- The pattern is either unimodal (all the season) or bimodal (the number of questing ticks temporarily decreases in summer) (91).

Tick Transmission of *Borrelia* to Hosts

When an infected tick attached to a host begins its blood meal, *Borrelia* move from the midgut and invade the whole body of the *Ixodes* tick and mainly the salivary glands (89). This process requires at least 48 hours and it has been told that removing ticks during this interval prevents the disease (92). However, in some occasions, *Borrelia* dissemination may happen before the blood meal, decreasing up to 18 hours the latency period before infection (93).

The population count of each stage is highly different. A female may lay several thousands of eggs giving birth to a multitude of larvae. Nymphs are about 10 times more frequent than adults. However, infection rates also differ — 1.6 % in larvae, 20 to 40% in nymphs, 40 to 50% in adults (77). Nymphs are thought to play a major role, considering the co-feeding infection potential. An infected nymph attached on a rodent may infect many batches of healthy larvae by a single cutaneous passage (88). This process could take place whatever the tolerance of the rodent, owing to the immune system or competent compatibility toward *Borrelia* genotypes of species.

Borrelia and Hosts

Once in nonhuman hosts, spirochetes slowly migrate through the skin without using blood or lymph circulating system (89). Later on, by blood/lymph circulating system, they invade different organs such as the heart, joints, kidneys, spleen, liver, and urinary bladder (some *Borrelia* may be isolated from urine; 94,95). In North America, *Peromyscus leucopus* (white-footed mouse) is the main reservoir for *B.b.*s.s (96).

Host-*Borrelia* species relationships are of primary importance. The coexistence of distinct species among a single vector (*B.b.*s.s., *B.g.*, *B.a.*, for instance, in *I. ricinus*) is questionable because evolutionarily it is not expected that different species occupy a single niche.

Technical difficulties, such as low efficiency of the isolation procedures, and PCR, which amplifies both living bacteria and DNA from dead bacteria, prevent a clear insight into host-*Borrelia* species associations. *B. afzelii* has been isolated from many micromammals but never from birds. In Western Europe and in Asia (Japan), *B. garinii* is primarily associated with birds (93,97,98). The same is true for *B. valaisiana* that has been detected only from birds (99). In squirrels, the predominance of *B. afzelii* and *B.b.*s.s. has been reported. In Europe, *B.b.*s.s. has been isolated only from squirrels (100) and, on one occasion, was PCR detected from a vole but no strain has been grown (101). These associations, *B.g.* and *B.v.* with birds, *B.a.* with rodents, *B.a.* and *B.b.*s.s. with squirrels, could in Europe constitute the primary or specific maintenance cycles (77).

It has been suggested that the differential sensitivity of a given *Borrelia* species to the complement of different vertebrates could play a major role in defining these cycles (102). It has indeed been shown that *B.a.* was resistant to rodent sera, whereas *B.g.* was readily lysed by rodent sera. This lytic activity could occur in the host itself and within the tick during blood meal, contributing to a reduction in population diversity in ticks.

An incompetent reservoir for a given *Borrelia* species could destroy the *Borrelia* when disseminating in the blood system by the way its humoral immune response (102).

However, in the skin of the same host, transmission of *Borrelia* during co-feeding could occur (88). This has been observed in deer and sheep that do not exhibit systemic infection.

In humans, a dead end for *Borrelia*, the evolution of the infection seems to differ from what is seen in wild hosts (103). In most occasions, the infection would not settle or remain subclinical. Often it leads to a local centrifuge progression of the *Borrelia* within the skin, leading to EM. Rarely, it may lead to delayed and invasive clinical presentations. In this case, a blood dissemination occurs, the bacteria colonize distinct organs in which they persist for months or even years (ACA).

Tick Acquisition of *Borrelia* from Hosts

The definition of a competent reservoir implies two parts — to be able to acquire *Borrelia* from the corresponding vector and to be able to transmit them efficiently to some vector. Both terms must be accurately checked.

For instance, it was believed that blackbirds (*Turdus merula*) were not competent reservoirs because, after being infected by ticks fed on rodents, they were unable to transmit *Borrelia* to healthy ticks (104). In fact, rodents usually harbor *B. afzelii* that do not infect birds. Similarly, it is not possible to get infected ticks from laboratory mice infected by *B. garinii*.

There are two ways to demonstrate the competence of a reservoir:

1. to compare the prevalence in questing ticks and in feeding ticks on a given potential reservoir in the same area. This must be done considering a single *Borrelia* species and one should differentiate co-feeding transmission from blood transmission. This method, however, showed that red squirrel transmitted only *B.b.s.s.* and *B.a.* (100).
2. The most effective way is to feed experimentally healthy (laboratory colony) ticks on tested hosts, which is called xenodiagnosis. It allowed to show that only *B.b.s.s.* and *B. afzelii* were transmitted by rodents and only *B. garinii* by blackbirds and pheasants (105).

Discovery of Lyme Disease

In the 1950s, it was known that EM, LBC, and ACA were infectious syndromes caused by bacteria, possibly spirochetes transmitted by ticks, and could be treated by penicillin. Around 1975, this knowledge was more or less forgotten, probably because of the inability to isolate the pathogen and, consequently, the nonavailability of a biological help in diagnosis. However, in the 1970s, Kelly, an American biologist, developed culture media that can allow a certain level of growth of *Borrelia* that is associated with relapsing fever (106).

In 1975, two mothers of teenagers living in the Old Lyme County (Connecticut) called the Connecticut State Health Department. They informed intelligence officers that, in the Lyme area, many teenagers, including her son, suffered from quite a rare disease — Chronic Juvenile Arthritis (CJA). A clinician from Yale University, specialized in both epidemiology and rheumatology (A.C. Steere), led a careful inquiry in Old Lyme County showing (107):

- the frequency of CJA in Lyme was 100 times higher than in all of the United States.
- when considering only woody areas, the CJA frequency became 100,000 higher
- Clinically, the syndrome observed among Lyme residents differed from CJA by several features. Steere showed that this disease, which he named Lyme arthritis, was rather due to infectious causes than to genetic ones. He determined that Lyme arthritis occurred essentially in Autumn and was not contagious (nonconcomittant outbreaks in a given family). Nutritional or toxic causes were eliminated. An extensive serologic, viral, and bacterial study did not allow the incrimination of a known pathogen. The observation that a cutaneous rash (108) preceded the arthritic symptomatology by several weeks indicates a relationship between this chronic arthritis and EM that had been described years ago in Europe. In 1970, Lyme arthritis came to be considered Lyme disease in the United States (109). Steere also noticed that cutaneous rash often occurred in parts of the body that could be in contact with the ground, assuming that a nonflying arthropod should be considered a potential vector.

Two years later, a medical entomologist, William Burgdorfer, was looking for *Rickettsiae* in *Ixodes dammini*, now called *I. scapularis*. He observed spirochetes and thought they could be associated with Lyme disease. He performed slide films directly with tick midguts. An immunofluorescence test, done with sera of patients from Lyme and then with EM European patients, confirmed this hypothesis, leading to two articles: "Lyme disease, a tick-borne spirochetosis?" (1) and "Erythema chronicum migrans, a tick-borne spirochetosis?" (110).

In 1984, Allan Barbour succeeded in growing the spirochetes in a medium called BSK (1,111), which led to serodiagnosis tests that helped describe the disease extensively. This included the specifically European symptomatologies associated with *Borrelia* species which are absent from the United States, leading to "Lyme borreliosis" being the final denomination of the disease.

Incidence of Lyme Borreliosis and Descriptive Epidemiology (112)

In the United States, where Lyme borreliosis has been a notifiable disease from the beginning, incidence of the disease increased from 1982 to 1996. In 1985, the number of cases was 2,748, and rose to 16,802 in 1998. This increase, of course, reflects increased surveillance and better awareness of the disease by practitioneers as well as some changes in case definition (in 1991) and maybe a true increase in incidence in known endemic areas.

From the 88,967 cases in the period 1992 to 1998, 92% were reported by only 10 states (in decreasing order: New York, Connecticut, Pennsylvania, New Jersey, Wisconsin, Rhode Island, Maryland, Massachusetts, Minnesota, and

Delaware). In addition, in each state a restricted number of counties reported most cases (in New York state responsible for 32.8% of 1992 to 1998, 81.9% of cases were from 5 out of 62 countries), leading to highly contrasted maps of endemic rates.

Regarding ages, the distribution is bimodal: reported incidence is higher in 5- to 9-year-old children and adults about 45 to 54. 51.9% patients are male.

Although onset of the disease may occur all over the year (from February: minimum of 1.6% to a maximum of 30.8% in July), summer is clearly the preferential season of onset (56.9% of cases occur between June and August). The onset of the disease out of the activity period of ticks reflects the cases diagnozed directly at chronic stages.

Lyme borreliosis in the United States is the most prevalent vector-borne disease. However, there are some controversies on the accuracy of the surveillance system. Indeed, as most diseases reported through a passive surveillance system, under-reporting is usual. However, considering Lyme disease, which is a highly polymorphic and chronic disease with a deficit in biological diagnosis, there is also a significant risk of overestimation. Several syndromes with not-yet elucidated etiology, such as chronic fatigue syndrome or "Yuppie's disease" in the United States, multisclerosis or Alzheimer disease in Europe, have been wrongly attributed to *B.b.s.l* (113). A wrong use of the diagnosis tests and unscientific information diffused by patients' associations, could be partially responsible for this misdiagnoses that lead to inaccurate treatments (several antibiotics over months ...). Despite these excesses, which usually are not reported, the reporting system provides an efficient picture of the disease in the United States. In addition, CDC made available a rationale for the correct use of biological tests, constituting a simple and efficient guideline.

In Europe, there are large heterogeneities within both the endemic rates and the surveillance system of Lyme borreliosis. A consensus on case definition was not obtained before 1996 (114), and in less endemic countries, no compulsory reporting system has been established.

It seems that the incidence of Lyme borreliosis is low in Western (Atlantic coast) and Southern parts (Mediterranean border characterized by drought) of Europe. In opposition, incidence is important in the North East and mainly Central Europe. Probably, Austria, Germany, and Slovenia are among the countries that report the higher number of cases.

Simultaneously, the *Borrelia* species are not equally distributed in Europe (115,42). In Western and Central Europe, *B. garinii* predominates. In Scandinavia, *B. afzelii* is predominant. In Southern Europe, the three pathogenic species are equally infrequent.

VIRULENCE AND PATHOGENESIS

Many virulence or pathogenesis mechanisms have been proposed. However, few, if any, have been confirmed.

It has been the case for hemolysins, adhesins, lectins, invasins .... Among those that are still under study (69) are

- An antigenic variation mechanism: Such an antigenic variation has been clearly characterized in relapsing fever *Borrelia* (116,117). However, *B.b.*s.l is not a blood bacterium as *Borrelia hermsii* or other *Borrelia* associated to relapsing fever. Anyway, a protein VlsE, belonging to the Vlp family (variable large protein), is the product of a plasmidic (*lp28.1*) gene *vls* that exhibits homology with the *vmp* system in *B. hermsii* (118). Multiple and independent recombinations occur between silent copies of *vls*. This phenomenon occurs in vivo independently of the immune response of mice. Such a system could lead to a selection of antigenic variants and constitutes a way to escape the immune response. Invariable regions (IR) of VlsE are useful as diagnosis antigens, one of them (IR6) being immunodominant (119).
- Similarly, it has been shown that proteins encoded by plasmidic genes *erp* analogous to ospE and F are expressed immediately after host infection eliciting early and partially protective antibodies. These proteins are expressed simultaneously to the temperature increase caused by the blood meal of the tick (from 20 to 35 °C). Again, these proteins are submitted to recombinations and could contribute to evasion from the immune system (120,121).
- One among the potential virulence factors, is well documented. It has been shown by several authors that *B.b.*s.s. was able to adsorb plasminogen on its surface (122). Plasminogen, when activated, gives birth to plasmin which is a host serine protease. These plasmin covered *Borrelia* have been shown to degrade the extracellular matrix (123). This mechanism could explain the ability of *Borrelia* together with the mobility with which it passes through membranes (invasiveness) (124).
- Finally, an imunological mechanism of pathogenicity has been evidenced which could explain chronic arthritis resistant to antibiotherapy. A nine amino acids peptide is shared in common by *ospA* from *B.b.*s.s. (125) and a *hLFA-1* peptide present on human leukocytes associated with allele DRB1 0401 from the major histocompatibility system. Antibodies elicited in certain conditions against *ospA* will act as autoantibodies responsible for articular lesions observed even after elimination of bacteria.

Regulation of the Expression of Two Lipoproteins, Potential Virulent Role and Applications (69)

Two outer membrane lipoproteins are of particular interest. OspA 31–32 kd and OspC 20–23 kd are both immunodominant and protective, which is a challenge for a bacterium (126,127). Each of these two lipoproteins illustrates a distinct strategy used by *Borrelia* to circumvent the immune response and have potential interest in diagnosis and vaccination. OspA is highly expressed within tick environment and repressed in the vertebrate host (128), whereas the opposite is true for OspC (129). The shift in regulation takes place very quickly: within the first 24 hours of the blood meal, the stimulus probably being the 37 °C of the host blood. This suggests a role for OspA restricted to tick environment.

Later on, its synthesis is quickly repressed to prevent elicitation of antibodies lethal for the bacterium. Recently, a role for OspA has been elucidated: it is an adhesin for an intestinal tick cells receptor (130). In opposition, OspC, expressed during and after the blood meal, probably should be useful in the initial host colonization because, in spite of its immunogenicity making it susceptible to initiate the killing of the bacterium, its expression is not repressed. However, after this initial phase, the bacterium survives in spite of the antibodies. The *ospC* gene exhibits a huge variability (because of mutations but also and overall to horizontal transfers of the highly variable central part of the gene) (131). This diversity could be interpreted as a mechanism to prevent serilisation of previously infected ticks by the blood of a previously infected host. The effects of the diversifying mechanism (analogous to the diversifying selection observed in immunoglobulins synthesis, for instance) is such that in a given place, there may be isolated bacteria bearing almost all the repertoire of *ospC* genotypes.

Recently, it has been shown that, among this whole repertoire, only a fraction of genotypes is able to infect humans. A further reduced fraction is able to lead to invasive (disseminating to deep organs after blood invasion) and chronic forms of the disease (132,103).

SYMPTOMATOLOGY (2)

Lyme borreliosis is often described in three phases. This model comes from syphilis and is sometimes artificial.

Presently, it seems more suitable to distinguish only an early and limited phase and a late and disseminated phase. These two stages usually are successive. However, each one may appear alone (133).

Early Phase

Erythema Migrans (EM). Although the blood meal of the tick requires at least a fixation of the parasitic acarian for five days, sometimes it remains unnoticed: larvae are very small and some localizations are hidden (folds, hair ...)

From 10 days to one week after the tick bite, a reddish unelevated spot may appear around the bite. It will enlarge in a centrifugal way, the edge is often redder and the center becomes normal again, giving it a "bull's-eye" aspect. It has been shown that bacteria are present ahead of the reddish edge that represents the inflammatory response following the intra dermal progression of the motile bacteria.

This lesion is the hallmark of the disease and, when typical, requires a treatment without any other confirmation.

In some occasions, EM may be multiple either directly or more often with a delay. This could represent a migration of bacteria using the lymphatic way.

Similarly, some general signs, such as a moderate fever, asthenia, and diverse pains (head, joints, muscles) ..., could indicate a certain level of diffusion of bacteria in the whole organism.

Another infrequent kind of lesion at this stage is very rare in the United States if at all: *Lymphadenosis Benigna Cutis (LBC)* or *Pseudolymphoma*. It appears on elective areas such as the ear lobe, nose, or forehead. It is a nodular and inflammatory lesion. Histologically, it is characterized by a uniform infiltration of poorly differentiated lymphocytes. It may be easily confused with a true lymphoma. In fact, this lesion is often caused by *B.a.*, which does not exist in the United States.

Late Phase

Several weeks or even months after the tick bite, several kinds of syndromes may appear. They are usually exclusive from one another (133).

Neurological Syndrome. Typically, it consists of a meningo radiculitis. The meningitis is subacute with a moderate increase of white cells and protein.

Nevritis concerns both cranial nerves, mainly the 7th with a facial palsy, and peripheral nerves. In these cases, the symptomatology predominates on sensitivity with pain.

Neuroborreliosis is twice as frequent in Europe than in the United States (134).

— *Cardiac symptoms*. They are rare and mainly consist of auriculo-ventricular block of stades 2 or 3. In exceptional cases endocarditis, myocarditis, or even pancarditis may occur. They are not as rare in the United States (134).

— *Late cutaneous symptoms*. Several months after the tick bite, a cutaneous inflammation appears which predominates at the limbs extremities. It evolves slowly over the years, to a considerable skin atrophy that makes the venous network visible. This lesion, *Acrodermatitis Chronicum Atrophicans (ACA)*, is rare in the United States and the described cases always concerned migrants from Europe where the frequency is higher in Northern and Eastern parts (134,135).

Biological Diagnosis

In fact, the biological tests are in limited number and their results must be interpreted with circumspection.

— *The unspecific tests*. relate to blood, CSF, and joint fluid: they look for infection or signs of inflammation. However, they are usually subnormal except in CSF where a moderate pleiocytosis or lymphocytosis, and an increased protein level, may be observed (2).

Specific Tests

— *Direct tests*. Bacteriology. Direct microscopic examination is of no interest. Culture is efficient on skin biopsies (20 to 50 % positive) (136). However, it is not quite necessary since the EM observation is sufficient for diagnosis. Positive results are rare on CSF and exceptional in joint fluid or synovial tissue.

— *PCR*. PCR has exactly the same indications and results as culture. It is therefore pathognomonic

when positive, which is rare except on skin biopsies (137).

— *The antigen "research" tests*. have been discarded because of insufficient reliability.

— *Indirect tests*. Serology is the most commonly used test but presents imperfections in terms of both sensitivity and specificity.

Its extensive use and interpretation led the CDC and the American College of Physicians to promote restrictive but rational rules (138,139):

- not useful at the EM stage (the lesion itself is a positive diagnosis and serology is often negative at this stage).
- later on, presumptive methods (Immunofluorescence or preferably ELISA) should be prescribed only in case of compatible symptoms in an area where Lyme disease is known to exist.
- the western blot must be prescribed only in case of a doubtful presumptive test, it is then confirmative.

The main problems with serology are in fact false positive results (140).

Finally, the limitations encountered in all the tests may be on account of the low level of bacteria in the host body, which is a characteristic in this disease.

PREVENTION AND TREATMENT OF LYME BORRELIOSIS

Collective

There are few collective (113) prevention means and they are usually hazardous to the environment.

Elimination of rodent populations is unrealistic but landscape modification can help in reducing them. Reduction of the deer population would be an efficient method to decrease the tick population. The increase in the population of deer has been responsible for the extension of risk areas in the United States.

If deer were prevented from freely circulating in the neighborhood of houses it would limit the tick infestation of the gardens.

Up to now, the collective use of acaricids in the wild has been proposed but they have been used only on a local scale.

The repartition of permethrin impregnated cotton by a self delivery system for mice in periods of rodent nesting is efficient but cannot be recommended everywhere because of environment concerns. A more efficient way is the automatic application of acaricid on deer in specially equipped deer-feeding stations.

A biological method has been proposed with an insect parasiting tick and laboratory colonies of this insect have been settled.

Finally, only an integrated prevention associating several means: host reduction, habitat modification, and insecticide application, may be efficient in the long term.

Individual Prevention (113)

1. Behavioural measures.

- The knowledge of the high-risk areas and periods is important.
- To wear long and covering clothes with sleeves and trouser legs (recovered by socks) tightly laced is quite useful.
- The use of repellents (the main ones are NN-diethyl-mtoluamide: DEET, and permethrin which is also acaricid) on impregnated clothes is efficient.
- A careful examination of the entire body (including the head) must be carefully carried out by an observer, after a walk in the forest.

2. Vaccine.

   A vaccine based on recombinant OspA has been studied by two companies. Their results have been published in the same issue of the New England Journal of Medicine in July 1998 (141,142). Results from both formulations were more or less similar. However, only one of these companies (Smith Kline Beecham) has been commercializing the vaccine from the beginning of 1999 under the name Lymerix.

Principle: The Lymerix vaccine is made of the lipid form of recombinant (expressed in *E. coli*), OspA: L-OspA. The injection dose is 30 μg of purified L-OspA adsorbed to aluminum hydroxide.

The originality of this vaccine is that its activity takes place not in the vaccinated human subject but in the biting tick body (143).

The anti-OspA antibodies are ingested by the tick during its blood meal and allow the *Borrelia* killing during the period OspA protein is still expressed in the outer membrane of the infecting *Borrelia*.

The vaccine has been made of OspA of a *B.b.*s.s. isolate and is therefore useless elsewhere than in North America where the only pathogenic species is *B.b.*s.s.

Formulations suitable for Europe and Asia are studied but the diversity of OspA among *B.g.* is an obstacle. It has been proposed to add a second antigen such as decorin-binding proteins (DBPs). However, it has been shown that a protective immunization with DBPA is reached in mice by needle inoculation challenge but not by tick inoculation challenge (71).

- Clinical trial:

  10,936 subjects living in endemic areas were randomly divided into two groups and received different schemes of Lymerix vaccine injection. They were examined clinically and biologically. The conclusions were the following ones: a global efficiency of 76 % (definite cases) after two years (3 injections) (141).
- Conclusion

  Indeed the efficacy is not complete and the repetition of injections a constraint. Probably, a cause for immune escape of *Borrelia* is caused by the fact that, during the first days (slow feeding phase) of the

tick attachment, only minute amounts of blood are sucked, although the tick temperature rises. When most of the blood meal is obtained in the late period (in fact, last day out of 5 to 7), OspA is usually no more expressed.

3. Treatment (144).

Early Lyme disease

- It has been demonstrated that 100 mg twice daily of doxycycline is as effective as amoxycline 500 mg three times daily and cefuroxixme 500 mg twice daily to treat early localized or disseminated Lyme disease (145). Additionally, docycycline is also efficient against erlichiosis also transmitted by *Ixodes* ticks. A three weeks treatment is recommended. For children above 9 years doxycycline may be used (100 mg twice daily). For children (whatever their age, amoxillin 50 mg/kg/day in three times (500 mg dose maximum) or cefuroxime 30 or 40 mg/day twice daily (maximum: 500 mg) during three weeks.

Late Lyme disease

- When central nervous system is involved, intravenous treatment is to be preferred: either penicillin IV (24 millions Unit daily or ceftriaxone: 2 mg daily IV). For children, either a single IV dose 50–60 mg/kg/day (2 g maximum) or 200,000–400,000/kg/day (20 M maximum) are suitable for three weeks (maximum duration with ceftriaxone).
- Lyme arthritis (146): oral regimens are usually as effective as intravenous ones and they may be administered longer, which is advantageous. In adults, doxycycline 100 mg twice daily or amoxillin 500 mg four times daily for four weeks are recommended. Concerning children, a treatment similar to EM one is suitable when administered 30 days.
- Treatment-resistant Lyme arthritis (146)
- When joint swelling persists after two normal courses of treatment, it is suitable to use intra-articular steroids and non steroids anti-inflammatory.
- On some rare occasions, arthroscopy synovectomy may be suitable.

CONCLUSION

Lyme borreliosis was not recognized in the United Stated before 1976. It is now the most prevalent vector-borne disease in that country. Its emergence is in part due to the recent desire of humans to live in close contact with nature and possibly to an increasing population of deer. In addition to individual prophylactic measure, only a vaccine is able to afford active protection against the disease. Such a vaccine already exists in the United States, but it is not suitable for Europe or Asia. The need for an efficient vaccine on a global scale is the actual challenge.

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MACHINING AND BIOAEROSOLS.

See Bioaerosols in Industrial Settings

MACROPHYTE COLONIZATION BY FUNGI.

See Water Fungi as Decomposers in Freshwater Ecosystems

MAGNETOTACTIC BACTERIA.

See Biomineralization by Bacteria

MANGANESE CYCLING.

See Metal (U, Fe, Mn, Hg) Cycling

MANGANESE OXIDATION.

See Biomineralization by Bacteria

MARINE BIOTECHNOLOGY

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The deliberate manipulation of living organisms to serve practical human needs has a long history, dating back at least as far as the rise of agriculture. Over the millennia, the tools available to humans for the control of biological processes have become more and more technologically sophisticated. Biotechnology, defined as "any technique that uses living organisms (or parts of organisms) to make or modify products, to improve plants or animals, or to develop microorganisms for specific uses (1)," is not a new concept. However, the modern biotechnology industry originated only three decades ago. Today, biotechnology combines commercial and scientific enterprises under the unifying principle of utilizing life to serve life. New tools, such as genetic engineering techniques, advanced microbial bioreactors, and natural product chemistry have contributed to rapid development in the fields of health, agriculture, manufacturing, and environmental remediation. It is therefore not surprising that "biotechnology is expected to have a dramatic effect on the world economy over the next few decades (2)."

The development of new biotechnological applications is often linked to the discovery of new organisms, novel compounds produced by organisms, or unique metabolic pathways of organisms. Marine ecosystems contain a major share of the biological resources of the planet and host highly diverse populations. Moreover, the vast majority of marine biota remains untapped as a resource for biotechnological applications. The practical application of marine organisms for the development of new products and processes comprises the burgeoning field of marine biotechnology. Although many conflicting definitions of this term may be found in the literature, the definition of marine biotechnology used here is "the application of scientific and engineering principles to the processing of materials by marine biological agents to provide goods and services (3)." Within this broad characterization lie processes involving the use of marine animals, plants, fungi, protists, algae, cyanobacteria, bacteria, and archaea; however, this article will emphasize the use of the microbes in marine biotechnology. An exhaustive review of the subject is not possible here, but selected examples from the literature will be presented. The term *marine* must also be qualified, as a number of important developments in marine biotechnology have actually been made using microorganisms from diverse aquatic environments including hot springs, hypersaline ponds, and ephemeral freshwater pools. Eight major subject areas are discussed:

- Marine natural products, including pharmaceuticals, pigments, nutritional supplements, enzymes, biomaterials, and specialty products.
- Energy production from marine organisms and the use of marine microbes to mitigate the introduction of carbon dioxide from fossil fuel combustion into the atmosphere.
- Marine bioprocesses, including environmental bioremediation and wastewater treatment, the formation and effects of biofilms, and the development of genetic engineering techniques utilizing marine microbes.
- Aquaculture, including the use of genetic engineering to enhance the growth and fecundity of cultured organisms, and the production of microbial feedstocks for aquacultured animals.
- Marine health and safety, including the prevention of marine animal diseases and human diseases of marine origin, and techniques for detecting pathogens and toxins in seafoods and marine waters.
- Marine ecology, including the identification of marine organisms, the assessment of marine ecosystem biodiversity, and the study of marine microbial metabolism.
- Marine bioprocess engineering and the steps taken to bring marine microbial products from discovery to commercialization.

- Legal aspects of marine biotechnology, including concerns over the use of genetically modified marine organisms.

MARINE NATURAL PRODUCTS

Biomass Protein

The production of foodstuffs through cultivation of marine macroalgae (seaweeds) has a long history, particularly in Japan and China (4). The red alga *Porphyra*, the brown kelps *Laminaria* and *Undaria*, and the green alga *Monostroma* are just a few food-grade examples of at least 221 seaweed species now cultivated worldwide (5,6). Tissue culture techniques are widely used for the production of enhanced strains capable of rapid growth and to control the life cycles of the farmed algae. Protoplast fusion is also used to create improved hybrid strains (5). Both the seaweeds and the microalgae grow rapidly, and are generally rich in protein, hence, both have been considered as potential sources of biomass protein for large-scale human and animal consumption. Some of the earliest attempts at the mass cultivation of microalgae were carried out to test the feasibility of low-cost high-protein food production from the green alga *Chlorella*. These efforts, carried out in the early 1950s, revealed the impracticality of using microalgae for food, mainly because of the production costs, which were far in excess of those associated with existing plant protein sources such as soybeans (4). However, the ability to mass-culture microalgae was demonstrated, and this has led to ever-improving technology and many successful efforts to commercialize the production of high-value microalgal products (7).

Pharmaceuticals and other Bioactive Compounds

Perhaps the most exciting application of marine biotechnology is the identification, production, and commercialization of novel bioactive compounds from marine organisms. Bioactive secondary metabolites have been discovered in a wide variety of marine animals, plants, and microorganisms. Some of the most significant discoveries have been natural products associated with marine invertebrates (sponges, corals, bryozoans, tunicates, etc.), including antitumor compounds, anti-inflammatory compounds, antibiotics and antiviral agents, and immunosuppressants and antiparasitic agents (8). Thousands of such bioactive compounds have been discovered, yet despite their vast potential, as of 1995 only the antiviral arabinose nucleosides ara-A and ara-C had been marketed and put into clinical use (9). Probably, the single greatest impediment to the commercialization of marine natural products is the lack of adequate supply. Natural product chemists are able to screen raw extracts for bioactivity and isolate and characterize promising compounds using relatively small quantities of source material, but clinical trials require much larger supplies and pharmaceutical production requires a renewable resource base. Environmental supplies of such metabolite-rich marine animals as gorgonians and bryozoans would quickly be wiped out if they were put into industrial-scale pharmaceutical production. One way to address this problem is to develop the requisite culture techniques for the organisms of interest. Using this approach with marine invertebrates can be very difficult, but in one notable casework is under way to reliably produce the promising anticancer drug bryostatin-1 from aquacultured bryozoans (10). Another approach to the supply problem is the chemical synthesis of the newly characterized molecules and related molecules that may show superior activity. For example, total chemical syntheses of prostaglandins created a sufficient supply of these chemicals for clinical trials, probably saving the prostaglandin-rich Caribbean gorgonian *Plexura homomalla* from being overharvested (11). Often, however, the complexity of bioactive marine compounds prevents the cost-effective development of chemical syntheses, precluding pharmaceutical companies from investing into their development as drugs.

Marine microorganisms hold enormous potential as sources for new drugs. Marine microalgae, cyanobacteria, bacteria, and fungi produce a wide range of secondary metabolites of possible pharmaceutical utility (Table 1). In some cases, interesting compounds originally isolated from the tissues of marine animals, plants, and macroalgae have later been shown to originate from commensal or symbiotic bacteria living on or in their tissues (12). In addition, several factors make production of bioactive compounds from marine microbes more attractive than production from marine animals (13). Unlike marine animals, many microbes may be cultivated readily in bioreactors under controlled environmental conditions. Such control is essential for industrial situations, where reproducible production of a metabolite is a must. Furthermore, microbes grow rapidly, allowing for strain selection of hyperproducers to be carried out on relatively short timescales, and reducing the time required for production of significant quantities of metabolites. Microbes are also more easily transformed genetically than higher organisms, enabling the cloning of genes encoding metabolite production from one organism to another whose growth can be controlled better. The hazards associated with release of transgenic organisms into the environment are also reduced when closed bioreactor technology is employed for cultivation, as opposed to the open-system outdoor technology commonly used for cultivating marine animals.

Bioactive compounds have been isolated from bacteria inhabiting a wide variety of marine environments (Table 1). For example, free-living water column *Alteromonas* sp. from the sea have yielded brominated pyrroles and other bromine-containing compounds with antibiotic properties, whereas bacteria from deep-sea sediments produced the novel cytotoxic and antiviral microlactins (12). Marine microalgae, too, have yielded bioactive compounds. A host of red-tide dinoflagellates produce cytotoxic compounds of pharmacological utility, including the antitumor and antibiotic macrolides, the antifungal goniodomin, and the sodium channel blocker saxitoxin (25). Other microalgae produce bioactive compounds, such as the green alga *Dunaliella primolecta*, which contains antiherpesvirus substances (26), and certain diatoms

Table 1. Some Useful and Potentially Useful Bioproducts from Marine Microorganisms

Product	Current/Potential Use	Organism	Type/Origin	References
Brominated pyrroles	Antibiotics	*Alteromonas* sp.	Marine water column bacterium	12
Majusculamides	Anticancer drugs	*Lyngbya majuscula*	Marine cyanobacterium	14
Hormothamnins	Anticancer drugs, fungicides	*Hormothamnion enteromorphoides*	Marine cyanobacterium	14
Cephalosporins	Antibiotics	*Cephalosporium acremonium*	Marine fungus	1
Polyunsaturated fatty acids	Dietary supplements	*Crypthecodinium cohnii*	Dinoflagellate from coastal waters	15
		Thraustochytrium spp.	Fungoid protists from various marine environments	16
β-Carotene	Dietary supplements, food colorings	*Dunaliella salina*	Green microalga from alkaline saline lakes and marine waters	17
Astaxanthin	Dietary supplements, animal feed additive	*Haematococcus pluvialis*	Green microalga from ephemeral freshwater pools	17
Thermostable polysaccharide-hydrolyzing enzymes	Starch hydrolysis, paper manufacturing	*Rhodothermus marinus*	Thermophilic bacterium from submarine hot springs	18,19
		Thermococcus hydrothermalis	Hyperthermophilic archaeon from deep-sea hydrothermal vent	20
Thermostable DNA polymerases	DNA amplification by polymerase chain reaction	*Thermus aquaticus*	Thermophilic bacterium from terrestrial hot springs	21
		Pyrococcus furiosus	Hyperthermophilic archaeon from geothermally heated marine sediments	22
Cold-adapted lipases	Cold water detergents	*Moraxella* spp.	Bacteria from Antarctic seawater	23
Polysaccharides	Gelling agents, oil recovery	*Porphyridium cruentum*	Red microalga from various marine and terrestrial systems	7, 24
Phycocyanin	Medical diagnostics	*Spirulina platensis*	Cyanobacterium from alkaline saline lakes and coastal flats	7

that produce powerful antibiotics (27). Marine fungi, while less studied than other marine microorganisms, show promise as a source of novel compounds (28); a marine fungus was the original source for the commonly used cephalosporin drugs (1). Finally, the cyanobacteria are perhaps the most intriguing group of marine microorganisms for drug discovery. Cyanobacteria produce diverse cytotoxins, potentially useful as antibiotics against gram-positive bacteria and as fungicides (29), as well as potential anticancer and HIV-inhibiting compounds (30). A number of compounds with promising anticancer and antifungal properties have been isolated from marine cyanobacteria, including the majusculamides and the hormothamnins (14). Furthermore, enhanced biomass and/or metabolite accumulation in cyanobacteria has been achieved through the careful control of environmental factors during culturing, making these organisms attractive potential candidates for pharmaceutical development (29).

Pigments and Nutraceuticals

For bioactive natural compounds, the progression from discovery to commercial pharmaceutical use can take many years, many millions of dollars, and faces substantial regulatory hurdles. The reward for success can be sole access to billion-dollar markets. On the other hand, marketing natural products as whole health foods, nutraceuticals, dietary supplements, or feed additives allows for more rapid and less expensive introduction into a less-regulated commercial arena. The industrial-scale production of marine bioproducts from both microalgae and cyanobacteria has been successfully carried out by following this commercial approach. Polyunsaturated fatty acids (PUFAs) have been shown to have beneficial health effects on human health, particularly with regard to the prevention of coronary heart disease and in supporting the proper brain development of infants (31). Fish oils are the primary dietary source of PUFAs, but recently, infant formulae rich in PUFAs

have been successfully produced from microalgae grown heterotrophically in bioreactors (15,32; Table 1). Potential new candidates for the marine biotechnological production of PUFAs include the thraustochytrids (16). Carotenoid pigments are another class of compounds that have been successfully mass-produced from microalgae, both in open ponds and raceways and in photobioreactors, to make human dietary supplements and feed additives for animal nutrition. Two prominent examples produced photoautotrophically from green algae, are β-carotene from *Dunaliella* and astaxanthin from *Haematococcus* (Table 1;17). Natural microalgal carotenoids are also becoming attractive as additives for cosmetics because of their UV light-absorbing properties. Probably the biggest success stories so far in the commercial biomanufacturing of marine microalgal products are the whole-biomass health food products from the green alga *Chlorella* and the cyanobacterium *Spirulina*, which both boast annual production figures in the thousands of tons dry weight (17).

Enzymes

One of the most significant contributions of marine biotechnology to date has been the isolation and production of novel enzymes for industrial processes and molecular biology. Many industrial processes can be improved through the use of enzymes that are active under conditions of high salt, high or low temperature, high or low pH, and/or high pressure. Marine microorganisms isolated from extreme environments have evolved the capacity to produce enzymes capable of being active under extreme conditions. For example, marine vibrio bacteria have been a source for salt-tolerant proteases used in detergents and membrane cleaning formulations (3). The bacterium *Rhodothermus marinus*, isolated from a marine hot spring, has produced industrially useful polysaccharide-hydrolyzing mannanase and cellulase enzymes that remain active at temperatures exceeding 80 °C (Table 1; 18,19). Similarly, a thermostable α-glucosidase is produced by the hyperthermophilic deep-sea hydrothermal-vent archaeon *Thermococcus hydrothermalis* (20). Thermostable DNA polymerases, such as that from the hot spring bacterium *Thermus aquaticus*, form the basis for the polymerase chain reaction (PCR), a widely used and powerful technique in molecular biology (21). A new thermostable DNA polymerase with unique properties was recently purified from the hyperthermophilic archaeon *Pyrococcus furiosus* (22). Cold-adapted enzymes from psychrophilic marine microorganisms, such as the lipases from the Antarctic marine bacteria *Moraxella* spp., are also of potential interest for use in cold water detergents and other industrial applications (23; Table 1). Some of these cold-tolerant enzymes can be cloned into yeasts for commercial production using conventional fermentation technology (33).

Polysaccharides, Biopolymers, and Biomaterials

An important area of marine biotechnology is the development and production of useful biomaterials from marine organisms. In some cases, materials derived from marine organisms have unique properties, whereas in others, bioproduction is seen as an economically viable alternative to synthetic sources. Marine macroalgae have long been used as an abundant natural source for phycocolloid polysaccharides, which are used as emulsifiers, solidifiers, stabilizers, and clarifiers in a wide variety of foods, drinks, and other products (5). Although some seaweeds are still harvested from the wild, there is an ever-increasing industry based around farming of macroalgae. Major products include agars and carrageenans from red algae and alginates from brown algae (4). Agars are of particular significance to the biotechnology industries, as they are used as substrates for microbial cell cultures and for DNA separations. Sulfated polysaccharides are another class of useful compounds, produced by the red microalga *Porphyridium cruentum* (7). Although *P. cruentum* has been successfully cultivated in large-scale photobioreactors (24), economically viable production has been elusive. Two other marine polysaccharide biomaterials of note are chitin and chitosan, both isolated from the exoskeletal wastes of crustaceans, but potentially yielded from marine fungi as well (34). These compounds are used as adhesives, biodegradable medical products, artificial membranes, and have a host of other applications because of their strength and flexibility. Natural laminated composite materials from marine mollusk shells, likened to "ceramic plywood," have been studied recently with transmission electron microscopy, potentially leading to the development of novel tough, lightweight structures (35,36). Finally, marine diatoms have been considered recently as a renewable source of high-purity silica with uniquely high surface area and porosity, for use as an alternative to zeolites and in other industrial applications (37).

Specialty Products

Three additional marine bioproducts with commercial applications, but small and specialized markets are pigment standards, isotopically labeled compounds, and fluorescent diagnostic markers. Each of these is produced from cultured microalgae. Pigment standards are used primarily for the calibration of high-performance liquid chromatographic (HPLC) analyses of pigments in environmental samples. A variety of organic compounds can be labeled with stable isotopic markers for research purposes by growing the source microalgae in a medium containing isotopically labeled nutrient salts. These compounds have potential applications as cell culture biochemicals and in diagnostic breath tests (38). Lastly, certain highly fluorescent water-soluble pigments, such as phycoerythrin and phycocyanin, are commercially produced from microalgae (7; Table 1). These compounds are useful in marker applications such as flow cytometry, fluorescence microscopy, and immunoassays.

ENERGY

Energy Production

The concept of using marine organisms as an energy source consists of two major themes: producing hydrocarbons from biomass and generating hydrogen from cultured

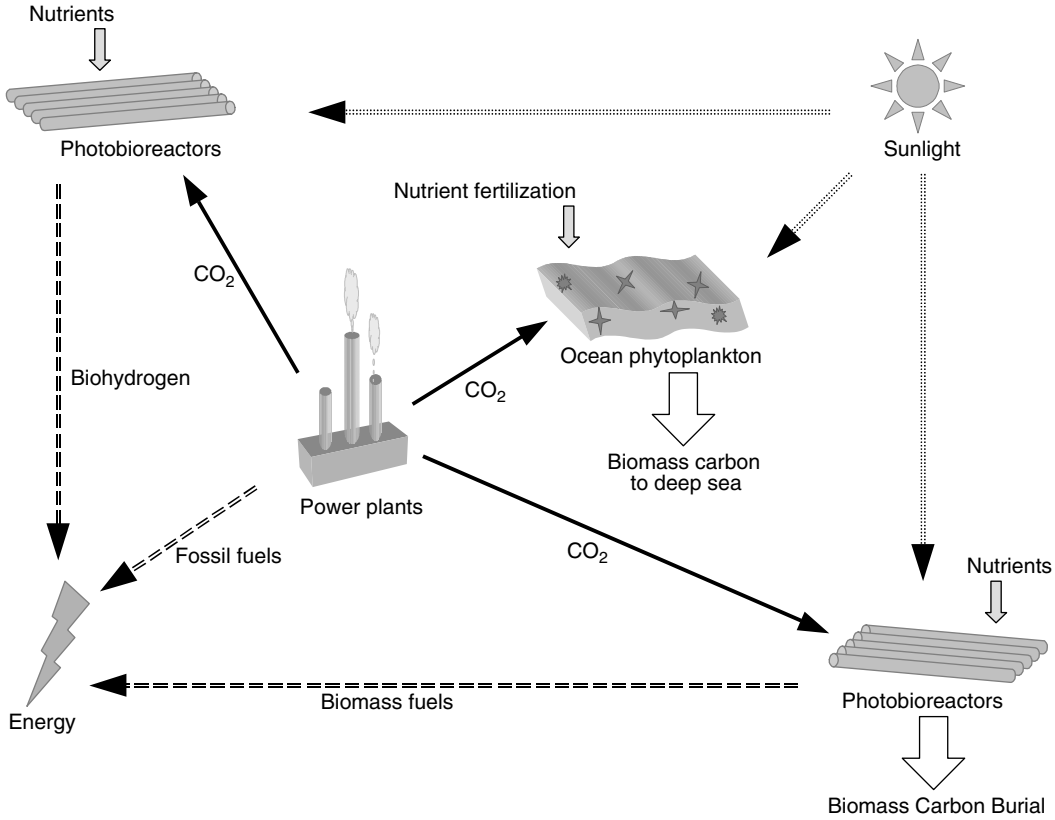

Figure 1. Schematic diagram of the potential uses of marine microorganisms in energy production and carbon dioxide mitigation.

microorganisms (Fig. 1). Neither of these approaches has yet led to commercial energy production, but because of the renewable nature of these energy sources and their relatively low impact on the environment when compared to fossil fuel combustion, research and development projects in these areas continue to increase.

Microalgae and seaweeds are the primary subjects of research into hydrocarbon production from marine biomass sources. These organisms grow rapidly by photosynthesis utilizing carbon dioxide and inorganic nutrient salts, thus could be considered as a source of renewable fuels synthesized using solar energy. Certain microalgae naturally produce energy-rich fuels. The biomass of the green alga *Botryococcus braunii*, for example, may consist of up to 86% isoprenoid hydrocarbons by dry weight (39). These compounds can easily be catalytically converted to gasoline-like fuels. Unfortunately, the mechanisms of isoprenoid production in this organism are not well understood, thus yields have been unreliable. Microalgae can also be induced to accumulate lipids via nutrient-deficient cultivation, but cost-effective conversions of these triglycerides into fuels have not been developed. The green alga *Dunaliella* will, under saline conditions, produce up to 85% by dry weight of glycerol. Glycerol is not a good fuel in itself, but could potentially be fermented to *n*-butanol and ethanol by bacteria. Production of methane via biological gasification of carbohydrates from macroalgae and microalgae has also been demonstrated, but this process has not been efficient enough to warrant large-scale development (39). Gaseous hydrocarbon production via vacuum pyrolysis of biomass, a relatively new process, has been used recently to generate fuels from the abundant and easily cultured calcifying marine microalga *Emiliania huxleyi* (40). Because of the high hydrocarbon output and the low energy input, this process holds great promise as an economically viable form of energy production for the future.

Hydrogen is attractive as a clean fuel, as it holds high potential energy and, when combusted, produces water and not carbon dioxide. Hydrogen can be produced by microorganisms via three principal processes (41). The first is through the fermentation of organic substrates by anaerobic bacteria such as *Clostridium*. This method of biological hydrogen production has the advantage of a high reaction rate, but relies upon energy-rich organic compounds as substrates, and does not completely decompose these compounds. The second process is the generation of hydrogen by photosynthetic bacteria, such as the marine bacterium *Rhodovulum*. This process also requires organic substrates as a source of hydrogen atoms, but utilizes light energy in addition to chemical energy to effect the evolution of hydrogen gas. Systems utilizing a close synergistic coupling of these first two reactions could potentially be designed for complete decomposition of organic wastes with simultaneous energy production in the form of hydrogen gas. Also, reactors utilizing immobilized enzymes from marine bacteria are possible, and preliminary data show that this technique can be carried out with a remarkably high yield of hydrogen (42). The third process for biohydrogen production is the

hydrolysis of water into hydrogen and oxygen gas by photosynthetic cyanobacteria and green algae (Fig. 1; 41). This process directly utilizes solar energy to produce clean energy-rich hydrogen gas, requires no previously existing organic substrates, and does not add to the atmospheric burden of carbon dioxide. The greatest hurdles to overcome in developing this process into an energy producing industry lie in improving the hydrogen yields from photosynthetic organisms, in designing suitable photobioreactor technologies for the cultivation of the organisms, and the efficient collection of the hydrogen they produce. Because of the recognition of the finiteness of fossil fuel resources and the climate-altering effects of their combustion, interest in the marine biotechnological development of biohydrogen production systems is active and growing (41).

Carbon Dioxide Sequestration

The steady rise of carbon dioxide in the atmosphere raises concerns about potential global warming and global climate change. Marine biotechnology has the potential to help slow the accumulation of anthropogenic carbon dioxide in the atmosphere in at least three ways (43). First, energy technologies based on marine microbes (discussed earlier) may allow for energy production with substantially reduced release of carbon dioxide. Second, mass cultures of marine microalgae and cyanobacteria may be used in carbon dioxide scrubbing schemes, to remove or recycle carbon dioxide emissions from power plants and other fossil-fuel consuming industries through photosynthesis and/or calcification (Fig. 1). Such schemes are currently the subject of novel research projects funded by the U.S. Department of Energy. Third, carbon dioxide may be removed from the atmosphere to the deep-sea. This can be accomplished through direct injection of liquid carbon dioxide to great depths via pipelines. Alternatively, stimulation of the photosynthetic productivity of oceanic phytoplankton after fertilization with iron and possibly other nutrients may result in greater export of sinking organic material to the deep sea, and genetic manipulation of other members of the marine food web might enhance such export (Fig. 1). None of these marine biotechnologies has been developed beyond the scale of pilot studies, but each has potential for helping to mitigate the global effects of fossil fuel combustion.

BIOPROCESSES

Bioremediation and Wastewater Treatment

The cleanup of pollutants from the marine environment is a pressing issue today, and one in which marine biotechnology is poised to play a vital role. The degradation of petroleum-based hydrocarbons has received the most attention in this area because of public awareness of oil spills from tankers, drilling platforms, and associated operations. The natural degradation of hydrocarbons in the marine environment is carried out principally by bacteria and fungi, but the controls on this complex process are not well understood. Nutrients, temperature, salinity, oxygen, and other environmental parameters play a role, as does the prior exposure (adaptation) of the microorganisms to hydrocarbons (44). Enhancing the activity of the natural microbial populations through the addition of fertilizers is one method of oil bioremediation. Seeding spills with laboratory-grown inocula of hydrocarbon-degrading bacteria, is another. The combination of inoculation and fertilization has also shown promise (3). Another approach is the addition of surfactants to the spill. Chemical surfactants such as synthetic detergents have been widely used to aid in the emulsification of water-hydrocarbon mixtures; however, concerns about the toxicity and lack of biodegradability of these compounds has sparked research into the use of biosurfactants for environmental cleanup operations (45). At least one marine bacterium, *Acinetobacter*, has been used for the commercial production of biosurfactants. Genetic modification of such microorganisms to enhance their petroleum-degrading capabilities may be an important future direction for bioremediation research, but introduction of such genetically modified bacteria into the environment is an issue of some concern and debate.

Two other areas of bioremediation benefit from marine biotechnological research: the degradation of pesticides, herbicides and other organic toxins, and the sequestration of toxic heavy metals. A number of marine and freshwater bacteria have been identified as having the potential to partially or completely degrade organophosphate pesticides, phenylurea herbicides, phenoxyacetate weed killers, and halogenated aromatics such as PCBs (45). Similarly, a recent study reported the isolation of a marine *Nocardioides* bacterium capable of growth on and degradation of the polyaromatic hydrocarbon phenanthrene (46). Heavy metals such as lead, uranium, silver, cadmium, and mercury are serious marine environmental problems because of their toxicity and persistence. Marine bacteria, actinomycetes, and microalgae that are resistant to such metals, and in some cases, which can accumulate the metals or convert them to less toxic forms, have been reported (3,47).

Organic toxin-degrading and heavy metal-accumulating aquatic microorganisms are of great interest not only for environmental remediation but also for the treatment of industrial liquid wastes in sludge reactors (45). Aquatic microorganisms also can be used in wastewater bioreactors for the removal of inorganic nutrients from industrial and domestic liquid wastes. Mass cultures of microalgae are used in this application in continuously mixed high-rate growth ponds at sewage treatment plants. The microalgae work in parallel with aerobic bacteria, consuming the nutrients produced from organic decomposition by the bacteria, and providing photosynthetically derived oxygen to support bacterial metabolism (48). Such synergistic bacterial-algal treatment of wastewater was one of the earliest practical applications of microalgal biotechnology.

Biofilms, Bioadhesion, and Biocorrosion

The formation of biofilms on submerged solid surfaces, and their subsequent colonization by attaching organisms, are normal marine biological processes (3,49). These films are

composed of layers beginning with organic molecules, followed by bacteria, then other microorganisms, and marine animals. Biofilms are important parts of natural aquatic ecosystems, but biofouling on the hulls of ships significantly reduces their performance and fuel efficiency, and biofilms also have been implicated as a contributing factor in corrosion of metals in marine systems. The application of marine biotechnology to biofouling research falls into three principle categories: creating an understanding of the ecological significance of biofouling and processes that control it, preventing biofouling from occurring on ships, pilings, and other submerged surfaces, and developing new compounds and materials from biofouling organisms. The interaction of biofilm-producing bacteria with marine invertebrates has been studied in some detail. For example, enzymes and polysaccharides produced by the biofilm-forming marine bacterium *Shewanella colwelliana* apparently act as cues for the settlement and attachment of oyster larvae (3). In this case, study of the bacteria and their chemical messengers could lead to microbial methods for enhancing settlement of oyster larvae in mariculture systems. Methods for the prevention of biofouling include the widespread use of antifouling paints, but these are generally toxic, and thus more environmentally friendly techniques are desired. Compounds are sought that either inactivate settlement cues or inhibit the initial colonization by bacteria. Two possibilities in this arena include the isolation and use of natural inhibitory compounds from marine organisms, and/or the development of nonstick paints that make settlement mechanically difficult (50). Finally, the study of bioadhesion in the marine environment may lead to unique water-resistant adhesives. One such adhesive already on the market, derived from the byssal threads of mussels, is being used to enhance cell attachment in tissue culture applications and may have future applications as an adhesive in surgical procedures (32).

Genetic Techniques in Marine Bioprocesses

The potential practical applications in marine biotechnology of recombinant DNA and other techniques for the genetic modification of organisms seem to be limitless. Some examples mentioned earlier include the genetic engineering of marine archaea to produce novel thermostable enzymes, and the genetic alteration of marine bacteria for the ability to degrade pollutants in the environment. Although there is no room here for an exhaustive review of the subject, a few other prominent examples of work in progress, specifically dealing with improved strains for production and/or distribution of natural products, are discussed here.

Cyanobacteria are attractive organisms for the introduction and expression of foreign genes (51). Although most attempts to genetically transform cyanobacteria have been used for basic research purposes or for use in bioremediation (52), two notable projects are under way to introduce and express genes coding for useful natural products. The first is the cloning of a mosquitocide-coding gene from a terrestrial bacterium into *Synechococcus*. The bacterium *Bacillus thuringiensis* has been known long for its insecticidal properties, stemming from several crystalline proteins. The gene coding for the most potent of these compounds (the protein CryIVD) was cloned into the cyanobacterium, which successfully expressed the gene and exhibited toxicity when eaten by *Aedes aegypti* mosquito larvae (53). This technique, when fully developed, could allow for inexpensive biocontrol of disease-carrying mosquitoes, without the use of chemical pesticides. A second notable project is the use of genetic engineering techniques to create a mutant strain of *Synechocystis* that overexpresses genes coding for the production of the carotenoid pigment zeaxanthin. Specific genes from a yeast and from *Synechocystis* were linked and expressed in the cyanobacterium, allowing zeaxanthin to become the dominant carotenoid pigment (54). These or similar mutant cyanobacteria could potentially be used to manufacture zeaxanthin for dietary supplements and other health applications.

Genetic techniques also have been applied to carotenoid production from green algae. In one example, mutants of the astaxanthin-producing alga *Haematococcus pluvialis* exhibiting resistance to carotenoid biosynthesis inhibitors were isolated and then formed into hybrids using protoplast fusion techniques (55). The new hybrid microalgae produced levels of carotenoids threefold higher than the wild-type strains. Another genetic technique for carotenoid production involved the cloning of a cDNA from *Haematococcus* into the easily cultivated *E. coli* bacterium. The resulting bacterium was able to produce the carotenoid canthaxanthin (56). Such cloning techniques hold promise for the development of bacterial fermentation-based carotenoid pigment production, a viable alternative to photosynthetic production by microalgae.

AQUACULTURE

Aquaculture is to the sea what farming is to the land. The cultivation of fish, shellfish, and crustaceans for food is a fast-growing industry. Aquaculture currently accounts for over one quarter of the fish supply for direct human consumption (57), and is likely to continue to expand as ocean fisheries decline worldwide. A variety of marine biotechnological techniques can be used to improve and enhance the aquaculture of marine organisms. Examples include genetic techniques for enhancing growth and cold tolerance, the hormonal manipulation of reproduction, the large-scale production of feedstocks through microalgal mass culture, and the integration of algal and animal production systems to increase productivity and reduce waste effluents.

Genetic Techniques in Aquaculture

At least three genetic approaches to enhancing the growth of fish have been demonstrated (3). First, foreign growth hormone genes have been fused with promoters and introduced into the eggs of commercially important fish species, resulting in transgenic offspring that grow larger than untreated control fish. Second, growth hormone genes from fish have been cloned into bacteria, which express the genes and produce large quantities of the hormone, which can then be injected into farmed fish to enhance

their growth. Third, fish gene constructs can be developed that cause overexpression of growth hormone genes when reintroduced into the fish. Similar techniques have been applied to other aquacultured animals such as abalone and oysters. Another application of genetic techniques to fish farming is the introduction of genes coding for antifreeze proteins from polar fish into fish from temperate waters, to enhance their resistance to cold. Hormones other than those promoting growth are also utilized in aquaculture, for example, to induce spawning of fish or the molting of lobster.

Microalgal Biotechnology in Aquaculture

Microalgae are a vital resource for the aquaculture industry. Various commercially important mollusks, fish, and crustaceans utilize live microalgae as food during at least part of their life cycle (58). Although natural phytoplankton populations are often used to feed aquacultured animals, this approach is fraught with problems. These problems are primarily related to the inability of the farmer to control wild phytoplankton species compositions (which can include toxic species), together with difficulties encountered in trying to exclude unwanted protists and other consumers. Microalgal production is therefore an integral component of many aquaculture operations (58). In addition to providing a food source for animals and their larvae, microalgae are also used within aquaculture ponds and tanks to help maintain oxygenation and to remove excess nutrients. If the algae are of commercial value themselves, they add to the economic potential of the operation. This sort of integrated aquaculture approach is useful for reducing wastes and for increasing the overall efficiency of the growth system. Similarly, the combined farming of fish (salmon) and seaweeds (*Porphyra* and *Gracilaria*) has been shown to be positive for optimizing the efficiency of aquaculture operations and for reducing the pollution of coastal waters with waste effluents (58,59).

HEALTH AND SAFETY

Marine biotechnology has a significant role to play in maintaining the health and safety of our marine resources. Disease prevention in wild and cultured marine organisms, as well as quality assurance in seafoods, are important areas for research and development. The rapid detection of biotoxins and human pathogens in marine environments are also critical foci.

Marine animals, particularly those raised in intensive aquaculture, are susceptible to disease. Bacteria, viruses, fungi, and protozoan infections are common problems faced by fish and shellfish. In aquaculture, diseases caused by marine vibrio and other bacteria are particularly common. The widespread use of antibiotics is undesirable, because of both high cost and the selection for resistant pathogenic strains. Because of this problem, a number of fish vaccines against bacterial pathogens have been developed using formalin-inactivated bacterial cultures (3). Also promising is the use of antimicrobial peptides isolated from natural sources to confer disease-resistance to marine animals. Certain peptides from insects and from the skin mucus of the flounder have been demonstrated to protect coho salmon from infection by the bacterial pathogen *Vibrio anguillarum* (60). Viral diseases of marine animals are also of concern in aquaculture. Important fish vaccines have been developed using recombinant DNA technology, to combat the infectious hematopoietic necrosis and infectious pancreatic necrosis viruses in salmonids (61). Diseases of shellfish caused by protozoa are also a subject of intensive biotechnological research. One such disease, caused by the parasitic protozoan *Perkinsus marinus*, nearly wiped out oyster production in Chesapeake Bay (3).

In addition to the various prevention approaches mentioned earlier, rapid early detection of pathogenic organisms is a significant weapon against marine diseases, both those affecting marine animals and those affecting humans. Most bacteria in natural samples resist attempts at culturing, thus standard plate-count methods will not reveal them. Notable human pathogens that frequently exist in a viable but nonculturable state in aquatic systems include *Vibrio cholerae* and *Salmonella enteritidis* (62). Recently, sensitive molecular methods for the detection of specific microbes in natural samples that do not rely on culturing have been developed. DNA probes, PCR amplification of specific genes, and monoclonal antibody-based tests have been successfully used for the detection of a number of human pathogens in aquatic environments, including those causing dysentery, cholera, and septicemia in humans (3). Similarly, DNA probes have been used to detect pathogens of aquacultured animals such as the shellfish parasite *Marteilia refringens* (63). A sensitive PCR assay for the salmonid pathogen causing bacterial kidney disease, *Renibacterium salmonarium*, also has been developed (64). In the near future, the use of these direct detection methods may well supplant traditional culture methods for bacterial enumeration.

Many marine toxins are produced by microalgae or bacteria and are concentrated in the tissues of organisms as they are passed up the food chain. Examples such as ciguatoxin and saxitoxin are capable of causing fatal poisoning in humans. The development of sensitive assays for marine toxins is thus of great practical interest. Puffer fish toxin (tetrodotoxin), a potent and sometimes lethal sodium channel blocker, is probably produced by bacteria associated with its namesake fish. A monoclonal antibody-based immunoassay has been recently developed to detect this toxin in biological samples (65). Similarly, a variety of bioassays, biochemical and immunoassays, and physicochemical methods are available for the detection of the diarrhetic phycotoxins responsible for shellfish poisoning (66). However, the sensitivity of such assays is only one of many important qualities. For fisherman and others with a strong stake in assuring the safety of seafood for consumption, the ease and rapidity of a toxin assay are critical parameters to consider. The simple commercially-available test kit for ciguatoxin is an excellent example of marine biotechnology research put to practical use (67). In this respect, an exciting avenue for research is the development of biosensors for the detection of marine toxins. Biosensors combine biological molecules such as enzymes or antibodies with a transducer that can convert

a biological response into an electronic signal (68). Applied marine biotechnological research in this area could lead to the development of real-time true toxicity sensors for use in the field to assess the safety of marine foodstuffs.

MARINE ECOLOGY

Fundamental research on the structure and functioning of marine ecosystems has been recently given a tremendous boost from the application of molecular biological tools. Such tools have allowed for the identification of species, and the assessment of the genetic variability within and between species, with unprecedented detail and accuracy. For example, the study of fish and invertebrate populations has benefited from the use of restriction endonuclease digestion of mitochondrial DNA. Changes in mitochondrial DNA have been used to trace genetic lineages in numerous marine animal species, from striped bass to humpback whales (69). This technique has allowed researchers to delineate and track discrete populations of animals that could not otherwise be differentiated. The phylogeny of marine animals can besimilarly determined through sequence analysis of ribosomal RNA, creating "family trees" that define the genetic relatedness of disparate species. The separation and immunological staining of individual proteins, with subsequent comparison of homologous proteins (isozyme analysis), can also be used to distinguish genetically distinct species that are morphologically identical.

The application of genetic techniques to marine microbial ecology has revolutionized the field. Evolutionary relationships between marine microorganisms are exceedingly difficult to ascertain from morphology alone, but through the use of DNA and RNA hybridization techniques, highly detailed phylogenetic trees that shed new light on microbial diversity in the sea have been created. For example, by cloning and sequencing 16S rRNA genes amplified by PCR, researchers have found that the ubiquitous bacterial genes recovered from seawater do not correspond to any cultured microbes (70). Similarly, marine archaea, previously thought to exist only in extreme environments, have been found to be abundant components of ocean plankton through the use of oligonucleotide primers specific for their rRNA genes (71). Genetic analyses of marine eukaryotes have demonstrated that the traditional separation of these organisms into algal, fungal, and protozoan groups is a gross oversimplification of the true relationships between them. For example, dinoflagellates, traditionally considered among the algae, are now thought to be more closely related to heterotrophic ciliates than to other algae such as diatoms or chlorophytes (72).

As genetic techniques in marine biotechnology are applied to microbial ecology, our view of the relationships between organisms continues to be improved. Beyond the assessment of microbial biodiversity, genetic techniques can also aid in the study of the metabolism of marine microorganisms in their environment. For example, variations in rRNA content via hybridization to 16S rRNA probes have been used to study the starvation response in marine bacteria (73). Also, gene expression in natural populations can now be assessed using mRNA analysis. This technique is now being applied to novel oceanic cyanobacteria, in order to assess their potential nitrogen-fixing activity (74). The ability to assay for gene expression should allow marine microbial ecologists to determine, better than ever before, which microorganisms in the sea are truly responsible for carrying out specific metabolic processes.

MARINE BIOPROCESS ENGINEERING

Marine organisms, particularly microorganisms, represent a vast store of genetic diversity that could potentially be used to generate thousands of unique and useful compounds. However, to date, the success rate for the progression of marine products from initial characterization through to mass production has been quite low (75). Marine bioprocess engineering represents the critical missing link between discovery and commercialization of marine bioproducts. Bioprocess engineering may be defined as "the translation of life-science discoveries into practical products, processes, or systems capable of serving the needs of society" (76). Marine bioprocess engineering is therefore an approach, based on sound scientific and engineering principles, to surmount technical and infrastructural barriers associated with the production and commercialization of marine bioproducts. The engineering of a marine bioprocess begins with compound characterization and screening, progresses through small-scale production and scale-up to industrial production, and ends with product marketing (Fig. 2). Along the way, issues of product separation and recovery, product formulation, production systems development, and market evaluation must be addressed.

Characterization and Screening

Engineering a marine bioprocess begins with the identification of a marine biological product of some potential commercial interest. In some cases, this may be a major product of the organism that can be easily identified, such as an algal polysaccharide or major pigment. In other cases, particularly with potential drug candidates, extracts from the organism are put through a series of in vitro screening procedures to identify bioactivity (Fig. 2). Screening may involve application of the extract to bacterial or fungal cultures or to various tumor cell lines or other test subjects. Wild collections or small-scale cultures usually suffice in terms of total material for this initial screening. Extracts exhibiting bioactivity are then subjected to chromatography or other separation techniques, to isolate small amounts of individual compounds. These compounds can then be characterized by GC-MS, NMR or other methods, to determine their molecular structures. Individual purified compounds are then subjected to further in vitro screening procedures to confirm their bioactivity. Promising compounds may be subjected to chemical or enzymatic combinatorial catalysis, to generate libraries of closely related compounds for additional screening.

To culture marine organisms, even at the bench scale, one needs information on the growth conditions

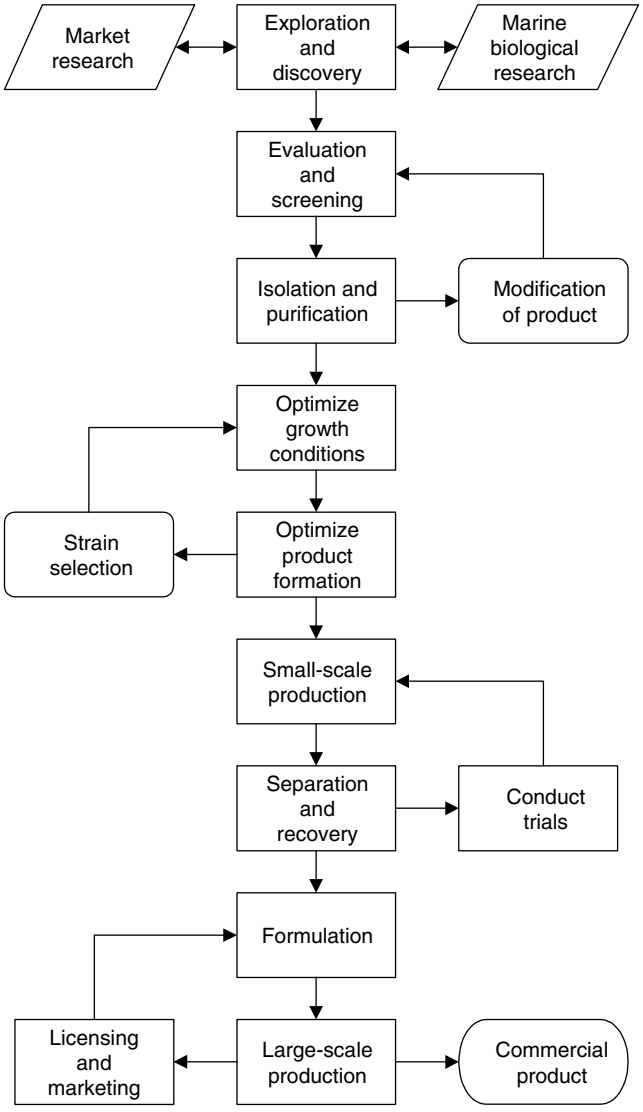

Figure 2. Conceptual flowchart for a marine bioprocess, leading from discovery to commercialization.

that the organisms require. Identifying the parameters controlling growth and affecting product formation can constitute a major research endeavor, and such attempts can sometimes prove fruitless. Product formation may require conditions very different from those favoring growth, as is the case with astaxanthin formation in the green microalga *Haematococcus pluvialis* (77). Once an organism is in culture, several techniques may be employed to generate strains capable of enhanced product formation. Classical strain selection techniques involving the handpicking of individual overproducing organisms is one approach, but can be difficult, especially with bacteria and small microalgae. In some cases, the use of automated cell sorting by flow cytometry can speed up the strain selection process, as with the selection of pigment-hyperproducing yeast mutants (78). Microbial antagonism can be used to elicit the production of bioactive compounds normally used in cell defense, by deliberately exposing cultures to foreign organisms. This strategy has been effective for the induction of antibiotic compound production by marine epibiotic bacteria (79).

Production

Marine animals, plants, and macroalgae that produce compounds of commercial interest may be harvested from the wild or cultivated in aquaculture "farms." The former approach is usually not viable for the maintenance of a steady supply of source material for industrial-scale bioproduct manufacture. The latter approach may at times be viable, but quite often the organisms of interest are difficult to reliably cultivate, and product yields are often highly variable. Marine microorganisms, on the other hand, may be cultivated in bioreactors that allow tight control over environmental parameters and isolation from potential contaminants. Marine bioprocess engineering is therefore generally more applicable to the microbes than to the multicellular life-forms. Two basic types of bioreactors are commonly employed: fermentors and photobioreactors. Fermentors are closed, environmentally controlled bioreactors, generally made of stainless steel, which are used for the heterotrophic culturing of microorganisms. Some specialized fermentors for culturing extremophiles are designed to operate at high temperatures and/or pressures. Photobioreactors are transparent versions of fermentors, usually made partly or entirely of glass or clear plastic cylinders, tubes, bags, or panels, to allow light penetration into the culture (80; Fig. 3). Photobioreactors are used for the culturing of photosynthetic organisms, principally microalgae and cyanobacteria, but in some cases other types of photosynthetic bacteria as well (81). In some instances, particularly when culturing in extreme conditions of pH or salinity, open ponds or raceways are adequate for the mass production of microalgal bioproducts (82). However, in general, the potential for close control of culture conditions and exclusion of contaminating organisms make photobioreactors the growth vessels of choice for microalgae. Light penetration is key in photobioreactor technology; hence, the ratio of surface area to volume in a large-scale photobioreactor is designed to be much greater than that of a typical fermentor. Moreover, photobioreactors must be exposed to a light source, either natural sunlight or artificial light. Artificial lighting is expensive, whereas natural sunlight, although free, is limited by latitude and weather.

Despite the wide variety of fermentor and photobioreactor designs now in use or in development, all microbial bioreactors share common features (Fig. 3). All bioreactors require some form of mixing to keep cells in suspension, some form of temperature control, and systems for introducing fresh growth medium and for effecting gas exchange (carbon dioxide and oxygen). Systems for the control of culture pH, cell concentration, and incident light levels are also frequently of use. Finally, all bioreactors must have some means for conducting harvests, either in batches or continuously. In a production plant, cultures must be effectively scaled up from the bench scale (milliliters to liters) to the full production scale (thousands of liters or more). Many aspects of environmental control that seem straightforward at bench scale become problematic at production scale, including the maintenance

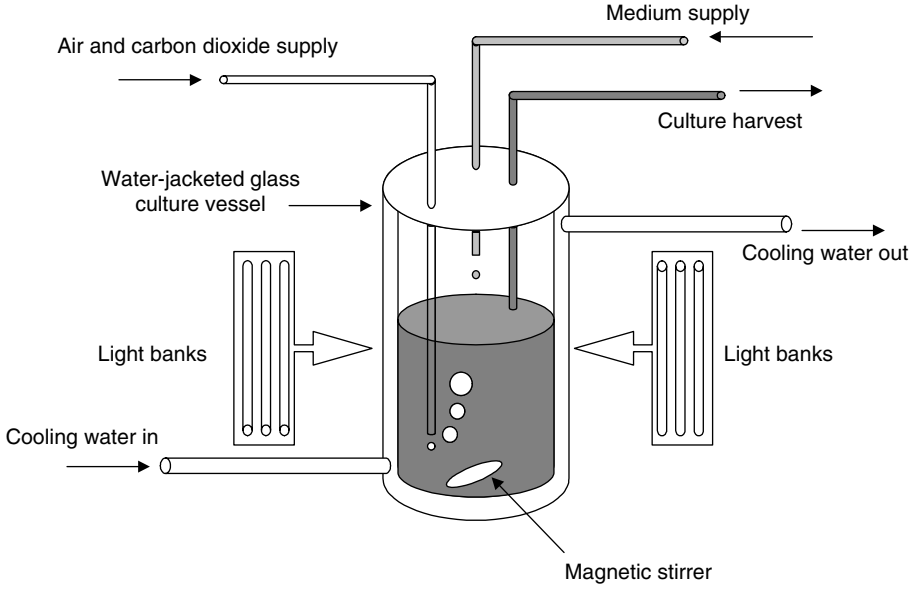

Figure 3. Schematic diagram of a simple continuous-culture laboratory-scale photobioreactor.

of sterility in bioreactors and their associated piping and pump systems, the achievement of adequate turbulent mixing, and the rapid exchange of gases. None of these problems is insurmountable, but the solutions must be carefully engineered.

Separation, Recovery, and Formulation

Once a marine microorganism is successfully growing at production scale, and is actively synthesizing the target compound, it must be processed (Fig. 2). Separating microorganisms from liquid media can be achieved by filtration, centrifugation, flocculation, or in some cases by passive settling. Each of these methods has advantages and disadvantages, and they are all expensive in practice, but in general, centrifugation is preferable for unicellular organisms. For organisms with tough cell walls, mechanical or enzymatic degradation may also be necessary to release the intracellular contents. Once the cells have been concentrated into a slurry, they can be dried and/or subjected to some sort of extraction procedure. Whole-cell products are typically dried, whereas specific compounds are usually extracted from the biomass paste with natural oils, chemical solvents, or supercritical fluids.

Formulation of marine bioproducts is an important step in the route to commercialization (Fig. 2). Microbial contamination, chemical purity, bioavailability, and consumer appeal are all characteristics to consider during the bioprocess engineering endeavor. The complete exclusion of bacteria and fungi from large-scale systems, especially outdoor photobioreactors, may not be cost-effective or may even be possible. The degree to which these organisms must be kept out of the product will depend on the end application; for example, products for animal feeds may not have regulatory requirements as strict as those for products intended for human consumption. Postprocessing sterilization or the sterile extraction of compounds may circumvent this issue entirely. The chemical purity of bioproducts also may be of great importance, particularly when compounds are extracted before packaging. For example, some drugs and nutraceuticals have geometric and stereoisomers of varying bioefficacy. Furthermore, in pharmaceutical applications, it is often critical that compounds related to the target compound not be present, as they could potentially confer toxicity upon the final product. In formulating the final product that will go to the consumer, the bioprocess engineer may also need to consider aspects of the bioavailability of the target compound in its delivery system. For example, the form (liquid/solid, oil/water, etc.) of the product and the composition of additives (stabilizers, preservatives, emulsifiers, etc.) may have a direct bearing on both storage life and on how well the target compound is assimilated into the body. Finally, the texture, odor, taste, and packaging of the end product should be appeal to the consumer.

Market Evaluation

Ultimately, the sales of the product will be the metric by which the bioprocess engineering activity is judged. It is therefore critical that careful market evaluation be carried out before too much investment is made into a new bioprocess. In a sense, market evaluation should be carried out simultaneously with product discovery (Fig. 2). Marine bioprocesses can be very expensive to engineer, thus the potential productivity of the planned operation must be commensurate with the expected demands for the product. Potentially less expensive alternative sources for the product, such as chemical syntheses or terrestrial organisms, must be scrutinized. For example, although certain microalgae are capable of synthesizing the carotenoid pigment lutein, no algal bioprocess has been developed that can compete with the inexpensive production of lutein from the petals of marigolds. Only if the marine bioproduct is destined to capture a substantial market share should

implementation proceed. Lastly, consideration of niche markets should be given in developing marine bioproducts. Potential drugs for which there is no alternative will be pursued before those of more general utility. Similarly, where currently only synthetic forms exist, a "natural" form may be perceived to be superior by the consumer.

LEGAL ASPECTS OF MARINE BIOTECHNOLOGY

Risk Assessment and Regulatory Issues

The safety of marine biotechnology for humans and for marine ecosystems is a subject of some discussion. Companies involved in marine biotechnology are both cultivating organisms introduced from foreign locations and creating genetically modified organisms with unique properties. The risks can be divided into two categories: those associated with the inanimate products of marine biotechnology and those associated with the organisms used in marine biotechnology. The former would appear to be no greater than risks associated with the products of any industry (3). No special hazards have been associated with the inanimate products of genetically modified organisms, so regulations governing their use essentially follow existing consumer safety guidelines. On the other hand, the safety of the genetically modified organisms themselves is at issue. Although the accidental generation of pathogenic organisms is exceedingly unlikely, the potential ecological consequences of the accidental release of genetically modified marine organisms into the wild are not well-understood. Marine organisms have the potential for rapid dispersal once released into the environment to a much greater extent than terrestrial organisms (83). Field tests of genetically modified marine microorganisms are likely to occur in the near future. The regulation of such modified microbes in the United States falls under the jurisdiction of the Environmental Protection Agency, whereas the Food and Drug Administration is responsible for issues regarding transgenic fish and shellfish. To date, there is no agency specifically tasked with the oversight of safety issues in marine biotechnology, although it has been proposed that such a unit be designated at the National Marine Fisheries Service (84).

Intellectual Property and Technology Transfer

Marine biotechnology, though grounded in science, is above all else a commercial enterprise. As such, much of the research and development in marine biotechnology is funded by venture capitalists or by large corporations. To these profit-minded groups, trade secrets must be jealously guarded and new technologies need to be rapidly patented. Maintaining rights to hard-won intellectual property is a key to success in the business of biotechnology, and marine biotechnology does not present an exception. Among the most significant policy issues facing government and the growing marine biotechnology industry is the issue of ownership of bioprocesses and biomaterials (85). Although apparatuses and processes have traditionally been the principle subjects of patents, the securement of intellectual property rights to both molecules and organisms is now possible. Intellectual property law, however, is not static, and is likely to be modified in coming years to adapt to rapid changes in biotechnology.

Despite the commercial emphasis of marine biotechnology, academic institutions are at the forefront of research into marine biology, ecology, and microbiology. If the combined forces of industry and academia can be brought to bear on marine biotechnological issues, society as a whole will benefit. To accomplish this, procedures and guidelines for technology transfer must be set up such that both industry and academia can feel content with their resource allocations. Excellent models for such industry/academia partnerships are to be found in the National Science Foundation's Engineering Research Centers. Among these is the newly founded Marine Bioproducts Engineering Center (MarBEC), a cooperative effort between the University of Hawaii, the University of California, and several industry partners with interests in marine biotechnology (75). Advisors from the private sector help to guide the directions for research in these centers, whereas key research results are shared with industrial sponsors. Most importantly, these centers provide the unique multidisciplinary education and training opportunities that students need to become tomorrow's leaders in the marine biotechnology industry.

CONCLUSION

The oceans cover most of the Earth's surface, yet the vast potential of their denizens as the sources for new products, processes, and research tools has hardly been tapped. The application of biotechnology to marine organisms, especially microorganisms, is beginning. Marine biotechnology is likely to yield a plethora of new drugs, health products, fuels, environmental remediation processes, advances in aquaculture, vaccines, and technologies for advanced marine research. To reach these goals will require the development of novel partnerships between academics, engineers, industrialists, legal experts, and financiers who share a common interest in finding practical uses for the unique resources of the sea.

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MARINE METHANOGENS. See METHANOGENESIS IN THE MARINE ENVIRONMENT

MATS, MICROBIAL. See AGGREGATES AND CONSORTIA, MICROBIAL; CYANOBACTERIA

MEMBRANE FILTER PROCEDURE FOR HETEROTROPHIC BACTERIA. See HETEROTROPHIC BACTERIA AS INDICATORS OF WATER QUALITY

MERCURY CYCLING. See METAL (U, Fe, Mn, Hg) CYCLING

MEROPLANKTON

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Meroplankton are autotrophic and heterotrophic organisms, which occur in the water column as part of the plankton but also settle or sink to the bottom sediments and thus spend a portion of their life cycle in the benthos. These temporary plankters, sometimes referred to as tychoplankton, have both vegetative cells and resting propagules that are important in phytoplankton dynamics in both freshwater and marine environments. Resting propagules include cysts of dinoflagellates, asexual spores, or akinetes of cyanobacteria, sexually produced zygotes of *Eudorina* and *Volvox*, vegetative colonies of *Microcystis*, and resting cells of diatoms (1). Alternation from planktonic to benthic phases range from diurnal (2,3) to decadal or longer timescales (4). On short timescales, settling of vegetative cells is an adaptation to diurnal changes in light and nutrient fields. On longer timescales, the benthic phase is likely to be in the form of resting propagules.

Resting cells or resting propagules are a physiological adaptation allowing vegetative cells to survive for long periods under conditions unfavorable for growth (1). Such resting stages are common among planktonic organisms and include cysts of chyrsophytes and dinoflagellates, akinetes (asexual spores) of cyanobacteria and resting cells of diatoms. Some vegetative cells of planktonic organisms sink to the sediments at aphotic depths where light is not available for photosynthesis, and benthic resting propagules are formed. This adaptation survival strategy has ecological and evolutionary advantages (5). The alternation from planktonic to benthic habitat provides a strategy that allows populations to survive conditions unfavorable for planktonic growth by settling and forming resting propagules in the benthic environment. These propagules ensure population survival until resuspension into a planktonic environment favorable for growth. This capability of a planktonic species to survive periods when environmental factors are unsuitable for growth and to maintain a potential inoculum for growth when favorable environmental factors return is fundamental to the planktonic way of life (1).

This article is restricted to organisms that are photoautotrophic in the planktonic phase (phytoplankton) and thus does not include invertebrates with meroplanktonic stages in their life cycle.

CYANOBACTERIAL AKINETES

Akinetes are resting propagules of cyanobacteria, which form and survive in the benthic environment and play an important role in maintaining meroplanktonic populations. Benthic akinetes of the cyanobacterium *Gloeotrichia echinulata* obtain phosphorus supplies after germination on the sediments; subsequent planktonic growth can be mostly or in part supported by these accumulated reserves (6). Resuspension or direct penetration of light to

the sediments may play a role in germination of cyanobacterial populations from akinetes and their introduction into the plankton (7). Phosphorus accumulated by vegetative cells of *G. echinulata* in the benthos can account for a significant portion of internal phosphorus loading into the overlying waters when the population is recruited into the plankton (8,9). For example, internal loading from recruitment of this species in Lake Erken, Sweden ranged from 0.4 to 0.6 mg P m^{-2} d^{-1} during July and August (10). Recruitment of vegetative cells of *Aphanizomenon flos-aquae* accounted for similar rates of internal loading from the sediments in a shallow, hypereutrophic lake (Agency Lake, Oregon). Motility of cyanobacterial populations is facilitated by gas vacuoles that increase buoyancy. Not all phytoplankton populations of cyanobacteria are maintained by meroplanktonic dynamics that involve akinetes. Many populations bloom, then are maintained in low numbers in the plankton during the remainder of the year (7,11).

DINOFLAGELLATE CYSTS

Encystment by dinoflagellates is a morphological adaptation for survival in the benthic environment. Such cysts have thick resistant walls of cellulose, and their protoplasts usually contain reserves of lipid and starch, which are accumulated during a stationary growth phase (1). Stimuli for encystment in two dinoflagellates were primarily nitrogen availability coupled with a secondary influence of irradiance (12). Cysts may accumulate phosphate during the period of dormancy, providing a competitive advantage after germination and resuspension into the pelagic (13). Algal cyst dormancy may also be a temporarily transient mechanism of avoiding herbivory in the plankton (14). These results suggest an important role for dinoflagellates in the translocation of nutrients from sediments to overlying waters. The alternation between vegetative populations and the distinct resting phases of *Ceratium hirundinella* provides a classic example of a meroplanktonic dinoflagellate (1).

DIATOM RESTING CELLS

Resting cell formation as a survival strategy for diatoms that sink to aphotic waters in both freshwater and marine environments was first noted more than 100 years ago. Whipple postulated in 1895 that dormant diatoms resuspended from surficial bottom sediments during seasonal turnover of the water column in lakes provide seed populations for seasonal population blooms (4). Hensen in 1887 recognized that resting stage formation was a survival strategy in some marine diatoms (5). In the resting phase, the cytoplasmic contents of vegetative cells condense in several stages and become physiologically dormant with the resting cells containing stored nitrogen, polyphosphate, and lipid reserves (4,15,16). Resting cells of diatoms and their intermediate stages have been described from a wide variety of aquatic habitats ranging from small lakes to the deep ocean (4,5,16,17).

FORMATION AND REJUVENATION OF RESTING PROPAGULES

How rapidly and frequently resting cells and other resting propagules actually form is open to conjecture. Formation of diatom resting stages is induced by darkness and accelerated at high temperature (4). In *Aulacoseira subarctica*, it occurs at very low light levels, about one $\mu mol\, m^{-2}\, s^{-1}$ (18), with several days required for resting cell formation at 20 °C. By contrast, photosynthetic capacity of *A. subarctica* declines during dark incubation in the laboratory with little loss of metabolic competence of cells for four weeks (18). Likewise, the transitory benthic assemblage in Lake Apopka, Florida is physiologically active when incubated at 100 to 300 $\mu mol\, m^{-2}\, s^{-1}$ in three-hour light-dark bottle experiments (19). *Aulacoseira italica* is the dominant species of the autotrophic assemblage subjected to periodic resuspension in this shallow, polymictic lake. After formation, resting cells of diatoms buried in sediments retain the capability of rejuvenation for decades (4).

Numerous studies report rapid rejuvenation of physiologically inactive resting phases of diatoms (4). Resting cells of *Aulacoseira granulata* buried in the sediments of Douglas Lake, Michigan, for approximately 20 years fix carbon photosynthetically in a matter of hours after exposure to moderate light (50 $\mu mol\, m^{-2}\, s^{-1}$) (16). Resting cells of diatoms rejuvenate at rates that generally increase with temperature and light but vary among species (4). Thus, resting cells of diatoms are adapted for survival in darkness and are rejuvenated to a physiologically active state within hours after exposure to a normal light and nutrient environment (4,16).

RESUSPENSION DYNAMICS

Studies show that induced resuspension increases nutrients, phytoplankton, and suspended solids in experimental enclosures (20). Elevated phytoplankton biomass persisted for at least four to six days, whereas increased suspended solids were maintained for less than one day. Phytoplankton that persisted in these studies were generally planktonic rather than meroplanktonic species. Unpublished experimental data for resuspended meroplankton in Lake Apopka, Florida, show that cells settle slower after resuspension than other sedimentary components. Meroplanktonic populations, therefore, are maintained in a suspension longer than resuspended, inorganic solids.

The effect of experimental resuspension on population dynamics of *Aulacoseira italica* was studied in a small lake in England (21). Two areas of Blelham Tarn, a 17-ha lake, were isolated with vertical polyvinyl chloride (PVC) screens. Compressed air was used as an "air-lift pump "to destratify the enclosed areas after the water column had stratified in the summer. The late summer increase in *Aulacoseira* caused by experimental destratification in 1967 was dramatic, and this bloom was greater than many spring blooms for the previous 22 years, a period of thoroughly documented population dynamics. Results, however, were confounded by water flowing over the top

and under the bottom of the enclosures. Consequently, the response may not have been associated entirely with destratification if cold, bottom water probably passed under the PVC screens. Increased numbers of *Aulacoseira* likely resulted from nutrient-enhanced growth of a seed population that was resuspended during artificial destratification.

Resuspension and its significance on biological and chemical dynamics in temperate and subtropical lakes depend on physical and morphometric factors. Resuspension of sediments in shallow lakes likely will be more frequent and important than in deep lakes, and will not occur in temperate lakes during periods of thermal stratification or ice cover. In deep stratified lakes, resuspension occurs seasonally only during periods of isothermal mixing and during upwelling events in large lakes.

Wind-induced resuspension of bottom sediments in shallow lakes depends on wave-generated turbulence (22). Benthic sheer stress calculated from wind speed, effective fetch, and depth predicts resuspension effects on nutrients (phosphorus and nitrogen) and chlorophyll better than wind speed alone in seven shallow, New Zealand lakes (23). Such empirical relationships do not consider differential effects of resuspension related to size, density, and types of particles being resuspended (24).

Wind-induced resuspension of sediments increases phosphorus and chlorophyll in the water column (19,23,25). The residence time of resuspended particles in the water column is relatively short, on average seven hours (25), and effects on phytoplankton are highly transient (26). Because of these short residence times, resuspended particles such as small phytoplankton or nutrients may have longer effective residence times than inorganic particles. Data from New Zealand lakes show that annual mean TP concentrations including resuspension events were four times greater than means during calm periods (23). In Lake Apopka, Florida, chlorophyll concentration in the water column doubled one day following a resuspension event (19). Whether such increases in phytoplankton biomass are related to new growth resulting from resuspended nutrients or to direct resuspension of algal cells or resting propagules can be ascertained from short-interval sampling before, during, and after a resuspension event. Automated sampling of the relevant variables in situ is needed to describe the associated dynamics adequately.

TIME SCALES OF RESUSPENSION AND RECRUITMENT

Life history and survival strategies for meroplankton involve resuspension or recruitment of vegetative cells and resting propagules from the benthic environment. Physical and chemical factors in the aquatic environment play important roles in the various strategies for meroplanktonic existence. The principal physical factors are interactions between water temperature and wind-induced turbulence. In deep, thermally stratified lakes, effects of turbulence are seasonal and most important when such monomictic or dimictic lakes are homothermic. Shallow, polymictic lakes differ in that effects of turbulence depend on short-term variability in local meteorologic conditions. Algal populations with a meroplanktonic phase are planktonic only during periods of relatively high wind-induced turbulence associated with a homothermic water column. Chemical factors are important because the benthic environment is generally richer in nutrients than are overlying waters.

In shallow lakes, diurnal mixing is an important factor in phytoplankton dynamics. Thermal stratification in Lake George, Uganda, develops during the day and is then obliterated by evening winds and cooling (3). The dominant cyanobacterial populations settle toward the bottom while the water column is stratified, then are resuspended and distributed uniformly over the water column during diurnal mixing (2). Chlorophyll concentrations vary during stratified conditions from 100 μg/L in surface waters to more than 400 μg/L at depth where settling algal cells are concentrated. When the water column is not thermally stratified, chlorophyll is distributed homogeneously in the 2-m water column.

Diurnal variation in environmental factors is also important in shallow lakes and reservoirs in Brazil (27). In such systems sediment/water interactions are accentuated by diurnal mixing. As a result, wind-generated turbulence affects nutrient availability and primary productivity of phytoplankton because nutrients regenerated in the benthic environment are entrained daily and become available for phytoplankton production. Seasonal changes in rainfall cause water depth to fluctuate 2 to 4 nm and thus influence turbulence and sediment/water interactions.

Direct inoculation of meroplankton from the benthic to planktonic environment occurs in Lake Apopka, a shallow, hypereutrophic lake in north central Florida (19,28). Chlorophyll *a* in Lake Apopka is characteristic of a hypereutrophic lake, averaging 104 mg/m^3 with a coefficient of variation of 44%. Water temperature varies seasonally, but no seasonal pattern is apparent in the standing crop of phytoplankton measured as chlorophyll *a*. Bimonthly data and data collected less frequently provide little evidence for pronounced seasonal variation in phytoplankton dynamics or standing crop in the lake (19,28). Chlorophyll *a* concentrations beginning in February or March and continuing until July ranged from 100 to 200 μg/L, exceeding the range of 50 to 100 μg/L for other months of the year.

The role of wind resuspension in controlling these dynamics is evident from a plot of average daily wind speed and chlorophyll concentration. Chlorophyll is positively correlated with wind speed because meroplanktonic algae are resuspended from the sediments. Effects of wind-induced resuspension are unusual in that light attenuation also is positively correlated with increased chlorophyll concentration (28) and not affected disproportionately by nonalgal turbidity that occurs in other lakes (29). Algal standing crop increases during and after windstorms in Lake Apopka because wind-induced turbulence in the water column resuspends loosely consolidated bottom sediments and sedimented algal cells. Recent research shows that small particles including algal cells may be resuspended more readily by wind-induced turbulence than larger cells and denser sestonic materials (24).

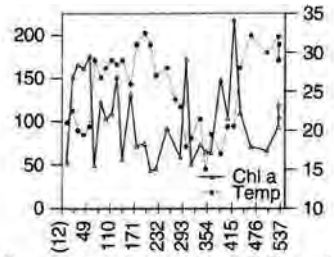

Figure 1. Surface water temperature (°C) and chlorophyll *a* concentration (μg/L) in Lake Apopka, Florida, February 1990 to July 1991 [Schelske et al. 2000 (28,47)].

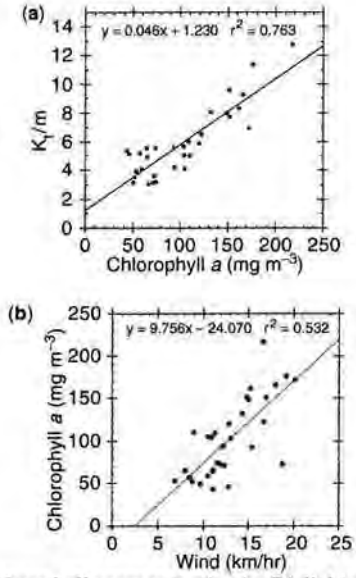

Figure 2. Chlorophyll *a* concentration (μg/L), light extinction and wind speed in Lake Apopka, Florida on data collected from February 1990 to July 1991 (28,47). (**a**) Linear regression of extinction coefficient (Kt of photosynthetically active radiation) on surface-water chlorophyll *a*. (**b**) Linear regression of surface-water chlorophyll *a* on average daily wind speed measured at Orlando International Airport [Schelske et al. 2000 (28,47)].

Phytoplankton, primarily diatoms, settle to the bottom in this lake during calm periods forming a nepheloid layer (used here as the layer of near-bottom materials that are suspended by turbulent mixing). Chlorophyll concentrations in a 5-cm thick flocculent layer of sediments near the bottom range from 2,940 to 4,230 μg/L (19), or at least 10- to 20-fold greater than the maximum in the surface water. If mixed into the water column, enough chlorophyll may be present in this layer to double the concentration found in the water column. Thus, wind resuspension plays an important role in phytoplankton dynamics in Lake Apopka.

No strong seasonal pattern in water transparency, chlorophyll, or nutrients (nitrogen and phosphorus) is apparent in Lake Apopka (28). Silica was an exception; the monthly average for 1977 to 1980 was lowest in February and March and highest in November. This seasonal pattern was related both to uptake of silica for the seasonal production of diatoms and to input of dissolved silica during periods of increased hydrologic loading.

Meroplanktonic algae in the near-bottom layer in Lake Apopka are physiologically viable and are adapted to periods of darkness or subsaturating irradiance characteristic of the lake bottom (19). Other species of diatoms with condensed cytoplasmic bodies also may be resting cells. Resting propagules in the benthic aphotic assemblage rejuvenate after short exposure to low irradiance, and when resuspended in the water column, are an important component of the phytoplankton.

Wind resuspension of nutrients and vegetative cells or resting propagules may be important in Lake Okeechobee, a shallow, subtropical, polymictic lake in Florida. *Actinocyclus normanni*, a diatom species with resting cells (4), may occur in phytoplankton samples following wind resuspension (28). *Staurosira construens*, another diatom species with resting cells, was among the ten most abundant taxa at most of the 21 stations sampled during an intensive two-year study of phytoplankton (30). Cyanobacteria, however, dominated the phytoplankton biovolume (30). *Lyngbya* sp. and pennate diatoms may be meroplanktonic and comprise a large portion of phytoplankton biomass in some regions of this shallow lake where resuspension also increases nutrients (31), inorganic turbidity and phytoplankton biomass (29) in the water column. Inorganic turbidity decreases mean light availability in the water column, resulting in lower algal production (29).

Wind resuspension of meroplankton and inorganic solids is also important in the shallow western basin of Lake Erie. The abundance of *Fragilaria crotonensis* Kitt. increased sixfold in surface waters two days after resuspension of sediments by wind-induced turbulence (32) and probably resulted from resuspension of a seed population that then expanded in surface waters. Inorganic turbidity presumably decreased soon after the wind event, allowing the planktonic population to respond to favorable nutrient and light conditions. Thus, wind-induced resuspension controlled by the frequency of storms on daily to weekly scales can be important in shallow, temperate lakes.

The role of meroplanktonic resuspension on longer timescales in deep, temperate lakes is apparent in Lake Michigan, one of the Laurentian Great Lakes. This monomictic lake (mean depth 87 m and surface area 58,000 km^2) has a large heat capacity and rarely freezes over completely. As a consequence, the period of isothermal mixing is long, extending at least from January until May in most years (28). As a result of periodic high turbulence, the water column is mixed completely during this period even at the greatest depths and is cooled below the temperature of maximum density ($<4^{circ}$C). Turbulence during storm events is adequate to resuspend sediments (33) and to mix the entire water column, entraining not only nutrients but also resting cells of diatoms and cyanobacteria (4,34).

Usually, resuspension and external loading of phosphorus in Lake Michigan tend to reset the total phosphorus concentration at approximately the same level between years, but during the unusually cold winter of 1976 to 1977, ice cover was nearly complete, and winter resuspension was minimized (33). In the spring of 1977, the average total phosphorus concentration in the water column was only five μg P/L compared to eight μg P/L in the previous year. Reduced turbulence during ice cover also influences

the abundance of meroplanktonic diatoms by shortening the period of isothermal mixing (35).

Wind resuspension of diatoms on daily to weekly scales affects phytoplankton biomass in coastal, marine environments where it is greater and more variable than in deeper, offshore waters (36). One important factor controlling variability is the frequency of wind events. Although nutrient enrichment from wind-induced upwelling and its effect on coastal phytoplankton blooms are well known, blooms of the diatom *Skeletonema costatum* develop in response to wind-induced vertical transport of diatoms from the aphotic zone to surface waters in the coastal plume of the Hudson River. Resuspended diatoms grow while being transported seaward in the plume, thereby increasing production 100 km beyond the zone of most active resuspension. Wind-induced resuspension, therefore, can affect diatom production on daily to weekly scales over a greater area than that affected directly by the wind.

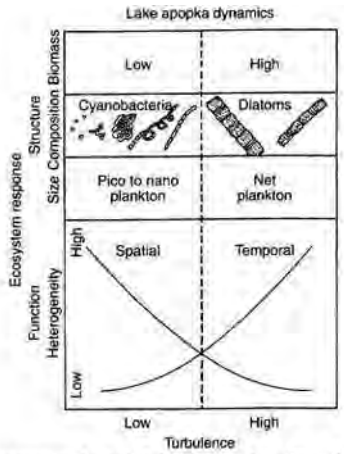

Figure 3. A conceptual model for dynamics of surface-water phytoplankton in Lake Apopka, Florida (from 19?). The model depicts ecosystem response to wind-driven, water-column turbulence in terms of function and structure of the phytoplankton community [Carrick et al. 1993, Limnology and Oceanography, (19)].

ECOLOGICAL SIGNIFICANCE OF MEROPLANKTON

The periodic appearance of meroplankton in the plankton, either from sediment resuspension or recruitment, enhances the diversity of the phytoplankton assemblage in aquatic ecosystems. The relationship between wave-induced turbulence and assemblage diversity in Lake Apopka, Florida, is illustrated with a conceptual model (Fig. 3), which has been confirmed by other studies. Wind-induced turbulence increases diversity by maintaining meroplanktonic species in the water column (37). Resuspension effects also increase the concentration of phytoplankton, bacteria, and nutrients in lakes (23,38,39). Inocula of dormant diatoms following wind-induced resuspension led to growth pulses of *Melosira* (*Aulacoseira*) in Lake Lanao, Philippines (40). After resuspension events, species are lost from the planktonic assemblage according to size and density as turbulence decreases. Small particles or picoplankton (1.5–6 μm) are resuspended by low turbulence, whereas greater turbulence is needed to resuspend larger microplankton (24). Phytoplankton populations that settle slowly under low turbulence may be maintained at low levels in the plankton throughout the year and provide an inoculum for growth when environmental conditions are favorable (11).

Sedimentation of diatoms is a life cycle strategy to enhance survivorship by incidental grazer avoidance or redistribution to more nutrient-rich waters (5). Likewise, algal cyst dormancy is also proposed as an adaptation to escape herbivory (13). Sedimentation of phytoplankton diatoms from the euphotic zone into the relatively nutrient-rich nepheloid layer in Lake Apopka, Florida, provides a mechanism to utilize relatively nutrient-rich waters at the sediment-water interface on short timescales (19, 28).

A number of studies show that diatom blooms in surface waters are an important energy source for benthic food webs. Rapid sinking of diatoms must be invoked for diatoms to reach the bottom so they can be preyed upon by benthic grazers in deep-water marine and freshwater environments. The literature reports that rapid settling of diatoms in fecal pellets of zooplankton is one mechanism for rapid transport to the bottom. A comprehensive literature review, however, concludes that the so-called "fecal pellet express "is relatively unimportant and that the most important process is aggregation of diatoms within the water column (5). Aggregated clumps of diatoms sink rapidly (sinking rates of 10 to 100 m/day) to the benthic community (41,42). These clumps are now thought to disaggregate within two weeks or less so cells can be resuspended with less turbulence than necessary for aggregated cells (43). Disaggregation also enables vegetative cells to assimilate nutrients in the benthic environment.

Mechanisms promoting rapid transport of diatoms to the benthos have not been studied as extensively in lakes as in the marine environment. Diatoms are an important food source for benthic organisms in the Laurentian Great Lakes where sedimentation of the spring diatom bloom provides a major food source for *Dioporeia affinis* (44). This amphipod apparently stores enough lipids from feeding on settled diatoms in the spring to fulfill a major component of its annual energy requirements. In these deep lakes, the spring bloom of diatoms that originates from resuspended seed populations or resting propagules is important. In shallow lakes, no adaptation for rapid sinking is required for diatoms to settle out of the euphotic zone to the bottom within a few days. In Lake Apopka, for example, most of the benthic environment is aphotic even though the mean depth is 1.7 m (28). Phytoplankton settling to the bottom may have an ecological advantage in this lake in that near-bottom waters are nutrient-rich compared to surface waters with relatively low levels of soluble inorganic nitrogen and phosphorus (28). Settling to the sediment–water interface, a site of nutrient regeneration, may be an adaptive strategy to enhance nutrient uptake and utilization by meroplanktonic populations.

LAKE MANAGEMENT IMPLICATIONS

Meroplanktonic vegetative cells or resting propagules may assimilate nutrients during the benthic phase and

store excess phosphorus (luxury uptake) that can be used for growth during the planktonic phase. The capability for luxury uptake is found also in other resting propagules. Benthic akinetes of the cyanobacterium *Gloeotrichia echinulata* utilize luxury uptake to store phosphorus supplies for planktonic growth (6). Vegetative cells of this species also accumulate phosphorus during the benthic phase of their life cycle (10,45). Phosphorus accumulation is not affected by alum treatment to reduce the exchange of phosphorus between sediments and water (9). Resting cysts of a marine dinoflagellate store phosphorus assimilated during the benthic phase, providing a competitive advantage in the planktonic phase (13), and resting cells of diatoms can be buried for decades or longer in sediments and remain viable (4).

Meroplanktonic diatoms also store phosphorus after settling to the benthic environment (28). The assimilated phosphorus, stored as polyphosphate in these cells, is a biological phosphorus sink that is not geochemically reactive (46). This biological sink is not a component of phosphorus loading models that are based only on total phosphorus concentration (46,47). If polyphosphate buried in the sediments is not reactive, loading models may underestimate sediment losses of phosphorus and over predict response times to reduced phosphorus loading (46). Burial of intact cells or propagules with stored polyphosphate, therefore, has management implications, as well as ecological significance because this process may ameliorate predicted effects of anthropogenic phosphorus loading.

Recognizing the benthic stages in meroplanktonic dynamics has additional implications for lake management. Akinetes, cysts, and diatom resting cells store phosphorus that also may be an unrecognized biological sink. Phosphorus uptake by cyanobacterial populations is not affected by alum treatment used to control the chemical exchange of phosphorus and overlying waters (9). Translocation of phosphorus from sediments to the water column occurs when cyanobacterial populations are recruited into the plankton from the sediments. Thus, controlling resuspension of akinetes is a possible lake-management strategy.

Dredging sediments containing large numbers of akinetes may help control nuisance blooms of obnoxious cyanobacteria (48) and remove phosphorus from the lake (49). Akinetes are found only in the upper sediments in lakes with a recent history of such nuisance blooms (48). Desiccation of sediments impairs the germination and recruitment of akinetes of *Anabaena circinalis* into the water column (50). Periodic drying of wetlands adjacent to lakes or drawdown of water level, therefore, may reduce cyanobacterial inocula for population growth in the water column. Relatively high sediment losses of phosphorus are important for the rapid response of Lake Apopka to decreased external loading of phosphorus (47). Polyphosphate stored in intact algal cells is the proposed mechanism for this response. These examples show that understanding meroplankton dynamics is an important component of lake management.

In summary, four mechanisms are proposed by which resuspension or recruitment of meroplankton organisms from the benthic environment affect the production and dynamics of phytoplankton in surface waters. First, entrainment of algal cells or resting propagules can increase algal biomass by direct inoculation of the water column without the response time lag required for an increase in biomass due to growth alone. Second, entrainment of algal cells or resting propagules can provide seed populations that then grow in the photic zone, provided physical and chemical conditions are favorable for growth. Third, entrainment of algal cells or resting propagules and sediments with their interstitial waters can increase the nutrient content in the water column and thereby stimulate nutrient-limited algal growth in a few days. Fourth, entrainment of either inorganic sediments or organic sediments during resuspension events can increase nonalgal turbidity, thereby decreasing light availability for phytoplankton growth and production. Fifth, translocation or recruitment of vegetative cyanobacteria from the sediment occurs when gas vacuoles develop and increase buoyancy. Four of these mechanisms depend on wind-induced turbulence and resuspension in the water column, a passive process, for development of phytoplankton populations (28). By contrast, translocation is an active process in cyanobacteria that depends on vacuole formation and increased buoyancy of vegetative cells for recruitment from the benthic environment. It is likely that more than one of these processes is important in many systems. These processes have important implications for ecological studies and management actions.

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MESSENGER RNA-TARGETED PROBES.

See Fluorescent Probes for in situ Analyses of Microbial Communities

METABOLIC PATHWAYS.

See Evolution of Metabolic Pathways for Degradation of Environmental Pollutants

METABOLISM OF MIXTURES OF ORGANIC POLLUTANTS

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Most of our concepts on the biodegradation of organic chemicals originate from laboratory studies of single chemicals supplied at high concentrations to pure microbial cultures of microorganisms. For example, most of the presently used kinetic models for the prediction of the biodegradation of pollutants are based on single substrate usage. However, in wastewater and the environment, pollutant-degrading microbes are confronted with mixtures of pollutants and carbon sources of natural origin, both of which are usually present at very low concentrations.

Recently, considerable information has been collected as to how microorganisms handle the biodegradation of mixtures of organic compounds, including pollutants. In this article the most important phenomena and emerging principles with respect to utilization patterns and growth (degradation) kinetics reported for growth of microbial cultures in the presence of mixtures of carbon substrates will be presented. There is now much evidence that especially under conditions as they pertain in most ecosystems, that is, at low concentrations, microbes utilize mixtures of substrates of natural origin simultaneously with pollutants. Cells benefit from mixed substrate utilization in several ways, such as from improved growth rates, competitive advantage at low nutrient concentrations, and a better dynamic metabolic flexibility to temporary fluctuations in the spectrum of available nutrients. This has consequences on our understanding of and approach to issues such as the extent to which pollutants can be degraded in the natural environment in the presence of natural carbon sources, and how we can best stimulate and enrich a pollutant-degrading microbial population in an ecosystem.

LABORATORY VERSUS NATURE: CONTRASTING SCENARIOS FOR POLLUTANT BIODEGRADATION

As a source of carbon and energy for growth, heterotrophic microorganisms use small molecular weight–reduced carbon compounds that originate from the death and subsequent decay of other cells. In their quest for food, this class of microbes has developed the ability to utilize for growth not only organic compounds of natural origin but also a wide variety of artificial organic chemicals. As a rule, only dissolved monomers in the range of a few hundred daltons can cross the cell envelope. However, most of the decaying organic matter is present in a polymeric form and heterotrophic microbes have to rely on the extracellular enzymatic or chemical hydrolysis of these polymers into smaller portions that can be transported into the cell by means of efficient transport systems. This

hydrolysis is slow and, therefore, except for the occasional local burst, the environmental pool of organic compounds available for growth for heterotrophic microbes is small. In oligotrophic aquatic systems, typical concentrations of readily utilizable carbon sources are in the range of 1 to 10 μg of carbon L^{-1} and in eutrophic aquatic compartments they can rise transiently to reach 100 μg of carbon L^{-1} (1,2). Concentrations of individual organic pollutants in aquatic environments, including xenobiotic chemicals, are usually in the low $\mu g\,L^{-1}$ or even $ng\,L^{-1}$ range. Even in wastewater treatment plants, compounds used in large amounts, such as laundry detergents, reach peak concentrations of only a few $mg\,L^{-1}$ (3). As a consequence, in the majority of ecosystems, heterotrophic microbial growth is limited by the availability of carbon energy substrates (in addition to temperature) for most of the time (4). This scenario illustrates the growth conditions that heterotrophic microorganisms typically experience in nature (but also in well-run wastewater treatment plants): it consists of a pool of dozens or even hundreds of different natural carbon compounds and pollutants with concentrations of individual components that are extremely low.

To study the biodegradation of organic chemicals two contrasting strategies have been typically followed. On one hand, a "real-world approach" is taken in which the disappearance of a pollutant is followed in the environment (or in soil or water samples brought into the laboratory). This "black-box" approach can give realistic information about the disappearance of a chemical in a particular environment, but gives little insight into principles and mechanisms that govern the biodegradation of a compound. On the other hand, defined physiological and kinetic biodegradation studies are conducted in the laboratory under batch conditions in which pure cultures of microbial strains are supplied with high concentrations of a single chemical that serves as the only source of carbon and energy. This "ivory tower" approach contrasts strongly with the conditions these microbes experience in ecosystems as outlined earlier, but it allows investigation of the genetics and biochemistry involved in the degradation pathways for a pollutant.

In the following sections, an overview will be given first on the information available for mixed substrate growth conditions in batch cultures in which (especially at high concentrations) both sequential and simultaneous utilization of mixtures of carbon substrates is observed. This information is relevant for the degradation of pollutants at high concentrations, that is, for the treatment of special pollutant-containing wastewater, or the bioremediation of heavily contaminated environmental compartments. Subsequently, degradation strategies followed by heterotrophic microbes to deal with mixtures of carbon substrates under carbon/energy-limited conditions, as they are found in most ecosystems, are discussed. There is now considerable evidence that, as a rule, microbial cells do not specialize on one particular carbon substrate at a time but simultaneously utilize as many of the different available carbon compounds as possible, a behaviour usually referred to as *mixed substrate growth* (5,6).

BATCH CULTURE SYSTEMS: DIAUXIC GROWTH VERSUS MIXED SUBSTRATE GROWTH

Jacques Monod was the first to investigate the growth of bacterial strains in batch culture with mixtures of carbon sources (7). He was particularly attracted by the phenomenon of the two-step (diauxic) growth pattern exhibited by *Escherichia coli* when he supplied this bacterium with the two sugars, glucose and lactose (other cases of obviously "simultaneous" utilization of two sugars seemed less interesting to him). In this case, glucose was utilized first and the utilization of lactose was completely repressed until glucose had been completely consumed. A short lag followed until the cells started to grow with lactose. Subsequently, Monod and his coworkers (8) investigated the phenomenon of diauxic growth in more detail (also referred to as *carbon catabolite* or *glucose repression*). The elucidation of the molecular mechanisms leading to the controlled expression of lactose-utilizing enzymes revealed that genes for catabolic pathways are clustered (the lactose operon) and their expression is controlled in a coordinate manner. The detection of the operon was certainly a milestone in biology, but it has directed the attention away from mixed substrate utilization.

Since the detection of the mechanisms leading to carbon catabolite repression, the diauxic/sequential utilization of mixtures of carbon sources is considered to be the rule under substrate-excess conditions, rather than the exception. Nevertheless, "mixed substrate growth," that is, the simultaneous utilization of two or more carbon sources by pure microbial cultures (not to be mistaken with "cometabolism") has frequently been documented in the literature, indicating that the concept of diauxic carbon source utilization was (and still is!) incorrectly presumed to predominate under batch growth conditions. A compilation of some 50 cases (6,9) indicates that mixed substrate utilization occurs at high- and low-substrate concentrations with a wide range of different substrates, during growth under aerobic and anaerobic conditions, in gram-positive and gram-negative bacteria, Archaebacteria, and yeasts. The observations reported can be classified into two different categories:

1. A simultaneous utilization of mixtures of carbon sources supporting, on their own, only medium to low maximum specific growth rates; this frequently results in improved growth rates during growth with the mixture, and
2. Substrate mixtures that cause catabolite repression at high initial concentrations but at low concentrations these substrates are utilized simultaneously.

Simultaneous Utilization at High Substrate Concentrations

This first category of mixed carbon source utilization is well-documented in the literature [see 6), but the list given there is incomplete; more examples for pure and mixed cultures can be found in Ref. (9)]. For example, in a mixture of glucose (the carbon source supporting the highest μ_{max}) and methanol, methylotrophic yeasts utilize the two carbon sources in a clearly diauxic manner,

with glucose utilized first. However, supplied with mixtures of glycerol/methanol or xylose/methanol the two substrates are utilized simultaneously (6). For a number of microorganisms, a clear improvement of μ_{max} was observed for growth with the mixture as against growth with the individual carbon sources. In the case of *Hansenula polymorpha*, μ_{max} increased from 0.18 h^{-1} (with methanol alone) and 0.21 h^{-1} (with xylose alone) to 0.36 h^{-1} when a mixture of xylose/methanol was supplied. Similar results were reported for *Leuconostoc oenos* supplied with mixtures of glucose/citrate and glucose/fructose, and *Thiobacillus versutus* (glucose/galactose). This effect suggests that rates of breakdown for such carbon sources are not high enough to saturate the anabolic pathways with precursors for biosynthesis and that every additional compatible substrate will lead to an improvement of rate of biomass synthesis. It should be added here that an enhanced maximum specific growth rate was also observed for batch growth of *Thiosphera pantotropha* with acetate/ammonia when it was supplied with both oxygen and nitrate as mixed terminal electron acceptors (10). This effect might be attractive for bioremediation processes using denitrifying strains. Unfortunately, only limited data are available and it is therefore not yet possible to judge whether the extent of stimulation in growth rate might be predictable. Additive growth yields during the utilization of multiple substrates are usually observed but cases of enhanced (i.e., greater than additive) growth yields on account of mixed substrate utilization have been reported (11).

It was also demonstrated for both bacteria and yeasts that more than two carbon sources may be simultaneously utilized. For example, *Pseudomonas fragi* consumed glucose, lactate, citrate, asparate, and glutamate simultaneously when these were present at concentrations of 0.5 to 1.0 g L^{-1}. An interesting example is shown in Figure 1

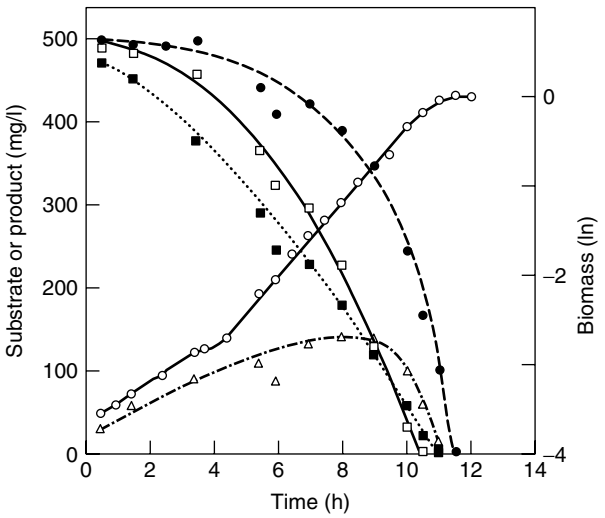

Figure 1. Batch growth of isolate NA 17 on a mixture of ethanol (●), isopropanol (■) and n-butanol (□) at 55 °C. Growth (○) is given as ln of dry weight (g L^{-1}) produced. Acetone (△) was produced during the cultivation and utilized again. Adapted from N. Al-Awadhi, Ph.D. thesis No. 8118, Swiss Federal Institute of Technology, Zürich, Switzerland 1989 with permission.

for an unidentified gram-negative thermophilic isolate, NA 17, isolated in our laboratory (12). When this bacterium was supplied with a mixture of the three solvents ethanol, isopropanol, and n-butanol, it utilized them all simultaneously. At the same time, acetone was produced from isopropanol in the early phase of growth, which was reutilized in the later phase of growth. Environmental conditions may affect the utilization pattern of substrates. For example, the yeast *Candida utilis* utilized acetate and xylose simultaneously at pH 6, but preferred acetate at pH 4.5 (probably because a significant fraction of the acetate entered the cell in the protonated form with no need for active transport). These examples demonstrate that microorganisms are able to handle quite complex combinations of substrates simultaneously.

Utilization of one substrate can enhance the degradation of a second substrate. For pure cultures, increased degradation rates for a pollutant have been reported for 2,4-dinitrophenol when glucose was added, or for benzene and *p*-xylene upon addition of toluene (9). These effects were also observed for natural populations, but it remains unclear whether or not it was based on mixed substrate utilization. However, it should be noted that the converse effect was also reported. The latter occurs probably with substrate combinations in which simultaneous utilization does not result in an enhanced growth rate, that is, in which the catabolic flux of one substrate, is able to saturate the biosynthetic capacity of a cell. Hence, whether fed with one or two substrates the total carbon assimilation rate will remain the same. Therefore, feeding a second carbon source will reduce the consumption rates for the first carbon substrate.

Many good carbon/energy substrates, including pollutants, also contain heteroatoms such as nitrogen, phosphorus, or sulfur groups, and when metabolized they can act as a source of both, carbon/energy and nitrogen, phosphorus, or sulfur. Because of the dual role of carbon in both dissimilation and assimilation, most of the compounds are carbon-deficient, and the cells have to excrete part of the heteroatom. In the case of C/N-compounds the degree of oxidation of the carbon skeleton, the position of nitrogen atom, and the catabolic pathway involved determine the fraction of nitrogen released (mostly in the form of ammonia). Supplied with an additional suitable carbon source, the two compounds are frequently taken up at rates such that the intracellular elemental demand is balanced and no nitrogen has to be excreted. A typical example has been reported for the utilization of the xenobiotic metal-complexing agent nitrilotriacetate (NTA) by *Chelatobacter heintzii*. When supplied with NTA as the only source of carbon, energy, and nitrogen, approximately 60% of the nitrogen in NTA was excreted in the form of ammonia and during balanced growth the release of ammonia was exactly proportional to the increase in biomass. When supplied with an additional carbon source, the uptake of both compounds was balanced, no ammonia was released and at the same time the specific growth rate increased from 0.07 h^{-1} to 0.13 h^{-1} (10). A similar effect has been observed for growth with organophosphonates and sulphonates in which surplus phosphorous and sulfur are released as phosphate, sulfite, or sulfate, respectively. This effect is

particularly important in the case in which excess ammonia, phosphorus, or sulfate represses the synthesis of the enzymes involved in the uptake and catabolism of these compounds (13).

It appears that many microorganisms are probably utilizing certain substrates only in combination with other substrates (there are many indications in the literature but rarely this has been followed up). The reasons for this phenomenon are largely unknown, perhaps, they lie in the balancing of catabolic and anabolic metabolite fluxes in the cell for avoiding the accumulation of inhibitory metabolites from one of the substrates. A typical example is the utilization of mixtures of amino acids by *E. coli* in which, for instance, serine cannot be utilized on its own but only in combination with glycine, leucine, and isoleucine (14). Therefore, testing the substrate utilization pattern of microbial isolates with the traditional single-substrate approach might not give us the full information about their catabolic capacity.

Release From Catabolite Repression At Low Substrate Concentrations

Mixtures of incompatible carbon sources, leading to diauxic growth when supplied under batch culturing conditions at high concentrations, are frequently consumed simultaneously when initial concentrations are lowered (6,10). An example is shown in Figure 2 for a culture of *E. coli* growing with a glucose/galactose mixture. This combination of sugars is utilized in a diauxic manner when supplied in the gram per liter range, however, when their initial concentration is lowered to 2 mg L^{-1} or less,

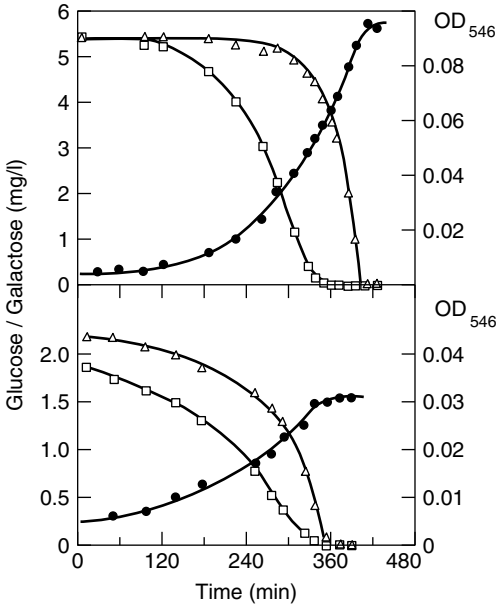

Figure 2. Batch growth of *E. coli* ML30. in a synthetic medium with different initial concentrations of glucose (□) and galactose (△). The upper panel indicates that the utilization of galactose starts as soon as the residual concentration of glucose is below approximately 2 mg L^{-1}. Growth (●) is given as optical density of the culture at 546 nm. Source of figure U. Wanner and T. Egli, *FEMS Microbiol. Rev.* **75**, 19–44 (1990) with permission.

they are consumed simultaneously (2 mg L^{-1} of a utilizable carbon source is a "feast" in the environment!). A number of other examples can be found in the literature in which the simultaneous utilization of incompatible carbon sources has been documented at low concentrations, however, only in a few cases has the transition from diauxic to simultaneous utilization been investigated systematically.

Many reports can be found in the literature in which the utilization of individual carbonaceous pollutants by a pure microbial culture has been investigated at "environmentally" low concentrations (i.e., in the low μg L^{-1} to ng L^{-1}-range) in batch culture. In contrast, mixtures of carbon compounds were considered only occasionally. For example, mixtures of acetate and phenol, or glucose and aniline were utilized simultaneously when supplied at concentrations between 1 and 10 μg L^{-1} by pure cultures of pseudomonads (9). In this context, a study reported for *Pseudomonas aeruginosa* is also interesting (15). This bacterium grew with a mixture of 45 carbon compounds added to tap water at a concentration of 1 μg L^{-1}, each, whereas none of these compounds supported growth when supplied on its own at this concentration. (However, note that it was not tested for which of the compounds were utilized and to what extent.) Such studies are usually difficult to interpret because of the interference of uncharacterized dissolved organic carbon, which is always present in cultivation media.

It has been pointed out that the effects of one substrate on the utilization of another are largely unknown (9). Nevertheless, from the information so far available, one has to assume that at the low environmental concentrations, in which growth of microorganisms also in batch systems is essentially carbon/energy-limited, carbon catabolite repression effects can be neglected and the simultaneous utilization of incompatible carbon substrates should be possible. Other effects, such as whether or not the cells are in a state in which catabolic enzyme systems are expressed sufficiently and whether or not these systems can be induced at the low concentrations, might play a far more important role than carbon catabolite repression (also illustrated later).

MIXED SUBSTRATE GROWTH IN CONTINUOUS CULTURE SYSTEMS LIMITED BY THE CARBON/ENERGY SOURCE

Although it was already recognized in the 1940s that laboratory media containing high concentrations of single carbon sources do not reflect environmental growth conditions, there were very few attempts to study the effects of low concentrations and mixtures of substrates by microbial cultures (5,6). This situation still prevails, despite the fact that it was pointed out that the concept of diauxic utilization of mixtures of carbon sources (as observed at high concentrations in batch culture) can probably rarely be applied to conditions in nature (5,16). As a more appropriate tool to investigate the behavior of microbial growth and degradation processes in the laboratory, cultivation in carbon-limited continuous culture has been suggested (4,16,17).

Within the last two decades, many studies have demonstrated that the mixtures of carbon substrates,

including pollutants, are utilized simultaneously under carbon-limited cultivation conditions in continuous culture (6). Also, for "incompatible," carbon catabolite repression–causing mixtures of carbon sources, simultaneous utilization during slow growth seems to be the rule. From these studies, a number of principles on the physiological and growth-kinetic behavior of microbial cells under environmental conditions, which are also relevant for the degradation of organic pollutants in ecosystems, can be deduced.

Substrate Utilization Patterns

The growth of methylotrophic yeasts with mixtures of glucose and methanol will be used here as an example of commonly observed microbial strategies for the utilization of diauxic substrate combinations in C-limited continuous culture. This system has been investigated in detail with respect to physiology and kinetics (6,11). Growth of methylotrophic yeasts with mixtures of glucose and methanol in batch culture results in a perfect diauxic utilization pattern with glucose utilized first and an extended intermediate lag before methanol is consumed. Repression of methanol utilization by glucose is also observed during fast growth in C-limited chemostat culture when the yeasts are fed with glucose/methanol mixtures. However, when the dilution rate is lowered, the two carbon sources are utilized simultaneously as shown for *H. polymorpha* in Figure 3a. When supplied with either methanol or glucose as the only source of carbon, a maximum specific growth rate of 0.19 h^{-1} ($\mu_{max(methanol)}$) or 0.51 h^{-1} ($\mu_{max(glucose)}$), respectively, was observed, in which the culture was washed out (D_{crit}). When the yeast was fed with a mixture of glucose/methanol (approx. 2 : 3, w/w), methanol was utilized simultaneously with glucose up to a dilution rate of 0.26 h^{-1} (i.e., considerably higher than the D_{crit} observed for growth with methanol alone!). At this dilution rate methanol started to accumulate, and at a D higher than 0.33 h^{-1}, only glucose was used for growth by the culture. Hence, growing with the "good" carbon source glucose, the yeast was able to utilize the "worse" carbon source methanol even at dilution rates exceeding $\mu_{max(methanol)}$. The dilution rate at which methanol consumption became repressed was dependent on the mixture composition fed, and this dilution rate increased with decreasing proportions of methanol in the mixture. For example, when methanol contributed only 20% of the total carbon, it was utilized up to dilution rates of almost 0.4 h^{-1} (18).

This behavior has been reported for several bacterial and yeast cultures fed with "diauxic" mixtures of carbon sources and only one exception to this rule has been observed (6). A closer inspection of the specific consumption rates for methanol during growth with single substrates or with mixtures explains this surprising effect of utilization of a substrate at dilution rates exceeding its μ_{max} (Fig. 3b). During growth with methanol alone, the maximum specific methanol consumption rate, $q_{methanol(max)}$, up to which normal growth was observed, was in the range of 0.40 to 0.45 g methanol (g dry biomass h)$^{-1}$ at a dilution rate of approximately 0.17 h^{-1}. At higher dilution rates, unbalanced growth occurred, unutilized

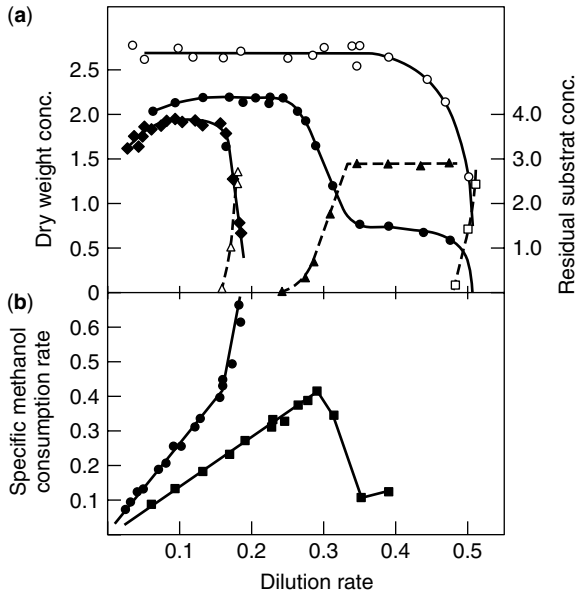

Figure 3. Growth of *H. polymorpha* as a function of dilution rate h^{-1} in carbon-limited chemostat culture supplied with either methanol, a mixture of glucose/methanol (38.2%/61.8%, w/w), or glucose as the source(s) of carbon and energy. Panel a: Dry biomass (g L^{-1}) formed with methanol alone (♦), the mixture (●), or with glucose (○); residual glucose concentration (g L^{-1}) in the culture during growth with glucose or the mixture (□); residual methanol concentration (g L^{-1}) during growth with methanol alone (△) or with the mixture (▲); panel b: Specific methanol consumption rates (g methanol (g dry biomass hour)$^{-1}$) during growth with methanol alone (●) or with the mixture (■). In all experiments, the total concentration of methanol and/or glucose in the inflowing medium was always 5 g L^{-1}. Adapted from T. Egli, C. Bosshard, and G. Hamer, *Biotechnol. Bioeng.* **28**, 1735–1741 (1986).

methanol accumulated, resulting in excessive oxidation of methanol until the system collapsed at $D = 0.19$ h^{-1}. When cultivated with the mixture at $D = 0.17$ h^{-1}, $q_{methanol}$ was only approximately half of $q_{methanol(max)}$ (Fig. 3b), and hence the methanol pathway had spare capacity to handle methanol. Consequently, the cells continued to utilize methanol when the dilution rate was increased until they consumed methanol at the maximum possible rate (with this mixture at $D = 0.28$ h^{-1}). Only when the dilution rate was further increased, and the culture was pushed beyond its methanol consumption limit, were the cells forced to switch to growth on glucose only. During growth with mixtures containing small proportions of methanol, its consumption was repressed at dilution rates above 0.35 to 0.4 h^{-1}, and the maximum capacity of the methanol catabolic pathway was never reached. This repression can be attributed to the increasing catabolite repression effect of glucose when approaching μ_{max} of 0.51 h^{-1}. Such consumption of a "slow" carbon compound at dilution rates exceeding its μ_{max} has been reported for a number of cases (6,11).

The example given was for a binary mixture of carbon sources. However, more complex mixtures containing up to 20 different substrates were shown to be degraded

simultaneously under carbon-limited continuous cultivation conditions by pure cultures (6).

Growth Kinetics With Mixed Carbon Substrates

The traditional kinetics describing microbial rates of substrate consumption and growth are based on the assumption that these processes are governed by the concentration of a single, growth-limiting substrate (19). The most widely used kinetic model (eq. 1) was proposed by Monod (7). It relates the specific rate of growth (μ) to the concentration of the growth-controlling substrate (s) via two parameters, the maximum specific growth rate (μ_{max}) and the substrate affinity constant (K_s) (the analogy to the Michaelis-Menten equation for enzyme reactions is obvious). Most of the models used for kinetic studies in the biodegradation of pollutants are based on Monod's model (19).

$$\mu = \mu_{max} \frac{s}{K_s + s} \quad (1)$$

The fact that, under environmental conditions, a microbial cell will probably utilize a variety of carbon substrates simultaneously raises the question of whether and how kinetic models based on growth rate controlled by a single substrate can be applied to a situation in the environment.

First experimental evidence for the influence of mixed substrate utilization on the kinetics of growth was observed for a culture of a *Corynebacterium* cultivated in a carbon-limited chemostat culture with mixtures of glucose and amino acids (19). When this culture was run at a constant dilution rate, the steady-state concentration of glucose was lowered when amino acids were included as carbon/energy sources in the medium. The observation that steady-state concentrations of substrates can be reduced during mixed-substrate utilization in carbon-limited chemostat cultures has been confirmed by numerous studies including various organisms and combinations of carbon substrates.

The most detailed kinetic analysis has been reported for the growth of *E. coli* with mixtures of up to six different sugars and mixtures of glucose and 3-phenylpropionate (3ppa) (20,21). An example of the response typically observed is shown in Figure 4a for growth at a constant dilution rate with different mixtures of glucose, fructose, and galactose in the feed. As expected, all three sugars were utilized simultaneously, independent of composition of the mixture. In this particular experiment, fructose always contributed around 33% to the total carbon concentration in the inflowing medium, whereas the contribution of glucose and galactose varied. With all mixtures, the steady-state concentrations of the individual sugars were reduced compared with the level measured during growth at the same dilution rate with single sugars. The concentrations of the individual sugars reflected their contribution to the total substrate supplied in the inflowing medium, that is, the steady-state concentration of fructose in the culture remained virtually constant, whereas the residual concentrations of

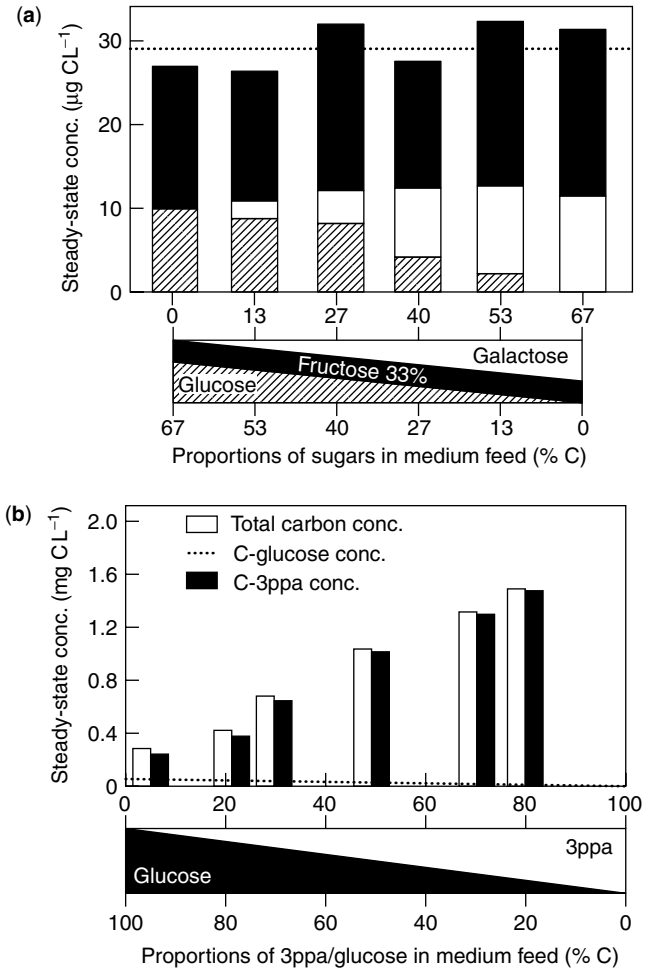

Figure 4. Mixed-substrate kinetics during growth of *E. coli* in carbon-limited continuous culture. (**a**) Growth with different mixtures of glucose, fructose, and galactose at a dilution rate of 0.30 h^{-1}. (**b**) Growth with different mixtures of glucose and 3-phenylpropionate (3ppa) at a dilution rate of 0.6 h^{-1}. In all experiments, the total carbon concentration from the mixture was such that it supported a total biomass of 45 mg dry cell weight per litre. Adapted from K. Kovářová-Kovar, and T. Egli, *Microbiol. Mol. Biol. Rev.* **62**, 646–666 (1998).

glucose and galactose were approximately proportional to the mixture fed.

Interestingly, the concentration of total organic carbon from all three sugars remained approximately constant (Fig. 3a), suggesting that the growth rate of the culture might be controlled by either the total sugar concentration, or the total available carbon in the environment. However, some of the data obtained in this series of experiments with six different sugars and for a methylotrophic yeast with glucose and methanol indicated that control of μ by a lumped parameter, such as total organic carbon, is probably only applicable to substrates with similar K_s constants and growth yields. This was confirmed for the growth of *E. coli* with mixtures of glucose and 3-phenylpropionic acid, two substrates for which the affinity constants differ significantly ($\sim$30 $\mu g\,L^{-1}$ and $\sim$700 $\mu g\,L^{-1}$

for glucose and 3ppa, respectively). In this case, the steady-state total carbon concentration in the culture originates mainly from 3ppa (Fig. 4b). Nevertheless, steady-state concentrations of both substrates were always lower during growth with single substrates and reflected their relative contribution in the feed.

It must be pointed out that the reduction of steady-state concentrations of individual substrates during mixed-substrate growth has been reported only for mixtures of sugars and the "model pollutant" 3ppa and for the solvent methanol. However, recently, this kinetic pattern was also observed for the growth of *Ralstonia eutropha* with mixtures of fructose and the pesticide 2,4-D, which indicates that it is likely that this kinetic behaviour is applicable to the degradation of pollutants in the presence of alternative carbon substrates (Rüegg, Füchslin and Egli, unpublished). Definitive statements addressing such issues will require further experimental confirmation.

A number of different kinetic models have been proposed in the literature to describe microbial growth and competition in a multisubstrate environment (21). Many of them are based on the assumption of sequential, or diauxic utilization patterns of substrates, and therefore contain inhibition terms. Nevertheless, several models have been put forward, usually derived by combining two or more Monod-terms, which can be applied to growth with mixtures of carbon sources in continuous culture. A comparison of different models using our experimental data obtained for the growth of *E. coli* with different sugar (22) and glucose/3ppa mixtures (21) was made. The model giving the most accurate description of the two sets of data is given in eq. 2,

$$\mu = \frac{\mu_{\max} \cdot \sum a_i \cdot s_i}{\mu_{\max} + \sum a_i \cdot s_i} \qquad (2)$$

where i is the number of substrates, μ is the specific growth rate predicted, $\mu_{\max}$ is the maximum specific growth rate of the best substrate, and a_i is the specific affinity for substrate i defined as $\mu_{\max,i}(K_{s,i})^{-1}$. Although such a model might be difficult to apply to a particular environmental situation (the number of substrates utilized by a cell and their concentration would have to be known), it provides a first approach to the understanding of principles of mixed-substrate growth kinetics.

Mixed-Substrate Growth and the Regulation of Pollutant-Degrading Enzyme Systems

Many enzyme systems involved in the degradation of organic pollutants are inducible and are only expressed in significant amounts in the presence of the pollutant. Hence, the questions arise as to how much pollutant is required to stimulate induction, what does the time course of this induction look like, and how are extent and rates of degradation influenced by the presence of alternative carbon substrates.

First answers to some of these questions have been obtained in an attempt to understand the degradation of the complexing agent, nitrilotriacetate (NTA), during wastewater treatment and in the environment in which a pure culture of *Chelatobacter heintzii* was grown in carbon-limited continuous culture with mixtures of NTA and glucose (23–25). The study has revealed some interesting regulatory aspects that are not only valid for this particular system but may be of use for understanding also the environmental regulation of other pollutant-degrading enzyme systems. In *C. heintzii*, NTA is degraded by an inducible NTA monooxygenase (NTA-MO), and during cultivation with glucose the activity of enzyme was close to the detection limit. Nevertheless, when a C-limited continuous culture of this bacterium was fed with glucose/NTA mixtures containing less than 1% of the carbon as NTA, the culture was able to degrade the NTA fed, although the enzyme levels did not increase significantly. Obviously, glucose did not interfere with the degradation of NTA, and the low expression level of NTA-MO was sufficient to ensure utilization of this chemical. Significant induction of NTA-MO protein and activity was observed only when the proportion of NTA in the medium feed exceeded 1% of the total carbon (Table 1).

Table 1. Expression of NTA Monooxygenase (NTA-MO) and Extent of NTA Degradation in a Carbon-limited Continuous Culture of *C. heintzii* ATCC 29,600 Grown at a Constant Dilution Rate of 0.06 h^{-1} with Different Mixtures of Glucose and NTA in the Feed. The Degree of Induction Was Assessed from Both, Specific Activity of NTA-MO in Cell Free Extract, and the Immunological Quantification of the Monooxygenase and the Oxidoreductase Proteins (Data from M. Bally, E. Wilberg, M. Kühni, and T. Egli, *Microbiology* 140, 1927–1936 (1994)

Carbon Sources in Feed	Conc. and % of Carbon Sources in Feed		Residual Conc. of Carbon Sources in Culture	Level of Expression of NTA-MO (in % of Final Level)
NTA	1 mg L^{-1}	(0.04% C)	~30 μg L^{-1}	Not induced
Glucose	2 g L^{-1}	(99.96% C)	~20 μg L^{-1}	<1%
NTA	10 mg L^{-1}	0.36% C)	~25 μg L^{-1}	not induced
Glucose	2 g L^{-1}	(99.64% C)	~20 μg L^{-1}	<1%
NTA	0.1 g L^{-1}	(3.6% C)	~30 μg L^{-1}	Induced
Glucose	1.91 g L^{-1}	(96.4% C)	~20 μg L^{-1}	~10%
NTA	1.0 g L^{-1}	(36% C)	~75 μg L^{-1}	Induced
Glucose	1.28 g L^{-1}	(64% C)	~20 μg L^{-1}	~70%

If this behaviour can be extrapolated to environmental conditions, one would expect no significant induction of NTA-utilizing enzymes in *Chelatobacter* cells growing in surface water where the contribution of NTA to the total carbon available is usually below 0.1 to 1%. However, in sewage treatment plants receiving a high load of NTA, one would expect to see an induction. Results from pure culture studies carried out in several Swiss treatment plants and model treatment plants fed with artificial sewage containing different proportions of NTA confirmed these observations (25). Although these studies are not perfect and leave considerable room for speculation, they indicate that for short-term fluctuations the degradation of NTA is probably regulated at the enzyme level rather than an enrichment of an NTA-degrading population.

It is obvious that growth conditions in wastewater treatment plants and the environment are rarely constant. Here pollutant-degrading microorganisms have to cope with changes in both concentrations and spectrum of available carbon sources. The ability to react to such changes in NTA availability was therefore also investigated for *C. heintzii* (24). When a culture growing with glucose only was suddenly switched to a medium containing NTA as the only source of carbon/energy and nitrogen, the cells exhibited a lag phase of more than a day before they started to express NTA-MO and resumed growth. This lag time was considerably reduced when the cultures were shifted from glucose to mixtures of glucose/NTA instead of NTA only. For example, shifting the cells to a mixture of 99% NTA and 1% glucose reduced the time needed for the induction of NTA-degrading capacity from some 25 to less than 10 hours, and shifts to media containing 90% glucose and 10% of NTA resulted in immediate induction. This demonstrates the important role of alternative carbon and energy sources for the metabolic flexibility of cells and their ability to induce pollutant-degrading enzyme systems. Microorganisms can be forced into a starvation response by suddenly providing a cell with a new substrate for which the enzymes are not sufficiently expressed. During starvation, all signals are set for the degradation of intracellular reserve materials, ribosomes and proteins for the generation of energy and building blocks, a state from which it takes considerable time to recover. Of course, it is essential to maintain carbon-limited conditions for a smooth induction process. Supply of high concentrations of easily degradable carbon could lead to catabolite repression of the pollutant-degrading enzyme system.

THRESHOLD CONCENTRATIONS FOR UTILIZATION AND INDUCTION

A central question concerning the fate of pollutants in the environment is that of their degradation at low concentrations. Frequently, the concentration of dissolved organic carbon of natural origin is 10 to 100 times higher than that of organic pollutants (9). On one hand, it has been demonstrated for a number of organic chemicals that they can be degraded and assimilated at concentrations of a few nano or even femto gram L^{-1}, which is far below the "threshold" concentration of 1 to 100 $\mu g\,L^{-1}$ carbon normally considered to be necessary for growth (9). On the other hand, threshold concentrations have been reported for a number of chemicals, some of them considerably higher than 100 $\mu g\,L^{-1}$, below which they were not degraded.

With respect to the utilization of pollutants at low concentrations, there is now evidence that threshold concentrations for utilization of (and growth with) particular carbon compounds are lowered by the simultaneous utilization of other carbon substrates (6,9). For example, *P. aeruginosa* grew with a mixture of 45 compounds, each present at concentrations of 1 $\mu g\,L^{-1}$, but was not able to grow with the individual compounds at this concentration. Similarly, *Salmonella typhimurium* failed to grow in a mineral medium with glucose supplied at 1 $\mu g\,L^{-1}$; however, when supported with 5 $mg\,L^{-1}$ of arabinose, glucose was still utilized at 0.5 $\mu g\,L^{-1}$. The aforementioned mixed sugar experiments for *E. coli* suggest that an individual sugar should theoretically be consumed even at minute concentrations.

With respect to threshold concentrations for utilization and induction, an interesting phenomenon was recently observed in our laboratory for the growth of *E. coli* with 3ppa (19). Cultured in glucose-limited chemostat culture, *E. coli* exhibited no detectable 3ppa-degrading activity. When low concentrations of 3ppa (0.3 and 3 $mg\,L^{-1}$) were added to the glucose feed medium (the glucose feed concentration was 100 $mg\,L^{-1}$), no degradation of 3ppa was observed. Even extended exposure for more than 100 generations did not lead to 3ppa degradation. However, if the feed concentration of 3ppa was raised to 5 $mg\,L^{-1}$ or more, degradation was induced. The data indicate a threshold concentration for the induction and utilization of 3ppa in the range of 4 $mg\,L^{-1}$. Most interestingly, once induced, the culture was able to degrade 3ppa below the threshold concentration required to trigger induction and 3ppa degradation continued, although the actual concentration of 3ppa in the reactor was always below 1 $mg\,L^{-1}$ (compare also Fig. 4b).

CONCLUSION

The data suggest a general kinetic principle, that is, multiple substrate utilization is widespread and the extent of substrate degradation depends on the number of substrates utilized, their ratio, and the growth rate. This principle has a number of important implications for the growth of heterotrophic microorganisms under carbon-limited conditions and for our conceptual thinking of pollutant biodegradation.

With respect to growth, the principle explains the puzzling observation that heterotrophic microbes can survive and even grow reasonably fast at the low substrate concentrations encountered in many oligotrophic ecosystems, such as the open ocean. Considering competition in the environment, the ability of a cell to utilize several substrates simultaneously should lead to an enhanced competitiveness. In comparison to a fastidious strain that is restricted to the utilization of one or only a few carbon substrates, the metabolically versatile strain would be able to support faster growth at the same substrate concentration (always assuming that the two strains utilize the common substrates with similar kinetic properties).

Or in other words, microorganisms could achieve the same growth rate at a lower substrate concentration and would therefore push the substrate concentration down to a lower level. There are indeed a number of examples in the literature in which nutritionally versatile strains were reported to outcompete specialist strains under mixed-substrate growth conditions, whereas the specialists won the competition for a single substrate (26). Hence, affinity for a substrate is only one component of the ecological competitiveness in the environment, another is a wide catabolic spectrum. It is frequently argued that it is not worthwhile for a cell to put efforts into the utilization of a compound that is present at too low a concentration. This argument certainly does not hold if one considers that cells utilize mixtures of substrates simultaneously. It will be the sum of carbon obtained by the cell that determines whether or not growth is possible. Therefore, it seems justified to conclude that the presence and simultaneous utilization of alternative carbon substrates enhances both the kinetics and the extent of the degradation of pollutants. However, clear data supporting this view are still scarce and a more systematic approach is required to obtain a comprehensive ecological understanding of the relationships between multiple substrate utilization, threshold concentrations, and cleanup goals.

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METALS. See METAL (U, Fe, Mn, Hg) CYCLING

METALS, BIOREMEDIATION OF. See BIOREMEDIATION: AN OVERVIEW OF HOW MICROBIOLOGICAL PROCESSES CAN BE APPLIED TO THE CLEANUP OF ORGANIC AND INORGANIC ENVIRONMENTAL POLLUTANTS

METALS IN SOILS. See METALS: MICROBIAL PROCESSES AFFECTING METALS

METALS: INTERACTION WITH BIOFILMS. See SORPTION PROPERTIES OF BIOFILMS

METALS, MICROBE RESISTANCE TO. See METALS: MICROBIAL PROCESSES AFFECTING METALS

METALS: MICROBIAL PROCESSES AFFECTING METALS

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The accumulation of metals in the environment due to anthropogenic activities has led to concern over the long-term fate of metal contaminants and the impact of metal accumulation on terrestrial and aquatic ecosystems. In nature, microorganisms carry out many different processes that influence the bioavailability of metals to other living organisms, and that affect their partitioning into organic and inorganic substances. Very often microbial transformations of metals are fortuitous reactions that occur as a result of microbial processes that change the physical and chemical conditions in the environment. However, various metals also have essential roles in microbial metabolism and must be carefully regulated in the cell to prevent deficiencies and toxicities. In addition to the role of metals in enzymes, some metals such as iron and manganese are used as electron acceptors for respiration by dissimilatory metal-reducing microorganisms under anoxic conditions. These redox transformations are particularly important because they change the solubility of metal ions and thus may be used to solubilize metals from ores and contaminated waste, or conversely, to precipitate metals and decrease their bioavailability. Lastly, microorganisms may carry out alkylation reactions with metals such as selenium, mercury, and arsenic that volatilize these metals. Together, all of these processes are fundamental not only for understanding the biogeochemistry of metals, but also for the development of bioremediation technology to treat metal-contaminated systems.

MICROBIAL PROCESSES AFFECTING METALS

There are many different biological processes that directly affect how metals will partition into various organic and inorganic forms that occur in nature. These include uptake of metals by living cells, sorption to the cell surface, and redox reactions that result in precipitation of solid-phase minerals or solubilization of metal ions. Prediction of the fate of metals is complicated because all of these biological processes can occur simultaneously at varying rates that depend on the species and activities of the microorganisms that are present and the particular environmental conditions. Microorganisms have a high surface-to-volume ratio, and although they comprise a very small portion of the total mass of soil and water media, they take up and adsorb significant quantities of metals over time. In reactions that are carried out by the heterotrophic microorganisms, the rates of the various reactions that are carried out by microorganisms are determined by the availability of reduced carbon in the organic substances that are used for growth. The relative amounts of metals that are taken up into living cells or that are adsorbed to the cell surface depend on the specific metal involved, the pH and ion composition of the medium, and the growth phase of the cells (1). Metals that are taken up by microbial cells may be further combined into various organic molecules that have different degradation rates and potential toxicities to other living organisms. For example, methyl mercury and organoselenium compounds represent highly toxic substances, whereas the fate and toxicities of metal ions bound to metalothioneins and cell wall components or to exopolysaccharides are less well understood. Selective precipitation of metal ions by specific functional groups on the surfaces of microbial cells can lead to the formation of nucleation sites for the precipitation of more metal ions and counter ions that result in the formation of mineral aggregates (2). The formation of metal–organic complexes also commonly leads to recalcitrance of organic molecules that may persist in the environment for long periods of time (3,4). In particular, microbial synthesis and secretion of extracellular polysaccharides affects the formation of primary organomineral complexes (<2 μm clay particles and silt size microaggregates 220 μm), which may interact with polyvalent metal ions. Because silt and clay size particles also harbor the more recalcitrant organic matter in the soil, these interactions with metal ions can help protect extracellular polysaccharides or other trapped organic matter in soil from decomposition (5). The role of microorganisms in producing and degrading organic–metal complexes is thus a key consideration in predicting the chemical form and fate of metals in the environment.

Uptake and Accumulation of Metals by Living Cells

Metal ions are taken up by cells by both passive and active transport processes. Divalent metal cations such as Mn^{2+}, Fe^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+}, and Cu^{2+} are all structurally very similar, having only a 14% difference in ionic diameters, and all carrying a double positive charge. To differentiate between structurally similar metal ions, heavy metal uptake systems must bind to these ions tightly. Thus, there are two general types of uptake systems for metal ions (6). The first are fast and unspecific, and are constitutively expressed because they are used for a variety of substances. These systems are usually driven by differences in the membrane potential, and are charged chemiosmotically by proton ATPase activity. The second type of heavy metal ion uptake system has high substrate specificity, and is inducible, but slower. These specific uptake systems often directly couple with ATP hydrolysis as the energy source for transport, in addition to using the chemiosmotic gradient. Usually, specific uptake systems are induced only under certain conditions such as during trace metal deficiencies. However, when these systems are expressed, they may also transport nonessential or toxic metal ions that are structurally similar to physiological ions. For example, oxyanions such as chromate (CrO_4^{2-}) and arsenate (AsO_4^{3-}), which resemble sulfate and phosphate, respectively, enter the cell by the sulfate and phosphate transport systems.

The two types of uptake systems, passive and active, can usually be distinguished by their different transport kinetics. Because there is no energy involved in the passive uptake, it occurs rapidly and metal concentrations between the outside and inside of the cell reach a steady state once equilibrium is achieved. Active uptake, on the other hand, takes longer because there is an energy requirement.

Metal Sorption to Microbial Cell Walls

Many different functional groups on the surfaces of bacterial and fungal cells can interact with metals, including carboxyl, amine, and phosphoryl groups, and various peptides. These negatively charged functional groups occur as components of structural materials in the cell wall, and are also present in the capsules of bacteria that produce exopolysaccharides. Experiments have shown that uncapsulated bacterial strains accumulate lower quantities of metals than capsulated bacteria (7). Nevertheless, capsulated cells may be better protected from metal toxicities because the production of capsular substances that adsorb metals may help prevent the entry of metals into the cell. Following a similar strategy to that of capsulated bacteria, many fungi contain melanin that binds with metals and thereby reduces their potential toxicity. Melanins contain an array of binding sites for metal ions that in melanin-pigmented fungi can increase the sorption capacity for nickel, copper, zinc, cadmium, and lead by 2.5- to 4-fold as compared with albino fungal strains without melanin (8).

Both pH and the chemical composition of the medium surrounding bacterial and fungal cells can strongly affect the accumulation of metals on the cell surface. This is because the functional groups that are responsible for the adsorption of metals are negatively charged and are protonated at low pH, which results in competition between protons and metal ions for the binding sites. In addition to protons, other cations also will interact with these anionic sites, including Na^{+}, Mg^{2+}, and Ca^{2+}, which are common in soil and aqueous media. Overall, the adsorption of metals is usually enhanced by an increase in pH, although this varies for different metal ions and microbial species (1). However, the maximum binding of metals may occur over an intermediate pH range near neutrality because at very high pH, microorganisms may change their cell surface and metals may form neutral and anionic hydrolysis species that have less affinity for negatively charged metal-binding sites.

Use of Microbial Biomass as Metal Bioadsorbents

Sorption of metals to cell surfaces has been investigated as a possible method to extract and recover metals from contaminated water. Microalgae, in particular, are being investigated for use in biofiltration because biosorbents produced from these microorganisms have a high adsorption capacity, which in some cases is comparable to that of activated carbon. Algal biomass may also be used as a source of organic acids and other soluble complexing agents that can be used as extractants. However, there are still many problems and considerations in the optimization of this technology. The sorption capacity of biomass prepared from different algae and blue-green algae can vary considerably. Adsorption characteristics also are influenced by processing the materials in different ways. For example, dried and partially mineralized biomass of the cyanobacteria *Anabaena* sp. has a 4-, 8-, and 10-fold increase in sorption capacity for cadmium, copper, and lead, respectively, as compared with the fresh biomass (9). In another example, the sorption of nickel by cyanobacteria can be increased by autoclaving (10). Thus, there are many empirical considerations in developing this technology.

Among the advantages of using microbiological materials as biosorbents, algal and cyanobacterial cells have a high surface area, are inexpensive to produce, and can be grown in large quantities in earthen ponds using sewage wastewater as a nutrient source (11). Since the early studies in the 1970s, a wide variety of cyanobacteria and green algae have been examined with the aim of identifying particular species that are effective in the adsorption of metals from wastewater. Parameters that influence sorption include the pH of the water being treated, the temperature of the medium, the presence of competing ions, the residence time, and the density of the biomass (12). To increase the selectivity of biosorbents for particular metals, it may be advantageous to use specific components of the biomass, or at least to identify the active fractions, which can subsequently be optimized through cultural procedures or appropriate processing of the biomass materials.

Biosorbents have been studied in relation to their ability to bind a wide variety of metals including zinc, cadmium, copper, nickel, and lead. They have also been studied for the sorption of radionuclides including technetium (13), cobalt and cesium (14), and americium and uranium from wastewater (15). In one investigation comparing 15 blue-green algae species, the biomass of 11 of the 15 species that were studied proved to be superior to activated carbon for the adsorption of cadmium, whereas the biomass of four other species was inferior (16). Most of the cadmium that was adsorbed by the effective algal biomass could be readily extracted using a dilute mineral acid (pH 2). This is highly advantageous because the biomass may be used repetitively to concentrate heavy metals from wastewater. Similar results have been obtained with americium and uranium, which were extracted from water using biomass produced from *Anacystis nidulans* (15). In this latter research, the optimum pH range for the sorption of both actinides was in the acidic region between 3.0 and 5.0. Under these conditions, the algal biomass was able to remove 92% of the americium (Am) and 85% of the uranium (U) from contaminated wastewater, from which 46% of the americium and 82% of the uranium originally present in the wastewater could be recovered by elution with a solution of ammonium carbonate.

Biosorbents can also be generated using fungal mycelium by-products that are generated from fermentation industries (17), and have been used to purify zinc, lead, silver, nickel, and cadmium from water in continuous-flow systems. Another application of microorganisms for the removal of metals from water effluents

is the use of constructed microbial mats. These mats contain mixed microbial communities that are grown and become embedded in glass wool supports (18). When contaminated water is passed over the mats, they rapidly remove toxic metals and metalloids including cadmium, lead, chromium, selenium, and arsenic. Metals that are removed by these mats are deposited in different forms that reflect the various microenvironments and redox gradients within the mats. In addition to the adsorption onto the cell walls that occurs in both oxic and anoxic zones, metals oxides are precipitated in the oxic zones, whereas sulfate-reducing bacteria precipitate metal sulfides in the anoxic zones.

Another process that has been investigated for the extraction of metals using microorganisms involves the use of metallothioneins and related metal-binding peptides. Metallothioneins are intercellular proteins that bind metals and contribute to cell homeostasis in the presence of potentially toxic metal concentrations. These proteins are widespread among animals and are also produced by fungi. Metal-tolerant cyanobacteria (blue-green algae) produce similar substances that are glutathionine-derived polypeptides. In developing metal-binding biomass materials, genetic engineering of microorganisms has been performed to obtain bacteria and cyanobacteria with improved metal-binding proteins that are expressed on the outer membrane of the cells (19–21). Eukaryotic metallothioneins have been expressed in *Escherichia coli* as fusions to membrane or membrane-associated proteins such as LamB, the peptidoglycan-associated lipoprotein protein (PAL), or a hybrid Lpp/OmpA carrier sequence (21). The use of different anchors enables the metallothionein to be targeted into various locations in the cell. In experiments examining different fusion proteins, each type of fusion differed in terms of expression, stability and ability to permit *E. coli* to accumulate Cd^{2+}. In similar studies with cells of a genetically engineered *E. coli* strain, JM109, which expresses metallothionein and a Hg^{2+} transport system, cells in suspension accumulated Hg^{2+} effectively at low concentrations (0–20 μM) over a broad range of pH (19). These results suggest that there is considerable potential for the genetic engineering of bacteria to remove metals from wastewater or from contaminated solutions.

MICROBIAL SOLUBILIZATION OF METALS

Factors Controlling Metal Solubilities

The solubility of inorganic metal ions and their hydrolysis species with other ions is controlled by pH, redox potential, and the ionic composition of the medium in which the metals occur. Metals may also form complexes with organic substances, and can be chelated by siderophores that are produced in response to metal deficiencies. In general, the solubility of metal ions increases by 10-, 100-, and 1,000-fold for every unit decrease in pH for mono-, di-, and trivalent metal ions, respectively. However, the total free activity in solution is also controlled by the ion-pairing with anions such as sulfate, phosphate, and carbonate. Thus, computer models are necessary to determine the speciation and solubility of metals in aqueous systems.

The solubilities of metal ions and the formation of metal complexes with organic substances have been modeled using thermodynamic models that take into account solution complexation and precipitation of solid-phase minerals. Organic acid anions and fulvic acid from dissolved organic matter, in particular, will significantly increase metal solubilities by forming metal complexes. Computer software programs that have been developed for modeling metal solubilities include GEOCHEM, MINTEK, and CHARON. The latter computer program, CHARON, takes into account interactions with both dissolved and solid organic materials that have complexing capacity for Zn^{2+}, Cd^{2+}, Cu^{2+}, Ca^{2+}, Mg^{2+}, and Al^{3+} (22).

Microorganisms directly affect the solubility of metals by their influence on pH. Acid formation by microorganisms can be accomplished by a variety of mechanisms, including respiration, release of organic acids and protons, and by mineral transformations. The most common mechanism involves simple release of carbon dioxide by heterotrophic microorganisms that use organic carbon for metabolism. Over the long term, production of carbonic acid results in the slow dissolution of rocks and minerals. In surface soil, carbonic acid production is also attributed to mycorrhizal fungi that produce carbon dioxide and enhance root-respiration rates (23). In contrast to carbonic acid, the involvement of organic acids in soil acidification is somewhat complex. Organic acids are commonly released as components of plant root exudates, but are also produced by microorganisms that grow on root exudates or that decompose organic detritus. Under aerobic conditions, organic acids such as citrate, malate, and succinate are excellent carbon sources for growth and do not accumulate (24). However, under low-oxygen conditions that commonly occur under wet soil conditions, microorganisms may transform plant-derived organic acids and other compounds into various fermentation products. These include acetic, lactic, and butyric acids that can accumulate to millimolar concentrations in microenvironments of high microbial activity. In addition to influencing pH, these substances also serve as carbon substrates that may be used to produce hydrogen or may be used by acetate-degrading anaerobic bacteria to drive dissimilatory reduction of metals (Fig. 1).

Still another process leading to the acidification of surface soil is the release of protons by microorganisms. Proton-release into the cell envelope is fundamental to microbial metabolism, and is used to generate an electrochemical potential across the cell membrane to drive synthesis of ATP. In surface soil, the relative contribution of plants and microorganisms to this process has not been easy to determine because certain microorganisms such as *Azospirillum* sp. can significantly increase proton efflux by plants and increase acidification of the rhizosphere (25).

Chemolithotrophic activity leading to soil acidification is largely because of the activity of *Thiobacillus ferrooxidans* and other iron- and sulfur-oxidizing microorganisms that produce protons as a result of the mineral-oxidation process. These bacteria typically thrive under extreme acid conditions, but are also active in normal soils that have a pH range from 3.5 to 9. The diverse bacteria and

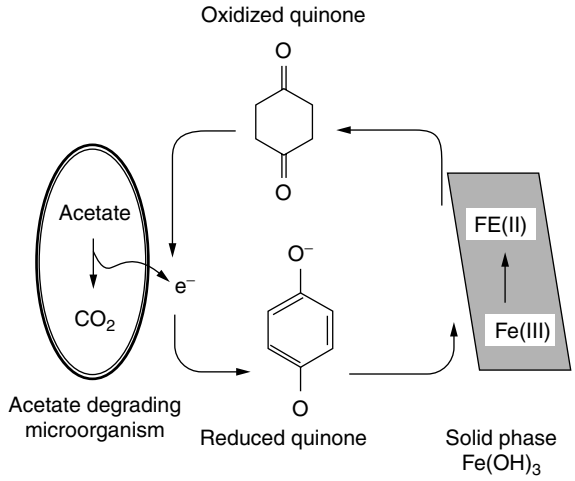

Figure 1. Dissimilatory reduction of solid phase iron minerals using quinones contained in soil organic matter as water soluble electron shuttles between bacteria and the mineral surface. Adapted after Lovely [25].

fungi that can grow well beyond this pH range include acidophilic bacteria such as *Thiobacillus thiooxidans*, which can grow at pH 1.0, and microorganisms such as *Bacillus alcalophilus*, which grows at pH 11. Recent studies on acid environments have revealed highly complex communities containing high microbial diversity including bacteria, fungi, archaea, eukaryotic algae, and even protozoa (26). The activity of acid-forming chemolithotrophs, however, generally is of concern only in soils that have exposed pyrite, or that have been amended with elemental sulfur. When large quantities of these elements are exposed, such as during mining, they can cause extreme acidification of the soil, lowering the pH to values less than 1.0 (27,28).

Bioacidification has been investigated for the leaching of metals from sewage sludge and provides a potentially useful and cost-effective method for reducing the heavy metal content of waste biosolids before application to agricultural fields or land disposal (29). In this process, elemental sulfur is added to the sludge and is converted to sulfuric acid by the oxidation of the sulfur. The lowered pH then permits solubilization and leaching of metals from the biosolids. Using an integrated process, metal-contaminated soil can be cleaned by a sequential process involving bioleaching, followed by precipitation of metal sulfides (30). In this process, soil is mixed with elemental sulfur and incubated under aerobic conditions to generate sulfuric acid. The metals contained in the leachate are subsequently stripped using an anaerobic bioreactor containing a mixed culture of sulfate-reducing bacteria that precipitate soluble metal species as solid metal sulfides. The efficacy of this treatment will vary for different metals. As shown by White and coworkers (30) in an experiment with an artificially contaminated soil, cadmium, cobalt, chromium, copper, manganese, nickel, and zinc were efficiently leached, whereas lead leaching was slow and remained incomplete over a period of 180 days. Similar results were obtained by these researchers in experiments with an industrially contaminated soil in which approximately 69% of the main toxic metals present, namely, copper, nickel, and manganese, were removed after 175 days.

Bioleaching of Metals

Microbes are increasingly being used in commercial mining operations for bioleaching of copper, uranium, and gold ores. As reviewed by Rawlings (31), direct leaching can be achieved by changing the redox state of the metal being collected to make it more soluble. In contrast, indirect leaching involves altering the redox chemistry of other metal cations that are then coupled to chemical oxidation or reduction of the harvested metal ion. This can also be combined with direct microbial attack on the mineral matrix in which the metal resides.

In addition to mining metal ores, bioleaching is being proposed as a method to treat metal-contaminated aquatic sediments. In this process, sulfur-oxidizing bacteria are used to generate sulfuric acid from elemental sulfur. As shown by Seidel and coworkers (32), in an oxic sediment with good permeability, about 60% of the total zinc, cadmium, nickel, cobalt, and manganese that was contained in a test sediment could be removed by percolation-leaching after 120 days, which was sufficient to enable the treated sediments to be reused as soil. However, the sulfur-oxidizing bacteria may take different periods of time to activate and begin producing acidity. In freshly dredged anoxic sediment, only 9% of the metals were removed. Similar processes have been used to treat metal-contaminated municipal sludges (33). A fully integrated strategy employing microorganisms it may be combine metal bioleaching with a collection of metals using bacterial biomass as biosorbents to collect the metals from the leachates. This could have many cost-advantages over the use of alternative processes that involve the use of industrially produced acids and metal chelators, followed by metal collection on cation exchange resins. Another interesting alternative is the use of activated sludge as a metal collector, which has been used with a countercurrent biosorption system for the removal of metals from aqueous effluents (34).

Metal Chelation by Organic Acids and Microbial Siderophores

Almost all microorganisms produce compounds called *siderophores* that are secreted into the environment under iron-limiting conditions that commonly occur in aerated systems at neutral to alkaline pH (35). Siderophores are produced by almost all bacteria and fungi, and there are well over 100 different types of siderophores, each of which has different metal-binding characteristics and chemical properties. Three broad classes of siderophores include the hydroxamate siderophores, catechol siderophores, and mixed ligand siderophores such as those produced by pseudomonads. Microbial siderophores are generally highly specific for iron, but as shown in some studies, may also mobilize other metals. This phenomenon has not yet been well investigated with respect to the uptake of heavy metals, primarily because very few siderophores are commercially available and they must be purified from laboratory cultures for use in experiments.

The possible function of siderophores for the deliberate uptake of essential trace metals other than iron has been investigated in only one bacterial species to date. Microorganisms require small amounts of certain transition metals that are used in metalloenzymes, including molybdenum, cobalt, nickel, and copper (36). In experiments with *Pseudomonas aeruginosa* that produces two different siderophores, pyochelin and pyoverdin, the former binds and transports Cu(II), Co(II), Mo(VI), and Ni(II), but has relatively low affinity for Fe(III) (37). In contrast, pyoverdin strongly binds iron, and its production and transport is regulated only by the availability of iron. These data suggest that siderophores may have a much broader role in metal solubilization and transport than simply for iron.

Other research has preliminarily examined the possible role of siderophores in transporting nonessential heavy metals, although the relative contribution of organic acid production, decrease in pH, and siderophores to metal solubilization in soil is not yet well understood (38). In a study with alcaligin E, the siderophore of the heavy metal-resistant *Ralstonia* sp. strain CH34 (formerly *Alcaligenes eutrophus*), the siderophore was shown to interact with cadmium and also affected its bioavailability and toxicity (39). In an alcaligin E-deficient CH34 derivative, the addition of alcaligin E stimulated growth in the presence of cadmium, which was attributed to a decrease in the bioavailability of cadmium in the presence of alcaligin E. However, alcaligin E had no influence on uptake or the cellular concentration of cadmium. Scanning electron microscopy further showed that the morphology of precipitated Cd crystals was altered by alcaligin E. Thus, alcaligin E appears to provide some protection against cadmium toxicity. In this particular study, a link between the *Ralstonia* sp. strain CH34 siderophore system and the *czc* -mediated Cd-efflux system was hypothesized.

MICROBIAL REDOX TRANSFORMATIONS OF METALS

Dissimilatory Metal Reduction

Many bacteria have the ability to reduce metals from their soluble, oxidized forms to reduced ion species that have decreased solubility or that precipitate from solution (Table 1). The energetics of metabolic reactions involving metals as electron acceptors has been reviewed (40) and can be predicted on the basis of the Gibbs free energy for the redox reaction. Metal reduction by microorganisms can involve either dissimilatory metal reduction in which the metal is used as an electron acceptor for respiration, or fortuitous processes involving nonspecific oxidoreductases (41). This latter process may be carried out by a variety of oxidoreductases such as cytochromes that function in respiration, with the difference that metal reduction does not support energy conservation and cellular growth.

Iron is used as an electron acceptor for growth of anaerobic bacteria including *Shewanella putrefaciens, Geobacter metallireducens*, and *Thiobacillus ferrooxidans*. However, with the exception of iron and manganese, heavy metals normally occur at low concentrations except in contaminated environments such as in metal ores, industrial waste, or mine tailings, and thus are not available at concentrations that would be physiologically relevant for use as electron acceptors during active growth. Even the direct use of iron as a terminal electron

Table 1. Biologically Driven Redox Transformations of Metal Ions

Metal	Reaction	Reductant	Microorganisms	Notes
As	$As^{5+} \rightarrow Ag^{3+}$		Gram positive	Reduced intracellularly before efflux by As resistance system (42)
Ag	$Ag^{+} \rightarrow Ag^{0}$			
Au	$Au^{3+} \rightarrow Au^{0}$		Fe(III)-reducing microorganisms	
Cr	$Cr^{6+} \rightarrow Cr^{3+}$	Glucose, acetate, amino acids	*Pseudomonas Bacillus* Streptomycetes Enterobacter	Cr(VI) not used for respiration.
Cu	$Cu^{2+} \rightarrow Cu^{+}$	S^{0}	Thiobacillus	Ferric ion oxidoreductase
Fe	$Fe^{3+} \rightarrow Fe^{2+}$	H_2, S^{0} organic acids alcohols	Geobacter Shewanella Desulfovibrio Thiobacillus Diverse bacteria	Fe(III) not used for respiration
Hg	$Hg^{2+} \rightarrow Hg^{0}$		Diverse bacteria	Detoxification mechanism plasmid-borne genes
Mn	$Mn^{6+} \rightarrow Mn^{4+}$ $Mn^{4+} \rightarrow Mn^{2+}$		Diverse bacteria	Mn reduction may be chemically coupled to oxidation of Fe(II)
Se	$Se^{6+} \rightarrow Se^{0}$ $Se^{6+} \rightarrow Se^{4+}$ $Se^{4+} \rightarrow Se^{0}$			Reduction of Se(VI) to Se(IV) can be used for respiration in some bacteria
Tc	$Tc^{7+} \rightarrow Tc^{4+}$		Sulfate-reducing bacteria	Fortuitous enzymatic
U	$U^{6+} \rightarrow U^{4+}$	Acetate, lactate, H_2	Geobacter, Desulfovibrio, Micrococcus,	Oxidizes *c*-type cytochromes, may be used for respiration by some bacteria
V	$V^{5+} \rightarrow V^{3+}$, V^{2+}			

acceptor for respiration by mesophilic bacteria appears to be relatively rare (43,44) because most of the aerobic bacteria that have so far been shown to reduce Fe(III) have been found to be unable to conserve energy while using this process (45). Recently, Francis and coworkers described a mesophilic bacterium that uses Fe(III) for respiration (44). In this case, a bacterium closely related to *Pantoea* (formerly *Enterobacter agglomerans*) was shown to couple the oxidation of either acetate or hydrogen to the reduction of Fe(III), Mn(IV), Cr(VI), and a quinone.

Role of Quinones in Metal Redox Transformations

For some time, metal reduction by microorganisms has been assumed to occur by direct contact of microorganisms with the surfaces of solid-phase minerals or with dissolved metal ions that are present in the solution. This view is changing rapidly because there is now much evidence that these reactions may be facilitated primarily by reduced humic substances or quinones that serve as electron shuttles for the transfer of electrons from oxidoreductase enzymes in the cell walls to nearby solid-phase minerals (46–48). After transferring electrons to metals in the vicinity of the cell, the quinones, or humic substances are regenerated to their oxidized state, and thereby can act catalytically to drive metal-reduction processes. As shown with Fe(III), even low concentrations of humic substances can significantly enhance the rate of iron reduction (49). Recent studies have focused on two Fe(III)-reducing microorganisms, *G. metallireducens* and *Shewanella alga*, which can substitute humic substances for Fe(III) as the terminal electron acceptor (49). Both *G. metallireducens* and *S. alga* conserve energy to support growth by reducing the humic substance analog, 2,6-anthraquinone disulfonate (AQDS), to 2,6-anthrahydroquinone disulfonate (AHQDS). The role of these electron-shuttles for the reduction of other metals such as chromium and uranium is now an active area of investigation. For example, the radiation-resistant bacterium, *Deinococcus radiodurans*, was recently shown to reduce Fe(III), U(VI), and Tc(VII) in the presence of anthraquinone-2,6-disulfonate (50). Recently, it has been shown that microbial reduction of metals, such as Fe(III) to Fe(II), can lead to subsequent nonenzymatic reduction of other metals, such as chromium by indirect processes (51). It remains to be investigated whether this process may also be enhanced by quinones.

Taking advantage of the fact that heavy metals have decreased solubility in their reduced forms, a variety of biological treatment processes have been proposed for the treatment of metal-contaminated soil and water. Many different organic carbon sources including glucose, organic acids, and amino acids can serve as electron donors for metal reduction. In addition to treatment of metal-contaminated sites, the ability to use metals as electron acceptors for respiration is particularly important with respect to bioremediation of organic contaminants under anaerobic conditions in sediments and subsurface environments. In the sections that follow, various methods for bioremediation of metals and organic contaminants using microbiologically driven metal-oxidoreduction reactions are summarized.

Microbiological Reduction and Detoxification of Chromium

Chromium is the seventh most abundant element on earth and is present in soil at concentrations between 5 and 3,000 $mg\,kg^{-1}$. As a result of its extensive use in industrial processes, chromium is a common metal pollutant in soil and water. Chromium can exist in the environment in different oxidation states, but most commonly occurs as Cr(III) and Cr(VI) (52). The oxidized form, Cr(VI), occurs as a water-soluble oxyanion, CrO_4^{2-}, which is a strong oxidizing agent and highly toxic to microorganisms (53,54). A variety of methods have been developed to remove chromium from water using both biosorption and bioreduction to Cr(III).

Biological reduction of the chromate ion, Cr(VI), to the chromic ion, Cr(III), can easily be achieved by indigenous soil bacteria and can be stimulated by providing an organic amendment (55). Following reduction, the chromic ion subsequently precipitates as insoluble $Cr(OH)_3$. Chromate reduction also has been studied under anaerobic conditions, in which case, Cr(VI) is reduced by bacteria that use acetate as a carbon source (56). Sulfate and nitrate both slightly inhibit this latter process, whereas oxygen causes complete inhibition. Studies aimed at the treatment of contaminated water show that nearly complete removal of Cr(VI) can be achieved by bacteria in flow-through systems containing 26 $mg\,L^{-1}$ Cr(VI) when acetate is provided at a ratio of 9 mg C mg^{-1} of Cr(VI) (57). In addition to this treatment method, there are a variety of other biological treatment methods. Among these, *Pseudomonas fluorescens* has been used to reduce Cr(VI) using citrate as a carbon source (58). Other researchers have studied *Bacillus* sp. in packed-bed bioreactors (59). In the latter case, reduction of Cr(VI) by *Bacillus* is achieved under aerobic conditions by a reductase that mediates the transfer of electrons from NAD(P)H to chromium (60). This reductase system is speculated to be a specific detoxification mechanism that differs from the dissimilatory electron-transfer mechanism used by other bacteria.

Still another application aimed at the treatment of mixed waste has been to couple the biodegradation of organic pollutants with chromium reduction. For example, a phenol-degrading consortium has been combined with a strain of *E. coli* that is capable of Cr(VI) reduction (61). In this manner, the phenol-degrading consortium degraded phenol into metabolites that were used by the *E. coli* strain as an electron donor for the reduction of Cr(VI).

In addition to organic substrates that serve as electron donors for Cr(VI) reduction, some bacteria can also use H_2, H_2S, and reduced Fe(II) as electron donors. For example hydrogen is used by *Desulfovibrio vulgaris* to drive the reduction of Cr(VI) to Cr(III) (62), in which case, the *c*-3 cytochrome from this organism functions as the Cr(VI) reductase. In experiments examining the use of H_2S as an electron donor, researchers have discovered a sulfate-reducing bacterial consortium that tolerates concentrations of 2,500 $mg\,L^{-1}$ of Cr(VI) and that indirectly reduces chromium by the production of H_2S gas (63). Cr(VI) reduction by an indirect mechanism also has been observed during dissimilatory iron reduction by the blue-green algae species *Shewanella*, which first

reduces Fe(III) to Fe(II), after which Fe(II) transfers electrons to Cr(VI) (51). In this process, the iron is cycled between the reduced and oxidized forms and appears to be working catalytically. Thus, there are a variety of practical methods for achieving detoxification of chromate in anaerobic soil and water.

In contrast to the reduction of Cr(VI), which depends mostly on the organic matter content of the system, the reverse reaction involving the oxidation of Cr(III) to more soluble Cr(VI) occurs most rapidly in soils or aqueous systems that are high in manganese [Mn(IV)] oxides and that contain low organic matter (64). In aqueous systems, Cr(III) appears to be oxidized chemically and occurs readily in the presence of light and Fe(III) minerals (65). In this case, chromium oxidation appears to be driven by photolysis of $FeOH_2^+$ complexes. However, this process is strongly inhibited by organic acids (66), which are thought to promote dissolution and reduction of manganese oxides to Mn(IV), such that manganese can no longer serve as an electron acceptor for the oxidation of chromium.

Redox Transformations of Manganese

Manganese can exist in several different valence states, but occurs in the environment primarily as Mn(IV) and Mn(II) minerals. Although manganese can be transformed abiotically, microorganisms are the major catalysts of manganese cycling in the environment (67). Manganese reduction is carried out by diverse microorganisms including various species of *Pseudomonas*, *Geobacter*, and *Shewanella*. For most of the bacteria that have been studied, manganese does not appear to be used as an electron acceptor for respiration and thus does not contribute to their energy metabolism (45). Nonetheless, this view may change as future studies determine whether or not microbial respiration using manganese oxides can be effectively coupled using quinones and other organic matter constituents. In addition to reduction that is driven directly by oxidoreductases, manganese also can be chemically reduced by Fe(II) and H_2S (68), and can be catalyzed by organic acids at pH values less than neutrality (69).

The opposing reaction, involving oxidation of Mn(II) minerals, can be achieved both biotically and abiotically. In contrast to most metal ions that have decreased solubilities on reduction, manganese solubility is increased by reduction of Mn(IV) to Mn(II). Thus, manganese-oxidizing bacteria are involved in the formation of manganese-precipitates, such as those that encrust iron pipes, and that generate manganese-oxides in the soil and manganese nodules in marine systems. The abiotic oxidation of Mn(II) to Mn(IV) occurs most rapidly under aerated conditions at neutral to alkaline pH, but is still very slow compared with biotic oxidation of manganese. Biological generation of manganese oxides is carried out enzymatically by a variety of manganese-oxidizing bacteria, fungi, and algae that use *c*-type cytochromes (70). As reviewed by Gounot (67), bacteria that carry out this process include the sheathed bacteria, *Leptothrix*, the budding and appendaged bacteria, *Pedomicrobium* and *Hyphomicrobium*, and many other common bacteria including *Bacillus, Pseudomonas*, and *Vibrio*. Biotically driven manganese oxidation also can occur indirectly by reaction with hydrogen peroxide, which is produced by certain bacteria.

Although manganese oxides are less abundant in soil and sediment than in iron oxides, they tend to accumulate in sites of high microbial activity and thus have a greater role in microbial processes than would be predicted simply on the basis of their relative abundance in the environment. Importantly, manganese oxides can abiotically facilitate the oxidation of a variety of carbon substrates, including organic contaminants such as toluene and atrazine. By-products of these substances may subsequently be degraded by various bacterial species and consortia that occur in the vicinity of manganese oxides. In studies on the use of Mn(IV) by a toluene-degrading consortium, toluene, an enrichment culture from a contaminated sediment was able to grow on toluene under strictly anaerobic conditions using manganese oxide as an electron acceptor (71). Of the different manganese oxides tested, the rate was slowest with crystalline manganese oxide as compared with amorphous manganese minerals. In the case of atrazine, which is now the most common pesticide pollutant in groundwater in the United States, manganese oxides can catalyze the complete degradation of the parent compound, but also generate dealkylated metabolites that can subsequently serve as substrates for atrazine-degrader organisms (72). The use of biogeochemical systems for the degradation of organic pollutants is a relatively new concept, but undoubtedly will become an increasingly important concept for bioremediation of organic contaminants in soil and sediment systems.

Methylation and Volatilization of Metals and Metalloids

As previously mentioned, microorganisms transform metals through oxidation and reduction reactions. Other transformation reactions carried out by microorganisms are methylation and demethylation. During methylation reactions, inorganic forms, are converted to organic forms and vice versa during demethylation reactions. The microbial methylation of heavy metals, such as selenium, arsenic, tin, lead, and tellurium results in the production of volatile and often toxic gases. In the early 1800s, several cases of arsenical poisoning occurred in Germany because of the use of wallpapers that contained arsenic in the pigments. The wallpapers were found to emit trimethylarsine when they became damp and moldy. The volatilization of heavy metals/metalloids from contaminated soil, sediment, and water is also of considerable interest as a remediation technique. Remediation methods, using microbially mediated volatilization, have been proposed for selenium (73,74), and mercury (75). These metals are also the most studied with respect to methylation reactions and, as such, will be the focus of this section (see Table 2 for a list of metal-alkylating microbial genera).

Methylation of Selenium. The environmental threat of elevated levels of selenium in soil and water has been recognized in many locations throughout the western United States (76). In California's San Joaquin Valley,

Table 2. Microorganisms Known to Alkylate Heavy Metals and Metalloids

Genera	As	Hg	Se
Algae			
Chlorella			X
Fungi			
Acremonium			X
Alternaria			X
Aspergillus	X	X	X
Candida	X		X
Cephalosporium			X
Fusarium			X
Gliocladium	X		
Neurospora		X	
Penicillium	X		X
Saccharomyces	X	X	
Schizophillum			X
Scopulariopsis	X		X
Trichophyton	X		
Bacteria			
Aeromonas	X		X
Acinetobacter			X
Alcaligenes			X
Bacillus		X	
Corynebacterium			X
Escherichia	X	X	
Flavobacterium	X		X
Klebsiella		X	
Mycobacterium		X	
Pseudomonas	X	X	X
Rhodocyclus			X
Rhodospirillum			X

elevated levels of selenium in Kesterson Reservoir were linked to the death and deformity of waterfowl. In soil, sediment, and water, bacteria and fungi are the predominant selenium-methylating organisms (77). The methylation of selenium is thought to be a protective mechanism used by microorganisms to detoxify their surrounding environment. The predominant selenium gas produced by most microorganisms is dimethylselenide (DMSe) (78). In general, the formation of alkylselenides from selenium oxyanions involves a reduction and methylation step; however, the pathway by which these reactions occur is still highly debated.

The biosynthesis of methionine from homocysteine is an important transformation in the methylation of selenium. During the activated methyl cycle, homocysteine is methylated by the coenzyme methylcobalamin (CH_3B_{12}, derivative of vitamin B_{12}), yielding methionine. Methylcobalamin has been isolated from bacteria (79) and is believed to donate methyl groups to selenium, resulting in the formation of volatile alkylselenides. Thompson-Eagle and coworkers (80) found that the addition of methylcobalamin promoted the methylation of selenate [SeO_4^{2-}, Se(VI)] by a fungal isolate, *Alternaria alternata*. McBride and Wolfe (81) found that cell-free extracts of a *Methanobacterium* sp. methylated SeO_4^{2-} when methylcobalamin was present. Cell-free extracts of an enteric bacterium, *Enterobacter cloacae* SLD1a-1, isolated from seleniferous drainage water, catalyzed the formation of DMSe from selenite [SeO_3^{2-}, Se(IV)] or elemental selenium [Se(0)] when methylcobalamin was the methyl donor (82). In addition to methylcobalamin, *S*-adenosylmethionine has been identified as a cofactor in the microbial methylation of inorganic selenium (83). Doran (78) found that cell-free extracts of the soil bacterium, C*orynebacterium*, were able to methylate SeO_3^{2-} or Se(0) when *S*-adenosylmethionine was present.

Methylation of Arsenic. The bacterial methylation of inorganic arsenic has been studied extensively using methanogens. These archae are present in large numbers in anaerobic ecosystems such as sewage sludge, freshwater sediments, and composts in which organic matter is decomposing (76). Under anaerobic conditions, the biomethylation of arsenic only proceeds to dimethylarsine, which is stable in the absence of oxygen, but which is rapidly oxidized under aerobic conditions. It has been shown that at least one *Methanobacterium* sp. is capable of methylating inorganic arsenic to produce volatile dimethylarsine. Arsenate [AsO_4^{3-}, As(V)], arsenite [AsO_2^-, As(III)], and methylarsonic acid can serve as substrates in dimethylarsine formation. Inorganic arsenic methylation is coupled to the CH_4-biosynthetic pathway and may be a widely occurring mechanism for arsenic detoxification.

Cell-free extracts of a *Methanobacterium* strain, MOH, produce volatile dimethylarsine when incubated under anaerobic conditions with AsO_4^{3-}, methylcobalamin, hydrogen, and ATP (81). Whole cells of methanogens also produce dimethylarsine as a biomethylation end product of arsenic under anaerobic conditions. Cell-free extracts of *D. vulgaris* strain 8,303 also produce a volatile arsenic derivative, presumably an arsine, when incubated with AsO_4^{3-}. Interestingly, another study found that resting cell suspensions of *Pseudomonas* and *Alcaligenes*, incubated with either AsO_4^{3-} and AsO_2^- under anaerobic conditions, produced arsine, but no other arsenic intermediates were formed (84). *Aeronomonas* sp. and *Flavobacterium* sp. isolated from lake water were capable of methylating arsenic to dimethylarsinic acid, whereas the *Flavobacterium* sp. additionally methylated dimethylarsinic acid to trimethylarsine oxide (85).

Several fungi have demonstrated the ability to transform arsenic. It is well established that the fungi are able to volatilize arsenic as methylarsine compounds, which are derived from inorganic and organic arsenic species. The volatilized arsenic dissipates from the cells, effectively reducing the arsenic concentration to which the fungus is exposed. In recent studies, three different fungal species, *Candida humicola, Gliocladium roseum*, and *Penicillium* sp., were capable of converting methylarsonic acid and dimethylarsinic acid to trimethylarsine (86). In addition, *C. humicola* used AsO_4^{3-} and AsO_2^- as substrates to produce trimethylarsine. Cell-free extracts of *C. humicola* transformed AsO_4^{3-} into AsO_2^-, methylarsonic acid into dimethylarsinic acid and trimethylarsine oxide, and dimethylarsinic acid into methylarsinic acid and trimethylarsine oxide (87). Although trimethylarsine formation from inorganic arsenic and methylarsonic acid is inhibited by the presence of phosphate, its synthesis from

dimethylarsinic acid is increased in the presence of phosphate (86). Recently, Huysmans and Frankenberger (88) isolated a *Penicillium* sp. from agricultural evaporation pond water capable of producing trimethylarsine from methylarsonic acid and dimethylarsinic acid.

Methylation of arsenic is thought to occur by the transfer of the carbonium ion from *S*-adenosylmethionine (SAM) to arsenic. Incubation of cells with an antagonist of methionine inhibits the production of arsines, thus supporting the role of methionine as a methyl donor (89). The addition of either methanearsonic acid or dimethylarsinic acid to cell-free extracts yields trimethylarsine oxide (87). Further reduction of trimethylarsine oxide to trimethylarsine requires the presence of intact cells (90). Various arsenic thiols (cysteine, glutathione, and lipioc acid) are thought to be involved in the reduction step of trimethylarsine oxide to trimethylarsine (91,92). The final reduction step is inhibited by several electron transport inhibitors and uncouplers of oxidative phosphorylation (90,93). Preincubation of cells with trimethylarsine oxide increases the rate of conversion to trimethylarsine, suggesting an inducible system (90). In addition, the rate of transformation of AsO_4^{3-} to trimethylarsine is increased by preconditioning the cells with dimethylarsinic acid (93).

Methylation of Mercury. Although a number of microorganisms are capable of methylating mercury under both aerobic and anaerobic conditions (94), field studies suggest that mercury methylation occurs most rapidly under anoxic conditions (95,96). Recent evidence suggests that sulfate-reducing bacteria are the dominant mercury methylators in estuarine (97) and lacustrine (98) anoxic sediments. Aerobic bacteria that are active in methylating Hg(II) include *Pseudomonas* sp., *Bacillus megaterium, E. coli*, and *Enterobacter aerogenes*, whereas fungi include *Aspergillus niger, Neurospora crassa, Scopulariopsis brevicaulis*, and *Saccharomyces cerevisiae*. The anaerobic bacterium *Clostridium cochlearium* was found to methylate a variety of mercury compounds including HgO, $HgCl_2$, $Hg(NO_3)_2$, $Hg(CN)_2$, $Hg(SCN)_2$, and $Hg(OOCCH_3)_2$ (99).

As with selenium methylation, methylcobalamin has long been suspected as a cofactor in the microbial methylation of mercury because it is known to donate methyl groups to Hg(II) (100). Methylcobalamin is also an important coenzyme in the biosynthesis of methionine (101). In *E. coli*, methylcobalamin was found to catalyze the transfer of a methyl group to homocysteine, resulting in the formation of methionine (102). In *N. crassa*, the formation of methyl mercury (CH_3Hg^+) was stimulated by the addition of homocysteine, but was inhibited by the addition of methionine (103). Choi and coworkers (104) recently confirmed that the sulfate-reducing bacterium, *Desulfovibrio desulfuricans*, methylates Hg(II) by the coenzyme methylcobalamin. It was additionally proposed that Hg(II) might be methylated during the acetyl coenzyme A synthase reaction.

Bioremediation of Metal-Contaminated Soil Using Bioaugmentation

A variety of bacteria have been examined for their potential use in the cleanup of metal-contaminated sites, and there are numerous studies in which bacteria and consortia have been identified for use under aerobic and anaerobic conditions. Some bacteria appear to be particularly promising, but there has not yet been much commercialization of this technology. One bacterium, *Pseudomonas maltophilia* strain, which was isolated from soil at a toxic waste site in Oak Ridge, Tennessee (105), catalyzes the transformation and precipitation of numerous toxic metal cations and oxyanions including Hg(II), Cr(VI), Se(IV), Pb(II), Au(III), Cd(II), Te(IV), and Ag(I). Effective removal of the toxic metals is accomplished within one to two days for most of the metals, with silver requiring seven days. With this particular microorganism, a variety of mechanisms are involved in each of the metal transformations. The NADPH-dependent reduction of Hg(II) to Hg(0) is catalyzed by an inducible mercuric reductase. Reduction of selenite and tellurite to their insoluble elemental forms appears to be mediated by an intracellular glutathione reductase that uses bis(glutathio)Se(II) or bis(glutathio)Te(II), respectively, as pseudosubstrates. Reduction of hexavalent chromium is catalyzed by a membrane-bound chromate reductase.

In addition to mesophilic bacteria, there are also diverse hyperthermophilic microorganisms that have the ability to reduce Fe(III) with hydrogen (H_2) as the electron donor, and other metal ions. *Pyrobaculum islandicum* uses poorly crystalline Fe(III) oxide as an electron acceptor for growth on hydrogen or peptone and yeast extract as the electron donor (106). Cell suspensions of *P. islandicum* grown on hydrogen can also reduce U(VI), Tc(VII), Cr(VI), Co(III), and Mn(IV). It is speculated that these hyperthermophilic microorganisms may contribute to the speciation of metals in hydrothermal environments and could account for the formation of various minerals such as magnetite and uranium deposits at high temperatures near 100 °C. Reduction of toxic metals by this and related microorganisms also might be applied to the remediation of metal-contaminated water or waste streams (106).

Although there are many potential applications of microorganisms for bioremediation, methods involving bioaugmentation remain problematic and include the cost for the production of large quantities of inoculum that need to be transported and incorporated into the contaminated medium on site. Other problems include poor survival after release into the environment, low activity, and toxicities to mixed contaminants. Nevertheless, progress has been achieved in developing bioaugmentation technology for bioremediation of organic contaminants, which may be extended to the treatment of metal-contaminated sites (107).

HEAVY METAL TOXICITIES TO MICROORGANISMS

Elements of the Periodic Table with a density of more than 5 $g\,cm^{-3}$ are known as *heavy metals*. Although this encompasses 65 different elements, there are relatively few heavy metals that are generally regarded to be of environmental concern. These are arsenic, cadmium, chromium, copper, lead, mercury, nickel, and zinc. Although each of these metals is toxic to most organisms at relatively high concentrations, copper, nickel, and zinc

also have important functions as essential trace elements (i.e., micronutrients). These and other metals such as iron and manganese are required cofactors of enzyme-catalyzed reactions. However, it should be noted that there is a fine threshold between heavy metal deficiency and toxicity, which may vary for different organisms depending on their tolerance and resistance mechanisms.

The contamination of soil with heavy metals usually occurs by direct application from sources including mine wastes, atmospheric deposition (a result of metal emissions to the atmosphere from metal smelting, fossil fuel combustion, and other industrial processes), animal manures, and sewage sludge. Surprisingly, some inorganic fertilizers contain significant quantities of heavy metal impurities. Sewage sludge, which is often used as a soil conditioner, contains useful quantities of organic matter, nitrogen, and phosphorus; however, it often contains heavy metals. The heavy metals are chelated or complexed by the organic matter, which are then released on decomposition. Heavy metal cations in soil may be present as several different forms: (*1*) as ions in soil solution, (*2*) as easily exchangeable ions, (*3*) organically bound, (*4*) coprecipitated with metal oxides, carbonates, phosphates, or secondary minerals, or (*5*) as ions in primary minerals (108–110).

Heavy metals in soils are found as various immobilized (e.g., organically bound) and nonimmobilized (e.g., in soil solution) forms. The heavy metal form is highly influenced by soil properties such as pH, oxidation-reduction state, clay content, iron oxide content, and organic matter content (111). Those metals that are immobilized are considered biologically unavailable, whereas those that are nonimmobilized are considered biologically available. Therefore, the toxicity of heavy metals to a soil microbial community will differ depending on their chemical state. A soil microbial population will experience a lower heavy metal concentration when a soil contains heavy metals largely in immobilized forms (e.g., carbonate complexes). Because all heavy metals in soil are not available, an important distinction must be made between the total extractable and biologically available heavy metal content in the soil. A heavy metal extraction from the soil recovers both biologically available and unavailable metals; therefore, the extractable heavy metal content does not reflect its biological effect.

It is well known that exposing microorganisms to heavy metals affects their growth and survival. In the early 20^{th} century, mercuric chloride ($HgCl_2$) was widely used as a general disinfectant, whereas the use of copper in Bordeaux mixture was used to control various fungal pathogens. Heavy metals inactivate cellular enzymes by combining with a component of the protein [e.g., $HgCl_2$ inactivates enzymes that contain sulfhydryl (–SH) groups]. In general, bacteria and actinomycetes (prokaryotes) are more sensitive to heavy metal–soil pollution than fungi (eukaryotes). However, bacteria are more sensitive than actinomycetes, and gram-positive bacteria are generally more sensitive than Gram-negative bacteria (112).

In soils, diverse communities of microorganisms, displaying a wide range of metabolic activities, carry out the cycling of nutrients such as carbon, nitrogen, phosphorus, and sulfur. It has been estimated that there are approximately 13,000 species of bacteria in a single gram of soil (113), and an unknown number of fungi and algae. The fertility of natural soil ecosystems is therefore highly dependent on nitrogen fixation and the turnover of soil organic matter (SOM) that is mediated by the soil microbial biomass. However, chemicals (e.g., heavy metals) that alter the biological activity of soil microorganisms can damage the functioning of the soil ecosystem for long periods of time. Affected soils often exhibit decreased microbial diversity, decreased microbial biomass, lower respiration rates per unit biomass, and manifest decreased enzyme activities.

A growing body of evidence suggests that microorganisms are far more sensitive to heavy metal pollution than the rest of the soil faunal (meso and macro) or floral community (114). Thus, the diversity and metal-tolerance of soil microbial communities is being examined as an indicator of heavy metal pollution, which could be used to define critical metal loadings for soil protection. As a result, there is considerable interest in the impact that heavy metals have on the soil microbial community. A heavy metal disturbance affects the growth, metabolism, and morphology of soil microorganisms through protein denaturation, functional disturbance, or cell membrane destruction. Several methods have been used in the past to investigate the response of microorganisms to heavy metal pollution including: (*1*) fatty acid composition, (*2*) microbial biomass size, (*3*) microfaunal composition, (*4*) respiration activity, (*5*) enzyme activity, (*6*) nitrogen fixation, (*7*) nitrogen and carbon mineralization, and (*8*) microbial carbon to soil carbon ratio.

However useful each of these microbial parameters may be in monitoring heavy metal pollution of soils no single parameter can be used universally. Combining one or more of these microbial parameters may provide a more sensitive indication of soil pollution. As mentioned previously, heavy metal affected soils show decreased microbial diversity. Decreases in biodiversity occur when species that lack tolerance to the heavy metals die off, whereas metal-tolerant species are enriched. Therefore, biodiversity of the soil microbial community could be used as a method to determine the impact of heavy metals on the environment. Classical methods involved isolating, and subsequently culturing microorganisms for identification. However, such attempts are hampered by sampling and culturing techniques, leading to bias toward certain groups within mixed microbial communities. The use of monoclonal antibodies has also improved the specificity of these methods, however, this technique is of limited applicability because of the heterogeneous nature of soil. Genetic diversity, which is always present within species, may be useful in determining the response of soil microbes to metal contamination. Individual species and strains of microorganisms have been identified by determining differences in the nucleotide sequences of the DNA. The study of population genetics is commonly known as *genetic fingerprinting* (115).

HEAVY METAL–RESISTANCE MECHANISMS

As previously mentioned, some heavy metal cations play an important role as trace elements in biochemical reactions, however, when a metal with unknown biochemical function replaces a functional metal, toxicity results. The heavy metal cations form unspecified complex compounds in the cell, which subsequently produce the toxic effects. Heavy metal cations such as Hg(II) and Cd(II) form strong toxic complexes. Even essential trace elements such as Ni(II), Zn(II), and Cu(II)become toxic at high concentrations. Therefore, intracellular concentrations of heavy metal ions must be highly regulated, and cellular heavy metal homeostasis is regulated through specific resistance mechanisms (116).

Bacterial resistance systems function by energy-dependent efflux of toxic ions. In many cases, these systems are encoded by plasmids that contain genes encoding resistance systems for toxic metal and metalloid ions (42). Plasmid-borne resistance systems include efflux systems for Ag^{+}, AsO_2^{-}, AsO_4^{3-}, Cd^{2+}, Co^{2+}, CrO_4^{2-}, Cu^{2+}, Hg^{2+}, Ni^{2+}, Pb^{2+}, TeO_3^{2-}, and Zn^{2+}. In some cases, such as with mercury and chromium, certain microorganisms employ enzymes that carry out redox transformations that result in a change in valence and reduced toxicity (117).

Arsenic

Arsenic is a metalloid of Group VA of the Periodic Table and exists in four oxidation states, +5, +3, 0, −3. Arsenic forms alloys with various metals, and covalently bonds with carbon, hydrogen, oxygen, and sulfur. Arsenate, a biochemical analog of phosphate, is transported by highly specific energy-dependent membrane pumps into the cell during assimilation of phosphate, whereas arsenite has a high affinity for thiol groups of proteins, resulting in the inactivation of many enzymes. Its similarity to phosphorus and its ability to form covalent bonds with sulfur are two reasons for arsenic toxicity. As a result, arsenic has no function as a trace element in bacteria, although some bacteria are known to use arsenate as a terminal electron acceptor during anaerobic respiration (118–121). The poisonous character of arsenic made it a very effective herbicide and insecticide.

In *E. coli*, resistance to arsenic can be achieved by two distinct mechanisms that include chromosomal and plasmid-encoded systems. Chromosomally encoded resistance occurs by the activation of a phosphate uptake pump with an increased selectivity for phosphate. In bacteria, two phosphate uptake systems are present, Pit (phosphate-inorganic transport) and Pst (phosphate-specific transport) (122). The Pit system is constitutive and does not discriminate between phosphate and arsenate. During periods of phosphate starvation or arsenatetoxicity, the Pst is activated, and the reduction in cellular arsenic is achieved by a higher affinity for phosphate. Thus, activation of the Pst system confers higher levels of resistance by virtue of the reduced uptake of arsenate.

Plasmid-determined resistance is a consequence of an accelerated efflux of arsenical and antimonal compounds but not phosphate from the cell. Three genes, named *ars* A, *ars* B, and *ars* C, are responsible for the export function in *E. coli* (123). The genes for resistance are clustered on plasmids R773 and R46. The ArsA protein is an arsenite- and antimonite-stimulated ATPase that forms a complex with the membrane-bound protein ArsB. The ArsAB complex actively exports As(III) and Sb(III) from the cell on the hydrolysis of ATP. To confer resistance to arsenate, the protein ArsC is required. The ArsC protein reduces As(VI) to As(III), which is subsequently exported by the ArsAB complex. Other *ars* C genes have been identified in the gram-positive staphylococcal plasmids pI258 and pSX267. However, the export of arsenic and antimony oxyanions is accomplished using membrane potential, rather than cellular ATP as an energy source.

Cadmium

Cadmium is a relatively rare element that is closely related to zinc, with which it is usually associated in nature. Cadmium is divalent in all its stable compounds. It has many industrial uses, including pigments in plastics, paints, and ceramics, and is used in the production of corrosion-resistant plating, alloys, and batteries. When compared with zinc, cadmium is more toxic because of its higher solubility. Poisoning through the contamination of food and drinking water by cadmium-plated containers has been reported, however, the principal human risk is from industrial exposure, primarily through the inhalation of cadmium fumes.

In *Ralstonia* sp. CH34, cadmium is accumulated by a magnesium uptake system (MIT) (124), which is also responsible for the accumulation of cadmium in other bacteria. However, resistance to cadmium in bacteria is based on cadmium efflux. Cadmium resistance in gram-negative bacteria appears to be regulated by RND (resistance, nodulation, and cell division)-driven systems such as the *Czc* and *Ncc*, which are mainly zinc (also cobalt in sp. CH34) and nickel exporters, respectively (6,125). The CadA pump from *Staphylococcus aureus* was the first example of cadmium-exporting P-type ATPase found in gram-positive bacteria (126). In other gram-positive bacteria, cadmium resistance is also mediated by proteins similar to CadA (127). Cadmium-resistance genes may also be conferred by plasmids and may co-occur with genes that confer resistance to chromium and some antibiotics (128).

Chromium

Chromium has many industrial uses and, as a result, large volumes of chromium waste in various chemical forms are discharged into the environment. Chromium can exist in oxidation states ranging from −2 to +6. However, only Cr(III) and Cr(VI) are normally found within the range of pH and redox potentials common in environmental systems. Hexavalent chromium [Cr(VI)] forms chromate (CrO_4^{2-}) and dichromate ($Cr_2O_7^{2-}$), which are toxic, mutagenic and carcinogenic, soluble over a wide pH range, and mobile in the environment. This form of chromium can

easily cross the membranes of eukaryotic and prokaryotic cells. The trivalent form, Cr(III), is considerably less toxic and is virtually nonmobile, largely because it precipitates as oxides and hydroxides at pH 5 and above. In humans, Cr(III) is incorporated into a small polypeptide, which is responsible for activating the insulin receptor tyrosine kinase. In microorganisms, no beneficial uses of chromium have been found.

Chromate is a competitive inhibitor of sulfate, and enters the cells by the sulfate-transport pathways, which is normal for many microorganisms (6). Once in the cytosol, Cr(VI) may be reduced to Cr(III), which in turn reacts with the DNA. Trivalent chromium is relatively innocuous, because biological membranes do not allow its passing. Resistance to chromium is often reported to be plasmid-related and has been found in *Pseudomonas* strains, *Streptococcus,* and *Alcaligenes*, and is also known to occur in yeast (129). Chromate resistance results from the decreased accumulation of chromium, through efflux mechanisms, by the resistant cells. A chromate resistance plasmid was isolated from *Pseudomonas mendocina* MCM B-180 (130). Incubation of the strain at 42 °C for 24 hours caused loss of chromate resistance and the plasmid pARI180. Transformation of *E. coli* DH5-alpha with the purified plasmid DNA resulted in the simultaneous acquisition of resistance to chromate and the ability to reduce hexavalent chromium. Although the microbial reduction of Cr(VI) to Cr(III) is not known to be a plasmid-determined process, it may act as an additional mechanism of resistance to chromate.

Copper

Copper easily interacts with radicals, especially that of molecular oxygen. The toxicity of copper is based on its ability to produce hydroperoxide radicals, which interact with the cellular membranes. This characteristic makes it very toxic, and many organisms are highly sensitive to copper. Copper has a role in copper/zinc superoxide dismutases, however, its most important function is in the cytochrome *c* oxidase and other related enzymes, which are oxygen-dependent, terminal oxidases in the electron-transport chain of many microorganisms (131). The purpose of copper in cytochrome *c* is to transfer electrons, which are used to reduce molecular oxygen to water. The energy that is derived during the electron transfer is used to pump protons across the cytoplasmic membrane.

Plasmid conferred resistance to copper has been described in *E. coli* (132,133), *Pseudomonas* (134), and *Xanthomonas* (135). Each of these systems is highly homologous and contain the same genes. *Pseudomonas* species contain two regulatory genes (*cop* R and *cop* S) and four structural genes (*cop* ABCD). In *E. coli*, the comparable genes are called *pco* RS and *pco* ABCD. CopS is a sensor protein found in the membrane, whereas CopR is the DNA-binding responder protein. Of the four structural proteins, CopD is an inner membrane protein, CopB is an outer membrane protein, and CopA and CopC are periplasmic proteins (Fig. 2). CopA and CopC are copper-binding proteins, which contain 11 and

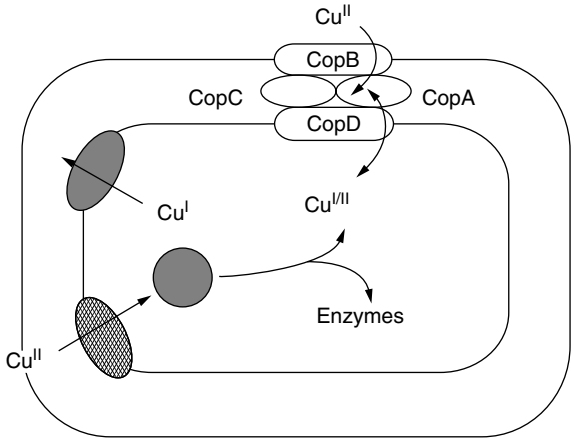

Figure 2. Copper transport and resistance in *Pseudomonas*. Hypothesized chromosomal uptake and efflux membrane transporters are shown, as well as intracellular copper-binding protein and plasmid-encoded CopABCD, the products of the copper resistance system of *P. syringae*. Adapted from Silver and Phung [123].

1 Cu^{2+} ions, respectively (134). At present, it is not clearly understood how the membrane proteins, CopB and CopD, move copper across the periplasm. In *E. coli*, plasmid copper–encoded resistance strongly interacts with chromosomally encoded functions (136). In addition, the actual resistance mechanism may depend on the growth phase (133). When copper-resistant *Pseudomonas* species are grown in the presence of high levels of copper, the bacteria turn blue (132). This occurs when copper is accumulated in the periplasm and the outer membrane, thus protecting the cell from toxic copper. However, *Xanthamonas* sp. and *E. coli* do not turn blue, implying that no copper storage is occurring in the periplasm.

In *Enterococcus hirae*, a gram-positive bacterium, resistance is conferred by the *cop* operon that contains two structural genes (*cop* A and *cop* B), both encoding a P-type ATPase. Compared with the gram-negative bacterial systems, that of *E. hirae* is the most understood copper transport and resistance system. Although it is suspected that CopA is responsible for copper uptake and nutrition, CopB is responsible for copper efflux and detoxification (137). Both proteins apparently transport silver as well (138). In addition, copper-transporting P-type ATPases have been found in a variety of organisms, including cyanobacteria, yeast, and eukaryotes. However, in *S. cerevisiae*, the P-type ATPase does not transport copper across the cytoplasmic membrane. To take up copper, Cu(II) is first reduced to Cu(I) by iron/copper-specific reductases. The Cu(I) is then bound to copper-specific protein transporters, which transport the Cu(I) into the cell (139).

Lead

Lead is a member of group IVA of the Periodic Table and exists mainly in two oxidation states, +2 and +4, and rarely occurs in its elemental state. Lead forms many salts, oxides, and organometallic compounds. Because of

its low solubility, it is largely biologically unavailable, and therefore is often not toxic to microorganisms. Lead is widely used in manufacturing, and the largest single use is the production of storage batteries. Additionally, it has been used as an automotive fuel additive, despite the fact that it is highly toxic to humans and animals. Lead affects the central nervous system, reproductive system, and blood pressure.

Although lead-tolerant gram-negative and gram-positive bacteria have been isolated, very little is known about their resistance mechanisms. Isolates belonging to the genera *Arthrobacter* and *Corynebacterium*, and species of *Alcaligenes, Bacillus*, and *Pseudomonas* have been identified (140). Lead-resistant bacteria isolated from lead-contaminated soil have been found to contain mercury- and copper-resistance genes (141). In lead-tolerant strains of *Citrobacter freudii* and *S. aureus*, lead is accumulated within the cell as lead phosphate (142,143).

Mercury

Mercury is noted for being one of the few metals that exists as a liquid at ambient temperatures and for being a potent human neurotoxin. Natural (e.g., volcanic eruptions) and anthropogenic (e.g., fossil fuel combustion) activities release large amounts of metallic mercury [Hg(0)] into the biosphere, however, it readily undergoes biotic and abiotic conversion to organic forms such as methylmercury (CH_3Hg^+). Methylmercury is water-soluble and fat-soluble, and therefore poses a threat to aquatic organisms such as fish and especially to fish consumers. Mercury pollution first received a great deal of publicity from the infamous Minamata Bay incident in Japan, where direct discharge of mercury-contaminated industrial waste led to extremely high levels of methylmercury in fish, which when eaten by humans, caused physical impairments and death.

In the environment, microorganisms are involved in the transformation of inorganic and organic mercury compounds, mostly as a detoxification mechanism. Numerous microorganisms avoid mercury toxicity by reducing ionic mercury [Hg(II)] to volatile Hg(0), which may be a potentially useful application to remove mercury from mercury-contaminated water. The reduction of Hg(II) to Hg(0) can be mediated by a number of microorganisms including enteric bacteria, *Pseudomonas* sp., *S. aureus, T. ferrooxidans, Streptomyces*, and *Cryptococcus* (144). The ability of bacteria to reduce Hg(II) is linked to mercury-resistance (*mer*) operons (122). The plasmid codes for a protein (merP) that initially binds to Hg(II) in the periplasm. The Hg(II) is then transported through the inner membrane into the cytoplasm by the membrane-bound protein merT. In the cytoplasm, Hg(II) is reduced to Hg(0) by a soluble, NADH-dependent, FAD-containing mercuric reductase. Mercuric reductase is active in the presence of excess thiols (R-SH) such as mercaptotethanol, dithiothreitol, glutathione, or cysteine. Intracellular Hg(0) is subsequently eliminated from the cell by enhanced diffusion.

Nickel

Nickel has oxidation states of +1, +2, and +3, but its preferred valence state is +3 in biological systems. In addition, it is chemically analogous to both iron and cobalt. As a result, the toxicity of nickel is similar to that of cobalt. Nickel is used principally in the production of stainless steel and other alloys because of its excellent corrosion resistance. However, nickel has carcinogenic properties and is involved in hypersensitivity reactions. Nickel dermatitis, a nickel allergy, is especially common among nickel platers and people wearing low-quality jewelry. Nickel is an essential micronutrient for many bacteria, including some acetogenic bacteria and methanogens. Nickel is a component of hydrogenase, urease, and carbon monoxide dehydrogenase, and is mainly bound to cysteine or histidine.

In bacteria, nickel enters the cell mainly by the CorA system (metal transport system), which is generally fast and unspecific (145). Two highly specific nickel transport systems are the HoxN and ABC transporters. The HoxN transporter, identified in *Ralstonia eutropha*, is part of the hydrogenase gene cluster. Uptake of nickel in this system is most probably driven by a chemiosmotic gradient. During the production of hydrogenase in *E. coli*, nickel is supplied by the ABC (ATP binding cassette) transporter and a periplasmic nickel-binding protein.

Through sequestration and/or transport, nickel can be detoxified. In *S. aureus*, nickel is bound to polyphosphate (146), and in *S. cerevisiae*, nickel is suspected to be disposed of and bound to the histidine in the vacuole (147). A proton-pumping ATPase transports nickel into the vacuole. It is suspected that other yeast and fungi probably detoxify nickel by similar mechanisms and also by mutation of the CorA uptake system (148,149). A well-known system is that of *Ralstonia* sp. strain CH34, in which an RND transporter drives nickel efflux (125,150).

Zinc

Zinc only occurs as the divalent cation Zn(II), which does not undergo redox changes under biological conditions. Zinc is a component in a number of enzymes and DNA-binding proteins, for example, zinc-finger proteins, which exist in bacteria. In humans, zinc toxicity may be based on zinc-induced copper deficiency; however, zinc is apparently less toxic than copper. In *E. coli*, the toxicity of zinc is similar to that of copper, nickel, and cobalt.

Three transporter groups contribute to the transportation of zinc. The first type of transporter system, known as CorA MIT, is responsible for transporting zinc in *S. cerevisiae* (151). This system is also present in *Archaea* and many other bacteria, but magnesium transport is not inhibited by the presence of Zn(II). The second type of transporter protein, MgtE, is chemiosmotically driven (152), and can be found in *Providencia stuartii* and several other gram-negative and gram-positive bacteria (153). The MgtE transporter is not as broadly distributed as CorA system. The third system is MgtA, which is a magnesium/zinc transporter found in *Salmonella typhimurium* (153). The MgtA system is a P-type ATPase,

which is suspected of transporting zinc better than magnesium. MgtA is regulated by magnesium starvation, which may be affected by zinc. However, this transporter is not the inducible high-specificity uptake system for Zn(II).

CONCLUSION

In the environment, unnaturally elevated levels of heavy metals exist as a result of a variety of anthropogenic activities. Heavy metals considered to be of concern are arsenic, chromium, cadmium, copper, lead, mercury, nickel, selenium, and zinc. Although a number of metals are toxic at excessive concentrations, all cellular life requires certain heavy metals as micronutrients. In aquatic and terrestrial environments, heavy metal and metalloid transformations are largely carried out as a direct result of microbial activities. Microorganisms influence the fate of metals by a number of processes including uptake into living cells, adsorption on cell walls, and chelation at the cell surface by microbially produced substances. These processes convert metals into organometal complexes that can persist in the environment and slow the degradation of organic substances, or may be taken up by higher organisms, leading to food chain transfer of metal ions. Microorganisms also carry out redox transformations of metals and produce acidity that can result in the solubilization of metals. Other microbially mediated metal transformations include methylation and demethylation reactions. The methylation and subsequent volatilization of metals, or selective precipitation of metals on the cell surface, often represent detoxification mechanisms. These and additional bacterial resistance mechanisms, which function by the energy-dependent efflux of toxic ions, are in many cases encoded by plasmids. The microbial transformation of heavy metals and metalloids into insoluble elemental forms or volatile forms may have applications in remediating metal-contaminated soil and water systems. Microbial processes also can be harnessed for biomining of metals from ores, and for the extraction of metals from contaminated water.

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METAL STRESSED ENVIRONMENTS, BACTERIA IN

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Bacteria are ubiquitous, found not only in nearly all surface environments, but in many subsurface environments as well (1–5). They are either found attached to surfaces, for example, as biofilms (6–9) or as complex associations of particles and cells (10), gluing sedimentary material together (11), or free-floating in the surrounding medium. In either case, they do not have direct control over the aqueous chemistry of the fluid phase of the environment. This fluid phase might include intrusions of heavy metal ions either from anthropogenic, for example, mining operations (12–16), or naturally occurring, for example, sources such as hydrothermal springs (17–21). If bacteria are to survive in these metal stressed environments, they must have the capacity to avoid or detoxify their surroundings.

From a bacterial perspective, a metal-stressed environment can be defined as an external aqueous chemistry that can inhibit cell metabolism and growth or can cause cell death for example, impair enzyme function (22) or entomb/fossilize bacterial cells (9). Unless the bacteria, either individually or as a population, are able to reduce the toxic effect of the metals.

Although bacteria do not have direct control over the chemistry of the fluid phase they encounter, they exhibit tremendous molecular and metabolic diversity (23–25), some of which allows them to respond to the substances dissolved in the medium, that is, metals, and to alter the chemistry of their surrounding microenvironment to their benefit (26–28). As a case in point, bacteria exploit a wide range of redox reactions with a negative $\Delta G'_o$ to support metabolism (24); these reactions often affect the solubility of metals. This article will focus on these mechanisms that allow bacteria to persist in metal stressed environments.

BACTERIAL RESPONSES TO TOXIC HEAVY METALS

Metal Transporters

Metal-resistant bacteria will be enriched and persist in anthropogenically or naturally "contaminated" ecosystems (29). When these bacteria encounter soluble, toxic heavy metals, they exert energy to remove the metals from the inside of their cells, thus preventing toxicity. The processes involved in metal transport out of the cell include enzymatic detoxification and transport (30), ATPase-based transporters, (31) and proton/antiport systems (32) (Table 1).

Schmidt and coworkers (32) identified similar nickel- and cobalt- resistance capabilities in various bacteria isolated from metal-contaminated soil and highly polluted domestic and industrial wastes. The information for these resistance mechanisms is typically contained on a plasmid (53), which allows for the exchange of metal-resistance properties between closely related bacteria. Perhaps, the best-characterized bacterial heavy metal resistance mechanism is the ability to form plasmid-mediated membrane porters (32,33). Membrane porters are produced as a direct response to toxic heavy metals and function by transporting soluble metals out of the cell (54). Plasmids often confer resistance to a variety of metals, for example, *Alcaligenes eutrophicus* plasmid pMOL28 confers resistance to chromate, cobalt, mercury, and nickel, and plasmid pMOL30 confers resistance to cadmium, cobalt, copper, mercury, and zinc (34,35). In a separate study, Schmidt and Schlegel (36) identified plasmid-mediated resistance to cadmium, cobalt, copper, nickel, and zinc. These bacterial transporters do not change the stability (redox property) of the metal or produce stable organo-metal complexes, that is, the metal remains soluble. Therefore, the metal is still available to exert a toxic effect on nonresistant bacteria.

Transformation of Metals into Volatile Forms

Some bacteria reduce the concentration of select toxic compounds by producing volatile, typically methylated metal forms (Table 1). Examples include the formation of Hg°, methyl- and dimethyl-Hg (37–39), methyl-Cd, methyl-Pb, methyl-Sn (40), dimethyl selenide (41), elemental selenium by dissimilatory selenate reduction (SeO_4^{2-} to Se°;42), arsine (43), and dimethyl arsine (44). In environments containing these metals, their transformation into a volatile form will reduce the metal concentration in the microenvironment immediately surrounding the bacterium because metals are transferred into soil gas and potentially lost to the atmosphere. The formation of volatile metals protects metal-resistant and unresistant bacteria.

Table 1. Specific Metal Resistance Mechanisms Initiating at the Genetic Level and Occurring as a Consequence of Microbial Metabolism

Toxic Heavy Metals	Mechanism
Hg^{2+}/organomercurials	Enzymatic detoxification + transport out of the cell (30)
AsO_4^{3-}/AsO_2^{-}	Arsenate is reduced to arsenite, which is pumped out of the cell by an ATPase (31)
Cd, Co, Cr, Cu, Hg, Ni, Zn	Pumped out of cells by an H^+-antiporter/metal-specific efflux (32–36)
Cd, Hg, Pb, Sn, Se, As	Formation of volatile metal forms (37–44)
Cd, Cu, Ni, Zn,	Protein-metal chelation (45–50)
Cu, Mn	Carbohydrate-metal chelation (51,52)

Metal Complexation

The bacterial formation of organic metal-complexing agents in response to the presence of toxic metals effectively chelates the metal in solution, reducing its toxicity (Table 1). Protein-metal chelate responses to cadmium (45,46), copper (47,48), nickel (49), and zinc (46,50) have been described. These metal chelators reduce metal bioavailability and limit mineral precipitation due to the formation of a stable organo-metal complex (49). They are produced by aerobic and anaerobic bacteria, including dissimilatory sulfate-reducing bacteria (SRB) and methanogens. In these systems, the protein-metal complexes are formed as a direct response to the metal and confer metal resistance by preventing the metal from entering the cell. Carbohydrate-metal complexes have also been described (51). In the study by Kidambi and coworkers (51), alginate, a copper-chelating, uronic acid–containing capsule, was produced by *Pseudomonas syringae* in response to the presence of copper. A capsule has also been synthesized by a *Rhizobium* sp. in response to the presence of manganese (52). Capsules are chemically and structurally complex organic macromolecules, which function as a hydrated cation-exchange resin that binds metals from solution (55,56). Like the bacterial formation of volatile metal forms, organic complexation of metals also protects nonmetal-resistant bacteria from the toxic effect of soluble, ionic heavy metals.

MINERAL PRECIPITATION

The greatest liability to bacteria in metal-stressed environments is the ubiquitous presence of iron that reacts with anionic charge groups on bacterial cell surfaces, which confer a net negative surface charge on most bacteria. This interaction results in the formation of fine-grained mineral precipitates, which over time, can coalesce into a mineral "armor" surrounding the bacterium. For an organism that interacts with its environment by diffusion processes, this represents a barrier to obtaining nutrients and is deleterious to the mineralized cell. Therefore, despite the elaborate resistance mechanisms toward base metals described in the preceding sections, bacteria must be able to "deal" with mineral formation, which typically occurs in metal-containing systems.

The formation of many secondary minerals in natural and laboratory systems is catalyzed by microorganisms (57; see Tables 2 and 3). These precipitation reactions

Table 2. Secondary Minerals Known to Form on Bacterial Cells by Surface Catalysis, that is, Interaction with Net Negative Surface Charge on Bacteria

Minerals Formed
Iron-oxides (ferrihydrite, hematite, goethite; 7,60)
Hydroxy iron-sulfates (61)
Metal phosphates, phosphorite (62,63)
Fe-silicates, geothermal sediments (17)
Fe-Al-silicates, sediment bacteria (11)
Metal-sulfides, for example, millerite (11)

Table 3. Secondary Minerals Known to Form on Microbial Cell Surfaces as a Consequence of Dissimilatory Metabolic Activity and Surface Catalysis

Minerals Formed	Process
Gypsum/calcite/magnesite	Photosynthetic shift in (CO_2) from bicarbonate-containing solutions (64,65)
Iron-oxide/oxy-hydroxide	Enzymatic iron oxidation, Fe(II) to Fe(III) (66,67)
Manganese oxide	Enzymatic manganese oxidation, Mn(II) to Mn (IV) (68)
Ferromanganese Concretions (Fe-Mn oxides)	Combination of the aforementioned two systems (69)
Metal-sulfides	Dissimilatory sulfate reduction (15,16)
Metal phosphates	Phosphatase activity (70)
$Cr(OH)_3$	Dissimilatory metal reduction, chromium (VI) to chromium (III); (71)
UO_2	Dissimilatory metal reduction, uranium (VI) to uranium (IV) (59,72)

have been divided into two general categories, namely, passive and active mineralization. Passive mineralization or surface catalysis is caused by the net negative charge on most bacterial cell surfaces (58) that nucleates the precipitation of metallic cations from solution. Active surface-mediated mineralization occurs by the direct transformation of metals into unstable forms (59) or by the formation of metal-reactive by-products (15,16). These dissimilatory processes, combined with bacterial surface catalysis, are responsible for cell surface mineral formation in these systems.

Natural environments in which biomineralization occurs include freshwater (6,8,73–75), seawater (69,76), sediments (77), zones of groundwater discharge (25,26,78), mine tailings (79–85), acid mine–drainage environments (7,11,12,60,86,87), pyritic soils (88,89), bog iron deposits (90), and hydrothermal systems (17–21). The samples of metal-encrusted bacteria used in this article were adsorbed onto 200 mesh Formvar-coated copper grids or prepared for ultra thin sections by conventional embedding (91) without the addition of osmium tetroxide or uranyl acetate as heavy metal fixatives and contrasting agents. Therefore, the electron density in the samples is due to the naturally immobilized metals.

Cell Envelope Structure and Chemistry

Mineral formation generally does not occur within bacteria because of the space constraints caused by the high concentration of organic material within a cell. Therefore, the outermost surface of a bacterium, that is, the interface between a bacterium and its external environment, will typically be responsible for the precipitation of metals from solution. The chemical structure of the cell surface is related to the phylogenetic or taxonomic position of the microbe (24,92,93). The bacteria are divided into gram-positive or gram-negative (although gram-variable organisms also exist) groups on the basis of cell envelope structure and chemistry. The Archaea, like the Bacteria, have demonstrated both positive- and negative-staining characteristics using the Gram reaction. However, the Archaea possess a unique chemistry (94), antigenicity (95), and ultrastructure (96) when compared with the Bacteria. Prokaryotes, excluding those bacteria which do not possess cell walls (e.g., *Mycoplasma*, *Thermoplasma*, and *Methanoplasma*) can possess a variety of surfaces that can interact with soluble metals in the environment.

Gram-positive bacteria possess a thick (typically 15 to 25 nm), peptidoglycan-containing cell wall that provides the framework to which the secondary polymers (teichoic acids or teichuronic acids) attach (58). Peptidoglycan, the major shape-determining structure for the organism, consists of repeated β (1–4)-linked N-acetyl glucosamine-N-acetyl muramic acid dimers. The N-acetyl muramic acid residues of this polymer possess short peptide stems (4 to 5 amino acids), which may be covalently bound to other muramic acid residues on neighboring strands. This results in a three-dimensional macromolecule shaped like the bacterium (97). These carbohydrate matrices and peptide stems possess carboxylate groups, which dominate the charge density of this structure (98,99). Teichoic acids, a secondary polymer, are composed of polyalcohol-based chains joined by phosphodiester linkages (e.g., polyglycerol-phosphate in *Bacillus subtilis* 168). The phosphate moieties confer a net negative electrical charge on this polymer. Teichuronic acids, a secondary polymer produced under phosphate-limiting conditions, are composed of uronic acid polymers, which also possess anionic reactive sites (100). Gram-positive archaea can resemble gram-positive bacteria, possessing a single homogeneous layer external to the plasma membrane (101). This envelope consists of an N-acetyl talosaminuronic acid/N-acetyl glucosamine peptidoglycan-like polymer termed *pseudomurein* (found in *Methanobacterium* spp.) or of a proteinaceous layer and a heteropolysaccharide cell wall (found in *Methanosarcina* spp., which grows as irregularly shaped cells in packets).

Gram-negative bacteria possess cell envelopes, which are structurally and chemically more complicated than those of the gram-positive bacteria. External to the plasma membrane is a thin (2 to 3 nm) layer of peptidoglycan contained within a periplasm, possibly

having a gel-like consistency (102). The periplasm is bound by an outer membrane that is a lipopolysaccharide (LPS)-phospholipid-protein mosaic in which the LPS and phospholipid occur on the opposite membrane faces of the bilayer (58). The LPS is anchored to the outer membrane by its lipid moiety and extends its polysaccharide chains outward from the bacterial surface (103). The outer membrane is often cemented to the peptidoglycan by salt-bridging or covalent bonding of the outer membrane proteins. One class of these proteins forms hydrophilic pores or channels (58). The LPS and peptidoglycan of gram-negative bacteria possess a net electronegative charge, which allows interaction with soluble cations (104–106).

Additional wall layers (e.g., capsules, S-layers, or sheaths) can exist external to the gram-positive or gram-negative cell envelopes described earlier. Capsules, commonly produced by bacteria in natural systems, are highly hydrated, amorphous assemblages of polysaccharides or polypeptides that are chemically linked to the cell surface and may extend up to 1 μm from the cell (107). Because of their carboxylate groups, capsules usually possess a net negative charge, and they may also possess additional anionic reactive groups because of the presence of phosphate moieties in polysaccharide chains. The highly hydrated nature of bacterial capsules and their cell surface location allows extensive interaction between capsular material and soluble metal cations (5,56,108).

S-layers are paracrystalline cell surface assemblages consisting of proteins or glycoproteins with p2, p4, or p6 symmetry (109). They self-assemble and associate with the underlying wall through noncovalent interaction (110). S-layers are located external to the peptidoglycan-based cell wall of the gram-positive Bacteria, the LPS of gram-negative Bacteria, the pseudomurein of gram-positive Archaea (109), or the cell membrane of gram-negative Archaea (58,94,96,111–113). S-layer proteins generally possess an acidic isoelectric point (pI) and thus exhibit a net negative charge, which can interact with metallic cations.

Sheaths, which surround chains of cells, are less frequently encountered. In the bacteria, they are typically recalcitrant structures composed of homo- or heteropolymers (carbohydrate or carbohydrate and protein) and usually remain intact even after cell degradation (114). The role of these sheath structures in the enzymatic precipitation of iron and manganese will be described later. Sheaths in the archaea are covalently linked proteinaceous structures that are found on *Methanospirillum hungatei* and *Methanosaeta concilii* (115,116).

Passive Mineralization

Ion-exchange reactions play an important role in the initiation of mineral formation through competition between hydronium ions, alkaline earth ions, and heavy metals for anionic reactive sites on bacterial surfaces (117; Cell envelope structure and chemistry). An acid-base titration of *B. subtilis* cell wall demonstrated that carboxyl, phosphoryl, and hydroxyl groups could potentially interact with soluble heavy metals (118–120). Under normal growth conditions, divalent cations (usually Ca^{2+} and Mg^{2+}) contribute to the stability of teichoic and teichuronic acid polymers (100), LPS (121), and S-layers (122). It is these cation-stabilized anionic sites that are replaced by heavy metals and serve as nucleation sites for the formation of minerals outward from the bacterial cell surface. Planktonic and sessile bacteria nucleate metals from solution resulting initially in the precipitation of fine-grained minerals of 10 nm diameter (Fig. 1).

Figure 1. An unstained TEM micrograph of bacteria collected by filtration (0.45 μm) of water from the Golden Giant mine tailings pond (Hemlo gold region Marathon, Ontario, Canada). Note that some of these bacteria have precipitated fine-grained (of 10 nm size) iron-arsenic minerals (determined by EDS; data not shown) on their surface, whereas others are nonmineralized. Bar equals 1 μm.

Bacterial metal precipitation typically exceeds the stoichiometry expected per chemical reaction site within the cell envelope (123). Mineral formation results from neutralization of chemically reactive sites and proceeds by nucleation of additional metallic ions with these previously sorbed metals. These critical nuclei, stabilized by the wall, are less prone to remobilization by dissolution because the wall reduces the interfacial tension between the mineral nucleus and the bulk-water phase. Mineral growth, then, is most active at the outer surface of the bacterium where these nuclei are formed and where space constraints by the envelope polymers do not inhibit metal precipitation. Mineral growth at these nucleation sites initially prevents a bacterium from being completely encrusted in metal. This is clearly important to an organism that obtains its nutrients by diffusion. The eventual, complete mineralization of microbial surfaces produces hollow minerals that are generally the size (i.e., μm order) and shape of the cell (Fig. 2). As metal precipitation proceeds, larger aggregates of mineralized bacteria that can trap insoluble particulate material, for example, clays form (124). When iron is present, bacteria can interact with one another to form visible bacteria-iron-hydroxide flocs (125) that can enhance the immobilization of base metals (7,60,126).

Direct Red/Ox Transformation of Metals

Many bacteria possess the capacity to oxidize metals, channeling these electrons through the electron-transport chain to generate a proton-motive force (PMF) for ATP

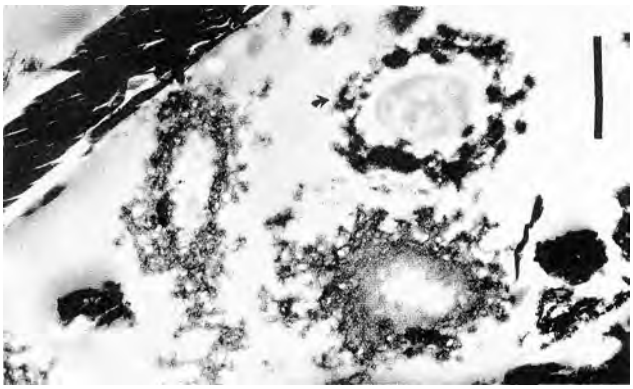

Figure 2. An unstained, ultrathin section TEM micrograph of a mineralized biofilm from the Copper Rand sulfide mine tailings pond (Chibougamou, PQ, Canada) demonstrating the presence of a mineralized bacterium (arrow), microfossils, and inorganic particulate material that has been trapped by the biofilm in this metal-stressed environment. Bar equals 1 μm.

synthesis. Other bacteria possess the capacity to use metals as electron acceptors, facilitating the oxidation of organic carbon in anaerobic respiration processes. In both physiological processes, bacteria often must deal with the inherent metal stress that results from using metals in dissimilatory metabolic reactions.

The oxidation of iron ($2Fe^{2+} + \frac{1}{2}O_2 + 2H^+ \rightarrow 2Fe^{3+} + H_2O$; 26,27,127) and manganese ($2Mn^{2+} + O_2 + 2H_2O \rightarrow MnO_2 + 4H^+$, net reaction of a two-step oxidation; 128,129) typically occurs within the aerobic/anaerobic interface in which the reduced metals encounter an oxidizing environment. In natural systems, sheaths of *Sphaerotilus* (66,67) and *Leptothrix* (68,127) are integral to the oxidative enzyme-mediated precipitation of iron and manganese because they contain the enzymes responsible for metal oxidation and they nucleate the oxidized mineral precipitates.

Dissimilatory metal-reducing bacteria are best known for their ability to use minerals for their electron acceptors resulting in the solubilization of Fe and Mn oxides (130–134) and any coprecipitated base metals. Dissimilatory metal reduction is also responsible for the bacterial cell surface precipitation of other metals, for example, $Cr(OH)_{3(s)}$ [Cr (VI) to Cr (III); 71,135] and $UO_{2(s)}$ [U (VI) to (IV); 59,72]. Although iron reduction can also result in the formation of magnetite (131) or siderite (3,136,137), reduced iron and other base metals are commonly precipitated as sulfides (138; see following text).

Reactive By-Product Catalyzed Mineral Formation

Cyanobacteria were found to precipitate gypsum or calcite in a freshwater environment, even when the bulk solution pH was too acidic to favor calcite precipitation (139). In this system, calcite formation was mediated by an increase in alkalinity at the bacteria cell surface caused by photosynthetic activity. It was also determined that the S-layer of *Synechococcus* sp., the cyanobacterium mediating this process, was responsible for nucleating these minerals from solution (64,65,140).

The basic biochemical (1) and geochemical (2) reactions mediated by dissimilatory SRB (15,16) are as follows:

$$2CH_2O + SO_4^{2-} \rightarrow H_2S + 2HCO_3^-$$

$$Fe^{2+} + H_2S \rightarrow FeS_{(s)} + 2H^+$$

The immobilization of FeS on SRB surfaces is promoted by a combination of the ionic interaction of Fe^{2+} with the anionic cell surface polymers and biogenic H_2S (Fig. 3; 49,141). Although the amorphous precipitates often found on bacterial surfaces represent the early stages in authigenic mineral formation (11,142), in SRB systems, a mineral transformation occurs rapidly after the initial metal immobilization occurs resulting in pyrite formation (143).

Bacterial phosphatase activity has been shown to release inorganic phosphate into solution and catalyze the formation of metal phosphates that nucleated on the bacterial cell surfaces perhaps with previously immobilized metals (70).

HOW DO BACTERIA SURVIVE IN A METAL-STRESSED ENVIRONMENT?

In laboratory metal binding experiments, living *B. subtilis* cells bound less metal than did non living cells (145). These experiments determined that the membrane-induced PMF, which pumps protons into the wall fabric, reduces the metal binding ability of the cell walls, probably through the competition of protons with metal ions for anionic wall sites. Therefore, the metabolic activity and the energized membranes of viable bacteria probably explains why one bacterium in a natural population is not mineralized, whereas a second adjacent bacterium that is dead or dying can be extensively

Figure 3. An unstained whole mount of a *Desulfovibrio desulfuricans* that has been cultured in the presence of 100 ppm Fe^{2+}, resulting in the precipitation of amorphous FeS (data not shown). FeS precipitation at this site is caused by the presence of a HS^-, released as a by-product of SRB metabolism, presumably forming a HS^- rich microenvironment around these bacteria. Note the extensive, electron-dense mineralization initiating at the bacterial cell surfaces. The nucleation and growth of minerals outward from the bacterial cell surface protects the cell from being completely encrusted with minerals allowing continued uptake of nutrients by diffusion. Bar equals 1 μm.

mineralized (6,9; see Fig. 1). Mineral precipitation reduces the cell surface area available for nutrient uptake (see Figs. 1–3). Consequently, those mineralized cells are starved, and as their PMF dissipates, they die. Without the competition from hydronium ions within the cell envelope, mineralization proceeds to completion, reducing the concentration of soluble, toxic heavy metals in the surrounding microenvironment. This would promote the survival of any remaining nonmineralized bacteria. The presence of nonmineralized and mineralized bacteria in a common environment may also correlate directly to the presence of metal transporters (30–36,53,54). In aerobic environments that are stressed by iron and base metals, the inability of a bacterium to pump toxic heavy metals out of the cell will result in cell death, a dissipation of its PMF, and nucleation of iron hydroxides possessing coprecipitated base metals. This overall process will protect bacteria possessing membrane transporters from biomineralization.

The high surface-to-volume ratio of bacteria facilitates their growth. Because access to nutrients is based on diffusion, the greater this ratio, the greater the diffusion of nutrients into cells (105,144–147). In terms of mineralization, high surface-to-volume ratios also provide a tremendous surface-biomineralization potential when compared with eukaryotic microorganisms. For example, the eukaryotic algae typically possess a surface-to-volume ratio that is one-tenth of that of bacteria. The implications of the differing mineralization potentials are twofold. First, bacteria can precipitate more metal than could a comparable measure of eukaryotic biomass, and second, in a mineral-precipitating environment, the ability of some bacteria to precipitate the bulk of the soluble metals increases the probability that the nonmineralized bacteria will survive. Therefore, from a evolutionary/ecological perspective, mineral formation represents a resistance mechanism to the presence of toxic soluble heavy metals.

In sediments, the decomposition of organic matter does not typically include organics (most probably bacterial cell envelopes; 148) that have bound heavy metals (149,150). Bacteria-metal-clay aggregates (10 to 100 μm) are even less likely than mineralized bacteria to remobilize metals (148). This is probably because of the extremely low surface area-to-volume ratios of metal-laden flocs compared with that of individual bacteria. The preservation of bacterial microfossils suggests that metals immobilized on bacterial cell envelopes are less available for remobilization and subsequent toxicity toward other viable bacteria.

WHAT HAPPENS WHEN THE METAL STRESS IS TOO MUCH?

Paleological Significance

Although individual bacteria are extremely small, the death of large populations of bacteria through mineralization has produced geologically significant deposits of carbonates (151), iron-silica deposits (152–155), sulfides (156–158), and phosphorites (63,159–161). Note that these bacteriologic processes are still occurring. The formation of bacterial microfossils results from the cell surface immobilization of soluble heavy metals (biomineralization) by ionic interactions with or without the formation and release of chemically reactive metabolic by-products. Metal encrusted cell surfaces are resistant to remobilization and are typically the only component of the cell that is preserved, possibly, as long as several billion years. The size and shape of the microfossils are determined by bacterial morphology, which includes spherical, rod-shaped, filamentous, vibrioid, helical, and stalked structures. The identification of bacterial microfossils as hollow mineral assemblages using ultrathin section TEM requires preservation of the original biomineralization phenomenon (Fig. 4). Preservation of microfossils will result when the metals are immobilized on bacteria in a chemically and biogeochemically stable environment. However, for long-term preservation, silicification of bacteria often in association with iron results in the formation of extremely stable microfossils (17,19,162–164,165–168). In their simplest form, bacterial microfossils consist of only a mineralized cell envelope, which preserves the original bacterial morphology. Even in rare cases when both the cell envelope and the cytoplasm are mineralized, the cell envelope can be easily differentiated from the cytoplasm preserving the original cell morphology.

Contemporary Systems

Mineral precipitation is occurring in natural systems and is being exploited for bioremediation and industrial purposes. In aquatic systems, mineral growth cross-links individual bacteria-forming bacteria-mineral flocs (9) that settle through the water column (125). This promotes the transfer of once soluble metals into the sediment producing sediments rich in these metals (11). SRB also enrich sediments with base metals as metal sulfides (12). The enrichment of base metals in sediments was recognized by Timperley and Allan (169) who advocated the examination of sediments, presumably containing metal-encrusted

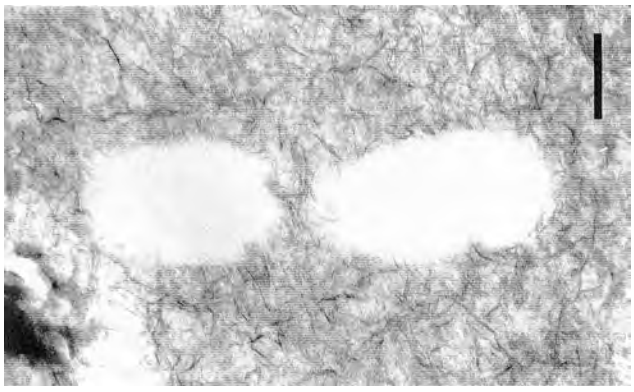

Figure 4. An unstained ultrathin section of TEM micrograph of a mineralized biofilm from the Copper Rand mine tailings pond (Chibougamou, PQ, Canada) demonstrating the presence of bacterial microfossils possessing no obvious bacterial remains. The outward growth of minerals from bacterial surfaces is inferred because the formation of hollow, bacteria-shaped structures within a geochemically precipitated iron-manganese oxide is not possible. Bar equals 0.5 μm.

bacteria, during exploration geochemistry to determine the presence of anomalous metal concentrations.

Cyanobacteria have been implicated in open ocean-whiting events (calcite precipitation; 170) by the same photosynthesis generated alkalinity and bacterial surface-active nanoenvironment described by Thompson and coworkers (65). Bacteria are also actively involved in the precipitation of silica in ambient and hydrothermal current-earth systems (11,75,163) conceivably through the precipitation of silicic acid as amorphous silica that is coprecipitated with iron (166).

CONCLUSION

Bacteria possess a wide range of responses toward toxic metals, for example, transporters, the ability to produce volatile or organic metal forms, and biomineralization. Mineral formation, resulting from surface catalysis or a combination of dissimilatory processes and surface catalysis, has produced microfossils in ancient geologic formations and is occurring in current-earth systems. Although mineralization is deleterious to individual bacteria, they are still able to persist in these mineral-forming environments. This inherent ability of bacteria to precipitate metals from solution without killing the entire population has been exploited for the reclamation of metals from contaminated water (13,14,171,172) and will no doubt play a role in the bioremediation of metal-contaminated environments in the future.

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METAL TRANSFORMATIONS. See METAL (U, Fe, Mn, Hg) CYCLING

METAL (U, Fe, Mn, Hg) CYCLING

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Metal transformations in the environment are dominated by two fundamental processes: redox reactions and complexation by organic and inorganic compounds. By these two processes, metals are cycled among soluble, insoluble, and volatile states as well as organic and inorganic forms. In addition to strictly abiotic (chemical) reactions, metals are transformed by microbially mediated pathways. These consist of direct (enzymatic) reactions or indirect (chemical) reactions with microbially produced end-products. Microbial metal transformations represent a key component to metal cycling in natural systems.

Metal cations are classified according to their electron configuration (1,2). A-type metal cations possess electron configurations of inert gases, are distinguished by low polarizability and are termed "hard spheres" or "hard acids". A-type metal cations include elements in group I (alkali metals) and group II (alkaline earth metals), and the lanthanides and actinides, such as uranium (as $(UO_2)^{2+}$). B-type metal cations possess electron configurations that contain 10 to 12 outer-shell electrons, are highly polarizable, are termed "soft spheres" or "soft acids" and include Hg^{2+}, Au^{+}, Ag^{+}, Cu^{+}, Cd^{2+}, Zn^{2+}, and Pb^{2+}. In general, A-type metal cations are O-seeking, whereas B-type metal ions are N- and S-seeking. Some A-type metal cations (e.g., Na^{+}, Ca^{2+}, K^{+}, and Mg^{2+}) are required nutrients for cell growth. In contrast, B-type metal cations are often toxic to organisms at relatively low concentrations. Transition metal cations (e.g., Fe^{3+} and Mn^{4+}) contain one to nine outer-shell electrons, are highly redox active, and exist in multiple oxidation states in the environment. The chemical characteristics of transition metal cations lie between A- and B-type metal cations (1,2). This chapter outlines the chemical and biological pathways that contribute to the cycling of representative A-type (uranium), transition (iron, manganese), and B-type (mercury) metal cations. They also serve to illustrate the central role of metal cycling in other environmentally significant processes, including the mobilization and transport of hazardous metals and the cycling of other elements including trace metals, carbon, nitrogen, phosphorus, and sulfur.

URANIUM CYCLING

Overview

Uranium is a radioactive constituent of more than 150 naturally occurring minerals, including the most commonly found forms, uraninite (UO_2) and coffinite ($USiO_4 \cdot nH_2O$) (3). Uranium concentrations in marine waters (10 $\mu g\,kg^{-1}$) are approximately 10-fold greater than in freshwaters (4). Uranium is found in the 0, +III, +IV, +V, and +VI oxidation states, although the +IV and +V oxidation states predominate at circumneutral pH. Redox transformations and complexation reactions are important to predicting the fate and transport of uranium in contaminated subsurface aquifers. Uranyl ion (U(VI)) is highly soluble in aqueous environments, whereas uraninite (U(IV)) is highly insoluble (3). Uranium solubility is also greatly affected by complexation with a variety of organic and inorganic compounds. In aerobic environments, U(VI) is complexed by carbonates, forming highly mobile U(VI)-carbonate complexes (5). On the other hand, U(VI) is also strongly complexed by inorganic phosphate, forming highly insoluble U(VI)-phosphate complexes. Uranium mobility is also retarded by U(VI) sorption to particulate organic matter and Fe(III)- and Mn(IV)-oxides (6,7).

Uranium Oxidation

Under anoxic conditions, particulate U(IV) is thermodynamically favored. Particulate U(IV) oxidation to soluble U(VI) is rapid under aerobic conditions, with a half-life on the order of seconds at circumneutral pH (6). In Chesapeake Bay sediments, for example, particulate U(IV) is released to pore waters and bottom waters by U(IV) oxidation processes. Anoxic conditions during the summer result in reductive precipitation of U(IV) particles and minimal exchange of uranium to the bottom waters. Bay turnover in the fall, however, results in increased bottom water O_2 concentrations, enhanced U(IV) oxidation rates and subsequent release of U(VI) to the water column (8).

Neutrophilic U(IV)-oxidizing bacteria (UOB) are yet to be discovered, indicating that U(IV) oxidation in marine and freshwater environments is primarily the result of O_2-catalyzed oxidation pathways. Two acidophilic UOB, *Thiobacillus ferrooxidans* and *T. acidophilus*, oxidize U(IV) enzymatically under aerobic conditions, yet are unable to grow with U(IV) as the sole electron donor (9). Microbial U(IV) oxidation may therefore only be an important component of uranium cycling in uranium-contaminated acidic environments, such as acid mine drainage.

Uranium Reduction

In anaerobic aqueous solutions, abiotic reduction of U(VI) by inorganic reductants such as hydrogen sulfide and Fe(II) proceeds at extremely slow rates (10). However, under anaerobic conditions, U(VI) is chemically reduced to U(IV) by Fe(II) sorbed to mineral surfaces, such as hematite (10). Fe(II)-containing minerals such as pyrite (FeS_2) also reduce U(VI) under anaerobic conditions (11). Sulfidic marine sediments are therefore considered the main repository for uranium in the global uranium budget. Organic compounds are not effective chemical reductants of U(VI) at temperatures below 45 °C (10).

Sulfate-reducing bacteria (SRB) such as *Desulfovibrio desulfuricans* and *D. vulgaris* reduce U(VI) under anaerobic conditions, yet are unable to grow on U(VI) as the sole

terminal electron acceptor. U(VI)-reducing bacteria (URB) *Shewanella putrefaciens* and *Geobacter metallireducens*, on the other hand, couple U(VI) reduction to anaerobic growth (12,13). U(VI) chemical speciation is an important component to microbial U(VI) reduction. *Desulfovibrio desulfuricans* reduces U(VI) complexed to multidentate ligands (citrate, oxalate) at rates faster than U(VI) complexed to monodentate ligands (acetate). Interestingly, the opposite trend is observed with U(VI)-reducing *Shewanella alga* (14).

The contribution of microbial U(VI) reduction to uranium cycling is greatly affected by the presence of competing terminal electron acceptors. In redox-stratified marine sediments, the U(VI) reduction zone is found below the zone of Fe(III) reduction, suggesting that Fe(III) is used as a preferred terminal electron acceptor (15). U(VI) is released from estuarine sediments only after the Fe(III) and Mn(IV) oxides are reductively dissolved (16). In pure culture, U(VI) reduction by *S. alg*a (an Fe(III)- and U(VI)-reducing bacterium) is inhibited by the presence of Fe(III) oxides, which divert electron transfer from U(VI) to Fe(III) (17).

Uranium Precipitation and Biosorption

In addition to microbially mediated reductive precipitation pathways, microorganisms also immobilize U(VI) via phosphatase-associated pathways. U(VI) is precipitated at the cell surface as polycrystalline HUO_2PO_4 by complexation with inorganic phosphate liberated enzymatically from organophosphate compounds (3,18). Phosphatase-associated precipitation pathways may provide the basis for a microbial uranium detoxification system. In another indirect mechanism, U(VI) is scavenged by microbially produced siderophores that otherwise sequester low levels of Fe(II) in natural waters (18). Other microbially mediated mechanisms for uranium immobilization include direct uranium uptake or biosorption to cell surfaces (3). Direct uranium uptake results in the formation of intracellular uranium crystals (3). Extracellular uranium biosorption results from interactions between anionic sites on cell surfaces (e.g., carboxylic acid groups) and positively charged U(VI) species (19). In this manner, all microorganisms with negatively charged cell surface groups possess potential to biosorb uranium.

Hypothetical Uranium Cycle

A hypothetical uranium cycle is depicted in Figure 1. This hypothetical cycle is based on known uranium transformation pathways and does not reflect the relative contribution of each pathway to overall uranium cycling. The primary sources of U(VI) in the environment include weathering of U(VI)-bearing minerals and anthropogenic inputs. In aerobic surface waters, U(VI) is complexed to carbonates or sorbed to inorganic and organic particulate matter, which is then transported through the redox transition zone to anoxic bottom waters and sediments. Under anoxic conditions, URB produce particulate U(IV), which is stably maintained in the sediment solid phase. URB activity is inhibited by competing terminal electron acceptors, such as Fe(III) and Mn(IV). Pyrite and Fe(II) sorbed to catalytic mineral surfaces mediate the abiotic (chemical) reduction of U(VI). U(IV) is reoxidized upon exposure to oxygenated pore waters and the resulting U(VI) products freely diffuse into the water column to complete the uranium cycle.

IRON CYCLING

Overview

Iron is a transition metal that undergoes a variety of microbially and chemically mediated redox transformations and is complexed by a wide range of organic and

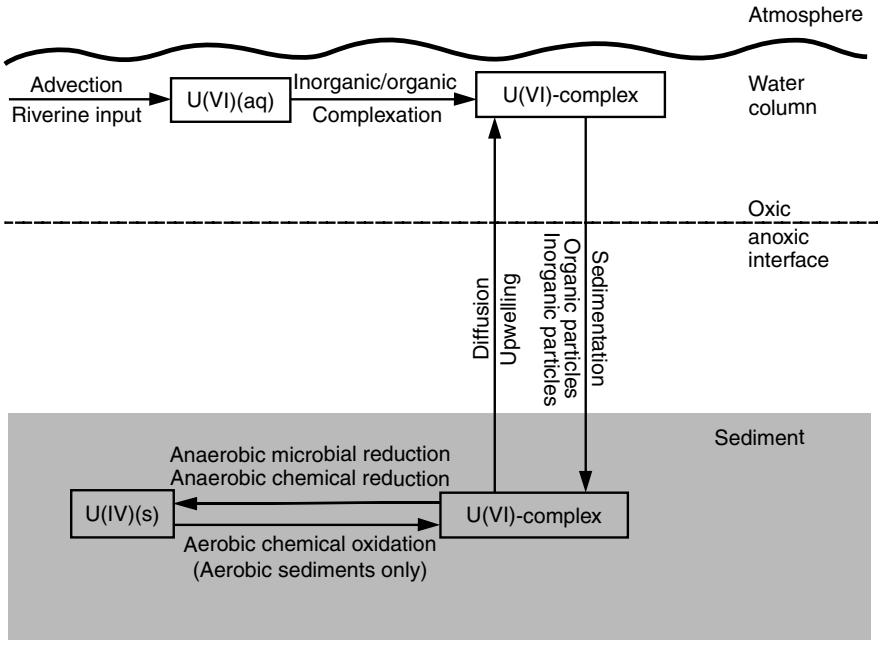

Figure 1. Hypothetical uranium cycle.

inorganic compounds. Iron redox transformations are critical to iron cycling in aqueous environments because Fe(III) is sparingly soluble at circumneutral pH, whereas Fe(II) is highly soluble (1). The predominant iron oxidation states are 0, +II and +III. Table 1 lists several common Fe(II)- and Fe(III)-bearing minerals. Iron enters marine environments via riverine input and atmospheric deposition (20). In aqueous environments at pH less than 5, Fe(II) is thermodynamically favored and is used as an electron donor by Fe(II)-oxidizing bacteria (FOB). In aqueous environments at pH greater than 5, Fe(III) is thermodynamically favored and is used as a terminal electron acceptor by Fe(III)-reducing bacteria (FRB). Fe(II) is chemically oxidized to Fe(III) by O_2 and other oxidants, including Mn(IV), whereas Fe(III) is chemically reduced to Fe(II) by organic and inorganic reducing agents. Fe(III) and Fe(II) are readily chelated by organic (e.g., EDTA, citrate, oxalate) and inorganic (e.g., PO_4^{3-}, HCO_3^-) compounds. In the following section, the biotic and abiotic pathways of iron cycling are outlined, with emphasis on microbial Fe(II) oxidation and microbial Fe(III) reduction.

Microbial Fe(II) Oxidation

FOB are isolated from aerobic environments at pH less than 5 and microaerobic environments (<10% O_2 saturation) at circumneutral pH (4). FOB are generally not found in high pH environments where Fe(III) is thermodynamically favored over Fe(II). At pH less than 5, aerobic FOB outcompete O_2 for Fe(II) substrates. At circumneutral pH, however, aerobic FOB outcompete chemical Fe(II) oxidation pathways only at low O_2 concentrations (4). Acidophilic FOB that grow by coupling Fe(II) oxidation to aerobic respiration include *T. ferrooxidans*, a common isolate of acid mine drainage (21). Leachate originating from many acid mines contains high concentrations of acid-soluble Fe(III), an indication of FOB activity (22). *Thiobacillus ferrooxidans* is also able to oxidize H_2S, S(0), $S_2O_3^{2-}$, FeS, H_2 and formate under aerobic conditions. Fe(III) oxyhydroxide precipitates are often found in spent media of Fe(II)-oxidizing *T. ferrooxidans*, suggesting that Fe(II) oxidation occurs either extracellularly or in association with the outer cell membrane. Other acidophilic FOB include *Leptospirillum ferrooxidans*, members of the genus *Mettalogenium* and the thermophilic acidophiles *Sulfolobus acidocaldarus* and *Sulfobacillus thermosulfidooxidans* (4). An as-yet uncharacterized acidophilic Archeon isolated from acid mine drainage also oxidizes Fe(II) (23). The ability to oxidize Fe(II) appears widespread in prokaryotes and may be an important component of iron cycling in aerobic/low pH, microaerobic/circumneutral pH or high-temperature environments.

Table 1. Common Iron-Containing Minerals

Mineral Name	Composition	Iron Oxidation State
Siderite	FeC_2O_3	+2
Pyrite	FeS2	+2
Hematite	α-Fe_2O_3	+3
Goethite	α-FeOOH	+3
Lepidocrocite	β-FeOOH	+3
Magnetite	Fe_3O_4	+2, +3

The neutrophilic FOB *Gallionella ferruginea* resides in Fe(II)-rich freshwaters that contain low O_2 concentrations (24). At low O_2 concentrations, *G. ferruginea* outcompetes O_2-catalyzed chemical oxidation pathways for Fe(II) substrates. During microaerobic Fe(II) oxidation, *G. ferruginea* forms stalks of twisted, bundled fibrils encrusted with Fe(III) oxyhydroxides (25). Neutrophilic FOB phylogenetically unrelated to any known lineage are enriched from iron-containing groundwaters by incubation in opposing Fe(II) and O_2 gradients (26). Other neutrophilic FOB include *Leptothrix discophora*, a freshwater isolate that contains a soluble Fe(II)-oxidizing protein (27). In the neutrophilic FOB *Metallogenium* and *Arthrobacter siderocapsulatus*, Fe(II) is oxidized by O_2 produced during H_2O_2 disproportionation by catalase. Regardless of the mechanism for Fe(II) oxidation, FOB are generally encased in precipitated Fe(III)-oxide crusts (24), which may sink to underlying sediments.

Microbial Fe(II) oxidation is also an important metabolic pathway in anoxygenic photosynthesis. Fe(II) oxidation by anoxygenic, photosynthetic FOB is postulated to be one of the first cellular energy transduction pathways on early Earth (28). In the modern biosphere, Fe(II) is oxidized by anoxygenic phototrophs, which couple reverse electron transport to carbon dioxide fixation (28). Nonphotosynthetic, anaerobic FOB derive metabolic energy by coupling Fe(II) oxidation to anaerobic respiration. For example, strains isolated from brackish-water sediments grow anaerobically by coupling Fe(II) oxidation to NO_3^- reduction (29). Geochemical evidence for NO_3^--reducing FOB is found in the suboxic zone of the Black Sea where Fe(II) and nitrate are depleted in the same depth horizon (30). Microbial Fe(II) oxidation may therefore be a significant component of iron cycling in the photic zone of anaerobic waters and in the suboxic zone of Fe(II)- and NO_3^--rich waters and sediments.

Chemical Fe(II) Oxidation

Chemical (O_2-catalyzed) Fe(II) oxidation contributes significantly to iron cycling because Fe(II) is rapidly oxidized by O_2 at pH greater than 5 (half-life on the order of seconds to minutes; (1)). In the Santa Barbara Basin, for example, Fe(II) diffusing from anaerobic sediments into the overlying anaerobic water column is rapidly oxidized by O_2 upon reaching the oxic/anoxic interface (31). The resulting Fe(III) oxide particles settle through the water column and re-enter the anaerobic sediments where they are re-reduced, released to the overlying water column and again precipitated at the oxic/anoxic interface. In this way, iron atoms are recycled hundreds to thousands of times before burial.

Chemical Fe(II) oxidation is enhanced by chemical reactions that raise pH. These reactions include NH_4^+ production during protein degradation, organic acid oxidation during microbial metabolism and CO_2 fixation (HCO_3^- removal) during photosynthesis (4). O_2-catalyzed, Fe(II) oxidation coupled to photocatalyzed, Fe(III) reduction (see following text) may form the basis of a local Fe cycle in

the photic zone of aquatic environments. Fe(II) is protected from oxidative precipitation at elevated pH and E_h by chelating agents, such as oxalate, citrate, and humic acids. Conversely, Fe(II) oxidation is promoted by microbial breakdown of Fe(II)-chelating compounds. Photochemically produced Fe(II) is also readily oxidized in a Fenton reaction with hydrogen peroxide, producing hydroxyl radical ($OH^{\bullet}$) and particulate Fe(III). $OH^{\bullet}$ produced by the photo-Fenton reaction is a potential oxidant of complex, natural organic matter that is otherwise resistant to degradation (32). The photo-Fenton reaction is postulated to be a dominant pathway for Fe(II) oxidation and organic matter degradation in the photic zone of oligotrophic seawaters (33).

Chemical Fe(II) oxidation is also an important component of iron cycling in anaerobic environments. Under anaerobic conditions at pH greater than 5, Fe(II) is rapidly oxidized to Fe(III) by Mn(IV) oxides (34). Fe(III) reduction in redox-stratified environments may therefore be masked by Mn(IV)-catalyzed (chemical) oxidation of Fe(II). Iron cycling is also tied to uranium cycling. Under anaerobic conditions, Fe(II) sorbed to Fe(III) oxide surfaces is rapidly oxidized by U(VI) to produce particulate Fe(III) and particulate U(IV) oxidation products (see preceding section on uranium cycling; (10)).

Microbial Fe(III) Reduction

The contribution of other anaerobic respiratory processes (methanogenesis, denitrification, and sulfate reduction) to carbon, nitrogen, and sulfur cycling is well documented (22). Only recently has the role of microbial (dissimilatory) Fe(III) reduction in the cycling of iron and other elements been recognized. Geochemical evidence for FRB is found in both freshwater and marine environments where Fe(II) is detected in high concentrations (35–37). In Fe(III)-rich, anaerobic marine sediments, FRB activity can account for up to 80% of the in situ rate of organic carbon oxidation. Laboratory-controlled sediment incubations demonstrate that Fe(II) production is inhibited by heat pasteurization or by addition of metabolic inhibitors and energetically more favorable terminal electron acceptors, such as O_2 and nitrate (35). Microbial Fe(III) reduction, on the other hand, suppresses less energetically favorable terminal electron-accepting processes such as sulfate reduction and methanogenesis in Fe(III)-rich, anaerobic freshwater sediments (38).

FRB couple the oxidation of inorganic substrates, such as H_2 and S(0), as well as a variety of organic carbon compounds to the reduction of Fe(III) (35,36). Respiratory electrons pass through an electron transport chain, which may terminate with Fe(III)-specific reductases. The energy released during electron transfer is coupled to generation of a proton motive force and ATP. To transfer respiratory electrons to particulate Fe(III) oxides, FRB must contact the oxide surface directly or employ electron shuttles to transport electrons to the oxide surface (39–41). Although amorphous Fe(III) oxides are more readily reduced by FRB than crystalline Fe(III) forms (e.g., hematite and goethite), differences in reduction rates are attributed to differences in reactive surface areas (42).

The ability to reduce Fe(III) is widespread in the prokaryotic world (43). FRB are found in the epsilon (e.g., *Geospirillum*) and alpha (e.g., *Rhodobacter capsulatas*) subdivisions of the proteobacteria as well as in the thermophilic gram-positive group (*Bacillus infernus*). *Geovibrio ferrireducens* and *Geothrix fermentens* form two novel FRB lineages within the Bacteria. Hyperthermophilic bacteria capable of reducing Fe(III) include *Archaeoglobus fulgidus*, a sulfate-reducing member of the Archaea (44). Microbial Fe(III) reduction is therefore a potentially important component of iron cycling in iron-rich, high-temperature environments, such as hydrothermal vents. These findings also strengthen the hypothesis that microbial Fe(III) reduction was one of the first respiratory processes to have evolved on early Earth (44).

The best-studied FRB are found in two groups of proteobacteria: facultative anaerobes in the gamma subdivision (e.g., *Shewanella putrefaciens* and acidophilic *T. ferrooxidans* (45,46)) and strict anaerobes in the delta subdivision (e.g., *Geobacter metallireducens* (46)). Most delta proteobacterial FRB completely mineralize organic carbon substrates under Fe(III)-reducing conditions, whereas their gamma proteobacterial counterparts only partially oxidize them (43). Gamma proteobacterial FRB, however, are noted for their ability to respire on a wide battery of compounds as alternate terminal electron acceptors. *Shewanella putrefaciens*, for example, respires on O_2, Fe(III), nitrate, nitrite, fumarate, Mn(IV), Cr(VI), U(VI), Tc(VII), sulfite, bisulfite, S(0), and potentially several other compounds (13,47,48). In the presence of competing terminal electron acceptors, the energetically favorable one is preferentially reduced by *Shewanella* species. For example, O_2 and nitrate are preferentially reduced before Fe(III) (49,50), whereas Fe(III) is preferentially reduced before U(VI) (17). The contribution of microbial Fe(III) reduction to the cycling of iron, uranium, and other elements in anaerobic environments is therefore greatly affected by competing terminal electron acceptors.

Microbial Fe(III) reduction is important to other environmental processes associated with iron cycling, including reductive dissolution of Fe(III)-containing clays, gleying of waterlogged agricultural soils, biomineralization of Fe(II)-containing minerals and mobilization of phosphate and trace metals sorbed to Fe(III)-oxide surfaces (35). Although iron is an essential nutrient for most microorganisms, little is known about the impact of assimilatory Fe(III) reduction on iron cycling in natural waters and sediments.

Chemical Fe(III) Reduction

Several organic compounds (e.g., cysteine and some phenols) reduce Fe(III) chemically (abiotically) at circumneutral pH (35). These compounds are generally not found in significant concentrations in the environment, however, and their role in iron cycling is minimal. Sulfide, on the other hand, is found in high concentrations in anaerobic marine environments. Fe(III) is rapidly reduced by sulfide at circumneutral pH (51) and solid phase iron monosulfides (FeS) or FeS_2 are generated via the following series

of reactions:

$$2FeOOH + H_2S + 4H^+ \rightarrow 2Fe^{2+} + S(0) + 4H_2O \quad (1)$$

$$Fe^{2+} + H_2S \rightarrow FeS + 2H^+ \quad (2)$$

$$FeS + H_2S \rightarrow FeS_2 + 2H^+ \quad (3)$$

In anaerobic sulfate-rich sediments, sulfide produced by SRB may diffuse into the Fe(III) reduction zone and outcompete FRB for Fe(III) substrates that otherwise are available for use as terminal electron acceptor (52). A more energetically favorable terminal electron-accepting process (Fe(III) reduction) is thus replaced by a less favorable one (sulfate reduction).

Photoreductive dissolution pathways may account for a significant amount of Fe(II) produced in the photic zone of the open ocean (1). In photoreductive dissolution, UV radiation catalyzes electron transfer from organic ligands to complexed Fe(III) and soluble Fe(II) is released. FRB are generally not active in the photic zone because O_2, an energetically more favorable terminal electron acceptor, is produced at high concentrations during photosynthesis. Photoreduced Fe(II) is reoxidized by photosynthetically derived O_2 in the photic zone of surficial waters, thereby driving a local iron cycle (1).

Microbial Iron Assimilation

With only two known exceptions (e.g., lactic Streptococci and *Borellia burgdorferi*) (53), all organisms require iron for use as protein cofactors. Iron-containing proteins include cytochromes, FeS proteins, catalases, peroxidases, ribonucleotide reductases, ferredoxin and transcriptional activation factors. Microorganisms have evolved numerous mechanisms for Fe assimilation. In general, these strategies include biosynthesis and secretion of low molecular weight, Fe(III)-chelating compounds (generally siderophores) that complex and solubilize Fe(III). More than 500 siderophores have been discovered in bacteria and fungi. Microorganisms that do not produce siderophores appear to be a rare exception (54). Fe(III)-siderophore complexes are transported into the cell via active transport pathways mediated by cell surface receptors with high specificity for individual Fe(III)-siderophore complexes. Iron is removed from the Fe(III)-siderophore complex either by direct reduction of the complexed Fe(III) or via degradation of the siderophore followed by Fe(III) reduction. The released Fe(II) is incorporated into proteins or stored within the microorganism for future use. Some microorganisms reduce Fe(III) prior to transporting it into the cell, whereas others take up soluble Fe(II) or Fe(III) directly from the environment (55). Microbial iron assimilation may have a significant impact on iron cycling in environments where iron is limiting or biomass concentrations are high.

Iron Cycling: A Hypothetical Model System

A hypothetical iron cycle is depicted in Figure 2. This hypothetical cycle is based on known iron transformation pathways and does not reflect the relative contribution of each pathway to overall iron cycling. The natural water

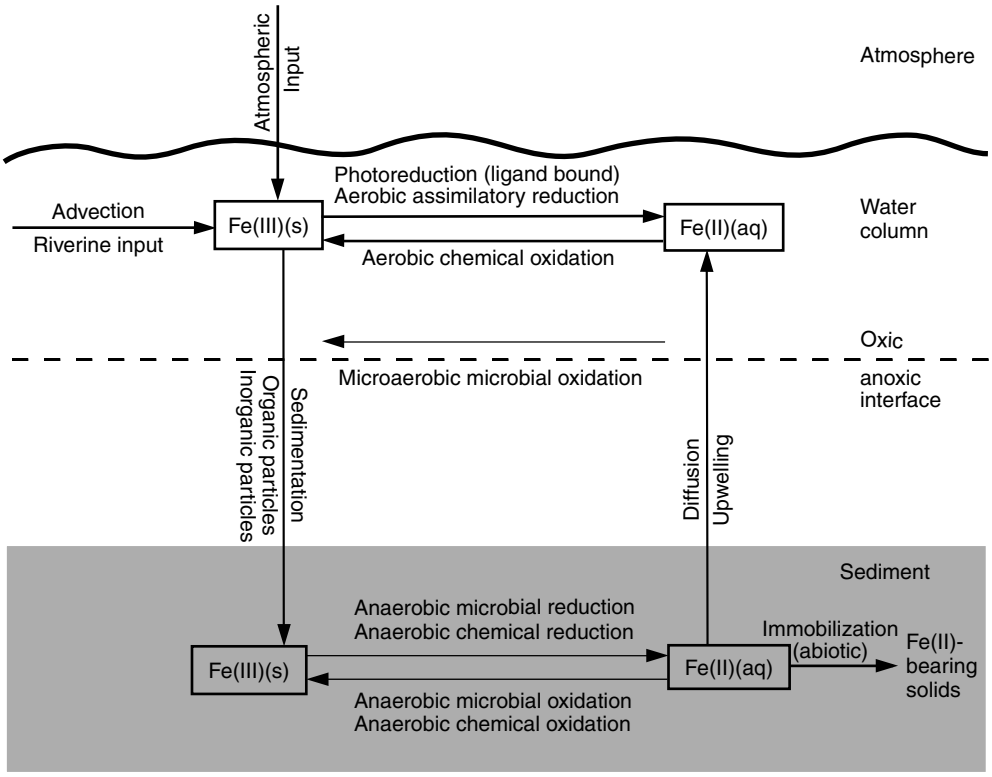

Figure 2. Hypothetical iron cycle.

system consists of aerobic surface waters, an oxic/anoxic interface and underlying anaerobic sediments. Fe(III)-bearing particles enter the system from rivers or the atmosphere. In the photic zone, Fe(III) is complexed to organic ligands and photoreduced to Fe(II). Fe(II) is oxidized to particulate Fe(III) by photosynthetically derived O_2. Fe(II) or Fe(III) may also be assimilated by the resident microbial community. Particulate Fe(III) or Fe(III) complexed by high molecular weight organic matter settles through the oxic/anoxic interface into the anoxic bottom waters. Particulate Fe(III) is reductively dissolved to Fe(II) via microbially or chemically driven pathways, releasing previously sorbed phosphate and trace metals to the water column. Fe(II) is found in a freely soluble form or is immobilized via reaction with bicarbonate or hydrogen sulfide and found as siderite ($FeCO_3$), FeS or FeS_2. Soluble Fe(II) is oxidized at the oxic/anoxic interface by microbially or chemically driven Fe(II) oxidation pathways. Particulate Fe(III) in anaerobic sediments remains buried until microbial or chemical Fe(III) reductive dissolution processes release soluble Fe(II) to the pore waters. Fe(II) is oxidized by anaerobic microbial and chemical pathways or transported upward by molecular diffusion and upwelling currents. If light penetrates the anoxic zone, anoxygenic phototrophic microorganisms oxidize Fe(II). At the oxic/anoxic interface, low dissolved O_2 concentrations provide an ecological niche for microbial Fe(II) oxidation. Photosynthetically derived O_2 drives Fe(II) oxidation in the surficial waters, whereas photocatalyzed Fe(III) reduction completes the hypothetical iron cycle.

MANGANESE CYCLING

Overview

Manganese is a redox-active transition metal that constitutes approximately 0.1% (dry weight) of the Earth's crust; it is approximately 50 times less abundant than iron. Table 2 lists common Mn-containing minerals (56). Manganese enters marine environments via riverine input and atmospheric deposition (57). The most common manganese oxidation states in the environment are +II, +III and +IV. Mn(II) is highly soluble at circumneutral pH, although not as redox reactive as Fe(II) (1). In contrast to Fe(II), Mn(II) is not readily oxidized by O_2 at pH less than 8. Under aerobic conditions and at circumneutral pH, Mn(II) is used as an electron donor by Mn(II)-oxidizing bacteria (MOB). Mn(II) is immobilized by complexation with inorganic compounds, such as bicarbonate and sulfide, producing the Mn-bearing minerals albandite and rhodocrosite:

$$Mn(II) + HCO_3^- \rightarrow MnCO_3(s) \quad \text{(albandite)} \quad (4)$$

$$Mn(II) + HS^- \rightarrow MnS\,(s) + H^+ \quad \text{(rhodocrosite)} \quad (5)$$

Mn(II) oxidation by O_2 is inhibited if Mn(II) is complexed to bicarbonate, sulfide, or humic acids (4). Mn(III), on the other hand, is highly unstable at circumneutral pH and, in the absence of complexing ligands, is rapidly disproportionated to Mn(II) and Mn(IV) via the following reaction:

$$2Mn(III) + 2H_2O \rightarrow Mn(II) + MnO_2(s) + 4H^+ \quad (6)$$

Mn(IV) is highly insoluble at circumneutral pH. Particulate Mn(IV) is used as a terminal electron acceptor by Mn(IV)-reducing bacteria (MRB). Heavy metals such as cobalt, nickel, and copper sorb to Mn(IV) oxide surfaces and are released following Mn(IV) reductive dissolution. Iron is also readily sorbed to Mn(IV) oxide surfaces, often leading to the formation of ferromanganese nodules in Fe- and Mn-rich sediments (4).

Table 2. Common Manganese-containing Minerals

Mineral Name	Composition	Manganese Oxidation State
Pyrolusite	MnO_2	+4
Hausmanite	Mn_3O_4	+2, +4
Manganite	MnOOH	+3
Rhodocrosite	$MnCO_3$	+2
Abandite	MnS	+2
Rhodonite	$MnSiO_3$	+2

Microbial Mn(II) Oxidation

Microbial Mn(II) oxidation is a potentially important component of manganese cycling in aerobic environments. In contrast to Fe(II), Mn(II) is not readily oxidized by O_2 at circumneutral pH. Mn(II) is therefore available for use as an electron donor by aerobically respiring MOB (e.g., *Hyphomicrobium manganoxidans* (58)) via the following redox reaction:

$$2Mn(II) + O_2 + 2H_2O \rightarrow 2MnO_2(s) + 4H^+ \quad (7)$$

Geochemical evidence for MOB is found in both freshwater and marine environments. For example, Mn(II) oxidation activity is detected just above the oxic/anoxic interface in Saanich Inlet (59). In other environments (4,58), Mn(IV) concretions are coated with aerobic MOB that display Mn(II)-oxidizing activity in pure culture. In addition, fossilized cells morphologically resembling extant MOB are found within Mn(IV)-bearing ores. Furthermore, Mn(II) oxidation ceases in sediment microcosms amended with metabolic inhibitors, such as azide or Hg. Aerobically respiring MOB are not detected in acidic environments: only small amounts of free energy are released during Mn(II) oxidation by O_2 at pH less than 5 (4).

Mn(IV) is oxidatively precipitated on the outer cell surface, in the periplasmic space or in the peptidoglycan layer of MOB (4). Not all MOB carry out Mn(II) oxidation for energy transduction purposes. In some MOB (e.g., *A. siderocapsulatus* and *L. discophora*), Mn(II) is oxidatively precipitated on the cell surface, forming a sheath that may protect the cell against Mn(II) toxicity, UV radiation, viral attack or heavy metal uptake. These bacteria also oxidize Mn(II) using catalase. Electrons are transferred to hydrogen peroxide, providing protection

from the excess hydrogen peroxide produced during aerobic respiration:

$$Mn(II) + H_2O_2 \rightarrow MnO_2 + 2H^+ \quad (8)$$

In spore-forming MOB (e.g., *Bacillus* SG-1), Mn(II) is oxidized by spores (and not vegetative cells), forming Mn(IV)-oxide sheaths that serve as anaerobic terminal electron acceptors for vegetative cells after germination. Freely soluble Mn(II) is not always the preferred MOB substrate. For example, *Oceanospirillum* strains only oxidize Mn(II) that is prebound to inorganic solids, such as Mn(IV) -oxides, ferromanganese nodules or clays. In contrast to their FOB counterparts, NO_3^--reducing MOB are not found in anaerobic environments. MOB are most likely unable to grow anaerobically on nitrate as terminal electron acceptor because the Mn(II)/NO_3^- redox couple yields little free energy. In addition, an anoxygenic phototrophic MOB that utilizes Mn(II) as an electron donor to drive carbon dioxide fixation is yet to be discovered.

Chemical Mn(II) Oxidation

In contrast to Fe(II), Mn(II) is oxidized slowly by O_2 at circumneutral pH (half-life on the order of months (1)). Mn(II) chemical oxidation rates are highest at high E_h (> +500 mV), pH (>8) and Mn(II) concentrations (>0.01 ppm). Mn(II) chemical oxidation rates are enhanced at circumneutral pH by the presence of Fe(III) oxide and silicate surfaces (1,60). Mn(II) chemical oxidation rates are also enhanced by pH increases associated with microbial metabolic activities, such as NH_3 production, organic acid oxidation, and carbon dioxide fixation.

Microbial Mn(IV) Reduction

Mn(IV) oxides are reduced by anaerobically respiring MRB, with Mn(II) produced extracellularly in large quantities. Geochemical evidence for MRB is found in both freshwater and marine environments where Mn(II) is detected in high concentration (35–37). Approximately 75% of organic carbon oxidation in anoxic Black Sea sediments is coupled to microbial Mn(IV) reduction (61). Mn(II) production in sediment incubations is inhibited by amendment with O_2 or metabolic inhibitors. Geochemical evidence for anaerobic NH_3 oxidation (AnAmOx) coupled to Mn(IV) reduction is also found in marine sediments (62), although a pure culture carrying out this type of metabolism is yet to be discovered.

As in dissimilatory Fe(III) reduction, anaerobic electron transport to Mn(IV) is coupled to proton translocation and ATP generation (63). Microbial Mn(IV) reduction is inhibited by O_2, which diverts electrons to aerobic respiratory pathways (facultative anaerobes only) or initiates oxidative cell damage. Facultative anaerobes capable of reducing Mn(IV) include *S. putrefaciens* and *Geospirillum barnesii*, whereas strict anaerobes include *G. metallireducens*, *D. acetoxidans*, *D. desulfuricans*, *G. fermentens*, and *B. infernus* (43). MRB couple the oxidation of H_2 and a wide range of organic carbon compounds to Mn(IV) reduction. The strict anaerobic group mineralizes organic carbon substrates, whereas the facultative group only partially oxidizes these compounds. The strict anaerobic group also couples S(0) oxidation to Mn(IV) reduction (64).

Shewanella putrefaciens reduces Mn(III) substrates under anaerobic conditions, although the role of this process in manganese cycling is unknown (65). In oligotrophic Antarctic lakes, Mn(IV) oxides are reduced under aerobic conditions by aerobic MRB belonging to the genus Carnobacteria (66). Aerobic Mn(IV) reduction is postulated to release trace metals and other nutrients that are otherwise sorbed to Mn(IV) oxide surfaces. Mn(IV) is also reduced by microorganisms during manganese assimilation. Although manganese is an essential nutrient for most microorganisms, little is known about the impact of assimilatory Mn(IV) reduction on manganese cycling in natural waters and sediments.

Chemical Mn(IV) Reduction

Mn(IV) is reduced chemically by a set of organic carbon compounds (e.g., oxalate, pyruvate, and substituted phenols) that are relatively unreactive with Fe(III) at circumneutral pH (67). These organic compounds, however, generally exist in low concentrations in the environment and their role in manganese cycling is minimal (35). In anaerobic marine sediments in which sulfate reduction is a major terminal electron-accepting process, Mn(IV) is reduced chemically by microbially produced sulfides (68):

$$H_2S + MnO_2 + 2H^+ \rightarrow Mn(II) + S(0) + 2H_2O \quad (9)$$

In addition, Fe(II)- and NO_2^--catalyzed Mn(IV) reduction reactions are potentially important to manganese cycling. Mn(IV) is reduced by Fe(II) at circumneutral pH and by nitrite under acidic conditions (34,69):

$$2Fe(II) + MnO_2 + 6H^+ \rightarrow 2Fe(OH)_3 + Mn(II) \quad (10)$$

$$NO_2^- + MnO_2 + 2H^+ \rightarrow Mn(II) + NO_3^- + H_2O \quad (11)$$

Furthermore, as Fe(III) is reduced by most if not all MRB, distinguishing between microbial and chemical Mn(IV) reduction pathways in Mn(IV)- and Fe(III)-rich environments is difficult (70). Similar to iron cycling, photocatalyzed Mn(IV) reduction is potentially significant to manganese cycling in oxygenated surface waters (1). Electron transfer to Mn(IV)-organic ligand complexes is catalyzed by UV radiation. Photoreduced Mn(II) may subsequently be reoxidized by MOB in surficial waters, thereby driving a local manganese cycle.

Manganese Cycling: A Hypothetical Model System

A hypothetical manganese cycle is depicted in Figure 3. This hypothetical cycle is based on known manganese transformation pathways and does not reflect the relative contribution of each pathway to overall manganese cycling. The natural water system is similar to the one used for the hypothetical iron cycle and is composed of a redox-stratified water column, an oxic/anoxic transition zone and

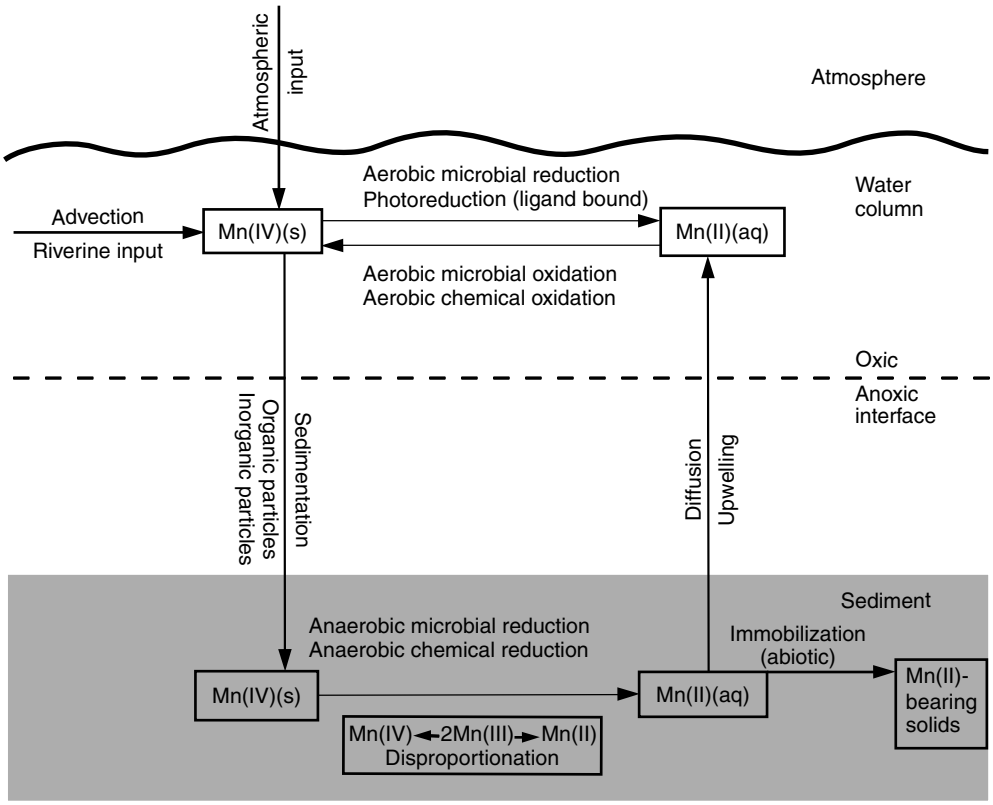

Figure 3. Hypothetical manganese cycle.

an underlying anoxic water column and sediment. Mn(IV) oxide particles are introduced into the system via riverine and atmospheric inputs. Mn(IV) oxides are complexed by organic ligands and photoreductively dissolved. Mn(IV) oxides are also reductively dissolved by aerobic MRB, with sorbed phosphate and trace metals subsequently released to the water column. Mn(IV) particles that settle through the oxic/anoxic transition zone are reduced by anaerobically respiring MRB or organic and inorganic reductants. In redox-stratified waters or sediments, the Mn(IV) reduction zone is located below the nitrate reduction zone and above the iron reduction zone. Pore water Mn(II) is either immobilized via reaction with sulfide or bicarbonate or transported back into the water column via pore water diffusion and upwelling. The manganese cycle is completed in oxygenated surface waters in which Mn(II) is oxidized by MOB to produce Mn(IV) oxides. In the absence of MOB, Mn(II) is slowly oxidized by chemical pathways. In the photic zone of surficial waters, the Mn(II) pool is fairly stable as photosynthetically derived O_2 is relatively unreactive with Mn(II).

MERCURY CYCLING

Overview

Mercury is a highly toxic metal that undergoes a myriad of microbially and chemically mediated transformations in aquatic environments and the atmosphere (71–74). Chemical speciation plays an important role in mercury toxicity, mobilization and fate. Predominant forms of mercury in natural systems include both organic and inorganic compounds, such as Hg(II), Hg(0), HgS (cinnabar), CH_3Hg^+ (monomethyl mercury; MMM), and $(CH_3)_2Hg$ (dimethyl mercury; DMM). Hg(II) and MMM are water soluble, HgS is an insoluble mineral, and both Hg(0) and DMM are highly volatile mercury gases. Mercury toxicity is magnified in natural waters as MMM is biomagnified from lower to higher trophic levels within the food chain. MMM accumulates in fish to levels one millionfold greater than ambient concentrations in the water column. Human exposure to MMM through consumption of freshwater and marine fish is now a major health concern. Cinnabar, the predominant form of mercury in nature, is formed under sulfidic conditions by Hg(II) complexation with hydrogen sulfide.

Mercury Methylation

Microbial Hg(II) methylation is a common detoxification strategy employed by microorganisms to remove mercury from their local environment (Table 3) (4,72). Mercury is removed by methylating Hg(II) in a stepwise fashion to MMM and DMM. Hg(II) is methylated to MMM at a rate three to four orders of magnitude faster than MMM is methylated to DMM. Paradoxically, mercury toxicity increases with the level of methylation (i.e., DMM > MMM > Hg(II)). MMM, however, is more easily sequestered outside the cell by sorption to clays and other negatively charged particles. DMM is extremely volatile

Table 3. Mercury-transforming Microorganisms

Hg Methylation:	Hg(II) Reduction:
Pseudomonas spp.	*Pseudomonas* spp.
Bacillus megaterium	*Staphylococcus aureus*
Escherichia coli	*Thiobacillus ferroxidans*
Enterobacter aerogenes	*Vibrio* spp.
Desulfovibrio spp.	*Bacillus* spp.
Desulfobulbus spp.	*Alcaligenes* spp.
Desulfobacter spp.	*Streptomyces* spp.
Aspergillus niger	*Acinetobacter* spp.
Saccharomyces cerevisiae	*Streptococcus* (group B)
Neurospora crassa	Enteric bacteria
	Coryneform bacteria
Hg(0) Oxidation:	Organomercurial Decomposition:
Pseudomonas aeruginosa	*Pseudomonas* spp.
Pseudomonas fluorecens	*Escherichia coli*
Escherichia coli	*Staphylococcus*
Bacillus subtillis	
Bacillus megaterium	

and is degassed to the atmosphere. Hg(II)-methylating microorganisms also methylate other metals including cadmium, lead, tin, selenium, and tellurium (4). In marine sediments, SRB (and potentially several others) methylate Hg(II) via direct (enzymatic) or indirect (chemical) pathways (72,75). In the former reaction, the cobalt-containing compound cobalamin is the methylating agent, whereas in the latter, the end product of sulfate reduction (hydrogen sulfide) catalyzes the reductive methylation of MMM in the presence of humic acids. Abiotic methylation of Hg(II) to MMM by catalytic metals and humic acids is a potential pathway for mercury cycling in organic matter-rich environments (71).

Mercury Demethylation

Organomercurial compounds are demethylated to Hg(II) (and ultimately Hg(0)) via microbially and chemically mediated pathways (72). In a variety of bacteria including Staphylococci, Pseudomonads and *E. coli*, MMM is demethylated to Hg(II) and methane by mercuric lyase. Phenyl-Hg^+, a slimicidal agent used in the paper pulp industry is likewise demethylated to Hg(II) and benzene by mercuric lyase. After removal of the methyl or phenyl groups, Hg(II) is reduced to Hg(0) by Hg(II) reductase (see following text). Genes encoding mercuric lyase are often found on plasmids or transposable elements that are transferred horizontally within natural microbial communities (76). Mercuric lyase demethylates other hazardous organomercurial compounds, including ethyl-Hg, flourescein-Hg, thimerosol, and merbromin (4). MMM is decomposed by photodegradation in lake waters to unknown end-products that may or may not produce Hg(0) (77). In the atmosphere, DMM is chemically demethylated to MMM or inorganic mercury via reactions with radical species, such as OH•, Cl•, and NO_3• (73). Atmospheric DMM is also photochemically decomposed to Hg(0) and methane by UV-catalyzed reaction pathways (4).

Mercury Reduction

Microbial Hg(II) reduction is a common detoxification strategy employed by a variety of microorganisms, including strict aerobes, strict anaerobes and facultative anaerobes (Table 3). Hg(II)-reducing microorganisms produce Hg(0), a highly volatile form of mercury that is readily degassed to the atmosphere. A large body of literature is available concerning ecological and mechanistic aspects of microbial Hg(II) reduction (72,74,78). Hg(II) reduction is catalyzed by Hg(II) reductase, yet the electron transfer reaction is not linked to energy generation. Hg(II) reductases also reduce other metals including Ag(II) and Au(II). Hg(II) reductase (*mer*) genes are often found on transposons and mobilizable plasmids that are transferred horizontally within natural microbial communities (79).

In aquatic environments, Hg(II) is reduced chemically by naturally occurring humic acids in a reaction that is enhanced by exposure to natural sunlight (80). Although not yet demonstrated, humic acid-reducing microorganisms may drive Hg(II) reduction indirectly by initiating the first step in the reduction process. In the atmosphere, Hg(II) in the aqueous phase is reduced chemically by sulfite (SO_3^{2-}) and hydroperoxyl radical (HO_2•) (81). UV-catalyzed photoreduction of Hg(II)-organo or -halide complexes is not likely to occur under atmospheric conditions.

Mercury Oxidation

Chemical oxidation of Hg(0) to water-soluble Hg(II) or particulate HgO is a critical pathway for mercury cycling in the atmosphere (73,81,82). More than 95% of atmospheric mercury is in the form of Hg(0). Hg(I) is nearly undetectable in the atmosphere because it undergoes rapid disproportionation to Hg(0) and Hg(II) in the presence of common atmospheric ligands (chloride, hydroxide, sulfite). Atmospheric Hg(0) is oxidized in the aqueous phase by ozone (O_3), hydroxyl radical (OH) and chlorine ($HOCl/OCl^-$), and in the gaseous phase by nitrate radical (NO_3•) and chlorine (Cl_2). Mammals, plants and microorganisms (including *E. coli* and several soil bacteria) oxidize Hg(0) to Hg(II) using catalase and possibly other peroxidases. In *E. coli*, Hg(0) is oxidized enzymatically by KatG, a hydroperoxidase-catalase (83). The contribution of microbial Hg(0) oxidation to mercury cycling in natural waters and sediments is presently unknown.

Mercury Cycling: A Hypothetical Model System

A hypothetical mercury cycle is depicted in Figure 4. This hypothetical cycle is based on known mercury transformation pathways and does not reflect the relative contribution of each pathway to overall mercury cycling. Hg(II) (as water-soluble Hg(II) or particulate HgO) enters the natural water system via atmospheric deposition. In the water column or sediments, Hg(II) is (*1*) complexed by sulfide to form HgS; (*2*) microbially, chemically, or photochemically reduced to Hg(0); or (*3*) microbially methylated to MMM. Hg(0) is (*1*) degassed to the atmosphere, or (*2*) microbially oxidized to Hg(II). MMM is (*1*) taken up by biota, (*2*) immobilized by sorption to negatively charged particles, (*3*) microbially demethylated

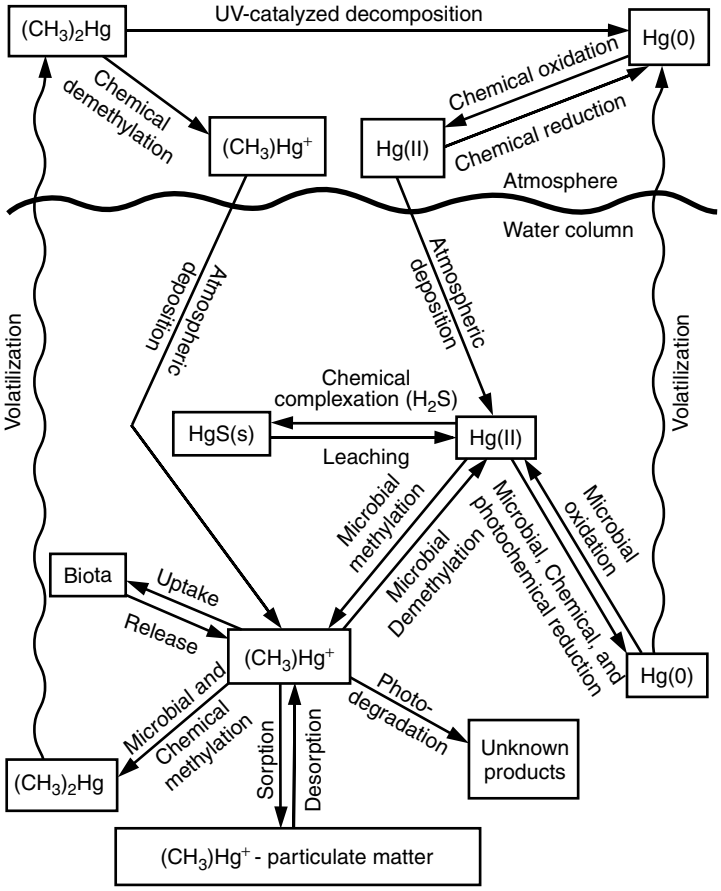

Figure 4. Hypothetical mercury cycle.

to Hg(II), (*4*) photodegraded to unknown end-products, or (*5*) microbially or chemically methylated to DMM. DMM is degassed to the atmosphere where it is chemically demethylated to (*1*) Hg(0) by UV-catalyzed pathways, or (*2*) MMM by reaction with various radical species. MMM is transported to the surface waters via atmospheric deposition. Atmospheric Hg(0) is oxidized to Hg(II) by ozone and other oxidants in the aqueous and gaseous phases. Atmospheric Hg(II) is reduced to Hg(0) by sulfite and other reductants in the aqueous phase. The mercury cycle is completed when Hg(II) re-enters the surface waters via atmospheric deposition.

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METHANE. See TRACE GASES SOIL

METHANOGENESIS. See BIOSOLIDS: ANAEROBIC DIGESTION OF

METHANOGENESIS IN THE MARINE ENVIRONMENT

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The biological production of methane is part of a microbial process that catalyzes the degradation of organic compounds as part of the global carbon cycle. Methanogenesis, as the process is called, occurs in the absence of oxygen by prokaryotic microorganisms that belong to the Domain *Archaea*, which are distinguished from the *Bacteria* and the *Eukarya* by evolutionary divergence of their genomes and phenotypic characteristics such as their ability to grow in extreme environments (1). In the methanogenic process, bacteria and the methanogenic *Archaea* function as a consortium in which the bacteria catalyze the degradation of plant and animal polymers and monomers to smaller intermediate compounds that are utilized for methane production by the methanogenic *Archaea*. Methane generated from the process is oxidized back to carbon dioxide by other microbial processes and released into the atmosphere, thus completing the carbon cycle by making the carbon available for photosynthetic fixation. This process complements microbial aerobic degradation by catalyzing degradative processes in regions that lack oxygen, which include such habitats as ocean, lake, and river sediments, rice paddies, arctic tundra, submarine hydrothermal vents, sewage digestors, digestive tracts of ruminant animals, human large intestines and termite hindgut. The distribution of methanogenesis in the oceans is discussed along with the diversity, habitats, and physiology of these microbial catalysts and their potential biotechnological applications.

THE ROLE OF OCEANS IN THE GLOBAL METHANE CYCLE

Global Distribution of Methanogenesis

Biogenesis of methane in the marine environment originates from several sources including the decomposition of organic matter settling in sediments and accumulating in submarine canyons, digestive tracts of marine fauna, and from geothermal sources of hydrogen (Fig. 1). Shallow continental shelves support large populations of phototrophic macroalgae, plants, and fish that contribute to high organic loading. Methane concentrations in these regions are severalfold greater than estimated concentrations at equilibrium with air (2,3). The equilibrium concentration of methane in the water column should be approximately 2.6 nM at 19 °C on the basis of the average partial pressure of methane in the atmosphere of 2×10^{-6} atm (4). Because there are no physical barriers to prevent convection, the methane concentration at all depths should be equal to the estimated equilibrium concentration with the atmosphere if this is the sole source of methane. In one example, Scripps Canyon, a nearshore submarine canyon with high methanogenic activity due to accumulation of organic debris, the methane profile in the overlying water column ranged from 4.7 nM at the surface to 870 nM at 23 m, concentrations that are 2- to 350-fold greater than the predicted solubility equilibrium with the atmosphere (Fig. 2a). These results, in conjunction with the observation that the methane concentration increases linearly with depth, indicate that dissolved methane in the water column originates from the sediments. The inverse relationship between depth and methane concentrations probably results from two processes: diffusional fluxes as the distance between the water column and methane source increased, and biological oxidation of methane in the aerobic zones of the water column (5). The nearly twofold saturation of methane concentration at the surface above Scripps Canyon indicates that there is a net flux of methane into the troposphere as a result of biodegradation in canyon sediments by methanogenic consortia.

Estimates of the total annual methane production from the combined global continental shelf regions at less than 10 m depth range from 0.7 to 14×10^9 kilograms annually, which is equivalent to the estimated range for freshwater lakes (3,6). These regions include tidal marine marshes (7), the lower depths of sediments (8) and

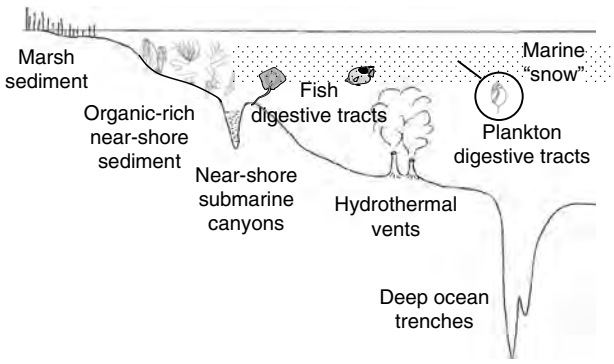

Figure 1. Sources of biological methane in the marine environment. See color insert.

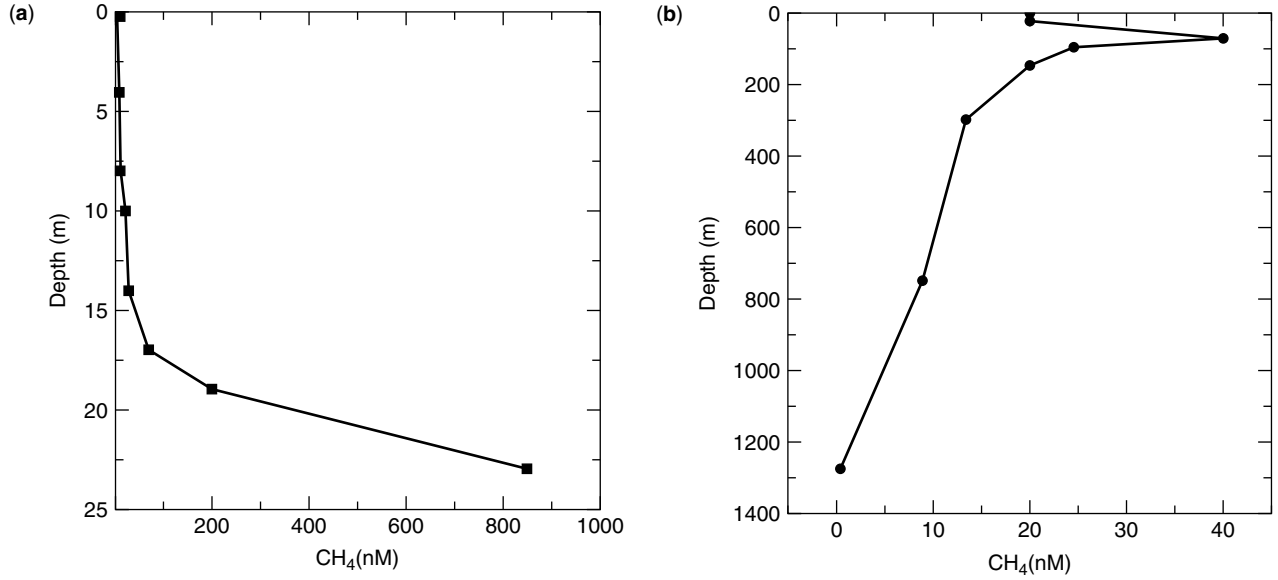

Figure 2. Dissolved methane profile in the water column above a nearshore submarine canyon near, La Jolla, CA (**a**) and in the open ocean (**b**). On the basis of data from (45) and (2).

submarine canyons, all regions that receive high organic loading (9).

Open ocean waters also show a supersaturation of methane in surface waters relative to atmospheric methane and a general decrease in dissolved methane with depth, which suggests that bacteria consume methane at lower ocean depths (Fig. 2b). Higher methane concentrations observed in the region below the surface likely result from methanogens associated with phytoplankton populations. On the basis of surface supersaturation of 1.3 and a concentration of 4.7×10^{-5} ml methane liter^{-1} seawater at the surface, the estimated total flux into the atmosphere from open oceans is 4 to 6.7×10^{9} kilograms annually (2,6). This may be an underestimate because upwelling may sweep deep, methane-rich water to the surface in some regions. Although estimates of methane release from marine sources are less than 4% of the total biogenic production released into the atmosphere annually, it is an underestimate of the total marine production because most of the methane is either oxidized to carbon dioxide or sequestered as gas hydrates.

Fate of Biogenic Methane

Most methane produced below 10 meters by marine anaerobic consortia is consumed or sequestered from the atmosphere. More than 80 % of the methane generated in marine sediments is oxidized in sulfate-rich anoxic marine upper sediments by a consortium of methanogens and sulfate-reducing bacteria closely associated in aggregates (10–12). Current reports suggest that the reaction is catalyzed via reverse methanogenesis by the methanogenic *Archaea* as a result of a thermodynamic shift, which results from sequestering of acetate and hydrogen by the sulfate-reducing bacteria. Although methane oxidation by the archaeon has been confirmed by isotopic and phylogenetic analysis, the proposed reaction mechanism has not been confirmed biochemically (12). As the remaining methane is released into the aerobic sediment zone and water column, most of it is oxidized as an energy substrate to CO_2 by the aerobic methanotrophs (13). Oxidized methane reenters the carbon cycle as bicarbonate anions dissolved in seawater or by direct release as CO_2 into the atmosphere. Although some methane escapes oxidation in eutrophic marshes and coastal waters that are supersaturated with methane or by direct release into the atmosphere through the vascular systems of aquatic plants, only a small proportion of the total global biogenic methane released annually originates from the oceans (<4%) (6,14,15). Biogenic methane is also sequestered as crystalline methane-hydrates in deep anaerobic ocean waters and sediments below 260 meters where methane can become saturating. Because the stability of methane sequestered in this form is dependent on low temperatures and high pressures, the distribution of gas hydrates is primarily restricted to deep oceans and Polar Regions. Gas hydrates are estimated to represent the largest hydrocarbon fuel reservoir on earth. However, methane sequestered as hydrates also have the potential to cause geologic instability of submarine slopes, which could lead to phenomenon such as tsunamis in the event of global changes that result in ocean warming (16). The combined processes of methane oxidation and sequestration as gas hydrates minimize the net release of biogenic methane from the oceans that would otherwise enter the atmosphere as a greenhouse gas.

DIVERSITY AND PHYLOGENY OF MARINE METHANOGENS

Isolates from the Oceans

The methanogens are classified as *Archaea*, which together with the *Bacteria* and *Eukarya* compose the three domains

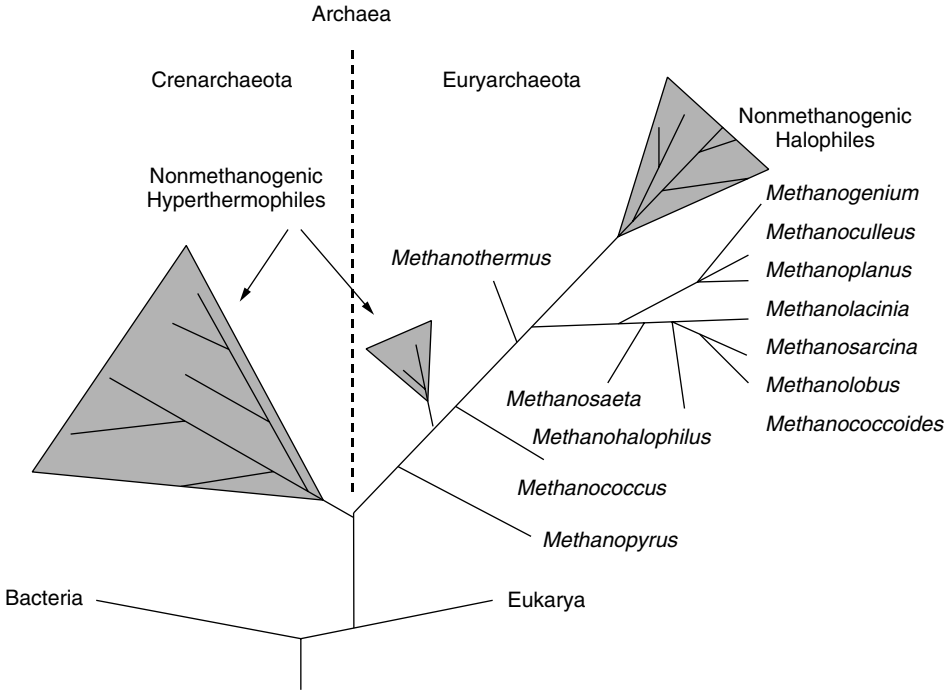

Figure 3. Phylogenetic tree of *Archaea* based on 16S rRNA gene sequence showing genera of methanogens from marine environments.

of life proposed by C. Woese on the basis of 16S rRNA sequence (Fig. 3; 1). The morphological features of the *Archaea* are similar to those of the *Bacteria*; they are unicellular microorganisms that lack a nuclear membrane and intracellular compartmentalization. However, many molecular features of the *Archaea*, which include histone-like DNA proteins, a large multicomponent RNA polymerase and the transcription initiation components, have similarity to the *Eukarya*. The *Archaea* also have characteristics that are unique from the *Bacteria* and *Eukarya*, such as nonpeptidoglycan cell walls, membranes composed of isoprenoids ether-linked to glycerol or carbohydrates and synthesis of unique enzymes and enzyme cofactor molecules. The requirement by all *Archaea* for extreme growth conditions, which include high temperatures, extreme salinity, or in the case of the methanogens, highly reduced, O_2-free environments, has redefined our perception of the boundaries of life. These revelations have fostered a renewed interest in searching for life in environments previously thought to be sterile, both on earth and extraterrestrially.

The methanogenic *Archaea* have one common attribute: they all use a methane-generating pathway for growth. However, despite their phylogenetic and catabolic coherence as a group, the methanogens are morphologically and physiologically diverse. In the marine environment, they include a wide range of morphological shapes (Fig. 4) with physiological tolerances that include psychrophiles from an Antarctic meromictic lake of marine origin that grow at 1.7 °C to extreme thermophiles from deep submarine thermal vents that grow at 113 °C; acidophiles from submarine thermal vents that grow at pH 5.0 to alkaliphiles from sediments that grow at pH 9.0; species that grow at marine saline concentrations to extreme halophiles that grow at nearly saturated NaCl concentrations in solar salterns; autotrophs that use only CO_2 for cell carbon and methyltrophs that use reduced carbon compounds.

Over one-third of more than 60 described species of methanogens are of marine origin and occur in four of the five orders within the archaeal kingdom *Euryarchaeota* (Fig. 3). Characteristics of marine methanogenic *Archaea* are described in Table 1. Species within the order *Methanobacteriales* have not been detected in marine environments. These species have a rigid cell wall composed of pseudomurein, which is chemically similar to bacterial murein and has an ultrastructure that resembles the dense monolayer cell wall of gram-positive bacteria. These species require a rigid cell wall for protection from osmotic lysis that would otherwise result from the osmotic differential between their intracellular milieu and surrounding freshwater environment. However, these species have only a limited ability to osmoregulate and become dehydrated when exposed to the higher solutes concentrations in estuarine and marine environments.

All described species in the order *Methanococcales* are marine autotrophs that grow exclusively by CO_2 reduction with H_2. These species form irregularly shaped cocci that synthesize an S-layer cell wall composed of a paracrystalline array of glycoproteins. Unlike *Methanobacteriales* that synthesize a rigid cell wall, these species have intracellular solute concentrations that are similar to seawater and are subject to osmotic lysis at "freshwater" solute concentrations. This order includes mesophilic species, as well as moderately and extremely thermophilic species.

The order *Methanomicrobiales* contains both nonmarine and marine species that are diverse in morphology and physiology. Most marine species grow as cocci and rods. In addition, *Methanoplanus* forms flat disk-shaped cells with characteristically angular ends. The cell walls in this group

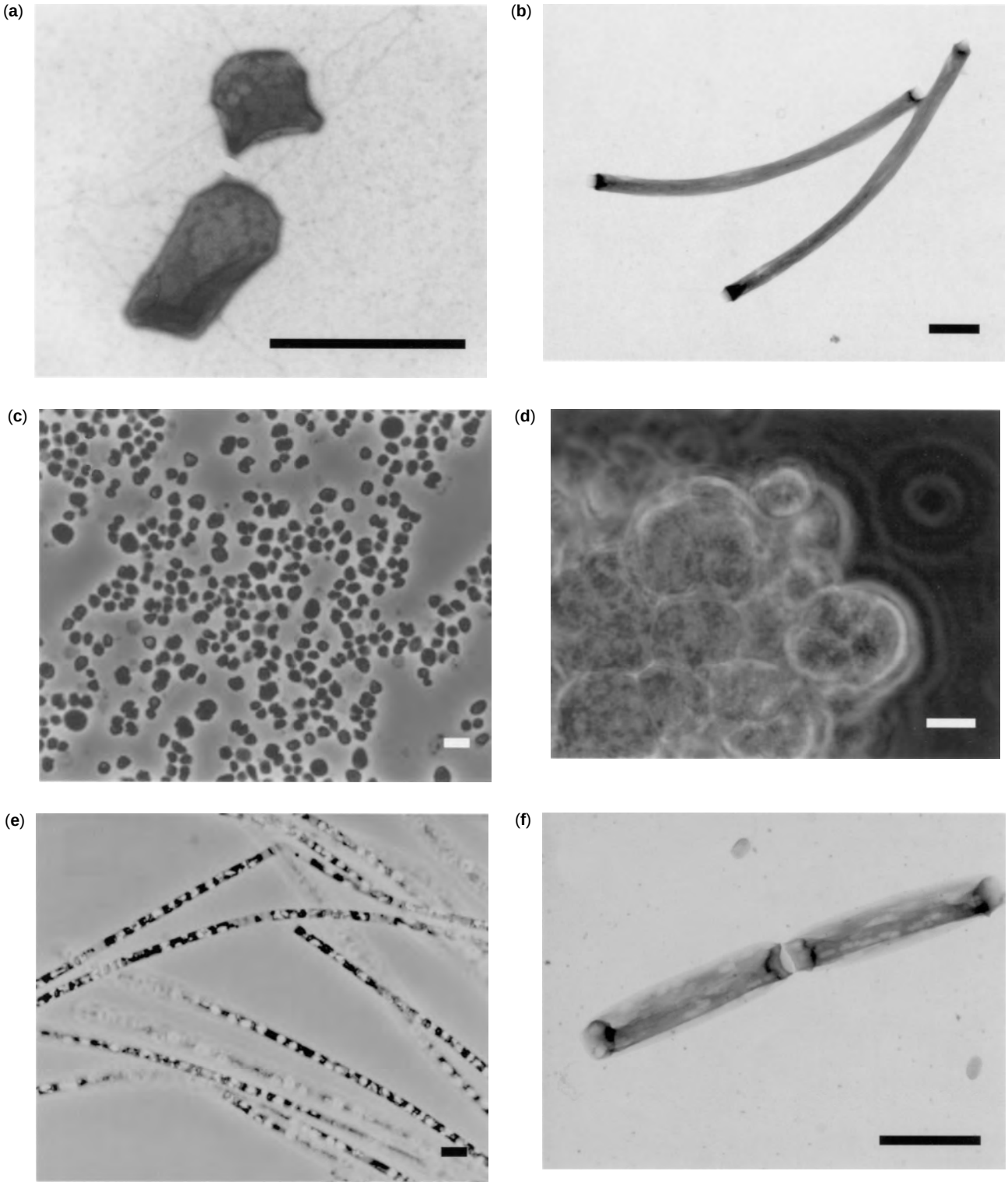

Figure 4. Phase-contrast light and electron micrographs showing diverse morphology of methanogenic *Archaea* isolated from marine sediments. Shown are negatively stained electron micrographs of hydrogen-utilizing irregular-shaped rod (**a**) and spirillum-shaped cells (**b**); light micrographs of acetate-utilizing cocci (**c**), sarcinal aggregates (**d**) and rods with intracellular refractile bodies (**e**); a negatively stained electron micrograph of an acetate-utilizing rod with internal gas vacuoles. Bar = 2 μm. (K. Sowers, unpublished data).

Table 1. Description of Methanogenic Archaea Isolated from Marine or Estuarine Sources[a]

Taxonomic Epithet	Morphology	Substrates[b]	Optimum Growth Conditions[c] pH	Temp (°C)	Isolation Source
Order *Methanobacteriales*					
No marine species reported					
Order *Methanococcaales*					
Family *Methanococcaceae*					
Genus *Methanococcus*					
maripaludis	irreg. coccus	H,F	6.8–7.2	35–39	marine marsh sediment
vannielii	irreg. coccus	H,F	7.0–9.0[d]	36–40	marine sediment
voltae	irreg. coccus	H,F	6.7–7.4	32–40	estuarine sediment
Genus *Methanothermococcus*					
thermolithotrophicus	irreg. coccus	H,F	6.5–7.5	65	thermal coastal sediment
Genus *Methanocaldococcus*					
fervens	irreg. coccus	H	6.5	85	marine hydrothermal vent
infernus	irreg. coccus	H	6.5	85	marine hydrothermal vent
jannaschii	irreg. coccus	H	6.0	85	marine hydrothermal vent
vulcanius	irreg. coccus	H,F	6.5	80	marine hydrothermal vent
Genus *Methanotorris*					
igneus	irreg. coccus	H	5.7	88	marine hydrothermal vent
Order *Methanomicrobiales*					
Family *Methanomicrobiaceae*					
Genus *Methanoculleus*					
marisnigri	irreg. coccus	H,F,2P,2B	6.2–6.6	20–25	marine sediment
thermophilicus	irreg. coccus	H,F	7.0	55	thermal marine sediment
Genus *Methanolacinia*					
paynteri	irreg. rod	H,F,2P,2B,CP	6.6–7.2	40	marine sediment
Genus *Methanogenium*					
cariaci	irreg. coccus	H,F	6.8–7.3	20–25	marine sediment
frigidum	irreg. coccus	H,F	6.5–7.9	15	antarctic saline lake[f]
organophilum	irreg. coccus	H,F,E,1P,2P,2B	6.4–7.3	30–35	marine sediment
Genus *Methanocalculus*[e]					
halotolerans	irreg. coccus	H,F	7.6	38	oil field
Genus *Methanoplanus*					
endosymbiosus	irreg. disk	H,F	6.6–7.1	32	marine ciliate
limicola	plate	H,F	7.0	40	drilling swamp
petrolearius	plate	H,F,1P	7.0	37	offshore oil field
Family *Methanosarcinaceae*					
Genus *Methanosarcina*					
acetivorans	irreg. coccus, pseudosarcina	AC,ME,MA, DMS,MMP	6.5–7.5	35–40	marine sediment
siciliae	irreg. coccus	AC,ME,MA, DMS,MMP	6.5–6.8	40	marine sediment
Genus *Methanolobus*					
bombayensis	irreg. coccus	ME, MA, DMS	7.2	37	marine sediment
taylorii	irreg. coccus	ME, MA, DMS	8.0	37	estuarine sediment
tindarius	irreg. coccus	ME,MA	6.5	37	marine sediment
vulcani	irreg. coccus	ME,MA	7.2	37	submarine fumarole
Genus *Methanococcoides*					
burtonii	irreg. coccus	ME,MA	7.7	23.4	antarctic saline lake[f]
methylutens	irreg. coccus	ME,MA	7.0	30–35	marine sediment
Genus *Methanohalophilus*					
halophilus	irreg. coccus	ME,MA	7.4	26–36	marine cyanobacterial mat
portucalensis	irreg. coccus	ME,MA	6.5–7.5	40	solar marine salt pond
Genus *Methanohalobium*					
evestigatum	irreg. coccus	ME,MA	7.4	50	salt lagoon sediment
Order *Methanopyrales*					
Family *Methanopyraceae*					
Genus *Methanopyrus*					
kandleri	sheathed rod	H	6.5	98	geothermal marine sediment

[a] type strain descriptions. For additional information see (44).
[b] H = hydrogen/carbon dioxide; F = formate; AC = acetate; ME = methanol; MA = methylamines; H/ME = methanol reduction with hydrogen; E = ethanol; 1P = 1-propanol; 2P = 2-propanol; 2B = 2-butanol; CP = cyclopentanol; DMS = dimethylsulfide; MMP = methylmercaptopropionate
[c] nr = not reported
[d] only a range reported
[e] family epithet currently uncertain
[f] marine origin

are composed of a protein S-layer and these species are sensitive to osmotic shock or detergents. Species include mesophiles, moderate and extreme thermophiles. Most species grow by CO_2 reduction with H_2, but some species also use formate or secondary alcohols as electron donors for CO_2 reduction. Psychrotolerant, mesophilic and thermophilic species are included within this order.

The order *Methanosarcinales* is the most catabolically diverse phylum of methanogens. Members of this phylum grow by the dismutation or "splitting" of acetate and pyruvate, and by catabolism of the methyl groups in methanol, methylated amines, pyruvate, and dimethylsulfide. Although species of *Methanosarcina* can grow by both catabolic pathways, other genera in this order are obligate methylotrophs. All species have a protein S-layer cell wall and most species grow as irregularly shaped cocci. However, several species of *Methanosarcina* also synthesize a heteropolysaccharide matrix external to the S-layer. This external layer can be up to 200-nm thick and is composed primarily of a nonsulfonated polymer of *N*-acetylgalactosamine and D-glucuronic or D-galacturonic acids. The matrix is called methanochondroitin because of its chemical similarity to a mammalian connective tissue component, known as chondroitin. At freshwater NaCl concentrations *Methanosarcina* spp. that synthesize methanochondroitin grow in multicellular aggregates rather than as single cells, but when grown at marine salt concentrations or with high concentrations of divalent cations such as Mg^{+2}, they no longer synthesize methanochondroitin and grow as single cells. Although all species within the genus *Methanosarcina* are capable of osmoregulation that enables them to grow in both nonmarine and marine environment, only two species have been isolated from marine environments. Species within this order are psychrotolerant or mesophilic.

The order *Methanopyrales* is the most deeply branching methanogenic archaeon and presently includes only one species, the extreme thermophile *Methanopyrus kandleri*. This species is an obligate hydrogenotroph and grows as a rod with a pseudomurein cell wall surrounded by a protein S-layer, similar to that described earlier for *Methanothermus*.

Distribution of "Nonculturable" Microorganisms

In the past few years molecular probes based on 16S rRNA gene sequences have been used as phylogenetic markers to provide information on microbial populations in natural systems (17). This approach has revealed that the majority of microbes in the environment have not been isolated and described. In addition to *Bacteria*, 16S rRNA sequences with high similarities to the archaeal kingdoms *Crenarchaeota* and *Euryarchaeota* have been detected throughout the marine water column. Cultured crenarchaeotes are hyperthermophilic, sulfur-dependent and usually strict anaerobes. Cultured euryarchaeotes include methanogens, extreme halophiles, and some hyperthermophilic, sulfur-dependent species. In surveys of temperate oceans euryarchaeotes predominated near the surface and crenarchaeotes were more predominant at depth (18). In contrast, euryarchaeotes were nearly absent in Antarctic and subantarctic waters. Although reports suggest that these *Archaea* represent a significant fraction of marine phytoplankton, their physiology and ecological roles are currently unknown. Despite their requirement for anoxic, reduced environments, transient anoxic microzones in particles and or endosymbionic niches could support methanogenic euryarchaeotes within the aerobic water column. Alternatively, the picoplanktonic euryarchaeotes detected by 16S rRNA sequence analysis may consist of undescribed, mesophilic, nonmethanogenic aerobes that successfully compete with eubacterial picoplankton in the water column. The identity and physiology of archaeal picoplankton has yet to be determined.

THE METHANE CARBON CYCLE IN MARINE HABITATS

Interspecies Hydrogen Exchange

In many marine habitats methanogens depend on other anaerobes to convert complex organic matter into substrates that they can catabolize. Unlike aerobic habitats, where a single microorganism can catalyze the mineralization of a polymer by oxidation to CO_2, degradation in anaerobic habitats requires consortia of interacting microorganisms to convert polymers to CH_4. These interactions are dynamic with hydrogen-utilizing microorganisms such as methanogens affecting the pathway of electron flow, and consequently carbon flow, by a process called interspecies H_2 transfer (19). In this association, the H_2-utilizing methanogens maintain a low H_2 partial pressure that enables oxidative pathways linked to proton reduction to be thermodynamically favorable. One physiological group of microorganisms affected by interspecies H_2 transfer is the polymer degrading fermentative anaerobes. In many of these microorganisms, substrate oxidation is linked to the electron carrier nicotinamide adenine dinucleotide (NAD), which has higher redox potential (-320 mv) than H_2 (-414 mv) under standard conditions. However, if the H_2 partial pressure is maintained at a low level (<10 Pa) by H_2-utilizing microorganisms, then H_2 production from NAD by a hydrogenase becomes thermodynamically favorable. This enables the fermentative microorganisms to reoxidize NADH by reducing protons to form H_2 rather than reducing pyruvate to form dicarboxylic acids and alcohols. This synergistic process enables the fermentors to conserve ATP by synthesizing more acetate and less reduced products. In addition, the net products, acetate and H_2, serve as substrates for growth by methanogens and sulfate-reducing bacteria. The result is that carbon and electron flow is directed toward more efficient degradation by the consortium to CH_4 and CO_2.

Another physiological group of microorganisms affected by this process is the H_2-producing acetogens. These microorganisms use partially oxidized polymer products such as fatty acids and alcohols generated by fermentative microorganisms and use protons as electron acceptors. The reactions carried out by these microorganisms for growth are not thermodynamically favorable (i.e., $+\Delta G^{\circ\prime}$ under physiological growth conditions) because the H_2 they generate accumulates and growth is subsequently inhibited. However, in association with H_2-consuming

microorganisms such as the methanogens, the H_2 partial pressure is maintained at levels low enough to make the reaction thermodynamically favorable (i.e., $-\Delta G^{\circ'}$ under physiological growth conditions). Because of their dependence on the H_2-consuming microorganisms, the H_2-producing acetogens are often referred to as obligate syntrophs. The ultimate products of these consortia are H_2S or CH_4 and CO_2. CH_4 is either oxidized by methylotrophs to CO_2 as it diffuses into the aerobic zone, or it enters the atmosphere by mechanisms described below.

Competitive and Noncompetitive Substrates

In marine habitats, there is a hierarchy in the competition for H_2 as an electron donor. Because oxygen is depleted by the activities of aerobic microorganisms, an anaerobic region occurs immediately below the sediment surface if the activities of the aerobic microorganisms exceed the rate of oxygen diffusion. An anaerobic zone can even extend into the water column in regions with high organic loading. Once anaerobic, sulfate reducers and methanogens become the predominant competitors for H_2 generated by fermentative and acetogenic bacteria. In marine sediments with limited organic loading, the sulfate-reducing bacteria outcompete the CO_2-reducing methanogens by virtue of their ability to use hydrogen and acetate at lower concentrations than the methanogens (20–23). Sulfate-reducing bacteria have an apparent K_m and H_2 threshold as low as 250 and 0.9 Pa, respectively, compared with methanogens that have an apparent K_m and H_2 threshold as low as 670 and 3 Pa, respectively. At a typical H_2 threshold concentration maintained at 1 Pa by a sulfate-reducing bacterium, a methanogen would be unable to use H_2 for growth and methanogenesis. In the marine environment, in which the sulfate concentration in seawater exceeds 0.02 molar and organic nutrients are typically limited, sulfate reducers outcompete methanogens. However, in marine habitats with high organic loading, such as elevated coastal marshes, eutrophic coastal waters and submarine trenches, the high rate of hydrogen production by fermentative and acetogenic bacteria can cause the rate of SO_4^{-2} reduction to exceed the rate of SO_4^{-2} diffusion from the water column (24,25). In these environments, sulfidogenesis and methanogenesis occur concurrently.

Methanogens also generate CH_4 from methanol, methylated amines, and thiols, which are readily available in the oceans as metabolites of marine fauna and flora (Table 2). Methanol results from bacterial degradation of pectin from marine plants. Methylated amines are available from decomposition of choline and creatine or by bacterial reduction of glycine betaine and trimethylamine N-oxide, which are osmolytes excreted by marine plants and animals. The thiol dimethylsulfoxide, an osmolyte synthesized by marine macroalgae, phytoplankton, and cyanobacteria, is converted to the methanogenic substrate dimethylsulfide by bacterial reduction. Although there is evidence that some of these compounds are utilized by sulfate-reducing bacteria, they support methanogenesis in habitats that contain high SO_4^{-2} concentrations (26).

Marine Sediments

The flow of carbon in anaerobic marine sediments is shown in Figure 5. Fermentative bacteria that synthesize hydrolytic enzymes such as cellulases, proteases, amylases, and lipases catalyze degradation of complex polymers to soluble monomers. The fermentative bacteria then ferment the soluble products to H_2, CO_2, simple alcohols, and fatty acids, including significant generation of acetate due to interspecies H_2 transfer. The H_2-producing acetogenic bacteria, the second consortium group, catalyze the oxidation of alcohols and fatty acids to H_2, CO_2 and acetate. The third group, the methanogenic *Archaea* and sulfidogenic bacteria, use the simple substrates H_2, CO_2, and acetate generated by the fermentative and acetogenic bacteria, to generate CH_4 and H_2S, respectively. In habitats with limiting amounts of organic substrates, sulfidogenesis is the predominant terminal process; in eutrophic habitats, methanogenesis is predominant (27). Although the acetate-utilizing methanogens *Methanosarcina* and *Methanothrix* have been identified from marine methanogenic enrichments, isotope studies performed in sediment suggests that most of the acetate is oxidized by a H_2-producing syntroph rather than by splitting to CH_4 (28). The net result of this marine consortium is that carbon and electrons are directed toward the synthesis of CH_4 or H_2S and CO_2, which then reenter the global carbon cycle.

Table 2. Reactions and Free Energy Yields from Methanogenic Substrates

Substrate	Reaction	$\Delta G^{\circ'}$ (kJ/mol CH_4)
Carbon monoxide	$4CO + 5H_2O \rightarrow CH_4 + 3HCO_3^- + 3H^+$	−196
Formate	$4HCOO^- + 4H^+ \rightarrow CH_4 + 3CO_2 + 2H_2O$	−145
Hydrogen/carbon dioxide	$4H_2 + HCO_3^- \rightarrow CH_4 + 3H_2O$	−135
Ethanol[a]	$2CH_3CH_2OH + HCO_3^- \rightarrow 2CH_3COO^- + H^+ + CH_4 + H_2O$	−116
Hydrogen/methanol	$CH_3OH + H_2 \rightarrow CH_4 + H_2O$	−113
Methanol	$4CH_3OH \rightarrow 3CH_4 + HCO_3^- + H_2O + H^+$	−105
Trimethylamine[b]	$4CH_3NH^+ + 9H_2O \rightarrow 9CH_3 + 3HCO_3^- + 4NH_4 + 3H^+$	−76
Dimethylsulfide[c]	$2(CH_3)_2S + 3H_2O \rightarrow 3CH_4 + HCO_3^- + 2H_2S + H^+$	−49
Acetate	$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$	−31
Pyruvate	$4CH_3COCOOH + 2H_2O \rightarrow 5\ CH_4 + 7CO_2$	−31

[a] other short chain alcohols utilized include propanol, isopropanol, butanol, cyclopentanol
[b] other methylated amines utilized include methylamine, dimethylamine, dimethylethylamine
[c] other methylated sulfides utilized include methylmercaptan, methylmercaptopropionate

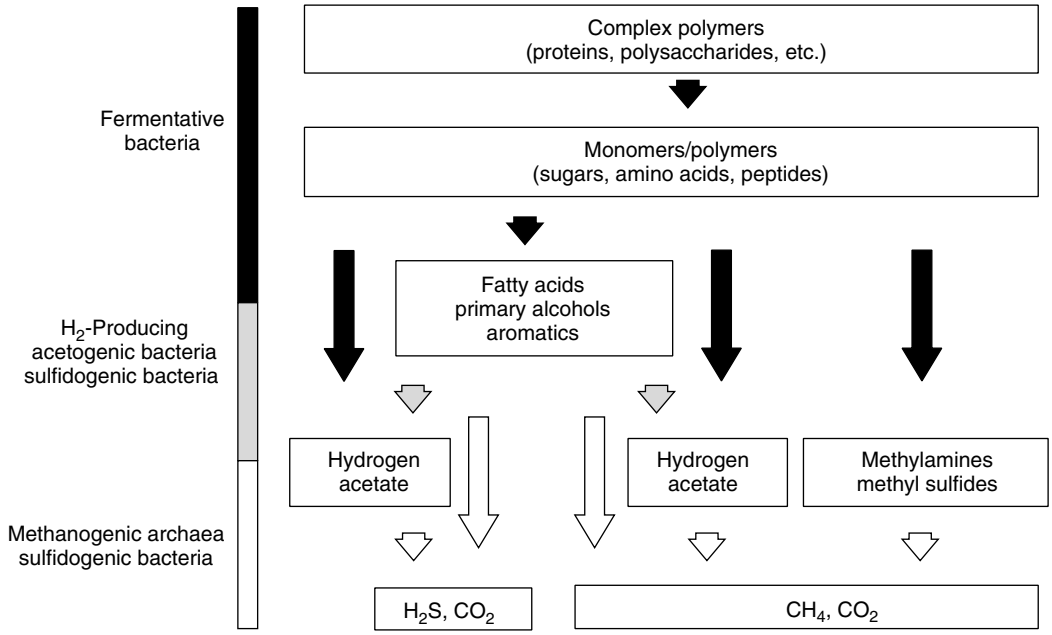

Figure 5. Carbon flow in a marine methanogenic consortium.

Hydrothermal Vents

Marine hydrothermal vents are regions in oceans where hot basalt and magma near the sea floor cause the seabed to slowly fragment (29). Seawater seeps into the fissures, mixes with hot minerals and is emitted from the vents as mineral-rich plumes. The types of vents range from warm vents that emit hydrothermal fluids above ambient temperatures to hot vents known as "black smokers" because they emit a characteristic black cloud as metal sulfides and other minerals in the 270 to 380 °C superheated plume precipitate upon mixing with the cooler seawater. Although reports on the stable isotope ratios of CH_4 ($^{13}C/^{14}C$) indicate that most methane generated from several hydrothermal vents is from geothermal decomposition of organic matter (30), several hydrogen-utilizing hyperthermophilic methanogens have been isolated near hydrothermal vents, possibly using geochemically produced H_2 that is released from the plume along with other reduced inorganic compounds or generated by bacterial decomposition of organic material in the surrounding vent community. The methanogenic *Archaea* and other lithotrophs that use hydrothermal inorganic materials serve as primary producers in this deep-sea nonphotosynthetic food chain. These microbes may be used directly as food by plankton and larger filter feeders that populate the vents. Dissolved geochemical and biogenic methane that remains in the vicinity of the vent can be utilized by methane-oxidizing bacteria associated with bivalves and sponges (31,32). The methanotrophic bacterial symbionts enable these nonfilter feeding animals to survive in the vent communities.

Ocean Water Column

Dissolved methane in the open ocean water column frequently has subsurface concentrations that are greater than the surface concentrations, which indicates that methane is generated within the water column (33). Although methane concentrations in shallow eutrophic coastal waters would be expected to be supersaturated as a result of methanogenic activity in sediments, supersaturated methane gradients in deep open waters cannot be explained by advection from submarine point sources such as anoxic sediments and abiotic hydrocarbon seeps. The only known source of methane in the open water is by methanogenic activity. It has been postulated that methane is produced in oxygenated ocean waters from the digestive tracts of fish and zooplankton, which provide anaerobic microniches for the obligately anaerobic methanogens (33). Methanogens have also been identified in close association with phytoplankton and aggregations of planktonic organic particles known as marine snow in the water column (34). Biogenic methane associated with the particles is exchanged with the water column as the particles sink causing it to become supersaturated with methane (35).

BIOTECHNOLOGICAL APPLICATIONS OF MARINE METHANOGENESIS

Natural Products

Species of methanogenic *Archaea* have adapted cellular mechanisms that enable them to survive and proliferate in marine temperature extremes ranging from sub zero in the Arctic and Antarctic oceans to near boiling in submarine hydrothermal vents. Archaeal proteins adapted to function in extreme temperatures could be a potential boon to industrial processes. Restriction endonucleases with unique recognition sequences from *Methanococcus aeolicus* have been marketed (36). As demonstrated by the commercial development of the DNA polymerases Vent™ and DeepVent™, archaeal enzymes can have

unique characteristics such as a lower error rate than conventional Taq polymerase and the ability to produce blunt end-products that can be readily ligated into any cloning vector with a blunt-end restriction site (37). As more hyperthermophilic marine vent methanogens are characterized, additional enzymes with unique features may be discovered. Hyperthermostabile proteases from methanogenic vent species have potential applications in high-temperature detergents. Only one psychrotolerant and one psychrophilic species have been described, but as additional cold-adapted methanogens are characterized, enzymes applicable to cold temperature industrial processes may also be discovered (38,39).

Archaeal bipolar lipids, synthesized by several species of marine methanogens, have unique properties because of their exceptionally high mechanical and chemical stability (40). As a consequence, the lipids have a natural resistance to oxidation and esterase (because of their ether glycerol linkage) and can be used to make liposomes that are stable over a broad range of conditions, including temperature and pH. This stability and characteristic low permeability to molecules and ions make these lipids potentially useful as vesicles in drug delivery systems and novel membranes for separation processes. Because of the thermostability of lipids from hyperthermophilic marine methanogens, the liposomes can be autoclaved to remove microbial contaminants.

Other unique biosynthetic products of the marine methanogens include small organic molecules produced as osmoprotectants (41). Unlike the extremely halophilic *Archaea*, which synthesize proteins that require high intracellular salt concentrations similar to their environment, all marine methanogens isolated thus far are halotolerant and maintain an intracellular milieu with a salt content lower than the environment. Because their proteins are denatured by salt concentrations found in seawater, these cells must remain equiosmotic with their environment by the synthesis of small organic molecules known of osmoprotectants. These molecules are often neutrally charged to minimize denaturing of proteins and often have a structure that is different from homologs used for biosynthesis to minimized perturbation of biosynthetic pathways. Both these characteristics enable marine methanogens to accumulate high intracellular concentrations of these compounds in response to the relatively high solute concentration of seawater. Some of the unique methanogen osmoprotectants include β-amino acids such as β-glutamate, β-glutamine, and N^{ε}-acetyl-β-lysine, and the phosphorylated inositol, di-*myo*-inositol-1,1'-phosphate. Besides the synthesis of these unique compounds, the biosynthetic pathways for their synthesis may be useful for in vitro biotechnological production of biopolymers.

Bioremediation

Many industrial wastes contain high concentrations of salts that can inhibit methanogenic digestors containing "freshwater" inoculum. Consortia of marine bacteria and methanogenic *Archaea* are already adapted for growth at greater solute concentrations. Because many species of marine methanogens have the ability to adapt to fluctuating solute concentrations by intracellular osmoregulation, digestors containing marine inocula are less likely to stall as a result of changes in effluent salt concentrations (42).

Marine methanogenic consortia can also degrade halogenated alkane hydrocarbons, and individual species of methanogens have been reported to dehalogenate brominated alkanes such as brominated benzoate and phenols (43). Because halogenated compounds are prevalent in the marine environment as a result of biosynthesis by a variety of marine plants and animals, this environment may be a virtually untapped source of naturally dehalogenating consortia and species for halogen waste treatment.

Alternative Energy and Chemical Sources

Marine plant biomass is a virtually unexploited renewable resource for biosynthesis of fuel and industrial chemicals. The higher water and soluble sugar content of marine biomass such as macroalgae, relative to the composition of terrestrial plants, make these resources amenable to relatively rapid rates of methanogenesis and high-energy yields by methanogenic consortia. In addition, species such as the giant marine brown kelp *Macrocyctis pyrifera* have rapid growths rates of up to 0.6 meters day^{-1}. As a result, this species could be exploited as a renewable "energy sink" for harvesting solar energy as plant biomass for microbial conversion to methane without a requirement for arable land. Another advantage of marine methane biogenesis is that microbial consortia occur naturally that can convert plant biomass to methane at efficiencies greater than 90% (Fig. 6). As an end-product for biomass conversion, the low solubility of methane in water facilitates its collection from an aqueous environment such as a fermentor and it can be transported directly as a fuel in the existing infrastructure for distributing natural gas. Furthermore, methane can be used as a precursor for biosynthesis of a variety of solvents and industrial chemicals by methanotrophic bacteria. Because depleted reserves will increase the cost of processing petroleum-based fuels and

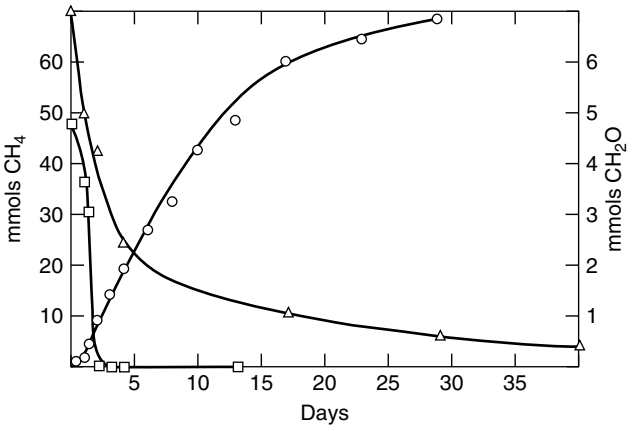

Figure 6. Methane generation catalyzed by a kelp-degrading microbial consortium in a methanogenic digestor. Symbols: soluble algal sugars alginate (△) and mannitol (□); methane (○). On the basis of data from (45).

chemicals in coming decades, synthesis of fuel and solvents from biogenic hydrocarbon sources such methane has the potential to become economically viable.

CONCLUSION

Marine methanogensis is less understood than in other habitats such as freshwater sediments, digestors, and ruminants. This is largely due to the general misperception that methanogenesis does not have a significant role in the marine environment. First, the apparent role of marine methanogenesis in the global carbon cycle is frequently underestimated because much the product synthesized by this process, methane, is oxidized as part of a microbial food chain before it can measured. Second, in many environments, sulfate reduction can outcompete methanogens for limited substrates. However, marine methanogens compete successfully in marine habitats that contain excess substrate or are sulfate depleted, which include eutrophic coastal sediments, fish, and plankton digestive tracts and in planktonic organic particles. Thus, they have specific ecological roles in the marine carbon cycle. Marine hydrogenotrophic methanogens also have a unique role as primary producers in hydrothermal vent communities that exist in the total absence of light. Methanogens isolated from marine sediments exhibit a wide range of phylogenetic diversity and 16S rRNA molecular analysis indicates that methanogenic species are ubiquitous throughout the oceans and coastal regions. Because only a small portion of these *Archaea* have been described, the oceans represent a largely untapped reservoir for natural products and processes generated by the marine methanogens.

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METHANOGENS.

See Trace Gases Soil

METHANOGENS: BIOTECHNOLOGICAL APPLICATIONS.

See Methanogenesis in the Marine Environment

METHANOGENS IN PETROLEUM RESERVOIRS.

See Petroleum Reservoirs, Influence, Activity and Growth of Subsurface Microflora in

METHANOGENS IN SOILS.

See Soil Bacteria

METHANOTROPHIC BACTERIA

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Methanotrophic bacteria, also called *methanotrophs*, are aerobic prokaryotic microorganisms that oxidize methane for energy and growth. They are ubiquitous in nature, living in any location where air and methane are simultaneously present. Habitats include soil, sediment, wetland, lakes, and marine systems that have distinct anaerobic–aerobic interfaces where anaerobic methane production occurs on one side of the interface and methane oxidation occurs on the other side. More recent work has shown that "high-affinity" methanotrophs, which consume methane at atmospheric methane levels (~1.7 parts per million by volume), are also present in many locations in the environment where no clear methane enrichment exists. Methanotrophic bacteria are of great ecological significance because they play a major role in the global carbon cycle (1–5). Additionally, their activity in nature significantly reduces "greenhouse gas effects" by mineralizing methane, a gas that absorbs ultraviolet wavelengths more strongly than carbon dioxide (2,6–8). They have also been shown to have many biotechnological applications, such as contaminant transformation (9) and chemical product formation in biochemical engineering (10).

A number of excellent review articles exist on methanotrophic bacteria. Hanson and Hanson (11) provided an overview of methanotroph taxonomy, the role of methanotrophs in global carbon cycling, methanotroph ecology, and a summary of their contaminant transformation capabilities. Lidstrom and Stirling (12), Murrell (13), Murrell and coworkers (14), Murrell and coworkers (15), and Murrell and Radejewski (16) published further reviews on the molecular biology of the organisms and the use of molecular methods for studying methanotroph ecology. Methanotroph biochemistry was summarized in various publications, including Colby and coworkers (17), Higgins (18), Anthony (19), Lidstrom (20), and Lipscomb (21), with recent emphasis on the enzymology of soluble (sMMO) and particulate (pMMO) methane monooxygenases, the enzymes responsible for methane oxidation in these organisms.

Because of the extensive existing literature on these important organisms, this article will concentrate on recent results, especially associated with the molecular ecology of methanotrophs, their role in the nitrogen cycle, and the biochemistry of monooxygenases and related enzymes, although a general background will also be provided for completeness. The article is divided into four major topics including methanotroph classification systems (phenotypic and genotypic), methanotroph biochemistry and molecular biology, microbial ecology of methanotrophs, and biotechnological applications.

CLASSIFICATION OF METHANOTROPHS

Methanotrophs belong to a broad group of microorganisms known as *methylotrophs*. Methylotrophic bacteria can use as their sole source of carbon and energy reduced carbon substrates with no carbon–carbon bonds (20). There are two traditional subgroups of methylotrophs, namely, obligate and facultative. Obligate methylotrophs grow only at the expense of compounds without carbon–carbon bonds, and all methanotrophs belong to this subgroup. Facultative methylotrophs grow on a variety of carbon sources that include C_1 compounds; however, they cannot use methane for growth (22). Table 1 summarizes growth and cometabolic substrates used by methylotrophic bacteria.

A second more recent subgrouping for methanotrophs, low-affinity and high-affinity organisms (where "affinity" refers to their K_S for methane consumption), has been developed on the basis of results from ecological studies (8,11). The discovery of distinct high-affinity organisms has resulted from studies on ecosystems that have no methane enrichment other than atmospheric methane. Because very little is currently known about high-affinity methanotroph species, descriptions presented here largely are associated with low-affinity organisms.

Known methanotrophs are strictly aerobic, catalase- and oxidase-positive, and possess cytochromes *c*, *a*, and either *o* or *b*. With the exception of one gram-positive

Table 1. Substrates of Methylotrophic Bacteria*

Growth Substrates	Examples of Cometabolic Substrates
Methane, methanol, methylamine, dimethylamine, trimethylamine, tetramethylammonium, trimethylamine N-oxide, trimethylsulfonium, formate, formamide, carbon monoxide, dimethyl ether, dimethyl carbonate, dimethyl sulfoxide, dimethylsulfide	Ammonia, ethylene, chloromethane, bromomethane, higher hydrocarbons (ethane, propane, trichloroethylene, etc.)

*At least one methylotroph has been reported to oxidize each compound presented.

microorganism (*Mycobacterium* species ID-Y), all methanotrophs are gram-negative (20). All methanotrophs possess intracytoplasmic membranes (ICMs) and are able to form cysts, exospores, or lipid cysts as resting stages under adverse environmental conditions. Methanotrophs have a complete pathway for methane oxidation and have the ability to assimilate cell carbon as formaldehyde. All methanotrophs can use ammonia as the sole source of nitrogen; many are capable of using nitrate and nitrite; and, as discussed in the following section, some methanotrophs are capable of N_2-fixation, providing them a significant competitive advantage in some environmental settings (5,11,23–28).

Despite the aforementioned similarities, differences in morphology, fine structure, resting stages, and biochemical pathways have allowed methanotrophs to be classified into three generic groups, namely, types I, II, and X (6,21,24,25,29–31). Some features that distinguish the three major classes of methanotrophs are summarized in Table 2. Six Type I genera (*Methylococcus*, *Methylomonas*, *Methylomicrobium*, *Methylobacter*, *Methylocaldum*, and *Methylospaera*), three Type II genera (*Methylosinus*, *Methylocystis*, and *Methylocella*), and one Type X genus (*Methylococcus*) have been recognized (16). Although the cell size and shape vary within species, Type I methanotrophs generally have rod, coccoid, or ellipsoid shapes, Type II methanotrophs have rod, crescent, or pear shapes, and the Type X *Methylococcus capsulatus* (Bath) has a coccoid shape. Most isolated methanotrophs grow optimally at approximately neutral pH and at a temperature near 30 °C, whereas some methanotrophs, including *M. capsulatus* (Bath) and a few recent isolates, can grow under more extreme conditions (29,30,32–36).

Some strains of Type I and X form cysts with a fine structure that is similar to that formed by *Azotobacter*, whereas Type II methanotrophs form either exospores or lipid cysts with some exospores being resistant to pasteurization (37–41). Some Type II methanotrophs form lipid cysts, or poly-β-hydroxybutyrate structures, during periods of excess methane. These storage polymers can make up to 20 to 25% dry mass and are used as a source of reducing power under methane-limiting conditions (42). The formation of rosettes at the end of the exponential phase of growth is characteristic of some Type II species, including *Methylosinus trichosporium* and those of genus *Methylocystis* (31,38).

Other characteristics that provide fingerprints of the different methanotroph phenotypes include the mole percentage of guanine and cytosine (G + C) in the DNA, major extracted phospholipid fatty acids (PLFAs), and the type of intracytoplasmic membrane (ICM) structure. Generally, the mole percentage G + C is higher in Types II and X than in Type I methanotrophs with reported values between 62 to 67% and 59 to 65%, respectively. Type I methanotrophs, on the other hand, have been reported to contain between 49 to 60 mol% G + C (43,44). Types I and X methanotrophs group similarly in terms of PLFAs, with those containing 16 carbons being the most predominant, whereas Type II methanotrophs have a predominance of 18-carbon PLFAs (45–53). Although C-16 and C-18 PLFAs are in larger percentage in Types I and II methanotrophs, respectively, the specific C-16 and C-18 fatty acids that are most dominant vary among the various genera of each. The ICM structure of Type I and Type X genera has been observed as vesicular disk-shaped bundles stacked throughout the center of the cell, whereas the ICM structure of Type II genera consists of paired membranes that are parallel to the cytoplasmic membrane or extend throughout the cytoplasm.

Methane monooxygenase (MMO) is the first enzyme involved in methanotroph catabolism. In general, type II and type X organisms express two forms of MMO, a soluble or cytoplasmic form (sMMO) and a membrane-associated particulate form (pMMO). Alternatively, Type I organisms, with one exception, express only pMMO. The biochemistry of sMMO and pMMO and the regulation of expression between the two MMOs are central to most studies of methanotrophs and will be presented in detail later. The existence of a complete TCA cycle and associated enzymes also has been used to distinguish between methanotrophic types. With the exception of one Type I methanotroph (54), only Type II methanotrophs have a complete TCA cycle. However, all groups of methanotrophs possess isocitrate dehyrogenase, and their need for NAD^+ versus $NADPH^+$ (or both) is a useful means of separating different bacteria into the types (Table 2; 25). Although all methanotrophs assimilate carbon at the formaldehyde level, Type I strains use the ribulose monophosphate pathway (RuMP) and Type II strains use the serine pathway. Type X methanotrophs possess enzymes for both pathways, 3-hexulose phosphate synthase and hydroxypyruvate reductase, as shown in Table 2. The ability to fix molecular nitrogen (and express nitrogenase) is a characteristic feature of Types II and X methanotrophs, whereas only Type X *M. capsulatus* (Bath) has been shown to express ribulose-bisphosphate carboxylase, the enzyme responsible for catalyzing autotrophic carbon dioxide fixation (25).

Phylogenetic analyses using 16S and 5S rRNA sequence techniques have provided valuable information on the relationships between methanotrophic bacteria

Table 2. Characteristics of Methanotroph Types (6,11,20,25)

Characteristic	Type I	Type II	Type X
Recognized genera	*Methylococcus* *Methylomonas* *Methylobacter* *Methylomicrobium* *Methylocaldum* *Methylospaera*	*Methylosinus* *Methylocystis* *Methylocella*	*Methylococcus*
Cellular Shape	Short rods, some cocci or ellipsoids	Rods, crescent- or pear-shaped	Cocci
Growth:			
• 45 °C	−	−	+
• pH (optimum)	7	7	7
• Resting stages	*Azotobacter*-type cysts	Exospores or "lipid" cysts	*Azotobacter*-type cysts
• Rosette formation	No	Yes	No
G + C content (mole %)	50–55	62.5	62.5
Predominant phospholipid fatty acids	16	18	16
Membrane type:			
• Vesicular disk-shaped bundles	+	−	+
• Paired membranes at periphery	−	+	−
Complete TCA Cycle	−	+	−
Carbon assimilation	RuMP*	Serine	RuMP/Serine
Key Enzymes:			
• Methane monooxygenases	Usually pMMO* only	pMMO and sMMO*	pMMO and sMMO
• 3-Hexulose phosphate synthase	+	−	+
• Hydroxypyruvate reductase	−	+	+
• Nitrogenase	−	+	+
• Ribulose-bisphosphate carboxylase	−	−	+
• Isocitrate dehydrogenase	NAD^{+}*/$NADP^{+}$*	$NADP^{+}$	NAD^{+}

*RuMP = ribulose monophosphate cycle;
pMMO = particulate methane monooxygenase;
sMMO = soluble methane monooxygenase;
NAD^{+} = nicotinamide adenine dinucleotide;
$NADP^{+}$ = nicotinamide adenine dinucleotide phosphate.

and have verified the taxonomic grouping of these bacteria into the three types (16,25,43,44,55–60). These studies have shown that bacteria using the serine pathway for formaldehyde assimilation (including Type II methanotrophs) are grouped into the α-subclass of *Proteobacteria*. Type I methanotrophs are found within the γ-subclass of *Proteobacteria*, and those methylotrophs that use the RuMP pathway for formaldehyde assimilation but do not use methane, are located in the β-subclass. Figure 1 presents the phylogenetic relationship between different representative methanotrophs (16).

BIOCHEMISTRY AND MOLECULAR BIOLOGY OF METHANOTROPHIC BACTERIA

Overview

The biochemistry and molecular biology of methanotrophic bacteria have been presented in detail elsewhere (11,13,15,19,21,22); therefore, only a summary will be presented here. All methanotrophs oxidize methane to methanol catalyzed by MMO (Fig. 2). NADH is required in this first oxidation step and acts as the electron donor when the reaction is catalyzed by sMMO. pMMO uses a higher potential electron donor, cytochrome *c*, resulting

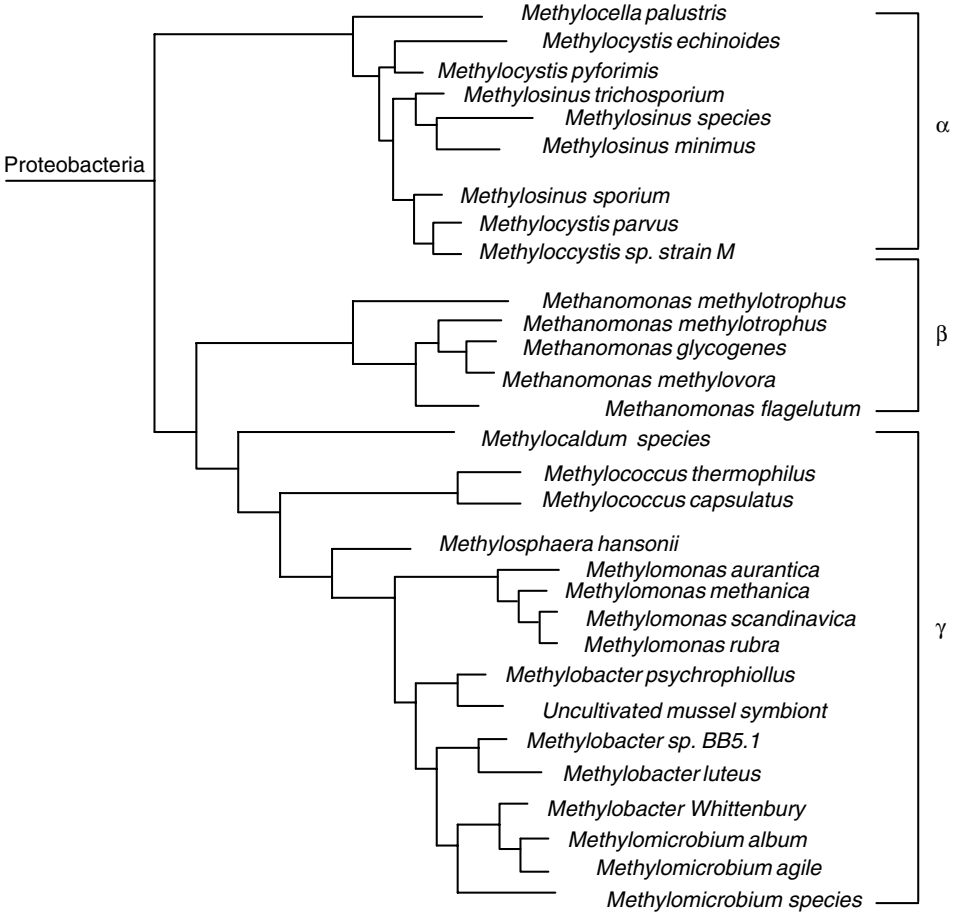

Figure 1. Detailed unrooted phylogenetic tree showing similarity relationship between different types of methanotrophic bacteria with respect to the α-(Type II methanotrophs), β-(nonmethane utilizing methylotrophs employing RuMP pathway), and γ-(type I methanotrophs) subdivisions of the *Proteobacteria* (11,16).

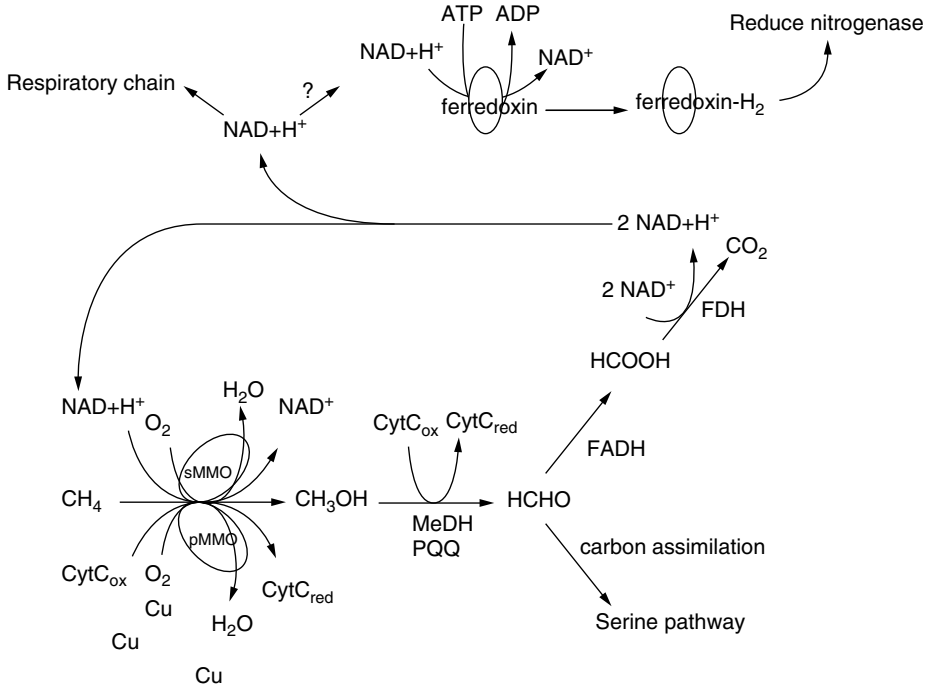

Figure 2. Oxidation of methane to carbon dioxide catalyzed by either pMMO (in the presence of copper) or sMMO (in the absence of copper) in Type II methanotrophs. The oxidation of formaldehyde to carbon dioxide proceeds in two NAD^+-linked steps. One of the NADH formed is invested for the MMO reaction, whereas the other is fed into the respiratory chain or possibly to the nitrogenase system. Carbon assimilation proceeds by the serine pathway and diverges at the level of formaldehyde. Abbreviations: CytC, cytochrome *c*; MDH, methanol dehydrogenase; FADH, formaldehyde dehydrogenase; FDH, formate dehydrogenase; PQQ, pyrrolo-quinoline quinone (11,19,22,63,64).

in higher growth yields as the supply of NADH usually becomes nonlimiting (61,62). Electrons from this first oxidation step are then transferred to the second reaction in which methanol is converted to formaldehyde by a periplasmic methanol dehydrogenase (MDH) along with a novel coenzyme methoxatin, or pyrrolo-quinoline quinone (PQQ) (22,63,64). Cytochrome *c* acts as the electron acceptor as electrons are shuttled from the first reaction by way of the PQQ prosthetic group. Formaldehyde can then either be further oxidized completely to carbon dioxide or diverge from the oxidative pathway to form various intermediates of the metabolic pathway that can subsequently be used to synthesize biomass. This central role of formaldehyde is a common feature of methanotroph metabolism.

In the oxidative pathway, formaldehyde is further oxidized to formate and then to carbon dioxide by formaldehyde dehydrogenase (FdDH) and a NAD^+-dependent formate dehydrogenase (FDH), respectively. The oxidation of formate to carbon dioxide is coupled with the reduction of two NAD^+ molecules that results in the generation of two NADH molecules, in which one of the two is reinvested back into the methane-oxidation reaction. The second NADH molecule is fed into the respiratory chain as a source of reducing power (19,22).

Assimilatory pathways are more diverging with the synthesis of multicarbon compounds from formaldehyde (carbon fixation) proceeding either through the serine-isocitrate lyase pathway (for Type II methanotrophs) or the ribulose monophosphate (RuMP) pathway (for Type I methanotrophs). In the former pathway, 2 moles of formaldehyde and 1 mole of carbon dioxide are used to form 1 mole of acetyl-CoA. In the latter pathway, 3 moles of formaldehyde are used in a complex cycle that ultimately yields pyruvate. Type X methanotrophs appear to possess enzymes from both major pathways.

Many of the aforementioned biochemical pathways are not unique to methanotrophs. A number of methylotrophs that do not use methane employ similar pathways for the generation of reducing power and carbon assimilation. In reality, the unique biochemical trait of methanotrophs is the use of MMO to catalyze the first step in methane oxidation. The goal of this section is to present the current knowledge of properties of sMMO and pMMO and the regulation of the MMO system, to discuss the biochemistry of nitrogen-fixation in methanotrophs, and to briefly review the biochemistry of methanol dehydrogenase because of its relevance in assessing the ecology of methanotrophs in the environment.

Biochemistry of Methane Oxidation

The initial step in methane metabolism, that is, MMO-catalyzed oxidation to methanol, is highly energy-demanding in that two reducing equivalents are required to split the di-oxygen molecule and oxidize methane (19,65). One of oxygen atoms is directly incorporated into methane, whereas the other is reduced to water. Although this initial priming reaction is energetically demanding, it permits the net positive yield of reducing power (NADH) through subsequent reactions in the catabolic pathway (Fig. 2).

As stated previously, two general types of MMO are observed in methanotrophs; Type I methanotrophs express only a pMMO (with one known exception) and Type II organisms and the Type X organism, *M. capsulatus* (Bath), express both a pMMO and a sMMO. The copper-to-cell ratio in the growth environment largely determines whether sMMO or pMMO is expressed in Type II and X methanotrophs (66–69). The regulation of expression of the two MMOs in organisms that express both enzymes is a topic of current intereeview of the biochemist; however, only a superficial understanding of this process exists (15). Current knowledge in MMO expression systems will be presented after a rstry of the sMMO and pMMO.

Properties of sMMO. sMMO is a multicomponent, nonheme iron protein. The enzyme has been highly studied because of its unique biochemistry and its ability to oxidize a large number of environmental pollutants resulting from its broad substrate specificity. The enzyme was first isolated from *M. capsulatus* (Bath) by Colby and Dalton (70). It has subsequently been purified from many other methanotrophs, including from the Type II *M. trichosporium* OB3b (71,72), from *Methylocystis* species M (73), from a bacterium of uncertain taxonomic status of *Methylobacterium* species CRL-26 (74), and, recently, from a Type I strain *Methylomonas methanica* 68–1 (75). All of the purified sMMOs consist of three components: a 245 kDa hydroxylase, containing nonheme iron (component A), a 15.8 kDa regulatory protein (component B), and a 38.4 kDa reductase (component C) (21,71). The genes corresponding to all three of these subunits have been cloned and sequenced from *M. trichosporium* OB3b (76) and *M. capsulatus* (Bath) (77) and have been used for probe construction for sMMO-bearing methanotrophs in natural environments (75,78,79).

The hydroxylase component of sMMO is a dimer of three subunits in an $\alpha_2\beta_2\gamma_2$ arrangement with molecular weights of ~60, 45, and 20 kDa, respectively (15). The active site of sMMO is associated with the dinuclear iron cluster of the hydroxylase subunit and is at a location where oxygen and methane interact to form methanol (71,80). Each of the two $\alpha\beta\gamma$-protomers contains a di-iron cluster, which can exist in three different oxidation states and facilitates the insertion of an oxygen atom into methane through a radical-associated mechanism.

The reductase component of sMMO contains the flavine adenine dinucleotide and a Fe_2S_2 cluster (17). This component transfers electrons from NADH to the active site of the di-iron cluster of the hydroxylase. Component B functions to increase the rate of electron transfer and also to increase the efficiency of coupling substrate oxidation to NADH consumption. Component B also can stop the flow of electrons in the absence of a substrate, which can be hydroxylated (e.g., methane), thus preventing the wasteful oxidation of NADH (81). It is postulated that components B and the reductase subunit are part of a complex regulatory system designed to allow both efficient transfer of electrons and oxygen activation, although simultaneously maintaining a tight coupling of energy used and substrate oxidized (82).

Properties of pMMO. pMMO is not as well characterized as sMMO. pMMO is highly sensitive to oxygen, is easily inactivated on removal from the membranes, and has proven to be difficult to purify and study. Only recently has the enzyme been purified with significant retention of activity observed (83); therefore, less information is available on the pMMO than sMMO. In general, pMMO is a multiple copper-containing enzyme that requires copper for regulation of expression and activity and possibly for coupling to the electron transport system (66,84,85).

The pMMO of *M. capsulatus* (Bath) has three main subunits of ~45, ~27, and 23 kDa (83), with the 45- and 27-subunits probably containing the enzyme-active site. Active pMMO contains 2 iron atoms and 15 copper atoms per mole of enzyme. pMMO also has small copper-binding compounds (CBC) associated with it (86,87). The specific role of the CBC is not known; however, they may play a role in catalysis and enzyme stabilization, act as a copper-sequestering agent associated with enzyme construction and activation, or influence redox conditions around the enzyme (15). The physiological reductant for pMMO is also not known, although cytochromes $b_{559/569}$, c, and c_{553} are possible candidates (15). pMMO has a higher specificity for methane and exhibits higher CH_4-oxidation activities than sMMO (88), thus leading to increased growth yields for organisms expressing pMMO (61,62). pMMO is, therefore, more specific in terms of its ability to oxidize methane, which may partly explain its greater prevalence in nature than sMMO.

Regulation of Expression of the MMO System. Murrell and coworkers (15) recently summarized what is known about the control of sMMO and pMMO expression in Type II and Type X organisms. All evidence suggests that the MMO system is primarily regulated by the copper-to-cell ratio in the growth environment, with low ratios supporting sMMO expression and higher ratios supporting pMMO expression (66). Recent evidence, however, has indicated that oxygen may also play a role in MMO expression and activity, although the exact role of oxygen is unknown (28).

The genes that encode sMMO have been identified and sequenced in various methanotrophs. The genes are all chromosomal and clustered as *mmoX*, *mmoY*, and *mmoZ* for the α-, β-, and γ-hydroxylase subunits, respectively, and *mmoB* and *mmoC* for proteins B and C, respectively (15). An undefined open reading frame, *orfY*, exists between the *mmoY* and *mmoB* (89) (Fig. 3). This gene arrangement appears to be highly conserved between methanotrophs both in terms of amino acid and DNA identities. Active *mmoB* and *mmoC* gene products have been successfully expressed in *Escherichia coli*; however, all attempts to express active hydroxylase subunits have been unsuccessful (90), except in *Methylomicrobium album* BG8 and *Methylocystis parvus* OBBP hosts, which usually possess only a pMMO under low-copper-to-cell ratio growth conditions (91).

Genes associated with pMMO have only recently been cloned and sequenced in one methanotroph, *M. capsulatus* (Bath) (92), although preliminary information on the molecular genetics of pMMO was identified earlier (84).

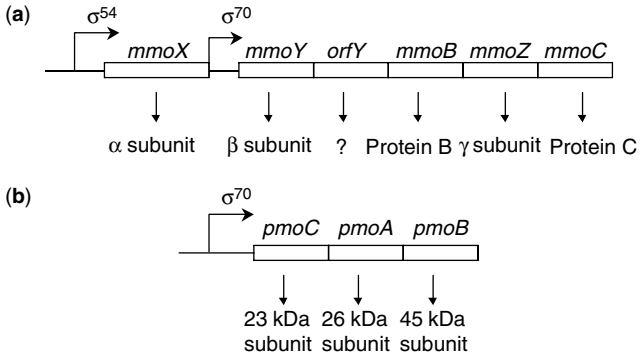

Figure 3. Operon regions for (**a**) sMMO and (**b**) pMMO and their gene products in some methanotrophic bacteria such as *M. capsulatus* (BATH) and *M. trichosporium* OB3b. Under copper-limited conditions, the sMMO genes are expressed from two transcriptional start sites, directed by the two (a σ^{54}-like and σ^{70}) promoters. The product of the open reading *Y* is unknown. Under high copper-to-biomass ratios, the pMMO gene cluster is expressed from a single transcriptional start site directed by σ^{70} (15,84,92).

Semrau and coworkers (84) reported that methanotrophs contained multiple copies of the genes encoding the 45 and 269 kDA subunits (identified as *pmoB1* and *pmoA1*, respectively) and were found to be in the order *pmoA1-pmoB1* on the chromosome. Nguyen and coworkers (85) further showed that the gene encoding the 23 kDa subunit (*pmoC*) was located upstream of *pmoA1*. Stolyar and coworkers (92) showed that the complete gene cluster *pmoCAB* (Fig. 3) was present in *M. capsulatus* (Bath). In fact, two almost identical copies of *pmoCAB* were found in *M. capsulatus* (Bath), and a third separate copy of *pmoC* was also identified. This gene cluster was found to be very similar to the equivalent gene cluster in ammonia-oxidizing bacteria (AOB) that express an ammonia monooxygenase (AMO), suggesting that pMMO and AMO might be evolutionarily linked (93–95). The *pmoCAB* gene arrangement has recently been observed in *M. trichosporium* OB3b and in *Methylocystis* sp. Strain M (96), which suggests that it may be common among other methanotrophs.

Nielsen and coworkers (97) used Northern Blotting and primer extension analysis to show that a full 5.5-kb transcript encoding the entire sMMO operon was expressed only under low-copper growth conditions in *M. capsulatus* (Bath). Subsequent experiments using *M. trichosporium* OB3b provided similar results and showed that the addition of 50 μM copper sulfate fully repressed the expression of the sMMO-related transcript (98). Neilson and coworkers (98) further showed that three major sMMO-related transcripts were observed in *M. trichosporium* OB3b, including *mmoX* only, *mmoY*, *mmoB*, and *mmoZ*, and *mmoY*, *mmoB*, *mmoZ*, *orfY*, and *mmoC*, and they also identified a σ^{54}-like promoter sequence upstream of the *mmoX* gene. Sigma factors (e.g., σ^{70}, σ^{54}, σ^{32}, etc.) are proteins that join with RNA polymerase to allow the enzyme to bind to specific DNA promoter regions. The presence of a σ^{54}-like promoter sequence upstream of *mmoX* gene indicates that the

expression of *mmoX* may be regulated by σ^{54}, a protein that is usually associated with the transcription of nitrogen-regulated genes (discussed later). A similar promoter sequence was found in *Methylocystis* sp. Strain M (89) and in *Methylomonas* sp. KSWIII (99).

A putative σ^{70} promoter sequence has also been identified between *mmoX* and *mmoY* in *M. trichosporium* OB3b, suggesting that sMMO expression may be actually regulated at multiple points. Associated with the repression of sMMO expression and activity after exposure to copper, *pmo* transcripts of 1.2 and 4.0 kDa have been noted in various methanotrophs (15). Evidence suggests that these are transcripts of *pmoCAB* and that they are transcribed from a single start site upstream of *pmoC* initiating at a putative σ^{70} promoter that is negatively regulated under low-copper conditions.

The exact mechanism of the coregulation of sMMO repression and pMMO expression is not yet understood. Various options are being considered, although the presence of σ^{54}-like promoter sequence upstream of the sMMO gene cluster indicates that activator proteins probably play an important role in sMMO repression and pMMO activation in the presence of copper (15). The presence of a σ^{54}-like promoter sequence upstream of the sMMO gene cluster might also explain the observed constitutive nitrogenase expression and activity associated with constitutive sMMO expression in *M. trichosporium* OB3b $sMMO^{C}$ mutants (28).

Nitrogen-Fixation in Methanotrophs

Nitrogen-fixation systems in methanotrophs have not been studied to the extent as MMO systems because early investigations suggested that methanotrophs had a nitrogenase system that was similar to other, well characterized systems (26,77,100,101). Nitrogen-fixation is highlighted because it has been shown that methanotroph ecology is strongly influenced by nitrogen-supply conditions and recent results have suggested that nitrogen fixation in Type II organisms may be coregulated with the MMO systems, and, as such, deserves special attention.

Nitrogen-fixation on all organisms is catalyzed by nitrogenase, a multicomponent enzyme complex. The enzyme catalyzes the reduction of molecular nitrogen (N_2) to ammonia (NH_4^+) in the presence of ATP and an electron source according to the equation (1):

$$N_2 + 10H^+ + 8e^- \longrightarrow 2NH_4^+ + H_2 \qquad (1)$$

This reaction requires the hydrolysis of 16 moles of $MgATP^{-2}$ to $MgADP^-$ and P_i^- (per mole of N_2) making it a very energy-demanding process (102). As a result, organisms must tightly regulate the synthesis of nitrogenase and its subsequent activity to prevent wasteful use of energy when other soluble nitrogen sources are available. Furthermore, because of the extreme sensitivity of nitrogenase to oxygen, N_2 fixation must also be regulated in response to extracellular oxygen levels. Synthesis and activity of nitrogenases in methanotrophs (and in other aerobic diazotrophs) must therefore be carefully regulated in response to the interrelationship between both nitrogen and oxygen. Adding to the complexity of nitrogenase regulation in *M. trichosporium* OB3b, there is evidence that sMMO genes may be expressed from the same promoter that controls nitrogenase expression under low-copper growth conditions (13). Thus, it appears that the regulation of the nitrogenase system in *M. trichosporium* OB3b is complex and might serve as an excellent model for studying the regulation of multiple genes from a single promoter.

Biochemistry of Nitrogen Fixation in Methanotrophs. Nitrogenases from methanotrophs have not been purified to homogeneity (26). However, there is genetic evidence that its protein components are homologous to other characterized nitrogenases. *nifH* gene probes containing universal sequences coding for the structural genes of the Fe protein have been successfully used to screen 13 methanotroph strains. All Type II strains, including *M. trichosporium* OB3b, and the Type X *M. capsulatus* (Bath), were found to contain homologous gene sequences (101) indicating that at least the structural polypeptides of the Fe protein in some methanotrophs are similar to nitrogenases from other diazotrophs. Both strains exhibited the typical sensitivity to oxygen as indicated by the characteristic bell-shaped curves for nitrogenase activity to increasing O_2 tension (26,27,100), although *M. trichosporium* OB3b appears to be more tolerant to oxygen than the Type X strain (100). It seems that *M. trichosporium* OB3b can modify its Fe protein in the presence of elevated O_2 levels, which can permit aerotolerant nitrogen-fixation (28).

Although little structural information is available on methanotroph nitrogenases, some information is available on enzyme function and regulation. For example, in addition to oxygen, fixed nitrogen sources, such as nitrate and ammonia, inhibit nitrogenase expression and activity in methanotrophs so far studied. Both *M. trichosporium* OB3b and *M. capsulatus* (Bath) exhibit the ammonia switch-off response similar to other free-living diazotrophs, although the mechanism of this response appears to differ from other diazotrophs (100,103). Recent results have further shown that *M. trichosporium* OB3b $sMMO^{C}$ mutants also express nitrogenase constitutively, relative to oxygen, whereas fixed nitrogen sources repress nitrogenase in these organisms (28). This indicates that nitrogenase expression and activity might be coupled with sMMO expression and activity (although the basis is unknown); however, the coupling appears to be related to oxygen rather than nitrogen influences.

Most nitrogenases consist of two proteins, namely, a molybdenum-iron (MoFe) protein, an approximately 220-kD protein of subunit structure ($\alpha_2\beta_2$) that contains iron and molybdenum, and an Fe protein, an approximately 64-kD dimer of identical subunits that contains iron. Recently, two Mo-independent nitrogenases have been identified and purified from the *Azotobacter* bacterium, although there is circumstantial evidence that these enzymes are also present in other N_2-fixing organisms. The first of these nitrogenases, often called *V-nitrogenase* or *nitrogenase 2*, contains vanadium instead of molybdenum (104,105), whereas the other nitrogenase, called the *alternative nitrogenase*, or *nitrogenase 3*, contains only trace amounts of either molybdenum or vanadium (106–109). It is

presently unknown whether methanotrophs possess either of these two Mo-independent nitrogenases, although studies are currently under way (28).

Because of the extreme stability of the N_2 molecule resulting from its triple bond, nitrogen fixation is an energy-intensive process. As a result, N_2 fixation requires a continuous supply of two factors in addition to nitrogen and nitrogenase, namely, a source of electrons with low redox potential and ATP. In methanotrophs, there is evidence that the supply of ATP is supplemented by recycling the hydrogen generated during nitrogen fixation in which its oxidation is coupled to ATP synthesis (64). With respect to the supply of electrons, it is suspected that they are supplied by NADH generated from the oxidation of formate, which is coupled to the reduction of nitrogenase through a NADH-ferredoxin reductase (110). This electron transport system appears to be specific for methanotrophs. The supplied electrons are transferred to the electron carrier ferrodoxin, a [4Fe-4S]-containing protein that transfers an electron to the Fe protein of nitrogenase, initiating the nitrogen-fixation process (see Fig. 2). The electron is then passed to the MoFe protein as two molecules of ATP are hydrolyzed, which superreduces the MoFe protein and binds nitrogen. The actual nitrogen reduction occurs on this superreduced MoFe protein in three discrete steps, each involving a pair of electron transfers that reduces nitrogen stepwise to ammonia.

Regulation of Nitrogenase Expression and Activity. The regulation of nitrogenase expression and activity in diazotrophs is very elaborate and involves the regulation of transcription of many linked genes (111). All nitrogenase expression systems studied thus far are controlled by interrelationships between at least oxygen, extracellular fixed nitrogen, and ADP supply levels. In methanotrophs, the role of copper in the regulation of the MMO system, especially in Type II and X organisms, further complicates nitrogenase expression and activity. It is not surprising that copper might impact a nitrogenase expression because copper is frequently associated with antioxidant enzymes, such as superoxide dismutase (SOD), and copper itself has significant antioxidant capabilities (112,113).

All nitrogen-fixing organisms studied thus far control nitrogen fixation at the transcription level of the nitrogen-fixation (*nif*) genes (114). Some regulation also occurs posttranslationally in certain organisms. The genetics of this regulation has been studied extensively in the facultative anaerobe *Klebsiella pneumoniae*. In general, the *nif* gene cluster is subject to regulation through the nitrogen-regulation (*ntr*) system, which involves the products of three genes *ntrA*, *ntrB*, and *ntrC* and, in turn, is regulated by various activators and repressors, such as fixed nitrogen source, oxygen, and ATP-to-ADP ratios. Genetic and molecular analyses of the three nitrogen-regulatory genes (*ntr*A, *ntr*B, and *ntr*C) in methanotrophs by DNA-complementation analysis with *ntr* genes from *K. pneumoniae* has suggested that nitrogen metabolism in methanotrophs is regulated by a similar global *ntr* system as found in most of the other nitrogen-fixing organisms (114). Further, the gene product of the *nifH* gene (the Fe protein) of *Rhodospirillum rubrum* was successfully used to monitor nitrogenase expression in *M. trichosporium* OB3b, suggesting that structural genes in methanotrophs may also be similar to other diazotrophs (28). The product of the gene *ntrA* is a sigma factor (σ^{54}) that recognizes specific *ntr*- and *nif*-promoters that also may recognize promoters responsible for sMMO gene expression under low-copper growth conditions (13,89,98,99). In the presence of a σ^{54}, RNA polymerase interacts with genes containing *ntr*- and *nif*-type promoters, but transcription of these genes requires positive activation by the general nitrogen regulatory protein NtrC (the product of *ntrC*) or by the *nif*-specific activator NifA (the product of *nifA*). The expression of these activating proteins is controlled by various factors, such as the availability of fixed nitrogen (notably NH_4^+) and, in many diazotrophs, by oxygen (115). Kim and Graham (28) provided suggestive data that the uncoupling of the effect of copper on sMMO expression also uncouples the effects of oxygen on nitrogenase expression. This observation may aid in better understanding the central regulation of MMO and nitrogenase expression and the activity in these organisms.

Methanol Dehydrogenase in Methanotrophs

Methanol dehydrogenase (MDH) is the second enzyme in the catabolic pathway of methanotrophic bacteria. Although MDH is not exclusive to methanotrophs, it is highly conserved in methanotrophs and common to almost all methylotrophs, and, as a result, MDH genes have proven useful in molecular ecological applications (116–118). It will only be mentioned briefly because detailed descriptions of MDH biochemistry and molecular biology have been provided elsewhere (12,63,119,120).

MDH is a $\alpha_2\beta_2$ tetramer comprised of large and small subunits of molecular weight, 60 to 67 kDa and 8.5 kDa, respectively (119). It is a quinoprotein with each tetramer containing 2 moles of pyrrolo-quinoline and one mole of calcium; electrons from MDH are transferred to cytochrome c_L, a cytochrome that is specific to the oxidation of methanol in methylotrophs (11). MDH and associated cytochromes are soluble and found in large quantities in the periplasm space of methylotrophs (119). The gene encoding the large subunit and the active site of MDH has been identified as *mxaF* (119) and is highly conserved in methanotrophs (11,15). The regulation and molecular genetics of MDH expression and activity is highly complex and has been reviewed by others (11,12,120,121).

ECOLOGY OF METHANOTROPHIC BACTERIA

Overview

Although methanotrophic bacteria are ubiquitous in nature, the true ecological versatility of these organisms has only recently become apparent. New molecular and other techniques have lead to the discovery of previously unidentified habitats for methanotrophs, including psychrophilic (32,122), thermophilic (35,123,124), (hyper) saline (125,126), acidic (33), and alkaline environments (126–128). These diverse organisms also have been found associated with plant root systems fulfilling a mutualistic

role with the plants analogous to that of rhizobia (129), have been implicated in soil denitrification (130), and are potentially involved in anaerobic CH_4 oxidation in marine systems (131).

The goal of this section is to provide a brief overview of methanotroph ecology, with emphasis to the relative ecological roles of Type I and Type II organisms. Particular attention will also be paid to the results available from the recent advancements in molecular biological monitoring of methanotrophs in the environment.

Classical Methanotroph Ecology

Methanotrophs have been found in almost all environments that contain both oxygen and methane (11). Early studies on methanotroph ecology focused on oxic–anoxic interfacial zones, typically in freshwater and marine systems, and, in most efforts, methanotrophs were successfully enriched from such environments. Unfortunately, many methanotrophs are not readily enriched and grown on laboratory media and, as a result, only few methanotrophs (even in CH_4-rich environments) have been identified using traditional microbiological techniques. This problem was further exacerbated by the fact that even organisms that grew on artificial media did not grow well. Early understanding of methanotroph ecology, therefore, was limited to a few organisms that grew on laboratory media under comparatively rich nutritional conditions, reflecting only a small fraction of methanotrophs actually present in the environment.

Despite these problems, general rules that still largely hold true were developed to define methanotroph selection in the environment. For example, methanotrophs were almost always found at oxic–anoxic interfaces; however, the specific type of methanotroph was dictated by a series of key chemical parameters, including nitrogen speciation and level, the relative levels of oxygen, copper, and methane, and the presence of other one-carbon compounds, such as methanol (11). In laboratory studies, Graham and coworkers (27) showed that a Type II strain *M. trichosporium* OB3b, was competitively successful over the Type I strain *Methylomicrobium album* BG8 when copper, nitrogen, and oxygen levels were low and CH_4 levels were high, whereas *M. album* BG8 was more competitive under the opposite conditions. These results were attributed to the Type II strain's ability to express sMMO and nitrogenase when copper and nitrogen levels were growth-limiting and to the apparent sensitivity of some Type II strains to elevated oxygen levels. Amaral and Knowles (132) confirmed these results using semisolid diffusion columns and showed that Type I strains primarily prevailed where oxygen levels were higher and CH_4 levels lower, and Type II strains prevailed where methane was higher and oxygen was lower. Similar results by others provide the same general picture (133,134); Type I organisms apparently prefer high-oxygen, high-soluble nitrogen, and low-CH_4 environments, whereas Type II strains prevail under the opposite conditions, especially when bioavailable copper levels are also low (11).

Although these general observations are still believed to be correct, the study of methanotroph ecology has been recently revolutionized by the development of new molecular biological tools for studying in situ methanotroph populations (14,16,60,135–137). These new techniques have shown that methanotroph diversity is much broader than was originally believed and that there are more atypical methanotrophs in the environment than was previously realized. For example, a new group of "high-affinity" methanotrophs that effectively grow under atmospheric CH_4 conditions has been identified (116). The identification of this group has changed much of the study of methanotrophs because these methane "scavengers" appear to be ubiquitous in nature and may play the major role in CH_4 oxidation that counterbalances CH_4 accumulation as a greenhouse gas (8,11).

New Techniques for Studying Methanotroph Ecology

The study of methanotroph ecology in situ has expanded on the advent of newly available techniques from molecular biology. Such techniques are particularly useful with methanotrophs because these organisms typically grow poorly on laboratory media, and, therefore, such methods allow the organisms to be studied without the need of subculture techniques. There are two excellent recent reviews on the molecular ecology of methanotrophs (14,15), thus only a summary will be presented here.

Two general strategies for studying methanotroph ecology using molecular techniques have been applied: (*1*) those that rely on polymerase chain reaction (PCR) technology and (*2*) those that directly use phylogenetic or functional gene probes to monitor community conditions. PCR methods are very sensitive and can detect target DNA at very low levels; however, such methods are innately nonquantitative because of the many uncertainties that arise during gene amplification (138). Alternatively, phylogenetic and functional gene probes are quite quantitative, but are somewhat less sensitive. The primary PCR-based protocols that have been used to study methanotroph ecology have included denaturing gradient gel electrophoresis (DGGE) using phylogenetic and functional gene primers, and reverse transcriptase PCR (RT-PCR).

DGGE uses the fact that DNA sequences of similar length, but different composition, migrate differently on gradient gels because of different $\%G + C$ contents. This technique has been used to assess genetic diversity in samples and also to identify major DNA sequences in samples that might be excised and sequenced for probe development and phylogenetic characterization. Alternatively, RT-PCR uses reverse transcriptase to convert mRNA to DNA and then uses PCR to amplify the DNA to determine the genetic message that is in a given sample. In conjunction with the use of appropriate PCR primer sets for the function genes, this method can indicate active gene expression within an environment sample. Both DGGE and RT-PCR can be used to describe broad community characteristics or activities, or they can be very specific, depending on the primers used in PCR amplification.

Phylogenetic and functional gene probes have been used in three general protocols: (*1*) fluorescent in situ

hydridization (FISH), (*2*) small subunit (SSU) rRNA analyses using P^{32}-labeled DNA probes, and (*3*) the tracking of functional genes (e.g., *mxaF*) using PCR primers aimed at those specific genes. FISH is a relatively simple technique that uses the microscopic identification and quantification of specific organisms or groups of organisms by the use of fluorescently-labeled DNA probes that bind to DNA-target sequences in whole cells. At this time, FISH is highly labor-intensive; however, it is the best method available at present for the direct quantification of organisms in environmental samples. ssu rRNA techniques also permit the quantification of organisms; however, this method relies on the extraction of rRNA from the sample and subsequent hydridization of specific DNA probes with the extracted sample rRNA. Quantification is usually performed by autoradiography. Finally, the use of PCR primer sets for functional genes allows the qualitative comparison of the microbial diversity of function in and between the ecosystems. For example, functional genes for sMMO, pMMO, and MDH might be used to compare the potential distribution of organisms containing these three enzymes within a given sample.

Insights into Methanotroph Ecology Using Molecular Techniques

Three major new sets of results have been attained using molecular techniques regarding methanotrophs in the environment, that is, (*1*) a more comprehensive picture of methanotroph biodiversity has been developed, (*2*) many new species have been identified (including many previously unculturable species), and (*3*) new methanotroph habitats have been observed. For example, DGGE has been used to assess diversity of the methanotroph community in landfill soils (136), groundwater impacted by TCE contamination (139), agricultural soils in Norway (135), in the rhizosphere (140), and in soil from rice fields (117). Further, functional gene probes for *pmoA* and *mmoX* and PCR gene amplication has been successfully used to assess methanotroph diversity in freshwater lake sediments (141) and in other environments (118,142–144).

All of these studies showed a greater array of putative methanotroph gene sequences than observed previously, although each separate study presented ancillary information on methanotroph ecology. For example, Wise and coworkers (136) used both molecular and traditional culture-based approaches to assess the influence of pH, air/CH_4/CO_2 level, and media richness on methanotroph community composition in landfill soil cover material. They found that rich CH_4 and nutrient conditions were selected for Type I strains and opposite conditions were selected for Type II strains. Interestingly, results were fairly similar to molecular and traditional detection methods, although the molecular procedures did identify one new phylotype related to the *Methylobacter-Methylomicrobium* group and a new group of related Type II species from the *Methylosinus* and *Methylocystis* genera.

Jensen and coworkers (135) showed that CH_4-enriched organic soil had broader array of methanotroph-associated 16S rRNA gene sequences than observed in sandy soil using DGGE, suggesting that the soil organic content influences the methanotroph diversity. However, Iwamoto and coworkers (139) found that biostimulation by methane of TCE-contaminated soil caused a major change in the phylogenetic community dominance in the soil and observed a shift from putative Type I strains to Type II strains in the system. Auman and coworkers (141) found both Type I and Type II methanotrophs in the top 0.8 cm of lake sediments and showed that sMMO-containing *Methylomonas* strains were more prevalent in the environment than was previously believed. In summary, these examples show that a broad variety of "types" of methanotrophs are present throughout the environment and, in most cases, the different methanotroph types were less systematically present in the environment than previous culture-based methods had indicated.

Recent work by Henckel and coworkers (117,118) is particularly noteworthy because they examined methanotroph community structure, using primers for functional genes and PCR and DGGE, in forest soil and rice fields not significantly enriched with methane (i.e., high-affinity methanotrophs). Henckel and coworkers (117) found that atmospheric CH_4 oxidation occurred only in narrow vertical bands in the soil in winter, whereas CH_4 oxidation was noted in the whole profile in summer (in forest soil). They further observed that total DNA increased with soil depth, but that methanotroph gene sequences were found only in the zones of higher CH_4 oxidation (although the methanotroph community did not change dramatically between summer and winter). Excised and sequenced DNA from parallel DGGE gels showed that the atmospheric CH_4-oxidizing methanotrophs present in these soils were only distantly related to previously identified methanotroph strains.

Henckel and coworkers (118) found similar vertical differences in methanotroph composition in rice fields, but noted that water content significantly impacted methanotroph dominance in the soils. Type I and Type II strains were noted at all depths, although Type II organisms tended to prevail in poorly drained soils. These data indicate that methanotroph community composition is temporarily and spatially dynamic even in soils without major CH_4 enrichment and that such populations are continually shifting on the basis of local variations in habitat conditions. These results also show that there is a pool of methanotrophs in the environment that appear to be quite distinct from previously identified methanotrophs and that they are responsible for CH_4 oxidation in locations other than oxic–anoxic interfaces. The global contribution of this potentially large group of methanotrophs to the carbon cycle is not, as of yet, known (11). However, results indicate that their contribution may be significant, and better defining the numbers, activity, and spatial distribution of these organisms is critical in understanding the balance of methane in the environment and the impact of methane as a greenhouse gas (8).

Recent work of Edwards and coworkers (145), Holmes and coworkers (5), Dunfield and coworkers (116), and Henckel and coworkers (118) provide further confirmation

of high-affinity methanotrophs in the environment. High-affinity methanotrophs have been suspected as important in the environment for many years (8,11,146); however, they have proven very difficult to characterize. This unique group of organisms has very low K_S values for CH_4 oxidation (as low as 10.0 nM), levels that are lower than what has been traditionally considered possible to permit extended survival (8,147,148). Regardless, the biochemistry and genetics of these potentially significant organisms have not been studied because they cannot be readily enriched ex situ because of their unique kinetic properties. As previously noted, evidence from in situ molecular ecological characterization suggests that they may be highly distinct from low-affinity methanotrophs, although the high-affinity organisms do appear to still fall under the classical "type" system of nomenclature. Alternatively, it is possible that other high-affinity methanotrophs have yet to be identified because they do fall within any of the existing primer- and probe-sequence databases.

METHANOTROPHS AND BIOTECHNOLOGY

As discussed earlier in this article, methanotrophs play an essential role in the cycling of carbon and nitrogen in the natural environment. Because of their ubiquity in the environment and of the unique ability of MMO to oxidize methane and a wide range of other chemicals, many have attempted to exploit their catalytic properties for biotechnological applications. Examples of such applications include biodegradation and bioremediation of various hydrocarbons, single-cell protein and polymer production from methane, biotransformations in synthesis pathways, and bioconversion of methane to methanol. In fact, there are numerous patents that address the use of methanotrophs in industrial applications (149–155). Although not intended to be all-inclusive, this section summarizes some of the potential applications of methanotrophic bacteria that have been studied.

Methane is considered a major greenhouse gas and is 26 times more efficient in absorbing and reemitting infrared radiation than carbon dioxide (2,6,11). Because methanotrophs oxidize between 50 and 75% of all methane biologically produced, they naturally play a very important role in our environment. Some studies have focused on optimizing the oxidative capacity of methanotrophs in natural environments as a means of controlling CH_4 release to the atmosphere (such as from landfills) (134,156), and many have focused on determining the conditions for their optimum growth and CH_4 oxidation (157–161).

Use of methanotrophs for in situ and ex situ bioremediation applications for environmental restoration has increased in recent years. Most studies have focused on their use in cometabolizing aliphatic compounds, such as trichloroethylene (TCE), a common contaminant in Resource Conservation and Recovery Act (RCRA) and superfund sites; however, some recent studies have focused on their ability to transform aromatic compounds. A thorough discussion of the benefits of methanotrophic bacteria in remediating TCE-contaminated sites is provided elsewhere in this Encyclopedia (162). Because sMMO has been shown to mineralize more than 90% of TCE (163) and has exhibited a broader substrate range than pMMO (including aromatic compounds), it is desirable to use sMMO-expressing methanotrophs for remediation purposes if possible. For this end, isolation of methanotrophic mutants that constitutively express sMMO, regardless of the copper concentration, has been reported (164). Other studies have focused on the potential of methanotrophs to play an integral role in the degradation of aromatic compounds, including linear alkyl sulfonates, substituted benzenes and biphenyls, by mixed methanotrophic-heterotrophic cultures that are routinely isolated from environmental samples (165–168).

The production of methanol and various alkene epoxides using methanotrophs relating to the use of these microorganisms in industrial applications has been a subject of studies (10,169–171). Large-scale production of methanol has been considered because of its potential to serve as an alternative cleaner-burning fuel, and propene oxide, for example, is a convenient model for gas-solid bioreactor systems and has significant commercial value (172,173). The exploitation of thermophilic methanotrophs is of particular interest in industrial applications. As previously described, CH_4- and methanol-oxidizing bacteria that grow in thermotolerant temperature ranges (45 to 60 °C) and thermophilic ranges (>60 °C) have been isolated (35,123,124). Thermophiles would offer several advantages in biotechnology, including reduced cooling costs, reduced contamination with human pathogens, the possibility of novel biodegradation reactions because of decreased stability of substrates at higher temperatures, and simple recovery of methanol (35).

Despite the earlier examples, the widespread use of methanotrophs in biotechnological applications has not been fully realized. Some reasons for this include the relatively slow growth of these microorganisms, the ability to chemically synthesize methanol relatively inexpensively, and, in the case of bioremediation of substrates more amenable to attack by sMMO, the prevalence of copper concentrations at levels high enough to inhibit sMMO expression by methanotrophs in the natural environment. However, despite these potential drawbacks, the findings reported earlier suggest that these ubiquitous CH_4 oxidizers may play a significant role not only in the natural cycling of carbon and nitrogen in the environment, but also in the cycling of carbon in environmental restoration and industrial applications.

CONCLUSION

Methanotrophic bacteria are ubiquitous in nature, living in any location where air and methane are simultaneously present. They reside in many different habitats and are now being found in locations and performing metabolic functions that were not even considered fifteen years ago. Further, as more novel methanotrophs are identified, particularly thermophiles and high-affinity CH_4 oxidizers, their apparent significance as biotechnological agents should expand further.

It is hoped that this article has provided a brief introduction to classical and emerging topics in methanotroph classification, biochemistry, molecular biology, ecology, and the application of these key organisms in biotechnology. It is also hoped that future efforts, especially related to the role of methanotrophs in the global carbon cycle and greenhouse gas reduction, will be stimulated by this article and further efforts will be expended on this key and environmentally significant group of microorganisms.

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METHANOTROPHIC BACTERIA: USE IN BIOREMEDIATION

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Specific types of anaerobic microorganisms produce methane in environments in which oxygen is limited. These include swamps, landfills, wetlands, peat bogs, sediments, and the intestinal flora of animals. Methane-oxidizing bacteria (methanotrophs) in turn remove methane from these environments. Therefore, anaerobic sediments are considered to be aerobic sink for methane because of the activities of methanotrophs.

Methanotrophs are a diverse group of bacteria that utilize methane as their sole carbon and energy source (1,2). These bacteria have been isolated from a wide range of environments including soils landfills (3), sediments (4), groundwater (5), salt reservoirs (6), and plant rhizosphere (7).

Methanotrophs were initially classified on the basis of their morphology, type of resting stage, intracytoplasmic membrane structure, and physiological characteristics (8). In recent years 16S rDNA sequence analysis has further clarified these phylogenetic relationships and defined eight genera of methanotrophs, namely, *Methylococcus*. *Methylomonas*, *Methylomicrobium*, *Methylobacter*, *Methylocaldum*, *Methylosphaera*, *Methylocystis*, and *Methylosinus* (9). These genera are divided into two distinct physiological groups. Type I methanotrophs that assimilate formaldehyde that was produced from the oxidation of methane through methanol using the ribulose monophosphate pathway have cellular membranes that are composed of predominantly 16-carbon fatty acids and possesses bundles of intracytoplasmic membranes. Type II methanotrophs utilize the serine pathway for formaldehyde assimilation, have distinct intracytoplasmic membranes arranged around the periphery of the cell, and contain predominantly 18-carbon fatty acids (1). Membranes of the genus *Methylococcus* possess a combination of characteristics of both type I and type II methanotrophs.

Methanotrophs form coherent phylogenetic clusters that share the common physiological characteristics described earlier (9). Type I methanotrophs cluster in the γ-subdivision of the Proteobacteria, whereas type II methanotrophs are grouped within the α-subdivision of the Proteobacteria. The tight phylogenetic clustering of these groups has allowed the design of a range of oligonucleotides, which target a broad range of both

methanotrophs and methylotrophs (1,10,11). A number of 16S ribosomal and gene functional probes are now available for culture-independent detection of methanotrophs and methylotrophs (3).

Several species of methanotrophs that produce methane monooxygenase and participate in the cometabolic degradation of TCE, have been isolated (12). However, soluble methane monooxygenase (sMMO) present in some methanotrophic bacteria has been found to be responsible for oxidizing a wide range of carbon substrates of interest for bioremediation applications (13). No sMMO has been isolated or observed in type I methanotrophs. Moreover, it appears that the sMMO is limited to a few species of type II and type X? methanotrophs. The sMMO has been found, through its nonspecific activity, to affect oxidations, dechlorinations, condensations, and rearrangements of meta-chlorotoluene, phenol, chlorofluorobenzenes, and mono and dichlorobiphenyls via it's nonspecific enzymatic activity. Soluble MMO is believed to be found only in type II and type X methanotrophs during copper-limiting conditions (14). Although membrane and particle-associated MMO has been reported, the rates of chlorinated solvent degradation with these organisms is significantly less than sMMO bacteria (15). These solvents have included halogenated aliphatic compounds such as trichloroethylene (TCE). Methanotrophs can utilize nitrogen as nitrate, ammonia, and molecular nitrogen while demonstrating TCE transformation capacity (16). This activity has been applied in subsurface bioremediation activities with beneficial results. This article concentrates primarily on methanotrophic bacteria applications shown to be effective in chlorinated solvent bioremediation with a focus on TCE.

BACKGROUND

In recent years there has been an increased interest in the use of microorganisms for environmental restoration. The usefulness of microorganisms with a diversity of metabolic activities in wide-ranging applications coupled with advances in the technology has lead to successful demonstrations in the ever-expanding bioremediation field. The application of environmental biotechnology as a successful remediation tool depends on the ability to stimulate or enhance specific activity of indigenous or introduced microorganisms. The challenge has been to enhance the activity of these microorganisms and develop means to bring the contaminant into direct contact with the organisms to achieve optimum bioremediation. Methanotrophic bacteria have a ubiquitous distribution in the environment and the use of natural gas or methane with other nutrients to stimulate their bioremediation activities through methane monooxygenase is a remediation option. These two features allow for a relatively efficient, inexpensive, and safe means to manipulate the environment to accelerate bioremediation. This entry describes the great interest in methanotrophic bacteria in bioremediation.

TCE is a volatile chlorinated organic compound that has been widely used as an organic solvent and degreasing agent and disseminates over large areas in the subsurface at contaminated sites. TCE is the most frequently observed volatile organic compound (VOC) at Resource Conservation and Recovery Act (RCRA) sites (17) and in groundwater (18). TCE is a Voc that is regulated in the United States as an air pollutant under the Clean Air Act Amendments Title III. The U.S. Occupational Safety and Health Administration is setting worker exposure limits for this solvent because of it's toxicity. Dichloroacetate and chloral hydrate are potentially produced in the groundwater in small amounts when TCE is metabolized and can be present in drinking water. Both these compounds have potential health effects because dichloroacetate is an experimental drug used to treat children with lactic acidosis, and chloral hydrate is a sedative (19).

Removing TCE from contaminated groundwater can pose risks while using conventional engineering methods such as vacuum extraction or pump and treat remediation. These risks include transfer of TCE to air through volatilization and handling issues. Because of increasing environmental concerns and federal regulations there is a growing preference for bioremediation applications that reduce risk and produce minimal toxic residuals. Although investigations of the microbial degradation of chlorinated ethenes have been ongoing since the 1950's (20,21), as recently as the 1980's it was believed that TCE could not be successfully biodegraded either aerobically or anaerobically (22). This belief was in part because of the widespread groundwater contamination by TCE. In 1980 alone, California closed 39 public water supply wells in the San Gabriel Valley because of TCE pollution, and New York, New Jersey, and Pennsylvania condemned wells because of the same contaminant (18).

Wilson and Wilson (23) demonstrated that TCE is susceptible to cometabolism by soil communities enriched with natural gas. Fliermans and coworkers (5) and others demonstrated that cultures enriched with methane and propane could cometabolically degrade a wide variety of chlorinated aliphatic hydrocarbons including ethylene, 1,2-cis-dichloroethylene (c-DCE); 1,2-trans-dichloroethylene (t-DCE); and vinyl chloride (VC); toluene, phenol, and cresol. Investigations that utilized methods to stimulate microorganisms in the subsurface vadose zone and groundwater found that both aerobic and anaerobic processes contributed to the removal of TCE as revealed by detection of TCE oxidation and anaerobic transformation products, c-DCE, t-DCE, and VC (24,25). These studies proved that bioremediation provides a powerful means of restoring contaminated aquifers. Mccarty and Semprini (26) concluded from laboratory and field studies that cometabolic transformation of TCE was strongly tied to methane utilization. When methane additions were stopped TCE transformation ceased. Figure 1 outlines the oxidation of methane by methane monooxygenase and the associated cometabolism of trichloroethylene.

It is now well recognized that TCE and other chlorinated aliphatic compounds can be degraded by a diversity of bacteria including methanotrophs (27), selected methanogens (28), and species of *Pseudomonas* (*P. cepacia*, *P. mendocina*, and *P. putida*) capable of also degrading aromatic compounds (29). Ensley (30) has

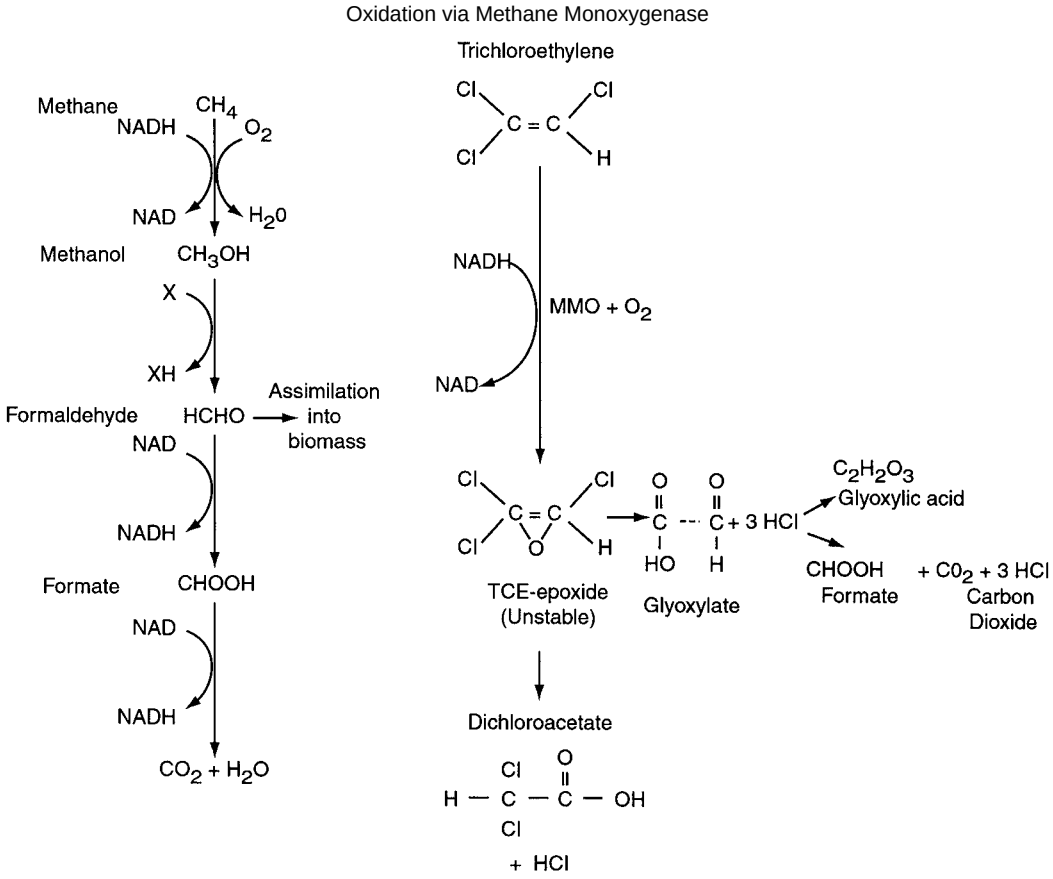

Figure 1. Oxidation of methane by the methane monooxygenase enzyme and the associated cometabolism of trichloroethylene.

demonstrated a linkage between TCE degradation and aromatic metabolism in *P. cepacia* G4, *P. mendocina*, and *P. putida*. Ensign and coworkers, (31) reported that pure cultures of *Xanthobacter* spp. cometabolized TCE with the utilization of propylene as a carbon and energy source presumably using the enzyme alkene monooxygenase. Fliermans and coworkers (5) and Bowman and coworkers (4) have shown that enrichments for methanotrophs in subsurface samples collected from the Savannah River Site in South Carolina stimulated the microbial degradation and complete mineralization of TCE and other chlorinated aliphatic compounds both in the laboratory and in situ. Propane utilizers or propanotrophs that also exhibit nonspecific oxidase activity may also be used for bioremediation of VOCS. Where mixtures of chlorinated aliphatic hydrocarbons including 1,1,1-trichloroethane are present, propane may be the stimulant of choice using air-sparging technology (32). However, methanotrophs are optimum in bioremediation when TCE is the primary contaminant of concern.

An advantage of utilizing methanotrophs for bioremediation is that aerobic conditions do not appear to support the formation of undesirable metabolites, such as c-DCE, t-DCE, or Vc that are partially dechlorinated by-products of anaerobic degradation of TCE. Vinyl chloride, a known animal and human carcinogen, is considered more hazardous than the parent compounds TCE and/or PCE. Methane oxidizers are likely to be found in zones that fluctuate between aerobic and anaerobic conditions such as soils that periodically flood and drain. Enzien and coworkers (33) has suggested that both anaerobic and aerobic populations may both be stimulated to biodegrade TCE in an aquifer under bulk aerobic conditions.

Although there appears to be a diversity of natural microorganisms that degrade TCE, the compound remains the most prevalent organic contaminant in the United States (34). The ubiquity of the TCE contamination suggests that either the environmental conditions do not support biodegradation, or that the correct consortia of microorganisms is not present, or at sufficient cell densities in the ecosystem. Thus, it is important to understand both the biological and the physical parameters associated with biodegradation of TCE. Despite these possible problems, the promise of bioremediation is revealed by the observation mixed methane-grown communities that were once stimulated have been found to metabolize TCE completely to harmless end products with a half-life of less than a day (35).

Although sediment methanotrophic bacteria can be efficient in degrading TCE from contaminated groundwater (4), certain methanotrophs are more efficient at TCE degradation than others (12). It has been suggested that mixed microbial populations are more efficient in TCE degradation than pure culture (36). Field

applications of methanotrophs for removal of TCE from the environment have proven that further studies of these bacteria are needed. Specific areas that need to be explored are associated microbial communities, population dynamics, and potential for biodegradability to decrease or eliminate the contaminant. Thus, there is a need for a better understanding of the microbial ecology of TCE-contaminated subsurface systems for biodegradation applications.

IN SITU BIOREMEDIATION

In situ bioremediation means that the environmental restoration of contaminated sediments are not moved from the site or that groundwater is not pumped and treated at the surface. When an in situ bioremediation technology is employed the relocation and transport of materials may be avoided. This makes in situ bioremediation, where applicable, a highly attractive technology for remediation because contaminants are removed on-site, not simply moved to another location or volatilized (37). In situ bioremediation of chlorinated solvents with methane injection is a site-specific application. This application results in decreased remediation costs, lower contamination risks, shorter restoration time, and increased efficiency, as well as enhanced public and regulatory acceptability. Public relations are enhanced because much of the action occurs with minimum aboveground activity and equipment and bioremediation can be among the least expensive remedial technologies in which its application is feasible.

Bioremediation of chlorinated solvents through stimulation of methanotrophic bacteria has been demonstrated at the Savannah River Site (SRS). The SRS is a 320 square mile facility owned by the U.S. Department of Energy and operated by Westinghouse Savannah River Company. The SRS, located in a rural area along the Savannah River in Aiken and Barnwell counties of South Carolina, has generated nuclear materials for defense, medical, and space applications since the 1950s. During the first 20 years of operation, most of the waste generated at the SRS, including millions of pounds of chlorinated solvents, was handled via burning rubble pits, evaporation ponds, and waste pits resulting in extensive soil, sediment, and groundwater contamination.

The SRS has now completed several successful bioremediation demonstrations on soil and groundwater contaminated with chlorinated ethenes utilizing methanotrophic biostimulation (25,38). The deployment of two projects at SRS designed for field demonstrations of in situ treatment of groundwater contaminated with chlorinated solvents by gaseous nutrient injection have proven the effectiveness of in situ bioremediation.

Demonstration 1

The first demonstration was near the 300-M operations area where fuel and target elements were degreased in processing. An estimated 13 million pounds of solvents were used in this processing from 1952 to 1982. Although evaporation was used to reduce much of the solvents, an estimated two million pounds was released to the M Area Settling Basin (24). These solvent discharges to the M Area settling basin consisted primarily of TCE, perchloroethylene (PCE), and trichloroethane (TCA).

The M area in situ bioremediation demonstration consisted of two horizontal wells for injection and extraction at the process sewer line leaking PCE and TCE (25). Figure 2 illustrates a side view of the 160-ft deep horizontal wells in relation to the surface nutrient injection and extraction systems. Groundwater, extracted air, and sediment samples were taken before, during, and after testing of the system that ran for more than one year. Subsurface gaseous nutrient injection including methane, nitrogen, and phosphorus was found to be an effective in situ bioremediation treatment for TCE-contaminated groundwater at the M Area site of SRS (25). Gene probe analysis demonstrated an increased MMO activity corresponding to significant TCE degradation rates among a high proportion of the indigenous methanotrophic bacteria in the M Area groundwater (4). Species-specific methanotrophic bacteria DNA probes have revealed that perturbations associated with biostimulation resulted in preferential changes in the structure and physiological status of microbial communities (37). Direct evidence of TCE mineralization in this project was demonstrated by increased groundwater chloride concentrations that correlated with severalfold increases in the density of methanotrophic bacteria (39). The rate of TCE biodegradation also correlated with growth of specific species of methanotrophs and nutrient growth factors including injected methane, phosphorus, and nitrogen (38). These increases correlated with methane groundwater concentrations and the pulsed injection regime over the year test period.

The observation that methanotrophic population increases correlated with TCE biodegradation indicated that the zone of influence of gaseous nutrient injection extended at least 60 ft from the injection well in both the horizontal and vertical directions (37). Groundwater monitoring of both bacteria and contaminant concentrations provide information on the efficiency of this technology (25). Application of these methane or air mixtures demonstrated a three to five order of magnitude increase in TCE-degrading methanotrophic bacteria during the methane and nutrient injection (39).

A subsequent remediation project was conducted in the same M Area aquifer to determine the effect of chemical oxidation on subsurface microbiology and cometabolic biodegradation capacity in the same aquifer after treatment with Fenton's reagent (40). The groundwater pH declined from 5 to 2.4 immediately after the Fenton's treatment indicating release of Cl^- ions from TCE/PCE. The pH subsequently rose to a range of 3.4 to 4.0 after 17 months. Limited methanotrophic bacteria growth and TCE degradation were detected in the treated zone (pH 3.4 and TCE 5 $mg\,L^{-1}$) with methane addition. Methane addition to groundwater from the control well without Fenton's Reagent (pH 4.9 and TCE 0.7 $mg\,L^{-1}$) stimulated methanotrophic growth. This was indicated by methane consumption and microbial characterization with fluorescent antibody analysis, phospholipid-based markers, and rDNA probes. Higher TCE concentrations in the Fenton's

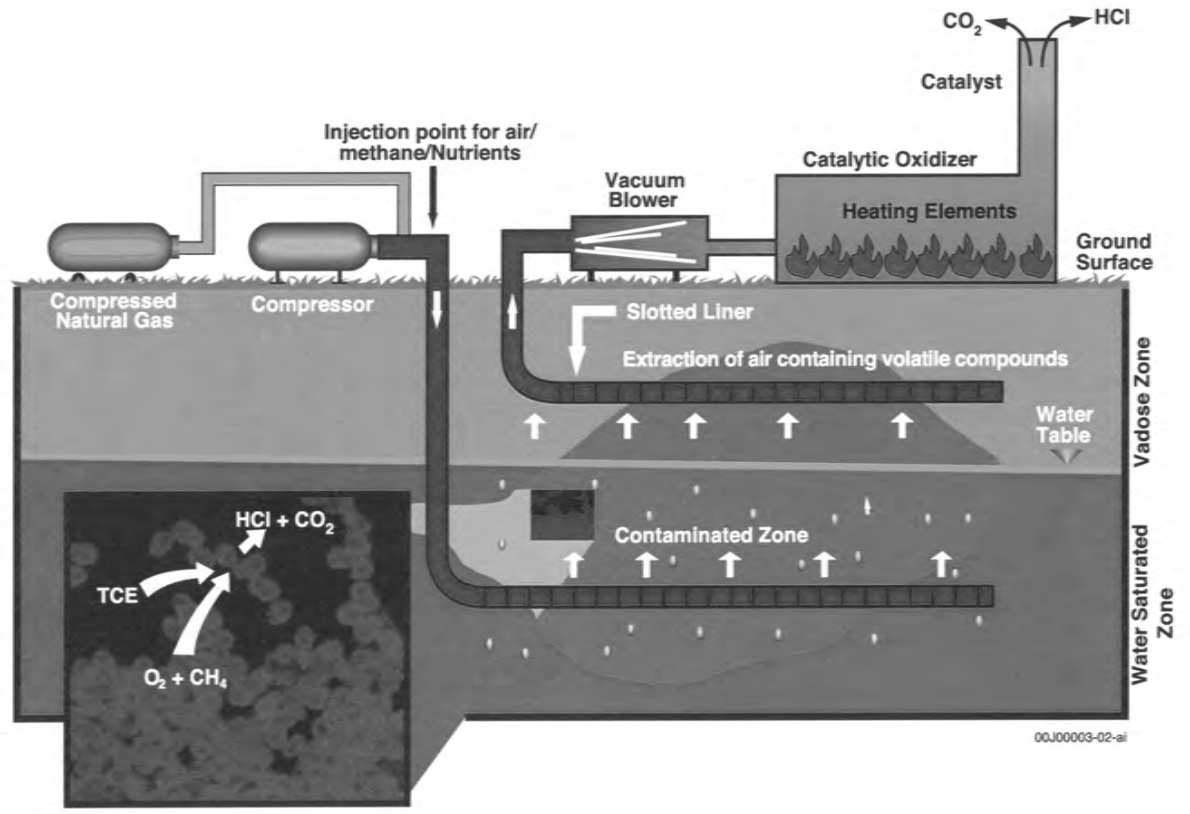

Figure 2. A side view of the horizontal wells in relation to the surface nutrient injection and extraction systems Modified from WSRC, Test Plan for In Situ Bioremediation Demonstration of the Savannah River Integrated Demonstration Project DOE/OTD TTP No.: SR 0566-01 (U), WSRC-RD-91-23, Westinghouse Savannah River Company, Aiken, SC, 1992. See color insert.

Reagent treated zone (16–21 mg L^{-1}) might have inhibited TCE cometabolism. These results also indicate that low groundwater pH resulting from the chemical oxidation process (pH 3.3 vs. 4.9) inhibited TCE biodegradation. Methanotrophic growth and TCE biodegradation may be possible because pH increases both in the treated zone and at the leading edge of the plume if sediments are able to buffer groundwater pH. Treatment with Fenton's Reagent limits the potential for subsequent monitored natural attenuation to occur. However, the Fenton's reagent process could be designed to operate at a higher pH (e.g., $\geq$4.5) and/or lower H_2O_2 concentration to minimize detrimental long-term biological effects, providing an optimum environment to couple advanced oxidation processes with bioremediation technologies.

Demonstration 2

A second demonstration of in situ bioremediation at SRS is at the Non Radioactive Waste Disposal Facility (NRWDF) formerly known as the Sanitary Landfill. The NRWDF began receiving solid waste from construction areas, offices, shops, and cafeterias in 1974. During the course of its operation, the Sanitary Landfill received numerous materials that can leach or generate hazardous compounds, for example, paints, thinners, solvents, batteries, rags, and wipes used with organic solvents. Wastes were cataloged but not segregated within the landfill. As a result, in 1988, recurring evidence of contaminants of concern (COCs) were detected in the groundwater including TCE, PCE, TCA, DCE, VC, and chlorobenzene (CB).

Initially, a treatability study to evaluate the NRWDF bioremediation potential using soil columns was employed to simulate both vadose and groundwater conditions using NRWDF sediment and groundwater (33). The results of the treatability study proved that cometabolic methanotrophic bioremediation of the COCs was possible at the NRWDF. A subsequent in situ optimization test demonstrated that biostimulation by addition of oxygen, nutrients, and methane at two sites within the NRWDF resulted in undetectable levels of contaminants and other organics in both the groundwater and vadose zone (X). Additionally, chloride concentrations in the groundwater at both sites increased significantly as methanotrophic bacteria densities increased, reaching a maximum population in three to four days, whereas contaminant levels decreased.

Figure 3 demonstrates methanotrophic bacteria labeled with direct fluorescent antibodies from NRWDF groundwater concentrated on a 0.2-μm filter (40). The total number of groundwater microorganisms did not change, indicating a selective stimulation of the methanotrophic

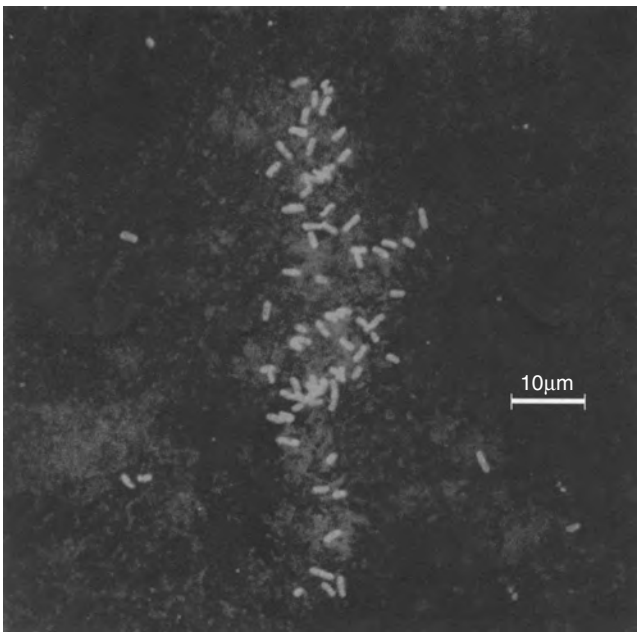

Figure 3. Concentrated groundwater methanotrophic bacteria on 0.2-μm filter labeled with fluorescent monoclonal? antibodies. See color insert.

Figure 4. In situ gaseous nutrient injection system at the Savannah River Site 70-acre nonradioactive waste disposal facility depicting the 400 ft and 600 ft horizontal wells. Insert is gas-nutrient pumping station.

population (40). The loss of contaminants appears to be because of cometabolic biodegradation through biostimulation as loss by volatilization was minimal. This work again clearly demonstrates that one can effectively change the subsurface bacterial population in a relatively short period. A larger scale (400 and 600 ft) set of horizontal wells at the NRWDF site is now being tested with nutrient injection for long-term containment of chlorinated ethenes (Fig. 4). Results to date indicate the spread of the associated TCE plume is being limited by the nutrient injection system.

Similarly, groundwater concentrations, another NRWDF contaminant, have significantly decreased in recent years at the NRWDF with a concomitant increase in chloride ions. Chlorobenzene can undergo microbiological dechlorination and the benzene ring can be converted to catechol, followed by ring fission or oxidation of the side chain. Geochemical data confirm that TCE and CB concentrations are decreasing at a greater rate than would be expected because of NRWDF groundwater transport or dilution.

These demonstrations represent another approach in the development of in situ bioremediation technologies. In both applications, a pulsed application of methane coupled with other gaseous nutrients worked best for methanotroph biostimulation and contaminant degradation. Through the use of the gaseous nutrient injection system (Fig. 4), the NRDWF groundwater COC concentrations are approaching minimal detection limits (5 ppb).

MONITORED NATURAL ATTENUATION

Monitored Natural Attenuation (MNA) is a risk management option that relies on natural biological, chemical, and physical processes to contain the spread of contamination from a source. Containment by MNA relies on sorption, volatilization, dilution, destruction, or biotransformation of contaminants. Comparing rates of contaminant transport to rates of MNA to other methods including groundwater transport models can quantitatively assess the efficiency of this option to prevent contaminant migration in groundwater systems. If groundwater movement is faster and relative to rates of contamination removal, contaminants have the potential to reach points of contact with human or wildlife populations. Conversely, if transport rates are slow relative to removal rates, contaminant migration will be more confined and less likely to reach a point of contact. Evaluating the factors mitigating contaminant transport to predetermined points of contact can assess the efficiency of MNA. Thus, this assessment includes hydrological (rates of groundwater flow), microbiological (rates of biodegradation), and sociopolitical (points of contact) considerations.

The MNA of TCE is associated with anaerobic dechlorination and cometabolism. Under specific anaerobic conditions TCE can be reductively dechlorinated to less chlorinated ethenes including DCE and VC. The presence of these compounds is evidence of TCE intrinsic bioremediation. It has been reported that if the amount of cis-DCE is greater than 80% of the total DCE, which it is in the case of the NRWDF, then it is a biodegradation product of TCE. The MNA of TCE is associated with the accumulation of these daughter products and the increase in chloride ions (39).

Other microbial processes involving methanotrophs may also be of value to bioremediation efforts. For example, denitrification rates in anaerobic environments have been found to be directly dependent on the methanotrophic activity and the percentage of soluble carbon produced during methane oxidation. Any of the three intermediates

(methanol, formaldehyde, or formate) from the methane oxidation (Fig. 1) could be used as electron donors by anaerobes.

A number of MNA investigations involving chlorinated ethene degradation have demonstrated that, although anaerobic dechlorination is occurring, the anaerobic processes alone cannot fully account for the observed contaminant reduction. Sequential anaerobic-aerobic chlorinated ethene degradation field and laboratory studies were carried out at three different remediation sites (39). In all three waste sites, methane generated from associated waste was proven to be stimulating the cometabolism of TCE. Microcosm studies confirmed the aerobic and anaerobic processes that were impacting the distribution and concentration of contaminants at these sites.

Indigenous sources of carbon associated with soils, sediments, and groundwater may be supportive of TCE degradation although certain soils may not support such activities (41). Walton and Anderson (42) demonstrated that TCE-degradation was carried out by indigenous microbial populations in the absence of an added cosubstrate in rhizosphere and nonrhizosphere soil slurries and groundwater samples from a TCE-contaminated site. Although significant differences existed in the rates of TCE cometabolism among methanotrophic microbial communities in these three habitats, similarities existed between the behavior of pure cultures and the indigenous soil microbial populations that cometabolize TCE. Questions regarding the specific populations of microorganisms that are responsible for aerobic and anaerobic degradation of TCE in various habitats still remain.

The growth of vegetation encourages the proliferation of microorganisms in the root zone by providing an environment conducive to microbial growth. The density and diversity of microorganisms in the root zone (rhizosphere) is enhanced when compared with bulk soil. Plants also provide an environment that often leads to increased rates of microbial degradation in the rhizosphere of organic contaminants (42). Enhanced biodegradation rates may be related to nutrient availability as reflected in the increased numbers of microorganisms and/or the increased microbial activity (metabolic or cometabolic) caused by root exudates. In some cases the TCE groundwater contamination is close to surface soils or impacts seeplines where plumes of contaminated groundwater can emerge as a result of geologic and topographical interactions. Significantly higher numbers of methanotrophic bacteria were observed in rhizosphere soils and on roots of *Lespedeza cuneata* (a legume) and *Pinus taeda* (Loblolly Pine). It was previously demonstrated that rhizosphere soils from these two types of plants showed higher rates of ^{14}C-TCE mineralization compared with nonvegetated soils (43). Methanotrophic bacteria activity is observed in organic sediments with aerobic and anaerobic microbial interactions. Seeplines that contain such conditions could be an important mechanism for bioremediation of chlorinated ethenes as a natural area for cometabolism to occur. Plumes of contaminated water are often observed in seeplines discharging to surface streams and rivers. The sediments in these seepline systems often contain highly active microbial communities. Streambeds are another area where contaminated subsurface water interacts with surface water. Complete oxidation of chlorinated ethenes has been observed in organic-rich-stream bed sediments (44). Future work could be focused on in situ microbial distribution and densities, and the role of specific populations including methanotrophs in response to TCE-contaminated groundwater seepage through the rhizosphere, streambeds, and seeplines.

EX SITU: BIOREACTORS

It is evident that significant progress has been made in the application of bioremediation utilizing methanotrophic bacteria. Methanotrophic bacteria have also been used in bioreactors as a means of complete removal of contaminant rather than transferring it from one form to another. Bioremediation with methanotrophic bacteria in bioreactors has been tested with free, immobilized, and attached cells. Studies using methanotrophs for TCE removal have been carried out in bioreactors with bacteria attached to carbon (45), diatomaceous earth (46), glass, and ceramic packing material (47). Both aquatic and air bioreactors utilizing methanotrophic bacteria have been developed to remove TCE.

Because methane is the only nutrient source to the microorganisms, sufficient quantities must be supplied resulting in high flow rates through the bioreactors. Because of the large gas demand relative to the poor solubility of methane and oxygen, this mass transfer can present a design challenge. In addition, pure oxygen and methane can potentially form explosive mixtures, therefore appropriate handling methods are necessary. The medium size or design used should be tailored to the velocity of flow. Bioreactors with methanotrophic bacteria have been successfully maintained with both mixed cultures (17) and pure cultures (36).

As with all microorganisms, maintenance of methanotrophs in a bioreactor or culture requires specific nutrient conditions. Growth factors that can influence methanotrophic activity are methane concentrations, copper concentration, nitrogen source (NO_3, NH_4^+), oxygen supply, pH, temperature, and the origin of the culture or inoculum used. Although methanotrophs have been isolated in culture conditions containing 50% methane (3), optimum conditions for TCE degradation in methanotrophic bioreactors are between 4 and 20% (46). Bioreactors are useful for further physiological studies of methanotrophs and have added to our knowledge of TCE biodegradation.

CONCLUSION

Microbial communities are highly diverse and capable of conducting an extensive range of metabolic activities (37). Irrespective of depth or geologic formation, subsurface microorganisms carry out all the major nutrient (carbon, sulfur, nitrogen, manganese, iron, and phosphorus) cycling. Although each geologic formation appears to have its own microbial structure, sandy formations

that are highly permeable to air or water flow have a higher microbial activity. Considering a generally large subsurface microbiota there is considerable interest for the prospect of degrading hazardous contaminants in situ by stimulating selective bacterial populations (biostimulation) or by the addition of organisms to contaminated sites (bioaugmentation). Stimulation of an indigenous population of methanotrophs by methane is likely to enrich species that are well adapted to their environment, whereas the deliberate addition of more microorganisms into such an environment may be compromised because the introduced organisms are not as likely to be able to compete.

Defining temporal and spatial relationships and population dynamics or interactions of selected microorganisms such as methanotrophs in the natural setting is important for the evaluation of bioremediation potential and its effectiveness. It has become increasingly evident that indigenous microbial systems are able to facilitate the degradation and mineralization of a wealth of compounds that were thought to be biologically recalcitrant twenty years ago. This realization has necessitated technologies whereby defined microbial types can be followed in situ in real time by techniques that are designed to be selective, sensitive, and easily applicable to soils, sediments, and groundwater.

Methanotrophs are physiologically versatile in their ability to exist in a variety of habitats and to live in hostile environments having a wide range of pH, temperature, heavy metal concentrations, oxygen concentrations, barometric pressures, salinity, and radiation. Under these diverse conditions a number of methanotrophs have been isolated that facilitate the degradation of TCE and its daughter products.

Evaluation, characterization, and utilization of microbial communities associated with in situ bioremediation of subsurface and groundwater contamination is a technological necessity for environmental restoration and assessment (48). In the past sixty years both industrial and government nuclear production and waste management facilities have generated a significant quantity of organic wastes. These wastes have found their way into the vadose zones and groundwater resulting in unacceptable environmental impacts. The adaptability and manageability of indigenous microorganisms such as methanotrophs make them ideal for the remediation of hazardous environmental wastes in a diverse range of habitats (49).

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METHANOTROPHS.

See TRACE GASES SOIL

METHODS FOR FLOW CYTOMETRY AND CELL SORTING

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Each day, throughout the world, samples are analyzed to determine the number and types of microorganisms present in the environment, and indirectly from the environment to foodstuffs and beverages. Measurements are made to estimate total cells, total viable cells, and specific pathogens or indicator cell types of interest. It has become apparent, however, that many microbes are not responsive to cultivation techniques (1–5). Therefore, conventional enumeration on solid or liquid media may severely underestimate the microbiota of interest. The aim of this article is to present an overview of the principles and the role that flow cytometry (FC) can take in the direct monitoring of microorganisms from various environmental liquids.

FC is now widely used as a means of perceiving, and sometimes also sorting cells (flow cytometric cell sorting — FCCS). It is well suited for the identification of single cells in suspension, ranging from aquatic samples with particulate debris through to aerosols (6,7). Although FC has been recommended several times for environmental microbiology (8–12) and is becoming more common in clinical microbiology (13), it is a grossly underutilized technique owing to the problems encountered with environmental samples and that instruments are largely designed for mammalian cell analysis (14).

Therefore, we begin this article with an introduction to the basics of FC and describe some current microbiological applications. This is followed by an overview of the current state of the art in FC methods used in analytical microbiology, and the most suitable applications of this technology. The remainder of the article outlines the various elements in any FC method, these being *(1)* sample preparation, *(2)* staining, *(3)* instrumentation, and *(4)* data analysis. Finally, predictions regarding future directions in FC are discussed. For general information and free FC software, the reader is also directed to various sites on the Internet (cytometry network sites, *http://nucleus.immunol.washington.edu/ISAC/network_sites.html*; JCSMR FC software, *http://jcsmr.anu.edu.au/facslab/facs.html*; ISAC WWW home page, *http://www.10.uniovi.es/ISAC.html*).

BASICS OF FC

Detailed discussions on FC have been provided previously (15–17) and only a brief outline is provided here. The earliest "modern" flow cytometers were used by the U.S. Army during World War II to detect bacteria and spores (18). These devices incorporated a sheath of filtered air to limit the air sample stream to the central portion of a flow chamber, and the detector was the recently developed photomultiplier tube. The next generation of flow cytometers was built by Kamentsky and coworkers (19), using spectrophotometric techniques to detect and measure nucleic acids and light scattering of unstained cervical cells in a flow stream. At the same time, the first flow cytometer with cell sorting (FCCS) capability was developed by adapting the ink-jet printer principle, using electrostatic deflection of charged droplets (20). Fulwyler's flow cytometer worked by measuring cell volumes obtained by the Coulter orifice principle. However, it was not until the development of an arc lamp-based instrument by Steen's group in 1979 that a flow cytometer with sufficient resolution for bacterial characterization was generally available (21,22) (commercially as the Skatron Argus FC, briefly during the mid-1990s as the Bio-Rad Bryte HS). Nevertheless, while the use of FC has become routine in biomedical applications (the most common area of routine use being in the analysis of blood cells), it is only now emerging from a research-only tool in microbiology (23). Portable flow cytometers specifically designed for microbiology, such as the Microcyte ($33 \times 43 \times 16$ cm and weighing about 15 kg) should

also improve their utility (24), but other modular systems such as the Partec PAS II (Partec GmbH, Munster, Germany) (*http://www.partec.de/indexre.html*) also offer systems for bacterial and yeast applications.

All flow cytometers quantitatively measure the optical characteristics of particles, such as cells, as they are presented, in single file, into a focused light beam. The light source used is either a high-pressure mercury vapor lamp or an assortment of different lasers. Cells can be analyzed at rates of over 100,000 per second as they are carried within a fast flowing air or fluid stream termed the *sheath flow* (Fig. 1(left)).

As particles pass through the light beam three parameters are measured: forward scatter (also called *FSC* or *forward angle light scatter* [FALS]), side scatter (also called *SSC* or *side angle light scatter* [SALS]), and fluorescence (FL). The amount of light scattered forward and at right angles by any particle tends to increase with cell size. In addition, cell refractivity is related to surface properties and internal structure and these also affect FSC and SSC. Natural fluorescence (autofluorescence) is emitted by cellular components, such as flavin nucleotides, pyridine, and photosynthetic pigments. However, most examples of the use of FC for microbial analysis make use of fluorescent dye labels rather than autofluorescence. A typical flow cytometer measures fluorescence in three wavelength ranges. Light of defined wavelengths is channeled to particular detectors; for example, detector FL1 will typically measure green fluorescence, FL2, orange fluorescence, and FL3, red fluorescence (Fig. 1(right)).

The least expensive and simplest flow cytometers are analysis-only instruments; their operating simplicity and reproducibility make them ideal for use in quality control procedures. For more than double the cost of an analysis-only instrument, a flow cytometric cell sorting instrument (FCCS) can be purchased (Fig. 1(right)). The FCCS uses one of two mechanisms to physically collect particles with particular characteristics determined by the operator. The most commonly used mechanism involves the use of a bimorph crystal to vibrate the flow cell. The vibration causes the stream of sheath fluid to form undulations and then break up into droplets. The stream of droplets passes between two deflection plates, which can be positively or negatively charged. Individual droplets can be charged with a negative or positive charge by charging the sheath stream at the nozzle, for the fraction of the second that it takes for a single droplet to form at the end of the sheath stream. This results in a single charged droplet being formed, which then passes between the two charged electrodes. The charged droplet is attracted to either one of the electrodes where it can be collected into a test tube, multiwell plate, or onto a microscope slide. The advantage of this droplet deflection sorting method is that it can operate at very high speed, as fast as 70,000 sorts per second. Also, by charging one droplet with a negative charge and another with a positive charge it is possible to sort two different types of particles simultaneously. The major problem with this type of sorting is that it is very complicated to set up and align the instrument.

The second sorting mechanism is employed by the Becton Dickinson FACScalibur and involves deflecting particles into a side stream by a mechanical arm. After the particles in the sheath stream have passed through the laser, the sheath stream approaches a "Y" shaped intersection. The majority of the sheath fluid and all the particles within the sample travel via the right arm of the "Y." A small amount of the sheath fluid, but no particles within the sample, travels down the left arm of the "Y." A mechanical arm at the "Y" intersection can come out into the stream and deflect particles down the left arm of the "Y." The particles can then be collected into a tube or onto a membrane. The advantage of this sorting mechanism is that it requires no setting up or alignment prior to use. It is very simple to perform and requires little or no experience. However, this sorting mechanism is not suitable for many applications because it is only capable of sorting up to 200

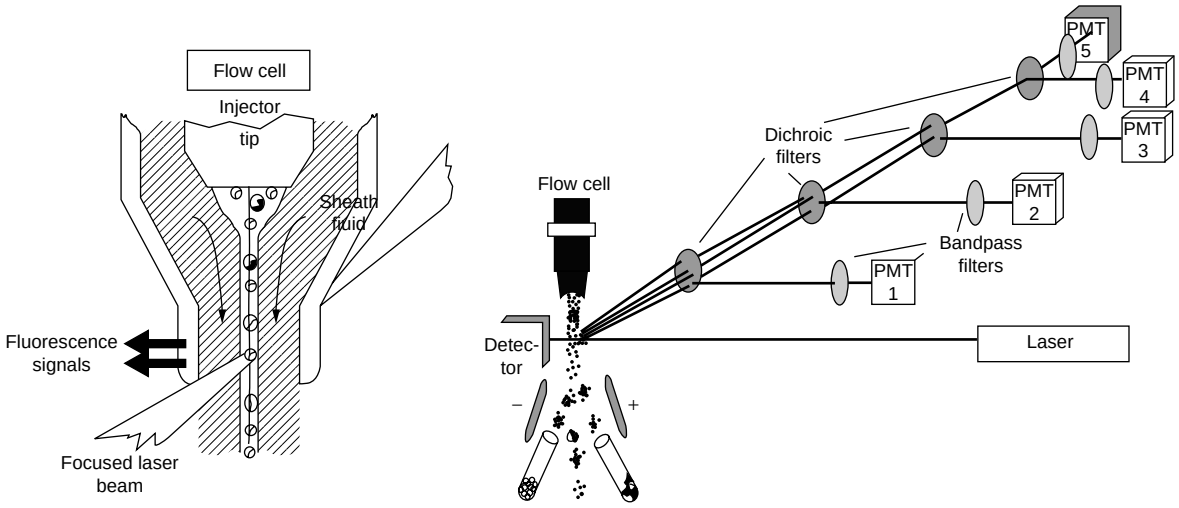

Figure 1. Flow cell illustrating sheath fluid and analysis of light scatter (left). Light-scattering and fluorescence signal production of the flow cell analysis point of the flow cytometer and cell sorting resulting from charge manipulation of droplets (right). From Purdue Cytometry CD-ROM vol. 1 (adapted with permission of the publisher).

particles per second. A further disadvantage is that the particles are sorted into a continuous stream of fluid rather than in a small droplet. This means that all the fluid is also collected, so particles typically require concentration by either centrifugation or filtration.

The large range of user changeable options on some sorting instruments make them considerably more difficult to operate than analysis-only models. Wavelength-scanning flow cytometers are also being investigated (25), and the so-called solid-state cytometers have recently been marketed for microbiological examination of water and food, where microbes have been collected on slides or membranes. These latter cytometers work by scanning the laser light over the flat surface and recording light scatter and fluorescent signals from cells in a similar way to that used in analyzer flow cytometers. Examples of such instruments include the ChemScan RDI instrument (Chemunex, Maisons Alfort, France) (Fig. 2) and CompuCyte (CompuCyte Corp., Cambridge, Massachusetts, USA).

A range of instruments are available that can detect cellular microorganisms and measure their light scatter and fluorescence properties. Particles that are sufficiently different in measurable properties can be discriminated and, using cell sorting instruments, physically separated from one another. For example, it is possible to differentiate some taxa of microorganisms, such as algae from yeasts from bacteria, simply by measuring the light scatter and autofluorescence of the individual cells (24). However, even with the best resolution available with conventional FC, most microorganisms within any particular phylum would be impossible to tell apart without artificially introducing some difference. For example, the majority of bacterial species, incredibly diverse although they are, appear very similar in terms of their light scatter and autofluorescent properties. To overcome this limitation, labeling techniques are used that discriminate groups

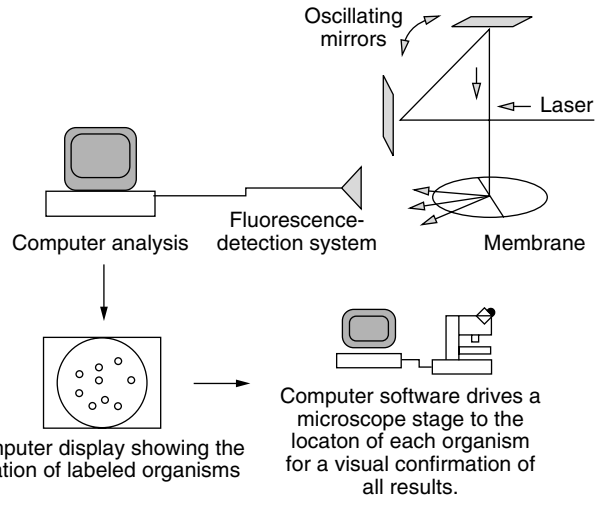

Figure 2. Detection of labeled organisms using the ChemScan RDI instrument. After analysis, the system permits all fluorescent events detected on the membrane to be visually validated by an automated microscope (from Ref. 26).

of microorganisms, typically by making them fluorescent. Various fluorescently labeled antibodies, lectins, and nucleic acid probes or fluorescent dyes are available (27). A considerable range of fluorescent dyes (fluorochromes) are available, for example, ones that bind to specific cell compounds, such as proteins (fluorescein isothiocyanate [FITC]), nucleic acids (propidium iodide [PI]), and lipids (Nile Red); those whose fluorescence depends on cellular physiological parameters (pH, membrane potential, etc.); and those whose fluorescence depends on enzymatic activity (fluorogenic substrates), such as esterases, peroxidases, and peptidases (Table 1) (13). Nonetheless, the most common application of fluorochromes is through their uses when conjugated to antibodies or nucleotide probes to directly detect cell antigens or sequences of DNA or RNA.

Flow cytometers count thousands of cells for each sample and for each cell the amount of forward and side angle light scatter and fluorescence collected by the various detectors is recorded. The actual amount of light (fluorescence or scatter) that is detected is recorded as arbitrary numbers, called *channels*. Channel values range from 0, corresponding to the lowest intensity, up to the highest intensity that can be a number from 64 to 9,999 depending on the setup of the analysis software. The raw data from the flow cytometer could be tabulated, as shown in Table 2.

It is possible to utilize all the data presented, as presented in Table 2, and look at the properties of each cell individually, as one might do when looking down a microscope. For example, in Table 2 the first cell had brighter green fluorescence (FL1) than the second, but that the second cell had brighter red fluorescence than the first and may be a different cell type. The third cell had almost no fluorescence at all and may be different again. With so many thousands of particles to consider however, flow cytometric data is not analyzed in this way, but is plotted in a number of readily interpreted formats. The most common data formats presented are histograms and dot-plots. Histograms show one property, perhaps FL1 intensity, against the number of cells that had a particular intensity for that property. Dot-plots display two properties against one another, for example, SSC against FL1. On a graph of SSC versus FL1, every cell would be shown as one dot, hence the term dot-plot. The limitation of dot-plots is that cells with the same measured optical properties are piled up on top of one another. Three-dimensional isometric plots and contour plots are variations on the normal dot-plot and are used to indicate the number of cells for each optical property coordinate (Fig. 3).

FC can be used to provide multiparametric data for each cell in the sample of interest. Hence, rather than visual comparison of two- or three-dimensional plots, more efficient methods to reduce the dimensionality of the data (such as principal components analysis or supervised multivariate data analysis) allows fewer graphs to be examined, and gives a statistical prediction of the identity of the analyzed particles. In this regard, artificial neural networks may prove to be the most suitable method of data analysis (28).

Table 1. Some of the Fluorescent Molecules Used to Study Microorganisms by FC (13)

Dye	Excitation Wavelength (λ_{max}) (nm)	Emission Wavelength Wavelength (λ_{max}) (nm)	Ligand or Substrate	Applications
TOTO-3	642	660	DNA, RNA	DNA quantification, cell cycle studies
SYTOX Green	504	525	DNA, RNA	Viability, DNA quantification
PI	536	625	DNA, RNA	Viability, DNA quantification, cell cycle studies
Ethidium bromide	510	595	DNA, RNA	DNA quantification, cell cycle studies
Hoechst 33258/33342	340	450	DNA (GC pairs)	Cell cycle studies
SYTO 13	488	509	DNA, RNA	Viability, DNA quantification, cell cycle studies
Mithramycin	425	550	DNA	Cell cycle studies
Pyronine Y	497	563	RNA	RNA quantification
FITC	495	525	Protein	Microbe detection
Texas Red (sulforhodamine isothiocyanate)	580	620	Protein	Microbe detection
Oregon Green isothiocyanate	496	526	Protein	Microbe detection
Indo-1	340	398–485	Ca^{2+}	Ca^{2+} mobilization
Fura-2	340	549	Ca^{2+}	Ca^{2+} mobilization
Fluor-3	469	545	Ca^{2+}	Ca^{2+} mobilization
BCECF	460–510	520–610	pH	Metabolic variations
SNARF-1	510	587–635	pH	Metabolic variations
$DIOC_6(3)$	484	501	Membrane potential	Antibiotic susceptibility, metabolic variations
Oxonol [$DiBAC_4(3)$]	488	525	Membrane potential	Antibiotic susceptibility, metabolic variations
Rhodamine 123	507	529	Membrane potential (mitochondria)	Antibiotic susceptibility, metabolic variations
Fun-1	508	525–590	Yeast vacuolar enzyme activity	Yeast metabolic state
Nile Red	490–550	540–630	Lipids	
Lectins	Depends on fluorochrome conjugated	Depends on fluorochrome conjugated	Membrane oligosaccharides	Cell wall composition, microbe detection
Fluorescently labeled oligonucleotides	Depends on fluorochrome conjugated	Depends on fluorochrome conjugated	Nucleotide sequences	Microbe identification
Calcofluor white	347	436	Chitin and other carbohydrate polymers	Fungal detection
Substrates linked to fluorochromes			Enzyme activities	Metabolic activity
Antibodies labeled with flurochormes			Antigens	Microbe detection

Table 2. Example of Possible Raw Data Output from a Flow Cytometer

Detector Measures	FSC Forward Scatter	SSC Side Scatter	FL1 Green Fluores-cence	FL2 Orange Fluores-cence	FL3 Red Fluores-cence
Data					
First cell	50	70	921	310	105
Second cell	120	90	191	250	940
Third cell	38	94	2	2	4
⋮	⋮	⋮	⋮	⋮	⋮
*n*th Cell	80	52	690	500	321

APPLICATIONS

Conceptually, there are two approaches used in analytical FC. The most common is population detection and enumeration, in which the clustering of numerous events forms a population. In these circumstances, there may be some overlap between the optical properties of the target population and that of other particles, but by virtue of the shear weight of numbers the presence of the target cell type is perceived. On the other hand, rare or single target cells would either be impossible to discriminate from the background noise or be considered negligible in number.

Therefore, the second conceptual area is the detection of rare events, in which a single event may need to be

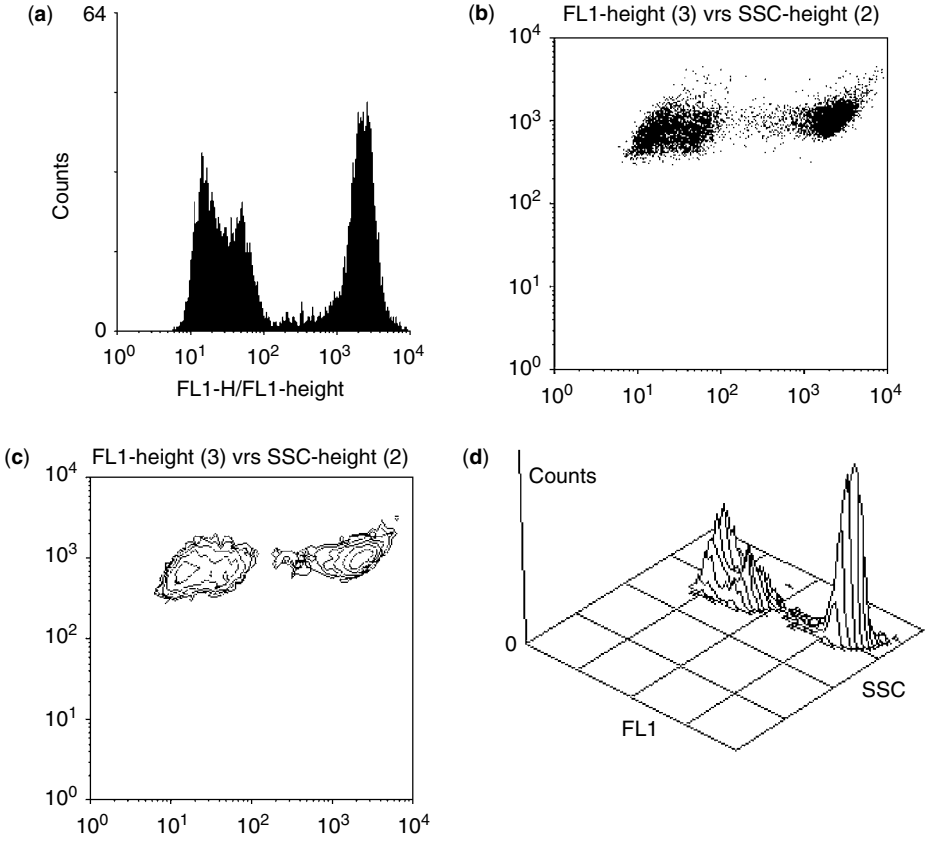

Figure 3. Some common plot types used for flow cytometric data presentation. Panel (**a**) shows a histogram plot for which the *X*-axis shows green fluorescence (FL1) and the *Y*-axis shows the number of cells with a particular fluorescence values (counts). Panels (**b**), (**c**), and (**d**) show a dot-plot, a contour plot, and an isomeric plot, respectively. In these cases, the *X*-axis shows green fluorescence (FL1) and the *Y*-axis shows SSC. Note that for (**b**), the number of cells at each intensity coordinate cannot be determined, whereas this information is given by contours for (**c**) and by a 3D projection for (**d**). Furthermore, the FL1 and SSC axis scales are logarithmic. Such logarithmic scaling is often used to fit cells with very different fluorescent or light scatter properties onto the same plot.

detected and considered to be a positive target (Fig. 4). This requires the target cell type to have optical properties that are very different from any other particle that may be encountered in the samples of interest, a very challenging test for microbiological analysis of complex and highly variable real-world samples.

Simple cytometers can be used to perform total counts of microbial cultures, such as the Chemunex Autosystem (29) or Microcyte (24). There is, however, an advantage in using more complex instruments, such as the Becton Dickinson FACSCalibur or Coulter XL, in that multiparameter analysis permits several measurements to be made simultaneously. For example, Deere and coworkers (30) used a live cell stain, a dead cell stain, and standardized fluorescent counting beads in a three-color assay. As the machine setup can be recorded and reagents combined into a one-tube kit, the three-color assay is as simple to perform as the one-color equivalent but provides additional data.

Total and Viable Counts of Environmental Communities

A first step in the microbial analysis of nonsterile samples is the determination of the total cell count and total viable count of microorganisms present. This gives a measure of the microbial loading and activity (bioburden) of a sample. In assessments of environmental health, determinations of the environmental bioburden provide valuable data. For example, clean waters tend to have lower microbial loadings than those polluted with organic matter. There are many circumstances under which water used in the production, for example, of foodstuffs and pharmaceuticals may not have to be sterile but the bioburden must be within acceptable limits. The effectiveness of routine processes to destroy microorganisms is also assessed by the determination of the bioburden (viz. heterotrophic count in treated waters).

Total Counts. Owing to the nonculturability of most environmental microorganisms, and the labor involved in microscopic techniques, FC is a first choice method for making bioburden determinations in environmental water samples, and staining and analysis methodologies are at an advanced stage. Total counts of waterborne microorganisms have traditionally involved epifluorscence microscopic enumeration of microorganisms supported on

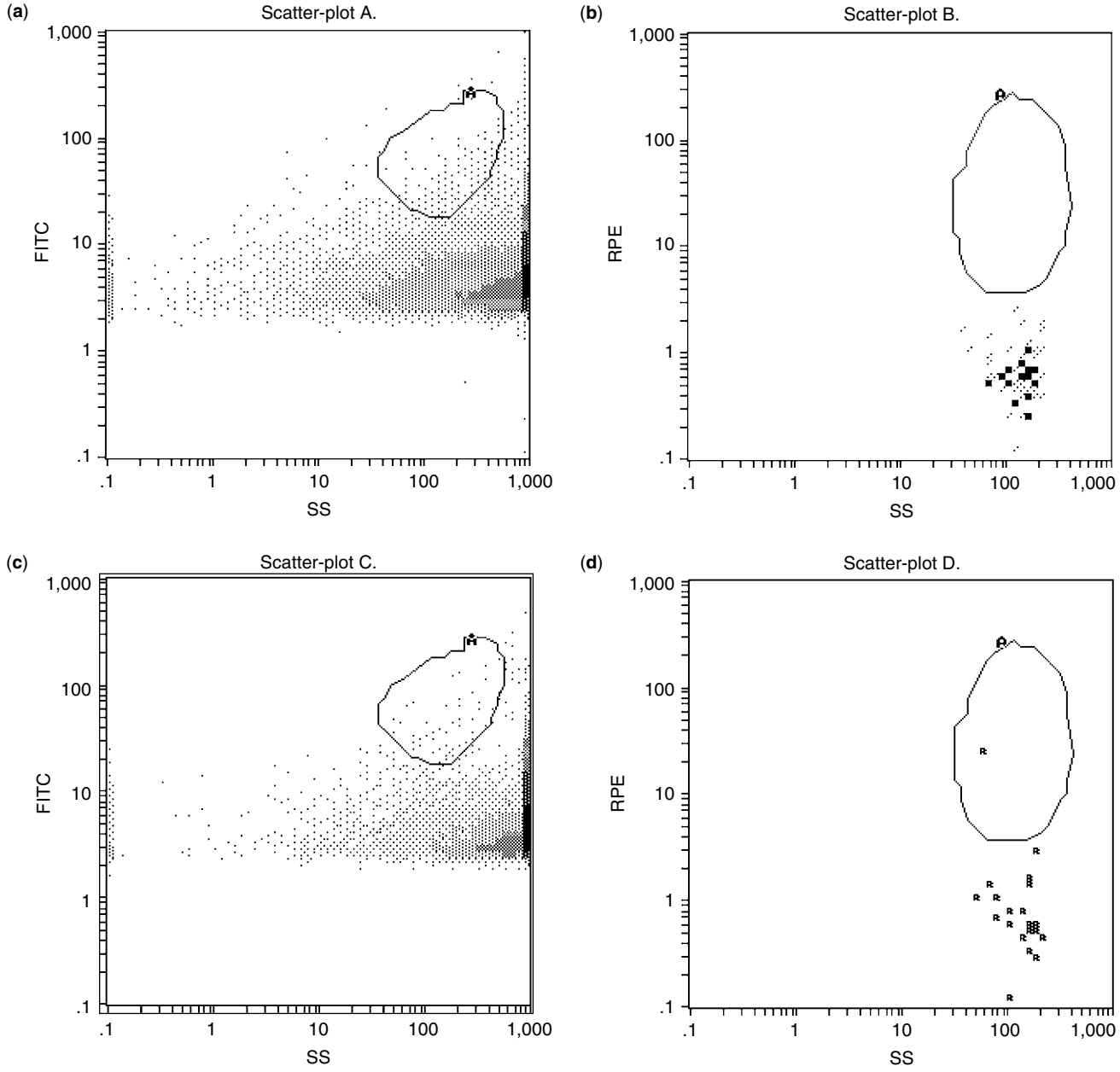

Figure 4. Analysis of river water samples for *D. discoideum* spores. Spores were stained with FITC and PE using two different spore coat mAbs. Axes are Log 90LS (SS) and either Log FL1 (FITC) of Log FL2 (RPE). Scatter plots A and B represent a negative (unseeded) sample. The area in scatter plot B is used to gate scatter plot B. All spores will appear in the area on scatter plot B. Note that there was no spore in this area on scatter plot B. Scatter plots C and D represent the same analysis performed on a similar sample seeded with an estimated single spore. Note the single particle in the area on scatter plot D.

a filter after staining with nucleic acid intercalating dyes, such as acridine orange, DAPI, or Hoescht (31). Various UV excitable dyes have also been used in FC (Table 1). However, the visible excitable nucleic acid stains exhibit significant staining of noncellular particles. Furthermore, the use of DAPI or Hoescht dyes is limited to the handful of microbiology laboratories with access to cytometers configured with UV excitation sources (most use 488 nm lasers). Nonetheless, new generation dyes that have superior nucleic acid specificity compared with acridine orange and are excited by a 488-nm light source have recently become available. For example, Gasol and Del Giorgio (32) have reviewed a range of new SYTO dyes and shown them to give similar results to those obtained with DAPI and direct epifluorscence microscopic counts. Pioneering work by Marie and coworkers (33) involved using the SYTO dyes and many other nucleic acid stains in an attempt to develop methods for the analysis of marine samples. Initial attempts proved unsuccessful because of the delicate biota of these marine samples necessitating

staining directly in the seawater, Hoescht remained the most appropriate dye for this purpose. More recently, 488 nm excitable nucleic acid stains were found to perform adequately in lightly buffered seawater (34). Analysis of both marine samples and pure microbial cultures labeled with the new dye yielded staining patterns, subpopulation distributions and counts that were similar to those obtained after Hoescht staining.

Viable Counts. Rapid bacterial detection and viability measurements have been greatly enhanced by recent advances in the use of fluorescent stains in cytometry. It has previously been shown that four physiological states can be distinguished: reproductively viable, metabolically active, intact, and permeabilized. Previous sorting experiments have shown that not all intact cells readily grow, but some intact cells can grow even when they fail to show metabolic activity, as determined by esterase turnover. To circumvent the limitations imposed by active dye extrusion or cell dormancy on viability measurements used to date (e.g., enzyme activity or cell polarization), a fast triple fluorochrome staining procedure has been developed that takes account of these problems. This allows further cellular characterization of intact cells by active exclusion of ethidium bromide (EB) (metabolically active cells), uptake of EB but exclusion of bis-oxonol (de-energized but with a polarized cell membrane), and uptake of both dyes (depolarized). Permeabilized cells were identified by PI uptake. The method was validated using an electronically programmable single cell sorter (EPICS Elite®) and aged *Salmonella typhimurium* cells. Reproductive viability was determined by sorting single cells to their staining pattern directly onto agar plates. Most polarized cells could be recovered as well as a significant fraction of the depolarized cells, demonstrating that depolarization is a sensitive measure of cell damage but a poor indicator of cell death (35).

There is considerable discussion in the literature on the possible existence of a viable but nonculturable (VBNC) state in many pathogenic and environmental bacteria (36–40) (see Viable but Not Culturable Microorganisms, this Encyclopedia). FC was used directly by Kell and coworkers (38) to investigate, for each cell, attributes, such as apparent cell integrity and the possession of some form of measurable cellular activity and compared to growth on artificial media (culturability). They clearly showed that putative VBNC cells were present, but suggested that most of the reports claiming a return to culturability have failed to exclude the regrowth of a limited number of cells that had never lost culturability. Hence, failure to clearly differentiate between the terms "viability" and "culturability" is fuelling the current debate, and Kell's group along with Barer and Harwood (41) suggest the replacement of the term "VBNC" with expressions that are internally consistent.

An investigation by Domingo and coworkers (42) on the survival of four *Salmonella* strains in river water microcosms included comparisons to standard culturing techniques, direct counts, whole-cell hybridization, scanning electron microscopy, and resuscitation techniques via the direct viable count method and FC. Plate counts of bacteria and in situ hybridization studies suggested a rapid decrease in ribosomal content and viability in filtered and untreated river water (several orders of magnitude within the first week of incubation). In contrast, direct counts remained relatively constant during 45 days in all microcosms. Although the culturable counts of two bacterial strains in filtered water after 31 days represented approximately 0.001% of the total counts, direct viable counts and resuscitation studies with a dilution series suggested that the number of viable bacteria was at least four orders of magnitude higher. Additionally, notable changes in forward scatter and in nucleic acid content were observed by FC only 4 hours after nutrient amendments. However, cells from the resuscitation experiments did not grow on solid media unless cell-free supernatant from viable cultures was added during the resuscitation period. The authors concluded that no immediately culturable *Salmonella* were present, which is similar to the conclusions drawn by others (40).

Total viable bacteria in marine aquaculture have also been determined, using a fluorescein diacetate (FDA) FC method (43). Within a few minutes the difference in the fluorescence scattergram between viable and dead was determined, and with the addition of an antibody, cells of the fish pathogen *Lactococcus garvieae* could be enumerated. One FC assay could be completed within 2 minutes and the total assay time including the preparation of bacterial sample was within 3 hours.

In many circumstances, microbial viability assessment is just as important as the determination of total microbial numbers. However, as is evident from the above discussion, a generally applicable probe for assessing total bacterial viability has not been conclusively identified. One difficulty is that any assessment of viability would require the measurement of some degree of activity and microorganisms with very low levels or in states of dormancy would be very difficult to distinguish from dead ones. Nonetheless, FC in combination with fluorogenic assays of enzyme activity has been shown to be appropriate, and FC can even be employed in difficult media, such as milk (44).

Determinations of Proportions of Major Subpopulations

The discovery of a major genus of marine photosynthetic prokaryotes by FC (45) demonstrated the power of FC for enumerating subpopulations of aquatic microorganisms. Fluorescent emission from endogenous pigments (Chlorophyll-A and phycoerythrin) is used to detect the phototrophs and fluorescent DNA stains are used to determine total microbial numbers. The information is combined with light scatter data to discriminate several ecologically diverse microbial groups. Using bivariate plots, as many as six taxa can be conclusively discriminated and results have demonstrated that the heterotrophic subpopulation had been grossly overestimated in the past owing to their increased culturability (46). More complex statistical techniques discriminated even more taxa; Carr and coworkers (47) used a multivariate approach to group 32 distinct populations of phytoplankton. Neural networks have also been trained to discriminate naturally fluorescent planktonic subpopulations (48). However, although these studies are useful and powerful,

they are ultimately limited by their reliance on discrimination based on inherent optical properties.

A more detailed and rational assessment of the true phylogenetic biodiversity and constitution of environmental samples is extremely difficult to achieve owing to the nonculturability and optical similarity of most microbial species. At present, quantitative direct methods for phylogenetic discrimination of microorganisms can be achieved by hybridizing fluorescent oligonucleotides of defined specificity to the ribosomes in situ within permeabilized whole cells (49,50). For example, FC was used to directly analyze the microbiota of activated sludge (the microbial consortium used to treat most of the sewage and wastewater in the developed world) to quantitatively assess the contributions of different phylogenetic groups to the sludge floc community. Data analysis revealed phylogenetic distributions substantially different from those found using culture based techniques leading to an improved understanding of floc microbiology (51). Similar problems were identified for marine bacterioplankton analyses, where even relatively short-term dilution-culture experiments do not measure in situ growth, but rather growth patterns of a selected enrichment (52). Furthermore, Fuch and coworkers demonstrated that the combination of flow cytometric analysis and sorting combined with fluorescence in situ hybridization (FISH) and degrading gradient gel electrophoresis analysis presented a fairly rapid method of analyzing the taxonomic composition of marine bacterioplankton. The FISH procedure has also being developed as a tool for species level phylogenetic tagging of protozoan pathogens detected in water samples, as the antibodies used are only genus-specific (53–55).

Detection of Specific Cell Types

In the FC detection of individual species or strains of microorganisms, one is often looking for rare events in a background of many nontarget organisms. Prepurification protocols may be possible, but are not preferred, owing to the complexity of the method and potential losses. Hence, key issues discussed next include FC sensitivity and thresholding, both necessary instrument limitations that need to be understood for single event monitoring.

Sensitivity. The detection of low densities of a precisely defined target group of microorganisms is essential for routinely monitoring microbial pathogens and studies of epidemiology and microbial ecology. The specificity of the target group ranges from very general (e.g., wild-type yeasts in a wine production culture), up to strain specific (e.g., *Escherichia coli* serotype 0157 : H7 in meat products). The density of the target microorganisms that it is desirable to detect also varies. For example, for practical monitoring, organisms that have a very high probability of infection per organism, such as the protozoan *Cryptosporidium parvum*, require analysis that can detect one organism per 100 L of water. In comparison, bacterial pathogens, such as *Legionella pneumophila* that have a very low probability of infection per organism, only require analysis that can detect several hundred per milliliter of water. Nonetheless, what these applications have in common is the relative rarity of the target population, typically constituting much less than 0.1% of the microbial community.

Furthermore, to the flow cytometer, the target cells are just another particle and the inherent scattering and fluorescent properties of microbial cells are very similar both to one another and to many noncellular particles. International food and water guidelines often suggest detection limits of one cell in extracts from 25 g of food or concentrates from 100 mL of water, a target cell could be just one of say 10^{11} particles that would be detected above the maximum triggering threshold appropriate for the inherent cellular properties of the target cell type.

Thresholding. A quick calculation reveals that for a flow cytometer to analyze 10^{11} particles at a rate of 10,000 per second (the maximum rate for most cytometers) would require an analysis time of about 4 months! Flow cytometers can, however, analyze samples at rates much greater than 10,000 events per second if the cytometer is set up so that it ignores most of the particles; this is called *thresholding*. The cytometer is set up with a threshold level on one parameter (e.g., forward angle light scatter or a fluorescence detector). Any particles that produce a signal on the threshold parameter below the threshold level are ignored by the cytometer. In most traditional applications of flow cytometry, light scatter is used as the threshold. When attempting to detect a single particle in among 10^{11} other particles with similar light scatter properties this is obviously not appropriate. Therefore, some optical difference must be artificially created between the target and extraneous particles to enable threshold determination. The degree and specificity of this difference is proportional to the threshold level that can be applied and in turn to the speed at which the sample can be analyzed. In almost all applications of flow cytometry to rare cell detection, the optical discriminator used to resolve target from nontarget microorganisms is a fluorescent intensity measurement resulting from a specific fluorescent label. However, it would also be possible to resolve emissions using phase, time, or spectral fingerprint. In practice, the degree of specificity required depends on the desired detection sensitivity. The ultimate sensitivity achievable is the conclusive detection of one microorganism from a large and complex sample, for example, 100 mL water or homogenate of 25 g food.

Detection of Single Cells. The development of a detection method simply involves defining one or more regions, on dot-plots, which enclose all target cells. No events should appear in that region for samples from which that cell type is absent. Furthermore, the number of seeded or naturally occurring target cells present in a sample should equate to the number of events in this region. However, the attainment of such simple methodology and exquisite sensitivity requires a degree of specificity that is very difficult to achieve.

Negative samples often contain interfering particles that will appear within the defined target region. These interfering particles can be either mineral or biological in nature and fluoresce owing to association with the fluorescent label or inherent autofluorescence. Some of the interference may not be from a real particle at all, but

generated by internal electrical noise from the cytometer circuitry that the cytometer mistakes for a particle. Despite these difficulties, detection of single specific microorganisms has been demonstrated. For example, in concentrates prepared from 5 L of turbid water samples Vesey (56) detected the spores of a protozoan slime mould, *Dictyostelium*, at a level of one spore per sample. The organism was labeled with phycoerythrin (PE) and FITC using two monoclonal antibodies that bind to the surface of the spores. The region enclosing stained spores was determined from their light scatter, green fluorescence, and orange fluorescence characteristics. The analysis protocol consisted of enumeration of events within the region representing FITC labeled spores on a dot-plot then gated by the region delineating PE labeled spores (Fig. 4). This level of sensitivity required two highly specific labels and was achieved, without need for sorting and confirmation, using an analysis-only flow cytometer.

Although macromolecular stains, such as nucleic acid intercalating dyes, exhibit excessive nonspecific binding, they are at least partially selective. A number of studies have used this selectivity to help reduce the background count from negative control samples by discriminating dye stained (including cells) from nondye stained particles. For example, using PI, Page and Burns (57) were able to significantly reduce, although not eliminate, background noise. The additional parameter of PI staining was useful when using FC to enumerate flavobacteria introduced into soil. Nonetheless, all methods, including FC, are limited for determining whole-cell numbers in soils and sediments owing to the difficulty of recovering cells bound to particles and aggregates (57).

High Sensitivity Analysis Using Cell Sorting. If a highly specific labeling regime suitable for FC is unavailable, the analysis-only flow cytometer will detect some false-positives and will not conclusively detect rare cell types. Under these circumstances, FC may still be useful in the detection of extremely rare cell types when applied as a means to physically select suspect particles (target cells and false-positives) by sorting. Flow cytometric cell sorting (FCCS) rapidly purifies and concentrates a sample for subsequent analysis using methodologies such as culture, microscopy, or gene probes. For example, Porter and coworkers (58) used FCCS to sort *E. coli* from polluted lake water. Positive events were selectively sorted and subsequently cultured for confirmation and enumeration in the presence of a much-reduced level of competing microorganisms.

During the mid-1990s, water utilities across the globe took to FCCS as part of routine monitoring of water for the presence of cysts and oocysts of the protozoan pathogens *Cryptosporidium* and *Giardia* (59,60). The protocols used could detect one cyst or oocyst in concentrates prepared from 10 to 1,000 L of water, depending on turbidity. Suspect particles were identified by the cytometer, sorted onto slides and enumerated by microscopy. These protozoan pathogens are the most commonly identified cause of waterborne disease in developed countries (61). The cysts and oocysts are robust enough to survive conventional water purification procedures, such as chlorination. This robustness also leads to their prolonged persistence in environmental samples such that they remain viable far longer than the indicator organisms normally used to detect fecal contamination. The probability of infection by very low doses is appreciable. This means that, for practical purposes, any detection method for treated waters would need to be able to find one cyst or oocyst in 10 to 1,000 L of water (62). Of all the methods available, those using flow cytometric sorting are the most effective currently developed (59,63), but methods using less expensive hardware are generally preferred (64).

RAPID METHODS USING FC

FC is probably the method of choice for reliable, rapid, and automatic microbiological analysis. If direct detection is not used, then selective concentration of target-cells, either aids microscopic or molecular analysis. In such a case, FC behaves as an advanced sample preparation method. For detection of specific culturable or nonculturable cells, sensitivities may need to be in the range of one cell per 25 g food or 100 mL water. Emerging microarray technologies (65), are currently less sensitive and require field testing for environmental samples.

Of a range of possible techniques, viability assessment in combination with immunolabeled bacteria with two/three color fluorescence flow cytometric analysis offers excellent activity measurement of specific cell types (66). For example, with the appropriate filter sets on commercial cytometers (Bryte-HS, Bio-Rad, Hercules, CA and FACScan, Becton Dickinson, San Jose, CA), the measurement of separated green (SYBR Green I), orange–red (PI), and far red (RPE-Cy5) fluorescence is possible, allowing the enumeration of viable immunodetected bacteria. Such a technique is quick (<3 hours), and offers numerous possibilities for rapid and precise analyses in sanitary, industrial, and environmental microbiology (43). Rapid methods can also be applied to the detection of human viruses in the environment, with infectious viruses being stained within host cells (11,67) or by detecting changes in the host cell morphology (cytopathic effect), both at an earlier stage than by conventional methods (68,69). Marie and coworkers (70) also describe the use of FC for direct detection of aquatic viruses in the marine environment, and a range of viruses more generally (71).

Sample Preparation for FC

In sample preparation, there are four important issues:

1. *Retention of target cells*, particularly important in quantitative assays, as, unknown losses of target cells will contribute to the analysis yielding an inaccurate, and probably highly variable result. The most common processes that impact on the full retention of target cells are concentration and purification. Commonly used concentration methods, such as centrifugation or filtration, often result in portions of a sample (and therefore target cells contained within the sample) being lost. Also, the forces applied to cells in samples processed these

ways can cause physical damage, which means that they are not recognized as target cells by the flow cytometer, and ultimately are incorrectly omitted from the result. Where concentration and/or purification of a sample is performed as part of a quantitative assay, losses of target cells must be assessed and accounted for accurately.

2. *Size limitation of particles in a sample*, particles contained in a sample analyzed by a flow cytometer must pass wholly through the flow orifice (typically less than 100 μm in diameter), and not disrupt the laminar flow when it does. To avoid the possibility of two particles jamming together in the flow orifice, and thereby blocking the orifice, the maximum size of particle in the sample should be at most half the diameter of the flow orifice. In practice, particles larger than half the diameter of the flow orifice can be analyzed by a flow cytometer; however, blockages do occur frequently the larger the inherent sample particle size becomes. The size of particles contained in a sample can be limited by passing the sample through a mesh filter of specified weave size. However, when processing a sample in this way prior to flow cytometric analysis, care must be taken to ensure that the number of target cells lost from the sample is assessed.
3. *Ensuring a monocellular sample*, as flow cytometers can only analyze discrete entities within a sample. A floc of particles is recorded by the cytometer in the same way as a large particle. This means that the instrument cannot discriminate between a large particle and a clump of particles. If clumps are not dispersed, target cells may not be analyzed by the flow cytometer because they have been part of a clump of particles. Thus, it is important that the particles within a sample are monodispersed prior to flow cytometric analysis, such as by the introduction of surfactant agents (e.g., detergents), or physically (e.g., vigorous vortex or passing through a mesh filter).
4. *Removal of fluorescent staining inhibitors*, which can dramatically affect the performance of immunofluorescent staining methods used to selectively label target particles. This is mostly owing to substances inhibiting the monoclonal antibodies from binding to the target epitopes. These substances can include soluble organic and inorganic compounds (e.g., alcohols or salts). It is for this reason that the suspension fluid of the sample must be compatible with antibodies used to perform the immunofluorescent staining. Replacing the suspension fluid with a suitable buffer can be done by concentrating the sample and re-suspending in a new buffer solution. However, throughout the concentration steps, attention must be given to the guidelines given above for sample preparation in order to preserve the other properties of the sample.

Labeling of Cells for FC

For FC, discrimination of different cell types and particles is generally achieved using fluorescent labels, as described in the previous sections. Fluorescent probes exist for a variety of cellular functions; here, we restrict the discussion to those that reflect cell viability (72). Although the debate as to the efficacy of these probes (i.e., whether they truly reflect viability) is likely to continue as a subject of some controversy, they can be unequivocally validated for particular applications. For example, Porter and coworkers (73), Diaper and Edwards (74), and Deere and coworkers (30) applied this principle to validate a range of dyes as alternatives to colony counts by measuring the culturability of stained and unstained cells after sorting. Assay confidence can be further improved by using combinations of probes that preferentially stain either live cells or dead cells. Even the protein content of cells has been determined using fluorescence and FC (75).

Physiological Labels. To flow cytometrists, the most familiar method of determining viability of leukocytes is the use of dye exclusion by intact membranes. These methods are also applied in microbiological studies to bacteria (76–78) and yeast (30,79). Cells with intact membranes are effectively impermeable to charged dyes, such as PI, EB, and SYTOX. However, if membrane integrity is lost, these dyes can enter and concentrate in association with the nucleic acid within cells, causing permeable cells to become fluorescent. PI is the preferred choice for dye exclusion assays because it has two positive charges making it less permeable than dyes, such as EB or 7-amino-actinomycin D (7AAD) with only one. Emission maxima are green for SYTOX dyes and red for PI, 7AAD, and EB. Problems of nonspecific binding may limit the live/dead discrimination accuracy of dye exclusion methods in highly particulate samples.

Membrane potential studies rely on the presence of an electrochemical transmembrane potential (more negative inside) to preferentially concentrate positively charged dyes, such as rhodamine 123 (Rh123), or exclude negatively charged dyes, such as those from the oxonol group (80–82). Rh123 has been used successfully to determine the viability of pure cultures of bacteria and yeast cells. Staining of dead cells has been investigated using oxonols alone with bacteria (81) or in conjunction with other indicators such as calcofluor white for yeast (82) and protozoa (83), PI for bacteria (80,84), esterase substrates for bacteria (85), or esterase substrates and PI for yeast (30). Rh123 and oxonols are inherently fluorescent, requiring selective concentration within the cell for detection. Whilst this may be acceptable for pure culture applications, nonspecific binding of fluorescent dyes is noted in environments laden with particles (73). In our experience, oxonol is the preferred dye for membrane potential measurements because Rh123 gives comparatively poor discrimination. Both dyes have green emission maxima and are therefore not spectrally compatible in dual stain assays, but can be used in conjunction with dyes such as PI (30).

Many esterified fluorochromes remain nonfluorescent until cleaved by intracellular enzymes, whereupon a fluorescent product is released. Cells only become fluorescent after substrate cleavage by functional cytoplasmic enzymes and product retention by intact membranes.

Therefore, two cellular functions are measured for added assay confidence. Dyes measuring viability using a similar principle are marketed commercially, one for bacterial cells and the other for fungal cells (e.g., Chemunex SA, Paris). The first studies using these types of dye, using FDA, showed poor product retention (86). However, derivatives are now available that have improved retention resulting from additional functional groups. For example, carboxy fluorescein diacetate (CFDA, 27) uses a carboxy group to reduce membrane permeability whilst chloromethyl fluorescein diacetate (CMFDA, 27) includes a chloromethyl moiety that covalently links the esterase substrate to intracellular molecules. A number of these derivatives were investigated using a range of species to monitor the viability of bacterial cultures (74). All were selective to some degree, the most widely applicable being CFDA or ChemChrome B. The validity of these dyes for reflecting true viability during stress has been reported for two bacterial species, *Klebsiella pneumoniae* (74) and *E. coli* (73). Both reports show that during stress incurred by incubation in lake water, the viable count using these fluorescent probes correlated well with the generally accepted standard for viability determination, the direct viable count (DVC, 87). As the DVC measures cell elongation, there is little question that responsive cells are viable. The correlation of the two approaches provided firm evidence that the more rapid fluorescent ester method genuinely reflected viability, despite the stressed cells entering a nonculturable state. These esterase substrates are less susceptible than inherently fluorescent dyes to limitations resulting from nonspecific binding. For example, Porter and coworkers (73) reported that esterase substrates were the dyes of choice to use for assessing total community viability directly in freshwater samples. Their green emission maximum makes them compatible with dyes such as PI.

The electron transport chain of respiring (viable) cells is capable of reducing the membrane permeant, nonfluorescent tetrazolium derivative 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) to a red fluorescent insoluble formazan (88,89). If a cell is sufficiently active, enough precipitate will form for its discrimination from the background. One advantage of this method is that the precipitate may be fixed so as to provide an increased choice of secondary stains. Although this dye has proved useful for pure cultures, it is less generally applicable than esterase substrates for analysis of mixed populations (such as ChemChrome B and V6 dyes, 90) because different cell types require different optimum staining conditions, and abiotic production is also possible.

Enzymatic Labels. Many culture-based methods used specific biochemical assays to identify cell types possessing particular enzymes. For example, the enzyme β-galactosidase is produced by coliforms, where it is involved in fermentation of the sugar — lactose. Flow cytometric assays for β-galactosidase have been described (27) that are applicable to bacterial and yeast cells with active β-galactosidases, either naturally present or transfected. The methods are simple, requiring less than one hour, but specificity must be thoroughly tested in the appropriate sample type because most species of microorganisms in the environment have yet to be biochemically typed and false-positives may result.

Immunological Labeling. Immunological labeling methods are the most familiar to the flow cytometrist. They have demonstrated their utility as specific labels for flow cytometric detection, enumeration, and purification of specific microorganisms. Their scope is limited by the cost of developing specific antibodies. However, once the antibody has been developed production of reagents is inexpensive.

Antibodies must bind epitopes that are always present on, but limited to, the target cell type; if necessary, internal epitopes can be targeted. It is important to realize that epitopes expressed during culture may differ elsewhere, with consequent failure of the detection protocol. For example, during pure culture studies, a sewage isolate of *Ochrobactrum anthropi* bound antibodies during the exponential but not the stationary growth phase (72). It has also been shown that epitopes recognized by antibodies that bind to the surface of *Cryptosporidium* oocysts can be removed by environmental pressures such as water disinfection (91).

Antibodies are large protein molecules and will inevitably bind to contaminating particles present in some samples. These particles may possess the epitope (cross-reactive particles), or associate with antibodies through nonspecific binding. Therefore, the specificity and sensitivity is limited by that of the antibody. For example, antibodies used to detect *Cryptosporidium* are only genus-specific and cross-react with strains that are not harmful to humans (55). Micelles found in milk form globules that bind excessive amounts of antibody leading to very high levels of background staining, reducing sensitivity (44).

Immunofluorescent reagents include either a directly conjugated dye labeled antibody or a labeled secondary antibody that is used to label the primary. Monoclonal antibodies (mAbs) or polyclonal antibodies (pAbs) can be used. For specific detection, direct conjugation is preferable to using indirect secondary staining and mAbs are strongly preferred to pAbs. Furthermore, mAbs of the IgG1 or IgG2 subclass often produce the least nonspecific staining (54). Staining protocols are well understood and require less than 1 hour. However, if suitable antibodies are unavailable, many months or even years may be required for their successful production.

Conjugation of antibodies with fluorochromes is now a routine procedure. Methods are well described for clinical applications (92,93) and many antibody conjugates are commercially available. However, antibody conjugates prepared for an application such as staining clinical samples are often not suitable for staining environmental samples. A small amount of nonspecific binding of an antibody conjugate may go unnoticed when analyzing a stained blood or fecal sample, whereas analysis of an environmental sample stained with the same conjugate is likely to result in the target cells being undetectable as a result of the high background staining. Variation in the binding specificities of antibody conjugates can be due to a number of reasons. The purity of an mAb prior to conjugation with the fluorochrome can

affect its binding specificity (54). Insufficient purification can result in contaminating proteins being conjugated with fluorochrome; these fluorochrome/protein conjugates may bind nonspecifically to particles. The amount of fluorochrome conjugated to a mAb will also effect its binding specificity and brightness after labeling. If too much fluorochrome is conjugated to the mAb, any advantage gained from increased brightness of labeled oocysts may be counterbalanced by an increase in nonspecific binding. Conversely, if too little fluorochrome is conjugated to the mAb, it may not stain target cells with sufficient brightness. Also, the formulation of the mAb conjugate, the working concentration, and the staining procedure recommended by the manufacturer may not be optimum for the analysis of all sample types. Nonspecific binding of antibodies can often be significantly reduced by use of appropriate blocking agents and buffers (94,95).

An alternative to conjugating antibodies with fluorochromes is to conjugate the antibodies to fluorescent beads (96). Suitable beads are commercially available with a wide range of fluorescence excitation and emission properties. The antibody-labeled beads are mixed with a sample to allow the beads to capture the target organism. The target organism can then be labeled with a second specific fluorescent probe that has fluorescence properties that can be distinguished from the bead.

Phylogenetic Labeling. FISH methods, which label specific ribosomal RNA (rRNA) sequences inside intact cells, have been described ("phylogenetic stains"; 97). First, cells are fixed and permeabilized to permit entry of the probe to its target within morphologically preserved cells. Then, conditions of probe binding and washing are selected such that the probe will bind only to its rRNA target sequence. Finally, labeled cells can be visualized by FC. As ribosomes are found in such large numbers within bacterial cells, this naturally amplifies the fluorescent signal. Further, the sequence divergence between the rRNAs of different species has formed the basis of a classification system for microorganisms (98). Portions of rRNA sequences have evolved at different rates and target sequences range from highly conserved (throughout a phylogenetic kingdom) to highly variable (up to strain specific). Information on these sequences, combined with ribosome structural studies, give sufficient information for rational design of selective oligonucleotide probes (see review in Ref. 99). Flow cytometric discrimination of FISH-target bacteria (100) and HIV pro-virus within cell cultures (101) have been described. Wallner and coworkers (10) critically evaluated and optimized protocols for FC and more recently, a modified method was developed for fluorescent labeling of *Cryptosporidium* oocysts (102).

Oligonucleotide probe-conferred fluorescence appears to correlate to cellular rRNA content, which in turn may relate to growth rate, activity, or viability (102–105). In *Cryptosporidium*, FISH has been shown to provide a useful indicator of oocyst viability (102), which antibody labeling methods do not. This is probably owing to the short life of rRNA within nonviable oocysts. Further, FISH techniques can label oocysts with species specificity and so discriminate the species that infects humans (*C. parvum*) from other *Cryptosporidium* species that bind to the genus-specific antibodies used for routine monitoring.

Under conditions of nutrient limitation, such as following pathogen entry into surface or drinking water, however, faecal bacteria undergo a rapid drop in growth rate. The corresponding drop in cellular rRNA content causes hybridization signals to decrease to background levels of fluorescence. It is possible that microorganisms entering into dormant life-cycle phases, such as encystation or sporulation, may similarly undergo some reduction in rRNA levels. For example, we have observed that the fluorescence of oocysts labeled using FISH with FITC conjugated probes is typically more than an order of magnitude less intense than that of oocysts labeled with a FITC conjugated antibody. Therefore, for most species, FISH is limited to the analysis of populations from culture, clinical samples, or environments favoring the target cell type's activity. To date, only modest increases in fluorescent signal-to-background ratio have been achieved using techniques such as multiple probes to different sites on the ribosome (106), enzymatic labeling of probes (107), the use of polynucleotide probes with multiple labels (50,108), and peptide nucleic acid probes (109). A procedure of cell incubation in the presence of a DNA gyrase inhibitor but nutrients (to activate but not allow division of cells, called *probe activated count*, 110) may improve signal strength, but additional signal-to-noise may be necessary for routine FC monitoring of FISH-labeled cells.

Choice of Fluorescent Dye. The choice of fluorochrome used for conjugating reagents used to label microorganisms can have considerable effect on the sensitivity of flow cytometric detection. Different sample types may require different fluorochromes to achieve optimum sensitivity and should be analyzed accordingly. Detection sensitivity is determined by the degree of discrimination of target from nontarget particles. Some nontarget particles will have similar optical properties to labeled target cells owing to nonspecific binding of the fluorescent labels used.

In environmental samples, however, many of the interfering particles are naturally fluorescent (autofluorescent) and include biological debris, algal cells, and inorganic matter. For example, a FITC labeled antibody is used to discriminate *Cryptosporidium* oocysts from other particles. However, particles, such as autofluorescent algae, with similar fluorescence and light scatter characteristics to labeled oocysts are also detected and is one of the reasons that sorting followed by microscopic observation is used to confirm results. A recent study used flow cytometry and fluorimetry to analyze particles present in water to determine the optimum excitation sources and fluorescent labels to use for detection (63). The spectral regions with minimal interference caused by other waterborne particles were identified. The study looked at nine commercially available fluorochromes and four excitation sources covering the range 300 to 700 nm. The longer the Stokes shift (difference in excitation wavelength and emission wavelength) of a fluorochrome, the less the background emission present at its emission peak, and therefore, the more suitable it would be for labeling microorganisms. There was

a trend of decreased autofluorescence intensity at longer excitation wavelengths with two major bands of autofluorescent emission, the shorter beginning at 390, peaking at 440, and ending at 510 nm, and the longer beginning at 640, peaking at 675, and ending at 700 nm. Thus, particles naturally present in water samples would emit detectable autofluorescent noise within these spectral regions. For the detection of microorganisms in water samples, optimum sensitivity would be achieved by avoiding regimes that depend on excitation sources and fluorochromes that require detection in the spectral regions covered by these two autofluorescent bands. In contrast, the emission from waterborne particles was least intense when using an excitation wavelength of between 550 and 610 nm. This implies that the use of fluorochromes excited by wavelengths within this range would be the most appropriate for labeling microorganisms for their detection in water samples. Fluorochromes excited by UV light (351 nm) or red light (633 nm) appeared to be the least useful for labeling microorganisms in both drinking water and untreated water.

There are two main criteria for determining the best fluorochrome for fluorescent labeling of microorganisms in environmental samples. The first is that a labeled microorganism is sufficiently more fluorescent than the autofluorescent particles naturally present in the sample. On the basis of this first criterion, the 488-nm excited FITC fluorochrome is excellent for labeling oocysts in untreated water samples, while the 542-nm excited aminolink carboxymethylindocyanine dye (Cy3), PE, and tetramethylrhodamine B isothiocyanate (TRITC) were the best in drinking water samples (63). The second criterion is that the fluorochrome should not bind to (and cause an increase in the fluorescence of) the particles naturally present in the environmental sample.

The charge and surface hydrophobicity distributions differ between fluorochromes and this will effect the nonspecific binding properties of conjugates. There are reports, however, of cyanine dyes showing excessive binding to monocytes (15). Further advantages of FITC and CY3 are the excitation sources required. The 488-nm lasers are fitted to all conventional cytometers, with the 542 laser required to excite CY3 being the smallest and least expensive of alternatives.

CONCLUSION

The application of FC to environmental microbiology started with the paradigm of the instrument representing a type of automated microscope. The advent of a large array of possible fluorochromes and biochemicals to label target species has now reached a stage where direct analysis FC can be used not only to identify total counts of active cells, but also the presence of low occurrence microbiota within environmentally challenging samples.

As with the advent of other noncultivation techniques in microbiology, FC has both highlighted deficiencies in traditional methods as well as expanded our options for improved analysis of environmental samples. Nevertheless, while FC has taken off in the food and beverage industry, its application to environmental microbiology seems to have been hampered by the cost of instruments. Yet, FC is rapidly becoming a routine methodology in aquatic microbial ecology. The combination of simple to use bench-top flow cytometers and highly fluorescent nucleic acid stains allows fast and easy determination of microbe abundance in lakes and oceans. One of the main advantages of FC over epifluorescence microscopy is the ability to obtain cell-specific measurements in large numbers of cells with limited effort. This characteristic has been used for differentiating photosynthetic from nonphotosynthetic prokaryotes, for measuring bacterial cell size and nucleic acid content, and for estimating the relative activity and physiological state of each cell.

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METHODS FOR THE IDENTIFICATION OF MICROBIAL ISOLATES

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Isolation and identification of microbial isolates is critically important for industrial microbiology (biotechnology), understanding the ecological role of microorganisms and worldwide microbial diversity, and understanding of disease-causing microbes, both current and emerging. Isolating bacteria involves recreating the natural environment or mimicking it as closely as possible so that isolates change minimally during the process. Fresh-stock cultures should be preserved to provide cells that reflect natural characteristics for future use. Identification methods are currently based on classical techniques that have been used for more than a century, and new genetically based techniques developed over the last two decades. This chapter presents an overview of factors that should be considered when isolating and characterizing microbial isolates.

BACKGROUND

One of the great frustrations of microbiologists is the difficulty associated with naming and classifying microorganisms. Because they are so small, it is difficult to differentiate them by a mere handful of physical or morphotypic characteristics that can be gleaned from colony growth and the shape and arrangement of their cells viewed under a microscope. Therefore, traditional classification schemes have been based on physiological tests, chemical content, shape, color, and so on (1,2). These characteristics may vary because of mutation, genetic plasticity, or environmental influences, resulting in confusion and errors in identification. For example, *Serratia marcescens* produces a pink colony at low growth temperatures and a white colony above 30 °C, owing to inhibition of pigment formation. In this case, taxonomic classification based on colony pigment might be unduly influenced by growth temperature. The lack of a metabolic trait might also serve as the basis for a major taxonomic decision. However, a colony that is incapable of expressing one trait, for example, pigment formation, might merely be a mutant strain and not an unrelated species. In addition, not all isolates of a single species respond in exactly the same manner when tested for metabolic capabilities such as substrate utilization. For example, *Hyphomicrobium*, a stalked bacterium found in aquatic environments, might be classified into species, in part, by a specific trait that appears only in 90% of the isolates, for example, growth on lactate. However, the remaining 10% of the *Hyphomicrobium* isolates would respond differently and taxonomic confusion would result (3).

The identification of microbes has rapidly advanced in the last two decades. This can be evidenced by the change in *Bergey's Manual of Determinative Bacteriology*, the foundational book for bacterial taxonomy and classification. The eighth edition (2) was based primarily on classical testing of physiological or metabolic and structural components of microbes; it consisted of one volume. The newest version of the manual, *Bergey's Manual of Systematic Bacteriology* (4), was expanded and published as a four-volume set based on genetic and metabolic/structural characteristics of microbes. Interest in reclassifying microbes of all kinds took shape with the development of new techniques for classifying bacteria based on molecular systematics. Because of efforts to preserve biological diversity, there has been a resurgence of interest in microbial diversity and systematics as well (5). At the centennial meeting of the American Society for Microbiology held in Chicago, Illinois, in 1999, several symposia were presented on just this topic. One of the most exciting advances resulting from new molecular

techniques is that although it may not be possible to "see" microbes using classical techniques, it is possible to "know" about them through the use of molecular methods, often without even culturing them.

This chapter will concentrate on identifying microbes that have been cultured, although culturing microbes from many environments presents a considerable challenge. Microbiologists report that less than 10% of the microorganisms in natural environments are culturable (6,7). This is probably true in many natural environments; however, in those where sufficient nutrient and a supporting physical factors exist, a higher percentage of viable microbes may be culturable (8). Additionally, some bacteria may be viable but nonculturable (see following section), moribund, dormant, or dead (9), especially in environments that are either geologically isolated or nutrient-limited in some manner (10,11). There is a need for a clear picture of the total biodiversity of natural environments; however, some information can only be gleaned from culturing cells. For example, if one is interested in the physiology of microorganisms, it may be important to grow them for experimental manipulation. If one needs a strain for use in biotechnology or another industrial application (12,13), one needs a culture. If one wants to construct genetic variants of the organism or transfer genetic material between closely related strains, one needs the isolates. Microbial banks needed to preserve the diversity of microbial life will need to be based on isolates; otherwise, they are merely gene banks.

OBTAINING ISOLATES

Many techniques are available for the isolation and purification of microbial isolates (14,15). Direct isolation from the environment is the most common means of isolating bacterial strains. Spread, streak, and pour plates, using agar or other solidifying agents, have the potential for separating cells from a mixed culture into individual colonies (Fig. 1). Before the use of agar for solidifying nutrient mixtures, slices of potato and other fruits and vegetables were used for nutrient and support media (16,17). When solid media are not suitable for microbial isolation, and when enumeration is not necessary, enrichment cultures can be produced by mimicking the natural environment or by providing favorable conditions for the organisms in question. For example, enrichments of iron-rich sediments from southern Nevada gave rise to large numbers of iron-oxidizing thiobacilli (Fig. 2) when enrichment cultures supported by a reduced iron source and acid conditions were provided (18). Another example is enrichment for halophilic bacteria from natural salterns, soil, lakes, or oceans. In this case, an aerobic environment containing a solution of 25% sodium chloride with a rich variety of amino acids and proteins will best select for halophiles. Enrichment and enumeration can be accomplished simultaneously using enrichment most probable number techniques (MPN) (19). The MPN technique involves inoculation of a microbial sample into a series of tubes or wells representing 10-fold dilutions. Positive reactions are used to estimate the "most probable number" of organisms of a specific type in a sample from a statistical table. Recipes for media to support a myriad of microbial types can be found in *The Handbook of Microbiological Media* (20). Enrichment cultures often do not enrich for just a single species or strain. Therefore, further purification must be attempted by direct plating on solid media or multiple transfers resulting in dilution to extinction of unwanted strains.

All decisions about what conditions are of use for growth of isolates should provide for the physiological needs of the microbe in question; these conditions will often mimic the natural environment. Microbial cultures can be incubated in a variety of wavelengths and intensities of light, or darkness, temperature regimes,

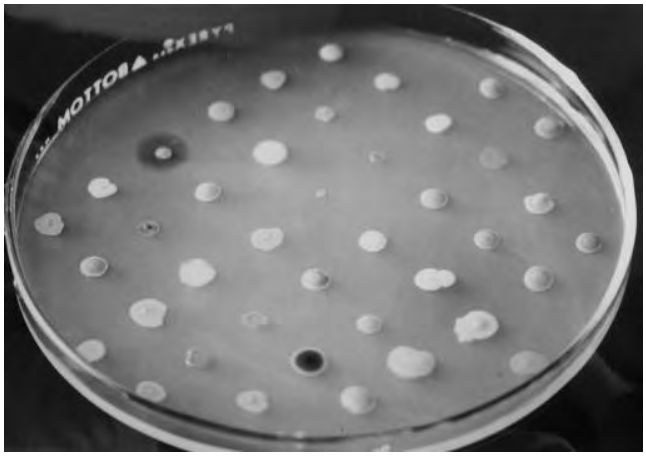

Figure 1. A bacterial garden of subsurface isolates applied to a grid after initial isolation from spread plates. Various colony morphologies and pigment production (e.g., dark colony surrounded by a light ring) can be useful during the identification process. Note: the colony surrounded by a dark ring demonstrates a physiological characteristic, such as agar degradation, that is potentially useful in identification. See color insert.

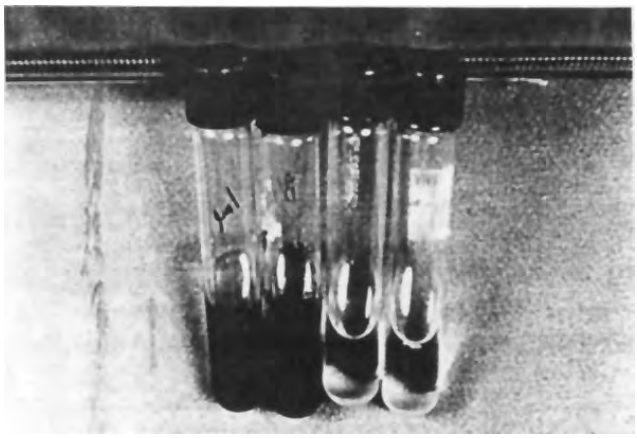

Figure 2. Iron-oxidizing (the two tubes on the left) and sulfate-reducing (the two tubes on the right) bacteria obtained through enrichment from Yucca Mountain, the U.S. proposed high-level nuclear waste repository. Courtesy of Dr. Beth Pitonzo, Characterization of Microbes Implicated in Microbially Influenced Corrosion from the Proposed Yucca Mountain Repository, *Ph.D. dissertation*, University of Nevada, Las Vegas, 1996.

pressures, salinities, and atmospheric conditions. Some cells are strictly aerobic, some are strictly anaerobic, and yet others are microaerophilic, requiring a reduced oxygen level. Cells that are aerobic or oxygen-tolerant can be grown under "room air" conditions. Those with oxygen sensitivity or intolerance require enhanced carbon dioxide incubation or an anaerobic chamber containing oxygen-free gases (Fig. 3). Roll-tubes, Balch pressure tubes, serum vials, and anaerobic/candle jars can also be used to grow anaerobic bacteria (17). Additionally, some microbes may be capable of growth using alternative electron acceptors to oxygen as in the case of nitrate, regardless of the gaseous environment provided; thus these molecules must be added to the growth medium.

Another consideration in obtaining microbial isolates is their interaction with surfaces. Microbes tenaciously stick to particles because of the production of exopolysaccharide (EPS), flagella, fimbrae, and other structures designed for attachment (Fig. 4). Electrostatic forces, hydrogen bonding, and hydrophilic and hydrophobic interactions between cells and surfaces may all play a role in the adhesion of microbes to surfaces (21). If the goal is to remove microbes from surfaces for isolation, such as soil particles, one approach is to interfere with the natural ionic bonding patterns of bacterial cells by shaking them in 0.1% sodium pyrophosphate (22). Centrifugation and blending techniques can also be used to promote the removal of microorganisms from samples and to concentrate biomass for enumeration (23,24). Sonication may separate cells that adhere to one another through biofilm production (25).

Other issues that influence organism isolation include the time of sample analysis and the physiological state of the organisms. Researchers have shown that the microbial communities present in natural samples change during storage (26–28). Immediate analysis of natural samples is critical for culturing some microbes, as faster growing or resuscitated microbes may overgrow those present in

Figure 3. Anaerobic chambers can be used to grow bacteria under defined atmospheric conditions. The gaseous composition can be manipulated to mimic natural environmental conditions. See color insert.

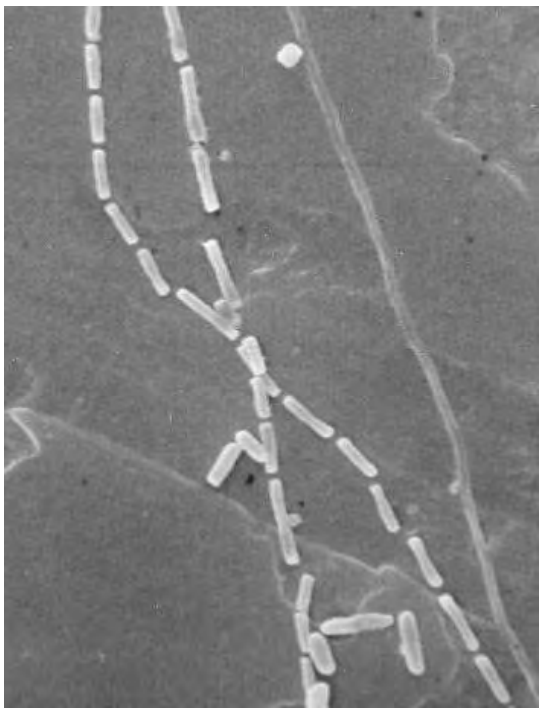

Figure 4. The initial stages of biofilm production on the surface of a polyethylene (plastic) coupon. Individual cells and chains of bacilli are seen attached to the coupon surface in this scanning electron micrograph. Magnification 5,000x. Courtesy of Dr. Gordon Southam.

only low density (29). If cells are injured (30) or viable but nonculturable (VBNC) (31), they may require special resuscitation measures, that is, recovery on a dilute nutrient medium (32) or exposure to distilled water (33). VBNC microbes were first described by Kogure (34) in marine systems where bacteria are nearly all gram-negative and sensitive to the antibiotic nalidixic acid. The resultant nondividing "snakes" were counted and compared to the culturable number from the same sample. The number that could elongate in the presence of nalidixic acid (an inhibitor of division) but could not be cultured were dubbed "viable but nonculturable" or VBNC. Often the previously nonculturable cells are the target of isolation attempts because of their important roles in natural environments. Rita Colwell and her colleagues reported instances where *Vibrio cholerae* and other pathogens have given rise to disease from sources containing VBNC cells (35–37).

Some microbes have not been isolated from natural environments, yet their existence is known because of their activities and they have been observed under the microscope. Techniques are currently emerging to identify these organisms without culturing. These techniques are based on using polymerase chain reaction (PCR) for amplification of indigenous ribosomal DNA (rDNA) (See following section; 38–40). Comparison of cultured isolates and DNA extracted directly from one subsurface rock environment yielded disparate results. The cultured cells were predominately gram-positive species, whereas, the extracted, amplified, cloned, and sequenced rDNA

suggested a community composed predominately of gram-negative bacteria (41).

Once isolates are obtained, it is important that they be maintained as frozen or lyophilized pure cultures. Repeated transfer of natural isolates often leads to loss of characteristics (42–44). Cells can be grown in a nutrient medium and diluted 1 : 1 with 20% sterile glycerol, and can be preserved at −70° to −80 °C. These cultures can be regrown from a few ice-crystal scrapings with a sterile transfer loop or sterile toothpick. Other preservation techniques are described in Gerhardt (15).

APPROACHES TO THE IDENTIFICATION OF ISOLATES

Visual Traits

For some isolates, looking at the colony or the cells under the microscope will lead one to a family or perhaps even a genus designation. However, for many bacteria, for example, the vast number of gram-negative rods, one colony and/or one cell look much like another. In the mid-1800s, not much more than these few observations were available to microbiologists. They extensively stained and described cell shape, cellular arrangement, the presence–absence and cellular location of spores, the presence–absence and types of granules, and motility. Many of these descriptive observations are used in *Bergey's Manual of Systematic Bacteriology* (4). Although this same detail is still available, most microbiologists use more sophisticated techniques when identifying isolates. One important reason to continue to use the older visual methods is that the purity of an isolate needs to be monitored during experimentation or identification. Contaminated cultures are often visible on agar plates if time is taken to observe them carefully. Contamination can also be confirmed using a microscope when the two cultures have different cellular shapes and sizes.

Electron microscopy has provided interesting visual images of bacteria and an ability to visualize cellular projections such as flagella and fimbriae. Cross sections have allowed for confirmation of cell wall structure, capsules, and other layers surrounding cells. Various equipment associated with electron microscopy can also measure the content of structures, for example, crystals, produced by cells (Fig. 5). In addition, special stains can be used to visualize specific structures that may be useful in identification. Much can be learned about individual isolates from microscopy, staining differences, and other visual techniques; however, identification of the organism is not generally possible by these techniques alone.

Physiological/Metabolic Testing

For decades, metabolic testing served, along with visual characterization of isolates as nearly the sole means for bacterial identification. Metabolic capabilities included utilization of various carbon or inorganic substrates, for example, carbohydrates, alcohols, amino acids, ammonium, or sulfate ions, either through aerobic or anaerobic respiratory processes, and enzymatic functions such as catalase and phosphatase. Tables of substrate utilization

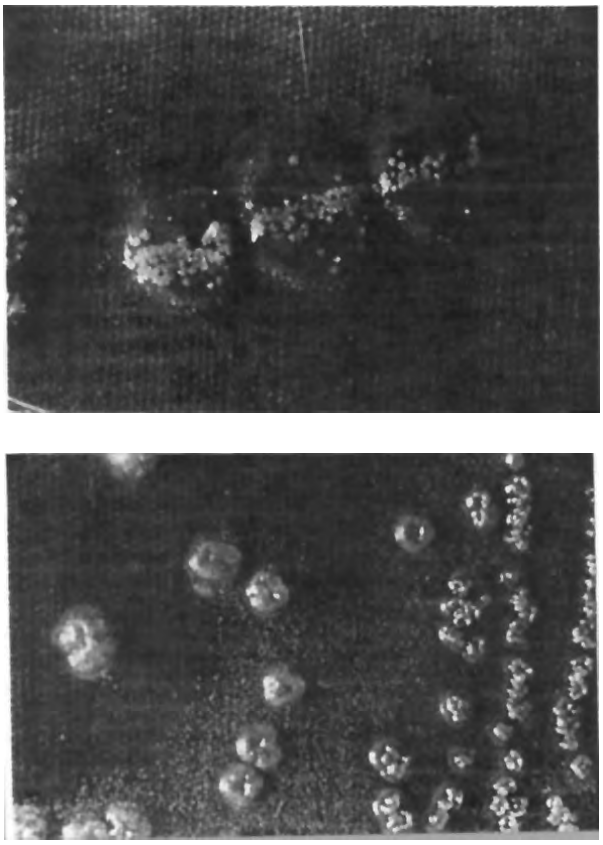

Figure 5. Crystals, visible to the naked eye, were precipitated within the agar matrix (**a**) and/or on the surface of bacterial colonies (**b**) when grown on calcium-containing medium. The ability to precipitate calcium is species or strain-specific. Courtesy of Ms. Mona Khalil.

continue to be an important part of bacterial identification (4). Automated approaches to bacterial identification are based on metabolic characteristics of isolates. These include rapid test strips supplied by API and microtiter plates created for BIOLOG (10) that test for multiple metabolic traits simultaneously. The results are read after incubation of a suspension of a pure culture under specific conditions, and they serve as a means of identifying specific bacterial groups such as enteric bacteria, yeast, anaerobes, and so on. BIOLOG testing is based on a 96-well microtiter plate containing 95 different carbon substrates. Oxidation of the substrate results in the development of a purple color (via a dye complex), the intensity of which is related to the amount of bacterial activity (Fig. 6). One drawback of metabolic techniques is that environmental microorganisms may require a different incubation temperature than clinical isolates. Another drawback for each of these approaches is that a gram stain, or an alternative test to determine cell wall structure (45), is required before the appropriate test strip or microtiter plate can be selected for inoculation. Many environmental microbes are gram variable and thwart attempts at choosing the appropriate metabolic test(s). An example of how important this can be was discovered during characterization of isolates from the subsurface in Rainier Mesa, Nevada

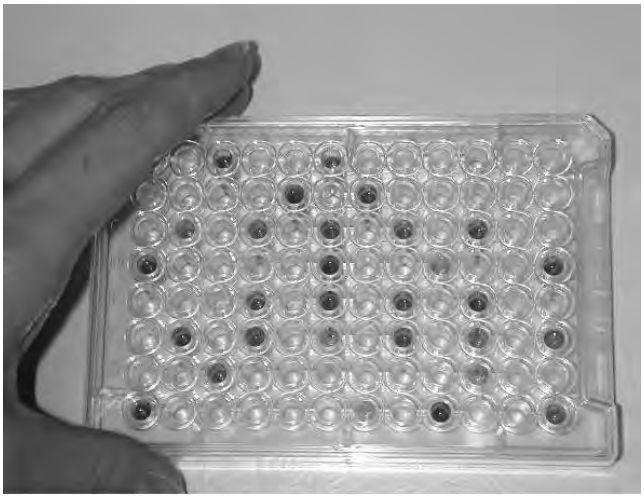

Figure 6. BIOLOG microtiter plates are used to create a metabolic fingerprint useful in the identification of bacterial isolates. Dark-colored wells indicate oxidation of individual carbon substrates. See color insert.

Test Site, Nevada. The isolates appeared to stain gram-negative. Using the API rapid nonfermenting strip, the metabolic profile matched that of *Pasteurella hemolytica*. However, the organisms were actually gram-positive *Arthrobacter* spp. (10). The significance of this error is not just the fact that a strain was mistakenly identified from the environment, but also the implication that pathogenic strains were present when they were not. One advantage to metabolic screening processes such as BIOLOG that contain numerous tests is that even if environmental organisms are not definitively identified, it is possible to develop a tailor-made database within which new isolates can be compared. In fact this may be necessary because most databases created from metabolic systems are based on the identification of isolates of medical importance, but not from natural environments. Additionally, at a general meeting of the American Society for Microbiology held in the year 2000, BIOLOG introduced an automated system utilizing multiple plates that are robotically inoculated, designed primarily for combinatorial analyses.

The importance of other metabolic activities in the identification of specific microbial types are under development but are not yet widely used. RNA provides evidence for metabolic activity and can be used to identify microorganisms in nature, in some cases in situ. The exact placement of these cells in the natural environment may be instructive. Probes can be constructed that target specific mRNA molecules demonstrating which specific cells in soil or biofilms, for example, are metabolically active in situ (46,47).

The presence or absence of resistance markers are traditional characteristics used to profile microbial isolates. Although the information may be interesting and useful in some studies, resistance markers are often located on plasmids and therefore, are in danger of being lost over time (5,48). Additionally, few markers are present in 100% of any one species or strain of bacteria. For example, variable zones of antibiotic and metal inhibition were measured in an investigation to determine diversity within a colony morphotype. Using other criteria, these same organisms were classified as the same species (49).

Although metabolic capabilities have been classical tools for microbial identification, they are not always reliable because of preparation and interpretation of metabolic tests, the fraction of any one species that is positive for a specific activity, and genetic plasticity. For these reasons, the use of metabolic tests to identify organisms have often been replaced by analysis of characteristics that are less variable, such as structural components (chitin, proteins, lipids) or genetic material.

Structural Components

Because some structural components of cells, such as the structure of cell walls, are relatively unchanging, they serve as good characteristics for broad-level identification of microbes. For example, archaebacterial cell walls are not made of peptidoglycan, as are those of eubacteria, rather a complex protein structure and fungal cell walls are composed of chitin. Because of this, suspected archaeal or fungal isolates can be classified into domains without difficulty. Eubacteria can be further classified by the layered structure of their cell walls. One of the first tests conducted on eubacterial isolates is the gram stain. This stain, devised by Christian Gram around the turn of the century, determines placement of bacteria into one of two broad categories based on cell wall structure. Later it was recognized that the gram stain could group cells with many like characteristics together. The principle of the gram stain in identification of bacterial strains is still in use, although an electron micrograph is often used to visualize the cell wall rather than relying on the original staining method.

Lipids are important structural components of microbial cells. Utilizing multiple lipids and components of specific lipids (See SUTTON, NICHOLS chapters), deeper insight into community composition and into metabolic activities can be gained. Although the composition of phospholipids in the cell membrane may change with culturing conditions, under a standardized set of conditions a comparison can be made between microbial isolates. A pioneer in this area, Dr. David C. White laid the foundation for a commercial application that used microbial lipid profiles as identification criteria. Microbial Identification Systems, Inc. (MIDI) uses types and abundances of fatty acid methyl esters (FAME analysis) as an identification tool. FAME profiles are extracted from the cell by saponification, chemically modified (methylated), separated, and identified on a gas chromatograph, or by using gas chromatography/mass spectroscopy. As with metabolic databases, the original database for MIDI was based on pathogenic or medically important isolates and thus was not as useful for environmental microbiology. Over the last decade, isolates from the deep subsurface and from industry have become an important part of the MIDI database. Like BIOLOG, data from the MIDI system can be used to determine the relatedness of isolates even if they are not specifically identified (Fig. 7) (50,51).

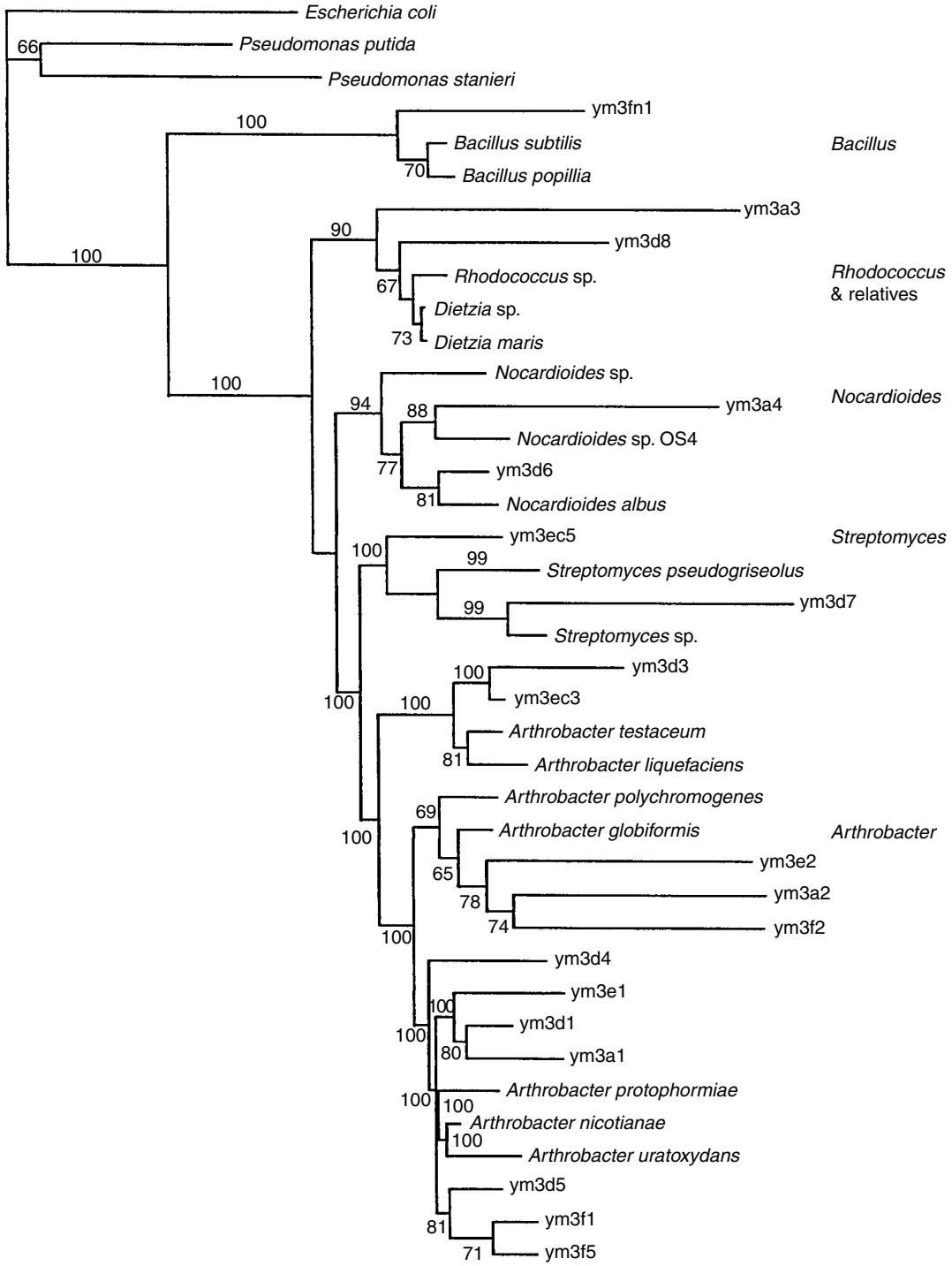

Figure 7. Phylogenetic tree based on MIDI FAME profiles of membrane lipid content. Notice that even unidentified isolates can be clustered near known control organisms. Values represent the percentage of time that the tree would be configured as shown in 100 separate analyses of the data (bootstrapping analysis) (52). Courtesy of Dr. Yingchun Wu, Molecular Analysis of Microbial Diversity Within Yucca Mountain, Ph.D. dissertation, University of Nevada, Las Vegas, 1998.

Other structural components can be used to identify cells by using labeled antibodies against those structures. Antibodies provide for very specific presence–absence and locational information about structures. Although antibodies have commonly been used to detect the presence of specific bacterial types in food and medical applications, they have not commonly been used for environmental isolates or in natural settings. Other structural components of cells, less commonly used for identification, such as flagella, lipopolysaccharide

components, and proteins G and A, and so on are described by others (1,5,15).

Genetic Analysis

By far the most reliable means of identification, and relatedness clustering of isolates, comes from more recent techniques associated with genetic analysis. The eighth edition of *Bergey's Manual of Determinative Bacteriology* (2) was the first edition containing DNA-based identification of bacteria. Individual isolates were described by the traditional means of classification and a percent of the genome accounted for by nucleic acid bases guanosine and cytosine (%G + C). The hypothesis was that cells that were similar would have a similar G + C percentage in their DNA makeup. Therefore, *Escherichia coli* and *Salmonella typhimurium* were expected to have similar G + C percentages because they are closely related. However, a dissimilar genus such as *Arthrobacter* would most likely contain a different G + C percentage. The percentage G + C was determined by a thermal melting curve of purified DNA from the bacterium in question (15). The method worked well for closely related bacteria but the danger that grossly unrelated bacteria might still have similar G + C percentage content was problematic. For example, the G + C percentage content of bacteria ranges from about 25 to about 75%; this range covers the G + C percentage content of all other living things (53). Therefore it is impossible to relate higher organisms to microbes through their G + C percentage content.

A subsequent development that addressed the sequence of nucleotides, not just the G + C content, was that of DNA hybridization. In this method, single-stranded DNA fragments from a microbe of interest were affixed to a filter and then mixed with labeled single-stranded DNA fragments from another bacterium. The strands were allowed to reanneal, and the percentage of DNA homology was determined by the extent to which the label was retained on a filter as double-stranded DNA. The mixed DNA molecules were compared to homologous DNA hybridizations (considered 100% hybridization) (15). This method worked well, but was tedious because many comparisons had to be made and each comparison had to be prepared separately, requiring large amounts of purified DNA.

The next development associated with genetic identification of microbes arose with the discovery of restriction enzymes by researchers at Stanford University. These naturally occurring bacterial enzymes help protect cells from foreign DNA, such as in the case of viral infection. Restriction enzymes cut DNA at sites that are specific for each enzyme. For example, EcoRI, the first restriction enzyme in common use, came from *E. coli* and cut DNA where the following sequence of nucleotides occurs in a strand of DNA:

$$5'\text{G} \downarrow \text{AA TTC } 3'$$

$$3'\text{CTT AA} \uparrow \text{G } 5'$$

Fragments of DNA resulting from restriction enzyme activity could be isolated, separated by gel electrophoresis, and compared to one another. Restriction fragment length polymorphisms (RFLPs) were created by this process and helped compare closely related species to one another without the necessity of harvesting and purifying large amounts of DNA. During electrophoresis, a single gel can hold many samples so that multiple comparisons can be accomplished at one time (14). The process is often repeated using several restriction enzymes to delineate more clearly the differences between microbes. If the entire genome is used, labeled DNA probes to specific areas within the genome may be used for visualization of banding patterns. Alternatively, amplification of small sections of the genome (see PCR in the following section) can provide RFLP banding patterns that can be visualized without probes. Automation of this process, using the Riboprinter by Qualicon, has assisted in epidemiological studies of microorganisms in medical settings and in bioremediation research on fate and transport of microbes.

Currently, the most definitive means of identifying microbial isolates is sequencing the 16S fragment of rDNA from an individual bacterium. Sequencing is conducted on the gene for the 16S RNA fragment of bacterial ribosomes and the 18S RNA fragment of eukaryotic ribosomes. Sequences are compared between isolates to determine their identification (based on extensive databases) and how closely they are related to each other. The historical development of these methods began in the 1980s when Carl Woese of the University of Illinois, Urbana-Champaign, manually sequenced ribosomal fragments of organisms throughout the entire realm of biology. He developed a master "tree of life" that showed how organisms are related to one another throughout all of biology. This Herculean effort gave way to the current scheme of biological inheritance and has revealed so much about the tree of life that classical methods could not address (54). Automation of the sequencing process has provided the ability to rapidly sequence the rDNA of any microbial isolate and compare it to others that have been deposited in sequence databanks (Ribosomal Database Project, RDP, or Genbank). These databases are now available to anyone over the internet and are constantly updated as inputs are made on a daily basis. Because of the revolution in molecular techniques and information technology, the taxonomy of bacterial groups is undergoing rapid evolution. For example, the genus *Pseudomonas*, once included all nonfermentative, gram-negative rods with polar flagella with a percentage G + C that spanned from 58–70 (2). The broad G + C percentage, the large number of species, and the variety of metabolic capabilities suggested that not all these organisms should be confined within a single genus. However, the basis on which to divide them was not clear. Today, this genus has been divided into numerous genera based on the 16S rDNA sequences of these organisms. One entire volume of the 1989 edition of the *Bergey's Manual of Systematic Bacteriology* (4) is based on gram-negative bacteria, many of which were once classified in the genus *Pseudomonas*.

In addition to automated sequencing, another technique has revolutionized taxonomy by allowing the rapid

amplification of minuscule amounts DNA, in some cases as few as 10 cells (55). This process is called polymerase chain reaction (PCR) and can be used both on the DNA of individual isolates or on DNA fragments isolated directly from environmental samples. Before the advent of PCR technology, large volumes of cells had to be grown to extract enough DNA/RNA for sequencing. This process could only be applied to culturable isolates. However, PCR made it possible to understand much more about microbes, even those that could not be cultured. For example, ribosomal DNA fragments have been amplified and sequenced, and were used to determine phylogenetic relationships of the uncultured microbial symbionts of a hydrothermal vent community (56). Short sequences of DNA, called primers, are required for PCR amplification. Specific universal primers have been developed for eubacteria and for archaebacterial groups, as well as other taxonomic groups. These universal primers are based on conserved sequences within the 16S rDNA molecule that abut variable regions in the molecule (14). Because primers are so specific, DNA can even be amplified from fossils (57,58).

Using specific labeled sequences of DNA or RNA as probes, one can determine the presence of a bacterial type (from its DNA) or a metabolic activity (from the presence of mRNA), and locate where in a microbial community that particular bacterial type resides by visualizing those particular labeled bacteria under the microscope. Probes can be radiolabeled or chemically tagged to allow visible or spectrophotometric detection of cells. Recently rDNA probes, domain to strain specific, have become increasingly popular for detection and quantification of specific microbial populations without the necessity of culturing organisms (59–63).

These techniques have taken the process from learning only limited information about culturable isolates to learning quite a lot about even uncultured microbes. With sequences known for many genes, not only can the identity and relatedness of one bacterium to another be rapidly determined, but its metabolic capabilities and structural components can also be discerned without even growing the cells.

Future Considerations

No matter how (relatively) easy it has become to sequence and compare cultured isolates or individual rDNA fragments from uncultured microbes, it is a daunting prospect to consider tabulating all microbial diversity. It is not known how many species or strains there are. However, some have estimated that microbial species alone surpass all other biological diversity (65). Until all species are placed on the microbial branches of the tree of life, one means of relating isolates to one another is through dendrograms, or trees of related microbes. These can be based on 16S rDNA sequences or other group traits, for example, the amino acid sequence of a common enzyme that has been highly conserved throughout bacterial evolution. Examples of conserved molecules might be DNA or RNA polymerase or cytochrome molecules. Trees such as these depict relationships between bacterial isolates and are the current mainstay of taxonomy and systematics research (Fig. 8); they will remain so until the systematics of bacteria is eventually described.

One topic about which little is known is that of how microbes change under varied environmental conditions. Even less is known about how this variability would affect taxonomy. It does not matter what type of bacterial cell is considered, in general, microbes survive a fast or famine existence (66). Organisms as diverse as soil microbes and human pathogens must survive between growth periods and exhibit a plethora of adaptations to meet such challenges. It is clear that organisms cultured in the laboratory (even once) may significantly differ from those in nature. This is no secret to microbiologists. However, bacteria continue to be cultured on media and under conditions unlike those from which the organisms were isolated. Gill Geesey and Bill Costerton revealed one such fundamental difference between nature and the laboratory in their Scientific American article, "How Bacteria Stick" (67). It was observed that the nature of extracellular polymers changed with repeated culture on rich media. Likewise, endolithic isolates from the subsurface have shown exopolysaccharide (EPS) production upon initial culture and lost or repressed the ability within two transfers on even a relatively low nutrient medium. EPS-producing ability, antibiotic production or enhanced corrosion ability was reestablished by contact with crushed rock from their native environment (68,69). In some cases, the same organism may produce different surface components (or different amounts of a surface component), depending on the physiological state of the organism, or the nutrient sources and growth conditions provided the organism (70–72). EPS production is a relatively obvious trait. However, equally dramatic but less obvious changes may occur within organisms when they move from one environment to another. For example, the physiology of *E. coli* in a human lower intestine and in a cold freshwater stream are undoubtedly very different. More is known about *E. coli* than any other living organism; however, it is not known how those two environments affect its activities.

Time is spent on standardizing growth and test conditions while microbes spend their time adapting to a changing environment in ways not yet imagined. Understanding their roles in natural settings will be the challenge of the new century.

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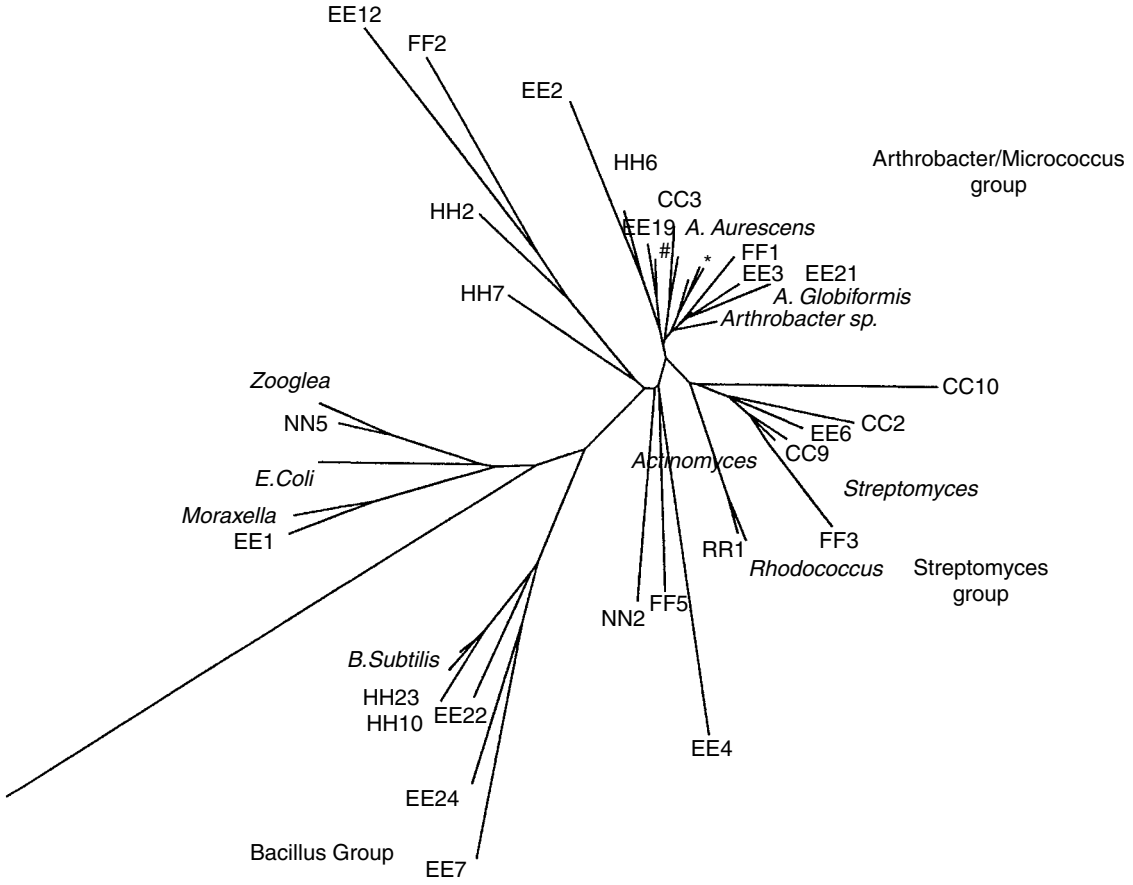

Figure 8. Phylogenetic tree of calcium-precipitating bacteria from paleosols in Eastern Washington state. Most calcium-precipitating bacteria clustered into one of three groups: *Bacillus, Streptomyces*, or *Arthrobacter/Micrococcus*. Note: isolates EE22 and HH6 are nonprecipitating strains. Sequences for the listed genera and/or species were obtained from GenBank or RDP (see text for explanation) and compared to the sequence data from calcium-precipitating isolates designated by letters. # on the figure designates *Micrococcus* sp. and * designates isolates HH1, NN3, and NN4. In time, it may finally be possible to find a basis for a definition of "species" for bacteria and other microbes. This definition has eluded scientists because of the lack of sexual reproduction in bacteria, often the mainstay of a macrobiological species definition. Additionally, much has to be learnt concerning genetic plasticity and gene expression in prokaryotes. Perhaps, with enough information about (bacterial) ribosomal sequences, a formal definition will be proposed. This would allow macro- and microbiologists to better place organisms on the tree of life.

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METHYLATION OF METALS.

See METALS: MICROBIAL PROCESSES AFFECTING METALS

MICROARRAYS.

See BIOCHIP-BASED DEVICES AND METHODS IN MICROBIAL COMMUNITY RIBOTYPING

MICROARRAYS: APPLICATIONS IN ENVIRONMENTAL MICROBIOLOGY

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The availability of entire genome sequences from many different organisms marks a new age in biology. This is the first time in history that the entire genetic content of living creatures can be accessed. This is also the first time that one can view a comprehensive and dynamic molecular picture of the living cell under various physiological states using state-of-the-art genomic technologies such as microarrays. The widespread and routine use of genomic technologies will shed light on a wide range of important research questions from how cells grow, differentiate, and evolve to the medical challenges of pathogenesis, antibiotic resistance, and cancer, from agricultural issues of seed breeding and pesticide resistance to the biotechnological challenges of drug discovery and the remediation of environmental contamination.

Microbial genomic information is increasing at an exponential rate. To date, about 40 microbial genomes have been completely sequenced, and at least 100 microbial genomes are currently being sequenced (see TIGR's web site: *www.tigr.org*). The most critical issue in the postgenomics era will be to elucidate the biological function and regulation of sequenced genes. It is expected that developments in microbial functional genomics and associated genomic technologies will have a tremendous impact on environmental microbiology. This article provides a broad description of microarray-based genomic technologies and a review of their applications for monitoring gene expression and for detecting microbial populations within the context of the natural environment. The advantages and disadvantages of the various microarray formats are emphasized here rather than the technical details of the technology.

MICROARRAY BASICS

Concepts, Principles, and History

Microarrays are microscopic arrays of large sets of DNA sequences that have been attached to a solid substrate using automated equipment (1). These arrays are also sometimes referred to as microchips, biochips, DNA chips, or gene chips. To avoid confusion with computer microchips, the term microarrays is preferred.

Microarray assays are based on the hybridization of a single-stranded molecule labeled with a fluorescent tag, or fluorescein, to a complementary molecule attached to a solid support, such as glass. In principle and practice, microarrays are extensions of membrane-based hybridization methods that have been used for decades to detect and characterize nucleic acids in biological samples. In microarray assays, an unknown sample is hybridized to an ordered array of immobilized DNA molecules of known sequence to produce a specific hybridization pattern that can be analyzed or compared to a given standard. The fluorescein-labeled DNA strand in solution is generally called *the target*, whereas the DNA strand immobilized on the microarray surface is referred to as *the probe*. Because the sequence of the immobilized molecule is usually known, it is used to "probe" or investigate the target molecule in solution. However, this terminology is opposite to the convention that originated with Southern blot hybridization, in which the target molecules on the membrane are interrogated by solution-phase probes.

Microarray assays are rooted in early biochemical experiments on solid substrates. In contrast to traditional hybridization assays that utilize flexible membranes (e.g., nitrocellulose) and radioactivity, microarray assays generally utilize nonporous solid surfaces and fluorescent detection. Compared to the macroscopic format of filter-based assays, the miniaturized microarray format represents a fundamental revolution in biological analysis (2). This is primarily due to the use of a nonporous solid surface that allows small amounts of biochemical molecules to be deposited at precise predefined locations on the surface with little diffusion, whereas porous substrates such as nylon allow diffusion of the applied materials and are not amenable to microarray preparation.

The basic principle behind microarrays was first proposed in the late 1980s. Augenlicht and his colleagues provided one of the first descriptions of DNA microarrays for simultaneously monitoring the expression level of thousands of human genes on nitrocellulose with radioactive labeling (3–5). They spotted 4,000 complementary DNA (cDNA) sequences on nitrocellulose and used them to analyze differences in gene expression patterns among different types and stages of colon tumors. At the same time, four groups independently developed the concept of determining a DNA sequence by hybridization to a comprehensive set of oligonucleotides, i.e. sequencing by hybridization or SBH (6–9). Although the concept of SBH is extremely elegant, there are several inherent problems, such as repeated sequences and the imperfect specificity of hybridization, that limit the use of SBH for sequence determination. Therefore, such challenges have motivated most researchers to shift the emphasis to applications that need to be addressed more immediately, such as profiling gene expression.

By the mid-1990s, the reverse dot blot scheme for monitoring genome-wide gene expression was recast by several different groups. Both DNA fragments and synthetic oligonucleotides were arrayed on various substrates, including nylon membranes, plastic, and glass (10,11). All of them depended on sequence-specific hybridization between the arrayed DNA and the labeled nucleic acids from cellular mRNA. Later studies in yeast with both DNA and oligonucleotide microarrays clearly indicated that microarrays are powerful tools for monitoring gene expression (12,13).

Microarray-based genomic technology has greatly benefited from many parallel advances in other fields. Without such advancements, the development of high-density microarrays and the various applications that we see today would not be possible (2,14). First, many genome projects have produced large-scale sequence information

and resources for microarray-based analysis. Second, technical advances have made it possible to fabricate high-density microarrays in a very small area. Finally, recent advances in fluorescent labeling and detection offer significant advantages in speed, data quality, and user safety for microarray-based assays.

Microarray Types and Their Advantages

Types of Microarrays. Microarrays can be divided into two major formats based on the immobilized probe: (*1*) *DNA microarrays* are constructed with DNA fragments typically generated with the polymerase chain reaction [PCR] (11,12,15) and (*2*) *oligonucleotide microarrays* are constructed with short (10- to 40-mer) or longer (up to 75-mer) oligonucleotide sequences that are designed to be complementary to specific coding regions of interest.

DNA microarrays have certain advantages over oligonucleotide microarrays, especially when monitoring gene expression patterns. While oligonucleotide microarrays are limited to array elements of low sequence complexity, the specificity of hybridization for a complex probe is improved with DNA microarrays using DNA fragments substantially longer than oligonucleotides (16). Also, oligonucleotide synthesis requires prior sequence knowledge, but this is not the case for DNA arrays. Nucleic acids of virtually any length, composition, or origin can be arrayed (16). However, oligonucleotide-based microarrays have the advantage of minimizing the potentially confounding effects of occasional cross-hybridization (13), and are uniquely suited for detecting genetic mutations and polymorphisms.

There are two general types of oligonucleotide microarrays based on the strategy used for oligonucleotide immobilization: (*1*) direct parallel synthesis on solid substrates by light-directed or photoactivatable chemistries (17,18) or standard phosphoramidite chemistries (Southern et al., 1994); and (*2*) chemical attachment of premade oligonucleotides to solid supports (8,19–30). Glass surfaces (9,31,32), glass pores (20), polypropylene sheets (33), and gel pads (8,27) have all been used as solid supports for oligonucleotide attachment.

Each strategy for oligonucleotide immobilization has specific advantages and disadvantages (1,34). There are two major advantages of the direct synthesis approach. First, the photoprotected versions of the four DNA bases allow microarrays to be manufactured directly from sequence databases, thereby removing the uncertain and burdensome aspects of sample handling and tracking. Second, the use of synthetic reagents minimizes chip-to-chip variations by ensuring a high degree of precision in each coupling cycle. The photolithographic approach, however, requires the use of photomasks that direct light to specific areas on the array for localized chemical synthesis; these photomasks are very expensive and time-consuming to design and build. Also, the yield and length of synthesized oligonucleotides are subject to wide variation and uncertainty that could lead to unpredictable effects on hybridization across the microarray. For the attachment of presynthesized probes, the concentrations and length of each oligonucleotide on the array can be controlled before immobilization. Standard synthesis chemistry is also well established for many nucleotide derivatives for which no light-inducible monomer equivalents are available. In addition, the post-synthesis approach is less complicated and can be customized according to the needs of the laboratory. However, the critical drawback of the post-synthesis approach continues to be the need for the external synthesis and storage of different oligonucleotides before array fabrication.

The use of polyacrylamide gel pads as an immobilization support for oligonucleotide microarrays offers significant advantages over the use of probes attached to solid supports (35). Three-dimensional immobilization of probes in gel pads may provide higher capacity and a more homogeneous environment than heterophase immobilization on glass, leading to higher sensitivity and faster hybridization kinetics (36). However, like nylon membrane-based supports, gel pads may cause higher background (37).

Advantages of Microarrays. Microarrays offer a number of advantages over conventional nucleic acid–based approaches.

1. *High Throughput and Parallel Analysis*. The attachment surface of nonporous substrates allows thousands of array elements or probes to be uniformly deposited on a very small surface area. As a result, gene expression can be monitored at the genomic level, or many constituents of a microbial community can be simultaneously assessed in a single experiment using the same microarray. This is very important for studying gene expression at the genome-wide level because the large amount of expression data generated from a single microarray experiment can allow researchers to begin to build a comprehensive, integrated view of a cellular system.

2. *High Sensitivity*. High sensitivity can be achieved in probe-target hybridization because microarray hybridization uses a very small volume of probe and the target nucleic acid is restricted to a small area (16,25). This feature enables high sample concentrations and rapid hybridization kinetics.

3. *Differential Display*. Different target samples can be labeled with different fluorescent tags and then hybridized in parallel to the same microarray, allowing the simultaneous analysis of two or more biological samples in a single assay. Multicolor hybridization detection minimizes variations resulting from inconsistent experimental conditions and allows direct and quantitative comparison of target sequence abundance among different biological samples (16,38).

4. *Low Background Signals*. Nonspecific binding to a nonporous surface is very low; as a result, organic and fluorescent compounds that attach to microarrays during fabrication and use can be rapidly removed, resulting in significantly less background than is typically encountered with porous membranes (16).

5. *Real-Time Data Analysis*. Once the microarrays are constructed, hybridization and detection are relatively simple and rapid, allowing real-time data analysis in field-scale heterogeneous environments.

6. *Automation*. Microarray technology is amenable to automation and therefore has the potential of being cost-effective compared to conventional detection methods (16).

Microarray Fabrication

A critical step in using microarrays is the fabrication procedure, which involves attaching or printing the DNA probes on the array (Fig. 1). The microarray format is compatible with many advanced fabrication technologies. The most widely used printing technologies are photolithography, mechanical microspotting, and ink-jet ejection. Each technology has advantages and disadvantages in microarray fabrication (1,2).

In photolithography, oligonucleotides are synthesized in situ on a solid surface in a predefined spatial pattern by using a combination of chemistry and photolithographic methods borrowed from the semiconductor industry (31). Photomasks direct DNA synthesis through the use of light, which serves to activate modified phosphoramidite versions of the four DNA bases for DNA synthesis (31).

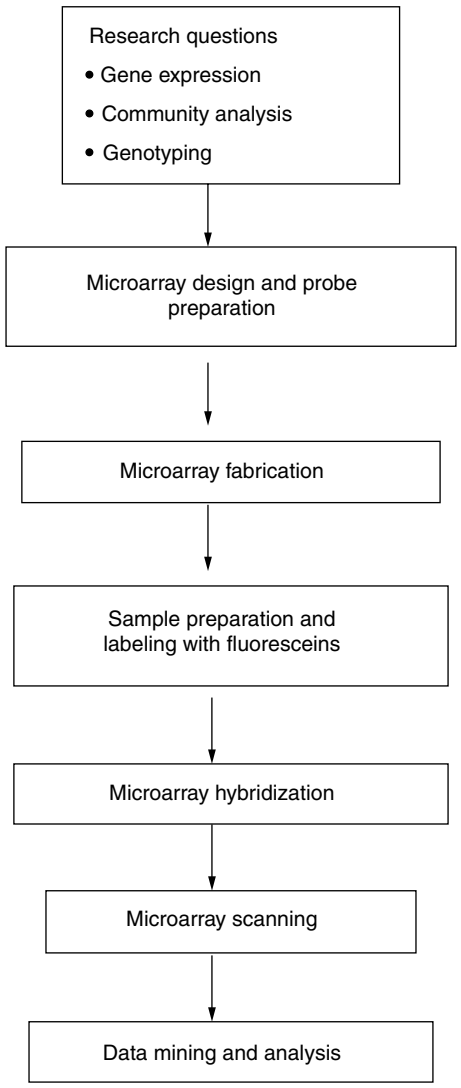

Figure 1. Flowchart of microarray-based experimental approaches.

A single base is added after each coupling step to growing oligonucleotide chains at thousands of defined locations (39). Affymetrix chips currently contain approximately 250,000 oligonucleotides in an area of 1 cm^2. One of the main advantages of this approach is that microarrays of extremely high density can be constructed (38). However, photolithography can only be used with oligonucleotides, not cDNA.

Mechanical microspotting uses an array of pins, tweezers, or capillaries to deliver picoliter volumes of premade biochemical reagents (e.g., oligonucleotides, cDNA, genomic DNA, antibodies, and small molecules) to a solid surface. Printing is accomplished by direct contact with the surface using a computer-controlled robot (14). Currently, more than 1,000 individual cDNA molecules can be deposited in an area of 1 cm^2 (40). The advantages of microspotting are ease of implementation, low cost, and versatility. One disadvantage of this method is that each sample to be arrayed must be prepared, purified, and stored before microarray fabrication. Also, microspotting rarely produces the densities that can be achieved with photolithography.

Ink-jet ejection technologies provide another way to manufacture microarrays. They utilize piezoelectric and other forms of propulsion via a robotic control system to transfer nanoliter volumes of reagents from miniature nozzles to defined locations on a solid substrate without surface contact. Similar to microspotting approaches, ink-jet ejection allows the spotting of virtually any biological molecule of interest, including cDNA, genomic DNA, antibodies, and small molecules. In contrast to microspotting, ink jets have the advantage of avoiding direct surface contact. Ink-jet ejection, however, cannot manufacture microarrays as dense as those prepared by photolithography or microspotting approaches.

All three technologies allow the manufacture of microarrays with sufficient density for genetic mutation detection and gene expression applications. The key considerations in selecting a fabrication technology include microarray density and design, biochemical composition and versatility, reproducibility, throughput, density, and cost. Because of its versatility, affordability, and wide applications, microspotting may become the microarray technology of choice for the basic research laboratory.

Microarray Hybridization and Detection

After constructing the microarrays, the most important issue in microarray-based analysis is the successful performance of target hybridization. Conceptually, microarray hybridization and detection are quite similar to traditional membrane-based hybridization (14). The target DNA or RNA from a biological source is labeled with fluorescent dyes [e.g., Cy3 (green)- or Cy5 (red)-labeled deoxycytidine triphosphate (dCTP)]. The labeled target DNA or RNA is then purified and hybridized with the microarrays. The unbound material is washed away, and the probe-target hybrid on the array is visualized by fluorescence detection with confocal scanning devices or a CCD (charged coupled

device) camera (41). The purity of RNA and DNA is a critical factor in the hybridization process, because cellular proteins, lipids, and carbohydrates can mediate significant nonspecific binding of fluorescently labeled cDNA to glass surfaces.

Fluorescent labeling and detection use fluorescently labeled DNA bases that absorb and emit light at distinct and separable wavelengths. The inherent low level of intrinsic fluorescence of glass and other microarray substrates permits the use of fluorescent labeling and detection schemes that enable a great leap in terms of speed, data quality, and user safety (14). Fluorescence-based assays make it possible to utilize advanced data acquisition technologies such as confocal scanners and CCD cameras.

Generally, a confocal scanner uses laser excitation of a small region of the glass slide ($\sim$100 μm^2), and the entire image of the array is collected by moving the glass slide, the confocal lens, or both across the slide in two directions (41). The fluorescence emitted from the hybridized target molecule is gathered with an objective lens and converted to an electrical signal with a photomultiplier (PMT) or an equivalent detector. The CCD camera exploits many of the same principles as a confocal scanner, but the CCD camera utilizes substantially different excitation and detection technologies (41). One key difference is that the CCD camera-based imaging system often illuminates and detects a large portion of the slide (1 cm^2) and hence does not require moving stages and optics. This reduces cost and simplifies instrument design. However, because the CCD camera does not move the optics or stages, several images need to be captured from different fields of the slide and then combined to represent the entire information on the slide.

Microarray Data Acquisition and Analysis

After the fluorescent signal is converted into digital output by the detection system, the images can be processed by a variety of commercial and free software (42). Signal quantification is usually accomplished by superimposing a grid over a microarray image to specify the target locations, and the likely shape and placement of the hybridization signal is predicted using mathematical methods. Local sampling of the background is generally used to specify a threshold that the true signal must exceed. By using these methods, it is possible to detect weak signals and extract an average density above the background for each array element (43).

Hybridization signals from microarrays should be normalized before comparing data from a single array or among multiple arrays because of differences in labeling efficiencies among fluorescent dyes and other experimental variations (14,44). Basically, two general approaches can be used for data normalization. One is to add a known amount of control RNA or DNA to the samples before labeling. The signal intensity from the controls can be used to normalize the data from a single array among different dyes or multiple arrays. The second approach is to use a set of housekeeping genes that are expressed consistently under certain experimental conditions. The average fluorescent intensity for these housekeeping genes can be used for normalization (44).

On the basis of the spiked controls, the intensity value for each array element can be converted into biologically relevant outputs (e.g., the number of mRNA transcripts per cell, micrograms of DNA per gram of soil). Quantitative gene expression, genotyping, and other outputs can then be correlated with the gene sequences on the microarrays, and higher order relationships, such as coregulation, can be identified using a variety of statistical methods such as cluster analysis, which has been the most widely used statistical technique for analyzing gene expression data (45).

USING MICROARRAYS TO MONITOR GENE EXPRESSION

Using Gene Expression Data to Define Gene Function

Determination of entire genome sequences is only the first step in the complete characterization of an organism. The next step is to elucidate the functions of these sequences and provide physiological and ecological meaning to this information. However, defining the role of each gene in a genome is a daunting task (12,46,47), and understanding how the genome functions as a whole in the complex natural history of living organisms presents an even greater challenge (12).

Both computational and experimental approaches can be used to study gene function and cellular regulatory networks. Sequence homology provides clues about gene function, but homology is not function, and sequence comparison to known genes in public databases has a limited role (46,48,49). One reason is that only 40–60% of the open reading frames (ORFs) in most sequenced microbial genomes have a postulated function (50–56). Even for the most extensively studied microorganism, *Escherichia coli* K-12, 38% of the ORFs have no attributable function (57). Also, the functional assignment of genes by similarity comparison is sometimes misleading or incorrect because of the complicated evolutionary and structure–function relationships among different genes (46). Therefore, experimental analysis must be performed to understand the biological role of a gene product. One of the key experimental approaches to defining gene function and regulation is to identify patterns of gene expression associated with various physiological states.

Gene expression is the process by which messenger RNA (mRNA) and then proteins are synthesized from DNA templates. RNA transcript levels are of great value for studying gene function and regulation for the following reasons (58,59).

1. *Correlation of Gene Expression and Function.* Natural selection dictates that genes expressed in specific cells under specific conditions must contribute to the fitness of the organism. Similar to the phenomena that natural selection has precisely fine-tuned the biochemical properties of gene products, it has also fine-tuned the regulatory networks that control when and where the product is made and in what quantity. Thus, it is believed

that there is a strong connection between gene expression and the function and regulation of the encoded product.

2. *Connection Between Gene Expression and Physiological States*. The set of genes expressed in a cell determine what the cell is made of, how the cell is built, what biochemical and regulatory systems are operative, and what the cell can do. Gene expression patterns can provide information about the dynamic changes in physiological states and functional activities of a cell under different environmental conditions.

3. *Guilt by Association*. It is generally believed that genes that are expressed together may function together. Sets of genes that share similar functions can be grouped on the basis of the similarities in their expression patterns. Also, common regulatory mechanisms can be inferred for genes with similar expression profiles, and conserved regulatory elements can be identified. Thus, based on the expression patterns of known genes, the function of hypothetical proteins can be predicted if such genes are coexpressed with genes of known function.

4. *Importance of Transcriptional Regulation*. While regulation of protein abundance in a cell is by no means achieved solely through the regulation of mRNA, almost all of the differences in cell types or functional states are correlated with changes in the mRNA levels of many genes. Thus, mRNA levels can be used as a measure of gene expression. High-density microarrays containing whole-genome sequence information are powerful and indispensable tools for the analysis of gene expression and regulation. Once one learns the biological consequences of gene expression patterns as a result of the growing knowledge of the functions of individual genes, it should be possible to use microarrays as a "microscope" for visualizing the complex and dynamic nature of living cells (58).

General Approaches to Revealing Differences in Gene Expression

Information about when and where genes are turned on or off under different environmental conditions is fundamental for understanding gene function and regulation. Three comparative approaches have been used for the display of differential gene expression.

1. *Differential Display of mRNA Under Different Physiological Conditions*. Cells of interest are cultured under different physiological conditions, and the differences in mRNA abundance between the test and reference samples are compared using high-density microarrays. This is the most straightforward and widely used approach for identifying patterns in gene expression associated with various physiological states (12,60,61).

2. *Differential Display of Temporal Gene Expression*. Cells of interest are grown under a specific physiological condition and then harvested at different time points during growth. Changes in mRNA levels are revealed using microarrays. Information on the temporal dynamics of gene expression is very useful in understanding when genes are turned on or off and how genes interact with each other.

3. *Comparison of Gene Expression Patterns Between Wild-Type and Mutant Cells*. The expression of genes in response to changing environmental conditions can be very complicated, and oftentimes the expression profiles of many genes are altered as a result. This presents a great challenge to understanding the underlying regulatory mechanisms. The most effective approach to defining the contributions of individual regulatory genes in a complex metabolic process is to use DNA microarrays to identify genes whose expression is affected by mutations in putative regulatory genes (12).

The basic scheme for microarray-based gene expression studies is outlined in Figure 2. In a typical microarray experiment for monitoring gene expression, gene-specific PCR primers are designed based on whole-genome sequence information and synthesized. Gene-specific fragments are then amplified with specific primers, purified, and arrayed on solid substrates. Once the

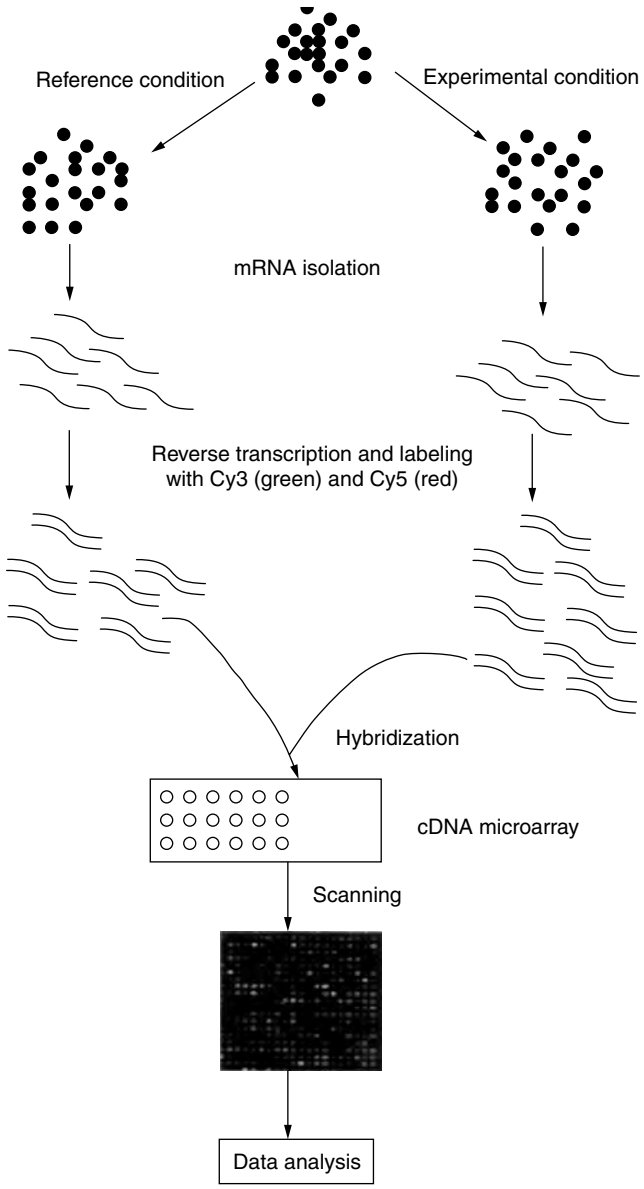

Figure 2. General scheme of microarray experiments for monitoring gene expression. See color insert.

microarrays are ready, total cellular RNA isolated from bacterial cells grown under two different conditions is fluorescently labeled with different dyes (Cy3 or Cy5) via the enzyme reverse transcriptase. The microarray is then simultaneously hybridized with fluorescently tagged cDNA from the test and reference samples. The signal intensity of each fluorescent dye on the array is then measured with a confocal laser-scanning microscope or CCD camera. The quantitative ratio of red (Cy5) to green (Cy3) signal for each spot reflects the relative abundance of that particular gene in the two experimental samples. With appropriate controls, the intensity can be converted into biologically relevant outputs (e.g., the number of transcripts per cell). A series of samples can be compared with each other through separate cohybridizations with a common reference sample, and the data can be analyzed with various statistical methods. A detailed discussion of the technical aspects of microarray experiments for monitoring gene expression can be found in the review by Eisen and Brown (14).

Specificity, Sensitivity, and Quantitation of Microarray-based Detection for Monitoring Gene Expression

Specificity, sensitivity, and quantitation are the three major concerns for any detection technology. Microarrays were initially used to measure gene expression levels in *Arabidopsis thaliana* (11). The microarrays of 48 duplicate cDNA sequences were constructed and simultaneously hybridized with a mixed set of fluorescently labeled probes that also contained cDNA from rat and yeast as negative controls. There was no detectable response from the rat and yeast controls, suggesting that microarray hybridization was very specific. DeRisi and coworkers (12) showed that microarray hybridization could differentiate homologous gene sequences that were less than 75% similar, and oligonucleotide microarrays can, in theory, distinguish single base pair differences among probes (62,63).

The detection limit, or sensitivity, can be evaluated by spiking labeled samples with a known amount of labeled control mRNA. The current detection limit of DNA microarrays used in human genome research allows monitoring of transcripts that represent $1:500{,}000$ (w/w) of the total mRNA. A similar detection limit was obtained using oligonucleotide microarrays (10). Such a detection limit is higher than that obtained with conventional Northern hybridization methods. For yeast, mRNA transcripts present at a level less than one molecule in 100,000 can be detected, which is equivalent to one copy per 20 yeast cells (64). This result suggests that microarray hybridization is very sensitive for detecting gene expression.

The quantitative aspects of microarray hybridization have not been well established. The variability of hybridization signal intensity as a result of variations in array fabrication, labeling, target concentration, and scanning was less than 15% (65). Because of the inherent variations of microarray hybridization and the massive amounts of data that need to be analyzed, genes generally showing a greater than 2-fold change in expression using two-color fluorescence detection were considered to be significantly different in their expression level (66). Comparison of microarray hybridization results with previously known results suggested that microarray hybridization appears to be quantitative enough for detecting differences in gene expression patterns under various conditions (10,12,66). Recently, DNA microarrays were used to measure differences in DNA copy number in breast tumors (67,68). Single-copy deletions or additions can be detected (68), suggesting that microarray-based detection appears to be quantitative.

Using Microarrays to Detect Gene Expression in Microorganisms

Both DNA and oligonucleotide microarrays have been successfully used for detecting differentially expressed genes in yeast (12,13,16,69,70) and bacteria (60,61,71–73). These studies, along with those in plants (11,66) and human cells (10,12,66–68,74–78), convincingly demonstrated that microarrays allow researchers to switch from the very focused view of a single gene or promoter to the global view of a complex regulatory network.

One of the most comprehensive studies demonstrating the feasibility and utility of DNA microarrays for genome-wide exploration of gene expression was carried out with yeast genome sequences (12). In this study, DNA microarrays containing virtually all of the genes of *Saccharomyces cerevisiae* ($\sim 6,000$) were used to study the effect of the diauxic shift from anaerobic to aerobic metabolism under glucose limitation and the concomitant switch to ethanol as a carbon source. By comparing the global view of changes in expression of genes of known functions with their metabolic pathways, DeRisi and coworkers (12) showed the metabolic pathways that were reprogrammed by this shift. The expression patterns of many previously unknown genes were also obtained, and this may provide a clue to their possible functions. In addition, to understand the underlying regulatory mechanisms, microarrays were used to compare the gene expression patterns between wild-type cells and mutants containing deletions in key regulatory genes. DeRisi and coworkers (12) demonstrated that this is a very effective approach to dissecting complicated regulatory networks and defining the functions of individual regulatory genes.

Using microarrays for analyzing gene expression profiles in procaryotic genomes may present some unique challenges because bacterial mRNAs do not have poly A tails, so they cannot be enriched by poly T-based reverse transcription. Because of the low abundance of mRNAs ($\sim$5% of total cellular RNA), rRNA ($\sim$95%) needs to be removed from the total RNA pool to reduce the hybridization background. However, quantitative and reproducible removal of rRNA without losing mRNA is difficult to achieve. Saizieu and coworkers (73) demonstrated that an oligonucleotide microarray containing probes representing approximately 100 genes from both *Haemophilus influenzae* and *Streptococcus pneumoniae* hybridized well with total RNA labeled chemically by a photocoupling reaction using psoralen-biotin. In this study, one to five transcripts per cell were detected, and mRNA quantitation was in good agreement with data from Northern blot analysis. Oligonucleotide microarrays, however, did not work

with total RNA labeled by reverse transcriptase and random priming. Recently, several studies have shown that labeling total RNA by reverse transcription and random labeling in the presence of fluorescent dyes (Cy3-dCTP or Cy5-dCTP) or ^{33}P have worked well with glass-based microarrays for *Mycobacterium tuberculosis* (72) and *Shewanella oneidensis*, and with membrane-based microarrays for *E. coli* (60,61,79). These studies demonstrated the feasibility and utility of microarrays for displaying genome-wide expression patterns in bacteria.

APPLICATIONS OF MICROARRAYS FOR MICROBIAL DETECTION IN NATURAL ENVIRONMENTS

Limitations of Conventional Molecular Methods for Microbial Detection

The detection, characterization, and quantification of microbial population diversity are formidable tasks for microbial ecologists. Traditional culture enrichment techniques for studying microbial communities have proven difficult and ultimately provide an extremely limited view of microbial diversity because the majority of naturally occurring species are not culturable (80). The development and application of nucleic acid–based techniques largely eliminated the reliance on culture-dependent methods, and consequently greatly advanced the detection and characterization of microorganisms in natural habitats (80–82). However, the limitations of conventional nucleic acid–based detection methods prevent them from being readily adapted as high-throughput, cost-effective assessment tools for monitoring microbial communities.

To assess microbial community dynamics and activities in natural environments, microbial detection tools need to be (*1*) simple, rapid, and hence real-time and field-applicable; (*2*) specific and sensitive; (*3*) quantitative; (*4*) capable of high throughput; and (*5*) cost-effective. Although conventional nucleic acid detection approaches [e.g., 16S rRNA gene-based cloning methods, denatured gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP), quantitative PCR, and mass spectrometry–based detection methods] remain vital to studies of microbial communities, they meet these requirements with difficulty. They are slow, labor-intensive, insensitive, nonquantitative, or expensive. Microarray-based technology has the potential of overcoming the limitations of traditional molecular methods for studying microbial community structure.

Advantages and Challenges in Using Microarrays for Environmental Studies

In addition to the advantages previously mentioned, microarray-based technology is well suited for detecting microorganisms in natural environments. Many target functional genes in environments are highly diverse, and it is difficult, sometimes even impossible, to identify conserved regions for designing PCR primers or oligonucleotides. The microarray-based approach does not require such sequence conservation because all of the diverse gene sequences from different populations of the same functional group can be fabricated on arrays and used as probes to monitor their corresponding populations.

In contrast to studies using pure cultures, microarray-based analysis of environmental nucleic acids presents a number of technical challenges. First, in environmental studies, the target and probe sequences are very diverse, and it is not clear whether the performance of microarrays with diverse environmental samples is similar to that with pure culture samples and how sequence divergence affects microarray hybridization. Also, environmental samples are generally contaminated with other substances, such as humic materials, organic contaminants, and metals, which may inhibit DNA hybridization on microarrays. No information is currently available on the performance of microarrays with complex environmental samples. In addition, unlike pure cultures, the biomass in environmental samples is generally low, and consequently, it is not clear whether microarray hybridization is sensitive enough for detecting microorganisms in environmental samples. Finally, it is not certain whether microarray-based detection can be quantitative. Environmental and ecological studies require experimental tools that not only detect the presence or absence of particular groups of microorganisms but also provide quantitative data on their biological activities.

Unlike microarray-based monitoring of gene expression, the application of microarray technology to environmental studies is still new and therefore, limited. It is possible to envision several ways in which microarrays can be applied to advance research in environmental microbiology. The following sections provide a brief description of the various microarray formats that have been used to study microbial populations and some preliminary unpublished data from work conducted primarily in our laboratory.

Functional Gene Arrays (FGAs)

The genes encoding functional enzymes involved in various biogeochemical cycling processes (e.g., carbon, nitrogen, sulfate and metals) are very useful as signatures for monitoring the physiological status and functional activities of microbial populations and communities in natural environments. Microarrays containing functional gene sequence information are referred to as functional gene arrays (FGAs) because they are primarily used for functional analysis of microbial community activities in environments. Similar to the microarrays used for monitoring gene expression, both oligonucleotides and DNA fragments derived from functional genes can be used for fabricating FGAs. Because no data are available for FGAs fabricated with oligonucleotides, the focus is mainly on recent studies using FGAs composed of DNA fragments.

Selection of Gene Probes. FGAs are designed for studying functional gene diversity in environments. To construct FGAs, the gene probes should be carefully defined and selected based on the specific research questions to be addressed. For example, microarrays can consist of gene probes that are involved in various biogeochemical

processes, including nitrification (ammonia monooxygenase, *amoA*), denitrification (nitrite reductases, *nirS* and *nirK*), nitrogen fixation (nitrogenases, *nifH*), sulfite reduction (sulfite reductase, *dsv*A/B), methanogenesis (methyl coenzyme M reductase genes, *mcrA*), methane oxidation (methane monooxygeneases, *mmo*), and plant polymer degradation (cellulases, xylanases, ligin peroxidases).

There are two general approaches for obtaining FGA probes. The first approach is to amplify the desired gene fragment from genomic DNA from pure bacterial cultures with specific primers or from cloned plasmids containing the desired gene insert with vector-specific primers. However, the availability of pure cultures and plasmid clones can be limited. The second approach is to recover the desired gene fragments from natural environments using PCR-based cloning methods (Fig. 3; 83). First, microbial community DNA is extracted and purified from environmental samples using a method described previously (84). The desired genes are then amplified with conserved primers specific to various groups of functional genes and cloned into plasmids, such as TA cloning vectors, which are then transformed into *E. coli* cells. The colonies containing the desired gene inserts are generally identified by PCR using vector-specific primers (83). Unique clones are typically distinguished by differences in digestion patterns generated by two pairs of restriction endonucleases (*Msp*I plus *Rsa*I and *Hha*I plus *Hae*III), followed by DNA sequencing. Finally, the sequences are compared and the clones that show more than 85% similarity can be used as specific probes for FGAs. These two approaches have been used to construct FGAs containing nitrite reductase genes and ammonia monooxygenase genes for monitoring bacteria involved in nitrification and denitrification (85).

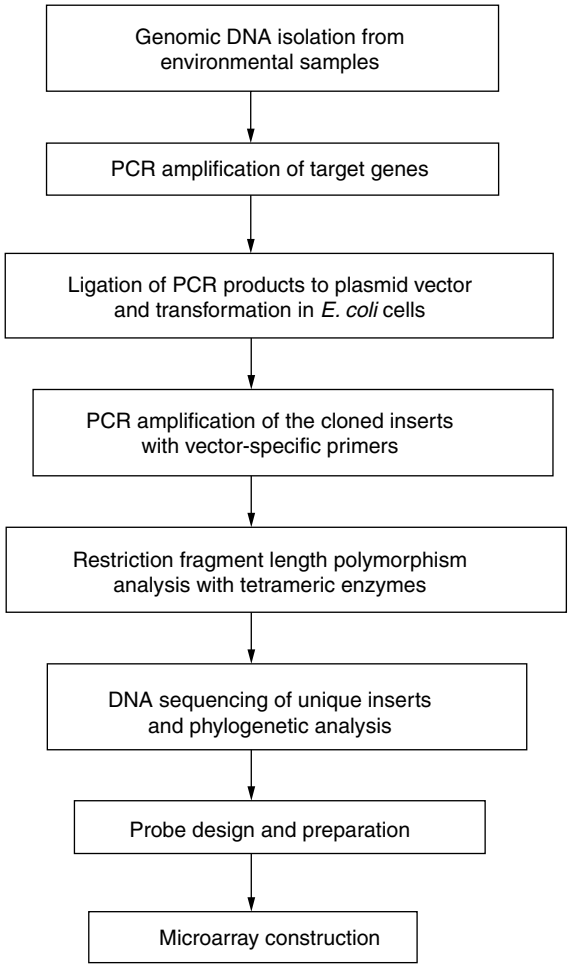

Figure 3. Molecular approach for recovering signature sequences from environmental samples.

Specificity, Sensitivity, and Quantitation. Hybridization specificity is influenced by many factors, such as G + C content, degree of sequence divergence, length, secondary structure of the probe, temperature, and salt concentrations. To determine the specificity of DNA microarray hybridization, functional gene arrays were constructed consisting of heme- and copper-containing nitrite reductase genes (27 *nir*S and 9 *nir*K, respectively), 7 ammonia monooxygenase genes (*amo*A), and 11 methane monooxygenase genes [*pmo*A] (85). 16S rRNA genes and five yeast genes were used as positive and negative controls, respectively. Cross-hybridization among different gene groups was not observed at both low (45 °C) and high (65 °C) stringency. No hybridization was observed with any of the five yeast genes on the array. These results indicate that specific microarray hybridization can be achieved when using DNA extracted from environmental samples.

The detection sensitivity of FGA hybridization was determined using genomic DNA from both pure cultures and soil community samples (Fig. 4). It was found that the arrays have a detection limit, or sensitivity, that was approximately 1 ng for pure genomic DNA and 25 ng for soil community DNA. This level of detection should be sufficient for many studies in microbial ecology. These results suggest that microarray hybridization can be used as a sensitive tool for analyzing microbial community structure in environmental samples.

To evaluate whether microarray hybridization can be used as a quantitative tool, the relationship between target DNA concentration and hybridization signal was examined. A strong linear relationship was observed between signal intensity and target DNA concentration in the range of 1 to 100 ng, suggesting that DNA microarrays can be potentially used as tools for quantitative analysis of environmental samples. Experimental variation between array slides can be reduced to below 15%, which is similar to the results obtained by Bartosiewicz and coworkers (65) in their study on monitoring gene expression. However, sequence divergence has significant effects on hybridization signal intensity (85). The difficult challenge for quantifying the abundance of microbial populations in natural environments is to distinguish differences in hybridization intensity caused by population abundance from those caused by sequence divergence.

Applications. Because functional gene arrays for microbial detection are presently in the development stage, their applications are still being explored. To demonstrate the applicability of DNA microarrays for microbial community analysis, functional gene arrays have been used

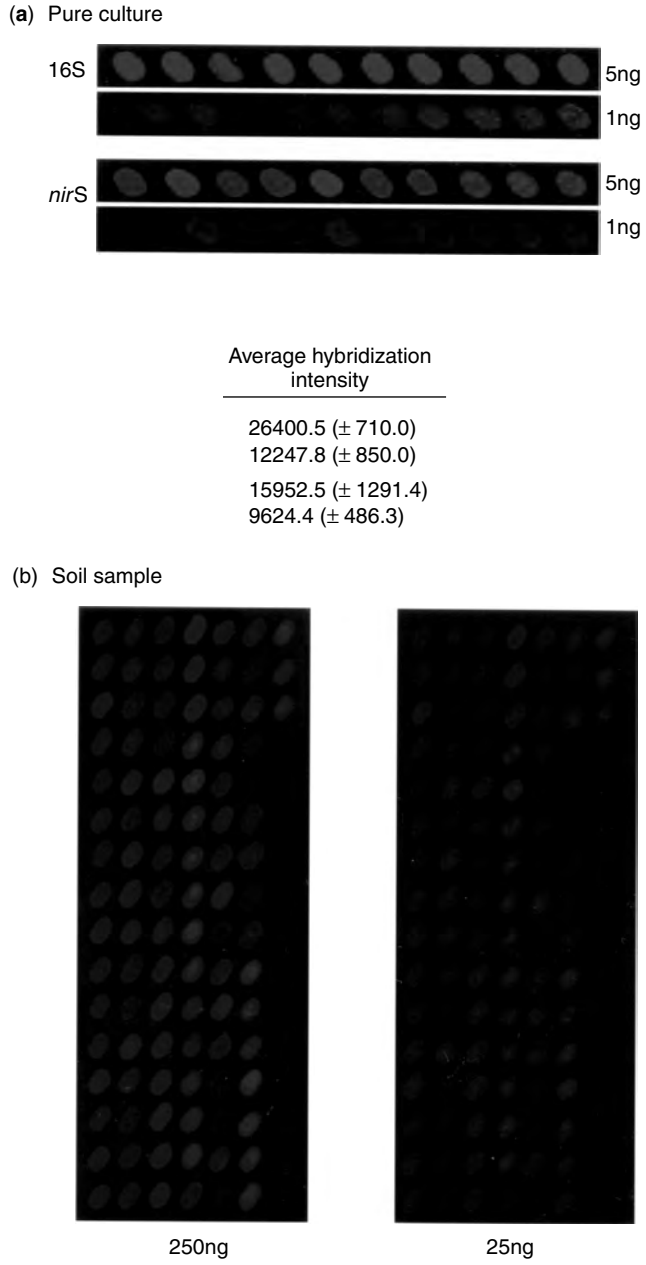

Figure 4. Array hybridization images showing the detection sensitivity with labeled pure genomic DNA and bulk community DNA from soil. (**a**) Genomic DNA from a pure culture of *nir*S-containing *Pseudomonas stutzeri* was labeled with Cy5 using a random primer labeling method. The target DNA was hybridized to nitrogen cycle microarrays at total concentrations of 1 and 5 ng. The average hybridization intensity at each target DNA concentration is presented. (**b**) Genomic DNA from surface soil was labeled with Cy5 as described in (**a**) and hybridized at total concentrations of 25 and 250 ng with the nitrogen cycle microarrays. See color insert.

to analyze the distribution of denitrifying and nitrifying microbial populations in marine sediment and soil samples (85). DNA from two marine sediment samples and four chromium-contaminated surface soil samples was fluorescently labeled using a random primer labeling method and hybridized with FGAs in triplicate. Strong hybridizations were obtained with both marine sediment and soil samples. Although most of the functional gene probes were derived from marine sediment environments, these probes also hybridized well with community DNA from soil samples, suggesting that the types of nitrifier and denitrifier genes on the array are common in both environments. The abundance of *nir*S genes appeared to be higher than that of *nir*K genes in both marine and soil environments. In addition, microarray analysis using labeled genomic DNA demonstrated that *nir*S genes found in pure cultures were dominant in marine sediment environments, whereas *nir*S and *nir*K pure cultures isolated from marine environments were not found in an environmental clone library generated by PCR-based cloning (86), suggesting that the diversity patterns revealed by PCR amplification may not accurately represent the population distribution in these environments.

Phylogenetic Oligonucleotide Arrays (POAs)

Ribosomal RNA genes are powerful molecules for studying phylogenetic relationships among different organisms and for analyzing microbial community structure in natural environments, because these genes exist in all organisms and contain both highly conserved and highly variable regions, which are useful for differentiating microorganisms at different taxonomic levels (e.g., kingdom, phyla, family, genus, species, and strain). Also, rRNA genes have the largest representative database, making them ideal molecules for developing microarray-based detection tools. Oligonucleotide microarrays containing information from rRNA genes are referred to as *phylogenetic oligonucleotide microarrays* (POAs) because such microarrays are used primarily for phylogenetic analysis of microbial community composition and structure.

POAs can be constructed for different phylogenetic taxa and used in community analysis studies. The oligonucleotide probes can be designed in a phylogenetic framework to survey different levels of sequence conservation, from highly conserved sequences giving broad taxonomic groupings to hypervariable sequences giving genus- (and potentially species-) level groupings. Such microarray assays do not require samples of high biomass if PCR or other signal amplification techniques are applied.

Challenges of Phylogenetic Oligonucleotide Arrays. One of the biggest potential problems for microarrays in general is probe accessibility (34). It is not clear how much of the probe is really accessible to hybridization because hybridization on microarrays is not simply a solution-phase reaction in which DNA strands readily associate together. This may depend on target and probe sequences and intrastrand secondary and tertiary structure. Preliminary results suggest that FGAs are less problematic. However, oligonucleotide arrays, especially 16S rRNA-gene-based oligonucleotide microarrays, present more difficult technical challenges.

There are two unique challenges for POAs.

1. *Specificity*. Because the rRNA gene is highly conserved and present in all microorganisms, specific detection with rRNA-targeted oligonucleotide microarrays can

be difficult. First, the probe length and G + C content can significantly impact microarray hybridization (26). Also, probe selection is limited by the sequence differences among the target genes, and cross-hybridization can be a problem for oligonucleotide arrays. In addition, oligonucleotide microarrays typically contain many probes. Ideally, all of the oligonucleotides should have similar or identical melting kinetics, so that all of the probes on an array element can be subjected to the same hybridization conditions at once. This can be difficult to achieve, because the melting temperature depends on the length and composition of the oligonucleotide probe as well as the target 16S rRNA molecules in the samples.

2. *Secondary Structure*. The hybridization of oligonucleotide probes to target nucleic acids possessing stable secondary structure can be particularly challenging because low stringency conditions (i.e., hybridization temperatures between 0 and 30 °C) are required for stable association of a long target nucleic acid with a short immobilized oligonucleotide probe (25,26,87,88). Any stable secondary structure of the target DNA or RNA must be overcome to make complementary sequence regions available for duplex formation. The stable secondary structure of 16S rRNA will have serious effects on hybridization specificity and detection sensitivity.

Applications. Oligonucleotide microarray technology for studies in environmental microbiology has not been rigorously developed. Although gel pad–based oligonucleotide microarrays (26) and the in situ fabricated oligonucleotide microarrays made by Affymetrix (Gary Andersen, personal communication) can be used for monitoring microbial populations, no study, as yet, has shown that microarrays consisting of oligonucleotides attached to glass slides work well in analyzing microbial community structure and function.

In the study by Guschin and coworkers (25), gel-pad oligonucleotide microarrays were constructed with oligonucleotides complementary to 16S rRNA sequences from key genera of nitrifying bacteria. The results showed that specific detection can be achieved with this type of microarray. However, the probe specificity depends on various factors such as probe length. They showed that, as the length of the oligonucleotide probe increases, mismatch discrimination is lost; conversely, as the length of the probe decreases, hybridization signal intensity (i.e., sensitivity) is lost. In addition, they illustrated that hybridization signal variations can be improved by varying probe concentrations and that multiple probe-target populations can be quantified simultaneously using multiple color detection.

Community Genome Arrays (CGAs)

Decades of scientific investigations have led to the isolation of many microorganisms from a variety of natural habitats. However, little or nothing is known about the genomic sequences for the majority of these microorganisms. Such a large collection of pure cultures should be very useful for monitoring microbial community structure and composition in natural environments if microarrays could be developed that did not require prior knowledge of gene sequences. The process of developing community genome arrays (CGAs) or microarrays constructed with whole genomic DNA from many individual pure cultures has commenced. The utility of CGAs depends on sample complexity, hybridization kinetics, and the cultivation of important organisms in the environment to be studied.

Community genome arrays consisting of 54 pure genomic DNAs isolated from different groups of bacteria have been constructed. Both gram-positive and gram-negative bacteria were represented on the array as well as members of the α-, β-, and γ-proteobacterial divisions and various strains of *Pseudomonas stutzeri*. Preliminary results indicated that as little as 50 ng/μl of probe genomic DNA can be clearly detected with fluorescently labeled target genomic DNA, whereas no substantial increase in hybridization signal intensity was observed for arrayed genomic DNA that was greater than 200 ng/μl. Clearly, CGAs can be used to distinguish bacteria at the genus level. Although some of the results suggest that they can also differentiate species of the same genus, more work is required to enhance the specificity of CGAs by evaluating different hybridization parameters, such as temperature. Nevertheless, because they circumvent the need for prior sequence information, genomic DNA microarrays hold considerable promise as a sensitive, specific tool for analyzing the structure and composition of microbial communities in natural environments.

CONCLUSION

Genomics and associated genomic technologies have revolutionized the biological sciences. The availability of whole-genome sequences from numerous bacteria, fungi, plants, and animals will pave the way for functional genomics by providing the information and resources for microarray construction and analysis. Microarrays will be the focal point for functional genomics by allowing the parallel analysis of gene expression profiles, mutations, and genetic polymorphisms. With this technology, it is possible to study the expression of all genes in an organism at once. However, the biggest challenge is how to analyze and interpret the massive amounts of data generated with microarrays.

Environmental microbiology will greatly benefit from the advances in functional genomics and genomic technologies because many microorganisms important to the environment have been completely sequenced or are in the process of being sequenced. It is expected that, in the near future, whole genome microarrays for numerous environmental microorganisms will be constructed and used in functional genomics research to determine patterns of gene expression under various environmental conditions and to identify novel metabolic pathways and regulatory networks. These discoveries at the basic research level will provide invaluable information for environmental studies.

The aim of microbial ecology is to derive the principles capable of explaining microbial community diversity, structure, and function. Such principles can only be

discovered by examining the structure and activities of populations and the properties of individual species (89). However, characterizing the diversity, structure, and function of microbial populations within an environmental context is a great challenge. Various types of microarray-based genomic tools will be very valuable for such a purpose because the interactions between microorganisms and the environment most often occur at the molecular level. With these tools, thousands of different microbial populations in a community can be examined at once and in real time. Although significant progress in applying microarray-based genomic tools to environmental studies has been made recently, these tools are still in the early stages of development. More effort is needed to recover signature sequences from different environments and improve the specificity, sensitivity, and quantitation of microarrays within the context of various environmental samples.

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MICROBIAL DEGRADATION OF EXPLOSIVES

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Explosives are highly energetic chemicals that rapidly release large amounts of energy and gaseous products upon detonation. The history of explosives dates back to the development of black powder long before even the industrial revolution started in Europe. More information on the discovery, use, and development of explosives can be found in a treatise by Linder (1). Explosives can be chemical, mechanical, electrical, or nuclear, but the following discussion covers only the microbial degradation of secondary chemical explosives. Primary explosives (also called initiator explosives) have low energies of activation and are sensitive to low-pressure shock waves or friction. They are used to initiate the detonation of secondary chemical explosives, which are relatively stable, easily manipulated, and make up the bulk of reactive material in most explosive formulations.

Presently some of the most frequently manufactured and used secondary explosives include 2,4,6-trinitrotoluene (TNT), dinitrotoluenes (DNT), 1,3,5-trinitrobenzene (TNB), N,2,4,6-tetranitro-N-methylaniline (tetryl), trinitroglycerine (TNG), nitroguanidine (NQ), ethylene glycol dinitrate (EGDN), nitrocellulose (NC), pentaerythritol tetranitrate (PETN), glycidyl azide polymer (GAP), hexahydro-1,3,5-trinitrio-1,3,5-triazine (RDX), octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) and 2,4,6,8,10,12-hexanitrohexaaza-isowurtzitane, CL-20 (Fig. 1).

Past and present practices with explosives such as manufacturing, formulations, testing and training, demilitarization, and open burning/open detonation (OB/OD) have lead to severe soil and groundwater contamination (2 to 4). It has been estimated that TNT alone is produced in amounts close to 2 million pounds a year (5) and a

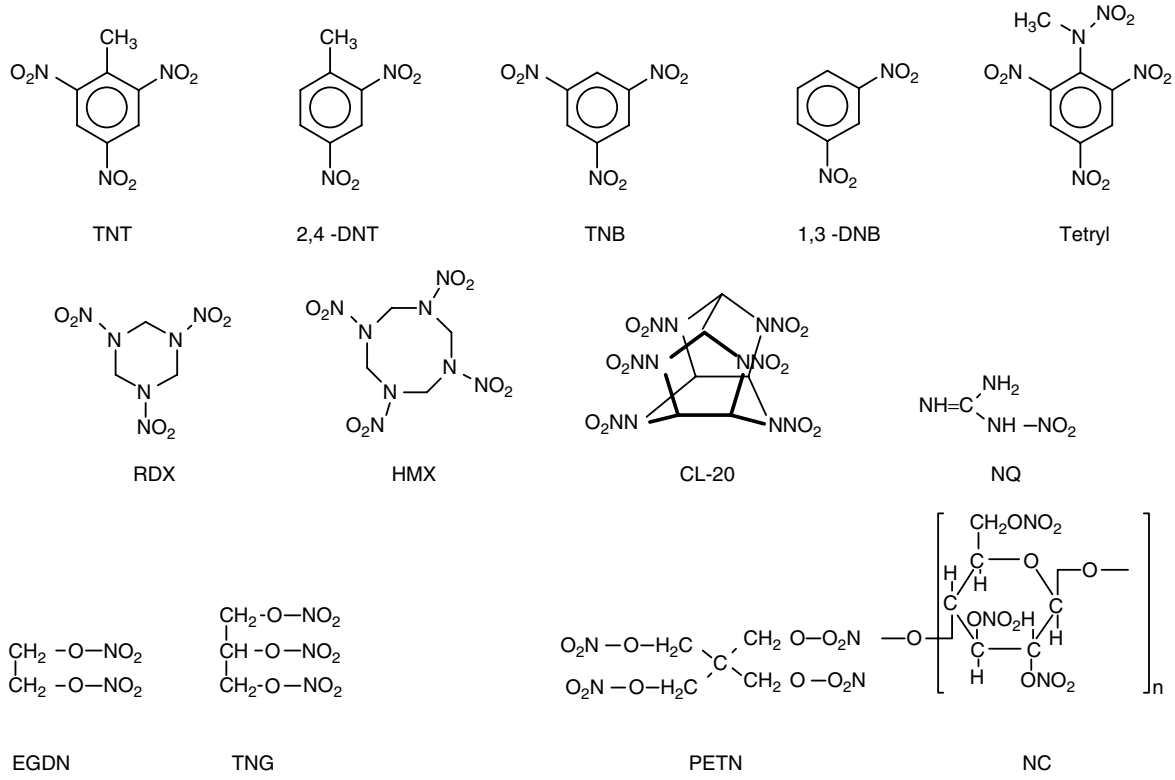

Figure 1. Structures and common names of most commonly used secondary explosives.

single TNT manufacturing plant can generate more than 1.8×10^3 m^3 of wastewater per day (6). During the manufacture of RDX, up to 12 mg/L may be discharged to the environment in process wastewater (7). Presently soil and groundwater contamination by explosives is a worldwide environmental problem that started following intensive military activities in World Wars I and II and the subsequent cold war that lasted until the turn of the century. For example, the widespread contamination of soil and water by explosives in the United States is attributed to activities related to the Second World War and the Korean War (8). Subsequent demilitarization activities caused most of the contamination problem in Germany (9). In Canada, approximately 100 OB/OD and training (air and land force bases) sites are known to be associated with activities involving RDX, HMX, and TNT (10).

Several countries such as United States, Canada, and Germany have initiated programs to determine the level of soil and water contamination by explosives and to develop cost-effective bioremediation technologies to clean their contaminated sites. Other countries whose territories (land and water) have experienced extensive military activities and some of the worst armed conflicts in human memory (First and Second World Wars, Vietnam and Korean wars, wars in the Middle East, conflicts in Africa and the civil wars in East Europe and in the breakaway republics of the former Soviet Union) have no available data on the environmental impact of explosives and the need to decontaminate affected areas.

The physicochemical properties (solubility, octanol/water partition coefficient (k_{ow}) soil/water partition coefficient (k_d), volatility) of some of the most frequently encountered explosives are presented in Table 1 (11–31). Knowledge of the physicochemical properties of explosives can help understand their fate and environmental impact. For example, k_{ow} and k_d values can tell how an explosive interacts with soil and gives an indication of its capacity for subsurface soil migration and groundwater contamination. Groundwater contamination indicates whether explosives and their degradation products leach through subsurface soil to reach the water table and is a useful indicator of the extent of site contamination.

Most energetic chemicals are toxic to aquatic and terrestrial species (32,33). Increased public awareness of the toxicity and risk associated with energetic compounds necessitates the development of cost-effective bioremediation technologies for their removal. The conversion of contaminants to water and gaseous nitrogen or carbon dioxide is called *mineralization*. As far as we are aware, no in situ microbial mineralization–based technologies exist for any explosive, but ex situ bioremediation technologies such as composting (recently reviewed by Burns-Nagel and coworkers (34)) and bioslurry technology (35) have been reported. In almost all reported studies, little to no mineralization is produced, and the fate of the compound and its (bio)transformed products are undetermined. The present document provides an overview of the scope of the contamination problems related to secondary explosives and summarizes relevant key biodegradation studies undertaken under both aerobic and anaerobic conditions to degrade these compounds.

BIODEGRADATION OF EXPLOSIVES

The nitro $-NO_2$ functional groups that define the chemical and energetic properties of explosives also characterize their biochemical and microbial reactivity. For example the frequent observation of reduced amine products during TNT biodegradation is attributed to the presence of highly oxidized $-NO_2$ groups directly attached to the aromatic benzene ring in the parent compound. The π-electrons and the carbon atoms located in the aromatic ring of TNT are shielded from any external attack, particularly electrophilic, by the steric effects caused by the presence of the four functional groups ($3\ -NO_2$ and $1\ -CH_3$) and by the electron withdrawing properties of the nitro groups (10). Consequently, any enzymatic attack on TNT would preferably take place at the external $-NO_2$ functional groups leaving the stable aromatic system intact. As is described later, TNT, under both aerobic and anaerobic conditions, undergoes reduction of the highly electrophilic $-NO_2$ groups to produce the corresponding hydroxylamino derivatives ortho-hydroxylamino-4,6-dinitrotoluene (2-HADNT) and para-hydroxylamino-2,6-dinitrotoluene (4-HADNT). The characteristic functional group $-$NHOH is extremely reactive and can participate in several abiotic and biological reactions such as rearrangement (Bamberger) to produce the corresponding phenolamines ortho-amino-5-hydroxy-4,6-dinitrotoluene, 2-A-5-OH-4,6-DNT, and para -amino-5-hydroxy-2,6-dinitrotoluene, 4-A-5-OH-2,6-DNT (36). Also the $-$NHOH group can undergo further reduction to produce the corresponding amines under both aerobic and anaerobic conditions. Under strictly anaerobic conditions, ADNT (amino-dinitrotoluene) is reduced further to produce the reactive product 2,4,6-triaminotoluene (TAT) (37–41), which can irreversibly bind to soil (38,42).

In the case of the two nonaromatic cyclic nitramines RDX and HMX, which lack the aromatic stability of TNT, it was shown that once an initial successful microbial (or chemical) attack occurs at RDX or HMX, the molecule becomes unstable and undergoes spontaneous decomposition to eventually produce nitrous oxide, formaldehyde, and carbon dioxide (43,44).

General Procedure for Laboratory Test Studies

Contaminated soil (or water) samples can be obtained from either a firing range, a OB/OD, or a manufacturing site and are analyzed for explosives content using a validated method such as the U.S. Environmental Protection Agency's EPA Method #8330 (45). The contaminated soil is then treated with a source of microorganisms (domestic anaerobic sludge, soil indigenous degraders, microbial consortia in manure, or specific isolates) in low volume (50 to 100 ml) serum bottles (microcosms) (Fig. 2; 43,44). Some microcosms are supplemented with either a uniformly labeled [UL-^{14}C] explosive and then fitted with a small test tube containing KOH (0.5 M) to trap liberated carbon dioxide ($^{14}CO_2$). In some cases heavy isotope ^{15}N labeled compounds are used in conjunction with mass spectrometry to confirm the origin of N_2, N_2O, and other N-containing intermediates produced during the biodegradation of explosives such as RDX and

Table 1. Physicochemical Properties of Some of the Most Used Explosives

Compound	Abbrev.	Type of Nitration	Chemical Formula	Molecular Weight g/mol	Melting Point °C	Water Solubility mg/L at 25 °C	Partition Coefficients log K_{ow}	Adsorp. Coeff. K_d L/kg	Henry's Law Constant atm-m^3/mole at 25 °C	Vapor Pressure mm Hg at 25 °C
2,4,6-Trinitrotoluene	TNT	C–NO_2	$C_7H_5N_3O_6$	227.13	80.1	130* (11)	1.6, 2.2, 2.7 (14)	4.0 (12) 6.38 (15)	4.57×10^{-7}* (13)	1.99×10^{-4}* (13)
2,4-Dinitrotoluene	2,4-DNT	C–NO_2	$C_7H_6N_2O_4$	182.15	71	280 (16)	1.98 (16)	7,400 (17)	1.86×10^{-7} (16)	2.17×10^{-4} (16)
1,3,5-Trinitrobenzene	TNB	C–NO_2	$C_6H_3N_3O_6$	213.12	122.5	340* (18)	1.18 (16)	60,000 (17)	2.21×10^{-9} (16)	3.03×10^{-6} (16)
1,3-Dinitrobenzene	1,3-DNB	C–NO_2	$C_6H_4N_2O_4$	168.11	90	200* (19)	1.49 (16)	4,500 (17)	8.01×10^{-7} (16)	1.93×10^{-4} (16)
N,2,4,6-Tetranitro-N-methylaniline	Tetryl	C–NO_2 N–NO_2	$C_7H_5N_5O_8$	287.17	129.5	75.0* (20)	1.65 (21)	5.8 (17)	2.69×10^{-11} (16)	5.69×10^{-9} (16)
Hexahydro-1,3,5-trinitro-1,3,5-triazine	RDX	N–NO_2	$C_3H_6N_6O_6$	222.26	204.1	42.3* (22)	0.87 (23)	1.2 (17) 0.2–7.8 (24)	1.96×10^{-11} (16)	4.03×10^{-9} (16)
Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine	HMX	N–NO_2	$C_4H_8N_8O_8$	296.16	286	6.63* (25)	0.13 (26)	0.0–1.2 (24)	2.60×10^{-15} (16)	3.33×10^{-14} (16)
2,4,6,8,10,12-Hexanitro-2,4,6,8,10,12-hexaaza-isowurtzitane	CL-20	N–NO_2	$C_6H_6N_{12}O_{12}$	438.19	260** (27)	50.0 (28)	ND	ND	ND	ND
Nitroguanidine	NQ	N–NO_2	$CH_4N_4O_2$	104.07	245	4,200 (16)	−0.83 (16)		4.67×10^{-16} (16)	1.43×10^{-11} (16)
Pentaerythritol tetranitrate	PETN	O–NO_2	$C_5H_8N_4O_{12}$	316.17	141.3	2.1 (29)	3.71 (16)		1.07×10^{-9} (16)	5.38×10^{-9} (16)
Trinitroglycerol	TNG	O–NO_2	$C_3H_5N_3O_9$	227.11	13.2	1,950 (16)	1.77 (21)		2.71×10^{-7} (16)	1.77×10^{-3} (16)
Ethylene Glycol Dinitrate	EGDN	O–NO_2	$C_2H_4N_2O_6$	152.08	−22.8 (29)	5,600 (16)	1.28 (16)		2.52×10^{-6} (16)	7.06×10^{-2} (16)
Nitrocellulose	NC	O–NO_2	$(C_6H_7N_3O_{11})_n$	10^5–10^6 (30)	206*** (31)	insoluble (16)	ND	ND	ND	ND

*at 20 °C; **with decomposition; ***decomposes partly before melting.
Source: Modified from several references.

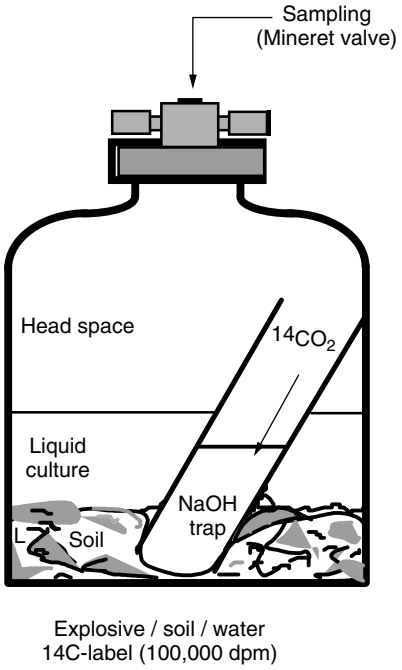

Figure 2. A typical laboratory microcosm used to study the microbial degradation of pollutants. See color insert.

HMX (43,46). Microcosms with the [UL-^{14}C] explosive are sampled for the determination of $^{14}CO_2$ (mineralization) in the KOH trap using a Packard, Tri-Carb 4530 liquid scintillation counter. Microcosms that did not receive ^{14}C-labeled RDX are reserved for the analysis of intermediate degradation products using analytical techniques such as gas chromatography-mass spectrometry (GC-MS), high performance liquid chromatography-UV detection (HPLC-UV) and liquid chromatography-mass spectrometry (LC-MS) (43,44).

Biodegradation of TNT and Other Related Polynitroaromatic Explosives

TNT, which is considered as one of the most widely used explosives, is selected as a model compound to review the microbial degradation of polynitroaromatic energetic chemicals. TNT is a reactive molecule and has been repeatedly reported to biotransform readily to give monoamino-dinitrotoluenes (ADNT) and diamino-mononitrotoluene (DANT) under both aerobic and anaerobic conditions (9,47–49). The resulting amines contain reactive $-NH_2$ functional groups that (bio)transform further to several other products including azo and acetyl derivatives, with little to no mineralization (9,39). In general no significant mineralization has been generated thus far during bacterial treatment of TNT. Fungi can produce relatively high amounts of mineralization (40%) (9) in a liquid culture but no significant mineralization is observed in soil. Tables 2 and 3 (50) summarize the microorganisms (bacteria and fungi) that have been studied for their degradation potential of TNT.

Table 2. Biodegradation of TNT with Bacteria Under Aerobic and Anaerobic Conditions

Bacterial Strain	[TNT] (mg/L)	Biotrans-formed (%)*	Bacterial Strain	[TNT] (mg/L)	Biotrans-formed (%)*
Gram-negative aerobic:					
Acinetobacter johnsonii	<10	100	*Agrobacterium* sp. 2PC	>60	100
Alcaligenes eutrophus	>60	27	*Cytophaga pectinovora*	<10	100
Flavobacterium odoratum	<10	100	*Klebsiella* sp. 1PC	<10	100
	>60	0		>60	58
Pseudomonas aeruginosa	<10	97	*Pseudomonas* sp. JLR11	100	100
	>60	52	*Pseudomonas* sp. Tol1A	>60	46
Pseudomonas fluorescens	>60	73	*Rahnella aquitilis* BFB	>60	100
Gram-negative facultatively anaerobic					
Enterobacter cloacae PB2	113	95	*Escherichia coli*	>60	100
	>60	8		100	100
Gram-negative anaerobic:					
Veillonella alkalescens	100	100			
Sulfate-reducing bacteria:					
Desulfobacterium indolicum	100	82	*Desulfovibrio vulgaris*	100	69
Desulfovibrio sp. (B strain)	100	100	*Desulfovibrio* spp.	100	100
Gram-positive aerobic:					
Rhodococcus erythropolis	>60	15	*Staphylococcus* sp.	100	98
Gram-positive anaerobic:					
Clostridium acetobutylicum	50	100	*Clostridium pasteurianum*	100	100
Methane-producing bacteria:					
Methanococcus strain B	113	100	*Methanococcus deltae*	113	20

Source: Data from J. Hawari, S. Beaudet, A. Halasz, G. Ampleman and S. Thiboutot, *Appl. Microbiol. Biotechnol.* **54**, 605–618 (2000).; *Mineralization either not reported or less than 1%.

Table 3. Biodegradation of TNT with Fungi

Fungal Strain	[TNT] (mg/l)	CO_2 (%)	Biotransformed (%)
Wood-rotting/white-rot basidiomycetes:			
Phanerochaete chrysosporium ATCC 1767	100.0	13.7	100
Phanerochaete chrysosporium BKM-F-1767	100.0	18.0	100
Phanerochaete chrysosporium BKM-F-1767	40.0	10.0	100
Phanerochaete chrysosporium BKM-F-1767	20.0	39.0	100
Phanerochaete chrysosporium	10.0	40.0	100
Fomes fomentarius MWF01-4	22.7	8.0	93*
Trametes versicolor TM5	22.7	28.0	100*.
Litter-decaying basidiomycetes:			
Agaricus eastivalis TMAest1	22.7	9.0	100*
Agrocybe praecox TM70.3.1	22.7	14.5	100*.
Clitocybe odora TM3	22.7	5.1	88*
Coprinus comatus TM6	22.7	0.8	82*
Micromycetous fungi:			
Alternaria sp. TMRZ/WN2	22.7	0.4	74*
Aspergillus terrus MWi458	22.7	0.2	100*
Fusarium sp. TMS21	22.7	0.6	100*
Mucor mucedo DSM810	22.7	0.1	95*
Neurospora crassa TM	22.7	0.6	100*
Penicillium frequentans ATCC96048	22.7	0.5	100*
Rhizoctonia solani MWi5	22.7	0.2	90*

Note: Data from J. Hawari, S. Beaudet, A. Halasz, G. Ampleman, and S. Thiboutot, *Appl. Microbiol. Biotechnol.* **54**, 605–618 (2000).;
n.d.: no data; *[TNT] = 56.8 mg/l.

The addition of a hydride anion (H^-) to the aromatic π-system of TNT can lead to the formation of a Meisenheimer complex that has the potential for mineralization. Vorbeck and coworkers (51) reported the formation of monohydride and dihydride Meisenheimer complexes in the treatment of TNT with *Rhodococcus erythropolis* strain HLPM-1, whereas Lenke and Knackmuss (52) observed a Meisenheimer complex during conversion of picric acid (2,4,6-trinitrophenol) to 2,4-dinitrophenol. In addition, Duque and coworkers (53) reported that denitration of the resulting complex formed from the treatment of TNT with *Pseudomonas* sp. clone A lead to mineralization. Nishino and coworkers (54) showed that 2,4-DNT, a partially denitrated TNT, could mineralize under aerobic conditions using *Burkholderia* sp. An enzymatic denitration process yielding partially or totally denitrated TNT products would thus create a pathway for mineralization if coupled to the process reported by Nishino and coworkers (54).

Biodegradation with Anaerobic Bacteria

There has been extensive reporting on the biodegradation of polynitroaromatic explosives such as TNT under anaerobic conditions. In almost all studies, the initial products are the amine derivatives that biotransform further to other aromatic products without being mineralized. Most reported studies on the anaerobic treatment of TNT have been recently reviewed by Hawari and coworkers (50). Thus far, TNT biodegradation under anaerobic conditions lead to the initial production of 4-amino-2,6-dinitrotoluene (4-ADNT), 2-amino-4,6-dinitrotoluene (2-ADNT), 2,4-diamino-6-nitrotoluene (2,4-DANT), and 2,6-diamino-4-nitrotoluene (2,6-DANT). Reduction of the $-NO_2$ groups in TNT is found to be regioselective and normally favors reduction at the para $-NO_2$ group to produce the corresponding mono- and diamino derivatives 2-ADNT and 2,4-DANT. 2,4,6-Triaminotoluene (TAT) is only observed under strictly anaerobic conditions (Fig. 3; 37,41,55,56). TAT is a very reactive molecule and can undergo several abiotic and biological reactions. The triamine was reported to undergo deamination to produce ammonium ions using a sulfidogenic isolate (41), whereas Funk and coworkers (55) reported the formation of para-cresol from TNT biodegradation. More recently Hawari and coworkers (40) showed that when [$^{13}CH_3$]TNT was treated with the sludge, neither [$^{13}CH_3$]toluene nor [p-$^{13}CH_3$]cresol were detected as TNT metabolites during biodegradation, indicating the absence of denitration or deamination. Table 2 clearly shows that TNT biotransforms effectively without significant mineralization. Figure 3 shows the transformation of TNT to TAT, which accumulates in the system before being transformed to other unidentified products without mineralization.

Biodegradation by Fungi

The interest in using fungi in bioremediation stems from their widespread presence in nature and their ability to produce several extracellular enzymes including lignin peroxidase (LiP), manganese-dependent peroxidase (MnP), and laccase, all capable of degrading several types of chemical bonds. The most recent and comprehensive reports on the biotransformation of TNT with fungi have been recently reviewed by Fritsche and coworkers (9), who described the existence of close to

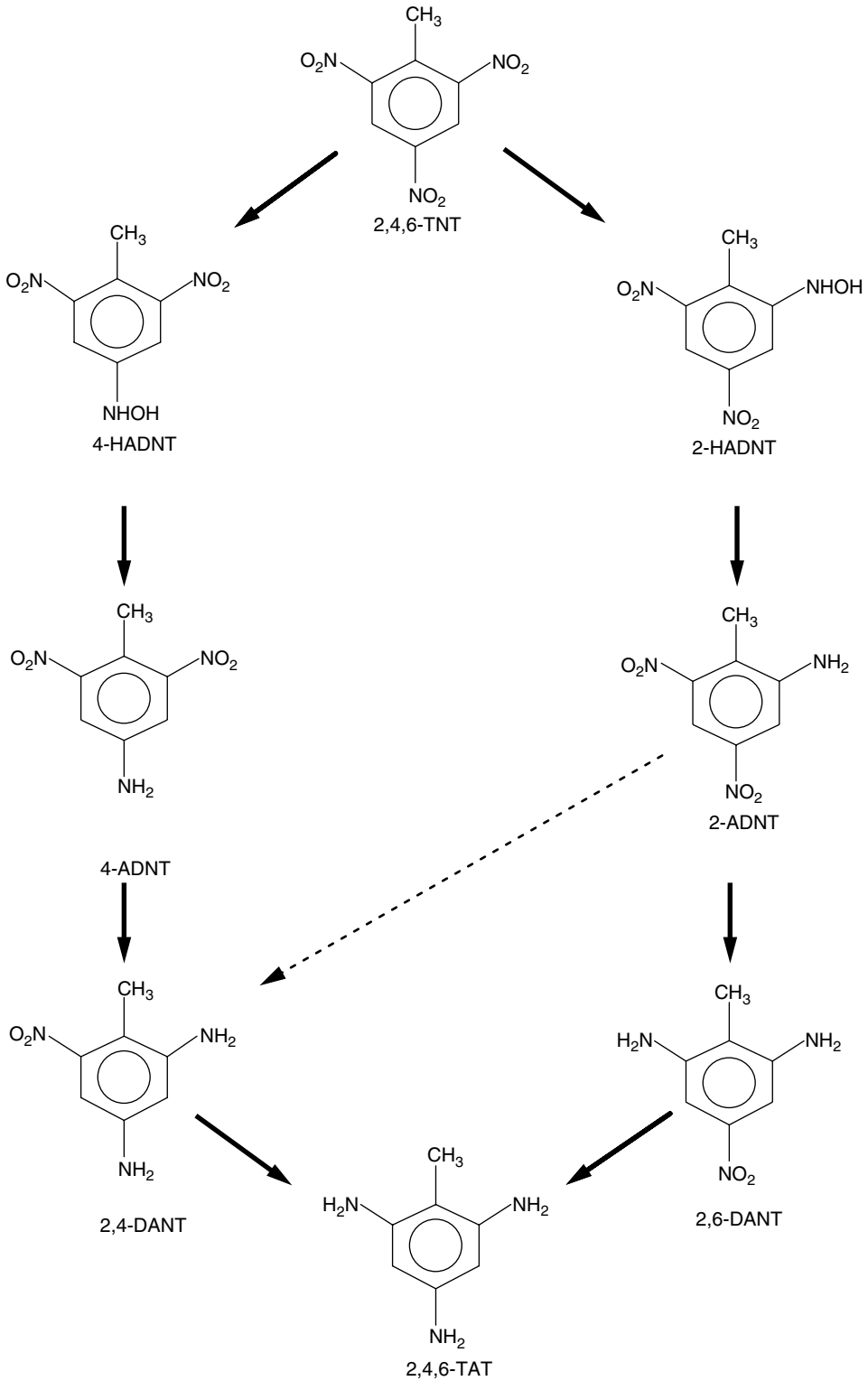

Figure 3. A comprehensive pathway summarizing biotransformation routes of TNT under anaerobic conditions. Constructed from papers that have been recently reviewed in References 37–41,49,50,55,120.

1.5 million different fungal species that colonize a wide range of habitats. Ninety-one fungal strains were tested and most were found capable of biodegrading TNT. In the case of the wood rot fungi, *Phanerochaete chrysosporium*, mineralization of TNT is found to be dependent on the organism's physiological condition being ligninolytic or nonligninolytic (9,46,57). TNT is reported to mineralize under the ligninolytic condition of the fungi, a state identified by the presence of peroxidase enzymes such as lignin peroxidase (LiP) and manganese-dependent

peroxidase (MnP). For example, more than 30% mineralization has been reported for the degradation of TNT with *Phanerochaete chrysosporium* (58) and *Clitocybula dusenii* TMb12 (59) in liquid cultures under ligninolytic conditions. Several other authors (60–62) reported that MnP from basidiomycetous fungi is capable of converting TNT and its reduction products (hydroxylamino- and amino-dinitrotoluenes) to carbon dioxide in relatively high yield.

The initial products from TNT biotransformation with *Phanerochaete chrysosporium* were nitrosotoluene (NsT), ortho-hydroxylamino-4,6-dinitrotoluene (ortho-HADNT), para-hydroxylamino-2,4-dinitrotoluene (para-HADNT) and mono- and diaminonitrotoluenes (ADNT and DANT) (46,63). The initial products NsT, HADNT, ADNT, and DANT were formed under nonligninolytic conditions (no LiP) and biotransformed further to give several other secondary products including azo, azoxy, phenolic, and acylated (acetylated and formylated) derivatives (Fig. 4). Some of the detected azoxy compounds, possibly produced as condensation products between HADNT and NTs, include 4,4′,6,6′-tetranitro-2,2′-azoxytoluene (TN-2,2′-AzoxyT) and 2,2′,6,6′-tetranitro-4,4′-azoxytoluene (TN-4,4′-AzoxyT). The azoxy compounds did not accumulate and they biotransformed to the azo derivatives 4,4′,6,6′-tetranitro-2,2′-azotoluene (TN-2,2′-AzoT) and 2,2′,6,6′-tetranitro-4,4′-azotoluene (TN-4,4′-AzoT), which later reduced to give the hydrazo-derivatives 4,4′,6,6′-tetranitro-2,2′-hydrazotoluene (TN-2,2′-HydrazoT) and 2,2′,6,6′-tetranitro-4,4′-hydrazotoluene (TN-4,4′-HydrazoT), respectively (Fig. 4). In a more recent study, Hawari and coworkers (46) detected nine different acylated products as shown in Figure 4. They included 2-N-acetylamido-4,6-dinitrotoluene, 2-N-AcDNT, and its p-isomer, 4-N-AcDNT, 2-formylamido-4,6-dinitrotoluene, 2-N-FmDNT, and its p-isomer, 4-N-FmDNT, 4-N-acetylamino-2-amino-6-nitrotoluene, 4-N-AcANT, and 4-N-formylamido-2-amino-6-nitrotoluene, 4-N-FmANT, 4-N-acetylhydroxy-2,6-dinitrotoluene, 4-N-AcHDNT, and 4-N-acetoxy-2,6-dinitrotoluene, 4-N-AcoxyDNT, and finally 4-N-acetylamido-2-hydroxylamino-6-nitrotoluene, 4-N-AcOHDNT.

In general, biodegradation of TNT by ligninolytic fungi (*Clitocybula dusenii* TMb12 and *Phanerochaete chrysosporium*) produced higher mineralization amounts than those normally obtained by bacteria (Tables 2 and 3). It has been suggested that the presence of very reactive free radicals, created by an electron transfer process to TNT by either LiP or MnP (9,57,63,64) might enhance the degradation of the aromatic ring. The involvement of highly reactive free radicals in the degradation of TNT by fungi, which can be verified using electron spin resonance spectroscopy (ESR), creates a problem in soil because of the potential quenching of free radicals in soil.

In summary, TNT biotransforms readily under both aerobic and anaerobic conditions with little to no mineralization. Therefore the discovery of new microorganisms capable of mineralizing TNT and other polynitroaromatic explosives such as tetryl or enhancing their irreversible binding (immobilization) to soil is receiving considerable attention from the scientific community. As we will describe later several authors have recently addressed the importance of understanding the mechanisms of interactions between the energetic chemicals and their degradation products with their matrix (soil and biomass) as an alternative soil remediation technology (52,65–67).

Biodegradation of the Heterocyclic Nitramine Explosives RDX, HMX and CL-20

Heterocyclic nitramine explosives such as RDX, HMX, and CL-20 lack the aromatic electronic stability of TNT and its transformed products. Predictably, a successful enzymatic transformation of one of the $N-NO_2$ or the inner C–N bonds of the cyclic nitramine would lead to ring cleavage because the inner C–N bonds in RDX become very weak (<2 Kcal/mole) (68). Recently this hypothesis was tested and proved feasible when RDX produced high amounts of carbon dioxide and nitrous oxide following its treatment with either municipal anaerobic sludge (43,44) or the fungus *Phanerochaete chrysosporium* (69).

Biodegradation Under Anaerobic Conditions

McCormick and coworkers (70) is the first group of workers to describe biodegradation of RDX using microorganisms in anaerobic sludge. They postulated a pathway for the degradation of RDX based on the sequential reduction to hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine, (DNX), and hexahydro-1,3,5-trinitroso-1,3,5-triazine, (TNX) prior to cleavage to form formaldehyde, methanol, hydrazine, and dimethyl hydrazine. Subsequent reports on the biodegradation of RDX and HMX, reviewed recently by Hawari and coworkers (43), did not provide additional information on the initial and intermediate metabolites and the enzymes associated with their formation. For instance several soil isolates *Providencia rettgeri*, *Citrobacter freundii,* and *Morganella morganii* (71) and consortia from horse manure (72) were found to successfully remove RDX, but apart from the nitroso derivatives (MNX, DNX, and TNX) no other ring cleavage products were reported. Recently, Hawari and coworkers (43,44) reported detailed time course studies for the biodegradation of RDX and HMX by domestic anaerobic sludge and provided evidence for the effective mineralization of both explosives to nitrous oxide (N_2O) and formaldehyde (HCHO), which later biotransforms to carbon dioxide. Using LC/MS, Hawari and coworkers (43,44) provided experimental evidence signifying the formation of two important ring cleavage products, identified as methylene dinitramine, $O_2NNHCH_2NHNO_2$, and traces of dimethanolnitramine, $(HOCH_2)_2NNO_2$ (Fig. 5). Methylenedinitranmine was detected in almost stoichiometric amounts and the suggestion was thus made that RDX underwent ring cleavage via MNX or via a direct attack with a hydroxylase or hydrolase enzyme because neither DNX nor TNX can give methylenedinitramine (Fig. 5). In general, alkyldinitramine and hydroxyalkylnitramines, such as the present $(HOCH_2)_2NNO_2$, are unstable in water and can undergo spontaneous decomposition to eventually produce N_2O. The presence of nitrous

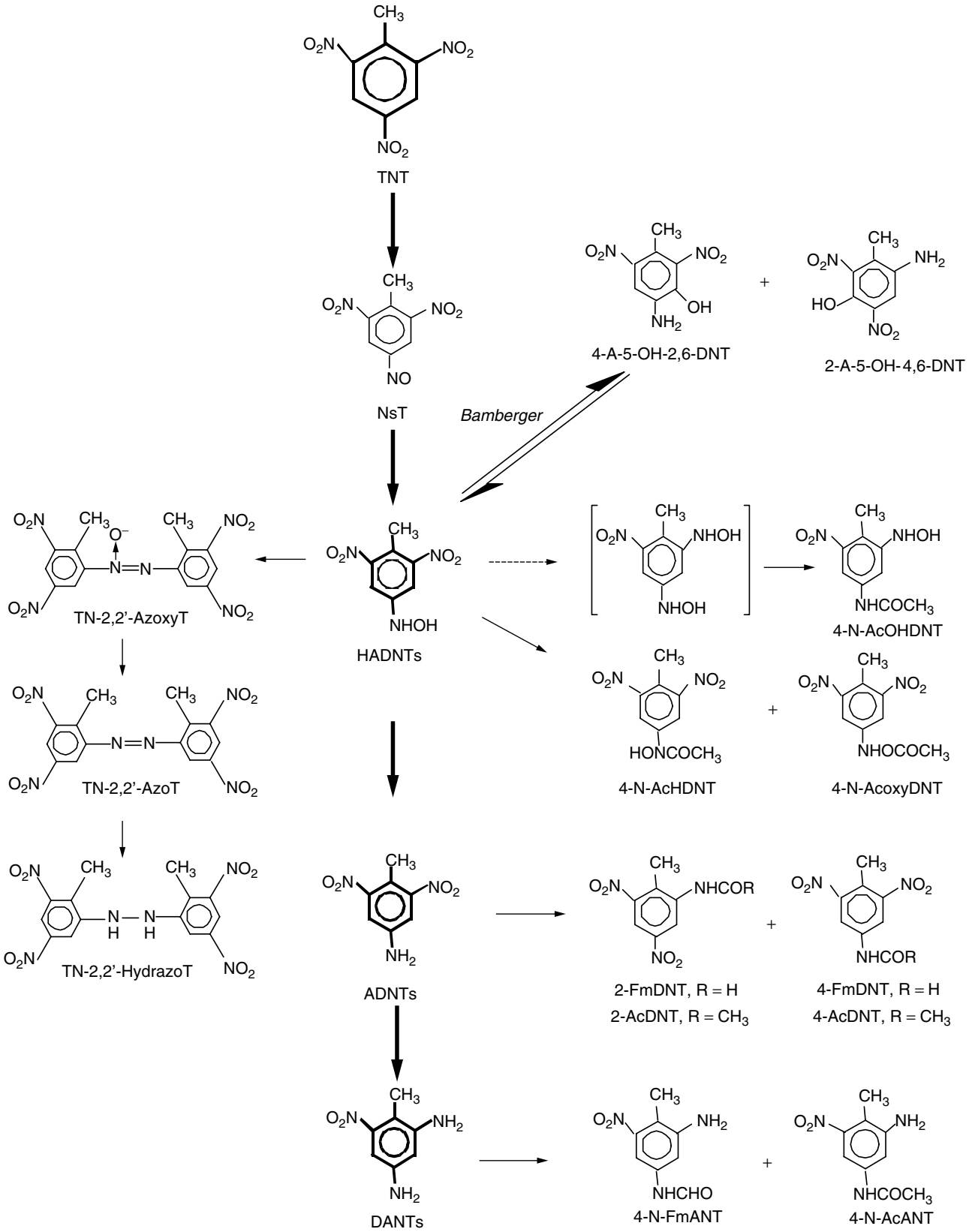

Figure 4. A comprehensive pathway summarizing biotransformation routes of TNT by fungi. Constructed from earlier papers published in the field in References 9,46,57,61,121.

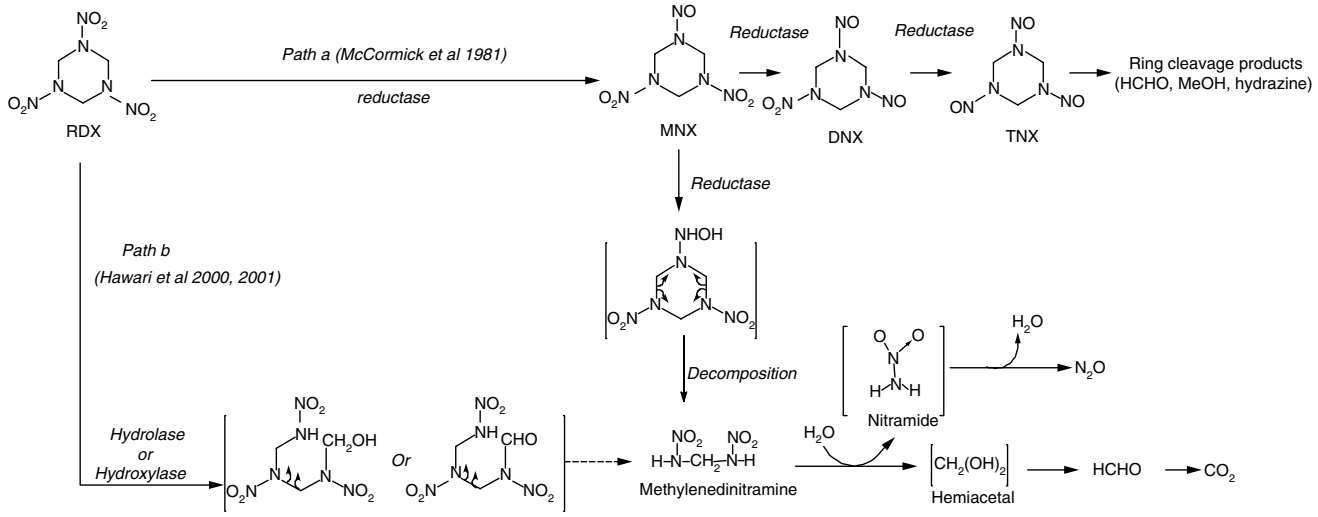

Figure 5. Postulated degradation pathways of RDX with domestic anaerobic sludge in a liquid culture. The observation of methylenedinitramine in stoichiometric amounts might favor ring cleavage as shown in the Figure. Square brackets signify undetected compounds. Modified from J. Hawari et al., *Environ. Sci. Technol.* **35**, 70–75 (2001).

oxide as an RDX metabolite was confirmed by using ring labeled [^{15}N] and the formation of $^{15}N^{14}NO$ detected by mass spectrometry at a mass ion of m/z 45 Da (43).

Interestingly anaerobic degradation of RDX in soil gave the three nitroso derivatives MNX, DNX, and TNX, with preferential accumulation of TNX. The formation of nitroso products from both RDX and HMX is considered to be enzymatic and produced by stepwise 2 electron transfer processes leading to the reduction of $-NO_2$ groups to the corresponding $-NO$ groups (Fig. 5). The resulting nitroso products did not accumulate but the mechanism of their subsequent transformation is not established yet. Degradation initiated by nitroreductases has been suggested as a potential degradation route for cyclic nitramines under anaerobic conditions (73). The authors reported the degradation of RDX by the enterobacterium *Morganella morganii* via the oxygen insensitive Type 1 nitroreductase (a two electron transfer process). No further details on the nature of intermediate products or metabolic pathways were provided. On the other hand, several studies reported the effective biodegradation removal of RDX and HMX in soil bioslurries (74–77), but in most cases no rigorous product analyses or mass balances were given. This information is very important when laboratory data are scaled up for further field application.

Biodegradation Under Aerobic Conditions

Experimental evidence gathered thus far on the aerobic metabolism of RDX and HMX has shown potential for their mineralization (10). Several strains of bacteria including *Stenotrophomonas maltophilia* (78), *Rhodococcus* sp. strain A (79) and *Rhodococcus* sp. strain D22 (80) biodegraded RDX when used as nitrogen source in pure culture. Most of these studies showed the initial formation of nitrite, but no other initial or intermediate metabolites were reported. Binks and coworkers (78) reported the cleavage of RDX with *Stenotrophomonas maltophilia*, but apparently misidentified the degradation products (43). Later Coleman and Duxbury (81) suggested the involvement of cytochrome P-450 in the metabolism of RDX by *Rhodococcus* sp. strain DN22. Recently, Sheremata and coworkers (69) employed the fungal strain *Phanerochaete chrysosporium* to degrade RDX using glycerol as a carbon source and detected the mononitroso derivative (MNX) as the sole RDX intermediate product, which did not accumulate. At the end of the experiment nitrous oxide (60%) and carbon dioxide (55%) were obtained as end- products. Mineralization of RDX (0.028 mg/L) by *Phanerochaete chrysosporium* has been reported earlier by Fernando and Aust (82). Using [^{14}C] RDX the authors reported high levels ($66.6 \pm 4.1\%$) of mineralization in liquid culture. No other metabolites were observed. In summary, despite the recent success in mineralizing cyclic nitramine explosives, the degradative pathways and enzymes involved in the process are unknown and warrant further research in this area.

Biodegradability of CL-20

CL-20 is a newly released energetic chemical whose microbial and environmental behavior is not yet known. However, the energetic chemical is a strained heterocyclic nitramine, which like RDX and HMX contains the $N-NO_2$ functional groups that characterize the chemical and microbial properties of the explosive (83). CL-20 was originally synthesized by Nielsen (84) and later adopted by Thiokol for large-scale production (83). In addition, both RDX and HMX are cyclic oligomers of methylenenitramine, CH_2-N-NO_2, $((CH_2NNO_2)_3$ for RDX and $(CH_2NNO_2)_4$ for HMX), whereas CL-20 is a caged rigid structure that contains the repeating unit

$CH{-}NNO_2$ (27,83). The three energetic chemicals RDX, HMX, and CL-20 are expected to show similar chemical and enzymatic reactions but with different kinetics. Earlier work indicated that initial enzymatic or chemical attack on either RDX or HMX leads to a ring cleavage with the eventual production of N_2O, HCHO, and CO_2 (10). The fact that CL-20 is a heterocyclic nitramine that also contains $CH{-}NNO_2$ and C–N functional groups (83), might indicate that the chemical could degrade by a mechanism similar to that of RDX and HMX (43,44). In fact, it has been reported that the characteristic bond cleavage of $N{-}NO_2$ in both RDX and HMX also occurs in CL-20 (85).

Other Explosives

There has been no extensive reporting on the microbial degradation of other explosives such as PETN, NC, NG, GAP, and CL-20 because the explosive is either a new release (CL-20) to the environment or research is hampered by very poor water solubility or steric effect such as the case with nitrocellulose (NC) (16,86). In the case of GAP, no extensive contamination has been reported in Canada because the explosive is not used by their military. Other explosives such as TNG (very reactive) and PETN are frequently hydrolyzed, water-assisted cleavage of the $-C{-}O{-}NO_2$ bond, to release the nitrite ion with the subsequent formation of a hydroxylated organic (R–OH) product instead.

(BIO)REMEDIATION TECHNOLOGIES

The most recent and important review on the applications and costs of biological treatments of explosives-contaminated soil has been published by Jerger and Woodhull (35). Several field-scale remediation studies have been conducted, but the actual fate of explosives is generally unknown. Table 4 outlines some of the reported bioremediation-based technologies that has been recently reviewed by Jerger and Woodhull (35).

Composting

Composting is a well-known biochemical process frequently used for waste reduction. Because of the presence of a wide diversity of biological activity within the compost, the technique can thus be used to biodegrade pollutants. Several groups of workers have reported the use of composting as laboratory and field-treatment processes for explosives (34,86,87). Composting received a wide public acceptance and the technology is frequently used in Germany and the United States. However, composting usually requires a large volume of organic amendments, making it difficult to trace the fate of the pollutant, particularly TNT and its biotransformed products in the compost. TNT transformed products such as HADNTs and their reduced amino derivatives can bind strongly to biomass or to soil humic material through the formation of amide linkages (–NH–CO–) (immobilization). Several groups of researchers (34,38,65,67) developed diagnostic tools such as NMR spectroscopy and immunoassay kits to determine the environmental fate of the explosive in soil and subsurface soil during composting. More details on the various types of practiced composting technologies including static piles, agitated composting and windrow composting can be found in a recent review written by Burns-Nagel and coworkers (34). These authors described in detail the transformation pathway of TNT under anaerobic/aerobic composting conditions and its eventual fate and environmental impact after composting. Presently composting, particularly windrow composting is accepted in the United States (35,88).

Slurry Phase Process Sabre (Simplot Anaerobic Bioremediation)

This process treats the explosives (RDX, HMX, and TNT) in soil in above ground bioreactors (biocells) under anaerobic conditions (89). In bioslurry technologies, several parameters must be adjusted including temperature, pH, redox potential, soil/water % and the organic source that

Table 4. Comparison of Technologies for the Remediation of Explosives Contaminated Soil (100,000 tones)

Technology	$/Ton	Advantage	Disadvantage
Windrow composting	133	Fast, self-heating, compost as a soil conditioner	Bulking agent and volume, cost for air monitoring (production of ammonia)
WMI TOSS	162	Currently offered as a commercial service	Transportation cost, external heat source,
EarthFax Fungal-based remediation	115	Rapid, extensive explosive reduction	Ambient temperature, inoculum, forced-aeration soil pile,
WR Grace-DRAMEND	130	Smaller final volume, no specialty equipment required	External heat source, limited depth of operation (<18 in.), long treatment time,
Simplot-SABRE bioslurry	125	Proven, treat high concentration, field application	Nature of soil (clay and plant material) Slurry preparation (soil washing), Slurry disposal (slurry dewatering)

Source: Modified from Jerger and Woodhull (35).

serves as a carbon source for the degrading microorganisms. The bioslurry-based technology is licensed by the University of Idaho Research Foundation. Earlier Guiot and coworkers (76) reported that very high concentrations of RDX (24,060 mg/kg) and HMX (7,860 mg/kg) can be effectively removed from contaminated soil in 8 L bioslurry reactors using a cement mixer as a bioreactor. Several other laboratory and field experiments for the treatment of explosives using bioslurry technology have been reported and reviewed by Hawari (10).

Daramend Remediation Technology

The technology is similar to land treatment; however, proprietary amendments are added to the soil to maintain operational cycles under anaerobic, anoxic, and aerobic conditions. The technology has been tested at several U.S. military sites containing soil and sediment contaminated with organic explosives. The technology is patented (U.S. Pat # 5,411,664; 5,480,579 and 5,618,427) and owned by Grace, Mississauga, ON, Canada.

Soil Pile Two-Stage Technology (TOSS)

A two-stage soil (TOSS) process was developed by Waste Management Inc., Cincinnati, OH. In the first stage of the process, the soil is treated with manure or anaerobic sludge in a biopile. The treated soil is then subject to a second treatment using composted wastes under aerobic conditions.

Fungal Treatment in Soil Piles

The technology is developed by EarthFax Engineering, Logan, VT, which is based on the use of extraneous source of fungi with organic amendments to remove explosives from soil. The technology has been tested on soil highly contaminated with TNT and tetryl (35).

Natural Attenuation

The term natural attenuation is a collective terminology that sums up the effect of several natural attenuation processes including transformation (abiotic and biotic) and transport (soil sorption, immobilization, and evaporation) on the environmental fate of pollutants. As the name implies, immobilization is a stabilization process wherein the pollutant and/or its degradation products irreversibly bind to the soil to produce complexes with long-term stability. To be able to understand and enhance immobilization we must first understand the application of the physicochemical properties that determine the binding mechanisms of the energetic chemical to soil (Table 1). For example TNT is relatively soluble in water (145 mg/L) with a soil-water partition coefficient (K_d) >4, whereas RDX and HMX are weakly soluble (45 mg/L and 5 mg/L, respectively) with K_d values around 1. K_d values provide us with quantitative evidence on the mobility and migration of explosives from soil through subsurface soil to cause groundwater contamination. The other physicochemical parameter is the octanol/water partition coefficient, K_{ow}, which indicates the chemical distribution between soil organic matter and water or between soil and plant tissue. Thus K_{ow} values can provide insight on important natural processes such as natural attenuation and phytoremediation. It has been shown that compounds with $1 < K_{ow} < 3$ can transport into the plant tissues.

The ability of TNT to (bio)transform to products that contain reactive amino functional groups ($-NH_2$) capable of binding to soil constituents could have potential remedial applications (natural attenuation) (52,90). The irreversible binding of TAT, a reduced TNT product, to soil and the long-term stability of the resulting complexes are considered important parameters in the enhancement of natural attenuation (41,91,92). Therefore, mechanisms to engineer and enhance the complexation of TNT and its degradation products with soil and improvement of the stability of these complexes would naturally attenuate and stabilize these contaminants in their matrix.

Phytoremediation

The use of plants and their associated microbial populations to remove contaminants from soil and water is an attractive in situ remediation method (93–95) that appears to be more cost-effective (96,97) than existing excavation, incineration, or other physical/chemical extractive methods. The process is also thought to be less disruptive to ecosystems as contaminants are selectively extracted without disruption to matrix organic content or soil microflora. Further insight into market overview of phytoremediation and the International industrial activities in this area can be found in Glass (98).

Terrestrial plants often have specialized microbial environment associated with their roots known as rhizosphere. The rhizosphere can be influenced by the plant through the secretion of nutrients and exudates, compounds that enhance the microbial populations' capacity to degrade a specific contaminant (99). Often the soil microbial population serves to protect the plant from a herbicide or other contaminant. For example, Siciliano and Greer (100) demonstrated the growth of meadow bromegrass (*Bromus erectus* Huds.) associated with a TNT transforming *Pseudomonas* sp. soil microbe on soil containing 46 g/kg TNT. However, more commonly in the case of explosives, the water-soluble contaminant fraction is passively transported in ground water directly into the plant tissue with no immediate transformation. In fact, phytoremediation experiments involving TNT and RDX often make use of constructed wetlands populated by aquatic plants (101–104) or arboreal species with high water demand (105), as the dominant mechanism for the uptake of TNT and RDX appears to be aqueous solubilization and transpirational flux. Furthermore, while TNT and other aromatic explosives are enzymatically modified and sequestered in root tissues (106–109), RDX appears not to be readily transformed and is deposited in leaf tissues following the evaporative loss of water (110). Recently we monitored several indigenous plant species at a Canadian antitank

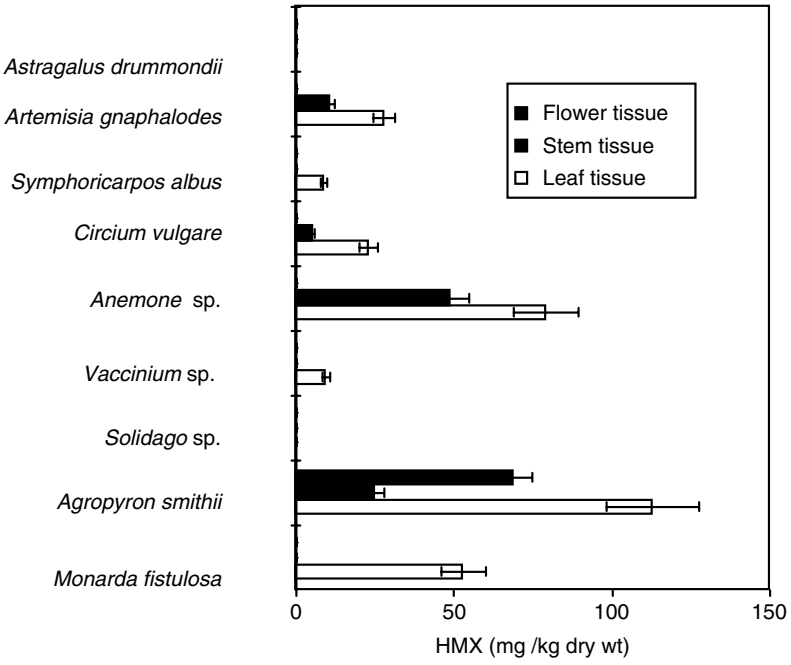

Figure 6. Prairie indigenous plants accumulating HMX in their leaves tissues.

firing range in central Alberta, where the melt-cast explosive Octol (70:30 wt % mix of HMX to TNT) was used and found the accumulation of HMX in the leaves of all tested plants, indicating the potential use of plants as a in situ clean-up option for military-contaminated sites. Rye grass and wheat were found to be the best species to phytoextract HMX from the site, but the heterogeneity of the soil and the lack of uniformity in HMX distribution at the site did not allow the determination of the percentage of HMX removal form the soil. Some of the analyzed plant tissues and their potential to phytoextract the explosive HMX are shown in Figure 6.

In a study involving the uptake of ^{14}C labeled RDX (111) into terrestrial plants gel permeation chromatography fractions with molecular weights of 800 to 1,000 daltons were observed to contain ^{14}C radioactive label. Similarly, in bush bean hydroponic studies involving ^{14}C labeled RDX (112) small quantities of radioactive component began to appear in basic aqueous leaf tissue extracts, which indicated modification of RDX. In both studies no radioactive gaseous products were detected. It is unclear if the labeled products in the studies of Harvey and coworkers (112) and Larson and colleagues (111) were direct conjugates of intact RDX species, or the high molecular weight products resulting from the incorporation of ring fragments or low molecular weight products.

ENVIRONMENTAL AND ECOLOGICAL RISK ASSESSMENT

In addition to their explosive characteristic, energetic chemicals such as RDX, HMX, tetryl, DNT, and TNT are toxic to terrestrial and aquatic species (32,33,113). They are also recalcitrant ($t_{1/2}$ for RDX 1.7 years) (114) and some are classified as carcinogens with adverse effects on the central nervous system (25,115). An ambient water quality criterion for the protection of human health of 105 μg/L has been proposed for ingestion of drinking water (116). The toxic and carcinogenic effects of explosives necessitate their remediation, but their (bio)transformation does not necessarily mean that the technology is working. Because explosives can biotransform to even more dangerous chemicals, a soil remediation technology can be considered successful if both chemical and toxicological indicators confirm that the soil is clean. Integrated chemical and toxicological monitoring methods must thus become available so that valuable information on the initial state of a contaminated site, as well as the fate of these chemicals after remediation can be obtained. Extensive toxicological assays (Ames, acute toxicity and earthworm tests) of composted soil revealed that composting, one of the most widely applied soil remediation technologies, particularly in both United States and Germany, is effective in the reduction of the toxicity of contaminated soil (34,117–119).

SAFETY PROCEDURES

Because of their explosive characteristics, careless handling of sampling procedures can lead to detonation, deflagration, or burning under various stimuli such as friction and electrostatic discharge. Thus, specific safety procedures must be followed while sampling at any explosive-contaminated site, particularly those that have unexploded ordinances (UXOs). It is expected that contaminated sites may contain more than one explosive, and in the case of nitroglycerine (NG), an organic vapor protective respiratory mask should be worn at all times during sampling and sample preparation. Although for other explosives this protective equipment is not needed, explosive powder might be carried away with soil dust. Under these circumstances, a dust mask must

be worn during sampling and sample preparation. Also protective clothing, gloves, and glasses should be worn at all times while working with explosives contaminated materials to avoid dermal contacts and harmful injuries.

CONCLUSION

In general, explosives are labile molecules and once in the environment they can (bio)transform, migrate through subsurface soil to reach groundwater and/or transport to plant tissues. Polynitroaromatic explosives such as TNT biotransforms quite readily under both aerobic and anaerobic conditions to initially produce amines that can react further to give the acyl-, azo-, and azoxy-derivatives with little mineralization. Fungi, however, can mineralize TNT in liquid culture medium (9). No mineralization-based bioremediation technologies are known for TNT or any other explosive and therefore there is a need for the discovery of new and engineered enzymes and microorganisms to promote mineralization of TNT and its biotransformed products. On the other hand the nonaromatic RDX and HMX explosives can biodegrade to produce nitrous oxide and formaldehyde. A potentially useful and emerging cost-effective in situ remediation technology for the clean-up of soils at contaminated military sites is phytoremediation. Another important approach for the management of sites contaminated with explosives is to engineer and create mechanisms to irreversibly bind explosives and their degradation products with soil (humification and immobilization).

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MICROBIAL DEGRADATION OF FUEL OXYGENATES

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INTRODUCTION AND STATEMENT OF THE PROBLEM

In 1990, the U.S. Environmental Protection Agency promulgated the Clean Air Act Amendments, which, in part, regulated the composition of gasoline motor fuels sold in cities that did not meet the federal ambient air quality standards for carbon monoxide and trophospheric ozone levels (1). The resulting oxygenated fuels and reformulated oxygenated gasoline (RFG) programs required that gasoline contain 2.7 and 2% by weight of oxygen as the oxychemicals methyl *t*-butyl ether (MTBE) or ethanol for reducing tailpipe emissions of carbon monoxide and ozone-forming compounds, respectively. Although other modifications of RFG were being implemented (e.g., reduction in sulfur, olefins, and aromatics content), MTBE was the primary oxygenate used by refiners to comply with the 2 or 2.7 wt.% oxygen (11 or 15% by volume) requirement. Ethanol was also accepted as a fuel blending oxygenate and used in some of the Midwest gasoline markets. MTBE blended RFG was sold in the eastern, western, and southern states. Other branched alkyl ethers and alcohols in addition to MTBE and ethanol have also been used as oxygenates (see Fig. 1) in gasoline blends, but less frequently depending upon hydrocarbon feedstocks and refining operations. For example, MTBE is made from isobutylene and methanol whereas tertiary amyl methyl ether (TAME) is made from isopentylene. Although there were apparent reductions observed in air pollutants in major cities of the United States during the 1990s, a U.S. Geological Survey showed the presence of detectable MTBE in 16.9% of the shallow urban monitoring wells and 3.4% of the rural wells samples (2). In addition, many public well water systems in California have been impacted with MTBE (3). It is now clear that MTBE is present in groundwater from the accidental release of gasoline from the underground storage system at fuel service stations. Gasoline releases from buried tanks or lines can migrate to subsoils and water-soluble compounds like MTBE can disperse into the water table and be transported with the local hydrogeologic flow of groundwater. MTBE is the most water-soluble component of motor fuel and is biodegraded very slowly in subsoils compared to the rapid metabolism of other gasoline organics (alkanes, isoalkanes, and aromatics). MTBE, therefore, has become a groundwater contaminant of concern, which continues to threaten public and private drinking water supplies. The severity of the MTBE groundwater problem has been addressed by an EPA Blue Ribbon Panel (4), which recommended a reduction in the use of the ether, a phase-out, and a replacement by an alternate oxygenate (e.g., ethanol) without compromising air quality standards.

It is widely recognized that aromatic hydrocarbon groundwater plumes (e.g., of benzene, toluene, ethylbenzene, and xylenes; BTEX compounds) from fuel spills are readily biodegraded by microbes associated with most subsoils (5,6). Microbial degradation of these compounds in groundwater is part of the overall intrinsic (natural attenuation) mechanism commonly observed in aquifers and is responsible for the observed reduction or control of groundwater BTEX plumes. In contrast, there is no overwhelming evidence currently that MTBE (or other ether oxygenates) plumes are intrinsically biodegraded (aerobic or anaerobic) in source spill zones or in the soluble portion of plumes. Some of the experimental research underway to address the problem of MTBE biodegradation in cultures, enrichments, and soil, and prospects for enhancing bioremoval mechanisms in aquifers is summarized in what follows.

PROPERTIES OF OXYGENATES

Much of what we know of the biodegradability of gasoline components (especially hydrocarbons) in subsoils and aquifers comes from decades of research on the metabolic capabilities of microbial cultures isolated from soils, sediments, and biosolids (7). The aromatic hydrocarbons and fuel oxygenates given in Table 1 are the most

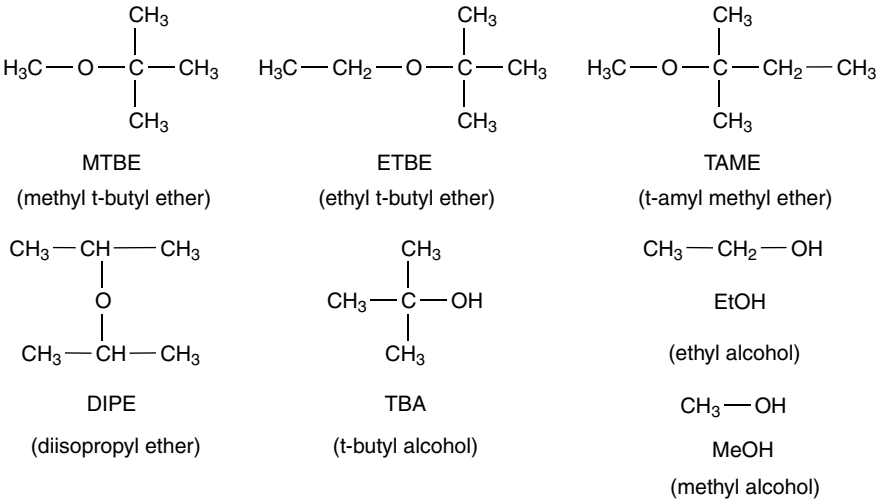

Figure 1. Fuel oxygenates used in gasoline blends.

Table 1. Physicochemical and Biological Properties of Water-Soluble Fuel Hydrocarbons and Oxygenates in Gasoline

Feature[a]	BTEX[b]	MTBE	ETBE	TAME	DIPE	Methanol	Ethanol	TBA
Molecular weight (g/mol)	78.1–106.2	88.1	102.2	102.2	102.2	32.0	36.1	74.1
Water solubility (mg/L)	110–1,780	43,000–54,000	26,000	20,000	2,000–9,000	Miscible[c]	Miscible	Miscible
log K_{oc}[d]	1.5–3.1	1.1	1.0–2.2	1.3–2.2	1.5–1.8	0.4–0.9	0.2–1.2	1.6
Henry's law constant[e] (dimensionless)	0.22–0.35	0.02–0.12	0.11	0.05	0.2–0.41	2×10^{-4}	1×10^{-4}	5×10^{-4}
Biodegradability	+	+/−	+/−	+/−	+/−	+	+	+/−
Bacterial growth[f]	+	−	−	−	−	+	+	−
Aquatic toxicity[g] EC^{50} (mg/L)	1–48[h,i,j]	100,450–720[k]	37, > 2500[l]	0.11, 100[m]	476[n]	2–26[o]	13,000–6,000[o]	4,430–5,000[o]
Percent in gasoline (average weight)	1.9–10.7[p]	11, 15[q]	12.9, 17.5[q]	12.4, 16.8[q]	12.9, 17.4[q]	NP[r]	6–10[s]	0–0.75

[a] Data on water solubility, log K_{oc}, Henry's law constant, and aquatic toxicity were taken from Ref. 8 unless noted otherwise. Values given are at 20 to 25 °C.
[b] Benzene, toluene, ethylbenzene, and xylenes.
[c] Completely soluble in water.
[d] Relative partitioning of compound between aqueous and organic phases.
[e] Relative partitioning of compound between vapor (air) and aqueous phases.
[f] *Biodegradation:* +, microbial degradation in most soils, sediments, and biosolids usually demonstrated; +/−, biodegradation not always observed or is incomplete. *Bacterial growth:* +, bacterial growth refers to the ability of organisms to produce significant cell mass on the organic compound (e.g., ≥ 0.5 g cells/g compound);−, refers to little or no biomass formed (≪ 0.5 g cells/g compound) through the metabolism.
[g] EC_{50} concentration affecting growth, reproduction, or immobilization of 50% of the test animals unless otherwise indicated for *Daphnia* (48-hour test) and fathead minnow (48- to 96-hour test); aquatic toxicity for aromatic hydrocarbons are from Ref. 9.
[h] LC_{50}, concentration affecting survival of 50% of the test animals (96-hour test).
[i] First and second values are for *Daphnia* and fathead minnow.
[j] Concentration for *Daphnia* is a 24 hour IC_{50}.
[k] Lowest observed effect concentration for *Daphnia* and fathead minnow, respectively, from Ref. 10.
[l] LC_{50} for mysid shrimp and sheepshead minnow, respectively.
[m] Algae and *Daphnia*, respectively.
[n] Fathead minnow.
[o] Various species.
[p] Data from Ref. 11 in wt./wt.% of gasoline; current RFG may contain less than or equal to 1% benzene.
[q] Wt.% by volume for reformulated gasoline containing 2 or 2.7 wt.% of O_2 (first and second value, respectively).
[r] NP, not present. Methanol is not usually added to gasoline but may be present in MTBE — formulated gasoline as a byproduct of MTBE production.
[s] Wt.% by volume as formulated in the gasoline market of the midwestern states of the United States.

water-soluble components of motor fuel and represent the predominant groundwater contaminants detected in monitoring well water samples from accidental gasoline releases at retail sites. Table 1 also is a comparative summary of the physical/chemical (solubility, sorption, and volatility) and biological (biodegradability and aquatic toxicity) features for the most water-soluble components of current formulated gasolines (8). In general, BTEX compounds are the least water-soluble, more volatile (higher Henry's law constant), the most biodegradable, and more toxic to aquatic species. BTEX compounds represent about 20% by weight of the gasoline fraction whereas the dominant oxygenates such as MTBE and ethanol are present at 15 and 10%, respectively. The octanol water partition coefficient (a measure of sorption to soil organic carbon) for BTEX and oxygenates indicates that they have similar but low tendencies to sorb to soils ($\log K_{oc} = 1$ to 2). Henry's law constant, given as a dimensionless value, indicates that the alkyl ethers are at least five times less volatile from aqueous solutions than BTEX. The alcohols, methanol, ethanol, and *t*-butanol (TBA), in contrast, are several orders of magnitude less volatile than BTEX and the ether compounds. BTEX and ethers are more readily analyzed from water samples by gas-liquid and headspace chromatography and purge and trap gas chromatography (P/T GC) and mass spectrometry methods (12). Because of the low volatility of alcohols, samples may need to be analyzed by heated P/T GC.

Some of the main reasons for the persistence of organic chemicals in soil and groundwater, even at low concentrations (μg/L or mg/L), are the (*1*) poor biodegradability by indigenous microbial populations, (*2*) absence of active organisms in soil, (*3*) inability of microbes to grow on the compound and produce actively degrading biomass, and/or (*4*) availability of insufficient concentrations of electron acceptor (e.g., O_2 to promote aerobic degradation). In general, BTEX compounds are rapidly biodegraded by the ubiquitous presence of aerobic hydrocarbon-degraders in most soils. There is now good evidence that this hydrocarbon degradation in aquifers is part of the containment of BTEX plumes under aerobic, and in some cases, anoxic (low oxygen) or anaerobic conditions (5,6,13).

The biodegradation of the fuel ether oxygenates, MTBE, ETBE, TAME, DIPE, and TBA, however, is not always observed in aquifer sediment biodegradation assays.

Studies on the characteristics of isolated cultures and enrichments have indicated that microbes grow poorly (low cell yield) and slowly (long doubling time) on these ethers and TBA. The poor biodegradability property of these oxygenates is, in part, responsible for their persistence in groundwater. The alcohols, methanol and ethanol, are very biodegradable and microbes metabolizing these chemicals are widely distributed in soils, sediments, and biosolids (wastewater activated sludges) (9,10). Ethanol and methanol are readily oxidized to carbon dioxide and methane under aerobic and anaerobic conditions, respectively.

The aquatic toxicities of the aromatic hydrocarbons (BTEX) expressed as EC_{50} (e.g., the concentration affecting 50% of test organisms in growth or survival) to the common aquatic species, *Daphnia*, algae, and fathead minnow are in the range of 15 to 50 mg/L. EC_{50} toxic doses of the alkyl ethers to these same organisms are much higher, usually greater than or equal to $\geq$100 to 2,500 mg/L. Methanol is significantly more toxic (EC_{50}, 2–26 mg/L) than ethanol (EC_{50}, 13,000–16,000 mg/L) or TBA (EC_{50}, 4,430–5,500 mg/L). The aquatic toxicity is critical when assessing the environmental impact of a pure chemical or RFG spill into a water body (river, lake, and stream) and the subsequent effects of eutrophication on survival, growth, and reproduction of affected species.

OBSERVATIONS ON THE MICROBIAL METABOLISM OF MTBE

As indicated previously, MTBE is the predominant oxygenate in the current formulations of gasoline and is a common groundwater contaminant from fuel storage tank spills to subsoils. Research over the last 7 years has focused on assessing the biodegradability of MTBE under aerobic and anaerobic conditions in single or mixed cultures derived from soil and sediment enrichments. Table 2 is a summary of these studies. Under aerobic conditions, three types of bacterial cultures that degrade MTBE have been isolated. Cometabolic cultures of mixed or single species that are able to grow on alkanes (e.g., propane, pentane, hexane) or isoalkanes (e.g., 2-methyl butane and 2-methylpentane) as primary substrates appear to stimulate one or more *monooxygenase* enzymes involved in MTBE metabolism (15–19). However, in some cultures grown on alkanes/isoalkanes, MTBE is cleaved to only TBA (15,16). Metabolic studies with these cultures have shown that uniformly labeled (UL) ^{14}C-MTBE is oxidized 45 to 60% to $^{14}CO_2$, 20% of the label is incorporated into the cells and the remaining 20% is associated with a soluble but less degradable or slowly degrading fraction. Cyclohexane can also serve as a primary substrate in a mixed culture derived by Corcho and coworkers (20), which can degrade MTBE to carbon dioxide.

Several single (pure) cultures have been isolated from soils and biosolids that degrade MTBE but which also grow poorly on the ether. Isolates of gram-positive (*Rhodococcus* and *Arthrobacter*) and gram-negative (*Rubrivivax*, *Hydrogenophaga*, and *Methylobacterium*) bacteria have been obtained from primary mixed cultures that were selected on MTBE (21–24). These organisms grow on many other substrates (e.g., alkanes, alcohols, and sugars) and have the ability to completely or partially degrade MTBE.

Mixed consortia of bacteria, which also degrade MTBE, have been enriched from biosolids obtained from refinery, chemical, and municipal wastewater treatment

Table 2. Microbial Cultures and Enrichments Degrading MTBE

Metabolism	Culture Type[a]	Substrate[b]	Reference
Aerobic	(1) Cometabolic (mixed or single)	Alkanes and/or isoalkanes Cyclohexane	15–19
	(2) Single species	MTBE, other substrates[c]	
	Rhodococcus sp. nov.		21
	Rubrivivax (PM1)[d]		22
	Hydrogenophaga flava		23
	Methylobacterium, *Rhodococcus*, and *Arthrobacter*		24
	(3) Mixed (biosolids and biofilter solids)[e]	MTBE	25–28
	(4) Soil, sediment enrichments[f]	MTBE	30–32
Anaerobic	Soils and sediments	MTBE	
	(a) Methanogenic		34[g], 35[h]
	(b) Iron-reducing		36
	(c) Nitrate-reducing		37

[a] Mixed or single refer to multiple or single species cultures, respectively. Cultures were isolated from soil, biosolids, or obtained from culture collections.
[b] Growth of organisms on MTBE is usually poor compared to most other substrates.
[c] Alcohols, hydrocarbons, or sugars.
[d] A member of the *Leptothrix* group of the β-Proteobacteria subclass.
[e] Activated sludges from refinery or chemical biotreaters or wastewater plants.
[f] Aquifer or surface soils or river sediments.
[g] MTBE transformed to *t*-butyl alcohol only with methane formed from methoxy-carbon of MTBE.
[h] Extent of MTBE degradation not determined; methanogenic conditions produced from other substrates.

plants (25–28). These cultures also do not grow well on MTBE (cell yields are 0.05 to 0.2 g cells/g MTBE) but degrade the ether rapidly with the transient formation of TBA (25). It should be mentioned that the other branched alkyl ethers (ETBE, TAME, and DIPE) occasionally detected in fuels and groundwater are also degraded by these same mixed enrichments (25,26) and single species isolates (15,19). This suggests that there are common ether-cleaving and branched alcohol oxidizing enzyme systems in these cultures.

Laboratory-scale biotreaters of these cultures usually need to be operated with high cell solids retention because of the slow growth and low cell yield on MTBE. Concentrations of MTBE that can be degraded and reported removal rates with these mixed cultures have varied from 5–200 mg/L to 5–70 mg MTBE/g cells/hour, respectively. The presence of other fuel compounds (e.g., BTEX) usually does not affect biotreatment of MTBE in these systems because mixed cultures are not limited in numbers of aromatic hydrocarbon-degraders. A recent study by Deeb and coworkers (29) suggests that 20 mg/L levels of ethylbenzene and xylenes inhibits MTBE degradation in a pure culture of an MTBE degrading organism (*Rubrivivax* PM1). It is not clear, however, that these hydrocarbons would affect MTBE biodegradation in portions of the contaminant plume in which soluble fuel organics and MTBE are present at low concentrations (μg/L to mg/L) and BTEX-degraders are ubiquitous.

Evidence for the apparent aerobic degradation of MTBE by indigenous ether-degrading microbes in aquifer soils (30,31) and streambed sediments has also been shown (32,33). UL-^{14}C-MTBE was degraded to 15 to 65% $^{14}CO_2$ in laboratory microcosms of sediments. Although experiments were done at less than or equal to 1 mg/L MTBE, only 3 of 12 surface or aquifer sediments showed significant mineralization ($\geq$50%) to $^{14}CO_2$. It is not clear whether the ether was degraded beyond TBA in the other site samples in which the amount of $^{14}CO_2$ formed was 5 to 30% of the total isotope used in the experiment.

The degradation of MTBE under the anaerobic conditions of methanogenesis and iron - or nitrate-reduction has recently been reported (34–37). Soil microcosms prepared with anaerobic river sediment that formed methane degraded MTBE to TBA only after several months (34). The degradation of UL-^{14}C-MTBE and ^{14}C-TBA (50 mg/L) was assessed in iron-reducing microcosms containing aquifer material or river sediment from a fuel spill site and amended with Fe^{3+} and humic acid as electron acceptors (35). $^{14}CO_2$ was formed after a long lag period of 250 to 300 days. The maximum percentage of $^{14}CO_2$ formed, however, was 20 to 30% of the labeled MTBE, suggesting that only the methoxyl-carbon of MTBE was mineralized and metabolism terminated at TBA. Similarly, the experiments by Bradley and coworkers (37) on the biodegradation of ^{14}C-MTBE in NO_3-reducing microcosms of stream bed sediments showed that about 20 to 25% of the ether was mineralized to $^{14}CO_2$ indicating that MTBE was only degraded to TBA.

In summary, data on the isolation of mixed and single cultures of naturally occurring bacteria that degrade MTBE indicate that growth on the ether is slow and generates a low cell yield. The single culture strains can grow on other substrates (e.g., alkanes and isoalkanes) which may induce ether and branched alcohol oxygenase-like enzymes that are, in part, responsible for the complete degradation of MTBE to carbon dioxide. Although there is preliminary evidence by some workers that suggest MTBE may be degraded in soils and sediments under induced methanogenic and iron- and nitrate-reducing conditions, it is not certain that the ether has been degraded beyond TBA.

PROPOSED BIODEGRADATION PATHWAY FOR MTBE

A current scheme for the metabolism of MTBE based on our understanding of its degradation by microbial cultures, animal tissues, and chemical oxidation (ultraviolet/air or UV/H_2O_2) mechanisms is given in Figure 2. Studies on UV/H_2O_2 decomposition of MTBE have shown that the major products of hydroxyl radical attack on the ether are TBA, formaldehyde, acetone, acetol (pyruvaldehyde), and methyl acetate; other intermediates include hydroxyacetone, 2-hydroxyisobutyric (HIB), pyruvic, acetic, and formic acids (38,39). Tertiary butyl formate (TBF) has also been proposed as an initial metabolite from the enzymatic attack on the ether in microbial cultures (15,17). The abiotic hydrolysis of TBF is slow ($t_{1/2} = 5$ days) (40) suggesting that the rapid formation of TBA from TBF observed in microbial and rat liver tissues must be enzymatic. TBA is oxidized to acetone and formaldehyde in chemical oxidation reactions but is metabolized by bacteria (15) and mammalian tissue (41–43) to 2-methyl-1,2-propanediol and 2-methyl-2-hydroxy propionic acid (HIB). In one report, however, on the metabolism of TBA by a rat liver P-450 enzyme preparation, the branched alcohol was apparently metabolized directly by a hydroxyl radical oxidation to acetone and formaldehyde (44). For many years, tertiary alcohols like TBA (and tertiary amyl alcohol) were known to be recalcitrant and/or biodegraded slowly in most environmental media (soils and biosolids) containing diverse microbial populations (45,46). The remainder of the MTBE biodegradation pathway for acetone is presumed to occur via the formation of pyruvate, acetate, and then carbon dioxide. In this regard, acetone and isopropanol soil enrichments and isolates derived from these cultures have been shown to metabolize acetone in the sequence: acetone $\rightarrow$ hydroxyacetone $\rightarrow$ pyruvaldehyde $\rightarrow$ pyruvate $\rightarrow$ acetate (47). Pyruvate metabolism to acetate and carbon dioxide is a common biochemical transformation in microbial and animal cells.

PROSPECTS AND CHALLENGES FOR THE BIOREMEDIATION OF FUEL ETHERS

Cleanup goals for MTBE in groundwater supplies vary among states (20–400 μg/L). Much higher concentrations are allowed in groundwater in which drinking water supplies are not at risk (48). MTBE enters aquifers from gasoline spills to the subsoil environment and is transported, in some cases, unattenuated through the local groundwater flow paths because of its high water solubility, low soil sorption, and poor biodegradability. Dissolved

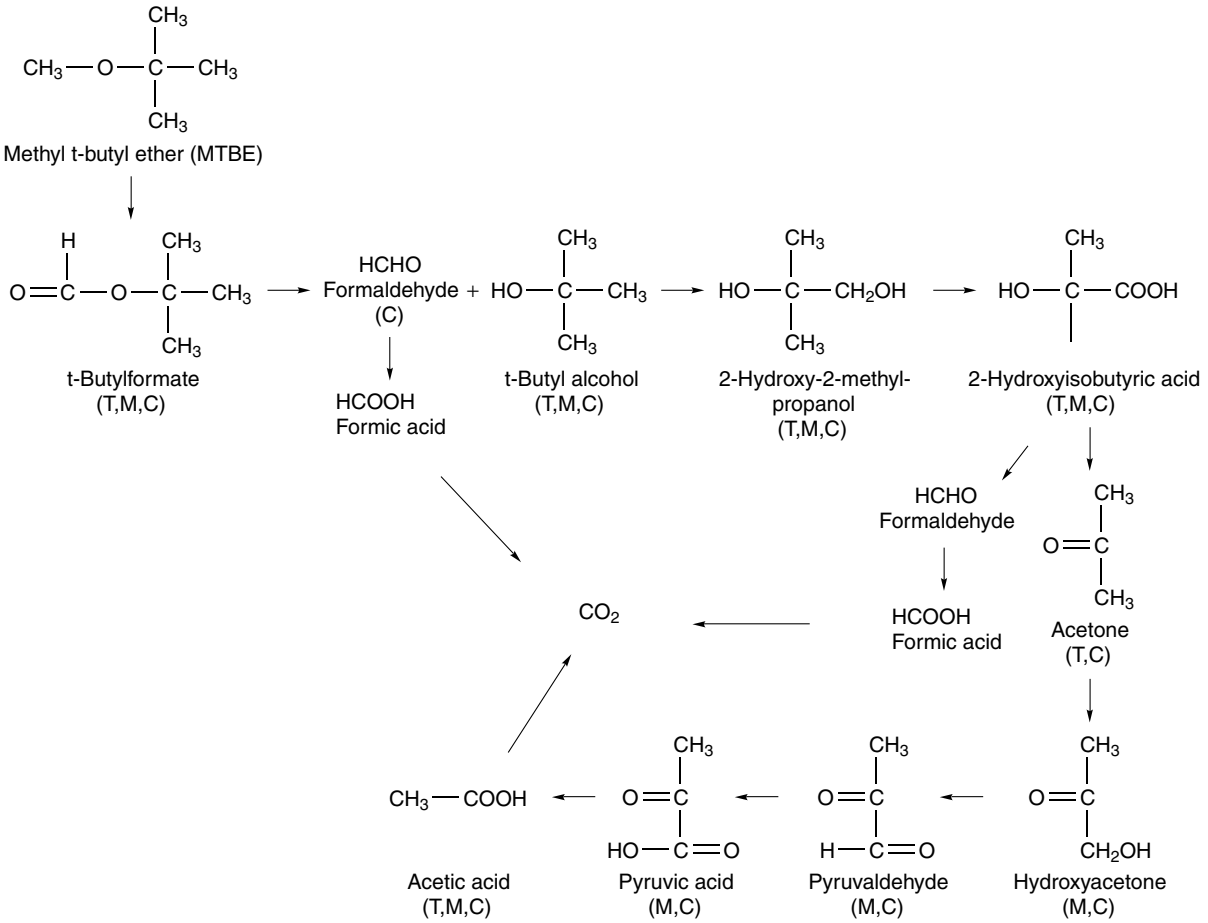

Figure 2. Proposed biodegradation pathway for MTBE. Metabolites shown have been identified from microbes (M), animal tissues (T), or from chemical oxidation (C) experiments.

oxygen concentrations are usually low within the MTBE plume and this also limits microbial metabolism of the ether. In a study to evaluate the potential extent of groundwater contamination by MTBE, researchers have argued that many existing plumes may persist and become even larger and impact more potable supplies owing to uncertainties in vertical and horizontal groundwater transport (49). Unassisted natural attenuation mechanisms and the use of indigenous microbes to biodegrade ethers in groundwater, therefore, may be limited. Engineered in situ MTBE bioremediation systems have been tested in which oxygen and/or cosubstrates (e.g., propane) are sparged into aquifers to stimulate naturally occurring ether-degraders (23,30). In one such field pilot study at the Port Hueneme Naval Base in California, oxygen sparging and the injection of specialized ether-degrading cultures (biobarrier) were used to evaluate the effectiveness of controlling the migration of an MTBE plume (30). In this study, biodegradation of MTBE in the bioaugmented plot occurred faster and was more extensive (no accumulation of TBA) than in comparable plots containing only O_2 or no amendments. Bioaugmentation technology for remediation of aquifers has also been used with other groundwater contaminants such as chlorinated solvents under aerobic- and anaerobic-induced conditions (50,51). This biobarrier concept of nutrient stimulation and/or aquifer seeding of microbial cultures appears to be one tool for controlling the migration of groundwater contaminants. Microbial inoculation of subsoils may be required for compounds like MTBE for which growth on the ether by indigenous microbes is poor and naturally occurring organisms are present in low numbers. However, an understanding of the long-term performance of active groundwater biobarriers will be required for controlling the migration of MTBE plumes. Nutrient limitation, distribution of seed culture, decay of inoculant, reseeding frequency, soil-type effects on activity, and the migration of inoculant are challenges to the long-term stability and function of groundwater biobarriers.

The use of ex situ technologies for treating fuel-contaminated groundwater in bioreactors containing specialized MTBE-degrading cultures has been pilot-tested in laboratory-scale units and biofilters (27,28,30,52). Such studies indicate that these systems are less robust at low pH ($\leq$pH 6.0), low temperature ($<20\,^{\circ}C$), low dissolved oxygen ($\leq$1 to -2 mg/L), and high input MTBE concentrations ($>$150 to -200 mg/L). Also, bioreactors need to be operated under high biomass retention to sustain good removal efficiencies ($\geq$95%). There have been no well-documented field-scale uses of bioreactors for treating

groundwater containing MTBE or other fuel oxygenates. Ex situ biotreatment competes with known "pump and treat" engineering technologies such as air-stripping and activated-carbon adsorption for removing fuel contaminants (ether oxygenates and aromatic hydrocarbons). It should be emphasized that contaminant removal by continuous pumping of groundwater may not be cost-effective for removing high concentrations from source zones. Challenges to the development of efficient ex situ biotreatment technologies for groundwater containing MTBE and other fuel oxygenates will require additional studies to assess reactor size for optimizing and treating various groundwater flow rate scenarios, maximum contaminant concentrations, and efficient biosolids retention.

CONCLUSION

Current knowledge of the biodegradation of the two primary motor fuel oxygenate additives MTBE and ethanol indicates that: (*1*) aerobic MTBE biodegradation (completely to carbon dioxide) can be demonstrated in isolated single and mixed bacterial cultures and enrichments from soils and biosolids, although there have been few reports of partial ether metabolism under anaerobic conditions; (*2*) the metabolism of MTBE in microbes and animal tissues suggests a pathway in which the predominant intermediates are MTBE $\rightarrow$ TBA $\rightarrow$ 2 – hydroxyisobutyrate $\rightarrow$ acetone $\rightarrow$ pyruvate $\rightarrow$ acetate $\rightarrow$ carbon dioxide; and (*3*) ethanol is readily degraded by various microbial populations in soil and sewage biosolids under aerobic and anaerobic conditions. The poor growth of microbial cultures on ether oxygenates remains a biochemical enigma but may explain their recalcitrance (relative to other fuel hydrocarbons) in soil and groundwater systems. In situ biostimulation and bioaugmentation of aquifers and the use of ex situ bioreactors for MTBE remediation will continue to be a challenge for environmental scientists and engineers to make these technologies more robust and reliable.

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MICROBIAL ENHANCED OIL RECOVERY (MEOR), USE OF AND BIOSURFACTANTS IN.

See BIOSURFACTANTS: TYPES, SCREENING METHODS, AND APPLICATIONS

MICROBIAL FLOCS SUSPENDED BIOFILMS

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Microorganisms as suspensions within engineered and natural aqueous systems are typically present as flocs. Microbial flocs represent a complex aggregate of microbial cells, bioorganic and inorganic material forming stable associations (1–3). An arbitrary number of 10 cells has been proposed as a minimum requirement for a group of cells to be considered an aggregate or floc (4). The simplest flocs, however, are sedimenting units that can be typically isolated by cascade filtration and ultracentrifugation (5) and are composed of two or more primary particles (e.g., bacterial cell and an inorganic particle) (6).

Floc properties reflect the combined interactions of the physicochemical and microbiological conditions in a given system. As a result of the complexity of the relationships, it is difficult to precisely predict the behaviour and properties of microbial flocs. Nonetheless, floc formation or aggregation can significantly alter the hydrodynamic properties of the constituent particles, and is important to a number of significant environmental processes. This particularly applies to flocculation, settling, and nutrient and contaminant transport properties in biological wastewater treatment and natural aquatic systems (3,7,8). At the gross scale (sizes > 1 μm), the floc is viewed as a structural entity in which emphasis is focused on size, shape, density, porosity, and settling velocity. Although the structure of flocs controls many of their properties including settling and aggregation, structural detail at this resolution yields limited information.

At the fine scale (<1 μm), the importance of the microorganisms and bioorganic material to floc development and properties is well recognized. Visual observations of natural flocs with high-resolution electron-optical microscopy techniques reveal cross-linkages among primary particles by bridges of fibrillar extracellular polymeric substances (EPS or fibrils) (3). EPS are important bridging and stabilizing structures in pelagic associations between microbes and abiotic particles, in activated-sludge flocs and anaerobic granules, and are common components of aquatic systems (freshwater and marine) and sediment (3,6,7,9–11,166).

Understanding the function-structure relationships of microbial flocs is important to microbiologists and engineers. The goal is to be able to solve practical problems more effectively and to provide better information in order to model the ecology and contaminant transport in aquatic environments, and the operation of wastewater systems. This may be achieved as a result of the developments and application of molecular methods used in identifying microorganisms, new methods for examining the microbial community and activities in situ, and novel approaches to examining microbial-floc architecture, including composition, properties, and function relationships.

Figure 1 illustrates and summarizes the complex nature of flocs, that is, factors influencing flocculation and floc properties. The relationship to biofilms is clear but is usually inferred. In many ways, flocs can be defined as biofilm communities as these structures at the microscopic level reveal structural and compositional similarities to fixed films particularly with respect to EPS and its association with either abiotic or biotic surfaces (3,12). Flocs have been described as individual microecosystems with autonomous and interactive physical, chemical, and biological functions or behaviors operating within the floc matrix (2). However, microbial floc are not typically sessile structures. This article focuses on specific aspects of microbial floc structure, bioflocculation, and floc behaviour in the context of a biofilm and as a floating and mobile structure.

ENVIRONMENTAL SIGNIFICANCE OF FLOCS IN NATURAL AND ENGINEERED SYSTEMS

Freshwater Floc

Lacustrine and riverine flocs form flocculated fine-grain sediment found in the benthic regions of freshwater

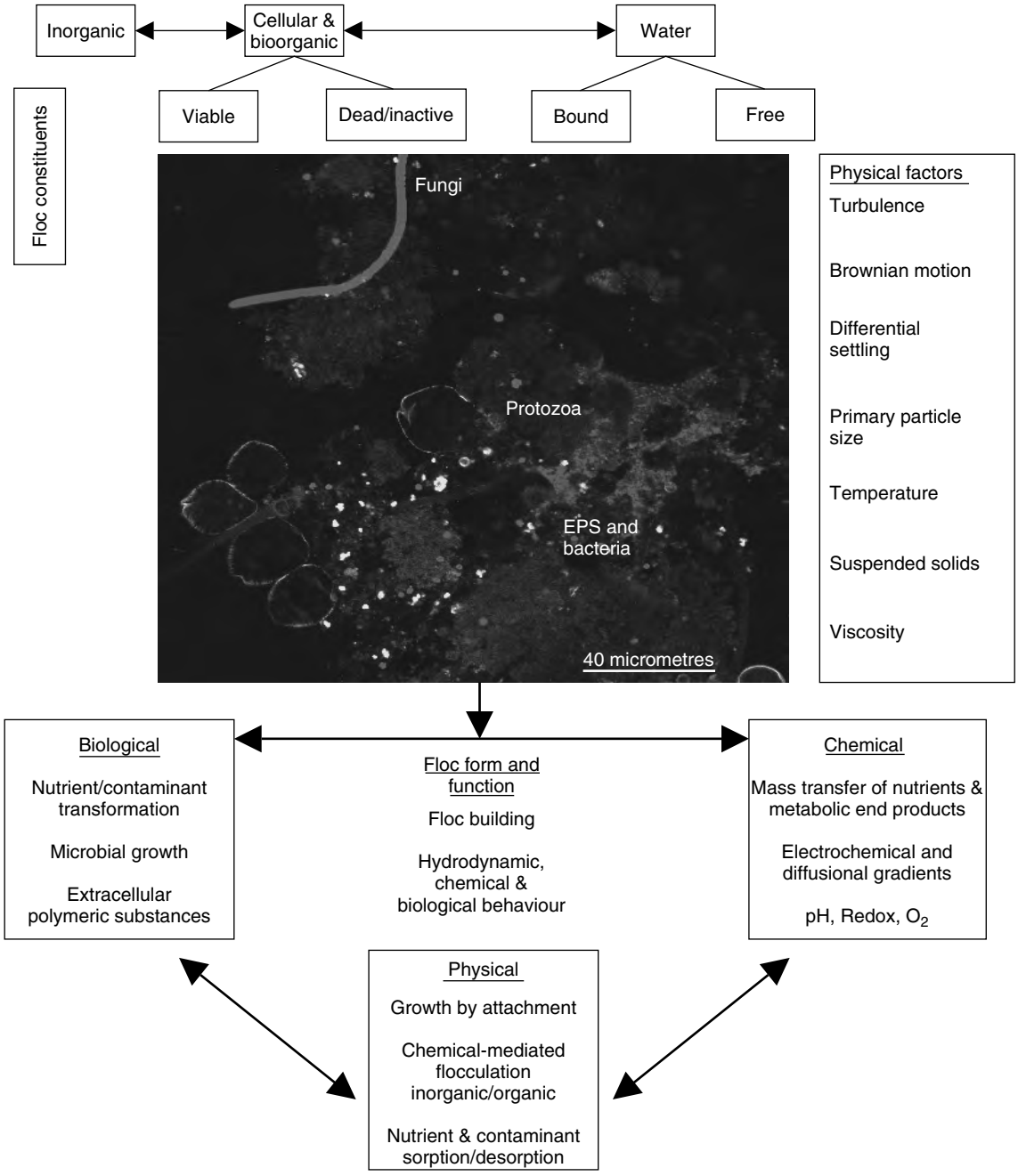

Figure 1. Conceptual description of floc form and function illustrating the physical, chemical, and biological factors influencing microbial floc development (Adapted from I. G. Droppo et al., *Water Air Soil Pollut.* **99**, 43–53 (1997) (2). The microbial floc shown is from activated sludge visualized by scanning confocal laser microscopy following staining with fluorescently labeled lectin stains.

environments (1–3). This sediment is a complex matrix of microbial communities and organic (detritus, cellular debris, and EPS) and inorganic material. It is structurally and functionally dynamic because of multiple interactive processes operating among the various physical, chemical, and biological factors associated with floc properties (1,2,6,8,13). Although flocs can regulate their own environment, they also have the ability to regulate the surrounding water quality by their physical, chemical, and/or biological activity (3,7,8). Microbial flocs in the freshwater fluvial environment can have potentially significant consequences on sediment formation and transport and, consequently, on the fate of contaminants entering this environment.

Aggregation of inorganic particles appears to be primarily associated with biological flocculation (2). Clay surfaces readily sorb nutrients and contaminants on their surfaces and are important sites of microbial colonization in aquatic systems. Ultrastructural studies show that EPS are an important structural component of microbial

flocs with direct association with the inorganic clay particles. The inorganic material typically consists of iron oxyhydroxides, clay minerals (Al/Si), and silicates (5). EPS appears to be the dominant material for the development and stabilization of natural floc (6). This does not exclude electrochemical flocculation completely (Fe,Al/Si,Si), but rather appears to be less significant than biological flocculation within the natural systems observed.

Marine Organic Colloids and Marine Snow

Organic-rich particles produced by microorganisms in marine systems can aggregate often developing into the largest colloids known as *marine snow* (14,15). The most numerous of these consists of bacteria, many of which possess capsules and sheaths composed of EPS. Three principal types of flocs are observed: (*1*) small flocs composed of heteroaggregates of bacteria, EPS, and other colloids, (*2*) disintegrating microzooplankton fecal pellets, and (*3*) marine snow (flocs $>500\ \mu m$). Microbe-mineral associations are not uncommon in marine environments. Mineral ions serve as a nutrient source and a site for colonization. These associations also reflect biogeochemical processes. Flocs enriched in iron and silica are found to be prevalent near deep-sea hydrothermal vents (16).

Marine flocs oscillate through states of disaggregation and aggregation as the EPS matrix degrades or reforms. The composition of the EPS of marine films and flocs has been well-studied (7,9,17,18). The EPS is absorptive and contributes to trophic interactions and biogeochemical processes through the concentration of nutrients and metals in highly oligotrophic environments. Marine snow is represented by diverse structures (15,18,19) and the carbohydrate and protein composition of the floc matrix is highly variable and related to floc structure (18).

Epsiodic blooms of photosynthetic diatoms, cyanobacteria, and flagellates can occur in response to nutrient inputs of carbon (as carbonate) with concomitant limitation of other nutrients such as nitrogen, silicon, or phosphorous (20). Significant excess production of microbial cells and EPS may arise resulting in substantial transformation of environmental conditions when this material begins to biodegrade and oxygen is depleted. The EPS may form dense mats that float and wash up on shores presenting risks to humans depending on the microorganism and causing significant economic loss to regions affected. Aggregation of cells and EPS may result in flocculation and settling to the underlying sediments (21).

Anaerobic Methanogenic Granules

The formation of a dense microbial aggregate, referred to as *granules*, is a prerequisite for the operation of the up-flow anaerobic sludge blanket (UASB) bioreactors. Considerable attention has been given to the granulation process, including the role of EPS, microbial community, and the structure and stability of the granule (see Raskin, Granules (Anaerobic) and Granulation Processes in Biosolids, in this Encyclopedia). Granulation has been observed to occur aerobically under certain conditions in bench-scale sequencing batch reactor (SBR) (22). These granules, similar in size (1 to 3 mm) and stability to anaerobic granules, were composed of bacteria that were attached to a fungal backbone (*Geotrichum* sp.).

Activated Sludge

Considerable attention has been given to the study of activated-sludge flocs (see Kampfer and Wagner, Filamentous Bacteria in Activated Sludge: Current Taxonomic Status and Ecology, Nielsen, Activated Sludge–The Flocs, and Wanner, *Filamentous Bulking in Activated Sludge*: *Control of*, this Encyclopedia). Microbial floc formation and gravity sedimentation of the synthesized biomass in secondary clarifiers of activated-sludge plants are considered to determine the overall efficiency of this secondary wastewater treatment process (23–25). Solid-separation problems, such as microbial bulking and foaming, settling difficulties of microbial flocs, and difficult dewatering of the sediment sludge, however, have plagued the activated-sludge process since its inception (23,26).

Sludge flocs consist of microorganisms, EPS, organic, and/or inorganic colloidal particles (27–30). EPS, salt bridges, a backbone of filamentous bacteria, or a combination of all three account for the integrity and mechanical stability of sludge flocs (31–33). A multitude of factors (e.g., nutrient regime, oxygen, and pH) can affect both the population dynamics and floc structure in a wastewater-treatment system. More emphasis is being placed on the structure and the physicochemical properties of flocs and the suspending medium because these may strongly influence the behaviour (e.g., settling properties) of biomass (34–38). This is not only true for settling properties but may apply to the reduction in biochemical oxygen demand, nutrient removal, sludge dewatering characteristics, and sludge management.

STRUCTURAL AND PHYSICOCHEMICAL PROPERTIES OF FLOC

Microbial flocs are usually not spherical but possess highly irregular shapes characterized by a large range of particle sizes, from single bacterial dimensions (ca. 1 to 3 μm) to large aggregate sizes of more than 1,000 μm (2,11,39). Median shape-factor values range from 0.6 to 0.7 (1.0 = a perfect circle). A bimodal floc-size distribution is often found (11,39,40,167). The percentage by number distributions tend to be positively skewed, whereas the percentage by volume distributions are negatively skewed. Although the majority of the mass is in the larger-size classes, the majority of the particles are smaller. Longest dimensions and widths yield similar distributions of the equivalent spherical diameters with slight variations reflective of the nonspherical shape of flocs. Floc size has significant implications for modeling of contaminant transport because the larger flocs will probably settle down much faster than the finer flocs, which will travel with their associated contaminant much further within the system.

The apparent density of flocs (about 1.03 g/cm^3) is close to the density of water (41,42). Consequently, the small difference in densities between flocs and bulk water leads to low settling velocities. Although a positive

relation between floc size and settling velocity is observed, there is a significant level of variability. Factors that can affect settling rates and impose variability in the results include floc composition, shape, porosity, and water content (2,42–44).

Increasing porosity leads to an increase in water content that forces the density of the floc toward the density of water, thus reducing the settling velocity. Porosity is complex as the definition or identification of pores is highly dependent on the resolving power used for their observation. Pores observed with conventional optical microscopy (e.g., phase or bright field optics) have been assumed to be devoid of materials and appear to be significant open channels for the movement of water through floc as it is settled or transported. These pores, are however, filled with a complex network of cells and EPS (1–3). Calculations of pore water movement that are important for understanding floc settling and contaminant transport that do not consider the internal structure of the pores may be erroneous.

The EPS within the floc matrix may be unattached or loosely attached to the floc surfaces (slime), or tightly bound to the exterior of cell walls (capsules) (3,27,45,46). EPS tend to have a sticky fibrillar nature (particularly the acidic polysaccharides), which may be the main component that gives flocs their strength and psuedoplastic nature (3,6,12). EPS may provide a framework for the floc itself. In activated-sludge flocs, a certain amount of filamentous microorganisms are desirable and thought to be necessary for the formation of larger, denser flocs (backbone theory) (23,33).

The chemical composition of microbial flocs is summarized in Table 1. Accumulation of EPS within the floc matrix probably occurs by metabolic synthesis, cell lysis, and/or adsorption of organic material (e.g., humic substances). The EPS content from metabolic synthesis is strongly related to environmental and microbiological conditions (34,35,47,48). The contribution of cell lysis is often attributed to the physiological status of cells or the EPS-extraction methods employed. The adsorption of organic material (e.g., humics) that contributes to EPS from aquatic systems or wastewater is related to the composition and properties of these organic materials. At present, it is difficult to distinguish the relative importance of the contributions of the three mechanisms to EPS content. The strong correlation between protein and DNA accumulation within the EPS indicates that cell lysis is the primary mechanism (34,35).

Table 1. Chemical Composition of Flocs

Composition	References
Total Mass	49,50
Dry weight 60–90% cellular organic material	
Wet weight 90–98% water	
Extracellular polymeric substances (15–33% of cellular organic material)	30,32,45, 47,51–61
Carbohydrates and acidic polysaccharides (5–40%)	
Protein (20–80%)	
Lipids (13–89%)	
DNA (1–10%)	
Humic substances (0–40%)	
Inorganic salts and particles (10–30%)	
Molecular weight distribution	
500–100,000 daltons (ultrafiltration studies)	56,62
10,000–2,000,000 daltons (chromatographic separation)	57,58,63

Microbial flocs are naturally hydrated because of the presence of large numbers of hydroxyl, carboxyl, and phosphate groups. However, floc surfaces do possess hydrophobic areas (30,64). Side chains in amino acids, the methyl groups in polysaccharides, and the long-chain carbon groups in lipids contribute to the hydrophobic properties of sludge flocs. Flocs are negatively charged under neutral pH conditions. The presence of ionizable groups such as carboxyl, phosphate, and amino groups in the EPS and cell surfaces is responsible for the density of surface charge. The zeta potential of sludge flocs is usually in the range of −10 to −30 mv (55,65,66).

DIFFUSIONAL GRADIENTS IN MICROBIAL FLOC

Ultrastructural studies of microbial flocs indicate a biofilm structure (3). Flocs are essentially biofilms that are turned back on themselves so that the biofilm/substratum interface is internalized as the core of suspended flocs. Large pores within the floc observed by conventional optical microscopy (COM) are not necessarily open channels devoid of structural material, but rather contain a complex matrix of "transparent" EPS (3,18). In sessile biofilms, microbial cells form dense layers of cells, or microcolonies, which are bound to each other by EPS (67–71). However, within these biofilms, there are voids and interstitial spaces between the microcolonies, which form channels and allow transport of material through the matrix. These channels are filled with water and low concentrations of EPS, thereby making the biofilm very absorptive and porous. The microbial floc is more similar to the microcolony within the biofilm. Gradients typically observed in sessile biofilms such as pH, oxygen, redox and substrate concentration may be present within the floc and may support sequential (aerobic/anaerobic) processes. Recent reviews on EPS produced by microorganisms (12,48,72) further supports the notion that the nature and prominence of EPS associated with the floc structure defines the flocs as biofilms.

Transport through porous structures can be attributed to both advection/convection and diffusion. The influence of the bulk-liquid velocity on convection in the voids within sessile biofilms can be interpreted from interfacial transport phenomena (68–71). In a biofilm system, substrate transport involves convection from the bulk liquid to the vicinity of the microcolonies, diffusion through the mass-transfer boundary layer, and finally diffusion through the microcolony to the individual cell (67,68,73). A similar process might be expected to occur in microbial flocs (74,75).

As a result of the high water content of microbial floc, these structures are considered to have a high porosity. The development of porous flocs may be advantageous to microbial communities because substrate removal can

be 60% greater than that for dispersed bacteria (43,76). Flow through the floc pores has been calculated to be up to 100 $\mu m.s^{-1}$. Microbial aggregates would be so porous that these might be permeable to fluid flow within certain shear rates or during gravitational settling. Mass-transfer by diffusion in this situation would be negligible because the flow through the floc is predicted to result in little difference in concentrations of nutrients between the bulk water and within the floc. Li and Ganczarczyk (77) proposed that if there is significant flow through pores, the floc-settling velocity would increase rather than decrease.

Alternatively, flow through flocs could be viewed as being negligible and water could be retained as an integral part of the floc structure owing to EPS having a high water-binding capacity (49,78,79).

Resistance within the floc due to the complex EPS matrix may affect the flux of water in and out of the floc. The EPS, as observed by Liss and coworkers (3), suggests that flocs probably retain a great amount of water as a result of the large surface area it forms and surface water tension. The floc would probably assimilate nutrients and contaminants primarily through diffusional gradients under these conditions.

Where diffusional gradients dominate, the gradients around and inside the flocs would be maintained by a balance between microbial metabolic rates (e.g., uptake, metabolism, end product formation) and diffusion of materials from and to the bulk solution. A floc with an anaerobic/anoxic core and aerobic surface would be consistent with this process. de Beer and coworkers have pioneered the application of biosensors to the investigation of chemical microgradients and activity in biofilms and flocs (79–81). Anoxic microniches have been found to form within aerobic activated sludge, particularly with respect to denitrification and sulfate-reduction. Microsensor measurements revealed that most of the large and bulking flocs examined lacked an anoxic zone and possessed low denitrification and negligible sulfate-reduction activity, although sulfate-reducing bacteria were observed by fluorescence in situ hybridization. Flocs that were reported to be denser, however, were found to contain anoxic zones. It is likely that both advection and diffusion account for nutrient transport through the floc as it does in biofilm.

INTERFACIAL FORCES IN FLOC FORMATION

Forces acting on and between particles include gravitational, hydrodynamic (drag) forces and thermal energy, and interfacial forces. Noncovalent interfacial forces are primarily involved in microbial floc formation. Ionic bonds are also involved in controlling floc formation and its stability. Bos and coworkers (82) have reviewed the physicochemistry of microbial adhesive interactions, particularly as it applies to sessile biofilms.

van der Waals Forces

These forces are attractive forces and depend on the geometry and nature of the interacting bacteria. Typically, energies of van der Waals interactions vary with the inverse sixth power of the particle separation. They contain three terms, namely, permanent dipole–permanent dipole interactions, permanent dipole-induced dipole interactions, and induced dipole-induced dipole interactions. A number of methods have been described for evaluating the values of van der Waals forces among the interacting bacteria (66,83).

Electrostatic Double-Layer Forces

Charged bacteria in an aqueous medium attract oppositely charged ions from the solution leading to the establishment of an electrostatic double layer at the solid–liquid interface (83,84). Electrostatic interactions arise as the electrical double layer of two approaching bacteria overlaps. They are usually repulsive forces and are responsible for the stable dispersion of charged particles. Under neutral pH conditions, bacterial surfaces are negatively charged because of the dissociation of anionic groups associated with the bacterial surface.

Hydrophobic/Hydrophilic Forces

Hydrophobic forces among microbial surfaces depend in large part on the unique properties of the thin layer of water associated with the surfaces and forces at a short distance (<2 nm). Typically, energies of hydrophobic interaction decrease exponentially with the particle-separation distance. They are attractive forces, whereas hydrophilic forces are repulsive. Hydrophobic/hydrophilic forces are of a polar nature and may be two orders of magnitude higher than van der Waals and electrostatic forces (85) at short distances (<2 nm).

Steric Forces

This type of force arises among polymer-coated surfaces. The presence of EPS on sludge surfaces may prevent the approach of flocs, allowing only a loose contact. The situation is even more complicated for charged polymer-coated surfaces. Generally, it is difficult to quantify steric forces in biological systems on account of their complexity (86,87).

MECHANISMS OF FLOC FORMATION

Proposed mechanisms for floc formation emphasize the importance of surface properties in floc interactions. The differences lie where the surface properties is considered to be most important, and how a particular parameter is affected by nutritional and environmental conditions.

Charge Neutralization

On the basis of size and charge properties, aggregation of microorganisms has been described most easily in terms of Derjaguin and Landau (88) and Verwey and Overbeek (89) DLVO theory, which was originally developed to explain the stability of hydrophobic, inorganic colloid suspensions. This theory describes the interaction energies between surfaces and is the sum of van der Waals (attractive) and electrostatic (repulsive) interaction potentials. If cells can get sufficiently close to each other at a distance at which

the van der Waals forces are effective, these flocculate and yield stable structures.

The failure of DLVO theory to explain bioflocculation under certain conditions (29,90) may be related to the complexity of biological systems. The microbial cell surface is not smooth, and hydrophobic/hydrophilic groups and EPS are also found at the cell surface (hydrophobic and steric forces) (91). Therefore, neglecting the hydrophobic/hydrophilic interaction at the surfaces of bacteria, as treated in classic DLVO theory, is probably not reasonable in understanding floc formation and floc strength. Because DLVO theory assumes smooth surfaces on the interacting particles and considers only the van der Waals and electrostatic forces, a consideration of other physical forces, such as hydrophobic interactions, may extend its application [extended DLVO theory (82,85)].

Hydrophobic Interactions

The presence of a shell layer of bound water near the cell surface accounts for hydrophobic/hydrophilic interactions. When two similar surfaces approach each other at a short distance, the bound-water layers surrounding the surfaces will overlap, forming a displacement of the bound-water layer into the bulk water. This leads to a decrease in the free energy (i.e., attraction) in the case of a hydrophobic surface, but to an increase in the free energy (i.e., repulsion) in the case of a hydrophilic surface (92). Any alteration of the cell surface that increases the surface hydrophobicity will enhance the formation of flocs. Increasing experimental evidence indicates that hydrophobic interactions play an important role in bioflocculation (30,34,93–96).

Polymer Bridging

The polymer bridging mechanism has long been used to interpret microbial adhesive interaction (23,32,47,73,82,91,97,98). Floc growth through attachment of single cells or smaller flocs may occur through EPS. EPS constituents including cellulosic polysaccharides and DNA have been described as effective flocculating agents (61,99). Whether the amount or the specific composition of EPS, or both, is crucial to sludge-floc formation has been debated (32,97). The widely held view that the total EPS content is directly related to bioflocculation no longer applies because increasing emphasis is being placed on the type of polymer and its physicochemical properties (34).

Salt Bridging

Both the bacterial surface and EPS provide negatively charged adsorption sites. Inorganic ions, such as divalent cations (Ca^{2+} and Mg^{2+}), have been found to be strongly associated with the chemical structure of flocs and EPS (28,30,31,54,100). Removal of polyvalent cations from the floc matrix by ionic-chelating reactions leads to the disintegration of microbial flocs. Salt bridging agents that lead to EPS–metal complexes are probably needed to bind bacterial surfaces and EPS constituents to maintain floc stability and to increase the structural strength of EPS.

Surface Thermodynamics Approach

The initiation of microbial-floc formation can be interpreted in terms of system-free energy. The basic concept of the thermodynamic model is that the system-free energy is minimized at equilibrium. Consequently, the process of microbial-floc formation will be thermodynamically favored if the process itself causes the system-free energy to decrease. Electrostatic interactions and other specific bindings are ignored. The free energy of the interaction between two identical bacterial cells (B) immersed in a liquid (L) can be described as follows:

$$\Delta G_{\text{flocculation}} = -2\gamma_{\text{BL}}, \tag{1}$$

where $\Delta G_{\text{flocculation}}$ is the free energy of floc formation and γ_{BL} is the interfacial tension for the bacteria–liquid interface. If the total free energy of a system is reduced ($\Delta G_{\text{flocculation}} < 0$) by cell interactions, microbial-floc formation will be thermodynamically favored (101–103).

According to Neumann's equation-of-state, the interfacial tension γ_{BL} is correlated to the surface tension terms of bacterial cells γ_{B} and suspending solution γ_{L} as follows (103,104):

$$\gamma_{\text{BL}} = \frac{(\gamma_{\text{B}} - \gamma_{\text{L}})^2}{(1 - 0.015\gamma_{\text{B}}\gamma_{\text{L}})}. \tag{2}$$

Substituting Equation 2 into Equation 1, the free energy of floc formation can be calculated if the water-contact angle on cell surfaces and water surface tension are known (103).

PHYSIOLOGICAL FACTORS AFFECTING FLOC FORMATION AND PROPERTIES

Significant changes in the internal and surface properties of microbial flocs under various nutrient growth conditions have been observed particularly under nitrogen (N) and phosphorus (P) limitation (31,47,105–107). Generally, N-limited microbial cultures are high in cell polysaccharide and low in cell protein. In P- and N-restricted media, flocs possess large capsules and produce a higher surface charge per unit of dry weight. P limitations have been found to improve metal adsorption capacity of flocs. An increase in the carbon : Phosphorus ratio can result in the production of extracellular polysaccharides (108), and lead to proliferation of filamentous bacteria in sludge flocs (109). Low organic load conditions and a lack of nutrients can cause formation of pinpoint flocs and changes to properties of the flocs including poorer settleability (23,106,165). Short-term nutrient starvation of marine bacteria was found to cause a decrease in cell volume during the starvation period (110).

Other factors that can affect floc properties in engineered and natural systems include agitation, dissolved oxygen (DO) concentration, sludge age, and degradation of the EPS matrix. Agitation may disaggregate flocs and cause surface damage to or disruption of individual cells (90,111,164). Impacts on the metabolic state of floc caused by low DO concentration, anaerobic conditions, or the absence of a suitable electron acceptor may affect

floc stability and result in smaller flocs, which disaggregate (36,109).

Not all microbes aggregate and there is a variation in this ability between strains (4). Among the various bacteria in sludge, some are floc-forming microorganisms that are important in floc formation. They include cellulose (or cellulose-like) producing bacteria such as *Pseudomonas*, *Aerobacter*, *Agrobacterium*, *Azotobacter*, and *Zooglea ramigera* (24,25,98,112). Physiological age of cells is one of the primary factors related to the formation and properties of sludge flocs. Early experimental evidence indicated that large amounts of EPS were accumulated in the endogenous phase, and floc formation depends on the physiological age of cells (32,78,113). EPS is also produced in rapid-growth situations (114–116). Solids retention time (SRT), or sludge age, is a key process variable that can affect microbial growth rates. In general, low SRTs are associated with rapid rates of microbial growth and high rates of sludge production and wastes; high SRTs are related to slow growth and low rates of sludge production. On the basis of kinetic selection principles, a change in the microbial composition with respect to the SRT is expected. A high SRT will favor the accumulation of slow-growing microorganisms, whereas a low SRT enhances the domination of fast-growing microorganisms (117,118).

The SRT is defined as the average residence time of sludge within a bioreactor, whereas the F/M is defined as the ratio of the feed (carbon load) to the microorganisms (biomass concentration) in the bioreactor. These two parameters are not independent, but at steady state are correlated through the following equations:

$$\frac{1}{\mathrm{SRT}} = Y_{\mathrm{yield}}\left(\frac{\mathrm{F}}{\mathrm{M}}\right)E - K_{\mathrm{d}}, \tag{3}$$

$$\frac{\mathrm{F}}{\mathrm{M}} = \frac{S_{\mathrm{o}}Q}{(V\,X)}, \tag{4}$$

where Y_{yield} is the yield coefficient of biomass, E is the COD removal efficiency, K_{d} is the decay coefficient, S_{o} is the influent COD concentration Q is the influent wastewater flow rate, V is the aeration tank volume, and X is the concentration of biomass in the aeration tank. In general, a lower SRT is related to a higher F/M.

The flocculating ability of sludge flocs generally increases with an increase in the SRT (34,114,119–124). Surface charge and hydrophobic interactions are significant determinants in bioflocculation and floc stability, and the type and physical characteristics of EPS are important in biosolid-liquid separations (34,35,38,48,94). This is reflected in observations of floc structure. Flocs at lower SRTs (4 and 9 days) have been observed to be irregular in shape, whereas flocs at higher SRTs (16 and 20 days) had a more spherical, compact structure (125). Flocs at higher SRTs were less hydrated and covered in a dense EPS layer rich in osmiophilic granules indicative of lipidlike material that may account in part for the increased hydrophobicity of these flocs (34). Total EPS content as a sole parameter has limited value in understanding bioflocculation.

There has been considerable attention given to the role of quorum-sensing molecules in biofilm formation and stability (9,12). To date, there is no information on the role of these on microbial aggregation and bioflocculation processes for microbial floc.

METHODS FOR ANALYZING FLOC

The measurement and analytical methods used to study sludge surfaces are important for understanding the effects of physical, chemical, and microbiological conditions on microscopic properties of sludge flocs, and for relating microscopic properties of flocs to their behaviour. Few standard methods with good reproducibility are available, although various physical, chemical, and microbiological measurement and analytical techniques have been developed and used in floc research.

Floc Size and Sample Handling

Flocs are highly irregular in shape, porous, and three-dimensional; therefore, there is really no ideal way to characterize floc sizes. Equivalent spherical diameter (ESD) is the most frequently used term to represent floc size because of its simplicity, and the widely used Stokes' law equation is applied to estimate floc density from the ESD and settling-velocity data. In general, flocs range in size from a few μm to a few *mm* when measured by ESD (13,126). The effective diameter has also been determined as the geometric mean $\sqrt{(d_{\min} * d_{\max})}$ using the maximum ($d_{\max}$) and minimum ($d_{\min}$) dimensions across a two-dimensional floc image (127). Barbusinski and Koscielniak (128) and Li and Ganczarczyk (77) determined an average floc diameter defined as one half of the sum of the longest and shortest dimensions of the flocs to describe floc size. Researchers have also used fractal geometry to describe floc structure (129–133). Many methods and instruments have been developed in the past to measure floc-size distributions of natural and engineered systems. These methods include automated image-analysis systems (11,13), microscopic observations (33), and photographic techniques (28,126). The photographic size measurement, although easy to employ, does not allow measurement of very small flocs. The automated image-analysis systems usually comprise a microscope and a computerized digitizer, which allow for more accurate, reproducible, and fast estimates of floc morphological parameters. The Coulter counter has been used to measure floc size (75,119), but this method is destructive because of its impact on breakage and compression of larger flocs. Other instruments developed include a field-portable laser backscatter particle analyzer (134) and an in situ settling velocity instrument (135). Other less common floc-sizing methods, including filtration, centrifugation, and image-projection techniques (136), usually do not work because of artificial floc breakup or aggregations and are time consuming.

The critical step in floc size measurements is the sample handling and preparation. Considerable efforts have been given to overcome perturbation that may be associated with sampling and specimen preparation. Floc samples are usually collected as bulk suspensions and transported to laboratory for analysis. Subsampling of

flocs in the laboratory is normally done using a pipette. The opening of pipettes used to collect floc samples has to be wide enough (2 to 3 mm) to prevent floc breakage and disaggregation (39). Floc stabilization in low melting point agarose, before any further sample handling, has been found to minimize effects on floc-size distributions (39,137).

Floc Settling Velocity

The most common way to measure floc-settling velocity is by the multiple exposure photographic technique (42,44,126). This technique is effective in measuring floc size and settling velocity, but it lacks the precision in measuring fine flocs. A stereoscopic microscope and a video camera can be used to capture images of settling floc in a column filled with a media similar to the native environment of the samples (6). A small quantity ($\sim$ 1 mL) of floc samples is introduced at the top of the column. A sufficient travel distance is allowed for flocs to reach terminal velocity. Settling images of flocs are then recorded on a VCR as they pass though the focal plane of the microscope. These images are then analyzed using a computer-imaging software for size and settling velocity.

Ganczarczyk and his coworkers have used a power function of the form, $v = AL^n$, and a linear function, $v = A + BL$, to correlate floc-settling velocity (v) with its longest dimension as a characteristic size (L), where A, B, and n are the equation coefficients determined experimentally (42,131,138,139). The power function is considered to be a better way to describe the relationship because the power function predicts that the velocity will be zero when floc size approaches zero, whereas the linear function does not. Measured settling velocities have coefficients lower than that predicted by Stokes' law ($n = 2$). The power law coefficients (n) calculated from the power function generally range from 0.7 to 0.9. Ideally, in measuring floc-settling velocity, the number of flocs measured should be as large as possible and the size range included should be as broad as possible (140). A modified linear model incorporating the floc-settling shape factor has been found to improve the correlation coefficient (R^2) of the linear relationship (138).

Floc Density and Porosity

Density determinations for aggregates are usually on the basis of observations of terminal velocity, although methods based on settling flocs to their isopycnic levels using density-gradient techniques have been used (141–143). This technique, however, does not measure floc size concurrently with its density, thus, a size and density relationship might not be established easily. In addition, the ionic strength of the suspension medium and the nature of the medium itself have to be compatible and nontoxic to the biological flocs.

Stokes' law or modified Stokes' law are commonly used to calculate density from settling-velocity and size measurements (42,44,126,131,139,140,144). Stokes' Law is defined as follows:

$$v = \frac{1}{18}\frac{g \cdot d^2(\rho_f - \rho_w)}{\mu} \tag{5}$$

where v = terminal settling velocity
ρ_f = wet density of particle
ρ_w = density of water (assume settling in water)
g = gravitational constant
μ = viscosity of water (assume settling in water)
d = diameter of particle

Floc porosity from density is calculated as follows:

$$\varepsilon = \frac{\rho_s - \rho_f}{\rho_s - \rho_w} \tag{6}$$

where ρ_s and ρ_f are the dried sludge density (1.34 to 1.69 g/cm^3) and floc density, respectively, and ρ_w is the liquid density. The density calculation is considered to be an approximation because Stokes' law is best applied to spherical particles (139,140).

Variations of the floc-density model have been proposed that take into account the liquid viscosity, settling velocity, pH, and size of the flocs, and making assumptions concerning spherocity, a drag coefficient and the dried-sludge density (44,119,139,140,144). These are based on Stokes' law but there is no universal model. This is simply because these models were developed from their specific conditions such as the type of floc, the type of microorganisms, the hydrodynamic conditions, and the experimental techniques used. Therefore, floc density and porosity must be experimentally determined in all situations.

Extracellular Polymeric Substances

A number of methods have been investigated and applied to separate EPS from sludge flocs. They include physical (centrifugation, sonication, and thermal extraction), chemical (hydroxide addition, acidic stripping, ion exchange, and ethanolic extraction), and combined physicochemical methods (28,32,46,52,145). A more recently developed method involves the use of an ion exchange resin (Dowex resin) for EPS extraction from sludge flocs (45,57) at a low temperature (4 °C). This method yields a higher protein concentration than the widely used thermal extraction–solvent precipitation method, and minimizes lysis of cells at high temperatures.

Hydrophobicity

Techniques for determining hydrophobicity measure the hydrophobic properties of the outer cell surface as a whole or the colonial hydrophobicity of multicellular aggregates (146). Three techniques have been typically used in floc research, namely, microbial adhesion to hydrocarbons (MATH) contact angle measurement (CAM), and salt aggregation test (SAT).

MATH is based on bacterial cells possessing hydrophobic surface characteristics adhering to an oil–water interface, whereas hydrophilic cells do not (147,163). MATH is a simple and general technique for studying cell surface hydrophobicity and has been largely applied to suspensions of pure cultures, but it has also been widely used to measure the hydrophobicity of flocs (86,94,148,170). A limitation of this technique includes cell clumping.

CAM is a useful measurement of hydrophobicity of flocs because the surface-free energy can be estimated from the measurement (66,101,116,149). All measurements involve the preparation of a thin lawn of biomass through the vacuum filtration of a microbial suspension and the determination of sessile drop-contact angles on the lawn of biomass. Axisymmetric drop-shape analysis (ADSA) is an effective technique for determining contact angle measurements of biomass (34,150,151).

SAT is a simple technique in which the order in which cells are aggregated and settled when exposed to salting-out agents (e.g., ammonium sulfate) at increasing concentrations is a measure of their surface hydrophobicity (30,152,169). The most hydrophobic cells are aggregated first at a low salt concentration. The SAT technique has several limitations. Many hydrophobic bacterial cells will clump in the absence of salts. The electrostatic interaction may affect the results of SAT more than other hydrophobic measurement techniques (146).

Surface Charge

Colloidal titration is the most common method for determining the surface-charge density of flocs (34,113) and is routinely applied to determine polymer dosage in biosolids management. An excess amount of positively charged polymer is added to a floc suspension. The excess amount of positively charged polymers is titrated with a negatively charged polymer in the presence of an indicator dye; the surface-charge density of flocs is calculated from this titration. Electrophoretic mobility is particularly valuable because it allows the estimation of the zeta potential. This determination is related to the water chemistry of the suspension solution, and the type, size, and shape of the cells. An accurate measurement is difficult, and requires skill, experience, time, along with the use of specialized apparatus. Other methods include attachment to charge-modified polystyrene and fluorescent probe ion exchange resin, and electrophoretic mobility (153).

Microscopic, Molecular, and Microenvironmental Analysis

Correlative microscopy (CM) is a strategy of using multiple microscopes (3,154) or multipreparatory techniques (3), which collectively enable the microscopist to potentially visualize microbial flocs and their components and identify physicochemical parameters, three-dimensional arrangements of constituents, topography, chemical composition, and forces governing interfacial phenomena. Microscopes include conventional optical microscopy (COM) (1,13,167), scanning confocal laser microscopy (SCLM) (17,1,18,155), two-photon laser scanning microscopy (2P-LSM) (17), atomic force microscopy (AFM) (156–158) and Raman confocal microspectroscopy (159), transmission electron microscopy (TEM) (3,168), and environmental scanning electron microscopy (ESEM) (1,125). Applying CM permits one to detect, assess, and minimize artifacts, which might arise from using only one technique. The use of only one microscopic technique can bias or limit the information acquired because of the artifacts that arise in specific sample preparations and the resolution constraint associated with a particular technique (154,3).

Minimizing perturbations associated with manipulations while preparing material for CM can be achieved through sample stabilization in low melting point agarose (39). The structural integrity of the samples can be maintained through the stabilization, staining, and washing procedures. Nanoplast resin is particularly effective as a stabilization medium because it is a hydrophilic embedding resin, which holds the fibrillar EPS (3). Measurements of the dimensions of colloidal matrix material and their three-dimensional disposition are realistic. Nanoplast has been recently shown to be useful for stabilizing sediment biofilms and the EPS matrix of these structures for observation by SCLM (17). SCLM is a particularly useful technique in bridging the resolution gap between COM and TEM for the study of flocs (125,132,133,160). More detailed description of SCLM is provided by Neu and Lawrence (this publication). SCLM and 2P-LSM can be used in combination with a variety of fluorescent molecular probes to study the spatial distribution of floc constituents and the microenvironment nondestructively (17,18,160).

Molecular tools enabling the amplification, isolation, and sequencing of 16S rRNA genes from environmental samples [e.g., amplified ribosomal DNA restriction analysis (ARDRA), denaturing-gradient gel electrophoresis (DGGE)] are widely used in environmental microbiology to examine complex communities and to establish phylogenetic associations without having to culture individual isolates (161). Whole-cell fluorescent in situ hybridization (FISH) with taxon-specific, rRNA-targeted fluorescent oligonucleotide probes and detection by SCLM permit the in situ exploration of the structural distribution of individual community members (e.g., nitrifying bacteria) in microbial floc (92,79,160). Combining FISH with functionality through microautoradiography and/or differential gene expression (double-probing techniques) may provide a better understanding of processes and factors, that affect the ecology of complex microbial processes in floc associated with engineered systems and natural environments (41).

Information about the in situ activity of bacteria and the microenvironmental conditions within flocs has been possible with the development of microsensor technology (80,81,79). It has been possible to examine diffusional gradients in flocs and the constraints imposed by structure. A variety of electrochemical and fiber-optical microsensors can measure a variety of chemical and physical parameters (NO_3^-, NO_2^-, NH_4^+, CO_2, H_2, H_2S, CH_4, O_2, temperature, and pH) (81). Holding flocs in place in order to insert a microsensor is not trivial compared with a sessile biofilm. A vertical net-jet flow cell has been developed for microenvironmental analysis of flocs by microsensors (74). Flocs are retained above a piece of nylon stocking separating two tubes and an upward flow is applied.

CONCLUSION

Microbial flocs and bioflocculation processes are important to a variety of environmental processes associated with engineered and natural aquatic systems. Morphologically, most flocs are similar with respect to the association of

cellular, bioorganic, and inorganic constituents, although the proportions may vary. EPS contribute significantly to the floc structure and appear to bind the primary particles together. The EPS are arranged differentially within the floc matrix, including the large porous regions as seen by conventional optical microscopy, giving rise to a complex structure. This contributes to an extensive surface area, and owing to the properties of the EPS, a highly absorptive capacity and the ability to retain a considerable amount of water. Advanced optical microscopy and ultrastructural studies reveal a structure indistinguishable from sessile biofilms. Microbial flocs are biofilms, but as suspended structures they are subjected to a range of physical forces and chemical conditions, which may be of less importance in our understanding of sessile biofilms (e.g., settling, porosity, density, colloidal stability). Developments in the methods and techniques in microscopy, molecular biology, and microenvironmental analysis are permitting more detailed examination of microbial flocs. A more detailed and realistic understanding of the internal microenvironment and outward behaviour of flocs will be achieved, as will our ability to better model important environmental processes, such as sediment dynamics and contaminant transport, and better manage engineered systems.

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MICROBIALLY-INFLUENCED CORROSION (MIC).

See BIOCORROSION: ROLE OF SULFATE REDUCING BACTERIA; NUCLEAR WASTE RESPOSITORY IN YUCCA MOUNTAIN: MICROBIOLOGICAL ASPECTS

MICROBIAL REMOVAL BY PRETREATMENT, COAGULATION AND ION EXCHANGE

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For centuries, the process of providing safe drinking water has relied on the application of the "multiple barrier concept." Hippocrates (460–354 B.C.) writes in *Air, Water and Places* — the first treatise on public hygiene — that "qualities of the waters differ from one another in taste and weight,"... One should "consider the waters which the inhabitants use, whether they be marshy and soft, or hard and running from elevated and rocky situations, and then if saltish and unfit for cooking... for water contributes much to health." (1)

Today, the concept of multiple barriers for water treatment is the cornerstone of sanitary engineering. These barriers are selected to duplicate removal capabilities by succeeding process steps so that sufficient backup system are available to permit continuous operation in the face of normal mechanical failures. Traditionally, the barriers have included:

- source water protection,
- coagulation, flocculation, sedimentation,
- filtration,
- disinfection, and
- protection of the distribution system.

This section deals with the pretreatment and chemical conditioning of water for optimum removal of microbes within the drinking water treatment process. When these processes are combined with disinfection, filtration, and protection of the distribution system; high-quality potable water can be achieved. Alternatively, water source of very high quality may rely only on watershed protection and disinfection. The reader should consult each of these sections to gain a comprehensive view of the microbiology of drinking water treatment.

An example of the benefit of multiple treatment barriers is illustrated by a recent epidemiological study of a karstic groundwater system, in which one well was filtered and chlorinated while the second was only chlorinated (2). A study of antidiarrheal drug sales showed a strong correlation to lapses in chlorination of the well that had disinfection as the only treatment, whereas no effect could be traced to lapses in chlorination of the filtered well. The authors speculated that combination of filtration and chlorination and provided sufficient redundant treatment so that temporary lapses in disinfection did not generate a measurable outcome (2).

PRETREATMENT

Pretreatment can broadly be defined as any process to modify microbial water quality before the treatment plant. Pretreatment options may be compatible with a variety of posttreatment processes ranging in complexity from simple disinfection to membrane processes. Pretreatment can have the advantage of reducing or stabilizing the microbial load to the treatment process.

Roughing Filters

A roughing filter is a coarse media (rock or gravel) filter used to reduce turbidity levels before downstream processes, such as slow sand filtration, diatomaceous earth, or possibly membrane filtration. A review of design variables for roughing filters has been published by the American Water Works Association Research Foundation (AWWARF) (3). Roughing filters typically have a filter box divided into multiple sections containing gravel beds of decreasing size, inlet and outlet structures, and flow control devices. Examples of common configurations are shown in Figure 1.

Roughing filters have achieved peak turbidity removals ranging from 60 to 90%, with higher reductions generally associated with higher turbidity loadings (4,5). Coliform bacteria have been removed to a similar extent. Galvis and coworkers (5) reported pilot studies of various roughing filter configurations (horizontalflow, upflow, and downflow). They achieved 93 to 99.5% reduction in fecal coliform bacteria. When these filters were combined with a dynamic roughing filter (a filter with a thin layer of fine gravel on top of a shallow bed of coarse gravel with a system of underdrains) to pretreat high turbidity events, and subsequent slow sand filtration, fecal coliform removals of 86.3 and 99.8%, respectively, were achieved with an overall combined treatment efficiency of 4.9 to 5.5 log units. In a five-month pilot study of a medium gravel (5.5 mm), horizontal roughing filter in Texas City, Texas, Collins and coworkers (3) reported an average 47% removal of total bacteria measured by epifluorescence microscopy. In the same study, the roughing filter removed

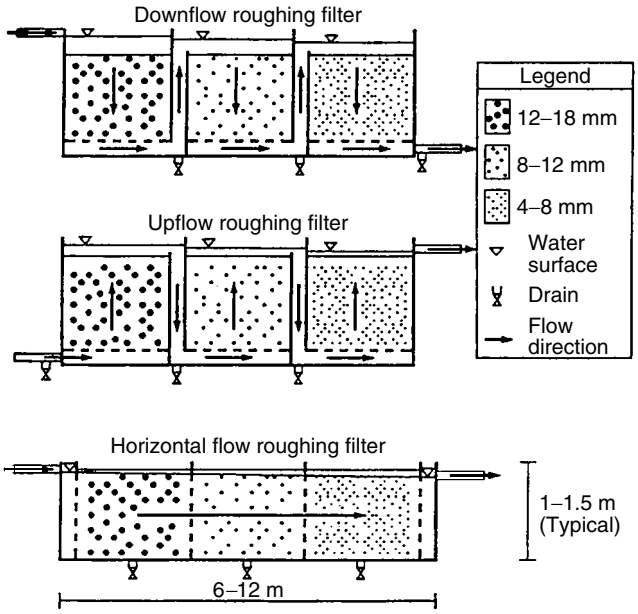

Figure 1. Typical roughing filter configuration loadings (Collins et al. 1994.

37% of the source water algal cells and 53% of the total chlorophyll. The researchers found that the roughing filters performed better for removal of clay particles when the filter was ripened with algal cells. Ahsan and coworkers (4) reported that addition of alum coagulant before a horizontal roughing filter could improve its performance for turbidity, color, organic carbon, head loss, and filter run length.

Microstrainers

Microstrainers are fabrics woven of stainless steel or polyester wires with apertures ranging from 15 to 45 m (usually 3,035 m). Because of the mesh size, microstrainers are useful for removal of algal cells and large protozoa (e.g., *Balantidium coli*), but does not show significant impact on bacteria or viruses. The overall rate of algae removal achieved for microstraining is usually between 40 and 70%, with simultaneous reduction in turbidity of 5 to 20% (6). The performance of microstrainers for specific applications varies depending on the type of algae present. Table 1 summarizes the performance of microstrainers for various types of algae. Although microstrainers can reduce the coagulant demand, it does not reduce smaller species or reproductive forms of algae.

Off-Stream Storage

The effects of off-stream storage are difficult to generalize because important physical, biological, and chemical processes are influenced by hydrological and limnological characteristics of the reservoir. For example, "round" reservoirs and lowland impoundments under strong wind influence can be regarded as homogeneous biotypes because of effective mixing, whereas long-stretched reservoirs whose depth increases with length can be considered as a series of interconnected individual basins (7). Reservoirs created by construction of a dam will have characteristics different from a natural or artificial lake. For the purposes of this discussion, off-stream storage refers to a pumped storage reservoir that feeds directly or indirectly a potable water intake.

Table 1. Performance of Microstainers for Various Algae

Diatoms		
Cyclotella	unicellular	10– 70
Stephanodiscus	unicellular	10–60
Melosira	filamentous	80–90
Synedra	unicellular	40–90
Asterionella	colonial	75–100
Fragilaria	filamentous	85–100
Chlorophyceae		
Chlorella	unicellular	10–50
Scenedesmus	cenobia (4–8 cells)	15–60
Pediastrum	cenobia (4–64 cells)	80–95
Blue-green algae		
Oscillatoria	filamentous	40–50
Anabaena	filamentous	50–70

Note: Adapted from Mouchet and V. Bonnelye, *Aqua* **47**, 125–141, 1998.

Oskam (8) summarized the self-purification processes that improve water quality in off-stream reservoirs (Table 2). The major factors that influence these processes are the degree of compartmentalization, the hydraulic residence time, the shape and flow through the reservoir, and the quality of the source water. In some cases, some of the processes can also degrade water quality. Failure to manage algal growth, control influx of nitrogen or phosphorous or other contaminants, or limit fecal contamination from runoff of surrounding areas or roosting birds can result in poorer quality of the impounded water.

Bernhardt (4) reported an elimination efficiency of coliform bacteria in dammed reservoirs of 8,099 percentage, when residence times were greater than 40 days, and 90–99% reduction of allochthonous bacteria, when retention times exceeded about 100 days. Similarly, reductions of enteroviruses (1.5 logs), Kjeldahl nitrogen (50%), total phosphorus (60%) and ammonium (70%) were reported for a pumped, off-stream reservoir after approximately 100 days retention time (9). Stewart and coworkers in 1997 (10) examined storm events that washed high levels of Giardia cysts (up to 17,000 cysts/100 L) and Cryptosporidium oocysts (up to 42,000 oocysts/100 L) into receiving reservoirs, with only one of 29 reservoir effluent samples, positive. Presumably, the cysts and oocysts were trapped in sediments that settled to the bottom of the reservoir because unattached organisms settle slowly (11). In a study of three Biesbosch reservoirs, storage with long residence times (average 24 weeks) resulted in reductions of 2.3 logs for *Giardia*, 1.4–1.9 logs for *Cryptosporidium*, 2.2 logs for *Escherichia coli*, and 1.7 logs for fecal streptococci (12,13)

Oskam (8) reported that die-off kinetics of microbes could be modeled as first-order reactions dependent on the residence time and short circuiting. For relatively rapid reactions (k-values > 0.05/d), the degree of compartmentalization had a positive effect on water quality. Therefore,

Table 2. Self-purification Processes that Improve Off Stream Reservoir Water Quality

Physical Processes
Equalization of peak concentrations (e.g., chemicals, microbes)
Exchange of oxygen and carbon dioxide with the atmosphere
Evaporation of volatile substances (e.g., solvents)
Settling of suspended solids and adsorbed substances (e.g., turbidity, heavy metals)
Biological Processes
Biodegradation of organic substances
Die-off of fecal bacteria and viruses
Nitrification of ammonium to nitrate
Denitrification of nitrate to nitrogen
Phosphorus elimination by phytoplankton uptake (in pre-reservoirs)
Chemical Processes
Oxidation of divalent iron and manganese
Hydrolysis of polyphosphates and organic esters (e.g., phthalates)
Photolysis of humic substances and polynuclear aromatic hydrocarbons

Note: Adapted from G. Oskam, *Aqua* **44**, 23–29 1995.

it would be better to have a series of three or four smaller reservoirs rather than one large impoundment. With estimated k-values of 0.07/d for removal of *Giardia* and *Cryptosporidium*, and 0.13/d for enteric viruses, compartmentalization in three or four reservoirs would increase the removal effect 15 to 230 times better than that achieved from a single basin (8).

For reservoirs with short retention times (and therefore limited self-purification), management of the raw water pumping schedule can improve water quality by avoiding periods of source water contamination. In a study of the Delaware River (U.S.A.), peak levels of microbial contaminants were associated with turbidity spikes following rainfall events (14). By operating the source water pumps to avoid these peak events, spikes of *Giardia* and *Cryptosporidium* that were 12 to 16 times higher than normal baseline levels could be avoided.

Bank Infiltration

Bank-filtered water is surface water seeping from the bank or bed, or a river or lake to the production wells of a water treatment plant. During the ground passage, water quality changes due to microbial, chemical, and physical processes, and by mixing with groundwater. The process can also be described as *induced infiltration* because the well field pumping lowers the water table causing surface water to flow into the aquifer under a hydraulic gradient. Bank infiltration has been widely used in European countries and is of great interest in many other countries. Variations on the underground passage concept include soil aquifer treatment, injection of surface water for underground passage, and aquifer recharge. Bank infiltration can be accomplished through natural seepage into receiving ponds, shallow vertical or horizontal wells placed in alluvial sand, and gravel deposits adjacent to surface waters, or infiltration galleries.

The advantages of bank infiltration are summarized in Table 3. The efficiency of the underground passage process depends on a number of factors: the quality of the surface water (turbidity, dissolved organic matter, oxygen, ammonia, and nutrients), the composition and porosity of the soil, the residence time of the water in the soil, and temperature. This efficiency can vary over time depending on the difference between the source water (e.g., river stage) and groundwater levels. This difference can influence the degree of groundwater mixing and residence time of the infiltrated surface water.

In the United States, and elsewhere, concern about groundwater under the direct influence of surface water (GWUDI) has caused some confusion on how to regard bank infiltration processes. Clearly, these processes are under the direct influence of surface water. However, the U.S. Surface Water Treatment Rule (USEPA, 1989) (16) ascribes no treatment credit to the infiltration process. Clancy and Stendahl (17) reported that removal of algae and diatoms ranged from 4.8 to 7.2 logs when the quality of the collection well was compared to when the raw water of the Grand River in Ontario, Canada. No *Giardia*

Table 3. Advantages of Bank Infiltration

- A natural pretreatment step requiring little chemical addition
- Reduced turbidity and particles
- Removal of biodegradable compounds
- Reduction of natural organic matter and lower disinfection by-product formation
- Reduction of bacteria, viruses and protozoa
- Equalization of concentration peaks (e.g., spills, temperature, etc.)
- Dilution with groundwater

Note: Adapted from W. Kuhn, International Riverbank Filteration Conference, Louisville, Kentucky.

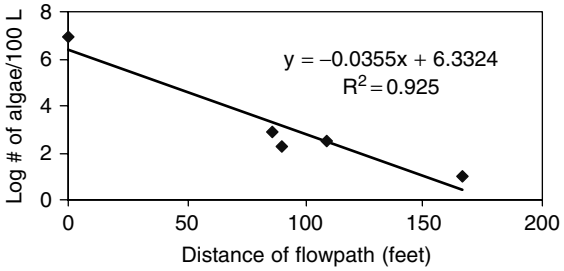

Figure 2. Relationship between algae reduction and theortical flow path. Adapted from Gollnitz et al. 1997 (18).

or *Cryptosporidium* were detected in the collector wells, although they were frequently detected in the river water. A relationship (Fig. 2) between the measured reduction of algae and the theoretical flow path for wells along the Great Miami River at Cincinnati, Ohio demonstrated approximately 1 log reduction for every 28 ft (8.5 m) of separation from the source water (Gollnitz and coworkers 1997) (18). Schijven and Ritveld (1997) (19) measured the removal of male-specific coliphage, entero- and reoviruses at three infiltration sites and compared the measured values to those predicted by a virus transport model. They found 3.1 logs reduction of bacteriophage within 2 m (6.6 ft) and 4.0 logs reduction within 4 m (13.2 ft) of very fine dune sand. Phages were reduced 6.2 logs through river bank infiltration greater than 30 m (98 ft) of sandy soil. In all cases the entero- and reoviruses were eliminated to a value lower than the detection limits (>2.6 to >4.8 logs removals). The virus transport model corresponded reasonably well with the measured results, producing calculated removals ranging from 2.5 to 15 logs. In ongoing studies being conducted by the American Water Works Service Company, Inc. and the Johns Hopkins University, monitoring of three river bank in filtration system along the Wabash (Indiana), Ohio, and Missouri Rivers have shown complete removal of *Clostridium* and bacteriophage indicators (Table 4) and substantial reductions in biodegradable dissolved organic carbon (BDOC) and assimilable organic carbon (AOC), which can stimulate bacterial growth in distribution system pipelines. All these data indicate that bank filtration can be highly effective for removal of microbial contaminants.

COAGULATION/FLOCCULATION/SEDIMENTATION

Coagulation is a process for promoting the interaction of small particles to form larger particles. In practice, coagulation refers to the process of addition of a coagulant and the formation of hydrolysis products, which causes coagulation, particle destabilization, and inter-particle collisions. The physical process of producing inter-particle contacts to form large particles is termed *flocculation*. Sedimentation is a solid-liquid separation process where particles settle under the force of gravity. Excellent reviews of these processes are available (20,21). With respect to coagulation and flocculation, microbes can be considered as particles (e.g., most bacteria and protozoa) or as colloidal organic particles (e.g., viruses).

Conventional

Conventional clarification typically refers to the process of chemical feed, rapid mix, flocculation, and sedimentation (usually in a rectangular basin). The efficiency of the sedimentation process may be enhanced using inclined plates or tubes. Removal of particles is chiefly governed by the particle terminal settling velocity, and the basin surface loading, or overflow rate. In the absence of a chemical coagulant, microbial removals are low because of low sedimentation velocities (11). The addition of coagulants neutralizes (or reduces) the surfaces electrical charge on the microbe and allow colliding particles to contact. Flocculation of these particles results in aggregates with sufficient settling velocities to be removed in the sedimentation basin.

Most studies of microbial removal by conventional clarification are estimated using jar test reactors. In these tests, removal of bacteria (*E. coli* vegetative cells and *Clostridium perfringens* spores) and parasites (Giardia cysts and Cryptosporidium oocysts) typically range between 1–2 logs (Fig. 3) (22). Overall, iron-based coagulants performed slightly better for microbial removal than alum or polyaluminum chloride (PACl); however,

Table 4. Reduction in BDOC, AOC *Clostridium*, and Bacteriophage Levels by Bank Infiltration

Site	Sample	Distance from River (ft)	BDOC (mg/L)	Total AOC (μg/L)	*Clostridium* cfu/100 ml	Bacteriophage	
						E. coil C pfu/100 mL	Famp pfu/100 mL
Terre Haute	Wabash river	—	1.12	191.5	164.0	191.0	17.5
	Collector	70–90	0.20	27.9	0.1	0.0	0.0
	Well#3*	400	0.09	20.7	0.0	0.0	0.0
Jeffersonville	Ohio river	—	0.39	56.7	140.8	55.3	16.3
	Well#9	200	0.05	32.1	0.0	0.0	0.3
	Well#2	580	0.03	15.2	0.0	0.0	0.0
Parkville	Missouri river	—	0.36	268.7	137.8	31.0	8.5
	Well#4	120	0.33	258.2	0.0	0.0	0.0
	Well#5	120	0.15	149.5	0.0	0.0	0.0

*Water from this well is not dominated by infiltration.

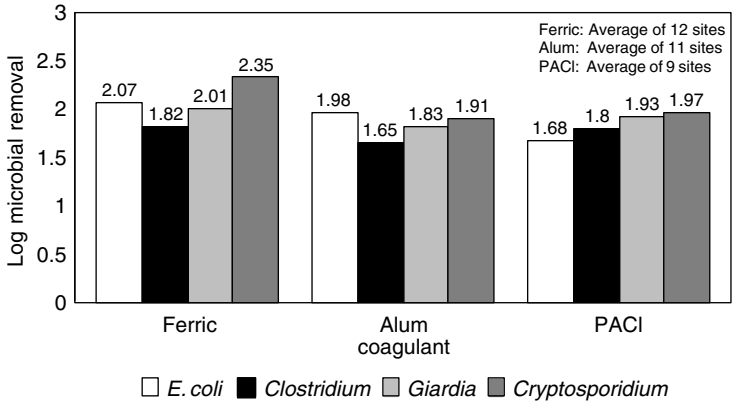

Figure 3. Removal of bacteria and protozoa under optimal coagulation conditions. Adapted from Bell et al. (22).

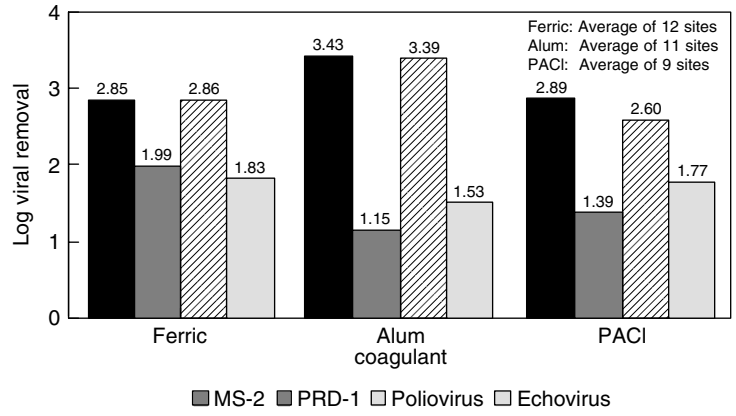

Figure 4. Removal of viruses under optimized coagulation conditions. Adapted from Bett et al. (22).

site-specific water quality conditions caused removal of efficiencies to a greater extent than did the choice of coagulant. Coagulation conditions (dose, pH, temperature, alkalinity, turbidity and the level and type of natural organic matter) impact the efficiency of removal, with slightly better overall microbial reductions under pH conditions (5–6.5) optimum for removal of total organic carbon (22).

Figure 4 shows that different viruses may respond quite differently to coagulation conditions. The bacteriophage MS2 and human enteric Poliovirus had similar removal efficiencies (2.6 to 3.4 logs), whereas the phage PRD-1 and enteric Echovirus were removed at a much lower rate (1.1 to 1.9 logs). Moreover, the differences in virus removal were most pronounced for alum [$Al_2(SO_4)_3$•18 H_2O]. Similar differences in virus adsorption have been observed in granulated gels (23). These results indicate that the performance of coagulation differs for various viruses and caution against extrapolating the data for other untested viruses.

Haas and coworkers (24) reviewed data from four bench-scale or pilot plant studies for coagulation, flocculation, and sedimentation of Cryptosporidium oocysts and chose data where the coagulant type, coagulant dose, pH, temperature, and mixing conditions were described. Using 24 data points, they found that the log removal of oocysts was dependent on coagulant concentration, polymer concentration, and process pH. The model had an excellent fit to the data (R^2 of 0.94). However, when data from other studies were added to the model, the fit of the model decreased. They concluded that additional data are needed, especially where the conditions of coagulation and flocculation are well described (methods of chemical addition, mixing conditions, and time, velocity gradient, etc.).

Performance of full-scale, conventional, clarification processes may be much more variable, depending on the degree of optimization. In an international report summarizing the performance of treatment plants from various countries, Gimble and Clasen (1998) (25) indicated that average microbial removals for coagulation and sedimentation ranged between 27 to 74% for viruses, 32 to 87% for bacteria (total coliforms or fecal streptococci), and 0 to 94% for algae. Low protozoan levels and limitations of the analytical methodology, limit full-scale data for *Giardia* and *Cryptosporidium* (26). Research has emphasized the need for an optimum coagulation dose as the most important factor to ensure good removals of cysts and oocysts by sedimentation and filtration (27–30). Impaired flocculation was one of the factors in the 1987 outbreak of cryptosporidiosis in Carrollton, GA (28). In a study of eight water filtration plants, Hendricks and coworkers in 1988 (31) concluded:

> ... without proper chemical pretreatment Giardia cysts will pass the filtration process. Lack of chemical coagulation or improper coagulation was the single most important factor in the design or operation of those rapid rate filtration plants where Giardia cysts were found in finished water ... with

proper chemical coagulation, the finished water should be free of Giardia cysts, have few microscopic particles, and have turbidity levels less than 0.1 NTU

Other factors that can influence the efficiency of the clarification include: variable plant flow rates, improper dose, poor process control with little monitoring, shear of formed floc, inappropriate mixing of chemicals, poor mixing and flocculation, and inadequate sludge removal (32). In addition to metallic coagulants (e.g., alum or ferric), polymeric coagulation or filter aids, or both, may be necessary to produce low turbidity levels (<0.1 NTU), especially for high-rate filtration (>4 gpm/sq ft or >10 m/h). Preoxidation with chlorine or ozone has been shown to improve particle removal by sedimentation and filtration (33,34). In some cases, current treatment plants are being designed with intermediate ozonation specifically to aid in particle removal by sedimentation and filtration (35).

Coagulation and sedimentation can be effective for removal of algal cells, and in most cases researchers have reported that coagulation conditions do not cause the release of algal toxins, provided oxidants are not added (36). Removal of algal toxins directly by coagulation and sedimentation has resulted in low removal levels, ranging from 0 to 49%, although addition of powdered activated carbon to the clarification process increased removals to 90% or better, depending on the carbon dose, type of carbon, toxin level, and organic matrix (36). A natural coagulant derived from shrimp shells (termed *chitosan*) was shown to be effective (>90% reduction) for removal of the algae *Chlorella* and *Scenedesmus quadricuda* at neutral to alkaline pH conditions and chitosan doses greater than 10 mg/L (37).

High Rate Clarification

Beginning in the 1930s, and increasing in usage in the 1970s and 1980s, were application of technologies to achieve high-rate clarification. Floc-blanket sedimentation or more generically, solids contact clarification, enhances particle removal by increasing particle concentration in a fluidized blanket so that flocculation and the rate of sedimentation is increased. Ballasted-floc systems combine coagulation with sand, clay, or carbon to increase the particle sedimentation rate. Adsorption or contact clarification is a process in which coagulated water is passed through a bed material where particles attach to previously adsorbed material. Because all these processes occur in smaller basins with higher surface loading rates than conventional clarifiers they can be generically termed as *high-rate clarification*.

When properly operated, high rate clarifiers can be as, or more, effective than conventional basins for removal of microbes. The choice of an appropriate blanket polymer is important for optimum operation (20). Bell and coworkers in 1998 (38) reported turbidity removals of 98% for Superpulsator® clarification (raw water turbidity 20–50 NTU, settled water 0.6 to 0.75 NTU), 89% for Accelator® clarification (raw water turbidity 4–10 NTU, settled water 0.5 to 0.9 NTU) and 61% for circular floc-blanket purification unit clarification (raw water turbidity 1.2–16 NTU, settled water average 0.97 NTU). Baudin and Laîné (39) evaluated three full-scale treatment plants and found complete removal (>2 to 2.8 logs) of *Giardia* and *Cryptosporidium* by pulsator clarifiers. The units produced between 1.0 and 2.7 logs of turbidity removal. Other investigators (40) have reported similar efficiencies for floc-blanket clarifiers. A combination of preozonation and Superpulsator® clarification reportedly improved clarification of *Giardia* and *Cryptosporidium*-sized particles from approximately 1.5 to 2.5 logs removal (41). Pilot plant studies of the sand ballasted-floc (actiflo system) showed effective removal of turbidity and particle counts (42). In addition, microscopic particulate analysis of raw and settled water showed average algae removal of 3.9 logs and 4.5 logs removal of diatoms (42). Floc formed on magnetic particles can be rapidly removed by using magnets within the sedimentation process (43–45). The particles can be collected and regenerated for reuse.

Dissolved Air Flotation

In dissolved air flotation (DAF), bubbles are produced by the reduction in pressure of a water stream saturated with air and the bubbles attach to floc particles causing the agglomerate to float to the surface where the material is skimmed off (20). DAF is most applicable to waters with heavy algal blooms or contains low turbidity, low alkalinity, and highly colored water. These waters are difficult to treat by sedimentation because the floc produced has a low settling velocity.

The effectiveness of DAF for treating algal-laden, humic colored, water is illustrated by the comments of Kiuru (46) a Finish researcher, who indicated that only DAF treatment plants have been built in his country since the mid-1960s. A 1.8 log removal of the algae *Aphanizomenon* and *Microcystis* was achieved by pilot-scale DAF. Similar results (1.4 to 2.0 log removals) have been obtained in full-scale studies (6). The effectiveness of DAF for treatment of algae also results in good removal of cell-associated toxins (6).

Plummer and coworkers (47) reported that depending on the coagulant dose DAF achieved between 2 and 2.6 logs removal of Cryptosporidium oocysts when conventional sedimentation resulted in 0.6 to 0.8 log removal. The performance of DAF for oocyst removal depended on the pH, coagulant dose, flocculation time, and recycle ratio. Other researchers have confirmed the effectiveness of DAF for oocyst removal, particularly when polyelectrolyte coagulant aids were added to help stabilize the floc (40).

Lime Softening

Precipitative lime softening is a process in which the pH of the treated water is increased (usually through the addition of lime or soda ash) to precipitate excessive concentrations of calcium and magnesium. Reduction of calcium can be achieved at pH values typically between 9.5 and 10.5 although removal of magnesium hardness requires pH levels between 10.5 and 11.5. This distinction is important because the pH of lime softening can inactivate many microbes at the higher end (10–11),

but may have less impact at more moderate levels (9.5). In precipitative lime softening, the calcium carbonate and magnesium hydroxide precipitates are removed in a settling basin before the water is filtered. Therefore, the microbial impact of lime softening can be a combination of inactivation by elevated pH and removal by settling.

Logsdon and coworkers performed a comprehensive evaluation of the effects of lime softening on the removal and disinfection efficiency of *Giardia*, viruses, and coliform bacteria (48). Coliform bacteria in river water (spiked with raw sewage) were inactivated 0.1 log at pH 9.5, 1.0 log at 10.5 and between 0.8 to 3.0 logs when exposed to a pH of 11.5 for six hours at 2–8 °C. Bacteriophage MS2 was sensitive to lime softening conditions, demonstrating more than 4 logs inactivation in the pH range of 11–11.5 within two hours. Hepatitis A virus showed a 99.8% reduction when exposed to pH 10.5 for six hours. Poliovirus was the most resistant virus tested, requiring exposure to pH levels of 11 for six hours before showing 2.5 logs inactivation. Reductions were less than 1 log when exposed for six hours to pH levels less than 11. The viability *Giardia muris* cysts (measured by excystation) were not significantly affected by exposure to pH 11.5 for six hours. *Cryptosporidium* viability (measured using dye exclusion) was not affected by exposure to pH 9 for five hours (49).

When the microbial removal by sedimentation is added to the microbial inactivation because of exposure to elevated pH levels, jar tests of precipitative lime softening at pH 11.5 resulted in 4 logs treatment of viruses and bacteria and 2 logs treatment of *Giardia* and *Cryptosporidium* (22). Limited full-scale data suggests that 2 logs removal can be achieved through sedimentation by precipitative lime softening (48).

In-Line Coagulation

For high-quality source waters (e.g., those waters where turbidity and other contaminant levels are low), coagulation may be performed within the raw water pipeline before direct filtration. In this case, coagulants are added directly to the pipeline, typically before an in-line static mixer, and a basin for sedimentation is not used. This method of in-line coagulation permits particle destabilization (charge neutralization) necessary for proper particle removal by filtration, but microbial removal by sedimentation does not take place.

ION EXCHANGE

Ion exchange is a treatment process in which a solid phase presaturant ion is exchanged for an unwanted water ion. The process is used for water softening (removal of calcium and magnesium), treatment of some radionucleides (radium and barium), and various contaminants (nitrate, arsenate, chromate, selenate, dissolved organic carbon). The effectiveness of the process depends on the background water quality, competing ions, and total dissolved solids. Although some ion exchange systems can be effective for adsorbing viruses and bacteria (50) they are not generally considered a microbial treatment barrier because the organisms can be released from the resin by competing ions. Therefore, breakthrough of the system is possible. Alternatively, ion exchange resins may become colonized by bacteria whose growth can contaminate treated effluents (51,52). Backflushing and other rinsing procedures, even regeneration, will not remove all of the attached microbes. Impregnation of the resin with silver suppresses bacterial growth only until a silver-tolerant population develops. Disinfection of ion exchange resins using 0.01% peracetic acid (one hour contact time) has been suggested (51).

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MICROBIAL STARVATION SURVIVAL IN SUBSURFACE ENVIRONMENTS

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Abundant research findings in the 1980s and 1990s firmly established the existence of diverse microorganisms in a variety of subsurface environments (1–5). Subsurface habitats include sediments and rocks, and they range from shallow subsoils to rocks lying at depths greater than 3 km below land surface. The depth limit of the biosphere has not been determined; but if we assume that temperature constrains the distribution of microorganisms with depth and that the upper temperature limit for life is approximately 110 °C, then the biosphere extends to average depths of 4 and 6 km into the oceanic and terrestrial crust, respectively (6). Calculations of this type have led several investigators to conclude that the total subsurface microbial biomass outweighs all other forms of life on Earth (6–8). Microorganisms in the subsurface represent a diversity of phylogenetic types as well as a diversity of metabolic capabilities (9,10). The diversity of

metabolic types no doubt reflects the range of energy sources in the subsurface. Although microorganisms, primarily prokaryotes, abound in the deep subsurface, theirs is not an easy life. Energy sources and other nutrients are almost never present in sufficient quantities to support rapid reproduction. Nutrient fluxes are among the slowest in the entire biosphere, and thus rates of microbial activity are infinitesimally slow (6,10,11). Most subsurface microorganisms are subject to extreme long-term nutrient stress and may remain viable in a relatively inactive, starved, quiescent state for intervals that can be measured most appropriately on a geologic timescale. Deep, isolated subsurface environments may be among the most extreme on Earth, and the ancient microorganisms that persist under these extreme conditions may possess unique adaptations for long-term survival.

This article addresses energy sources available in the subsurface and the responses of subsurface microorganisms to the generally low, or even nonexistent, nutrient fluxes in deep subsurface terrestrial environments, with emphasis on the persistence of microbial populations for thousands to millions of years. There is a large body of literature, including many excellent reviews, on starvation survival of microorganisms in other environments, especially the open ocean (12–18). This information is not reviewed here, except as it relates to survival in the deep subsurface. Starvation of subsurface microbes has received considerably less attention and so correspondingly less is known about starvation capabilities and responses of subsurface microorganisms. Other recent reviews on this or related topics include those of Amy (19), Kieft and Phelps (10), Kieft (20), and Morita (17).

ENERGY SOURCES IN THE SUBSURFACE

Organic and inorganic energy sources support microbial growth and activity in subsurface environments. Organic energy sources can be photosynthetically generated organic matter transported from the surface by groundwater flow or organic matter that has been sequestered at the time of geological deposition. For example, in the coastal plain aquifer sediments of the southeastern United States, most of the microbial activity is based on catabolism of low concentrations of dissolved organic carbon (<0.5 mg/L in the Middendorf aquifer) that is transported from the recharge zone via groundwater flow; however, there are also localized residual organic carbon deposits in the form of lignite (21). Where the energy sources are primarily surface-derived organic matter that is transported through an aquifer, it is possible to estimate rates of microbial activity based on changes in aquifer chemistry along a flow path. Biogeochemical models of this type have estimated microbial activities in groundwater aquifers to be orders of magnitude slower than in nearly all surface environments (10,11,22). Phelps and coworkers (22) estimated generation times of hundreds to thousands of years for microorganisms in the coastal plain aquifers of South Carolina. Although this seems like a relatively slow-paced life, it is probably faster than in many other subsurface environments. Rates of groundwater flow and, consequently, rates of nutrient flux are even slower in aquitards, tight rock formations, and in thick, unsaturated (vadose) zones in arid and semiarid environments. Extremely sluggish rates of metabolism in some fine-grained sediments can be inferred when viable microorganisms are detected along with significant quantities of organic carbon that have persisted since the time of geological deposition, for example, in 6- to 8-million-year–old lacustrine sediments (23), in 90- to 93-million-year–old shale (24,25), and in 230-million-year–old shale (26). Extremely slow rates of metabolism in thick vadose zones of arid regions can be inferred from slow rates of groundwater recharge, slow diffusion of nutrients through thin, discontinuous water films, and persistence of entrained organic carbon (27–29). These relatively static, vadose zone environments may be the most nutritionally extreme in the entire biosphere, and indigenous microorganisms may be the ultimate microbial survivors.

Not all subsurface life is dependent upon photosynthetically generated organic matter; inorganic energy sources can also be significant in the subsurface. Reduced, inorganic sources of energy (e.g., H_2, H_2S, Fe^{2+}) may be products of anaerobic microbial metabolism or they may be generated by purely geochemical processes. Fredrickson and coworkers (30) were among the first to demonstrate chemolithotrophic activity in the subsurface, with the finding of nitrifying, sulfur-oxidizing, and hydrogen-oxidizing bacteria in Atlantic coastal plain sediments. Since then, a number of subsurface environments have been shown to harbor chemolithotrophs. Microorganisms capable of oxidizing hydrogen may be especially important in the subsurface. Several thermophilic, hydrogen-oxidizing, metal-reducing bacteria have been isolated from deep, hot environments (31–33). These may be of particular evolutionary interest, as hydrogen oxidation coupled to metal reduction occurs in many thermophilic microorganisms that represent deep branches of the phylogenetic tree, and this form of respiratory metabolism has been posited to be that of the last common ancestor of all organisms (34). Entire subsurface ecosystems may exist that depend on geochemically derived sources of hydrogen for primary sources of energy and that are therefore independent of photosynthesis for electron donor or electron acceptor (7,35–37). Stevens and McKinley (35) presented evidence of chemolithoautotrophic ecosystems in deep basalt aquifers in which hydrogen-oxidizing methanogens are the primary producers. The hydrogen can be generated geochemically through basalt weathering (35,38). Pedersen (36,37) has proposed a similar mechanism supporting life in a deep, fractured granite subsurface environment in Sweden. Hydrogen can also be generated by radiolysis, that is, splitting of water by ionizing radiation; such radiolytically produced hydrogen has been hypothesized to serve as electron donor for microorganisms in deep environments with sufficient radiation flux (26,39). Regardless of the source, inorganic energy in forms such as H_2 is probably made available in only sparing amounts and chemolithotrophs in these environments probably lead just as impecunious an existence as their heterotrophic counterparts.

Although exogenous energy sources may not be adequate to support frequent cell divisions in most subsurface environments, they may be utilized at a slow rate to fuel cell maintenance and repair. Morita (17) recently made a case for H_2 as the universal energy source that enables long-term survival of microorganisms in otherwise energy-poor environments. Morita's case for H_2 is built on the following points: its ubiquity in the biosphere, its ability to penetrate membranes, its low energy of activation, its ability to dissociate into protons and electrons in the presence of Fe(II), and the widespread phylogenetic distribution of hydrogenases in microorganisms. Low-level consumption of H_2 could provide sufficient energy for cellular repair of damage to macromolecules, including racemization of amino acids in proteins and depurination of nucleic acids. In the absence of exogenous energy sources such as H_2, it is hard to imagine that endogenous metabolism of storage products could be sufficient to maintain essential macromolecules for thousands to millions of years.

THE NATURE OF STARVED SUBSURFACE MICROORGANISMS

The majority of microorganisms in subsurface environments are prokaryotic and most of these appear to be bacteria (9), although the Archaea are also represented, especially methanogens in deep, anaerobic environments (35,40,41). Surprisingly, spore-forming bacteria do not comprise a significant component of most subsurface communities, even in those with negligible energy inputs. A possible explanation for this is that spores are incapable of repairing the unavoidable, accumulated damage to nucleic acids by ionizing radiation (42). Vegetative cells, on the other hand, are capable of at least some metabolic activity to support repair mechanisms and/or occasional DNA replication and cell division. Successful cell division effectively "resets the clock" on accumulation of DNA damage. Therefore, endospores dominate only in subsurface environments of more recent origin. As an example, endospore formers were found to be common among relatively shallow Siberian permafrost buried for 5,000 to 8,000 years, whereas they were rare in deeper, older (1.8 to 3.0×10^6-year–old) permafrost (43). Spores of actinomycetes and fungi may be rare in deep, ancient subsurface environments for the same reason.

Thus, most microorganisms in the subsurface are vegetative forms that lack obvious morpholological adaptation for resisting environmental challenges such as nutrient deprivation. In the absence of sufficient nutrient flux to support active metabolism, they exist as dormant cells. The term "somnicells" has been coined for these quiescent bacterial forms (44,45). Many of them are nonculturable, perhaps in a viable but nonculturable state, and so they remain hidden from traditional culture techniques in a state that is variously termed *cryptobiosis* or *anabiosis* (45). Clearly, there exists a broad spectrum of physiological states of microorganisms. Live, rapidly multiplying cells occupy one end of the spectrum; at the other extreme are cells whose membranes are no longer intact, that are incapable of substrate uptake and metabolism, and that are thus truly dead. Within that spectrum, one can envision cells that have one or more characteristics of life, for example, the ability to take up and metabolize substrates, cellular maintenance and repair, and so on, without undergoing cell division. A majority of deep subsurface microbes may exist in a state somewhere in the middle of the spectrum, that is, slowly metabolizing exogenous or endogenous energy sources, but rarely if ever dividing.

Further evidence for the starved state of subsurface bacteria lies in the fact that they exist in situ predominantly as extremely small, spherical cells, that is, "dwarfs" or "ultramicrobacteria" (20). The same holds true for surface soils, where dwarf cells make up the bulk of the biomass. As in soils, these dwarf forms probably represent a combination of potentially normal-sized bacteria that have miniaturized in response to nutrient deprivation as well as intrinsically small cells, that is, dwarf bacteria that do not grow into larger cells even when resuscitated by introduction of nutrients. Small size generally denotes a Spartan lifestyle. Intrinsically small cells are likely oligotrophs with high surface to volume ratios that favor high substrate uptake capacity relative to the low maintenance requirements of a small cell. However, stable dwarf bacteria that are capable of rapid growth have been isolated from a surface soil (46).

The dwarfing response in starved bacteria has been well documented in bacteria isolated from a variety of environments, including the subsurface (20,47–50). Starved cells undergo rapid diminution in cell size during the initial stages of response to starvation and thereafter the cell size stabilizes. In many bacterial types, the decrease in cell size begins with reductive division, also known as fragmentation, which is cell division without cytoplasmic growth (50). After two to three rounds of reductive division, size reduction continues as individual cells metabolize endogenous substrates. Whereas initial rates of endogenous metabolism may be rapid to provide energy for synthesis of new proteins, replication of DNA, and so on, long-term starvation survival requires a slow, frugal use of endogenous substrates at a rate that is just enough to support cellular maintenance. Dwarfing in marine *Vibrio* strains has been shown to be a response specifically to carbon starvation and is not triggered by nitrogen or phosphorus starvation alone (51). This may hold true for other genera and other environments. The lower size limit for dwarf cells has not been firmly established, but theoretical considerations as well as empirical data suggest a minimum cell volume of $0.004\ \mu m^3$, corresponding to a 0.2-μm–diameter coccus (20). Reports of considerably smaller sized "nannobacteria" in geological materials (52) are likely artifacts or possibly fragments of microbial cells. Likewise, the putative "nanofossils" in Martian meteorite ALH84001 (53) are unlikely to be fossils of microorganisms as they are known on Earth.

Starvation survival is of no selective advantage if cells are unable to respond when an energy-rich substrate finally becomes available. Starved bacteria have been shown to have a high affinity for energy-rich substrates (16,54). Substrate uptake requires an energized

cell membrane, that is, an active proton pump (or sodium pump in marine bacteria) coupled to electron transport to maintain a pool of high-energy compounds, for example, adenosine triphosphate (ATP) (45,55). Starving cells maintain a minimal concentration of ATP and may even increase their ATP content as starvation proceeds (56). Adenylate energy charge (AEC) is a measure of the physiological status of cells. AEC = ([ATP] + 1/2[ADP])/([ATP] + [ADP] + [AMP]). High values (>0.8) are thought to indicate active metabolism; intermediate values (0.4 to 0.8) indicate resting cells, and low values (<0.4) indicate dead or moribund cells. Kieft and Rosacker (57) measured AECs that ranged from 0.23 to 0.76 in deep southeastern coastal plain sediments. Each of these values likely represents an assemblage of microorganisms that vary in their levels of metabolic activity. The highest AEC value occurred in the sediment that had the highest number of culturable bacteria. AEC values were correlated with laboratory-measured respiration rates. Response time following addition of an energy-rich substrate is another measure of the physiological status of microorganisms. Kieft and coworkers (58) measured lag times for glucose mineralization that increased with depth and sediment age in a series of vadose zone sediments. This is similar to the laboratory findings of Amy and Morita (59), who found that the lag time for growth of a marine *Vibrio* was directly related to the length of time of starvation.

As in most other natural environments, the total numbers of cells (counted by microscopy or estimated from chemical analyses) are orders of magnitude greater than the numbers that can be cultivated. Culturability percentage ranges from an anomalously high 90% in highly conductive, Atlantic coastal plain aquifers (60) to more typical values of 0.01 to 0.0001% in a variety of other subsurface environments (27,29,58,61). Reasons for nonculturability are varied. Many cells are likely not cultured by currently available methods simply because we fail to understand their nutritional needs. Other cells may be injured, moribund, or actually dead. Dead cells may outnumber live cells in many subsurface environments. One measure of this is the relative amounts of membrane phospholipid fatty acids (PLFAs) and diglyceride fatty acids (DGFAs) (62). DGFAs are thought to be generated from PLFAs by the action of phospholipases immediately following cell death. Kieft and coworkers (58) measured ratios of DGFA to PLFA that increased with depth in a vadose zone, from approximately 0.001 in near-surface sediments to greater than 10 in deep sediments that have been buried for 10^4 to 10^6 years. Since there is ample evidence that subsurface microorganisms are capable of altering the chemistry of their environments (given sufficient time), it is clear that a significant proportion is viable and metabolically active, albeit at glacially slow rates. These may be in a starvation-induced, viable, but nonculturable state (44), and/or in a viable but as yet uncultured state (63). Since they resist cultivation, they can only be characterized by indirect approaches using molecular techniques (e.g., extraction, cloning and sequencing of DNA, or probing with oligonucleotide probes) or chemical analyses (e.g., PLFA profiling).

Finally, if one can generalize about diverse organisms occupying a wide range of habitats and perhaps comprising the majority of the Earth's biomass, the typical subsurface microorganism exists in situ as a relatively inactive, non-spore-forming, vegetative bacterium that is severely nutritionally challenged. Its starvation diet is directly related to its diminutive size and apparent nonculturability. Prolonged starvation stress selects for organisms that can maintain cellular integrity by slow endogenous metabolism of intracellular substrates and/or metabolism of meager exogenous substrates, possibly including H_2. Despite prolonged nutrient deprivation, it remains energetically poised for action should energy-rich nutrients appear.

LABORATORY STUDIES OF MICROBIAL SURVIVAL

Most of what we know about adaptations to starvation stress is based on laboratory incubation experiments. The traditional approach is to cultivate a pure culture of microorganisms in a rich nutrient medium, wash and resuspend the cells in a nonnutrient buffer solution, and then monitor characteristics such as culturability, cell morphology, and cellular physiology over time. This approach has been used to good advantage to investigate starvation responses of gram-negative marine heterotrophic bacteria (48,49,56,59,63,64,65). Experiments of this type have demonstrated various patterns of decline in culturability, in some cases preceded by a brief period of cell division without growth, that is, fragmentation (50). Cell volumes of these marine bacteria diminish during the early stages of starvation survival (49). Chemical composition of the cells changes as they convert to endogenous metabolism. Cell lipids, carbohydrates, and storage products (e.g., poly-β-hydroxyalkonoates) are consumed first, followed by a slow metabolism of cellular proteins and RNA. Starvation-specific proteins make their appearance with the onset of starvation stress (59,66,67). Some of these may be DNA-binding proteins that confer long-term stability (68). Starvation stress also results in changes in membrane lipid composition, as indicated by the appearance of stress signatures in the profiles of membrane PLFAs (48,62,69). Adaptations for starvation stress survival also confer cross-protection against other environmental insults, as well (68,70), for example, osmotic shock (71,72) and high temperature (72–74). The molecular mechanisms involved during induction of starvation survival are becoming understood in increasing detail for organisms such as *Escherichia coli* and marine vibrios (68,75–77). The low molecular weight compounds cyclic adenosine monophosphate and ppGpp appear to be important in intracellular signaling of starvation stress (68). The heat shock protein and molecular chaperone DnaK appears to play a central role. Mutant *E. coli* lacking *dnaK* failed to undergo reductive cell division when challenged by carbon starvation (74). DnaK mediates levels of the sigma factor RpoS, which in turn regulates expression of various starvation response genes (72). It remains to be seen whether the same molecular mechanisms are responsible for long-term starvation survival in subsurface bacteria. Microbial genome studies will likely

add to our knowledge in this area as they are expanded to include subsurface microbes.

This laboratory incubation approach to starvation studies has also been applied to soil microorganisms. Gram-positive, non-spore-forming soil bacteria of the genus *Arthrobacter* have been shown to be exceedingly starvation resistant. Early studies by Boylen and Ensign (78,79) showed that *Arthrobacter crystallopoites* could survive extended periods of starvation through a slow, sparing metabolism of endogenous substrates, including a glycogen-like polyglucose. Boylen and Ensign (78) found that 100% of cells remained viable after a 30-day incubation in nonnutrient buffer and that they decreased their rates of endogenous metabolism 80-fold during the first 2 days. In an experiment combining starvation and desiccation stress, Boylen (80) found 50% viability after 6 months in air-dried soil and that endogenous metabolism was slowed to a rate at which it could be projected that 50% of cellular carbon would remain after 12 years. However, as impressive as this is, it still does not compare to persistence on a geologic timescale in buried rocks and sediments.

Only a few subsurface bacteria have been tested for their starvation survival responses using laboratory incubation methods. Amy and coworkers (47) tested six strains isolated from volcanic tuff at the Nevada Test Site by suspending them in nonnutrient artificial pore water for 100 days and found patterns of response similar to those found in marine bacteria, that is, a rapid initial decline in culturable cells followed by a near–steady-state survival of a constant number of cells. Five of the six strains increased the number of culturable cells during the first two days of starvation, evidently by fragmentation. All six strains declined in cell volume during the early stages of starvation.

Kieft and coworkers (69) tested a *Pseudomonas aureofasciens* and an *Arthrobacter protophormiae*, each isolated from a subsurface sediment, for responses to nutrient deprivation in a porous medium, silica sand. The *Pseudomonas* altered its membrane lipid PLFA profiles in patterns that were similar to other gram-negative bacteria undergoing stress. The *Arthrobacter* showed no change in PLFAs when challenged by nutrient stress. In a longer-term study, Kieft and coworkers (48) tested the starvation-survival capabilities of subsurface *Pseudomonas* and *Arthrobacter* strains and compared them to those of closely related strains of bacteria isolated from surface environments. Phylogenetic relatedness was evidenced by similarity in 16S ribosomal RNA gene sequences. Starvation was carried out in two different sediments. One was a fluvial silt and other was a similar silt that had undergone soil development and was subsequently buried, forming a paleosol. Sediments were incubated under two different moisture conditions: one saturated with water and the other unsaturated to mimic conditions in vadose zone sediments. All strains survived for over a year in all treatments. Interestingly, there were no significant differences in viability percentage (quantified by plate counts) between the surface and the subsurface strains. However, the *Arthrobacter* strains survived better than the *Pseudomonas* strains under all conditions, as might be expected, given the well-documented starvation-survival characteristics of Arthrobacters. Survival was better in the paleosol than in the silt that did not undergo soil development. Survival may have been enhanced in the paleosol by the presence of residual soil organic matter as an exogenous substrate. Greater survival in the paleosol is also consistent with the observation of higher numbers of microorganisms in paleosols than in other buried sediments (27,81). Survival was better in saturated sediments than in unsaturated sediments, which is also consistent with field observations. Although vadose zone environments are generally relatively moist (matric water potentials greater than, i.e., less negative than, −0.1 MPa), the thin discontinuous water films of unsaturated sediments severely limit the flow of nutrients and the mobility of microorganisms (27,29). Thus, microorganisms in unsaturated, vadose zone sediments have even less access to exogenous substrates than in saturated sediments. Cells of both genera miniaturized during the early stages of incubation, as is typical of starved bacteria from all environments. Both the surface and surface *Pseudomonas* showed membrane PLFA profiles that are typical of nutrient-stressed gram-negative bacteria: increased ratios of saturated to unsaturated fatty acids, increased ratios of *trans* to *cis* monoenoic fatty acids, and increased ratios of cyclopropyl fatty acids to their monoenoic precursors. Again, the subsurface isolate reacted in a similar manner to the surface isolate. The surface and subsurface *Arthrobacter* strains demonstrated no discernible changes in membrane lipid PLFAs, as observed previously in the short-term starvation experiment (69).

The general conclusion from these experiments is that subsurface isolates are adapted for surviving long periods without exogenous nutrients, but perhaps not any more so than isolates from aquatic or soil environments. However, these laboratory incubation experiments have two severe limitations: one is that they are limited to culturable microbes whereas the majority of subsurface microorganisms are not culturable by currently available methods. The second obvious shortcoming is that laboratory incubations are relatively short, generally no more than a year or two (or a funding period) whereas microbes may be sequestered in the subsurface with limited nutrient availability for thousands of years or more. To truly grasp the concept of microorganisms remaining viable with little or no nutrient input for thousands to millions of years, one has to consider microorganisms in their natural geologic setting.

FIELD-BASED STUDIES OF MICROBIAL SURVIVAL

Two explanations can account for the presence of microorganisms in the subsurface. One is that they have survived since the time of geological deposition and the other is that they have been transported with groundwater more recently than geologic deposition. Depending on the environment, one or both of these explanations may be valid. The survival explanation is of greatest interest here. In many subsurface settings, the transport of microbes may be precluded by physical

conditions. Cemented sediments or crystalline rocks may lack fractures or pores of sufficient size to allow passage of microbes. Fine-grained sediments may preclude transport in a similar fashion (23,24). Thick unsaturated zones in arid regions may have insufficient rates of groundwater recharge to allow microbial penetration to any significant depth. By determining the time of geological deposition (or time of the last previous sterilizing event by heating) and the groundwater age, one can constrain estimates of the time since microorganisms were introduced into an environment. In subsurface strata with very old pore waters (thousands of years or more), it can be concluded that the inhabitant microorganisms have remained sequestered for at least as long as the age of the groundwater. If there is not a residual supply of organic carbon or other energy sources to these microbes, then it is likely that they have undergone few cell divisions in the subsurface and that any living cells that can be detected have maintained their viability as individual cells for exceedingly long periods of time.

There is a growing number of examples of apparently ancient microorganisms that have been resuscitated in culture from microenvironments that have been sealed against the influx of modern microorganisms for thousands and even millions of years (82–84). The finding of viable *Bacillus* endospores in the gut of an insect sealed in amber for 25 to 40 million years, by Cano and Borucki (85), is an especially striking case. The subsurface offers cases of ancient microbes that have been entombed for thousands to millions of years; however, the evidence for true sequestration from outside influences (immigration and/or nutrient transport) is generally more equivocal than it is for ancient amber.

Kieft and coworkers (58) quantified sediment ages, pore water ages, and microorganisms in a series of buried sediments at two sites in the Channeled Scablands of eastern Washington State. The sediments are aeolian loess sediments ranging in age from modern to approximately 1 million years. The sediment series constitutes a chronosequence in that age since time of geologic deposition is the main variable. Each of the sediments underwent some degree of soil development while at the surface before being buried by subsequent layers of very similar sediments. The pore water ages in the deepest, oldest sediments were estimated by the chloride mass balance method to be 1,200 years at one site and 3,000 years at the other. Microbial abundance (measured by microscopic counts, plate counts, and total PLFAs) declined with depth and sediment age in a pattern that was similar to patterns of microbial survival in laboratory starvation experiments, that is, rapid decline in the early stages of the experiment (represented here by shallow sediments of recent origin) and much slower decline in the later stages (represented by deeper, older sediments). Culturability percentage declined with depth. Microbial activities (radiolabeled glucose uptake and mineralization, β-glucosidase activity) showed similar patterns. PLFA profiles indicated the presence of several different types of microorganisms even in very deep sediments. PLFA signatures representative of gram negatives (e.g., *Pseudomonas*), gram positives (e.g., *Arthrobacter*), actinomycetes, and microeukaryotes were present even in sediments greater than 500,000-years old, with pore water age greater than 1,000 years. These data demonstrate the persistence of a diversity of microorganisms for a minimum of 1,000 years and probably much longer. Transport of microbes into the deep sediments cannot be ruled out, but the slow rates of groundwater recharge and the fact that microbes are usually transported more slowly than water in porous media suggest that microbes have persisted for much longer than 1,000 years. Likewise, the nutrient fluxes are extremely slow, and thus it is probable that these populations have undergone very few cell divisions.

Similar studies were carried out by Balkwill and coworkers (86) and Brockman and coworkers (28) in other low-recharge vadose zone sediments in eastern Washington State. Balkwill found evidence of a diversity of bacteria, including gram negatives and gram positives, in sediments with pore water ages of 15,000 and 30,000 years. Brockman and coworkers (28) isolated spore-forming and non-spore-forming bacteria from 4-million-year-old unsaturated sediments that have had only minimal groundwater recharge (~15 μm/year) since Pleistocene flooding about 13,000 years ago. Given the low recharge rates and negligible nutrient fluxes, these vadose zone bacteria appear to have survived starvation for 10^4 to 10^6 years.

Studies of volcanic tuff at the Nevada Test Site have revealed diverse bacteria, apparently existing within pores or fractures in the rock matrix (87–89). The environment here consists of unsaturated tuff, some of it highly welded by heating. Recharge rates are extremely slow and the depth to the water table is hundreds of meters. Microbes in some of these unsaturated strata are thought to have been trapped in minute pores within the rock matrix for at least 250,000 years (19).

SPECIAL CASES

Bacteria in Ancient Salt Deposits

Halophilic archaea and bacteria have been isolated from large deposits of NaCl evaporite (halite) from the Permian (225 to 270 million years before present) and Triassic (195 to 225 million years before present) Periods (90–94). Modern salt beds typically contain viable archaea; and it has been shown that archaea can become trapped within brine inclusions as halite crystallizes during evaporation, that various species of haloarchaea can survive within fluid inclusions for up to 6 months, and that these halophiles can then be resuscitated when the salt crystals are dissolved in a suitable growth medium (95). Fluid inclusions can comprise as much as 1% of the volume of halite deposits (96), and so they could conceivably harbor a significant number of cells. The question then is whether the microorganisms isolated from ancient salt beds have survived within fluid inclusions or whether they are more modern cells that exist on the surfaces of salt crystals or within fractures or pores in the salt beds. Comparisons of 16S ribosomal DNA (rDNA) sequences from modern isolates

with those from Triassic and Permian salt bed isolates do not show significant differences (97). Fredrickson and coworkers (96) concluded from rates of amino acid racemization and DNA breakdown that cells likely could not survive and that macromolecules would not remain from the Permian or Triassic Periods. Moreover, they were unsuccessful at cultivating halophiles or amplifying haloarchaeal 16S rDNA from 200-million-year-old salt crystals after disinfecting crystal surfaces and then dissolving the crystals; when the same techniques were applied to modern salt beds as a control, viable cultures and archaeal rDNA were produced. Vreeland and coworkers (94) reported cultivating a *Bacillus* isolate from the interior of a 250-million-year-old Permian primary halite crystal collected from the Salado Formation at 569 m depth in the Waste Isolation Pilot Project in New Mexico. The crystal is thought not to have undergone recrystallization since deposition, suggesting survival of an individual cell, presumably as an endospore, for 250 million years. This is an extraordinary finding that appears to contradict standard ideas regarding the long-term stability of macromolecules and the limits of microbial starvation survival. It may be that low water activity and/or protective proteins allow the persistence of microorganisms in halite. It remains to be seen whether more isolates will be recovered from such ancient halite. Fluid inclusions in other minerals, for example, silica, could also be sources of ancient microorganisms (98).

Bacteria in Frozen Environments

Permafrost soils in Arctic and Antarctic environments are another fascinating source of ancient bacteria that have persisted over a geologic timescale through limited reproduction or by surviving as individual cells for millions of years (see PERMAFROST, this Encyclopedia). Permafrost soils typically have extremely stable subzero temperatures, and so microbes in these habitats are not subjected to freeze-thaw stress (99). Microbial abundance in permafrost soils is often surprisingly high, 10^7 to 10^8 total cells/g dry weight, even in soils that have been buried for 3 million years and that are thought to have remained permanently frozen (100–102). The percentages of culturable cells can also be high, typically 0.1 to 10% in permafrost soils of Siberia (101,102). Fewer Antarctic permafrost microbes can be cultured: 0.001 to 0.01%, possibly because of the colder temperatures (−20 to −27 °C) (103). A diversity of microorganisms, most of them psychrophilic or psychrotolerant bacteria, has been isolated from buried permafrost soils, including aerobic heterotrophs, nitrifiers, denitrifiers, iron reducers, and sulfate reducers (43,102–104). Viability percentage and diversity generally decline with age of the permafrost (43). Even though the bulk of Siberian permafrost soil is permanently frozen at −10 to −15 °C, a significant proportion of the water (2 to 7%) exists in thin films of supercooled liquid (100), and this liquid water is thought to provide a protective habitat for microorganisms. Solidly frozen ice samples generally have few viable microbes, probably because of the irreversible damage caused by intracellular ice crystals (105). Thick cell walls, gelatinous capsules, and intracellular compatible solutes may also contribute to survival (105). The degree of metabolic activity in the permafrost bacteria is unknown. Certainly the temperature severely limits the rates, but the liquid water may provide nutrients at a rate sufficient to support occasional cell replications. The high percentages of culturable cells also suggest active metabolism. The deep cold biosphere deserves further study.

Survival of Potential Pathogens in Groundwater

Survival of pathogens in groundwater can be considered to be a special case in the context of this article because they are not naturally occurring in the subsurface and their survival in groundwater for even a short period is of interest from a public health standpoint. The topic of survival and transport of pathogenic microorganisms has been reviewed elsewhere (106–109), and so it is given only a cursory treatment here. Types of pathogens that can occur in groundwater include enteric bacteria (e.g., *Campylobacter jejuni*, *E. coli*, *Salmonella* spp., *Shigella* spp., and *Vibrio* spp.), protists (e.g., *Entamoeba hystolytica*, *Cryptosporidium parvum*), and viruses (e.g., hepatitis A, coxsackievirus, poliovirus). Sources of contamination can be treated or untreated wastewater that is introduced into the subsurface by infiltration, via leach fields from domestic septic systems, and by artificial recharge of groundwater (106,108,109). Animal wastes can also be a source of groundwater contamination (110). Factors controlling survival of pathogens in groundwater are the physical and chemical characteristics of an aquifer, the presence of competing or predatory microorganisms, and the characteristics of the pathogen itself. Survival is generally prolonged by lower temperatures. High concentrations of dissolved organic carbon in groundwater may enhance survival. Survival is generally longer in sterile groundwater than in the presence of natural groundwater communities (111,112). Most enteric pathogens are poorly adapted for growth and survival outside human and animal gastrointestinal tracts, and so they generally survive for only a few days to a few months in groundwater environments (109). Enteric bacterial pathogens in groundwater are frequently the same species as (or close relatives of) many of the ones that have been tested in laboratory incubation studies, and thus many of their physiological characteristics, including adaptations for starvation survival, are relatively well understood. Many have already been shown to enter a viable but nonculturable state when incubated in surface waters, including *C. jejuni*, *E. coli*, *Salmonella* spp., *Shigella*, spp., and *Vibrio* spp. (113,114), and the same is undoubtedly true when they occur in groundwater aquifers. Viruses may remain infective in the subsurface for longer than pathogenic bacteria, especially when they are protected from inactivation by sorption to aquifer solids.

CONCLUSION

There is now a substantial body of evidence that microorganisms are able to persist in the subsurface under energetically challenging conditions for thousands to millions of years, and that many of them do so as vegetative

cells lacking any obvious features favoring survival. How they do it is still largely unknown. An open question is whether subsurface microorganisms have evolved unique adaptations for long-term survival, whether selective pressures in the subsurface have generated unusually effective versions of starvation survival adaptations that also occur in surface microbes, or whether subsurface microorganisms have exactly the same starvation survival characteristics as their surface counterparts. Of course, each of these possibilities may be embodied in various subsurface microorganisms. This is related to the larger question of whether subsurface microbial populations and communities are unique to the subsurface or whether they can also be found in surface habitats. Amy (19) made a case for the uniqueness of subsurface microbial communities, based on their patterns of substrate utilization. At least some microbes in the subsurface are slowly metabolizing and slowly reproducing, as evidenced by changes in groundwater chemistry along a flow path, and thus they undergo natural selection in subsurface habitats. Given sufficient time, they should evince this evolutionary separation in their phenotypes (e.g., in adaptations to nutrient deprivation) and in their genotypes (e.g., in the sequences of "molecular clocks" such as 16S rDNA). However, 16S rDNA sequences may not change rapidly enough to demonstrate separation of subsurface microorganisms from surface populations. Also, gene flow between the surface and subsurface habitats is not permanently stanched; microbes enter and leave the subsurface via groundwater flow, tectonic activity, and so on (115). Thus, more information is still needed to determine the extent to which subsurface microorganisms are adapted specifically to life in the subsurface, including unique adaptations for long-term survival. Related to this, we need a better understanding of the in situ physiological status of microbes in deep subsurface environments, including any molecular structural features that may favor extreme long-term stability of DNA and other macromolecules. More data on the environment will also help. Continuing geochemical characterization of subsurface habitats is expanding our understanding of the energy sources that are available to support long-term survival and activities of subsurface microorganisms.

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MICROBIAL TOXICITY TESTS.

See TOXICITY TESTING IN SOIL, USE OF MICROBIAL AND ENZYMATIC TESTS

MICROBIOLOGY OF ATLANTIC COASTAL PLAIN AQUIFERS AND OTHER UNCONSOLIDATED SUBSURFACE SEDIMENTS

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Prior to 1970, microorganisms were thought to be an insignificant biotic component below the root zone. However, the past quarter century has seen considerable research focused on the characterization of subsurface microorganisms in sediments and groundwater using a variety of microbiological and biochemical measurements. Research has been conducted to gain a better understanding of the types of microbial communities existing in the subsurface, their abundance, their activities, and their potential for in situ biostimulation or ex situ biotechnological applications. These investigations will continue to provide information with respect to use of the subsurface for acquiring drinking water and for waste disposal practices.

This article will focus primarily on sites along the Atlantic Coastal Plain (ACP) where the microbiology of the sediments has been investigated extensively. Descriptions of methodologies used to characterize microbial abundance, spatial and temporal variability, diversity, transport of microbial populations through the subsurface, and the metabolic and degradative capabilities of microbial communities will be presented.

SITES

The majority of sites discussed in this article are located along the United States Atlantic coast: Savannah River Site (SRS), South Carolina; Dover Air Force Base (AFB), Delaware; Cape Cod, Massachusetts; and Oyster, Abbott's Pit, and Taylorsville Basin, Virginia (Fig. 1). These sites share common features of interlaid sands, silts, and clay lenses. Other sites also representative of unconsolidated sediments are included in this article though they are not located on the ACP.

In 1986, a series of boreholes were drilled as part of the Department of Energy's (DOE) interdisciplinary Subsurface Science Program for long-term microbiological and geochemical studies. Three boreholes were drilled to a depth of 200–300 m below land surface (bls) at DOE's SRS in 1986 (1), and a fourth borehole near Allendale, South Carolina, was drilled to approximately 437 m in 1988. The subsurface geology in this region consists of about 400 m of unconsolidated sands, clayey sands, and sandy clays of Tertiary and Cretaceous ages, which overlie a basement of dense metamorphic rock, igneous intrusives, or consolidated sedimentary rock. A more detailed account of the geochemistry described below can be found in reference 2. Sediments at depths greater than 30 m bls were saturated. Major confining clays of regional extent typically contained greater than 20% clay and had low hydraulic conductivity ($k < 0.1$ mm/sec) and low permeability ($k(\mathrm{D}) < 0.1$) and were found within the Ellenton, Pee Dee, and Middendorf formations. Nonconfining layers contained mostly sands with hydraulic conductivity ($k > 100$ mm/sec) and higher permeabilities ($k(\mathrm{D}) > 10$). Sediments from the confining layers exhibited higher total organic carbon (TOC) concentrations (2 to 3.5 mg/kg) than did highly permeable aquifer sediments (0.1 to 0.6 mg TOC/kg sediment). Nitrogen (nitrate and/or ammonia), sulfate, iron, and sodium concentrations were typically greater than 10 mg/L in the pore water. Phosphate concentrations were less than 10 mg/L in pore water extracted from sandy sediments or in pumped groundwater. The dissolved oxygen content of the groundwater was generally between 0 and 5 mg/L. Redox potentials were greater than 250 mV for shallow sediments, whereas deeper and downgradient sediments exhibited lower redox potentials, indicating anaerobic environments. For example, the Cape Fear formation at 463 m bls had a redox potential of 150 mV.

The Cape Cod site is the United States Geological Survey's Toxic Substance Hydrology Program Research Site. Treated sewage effluent from Otis AFB has been

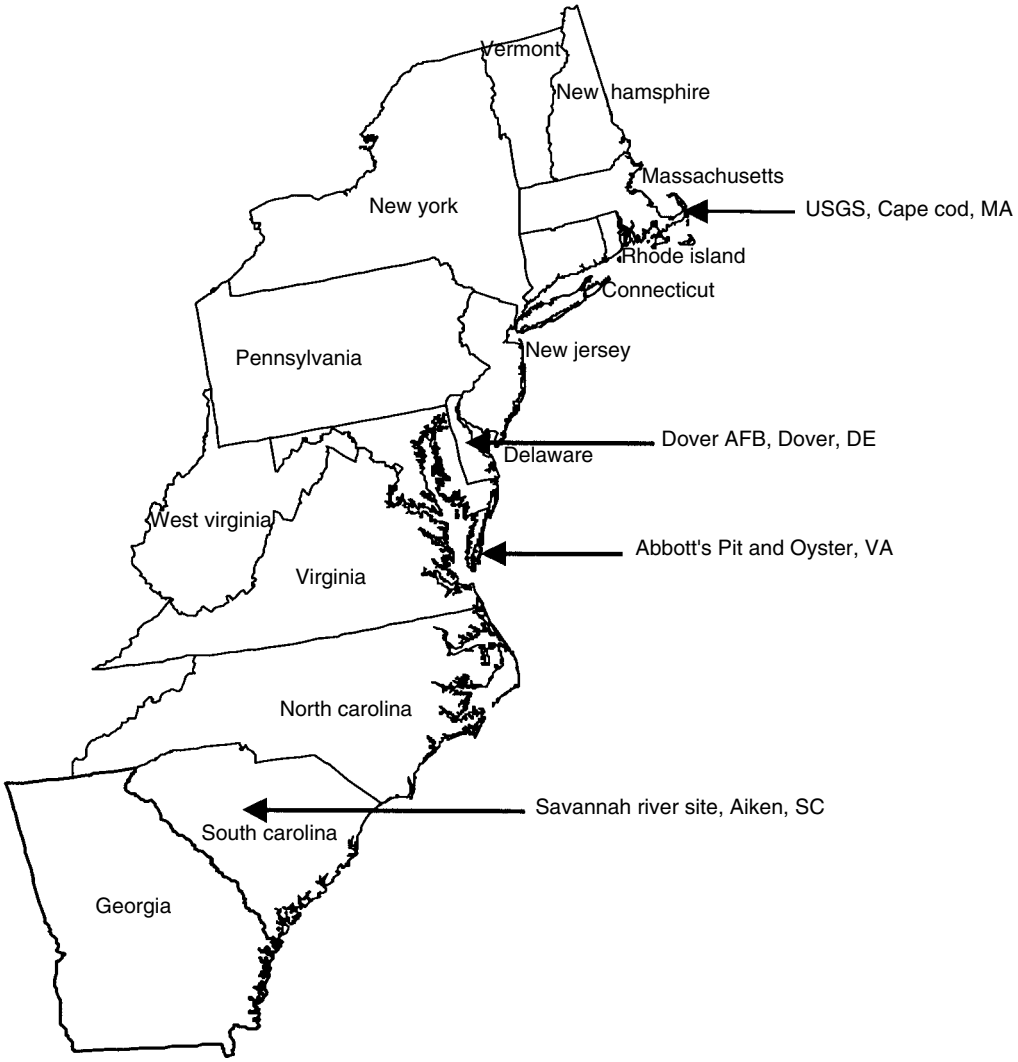

Figure 1. Map of Atlantic coastal states with the location of primary field sites. Additional work is described from other sites within and outside of this region.

disposed onto rapid-infiltration sand, resulting in a 5-km-long plume of organic contamination within the shallow unconfined sand and gravel aquifer (3). The groundwater flows southward toward Nantucket Sound. The plume is characterized by elevated temperature, specific conductivity, and dissolved organic carbon (up to 18 °C, 450 msec/cm, 4 mg/L) relative to uncontaminated zones of the aquifer (10 °C, <80 msec/cm, <1 mg/L) (4). Hydraulic conductivity is ~ 0.1 cm/s (5). Average porosity and mean grain size are 0.35 and ~ 0.59 mm, respectively (6).

The Delmarva Peninsula is the location for the Oyster and Abbott's Pit, Virginia, and Dover, Delaware, sites. The sediment at these sites (Oyster, Abbott's Pit, and Dover AFB) is predominantly shallow marine shoreface sediments deposited in the subtidal zone. The surficial aquifer is 24 to 30 m thick and the water table ranges in depth from 1 to 6 m bls. The pristine sediments at Abbott's Pit consist of medium- to fine-grained sands. The water table is typically at 3.75 m bls with fluctuations of a meter (7,8). Oyster has a shallow water table (1.5 to 2.2 m bls) and a narrow variation in grain size (0.12 to 0.25 mm) below the soil zone. Some sites at Dover AFB are contaminated with low levels of chloroethenes and petroleum hydrocarbons (9,10). The sediment texture at Dover AFB is more varied than at Abbott's Pit and ranges from coarse sand to clay. The water table varied from 3 m bls in the southeast portion of the base to 6 m bls in the northwest portion of the base (7,8).

The sites at Oyster are fields currently operated for agricultural operations and receive periodic fertilization (ammonia, phosphate). Ammonia is quickly converted to nitrate and has been detected in the groundwater. Phosphate has not been detected in the groundwater. At Oyster there are two distinct zones: an aerobic zone and an anaerobic zone (11). The aerobic zone has dissolved oxygen at greater than 1 mg/L, which is consistent with the regional groundwater. The anaerobic zone is located near an area of buried vegetable waste and the dissolved oxygen is less than 1 mg/L. Differences were noted in the geochemistry between the aerobic and anaerobic sites. The aerobic zone exhibited concentrations of 40 to 50 mg/L nitrate, less than 0.01 mg/L dissolved iron,

undetectable amounts of ammonia, and 18 to 40 mg/L carbonate (alkalinity). Conversely, the anaerobic zone exhibited concentrations of 0 to 1 mg/L nitrate, 10 to 40 mg/L dissolved iron, 0.4 to 2.4 mg/L ammonia, and 150 to 380 mg/L carbonate.

Dover AFB sediments generally exhibited concentrations of 5 to 15 mg/kg phosphorus, 1 mg/kg nitrate, 6 to 20 mg/kg sulfur, 13 to 31 mg/kg iron, and combustible organic matter of less than 0.1% (K. Deweerd, personal communication). Shallow groundwater wells typically showed dissolved oxygen at greater than 1 mg/L, dissolved iron at 0 to 2 mg/L, sulfate at 20 to 50 mg/L, and sporadic presence of both aliphatic and chlorinated hydrocarbons and methane. Deeper groundwater wells typically showed dissolved iron at 0 to 3 mg/L and sulfate at less than 10 mg/L, sporadic detection of aliphatic hydrocarbons and methane, and consistent detection of chlorinated hydrocarbons. Dissolved oxygen varied from greater than 1 mg/L in regions of little or no contamination to less than 1 mg/L in regions of higher contamination. In both the shallow and deep groundwaters, ammonia and phosphate concentrations were generally below detection limits (12).

SAMPLING THE SUBSURFACE

Sampling of subsurface sediments, rocks, and groundwaters for microbiological and geochemical analyses requires specialized technologies and equipment for sample collection. Different lithologies of sediments may also require different approaches (13, the author wants the article by W. T. Griffin in this Encyclopedia to be cited here). For example, methods appropriate for collecting cores from consolidated sediments may not retain samples of unconsolidated sediments such as "flowing" sands. When core sampling is prohibitive (i.e., costly with depth) then it may be necessary to rely on groundwater samples. Although the composition of the microbial community and activity of the groundwater samples may be vastly different from their host sediment, groundwater sampling is commonly used for routine monitoring, such as in situ bioremediation (14,15). An effect of groundwater sampling is smearing of local heterogeneities across a sediment column, thus representing a larger picture than would a sample from a discrete sediment interval. On the other hand, discriminating trends over time may be difficult to observe in less-frequent sediment coring regiments. Consequently, groundwater monitoring may prove advantageous over temporal and regional scales. In addition, the development of multilevel sampling provides a means to sample discrete groundwater intervals.

An overwhelming concern when examining subsurface environments is chemical and biological contamination of samples either by extraneous sediments or by the smearing of circulating drilling fluids during the coring, retrieval, and processing stages. Because of this it is important to employ tracers to detect and quantify the extent of bacterial and drilling fluid contamination. A variety of microbiological, chemical, and physical tracers have been used to evaluate biological or chemical contamination of subsurface samples (16, insert ref to an article in this Encyclopedia by W. T. Griffin here)

Another major concern and source of contamination occurs during sample processing, transport, and storage prior to sample analysis. Sample processing is typically conducted under an inert atmosphere minimizing biogeochemical alternations (17). Samples are generally stored under inert conditions and cooled during transport with all analyses initiated as soon as possible after core retrieval or within 72 hours. By understanding the limitations and shortcomings of subsurface exploration technologies and by employing rigorous procedures for sample recovery, QA/QC, and processing, representative subsurface samples of defensible quality can be obtained.

METHODS FOR COMMUNITY ANALYSIS

Quantification of microbial populations may be assessed by direct microscopy techniques. Acridine orange direct counts (AODC) provide a total estimate of microbial abundance, whereas INT [2-(*r*-iodophenyl)-3-(*r*-nitrophenyl)-5-phenyl tetrazolium chloride], LIVE/DEAD *Bac* Light, and nalidixic acid direct counts provide an estimate of the viable cell count (18). Fluorescent antibody direct counts provide an estimate of specific populations (e.g., nitrogen-transforming bacteria). Microbial assessment usually includes classical methods for the enrichment and isolation of bacterial strains. The most commonly used methods include counts of colony forming units (CFU) on solid media (plate counts) and determination of the most probable number (MPN) using replicate dilution series, which may employ selective and/or differential media to grow and enumerate bacteria, fungi, and protozoa (19–22). Whereas it is accepted that these cultivation methods underestimate in situ populations by one to three orders of magnitude, these methods can provide useful indications of the predominance or significance of specific microbial populations. The inability to recover specific populations in culture does not preclude their presence. Likewise, the presence of populations representing a high percentage of the total culturable microorganisms is not evidence of relative importance. For example, the presence of greater than 105 cell/g of obligate anaerobes (sulfate reducers, methanogens, and acetogens) in a sediment sample in which 107 aerobic heterotrophs/g were detected provides evidence that anaerobic processes may well be important.

It is well established that cultivation of microorganisms represents only 0.1 to 10% of the total microbial community. Methods for determining microbial biomass and community structure, which do not require cultivation, include phospholipid fatty acid (PLFA) and nucleic acid techniques that quantify cellular macromolecules (e.g., lipids or DNA). As these markers are typically decomposed soon after cell death they can be used to estimate viable microbial biomass and diversity (23). In the case of PLFAs, the phosphate is cleaved from the phospholipid molecule upon cell death producing diglyceride fatty acids (DGFA) (24). Therefore, the PLFA provides an estimate of recently viable cells, whereas the DGFA provides a measure of dead cellular residue. PLFA also provides an assessment of the nutritional and physiological status of the microbial community. Whereas both PLFA and nucleic acid techniques can estimate overall community

structure and detect changes and shifts in community composition, nucleic acids can target individual members of the community or a specific gene of interest.

Metabolic or respiratory capacities may be assessed to determine microbial community activity by analyzing the utilization and formation of metabolic intermediates (e.g., the conversion of nitrate to nitrite during denitrification). For example, rainwater entering the subsurface contains significant dissolved oxygen. The removal of dissolved oxygen from groundwater indicates altered physiological parameters and biogeochemical activity. Metabolic pathways are often assayed using radiolabeled electron donors or acceptors. One method of assessing the metabolic activity would be to supply ^{14}C-acetate as the carbon source and analyze for the respiratory end products of ^{14}C-carbon dioxide, ^{14}C-methane, or for the incorporation of ^{14}C-acetate into cellular components (e.g., total lipids) (25–27, Kicks and Fredrickson, 1989). Another method uses the Biolog^{TM} system for screening 95 different substrates simultaneously in a multiwell system containing an indicator dye that changes color upon utilization of the substrate. This method provides a community-level physiology profile (CLPP) (28,29). These metabolic methods can also be applied for the detection of contaminant degradation capabilities (e.g., BTEX, trichloroethylene [TCE]) (15,26,30).

These microbial assessment methods are based on laboratory experiments using retrieved sediment or groundwater samples. It is important to note that these retrieved samples are subjected to the redistribution of nutrients during sample collection and sample processing and the stimulation of microbial activity during sample storage and shipment (31–33). These disturbance artifacts may result in the overestimation of in situ microbial activities (2).

In addition to microbial assessment methods, various techniques are used to characterize sediment and groundwater chemistry (e.g., nitrate, pH, TOC, dissolved oxygen) for the purpose of determining electron donors, acceptors, and end products, type and age of sediment, age of groundwater, and groundwater flow regimes (34,35). Sediment physical parameters (e.g., particle size, water content, porosity) are investigated because they may impact the localized chemistry and microbial activity as well as constrain biogeochemical activity on regional scales.

Presence, Abundance, and Diversity of Microorganisms in Atlantic Coastal Plain Sediments

Direct and Viable Counts

At the SRS, the highest AODC values were observed in surface soils (10^8 cells/g) and decreased with depth to $\sim 10^6$ to 10^7 cells/g (22,36). Sandy aquifer formations had the highest AODC counts, whereas clayey interbed layers had the lowest counts (22). Viable counts (10^3 to 10^7 cells/g) were lower than the direct counts (36). Sandy sediment samples contained the highest viable counts (10^5 to 10^8 cells/g) as compared to the confining clayey layer ($<10^3$ cells/g) (19,22).

The highest viable counts were observed with dilute media; yet 10 to 50% of the bacteria could grow rapidly on nutrient-rich media, suggesting high metabolic flexibility. In the shallow sediments at Oyster, Virginia, the viable counts at the surface were 10^6 to 10^7 cells/g and declined to 10^4 cells/g at 1 m to less than 10 cells/g below 6 m depth (7). Similar results were seen at Long Island, New York, where 10^6 to 10^7 cells/g AODC counts were demonstrated for both shallow (1.5 to 5 m) and deep (10 to 18 m) sediments (27). Microbial communities of sediments from various sites at Dover AFB were estimated using MPN, PLFA (37), and DNA methods (10). Biomass estimates for subsurface sediments were 10^3 to 10^7 cells/g by MPN, 10^6 to 10^7 cells/g by PLFA, and 10^7 cells/g by 16S DNA methods, whereas surface sediments exhibited 10^7 to 10^8 cells/g for all methods. In shallower vadose zone sediments (at depths of 1.7 to 2.0 m) at Dover AFB, the viable counts were 10^2 to 10^5 cells/g (38,39). When groundwater and adjacent sediment were investigated, the groundwater samples generally exhibited direct and viable counts that were one to three orders of magnitude less than the sediment samples (36). Furthermore, the difference between the viable and direct counts for sediments or groundwater was two to three orders of magnitude.

Diversity may be judged based on colony morphology. Balkwill (19) showed that typically 11 to 62 distinct colony types were seen on enumeration plates for aerobic facultative heterotrophs. Furthermore, the diversity did not decrease with depth. However, the diversity varied extensively between geological formations. Similar results were demonstrated by Shanker and coworkers (40). Diversity based upon colony morphology was dependent on the degree of nutrient added to the medium. Subsurface samples were observed to have higher viable counts on diluted nutrient medium, yet colony morphology was difficult to distinguish. On nutrient-rich medium, colony morphology, colony size, shape, and pigmentation provided an estimate of greater diversity than the dilute nutrient medium (19,41). In Figure 2, the advantages of both nutrient-rich and diluted media for estimating viable counts and diversity are demonstrated. Species richness was also highest with nutrient-rich medium (41).

MPN and Enrichments

Several other microbial trophic groups besides the abundant aerobic heterotrophic communities exist in the ACP sediments, making for physiologically diverse microbial communities. In the same sediment sample, it is not unusual to detect aerobic heterotrophs, facultative heterotrophs, and obligate anaerobes, likely reflecting localized niches (21,31,42).

Methylotrophs (C-1 compound utilizers) have been observed in low populations (10^2 cells/g or less) for both SRS and Dover AFB sediments (15,37). These microorganisms were of particular interest for their potential to degrade TCE (43). At both SRS and Dover AFB, cometabolic bioremediation demonstrations utilized the addition of methane to stimulate methylotrophic populations to increase up to five orders of magnitude (15,38,39,44).

Nitrifiers, sulfur-oxidizers, and hydrogen-oxidizers were cultured from ACP sediments in South Carolina (20). Denitrifiers were detected by Francis and coworkers (42)

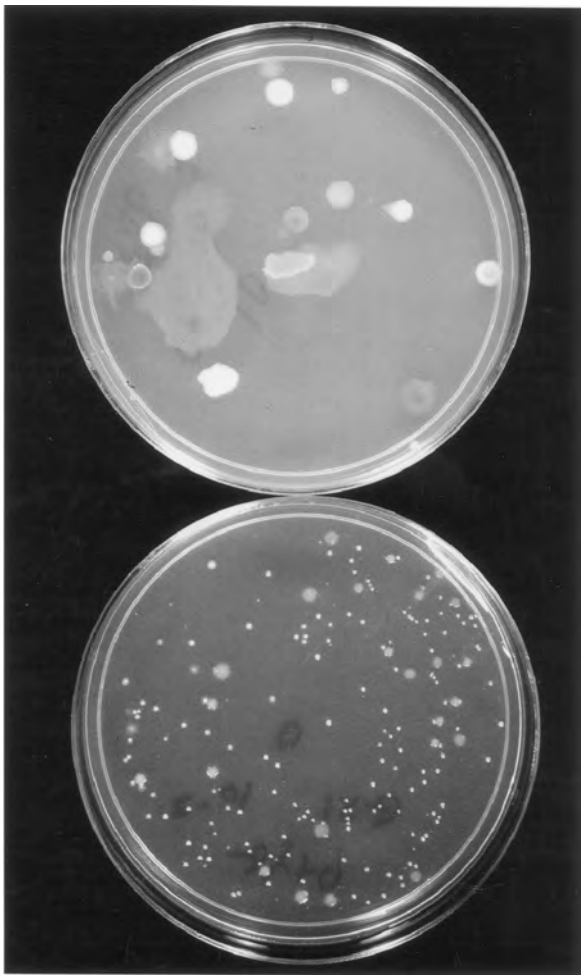

Figure 2. Colony-forming units on a dilute medium (left plate) and a nutrient-rich medium (right plate) for an SRS sandy sediment. For the same plating dilution of the sample, the dilute medium provides higher total numbers and the nutrient-rich medium provides more diversity.

and iron reducers were isolated by Lovley and coworkers (45). Based on geochemical data, the potential for iron reduction coupled to organic matter oxidation in ACP sediments was observed by Murphy and coworkers (35). Furthermore, Murphy and coworkers demonstrated that ferric oxide coatings were found in the sediments in which iron reduction was indicated by microbial enrichments. Sulfate-reducing bacteria were found in sediments having low redox potential and a sufficient concentration of sulfate. These bacteria were found in deep aquifers (21,34), in the distal portion of aquifer flow paths (35), and in subsurface areas of high organic matter concentrations, for example, organic contaminants, such as BTEX (46,47), and organic carbon deposits, such as lignite (35).

Other forms of naturally occurring organic carbon, such as simple organic acids, have been found in the ACP sediments. Jones and coworkers (21) detected short-chain organic acid accumulations (e.g., acetate) in microcosms derived from South Carolina coastal plain sediments, and Murphy and coworkers (35) detected acetic, formic, and oxalic acid near lignite particles within the Middendorf aquifer in South Carolina. It is the presence of these fermentation products (hydrogen, organic acids) that supports the anaerobic microbial activities found in ACP sediments. Furthermore the abilities of these resident microorganisms to produce carbon dioxide, change nitrogen, sulfur, and iron species, excrete volatile fatty acids, generate methane, and degrade chlorinated ethenes, petroleum, and polyaromatic hydrocarbons have impacts on water quality.

Eukaryotic microorganisms (protozoa, algae, lower fungi) may be important in groundwater aquifer communities, given their potential impact as heterotrophic feeders. Amoebae and flagellates were observed at concentrations of generally 10^1 to 10^2 cells/g in shallow SRS aquifer samples containing high bacterial viable counts, though ciliates were not detected. In addition, a variety of green algae, phytoflagellates, diatoms, and a few cyanobacteria were found. Fungi were widespread across the 30-m depth in SRS borehole profiles and usually ranged from 1 to 50 propagules/g (22). In pristine aquifers at the Cape Cod site, these eukaryotic microorganisms are usually present at concentrations of less than 10^2 cells/g dry weight aquifer material. The aquifer is dominated by small flagellates but amoebae and ciliates are occasionally present (48). In organically polluted aquifers at the Cape Cod site, where bacterial abundance was increased because of the pollution, the protist concentrations increased several orders of magnitude (3).

Microorganisms with unexpected or unique properties have been isolated from ACP sediments. Subsurface microorganisms have been shown to exhibit increased resistance to UV radiation (49) and tolerance to higher levels of hydrogen peroxide (50). Some microorganisms isolated from great depths (2,500 to 3,000 m) can grow at elevated temperatures (50 to 60 °C), for example, *Bacillus infernus* (51), and reduce iron under thermophilic conditions (52).

Microbial Community Profiles

Microbial community structure and metabolic potential have been investigated using the broad spectrum method of the API or Biolog™ technology. The latter method allows for high number of substrate utilization tests at the same time on one sediment sample. Substrate compounds have been expanded to test recalcitrant or toxic chemicals. Upon oxidation of the substrate by the microorganisms in the sample, the tetrazolium dye is reduced, producing a detectable color response. Aerobic chemoheterotrophs isolated from ACP sediments exhibited diverse metabolic capabilities where all but two of the API carbon sources were utilized by 40% of the isolates and three of the carbon sources were utilized by 75% of the isolates (53). Surface versus subsurface isolates exhibited different utilization responses.

Common names for this method when applied to the mixed communities of environmental samples are CLPP (28) and ecofunctional enzymes (29). ACP sediments showed a preference to utilize amino acids over carbohydrates and selected individual compounds, such as acetate as sole carbon sources (7,28,29). At the

Oyster, Virginia site, the aerobic utilization of carbon sources decreased significantly with depth. The surface samples exhibited the largest values for substrate utilization and the next highest values were seen at sample depths that coincided with the water table (7). Fliermans and coworkers (29) used the ecofunctional enzymes to distinguish the physiological patterns of the various microbial communities during groundwater remediation of TCE at SRS. Differences in function of microbial communities were observed among the bioventing treatments of 1 and 4% methane injections, pulsed injections of air, methane, and nutrients. The microbial communities in the groundwater changed their activity against certain substrates during the changes in remediation process. Community differences were also seen between and among the sampling wells and the demonstration site. With Taylorsville Basin samples, the CLPP community profiles were used to distinguish among surface soils, aquifer waters, drilling muds, and sediment cores. Similar results for distinguishing phenotypic changes in microbial communities between drilling operations and cores were obtained by PLFA analysis (28). Make-up waters showed PLFA patterns typical of gram-negative bacteria. The drilling mud had a combination of monounsaturated and terminally branched saturated fatty acids. The types and percentages of the terminally branched saturated fatty acids have been shown to be indicative of gram-negative obligate anaerobes, for example, sulfate-reducing bacteria. In addition, the PLFA results provided estimates for microbial biomass values, which ranged from 124 pmol PLFA/g in drilling muds to 7 to 13 pmol PLFA/g for outer and inner parings of the cores.

Metabolic and Degradative Activities

Potential rates of microbial activity are measured in laboratory microcosms. Sediment or groundwater material is placed in a closed container (with or without additional substrate amendments) and incubated under controlled conditions; disappearance of reactant and/or appearance of a product is measured. Typically, sterile or poisoned controls are used to distinguish between biotic and abiotic processes. The types of metabolic or degradative activity examined may include production of carbon dioxide (Kieft et al., 1991, 54,55), incorporation of acetate into lipids (56), thymidine incorporation into DNA (56), reduction of sulfate (40), formation of methane (21), or disappearance of the recalcitrant or toxic compounds (26). Denitrification activity has been quantified in ACP sediments using the acetylene-blocking technique, analyzing the production of N_2O, and quantifying nitrate disappearance (42). Madson and Bollag (57) measured the disappearance of glucose and indole under both aerobic and anaerobic conditions. Under anaerobic conditions, the disappearance of lactate, formate, acetate, phenol, and benzoate was measured, and potential rates of acetogenesis and methanogenesis were determined (21). These other activities of microorganisms in the subsurface, including denitrification, sulfate reduction, and methanogenesis, are covered in greater detail by Kieft and Phelps (58).

The use of radiotracers is potentially useful for measuring microbial activity, thanks to the high degree of specificity and sensitivity. By labeling a particular compound, with ^{14}C, ^{3}H, or ^{35}S, one can measure the rate of transformation of that substrate as well as the rate of the end product formation. As compared with aquatic or terrestrial environments, radiotracer experiments for subsurface environments may take incubation times of hours rather than minutes. It is important to note that the rates measured by these methods should be considered potential rates and not actually in situ rates. Thus, they are useful for comparing relative activities among subsurface samples. The potential for ACP subsurface microorganisms to degrade a diverse array of compounds has been demonstrated through microcosm studies testing a variety of ^{14}C-labeled organic compounds for degradation or transformation. Most CO_2 production assays have utilized either ^{14}C-glucose or ^{14}C-acetate and analyze for $^{14}CO_2$ (27,54,57,59,60).

Aerobic CO_2 production rates for ACP sediments were estimated at 80,50 to 70, and 0.6 to 4.9 nmol/g/day for surface, sandy aquifer, and clayey sediments, respectively (40). Anaerobic CO_2 production rates (mmol CO_2 per liter per year) in ACP sediments were estimated at 2.4×10^{-1}to9.0×10^{-3} for the shallower Black Creek and Middendorf Aquifers and at less than 10^{-5} for the Cape Fear aquifer (61). However, geochemical estimates of these in situ activities indicate metabolic rates of 10^{-4} to 10^{-6} mmol CO_2/L/year (2,61). Thus, the radiotracer laboratory microcosm studies overestimated microbial in situ activity by a factor 10^3 to 10^6. Alternatively, the differences in these microcosm studies may indicate that water, nutrient availability, and redox condition may have a dominant effect on the microbial activities. For example, CO_2 production rates in a jet fuel–contaminated aquifer were stimulated with the addition of nitrate (26); without the nitrate addition the production rate was negligible. Some of the more recalcitrant or toxic compounds tested include chlorobenzene, *r*-chlorophenol, BTEX, and trichlorobenzene (15,25,26,43,55).

Physical and Chemical Factors Limiting Microbial Abundance and Activities

Variability in measured microbial activities within many subsurface samples is, in part, caused by differences in water availability, hydrologic flow, sediment structure, and the associated biomass. In ACP sediments, population density had a moderate positive correlation (0.4 to 0.6) with sand content and a moderate negative correlation with clay content (22). This was also supported by Phelps and coworkers (17,31) who showed the effects of particle size and moisture content on the abundance of aerobic heterotrophs and on microbial activity, as measured by acetate incorporation into lipids. The trend is: at less than 20% clay content, lower abundance and lower activity were observed than with sandy samples. Samples containing greater than 70% sands had similar microbial abundances, but activities were much lower in samples that were at less than 70% saturation. In general, sandy samples had lower activity responses under conditions of low water content, low permeability, and low hydraulic conductivity as compared to the sands with higher values for these three parameters (17,31). Thus, these parameters of silts and

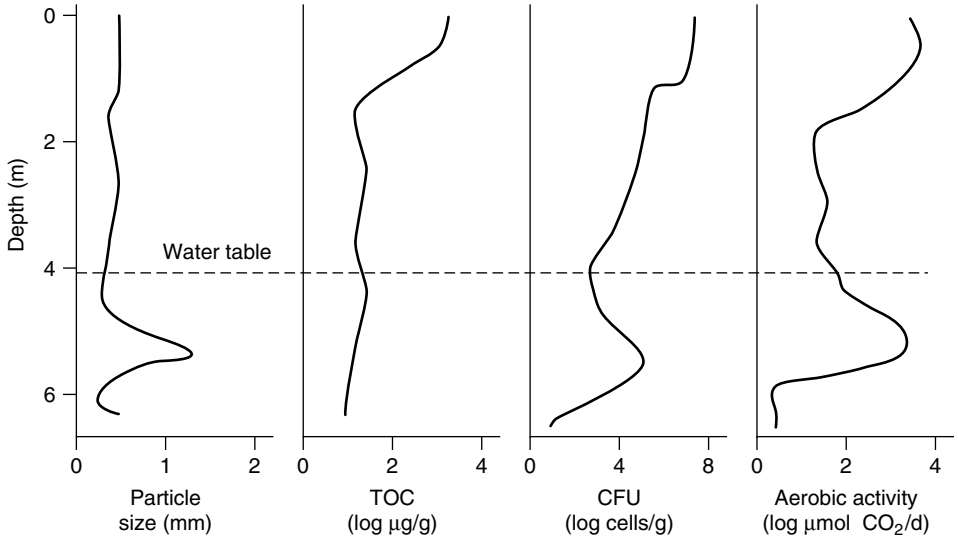

Figure 3. Schematic representation of changes in physical, chemical, and microbial parameters with depth.

clays may limit the flux of water and the flow of aqueous nutrients. Figure 3 shows a schematic representation of the changes in microbial abundance and activity to the changes in particle size and total organic carbon of ACP sediments across a vertical profile. Near the surface organic carbon, microbial abundance and activity are elevated. These parameters decrease with depth until the depth profile intercepts the water table and a responding increase in microbial activity is observed. A second change is shown when a zone of sediment containing larger particle size is met, which may allow for better flux of nutrients through the sediment. The subsequent microbial changes were observed as increases in both abundance and activity measures.

Nutrients may be limiting in the subsurface environment and this poses challenges for bioremediation of contaminated sites. In laboratory microcosm studies, the nutrients are mixed and redistributed, making them available to resident microorganisms. Similarly, in a field demonstration for TCE bioremediation at the SRS, vacuum extraction or air sparging caused a stimulation of subsurface microbial populations and activities (14,15). It was hypothesized that air sparging at 200 scfm or vacuum extraction would redistribute nutrients and/or toxicants, resulting in the increase of microbial populations and TCE degradation. However the total microbial community abundance did not increase (15,38,39).

Laboratory microcosms studies investigated potential limiting nutrients. Previous research on nutrient-limiting conditions in ACP sediments investigated combinations of nutrients under aerobic and anaerobic conditions. The effects of N (sodium nitrate), P (sodium phosphate), S (sodium sulfate), C (glucose), trace minerals, and water on the incorporation of ^{3}H-acetate into membrane cellular lipids of ACP sediments showed that each addition caused stimulation in at least some samples (31). Based on the degree of stimulation, nitrogen and phosphorus were found to be limiting for the ACP sediments. For the SRS bioremediation demonstration, ammonia, nitrous oxide, orthophosphate and triethylphosphate (TEP) were tested for potential field application by using microcosms containing site groundwater. Based on the greater stimulatory responses of TEP and nitrous oxide in the microcosm studies, they were chosen as supplements for the field operation (62). Furthermore, they had an additional advantage of being readily dispensed as a gas.

Investigators at the WSRS Integrated Demonstration of TCE bioremediation (14,15,38,39) decided to inject gaseous nitrous oxide and TEP along with the addition of methane with the intent of stimulating subsurface microbial activities, including the biodegradation for TCE. Within six weeks, TCE degrader populations increased and they were evidenced in greater numbers upon enrichment. Modeling, toxicant concentrations in groundwater, and chloride chemistry substantiated the observation that nitrous oxide and TEP stimulated subsurface microbial populations resulting in increased toxicant degradation. The results indicated that less than 0.1% of added TEP was likely incorporated into biomass and that the stimulated microbial activities were still less than micromolar levels per year, with less than 1% of total in situ microbial activity directed toward the cometabolic degradation of TCE and related chlorinated toxicants. Importantly, the laboratory and field experiments developed fundamental conceptual tools for assessing, predicting, and visualizing subsurface microbial activities that were capable of being stimulated and directed toward toxicant bioremediation (14,38,39). Furthermore, the observation from this applied bioremediation project was consistent with the conceptual understanding of subsurface microbial ecology of the Atlantic coastal plains.

Presence Abundance and Diversity Detected by Molecular Methods

Nucleic acid methods are only now coming into use for characterization of microbial communities and have had limited application in ACP sediments (10,63). In the past, specific microorganisms and populations have been monitored using techniques that rely on selective enumeration media and fluorescent antibody labeling.

These methods have been applied at sites on the Delmarva Peninsula (64). However, such methods are very limited in their application to the characterization of natural microbial communities because of the inability to cultivate the vast majority of microorganisms on standard microbiological media. The application of nucleic acid hybridization techniques to environmental microbiology has provided a new opportunity to investigate and quantify overall microbial community structure, detect changes and shifts in population composition, as well as target specific microbial groups and individual members of the community (e.g., 16S ribosomal RNA [rRNA]). Moreover, the use of labeled DNA probes, specific for a gene(s) of interest (e.g., genes responsible for degradative ability), can be used to characterize and monitor microbial activity.

Microbial Community Composition and Diversity

In ACP sediments, the primary published community information comes from comparative techniques such as denaturing gradient gel electrophoresis (DGGE) and randomly amplified polymorphic DNA (RAPD) techniques to analyze 16S ribosomal DNA (rDNA). In these techniques, nucleic acids (DNA and RNA) are extracted from environmental (sediment) samples, either by mechanical separation of microorganisms from soils (i.e., cell fractionation; 65,66) or through direct isolation of DNA (67). Differences in chemical and physical characteristics of sediments may require optimization of these procedures (68). To date, the majority of molecular studies fingerprinting microbial community structure and diversity in sediments and groundwater have used 16S rRNA as the molecular marker. The 16S rDNA fragments, obtained by reverse transcription of rRNA (69) or enzymatic (polymerase chain reaction [PCR]) amplification of DNA (69), are subsequently cloned and can be sequenced or subjected to restriction endonuclease digestions to generate restriction fragment length polymorphism (RFLP) patterns of microbial diversity. DGGE separates PCR-amplified 16S rDNA fragments according to sequence differences (reviewed in 70). The number of DGGE bands is presumed to correspond to the number of predominant members in the microbial community and this technique can detect shifts in community structure. DGGE bands can be hybridized with specific probes and excised to obtain DNA sequence information. Another PCR-based diagnostic method, RAPD, uses short oligonucleotide primers of an arbitrary sequence to fingerprint microbial community diversity (11).

Interpretable patterns have been obtained using the DGGE and RAPD techniques on ACP samples. DGGE has been used to monitor shifts in soil microbial community structure during natural attenuation and enhanced bioremediation during a controlled oil spill in Delaware plain sediment (71). Sequence analyses of DGGE bands indicated that oil treatment promoted growth of the alpha *Proteobacteria* subgroup and members of the *Flexibacter-Cytophaga-Bacteroides* phylum. Franklin and coworkers (11) employed RAPD to determine aerobic and anaerobic community diversity from a shallow ACP aquifer. Although RAPD cannot provide information on specific microbial taxa, genetically distinct microbial consortia were detected in the aerobic and anaerobic aquifer regions, and spatial and temporal differences were observed that were correlated with changes in groundwater chemistry.

Zhou and coworkers (72) cloned and partially sequenced eubacterial rRNA genes to examine the composition of a tundra soil community. Whereas no dominant RFLP patterns were detected, the majority of the clones belonging to the alpha and delta subdivisions of the *Proteobacteria* displayed considerable phylogenetic diversity. A similar molecular approach has been applied recently to characterize microbial community composition and diversity in low–carbon-saturated subsurface, surface, and vadose soils from the Delmarva Peninsula (73). Subsurface sediments from various sites in the ACP appear to have similar gross phylogenetic compositions (44,73,74, Zhou, personal communication). The *Proteobacteria* appeared to dominate both contaminated (Dover AFB) and uncontaminated subsurface sites (Abbott's Pit), representing over 80% of the clones. The gamma subdivision of the *Proteobacteria* represented the highest percentage of 16S clones examined. The alpha and beta subdivision of the *Proteobacteria* followed as the next most abundant group. However, the relative proportions of these varied among samples and sites. The next most prevalent, representing less than 10% of the clones, were the gram-positives, *Fibrobacter*, other groups, and unknown groups represented the remaining 10% of the clones.

A few specific types of bacteria appeared in the dominant groups at both the contaminated and uncontaminated sites. Many of the gamma subdivision 16S sequences were closely related to *Pseudomonas* and typically the closest matches were to species such as *stuzeri*, *mendocina*, and *putida*. There were also sequences related to *Acinetobacter*. In the alpha subdivision of the *Proteobacteria*, sequences closely related (up to 100% similarity) to strains of *Agrobacterium tumefaciens* consistently appeared, along with an occasional sequence related to *Sphingomonas*. There was a more diverse collection of beta subdivision-like sequences found that included *Rhodoferax*, *Azoarcus*, *Rastonia*, and *Burkholderia*. The most frequent gram-positive–like sequences (up to 95.5% similarity) were to related *Bacillus* spp.

Comparisons of the ACP results to those using similar approaches in cloning and sequencing of 16S rRNA indicates interesting patterns that may be related to water content and redox. Additional sampling at many more sites is needed before generalities can be made with confidence. However, it appears that the ACP communities and those from an arid paleosol differ to a much greater degree than do communities from spatially distinct areas of the ACP (e.g., Abbott's Pit and Dover AFB). Three of the most commonly occurring sequences (*Pseudomonas*, *Bacillus*, and *Burkholderia*) found from a study of deep (188 m bls) paleosols at an arid site (75) were also found in the ACP sites. However, many of the common clones from the arid site, including *Micrococcus*, *Clavibacter*, *Nocardioides*, *Comanonas*, and *Erythomicrobium* were not readily detected in the ACP sites examined (44,73,74, Zhou, personal communication). Interestingly, sequences recovered from Carolina Bay (a shallow freshwater pond)

sediments from under 18 cm of standing water at an ACP site in South Carolina (76) have many common features with the Abbott's Pit and Dover AFB subsurface sediment samples. Alpha, beta, and delta *Proteobacteria* sequences were common in both sets of samples. Also, *Fibrobacter* sequences were observed in both. Some of the major differences (e.g., the presence of green nonsulfur bacteria in the Carolina Bay samples) are probably related to anaerobic photosynthetic processes that would not be expected in the ACP subsurface sediments. The greater importance of anaerobic processes probably also accounts for many of the differences between sequences from the ACP sediments and sequences recovered from contaminated samples from Michigan (Dojka et al., 1997). Again, the similarities were the frequent occurrence of sequences related to alpha, beta, and gamma subdivisions of the *Proteobacteria*. The redox environment (with zones of methanogenesis, sulfate-reduction, and iron reduction) at the Michigan site is probably reflected in the occurrence of sequences related to the green nonsulfur bacteria, the Archaea, and *Syntrophus*.

Catabolic Gene Probes

Techniques that target specific components of the microbial community have been more broadly applied to ACP samples. Molecular-based approaches (i.e., DNA-DNA hybridization) have been applied for site assessment and characterization of specific microbial populations, changes in microbial community structure, and abundance of catabolic genotypes in contaminated subsurface sediments (10). A growing list of gene probes, specific for catabolic genes that encode the enzymatic ability to degrade many environmental contaminants (Table 1), are available as molecular diagnostic tools to monitor subsurface microbial responses. Gene probes have been effectively used in monitoring bioremediation in ACP sediment and groundwater.

Gene probes to monitor *tod*, *smmo*, and *mdh* (see Table 1) abundance were used to assess microbial community responses to injections of air, methane, and nutrients in saturated and unsaturated sediments during the integrated in situ demonstration at SRS. *Tod* genotype frequency exhibited a slight increase with air injection and a decrease upon methane and nutrient treatments (10). Whereas low biomass, poor DNA recovery, and coextracted contaminants precluded quantitative measurements, the *smmo* genotype increased with air injections and decreased with methane injections. The *mdh* gene frequency decreased upon air treatment, whereas methane and nutrient treatments corresponded with increased *mdh* genotypes. These results supported the changes observed in methylotrophic populations as determined by PLFA and MPN techniques (15,38,39,77). Gene probes (*smmo*, *mdh*, *tod*, *nahA*, *nahH*, and *codh*) were also used to characterize the intrinsic and accelerated anaerobic bioremediation of chlorinated solvents at the Dover AFB, Delaware, site. The overall trend observed was an increase in gene frequency in contaminated areas relative to uncontaminated control samples. Recently, a suite of gene probes (*alkB*, *nahA*, *nahH*, *todC1C2*, and *xylA*) were used to monitor indigenous subsurface aquifer sediment microbial community responses to hydrocarbon contamination at Columbus AFB, Mississippi (78). Significant increases in each of the catabolic genotypes were observed during the course of study and reflected shifts in microbial community structure associated with the hydrocarbon exposure and migration in groundwater.

Plasmid Incidence

The presence of extrachromosomal elements in bacteria in ACP sediments has been documented (79), but the data are sparse. Plasmids are autonomously replicating extrachromosomal elements ranging in size from a few kilobases to greater than 500 kb. The occurrence of plasmids has been well documented among Bacteria and Archaea or eubacteria and archaebacteria (80). The presence of such accessory elements typically confers a novel or advantageous trait to the host cell. Examples of some typical plasmid-encoded traits include protection from UV light damage, resistance to heavy metals, proliferation in the presence of antibiotics, and catabolism of xenobiotic compounds. Catabolic plasmids are widespread in nature (reviewed in 81) and plasmids have been reported to increase in frequency in contaminated environments (82).

Contaminated environments may increase plasmid frequency but the data are ambiguous. For example, in ACP samples Fredrickson and coworkers (79) reported conflicting data on plasmid occurrence in deep subsurface sediment bacteria isolated from SRS. A greater frequency of plasmid-containing bacteria was observed in uncontaminated deeper sediments relative to surface soil and upper aquifer sites. A whole-plasmid gene probe assay

Table 1. Examples of Catabolic Gene Probes Used to Monitor Microbial Activity

Gene Probe	Relevant Characteristic	Reference
AlkB	Alkane hydroxylase	Kok and coworkers (1989)
Codh	Carbon monoxide dehydrogenase	Jablonski and Ferry (1992)
NahA	Naphthalene dioxygenase	Simon and coworkers (1993)
NahH	Catechol-2,3-dioxygenase	Ghosal and coworkers (1987)
Mdh	Methanol dehydrogenase	Machlin and Hanson (1988)
Smmo	Soluble methane monooxygenase	Wackett and Gibson (1989)
todC1C2	Toluene dioxygenase	Zylstra and Gibson (1989)
XylA	Xylene monooxygenase	Suzuki and coworkers (1991)

indicated homology between numerous subsurface plasmids and the catabolic plasmid TOL in bacteria from both uncontaminated and putatively contaminated samples at this SRS site. However, Ogunseitan and coworkers (83) reported a greater than fourfold increase in plasmid frequency in bacteria isolated from pristine shallow aquifer materials relative to hydrocarbon-contaminated sites.

Spatial Distribution

The spatial distribution and the scale of microbial variability in the subsurface has been the subject of discussion and attention in the literature that focuses at the grain size level (84) and above (85). The measurement and description of spatial variability can be confusing, especially because there is no consensus on the methods for doing so. Numbers of samples, sampling intervals, site complexity, and the presence of contaminants vary considerably among studies. However, microbial heterogeneity and variability can be important in bioremediation (86). Investigators have found striking differences in activity that appear to be related to toxicity of and enrichment by contaminants in ACP sediments (87). However, if we examine the published evidence on uncontaminated sites, there are some general observations that can be made. It appears that, at the smallest scale of variability (e.g., grain to grain), samples from the ACP appear to be small (e.g., references). However, at scales of above 20 cm there can be considerable variability (8).

The majority of studies of spacial variability in the ACP have occurred at a limited number of sites, including the Abbott's Pit and Oyster sites (8,11,88) on the Delmarva Peninsula. As described elsewhere in this article, these sites are predominantly shoreface deposits (sands laid down just off shore of beaches). Thus, they are relatively uniform with low clay contents. Our conclusions on spatial variability may not hold for other types of sediments, particularly those containing clay lenses. In those sites, the geology and arrangement of the clays may play an important role in the spatial variability. Also, much of the high-density sampling that allows for appropriate evaluation of spatial variability has been done in the vertical dimension (7,53). This is likely because of the significantly reduced costs associated with intense vertical sampling from the same bore hole compared to sampling from excavations to depths or multiple boreholes at close intervals.

There are several methods that have been used to examine small-scale variability, and some of these have been applied to ACP sediments. The first method is direct microscopic examination or other imaging of spatial distribution at the grain scale (84,89). The second is the measurement of microbial properties using a variety of sample sizes (90). A third method that has apparently not been applied to ACP sediments is the characterization of communities on fractionated soil samples (91). Complicating all these methods is the potential for density-dependant factors to influence the measurement of microbial communities in sediments (90). Zhang and coworkers (8) found that sample size in the range of 0.1 to 100 g had no significant effect on CFU counts at the Oyster site. This contrasts with similar measurements at an arid subsurface site (less than 15 μm recharge/year) with generally low activity and biomass (92), where many of the smaller samples had no detectable activity. At those low activity sites larger sample sizes were required to sample the apparently rare patches of activity. However, they found that at similar sites with significantly higher recharge (e.g., 25 cm/year) the situation was more similar to those observed at the ACP sites, with patchness not effecting the measurement in the 0.1 to 100 g sample range. In a series of samples taken at close intervals for CFU counts at the Oyster site, Zhang and coworkers (7) found that the variability in these sediments at the centimeter scale was generally less than that reported in other studies (18). The ratio of the maximum/minimum CFU counts for samples 3–5 cm apart was 10 or less in eight of nine instances and never reached the factor of 220 seen by Beloin and coworkers (18). These CFU results were largely confirmed later at the Abbott's Pit site (8) and were similar for microbial activity (e.g., acetate incorporation). However, in samples near the water table the bacterial environment can change rapidly, and these changes may increase close interval variability (8).

Methods describing the spatial distribution and scale of microbial variability above the grain size level are still being developed, and one promising technique, geostatistics, has been borrowed from geology. Although not commonly applied in microbiology, geostatistical and other spatial analysis techniques are clearly useful in analysis of subsurface microbiological data. Their application gives us insight into the spatial distribution in ACP sediments. Geostatistical techniques are largely unfamiliar to microbial ecologists, except for some of those who deal with (surface) soil- and plant-associated microbial communities (93–95). However, the advantages of these techniques in analysis of spatial distribution of bacteria have been addressed (92). These spatial analysis techniques are now being applied at a variety of scales in many biological applications ranging from within cell spatial distribution (96) to epidemiology (97).

Conventional statistical techniques assume that the properties within a sampling unit are spatially uncorrelated. This implies that the sample mean is the best estimate of a property at any location within the sampling unit. Clearly in a functioning ecological system the assumption of uncorrelated samples is often violated as communities interact. Under the hypothesis that subsurface properties exhibit some spatial autocorrelation, conventional techniques (e.g., linear regression) that ignore spatial correlation will yield poorer results in estimating subsurface properties at unsampled locations than do geostatistical techniques.

Geostatistics provides a tool for incorporating the spatial autocorrelation between samples into the estimation of subsurface properties at unsampled locations. Central to most geostatistical methods is the construction and analysis of variograms. Variograms traditionally describe the correlation between a subsurface characteristic, such as hydraulic conductivity, as a function of the distance and direction separating measurements of the characteristic. However, we can apply this to a microbial property

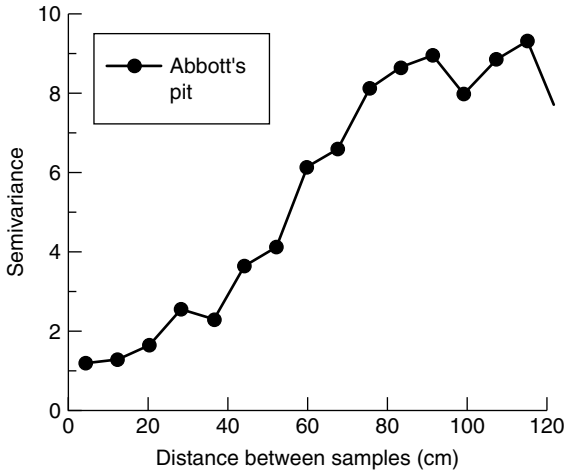

Figure 4. Evaluation of the scale of variability of CFU at the Abbott's Pit site. Comparatively low variability is present at 20 cm and below.

such as CFU (Fig. 4; 98). Analysis of the variogram provides important insights into the magnitude, direction, and scale of spatial autocorrelation.

The spatial distribution of subsurface microbial activity and community structure is hypothesized to be controlled by water transport, nutrient delivery, and the redox environment of the porous media. These latter variables, in turn, are likely dependent on the physical and chemical properties of the subsurface media. Therefore, the spatial structure of subsurface microbial communities should be strongly dependent on the spatial structure of key physical and chemical properties of the subsurface media. Geostatistical techniques offer approaches to testing these dependencies.

As indicated by geostatistics, the variability of CFU distribution with depth (41,98) at these sites increases rapidly at distances over 20 cm (Fig. 5) Thus, the distribution of CFU in closely spaced samples (e.g., 0 to 20 cm) is strongly correlated. This implies that predictions of CFU counts at closely spaced intervals

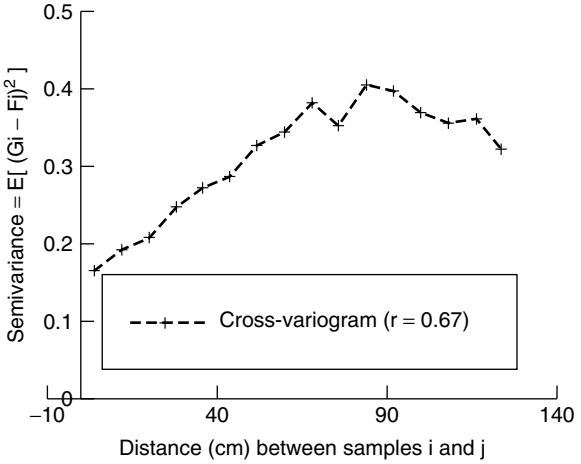

Figure 5. Variogram plot for CFU and microbial activity. Again there is considerably less variability at 20 cm and below.

from adjacent samples are relatively robust. However, at greater distances from a sample, the "representativeness" of the sampled location for the unsampled location becomes very poor. Thus, as in the ACP data presented in Figure 5, samples taken at intervals of 1 m in a vertical direction are likely to miss important features of the bacterial distribution. These can be seen in data from Zhang and coworkers (8) where a sample interval of 1 m in the ACP sediments could have missed a 1,000-fold increase in CFU and a significant increase in bacterial activity associated with a defined layer in a relatively uniform geological setting. One meter sampling spacing is not uncommon in subsurface microbiology (99), and it may be appropriate in some environments. However, it is apparent that closer sampling is desired for these ACP sediments.

The spatial relationships among variables can also be described using geostatistics (Fig. 6), and these relationships can be important in improving predictive ability and potentially in reducing sampling costs. For example, CFU counts have been found to be spatially correlated with microbial activity in these ACP samples (Fig. S2). The microbial activity (data not shown) varied over similar spatial scales as the CFU counts (Fig. S1). Thus, there was a reasonable cross correlation between the activity and the CFU data. This allows for better use of the CFU data in predicting activity in unsampled locations or better use of the activity data in predicting CFU in unsampled locations. This improvement in predictive ability can be important when there is a large cost difference among analysis for different variables, particularly when doing spatial analysis, such as geostatistics, that commonly requires a large number of samples (92). In those cases, identification of a good "proxy" variable can save time and effort and improve with little degradation (loss) in prediction of the other variable. This can be very important when applying some of the more costly and time consuming molecular methods described above (nucleic acid and PLFA methods).

There has been limited examination of horizontal heterogeneity with respect to microbial community structure in ACP sediments. For example, in a PLFA analysis (data not shown) of vadose zone microbial communities at Dover AFB, three samples taken within the 1.7- to 2.0-m interval from each borehole clustered together (Fig. 7). Boreholes C and E were the closest of those used in the analysis, at 30 m apart, and the community similarity was greatest between these two sites (they clustered together). The most divergent community was from borehole F, which was 40 m from E and 60 m from H but also had a higher clay content (data not shown). Thus, at the same sampling depths, there was stronger spatial discrimination among communities at the tens of meter scale than was evident within a borehole. This is important as it gives confidence that differences in communities seen at the tens of meters scale are not random fluctuations (if this were so we might expect the replicates from different boreholes to cluster).

At the Oyster, Virginia site, a study of the microbial community in groundwater indicated that there could be significant well to well differences in the microbial communities over fairly short distances (11) and over

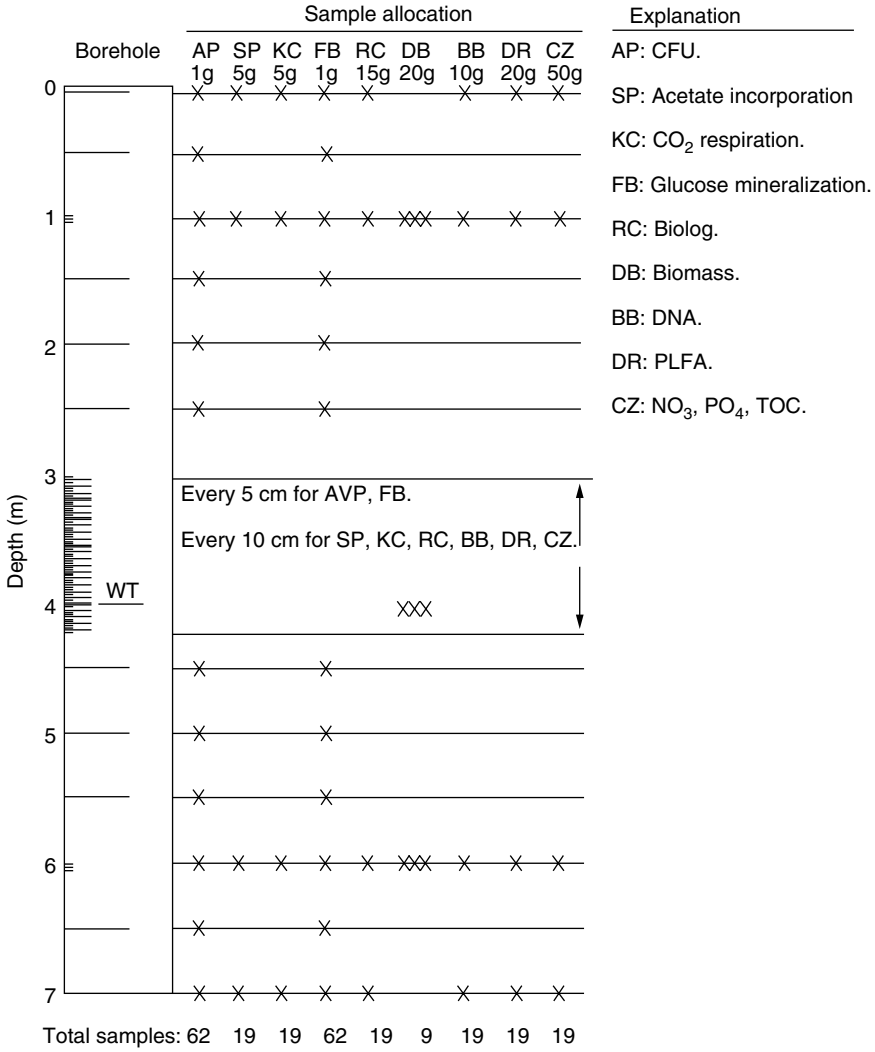

Figure 6. Field sampling plan for microbial and physical parameter analyses that could be used in geostatistical analysis of the data.

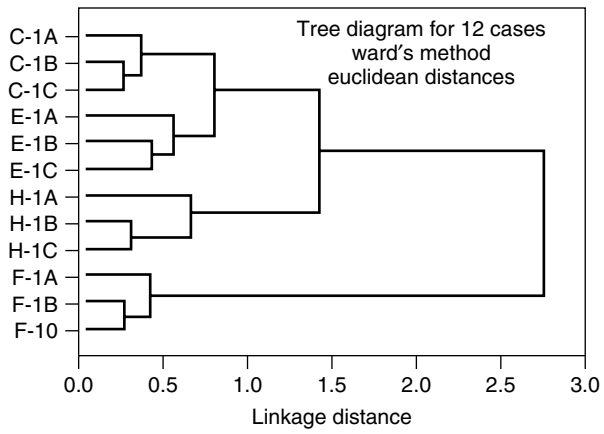

Figure 7. Hierarchical cluster analysis of PLFA microbial community profiles of triplicate vadose zone samples from each of four boreholes (C,E,H, and F).

time. These differences appeared to be related to redox gradients (anaerobic and aerobic zones) and shifts in the spatial location of the gradients over time. Groundwater chemistry apparently exerted considerable control of the community structure in these samples. The researchers concluded that there is considerable potential for variance in microbial community structure at scales of 10 to 100 cm, well within the spacing of wells at field sites. This would put the potential for horizontal heterogeneity where there are strong geochemical gradients in the groundwater at these ACP sites within the same scale as that for the vertical heterogeneity.

Bacteria Transport

To those unfamiliar with bacterial transport in the subsurface, the surprising aspect of bacterial transport in ACP sediments is the general lack of transport and high retention of microorganisms in sandy sediments with groundwater flow rates on the order of 20 m/year (8). For example, in a bacterial transport experiment at the Oyster Virginia site, bacteria were tracked 4 m from the injection point (100). However, it appeared that the majority of the biomass injected was retained within the first 0.5 μm of flow from the injection point. Similar results have been observed in similar sediments at Cape Cod (101). The Cape Cod site also is a largely sandy

site, with only 10% of the particulate mass falling below a grain size of 0.21 mm. Typically, peak breakthrough concentrations of bacteria sampled 6 m downgradient from the injection point were less than 10% of the peak bromide breakthrough concentrations (101). Numerous laboratory studies using sand from ACP sites have confirmed the large degree of retention of bacteria during transport though these porous sand (101). Thus, although water flows quite freely though these porous sediments, bacterial transport is very limited. Bacteria can only be transported long distances in very small numbers as the high degree of retention indicates.

The potential for bacterial transport in sediments can increase with increasing physical heterogeneity (102,103) where there is connected pore space (104), with reductions in geochemical interactions. In the Cape Cod study, there was one layer sampled where the peak bacterial concentrations reached 25% of the bromide concentration (101). The significant difference in retention of the bacteria in different sand layers points out the large degree of variability that can occur over 1 m vertical distances in these sediments. The presence of iron minerals can also contribute to absorption of the bacteria (105) and the ACP sediments typically contain iron-coated quartz (8, Parsons and Swift, 1995). Thus, heterogeneity in both physical and geochemical properties can be important in transport.

Heterogeneity in seemingly uniform ACP sediments has also been implicated as causing large differences in bacterial transport in the vadose zone (106,107). In laboratory experiments using intact blocks of ACP sediments from the Oyster site consisting of greater than 99% sand, flow and bacterial transport were extremely heterogeneous. Only 6 to 16% of the defined vertical regions in the block produced flow when simulated rainfall was applied to the surface of the block. Vertical regions producing the fastest water flow did not produce the highest peak bacterial concentrations. The effects on bacterial transport appeared to be related to soil particle size rather than to porosity. Bacteria were trapped in layers of the block containing smaller sand particles.

The high degree of attenuation of bacteria during transport in these sediments indicates that growth may be a key in the long distance transport of bacteria (108). Thus, although there are documented differences in bacterial properties related to attenuation even within a single strain (109,110) other bacterial properties related to growth and survival may also play a role in long distance transport.

CONCLUSION

The microbial communities in ACP subsurface environments are quite diverse. Microbial trophic groups commonly observed in these environments include aerobic heterotrophs, methylotrophs, nitrifiers and denitrifiers, sulfur or iron-oxidizers, anaerobic heterotrophs, sulfur or iron-reducers, methanogens, fungi, and protozoa. These microbial populations vary depending of the physical and chemical composition of their immediate environment, thus providing for the heterogeneity seen in the subsurface microbial communities. By their presence in the subsurface sediments and groundwater, these microorganisms can impact water quality by producing carbon dioxide, changing nitrogen, sulfur and iron species, producing methane, excreting volatile fatty acids and degrading chlorinated ethenes, petroleum, and polyaromatic hydrocarbons. The rate of impact may be limited by physical and chemical factors that limit microbial metabolism and growth; yet manipulations of the environmental conditions (i.e., oxygen and carbon addition) may alter microbial growth and metabolism to the advantage of reducing contamination in the subsurface environments.

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MICROBIOLOGY OF CRETACEOUS SHALES AND SANDSTONES OF THE SOUTHWESTERN UNITED STATES

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GEOLOGICAL BACKGROUND

Cretaceous rock formations of the southwestern United States originated as marine sediments deposited between 66 and 100 Ma (Ma = million years before present) (1). At that time, a large sea covered the western United States and often extended from Montana south into Mexico. The size of the sea depended on the worldwide fluctuations in sea levels. The series of advances and retreats in water levels resulted in the formation of extremely productive shallow marine and coastal ecosystems that contained considerable levels of plants, algae, and bacteria in addition to marine sediments. The influences of decomposition, burial, temperature, pressure, and chemical reactions worked in concert to convert the decaying plants, algae, and microorganisms to petroleum, coal, and organic carbon-rich shales.

In certain locations, the rapid changes in water levels created an intertonguing of sands and clays of relatively shallow thickness (to tens of meters) (2). Sands were deposited in shallow waters and finer grained silts, and muds at greater depths. Where the water was deep for longer periods, thicker shales were formed. Coastal systems associated with this sea exhibited fluvial (deposited in rivers) as well as deltaic formations, especially as the sea began to retreat during the late Cretaceous period. Through microbial, geochemical, and hydrological activities, sediments subsequently became cemented with quartz overgrowths as well as calcite, gypsum, and pyrite minerals. The biodegradation of organic materials and subsequent release of carbonate into solution resulted in precipitation with soluble calcium to form calcite. Reduction of sulfate to sulfide and the subsequent reaction of sulfide with Fe(II) formed pyrite. The net result was the hardening of sand into sandstones and of mud and silt into shales.

In the studies discussed within this review, shale and sandstones obtained as cores from the San Juan Basin (Cerro Negro), were originally deposited at alternating shallow intervals as described (3,4). The sandstone members from Paguate, Cubero, and Lower Oak Canyon formations represent near-shore (shore-face or near-shore-bar) depositional environments, whereas the shale members from Whitewater Arroyo, Clay Mesa, and Upper Oak Canyon represent adjacent, but deeper and less-energetic, near-shore ("toe-of-slope") environments (Fig. 1).

During the late Cretaceous period, as seas receded, fluvial sediments were often deposited. These tend to have similar characteristics to the earlier mentioned alternating intervals, except that rock are of freshwater origin and therefore, pore water concentrations of NaCl and sulfate are lower.

SULFATE AS ELECTRON ACCEPTOR

An important characteristic of sediments of marine origin is the abundance of sulfur oxidized in the form of sulfate or reduced in the form of sulfide minerals like pyrite (FeS_2). The importance of sulfate in marine and other environments is evident when one considers the microbial component. Groundwater flow is relatively slow in the vertical plane as a result of the confining nature of shale and clay formations. Consequently, in order that surface water enters the more permeable aquifers, organic-rich shales must often be traversed. The introduction of biodegradable organic compounds into the surface waters as they move down from the surface, often results in depletion of dissolved oxygen (present at only 250 μM at saturation) as microorganisms respire within the sediments. When oxygen is no longer available to act as respiratory electron acceptor, sulfate will be used by sulfate-reducing bacteria (if it is present). The presence of sulfate allows for the biodegradation of organic materials, including petroleum components (see review

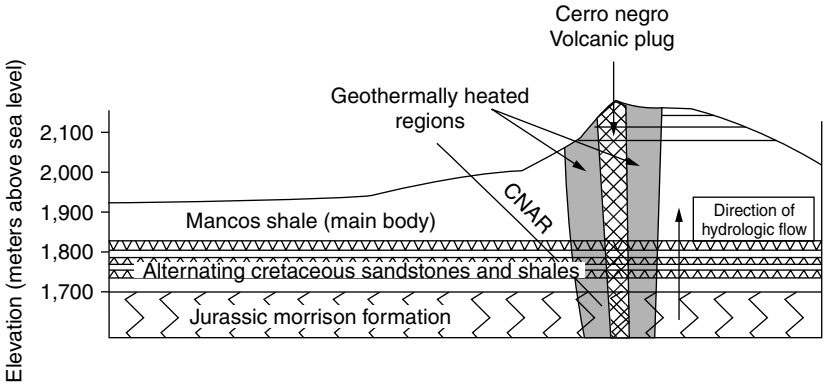

Figure 1. Diagram showing the geology of the drilling site in the San Juan Basin. CNAR represents the actual angled borehole from which cores were obtained. Alternating sandstones and shale formations are named in the text. Regions adjacent to the volcanic plug were sterilized at approximately 3.4 Ma (14).

on petroleum biodegradation (5)) that would be degraded slowly or not at all in its absence. It should be noted that the rate of biodegradation of the organic compounds would be considerably faster if oxygen were acting as the primary respiratory electron acceptor rather than sulfate. The equation below describes the degradation of organic materials by sulfate-reducing microbial communities.

$$\text{Buried organic material} + SO_4^{2-} \longrightarrow HCO_3^- + HS^-$$

Following the release of dissolved sulfide, it frequently reacts with iron to form pyrite as described in the following series of reactions:

$$Fe^{2+} + HS^- \longrightarrow FeS + H+$$
$$FeS + HS^- + H^+ \longrightarrow FeS_2 + 2H$$

If the sulfate is completely consumed at a particular organic-rich location, the only remaining respiratory electron acceptor will be bicarbonate. Bicarbonate is usually abundant in subsurface systems and its reduction by microorganisms with consequent methane production is commonly observed in organic-rich subsurface systems including petroleum-bearing sediments (6). Methane produced through methanogenesis as a result of the biodegradation of organic compounds is an important component of certain natural gas deposits.

THE SAN JUAN BASIN

Through a United States Department of Energy (DOE) sponsored project, the microbiology of the interfingered cretaceous sandstones and shales of the San Juan Basin was studied. These formations were drilled in the latter months of 1994, in a large effort that involved investigators from several universities and DOE's national laboratories. The drilling operation brought rock cores to the surface that were six centimeters in diameter. The cores were immediately placed in an anaerobic glove box to minimize air exposure of potentially oxygen-sensitive microorganisms and to prevent oxidation of reduced chemical species. The cores were then fractured into five to fifteen centimeter lengths before shipping them on ice to investigators.

Much of the core material was from sediments that are below the water table. Groundwater sampled early in the study was found to be devoid of oxygen, to contain low levels of reduced iron [Fe(II)], and was rich in sulfate and sulfide (2). A hypothesis was proposed, suggesting that sulfate reduction would be the most likely respiratory process for microorganisms living within this subsurface environment. To test the possibility of sulfate reduction in the subsurface rock cores, a technique involving silver foil was employed in which sheets of silver foil (7×7 cm), along with a solution containing radiolabeled sulfate, are placed on intact core faces and incubated for about six weeks (7). During incubation, labeled sulfide formed by the reduction of sulfate reacted immediately with the silver and became fixed. At the end of the incubation, the foil was removed and excess sulfate washed off leaving the labeled product attached to the foil as silver sulfide. The foils were then subjected to autoradiographic imaging to determine the location of sulfate-reducing activity within the cores (Fig. 2).

Although some cores showed no activity, the majority exhibited some activity that was localized within small patchy areas on the core face (Fig. 2). The most interesting results came when the total counts on each foil were determined and plotted as a function of depth. Several cores had activity that was extremely high, 200- to 1,000-fold greater than the others. These samples were found near sandstone–shale interfaces within the sandstone and below the interface (7). Examination of the organic carbon content of the rock samples suggested a hypothesis as to why activity was highest in these particular zones. When total organic carbon concentrations were plotted as a function of depth, the highest levels of organic carbon were also observed at these interfaces in the shale. In fact, the steepest gradients of organic carbon exist across the same interfaces in which the greatest level of sulfate-reducing activity exists. Along with these laboratory studies, multilevel sampling of well waters showed a decreased $\delta^{13}C$ for dissolved inorganic carbon and highest levels of dissolved sulfide within sandstones near shale sandstone interfaces (8). These results suggest that organic matter trapped in shales during deposition diffuses across the sandstone shale interfaces from the shales, and supports the existence of discrete microbial communities at and near these sandstone shale interfaces within the adjacent sandstones

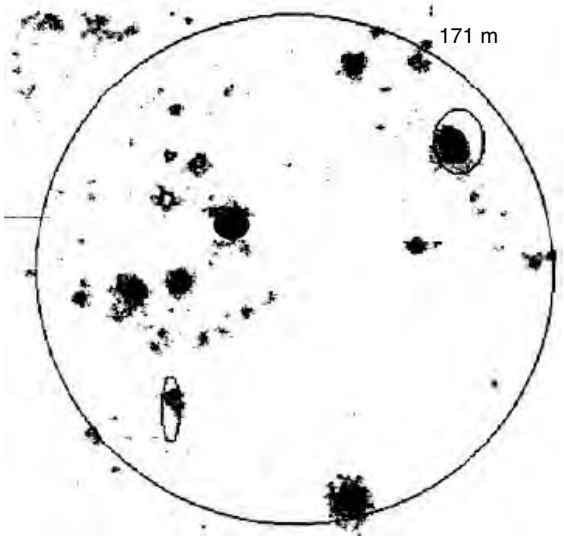

Figure 2. Autoradiographic analysis of sulfate-reduction activity on the freshly exposed face of a rock core obtained from 171 m depth of the Cerro Negro, N.M., exploratory drilling site. The image shows the localization of sulfate-reducing activity on a two-dimensional scale. This core shows the patchy distribution of microbial activity. The interior of the black circle represents the contact area of the cylindrical core with the foil. The level of radioactivity at that location on the foils is represented by darkness with some enhancement to improve contrast. Small circles represent points of reference and not necessarily microbial activity (7).

(Fig. 3). These microbial communities are apparently growing on organic matter laid down approximately 90 million years ago.

Experiments were designed, in which ground rock material was used to test the hypothesis that carbon-rich shales with low permeability supply nutrients for microbial communities living in higher permeability adjacent sandstones. To avoid contamination from the exterior of the core, cores were fractured and, with the aid of a sterile masonry drill bit, samples were ground from the center of the core. In these experiments, ground shales and sandstones were combined to determine the influence of shale organic matter on sulfate reduction as well as acetogenesis within these samples. When ground rock was incubated over time, sandstones carried out sulfate reduction and this activity was increased when ground shales were added to the incubations. Additionally, acetate was produced and this production was proportional to the shale addition. On the other hand, the shale did not reduce sulfate or produce any acetate when incubated separately. Therefore, it appeared that shales provide the energy needed to stimulate microbial activity within the sandstone. These results suggest that complex organic matter bound in shales can diffuse across sandstone shale boundaries (Fig. 3). This organic matter in turn, provides a source of nutrients and energy for heterotrophic bacteria living in a discrete microbial community within the sandstone at the sand–shale interface. Products of these microorganisms provide the reducing equivalents needed for sulfate reduction and possibly for acetogenesis. This model illustrates a novel mechanism for the long-term survival of microbial communities in the subsurface.

In another detailed study of these rocks, Fredrickson and colleagues (2) have shown that the distribution of pore throat diameters varied dramatically between shales and sandstones. The vast majority of shale pore throats were smaller than any known culturable bacteria (about 1–100 nm). In contrast, pore throats for sandstones were sometimes as large as 10 to 20 μm, which would likely allow transport of most known bacteria. A variety of techniques including acetate mineralization rates, sulfate-reduction rate, and culturable bacterial numbers, again showed that microbial numbers and microbial activity were far smaller in shales than in nearby sandstones. A rational hypothesis based on these data is that growth and metabolism of shale bound organisms is limited by slow diffusion of nutrients and/or by the microbes inability to easily migrate through the narrow pores.

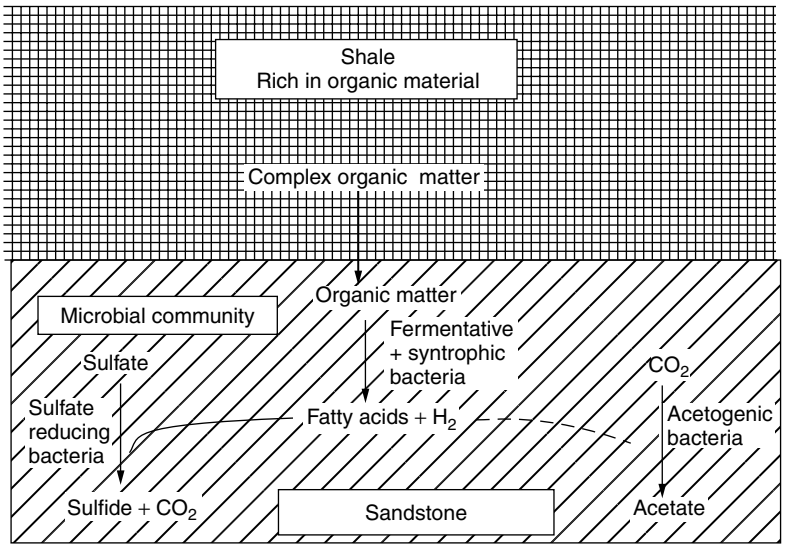

Figure 3. Drawing of the microbial processes postulated to occur near the interfaces of Cretaceous sandstones and shales (27).

The idea of ancient organic matter fueling microbial growth has been previously postulated for microorganisms living near oil deposits. Microorganisms appear to grow by catabolizing components of the buried petroleum. In recent years, several research groups have isolated microorganisms from both marine and continental oil reservoirs (9–11). Additional geochemical or geophysical characteristics, of these subsurface locations apparently limit the biodegradation process so that petroleum remains.

ISOLATION OF PURE CULTURES FROM THE SAN JUAN BASIN

A mineral medium was inoculated with a sandstone groundwater mixture and these were incubated with H_2 as electron donor and sulfate as electron acceptor (12). Under these conditions, both sulfide and acetate were produced and two pure cultures of organisms were isolated from these enrichment cultures. The hydrogen-utilizing sulfate-reducing bacterium was named *Desulfomicrobium hypogeium* and the autotrophic acetogen was named *Acetobacterium psammolithicum*. The two organisms were typical of members of their genera with the ability to use hydrogen as electron donor.

As H_2 is a critical intermediate in the biodegradation of complex organic compounds, it is important to understand which group of organisms is responsible for its consumption. Experiments were designed to examine competition between the two organisms to elucidate which was the dominant process for H_2 consumption; sulfate-reduction or acetogenesis. Studies which determined the lowest H_2 concentration utilizable for growth showed that the sulfate-reducer was capable of drawing H_2 concentration much lower than the acetogen. This in turn indicated that under the conditions tested, and likely under subsurface conditions that typically exhibit threshold H_2 concentrations, the sulfate-reducer is presumably responsible for the majority of H_2 consumption. In competition experiments in which both organisms were grown together in the presence of H_2, the sulfate-reducing culture was able to prevail over the acetogen, thereby indicating once again that sulfate reduction was likely to be the most important process for H_2 consumption.

ORIGINS OF MICROORGANISMS IN THE SUBSURFACE

Experiments at the San Juan Basin and Piceance Basin (see following section) were originally designed to test the following hypotheses. (*1*) Bacteria present today are descendents of organisms originally deposited within sediments. (*2*) Bacteria have migrated from the surface or other locations to subsurface sediments. This question was tested using samples obtained at the Cerro Negro site in the San Juan Basin where a volcanic neck protruded through the buried cretaceous sediments about 3.39 Ma (13), thereby sterilizing the sediments near the neck (Fig. 1). It was proposed that if hypothesis A was correct, then sediments adjacent to the volcanic intrusion would be currently sterile. In contrast, organisms would be discovered in this sterilized zone if existing organisms migrated to the sterilized sediments from the surface or from nearby sediments during the last 3.4 million years. To obtain samples from the sterilized region, an angled borehole was directed at the volcanic neck and samples collected throughout the drilled interval. Fission track analysis undertaken at the same time determined the temperature of rock during the volcanic intrusion to have reached a maximum of 210 to 320 °C (14). Hydrologic flow models of groundwater near the site suggested a generally vertical flow at this location from the underlying Triassic Chinle formation upward through the cretaceous sediments, indicating the possible transport of microorganisms from underlying sediments (14) (Fig. 1). The rock cores from the sterilized region were shown to contain active microorganisms based on phospholipid fatty acid (PLFA) analysis and microbial activity studies (2). It appears that rock colonization likely occurs continuously as microorganisms are transported in groundwater.

PICEANCE BASIN, COLORADO

Microbial analysis was carried out on rock cores from late cretaceous and tertiary sediments of the Piceance basin in western Colorado. The sediments were deposited during 73 to 35 Ma in a fluvial environment deeply buried between 35 and 5 Ma, with temperatures peaking between 120 and 145 °C (6,15) during this time. The result was interbedded sandstones, shales, and coal deposits. During the last five million years, temperatures have cooled, with current temperatures ranging from 43 to 85 °C for the sampled regions. Cretaceous sediments sampled in the lower part of the interval have current in situ temperatures of 81 °C for the 1,996-m depth interval and 85 °C for the 2,091 to 2,096-m interval. The harvested sediments were incubated at 65 °C, in microbiological media designed for the cultivation of a variety of different physiological groups of bacteria. Among the range of microbial types in which cultivation was attempted, both Fe(III) and Mn(IV) reducing (respiring) bacteria grew from the Cretaceous sandstones (16,17). Sulfate-reducing activities were detected in some of the core material (unpublished data, L. R. Krumholz and J. M. Suflita); however, it is likely that Fe(III) reduction and methanogenesis are also important respiratory processes in the sandstones. Positive enrichment cultures, plus the fact that the rock at the 1,996-m depth interval contained PLFA (microbial membrane components) at levels above the detection limit, suggest that the upper interval of these late cretaceous sandstones and coal material contain active metal-reducing microbial communities.

Energy Sources for These Communities

Cretaceous sediments in the Piceance Basin are important sources of fuel as they are rich in both methane and coal (6). Methane and hydrocarbons have recently been shown to be oxidized by methanogenic Archaea in a process coupled to the reduction of sulfate (18,19), although

this has not been demonstrated with pure cultures. It seems probable that methane and coal-derived organic compounds are the major energy sources for existing microbial communities in deep cretaceous sandstones from the Piceance Basin.

ORIGINS OF MICROORGANISMS

Sediments from the upper Cretaceous layer appear to be inhabited by metal-reducing organisms; however, the depth interval obtained from a somewhat more hydrologically isolated region a few hundred meters below did not yield detectable organisms (16). A similarity with the Cerro Negro site in the San Juan Basin is that both of these regions had been previously subjected to sterilizing temperatures and have now cooled to temperatures capable of supporting life. Only the upper region from the Piceance basin appeared to have an associated microbial community, suggesting that the lower, hydrologically isolated region has not been recolonized. The mechanism for colonization of the upper region is almost certainly via transport of microorganisms from nearby formations or from the surface.

Muds are typically used during drilling operations to lubricate drill bits and remove rock cuttings from boreholes. Thermophilic (60 °C) incubations of drilling mud from the described earlier operation produced Fe(III)-reducing enrichment cultures (17). The enrichment cultures coupled the oxidation of H_2 as well as a variety of low molecular weight organic acids to the reduction of amorphous Fe(III) oxide. Magnetite and siderite ($FeCO_3$) were the reduced products of iron reduction (20). The temperature range for the enrichment cultures was 45 to 75 °C. These enrichment cultures have been studied and the individual components have been characterized phylogenetically (17). The major components of the Fe(III)-reducing enrichment cultures from this site and enrichments obtained from Triassic Age Taylorsville Basin in Virginia represent a unique lineage of iron-reducing bacteria. The fact that these clones are very deeply branched within the phylogenetic tree of life has allowed the authors of the work to suggest that thermophilic iron reduction may have evolved early as a form of microbial respiration. The isolation of thermophilic bacteria capable of producing magnetic rock in ancient sediments also points to the possible biogenic origin of magnetic rock formations during these or earlier time periods.

RELATED STUDIES

Although the goal of this review is to discuss the microbiology of Cretaceous shales and sandstones of the southwestern United States, two additional analogous microbial ecosystems must also be mentioned. One occurs within the alternating cretaceous sand and clay layers of the Atlantic coastal plain where low levels of sulfate reduction have been observed in clays and much higher levels in adjacent sandy sedimentary layers (21). The apparent lack of sulfate-reduction activity in clays has resulted in a buildup of fermentation products within clays (22,23). The diffusion of microbial fermentation products across a sand-clay boundary apparently provides the organic electron donors needed for sulfate reduction in adjacent sand formations, in the same way that organic materials from shales fuel sulfate reduction in adjacent sandstones within the Cretaceous sediments of the San Juan Basin.

The other is a relatively well-characterized site in the Texas coastal plain represented by late Cretaceous and Eocene (~45 Ma) sediments. The aquifers studied were deposited in fluvial and/or deltaic environments (24–26). Eocene sediments are composed of sands and silts with interbedded clays and lignites. Lignite is a remnant of ancient plant material. Using the silver foil technique outlined earlier, and a direct measurement of sulfate reducing activity (26), it has been shown that sandy regions have sulfate-reducing activity, which clay regions do not have. The most active sulfate-reducing zones lie directly adjacent to clay and lignite deposits. Products from the fermentation of organic matter within the lignite-clay deposit appear to provide the energy for sulfate-reducing microbial communities that live at these lignite sand interfaces. These sulfate-reducing microbial communities, present in discrete regions can only be evaluated if the heterogeneity is examined at the centimeter scale in core material. Microbial activity occurs as a result of the diffusion of organic compounds originally trapped in clay and lignite into adjacent more permeable sand regions. Once in the sand, microorganisms are capable of growing on these organic compounds. This geochemical process could likely sustain these microbial communities over long periods of time.

CONCLUSION

Studies on cretaceous rock have illustrated the adaptability of subsurface microorganisms and their ability to grow in different environments. Subsurface bacteria will consume buried organic materials trapped in sediments over millions of years. Long-term growth of microorganisms in organic-rich subsurface formations appears to rely on a rich source of organic material trapped in a shale, lignite, or clay, which on their own suppress microbial activity. As the organic compounds migrate out of these rich materials into permeable sandy sediments, they appear to be degraded by the resident microorganisms. This process could maintain microbial communities over long periods of time.

Deeply buried organic-rich subsurface sediments are usually anoxic and the resident microflora rely on electron acceptors including sulfate and bicarbonate, resulting in the formation of sulfide minerals or biogenic methane.

Colonization of subsurface formations occurs during and following burial of those sediments. It seems likely that the microflora of subsurface formations is constantly in flux, with microorganisms migrating in groundwater from one location to another. Those microorganisms that are most efficient and/or adaptable will likely be the ones to prosper at any given location.

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MICROBIOLOGY OF DEEP HIGH TEMPERATURE SEDIMENTARY ENVIRONMENTS

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Economic wealth in today's world continues to depend on subsurface environments that provide societies with fossil fuels for energy to sustain industrial activities. During the twentieth century, geologic understanding of these subterranean realms has increased considerably and yet, we have only recently discovered that microbial communities exist in these oil- and natural gas-bearing deep terrestrial and marine sedimentary formations. For a thorough comprehension of the factors that contribute to the accessibility, quality, and origin of these natural resources, we are compelled to learn more about the life that coexists with this organic energy. Hydrogen sulfide in crude oils from sedimentary strata has corrosive and toxic effects, and this gas may be biogenic. Furthermore, because of the relatively well-defined geology of these environments and the chance for occasional access by drilling, microbiologists are attracted to opportunities to conduct discovery science in which they can learn more about these unusual communities. Knowing the geologic context of these austere habitats allows microbiologists to place conditional constraints on the presence of living cells in these deep-earth locations and test hypotheses related to such relevant issues as microbial transport through confined spaces in porous media or colonization of geologically sterilized strata. Through these studies we learn more about the edges of the biosphere in places where life was, if acknowledged to exist, not thought to be of great importance. The questions now frequently considered by microbiologists are "how deep? how hot?" (1). We are also led to consider the likelihood that microscopic life has been sustained under related conditions on other celestial bodies.

This article will consider the microbiological characteristics of deep sedimentary environments and identify common themes among the microorganisms that exist therein. Firm microbiological information for these habitats is still difficult to come by, largely because of the infrequent and expensive nature of well-designed and carefully conducted sampling opportunities. Any research

in this field, however, has the potential to illuminate our limited understanding of who is there, how they got there, and what they are doing there.

DEEP SEDIMENTARY BASINS CONTAINING MICROORGANISMS

The earliest study that considered the presence of microorganisms in deep sedimentary strata was conducted by Bastin and coworkers (2), who noted evidence of sulfate-reducing bacteria in oil reservoir fluids. More recently, a variety of microorganisms have been cultured from oil wells around the world (3). Research that has focused on the microbiology of subsurface environments since the 1980s has seen marked attention to the methods of sample collection and, therefore, an increased level of confidence that microbial communities are indigenous to deep-earth systems (4). The findings of several of these recent studies with regard to deep sedimentary rock environments in which microorganisms have been detected are detailed. The investigation of the Piceance Basin in western Colorado is highlighted because it combines various approaches to evaluate present and past conditions of this extreme microbial environment. Specifically, determinations were made for: (*1*) thermal histories by fluid inclusion microthermometry and vitrinite reflectance, (*2*) hydrologic patterns by isotope geochemistry, (*3*) pore throat dimension by mercury porosimetry, and (*4*) microbial colonization rates by numerical simulation of groundwater flow. By linking these methods, the presence of microorganisms in the subsurface and the nature of their existence can be more easily explained.

PICEANCE BASIN

One of the sedimentary basins of importance to gas production in the United States is the Piceance Basin in western Colorado. By some estimates, this coal-bearing basin contains more methane (84 trillion cubic feet) than any other coal bed resource in continental United States (5). These resources are contained within low-permeability strata of Cretaceous age and oil shales of Tertiary age. The gas- and coal-bearing formations in the Piceance Basin have been extensively surveyed for their gas and coal resources, yielding considerable information related to the geologic and hydrologic history of the subsurface. In 1994, this information was used by the U.S. Department of Energy's Subsurface Science Program to guide investigations of microbial communities in the deep strata (6). Selection of this site was based on the following criteria: (*1*) the sedimentary basin is hydraulically tight, so deep strata have minimal communication with near-surface microorganisms through transport in groundwater; (*2*) the basin has a well-characterized tectono-thermal history; and (*3*) the strata contain abundant trapped natural gas compartments that could potentially harbor microorganisms. The focus of this study was to determine how geologic parameters constrain the presence of microorganisms in deep sedimentary strata, specifically the degree to which geologically sterilized formations were subsequently colonized and the factors that might prevent or retard this colonization.

Geologic Setting

The Piceance Creek Basin is located on the Colorado Plateau in western Colorado (Fig. 1). The sedimentary rocks within the basin are composed predominantly of shale and sandstone (Fig. 2) deposited in marine, coastal, and nonmarine environments during the Late Cretaceous to Early Tertiary [about 73 to 35 million years ago (Ma)] (7). The maximum formation temperatures were achieved at 35 Ma. The uplift of the Colorado Plateau started as early as the Early Eocene (8). Apatite fission track analyses indicated that the last phase of erosion started about 9 (9) or 5 Ma (10). Since then, erosion has removed 1 to 1.5 km of strata along the Colorado River valley. The Piceance Creek Basin contains large volumes of natural gas within sandstone bodies intercalated with shale. Natural gas production and testing data indicated that the rock formations, although fracture-bearing, are hydraulically tight, with reservoir permeabilities that are typically tens of microDarcies (μD) and rarely reach tens of milliDarcies (mD) (11).

Core Description and Analysis

Mud and gas log analyses collected during the drilling of the deep gas-exploration well, 1M18 (Fig. 1), indicated that a sharp transition from low to high gas concentrations occurred in the sandstone bodies within the upper Mesaverde Group. Three coring intervals (above, within, and below this transition) were targeted for sampling and to capture shale/sandstone interfaces in which microbial activity was anticipated to be the highest.

By piggybacking the drilling of deep wells for gas resource development, cores were collected from rocks as deep as 2,096 m below land surface. Considerable care was used in obtaining, handling, and processing these cores before microbiological characterization (4,13). This involved the use of tracers to assure core quality, paring of contaminated core sections away from sections that were used for microbiological study, the shipping of blind controls and blanks, and overnight shipping of samples to microbiologists. Chemical and physical tracer measurements and phospholipid fatty acid (PLFA) analyses of the cores and the drilling mud identified core subsamples with little or no chemical or microbial contamination (6).

Two cores of sandstone and shale and one core of sandstone were obtained with a gel coring device (Baker Hughes, Houston, TX, U.S.A.) during the drilling of a gas-exploration well at the 1M18/1M8 site (Fig. 1). The coring depth intervals were 856 to 862 m (Core 1), 1,996 to 1,996.7 m (Core 2), and 2,090 to 2,096 m (Core 3) below land surface, mbls, (Fig. 2). Core 1 was obtained from the fluvial G-sandstone of the Wasatch Formation (Lower Tertiary). Cores 2 and 3 were obtained from the fluvial interval of the Mesaverde Group (Upper Cretaceous). Gas analyses performed on the drilling muds during the coring verified the transition from low to high methane concentrations between Cores 2 and 3 (14).

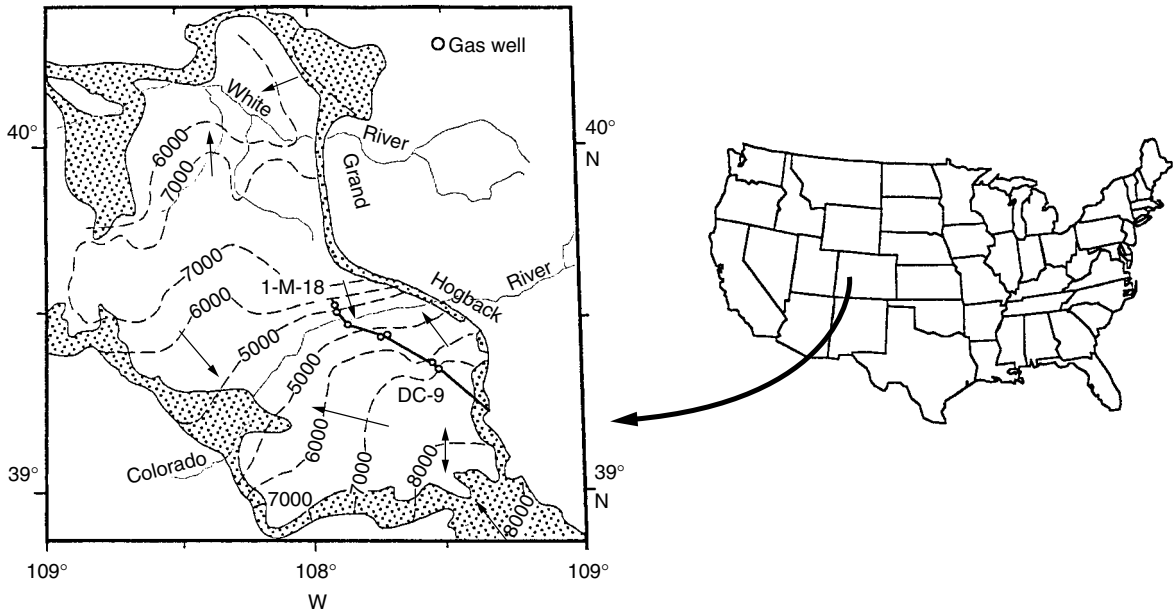

Figure 1. Groundwater flow direction (arrows) and potentiometric surface (contours in feet) in the Mesaverde group (simplified from G. W. Freethey, B. A. Kimball, D. E. Wilberg, and J. W. Hood, *General Hydrogeology of the Aquifers of Mesozoic Age*, Upper Colorado River Basin, U.S. Geological Survey, Reston, Virginia, 1988). The line connecting gas production wells is the location of the cross section shown in Figure 2.

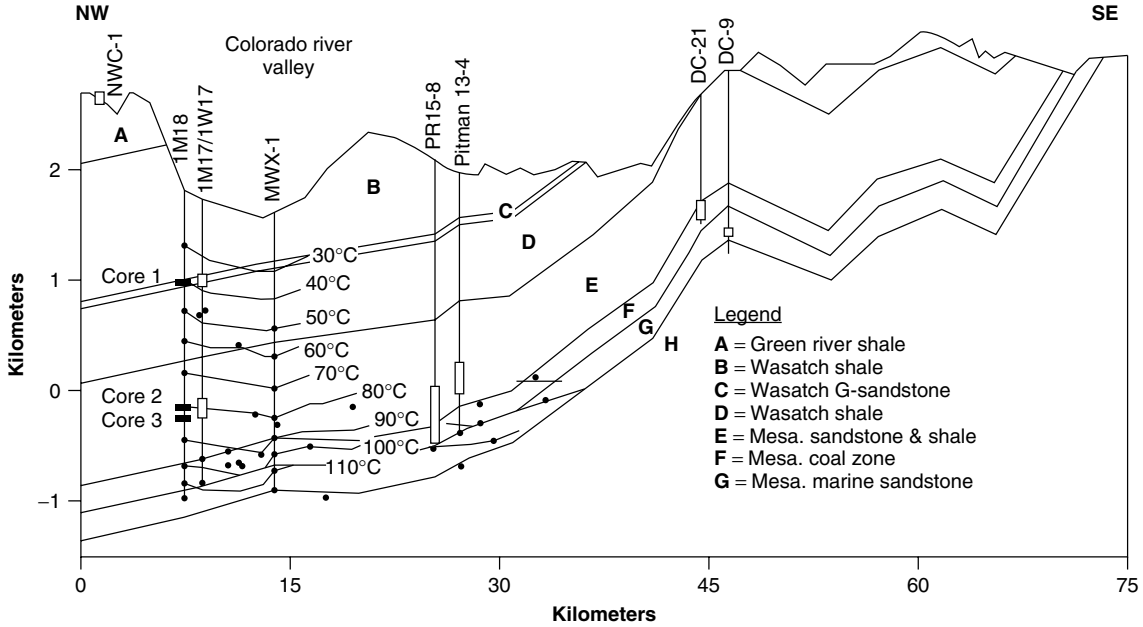

Figure 2. A NW-SE cross section along the maximum hydraulic gradient. The 1M18/1M8 is the microbial coring site. Also shown are the main stratigraphic units, locations, and depth intervals (open boxes) for the sampled wells and springs, and present-day isothermal contours interpolated from temperature logs and corrected bottom-hole temperature measurements (solid dots).

The present-day formation temperatures at the depths of Cores 1,2, and 3 were measured by logging three months after drilling was completed and were found to be 42, 81, and 85 °C, respectively. The maximum natural gas concentrations at the three coring intervals were 2.6×10^3, 2.1×10^4, and 8.8×10^5 ppm in order of increasing depth.

Core 1, consisting of sandstone and shale, had been pervasively cemented by quartz, calcite, pyrite, and various clay minerals, with gas porosities from 1.3 to 12.2%, permeabilities from 0.001 to 0.62 mD, and a maximum pore throat diameter of 0.5 μm, as measured by Hg injection porosimetry. Two nearly vertical natural

fractures with apertures on the order of millimeters were encountered within Core 1. Core 2 had gas porosities from 5 to 7% and permeabilities from 0.007 to 2.3 mD. Petrographic observations revealed large (up to 1 mm) connected pores that were partially filled with quartz, albite, illite, and chlorite cements. The pores in Core 2 were up to 1 mm in diameter, with a maximum pore throat diameter of 3.0 μm. Core 2 intersected a vertical, natural fracture. Core 3 was composed entirely of very homogeneous sandstone with gas porosities from 5.6 to 7.5% and permeabilities from 0.02 to 0.5 mD, and a maximum pore throat diameter of 2.0 μm. No fractures were encountered in this core. Generally, the cores were of low porosity, low permeability, and narrow pore throats as a result of diagenetic cementation.

Investigators used a battery of approaches to determine microbial biomass and different physiological types of microorganisms. Cores were scrutinized for culturable anaerobic bacteria, including Fe(III)- and Mn(IV)-reducing bacteria, fermenters, sulfate-reducers, nitrate-reducers, and methanogens. Ester-linked phospholipid fatty acid (PLFA) and selected enzyme and physiological activities were assayed as well. Acridine orange direct counts were generally at or below the limits of detection for this method, making this approach of limited use in estimating biomass.

Low but measurable microbial biomass was detected, however, by total PLFA analyses and by enrichments for anaerobic bacteria in the core obtained from 856 to 862 mbls (6). This core yielded anaerobic, thermophilic (growth up to 75 °C) Fe(III)-reducers and fermenters. A single positive enrichment and lower PLFA values were evident in samples from the core obtained from 1,996 to 1,997 mbls. Methanogens and sulfate-reducers were not detected in any of the samples, nor were bacteria that could grow with methane and any added electron acceptors. As with many deep subsurface samples that are low in biomass, attempts at direct extraction and amplification of microbial DNA from the samples were unsuccessful. However, 16S rRNA genes could be cloned from products of polymerase chain reaction (PCR) amplification of DNA extracted from a positive Fe(III)-reducing enrichment that resulted from incubation of core material from 858 mbls. The bacteria in this enrichment were related to bacteria in the genus *Desulfotomaculum*, characterized as gram-positive, spore-forming sulfate-reducing bacteria.

Geothermal History

The thermal history at the drilling site was reconstructed on the basis of fluid inclusions microthermometry (15) and vitrinite reflectance data (16; Fig. 3). Vapor-liquid inclusions (5 μm in diameter) found in crystalline quartz and calcite cements of the two natural fractures in Core 1 yielded homogenization temperatures from 75 to 125 °C (average, 95 °C), indicating that fluid at least this hot once migrated through the natural fractures. The salinity of the fluid was close to that of freshwater. Crystalline quartz filling the fracture in Core 2 contained vapor/liquid fluid inclusions that homogenized between 138 and 155 °C. The salinity was close to that of freshwater. The pressure correction for these homogenization temperatures was less than 5 °C on the basis of the maximum burial of the formations (<3 km).

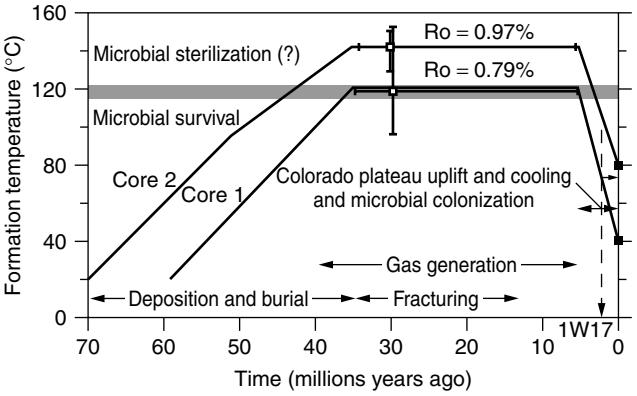

Figure 3. Geothermal history of cores 1 and 2. The solid circles with error bars are fluid inclusion data from fracture-filling minerals. The solid squares are data from borehole logging. The maximum temperature plateaus are based on vitrinite reflectance data.

The average vitrinite reflectance of centimeter-sized coal fragments in Core 1 was 0.79%. The average vitrinite reflectance value of carbonaceous shale from Core 2 was 0.97% (Fig. 3). The vitrinite reflectance values of three drill cuttings of the coal zone of the Mesaverde Group were determined to be 1, 1.67, and 1.77% at depths of 2,231, 2,677, and 2,753 mbls, respectively. Theoretical vitrinite reflectance profiles were derived with the model of Sweeney and Burnham (17) and were based on the burial history (7). The best match between the predicted profile and the five data points was obtained by assuming a geothermal gradient of 50 °C/km from 35 to 5 Ma and 35 °C/km during other times. The present-day geothermal gradient is around 35 °C/km. A higher paleo-geothermal gradient of 58 °C/km was estimated for a much deeper well drilled some 30 km to the southeast of the 1M18/1M8 site (10). At a geothermal gradient of 50 °C/km, the maximum geothermal temperature (T_{max}) for Core 1 was about 120 °C, assuming an average surface temperature of 20 °C. A T_{max} of 125 °C was calculated with the equation of Barker and Pawlewicz (18). The T_{max} of Cores 2 and 3 were calculated to be 140 to 145 °C and 145 to 150 °C, respectively.

The fluid inclusion homogenization temperatures and the vitrinite reflectance derived T_{max} are similar within error and indicate that the paleotemperatures of these formations almost certainly rendered these rocks lifeless within the constraints of our current understanding of life at high temperature. As recently as 5 Ma, through uplift and erosion by the Colorado River and a decline in the geothermal gradient, the formations cooled to present-day temperatures, which are more permissible for microbial colonization and survival. This suggests that the presence or absence of microorganisms in Cores 1 and 2 would be dictated by past thermal regimes that sterilized the formations at depth, the present thermal regimes that are comparatively permissible in terms of microbial survival, and by the microbial migration into these deep rock formations through the movement of groundwater.

Stable Isotopes of Formation Water

The chemical and isotopic compositions of water samples collected from wells producing from the Mesaverde Group and Wasatch Formation and from springs in the overlying Green River Formation (see Fig. 2 for sample locations) place constraints on the paleohydrology of these formations. The modern meteoric water, obtained from a shallow spring (NWC-1 in Fig. 2) on the high plateau above the Colorado River valley, is characterized by a $\delta^{18}O$ of -16.9% and a δD of -122%. In contrast, the $\delta^{18}O$ and δD of the deeper Mesaverde Group and Wasatch Formation waters were much heavier than the modern meteoric water. The water sample from Pitman 13–4, a Mesaverde Formation well, had a $\delta^{18}O$ value of -4.0% SMOW and a δD value of -47% (Fig. 4). The water from Mesaverde well, DC-9, had a $\delta^{18}O$ of -3.4% (its δD was not determined), whereas water from an adjacent but shallower (Fig. 2b) Mesaverde well, DC-21 (Fig. 2), had a lighter $\delta^{18}O$ value of -15.9%, relative to DC-9 and a δD of -109%. The 1W17 well producing water and gas from the Wasatch G-sandstone yielded a $\delta^{18}O$ value of -6.6% and a δD value of -58%. One other Wasatch Formation water sample from well 3W17 had isotope ratios of -10.6% for the $\delta^{18}O$ and -82% for the δD, both lighter than those for 1W17. The Wasatch and Mesaverde isotopic analyses yield a linear trend with a slope lower than the global mean meteoric water line (dashed line in Fig. 4), which is a relationship that is found in many intracratonic basins (19). These trends result from a variety of processes including H isotopic exchange between groundwater and hydrocarbon gases and O isotopic exchange between groundwater and diagenetic carbonates. The amount of exchange increases as the age of the groundwater increases. The trend may also be formed by mixing of meteoric water and a diagenetically altered groundwater of nonspecific age and isotopic composition at least as heavy as that of Pitman 13–4. In both instances, the intersection of this trend with the global meteoric water line yields the $\delta^{18}O$ and δD of meteoric recharge, which in turn reflects mainly the latitude and altitude of precipitation (20). This trend intersects the GMWL at a $\delta^{18}O$ of -13% and a δD of -100%, values that are distinctly heavier than the present-day precipitation or spring water. The North American plate has remained at the same latitude since at least the Cretaceous, but the altitude of the surface may have changed during the main uplift phase of the Colorado Plateau. The groundwater recharged before the uplift of the Colorado Plateau would have been isotopically much heavier than that of either modern recharge or of the intersection of the Mesaverde-Wasatch trend with the GMWL (arrow in Fig. 4). This suggests that the recharge age of the Mesaverde-Wasatch water occurred during the uplift of the Colorado Plateau and while the paleotemperatures of those formations were falling below the T_{max}. An alternative explanation is that the trend represents diagenetic modification of or a mixing line with modern precipitation (lower dashed line in Fig. 4), but that it has been displaced upward by 15% by isotopic exchange with hydrocarbon gases or hydrogen sulfide. The ^{36}Cl isotopic analyses of 1W17 (not shown) suggest a subsurface residence time of 1.6 Ma, which would

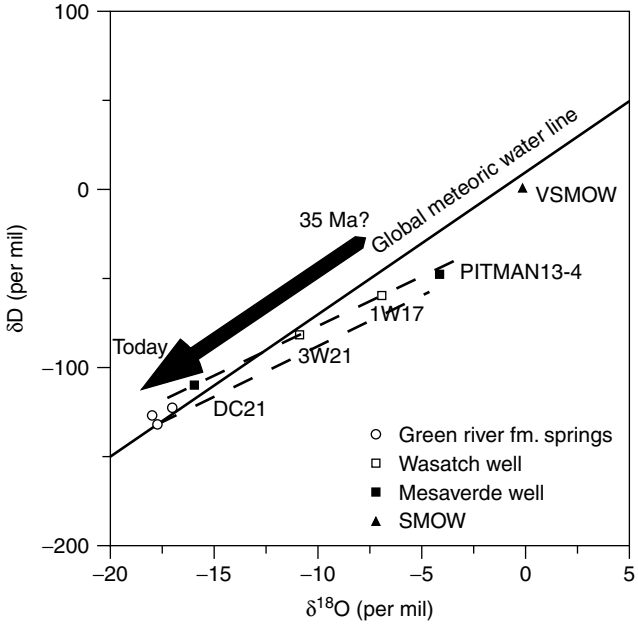

Figure 4. $\delta^{18}O$ and δD of water samples collected from springs and wells. The upper dashed line is the mixing line formed by data and the lower dashed line is the same trend displaced downward and anchored to present-day precipitation. VSMOW is Vienna Standard Mean Ocean Water. GMWL, the Global Meteoric Water Line, from [H. Craig, Science 133, 1,702–1,703 (1961)]. The arrow delineates the hypothetical migration of the isotopic composition of precipitation in western Colorado from 35 Ma since the beginning of uplift.

support the former explanation. The total dissolved solids of the Mesaverde Group water increases from about 0.8% at the DC-21 site to 2.8% at the 1M17 site, preventing accurate ^{36}Cl age determinations. When the age of 1W17 is compared with the thermal history (Fig. 3), it indicates that the temperature of the Wasatch G-sandstone was 75 °C, whereas that of the Mesaverde Group sandstone was greater than 100 °C. This indicates that the Wasatch was certainly habitable at the time the formation water was at the surface. To understand how rapidly the groundwater moved into the Wasatch strata requires more detailed modeling of the paleohydrology.

Numerical Simulation of Groundwater Flow

Groundwater flow and heat transport within a two-dimensional domain were simulated by using a finite element code (22; Fig. 5; Table 1). The cross section was discretized into 23 rows and 75 columns. The groundwater flow and heat transport in the basin were assumed to have reached steady states. The groundwater table was assumed to coincide with the upper boundary of the model domain because of the numerous streams on the surface. The surface temperature was set to 10 °C. The measured heat flux within the basin was set at 65 mW/m^2 (8) and assumed to be uniform on the bottom. Other boundaries were no-flow boundaries and nonconductive to heat. The permeabilities used in the model (Table 1) were within the range of measured permeabilities from well production and testing (11). The modeled temperatures were within

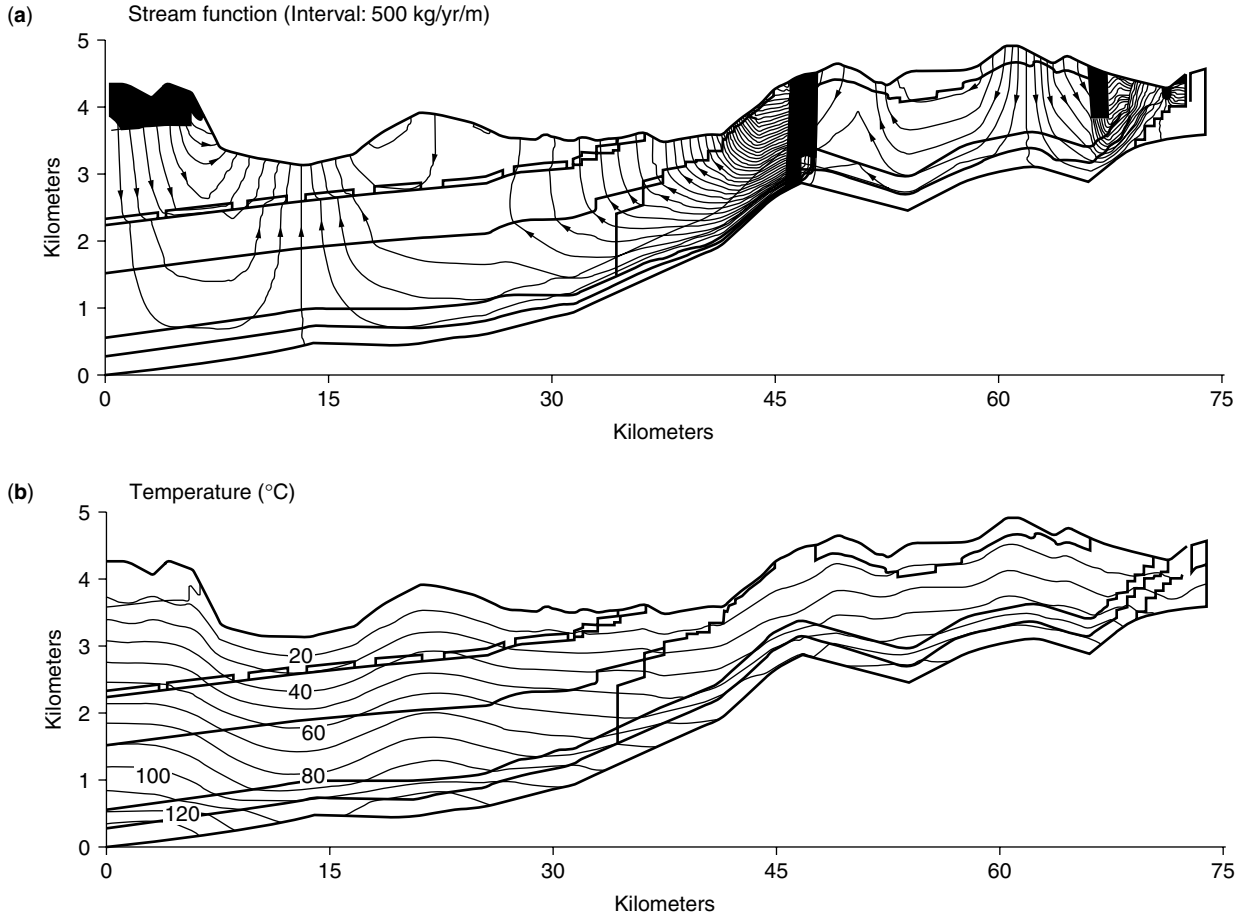

Figure 5. Numerical modeling results: (**a**) stream function and (**b**) temperature. See text for explanation.

Table 1. Material Properties used in Simulation

Rock Unit	1	2	3	4	5	6	7	8	9
Kx (m/yr)	10	0.01	0.5	0.01	0.05	0.1	0.05	0.1	0.1
Kx/Kz	1.0	0.001	1.0	0.001	0.5	1.0	0.5	1.0	0.5
ϕ (%)	0.20	0.10	0.10	0.10	0.10	0.10	0.10	0.15	0.10
λ (W/°C-m)	2.0	2.0	2.5	2.0	2.5	2.5	1.0	1.0	3.0

Note: Kx, Kz = horizontal and vertical hydraulic conductivity; λ = bulk thermal conductivity of the solid; ϕ = porosity.

5 °C of most corrected bottom-hole temperatures (compare Fig. 2 with Fig. 5b). The model correctly predicted that both the Wasatch Formation and Mesaverde Group are under hydrostatic pressure at the 1M18 site but are overpressured at the MWX-1 site. Simulated overall flow directions in the Mesaverde Group were consistent with those inferred from the potentiometric map constructed from drill stem tests of natural gas wells (compare Fig. 1 with Fig. 5a).

The model indicates that groundwater recharged on the plateau above the Colorado River valley can infiltrate vertically downward into the Wasatch Formation. The average linear velocity is 0.003 to 0.008 m/yr in the upper Wasatch Formation and 0.05 to 0.1 m/yr in the G-sandstone. Using the simulated average linear velocity and the flow path indicated by the stream function (Fig. 5a), the travel time of a particle from the oil-rich Green River shale to the G-sandstone is about 0.5 million years, neglecting the effect of diffusion, dispersion, and adsorption. The total groundwater flux into the Upper Wasatch Formation and the G-sandstone is about 3,000 kg/yr per meter of width of cross section. With this flux, several million years would be required to completely flush the Upper Wasatch Formation with fresh and isotopically light meteoric water. Thus, the simulated groundwater flux is consistent with the stable isotope ratios and ^{36}Cl data for the formation water in the Wasatch G-sandstone.

The average linear velocity is about 0.003 to 0.009 m/yr within the lower Wasatch and Mesaverde Group near the 1M18 site. The calculated particle travel time by advection alone is about 1.2 million years from the Green River Formation to the sampling depths of Cores 2 and 3. The smaller topographic relief at the beginning of the uplift and erosion could increase the travel times.

Piceance Basin Summary

Although low microbial biomass and infrequent culturable cells were the rule in samples from the Piceance Basin, indeed at the limits of detection in many cases, the microbiological characteristics of the samples were generally comparable to other deep sediment materials in being sparsely colonized by microorganisms (23). This paucity of microbial life is a common theme for investigations of cores from pristine deep strata and often contrasts with the results of studies that characterize microorganisms in aquifer or formation waters obtained through an existing well. Probably most deep strata are lightly colonized by hardy survivors of subsurface conditions although this may change once a well is drilled, bringing with it the changed conditions, including a flux of fluids surrounding the well installation.

That the bacteria found in these formations frequently exhibit metabolic flexibility has been demonstrated in the past (24,25) and may not be surprising given the need to exercise a range of metabolic strategies in order to survive. More difficult to understand would be the manner in which such organisms alter their metabolic approaches when they are probably so constrained by available energy. For example, a microorganism known to reduce both manganese and iron as terminal electron acceptors appears to have different terminal reductases depending on the metal being reduced (26). Whether both reductases would be maintained ready to function in cells surviving hostile subsurface conditions in which they may be deprived of electron donors is uncertain. The energy required for the conversion from one metabolic strategy to another would seem daunting to a cell existing under such circumstances. Hydrogen has been proposed as a subsistence energy source for subsurface microorganisms in which the primary objective of the cells is survival and not growth (27). Research studying the energy required for cell maintenance or physiological adaptation is hampered by the geologic timescales under which these cells must survive, and laboratory studies have not focused much attention on this point.

Two hypotheses can account for the presence of bacteria in the two uppermost cores: (*1*) the bacteria represent members of microbial communities that were entrapped during burial of the sandstone strata and survived the maximum paleotemperature of 120 to 145 °C for about 30 million years and (*2*) the maximum paleotemperature experienced by all three cores eradicated microbial communities entrapped during burial (Fig. 3) and bacteria infiltrated the strata during uplift and cooling of the formations over the last five million years.

The generally accepted upper temperature limit for the survival of bacterial vegetative cells and spores is 120 °C (28). Hedrick and coworkers (29) reported the presence of significant concentrations of ether lipids and phospholipid fatty acids in hydrothermal vent flanges exposed to fluid temperatures of 350 °C. This observation suggests that, within these environments, eubacterial survival at temperatures greater than 120 °C may be possible. If such hyperthermophilic bacterial survivors were present in Cores 1, 2, and 3, they should have been detected in the PLFA analyses of Core 3. The PLFA analyses are consistent with the lack of any positive microbial enrichments at incubation temperatures as high as 85 °C and suggest that bacteria did not survive the thermal history experienced by Core 3.

The hydraulically tight structure of the sandstone may have added to the demise of in situ microbial communities by limiting the supply of soluble nutrients required by the bacteria to maintain the cell and macromolecular integrity under the depravations of extreme heat. The high methane concentrations in the stratum sampled by Core 3 (14) suggest that fluid exchange was much more limited than in the strata sampled by Cores 1 and 2. This interpretation is consistent with the observed presence of fractures in Cores 1 and 2 and the absence of fractures in Core 3. If the sandstone that comprised Core 3 held pressurized gas, the entry of water and microorganisms would have been prevented. Column experiments indicate that gases in porous media form a relative permeability seal that prevents flow of water past the free gas (30,31).

Isotopic analyses of the formation of water and numerical simulations of the fluid flow indicate that meteoric water has penetrated these formations, particularly the sandstone bodies sampled by Cores 1 and 2, within the last few million years during which formation temperatures gradually dropped into the permissible range for microbial colonization. Numerical simulation yielded a water molecule travel time from 0.5 to 1.2 million years from the surface to the sampling depths. However, microbial travel time could be longer, given the small pore throats in the sandstone, if the microbial adhesion to the mineral surfaces was low and the closely spaced fractures acted as the main pathways for microbial transport during the uplift and cooling phase of the basin (Fig. 3).

Accordingly, sandstone proximal to fractures in Core 1 and 2 could be colonized, whereas small pore throats of the encompassing shales would impede colonization of the sandstone in Core 3, unless it was near the fractures. The overpressured nature of the sandstone compartment sampled by Core 3 indicates that gas transport is impeded, which certainly implies that it is impervious to microbial infiltration. Because the microorganisms cultured from these formations do not exhibit growth at temperatures greater than 75 °C, they more likely represent immigrants rather than survivors.

MICROBIOLOGY OF OTHER DEEP SEDIMENTARY STRATA

Taylorsville Basin

The Taylorsville Basin underlying Virginia, U.S.A., is another deep sedimentary basin where the microbiology has been studied through the analysis of cores. The basin is a Late Triassic-Early Jurassic synrift basin

that formed on the eastern edge of North America (32). The Mesozoic sandstone and shale of the basin are overlain by Coastal Plain sediments of the Miocene age (to 5 Ma) (33). The Coastal Plain sediments are relatively permeable and receive considerable recharge; however, the low-permeability Triassic strata are hydrologically more isolated and thus, more likely harbor microbial cells that have been removed from surface contact for long periods. This feature, along with infrequent drilling activity in the deeper, older strata made the Taylorsville Basin a target for studies of ancient subsurface bacteria. As with the Piceance Basin coring efforts, the degree of contamination as a result of the coring was minimized and quantified (34,35).

Several studies verified the presence of unique microorganisms in the strata of the Taylorsville Basin and one of these has been highlighted in the literature. *Bacillus infernus* was obtained from a rotary sidewall core sample taken from 2,799 mbls in the Taylorsville Basin (36). This microorganism exhibits several attributes that suggest that it is designed to survive under subsurface conditions. Like many others isolated from subsurface strata, it is metabolically versatile (24). Although it is strictly anaerobic, it can use a number of diverse electron acceptors, including Fe(III), MnO_2, trimethylamine oxide, and nitrate. Cells of *B. infernus* can ferment glucose or oxidize formate or lactate coupled to the reduction of oxidized iron or manganese. It is thermophilic, growing at temperatures up to 61 °C and halotolerant with strains growing at up to 2.1 M Na^+. The cells are able to survive starvation for at least 52 days at 50 °C. This survival capability, along with its placement in the genus *Bacillus*, suggests that the organism can form endospores, although they have not been observed. Phylogenetically, its affiliation as a species within *Bacillus* is quite interesting because no other strictly anaerobic species of *Bacillus* had been described.

Other microorganisms from the Taylorsville Basin include new species of the genus *Desulfotomaculum* (37). In contrast to *B. infernus*, these bacteria are nonmotile, endospore-forming, sulfate-reducing bacteria that do not use metals as terminal electron acceptors. Liu and coworkers (25) described thermophilic Fe(III)-reducing bacteria that were obtained from the Taylorsville Basin (and from the Piceance Basin). They too, are unique from *B. infernus*, based on the 16S rDNA phylogenies, and this study represents the first consideration of the importance of thermophilic metal reducers in the context of early earth history. Evidence of the wide phylogenetic distribution of iron reducers, these subsurface organisms constituting a new lineage apart from the mesophilic iron reducers, suggests an early evolution of the iron-respiring trait and subsequent conservation of the trait through the diverging lineages of the Bacteria (38). That these microorganisms were recovered from geographically and geologically isolated, ancient sedimentary basins and that they maintain the capability to reduce iron further suggests that this phenotypic attribute extends back at least to the Mesozoic (25).

Tseng and coworkers (15) attempted to constrain the mechanisms by which these microorganisms could exist in the subsurface of the Taylorsville Basin. To assess the likelihood of long-term microbial survival in place or transport into the Mesozoic strata, this group refined models of the thermal and burial history of the basin by characterizing aqueous and gaseous fluid inclusions and evaluating fission track data from materials derived deep in the basin boreholes. Their data suggest that at the time of maximum basin burial (ca. 200 Ma), temperatures at the microbial sampling depths probably exceeded 160 °C for several million years. Although we cannot verify that microorganisms did not survive these conditions, microbial biomass would be significantly reduced during this period of high temperatures in the basin. Furthermore, the authors suggest that these strata were not again habitable by microbes until 140 Ma, when the in situ temperatures at the microbial sampling depth cooled to around 100 °C, providing a habitat where at least hyperthermophiles could survive. However, for the microorganisms that could be cultured from these depths, none could grow at greater than 75 °C (25) and the model for the basin's thermal history developed by Tseng and coworkers (15) suggests that these lower temperatures were not characteristic of the sampling depths until around 60 Ma. Although conclusions based only on culturable microorganisms are tenuous, this could mean that the colonization front of viable cells advancing from the surface strata did not arrive in the deep sandstones and shales of the Taylorsville basin until the early Cenozoic. However, the in situ parameters of a cell's environment can shift the thermal limits for microbial growth and may influence cell survival in the subsurface. For example, high hydrostatic pressure can increase the maximum growth temperature in some microorganisms (39,40). Thus, the estimate that microbes became reestablished in the Taylorsville basin during the early Cenozoic is hypothetical and hinges on uncertainties in microbial survival characteristics.

Another study of the Taylorsville Basin proposes that the tectonic properties of a sedimentary basin determine when and at what rate microorganisms might colonize deep strata (41). Using two-dimensional paleofluid flow and heat transport modeling, Tseng and Onstott speculated on the rates and timing of microbial movement into the deep Taylorsville Basin rocks. This study indicates that during the Jurassic the basin topography, by virtue of regional tectonic uplift and erosion, forced groundwater into the deep strata at a rate of 1 to 100 mm/year. Given that rate of water movement, microorganisms from the surface could have reached the sampled depths in 1 to 20 million years. Thermophilic microorganisms such as those cultured from the cores could have reached the sampled depths even faster during Jurassic groundwater movement if they already resided deep in the formation at the 75 °C isopleth (ca. 1,300 mbls), at the edge of microbially survivable temperatures. More recently (from 140 Ma until today), groundwater flow rates are estimated to be 10 to 100 times slower than during the Jurassic. Because of these lower rates, a factor of the lower topographic relief in the region than in the Jurassic, the minimum time required for cells to make the trip from the ground surface to the microbially sampled intervals is between 50 and 180 million years.

Hydrocarbon-Rich Deep Sedimentary Formations

Because sulfate-reducing bacteria (SRB) can reduce the quality of crude oil by producing sulfide, considerable effort has focused on the presence of these microorganisms in oil reservoirs.

Culturable thermophilic and hyperthermophilic bacteria, including SRB, have been obtained from fluids emanating from newly developed crude oil reservoirs at several locations. In 1993, these organisms were obtained from drilling locations in the North Sea and the North Slope (42). Although novel methods were not used to collect the samples (the investigators relied on collection of reservoir fluids from oil production locations), this study was marked by the application of hyperthermophile cultivation approaches by one of the premier international laboratories for these types of investigations. The oil reservoirs that were investigated had all undergone waterflooding with seawater (up to 127,000 m^3 per day), indicating the potential that these organisms were introduced into these subsurface formations via oil production activities. That these microbial communities had been introduced through drilling or waterflooding is a conclusion that is consistent with characterization studies of microorganisms that were obtained from produced waters from souring oil reservoirs (43).

In contrast, the authors of another study of North Sea subsurface microbial communities concluded that the microorganisms in the reservoir were likely indigenous (44). In that study, thermophilic cells were isolated from formation fluids that were obtained before any water injected into the reservoir had reached the production well. Thus, the seawater used to flood this reservoir could not have served as a source of the microorganisms. The authors could not completely rule out drilling muds or drilling equipment as the source of these microorganisms. However, they contend that the large numbers of thermophilic sulfate-reducers present in the samples speak in favor of indigenous communities in the reservoir because the in situ conditions would have limited the populations of chance contaminants that were inoculated as a part of the original drilling activities. In another study of the North Sea region, a core was obtained from 1,000 meters below sea floor in a pristine location of the United Kingdom Continental Shelf (45). That a sustaining thermophilic enrichment culture of SRB was developed from the inner portions of the core further substantiates the contention that these bacteria are indigenous to deep sedimentary strata that are rich in crude oil.

Until recently, the presence of anaerobic microorganisms in deep oil-bearing strata was difficult to explain because of the limited evidence that such cells could sustain themselves through anaerobic degradation of the hydrocarbon characteristic of crude oils (e.g., long-chain alkanes and alkylbenzenes). However, studies within the last decade have led to a greater appreciation of the number of physiological mechanisms by which microorganisms can cause meaningful anaerobic degradation of higher hydrocarbons (46). Long-chain alkanes can be degraded by classical anaerobic physiologies such as denitrifiers (47,48), sulfate-reducing bacteria (49), and methanogens (50). Alkylbenzenes can be degraded by denitrifiers (48) and sulfate-reducing bacteria (51,52). Thus, although the conditions of many deep sedimentary formations may confine the metabolic activity or even survival of cells, multiple physiological strategies have appeared to evolve for the slow but efficient use of complex organic compounds in such environments.

CONCLUSION

Common themes that exist for microbial survival in deep strata include how cells can weather the enormous constraints associated with isolation from electron donor and electron acceptor supply that is dictated by deep burial and low fluence typical of low-porosity, low-permeability materials. Add to these issues the maximum temperature for microbial survival associated with deep sedimentary rocks and one could conclude that life would be sparse or nonexistent in remote underground locations. Nevertheless, the research summarized earlier indicates that meager populations of microorganisms are found residing in deep sedimentary strata for millions of years at temperatures less than 85 °C. Such systems rarely bear as much biomass as surface soils, but on a volumetric basis, the total biomass of these deep realms is globally significant (53).

An equally important question is how the microorganisms came to exist at great depths. The conclusions from the microbial studies of the Piceance Basin and the Taylorsville Basin are similar in that they ascertain that in the past these rocks were most likely too hostile for life to exist. In each case, the chemical and physical data from these basins indicate that the original cells deposited in prehistoric rivers or lake sediments were probably snuffed out by intolerably high temperatures. Additional data suggest that the current distribution of microorganisms in these deep rock environments is predicated on an uninterrupted route for water to travel from the surface to subsurface strata via a network of fractures, microfractures, and interconnected porosity at scales appropriate for microbial transport. These routes for colonization develop during periods of tectonic deformation, uplift, and the associated cooling that restores the temperatures of the deep strata to a habitable level. Even then, the microorganisms that first reenter these voids must be able to survive the austere surroundings.

Many questions remain unanswered regarding bacteria in these deep strata. What is their primary source of energy? Although many of them appear to be physiologically versatile, what can they really count on in terms of energy supplies? The ubiquitous and highly diffusive nature of hydrogen gas in subsurface environments makes this fundamental nugget of electrons a possible source of energy (27), but from whence does it originate? Are most of the cells that are found in these strata sustaining themselves by low levels of metabolic activity or are they zymogenous [i.e., those that persist but are inactive under present environmental conditions (54)]? And related to this, how long can they actually survive? Recent studies of subsurface salt deposits postulate microbial survival at 250 million years within saline fluid

inclusions (55). Understanding the mechanisms by which microorganisms survive over eons in deep rocks of any nature will continue to challenge scientists more than the investigations that aim merely to recover these cells and define their longevity.

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MICROCHIPS. See Biochip-Based Devices and Methods in Microbial Community Ribotyping; Microarrays: Applications in Environmental Microbiology

MICROGRAVITY EFFECTS ON MICROORGANISMS.

See Space Microbiology: Effects of Ionizing Radiation on Microorganisms in Space; Space Microbiology: Microgravity and Microorganisms

MICROORGANISMS IN SOIL: FACTORS INFLUENCING THEIR ACTIVITY

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The objectives of this article are to provide a brief overview of the physicochemical and biological factors that influence the activity, ecology, and population dynamics of microorganisms in soil. Soil differs from other microbial habitats in that it is dominated by a solid phase consisting of particles of different sizes surrounded by aqueous and gaseous phases, the amount and composition of which fluctuate markedly in time and space. The solid phase is a tripartite system composed of finely divided minerals (both primary and secondary); residues of plants, animals, and microorganisms in various stages of decay; and a living microbiota. These particles exist as independent entities and mixed conglomerates. The aqueous phase, in which inorganic and organic substances that serve as nutrients for or inhibitors of the microbiota are dissolved, is normally discontinuous, except when soil is saturated (e.g., after a heavy rain, snow melt, flooding, and extensive irrigation). This discontinuity restricts the movement of microbes, especially of bacteria and other nonfilamentous forms, and results in local accumulations of nutrients and toxicants, escape of cells from grazing predators, a low probability of gene transfer, and other phenomena that do not occur in habitats with a continuous water phase. The pore space not filled with the aqueous phase is filled with air and other gases and volatiles. Because of the low diffusion coefficients of most gases and the quiescent nature of soil, in contrast to the continuous movement and wave action of aquatic environments, some gases (e.g., CO_2, CH_4, CO, NO_x) and volatiles (e.g., short carbon-chain fatty acids, alcohols, and aldehydes, as well as aromatic compounds) may accumulate and other gases (e.g., oxygen) may be depleted. The concentration of gases, in turn, will affect the composition and activity of the microbiota (e.g., aerobes vs. facultatives, anaerobes, and microaerophiles; 1–3).

Despite the vagaries of the environmental conditions that are conducive to microbial growth (e.g., in available nutrients, especially in available carbon and energy sources, although limitations in other nutrients may be as or more restrictive, and in available water, temperature, radiation, nutrients, osmotic pressure), soil probably contains more genera and species of microorganisms than other microbial habitats, because soil is exposed to and eventually receives essentially all microbes present on earth. Some of these species are present in low numbers, probably because the conditions for their survival and growth are restricted to discrete sites where the nutritional and other physicochemical environmental factors necessary for their establishment, growth, and survival are located. This is one of the results of a structured environment with a high solid:liquid ratio. Consequently, on the basis of the numbers and diversity of microbes in soil, soil appears to be a good habitat for microorganisms (*good* being an anthropocentric concept) despite the apparent limitations for their survival and growth, probably because microbes indigenous (autochthonous) to soil are well adapted to this relatively austere environment. However, many exogenous microbes are not naturally adapted to and do not survive in soil. Most microbes indigenous to soil are oligotrophic or zymogenic, which may explain their higher numbers and greater diversity than in other habitats that are more copiotrophic (e.g., skin, oral and vaginal cavities, gastrointestinal and urinary tracts) and why many species from these habitats do not apparently establish, grow, and survive well in soil.

Microorganisms, perhaps more than any other organisms, are highly adaptable, both physiologically and genetically. They have to be as they have been around for more than 10^9 years. In addition, soil obviously selects and enriches for some microbes, and there is a homeostasis in soil that has probably existed for millions, if not billions, of years. It is this homeostasis that human beings are constantly attempting to alter for their presumed benefit (e.g., enhanced production of food and fiber). These alterations have involved primarily inoculation of soil (e.g., with *Rhizobium* sp., including genetically modified strains, to enhance nitrogen-fixation by legumes; with mycorrhizal fungi to enhance the uptake of phosphorus by plants; with white-rot fungi to enhance degradation of toxic and persistent organic pollutants; and with a variety of rhizobacteria and other microbial antagonists of soilborne, root-infecting pathogens).

Although some of these inoculations have been successful and appeared promising in the laboratory and even in the greenhouse, they have generally been less successful in situ, especially in the long term, probably because the emphasis has been directed toward altering — presumably improving — the physiology of the microbes. This emphasis incorrectly assumes that the

physiological characteristics of microbes, which have evolved for millions of years and have adapted to and have been selected by the soil environment, can be changed significantly to improve their establishment, growth, survival, and desired activities in soil. Even when microbes have been genetically engineered, which presumably simulates millennia of evolution, their survival in soil is seldom improved. In the case of genetically modified organisms, the introduced genes usually code for catabolic functions (e.g., degradation of xenobiotics), for markers (e.g., antibiotic resistance, *lux* genes) that enable monitoring their persistence in soil, or for some product (e.g., the insecticidal toxins produced by subspecies of *Bacillus thuringiensis*). However, even in the presence of the specific substrates on which, for example, the products of catabolic genes function, which would presumably provide an ecological advantage to the modified organisms, the introduced genetically modified organisms seldom survive longer in soil than the unmodified parental strains (4–8).

Consequently, the major emphasis should not be on the physiology of potential inoculants but, rather, on the physicochemical characteristics of soil that affect the establishment, growth, survival, and activity of the inoculants, and that of indigenous microbiota, whose importance should not be minimized. For example, control of pathogens (the term *pathogen* is another anthropocentric concept) occurs naturally in soil as part of the normal competitive, antagonistic, parasitic, and predatory interactions between microbes, although often at levels below those desired by human beings. Most organisms that have been introduced into soil as biocontrol agents were originally isolated from soil (e.g., nematode-trapping fungi, antibiotic-producing actinomycetes, antagonistic rhizobacteria, *B. thuringiensis*) in which they were exerting some level of control. The large-scale in vitro cultivations of these organisms and their subsequent reintroductions into soil have been, for the most part, unsuccessful. This lack of success is probably because the physicochemical and biological factors that kept the indigenous organisms at some low level of activity also reduced their numbers and biocontrol activity to the same low levels shortly after their reintroduction into natural soil, either with or without the target pathogen (1,2,9).

The same phenomena have been observed for the introduction of microbes that, for example, degrade xenobiotics, fix nitrogen as either nodule-formers or free-living microorganisms, or establish mycorrhizal associations: the colonization and subsequent activity of these inoculants is not restricted by their physiology but by the physicochemical characteristics of the recipient soil. However, the possibility that in vitro cultivation of microorganisms changes their physiology so that they lose their ability to survive and grow in soil after reintroduction needs to be studied.

If the specific physicochemical characteristics of soil that control the growth and survival of individual species were known, it might be possible to manipulate those characteristics to encourage the growth of desirable microorganisms (e.g., nitrogen-fixers, biocontrol agents, degraders of xenobiotics) and discourage the growth of undesirable ones (e.g., pathogens of plants and animals), which is the ultimate purpose of studying microbes in soil and other natural habitats. Different physicochemical factors differentially affect different groups of organisms (e.g., bacteria vs. fungi) and even different species. Control of organisms in soil by the appropriate manipulation of these factors could decrease the use of pesticides, fertilizers, and other xenobiotics, which have numerous deleterious effects on the biosphere.

The importance and effects of the physicochemical factors of soil, both with respect to the soil microbiota in general (1–3) and, more specifically, to the transfer of genetic information among bacteria in soil (4–8), have been discussed in detail. Consequently, they will be discussed here in only general terms.

MICROHABITATS IN SOIL

Microorganisms can be considered to be aquatic creatures, and their metabolism in soil is restricted to sites where water is available. Hence, their distribution in soil is restricted essentially to sites that contain clay minerals because sand and silt do not retain water against gravitational pull. Clays, because of their surface activity (which results from their unique crystalline structure because they are essentially secondary minerals), retain water against gravitational pull. The water adjacent to their active surfaces and coordinated with charge-compensating ions on the clays becomes sufficiently ordered to form a quasicrystalline structure (i.e., the strong attraction of water molecules to the negatively and positively charged loci on clays and to their charge-compensating ions enhances the hydrogen bonding of adjacent water molecules). The ordering of this clay-associated water decreases with distance from the clay surface until a distance is reached at which water is no longer under the attraction of the clay and is susceptible to gravity (2).

Clay minerals do not generally exist free in soil but exist primarily as coatings, or cutans, on larger sand and silt particles or as oriented clusters, or domains, among these particles. The clay-coated particles cluster together, primarily as the result of electrostatic attraction between the net negatively-charged faces and the net positively-charged edges of clay, into microaggregates, which, in turn, cluster together to form aggregates that can range from 0.5 to 5 mm in diameter and are stabilized by organic matter and precipitated inorganic materials. These aggregates retain water, the thickness and permanence of which depend on the type and amount of clay and organic matter within the aggregates, and this water may form "bridges" with the water of adjacent aggregates. These aggregates or clusters of aggregates, with their adjacent water, comprise the microhabitats in soil wherein microbes function (3).

As a result of the discreteness of microhabitats in soil, interactions between microbes, both positive (e.g., commensalistic, protocooperative, mutualistic, synergistic) and negative (e.g., competitive, amensalistic, parasitic, predatory), probably occur less frequently and more sporadically than in habitats where water is continuous. Even when the pore space is saturated with the aqueous phase

or where water bridges among adjacent microhabitats occur, movement of bacteria among microhabitats may be restricted because the surface tension of the ordered water around aggregates may be too great to allow passive movement of cells or even active movement of flagellated cells. There is no convincing evidence that bacteria are flagellated in soil, although they may have the genetic capability to produce flagella when isolated from soil and cultured in liquid media or on agar. Moreover, if motility was important to the survival of bacteria in soil, all autochthonous species would probably be flagellated. However, filamentous fungi are able to cross pore spaces among microhabitats, even when the spaces are not filled with the aqueous phase. Filamentous fungi grow by apical and lateral extension of hyphae from a food- and water-base in a microhabitat, and because they translocate nutrients and water internally, they are essentially independent of the ambient nutritional and aqueous conditions of the ramifying mycelia. In addition, the hyphae are surrounded by water films in which bacteria, bacteriophages, nutrients, genetic material, and so on, can be transported from one microhabitat to another.

The conditions within a microhabitat can also affect microbial activities. A bacterium that sticks, if it sticks (discussed later), on a clay cutan or domain after its chemotropic attraction to adsorbed organic substances may be nutritionally deprived after it consumes the adsorbed substances, if it can use the adsorbed substances (discussed later) because it will be dependent on the rate of diffusion of new substances for its nutrition. If the adsorbed substances to which it was attracted are toxic, the bacterium may die (3).

The mechanisms by which organic molecules are bound on clay will determine the tenacity with which they are held and the ability of microbes, usually with the aid of extracellular enzymes, to use these molecules as substrates. The presence of chaotropic ions (which decrease the structure of water and tend to disrupt hydrophobic interactions by increasing the accommodation of nonpolar compounds in aqueous solutions) and of antichaotropic ions (which increase the structure of water and, thereby, increase hydrophobic interactions by reducing the ability of aqueous solutions to accommodate nonpolar groups) will affect the nutritional status of the microhabitat. Although clay cutans and domains are relatively stable, some clay particles may become dislodged and get attached to the surface of microbes in the microhabitat. Such attachment reduces the effective surface area of microbes for transmembrane transfer of nutrients inward and of waste products outward (3,10).

In addition to their discreteness, microhabitats in soil are highly variable and heterogeneous. This heterogeneity is a result of the heterogeneity of the surfaces, which are coated, partially or completely, with clay minerals, hydrous metal oxides, and organic matter with pH-dependent and pH-independent charges (11), and of fluctuations in the types and concentrations of organic and inorganic solutes in the soil solution (1–3). Even over small distances, the composition and size of the particles, the amounts and types of solution, nutrients, and gases, and the pH, E_h, ionic strength, and other physicochemical characteristics can vary. This variability in abiotic factors is reflected in the heterogeneity of the microbiota, which is demonstrated by the simultaneous occurrence in the same soil sample of autotrophs and heterotrophs (both oligotrophs and copiotrophs), aerobes and anaerobes, vegetative cells and spores, prokaryotes and eukaryotes, and cells with different requirements for and tolerances to ambient and extreme conditions of pH, E_h, temperature, osmotic pressure, and so on.

These conditions in soil differ markedly from those in sediments of aquatic systems. Although clay minerals in sediments also occur as cutans on larger particles and as domains in aggregates, water-dependent microhabitats probably do not occur as they do in soil, because the water in sediments is continuous from one aggregate to the next. Moreover, microbes in sediments appear to colonize primarily sand and silt particles, rather than clay, because water surrounds these particles, and the need to overcome the electrokinetic repulsion between net negatively charged clay minerals and microbial cells is reduced or eliminated (2).

It is the task of the soil microbiologist to determine the abiotic and biotic factors that control the activity and population dynamics of microorganisms in soil and to devise methods for manipulating the responsible factors to enhance or attenuate the activities of specific components for some desired anthropocentric benefit. A brief discussion of each physicochemical or biological factor follows, along with speculations on how it may be manipulated. These discussions and speculations are not intended to be exhaustive (1,2). Because of limitations in space, references are primarily to reviews in which citations of numerous relevant original papers can be found. In addition, some recent texts on soil microbiology and microbial ecology, which provide more information on the effects of the physicochemical and biological characteristics of soil on microbial processes, are referenced (12–22), as is the classic volume of Brock (23) on microbial ecology.

FACTORS

Carbon and Energy Sources

Although most soils contain considerable amounts of organic matter, much of this material is relatively unavailable as a source of carbon and energy because it has been humified. The addition of a source of readily available carbon, such as fresh plant residues (green manures), bagasses, or sugars, will enhance the growth of the microbiota until the available carbon has been mineralized and the activity and density of the microbiota declines to its original level. Although this stimulation of the microbiota is generally nonspecific, it may result in the degradation of some recalcitrant materials (e.g., some xenobiotics), as a result of a general "priming action," and in some biocontrol of soilborne plant pathogens (1,2). The addition of specific compounds that only desired members of the microbiota can use could enhance degradation, biocontrol, and other beneficial activities. Unfortunately, compounds with such specificity have not been identified,

and many indigenous microbes share the same enzymatic capabilities. Nevertheless, the ability to enhance, even if only temporarily, the growth and survival in soil of genetically modified bacteria by amending the soil with the specific substrates on which the products of the novel genes function suggests that this use of substrates for enrichment of specific indigenous microbes should be explored further (4,8).

Mineral Nutrients

As with carbon and energy sources, most soils contain sufficient total amounts of mineral nutrients, but most are present in unavailable forms. The sequence of requirements of the major inorganic nutrients for optimum microbial growth is N > P > S (1,2). Fertilization of soil with these nutrients will enhance growth but usually nonspecifically. In some cases, too much of a mineral nutrient will suppress the growth or activity of a desired population. For example, the presence of elevated amounts of inorganic nitrogen will inhibit the lignolytic activity of *Phanerochaete chrysosporium* (24). The addition of materials with a high C : N ratio (e.g., saw dust, corn cobs) to immobilize the inorganic nitrogen may be an effective and inexpensive method to enhance the activity of this fungus and its degradation of various recalcitrant xenobiotics (9).

Growth Factors

Because the requirements for specific growth factors differ for different species, the addition of such factors may enhance the growth of desired species better than the addition of nonspecific carbon and energy sources. Unfortunately, too little is known about the specific requirements for the growth factors of specific species, although vitamin B_{12} (cyanocobalamin) and various siderophores (which are here considered to be growth factors) are good candidates. It is unfortunate that the early studies by Lochhead and colleagues (25,26) on the effects of growth factors on microbial events in soil have not been extended. However, even if specific growth factors for specific components of the soil microbiota can be identified, their persistence and activity in soil may be limited; for example, they may be rapidly mineralized, as are other added organic molecules, or, in the case of siderophores, they may bind on surfaces, such as clay minerals, and not perform their function (27).

Ionic Composition

The soil solution is essentially a weak electrolyte composed of a variety of organic and inorganic cations and anions. Because the interface between microbial cells and the soil solution is essentially ionic, even small changes in ionic composition and strength probably have a significant effect on the growth and activity of the microorganisms and on the binding of organic compounds on surfaces. Unfortunately, not enough is known about how the ionic composition of soil affects specific organisms to suggest the manner in which the ionic composition can be manipulated beneficially. However, care must be taken in any such manipulation because large changes in ionic composition may alter the osmotic pressure and the availability of water.

Available Water

Perhaps the most important physicochemical factor that affects microbes in soil is an adequate supply of available water. Microbes are essentially aquatic organisms and even in soil require a sufficiently high water activity (a_w) for growth. Except for short periods after rain, snow melt, flooding, or irrigation, soils usually contain insufficient available water to support a high level of microbial activity because the water is rapidly drained by gravity. The clay fraction has a major role in water retention because of its high surface charge, which holds water against gravity, and this bestows some stability to microbes present in this water. Consequently, the apparent correlation between microbial activity and the amounts and types of clay in soil may be the result primarily of the fact that this is where water is located, and the presumed importance of clays in concentrating nutrients, removing inhibitors, direct surface interactions, and so on, may be only secondary (2). However, some of the water retained by clay is so tightly bound that its a_w is too low for use by microbes (e.g., temperatures in excess of 160 °C are necessary to remove this water). The amount of such tightly bound water depends on the structure of the clay minerals: for example, 2 : 1, Si:Al clays (e.g., smectites) bind water much more tightly than 1 : 1 clays (e.g., kaolinite) (i.e., the matric potential of smectites is higher). Consequently, the total amount of water present in a soil (as determined by drying) or the amount of water that a soil can hold (the water-holding capacity) does not indicate accurately the amount of water available to microbes. Microbes function best at a water potential of −33 kPa (which is close to the field capacity, defined as the amount of water retained by soil 48 hours after its saturation), in which no more water will be removed by gravity and the balance between the amounts of available water and oxygen is optimum for growth (28). This water potential is most easily determined with a pressure plate extractor.

Particulate organic matter also retains ordered water, primarily as a result of the polar groups on the organic matter. This water is also tenaciously held and not all of it is available to microbes. For example, the water-holding capacity of an organic soil (85% organic matter) was 262% (w/w), but only when the water content was at the −33-kPa water tension of the soil (157% w/w) was the a_w sufficiently high for significant microbial activity (1–3). Moreover, drying of soil can render the organic matter difficult to rewet, as a result of its structural reorientation and exposure of hydrophobic regions. This development of hydrophobicity can also cause clay-organic aggregates to become difficult to rewet (29).

Different groups of microbes have different tolerances to a_w (e.g., fungi can metabolize at a significantly lower a_w than can bacteria). The water content of soil is easily controlled by irrigation, but “fine-tuning” of the manipulation of this physicochemical factor for the differential control of microbes will require considerably more study. In many cases, the effects on microbes of

changes in the water content of soil are the result of changes in the oxygen content.

Temperature

Extremes in temperature can result in the majority of the indigenous microbial populations being psychrophilic or thermophilic, depending on the geography of the soil. However, most soils worldwide contain predominantly mesophilic microbes. Regardless of the geographic location of the soils, the temperature of the soil at the surface, a few centimeters from the top, where most of the microbial activity usually occurs, does not fluctuate more than a few degrees centigrade in any season of the year. Consequently, the temperature of soils in situ cannot be conveniently manipulated for extended periods. Although the heating of soil for the control of some plant pathogens (e.g., solar heating or steam after covering the soil with black plastic) can be accomplished relatively easily, this is generally a nonspecific treatment because essentially all indigenous microbes are inhibited and then return to their original population levels after the soil reverts to its normal temperature range. These sites are then naturally inoculated with the same microbes, both pathogenic and nonpathogenic, from adjacent areas. To achieve long-term control, usually requiring the elimination or reduction of specific populations, other critical changes in the physicochemical characteristics of the soil (e.g., change in the clay mineralogy) need to be made before heating.

Pressure

Changes in atmospheric pressure are probably too small to affect the soil microbiota significantly, and the overall solute concentration of normal soil solutions is usually not high enough to suggest that osmotic pressure inhibits the microbiota. However, the osmotic pressure may increase sufficiently to affect some microbes within microhabitats where the solute levels are probably higher because of their concentration at solid–liquid interfaces, especially at charged surfaces, and during periods of drying. This is especially true in saline and alkaline soil. Most microbes in soil are probably stenohaline and, therefore, susceptible to damage by hypertonic osmotic pressures. Moreover, marked increases in solutes will affect the osmotic potential of water and decrease its availability. Altering the osmotic pressure of soil by the addition of organic or inorganic solutes is relatively easy. Some years ago, control of nematodes in soil was achieved by the addition of copious quantities of molasses, presumably because of the increase in osmotic pressure (W. Feder, personal communication). However, because large changes in solute concentrations can affect numerous other physicochemical characteristics (e.g., available water, pH, oxygen content, and mineral nutrients if the solute can be used as a carbon source), caution is recommended when the manipulation of this factor for the control of microbes in soil is considered, especially as the effects may be nonspecific.

Atmospheric Composition

Because air and water share the same pore space among microhabitats, the atmospheric composition in soil can be easily manipulated, at least on an overall gross basis, by altering the water content. Flooding has been used in an attempt to control some soilborne fungal pathogens of plants (e.g., Fusarium wilt of banana) because most filamentous fungi are obligate aerobes (2). However, the various spores of fungi, especially chlamydospores, appear to be able to resist long periods of anaerobiosis, and when the soil subsequently becomes aerobic again, the spores germinate and reestablish the fungal colonies. Moreover, many fungi — especially, but not restricted to, pathogens of animals — are dimorphic and can persist in the yeast form under anaerobic conditions.

The addition of large quantities of available substrates will also reduce the amount of oxygen and increase the levels of carbon dioxide in the soil. However, even under "normal" conditions, the content of carbon dioxide is higher and that of oxygen is lower in soil than in the overlying atmosphere, as a result of microbial metabolism and the slow diffusion rate of these gases. Even in well-aerated soils, anaerobic sites are present, as indicated by the ability to isolate obligate anaerobes from such soils. In addition to O_2 and CO_2, other gases (e.g., CH_4, NO_x) and volatiles (e.g., short carbon-chain organic acids, aldehydes, alcohols, esters, hydrocarbons, ethylene) are present in the atmosphere of soil and can serve as either substrates for or inhibitors of microbes (30,31).

Manipulation of the soil atmosphere for the control of specific components of the microbiota is the basis of the fumigation of soil with a variety of xenobiotics. In many cases, however, the effects of these pesticides are transient, and they often affect nontarget organisms also. Nevertheless, manipulation of the soil atmosphere appears to have a good potential for controlling specific portions of the microbiota in soil, especially if it can be fine-tuned and coupled with the manipulation of other physicochemical factors to enhance the duration of its effects.

Electromagnetic Radiation

Although light probably affects only microbes residing on the surface of soil, it can be important in arid and semiarid soils in which photosynthesis in algal crusts, both prokaryotic and eukaryotic, is probably the major source of carbon and energy (32). In such environments, resistance to ultraviolet radiation (e.g., pigments) is probably necessary. Microbes residing below the soil surface do not generally have such resistance, and manipulation of the soil that periodically exposes these microbes to direct solar radiation (e.g., cultivation) can exert some control, although relatively nonspecific, on the microbiota. Although radiation is a physicochemical factor of major importance in aquatic environments, it is generally of minor importance in soil and, hence, difficult to manipulate for the control of specific species.

pH

The hydrogen ion (H^+) concentration is a major physicochemical characteristic of soil that is amenable to manipulation in situ. In general, fungi predominate in acidic soils (below pH 5.5), whereas most eubacteria, including actinomycetes, predominate in near-neutral or moderately

alkaline soils. The apparent lower numbers of fungi in the latter soils is not because fungi are intolerant to these pH values but because bacteria are efficient competitors at these pH values and prevent the establishment and proliferation of fungi. In contrast, the lower numbers of bacteria in acidic soils is the result of their intolerance to the elevated concentrations of H^+ that fungi can tolerate, and therefore, the fungi can proliferate in the absence of bacterial competition. In addition to numerous effects of pH on a spectrum of physiological, morphological, and metabolic responses of the microbiota, the pH also affects the solubility, availability, and toxicity of mineral nutrients; the speciation of heavy metals (33); the sign of the net surface charge of amphoteric molecules and the negative charge of ionizable molecules, which will affect the adsorption and subsequent binding of cells and organic molecules on surfaces and, hence, their availability as nutrients and their bioactivity (2); and numerous other phenomena. Manipulation of the pH of soil is done routinely, although primarily to enhance the growth of plants, by the addition of lime, to raise the pH, or of gypsum or some other relatively inexpensive source of sulfur, to reduce the pH. A prime example of the manipulation of the pH of soil for biological control is the reduction in pH to control the scab of potato caused by *Streptomyces scabies*.

Oxidation-Reduction Potential

The E_h of soil is related primarily to the oxygen content of soil and, therefore, can be manipulated, to some extent, by cultural practices (e.g., cultivation, irrigation, improvement of drainage) (34). The E_h is also influenced by pH, temperature, pressure, and nutrients. The ability to isolate aerobes, facultatives, and anaerobes (including fermentors, methanogens, and sulfate-reducers) from the same soil sample indicates that microhabitats that differ in E_h by approximately 1,000 mV (e.g., from +700 to −300 mV) can coexist in the same soil. Consequently, fine-tuned manipulation of the E_h of soil to control specific components of the microbiota would appear to be difficult.

Surfaces

Some aspects of the importance of surfaces, especially those of clays, to microorganisms and their activities in soil were discussed earlier. The type of clay present in soil has been shown to have a profound influence on numerous microbial activities in soil (e.g., growth; heterotrophic, autotrophic, and mixotrophic metabolism; organic and inorganic nutrition; spore germination; competition, amensalism, predation, and parasitism; transfer of genetic information; pathogenesis) and provides protection against the toxicity of acid precipitation, heavy metals, organic compounds, gases, volatiles, hypertonic osmotic pressures, elevated temperatures, desiccation, ultraviolet light, and X rays (2). How clays affect these activities is not always clear. In many cases, the effects of clay appear to be indirect by modifying other physicochemical characteristics of the microhabitats (e.g., pH, water potential, nutritional status, activity of toxicants), which either enhances or attenuates the growth and metabolic activities of individual population, which in turn, influence the growth and activities of other populations. The indirect nature of these effects has been demonstrated in studies in which the same results were obtained whether or not the clays and the microbes were separated by a dialysis membrane (2).

The demonstration of direct effects, which involve surface interactions (e.g., adhesion) between clays and microbes, is more difficult. There is empirical evidence to suggest that such surface interactions occur in situ: for example, lack of movement of large numbers of microbes from surface to underlying soil layers and then to groundwater during heavy rains, snow melts, flooding, or irrigation; failure to wash substantial amounts of microbes from soil columns in perfusion or leaching experiments; partial removal of microbes from wastewater in percolation beds; increased release of microbes from soil by sonication, surfactants, and other methods to enhance the number of microbes enumerated. However, there is limited evidence for such surface interactions from carefully controlled studies (e.g., using electron microscopy and changes in particle-size distributions to detect surface interactions) (2). Moreover, on theoretical grounds, direct surface interactions are difficult to understand because the high electrokinetic potentials, of the same net negative charge, on both clays (and humic substances) and cells at the pH of most soils mitigate against such interactions. In fact, such surface interactions can be demonstrated only: (*1*) when the pH is lowered to levels at which most microbes cannot grow (e.g., pH 2 to 3) and the net charge on the microbes (and of some clays and humic substances) becomes positive, whereas the net charge on most clays remains negative because the charge is independent of pH and results from isomorphous substitution within the crystal structure; (*2*) when the electrokinetic potentials of the clay and microbes are reduced by polyvalent cations; or (*3*) when the net charge is reversed to positive at higher pH values (e.g., pH 6 to 8) by the first hydrolysis product of some heavy metal ions (2,35,36). These conditions seldom occur in soil. Moreover, as indicated earlier, it may be detrimental to a microbe if it sticks to a clay-containing aggregate. Consequently, microbes probably reside in the relatively permanent water films associated with such aggregates rather than on the surface of the aggregates. However, the apparent lack of movement of microbes through soil may be the result of hysteresis rather than of direct surface interactions.

Similar questions must be asked about the binding of organic molecules on clay. Because of their small size relative to microbial cells, organic molecules bind on clay, not only by ionic interactions, which are also dependent on the ambient pH and the isoelectric point of amphoteric molecules and the pK of nonampholytes, but also by multiple hydrogen bonds. Although such binding serves to concentrate these molecules at the solid–liquid interface, it also reduces their availability to microbes. Numerous studies of proteins, peptides, amino acids, polysaccharides, nucleic acids, nucleotides, and other organic molecules bound on clay minerals have demonstrated such resistance to biodegradation (2). Despite this resistance to biodegradation, the activity of some of these biomolecules [e.g., enzymes (11,37,38),

transforming DNA (39–42), insecticidal proteins from subspecies of *B. thuringiensis* (43–46)] is not eliminated, although it is usually reduced. Similar relations among binding, degradation, and activity have been reported for antibiotics and other inhibitors (2).

In contrast to organic molecules, inorganic ions on clays are readily exchangeable and can be used as nutrients by microbes. In addition, clays can scavenge H^+ and toxic heavy metal ions by cation and anion exchange and, thereby, reduce their toxicity (2,33,47).

The incorporation of mined clay minerals into soil, especially of montmorillonite (smectite), can affect the establishment, proliferation, and activity of fungal pathogens of plants and animals (i.e., help convert "conducive" to "nonconducive" soils; 2,48), probably by affecting the growth of and competition by bacteria and by binding siderophores necessary for the iron nutrition of the fungi (27). Although such changes may initially appear to be positive, the numerous direct and indirect effects that clay minerals have on many other physicochemical characteristics of soil and, therefore, on the soil microbiota indicates that all possible permutations of altering the clay mineralogy of a soil — a relatively permanent change — should be carefully evaluated before such additions of clay are made.

Many of the effects attributed to clay are probably also caused by organic particles [e.g., humic substances (49,50)] in soil because they share some of the characteristics of clay (e.g., high specific surface area and activity, ion-exchange capacity). However, because these substances are difficult to extract, purify, and characterize, these effects have been insufficiently studied. Moreover, because of their organic nature, the permanence of these particles generally is less than that of clay. However, the association of humic substances with clays and hydrous metal oxides probably increases their persistence in soil (51). Nevertheless, less concern is necessary, compared with clay, before the amount of organic surfaces is increased by the incorporation of plant and other organic residues into soil, as the permanence of such surfaces is more transient because of their susceptibility to biodegradation. However, the relative importance of surfaces of organic matter to microbial events in soil is not as well understood as that of clay.

Because of the profound and multifaceted effects that surfaces, both inorganic and organic, have on microbes in soil, their manipulation for the control of specific portions of the soil microbiota should be extensively investigated.

Spatial Relations

The spatial relations between microbes in soil are primarily influenced by the structure of the microhabitats, as discussed earlier. Although some cultural practices (e.g., cultivation, irrigation) affect these relations, these effects are probably nonspecific and transitory. Hence, manipulation of spatial relations may not be an easy or effective way to control specific microbes in soil.

Characteristics of the Microorganisms

As indicated earlier, autochthonous microbes in soil are highly adaptable, both physiologically and genetically, and they have established a homeostasis in soil. Until recently, attempts to alter this homeostasis have generally been unsuccessful. However, it is now possible to alter the genetics of microorganisms and to introduce these genetically engineered organisms to soil for performing specific functions. However, introduction is not always followed by establishment (i.e., colonization), growth, and survival of the altered organisms. Consequently, until the physicochemical characteristics of soil can be manipulated to ensure a significant and extended expression of the introduced genes, any effects of these genes will be transient. If and when such manipulation becomes possible, other problems, such as the transfer of the genes to the indigenous microbiota, which is probably more adapted to the soil environment than the introduced organisms, and potential ecological and health hazards of the products of the novel genes, need to be considered. Inasmuch as these and other aspects of the release of genetically altered organisms to the environment have been extensively discussed (4–8,42,52,53), they will not be discussed further here.

Interactions Between Microorganisms

Although the physicochemical factors briefly discussed earlier influence the growth and survival of microbes in soil, interactions, primarily negative ones, between microbes within a microhabitat have a marked effect. For example, there are numerous studies that show the survival and growth of a spectrum of microorganisms in sterile soil, including genetically engineered and nonengineered microbes and microorganisms that are not autochthonous to soil, whereas the same organisms do not survive or grow when introduced into the same soil when it is not sterile (4,5,8). Although various positive, negative, and presumably neutral interactions between and among microorganisms have been demonstrated, most studies have been conducted with model systems consisting only of a few species of interacting organisms. Inasmuch as the microhabitats in soil are heterogeneous and their inhabitants also display a high degree of heterogeneity, numerous interactions between inhabitants are undoubtedly occurring simultaneously and are in constant flux. By understanding the specific physicochemical factors that affect positive and negative interactions, it might be possible to manipulate these factors to enhance natural biocontrol of desired and undesired microbes.

CONCLUSION

Increased knowledge of how individual physicochemical factors affect microbes in soil may provide some clues as to the way in which such factors can be manipulated. Unfortunately, there have been few concerted research efforts to determine the influence of these factors, either alone or in various permutations, on organisms in soil, primarily because of the reluctance of funding agencies to support such basic research. Such support could reduce the nonproductive expenditures for trial-and-error (empirical) studies and produce important, unique, and focused information on the factors that control microbial activities

not only in soil but also in other microbial habitats, including animal systems. However, it is obvious that a change in one factor will result in changes in numerous other factors and that this cascade of changes may either augment or cancel the initial effect. Most of the factors, which are constantly fluctuating, are interrelated and, therefore, must be studied, understood, and manipulated in concert. In addition, the presumed effects of such changes must be carefully and critically evaluated (54). For example, the failure to recover an introduced microorganism from soil, especially from nonsterile soil, may only reflect a "viable but nonculturable" stage of the organism. Microorganisms in soil, especially introduced organisms, sometimes become so debilitated or otherwise altered that they cannot be recovered, especially on selective media, although they could be surviving and possibly even growing in soil (4). Consequently, it must be clearly and unequivocally established that the apparent lack of survival and growth is not an artifact of the experimental procedures.

Although soil is a fundamental environment because entire food webs ultimately depend on it, there is still much to learn about soil as an environment for microbial life. There is a plethora of questions, many of which are old but still unresolved and complex. Although some concepts and techniques of molecular biology will undoubtedly be helpful in answering some of these questions, it would be imprudent to forget and not to use older tested methods (e.g., respirometry, assays for enzymes and ATP, plating on selective media, X-ray diffractometry, Fourier-transform infrared spectrometry, electron microscopy, measurement of surface charges). In many cases, the methodology is secondary to asking the pertinent questions. Moreover, it must be remembered that because of the complexity of soil, the concepts and techniques of microbiology, chemistry (ranging from biochemistry to inorganic and physical chemistry), physics, mathematics, and agronomy, in addition to those of molecular biology, must be invoked.

Although the use of molecular techniques has provided new information on the microbiology of soil (22), there is no convincing evidence that the amount and value of this information is commensurate with its cost. Moreover, many paradoxes need to be resolved concerning the relations between these and more classical techniques. For example, the use of DNA probes and immunology is extremely helpful in monitoring the fate and ecology of individual species in soil. However, these techniques require that the species be cultured to obtain their DNA or antigens. The use of the polymerase chain reaction, DNA-DNA hybridization, flow-cytometry, or other methods to measure antigen–antibody reactions, and soon are secondary to being able to culture the organisms in vitro. Inasmuch as only 1 to 10% of the soil microbiota can presumably be cultured, the number of species that can be monitored is limited. Similarly, although 16S rRNA analyses can provide valuable information on changes in the apparent diversity of species of microbes in soil over time, culturing these species will eventually be necessary to identify them. Culturing helps to establish databases relating the RNA patterns to identifiable species, as well as the study of their physiology, functions, production of potential valuable (anthropocentric) metabolites, and so on. Despite these current limitations of molecular techniques in answering many relevant questions relating to microbial life in soil, the application of these techniques, in conjunction with other techniques and with posing correct and relevant questions, should provide much valuable information in the future (3).

In a time when tremendous advances are being made in many biomedical areas, similar advances must be made in the sustained production of food and fiber, especially in many parts of the world where production is too low to sustain the high and burgeoning human population. Many of the current advances in biomedical research have limited impact in these parts of the world because an adequate source of nutrients is the primary limiting factor to human life. Because of the fundamental role of microbes in the production of food, this limit to human life can only be solved by increasing the knowledge of the factors that affect soil as an environment for microbial life.

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MICROSPHERES AS TRACERS IN GROUNDWATER.

See TRACERS IN GROUNDWATER: USE OF MICROORGANISMS AND MICROSPHERES

MICROSPORIDIA: BASIC BIOLOGY

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The microsporidia are single-celled, obligately intracellular eukaryotic organisms that have caused significant economic losses in agricultural industries, have interfered with biomedical research, and are recently emerging as etiologic agents of disease in mammals, including humans. *Nosema bombycis* was the first named microsporidian and was identified by Nägeli in 1857 as the cause of pébrine (pepper) disease in the silkworm, *Bombyx mori* (1). *Encephalitozoon cuniculi* Levaditi, Nicolau, and Schoen 1923 was the first mammalian microsporidian described by Wright and Craighead in 1922 as causing motor paralysis in rabbits (2,3), and three *Encephalitozoon*

Table 1. Species of Microsporadia Identified in Humans

Species	Sites of Infection
Brachiola vesicularum Cali, Takvorian, Lewin, Rendel, Sian, Wittner, Tanowitz, Keohane, and Weiss 1998	Cornea, skeletal muscle
Brachiola connori (syn. Nosema connori) Sprague 1974	Disseminated
Encephalitozoon cuniculi Levaditi, Nicolau, and Schoen 1923	Disseminated
Encephalitozoon hellem Didier, Didier, Friedberg, Stenson, Orenstein, Yee, Tio, Davis, Vossbrinck, Millichamp, and Shadduck 1991	Disseminated
Encephalitozoon intestinalis(syn. *Septata intestinalis*) Cali, Kotler, and Orenstein 1993	Disseminated
Enterocytozoon bieneusi Desportes, Le Charpentier, Galian, Bernard, Cochand-Priollet, Laverne, Ravisse, and Modigliani, 1985	Duodenum, jejunum, bile tract
Microsporidium africanum Canning and Lom 1986	Cornea
Microsporidium ceylonensis Canning and Lom 1986	Cornea
Nosema algerae Vavra and Undeen 1970	Cornea
Nosema ocularum Cali, Meisler, Lowder, Lembach, Ayers, Takvorian, Rutherford, Longworth, McMahon, and Bryan 1991	Cornea
Pleistophora sp.	Skeletal muscle
Trachipleistophora anthropophthera Vávra, Yachnis, Shadduck, and Orenstein 1998	Disseminated
Trachipleistophora hominis Hollister, Canning, Weidner, Field, Kench, and Marriott 1996	Skeletal muscle, nasal sinuses
Vittaforma corneae (syn. *Nosema corneum*) Shadduck, Meccoli, Davis, and Font 1990	Cornea, urinary tract

species have been commonly reported to infect laboratory animals, farm animals, and companion pets (4–7). In humans, microsporidiosis was first documented in 1959 in a nine-year-old boy with headaches, fever, and convulsions (8). Since 1985, microsporidiosis has been recognized as an opportunistic infection associated with persistent diarrhea and systemic disease in immunocompromised individuals, particularly in persons with AIDS, organ transplant recipients, and malnourished children (7–12). In addition, microsporidiosis has been attributed as a cause of traveler's diarrhea. (10,13,14). To date, of more than 1,200 species of microsporidia, 14 have been identified as causing infections in humans (Table 1). The most commonly encountered species that infect humans include *Enterocytozoon bieneusi* Desportes, Le Charpentier, Galian, Bernard, Cochand-Priollet, Laverne, Ravisse, and Modigliani 1985, which is primarily associated with persistent diarrhea, and the *Encephalitozoon* species, *Encephalitozoon cuniculi*, *Encephalitozoon hellem* Didier, Friedberg, Stenson, Orenstein, Yee, Tio, Davis, Vossbrinck, Millichamp, and Shadduck 1991, and *Encephalitozoon intestinalis* (syn. *Septata intestinalis*) Cali, Kotler, and Orenstein 1993, which commonly cause systemic disease (7,15–18). This article purports to describe the biology of these microsporidia in relation to public health.

CLASSIFICATION

Nosema bombycis Nägeli 1857, the first named microsporidian, was originally classified with the Schizomycetes and included yeasts and bacteria. The order Microsporidia Balbiani 1882 was then created, and the microsporidia were later placed into the phylum Microspora Sprague 1977 of the subkingdom Protozoa within the kingdom Protista in 1980 (1,19). In 1998, Sprague changed the phylum name to Microsporidia Balbiani 1882 (20).

The taxonomy of the microsporidia has been based on the life cycle and ultrastructural characteristics such as size of the developing and mature organisms, nuclear arrangement (monokaryon or diplokaryon), the array and number of polar filament coils, interface with the host cell during development (e.g., direct contact with host cell cytoplasm, replication within a host cell–derived parasitophorous vacuole and replication of organisms surrounded by endoplasmic reticulum), the process of sporogony (occurring in the absence or presence of a sporophorous vesicle and the number of sporoblasts generated per sporont), and mode of cell and nuclear division (binary division and karyokinesis with delayed cytokinesis) (4,5,21). Opinions, however, tend to vary for classifying the microsporidia below phylum levels on the basis of structural characters (21).

Structure

Spore and Spore Wall. The spore is the mature and infectious stage of the microsporidia. Spores vary in shape from spherical to oval or rodlike. Mammalian microsporidian spores tend to be smaller, measuring 1.5–4.0-μm long and 1–3-μm wide, whereas some microsporidian species that infect fish, for example, may be as long as 40 μm (5,7,21). Unstained microsporidian spores appear refractile and green under light microscopy.

The general structural features of a typical mammalian microsporidian spore are shown in Figure 1. Each microsporidian spore is enclosed by a three-layer wall that protects the organism from the environment (21,22).

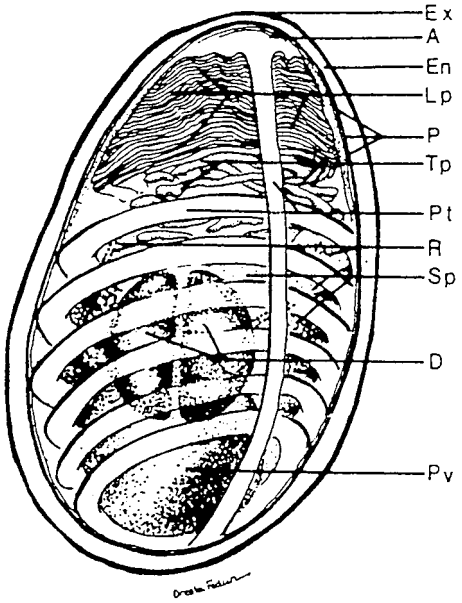

Figure 1. Diagram of a microsporidial spore. The spore wall consists of an electron-dense exopsore (Ex), an electron-lucent endospore (En), and a unit membrane (P). The extrusion apparatus includes an anchoring disc (A), polar tubule (Pt), lamellar polaroplast (Lp), and tubular polaroplast (Tp). Posterior vacuole (Pv), ribosomes (R), sporoplasm (Sp), and nucleus (D) are shown. Reprinted by permission of the publisher from A. Cali and R. L. Owen, in The Laboratory Diagnosis of Infectious Diseases: Principles and Practice, A. Balows, W. Hausler, Jr., and E. H. Lennette, eds., Springer Verlag, New York, 1988, pp. 928–949.

The outer electron-dense exospore is composed primarily of (glyco)proteins and varies in thickness (10–200 nm) and complexity (unstratified or multilayered). The middle electron-lucent endospore is approximately 100-nm thick (though slightly thinner in *E. bieneusi*) and is composed of an α-chitin based on infrared spectroscopy and X-ray diffraction studies (21). Fluorescent brighteners such as Calcofluor White M2R, Uvitex 2B, and Fungifluor have a high affinity for chitin and stained microsporidia fluoresce bright white or turquoise (23–26). The endospore is relatively thin at the apex or anterior region of the spore from where the polar filament everts during germination. The innermost layer of the spore wall is a trilaminar plasma membrane that encloses the spore cytoplasm (21).

Polar Filament/Tube. The polar filament, also referred to as the polar tube, is a tube coiled within the mature spore and is the structure that defines an organism as being a microsporidian (4,28,29). The function of the polar filament is to facilitate infection so that during germination, the polar filament everts or turns inside out and propels the contents of the spore into a host cell (30–33,41). The polar filament arises from the anchoring disc-polar sac complex at the anterior end of the spore and forms coils at the posterior region of the spore (21). The arrangement and number of polar filament coils varies among species and has been used as an aid for classification. A double-array of approximately six coils of the polar filament is characteristic of *E. bieneusi*, whereas a single-array of four to eight polar filament coils is typically observed in mature spores of the *Encephalitozoon* species.

The polar filament is generated from the endoplasmic reticulum and Golgi-like vesicles (21,34–38). In the mature spore, the polar filament is surrounded by a membrane, is filled with electron dense unassembled polar tube proteins(s), and consists of several concentric layers around a central core when viewed by transmission electron microscopy (4,39–42). During discharge, the electron-dense polar tube proteins appear to assemble at the growing tip of the tube so that the polar filament appears like a cylinder within a cylinder (4,39,43). The polar tube is flexible and bulges as the cytoplasmic contents pass through (32,33,41,42). After germination, the extruded polar tube appears hollow and extends approximately 50–100 μm and is approximately 0.1–0.15-μm wide (28,33,42,44,45). An immunofluorescent antibody-stained *E. hellem* spore that has germinated and extruded its polar filament is shown in Figure 2.

Anchoring Disc-polar Sac Complex. The anchoring disc is found in the anterior-most portion or apex of the mature spore and is believed to anchor the polar filament during germination. The anchoring disc appears continuous with the outer covering of the polar filament, and the polar sac, which is filled with electron-dense material, is found in the central region of anchoring disc (21,38). This complex is the most carbohydrate-rich structure in the microsporidia and is responsible for the reason that microsporidia stain acid fast (37).

Polaroplast. Just posterior to the anchoring disc-polar sac complex and surrounding the base of the polar filament is an array of lamellar membranes called the polaroplast that occupies up to one-half the volume of the mature spore. The unit membranes of the polaroplast, polar sac, and polar filament appear to be generated through the same membrane system, and also are carbohydrate-rich (4,21,36). The polaroplast becomes the outer membrane of the sporoplasm that is the sac that becomes filled with the spore contents during and just after polar filament extrusion (32).

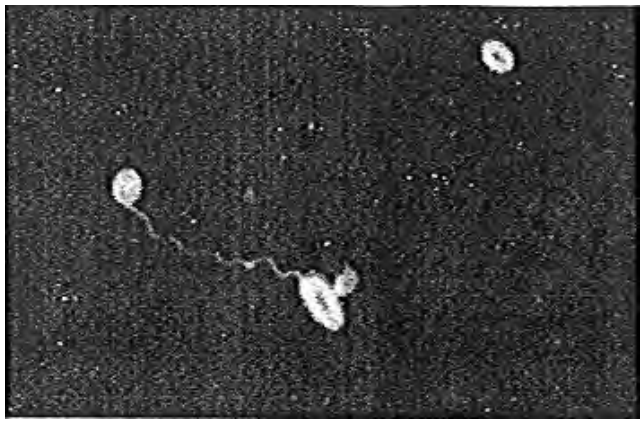

Figure 2. Immunofluorescent antibody-stained *E. hellem* germinated spore. The sporoplasm containing the spore contents is seen at the upper left end of the extruded polar filament. (X 600).

Nucleus. The microsporidian nucleus is found in the central region of the organism and is limited by a double-unit membrane separated by a perinuclear space typical of eukaryotes. The nucleus in microsporidia exists in a single nucleus structure, also termed the *monokaryon* (e.g., *Encephalitozoon, Enterocytozoon, Pleistophora*, and *Trachipleistophora*) or in a *diplokaryon* (e.g., *Brachiola, Nosema*, and *Vittaforma*) arrangement. The diplokaryon consists of two closely aligned nuclei that function as a single unit (4,29). Nuclei divide on mitotic spindles, but microsporidia lack centrioles (4,18). Although rarely observed in most stages, nucleoli may be seen just before spore formation (21).

Endoplasmic Reticulum and Ribosomes. Ribosomes are the predominant structures found in the cytoplasm of microsporidia, suggesting that a high rate of protein synthesis occurs in developing microsporidia (21). During merogony (parasite replication), the ribosomes are dispersed throughout the cytoplasm and during sporogony (parasite differentiation into spores), increasing amounts of endoplasmic reticulum develop to which the ribosomes then attach. Although microsporidia are eukaryotes, their ribosomes are prokaryote-like (46). Vossbrinck and coworkers found that *Varimorpha necatrix* Kramer 1965 (a microsporidian of butterflies) contains the smaller 70S ribosomes, which have 16S and 23S subunits, but lack a separate 5.8S subunit typically found in eukaryotes. The 23S subunit, however, contains sequences of the typical 5.8S subunit (47,48).

Golgi Apparatus. The microsporidia contain Golgi-like membranes consisting of small groups of sacs and opaque vesicles sometimes arranged in parallel arrays in the anterior region of the organisms (4,21). These membranes and sacs contain thiamine pyrophosphatase, an enzyme and histochemical marker that is specific for the Golgi apparatus (49). These structures appear to support polar tube formation, spore germination, and polar filament extrusion.

Posterior Vacuole. The posterior vacuole is a rather large membrane-bound structure formed from the Golgi-like vesicles that coalesce late in sporogony during spore maturation (4). The posterior vacuole provides a diagnostic characteristic for detecting microsporidia in clinical specimens using light microscopy methods (26,50,51). The function of the posterior vacuole is unknown but along with the polaroplast, it swells just before germination, suggesting a role in polar filament extrusion (30,65–67).

Sporophorous Vesicle. In some species of microsporidia, sporoblasts develop into spores within envelope structures called sporophorous vesicles. Often, the sporonts secrete the material that forms the sporophorous vesicles but in some cases the host cell contributes to producing this envelope. These vesicles may be transient structures that degenerate after spore release or they may persist. Freeze fracture studies indicate that the sporophorous vesicles do not form a unit membrane structure (21). Two species of microsporidia that infect humans and generate spores within sporophorous vesicles produced by both the sporonts and the host cell include *Trachipleistophora hominis* Hollister, Canning, Weidner, Field, Kench, and Marriott 1996 and *Trachipleistophora anthropophthera* Vávra, Yachnis, Shadduck, and Orenstein 1998 (55–57).

Genetics

Along with structural characteristics, molecular biology approaches are now being applied toward postulating phylogenetic or evolutionary relationships among the microsporidia that have subsequently affected the taxonomic classification of the microsporidia. On the basis of several gene sequence comparisons as described in the following sections, Cavalier-Smith published a revised six-kingdom system of life in 1998 and transferred the microsporidia to the kingdom Fungi (1,58). Additional changes have occurred in the classification of microsporidia that also infect mammals. At the genus level, *Septata intestinalis* was reclassified into the genus *Encephalitozoon* based primarily on rDNA sequence comparisons (59–61). At the species level, heterogeneity has been detected among isolates within a species by pulsed-field gel electrophoresis and sequence comparisons of rDNA ITS regions, and these variations have led to the designation of strains and genotypes within the species of *E. cuniculi*, *E. hellem*, and *E. bieneusi* (62–71).

Microsporidian Genome. Studies on the genetics of microsporidia are relatively recent, and of the species studied to date, microsporidia possess between 8 (e.g., *Vairimorpha* spp. and *Nosema costelytrae* Hall, Oliver, and Given 1977 that infect various insects) and 16 (e.g., *Glugea atherinae* Berrebi 1978 that infects fish) chromosomal DNA bands based on pulse-field gel electrophoresis (69,72–75). Of the microsporidian species infecting mammals whose chromosomes have been characterized, *E. cuniculi* has 11 chromosomal DNA bands and a genome size of 2.9 Mb (69,75,76). *Encephalitozoon hellem* and *E. intestinalis* each have 10 chromosomes and have genome sizes of 2.4 Mb and 2.5 Mb, respectively (69,75,77). The largest microsporidian genome size measured to date is 19.5 Mb in *Glugea atherinae* (69,74,75,78). It has been hypothesized that the relatively small genome size of the microsporidia reflects their early divergence as eukaryotes (75).

Ribosomal RNA Genes (rDNA). The earliest indication that the microsporidia may have been early branching eukaryotes arose from observations that the microsporidia are nucleated, that they lack mitochondria, and that they possess prokaryote-like ribosomes (47,79). Vossbrinck and coworkers observed that in general, sequences of the large subunit rDNA (LSUrDNA) did not vary greatly between species, that the small subunit rDNA (SSUrDNA) sequences varied moderately, and that the internal transcribed spacer (ITS) region sequences were highly variable between species. Nowadays, species identity between isolates of microsporidia seems to be based on SSUrDNA sequencing, whereas subspecies designations (e.g., strain and genotype) are based on ITS rDNA sequence differences and pulse-field

electrophoresis chromosome studies. For example, distinct genotypes of *E. bieneusi* isolates from humans, pigs, cats, farm dogs, cattle, and nonhuman primates have been identified based on gene sequence differences in the rDNA ITS region (62–66). *Encephalitozoon cuniculi* has a wide mammalian host range, and isolates from rabbits, mice, domestic dogs, and humans were found to differ slightly in their sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblot profiles (67,80,81). These isolates also differed in their number of 5′-GTTT-3′ repeats in the rDNA internal transcribed spacer sequences, resulting in the designations of strain I (rabbit prototype with three repeats), strain II (mouse prototype with two repeats), and strain III (dog prototype with four repeats) (67,82,83). These strains, however, are not host-specific and to date only *E. cuniculi* strains I and III have been identified in humans (70,81,84–86). Within these strains of *E. cuniculi*, additional karyotype diversity was identified by Biderre and coworkers using pulsed-field gel electrophoresis (69,75,78,87). *Encephalitozoon hellem* genotype diversity was also identified by pulse-field gel electrophoresis, rDNA ITS sequence data, and Western blot immunoassay profile variations (70,71,77,88). To date, no genetic diversity has been described among isolates of *E. intestinalis* (77,89,90).

Reclassification of Microsporidia from the Protozoa to the Fungi. A number of phylogenetic studies comparing specific microsporidian genes with homologous genes of other organisms have led to the reclassification of the microsporidia from the protozoa to the fungi (90). The α-tubulin and β-tubulin gene sequences of the microsporidia were found to be more closely related to those of fungi than to other protozoans (68,92–96). Studies on gene sequences encoding for translation elongation factors EF-1α and EF-2 and the largest subunit of RNA polymerase II also supported a close relationship between the microsporidia and fungi (97,98). Recently, mitochondrial-type HSP70 gene sequences for the microsporidia *Varimorpha necatrix* and *Nosema locustae* Canning 1953 were identified and found to be more closely related to HSP70 gene sequences of fungi than to protozoa (99–102). Furthermore, a phylogenetic analysis of the TATA box binding protein gene from *N. locustae* also indicated a close relationship between microsporidia and fungi (103). Both fungi and microsporidia contain chitin and trehalose in their cell walls, and both contain distinct genes for thymidylate synthase and dihydrofolate reductase when compared with protists and plants in which these activities are expressed by a single protein (96,104). These observations support the reclassification of microsporidia from the protozoa to the fungi and also indicate that the microsporidia once did possess mitochondria and therefore are not early-branching eukaryotes as first believed (101,102,105). As additional data are analyzed and compared, the criteria for further classifying microsporidia should likewise become clarified.

Biology

The microsporidian life cycle in mammalian hosts is relatively simple. Infections usually begin after ingestion or inhalation of spores. A shift in pH or osmolarity results in calcium influx and posterior vacuole swelling followed by spore germination during which the polar filament everts to propel the spore contents into a host cell (21,30–33,54,106). The spore wall provides some measure of resistance so that sufficient pressure can be maintained within the spore to facilitate polar filament extrusion (30,54). Alternatively, Foucault and Drancourt recently proposed that host cells may instead phagocytose spores under suitable conditions (107). After a host cell becomes infected, the microsporidia proliferate(a process named merogony) and then differentiate into spores (a process named sporogony). Released spores then infect adjacent cells, disseminate to other tissue sites — usually by infecting trafficking monocytes or macrophages — or spores are shed with urine, feces, or respiratory secretions (5,7,50). *Enterocytozoon bieneusi* and *Encephalitozoon* species are the most commonly detected microsporidia in humans and other mammals, and features of their respective life cycles are described below. The life cycles of other species of microsporidia infecting humans may be found in other publications (5,21,40,55–57,108).

Enterocytozoon bieneusi. *Enterocytozoon bieneusi*, the sole and type species in the genus *Enterocytozoon*, is the most commonly reported microsporidian species that infects humans. In addition, *E. bieneusi* has been detected in pigs, nonhuman primates, farm dogs, cattle, rabbits, and cats (62–66,109). *Enterocytozoon bieneusi* primarily causes localized infections of the small intestine and organisms replicates in the supranuclear region of enterocytes. All stages develop in direct contact with the host cell cytoplasm and possess nuclei in monokaryon arrangement throughout their development (16,21,57,110) (Fig. 3). Early stages undergo rapid nuclear division to generate multinucleated merogonial plasmodia that contain electron-lucent clefts as a result of expanding nuclear envelopes and endoplasmic reticulum membranes. With further development, vesicles associate with endoplasmic reticulum to form the polar sacs, and electron dense discs form precursors to the polar filament. Eventually, each nucleus associates with a membrane within which the polar filament precursors combine to form continuous tubes. The polaroplasts begin to flatten, and vesicles of each polaroplast appear to enclose the polar filament. The plasma membrane of the sporophorous plasmodium invaginates and separates into individual spores while simultaneously thickening to form the spore wall. Sporogony is considered polysporous and about 60 spores may develop from one sporogonial plasmodium. The entire life cycle is completed in about four to five days, and because *E. bieneusi* infections tend to remain localized to the small intestine and biliary tract, spores are primarily shed with the feces (11,111).

Encephalitozoon Species. *Encephalitozoon cuniculi* is the type species of the genus *Encephalitozoon*, that infects humans, and has the widest host range among mammals (4,5,18,112). Three other species are contained within this genus. *Encephalitozoon intestinalis* is the

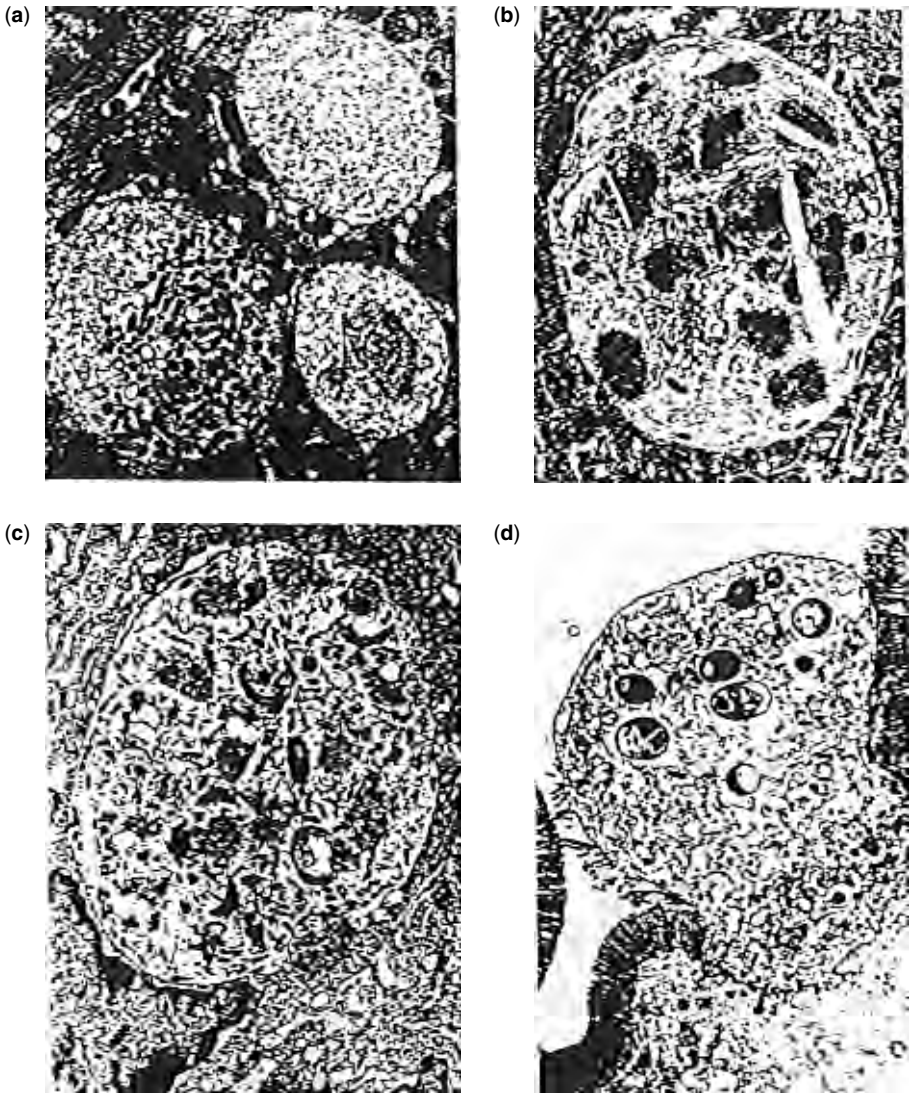

Figure 3. Transmission electron microscopy of *E. bieneusi*. (**a**) Two proliferative plasmodia, a very early one without a nucleus (upper right) and one with a single nucleus (lower right), and a sporogonial plasmodium (lower left) containing electron-dense discs and rod-shaped precursors of polar tubes. Not the intimate association of the cell's electron-dense mitochondria with the plasmodia (X 10,000). (**b**) An early sporogonial plasmodium with several electron-dense nuclei, discs, and prominent electron-lucent clefts. (X 10,000). (**c**) A single late sporogonial plasmodium molding the apical nuclear pole. Future spores are identified by the polar tubes coiling around nuclei and polar vacuoles. When cut on-end, the double layer of three turns is visible. (X 9,000). (**d**) A shedding cell, with few microvilli, contains several spores with polar vacuoles. (X 5,000). Reprinted by permission from the publisher of D. P. Kotler and J. M. Orenstein, in Infections of the Gastrointestinal Tract, M. J. Blaser, P. D. Smith, H. B. Greenberg, and R. L. Guerrant, eds., Raven Press, Ltd., New York, 1995, pp. 1,129–1,140.

second-most common microsporidian in humans (after *E. bieneusi*) and was recently detected in cows, dogs, goats, and donkeys (113). *Encephalitozoon hellem* was first described and isolated from three AIDS patients with ocular infections and has since been described in psitaccine birds, as well (114,115). The fourth species, *E. lacertae* Canning 1981 was recently described in skinks (African lizards) but has not been detected in mammals (116).

The *Encephalitozoon* organisms develop within membrane-bound parasitophorous vacuoles (PV) that are believed to be of host origin, and single nuclei in monokaryon arrangement are observed in all stages of development (4,5,21) (Fig. 4). Merogony occurs as binary division, and the meronts, which are rounder and larger than the mature spores, are found attached or closely aligned with the PV membrane. Ribosomes are predominant during early development, and endoplasmic

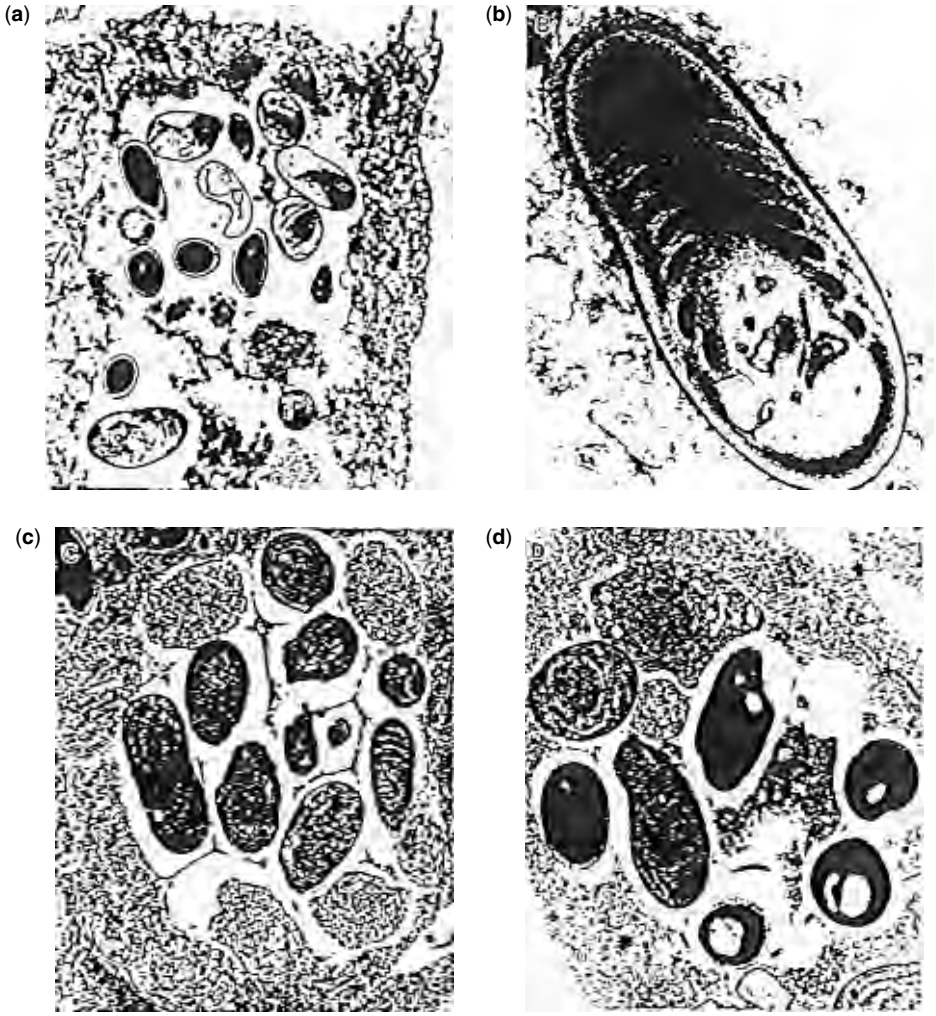

Figure 4. Transmission electron microscopy of clinical and cell culture isolates of *E. intestinalis*. (**a**) Microsporidia consistent with *E. intestinalis* in a cytoplasmic vacuole of necrotic epithelial cell from the bronchoalveolar lavage specimen. Note the few remaining microvilli in the upper right (X 6,000). (**b**) High magnification electron micrograph of a spore in the bronchoalveolar lavage specimen. Note the characteristic coiled polar tube seen in a single row, and the posterior vacuole (X 30,000). (**c**) Microsporidia from the bronchoalveolar lavage specimen and cultured in RK-13 cells develop within septated parasitophorous vacuoles. Meronts and sporonts are located near the edge of the vacuole and sporoblasts are centrally located (X 10,000). (**d**) The microsporidia cultured from the nasal lavage specimen are ultrastructurally identical to those cultured from the bronchoalveolar lavage fluid. This field show appendages (arrows) that were seen in both cultures (X 11,000). Reprinted by permission from the publisher of E. S. Didier et al., *J. Eukaryot. Microbiol.* **43**, 34–43 (1996).

reticulum can be observed during the later stages of merogony. Sporogony begins as the plasma membranes thicken and the polar filaments develop. The sporonts detach or separate from the PV membrane and continue to divide by binary division. Sporogony in *Encephalitozoon* is considered bisporous and sometimes tetrasporous so that two or four sporoblasts develop from one or two divisions of each sporont, respectively (4,21,57). The development of *E. intestinalis* differs slightly from that of *E. cuniculi* and *E. hellem* because an extracellular matrix substance is secreted into the PV that surrounds the developing organisms to form chambers or septa in the PV that is not as pronounced during development of *E. cuniculi* or *E. hellem* (17,39,118). Eventually, the PV membrane and host cell plasma membrane rupture to release spores. During intestinal stages of infection (particularly with *E. intestinalis*), spores are shed in the feces. However, all mammalian *Encephalitozoon* spores can disseminate by infecting trafficking monocytes and macrophages and thereby infect sites that include, but are not limited to, the kidneys and respiratory tract. Subsequently, *Encephalitozon* spores typically are shed in urine or respiratory secretions during the chronic stages of infection (4,5,7,11,50,111).

EPIDEMIOLOGY AND CLINICAL DISEASE

Clinical Symptoms

The clinical syndromes associated with natural and experimental microsporidia infections in mammals tend to be characterized into three groups according to immune status and responses expressed by the various hosts.

Immune-Deficient Hosts. Microsporidiosis is associated with disease and is sometimes lethal in immune deficient hosts (4–6,119). Among animals, immature or underdeveloped immune responses were probably responsible for the spontaneous abortions that were reported in a colony of squirrel monkeys, the renal failure and death of infected puppies, and the stillborn birth of a foal caused by microsporidial placentitis (5,120–125). In addition, chronic diarrhea was observed in neonatal gnotobiotic pigs experimentally infected with *E. bieneusi* (125). Immunodeficient SIV–infected rhesus monkeys (*Macaca mulatta*) that were naturally or experimentally infected with *E. bieneusi* developed chronic diarrhea, and the monkeys experimentally infected with *E. cuniculi*, *E. hellem*, or *E. intestinalis* often developed severe hepatic and/or nephrotic necrosis (127–130). Genetically immune-deficient animals such as athymic mice that lack functional T lymphocytes, and severe combined immunodeficient (SCID) mice that lack functional T and B lymphocytes, developed severe ascites and died after inoculation with *E. cuniculi*, *E. hellem*, or *Vittaforma corneae* (127,131–134). Athymic mice inoculated with *Trachipleistophora hominis* developed severe skeletal muscle disease (55), and interferon-gamma receptor knockout mice developed chronic infections after inoculation with *E. intestinalis*, whereas wild-type mice inoculated with *E. intestinalis* expressed no clinical signs and apparently cleared their infections (135,136). Chemically induced immunosuppression of rabbits chronically infected with *E. cuniculi* and treated with cyclophosphamide resulted in incontinence, loss of motor control, tremor, muscular weakness, and paralysis of the hind limbs prior to death (137).

Humans most susceptible to developing disease associated with microsporidiosis caused by *E. bieneusi* or *E. intestinalis* are AIDS patients with less than or equal to 100 $CD4^+$ T cells/μL blood. These individuals developed chronic or persistent diarrhea and possibly fever, loss of appetite, weight loss, and wasting disease (11,111,138). *Enterocytozoon bieneusi* infections tend to remain localized to the small intestine but biliary tract involvement leading to inflammation of the bile ducts and gall bladder is common. Organ transplant recipients undergoing immunosuppressive therapy to prevent rejection have been reported with microsporidiosis due to *E. bieneusi* and *Encephalitozoon* species and the clinical symptoms in these individuals included fatigue, fever, nausea, and diarrhea (111,139–142). Malnourished children are considered immune compromised and an increasing number of reports have been published describing the detection of microsporidia in the feces of such children with diarrhea (10,13,143–148). In otherwise healthy individuals, microsporidia infections in immunologically privileged sites such as the lens or cornea, can result in visual impairment, blindness, perforation of corneal ulcers, or spontaneous rupture of the lens, as has been described in rats, rabbits, cats, blue foxes, and humans (who were either infected or not infected with HIV) (123,149–154).

Enterocytozoon bieneusi organisms primarily infect enterocytes of the small intestine (jejunum and duodenum) and epithelial cells of the biliary tract. The development of diarrhea may be related to the replication of organisms that is associated with loss of villous enterocytes, villous atrophy (blunting), D-xylose malabsorption, crypt hyperplasia, mononuclear infiltration, and decreased brush-border disaccharidase activity (7,11,111). Tumor necrosis factor, a proinflammatory cytokine, was detected in relatively high levels in stools of AIDS patients with microsporidiosis and may contribute to the wasting associated with microsporidiosis in AIDS patients (155).

Encephalitozoon species often disseminate rather than remain in the small intestines, and may be associated with sinusitis, keratoconjunctivitis, hepatitis, myositis, peritonitis, nephritis, encephalitis (with seizures), and pneumonia. Lesions associated with *Encephalitozoon* infections include necrosis and lymphocytic infiltration of affected organs, particularly of small intestine, liver, kidney, nasal cavities, sinuses, and pancreas (6,7,11,111,119,123).

Hosts That Develop Hyperimmune Responses. Carnivores such as domestic dogs, foxes, and mink commonly become infected with *E. cuniculi* by transplacental transmission, often resulting in neonatal death. Surviving carnivores, or those that became infected after birth, subsequently developed hypergammaglobulinemia that progressed to renal disease via type III hypersensitivity immune responses (4,6,119,124,154,156–160). Perivascular granulomatous lesions were typically observed in infected carnivores and granular deposits containing IgM and IgG were detected in the glomerular basement membranes in *E. cuniculi*–infected mink (161). Lesions in these infected carnivores were characteristic for polyarteritis nodosum and included focal renal cortical lymphocytic and granulocytic infiltrates, fibrosis, and tubular dilatation with fibrinoid vasculitits. Kidney, brain, liver, lung and eye may be affected, and organisms may be found extracellularly in necrotic zones and within parenchymal and endothelial cells (6,119,123). In humans, hyperactive immune responses to microsporidia specifically have not been described.

Immunologically Competent Hosts. Spontaneous (i.e., natural) infections with *E. cuniculi* commonly have been reported in immune competent mammalian hosts that include, but are not limited to rodents, lagomorphs, ruminants, humans, and nonhuman primates (4,5,7,43). During the early or acute stage of infection in otherwise healthy animals, clinical signs of disease may be evident (6,7,119,123). A chronic phase ensues during which few clinical signs are evident, yet the microsporidian infections generally persist. In this context, microsporidiosis represents a balanced host-parasite relationship because the host survives with relatively few symptoms and the parasite persists.

The euthymic murine models for *E. cuniculi* have been particularly useful examples of microsporidiosis in competent hosts because mice are natural hosts of *E. cuniculi*, the organisms disseminate in mice similar to where they spread in humans, the immune responses can be easily studied in mice through the availability of inbred strains of mice and immunological reagents, and the clinical manifestations of *E. cuniculi* infections in immunologically competent and immunodeficient mice parallel those seen in humans infected with *E. cuniculi* (4,5,7,43,119,123). Early after infection with *E. cuniculi*, euthymic mice may develop ascites that resolves within two weeks, and infected rabbits may on occasion develop motor paralysis, convulsions, and torticollis. *Encephalitozoon cuniculi* infections apparently persisted in these hosts as evidenced by the continued expression of microsporidia-specific antibodies, sporadic shedding of organisms, and the observation of microsporidia-associated lesions at necropsy. In addition, microsporidia could later be recovered from chronically-infected mice that were injected with the immunosuppressant, cortisone (162,163). Rabbits similarly develop chronic, life-long infections with *E. cuniculi* and have been used to study the pathogenesis and diagnosis of microsporidiosis (4–6,123,124). Interstitial granulomatous inflammation of kidney, liver, and sometimes, brain, are quite common in animals with encephalitozoonosis (6,119,123).

It is less clear if immune competent humans infected with microsporidia eventually clear their infections or develop chronic life-long infections, as occurs in most other animals. (7,18,43,112,124). Seroprevalence data among various human populations range from 0 to 38% (4,10,164–166), but these earlier studies that utilized *E. cuniculi* as the antigen, and are difficult to interpret now that new species have been identified as causes of infection in humans. Recently, however, a stringent serological study on 300 Dutch blood donors and 276 pregnant French women indicated prevalences of 8% and 5%, respectively, for the presence of *Encephalitozoon*-specific antibodies (167). As diagnostic methods have improved for detecting microsporidian spores or DNA by histochemistry or polymerase chain reaction-based methods, respectively, cases of self-limiting diarrhea of approximately two weeks' duration have been reported in travelers and other persons not infected with HIV (13,143–145,147,148,164,168–173). In addition, microsporidia have been detected in the feces of an asymptomatic child in the Zambia, and in 20 fecal specimens out of 255 asymptomatic individuals in two communities in Mexico (144,174). Such findings suggest that microsporidiosis is probably more common in immune competent human populations than previously believed, but further studies are needed to determine if persons are persistently infected with microsporidia even after clinical symptoms resolve, or whether these individuals clear the microsporidia.

Modes of Transmission

Many infections in mammals occur after ingestion or inhalation of microsporidian spores. Though not reported in humans, transplacental transmission of *E. cuniculi* is common in carnivores, especially blue foxes and domestic dogs, and has occasionally been reported to occur in non-human primates, horses, rabbits, and rodents (4,5,175). Microsporidiosis can be transmitted to humans by trauma or direct contact. In laboratory animals, microsporidiosis has been transmitted by intraperitoneal, intravenous, intrarectral, intratracheal and intracerebral inoculations (6,7,119).

Zoonotic Transmission. Zoonotic transmission of microsporidial parasites from animals to humans has not been proven to date, but appears likely based on the wide range of hosts that become infected with species of microsporidia that also infect man. *Enterocytozoon bieneusi* has been identified in SIV–infected macaques (*Macaca mulatta*), pigs, cows, dogs, rabbits, cats (62,64,66,109,130), and the genotypes that infect pigs are most similar to those that infect humans. *Encephalitozoon intestinalis*, the second most commonly identified human microsporidial infection, has been identified in five different domestic animal species in association with a human survey conducted in a village setting in Mexico (113). *Encephalitozoon hellem* was first identified in AIDS patients as causing ocular, respiratory and systemic infections (11,150,153,176) and recently has been detected in several psittacine pet bird species (5,114,115,123). Anecdotally, at least two of the three AIDS patients from who *E. hellem* was first isolated had pet birds, but these birds were never tested for microsporidiosis (176). *Encephalitozoon cuniculi* has the widest host range among mammals, and isolates from various animals have been characterized as being identical to isolates from humans based on rDNA and western blot studies (70,81,84–86). The strongest evidence suggesting zoonotic transmission was reported in a single case where children were exposed to puppies with overt encephalitozoonosis and one of the three children seroconverted to express antibodies to *E. cuniculi* (123,176).

Vectorborne Transmission. Although no direct evidence presently exists, morphological and phylogenetic studies using small subunit rDNA sequence comparisons among microsporidia of humans and insects supported the possibility that microsporidia may be transmitted from insects to humans (178). Furthermore, a microsporidian species of mosquitoes, *Nosema algerae*, was recently identified in, and isolated from the cornea of an HIV-seronegative individual (179), and *T. hominis*, which was first isolated from a human, was able to infect larval stages of two species of mosquitoes (180).

Waterborne Transmission. Microsporidial spores are remarkably resistant to the environment, and water contact has been identified as a risk factor for microsporidiosis in epidemiological studies (10,164). Analysis of seasonal patterns of diarrhea cases in AIDS patients showed wet-season increases in cryptosporidial diarrheas, but there were no clear correlations between seasonality and microsporidial diarrheas (10,164,181). Drinking water has been identified as a source of exposure for other diarrhea-associated protozoans such as *Cryptosporidium parvum*,

Giardia lamblia and *Cyclospora cayetenensis*, and likewise, *E. bieneusi*, *E. intestinalis*, and *V. corneae* have been detected in sewage, ditch water, groundwater and surface water sources prompting the U.S. Environmental Protection Agency to include microsporidia on the Occurrences Priority List for drinking water safety (182–186). Swimming in pools was also considered a risk factor associated with an outbreak of microsporidiosis (187). Methods for improving detection of microsporidia in water sources are under development and will be important for determining the risk of waterborne transmission (164,185,188,189).

CONCLUSION

Microsporidiosis is a reemerging infection in animals and an emerging infection in humans, particularly in immunologically compromised individuals (190). As awareness of microsporidiosis has increased and as detection methods have improved, microsporidiosis became increasingly recognized in a wider range of animal hosts and human populations. However, much still needs to be learned about the epidemiology and natural history of microsporidiosis in immunocompetent and immunodeficient populations of people. The ubiquitous nature of these organisms and the potential risk for zoonotic, vectorborne, and waterborne transmission suggest that microsporidiosis represents a public health concern that further raises the need for improving surveillance, detection, and treatment of microsporidiosis.

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MICROSPORIDIA: OCCURRENCE, FATE AND METHODOLOGIES

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Microsporidia is a nontaxonomic term used to describe a group of protozoa that belong to the phylum Microspora. Microsporidia may be the most ancient branch of the eukaryotic tree (1). Although microsporidia have a nucleus, they lack mitochondria and peroxisomes (2), and their ribosomal characteristics are more closely related to bacteria than to higher eukaryotes (2,3). In addition, the human enteropathogenic microsporidia (HEM) are closer in size to bacteria (~1 to 2 μm in diameter) than to most other protozoan parasites such as *Giardia lamblia* (~15 μm diameter) and *Cryptosporidium parvum* (~5 μm diameter). Microsporidia have an environmentally resilient spore wall that consists of a proteinaceous exospore and a chitinous endospore (1)

The HEM cause chronic diarrhea and wasting in HIV-infected individuals. Estimates indicate that about 7 to 50% of these immunodeficient patients are infected with HEM, making it one of the most important intestinal pathogens in these populations (4). No reports on the infective dose of the HEM have been documented, but indications are that it is very low especially in the immunocompromised (4). In immunocompetent individuals, a form of diarrhea, similar to that seen in the immunocompromised, is evident but is self-limited (5). Asymptomatic infections have also been reported in both HIV infected and immunocompetent patients (6). Finally, as improved methods for clinical detection of HEM are developed, increasing reports of infection in the immunocompetent are being described, indicating that the HEM may also be an important pathogen in individuals not infected with HIV (5,7–13). However, as with most pathogens, AIDS patients, individuals on immunosuppressive drugs, the very young and the very old, and pregnant woman should be considered at risk for infection from these emerging pathogens.

At one time, the microsporidia were included in the phylum Sporozoa along with what is now the Apicomplexa. Subsequent research is beginning to show that the microsporidia are a well-defined group with no known relationships with other protists. Recent phylogenetic evidence has even suggested they are more closely related to fungi than protozoa. Modern taxonomies reflect this confusion by placing the microsporidia in its own phylum. For a more detailed history of microsporidian systematics, see Sprague (14), Issi (15), and Canning (16). Initial classification of microsporidia used the morphology of the germination and invasive structures for higher taxonomic characteristics. Another promising approach to systematics is based on molecular characterization. Phylogenetics or the use of genetic information to determine associations and evolutionary lineage appears to be the most promising of these, with particular emphasis given to ribosomal DNA gene sequencing (2,17,18). Interestingly, such analyses of ribosomal sequences tends to indicate that microsporidia are closely related to protozoa (3,17,18), whereas phylogenetic analyses of some other microsporidial genes such as β-tubulin tend to indicate that they are more closely related to fungi (19).

Having risen from relative obscurity to well-recognized human pathogens, HEM, which were most often identified as the etiological agent of disease in the immunodeficient (4,20–22), are increasingly identified with disease in immunocompetent populations (6,7,9–11,21,23–25). Human pathogenic microsporidia are ubiquitous in their geographic distribution, and they have been documented as disease agents on most continents including North and South America, Europe, Asia, Africa, and Australia (21).

Most of the 1,000 and more species of microsporidia are parasites of fish and insects (2), but beginning with the diagnosis of microsporidia in AIDS patients (20), they have become recognized as emerging human pathogens by the United States Environmental Protection Agency, the Center for Disease Control and Prevention, and the National Institute of Health. To date, there are at least six genera of microsporidia that have been identified as the etiologic agents of disease in humans: (*1*) *Enterocytozoon bieneusi*; (*2*) *Encephalitozoon* sp. (*Encephalitozoon intestinalis*, Encephalitozoon hellem, and *Encephalitozoon cuniculi*); (*3*) *Pleistophora* sp; (*4*) *Vittaforma corneae*; (*5*) *Nosema* sp. (*Nosema connori* and *Nosema ocularum*); and (*6*) *Trachipleistophora hominis* (21). These genera cause a variety of infections including infections of the respiratory tract, the urinary tract, and the gastrointestinal tract.

The first documented waterborne outbreak of microsporidia was described recently by Cotte and coworkers (24). They performed an extensive screening of 1,454 stool samples from 1993 to 1996. They found microsporidia in 338 persons: 61 of these were immunocompetent, whereas the rest were either HIV-positive or transplant patients. They also documented a waterborne outbreak involving 200 persons, which occurred in the summer of 1995. They identified that the major factor associated with this outbreak was the use of one of three local water distribution systems. Risk factors associated with infection by HEM have also been described by Watson and coworkers (26) and include swimming in rivers, ponds, and lakes, as well as drinking unfiltered tap water. Finally, Enriquez and coworkers (27) assessed the prevalence of *E. intestinalis* in two rural communities in Mexico. They found that at least one household member from 15 families was infected with this parasite (70 total households were screened) and that the primary factors associated with these infections were the use of untreated indoor faucets or the use of community wells for drinking. These data suggest the potential for waterborne transmission.

Microsporidia that are associated with human infection have also been documented to occur in animals other than humans. The primary microsporidia that may have a definite zoonosis is *E. cuniculi*, which is often associated with infection of several animals including mice, rabbits, foxes, and dogs. Other human pathogens may also have animal reservoirs including *E. bieneusi*, which has been detected in the feces of pigs (28) and primates (29–31), and *E. intestinalis*, which has been detected in the feces of a variety of animals including pigs and cows (32). *Enterocytozoon bieneusi* and *E. intestinalis* are of particular interest because they are associated with gastrointestinal disease. However, in addition to infections of the small intestine, they have also been associated with infection of the nasal (33–35) and bronchial epithelium (36,37). For these reasons, it has been postulated that there may be three probable modes for the transmission of *E. bieneusi* and *E. intestinalis*: (*1*) fecal-oral; (*2*) inhalation; and (*3*) ingestion of contaminated water.

A total of four studies have documented the presence of HEM in various water sources including primary and tertiary wastewater effluents, surface water, groundwater, and drinking water. Studies analyzing surface water, tertiary effluent, raw sewage, and groundwater have found species of microsporidia pathogenic to humans, such as *E. bieneusi*, *E. intestinalis*, *Pleistophora* spp., and *V. corneae*. It has been speculated that because of the small size of microsporidia, they could be transported in the subsurface water over long distances and could occasionally contaminate drinking water wells (38). Microsporidia including *Nosema*, *Pleistophora*, *Telomyxa*, *Vavraia*, and *Vairimorpha* have also been isolated from ditch water (39). Sparfel and coworkers (40,41), have detected *E. bieneusi* in river water in France. They also detected *V. corneae* and *Pleistophora* sp. Finally, Dowd and coworkers (42) detected *E. intestinalis* in drinking water samples taken in Guatemala. These water samples were collected from small reservoirs fed by springs or wells. Local communities use these reservoirs as sources of drinking water. A total of 12 water samples were screened in this study and five of these were positive for HEM.

The issue of the waterborne status of *E. bieneusi* and *E. intestinalis* still remains unclear (43). Occurrence data are slowly accumulating, suggesting that there is a definite possibility that these two microsporidia are waterborne. This combined with the first documented waterborne outbreak, the diagnosis of microsporidia in association with travellers diarrhea, and the risk factors associated with water tend to lean toward these two protozoa having the potential to be waterborne.

In addition to documentation related to the presence of the human enteropathogenic microsporidia (HEM) in water, there is increasing data related to environmental survival and disinfection. The survival of *E. cuniculi* spores suspended in distilled water and exposed at defined temperatures was investigated. Infectivity of *E. cuniculi* spores was tested by animal infectivity. There was no marked loss of infectivity of spores stored at 4 °C for two years or frozen at −12 °C and −24 °C for 1, 8, and 24 hours. Marked loss of infectivity was seen after heating at 60 °C and 70 °C for 5 minutes and 1 minute, respectively. These findings demonstrate that *E. cuniculi* spores suspended in water can survive freezing temperatures but were no longer infective at very high temperatures (44). Spores have been shown to survive for up to four weeks in a dry hospital environment at 2 °C (45) Disinfection with 2% lysol or 10% formalin or 70% ethyl alcohol for 30 minutes have been shown to inactivate spores (46,47). Labeau and coworkers (48) indicate that the HEM appear susceptible to readily achievable oxidant disinfectants. *Septata intestinalis*, was utilized to determine whether chlorine could be used as an effective disinfectant. A rabbit kidney cell culture system was used to determine 50% tissue culture infective dose (TCID) (49) and a minimal infective dose (MID) following exposure to a chlorine concentration of 2 mg/L after a minimum exposure time of 16 minutes. Data suggested that chlorine treatment may be an effective water treatment for *E. intestinalis* (50). Other than these studies, little work has been done related to the environmental occurrence and fate of HEM.

Available evidence suggests that microsporidia have potential environmental transmission routes including air and water. Recently, direct evidence has added credence to these hypotheses. Before this, there had been no documentation of the occurrence of microsporidia in the environment because there was a lack of effective methodologies with the ability to specifically detect and determine the species of the human pathogenic microsporidia. Current methods for the collection of water for recovery of protozoa (*Cryptosporidium* and *Giardia*) involve cartridge filtration of large volumes of water. Such cartridge filters are required to sample large volumes of water (>100 L) (8). Collecting large volumes of water is necessary to detect the low concentrations of protozoa that may be found in many water supplies. Such low concentrations of these pathogens in water sources are known to be significant from a human health perspective (8). After concentration of the water, methods for detection and confirmation of the microsporidia are necessary. The definitive method currently being used for the species determination of microsporidia in clinical samples is transmission electron microscopy (TEM) (49,51). However, the use of TEM for screening environmental samples is not a feasible approach. It is for this reason that other methods for environmental screening are required.

The major problems associated with detection and species determination of HEM in the environment relate to the fact that there are over 1,000 species of microsporidia, many of which can be found in environmental samples at any given time. Of these 1,000 plus species, only a handful are of relevance as human pathogens and only two (*E. bieneusi* and *E. intestinalis*) are considered potential waterborne pathogens. Another problem is the size of the HEM: *E. bieneusi* is approximately 0.5×1 μm, whereas *E. intestinalis* is approximately 1×1.5 μm in diameter. These protozoan pathogens are thus approximately the size of a bacteria, making confirmation by traditional environmental parasitology methods, such as immunofluorescent or light microscopy, difficult if not impossible. The primary methods to visually identify species of microsporidia rely on internal morphology of

the spore, especially the layout of the polar tubules. The visualization of such internal structures is well beyond the resolution of modern light microscopes. Thus, the development of novel methods for environmental detection of these much smaller protozoan pathogens is required.

One of the most promising methods used for environmental detection of the microsporidia is the polymerase chain reaction (PCR). The PCR is a molecular method that involves the enzymatic amplification of specific target DNA from HEM, allowing this target DNA of a known amplified size to be visualized in an agarose gel on staining with an appropriate dye. Thus, if DNA can be extracted from environmental samples and the use of the PCR reveals the correctly sized amplification product, it is a good indication that particular sample contained HEM. Here again the problem becomes that of differentiating the amplified HEM DNA from other microsporidia species potentially present in a given environmental sample. For this reason, the PCR should be designed to be highly specific for only the HEM and combined with a second method to provide confirmation and species determination. However, as with any such method, extensive validation on known isolates of HEM and non-HEM is required.

MICROSPORIDIA SPORES

Microsporidia spores for experimental research can be obtained directly from the American Type Culture Collection (ATCC). These spores included *E. intestinalis* ATCC 50506, *E. hellem* ATCC 50504, *E. cuniculi* ATCC 50503, *V. corneae* ATCC 50505, *Endoreticulatus sherburg* ATCC 50040, *Nosema necatrix* ATCC 30460, and *Nosema trachiplusieae* ATCC 30702.

WATER SAMPLING

Water sampling for microsporidia spores represents the first major obstacle that must be over come in the development of monitoring methods. The small size of the microsporidia as compared with other protozoans, namely, *C. parvum* and *G. lamblia* for whom these methods were developed provides novel challenges in relation to water sampling methods. Since with the other protozoan pathogens mentioned a volume of water between 10 and 1,000 L must be sampled to provide appropriate limits of detection sensitivity, it is necessary to concentrate water samples. This is done primarily through the use of filtration. Methods developed by and for the USEPA were designed for the larger protozoa *Cryptosporidium* spp (5 μm) and *Giardia* spp (15 μm), and typically they use a filter porosity in the range of 2μm. Because HEM are in the range of 0.5 to 2 μm, these methods are not ideal. Thus, methods specific for microsporidia would be able to sample large volumes of raw and finished water and retain particulates in the range of 0.5 to 2 μm. This requires a trade-off because by reducing the nominal porosity of the filters a much smaller volume of water can be effectively passed through the filters. If the porosity is such that larger volumes can be sampled, the porosity of the filters must be increased, reducing the efficiency with which these smaller particles can be retained within the filter matrix. Currently, only a handful of reports have been published related to water sampling for HEM. These reports detail several different methods used for water sampling. Yet, only two of these report sampling efficiencies.

As mentioned, several methods have been used to successfully concentrate water samples for HEM. These methods include the use of wound cartridge filters, pleated membrane filters, dipping rod for grab sampling, and cotton mesh. Other methods that are promising for successful water sampling for microsporidia will also be mentioned. Dowd and coworkers (52) evaluated the use of wound nylon cartridge filters for the concentration of water spiked with known concentrations of *C. parvum* oocysts and *E. intestinalis* spores. In side by side comparison, the efficiency of the Information Collection Rule (ICR) method (53) for protozoan detection in large volumes of water was assessed for its ability to concentrate and purify microsporidia spores.

The mean recovery percentage for *Cryptosporidium* oocysts was similar to the data obtained on the basis of blind laboratory studies performed by Clancy and coworkers (54). In her study, cartridge filters spiked with *Cryptosporidium* sp. oocysts and *Giardia* sp. cysts similar to those described here were prepared and sent to several commercial labs for analysis. These commercial labs displayed recovery percentages for *Cryptosporidium* oocysts, similar to those described here, on the basis of which, the recovery efficiency shown for microsporidia was deemed to be typical when using these methods. The smaller size of microsporidia spores compared with *Cryptosporidium* oocysts may explain the lower recovery efficiency. Thus, until other methods have been evaluated, the ICR method, although it may serve as a method for the collection of microsporidia spores from large volumes of water, is not deemed to be efficient enough for the routine use when screening for human pathogenic microsporidia.

To do this, a comparison was made between microsporidia spores and *Cryptosporidium* sp. oocysts. This provided the first insight into the problems inherent in concentration methods used for HEM. The nominal porosity of the cartridge filters used were 2 μm. The low flow rate was used to assist in retention of the spores in the filter matrix. However, this method proved far from efficient even for the large *C. parvum*. The recovery of *Cryptosporidium* oocysts was 12.5%, whereas the recovery of the microsporidia spores was 4.6%.

The second method, which evaluated recovery efficiency (42), utilized the Gelman Envirochek module, which is a 2 μm porosity absolute membrane that is pleated and contained within a disposable filter housing. Following similar methods described in the previous experiment, concentrated water spiked with known spore concentration were concentrated using the Gelman Envirochek cartridge. These spores were prestained using calcofluor white (Molecular probes, Eugene Oregon), which is a fluorescent label that shows specificity for chitin and reacts well with the chitinaceous endospore layer of the microsporidia. Filters were processed as described in the Envirochek product literature (found on the web at the *http://www.pall.com/gelman/micro_an/papers/*

envirochek.asp), filtered onto black polycarbonate membrane filters, and visualized using a DAPI and a fluorescent microscope.

This method provided improved recovery percentages over the standard wound cartridge method evaluated by Dowd and coworkers (52). The average of four trials with the Envirochek capsule showed 38.6% recovery. This is significantly higher than recovery using wound cartridge filtration. Another advantage is the sample handling time, which is reduced from 240 minutes to only 70 minutes. The cost per sample however is increased from around $20 to over $100. Another aspect to be considered is the volume of water that can be filtered using each method. With the wound nylon membrane, up to 1,000 L can be filtered, whereas with the Envirochek, 10 to 20 L can be filtered. Thus, the volume able to be filtered and the increase in recovery may be balanced out.

Other studies have also used additional methods for the sampling of surface and groundwater. Groundwater samples have been collected using the same filter cartridge apparatus through submersible pumps at the bottom of the wells, which provided sampling pressure. Wastewater samples were collected using dipping rods and sterile one-liter polypropylene bottles, which had been previously coated with elution solution [10 X phosphate buffered saline (PBS), 1.0% Tween 80, 1.0% sodium dodecyl sulfate (SDS), and 1% Antifoam A (Sigma Chemical, St. Louis, MO)]. Two liters of raw and six liters of treated effluent were collected. River samples have been collected using gauze filters (40).

Another method that shows promise for collection of water samples include the Filta-Max system (IDEXX, Inc., Westbrook, Maine). This system uses highly compressed reticulated foam filters. Up to 60 of these foam filters are compressed to provide a nominal porosity similar to previous methods (2 μm). These foam pad filters are placed inside a reusable membrane housing and are used for water concentration. One advantage is that these filters can easily be custom-manufactured using a few additional foam pads, which are compressed to the same size to fit the housing. The added compression lowers the nominal porosity to around 1 μm. Thus, the Filta-max system may be more versatile. Good recovery of *C. parvum* has been reported, and like the Envirochek filter system, the Filta-max is now USDA-approved for monitoring for *Cryptosporidium* oocysts.

DETECTION METHODS

Antibody Based Methods

The use of immunofluorescent assay (IFA) for detection of protozoa such as *Cryptosporidium* oocysts and *Giardia* cysts is routinely used as a primary detection method. Following this fluorescent detection method, there is a confirmation step involving Differential Interference Contrast (DIC) microscopy, which enables visualization of characteristic internal structures with the cyst and oocysts. The routine analyses of drinking water for microsporidia would require that such methods of detection be not only highly sensitive but also very specific. In addition, detection of protozoa on the basis of fluorescent characteristics is not considered a proof positive means of identification. An example of this is the "presumptive" IFA identification of *Cryptosporidium* sp. oocysts and *Giardia* sp. cysts based on the size and shape of their fluorescing cell wall and the subsequent "confirmation" of their identity based on visualization of their internal structures by differential interference contrast (DIC) microscopy. The small size of microsporidia makes the identification of internal structures beyond the resolution capabilities of light microscopy. Thus, any light microscopic assay would need to be extremely sensitive and very specific for routine use in the detection of human pathogenic microsporidia in environmental samples.

Dowd and coworkers (52) assessed two different commercially available antibodies (monoclonal and polyclonal) for their ability to specifically detect human pathogenic microsporidia without cross-reactivity with other environmentally relevant organisms. It was found that available antibodies were able to bind to three of the primary microsporidia pathogens but did not react with *E. bieneusi*. It also cross-reacted with *Endoreticulatus* sp. and was strongly reactive against *Staphylococcus aureus*. A second was found to cross-react with two other species, in addition to the human pathogens of interest. It also showed only sporadic cross-reactivity with *E. bieneusi* (32). Thus, neither of the two antibodies was deemed specific enough to provide confidence in IFA analysis of water. In addition to the cross-reactivity, neither antibody was able to effectively react with *E. bieneusi*.

A second use of antibodies is immunomagnetic separation (IMS), which is used for separation (IMS) of protozoan from complex matrices. As mentioned earlier, the concentration of large volumes of water result in concentration of all particulate matter contained within the water sample. This particulate matter, which also contains the parasites, contains up to 5 ml volume of sediment. In most cases, this is a very complex and heterogeneous matrix. To purify the sample, density gradient flotation (53). However, recent methods utilize IMS to purify the parasites from feces (55) and concentrated water samples (52). There were several problems associated with the spore separation, especially in unpurified water concentrate samples. The magnetic separation nonspecifically extracted a considerable amount of magnetic and paramagnetic materials contained in the sediments, in addition to the immunomagnetic beads. These copurified sediment particles inhibited all subsequent PCR analysis. After purification of water concentrates by Percoll-sucrose floatation, the IMS method showed promising results in initial spiked studies, allowing for amplification of microsporidia DNA. Therefore, a purified water sample, which was shown to be negative for microsporidia by IFA, was used in a limit of detection sensitivity study to determine how effective this method would be for routine use. This limit of detection study was performed with four replicates. Thus, the IMS method for spore separation shows promise for screening water samples for *Encephalitozoonidae*. This method indeed cannot be used for the detection of *E. bieneusi* because the antibodies tested to date do not show consistent cross-reactivity with this

species. This provides some concern because *E. bieneusi* may arguably be the more important human pathogenic microsporidia. Because of these limitations on the use of antibodies for the detection of microsporidia in water samples, another approach using DNA extracted directly from water samples was developed.

Community DNA Extraction

Because the use of IFA as a method of detection has not been shown to be specific nor sensitive enough for routine environmental monitoring, the use of the PCR is required. To use the PCR, it is necessary to extract DNA from concentrated water samples. Most concentrated water samples are turbid and contain large volumes of sediment and other particulate matter. The extraction of total DNA from environmental samples, especially soils and sediments, has always been considered to be difficult. This was because with classic methods much of the DNA from samples was often lost, either through sorption to colloids or in the extensive DNA purification process that is required. In addition, many PCR inhibitory substances are copurified with the DNA. On top of this, processing is time-consuming and labor-intensive. The DNA extraction from environmental water concentrates also presents additional challenges because the samples vary widely depending on the type of water sample collected and where it was collected.

Dowd and coworkers (52) developed a method optimized and standardized for routine screening of water concentrates for microsporidia. They developed a modified protocol using a QIAamp® Tissue Kit (QIAGEN Inc., Santa Clarita, California). Four limits of detection sensitivity experiments with replicates were performed using Percoll-sucrose floatation purified water concentrates and the DNA extraction procedure. The limit of detection was shown to be a mean of four spores. Additional findings suggest that this limit of detection sensitivity can increase to as high as 10^3 spores in some environmental water samples.

Dowd (42) has also evaluated a second method that utilizes the Bio101 soil DNA extraction kit (Bio101). This is a relatively new kit that has been optimized for use in soil samples. Because the sediment of a concentrated water sample is a matrix like soil, this kit shows promise for use in this method. In preliminary studies, the limit of detection sensitivity averaged 200 spores in four very different concentrated water samples. The low was 20, whereas the high was 2,000 spores. Although only preliminary, these results suggest that the use of this DNA extraction kit has the potential for use in detection of the microsporidia in concentrated water samples. Benefits include saving time and cost, increased efficiency, and reasonable detection sensitivity.

Polymerase Chain Reaction (PCR)

The major obstacle hindering the detection of microsporidia in the environment has been the small size of the human pathogenic microsporidia (<2 μm in diameter), which has made it virtually impossible to positively identify the species of isolates visually, without the use of transmission electron microscopy (TEM). Because of the difficulty in using TEM on environmental samples, there has been no confirmed documentation of human pathogenic microsporidia associated with water samples. Another important factor that makes it difficult to correctly identify microsporidia in the environment is the presence of other genera of microsporidia that are not associated with pathogenicity in humans but which do infect nonhuman targets including fish and insects. These other members of the Microspora have been detected in water and have been shown to cross-react with antibodies developed against the human pathogens, thus prohibiting the use of IFA analysis.

Three PCR primers sets have been utilized to analyze water samples for microsporidia (40). The first set is specific for *E. bieneusi* and was utilized to amplify DNA in river water samples. The second set is specific for all of the primary human pathogens (56). Dowd and coworkers (38), used these primers to amplify DNA in a variety of water samples. Forward primer (5′-CAC CAG GTT GAT TCT GCC TGA C-3′) and the reverse primer (5'-CCT CTC CGG AAC CAA ACC CTG-3') amplify the small subunit ribosomal DNA of microsporidia. PCR conditions used for environmental samples were as follows: *Taq* Gold (Perkin-Elmer Corp., Norwalk, Connecticut) induced hot-start cycling conditions, GeneAmp PCR buffer II and GeneAmp dNTPs (Perkin-Elmer Corp). The cycling profile was as follows: 10 minutes of denaturation at 95 °C, followed by 40 cycles of denaturation at 94 °C for 45 seconds, annealing at 55 °C for 20 s, and extension at 72 °C for 40 seconds. A final extension step consisting of 5 minutes at 72 °C was also included. These primers are specific for HEM but are problematic for environmental applications because of their tendency to form primer dimers. Thus, these primers are not as sensitive as they could be for environmental work.

A second set of primers also designed to be specific for the human pathogenic microsporidia, when combined with CDHC, have been shown to confirm identification of PCR-amplified microsporidia to the species level (42). These primers were also shown to be highly sensitive when tested in spiked environmental and clinical samples and do not exhibit serious primer or primer-primer dimerizations as predicted by primer design software (DNAstar, Madison, Wisconsin) or actual testing (data not shown). The forward HEM primer was designated MicF1 (5′ AGG TTG ATT CTG CCT GAC 3′), and the reverse primer was designated MicR1 (5′ GCG CCT GCT GCC RTC CT 3′). All PCR conditions were as follows: *Taq* Gold (Perkin-Elmer Corp., Norwalk, Connecticut)-induced hot-start cycling conditions consisting of 10 minutes of denaturation at 95 °C, followed by 40 cycles of denaturation at 94 °C for 1 minute, annealing at 56 °C for 30 seconds, and extension at 72 °C for 1 minute. A final extension step consisting of 5 minutes at 72 °C is also included. These primers have been shown to be highly sensitive and specific for use in environmental samples including concentrated water samples.

Species Determination

In addition, the use of molecular methods such as the PCR has provided the ability to quickly determine the species

of microsporidia — a task that traditionally required the use of TEM. Currently, there are two methods that show considerable utility. The first is the use of the PCR followed by restriction fragment-length polymorphic (RFLP) analysis to help differentiate species on the basis of varied restriction patterns (56). The second and the most promising is the use of PCR sequencing and computer database homology comparison (CDHC) (42). The use of restriction analysis is only slightly more cost-effective but still leaves considerable doubt at least in environmental samples as to the actual identity of the organisms from which the amplicon was derived. For instance, the *Encephalitozoonidae* (*E. intestinalis*, *E. hellem*, and *E. cuniculi*) can often be distinguished by restriction analysis of their PCR products. However, for environmental samples, there is the potential for other genera of microsporidia to be amplified, some of which could have the same restriction patterns as the human pathogens, thus lending uncertainty to such analyses. This is because a yet uncharacterized organism having the same restriction pattern but a completely different genetic sequence and taxonomic designation could as easily generate such a PCR product and subsequent restriction pattern. Thus, the use of the PCR sequencing and database homology comparison may be the method of choice for species determination of human pathogenic microsporidia, especially when screening environmental samples. This method has also been tested using a statistically relevant population of controls.

Sequencing of PCR products has been described previously (57). Following sequencing, CDHC is used to analyze the sequence. This procedure was briefly described by Dowd and coworkers (42). The initial database search was performed using BLASTn (58) provided by the National Center for Biotechnology Information (NCBI) at their web site (*http://www.ncbi.nlm.nih.gov*). The query sequence or PCR sequencing results from the environmental sample is entered into the BLASTn search engine and the database search is conducted. Results are carefully analyzed in graphic and statistical form as provided by the BLASTn search engine results page. The graphic form was used to ensure that the whole query sequence entered into the search was subsequently aligned with the high-scoring database sequence returned. Following this initial search, the database sequence is downloaded from GenBank and the database sequence and query or unknown sequence are aligned again using pairwise (Martinez-NW Method) alignment. This algorithm identifies regions of perfect match between two sequences, while the Needleman-Wunsch method then optimizes the alignments between regions containing perfect matches. This second alignment is performed separately from the alignment generated by BLASTn, which is often incomplete or inaccurate. This Martinez-NW alignment allows assessment of the entire query sequence alongside the known database sequence.

CONCLUSION

Microsporidia are emerging protozoan pathogens, which may be associated with waterborne transmission. Previously there has been a lack of published methodologies applicable to environmental research. Recently, an assessment of standard methods for protozoan concentration from large volumes of water and the development of improved detection methodologies have been documented. Several problems have been identified in the course of these studies that require further research. The major issue is the development of methods for determining the viability of spores isolated from the environment. Gelman Envirochek provides adequate recovery efficiencies allowing monitoring of finished water for the presence of these pathogens. PCR-detection methodologies appear adequate for detection and species determination. Finally, adequate occurrence, survival, and disinfection data is still lacking in spite of recent studies addressing these issues. This is highly significant, since especially with the recent detection of human pathogenic microsporidia in water, drinking water utilities should have available methods to assess if their facilities can remove or inactivate these pathogens before they reach the consumer. Microsporidia are smaller than other waterborne pathogens and may be more likely to penetrate filters used in conventional water treatment. The methods presented here could be used to assess the effectiveness of current water treatment processes in the removal of microsporidia from water.

There is a definite need for better understanding of the epidemiology HEM and for better management and control measures to ensure the safety of water used for drinking and water-washed produce. Confirmation of these water sources, as a potential source of disease, may rapidly lead to the development of better management practices being implemented by populations in these countries, in order to reduce the prevalence of these parasites in waters used for consumption and irrigation. Little is known about the occurrence or prevalence of HEM in relation to immunocompetent humans, wild, and domestic animals. HEM are being increasingly identified as the causative agent of traveler's diarrhea, most probably caused by the consumption of contaminated food or water. However, the environmental occurrence of these emerging pathogens has not been adequately documented, due to a previous lack of effective methodologies. This emphasizes the need for further research to document the occurrence of these protozoan pathogens in source and treated water.

The presence of human pathogenic microsporidia in surface water may suggest the presence of environmental reservoirs that include domestic and wild animals. Now, the detection of *E. intestinalis* and *E. bieneusi* in surface water may further lend credence to the potential for wild and/or domestic animal reservoirs. Like *Giardia* sp., which has environmental reservoirs including raccoons, and *Cryptosporidium* sp., which has cows as accessory hosts, microsporidia may also have wild and/or domestic animals that are natural or accessory hosts. In any case, the role of water in the transmission of infection to humans could play a significant role, and the microsporidia may be the newest waterborne emerging pathogens.

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MINE TAILINGS.

See BIOMINERALIZATION BY BACTERIA

MINES.

See CAVES AND MINES MICROBIOLOGICAL SAMPLING

MIXOTROPHS.

See PROTOZOAN CILIATES IN FRESHWATER ECOSYSTEMS

MODELING OF BIOFILMS

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The major difference between microbial systems with single cells in suspension and systems with biofilms or microbial flocs is that in the latter, transport processes usually are of primary importance. As a result of the high density of microbial cells in biofilms, high mass fluxes

are induced, which often make transport the rate-limiting process, and strong spatial gradients are formed, which create a variety of microniches in the biofilm interior (1,2).

There are a number of typical biofilm characteristics that are of relevance for biofilm modeling: (*1*) Biofilms are very complex systems. Various microbial species may coexist in a biofilm, and their behavior is determined by a large number of physicochemical parameters; (*2*) Biofilms are very dynamic systems. Typical time constants of biofilm processes range from seconds to weeks; (*3*) Biofilms have a high spatial heterogeneity. They grow in homogeneous layers of microbial cells or form mushroom-like structures; (*4*) Biofilms can be found in a variety of environmental and technical systems that differ considerably in geometry, hydrodynamic conditions, and properties of the substratum.

In biofilm modeling, this diversity is reflected by the numerous models that have been developed during the last thirty years. This development was paralleled by a dramatic increase in experimentally gained knowledge and a fast-growing computational capacity. The biofilm models existing today differ considerably in their complexity, resolution in time and space, database needed, and purpose for which they were developed. Some models are relatively simple and are used, for example, for the design of wastewater treatment plants, whereas some are rather complex and were developed as research tools to analyze experimental data or to test new hypotheses. Some are simple steady state models, whereas some describe the evolution of the biofilm structure in time, and in one, two, or three spatial dimensions.

In the following, biofilm modeling at three different levels is described. First, an example is given of one of the original, simple design models with one species and one substrate. Then, the one-dimensional, multispecies and multisubstrate models that are mostly used to solve practical problems, are discussed. Finally, new developments of models as tools for biofilm research are reviewed.

ONE-DIMENSIONAL, ONE-SPECIES AND ONE-SUBSTRATE MODEL

In wastewater treatment, numerous design formulas for biofilm reactors have been proposed over the past years (3). These formulas were mostly empirical, that is, they were based on data-fitting. Only in the 1970s, mechanistic models, that is, models derived from physical principles, started to evolve (4). The significance of transport of dissolved substrates in biofilms was then realized and it was found that this process could be described by Fick's first law of diffusion as

$$j = -D_F \frac{dS}{dz} \tag{1}$$

where j is the specific mass flux ($ML^{-2}T^{-1}$), D_F the molecular diffusivity in the biofilm (L^2T^{-1}), and S the concentration of the dissolved substrate (ML^{-3}), and z is a spatial coordinate in the direction opposite to the mass flux (L). To model consumption of the substrate diffusing into the biofilm, there are several alternatives. A very widely used model is the Monod equation for the substrate consumption rate r ($ML^{-3}T^{-1}$),

$$r = -r_{max} \frac{S}{K + S} \tag{2}$$

where r_{max} is the maximum substrate consumption rate ($ML^{-3}T^{-1}$), and K is the Monod or half-saturation constant (ML^{-3}). For a steady state situation, the change of the substrate flux is equal to the rate of substrate consumption at any location in the biofilm:

$$\frac{dj}{dz} = r \tag{3}$$

Substituting Equations 1 and 2 into 3 yields a one-dimensional mass balance equation for the substrate S,

$$\frac{d^2S}{dz^2} = \frac{r_{max}}{D_F} \frac{S}{K + S} \tag{4}$$

This is a nonlinear, second-order, ordinary differential equation, by which the concentration profile and the mass flux of the substrate in the biofilm can be calculated for a steady state situation. The solution has to be obtained by a numerical method and requires two boundary conditions. Usually, these are a no-flux condition at the interface between biofilm and substratum,

$$j = 0 \tag{5}$$

and a given substrate concentration S_{bulk} at the interface between biofilm and bulk fluid:

$$S = S_{bulk} \tag{6}$$

Simplifying Assumptions

The biofilm model as given by Equations 4 to 6 is based on a number of simplifying assumptions. These assumptions form an intrinsic part of the model and therefore are listed here:

- The substrate concentration is constant with time and throughout the bulk fluid of the system or reactor.
- The substrate considered is the only limiting substrate, that is, the concentrations of all the other substrates present are not significantly changed by the microbial activity in the biofilm.
- Transport of the dissolved substrate in the biofilm is by molecular diffusion only.
- There is one relevant microbial species forming a biofilm, that is attached to a plane substratum and consists of microbial cells evenly distributed throughout the biofilm.
- There is an equilibrium between net growth of microbial cells in the biofilm and net detachment of cells from the biofilm to the bulk fluid, that is, the biofilm thickness remains constant.
- The dimensions of the substratum area covered by the biofilm are large compared to the biofilm thickness,

which means that for symmetry reasons, the problem is one-dimensional in space and that the direction of mass fluxes is perpendicular to the substratum.

- The physicochemical conditions in the system or reactor do not change with time and space.

Data Requirements

For the solution of Equation 4 the values of the model parameters D_F, r_{max}, and K, as well as the substrate concentration S_{bulk} and the biofilm thickness L_F must be known. Usually, the spatial coordinate z is taken to be $z = 0$ at the substratum and $z = L_F$ at the biofilm surface. The diffusivity D_F of the substrate in the biofilm is affected by the microbial composition and biofilm density (5,6). Values for D_F given in the literature usually range from 40 to 100% of the diffusivity D in pure water (7–9). In most models, a value of $D_F = 0.8 * D$ is used. The maximum substrate consumption rate r_{max} is determined by

$$r_{max} = \frac{\mu_{max}}{Y} X \tag{7}$$

where μ_{max} is the maximum specific microbial growth rate (T^{-1}), X is the concentration of the microbial cells in the biofilm (ML^{-3}), and Y is the yield coefficient ($M_X M_{S^{-1}}$) that denotes the mass of X produced per unit of S utilized. For most species and substrates, numerical values for the kinetic and stoichiometric parameters μ_{max}, K and Y can be found in the literature (10). However, it becomes more and more clear that the kinetic parameters of a microbial species may vary considerably (11,12) and it seems that the values of the kinetic parameters of planctonic and sessile cells of the same species may be different (13). However, it remains unclear to what extent the observed variations are attributed to metabolic differences between the sessile and the planctonic state of the species.

Modifications and Extensions of the Model

A lot of experimental data can adequately be fitted by Equation 4 based on the Monod rate equation (Fig. 1). Studies of enzyme kinetics by Michaelis-Menten yield a potential theoretical explanation of the Monod Equation 2. With regard to modeling, the attractiveness of this equation is primarily due to its mathematical properties: for substrate concentrations S much larger than K, the rate r asymptotically reaches a maximum, which is reasonable because it must be expected that other substrates subsequently become rate-limiting. For values of S approaching zero, r converges to zero as well, which prevents numerical problems arising with rate equations for which S can become negative. Furthermore, the Monod equation represents a mathematical function, which is smooth for the whole range of substrate concentrations. Nevertheless, some researchers find zero-order rate expressions.

$$r = -r_0 \tag{8}$$

or first-order rate expressions

$$r = -r_1 S \tag{9}$$

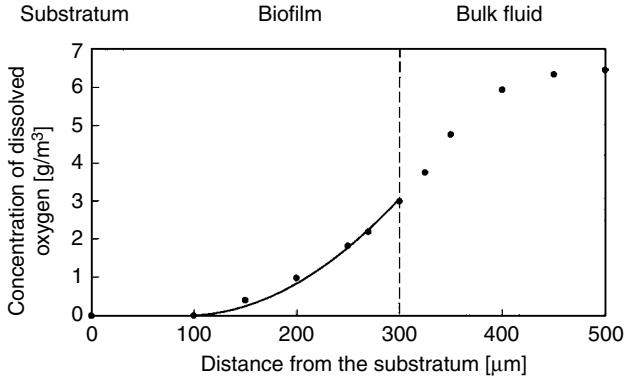

Figure 1. One-dimensional, one-species and one-substrate model: measured (14) and calculated spatial profile of dissolved oxygen.

with the rate constants r_0 ($ML^{-3}T^{-1}$) and r_1 (T^{-1}) adequately fit their data. The advantage of rate Equations 8 and 9, which can be considered to be special cases of the Monod Equation 2, is that they allow the analytical solution of the mass balance equation (15). However, with the computational capacities available today, it is not so advantageous to use Equations 8 and 9.

Mass transport limitation in a liquid boundary layer on the bulk fluid side of the biofilm surface often can have a significant effect on the spatial profile and consumption of the substrate in the biofilm (Fig. 1). Therefore, methods have been developed which allow inclusion of this process in the one-species and one-substrate model (16,17).

ONE-DIMENSIONAL, MULTISPECIES AND MULTISUBSTRATE MODEL

On the basis of the same mass balance principles used to derive the one-species and one-substrate model, a more general multispecies and multisubstrate model can be developed, if some of the simplifying assumptions that have been made in the former model are omitted. Microbial species and substrates will subsequently be referred to as particulate and dissolved components, respectively. For any component, a mass balance can be set up, which states that for any time and location the

change of the concentration in time	=	the change of mass transport in space	+	the transformation (production or consumption) rate

In mathematical terms, this is written as:

$$\frac{\partial C}{\partial t} = -\frac{\partial j}{\partial z} + r \tag{10}$$

where C is the component concentration (ML^{-3}). This mass balance equation is the basis for modeling of multispecies and multisubstrate biofilms. The equation can be applied to any microbial species and substrate, given that an adequate mathematical description of the transport and transformation processes of these components can be provided. Equation 10 describes a non–steady state situation, and as written here is one-dimensional in space.

Transformation Processes

Transformation processes are mathematically described by rate equations, which quantify the mass of a component produced or consumed per unit time and unit volume (18). These rate equations and their parameters have to be known for the dissolved as well as for the particulate components, that is, for all components that are to be considered in the model. Besides microbial species and dissolved substrates these can also include components such as pH, organic and inorganic particles, extracellular polymeric substances, or inactivated cells. In principle, the rate Equation 2, which is used in the one-species and one-substrate model, also applies here. However, because here a large number of interactions can occur between the various components considered, the rate equations in the multispecies and multisubstrate model usually are more complex (12,19). The so called stoichiometric matrix has proven to be a good means to represent the transformation processes and interactions of multispecies and multisubstrate microbial systems.

Stoichiometric Matrix. Table 1 shows an example, which has been taken from the literature (20) and is used here to describe the structure and use of stoichiometric matrices. In the first column of the matrix, the transformation processes considered in the model are listed, in the next columns, the stoichiometric coefficients of all particulate and dissolved components are included, and in the last column, the rate laws of the transformation processes are listed. According to the formula at the bottom of the matrix, the transformation rate r_i of component i is calculated as the sum of the stoichiometric coefficients v_{ij} multiplied by the process rate laws p_j of all transformation processes. The example given in Table 1 describes a biofilm system with two microbial species A and B, and the dissolved substrates acetate, glucose and dissolved oxygen. The processes numbered 1 to 4 describe growth and decay of the microbial species. The parameters Y and b denote the yield coefficients ($M_X\,M_{S-1}$) and decay rate constants (T^{-1}), respectively. The specific growth rates μ_i (T^{-1}) are modeled as

$$\mu_A = \mu_{\max A}\frac{C_{Ace}}{K_{Ace}+C_{Ace}}\frac{C_{O_2}}{K_{O_2}+C_{O_2}}F_{pH_A} \tag{11}$$

and

$$\mu_B = \mu_{\max B}\frac{C_{Glu}}{K_{Glu}+C_{Glu}}\frac{K_{O_2}}{K_{O_2}+C_{O_2}}F_{pH_B} \tag{12}$$

In these equations microbial growth depends on two rate-limiting substrates (12,19,21) and on pH, according to the expressions

$$F_{pH_A} = \frac{1}{(1+10^{5.5-pH})(1+10^{pH-9.5})} \tag{13}$$

and

$$F_{pH_B} = \frac{1}{(1+10^{5-pH})(1+10^{pH-9})} \tag{14}$$

Table 1. Stoichiometric Matrix

Process	Component[a] Particulate		Dissolved								Process Rate Law
	$i=1$	2	3	4	5	6	7	8	9	10	
j	A	B	Ace	Glu	O_2	CO_2	HCO_3^-	H^+	HPO_4^{-2}	$H_2PO_4^-$	$p_j(ML^{-3}\,T^{-1})$
1. Growth of A	1		$\frac{-1}{Y_A}$		$1-\frac{1}{Y_A}$	$\frac{2}{64}\left(\frac{1}{Y_A}-1\right)$		$\frac{-1}{64Y_A}$			$\mu_A X_A{}^b$
2. Decay of A	−1		1					$\frac{1}{64}$			$b_A X_A$
3. Growth of B		1	$\frac{1}{Y_B}-1$	$\frac{-1}{Y_B}$				$\frac{1}{64}\left(\frac{1}{Y_B}-1\right)$			$\mu_B X_B{}^b$
4. Decay of B		−1		1							$b_B X_B$
5. Production of CO_2						1	−1	−1			$\varepsilon_1 k_{f1} C_{H^+} C_{HCO_3^-}$
6. Consumption of CO_2						−1	1	1			$\varepsilon_1 k_{b1} C_{CO_2}$
7. Production of $H_2PO_4^-$								−1	−1	1	$\varepsilon_1 k_{f2} C_{H^+} C_{HPO_4^{-2}}$
8. Consumption of $H_2PO_4^-$								1	1	−1	$\varepsilon_1 k_{b2} C_{H_2PO_4^-}$
Transformation rate ($ML^{-3}\,T^{-1}$):			$r_i = \sum_j v_{ij} p_j$								

[a] Units for microbial species A and B, acetate, glucose and O_2 are gCOD m^{-3}; units for CO_2, HCO_3^-, H^+, HPO_4^{-2} and $H_2PO_4^-$ are mol m^{-3}
[b] μ_A and μ_B according to Equations 11 and 12.

The pH value in this example is the result of the two chemical reactions

$$H^+ + HCO_3^- \underset{k_{b1}}{\overset{k_{f1}}{\rightleftharpoons}} CO_2 + H_2O \tag{15}$$

and

$$H^+ + HPO_4^{-2} \underset{k_{b2}}{\overset{k_{f2}}{\rightleftharpoons}} H_2PO_4^- \tag{16}$$

where k_f ($L^3 M^{-1} T^{-1}$) and k_b (T^{-1}) are the forward and backward rate constants, respectively, of the chemical reactions, which in Table 1 are described by the processes numbered 5 to 8. The example demonstrates that biochemical and purely chemical reactions can formally be treated in exactly the same way. Because the biofilm is a multiphase medium, concentrations can be defined in two different ways. Usually, S and X refer to the concentrations of dissolved and fixed particulate components, respectively, defined as mass per unit biofilm volume, whereas C is the concentration of a dissolved component defined as mass per unit liquid phase volume, and ρ_s is the density of a cell or particle defined as its mass per unit of its volume. The two definitions are related to each other by

$$S = \varepsilon_l C \tag{17}$$

and

$$X = \varepsilon_s \rho_s \tag{18}$$

where ε_l and ε_s are the volume fractions (−) of the liquid phase and solid phases in the biofilm, respectively. The volume fraction ε_l is also referred to as porosity.

Stoichiometric matrices are increasingly used to describe the transformation processes of complex microbial systems (22). They readily allow one to check whether the kinetics and stoichiometry of a model have been set up correctly, that is, to verify, whether the sum of the stochiometric coefficients of each transformation process is zero, if the components are expressed in consistent units (23).

Transport Processes

Transport of dissolved substrates in the liquid phase of the biofilm is in this model also described as a diffusive process according to Equation 1. Transport of particulate species that are attached to the substratum and form the biofilm solid matrix, is the result of microbial growth and decay in the biofilm. Growing or shrinking cells lead to a volume expansion or contraction of the biofilm solid matrix, respectively, and to a displacement of neighboring cells (24). This displacement can be interpreted as advective transport and is formally described by

$$j = u_F X \tag{19}$$

where u_F is the distance by which the cells are displaced per unit time (LT^{-1}). The displacement velocity u_F of a cell at the location z is equal to the added net specific mass production of all particulate components of the biofilm matrix between the substratum and this location:

$$u_F(z) = \frac{1}{1-\varepsilon_l} \int_0^z \sum_i \frac{r_i}{\rho_{si}} dz \tag{20}$$

where r_i is the net transformation rate ($ML^{-3} T^{-1}$) of the particulate component i (20). In the one-dimensional, multispecies and multisubstrate model, the direction of the specific mass flux j is perpendicular to the substratum.

Transfer Processes

Mass-Transfer Resistance at the Biofilm Surface. The exchange of dissolved and particulate components between the biofilm and the bulk fluid is usually affected by the existence of a liquid boundary layer, which creates a mass-transfer resistance outside the biofilm surface and in which mass transport is due to molecular diffusion. This mass transport can be modeled as

$$j = \frac{D}{L_L}(C_L - C_B) \tag{21}$$

where L_L is the thickness of the liquid boundary layer (L), D is the molecular diffusivity of a dissolved or suspended particulate component in water ($L^2 T^{-1}$), and C_B and C_L are the concentrations of the component in the bulk fluid and at the biofilm surface (ML^{-3}), respectively. If flow in the bulk fluid is highly turbulent, mass-transfer resistance can usually be neglected; if it is almost stagnant, the boundary layer thickness may be on the order of several hundred micrometers, and C_L may differ significantly from C_B.

Detachment. In most cases, detachment is a very significant process, which limits biofilm growth (25). Detachment describes the loss of particulate material from the biofilm to the bulk fluid. This loss is primarily due to hydraulic shear forces (26,27) and occurs either as erosion of single cells or particles from the biofilm surface or as disruption of large chunks of biomass from the biofilm solid matrix. The latter phenomenon is referred to as sloughing. (See Biofilm Detachment, this Encyclopedia.) Modeling of both phenomena can be based on a velocity u_{de} (LT^{-1}), which yields a phenomenological description of the decrease of the biofilm thickness per unit time as a result of the detachment process. Because detachment was found to be caused by a variety of factors in addition to mechanical shear stress, such as changes of the physico chemical conditions (28–30), nutrient depletion (31), or release of specific enzymes in the biofilm depth (32), a universal expression for u_{de} does not exist. The expression

$$u_{de} = c_{de} L_F^2 \tag{22}$$

represents a purely empirical equation, chosen for its ability to exclude an infinitely increasing biofilm thickness (20). As already mentioned, two types of detachment are distinguished: erosion is modeled by small values of the detachment coefficient c_{de} ($T^{-1} L^{-1}$), which are constant or varying only slowly, and sloughing by

pulse-functions, that is, values of c_{de} which for short time periods become extremely large.

Attachment. This process refers to the adsorption of cells and particles suspended in the bulk fluid to the biofilm surface and is modeled by an attachment velocity as

$$u_{at} = \frac{1}{1-\varepsilon_l} \sum_i \frac{k_{at} X_{Li}}{\rho_{si}} \tag{23}$$

where k_{at} is the attachment rate coefficient (LT^{-1}) and X_L is the concentration of the suspended particulate component i at the bulk fluid side of the biofilm surface (ML^{-3}). Attachment is seldom explicitly included in biofilm models, simply because detachment usually exceeds attachment, and detachment thus is assumed to model the net mass transfer of cells and particles to the bulk fluid.

Mass Balance Equations

Dissolved Components. Substituting Equation 1 into Equation 10 yields a mass balance equation, which describes the development over time and the spatial profile in the biofilm of the concentration S of a dissolved component as:

$$\frac{\partial S}{\partial t} = D_F \left(\frac{\partial^2 S}{\partial z^2} \right) + r \tag{24}$$

with the boundary conditions

$$\frac{\partial S}{\partial z} = 0 \tag{25}$$

at $z = 0$ and

$$S = S_L \tag{26}$$

at $z = L_F$. Results as they are typically obtained by Equation 24 are shown in Figures 2 and 3, which display spatial profiles calculated for the example defined in Table 1 (20). Due to biofilm growth, the spatial profiles change their position in space (Fig. 3).

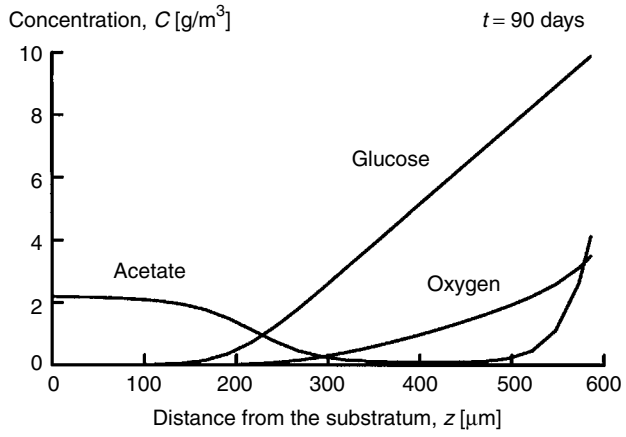

Figure 2. One-dimensional, multispecies and multisubstrate model: spatial profiles of dissolved oxygen, acetate and glucose.

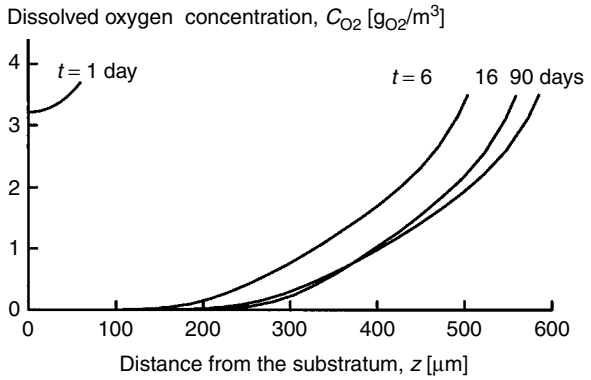

Figure 3. One-dimensional, multispecies and multisubstrate model: shift of the spatial profiles of dissolved oxygen as a result of biofilm growth.

Particulate Components. On the basis of the mass balance Equation 10 and Equation 19 for the mass flux, the development over time and the spatial profile in the biofilm of microbial species, inactivated cells, extracellular polymeric substances, and organic and inorganic particles is described as:

$$\frac{\partial X}{\partial t} = -\frac{\partial (u_F X)}{\partial z} + r \tag{27}$$

where X is the concentration of the particulate component in the biofilm (ML^{-3}) and u_F can be calculated by Equation 20. The boundary condition needed to solve Equation 27 is a no-flux condition at the substratum,

$$j = 0 \tag{28}$$

for $z = 0$. Equation 27 is used to calculate the relative abundance, spatial distribution, and development in time of the particulate components, as illustrated in Figure 4 for the microbial species, whose kinetics and stoichiometry are defined in Table 1. From the spatial profiles of the net specific growth rates

$$\mu_{net} = \mu - b \tag{29}$$

as shown in Figure 5, it can be understood why species A dominates near the biofilm surface, while species B primarily develops in the biofilm depth (Fig. 4).

Biofilm Thickness. The development over time of the biofilm thickness is the result of the net production of particulate components in the biofilm, as described by Equation 20, and of the exchange of cells and particles between biofilm and bulk fluid, as described by Equations 22 and 23. It is modeled as

$$\frac{dL_F}{dt} = u_F(z = L_F) - u_{de} + u_{at} \tag{30}$$

In situations for which only incomplete data is available for the terms on the right-hand side of Equation 30, u_{at} usually is set to zero and u_{de} equal to $u_F(z = L_F)$, having the effect that L_F remains constant at its initial value.

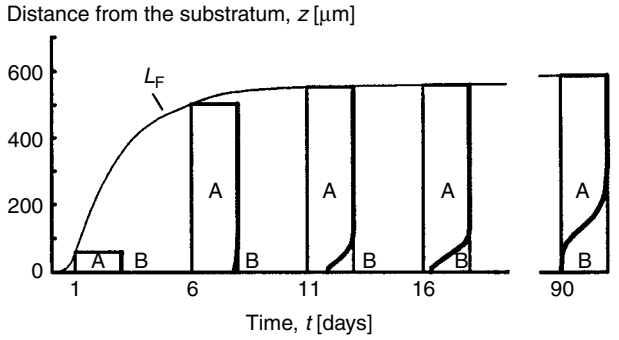

Figure 4. One-dimensional, multispecies and multisubstrate model: progression of the biofilm thickness L_F and the microbial composition of the biofilm solid matrix. Bars show the relative abundance of the two microbial species A and B between substratum (bottom) and biofilm surface (top).

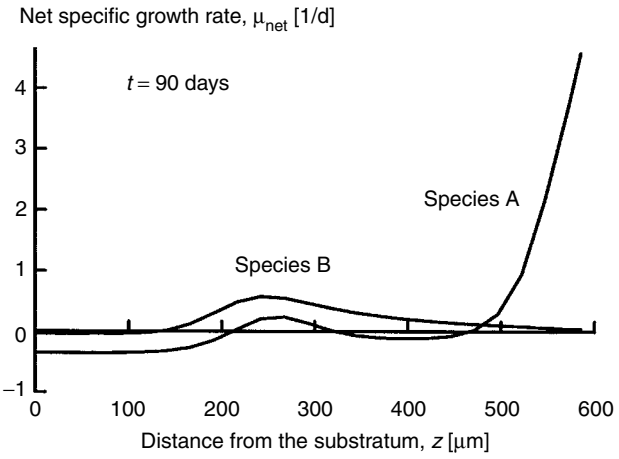

Figure 5. One-dimensional, multispecies and multisubstrate model: spatial profiles of the net specific growth rate of two microbial species A and B. Near the surface, A outgrows B; in the biofilm depth, B outgrows A.

Bulk Fluid. In general, the processes that take place in the biofilm also have an effect on its environment. Usually, this environment is modeled as a completely mixed volume of water, termed bulk fluid. Transformation processes in the bulk fluid can be equally important to those that take place in the biofilm. For each dissolved and suspended particulate component considered, an additional mass balance equation

$$\frac{d(V_B C)}{dt} = Q(C_{in} - C) + A_F j + V_B r \qquad (31)$$

is needed, where C_{in} and C are the influent and bulk fluid concentrations of each component (ML^{-3}), respectively, V_B is the bulk fluid volume (L^3), Q is the rate of flow through the bulk fluid ($L^3\,T^{-1}$), A_F is the biofilm surface area (L^2), j is the mass flux across the biofilm surface ($ML^{-2}\,T^{-1}$), and r is the transformation rate ($ML^{-3}\,T^{-1}$) of the component.

Numerical Solution of the Mass Balance Equations

For each dissolved and particulate component, mass balance equations for the biofilm and the bulk fluid have to be included in the model, together with Equation 30 for L_F and appropriate rate expressions for all the components considered. The numerical solution of the resulting set of nonlinear, partial differential equations requires some skill in numerical mathematics and a considerable computational effort.

Computer Simulation. AQUASIM is a computer program for the identification and simulation of aquatic systems (33–36) and includes the multispecies and multisubstrate model (37). The program is available for several types of computers and solves the mass balance equations for the components and processes, which are specified by the user.

Simplifying Assumptions

The one-dimensional, multispecies and multisubstrate biofilm model as given by Equations 24 to 28,30, and 31 is based on the following simplifying assumptions:

- The bulk fluid of the system or reactor is assumed to be completely mixed.
- The dimensions of the substratum area covered by the biofilm are large compared to the biofilm thickness, which means that for symmetry reasons, the problem is one-dimensional in space and that the direction of mass fluxes is perpendicular to the substratum.
- The biofilm is attached to a plane substratum and is formed by microbial cells evenly distributed throughout the biofilm.
- The volume fraction of the liquid phase in the biofilm, ε_1, does not change with time and space.
- Several microbial species that compete for nutrients and space in the biofilm, can be considered.
- The processes contributing to biofilm growth, that is, to the change of the biofilm thickness in time, are the net production of particulate components in the biofilm, and detachment or attachment of particulate components at the biofilm surface.
- Several limiting substrates can be considered, whose influent and bulk fluid concentrations may vary with time.
- Transport of the dissolved substrates in the biofilm is via molecular diffusion only.

Data Requirements

To set up the multispecies and multisubstrate model, it must be decided which dissolved and particulate components are to be considered. Criteria for this decision are the expected relative abundance of the components forming the biofilm matrix and significance of mass fluxes. Sometimes, specialized microbial species, degrading a xenobiotic organic chemical of interest, also are included. However, it is useless to include components for which rate equations or parameter values are not known. If this is the case, it is better to introduce, for example, components such as "heterotrophic organisms" or "readily degradable organic substrate" instead of several incompletely defined microbial species or substrates. The data required by

the model for all components considered, include the diffusivities in pure water and in the biofilm, and rate laws, kinetic parameters, and stoichiometric coefficients of the transformation processes. Sometimes the rate constants k_f and k_b, as used in Equations 15 and 16, are not known, but the equilibrium constant $K_e = k_f/k_b$ is available. If these equations describe processes that are fast compared to other processes included in the model, it is possible to choose an arbitrary (large) value for k_b and to calculate k_f as $k_f = k_b K_e$. Furthermore, the density of the particulate components, ρ_s, as well as expressions for their attachment and detachment velocities must be given. The latter can seldom be taken from the literature, because they depend on the properties of the reactor or environmental system, and are difficult to measure directly. However, net detachment rates can readily be determined experimentally, if the detached particulates are collected in the effluent of the reactor or system. The volume fraction of the biofilm liquid phase usually is chosen to be $\varepsilon_1 = 0.8$. The geometric and hydraulic data needed are the biofilm surface area A_F, the bulk fluid volume V_B, the thickness of the liquid boundary layer L_L, and the flow rate Q. To start a simulation, the influent and bulk fluid concentrations of all components, the initial spatial distribution of the particulate components in the biofilm, and the initial biofilm thickness must be known. The spatial profiles of the dissolved components in the biofilm develop so fast that the choice of their initial values usually is insignificant. In situations in which only a few dispersed cells are already attached to the substratum, it is no problem to start the simulation with a very small mass of particulate components, that is, with an initial biofilm thickness of a fraction of a micrometer.

Extensions of the Model

A more detailed description of the multispecies and multisubstrate model and examples of its application can be found in the literature (20). In recent years, new experimental techniques have revealed phenomena that cannot be taken into account by the model (38). Therefore, a number of extensions have been made to the original model:

- In the original, multispecies and multisubstrate model, there was either detachment or attachment of cells and particles from or to the biofilm surface, respectively, but the two processes could not occur simultaneously. In the extended model, detachment rates can be specified individually for each particulate species in the biofilm (37). This makes it possible that some species are detached slower than others or even accumulate at the biofilm surface, if attachment is a significant process. For mathematical reasons, simultaneous detachment and attachment is possible only if the particulate components, which form the biofilm solid matrix, are not only displaced by advection, but also by diffusion along the concentration gradient. The latter process allows that particulates that have attached to the biofilm surface can subsequently and slowly penetrate the biofilm (39).
- In the extended model, suspended cells and particles can also be transported in the biofilm liquid phase (39). Consequently, adsorption and desorption of these components to and from the biofilm solid matrix can occur and the kinetics of these processes have to be quantified.
- New experimental data clearly indicate that the physical properties of the biofilm change with time and space. The liquid phase volume fraction, ε_1, decreases with time, especially in the biofilm depth (40). The extended model includes a rate r_{ε_1}, which may be a function of time, space, or other process rates, and thus offers the possibility to test alternative hypotheses about the mechanism underlying the change of ε_1 (37). In the extended model, the diffusivity of dissolved components, which was found to vary considerably over the biofilm depth (9) can be any function of time or space.

With all the additional terms and processes (Fig. 6), the mass balance equations of the extended model have become rather complex (37). However, these additional terms are mainly needed to make the mass balance equations rigorous, and the extensions have little relevance for practice; rather they serve as tools for helping to interpret experimental data obtained in basic research.

The extensions of the multispecies and multisubstrate model are implemented in the computer program AQUASIM (35,36). By default, all the extensions are inactive, thus allowing one to choose the complexity of the model according to the data available. Additional features of AQUASIM are that the substratum may be flat, cylindrical, or spherical, and that the diffusivities in the biofilm may vary with time and over the biofilm depth, accounting for the observation that dissolved components in some cases are transported by advection or turbulent diffusion in parts of the biofilm.

NEW TRENDS IN MODELING

New experimental techniques for the analysis of the structure and function of biofilms have evolved rapidly in the last years, revealing that biofilms are systems that are much more complex and diverse than believed previously (41–43). In parallel to the development of the new experimental tools that provide data at the microscopic scale, new models have been developed to describe biofilm behavior at the level of cell colonies or even individual cells. The objective of these efforts is, on the one hand, to design models able to reproduce the experimentally observed phenomena, and on the other hand, to provide analytical tools to study hypotheses about the mechanisms underlying these phenomena. It is impossible to describe in detail all the modeling work that has been done during the last years. Therefore, in the following text only some of the concepts, objectives, and results of new trends in biofilm modeling are summarized.

Multidimensional Models of Biofilm Structure

Modeling Concepts. One group of new biofilm models primarily deals with the development of the biofilm

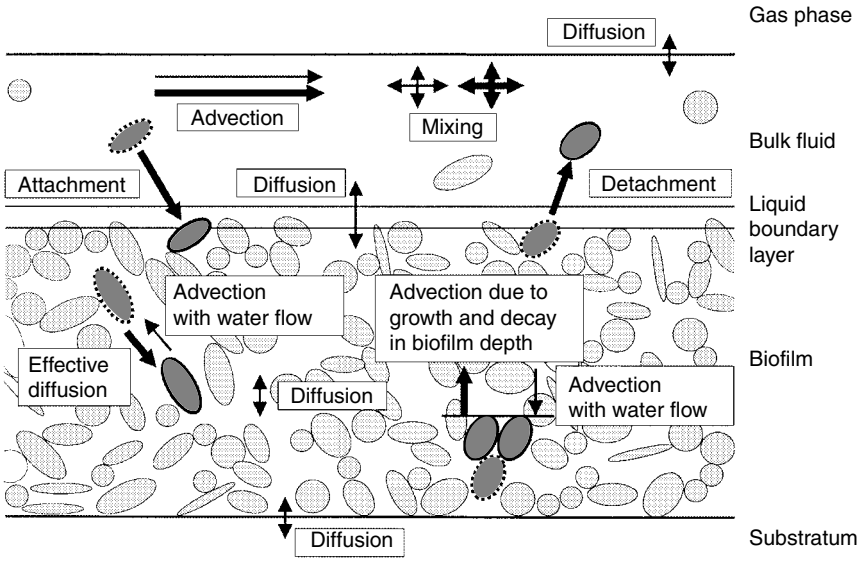

Figure 6. Extended one-dimensional, multispecies and multisubstrate model: transport processes considered in the model. Thick arrows refer to particulate components; thin arrows refer to dissolved components.

structure, which in many cases has been found to be an aggregate of distinct microbial colonies with large pores in between, and does not have the form of a smooth layer of homogeneously distributed cells. Most of these models are so called "cellular automaton" models (44–46). Their concept is to divide the space at the liquid side of the substratum into a large number of very small volume elements in which the changes in time of the concentrations of the dissolved and particulate components are calculated as the result of transformation and transport processes. These processes are the same as those considered in the one-dimensional, multispecies and multisubstrate model described earlier, but the major difference is that here transport is modeled in two or three dimensions. Transport of the particulate components that form the biofilm solid matrix, occurs when, because of the production of mass, the concentration of the particulates in a volume element exceeds a given maximum packing density and any mass produced beyond that limit is displaced to adjacent volume elements. Transport of dissolved and suspended particulate components is also modeled by the cellular automaton approach (45), or by numerical solution of the mass balance Equation 24 in two- or three-dimensional form, for example, by finite difference techniques (47). If the Navier-Stokes equations that provide a multidimensional description of the flow field, are solved simultaneously with the component mass balance equations, advective transport of dissolved and suspended particulate components can also be considered (46,48). Most of these models have been developed for one microbial species, but there are attempts to consider more than one species, interacting by transformation processes (49). To start the calculation, some mass of particulate components usually is attributed at random to a few volume elements, thus simulating the initial colonization of the substratum.

Formulation of Rules. The solution of the model equations for dissolved components primarily requires a lot of computational resources and therefore necessitates a careful selection of appropriate numerical methods (50). However, the most demanding step in the development of the cellular automaton models is the formulation of the rules that determine how the particulate mass is transferred between adjacent volume elements. After each integration step in time, all volume elements are sequentially checked for excess particulate mass, which then is displaced to adjacent volume elements, either selected at random (47,49) or by a given criterion, such as preferential displacement in the direction of the biofilm surface (51). Rules also have to be formulated if additional processes are considered. Detachment is attributed to liquid shear stress exceeding the mechanical strength of the biofilm solid matrix in a volume element (52), or is modeled by simple probability functions based on shear stress and mechanical strength (51), or on empirical coefficients (53).

Results. The new multidimensional models are able to reproduce biofilm structures as they are observed experimentally, even if these models are not based on identical rules, and also may differ in the number of processes and components they consider. It seems that introducing stochastic elements into biofilm models is the key factor for modeling their multidimensional structural development. An interesting model prediction is that dense biofilms are produced if their development is limited by the microbial growth rates, and that porous, mushroom-shaped biofilms are produced if the substrate transport rates are limiting (Fig. 7).

Models Based on the Behavior of the Individual Cells

Modeling Concepts. Inspired by observations of population dynamics made in ecology, it is argued that the development of the biofilm must be the result of the behavior of the individual cells of which it is composed. This idea has led to a number of new modeling approaches, by which properties of the individual cells

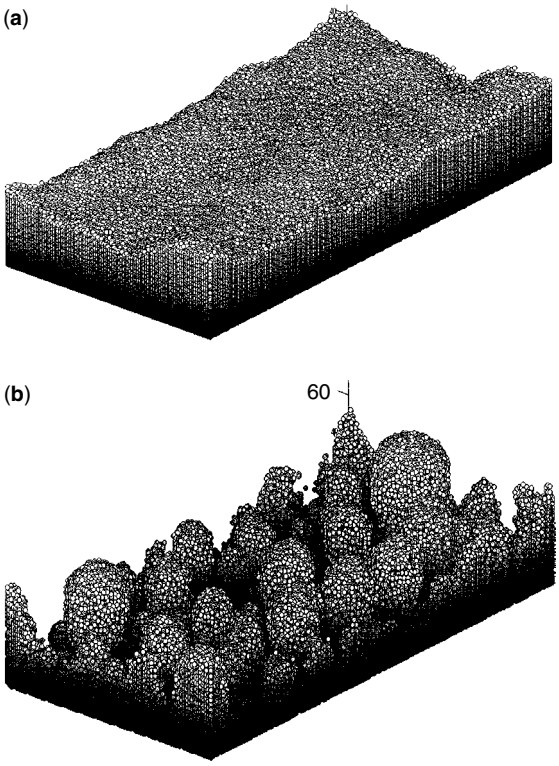

Figure 7. Three-dimensional cellular automaton model of biofilm structure: for high (**a**) and for low (**b**) substrate concentrations, different types of biofilm structures are developing (54).

and processes taking place at the cell level are considered. In one approach, intracellular transformation processes, mass transfer across the cell membrane and extracellular mass transport is modeled at the level of individual cells, and then by averaging, an expression for a growth rate is derived, which is representative for a larger volume element (55). In another approach, called "individual-based modeling," the usually made assumption is abandoned that the behavior of the cells of a microbial species can be modeled by kinetic rate laws and parameters which represent average values not changing with time and space (56). In this model, each cell of the biofilm is described as a distinct object with parameters obtained by random draws from a statistical distribution. As a result of this procedure, the cells of the population do not grow synchronously, leading to the biofilm heterogeneity as it is observed experimentally.

Results. The final objective of individual-based modeling is to explain the behavior of a biofilm at the macroscopic level as the result of the processes that take place at the microscopic level. So far, the approach has suffered from a lack of experimental data on the behavior of the individual cells and on the variability of their kinetic parameters, but with the rapidly increasing knowledge about cells and cell-cell interactions, individual-based modeling could become an important research tool.

Simplifying Assumptions

The multidimensional models of biofilm structure and the one-dimensional, multispecies and multisubstrate model are based on mass balance equations, that is, they all are derived from physical principles. However, knowledge on the mechanism determining the three-dimensional development of the biofilm solid matrix is incomplete. In the one-dimensional, multispecies and multisubstrate model this problem is solved by averaging all variables in planes parallel to the substratum. By doing so, the model complexity is reduced to one dimension in space and to one unknown structural parameter, the liquid phase volume fraction, ε_1. Alternatively, in the new multidimensional models, probability functions are used, and rules are formulated to describe the displacement of microbial cells in various spatial directions as a result of cell growth. This approach is empirical; however, the striking correspondence between observed and modeled development of biofilm structures is an indication that the functions and rules describe the underlying (unknown) physical mechanisms quite well. This also is true for the models describing biofilm behavior at the level of individual cells.

Data Requirements

Among the new models describing the multidimensional development of the biofilm structure and the behavior of individual cells, there are differences in the number of components and processes considered, as well as in the mechanisms by which the processes are modeled. However, for the mathematical description of the geometry of the system or reactor and of the transformation processes, the same data are used by all models, except for the individual-based model, which also needs data on the cell size and variability of the kinetic parameters (56). As compared to the one-dimensional, multispecies and multisubstrate model, the major differences are that in the multidimensional models, the initial and influent concentrations and the inflow do not only vary with time, but also with one to three space coordinates. The complete description of the flow field based on physical laws requires a high computational effort, but avoids the introduction of "artificial" parameters like "diffusivity in the biofilm" or "liquid boundary layer thickness." All models require information on the "packing density" of the microbial cells in the biofilm. In some models this parameter is specified explicitly; in others it is specified indirectly as "porosity" or "biofilm liquid phase volume fraction," or by simple rules such as "cells may not overlap" (56). As mentioned earlier, attachment of cells or particles to the biofilm surface is hardly ever considered, because detachment is assumed to model the net transfer of particulate material between bulk fluid and biofilm. Detachment is modeled as the result of shear stress locally exceeding the mechanical strength of the biofilm solid matrix, as an empirical first-order process, or by use of probability functions, which in one model also are applied to transformation and diffusion processes (53).

CONCLUSION

There are a large number of existing biofilm models. These models differ considerably in their complexity, that is, in the number of processes that they consider and in the accuracy with which these processes are described. Which of these models is appropriate to use for solving a given biofilm problem primarily depends on the objectives and the questions asked (57,58). To make a rough estimate of the amount of biomass needed to eliminate a specific substrate in a biofilm reactor, the simple one-species and one-substrate model may be adequate (17). To calculate the effluent concentrations of a wastewater treatment plant, the one-dimensional, multispecies and multisubstrate model has been applied successfully (59). To identify gaps in knowledge, to analyze differences between experimental observations and model predictions, and to formulate and test alternative hypotheses on processes for which the mechanisms are not yet fully understood, the new generation of biofilm models offers a large potential (46). These new models may serve as powerful research tools to analyze topics such as the role of extracellular polymeric substances, cell-to-cell interactions, gene transfer, and detachment. They also represent a unifying means for the indispensable interaction between biofilm researchers of the many disciplines needed to promote the understanding and control of biofilm systems.

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MODELING OF VIRUS TRANSPORT AND REMOVAL IN THE SUBSURFACE

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Pathogenic viruses are a significant cause for waterborne disease outbreaks attributed to the consumption of contaminated groundwater (1–3). Contamination originates from fecal contaminated sources such as failing septic systems, leaking sewer lines, land application of wastewater, or its mixing with infiltrated surface water. A groundwater well can sufficiently be protected from contamination with viruses, provided setback distances between the source of contamination and the well are great enough. Similarly, viruses can effectively be removed from surface water by passage through soil, provided travel times and travel distances are adequate (4). During passage through soil, viruses are removed from the aqueous phase because of adsorption and inactivation (5,6). Moreover, advection and dispersion affect spreading of viruses, and attenuation. The effectiveness of soil passage for protection of groundwater wells or treatment of surface water can be evaluated by calculating the concentration of viruses at the point of origin from the concentrations in source water by means of a computational virus transport model, which describes the removal processes.

Under field conditions, contamination levels of pathogenic viruses are usually too low to be detected and thus their behavior cannot be easily studied. Therefore, in most studies on subsurface transport of viruses, bacteriophages are used as model viruses. Bacteriophages closely resemble pathogenic viruses, but are not harmful to humans. Bacteriophages can be prepared in large quantities (10^{10}–10^{12} phages per ml) and can be injected or seeded in high numbers into an aquifer. Moreover, bacteriophages can easily be assayed compared to pathogenic viruses.

MODELING SUBSURFACE TRANSPORT OF VIRUSES

Conceptual Model

Processes that must be included in modeling of virus transport in soil and groundwater are adsorption, inactivation, advection, and dispersion. Adsorption of viruses to soil may be irreversible or reversible. In the case of irreversible adsorption, attachment exists and there is no detachment. In the case of reversible adsorption, one may have sites where attachment and/or detachment are fast relative to the flow velocity, allowing equilibrium to occur. For some other sites, adsorption is kinetically limited relative to flow velocity, with constant attachment and detachment rate coefficients. Under the condition of saturated flow, the governing equations of one-dimensional virus transport are as follows:

$$R\frac{\partial C}{\partial t} + \frac{\rho_B}{n}\frac{\partial S_{kin}}{\partial t} = D\frac{\partial^2 C}{\partial x^2} - \nu\frac{\partial C}{\partial x} - Q \tag{1}$$

$$R = 1 + \frac{\rho_B}{n}k_{eq} \tag{2}$$

$$Q = \left(\mu_l + \mu_{s,eq}\frac{\rho_B}{n}k_{eq}\right)C + \mu_{s,kin}\frac{\rho_B}{n}S_{kin} \tag{3}$$

$$\frac{\rho_B}{n}\frac{\partial S_{kin}}{\partial t} = k_{att}C - k_{det}\frac{\rho_B}{n}S_{kin} - \mu_{s,kin}\frac{\rho_B}{n}S_{kin} \tag{4}$$

Here, C is the free virus concentration, that is the number of free viruses per unit volume in the aqueous phase, $[L^{-3}]$; S_{kin} denotes the concentrations of viruses attached to kinetic sites in terms of number of viruses per unit mass of soil, $[M^{-1}]$; ρ is the bulk density of the saturated soil, $[M.L^{-3}]$; n is the porosity; D is the hydrodynamic dispersion coefficient, $[L^2.T^{-1}]$; ν is the pore water velocity, $[L.T^{-1}]$; k_{eq} is a distribution coefficient, $[L^3.M^{-1}]$; R is the retardation coefficient related to equilibrium adsorption; k_{att} and k_{det} are the attachment and detachment rate coefficients, respectively $[T^{-1}]$; μ_l is the inactivation rate coefficient for the free viruses, $[T^{-1}]$; $\mu_{s,eq}$ and $\mu_{s,kin}$ are the inactivation rate coefficients for viruses attached to equilibrium and kinetic sites, respectively $[T^{-1}]$.

Virus adsorption to soil is the most important process for attenuation. However, actual removal, that is, disappearance of viruses, is due to inactivation, and irreversible attachment, whenever it exists. Under steady state conditions, the relative contributions of inactivation and adsorption to the removal of viruses by soil passage

can be compared easily. A steady state situation occurs when input of virus is continuous. This is usually the case during bank filtration, dune recharge, deep well injection, or continuously leaking sewage pipes and may be seen as a worst-case situation. Considering a one-dimensional steady state situation, Equation (1) may be simplified to:

$$\alpha_L \frac{\partial^2 C}{\partial x^2} - \frac{\partial C}{\partial x} = \frac{Q}{\nu} \tag{5}$$

Here, $\alpha_L = D/\nu$ is the longitudinal dispersivity, [L]. Under steady state conditions, it follows from Equation (4) that

$$\frac{\rho_B}{n} S_{kin} = \frac{k_{att}}{\mu_{s,kin} + k_{det}} C \tag{6}$$

Substitution of Equation (6) into (3) gives

$$Q = \lambda C \tag{7}$$

where

$$\lambda = \mu_l + \mu_{s,eq} \frac{\rho_B}{n} k_{eq} + \frac{k_{att}}{1 + \dfrac{k_{det}}{\mu_{s,kin}}} \tag{8}$$

Now, the solution of Equation (5) can be written as

$$\log_{10}\left(\frac{C}{C_0}\right) = \frac{x}{2.3} \frac{\left(1 - \sqrt{1 + 4\alpha_L \dfrac{\lambda}{\nu}}\right)}{2\alpha_L} \tag{9}$$

where C_0 is the concentration at $x = 0$, and $\log(C/C_0)$ is a measure of virus removal. It can be shown that for small values of α_L:

$$\frac{\log_{10}\left(\dfrac{C}{C_0}\right)}{t} = -\frac{\lambda}{2.3} \tag{10}$$

where t is the travel time defined by $t = x/\nu$.

Equation (10) gives the removal rate. From Equations (8, 9), and (10), the relative contributions of adsorption and inactivation to virus removal can be deduced. The first term in Equation (8) gives the removal rate by inactivation of free virus. The second and last terms give the removal rate of virus by interaction with equilibrium and kinetic sites, respectively. Interaction means the combination of attachment, detachment, and inactivation of virus at a particular type of site of the porous medium.

Interaction with Equilibrium Sites

Equilibrium adsorption has been assumed in several column and field studies, whereby kinetic adsorption is neglected (7–10). In some studies, an extra sink term was also included to account for virus removal owing to irreversible attachment (11–13). To show the effect of equilibrium adsorption, some column breakthrough curves were simulated using the CXTFIT-code (14). This code is based on analytical solutions of equilibrium and kinetic transport models, including governing Equations (1) to (4).

For a period of ten days, a virus was seeded on top of a column at a constant concentration. Breakthrough was calculated and is displayed in Figures 1a and b (linear and semilog plots, respectively). The parameter values that were used for these simulations are given in Table 1. Dispersion is basically a characteristic of the porous medium, provided that no size and/or charge exclusion effects are present. Therefore, a virus entering the soil experiences the same dispersion as a conservative salt tracer. This is the case when considering curves A and B. A conservative salt tracer is not retarded and C/C_0 reaches a plateau of one shortly after breakthrough (curve A). A virus that does not attach to the soil shows the same breakthrough as the salt tracer, but the plateau is lower because of inactivation of the virus (curve B). If a virus is also retarded owing to equilibrium adsorption, it shows the same curve as B, but it is shifted to the right (curve C). In addition, the plateau is even lower because more time is available for inactivation to occur. If this virus is subject to a higher dispersion (Table 1), the shape of the curve becomes flatter as is illustrated by curve D. Here, a fraction of the virus breaks through earlier, but the time to peak breakthrough is concurrent with the middle of the plateau of curve C.

Retardation coefficients of about 2–5 have been reported (9,15–17). Retardation coefficients of 1.7–2 were found in a study with five different bacteriophages (18). In that study, it was also found that the fraction of equilibrium sites to the total number of adsorption sites depends on the type of phage. Bacteriophage PM2 did not interact with equilibrium sites, and bacteriophages MS2,

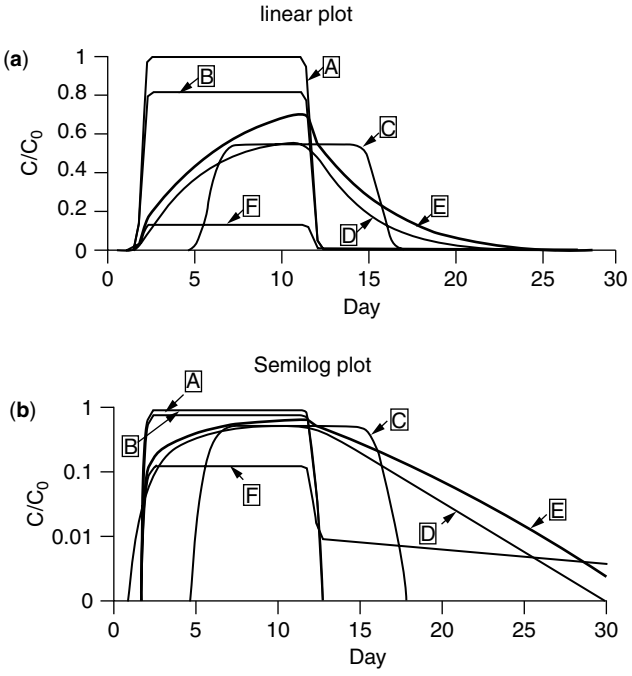

Figure 1. Simulated breakthrough curves of (**a**) a conservative salt tracer, (**b**) a virus that does not adsorb, but that is inactivated, (**c**) a virus that is retarded because of equilibrium adsorption and that is inactivated, (**d**) like (**c**) with higher dispersion, (**e**) a virus that adsorbs to kinetically limited sites, and that is inactivated.

Table 1. Parameter Values Used to Simulate Breakthrough Curves for (a) a Conservative Salt Tracer and (b)–(f) for a Virus Exhibiting Different Types of Adsorptive Behavior. Resulting Breakthrough Curves Are Shown in Figures 1a and 1b. Pore Water Velocity = 1.5 m/day and distance = 3 m.

Parameter	A Salt Tracer	B Virus + Inactivation	C Virus as in B + Equilibrium Adsorption	D Virus as in C + High Dispersion	E Virus as in B + Kinetic Adsorption	F Virus as in E But Slow Detachment
D	0.02	0.02	0.02	1	0.02	0.02
R	1	1	3	3	—	—
k_{att}	—	—	—	—	1	1
k_{det}	—	—	—	—	0.5	0.005
$\mu_l = \mu_s$	0	0.05	0.05	0.05	0.05	0.05

Dimension of D is [m^2.day^{-1}], that of k_{att}, k_{det}, μ_l and μ_s is [day^{-1}].

PRD1, and Qβ only for a minor part. However, a larger proportion (56%) of ϕX174 adsorbed to equilibrium sites. For this latter phage the value of k_{att} was low. Thus for ϕX174, the interaction with equilibrium adsorption sites contributes more to removal than the interaction with kinetic sites. In the case of other phages, the interaction with kinetic sites is far more important in determining removal. Generally, little or no retardation due to equilibrium adsorption of viruses is found (11,15,19–21). Because the value of k_{eq} is generally very low and the values of μ_l and $\mu_{s,eq}$ are of similar order, interaction with equilibrium sites will not contribute significantly to virus removal.

Interaction with Kinetic Sites

At field scale (16,21), attachment rates are relatively fast compared to advection ($k_{att}L/v > 1$, where L is a characteristic travel distance), whereas detachment rates are much slower ($k_{det}L/v < 1$). At very low flow rates, for example, under some natural gradient conditions, kinetically limited adsorption may successfully be described by equilibrium adsorption. To show the differences and similarities between equilibrium and kinetically limited adsorption, various breakthrough curves resulting from kinetic adsorption were simulated using the CXTFIT-code (14) and added to Figures 1a and 1b. Curve E simulates breakthrough of a virus, with the same dispersion as the salt, but now exhibiting kinetically limited adsorption, whereby $k_{att}/k_{det} + 1$ equals the value of R in curve D. The time to peak breakthrough is somewhat retarded. In fact, curves D and E in Figures 1a and 1b have a very similar shape. In some studies (15,22), equilibrium adsorption was assumed, but then an artificially large dispersion coefficient that is much larger than that of the coinjected salt tracer, was needed to fit virus breakthrough curves of shapes similar to curves E and D. Applying the same dispersion coefficient for the salt tracer and the virus, as well as assuming kinetic adsorption is more appropriate. Curve F simulates virus breakthrough, now exhibiting slow detachment. The shape of this curve on linear concentration-scale (Fig. 1a) is now the same as A and B, only the maximum concentration is lower because of attachment. However, when plotted on a semilog scale, a tail becomes visible, which makes it distinct from the other curves (Fig. 1b). Now, if measurements are stopped before the end of the plateau is reached, or if only linear plots are made, one may conclude that there is no reversible adsorption but a very high rate of irreversible adsorption (11,22). These simulations show that description of virus transport by an equilibrium model and a kinetic model may lead to similar results, and that investigation of tailing on a semilog plot is required to tell the difference. A shortcoming of many virus transport experiments is that the tail of the breakthrough curve is not measured, so that the kinetic behavior cannot be observed and quantified (8,9,11).

In several column experiments, adsorption of bacteriophages MS2 and PRD1 was shown to be reversible and kinetically limited; bacteriophages were not found to be retarded by equilibrium adsorption (15,19,23). The same was observed in field studies with MS2, PRD1, ϕX174, and poliovirus 1 (16,21,24,25). Figure 2 shows a typical breakthrough curve of MS2 from a column study (26). Open circles in Figure 2 show the measured breakthrough curve of MS2. A one-site kinetic model was applied to fit this curve. Values for k_{att1}, k_{det1}, and μ_{s1} were obtained by fitting the whole breakthrough curve. These values are given in Table 2 under column A and correspond to curve A in Figure 2. The value of k_{det1} is higher than that of k_{att1} and the value of μ_{s1} is much higher than that of μ. Curve A fits the measured breakthrough curve very well, except for the tail part; this is not considered as satisfactory. In another

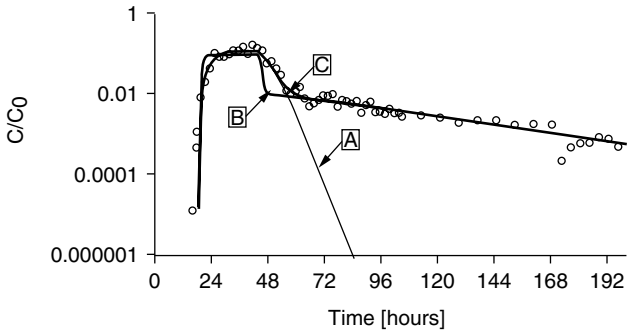

Figure 2. Measured breakthrough curve (open circles) of MS2 fitted with a one-site kinetic model (curves A and B) and a two-site kinetic model (curve C) (26).

Table 2. Parameter Values (in day^{-1}) Estimated from Breakthrough Curves of Column Experiments: A, B, and C in Column Headings Correspond to Curves A, B, and C in Figure 2 (26)

Rate Coefficients	A: One-site Model	B: One-site Model	C: Two-site Model
k_{att1}	4.80	2.64	1.94
k_{det1}	6.72	0.065	0.065
k_{att2}			3.36
k_{det2}			13.7
μ_1	0.07	0.07	0.07
μ_{s1}	5.76	0.43	0.43
μ_{s2}			0.43

set of simulations, again a one-site model was used, but this time, trying to fit both the peak and the tail of the breakthrough curve. The corresponding parameter values are given under column B in Table 2 and the corresponding curve is also shown in Figure 2. It was found that the value of μ_{s1} mainly determines the tail slope, whereas the value of k_{det1} mainly determines its intercept. The end of the rising limb and the start of the declining limb of the breakthrough curves cannot be simulated completely, probably owing to an another reversible adsorption process. The value of k_{det1} is now much less than that of k_{att1} (Table 2, column B). Finally, the breakthrough curve was fitted by applying a two-site kinetic model that is, assuming that there is interaction with two different kinds of kinetic sites. The estimated values are given in column C of Table 2. The resulting curve C, in Figure 2, clearly gives a very satisfactory fit of the measurements. The values of k_{att1}, k_{att2}, k_{det2}, and μ_l mainly determine the height of the breakthrough curve. The value of k_{det2} strongly affects the skewness of the climbing and declining limbs. After the pulse of viruses has passed, site 2 looses its influence on the shape of the curve because of the high detachment rate of this site. The values of k_{det1} and μ_{s1} mainly determine the tail of the breakthrough curve.

Similarly shaped breakthrough curves, that is, with the rising limb skewed to the right and with a smooth transition of the declining limb to the tail, have been found in other studies (15,18,19,23). Possibly, the involvement of more than one type of kinetic site is general. As for parameters that were found for the breakthrough curve in Figure 2, it appeared that δk_{attl} (Eq 8). In that case, attachment mainly determines virus removal, which appears to work as irreversible attachment. In some studies, such an approach was followed, that is, virus inactivation was neglected and virus removal was assumed to be solely because of irreversible attachment (20,25).

Colloid Filtration

According to colloid-filtration theory, the attachment rate coefficient of a colloid (e.g., a virus) can be expressed in terms of a single collector efficiency η and a sticking (or collision) efficiency α as follows (27):

$$k_{att} = \frac{3}{2}\frac{(1-n)}{d_c}\alpha\eta v \tag{10}$$

Here, d_c is the average diameter of the single collector (soil grain), [L]. The fraction of particles that collide with the collector is given by η, the single collector efficiency. Because viruses are small in size, their transport in the immediate vicinity of the collector surface is dominated by Brownian diffusion, and the single collector efficiency can be given by the Smoluchowski–Levich approximation (28):

$$\eta = 4A_s^{1/3}N_{Pe}^{-2/3} \tag{11}$$

Here, $N_{Pe} = d_c n v/D_{BM}$, is a Péclet number that accounts for diffusion; $D_{BM} = K_B(T + 273)/(3\pi d_p \mu)$ is the diffusion coefficient, [L^{2}T^{-1}]; $K_B = 1.38 \times 10^{-23}$ is the Boltzmann constant [J/K]; T is temperature; d_p is the virus particle size; μ is the dynamic viscosity [ML^{-1}T^{-1}]; $A_s = 2(1-\gamma^5)/(2-3\gamma+3\gamma^5-2\gamma^6)$ is Happel's porosity dependent parameter, with $\gamma = (1-n)^{1/3}$.

The sticking efficiency, α, represents the fraction of the particles colliding with the collector which remain attached to the collector (29). The sticking efficiency reflects the net effect of repulsive and attractive forces between surfaces of the particles and the collector. It depends on the surface characteristics of the virus and soil particles. Therefore it depends on pH, organic carbon content, and ionic strength. It is believed that α is independent of hydrodynamic effects (velocity and dispersion). So, if α is known for a given set of conditions, such as type of virus, type of soil, pH, organic carbon content, and ionic strength, then it is possible to calculate the value of k_{att} for a different set of hydrodynamic conditions using Equation (10). This emphasizes the importance of knowing the value of α under a given set of conditions.

Commonly, α is derived from experimental values of k_{att} and calculated values of the single collector efficiency using Equation (10) (15,19,21,23,28,30,31). Alternatively, α has been estimated from relative breakthrough (RB) of the mass of viruses relative to that of a conservative salt tracer (20,25,32) and assuming irreversible adsorption using the following equations:

$$\alpha = \frac{d_c\left[\left[1-2\left(\frac{\alpha_L}{x}\right)\ln(RB)\right]^2-1\right]}{6(1-n)\eta\alpha_L},$$

$$\text{where} \quad RB = \left[\frac{\int_{t_0}^{t_f}\frac{C_{virus}}{C_{0,virus}}dt}{\int_{t_0}^{t_f}\frac{C_{salt}}{C_{0,salt}}dt}\right] \tag{12}$$

FACTORS AFFECTING ADSORPTION OF VIRUSES TO SOIL

Virus Type

Viruses, even strains of the same type, adsorb soil to different extent under similar conditions (3–35). The behavior of different viruses in their interactions with solids is believed to be the result of differences in the electrical charge and the hydrophobicity of the virus surface (36). Virus surface charge is determined by the weakly acidic and basic groups in its protein coat and may vary not only by the type of the virus but also

by the strain (37). For example, at pH values above 5, the surface charge of bacteriophage MS2 is negative and remains constant (38). At pH values above 6, the negative surface charge of vaccinia virus, reovirus, and λ is also relatively insensitive to changes in pH (38). At higher pH, bacteriophage PRD1 (39) and recombinant Norwalk-like virus particle (30), however, show a further decrease in their negative charge. Bacteriophage λ (28) and Norwalk virus-like particles (30) have a stronger negative surface charge than MS2 at pH 5–7, despite the fact that MS2 has a lower isoelectric point (pI) than the other two. Viruses having a stronger negative surface charge will generally attach less owing to stronger electrostatic repulsion in soil at a pH 6–8. Nevertheless, the attachment coefficient of MS2 is found smaller than that of bacteriophage λ and Norwalk virus-like particle, possibly because of steric repulsion of hydrophilic loops that protrude from the surface of MS2 (28).

The coat proteins of a virus may contain spans of amino acids that are hydrophobic. Depending on the way these proteins are folded, such hydrophobic parts may be either on the inside or on the outside of the virus coat (37). Viruses differ in hydrophobicity: Echovirus 7 and ϕX174 exhibit little, if any, hydrophobic character, whereas, echovirus 5, MS2, T7, PRD1, and ϕ6 all are more hydrophobic to a similar extent (36,41). Under conditions of electrostatic repulsion, interaction of the more hydrophobic viruses with hydrophobic surfaces may be a significant factor (15,37).

Soil Type

Generally, soil grains have patchwise distributed surface charges that originate from inherent differences in properties of crystal faces of mineral grains and from minerals having bulk-bound or surface-bound chemical impurities (41). Oxides of iron, aluminum, and manganese are the most common sources of surface charge heterogeneity in the groundwater environment. These oxides carry a positive charge at near neutral pH and are generally present in minor amounts as surface coatings on mineral grains and form favorable sites for negatively charged viruses. It has been shown that minor degrees of charge heterogeneity on grain surfaces result in attachment rates that are orders of magnitude higher than similar surfaces having no charge heterogeneity (41).

The concept that viruses preferably attach to soil grain surfaces with favorable charge characteristics is supported by several studies. Solids having a high pI (isoelectric point) are better virus adsorbents than those with low pI (37). Also, favorable for attachment of viruses are a higher cation exchange capacity (33), the presence of exchangeable iron (42), and iron oxides (20,32,39,43–46). Attachment of viruses has been shown to be better to soils with higher specific surface areas (47). Generally, sandy soils are weaker adsorbents than clays and minerals (3,48). This is because clays may have surfaces with a very heterogeneous charge distribution (49).

pH

The most important factor that affects virus adsorption to soil is pH (34). Generally, viruses adsorb less at higher pH owing to stronger electrostatic repulsion (33,34,42,45,48,50). In fact at higher pH, the attachment rate is lower than the detachment rate. In Figure 3, the sticking efficiency of MS2 is plotted versus pH within the range of 4–8 and at different ionic strengths, using the data from several column studies (15,19,23,28). The pI of MS2 is 3.5, it is therefore negatively charged within this pH range. The figure shows that the value of α decreases with increasing pH, and becomes less than 0.01 at a pH of about 5.5 and above. The sticking efficiency of PRD1 has also been shown to be pH-dependent. In columns with silica beads at pH 5.5 (15), α for PRD1 was between 0.006 and 0.013, and at pH 7, PRD1 did not adsorb at all. In field studies, detachment was very slow compared to attachment at pH 5.4–7.4, but increased strongly at pH 8 or higher (16,32,52). At a pH that is lower than 2.5–3.5 pH units above the pI of the grain surfaces, PRD1 attaches irreversibly, but above this pH, PRD1 attaches reversibly (39).

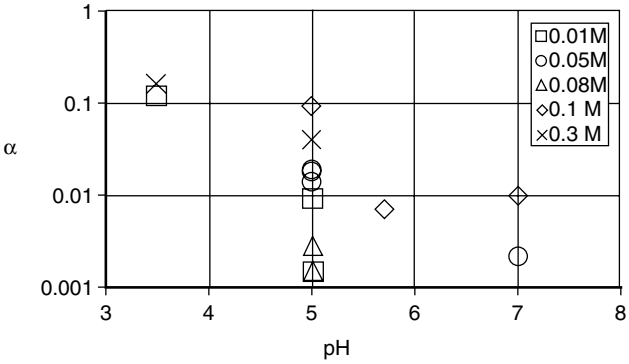

Figure 3. Sticking efficiency α versus pH of MS2 at different ionic strengths (7,16,20,24,29).

Hydrophobic Interactions

At high pH, when both the surfaces of soil grains and viruses are negatively charged, hydrophobic interactions may be the major factor mechanism for virus attachment (15,37,53). Hydrophobic interactions may be seen as a consequence of the thermodynamically unfavorable interaction of hydrophobic substances with water molecules and is not due to interactions among hydrophobic particles themselves (54). For example, the attachment of MS2 is very weak and decreases with pH; but when silica beads are coated with C_{18}-trichlorosilane (a hydrophobic substance), 400 times more attachment takes place, independent of pH (15).

Hydrophobic interactions of viruses with solid surfaces have been suggested to play a role in the interaction between poliovirus 1 and Millipore and Zeta plus filters (55). Similar results have been found for poliovirus 1, echovirus 1, and rotavirus SA 11 with highly organic estuarine sediments (53), for poliovirus 1 with nitrocellulose membrane filters (54), for MS2 and PRD1 with octadecyltrichlorosilane coated silica (15,19), and for MS2, ϕX174, T7, PRD1, and ϕ6 with nitrocellulose and cationic polysulfone membranes (40).

Ionic Strength

Viruses tend to strongly adsorb to various materials in solutions at high ionic strength (44,45). This is in agreement with DLVO (Derjaguin-Landau-Verwey-Overbeek) theory, which describes the attractive and repulsive forces between colloids (e.g., viruses) and grain surfaces (41). A higher ionic strength compresses double layers, thereby increasing attachment rates. As shown in Figure 3, the sticking efficiencies of bacteriophages MS2 and PRD1 were found to increase with ionic strength in the range of 0.01–0.1 M (molarity) NaCl (sodium chloride) at pH 3.5–5 (28).

A filamentous bacteriophage, SJC3, attached more to quartz grains at pH 7 as a result of an increase in the concentration of a particular salt from 1 to 100 mM (56). However, removal of SCJ3 was even higher at the low concentration of 1-mM NaCl that is at low ionic strength. It was suggested that under that condition the filamentous bacteriophage has stiffened and was consequently retained by straining.

If forces between the surfaces of grains and viruses are repulsive at all separation distances, then DLVO theory predicts increased detachment rates at lower ionic strength (41). In agreement with this prediction, detachment of poliovirus 1 was enhanced by deionized water that was applied to simulate heavy rainfall (58). Attached particles of bacteriophage SJC3 were shown to detach easily when the ionic strength was lowered by changing the salt solution from 1 mM $CaCl_2$ (calcium chloride) to 1 mM NaCl (56). However, if forces between the surfaces of grains and viruses are attractive at very short separation distances, DLVO theory will predict an increased detachment rate at higher ionic strength (41). With increasing ionic strength, both the attachment and detachment and detachment rate coefficients of MS2 were found to increase (28).

Multivalent Cations

Multivalent cations can link virus and adsorbents of like charge by forming salt bridges between them (44,47,48) or by charge reversal (45). For example, at pH 5, attachment of MS2 to silica beads was at least 10 times higher in the presence of Ca^{2+} (calcium ions) than without Ca^{2+} (15). Bacteriophage SJC3 attached more to quartz at high ionic strength by a multivalent (Ca^{2+} or Mg^{2+} (magnesium ion)) than a monovalent (Na^{+} (sodium ion)) cation (56). Generally, viruses with higher negative charge attach less to sand, but possibly, this is reversed at high concentrations of multivalent cations (46,58).

Organic Matter

Dissolved and/or suspended organic matter in groundwater exists mainly in the form of humid substances (59). Among dissolved and/or suspended organic matter in the aquifer matrix, humid substances have the highest affinity to nonionic hydrophobic organic compounds (59–60). Other forms of dissolved organic matter consist of proteins, polypeptides, and amino acids. Commonly, dissolved or suspended organic matter is, similar to viruses, negatively charged, and hence it competes with viruses for the same binding sites (37). Dissolved or suspended organic matter can also be used to elute previously attached viruses (48). Thus detachment of viruses may be strongly increased by dissolved or suspended organic matter. Dissolved surfactants increase virus detachment, possibly by disrupting hydrophobic bonds between the soil and the viruses (61) or by causing charge reversal (20,32). If the nature of the soil is more important for adsorption, that is, if there is an excess of adsorption sites available, there may be no effect of dissolved organic matter (57).

The effect of solid and/or bonded organic carbon content (f_{oc}) of the soil is found ambiguous. It depends on the combination of soil type, virus type, and nature of organic matter. On one hand, a soil with bonded organic carbon has fewer sites for virus adsorption, because organic matter already occupies adsorption sites for viruses. Consequently, adsorption of viruses will be less to soils with higher f_{oc} (20,42,43), but this effect may be significant only for soils with high values of f_{oc} (47). On the other hand, the bonded and/or solid organic matter may provide hydrophobic adsorption sites for viruses. This was demonstrated in a study where attachment of MS2 to negatively charged silica beads at pH 7 could be increased considerably by very small amounts of bonded hydrophobic C_{18}-chlorosilane (19).

FACTORS AFFECTING VIRUS INACTIVATION IN THE SUBSURFACE

Viruses may be inactivated because of disruption of coat proteins and/or degradation of nucleic acids (37). The most important factors that influence virus inactivation rates are temperature, adsorption to particulate matter and soil, unsaturated conditions, and microbial activity. These factors are discussed in the following sections. Factors such as pH, ammonia, calcium hardness, magnesium hardness, total hardness, nitrate, total dissolved solids, and turbidity do not significantly affect inactivation of MS2, poliovirus 1, and echovirus 1 (62). Inactivation of MS2, however, increases significantly with calcium hardness (62). The inactivation rates of coxsackievirus A9 and B1, echovirus 7 and poliovirus 1 were reported to be larger in deionized than in untreated groundwater (12). The type of water (groundwater, secondary or primary effluent) was not found to significantly affect inactivation rates of poliovirus 1, echovirus 1, HAV, MS2 and PRD1 (63–64). In some cases, however, a greater value of μ_1 in primary effluent than in groundwater was found for poliovirus 1, HAV, and F-specific RNA bacteriophages (FRNAPH's) (65).

Temperature

Temperature is the most important factor that influences virus inactivation (6,62,66). Inactivation rates increase with temperature (62,64–68). Figure 4 shows a number of bacteriophages for which μ_1 varies with temperature in nonsterilized groundwater (69). The temperature sensitivity of μ_1 depends on the type of virus. Regression analysis suggests a dependence of μ_1 on temperature in the form:

$$\ln \mu_1 = aT + b \tag{13}$$

Table 3. Dependence of μ_1 of Viruses with Groundwater Temperature T (°C) (7)

	Polio 1	Echo 1	HAV	MS2	FRNAPH's	PRD1
Number of observations	10	5	4	8	2	3
Slope a^*	0.033	0.12	−0.024	0.12	0.14	0.09
Intercept b^*	−2.4	−3.0	−1.4	−3.5	−7.6	−4.0
Correlation coefficient	7%	71%	8%	85%	100%	71%

*See Equation 13.

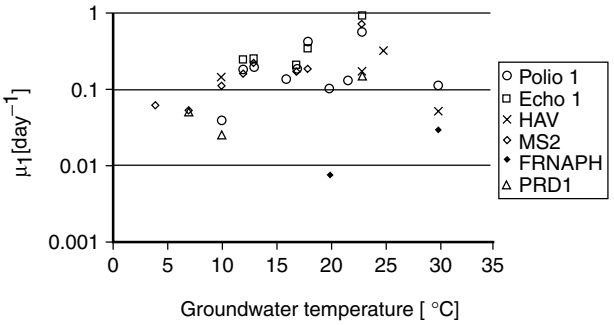

Figure 4. Inactivation rate coefficient μ_1 versus temperature for viruses in nonsterilized groundwater (7). See values in Table 3.

Values of coefficients a and b for six different viruses are given in Table 3. In the case of FRNAPH's and PRD1 these values are only an indication, because only few data were available (69). Its shows that temperature sensitivity of μ_1 depends on the type of virus. Poliovirus 1 is much less sensitive to temperature than MS2 or echovirus 1. HAV may be regarded as insensitive to changes in temperature. MS2 and other FRNAPH's are very sensitive to changes in temperature normally found in shallow groundwater. Estimates of μ_s-values at different temperatures for MS2, PRD1, and poliovirus 1 (64) suggest that μ_s-values change similarly with temperature as μ_1-values (69).

Particulate Matter and Soil

A substantial fraction of viruses is attached to colloidal particles in water with a size of less than 0.3 μm (70–72). These colloidal particles include clays, cell fragments, solids in wastewater effluents, and other miscellaneous debris (73). Virus association with solids has been observed to generally reduce virus inactivation (71,74–79).

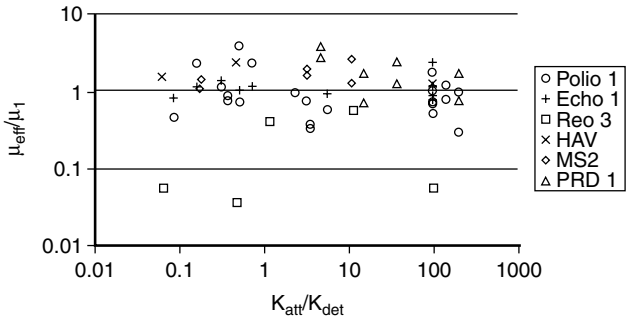

Figure 5. Reduction factor for inactivation rate μ_{eff}/μ_1 versus k_{att}/k_{det} for viruses in a batch system with soil (69).

Reduced inactivation is more pronounced in case of stronger attachment, especially to clays (80,66). However, at low clay concentrations, as in seawater, reduced inactivation has not been observed (78,81). Yet, in some other studies, a larger inactivation rate was measured in the presence of soil (48,64,82). Possibly, the effect is highly dependent on the virus type. This is supported by a large number of batch studies with various viruses and soil types (48,63–64). One may define an effective inactivation coefficient as μ_{eff} (69). This coefficient was calculated from the data of these batch studies and compared with corresponding values of μ_1, as a function of the ratio k_{att}/k_{det} (Fig. 5). A value of μ_{eff}/μ_1 less than one reflects reduced inactivation. It appears that for poliovirus 1, echovirus 1, and HAV the ratio μ_{eff}/μ_1 fluctuates around one, independent of adsorption. This implies that the values of μ_1 and μ_s are similar in these cases. Inactivation of reovirus 3 is reduced in all cases, but that of MS2 is enhanced in all cases, and that of PRD1 is enhanced in most cases. One may expect that in the case of reduced inactivation the ratio μ_{eff}/μ_1 decreases with an increasing ratio of k_{att}/k_{det} ($\mu_1 > \mu_s$), and the opposite in the case with enhanced inactivation ($\mu_1 < \mu_s$). However, Figure 5 shows no clear dependence of inactivation on adsorption.

Microbial Activity

Inactivation of viruses may be enhanced because of microbial activity (6). This is frequently found, when comparing inactivation rates of viruses in sterilized and nonsterilized wastewater (48,63,66). Inactivation of viruses may also be enhanced in toxic groundwater where aerobic bacteria are present (46,67).

Unsaturated Conditions

Under unsaturated conditions, the contribution of inactivation to virus removal is much more important and may be several times higher than under saturated conditions (9,10,23,83). It was suggested that the air–water interface may be retaining and/or inactivating viruses during transport through unsaturated soil. This is in agreement with observations from agitated batch suspensions of viruses. In strongly agitated batch suspensions of viruses, the inactivation rate can be reduced considerably by the addition of organic matter and even more by clay particles (82,84). Addition of organic matter forms a film on the water surface and thus lowers the chance of a virus particle contacting the air. When clay particles are added, viruses will rapidly attach to the clay particles, lowering the chance of entering the

air–water interface even more. Therefore it is believed that contact with the air–water interface increases inactivation. This protective effect of viruses attached to solid particles can even be stronger in a container with hydrophobic polypropylene walls (84,85), suggesting physical forces associated with the air–water–solid contact line that may be responsible for enhanced inactivation. The effect of unsaturated conditions on virus removal is highly dependent on the type of virus (22). MS2 is inactivated more in a container with polypropylene walls than in glass container, but this is not the case for ϕX174 (85). MS2 is more hydrophobic than ϕX174 (36,40) and will, therefore, enter more easily an air–water interface, and attach more to hydrophobic solid surfaces, and is subsequently inactivated because of the unfolding of coat proteins at the interface (22). Under unsaturated conditions in soil, removal of both MS2 and ϕX174 is increased, but that of MS2 is increased the most. The increased removal of MS2 is because of increased inactivation, but that of ϕX174 is due to increased adsorption (22).

ADVECTION AND DISPERSION OF VIRUSES

In relatively homogeneous sand, viruses are transported at the same velocity as a conservative salt tracer (20,21). In porous media with small-scale heterogeneities, for example, fractured media, some of the pore space may be accessible for solutes but not for microorganisms (86). In such heterogeneous soils, therefore, breakthrough of solutes will be partially retarded and much more dispersed than a colloidal particle such as a virus. Breakthrough curves of solute tracers in heterogeneous soils will typically have the shape of curve E in Figure 1a. Diffusion into a matrix with small pores has the same retarding effect as kinetically limited adsorption. Therefore, time to peak breakthrough of the solute will be later than that of the virus because the average flow velocity of viruses is higher than that of the water. By applying a stochastic model for virus transport, it can be shown that a high degree of aquifer heterogeneity can lead to virus breakthrough preceding that of a conservative tracer (87).

Bacteriophages are better tracers for transport of colloidal contaminants than solutes in fractured rock because they are not subject to matrix diffusion (88). Much higher flow rates of bacteriophages than that of solutes have been observed, because of the presence of more permeable pathways in clay (89,90), gravel (91), or limestone (92,93).

MODEL VIRUSES

Model viruses are needed to represent the behavior of pathogenic viruses during subsurface transport and to predict their removal. Bacteriophages MS2, PRD1, ϕX174, and naturally occurring FRNAPH's have been used extensively to study virus transport under various column and field conditions. In this section, the roles of these bacteriophages as model viruses are evaluated.

MS2

MS2 is an icosahedral phage with a diameter of 27 nm and a low pI of 3.5. The three-dimensional structure of its capsid is known at the atomic level (28). MS2 may be considered as a relatively conservative tracer for virus transport in saturated sandy soils with a low organic carbon content in the pH-range of 6–8. Under those conditions MS2 has shown little or no adsorption (8,11,21,23,46,88,94). In most soils, attachment of MS2 is less than or equal to that of most other viruses (11,19,25,28,30,34,94,95,96,97). Possibly because of the presence of multivalent cations, MS2 may attach more than the less negatively charged ϕX174 (18,58). MS2 is less stable than several pathogenic viruses. It is inactivated faster at temperatures of 10–25 °C. But, at temperatures lower than 7 °C, its inactivation rate is very low and similar to that of PRD1 (21,62,64,68). Under unsaturated conditions, MS2 is not a good choice as a relatively conservative virus tracer, because of its strong sensitivity to air–water interfaces and, consequently, strongly enhanced inactivation (22).

PRD1

PRD1 is an icosahedral bacteriophage with a diameter of 62 nm and with an inner lipid membrane (15,98). Its pI lies between 3 and 4 (39). Because of its larger size, PRD1 is of interest as a representative of rotaviruses and adenoviruses (99). Although, adenovirus and PRD1 may differ in surface charge, it is worth mentioning that PRD1 is much closer in structural design to adenovirus than any other known bacteriophage, and vice versa. The major capsid protein (P3) of PRD1 has the same double "jellyroll" fold as the human adenovirus type 2 hexon, despite their very different sequences and sizes. This implies that the basic structure as well as the assembly pathways of complex viruses were perfected very early in evolution and have been retained despite the high mutability of viruses (100). This observation further strengthens the validity of using bacteriophages as model viruses for the less-tractable animal viruses that cause human diseases.

With regard to attachment characteristics, PRD1 seems to behave less conservative than MS2 (15,17,23,58), possibly because it is more hydrophobic than MS2 (15,23,36,40). However, at field scale (21,25) removal of PRD1 is found to be similar to that of MS2. PRD1 may be considered as a relatively conservative model virus, similar to MS2, under field conditions (pH 6–8) in sandy soils with low organic carbon content and low concentration of multivalent cations. PRD1 may be considered as a worst-case model virus because of its low inactivation rate between 10–23 °C (64,68).

Bacteriophage ϕX174

Bacteriophage ϕX174 is less hydrophobic than MS2 (54). In studies on the retention of viruses by barrier materials, such as membranes, condoms, and testing gloves, ϕX174 is regarded as the best model virus, because it exhibits the least electrostatic and hydrophobic interaction (36,40,101). ϕX174 has essentially no charge at neutral pH (pI = 6.6–6.8) and its size is 27 nm (58,101).

Bacteriophage ϕX174 may be a relatively conservative model virus, because of its low hydrophobicity (36) and stability (24–25). In situations, where hydrophobic interactions would significantly increase virus removal, like under unsaturated conditions, ϕX174 could be a better choice as a model virus than MS2 or PRD1. In the presence of multivalent cations, ϕX174 was found to attach the least of five bacteriophages, including MS2 and PRD1 (18,58).

F-Specific RNA Bacteriophages

F-specific bacteriophages have similar physical properties as enteroviruses, especially with respect to size (102,103). MS2 belongs to group I of FRNAPH's (104). As naturally present model viruses, they are good candidates to represent enteroviruses in various treatment processes of surface water, including soil passage (transport through soil). Before entering a treatment like soil passage, enteroviruses and FRNAPH's largely have followed the same path, that is, both have passed the sewerage system, followed by sewage treatment, discharge into surface water, and some kind of pretreatment. It may be reasoned that along this path, from the sewerage system to the point of recharge into an aquifer, viruses that are less stable or that adsorb readily to solid surfaces, have disappeared already. This suggests that a selection has taken place of very stable and poorly adsorbing viruses, that is, worst-case viruses. This selection has been the same for FRNAPH's and enteroviruses.

In surface water, FRNAPH's occur in numbers of 10^2–10^4 times higher than enteroviruses (103). Therefore it has been possible to show 4 to 6 log units removal of FRNAPH's by riverbank filtration (105). Removal of FRNAPH's and MS2 has been shown to be similarly low at field scale (24,31,99).

VIRUS REMOVAL BY TRANSPORT TROUGH SOIL

From Equations (8,9), and (10) with constant rate coefficients for attachment, detachment, and inactivation, it can be deduced that in a saturated soil under steady state conditions, the degree of virus removal [calculated as $\log_{10}(C/C_0)$] should decline in a linear fashion with travel distance and travel time. In field studies, where bacteriophages were seeded as slug injection (16,20,24–25,32,52) or for a period of several days (21,46), steady state was not actually achieved. Nevertheless, simulations show that, for all practical situations, maximum breakthrough concentrations decline linearly with travel time or travel distance as well.

However, several column (57,106) and field studies (16,20–21,24–25,31–32, 46,52) have shown that removal rate often appears to be higher during the first meters of soil passage. Obviously, predictions of virus removal over larger travel times or distances can be severely overestimated if they are based on removal data from column or field experiments where transport was studied over short times and distances.

Higher initial removal of virus may be explained by soil and/or virus heterogeneity. Viral heterogeneity may be a cause for the higher initial removal, that is, the viruses in the suspensions that were used for seeding a column or a field site may have different affinities for attachment sites (20–21). Possibly, viruses that are attached to other colloidal particles behave as particles with different size and density and, therefore, have different single collector efficiencies and different sticking efficiencies (69). Transport of MS2 through sand columns has been shown to be greatly enhanced by its attachment to particles of Na-montmorillonite (107). The extent of such colloid-facilitated virus transport depends on the type and size of colloids, as well as the extent of virus attachment to the colloids (107). The extent of colloid-facilitated virus transport under various environmental conditions is unknown (107).

In a study on the removal of viruses by deep well injection (46), nonlinear removal appeared to have been caused by a change in surface charge of the porous medium. From geochemical mass balances, it was deduced that higher initial removal was caused by preferable attachment of viruses to patches of ferric hydroxides. These were precipitated as a consequence of pyrite oxidation within eight-meters distance from the point of injection, but not at larger distances. In other field studies, the reasons for higher initial removal are not understood (21).

A higher initial removal rate, followed by a residual removal rate can be described by the following empirical equation (108):

$$\log\left(\frac{C}{C_0}\right) = \frac{a}{b}(e^{-bt} - 1) - ct \qquad (14)$$

where, at $t = 0$, the initially high removal rate is determined by $-(a + c)$. In the case that $a = 0$ or $b = 0$, there is no higher initial removal rate. Parameter c gives the base removal rate, that is, there will always remain a certain contribution because of inactivation of free and attached viruses.

Equation (14) has been used to fit the field data and the results are shown in Figures 6a–6c. Values of parameters a, b and c were found by fitting Equation (14) to the field data are given in Table 4. Table 5 gives corresponding values for removal and sticking efficiencies of the bacteriophages, and Table 6 gives hydrologic and chemical properties of the aquifers. The aquifers are denoted A to F and are ordered according to grain size.

In some cases, only two to three measurements of removal with time were available (16,20,25,31,32,46). These removal data are shown in Figure 6d. For these data, Equation (14) was not applied, but for the sake of comparison, removal rates were calculated from the linear change in removal between adjacent pairs of observations in time.

Aquifer A contains very fine dune sand. The initial removal rate was found to be strongly and positively correlated with soil organic carbon content and to a lesser extent with the content of ferric oxyhydroxides. Because of the high pH, conditions for attachment are unfavorable. Aquifer B contains fine sand. Initially, removal rates are higher than in aquifer A because of the presence of favorable attachment sites in the form of

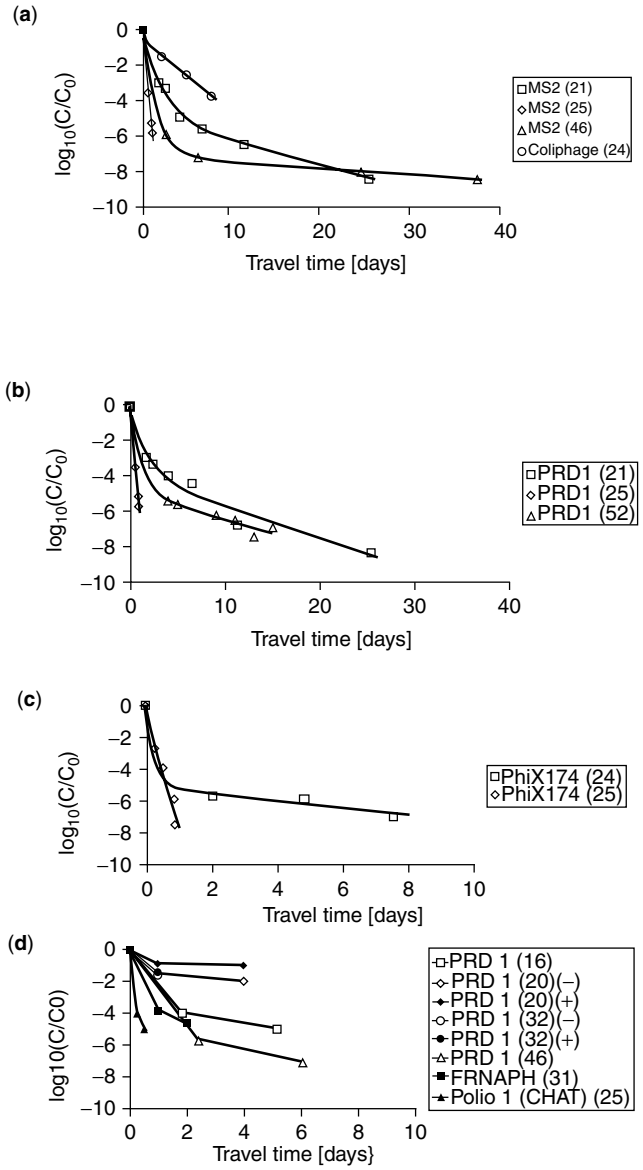

Figure 6. Removal of bacteriophages and poliovirus 1 in relation with travel time in sandy aquifers. See corresponding parameter values in Table 4 and aquifer properties Table 6.

ferric oxyhydroxides together with a lower pH. However, the removal rate coefficient λ in aquifer B is lower than that in aquifer A. This can only partially be explained by a two times lower pore water velocity in aquifer B compared to that in aquifer A, which leads to a lower value of the attachment rate coefficient. The different removal rates can be explained neither by the difference in pH nor by the difference in temperature. Higher pH is less favorable for attachment and at lower temperature the inactivation rate is lower. Therefore there must be fewer sites for attachment in aquifer B than in aquifer A. Note that similar collision efficiencies for aquifers A and B are reported, but these were based on average removal and pore water velocities from the source to each well. In fact, collision efficiencies in aquifer B are initially higher than in A, but finally lower. Eventually, 8-$\log_{10}$ removal was achieved in both aquifers. The sand in aquifer C is coarser but otherwise similar to A. Similar removal rates and collision efficiencies for PRD1 were found in aquifers

Table 4. Removal Rate Parameter Values from Field Studies

Figure	Virus	Aquifer	Ref.	a^*	b^*	c^*
6a	MS2	A	21	2.2	0.43	0.13
	MS2	F	25	28	25	5.2
	MS2	B	46	5.0	0.71	0.035
	Coliphages	E	24	6.5	9.8	0.40
6b	PRD1	A	21	3.7	0.53	0.18
	PRD1	F	25	45	32	4.7
	PRD1	D	52	4.2	0.88	0.15
6c	ϕX174	E	24	56	11	0.24
	ϕX174	F	25	0.27**		7.7**
					$c_1{}^*$	$c_2{}^*$
6d	PRD1	C	16		2.2	0.30
	PRD1	D-	20		1.5	0.17
	PRD1	D+	20		0.90	0.033
	PRD1	D–	32		1.7	
	PRD1	D+	32		1.4	
	PRD1	B	46		2.4	0.38
	FRNAPH	A	31		3.8	0.83
	Polio 1	F	25		16	4.0

*Parameter values a, b and c estimated by applying Equation (14); c_1 and c_2 calculated from the increase in removal in time between to adjacent points in time; dimension of a, b, c, c_1 and c_2 is day^{-1}.
**Parameters were estimated from a linear model with intercept a and slope b.

Table 5. Sticking Efficiencies and Removal of Viruses in Sandy Aquifers (A–F)

	Distance [m]	α	Aquifer	Ref.
MS2	2.4	0.0014	A	21
	30	0.00027	A	21
	7.5	0.004–0.18[a]	F	25
	19.4	0.004–0.20[a]	F	25
	8	0.0014	B	46
	38	0.00020	B	46
PRD1	0.94	0.0028–0.0030[c]	C	16
	0.94	0.00085–0.0016[d]	C	16
	1.0	0.009–0.013[e]	D–	20
	1.0	0.0014–0.0026[f]	D+	20
	2.4	0.0024	A	21
	30	0.00043	A	21
	7.5	0.004–0.182[a]	F	25
	0.9–1.0	0.032 ± 0.016[b]	D–	32
	0.9–1.0	0.016 ± 0.005[b]	D+	32
ϕX174	7.5	0.006–0.31[a]	F	25
Polio 1	7.5	0.047–2.1[a]	F	25
(CHAT)	19.4	0.019–0.87[a]	F	25
FRNAPH's	2	0.0020 (0.0015–0.0029)[b]	A	31
	4	0.00078 (0.0004–0.004)[b]	A	31

[a]Corresponding to a range in grain size from 0.00125–0.012 m;
[b]95% confidence interval;
[c]pH = 7.4;
[d]pH = 8.4;
[e]$f_{oc} < 0.01\%$;
[f]$f_{oc} = 1\%$;
See Table 5 for corresponding properties of the aquifers.

Table 6. Hydrologic and Chemical Properties of the Studied Sandy Aquifers (A–F)

	A	B	C	D+*	D−*	E	F
References	21,31	46	16	20,32,52	20,32,52	24	25
Grain size (mm)	0.20–0.24	0.27	Fine to medium	0.5	0.5	2.4	1.25–12
Porosity	0.35	0.32	0.3	0.39	0.39	0.2	0.15
Clay%	0.5–0.8	0.9	0–15	} <1	} <1		
Silt%	1–4	1.1					
Sand%	95–98	98		} >99	} >99		
Gravel%	0	0					
Hydraulic conductivity (m/d)	12	25	6	110	110	240–300	900–13,800
Pore water velocity (m/d)	1.2–2	1	1.9	0.2–1	0.2–1	1–2.9	27
f_{oc}%	0.1–0.4	0.17	0.03	1	<0.01		
Surface Fe (III) (g/kg)	0.7–1.7			0.20	0.26		
pH	7.3–8.3	6.6–6.8	7.4–7.5	5.8–6.7	5–5.8	6–6.4	7.2
Electric conductivity (μS/cm)	900	450		250–450	30–100	300–800	288
Temp	2–5	12		15	16	9–12	10.3
Dissolved oxygen (mg/I)	1.1–9.7	10 → <0.5		0–0.5	4.5–11		3.5
Nitrate (mM)		0.31 → <0.008		0.3	<0.01		
Sulfate (mM)		0.65 → 0.40		0.36	0.085		
Bicarbonate (mM)	2.4–2.8	5.6–6.2		0.64	0.028		
DOC (mg/I)	1.5–2.4	0.55		2–4.4	0.3–1		
Ca2+ (mM)	2.2–2.3	1.7–2		0.21	0.028		
Mg2+ (mM)	0.52–0.63	0.36–0.53		0.13	0.037		

*D+ and D−: the same aquifer, but D+ is a sewage contaminated zone, whereas D− is an uncontaminated zone. f_{oc} is soil organic carbon content. DOC is dissolved organic carbon.

A and C. The sand of aquifer D is also coarser than in A or B, and the hydraulic conductivity is much higher. Aquifer D consists of a sewage contaminated (D+) and uncontaminated zone (D-). In D+, organic matter occupies favorable attachment sites. Nevertheless, similar collision efficiencies for PRD1 were reported in aquifer D+ as in A and C. But, removal rates in D+ are lower than in A or C because of a combination of a lower transport velocity and coarser sand (fewer collisions). Organic matter limits attachment in D+ by occupying attachment sites, whereas, a higher pH is the limiting factor in A and C. In aquifer D−, removal rates and collision efficiencies are higher than in aquifer D+ because in D−, attachment sites are not blocked by organic matter. The sand in aquifer E is coarse, the hydraulic conductivity and pore water velocity are higher. Within eight days of travel time, the removal rate of coliphages is lower than the initial removal rates of MS2 in aquifers A and B. Also, the final removal rate of ϕX174 in aquifer E is only about twice that of MS2 in aquifer A. Bacteriophage ϕX174 is expected to attach much more than MS2 because its surface charge is less negative than that of MS2. The relatively low attachment rates in aquifer E are probably because of the coarseness of the soil, which lead to low single collector efficiencies. In aquifer F, we have the coarsest sand and, by far, the highest hydraulic conductivity and pore water velocity. In aquifer F we find the highest removal rates. This is also reflected by the relatively high collision efficiencies, suggesting the presence of favorable attachment sites. The Ground Water Rule of the US EPA (GWR) (2) requires 4-log removal of virus for adequate protection of groundwater, whereas drinking water companies may even require 8-log removal (21) based on a maximum risk of infection of 10^{-4} per person per year (109). From Figures 6a–6d it is apparent that a removal of 4 log or more was achieved within eight days (about 20 meter) in all the studies, with one exception, where measurements were carried out only for four days (20). Within about 40 days or less, 8-log removal of MS2 and PRD1 can be achieved (21,46).

From the data shown in Figures 6a and 6b, it is also possible to identify a relatively worst case situation, where interaction with the soil grains is very low because of the absence of preferable sites for attachment. In aquifers B and D+, very low residual removal rates of 0.035 $\log_{10}$ units per day were found for MS2 and PRD1, respectively. This is equal to a removal rate of 0.081 [day^{-1}] on a natural log scale. In aquifer D+, attachment of PRD1 was limited owing to the presence of organic matter. In aquifer B, a value of 0.024 [day^{-1}] for μ_1 of MS2 was measured at the monitoring well at 8 m, near the outer limits of the toxic zone. Thus, a value of k_{att} equal to 0.057 [day^{-1}], and a value of the collision efficiency equal to 1.5×10^{-5} can be estimated. In this situation, the contributions of attachment and inactivation to removal of MS2 are similar. MS2 may be considered here as a worst case virus tracer, as it attaches less to soil than most viruses. A value of μ_1 equal to 0.024 day^{-1} for MS2 in groundwater at 12 °C is relatively low and similar to that of several human pathogenic viruses in groundwater at 10 °C (69).

In contrast to the removal capabilities of sandy aquifers, removal of viruses in karst, fractured bedrock and gravel aquifers may be very low. Such aquifers are identified as sensitive to fecal contamination by the U.S. EPA's proposed Ground Water Rule (2). These aquifers have in common more permeable pathways that exist, which allow very high flow rates of viruses (91–93). In such pathways, the single collector efficiency will be very low, and consequently there will be little attachment. Because of the high transport rate, inactivation will also be minimal. In gravel, removal of slug-injected bacteriophages T7

and H40/1 was only 2 log over a travel distance of 50 meters (84,91). Nevertheless, considerable removal may also be found, for example about 6-log removal of MS2 over a distance of 20 m in limestone (93) or 1-log removal of MS2 and PRD1 over a distance of 0.5 m in a clay-rich till (90). It is obvious that preferred pathways, such as fractures and breaches, will contribute greatly to the uncertainty in the removal capabilities of a certain aquifer.

CONCLUSION

During subsurface transport, viruses are removed from the aqueous phase by inactivation of free viruses and by attachment to specific sites on the soil grains, followed by inactivation of the attached viruses. Major factors affecting virus attachment to soil and their inactivation that were described in the previous sections are summarized and listed in Tables 7 and 8.

At pH 7 –8, attachment of the viruses to the soil grains proceeds faster than detachment from the grains. At this pH range, as in many sandy aquifers, the net surface charge of most viruses and soils is negative, and thus conditions for attachment are generally unfavorable because of electrostatic repulsion. However, the grains in sandy soils contain patches on their surface that have favorable charge characteristics, like positively charged iron, aluminum, or manganese oxide coatings to which viruses preferably attach. because of the presence of such favorable sites for attachment, removal of viruses may be higher than 2.5 log per day (about one log per meter). In the case of few or absence of such patches of favorable sites, removal of viruses may be as low as 0.035 log per day (about 0.05 log per meter).

Dissolved organic matter may decrease virus attachment to soil because of competition for the same favorable attachment sites. Dissolved organic matter, like surfactants, may disrupt hydrophobic bonds between soil and virus, resulting in an increased detachment rate. At the same time, viruses and many organic materials contain hydrophobic groups on their surfaces. Therefore, once adsorbed, bonded organic matter may provide hydrophobic binding sites for viruses.

Depending on soil heterogeneity, viruses may travel faster than the average water flow and show a smaller dispersion than a solute, because they can be excluded from small pores and, therefore, preferentially follow more permeable pathways. Consequently, removal of viruses may be much less effective in media such as fractured rock or karstic aquifers.

Temperature is the most important factor that influences virus inactivation. Inactivation rates increase with temperature, but the extent depends on the type of virus. Inactivation of some pathogenic viruses, such as HAV, is very insensitive to temperature changes encountered in most groundwater. Probably, inactivation rate coefficients of free and attached viruses change similarly with temperature. Microbial activity may increase inactivation rates of viruses under aerobic conditions. Under unsaturated conditions, inactivation may be strongly enhanced because of physical forces at contact lines between air, water, and solid surfaces and may then be the major factor that determines virus removal.

Bacteriophages MS2 and PRD1 may be considered as suitable model viruses. Mainly owing to their negative charge, they are removed equally or less than most other viruses at pH 6–8 and at temperatures below about 10 °C, provided the soil do not contain too many hydrophobic sites or multivalent cations. PRD1 is more stable than

Table 7. Summary of Major Factors Affecting Virus Attachment to Soils

Factor	Effect on Attachment Rate Coefficient k_{att}
Virus type	k_{att} higher because of
	— a less negative surface charge of a virus
	— higher hydrophobicity of a virus
Soil type	k_{att} higher owing to
	— the presence of favorable attachment sites (patches of positively charged metal-oxides)
	— a less negative surface charge of soil
	— a high cation exchange capacity of soil
	— a high specific surface area of soil
	— the presence of hydrophobic sites
Groundwater	k_{att} higher because of
	— a decrease in pH
	— an increase in ionic strength
	— the presence of multivalent cations
Organic matter	k_{att} lower because of
	— the presence of dissolved, negatively charged organic matter
	— the presence of bonded organic matter occupying favorable attachment sites
	k_{att} higher owing to
	— presence of bonded organic matter providing additional attachment sites

Table 8. Summary of Major Factors Affecting Virus Inactivation in Groundwater

Factor	Effect on Inactivation Rate Coefficient μ_1
Virus type	— The extent of effects of all factors mentioned below depend on the virus type
Temperature	— μ_1 higher because of a higher temperature
Microbial activity	— μ_1 higher because of microbial activity under aerobic conditions
Unsaturated conditions	— μ_1 higher under unsaturated conditions

MS2 at higher temperatures. Bacteriophage ϕX174 may be a relatively conservative model virus, because of its low hydrophobicity and stability. FRNAPH's, as a group of naturally occurring viruses, behave relatively conservative, like MS2 that is also a FRNAPH.

Rate of removal of viruses by passage through soil (logarithmic reduction in concentrations) appears to decline with distance. The nonlinearity of removal with distance has important consequences for prediction of virus removal. Thus it is also important for the calculation of setback distances that are needed to adequately protect groundwater sources and to ensure adequate treatment of infiltrated surface water. Predictions of virus removal at larger distances are severely overestimated if they are based on removal data from column experiments or from small-scale field studies. To improve predictions on the removal of viruses by soil passage, knowledge of the soil heterogeneities at the location of interest is needed.

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MODELING THE TRANSPORT OF BIOAEROSOLS

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Bioaerosols can be generated in a number of ways, including the spray irrigation of vegetation using treated

domestic wastewater, humidification of indoor air using evaporative coolers, spray drift from industrial cooling towers, bursting of bubbles as a result of wave action, and rain splash. There has been considerable interest in being able to predict the atmospheric concentrations of microorganisms in the resultant bioaerosols for purposes such as assessing the potential human health risks associated with exposure to the microorganisms and transmission of plant pathogens. Predictive models range from fairly simplistic exponential models that are the results of fitting experimental data to more sophisticated particle-tracking models. In this article, several types of bioaerosol transport models are discussed.

BIOAEROSOL SURVIVAL MODELS

Exponential Decay Model

A very basic survival model can be developed by fitting experimental data to a simple mathematical expression, such as the exponential equation. For example, an experiment that measures the number of viable microorganisms in a bioaerosol as a function of the age of the bioaerosol is conducted. The resultant data are then plotted on a semilog plot. Assuming that the resultant plot produces a straight line, the viability of microorganisms at time t, V_t, can be expressed as:

$$V_t = V_0 e^{-kt} \tag{1}$$

where V_0 is the concentration of microorganisms at time 0 and k is an experimentally derived decay rate. Obviously, a model such as this does not allow one to predict the survival of the microorganisms under other environmental conditions, as there is no attempt to account for the individual effects of the various factors that affected their survival.

Regression Decay Model

A more complex model of microbial survival in a bioaerosol can be expressed using a multiple regression model. Such a model is based on the recognition that there are a number of factors affecting the survival of microorganisms in aerosols. A series of experiments is conducted to study the viability of microorganisms in a bioaerosol as a function of a variety of chemical and physical factors, including relative humidity, temperature, radiation, and oxygen (1,2). The results of these experiments will then be statistically analyzed and expressed as a multiple regression equation of the form:

$$V = a + bx_1 + cx_2 + dx_3 + ex_4 + \ldots zx_n \tag{2}$$

where x_1, x_2, x_3, x_4, $\ldots x_n$ are the measured effects of the varied parameters.

The regression decay model may be more useful than the exponential decay model, as it may be used to predict the survival of microorganisms under conditions that were not studied in the experiments performed to derive the equation. For example, knowing the values of relative humidity, temperature, radiation, and oxygen concentration for a given environment, one could calculate V using Equation 2. The validity of the calculated value of V would have to be verified independently to have a high degree of confidence in the value. It is likely that the results of such a prediction would be highly dependent on a number of factors. These factors include (*1*) the similarity of the microorganisms used in the experiments used to derive the equation and the microorganisms of interest, (*2*) whether the parameters of interest are within the numerical range of the parameters used in the original experiments, and (*3*) the numbers of values of each parameter examined in the original experiments.

DISPERSION MODELS

In order to predict the concentrations of microorganisms in the atmosphere some distance downwind form a source, it is necessary to use relatively complex models, which require one to have a great deal of information about the conditions of the environment being modeled. There are two main types of these models, the Gaussian plume model that is believed to be valid for the simulation of bioaerosol dispersion from 100 m to 10 km and particle-tracking (or random-walk) models, which are used for simulations at distances of 10 m to less than 1 km (3).

Gaussian Plume Model

The Gaussian plume model can be used to describe the statistical distributions of microbial concentrations in an aerosol downwind from a contaminant source. This model is used extensively to model downwind concentrations of atmospheric chemical contaminants from industrial sources. The Industrial Source Complex (ISC) Short-Term model is accepted by the U.S. Environmental Protection Agency (4) as it provides options to model emissions from a wide range of sources, which might be present at a typical industrial source complex.

The model uses the steady-state Gaussian plume equation for a continuous elevated source and assumes that the mean concentrations of particles in a bioaerosol are normally distributed (both vertically and horizontally) around the downwind axis of the plume from a point source. For each source and each hour, the origin of the source's coordinate system is placed at the ground surface at the base of the stack. The x-axis is positive in the downwind direction, the y-axis is crosswind (normal) to the x-axis, and the z-axis extends vertically. The fixed receptor locations are converted to each source's coordinate system for each hourly concentration calculation. The hourly concentrations calculated for each source at each receptor are summed to obtain the total concentration produced at each receptor by the combined source emissions.

For a steady-state Gaussian plume, the hourly concentration of microbes per unit volume of air at ground level, at downwind distance x (meters), and crosswind distance y (meters), χ, can be simply expressed as:

$$\chi = ABCD \tag{3}$$

where the variables A, B, C, and D can be verbally described as representing the following. A represents the concentration of particles on the plume axis at a distance x from the source; B describes the lateral dispersion (i.e., spreading out from the point source in the horizontal direction) of the particles; C describes the vertical dispersion (i.e., spreading out from the point source in the vertical direction) and the vertical settling of the particles; and D accounts for the inactivation of the microorganisms.

Mathematically, the Gaussian plume model is expressed as (5):

$$\chi = \left(\frac{bQ}{2\pi u\sigma_y\sigma_z}\right)\left[\exp\left(\frac{y_2}{2{\sigma_y}^2}\right)^2\right] \times \left\{\sum_i q_i \exp\left[\frac{-\left(H - V_{si}\frac{x}{u}\right)^2}{2{\sigma_z}^2}\right]\right\} \exp\left(-\lambda\frac{x}{u}\right) \quad (4)$$

where b is the number of microorganisms per unit volume of source (m^{-3}) at the source, Q is volume emission rate of the source (m^3s^{-1}), u is the mean wind speed for a 1-hour period of time (ms^{-1}), σ_y and σ_z are the standard deviations of the horizontal and vertical plume spread (m) and are functions of downwind distance and atmospheric stability, y is the lateral distance from the mean plume axis (m), q_i is the mass-weighted proportion of particles in the ith size category, H is the height above the ground of the plume axis after the initial rise of the plume (m), V_{si} is the settling speed of the average microorganism-containing droplets in the ith size category (ms^{-1}), and λ is the death rate of the microorganisms (s^{-1}).

This model makes the following assumptions (6): (*1*) there is a Gaussian distribution of the plume in both the horizontal and vertical direction; (*2*) particles are emitted from the source at a constant rate; (*3*) wind velocity and direction are constant; (*4*) the terrain is flat; (*5*) the particles are smaller than 20 μm, making the effects of gravity negligible; and (*6*) diffusion in the downwind direction is negligible.

Several of the critical parameters in the model will now be discussed in more detail.

Dispersion. The plume spreads or disperses as it moves downwind from the source (Fig. 1). The variability in plume spread is highly dependent on the turbulence of the air in the vicinity of the plume. The turbulence disperses the plume both vertically and horizontally; however, the dispersal in the horizontal direction tends to be greater than in the vertical direction, due to the lesser amount of energy needed to counteract the force of gravity in the horizontal direction. The amount of turbulence is dependent on the atmospheric stability, heating or cooling of the air by the ground, wind strength, and the roughness of the terrain (3). Rough terrain tends to increase turbulence, hence faster dispersion of the plume. Vertical spread varies much more as a function of turbulence than horizontal spread. Plume spread expressions are divided into several categories, 1–4 (3) or A–F (4), based on the level of turbulence present, with one being the highest level of turbulence.

The dispersion of the plume is expressed in the model in the form of parameters σ_y and σ_z. The equations used to calculate values for these parameters were derived empirically, based on available data of plume spread and approximately fit the Pasquill-Gifford curves (7). The equations used to calculate σ_y (in meters) are of the form:

$$\sigma_y = 465.11628(x)\tan(\mathrm{TH}) \quad (5)$$

where

$$\mathrm{TH} = 0.017453293[c - d\ln(x)] \quad (6)$$

In Equations (5) and (6), the downwind distance x is in kilometers, and the coefficients c and d are constants based on the level of turbulence in the system. The equation used to calculate σ_z (in meters) is of the form:

$$\sigma_z = ax^b \quad (7)$$

where the downwind distance x is in kilometers. The coefficients a and b are constants based on the turbulence of the system and the downwind distance. Values of a may range from 0.31 for relatively stable conditions to 0.40 for highly turbulent conditions; values of b range from 0.71 v for stable to 0.91 under highly turbulent conditions (3). These expressions were determined by Briggs as reported by Gifford (8) and represent a best fit to urban vertical diffusion data reported by McElroy and Pooler (9). Although the Briggs functions are assumed to be valid for downwind distances less than 100 m, the concentrations at receptors less than 100 m from a source may be suspect.

The U.S. EPA's ISC model (4) can be used to describe much more complex plume dispersion behavior, such as

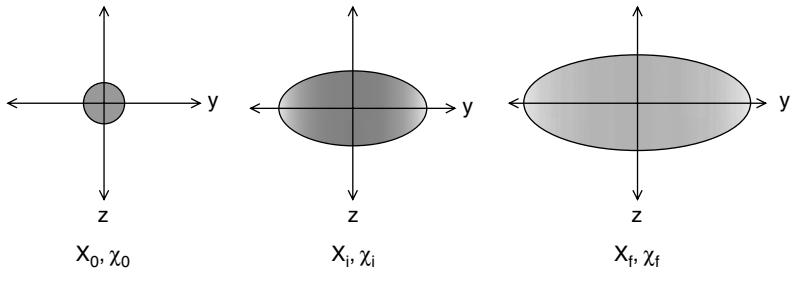

Figure 1

that imposed by the effects of downwind buildings, and so on.

Wind Speed. The wind power law is used to adjust the observed wind speed, u_{ref}, from a reference measurement height, z_{ref}, to the release height, H. The stack height wind speed,u_s, is used in the Gaussian plume (Eq. 4). The power law equation is of the form:

$$u_s = u_{ref}\left(\frac{H}{z_{ref}}\right)^{p} \tag{8}$$

where p is the wind profile exponent. Values of p depend on the turbulence of the system, and may vary based on whether the environment is rural or urban. Typical values of p have been found to vary between 0.07 and 0.55 for rural settings and between 0.15 and 0.30 for urban settings (4).

Plume Height. The maximum height to which the axis of the plume will rise is determined by a number of factors, including the characteristics of the source, the temperature of the bioaerosol, the wind speed, and the vertical stratification of the atmospheric temperature (3).

Calculation of the plume height can be a very complex matter because of the difference in behavior of the plume based on the temperature of the plume relative to that of the environment. The plume rise is considered to be buoyancy dominated if bioaerosol temperature is greater than ambient air temperature. Where stack gas temperature is less than or equal to ambient air temperature, it is assumed that the plume rise is dominated by momentum.

For buoyancy-dominated conditions, the distance downwind from the source to the receptor is assumed to be less than the final height of the plume axis. In this situation, the final height of the plume axis, H_e, can be calculated from the following equation (11):

$$H_e = h'_s + 1.60\left(\frac{F_b{}^{1/3}x^{2/3}}{u_s}\right) \tag{9}$$

where F_b is the Briggs buoyancy flux factor and can be calculated from the bioaerosol temperature, the ambient air temperature, the diameter of the source, and the initial velocity of the bioaerosol as it leaves the source.

For momentum-dominated conditions, the following equations (11) are used to calculate a distance dependent momentum plume rise:

(a) turbulent conditions:

$$H_e = h'_s + \left(\frac{3F_m x}{\beta_j{}^2 u_s^2}\right)^{1/3} \tag{10}$$

where F_m is the momentum flux parameter (calculated based on the bioaerosol temperature, the ambient air temperature, the diameter of the source, and the initial velocity of the bioaerosol as it leaves the source.), β_j is the jet entrainment coefficient (calculated from the initial velocity and the wind speed), and x is the downwind distance (meters).

(b) stable conditions:

$$H_e = h'_s + \left[3F_m\frac{\sin\left(\frac{x\sqrt{s}}{u_s}\right)}{\beta_j^2 u_s\sqrt{s}}\right]^{1/3} \tag{11}$$

where x is the downwind distance (meters).

Death Rate. The death rate term, D, in Equation (3) is a simple method of accounting for microbial removal by biological, physical, or chemical processes. As shown in Equation (4) it is of the form:

$$D = \exp\left(-\psi\frac{x}{u_s}\right) \quad \text{for } \psi > 0$$

or

$$= 1 \quad \text{for } \psi = 0 \tag{12}$$

For a microorganism that exhibits first-order inactivation rate kinetics, if $T_{1/2}$ is the microbial half-life in seconds, one can obtain λ from the relationship:

$$\psi = \frac{0.693}{T_{1/2}} \tag{13}$$

Another method that can be used to determine the value of λ is described by Lighthart (12). In this case, data from experiments examining the inactivation of 16 different bacteria as a function of temperature and relative humidity were analyzed. A linear regression relationship was then developed to enable a calculation of λ:

$$\log_{10}\lambda = -0.245 - 0.892\log_{10}(\text{aerosol age[hours]}) + 0.0055 \times \text{temperature (C)} - 0.15 \times \text{gram reaction} \times \text{RH(\%)} \tag{14}$$

where the value for the gram reaction is zero for gram-negative bacteria and one for gram-positive bacteria. When the calculations of the regression equation were compared with the reported experimental data, R values ranging from 0.33 to 0.98 were found.

Bioaerosol Particle-Tracking Model

In this model, the movement of the droplets in the bioaerosol are tracked over discrete time steps as they are moved through the air after being sprayed into the environment. The airflow can be either laminar or turbulent. After a specified time step, t, the position (x_i, y_i) of the bioaerosol and the number of microorganisms in the bioaerosol downwind from the source are calculated (3). The position of the bioaerosol is calculated by multiplying the horizontal or vertical droplet velocity at a given time step,V_t, by the time in that time step. The droplet velocity is calculated using the equation:

$$V_t = V_f - (V_f - V_0)E^{-t/\tau} \tag{15}$$

The various parameters in this equation are as follows.V_f is the wind velocity. V_0 is the initial spray droplet velocity and can be calculated using the pressure in the spray nozzle, P, and the density of water at ambient temperature, ρ_p:

$$V_0 = \sqrt{\frac{2P}{\rho_p}} \tag{16}$$

The parameter τ is the relaxation time, and describes the time necessary for an aerosol droplet to change from its initial velocity to its final velocity (13). The relaxation time depends on the size and density of the droplet and the density and viscosity of the atmosphere. It can be calculated using the following equation:

$$\tau = \frac{\rho_p {D_p}^2 C_c}{18\eta} \tag{17}$$

where C_c is the Cunningham slip factor (a correction factor for the velocity of particles less than 1 m in diameter whose molecular slip at the droplet's surface causes them to fall faster than would be predicted by Stokes' law), D_p is the diameter of the particle, and η is the coefficient of velocity of ambient air.

The number of microorganisms present in the bioaerosol after a given time step can be calculated as the packed bacterial particle diameter:

$$D_{pack} = 3\sqrt{\frac{v}{0.71}} \tag{18}$$

where D_{pack} is the residual packed bacterial diameter, assuming rhombohedral packing, and v is the volume of the mean number of bacteria, n, that would be suspended in an initial spray droplet with an initial diameter, D_p. The parameter v can be calculated from:

$$v = n\left[\frac{4}{3}\pi\left(\frac{D_p}{2}\right)^3\right] \tag{19}$$

The model uses the above equations to calculate the position (x_i, y_i) of the bioaerosol (i.e., $V_t \times t$) and the number of microorganisms in the bioaerosol (i.e., D_{pack}) for a given time period. Note that both the horizontal and vertical position of the plume must be calculated using Equation 15. This process is repeated until the desired length of time has been modeled or until there are no bacteria left in the aerosol (i.e., $D_p < D_{pack}$). A schematic view of the model output is given in Figure 2.

During each time step, the number of microorganisms remaining in the bioaerosol is determined by considering a number of factors. Evaporation has a large effect on the change in droplet size over time. This change is calculated using the following equation (14):

$$\frac{dD_p}{dt} = \frac{4D_v M}{R\rho_p D_p}\left(\frac{p_\infty}{T_\infty} - \frac{p_d}{T_d}\right) \times \left(\frac{2\lambda + D}{D_p + 5.33\left(\frac{\lambda^2}{D_p}\right) + 3.42\lambda}\right) \tag{20}$$

where D_v is the diffusion coefficient of water in air at ambient temperature, R is the universal gas constant, M is the molecular weight of water, p_∞ is the ambient water vapor pressure, p_d is the saturation water vapor pressure at the droplet surface temperature, T_∞ is the ambient temperature, T_d is the droplet surface temperature, and λ is the mean free path of the air molecules.

The droplet surface temperature, T_d, changes in each time step. This change is calculated using the equation:

$$dT = \frac{3t}{r^2\rho_p C_p}\left[\frac{D_v MH}{R}\left(\frac{p_d}{T_d} - \frac{p_\infty}{T_\infty}\right) - k(T_\infty - T_d)\right] \tag{21}$$

where r is the radius of the droplet, C_v is the specific heat of vaporization of water at ambient temperature, H is the latent heat of fusion of water at ambient temperature, and k is the thermal conductivity of the air.

The droplet temperature for each time step is calculated by subtracting dT from the current T_d. The resultant droplet surface temperature is then used as the initial temperature for the next time step.

The initial number of microorganisms in a droplet is modeled randomly as a function of the diameter of the droplet generated by the sprayer. The diameter of particles in the initial bioaerosol is assumed to vary and may be calculated based on information from the sprayer manufacturer. The number of microorganisms in the particles will change over time as a result of evaporation, as discussed earlier. Their survival has also been found to depend on the initial number of microorganisms in the droplets and therefore the initial size of the droplet (14).

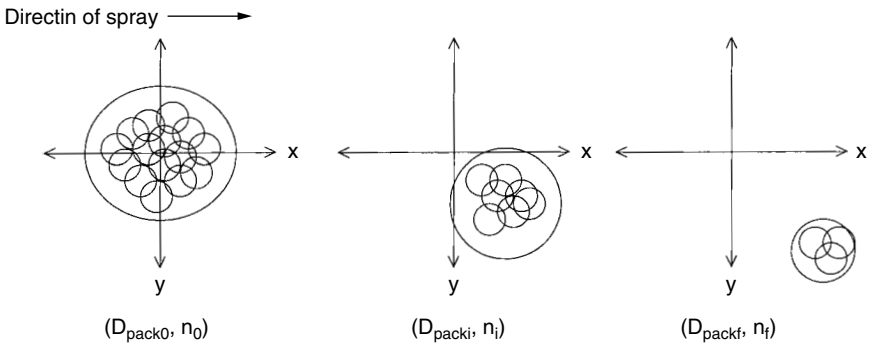

Figure 2

Microorganisms suspended in larger droplets have been found to survive for longer periods of time than those in smaller droplets. Presumably this can be explained by the larger surface area:volume ratio of smaller particles compared to that of larger particles, so that exposure to detrimental environmental factors would be enhanced in the smaller particles.

CONCLUSION

The dispersal of microorganisms in bioaerosols is well documented, and models to simulate their dispersal have been developed. However, the widespread application of these models has not been observed, probably due to the large number of input data that would be required to produce an output in which there could be a high degree of confidence. The use of the Gaussian plume model is widely used, however, for predicting the fate of chemical contaminants in the atmosphere. In addition, these models have been developed for the outdoor environment where large spatial scales are of interest. Data to support a large-scale field model of bioaerosol dispersal is very limited. As interest in the land application of biosolids increases, it is likely that the need for models such as this and interest in this area will increase.

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MODELING. See Activated Sludge Models: Microbiological Basis; Biodegradable Dissolved Organic Carbon in Drinking Water; Data Analysis and Modeling

MODELS, ACTIVATED SLUDGE. See Activated Sludge Models: Microbiological Basis

MODELS: ROLE OF PROTOZOA. See Protozoa in Marine and Estuarine Waters

MOLECULAR METHODS FOR SOILS. See Biodiversity in Soils: Use of Molecular Methods for its Characterization

MONOOXYGENASES. See Oxygenase Enzymes: Role in Biodegradation

MUSTARD GAS, BIODEGRADATION OF. See Chemical Weapons, Biodegradation of

MYCOBACTERIUM AVIUM COMPLEX

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THE *M. AVIUM* COMPLEX

M. avium and *Mycobacterium intracellulare* are members of the *M. avium* complex. Though separate species, they share 40 to 50% DNA relatedness (1) and many cultural, biochemical, enzymatic, and structural features.

Because there are so few phenotypic characteristics that distinguish the species, identification requires DNA probes or analysis of PCR products or restriction fragment length polymorphism (RFLP) analysis. Cells of the *M. avium* complex have a complex, multilayered outer membrane that is rich in lipid (2). The lipid-rich wall contributes to the hydrophobicity (3) and impermeability (2) of members of the *M. avium* complex. Both those factors contribute to the slow growth rate of members of the *M. avium* complex (i.e., one generation per day in rich laboratory media) as does the presence of a single chromosomal copy of the genes for ribosomal RNA (4). Hydrophobicity and impermeability are also major determinants of the antibiotic (5) and disinfection resistance (6) of the *M. avium* complex. The hydrophobicity of the *M. avium* complex also contributes to their aerosolization from water (7).

Though *M. avium* and *M. intracellulare* are related to *Mycobacterium tuberculosis*, they are not obligate pathogens requiring complex media for their growth. Members of the *M. avium* complex are environmental opportunistic pathogens that are free-living saprophytes found in water, soil, and aerosols. *Mycobacterium avium* complex strains can grow in drinking water (8) and natural water (9). They are intracellular pathogens, able to grow in human macrophages (10) and amoebae (11). Human and animal infection results from exposure to environmental compartments containing *M. avium* or *M. intracellulare*.

M. avium includes four distinct subspecies that share 100% DNA similarity (12). *Mycobacterium avium* subspecies *avium* is a human and animal pathogen. This review focuses on that subspecies because of its importance to public health. *Mycobacterium avium* subspecies *silvaticum* is common in animals (e.g., deer), rarely causes infection in humans (12), and is distinguished from subspecies *avium* by the presence of the insertion sequence IS901. *Mycobacterium avium* subspecies *paratuberculosis* is the causative agent of Johne's disease in cattle, and can be distinguished from other members of the species by the presence of multiple copies of the insertion sequence IS900 (12). *Mycobacterium avium* subspecies *paratuberculosis* has been implicated as the etiologic agent of Crohn's Disease in humans (13). It grows extremely slowly and requires mycobactin for growth (14). Finally, *M. avium* subspecies *lepraemurium* is the causative agent of a leprosy-like disease in mice. It is a fastidious microorganism and only limited *in vitro* multiplication of this subspecies has been observed (15). Throughout this review, *M. avium* refers to *M. avium* subspecies *avium*.

M. avium and *M. intracellulare* are just two species of environmental mycobacteria. There are many other species of the genus *Mycobacterium*, which are found in water and soil and are opportunistic pathogens of humans, animals, and birds (16). Many of the ecological and physiologic features of the *M. avium* complex are shared by those other environmental mycobacteria (e.g., *Mycobacterium kansasii*, *Mycobacterium marinum*, *Mycobacterium xenopi*, and *Mycobacterium chelonae*). Those features include hydrophobicity, impermeability, and antibiotic and disinfection resistance (16). This review focuses on the *M. avium* complex because of its widespread occurrence in the environment, and its importance as a pathogen of humans, animals, and birds. However, many of the principles taught here are applicable to the other mycobacterial species.

EPIDEMIOLOGY OF THE *M. AVIUM* COMPLEX

Infections in Humans and Risk Factors

Infection by the *M. avium* complex is usually associated with existence of a preexisting condition that disposes the individual to infection (Table 1). Among individuals with competent immune systems, *M. avium* and *M. intracellulare* pulmonary infections are found in patients with predisposing lung conditions that include silicosis and black lung (17), and patients with pulmonary alveolar proteinosis (18) and cystic fibrosis (19). Elderly women without any of the known risk factors for *M. avium* complex infection have also been described (20). *Mycobacterium avium*, but not *M. intracellulare*, infections are

Table 1. Patient Types and Risk Factors for *M. Avium* Complex Infection

Patient Type	Infection	Route of Infection
Nonimmune Deficient		
Children (1–3 years, erupting teeth)	Cervical Lymphadenitis	Oral
Pulmonary Alveolar Proteinosis	Pulmonary	Aerosol
Women (Chest Architectural Defects)	Pulmonary	Aerosol
Preexisting Lung Disease (Occupational)	Pulmonary	Aerosol
Cystic Fibrosis	Pulmonary	Aerosol
Trauma (surgical or injury)	Granuloma at Site	Liquid or Device
Immune Deficient		
Interleukin-12 Receptor Deficiency	Bacteremia	Oral to GI tract
Malignancy (e.g., Leukemia)	Bacteremia	Oral to GI tract
Immune-suppressive Therapy (Transplantation)	Bacteremia	Oral to GI tract
HIV-infection (AIDS)	Bacteremia	Oral to GI tract

found in (and limited to) the cervical lymph nodes of young children with erupting teeth (21). *Mycobacterium avium* and *M. intracellulare* infections have also been associated with contaminated medical equipment (22,23).

Immune-deficiency resulting from human immunodeficiency virus (HIV) infection (24), IL-12 deficiency (25), malignancy (26), or from immunosuppression associated with transplantation (27) are also risk factors for *M. avium* infection. Unlike the pulmonary infections, infections in the AIDS patients are almost entirely (i.e., 95%) due to *M. avium* (28,29). The reason *M. intracellulare* infections are rare among AIDS patients has not been identified. Infections in immunodeficient individuals are not limited to the lungs, but occur throughout the body and are commonly diagnosed by the appearance of *M. avium* in blood (bacteremia).

One source of *M. avium* infection in AIDS patients is water. DNA fingerprints of *M. avium* isolates from water to which the AIDS patient was exposed were identical with those of the patient (30). Further, an extraordinarily high frequency of *M. avium* infection in AIDS patients in Finland was associated with very high numbers of *M. avium* in drinking and environmental waters (31). In 10 to 30% of *M. avium*-infected patients with the acquired immunodeficiency syndrome (AIDS), more than a single strain has been isolated from individual patients based on differences in serotype (32) or DNA fingerprint pattern (33). That is not surprising, in light of the profound immunodeficiency of the patients and the ubiquity and diversity of *M. avium* types in the environment (33).

Infections in Animals and Risk Factors

Though *M. avium* was originally recovered and described as a pathogen of birds, infection is seldom encountered in poultry. This is probably due to the fact that broilers are six to seven weeks old at slaughter. That is insufficient time for infection to be manifest. More commonly, *M. avium* infection is found in pigs (34). Infection in pigs can appear in the cervical lymph nodes and then the mesenteric lymph nodes (34). Infection is diagnosed by the appearance of tuberculous lesions in the lymph nodes. Because tuberculous lesions result in loss of profit, factors leading to *M. avium* infection have been identified. Two factors associated with higher frequencies of *M. avium* infection are the use of pine bark mulch as bedding (35) and the presence of birds in the pig sties (36). Substitution of straw for bedding and erection of barriers to birds reduce the incidence of *M. avium* infection. Stress is also a likely factor in *M. avium* infection. By manipulating the level of stress of chickens, it was shown that chickens under either abnormally high or low levels of stress were more susceptible to *M. avium* infection (37). This might be an important factor in the morbidity and mortality of animals captured in the wild and transported to zoos (38).

Water can be a source of *M. avium* infection in captive animals. Macaques infected with simian immunodeficiency virus (SIV) were infected with *M. avium* strains that were present in their drinking water (39).

ECOLOGY OF THE *M. AVIUM* COMPLEX

Members of the *M. avium* complex are found in almost every environmental compartment. They have been isolated from natural waters (40–42). Because *M. avium* complex organisms can be isolated from freshwaters and those of moderate to high salinity (4) and grow in waters of moderate salinity (9), the *M. avium* complex may be estuarine. Drinking waters also yield the *M. avium* complex (42–45). Drinking water may be a more important source of the *M. avium* complex than natural water because of more possibilities of human exposure. Members of the *M. avium* complex have been shown to occur in high numbers in hospital hot water systems (46), and persist for as long as 18 months in hospital water systems (30). They have also been recovered from biofilms that form on pipes in drinking water systems (47), and from the dialysis membranes used in treatment of drinking water by reverse osmosis (48). Groundwater seldom contains the *M. avium* complex (49). *Mycobacterium avium* complex bacteria have also been recovered from biofilms collected at the air-water surface of natural bodies of water (50), droplets ejected from the surface of a river (50), and aerosols (51). Ejection of droplets enriched with cells of the *M. avium* complex is one mechanism for the aerosolization of the *M. avium* complex. Long-term exposure to aerosols generated in swimming pools may be responsible for granulomatous pneumonitis found in swimming pool attendants (52). *Mycobacterium avium* complex cells of higher hydrophobicity were more highly enriched in droplets than cells of mycobacterial strains of lower hydrophobicity (7).

In addition to water, *M. avium* and *M. intracellulare* have been recovered from soils (41,53–55). Soils rich in humic and fulvic acids have high numbers of *M. avium* complex bacteria whether they have been collected from the coastal acid, brown water swamps of the eastern United States (41) or from peatlands or boreal coniferous forests of Finland (55). *Mycobacterium avium* complex isolates have also been recovered from sphagnum vegetation (56) and pine bark mulch (35). Their presence in the latter undoubtedly contributes to the association of the use of pine mulch bedding and *M. avium* lymphadenitis in pigs (34). As noted earlier, members of the *M. avium* complex have also been isolated from medical instruments (22,23). It has been presumed that their presence in such devices is due to insufficient disinfection of these disinfection-resistant bacteria (22,23).

TRANSMISSION OF THE *M. AVIUM* COMPLEX

One route of *M. avium* complex infection is via aerosols. Cells of *M. avium* complex bacteria are readily aerosolized from water suspensions (57) and are associated with natural aerosol fractions containing particles of a size (i.e., 1 μm diameter) that can enter human alveoli (50,51). The route of *M. avium* complex infection leading to pulmonary infection is logically via the aerosol route. Aerosolization of *M. avium* complex cells can occur either through the natural process of ejection of droplets (57) or physical or

mechanical agitation (52). Cell surface hydrophobicity is the major determinant of aerosolization (7) with little or no contribution from cell surface charge (58).

It has not been determined whether the route of infection in immunodeficient patients is via the pulmonary or gastrointestinal tracts or both. Studies in AIDS patients have implicated both routes (59,60). The appearance of *M. avium* in blood (i.e., bacteremia) was equally likely to have been preceded by pulmonary or gastrointestinal tract infection or colonization (59,60). The difficulty of identifying a specific route is due to the fact that the required specimens (i.e., sputum and feces) contain microorganisms in addition to the *M. avium* complex. To detect, identify, and enumerate *M. avium* complex, specimens must be decontaminated to permit colony formation of the slowly growing mycobacteria. However, decontamination also reduces mycobacterial numbers such that the sensitivity of detection is reduced.

Drinking either natural or potable water containing *M. avium* is the likely route of cervical lymphadenitis in children (21). Because the age of the children coincides with the eruption of teeth, it is compelling to hypothesize that the trauma of the gums leads to entry of the mycobacteria and their migration to the cervical lymph nodes. What is interesting is that until 1975–1985, the majority of cases of childhood cervical lymphadenitis were caused by *Mycobacterium scrofulaceum*, another environmental opportunist (21). However, since 1985, almost all the cases are caused by *M. avium*. Further, recent environmental surveys (42,44,45) have not reported recovery of *M. scrofulaceum* although the methods of isolation and identification of mycobacteria had not changed. It appears that *M. scrofulaceum* has disappeared and its niche has been occupied by *M. avium*.

Introduction of *M. avium* complex microorganisms into patients via contaminated instruments (22,23) or kidney dialysis units (8) are additional routes of infection. In the cases published in the literature, disinfection resistance of the mycobacteria (8,22,23) has been suggested as the major factor leading to contamination of the vector.

MOLECULAR EPIDEMIOLOGY OF THE *M. AVIUM* COMPLEX

A variety of typing methods has been developed for studies of the epidemiology and ecology of members of the *M. avium* complex. These methods are necessary because it is insufficient to simply isolate *M. avium* or *M. intracellulare* from a particular sample, and say that the sample's origin is the source of human or animal infection. The isolated strains must be members of the same clone. Serotyping and restriction fragment length polymorphism (RFLP) based typing methods have proven the most useful. Serotypes are based on the presence of carbohydrate-containing antigens on the surface of *M. avium*, *M. intracellulare*, and *M. scrofulaceum* (61). Serotypes of the *M. avium* complex correlate with species assignments based on DNA probes (62) and certain serotypes are found more commonly in AIDS patients (63).

DNA fingerprinting of *M. avium* complex isolates has been performed using pulsed field gel electrophoretic (PFGE) separation of large restriction fragments (33,64). Though time-consuming, PFGE patterns are discriminatory and have led to the proof that *M. avium* isolates from patients share the same PFGE pattern with isolates from water to which the patient was exposed (30). They have also demonstrated that AIDS patients can be infected with more than a single clone of *M. avium* (33). One advantage of PFGE fingerprinting is that any species and isolate can be typed.

Typing of *M. avium* isolates can be performed rapidly by restriction fragment length polymorphism (RFLP) analysis, using the insertion sequence IS1245 as a target or probe (65,66). Between 5 and 25 copies of this insertion sequence are found in the genome of *M. avium* (65,66) Thus, IS1245 typing is quite discriminatory. IS1245 typing can also be carried out with the polymerase chain reaction (67). Primers have been designed, that amplify the DNA sequences between IS1245 copies, and the resulting patterns of amplified bands (i.e., intervening sequences) can be compared (67). Unfortunately, IS1245 is only found in *M. avium* strains (65,66) and there have been no reports to date of IS-elements suitable for typing *M. intracellulare* or *M. scrofulaceum*.

METHODS FOR WORKING WITH THE *M. AVIUM* COMPLEX

Recovery, Growth, and Isolation

Though cultivation of the *M. avium* complex is difficult because of their slow growth and the attendant risk of contamination, they are not fastidious. They grow on a variety of media and will even grow in water (8,9). Inclusion of oleic acid, either with albumin or as part of a detergent (e.g., Tween 80®) to reduce the toxicity of the fatty acid stimulates growth. Inclusion of a detergent in mycobacterial media has also been used to reduce the formation of cell aggregates. Not only does the detergent reduce the aggregation, but also it accelerates growth, probably through an increase in permeability (2). Unfortunately, detergent also increases the susceptibility of mycobacterial cells to disinfectants and antimicrobial agents (5).

To reduce the chance of overgrowth of *M. avium* colonies by those of other microorganisms, samples are often decontaminated. The success of decontamination relies on the relative resistance to the killing action of acid (e.g., oxalic acid), base (e.g., NaOH) or detergent (e.g., cetylpyridinium chloride, CPC). However, decontamination does reduce the number of viable mycobacterial cells (68). Cetylpyridinium chloride (0.005% for 30 min to 24 hr) appears to be most useful, based on its ability to reduce colony counts of non-mycobacterial cells while reducing mycobacterial numbers very little. Filtration following decontamination has been used to isolate mycobacteria in drinking water (44,45). In that approach, filters are placed on media and mycobacterial colonies grow on top of the filters (44). A number of publications have compared different methods for the recovery of *M. avium* complex microorganisms from water (69–71) and soil (54,68,72,73). Methods for the recovery of *M. avium* complex from aerosols, ejected droplets, air-water biofilms (50), and from biofilms on

pipe surfaces (47) have been published. Immunomagnetic beads coated with antimycobacterial antibody have been used to isolate *M. tuberculosis* from sputum samples (74), and there is no reason to believe that such an approach might not work for isolation of *M. avium* complex from water samples and soil suspensions. Based on the fact that *M. avium* survives following phagocytosis by *Acanthameoba castellani* (11), it might be possible to use phagocytic amoebae or protozoa to scavenge and concentrate mycobacteria in water or a soil suspension.

Identification of the *M. Avium* Complex

Commonly, the identification of putative mycobacterial colonies from a patient or environmental sample is initiated with the acid-fast stain. *Mycobacterium avium* complex strains can form interconvertible opaque (i.e., 1 to 2 mm diameter, domed cream, or yellow-pigmented colonies) or transparent (0.5 mm, flat, transparent) colonies. The transparent colonies are especially difficult to see, even on a transparent medium. Acid-fast isolates can be characterized as rapidly growing (i.e., colony formation in less than seven days at 37 °C) or slowly growing (i.e., colony formation in greater than seven days at 37 °C). The ability to form pigment in the dark (scotochromogenic), or only in the light (photochromogenic), can also be assessed with growth rate. Though, identification of mycobacteria is possible by cultural, biochemical, and enzymatic tests, current practice uses DNA probes or PCR amplification with or without restriction endonuclease digestion of PCR products. This is particularly important for the *M. avium* complex because *M. avium* and *M. intracellulare* cannot be distinguished reliably with cultural or biochemical tests. DNA probes are commercially available for *M. avium, M. intracellulare,* and other mycobacteria (Gen-Probe, San Diego, CA). A nested PCR amplification of the 16S rRNA gene can be used to identify members of the genus *Mycobacterium* and *M. avium* and *M. intracellulare* (75). PCR amplification of the *hsp-65* heat shock protein gene of mycobacteria followed by restriction endonuclease digestion with *Hae* III, and *Bst* EII has been used for identification of many mycobacterial species (76,77). It is also possible to use PCR to detect *M. avium* complex cells in water samples, though the limit of detection is only 10^4 per ml.

Measuring Virulence of the *M. Avium* Complex

Virulence of *M. avium* complex isolates can be measured in chickens (78), mice (79), or in the beige mouse (80). Animals can be infected by a variety of routes (e.g., intraperitoneal or intravenous), and the establishment and progression of infection assessed by measuring the number of colony-forming units (CFU) in lung, spleen, and liver (78,79,80) after different periods of time following infection. In beige mice, LD_{50} values can be measured (80). In the chicken, *M. avium* strains are considerably more virulent than are strains of *M. intracellulare* based on comparison of mortality and microbial numbers (as colony-forming units, CFU) in organs (81). In mice, *M. intracellulare* strains are more virulent than *M. avium* strains, again using the same criteria (82). Because members of the *M. avium* complex are intracellular parasites, it is possible to measure growth in alveolar or peritoneal macrophages (83) and epithelial cells (84).

The growth conditions of the *M. avium* complex strains influence virulence. The mortality and CFU per gm of organ was higher when strains were grown at 42 °C compared to 37 °C (85). *Mycobacterium avium* complex cells grown under reduced oxygen tension (i.e., microaerobic) were significantly more virulent compared to cells grown under normal oxygen levels (86).

Genetic differences in addition to species assignment are also determinants of virulence in the *M. avium* complex. *Mycobacterium avium* complex strains form interconvertible colony types (i.e., transparent and opaque) that differ in virulence. Transparent variants have been shown to be of greater virulence (i.e., mortality and CFU per gm of organ) in chickens (87–90), rabbits and guinea pigs (89), and mice (90) compared to their isogenic opaque variants. It has also been shown that infection with the more virulent transparent type does not trigger release of the interleukins IL-1 and IL-6 (91,92) and TNFα (92). That data suggests that the reduced virulence of opaque colonial variants of *M. avium* complex strains is due to their inability to suppress initiation of the host immune system (91,92).

Two factors have been identified that contribute to the survival and growth of *M. avium* complex cells in phagocytic cells of the mammalian immune system. Vesicles containing *M. avium* complex cells are not acidified as are the vesicles containing other bacteria that do not survive phagocytosis (93,94). In addition, the *M. avium* complex-containing vesicles do not fuse with lysosomes, thus preventing exposure of the cells to bactericidal proteins and lytic agents (95). It is likely that those characteristics also contribute to the survival of *M. avium* complex cells in phagocytic amoebae (11).

GROWTH AND PHYSIOLOGICAL ECOLOGY OF THE *M. AVIUM* COMPLEX

Members of the *M. avium* complex show evidence of adaptation to survival in the natural environment. In addition, those adaptations result in the ability of cells to persist in drinking water and drinking water distribution systems, resist disinfectant, and antibiotic action, and survive and grow in human and animal macrophages.

Members of the *M. avium* complex grow in natural and drinking water (8,9). They grow in fresh and relatively saline waters of 2% salt (9), in keeping with their isolation from estuarine environments (40). They grow over a wide temperature range (i.e., 10°–45 °C) (9). Optimum growth occurs at pH 4.5–5.5 (96,97). Because the isoelectric point of *M. avium* complex cells is near pH 4 (58), their cells would carry no net charge in mildly acidic environments such as encountered in brown water coastal swamps of the eastern United States and peat bogs and boreal coniferous forests of Finland. Highest numbers of *M. avium* complex bacteria are associated with reduced dissolved oxygen (41,53) suggesting they are microaerophilic. *Mycobacterium avium* complex numbers are also correlated with high levels of zinc (41,55),

suggesting that the group has a high requirement for this metal.

High numbers of *M. avium* complex microorganisms were associated with high concentrations of humic and fulvic acids (41,55). That discovery led to the demonstration that the growth of *M. avium* complex strains is stimulated by humic and fulvic acids (98). Growth stimulation by humic and fulvic acids and a preference for acidic, microaerobic environments are consistent with, and in fact explain, their high numbers in the acidic, brown water coastal swamps of the eastern United States (41), and in the waters and soils of boreal coniferous forests and peat bogs in Finland (55).

Members of the *M. avium* complex are the most hydrophobic of microorganisms (3,7). The high cell surface hydrophobicity is a major determinant of their enrichment in droplets ejected from the waters (57), and their concentration at the air-water interface (50). Though not proven, their hydrophobic surface (3,7) is likely a major contributor to the disinfection (6), antibiotic (5), and heavy metal resistance (99). It may also contribute to the formation of biofilms (47,48). The combination of high hydrophobicity and heavy metal resistance may allow *M. avium* complex cells to be pioneers of biofilm formation in drinking water distribution networks, and hot and cold water systems in hospitals, especially if galvanized (i.e., zinc-coated) pipes are employed.

In summary, members of the *M. avium* complex are adapted to life in marginal environments. Marginal environments include those of low nutrient or acidic pH. They also include three human-influenced environments, water subject to disinfection, medical equipment requiring sterilization, and patients undergoing antibiotic therapy. In each of those environments, rapidly growing microorganisms are killed and members of the *M. avium* complex and other mycobacteria can invade and proliferate.

DISINFECTION RESISTANCE OF THE *M. AVIUM* COMPLEX

Mycobacterium avium and *M. intracellulare* are resistant to disinfection. Strains of *M. avium* are at least 500-fold more resistant to chlorine, 100-fold more resistant to chlorine dioxide, and 50-fold more resistant to ozone than *Escherichia coli* (6). Though some *M. avium* strains were relatively resistant to chloramine (6- to 25-fold) compared to *E. coli*, some strains (2 of 5) were just as susceptible (6). Susceptibility of *M. avium* strains to chlorine was strongly correlated with growth rate. The slower growing strains were more resistant (6). Cells of *M. avium* grown in water were, on average, 10-fold more resistant to chlorine than were cells grown in laboratory medium (6). This could be due to the fact that growth rates in water are lower than rates in laboratory media. *Mycobacterium intracellulare* strains are slightly more chlorine-resistant and *M. scrofulaceum* strains much more sensitive to chlorine (fivefold) compared to *M. avium* strains. The relative susceptibility of *M. scrofulaceum* to chlorine may contribute, in part, to its disappearance from water.

Members of the *M. avium* complex are resistant to disinfectants that are used for surface and instrument sterilization. Concentrations of gluteraldehyde and phenolic-based disinfectants that kill other microorganisms have little effect on strains of *M. avium* complex and other mycobacteria (100).

ANTIMICROBIAL RESISTANCE OF THE *M. AVIUM* COMPLEX

Members of the *M. avium* complex grow at concentrations of heavy metals (e.g., Hg^{2+}, Cd^{2+}, and Cu^{2+}) that are approximately 10-fold higher than concentrations that inhibit the growth of other bacteria (99). In addition, some strains express specific resistance mechanisms. A mercury-resistant (grew at 1 mM Hg^{2+}) strain of *M. avium* isolated from a metal-polluted portion of the Chesapeake Bay was shown to produce a mercuric reductase (101). That same strain was shown to be resistant to Cu^{2+} (grew at 1 mM Cu^{2+}) by virtue of its ability to sequester copper in the membrane fraction as an insoluble sulfide (102). Not only do those two mechanisms result in survival of cells of the *M. avium* complex strain, but they also permit survival of metal-sensitive microorganisms in the immediate environment because the toxic heavy metal is removed or sequestered.

Members of the *M. avium* complex, along with the entire genus *Mycobacterium*, are relatively resistant to antibiotics. High cell surface hydrophobicity (3,5,7,103) and membrane impermeability (2,5) both contribute to resistance to a wide spectrum of antibiotics. Reduction of cell surface hydrophobicity through the use of detergents, significantly, reduces antibiotic resistance (5). Permeability of β-lactam antibiotics (e.g., penicillins) is significantly lower in mycobacteria than it is in other bacteria (2,104). To overcome these two cell surface barriers, cells have been exposed to an antibiotic whose target is the cell wall (e.g., ethambutol) and a second, whose target is not the cell wall (e.g., rifamycin) (105,106). In a number of cases, the combination resulted in killing above the level (i.e., synergistic) of either antibiotic alone (105,106). A second successful approach has been the development of hydrophobic or tissue-trophic variants of antibiotics. The erythromycin derivatives, clarithromycin and azithromycin, are more hydrophobic than their parent and have demonstrated effectiveness *in vitro* and *in vivo* against members of the *M. avium* complex (107,108). Amphipathic derivatives of isoniazid showed higher anti *M. avium* complex activity compared to isoniazid itself (109).

In addition to broad spectrum antibiotic resistance of the *M. avium* complex due to high cell surface hydrophobicity and membrane impermeability, strains of the *M. avium* complex have been isolated that have mutations leading to resistance to specific antibiotics. For example, clarithromycin- and azithromycin-resistant strains have been isolated from patients undergoing therapy with one of those drugs. Those strains have been shown to carry mutations in the sequence of the 23S rRNA, one of the targets of their action (110,111). Interestingly, there have been no reports of antibiotic-resistant *M. avium*

complex strains whose resistance is due to an antibiotic inactivation, though a strain of *Mycobacterium smegmatis* was shown to inactivate rifampicin by ribosylation (112). Such a mechanism of resistance is common amongst gram-negative, plasmid carrying bacteria. However, the modest increases in minimal inhibitory concentrations resulting from such antibiotic-inactivation mechanisms, may not be of adaptive utility to the mycobacteria with their high level of broad spectrum resistance.

CONCLUSION

Members of the *M. avium* complex are environmental opportunistic pathogens that are well adapted to survival and growth in natural and human-influenced habitats. Their ability to grow in natural waters and drinking waters, and drinking water distribution systems, coupled with their resistance to disinfection suggests that they will remain hazards to individuals with conditions that predispose for *M. avium* complex infection. Because the number of immunosuppressed individuals throughout the world is expected to continue to rise as a consequence of organ transplantation, malignancy, and chemotherapy, the number of *M. avium* complex susceptible individuals will also increase. Eradication from drinking water distribution systems will be difficult because of the ability of these microorganisms to form biofilms. Present disinfection practices also have the disadvantage that they result in selection of the *M. avium* complex, and other mycobacteria. Because the *M. avium* complex and the other environmental opportunistic pathogens of the genus *Mycobacterium* are ubiquitous, it is likely that they will emerge as a leading occupational hazard in the workplace. Already members of the *M. avium* complex and other mycobacteria have been implicated as causative agents of hypersensitivity pneumonitis among swimming pool attendants (52) and metal workers in the automobile industry (113).

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MYCORRHIZAE. See HOT DESERT SOIL MICROBIAL COMMUNITIES

MYCORRHIZAE: ARBUSCULAR MYCORRHIZAE

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WHAT IS AN ARBUSCULAR MYCORRHIZA?

Mycorrhizae form as a result of mutualistic symbioses between plants and fungi localized in the root or rootlike structures. Their major function is that the fungi form a fine, absorbing network of hyphae that transport resources in the substrate to the host in exchange for carbohydrates.

The arbuscular mycorrhiza is the most widespread type of mycorrhiza and it is the most ancient. This type of mycorrhiza has been described as an endomycorrhiza, a vesicular-arbuscular mycorrhiza (VAM), a phycomycetoid mycorrhiza, and, currently, an arbuscular mycorrhiza (AM). Generally, this type of mycorrhiza is named on the basis of the structures formed. Arbuscules are widespread and known to be important in resource exchange between host and fungus. These are presumably ubiquitous across taxa, but are very transitory and sometimes are not formed. Vesicles are formed by the *Glomus*/*Acaulospora* lineage, but again, are not found for all genera. The predominant features of an AM are shown in Figure 1. Vesicles and hyphal structures found in fossils from the Devonian are indistinguishable from modern structures.

The fungi forming AM are a monophyletic group, the Glomales, which appears to go back to an ancestral fungus forming an association with algae in the Ordovician or Silurian epoch. The divergence between *Geosiphon* (a related fungus forming a symbiosis with *Nostoc*) and *Glomus* occurred sometime during the Silurian or Devonian epochs. A further divergence between the *Gigaspora* and the *Glomus* lineages occurred sometime between 350 and 400 million years ago. Today, six genera are recognized: *Glomus, Acaulospora, Sclerocystis* and *Entrophospora* (Glomaceae), and *Gigaspora* and *Scutellospora* (Gigasporaceae).

AM are a particularly important linkage between plant and soil. They appear to have been a critical step in the invasion of land. Algae and plants in saturated

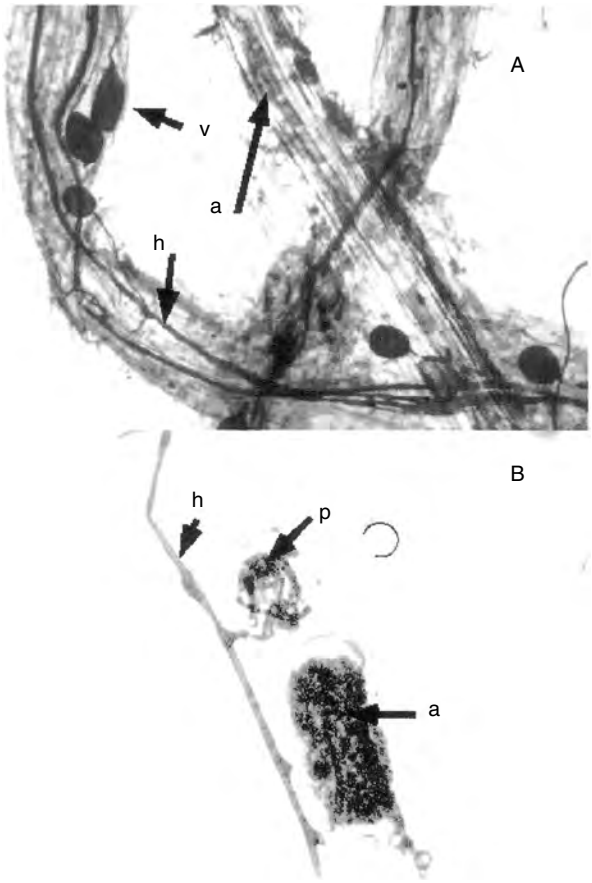

Figure 1. Illustrations of AM structures. **A**) Infected root segment with internal arbuscules (a) and extramatrical hyphae (h) and vesicles (v) extending from the root into the soil. These structures are involved in nutrient uptake and carbon storage. **B**) A greater detail of the intercellular hyphae (h), peletons or coils (p), and arbuscule (a). These structures are all involved in nutrient and carbon exchange between host and fungus.

environments are bathed in a nutrient solution and nutrients are taken up by diffusion. Rhizoids are relatively inefficient structures for obtaining soil resources as the soil dried out at long distances. Taking the symbiosis between the ancestral plant and fungus to the land enabled the hyphae to efficiently spread into soils extracting soil resources in exchange for carbon.

BASIC STRUCTURE/FUNCTIONING FOR AN INDIVIDUAL PLANT

The basic functioning of an AM is tightly coupled to both its internal (within the host) and external structures (within the soil). An AM forms as a runner hypha growing through the soil intercepts a susceptible root section. Susceptible roots are those uninfected segments located just behind the region of elongation (Fig. 2). This hypha penetrates a susceptible root and forms an appressorium. The internal hyphae then proliferate forming either an Arum-type mycorrhiza, in which intracellular arbuscules emerge from intercellular hyphae, or a Paris-type mycorrhiza, in which sequential coils and infrequent arbuscules emerge from

inter- or intracellular hyphae. In all cases, there remain intact membranes separating the plant and the fungus cytoplasm.

As the internal portion of an AM forms, carbon is transported back into the external hypha. The external hyphae appear to form two distinct architecture types. The first is the mycelial network of runner hyphae that continues to spread, looking for new root segments, or forming vesicles or spores accumulating carbon. Associated with the internal network is also a dichotomously branching, external hyphal network that spreads outward searching for soil resources. There are limits to this extension as at each branching, the hyphae decrease in diameter.

This means that there are three structural elements to the AM fungal mycelium. The first element is the internal hyphae. Carbon is taken up by the fungal hyphae within the plant. Evidence to date shows that much, if not all, of the internal hyphae can take up sugars, although adenosine triphosphatases are concentrated in the arbuscules. Importantly, fungi generally convert the simple sugars to trehalose or other complex sugar to prevent or reduce reabsorption. Within the root cortex, hormonal composition also changes. These are probably primarily associated with loading and unloading of carbon and nutrients. However, these hormone shifts also lead to other changes in rooting morphology, such as increased lateral root formation. These changes can affect the entire dynamics of root form and chemistry.

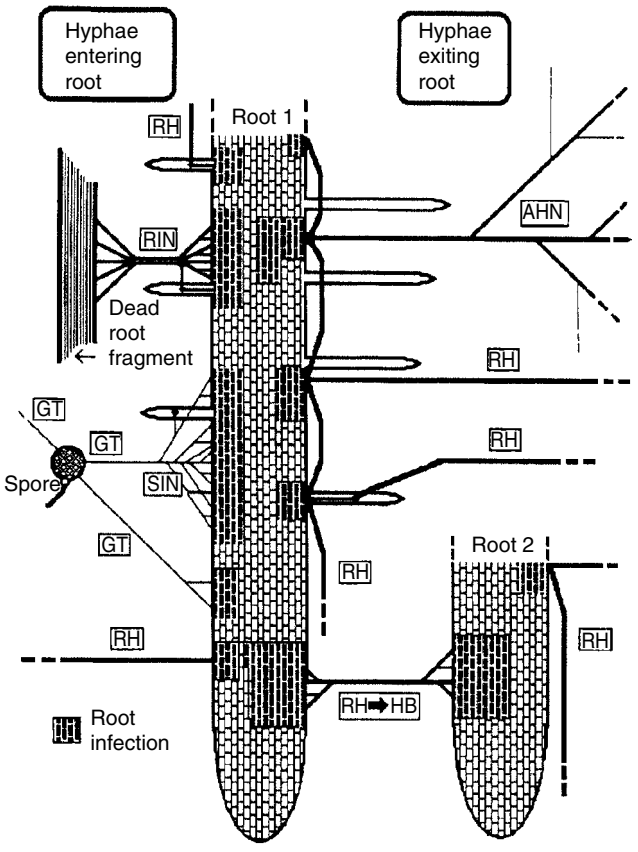

Figure 2. Architecture of the external hyphae forming AM. The primary hyphal types observed in soils include the runner hyphae (RH), hyphal bridges between plants (HB), and absorptive hyphal networks (AHN). New AM infections can be formed by germ tubes (GT) arising from spores either as individual hyphae, or as infection networks (SIN), or from infected root fragments (RIN). When many hyphal bridges are formed between plants, a common mycorrhizal network (CMN) is formed.

The second element is the external absorbing network. This is a dichotomous branching mycelium that starts as a single thick hypha extending out beyond the root hairs, then dividing into ever-finer hyphae. This network consists of 80 to 120 cm of hyphae per root penetration in up to an eighth-order branching structure. At the base, this hypha is 10 to 12 μm in diameter. Towards the hyphal tip, it may be as fine as 2 μm. The outward limit appears to range from 2 to 8 cm, corresponding to a depletion zone surrounding each individual mycorrhizal root. These external hyphae pick up soil resources and transport those resources to the host in exchange for carbon. Most soil resources are transported to the plant via this network, including phosphorus, nitrogen, water, iron, calcium, zinc, manganese, magnesium, and sulfur.

The third element is the runner hyphal network. These hyphae consist of single large (10 to 15 μm in diameter) hyphae, or multiple hyphae wrapped around each other in a primitive, rhizomorph-like structure. The actual functions of these hyphae are not well understood. These hyphae clearly are involved in searching out new roots to form new mycorrhizae. The external hyphae do not appear to have the enzymatic capacity to take up sugars, but they do move carbon and water. They transport nutrients depending on the flow rates, direction, and sink strength. Importantly, it is this mycelium that connects multiple roots of a single plant and multiple plants in a complex network of mycorrhizal partners, called the common mycorrhizal network (Fig. 2). We do not know the extent of any single mycelium, or the amount of transport of this network. Carbon, nitrogen, and phosphorus (and probably many other resources) are transported between plants through these hyphae but the amounts and significance remains controversial (Fig. 3). In some cases labeled carbon from one plant can be found in the fungal hyphae within a neighboring plant but appears not to be transported to the shoot. In other cases, the data show label appearing in the shoot. It could be directly transported or respired and refixed. Clearly, this is an important topic in need of additional research.

Physiologically, AM plants are very different from nonmycorrhizal plants. Some of these differences are a direct consequence of their increased resource uptake and ability to be a carbon sink, but others are indirect, caused by the mechanisms regulating resource transfer. An example is the hormonal balance of the host. Several studies have demonstrated changes in the host hormonal balance beyond that expected by direct production and transport from the fungus to the plant. This is likely because of the roles of phytohormones in carbon and nutrient loading and unloading. However, the resulting shifts can affect everything from stomatal opening to root branching. Generally, the shifting balances are beneficial to both the plant and the fungus.

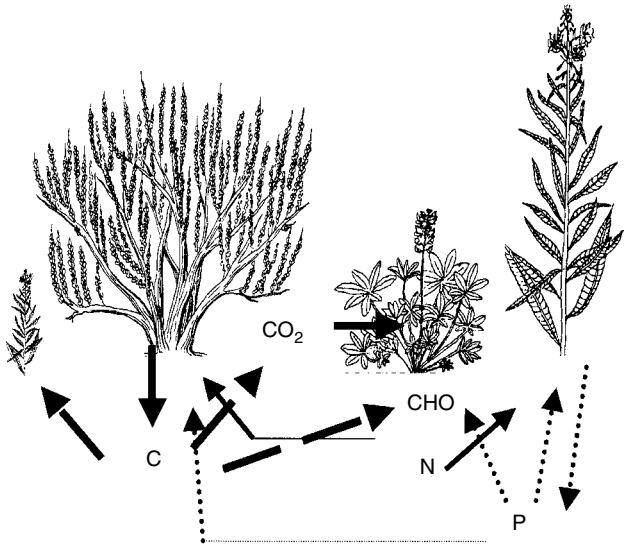

Figure 3. Complex resource transfers among common mycorrhizal networks in a community. Each arrow shows an elemental transport between plants via mycorrhizal hyphae demonstrated in the literature. Resources may be exchanged depending on the sink strengths, resource concentrations, and diversity of fungal and plant partners. The quantitative extent of resource transport is still an open research question.

Thus, our understanding of mycorrhizal dynamics and mechanisms at the individual plant/fungus scale is rapidly improving. What we know comes largely from pot, chamber, and petri dish experimental systems. However, there are subtle mechanisms that are associated with fungal growth and maintenance, infection, and plant and fungal genetic differences. In the field, these can add up to quite different roles when viewed at the larger scales of a stand, community, or landscape.

SCALING AND COMMUNITY AND ECOSYSTEM EFFECTS

AM are not evenly distributed along a root or across a field, they are also not benign sealed tubes extending into the soil from the roots. They are organisms with their own needs, physiology, and patterns. Thus, the generalized models of AM action (nutrient transport from the fungus to the plant and of carbon from the plant to the fungus), developed largely from pot culture studies, must be recast in the context of larger-scale field dynamics.

When plants occupy a stand, there are multiple individuals, and generally, several species. In many cases, the soil is rich in resources. In pot studies, mycorrhizal density declines with increasing fertility. However, in a competitive field environment, the level of nutrients available per plant can remain low. Mycorrhizal infection is regulated by the nutrient status of the leaves. Thus, plants in soils that appear to be high in nutrient levels may still have a high mycorrhizal infection. Just as important, roots can grow rapidly during certain conditions and outgrow the mycelial network. In these cases, the percentage of mycorrhizal infection can be low, but can occupy a large number of root segments. Thus, in the field, the percentage of root infection at the community level depends on a much larger number of variables than in pot cultures.

Research continues to focus on nutrient uptake by individual mycorrhizae. However, in many cases all plants in a community form AM, potentially with the same fungus. Why, then, do mycorrhizae matter? At the stand level, AM are often important to a lesser extent for the nutrient transport to an individual plant, and to a greater extent for the differing species of AM fungus select among the resident plants, favoring some but not others. In other cases, some plants have evolved to reject AM. AM fungi preferentially support plants forming mycorrhizae. A century ago, Stahl divided the world into mycotrophs (plants that are capable of forming mycorrhizae) and nonmycotrophs (those that do not). More recent research has clearly demonstrated that this is an advanced genetic trait mostly found in some weedy species that preferentially survive in highly disturbed lands.

Thus, the importance of AM is not in increasing productivity overall, but in altering the competitive outcomes among plants. These effects can range from minor shifts in nutrient allocation, to situations in which the hyphae colonize and reduce the growth of some species. For example, in some communities, AM increase the growth of mycotrophic plants in competition with nonmycotrophs, thereby increasing the total plant diversity. In other cases, when all plants form AM, as the richness of AM fungal species increases, up to five to eight species, more plant species survive, presumably occupying slightly different niches.

In extreme cases, AM fungi may actually inhibit the growth of certain species. In relation to competition between grasses and chenopodiaceous weeds, AM improve the growth of grasses and reduce the growth of weeds. This can affect succession outcome and overall plant production. This phenomenon was apparent in an experiment in the Great Basin where a dominant early colonizer is *Salsola kali* L., a chenopodiaceous shrub that does not form AM. We found that inoculated AM associated with grasses colonized and parasitized the *S. kali*, reducing the height, density, and productivity of the overall stand. However, the grass establishment was enhanced.

At the ecosystem scale, the extramatrical mycelium has multiple functions within the soil. The hyphae not only increase the surface area for uptake, but also affect nutrient availability. In some instances, nutrients in the soil solution can be increased. The hyphae themselves respire carbon dioxide that, upon contact with the soil solution, becomes HCO_3^-. This pH effect weathers phosphorus and other minerals from clay particles. Under some conditions that we do not understand, the hyphae also produce organic acids, such as oxalates, that bind cations that otherwise would immobilize the PO_3^-. This can, in turn, enhance phosphorus uptake.

The hyphae themselves require nutrients for their own growth, which could affect nutrient availability for plants.

In the case of nitrogen, its concentration in most fungi is approximately 10%. This is contrasted with plant leaves where it is present at 2 to 4%. When the hyphal matrix is dense up to 1 to 5% of the total "soil" nitrogen can be bound in fungal tissue. This amount is equal to or exceeds the available nitrogen in soil.

AM hyphae extend outward from the root into the soil. Some or all of these hyphae produce a glycoprotein called glomalin that is both relatively recalcitrant and sticky. This compound "glues" together many soil particles forming soil aggregates. At an ecosystem scale, this aggregate formation may be among the most important functions of AM fungi. These aggregates also appear to be important for carbon sequestration. With elevated carbon dioxide, more carbon flows into hyphae, and newly fixed carbon is found preferentially in aggregates. Preliminary calculations suggest that up to 25% of the recalcitrant soil organic carbon is made of glomalin.

At the scale of a stand of plants, there are multiple species of AM fungi. Whereas species richness varies among sites, estimates range from 8 to 50 taxa per site. This suggests that the soil community consists of a shifting matrix of AM fungi. Individual studies have shown that improvement in plant diversity approaches an asymptote at about five fungal species in a small sward. Plants establish and preferentially associate with and increase one or two species of fungi. When any of those individuals die, a gap is opened for the establishment of other plants or fungi. This can result in a legacy of plant/AM fungi in time that may affect the overall community structure.

In tillage agriculture, a simpler system develops. As the mycelia network does not remain intact following soil disruption, the dynamics of agricultural AM fungi and plants may be quite different from wildland ecosystems. The mycelium must re-form with each cropping season and, if crop rotation is practiced, with differing plant species.

MYCORRHIZAE AND OTHER SOIL ORGANISMS

AM do not exist in a vacuum. They perform three distinct functions in the soil food web. First, AM fungi comprise a large and rich resource of carbon and nitrogen. Grazing is indicated by holes in spores and nematodes curled up inside old spores. Bacteria cover dead and dying spores. Hyperparasites colonize and utilize these large, lipid-rich bodies. However, collembola and mites appear to prefer other fungi and there are hints that bacteria do not colonize the surface of living AM fungi as they would other fungi. The mechanisms of resistance are not known; they may include the presence of fast-sealing compounds, high C/N ratios, antibiotics, or other factors. This is an open research area. There are even hints in the literature that AM fungi can be predators of soil animals. Predation of collembola, nematodes, and other animals could be important in nitrogen-limited ecosystems.

Because AM are a distinct part of the soil food web, they play a crucial role in carbon cycling. With elevated atmospheric carbon dioxide, both hyphal production and turnover increase. This is reflected in the increasing glomalin and carbon within soil aggregates. Carbon loss from soil declines. With fertilization, particularly nitrogen, AM hyphal mass declines. In several data sets, these environmental changes result in reduced carbon through the AM channel and more through the saprophytic and facultative parasitic fungal web, and through the bacterial web. The increasing grazer activity means that carbon turnover remains high or can increase (Fig. 4).

AM fungi form a mycorrhizosphere extending outward from a root and creating a chemically and physically different environment than either the root surface or the bulk soil. In fact, in many ecosystems, there may be no truly bulk soil. In grasslands and many shrublands, the AM mycelial network completely extends between roots, colonizing all soils and changing the chemical environment. A myriad of bacteria and fungi, and their food webs, depend upon the carbon extending into these areas.

Finally, if AM fungi are largely involved in soil aggregation, then the entire structure of the soil physical/chemical composition of the soil is dependent on the formation, species composition, and survival of these interesting and crucial fungi.

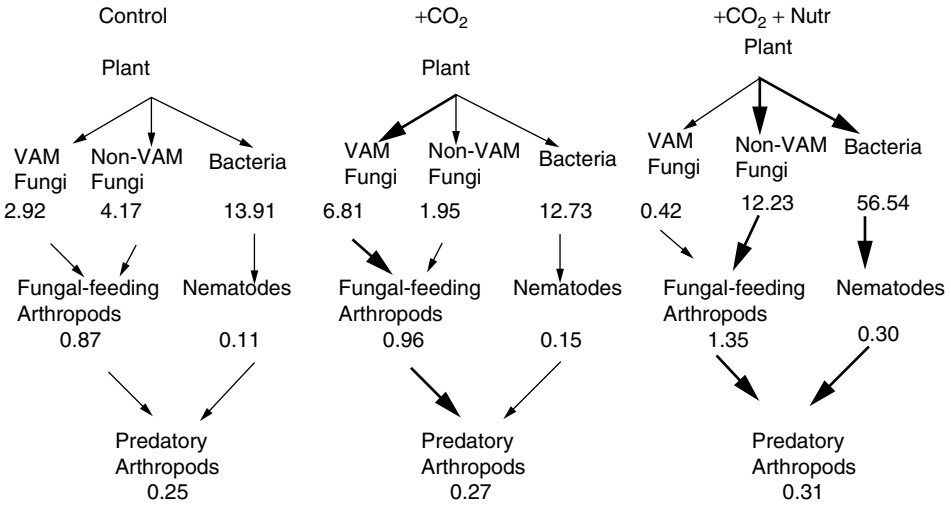

Figure 4. Standing crop of soil organism groups in soil trophic webs. AM are a major channel for carbon under ambient conditions. With added atmospheric carbon dioxide, more carbon is channeled into the AM fungi. With increasing nitrogen, more goes to saprophytic and facultative parasitic fungi, and bacteria. This also increases soil grazers respiring more carbon dioxide back into the atmosphere.

CONCLUSION

AM fungi are crucial components of soil environments. These organisms regulate much, if not the majority, of nutrient and carbon flow pathways. We basically understand how AM increase nutrient uptake from soil to host plant. The mechanisms of carbon transfer from plant to fungus are being studied in detail. However, there are many species of plants and fungi that interact with each other and with the other soil biota in complex and interconnected ways. These include soil aggregation, nutrient weathering, altered competitive outcomes, and altered food web dynamics. Together, the complexity of these interactions deserves considerably more research to understand how AM affect plant production in agriculture and regulate the structure and functioning of wildland ecosystems.

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MYCORRHIZAE: ECTOMYCORRHIZAL FUNGI

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"Mycorrhiza" literally translates as "fungus root" and represents the symbiotic association between plant roots and specialized soil fungi (mycorrhizal fungi). The fungi act as extensions of root systems and benefit host nutrition by their ability to extract nutrients, especially phosphorus and nitrogen, and water from a volume of soil hundreds to thousands of times greater than the roots alone can explore. Other general host benefits include protection of fine roots from pathogens, increased resistance to drought and heavy metal toxicity, and increased root development and longevity. In return, mycorrhizal fungi depend on the host for carbon, primarily in the form of simple sugars, and some vitamins. Mycorrhizal symbioses have strongly coevolved over the millennia such that each partner depends on the other for survival and fitness in natural ecosystems. There are four major classes of mycorrhizae: ectomycorrhiza, ericoid mycorrhiza, arbuscular mycorrhiza, and orchid mycorrhiza. In this Encyclopedia entry, we focus on ectomycorrhizae (EM) and briefly discuss four related subtypes, ectendomycorrhizae, arbutoid, pyroloid, and monotropoid mycorrhizae. We provide general reviews of hosts and fungal associations, structure and function, ecology, ecosystem function, physiology, and practical applications in forestry. Readers are referred to books by Allen (1,2), Harley and Smith (3), Safir (4), Smith and Read (5), and Varma and Hock (6) for greater detail on mycorrhizae. A two-volume, 42-chapter treatise edited by Norris and coworkers (7) and a book by Brundrett and coworkers (8) provide detailed methodological approaches in mycorrhiza research and applications in agriculture and forestry.

HOST–FUNGUS ASSOCIATIONS

Many tree species in temperate forests form EM, including all members of the Betulaceae, Fagaceae, Myrtaceae, Pinaceae, Salicaceae, and Tiliaceae. In tropical regions, EM hosts occur in the Caesalpiniaceae and Dipterocarpaceae. Additional genera in other families also form EM, for example, *Arbutus, Arctostaphylos, Albcausuarina*, and *Cercocarpus*. Some EM hosts form both arbuscular mycorrhizae and EM, for example, *Alnus, Eucalyptus, Populus, Salix*, and some Pinaceae species (9), but in these situations, EM is typically the dominant type, particularly on mature trees.

Ectomycorrhizal fungi number in the thousands and are primarily Ascomycotina and Basidiomycotina. Well-known genera include *Amanita, Astraeus, Boletus, Cantharellus, Cortinarius, Elaphomyces, Gomphidius, Hebeloma, Hymenogaster, Hysterangium, Inocybe, Laccaria, Lactarius, Leccinum, Paxillus, Piloderma, Pisolithus, Rhizopogon, Russula, Scleroderma, Suillus, Thelephora, Tricholoma*, and *Tuber*. Cairney and Chambers (10) provided an excellent review of the taxonomy, ecology, and physiology of several of these genera. A few species of *Endogone* in the Zygomycotina also form EM. Molina and coworkers (9) listed the major genera, number of species, and host ranges of EM fungi.

STRUCTURE AND FUNCTION

EM form primarily on the short, fine roots rather than on long, structural, lateral roots. EM are characterized by an often colorful sheath or mantle of fungus mycelium that envelops the root. The mantle varies in structure and can range from a thin, loosely interwoven structure to an organized pseudoparenchymatous or parenchymatous tissue, typically 10- to 100-μm thick. The fungus penetrates the root epidermis and cortex, growing intercellularly to form the Hartig network, a differentiated tissue composed of interwoven and anastomosed hyphae (Fig. 1). Nutrient and metabolite exchange occurs within this extensive zone of root cell–fungus mycelial contact. In angiosperms, the Hartig net surrounds only the epidermal cells. Ectomycorrhizal

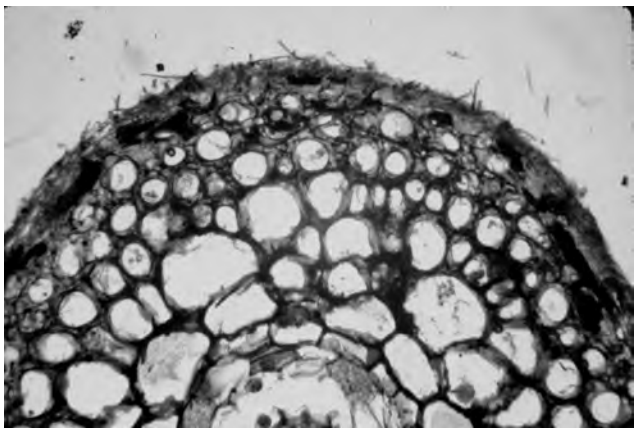

Figure 1. Crosssection of a Douglas-fir ectomycorrhiza showing the fungal colonization on the root surface (mantle) and growth between cortical cells (Hartig net). See color insert.

fungi do not penetrate past the endodermis into vascular tissue.

EM vary tremendously in size and branching patterns, characteristics determined by the host–fungus combination (11). EM begin as simple, unbranched structures, but most elongate and branch in various ways (Fig. 2). For example, *Pinus* EM branch dichotomously, sometimes repeatedly to form compound structures. Other host genera develop pinnately branched structures, and still others develop irregular, variously branched patterns. Ectomycorrhizal fungi produce several classes of plant growth promoting hormones (auxins, cytokinins, and gibberellins) that enhance root elongation and longevity. Such enhancement effectively increases root surface area for nutrient and water absorption.

Ectomycorrhizal fungi ramify extensively through the surrounding soil, forming fungal colonies of various size and structure (12). Many EM fungi develop complex mycelial strands or rhizomorphs that function in nutrient and water movement and storage. Mycelial growth is most prolific in the humus layer and in the humus–mineral soil interface; however, some fungus species specialize in specific substrate colonization, such as buried wood, deep humus, or bare, mineral soil. Sexual reproduction occurs within sporocarps produced by soil mycelium, and many EM fungi produce prolific fruitings of mushrooms or truffles (hypogeous sporocarps).

Figure 2. Douglas-fir EM showing pinnate branching pattern. See color insert.

Most EM hosts and fungi are obligate symbionts, depending on one another for survival and reproduction in natural ecosystems. For example, attempts to establish exotic plantations of EM trees often fail unless compatible EM fungi are present. Many EM hosts depend on the fungus for phosphorus uptake. Other host benefits from EM include nitrogen and potassium uptake, water uptake and enhanced drought tolerance, promotion of rooting and root longevity, detoxification of soil and heavy metal tolerance, and protection against root pathogens.

Many, but not all, EM fungi can be isolated and grown in pure culture. Thus, a wealth of information is available regarding their nutritional requirements and response to environmental conditions. Ectomycorrhizal fungi are most commonly isolated from sporocarp tissue. In this procedure, young sporocarps are brushed free of dirt, carefully broken to expose interior tissue, and then a small amount of tissue is aseptically removed and placed on artificial media in test tubes or culture plates. Various media are used, the most common being the modified Melin-Norkrans medium (13), a fully synthetic medium with glucose as the carbon source. Cultures also can be obtained from surface sterilized EM root tips, but the success rate is usually low. *Cenococcum geophilum*, one of the more common of EM fungi, is not known to produce sporocarps but can be easily isolated from its large, black sclerotia. Sclerotia are retrieved from the soil by wet-sieving and decanting, then are surface sterilized, and placed aseptically on nutrient agar in tubes or plates (14).

Ectomycorrhizal fungi grow slowly in culture compared to saprophytic or pathogenic fungi, and may take 4 or more weeks to cover a 10-cm diameter culture plate. Some EM fungi grow even more slowly and are difficult to maintain in culture. Still others have never been isolated, thus indicating strong differences in the obligate need for host association. Most EM fungi, however, have limited ability to degrade complex organic compounds and thus depend upon the host for their energy (carbon) (15), as well as for other metabolites, such as thiamin.

Ectendomycorrhizae

Ectendomycorrhizae are a subtype of EM and share in common the colonization of short fine roots, mantle, and Hartig net development, and an association with typically EM hosts. The mantle is typically sparse and thin, however, and the Hartig net may only irregularly penetrate between cortical cells. The primary difference from ectomycorrhizal fungi is their intracellular hyphal penetration into the cortical cells, at times completely filling the lumen with hyphal coils, although the plasma membrane is not breached.

Only a few fungal species are known to form ectendomycorrhizae and most are ascomycetes. Known genera include *Wilcoxina* (commonly called *E-strain fungi*), *Phialophora, Chloridium*, and *Sphaerosporella* (9). Known host genera include *Pinus, Larix, Picea, Abies, Pseudotsuga, Tsuga, Betula, Shorea*, and *Lithocarpus*.

Though less is known about the ecology of this group of mycorrhizal symbionts than EM fungi, they have been demonstrated to provide similar nutritional benefits as EM. Ectendomycorrhizae are most commonly reported on seedlings, particularly in bareroot nurseries in which they may be the dominant mycorrhizal type. They also inhabit forest soils but appear to be a minor component compared to EM.

Arbutoid and Pyroloid Mycorrhizae

Arbutoid mycorrhizae are those typical of two genera of Ericaceae, *Arbutus* and *Arctostaphylos*. These mycorrhizae resemble ectendomycorrhizae in that hyphae colonize both intercellularly and intracellularly. The Hartig net and intracellular coils, however, are restricted to the root epidermis as opposed to ectendomycorrhizae, in which hyphal colonization may extend to the endodermis. Most fungi that form arbutoid mycorrhizae on *Arbutus* and *Arctostaphylos* also form EM on other hosts (16–18).

Mycorrhizae on *Pyrola* (pyroloid) resemble arbutoid mycorrhizae anatomically, having an epidermal Hartig net, a disorganized fungal sheath, and intracellular penetration restricted to the epidermis (3,19). Both ascomycetes and basidiomycetes appear to associate with *Pyrola* (19).

Monotropoid Mycorrhizae

Monotropoid mycorrhizae form on achlorophyllous members of the Ericaceae (the Monotropoideae). They resemble arbutoid mycorrhiza, with a Hartig net restricted to the epidermis and a thick fungal sheath. In addition, fungal pegs develop in the epidermal cell walls, at least in *Monotropa, Sarcodes*, and *Pterospora* (19–21). As in the case of arbutoid and ectendomycorrhizae, the monotropoid type seems to be a host-mediated variant of EM.

Achlorophyllous monotropes are often mistakenly called *saprotrophs*; however, they are strongly mycoheterotrophic (22). They develop mycorrhizae with EM fungi that are connected to and functioning with neighboring forest trees. Transfer of labeled carbon has been demonstrated from tree photosynthate to the monotrope via the interconnecting EM fungus (23).

ECOLOGY OF ECTOMYCORRHIZAE

Generalizing about the ecology of EM is difficult because of the physiological diversity of fungi, the wide range of host–fungus compatibilities and specificities, and the various ecological conditions EM experience. Ectomycorrhizal fungi show strong differences between and within species in their tolerance of environmental extremes and production of degrading enzymes. Some fungi are also common to specialized soil niches or successional stages of community development, whereas others are less specific, occurring in a wide variety of habitats and developmental stages. Many EM fungi are broad-host-ranging and can form EM with taxonomically diverse hosts. Others associate with only angiosperms or gymnosperms. Still others will form EM only with a specific host genus as seen in repeated fruiting of sporocarps with particular hosts (9). EM hosts typically associate with many EM fungus species at any one time, and the composition of fungus associates changes as the plant matures and plant and fungal community develops. We poorly understand the interactions of EM fungi and hosts in natural soils and the functional ecologies of these diverse fungi in dynamic forest ecosystems. Given the coevolved nature of these symbionts, however, the dynamics of forest and fungal communities are likely strongly interrelated through the mycorrhizal links. Considerable research is needed to document the nature of these mycorrhizal links between plants and how they function in determining forest ecosystem resiliency after disturbance and maintaining long-term ecosystem productivity. In the next section, we summarize various aspects of ecology and ecosystem function.

Autecological Considerations

Ectomycorrhizal fungi respond to various biotic and abiotic factors, including vegetation, climate, topography, disturbance, and soil parent material. Responses are measured by observing changes in mycelial growth and EM development in the soil, and in the production of sporocarps, such as mushrooms and truffles (1,2,5). Disturbances such as fire affect fungal species through alterations to the plant community, as well as to the soil environment. The vegetative state of EM fungi exists as a mycelium exploring the soil substrate and via extensive colonization of root tips. As such, conditions in the soil environment likely impact fungi more than conditions above the ground. Climatic factors, such as temperature and precipitation, however, influence the soil environment and therefore influence fungal growth and reproduction. Generally, it is believed that EM fungi respond best to moderate temperatures and relatively high, but not saturated, soil moisture levels. Nevertheless, EM fungi also occur and function in arid forest ecosystems worldwide. Topographic factors, such as elevation, slope, and aspect are thought to influence the occurrence of fungi by moderating climatic factors. High levels of soil organic matter, including humus and coarse woody debris, positively influence the abundance of EM roots for many fungal species (24).

Community Dynamics and Succession

Changes in plant species composition during primary or secondary succession affect EM species composition owing, in part, to the host specificity phenomenon mentioned previously (9). Ectomycorrhizal plants and fungi influence each other in community development, and the dynamic nature of this influence involves feedback between the aboveground and belowground components of the system (25). Indeed, interspecies and intraspecies plant connections via fungal links have been demonstrated in the laboratory and in the field, and these links support nutrient transfer between plant individuals (5,26). Evidence of fungal succession often is observed in the occurrence of sporocarps as trees age under old field successional conditions (27), leading to interesting

hypotheses about where fungal species fall on the ruderal—competitor continuum of Grime (28). As belowground data are gathered, our understanding of fungal community dynamics and succession is necessarily being refined.

Biodiversity of Ectomycorrhizal Fungi

Biological diversity commonly means the number of species (species richness) in a particular location or habitat. Methods of measuring EM fungal species richness differ and provide a different understanding of fungal community dynamics. The biodiversity of fungi affects and is affected by the plant community structure (29,30). Bruns (31) hypothesized that EM fungal diversity is regulated predominantly by resource partitioning, response to disturbance, and interaction with other organisms.

Sporocarp and EM root tip sampling methods each provide knowledge of the vast diversity of EM fungal species. Sporocarp studies typically show a high diversity in a small area and are critical to our understanding of food web dynamics. In recent studies in old growth stands of Douglas-fir (*Pseudotsuga menziesii*) and western hemlock (*Tsuga heterophylla*) in the Pacific Northwestern United States, fungal species richness of epigeous EM sporocarps ranged from 19 to 70 (32, Smith, Molina, and Castellano, unpublished data). Sporocarps in these studies were collected over multiple years within stands 30 to 50 ha in size. Fungal species richness of truffle-like sporocarps ranged from 8 to 14 species per stand (Smith, Molina, and Castellano, unpublished data). Sporocarp studies, however, fail to estimate total EM fungal species richness. Direct sampling of EM fungus root symbionts, with molecular methods, is necessary to detect the many species of fungi that go undetected in sporocarp sampling because some fruit in hidden places, such as on the underside of wood, fruit infrequently, or may not fruit at all. Although insight into the biological diversity and community dynamics of EM fungi has been gained from both sporocarp and EM root tip sampling methods, we are a long way from understanding the functional roles of the many species.

Like many other biological systems, EM fungus communities are dominated by one or a few EM fungus species, with most species occurring with low frequency and abundance in the community. Interestingly, dominance of fungi, as revealed by sporocarp production, is not congruent with the dominant species indicated by EM root tips (5). Thus, species that are most important to the production of sporocarps for small mammals may be of little importance to the tree for nutrient uptake. Although we have progressed from our "black-box" approach in terms of how EM fungi are distributed and function in ecosystems, it remains risky to consider different species as redundant members of the same guild. It is increasingly clear that species matter, and that fungal diversity is a result of each species playing important roles in ecosystem function and resiliency.

ECOSYSTEM FUNCTIONS

Nutrient Cycling

It is well documented that EM fungi mobilize nutrients from the soil and transfer them to host plants (5). Initially, phosphorus was identified as the main nutrient that fungi acquired for plants, but increasing evidence reveals that fungi also mobilize nitrogen, calcium, potassium, and other nutrients. Fungi are also directly involved in carbon cycling, having access to approximately 15% of carbon assimilated by a tree (5). Ectomycorrhizal fungi are thus directly involved in cycling and retention of most nutrients in ecosystems. The physiology of these pathways is covered later in this article.

Food Webs

Ectomycorrhizal fungi are critical food members of soil webs in forest ecosystems and provide food for various microbes and animals. Fungal mycelium and exuded carbohydrates support diverse soil organisms, such as fungal-feeding arthropods, bacteria, and microbial-feeding nematodes (30). About 80% of soil microarthropods are fungivores; these small animals are the primary shredders and mobilizers of organic nutrients in forest soils (33). Sporocarps produced by EM fungi are important food for various wildlife, including small and large mammals, and mollusks (34,35). Some rodents, such as the California red-backed vole (*Clethrionomys californicus*) and northern flying squirrel (*Glaucomys sabrinus*), rely on EM fungal sporocarps, especially the truffle fungi, for as much as 90% of their food supply (36,37). Because small mammals are primary prey for many birds (e.g., owls, hawks, etc.) and larger mammals, the availability and consumption of truffle EM fungal sporocarps is a key link in forest ecosystem food webs.

Soil Structure

Ectomycorrhizal fungi contribute to soil structure through various mechanisms (5). Some carbohydrates from plants leak into the mycorrhizosphere, which increases populations of soil microorganisms. Soil particles adhere to mucilage on the surface of hyphae, thereby increasing the soil structure. Fungal hyphae physically bind soil particles, as do the roots of the plants. Siderophores and other chelators produced by fungi also cause soil particles to aggregate, which contributes to soil porosity and facilitates air and water movement. Because the roots of most plants and soil microbes are strongly aerobic, this is a vital function that EM fungi perform.

Ecosystem Resiliency

Ectomycorrhizal fungi perform critical processes at the ecosystem level that influence plant productivity and soil fertility (5), and therefore are essential to ecosystem resiliency (25,38). It is now clear from plant communities involving Douglas-fir in the Pacific Northwest that plant succession is directly influenced by EM fungi (26,39). This interaction between plant species via their EM fungi was

predicted by studies of Douglas-fir and members of the Ericaceae that form arbutoid EM (*Arctostaphylos* and *Arbutus*) that showed much overlap in their EM fungus associates (16). These plant communities undergoing secondary succession were found to be still developing decades after a disturbance event. Studies of fungal communities continue to provide data for how EM fungi influence the development and recovery of the plant communities. A critical phase in ecosystem resiliency immediately follows a disturbance event, such as that caused by a stand replacing fire. Here, recovery was driven less by interplant links via EM fungus hyphae discussed earlier than by the survival of resistant fungal inoculum, likely in the form of spores or sclerotia, which supported the growth of regenerating seedlings (40,41). Regeneration of Douglas-fir stands has been shown to fail after clear-cut harvest and if establishment of early successional shrub species that harbor suitable EM fungus inoculum is prevented. The soil microbial community in clear-cut sites is thought to have crossed a critical threshold such that the site can no longer support an EM community (25). Douglas-fir seedlings planted into such a site will fail without EM on the roots; however, the survival of the seedlings is increased if soil taken from beneath EM hosts is added to the planting holes. It is thought that fungal propagules in the introduced soil provide the EM fungus inoculum needed by the seedlings.

Additional evidence for the contribution of EM fungal propagules to ecosystem recovery comes from a plant community following a stand replacing fire. Here, *Pinus* seedlings established in the first year after the fire were largely colonized by fungi in the genera *Rhizopogon* and *Tuber* (41). A previous study by Taylor and Bruns (40) had shown that *Pinus* seedlings grown in soils collected before the fire were colonized by a different group of fungi than those observed after the fire, and included species of *Russula, Tomentella*, and *Amanita*. Species of *Rhizopogon* and *Tuber*, which were present in the prefire stand, but in low relative abundance, likely produced a resistant spore bank that responded to disturbance in much the same way seed banks do for plants.

PHYSIOLOGY

Early physiological studies have been extensively reviewed by Marks and Kozlowski (42), with chapters on mineral nutrition (43), carbohydrate physiology (44), and hormonal relations (45). This fundamental reference has been followed by other books with sections updating advances in EM physiology (2,3,5,6,10). Specific reviews also have been published on carbon and nitrogen metabolism (46–48) and physiological ecology (38,49–51). Cairney and Chambers (10) have recently summarized major taxonomic, physiological, and ecological characteristics of 15 major EM fungal taxa.

Because EM fungi represent such a diverse taxonomic and genetic base, there are many apparent contradictions reported in their physiology, which reflects the extreme functional diversity of the group.

Nutrition

Carbon. As heterotrophic organisms, EM fungi derive energy and carbon for growth from organic sources. In vitro growth studies of many EM fungi have demonstrated a wide diversity in the ability to use various simple sugars and polysaccharides (10,44,52,53). The most commonly used sugars are glucose, fructose, and mannose, and the most commonly reported polysaccharides are starch and pectin, although many EM fungi also can use cellulose. Growth responses of ectomycorrhizal fungi can be complicated when small "starter" amounts of glucose are available and allow substrate adaptation, significantly expanding the range and amount of carbohydrates used (44,54–56). Although sucrose is the main carbohydrate transferred from the host plant, EM fungi may not be able to use sucrose directly (44,52), but rely on host enzymes (57) for hydrolysis to glucose and fructose.

Evidence for use of phenolic compounds as a carbon source for respiration and growth has been limited. Although EM fungi can significantly modify various phenols (58–60), this reaction is thought to be a detoxification response. Production of $^{14}CO_2$ from ^{14}C-lignin (61,62) and xenobiotics (63,64) indicates that certain EM fungi can actually metabolize phenolic compounds. There is also indirect evidence that, based on decoloration of polymeric dyes, certain EM fungi can break down lignin (65,66); these results, however, have recently been questioned as possible artifacts (67).

Hydrolysis of fatty acid esters by various EM fungi (68) suggests that energy-rich fatty acids could serve as an additional source of carbon for EM hyphae and rhizomorphs distal to the host tree.

Nitrogen. Nitrogen is essential for the synthesis of proteins, including enzymes, nucleotides, and many minor cellular components. Of the inorganic forms, ammonium salts have been most frequently used, although nitrate is also used, but with slower uptake and growth rates (69,70). Organic nitrogen sources include amino acids (43,53,71,72), peptides and proteins (43,53,70,72,73), glucosamine (74), nucleic acids (74), and urea (53,72). Certain EM fungi use the nitrogen in proteins complexed with tannins (65,75), a trait that may become increasingly important when polyphenol complexation is thought to limit nitrogen availability (38). Both chitin (52,76) and humic materials (74) appear to be poor nitrogen sources for pure cultures.

Phosphorus. Phosphorus, like nitrogen, is also a major nutrient required for cellular components, mainly nucleic acids, membrane phospholipids, and energy-transferring nucleotide phosphates. Although inorganic phosphate is the most commonly used form in culture media, organic phosphorus forms, such as soluble and insoluble phytates (43,77) and nucleic acids (43,65,78) can also be used. Most of the work on organic phosphorus has involved measuring phosphatase enzymes (see section on Products, discussed later).

Other Compounds. Unlike nitrogen and phosphorus, sulfur metabolism of EM fungi remains largely unexplored, although certain EM fungi have been found capable of oxidizing elemental sulfur (79).

Growth Limits

In addition to the chemical form of various nutrients, growth rates and yields of EM fungi also can be affected by nutrient concentrations (80,81), pH (82), temperature (83–85), and moisture (86,87). Examples of environmentally significant compounds that can affect the growth of EM fungi include natural polyphenols (88) and applied fungicides (89) and herbicides (90). The effects of "heavy" or toxic metals on EM fungal growth, EM formation, and plant uptake have recently been summarized (91).

Products

Although the in vitro traits described earlier provide insight into the nutritional requirements and physiological potentials of EM fungi, the extent to which EM fungi interact with their soil environment is of critical importance to the symbiosis. Ectomycorrhizal fungi affect their nutritional environment by releasing extracellular enzymes to utilize detrital nutrient pools and mineral weathering agents to obtain mineral nutrients. Ectomycorrhizal fungi also can affect their biotic environment by production of substances that either inhibit or stimulate the activities of competing or associative microorganisms.

Interactions with Organic and Mineral Resources

Production of polysaccharide- and glycoside-hydrolyzing enzymes and polyphenoloxidases with the potential to break down detrital plant cell wall barriers has been measured in pure cultures of EM fungi (10,51), EM-containing microcosms (92,93), and field samples in which EM are prevalent (65). Production of proteolytic enzymes to obtain nitrogen from peptide and protein sources also has been reported for cultures (94,95), microcosms (92,93), and field samples (65). Phosphatase enzymes, which release inorganic phosphorus from organic sources, are the most extensively studied group of EM enzymes. Well over 40 publications have reported enzyme production in culture (95–99), tree syntheses (100–102), microcosm studies (92), and field samples (65,77,103–105).

Ectomycorrhizal fungi are involved in weathering minerals and solubilizing mineral phosphates (106–108) by production of organic acids (109) and iron-chelating siderophores (110).

Interactions with Other Organisms

Ectomycorrhizal fungi secrete various plant-growth regulating compounds to control host plant root development and morphology, including auxins, cytokinins, gibberelins, and indole acetic acid (45,111–113). Vitamin production by various EM fungi may stimulate associated decomposer microorganisms in the mycorrhizosphere (45,114). Alternatively, EM fungal cultures also have been shown to inhibit bacteria, other fungi, and grazing fungivores (115–118).

PRACTICAL APPLICATIONS IN FORESTRY

Nursery Inoculation

In the late 19th century, the failure to establish introduced pines in the Southern Hemisphere stimulated research into inoculation of seedlings with EM fungi (119). The premise was that forest seedlings destined for field sites lacking apparent adequate EM fungi would benefit from introduction of appropriate EM fungal partners in the nursery. Early inoculations predominately introduced forest soil containing EM fungus propagules (120,121). Nursery inoculations with pure cultures of EM fungi did not begin until the 1950s, specifically to address the difficulties in reforestation of steppe lands and mine spoils. Subsequently, large-scale nursery inoculations in the developed world began to exclusively use single-species fungal inoculum from pure cultures or spore-bearing tissues (121). More than 70 different EM fungal species have been tried as inoculum, either as pure culture or spore inoculum (122,123). Earlier, it became apparent that specific fungal species are considerably more effective than other fungal species for particular plant species (124). Individual EM fungal species and even ecotypes (strains) can differ strongly in physiological characteristics and response to environmental conditions that affect inoculum performance. Matching EM fungal ecotypes, host species, and abiotic conditions is important to a successful inoculation program.

Currently, several commercial companies in North America produce pure mycelial cultures or spore inocula (125). Each inoculum type has benefits (both economically and performance-wise) and restrictions as to its use. For some fungal species, only spore inoculation works successfully; for other species, only inoculation with pure cultures of vegetative mycelium works (125). Benefits of EM inoculation programs in commercial forest nurseries include reduction in percentage of culled seedlings, increases in stem caliper or leader growth, protection against seedling root pathogens, rapid EM colonization to reduce stunting, and improved performance of seedlings upon field planting (122,123,125). Additional work is needed in multispecies inoculation and expanding the cadre of EM species that work effectively in nursery inoculation programs, particularly on a regional basis.

Field Performance of Inoculated Seedlings

Enhancing field performance of planted seedlings has been the overriding catalyst for EM inoculation programs. Over the last 30 years, more than a 100 papers have been published on the field performance of EM-inoculated seedlings (123). Experiments include nearly 600 unique combinations among 66 host plants, 70 EM fungal species, and 5 different types of inoculum. Of the many field trials, only one-half showed improved seedling performance after inoculation. Successful trials occurred on difficult sites to reforest such as mine spoils and hot, dry sites. Worldwide, *Pisolithus tinctorius* is the most extensively studied EM fungus, and *Pinus* spp. have received the most attention for host plants (123). In western North America, *Rhizopogon* species have received considerable

attention. Likewise, in Europe, *Rhizopogon* species and other species, such as *Laccaria bicolor*, have received significant attention. Additional work is needed to discover under what circumstances any particular fungus helps seedling field performance. Finding one fungus that will benefit numerous hosts in diverse ecological settings is not a reasonable expectation. Even where the specific attributes of a fungus seem desirable, that is, drought resistance, an ecotype or ecostrain of the target fungus may perform better under local conditions than a superstrain.

CONCLUSION

As noted throughout this encyclopedia entry, mycorrhizal fungi contribute to healthy ecosystems by forming mutualistic, symbiotic associations with plants, contributing to nutrient cycling, providing food for animals, and creating habitat diversity for many forest organisms. While the basic structure and functions of EM fungi are delineated, species-level functions of EM fungi, habitat requirements, and interactions with particular plant species represent large knowledge gaps needing research attention. Knowledge of the ecological requirements of EM fungi, particularly their relation to forest community succession and disturbance events, is essential for making science-based decisions about issues in fungal conservation worldwide.

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MYCOTOXINS. See AIRBORNE TOXIGENIC MOLDS

N

NATURAL ORGANIC MATTER (NOM) IN AQUATIC ENVIRONMENTS.

See ACTIVITY AND CARBON TRANSFORMATIONS IN BIOFILMS

NEURAL NETWORKS.

See DATA ANALYSIS AND MODELING

NEUSTON MICROBIOLOGY: LIFE AT THE AIR–WATER INTERFACE

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One of the first things that can be noted when examining the air–water interface as a habitat for microorganisms is its size, water covers 71% of the Earth's surface. Oceans, lakes, rivers, ponds, and even puddles make a contribution to the habitat, although briefly in some of the latter cases. However, widespread as the habitat may appear, it is limited in its thickness to just a few micrometers for microorganisms. An interface has been defined in physicochemical terms as the boundary between two phases in a heterogeneous system (1). As such, the physicochemical properties of the air–water interface are different from those found in either of the phases that meet there: the atmosphere and the water column. It is these properties that make this a unique habitat for neuston microorganisms.

Although it is unique, it has been described as an exposed habitat (2), subject to a variety of forms of intense environmental stress, including solar radiation and toxic substances (3–5), and has been considered an extreme environment (6). Ecologically, habitats have been considered extreme when they are at the ends of environmental gradients such as temperature, pH, salinity, nutrients, or pressure (7), or when they had a low diversity of organisms (8). However, with the advent of noncultural, molecular techniques, these extreme environments have been demonstrated to be more diverse than originally believed (9). The bottom line is that it is hard to come up with a good definition of extreme environment (10).

Shilo (11) presented an alternative to the aforementioned considerations suggesting that habitats that are subjected to regular and sporadic fluctuations in environmental conditions, many of which could be extreme in themselves, merit consideration as extreme environments. Furthermore, he suggested that organisms surviving in these habitats would require great plasticity to accommodate the changes that occur. This idea, combined with the presence of a variety of forms of environmental stress, led to the consideration of the air–water interface as an extreme environment for microorganisms (6). Because of the potentially hostile nature of this habitat, the characteristics that would aid microorganisms, particularly bacteria, to be successful there will be considered.

THE NEUSTON

The term *neuston* (from the Greek $\nu\varepsilon\omega$, to swim) was introduced by Naumann (12) to indicate that the microorganisms associated with the air–water interface. This was in contrast to the word *pleuston* (from the Greek $\pi\lambda\varepsilon\omega$, sail, float, swim in a semisubmerged condition) that was introduced by Schröter and Kirchner (13) to encompass the whole assemblage of floating plants that are both submerged and interfacial. Modifications to the definition of neuston were made by Geitler (14) who separated them into the epineuston, to distinguish organisms on the upper side of the air–water interface, and the hyponeuston, to distinguish organisms that are on the underside. More recently, the term *neuston* has expanded to include a wide range of organisms associated with the air–water interface, particularly in marine environments (15,16) including organisms large enough to be collected by nets. Size ranges of the expanded concept include the following: piconeuston (<2 μm) = bacterioneuston, nano- and microneuston (2 to 200 μm) = phytoneuston and protozoan zooneuston, mesozooneuston (0.2 to 20 mm) = copepods, isopods, amphipods, fish larval stages; macroneuston (>2 cm) = coelenterates and floating organisms, also referred to as the *pleuston* (16). For the purposes and limitations of this discussion, the terms *bacterioneuston*, *phytoneuston*, and *zooneuston* (for protozoa) will be used. Larger organisms will not be directly addressed.

SAMPLING

The information gathered on the microbiology and chemistry of the air–water interface has been largely dependent on the type of sampler employed for collection. In general, the aim of sampling this habitat is to collect the microorganisms and molecules associated with the interface and none or the least possible quantity of the underlying bulk-phase water (17). This has led to a fairly large number of samplers or sampling techniques being designed for collection (Table 1). The thickness of the water sample that is collected at the air–water interface depends on the collecting technique employed and the type of material that the sampler is constructed from. Many of the sampling devices function through adsorption, and samplers composed of different materials will adsorb different air–water interface components (18). Thus, samplers can have both qualitative and quantitative

Table 1. Sampling Methods for the Air-Water Interface

Sampling Method	Thickness of Sample	Reference
Paper filter	8–100 μm	Amies (22)
Calcium alginate gauze		Amies (22)
Screen sampler	300–400 μm	Garrett (23)
Rotating drum	34–100 μm	Harvey (24)
Bubble microtome	0.5–10 μm	MacIntyre (25)
Germanium prisms	30 nm	Baier (26)
Glass plate	20–100 μm	Harvey & Burzell (27)
Teflon plate	50–100 μm	Larsson and coworkers (28)
Teflon disk		Miget and coworkers (29)
Membrane filters	8–100 μm	Crow and coworkers (30)
Electron microscope grids		Young (31)
Freezing probe	1,000 μm	Hamilton and Clifton (32)
Polyvinylchloride film	1 μm	Hamilton and Clifton (32)

differences in their ability to collect material at the air–water interface, and direct comparisons between samplers are at best difficult and at worst should not be contemplated (19). To compare the sample collected at the air–water interface to the corresponding bulk-phase sample, the term enrichment has been used. Determining enrichment is one way that the samples collected at the air–water interface have been compared to the bulk phase. Enrichment (E) is calculated by the ratio between a component (X) at the air–water interface and in the bulk phase: $E = X_{\text{interface}}/X_{\text{bulk}}$. When E is greater than one, the interface has a positive enrichment, and when E is equal to one there is no accumulation. However, E less than one indicates that the interface is depleted in the component compared with the bulk phase. E will depend on the type of sampler used and the thickness of the layer that it collects. Further discussions of sampling the air–water interface, which cover this subject wholly or in part, are presented by others (6,16,18,20,21).

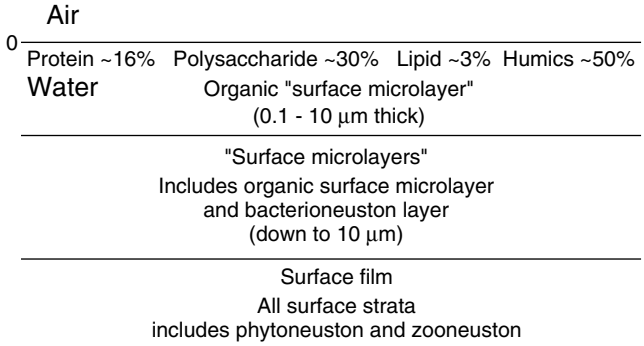

Figure 1. Model of the structure of the water surface strata at the air–water interface showing an "organic surface microlayer" of a gel-like matrix composed of proteins, polysaccharides, lipids, and humic substances. A "surface microlayer" consisting of the organic surface microlayer and the bacterioneuston layer can be up to 10 μm thick. Surface film refers to these layers and all additional surface strata. Adapted from J. McN. Sieburth, in P. S. Liss and W. G. N. Slinn, eds., *Air-Sea Exchange of Gases and Particles*, D. Reidel Publishing, Hingham, Mass., 1983, pp. 121–172.

THE NATURE OF THE HABITAT: MODEL OF THE STRUCTURE

As a habitat, the structure of the air–water interface has been modeled on the basis of the chemical components and the biological organisms that have been collected and characterized from it. As pointed out earlier, sampling and samplers have a bias to what and how much interface material is collected. Thus, the actual thickness of the various layers is only estimated, always doubtfully and should be treated with caution. One model of the air–water interface structure was proposed by Sieburth (33; Fig.1). Several terms are important in discussing the structure: the organic layer has been termed the *organic surface microlayer* (3,5) or alternatively, the *surface nanolayer* (16) and may be on the order of micrometers thick in its hydrated form; an additional thickness, down to approximately 10 μm, is the bacterioneuston layer (33); layers of additional thickness, including the phytoneuston and protozoan zooneuston, have been termed the *surface film* (3,5) or alternatively, the *surface microlayer* (16). The devices and techniques used to sample the air–water interface (Table 1) collect a much thicker layer than the organic surface microlayer or the bacterioneuston layer. Thus, air–water interface samples generally are diluted by underlying bulk-phase water.

The organic surface microlayer was initially considered to be largely composed of lipid material (3). However, subsequent investigations revealed that lipids were not present in concentrations high enough to form a distinct layer (33–35). The model proposed by Sieburth (33; Fig. 1) indicates that the organic surface microlayer consists of a matrix of lipids, proteins, polysaccharides, and humic substances. The source of this material at the air–water interface in open areas of large bodies of water is considered to be largely autochthonous, mainly from planktonic sources. In contrast, in coastal areas and smaller bodies of water, there is a considerable terrestrial, or allochthonous, contribution. The smaller the body of water, the greater may be the terrestrial input. The concentrations of these components at the air–water interface vary both spatially and temporally from one water body to the other and also within each water body (35–37). The organic surface film material

may be compressed by wind action or internal waves into surface slicks that are visible (areas that show damping of capillary waves) to an observer (38–40). These occur frequently in coastal waters and lakes. However, whether a visible slick is present or not, there is an organic surface microlayer and a surface film on all waters (33). A wind velocity less than 5 to 7 m ms^{-1} but greater than 2 to 3 m ms^{-1} can create surface slicks (41,42). As the wind speed increases, the slicks will continue to be compressed to a point at which they will collapse and form fibers and particulate material that can be carried down into the bulk phase (43,44). Along with wind, Langmuir circulation will also cause a concentration of surface film material into bands (45). Wind and wave action will be more important to surface slicks and chemistry in larger bodies of water (46).

The air–water interface and adjacent surface films are dynamic, continually accumulating, and losing material to and from both the atmosphere and the bulk-water phase through a number of mechanisms (Fig. 2). Inputs to the surface film from the atmosphere include precipitation and dry deposition, whereas wind-generated aerosol, bursting bubbles, and evaporation are outputs from the surface film back to the atmosphere. The bulk-water phase contributes to the surface film through upwelling, convection, and bubbles, whereas wave action, dissolution, and sedimentation can transfer surface film material back to the bulk phase. Both Brownian and eddy diffusion can work to input or output material to or from the surface film. The main mechanisms for transformation of material within the surface microlayer are photodegradation and biological action.

Of the neuston microorganisms, the bacteria, once they arrive at the air–water interface, are most unlikely to be able to escape under their own power. This would suggest that they must adapt to the extreme conditions or perish, unless they are transported away by physical means into the bulk phase or the atmosphere (Fig. 2). As a result, most of the following discussion will concentrate on the bacterioneuston. Although some phytoneuston may also be found immobilized, motile forms of phytoneuston and protozoa may play a more transient role in surface films and be able to choose the times when they are members of the neuston.

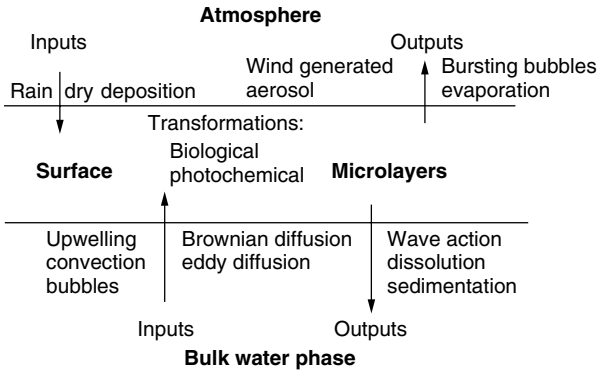

Figure 2. Factors involved in the input and output and transformation of dissolved and particulate material in surface films. Adapted from K. A. Hunter, in P. S. Liss and R. A. Duce, eds., *The Sea Surface and Global Change*, Cambridge University Press, Cambridge, U.K., 1997, pp. 287–319.

BACTERIONEUSTON (PICONEUSTON)

The thickness of the bacterioneuston layer will depend on how the bacteria are distributed at the surface (33). The bacteria at the air–water interface can be epineustonic or hyponeustonic, can be spread out as a single layer of cells (Fig. 3a), exist as microcolonies (Fig. 3b), or be attached to particulate matter (Fig. 3c), the size of which will effectively increase the thickness of the bacterioneuston layer (Fig. 1).

The microenvironment of an aquatic bacterium is dominated by low Reynolds numbers, and thus, viscosity (49,50). Because of the viscosity, and surface tension, the air–water interface for a bacterium could have characteristics similar to a solid substratum (17,33). It follows then that the cell surface characteristics that can play a role in the attachment of bacteria to solid substrata (51) could be of importance at the air–water interface. Cell surface hydrophobicity and charge have been demonstrated in the laboratory to be important to bacterial accumulation at the air–water interface in situations both with (52,53) and without (54) artificial organic surface films. In the presence of organic films, the architecture of the film and its thickness influenced the enrichment of certain bacteria (52,55). Without an organic film, accumulation by hydrophilic bacteria was particularly influenced by cell surface charge (54). In a study comparing bacterioneuston and bacterioplankton isolated from natural samples, Dahlbäck and coworkers (56) demonstrated that the bacteria from the air–water interface had a higher degree of cell surface hydrophobicity than the corresponding planktonic cells.

Firm adhesion of bacteria to solid substrata is generally mediated by extracellular polymers, fibers, and fimbriae (51). Studies using either scanning or transmission electron microscopy of bacterioneuston from natural environments have indicated the presence of these materials or structures associated with the bacteria (48,57–60). Thus, it appears that the bacterioneuston use similar mechanisms to get attached to the air–water interface as they would to get attached to a solid surface. In fact, bacterioneuston readily get attached to solid materials that are passed through the air–water interface (61), although in some situations this may only be reversible attachment (62). Although attachment may be an important factor for the bacteria at the air–water interface, there is the possibility of an exchange between the interface and bulk phase (63), suggesting that motile bacteria may be able to move in and out of the surface microlayer, perhaps to scavenge nutrients. This is supported in freshwater habitats by reports of changes in the numbers of bacterioneuston over a diel period (64,65). However, this exchange may be masked by cells trapped in thick organic films at the air–water interface (66). In larger bodies of water, wind can compress organic material and cells in surface slicks resulting in higher enrichment of bacterioneuston (67). The bacteria in the slicks can be

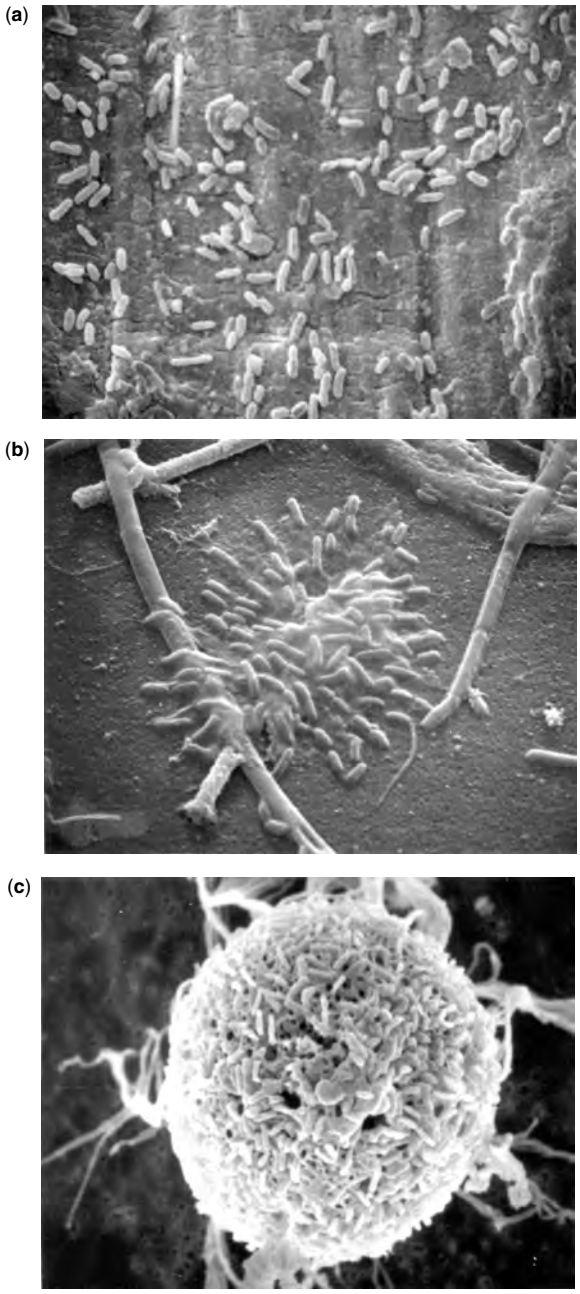

Figure 3. Scanning electron micrographs of freshwater bacterioneuston. (**a**). Assemblage of bacterioneuston in a single layer from the surface of a freshwater pond. Bar = 2 μm. With permission from J. S. Maki and M. Hermansson, in R. S. Wotton, ed., *The Biology of Particles in Aquatic Systems*, 2nd ed., Lewis Publishers, Boca Raton, Fla., 1994, pp. 161–182. (**b**) Microcolony of bacterioneuston. Bar = 2 μm. With permission from J. S. Maki and C. C. Remsen, Microb. Ecol. 9 177–183 (1983). (**c**) Bacterioneuston attached to a particle at the air–water interface. Bar = 1 μm. For details see reference 48.

readily transported by wind (68), possibly over long distances (69).

The major advantage for bacterioneuston at the air–water interface is the availability of nutrients. The model depicted in Figure 1 indicates the presence of proteins, carbohydrates, and lipids. Dissolved organic carbon (DOC) has often found to be enriched in the surface microlayer in both marine (35,70–73) and freshwater samples (47,66,74). In addition, dissolved phosphorus and inorganic nitrogen have also been reported to be enriched in both marine (35,75–78) and freshwater (66,74,79–84) surface films. It has been suggested that by adjusting these nutrients to a microlayer thickness of 0.1 μm, the concentrations approach those found in laboratory culture media (70). Furthermore, starving bacteria resume growth when they come in contact with the nutrients at an air–water interface in the laboratory (85) and greater enrichments of bacteria have been found in surface films in oligotrophic lakes rather than in eutrophic ones (86).

However, the presence of high levels of nutrients at the air–water interface does not always guarantee a high level of bacterial activity in comparison with the plankton. Uptake or turnover of labeled compounds has been reported by investigators in some situations to be similar in both the neuston and the plankton (35,77,87–89) and in others to be lower in the neuston than the plankton (65,90–92). Higher neuston activity has been observed in the presence of surface slicks (93), in more oligotrophic waters (35,87), and, in one case, dependent on the device used to collect the air–water interface sample (84). In addition, use of direct microscopic counts of total bacteria and either formazan dyes to determine respiring bacteria or microautoradiography to determine active bacteria showed enrichment of either respiring or active bacterioneuston at the air–water interface in marine or freshwater habitats (63,66,93). The conclusion appears to be that there is always some activity in the bacterioneuston, indicating that irrespective of the kinds of environmental stress that are present, there are some cells that can cope with them. The mechanisms they may use to cope, although not always directly assessed using bacterioneuston, will be examined in the following sections.

BACTERIONEUSTON AND ENVIRONMENTAL STRESS

Many of the forms of stress that will be discussed can and will have effects on microorganisms throughout the water column. However, with its exposed position at the top of the water column, the air–water interface and the associated neuston microorganisms will often face the brunt of these forms of stress. Thus, the neuston must have mechanisms to contend with the stress to remain viable (Fig. 4). In addition, fluctuations of these various environmental parameters may only affect the air–water interface and not the rest of the water column.

Solar/UV Radiation

Of the different forms of environmental stress that the neuston at the air–water interface are exposed to, the most obvious is intense solar radiation (3,46) with its visible (400 to 750 nm) and ultraviolet (UV, approximately 100 to 400 nm) components. The most biologically important are UV-A (320 to 400 nm) and UV-B (280 to 320 nm),

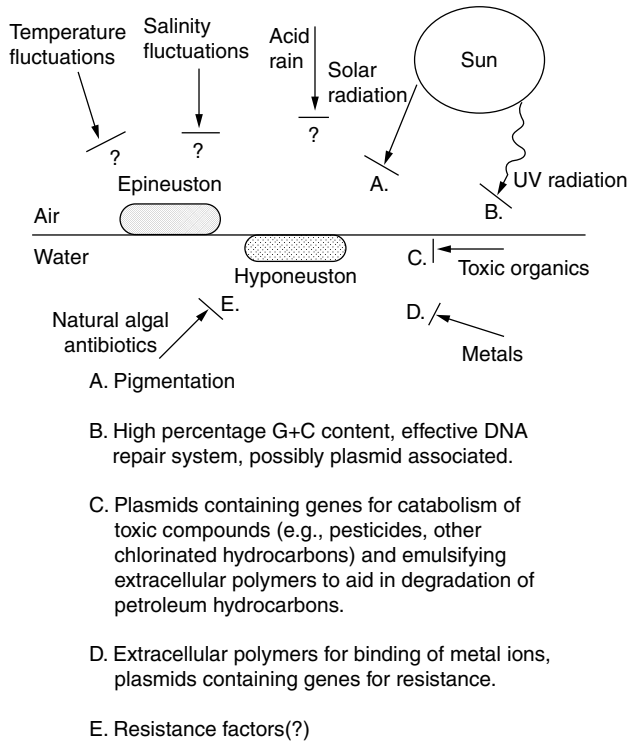

Figure 4. Compilation of the various forms of environmental stress that microorganisms encounter at the air–water interface and possible adaptive mechanisms for the survival of bacterioneuston. Adapted from J. S. Maki, in T. E. Ford, ed., *Aquatic Microbiology*, Blackwell Scientific Publications, Boston, Mass., 1993, pp. 409–439.

although harmful effects can be caused by wavelengths up to 700 nm (94). These wavelengths can act directly on the bacterioneuston or indirectly on them through photodegradation of organics and its resulting products. The overall effect of solar radiation on neuston can be lethal or nonlethal. The direct lethal effects are primarily caused by lesions in the DNA. Nonlethal effects include growth delay, growth inhibition, inhibition of induced enzyme synthesis, reduced active transport, and mutagenesis (95–98).

The effects of solar radiation on the viability and activity of bacterioneuston or very near surface bacteria have been indicated to be deleterious by some investigators (99–101). However, additional investigations have shown little or no difference in activity between intense sunlight and cloudy conditions (63) and little effect of UV and visible light on the total activity (35,87–89,102). These results indicate that in spite of the negative effects of solar radiation, metabolically active bacterioneuston exist. The implication is that they are capable of resisting or are tolerant to these negative effects.

Mechanisms for tolerance and/or resistance in bacterioneuston could include: (*1*) physical screening by UV-absorbing molecules, (*2*) interference or protection of hazardous photoproducts like singlet oxygen by carotenoid pigments, and (*3*) systems for the repair of DNA damage (95). Among UV-absorbing molecules, mycosporine-like amino acids (MAAs), which have absorbance maxima in the range of 310 to 360 nm, have received a lot of attention (103). These types of molecules have not been reported in bacteria. However, a recent report has indicated that some dinoflagellates secrete MAAs and that they can be quantified in dissolved organic material (104). Whether these can be found at the air–water interface where they could potentially protect bacterioneuston has not been reported.

The heterotrophic bacterioneuston from both marine and freshwater habitats has been reported to contain a large portion of pigmented cells (61,105–110), but not always in the highest frequency (30,111). The proportion of pigmented bacteria can increase after exposure to UV-B (112), which may be one reason pigmented bacteria have been isolated at the air–water interface. Generally, carotenoid pigments can protect from the effects of visible light by quenching the products of photochemically induced oxidations (113) but are not effective in blocking UV-caused DNA damage (114).

As stated earlier, the lethal effects of UV light involve DNA damage and the formation of thymine dimers on strands of DNA. One possible protection mechanism would be to possess DNA with a high guanosine with cytosine (G + C) content to avoid thymine damage from UV radiation (115). An examination of common bacteria isolated from surface films showed that 10 out of the 16 genera represented had mol% G + C in excess of 50 (6). In addition, DNA repair systems (photoreactivation, excision repair, recombination repair, and SOS repair) would be important to bacterioneuston survival. Resistance to UV radiation may be encoded on plasmids (116) and the presence of plasmids has been reported in both marine and freshwater bacterioneuston (108,110,117,118).

Photodegradation is one of the major mechanisms for the transformation of materials at the air–water interface (Fig. 2). It can also can affect bacterioneuston indirectly. On the positive side, it can cause the breakdown of recalcitrant compounds making more usable compounds for the bacterioneuston. On the negative side, photolysis of crude oils was shown to have a toxic effect on bacterial activity (119).

Toxic Substances

The surface film is known to accumulate toxic substances including hydrocarbons (Table 2) and heavy metals (Table 3). Materials collected from the air–water interface from marine and estuarine habitats have been shown to be toxic to eukaryotic organisms in a variety of bioassays (120–124). These substances may also have an effect on the bacterioneuston.

Among the organics, petroleum, pesticides, and other chlorinated hydrocarbons have been reported from air–water interface samples from both marine and freshwater habitats (Table 2). By themselves, they may affect cell membranes, nucleic acid synthesis, amino acid and protein synthesis, enzyme activity, and may cause mutations (126). It has been demonstrated that the presence of a number of different organic compounds at the air–water interface can inhibit the growth of heterotrophic bacterioneuston (Fig. 5). There are also interactions between these organics and UV radiation. As stated

Table 2. Examples of Petroleum, Polyaromatic, and Chlorinated Hydrocarbons Reported From Air–Water Interface Samples[a]

Compound[b]	Marine	Freshwater
Hydrocarbons	X	X
Normal Alkanes	X	
PAHs	X	
PCBs	X	X
Pesticides	X	
DDT	X	X
DDE	X	X
DDD	X	X
Dieldrin	X	
Aldrin	X	X

[a]Adapted from J. S. Maki, in T. E. Ford, ed., Aquatic Microbiology, Blackwell Scientific Publications, Boston, Mass., 1993, pp. 409–439 and J. S. Maki and M. Hermansson, in R. S. Wotton, ed., *The Biology of Particles in Aquatic Systems*, 2nd ed., Lewis Publishers, Boca Raton, Fla., 1994, pp. 161– 182.
[b]PAH, polyaromatic hydrocarbon; PCB, polychlorinated biphenyl; DDT, dichlorodiphenyltrichloroethane; DDE, dichlorodiphenyldichloroethylene; DDD, dichlorodiphenyldichloroethane.

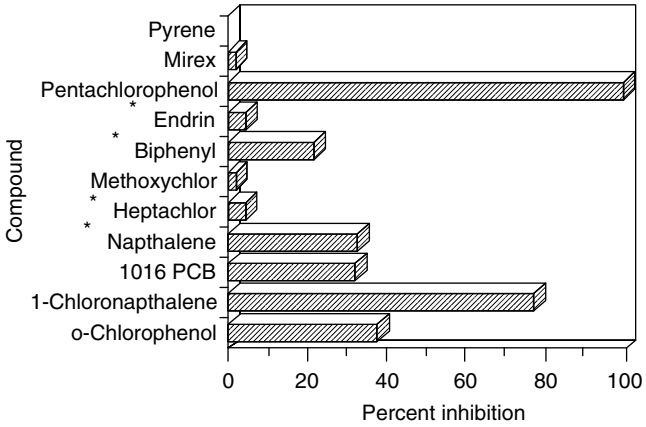

Figure 5. Percentage growth inhibition of up to 53 bacterioneuston strains by chlorinated and aromatic hydrocarbons (concentration of 100 μg in a modified antibiotic sensitivity test. *Indicates only 49 strains were tested. Adapted from D. G. Ahearn, S. A. Crow, W. L. Cook, and A. W. Bourquin, Report No. EPA-R-803141, U.S. Environmental Protection Agency, Gulf Breeze, Fla., 1977, p. 22. (130).

Table 3. Examples of Essential and Toxic Metal and Metalloid Types Measured in Surface Film Samples from Marine, Estuarine, Salt Marsh, and Freshwater Ecosystems[a]

Type[b]	Metal	Marine	Estuarine /Salt Marsh	Fresh -water
Essential	Vanadium	x		
	Chromium	x	X	X
	Manganese	x		X
	Iron	x	X	X
	Nickel	x	X	X
	Copper	x	X	X
	Zinc	x	X	X
Toxic	Selenium		X	
	Silver	x	X	
	Cadmium	x	X	X
	Mercury		X	
	Lead	x	X	X
	Aluminum	x		X
	Tin/Organotin	x		

[a]Adapted from J. S. Maki, in T. E. Ford, ed., *Aquatic Microbiology*, Blackwell Scientific Publications, Boston, Mass., 1993, pp. 409–439 and J. S. Maki and M. Hermansson, in R. S. Wotton, ed., The Biology of Particles in Aquatic Systems, 2nd ed., Lewis Publishers, Boca Raton, Fla., 1994, pp. 161– 182.
[b]Metal and metalloid type according to T. J. Beveridge et al., Adv. Microb. Physiol. 38, 177–243 (1997). (125).

earlier, photolysis of crude oil was shown to result in a toxic effect on bacterial activity (119). UV light has also been shown to increase the photoinduced toxicity of PAHs (127), although this can be improved by the presence of humic acids (128). The presence of humic acids also has been reported to increase the photodegradation of pesticides (129).

Many of these organic compounds, in addition to photodegradation, may also be subject to biodegradation. Actually, the microorganisms may more rapidly deal with the products of photodegradation than with the hydrocarbons themselves (131). The presence of petroleum hydrocarbon degrading bacterioneuston has been demonstrated in a variety of situations (132–135), although they may be inhibited by the presence of pesticides (136). The biodegradation of hydrocarbons and PAHs by the neuston may be less than in the plankton (109,134,137). When bacterioneuston that degrade hydrocarbons have been examined, they generally possess extracellular polymers (59,138), which may have an emulsifying effect on petroleum hydrocarbons and aid in their degradation (135,138). Exopolymers may also act to accumulate pesticides (139).

Metals have álso been measured in air–water interface samples (Table 3). Some of these metals are essential for growth, reproduction, and survival of bacteria (125). Inspite of the with measurements of dissolved trace metals at the air–water interface, there has been some concern about contamination because of the sampling techniques (21). However, even high levels of essential metals can be toxic. Metals can affect microbial growth, morphology, and metabolic activity (140) and can alter microbial community structure quantitatively and qualitatively (141). Metal toxicity is caused by the ionic form and for the most part metals at the air–water interface are associated with organic molecules and/or particulates. Obviously, this may reduce the toxicity to microorganisms, but enough ionic forms may be present in concentrations high enough to cause problems (142). In addition, many bacterioneuston are associated with particulates and may be exposed to particulate-bound metals (143,144).

Bacterioneuston have been shown to be more resistant or tolerant to metals than bacteria from the bulk phase (108,110,145), but not in all cases (117). Metal resistance mechanisms have been recently reviewed (146–148) and many resistance factors are plasmid-associated. As pointed out earlier, many bacterioneuston possess plasmids (108,110,117,118). However, there has been no direct

link established between the presence of these plasmids and any respective metal resistance. In the future, use of specific gene probes may be able to establish this relationship.

Temperature Fluctuations

Microbial neuston are exposed to heat from the water and from direct absorption (3). Changes in temperature include regular diel and seasonal fluctuations (3) and rapid sporadic changes due to factors like cloud cover (149). The effects of these changes have not been extensively studied regarding bacterioneuston. The number of total bacteria positively correlated to changes in air temperature and colony-forming units positively correlated to surface water temperature in the neuston of an Antarctic freshwater pond (65). Short-term temperature fluctuations increased the bacterial diversity in chemostat enrichments of sediment bacteria, indicating that separate temperature niches may be used by different bacteria (150). Thus, temperature fluctuations at the air–water interface may affect single types of bacteria but may not adversely affect the bacterioneuston population as a whole.

Ice formation is one seasonal temperature effect that bacterioneuston may have to deal with. It is clear that some bacteria possess the ability to survive in ice habitats (151). However, it is unknown if any of these bacteria are neustonic. In one study, nocturnal ice formation was suggested to have an inhibitory effect on the activity and community size of the bacterioneuston of an Antarctic freshwater pond (65). They suggested that the bacterioneuston needed time to recover after the nocturnal freezing. The direct effects of ice formation on bacterioneuston have not been reported.

Precipitation

Precipitation in the form of rain can disrupt surface films (152). In addition, precipitation can have effects on neuston microorganisms. First, in marine systems, it can cause changes in salinity. Second, probably more noticeable in freshwater environments, acid precipitation could cause changes in the pH. In each case, the air–water interface faces the initial brunt of the precipitation.

Not much is known about the effects of precipitation and salinity changes on marine bacterioneuston, although some marine bacteria are sensitive to changes in salinity (3). In one study, it was noted that rain caused a decrease in bacterioneuston densities, but a stimulation in activity (153). The changes in salinity were not determined and whether or not this had any effect on the increase in activity was unknown. However, others have reported that in marine coastal water, many bacterioneuston isolates did not require added salt for growth, suggesting that they were of terrestrial or freshwater origin (107,135). Whether these could respond to a decreased salinity brought about by precipitation is unknown.

Acid rain has been a long-term problem for many freshwater ecosystems (154). Acid precipitation may have effects on the chemistry of the surface microlayer by increasing particle formation and by acid degradation of the higher molecular weight organics (155,156). Lower pH will also work to make metal ions more available, perhaps to more toxic levels (157). pH stress on bacteria can result in acid damage and/or cell death (158). Mechanisms to avoid pH stress include (*1*) decreased membrane permeability, (*2*) internal buffering, (*3*) modification of external pH, (*4*) proton extrusion or uptake, and (*5*) prevention of metal ion toxicity (157). Bacterioneuston affected by acid rain may use some or all of these mechanisms to remain successful. However, the specific effects of acid rain on bacterioneuston viability and activity have not been addressed.

Natural Algal Antibiotics

Cases of inhibition of bacterioneuston in the marine environment have been reported (70,159). Although anthropogenic materials may have caused this inhibition, it is also possible that it may have been as a result of the production of natural toxic substances from marine algae (33).

A Freshwater Bacterioneuston Example: *Nevskia ramosa*

A number of different bacteria have been found enriched at the air–water interface under various circumstances. Examples include bdellovibrios (160) and *Aeromonas salmonicida* (161,162). In the case of *A. salmonicida*, its presence in the air–water interface may be related to its high cell surface hydrophobicity (161). One of the best examples of a freshwater bacterioneuston and its adaptations to its habitat is the gram-negative, slightly bent rod-shaped bacterium *N. ramosa* (Figs. 6a and 6b). This epineustonic bacterium was first described by Famintzin (163) from the water surface of an aquarium and since then has been repeatedly established in enrichment cultures (105). Recently, it has been isolated in pure culture (164) allowing physiological experiments to be conducted. Phylogenetically, on the basis of 16S rRNA sequencing (165), *N. ramosa* is the only member of a deep branch of the γ-subclass of the class *Proteobacteria*.

Nevskia ramosa is not obligately a member of the neuston. Motile cells develop in the bulk water and become neustonic, losing their flagella, when the availability of combined nitrogen (ammonia, nitrate, or amino acids) is depleted (164). The cells form rosettes of dichotomously branched slime stalks with single cells at the tips (Fig. 6) and live outside the water phase (i.e., epineustonic). When the cell divides, the stalks form branches. The stalk is secreted from the concave side of the cell and analysis of its exopolysaccharides indicates that it is primarily rhamnose with small amounts of glucose and mannose (164). The metal binding capacity of these exopolysaccharides has not been determined. These films with exopolysaccharide stalks appear to have a high hydrophobicity (164). The bacterium has a high G + C content (67.8 to 69 mol% G + C) and, although sensitive to UV radiation, can overcome damage through photoreactivation (164). Thus, it appears that this bacterium is well-adapted to existence in the neuston. It is also worthy of note that it takes a form of stress (i.e., combined nitrogen depletion) for what could be considered a planktonic organism, to change its habitat to the air–water interface and the neustonic life. This would suggest a certain amount of genetic plasticity

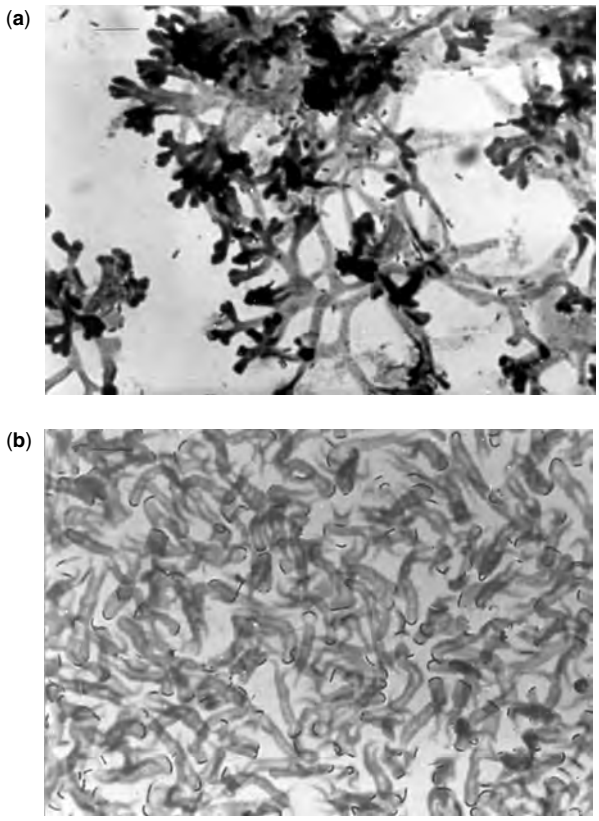

Figure 6. Photomicrographs of *N. ramosa*. (**a**) From the air–water interface of a bog lake. Bar = 15 μm. (**b**) From a formaldehyde-fixed and toluidine blue-stained enrichment culture. Bar = 15 μm. The thin bent rods are at the tips of the branching slime stalks. Photomicrographs courtesy of H.-D. Babenzien (unpublished).

to adapt to the different conditions that are present in both types of habitat.

OTHER MICROORGANISMS

The bacterioneuston are not the only microorganisms that are found at the air–water interface. Each type of microorganism that resides or can be found in the surface microlayer faces the same inhibitory conditions that the bacterioneuston do and must have strategies for dealing with each one. Both the effects and the strategies for dealing with them have not always been investigated directly with neuston organisms.

Phytoneuston and Protozoa (nano- and Microneuston)

Because of their size, algae (phytoneuston) and protozoa in the neuston are normally excluded from the thinnest layers at the air–water interface. As pointed out earlier, when they are at the air–water interface, they are subject to the variety of conditions that exist there. For example, when the effect of UV-B was examined on pelagic microbial communities, it severely limited both ciliates and large phytoplankton (166). This would most probably have its greatest effect on neuston microorganisms. However, because of their mobility, they may be able to avoid the hazardous effects of solar radiation. A diurnal migration of motile microorganisms in and out of the neuston has been reported for both marine and freshwater environments (167–169).

Algae in the neuston (phytoneuston) have two potential roles in surface films: (it 1) they are involved in transfer of carbon dioxide from the atmosphere and (it 2) they produce dissolved organic material (47). Their presence has been reported in surface films either through cell counts or by measurements of chlorophyll *a* (81,82,170–175). There can also be seasonal changes in the phytoneuston populations (173,175–177), which may be linked to changes in the phytoplankton or benthic algal communities (175). The activity of these populations has been under some question. Reports have indicated the presence of primarily dead cells (99) and lower photosynthetic activity at the air–water interface compared with bulk-water samples (35,178,179), although the latter may be influenced seasonally, by the presence of contaminants or of surface slicks (172,180–182). Alternatively, one study has shown higher primary production per unit volume at the air–water interface than in the bulk phase when membrane filters were used to obtain a very thin sample (182). Photoinhibition has been suggested to be one of the main causes of lower viability and activity of phytoneuston at the air–water interface (35,171,174,182). However, Cullen and coworkers (183) reported that the phytoneuston at the air–water interface were physiologically photosynthetically competent like cells examined at a 10-cm depth. They suggested that there may have been a possible mild inhibition of photosynthesis, because of these populations being well-adapted to bright light (183).

The protozoa may provide the link between the bacterioneuston and the rest of the community of organisms associated with the air–water interface (15,33,184). Both ciliate protozoa (167,185,186) and amoebae (70,187) have been reported to be present and at times more concentrated in the neuston of both marine and freshwater ecosystems. They have been observed to have a spatial heterogeneity in surface films (185,186). This patchy distribution by the protozoa has been shown to be related to the distribution of surface film bacteria (186).

Viruses

Viruses have been demonstrated to be present in high numbers in aquatic habitats (188) and could play a negative role in affecting both microbial neuston viability and activity. In addition, lysogenic viruses can be involved in gene transfer through transduction (189). Virions have been reported to adsorb to rising bubbles and when the bubble bursts can be transferred to the atmosphere in jet drops (190,191). This could also act as one mechanism for virus enrichment at the air–water interface. Other factors at the air–water interface, including high levels of UV light and the presence of mutagenic/carcinogenic compounds, could inactivate the repressors, which maintain the temperate virus in a lysogenic state (189) and cause a release of virions. In addition, UV radiation has been demonstrated to result in the destruction of viral particles in marine surface

water, with a decrease in destruction with increasing depth and corresponding attenuation of UV-B (192). This would suggest that the virions at the air–water interface would be the most affected by solar radiation.

In a study of viruses at the air–water interface in Lake Superior, Tapper and Hicks (193) found that free virus particles at the surface ranged from 0.7 to 9.2×10^6 ml^{-1}, between 2 and 15 times more abundant than in bulk-phase samples. They attributed this in part to the increased number of bacteria that were also more abundant at the surface. After treatment of samples with UV light or mitomycin C to induce temperate phage into a lytic state, an increase in viral particles and a decrease in bacterial number was observed. They estimated that three times more bacteria in the surface film may contain temperate viruses compared with those in the bulk phase in this environment. It is also interesting to note that exposure to air liquid interfaces (194) and solar radiation (195) have been reported to lead to the inactivation or removal of viruses. So, although increased numbers of phage may be associated with the air–water interface (193), their ability to be infective is questionable.

CONCLUSION

The air–water interface is subject to a number of different forms of environmental stress that undoubtedly limits the survivability of all the different types of microorganisms that may end up there. The air–water interface is also a dynamic and patchy habitat and, although it appears easy to make generalizations, it must be emphasized that each individual situation must be examined on its own. However, it is clear from the aforementioned text that some organisms are capable of not only surviving there but may also be able to flourish. Shilo (11) pointed out that microorganisms in extreme environments would have to possess a certain amount of plasticity to survive, which would be evident from the earlier discussions of neuston microorganisms. The studies of *N. ramosa* provide an intriguing example of how a bacterium can adapt to a neustonic life. More examples of this type, not only of bacteria but of other microorganisms as well, are needed to be able to provide a greater understanding of how they cope with the various forms of stress in this extreme environment.

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NITRIFICATION.

See TRACE GASES SOIL

NITRIFICATION IN ACTIVATED SLUDGE.

See ACTIVATED SLUDGE — MOLECULAR TECHNIQUES FOR DETERMINING COMMUNITY COMPOSITION

NITRIFICATION IN AQUATIC SYSTEMS

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Nitrification is the process whereby ammonium is oxidized to nitrite and then to nitrate. It, thus, links the most oxidized and most reduced forms of nitrogen, and helps determine their overall distributions. Ammonium rarely occurs at significant concentrations in oxygenated habitats. It is recycled rapidly between heterotrophic organisms (which excrete ammonium directly, or release organic nitrogen that is microbially degraded to ammonium) and photosynthetic phytoplankton (which use ammonium as a nitrogen source) in the surface ocean. Ammonium can accumulate in anoxic sediments and in stratified water in which oxygen concentrations are very low.

Similarly, nitrite rarely accumulates in oxygenated habitats (see the following text for exceptions), although nitrite is an essential intermediate in several oxidation and reduction processes in the nitrogen cycle. Nitrate, the end product of nitrification, however, accumulates in the deep ocean, and seasonally in the deep water of lakes, where there is no demand for inorganic nitrogen by phytoplankton.

The oxidation of ammonia to nitrite and of nitrite to nitrate, although thermodynamically favorable when linked to reduction of oxygen, has long been considered a biological process, with no known abiotic contributions. Oxidation of ammonia and amino-level nitrogen in organic compounds to nitrogen gas linked to the reduction of manganese oxide has been demonstrated in sediments, and this pathway may have significant ramifications for the nitrogen cycle of sediments. Whether this represents an example of abiotic nitrification or anaerobic biological nitrification (discussed in the following text) remains to be resolved.

The most important organisms in nitrification are the so-called nitrifying bacteria. This group includes several genera of bacteria, all within the Proteobacterial phylum. These genera are not all closely related to each other, but appear to have arisen from a photosynthetic ancestor, diverging before the ability to nitrify was developed in the various groups (1). There are two functionally distinct groups of nitrifiers: those that oxidize ammonium to nitrite [ammonia-oxidizing bacteria (AOB)] and those that oxidize nitrite to nitrate [nitrite-oxidizing bacteria (NOB)]. No organism is known to carry out both reactions. These unique metabolic traits are not without costs; the nitrifiers are chemolithoautotrophic for the most part, a lifestyle that enables them to exploit a unique niche in natural systems, but apparently confers a constraint of slow growth and inflexible nutritional requirements.

Nitrification is closely coupled with other important steps in the nitrogen cycle, such as nitrogen assimilation by phytoplankton and loss of fixed nitrogen by denitrification. Thus nitrification is part of the linkage between the carbon and nitrogen cycles at several levels. These and other aspects of nitrification and nitrifying bacteria in marine systems are explored in this article. The emphasis is on the last 10 to 20 years. Other useful reviews are found in References (2–5).

PHYSIOLOGY AND ENERGETICS OF NITRIFYING BACTERIA

Autotrophic Nitrification

Obligately autotrophic, or in a few cases, mixotrophic bacteria are thought to perform most of the nitrification

that occurs in natural habitats, but the diversity of known nitrifying physiologies is rapidly expanding. We shall consider the classical autotrophs first.

Most of our knowledge of autotrophic nitrifiers derives from studies on cultivated strains, and the best-known of those are the ammonia-oxidizing genus *Nitrosomonas* and the nitrite-oxidizing genus *Nitrobacter*. The cultured autotrophic nitrifiers, both ammonia-oxidizers and nitrite-oxidizers, depend on carbon dioxide as their major carbon source, and fixation is carried out by the Calvin cycle. It is estimated that carbon dioxide fixation, accounts for about 80% of the energy budget of an autotroph (6,7). In addition, the use of ammonium or nitrite as the sole source of reducing power for this autotrophic growth is relatively inefficient: about 35 moles of ammonium or 100 moles of nitrite must be oxidized to support fixation of a single mole of carbon dioxide (8). This "perverse insistence "on fixing their own carbon dioxide, using a unique but low-yield energy source (9), accounts for their well-deserved reputation for slow growth, even under optimum conditions in the laboratory. Minimal generation times of 7 to 13 hours are usual for *Nitrosomonas* and *Nitrobacter* (10).

The overall reaction for ammonia oxidation (Eq. 4) shows that the process consumes molecular oxygen and produces hydrogen ions, in addition to nitrite. A requirement for molecular oxygen occurs in the first step of the oxidation (Eq. 1), which is catalyzed by a monooxygenase [ammonia monooxygenase, (AMO)]. The uncharged gaseous ammonia is the actual substrate for AMO, as demonstrated by the pH dependence of the reaction rate (11,12). AMO has never been completely purified and assayed in cell-free conditions, although its gene sequence has been derived for both *Nitrosomonas* and *Nitrosococcus* type AOBs (13–15). AMO contains copper and probably also iron in its active form (16).

The immediate product of AMO is hydroxylamine, which is further oxidized by hydroxylamine oxidoreductase (HAO) to nitrite (Eq. 2). Oxygen is also consumed by the terminal oxidase (Eq. 3), as a result of electron transport generating ATP for cellular metabolism.

$$NH_3 + O_2 + 2H^+ + 2e^- \rightarrow NH_2OH + H_2O \quad (1)$$

$$NH_2OH + H_2O \rightarrow NO_2^- + 5H^+ + 4e- \quad (2)$$

$$2H^+ + 0.5O_2 + 2e^- \rightarrow H_2O \quad (3)$$

$$NH_3 + 1.5O_2 \rightarrow NO_2^- + H_2O + H^+ \quad (4)$$

Although the overall reaction is energy yielding, the initial oxidation of ammonia to hydroxylamine involves the consumption of reducing power, which is supplied by linkage to the second step, hydroxylamine oxidation, which yields four electrons. The nitrogen oxidation and electron transport pathways in *Nitrosomonas* are incompletely known, but involve several unique and interesting components that are linked in the cytoplasmic membrane and periplasmic space (17). Depending on conditions (and enhanced at low-oxygen concentrations), nitric oxide (NO), nitrous oxide (N_2O), and even dinitrogen gas (N_2) have been reported as secondary products in autotrophic ammonia oxidation. Although nitrous oxide and nitric oxide can be produced in vitro by HAO from hydroxylamine (18), reduction of nitrite appears to be the dominant pathway in whole cells (17,19,20). Nitrite reductase activity has been demonstrated in *Nitrosomonas europaea* (21,22), and a gene with homology for the copper type nitrite reductase of heterotrophic denitrifiers was recently detected in a group of marine *Nitrosomonas* isolates (23).

The biochemistry of nitrite oxidation is simpler than ammonia oxidation because it is only a two-electron transfer and involves no intermediates. The additional oxygen atom in nitrate is derived from water (Eq. 5), and the molecular oxygen that is involved in the net reaction (Eq. 7) results from electron transport involving cytochrome oxidase (Eq. 6).

$$NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ \quad (5)$$

$$2H^+ + 0.5O_2 \rightarrow H_2O \quad (6)$$

$$NO_2 + 0.5O_2 \rightarrow NO_3^- \quad (7)$$

The energy yield of nitrite oxidation is even less than that of ammonia oxidation, necessitating the oxidation of vast amounts of nitrite to fix small amounts of carbon dioxide. For both processes, therefore, the biogeochemical impact is greater on the nitrogen cycle than on the carbon cycle.

The biomass produced by primary fixation of carbon dioxide by nitrifiers is probably not significant, except in a few unusual habitats. Areas under the sea ice in the Antarctica (24), an anchialine cave in the Yucatan Peninsula, Mexico (25), and the Mississippi River Plume in Gulf of Mexico (26) are cited as exceptions. In all the three cases, the significance of autotrophic nitrification as an in situ source of organic carbon is enhanced relative to the production of organic matter by photosynthesis. Photoautotrophic carbon production is minimal, because of the absence of light caused by the ice cover, the geometry of the cave itself, and in the Mississippi River system, high levels of suspended matter, respectively. Chemoautotrophy might also be a significant source of in situ production in hydrothermal plumes in which elevated ammonium (and perhaps methane) concentrations could support ammonia-oxidizing bacteria (27).

Heterotrophic Nitrification

Many denitrifying bacteria, other heterotrophic bacteria and fungi have been reported to perform heterotrophic nitrification. Heterotrophic nitrification refers to several different oxidation reactions that parallel parts of the autotrophic nitrification process, including reactions, which release nitrate or nitrite from the breakdown of organic nitrogen compounds. It has been argued that heterotrophic nitrification involves quite different enzyme systems (28), and that it cannot serve as an energy-generating mechanism (29), as does the autotrophic process. In the aerobic denitrifiers, which are also capable of nitrification, the initial enzyme, AMO, appears to be quite similar to the enzyme in autotrophic nitrifiers. However, HAO differs significantly and in

the heterotrophs is a smaller, simpler enzyme that performs a two-electron transfer (instead of the four-electron transfer of the autotrophic HAO), and releases nitroxyl as the product, rather than nitrite (30). Thus, no electrons are available for reverse electron transport in the heterotrophic system, and the purpose of the process appears to be a means of disposing of excess reductant in times of redox stress, rather than harvesting that reductant for cellular energy.

In aquatic systems, the most important heterotrophic nitrifiers are thought to be a class of denitrifiers, which are capable of aerobic denitrification, and which can also oxidize ammonia. The organism in which these physiologies were first described is now known as *Paracoccus pantatrophus* (formerly known as *Thiosphaera pantotropha*). *Paracoccus pantotrophus* oxidizes ammonia to nitrite using an enzyme that exhibits important similarities and differences compared with AMO from autotrophic nitrifiers (31). The nitrite so generated can be released into the medium or denitrified to dinitrogen gas. Denitrification of nitrite or nitrate can occur under atmospheric levels of oxygen (32). Aerobic denitrifiers have mostly been isolated from wastewater treatment systems, and their prevalence and ecological significance is unknown in natural aquatic systems.

Because autotrophic nitrification is such a hard way to make a living, it seems strange that heterotrophic nitrification is not more common. Thermophilic heterotrophic nitrifiers, capable of oxidizing ammonia to nitrite, were isolated from several hydrothermal vent habitats (33). Most of the isolates were nitrate reducers or denitrifiers, and exhibited a wide diversity related to the genera *Thermus* and *Bacillus*. Because they are able to ammonify, nitrify, and reduce nitrate, they would seem to possess most of the physiologies of importance in the nitrogen cycle. Such organisms may be common in mesophilic aquatic habitats, but have not been quantitatively investigated.

Heterotrophic strains capable of oxidizing nitrite have also been reported. Sakai and coworkers (34) described several classes of heterotrophic strains that oxidized nitrite to nitrate with variable amounts of nitrate accumulation, depending partly on the denitrification capabilities of the strains. Strains that were capable of both oxidation and reduction of nitrogen oxides were shown to switch between the two directions of conversion depending on the oxygen tension of the culture (35).

Heterotrophic nitrification has been studied in terrestrial systems, especially acid forest soils, in which it has been difficult to document autotrophic nitrification. Experiments using isotopes to differentiate production of nitrate from inorganic and organic substrates in a forest system found that heterotrophic nitrification accounted for less than 10% of the total nitrification rate (36). No information of this sort is available on the occurrence or significance of heterotrophic nitrification in aquatic systems. The potential for ammonia and nitrite oxidation by heterotrophic bacteria in aquatic systems warrants further exploration, and the capability may be present in many strains already in culture. If heterotrophic nitrification is common in nature, a focus on autotrophic nitrification as the model system and the basis for estimation of rates is too narrow and unrealistic. On a per cell basis, heterotrophic nitrification is much slower than is autotrophic nitrification, and the relative impact on nitrogen turnover must be greater by autotrophs because of their total dependence on nitrogen oxidation for energy. Nevertheless, widespread abundance of more numerous heterotrophic nitrifiers could compete in importance with the smaller number of slow-growing autotrophs.

Anaerobic Nitrification

The interest in anaerobic nitrification and aerobic denitrification arises mainly from the necessity to treat large volumes of wastewater to reduce nitrogen loading before release into natural water. Classical nitrification and denitrification are environmentally incompatible processes, the first being obligately aerobic and the second induced only under conditions of anoxia. It is, therefore, usual to involve two steps in wastewater treatment: an aerobic step to convert ammonia to nitrate and a subsequent anaerobic step to convert the nitrate to dinitrogen. If the two steps could be combined in one organism under either aerobic or anaerobic conditions, much time and money would be saved in wastewater treatment. And clearly, if a single organism is capable of combined nitrification and denitrification in a bioreactor or water treatment plant, such an organism could be of considerable importance in the natural environment as well.

The heterotrophic nitrifier mentioned earlier, *P. pantatrophus*, carries out at least part of both processes under aerobic conditions. *Paracoccus pantatrophus* was originally isolated from wastewater and its ability to denitrify aerobically in several other conventional heterotrophic denitrifiers has been confirmed (32).

Autotrophic nitrifying bacteria exhibit some abilities for anaerobic metabolism as well. Enrichment cultures under chemolithotrophic conditions and very low oxygen concentrations catalyzed the net removal of ammonium as nitrogen gas (37). Bock and coworkers have shown that *Nitrosomonas eutropha* produces gaseous products, mainly nitric oxide and dinitrogen gas, during growth on nitrogen dioxide gas (NO_2) and ammonia (38). The process proceeds at a slower rate than ammonia oxidation in the presence of a normal air atmosphere and supports cell growth. Additions of nitrogen dioxide gas and nitric oxide enhanced the complete removal of nitrogen in the form of ammonia and organic nitrogen, without the addition of organic carbon substrates (39).

A completely novel process in which ammonia and nitrite are converted, anaerobically, to dinitrogen gas has recently been reported, the process has been quantitatively described (40,41), and the organisms responsible for this novel metabolism have been identified as relatives of *Planctomyces* (41). Referred to as *anammox*, the process probably involves a consortium of the planctomycete organism and an autotrophic ammonia-oxidizer such as *N. europaea* or *N. eutropha*. Ammonia is oxidized to nitrite by the autotroph under microaerophilic conditions. The nitrite so produced is reduced to dinitrogen gas by the planctomycete. Both oxygen and nitrite concentrations

are maintained at nearly undetectable levels by the metabolism of the members of the consortium, and although both organisms grow quite slowly (generation times, for the planctomycete, of two weeks are reported), the net removal of ammonium occurs at a rate 25 times faster than that reported for nitrogen removal by anaerobic autotrophic nitrification alone (40).

The anammox process was discovered and characterized in anaerobic wastewater treatment systems, and 16S rRNA sequences identified as belonging to the planctomycete member have been detected in several such systems. Research into the occurrence of the process and presence of the organisms in natural aquatic systems has not yet been reported. Anammox would constitute a shortcut in the conventional nitrogen cycle similar to that proposed by Codispoti and Christensen (42), who suggested that nitrous oxide produced by nitrifiers might be denitrified by denitrifiers, thus linking nitrification and denitrification without going through nitrate. In the anammox process, the linkage occurs at the level of nitrite. It is suggested, therefore, that places where ammonium and nitrite both occur in the presence of low oxygen concentrations might be suitable habitats for anammox. Such environments include oxic–anoxic interfaces, such as those found at sediment–water interfaces in hemipelagic and shallow sediments, and in stratified lakes and in the water columns of stratified basins, such as the Black Sea and the Cariaco Basin. At first consideration, the anammox consortium seems unlikely to dominate processes in these environments, on the basis of the observation of chemical distributions. Ammonium builds up to very high levels in anoxic sediments and stratified water column, in both freshwater and marine environments. Microaerophic autotrophic nitrification, linked to anaerobic denitrification across the oxic–anoxic interface, has been used to interpret the chemical distributions that typically show depletion of oxygen and nitrate above the interface and accumulation of ammonium below. Denitrification produces a net loss of fixed nitrogen, which would presumably be much larger if anammox were also involved. Mass balance measurements might provide insight into the potential for anammox involvement in natural systems.

In addition to the unconventional activities of conventional nitrifiers and denitrifiers and the discovery of novel nitrogen metabolisms in new organisms, it has also been recently proposed that a short circuit of the nitrification/denitrification couple can also be accomplished abiotically. In marine sediments that typically contain relatively high manganese levels, dinitrogen gas can be produced by the oxidation of ammonia and organic nitrogen by manganese dioxide in the air. The reduced Mn^{++} thus formed can be reoxidized by oxygen to continue the oxidation of ammonium, or can reduce nitrate to dinitrogen gas (43). Although the free energy of these coupled reactions is shown to be favorable, it remains to be seen whether the abiotic process can be unequivocally identified in natural systems, and the degree to which it may compete with the biologically catalyzed processes. Hulth and coworkers (44) detected anoxic production of both nitrite and nitrate in marine sediments, concurrent with the production of reduced manganese. The nitrate production was directly proportional to the initial manganese oxide content, and the nitrate was subsequently depleted, apparently by denitrification. Hulth and coworkers (44) proposed a series of linked redox cycles in which anoxic nitrification, coupled to manganese reduction, was linked in series to anoxic organic matter oxidation through several biogeochemical reductants, including iron and hydrogen sulfide. These authors proposed that the lithotrophic nitrification they observed is biologically mediated, and potentially of significance in the nitrogen cycle at sites where oxidized metals are reworked into anoxic sediments.

Anoxic ammonia oxidation, whether it results directly in dinitrogen gas formation (as in anammox) or in nitrate production (when linked to manganese reduction), would introduce new links to the aquatic and sediment nitrogen cycle. Failure to account for anoxic ammonia oxidation might lead to an underestimate of ammonium removal, because the products do not accumulate; they are either lost to the atmosphere immediately, or rapidly reduced by the next step in the anaerobic cycling of organic matter.

PHYLOGENY OF BACTERIA INVOLVED IN NITRIFICATION

Discovery and Description

Autotrophic nitrifiers were first isolated and characterized by Winogradsky (45), the "father of chemolithoautotrophy", in the late 1800s (1890). Winogradsky's organisms were derived from soils; the first reported isolation and characterization of marine nitrifying bacteria was by Stanley Watson in the 1960s (46). Nitrification had been detected in seawater before that time and nitrifiers had been implicated in the production of nitrite in seawater (47,48). The first marine ammonia oxidizer to be cultured was originally called *Nitrosocystis oceanus*, and is now referred to as *Nitrosococcus oceani*, the lone ammonia-oxidizing species in the γ-subdivision of the Proteobacteria. Watson also isolated marine strains of the best-known terrestrial genus of ammonia-oxidizing bacteria, *Nitrosomonas*, and he also reported the first isolations and characterizations of marine nitrite-oxidizing bacteria (49,50). His culture collections have formed the basis of much of our modern knowledge of the biochemistry, physiology, phylogeny, distribution, and activity of nitrifiers in seawater and terrestrial systems. Koops and coworkers recently summarized the history and general characteristics of these genera (51). Isolates, identified as *Nitrosomonas* and *Nitrobacter*, are the nitrifiers most commonly obtained from lake water using classical enrichment techniques (52). When PCR and DNA sequencing techniques retrieved a preponderance of *Nitrosospira*-like sequences from freshwater habitats (53), the representativeness of these isolates was seriously questioned (discussed in the following text). Later isolations and descriptions of new species have increased the physiological and phylogenetic diversity of cultured nitrifiers to include halophilic, alkaliphilic, and psychrophilic strains, for example, *Nitrosococcus halophilus* (54), *Nitrobacter alkalicus* (55), *Nitrospira moscoviensis* (56), *Nitrosomonas cryotolerans* (57). In fact, it is relatively simple, although also relatively tedious, to isolate nitrifiers using standard techniques in liquid

culture (growth on plates is very slow and not very reproducible). So far, there have been no isolations reported that were accomplished under very low substrate conditions or "trace-metal clean "conditions, which might be more realistic for many aquatic environments.

Phylogeny of Nitrifying Bacteria

The number and diversity of bacterial strains that are identified as autotrophic nitrifying bacteria is rather limited, in comparison with the number and diversity of organisms that are capable of denitrification or of heterotrophic growth. A description of the species of ammonia oxidizers and nitrite oxidizers recognized on the basis of morphology (cell shape and distribution of internal membranes) and physiology can be found in References 51,58, respectively.

The generic and species' affiliations of nitrifiers were reassessed on the basis of DNA sequences of the 16S ribosomal RNA genes from the cultured strains (1,59). The phylogeny of nitrifiers (1) shows that most of them are descendents of a common ancestor that was photosynthetic, rather than descending from a common ancestral nitrifier. The ammonia oxidizers are found in the β- and γ-subdivisions of the Proteobacteria (Fig. 1). The nitrite oxidizers are found in the α-, β-, and γ-subdivisions.

The ammonia-oxidizing bacteria (AOB) comprise two main groups in the Proteobacteria, one in the β-subdivision and one in the γ-subdivision. The β-subdivision species, containing genera known as *Nitrosospira* and *Nitrosomonas*, have been the subject of many recent studies. The main tool for investigating them in both culture and field conditions is polymerase chain reaction (PCR) followed by DNA sequencing. On the basis of 16S rRNA sequence analysis, several clusters, and a large amount of microdiversity within clusters, has been detected in a wide variety of environments, both terrestrial and aquatic. Few generalities linking species or strains to environmental conditions have emerged. For example, McCaig and coworkers (60) investigated the community structure of β-subdivision AOB in marine sediments underlying fish farms and found a novel *Nitrosomonas* group whose distribution correlated with elevated ammonium concentrations. Hiorns and coworkers (53) could detect *Nitrosospira* in lake water and lake sediments by direct PCR, but could detect *Nitrosomonas* only after enrichment culturing, suggesting that *Nitrosomonas* preferred the higher ammonium concentrations of batch culture to the variable and lower concentrations in the lake. Hastings and coworkers (61), working in the same system, found evidence for distinct sediment and lake water population, with high and low ammonium preferences, respectively. However, it was *Nitrosopsira* sequences that were associated with high ammonium concentrations derived from sediments. Following up this study, Whitby and coworkers (62) were able to retrieve *Nitrosomonas* sequences directly from lake samples without enrichment, but only during the summer months, and different sequence clusters were associated with different sediment regimes. Stephen and coworkers (63) surveyed enrichment cultures and gene libraries of soils and marine sediments and found that *Nitrosomonas* sequences were more often associated with enrichment cultures and *Nitrosospira*s with libraries. *Nitrosospria* -like sequences were dominant in ammonia-oxidizer sequences retrieved

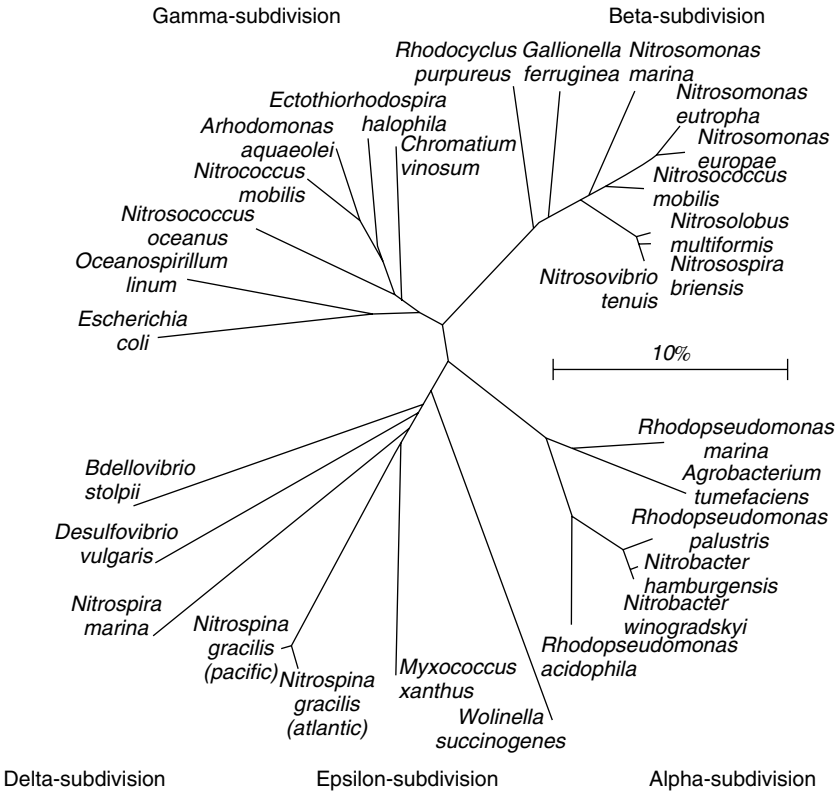

Figure 1. Distance tree for the proteobacteria, including ammonia and nitrite-oxidizing isolates. Scale bar corresponds to 0.1 estimated fixed mutation per sequence position. With permission from A. Teske, *J. Bacteriol.* **176**, p. (1994).

from the Arctic Ocean (64). These findings suggest that the *Nitrosomonas* lineages are less common in natural samples than *Nitrosospira*s, and raise concerns about biases introduced by relying on pure culture techniques. *Nitrosomonas* sequences, however, have been derived directly from the environment including freshwater lakes (62,65) and an alkaline lake. Hovanec and DeLong (66) were unable to detect *Nitrosomonas*-like AOBs in fish aquaria, and concluded that novel, as yet unidentified and uncultured, AOBs must be present.

The γ-subdivision ammonia oxidizers are represented by a single genus and species, *N. oceani*, which has only been reported from marine or saline environments. Several different strains exist in culture, but they all appear to be closely related. They have been detected by PCR in saline lakes in Antarctica (67) and by immunofluorescence in various marine systems (68,69) and in the Mediterranean Sea (70).

The new phylogeny of nitrite-oxidizing bacteria, based on 16S rRNA sequences, shows that the best-known autotrophic nitrite oxidizer, *Nitrobacter*, comprises a coherent genus in the α-subdivision of the Proteobacteria. This genus is most closely related to the genera that contain autotrophic members and strains capable of denitrification (1). Like *N. oceani*, *Nitrococcus mobilis* belongs to the γ-subdivision of the Proteobacteria, the only example of both ammonia- and nitrite-oxidizing phenotypes occurring in the same subdivision. *Nitrospira* and *Nitrospina*, two strains originally isolated from marine environments, are assigned to the γ-subdivision of the Proteobacteria. A novel *Nitrospira* strain, *N. moscoviensis*, isolated from a heating system in Moscow, Russia, was assigned to a new genus outside the Proteobacteria (56). These authors reanalyzed the *Nitrosomonas marina* sequence that Teske and coworkers (1) had placed in the γ-Proteobacteria, and concluded that *Nitrospira* belonged outside the Proteobacteria, in a deeply branching cluster related to *Leptospirilla*.

Except for *Nitrospira* and *Nitrospina*, all the autotrophic nitrifiers cluster in phyla that are characterized by photoautotrophic ancestry, and are themselves characterized by the possession-complex intracytoplasmic membranes. These membranes are thought to be the site of the redox proteins involved in nitrogen oxidation, and therefore homologous with the photosynthetic membranes of photosynthetic bacteria and cyanobacteria. Nevertheless, the autotrophic nitrifiers are polyphyletic, and the phenotype has apparently arisen independently numerous times. The homology of the functional genes (*amo, hao*) involved in their physiology implies gene transfer events, however, rather than independent evolution of these enzymes. An understanding of the phylogeny of nitrifying bacteria is relevant to the study of nitrification in aquatic habitats because it has implications for detection and quantification methods. Although the nitrifiers are polyphyletic, they are not so diverse as to be unmanageable; their affiliation within a small group of lineages makes them amenable to identification and detection using a relatively small suite of molecular probes. This approach forms the basis of much current knowledge on the diversity and distribution of autotrophic nitrifying bacteria. A comprehensive reevaluation of the phylogeny and evolution of nitrifying bacteria should appear in the new edition of Bergey's Manual of Determinative Bacteriology, which is being prepared for publication at the time of this writing.

The phylogeny of heterotrophic bacteria is much more complex, and less well known. Various forms of heterotrophic nitrification have been reported for bacterial genera including *Bacillus*, *Paracoccus*, *Pseudomonas*, *Thermus*, *Azoarcus*, and the fungus, *Aspergillus*. The ability appears to be widespread, but no phylogenetic pattern has yet emerged, nor is there a consensus on the biochemistry of heterotrophic nitrification among these different groups.

The autotrophic ammonia oxidizers show significant metabolic and morphological similarities with another group of autotrophic bacteria, the methane oxidizers. They are also closely related phylogenetically to the methane oxidizers, in both the γ- and β-subdivisions (1). Before the availability of ribosomal RNA sequence data for determination of phylogenetic relationships, it had been reported that ammonia-oxidizing nitrifiers were capable of methane oxidation and vice versa (12,71,72). Thus, it was an interesting verification of the metabolic studies when it was reported that the central genes in the two pathways, that is, genes encoding AMO in the nitrifiers and methane monooxygenase in the methanotrophs, were evolutionarily related (73). It has not been possible to unequivocally determine the process that is the natural or predominant one for some nitrifiers and methanotrophs in nature (discussed in the following text).

ROLE OF NITRIFICATION IN THE NITROGEN CYCLE

The role of nitrification in the nitrogen cycle, whether on land or in aquatic systems, is to convert ammonium, a common nitrogenous waste product of heterotrophic metabolism, to nitrate, the most oxidized form of fixed nitrogen. Nitrate is the most abundant form of nitrogen in the ocean, and depending on the season and thermal stratification of the water column, can accumulate to very high levels in freshwater as well. Plants can use many forms of organic and inorganic nitrogen, and the transformation of ammonium into nitrate does not change the absolute availability of nitrogen for plant or algal nutrition. In soils, the different ionic properties of ammonium and nitrate are important in determining the availability of inorganic nitrogen in the soil solution, but in aquatic systems, the properties of these ions are less important to their distributions. Unlike nitrogen fixation, which introduces new fixed nitrogen into the system, and denitrification, which removes it from the system, nitrification simply changes the chemical nature and oxidation state of inorganic nitrogen. Because of the different chemical properties and varying preferences, abilities, and metabolic costs of using ammonium versus nitrate, and the role of nitrate as a substrate for denitrification, however, this transformation is very important in both agricultural and natural systems.

In aquatic systems, ammonium is often the first metabolic product of the breakdown of organic nitrogen; it is an excretory product of zooplankton and protozoans, and is released from the microbial degradation of

complex organic compounds. Because ammonium contains nitrogen at the oxidation level of proteins, it is readily assimilated by both phytoplankton and bacteria, and is a preferred nitrogen source. Therefore, it rarely accumulates in surface waters, it is assimilated as rapidly as it is produced by various members of the microbial food web. In fact, ammonia-oxidizing bacteria may be in competition for ammonium with other planktonic organisms. The different physiological requirements of phytoplankton and nitrifying bacteria, probably, play a role in determining exactly where in the water column ammonium assimilation and ammonium oxidation occur. As explained later, most nitrification occurs near the base of the euphotic zone in the upper 100 or so meters of the ocean. However, there is usually very little nitrate in the surface ocean, because of utilization by phytoplankton. The nitrate in the deep water of oceans and lakes has accumulated from nitrification in the absence of phytoplankton assimilation. It is because of nitrifying bacteria that nitrogen accumulation in the deep water is in the form of nitrate, rather than ammonium. The deep nitrate reservoir can be made available to phytoplankton by mixing and upwelling, and in lakes by seasonal overturn. These physical processes bring cold, deep nitrate-rich water up to the surface where, in the presence of light, phytoplankton can assimilate the nitrate. Thus, although nitrate is not usually abundant in surface water, it is a very important nitrogen source for phytoplankton.

The nitrate, which is produced by nitrification, especially that which accumulates below the euphotic zone, may have a fate other than assimilation by phytoplankton. Denitrification is another step in the nitrogen cycle that is also controlled by bacteria. Denitrifying bacteria are usually heterotrophs, and the capability for denitrification is widespread across the bacterial and archaeal domains (74). Denitrifiers use oxides of nitrogen as electron acceptors in the place of oxygen. In the process, nitrate is reduced sequentially to nitrite, then to nitric oxide and nitrous oxide, and finally, to dinitrogen gas. The last three products are all gases. Most organisms, which require nitrogen for nutrition, are unable to assimilate the gases; thus, denitrification results in a net loss of fixed nitrogen, that is, biologically available nitrogen, from the system. Although denitrification involves several semi-independent steps that need not function together, it is common for denitrifiers to begin the sequence with nitrate and to produce varying amounts of the other products depending on the environmental conditions. Thus, although denitrifiers might appear to have little in common with nitrifiers, the former are in fact dependent on the latter; other than lightning and fertilizers, nitrifiers are the only source of nitrate. The links between nitrification and denitrification will be discussed further in the context of the environments in which they occur (illustrated in the following text). The link is mentioned here to emphasize that, although nitrifiers by themselves do not change the fixed nitrogen inventory of the ecosystem, their activity makes possible the activity of denitrifiers, which are responsible for the major loss term in the global nitrogen budget.

Thus, the role of nitrifiers in the nitrogen cycle of aquatic systems is to link the oxidizing and reducing processes of the nitrogen cycle by converting ammonium to nitrate. This conversion is responsible for the fact that the major fixed nitrogen pool in the ocean is in the form of nitrate. The deep nitrate reservoir of oceans and lakes is a huge pool of nitrogen whose availability to phytoplankton is controlled largely by physical processes. The nitrification link also makes possible the loss of fixed nitrogen by denitrification, by converting nitrogen released as a waste product of animal metabolism into a form that can be respired by denitrifiers.

DISTRIBUTION AND ABUNDANCE OF NITRIFIERS IN AQUATIC HABITATS

Although most strains of ammonia-oxidizing and nitrite-oxidizing bacteria have characteristic intracytoplasmic membrane structures that can be visualized by electron microscopy, it is not possible to distinguish the otherwise nondescript cells from other bacteria in water samples using microscopic techniques widely used to visualize and enumerate bacteria, that is, epifluorescence microscopy with DNA fluorochromes. Most probable number (MPN) methods, relying on the appearance or disappearance of nitrite in dilution media, have been used to estimate the abundance of ammonia oxidizers and nitrite oxidizers, respectively, in aquatic environments. MPN approaches are widely used to estimate abundances of nitrifying bacterial population in soil, but there are very few modern reports using this technique in the water column of aquatic systems. Its application is only marginally more common in sediment studies (Table 1); McCaig and coworkers (60) used MPN to estimate the abundance of ammonium- and nitrite-oxidizing bacteria in polluted sediments underlying marine fish farms. In the sample taken from directly underneath the fish cage, nitrite oxidizers were abundant and ammonia oxidizers were not detected. Further away from the cage, ammonia oxidizers were detected, but nitrite oxidizers were not present above detection level at these stations. Using MPN in freshwater eutrophic lake sediments, Smorzewski and Schmidt (75) found that nitrite oxidizers always outnumbered ammonia oxidizers, by up to a factor of 8.1. Pauer and Auer (76), using MPN, found very low nitrifier population in the water of a hypereutrophic lake, whereas sediments harbored much higher population densities. Hall (52) estimated from the data in a number of lake studies that the efficiency of recovery for MPN was 0.05 to 0.001% for ammonia oxidizers. He also concluded that MPN abundances showed little correlation with observed nitrifying activities. This technique should, however, yield isolates of the most abundant cell type present, assuming it can grow under the enrichment culture conditions.

Schmidt and coworkers applied immunofluorescence (IF) to the study of nitrifying bacteria in soil and lake systems (83, 84,77). In many cases, IF was used to study the diversity of population (75,83) and not directly for enumeration. IF did provide, however, one of the first methods for the enumeration of specific populations that did not require enrichment culturing. The specificity

Table 1. Abundances of Nitrifying Bacteria Reported from Aquatic Systems

Location	Depth (m)	AOB Cell Abundance	NOB Cell Abundance	Method	Reference
		Sediments			
Freshwater lake sediments	Surficial sediments	$8–37 \times 10^5$ cells g^{-1}	$21–67 \times 10^5$ cells g^{-1}	MPN	75
sediments under marine fish farm cages	0.5-cm core top	Up to 0.2×10^6 cells m^{-2}	Up to 25×10^6 cells m^{-2}	MPN	60
hypereutrophic lake	Water column	10 cells mL^{-1}		MPN	76
	Sediments	10^5 cells mL^{-1}			76
Water Column Environments		*Cells mL^{-1}*	*cells mL^{-1}*		
Lake Itasca (MN, U.S.A.)	Oxic water column		*Nitrobacter* up to 1,000	IF	77
	Anoxic water column		*Nitrobacter* up to 100	IF	77
Rhine River	Wastewater effluent		*Nitrobacter* $10^6–10^8$	IF	78
Tamar River	0.5 m	Up to 90		MPN	79
Lake Plussee (Germany)	Oxic water column	*Nitrosomonas* 2.5×10^3		IF	80
Belauer Sea (Germany)	Oxic water column	*Nitrosomonas* 5×10^3		IF	80
Lake Bonney (Antarctica)	Oxic water column	*Nitrosomonas* 2×10^3		IF	81
	Oxic water column	*Nitrosococcus* 0.8×10^3		IF	81
Mediterranean lagoon	Oxic water column	*Nitrosococcus* $1–10^3$		IF	70
Peru upwelling	Ave over water column	*Nitrosomonas* + *Nitrosococcus* 0.3×10^3	*Nitrobacter* + *Nitrococcus* 0.2×10^3	IF	69
Southern California Coastal waters	Ave over water column	*Nitrosomonas* + *Nitrosococcus* 0.3×10^3	*Nitrobacter* + *Nitrococcus* 0.3×10^3	IF	68
Chesapeake Bay	Ave over water column	*Nitrosomonas* + *Nitrosococcus* 10^7		IF	82

Note: AOB — Ammonia-oxidizing bacteria, NOB — Nitrite-oxidizing bacteria, IF — Immunofluorescence, MPN — Most Probable Number.

of the assays could be optimized to distinguish among serotypes of the same species or to detect all serotypes within a genus. Stanley and coworkers (77) used a cocktail of antisera specific for different serotypes to enumerate *Nitrobacter* in the oxygenated-near surface and the anoxic deep layers of Lake Itasca (Minnesota, U.S.A.) (Table 1). Similarly, Montuelle and coworkers (78) detected several serotypes of *Nitrobacter* in wastewater effluent enter the Rhine river, and reported that the diversity of serotypes in the river varied with wastewater input. Feray and coworkers (85) compared IF with a standard MPN technique and an MPN/PCR method for enumeration of nitrite-oxidizing bacteria in freshwater sediments and found that immunofluorescence performed the best overall, although all of the methods suffered from low recovery of added cells. Sediments may be more problematic for the enumeration of bacteria than water samples, but such quantitative evaluations of enumeration techniques have not been routinely reported. Use of IF in soils has been less successful, and low detection levels have been attributed to the specificity of IF assays, such that many different antibody preparations are required to detect the multitude of serotypes present. PCR-based methods might be an improvement, if their specificity could be manipulated to include all variants within the physiological type of interest (e.g., all *Nitrobacter* strains) and no cells of any other group (discussed later).

Ward and coworkers have used IF to enumerate nitrifiers in several different aquatic habitats (Table 1), having shown initially that IF enumeration was accurate and precise when compared to acridine orange counts at dilute cell concentrations (86). In Lake Bonney, a permanently ice-covered lake in Antarctica, *Nitrosomonas* serotype cells were present at maximum concentrations just above the oxic–anoxic interface, and *Nitrosococcus* serotype cells were present in a similar distribution but at lower levels (81). *Nitrosomonas* type cells were also enumerated by fluorescence in situ hybridization (FISH) using oligonucleotide probes designed to be specific for the *Nitrosomonas*/*Nitrosospira* group of ammonia oxidizers (87). Maximum abundances detected by FISH were slightly higher than those detected by IF (81), and the distribution of cells detected by FISH differed slightly from that detected by IF, perhaps reflecting the different specificity of the antibody and nucleotide probes. FISH has been applied in systems in which higher cell numbers and higher growth rates are found [e.g., sewage sludge; (88)], but has not been widely reported for nitrifiers in natural waters.

In a small survey of marine and estuarine sites, Ward (82) reported that *Nitrosomonas* serotypes were more abundant than *Nitrosococcus* and that total abundance of ammonia oxidizers (*Nitrosomonas* and *Nitrosococcus* serotypes) ranged from 10^4 cells mL^{-1} in Chesapeake Bay to 10^2 cells mL^{-1} in inshore ocean waters and 10 to 10^2 cells mL^{-1} offshore. Maximum abundances occurred near the bottom of the photic zone in the vicinity of the primary nitrite maximum in some environments (69), but such characteristic patterns are not always detected (Table 1). *Nitrosococcus oceanus* abundance, determined by IF, was positively correlated with temperature, particulate organic carbon and nitrogen, and total bacterial abundance, and negatively correlated with dissolved oxygen, in brackish Mediterranean lagoons (70). There was no relationship between IF and MPN counts over the 20-month sampling period of this study.

The results from FISH and IF measurements show that nitrifiers, generally, do not contribute a very significant portion of the total bacterial assemblage to natural water, leading to the conclusion that individual species of nitrifiers represent on the order of 0.1% of the total bacterial assemblage in the water column. Even in lakes where nitrifying abundances were higher, nitrifying cells contributed less than 0.01% of the total bacterial abundance. Even in recirculating aquaculture biofilters, supplied with high levels of ammonia, nitrifiers represent only 20% of the total eubacterial rRNA (88). This generalization is consistent with the autotrophic nature of nitrifiers, which requires that individual cells process relatively large amounts of nitrogen for minimal return in terms of carbon assimilation.

METHODS FOR MEASURING NITRIFICATION IN WATER AND SEDIMENTS

Direct measurement of the rate of nitrification is problematic for several reasons related to the sensitivity of the methods and potential artifacts introduced by incubation methods. The easiest experimental design might be simply to incubate samples and measure the concentrations of nitrite or nitrate over time. This approach provided some of the earliest evidence for the occurrence of biologically mediated nitrification (47,89). Vaccaro (48) estimated nitrification rates by measuring changes in nitrite and nitrate in incubated samples, from depths up to 800 m in the Sargasso Sea, in the presence of added ammonium or mixed whole plankton. He reported that both additions stimulated the net production rate of nitrite or nitrate. In such an experiment, accumulation of nitrite or nitrate indicates net nitrification. A decrease in the concentration over time could be observed, however, even when nitrification is occurring, if consumption of nitrate or nitrite exceeds production in the incubation bottle. The low concentration relative to high biological demand for fixed nitrogen in the surface water of the ocean and many lakes means that tight coupling between production and consumption terms can obscure large fluxes.

The addition of specific inhibitors has been used as a modification of the simple nutrient-measurement approach just described. In this approach, chemicals that specifically inhibit either ammonium oxidation (e.g., acetylene, allylthiourea, methyl fluoride, N-serve) or nitrite oxidation (chlorate) are added to replicate incubation bottles (90,91). The method assumes that the nitrite concentration is at a steady state in the sample, and that nitrification is the only process that produces or consumes nitrite. Clearly, the bottles must be incubated in the dark to prevent consumption by phytoplankton. One needs to only measure the concentration of nitrite over time in the bottles in which ammonium oxidation was inhibited to estimate the nitrite-oxidation rate (equal to the rate of nitrite decrease). The rate of nitrite increase in the bottles to which the nitrite-oxidation inhibitor was added equals the rate of ammonium oxidation. There are some potential problems with this approach: (*1*) preventing production by phytoplankton, probably, has cascading effects on the activities of other microbes in the bottle, such that the rate of ammonium mineralization is reduced, thus changing the source term for the nitrification substrate. (*2*) Incubating in the dark may release the nitrifying bacteria from light inhibition such that the measured rate exceeds the in situ rate. (*3*) Incubations typically last 48 hours, which is sufficient to overcome the lag induced by light inhibition, but is also long enough to create quite unnatural conditions. The search for precisely specific inhibitor compounds has been extensive and has resulted in a plethora of potentially useful compounds. Many are problematic, for reasons not directly related to nitrification. For example, acetylene inhibits both nitrifiers and denitrifiers (92,93). Thus, its use to measure one process will also inhibit the other, and when one depends on the other (as is the case when denitrification depends on nitrification for nitrate), both rates are affected and the independent measurement of one is not possible. It is reported that the length of exposure to acetylene can be optimized to differentiate between its effects on nitrification and denitrification (94). N-serve is a commercial preparation that specifically inhibits ammonia-oxidizing bacteria (95) and it serves as the basis for the sensitive $^{14}CO_2$ method for the measurement of nitrification rates (discussed later). Being chemolithoautotrophs, nitrifiers fix carbon dioxide while oxidizing nitrogen. The amount of carbon dioxide fixation due to nitrifiers can be computed by difference between incubations, with and without addition of an inhibitor that specifically removes the contribution of nitrifiers (90,96,97). Then, a conversion factor is used to translate the carbon dioxide fixation into ammonium and nitrite-oxidation rates. This conversion factor has been shown to vary in the case of ammonia oxidizers by a factor of five in pure cultures (90,98), and thus its use introduces some uncertainty because the factor cannot be directly determined in field samples.

In addition, N-serve is not soluble in water, and so its addition to samples requires that it be dissolved in an organic solvent. This solvent can affect the other members of the community, and the use of ethanol has been shown to stimulate dark carbon dioxide incorporation by heterotrophic bacteria (3,79). Thus, in systems in which

nitrifiers are a very small part of the overall assemblage, the N-serve approach may yield artifacts (3,99,100).

Unfortunately, a direct radioisotope-tracer method is not very useful for measuring rates of nitrification in the environment. Capone and coworkers (101) demonstrated the use of ^{13}N to quantify nitrification rates, but the isotope is so short-lived (10 minutes) that its use is usually impractical. The other main approach to measuring nitrification rates directly is to use the stable isotope, ^{15}N, as a tracer (102,103). This approach is not without its problems, mainly due to the facts that ^{15}N has a significant natural abundance and that it must be measured using a mass spectrometer or emission spectrometer, both more expensive and difficult than using a scintillation counter for radioisotopes. Somewhat shorter incubations are possible (a few hours to 24 hours are commonly used). The signal of transfer of the tracer from substrate to product pool (e.g., $^{15}NH_4$ to $^{15}NO_2$) can be detected regardless of what other processes are occurring in the incubation (so in situ light conditions can be used) and no assumptions of steady state need be made. The major drawback of this method is the necessity to add a tracer, sometimes in excess of the natural concentration of substrate. This problem has been largely overcome by the advent of more sensitive mass spectrometers, however, and estimates obtained under conditions approaching in situ may be possible.

The ^{15}N approach is most useful in water samples because complete mixing of the tracer is possible. In sediments and soils, rate measurements are constrained by the inhomogeneous nature of the sample and the dependence of rates on the structure of the environment. In this situation, fluxes between overlying water and sediment cores can be analyzed to obtain areal rates. In conjunction with ^{15}N tracer addition, estimates of nitrification rates can be obtained from the dilution of nitrite or nitrate in the overlying water because of its production in the sediments (104). The isotope-pairing method for the measurement of denitrification (105,106) is essentially a modification of an isotope-dilution approach and provides information on nitrification rates as well. Inhibitor approaches similar to those described earlier for water samples have been used in sediments (107,108). The methylfluoride method (108,109) seems particularly promising because the gas can diffuse thoroughly into the core with minimal disturbance of microzones and gradients. This ammonium oxidation inhibitor is added to cores and the accumulation of NH_4^+ over time is assumed to represent the net rate of nitrification. Other processes that consume ammonium would lead to an underestimate of the rate.

To overcome the biasing resulting from uneven dispersal of tracer or inhibitor, sediment rate measurements are often made in slurries, which destroy the gradient structure of sediments, which is essential to the in situ fluxes. Slurries may provide useful information on potential rates, but not in situ rates. Even if rate measurements in sediments are made using whole-core incubations, for example, when the inhibitor is a gas, it is still difficult to obtain a depth distribution of the rate (usually, an areal rate is obtained). A sophisticated measurement and model-based system that avoids direct rate measurements has been used to overcome this problem. Microelectrodes that have very high vertical resolution are used to measure the fine-scale distribution of oxygen and nitrate in freshwater sediments. By assuming that the observed vertical gradients represent a steady state condition, reaction-diffusion models can be used to estimate the rates of nitrification, denitrification, and aerobic respiration, and to compute the location of the rate processes in relation to the chemical profiles (110,111). The major drawback to this approach is the lack of microelectrodes for nitrate that can be used in seawater (although recent developments in biosensors may overcome the carbonate interference that is problematic in seawater).

Incubation methods, whether enclosing water in a bottle or bag or sediment in a coring tube, may introduce unavoidable artifacts. Approaches that avoid incubations altogether are therefore attractive, but they are largely limited to measuring changes in chemical concentrations over time, and therefore can detect net transformation rates only. The compromise made by most investigators is to maximize the size and minimize the length of incubations to minimize artifacts associated with wall growth or preferential inclusion or exclusion of grazers.

DISTRIBUTION OF NITRIFICATION IN WATER AND SEDIMENTS

It was long ago recognized that nitrification must be a process of some consequence in aquatic systems; the major nitrogen product of organic matter decomposition is ammonium, but the huge volume of the deep ocean contains nitrate at 40 μM concentrations and ammonium at trace or undetectable levels. Therefore, it seemed obvious that nitrification must be occurring in the deep ocean. Nitrate concentrations in the surface ocean are usually maintained at low levels because phytoplankton assimilate nitrate more rapidly than it can be supplied by mixing or diffusion from the deep nitrate reservoir. Ammonium, which is produced in the photic zone by heterotrophic processes, is also usually immediately assimilated by phytoplankton before it can be nitrified. The recognition that ammonium and nitrate have different sources, and that the differences are important both physically and biologically, led to the application of the New Production Paradigm (112,113) as a way to understand phytoplankton nitrogen demand and growth in the surface ocean, and the subsequent flux of nitrogen to the deep sea and ocean floor (Fig. 2). Ammonium is considered a "regenerated" source of nitrogen because it is produced largely in situ, in the same water where phytoplanktons live and use it. The nitrogen in ammonium is recycled rapidly and repeatedly between living biomass (phytoplankton, the zooplankton which graze on them, protozoans, bacteria) and the inorganic nutrient form, which is released from heterotrophic metabolism and grazing.

Nitrate, on the other hand, is considered to be "new" nitrogen because it is not present in the euphotic zone most of the time. For phytoplankton to use it, nitrate must be

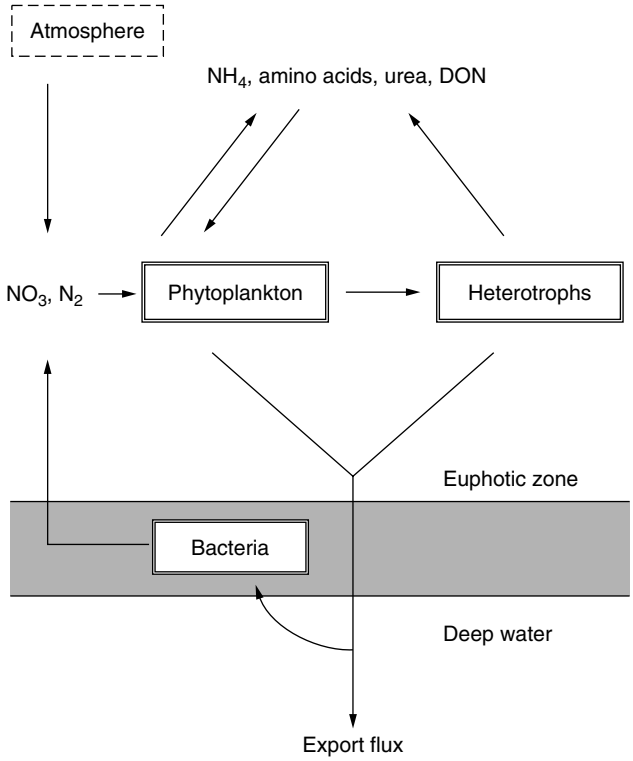

Figure 2. Schematic representation of the New Production Paradigm to explain the processes, which supply nitrogen to the euphotic zone of the ocean (after Eppley and Peterson, 1979). If the shaded area is below the euphotic zone, the nitrate produced there will enter the surface layer as a new nutrient (by physical processes). If the shaded area is within the euphotic zone, the nitrification occurring there will supply regenerated nitrate.

transported into the system by physical means, that is, by mixing or upwelling from deep water or falling in rain, and the rate of nitrate supply can be equated with the steady state rate of primary production on the basis of nitrate as a nitrogen source (113). This equality makes it possible to measure "new production," and by inference, the sinking flux of nitrogen, by measuring the assimilation of nitrate in incubated samples. This perception of nitrate as a new nutrient is consistent with the idea that nitrification, leading to the accumulation of nitrate, occurs in deep water, and not in the euphotic zone itself.

Nitrification Rates in Marine Systems

Actual measurements of the depth distribution and rate of nitrification, however, showed this picture, with most of the nitrification occurring in the deep ocean, to be an oversimplification. The greatest nitrification rates, both ammonium oxidation and nitrite oxidation, occur not in the deep ocean, but in a region near the bottom of the euphotic zone. In this depth interval, the light intensity is very reduced and phytoplankton are light-limited, and their rates of nutrient assimilation are therefore reduced. It is in this interval that nitrifying bacteria can compete with phytoplankton for ammonium; one often observes a sharp peak in nitrification rate at a depth in the water column where light intensity has been reduced to 5 to 10 % of surface light intensity (114,115).

Rates of nitrification reported for the open ocean are in the range of a few to a few hundred nanomolar per day, and have been detected as deep as 3,000 m (116). Rates of nitrification reported in various aquatic environments are presented in Table 2. Where profiles extending to a depth of several hundred to a few thousand meters are available, the main pattern that emerges is the association of the highest rates of ammonia oxidation with the lower region of the photic zone (Fig. 3). In the eastern tropical North Pacific, ammonia-oxidation rates at the maximum were no more than 20 nM d^{-1} (116). In the Peru upwelling region, a maximum rate of 747 nM d^{-1} was reported (115). In the temperate eastern Pacific Ocean, off western North America, maximum rates of 45 nM d^{-1} were reported (114). Nitrite oxidation shows a less predictable distribution with depth; in the Peru upwelling system, maximum rates of 600 nM d^{-1} were observed near the lower boundary of the euphotic zone, but high rates (e.g., nearly 300 nM d^{-1}) were observed within the oxygen-minimum zone (115).

Several studies of nitrification rates have focused on the primary nitrite maximum, rather than attempting complete depth profiles. Dore and Karl (97) reported a few rate measurements on the basis of inhibitor experiments from the central Pacific Ocean. Ammonium- and nitrite-oxidation rates were usually comparable, and were maximal just below the primary nitrite maximum. Bianchi and coworkers (128) reported nitrification rates up to 1 to 2 μM d^{-1} in the Rhone River Plume, with rates decreasing to the usual oceanic levels with increasing distance from shore in the Mediterranean Sea.

The rate of nitrification in deep ocean water is minimal, because of the decreasing flux of ammonium from organic matter decomposition with increasing depth. Thus, in many parts of the ocean, the typical depth-distribution of nitrification shows a subsurface maximum that occurs near the bottom of the euphotic zone, and very low rates persisting to great depths. The great accumulation of nitrate in the deep sea is, therefore, due to a small production term and a lack of any significant consumption terms.

Based partly on anecdotal evidence from culture work, observations in waste water treatment systems with very high particulate loads, and the prevalence of small particles in natural water, it has been suggested that nitrification occurs mainly on particles and is mediated by particle-attached bacteria (5). Phillips and coworkers (129) found nitrifier sequences associated both with particles and in the water (planktonic) in the northwestern Mediterranean Sea. The relative distribution of *Nitrosomonas* versus *Nitrosospira* clones differed between particles and planktonic samples, indicating niche preference by different species. The relative contribution to nitrification rates by particle-associated versus truly planktonic-nitrifying bacteria is difficult to assess. The distribution of rates as a function of depth indicates that rapidly sinking, that is, large, particles cannot be the main site of the process; nitrification associated with suspended small particles could be consistent with the typical subsurface rate maximum that is characteristic of oceanic water. Lavrentyev and coworkers (130) found that nitrifiers appear to escape predation

Table 2. Nitrification Rates Reported from Aquatic Systems

Location	Depth (m)	NH_4^+ Ox Rate (nmol l^{-1} d^{-1})	NO_2^- Ox Rate (nmol l^{-1} d^{-1}	Method	Reference
Water Column					
Sagami Bay, Japan	Photic zone ave, June light	50,000		^{15}N Tracer	117
Sagami Bay, Japan	Photic zone ave, June dark	26,000		^{15}N Tracer	117
Sagami Bay, Japan	Photic zone ave, October, light	38,000		^{15}N Tracer	117
Sagami Bay, Japan	Photic zone ave, October, dark	19,000		^{15}N Tracer	117
Western North Pacific	10–20 m	4.8–29		^{15}N Tracer	118
Western North Pacific	100–125 m	4.1–8.9		^{15}N Tracer	118
Southern California Bight	0 m	0–0.7		^{15}N Tracer	102
Southern California Bight	Subsurface max (approx. 70 m)	Up to 140		^{15}N Tracer	102
Central North Pacific Gyre	109–148 m	2.24–7.3		^{15}N Tracer	102
North Pacific, coastal	0 m	0.2–0.7		^{15}N Tracer	103
North Pacific, coastal	30–60 m	20–30		^{15}N Tracer	103
Southern California Bight	100 m	2–40	25–50	^{15}N Tracer	12
Southern California Bight	400 m	1–5	10–100	^{15}N Tracer	12
Eastern Tropical South Pacific	Subsurface maximum (50–79,81–101)	20–100	100–600	^{15}N Tracer	115
Eastern Tropical South Pacific	400 m	0–2	0–20	^{15}N Tracer	115
Eastern Tropical North Pacific	Subsurface maximum (80–100 m)	4–58		^{15}N Tracer	116
Eastern Tropical North Pacific	1,000 m	0–2		^{15}N Tracer	116
Eastern Tropical North Pacific	2,000 m	0–2		^{15}N Tracer	116
Cariaco Trench	0 m	0		^{15}N Tracer	119
Cariaco Trench	150 m	24–50		^{15}N Tracer	119
Cariaco Trench	>250 m	0		^{15}N Tracer	119
Black Sea	30	4.8	48	^{15}N Tracer	119
Black Sea	60–80 m	60	240	^{15}N Tracer	119
Central North Pacific	Approx. 160 m	Up to 120	Up to 125	Inhibitor, ^{14}C	97
Rhone River Plume NW Mediterranean	Surface plume summer	3,408 –4,200	1,536 –2,304	Inhibitor	117
Rhone River Plume NW Mediterranean	Surface plume winter	120–720	120–720	Inhibitor	120
Rhone River Plume NW Mediterranean	Nepheloid layer	720 ± 192	528 ± 144	Inhibitor	120
NW Mediterranean	Nitrite max, 40–60 m	120–270	50–160	Inhibitor	120
Southern Ocean	0 m	24	14.4–36	Inhibitor	91
Southern Ocean	50 m	24–43.2	28.8–48	Inhibitor	91
Southern Ocean	100 m	24–84	9.6–72	Inhibitor	91
Gulf of Mexico	70 m		4,800–12,000	$\Delta[NO_3^-]$, drogue	121
Grasmere (U.K.)	Hypolimnion, integrated		393–493 μmol N m^{-2} d^{-1}	$\Delta[NO_3^-]$	4

(*continued overleaf*)

Table 2. (*Continued*)

Location	Depth (m)	NH_4^+Ox Rate (nmol l^{-1} d^{-1})	NO_2^- Ox Rate (nmol l^{-1} d^{-1}	Method	Reference
Rivers					
Delaware, USA	2 m		Up to 400	^{15}N Tracer	122
York, U.S.A.	2–18 m		Up to 1,200	^{15}N Tracer	123
Tamar, U.K.	0.5 m		Up to 180	Inhibitor, ^{14}C	79
Chesapeake Bay	10 m		Up to 3,750	^{15}N Tracer	123
Sediments			*Areal Rate*		
Pond, Denmark (core incubation)	2 mm		1.3×10^4 nmol cm^{-3} d^{-1}	Microelectrode, diffusion model	124
Lake Vilhelmsborg, Denmark	2.5 mm		4,100 nmol cm^{-3} d^{-1}	Microelectrode, diffusion model	125
Onondaga Lake	Integrated		2.64×10^4 μmol N m^{-2} d^{-1}		76
Coral Reef Sediments, Australia	Integrated		Up to 1.6 mmol N m^{-2} d^{-1}	^{15}N dilution	104
Coral Reef, Enewetak Atoll	Surface, integrated				126
Sponges, Caribbean Sea	Surface, integrated		Up to 413 mmol N m^{-2} d^{-1}	DIN flux	127

by protozoans by aggregating into particles that are too large to be grazed. Thus, a trophic effect on nitrification is implied, which also has ramifications for the planktonic versus particle-associated question.

Distribution of Nitrification in Lakes

Nitrification is also recognized as an important nitrogen flux in lakes, but seasonality and individuality of lakes makes impossible generalizations, such as those that apply to the ocean. Hall reviewed studies before 1986 (52), and relatively few studies focused on rate measurements in lakes have been published since that time. Hall referred the reader to the review (52) and a short summary of that work is presented here. For typical temperate lakes that undergo seasonal stratification, nitrification rates are maximal at overturn or during the winter circulation period. Thus, nitrate builds up in the water column, and is subsequently depleted in the surface water after summer stratification sets in. Nitrification activity tends to be low in the summer, and to increase with depth. All of these aspects of the pattern of nitrification activity can be at least partially explained by ammonium availability. During stratification, ammonium is depleted in surface water by algal assimilation, and it builds up in the deep water as a result of organic decomposition. If the decomposition depletes oxygen in the deep water, nitrification is inhibited. When overturn occurs, ammonium and oxygen are mixed throughout the water column, stimulating nitrification. Ammonium concentrations may be greatest near the sediment–water interface, even in hypolimnions where oxygen is not completely exhausted, thus explaining the increase of nitrification rates with depth. Light inhibition has also been invoked to explain the increase in nitrification rates with depth (131) (illustrated later). The hypolimnion of seasonally stratified lakes is relatively isolated for months at a time, during which nitrate often accumulates. Nitrification is, generally, accepted as responsible for the accumulation, but whether it is mediated by planktonic bacteria or by bacterial population in the sediments apparently varies with ecosystem type. In more eutrophic lakes, the accumulation of nitrate is rapid after stratification, but a long period of stratification promotes depletion of the nitrate by denitrification.

Much recent work on nitrification in lakes and shallow systems has focused on the sediments in which where most of the nitrification activity is believed to occur. Sediments are typically layered systems in which nitrification and denitrification are closely coupled across the oxic/anoxic gradient. Pauer and Auer (76) concluded from their work in a hypereutrophic lake that the water column did not contribute significantly to nitrification in the system, and that the major site for nitrification was the sediment. Use of microelectrodes and isotope-pairing studies and reaction-diffusion models (106,110,111,132,133) mainly intended to quantify denitrification, and have quantified the close linkage between the two processes in lake and estuarine sediment systems. The main role of nitrification is to oxidize ammonium to nitrate, which is then consumed by denitrification. Nitrate does not buildup in the system. Concentrations of oxygen, ammonium, and nitrate are all important in regulating rates of nitrogen cycling, often in counterintuitive ways (discussed later).

The magnitude of nitrification rates in sediments can be much higher, and is certainly more variable than those reported from water column measurements (Table 2). The variability arises not only from the small-scale heterogeneity inherent in sediments (partly also because of bioturbation and association of nitrification with the walls of faunal tubes in the sediments), but from the wide range in the level of organic matter input into sediments in shallow water. Irrigation by worm tubes and sediment infauna has been shown to increase nitrification rates

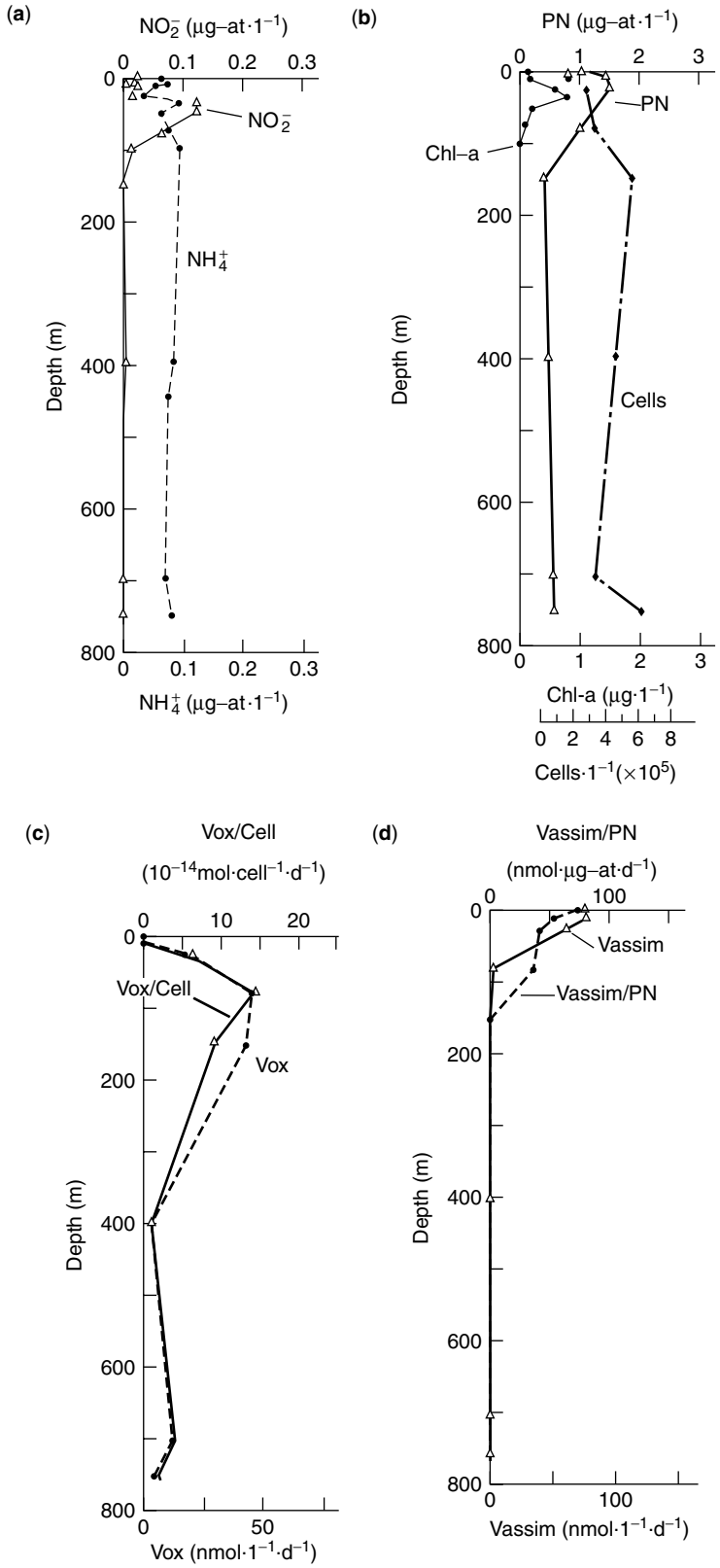

Figure 3. Distribution of variables at a station in the Southern California Bight. (**a**) Ammonium and nitrite. (**b**) Particulate nitrogen (PN), chlorophyll a (Chl-a), and ammonia-oxidizing bacteria (cells) enumerated by IF. (**c**) Ammonium-oxidation rate (Vox) and ammonium-oxidation rate normalized to cell number (Vox/cell). (**d**) Ammonium-assimilation rate (Vassim) and assimilation rate normalized to particulate nitrogen (Vassim/PN). With permission from B. B. Ward and A. F. Carlucci, *Appl. Environ. Microbiol.* **50**, (1985).

by increasing oxygen availability (134–136). In both deep and shallow sediments, nitrification can be one of the main sinks for oxygen in sediments (137,138). Very high nitrite and nitrate fluxes to the water column from sediments, including coral reefs (101,126) and sponge-dominated reef surfaces (127), have been attributed to nitrification associated with invertebrates and biotic sediments.

ENVIRONMENTAL VARIABLES THAT AFFECT NITRIFICATION RATES AND DISTRIBUTIONS

As mentioned earlier, in the general discussion of the depth distribution of nitrification rates, variables such as light intensity and substrate concentration are important determinants of the magnitude and location of nitrification rates. These are the kinds of variables that determine much of the biogeochemical cycling in the environment, and so their influence on nitrification is not surprising. Their effects have been studied, in both laboratory culture experiments and in field samples, using incubations and measurements of natural assemblages. Two useful overviews of research into the ecological and environmental factors that influence nitrification were published (2,5).

Temperature

The effect of temperature, although of potential importance in wastewater systems in which nitrifying bacteria are cultured under artificial conditions, is not generally considered to be an important environmental variable for nitrification because bacterial populations are generally adapted to the temperature of their environments. Thus, one can demonstrate a classical dependence of the rate of nitrification on temperature in any particular environment, but temperature is not generally the limiting factor. The optimum rate is usually attained at temperatures exceeding ambient temperature, but the observed rate of nitrification indicates that the in situ population is adapted to its ambient temperature. In other words, nitrifiers adapted to low temperature can nitrify under low-temperature conditions at rates comparable to the rates attained by nitrifiers adapted to higher temperatures living under high-temperature conditions. Thamdrup and Fleischer (139) found that the nitrification rate was highly adapted to temperature in arctic sediments, with temperature optima ranging from 14 to 40 °C, depending on the temperature of the environment. In the sediments of a eutrophic lake, bioturbation-stimulated nitrification rate and associated N_2O release at 15 °C, but there was no stimulation at 10 °C, implying perhaps complex trophic interactions and temperature in determining biogeochemical rate processes (136). Although temperature is an important master variable for biological processes, nitrification is, if anything, less sensitive to regulation by temperature than other processes, and is usually regulated in the environment by some other variable.

Inhibitory Compounds

Nitrifying bacteria, both ammonia oxidizers and nitrite oxidizers, but especially the former, are susceptible to inhibition by a wide range of compounds, and several different modes of action have been suggested (140). The two most common modes of action are (*1*) interference with the active site of the primary enzyme (i.e., AMO in ammonia oxidizers) by compounds that share structural homology with ammonia and (*2*) metal-binding compounds, which interfere with the action or availability of copper in the ammonia-oxidizing enzymes. In both ammonia and nitrite oxidizers, the susceptibility of key enzymes in the nitrification pathways form the basis of methods used to measure the rate of nitrification (as mentioned earlier).

In terrestrial systems, the presence of certain organic compounds (e.g., monoterpenes produced by plants) has been proposed to limit the rate of nitrification and the inhibition of nitrification in acid soils has long been of concern. The potential of naturally occurring organic compounds to inhibit nitrification in seawater has not been considered very much. Inhibition by organosulfur compounds has been demonstrated in cultured marine ammonia oxidizers (141) and the inhibitory effect of sulfide on nitrification is thought to limit nitrification and coupled nitrification/denitrification in marine sediments [discussed later (142)]. Although naturally occurring organic compounds have not been investigated as potential inhibitors in seawater, the product of their photodecomposition, carbon monoxide, has been implicated (143). Although carbon monoxide, like methane, acts as a substrate analog or a suicide inhibitor for ammonia oxidizers, the direct inhibitory effect of light on nitrifiers (see in the following text) is considered to outweigh the potential effect of carbon monoxide inhibition in surface water. As a substrate analog, carbon monoxide can be oxidized by ammonia-oxidizing bacteria, and R. D. Jones (144) proposed this method for quantification of ammonia-oxidation rates in natural systems. Like methane oxidation (12), however, the rate of carbon monoxide oxidation by nitrifiers depends on the relative concentration of alternative substrates, which may cause artifacts in relating overall rates of carbon monoxide oxidation to nitrification.

Light

The inhibitory effect of light was reported by German researchers in the 1960s (145,146), verified in enrichment cultures in seawater (147) and later described in more detail (148). Horrigan and coworkers (147) showed that even in enrichment cultures of nitrifiers derived from the sea surface film, nitrification was severely inhibited by light, such that periods of more than 12 hours darkness were necessary to allow net nitrification to occur over a 24-hour period. Vanzella and coworkers (148) found evidence that nitrite oxidizers were more sensitive to sunlight than were ammonia oxidizers, on the basis of single culture studies, but Guerrero and Jones (149) showed that species-specific responses might obscure any generalizations among major groups. Horrigan and Springer (150) reported that oceanic strains of ammonia oxidizers were, generally, more sensitive to light inhibition than were estuarine strains. Degree of inhibition was inversely correlated with ammonia-oxidation rate, but the mechanism of this relationship could not be determined.

Horrigan and Springer (150) argued that the greater sensitivity of oceanic ammonia oxidizers might be responsible for the generally lower rates of ammonia oxidation observed in oceanic versus river or estuarine environments, but the complex of other factors such as overall rates of material processing could not be independently evaluated.

Ammonia- and nitrite-oxidation rates are often reported to be highly coupled and to occur at about the same rate. This coupling would be consistent with the observation that neither ammonium nor nitrite accumulates at high levels in most natural waters. For nitrite, the exceptions to this rule are the primary nitrite maximum of near-surface water, and the secondary nitrite maximum characteristic of oxygen-minimum zones. The surface water feature has been attributed to two possible processes. Because the primary nitrite maximum is usually associated with low light intensities at the bottom of the euphotic zone, some interaction between biological processes and light is suspected. In one scenario, phytoplanktons are responsible; assimilation of nitrate (by reduction to nitrite and then to ammonium) requires energy, especially the step at which nitrite is reduced. Under low light intensity, phytoplankton might not have sufficient energy to reduce the nitrate completely, and some of the intermediate nitrite is allowed to leak out of the cell. In the alternative scenario (151), nitrifying bacteria are responsible; nitrite oxidizers are more sensitive to light inhibition than are ammonia oxidizers, and so ammonia oxidizers are able to be active at slightly shallower depths in the water column than are nitrite oxidizers. This leads to an accumulation of nitrite in the interval between the depths at which ammonia- and nitrite-oxidizers are released from light inhibition.

Several studies of nitrification rates in surface seawater from various geographic regions show profiles that are consistent with light inhibition (68,103,114,115,119). Simulated in situ rate measurements (i.e., measured under simulated in situ light conditions) show a clear negative relationship with ambient light intensity (Fig. 4). Nitrifiers may be somewhat protected from light inhibition in surface water by the presence of absorbant organic compounds in seawater. However, the data from simulated in situ rate measurements are consistent with light inhibition in surface water, and the well-verified sensitivity of nitrifiers in culture strongly suggests an influence of light in the environment. Dore and Karl (97) did not directly assess the effect of light on nitrification rates (all rates were measured in the dark) but they attributed two distinct features within the primary nitrite maximum to phytoplankton (the upper primary nitrite maximum) and nitrifiers (the lower primary nitrite maximum). Mortonson and Brooks (152) concluded that phytoplankton were responsible for a primary nitrite maximum in Lake Michigan, on the basis of seasonal correlations between chlorophyll and nitrite; however, direct experimental evidence was not available. The relative contribution of phytoplankton versus nitrifying bacteria to the maintenance of this feature is apparently still unresolved and both may be involved.

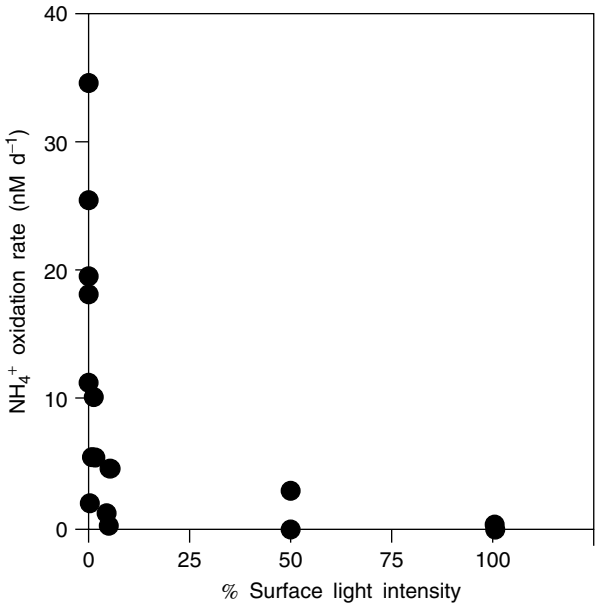

Figure 4. Ammonium-oxidation rate versus surface-light intensity for combined data from three stations in the Southern California Bight (approximately 33°20 N', 118°30′ W). Rates measured using ^{15}N tracer methods. With permission from B. B. Ward and A. F. Carlucci, *Appl. Environ. Microbiol.* **50**, (1985).

Lipschultz (153) documented the light inhibition of ammonia oxidation in the Delaware River, and concluded that this effect influenced the spatial distribution of nitrification in the estuary. Depending on their depth, light is not usually a problem for nitrification in sediments. In shallow sediments, light may have an indirect positive effect on nitrification rates by increasing photosynthesis, and thus increasing oxygen supply to the sediments [see in the following text; (124)].

Nitrification in the Euphotic Zone. Independent of their potential involvement in the primary nitrite maximum, it is significant for other reasons that nitrification rates are often maximal in the vicinity of the bottom of the euphotic zone. The important distinction made in the new production paradigm (as mentioned earlier), between ammonium, which is the "regenerated" nutrient and nitrate, the "new" nutrient is the basis of ^{15}N-tracer methods to assess new and regenerated primary production. However, if nitrification occurs in the same depth interval in which nitrate assimilation occurs, then nitrate too is a "regenerated" nutrient. Then, total nitrate assimilation would depend on a combination of nitrate supplied by nitrification and that supplied by mixing from the deep reservoir. Several studies have addressed the question of whether significant nitrification occurs in the euphotic zone and have concluded that in situ nitrification could supply 100% or more of the phytoplankton nitrate demand (91,97,154). Thus, nitrification and nitrate assimilation by phytoplankton can be closely coupled, although the two processes are favored by quite different environmental conditions.

Substrate Concentration

The influence of light may be compounded by the necessity for ammonia oxidizers to use ammonium, which is in short supply at those depth intervals in the water column where light is most intense. When ammonium assimilation and ammonium oxidation are measured in the same incubation, it is seen that assimilation occurs in the upper portion of the euphotic zone and nitrification in the lower portion (Fig. 3). This pattern suggests that in the well-lit upper water, phytoplankton are able to assimilate ammonium but nitrifiers are either unable to compete for ammonium in the presence of phytoplankton or else, light inhibition prevents them from using ammonium in that environment. In the lower portion of the euphotic zone, phytoplankton may be light-limited and unable to assimilate ammonium, whereas nitrifiers are released from light inhibition and able to use the ammonium being released by heterotrophic decomposition in that interval.

The influence of substrate concentration on nitrification rates is expected to be a first-order dependence and, indeed, this response is usually observed in culture. The instantaneous rate of ammonium oxidation or nitrite oxidation increases predictably with increasing ammonium or nitrite concentration in culture experiments. However, researchers often have been unable to demonstrate consistent substrate dependence for ammonia oxidizers in natural assemblages. A general relationship between measured ammonium-oxidation rate and ambient ammonium concentration supports the importance of substrate concentration (Fig. 5). Vaccaro (48) found that net nitrite and nitrate accumulation in samples from the Gulf of Mexico were stimulated by the addition of ammonium. In ^{15}N tracer experiments on water samples, when substrate additions are made, the micromolar level representative of the environment, ammonium oxidation, is usually independent of substrate concentration. Lack of response to substrate perturbation (102,155) has led to speculation that the affinity of ammonia oxidizers for ammonium may be so great that current experimental methods cannot detect a response to enhanced substrate concentrations. In contrast to ammonia oxidation, nitrite oxidation in natural samples usually shows a more conventional response to added substrate (102), but this second step in the nitrification process has received somewhat less attention.

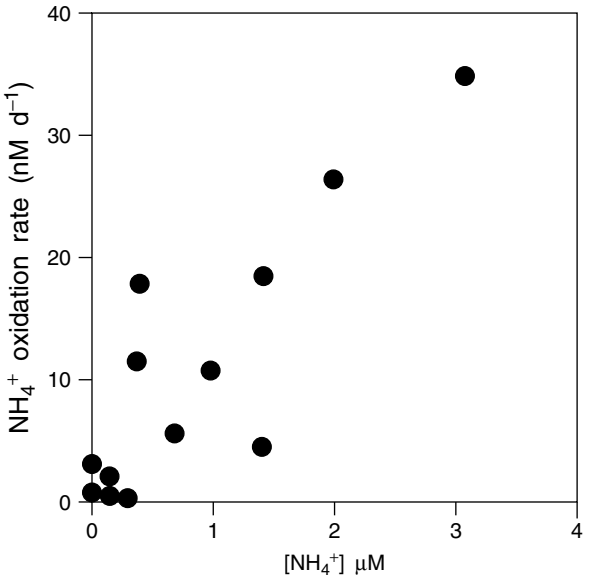

Figure 5. Ammonium-oxidation rate versus ambient ammonium concentration for combined data from four stations in the northeastern Pacific Ocean (approximately 47°07′ N, 125°W). Rates measured using 15 N tracer methods. With Permission from B. B. Ward and A. F. Carlucci, *Appl. Environ. Microbiol.* **50**, (1985).

Ammonium has been shown to have a direct effect on nitrification rates in freshwater sediments. In the absence of added ammonium, nitrification was restricted to a narrow interval in the sediment just above the denitrification zone. When ammonium was added to the core, oxygen became limiting and nitrification occurred throughout the oxygenated region of the core (125).

Oxygen Concentration

Oxygen concentration is also an important variable in determining nitrification rates; nitrifiers are reputed to be microaerophiles. Although requiring molecular oxygen for reactions in the nitrogen-oxidation pathways and for respiration, they are considered to thrive best under relatively low oxygen conditions. For example, Goreau and coworkers (156) reported that *Nitrosomonas* sp. marine grew best under an oxygen concentration of 1% in the headspace (compared with 20% in air) and this result has been extrapolated to nitrifiers in general. The proportion of ammonium that is oxidized to nitrite versus released as gaseous intermediates also varies with oxygen tension (see in the following text).

Microaerophily may be important in interface environments such as the sediment–water interface and in the oxygen-minimum zones of the ocean. In the sediment environment, for example, ammonium (derived from anaerobic decomposition in deeper layers) diffuses up toward the sediment–water interface and oxygen diffuses into the sediments from the overlying water. At the interface, nitrifiers find an optimum environment. In such an environment, nitrification can be directly linked to denitrification through their common intermediates (including nitrite, nitrate, nitrous, and nitric oxides). The nitrate produced during nitrification, along with the nitrate that diffuses into the sediments from the overlying water, helps support denitrification in subsurface sediments. In the sediment interface environment, the zone in which nitrification occurs may be less than a millimeter thick, or it may extend for several centimeters into the sediments, depending on the environment and the organic loading to the system.

The coupling between obligately aerobic nitrification and facultatively anaerobic denitrification is the classical way in which oceanographers view the linkage between the two processes in the environment. Vertical gradients of oxygen, nitrate, nitrite, and ammonium are consistent with the stratification of the processes imposed by the physiological constraints of the organisms in relation to the distribution of variables in the environment. The dependence of the depth distribution of nitrification and

denitrification on oxygen distribution has been shown elegantly in freshwater sediments (124). In this experiment, the oxygen penetration into microbial mat sediments varied on a diel light/dark cycle as photosynthesis proceeded at day and ceased at night. Oxygen and nitrate concentrations were measured using microelectrodes or biosensors and nitrification and denitrification rates were modeled using a reaction/diffusion model. Nitrification occurred only in the daylight when oxygen was available from photosynthesis. At night, photosynthesis ceased and oxygen penetration into the mat was insufficient to support nitrification. The counter effect of light potentially inhibiting nitrification was apparently not a problem; light penetration in sediments even during the day was not sufficient to inhibit. The absence of nitrification in surface sediments at night was mysterious, and was attributed to inhibition of nitrifiers by unknown chemical components of the sediments and pore water. Such direct demonstration of these interactions among photosynthesis and nitrification has not been possible in marine sediments to date, but it seems likely that the same relationships pertain, except for the confounding effect of sulfide.

Nitrifying bacteria living in marine sediments that are periodically or persistently exposed to anoxic conditions would need to be able to survive periods of inactivity or even serious inhibition to recover when conditions improved. The physiological basis of this survival or tolerance of anoxic conditions is unknown, and the degree to which nitrifiers can recover from serious anoxia is questionable. Joye and Hollibaugh (142) showed in microcosm studies that nitrification was almost completely inhibited by sulfide, the end product of bacterial sulfate reduction that occurs widely in anoxic marine sediments. A pulse of sulfide, which was detectable in the sediments for only a few hours, inhibited nitrification for at least 24 hours. Thus, in marine sediments in which sulfate reduction occurs, the ability of nitrifiers to respond to daily oxygen fluctuations may be impeded. Even when the oxygen–sulfide interface deepens during the day, nitrifiers may be unable to recover from the sulfide poisoning. In that case, both nitrification and denitrification (which is partially dependent on nitrate supply from nitrification) may occur at slower rates than would be predicted for similar environments in freshwater sediments (in which sulfate, and therefore sulfide, release from sulfate reduction is much less prevalent). In contrast, Jensen and coworkers (125) reported that nitrification could be stimulated essentially instantaneously by the addition of oxygen to sulfidic freshwater sediments. Using microelectrodes to measure oxygen and nitrate profiles in a sediment core, Jensen and coworkers (125) detected an almost immediate increase in nitrification rate when anaerobic sediments were aerated, implying that nitrifiers are inhibited but not poisoned by sulfide. When sediments were allowed to equilibrate with overlying water of differing oxygen concentrations, the zones of nitrification and denitrification stabilized at greater depths with increasing oxygen concentration, reflecting oxygen penetration into the core (Fig. 6; 111).

This picture may be further complicated by the suggestion that nitrifiying bacteria are in fact not obligate aerobes (as mentioned earlier). Although net nitrification and growth at the expense of inorganic nitrogen occurs only under aerobic conditions in autotrophic nitrifiers, both ammonia- and nitrite-oxidizing nitrifiers are apparently capable of partial or even complete denitrification. Loss of fixed nitrogen has been observed in cultures of nitrifying bacteria growing on reduced oxygen tension, including the report by Goreau and coworkers (156) cited earlier. Not only did the ammonia oxidizers grow best at 1% oxygen on a lithotrophic medium (no organic substrates), but they also produced the greatest amount of nitrous oxide relative to nitrite under those conditions. The annamox process leads to loss of fixed nitrogen as dinitrogen gas production from ammonium. Production of nitrous oxide, nitric oxide, and dinitrogen was reported for *Nitrosomonas* growing in the presence of organic compounds in the absence of oxygen (157). *Nitrosomonas* could also grow using hydrogen as an electron donor and nitrite as its electron acceptor (158). Nitrite oxidizers can grow by dissimilatory nitrate reduction in the presence of organic matter and the absence of oxygen (159).

The potential ecological impact of this physiological versatility in nitrifying bacteria has not been widely investigated in natural systems. (They have received much more attention in connection with sewage and wastewater treatment, in which there is economic incentive to enhance the conversion of ammonium to nitrogen gas under totally anaerobic or totally aerobic conditions.) The conditions that are conducive to denitrification by nitrifying bacteria are the same ones that induce denitrification in classical denitrifying bacteria. It is conceivable that both metabolic types are involved in the process. Thus, the net inorganic nitrogen distribution that we observe might be a more complex function of multiple processes than presently appreciated.

NITROUS OXIDE AND NITRIC OXIDE PRODUCTION DURING NITRIFICATION

The aspect of the nitrifiers' reductive metabolism that has received the most attention is the potential contribution of nitrifying bacteria to the production of trace gases such as nitrous and nitric oxide in the water column and sediments. Ammonium oxidizers produce both N_2O and nitrogen monoxide in culture, and the relative proportion of product that appears as gas increases at low oxygen concentrations (156,160). These gases are also intermediates in denitrification, but could derive from nitrification under low or zero oxygen concentration. Both N_2O and NO are involved in important atmospheric processes; they contribute to greenhouse warming and to catalytic destruction of stratospheric ozone. Thus, understanding processes that are responsible for their production could prove to be important for understanding or potentially regulating their fluxes.

A significant positive correlation between apparent oxygen utilization (AOU) and N_2O accumulation is often observed in marine systems (161,162). The relationship implies that nitrification is responsible for N_2O accumulation in oxic water, where it is released as a by-product

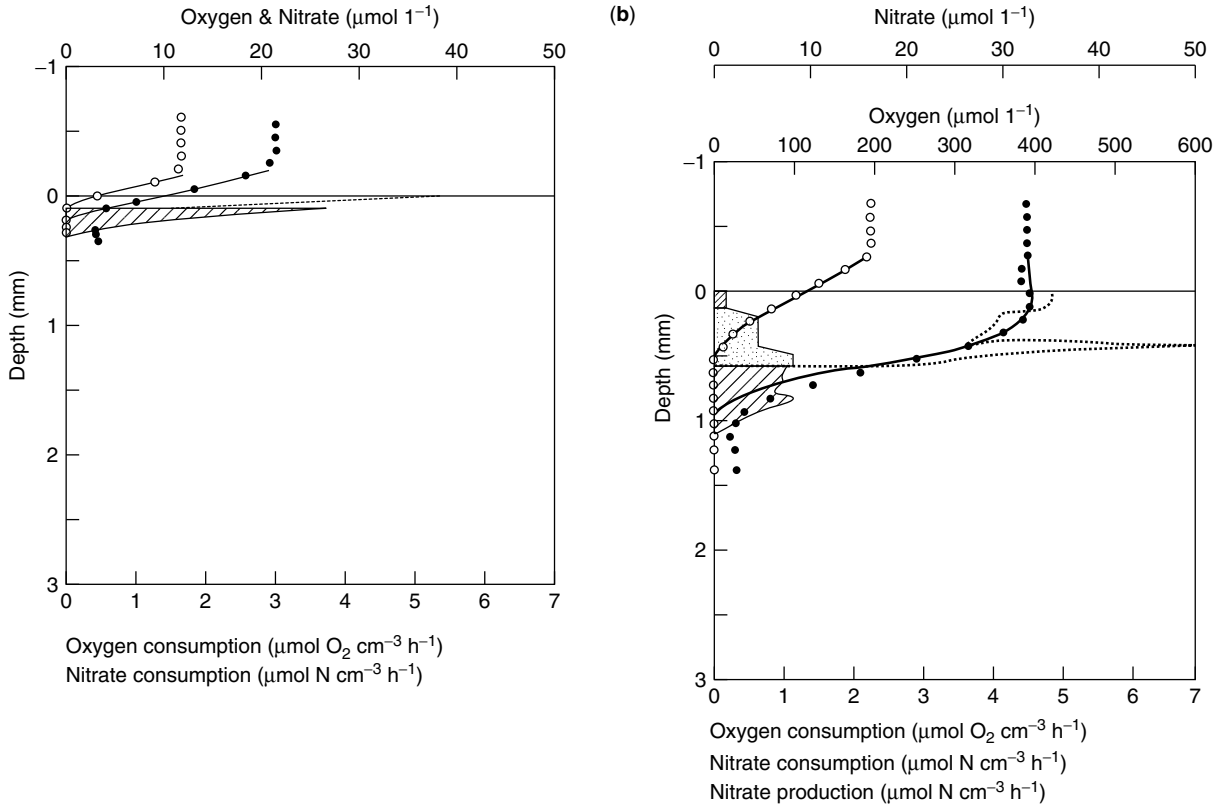

Figure 6. Steady state concentration profiles of oxygen (O) and NO_3^- (•) measured in sediment incubated in a continuous flow-through system with 10 (A), 200 (B), 400 (C), and 600 (D) μ MO_2 in the overlying water. The modeled oxygen and NO_3^- profiles are indicated by solid lines (diffusion-reaction model). The distribution and magnitude of the volume-specific activities of oxygen consumption, net NO_3^- production, and NO_3^- consumption are indicated by the white (enclosed by the broken line and the axes), the stippled, and the hatched areas, respectively. Note the differences in scales. The sediment was incubated with no NH_4^+ in the overlying water. With permission from K. Jensen et al., *Appl. Environ. Microbiol.* **60**, 2,094–2,100 (1994).

of mineralization going to completion by nitrification. The relationship breaks down at very low oxygen concentrations, in which N_2O is usually below atmospheric saturation because of consumption by denitrification. In a few special places in the open ocean, oxygen concentration is depleted to a level low enough to allow denitrification to occur in the water column. These regions, referred to as *oxygen-minimum zones*, occur off the coast of Peru (eastern tropical South Pacific), in the Arabian Sea, and the eastern tropical North Pacific Ocean (off the west coast of Mexico). The coupling between nitrification and denitrification has also been studied in these systems, which are essentially analogous to the sediment environments described earlier, except that the oxygen and nitrate gradients extend over tens to hundreds of meters. Suboxic and anoxic water and sediments tend to have large fluxes, and sometimes large accumulations, of the gaseous intermediates of nitrification and denitrification. This is probably caused by the sensitivity of the various organisms and enzymes involved in their production and consumption to oxygen concentration in the local environment of the microorganism.

In studies of nitrogen cycling in oxygen-minimum zones, nitrification and denitrification appear to be linked, as might be expected from analogy with sediment systems. A schematic that describes the approximate locations of nitrifying and denitrifying activities relative to the chemical distributions typical of an oxygen-minimum zone are shown in Figure 7. Ammonium oxidation typically is maximal near the bottom of the euphotic zone close to the upper boundary of the oxygen-minimum zone (115), but nitrite oxidation is detected within the oxygen-minimum zone itself. This finding is consistent with the ability of nitrite oxidizers to persist and metabolize at very low or zero oxygen conditions, but their metabolism under these conditions would be expected to be dissimilatory rather than oxidative. The nitrous oxide that typically accumulates in the suboxic (low but not zero oxygen concentrations) regions of the oxygen-minimum zones is thought to be caused by nitrification because nitrous oxide is depleted in the core of the oxygen-minimum zone where denitrification rates are thought to be greatest. A direct tracer confirmation of this pathway in nature has not yet been accomplished. Stable isotope measurement of nitrous oxide from oxygen-depleted water in the Arabian Sea imply that both nitrification and denitrification may contribute to the signal (116,163) attributed the nitric oxide production they observed in the oxygen-minimum

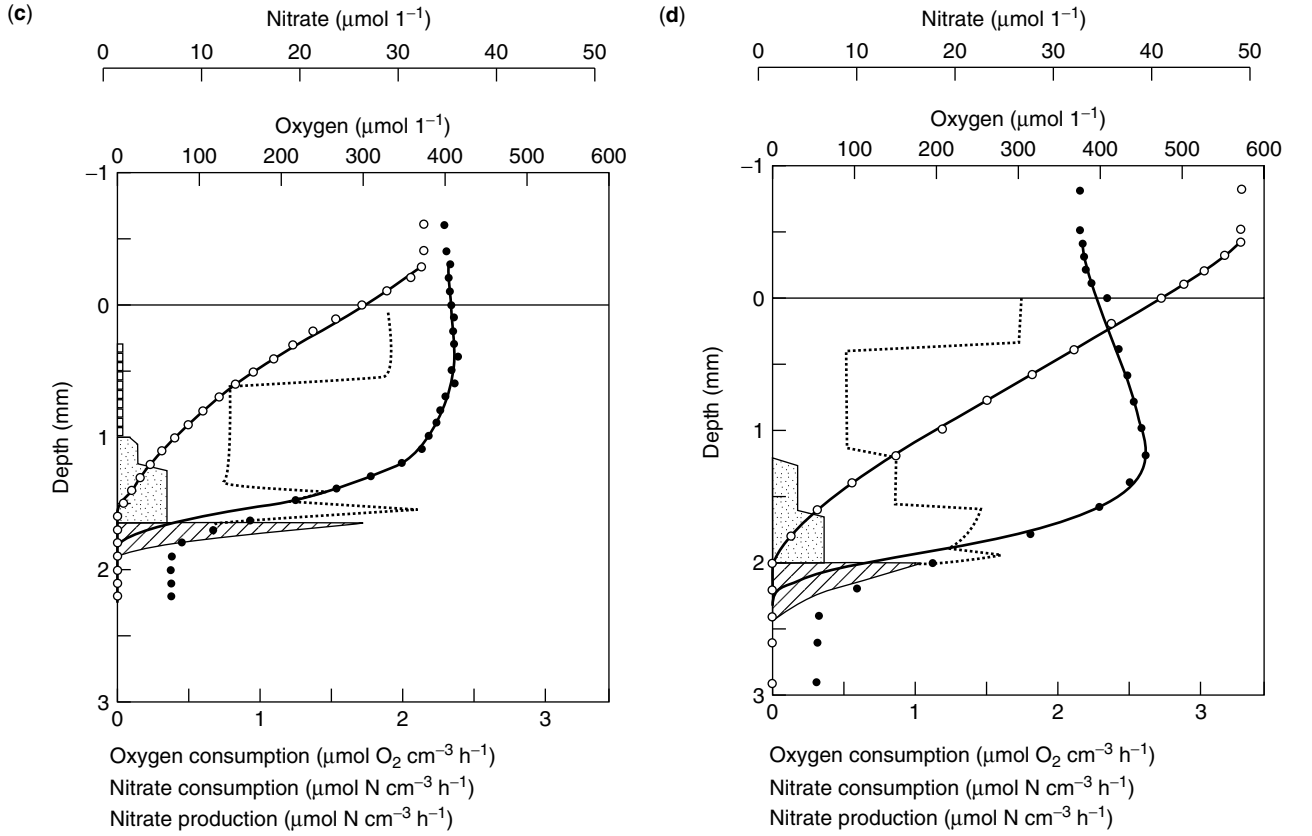

Figure 6. (*Continued*)

zone of the eastern tropical North Pacific to nitrification and found that it was equivalent to 18% of the total ammonia-oxidation rate.

Nitrous oxide accumulations have also been reported in lakes; the most notorious of these are the lakes in the McMurdo dry valley region of Antarctica. Priscu and coworkers (164) reported concentrations of N_2O in Lake Bonney that were equivalent to 580,000% saturation with respect to the global average atmospheric N_2O concentration. In incubations of several days duration with added acetylene, the concentration of N_2O did not change, leading Priscu and coworkers (164) to conclude that nitrification, rather than denitrification, was responsible for the buildup of the gas. Nitrous oxide is present in several of the dry valley lakes at much lower, but still supersaturated, levels, and the source is likely to be nitrification. These lakes show a relationship between nitrous oxide accumulation and AOU similar to that observed in the ocean, except that the ratio of N_2O production to AOU is much greater, implying that a higher proportion of ammonium is nitrified to the trace gases, rather than completely to nitrate (165).

NITRIFICATION AND METHANE OXIDATION

As mentioned earlier in connection with the physiology of nitrifying bacteria, the ammonia oxidizers and the methanotrophs have important biochemical similarities. These similarities extend to the nature of the primary enzyme in the ammonia- and methane-oxidation pathways, the sensitivity of the enzymes to a wide range of metabolic inhibitors, the metabolic capabilities of the cell and to the ultrastructure of the cell (140). Methanotrophs, like ammonia oxidizers, depend on two substrates with, generally, opposite sources. Methane, like ammonium, accumulates in anoxic habitats where it is produced by strictly anaerobic methanogens. Oxygen diffuses into surface water or surface sediments from the overlying oxygenated habitats. The classical environment for significant contributions by methanotrophs to system-wide carbon cycling is stratified lakes, in which a large fraction of the annual carbon fixation is cycled through methanogenesis and methane oxidation. The methanotrophic activity is highest at the interface where oxygen and methane coincide. As described earlier for sediments, such an interface would also be an interface for ammonium and oxygen and might be expected to harbor high nitrification activity as well.

Because of the presence of high sulfate concentrations in seawater, methanogenesis is not as important in marine sediments and seawater as it is in freshwater systems. Nevertheless, there are a few marine environments in which methane is found. The situation in Scan Bay or Cape Lookout Bight sediments, or in the water column of the Black Sea or the Cariaco Basin, is largely analogous to that in stratified lakes (as mentioned earlier).

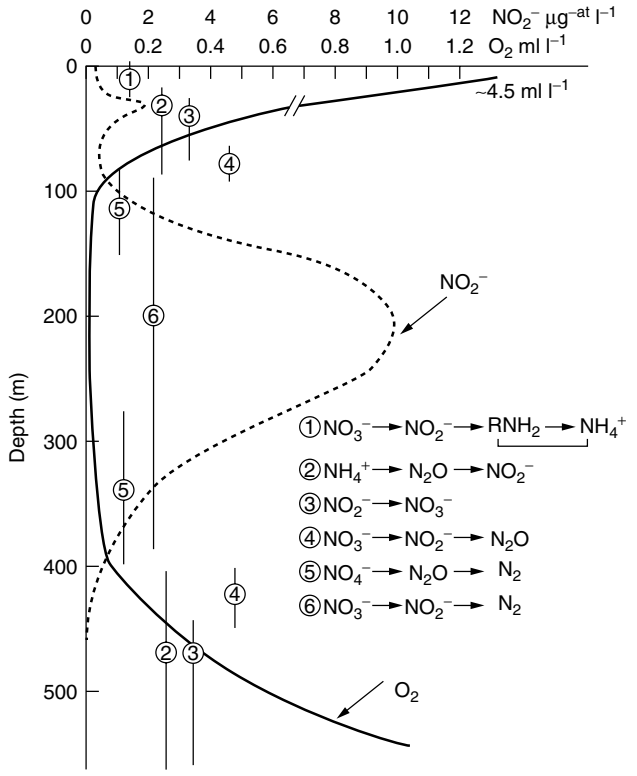

Figure 7. Schematic or expected chemical and activity distributions in the oxygen-minimum zone off Peru. Numbers in circles represent simplified pathways (shown in the figure) and the lines associated with each circled number denote the depth interval over which the activity is expected to occur. With permission from L. A. Codispoti and J. P. Christensen, *Mar. Chem.* **16**, 277–300 (1985).

The observation from culture studies that methane oxidizers are capable of oxidizing ammonia, and that ammonia oxidizers are capable of oxidizing methane, has led to uncertainty about the organisms that are responsible for observed methane and ammonium fluxes in nature. Although both groups of microorganisms show similar regulation by environmental variables and similar sensitivities to a variety of inhibitors (140), the possibility for differential regulation based on substrate affinity or competition suggested that "cross oxidation" might have important implications for the rate of ammonium or methane oxidation in nature. On the basis of a combination of simulated in situ rate measurements, inhibitor studies, and kinetic experiments with natural assemblages, it has been concluded largely that methanotrophs are mostly responsible for methane oxidation and nitrifiers for ammonium oxidation in both freshwater and marine environments (155,166). These conclusions do not rule out a role of cross oxidation or participation by both groups in some environments, and the two processes remain problematic to separate entirely in the environment.

CONCLUSION

The nitrogen cycle warrants study in aquatic environments because of this element's role as the macronutrient most likely to be limiting for primary production. The diverse compounds and wide range of oxidation states in which nitrogen occurs allow this element to play many different roles in the chemistry of the atmosphere and aquatic systems, and in the biochemistry of organisms. Most of the transformations in the nitrogen cycle are solely the domain of microorganisms, and classical autotrophic nitrifying bacteria exemplify this. The diversity and phylogeny of microbes involved in nitrification includes a variety of heterotrophs, and may include as yet unidentified organisms with novel metabolisms, yet to be elucidated. This is particularly true for sediment systems in which anaerobic reactions, either biotic or abiotic, may have been underestimated in the past.

In recent years, our understanding of the rates and distribution of nitrification in the open ocean has converged on a model in which the rates are greatest in the upper water column, within the euphotic zone, such that nitrification is linked to nitrogen assimilation by phytoplankton. The other major site for nitrification in the ocean is the sediment–water interface and surficial sediments, where nitrification is tightly linked to denitrification. Sediments are, usually, the dominant site for nitrification in many freshwater habitats. The metabolic diversity of nitrifiers, although limited compared with many other microorganisms, is greater than that usually admitted by simple models of processes in relation to environmental variables such as oxygen. These aspects of environmental regulation of nitrification and the diversity of organisms involved in nitrification remain interesting areas of fruitful research.

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NITRIFICATION IN SOILS.

See Soil Nitrogen Cycle

NITRIFICATION IN THE MARINE ENVIRONMENT.

See Denitrification in the Marine Environment

NITRIFIERS TYPING USING MICROCHIPS.

See Biochip-Based Devices and Methods in Microbial Community Ribotyping

NITRIFYING BACTERIA IN DRINKING WATER

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Nitrification is an important step in the global nitrogen cycle. During nitrification, ammonia is converted sequentially to nitrite and nitrate. This process can be performed either by heterotrophic or lithotrophic organisms. In the case of lithotrophic nitrification, the genera of bacteria that convert ammonia to nitrite are referred to as ammonia-oxidizing bacteria (AOB), and those that oxidize nitrite to nitrate are termed nitrite-oxidizing bacteria (NOB). These bacteria, collectively referred to as nitrifiers, are ubiquitous in aquatic and soil habitats and can grow under a variety of environmental conditions. Heterotrophic nitrification is carried out in soil and freshwater environments by fungi and several bacterial genera.

Nitrification via the growth of AOB and NOB in drinking water can have a deleterious effect on water quality. Studies have shown that many of the environmental conditions favoring the growth of nitrifying bacteria also occur in many drinking water distribution systems. These include the presence of ammonia as a nutrient source, warm temperatures, and neutral or slightly basic pH conditions. The proliferation of these bacteria in the distribution system can result in the production of nitrite and nitrate from ammonia, the breakdown of the ammonia-based disinfectant monochloramine, and an increase in heterotrophic plate-count (HPC) bacteria.

Although water agencies have observed the effects of nitrification, relatively sparse information exists in the literature on the ecology and occurrence of nitrifying bacteria in water systems. This topic is gaining importance with the growing number of water agencies converting from chlorine disinfection to monochloramine disinfection in the distribution system in order to limit the production of chlorinated by-products. Identification of favorable growth conditions can lead to the development of prevention and control measures. Improved methods for the measurement of bacteria are also needed because current culture-based methods greatly underestimate the actual numbers of nitrifiers present, are cumbersome, and require weeks of incubation before results are available. Early identification of nitrification symptoms in water is important for a utility to prevent the occurrence of nitrification and its subsequent effects on water quality.

BIOLOGY AND PHYSIOLOGY

Lithotrophic nitrifying bacteria are found in a variety of shapes, including straight or curved rods, spheres, spiral, and lobular forms, ranging in size from approximately 0.3 to 11.7 μm (Fig. 1). They typically form aggregates in nature and are motile by polar or peritrichous flagella (1–3). They are gram-negative facultative aerobes and are characterized by intracellular membranes. These bacteria, once classified in the family *Nitrobacteraceae,* have been reclassified into three separate subdivisions (alpha, beta, and gamma, under the division *Proteobacteria*), based on 16S ribosomal RNA analysis (1,2).

There are five genera of AOB and four genera of NOB, which are classified by the cell shape or arrangement of intracytoplasmic membranes (Table 1). All AOB except *Nitrosococcus oceanus* are categorized in the beta subgroup of purple photosynthetic bacteria. *Nitrosococcus oceanus* is in the gamma subdivision. NOB are found in the alpha, beta, and gamma subdivisions (2).

Table 1. Lithotrophic Nitrifying Bacteria Genera

Ammonia-Oxidizing Bacteria
Nitrosomonas — straight rods, intracellular membrane in peripheral cytoplasm
Nitrosococcus — spheres, intracytoplasmic membranes in peripheral or central cytoplasm
Nitrosospira — tightly wound spirals
Nitrosovibrio — slender, curved rods; lacking extensive intracellular membranes
Nitrosolobus — pleomorphic lobate cells partially compartmentalized by the cytoplasmic membrane
Nitrite-Oxidizing Bacteria
Nitrobacter — pleomorphic rods to coccoid cells
Nitrospina — straight slender rods
Nitrococcus — spheres
Nitrospira — spiral to vibrio

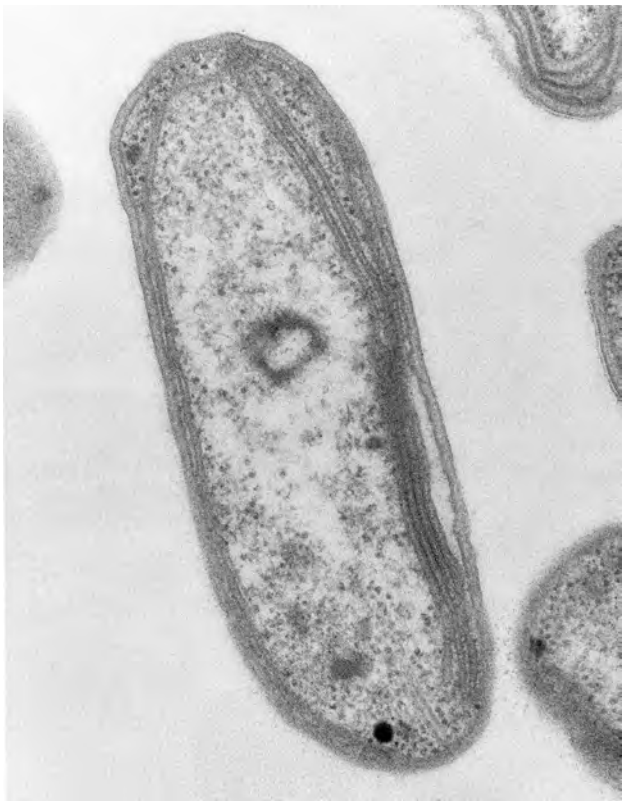

Figure 1. Transmission electron micrograph of nitrifying bacteria (4). Reprinted by permission of the American Water Works Association copyright © 1993.

AOB are obligate chemolithoautotrophs and obtain their energy source by oxidizing ammonia (NH_3) to nitrite (NO_2^-) through the intermediate step, hydroxylamine (NH_2OH). NOB then oxidize nitrite to nitrate.

Ammonia-oxidation reactions are illustrated in the following equations (5):

$$NH_3 + O_2 + 2H^+ + 2e^- \rightarrow NH_2OH + H_2O \quad (1)$$

$$NH_2OH + H_2O \rightarrow NO_2^- + 5H^+ + 4e^- \quad (2)$$

Conversion of NH_3 to NH_2OH is believed to be catalyzed by a monooxygenase (3). The oxidation of NH_2OH to NO_2^- is catalyzed by hydroxylamine oxidoreductase (HAO). The energy produced during the oxidation of NH_2OH to NO_2^- is NADH (3).

AOB use carbon dioxide as the main source for cellular carbon via the reductive pentose phosphate cycle. However, all ammonia oxidizers are able to metabolize selected organic compounds to a limited extent in the presence of an inorganic energy source. In media supplemented with formate and acetate and, to a lesser extent, pyruvate, the growth of AOB was enhanced by 130% (3). However, these compounds may inhibit growth at elevated concentrations (3).

With the exception of *Nitrobacter*, NOB are obligate lithoautotrophs. In the presence of organic substances, some strains of *Nitrobacter* can grow mixotrophically or heterotrophically (2,3,5), but at a slower rate and with less efficiency. *Nitrobacter* have been shown to be closely related to *Photopseudomonas palustris* by cell morphology, ultrastructure, and 16S rRNA. Some strains of *Nitrobacter* have been found to be facultative aerobes (3).

Nitrite oxidation occurs via the following reaction:

$$NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^- \quad (3)$$

Energy is derived from the oxidation of nitrite to nitrate, providing NADH and ATP for carbon dioxide fixation. Nitrite oxidoreductase (NO_2^-–OR) catalyzes this process. NOB fix carbon dioxide via the reductive pentose phosphate cycle (2,3,5). In the genus *Nitrobacter*, ribulose-1,5-bisphosphate carboxylase/oxygenase (Ribulose BisCO) is responsible for fixing carbon dioxide. Interestingly, nitrite oxidation appears to be reversible; NO_2^-–OR can reduce nitrate to nitrite under anaerobic conditions (3,5). In addition to growing heterotrophically on acetate, formate, and pyruvate, *Nitrobacter* can utilize glycerine (3,5).

Environment

Nitrifiers are ubiquitous in nature and can grow in a variety of habitats under a range of conditions. They are found in freshwater, soil, brackish water, sewage, and potable water. Nitrifiers can be found in temperatures ranging from 4 to 60 °C and at pH levels ranging from 4.6 to 9.0. Optimum growth, however, occurs at temperatures between 25 and 30 °C, at pH levels between 7.5 and 8.1,

and at substrate concentrations between 1 and 25 mM (6). The generation time for AOB ranges from 8 to 24 hours (3), depending on the genus and environmental conditions. For NOB, the generation time is slower, typically between 10 and 150 hours. The optimum pH and temperature for NOB are 7.6 to 7.8 and 28 to 30 °C, respectively. *Nitrobacter* is very sensitive to high oxygen partial pressure and to the presence of ammonia and nitrite. In the nitrification process, acid is produced by AOB, thus reducing the pH, making an enclosed environment (i.e., batch culture) less desirable for AOB, and inhibiting growth (3).

Nitrifiers are very sensitive to visible and ultraviolet (UV) irradiation and even fluorescent lighting (5). Alleman and coworkers (7) found that *Nitrosomonas* was completely inhibited after 10 minutes of exposure to light, but recovered 40% by excision repair.

Identification and Enumeration

Nitrifiers can be identified by function, morphology, and location of intracellular membranes. Transmission electron microscopy can be used to determine the location of the membrane characteristic of each species. Because nitrifiers grow slowly, the culture or enumeration of nitrifiers is a lengthy and tedious procedure, requiring several weeks to several months of incubation. Colony formation on agar plates is typically small (0.2 mm) and tan-brown in color. Enrichment media containing either ammonia or nitrite are used to confirm AOB or NOB metabolic processes. Selective media are also used in enumeration by most-probable-number (MPN) methods (1,2,6,8). After incubation on enrichment media, wells or tubes inoculated with AOB or NOB samples are spot-checked for the presence of nitrite or nitrate. Recovery with selective media has been reported to have a low recovery efficiency of 0.1 to 1% for environmental samples (9).

Other methods of detection include immunofluorescent and molecular-based technologies. Labeled bacterial cells can be detected by epifluorescent microscopy or enzyme-linked immunosorbent assay (ELISA). Quantification may be obtained by estimating positives from serial dilutions or MPN methods. Because of the strong strain specificity of the antibodies, the practicality of immunobased methods is limited for environmental monitoring, as numerous serotypes of nitrifying bacteria are found in nature. Future development of these techniques may involve monoclonal antibodies with more general specificity (5). Molecular methods for detecting nitrifiers target either the genes for the 16S ribosomal DNA or a specific functional gene such as ammonia monooxygenase or nitrite oxidoreductase (5). Because nitrifiers are in three subdivisions (based on 16S ribosomal DNA), group-specific probes based on 16S ribosomal sequences are more difficult to make. However, molecular methods do have a number of advantages for detection of nitrifiers in the environment. Polmerase chain reaction (PCR) technology can be used to detect low levels of nucleic acid in the environment, and targeting mRNA makes it possible to detect viable or metabolically active nitrifying cells.

ECOLOGY AND OCCURRENCE IN POTABLE WATER

Ecology in Water

Depending on the treatment application and the disinfectant, biological nitrification can be either a benefit or a nuisance in potable water. Nitrification is beneficial whenever the elimination of natural sources of ammonia or nitrite is desirable because ammonia and nitrite exert considerable chlorine demand and produce biologically unstable water in distribution systems (10). Under this scenario, nitrifying bacteria are allowed to increase to high levels in the filter beds or in the clarifiers, where they convert the ammonia to nitrate (10,11). Because nitrate does not exert an appreciable chlorine demand on chlorinated water, nitrification results in lower chlorination costs.

Partial or incomplete nitrification in distribution systems that use chloramines can adversely affect water quality. Inorganic chloramines are disinfectants formed by combining chlorine and ammonia. Inorganic chloramines form fewer chlorinated by-products and have a more persistent residual in the distribution system than chlorine. When nitrification occurs in chloraminated water, the total chlorine residual and ammonia residual decay rapidly, and an increase in nitrite levels is observed (Fig. 2; 10,12,13). This is because AOB oxidize the excess ammonia to nitrite, which has significant chlorine demand (10). Nitrite has also been shown to accelerate breakdown of the chloramine residual (14). Other evidence of nitrification may include a decrease in pH and dissolved oxygen level in the water (10,15). A loss of disinfectant residual can lead to a substantial increase in HPC bacteria (12). Nitrite accumulates because the conversion of nitrite to nitrate appears to occur slowly in chloraminated potable water. The oxidation of nitrite to nitrate has been observed to occur when the chloramine residual is lower than that for ammonia oxidation; this suggests that NOB are more susceptible than AOB to disinfection by chloramines.

Occurrence in Water

Nitrification has been reported to occur in water distribution systems using chloramines throughout the United States, Europe, and Australia. In 1993, a survey of water utilities was conducted to determine the extent of nitrification in the United States (15). The criteria for nitrification indicators included loss of chloramine residual, elevated heterotrophic bacteria, increased nitrate or nitrite, depletion of oxygen, and other evidence. The results indicated that 63% of the 42 utilities surveyed that serve over 50,000 customers had some indication of nitrification (Fig. 3). Region or climate did not seem to make a difference in repeated occurrence of nitrification problems.

Although many utilities may have nitrifiers in source waters and in effluents from the treatment plants (11,16), nitrification episodes tend to occur in the distribution systems, where conditions are optimal for the growth of AOB (12,16,17). Because AOB grow relatively slowly, they are frequently found in those areas of the distribution

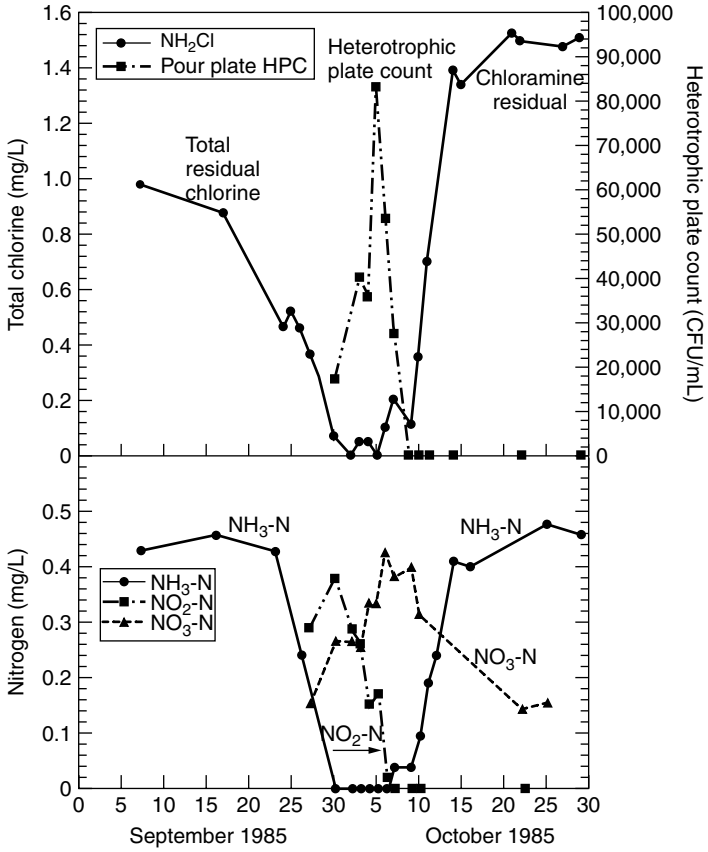

Figure 2. Heterotrophic bacteria, chlorine, and nitrogen species during 1985 nitrification episode in a southern California finished-water reservoir (12). Reprinted by permission of the American Water Works Association copyright © 1993.

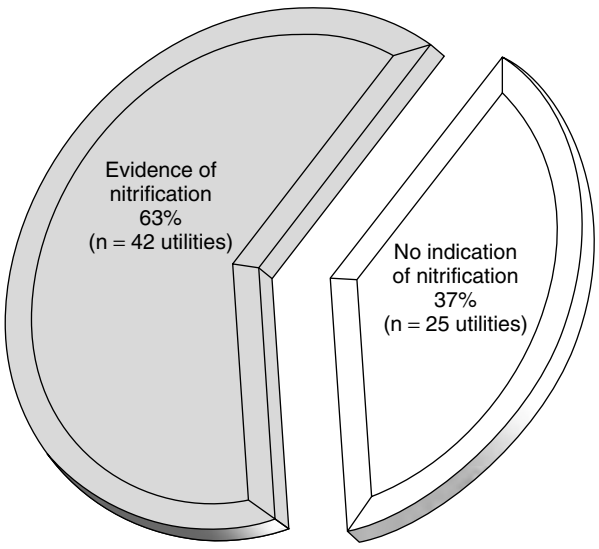

Figure 3. 1991 telephone survey of large water utilities in the United States (serving over 50,000 customers) experiencing nitrification (15). Reprinted with the permission of the AWWA Research Foundation.

systems that have extended detention times (e.g., finished-water reservoirs, storage tanks, and dead ends). During several nitrification episodes in a southern California distribution system, it was observed that AOB levels had increased up to 2,400 MPN/mL (Table 2; 12,16,18,19). In comparison, during periods when nitrite was not detected, the AOB levels ranged from less than 0.2 to 20 MPN/mL (16,18,20). In both cases, actual levels are likely to be much higher, given the low recovery efficiency of the MPN technique (9).

In the distribution system, AOB have been recovered from water in covered reservoirs and distribution system pipelines (12,13,16,19). They have been detected in biofilms attached to surfaces such as Hypalon^{TM} (52 to 1,500 MPN/cm^2) and polypropylene floating covers of reservoirs and epoxy-coated steel tank reservoirs (11 to 860 MPN/cm^2) (Table 2; 16,19) (Table 2). These results suggest that AOB are more densely concentrated in the biofilms than in the water column. Nitrifiers have also been recovered from tubercles on pipelines made of iron, old cement-lined cast-iron pipes, sediment on the bottoms of reservoirs or pipelines, and filter media — for example, sand, anthracite, and granular activated carbon (GAC) — in filter beds (11,19,21). Attachment to surfaces offers the bacteria protection from inactivation and serves as a sink for seeding the water column.

Nitrification Indicators. Because of the difficulties in cultivating and enumerating AOB, most water utilities use several surrogate parameters to detect nitrification. The monitoring parameters include nitrite, ammonia,

Table 2. Levels of Ammonia-Oxidizing Bacteria in the Distribution System

Source	Site	Ammonia-Oxidizing Bacteria MPN/mL	MPN/mg	MPN/cm^2	Reference
Raw		<0.2—70			12,16
Plant effluent		<0.2—11			16,19
Wells		<0.2			19
Distribution system					
Pipeline		0.84–570	1.7–9,700	0.15–1,300	19
		0.5–2,400			18
Finished-water reservoir	Influent	<0.2–2			16
	Water column	280–340			12
		<0.2–400			16
		0.18–6.7			19
	Effluent	360–820			12
	Hypalon cover biofilm			52–1,500	19
	Bottom sediment		7	39,000–420,000	16
Storage tank	Water column	<0.2	380–23,000	11–860	19
	Sludge	81,000	4,000	N/A	19

HPC bacteria, and chloramine levels. Once AOB have been established within the system, nitrite is one of the primary parameters measuring the nitrification process. During nitrification episodes, nitrite-nitrogen (NO_2^-–N) levels of 5 to 500 μg/L have been reported, with levels more frequently ranging from 15 to 100 μg/L (12,13,15,17,18). Any nitrite level distinctly above background (typically 0.005 mg/L) is considered evidence of nitrification and warrants further analysis by the water utility to ensure that water quality does not deteriorate. Ike and colleagues (20) observed nitrite levels increasing from the detection limit (5 μg/L) to 30 μg/L NO_2^-–N within six days, resulting in nitrification and general deterioration of water quality in a 65-million-gallon finished-water reservoir. At other utilities, such as that in Ann Arbor, Michigan (13), where the finished water leaving the plant averaged 16 μg/L NO_2^-–N, it is important to evaluate other nitrification parameters (e.g., HPC and chloramine levels) to determine nitrification progression.

The chloramine residual is another frequently monitored parameter, and residual degradation rates beyond normal chloramine demand may be further evidence of ongoing nitrification. Wolfe and associates (12) reported a chloramine residual decline of 0.6 mg/L (from 1.4 to 0.8 mg/L in a southern California finished-water reservoir) within one day, which suggested that nitrification was occurring. Skadsen (13) determined that within the Ann Arbor system, chloramine decreases of 0.5 to 1.5 mg/L chloramines were a result of normal demand. Anything beyond this was suspect for nitrification. In 1991, Skadsen (17) observed an average chloramine residual decline of 3.2 mg/L chloramine in the distribution system; this correlated with a nitrification occurrence.

Increased HPC bacteria levels may also be observed during nitrification episodes, providing additional evidence that water quality is changing. During nitrification episodes, utilities have reported HPC levels ranging from 100 to 85,000 HPC bacteria/mL (12,13,16,18). Detection of coliforms, however, does not appear to be correlated with nitrification episodes, although it may occur simultaneously. Oxygen and pH have been reported to provide additional early warning information (10); however, most utilities do not routinely measure oxygen levels, and if the water is well buffered, as in hard water, pH is not noticeably affected.

Factors Influencing Occurrence. The basic constituents and conditions necessary for nitrifier growth include a source of ammonia and nitrite, carbon dioxide, oxygen, a dark environment, moderate temperatures, and a slightly basic pH. Rapid growth of nitrifiers occurs when the water is warm and relatively nonturbulent. Factors affecting the likelihood of nitrification in the distribution system can be grouped into the following categories: physical, biological, chemical, and operational.

Physical. Nitrification episodes appear to be more pronounced during the warmer months (Fig. 4; 12,13,16,20, 22,23). In chloraminated water, AOB may proliferate rapidly (to problematic concentrations) above a temperature threshold range of 15 to 18 °C (16). Wolfe and coworkers (16) found that AOB levels remained at or below 0.2 MPN/mL when temperatures were lower than 18 °C in a southern California distribution system (Fig. 5), and an increase was loosely linear with temperatures above this value. Kirmeyer and colleagues (15) found that water samples obtained from selected water utilities in the United States had nitrite levels of 50 μg/L or more (as NO_2^-–N) when the water temperatures were at or above 15 °C (Fig. 6). In a bench-scale study, Lieu and associates (4) found that logarithmic growth — from 40 to more than 1,000 colony-forming units (CFU)/mL — took 28 days at 10 °C but occurred two and three times at 15 and 25 °C, respectively, in dechloraminated tapwater. On corresponding days, nitrite levels were considerably higher (≥0.1 mg/L NO_2^-–N) at the warmer temperatures

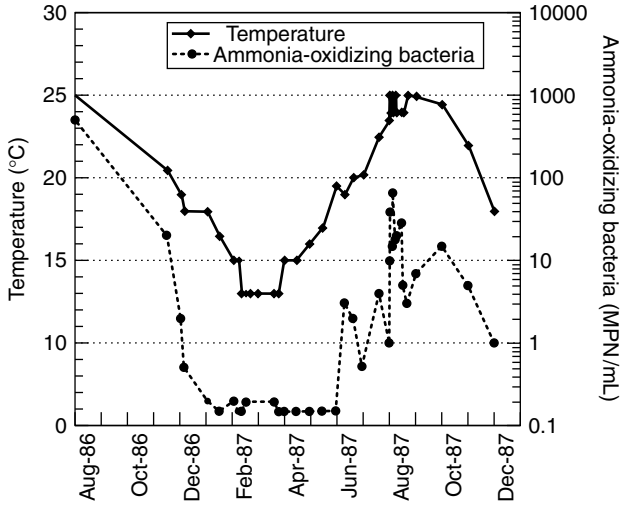

Figure 4. Seasonal relationship between AOB and temperature in a finished-water reservoir in southern California (20). Reprinted with permission from the copyright holder, the International Water Association.

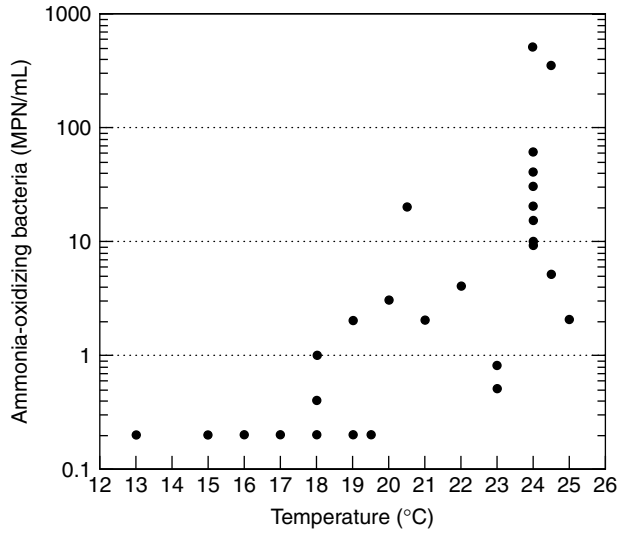

Figure 5. Relationship between AOB occurrence and temperature (16). Reprinted by permission of the American Society for Microbiology.

than at 10 °C. Clearly, at warmer temperatures and with a shorter lag phase, AOB can be more problematic in the distribution system.

Nitrifiers are very sensitive to near-UV, visual, and fluorescent light; consequently, nitrification episodes in distribution systems only occur in the dark (in covered reservoirs, pipelines, taps, etc.). Nitrifiers, however, do have an excision repair mechanism for DNA repair; therefore, low levels of nitrifiers may be recovered from partially shaded reservoirs or channels.

Biological. In addition to interactions with chemical and physical environments, nitrifiers may have a synergistic relationship with HPC bacteria. During growth, AOB produce metabolic by-products that may enhance

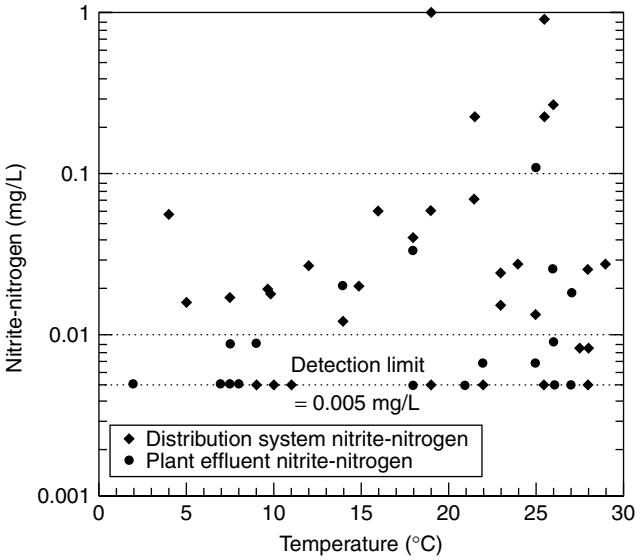

Figure 6. Relationship between nitrite levels and temperatures from water samples from water utilities in the United States (15). Reprinted with the permission of the AWWA Research Foundation.

the growth of HPC bacteria (6). Likewise, HPC bacteria may produce by-products such as acetate, formate, and pyruvate, which enhance AOB growth (3). In a southern California distribution system, Wolfe and coworkers (16) found a positive correlation between AOB and HPC bacteria when HPC levels exceeded 100 CFU/mL (Fig. 7). A positive correlation was also found between HPC bacteria and nitrite (13,15,17). It is not clear whether there is a synergistic relationship between nitrifying bacteria and other bacterial groups such as coliforms or pathogens.

Chemical. To study the effects of monochloramine residual and temperature on the inactivation of AOB, Lieu and

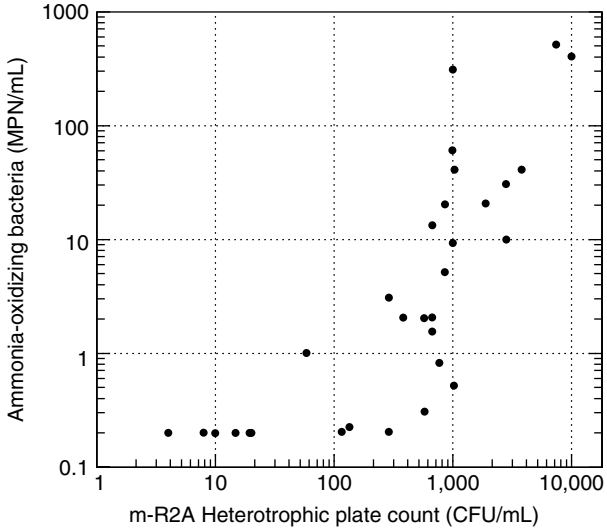

Figure 7. Relationship between AOB occurrence and HPC bacteria recovered on R2A medium (16). Reprinted by permission of the American Society for Microbiology.

colleagues (4) exposed a naturally occurring population of AOB to three chloramine residual concentrations — 1.7, 2.0, and 2.5 mg/L chloramines at a 4 : 1 chlorine-to-nitrogen (Cl_2 : N) ratio by weight — in a bench-scale study for an eight-day contact time at temperatures of 10, 15, and 25 °C (Fig. 8a–c). At 10 °C, the AOB were inactivated to below the detection level (<0.2 MPN/mL) and did not recover. At 15 °C, the AOB were able to recover at different rates following exposure to 1.7 and 2.0 mg/L chloramines, but not following exposure to 2.5 mg/L chloramines. At 25 °C, only the AOB exposed to 1.7 mg/L chloramines were able to recover, following two logs of inactivation and regrowth. Importantly, the rate of inactivation and regrowth was found to be faster at 25 °C than at 15 °C (Fig. 8b,c). Also, it is interesting to note that many utilities operate with chloramine residuals of 2 to 3 mg/L leaving the plant (15,24).

In another laboratory assay, Cunliffe (25) showed that 99% inactivation of a naturally occurring population of AOB was achieved with exposure to 0.6, 1.85, 3.05, 4.05, and 5.2 mg/L total chlorine in approximately 2,000, 250, 100, 75, and 40 minutes, respectively, in 30 °C tapwater.

When the monochloramine residual is at threshold levels of effectiveness, the Cl_2 : N ratios can enhance or hinder the inactivation and regrowth of AOB (12). Field and bench studies conducted independently by Knudsen — as discussed in Kirmeyer and coworkers (15) — and by Lieu and coworkers (4), respectively, have indicated that AOB and nitrite were more likely to increase at lower Cl_2:N ratios (e.g., a 3:1 Cl_2:N ratio, by weight) than at higher chloramine ratios (e.g., a 5:1 Cl_2:N ratio, by weight), presumably because of the available free ammonia present at Cl_2:N ratios of less than 5:1.

Potable waters high in organic matter, humic material, and color not only enhance nitrifier growth, but also have a high chlorine demand, creating unstable water. Under these conditions, nitrification can lead to pH changes in the system, growth of other bacteria (e.g., HPC, sulfate-reducing, or iron bacteria), and pipeline corrosion. Other chemical parameters, such as alkalinity, hardness, and electrical conductivity, are not thought to be significant to the survival and growth of AOB.

Operational Practices. Operational practices also affect the likelihood of nitrification in drinking water systems. In the distribution system, practices promoting degradation of chloramines, low flow rates, and long detention times can lead to proliferation of AOB.

Results from surveys of distribution systems indicate that higher levels of AOB, nitrite, and HPC bacteria were found in water with lower chloramine levels (13,15,25,26). Kirmeyer and colleagues (15) obtained water samples from selected utilities across the United States. Their findings indicated that samples with chloramine levels of 0 to 1 mg/L had an average NO_2^-–N level that was 30 times higher than samples with chloramine levels above 2 mg/L (Table 3). In Australia, Cunliffe (25) found AOB levels in the distribution system to be inversely correlated with total chloramine levels (Table 3). Total chlorine levels of less than 1 mg/L had median nitrifier levels 65 times greater than those for total chlorine levels of 1 to 2 mg/L.

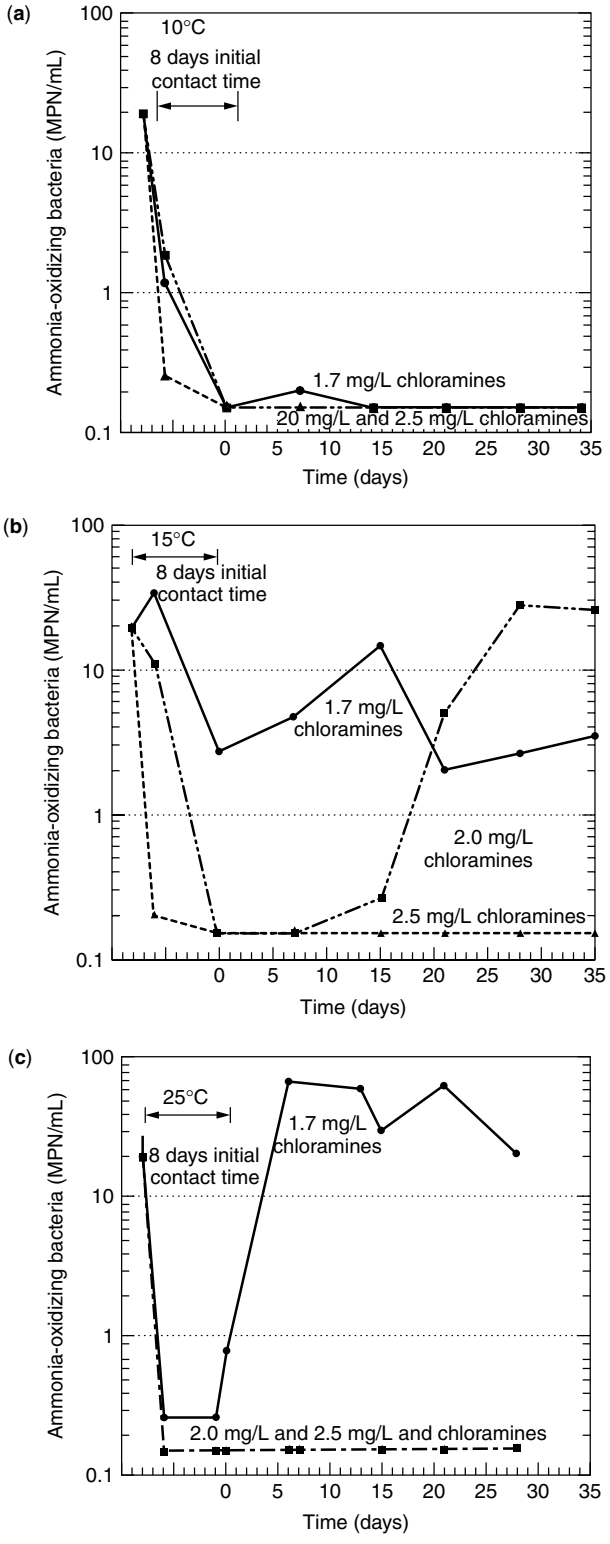

Figure 8. Regrowth of AOB following exposure to chloramines (4:1 Cl_2:NH_3–N) at (**a**) 10 °C, (**b**) 15 °C, and (**c**) 25 °C (4). Reprinted by permission of the American Water Works Association copyright © 1993.

Regrowth of nitrifiers to problematic levels is also highly dependent on residence time. Direct correlations between detention times and nitrification symptoms

Table 3. Average AOB and Nitrite Levels in Chloraminated Distribution Systems

Total Chlorine (mg/L)	AOB (nitrifiers/mL) (25)	NO_2–N (mg/L) (15)
0–1 mg/L	130	0.3
1–2 mg/L	2	0.075
>2 mg/L	0.5	0.01

have been observed in the distribution system (12,17,25). In a survey of nine United States water utilities during the summer, water samples with detention times of three days or less exhibited low or no nitrification symptoms as compared with water samples with detention times of more than three days, which did exhibit nitrification symptoms (15). Similarly, Wolfe and associates in southern California (12) and Skadsen in Michigan (17) found that mean detention times of three and two days, respectively, seemed to deter nitrification episodes in their systems. Other water utilities may have longer detention times in their distribution system (e.g., reservoirs without, experiencing nitrification; in such cases, although the longer detention times do heighten the potential for nitrification, this effect is apparently counterbalanced by other factors (e.g., cooler temperatures, higher residuals) that tend to inhibit nitrification.

The type and condition of the distribution system pipelines, reservoirs, and tanks can influence nitrification. For example, cast-iron pipes or old mortar-lined iron pipes with heavy turberculation are notorious for nitrification. Poorly maintained reservoirs and tanks not routinely cleaned or in need of a new inner surface coating are also excellent sources for AOB growth. Stewart and Lieu (19) reported that concrete materials appear to yield fewer AOB, presumably because of the high alkalinity leached from the concrete.

Treatment technologies may also affect nitrification occurrence. Oxidants such as ozone and chlorination increase the assimilable organic carbon (AOC) levels of the water and may thus increase the potential for nitrification (27). GAC has also been shown to have promoted nitrification when chloramines, at a low Cl_2:N ratio by weight, were added across the filter, resulting in growth of nitrifiers in the filters (13). Interestingly, low levels of chlorite, a product of chlorine dioxide, may inhibit nitrification (28,29). A survey conducted in Texas showed that utilities using chlorine dioxide as a predisinfectant and chloramines as a postdisinfectant had less nitrification symptoms than those utilities that used only chloramines (28).

PREVENTION AND CONTROL

Successful management of nitrification requires a thorough understanding of factors enhancing the nitrification process, early warning monitoring, and measures to prevent nitrification from increasing to problem levels.

Prevention and Control Plan

An optimization plan should be in place to minimize conditions favoring nitrification, and the actions needed to control nitrification should be identified before problems begin to occur. The plan should be both flexible and quick to implement. Speed is essential because degradation of water quality from nitrification can occur within days and increase within hours. The plan should include information on staff (i.e., knowledge, responsibility, and training), monitoring, and action levels (24,30).

Monitoring

Common parameters to be monitored as a part of early warning monitoring include nitrite, total and free chlorine (possibly monochloramine) residuals, HPC bacteria, total and free ammonia, and, particularly, nitrite. Monitoring of pH and oxygen levels may also be useful. These parameters will provide a picture of the status of water quality and nitrification symptoms. Each utility should determine acceptable background levels and implement action if levels increase beyond this. The type of action items called for should be established on the basis of the stage to which nitrification has progressed, as determined by the level of key parameters.

Prevention Methods

Nitrification prevention methods include optimizing the chloramine residual, chlorine-to-ammonia ratio, and system detention time or flow rate.

Maintaining a good chloramine residual throughout the distribution system is a key part of prevention. On the basis of surveys and reports, a chloramine residual of 2 mg/L or more is commonly used in the distribution system to control nitrification (15,24,25). However, the residual levels selected for use in a specific distribution system must be based on the chloramine demand, the water matrices, the physical parameters, and the water system. Utilities with high chloramine demand (e.g., Ann Arbor, Michigan; Broward County and Key West, Florida), use chloramine residuals of 3 to 5 mg/L (15,31).

Another method of prevention for early stages of nitrification is a distribution system flushing program. The flushing action removes stagnant water, scours biofilm, and provides freshwater with increased disinfectant dose. In many cases, this method helps for a short time, but it may have to be implemented frequently and regularly to be effective (17,22).

In areas of low flow, AOB have a greater chance to proliferate. Common inlet-outlet ports on storage tanks are well recognized for creating dead zones within the tanks. A solution is to enhance mixing, thereby increasing turnover rate.

Another method involves temporary switches to free chlorine, scheduled just before peak periods of seasonal nitrification, to oxidize biofilm; this has been done in Broward County (Florida)—as discussed by Kirmeyer and coworkers (15)—and elsewhere (18,24). In southern California, this method was used successfully for 10 years and applied during early summer for one month before peak nitrification periods, which occurred in midsummer.

However, some utilities have reported that when the system disinfectant is switched to free chlorine, elevated coliform levels are initially detected, presumably from dislodging biofilm, as was the case in Broward County (15).

Other options for prevention may include boosting the chloramine residual in some areas of the distribution system by adding booster stations at strategic locations, such as at reservoir effluents, where residuals tend to degrade. Also, the chloramine level leaving the treatment plant can be elevated to create an overall higher residual in the distribution system (24).

Two water utilities reported success in controlling nitrification by increasing the pH of their water to 9.0 (15,31,32). Although nitrification was not eliminated, one utility reported that nitrification was reduced and controlled in three out of four years (32).

Control Methods

When nitrification symptoms are detected early, less aggressive methods such as flushing and increasing circulation and flow rates may be sufficient. During this time, key parameters should be monitored daily or even more frequently. If nitrification symptoms become more severe, boosting the chloramine residual or increasing the Cl_2 : N ratio may eliminate the problem. If nitrification persists, switching disinfectants to free chlorine will eliminate nitrification problems almost immediately. However, switching to free chlorine would temporarily increase trihalomethane levels and is not without operational difficulties (18). As new experience is gained, it is important to reevaluate the action levels and the control plan and to readjust the parameter levels as needed.

CONCLUSION

Since the early 1980s, water utilities have switched from free chlorine to cloramines as one alternative to meeting trihalomethane regulations. A major disadvantage of chloramines, however, is the potential for nitrification. Nitrifying bacteria are found in drinking water distribution systems, and, under conditions that enhance the growth of these bacteria, nitrification may adversely affect water quality. Some water utilities have developed and successfully used prevention and control measures that minimize the impact of nitrification on water quality. Improved methods of identification and enumeration are needed, however, to enhance tracking and understanding of the occurrence and ecology of nitrifying bacteria. In the future, molecular methods may offer the best approach for studying these organisms in the distribution system.

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NITROGEN CYCLE.

See DENITRIFICATION IN THE MARINE ENVIRONMENT

NITROGEN CYCLE IN AQUATIC SYSTEMS.

See NITRIFICATION IN AQUATIC SYSTEMS

NITROGEN CYCLE IN SOILS.

See SOIL NITROGEN CYCLE

NITROGEN CYCLE IN THE MARINE ENVIRONMENT

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Nitrogen (N) is an essential element required by all organisms in stoichiometric proportions to carbon and other essential elements for balanced growth. It occurs in a complex array of different chemical pools and states in the ocean. Microorganisms participate in, and in fact dominate, most of the transformations of nitrogen in the sea. They are intimately involved in the uptake of nitrogen into organic forms, its rerelease during regeneration, conversions between combined forms and the large gaseous pool of dinitrogen gas (N_2), and the production and consumption of nitrogenous greenhouse gases. Articles on the specific processes of nitrogen fixation (NITROGEN FIXATION IN THE MARINE ENVIRONMENT, this Encyclopedia), nitrification (NITRIFICATION IN AQUATIC SYSTEMS, this Encyclopedia), and denitrification (DENITRIFICATION IN THE MARINE ENVIRONMENT, this Encyclopedia) in the Encyclopedia, as well as on microbial nutrition (INORGANIC NUTRIENT USE BY MARINE MICROORGANISMS, this Encyclopedia) are included. [See also articles in Carpenter & Capone (1), Blackburn & Sorensen (2), and Kirchman (3).]

Nitrogen is thought to limit primary production through much of the world's oceans (4,5). The organic productivity of many marine ecosystems is intimately tied to the delivery and availability of nitrogen. Further impetus to better understand the relationship between nitrogen cycling and marine productivity has arisen from the observation of anthropogenically induced perturbations at regional and, possibly, global scales in the marine nitrogen cycle, a result of massive increases in fertilizer production. Concerns about greenhouse warming and climate change (6) have also focused attention on the marine nitrogen cycle as nitrous oxide (N_2O), a product of nitrification and a substrate and metabolite of denitrification, has been increasing in the atmosphere (7). Nitrous oxide can react with and destroy stratospheric ozone and is also a potent greenhouse gas (7).

NITROGEN INPUTS AND OUTPUTS

Nitrogen enters the sea by several pathways. Organic and inorganic forms of nitrogen arrives from land by riverine delivery (8), through runoff, and by wet and dry atmospheric deposition of inorganic and organic N (9–11). Some gaseous forms of combined nitrogen, for instance NH_3 and NO_x, may enter the coastal oceans from adjacent land areas (12). Soluble forms of nitrogen can also arrive through submarine groundwater discharge in coastal areas (13,14). The relative importance of each of these pathways of nitrogen delivery will vary with respect to location and climate — for example, the influence of the Amazon in the tropical Atlantic is profound (15), whereas riverine delivery may represent only minor inputs in other locations (e.g., southwest Pacific Ocean). Atmospheric inputs may be greater near landmasses, and the form of atmospheric input will vary as a function of land use and population density.

Nitrogen is removed from the sea by biological denitrification (DENITRIFICATION IN THE MARINE ENVIRONMENT, this Encyclopedia) and gaseous evasion (16), sedimentation to the seafloor (17), and biomass harvest.

MARINE NITROGEN POOLS

Once in the sea, nitrogen occurs in a variety of molecular forms, redox states, and phases (Table 1; 18). Specific pools of nitrogen show various and distinct spatial and temporal patterns of abundance, thereby providing important clues to the underlying biogeochemical dynamics crucial to our understanding of this cycle.

The largest reservoir of nitrogen in the sea is dissolved dinitrogen gas (N_2), which occurs in concentrations of about 1 mM (Table 1). Concentrations are relatively uniform and vary largely as a function of temperature and salinity dependent solubility (20,21). Nitrous oxide, which occurs as a trace constituent in seawater and is typically from 10 to 50 nM in seawater (22), arises from several microbial pathways (see in the following text). In the sea, one may observe relatively high transients in N_2O concentrations at redox boundaries (23). Nitric oxide (NO) also occurs in trace concentrations in seawater,

Table 1. Primary Pools and Concentrations of Nitrogen in the Ocean

Formula	Form/Pool	Typical Concentrations μM Oceanic	Coastal	Global Marine Pools Pg
Gaseous				
N_2	Dinitrogen	900–1,100	900–1,100	$22–23 \times 10^3$
N_2O	Nitrous oxide	0.006 –0.07	0 –0.25	0.2 –0.8
NO	Nitric oxide	?	?	?
Inorganic				
NO_{3-}	Nitrate	<0.03–40	<0.1–200	570–677
NO_{2-}	Nitrite	<0.03–0.1	<0.03–10	?
NH_{4+}	Ammonium	<0.03–1	<0.03–100	7–8
$(NH_2)_2CO$	Urea	<0.1–0.5	0–2	
Organic				
DON	Dissolved organic	3–7	3–20	63–530
PON	Particulate organic	0.07–0.5	0.1–30	3–24
Phyto biomass	Particulate organic			0.15–3
Animal biomass	Particulate organic			0.17

Source: (Adapted from J. H. Sharp, in E. J. Carpenter and D. G. Capone, eds., *Nitrogen in the Marine Environment*, Academic Press, New York, 1983, pp. 1–35 (19)).

has biological sources and sinks and is highly reactive (NITRIFICATION IN AQUATIC SYSTEMS, this Encyclopedia). However, far less is known about the importance and cycling of NO in the sea.

Nitrate (NO_3^-) is the dominant inorganic form of nitrogen in runoff, riverine input, groundwater discharge, and atmospheric deposition to the ocean. In the sea, concentrations of NO_3^- can vary widely in space and time. Relatively high concentrations (10 s–100 s μM) are characteristic of eutrophic coastal and upwelling environments, whereas lower concentrations occur in the surface waters of the tropical gyres (19) and in suboxic and anoxic zones (Table 1).

Nitrite (NO_2^-) generally occurs in the sea in much lower concentrations than nitrate (Table 1), although higher concentrations are associated with redox interfaces both in the water column and sediments. The primary nitrite maximum of the open ocean occurs near the base of the euphotic zone and is generally associated with assimilatory NO_3^- reduction (and release of the NO_2^- intermediate), whereas a deeper, secondary NO_2^- maximum is associated with the differential inhibition of nitrification (24) (also, see NITRIFICATION IN AQUATIC SYSTEMS, this Encyclopedia).

Ammonium (NH_4^+) concentrations also vary widely among environments. In oligotrophic regions, ammonium concentrations are usually near the limit of detection by conventional colorimetric methods (<0.03 μM). In suboxic and anoxic environments NH_4^+ concentrations increase. In highly reducing nearshore sediments, mM levels of NH_4^+ can be observed. High concentrations of NH_4^+ are also associated with nearshore waters subject to sewage and waste discharges (25,26).

Dissolved organic nitrogen (DON) also represents a major nitrogen pool in marine systems (Table 1). Analyses of organic nitrogen have focused on both specific compounds such as individual amino acids and nucleic acids (19) as well as polymeric compounds (protein, DNA) and specific classes of DON (<0.3 to 0.7 μM fraction) (27) and particulate organic nitrogen (PON)(>0.2 to 0.7 μm). Amino and nucleic acids, although typically very low in concentration, are highly dynamic pools in many marine ecosystems (28). The bulk of the organic nitrogen pool remains yet to be characterized. Recent evidence indicates that much of the uncharacterized fraction may consist largely of refractory biopolymers, possibly peptidoglycan remnants of bacterial cell walls (29).

PROCESSES

Most of the transformations of nitrogen in the ocean are performed by bacteria, although even those that also occur in higher organisms are still dominated by microorganisms. The principal transformations of the marine nitrogen cycle include the uptake and incorporation of inorganic forms of nitrogen into organic nitrogen; the regeneration and release of inorganic nitrogen, primarily as NH_4^+, from organic forms of nitrogen; the oxidation of ammonium and nitrite in nitrification; the reduction of nitrate or nitrite to the gaseous end products, N_2 and N_2O in denitrification; and the reduction ("fixation") of N_2 to NH_4^+ (Fig. 1).

Inorganic nitrogen, as nitrate, nitrite, ammonium, and organic nitrogen, such as urea and other small molecular weight organic molecules (e.g., amino acids), often termed utilizable or combined nitrogen, can be taken up and incorporated into organic matter by a variety of organisms. Very high rates of nitrogen uptake and assimilation into organic matter occur in photic environments in conjunction with photosynthetic carbon assimilation (see PLANKTONIC ALGAE IN THE MARINE ENVIRONMENT, this Encyclopedia). These reactions are largely catalyzed by photoautotrophs including cyanobacteria, eukaryotic micro- and macroalgae and sea grasses. Heterotrophic bacteria and archaea (as well as some fungi) can also assimilate inorganic nitrogen and may compete with photoautotrophs for these resources (see INORGANIC NUTRIENT USE BY MARINE MICROORGANISMS, this Encyclopedia).

The capacity for nitrogen fixation, the reduction of N_2 gas to NH_4^+ for biosynthesis, occurs in a disparate

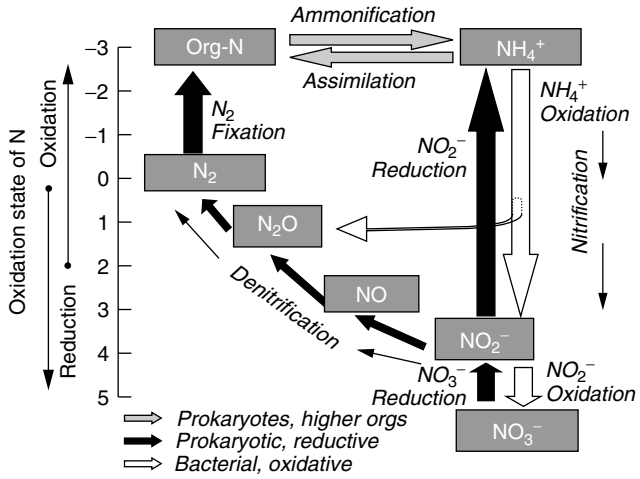

Figure 1. Marine nitrogen cycle emphasizing oxidation-reduction reactions and biological transformations. Adapted from D. G. Capone, in D. Kirchman, eds., *Microbial Ecology of the Oceans*, Wiley-Liss, New York, 2000, pp. 455–493.

array of prokaryotes, including representatives of both eubacteria and archaea. Organisms that can fix nitrogen are at an advantage in environments with low levels of combined nitrogen. Through this process, N_2 fixers provide a source of utilizable nitrogen to the biosphere from the large pool of N_2 (Table 1) and thereby balance losses of NO_3^- by denitrification. Marine N_2 fixers are found in greatest abundance in the tropical open ocean and in shallow tropical environments (see NITROGEN FIXATION IN THE MARINE ENVIRONMENT, this Encyclopedia).

Oxidized forms of nitrogen, such as NO_3^- and NO_2^-, that are taken up by cells must first be reduced to NH_4^+, at the −3 oxidation level, by assimilatory NO_3^- and NO_2^- reductase before being assimilated into cell biomass (30). In most marine organisms utilizing inorganic nitrogen, including nitrogen fixers (31), the assimilation of NH_4^+ is catalyzed by the enzymes, glutamine synthetase and glutamate synthase (GS/GOGAT pathway) with glutamate as the major end-products. Glutamate is subsequently utilized in the cell to synthesize other nitrogen-containing compounds.

Nitrogen is a constituent of a wide variety of simple and complex organic molecules, and the pathways of organic nitrogen breakdown largely remain to be detailed. The release of inorganic nitrogen from organic forms can occur through the metabolism of higher organisms by the urea cycle with release of various urea cycle end-products (e.g., NH_4^+, urea; 32). The degradative catabolism of organic nitrogen by heterotrophic microorganisms including many bacteria and fungi, often termed ammonification, also results in the release of NH_4^+ (33). Labile DON that is released by excretion or cell lysis can also be further catabolized by heterotrophs or assimilated by heterotrophs or autotrophs (32,34). A variety of algal taxa and bacteria possess cell surface oxidases (35). These may be important in liberating NH_4^+ from organic compounds and the NH_4^+ released is subsequently available for uptake.

Nitrification is a two-step process involving first the conversion of NH_4^+ to NO_2^- followed by the oxidation of NO_2^- to NO_3^- (see NITRIFICATION IN AQUATIC SYSTEMS, this Encyclopedia). These reactions are catalyzed by two specialized groups of aerobic, autotrophic bacteria: the ammonium oxidizers and the nitrite oxidizers. The fact that large reserves of NO_3^- are maintained in the deep sea attest the major role of nitrifiers in the oceans. However, nitrification occurs at relatively low rates in the deep sea (36) (also see NITRIFICATION IN AQUATIC SYSTEMS, this Encyclopedia). Direct measurements of nitrification are among the most challenging to make and therefore the scarcest among the major processes involved in the marine nitrogen cycle. Pathways of heterotrophic nitrification have been described for soils but have not been well characterized in aquatic environments. Autotrophic nitrifiers have been implicated in the production of N_2O in many marine environments (37,38).

Biological denitrification is the sequential conversion of oxidized forms of nitrogen to gaseous end-products (39) (see DENITRIFICATION IN THE MARINE ENVIRONMENT, this Encyclopedia; Fig. 1). These oxidized nitrogen substrates, including nitrate and nitrite, serve as terminal electron acceptors in respiration. Denitrification is generally coupled to the oxidation of organic compounds. The first step of the process, the reduction of NO_3^- to NO_2^- is termed dissimilatory NO_3^- reduction (to distinguish it from the assimilatory pathway — see preceding text) and occurs in a relatively wide variety of bacteria. The capacity to reduce NO_2^- to N_2O or N_2 — true denitrification — occurs in a smaller subset of these bacteria (39). These reactions generally occur where O_2 is low. In some bacteria, NO_3^- reduction is followed by dissimilatory reduction of NO_2^- to NH_4^+, and NH_4^+ released into the environment (40), a process referred to as dissimilatory nitrate reduction to ammonium.

The denitrification pathway represents a loss of utilizable (combined) nitrogen for marine ecosystems. When the first two reduction steps in either pathway are temporally or spatially uncoupled, NO_2^- may accumulate. Thus, $NO_2^-$is a key branch point in the nitrogen cycle as an intermediate in nitrification, assimilatory and dissimilatory NO_3^- reduction. Denitrification occurs at substantial rates in a variety of benthic and pelagic marine environments, including midwater areas of the Indian and Pacific Oceans (41), as well as estuarine, coastal and shelf sediments (42–44).

CONCEPTS IN MARINE NITROGEN CYCLE DYNAMICS

The multiplicity of chemical species of nitrogen, their distribution among different phases, and various biotic transformations among pools, makes the biogeochemistry of nitrogen relatively complex. In comparing the differences between systems, several key concepts regarding nitrogen distributions and their relationships to plant and animal production have emerged over the last several decades. These concepts are dynamically evolving and being refined as we learn more about marine systems.

Deep Nitrate, Redfield Ratios, and the Conveyor Belt

While surface waters are often depleted in inorganic nutrients such as NO_3^- and PO_4^{-3} throughout much of

the open oceans, relatively high nutrient concentrations are found in deep waters beneath the thermocline. Assimilation of nutrients by phytoplankton accounts for the depletion of nutrients in the upper water column in which light is available for photosynthesis (i.e., the euphotic zone), whereas the heterotrophic metabolism of bacteria is largely responsible for the regeneration of nutrients in deep water. The concentration of NO_3^- below the thermocline is greater in the Pacific Ocean than in the Atlantic Ocean, because of the large-scale circulation pattern of deep waters.

Redfield and coworkers (45,46) developed a geochemical model relating the increase of nutrients at depth in the sea to the aerobic bacterial regeneration, coupled with nitrification, of planktonic organic matter sedimenting through the subeuphotic zone. The stoichiometric relationships, termed the Redfield Equation, between the composition of generic plankton organic matter and the macronutrients released during aerobic decomposition is:

$$(CH_2O)_{106}(NH_3)_{16}(H_3PO_4) + 138O_2 \Leftrightarrow 106CO_2 16HNO_3 + H_3PO_4 + 122\ H_2O \quad (1)$$

Using the Redfield ratio (106:16:1 for the mole ratio of CO_2: NO_3: PO_4) one can predict the inorganic nutrient concentrations resulting from regeneration of plant material based on the relative degree of O_2 utilization in deep water. Briefly put, the longer a parcel of water is at depth (i.e., removed from the euphotic zone), the greater the (*1*) decomposition of organic matter, (*2*) the O_2 consumed, and (*3*) the concentration of inorganic nutrients in that parcel of water.

Deep waters largely form in the North Atlantic Ocean and flow south through to the South Atlantic, whereas new deep water is entrained in the circum-Antarctic region. A portion of this water feeds the Indian Ocean deep-water flow before finally arriving in the Pacific Ocean. This large-scale interbasin circulation is often referred to as the Conveyor Belt (47). Upwelling occurs along the way, but waters entrained at depth the longest eventually upwell in the Pacific. The oldest waters, in equilibrium with atmospheric oxygen when they first sink, can take upwards of 2000 y to make this transit. Along the way, organic carbon sedimenting from the surface fuels heterotrophic oxygen consumption and the regeneration of nutrients in these waters. Consequently, the highest concentrations of NO_3^- are found in midwaters of the Pacific Ocean that have remained at depth the longest.

Indeed, waters found at middepths in the Indian Ocean and in the tropical Pacific Ocean have very low O_2 levels and lower-than-predicted concentrations of NO_3^- due to initiation of bacterial nitrate reduction and denitrification processes that consume NO_3^-. Large expanses of anoxic midwaters in the Indian Ocean, and eastern tropical North and South Pacific are major global sites of denitrification (41).

Recently, a new parameter, termed N*, which considers departures from Redfield stoichiometry occurring during the decomposition of organic matter and regeneration of nutrients has been derived (48,49). It is defined as:

$$N* = (NO_3^- + 16PO_4^+ + 2.90) * 0.87 \quad (2)$$

The value of 2.90 adjusts N* to 0 when considering global pools of nitrogen and phosphorus. The N* parameter has been used to identify both negative and positive deviations from canonical Redfield values. For example, large areas of the tropical North Atlantic have been found to exhibit an excess of inorganic nitrogen relative to phosphorus at middepths (49,50). It has been suggested that this excess of regenerated N, relative to P at these depths, results from the sedimentation of material enriched in nitrogen by nitrogen fixation in near surface waters.

Nitrogen as a Limiting Nutrient

Much of the interest in the nitrogen cycle derives from the general observation that N is often the nutrient factor limiting plant growth and/ or biomass accumulation in the ocean (4,5,25). The concept of limiting nutrients, still actively debated today, was originally derived from the application of Liebig's "law of the minimum." Liebig first proposed that the chemical factor in shortest supply would limit plant growth in agricultural systems. Oceanographers, observing the reciprocal relationship between the increase in phytoplankton biomass in the spring in the temperate North Atlantic Ocean with the decrease in NO_3^- (and PO_4^{-3}) concentrations, inferred nitrogen as the limiting nutrient factor resulting in the termination of the spring bloom (51). Biotic factors, such as grazing, also contribute to bloom termination and this has promoted considerable discussion about the relative extent of "top-down" (i.e., herbivore) versus "bottom-up" (i.e., nutrient) control of plant biomass and production (52). The observation that coastal eutrophication frequently results from nitrogen enrichment of systems (25,53), see following text) also provides additional evidence for a controlling role of nitrogen in these systems.

Various approaches have been used to assess nutrient limitation and can give widely divergent results, depending on the ecosystem component or process considered (e.g., biomass limitation versus net plant production versus net ecosystem production), the timescale of the observation or the particular parameter assessed as an index of limitation and cellular capacity for growth (5,54). Nutrient bioassays in which nutrients (N or P) are added to incubations of natural water are commonly used to assess nutrient limitation. Short-term (e.g., hours) nutrient bioassays often focus on physiological responses, for instance in gross photosynthetic capacity as determined by $^{14}CO_2$ uptake, whereas somewhat longer (days) term assays are needed to monitor biomass (often as chlorophyll *a*) accumulation that approximates net production. Biologists generally focus on nutrient limitation of growth on timescales of hours to days, whereas geochemists are typically more interested in net ecosystem production at larger spatial dimensions (e.g., basin scale) and often on much longer timescales (54).

The simplistic view that nitrogen is the key limiting nutrient throughout most of the ocean is being rapidly

replaced with a much more dynamic view of seasonal variation in limiting nutrients in estuaries (55,56) and coastal waters (57). The high nitrate/low chlorophyll (HNLC) concentration oceanic regions are primarily Fe-limited (58). Phosphorus may be a key limiting nutrient in shallow tropical ecosystems (54,59,60) and even the North Atlantic (61). Moreover, we have also come to recognize that nutrient limitation may differ between contemporaneous species within a system (e.g., Si or Fe limitations of diatoms (57,62), or Fe (63,64) or P (65) limitation of nitrogen-fixing cyanobacteria in ostensibly nitrogen-limited ecosystems).

New Nitrogen and Oceanic Productivity

A useful conceptual framework for placing oceanic nitrogen-cycle studies in the broader context of carbon productivity is the "New Production Model" (66). This model recognizes two types of phytoplankton primary production: (*1*) "recycled" production supported by nitrogen (termed "recycled nitrogen") regenerated from organic matter within the euphotic zone, and typified by NH_4^+ and (*2*) "new production" that is supported by nitrogen imported from outside the euphotic zone and exemplified by NO_3^- diffusing or advecting up from deep pools, nitrogen fixation, atmospheric nitrogen deposition as well as nitrogen derived from rivers and runoff in nearshore areas, all representing sources of "new nitrogen" (Fig. 2).

One very useful aspect of the concept is to provide a constraint on organic export from the euphotic zone, independent of more conventional (and controversial) procedures such as direct measurement of export flux (67). Interest in the capacity of the upper water column to draw down and export to depth atmospheric CO_2 reinvigorated interest in estimating new production (68). Initial attention was focused on the role of deep NO_3^- in promoting atmospheric CO_2 drawdown, but the recognition that NO_3^- codiffuses or advects from depth with greater than stoichiometric amounts of CO_2 with respect to phytoplankton needs, mitigates the potential for deep NO_3^- to affect atmospheric CO_2 sequestration (69). On the other hand, new production dependent on nitrogen fixation or atmospheric combined nitrogen deposition can support CO_2 sequestration (70,71).

Benthic-Pelagic Coupling

Although the deep benthos of the open ocean receives organic debris from overlying waters and may exhibit seasonal responses to input (72), only a small fraction of the export production reaches the sea bottom (73), and the benthos is sufficiently far removed that it has essentially no affect on processes on the upper, photic layers on short (annual-decadal) timescales. However, as one moves closer to the continents, the ability of the benthos to interact on biologically relevant timescales with the euphotic zone increases dramatically. In general, the higher levels of new production found in coastal surface waters (68) results in a higher delivery of exported production to the sediments (73) and higher sediment organic content (17). Low O_2 and higher concentrations of NH_4^+ are often associated with nearshore and coastal sediments, both a result of the intense heterotrophic metabolism by the dense populations of bacteria in the sediments. Fluxes of inorganic nutrients from the benthos, in turn, can support a large fraction of the nutrient demand of water column primary productivity (53). The high levels of spring production fueled by high nutrients in spring freshets in estuaries, or winter overturn in coastal waters often result in organic accumulation in sediments that are metabolized through the year providing a steady supply of inorganic nutrients to overlying waters (74).

Coupling of Biogeochemical Cycles

In the sea as elsewhere, the nitrogen cycle does not operate in isolation but is directly coupled to the cycling

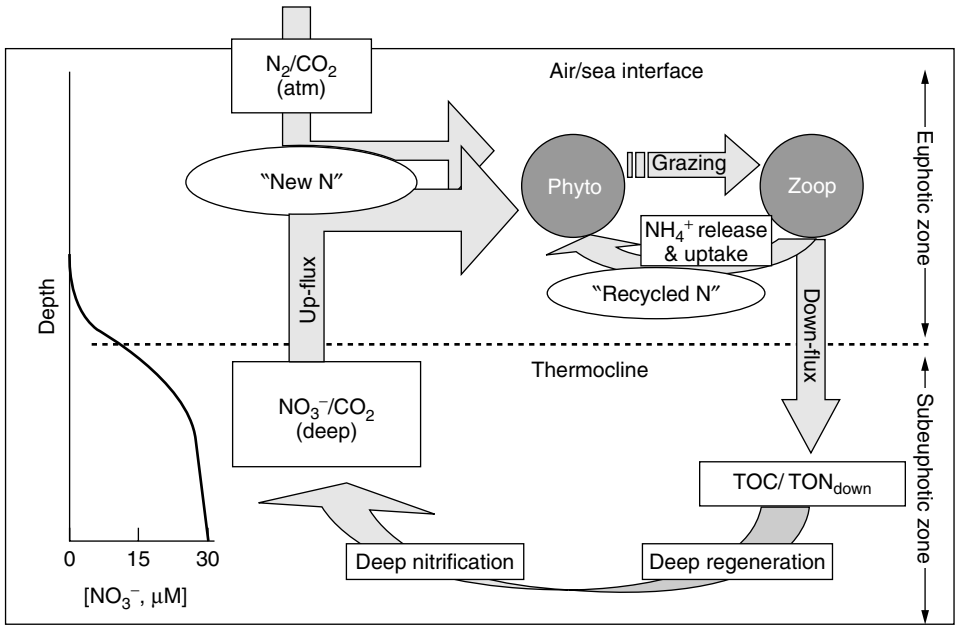

Figure 2. Conceptual representation of the new nitrogen model after R. C. Dugdale and J. J. Goering (66). Abbreviations: TOC = total organic carbon; TON = total organic nitrogen.

of carbon and other biologically relevant elements. In addition to a carbon source, all organisms have a biosynthetic requirement for a suite of essential nutrients (e.g., N, P, S, Mg, Fe, etc.; see INORGANIC NUTRIENT USE BY MARINE MICROORGANISMS, this Encyclopedia), needed in stoichiometric proportions for balanced growth (33). Nitrogen is a macronutrient because it often constitutes several percent of cell biomass. Hence, all organisms regardless of their physiology devote portions of their overall energetic metabolism to nitrogen acquisition and incorporation. Furthermore, forms of inorganic nitrogen are directly involved in the energetic metabolism of certain heterotrophic (e.g., NO_3^- reducers and denitrifiers), and autotrophic (e.g., nitrifiers) bacteria.

Within particular biogeochemically significant species of bacteria and archaea, one can find the capacity to link sulfate respiration and methanogenesis with nitrogen assimilation and ammonification, to couple sulfide, NH_4^+ or H_2 oxidation to denitrification (75), or methane oxidation, sulfate reduction or denitrification to nitrogen fixation. Interestingly, bacteria and archaea of different presumed physiologies can exhibit considerable plasticity in metabolism: CH_4 oxidizers often have a capacity to nitrify although many nitrifiers can oxidizers CH_4 (76); some bacteria classified as denitrifiers can reduce metals (and vice versa) (77).

Controls on Nitrogen Cycle Pathways

Controls on nitrogen cycle processes range from molecular and physiological regulation (e.g., product feedback inhibition and substrate limitation of enzyme activity and gene transcription), to broad-scale physical phenomenon (light, temperature, diffusion, convective mixing; see NITROGEN FIXATION IN THE MARINE ENVIRONMENT AND NITRIFICATION IN AQUATIC SYSTEMS- this volume). The mix of controlling factors determining the processes that dominate various environments result in the characteristic nitrogen cycle of particular systems.

As alluded to earlier (in *N as a Limiting Nutrient* section), the availability of elements other than nitrogen are often controlling factors in particular ecosystems, or of subpopulations within ecosystems. For instance, although the concentration of a particular substrate or product of a nitrogen transformation pathway may directly modulate a reaction rate in nature, the rate of transformation of specific forms of nitrogen can also be constrained by the availability of other required elements. Iron availability, which has been found to limit primary productivity in certain areas of the ocean (58), may constrain the rates of NO_3^- assimilation or nitrogen fixation because the enzymes responsible for the utilization of these compounds are Fe-containing (i.e., NO_3^- reductase and nitrogenase, respectively) (30,63). Atmospheric deposition of aeolian dust is thought to be a key pathway of Fe flux into the sea (78) and appears to stimulate primary productivity in some areas of the oceans (see following text), possibly through enhancement of NO_3^- assimilation and N_2 fixation (64).

The availability of other chemical constituents may also limit or determine the relative importance of particular nitrogen cycle pathways. Organic substrates can limit denitrification (39) or heterotrophic nitrogen fixation (79). Oxygen concentration can profoundly influence the relative importance and rates of nitrogen cycle reactions such as nitrification, denitrification and nitrogen fixation. The source of phosphorus for planktonic nitrogen fixers, such as the cyanobacterium *Trichodesmium*, which proliferate in highly oligotrophic tropical oceanic waters remains to be identified (65,80). Phosphorus can be an important controlling factor in benthic nitrogen fixation in shallow carbonate systems (59). Most recently, important interactions of silicon, nitrogen, and iron have been shown for oceanic systems dominated by diatoms (57,62,81).

Increased nutrient input into nearshore environments from anthropogenic sources can uncouple controls on natural biogeochemical processes. Such impacts can cause direct perturbations on rates of nitrogen cycling, with dramatic and profound effects on nearshore ecosystems. Examples of anthropogenic disturbance include the effect of duck excreta in stimulation of plankton blooms in Great South Bay, New York (25), and the overgrowth of coral reef ecosystems by macroalgae resulting from increased nitrogen loading (82,83). More dramatic examples are the apparent increases in the extent of anoxia over the last few decades in systems such as the Chesapeake Bay (84,85) or coastal waters of the Mississippi plume (86), which have been related to increased nitrogen fluxes to these systems.

CONTRASTING SYSTEMS

The details of the nitrogen cycle vary greatly among ecosystems with respect to the mode of N delivery and removal, the relative sizes of nitrogen pool and species and the relative importance of particular nitrogen transformation pathways. Contrasts with respect to nitrogen distributions and processes are easily seen along the various spatial gradients that define the major oceanic provinces (87,88)—for example, with respect to distance offshore, mixed layer, and photic zone depth, and as a function of overall depth to the sea floor. Sharp contrasts in nitrogen distributions and transformation processes are evident along latitudinal gradients. Relatively nutrient-poor waters in the oceanic tropics grade into nitrogen-rich waters at temperate and boreal latitudes. In surface waters at higher latitudes, the processes of the nitrogen cycling exhibit strong seasonality. Substantial variations in nitrogen cycling and nitrogen distributions occur with depth in the water or sediment column.

Temperate Estuarine and Coastal Shelf Waters

In nearshore environments, such as estuaries and, to a lesser extent, coastal shelf waters, inorganic nutrient fluxes are often dominated by external inputs, arriving from riverine flow, runoff, atmospheric deposition and groundwater discharge (53). Large inorganic nutrient inputs and pools, combined with a high capacity for primary production, result in rapid incorporation of inorganic nitrogen into organic material. Subsequently, this dissolved and particulate organic material can be exported to the sediments or advected from the system. Because of high plant biomass and suspended inorganic

particles from runoff and resuspension, nearshore systems are typically characterized by a shallow euphotic zone, with relatively strong benthic-pelagic coupling (see earlier). Fluxes of nutrients and biological processes are strongly phased by season and vary spatially along gradients from land to sea. Primary productivity is low in winter. Spring freshets bring large nutrient pulses to estuaries and nearshore waters (74) and promote intense primary production and nutrient cycling in mesohaline regions. Maxima in primary production, nutrient uptake and drawdown, and organic export often occur in the spring or early summer, with recycling becoming more prominent in the summer and fall.

In such organic-rich systems, high NO_3^- concentrations can promote considerable levels of denitrification (42,43), which may otherwise be constrained by in situ nitrification rates (40,89). In general, because relatively high supplies of externally loaded and recycled inorganic and organic nitrogen are available for plant growth, nitrogen fixation is not generally of significance in temperate estuaries or shelf waters (79,90).

Temperate and Boreal Oceanic Waters

The open ocean receives substantially less continentally derived nutrient input (48), and exhibits a far greater dependence on the seasonal injection of deep nutrients into the euphotic zone. In temperate and boreal areas, plant production and nutrient drawdown are a cyclic, seasonal phenomena (51). During the winter, convective mixing of deeper waters recharges the euphotic zone with nutrients. The classical spring diatom bloom of the North Atlantic is initiated when day length increases and a seasonal thermocline is established (88,91). Phytoplankton are confined to the upper mixed layer in which there is abundant NO_3^-. As plant biomass increases, there is a corresponding drawdown of NO_3^- concentration. The bloom is terminated when NO_3^- is drawn down and, concurrently, active grazer populations have developed. Populations shift to a dinoflagellate-dominated community in the summer, which depends largely on recycled nitrogen, vertical migration to deep NO_3^- and the slow diffusion of NO_3^- across the thermocline (51).

Coastal and Circum-Antarctic Upwelling Regions

Wind-driven circulation patterns in ocean gyres result in strong regional upwelling along the eastern flank of the major oceanic basins and in the circum-Antarctic region. NO_3^- and PO_4^{3-}-rich deep waters are upwelled and create areas of intensive water column primary production (92). Phytoplankton populations are often dominated by diatoms and uptake of NO_3^- is a predominant process in the nitrogen cycle. However, iron and silicon may play key roles in controlling overall export production (93,94) (see preceding text). Much of this production can reach the sediments (68). Although these areas represent only a small fraction of the surface area of the global ocean, they account for a disproportionate fraction of global productivity and are often associated with high fish production (92,95). Shelf sediments underlying upwelling areas can receive large amounts of sedimenting organic matter, which supports high levels of denitrification (42).

Equatorial Upwelling

In the equatorial Pacific, there are large areas of equatorial divergence that drive weak upwelling. These zones exhibit enhanced productivity relative to the surrounding oligotrophic gyres (96). Despite the upwelling of NO_3^- in these HNLC areas, levels of phytoplankton biomass do not attain the concentrations expected, given the ambient nutrient concentrations. Among key nutrients, upwelled NO_3^- is not drawn down completely (58,97,98). Martin and coworkers (58) hypothesized that export production and nitrogen cycling in these systems far removed from continental sources was in fact be Fe-limited. Recent experimental research has confirmed that iron is indeed a major factor in limiting phytoplankton populations in HNLC areas (99,100). Silicate also plays a key role in regulating the uptake of nitrate by diatoms and their export in these regions (81).

Subtropical/Tropical Oceanic Gyres

The subtropical and tropical oligotrophic gyres are vast areas of the worlds' oceans, representing 40 to 60% of the total ocean area. In these systems, continental influences are at a minimum, surface water temperatures are high and relatively constant seasonally, the upper water column is permanently stratified and chronically depleted in nutrients and, consequently, primary productivity is also low. Primary production is largely dependent on recycled nitrogen (68). The euphotic zone is typically very deep ($\geq >80$ m), and there is generally a chlorophyll *a* and algal biomass maxima toward the base of the euphotic zone (typically at 60 to 80 m). One source of "new" nitrogen for these systems is the slow vertical diffusion of NO_3^- across the thermocline (4,101) and, possibly, periodic eddy pumping (102). Some NO_3^- transport from depth to the near surface is effected by vertically migrating diatom mats (*Rhizosolenia* nitrogen) (103). Most of the NO_3^- moving upward is intercepted by the phytoplankton populations near the base of the euphotic zone. Atmospheric deposition of dissolved inorganic nitrogen (DIN) (104) and dissolved organic nitrogen may also provide inputs of new nitrogen to these very nutrient-poor systems (10). Nonetheless, the upper mixed layers are generally typified by very low biomass and nutrient concentrations well below the limits of detection by conventional colorimetric measurements (typically estimated to be about 30 nM for NO_3^- and NH_4^+).

Nitrogen fixing organisms, such as the filamentous cyanobacteria *Trichodesmium* spp., inhabit the upper water column in these environments (71). In the subtropical portions of these gyres, nitrogen fixers may only flourish for a few months each year and are of limited significance in nitrogen cycling (105). However, recent research has indicated a much more significant role of nitrogen fixers in tropical systems (48,49,80,106) and a much greater diversity of diazotrophs than previously thought (107) (see NITROGEN CYCLE IN THE MARINE ENVIRONMENT, this Encyclopedia). In the tropical Atlantic Ocean, nitrogen fixation can be of quantitative significance as a source of new nitrogen through the year (71). Moreover, *Trichodesmium* and the diatom endosymbiont

Richelia, which can be observed by ocean color satellite (e.g., SeaWiFS), can bring large pulses of nitrogen into these systems during episodic blooms (108,109).

In the tropical waters of the Indian Ocean, and in the eastern tropical North and South Pacific Ocean, anoxia develops in subsurface waters that have been out of contact with the atmosphere for long periods (see preceding text). Areas of globally significant denitrification occur at the oxic–anoxic interfaces of the intermediate waters of these areas (41). At these interfaces, substantial N_2O production can also occur (110).

Our understanding of the subtropical oligotrophic ocean has greatly benefited over the last decade from the establishment of two time series stations under the U.S. Joint Global Ocean Flux Study (JGOFS): the Hawaiian Ocean Time (HOT) series station north of Oahu in the subtropical Pacific and the Bermuda Atlantic Time Series (BATS) station have provide unmatched data sets with which to discern complex interactions between biogeochemical processes and to detect subtle changes and shifts in major processes over time. Karl and coworkers (70,111) have noted fundamental shifts in phytoplankton populations and predominant nitrogen cycle processes at HOT over the last decade, leading from a previously eukaryote-dominated nitrogen-limited system to a prokaryote-dominated phosphorus-limited system with a substantial input of nitrogen by nitrogen fixers in the present state. Similarly, Michaels and coworkers (112) have detected large drawdown of dissolved inorganic carbon at the BATS site each summer, which cannot be sustained by available pools of inorganic nitrogen or phosphorus. Along with large positive N* anomalies at middepths, they have concluded that a substantial amount of nitrogen fixation must be occurring at this station (48).

Coral Reef, Algal Mats, and Large Plant-Dominated Communities

In shallow marine systems, a variety of benthic plant communities proliferate. These systems exhibit particularly intense rates of primary production and nitrogen cycling and include temperate salt marshes (113), the rooted seagrasses of the tropics and temperate zones (114), tropical mangroves (115), kelps (116) and coral reefs (117). Macroalgal communities (118) and algal mats often occur in association with each of these communities.

Rooted plant systems such as the seagrasses and salt marshes, are very productive and, therefore, plant nitrogen demand is high (see SEAGRASSES COMMUNITIES AND PLANT–MICROBE INTERACTIONS IN THE MARINE ENVIRONMENT, this Encyclopedia). Much of this demand is met by root uptake of nitrogen from sediment pore waters (119). Intensive nitrogen regeneration and nitrogen fixation in the sediments provide ample dissolved nitrogen in these systems to meet demand (120). Algal mats are notable for their vertical compression of processes. Coral reefs, which are largely defined by the coral-algal symbioses (but which may also be dominated by macroalgae) are generally thought to proliferate in nutrient-poor tropical waters because of high levels of nitrogen fixation occurring in these environments (117,120).

GLOBAL PERSPECTIVES

Nitrogen Budgets and Balances

Processes and pools in the marine nitrogen cycle are often placed in the context of the terrestrial and global nitrogen cycles. Estimates of the gaseous nitrogen pools and some inorganic nitrogen (e.g., NO_3^-) pools have been refined on the basis of results from diverse and far-ranging oceanographic studies such as the GEOSECS (Geochemical Sections) and WOCE (World Ocean Circulation Experiment), as well as intensive monitoring programs in many nearshore and coastal areas. Because of the difficulty in the analytical approaches involved in measuring organic DON, PON pools, far fewer measurements are available for these nitrogen compounds. Consequently, our projections of global pools of DON and PON are less robust. Although we broadly understand the distribution and relative importance of nitrogen processes, we still lack quantitative estimates of the fluxes and internal transformations of nitrogen within many systems.

With increasing evidence of large-scale perturbations of the nitrogen cycle (see following text), it has become more pressing to develop capabilities to predict changes that may occur in the near term. Estimating the overall current size of the various pools and magnitude of specific processes of the nitrogen cycle in the sea, improving our estimates as new data become available, and attempting to retrospectively infer changes that have occurred in earlier earth history will foster that ability.

Current estimates of the major fluxes of nitrogen in the ocean indicate that riverine inputs into the oceans are relatively well constrained and are thought to account for an input of between 21 and 110 Tg N/y. Atmospheric deposition to the sea surface accounts for an input of about 56 to 154 Tg N/y (Table 2). This value has recently been revised upward to include DON deposition (10). Current estimates for pelagic nitrogen fixation range from about 80 to more than 190 Tg N/y (121) (Galloway and coworkers in prep.). Benthic nitrogen fixation has been estimated to account for an input of about 15 Tg N/y (122).

The demand for nitrogen by primary producers can be inferred from extensive surveys of primary production in the world's oceans. Current estimates suggest that nitrogen demand for total oceanic primary productivity to be about 7,200 Tg N/y (123). Of this total, export ("new") production is thought to account for about one-third, or 2,400 Tg N/y. In theory, in a steady state ocean, the demand for new nitrogen should be met by the sum of external inputs (atmospheric deposition and sources from land), nitrogen fixation and NO_3^- from depth (22,38). Even assuming maximum values for the other sources of nitrogen (Table 2), NO_3^- from depth (arising from regeneration and nitrification) must account for the bulk of new nitrogen demand (Fig. 2).

The oceans are currently estimated to be taking up atmospheric carbon dioxide at a rate of about 2 Pg/y (124). This occurs through both chemical solution of CO_2 (referred to as the solubility pump) and by biological uptake (the biological pump) (125). The amount of nitrogen that would be required to biologically assimilate 2 Pg

Table 2. Major Nitrogen Fluxes in the Ocean

Process	Flux Tg N/y	Source
Inputs		
Riverine & runoff	21 to 110	8, 126, 163
Atm deposition	56 to 154	10
N_2 fixation Water column	80 to 190	49, 71, 122
Benthic	15	122
Total	172 to 469	
Production demand		
Planktonic		
Total Prod	7,200	123
Export Prod	2,424	123
Benthic-net annual	160	164
Outputs		
Sedimentation	20 to 38	127, 165, 166
Denitrification Water column	64 to 290	41, 129, 130
Benthine	60 to 300	41, 42, 43, 44
Total	144 to 628	

of carbon dioxide assuming Redfield stoichiometry is about 350 Tg. This is approximately equivalent to upper projections for all external inputs including nitrogen fixation (Table 2).

Estimates of rates of nitrogen losses from the sea through sedimentation of PON and burial over the vast expanses of the sea floor are difficult to make and are therefore also rare. However, given the great depth and relatively low productivity over much of the oceans, little detritus makes it to deep sediments (73). Continental margins are thought to be key areas of organic burial in the current ocean (126,127). Current estimates of N burial range from 20 to 38 Tg N/y (Table 2).

Denitrification in the oceans has been examined more intensively, particularly in the Indian (128) and tropical Pacific Oceans (41,129), and on the continental shelves (42,44) (see preceding text) over the last few decades, and there have been several upward revisions of its quantitative significance (130). Water column denitrification has been estimated to account for losses of about 64 to 290 Tg N/y, whereas denitrification losses from coastal and shelf sediments has been estimated to be approximately 60 to more than 300 Tg N/y (42,44). Other mechanisms, whereby nitrogen may be lost from the system include gaseous evasion of nitrogen (16) and fisheries harvest.

Although lower end estimates are about equivalent, current upper end values indicate total nitrogen inputs are about 160 Tg N/y less than estimates for its removal, suggesting an imbalanced nitrogen cycle. This may be because of underestimates of nitrogen inputs or overestimates of nitrogen losses. However, there is no a priori reason to assume a balanced nitrogen cycle, which may only be achieved over longer timescales (e.g., timescales of oceanic mixing; 131).

Large-Scale Perturbations of the Nitrogen Cycle

Current evidence indicates large-scale natural (70) and human-induced (6) perturbations in the marine nitrogen cycle, paralleling those occurring in the carbon cycle (132). River loads of nitrogen to the sea have increased dramatically over recent time (133). Resultant coastal eutrophication and increasing hypoxia have been mentioned earlier (see Controls on N cycle pathways, this Encyclopedia). Increasing nutrient loading in coastal areas may account for stimulated uptake and burial of carbon dioxide on the shelf in these areas (126).

Nitrous oxide has been implicated in atmospheric reactions leading to the destruction of ozone in the stratosphere (7). It is also a potent greenhouse gas with a several hundredfold greater capacity for absorption of radiation per molecule than carbon dioxide and can thereby contribute to radiative heating in the troposphere (134). Direct manifestation of global perturbation has also been detected in atmospheric N_2O pools. The atmospheric concentration of N_2O, which is about 330 ppb at present, has been increasing at about a rate of 0.2 to 0.3% per year (134). The role of the oceans as a source or sink of N_2O to the atmosphere is uncertain (21,38). As indicated earlier, the seas play a central role in N_2O cycling (38) and may act as source (16,135) or sink for this gas.

The suggestion by Martin and coworkers (93), that fertilization of HNLC areas of the ocean with Fe to stimulate oceanic primary production and carbon export in these areas could help ameliorate increasing atmospheric CO_2, created a lively discussion during the early 1990s about the merits of such a scheme (136). Although much of the debate centered on the factors controlling phytoplankton populations in these areas, discussions of the feasibility and merits of large-scale environmental engineering also entered the debate. The possibilities that such a scheme would work was met with skepticism by many oceanographers (69,137). Among possible scenarios proposed, Fuhrman & Capone (138) proposed that fertilization could stimulate the production of other gases with potential impacts on climate, such as N_2O and dimethyl sulfide (DMS), through the production of suboxic waters with increased surface production and flux. Although N_2O wasn't analyzed during the IRONEX experiment, Turner and coworkers (139) did observe increases in DMS.

Interest in ocean Fe fertilization has been reinvigorated with the positive results of several more open-ocean iron enrichment experiments in two HNLC regions, the IRONEX II experiment in the equatorial Pacific (100), and the SOIREE experiment in the Southern Ocean (140). Furthermore, the proposition that nitrogen fixers are largely nitrogen limited in low-nutrient low-chlorophyll (LNLC) (141) regions presents the prospect of stimulating this process by Fe fertilization as well.

HOW WE STUDY THE NITROGEN CYCLE

The approaches to study the marine nitrogen cycle are many and varied, and new methods are constantly evolving. As in all analytical fields, we are ever seeking to improve our sensitivity for detecting analytes and for specific transformations, to develop methods to detect new compounds and pathways, to minimize our perturbations of systems studied, to reduce the

introduction of experimental artifacts and to increase the efficiency, frequency, and coverage of our sampling (142,143).

It is beyond the scope of this article to detail and critique the various analytical methods involved in assessing nitrogen cycling. Parsons and coworkers (144) publication serves as a starting point for methods involved in routine determination of major inorganic and organic pools. D'Elia (145) has reviewed methods for determination of nitrogen compounds in seawater.

Methodology for the various microbiological and biogeochemical assays involved in nitrogen cycling may be found in several recent compendia. See articles in: Kemp and coworkers (146), including Capone (147,148) and Seitzinger (149); in Hurst et al. (150) including Capone (151); and in Knowles & Blackburn (152) including (153). See also (154).

At the broadest level, our approaches to studying the marine nitrogen cycle can be regarded as either observational or experimental, and the earliest insights into the marine nitrogen cycle came from observations of the spatial and temporal distributions of key nutrients such as NO_3^- in marine waters and sediments. The application of the natural abundance of stable isotopes of biologically relevant elements has greatly enhanced the utility of observational approaches (155). Using the small differences in the ratio of the stable isotopes of nitrogen, ^{14}N and ^{15}N, with a knowledge of source materials and the extent of fractionation by particular biological and chemical transformations, one can glean substantially more information than from just the distributions of particular pools of the nitrogen cycle.

In parallel with observational studies, experimental studies using natural and cultured populations have expanded our understanding of microbially mediated nitrogen transformations. The isolation and characterization of nitrifying (156), denitrifying (41) and nitrogen-fixing (79) marine bacteria and archaea has allowed us to identify and characterize the microbiological basis of the major nitrogen transformations. Experimental approaches examining nitrogen transformations have allowed direct confirmation of inferences and hypotheses developed using observational and classical microbiological methods. Direct tracer methods are preferred to directly quantify specific pathways of biological nitrogen transformation (157). Although nitrogen does have a radioisotope, ^{13}N, its very brief half-life and the sophisticated facilities necessary to generate it, precludes its usefulness in field applications (148,151). In contrast, the stable isotope of N, ^{15}N, is available in a variety of forms useful for determining rates of specific pathways.

The enzymes mediating various nitrogen transformations (e.g., assimilatory NO_3^- reductase, glutamine synthetase) have been characterized and assays developed to estimate the activity in natural populations of marine microbes (30). Recently, immunological and molecular methods have provided sensitive means to detect proteins and genes involved in nitrogen cycle transformations (158,159).

Providing mathematical formalisms, which describe the cycling of nitrogen in the sea and which test our conceptualizations of processes is an activity dating back to Redfield (160), Riley (161) and Dugdale (162). "Modeling" has become an indispensable tool with which to integrate our knowledge of the marine nitrogen cycle and to place this knowledge in a broader framework.

CONCLUSION

The biogeochemical cycle of nitrogen is relatively complex, with forms of nitrogen occurring over a range of oxidation states, and in a variety of chemical forms and phases. A diversity of biological nitrogen transformations are linked to reactions of energetic metabolism and basic nutrition. Many of the reactions are restricted to prokaryotes. The uptake and release of nitrogen to and from organic pools are quantitatively important pathways in many plant-dominated environments. The bacterial nitrogen transformations of nitrogen fixation, nitrification and denitrification, largely determine the form and relative availability of key nitrogen species and there is considerable variation among systems with respect to the relative importance of these pathways. The nitrogen cycle is an important feature of all marine ecosystems and is a factor affecting the net productivity of many of these systems: it is susceptible to perturbation at various levels. Components of the marine nitrogen cycle may provide important feedbacks to marine carbon cycling and to global climate change.

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NITROGEN FIXATION. See SOIL NITROGEN CYCLE

NITROGEN FIXATION IN METHANOTROPHS. See METHANOTROPHIC BACTERIA

NITROGEN FIXATION IN SOILS — FREE-LIVING MICROBES

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Biological dinitrogen (N_2) fixation, the conversion of atmospheric dinitrogen to ammonia for incorporation into cell components, is often described as the second most important biological process on earth after the process of photosynthesis. In fact, it would be seen as equally important from certain points of view. It is true that life on earth depends on the use of solar energy and the fixation of carbon dioxide by photosynthetic plants and microbes to produce the biomass that is at the base of all food webs. However, primary production (i.e., CO_2 fixation and biomass production) is generally limited by the availability of fixed nitrogen (N), for example, ammonium (NH_4^+) and nitrate (NO_3^-). Thus, high rates of photosynthesis and plant production are dependent on a ready supply of fixed nitrogen.

Before the advent of industrial fertilizer factories, agricultural production relied on the use of animal manures, green manures (crops such as legumes grown to enrich the soil), and the use of dinitrogen-fixing grain and forage legumes. Natural, unfertilized ecosystems rely on nitrogen from wet and dry deposition and from any of a number of associations that involve free-living (nonsymbiotic) dinitrogen-fixing microbes (bacteria and cyanobacteria) or symbiotic plant-microbe associations involving root-nodule bacteria such as *Rhizobium* and *Bradyrhizobium* or the actinomycete *Frankia* (1).

This review explores the fundamentals of the process of biological dinitrogen fixation and the role of free-living dinitrogen-fixing microbes in soil. Root-nodule symbioses are covered in detail in NITROGEN FIXATION (IN SOILS, SYMBIOTIC) this Encyclopedia.

THE SIGNIFICANCE OF DINITROGEN FIXATION

Global agricultural consumption of synthetic nitrogen fertilizer increased 27-fold between 1950 and 1990 to approximately 80 million metric tons per year, which is about 50% of the total amount of nitrogen fixed by biological and physical processes (2). Nitrogen is absolutely essential for microbes and higher organisms to synthesize proteins and other important biochemicals (e.g., nucleic acids), and it is a fundamental tenet of agriculture and plant physiology that plants require relatively high levels of nitrogen to produce abundant biomass (yield). Nitrogen is generally the most limiting nutrient for plant and microbial growth (assuming adequate available carbon for microbes) in soils not receiving supplemental fertilizer nitrogen. In natural systems, nitrogen for plant growth comes from the mineralization of soil organic matter, from rainfall or other atmospheric deposition, or through biological dinitrogen fixation.

About 85 million metric tons of nitrogen is fixed annually through industrial processes (3–5). The expense

factor of nitrogenous fertilizers is derived in part from the requirement for substantial inputs of energy, usually in the form of natural gas (see Section Energy costs of nitrogen fixation). However, industrial fixation provides only about 1/3 to 1/2 the amount of fixed nitrogen acquired through biological dinitrogen fixation. Accurate estimates of the magnitude of biological dinitrogen fixation are hard to derive but reported values range from 100 to 180 million metric tons per year. About 65% of the nitrogen used in agriculture is biologically fixed nitrogen (6). Most of this is through symbiotic dinitrogen fixation (see NITROGEN FIXATION IN SOILS (SYMBIOTIC)) but nonsymbiotic and associative dinitrogen fixation is reportedly of significance in crops such as sugarcane and sorghum, which have a C_4 photosynthetic pathway and may be grown in ecosystems where nitrogen for plant growth is a limiting factor. Paddied rice, because of its cultivation in flooded soils, derives significant benefit from the activities of free-living diazotrophic bacteria and cyanobacteria that find favorable habitats (root exudates, low oxygen concentrations, etc.) in the flooded soils.

Biological dinitrogen fixation remains an effective alternative to the use of expensive ammonium-based fertilizer nitrogen but it must be emphasized that the high-yielding agricultural systems of the U.S. and elsewhere are difficult to sustain by relying only on biological dinitrogen fixation and the use of animal manures. The growing world population will probably dictate production systems that will require judicious use of synthetic and, where possible, organic fertilizers. Legumes, other dinitrogen-fixing systems (e.g., the *Azolla-Anabaena* symbiosis), and perhaps genetically modified plants yet to be developed will continue to play an important role in sustainable agricultural production. Nitrogen-fixing plants are also of high value in restoring disturbed or impoverished soils. They serve as excellent cover crops, green manures, and forage crops for livestock production. Use of dinitrogen-fixing crops also has the potential to reduce the contamination of groundwater with nitrate. Further aspects of the utility of symbiotic nitrogen fixation in agriculture and other ecosystems are discussed elsewhere in this volume (NITROGEN FIXATION (SYMBIOTIC) IN SOILS).

THE NITROGENASE ENZYME COMPLEX

It is believed that the process of biological dinitrogen fixation is restricted to procaryotic microbes, including many genera of soil bacteria, cyanobacteria (formerly the "blue-green algae"), and a few actinomycetes (most notably, *Frankia*). Microbes that can use dinitrogen as their sole nitrogen source are often referred to as "diazotrophs" (a term coined by R.W.F. Hardy in the early 1970s). When used in this article, the term applies to any dinitrogen-fixing microbe (bacterium or cyanobacterium) that is capable of fixing dinitrogen independent of any higher-plant host if given sufficient supplies of energy and carbon sources. These microbes can fix dinitrogen as free-living forms (i.e., not plant associated) in loose associations with higher plants called associative symbioses (e.g., many grass-bacteria associations) (1) and in truly symbiotic partnerships with higher plants called root-nodule symbioses, involving rhizobia and *Frankia*.

A trait shared by all the microbes mentioned earlier is that they produce the enzyme complex called "nitrogenase," which mediates biological dinitrogen fixation. It is most appropriately called the "nitrogenase complex" because it is actually composed of two protein components (dinitrogenase and dinitrogenase reductase), each of which is composed of multiple subunits. The nomenclature of the nitrogenase complex is as follows (7,8):

- The overall complex is known as nitrogenase.
- The MoFe protein is dinitrogenase (the "type species" substrate is dinitrogen; enzymes conventionally are named relative to their substrate).
- The Fe protein is designated dinitrogenase reductase; the general consensus is that its function is the reduction of dinitrogenase.
- Functional aspects of the nitrogenase complex are shown schematically in Figure 1. Unusual properties of the nitrogenase complex (8) are summarized in the following section:
- It consists of two proteins; the MoFe protein (dinitrogenase) and the Fe protein (dinitrogenase reductase);
- it is destroyed by oxygen;
- it contains iron and molybdenum or vanadium;
- it needs Mg^{2+} ions to be active;
- it converts ATP to ADP when functioning;
- it is inhibited by ADP;
- it reduces dinitrogen and several other small triply bonded molecules;
- it reduces H^+ to H_2 even when dinitrogen is present.

The overall reaction for biological dinitrogen fixation using nitrogenase is as follows:

$$N_2 + 8H^+ + 8e^- + 16\text{Mg-ATP} \xrightarrow{\text{Nitrogenase}} 2NH_3 + 16\text{Mg-ADP} + \text{nPi} + H_2 \uparrow \quad (1)$$

Two MgATPs are required for each electron transferred from dinitrogenase reductase to dinitrogenase. Thus, the reaction, as written, shows a requirement for 16 molecules of ATP (470 kJ; 112 kcal). This is the equation for the reaction as mediated by cell-free enzyme extracts. Under natural conditions, however, probably 20–30 MgATPs may be needed as the process is less efficient than when observed under optimum laboratory conditions (9,6). A consensus on the general model for the mechanism of nitrogenase has evolved over quite a few years. The mechanism can be summarized as follows:

- Dinitrogenase reductase (the Fe protein) accepts electrons from a low-redox donor such as reduced ferredoxin (Fd_{red}) or flavodoxin (Fld) and it binds two MgATPs (Fig. 1).
- It transfers electrons, one at a time, to dinitrogenase (the MoFe protein).

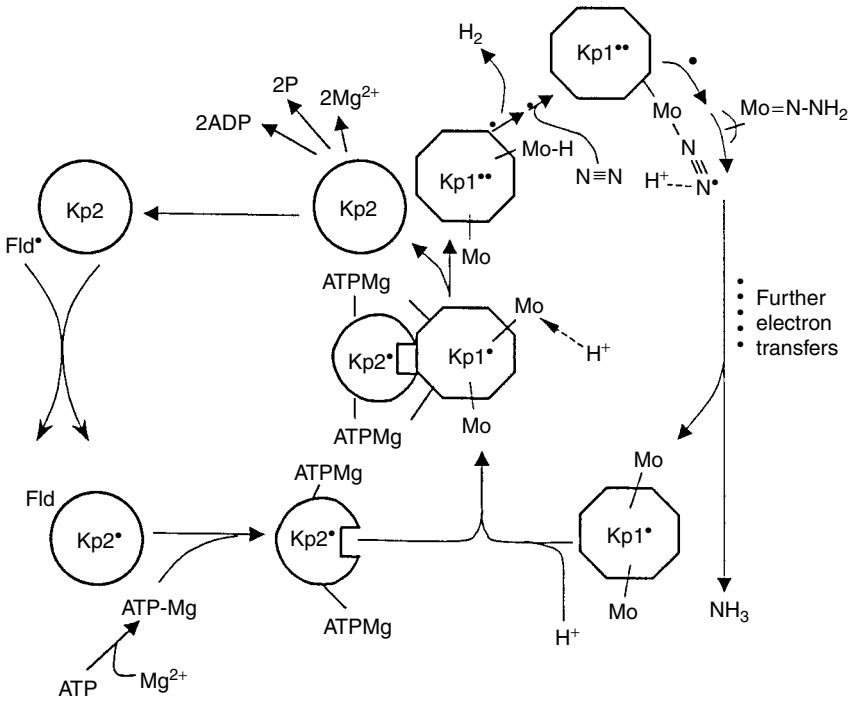

Figure 1. A scheme for the action of the Klebsiella nitrogenase complex. Kp1 is the larger protein, containing iron and molybdenum atoms (dinitrogenase, the MoFe protein); Kp2 is the smaller protein with iron atoms only (dinitrogenase reductase, the Fe protein). Fld is flavodoxin, which donates an electron (black dot) to iron atoms in Kp2. ATP reacts with magnesium ions to produce a compound which activates the reduced form of Kp2. Meanwhile Kp1, with a "spare" electron among its iron atoms, has bound a hydrogen ion from water at a transition metal atom depicted as molybdenum. Activated Kp2 joins Kp1 carrying substrate to form a complex, assisted by ATPMg, within which an electron from the iron atoms in Kp2 is transferred to the iron atoms in Kp1, prior to reaching the bound substrate. After two such electron transfer events, the two bound hydrogen atoms are displaced by N_2 and released as hydrogen gas (H_2). Further electron transfers lead to hydrogen ions binding to the bound N_2 and so by way of at least one metal-bound intermediate stage (depicted as $-Mo-N-NH_2$) the product, NH_3, of enzyme action is released. Each electron transfer requires a new electron from Kp2 and each time the transfer takes place two molecules of ADP are formed from the ATP. The two proteins have to come together and separate eight times to reduce one N_2 molecule. The scheme is one of several that could be proposed based on the experimental findings that Kp2 is concerned with ATP consumption and electron transfer, Kp1 with substrate binding and reduction. Redrawn from J. R. Postgate, Nitrogen Fixation. 3rd Ed., Cambridge University Press. Cambridge, UK, 1998. Reprinted with permission of Cambridge University Press.

- Dinitrogenase reductase and dinitrogenase form a complex, the electron is transferred, and two MgATPs are hydrolyzed to two MgADP + Pi (phosphate).
- Dinitrogenase reductase and dinitrogenase dissociate and the process is then repeated.
- When dinitrogenase has collected enough electrons, it binds a molecule of dinitrogen, reduces it, and releases NH_3.
- The MoFe protein (dinitrogenase) then accepts additional electrons from dinitrogenase reductase (the Fe protein) to repeat the cycle.

In each cycle of dinitrogen fixation, dinitrogenase and dinitrogenase reductase bind together, MgATP is hydrolyzed and an electron is transferred. The dissociation of the MoFe protein–Fe protein complex is the rate-limiting step of the process. In fact, the nitrogenase complex is "remarkably slow — it takes 1.25 seconds for a molecule of enzyme to form two of NH_3. The two proteins have to come together and separate eight times to reduce one dinitrogen molecule" (8). A consequence of the slowness of nitrogenase is that dinitrogen-fixing bacteria must synthesize a lot of the protein. Nitrogenase can commonly account for 10% of the cell's proteins, and levels up to 40% have been recorded (10).

Returning to the reaction of biological dinitrogen fixation described earlier, we can see that for every eight electrons (e^-) transferred via "nitrogenase," $2e^-$ are consumed in the formation of H_2. The production of H_2 that accompanies the fixation of dinitrogen is obligatory. One H_2 (requiring 4 MgATP) is released for each dinitrogen reduced to $2NH_3$ (requiring 12 MgATP). Thus, 25% of the energy from MgATP is "lost" in the production of H_2. Interestingly, some diazotrophs contain an uptake hydrogenase that allows them to oxidize some of the H_2 and to generate a reduced electron carrier or MgATP. This can then be used in the dinitrogen fixation reaction, thereby recapturing some of the energy lost.

The Alternative Nitrogenases

Before the early 1980s, researchers believed that there was only one type of nitrogenase and that molybdenum was essential for dinitrogen fixation. However, in the early 1980s, Dr. Paul Bishop and his colleagues in North Carolina isolated a second nitrogenase (nitrogenase 2) from *Azotobacter chroococcum*, which was produced only under conditions of molybdenum starvation (11). With this

discovery it became evident that molybdenum was not an obligatory requirement for dinitrogen fixation in all bacteria but that it could be substituted with vanadium. Nitrogenase 2 is like nitrogenase 1 in that it consists of two proteins, a VFe protein and an Fe protein, produces H_2, and is sensitive to O_2. Nitrogen binds to the VFe protein, suggesting that metals other than Mo can participate in binding the nitrogen to the protein for reduction. Subsequently, a third nitrogenase, nitrogenase 3, was described (8). This complex does not appear to contain either Mo or V and is synthesized under starvation for both of the metals.

All of the "nitrogenases" are complexes of a dinitrogenase reductase and a dinitrogenase component. Nitrogenases 2 and 3 appear to produce more H_2 than nitrogenase 1 and reduce acetylene to ethane (C_2H_6) rather than ethylene. Quite recently, yet another nitrogenase has been described. *Streptomyces thermoautotrophicus* is a unique, thermophilic (it can grow at 65 °C) actinomycete that can use carbon monoxide (CO) as an energy source and oxidize it to CO_2 or can use H_2. It fixes dinitrogen and is unique in that its nitrogenase is not inhibited by CO and neither reduces acetylene nor is poisoned by O_2. It also relies on a manganese-containing protein that apparently functions as the dinitrogenase (12,8).

The finding that dinitrogen can be fixed in the absence of molybdenum (Mo) is interesting because prior to the discovery of the alternative nitrogenases it was believed that Mo was mandatory for dinitrogen fixation. It now seems that the predominance of bacteria containing nitrogenase 1 (the Mo-containing enzyme) may have been due to the fact that researchers habitually used Mo-containing media to isolate dinitrogen-fixing bacteria. The other nitrogenases may be quite common in nature although this is not yet known. The study of alternative nitrogenase systems is quite young and many fundamental questions remain unanswered.

Substrates for Nitrogenase

The natural primary substrate for nitrogenase is dinitrogen (N≡N). In addition to reducing N_2 to NH_3 and protons to H_2, nitrogenase also reduces several other small, triple-bonded molecules (Table 1). Of particular interest among this list of substrates is the gas acetylene. In the late 1960s, it was discovered that nitrogenase could reduce acetylene (HC≡CH) to ethylene ($H_2C{=}CH_2$) (13). The significance of this finding lay in the fact that acetylene and ethylene can be very easily measured using a gas chromatograph to separate the two gases. This gave rise to the simple, sensitive, and rapid acetylene-reduction assay for nitrogenase activity, the development of which led to an explosion in research on dinitrogen fixation. It eliminated the need to have an expensive mass spectrometer for studies using ^{15}N or to deal with the tedious and insensitive Kjeldahl analyses to measure nitrogen in soil or biological materials. The technique is currently widely used, but one should become familiar with the limitations of the technique before extrapolating its results to actual rates of dinitrogen fixation in field samples (14,15).

Table 1. Substrates for Nitrogenase: The Triple Bond Connection

Name	Formula	Major Products
Proton	H^+	H_2
Dinitrogen	N≡N	$NH_3 + H_3$
Nitrous oxide	$N{\equiv}N^+{-}O^-$	$N_2 + H_2O$
Azide	$[N{\equiv}N^+{-}N^-]$	$N_2 + NH_3 + N_2H_4$
Acetylene	HC≡CH	$H_2C{=}CH_2$
Cyanide	$[C{\equiv}N]^-$	$CH_4 + NH_3$
Carbon monoxide	C≡O	C≡O apparently binds to the N-binding site but is not reduced. It blocks reaction with other substrates.

The Genetics of Nitrogenase

As one might surmise from the complexities of the system described earlier, the genetics of the process are equally complex. Much of our knowledge of the genetics of the nitrogenase system has come from the intensive study of the bacterium *Klebsiella pneumoniae*, a member of the family Enterobacteriaceae and a close relative of *Escherichia coli*. Thirty years of research on this bacterium and, more recently, other diazotrophic bacteria has shown that the nitrogenase complex and supporting systems are under the control of 20 or more genes (Fig. 2); Table 2). The functions of the nitrogen-fixation genes are summarized in Table 2, starting with the gene on the right-hand side of Figure 2. Clearly, the complexity of this system does not make it an easy target for genetic manipulation and transfer to higher organisms. Still, some hold out the prospect that we may someday see genetically engineered crops such as corn, wheat, and other cereals that fix their own nitrogen. However, this is one of the more elusive goals facing nitrogen-fixation researchers.

Physiological Prerequisites for Dinitrogen-Fixing Microbes

The physiological requirements for a free-living diazotroph to fix dinitrogen (8) are summarized in Table 3. These stem

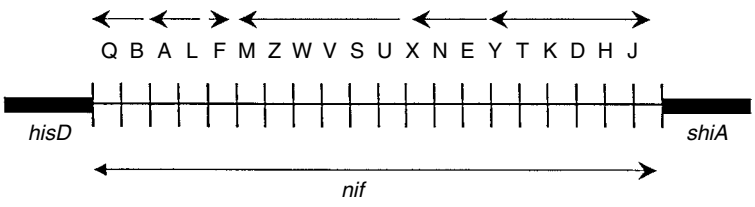

Figure 2. Map of the nitrogen fixation genes of *Klebsiella pneumoniae*. The capital letters denote the separate genes that make up the *nif* cluster. The upper arrows indicate subclusters (operons) in which genes are sequentially transcribed as a group and the directions in which they are read. Brief descriptions of the function(s) of each gene are given in Table 2. Redrawn from J. R. Postgate, Nitrogen Fixation, 3rd ed., Cambridge University Press, Cambridge, U.K., 1998. Reprinted with permission of Cambridge University Press.

Table 2. Currently Recognized or Postulated Functions of the Genes for Nitrogen Fixation in Diazotrophic Procaryotes

nifJ codes for an enzyme (pyruvate oxido-reductase) that generates from pyruvate the electrons used for the reduction of dinitrogen.
nifH codes for a peptide molecule, two of which combine to form dinitrogenase reductase, the smaller of the proteins constituting nitrogenase.
nifD codes for a peptide molecule (called the α-subunit), two of which combine to form part of dinitrogenase, the molybdoprotein of nitrogenase.
nifK codes for a somewhat different peptide molecule (called the β-subunit). Two of these combine to form the other part of the dinitrogenase protein, which is thus an $\alpha_2\beta_2$ tetramer composed of two pairs of similar peptides.
nifT codes for a very small protein molecule of unknown function.
nifY codes for a protein of uncertain function.
nifE codes for a peptide molecule, two of which combine to form the NifE product.
nifN codes for a peptide molecule, two of which combine to form the NifN product. The NifE and NifN proteins combine together to form a protein that somewhat resembles dinitrogenase, which appears to act as a template for synthesizing FeMoco.
nifX codes for a protein, which may have a regulatory function.
nifU codes for a protein possibly concerned with the iron-sulfur center of dinitrogenase reductase.
nifS codes for a protein, which may also be concerned with the Fe-S center of dinitrogenase reductase.
nifV codes for a peptide, two molecules of which combine to form an enzyme which makes homocitrate, a part of FeMoco, from 2-oxoglutarate and acetyl-coenzyme A.
nifW codes for a protein necessary for full activity of dinitrogenase.
nifZ codes for a protein, which seems to be involved in the insertion of FeMoco into dinitrogenase.
nifM codes for a protein concerned with rendering dinitrogenase reductase active.
nifF codes for the flavodoxin, which accepts electrons from pyruvate, via the *nifJ* product, and passes them to dinitrogenase reductase.
nifL codes for a regulator protein, which switches off the whole *nif* cluster.
nifA codes for a regulator protein, which switches on the whole *nif* cluster.
nifB codes for a protein involved in the synthesis of FeMoco.
nifQ codes for a protein involved in the uptake of molybdenum for FeMoco synthesis.

Note: J. R. Postgate, Nitrogen Fixation, 3rd ed., Cambridge University Press, Cambridge, U.K., 1998. Reprinted with permission of Cambridge University Press.

Table 3. Physiological Requirements for Nitrogen Fixation by Free-living Diazotrophic Microbes

- The need for trace elements
  - — Molybdenum or Vanadium, Iron — for "nitrogenase"
  - — Magnesium — for production of "MgATP"
- The need for ATP
  - — A minimum of 16 ATP per N_2 fixed. Probably 20–30 under natural conditions.
  - — Needed for nitrogenase activity and for nitrogenase synthesis.
  - — The high ATP requirement means an abundant supply of energy-yielding substrates must be readily available for vigorous N_2 fixation.
- The need to minimize H_2 evolution
  - — Up to 35% of the ATP diverted to nitrogenase may be consumed in H_2 evolution.
- Acceptable Temperature
  - — Most diazotrophs are mesophiles.
  - — Many will not grow on media at 37 °C.
  - — Nitrogenase activity falls off rapidly at about 40 °C.
- The need to exclude O_2 from the enzyme complex
  - — Nitrogenase is destroyed by O_2
- A source of low-redox reductant
  - — This is restricted to the naturally occurring ferredoxins and flavodoxins.
- Low concentrations of "fixed N"(NH_4^+, NO_3^-, organic N)
  - — Nitrogenase synthesis is repressed by very low levels of combined N and activity of existing enzyme is inhibited.

Note: Adapted from J. R. Postgate, Nitrogen Fixation, 3rd ed., Cambridge University Press, Cambridge, U.K., 1998.

primarily from the unique properties and intricacies of the nitrogenase complex, including its exceptional sensitivity to molecular oxygen, the metal content of the complex (Fe and Mo or V), and the need for adequate supplies of reducing power and MgATP.

As is the case with all soil microbes, diazotrophs are also subject to the limitations imposed by physical, chemical, and biotic factors in their environments. Thus, factors such as moisture content, pH, soil nitrogen (through its effects on enzyme synthesis and activity), temperature, and competition with other microbes, especially for limited supplies of energy sources, can adversely affect their abilities to grow and fix dinitrogen.

Most of the diazotrophs listed in Table 4 are mesophiles (microbes growing at moderate temperatures) and fail to fix appreciable quantities of nitrogen above 37°–40°C.

Table 4. List of Genera of Microbes That Include Dinitrogen-Fixing Species or Strains. The List Is Not Intended to Be All-Inclusive. [Adapted from J. R. Postgate, Nitrogen Fixation, 3rd ed., Cambridge University Press, Cambridge, U.K., 1998. See also Eady (17) and Young (18)

	Genus or Type	Species (Examples Only)
Aerobes	*Azotobacter*	*A. chroococcum**, *A. vinelandii**
	Azotococcus	*A. agilis**
	Azomonas	*A. macrocytogenes**
	Beijerinckia	*B. indica**, *B. fluminis**
	Derxia	*D. gummosa**
	Pseudomonas	*P. stutzeri P. saccharophila*
	Azoarcus	*A. communis*, *A. indigens*
	Acetobacter	*A. diazotrophicus*
Facultative (aerobic when not fixing N_2)	*Klebsiella*	*K. pneumoniae K. oxytoca*
	Paenibacillus	*P. polymyxa*, *P. macerans*
	Enterobacter	*E. agglomerans* (*Erwinia herbicola*)
	Citrobacter	*C. freundii*
	Escherichia	*E. intermedia*
	Propionibacterium	*P. shermanii*, *P. petersonii*
Microaerophiles (normal aerobes when not fixing N_2)	*Xanthobacter*,	*X. flavus**, *X. autotrophicus*
	Thiobacillus	*T. ferro-oxidans*
	Azospirillum	*A. lipoferum**, *A. brasilense**, *A. amazonense*, *A. halopraeferens* *A. irakense*
	Aquaspirillum	*A. perigrinum**, *A. fascicilus**
	Methylosinus	*M. trichosporum*
	Bradyrhizobium	*B. japonicum*
	Herbaspirillum	*H. seropedicae*
	Burkholderia	*B. brasilense*
	Azoarcus	*A. indigens*, *A. communis*
Strict Anaerobes	*Clostridium*	*C. pasteurianum**, *C. butyricum*
	Desulfovibrio	*D. vulgaris*, *D. desulfuricans*
	Methanosarcina	*M. barkeri*
	Methanococcus	*M. thermolithotrophicus*
Phototrophs (aerobic cyanobacteria)	*Anabaena*	*A. cylindrica*, *A. inaequalis*
	Nostoc	*N. muscorum*
	Gloeothece	*G. alpicola*
	Calothrix (7 other genera of heterocystous cyanobacteria)	
Phototrophs (microaerophilic cyanobacteria)	*Plectonema*	*P. boryanum*
	Lyngbya	*L. aestuarii*
	Oscillatoria	
	Spirulina	
Phototrophs (facultative bacteria)	*Rhodospirillum*	*R. rubrum*
	Rhodopseudomonas	*R. palustris*
Phototrophs (anaerobic bacteria)	*Chromatium*	*C. vinosum*
	Chlorobium	*C. limicola*
	Thiopedia	
	Ectothiospira	*E. shapovnikovii*

*Signifies that all reported strains of the species fix N_2. Reprinted with permission of Cambridge University Press.

It is noteworthy that three diazotrophic microbes have now been confirmed as thermophiles (microbes growing at high temperatures) (8). These include *Methanococcus thermolithotrophicus*, a methanogenic bacterium that fixes dinitrogen at 64 °C; *Mastigocladus*, a cyanobacterium from hot springs that can fix dinitrogen at 60 °C; and *Streptomyces thermoautotrophicus,* an actinomycete isolated from a smoldering charcoal heap that can fix dinitrogen at 65 °C.

The Energy Costs of Dinitrogen Fixation

Dinitrogen is described as the most stable diatomic molecule known. The two atoms of nitrogen in the diatomic molecule are joined by a very stable triple bond. Breaking the triple bond requires a large amount of energy (945 kJ (226 kcal) per mole) and perhaps therein lies the greatest challenge of fixing dinitrogen chemically or biologically. Dinitrogen fixation is "energy expensive" because it requires much energy to break the triple bond of dinitrogen and also to provide the hydrogen necessary to reduce dinitrogen to $2NH_3$. The chemical fixation of nitrogen is accomplished most widely through the high-pressure catalytic method called the Haber-Bosch process named after the German scientists who developed the process in 1914. The process served as a source of ammonium and nitrates for manufacture of explosives during World War I. The reaction for chemical fixation of nitrogen can be written as follows:

$$N_2 + 3H_2 \xrightarrow[K_2O,\ Al_2O_3]{} 2NH_3(\text{aqueous})$$

$$\Delta G = -53 \text{ kJ}(12.7 \text{ kcal})$$

To obtain high yields from this reaction in reasonable time, high pressures (approximately 200 atm; 20,265 kPa.) are applied to the reaction vessel and the temperature is raised to 400–500 °C. Natural gas (methane) is usually employed as the feedstock for the hydrogen needed to reduce the dinitrogen and is also required to heat the reaction vessel. That is, it takes about 31,000 cubic feet of natural gas, 5.59 barrels of oil, or two tons of coal to fix one ton (2,000 lbs) of ammonia (16). About 40% of this is used to provide the necessary heat and the remainder to provide the H_2 needed for the reaction. It is because the production of ammonium-based fertilizers relies on fossil fuel (natural gas, etc.) that fertilizer costs are high and are, at present, somewhat inextricably linked to fossil fuel prices and the whims of that market. For example, an oil embargo against the United States in the early 1970s resulted in long lines at gas pumps and quadrupled fertilizer prices. The high fertilizer prices were due also, in part, to short supplies at a time of peak demand. Currently, the industrial production of fertilizer nitrogen is a major consumer of fossil energy and "a huge and growing economic cost of more than U.S. $20 billion per year" (2).

The chemical fixation of dinitrogen is energy-intensive, as is its fixation in biological systems (Table 5). The principal differences lie in the sources of reductant and energy and the fact that biological dinitrogen fixation takes place at ambient pressures and temperatures — quite a feat when one considers the rigors of the Haber-Bosch process described earlier. Energy for biological dinitrogen fixation comes from the oxidation of organic carbon sources such as glucose or from light in the case of photosynthetic diazotrophs. It can take several

Table 5. Carbon and Energy Source Requirements for Heterotrophs Grown in Chemostats Limited by the Carbon and Energy Source

Organism	C and Energy Source	Efficiency of N Incorporation During Growth (mg N g^{-1} C and Energy Source Used)		Carbon and Energy Expenditure for N_2 Fixation	
		N_2	NH_4^+	(g C $g^{-1}N_2$)	lb C used per 100 lb of N_2 fixed
		Anaerobic growth by fermentation			
Clostridium pasteurianum	Sucrose	11	22	46	4,600(2,054)[†]
Klebsiella pneumoniae	Glucose	8	19	72	7,200(3,214)
		Aerobic growth			
Klebsiella pneumoniae (O_2-limited)	Glucose	15	35	38	3,800(1,696)
Azospirillum brasilense (9 μM O_2)	*Malate*	26	48	19	1,900(848)
Azotobacter vinelandii (2–10 μM O_2)	Sucrose	16	63	47	4,700(2,098)
Azotobacter vinelandii (180 μM O_2)	Sucrose	7	38	117	11,700(5,223)

[†]The 100-lb figure is chosen as representative of a typical amount of fertilizer N that might be applied to a crop. Numbers in () are values in kg.

Note: Adapted from S. Hill, G. Stacey, R. H. Burris, and H. J. Evans, eds., *Biological Nitrogen Fixation*, Chapman Hall, New York, 1992, pp. 87–134. See also Giller and Day (28). Reprinted with permission of Kluwer Academic Publishers.

Table 6. Estimated Average Rates of Biological Dinitrogen Fixation for Specific Organisms and Associations

Organism or System	N_2 fixed ($kg\,ha^{-1}\,yr^{-1}$)
Free-living microorganisms	
Cyanobacteria ("Blue-green algae")	25
Azotobacter	0.3
Clostridium pasteurianum	0.1–0.5
Grass-bacteria associative symbioses	5–25
Plant-cyanobacterial associations	
Gunnera	12–21
Azolla	313
Lichens	39–84
Legumes	
Soybeans (*Glycine* max L. Merr.)	57–94
Cowpeas (*Vigna, Lespedeza, Phaseolus*, and others)	84
Clover (*Trifolium hybridum* L.)	104–160
Alfalfa (*Medicago sativa* L.)	128–600
Lupines (*Lupinus* sp.)	150–169
Nodulated nonlegumes	
Alnus (Alders e.g., Red and Black alders)	40–300
Hippophae (Sea Buckthorn)	2–179
Ceanothus (Snow brush, New Jersey tea, California lilac)	60
Coriaria ("tutu" in New Zealand)	60–150
Casuarina (Australian pine)	58

Adapted from Stevenson and Cole (20). Reprinted by permission of Wiley & Sons.

thousand pounds of carbon substrate to fix 100 pounds of nitrogen!

NONSYMBIOTIC NITROGEN-FIXING BACTERIA

Biological dinitrogen fixation is restricted to procaryotic organisms such as bacteria (cyanobacteria and actinomycetes are specialized bacteria). Of the 10,000 or so bacterial species named, only about 100 currently contain bonafide diazotrophic species. While this may appear to be a somewhat limited representation, it encompasses species representing all sorts of physiological types and occupying all sorts of ecological niches. Some of the genera of free-living diazotrophs are listed in Table 4 (8,17,18). With respect to carbon metabolism, these organisms include heterotrophic and chemoautotrophic bacteria, photoautotrophs (bacteria and cyanobacteria), and photoorganotrophs. With regard to Oxygen (O_2) requirements, they are well represented by aerobes, microaerophiles (growing best at low O_2 tension), facultative anaerobes, and obligate anaerobes. Such metabolic diversity enables some type of diazotroph to colonize almost any conceivable microhabitat. In fact, diazotrophs occur widely in nature as free-living microbes and also in a large variety of associations with plants and animals. The great range of metabolic diversity suggests that diazotrophs contribute to the supply of "fixed" nitrogen for growth of nonfixing microbes and higher forms of life in all sorts of environments.

Nitrogen Fixation in Soil

Soils may contain several thousands to millions of diazotrophic bacteria in a single gram of soil. These can represent a wide range of physiological types as discussed previously. It can be surmised that substantial quantities of nitrogen are fixed biologically in the soil. However, this is far from the actual situation. Early estimates of nitrogen fixation in soils free of legumes ranged from tens to hundreds of kg $ha^{-1}\,yr^{-1}$ (see the extensive review of early literature by Moore) (19). These early studies were based on short-term (<1 yr) accumulations of nitrogen in soils incubated under a variety of laboratory conditions, with or without carbon amendments, or on nitrogen-balance studies of field soils in a range of cropping systems from grasslands to cultivated row crops. There is a strong consensus, backed by decades of research that rates of dinitrogen fixation in soil are quite low and fixation rates in intensively managed agricultural soils are often considered insignificant. In fact, Postgate (8) states "It is important to be clear that free-living nitrogen-fixing bacteria, at least when in the free-living state, are rarely of great importance in the terrestrial nitrogen economy." This is not to say that dinitrogen fixation by free-living bacteria does not

make meaningful contributions in some ecosystems and even in some agriculturally important crops. In some semi-arid soils, the contribution of biological dinitrogen fixation may be as much as 50% of the annual nitrogen inputs (20).

Currently, there is general agreement that the quantities of dinitrogen fixed by free-living heterotrophic bacteria (i.e., nonsymbiotic dinitrogen fixation) in soil are on the order of 1 to 5 kg ha^{-1} $year^{-1}$ (5,20) (Table 6). Higher rates may occur in flooded soils in which the diazotrophic cyanobacteria can be quite active until they are "shaded out" by the crop canopy. Estimates of as much as 25–50 kg nitrogen fixed per hectare annually in rice paddies appear frequently in the literature. Also, there are reports of high rates of dinitrogen fixation in cryptobiotic crusts containing diazotrophic cyanobacteria. Such crusts are usually active for relatively short periods following rainfall. However, their inputs of fixed nitrogen are ecologically significant in these fragile communities. In comparison, nitrogen fixation rates in symbiotic systems [*Rhizobium, Bradyrhizobium*/legumes, *Frankia* in the actinorhizal symbioses, *Azolla* (aquatic fern)/*Anabaena* (cyanobacterium)] may be one to several orders higher in magnitude (Table 6).

Factors Limiting Dinitrogen Fixation by Free-Living Diazotrophs

It is clear from this discussion that free-living heterotrophic diazotrophs make relatively small contributions to the nitrogen budget of most soils. One might reasonably ask why, with nitrogen being such a critical soil nutrient, the free-living bacteria do not fix greater quantities of nitrogen in soil. The answer to this question most likely lies in the nature of the nitrogen fixation process itself. Recall from Table 3 that there are very specific physiological requirements for nitrogenase function in intact cells. It is these requirements which most often constrain the diazotrophic bacteria in soil, especially soils not receiving abundant plant remains (senescent roots, leaves, etc.) or supporting an active plant population. Primary factors affecting free-living diazotrophs in soils are listed in Table 7 and discussed later. Briefly, these include a supply of available carbon and energy sources, limitations imposed by oxygen, combined nitrogen and competition from nonfixing microbes. Thus, dinitrogen-fixing bacteria face a variety of biotic and abiotic forms of stress in most upland soils.

Supply of Readily Utilizable Carbon/Energy Sources

The most limiting factor for nonsymbiotic dinitrogen fixation in soil is probably the lack of abundant, readily available supplies of organic carbon. Little dinitrogen fixation occurs in habitats low in carbon. Numerous studies show that dinitrogen fixation rates increase markedly upon the addition of useable carbon sources such as carbohydrates, cereal straws, and so on, to soil.

As a rough approximation, it takes one to several tons of glucose to fix 100 lbs of nitrogen biologically (see Table 5). Free-living diazotrophic bacteria typically fix about 10 to 15 mg of nitrogen per gram of carbon consumed. In most natural soil systems, organic inputs are limited and there is intense competition among soil microbial populations for the limited resource. Estimates of the amounts of carbon contributed to soils from crops and grasslands have been expanded over the years. As much as 24 to 50% of the carbon fixed in photosynthesis may appear in soil as roots and root exudates (21). Barber (22) estimated a fine root production under corn to be 1.2 to 1.5 tons ha^{-1} yr^{-1} and Gregory and coworkers (23) reported 1.2 to 1.3 tons ha^{-1} yr^{-1} under winter wheat. Fine root production in a native tallgrass prairie was reported to be as much as 5.6 tons ha^{-1} yr^{-1} (24). Thus, several tons of carbon may be translocated below ground (Table 8), where it is potentially available for microbial growth. However,

Table 7. Biotic and Abiotic Factors Affecting Dinitrogen Fixation by Free-living Soil Bacteria

Factor	Biotic or Abiotic	Effect on N_2 Fixation
Carbon/Energy source	Biotic/ Abiotic	Lack of an abundant supply of available organic C is considered the principal limiting factor for N_2 fixation by free-living soil diazotrophs. (Recall the need for large quantities of ATP for nitrogenase activity.)
Oxygen	Abiotic	Nitrogenase is in most cases irreversibly damaged by exposure to O_2. N_2 fixation by aerobes is usually most vigorous when the O_2 level is much reduced and it must generally be absent for the anaerobes and facultative anaerobes.
Combined nitrogen	Abiotic	Nitrogenase is strongly controlled by the presence of combined nitrogen [i.e., ammonium, nitrate and organic N compounds (amino acids, etc.)] in soil.
Competition	Biotic	N_2-fixing bacteria must compete with all other soil microbes for carbon supplies, etc. It is generally agreed that diazotrophs constitute 1–10% of the cultivable bacterial population.
Others	Biotic	Like all other soil bacteria, diazotrophs are subject to predation by protozoa and lysis by bacterial viruses (bacteriophages).
	Abiotic	pH, temperature, trace element availability, etc.

Table 8. Quantities of Carbon Lost from Roots (Rhizodeposition) of Prairie Plants Under Field Conditions

System	Rhizodeposition (root C + Soil C + CO_2 − C) tons ha^{-1} yr^{-1}	Reference
Agropyron/*Koeleria*-dominated mixture	1.3	Warembourg and Paul (26)
Tall grass prairie (large mixture of species)	2.1*	Kucera et al. (27)
Tall grass prairie	2.0*	Dahlman (24)

*Probably underestimates since these values do not include CO_2 respired.
Note: After Whipps (25).

Table 9. Mechanisms Through which Dinitrogen-fixing Microbes Protect Nitrogenase from Exposure to Oxygen

Avoidance — Anaerobes and facultative anaerobes fix N_2 only in the absence of O_2 with the exception of Klebsiella *pneumoniae*, which can tolerate very low levels of O_2. In fact, O_2 is one of the factors that regulates the synthesis of nitrogenase in this bacterium.

Microaerophily — Most aerobic diazotrophic bacteria fix nitrogen maximally at low partial pressures of O_2 thereby lessening the exposure of nitrogenase to O_2. For example, it has been reported that *Azospirillum* fixes N_2 most rapidly at 0.007 atm O_2 (29).

Respiratory protection — Respiration functions in all aerobes to divert O_2 away from nitrogenase to some extent. In certain *Azotobacter* species, an exaggerated form of respiratory protection is observed. In fact, this bacterium exhibits one of the highest respiration rates of all life-forms. The high respiration rate serves to scavenge O_2 and to keep it away from nitrogenase. As a consequence, the bacterium must consume large amounts of substrate to scavenge O_2 and growth is very inefficient in terms of carbon consumed under these conditions.

Conformational protection — Some *Azotobacter* species produce a protein that binds to the nitrogenase and protects it from damage by O_2. In the presence of O_2, and when respiration cannot keep up with the incoming O_2 supply, the protein binds to the nitrogenase complex and alters its conformation (shape) in such a way that it is protected from O2. Upon exposure to O_2 the organism stops fixing N_2 abruptly but when conditions of oxygenation return to a more favorable state, the organism resumes N_2 fixation. The enzyme is not destroyed.

Production of specialized cells — Many diazotrophic cyanobacteria produce specialized, thick-walled cells called heterocysts in which the nitrogenase is compartmentalized. These cells do not evolve O_2 in photosynthesis and the thick wall excludes external O_2. The diazotrophic actinomycete, *Frankia*, produces vesicles in which nitrogenase is protected from O_2. The vesicle wall becomes thicker as the O_2 concentration increases in the medium.

Slime — Aerobic diazotrophs grown on nitrogen-free agar media frequently produce large gummy colonies due to the production of extracellular polysaccharides. The gum serves as a diffusion barrier to the free flow of O_2 into the colony such that cells in the interior of the colony are not as exposed to O_2. The efficacy of this mechanism is questionable.

Temporal or spatial separation of dinitrogen fixation and oxygen-evolving processes — Non-heterocystous cyanobacteria solve the O_2 problem by fixing N_2 primarily during the dark phase of growth (i.e., temporal separation) when O_2 evolution is not occurring and respiration serves also to scavenge O_2 away from nitrogenase. Others form aggregates of cells and some of these cells then function in a microaerophilic environment (i.e., spatial separation).

After J. R. Postgate, Nitrogen Fixation, 3rd ed., Cambridge University Press, Cambridge, U.K., 1998.

there is not enough carbon channeled to the diazotrophs to support the fixation of substantial quantities of dinitrogen. Consider the following calculation put forth by Giller and Day (28) for nitrogen fixation under a wheat crop. They assumed (*1*) an efficiency of 10 g of carbon for fixation of one gram of nitrogen; (*2*) dinitrogen-fixing bacteria comprised 10% of the total population (they stressed this was "a high estimate"); and (*3*) all the carbon translocated below ground was equally available for use by all bacteria. On the basis of these assumptions, 1,500 kg carbon ha^{-1} translocated below ground by a wheat crop would allow for a maximum potential nitrogen fixation of 15 kg nitrogen ha^{-1} yr^{-1}.

The limited availability of substrates in most systems means that diazotrophic bacteria are frequently found in loose associations with plant roots because roots release carbon in the form of root exudates, lysates, and sloughed cells. These loose associations between roots of plants, especially the grass species that include major cereal crops, have been the subject of much research since the 1970s and are still under active investigation. Estimates of dinitrogen fixation by these "associative symbioses" (see

following section) range from about 5 to 15 kg of nitrogen fixed per hectare per year. While this does not strictly represent "dinitrogen fixation in soil," the nitrogen fixed in these associations does enrich the soil upon decomposition of plant and microbial cells.

Oxygen Concentration versus Dinitrogen fixation

As mentioned earlier, nitrogenase is "poisoned" by molecular oxygen (O_2). For this reason dinitrogen fixation rates tend to be highest in environments where the O_2 tension is considerably reduced. Most aerobic diazotrophs fix dinitrogen most efficiently at O_2 concentrations that would be described as microaerophilic. For example, *Azospirillum* fixes dinitrogen most rapidly in culture when the pO_2 has been reduced to 0.007 atm (29). Similarly, *Azotobacter* is much more efficient under low-oxygen conditions (Table 5). Alexander and Zuberer (30,31) demonstrated that bacteria-associated roots of intact maize and sorghum plants exhibited maximal nitrogenase activity (acetylene reduction) when the O_2 content surrounding the roots was lowered to 2 kPa (2% O_2). Similar results have been shown for soils incubated under a range of O_2 conditions. Interestingly, dinitrogen-fixing bacteria tolerate higher levels of O_2 when there is more carbon substrate available to them, presumably because high respiration rates divert O_2 from nitrogenase.

The sensitivity of nitrogenase to molecular O_2 has led to some unique adaptations or strategies allowing diazotrophs of one kind or another to deal with exposure to it (Table 9) (8). These range from simple avoidance to complex physiological mechanisms and morphological adaptations. Anaerobic diazotrophs are not faced with the oxygen problem because, to begin with, they do not grow in its presence. Most of the facultatively anaerobic diazotrophs only fix dinitrogen when they are functioning as anaerobes, with the exception of *Klebsiella*, which appears to tolerate very low levels of O_2 (8,9). It is the aerobic bacteria and cyanobacteria that are faced with the larger challenge of managing exposure to O_2 while operating the nitrogenase complex. *Azotobacter* engages in respiratory protection in which very high rates of respiration are thought to keep O_2 away from the enzyme complex. In fact, *Azotobacter* exhibits perhaps the highest respiration rate among living organisms. *Azotobacter* is also capable of a process called "conformational protection" in which nitrogenase is protected by a separate protein during exposure to too much O_2. Upon return to lower O_2 levels, the nitrogenase complex resumes activity, having avoided the usual destruction by O_2. Other diazotrophs protect nitrogenase by packaging it in specialized cells that exclude O_2. Many dinitrogen-fixing cyanobacteria produce thick-walled cells called "heterocysts." These cells contain the nitrogenase complex and lack Photosystem II, which catalyzes the oxygen-yielding reactions of photosynthesis. Thus, compartmentalization keeps O_2 away from the sensitive complex. The root-nodulating actinomycete, *Frankia*, produces thick-walled structures called "vesicles" that protect nitrogenase from O_2. Some diazotrophs produce copious quantities of slime (extracellular polysaccharides) thought to create a diffusion barrier to O_2 and some produce aggregates of cells in which some individuals find conditions suitable for expressing nitrogenase activity. Finally, some diazotrophic cyanobacteria fix dinitrogen during periods of darkness when photosynthetic O_2 production does not interfere with nitrogenase activity.

It is apparent that O_2 plays a significant role in governing the occurrence of dinitrogen fixation in various environments. It is not surprising that the higher rates of nitrogen fixation reported in the literature are generally associated with seasonally or chronically wet soils because here the O_2 levels tend to be lower due to saturation. It is for these reasons that paddied rice is one of the few agricultural crops that derives significant benefit from the activities of nonsymbiotic diazotrophs and dinitrogen-fixing cyanobacteria.

Combined Nitrogen

Since nitrogenase activity is very costly to the bacterium in terms of energy consumption, it is carefully regulated by the availability of nitrogen in the culture medium or in the soil. Generally, nitrogenase activity does not occur unless there is an acute shortage of combined nitrogen (ammonium, nitrate, organic nitrogen). Thus, in most fertilized agricultural soils, there is usually sufficient nitrogen available to repress nitrogenase synthesis at least during most of the growing season. Alexander and Zuberer (30) showed that as little as 4 mg $L^{-1}NH_4^+$–N in a hydroponic system was sufficient to stop dinitrogen fixation associated with roots of whole maize plants exposed to 2 kPa (2% v/v) O_2. However, it has been proposed that nitrogen fixation could be of significance late in the growing season when nutrient-depletion zones may have established around roots. Nitrogen fixation might also occur in nitrogen-deficient microsites such as those that might occur in rhizosphere soils in which root and microbial nitrogen uptake deplete the soil solution of available nitrogen. In soils and grasslands not receiving fertilizers, available nitrogen is usually quite low and conditions would be more favorable for nitrogen fixers. In fact, it is in ecosystems such as these that the contributions of nonsymbiotic dinitrogen fixers are most significant, possibly representing as much of 50% of the annual nitrogen inputs (20).

Other Environmental Factors

Soil Moisture. As with all other bacteria, free-living diazotrophs require adequate moisture for growth and reproduction. Besides, moisture is probably most influential in terms of its effects on the O_2 tension of the soil. Waterlogged soils with low O_2 contents and containing available carbon generally show higher rates of dinitrogen fixation than drier soils. It is also likely that dinitrogen fixation occurs in seasonally flooded soils, for example, pastures during wet seasons. In such situations, both nonsymbiotic bacteria and diazotrophic cyanobacteria might make an important contribution.

pH. Most soil microbes are sensitive to extreme pH levels, with the majority favoring environments ranging

from 6.8 to 7.2. Nitrogen-fixing bacteria are no exception. However, the pH of the environment can have marked effects on the distribution of diazotrophs. *Azotobacter* is characteristically absent from acid soils whereas *Beijerinckia* may be particularly abundant. The latter appears to be more tolerant of the higher iron and aluminum contents of acid soils. Cyanobacteria are also sparse or absent in acid soils and tend to be favored by soils of slightly higher pH (7.5 to 8.5) (32).

Temperature. Temperature affects microbial growth in the usual manner. Growth and activity tend to be slow at cool temperatures and increase up to a point with increasing temperatures. Most diazotrophs are mesophilic, preferring temperatures in the range of 15° to 35 °C. Nitrogenase activity tends to decrease rapidly beyond 37 °C; however, there is at least one species of thermophilic cyanobacteria that grows in hot springs. *Methanococcus thermoautotrophicus* fixes dinitrogen at 64 °C and the recently discovered *Streptomyces thermoautotrophicus* fixes dinitrogen at 65 °C (8,12).

Other Nutrients. Recall that iron and molybdenum or vanadium are essential for the nitrogenase enzyme complex. Magnesium is needed to form the MgATP complex required for nitrogenase activity. Also, it is probable that the "nitrogenase" system of *Streptomyces thermoautotrophicus* requires manganese for its activity. In general, soils contain sufficient quantities of these elements to not limit dinitrogen fixation. One possible exception might occur in acidic soils in which the availability of molybdenum is decreased because it is a metal that becomes less soluble (less available) at low pH. Currently, however, we know that not all diazotrophs have an obligate requirement of Mo so its deficiency in soil does not necessarily preclude the occurrence of dinitrogen fixation. Alternatively, high concentrations of soluble iron and aluminum in acid soils might be inhibitory to some bacteria. Phosphorus (P) is required in large quantities by dinitrogen-fixing microbes because of the high demand for ATP to support nitrogenase activity. Thus, soil P must also be adequate for microbial growth.

The Associative Symbioses — Grass-Bacteria Associations

This section explains why rates of dinitrogen fixation in soils, especially those free of significant vegetation, are in general quite low. The requirements for an operative nitrogenase system place substantial demands on a bacterium, whether in culture or in natural habitat. As discussed earlier, chief among these requirements is provision of an abundant supply of carbon or energy sources. Available organic carbon, assuming moisture for growth is adequate, is the most limiting factor for growth of heterotrophic bacteria in soil.

Almost throughout the history of soil microbiology (since 1900) scientists have known that there is pronounced stimulation of microbial growth in the rhizosphere (the zone of soil immediately surrounding the root) of most, if not all, plants. This so-called rhizosphere effect is due to the provision of carbon and energy sources for heterotrophic soil bacteria among which are a large number of genera of dinitrogen-fixing bacteria, many of which are listed in Tables 4 and 5. It is a reflection of the carbon limitation of microbial growth in soil. To the soil microbe, the root is an oasis of sorts, a source of nutriment in an otherwise nutrient-poor environment.

In the late 1960s and the early 1970s, considerable interest was rekindled in the possibility that important agricultural crops, mainly cereals such as corn, sorghum, and millet and forage grasses, might derive significant inputs of nitrogen from dinitrogen-fixing bacteria associated with their roots. These more or less loose associations between roots of higher plants and free-living diazotrophs are now commonly referred to as "associative symbioses." In these associations, the bacterium obtains carbon for growth and dinitrogen fixation in the forms of root exudates, lysates, sloughed cells, and root-derived mucilage, which is sometimes called "mucigel." Recall from the earlier discussion that plants translocate substantial quantities of carbon below ground (Table 8). At least part of this carbon supports dinitrogen fixation by root-associated diazotrophs.

Considerable interest was focused on the soil bacterium *Azospirillum*, which was isolated from the roots and rhizosphere of a number of important tropical C_4 grasses. Early reports suggested that as much as 90 kg of nitrogen $ha^{-1}\,yr^{-1}$ were being fixed by bacteria associated with grasses such as *Paspalum* (bahia grass) and *Digitiaria* (digit grass) and cereals such as millet (*Pennisetum*) and sorghum (*Sorghum*). On closer examination, over 10 to 15 years of further research, it became apparent that rates of dinitrogen fixation in these associations are more on the order of 10 to perhaps 25 $kg\,ha^{-1}\,yr^{-1}$. Early estimates of high rates of dinitrogen fixation in these systems were based mainly on the use of the acetylene reduction assay using excised roots incubated under ideal conditions and after prolonged incubations. It soon became apparent that rates determined using such methods could not be extrapolated to rates of fixation under field conditions and across entire growing seasons. Later studies using the stable isotope ^{15}N led to estimates in the range of 5 to 25 $kg\,ha^{-1}\,yr^{-1}$, which are widely accepted today. It should be mentioned that the enthusiasm for the study of the associative symbioses was at least partly fueled by a worldwide oil embargo and high fertilizer prices at the time. Although nitrogenous fertilizers are not directly linked to fossil fuels other than methane, part of their expense is due to transportation and application costs, which are petroleum-dependent. Thus, because of an unusual set of circumstances, this research area was catapulted to the forefront along with the intensified study of many aspects, both basic and applied, of symbiotic dinitrogen fixation (see NITROGEN FIXATION IN SOILS (SYMBIOTIC)).

While the optimism about dinitrogen-fixing cereals may have subsided somewhat since the earlier years, nitrogen fixation associated with two agriculturally important crops, rice and sugarcane, deserves special mention. Each of these nonleguminous crops, one a C_3 plant (rice) and the other a C_4 plant, derive benefit from nonsymbiotic diazotrophs in different ways. As

was mentioned earlier, rice crops receive about 25 to 50 kg of nitrogen $ha^{-1}\,yr^{-1}$ as a result of the combined activities of diazotrophic cyanobacteria and free-living heterotrophs in and on the flooded paddy soils. Here, the cyanobacteria find favorable conditions, especially during the early growth of the crop when shading is minimal. Bacteria in the flooded soils use root- and possibly algae-derived carbon to derive dinitrogen fixation. The low pO_2 associated with submerged soils also tends to favor the diazotrophic bacteria by lowering the O_2 stress on nitrogenase.

Some varieties of sugarcane have been found to support populations of the bacterium *Acetobacter diazotrophicus* in the vascular tissues. This bacterium is unique in occupying these tissues as an endophyte. Here, the bacterium has direct access to a ready supply of sugars and is free from the competition that it would have were it located on the outside of the roots. Reliable estimates of dinitrogen fixation by these sugarcane-*Acetobacter* associations are in the range of 100 to 200 kg of nitrogen $ha^{-1}\,yr^{-1}$ (1,33).

CONCLUSION

This article attempts to introduce the nuances of the process of biological nitrogen fixation by free-living, that is, nonsymbiotic, soil bacteria. An understanding of the potential and limitations of these bacteria can only be gained by appreciating the intricacies of the enzyme complex nitrogenase, which I hope I have presented at a level that is neither too simplistic nor too complex. It is a remarkable enzyme system that mediates a remarkably important process for life on earth. Given the list of prerequisites for dinitrogen fixation is so long, it is almost a marvel that the process is so pervasive in aquatic and terrestrial habitats.

Also described is the diversity of the microbes, bacteria, and cyanobacteria, which are known to carry out nonsymbiotic dinitrogen fixation. This list is, no doubt, far from complete and it is exciting to think of what lies ahead in the discovery of new, previously undescribed, organisms and possibly even different mechanisms for dinitrogen fixation. The discovery of the alternative nitrogenases and even more recently, the chemoautotrophic thermophilic actinomycete, *Streptomyces thermoautotrophicus*, give reason to believe there is yet much to discover.

I have included information about the associations between dinitrogen-fixing bacteria and higher plants such as the grasses. The intent is to emphasize the importance of vegetation in natural as well as agroecosystems in providing the carbon sources needed to "fuel" nitrogen fixation by soil bacteria. It is only in environments receiving abundant supplies of carbon and energy sources that one can expect to find significant rates of dinitrogen fixation. Only the photoautotrophic dinitrogen-fixing cyanobacteria and perhaps a few chemoautotrophic bacteria are free of this constraint. Nonetheless, it is encouraging to realize that at least two major crops, rice and sugarcane, are at least in part "fertilized" by dinitrogen-fixing bacteria.

No one can accurately predict the future, especially with regard to the role of biological dinitrogen fixation in feeding an expanding global population. Undoubtedly, dinitrogen-fixing crops, especially the legumes, will figure prominently in food-production systems as we strive to reduce dependence on fossil energy supplies and to minimize the negative impacts of excessive use of nitrogen fertilizers. It is tantalizing to think that the secrets of the nitrogenase complex might be unveiled and put to use for a less energy-intensive industrial process to produce nitrogen for crop growth. Or perhaps that molecular biologists may someday figure out how to transform nonfixing cereal crops into symbiotic systems such as the legumes. No matter what, it is reasonably certain that nitrogen fixation will probably continue to be viewed as "the second most important biological process on earth."

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NITROGEN FIXATION IN SOILS (SYMBIOTIC)

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Symbiotic nitrogen (N_2) fixation involves the association between specific nitrogen-fixing prokaryotic microorganisms and eukaryotic, usually photosynthetic, hosts. In these often long-term relationships, the microsymbiont provides the host with nitrogen (N) from a source that the host could not otherwise access, while the microbe receives carbohydrates and organic acids from the host for growth and nitrogen fixation. In some cases, symbiosis also results in the development of a special structure within which the microbe is housed and protected from external stresses or from the negative effects of oxygen on nitrogen fixation. Symbiotic nitrogen-fixing associations that are of significance in soil include:

- leguminous plants and root- or stem-nodule bacteria from the genera *Allorhizobium, Azorhizobium, Bradyrhizobium, Mesorhizobium, Rhizobium,* and *Sinorhizobium* (collectively called "rhizobia");
- actinorhizal plants in association with the actinomycete *Frankia*, and
- the water fern *Azolla* and cyanobacterial microsymbionts belonging to the species *Anabaena azollae*.

This article emphasizes the more extensively studied legume/rhizobial and actinorhizal/*Frankia* symbioses. Readers are referred to articles by Lumpkin and Plunkett (1), Roger (2), Wagner (3), and Lejuene and coworkers (4) for more information on the *Azolla*/*Anabaena* symbiosis.

THE IMPORTANCE OF SYMBIOTIC NITROGEN FIXATION IN SOIL

Symbiotic nitrogen fixation is, after photosynthesis, the second most important biological process on earth, and the single greatest contributor to the global nitrogen economy. Nitrogen fixation adds about 90 Tg of nitrogen annually to the soils of the world, and accounts for more than two-thirds of all the N used in agriculture (5).

Legumes have been used in crop rotation and agriculture since the time of the Romans, but an understanding of their potential for soil improvement is of relatively recent origin. Thus, it was only in the 1800s that such plants were shown to accumulate nitrogen from sources other than soil and fertilizer, and in 1886 that Hellriegel and Wilfarth associated this ability with the presence of swellings or nodules on the legume root (6). Isolation of the microsymbiont responsible for this nitrogen fixation followed soon thereafter. Rates of nitrogen fixation in the legume/rhizobial symbiosis vary with growing season, soil fertility, and water supply, but range from essentially 0 to 450 $kg\,ha^{-1}$ $annum^{-1}$. Nitrogen fixation can meet as much as 97% of the plant's nitrogen needs (7). Nitrogen left in the field following the harvest of crops such as soybeans or peas can result in a buildup of the organic nitrogen content of the soil, can limit soil acidification, and substantially reduce the nitrogen-fertilization needs of a subsequent nonlegume crop. Ley farming systems in which regenerating pasture legumes replace the fallow period of traditional dry-land agricultural systems are also a major contributor to soil nitrogen in some regions (8).

Nitrogen is also a key limiting element in many natural ecosystems, with legumes required to maintain productivity (9). Towne and Knapp (10) found annually burned prairies to be often nitrogen-limited, supporting diverse leguminous plants; Leach and Givnish (11) noted legumes in remnant oak savannahs present across much of the light and soil gradients, with best growth in well-lit sandy locations. Knops and Tilman (12) followed the

dynamics of carbon and nitrogen in sand-plain agricultural fields abandoned between 1927 and 1982, noting that carbon accumulation after abandonment was dependent on nitrogen deposition and symbiotic nitrogen fixation. These abandoned fields essentially retained all nitrogen, meaning that even minor inputs from fixation could have significance.

Sprent and Parsons (13) recognized 25 genera and 225 species of actinorhizal plants, coming from eight plant families. All are perennial dicotyledons, and most are woody trees and shrubs. *Casuarina* and alder species are the predominant commercial species, but a number of species are important as pioneers under low temperature or arid soil conditions. Documentation of rates of nitrogen fixation in tree species is highly variable, with only 8 to 32% of plants in an arid environment bearing nodules (14). Silvester (15) reported average annual nitrogen-fixation rates for *Coriaria* over a 20-year period as 90 $kg\,ha^{-1}$, Dommergues (16) noted rates of 15 to 94 $kg\,ha^{-1}\,y^{-1}$ or 6 to 47 $kg\,N\,tree^{-1}\,y^{-1}$.

Use of *Azolla* as a green manure in rice cultivation dates back to the 11^{th} century (2). Wagner (3) reported rates of nitrogen fixation under propagation conditions in a rice paddy of 1.10 $kg\,ha^{-1}\,day^{-1}$, Roger (2) rates of nitrogen fixation of 224 $kg\,N\,ha^{-1}$ crop $cycle^{-1}$. Both authors, however, noted marked variation in nitrogen fixation under field conditions. Thus, Roger (2) found only 10 to 50 $kg\,N$ fixed ha^{-1} across 37 sites in 10 countries. This author also noted a marked decline in *Azolla* usage in Vietnam and China during the 1980s, with 6.5×10^6 ha planted before 1978, but only 0.7×10^6 ha in 1982, attributing this to inexpensive sources of urea and the disbanding of many agricultural communes.

Graham and Vance (17) have contrasted the declining role for biological nitrogen fixation in American, European, and Chinese agriculture with the continued dependence on nitrogen fixation in countries such as Brazil, Australia, and Southern Africa. For the former, the availability of inexpensive fertilizer nitrogen, the need to land-farm animal wastes, and even population pressures that limit the land available for *Azolla* propagation are factors affecting adoption of nitrogen-fixation technologies. Indicative of these changes, Brockwell and Bottomley (18) have noted static or declining production of legume inoculants worldwide, and van Kessel and Hartley (19) have noted a gradual decline in the rates of nitrogen fixation reported in the field studies of bean and soybean. Graham and Vance contrast this with the need for improved nitrogen fixation technologies in areas of extensive agricultural production where rainfall is erratic and unreliable, or for production under conditions of subsistence agriculture. How these needs will change over the next 30 years remains in question. Mannion (20) and others have suggested that an increase in the world's population to 8.3 billion by 2025 will necessitate significantly enhanced food production, while Tilman (21) has projected a threefold increase in nitrogen-fertilizer usage and an 18% increase in land for cultivation over this period. The rapid consumption of fossil fuels, the pollution and land degradation associated with such increased fertilizer usage, and declining soil quality (22) are likely to be major issues. Tilman and others have argued for a more logical and efficient use of nitrogen in agriculture, with the development of improved nitrogen-fixation and green manure technologies for subsistence farmers being a major thrust.

THE LEGUME-RHIZOBIAL SYMBIOSIS

Mechanisms of Infection

Mechanisms used by rhizobia to gain entry into their hosts and establish symbiosis include:

- root hair penetration and infection-thread formation as occurs in root-nodule formation on peas and clovers
- entry by wounds or sites of lateral root-emergence as found in root nodule formation in peanuts and the tropical pasture legume *Stylosanthes*, and
- penetration of the root primordia found on the stem of plants such as *Sesbania* and *Neptunia*.

A single rhizobial strain may infect one host species by root hair penetration and infection thread formation, another by wounds or sites of lateral root emergence. In this article, we will emphasize root hair infection. Additional details of these processes are provided by Hirsch (23), Boogerd and van Rossum (24), and Boivin and coworkers (25).

The Fahraeus slide technique (26,27) and the root tip marking procedure (28) are seminal to our knowledge of root hair infection. The former allowed repeated observations of the infection process in small-seeded legumes such as clovers, whereas the latter showed differences in the susceptibility to infection of root hairs according to their age, and allowed research to focus on those parts of the root where infection was in process. Compatible rhizobia begin to attach to the immature root hair of their host within minutes of contact. Characteristic deformation and curling of the root hair follows (Fig. 1a) with the root hair cell wall at the point of infection degraded to permit rhizobial penetration. Rhizobia never gain intracellular access into their host. Fresh plant-derived material is deposited around them as they penetrate the root hair, and as they move down the root hair in the direction of the cortex they remain enclosed within a plant-derived infection thread (Fig. 1b). Even when released into cells of the host root cortex, the rhizobia remain enclosed within a membrane that serves to minimize host recognition of invasion and initiation of defense responses.

Biochemical, genetic, and mutational studies have shown that initial infection events are accompanied by molecular signaling between host and rhizobia. These are reviewed in detail in the papers of Long (29) and Schultze and Kondorosi (30), with some 50 rhizobial genes thought to be involved in the nodulation process. Some affect host recognition, or the regulation of nodulation, but most function in the synthesis of specific lipo-chitooligosaccharide "nodulation factors." Depending on the species of rhizobia, these genes may be located on the bacterial chromosome (*Bradyrhizobium*) or associated with nonchromosomal plasmid DNA (*Rhizobium)*.

(a)

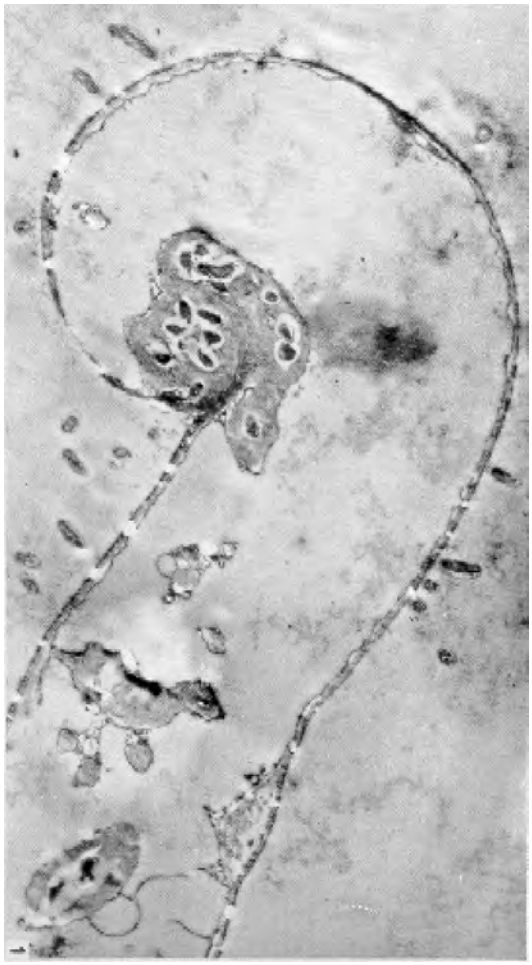

Figure 1a. Root hair deformation, curling, and infection; an early stage in the nodulation of clover by *Rhizobium leguminosarum* bv *trifolii* (From K. Sahlman and G. Fahraeus, *J. Gen. Microbiol.* **33**, 425–427 (1963) used with permission). See color insert.

(b)

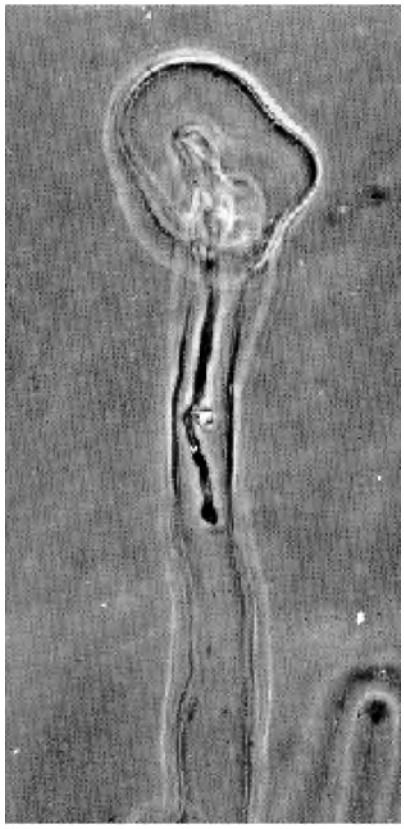

Figure 1b. Rhizobia contained within a plant-derived infection thread move down the root hair in the direction of the root cortex (From G. Fahraeus, *J. Gen. Microbiol.* **16**, 374–381 (1957) used with permission). See color insert.

Organisms living saprophytically in soil do not express the majority of their nodulation genes. However, flavonoid excretion by a suitable host leads to *nod*-gene expression, followed by synthesis of the nodulation factor. Legumes differ in the spectrum of flavonoids that each produces, whereas species of rhizobia react differently to specific flavonoid compounds. Thus, naringenin and daidzein stimulate *nod*-gene expression in soybean rhizobia; luteolin is required for the rhizobia from alfalfa. Variation in the structure and range of *nod*-factors produced by rhizobia also contributes to specificity in nodulation. These substances have the same core structure, but vary in the side chains that each carries, affecting host range (Fig. 2).

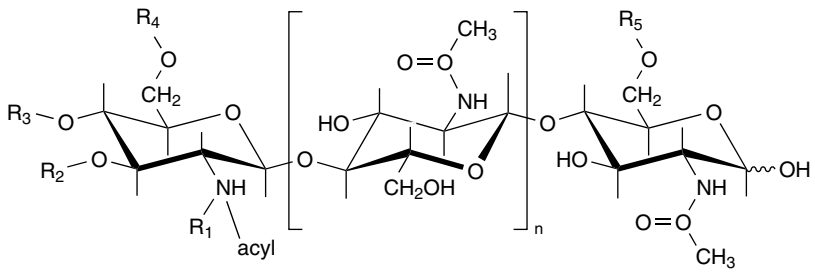

Figure 2. The general structure of *nod* factors elicited by rhizobia. In this structure $N = 2$ or 3, and the substitutions possible at each position are:

R_1	H or methyl
R_2 and R_3	H, carbomoyl, or 4-O-methylcarbomoyl
R_4	H, acetyl, or carbomoyl
R_5	H, sulfate, acetyl, 2-O-methylfucose, 4-O-sulfo-2-O-methylfucose 3-O-acetyl-2-O-methylfucose, 4-O-acetylfucose or D-arabinose
Acyl	C16 to C20

Modified from Schultze and Kondorosi M. Schultze and A. Kondorosi, *Curr. Opin. Genet. Develop.* **6**, 631–638 (1996) and used with permission.

Table 1. Some Parameters of Nodule Infection and Nitrogen Fixation in Legumes

With pure cultures of rhizobia/bradyrhizobia	
Attachment to root hairs	~60 seconds
Increasing attachment with time	Up to 4 h
Root hair curling	3 to 5 h
Visible infection-thread formation	~72 h
Nodules visible to the naked eye	6 to 12 d
With purified *nod* factor at 10^{-9} to 10^{-12} M	
Depolarization of root hair plasma membrane	~15 seconds
Modifed root hair pH	~15 seconds
Calcium spiking in root hairs	10 min
Expression of early nodulin genes	6 to 24 h
Root hair curling	~18 h
Cell division in the root cortex	days

Nod factors act as powerful plant hormones that alone, and at concentrations as low as 10^{-9} M, can bring about many of the physiological and developmental responses associated with nodule formation. These include depolarization, calcium spiking, and pH changes within the root hair (31,32,33) and break down and rearrangement of actin filaments at the root hair tip (34,35). Table 1 lists some of the changes induced by rhizobia and their nodulation factors, and the time frame against which these changes can occur. Prithiviraj and coworkers (36) have also noted enhanced germination and early plant growth in a number of crop species, including some non legumes, following treatment with nod factor.

Nodule Development and Function

Stimulation of plant cell division and enlargement in the root cortex results in the formation of a visible nodule. The shape of this nodule is a characteristic of the host, and regulated by the pattern of cortical cell division following infection. Thus, pea, medic, and clover nodules are elongated, with a pronounced meristematic region, and increase in length over the course of the growing season. As shown in Figure 3a, such indeterminate nodules contain four quite distinct regions:

- A meristematic zone, with little evidence of infection by rhizobia, but where host cells are undergoing active division;
- A region where many plant cells are infected, but the rhizobia they contain have not yet undergone significant change in size, shape, or surface chemistry. Rhizobia in this region of the nodule fix little, if any, nitrogen;
- An area of active nitrogen fixation, which is usually red or pink in color because of the presence of leghemoglobin. Host cells in this region may contain many rhizobia, and a number of these may be misshapen and modified in surface composition. They are referred to as *bacteroids*; and
- A region of nodule senescence where the symbiosis is degenerating. In this region, proteolytic activity is high, bacterial cells may be undergoing lysis, and the degradation of leghemoglobin often results in a green or brown coloration.

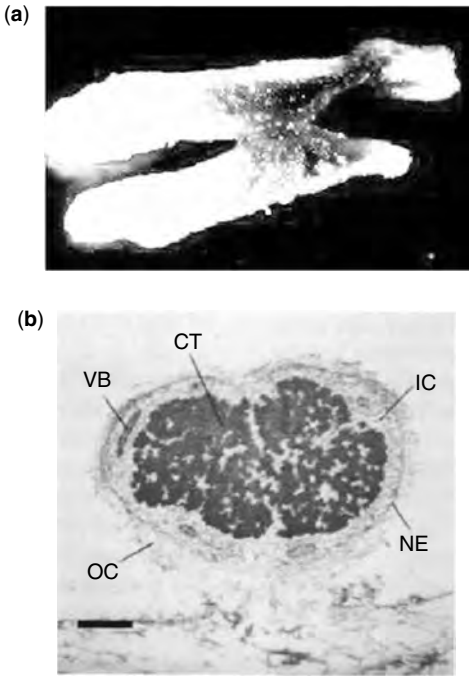

Figure 3. (**a**) The internal organization of mature nodule from *Medicago sativa*. Note the progression from the white uninfected meristematic region, through zones of active nitrogen fixation and leghemoglobin production, to the onset of nodule senescene on the right. (Photo C.P. Vance, used with permission). (**b**) The internal organization of a mature nodule from *Phaseolus vulgaris*. The determinate bean nodule is shown in transverse section. It has been stained with toluidine blue and includes central tissue (CT), an inner cortex (IC) with vascular bundles (VB), the nodule endodermis (NE), and outer cortex (OC). (From R. Tate et al., *MPMI 7*, 582–589 (1994) used with permission.) See color insert.

Nodules that are actively fixing nitrogen, with a large region that is pink or red in color, are termed *effective*, whereas those in which the nitrogen fixation is limited and the nodule green or white in internal color are termed *ineffective*. In contrast to the nodule morphology described earlier, the nodules found on hosts such as *Phaseolus* and *Glycine* are rounded and determinate, and lack a persistent meristem (Fig. 3b).

Nodule number plant^{-1} is influenced by the host genotype, by the number and efficiency of the inoculant rhizobia, and by nutritional and soil stresses. It can vary from very few to more than 300 nodules plant^{-1}, and nodule number and nodule size are usually inversely related. Because of this, nodule mass plant^{-1} measured several times throughout the growing season is probably the best simple indicator of nodulation success.

The indeterminate alfalfa nodule shows a progression from the white uninfected meristematic area on the left, through stages of infection by *Sinorizhobium meliloti,* active nitrogen fixation and leghemoglobin production,

to the onset of nodule senescence on the right. (Photo; C. P. Vance, used with permission)

The determinate bean nodule is shown in the transverse section. It has been stained with toluidine blue and includes central tissue (CT), an inner cortex (IC) with vascular bundles (VB), the nodule endodermis (NE), and outer cortex (OC). (From R. Tate, E. J. Patriaca, A. Riccio, R. Defez, and M. Iaccarino, *MPMI* **7**, 582–589 (1994)) used with permission)

Nodule-Specific Gene Expression

Interaction of host and rhizobia is accompanied by the expression of nodule-specific proteins or "nodulins", which were initially thought to be specific to infected root hair or nodules and required for nodule initiation, formation, or function. Some nodulins have now been detected in other tissues, and some have also been identified in actinorhizal or mycorrhizal symbioses. Nodulin expression can vary both temporally and spatially. "Early" nodulins expressed within six hours of rhizobial addition are more likely to be involved in infection and nodule development; later nodulins are more generally related to nodule function and nitrogen fixation. At least 50 putative nodulins have been identified at the protein and mRNA level but functions for many of these remain to be determined. Nodulins that have been characterized include leghemoglobin, phosphoenol pyruvate (PEP) carboxylase, and a number of enzymes involved in allantoic acid synthesis. More detail is provided by Pawlowski (38). Spatial differences in the distribution of leghemoglobin and uricase within the nodules of *P. vulgaris* is shown in Figure 4 (37).

Chemistry of Nitrogen Fixation

The enzyme nitrogenase that catalyzes the reduction of atmospheric nitrogen in nodules comprises two oxygen labile proteins (39,40):

- Dinitrogenase (MoFe protein or component 1) an $\alpha_2\beta_2$ tetramer of MW ~220,000, which contains two [$MoFe^6S^8$] clusters per molecule,
- Dinitrogenase reductase (Fe protein or component 2) an α_2 dimer of MW ~60,000, and containing a single [4Fe-4S] cluster and two Mg. ATP binding sites. The Fe protein donates electrons to the MoFe protein.

In studies of nitrogen fixation a distinction is usually made between *nif* genes needed for the synthesis of the MoFe and Fe proteins, and *fix* genes involved in the regulation of nitrogen-fixing activity (41). Alternative dinitrogenases in which vanadium replaces molybdenum or iron alone is present have been identified in some organisms.

Both dinitrogenase and dinitrogenase reductase are oxygen labile, with all nitrogen-fixing organisms needing mechanisms to avoid exposure of these enzymes to oxygen. In the case of the legume nodule, these mechanisms include barriers to oxygen diffusion and the presence of leghemoglobin as an oxygen carrier (42).

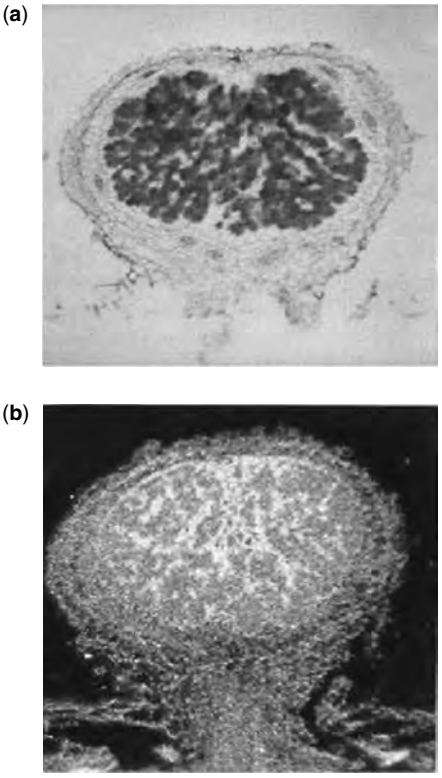

Figure 4. Longitudinal section of a mature *P. vulgaris* nodule showing the in situ localization of the (**a**) leghemoglobin and (**b**) uricase II transcripts. Figure 4a was taken using bright-field and Figure 4b using dark-field microscopy. Leghemoglobin is synthesized in invaded cells of the central tissue, and is not detected in uninfected cells of this region or in peripheral tissues, whereas uricase II is found only in uninvaded cells. (From R. Tate, E. J. Patriaca, A. Riccio, R. Defez, and M. Iaccarino, *MPMI* **7**, 582–589 (1994) used with permission). See color insert.

Specificity in Legume Nodulation

Given the complex signaling involved, specificity in nodule formation by legumes is to be expected. This can be evident at a number of levels:

- Many caesalpinioid legumes, including all species of *Cassia* never form nodules,
- Each rhizobial isolate has the ability to nodulate some but not all legumes. Cross-inoculation groups comprising legume species nodulated by the same rhizobia can often be further subdivided into effectiveness subgroups that differ in their ability to fix nitrogen with particular rhizobia.
- Cultivars of the same plant species can vary in the level of nitrogen fixation achieved.
- Mutation can lead to loss of nodule-forming ability in the host or rhizobia.

Legume collection from different regions of the world is often complicated by the need to match collections of the host with those of suitable rhizobia. Thus, the pea cultivar Afghanistan nodulated normally with pea rhizobia from

the Middle East, where it was collected, but carried a single recessive gene-limiting nodulation with most pea rhizobia from Europe. Similarly, the narrow germ plasm base used in breeding American soybean varieties has affected their ability to symbiose with indigenous soil rhizobia in other areas of the world. When the variety Bossier was introduced into Africa, it nodulated poorly, and had to be inoculated, whereas landraces such as Orba and Malayan nodulated with the indigenous rhizobia and benefited little from inoculation.

This specificity means that legumes introduced into new areas of production will often require artificial inoculation to ensure good nodulation and effective nitrogen fixation. Many such inoculant preparations are now sold commercially, with more than 100 different preparations needed to satisfy the requirements of all currently important crop, pasture, and tree legumes. The *Rhizobium* Research Laboratory website at *http://www.rhizobium.umn.edu* (43) details strain requirements and types of inoculants available.

Legume Inoculation

Where legume inoculation is required, the strain or strains of rhizobia employed must meet the following specific criteria:

- Form highly effective nodules with all commonly used cultivars of the legume species for which it is intended;
- Be competitive in nodule formation with such indigenous rhizobia as may occur, and persist well in the soil;
- Be tolerant of soil environmental stresses such as pH and high temperature;
- Grow well in simple, inexpensive culture media;
- Be genetically stable; and
- Survive well on the seed before sowing and in the soil.

Once identified as being of suitable quality, a rhizobial strain(s) used in inoculation, must be grown to a suitable cell density in culture vessels, and then inoculated into one of the several carrier materials. It survives in this carrier until used by the farmer (44). Initially sterile peat flour preparations are usually preferred as the carrier, but nonsterile peat flour, granular peats, other granular preparations, and even frozen liquid concentrates may be used. Essential features of such carriers include:

- high water-holding capacity;
- nontoxicity to rhizobia;
- ready availability, low cost, and easily processed;
- sterilizable; and
- good buffering capacity.

Such preparations should be properly prepared, inoculated, and stored and should maintain counts in excess of 10^8 to 10^9 rhizobia per g^{-1} or mL^{-1} for periods of up to a year. When appropriately used the expectation is that they will supply 10^3 to 10^5 rhizobia $seed^{-1}$ when applied to the seed, or 10^{11} cells ha^{-1} when applied to the soil (45). Quality control for such inoculant preparations can be quite variable, but is strictly regulated in countries such as Australia, Canada, France, and Uruguay.

Four alternate procedures can be used to introduce rhizobia into the soil with the method used determining the type of inoculant preparation needed. Procedures include:

- seed inoculation, with the inoculant mixed with milk, sugar solution, or other adhesive sticker and then applied to the seed to provide uniform coverage. Ideally, seed should be inoculated, dried in the shade and planted the same day, a procedure that is often irksome for larger scale farmers.
- seed pelleting using a stronger adhesive (40% gum arabic, 10% methyl ethyl cellulose), with the seed first inoculated as described earlier, and then rolled in finely ground limestone or rock phosphate. Seed pelleting has been used extensively to counteract the impact of unfavorable soil conditions or in range-seeding. Commercial preinoculation of seed for subsequent sale is also common, but decline in rhizobial numbers during seed storage is significant, and preinoculated seed only rarely meets inoculation standards.
- soil inoculation with granular peat or liquid preparations. The inoculant is banded in soil below the seed and makes contact with the emerging radical. Soil inoculation is most useful when the seed to be planted has been treated with fungicide, or soil conditions dictate higher than normal levels of inoculation.
- cover inoculation in which inoculant rhizobia are introduced during irrigation, and at high cell numbers.

Inoculation of legume seed by sprinkling it with inoculant in the planter seed box, often used as an insurance procedure when soils are thought to already contain some rhizobia, provides very patchy nodulation, and cannot be recommended.

Production of inoculants and legume inoculation is not a difficult procedure. Properly carried out, the results can be spectacular, with yield increases of more than 50% compared with uninoculated controls (Fig. 5). However, inoculation can fail, especially where inoculation quality is not well regulated. Lupwayi and coworkers (45) reported a steady improvement in inoculant quality in Canada following regulation of inoculant production in 1975. In contrast, Gomez and coworkers (46) evaluated 18 soybean inoculants produced in Argentina, and found rhizobial numbers to vary from 0 to $\log_{10}$ 9.94 rhizobia g^{-1} product. Many of the products were contaminated with other organisms, and only 5 of the 18 would have met Canadian standards. Catroux and coworkers (47) assessed the current status of legume inoculants, and concluded "We enter the era of biotechnology knowing more and more about the mechanisms of nitrogen fixation at the gene level, but except for some ..... developed countries still lacking good quality and reliable inoculants."

Figure 5. Response to inoculation in soybean planted in newly cultivated production areas in Puerto Rico. (photo: R Stewart Smith, used with permission). See color insert.

Unsatisfactory products aside, poor response to inoculation can be because of:

- delays between inoculation and seeding;
- use of inoculant rhizobia preparations that will not nodulate the legume in question;
- use of old, dessicated, or improperly stored products in which most of the rhizobia have died;
- high soil temperature or acid-soil conditions leading to the death of inoculant rhizobia in the soil;
- interaction between inoculant rhizobia and seed-applied pesticides or fertilizers;
- deviation from inoculant manufacturer's recommendations resulting in too few rhizobia added or poor contact between seed and inoculant bacteria;
- competition for nodulation with indigenous rhizobia; and
- inadequate extension information for, especially, third world farmers.

Once a particular plant species has been correctly inoculated, it is not usually necessary to reinoculate in subsequent years. Rhizobial numbers released from the nodule following senescence can exceed Log_{10} 10 rhizobia g^{-1} nodules (48), with sufficient rhizobia persisting in the soil such that reinoculation is not necessary. Diatloff (49) and others have returned to sites previously used in inoculation experiments, and shown that the original inoculant rhizobia still dominated nodulation some 10 to 15 years later. In contrast, Hungria and Vargas (50) have recommended annual reinoculation for legumes seeded into the acid and high-temperature soils of the Brazilian cerrados, where the rate of die-off of rhizobia in soil is much greater.

Factors Affecting Legume Nodulation and Nitrogen Fixation

A number of factors, including several already mentioned, can affect successful nodulation and nitrogen fixation. These include:

Host Cultivar. As indicated earlier, it is usual for different cultivars of the same species to differ in nitrogen-fixing ability with specific rhizobia. With crop legumes, this often relates to flowering and maturity differences (51) with early flowering varieties more likely to be limited in ability to fix nitrogen. However, genetic differences in earliness of nodulation, nodule mass $plant^{-1}$, onset of nodule senescence and enzyme function have been reported, and appear amenable to genetic manipulation (52–54). In several situations, coadaptation of host and rhizobia has been reported, with hosts from a particular region likely to perform better with local, than with introduced, rhizobia (55,56).

A simple approach to improve nitrogen fixation in crop plants is to select for both seed and biological yield in plants grown under conditions of low nitrogent content in soil. The situation with soybeans suggests that even here, however, more than one approach might be possible. Thus, in the Cerrado of Brazil where soils are nitrogen-deficient and response to inoculation is dramatic, an emphasis on inoculation has resulted in cultivars deriving a high percentage of their nitrogen requirements from symbiosis (57). By contrast in Africa, Mpepereki and coworkers (58) have advocated the use of symbiotically promiscuous cultivars that are able to nodulate and fix nitrogen with indigenous bradyrhizobia as better suited to the needs of subsistence farmers with limited access to inoculants.

Soil Acidity. There are more than 800 million ha of Oxisols and Ultisols with a pH less than 5.0 in Latin America alone. Progressive soil acidification is also a common consequence of ley farming and specific nitrogen-fertilization practices. Such low pH levels limit rhizobial survival in soil, and affect various steps in nodulation and nitrogen fixation. Their influence may be related to H^+ concentration per se, to the presence of toxic levels of aluminum and manganese, or to induced deficiencies of phosphorus, calcium, or molybdenum.

Species of rhizobia differ sharply in pH tolerance, with *R. tropici, R. loti*, and some bradyrhizobia known for their acid-tolerance (59), and *S. meliloti* being particularly acid sensitive. Brockwell and coworkers (60) recovered an average of 89,000 *S. meliloti* g^{-1} from soils with near-neutral pH, but only 37 cells g^{-1} from soils of pH below 6.0. Even the latter rhizobia are more likely to have come from microsites of more favorable pH in the soil, than to be pH tolerant. Failure to nodulate under such conditions can be related to poor survival of the rhizobia, or to inhibited attachment of the rhizobia to their host at low pH. Although lime is commonly applied to alleviate the pH problems of soil in the United States, the areas involved and the cost and availability of limestone can limit the use of lime in other regions. Further, where minimum tillage approaches are needed to maintain soil organic matter content, as in large areas of Brazil (50), lime is often surface-applied, and must infiltrate the soil over time, leading to variation in soil pH with depth. Practices that mitigate the need for high rates of lime application in acid soil include the pelleting of seed with finely ground lime or rock phosphate to create microenvironments of more suitable pH, and the use of more acid-tolerant cultivars and strains. In Australia, the use of the somewhat acid-tolerant strains such as WSM419 together with annual

medics collected from acid-soil areas in Sardinia has extended the area sown to *Medicago* species by some 350,000 ha since 1985 (61). The genetic basis for some differences in acid-tolerance among strains of *S. meliloti* are reviewed by Dilworth and coworkers (62). The acid-tolerant *R. tropici* strains CIAT1899 and PRF81 also have been extensively used for bean inoculation in Brazil.

Mineral Nutrition. Several elements have functions in nodulation and/or nitrogen fixation above and beyond those associated with nitrogen-fertilized plants. These include:

- molybdenum, iron, and sulfur as components of the nitrogenase enzyme;
- iron needed for leghemoglobin and in nodule development;
- calcium required for rhizobial attachment and for cell wall integrity in both plant and *Rhizobium*;
- phosphorus needed for energy transformation and supply to the microsymbiont;
- cobalt for nodule coenzyme function;
- boron needed for cell wall formation and the development of the diffusion barrier to oxygen in the inner cortex; and
- nickel as a component of the hydrogen uptake system present in some rhizobia, and used to recapture hydrogen released during fixation.

Legumes deficient in one or more of these elements may be limited in their ability to nodulate or fix nitrogen, and have the generalized yellow chlorosis typical of nitrogen-deficient plants. The need for phosphorus in symbiosis is such that the nodulated plants have a much higher requirement for phosphorus than do those supplied fertilizer nitrogen. A consequence is that legumes are commonly dependent on a three-way symbiosis with both rhizobia and AM mycorrhizal fungi.

THE ACTINORHIZAL-*FRANKIA* SYMBIOSIS

Mechanisms of Infection

Because *Frankia* were not isolated in pure culture until 1978, and in some cases are still difficult to isolate, knowledge of the actinorhizal-*Frankia* symbiosis lags behind that available for the nodulated legume. The suggestion by Soltis and coworkers (63) of a single phylogenetic clade of plants predisposed to nodule formation, and including both legume and actinorhizal species, has focused studies toward overlaps and differences in these two systems, with significant recent progress.

Actinorhizal plants group into four clusters (64). In the Betulaceae, Casuarinaceae, and Myricaceae (cluster 4), infection proceeds intracellularly through root hairs, with mitotic activity in cortical cells adjacent to infected root hairs leading to a slight but visible protruberance called the *prenodule* (65). *Nif*-gene expression in *Frankia* cells from the prenodule has been reported, but no indication was given of relative rates of nitrogen fixation. A nodule primordium separately formed in the root pericycle then becomes infected with *Frankia* from the prenodule. Multilobed *Alnus* nodules are shown in Fig 6. In this host, lobes arise from modified lateral roots without root caps, have a significant periderm, a central vascular cylinder, and infected cells in the cortex (66). Nodule induction by rhizobia in the nonlegume *Parasponia* follows a similar sequence, although the rhizobia enter the plant intercellularly and not through root hairs.

Figure 6. Nodules of *Alnus* showing a typical multilobed structure that is derived from modified lateral roots. (Photo: P.O. Lundquist, used with permission). See color insert.

Infection in the Eleagnaceae, Rhamnaceae, and Rosaceae (clusters 1 and 2) is predominantly intercellular. Thus, in *Discaria trinervis*, infection involves intercellular penetration between epidermal and cortical root cells, and root hair deformation is not common (67). Nodule primordia can be detected in young roots around six days after inoculation, with invasion of host cortical cells occurring some seven to nine days after inoculation, and associated with hypertrophy of the primordium cells. Differentiation of nitrogen-fixing vesicles of the microsymbiont occurs within already infected cells, with numerous uniformly distributed vesicles evident within cells of the host by 16 days after inoculation. Nitrogen fixation begins at about the same time. Berg and coworkers (68) described the intercellular infection process associated with *Datisca* and *Coriaria* (cluster 3).

Infection by *Frankia* provides some interesting similarities and contrasts with that found in legumes. Thus,

- flavonoid-containing preparations from seed washes of *Alnus rubra* influenced nodulation by *Frankia* (69), although in *Alnus glutinosa*, van Ghelue and coworkers (70) found no correlation between root hair deformation and infective ability.
- root hair–deformation bioassays developed for the study of the *Alnus-Frankia* infection process, suggest involvement of "*nod*-factors" analagous to those found in *Rhizobium* (71). In at least some *Frankia* strains, however, these *nod* factors are constitutive, heat-stable, hydrophilic, and chitinase-resistant, suggesting structural differences with those found in rhizobia. As a further indication of this, a *Frankia* genomic DNA library-tested by Ceremonie

and coworkers (71) failed to complement nodulation mutants of *Sinorhizobium*.

- infection threads produced in the *Comptonia, Casuarina, Alnus, Datsica*, and *Myrica* symbioses (68) do not result in release of *Frankia* into cells of the host (72). Instead, host cells are filled with branched infection threads.
- regulation of nodulation in some symbioses with *Frankia* parallels that found with *Rhizobium*. Thus, Valverde and Wall (73) noted nodules in *Discaria* mainly located around the position of the root tip at the moment of inoculation, showed nodule distribution affected by culture age and inoculum dosage, and found that taproot nodule initiation resulted in distal suppression of nodulation. Similar regulation of infection occurs in the legume *Rhizobium* symbiosis.
- although nodule-specific gene expression occurs both in the legume-*Rhizobium* and actinorhizal-*Frankia* symbioses, it has been more extensively studied in legumes. Known nodulin genes in the *Frankia* symbiosis include a serine protease expressed early in symbiosis (65) and a nodule-specific hemoglobin (72).
- *Frankia* nitrogenase, like that of *Rhizobium*, must be protected from oxygen. In most of the hosts with which *Frankia* associates, the microsymbiont produces vesicles, and it is within these structures that nitrogenase activity is found. The vesicular envelope contains layers of hopanoid lipids that increase or decrease in thickness with oxygen tension and stress (74), and with oxygen regulation, thought to be a function of envelope-thickness. In *Casuarina* and *Myrica*, vesicles are not found in the nodule, and oxygen diffusion is limited by lignin and suberin-like materials that gird the nodule. In these nodules, as in legumes, hemoglobin facilitates the required oxygen supply to the microsymbiont (75).

Specificity in the Actinorhizal Symbiosis

Data on cross-inoculation specificity among actinorhizal plants is incomplete, and particularly affected by the limited availability of pure cultures from the Rhamnaceae, Rosaceae, and Coriariaceae. As with legumes, many plant species of lesser importance have not been studied.

16S rDNA sequence analysis (76–78) has permitted identification of four main clusters within the genus *Frankia*:

- A large group including isolates from both *Alnus* and *Casuarina*;
- Uncultured isolates from *Dryas, Coriaria*, and *Datisca*;
- Strains from *Eleagnus, Hippophae*, and *Gymnostoma*;
- Atypical and non- nitrogen-fixing isolates.

Cross inoculation somewhat parallels these groupings, although *Alnus* and *Casuarina* require separate inoculant strains, and *Myrica* spp. are promiscuous and nodulate with isolates from *Alnus, Casuarina*, and the *Eleagnus–Gymnostoma* complex. *Atriplex cordobensis* is also promiscuous, nodulating with *Frankia* strains from *Colletia, Ceanothus, Hippophae*, and *Casuarina* (79). Many additional studies need to be undertaken to resolve infective and effective subdivisions among these organisms. Thus, Simonet and coworkers (80) found seven phylogenetically distinct groups of *Frankia* in the nodules of Australian *Casuarina* and *Allocasuarina* species, but noted that these species require inoculation when planted in other countries. They examined *Casuarina* nodules from plants grown outside Australia, and recovered only one of the seven phylogenetic groups. Because these *Frankia* were more easily cultured, had a wider host range, and appeared to survive in soil apart from their host, saprophytic competence is likely to be as important for *Frankia* as for the rhizobia.

Inoculation

Frankia inoculation may be by cultured cultures, crushed nodules, or infective soil (13). Encapsulated, alginate bead formulations have also been used (81), but inoculation procedures for actinorhizal plants are much less satisfactory than for legumes. Best results are achieved by transplantation from inoculated nursery stocks (82,13) but the inability to culture specific *Frankia* is still a major impediment.

CONCLUSION

This presentation has emphasized recent improvements in our knowledge of nodulation and symbiotic nitrogen fixation. It is clear that these processes contribute significantly to crop and pasture production in both the developed and developing world. What is less obvious, and what needs to be pointed out in conclusion, is that the role for symbiotic nitrogen fixation and the consequent research requirements differ markedly among regions.

For countries such as the United States where fertilizer nitrogen is relatively inexpensive and animal manure plentiful, symbiotic nitrogen fixation is not emphasized for crop production, and dependence on symbiotically-fixed nitrogen is only critical for range, forage, revegetation, and natural environments. Applied research requirements are limited, allowing emphasis on the molecular and genetic basis of nodulation and on host and *Rhizobium* biodiversity. It is not surprising that inoculant production is stagnant or in decline, nor that the percentage of legume crop nitrogen derived from nitrogen fixation is also dropping.

In contrast, developing countries with burgeoning population and extensive soil degradation have urgent applied requirements. In these countries, small landholders are frequently unable to afford or obtain fertilizer nitrogen, edaphic factors (low soil pH, high soil temperature, drought, and nutrient deficiencies) constrain nitrogen fixation, and inoculant quality is often poor. For these regions, varietal improvement in the ability to fix nitrogen in symbiosis, cultivar and strain selection for the tolerance of edaphic constraints, a better understanding of the factors

affecting rhizobial strain survival in soil, and above all, improved inoculant quality and availability are urgently required. It has been suggested that nitrogen fixation worldwide will have to increase threefold over the next 30 years to meet the increasing food requirements. Much of that increase will have to occur in developing countries, emphasize crop as well as other legumes and nonlegumes, and stress the more applied side of symbiotic nitrogen fixation.

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NITROGEN FIXATION IN THE MARINE ENVIRONMENT

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Nitrogen (N) is one of the most abundant elements in biological materials and is used by most organisms in its inorganic chemical forms, nitrate (NO_3^-) and ammonium (NH_4^+), or as organic nitrogen (e.g., urea, amino acids, protein). These chemical forms of nitrogen can be rapidly depleted in many marine environments, and the availability of nitrogen often limits the productivity of marine and estuarine habitats (see NITROGEN CYCLE IN THE MARINE ENVIRONMENT). Nitrogen has a complex biogeochemical cycle that is largely mediated by biological transformations. Chemical forms of nitrogen range in oxidation state from $+5(NO_3^-)$ to $-3(NH_4^+)$, with N_2 having an oxidation state of 0 (1). Briefly, major components of the nitrogen cycle are ammonification (remineralization, liberation of NH_4^+) resulting from decomposition of organic materials, oxidation of NH_4^+ ultimately to NO_3^- by nitrifying bacteria, denitrification of NO_3^- to N_2 by denitrifying bacteria, and fixation of N_2 into ammonium and ultimately organic matter by nitrogen-fixing bacteria (including cyanobacteria) (1). Plants, both multicellular and unicellular, are the organisms responsible for fixation of carbon dioxide into organic matter (primary production) and are dependent upon the availability of NH_4^+, NO_3^-, and some simple organic forms of nitrogen. Thus, the interplay between the different components of the nitrogen cycle, in particular the balance between nitrogen fixation and denitrification, can control the productivity of aquatic environments.

Nitrogen gas (N_2), readily dissolved in seawater, is not available to most organisms, except for "nitrogen-fixers" (also commonly referred to as diazotrophs), which reduce nitrogen gas to ammonium that is then assimilated into organic matter. Thus, N_2 is an important potential source of nitrogen in nitrogen-depleted waters, and can be a quantitatively important process in the nitrogen cycle that contributes to the productivity of marine systems. In this section, we will review the basic aspects of biological nitrogen fixation and discuss the significance of nitrogen fixation in open oceans and estuaries, and touch on coral reefs.

BRIEF REVIEW OF NITROGENASE BIOCHEMISTRY AND GENETICS

Nitrogen can be fixed into combined forms abiotically: industrially reduced to ammonium by the Haber process, or naturally to nitrogen oxides by lightning. Although the nitrogen fixation reaction is thermodynamically favorable and nitrate is the most stable form, the transition state requires a large input of energy to break the triple bond between the nitrogen atoms of N_2. The ability to fix N_2 biologically appears to be restricted to prokaryotes, and is catalyzed by the enzyme nitrogenase. This enzyme is very similar (in both its gene and amino acid sequences) among very distantly related microorganisms, which implies that the enzyme may have evolved early (2). The chemical reaction for biological nitrogen fixation is:

$$N_2 + 8e^- + 8H^+ + 16\text{MgATP} \rightarrow 2NH_3 + H_2 + 16\text{MgADP} + 16P_i$$

Thus, the physiological costs of N_2-fixation are high with respect to adenosine triphosphate and reducing equivalents. Most N_2-fixing microorganisms will preferentially use ammonium, if it is available, and the synthesis of the nitrogenase enzyme is regulated in response to

ammonium availability (enzyme synthesis is repressed in the presence of high concentrations of ammonium). Elaborate molecular and biochemical mechanisms control the transcription of the nitrogenase genes and activity of the proteins themselves (3). In addition to being energetically expensive, nitrogenase is rapidly inactivated by oxygen, at least in vitro (4). Recently, a nitrogenase has been described that is not sensitive to oxygen inactivation (5), but its relationship to "conventional" nitrogenases, or its prevalence in nature are not yet known.

The nitrogenase of most nitrogen-fixing microorganisms is composed of two metalloproteins, the molybdenum (MoFe) protein and the iron (Fe) protein (6,7). The genes that encode proteins necessary for nitrogen fixation are called *nif* genes. The *nifD, nifK*, and *nifH* genes encode the subunits of the MoFe protein (*nifD* and *K*) and the iron protein (*nifH*) of the enzyme nitrogenase. These genes are found in diverse prokaryotes, representing most, if not all, major evolutionary lineages (including both Archaea and Bacteria), and many different physiological groups (e.g., phototrophs, chemoautotrophs, chemoheterotrophs) (8). Two additional nitrogenases are very similar to the conventional nitrogenase, but molybdenum is replaced by vanadium ("first alternative" encoded by *vnfHDGK*) or by iron ("second alternative, *anfHDGK*) in these nitrogenases. Other multiple copies of *nif* genes have also been reported (there are six copies of *nifH* in *Clostridium pasteurianum*). In some organisms different copies of the molybdenum nitrogenase are regulated differently (e.g., 9). Microorganisms can contain one or multiple copies of nitrogenase. Although the alternative nitrogenases have been detected in the environment (10), the ecological and paleoecological significance of the different nitrogenases is not known.

There are about 20 known nitrogen fixation genes that are involved with various functions of nitrogen fixation or assembly of nitrogenase, including molybdenum uptake, providing reducing equivalents, providing scaffolding for assembly of MoFe cofactor, and so on. The functions of these genes are beyond the scope of this article but are discussed elsewhere (11).

THE DIVERSITY OF MARINE NITROGEN-FIXING MICROORGANISMS

Many different types of microorganisms can fix nitrogen, but the genetic capability is not uniformly distributed throughout evolutionary lineages such that not all representatives of an evolutionary lineage that contain nitrogen-fixers fix nitrogen. For example, some cyanobacterial species can fix nitrogen, but other morphologically very similar cyanobacteria cannot. Taxonomic information alone is not sufficient to predict whether a given species is able to fix N_2. There is a large number of known N_2-fixing species, but there are undoubtedly many more that have simply not been shown to fix nitrogen, either because they have never been tested or because the right conditions have not been used (8).

There are nitrogen-fixing representatives in most branches of the Prokaryotic tree, including representatives of Archaea and Bacteria (Fig. 1). The Archaea include the major clades Crenarchaeota and Euryarchaeota. Thus far, nitrogen fixation has only been found in the methanogens that are included in the Euryarchaeota (12). Given that it is now recognized that there are many uncultivated Archaea in marine systems (13), it is possible, if not likely, that other Archaea phyla will be detected as more organisms are brought into culture.

Nitrogen-fixing microorganisms are scattered throughout the proteobacteria and cyanobacteria groups within the Bacteria (Fig. 1). N_2-fixing bacteria have been isolated by conventional microbiological techniques from many marine environments. Common diazotrophic marine groups in the γ proteobacteria are *Azotobacter* and *Klebsiella* (Table 1). Representatives of N_2-fixers in the δ proteobacteria are the sulfate reducers, such as *Desulfovibrio* (Table 1). Diazotrophic α proteobacteria include relatives of *Rhodobacter* and *Rhizobium*.

The diverse array of diazotrophs represents many modes of metabolism (Table 1). Cyanobacteria, major N_2-fixers in many marine environments, from the water column of the open ocean to mats occurring in shallow-water coastal intertidal areas, are oxygenic phototrophs. In certain habitats, such as marine mats and anoxic water columns within the euphotic zone, anoxygenic purple and green sulfur and purple nonsulfur bacteria can be abundant and are potentially diazotrophic.

There are many different types of heterotrophic microorganisms that fix nitrogen, spanning a wide range of heterotrophic modes of metabolism, including aerobes, microaerophiles, facultative and strict anaerobes (*Clostridium*) (14). It is unclear whether heterotrophs are important in N_2-fixation in the water column, but they are undoubtedly more active in sediments (15) and in association with invertebrates (16). N_2-fixers have been isolated from sea urchins and other marine invertebrates (17) and from estuarine waters (18,19).

Molecular phylogenetic approaches have shown that marine microbial cultures are not in general representative of natural marine open ocean microbial assemblages (20,21), which implies that probably our N_2-fixing culture collections also are not representative of natural assemblages. There are two general kinds of microbiological information from marine systems: experiments and enumerations using cultivation or enrichment, and culture-independent information. This is particularly relevant to the discussion of nitrogen fixation. Molecular phylogenetic information is usually obtained from 16S ribosomal RNA genes, which provides no direct information on the ability to fix nitrogen. Cultivation studies, on the other hand, do not necessarily reflect the microorganisms that are most active or most important in situ. Culture-independent methods that target nitrogenase genes provide some information on the organisms present in situ (22,23), but do not provide as high resolution phylogenetic information as studies with ribosomal genes, as all microorganisms have ribosomal genes. Results of culture-independent studies targeting *nif* genes have provided evidence for the presence of many N_2-fixing microorganisms that have not previously been described. Diverse N_2-fixing microorganisms have been detected from marine

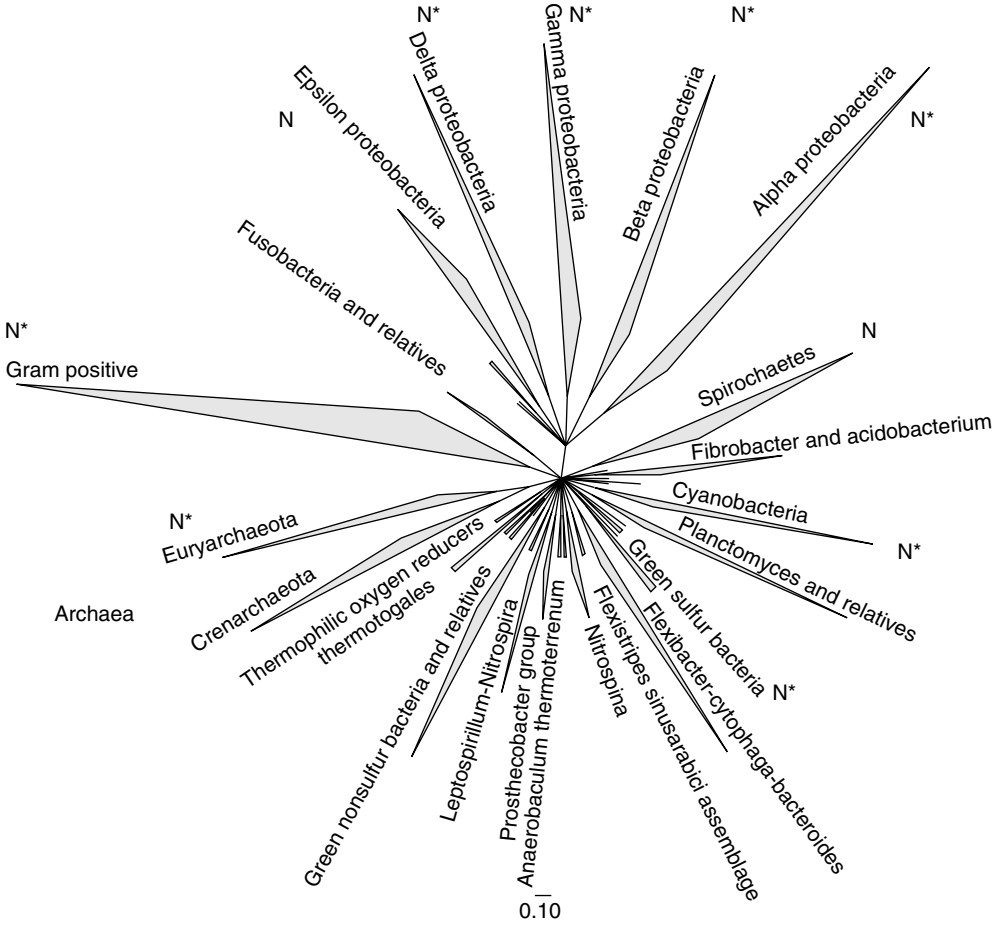

Figure 1. Cartoon of phylogenetic tree based on 16S ribosomal RNA sequences showing the distribution of N_2-fixing capability (N) and groups for which *nif* genes have been sequenced (*) among the major prokaryotic lineages. Tree derived from the ARB database (*http://www.biol.chemie.tu-muenchen.de/*).

environments ranging from cyanobacterial mats (24), sediments (25), and seagrass sediments (26,27) to the open ocean (28). Nitrogenase gene sequences recovered from these environments cluster in the γ proteobacteria, α proteobacteria, and cyanobacterial clades. In environments such as mats and in association with invertebrates, sequences have been recovered that cluster (cluster III of nitrogenases) with typical anaerobes (*Clostridium* and sulfate reducers) (28,29). These N_2-fixing microorganisms cannot be identified to the genus, as the sequences are not similar enough to sequences from cultivated isolates. This molecular technique can also be used to determine which microorganisms are expressing the nitrogenase apparatus (23), and these studies are currently in progress.

FACTORS THAT REGULATE NITROGEN FIXATION RATES

Nitrogen fixation in marine environments is controlled by a complex suite of chemical and physical factors (30). The major factors are oxygen (nitrogenase is rapidly inactivated by oxygen), iron, and perhaps molybdenum availability (trace metal components of nitrogenase), and availability of other forms of nitrogen (which are used by non-N_2-fixing microorganisms and preferentially used by N_2-fixing microorganisms).

Nutrient availability is probably one of the most important factors controlling nitrogen fixation. When fixed nitrogen forms are present, N_2-fixing microorganisms do not have an ecological advantage and non-N_2-fixing microorganisms are more competitive. Most N_2-fixing microorganisms will preferentially use ammonium, and sometimes nitrate, rather than fix N_2. Thus, ammonium or nitrate availability tends to inhibit N_2-fixation. Other important nutrients are phosphorus and trace metals. The growth rate of N_2-fixing organisms can greatly exceed that of competing microorganisms that are dependent upon other forms of nitrogen when the supply rate of nitrogen is low, when the ratio of nitrogen to phosphorus supply is low (31), or when phosphorus loading is high (32). These conditions can lead to blooms of N_2-fixing cyanobacteria, which are often nuisances because of taste, odor, and toxicity problems (33). Such blooms have been observed worldwide in coastal and estuarine regions. Iron and molybdenum are important components of conventional nitrogenases, and therefore, N_2-fixation is dependent upon the availability of these trace elements as well (30).

Table 1. Some Representative Marine N_2-fixing Species Showing Breadth of Phylogenetic Distribution of Marine Prokaryotes

Phylogenetic Group	Mode Metabolism
α Proteobacteria	
Rhodobacter sp. ATCC 35206	Aerobic heterotroph or facultative anaerobic phototroph
δ Proteobacteria	
Desulfovibrio africanus ATCC 19997	Anaerobic heterotroph
Desulfobacter curvatus ATCC 43919	Anaerobic heterotroph
γ Proteobacteria	
Chromatium purpuratum ATCC 700430	Anaerobic phototroph
Vibrio diazotrophicus ATCC 35885	Aerobe, facultative anaerobe
Methylophaga thassica ATCC 33146	Aerobic chemotroph
Roseobacter denitrificans ATCC 33942	Heterotroph
Gram-Positive	
High GC (Thallobacteria)	
Deleya marina	Aerobe, facultative anaerobic
Low GC (Firmibacteria)	
Clostridium oceanicum	Anaerobic heterotroph
Bacillus marinus	Aeribem facultative anaerobe
Cyanobacteria	
Trichodesmium sp. IMS101 WH9601 filamentous nonheterocystous	Phototroph, oxygenic
Richelia sp. uncultivated heterocystous symbiotic	Phototroph, oxygenic
Anabaena sp. CA ATCC 33047 Filamentous heterocystous	Phototroph, oxygenic
Cyanothece sp. ATCC 51472 unicellular cyanobacterium	Phototroph, oxygenic
Arclaea	
Methanococcus maripaludis	Anaerobe, chemoautotroph

Note: See Young (1990) for extensive list of prokaryotic N_2-fixers, and Pearl and Zehr (2000) for more extensive list of cultivated marine N_2-fixing strains available in culture collections.

The relative availability of fixed nitrogen, phosphorus, and iron varies among freshwater, coastal, and open ocean habitats and may be some of the primary factors determining when and where N_2-fixation occurs. Blooms of N_2-fixing cyanobacteria are often found in lakes, but less frequently found in temperate coastal regions and oceans even though fixed nitrogen is generally thought to be the primary limiting nutrient (in shorter supply relative to phosphorus) in many of these areas (30). The reasons for this are still not fully understood, but could be related to the relatively high mass of combined nitrogen often found in coastal waters and estuaries, despite relatively low N/P ratios (34). Other factors such as turbulence or iron availability could also limit marine N_2-fixation.

Temperature and light can have direct effects on the growth and activity of microorganisms. The distributions of some diazotrophs appear to be constrained by temperature. For example, *Trichodesmium*, an important open ocean marine diazotroph, has been observed primarily in waters with temperatures greater than 20 °C (35).

Phototrophic microorganisms such as cyanobacteria and phototrophic bacteria are dependent upon light to obtain energy to support nitrogen fixation. In microbial assemblages composed of both phototrophs and heterotrophs, light-stimulated carbon fixation by phototrophs can secondarily stimulate nitrogen fixation via associated heterotrophs. Some cyanobacteria, such as *Trichodesmium*, contain gas vacuoles that allow them to migrate up and down the water column. This buoyancy mechanism may allow cyanobacteria to optimize photosynthetic carbon fixation and nitrogen fixation.

Although salinity can affect the growth and activity of microorganisms along salinity gradients in estuaries (36), and high salinities have been shown to affect certain N_2-fixing microorganisms (37), at least some N_2-fixing bacteria can have wide salt tolerances (15). Furthermore, there are many examples of N_2-fixing systems in coastal regions, implying that salinity does not ultimately constrain N_2-fixation.

Nitrogenase is sensitive to inactivation by oxygen, and protecting nitrogenase from oxygen is important for diazotrophic microorganisms. Microorganisms use a diverse array of strategies to avoid oxygen inhibition or inactivation. Anaerobes do not need to protect nitrogenase from aerobic conditions, but facultative microorganisms, aerobes, and oxygenic phototrophs need specific mechanisms to prevent nitrogenase from being damaged by oxygen. Facultative bacteria often repress expression of nitrogenase under aerobic conditions, and some aerobic

heterotrophic bacteria are believed to maintain high respiratory rates to keep intracellular oxygen levels low. Oxygenic phototrophs have the biggest disadvantage as they evolve oxygen through photosynthesis. Cyanobacteria usually prevent oxygen from inhibiting nitrogen fixation either by fixing nitrogen at night or by using specialized cells called *heterocysts* that fix nitrogen but do not perform oxygenic photosynthesis (38). There are a few cases that remain enigmatic, most notably in *Trichodesmium*, where it appears that N_2-fixation occurs at the same time as photosynthesis without heterocysts (39).

Physical and chemical factors can control N_2-fixation because of indirect effects. For example, turbulence of water can affect the rate of oxygenation of water, causing indirect effects on oxygen-sensitive diazotrophs such as heterotrophs. The chemical form of iron (Fe) is affected by the presence of other ions and pH that determines the solubility of iron. Iron can precipitate, effectively limiting the availability of iron for diazotrophs (and other organisms). Iron can also be complexed with organic materials (30), and so there may be an interaction between the concentration of dissolved organic matter and the biological availability of iron. It may be that iron availability ultimately limits the rates of N_2-fixation in the oceans and that global climate factors controlling dust inputs to the oceans over long timescales set the balance between global N_2-fixation and denitrification, and ultimately the productivity of the oceans (40,41). There is some evidence the iron can limit the growth of the cyanobacterium *Trichodesmium* (42).

ECOLOGY OF NITROGEN FIXATION

N_2-fixing microorganisms can be found in a multitude of habitats throughout the marine realm, but nitrogen fixation activity is usually restricted to times and places where other more easily assimilated forms of nitrogen are depleted. N_2-fixation is ecologically advantageous when competing microorganisms are limited by the availability of dissolved inorganic nitrogen (ammonium or nitrate), but when other forms of nitrogen are available in high enough concentrations, or are rapidly cycled, N_2-fixation is not competitive because of the costly energetics of the nitrogen fixation process. However, in some environments, N_2-fixation appears to persist, even when high ammonium concentrations are present, such as in some sediment environments (14).

The physiological costs can mean slower growth rates when microorganisms are dependent upon N_2-fixation, compared with growth on fixed inorganic forms (30,43). The higher growth rate of non-N_2-fixers has been interpreted as being the result of increased competitive ability for other resources such as light or phosphorus according to resource competition theory (44).

In terrestrial environments there are many examples of symbiotic relationships between plants and N_2-fixing microbes. Undoubtedly there are diverse symbiotic relationships in the marine realm that are yet to be uncovered. One notable symbiotic relationship is between eukaryotic diatoms (of the genera *Rhizosolenia* and *Hemiaulus*) and *Richelia*, a filamentous heterocystous cyanobacterium (Table 1). Many associations between cyanobacteria and eukaryotes have been observed microscopically, but it remains to be seen how many of the interactions involve N_2-fixation (45).

Interactions among microorganisms that stimulate N_2-fixation span the entire range from loose associations to mutualistic symbiosis. In addition to N_2-fixing cyanobacterial symbioses with eukaryotes, there are symbiotic associations between bacteria and cyanobacteria. Bacteria have been shown to be associated with heterocysts of cyanobacteria, and there may be mutual benefits for both microorganisms. Bacteria can help to keep oxygen concentrations low and may benefit from nitrogenous compounds leaked or excreted from the heterocyst. Some bacteria that fix nitrogen are associated with non-N_2-fixing species of cyanobacteria (25).

MEASURING NITROGEN FIXATION RATES

Procedures for determining the presence of nitrogen fixers and their contribution to nitrogen inputs are varied and steadily evolving (1). Nitrogen-fixing bacteria and cyanobacteria have been isolated from diverse marine ecosystems (46,47) and the number of nitrogen fixers in particular environments may be estimated by classical microbiological procedures involving serial dilution and growth on nitrogen-free media (48). However, both isolation and conventional enumeration methods rely on selective media and cannot provide an unbiased or comprehensive analysis of the physiological diversity of organisms, such as nitrogen fixers, or a quantitative evaluation of their numbers (49). Conventional procedures are being largely supplanted by molecular methods that aim to determine the presence of specific genes of the nitrogen-fixing complex (22). Besides the potential to quantify the abundance of *nif* genes as a proxy for abundance, extraction, cloning, and sequencing of these genes may provide a less-biased assessment of the true diversity of diazotrophs in particular environments.

Methods for determining the rate of nitrogen fixation are key to evaluating whether nitrogen fixers are active in situ as well as for assessing the quantitative importance of this process in specific ecosystems. There are two common procedures for determining nitrogen fixation: the uptake of $^{15}N_2$ into microbial biomass, and the fortuitous conversion of acetylene to ethylene by the nitrogenase enzyme (50). The uptake of dinitrogen gas isotopically enriched with the heavy isotope of nitrogen, ^{15}N, provides a direct measure of this process to the level of incorporation of the recently fixed N_2 into organic matter and biomass. However, the analysis of the amount of $^{15}N_2$ does require relatively sophisticated and specialized sample preparation and instrumentation, either an emission spectrometer or mass spectrometer capable of resolving the isotopes of nitrogen. The "acetylene reduction method" is an indirect enzymatic method that takes advantage of the capacity of the nitrogenase enzyme to convert acetylene (C_2H_2) to ethylene (C_2H_4). In the presence of added acetylene, actual N_2-fixation is inhibited, and the increase over time in ethylene is taken as a measure of the enzyme activity. Acetylene reduction can be expressed in terms of nitrogen

fixation by assumption of a conversion factor based on the stoichiometries of the two reactions (typically given as three or four molecules of acetylene reduced per molecule of N_2), or derived by direct comparisons with $^{15}N_2$ uptake. The acetylene reduction procedure is relatively simple and sensitive. Analysis of acetylene and ethylene requires only a simple gas chromatograph equipped with a flame ionization detector.

Another approach gaining wider use for detecting active nitrogen fixation in marine ecosystems is the analysis of the natural abundance of the stable isotopes of nitrogen, which relies on the subtle differences among different biological reactions to discriminate against the heavier (^{15}N) isotope of nitrogen relative to the more abundant lighter isotope, ^{14}N. The natural abundance of ^{15}N nitrogen in the largest reservoir of nitrogen, atmospheric N_2 gas, is generally given as 0.3663 atom% ^{15}N. ^{15}N isotope abundance is most often expressed in $\delta^{15}N$ notation:

$$\delta^{15}\mathrm{N}(‰) = \left[\frac{(^{15}\mathrm{N}/^{14}\mathrm{N})_{\mathrm{sample}}}{(^{15}\mathrm{N}/^{14}\mathrm{N})_{\mathrm{air\ standard}}} - 1\right] \times 1,000$$

where atmospheric N_2 is defined as 0. The largest reservoir of combined inorganic nitrogen in the sea is nitrate, which has a $\delta^{15}N$ of about 4.5 to 5‰, somewhat higher than atmospheric N_2. Whereas most processes of uptake discriminate slightly in favor of the lighter isotope, the base of marine food chains dependent upon nitrate is typically about 2 to 3‰, and the $\delta^{15}N$ increases up the food chain about 3.5‰ per trophic step (51). Nitrogen fixation, however, effects little fractionation, and results in biomass with an isotopic signature similar to that in atmospheric N_2, or near 0. Food webs that have a substantial input of nitrogen through nitrogen fixation are generally "lighter" (i.e., have a lower content of the heavy isotope, ^{15}N) at comparable trophic levels than those dependent upon nitrate (e.g., from deep water sources).

Lastly, recent advances in the application of molecular techniques to environmental systems have demonstrated the feasibility of detecting the presence of transcripts of functional genes, such as *nifH*, as might be expected in actively nitrogen-fixing organism (23).

Nitrogen Fixation in the Open Ocean

The biological productivity of the oceans is based largely on the primary producers, single-celled eukaryotic and prokaryotic phototrophic microorganisms collectively called *phytoplankton*. These organisms obtain their nitrogen predominately from inorganic nitrogen in the form of nitrate and ammonium. The production of the phytoplankton and the rest of the food chain that ultimately derives nitrogen from the phytoplankton is based on the use of nitrate, ammonium, and other forms of nitrogen, including atmospheric nitrogen gas. Because different forms of nitrogen have different sources and fluxes, a model of nitrogen-based production in the oceans was constructed that described production as "new" or "regenerated," depending upon whether the production was based on new inputs of nitrogen (which includes nitrate in rain, runoff, diffusion and upwelling from deep water, and atmospheric nitrogen gas) or regenerated

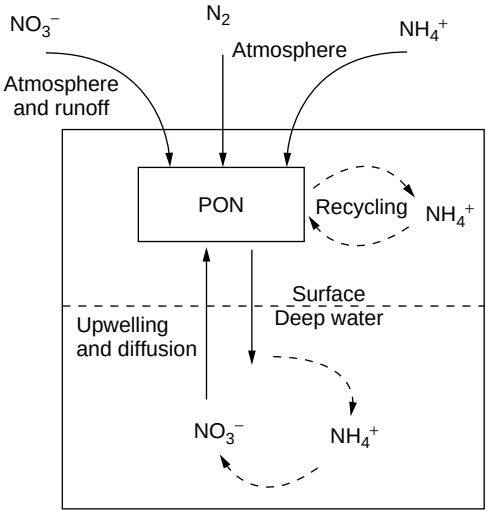

Figure 2. Nitrogen cycle in the water column of the oceans showing major inputs and sinks of dissolved inorganic nitrogen. The cycle through the dissolved organic nitrogen pool is ignored in this simplification, to highlight the importance of N_2 as new nitrogen and to demonstrate the fate of nitrogen in biological materials (PON, particulate organic nitrogen).

(largely ammonium derived from the decomposition and recycling of nitrogen in organic matter) (52). Inputs of new nitrogen can increase ecosystem production, whereas regenerated production is supported by internal recycling (Fig. 2). Nitrogen fixation, therefore, represents a new input of nitrogen to the open ocean, as it can support production beyond that supported by internal decomposition and recycling of organic matter (53). This concept is important for understanding the importance of N_2-fixation in the oceanic realm, as it can enhance the productivity of the oceans, which is analogous to the use of nitrogen-fixing legumes in agriculture to enhance crop yields in nitrogen-poor soils and rice paddies.

N_2 in seawater in equilibrium with the atmosphere is about 800 μM, whereas nitrate and ammonium in nutrient-poor waters are often less than 1 μM and substantially less (nM) in the oligotrophic gyres (1). Relatively low bioavailability of combined forms of nitrogen that constrain biological growth and productivity is termed *nitrogen limitation* (for a review, see 40). Under these conditions, N_2-fixation can alleviate nitrogen limitation until the availability of some other nutrient (e.g., phosphorus) or trace element (e.g., iron) limits the growth of N_2-fixing microorganisms.

In some regions of the open ocean, nitrate is present in relatively high concentrations as a result of upwelling of nutrient-rich deep water (which contains nitrate resulting from decomposition of particulate matter from the surface ocean, and nitrification of ammonium to nitrate) (Fig. 3). In these regions, N_2-fixation is not important from an ecosystem perspective as nitrogen fixation is not ecologically advantageous and N_2-fixation rates are low relative to rates of uptake of nitrate. In large regions of the oceans, including the major gyres of the Atlantic and Pacific oceans, however, there is minimal upwelling, and N_2-fixation can, at times, be significant relative to

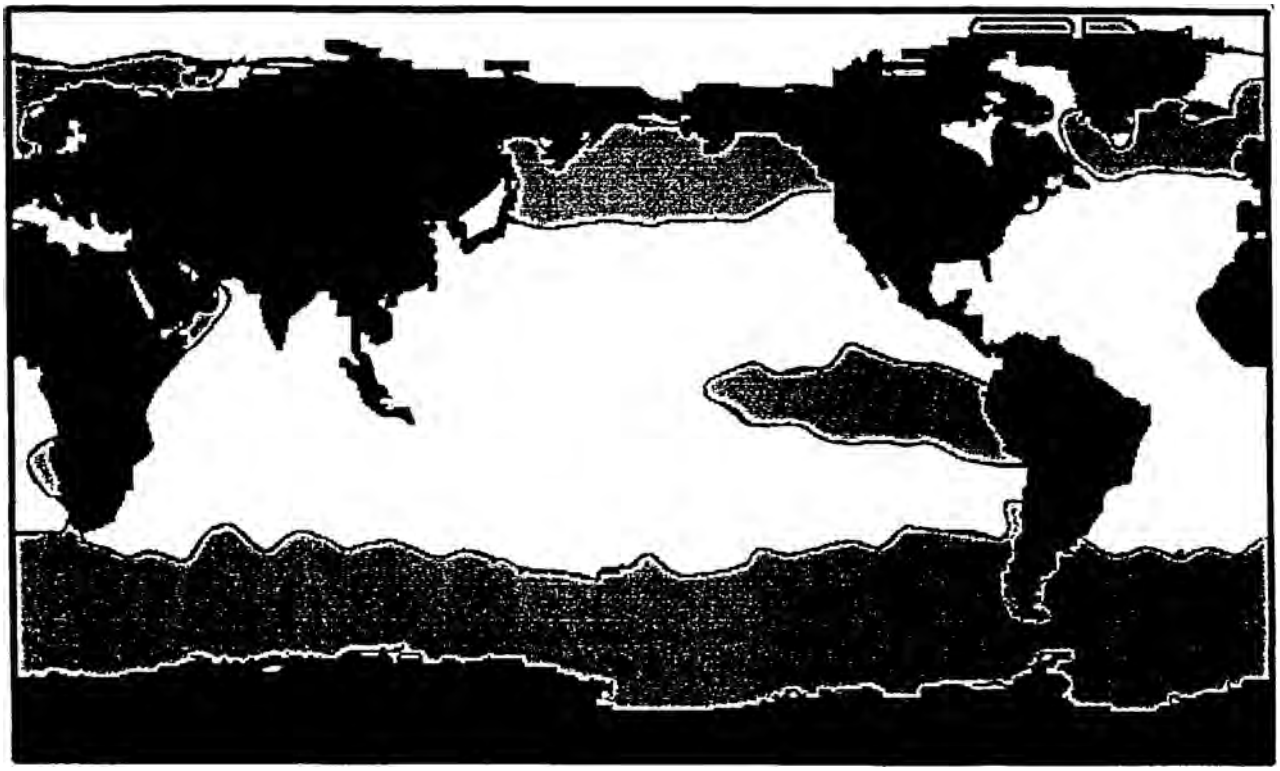

Figure 3. Map of nitrate in surface waters of the world's ocean. Data obtained from the NOAA World Ocean Atlas 1994 (*http://dpo.ori.u-tokyo.ac.jp/ocean/toolmap/Levitus-zonal.html*). Land masses are shown in black. Ocean areas in white correspond to waters with less than 0.5 μM concentrations of nitrate in surface water, and gray areas indicate regions with nitrate concentrations greater than 0.5 μM.

sources of nitrate (54). Dissolved inorganic nitrogen and phosphorus are depleted to depths below 100 m in these areas (Fig. 4). *Trichodesmium*, the best-known diazotroph in oligotrophic oceans, is usually found predominantly in the upper 50 m (Fig. 4).

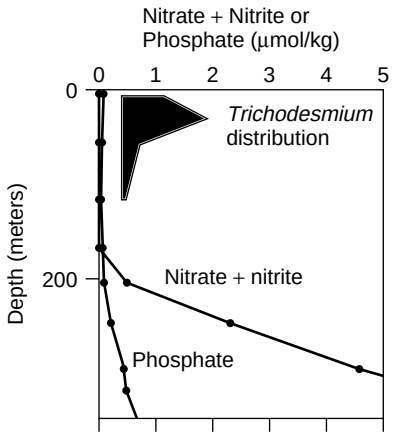

Figure 4. Depth profile of dissolved inorganic nitrogen (nitrate + nitrite) and dissolved inorganic phosphorus in a central oligotrophic ocean gyre. Data from the Hawaii Ocean Time series station ALOHA in July 1999. A typical depth distribution of *Trichodesmium* (see 55) is shown relative to the depletion of inorganic nitrogen in the mixed layer.

The seemingly consistent ratio of major elements in marine plankton, compared to the ratios of inorganic forms of nutrients dissolved in seawater was an early observation that has been called the *Redfield ratio* (40,56). This ratio, a C/N/P of 106 : 16 : 1, is often assumed to be a constant, at least when integrating the biological activities and nutrient fluxes over long timescales. The significance of the constant nutrient ratios with respect to N_2-fixation is that N_2-fixation and denitrification can add nitrogen to or remove nitrogen from the biological pools involved in nutrient cycling in the sea (Fig. 5), uncoupling the ratios of supply of nutrients through mineralization of organic matter.

Nitrogen is only one of a number of nutrients that are important for the growth of microorganisms. In general all organisms have an elemental composition (e.g., C/N/P ratios) that reflects a balance between the optimal composition for maximum growth rate and the ratio of the elements supplied in the environment (Fig. 5).

Deviations from the general constancy and correspondence of elemental ratios in the oceans with respect to marine biological materials have been used to identify regions where excess nitrogen may be formed through N_2-fixation or removed by denitrification. One derived parameter, termed N^*, compares the relative concentrations of inorganic nitrogen and phosphorus along surfaces of equal density (isopycnals) discerning regions of excess nitrogen relative to phosphorus (57,58). Using GEOSECS

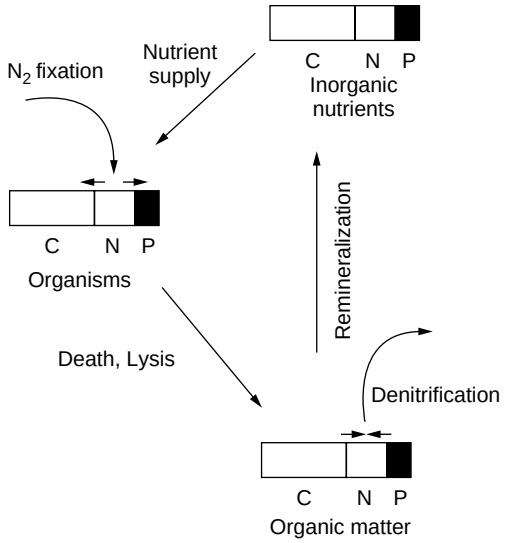

Figure 5. Diagram of cycling of nutrients and the effects of nitrogen fixation and denitrification on the stoichiometry of carbon, nitrogen, and phosphorus in organisms, remineralized nutrients, and nutrient supplies.

data, Gruber and Sarmiento (58) found evidence for N_2-fixation in the North Atlantic Ocean, as well as in the Mediterranean Sea ($N^* > 2.0\ \mu mol\ kg^{-1}$). These data indicate very large inputs by nitrogen fixation in the North Atlantic, exceeding most measured rates of N_2-fixation.

Hence, there are still major uncertainties in these N_2-fixation estimates (59). Ground truthing of these measurements is difficult for a number of reasons. First, N_2-fixation is currently assumed by most biogeochemists to be caused by primarily the filamentous nonheterocystous cyanobacterium *Trichodesmium*, but other diazotrophs clearly are present in the open ocean (45) and are probably contributing to N_2-fixation over different time and spatial scales than *Trichodesmium*.

Including these microorganisms in the nitrogen budgets may require a major change in the way we perceive the nitrogen cycle to operate in the open ocean (41). The sporadic nature of the growth and bloom formation of *Trichodesmium* (and other organisms) makes it hard to obtain statistically reliable data on the distribution and in situ N_2-fixation rate that can be used to assess N_2-fixation over long, integrative time scales.

Nitrogen Fixation in Coastal Zones

Nitrogen fixation occurs in a range of habitats in the coastal zone, from estuarine waters to benthic sediments, salt marshes, and cyanobacterial mats (14,46). Rates can be high in some of these environments, cyanobacterial mats in particular, because of the high concentration of microorganisms, but N_2-fixation in these systems often contributes only a small fraction of the overall nitrogen budget (14).

Estuarine Water Column. The biology and chemistry of estuaries is complex because of variations in hydrologic features, tidal cycles, and extent and characteristics of human development and use of the watershed. Large temporal and spatial variations in nutrient inputs, including both nitrogen and phosphorus, are observed throughout the world's estuaries, depending on geological factors, but more importantly, the types of land use and regulations and policies controlling water quality. Many of the world's estuaries are affected by anthropogenic factors, one being increases in nitrogen and phosphorus runoff (60,61). In many estuaries nitrogen concentrations can be high (in the form of nitrate, dissolved organic nitrogen, and ammonium), but sometimes the ratios of nitrogen to phosphorus still can stimulate nitrogen-fixing microorganisms and even blooms of cyanobacteria (62). Blooms of nitrogen-fixing cyanobacteria often have important implications as a result of the toxicity of some cyanobacteria (harmful algal blooms) (63). Even in estuaries with high nutrient loading, N/P ratios can decrease substantially over the length of the estuary, setting up conditions that could stimulate the growth of N_2-fixing microorganisms (Fig. 6). Blooms of N_2-fixing cyanobacteria such as *Anabaena, Aphanizomenon*, or *Nodularia* have been observed in estuaries throughout the world (62).

Cyanobacterial Mats. Cyanobacterial mats occur in many intertidal regions, but are often localized in restricted areas. Cyanobacterial mats are composed of complex assemblages of phototrophs, dominantly cyanobacteria and heterotrophs. These can be highly structured, with layers of cyanobacteria, phototrophic bacteria, and heterotrophic microorganisms (29). The mat assemblages demonstrate complex interactions among microorganisms that result in strong diel patterns of N_2-fixation (Fig. 7). As both phototrophs and heterotrophs in the mats can fix nitrogen, it is sometimes difficult to determine which organisms are fixing N_2. The cyanobacteria often fix N_2 at high rates during the day when heterocystous species are involved, and during the night when nonheterocystous species are the primary fixers (64). However, dark fixation by heterotrophs can also potentially be fueled by photosynthates synthesized by phototrophs during the day.

Sediments. Nitrogen fixation is associated with marine sediments from shallow estuaries to the deep sea (46). Sediments in estuaries and coastal areas are often organically enriched through deposition of material, either imported from terrestrial environments or formed by microplankton or macrophytes, or through growth of rooted macrophytes such as seagrasses and macroalgae. Intense microbial activities associated with organic decomposition promote anoxia within millimeters of the sediment–water interface, a feature that should favor anaerobic diazotrophs. Sulfate respiration is often the predominant anaerobic process in such sediments, and many sulfate respirers are capable of N_2-fixation (Fig. 1). Similarly, fermentative bacteria such as clostridia, known diazotrophs, have been isolated from marine sediments. Organic-rich sediments are also associated with high concentrations of ammonium, which should inhibit the development of active nitrogen fixers. Nonetheless, low

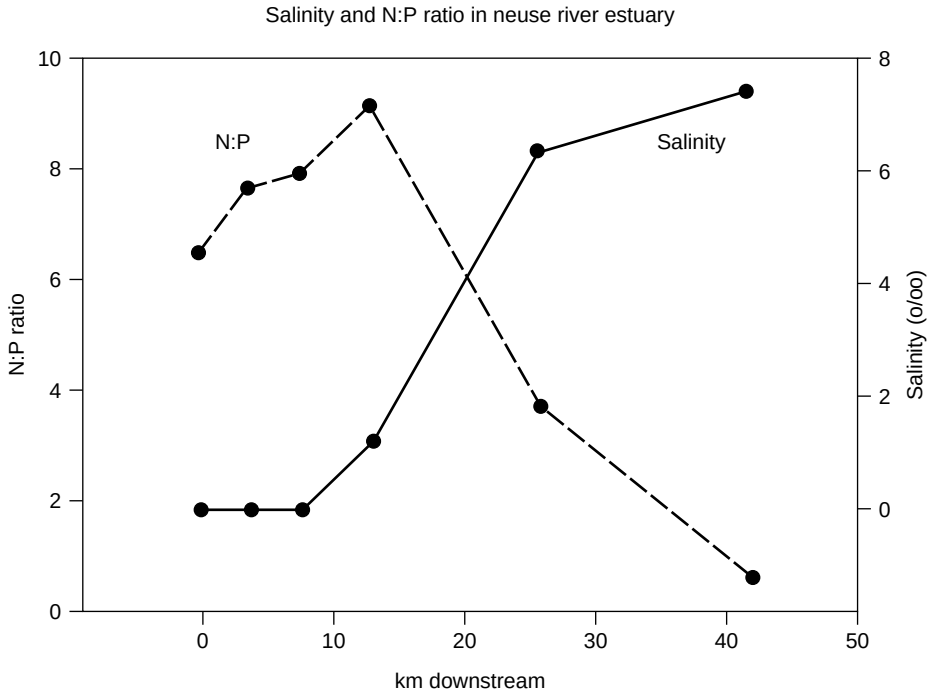

Figure 6. Transect of N/P ratio (nitrate:phosphate) and salinity along an estuary (the Neuse River, North Carolina, data courtesy of H. W. Paerl, University of North Carolina at Chapel Hill), showing how nitrogen depletion along the length of the estuary may provide nitrogen-fixing conditions.

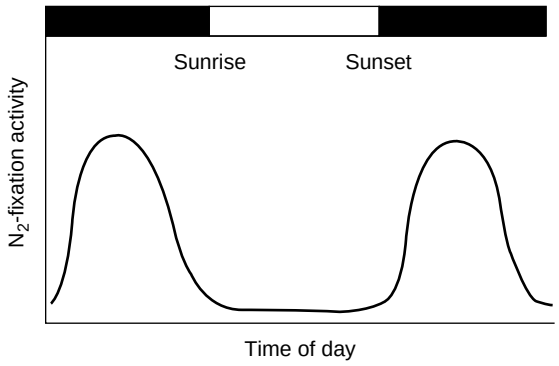

Figure 7. Daily pattern of nitrogen fixation in a marine mat dominated by nonheterocystous cyanobacteria (see 64).

levels of nitrogenase activity are detectable in shallow sediments, and this activity can be stimulated by removal of ammonium pools or addition of organic substrates (46). Moreover, the *nif* genes can be recovered from shallow, organic-rich sediments, demonstrating the existence of populations of bacteria with this metabolic potential.

Sediments vegetated by seagrasses can have much higher levels of nitrogen fixation than surrounding unvegetated sediments (46). This may be a result of the enrichment of the rhizosphere of these sediments by organic exudates from the seagrass roots as well as potential associative symbioses between the seagrass and rhizosphere bacteria as are found in terrestrial grasses. Although relatively little is known about the nitrogen-fixing microbes in vegetated sediments, a few isolates and *nifH* sequences have been obtained from seagrass root–associated sediments (26) and from vegetated salt marsh sediments (65). These communities are diverse, yet remarkably similar between vegetation types and stable through the season (27).

Coral Reefs. Coral reefs are biologically diverse and highly prolific ecosystems that exist as shallow oasis bathed by the nutrient-poor waters of the tropical oceans. An important role for nitrogen fixation formed part of an early hypothesis to explain their richness (66), and subsequent research has confirmed this in identifying various sites of intensive nitrogen fixation on coral reefs (67). These include monospecific mats of heterocyctous cyanobacteria, epiphytes on reef macroalgae, symbionts of giant clams, bacteria and cyanobacteria associated with sponges, coral rubble, as well as the carbonate sediments interspersed within and surrounding the reef (46).

Recent analysis of the natural abundance of nitrogen in various components of the reef has found a generally low signature, indicative of the overall quantitative importance of nitrogen fixation to the overall nitrogen budget of the reef (68).

CONCLUSION

Our perspectives on nitrogen fixation in the marine environment are rapidly changing as a result of our understanding of the biochemistry, biology, and ecology of its role in global carbon cycling. The capability of nitrogen fixation is found in a diverse array of microorganisms and in a wide array of habitats. Recent advances in molecular biology techniques and data obtained from genomic sequencing, are providing new information on the diversity of diazotrophs, the evolution of nitrogenase, and the regulation of the nitrogen fixation apparatus. Diverse nitrogen-fixing microorganisms are found in many benthic and near-shore habitats, and rates can be very high in these localized habitats. Nitrogen fixation in the open ocean, previously believed to occur at low rates in only

a handful of organisms, now appears to be significant in the oceanic nitrogen budget, and is caused by a diverse array of bacteria and cyanobacteria. Increasing numbers of studies are targeting the nitrogenase gene in ecological studies of the factors controlling nitrogen fixation in the marine environment. These studies will help to understand nitrogen fixation in the marine environment in a new way, one based on linking the diazotroph diversity and the microbial population dynamics to the observed nitrogen fixation rates.

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NITROGEN FIXATION MEASUREMENT.

See NITROGEN FIXATION IN THE MARINE ENVIRONMENT

NITROGEN GASES.

See TRACE GASES SOIL

NITROGEN IN MARINE MICROORGANISMS.

See INORGANIC NUTRIENT USE BY MARINE MICROORGANISMS

NITROGEN REMOVAL IN ACTIVATED SLUDGE.

See ACTIVATED SLUDGE — MICROBIOLOGY OF NITROGEN REMOVAL

NORWALK-LIKE VIRUSES: DETECTION METHODOLOGIES AND ENVIRONMENTAL FATE

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Norwalk-like viruses (NLVs), the most common type of human caliciviruses, are important agents of acute nonbacterial gastroenteritis. NLVs are regularly implicated in food and waterborne outbreaks. The U.S. Environmental Protection Agency has listed caliciviruses on their contaminant candidate list for regulatory consideration in drinking water (1). The lack of a cell culture system has hindered investigation of the basic virology of NLVs and their fate in environmental systems. However, immunological and molecular methods have been developed for detection and characterization of NLVs. In this article, methodologies for detection (including application to environmental and epidemiological investigation) and the fate and persistence of NLVs in environmental systems will be reviewed.

The basic virology and epidemiology of NLVs is reviewed in a companion article (see HUMAN CALICIVIRUSES: BASIC VIROLOGY AND EPIDEMIOLOGY, this Encyclopedia). In short, NLVs are a genetically and antigenically diverse group of nonenveloped, RNA viruses. NLVs have a positive sense, single-stranded RNA genome, approximately 7.5 kB in length. Their protein capsid is composed of 90 archlike dimers of a single major capsid protein. Phylogenetic analysis of the genetic sequences of NLV strains supports their division into at least 12 specific genetic clusters within two distinct genogroups (2–6). As many as 14 distinct antigenic types of NLV have been suggested (7–9). Although several attempts have been made to separate the NLVs into antigenic types, there is currently no universally accepted scheme for antigenic typing of human caliciviruses. The genetic and antigenic diversity of NLVs poses a particular challenge to detection methodologies and the study of environmental occurrence, transport, survival, and fate.

ISOLATION AND RECOVERY

The basic framework for isolation, recovery, and detection of NLVs in any sample is: (*1*) primary concentration, (*2*) separation, (*3*) secondary concentration, (*4*) purification, and (*5*) detection. The techniques used at each step are sample type dependent. Indeed, processing of some sample types may not require one or more steps for effective processing, whereas other sample types may require multiple techniques at each step of the framework. This section will discuss specific aspects of sample collection, transport, and storage, followed by a discussion of general approaches to processing and preparation of different clinical and environmental samples before detection. Additionally, individual concentration, separation, and purification techniques at each step of the basic framework will be reviewed.

Sample Collection, Transport, and Storage

There are major differences between clinical and environmental samples with respect to isolation, recovery, and detection of NLVs. Positive clinical samples have relatively high NLV titers [up to 10^8 reverse transcription-polymerase chain reaction (RT-PCR) detectable units/g stool or vomitus]. Consequently, a clinical sample of as little as 100 mg of stool or vomitus can be sufficient for molecular detection of NLV. However, CDC guidelines recommend collection of sample volumes of at least

10 mL (10). Samples should be collected within the first 48 hours of clinical illness to maximize at possibility of NLV detection because this is often the peak time for virus excretion (11). However, samples collected within the first week of illness are likely to be positive for NLVs (12). Stool specimens should be stored at 4 °C, and shipped in specimen cups (or other secure container) on ice or cold pack to the laboratory performing the viral analysis. Specimen cups should be sent clearly labeled with waterproof label and placed in a waterproof bag with tissue or other blotting material to absorb any leakage during shipment (10). If possible, paired serum samples (acute and convalescent) should be obtained from people submitting stool specimens and also sent to the laboratory for performing analysis. Acute serum specimens can be obtained as long as five days after onset and convalescent serum can be collected as soon as 13 days after onset (13).

An early study of the storage of Feline Calicivirus (FCV) particles at −70 °C, −20 °C, and 4 °C, indicated that storage at 4 °C better preserved the integrity and infectivity of the viral particles over an eight week period (14). However, a later study examining stool specimens that were stored at −70 °C for 6 to 10 years indicated that long-term storage at ultracold temperatures does not pose a serious problem for microscopic identification of viruses (15). If samples are to be stored for very long periods and assayed by molecular methods, freezing at ultracold (−70 °C) temperatures may be preferable to prevent degradation of the viral nucleic acid.

In contrast to clinical samples, environmental samples typically have very low virus titers and may require large sample sizes for effective detection. Source water and treated drinking water may require 10 to 1,000 L per sample for detection. The U.S. Environmental Protection Agency (U.S.EPA) guidelines for enteric virus sample collection under the Information Collection Rule (ICR) called for 200 to 300 L of raw water and 1,200 to 1,500 L of finished water to be sampled (16). However, in field studies, NLVs have been detected in 1 to 40 L of water at contaminated sites (17–19). Research in the Netherlands suggests that as little as a few milliliters may be sufficient to detect NLVs in raw or primary treated sewage (20). The sample size of implicated food should be as large as possible, taking into consideration the limitations of processing and detection methods. Typical sample sizes are 10 to 25 g (with 4 to 10 mL of liquid/g food sample) (21). For sewage biosolids, sample sizes typically range from 0.5 to 1.0 L or about 50 g dry-weight solids (22,23). The sample-size range for enteric virus detection in soils and aquatic sediments is generally between 10 to 500 g (22).

Guidelines for water and sewage sample collection and transport in the U.S.EPA Manual of Methods for Virology state that samples must be placed on ice immediately after collection and should be shipped under conditions that maintain viability. All samples that cannot be processed within 24 hours are to be frozen at −70 °C, with subsequent freezing and thawing kept to a minimum (24).

Multistep Concentration, Separation, and Purification Methods

Several protocols for concentration and purification of NLVs from clinical and environmental samples have been reported and these are summarized in Figure 1 (18,25–33).

Foods. The general approach for separation and concentration of NLVs from various food items consists of homogenization, acid adsorption/elution, phase extraction, one or more polyethylene glycol (PEG) precipitations for concentration, and various purification steps (precipitate adsorption/elution, immunocapture, etc). This approach has been most extensively described for shellfish (26,30,31,34–36). A feature of this approach is the purification and concentration of intact virions from oyster extracts. Application of this method to 50 g quantities of artificially contaminated oysters resulted in an estimated detection limit of 10 to 100 NV particles (26,34). A minor modification of this method also was effective for simultaneous concentration and purification of additional enteric viruses [in particular hepatitis A virus (HAV)] from hard-shell clams that were assayed for both infectivity and RT-PCR (30). Modifications of the general protocol include addition of various RNA extraction methods for greater removal of RT-PCR inhibitors (31,35).

A second approach for separation and concentration of NLVs from food samples consists of an initial washing/elution step, PEG concentration, and RNA extraction. This approach attempts to minimize the corecovery of inhibitory compounds and the loss of virus by eliminating the homogenization and additional purification methods. Two methods using this approach were compared for the detection of seeded NLV and HAV on delicatessen foods. One method involved washing a 20 to 40 g sample of deli meat with phosphate buffered saline (PBS) several times, followed by organic extraction (fluorocarbon), PEG precipitation, and RNA extraction of the PEG pellet. The other method involved merely washing the deli meat twice with a guanidinium phenol–based reagent. Recovered RNA from both methods was assayed for seeded NV and HAV by RT-PCR. Both HAV and NV were detectable by either method, however, undiluted RNA extractions from first method were inhibitory to RT-PCR. Amplification was observed in tenfold and 100-fold dilutions of the extracts. For the simpler method, RT-PCR inhibition was observed for 62% of the samples at the tenfold dilution, but there was no inhibition at the 100-fold dilution. Inhibition was determined by failure to amplify an internal control (36,37).

Another direct RNA extraction method for separation and concentration of NLVs from food items was adapted from a protocol for recovery of rotavirus dsRNA in shellfish and other foods. The method involves repeated extraction of food samples with guanidinium isothiocyanate (GITC), followed by fluorocarbon extraction of pooled supernatants, viral RNA adsorption to and elution from hydroxyapatite or iodized glass powder, and RNA recovery from the eluate by cetyltrimethylammonium bromide (CTAB) (38). This method, or a slight modification, has been demonstrated to be effective for shellfish, fruit and vegetable coleslaw, milk and orange juice (38).

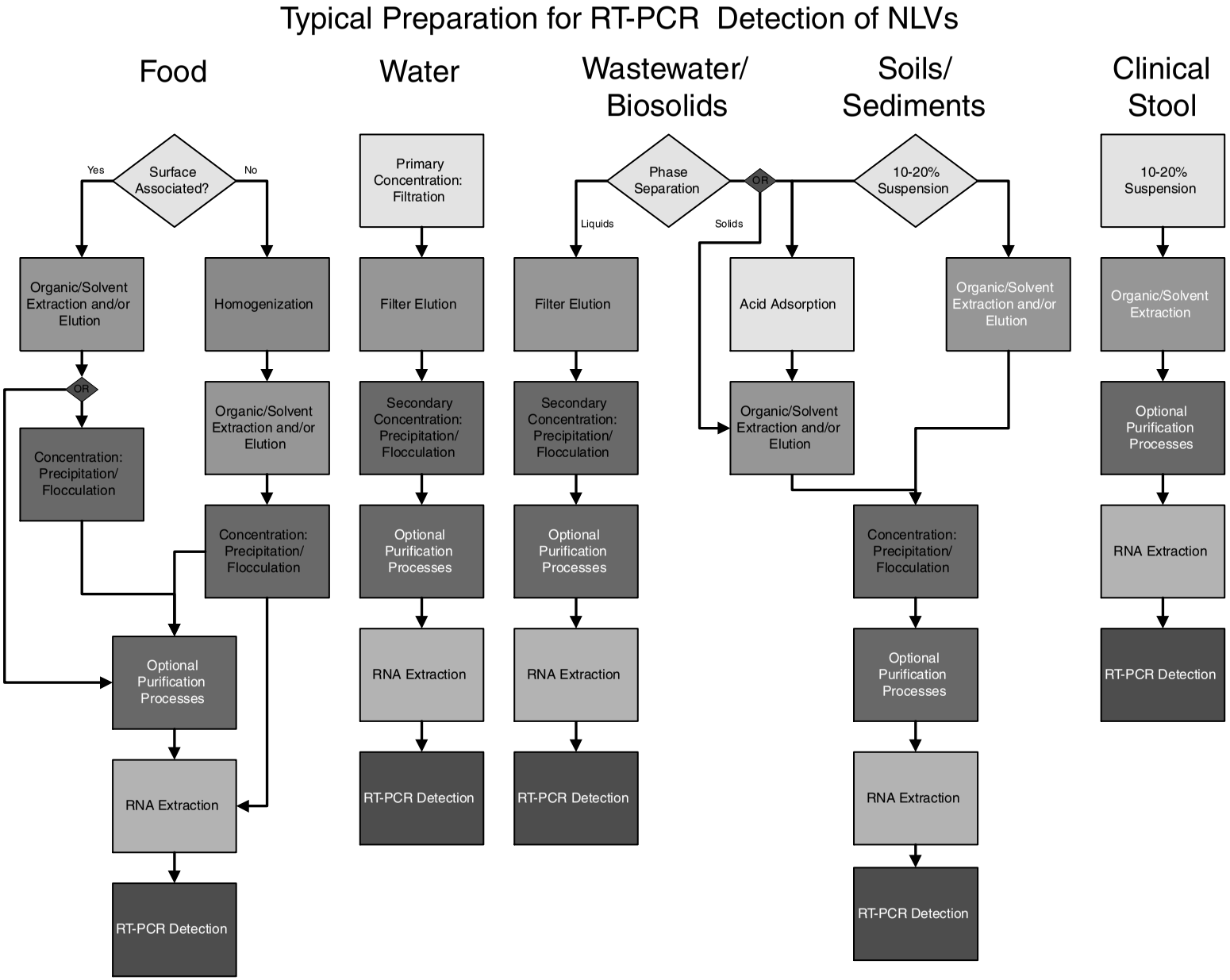

Figure 1. Typical preparation of various samples for RT-PCR detection of NLVs. Corresponding steps between samples types are indicated by the same colored polygon. See color insert.

Water. The general approach to processing of water samples involves a primary concentration step, usually by adsorption/elution to one of several types of filters. Primary concentration is generally followed by one or more secondary concentration steps using PEG or organic flocculation. One or more purification steps [immunomagnetic separation (IMS), organic extraction, RNA extraction, etc.] and, finally, detection by RT-PCR typically complete the protocol. Seeded virus studies, in which 10 L river water samples were filtered through glass wool columns, eluted, ultracentrifuged, and assayed by RT-PCR, demonstrated that NLVs could be concentrated from relatively large volumes of water (39).

A filter-elution method using 47 mm diameter flat filters (0.45 μm pore size) for NLV concentration from deionized water, raw water, and humic-rich bog water samples has been described (27). Samples were supplemented with $MgCl_2$ and pH adjusted to adsorb the viruses to the filter by hydrophobic and electrostatic forces. Filters were eluted with a urea arginine phosphate buffer, viruses in eluates were flocculated, the floc was centrifuged, and the pellets were RNA-extracted for RT-PCR detection. The method was able to detect low levels of NLV in 500 ml of deionized water, but sensitivity decreased as sample volume increased. In raw water, 0.5- to 5-seeded viral particles per milliliters could be detected. Number of viral particles seeded was estimated by comparison of end point RT-PCR with electron microscopy. Humic acid and metal ions prevented detection of NLV in the bog water (27).

A study using a culturable primate calicivirus (Pan-1) as a model virus for NLVs evaluated 1 MDS positively charged filters (Cuno, Inc., Meriden, Connectient) for concentration of seeded virus from 40 L water samples (40). Initially 200 mL volumes of deionized water, finished drinking water, groundwater, and surface water were each seeded with high concentrations of Pan-1, filtered through 1 MDS disk filters (Cuno, Inc., Meriden, Connectient), eluted with 3% beef extract (BE) at pH 9.0, PEG-precipitated, and then assayed by plaque assay. Recoveries ranged from 94% (deionized water) to 64% (surface water). Studies were repeated with 40-L volumes seeded with low levels of virus. Processed samples were assayed by plaque assay and RT-PCR. Recoveries in the larger volume experiments ranged from 38% (finished water) to 14% (surface water) by plaque assay. The detection limit by RT-PCR was estimated to be 0.75 to 1.5 PFU in 40 L (40). A similar method demonstrated recovery of NLVs from 1-L river water samples using positively charged Zetapore filters (0.45 μm nominal pore size) (18).

Negatively charged cellulose nitrate membranes have been used to evaluate 10-L tap water, river water, seawater, and sewage effluent samples for NLVs (17). Seeded studies demonstrated that the negatively charged filter/elution/flocculation protocol was capable of recovering NLVs from tap water and other waters. Subsequent detection of NLVs in analysis of unseeded water samples from sites with expected contamination validated this protocol. Briefly, water samples were filtered through a composite filter stack including a fiberglass prefilter and negatively charged membranes of decreasing pore diameter (5.0, 1.2, and 0.45 μm). Filters were eluted with 200 ml of 0.1% skimmed milk at pH 9.5. The eluates were then flocculated at pH 4.5, centrifuged, and the floc dissolved in 10 mL of neutral disodium hydrogen orthophosphate buffer. Similarly, 100 mL of raw sewage was directly eluted with 1% skimmed milk at pH 9.5 in a ratio of 10 : 4, centrifuged, supernatant-collected, flocculated, and floc-dissolved as described in the preceding text. Dissolved flocs were assayed by nested RT-PCR (17).

A seeded virus study described a volume reduction from 1 L to 20 to 50 μL in mock eluates (28). The protocol evaluated in this study precipitated mock 1-L BE-glycine eluates with a PEG precipitation (13% PEG, 0.2 M MNaCl), followed by chloroform extraction, followed by another PEG step and ultrafiltration (28).

NLV recovery from sewage has been described for 10-L samples by a conventional adsorption-elution method, followed by a modified two-phase polymer extraction using Dextran T40 and 10% PEG 6,000. After phases were separated, the bottom phase and interphase were additionally purified by Sephadex spin column chromatography and finally concentrated by centrifugal ultrafiltration (20).

Individual Techniques/Steps for Virus Recovery

Homogenization. For food items in which viral contamination is not surface-associated (e.g., shellfish, meat), an initial homogenization is used to disperse the sample, uniformly distribute the viruses, and thereby maximize recovery by subsequent processing steps. However, homogenization may release many compounds that may be inhibitory to detection methods. Researchers have found that processing only that portion of a sample most likely to be contaminated, for example, the stomach and digestive diverticula in shellfish, minimizes amount and release of RT-PCR inhibiting compounds. Processing of dissected stomachs and digestive diverticula rather than whole shellfish meat resulted in consistently better NLV detection limits by reducing RT-PCR inhibitory compounds (36,41). For food items and other samples in which viral contamination is surface-associated, homogenization may not be advisable. Instead, elution of viruses from the surface may be preferred. The most common method of homogenization is mechanical, by simply blending in an industrial blender approximately seven sample volumes of a diluent such as phosphate-buffered saline (PBS). However, a simple method has been described for extraction of viruses from shellfish by enzymatic (protease) homogenization (42).

Extraction. Organic solvent extraction is used for selectively causing the virus to preferentially distribute in the aqueous phase and cause impurities to enter an organic phase. This technique has been applied to both clinical and environmental samples for NLVs (26,28,34,43). The organic solvent, typically Freon (1,1,2-trichloro-1,2,2-triflouroethane) or chloroform, extracts lipids and other hydrophobic constituents from the sample, leaving the hydrophilic viruses in the aqueous phase. Environmental and health concerns have prompted evaluation of Freon substitutes of purification of NLV from

samples, including dichloromethane, 1 : 1 chloroform-butanol, Vertrel XF (1,1,1,2,2,3,4,5,5,-decaflouropentane), and a 7 : 3 mix of isopentane/1-chlorobutane.

Adsorption/Elution. In the case of food or sewage biosolids, adsorption/elution typically refers to the process of lowering the pH below the isoelectric point of the viruses such that the viruses will more efficiently adsorb to the negatively charged solids. The sample may then be centrifuged to pellet the solids and adsorbed viruses. The pellet can then be resuspended in a neutral eluant to desorb the viruses with subsequent removal of solids by centrifugation. In some cases, viruses are solids adsorbed initially, in which case, the adsorption step is skipped and viruses directly eluted from the sample. In a study comparing acid adsorption-elution (with glycine buffer at pH 7.5) to direct elution (with glycine buffer at pH 9.5) in shellfish samples, the former was shown to retain fewer inhibitors of RT-PCR than the latter (31).

In water samples, adsorption/elution refers to the process by which viruses are initially retained by and then recovered from filters. Typically filters for primary concentration in water have a nominal pore size that is 10 to 100 times the diameter of NLVs. Adsorption to filters is by both hydrophobic and electrostatic interactions (44).

Precipitation/Flocculation. PEG precipitation is an effective method for concentration of NLVs from volumes up to a few liters (28–30,45). Flocculation methods also have been described for concentration of NLVs (17,27,32). One study used PEG instead of organic flocculation for reconcentration of the viral particles in eluates. The authors cited a concern about coprecipitation of constituents in BE that would interfere with RT-PCR (40).

IMS/IC. Immunomagnetic separation (IMS), which is also known as *immunocapture* (IC), has been demonstrated as an effective secondary concentration step for NLVs in environmental samples. This technique removes many of the compounds inhibitory to RT-PCR (46,47). Rabbit polyclonal antibodies, raised against recombinant NLV antigens and attached to magnetic beads, have been used to separate NLVs from water concentrates (47). Efficiency of IMS concentration was reported to be volume dependent due to complications with collection of beads and possibility of antigen-antibody contact in larger volumes (47). The effectiveness of IMS is dependent on the quality of the antibodies, which in turn is influenced by the considerable antigenic diversity of NLVs. Unfortunately, the utility of IMS for NLVs has limited effectiveness because of the lack of broadly reactive antibodies. An exception to this is the use of pooled human serum IgG (HSIG) rather than mono- or polyclonal antibodies specific to NLV. This technique has the added benefit of broad immunocapture of enteric viruses; however, the sensitivity and recovery efficiency is typically less than virus-specific antibodies (29,30).

Indirect immunomagnetic capture (IC) coupled with guanidinium isothiocyanate (GITC)-RNA extraction was reported to be as effective in removing inhibitory substances from clam extracts as GITC alone. Recoveries with the combined method were lower than those with GITC alone, although more consistent. However, the difference in recoveries between GITC alone and IC/GITC was offset by the ability of IC/GITC to effectively assay ten times the volume of clam tissue as GITC alone (30). Immunomagnetic capture with virus-specific antibodies has also been evaluated for reduction of inhibition of RT-PCR in clinical stool specimens (46). The IC/RT-PCR method outperformed direct heat-release RT-PCR and antigen-detection enzyme-linked immunosorbent assay (ELISA) on a panel of 100 stools. Also, the IC step greatly reduced the number of stools in which RT-PCR was inhibited (46).

Spin Column Chromatography. Dextran gel (Sephadex) spin-column chromatography has been used for the removal of RT-PCR inhibitors in both clinical and environmental samples (27,28,43). However, this method has been reported to cause unacceptable losses of virus from the sample (28,48). Two factors, particle diameter and water-regain value, are important in determining the fractionation range of a Sephadex column. The water-regain value refers to the swelling capacity of the gel and is related to the fractionation properties of the gel. A low water-regain value corresponds to a low molecular-mass fractionation (49). The dry-particle diameter is important in determining the range of fractionation, especially for Sephadex with a higher water-regain value (28,49). Column to sample-volume ratios of 4 : 1 to 8 : 1 are reported to maximize removal of inhibitory substances and minimize virus loss (28).

Ultrafiltration. This method reduces sample volume by about 10- to 100-fold (e.g., from 0.5 to 5 mL to 20 to 100 μL) (18,28). Virus losses by adsorption to the ultrafilter are minimized by pretreatment of the filter. Samples must be relatively clean (low in particulates and high molecular weight macromolecules) before centrifugation to prevent clogging of the filter.

Gradient Centrifugation. Sucrose velocity gradient centrifugation and CsCl buoyant density gradient centrifugation have been described for purification of NLVs from stool specimens (50). However, these techniques typically are not recommended for samples containing low virus concentrations, such as food and water samples, because of the potential for virus losses by adsorption and other mechanisms.

RNA Extraction. Several methods have been described for extracting NLV RNA from the virion for subsequent analysis by RT-PCR. The four main techniques are: (*1*) heat release, (*2*) PEG/CTAB method (polyethylene glycol/cetyltrimethylammonium bromide), (*3*) GITC/silica, and (*4*) the single-step GITC method of Chomsczynski and Sacchi (43,51–53). Heat release of NLV RNA is accomplished as a first step in a RT-PCR reaction by heating the viral sample to 95 °C for five minutes (43). The benefits of heat release are simplicity, speed, and protection of viral RNA from degradation by RNAses. The primary drawback is its failure to remove RT-PCR inhibitors. A comparison of heat release and a conventional

RNA-extraction method on a panel of 45 stool specimens indicated that heat release had a similar sensitivity to the RNA extraction (54).

CTAB is a cationic detergent used to remove inhibitory factors in stool extracts during RNA extraction (53). The PEG/CTAB method has proven effective for processing of shellfish samples (36). Wolfaardt and coworkers found a modified PEG/CTAB method superior to the GITC/silica method on NLV-positive stool samples, and seeded samples of sewage, sludge, and tap water. However, on spiked river water concentrates, the effectiveness of these methods was comparable (39).

Single-step RNA isolation by GITC/phenol chloroform extraction has proven effective for isolation of calicivirus RNA in stools and from environmental samples (delicatessen meat) (37,55). The drawbacks of the method are related to RNA recovery by ethanol precipitation, which may cause the recovery of small quantities of viral RNA, as found in environmental samples, to be difficult.

The GITC/silica method improves recovery of RNA by adsorbing it to and eluting it from silica. Comparison of the PEG/CTAB and GITC/silica methods found PEG/CTAB to be 2- to 50-fold less sensitive than GITC/silica (56). A simple modification of the GITC/silica method incorporated the silica into a gel membrane within a spin column. This modified method has been shown to be simple and capable of removing RT-PCR inhibiting substances from environmental concentrates and clinical samples (18,31,35). In a comparison of single-step RNA extraction, RNA extraction incorporating Sephadex spin column chromatography, and silica gel–based extraction, the silica gel protocol was most efficient at removing RT-PCR inhibitors and yielding the most consistent viral RNA templates (31). Other modifications of the GITC/silica method include the use of a vacuum-manifold system, and binding the viral capsids to the silica in suspension, followed by centrifugation and resuspension in the GITC solution (17,42).

DETECTION METHODOLOGIES

Several methods have been described for the detection and diagnosis of NLVs, including microscopic, immunological, and molecular (nucleic acid) methods. Detection of NLVs in clinical samples is more reliable than detection in environmental samples for several reasons. First, in comparison to environmental samples, positive clinical samples have much higher virus titers. In addition, clinical symptoms of a patient may be indicative of NLV infection, which increases the possibility of a causal relationship between virus presence and a health effect. Detection of NLVs in the environment is made difficult by the very low levels, and perhaps sporadic presence of the viruses, and when investigating outbreaks, by the inevitable delay in initiating virus examination until after health effects have been observed such that implicated samples are no longer available.

Microscopic Methods

Because NLVs are much too small (~27 nm in diameter) to be visualized by light microscopic methods, electron microscopic (EM) techniques are required to visualize them. Electron microscopic detection and conclusive identification of NLVs is difficult because of the lack of a distinctive morphology, low virus titers (EM requires a minimum of 10^6 viral particles/mL and 10^8 preferred), and the need for relatively clean preparations for virion visualization (57). None of the microscopic methods described in this section are sensitive and specific enough to reliably detect NLVs in environmental samples, although they continue to be valuable and perhaps essential in clinical diagnostic virology and epidemiology.

Immune electron microscopy (IEM) take advantage of antigen–antibody interactions to aggregate virus particles, concentrate the immune aggregates, and facilitate virus identification (58). IEM also has been adapted for quantification of serological response to infection. Paired (acute and convalescent) sera from infected individuals are incubated with the virus particles, centrifuged, and the pellet resuspended and negatively stained. The antigen–antibody reaction typically is rated on a 1+ to 4+ scale, with a higher positive rating indicating a stronger reaction and higher antibody titer (58,59).

Solid phase immune electron microscopy (SPIEM) is a variation on IEM, in which the viral particles are immunocaptured on the EM grid. Grids are prepared by binding high-titer convalescent serum to a carbon-coated copper grid using antihuman IgG or protein A (60,61). SPIEM increases the sensitivity of diagnostic EM up to 100-fold (61). Other variations to EM include the use of electron dense markers, such as ferritin or colloidal gold attached to the antibodies to improve locating and visualizing viruses (62,63).

Norwalk virus, the prototype genogroup I NLV, was originally observed using IEM on stool material from a volunteer infected with a bacterial free stool filtrate derived from an outbreak in Norwalk, Ohio. When stool material from the infected volunteer was incubated with the volunteer's convalescent sera, centrifuged, negatively stained, and visualized by EM, and the pellet was resuspended, "fuzzy," 27-nm diameter, antibody-coated virus particles were observed (59). The isolated virus particles were used to determine serological response of other experimentally infected volunteers and individuals naturally infected during the outbreak. The serological response of these individuals to the virus particles suggested that the virus particles were the etiologic agent responsible for the outbreak. Other NLVs have since been identified using the same approach, including the Hawaii virus (HV), Montgomery County virus (MCV), and Snow Mountain virus (SMV) (64,65). IEM remains the basic clinical diagnostic examination method for NLVs and other diarrheal viruses. The continued value of EM for NLV clinical diagnosis and epidemiology was demonstrated by a recent report in which specimens from two outbreaks were positive for NLVs by EM, but negative by RT-PCR, although EM had the lower overall detection rate (66).

IEM has also been used to demonstrate antigenic relatedness among NLVs and to investigate outbreaks. Montgomery County virus was found to be related to NV, Hawaii virus was antigenically distinct from NV, and Snow Mountain virus was found to be distinct from

both HV and NV (64,65). Studies in the United Kingdom have described a SPIEM method using whole convalescent sera and purified IgM that distinguishes at least six NLV antigenic types. These studies also have demonstrated specific IgM responses to these antigenic types in infected individuals, although there was some cross-reactivity between certain serum pairs and at least three antigenic types (67–69). Separate studies in Japan have described as many as nine antigenic types of NLVs by cross-reactive IEM (70).

The benefits and advantages of EM techniques for detection of NLVs are rapid diagnosis and broad detection (61). The time for completion of EM detection techniques ranges from 15 minutes for direct EM to a few (two to four) hours for IEM techniques. Other techniques for NLV detection based on antigenic and nucleic acid methods may also be rapid, but they may not have the breadth of detection that EM techniques have. EM visualization also detects unexpected or novel viruses that may be missed by specific molecular or antigenic methods. The major disadvantage of EM techniques for detection of NLVs is the requirement of a highly purified and/or high-titer sample. Another disadvantage of EM techniques is the lack of readily available sera that are reactive to all antigenic types of NLV. In addition, EM requires a highly skilled technician and expensive equipment. For these reasons, EM techniques have little utility for detection of NLVs in environmental samples and are better suited for diagnosis of clinical illnesses and epidemiological investigations.

Immunologic Methods

As for the EM methods, immunologic methods lack the sensitivity to reliably detect low levels of NLVs in environmental samples, but they are very useful for clinical diagnosis of NLV infections and for epidemiological investigations. Early immunologic techniques for NLVs were severely limited by the lack of availability and poor quality of reagents. However, the production of recombinant, viruslike particles (rVLPs) generated from the baculovirus expression of NLV capsid genes has allowed an unlimited supply of reagents, both as antigen and for the production of antisera/antibodies (71). At least seven NLV capsid genes have been cloned into baculovirus vectors (71–79). The baculovirus-expressed NLV capsid gene protein self-assembles into VLPs with a yield of up to 12.5 mg of protein per 100 mL of culture medium. VLPs are purified typically by rate-zonal centrifugation in a sucrose gradient and perhaps further purified by CsCl density-gradient centrifugation (80). Recombinant NLV capsid protein expression has also been described in *Escherichia coli* and mammalian cell lines (81,82). So far, no intact VLPs have been generated using *E. coli* or mammalian expression of NLV capsid proteins, but *E. coli* expressed proteins have been used to produce anti-NV monoclonal antibodies (81,82).

RIA/BAI. Solid-phase microtiter radioimmunoassays (RIAs) have been described for both NV and SMV antigens in crude stool and for their antibodies in sera (83,84). They were used in early epidemiological studies of seroprevalence, seroconversion in volunteers, and outbreak investigations (85–87). Studies have reported RIA to be either 10 to 80 times more sensitive than, or at least as sensitive as, IEM, and more sensitive than an immune-adherence assay (84,88). Still, the sensitivity of RIA is not as great as newer EIA/ELISA techniques, and it has the additional drawbacks of requiring six days to complete and the use of radioisotopes (89).

A simplified technique, called *biotin-avidin immunoassay* (BAI), was developed that has the following advantages over the RIA: (*1*) added sensitivity in detecting Norwalk antibody, (*2*) less time consuming, (*3*) not requiring frequent labeling of antibody, and (*4*) no use of radioisotopes (90). BAI detects NLV infections by a fourfold titer rise in serum antibody titer or by antigen in stool, with a sensitivity similar to or greater than that of the RIA, and BAI appears to be more sensitive than the RIA for detecting antibody in single serum specimens.

EIA/ELISA. RIA and BAI techniques have been succeeded by more sensitive enzyme immunoassays, particularly with recent advances in production of immune reagents. Antigen and antibody detection enzyme immunoassays (EIAs) have been described for several NLVs (12,71,73,81,91–98). Early EIA/ELISA relied on human sera and viral antigen from human stool specimens (95,98). Current antigen and antibody detection EIAs employ rVLPs for the generation of hyperimmune antibodies in laboratory animals and as a source of antigen (99–102).

A typical antigen-detection protocol involves sandwiching the target antigen, generally from purified stool, between antibodies to the target antigen generated from two species of animal (one of which is used to coat the plate and the other labeled for detection) (80). Assays using monoclonal antibodies for NLV rather than polyclonal hyperimmune sera have also been described (96). Epitope mapping of NLVs using monoclonal antibodies against rVLPs have led to the production of unlimited quantities of reagents that recognize epitopes on regions of the capsid protein that are highly conserved among different NLV capsid proteins (103). Analysis of a panel of monoclonal antibodies to genogroup I NLV has identified a common epitope not found in genogroup II NLVs (104).

Antibody-detection EIAs that are used to examine serum specimens are very sensitive and more broadly reactive than antigen-detection EIAs. Initial protocols used recombinant NLV capsid proteins to coat wells of a microtiter plate to measure total NLV immunoglobulin in human sera. Later modifications of the protocol allowed detection of individual isotypes of immunoglobulin. Typically, isotype-specific, antihuman antibodies are used to coat the microtiter plate, thus selecting a specific isotype in human sera for reaction with recombinant capsid antigens (92,93). Monoclonal, antibody ELISAs for IgM have been described for genogroup-specific NLV detection (99,105). Detection of IgM-class antibody has proved useful for diagnosis of recent infection, especially complementary, paired sera immunoassays when sera and stool specimens have not been collected early in an outbreak (99,106).

Antigen-detection EIA was demonstrated to be nearly as sensitive as RT-PCR and dot blot hybridization for virus detection in the stools of volunteers infected with homologous virus, but one study reported unexpectedly a low detection in field specimens (80). An EIA, based on the capsid of Lordsdale virus (a genogroup II NLV), compared favorably with IEM and RT-PCR, with detections of 71%, 63%, and 84% of 213 outbreaks, respectively (107). However, the high specificity of antigen-detection EIA also limits its application because of the antigenic diversity of the NLVs. The more broadly reactive antibody-detection EIAs have important application in epidemiological investigation, in particular for determining the seroprevalence, geographic, and temporal distributions of different groups of NLV (80).

Molecular Methods

RT-PCR. The first RT-PCR assays were described for NLVs within two years of cloning of the NV genome (43,53). Early RT-PCR assays were very specific to NV and proved to be very sensitive (53). RT-PCR of serial tenfold dilutions of NV demonstrated that RT-PCR sensitivity was similar to the actual virion titer by EM and at least 100 times as sensitive as RIA (43). Examination of outbreak stool samples by IEM, ELISA, and RT-PCR suggest that RT-PCR is more sensitive than IEM or ELISA (39). A Norwegian study found RT-PCR to be more sensitive in detection of NLV in stool specimens than direct EM (55). Numerous protocols have been described for RT-PCR amplification of NLV RNA, and no single standardized method is currently accepted. Early application of RT-PCR to specimens from a series of outbreaks indicated that efficiency of detection was affected by the sequence diversity of the NLVs (108,109).

RT-PCR assays targeting the NLV capsid of the genome have been described (110,111). However, the majority of assays have targeted the more conserved polymerase region (43,53,112–119). Several approaches have been described for broad RT-PCR detection of NLVs. A multiplex approach to broad detection has been described using a mixture of several primers (120). Another approach has been the selection of primers from a highly conserved region of the NLV genome (113,114). A lowered annealing temperature has been incorporated into this approach to increase overall detection by allowing mismatching of base pairs (113). Another approach is the use of primers with equimolar degeneracies to facilitate less-specific template annealing (115–117). The incorporation of inosine into the primers to allow mismatches at specific locations has also been described (110,118,121). None of these approaches has consistently detected all NLV strains. Experimental comparison of several primer sets found at least three primer pairs, G1 set, G2 set, and Sapp35/Sapp36, which were required to detect viruses in the NV and SV clades (119).

Both random and specific priming have been described for reverse transcription (RT) of NLVs. A recent study found no significant difference in sensitivity or specificity when using random priming and specific priming during the RT step (122). Other studies have reported slightly higher sensitivities when using specific priming (53).

Several groups have examined different RT and polymerase enzymes for NLV RT-PCR. The most commonly used RT enzymes are AMV and MULV, originating from avian myeloblastosis virus and murine leukemia virus, respectively (43,53). Additionally, modified versions of MULV that are rendered RNAse H^- and more thermostable by a point mutation have proved effective for RT of longer regions of viral RNA (123). Bidirectional enzymes for RT and PCR steps also have been evaluated (41,124). Willcocks and coworkers compared the amplification efficiency of the combination of AMV reverse transcriptase and taq polymerase with a bidirectional polymerase, Tet-z, and found that the two enzyme system give more efficient detection than the Tet-z (124). A traditional two-enzyme protocol and a single-enzyme protocol using rTth were found to have comparable sensitivity for NLV detection (41). The benefit of a bidirectional enzyme is that it allows a higher-temperature RT than possible with normal RT enzymes, thus maximizing specificity of reverse-primer annealing and minimizing failure of annealing because of secondary structures. Cocktails of polymerases have also been evaluated, generally to maintain the activity of the Taq, and add a proofreading component (3′ to 5′ exonuclease activity that discriminates between correctly and incorrectly paired bases at the 3′-OH end of an extending strand), as with Pwo (123). The use of Tfl in a one-step system in concert with AMV has also been described for the amplification of NLV RNA transcripts (42). Additives, such as Triton X, bovine serum albumin (BSA), 2-mercaptoethanol, dithiothreitol (DTT), and dimethylsulfoxide (DMSO), have been incorporated into several RT-PCR assays to enhance the performance (18,112,114).

A multiplex RT-PCR method has been developed for the simultaneous detection of the human enteroviruses, HAV and NV. Three different sets of primers were used to produce three size-specific amplicons of 435 bp, 270 bp, and 192 bp for PV1, NV, and HAV, respectively. When tested on mixed, purified virus suspensions, the multiplex method was able to detect all three viruses at levels of detection similar to monoplex RT-PCR methods (125).

Current methods for the detection of nucleic acid from enteric viruses in environmental samples usually involve extensive concentration and purification of target viruses before detection by RT-PCR. Environmental samples may contain certain compounds (such as organic acids, proteins, and polysaccharides) that may inhibit or completely shutdown RT-PCR. Many of these compounds will coconcentrate with the isolated viruses or nucleic acids. Several methods have been described to overcome or eliminate inhibitory compounds including spin-column chromatography, modified nucleic acid extraction, and immunomagnetic separation. However, none of these methods completely and efficiently remove inhibitory substances without reducing viral titer. Some of the previously mentioned additives have also been incorporated into RT-PCR assays to minimize inhibition and enhance amplification (18,112,114).

Recent studies have described the incorporation of internal control template RNAs into the RT-PCR to indicate whether the samples contain inhibitory substances (36,40, 54,126). Failure of the internal standard

control to be amplified in samples confirms the presence of RT-PCR inhibitors (36). The internal controls are typically constructed as a deletion or insertion of the target RNA and are similar in size to the target RNA, such that it will not significantly affect the amplification. One study developed an internal RT-PCR standard allowing differentiation of the internal standard amplicon from wild-type NLV amplicons during southern hybridization and by size difference on a gel (54).

Highly sensitive, seminested RT-PCR systems for the specific detection of genotype I and II NLVs have been described (18,38,122,127–131). The estimated sensitivity of a nested RT-PCR method for detection of genogroup 1 NLV is less than 10^2 to 10^3 particles per milliliter of feces (27).

Typical RT-PCR protocols for NLVs amplify targets of a few hundred base pairs. However, a protocol has been described to amplify 3-kb products from 24 NLV strains previously characterized into four genetic groups, and overcome stable secondary structures between the RNA polymerase gene and the 5′ end of the second open-reading frame that made long amplifications difficult (123).

The use of thermolabile uracil N-glycosylase (HK-UNG) (Epicentre Technologies, Madison, WI) has been described for the prevention of carryover contamination in NLV RT-PCR. Efficacy of carryover prevention was validated by seeding NV amplicons containing dUTP (by replacing dTTP during amplification) into subsequent RT-PCR reactions (41).

Confirmation/Sequence Analysis. It is crucial that presumptive, positive RT-PCR results from agarose gel electrophoresis be confirmed by another method. RT-PCR analysis on environmental samples often results in the generation of multiple nonspecific product bands (37,124,132). Although one study suggests that these additional bands may be completely removed by DNAse treatment just before cDNA synthesis, presumptively positive amplicons still must be verified (122).

DNA sequencing and phylogenetic analysis is still the gold standard for confirmation of NLV PCR amplicons (108,133–137). Because it is expensive and time consuming, alternatives to DNA sequencing have been described for confirmation and analysis of NLV amplicons. Restriction fragment length polymorphism (RFLP) has been used to confirm NLV-positive RT-PCR results and to establish that all isolates from an outbreak were identical (18,38,138). A heteroduplex mobility assay (HMA) has been described as a presequencing screen for characterization of PCR amplicons among the commonly circulating NLV strains. Amplicons are mixed with and annealed to similar amplicons from reference strains and those of less than 90% sequence identity form visible heteroduplexes, allowing the strains to be categorized (139).

Oligoprobe hybridization is a convenient and sensitive way to confirm RT-PCR positives (37). It further offers the advantage of an increased sensitivity in addition to confirmation of RT-PCR product. Oligoprobe hybridization has detected positive RT-PCR samples that were not visible by ethidium bromide staining of an agarose gel (132,140). Additionally, probing may detect a product when a smear appears on the gel and sequencing is not possible.

The most common format described for oligoprobing is southern hybridization (43,112). The benefit of this method is discerning positives of the appropriate amplicon size. Its main drawbacks are the length of time required and the genetic diversity. Overcoming the genetic diversity is attempted using diverse sets of probes either in a cocktail, in parallel, or in sequence (112,116,117,136). Minimizing the length of time has been attempted by skipping the agarose gel step. Reverse line blot, a variation of a dot-blot hybridization, allows simultaneous confirmation of RT-PCR and genotyping of NLVs using a large set of probes in an array format (141). A liquid-format oligoprobe hybridization protocol using microplate hybridization provides a rapid confirmation of PCR products than does agarose gel electrophoresis (136).

Hybridization Assay. A dot blot hybridization assay has been described using ^{32}P labeled cDNAs to directly probe for NV RNA. However, this assay never gained favor because it was described the same time as the first RT-PCR assays and was reported to be about 100 times less sensitive (53).

APPLICATION OF METHODS

Outbreak Investigation

Newer methods have allowed sensitive detection of NLVs in clinical and sometimes environmental samples and have improved the ability to track specific NLV strains or antigenic types during the course of an outbreak or series of outbreaks. For example, an investigation of a large, multistate outbreak linked to contaminated oysters used molecular epidemiology to determine the dominant outbreak strain and a cocirculating strain (120).

Monitoring. Although methods now exist for the detection of NLVs in environmental samples, application of these methods to routine monitoring is still impractical in most situations. The available methods of NLV concentration, purification, and detection are not well standardized. Considering the range of environmental samples and conditions, and the numerous strains of NLVs, the accuracy and precision of NLV concentration, purification, and detection techniques have not been adequately established for routine application to field studies. Current methods are also expensive, with costs approaching $1,000 per sample for water, and they require experienced, highly trained personnel working in specialized laboratories. The widespread and diffuse nature of NLV contamination of environmental vehicles, such as water and food, suggests that the number of samples for effective monitoring within a surveillance framework would have to be numerous. Additionally, the time required to complete analysis of a single sample may be several days for confirmation of results, even with rapid molecular detection techniques. Further, none of the current molecular techniques can adequately determine whether a detected viral particle is infectious.

Recent advances in microarray technology, real-time RT-PCR, increased availability of NLV immunoreagents, an expanding database of NLV sequence data, and the development of ligand binding assays employing the cellular receptors for virus infectivity may eventually lead to reliable and definitive methods suitable for routine monitoring of NLVs in environmental samples.

ENVIRONMENTAL OCCURRENCE, FATE, AND PERSISTENCE

NLVs are excreted in the feces of infected individuals, and have been isolated from a wide variety of fecally contaminated samples. As evidenced by point-source outbreaks, NLVs may persist for long periods in the environment. This section discusses the environmental occurrence, fate, and persistence of NLVs.

Early Studies (Heat, Ether, Acid Sensitivity)

Studies in human volunteers indicate that NLVs are relatively heat-stable, ether-resistant, and acid-stable (8). Volunteer-feeding studies demonstrated that NV remained infectious after three hours at pH 2.7 at room temperature, after treatment with 20% ether at 4 °C for 18 hours, or after heating to 60 °C for half an hour (8).

Water. Studies with seeded virus in sea, river, and tap water have demonstrated the ability of available methods to recover and detect NLVs (17). NLVs have been detected in a range of water types including sewage-effluent, receiving water (fresh and seawater), seawater samples from swimming beaches, untreated well water, and occasionally municipal tap water (17,19).

RT-PCR analysis for NLVs on six 1-L samples from various sites on the River Aare near Berne, Switzerland, identified genogroup I NLV in every sample and genogroup II in two samples (18). In another study, NLVs were presumptively detected in three of six seawater samples tested and river water from downstream of a wastewater treatment plant (17). However, detection in two of the seawater samples was not confirmed by the nested reaction. It is not clear whether this was caused by false positive reactions in the initial reaction or lack of primer specificity for the nested reaction (17).

Investigation of a municipal water supply linked to an ongoing outbreak resulted in detection of genogroup II NLVs in untreated source water, finished water, and four tap water samples from various points in the distribution system. Additionally, both genogroup I and II NLVs were identified in the municipal sewage and genogroup II in the sewage effluent (19). Additional investigation linked the genogroup II NLV to an outbreak four months earlier in a city 70 km upstream. The time calculated for sewage effluent from the upstream outbreak to reach the downstream water supply and cause the described outbreak corresponded to the four-month period separating the outbreaks. During this period lakes and rivers were covered with ice, which may have increased the persistence of the viruses in the water (19).

A waterborne outbreak in Alaska and the Yukon Territory was associated with a well used as a restaurant's water supply, which had been contaminated by connection with a septic pit. NLV genetic sequences isolated from the well and from stool specimens of infected individuals were identical (142).

A survey of the presence of NLV in 63 types mineral water of 29 different brands found NLV sequences in 21 types mineral water by seminested RT-PCR. Representatives of both NLV genogroups I and II were randomly found in the contaminated samples, and NLV presence did not correlate with bottle characteristics or chemical properties (143).

Epidemiological evidence often implicates a water source, even when NLVs cannot be identified in the water source. For example, an outbreak of NLV was linked to a community water system in which surface runoff from heavy rainfall may have contaminated a spring, which served as the water source for the community system (144). A 1995 waterborne outbreak of NLV at a Wisconsin high school was linked to contamination of the drinking water supply by a back siphonage that occurred through submerged hoses on a flooded football field (145).

A large NLV epidemic in a Finnish municipality (1,500 to 3,000 people, i.e., 25 to 50% of the population) was most probably associated with contaminated drinking water. Although multiple pathogens were identified, Norwalk virus was the main cause of the outbreak. The source of contamination was a groundwater well situated in the embankment of a river. The well was contaminated by polluted river water during the spring flood by a back flow from the river through a forgotten drainage pipe (146). Waterborne outbreaks of NLVs in 1979, 1987, and 1996 were associated with recreational use of fecally contaminated lake water (145,147,148).

A Canadian epidemiological study on drinking water implicated conventionally treated tap water, meeting water quality requirements, in sporadic gastrointestinal illness (GI). Using a specific EIA, NV was evaluated as a possible agent responsible for the 35% excess of GI symptoms in tap water drinkers versus reverse osmosis treated water drinkers. It was concluded that NV was not responsible for the excess 35% (149). However, only the Norwalk antigen, and not other NLVs, was used to evaluate the sera. Because NV has been relatively uncommon compared with other NLVs and much of the observed GI illness was consistent with NLVs, it is possible that NLVs other than Norwalk were at least partly responsible for the 35% excess in illness attributed to tap water.

Wastewater

NLVs have been detected in raw sewage (17,20). In a seeded study, NLV was detected in raw sewage at low levels. Three volumes of a NLV-positive stool extract (200 μL, 20 μL, and 2 μL) were added to 20 mL aliquots of raw sewage. NLVs were detectable in the seeded sewage for all three seed volumes. Interestingly, analysis of 100 mL of unseeded raw sewage by the same method did not detect NLV, although NLVs were detectable in 10 L of unseeded final sewage effluent by seminested RT-PCR (17). Under outbreak conditions, 10 L sewage samples from three cities with ongoing NLV outbreaks

were positive for NLV. RT-PCR analysis of serial tenfold dilutions of extracted viral RNA from each of the sewage samples indicated that NLVs were present in high concentration (positives observed at up to 10^{-7} dilution). Follow-up analysis on additional sewage samples from one of the cities detected NLV in 10 of 11 samples over a period of three months. NLVs detected at two of the sites were closely related, whereas the virus detected at the third site was another genotype. The outbreak strains of NLV corresponded to those detected in the sewage at each site. Several additional strains of NLV (from both genogroup I and II) were isolated from follow-up samples (20).

Soils

Laboratory studies using seeded NV in soil and groundwater determined that NV was strongly adsorbed to clay soils (>4 $\log_{10}$ adsorption), moderately adsorbed to organic soils (1 $\log_{10}$ adsorption), and poorly adsorbed to sandy soils (<1 $\log_{10}$ adsorption). These studies, using poliovirus type 1 (PV1) and coliphage MS2 as benchmarks, determined that NV was less strongly adsorbed than PV1 (a strongly adsorbing virus) and equally or more adsorbed than MS2 (a poorly adsorbing virus). Additionally, these studies examined the persistence of NV, PV1, and MS2 over a six-month period in soil and groundwater. PV1 was the most persistent of the three viruses. NV was found to be similarly or less persistent than MS2 (150). Other studies on soil removal of NV in treated wastewater demonstrated similar results (151). Another study investigated the filtration and surface charge of NV in quartz sand, using recombinant VLPs. The surface charge of rNV particles and their filtration through quartz sand were demonstrated to be strongly influenced by pore water pH, suggesting that filtration of NV is dependent on electrostatic interactions between the virus and the quartz media (152).

Inactivation/Disinfection Studies

Early chlorine disinfection studies indicated that NV was more resistant to chlorine than poliovirus, rotavirus, or bacteriophage f2 (153). A study using seroconversion of volunteers to assess the disinfection efficacy of free chlorine on NV indicated that doses up to 10 mg/L were necessary to inactivate the virus in water (153). However, the NV preparation used in that study had a very high infectivity titer, exerted appreciable free chlorine demand, and caused free chlorine residual to disappear completely at the lower doses tested (3.75 mg/L). Parallel studies with bacteriophage f2 also indicated virus survival under similar experimental conditions. More recent studies using purified NV and sensitive RT-PCR detection suggest that NV is not more resistant than poliovirus (PV1) to free chlorine and a variety of other disinfectants (154,155). NV was found to be more susceptible than PV1 and coliphage MS2 to monochloramine (154). Free chlorine (1 mg/L), chlorine dioxide (1 mg/L), and ozone (0.37 mg/L) were shown to be as effective or more so on NV than PV1 and MS2 (155).

No cell culture or animal infectivity assay exists for human NLVs. However, certain animal caliciviruses may be grown in cell culture. Feline calicivirus (FCV) has been used as a surrogate for NLVs to study inactivation under environmental conditions (156,157). In one study, FCV was used to estimate the heat inactivation of NLVs in cockles and was found to be more sensitive to heat than hepatitis A virus (HAV) (157). When cockles were heated to an internal temperature of approximately 78 °C (by immersion in boiling water), infectious FCV was recovered after immersion for 30 seconds but not after 1 minute, and RT-PCR detection was positive at 1 min 30 seconds (157). In a study examining the inactivation of FCV by commonly employed disinfectants, such as gluteraldehyde (0.5%), hypochlorite (5,000 ppm), iodine (0.8%), gave 5 $\log_{10}$ reduction at one minute of contact time (156). A quaternary ammonium compound (1 : 10), 75% ethanol, 1% anionic detergent, and lower levels of hypochlorite gave no or only low levels of inactivation after one minute of contact time, ranging from 0 to 2.75 $\log_{10}$. Studies on heat inactivation of FCV in suspension demonstrated no titer reduction at 56 °C for one or three minutes, 3 $\log_{10}$ and 6 $\log_{10}$ at 70 °C for one or three minutes, and complete inactivation after boiling for one minute (156). FCV survived either dried or in suspension at 4 °C, with only a 4 to 5 $\log_{10}$ reduction in titer after about 60 days. At room temperature, 9 $\log_{10}$ reduction was observed in suspension between two and three weeks and in a dried state between three and four weeks. At 37 °C, infectivity reduction in suspension was approximately 1 $\log_{10}$ per day, but in a dried state there was complete inactivation within one day (156).

It should be noted that the appropriateness of FCV as a surrogate for NLVs in inactivation studies is questionable because NLVs are enteric pathogens and FCV is a respiratory pathogen. Although they do have similar capsid structures, the obvious differences in the route of infection calls into question the similarities of their environmental stability. Analogously, enteroviruses, which are enteric pathogens, and rhinoviruses, which are respiratory viruses, are both members of the picornaviridae, but they have very different stabilities under a variety of conditions.

Shellfish

Outbreaks of gastrointestinal illness associated with NLVs in shellfish are well-documented, and the risk of NLV illness has been shown to increase with the amount of contaminated seafood eaten (111,130,158–171). Steaming or cooking oysters does not appear to prevent NLV illness (164,171).

Outbreaks have occurred even when bacterial indicators of fecal contamination were within acceptable levels. The importance of adequate sanitation practices to protect shellfish and their harvest water was highlighted by a Florida outbreak of oyster-borne NLV gastroenteritis linked to overboard dumping of feces from harvest boats (164). Occurrence of NLV in shellfish has been shown to correlate to bacteriophage indicator levels, but not fecal coliform levels (172,173). In 48-hour oyster depuration experiments, 95% of bacteria were removed, but only 7% of NV titers were removed from the digestive diverticulum tissues (174). Occurrence of enteric virus

contamination, including NLVs, in shellfish harvesting areas has been shown to correlate with the incidence of epidemics of gastroenteritis in coastal population consuming bivalve mollusks (135,175). Additionally, heavy rainfall may be a contributing factor in the level of viral contamination (176).

A seeded study on the uptake, localization, and removal of NV in shellfish (oyster and clam) tissues found NV in the digestive diverticulum and stomach for all concentrations of seeded virus (174). At higher levels of seeded virus, NV was also detected in adductor muscle and hemolymph tissues. Another study reported marked differences of NLV detection in different seafoods. NLV detection in mussels was 10 to 100 times more sensitive than in shrimps and oysters (35).

NLVs have been identified in clam and oyster specimens by RIA (165). Using advanced molecular methods, NLVs have been detected in shellfish linked to outbreaks and shellfish from high contamination areas and commercial harvest areas (31,131,140,163). Analysis of sequence data from viruses detected in outbreak-associated shellfish have shown them to be the same or very similar to viral sequences found in outbreak stools (31,140,163). Some shellfish samples have been shown to contain a mixture of NLV strains (up to three different strains) with both genogroups I and II represented (135). Multiple genotypes of NLV in a single-oyster specimen or stool specimen also has been reported by others (130).

Other Foods

A seeded virus study demonstrated that NLVs could be recovered from fruits and vegetables in addition to clams and oysters. The study showed effective recovery and detection by nested RT-PCR of NLVs in milk, orange juice, coleslaw, fresh-cut melon and lettuce (38). Green salad has often been linked to NLV outbreaks (177,178). A simple method has been reported in seeded studies for consistent recovery and detection of NLVs from deli meat. Application of this method to an outbreak implicating deli meat from a university cafeteria allowed detection of a genogroup II NLV on sliced ham (37).

Other Exposure Vehicles and Settings

NLV outbreaks result in a high level of secondary cases as a result of low infectious dose, environmental persistence, and transmission by person-to-person contact. Direct person-to-person transmission, aerosol transmission, and fomite transmission of NLVs are a high risk in situations of close contact, such as on shipboard, in schools, or in nursing facilities (179–183). Under such conditions, outbreaks may persist or repeat (179,184). Investigators documented person-to-person contact as the means of transmission between members of two college football teams on the playing field (185). One team suffered a food-borne outbreak epidemiologically associated with turkey sandwiches from box lunches. That team passed the NLVs to the other team during the game. Other than contact on the field, there was no contact between the players on the two teams. NLVs, with identical genetic sequences, were detected in the stools of players from both the teams (185).

NLVs have been detected in RT-PCR analysis of environmental swabs collected during a hospital outbreak, with 11 of 36 swabs positive. Positive swabs were confined to the immediate environment of symptomatic patients, but were from items as diverse as lockers, curtains, and commodes. In the ninth week of an outbreak in a United Kingdom hotel, 144 environmental swabs were taken from around the hotel and tested for NLV by nested RT-PCR. Directly contaminated carpets had the highest rate of positive samples. NLVs were also detected from swabs of sites above 1.5 m, which are unlikely to have been contaminated directly, but could have been contaminated by NLV-containing aerosols. Retesting of the same sites, five months after the outbreak ended, yielded no positive RT-PCR results (186).

CONCLUSION

NLVs are environmentally persistent and are infectious at low doses. Worldwide, NLVs are an important cause of viral gastroenteritis. Outbreaks are explosive and are frequently linked to common sources of food or water. A wide variety of contaminated food items and water sources have been documented as the source of NLV outbreaks. Microscopic and immunologic methods have been described for the detection of NLVs and NLV antibodies in clinical samples. However, these methods are not sensitive enough for reliable detection of NLVs in environmental samples. Current molecular methods targeting viral nucleic acids, with RT-PCR and oligoprobe hybridization being most widely used, offer sensitive detection of NLVs in both clinical and environmental samples. However, these methods are frequently affected by inhibitory compounds associated with the samples that concentrate from environmental samples along with the viruses and their nucleic acids. Therefore, the most robust recovery and detection methods include processing steps to separate the viruses or their nucleic acids from these inhibitory materials before nucleic acid amplification

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NORWALK-LIKE VIRUSES (NLVS).

See HUMAN CALICIVIRUSES: BASIC VIROLOGY AND EPIDEMIOLOGY

NOSOCOMIAL INFECTIONS

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Nosocomial infections (NIs) have been recognized for more than a century as a critical problem adversely affecting patients' outcomes and quality of health care (1). Approximately 2.5 million NIs occur annually in the United States, meaning that approximately 5% of hospitalized patients will develop a documented infection acquired from the hospital (2,3). The rates may be even greater in intensive care units (ICUs). Even though they account for only 5 to 10% of hospital beds, the prevalence of NIs in ICU patients represents over 20% of hospital-acquired infections (4). Age and underlying diseases are established intrinsic risk factors for acquiring NIs (5,6); however, it is noteworthy that a number of current patient-care procedures in hospitals, such as immunosuppressive agents and broad-spectrum antibiotics, invasive devices, surgical procedures, and nutritional support also increase the risk for developing those infections (7). Of all the major complications of hospitalization, NIs account for 50% (8), resulting in prolonged hospital stays with an excess in health care costs of $ 4.5 billion per year (9–12), as well as high mortality rates (more than 88,000 deaths annually in the United States — one death every 6 minutes) (10,13). One-third of NIs are preventable, yet several infection-control measures should be taken in order to meet this level (14), and hand washing is still the cornerstone strategy for controlling transmission of infections in hospitals (10).

DEFINITIONS

From the combination of the Greek *nosos* (disease) with *komein* (to take care of) as *nosokomeion* (hospital) and through the Latin *nosocomium* (hospital) came the English *nosocomial* (pertaining to a hospital). Today, the term nosocomial infection (NI) is used worldwide (15).

The National Nosocomial Infection Surveillance (NNIS) is a 30-year surveillance system used by the Centers for Disease Control and Prevention (CDC, the former Communicable Disease Center) in Atlanta, GA. This program allows personnel from CDC to standardize and compare data routinely reported to them by over 200 affiliated American hospitals with regard to their nosocomial infection rates (16). The NNIS system defines NI as a localized or systemic condition that results from an adverse reaction to the presence of an infectious agent or its toxin and which was neither present nor incubating at the time of admission. For many bacterial nosocomial infections, this means the infection usually becomes evident 48 hours or more after admission, although this time may vary from agent to agent (17,18). Likewise, any infection acquired in the hospital, but which only becomes evident after hospital discharge, as well as any newborn infection resulting from the passage through the birth canal, is included under the subject of nosocomial infections (19). There are two conditions — colonization and inflammation — which have not been considered infectious. The former has been understood as the presence of microorganisms on the skin, mucous membranes, open wounds, or secretions that are not causing adverse clinical signs or symptoms. The latter is a condition that results from tissue response to injury or stimulation by noninfectious agents (17).

Table 1. Nosocomial Infection Rates from Different Intensive Care Units in the United States

Reference	Author	Unit	Rates (%)
31	R. P. Wenzel	Coronary	1.5
31	R. P. Wenzel	Burns	64
32	P. H. Chandrasekar	Medical	14
32	P. H. Chandrasekar	Surgical	35.2
11	N. A. Khuri-Bulos	Neuro-surgical	18.5

INCIDENCE

In 1964, the first hospital-acquired infection (HAI) rates from U.S. hospitals were collected and reported to be as high as 13.5% (20) (see Table 1). In the late 1960s and 1970s, some survey programs developed by CDC-enrolling community and tertiary hospitals revealed lower rates of nosocomial infections, which were between 3 and 6% (21–23). The current data shows that approximately 2.5 million NIs occur annually in the United States, meaning that approximately 5% of the hospitalized patients will develop some documented infection acquired from the hospital (2,3). Among these 2.5 million patients, almost 250,000 will suffer from bloodstream infections (2). The Second National Prevalence Survey conducted at teaching and nonteaching hospitals in the United Kingdom and Ireland revealed an overall NI rate of 0.9% (24). The prevalence shown by survey studies among other European countries varied between 6 and 10% (25,26). By contrast, the incidence of HAIs can be considerably higher in developing countries, reaching up to 25% (11,27). In a five-year review of published articles on NIs originating from developing countries (such as India, Brazil, Mexico, Chile, Nigeria, Panama, and Zimbabwe), the overall rates varied from 3 to 13.5% (28,29). However, rates as high as 35.4 and 68% have been observed in Burn and Surgical Units, respectively (30).

Rates of NIs differ greatly according to the site of infection, the population of patients, and the clinical service involved (26,33,34). Although ICUs account for only 5 to 10% of all hospital beds, currently the rates of NIs in ICU patients represent over 20% of all HAIs (4). In a surveillance study performed with four Swiss University Hospitals, the prevalence of NIs in ICUs was almost three times higher than the rate observed in medical wards and two times the rate in surgical wards (35).

HISTORICAL BACKGROUND AND TRENDS IN MICROBIOLOGY OF NOSOCOMIAL INFECTIONS

The first nosocomial outbreak of staphylococcal disease throughout the world occurred in the mid-1950s. In 1957 by the time of the viral influenza pandemic, rates of nosocomial staphylococcal infection reached high numbers, presenting mainly as pneumonia (36). By the end of the 1960s, a shift toward gram-negative organisms was noted. One of the first survey studies in the United States showed an overall HAI rate of 3.5%, with gram-negative bacilli accounting for 60% of all infections recorded (23). In the late 1960s and early 1970s, gram-negative organisms, such as *Klebsiella* sp. and *Proteus rettgeri*, emerged as multidrug resistant pathogens in tertiary hospitals all over the United States (37,38). During the following years, cases of aminoglycoside-resistant, gram-negative organisms, such as *Escherichia coli*, *Serratia marcescens*, *Pseudomonas aeruginosa*, and *Proteus* sp. were observed (39,40). Nevertheless, *Staphylococcus aureus* became the main focus of attention during that decade owing to its increased resistance to methicillin (from 2 to 5%) and gentamicin (from 1 to 13%) between 1975 and 1979 (40,41).

In contrast to the 1970s, major shifts in the etiology of NIs occurred during the 1980s (42). In 1986, data published by the CDC revealed overall NI rates between 2.4 and 4.1% and found *Enterococcus* sp. to be the third most common isolate from HAIs (43). Moreover, the increased use of vancomycin to treat enterococcus infections in the 1980s, as well as the overuse of in-dwelling vascular devices in immunocompromised patients, were closely correlated with an increased incidence of nosocomial infections caused by coagulase-negative staphylococci (CNS) (42,44). The CNS organism, previously considered a skin contaminant, has emerged as an important blood and prosthesis pathogen (45–47). By 1988, the first vancomycin-resistant *Staphylococcus hemolyticus* was isolated from a blood culture (48). Similar to CNS and enterococci, detection of *Candida* sp. in blood cultures, as well as in cultures from other sites, increased between 1980 and 1990 (48,49). During the seven-year period between 1978 and 1984 of statewide surveillance by Wenzel and coworkers (50), a significant rise in nosocomial *Candida* bloodstream infections from 0.1 to 1.5 infections per 10,000 discharges was noted. Analysis of data from NNISS hospitals showed that the total number of fungal infections increased from 6% in 1980 to 10.4% in 1990, and approximately 80% of these infections were caused by *Candida* sp. (51). Moreover, *Candida albicans* accounted for 76% of 25,000 primary bloodstream infections reported to the CDC during the same period (52).

The ten most commonly isolated pathogens accounting for NIs during the decade between 1980 and 1989 were as follows: *E. coli*, *P. aeruginosa*, *Enterococcus* sp., *S. aureus*, *Klebsiella* sp., CNS, *Enterobacter* sp., *Candida* sp., *Proteus* sp., and *Serratia* sp. (49,53,54).

In the 1990s, changes in the etiologic pattern of nosocomial infections were noticed. Studies showed progressive increases in the prevalence of organisms, such as *Stenotrophomonas* (formerly *Xanthomonas* and *Pseudomonas*) *maltophilia* and, more recently, *Burkholderia* (formerly *Pseudomonas*) *cepacia* in cultures from the respiratory tracts of cystic fibrosis (CF) patients (55–57).

Isolation of *Acinetobacter* sp. and *P. aeruginosa* has increased markedly, primarily in cases of ventilator-associated pneumonia in ICUs (58). Although recent publications demonstrated a tendency to a decreased incidence of nosocomial *P. aeruginosa* bacteremia (59,60), it is still the third most common nosocomial pathogen among gram-negative rods, accounting for up to 15% of all nosocomial bacteremias (58). There has been an increased observation of *Candida* sp. as a urinary tract infection pathogen, which has been isolated almost as frequently

Table 2. Species Distribution of *Candida* Blood Stream Isolates (SENTRY Program, January Through December 1997)

Species	% Isolates by Geographic Area (# Isolates Tested)			
	United States (203)	Canada (61)	South America (42)	All (306)
C. albicans	56.2	52.5	40.5	53.3
C. glabrata	18.7	11.5	2.4	15.0
C. parapsilosis	8.9	22.9	38.1	15.7
C. tropicalis	6.9	8.2	11.9	7.8
C. Krusei	2.5	1.6	—	2.0
C. guilliermond	0.5	—	2.4	0.7
Candida spp.	6.4	3.3	4.7	5.8

Source: Taken from Reference (69).

as *E. coli* (61,62). Studies from the United States and other countries have shown that *C. albicans* is the leading pathogen in all episodes of candidemias (63–65). Nevertheless, recent data also showed an increase of non-*albicans Candida* species causing bloodstream and other significant infections (66–68). An international surveillance of BSI owing to *Candida* sp. in the United States, Canada, and South America published in 1998 detected 306 episodes of candidemia among 34 medical centers. The overall rates of *C. albicans* and non-*albicans* BSI were 53 and 47%, respectively (69) (see Table 2). In some institutions, the proportion of non-*albicans Candida* isolates from blood cultures has equaled (70,71) or exceeded that of *C. albicans* (64) (see Table 3).

New gram-positive and gram-negative organisms have emerged among a special population of patients — patients with neutropenia (an abnormally small amount of mature white blood cells) and patients undergoing chemotherapy for cancer (72) (see Tables 4 and 5). Intestinal colonization with *Enterococcus* sp., has become a greater concern among these patients as well, because of its resistance to vancomycin and high mortality rates. A recent report described an outbreak of bacteremia owing to vancomycin-resistant *Enterococcus faecium* in the oncology unit of a teaching hospital where the mortality rate was 73% (73).

Surveillance studies have shown that the most commonly isolated organisms may vary depending on the infection site (see Table 6 and references). During the 1990s, the three most common, gram-positive pathogens (*S. aureus*, CNS, and enterococci) accounted for 34% of NIs, whereas the four most frequent gram-negative organisms were *E. coli*, *P. aeruginosa*, *Enterobacter* sp., and *Klebsiella pneumoniae*, accounting for 32%.

Table 3. Etiologic Agents in 145 Cases of Candidemia

Etiologic Agent	# Isolates	% Isolates
Candida albicans	52	37
Non-*albicans*	92	63
Candida parapsilosis	36	25
Candida tropicalis	35	24
Candida rugosa	8	5
Candida glabrata	6	4
Candida guilliermond	3	2
Candida Krusei	2	1
Candida lipolytica	1	1
Candida famata	1	1

Source: Taken from Reference (64).

Table 4. "New" Gram-Positive Pathogens in Patients with Neutropenia

Viridans streptococci, e.g., *Streptococcus mitis*, *S. milleri*
Leuconostoc species
Enterococcus species, especially vancomycin-resistant
Corynebacterium jeikeium, *C. urealyticum*
Rhodococcus equi
Stomatococcus mucilaginosus
Lactobacillus rhamnosus
Bacillus cereus
Clostridium septicum , C. tertium

Source: Taken from Reference (72).

Table 5. "New" Gram-Negative Pathogens in Patients with Neutropenia

Stenotrophomonas maltophilia
Alteromonas (Pseudomonas) putrifaciens
Legionella pneumophila, *L. micdadei*
Vibrio parahemolyticus
Capnocytophaga species
Alcaligenes xylosoxidans
Chryseobacterium meningosepticum
Burkolderia cepacia
Fusobacterium nucleatum
Leptotrichia buccalis
Methylobacterium species
Moraxella-like organisms

Source: Taken from Reference (72).

RISK FACTORS FOR NOSOCOMIAL INFECTIONS

Age and Gender

Studies have shown that host characteristics, such as age and underlying diseases, are intrinsic risk factors for acquiring nosocomial infections (5,6). As human life expectancy increases, more elderly people have been hospitalized with chronic diseases, resulting in longer exposure to nosocomial environments and antibiotic-resistant pathogens (76,77). Moreover, older patients have

Table 6. Most Frequent Organisms According to Site of Infection

Reference	BSI	Pneumonia	UTI	SSI
74	*Staphylococcus aureus*, *Enterococci*, CNS	*Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterobacter* spp.	*Escherichia coli*, *Enterococci*, *Pseudomonas aeruginosa*	*Staphylococcus* spp., *Enterococci*
35	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Escherichia coli*	*Staphylococcus aureus*
75	*Staphylococcus* spp., *Candida*	*Pseudomonas aeruginosa*	*Acinetobacter*	*Staphylococcus aureus*, gram-negative rods
62	*Staphylococcus aureus*, CNS	*Pseudomonas aeruginosa*, *Staphylococcus aureus*	*Candida albicans*	—

Note: BSI, blood stream infection; UTI, urinary tract infection; SSI, surgical site infection.

a greater need for intravenous therapy and critical care settings, which carry poorer prognoses (78,79). Likewise, children may suffer from high morbidity and mortality owing to HAIs (80). In particular, it is low birth weight infants and preterm newborns who may have their clinical course complicated by opportunistic, life-threatening pathogens (81,82). The site of infection differs between these two extremes of age. Respiratory infections in children and urinary tract infections in the elderly are the most common HAIs diagnosed (6). Although gender has not been established as a risk factor for HAI, most of the survey studies have shown predominating rates in men (83–85).

Ganguly, and coworkers (86), assessed 422 prospective patients and found higher rates of NIs in patients with underlying diseases, such as anemia (53.7%), diabetes mellitus (85.2%), hypertension (82%), and obesity (51.5%), than in controls with no comorbidities. These underlying diseases reduce the immunological resistance significantly, making the patient more vulnerable to infection (86). Among 100 diabetic patients studied who were undergoing elective surgery, the risk for development of early postoperative nosocomial surgical site infections was 2.7 times higher when it was related to perioperative hyperglycemia (31.3% vs. 11.5%) (87). Hematologic and oncologic patients may present with periods of extreme neutropenia (with less than 500 neutrophils per mm^3) because of powerful chemotherapy and radiotherapy, which also make them vulnerable targets to life-threatening HAIs (88). During a 42-month period of surveillance in a cancer center, 920 neutropenic patients were detected. A total of 444 NIs was identified for an overall rate of 48.3 HAIs per 100 neutropenic patients (89).

It is noteworthy that a number of current patient-care procedures in hospitals, such as immunosuppressive agents and broad-spectrum antibiotics, invasive devices (intravenous and urinary catheters and mechanic ventilation), surgical procedures, and nutritional support — so-called extrinsic risk factors — also greatly increase the risk for developing NIs (7).

Parenteral and Enteral Nutrition

Although enteral nutrition has an important task in the maintenance of gastrointestinal immunological function, thereby decreasing bacterial translocation and the risk of infection, cases of NI have been reported owing to contaminated feeding solutions (90,91). The stomach alkalinization induced by the enteral nutrition may allow gastric microbial growth, which can be translocated to the trachea resulting in tracheal colonization and, ultimately, pulmonary aspiration and nosocomial pneumonia (92). Total parenteral nutrition has been associated with HAIs, as well (93,94). A prospective study in a pediatric ICU showed that patients on parenteral nutrition support were 22 times more likely to develop NI than those patients not receiving it (95).

In contrast to healthy people, critically ill, hospitalized patients become colonized more rapidly with gram-negative bacteria during mechanical ventilation and carry a higher risk for ventilator-associated pneumonia (VAP) caused by such bacteria (96–98). The stomach is normally almost sterile because of the bactericidal activity of the hydrochloric acid that prevents colonization of the gastrointestinal tract with ingested pathogens (99). However, gastric bacterial colonization is associated with a number of factors frequently present in hospitalized patients, including advanced age, underlying gastrointestinal disorders, achlorhydria (an absence of hydrochloric acid from the gastric juice), malnutrition, and administration of antacids (100,101). Direct aspiration of small volumes of secretions from the trachea or gastrointestinal tract with large concentrations of bacteria is the most common event responsible for development of VAP (97). In addition, endotracheal tubes probably play an important role in predisposing such patients to aspiration, bypassing the normal protective mechanisms of the upper airway and allowing secretions to pool into the upper part of the trachea. This is particularly important in the presence of impaired levels of consciousness (102) (see Fig. 1). It has been speculated that *Pseudomonas* sp. may also cause VAP by a route different than other gram-negative bacteria, since this organism has an enhanced capacity

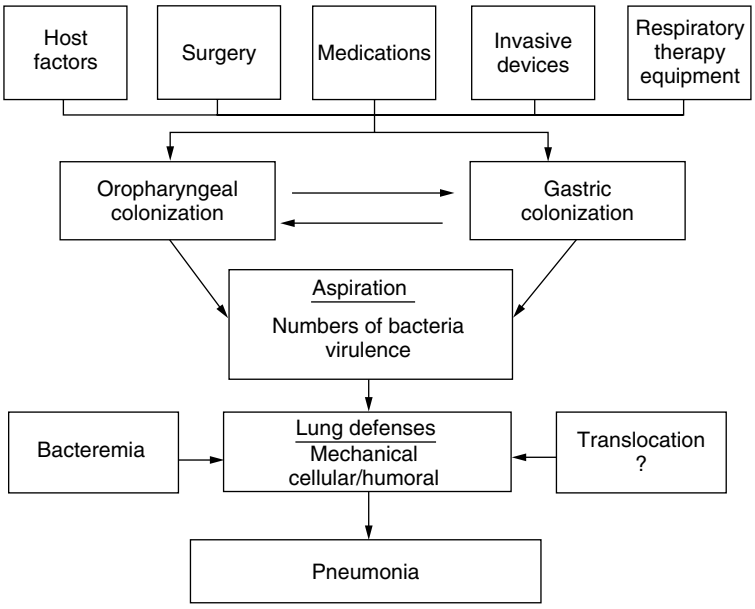

Figure 1. Factors related to the development of ventilator-assisted pneumonia. Taken from Reference 97.

for binding to the tracheobronchial tree of patients on mechanical ventilation without previous colonization of the oropharynx (103).

Intravenous Devices

Each year in the United States, 150 million intravascular devices are used by hospitals and clinics. The vast majority are peripheral venous catheters; however, about 5 million central venous devices of various types are sold in the United States (104). As early as 1977, Maki (105) suggested that device-related bacteremia develops in more than 25,000 patients in the United States each year. These numbers have continued to increase (106,107). Cases of device-associated bacteremia are referred to as primary bacteremia because there is no obvious infection focus (108). Primary bacteremia accounts for one-fourth of the sporadic cases of nosocomial primary bacteremia, whereas more than three-fourths of the nosocomial bacteremia occur in clusters. Approximately 75% of the primary bacteremia cases are reported to be device-associated (108). Bacteria may gain access to an intravascular device at several points during the entire process of infusion therapy (see Fig. 2) (109). In a prospective study by Maki and Ringer (110), colonization around the catheter insertion site was the most strongly associated risk factor for catheter infection. Hub contamination was the second most heavily weighted risk factor.

There are many patient- and hospital-related factors, which have been incriminated as a risk for increasing device-associated infections (see Tables 6 and 7). Staphylococci account for one-half to two-thirds of the episodes of catheter-related bacteremia, and most of those are because of contamination at the junction or at the device insertion site (110,111). Although *S. aureus* is still a frequent pathogen, coagulase-negative *Staphylococcus* has become predominant, especially among immunocompromised patients and those in whom long-term central venous access is required (42,44). When unusual isolates are identified, including gram-negative bacteria or fungi, the possibility of contamination of the infusion product rises (112—114).

Urinary Catheters

At least 25% of patients hospitalized in the United States annually are placed with an in-dwelling transurethral urinary catheter (115). Urinary catheters have been used since the 1800s for accurate measurement of urinary output, to relieve urinary retention or incontinence, to facilitate urinary flow in some neurologic patients, and for instillation of drugs or irrigation of the bladder (116). The duration of catheterization is directly related to the development of asymptomatic bacteriuria, which may result in nosocomial urinary tract infection (UTI), bacteremia, and sepsis (117,118). The incidence of bacteriuria in short- and long-term catheterized patients is 3 to 10% and 8 to 10%, respectively, per day of catheter placement. This means that patients who are catheterized late in their hospitalization have an increased rate of bacteriuria (115,119,120). Other risk factors for bacteriuria have been reported, including diabetes mellitus, abnormal serum creatinine, renal stones, and inadequate catheter care (121). In females, between 70 and 80% of UTIs are caused from a periurethral route contaminated with rectal flora (122,123). After removal of a catheter, the patient may remain at risk for bacteriuria up to 24 hours, possibly because of the increased urethral colonization associated with an in-dwelling catheter (124). In males, the predominant route is intraluminal. Bacteria may grow in the urine in suspension and ascend via lumen or they may grow in so-called biofilm outside of the catheter and then infect the bladder (122,125). Biofilm formation is a pathogenic process triggered when a bacteria attaches to the catheter surface resulting in encrustation of the catheter lumen by layers of organisms (125,126). In addition, microorganisms that are present on the meatus or

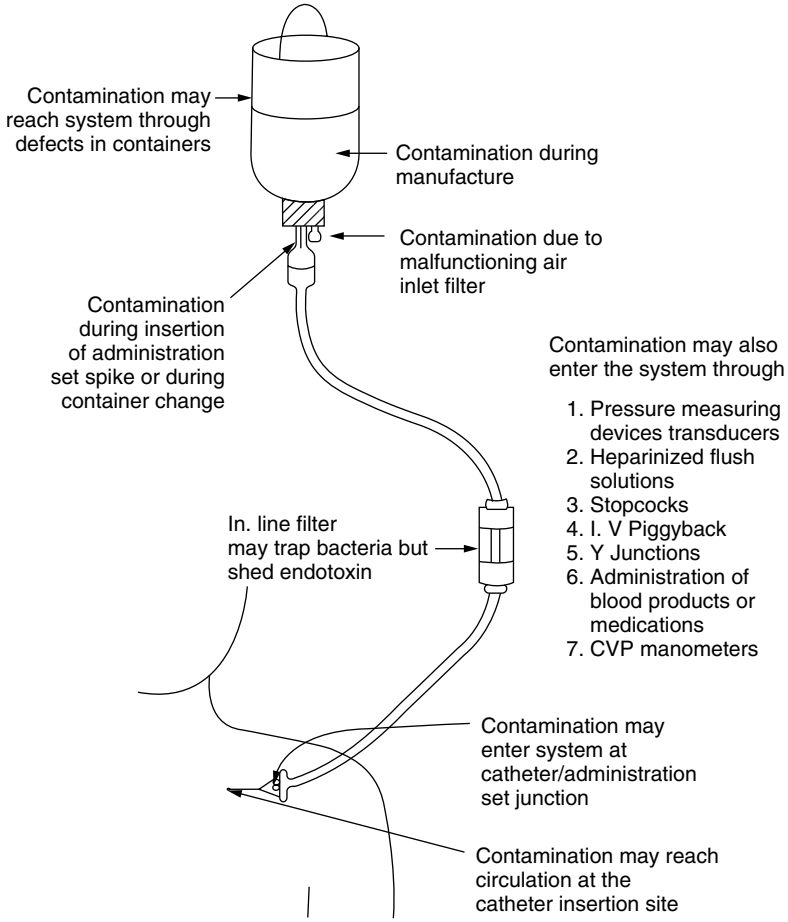

Figure 2. Bacteria may gain access to an intravascular device at several points during the process of infusion therapy. Taken from Reference 109.

Table 7. Patient-Related Risk Factors for Device-Associated Bacteremia

Age 1 year old or younger, 60 years old or older
Granulocytopenia
Immunosuppressive chemotherapy
Loss of skin integrity
Severity of underlying disease
Presence of distant infection

Source: Taken from Reference (109).

distal urethra can be transferred directly into the bladder during the insertion of the catheter (127). Among short-term catheterized patients, *E. coli* is usually the bacteriuric species most frequently isolated. Other common organisms are *P. aeruginosa*, *K. pneumonia*, *Proteus mirabilis*, *Staphylococcus epidermidis*, *Candida* sp., and enterococcus (118,128).

Use of Antimicrobial Therapy

Many years ago, studies already showed that prior exposure to antibiotic therapy was a decisive factor in selecting normal gastrointestinal flora, facilitating colonization and infection of patients (37,38,129,130). Rose and Schreier (131) compared 50 hospitalized patients treated with antibiotics with 50 untreated patients and noted a significant increase in the intestinal carrier rate of *Klebsiella* sp. among those patients receiving antibiotics. A retrospective survey at Boston City Hospital between 1935 and 1957 showed a favorable impact of new antimicrobial drugs in controlling primary bacterial pathogens and reducing the number of deaths by organisms such as pneumococcus and hemolytic streptococcus. On the other hand, there was a greater increase in the incidence and mortality caused by opportunistic agents such as *S. aureus*, *Pseudomonas* sp., *Enterococcus* sp., and *Proteus* sp. — organisms, which had not previously been considered highly pathogenic (129). A few years later, it was observed that the widespread use of antibiotics led to enhanced rates of carriage of resistant bacteria among both sick patients and healthy individuals from the community (132). Caugant and coworkers (133), found a genetic relationship between resistant strains of the *E. coli* that colonized and infected people in a specific region, demonstrating that excess resistance in the bacteria that colonize may also be an indicator of excess resistance in the bacteria that infect.

Pallares and coworkers (48), showed that the percentage of in-patients receiving at least one antibiotic increased from 23% in 1978 to 44% in 1992 in a tertiary hospital. Following the changes in antimicrobial usage, the susceptibility patterns of some microorganisms has changed, as well. The emergence of antibiotic resistance has been a global problem (134) since the

1940s when the first cases of penicillin-resistant staphylococci were identified (135). By the late 1950s, approximately 50% of the staphylococcal strains were resistant to penicillin (136). Methicillin and other semisynthetic penicillins were introduced in the 1960s and successfully treated penicillinase-resistant *S. aureus* until the mid-1970s, when methicillin-resistant *S. aureus* (MRSA) strains became endemic in many European and U.S. hospitals. This resistance mechanism was characterized by a low-affinity of penicillin-biding protein (PBP) (137 — 139).

Data from NNIS hospitals showed that the overall percentage of MRSA among the U.S. hospitals rose from 2.4% in 1975 to 29.0% in 1991 (140). The glycopeptide vancomycin has been the only uniformly effective treatment for methicillin-resistant staphylococcal infections during the past 30 years (141). However, the recent emergence of glycopeptide resistance among coagulase-negative staphylococci (142–144) and enterococci (145,146) has heightened concern about whether *S. aureus* could acquire such resistance. To this end, the morbidity and mortality produced by this organism would be alarming (147). In fact, all MRSA strains demonstrated sensitivity to vancomycin until 1996, when the first isolate of *S. aureus* with reduced sensitivity was reported in Japan (136). At least four new cases of infection caused by *S. aureus* with minimum inhibitory concentration (MIC) higher than 4 meg/mL to vancomycin have been isolated in the United States (147–149).

Enterococcus strains typically have intrinsic low-level resistance to aminoglycoside (150,151). High-level streptomycin- and kanamycin-resistance have been shown as early as the 1970s (152); whereas, high-level gentamicin-resistance has only been detected more recently (153–155). Studies have suggested that hospitals may be the major source for the spread of clonal-resistant strains (156,157). Highly resistant *Streptococcus pneumoniae* isolates recovered from European, African, and U.S. cities (158–160) were shown to be clonally related, suggesting intercontinental spread of such multiresistant bacteria (161).

Elaboration of inducible, chromosomal- and plasmid-mediated ß-lactamase among gram-negative bacteria has also been reported (162–167). In 1983, the first case of extended-spectrum ß-lactamase (ESBL) was described in Europe (168). The mechanism was recognized as either a plasmid- or chromosomal-mediated resistance present among Enterobacteriaceae, which conferred resistance to broader spectrum antibiotics than ß-lactamase enzymes can confer (169). ESBL enzymes initially became increasingly prevalent in Europe and later in the United States (170,171). Now, the ESBL represent a major group of ß-lactamases known worldwide (172,173).

Normally present in the digestive tract of healthy people, the frequency of nosocomial yeast infections has increased dramatically (174). Therapy for serious yeast infections has been difficult because of the limited number of available antifungal agents and the treatment toxicity associated with drugs such as Amphotericin B and Flucytosine® (fluconazole) (175). Because of its widespread prophylactic and therapeutic use, mainly in patients with AIDS, reports about the emergence of resistance by some species of *Candida* appeared very quickly (176,177). One particular *Candida* species, *C. krusei*, is intrinsically resistant to fluconazole, whereas isolates of *C. glabrata* and *C. albicans*, which are initially susceptible, may become resistant during treatment (178,179).

SPECIAL PATIENTS AND NOSOCOMIAL INFECTIONS

Elderly Patients

Higher incidence rates of HAIs have been documented among patients older than 60 years of age (5,6). The period between birth and the sixth decade has a constant and estimated risk for NI at approximately 10 per 1,000 discharged patients. After age 70, the risk rises sharply to 100 per 1,000 discharges (180). Gross and coworkers, showed that although the elderly (over 60 years old) made up 23% of hospitalized patients, they accounted for 64% of all NIs recorded. Moreover, when the group size was narrowed to patients over 70 years of age, the numbers were even more amazing, comprising only 9.9% of the patient population but accounting for 43.4% of the HAIs (180). Another study showed that more than 40% of NIs occur in persons 70 years of age and older; however, this age group accounts for only 10% of the discharges (77). One probable reason for progressive impairment of host defenses with aging leading to elevated rates of NIs, is the diminution in cell-mediated and humoral immune responses in the elderly population (77). In addition, many age-associated physiologic and anatomic changes, such as diminished ciliary function in clearing secretions, alteration of oral flora, decline of gastric acidity, and drier and thinner skin with less circulation, may predispose these patients to potentially severe nosocomial infections (181). Urinary and respiratory tract infections are the most common HAIs in the elderly (182). Most cases of UTI among elderly patients, whether or not symptomatic, are related to the use of an in-dwelling urinary catheter, particularly in females (5). In a surveillance study, 123 out of 300 elderly patients had in-dwelling urinary catheters, and 44% of these developed UTIs (183) (see Tables 7 and 8).

Table 8. Hospital-Related Risk Factors for Catheter-Associated Infection

Type of catheter (more risk with plastic than with steel)
Location of the catheter (central more than peripheral; femoral more than jugular/subclavian)
Type of placement (cutdown associated with greater risk than percutaneous placement)
Duration of placement (at least 72 hours is associated with greater risk than placement less than 72 hours)
Emergence (emergent placement carries more risk than elective)
Skill of venipuncturist (others associated with greater risk than IV team)
Type/use of catheter (percutaneously placed central venous catheters [CVC] carries greater risk than implanted CVC)

Source: Taken from Reference (109).

HIV-Infected Patients

The overall hospital-acquired rates for HIV-infected patients and patients with Acquired Immunodeficiency Syndrome (AIDS) fall within the range of 6 to 10% (184–186); however, this data may be underestimated owing to delayed or atypical presentation of nosocomial infection in that particular category of patients (187). When patients with AIDS are hospitalized, they carry greater risk for NIs and higher mortality rates by virtue of their compromised host status (184,186,188). Although no established relationship between $CD4^+$ cell counts and the rate of NI has been recorded in these patients to date, the depletion of $CD4^+$ lymphocytes below 250 per mm^3 may increase their risk for HAIs (189). The risk for HAIs may be even more significant when those patients have their immune status impaired because of non-Hodgkin's lymphoma, CMV infection, and wasting syndrome (188). In comparison to non-HIV-positive patients, bloodstream, urinary, and respiratory tracts are demonstrated to be more often associated with NIs in HIV-infected patients (186,190). At least five opportunistic diseases (tuberculosis, cytomegalovirus, herpes simplex virus, bacterial pneumonia, and cryptosporidiosis) are known to be transmitted nosocomially among immunosupressed patients, affecting HIV-infected patients in particular (191).

Mycobacterium tuberculosis infects one-third of the world's population, with a mortality rate of three million deaths per year (192). The incidence of HIV-associated tuberculosis has been increasing worldwide since the beginning of the AIDS pandemic in the 1980s (193), particularly because HIV-infected patients seem to be at increased risk for tuberculosis infection and tuberculosis patients are at increased risk for HIV infection (194,195). The World Health Organization estimated there will be approximately 10.2 million new cases of active tuberculosis among HIV-infected patients by the year 2000 (196). Africa comprises 77% of the world's coinfected patients, with Asia accounting for 10%. In the United States, rates range from zero (Montana) to 47% (Florida) (197). The coexistence of *M. tuberculosis* and HIV-1 accelerates the development, worsens the severity, and amplifies the transmission of tuberculosis (198). During an outbreak documented in a chronic-care facility in San Francisco, 35% of patients with AIDS who were exposed to the index case developed active tuberculosis within four months (199). Among exposed HIV-infected patients, high attack rates of active disease associated with high mortality rates justify the concern of early diagnosis and the isolation of suspected new cases before nososcomial transmission can occur, in particular, when there is a chance for the spread of multidrug-resistant tuberculosis (MDR-TB) cases (191). In an outbreak affecting 27 HIV-infected patients in a New York hospital within a median time range of 7 weeks after the diagnosis of MDR-TB, 21 had died (200).

In a setting of nontuberculous nosocomial respiratory diseases, bacterial pneumonia represents 21% of the NIs diagnosed in HIV-infected patients (185,187,201), and *S. pneumoniae*, *S. aureus*, and *Pseudomonas* spp. are the most common pathogens isolated from respiratory specimens (185,202). In fact, among all gram-negative organisms, *Pseudomonas* spp. accounts for 16 to 67% of nosocomial pneumonias. Nosocomial bacteremia is another clinical problem diagnosed in patients with AIDS (203). *Staphylococcus* sp. and *Pseudomonas* sp. account for a higher proportion of the bacteremias seen in patients with AIDS, most of which appear to be related to in-dwelling venous catheters (204). Urinary tract infection is also a common NI in HIV-infected patients related to the use of plastic devices. Goetz and coworkers (185), showed that among 32 NIs in HIV-infected patients, the urinary tract accounted for nine infections, and eight of these patients had a urinary catheter.

Oncologic Patients

Oncologic patients account for as many as 12% of HAIs (205). When the concept of empiric antibiotic therapy was first introduced, bacteremia was reported to affect approximately 20% of febrile patients with neutropenia (206). The advances in antimicrobial therapy and supportive measures (such as blood and nutrition supplies), as well as more potent antineoplastic chemotherapies and bone marrow transplantation, have improved the long-term survival and quality of life in patients with hematological malignancies and solid tumors (205,207,208). Nevertheless, infection remains the most significant complication of therapy in these patients, particularly when they had undergone myelosuppression (reduction of blood cell production by the bone marrow). Neutropenia, regardless of whether induced by therapy or as a consequence of the underlying disease, is the main risk factor for the development of serious and life-threatening infection (89,209). The cytotoxic chemotherapy can decrease the absolute neutrophil count below 500 cells/mm^3 and worsen its function, therefore dramatically increasing the patient's susceptibility to infection (207,210). A prospective study of bacteremia in neutropenic patients from a single institution showed an increase in the rate of nosocomial infection over an eight-year period by 40% (211).

Until the 1990s, gram-negative bacteria, especially *P. aeruginosa*, were responsible for 60 to 70% of the infections in oncologic patients with an elevated mortality rate (212,213). Among 22 patients with a diagnosis of lymphoma, leukemia, or solid tumor who developed *P. aeruginosa* bacteremia, half died within 72 hours from the time the first positive blood cultures were drawn (206). Likewise, in nononcologic patients, there was a clear shift in the etiologic agents responsible for NIs among patients with cancer during the 1980s, with an upward trend in gram-positive organisms (214). Of these gram-positive organisms, *S. aureus* and coagulase-negative *Staphylococcus* were the most frequent isolates (215,216), followed by *V. streptococci* and *Enterococccus* sp. (76,217). Since the 1990s, and continuing recently, 60 to 70% of bacteremias with a single organism affecting granulocytopenic patients with cancer have been because of gram-positive cocci (218–220) (see Table 9).

Extensive use of in-dwelling intravenous catheters, potent chemotherapeutic regimens resulting in oral and gastrointestinal mucosal damage and neutropenia, and the use of antacids, fluoroquinolone, and

Table 9. Bacterial Isolates from Cases of Bacteremia Owing to a Single Organism from the IATC of the EORTCT

Trial	Total No. of Isolates	Gram-Negative	Gram-Positive
I: 1973 to 1976	145	103 (71%)	42 (29%)
II: 1977 to 1980	111	74 (67%)	37 (33%)
III: 1980 to 1983	141	83 (59%)	58 (41%)
IV: 1983 to 1985	219	129 (59%)	90 (41%)
V: 1986 to 1988	213	78 (37%)	135 (63%)
VIII: 1988 to 1991	151	47 (31%)	104 (69%)
IX:1991 to 1993	161	53 (33%)	108 (67%)

Note: IATC, International Antimicrobial Therapy Cooperative Group; EORTCT, European Organization for Research and Treatment of Cancer Trails.
Source: Taken from Reference (72).

trimethroprim-sulmethoxazole prophylaxis are factors that have been associated with the high frequency of infections caused by gram-positive bacteria in oncologic patients (217,221–223). Moreover, the use of empiric, broad-spectrum antibiotic therapy and antifungal prophylaxis on febrile neutropenic patients has predisposed them to fungal infection, mainly caused by *Candida* sp. (224,225). *Candida albicans* is the most prevalent (70), yet other more resistant non-*albicans* species of *Candida* have been found colonizing and infecting these patients (226). Although the outcome in these patients have improved more recently, the mortality rate of NI is still around 20 to 25% (227,228) and varies according to the pathogen responsible for the infection (211,226,229). Poor prognostic factors such as uncontrolled cancer, shock at the onset of bacteremia, and prolonged neutropenia have been reported (227–230).

Intensive Care Unit Patients

Patients in ICUs represent only a very small percentage of hospital admissions (231); however, these patients suffer a disproportionate percentage of NIs compared with patients in noncritical care areas (31,62). Nosocomial bloodstream infections may occur two to seven times more often in ICU patients than in general ward patients (47). Usually, patients in ICUs are more severely ill and more often require invasive procedures and life support (62). Intravenous central-line and cardiovascular monitoring, mechanical ventilation, in-dwelling urinary catheters, and surgery can breach normal host defenses allowing progression of endogenous flora from colonization to infection in individual patients, as well as exogenous flora transferred from other patients or the environment (231–233). The crescent use of empirical, broad-spectrum antibiotic therapy in ICU patients is another important issue influencing their outcomes. Among 6,250 ICU patients studied in a point prevalence survey over 17 countries in Europe, 62.3% were receiving at least one antimicrobial on the day of the study (233).

Survey studies in ICUs in the United States have shown different pathogen distribution according to the site of infection (47,231). Richards and coworkers (62), found that 65% of bloodstream infections in adult medical ICUs were caused by gram-positive agents (coagulase-positive staphylococci, *S. aureus*, enterococci), whereas gram-negative aerobes (*K. pneumonia* and *P. aeruginosa*) and *C. albicans* were reported in only 17 and 11% of the cases, respectively. On the other hand, gram-negative organisms accounted for 64% of the nosocomial pneumonias and 40% of urinary tract infections, whereas gram-positive bacteria were less frequently identified at approximately 20% of each. A sharp increase in *Candida* sp. isolation was noticed when it was related to nosocomial UTIs. Species of *Candida* accounted for more than 30% of the nosocomial UTIs.

PROGNOSIS

HAIs have been recognized for more than a century as critical problem adversely affecting a patient's outcome and the quality of health care (1). In the United States today, NIs affect more than two million patients annually and contribute to more than 88,000 deaths — one death every 6 minutes (10,13). Of all the major complications of hospitalization, NIs account for 50%; the other 50% is related to medication errors, falls, and other noninfectious adverse events (8).

The adverse effects of HAIs have been measured by either increased mortality rates or prolonged hospitalization leading to an excess of health care costs of $4.5 billion per year (9–12) (see Table 10 with references). A survey study in France showed that the cost of antibiotic treatment for NIs represented a significant part of the hospital expenditure and, among the currently available drugs, third generation cephalosporins and parenteral fluoroquinolones are the most expensive (234). Pittet and coworkers (12), reported approximately 24 days extra length of hospital stay time and extra costs of $40,000 per survivor attributable to the average nosocomial bloodstream infection.

Despite the entire relevant progress in the management of critically ill patients, the fatality rates associated with HAIs remain very high (35). The mortality attributable to NI (which means the mortality associated with the infection and apart from the mortality related to underlying disease) averages between 7 and 9% (35,236) and may vary according to the organism involved (83,229,236–240) and site of infection (see Tables 11 and 12 with references). Shock is another variable that has been found to be independently associated

Table 10. Overall Estimated Morbidity, Mortality, and Cost of Nosocomial Infections

Author	Location	Number of Infected Patients	Infection Rate (%)	Mortality (%)		Excess Length of Stay (days)	Average Cost per Patient ($)
				Overall Mortality	Attributable Mortality		
Haley	All patients	9,423	5	—	—	4 (max. 68)	1,833 (max. 41,628)
French	All patients	—	—	—	7.4	23	330,000 annually[a]
Craven	ICU	372	51	31	—	—	—
Diaz-Molina	ICU	88	—	—	—	4.3	1,909

Note: ICU, intensive care unit.
Source: Taken from Reference (3).
[a]Estimate includes cost of antimicrobials only.

Table 11. Estimated Increase in Length of Hospital Stay, Cost, and Mortality Associated with Nosocomial Bloodstream Infections and Pneumonia

Site	Author	Location Numbere of Infected patients	Infection Rate (%)	Mortality (%)		Increased Length of Stay (days)	Average ($)
				Overall Mortality	Attributable Mortality		
Blood stream infection	Haley	All patients (167)	0.27	—	—	7 (max. 17)	3,061 (max. 9,027)
	Pettet	SICU (86)	2.7	50.0	35.0	24	40,000 per survivor
	Fagon	ICU (222)	—	23.8	16.3	—	—
	Townsend	NICU (49)	4	27.0	21.0	20	—
Pneumonia	Haley	All patients (770)	—	—	—	6 (max. 44)	4,947 (max. 41,628)
	Leu	All patients (100)	8.6	20.3	6.8	9.2	—
	Craven	ICU (49)[a]	21	55	15	—	—
	Fagon	ICU (49)[a]	9	71	—	—	—
	Driks	ICU (23)	18	56	—	—	—
	Torres	All patients (78)	24	33	—	—	—
	Kollef	SICU (43)[a]	15.5	37	28.7	—	—
	Craig	ICU (670)	8.8	20.3	14.7	7.7	—
	Fagon	ICU (48)[b]	11	54.2	27.1	20	—
	Fagon	ICU (328)[b]	16.6	52.4	30	—	–

Note: SICU, surgical intensive care unit; ICU, intensive care unit; NICU, neonatal intensive care unit.
Source: Taken from Reference (3).
[a]Includes only patients mechanically ventilated >48 hours.
[b]Includes only patients mechanically ventilated >72 hours.

Table 12. Estimated Increase in Length of Hospital Stay and Cost of Nosocomial Urinary Tract or Surgical Site Infections

Site of Infection	Author	Country	Study Population	Number of Infected Patients	Increased Length of Stay (days)	Average Cost
Urinary tract infection	Givens	USA	Surgery patients	32	2.4	$ 558
			Bowel surgery	—	4.0	$ 931
			Cesarean sections	—	1.4	$ 325
			Coronary artery bypass graft	—	1.5	$ 582
			Total hip replacements	1.0	0	—
	Coello	UK	All patients	36	3.6	£ 476
	Haley	USA	All patients	3,825	1 (max. 28)	$ 593 (max. $ 8,280)
Surgical site infection	Coello	UK	All patients	12	8.2	£ 1,041
	Haley	USA	All patients	233	7.0 (max. 68)	$ 2,734 (max. $ 26,019)

Note: USA, United States; UK, United Kingdom.
Source: Taken from Reference (3).

with increased mortality, especially among patients with gram-negative bacteremia, even though gram-positive organisms are the most common causes of nosocomial bloodstream infections (228,230,239,241,242). Other issues significantly associated with poor prognosis include sepsis, inappropriate empiric antimicrobial use, severity of the underlying disease, polymicrobial bacteremic episodes, hospitalization in ICUs, multiorgan failure, and adult respiratory distress syndrome (243–246). Moreover, it has been observed that disseminated nosocomial candidemia can itself be an independent predictor of mortality, overcoming the expected mortality rate of severe underlying diseases (224,229).

ENVIRONMENT AND NOSOCOMIAL INFECTIONS

Many reports have demonstrated the important role played by the hospital environment on the development of NIs among both sick patients and healthy people (247,248). The hospital environment is the most significant reservoir of resistant microorganisms (249). In the 1950s, extensive contamination of the environmental surfaces by *S. aureus* was documented in the rooms of patients with staphylococcal infections (247). At that time, investigations showed that healthy adults exposed to a hospital environment had a four time greater chance of developing NIs by *S. aureus* than those not exposed (247). Even higher indices of environmental contamination occur when patients have methicillin-resistant *S. aureus* in a wound or urine. They frequently contaminate objects in their rooms including bed lines, over-bed tables, patient gowns, and pressure cuffs (250). *Staphylococcus aureus*, *P. aeruginosa*, and *E. coli* can survive for approximately four weeks on a clean, dry surface. In addition, *S. aureus*appears to be viable on cotton strings and blood protein coagulum for up to six months, whereas the *P. aeruginosa* and *E. coli* survival can be even longer on similar wet and fibrous surfaces (248). Vancomycin-resistant enterococcus (VRE) is another microorganism with prolonged survival on hands, gloves, and environmental surfaces, predisposing patients to nosocomial transmission (146). In a survey study, Enterococcus was recovered from countertops, bedrolls, telephones, and the diaphragmatic surface of stethoscopes after 7 days, 24 hours, 60 minutes, and 30 minutes after inoculation, respectively (251).

An estimated 8,000 to 18,000 cases of Legionnaires' disease occur each year in the United States (252). Of the cases reported to CDC, approximately 23% are nosocomial with a mortality rate reaching as high as 40% (253). Even before the first bacterium was isolated, the first cases of nosocomial Legionnaires' disease dated back to the 1960s and 1970s (254) in patients suffering from underlying chronic disease, immunosuppression, or who were on corticosteroid therapy (255–257). Since then, numerous nosocomial outbreaks have been frequently described among transplant patients (258,259). The hospital water distribution systems have been implicated as the source of transmission because there are restrictions on maximal hot-water temperatures, making these systems ideal for amplification of *Legionella* growth (260,261). Cooling towers and tap water used for cleaning respiratory-therapy equipment have been linked to some outbreaks, as well (262–264). One study conducted in a transplant center showed that *Legionella* can colonize a hospital potable water system for long periods of time, resulting in a silent nosocomial transmission for as long as 17 years (265).

The hospital environment also plays a crucial role in the spreading of *Aspergillus* spp. (266). This fungus releases spores, which remain airborne or on dry surfaces for prolonged periods (267–269). In the presence of water, spores will germinate and mycelial growth will occur with subsequent sporulation (270). The primary route of acquiring *Aspergillus* infection is by the inhalation of spores and their deposition in both the upper and lower respiratory tract (271). Several potential sources for *Aspergillus* spores in hospital air have been described as follows: inadequate filtration of outside air (272,273), dust with high concentrations of spores (274), spices that are used for food preparation (275), the soil of potted ornamental plants, and fresh fruits (276,277). Immunocompromised in-patients, especially neutropenic patients, may develop nosocomial invasive aspergillosis present with pneumonia when exposed to high concentrations of airborne *Aspergillus* spores, with *Aspergillus fumigatus* being the most clinically relevant species identified from these patients (271,278). There have been many studies addressing the relationship between the concentration of airborne spores and the risk of invasive disease, especially in neutropenic patients; however, a dose-response relationship has not been established. Arnow, and coworkers (275), showed that an increase in mean concentrations of *A. fumigatus* and *Aspergillus flavus* spores from less than 0.2 to greater than 1 spore cubic meter of air was accompanied by a fourfold increase in the incidence of invasive aspergillosis from 0.3 to 1.2% in immunocompromised patients. Yet, in a more recent study, the occurrence of six cases of invasive aspergillosis could not be linked to changes in the recovery of airborne *Aspergillus* spp. spores (268).

PREVENTION AND CONTROL MEASURES

Infection control and prevention has been proven not only effective in reducing the morbidity and mortality associated with NIs, but to also be cost-effective (279–281). Modern infection control is grounded in the work of Ignaz Semmelweis who, in the 1840s, demonstrated the importance of hand hygiene for controlling transmission of infection in hospitals (10). Hand washing with antiseptic soaps remains the cornerstone of infection control, especially in the ICUs where the most severely ill, and therefore more susceptible, patients are located (282,283). A survey study of physicians' stethoscopes showed that bacteria can survive on its diaphragmatic surface for long periods of time, suggesting that stethoscopes may be a vector of cross-transmission. In addition, the study also showed that no bacteria could survive after disinfection with 70% alcohol or liquid soap (284).

Approximately one-third of NIs are preventable; however, several strategies other than changing human

behavior about hand washing should be pursued in order to meet this level (281). Some of these measures represent improvement of the national surveillance of NIs among in-patients and post-discharge patients (10), implementation of antibiotic control programs in order to decrease the emergence of highly resistant strains (285), and development of newer microbiologic methods (mainly molecular epidemiology) for the investigation of and understanding about multiresistant pathogens (286).

Moreover, it is worth noting that some groups of pathogens need specific control measures. Filtration of air coming into patients' rooms by Hepa filters, laminar airflow, well-sealed rooms capable of achieving high rates of air changes, and positive room air pressure relative to the corridor are all crucial measures implemented by infection control personnel in order to avoid air contamination and cross-infection with *Aspergillus* spp., principally in transplant units (287,288). Likewise, effective *Legionella* eradication methods in the hospital water distribution systems with the superheat-and-flush method, hyperchlorination, and, more recently, copper-silver ionization, have proven to prevent most of the hospital-acquired Legionnaires' disease infections (289).

CONCLUSION

The relationship governing infection can be described as follows:

$$\text{Infection} = \frac{[\text{number of microbes encountered}] \times [\text{virulence characteristics}]}{\text{Immune status of the host}}$$

Environmental microbes in a hospital setting can pose a significant risk. Whereas the concentrations of environmental microbes found naturally in the environment tend to be low, there are abundant opportunities for amplification. For example, small numbers of microbes can colonize a faucet aerator and significantly multiply in numbers. Whereas the virulence factors of environmental bacteria tend to be low, during amplification those with human pathogenic potential may be selected. Hospitalized patients tend to lose levels of both passive (e.g., intact skin through intravenous device) and active, (e.g., through medical therapy) immunity. Accordingly, care and vigilance must be constant to avoid hospital-induced infections from environmental microbes.

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NUCLEAR WASTE RESPOSITORY IN YUCCA MOUNTAIN: MICROBIOLOGICAL ASPECTS

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Yucca Mt. is the proposed site for the United States' first permanent high-level nuclear waste repository. The mountain is composed of volcanic ash, compressed and geochemically modified over millions of years. It is located approximately 125-km northwest of Las Vegas, Nevada. Yucca Mt., rests on land owned by a variety of federal agencies, and is protected by the isolation afforded by the federal government. The mountain itself is considered to be a safe disposal site because of the arid climate of the region, a geologic structure composed of hundreds of meters of vadose zone (unsaturated rock), and a deep groundwater level. The site is now perhaps one of the best studied physical structures on earth, having been the center of concerted research efforts since the 1980s. Life abounds within the mountain, but it is invisible and unknown to the casual observer. This article describes the microbial life within, and its potential effects upon, the Yucca Mt. repository.

THE REPOSITORY

Yucca Mt. is a long sloping mountain that abuts the southwestern boundary of the Nevada Test Site (NTS) (Fig. 1). The mountain is composed of multiple layers of bedded or nonwelded, and welded, volcanic ash (tuff) that was deposited 9 to 13 million years ago (1). Welding refers to the high temperature at which a geologic stratum was deposited, making the rock extremely hard and of low permeability. In addition to the welded nature of the tuff, it also holds very little water. The repository layer is approximately 300 m from the mountain surface and 275 m above the water table (2). The welded nature of the tuff, the deep dry vadose zone, and the isolation from large population centers make Yucca Mt. a desirable site for the permanent burial of high-level nuclear waste.

In 2002, the Department of Energy (DOE) will apply to the Nuclear Regulatory Commission for a license to excavate the repository, transport nuclear waste from remote sites, and deposit it at Yucca Mt. High-level nuclear waste is currently located throughout the United States and consists of spent nuclear fuel from submarines and nuclear power plants. The development of a permanent repository site is critical because waste has been placed in temporary facilities in which long-term storage may not have been considered (Fig. 2).

The design of the repository is quite unique. It will be built at the midpoint of a large "U" shaped tunnel of approximately 8 km in length (Fig. 3). The tunnel, called the Exploratory Studies Facility ESF, is 7.6 m in diameter, with both north and south portals. As you enter the

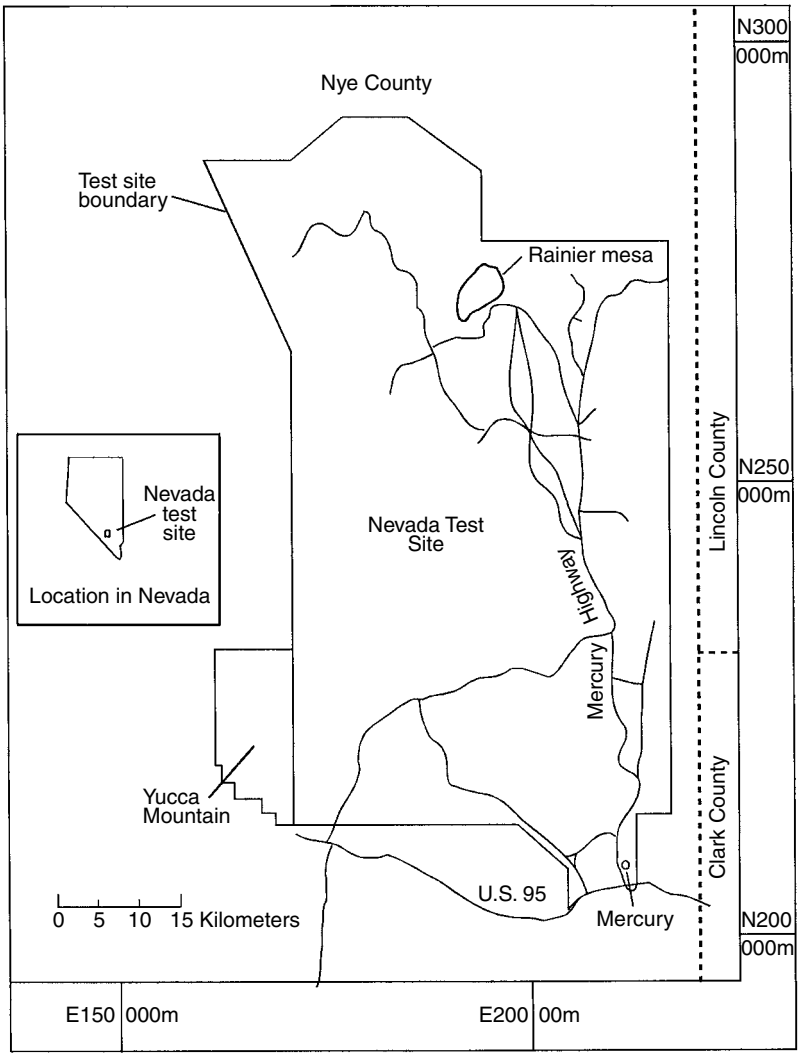


Figure 1. Location of Yucca Mountain relative to the Nevada Test Site. Reprinted from (3).

ESF, the tunnel slopes downward until reaching a welded tuff formation at the proposed repository depth: in the Topapah Springs member of the Paintbrush Tuff (Fig. 3). The ESF was mined using a tunnel boring machine (TBM) that was assembled just outside the north portal and disassembled outside the south portal when the shaft was completed. The TBM was electrically powered to reduce contamination of tunnel walls by diesel combustion products.

One aspect of repository design has been directed to the "near field," which is the environment immediately surrounding the waste packages. The near field is expected to initially experience high levels of radioactivity and heat following emplacement of high-level waste materials. As a result, water will be driven out of the near field by the intense heat. Desiccation of the immediate area will occur for an extended period of time. A second aspect of repository design concerns the "far field," an area surrounding the repository at a greater distance from the waste canisters. The far field merits investigation because it is a potential safe haven for microbes during the initial phase of the repository. This area is expected to be heated to a lesser degree and to contain higher than usual moisture content, conditions that will support microbial survival (6). In this article, we report on investigations that affect both the near- and far-field portions of the repository.

YUCCA MOUNTAIN

Yucca Mt. has been under investigation as a potential repository site since the mid 1970s. An extensive site characterization effort took place in the 1990s to investigate the geology, geochemistry, hydrology, and biology of the mountain. Microorganisms were known to be ubiquitous in subsurface environments (7–10), and therefore, investigation of their presence, abundance, metabolic potential, and taxonomy were of interest to the characterization effort. Characterization of the microbiology of Yucca Mt. was undertaken by scientists at several universities in conjunction with those at Los Alamos National Laboratory, a DOE facility in New Mexico.

Microbes are important to repository design because their activities may impact the long-term stability of the repository structures. Microbes can facilitate the

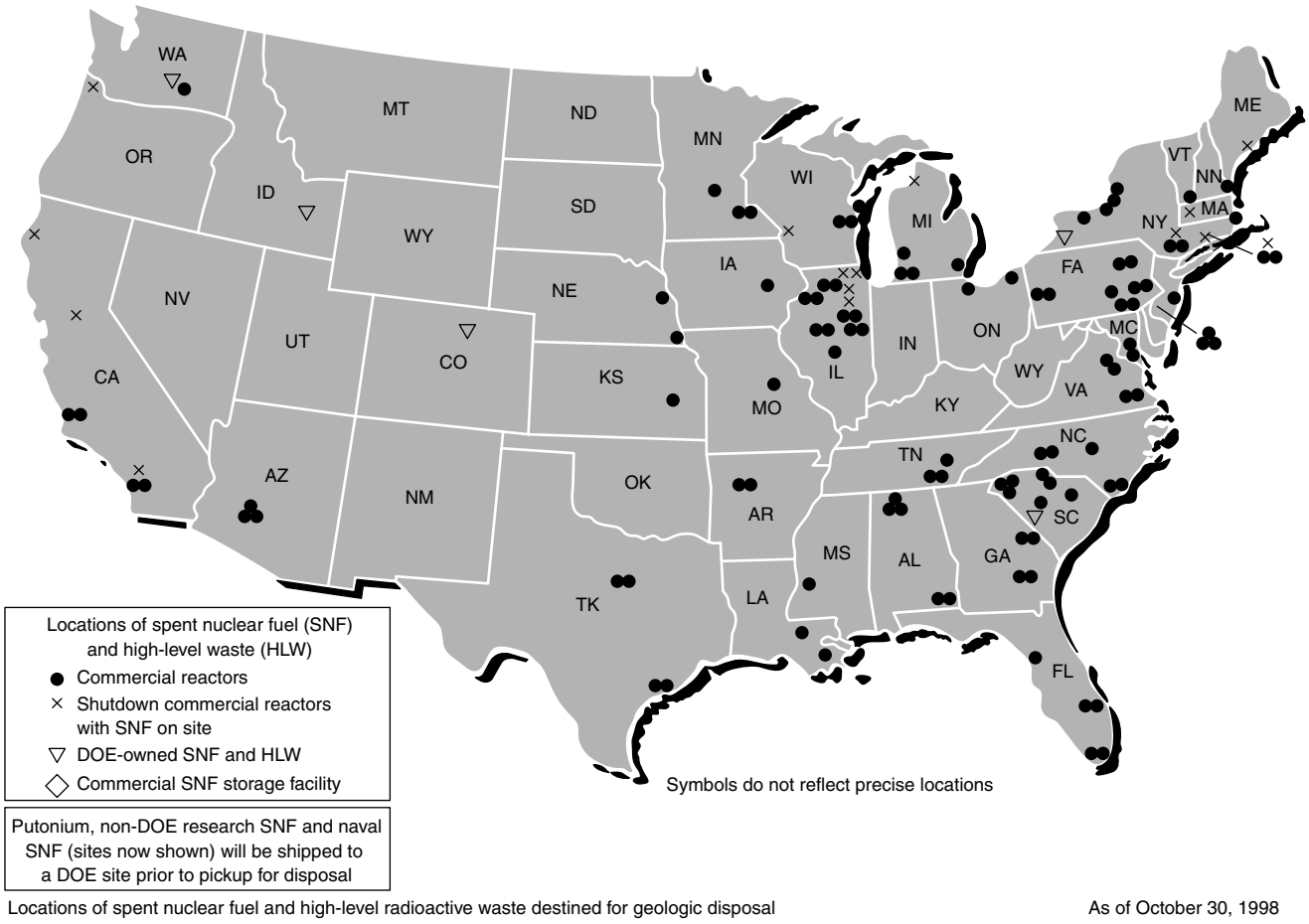


Figure 2. Locations of spent nuclear fuel and high-level radioactive waste destined for geologic disposal, as of October 1998. Reprinted from (4).

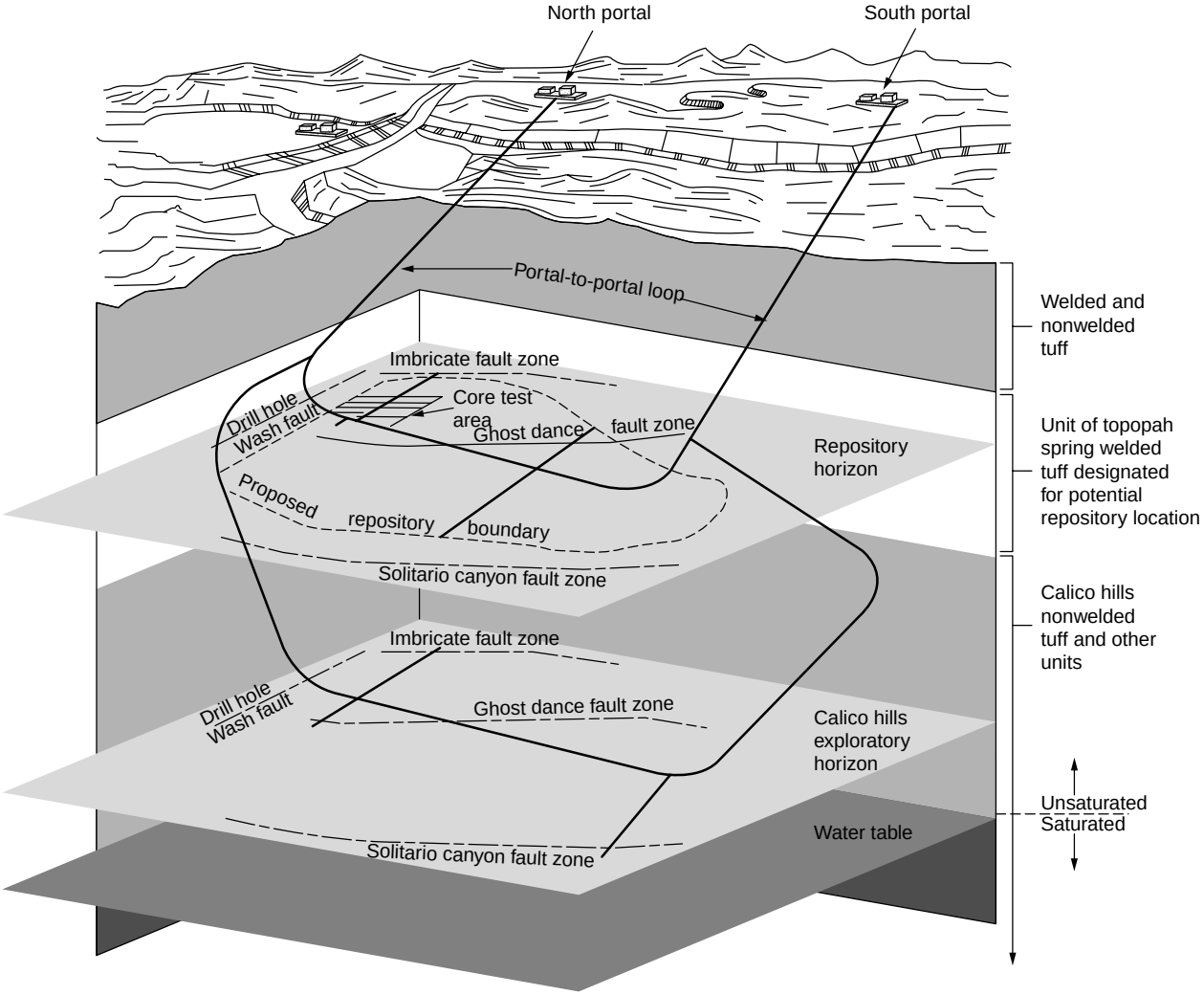
corrosion of waste-containment canisters (packages) and may bind radionuclides, enhance, or retard their transport. If microbes are present, they will impact repository stability. It is necessary to consider the potential activity of microbes, whether their actions are immediate or long-term, when building a repository that must last 10,000 years.

Site Characterization

During the excavation of the ESF in 1995, a team of investigators, led by Larry Hersman of the Los Alamos National Laboratory in New Mexico, sampled at nine locations in three geologic formations. The sampling scheme spanned the distance from the ESF portal to the repository depth (Fig. 4). The samples were obtained as the TBM progressed through the ESF; from rock remote to, adjacent to, and within large fractures shortly after the passage of the TBM (11). An extensive sampling and quality assurance plan was followed during the investigation (11,12).

The goal of this project was to determine bacterial abundance and diversity. Enumerations included total direct and culturable aerobic heterotrophic cell counts. Further analyses determined factors limiting microbial growth and activity and included enrichments to determine chemoautotrophic metabolic potential. Numbers of total and culturable aerobic heterotrophs were highly variable among samples and ranged from 3.2×10^4 to 2.0×10^5 and 1.0×10^1 to 3.2×10^3, respectively, per gram dry weight (11). Phospholipid fatty acid (PLFA) and diglyceride fatty acid (DGFA) analyses were used as a second measure of microbial abundance. This was particularly useful because the amount of PLFA is indicative of living biomass, whereas DGFA provides a measure of dead biomass. By comparison, total direct counting does not discriminate between live and dead cells and is less useful in determining community composition and metabolic status. The PLFA values were low compared with a similar subsurface environment at the NTS in which higher water content supported greater bacterial biomass (9,13). The nature of welded tuff and the lack of water in the vadose zone of Yucca Mt. were critical in repository site selection; nonetheless microbes were present in substantial numbers.

Experiments to determine factors that limit microbial growth involved nutrient addition to crushed rock; unamended crushed rock was used as a control. The act of perturbing the environment has been shown to stimulate microbial proliferation and perhaps resuscitation (14–16).



Note: Configuration of fault zones at depth is inferred.
Source: Presentation to the NWTRB, September 18–19, 1991. RERGBV5 P. 125. NWTRB/9–18/19–91 (RSN 1991).

Figure 3. Schematic of Yucca Mt. geologic structure showing current tunneling and proposed repository location, as well as a future monitoring tunnel below the repository. Reprinted from (5).

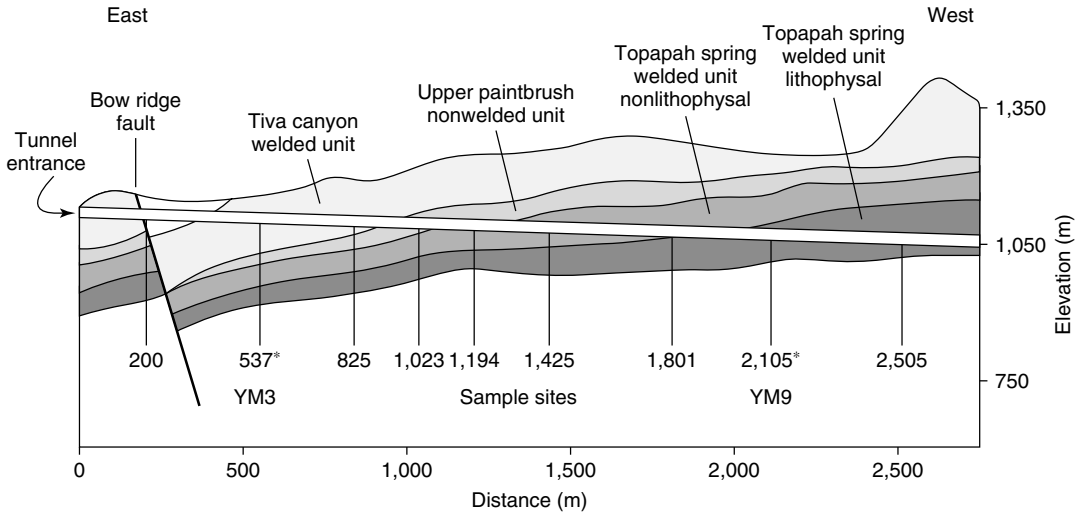


Figure 4. Cross section of Yucca Mountain showing the portal entry and proposed repository depths. Modified from (11) and reprinted from (3).

Crushed rock with no nutrient or water additions supported an increase in bacterial numbers. The original design of the repository placed crushed, mined rock around the packages as backfill in the repository structure. Because crushed rock demonstrated increased microbial numbers and activity, current design takes the potential for microbial activity into account and provides for quartz sand backfill and a protective drip shield over the packages (Fig. 5).

The addition of carbon substrates to the crushed rock further enhanced microbial growth compared with controls, a nitrogen source (ammonium nitrate), or a phosphorus source (potassium phosphate) during a 24-hr incubation (11). Mineralization of carbon substrates (glucose, glutamate, and acetate) was significantly increased by the presence of water but not further increased by nitrogen or phosphorus (11). It appears that lack of water is the most significant limiting factor to microbial growth and activity in the subsurface at Yucca Mt. Ironically, the TBM uses large amounts of water during the mining process so that previously dry rock increased in water content during the creation of the ESF. Additionally, water present in

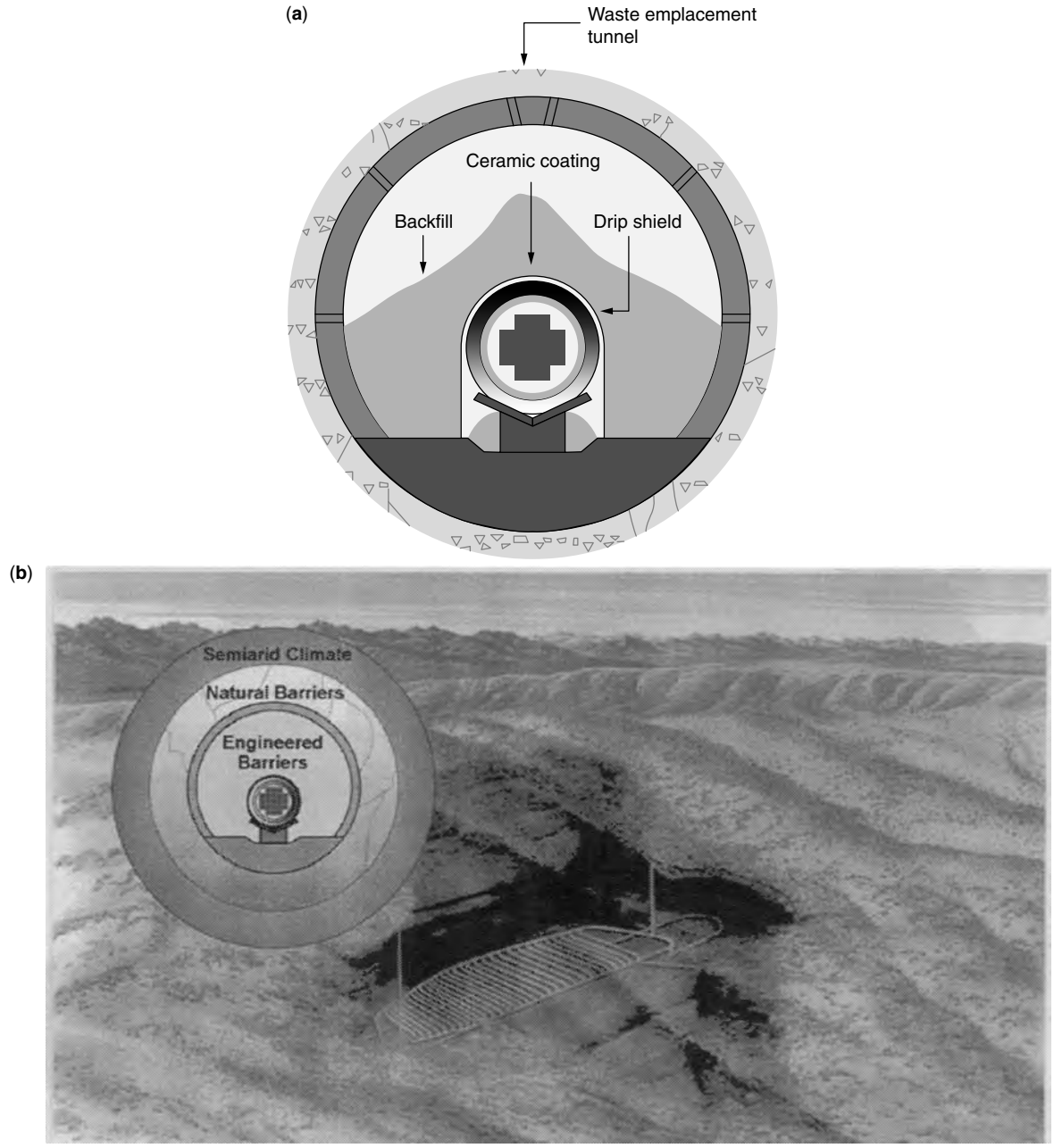


Figure 5. Engineered barrier system for high-level nuclear waste disposal. (**a**) Waste-containment package design with the drip shield separating the canister surface from the crushed backfill. (**b**) Schematic of the repository within the mountain. The three levels of barriers, engineered, natural, and climatic, are shown at the top left of the diagram. Reprinted from (17).

Table 1. Metabolites of Bacteria

Microbial Types	Carbon Source	Oxidizable Substrate	Oxidized Product	Terminal Electron Acceptor
Ammonia oxidizers	CO_2	NH_4^+	NO_2^-	O_2
Nitrite oxidizers	CO_2	NO_2^-	NO_3^-	O_2
Sulfur oxidizers	CO_2	H_2S, S, $S_2O_3^{-2}$	SO_4^{-2}	O_2; sometimes NO_3^-
Iron Oxidizers	CO_2	Fe^{+2}	Fe^{+3}	O_2
Aerobic heterotrophs	Organic Carbon	Organic Carbon	CO_2	O_2

the repository structure and augmented by the TBM mining process will be concentrated during the initial heating phase of the completed repository as it is driven out of the near-field environment.

Using the same samples taken during the site characterization described above, personnel in the Environmental Microbiology Laboratory at UNLV prepared enrichment cultures for autotrophic microorganisms (Table 1). Autotrophic enrichments for nitrifying bacteria (nitric acid production), sulfur-oxidizing bacteria (sulfuric acid production) and iron-oxidizing bacteria [ferric oxide (rust)] production were prepared in appropriate media (18,19). Sterile rock served as a negative control and American Type Culture Collection (ATCC) cultures of *Nitrobacter* and *Thiobacillus* spp. served as positive control cultures. These bacterial types were investigated because of their importance to microbially influence corrosion. Nitric acid produced by nitrifying bacteria has been shown to degrade concrete (20,21), and iron and sulfur-oxidizing bacteria have been implicated in the corrosion of metals (21–23). In fact, microbes are so proficient at degrading some structural materials that researchers have recently used them to degrade radioactively-contaminated concrete in a purposeful manner (24). Heterotrophic bacteria are also implicated in microbially influenced corrosion (MIC) (see Section Microbially Influenced Corrosion).

Results indicated that all three types of autotrophic bacteria were present, although not in every sample. Iron-oxidizing bacteria were most common, being enriched from nearly all samples. In fact, iron-oxidizing bacteria are common across the entire NTS. They were easily cultured from Yucca Flats (25,26) and Yucca Mt. (28) and, therefore, if some sites do not currently contain iron-oxidizing bacteria, they have the potential to become contaminated with them as the repository is constructed. Sulfur-oxidizing bacteria were not as common, but were present in many samples, whereas nitrifying bacteria were more infrequently found, often in only one of several replicates (3).

Heterotrophic bacteria from the same rock samples were enumerated using the method of Haldeman and coworkers (13). Isolates were obtained, purified, and tested by the MIDI system (Microbial ID Inc., Newark, DE). MIDI uses a fatty acid methyl ester profile to render the identification of an unknown bacterial culture. MIDI analysis often led to only a tentative identification of individual isolates from heterotrophic platings; approximately 50% of the isolates were identified. Because environmental microorganisms have not traditionally been characterized to the same extent as those of medical importance, many have not yet been added to the MIDI database. However, the relationship of unknown or unidentified isolates to those that were known was provided by dendrograms of the isolates (Fig. 6).

Although attempts were made to isolate anaerobic bacteria from each of the strata, none were isolated (11). The endolithic environment of Yucca Mt. and surrounding tuffs is highly aerobic and perhaps shipment of samples to another site contributed to the loss of anaerobes after sampling.

Phylogenetic Study

Following the general site characterization of microbiology of Yucca Mt., a phylogenetic study of the types of culturable and uncultured bacteria from two rock strata, YM3 and YM9, was initiated (3). YM3 rock samples were obtained from the highly fractured Tiva Canyon welded unit located 537 m into the ESF from the portal. The second sample, YM9, was obtained from the Topapah Springs welded unit more than 2,105 m into the ESF. YM9 is at the repository level, approximately 300 m below the mountain surface, and is composed of unsaturated tuff with relatively low permeability (Fig. 4). The samples were obtained in the same fashion as those described above in the general site characterization study. Because under typical conditions a mere fraction of the total microbial community can be cultured using plating or enrichment techniques (29–34), DNA was also extracted directly from rock at the two sites for analysis of uncultured and cultured microbial types. A complex series of manipulations, including amplification of DNA fragments using polymerase chain reaction technology (PCR), cloning techniques, transformation into *Escherichia coli*, and sequencing of the ribosomal RNA gene (rDNA), was undertaken to compare recovered rDNA sequences with those of cultured isolates.

The rDNA of all 19 of the YM3 cultured isolates revealed that they were from gram-positive genera. Seven of the 19 isolates were *Arthrobacter* spp., and all but one of the 19 appeared to fit into either the high G + C gram-positive bacterial group or the Actinomycete group; one isolate matched the low G + C *Bacillus* group most closely. MIDI analysis of the 19 isolates also identified them as belonging to gram-positive genera. Similar results were obtained from the YM9 site. Of the 27 isolates from the YM9 sample, 24 were clustered with the Actinomycete group, a broad grouping of bacterial genera. Among them, *Arthrobacter* spp. were the most common (6 isolates). The remaining three isolates matched gram-negative bacterial sequences of the genera *Pseudomonas* and *Acinetobacter*. Unlike isolates from the YM3 sample, nearly half of these isolates were unmatched to the MIDI database.

Direct DNA extraction, cloning, and sequencing yielded a somewhat different picture of bacterial community composition than that provided by heterotrophic plate counting. After restriction fragment length polymorphism (RFLP) screening to ensure that direct DNA clones were

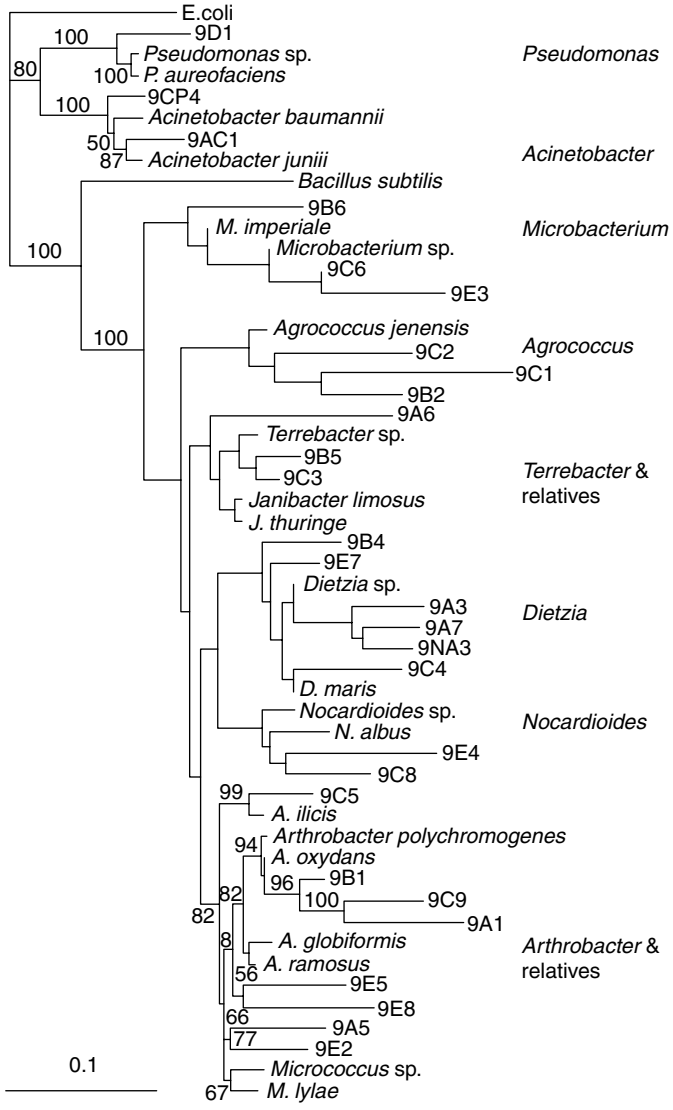


Figure 6. Dendrogram of isolates recovered from Yucca Mt. and analyzed by phospholipid fatty acid profiles (3). Dendrogram based on similarity indices. Euclidean distances of less than or equal to 25 are considered to be of the same genus (27).

each unique, cloned sequences were analyzed. Direct DNA clone sequences from YM3 suggested that the rock contained both gram-positive (43.5%) and gram-negative (56.5%) bacteria. Although gram-negative bacteria were not cultured, direct DNA analysis implied that they were abundant. Gram-positive cell types may be more capable of remaining culturable during long periods of isolation in extremely limited nutrient environments such as those found in the subsurface of Yucca Mt. The contrast between culturable isolates and direct DNA clones was even more dramatic in YM9. Here, only clones resulting from gram-negative bacterial DNA were recovered. Of 84 clones, 90% were from the γ proteobacteria and the remaining 10% were β proteobacteria. Obviously, the full nature of the microbial community was not appreciated when only culturable isolates were considered. For example, in YM9, gram-positive bacteria were cultured, however, they must have represented a minor portion of the bacterial DNA present in the rock because gram-positive sequences were not represented in cloning efforts. At first glance, one might think that perhaps gram-negative bacteria were preferentially amplified from direct rock extractions, however, bias in amplification was ruled out by experiments involving seeding of carbon-free rock with 10 known bacterial isolates, including both gram-negative and positive genera.

The discrepancy between culturable isolates and the nature of the community demonstrated by direct DNA extraction leads to the question: are gram-negative bacterial genes present in dead biomass or living biomass that is viable but nonculturable (VBNC)? Because of the extreme isolation and age of the rock under consideration (pore water age estimated by chlorine dating to be between 5 and 7×10^5 years (11,35); either scenario, or a combination, is possible. Gram-negative cells are well known to enter the VBNC state (28,34,36–39), therefore, some gram-negative DNA represented by direct DNA extraction may be present as VBNC. Alternatively, an indication that some of the microbial DNA is represented by dead biomass comes from the phospholipid fatty acid (PLFA) and diglyceride fatty acid (DGFA) analysis performed during the general site

characterization (samples taken before this study in the same rock formations). Approximately equal values of living (PLFA) and dead (DGFA) biomass lipids were found at YM3 and slightly more PLFA at YM9. In each case, if DNA in dead cells had been preserved, then much of the cloned DNA may have been from dead cells that lived during times up to 7×10^5 years ago.

APPLIED MICROBIOLOGY

Several issues related to the integrity of the repository at Yucca Mt. are considered in this section. The effect of radiation on bacterial survival is a primary concern for long-term stability of the packages and structural materials. Bacterial survival presents the potential for corrosion of the metal packages; whether bacteria survive in the near field or far field and return with condensing water as the near field cools. Biofilm formation, an initiator of the corrosion process, is a second area of concern. A third issue of concern is the potential of repeated upwelling of geothermally heated water within the mountain.

Effects of Radiation on Yucca Mt. Bacteria

Because most work concerning the effects of radiation on bacteria has been designed to deliver lethal doses of radiation for food preservation, little work has been done under conditions of low-dose exposure and with endolithic microbes. An experiment was conducted to supply relatively low doses of gamma radiation (1.63 Gy/min, 0 to 9.34 kGy total, over a six-day period) to native Yucca Mt. bacteria to test their ability to survive. The dose rate was designed to approximate that which might be experienced by bacteria in the far field (for unshielded canisters) or outer near field (if the canisters are shielded). Native bacteria were within rocks that had been aseptically sampled from Yucca Mt. and crushed before packing in sterile graduated cylinders (microcosms). Each microcosm began radiation treatment with approximately 10^7 CFU/gm wet weight. The number of culturable cells had been elevated by incubating the perturbed rock microcosms at room temperature for 14 days. Although culturable cells were obtained at time zero, by the time a dose of 2.33 kGy had been administered, no culturable cells remained. There were three lines of evidence that VBNC bacteria resulted from gamma irradiation doses of 2.33 to 9.34 kGy. Despite the lack of culturability, total direct and respiring cell counts remained relatively high in all samples, indicating that the cells may have lost culturability but not cellular integrity (direct cell count) or viability (respiring cell count). Additionally, rock slurries from irradiated microcosms contained microbes capable of utilizing carbon substrates in BIOLOG (40) carbon substrate microtiter plates through 7.01 kGy of irradiation. Likewise, PLFA and DGFA analysis revealed that although some cell death occurred at the early dosage points, considerable living biomass was still present at 2.33 kGy and remained stable throughout the remainder of the experiment (37).

VBNC cells have been resuscitated from the nonculturable state by a number of environmental stimuli, including changes in temperature, osmotic conditions, or nutrient addition (34,39,41–43). The VBNC cells produced during gamma irradiation were resuscitated by a 2-month long incubation at 4 °C. Culturable numbers of cells from each radiation time point were similar following resuscitation, that is, 10^3 to 10^4 CFU/gm wet weight. Nonirradiated controls, before and after resuscitation, contained 10^8 CFU/gm wet weight and maintained the considerable diversity seen in the original microcosms before irradiation. Irradiated microcosms lost diversity through cell death, and/or creation of VBNC cells. Although nonirradiated cells were capable of utilizing approximately 80% of 128 BIOLOG carbon substrates, irradiated microorganisms were only capable of utilizing 0 to 12% of the same substrates after irradiation. The same microcosm bacteria were capable of utilizing 25 to 30% of the substrates after radiation and subsequent resuscitation. There was no trend in substrate utilization associated with increasing radiation dose, however, carbohydrates and amino acids were most commonly metabolized following resuscitation.

MIDI analysis of isolates placed them into three general groups: approximately 50% mixed gram positives, approximately 30% mixed gram negatives, and approximately 20% *Gordona*/*Rhodococcus*. The percentage of culturable isolates falling into each broad group remained approximately the same between nonirradiated and resuscitated treatments, although the number of recovered colony types was reduced.

Although the time course of the experiments may seem brief compared with the life span of a functional repository, the experiments described here represent a first approach at investigating long-term radiation effects on environmental bacteria. That bacteria experienced a seemingly lethal radiation dose (by culturable counts) and resuscitated in just two months under a change in temperature is significant because cells will most likely remain viable and resuscitate in the repository at some point in the future. Perhaps cells closest to the repository will encounter radiation doses high enough to kill them, however, those more distant to the radiation sources may have the opportunity for resuscitation at a later date.

Isolation of Microbially Influenced Corrosion-Related Isolates from Yucca Mt.

Microorganisms have been implicated in the deterioration of organic, geologic, and metal materials in natural ecosystems (44). When the process involves metals, it is called microbially influenced corrosion or MIC. The bacterial communities associated with MIC can be divided into four metabolic categories: (*1*) sulfate-reducing bacteria (SRBs); (*2*) iron-oxidizing bacteria (FeOx); (*3*) exopolysaccharide-producing bacteria (EPS) and acid-producing bacteria (45,46). In an effort to evaluate the MIC corrosion potential of deep subsurface environments at the NTS, rock and water samples were taken from three locations to characterize and compare their indigenous microbial communities and determine which, if any, of three target MIC bacterial groups were present, that is, SRB, FeOx, and/or EPS-producing bacteria. Bacteria were isolated from Rainier Mesa, Yucca Flats, and Yucca Mt. on the NTS. Both Rainier Mesa and Yucca Mt. were

formed as the result of repeated volcanic ash deposition 9 to 13 million years ago (1) whereas Yucca Flats are the result of ancient alluvial deposits.

Iron-oxidizing bacteria are strictly aerobic and cause biologically initiated rust formation through the oxidation of reduced iron in metals; they were found in six of the eight samples analyzed. Sulfate-reducing bacteria are strictly anaerobic and result in the formation of hydrogen sulfide, which is directly corrosive to metals. Sulfate-reducing bacteria (SRBs) were found in only three of the samples; one from each site. Phospholipid fatty acid (PLFA) analysis of Yucca Mt. rock was indicative of sulfate-reducing bacteria and gram-negative bacteria in general. DNA extracted directly from Yucca Mt. rock also supported the idea of a predominantly gram- negative bacterial community (see earlier text).

Exopolysaccharide (EPS) producing bacteria may participate in both aerobic and anaerobic processes but function as the structural glue for the formation of biofilms on metal or other surfaces. Because of the nature of biofilms, there may be a very different electrochemical differential among parts of the biofilm, allowing for formation of microscale batteries and resulting in the dissolution of metal ions at the anode and deposition of same at the cathode (22,25,46). These activities are best known in aquatic systems in which metal corrosion is a serious problem (47).

Before human intervention at Yucca Mt., the repository level environment consisted of relatively undisturbed highly welded tuff containing very small amounts of water (4%) (25) and nutrients. Despite this, FeOx, SRBs, and EPS-producing bacteria have been cultured from Yucca Mt. We know that microbes of considerable diversity are present even within the deep repository level and that a significant number culturable even under the unfavorable conditions found in the welded tuff. Because of this, the potential for biofilm production and MIC exists under suitable conditions for growth and activity. Construction of the repository has and will continue to perturb the endolithic microbiota, provide considerable water through the drilling process, and add nutrients, either through the process of nutrient rearrangement by physical disruption, water flow, and/or the addition of nutrients (and additional microbes), simply because of human activity and equipment presence.

The next section describes work that has been done to date on MIC of metal candidates for repository packages.

Microbially Influenced Corrosion

Microorganisms known to produce MIC of metals were isolated from an alcove near the north portal of Yucca Mt. before the excavation of the ESF (25). These organisms included bacterial species capable of iron-oxidation, sulfate-reduction, and EPS production. Single bacterial types, combinations of two, and a combination of all three were placed in electrochemical cells designed to test for MIC (Fig. 7; 36). The test system consisted of a 1,020 carbon steel coupon (9.7 cm^2 surface area) embedded in soft R2A agar (0.5%, Difco), prepared using simulated Yucca Mt. pore water (27), in a 40×130 mm glass cylinder. Also, embedded in the soft agar was a platinum wire used as an electrode to supply electrical current to the

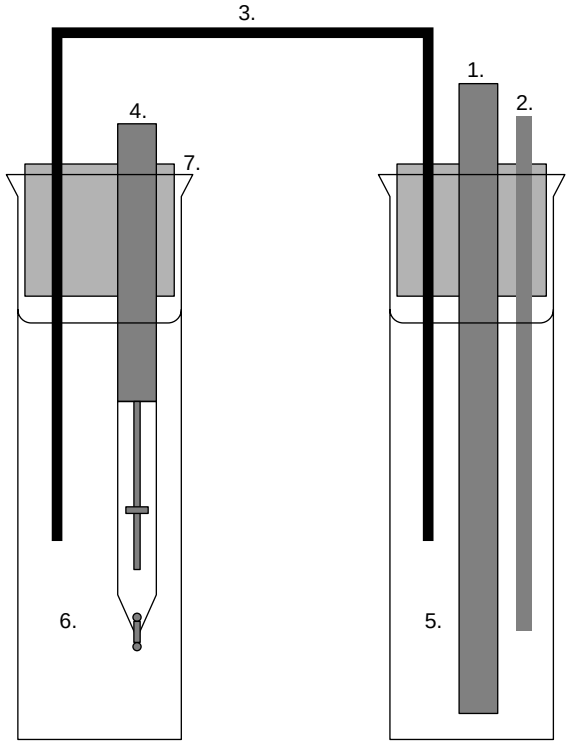


Figure 7. Schematic representation of the electrochemical corrosion cell apparatus (25).

metal coupon. The soft agar cylinder was connected to a reference electrode in a second cylinder containing 5% KCl by a salt bridge consisting of 1% KCl and 1.5% Bacto agar. The entire apparatus was sterilized before construction and monitored for bacterial contamination for three days before inoculation. One milliliter of each bacterial solution (10^7 CFU/ml) was deposited along the surface of the metal coupons just before sealing triplicate cylinders.

Corrosion potential was monitored using hardware from Gamry Instruments, Inc. (48,49). Using polarization resistance measurements, corrosion rates were calculated. Measurements were taken at multiple time points for up to 109 days. When acting independently, each bacterial type demonstrated a corrosion rate that was significantly higher than that of the uninoculated control: SRB > EPS-producers > FeOx. Combinations of microbial types demonstrated even higher corrosion rates with the highest rates occurring when all three bacteria acted in concert. A conditioning film formed on the uninoculated control coupons, although it represented a small fraction of the corrosion demonstrated when bacteria were also present.

In an attempt to measure corrosion potential in an environment more closely matching the Yucca Mt. repository, experiments using the electrochemical cell

apparatus were initiated using crushed Yucca Mt. rock and soft R2A inoculated with all three bacterial types. In addition, because placement of radioactive materials will increase the overall temperature of the surrounding rock environment, the electrochemical cells were incubated at ambient (25 °C), elevated temperature (55 °C), or ambient for three weeks followed by transfer to the higher temperature. Following incubation, biofilms were removed from the coupon surfaces by a freeze-fracture method and were sectioned for electron microscopy (50). Biofilm formation was seen in all inoculated tests at all temperatures. The uninoculated control produced a nonbiological film on the surface of the metal that resulted in low rates of corrosion. Elevated temperatures resulted in higher corrosion rates than the ambient treatment; however, the highest rates were observed when cells were allowed to colonize the coupon surfaces at ambient before a temperature upshift (51,52).

Investigations of MIC at Lawrence Livermore National Laboratory also confirmed the ability of Yucca Mt. microbes to corrode candidate materials for waste-containment packages in the repository. Under conditions similar to those described earlier, their studies demonstrated that titanium alloy, Alloy22, resisted corrosion induced by microbes to a much greater extent than other metals tested (53,54). They too have isolated a number of microorganisms from the endolithic environment of Yucca Mt.'s subsurface, many of which are responsible for MIC (55).

Characterization of microbial isolates and corrosion potential experiments have demonstrated that corrosion-associated microbes are present in Yucca Mt. and are capable of corroding 1,020 carbon steel (a material proposed as the outer shield for waste-containment packages). Elevated temperature enhanced corrosion effects and was particularly evident with the addition of crushed native rock. Hypotheses concerning the movement of water in the repository suggest that heat production will drive water out of the near field and into the far field for some period of time. The exact model for this depends on how the packages are made; some allow for a hotter repository and others a cooler one depending on the design of waste-containment packages. Meanwhile, high radiation levels will undoubtedly inactivate bacteria near the packages for a very long time. As the radiation decays, however, the near field will be cooler, becoming more hospitable for microbial life, and probably allowing surviving microbes to colonize the near field (56). Because it is known that Yucca Mt. bacteria can survive radiation (36,37) and corrode metal at elevated temperatures (51,52), it is probable that bacteria will colonize canister surfaces and be capable of corrosion at some time in the future.

If conditions are right, that is, water, nutrients, and permissive temperature, biofilms will form on canister surfaces. Biofilms formed with each bacterial type and the consortium from Yucca Mt., and therefore, it seems just a matter of time until they will form on metal canisters in the Yucca Mt. repository. The question is, how long will that take? Biofilm corrosive activity will require fairly short periods of time to breach standard carbon steel; however, the inner layer of the package is planned to be made of a titanium alloy.

Biofilms can also form on structural materials other than metal surfaces and are known to deteriorate virtually all materials, including concrete and synthetic polymers (44). To maintain structural integrity, MIC of other repository materials needs to be considered as well. It is difficult to predict what will happen in the next 10,000 years, and therefore, our predictions can be nothing more than educated guesses.

FUTURE STUDIES

Researchers in the Environmental Microbiology Laboratory at UNLV continue to investigate issues of interest to the stability of the Yucca Mt. repository. Biofilms are the most common cause of MIC. Their presence announces the eventual deterioration of a surface, whether it be of metal or other material. If boundaries for biofilm formation can be set using factors of heat and humidity, then models of the repository can more accurately predict the time needed for breaching the packages. Experiments using native rock microcosms with embedded metal coupons are currently under investigation.

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NUCLEIC ACIDS EXTRACTION FROM SOILS.

See BIODIVERSITY IN SOILS: USE OF MOLECULAR METHODS FOR ITS CHARACTERIZATION

NUTRIENT CYCLING: ROLE OF BACTERIOPLANKTON.

See PLANKTONIC MICROORGANISMS: BACTERIOPLANKTON

NUTRIENTS USE BY MARINE MICROORGANISMS.

See INORGANIC NUTRIENT USE BY MARINE MICROORGANISMS

O

OCCURRENCE OF PROTOZOA IN SPENT FILTER BACKWASH WATER

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In a conventional water treatment plant, raw water is treated by destabilizing the electrostatic charge on particles (coagulation), agglomeration of the destabilized particles (flocculation), settling of the heavier particles, and by filtration of the remaining particles. Filters have a limited capacity to retain solids, and therefore, must be backwashed to remove the retained solids. Accumulation of solids in a filter bed also creates resistance to the flow of water (measured as loss of head for the filter bed). Backwashing may be performed based on a number of different criteria — a terminal headloss, a fixed time interval, or breakthrough of solids (measured as turbidity or particle counts). Generally, plant finished water is used for backwashing the filters. The spent filter backwash water (SFBW) may be treated before its reuse, or disposal. Options for disposal may include discharge to a sewer or a receiving stream. Because backwash water may contain disinfectants and other chemicals, which may be harmful to a stream's biological life, direct discharge to streams is generally restricted. Similarly, discharge to sewers may be restricted based on the constituent and total quantity of the SFBW. However, for many water treatment plants, particularly in arid or water-scarce areas with limited raw water resources, reuse of the backwash water is a necessity.

REGULATION OF WASH WATER RECYCLE

The 1996 Safe Drinking Water Act Amendments require that the U.S. Environmental Protection Agency (USEPA) regulate the recycle of spent filter backwash water within the treatment process for public water systems by fall, 2000. In the Filter Backwash Recycling Rule (FBRR) (1), the USEPA has outlined the following provisions for all public water systems using surface water or ground water under the influence of surface water:

- The FBRR requires that recycled filter backwash water, sludge thickener supernatant, and liquids from dewatering processes must be returned to a location such that all processes of a system's conventional or direct filtration including coagulation, flocculation, sedimentation (conventional filtration only) and filtration, are employed. Systems may apply to the State for approval to recycle at an alternate location.
- Systems that practice recycle, must notify State (primacy) agencies and provide information on the origin of all recycle flows, the hydraulic conveyance used to transport them, and the location where they are recycled back into the plant; and the typical recycle flow (gpm), highest observed plant flow experienced in the previous year (gpm), the design flow for the treatment plant (gpm), and the State-approved operating capacity for the plant.
- Systems must collect and maintain information on recycle practices, for review by the State, on the all recycle flows and the frequency with which they are returned, the average and maximum backwash flow rate through the filters and the average and maximum duration of the filter backwash process in minutes, the typical filter run length and a written summary of how filter run length is determined (headloss, turbidity, time etc.), the type of treatment provided for the recycle flow, and data on the physical dimensions of the equalization and/or treatment units, typical and maximum hydraulic loading rates, type of treatment chemicals used and average dose and frequency of use, and frequency at which solids are removed where such units are used. The State after evaluating the information may require a system to modify their recycle location or recycle practices.

The concern for EPA and water purveyors is that recycling of SFBW (and other waste streams generated within a water treatment plant) can concentrate contaminants (microbes and chemicals) to a level that may be beyond the treatment capability of a plant, and thereby, potentially impact public health. The microbe of current concern in water treatment is *Cryptosporidium*, which is resistant to conventional water treatment disinfectants, and therefore, its physical removal during the treatment is very important. Craun and coworkers (2) reported 19 outbreaks of cryptosporidiosis in the United States with ten of them associated with community, noncommunity and private water systems (2). A number of these outbreaks have occurred at water utilities where recycle of a waste stream was identified as a contributing cause (3).

CURRENT SFBW RECYCLE PRACTICES

A survey of treatment schematics obtained from 362 water treatment plants serving populations greater than 100,000 people, showed that 226 plants practiced recycle of a waste stream (1). Of the plants that recycle, 88% used surface water as a source of supply and 65% provided treatment before recycle of the waste stream. Water was recycled to a point before the rapid mix process by 70% of these plants, before the sedimentation process by 15%, and before the filtration process by 10%.

Another survey conducted by the American Water Works Association (4) showed that 78% of 335 plants that recycle SFBW used surface water as a source of

supply. Conventional treatment was used by 51% (coagulation, sedimentation, and filtration), 21% used upflow clarification, 14% used lime softening, 10% used direct filtration, and 4% used other type of treatment schemes. SFBW was recycled in 83% of these plants to the head of the plant, 11% to a point before the sedimentation basin, 4% to the sedimentation basin, and 2% before the filtration process. The average generation of SFBW was 2.5% (2–10%) of the average water production. No treatment was provided in 30% of these plants before recycling SFBW; whereas, the others practiced sedimentation (54%), equalization (20%), sedimentation and equalization (14%), lagoon (4%), or other processes (7%) before the recycle of SFBW.

Occurrence of *Cryptosporidium* and *Giardia* in SFBW

Giardia lamblia is a flagellated protozoan first documented in 1966 as a causative agent of waterborne intestinal disease in the United States (5). *Giardia* forms an environmentally resistant cyst, which allows for the extended survival of the parasite in surface and treated drinking water. *Cryptosporidium parvum*, the protozoan responsible for cryptosporidiosis, is ubiquitous in surface water supplies and is resistant to conventional disinfectants (chlorine and chloramine) used in water treatment (6–12). The organism forms an environmentally resistant oocyst that permits prolonged survival in the aquatic environment. The Interim Enhanced Surface Water treatment Rule (13) lowered the turbidity limits to 0.3 nephelometric turbidity units (NTU) (in 95% of four-hour samples) as a means to control (by physical removal) *Cryptosporidium* in treated drinking water. A well-operated conventional water treatment plant is thought to remove 99–99.9% or more of the Cryptosporidium oocysts in water. Sedimentation (or clarification) processes play an important role in removing the majority of oocysts during the treatment process, with the remaining organisms removed by filtration. Backwashing of filters result in a major recyclable waste stream within a plant that can impact the source water quality. Solids (including microbes) retained on a filter can be recycled back to the source water if no further treatment is provided for the SFBW.

Table 1 summarizes the occurrence of protozoa reported by various studies. Rose and coworkers (14) reported *Cryptosporidium* at 686,900/100 L and 2,430,600/100 L in two samples collected from backwash water of rapid sand filters. Colbourne (15) reported *Cryptosporidium* at 1,000,000/100 L in the SFBW during an investigation following an outbreak of cryptosporidiosis in England. Supernatant from a settling tank treating the SFBW still had *Cryptosporidium* in the range of 100,000/100 L a few days after the outbreak. During this outbreak, 30% of the distribution system drinking water samples were positive for *Cryptosporidium*. The outbreak was attributed to dumping of animal wastes containing *Cryptosporidium* that were concentrated on the filters because of the continued recycle of spent filter backwash water.

It should be noted that measurement of Cryptosporidium oocysts in SFBW is very difficult as a result of the presence of large amounts of particulate material and interfering chemicals. Because Cryptosporidium oocysts are measured using an immunofluorescence microscopic technique, the large amount of particulate material minimizes the sample that can be analyzed to volumes typically less than one Liter. The small volume analyzed results in large multiplication factors when reporting counts per 100 L. The presence of interfering chemicals, particularly coagulation polymers, causes the fluorescent antibodies used in the immunofluorescence assay to nonspecifically stick to particle surfaces, increasing the likelihood that algal cells or other particles to be mistaken for Cryptosporidium oocysts. The reader, therefore, should be aware that the variability in the results that follow is highly influenced by the analytical methodology and the experience of the microscopist.

A survey of 66 water treatment plants in 14 states, found *Cryptosporidium* levels 57 to 61 times higher in the initial backwash water than in the corresponding raw water (7 to 108 oocysts/100 L) (8). *Giardia* was 12 to 16 times higher in the initial backwash water than compared to the corresponding raw water levels (4 to 32/100 L). *Cryptosporidium* was found in SFBW samples; the same time as it was found in the filtered plant effluent samples. This observation confirms the finding of Colbourne (15) that a filter can continue to concentrate protozoa, a few of which can be released into the filter effluent. On the basis of a survey of water treatment plants in 17 states, Rose and coworkers (7) reported a geometric average *Cryptosporidium* concentration of 217/100 L in SFBW samples.

Cornwell and Lee (16,17) documented the fate of *Giardia* and *Cryptosporidium* in two plants in Pennsylvania employing different type of treatment trains. One plant (Plant 1), employing direct filtration treated raw water from a combination of surface water, deep and artesian wells, and springs. SFBW at Plant 1 was treated in a clarifier before it was recycled back to the head of the plant. For the first sampling event, raw water *Cryptosporidium* level of 6/100 L increased to 40/100 L after addition of the clarified SFBW recycle stream that contained 141/100 L oocysts. The SFBW contained 902/100 oocysts indicating an 84 percent removal of oocysts in the backwash clarifier. *Giardia* in raw water at 3/100 L increased to 7/100 L after the addition of clarified SFBW recycle stream containing 86/100 L cysts. The SFBW contained 1,350/100 L cysts resulting in a 94% removal in the clarifier. For the second sampling event, *Giardia* was not found in raw water, SFBW, or clarified SFBW samples. However, raw water *Cryptosporidium* level of 140/100 L reduced to 45/100 L after the addition of clarified SFBW stream consisting of 750/100 oocysts. The low levels of oocyst in the clarified water compared with both the raw water and recycle stream, is attributed to the inaccuracies of the immunofluorescence method. SFBW contained 850/100 L oocysts, indicating only a 12% removal of oocysts in the backwash clarifier.

The Plant 2 received source water from an impounded reservoir, a spring and three wells, and employed adsorption clarification, and mixed media filtration. In addition to SFBW, a wastewater clarifier treated other

Table 1. Summary of Protozoa Occurrence in Filter Backwash Water

Reference	Location	No. of WTP Sampled	Sample Type (No. of Samples)	*Cryptosporidium* Level, #/100 L		Giardia Level, #/100 L	
(14)	U.S.A.	2	SFBW (2)	Sample1: 686,900 Sample2: 2,430,600		NR	
(15)	Thames Water, U.K.	1	Raw (unknown) SFBW (1) Supernatant[1] (1)	0.2 to 1,400 >1,000,000 >100,000		 NR	
(7)	U.S.A.	17 states	SFBW (subset of 257 Samples)	217[2]		NR	
(8)	U.S.A.	66 in 14 states	Raw Initial SFBW	7 to 108 57 to 61 times raw water level		4 to 32 12 to 16 times raw water level	
(16)	U.S.A.	2	Plant 1: Raw (1) Mixed influent[3] (1) SFBW (1) Supernatant[1] (1)	*Round* 1 6 40 902 141	*Round* 2 140 45 850 750	*Round* 1 3 7 1,350 86	*Round* 2 BDL NR BDL BDL
(17)	U.S.A.	2	Plant 2: Raw (1) Mixed influent[3] (1) SFBW (1) Supernatant[1] (1)	*Round* 1 13 30 16,613 80	*Round* 2 20 476 NR 420	*Round* 1 290 160 16,513 70	*Round* 2 60 79 NR BDL
(18)	Germany	1	*Centrifugation Method* Raw (8 positive out of 12) SFBW (8 positive out of 11) *Cartridge Filter* SFBW (33 positive out of 39)	 0.8 to 109 1 to 69 0.8 to 252		 NR	
(19)	Germany	1	SFBW[4] (1)	150		NR	
(20)	Pittsburg, U.S.A.	1	Raw (11 positive out of 15) Filtered (2 positive out of 15) SFBW (8 positive out of 15) SFBW (2 positive out of 15)	43 0.4 321		42 BDL 59	
(21)	U.S.A.	25	*IFA Method* Raw (17 positive out of 146) Raw (44 positive out of 146) SFBW (7 positive out of 148) SFBW (12 positive out of 148) *CC-PCR Method*[5] Raw (6 positive out of 122) SFBW (9 positive out of 121)	 108 175 Qualitative method Qualitative method		 89 203 NA NA	

Notes: [1]Supernatant from settling basin treating spent filter backwash water.
[2]Geometric mean concentration.
[3]Sample after addition of recycle stream.
[4]Sample taken 10 minutes after start of backwash cycle.
[5]Cell culture polymerase chain reaction method identifies live, infectious *Cryptosporidium*.
NR — not reported, NA — not applicable, BDL — below detection level.

waste streams (clarifier flush water, sand bed filtrate, filter-to-waste, and clarifier- to- waste) generated with in the plant. Supernatant from the wastewater clarifier was recycled back to the head of the plant. As was the case in Plant 1, monitoring at Plant 2 consisted to two sampling events. The raw water *Cryptosporidium* level of 13/100 L increased to 30/100 L after addition of wastewater clarifier recycle stream containing 80/100 L oocysts. The influent to wastewater clarifier contained 16,613/100 L oocysts in the SFBW and 2,600/100 L oocysts in the adsorption clarifier flush. *Giardia* in raw water at 290/100 L decreased to 160/100 L after the introduction of wastewater clarifier supernatant that contained 70/100 L cysts. The SFBW contained 16,513/100 cysts, whereas the adsorption clarifier flush contained 5,200/100 L cysts.

For the second sampling event, protozoa were analyzed in the individual and combined raw water and wastewater clarifier supernatant samples only. Raw water *Cryptosporidium* and *Giardia* levels of 20/100 L and 60/100 L, respectively, increased to 476/100 L and 79/100 L, respectively, after the addition of the wastewater clarifier supernatant stream. *Cryptosporidium* in the

Table 2. Occurrence of *Giardia* and *Cryptosporidium* in SFBW Measured by the IFA Method

Sample Location	*Giardia*			*Cryptosporidium*		
	Positive, %	Observed Level, #/100 L	Adjusted* Level, #/100 L	Positive, %	Observed Level, #/100 L	Adjusted* Level, #/100 L
			Geometric Mean			
Raw Water ($n = 146$)	30.1 ($n = 44$)	89	420	11.0 ($n = 17$)	108	470
SFBW ($n = 148$)	8.1 ($n = 12$)	203	3,200	4.7 ($n = 7$)	175	3,100
			90th Percentile			
Raw Water ($n = 146$)	30.1 ($n = 44$)	360	1,710	11.0 ($n = 17$)	310	1,350
SFBW ($n = 148$)	8.1 ($n = 12$)	890	13,900	4.7 ($n = 7$)	570	10,200

*Counts were adjusted for recovery efficiency. Data are based on samples positive for cysts or oocysts.

clarifier supernatant was observed at 420/L; whereas, *Giardia* was below detection level.

Karanis and coworkers (18) reported the occurrence of *Cryptosporidium* in raw and SFBW samples by using two separate sample collection techniques at a treatment plant in Germany. By using a continuous flow centrifugation method, eight out of 12 raw water samples showed *Cryptosporidium* in the range of 0.8 to 109/100 L. Using the same method, *Cryptosporidium* was observed in 8 out of 11 the SFBW samples ranging from 1 to 69/100 L. Using cartridge filters, 33 out of the 39 SFBW samples were positive for *Cryptosporidium* ranging from 0.8 to 252/100 L. At another plant in Germany, *Cryptosporidium* was reported at a level of 150/100 L in a SFBW sample that was collected 10 minutes after the start of the backwash cycle Karanis and coworkers (19).

States and coworkers (20) reported results of a fifteen-month protozoa monitoring study of raw, settled, filtered, and SFBW samples at the Pittsburg Drinking Water Treatment Plant. In raw water, *Cryptosporidium* (11 out of 15 samples) and *Giardia* (9 out of 15 samples) were observed at geometric mean levels of 43/100 L and 42/100 L, respectively. In contrast, only *Cryptosporidium* (2 out of 15 samples) was found at a geometric mean level of 0.4/100 L in filtered water. For the SFBW samples, *Cryptosporidium* (8 out of 15 samples) and *Giardia* (2 out of 15 samples) were detected at geometric mean levels of 321/100 L and 59/100 L, respectively. On the basis of the data, the level of *Cryptosporidium* in SFBW samples was approximately 7.5 times higher than that observed in raw water samples.

In the most comprehensive study of protozoa in SFBW waters, Arora and coworkers (21) collected paired raw and SFBW samples six times from 25 water systems across the United States. Source waters included: rivers (18 sites), creeks (3 sites), and reservoirs or lakes (4 sites), and were collected on the same day as the SFBW samples. SFBW samples were collected either from holding tanks (immediately following a filter backwash to avoid settling of solids) or by compositing a number of equally spread samples during the backwash cycle. *Giardia* and *Cryptosporidium* were analyzed by the immunofluorescence assay (IFA) (10) and, *Cryptosporidium* was analyzed by the cell culture polymerase chain reaction (CC-PCR) method that measures live and infectious oocysts (22).

Giardia and *Cryptosporidium* were detected using the IFA method in 30% and 11% of the raw water samples, respectively (Table 2). The adjusted (for recovery) geometric mean for *Giardia* and *Cryptosporidium* in the raw water samples was 420 and 470 per 100 L, respectively. *Giardia* and *Cryptosporidium* were detected in 8.1% and 4.7%, of the 148 SFBW samples, respectively. The adjusted geometric mean for *Giardia* and *Cryptosporidium* in SFBW samples was 3,200 and 3,100 per 100 L, respectively. The adjusted SFBW protozoa levels (geometric mean for positive samples) were approximately seven times higher than raw water levels.

To account for peak occurrences of protozoa (that might overwhelm treatment), the 90th percentile of *Giardia* and *Cryptosporidium* occurrence in raw water, and SFBW samples was determined. Although these levels were approximately 3 to 4.5 times higher than the geometric mean, the ratio of protozoa in SFBW was still approximately seven to eight times higher than in the raw water. This ratio is reasonable if, for example, a plant uses 2% of its total production to wash the filters, then the expected protozoa levels would be 50 times higher in the SFBW compared with the raw water (assuming no removal of cysts or oocysts during the coagulation/sedimentation process). However, if one assumes that coagulation and sedimentation removes between 1 and 2 logs of protozoa, then levels in the backwash water would range between 0.5 and 5 times the raw water level.

A total of 122 raw water and 121 SFBW samples were analyzed by both the IFA and CC-PCR methods. Infectious *Cryptosporidium* were detected in six raw water (4.9%) and nine SFBW samples (7.4%) (Table 3). Occurrence of live *Cryptosporidium* in the SFBW samples indicates the organism can penetrate treatment barriers to at least the

Table 3. Summary of *Cryptosporidium* Detection for Paired SFBW Samples

Assay	Raw $n = 122$	Backwash $n = 121$
Positive flotation IFA for *Cryptosporidium*	13.1%	5.8%
Positive CC-PCR for infectious *Cryptosporidium*	4.9%	7.4%

point of filtration and be present in the filter backwash water. The results highlight the need to carefully handle the disposal or recycle of SFBW to minimize the public health impact from infectious protozoa.

A comparison of detection of *Cryptosporidium* oocysts in raw water showed that 11% of the samples positive by IFA and 4.9% positive by CC-PCR (Table 3). This result is not surprising since IFA detects all oocysts, dead or infectious. However, infectious *Cryptosporidium* were detected in more SFBW samples (7.4%) than total oocysts detected by IFA (4.7%). These results probably are a reflection of the splitting of samples containing very low numbers of oocysts between the IFA and CC-PCR assays; the additional sample handling and potential loss of oocysts required for IFA method; and possibly, the higher sensitivity of the CC-PCR assay than the IFA technique (22).

Overall, *Giardia* was detected in raw water samples at least once for 18 sites (72%), and for 78% of the positive sites, *Giardia* was observed more than once. *Giardia* was detected in SFBW samples at seven sites (28%) with two sites positive, more than once. *Cryptosporidium* was detected in raw water samples from 16 sites (64%) at least once by either the IFA or CC-PCR methods, and a number of times, at 25% of the positive sites. *Cryptosporidium* was detected in the SFBW samples by IFA or CC-PCR methods at 10 sites (40%), with three of these sites positive, more than once.

OCCURRENCE OF OTHER WATER QUALITY PARAMETERS

Besides protozoa, other water quality parameters (dissolved organic carbon, disinfection by-products, metals, etc.) can also impact water treatment and/or water quality upon recycle of the SFBW. Because finished drinking water is typically used for filter backwashing, the presence of disinfectants and disinfection by-products (DBPs) can increase the overall DBP levels in treated water upon recycling of the SFBW. These increased DBP levels can impact water utilities at the borderline for the new DBPs maximum contaminant limits (23). Use of metallic coagulants in water treatment can introduce aluminum and iron to the raw and other metals such manganese and zinc can increase upon continual recycling. Recycling of these metals can be either beneficial (e.g., coagulation) or harmful (e.g., increased zinc loading on wastewater treatment plants), depending on site-specific water quality and treatment. Table 4 summarizes the average and 90th percentile results for a number of water quality parameters measured in raw and SFBW samples. The impact of wash water recycle ranged from an average threefold (for dissolved organic carbon) to a 92-fold (for trihalomethanes) compared to raw water samples. Peak levels (measured as the 90th percentile of occurrence) could further impact recycled water (Table 4).

TREATMENT STRATEGIES FOR SFBW

The level of treatment required for SFBW before recycle will vary from site to site, depending on the treatment process and water quality objectives. A reasonable assumption may be to treat the SFBW to the level of the raw water quality. Various treatment options are available to water treatment plants, which include equalization, sedimentation, filtration, and chemical oxidation.

Equalization

Equalization of the recycle flow involves reusing a constant amount of backwash water over a period. To achieve equalization, a backwash holding tank of sufficient size is necessary to store all the water used to wash the treatment filters until it can be recycled. The size of this holding tank will depend on the projected number of filters to be washed, the volume of water used to wash a filter, and the rate of recycle. If a plant, for example, used 2% of the total water produced to backwash the filters, and metered the recycled backwash water at a constant 2% of the plant production, these rates would be equalized. The advantage of this approach is that (*1*) changes in raw water quality (related to recycle) are minimized because the treatment process always experiences the same mix of raw and backwash

Table 4. Chemical Parameter of Raw and SFBW Samples

Parameter	Raw Water			Backwash Water			Increase (fold)	
	Range	Average	90%	Range	Average	90%	Average	90%
Dissolved organic carbon, mg/L	0.7–5.4	2.4	4.0	0.8–191	8.0	15.6	3.3	3.9
Trihalomethanes, μg/L	ND-21.8	0.6	0.7	ND-198	55.0	95.5	91.7	136.4
Haloacetic acids-6, μg/L	ND-21.5	1.9	3.4	ND-211	45.5	94.7	24.0	27.9
Bromide, mg/L	ND-0.68	0.033	0.04	ND-0.46	0.027	0.06	0.8	1.5
Aluminum, mg/L	ND-8.7	0.57	1.35	ND-145.8	14.8	44.2	26	32.7
Iron, mg/L	ND-1.8	0.83	1.78	ND-132	9.1	27.6	11	15.2
Manganese, mg/L	ND-3.0	0.09	0.17	0.01-17.9	1.4	3.9	16	21.8
Zinc, mg/L	ND-0.2	0.02	0.09	ND-1.0	0.1	0.30	5.0	3.2

water and (*2*) contaminants in the backwash water are diluted (about 50-fold) with the source water. However, the cost and space requirements for the holding tanks can be expensive.

Sedimentation

Once a holding tank is constructed, removal of solids from the backwash water can be achieved by: (*1*) allowing sufficient time for settling or (*2*) accelerating settling through the addition of polymeric coagulants. In the absence of chemical treatment, there was no reduction of SFBW particles with a detention time of 36 minutes and a overflow rate of 1.3 gpm/ft^2 (3.3 m/h) (21). When chemical treatment was applied (as either an anionic, cationic polymer, or ferric chloride), particle counts were reduced by about 1 log and turbidity levels were lowered by more than 90%. Polymeric pretreatment reduced *Giardia* and *Cryptosporidium* levels by greater than 2 log and *Clostridium* and *Bacillus* levels by more than 2.5 and 1.5 log, respectively. On the basis of the 7- to 8-fold increase in protozoa levels observed in SFBW, the authors conclude that sedimentation with chemical addition is adequate treatment to control the risk of *Cryptosporidium* in recycled wash water. These results were similar to Cornwell and Lee (16) who reported that a nonionic polymer was effective in increasing particle removals to more than 90% at overflow rates of 0.2 to 0.3 gpm/ft^2 (0.5 to 0.75 m/h).

Dissolved Air Flotation

Dissolved Air Flotation (DAF) may be a viable clarification process depending on the quantity of solids in the SFBW. McTigue and coworkers (24) reported a 1 log turbidity removal from SFBWs ranging in turbidity from 20 to 200 NTU without any addition of a polymer. Overall, DAF with polymer worked as well, or better than sedimentation with polymer. On an average basis, DAF with polymer removed 2.5 log of turbidity and particles from SFBWs.

Filtration

Removals of *Giardia* from 1.5 to 5.1 log and *Cryptosporidium* removal of 1.5- to 5.2-log have been reported for conventional coagulation/sedimentation/granular media filtration studies (10,25–27). Similar removals (*Giardia:* 2.9 to 5 log; *Cryptosporidium:* 1.3 to 5.9 log) have also been reported for direct filtration (25,26,28,29). However, because of the particle concentration of SFBW, a filtration option would have to be preceded by clarification.

Other filtration processes have been used of treatment of SFBW, including a backwashable depth filter, bag filters, and membrane filters (21,24,30–33). Using nylon (2.0 μm nominal rating) filters in the backwashable depth filter, removal of *Cryptosporidium* (3 log), *Giardia* (1.2 log) and *Clostridium* (1.5 log) was achieved at a flow rate of 6 gpm (23 L/min). A polybutylene terephthalate (PBT) filter with a nominal rating of 5 μm resulted in lower removal efficiencies (*Cryptosporidium*, 3 log; *Giardia*, 0.5 log; and *Clostridium*, 1-log removals). Goodrich and coworkers (31) reported *Cryptosporidium* removal ranging from 0.3 to 3.2 log for bag filtration. Membrane filtration (micro- and ultrafiltration) can remove protozoa more than 3.5 log (24,30–33) reported over a 3-log removal of particles and more than 2.5 log of turbidity from six separate SFBW samples by filtration and microfiltration. Again, because of the particle concentration of SFBW, these options would have to be preceded by clarification.

Disinfection

Because of the increased dissolved organic carbon and other oxidant demand-producing compounds in SFBW samples, clarification is typically necessary before a disinfection option. Arora and coworkers (21) examined the oxidant demand for potassium permanganate, chlorine dioxide, and ozone for SFBW samples from five water utilities and showed that the potassium permanganate demand was approximately 5.5 times higher for SFBW with particles than in samples without particles. The presence of particles in the SFBW samples increased the chlorine dioxide demand by a factor of 4. Maintenance of an ozone residual was not possible in nonclarified SFBW samples. Because clarification alone is sufficient to control concerns regarding recycle of *Cryptosporidium* oocysts in SFBW in most circumstances, the addition of a disinfection option would only be necessary in specialized cases.

Once oxidant demand-causing particles have been removed from the SFBW, inactivation of *Cryptosporidium* oocysts is possible with ozone, chlorine dioxide, or ultraviolet light. A 2-log reduction of *Cryptosporidium* with ozone may require 5 mg min/L (3) of disinfection at pH 8 and temperature of 20 °C, but may require 115 mg min/L (34) of chlorine dioxide disinfection under similar conditions. Ultraviolet (UV) technology for control of *Cryptosporidium* has shown that UV doses as low as 3 mJ/cm^2 resulted in inactivation greater than 4 logs (35).

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OCHRATOXINS.

See Airborne Toxigenic Molds

OIL RESERVOIRS.

See Petroleum Reservoirs, Influence, Activity and Growth of Subsurface Microflora in

OLIGOTROPHIC BACTERIA

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PAUCITY OF NUTRIENTS AS A SEVERE ENVIRONMENT

The inhabitants of severe environments are the products of evolutionary processes. For the earliest life on this planet, only where conditions were favorable could life be successful, but after the development of energy trapping mechanisms, great diversity arose. This variety of life-forms included organisms that had adapted or evolved to grow in harsher environments; these would have a niche with little competition from the not so specialized forms of life. To the extent that it is physically and chemically possible now that the world biomass is high, severe environments have become inhabited by evolved and adapted specialist organisms. Prokaryotes have invaded severe environments of different kinds very effectively. For example, they have invaded environments that are too hot or cold for the majority of organisms. Living when the temperature is extreme requires a series of special adaptations. A larger variety of special adaptations are needed for the range of nutritional deficiencies that are found in nature. Many such habitats are occupied with prokaryotes, and primarily and consistently by oligotrophs.

To understand the oligotrophic state, two topics are especially important and are generally not covered as part of microbial ecology because of their physical and

chemical nature; these will be treated here. One concerns the thermodynamics of a cell because a living organism must be viewed as an engine that needs an external supply of reactants to provide energy that can be manipulated for cellular use; and the other concerns the diffusion of small molecules up to a cell.

WHAT IS AN OLIGOTROPH?

Oligotrophic bacteria live most of the time in nutrient-deficient environments. Of organisms inhabiting such environments, the prokaryote oligotrophs are the most numerous. As compared with the multicellular Eukarya, the domains of Bacteria and Archaea and single-celled Eukarya have many more members that inhabit severe environments. This is no doubt because the lifestyles of single cells are less restricted by such an environment, and these cells are capable of adapting more easily than multicellular organisms to nutritional limitation. Let us compare oligotrophs with a very different extremophile, a pathogen. Both inhabit severe environments, one dealing with poor nutrition and one dealing with a host's sophisticated defense mechanisms. The pathogen's way of life is just as relevant as the oligotroph's to the topic of life in extreme environments, but each copes with totally different problems. Obviously, if some essential nutrient is completely missing, the oligotroph cannot grow, but its forte is surviving if nutrients are sometimes missing or if they are generally present in only very low concentration. Life may be possible, but only if the inhabitants have the physiological adaptations needed to endure such conditions.

Evolutionary theory designates the precursor of the three domains of Bacteria, Archaea, and Eukarya as the Last Universal Ancestor (LUA). In its time, the development of methanogenesis and oxygen-producing photosynthesis had not occurred. Then carbon compounds of intermediate oxidation state were formed abiotically either by geologic and astrophysical forces or by fallout from interplanetary material. At that time, life on this planet must have been sparse (1–4). This was an oligotrophic world. In today's biosphere, oxygen-producing photosynthesis supplies both the organic material and the oxidant so that many trophic levels in the food chain are possible and the biomass of the world is many orders of magnitude higher than that and much of the world is eutrophic. Still there remains a vast habitat that has little exploitable fixed carbon. Although an oligotrophic environment refers to all nutrients, most usage only refers to insufficiencies of oxidizable carbon compounds.

The largest habitat of a carbon-sparse is the deep seas (5). At the surface where there is light and carbon dioxide, there is usually a deficiency of nitrogen and phosphate, because the nitrogen and phosphorus are ordinarily depleted through consumption by photosynthetic organisms. These elements then pass through the food chain and eventually fall to the oceanic depths. This process maintains oligotrophic conditions throughout the water column. On the other hand, eutrophic conditions are usually prevalent near shores due to runoff. However, where there are upwelling currents of the deep ocean waters or when and where the thermal stratification of water layers ceases (and the thermocline temporarily disappears), then the mixing (turnovers) brings nitrogen and phosphate to the illuminated surface and growth is perfuse. In contrast, the bulk of the oceans have been described as biological deserts. Although these waters are clear, they still may contain more biomass than the rest of the world because their volume is so large. There, many varieties of small bacteria eke out their existence and persist. These generally are small marine bacteria and have been called ultramicrobacteria, oligobacteria, nanoplankton, picoplankton, and a number of other names. Deep freshwater lakes may also be oligotrophic and provide habitat for freshwater oligotrophs, depending on the geology of the surrounding watershed and on other factors, such as the absence of agriculture in the adjacent terrain.

Agricultural fields generally are regularly fertilized and much biomass is generated so that the food chain leading from the primary producers may have enough active stages that only a few recalcitrant compounds remain (6,7). During agricultural cultivation, nitrogen and phosphorus are replaced by fertilization, although they may be washed away (or metabolized to gaseous N_2). Typically, when agricultural activities cease, the environment becomes largely oligotrophic and the succession in the resulting ecosystem depends on nitrogen fixation, carbon availability, and soil formation. But even with land in crop production or in mature climax forests, oligotrophic bacteria persist, growing slowly on compounds left behind by organisms closer (metabolically) to the site of primary production.

A most interesting oligotrophic environment is the distilled water reservoirs in chemical and biological laboratories (8–10). In many cases, *Caulobacter crescentus* can be found there. This heterotroph must be able to find enough carbon compounds and enough nitrogen, phosphorus, sulfur, and trace elements coming by elution from the glass, by failures of the water purification processes, from resources contains in the gas phase, and from plasticizer and are able to survive and grow. Their persistence shows that they have attacked and solved the formidable biochemical and bioengineering problems of such an existence.

HISTORY OF THE FIELD

Nomenclature

The term "oligotroph" has been used since Weber coined the term in 1907 (11), but was brought to the attention of microbiologists when Jeanne Poindexter (8,9) used it to contrast with her new term "copiotrophs." In concept, the former class of bacteria is devoted to a life in a perennially sparse environment and the latter to environments with generally higher level of nutrition.

"Copiotroph" and "eutroph" are terms more or less equivalent to "zymogenous" or "allochthonous," as used by Winogragsky in 1924. His term "autochthonous" is approximately equal to "oligotrophic bacteria" (but it actually means of indigenous origin). These organisms are characterized, in large part in modern usage, by their

inability to grow in high levels of nutrients. They are not characterized solely by an exceptional ability to use low concentrations of substrates. In fact, the ability to use a substrate has a lower limit imposed by the physics of diffusion and not by biological constraints (see following text).

From Bacteriology to Microbial Physiology to Molecular Biology and Microbial Ecology

Microbial physiology had its start from the work in the last century of Pasteur and colleagues at the Institut Pasteur in Paris and from Robert Koch and colleagues in Berlin. During the first half of the twentieth century, progress shifted to Holland and then to America with Henrici at Minnesota. But the real shift occurred at the time of World War II and shortly thereafter with the development of molecular biology [stemming from Luria and Delbrück's work (12)], the principles of microbial genetics, and the appropriate methods needed for the study of bacterial genetics. At the same time, the studies of microbial ecology developed built on the earlier studies primarily of the Dutch school. In the postwar era the progress was fantastic, but it is only since the 1990s that the two microbiology branches of molecular biology and microbial ecology have fused together. Now, molecular biological tools and concepts allow microbiological ecology to be studied effectively. With respect to the so-called oligotrophic bacteria, there is much left to be done, but much is shortly to be accomplished given the state and applicability of the complementary sets of tools now available from molecular biology and microbiological ecology.

Work in the Last Half Century

Before discussing the problems of life in low nutrient conditions and oligotrophy, it is necessary to highlight several research areas investigated since 1940, in which the concept of the "oligotroph" would be appropriate. This section is just to give the highlights, key conclusions from the studies, and the key researchers; further discussion will be presented later.

1. Morita, who was Zobell's student, spent his career in studying marine organisms that persisted for a very long time under starvation conditions. Although there were some changes of the starving vegetative organisms, it was particularly significant that the cells became smaller and that the survival remained high. Most of his studies were of a marine vibrio (see section The Marine *Moritella* Ant-300; 13).
2. Jeanne Poindexter studied of the survival of a freshwater organism, *C. crescentus* (8–10). It grows in extremely low nutrient conditions. She was the first investigator to unearth a number of biological facets during her studies of *Caulobacter* growth in a variety of conditions. This organism differentiates into a swarmer cell and a stalked cell during every cell generation. This circumstance gave the opportunity to study differentiation of a bacterium to give insight to the developmental processes that take place in metazooans and plants. It was the utilization of this organism by Shapiro (14,15) that was important in meshing the ecological biology with the molecular biology of differentiation. (see section The Prosthecate Bacterium *C. crescentus*).
3. Ensign and his students studied a gram-positive soil microorganism that undergoes morphological changes during its culture cycle. In the stationary state it becomes a coccus instead of a rod. (15; see section The Jointed Bacteria, *Arthrobacter* spp).
4. Button and his group (16–21) are marine microbiologists. They isolated a number of marine organisms that appeared initially to be obligate oligotrophs. *Cycloclasticus oligotrophus* RB1 was the organism chosen for detailed study, and although it could not be grown when isolated, it was cloned in an interesting way. This was done by an MPN (most probable number) method that they called the dilution culture technique. (see section A Marine Ultramicrobacterium *C. oligotrophus* RB1).
5. The fifth development came from my laboratory (22–25) and concerned *Escherichia coli*, a nominal copiotroph. We developed a way to study bacterial growth very sensitively through a long light path cuvette in a spectrophotometer linked to a computer. With it we could study growth at very low substrate concentrations. We were able to show that even the organism *E. coli*, which was not thought to be an oligotroph and in fact had been touted as the premier example of a copiotroph, had actually no lower limit to the concentration of glucose in which it could grow. As efficiently as diffusion kinetics would permit, it takes up a monosaccharide, glucose, from the environment and converts it into cell mass. This was important in redefining the contrast between an oligotroph and a copiotroph. The implication is that even a copiotroph's uptake system is so efficient that a sugar molecule that collides with the cell is taken up before it can diffuse away and thus uptake is close to 100% efficient (see section Uptake at Low Concentrations by a Presumptive Copiotroph, *E. coli*).

CLASSES OF OLIGOTROPHS

The first review of the oligotrophs was by Kuznetsov and coworkers (26). They divided oligotrophs into four classes: (*1*) those that are only initially cultivatable; (*2*) those that could be isolated initially on poor medium, but then become culturable on rich medium; (*3*) those that are only cultivatable on special "poor" medium; and (*4*) those that cannot be cultivated but can be observed with a microscope.

Because of the recent studies by Pace and by those who learned techniques in his laboratory (5,27,28), it is quite clear that there are myriads upon myriads of organisms in nature that we cannot grow in the laboratory and therefore belong to Kuznetsov's class 4. These insights were obtained mainly by using hybridization with probes against portions of the 16S rDNA to identify the

phylogenetic location of the bacterium under inspection. Many of these are quite different from microorganisms that have been cultured. Electron micrographs show that the morphology of these is highly variable (5). Most of these "unculturables" would be true "obligate oligotrophs." Although some DNA molecules may have come from dead organisms that had grown elsewhere, most must have come from in situ obligate oligotrophs. Although we do not understand their basic physiology, we can infer that, with few exceptions, these organisms must have grown in the environment in which they were found.

WHAT PROPERTIES MUST AN OLIGOTROPH HAVE?

In 1978 a workshop was held (29) to consider "Strategies of microbial life in extreme environments" and the subcommittee on "Life under conditions of low nutrient concentrations" summarized from available knowledge and from just thinking about the problem what such oligotrophs must be like. I must summarize these considerations here and add more from work in the subsequent 25 years in the laboratories of Poindexter (8–10), Morita (13), Colwell (30), Button (16–21), Egli (31–36), Pace (5,27,28), and Koch (22–25).

- Oligotrophs should be able to efficiently scavenge their environment of nutrients. For this they should have a high surface-to-volume ratio. Not only should they be small for this reason, but also they should be nonspherical (37). Actually, some are spherical, possibly for simplicity in formation during growth. They should have a high volume-to-biomass ratio and active transport systems that can accumulate substances against a high concentration gradient. These "permeases" generally should be ready to function and the range of transport systems should suffice for the range of types of molecules that are likely to be encountered in the relevant oligotrophic environment. The energetics of active transport systems can depend on the type [respiration and ATP-linked generation from proton motive force (PMF)] and on the degree of coupling of the symporters or antiporters to the relevant ion gradients. Again, the choices depend on a balance of cost and upkeep versus utility to the organism. The prime problem of the cell is to acquire substrates, and developing enzymes to metabolize them is probably a less immediate problem. To summarize, they should have the ability to consume a variety of substrates that *might* occur and be able to do so promptly at any time. They must do so efficiently and waste little in maintaining and assembling an adequate transport system during the time a particular transport system is not needed.
- Oligotrophs should be able to husband their resources. Although suspended in an aqueous environment as in the high-seas environment mentioned in the preceding text, nutrition may be almost continuous but infinitesimal, but in most oligotrophic environments life is one of feast and famine. However, not as suggested for the life of *E. coli* (38), but one where the feast is very spartan and the famine supplies poor resources in terms of both quality and quantity (Poindexter chooses the word "fast" instead of a limited "famine"). To the degree that it is biologically possible, the oligotroph should (*1*) decrease its metabolism, (*2*) decrease any turnover, and (*3*) block any futile cycles. In addition, it should (*4*) have stored up some reserves to help it through the severest part of the famine. It should do this even at the expense of not engaging in certain amount of cellular growth. Clearly, all of these prohibitions are "judgment calls," but they appear to be built into the physiology and genetics of the species and kind of oligotroph being considered. The oligotroph must engage in metabolic processes, and their kinetic control to conserve resources is much more sophisticated than the mechanisms of copiotrophs. *Bacillus subtilis*, in an all-or-none way, carries out the sporulation pathway at great expense to itself and then, also in an all-or-none way, can revert to vegetative growth. *Escherichia coli* responds to a rapidly imposed starvation. The apparently simple choice of how not to grow too fast is seemingly both with and against the dictum of Darwin and the dogma of ecologists that an organism's goal is to increase its inclusive fitness. If it grew at the limit of its current resources, then it would not be able to form reserves for future needs, but the organism has no way of knowing the time when the near-famine state will become a total-famine state. In principle, evolutionary processes have selected for those varieties of organisms that do not grow too fast and do not extensively store accumulated resources as reserves. Somehow they must have a strategy, encoded in their genes, based on the past experience of earlier generations.
- Among the endogenous resources consumed during starvation, ribosomes and associated factors are generally used, whereas poly-hydroxy-butyrate (PHB) and glycogen are stored only in some bacteria. Degradation of ribosomes is a general strategy because a cell during growth needs many ribosomes, but during starvation a cell possesses an excess over its needs. When little protein synthesis is ongoing, degradation of currently unneeded proteins and ribosomes can permit specific proteins to be produced that can enable the cell to prolong its survival (see section Conservation of Current Resources for Future Use).
- Although a bacterium cannot pursue multiple strategies, a bacterial population can. I have discussed (39) reasons for believing that bacteria have built-in mechanisms to increase the rate of special mutations in regions of specific genes that are easily reversed when the need has passed. This can be done by having a tandem series of doublets or quadruplets in relevant genes. Thus, in a population of millions or more bacteria, there are mutations that are due to slippage and mismatch repair in these tandem repeats that turn on or off genes in which they are present and allow an individual organism to cope with an environmental challenge. When the environmental challenge is no longer present, the culture can revert in the same mutational way, and

the original genotype can be selected. So this type of mutational mechanism provides temporary help.

- Many organisms that appear to be obligate oligotrophs can be "coaxed" into becoming facultative oligotrophs or copiotrophs (these would be a subset of Kuznetsov's second category). In the reverse direction, copiotrophs seem to be converted to oligotrophs during slow growth. I have the impression from the literature and from my own experiments (25) that copiotrophic cells growing in slow chemostat culture slowly become converted to having a slower maximal rate of growth but also a higher efficiency of uptake. Although the topic of viable but nonculturable (VBNC) cells is currently in a state of flux with supporters and detractors, it would seem to be merely a difference in degree and strategy from characters expressed in other organisms, particularly oligotrophs.

WHO LIVES IN OLIGOTROPHIC ENVIRONMENTS AND WHO DOES NOT?

The biology of four oligotrophs and one copiotroph are explored in this section, in large part, to document and extend the conclusions mentioned in the previous section.

The Marine *Moritela* Ant-300

The in-depth study of Ant-300 in the laboratory of Morita involved many students and many years (13,40,41) and is probably the most completely documented case of an oligotroph. The starting point for Morita's studies was the observation that this vibrio on starvation for a carbon source changed progressively to small coccal-shaped form. Obviously, two processes are ongoing during the early phases of starvation. One process was that the starving rods divided to form smaller coccal-shaped organisms; this is similar to what is seen with *Arthrobacter* species (see following text). Moreover, similar behavior was observed with *E. coli* (3). In the latter case, the cells are long and wide in rich medium, whereas they become smaller in poor medium, although they maintain their proportions in the length and width dimensions. In the case of this vibrio, the culture increases in both viable and total count about fourfold in the first week of starvation as the cells become smaller. Thus, although the size of the individual cell decreases as cell division takes place, they are substantially all alive.

Thus, the first response is that the cells become smaller but still can form a colony and are alive by all the criteria used in microbiology. Subsequently, after the first week, some cells died using these assays, but the number of small round particles remained constant for many weeks, indicating little cell lysis. Therefore, there is an increase in the proportion of particles of the dimensions of starved living cells that cannot be induced to give rise to a colony. The viable count eventually plateaus after the decrease in viability of 1,000-fold (41). This is typical of the response of many bacteria to environmental challenges. It is speculated from electron microscopic studies (40) that the live particles are the ones that retain a less dense region in the center of the cell than do the bulk of the particles. This lighter appearance in electron microscopes is usually characteristic of DNA-containing regions. Moreover, the cells that appear not to form colonies have a periplasmic space that is a higher percentage of the total cell volume.

These same cellular changes could be duplicated by growing the organism in continuous culture at progressively slower dilution rates. During the starvation of particles of the dimensions of coccal cells, various cellular components generally decreased.

The Prosthecate Bacterium *C. crescentus*

Caulobacter is a genus of prosthecate bacteria. This aerobic heterotroph occurs in freshwaters and soils of low organic content. The dividing cell is asymmetric. One part, the stalked half, remains attached to a surface and continues to grow and divide; but the other part, the swarmer cell, carries out few biosyntheses. Instead it swims around, until it finds a place to reside. Then, it sheds its flagellum and in its place grows a stalk with holdfast material to hold it to the substratum. In poor, usually phosphate-deficient medium, the stalk continues to grow, generation after generation. The new region formed with every generation is separated by a partition from the earlier portion (42). The stalk appears to be an extension of the cell envelope, and two functions have been assumed for it: to function as an absorptive organ with a surface containing very little volume and to allow the cell body to be further from the surface of attachment. With a long stalk attaching it to a solid surface, the flow around the cell body bathes it with a fresh environment and thus the cell is better able to sequester resources (42).

Much of the behavior and the kinds of habitats in which *C. crescentus* is found are consistent with the niche of specialization to grow in a severely nutritionally limited environment. However, *C. crescentus* is not a strict obligate oligotroph. There is reason to believe that even fresh isolates from nature are not truly obligate oligotroph. This comes from the use of an effective technique used by microbiologists for isolating the species. The sample from the oligotrophic environment is plated on an agar that has a certain small amount of peptone, tryptone, and so on. Colonies that grew rapidly are identified the next day and colonies that have subsequently emerged are picked several days later. Many of these turn out to be *C. crescentus*. Consequently, it can be concluded that these organisms had obviously not been killed or destroyed by the nutritional supplement. Many other species of genus *Caulobacter* are more difficult to grow and thus some of them may be oligotrophs that are more obligate than *C. crescentus*.

Another conclusion that *C. crescentus* has copiotrophic tendencies can be drawn because the organism is currently under intense molecular biological study in the laboratories of Shapiro and those of her erstwhile students (14,15). The studies in these laboratories depend on the ability to grow cultures to quite a high density to allow molecular biologists to perform the many kinds of experiments and measurements of their trade. Because it now grows to high density, it can be concluded that the organism never really had an obligate oligotrophic status

or has modified its apparent obligate character during culture.

The Jointed Gram-Positive Bacteria, *Arthrobacter* spp.

Study of this oligotroph has been carried out in Ensign's laboratory (43–45). Arthrobacters grow as a chain of rods and have received their name because the junction between rods is usually at an angle. Consequently, they resemble the joints in a human limb or, more realistically, the legs of a spider. This articulation between chains is a result of the division process in which a septum is completed across the cell and then split into two, but the last few bonds may be split more slowly. Other gram-positive rods under certain conditions do the same thing. The organisms are found mainly in soils. Although the jointed chains of rod-shaped cells are useful for taxonomy, it is argued that this is not its characteristic form in its natural environment, which is usually nutritionally deficient. Under such conditions, the cells continue to divide until they appear coccoid. These coccoid cells are small, but not of a full resting form like a spore because they appear to grow and divide as coccoids, although they are more resistant to environmental stresses. Enough is known about wall growth in *E. coli* so we could engineer a recombinant form to convert it from a rod to a coccus, as occurs naturally for the *Arthrobacter*. Probably it could be done in several different ways. But it is not clear why this change in *Arthrobacter* takes place. I have argued that the change in surface-to-volume ratio would not increase the effectiveness of diffusion very much, although a small change could be biologically important. The more likely explanation is that in dividing to become smaller, the same amount of cytoplasm and nucleoplasm will be present in more "propagules" in the small coccoid form.

Their maximum growth rate in rich medium is not very fast, say a doubling every two to three hours. The low maximum rate is usually attributed to their inability to deal with saccharides. This makes some sense. In general, in the decomposition chain of vegetable biomass, the acid-soluble fraction of the cells is consumed first, then the reserve polysaccharides and proteins, and lastly the lignins. In these stages, the more and more recalcitrant compounds are enriched and organisms growing at the later stages have to deal with a different distribution of resources. So the arthrobacter, like *B. subtillis*, may commonly meet succinate in its environment and not sugars simply because the latter has been consumed first and the dicarboxylic acid, succinate, is a product of the degradation of aromatic and fatty compounds. Conversely, *E. coli* grows faster on certain sugars than it does on succinate. So the question of why the growth rate is slower cannot be explained on a biochemical basis. Poindexter's review (9) suggests that their inability to grow rapidly may be the reason that they can thrive in soils compared with the failure of an organism like *E. coli* to grow in this environment.

During starvation RNA and protein are degraded. This process is discussed further in the section Conservation of Current Resources for Future Use.

A Marine Ultramicrobacterium *C. oligotrophus* RB1

The fourth organism to be discussed is *C. oligotrophus* RB1—it was isolated and studied by Button and coworkers (16–21). He is a marine microbiologist and his group isolated marine oligotrophs that appeared to be obligate, at least initially. Cloning was performed by an MPN (most probable number) method that he called the dilution culture technique. In this technique, seawater was filtered and sterilized. Seawater contains a low level of nutrients; this could be demonstrated when the sterilized seawater was inoculated with a small fraction of the unsterile seawater and the number of organisms increased and in some weeks, the cell count returned to its original value as measured by flow cytometry. With this as a start, they used smaller inocula and subcultured by the same technique until they had a monoculture. One of the organisms generated by this type of subculture was named *C. oligotrophus*; it was studied extensively and it was found that it could be grown to high titers with aromatic compounds as carbon substrate (19).

Button's group isolated many cultures in this fashion from oceans in several parts of the world. Many of the strains could not be grown initially on high nutrients, but after culture and subculture in the laboratory, most could grow to higher titers. When cloned, their nutrition could be studied, and it was clear that a number could grow to high density on the appropriate substrates. Many of these cells appear to be less dense than a typical vegetative cell of a growing copiotroph. Thus the main point is that this organism uses low levels of carbon sources more effectively than many other organisms and has designed its biology to yield a small cell with a smaller, simpler, and specialized genome, little protein, and a high proportion of water so that the small cell is larger than would be expected from its protein content.

Fast Rate of Uptake at Low Concentrations by *E. coli*, the Presumptive Copiotroph

Escherichia coli is a gram-negative rod and has been considered the prime example of a copiotroph. When it is cultured in a glucose-limited chemostat and diluted into very low glucose minimal medium, it takes up glucose at such a fast a rate that it can be calculated that if it had no outer membrane the uptake would be the theoretical maximal rate limited by physical diffusion (23–25).

Consequently, the important experimental question is how close can bacteria come to this theoretical limit. To test this experimentally, I used the apparatus that Wang and I (22) had constructed to attack a related question. It was concluded from the uptake kinetics and by fitting the data to a variety of models that the cells of strain *E. coli* ML308 when cultured in a glucose-limited low dilution-rate chemostat for a month were, indeed, very efficient during batch growth in very low concentrations of glucose. The measurements and the subsequent calculations showed that if the outer membrane could have been stripped off the cells, then uptake at the cytoplasmic membrane would be at the theoretical level of the physically fastest possible consumption. But because of the extra obstacle to diffusion due to the diffusion resistance in the penetration

of the porins of the outer membrane, the ultimately fastest limiting rate for intact gram-negative cells is a little less than the theoretical limit. For more details, see (25).

PHYSICAL CONSIDERATION FOR SURVIVAL UNDER STARVATION OR OLIGOTROPHIC CONDITIONS

Two major topic are considered in this section. The first is the necessity of maintaining the energy within the cell. This mainly depends on the cell possessing and using ways to block dissipation of energy sources. The other is to consider the role of diffusion in the oligotrophic environment.

Control of Energy Metabolism

Consider a stripped down bacterium with no regulatory or control mechanisms. This hypothetical entity is like a car without a steering wheel, brakes, or accelerator. As long as energy is available, the bacterium can generate protonmotive force and ATP, say, from the respiration of glucose with oxygen and grow while the car engine could burn gasoline and the wheels would turn. The cell can carry out synthetic biochemistry and grow, synthesizing or importing from the environment all the necessary macromolecules for the cell's life, and the car would move. When the energy supply is no longer available, thermodynamic laws will require that the system should approach the equilibrium state. This, for the bacterium, means that almost all macromolecules would hydrolyze to their constituent amino acids, monosaccharide, fatty acids, nucleic acid bases, and phosphate. The reactions go toward dissolution because the concentration of water is high in living systems and provides the water that splits the macromolecules to their constituents. The car would just coast to a stop.

Although any system spontaneously moves toward equilibrium, net synthesis of a part of a system can occur only when the overall free energy of formation of the system is negative; that is, if the positive free energy for the synthesis (uphill part) is overcome by the negative free energy (downhill part) of the energy-supplying portion of the cell's metabolism. For biological systems, this is when an adequate chemical energy source is present within the cell at a sufficient concentration. When the high-energy sources are absent or only present at an infinitesimal concentration, the net reaction is still "downhill" as it always must be, but now the equilibrium-free energy state to which the system is driven is toward hydrolysis and the state of nonlife.

This is the issue relevant here. When the concentration of the internal energy source, ATP, is near zero and in the absence of a membrane protonmotive force, the system will spontaneously proceed toward the reversal of the synthetic paths. For example, if the forward direction of a particular reaction is the addition of an amino acid to a growing peptide chain by virtue of energy from a charged tRNA and from GTP, these two substances will not be present in the absence of metabolic energy, but uncharged tRNA and GDP will be available and the reverse process could happen. It might be slow. More extreme hydrolysis of a protein by ribosomes and associated factor would charge the tRNA and other steps would generate free amino acids. Because there are many steps, including sequential reactions, the reversal of most or many metabolic reactions during starvation would be slow, but eventually would drive the system backward. For a whole-cell this must lead to death and destruction. The relevant point here is that bacterial cells must have active mechanisms equivalent to brakes and clutches. This is an entirely unstudied aspect of microorganisms in general and of oligotrophs in particular.

Possible Processes That Would Speed Destruction of a Cell Faster Than the Default Rate

Enzymes are very efficient and effective machines, but they are not perfect. The default situation of reversal of the synthetic machinery in the absence of metabolic energy would occur if each energy-coupling enzyme had a single specificity and transduced energy with 100% efficiency. On the other hand, if the specificity and the coupling to energy source was not complete, there would be some spontaneous downhill hydrolysis reactions and the system would dissipate its macromolecular structures faster.

Probably much more important, the cell does possess energy-dissipating hydrolases (proteinases, nucleases, etc.). Their purpose to the cell would be to degrade and turn over cellular constituents as a proper aspect of cell metabolism and as part of the normal cell cycle. There are also enzymes that were made for export to the periplasm or to the environment to break down resources found there to create nutrient molecules that the saprophytic cell could use. However, portions of these enzymes may fail to be extruded from the cell and remain in an active state inside the cell; in this case degradation would be enhanced. There are hydrolytic enzymes residing in the periplasmic space of gram-negative organisms that have a nutritional function, but if given time during starvation, they may degrade some other constituents of the periplasmic space and its surfaces.

Bacteria Have Genetic Factors That May Function to Enhance Survival in a Given Environment

The point has been made that most of the genome of a typical organisms such as *E. coli* is not used under a single given growth condition (46–49). In fact, the majority of the genome may represent temporarily nonfunctioning DNA. This includes sequences left by genetic pathogens that inhabited and functioned in an ancestor and are no longer viable. In this age of complete sequencing of genomes, it is clear that there is much genetic information in cells that is not being used at any time. Probably most of it collectively serves a role when the cell is subject to one of many challenges that have occurred many times in the history of any species, including starvation and coping with a nutritionally deprived environment. Work from a number of laboratories with particular organisms and particular environmental challenges have been presented in the literature. This, of course, includes responses to starvation. But although many genes and many enzymes have been located in these studies, it is clear that the microbiologist is nowhere near a full accounting for the functions of the bacterial genome.

DIFFUSION LIMITATION OF UPTAKE

A bacterium is much more nutritionally limited than an animal in being able to seek out nutrients. Motile chemotrophic microorganisms do some hunting, but only to a limited extent. In fact, coliforms cannot move faster toward nutrients than small molecules can diffuse to the cell (38,48,50).

The kinetics of diffusion (50) of nutrients to the cell from the bulk medium depends on Fick's law. His law can be expressed in either of two forms. If we idealize the shape of a cell into a sphere, the mathematics using Fick's second form becomes simpler. The first biological application of Ficks's law, applied in the 1930s, concerned the adsorption of virus onto bacteria (51). Later, Delbrück (52) extended the treatment and obtained

$$C = C_o\left(\frac{1-a}{R}\right)$$

for the concentration, C, at a distance r from the cell center, where C_o is the concentration in the bulk phase at a long distance from the cell. The distance at which C becomes zero is a. The radius of the spherical cell is indicated by R. If there is complete consumption of every molecule that diffuses to the surface (which is the best that a cell can do), then at the surface of the cell, $C = 0$ when $a = R$. If uptake is less efficient than this physical limit, then a is less than R and C will be greater than zero at the surface, and the gradient of concentration at the cell surface in this case is less.

The rate of uptake of the cell is the quantity of interest. To obtain this information, we substitute the equation for concentration, given in the preceding text, into Fick's second law written for spherical geometry, carry out the mathematics, and end up with:

$$\frac{dq}{dt} = 4\pi DaC_o$$

where dq/dt is the rate of substance uptake by a cell and D is the diffusion constant of the nutrient in the medium. In an earlier paper (37), I have rearranged this equation to calculate the theoretical efficiency of the cell, E, as D/a^2. The efficiency is defined as the equivalent number of cell volumes of medium that are depleted of resource per second by diffusion to the surface if there is complete consumption by the cell.

BIOLOGICAL CONSIDERATIONS FOR SURVIVAL IN A NUTRITIONALLY POOR ENVIRONMENT

Both oligotrophs and copiotrophs must survive in a poor nutritional environment, but the former have evolutionarily "chosen" to specialize and persist in the chronic starvation condition and may not persist in other nutritionally richer environments for extended periods. The copiotrophs on the other hand will not successfully compete and survive indefinitely in a low nutrient environment. Consequently, each type must have been "tuned" to their particular environments by evolution to be better. So we should next consider those factors that would aid in either endeavor. It must be noted that except for chemostat or turbidostat culture, there are no environments that are truly constant and consequently organisms can reasonably "expect" upswings and downswings in the nutritional state of their immediate environment and as the result of evolution have "planned" to capitalize on and exploit the upswings and survive starvation and other down swings.

Conservation of Current Resources for Future Use

Many oligotrophs and copiotrophs, but not all, "choose" to synthesize polymers and form inclusion bodies to store carbon, nitrogen, and/or phosphorus, presumptively for later leaner times. This makes good sense if the cell could not use available resources for immediate growth because of some other constraint. This is the problem of heterotrophic dual nutrition (39,53), which has been discussed in a number of papers by Egli (31) and has been mathematically modeled (54). An example of this is cases in which some, but not all, of the needed nutrients are available in the environment at a given time. However, if the resource could have been used for immediate growth and the production of additional propagules and instead is used for storage material this would appear, at first, to be a bad strategy. But maybe not! Specifically, if future starvation could lead to cell death, additional metabolic reserves might postpone death enough to survive the starvation period. Parahydroxy benzoic acid is a common storage form. There is an alternative strategy to storing PHB. This strategy is used in some cases and may be a better approach sometimes. This was first discovered in *E. coli* (55). It was found that on starvation, the cells would break down ribosomes and use them for purines, pyrimidines, amino acids, and for carbon, nitrogen, and phosphorus for growth and metabolic energy, while keeping only a small fraction of functional ribosomes for new protein synthesis for the time when adequate resources might materialize.

A related phenomenon is that in slow dilution rate chemostat culture in the steady state, the organism keeps many more ribosomes than it can currently use. However, if a fluctuation occurs to greatly enrich the environment, these ribosomes come to function in seconds, presumably so that the organism can grow quickly after a shift up (38). So for multiple reasons, having extra an ribosome, but not too many, is an effective hedge against either future condition becoming either poorer or better.

Multiple Pumps

Cells can have a variety of mechanisms present in the cytoplasmic membrane (and in the outer membrane of gram-negative cells) for transport. The simplest class would be the transport mechanisms called "facilitate diffusion." These equilibrate the concentration of specific compounds across the membrane so that the concentration on both sides of the membrane tend to approach each other. Secondly, there are mechanisms that use metabolic energy to actively bring into the cell against a concentration gradient molecules from the environment. These active transport mechanisms are generally necessary under

nutritionally poor conditions. But then the cost of manufacture of the more complex mechanism, the necessary additional genetic apparatus, and the energy expenditure for function must be taken into account. Moreover, there may be energy consumption even if there is no substrate present (idling). This would constitute a continuing loss to the cell. There are other considerations as well; the transport units must be numerous and distributed on all sides of the cell surface. Because the cell frequently has many substances to import, this leads to competition for the space on the cell surface.

There is another quite different aspect — in most oligotrophic environments in addition to the total concentration of carbon compounds being low, it is the fact that it is usually composed of multiple and varied substances. So resident organisms must again choose to adopt different strategies depending on whether there are many kinds of substances or whether there is a single or only a few kinds of homologous resources. It may happen that a class of similar chemical species is present and can be accumulated by a single kind of pump. Egli has emphasized that multiple pumps for carbon compound are generally needed, but it must be emphasized here that different environmental habitats call for different allocation of membrane surface and competing bacteria may succeed or fail on this basis. It was pointed out earlier that oligotrophs should be constitutive in their synthesis of pumps so that they will be ready when the substrate enters their environment and not waste the time to induce the permeases and incorporate them into the membrane in response to sensing the compound in the environment, but this can be overdone.

Failure to Use Certain Environmental Sources

A cell that has many pumps has the costs discussed in the last paragraph, but there are more issues. A pump not functioning because of lack of exogenous substrate may act to allow cellular resources to exit the cell. A way to avoid this problem is to not form the pump or not install it in the cellular membrane. This may protect the cell, but may also prevent it from growing rapidly in a richer environment. This may be the critical reason also why oligotrophs do not grow fast in rich medium.

The Necessity of Surviving in Multiple Habitats

There are many species of bacteria that form the basis of Bergey's manual. Modern molecular biology and microbial ecology have shown that these known types are only a small fraction of what is "out there," as most of the world's organisms have not been cultivated. Most of these, no doubt, represent organisms adapted toward particular habitats quite different from that provided by the usual culture media in the laboratory.

There is a particularly important class of sequential adaptations: these are multiple adaptations that serve to aid an organism to grow because its habitat must necessarily vary from time to time. Many microorganisms must adapt serially to a number of habitats as aspects of their growth strategy. For example, *E. coli* must get to a new host before the current host dies of old age. To survive in nature, *E. coli* must adapt: (*1*) to the environment of the mammalian colon; (*2*) to that of wastewater and soil; (*3*) to the upper gastrointestinal tract of some other mammal, and (*4*) finally, to the large intestine of the new host. *Escherichia coli* must succeed in all to be passed from host to host and survive indefinitely. Although I have argued that much of what is known of the molecular biology of this organism bespeaks of adaptation to the ileum (the most distal part of the small intestine; 63), it must necessarily do well enough in each of these stages to persist in nature. Recently, there has been interest in organisms that have two alternate lifestyles: living in an environment as a saprophyte (nonpathogen) versus their living as pathogens inside of an animal or a plant host. We are beginning to understand the ways they switch from one growth mode to another.

References to Reviews in Related Areas

The study of oligotrophs overlaps the field of starvation survival. I will take the term "starvation survival" to include not only starvation of particular nutrients, but also survival in the stationary state, and the growth of organisms at low dilution rates in continuous culture and life in an oligotrophic nutritional environment. The key reviews and monographs of the field of starvation survival are Fletcher and Floodgate (56), Kjelleberg (57), and Morita (13). Related fields have to do with organisms that are in the shutdown state (58) and the viable but not culturable (VBNC) (30) state. The issue of VBNC is current and important. Particularly relevant are the studies of Matin (59) and Kolter (60). See References 61 and 62 for how *E. coli* survives during starvation.

Questions Not Answered Here That Need to be Addressed

This article has delved into the evolutionary principles, biophysics, biochemistry, and microbiological physiology of oligotrophs. This article has not provided answers to the following question areas:

1. Why don't all bacteria grow in any environment that has, at least, an infinitesimal concentration of necessary nutrients?
2. What are the mechanisms that increase chance of death when, at best, only slow growth can occur?
3. Are there threshold phenomena such that at too low nutrient concentrations, no growth can occur because all energy metabolism is spent in maintenance?
4. Are some organisms that appear to be obligate oligotrophs really experimental artifacts (48) because the organisms are destroyed, for example, osmotically when nutrients are added by the microbiologist? Does death occur because uptake is then too rapid that the cell's osmotic pressure becomes too large and causes rupture of the cell? Alternatively, does a rich medium under aerobic conditions produce lethal free radicals?
5. Are there two special biologies for oligotrophs and for copiotrophs or is there a continuum biologies for a continuum of habitats and niches? This is of basic microbiological interest.

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OPPORTUNISTIC PATHOGENS.

See *PSEUDOMONAS*

ORGANIC MATTER IN WATER.

See BIODEGRADABLE DISSOLVED ORGANIC CARBON IN DRINKING WATER

ORGANIC MATTER REMOVAL IN WATER TREATMENT.

See BIODEGRADABLE DISSOLVED ORGANIC CARBON IN DRINKING WATER

ORGANIC SUBSTANCES: INTERACTION WITH BIOFILMS.

See SORPTION PROPERTIES OF BIOFILMS

ORGANOPHOSPHORUS NERVE AGENTS, BIODEGRADATION OF.

See CHEMICAL WEAPONS, BIODEGRADATION OF

OSMOPROTECTION OF MICROORGANISMS.

See SALINITY EFFECTS ON THE PHYSIOLOGY OF SOIL MICROORGANISMS

OXYGEN: EFFECT ON MARINE MICROBIAL COMMUNITIES

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Oxygen is a master variable in nature that governs the distribution of organisms. Oxygen is produced by the oxygenic photosynthesis of cyanobacteria, algae, and plants. It is consumed by the, predominantly biotic, oxidation of organic matter previously formed by photosynthesis. A significant amount of oxygen is also used for the oxidation of reduced inorganic compounds such as ammonium, ferrous iron, reduced manganese, and sulfide; oxygen thus has an important impact on the major element cycles. Animals and plants almost exclusively depend on the presence of oxygen for their respiration, and microorganisms that do not require oxygen therefore dominate the anaerobic world. The effect of oxygen distribution on the biota is most easily seen in waterlogged systems where the low solubility and diffusivity of oxygen in water results in steep oxygen gradients in stagnant environments such as sediments. The low solubility also means that water bodies with a high consumption rate of oxygen periodically may turn anoxic with dramatic effects on fish and other organisms.

Microorganisms have adapted to different oxygen regimes and on this basis are defined as aerobic, microaerophilic, or anaerobic (1,2). Aerobic bacteria thrive at oxygen concentrations of 200 to 300 μM (equal to air saturation) while microaerophilic bacteria proliferate at lower oxygen concentrations, and they are often inhibited when concentrations approach air saturation at 200 to 300 μM O_2. Anaerobic bacteria, in contrast, are harmed by oxygen and are unable to grow with oxygen as electron acceptor. The presence or absence of oxygen also governs the mineralization pathways of complex organic matter, such as polysaccharides, and on the kinds of bacteria involved. While some pure cultures of aerobic bacteria can completely oxidize cellulose to CO_2, the same process in anoxic marine environments requires at least three physiologically specialized kinds of bacteria. The energy available to these anaerobes is much less than when oxygen is used as electron acceptor as oxygen reduction is the thermodynamically most favorable process (3). The electron acceptors used by anaerobes (e.g., NO_3^-, Mn^{4+}, Fe^{3+}, SO_4^{2-}, CO_2) are transformed to reduced products that may be eventually oxidized with oxygen. These products include ammonium, which is produced by dissimilatory nitrate reducers (4); hydrogen sulfide, which is produced by sulfur- and sulfate-reducers (5); methane, which is produced by methanogens (6); and reduced metals such as ferrous iron and reduced manganese, which are produced by metal reducers (7,8).

Historical Record of O_2 in the Sea

Considerable controversy exists about the evolution of oxygenic photosynthesis, but there is general agreement that the first oxygenic phototrophs were cyanobacteria-like organisms, as all oxygenic photosynthesis in the present world is performed by cyanobacteria (including the prochlorophytes) and the chloroplasts of eukaryotes that are closely related to cyanobacteria. Well-preserved fossil stromatolites usually thought to be affiliated with ancient cyanobacterial communities have been dated as far back as 3.5 Ga, but it should be kept in mind that even some modern cyanobacteria may perform anoxygenic photosynthesis (9). Cyanobacteria-like filamentous microfossils in stromatolites could be ancient relatives of

Chloroflexus-like organisms that perform only anoxygenic photosynthesis, but the morphological variation in filamentous and unicellular bacteria in stromatolites as old as 2.6 Ga (10) points to an origin of cyanobacteria older than that.

The precipitation of large banded iron formations (11) at about 2.7 to 1.8 Ga (i.e., 2.7 to 1.8 billion years ago) indicates that the oceans were being oxidized during that period, and the change in isotopic composition of carbon (12) and sulfur (13) at 2.8 to 2.7 Ga support the initiation of a considerable O_2 production. Banded iron formations older than 2.7 Ga are also found, and even the oldest known rocks (3.8 Ga) from the Isua formation in Greenland contain small banded iron formations (11). The highest rates of iron deposition occurred around 2 Ga. It is possible that some of the older banded iron formations were caused by the activity of iron(II) oxidizing phototrophic bacteria (14), but some scientists also assume that oxygenic phototrophs were present in the ancient microbial communities at about 3.8 Ga (15). Although there is good evidence for initiation of oxygenic photosynthesis no later than 2.8 Ga this does not mean that the oceans were predominantly oxic. The continued extensive precipitation of banded iron formations until about 1.8 Ga indicates that they were in fact predominantly anoxic at least until that time, and it is possible that only a thin surface layer of the oceans were oxic until the oxygen concentration in the atmosphere rose to considerable levels. It is even possible that the bottom waters of the oceans became sulfidic after the precipitation of dissolved iron and remained so until the O_2 concentration in the atmosphere presumably reached >10% of present day levels at about 1.0 to 0.6 Ga (12–13).

OXYGEN TOXICITY

In general, organisms that respire with oxygen grow faster and to higher densities than organisms that use alternative electron acceptors. However, the advantages of aerobic respiration come with a price. Oxygen reduction forms intermediates of extremely high reactivity that cause damage to many cell constituents, including lipids, sugars, amino acids, and polynucleotides. These intermediates are superoxide radicals, hydrogen peroxide, and hydroxyl radicals. The hydroxyl radicals, which are produced when superoxide radicals react with hydrogen peroxide, are responsible for most of the damaging effects of oxygen. The extreme reactivity of these compounds supported the evolution of a suite of detoxification and repair mechanisms (16–18).

Despite the devastating effect of oxygen metabolites, multicellular eukaryotes have "learned" to exploit them to their own benefit. One example is the so-called "respiration burst" of phagocytically active cells, during which the massive production of oxygen radicals helps kill invasive bacteria. Secondly, plants use hydrogen peroxide in the oxidation of phenols, which serves for the synthesis of lignin (17). However, in the prokaryotic world reactive oxygen intermediates generally represent a threat to cell integrity and consequently cells respond by means of several integrated detoxification systems. The different systems are discussed in detail elsewhere (17) and will only be described very briefly here.

Superoxide dismutase (SOD) catalyzes the dismutation of superoxide radicals into O_2 and H_2O_2:

$$O_2^- + O_2^- + 2H^+ \rightarrow O_2 + H_2O_2$$

The presence of SOD in organisms was originally interpreted as a major adaptation to aerobic respiration, and thus as a key to distinguish between aerobic and anaerobic organisms (19). Early studies supported this hypothesis as SOD activity was exclusively found with aerobic or facultatively aerobic bacteria. However, later investigations identified SOD activity in anaerobes such as *Desulfovibrio desulfuricans* (Norway 4), *Desulfotomaculum nigrificans*, and *Clostridium perfringens* (20,21). Recently, it has been proposed (22) that the SOD activity, which observed in many anaerobes, is only putative, and actually represents superoxide reductase activity instead (see below).

The possible role of H_2O_2 splitting enzymes (e.g., catalase) in oxygen detoxification was recognized by Gottstein in 1893 (23), who observed the production of gas when certain bacteria were treated with H_2O_2. The reaction is described by the following equation:

$$H_2O_2 \rightarrow H_2O + 0.5O_2$$

Catalase thus detoxifies a product of the SOD reaction. As for SOD, early reports correlated the presence of catalase with the ability to use oxygen, but as for SOD this generalization was overruled by later discoveries.

In contrast to SOD, superoxide reductase (SOR) detoxifies superoxide radicals without formation of molecular oxygen and thus avoids production of a potential source of new superoxide radicals. Instead, SOR reduces superoxide radicals to hydrogen peroxide that is further reduced to water by peroxidases. It seems that this detoxification reaction is particularly well suited for the needs of anaerobes (22), and thus SOR rather than SOD is expected to be found in anaerobes. The presence of SOR in organisms that lack SOD was confirmed by complete genome analysis. It was concluded (22) that the presence of SOR in anaerobes "indicate(s) that anaerobic microorganisms possess a mechanism for detoxifying reactive oxygen species that is independent of the SOD-catalase-based system of the aerobic world."

THE DISTRIBUTION OF OXYGEN IN WATER AND SEDIMENT

The vast majority of the oceanic water column is well oxygenated, and the water circulation suffices to keep even the deepest parts oxic with some exceptions such as the Cariaco Trench. Some open-water areas such as the upwelling areas outside Peru and Namibia may, however, experience periodic anoxia. Because of high primary production in these areas anoxia or very reduced oxygen concentrations may occur either at some depth in

the water column (the so-called oxygen minimum zone), or in the bottom waters (24). Isolated water bodies such as the Black Sea and fjords with a sill may have permanently anoxic deep waters (25), while estuarine areas that are stratified by gradients in temperature and salinity as, for example, the Danish straits, may experience periodic anoxia (Fig. 1; 26).

While marine waters are predominantly oxic, the reverse holds true for the sediments. Oxygen usually penetrates to a depth of a few mm in coastal sediments (27), and only sediments from deep oligotrophic oceans have homogeneous oxygen penetrations exceeding a few centimeters (Fig. 2; 28). It is, however, important to realize that oxygen locally may penetrate to considerable depths because of infauna. Burrowing fauna may maintain a thick layer of oxidized sediment (i.e., with a high content of particulate oxides and hydroxides of Fe^{3+} and Mn^{4+}) while the majority of this oxidized sediment at any given time is anoxic (29). The difference between oxic and oxidized conditions in a bioturbated sediment is illustrated in Figure 3 by profiles of redox potential and oxygen. As illustrated to the right in Figure 3, such sediments are also characterized by a large spatial variability in the distribution of oxic and oxidized conditions. The effect of the fauna on the reoxidation processes depends on the feeding and burrowing strategy of the animals. Some animals may make burrows down to a sediment depth of 2.5 m (30), but usually the affected layer is considerably thinner (0.5 m or less).

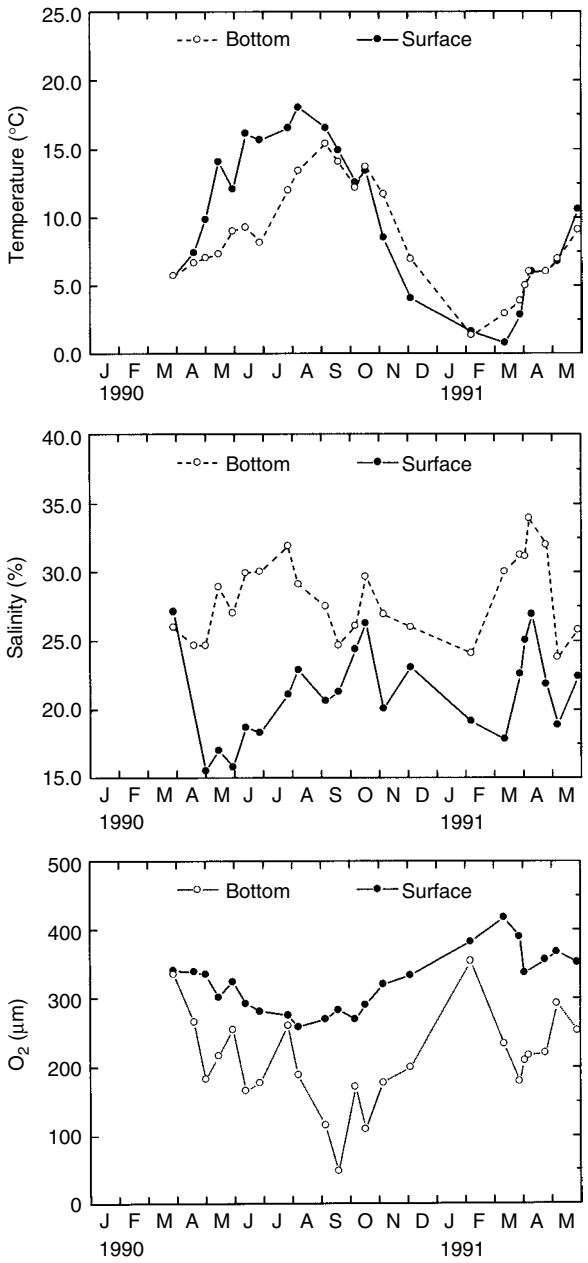

Figure 1. Changes in surface and bottom water temperature, salinity, and oxygen concentration through the seasons in the middle of Aarhus Bay at a water depth of 16 m. Owing to the density gradient caused by the differences in temperature and salinity, the water column was permanently stratified and the bottom water went almost anoxic in September. Redrawn with permission from B. Aa. Lomstein and T. H. Blackburn, Havforskning fra Miljøstyrelsen **16**, 1–74 (1992).

OXYGEN AND PHOTOSYNTHESIS

Oxygenic Photosynthesis and Photorespiration

The activity of oxygenic phototrophs may lead to elevated concentrations of oxygen in water masses with high photosynthetic activity (31). The effect of photosynthesis on the chemical microenvironment is, however, much more pronounced in shallow-water sediments. Profiles of oxygen, pH, and gross photosynthetic activity (for a description of methods see 29) are shown in Figure 4 for an illuminated marine cyanobacterial mat (Fig. 4a) and for a diatom-inhabited sandy sediment (Fig. 4b). Although oxygen reached a peak concentration of 1,540 µM (corresponding to supersaturation with pure oxygen gas) at a depth of 0.2 mm in the cyanobacterial mat, the sediment was anoxic below 1.6 mm depth. The high photosynthesis (CO_2 assimilation) rates in the upper 0.4 mm resulted in a pH as high as 10.3, although the pH at 3 mm depth was about 7. The effects of the photosynthetic activity in the diatom community (Fig. 4b) were less dramatic than in the cyanobacterial mat because of a lower population density.

The effect of very high oxygen concentrations on marine heterotrophic bacteria is poorly studied, but high oxygen concentrations affect phototrophs themselves as high oxygen promotes photorespiration. The ribulose-bi-phosphate-carboxylase (RUBP) that mediates carbon dioxide fixation in the Calvin cycle of all oxygenic phototrophs is also an oxygenase. When RUBP functions as an oxygenase ribulose-bi-phosphate is oxidized and no carbon is fixed. The dual function of the "most important enzyme in the world" may serve as a protection against photooxidation. Many marine photosynthetic organisms can accumulate bicarbonate. In combination with an intracellular carbonic anhydrase, such an active accumulation of bicarbonate may suppress photorespiration at even very low external concentrations (32,33).

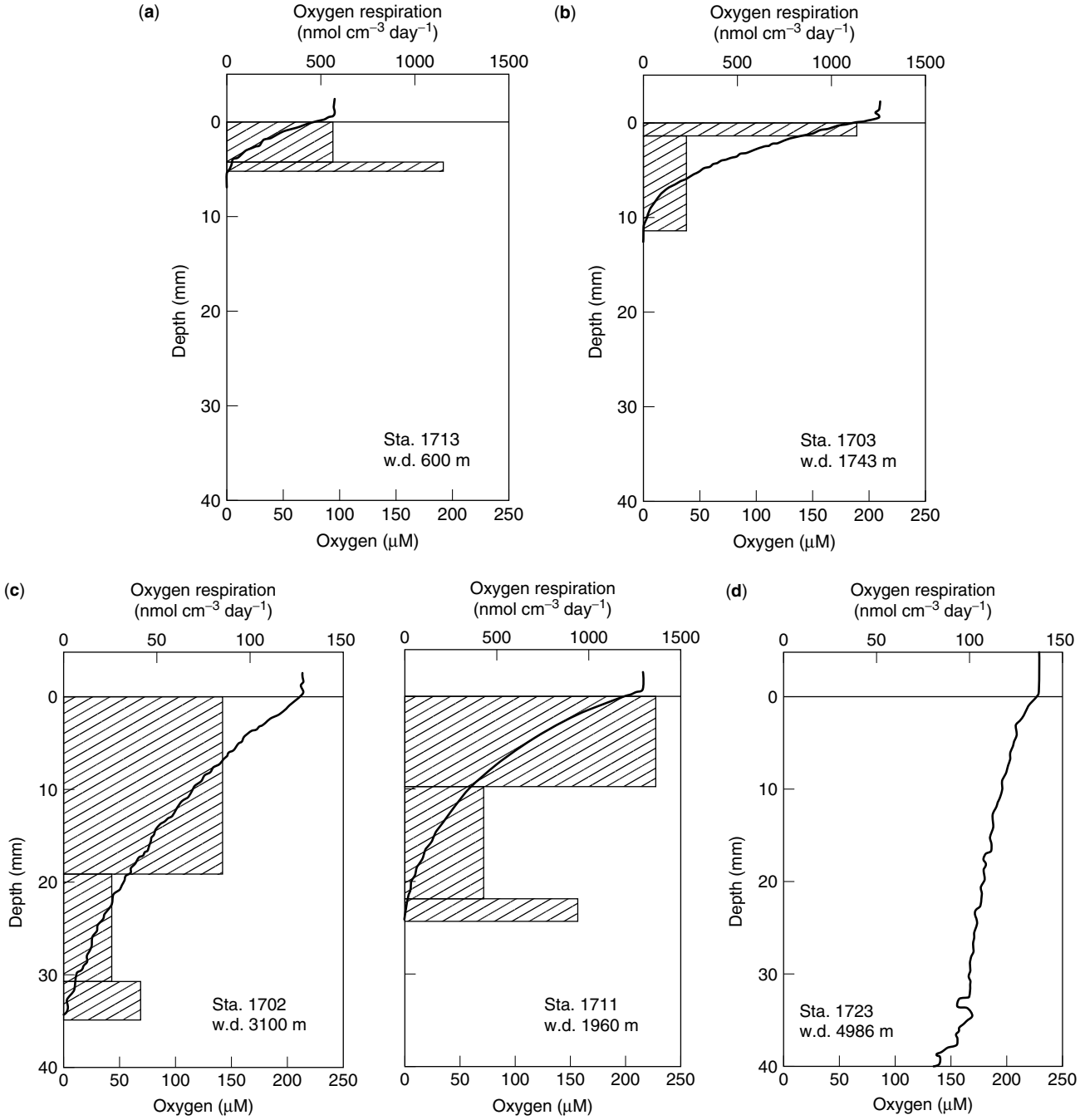

Figure 2. Oxygen microprofiles as measured *in situ* by oxygen microsensors in sediments along a transect in the Atlantic Ocean with water depths ranging from 600 to 4,986 m. The hatched bars illustrate oxygen uptake rates as calculated from the oxygen profiles by a diffusion-reaction model. Reproduced with permission from R. N. Glud et al., Deep-Sea Res. **41**, 1,767–1,788 (1994).

Experimental evidence (34,35) and diffusion-reaction models (36) indicate that photorespiration may balance a major part of the gross photosynthetic activity in benthic photosynthetic communities (Fig. 4), where conditions of high O_2 and low CO_2 (high pH) favor this process.

Anoxygenic Photosynthesis

Some cyanobacteria can perform anoxygenic photosynthesis in the presence of sulfide and then shift to oxygenic photosynthesis when sulfide is depleted (37,38). The oxidized sulfur product of this anoxygenic photosynthesis is elemental sulfur (37) or thiosulfate (39). Most anoxygenic phototrophs are, however, dependent on a reduced electron donor such as hydrogen sulfide to perform photosynthetic activity. Anoxygenic phototrophs are thus often found in a an anoxic layer below the oxygenic phototrophs where some infrared light is still present (40). Microbiology textbooks often show a typical zonation of

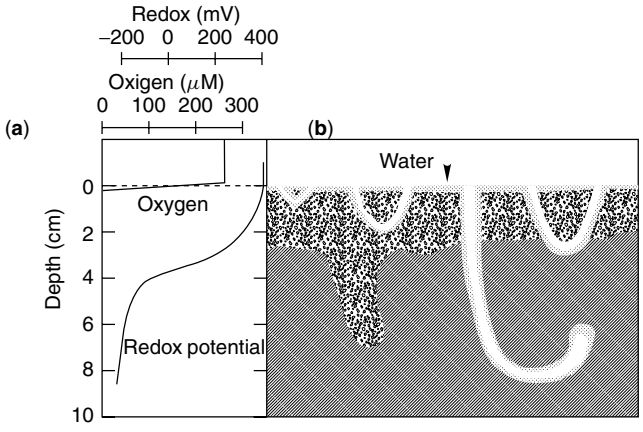

Figure 3. A: Oxygen and redox profiles in a coastal marine sediment as measured by electrodes. The sediment had a thick (but heterogeneous) oxidized layer because of the activity of the infauna, which periodically and locally oxidized the reduced ferrous and manganese minerals. In insert B are shown oxic sediment (dotted areas near sediment surface and around burrows), oxidized (redox potential > 0) but anoxic sediment (cloudy areas), and reduced (redox potential < 0) sediment (dark hatched areas). The oxygen and redox profiles are representative for the position shown with an arrow. Reproduced with permission from N. P. Revsbechand B. B. Jørgensen, *Adv. Microb. Ecol.* **9**, 293–352 (1986).

cyanobacteria followed by a purple layer covering a black sulfidic sediment. However, often the picture in nature is less clear. As an example, immobile purple bacteria (*Thiocapsa* sp.) are often found mixed with diatoms at sandy beaches. It has long been known that purple nonsulfur bacteria are facultative anaerobes that grow well in the dark as ordinary chemoorganoheterotrophs, but it was assumed that anoxygenic purple sulfur bacteria were strict anaerobes that could only be active under anaerobic conditions. The work of Kämp and Pfennig (41) showed that these bacteria are much more versatile, and that purple sulfur bacteria may even respire with oxygen using either organic or inorganic electron donors. The presence of purple sulfur bacteria in periodically oxic microenvironments is thus not surprising. Like many other microorganisms with a diversified metabolic potential the purple sulfur bacteria cannot compete with more specialized bacteria under constant environmental conditions. Grown under constant chemolithoautotrophic conditions in the dark *Thiocapsa* was thus outcompeted by thiobacilli (42). Anoxygenic phototrophs can generally not synthesize bacteriochlorophylls when oxygen is present, but planktonic phototrophs of the genus *Erythrobacter* (43) actually require oxygen to be active. Bacteriochlorophylls from such anoxygenic phototrophs are quite abundant in fully oxic oceanic waters (44). However, there are also reports of benthic anoxygenic phototrophs that produce pigments under oxic conditions (45). Because of the general inability to produce bacteriochlorophylls under oxic conditions, it was assumed that the vast majority of anoxygenic phototrophs could not perform photosynthesis when oxygen is present. This assumption was, however, based on a traditional culture approach with constant illumination and continuously reduced conditions. Experiments with purple sulfur bacteria grown under cycles of illumination and oxygen mimicking the conditions in the surface layer of sediments with presence of

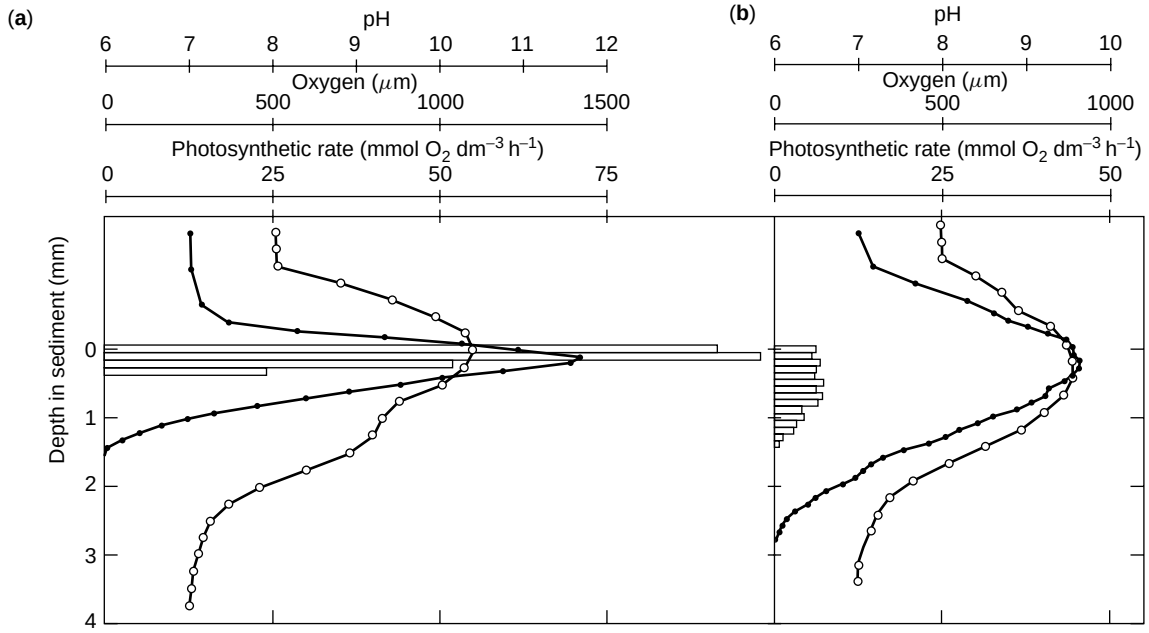

Figure 4. Profiles of oxygen (●), oxygenic photosynthesis (bars), and pH (ϒ) in a marine cyanobacterial mat (**a**) and a diatom-inhabited sandy sediment (**b**) during illumination at 1,000 μmol photones $m^{-2}s^{-1}$. Note the extreme pH values and concentrations of oxygen in the only 0.4-mm thick photic zone of the cyanobacterial mat in Figure 4a.

oxygenic phototrophs have shown that anoxygenic photosynthesis based on bacteriochlorophyll synthesized during anoxic periods continued during aerobic periods (46). Purple sulfur bacteria living in close association with diatoms on sandy beaches may thus be active during the oxic daytime hours, oxidizing elemental sulfur accumulated during the anoxic periods, or oxidizing periodic pulses of reduced sulfur compounds originating from deeper strata (47).

Oxygen and the Nitrogen Cycle

Nitrogen Fixation. Nitrogen fixation is restricted to the prokaryotic domains *Bacteria* and *Archaea*. No convincing evidence has been given for eukaryotic nitrogen fixers. Almost (48) all known nitrogen-fixing enzymes, the nitrogenases, are highly oxygen-sensitive (49,50) and it was consequently believed that nitrogen fixation was associated with bacteria that live under anoxic conditions or create anoxic conditions at the sites where nitrogen fixation takes place. Some examples are (*1*) anoxygenic phototrophs including species of the genera *Chlorobium* and *Chromatium*; (*2*) obligately anaerobic bacteria such as sulfate-reducers, clostridia, and methanogens, (*3*) oxygenic cyanobacteria that protect their nitrogenases within specialized anoxic cell structures (heterocysts), and (*4*) bacteria that are protected from a generally oxic environments by the action of a host organism such as the symbiotic association between the terrestrial *Rhizobium* sp. and legumes.

From soils it has been known for a century that *Azotobacter* may fix nitrogen under fully aerobic conditions (51). Until recently this ability has been explained by intracellularly near-anoxic conditions, caused by a combination of high respiration rates and a thick layer of extracellular polymeric material, which was supposed to slow down the diffusive supply of oxygen to the cell. Recently, however, it has been shown that protective proteins are responsible for the insensitivity to oxygen of the nitrogenase in *Azotobacter* (52).

The root-symbiont *Azoarcus* sp BH72 uses different respiratory pathways at high and low oxygen concentrations, respectively, to protect the nitrogenase (53). Apparently, nitrogen-fixing cells use a cytochrome oxidase under fully aerobic conditions, whereas cells that fix nitrogen under microaerophillic conditions use a quinol oxidase instead. Whether there is interdependence between the oxygen regime, the type of terminal oxidase, and the nitrogen-fixing state needs to be elucidated. Oxidases may also play an important role in regulating the oxygen concentration in nitrogen-fixing nonheterocystous marine cyanobacteria (50).

Nonheterocystous cyanobacteria have developed a series of different mechanisms to minimize the deleterious effects of oxygen on nitrogen fixation. These include behavioral and metabolic strategies as well as physical barriers (50). Additionally, nitrogenase itself may have the capacity to reduce oxygen. It has been shown that Fe-protein of the nitrogenase could reduce oxygen to H_2O_2 and water *in vitro* when the concentration of the Fe-protein was at least four times higher than the oxygen concentration. When the concentration of oxygen exceeded the concentration of the Fe-protein, hydroxyl radicals were formed that inactivated the nitrogenase (50).

A behavioral strategy is utilized for O_2 protection by the nonheterocystous cyanobacterium *Plectonema boryanum*, which fixes nitrogen during illumination by separating the process temporarily from photosynthesis (54). It has been demonstrated that two different kinetics of oxygen uptake were expressed during the photosynthetic (P) and nitrogen-fixing (N) phases, respectively. The K_m value for oxygen in P was 71.4 μM, while the K_m value for oxygen in N was only 13.5 μM. The organism was not able to fix nitrogen in the absence of oxygen. Thus, oxygen-dependent respiratory energy generation was obligatory for diazotrophic growth of this species, and the lower K_m for oxygen uptake during nitrogen-fixation indicates intracellularly low oxygen concentrations that would allow a functional nitrogenase. Other nonheterocystous cyanobacteria use the dark phase for nitrogen fixation and the light phase for photosynthesis (49,50).

In the open-ocean environment, *Azotobacter*-type organisms are not known and the absence of planktonic cyanobacteria with heterocysts suggested that cyanobacterial nitrogen fixation apparently did not occur. It was therefore a revolutionary finding when it was reported that the marine filamentous cyanobacterium *Trichodesmium* fixes nitrogen under oxic conditions (55,56). *Trichodesmium* may occur in gigantic blooms in the oceans, and the discovery of its nitrogen fixation was crucial for the understanding of the global nitrogen budgets. The nitrogen fixation in *Trichodesmium* is only taking place during illumination, which could be expected to suppress the process by oxygenic photosynthesis. *Trichodesmium* grows in loose aggregates, but microsensor investigations have not shown anoxic sites in the aggregates, and it is likely that *Trichodesmium* protects its nitrogenase in a similar way as *Azotobacter*, but other mechanisms are also possible (55). Recently, it was shown that nitrogen fixation in *Trichodesmium* is under circadian control (56). It was discovered that the transcription of the *nifHDK* gene (nitrogenase), the transcription of the *psbA* gene (constituent of PSII), and the transcription of the *psaA* -gene (constituent of PSI) are under control of a circadian rhythm, and that they are temporally separated over the diel cycle. It was concluded that a phase separation between the expression of photosynthesis genes and nitrogen fixation genes, which is clearly maintained by the circadian rhythm, could be one of the underlying mechanisms that allows oxygen-sensitive nitrogen fixation to proceed in *Trichodesmium* IMS 101 during illumination. Both *Trichodesmium* and *Plectonema* (see earlier section) thus seem to be able to accomplish a temporal separation of oxygenic photosynthesis and nitrogen fixation during the light period.

The nitrogenase from the thermophilic *Streptomyces thermoautotrophicum* is very different from all other known nitrogeases and the mechanism of nitrogen fixation mechanism is also highly unusual (48). The perhaps most striking difference is that this nitrogenase is not adversely affected by oxygen, and the mechanism of nitrogen fixation actually involves oxygen and superoxide. Reduction of acetylene to ethylene by nitrogenase is usually used

to measure the activity of nitrogen fixation in natural samples, but this organism does not produce ethylene. The question of whether similar unusual nitrogenases are found also by mesophilic organisms remains to be answered.

Nitrification and Denitrification

The oxidation of ammonium to nitrite by ammonium-oxidizing bacteria, such as *Nitrosomonas* and *Nitrosospira*, and the subsequent oxidation of nitrite to nitrate by *Nitrobacter*- type bacteria are oxic processes. Ammonium may also be oxidized by nitrite by the so-called anammox process (57) originally discovered in a wastewater treatment plant, but the end-product of this process is nitrogen gas. It is not yet known how important the anammox precess is in nature, but there are strong indications that it may occur in some marine sediments.

As nitrification is an aerobic process, nitrite and nitrate may be produced in both the pelagic environment and in the oxic surface layer of sediments. An example of a NO_x^- (i.e., $NO_2^- + NO_3^-$) profile in a marine sediment as measured with a microscale NO_x^- biosensor (58) is shown in Figure 5 together with an oxygen profile. The oxygen penetration in this coastal sediment was about 3 mm, and nitrification in the layer caused an elevation of the NO_x^- concentration from 4 μM in the overlying water to almost 10 μM at 2-mm depth. Nitrate consumption in the anoxic layers below 3-mm depth caused a decrease to zero at a depth of about 5 mm (59). In most nonphotic sediments, the majority of the NO_x^- consumption is due to denitrification. Denitrification is a predominantly anoxic process, but calculations performed on many profiles indicate that some denitrifying activity may occur at oxygen concentrations of a few micromolar (60). Denitrifying bacteria are facultative anaerobes (although exceptions to this general statement my be found (61)),

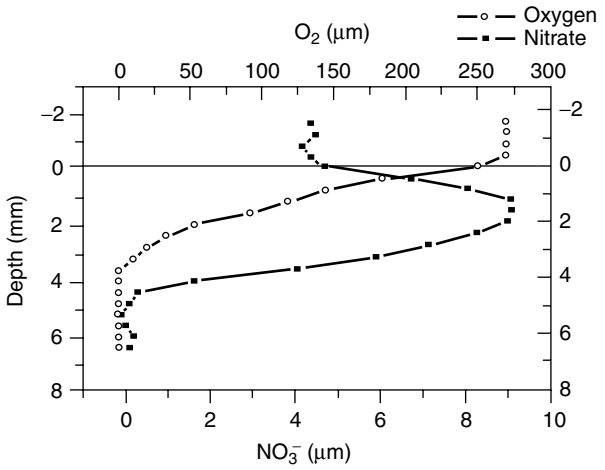

Figure 5. Profiles of O_2 and NO_x^- in a Danish coastal marine sediment. There was a production of NO_x^- in the oxic layer down to 3-mm depth producing a small peak in NO_x^-, and denitrification depleted NO_x^- at a depth of 4 to 5 mm. Redrawn with permission from N. P. Revsbech et al., in J. Buffle and G. Horvai, eds., In situ monitoring of aquatic systems: Chemical analysis and speciation, John Wiley & Sons, **2000**, pp. 195–222.

and as the energy yield by oxygen respiration is much higher than by denitrification, only few bacteria exhibit denitrification at high oxygen concentrations when grown in pure culture (62). It is possible that the aerobic denitrification exhibited by a few laboratory strains is caused by mutations that would have reduced competitiveness under natural conditions.

The highest nitrifying activity is often found near the oxic-anoxic interface (e.g., 63) where there is a high diffusive supply of ammonium from anoxic layers. It should therefore be expected that nitrifying bacteria are microaerophilic and exhibit a chemotaxis toward low oxygen as is well described for some sulfide-oxidizing bacteria (64). However, nitrifying bacteria predominantly live in immobile aggregates and do not follow changes in gradients of oxygen and ammonium. One possible reason that nitrifiers do not optimize their metabolism by chemotaxis is that they may rely on density-dependent intracellular signaling with homoserine-lactones within aggregates to be able to respond to changing availability of ammonium (65).

EFFECT OF OXYGEN ON THE IRON AND MANGANESE CYCLES

Much work has been conducted on microbial iron reduction in marine sediments (7), but the importance of microbial oxidation of Fe^{2+} in this environment has only been poorly explored because of methodological difficulties in distinguishing between chemical and biological contributions. At neutral pH iron oxidation may be biologically mediated, but the purely chemical oxidation of Fe^{2+} is so rapid at neutral pH that much and probably most Fe^{2+} oxidation in the marine environment is nonbiological. Bacteria-like *Gallionella ferruginea* overcome competition with rapid chemical iron oxidation at circumneutral pH by microaerophilic growth in environments with opposing oxygen and iron gradients where stable diffusion conditions for oxygen and ferrous iron prevail (66) or in environments with an advective supply of reduced iron (67,68). In marine environments biological iron oxidation has only been reported from hydrothermal vent sites (69,70), where the vent water may contain up to 1,000 μM Fe^{2+} and 20 μM Mn^{2+}. Large fluffy mats of precipitated iron oxides, which often consist of sheaths and filaments reminiscent of *Leptothrix ochracea* have been found in the surrounding of the vents. This suggests that the precipitated iron oxides may be of biogenic origin.

Ferrous iron oxidizing bacteria are extremely difficult to isolate in axenic culture (68). A recent publication (71) report on the isolation of a facultatively aerobic nitrate respirer from brackish sediment that oxidizes ferrous iron with nitrate as electron acceptor. It could be demonstrated that the strain also was able to grow by aerobic iron oxidation in opposing ferrous iron and oxygen gradients. Facultative iron oxidizers may be preferentially isolated with nitrate as electron acceptor instead of oxygen as chemical oxidation of iron with nitrate does not occur.

Reduced manganese in the form of Mn^{2+} ions is quite soluble at circumneutral pH, but in contrast to ferrous

iron, the spontaneous chemical reaction of Mn^{2+} with oxygen is very slow. Only above pH 8 does the chemical oxidation of Mn^{2+} become significant (72). Comparison of Mn^{2+} fluxes toward the oxic zone of a sediment and Mn^{2+} fluxes from the sediment and into the overlying water implied a 1,000 times higher oxidation rate in the sediment than would be the case by purely chemical oxidation with oxygen (73). Thus, at circumneutral pH, Mn^{2+} oxidation in sediments may be biologically mediated. Many bacteria catalyze the oxidation of Mn^{2+} with oxygen (74) and insoluble manganese oxide precipitate on the outside of Mn^{2+}-oxidizing cells (68). Manganese oxide encrustations may protect cells against grazing by protozoa, viral attack, or detoxify oxygen radicals (68). Despite the active involvement of bacteria in Mn^{2+} oxidation, growth-linkage to this process has not been observed. Manganese-oxidizing bacteria may be important for the formation of manganese nodules that are found on the surface of many deep-water sediments (75).

OXYGEN AND THE SULFUR CYCLE

Sulfide-Oxidizing Organisms

We currently have only a limited understanding of sulfide oxidation in the surface layers of marine sediments, where the overwhelming part of the sulfide formed by sulfate reduction is oxidized. Some hydrogen sulfide is oxidized chemically with oxygen or by aerobic chemolithotrophs and anoxygenic phototrophs, and these oxidations have been studied extensively (42). In most marine areas only little sulfide is, however, oxidized directly by oxygen. It reacts instead with oxidized iron and manganese compounds that are subsequently reoxidized with oxygen by either burrowing fauna (Fig. 3) or by strong turbulence in the overlying water and resuspension and repositioning of the sediment (76). In some areas the predominant electron acceptor for sulfide oxidation seems to be nitrate that is either used directly near the sediment-water interface or is transported to deep layers by gliding bacteria of the genera *Thioploca* or *Beggiatoa* (24,77).

Chemolithotrophic thiobacilli are probably the major aerobic sulfide oxidizers in nature. Many species of this genus are well characterized physiologically and phylogenetically, and the major biochemical pathways of sulfide oxidation have been elucidated (78,79). However, much interest has been attached to the larger sulfide-oxidizing bacteria of the microaerophilic genera *Beggiatoa* and *Thiovolum*, as they exhibit unique physiological and tactic characteristics (80). However, only a limited number of *Beggiatoa* strains have been cultured, and no *Thiovolum* isolates have been obtained yet. Our knowledge about the physiology of these large sulfide-oxidizing bacteria is thus still very incomplete.

Beggiatoa spp. often covers the surface of reduced marine sediments, where they oxidize sulfide and organic compounds diffusing upwards with oxygen diffusing downwards. The sediment surface may be totally white because of the mats of *Beggiatoa*, but often only depressions in the sediment are covered, resulting in white flakes on a gray-brown sediment surface. However, *Beggiatoa* is also present in the areas with an oxidized surface, but most filaments there are found at the oxic/anoxic interface located at some depth in the sediment. At least the cultivated *Beggiatoa* strains are microaerophilic, and exhibit a tactic response toward low oxygen (64,81). The fact that beggiatoas in nature have the ability to accumulate at the oxic/anoxic interface rather than "getting lost" in the anoxic sediment may be a result of negative sulfide taxis (64). *Beggiatoa* spp. do not have the enzyme catalase to protect them against hydrogen peroxide formed as a reduction product of oxygen, and they actually do not tolerate high oxygen concentrations for extended periods of time (82). There is evidence that some (uncultured) *Beggiatoa* sp. may reduce nitrate (83), and such a capability may explain some observations of occurrence that do not seem to be associated with an oxic/anoxic interface (84). The taxis toward low oxygen concentrations makes it possible for *Beggiatoa* to maintain a very high density at the oxic/anoxic interface and thereby to be very efficient in the oxidation of hydrogen sulfide. Profiles of oxygen and sulfide measured in sediment covered with *Beggiatoa* (Fig. 6) show how oxygen and hydrogen sulfide overlap in an $<$ 100 μm thick layer. Oxygen and sulfide are continuously supplied to this thin layer by diffusion from above and below, respectively, and the turnover-time for the sulfide and oxygen pools within the overlap zone is about 1 s, which is several orders of magnitude faster than by a purely chemical oxidation. Comparable studies with pure *Beggiatoa* cultures in an agar-stabilized gradient system showed that the sulfide-oxygen overlap zone was almost 1 cm in the absence of bacteria and about 0.1 mm in the presence of *Beggiatoa* (82).

Theoretically, an organism such as *Beggiatoa*, with a typical diameter of about 10 μm, should experience a relatively lower diffusive supply of oxygen and electron donors (85) than, for example, thiobacilli, with a typical size of 1×2 μm. The explanation for the success of some large bacteria in nature is always that the large size confers some advantage that is greater than the loss in fitness owing to inferior diffusive supply. The largest bacteria known (up to 750 μm in diameter) are thus nitrate-accumulating sulfide oxidizers that, by virtue of their size, can store a large amount of nitrate in an internal vacuole (86). The advantage of large size for *Beggiatoa* is poorly studied, but it may involve factors as the ability to store large amounts of sulfur intracellularly, rapid gliding mobility, reduced predation, and increased tolerance of the community to hydrodynamic stress. A study of a sulfidic coastal sediment showed that grazing resulted in a succession from unicellular motile bacteria-like *Thiobacillus* and *Thiovolum* over thin *Beggiatoa* species to large *Beggiatoa* with a diameter of about 30 μm (87).

Thiovolum has a somewhat different strategy than *Beggiatoa*, with which it is relatively closely related (86). Both rely, at least partly, on the oxidation of sulfide, but where *Beggiatoa* may form a well-defined oxidation zone within the sediment or at the sediment surface (Fig. 6), *Thiovolum* is able to move this oxidation zone up into the water-phase. It is able to form veils, which separates

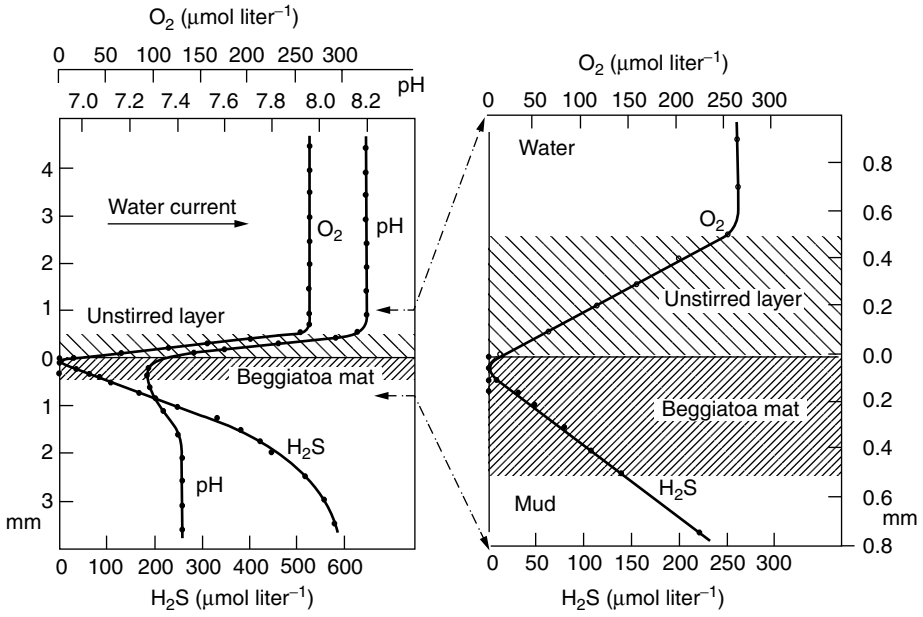

Figure 6. Oxygen and hydrogen sulfide distribution in a marine sediment covered with a Beggiatoa mat. Panel A to the left shows O_2, pH, and dissolved sulfide (H_2S) over a depth scale of several mm, and the enlarged panel (B) to the right shows that the overlap zone between sulfide and oxygen was < 0.1 mm. Reproduced with permission B. B. Jørgensen and N. P. Revsbech, *Appl. Environ. Microbiol.* **45**, 1,261–1,270 (1983).

anoxic, sulfide-containing water on one side of the veil from oxic water at the other side (80). The veils are kept together with filaments of extracellular polymeric material. By forming veils in the water the *Thiovolum* cells avoid competition from other bacteria. The veil can disintegrate into individual cells that may subsequently aggregate to form a new veil at another location when the conditions for *Thiovolum* deteriorate as a result of competition from colonizing organisms or because of lowered oxygen or sulfide availability. Colony formation in *Thiovolum* is unique. It is used not only to separate sulfide and oxygen so that a well-defined oxidation zone can be established, but also to create a water current through the veil by flagellar movement of the individual bacteria. Consequently, substrate availability is increased by a factor of about 40 above that possible by molecular diffusion (88).

Oxygen and Sulfate-Reducing Bacteria

Sulfate-reducing bacteria were originally described as highly oxygen-sensitive obligate anaerobes, and their distribution was asserted to be restricted to permanently anoxic environments. However, this has proven untrue (86). Over the last two decades reports have accumulated that document the presence of large numbers of viable sulfate-reducing bacteria in permanently or temporally oxygenated environments such as oxidized surface sediments (87), seawater (88), photosynthetic microbial mats (89–91), and associations with the rhizoplane of marine macrophytes (92,45). These observations indicate that sulfate-reducers must have developed mechanisms to cope with toxic oxygen radicals (see earlier section). Indeed superoxide dismutase and catalase were detected in a wide variety of "strict" anaerobic bacteria, including sulfate-reducing bacteria (93,13,94). In addition, the presence of polyglucose in several species of sulfate-reducers was interpreted as an adaptation to the periodic occurrence of oxic conditions in the environments from which they were isolated, typically the oxic–anoxic interface of microbial mats (95). The mechanism of polyglucose mediated oxygen tolerance has not yet been identified. Recently, it has been proposed (96) that polyglucose may stabilize NADH oxidases and prevent them from rapid inactivation in the presence of oxygen. It was also shown (96) that polyglucose containing *Desulfovibrio*-species were able to reduce oxygen at much higher ambient concentrations than *Desulfovibrio*-species that lack the ability to synthesize polyglucose.

However, sulfate-reducing bacteria may not only tolerate oxygen (97), they may also use it as an electron acceptor (98,99). It has thus been shown that several stains from different genera are able to use oxygen. Although energy is conserved (ATP formed) by the electron transport, none of the strains examined thus far can grow with oxygen as sole electron acceptor (100). Results from experiments with *Desulfovibrio vulgaris* CNS (101–103) even suggest that some sulfate-reducers can oxidize sulfide to sulfate in the presence of oxygen. The mechanism proposed involves an initial biologically mediated oxidation of sulfide to elemental sulfur and concomitant reduction of oxygen followed by the bacterial disproportionation of elemental sulfur into sulfate and sulfide. Recent investigations have also demonstrated that some sulfate-reducing bacteria are microaerophilic and exhibit a positive O_2 taxis toward low oxygen concentrations (104,105). The reason for such a positive aerotaxis is obscure, as no hitherto isolated sulfate-reducer can grow with oxygen as electron donor. However, a positive chemotaxis toward oxygen may enable freshwater sulfate-reducers, which naturally live under sulfate-limited conditions, to find the zone (oxic-anoxic interphase) where intermediately oxidized sulfur compounds, such as thiosulfate or elemental sulfur are formed. As mentioned above, sulfate-reducers may even actively participate in

the formation of these compounds, which then serve as substrates for growth.

There have been reports of extremely high sulfate reduction rates in the oxic layers of microbial mats, where a high supply of electron donors could originate from oxygenic photosynthetic activity of the cyanobacterium *Microcoleus chtonoplastes* (106). The initial findings of high aerobic sulfate reduction rates have not, however, been supported by more controlled experimental evidence. It is difficult to explain how an energetically inferior process could occur at high rates when it has to compete with oxygen-respiring microorganisms for the same substrates.

ANALYSIS OF OXYGEN AT A SCALE RELEVANT TO THE WORLD OF MICROORGANISMS

This review has shown several examples of microscale profiles of oxygen in microbial communities (Figs. 2–6), without going in any detail about the techniques available to obtain such information. These techniques have just been reviewed extensively (107,108), and only a very short description will therefore be given here. All the oxygen profiles shown in the figures were recorded with electrochemical oxygen microsensors such as the one shown in Figure 7. The gold cathode within such a sensor reduces oxygen entering through the silicone membrane to OH^-, and the current resulting from this reduction is proportional to the amount of oxygen being reduced. Because of a tip diameter of only a few micrometers, diffusion of oxygen to the tip is a very efficient means of transport as compared to advection, and the signal from such sensors is therefore almost ($< 2\%$) unaffected by changes in water flow. Even changes in diffusion coefficient of the analyzed medium do ordinarily not affect the reading to any significant extent. Sensors can be made with tip diameters down to 1 μM, and 90% response times can be down to 0.1 to 0.2 s. Commercial suppliers of such oxygen microsensors are Unisense A/S, Aarhus, Denmark, and Diamond Electrodes Inc, Ann Arbor, Michigan. An alternative is to use optic fibers with a tip coating that exhibits an oxygen-dependent fluorescense. Such sensors cannot be made as small as the electrochemical sensors, and they also react considerably slower to changes in oxygen concentration, but they have the advantage of being completely insensitive to water flow and diffusivity. Such optical sensors (optodes) are sold by Presense, Regensburg, Germany. A two-dimensional set of information about the distribution of oxygen may be obtained by use of so-called planar optodes, where a transparent sheet is covered with the same fluorescent material as used in ordinary oxygen optodes. The planar optodes may, for example, be helpful when the oxygen dynamic at the bottom of a biofilm (growing on the planar optode) is studied. Planar optodes are at present not commercially available.

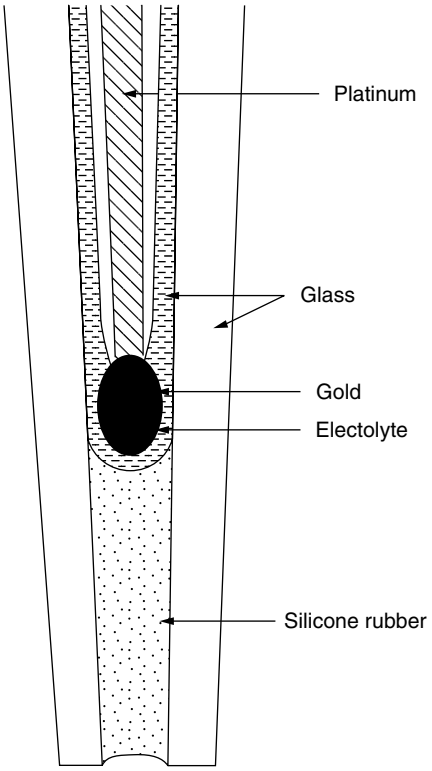

Figure 7. Oxygen microsensor with a tip diameter of about 5 μm. This type of sensor and similar microsensors for other chemical species may be used to analyze the chemical microenvironment in microbial communities.

CONCLUSION

Oxygen is a crucial chemical species for all microorganisms, whether it functions as the optimum electron acceptor for energy generation or has the role of a dangerous poison that has to be detoxified. Microbiologists generally distinguish between aerobic and anaerobic microorganisms, but it is becoming increasingly clear that the previous dogmas about the detrimental effects of oxygen on organisms such as sulfate-reducers that were thought to be strict anaerobes have to be reconsidered. Many "strict" anaerobes are actually equipped with defence mechanisms against oxygen or may even to some extent be able to use oxygen as an electron acceptor. The dogma about the detrimental effect of oxygen on the nitrogenases of nitrogen-fixing prokaryotes also has to be modified, as nitrogen fixation has been described to occur under fully aerobic conditions. The development of analytical techniques that allow for resolution of microscale chemical gradients of oxygen (and other relevant chemical species) has made it possible to observe exactly where oxygen is present and also at which rates it is being metabolized. The rise of molecular microbial ecology has at the same time enabled visualization of the presence and distribution of specific kinds of microorganisms, even when these microorganisms cannot be cultured. Together these powerful in situ techniques offer an outstanding opportunity to study the ecological niches of microorganisms, but whenever possible such studies should be combined with detailed physiological studies of pure cultures.

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OXYGENASE ENZYMES: ROLE IN BIODEGRADATION

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The global carbon cycle is necessary for life on earth. Inorganic carbon that is fixed and transformed into biochemical compounds by metabolic processes must eventually be mineralized to carbon dioxide, so that it will be available to subsequent generations of living organisms. Biodegradation and mineralization of natural and artificial organic compounds through catabolic pathways occurs both in the presence (aerobic) and in the absence (anaerobic) of molecular oxygen (O_2), producing energy and carbon for metabolism and growth of prokaryotic and eukaryotic microorganisms. Molecular oxygen can support aerobic biodegradation in two ways: (*1*) as a terminal electron acceptor for oxidation of organic compounds via pathways linked to aerobic respiration and (*2*) as a reactant in catabolic reactions resulting in incorporation of one or both oxygen atoms into organic substrates. The latter function is important for biodegradation of relatively inert organic compounds and is catalyzed by a class of enzymes known as oxygenases (1,2). Oxygenases act in conjunction with other enzymes such as dehydrogenases, hydrolases, and aldolases, by catalyzing one or more steps of the microbial biodegradative pathways that channel substrate carbon

into the central metabolic pathways. Since the discovery of the first oxygenase by Hayashi approximately half a century ago (3), the functions of these enzymes in numerous biodegradative pathways have been well established and their properties extensively described. An appreciation of the scope of the interest in oxygenases can be gained by consulting the many excellent review articles, book chapters, and books that have been published (4–16). In addition, comprehensive information about the roles of oxygenases as well as other enzymes of the biodegradative pathways can be found on the web site of The University of Minnesota Biocatalysis/Biodegradation Database (*http://www.labmed.umn.edu/umbbd*).

An oxygenase can be defined as an enzyme that catalyzes a chemical reaction between molecular oxygen (O_2) and a substrate, usually an organic compound, resulting in the formation of a product that contains one or both of the oxygen atoms. Oxygenases are found in many species of prokaryotes (bacteria) and eukaryotes (e.g., yeast, fungi, algae, plants, and animals), where they perform a variety of metabolic functions. In higher eukaryotes, they play important roles in detoxification and excretion of organic pollutants, metabolism of drugs, and biosynthesis of sterols, hormones, and other physiologically active compounds. In some species of yeast, fungi, and bacteria, oxygenases act in concert with other enzymes in catabolic pathways that catalyze reactions resulting in the breakdown and mineralization of stable organic compounds that enter the environment through natural processes and human activities. Major sources of these compounds include natural seeps and accidental spills of unrefined and refined petroleum, lignin, terpenes, and other secondary metabolites produced by plants and artificial organic compounds produced via synthesis of industrial chemicals. Contributions from the latter source increased dramatically during the twentieth century with the industrial revolution and human population explosion. Deliberate application of agricultural pesticides, release of industrial and domestic wastewater, exploitation of fossil fuels and leachates from industrial waste dumps, leaking pipelines, and chemical storage tanks have contributed to the presence of large amounts of recalcitrant toxic organics in the environment. Oxygenases that participate in the biodegradative pathways of microorganisms allow their cells to obtain carbon and energy for growth and metabolism of organic compounds that would otherwise be useless or even harmful to the organism.

Artificial organic compounds are produced by chemical synthesis, rather than by biochemical reactions, and many possess combinations of functional groups and chemical bonds that are not found in nature. Biodegradative pathways of these xenobiotic compounds may not exist in nature or mineralization may be incomplete and dead-end metabolites may be released into the environment. Research is currently being conducted to speed up the evolution of biodegradation pathways using the recently developed tools of molecular biology to produce oxygenases and other biodegradative enzymes with broader specificities for their substrates (17). The goal is to increase the rate of mineralization of troublesome organic pollutants such as atrazine and polychlorinated biphenyls (PCBs) (18,19).

The two main functions of oxygenases in catabolic pathways are activation of otherwise biochemically inert compounds via catalysis of hydroxylation reactions and cleavage of cyclic compounds so that they can be metabolized by central metabolic pathways (20). Biodegradation of cyclic hydrocarbons requires both hydroxylation and ring cleavage. Substituents (e.g., chloro, nitro, and sulfono groups) that chemists add to already stable organic compounds may retard biodegradation of a compound by inhibiting the activity of oxygenases as well as other enzymes of the biodegradative pathway. In some instances, the activity of oxygenases results in the removal of the inhibitory substituent and enhanced degradation of the product. For example, biphenyl 2,3-dioxygenase is the first enzyme of a catabolic pathway for degradation of biphenyl (21,22). In *Burkholderia* sp. strain LB400 this enzyme catalyzes the addition of molecular oxygen to some *ortho*-substituted PCBs, resulting in the spontaneous elimination of the chloro substituent from the *ortho* position of the ring (23). Other examples include dioxygenase-mediated elimination of fluoro, chloro, bromo, iodo, and amino substituents from aromatic rings of *ortho*-substituted benzoates (24–26), *para*-chloro substituent from 4-chlorophenylacetate (27), and monooxygenase-mediated elimination of *para*-chloro and *ortho*-chloro substituents from chlorophenols (28).

Biodegradation pathways involving oxygenases are utilized by bacteria, yeast, and fungi, but the broad diversity of biodegradative enzymes in bacteria has attracted the most attention because of the potential for biotechnological applications involving bioremediation and industrial uses such as production of chiral synthons for synthesis of single-enantiomer drugs and industrial chemicals (13,29). This article will highlight the activities of bacterial oxygenases that catalyze the incorporation of molecular oxygen into naturally occurring and artificial organic compounds. The types and basic functions of oxygenases will be discussed first, followed by an examination of their roles in the degradation of various classes of organic compounds.

Traditionally, oxygenases have been described as either monooxygenases or dioxygenases, on the basis of the number of molecular oxygen atoms contained in the product of the reaction (Table 1). Monooxygenases catalyze the addition of one oxygen atom, whereas dioxygenases catalyze the addition of both oxygen atoms to the substrate (S) as shown (30).

$$S + \frac{1}{2}O_2 \xrightarrow{\text{monooxygenase}} SO \quad (1)$$

$$S + O_2 \xrightarrow{\text{dioxygenase}} SO_2 \quad (2)$$

One limitation of this classification scheme is that some oxygenases do both, depending on the substrate. For example, the toluene dioxygenase of *Pseudomonas putida* F1 catalyzes the addition of one atom of molecular oxygen to the methyl group of 2- and 3-nitrotoluene (38), whereas

Table 1. Examples of Biodegradative Oxygenases

Reaction and Representative Enzyme	Enzyme Cofactor	EC Number[a]	Reference
I. Monooxygenation			
A. Hydroxylation			
Pentachlorophenol monooxygenase	Flavin	1.14.13.50	31
Camphor 5-monooxygenase	Heme iron	1.14.15.1	32
Alkane monooxygenase	Nonheme iron	1.14.15.3	33
B. Baeyer-Villiger monooxygenation			
Cyclohexanone monooxygenase	Flavin	1.14.13.22	34
II. Dioxygenation			
A.1. Hydroxylation			
Toluene dioxygenase	Nonheme iron	1.14.12.11	35
A.2. Hydroxylation/decarboxylation			
2,4-D/α-ketoglutarate dioxygenase[b]	Nonheme iron	1.14.11.-	36
B.1. Aromatic ring cleavage, intradiol			
Catechol 1,2-dioxygenase	Nonheme iron	1.13.11.1	3
B.2. Aromatic ring cleavage, extradiol			
Catechol 2,3-dioxygenase	Nonheme iron	1.13.11.2	37

[a]Enzyme Commission number (Nomenclature Committee of the International Union of Biochemistry and Molecular Biology).
[b]2,4-D, 2,4-dichlorophenoxyacetic acid.

the 4-methoxybenzoate monooxygenase of *P. putida* DSM1868 catalyzes the addition of both atoms of molecular oxygen to the vinyl group of 4-vinylbenzoate (39). However, in these and other examples (40), the atypical activity of the enzyme occurs with nonphysiological substrates, whereas with physiological substrates (usually the one that lends its name to the enzyme) one or both atoms of molecular oxygen are added when the reaction is catalyzed by a mono- or dioxygenase, respectively.

Mono- and dioxygenases may be further described based on whether the oxygen atom(s) of the product is present as a hydroxyl group(s) substituent of one of the carbon atoms (hydroxylase) or whether oxygen is inserted between a carbon-carbon bond (Table 1). A monooxygenase that inserts an oxygen atom between a carbonyl carbon and an adjacent carbon atom of aliphatic compounds (open chain and cyclic) is called a *Baeyer-Villiger monooxygenase* (BVMO) because the product is the same as that produced by the Baeyer-Villiger reaction familiar to organic chemists. Dioxygenases that insert both oxygen atoms between two carbon atoms of an aromatic ring are called *ring-cleavage dioxygenases* because the product is acyclic with the oxygen atoms present in two carboxylate groups or in a carboxylate and a carbonyl group of the product. Two hydroxyl groups must already be present as substituents of aromatic substrates for ring cleavage to occur, however. Intradiol ring-cleavage (also called *ortho ring cleavage*) dioxygenases catalyze the cleavage of the ring between two adjacent (vicinal) hydroxyl groups, whereas extradiol ring-cleavage (also called *meta ring cleavage*) dioxygenases catalyze the cleavage of the ring adjacent to only one of the hydroxyl groups. The first oxygenase discovered was a bacterial intradiol ring-cleavage dioxygenase that catalyzed formation of *cis*, *cis*-muconic acid from catechol (1,2-dihydroxybenzene) (30).

Mono- and dioxygenases employ cofactors at their active sites, which are involved in binding molecular oxygen prior to its incorporation into the organic substrate (Table 1). These cofactors also perform a mechanistic role in catalysis by activating molecular oxygen or the substrate, thus facilitating the reaction between the two. Flavin adenine dinucleotide (FAD) or flavin mononucleotide (FMN) or some form of iron (heme or nonheme) is the most common cofactor. Additional reactants and products may also be involved; these will be described with the specific examples discussed later.

MONOOXYGENASES AND THEIR ROLES IN BIODEGRADATIVE PATHWAYS

Most monooxygenases found in biodegradative pathways catalyze the addition of one atom of molecular oxygen to their substrates, whereas the other oxygen atom is reduced to a molecule of water. Two reducing equivalents (electrons) are also required and are usually supplied by the coenzymes reduced nicotinamide adenine dinucleotide (NADH) or reduced nicotinamide adenine dinucleotide phosphate (NADPH).

Monooxygenases are involved in the degradative pathways of open-chain and cyclic aliphatic compounds and aromatic compounds. The enzymes are structurally diverse in that the number of protein components and the number and type of cofactors vary, depending on the source of the enzyme. The following discussion will focus on only a few of the monooxygenases that have been described, but will illustrate that a range of catalytic activity and diversity of composition of the enzyme protein components and cofactors exists in different microorganisms.

Hydrocarbons are chemically inert and addition of oxygen activates the compounds for further degradation, as illustrated for linear alkanes, compounds that

are major constituents of petroleum. Monooxygenase-catalyzed hydroxylation of alkanes often occurs at a terminal methyl group (41). (In the following reactions, all reactants and products may not be depicted.)

$$R{-}CH_3 + O_2 + 2e + 2H^+ \longrightarrow R{-}CH_2OH + H_2O \quad (3)$$

The products are water and an alcohol that is oxidized to a fatty acid in reactions catalyzed by an alcohol dehydrogenase and an aldehyde dehydrogenase.

$$R{-}CH_2OH \longrightarrow R{-}CHO \longrightarrow R{-}COOH \quad (4)$$

The fatty acid is then coupled to coenzyme A (CoA) and mineralization proceeds via the β-oxidation pathway and the Krebs cycle.

$$\begin{array}{l} R{-}COOH \longrightarrow R{-}COCoA \longrightarrow Acetyl{-}CoA \\ \qquad\qquad\qquad\qquad\qquad\qquad\quad \downarrow \\ \qquad\qquad\qquad\qquad\qquad\qquad\quad CO_2 + H_2O \end{array} \quad (5)$$

Some alkane monooxygenases also hydroxylate the methyl group of straight-chain fatty acids, producing ω-hydroxy fatty acids.

$$\begin{array}{l} CH_3{-}(CH_2)_n{-}COOH + O_2 \\ \qquad\qquad\qquad\quad \downarrow \\ CH_2OH{-}(CH_2)_n{-}COOH + H_2O \end{array} \quad (6)$$

The hydroxylated carbon is then oxidized producing a diterminal carboxylic acid. Further degradation proceeds via β-oxidation and the Krebs cycle as described in equation (5). Branched alkanes are also degraded by alkane monooxygenase–mediated pathways, but less efficiently because branching hinders subsequent degradation reactions (42).

Less commonly, degradation pathways for alkanes involve a monooxygenase-catalyzed attack at a subterminal carbon position producing a secondary alcohol (7,41). Oxidation of the subterminal hydroxyl group by a dehydrogenase-catalyzed reaction produces a ketone.

$$\begin{array}{l} R{-}CH_2{-}CH_3 + O_2 \longrightarrow R{-}CHOH{-}CH_3 \\ \qquad\qquad\qquad\qquad\quad \downarrow \\ \qquad\qquad\qquad\qquad\quad R{-}CO{-}CH_3 \end{array} \quad (7)$$

The ketone is a substrate for a BVMO that inserts one atom of molecular oxygen between the carbonyl carbon and the adjacent carbon on the side with the longest alkyl group, producing an ester (43).

$$R{-}CO{-}CH_3 + O_2 \longrightarrow R{-}O{-}CO{-}CH_3 + H_2O \quad (8)$$

The ester is then hydrolyzed in a reaction catalyzed by an esterase producing an alcohol and acetic acid.

$$R{-}O{-}CO{-}CH_3 + H_2O \longrightarrow R{-}OH + CH_3{-}COOH \quad (9)$$

These products are then mineralized through pathways involving β-oxidation and the Krebs cycle as shown in equations (4) and (5).

Two types of monooxygenases that hydroxylate linear alkanes have been characterized. The alkane monooxygenase of *Pseudomonas oleovorans* is a multicomponent enzyme system consisting of the catalytic hydroxylase, an electron transport protein (rubredoxin) and an NADH: rubredoxin oxidoreductase (reductase) (44) (Fig. 1a). The three components together constitute a short electron transport chain containing flavin and iron redox centers that are reduced and oxidized as electrons are transferred from NADH to the enzyme's active site. The reductase, which contains FAD, oxidizes NADH and passes the electrons to rubredoxin, which contains two ferric iron ions that accept the electrons from FAD (45,46). The electrons are then transferred from rubredoxin to the hydroxylase that contains a diiron cluster at the active site, which binds and activates molecular oxygen during hydroxylation of alkane substrates (47). The hydroxylase is associated with the cell membrane, whereas the electron transport components are located in the cytoplasm. This enzyme system initiates the degradation pathways of $C_6{-}C_{14}$ *n*-alkanes (48). In addition, the enzyme hydroxylates the methyl group of $C_6{-}C_{12}$ fatty acids (Eq. 6). The alkane monooxygenase of *Corynebacterium* sp. strain 7E1C (49) has a different structure than that of *P. oleovorans*, though the product of the reaction is the same for both enzymes. In *Corynebacterium* the enzyme appears to consist only of a reductase and a hydroxylase component (Fig. 1b). The reductase is a flavoprotein, whereas the hydroxylase is a cytochrome P450, which contains iron bound to the protein as heme. Nonheme iron is also present in the hydroxylase component. The heme iron binds and activates molecular oxygen during catalysis. This type of monooxygenase

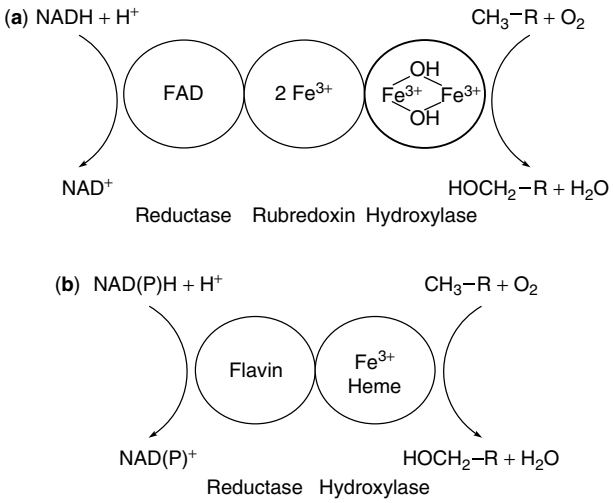

Figure 1. Proposed arrangement of the protein components of alkane monooxygenases of: (**a**) *Pseudomonas oleovorans* and (**b**) *Corynebacterium* 7E1C. R-alkyl or carboxyalkyl.

is also present in some species of *Candida*, a yeast that grows at the expense of alkanes (41).

Methane monooxygenases of methylotrophic bacteria (bacteria that utilize methane as a growth substrate) also hydroxylate longer chain *n*-alkanes in a process called *cooxidation* (50). These bacteria utilize methane but not the longer chain *n*-alkanes for growth. Methane monooxygenase catalyzes the oxidation of methane to methanol as the first step in the methane degradation pathway, but it has a relaxed substrate specificity and fortuitously oxidizes a wide range of substrates in addition to alkanes, including chlorinated alkanes, alicyclic, and aromatic hydrocarbons (51,52). The hydroxylated products are not further metabolized by the methylotrophs and further degradation is dependent on the enzymes of pathways expressed by other microorganisms in the environment. The methane monooxygenases of *Methylosinus trichosporium* OB3b and *Methanococcus capsulatus* (Bath) are three-component enzyme systems consisting of a reductase containing FAD and a 2Fe-2S cluster, a small protein without cofactors and a hydroxylase with properties similar to those of the alkane monooxygenase described earlier (14).

A BVMO that inserts an oxygen atom into C_4-C_{14} methylketones has been purified from *Burkholderia* (*Pseudomonas*) *cepacia* (43).

$$\begin{array}{l} CH_3-(CH_2)_{1-11}-CO-CH_3 + O_2 \\ \qquad\qquad\qquad\quad \Big\downarrow \\ CH_3-(CH_2)_{1-11}-O-CO-CH_3 + H_2O \end{array} \qquad (10)$$

This enzyme may participate in alkane degradation pathways involving subterminal hydroxylation as the first step (see earlier). The enzyme is a homodimer with a molecular weight of 123,000, contains FAD as a cofactor, and is specific for NADPH as the source of reducing equivalents.

Alkenes, also known as olefins, are unsaturated hydrocarbons containing a double bond between two carbon atoms of a hydrocarbon chain, which may also be degraded by pathways involving alkane monooxygenases (41,53). As with alkanes, terminal and subterminal hydroxylation of alkenes have been shown and a variety of hydroxylated compounds are produced, depending on the site of attack by oxygen. Degradation following subterminal hydroxylation of alkenes may involve a BVMO and an esterase as described for subterminal hydroxylation of alkanes (41). An epoxide is produced when the double bond is the site of attack, however.

$$R-CH_2-CH=CH_2 + O_2 \longrightarrow R-CH_2-\underset{\diagdown\,\diagup}{\overset{O}{CH}}-CH_2 + H_2O \qquad (11)$$

Epoxides may be hydrolyzed to diols spontaneously or via a reaction catalyzed by an epoxide hydrolase.

$$R-CH_2-\underset{\diagdown\,\diagup}{\overset{O}{CH}}-CH_2 + H_2O \longrightarrow R-CH_2-COH-CH_2OH \qquad (12)$$

In contrast, the epoxide produced from propene by *Xanthobacter* sp. strain Py2 is carboxylated producing acetoacetate which enters central metabolic pathways via acetoacetyl-CoA (54).

$$CH_3-\underset{\diagdown\,\diagup}{\overset{O}{CH}}-CH_2 + CO_2 \longrightarrow CH_3-CO-CH_2-COOH \qquad (13)$$

Alicyclic hydrocarbons (cycloalkanes) contain three or more carbon atoms arranged in a cyclic structure. They are major components of petroleum and are the fraction most resistant to biodegradation (55). Isolation of pure cultures of microorganisms capable of utilizing alicyclic hydrocarbons as sole carbon sources is difficult (56). However, a *Nocardia* sp. (57), a *Pseudomonas* sp. (58), and a *Xanthobacter* sp. (59) capable of growth on cyclohexane have been isolated. The aerobic degradation pathway for cyclohexane involves initial hydroxylation to cyclohexanol in a reaction catalyzed by a monooxygenase (Fig. 2). The soluble (i.e., not membrane bound) *Xanthobacter* sp. enzyme was partially purified and appeared to be composed of three components, including a cytochrome P450 type hydroxylase (hemoprotein), a ferredoxin, and a ferredoxin reductase (59). The latter two components transfer the required reducing equivalents to the catalytic hydroxylase component. These characteristics are similar to those of cytochrome P450 camphor 5-monooxygenase (32). In the second step of the pathway, cyclohexanol is oxidized to cyclohexanone in a reaction catalyzed by a dehydrogenase, then cyclohexanone 1,2-monooxygenase inserts one atom of molecular oxygen into the ring producing ε-caprolactone (55). The purified cyclohexanone monooxygenase, a BVMO of the *Xanthobacter* sp., contains FAD and FMN and is a monomeric protein with a molecular weight of 50,000. The enzyme is specific for NADPH as a source of reducing power, but has a broad substrate specificity for cyclic alkanones containing four to eight carbons. Straight-chain alkanones are not substrates (34).

Whereas a few bacterial strains capable of growth on alicyclic compounds in pure culture have been described, the predominant pathway for degradation of alicyclic hydrocarbons in the environment is thought to involve cooxidation and commensal interactions (60). Cooxidation occurs when a carbon source that does not support growth of an organism is partially oxidized by catabolic enzymes of a biodegradative pathway for a growth substrate having a similar chemical structure (50). Commensal interactions among the members of a mixed microbial population may then complete degradation of the cooxidation product(s) (56). Thus, the complete biodegradative pathway is not present in one organism and two or more organisms acting in concert are needed for mineralization of the starting compound. In the case of alicyclic hydrocarbons, an organism that contains a broad-specificity hydroxylase that allows it to grow on alkanes may initiate degradation by hydroxylating

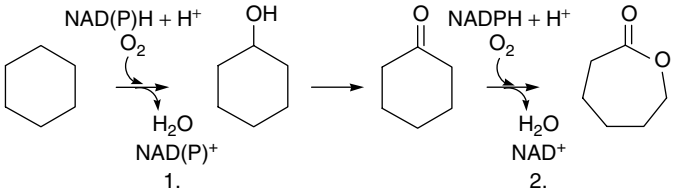

Figure 2. Involvement of monooxygenases in the degradation of cyclohexane. (*1*) Reaction catalyzed by cyclohexane hydroxylase. (*2*) Reaction catalyzed by cyclohexanone 1,2-monooxygenase. Hydrogen substituents on the ring carbon atoms are not shown.

the alicyclic hydrocarbon to a cyclic alcohol, which is then oxidized to the corresponding cyclic ketone (cycloalkanone), as describe earlier for cyclohexane. The cycloalkanone then serves as a growth substrate for other organisms containing a BVMO, esterase, and enzymes involved in β-oxidation (61). Cooxidation of alicyclic hydrocarbons has been studied in bacteria such as *Mycobacterium vaccae* JOB-5 (62) and *Pseudomonas aeruginosa* 473 (63), which do not utilize them for growth, but can initiate their degradation via hydroxylation and oxidation to cycloalkanones while growing on linear alkanes. Methylotrophs that contain a broad-specificity methane monooxygenase also carry out the initial hydroxylation of alicyclic hydrocarbons, as described earlier for alkanes (51,64). Alkyl-substituted alicyclic hydrocarbons are more predominant in petroleum than unsubstituted alicyclics and their degradation may be initiated by monooxygenases that hydroxylate either the alkyl group or the ring (55).

Terpenes are produced by plants and released into the environment on a large scale. They constitute a class of hydrocarbons containing many different acyclic, monocyclic, and bicyclic monoterpenes, which contain 10 carbon atoms (Fig. 3), as well as more complex structures such as diterpenoids (C_{20}) and triterpenoids (C_{30}). Terpenes have been shown to be degraded primarily through monooxygenase-mediated pathways. Hydroxylases and BVMOs similar to those of the degradation pathways of petroleum hydrocarbons are often involved (65). However, a pathway has been proposed for the degradation of abietic acid, a diterpenoid present in paper pulping wastewater, by *Pseudomonas abietaniphila* BKME-9, which involves hydroxylation of the aromatic ring of a proposed intermediate by a dioxygenase (66).

Hydroxylation of the C-8 methyl group of linalool by a soluble P450 monooxygenase ($P450_{lin}$) of *P. putida* PpG777 initiates the degradation of this acyclic monoterpene (Fig. 4) (67). The product of the reaction is an alcohol that can be also be oxidized to the aldehyde, 8-oxo-linalool, by the hydroxylase. The second hydroxylation reaction was hypothesized to produce an unstable gem

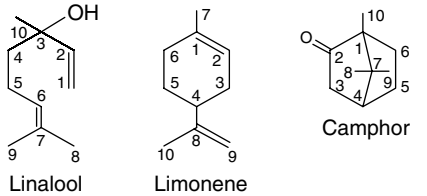

Figure 3. Examples of acyclic (linalool), monocyclic (limonene), and bicyclic (camphor) monoterpenes. Numbering schemes for the carbons atoms are shown.

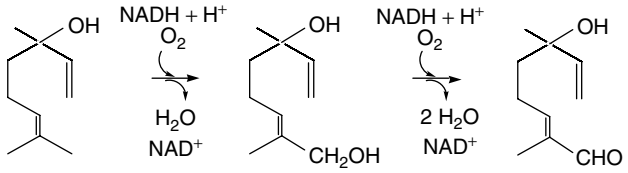

Figure 4. Hydroxylation and oxidation of linalool in reactions catalyzed by linalool 8-methyl hydroxylase of *P. putida* PpG777 (after Ullah and coworkers (67)).

diol that dehydrates spontaneously to the aldehyde. An alcohol dehydrogenase, which oxidizes the alcohol to the aldehyde, is also present in this organism. The aldehyde is likely oxidized to a carboxylic acid followed by β-oxidation. Linalool 8-methyl hydroxylase consists of three separate protein components containing redox centers: a reductase oxidizes NADH and passes the two reducing equivalents to the catalytic hydroxylase component via a small molecular weight electron carrier named *redoxin*. The reductase contains FAD as a redox center, the redoxin contains a 2Fe-2S iron-sulfur redox center, and the P450 hydroxylase contains ferric iron (Fe^{3+}) coordinated by a molecule of heme and a thiolate ligand at the active site. The properties of this enzyme are very similar to those of camphor 5-monooxygenase (see later). There is evidence for an alternate pathway that involves hydroxylation of the C-10 methyl group in this organism (65).

Several pathways have been described for the bacterial degradation of limonene (68). One pathway involves hydroxylation of the C-7 methyl group in a reaction catalyzed by a membrane-bound hydroxylase (69). A less-common pathway involves two monooxygenases: one that produces an epoxide and one with BVMO activity (68,70) (Fig. 5). The activity of limonene 1,2-monooxygenase in cell extracts of *Rhodococcus erythropolis* DCL14 was dependent on FAD, suggesting that the enzyme utilizes it as a cofactor during catalysis.

One pathway for biodegradation of camphor involves hydroxylation at C-5 and two BVMO-catalyzed reactions (Fig. 6). Hydroxylation is catalyzed by camphor 5-monooxygenase, also known as cytochrome $P450_{cam}$. This soluble enzyme has been purified from *P. putida* PpG786 and has been studied as a model for mammalian cytochrome P450s involved in a variety of metabolic reactions (32). The enzyme consists of a flavoprotein reductase, a 2Fe-2S iron-sulfur protein, and a cytochrome P450 hydroxylase with properties similar to those described earlier for linalool 8-methyl hydroxylase (67). Two different BVMOs catalyze the insertion of oxygen into the two rings, leading to cleavage of both rings. The

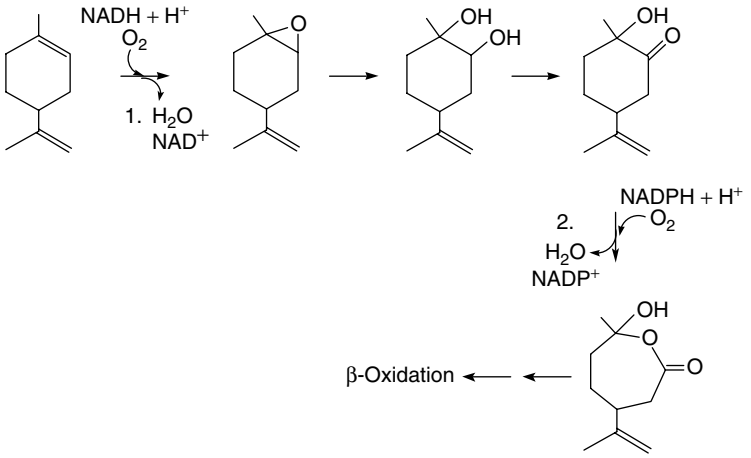

Figure 5. Involvement of monooxygenases in the degradation of limonene by *Rhodococcus erythropolis* DCL14 (68). (*1*) Reaction catalyzed by limonene 1,2-monooxygenase. (*2*) Reaction catalyzed by 1-hydroxy-2-oxolimonene 1,2-monooxygenase.

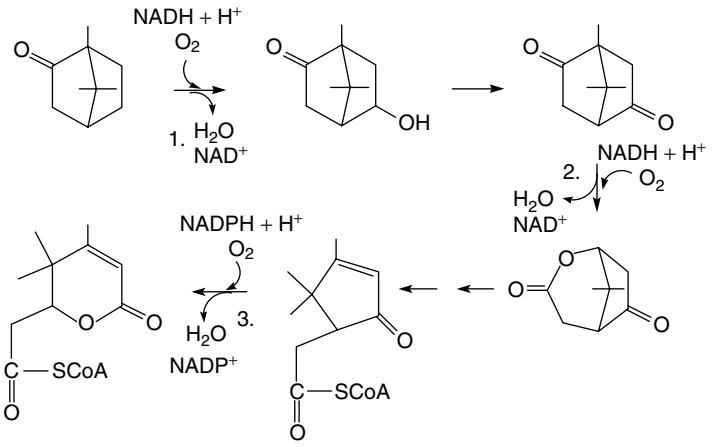

Figure 6. Involvement of monooxygenases in the degradation of camphor by *P. putida* (after Ougham and coworkers (72)). (*1*) Reaction catalyzed by camphor 5-monooxygenase (cytochrome $P450_{cam}$). (*2*) Reaction catalyzed by 2,5-diketocamphane 1,2-monooxygenase. (*3*) Reaction catalyzed by 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-CoA 1,2-monooxygenase.

first enzyme, 2,5-diketocamphane 1,2-monooxygenase, is a two-component enzyme consisting of a reductase with a molecular weight of 36,000, which oxidizes NADH, and a monooxygenase, containing FMN, which is a homodimer with a molecular weight of 78,000 (71). The second BVMO of the pathway, 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-CoA 1,2-monooxygenase, consists of a single protein component containing FAD, is specific for NADPH, and is a homodimer with a molecular weight of 106,000 (72).

Monooxygenases are also involved in the degradation pathways of aromatic ring compounds. The benzene ring is one of the most abundant and stable chemical structures present in organic compounds, plant lignin being a major source in nature (2). Biodegradation of aromatic compounds requires cleavage of the ring and metabolism to products that enter the central metabolic pathways. However, aromatic ring cleavage catalyzed by dioxygenases is a reaction that requires the presence of two hydroxyl group substituents on the ring. Thus, many of the biodegradation pathways involve hydroxylation by monooxygenases and/or dioxygenases and transformation to the dihydroxylated aromatic compounds (ring-cleavage substrates) such as catechol, protocatechuic acid or gentisic acid (Fig. 7b). For example, phenol, 2-hydroxybenzoic acid (salicylic acid), 3-hydroxybenzoic acid, and 4-hydroxybenzoic acid (Fig. 7a) are transformed to ring-cleavage substrates in reactions catalyzed by phenol hydroxylase (73), salicylate hydroxylase (74), 3-hydroxybenzoate hydroxylase (75), and 4-hydroxybenzoate hydroxylase (76), respectively. These enzymes utilize FAD as a cofactor and oxidize NADH to provide the necessary reducing equivalents for addition of one atom of molecular oxygen to the substrate and reduction of the other oxygen atom to water. Cleavage of aromatic rings will be discussed later in the section on dioxygenases.

In addition to hydroxylation of an aromatic ring, monooxygenases may hydroxylate alkyl substituents attached to the ring. Catabolism of toluene provides examples of the different ways that monooxygenases (and dioxygenases) participate in biodegradation pathways of aromatic compounds. Five different pathways have been described for the aerobic biodegradation of toluene (77). One pathway is initiated by a monooxygenase that catalyzes hydroxylation of the methyl group (Fig. 8), three pathways involve monooxygenases that hydroxylate the aromatic ring at the *ortho*, *meta*, and *para* positions, respectively (Fig. 9), whereas only one pathway is initiated by a dioxygenase that catalyzes dihydroxylation of the ring (Fig. 10).

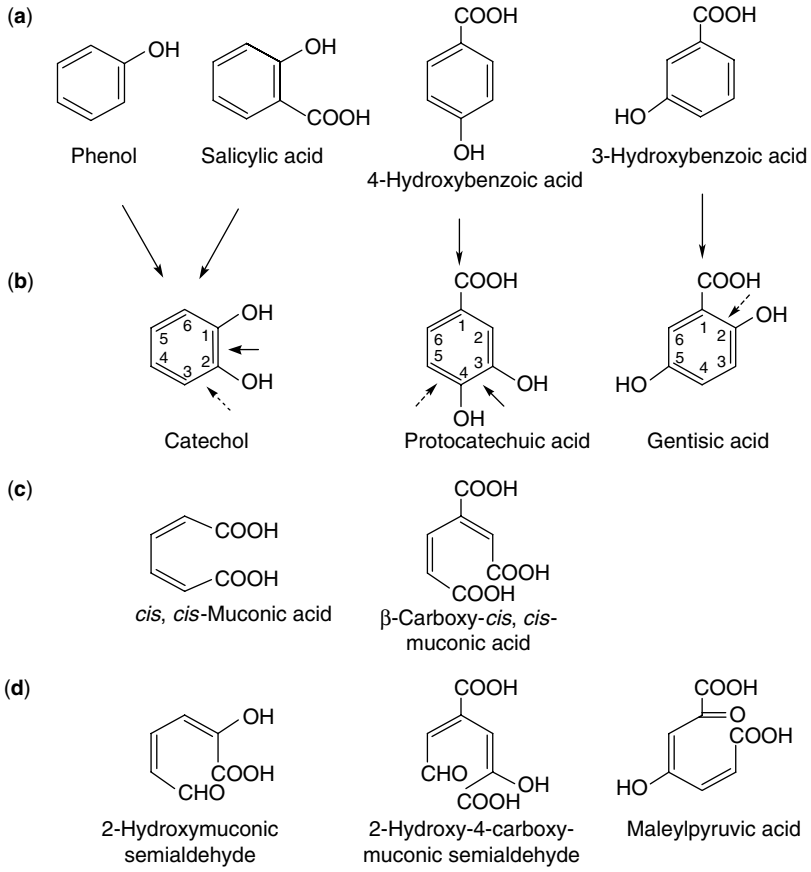

Figure 7. Transformation of aromatic hydrocarbons to common ring-cleavage substrates. (**a**) Examples of compounds that are hydroxylated in reactions catalyzed by monooxygenases. (**b**) Ring-cleavage substrates common to many pathways for degradation aromatic compounds. The positions in which intradiol (solid arrows) and extradiol (dashed arrows) ring cleavage occurs is shown. (**c**) Intradiol ring-cleavage products of dioxygenase-catalyzed attack on catechol and protocatechuic acid. (**d**) Extradiol ring-cleavage products of dioxygenase-catalyzed attack on catechol, protocatechuic acid, and gentisic acid.

The TOL plasmid of *P. putida* mt-2 encodes a pathway for biodegradation of toluene as well as xylenes and some substituted derivatives (78). Hydroxylation of the methyl group is the first step of the TOL pathway for biodegradation of toluene and *meta*- and *para*-xylenes (Fig. 8). The reaction is catalyzed by xylene monooxygenase, a two-component enzyme consisting of a reductase and a hydroxylase. The reductase is a monomer with a molecular weight of 42,000 and contains FAD and a 2Fe-2S iron-sulfur cluster as redox cofactors (79). The size of the membrane-bound hydroxylase component was calculated to be 41,650 from the deduced amino acid sequence (80). It probably contains a catalytic diiron cluster like that of the hydroxylase component of the related alkane monooxygenase (47,81) (Fig. 1). Two dioxygenases catalyze dihydroxylation and cleavage of the ring in subsequent steps of the pathway (82).

Burkholderia cepacia G4 contains a toluene 2-monooxygenase that catalyzes hydroxylation of the ring at the *ortho* position, producing *ortho*-cresol (83) (Fig. 9, reaction 1a). This enzyme also catalyzes the second ring hydroxylation (reaction 1b). The soluble enzyme was purified and shown to consist of a multisubunit hydroxylase ($\alpha_2\beta_2\gamma_2$, molecular weight 211,000) containing diiron clusters, a reductase containing FAD and 2Fe-2S iron-sulfur cluster, and a small molecular weight protein, lacking metals or organic cofactors, that enhanced enzyme activity 10-fold (84). These properties are similar to those described (or predicted from gene sequences) for alkane hydroxylase (47), soluble methane monooxygenase (85), and xylene monooxygenase (80). Ring cleavage in this pathway is catalyzed by an extradiol ring-cleavage dioxygenase (reaction 1c) (86).

Toluene 3-monooxygenase of *Ralstonia* (*Pseudomonas*) *pickettii* PKO1 catalyzes hydroxylation of the ring at the *meta* position, producing *meta*-cresol (Fig. 9, reaction 2a). Analysis of the genes encoding the enzyme suggests that it is comprised of a catalytic hydroxylase component plus a reductase and a ferredoxin (87). The second ring hydroxylation reaction of strain PKO1 is catalyzed by phenol/cresol hydroxylase (reaction 2b), a simple monooxygenase that contains only FAD as a cofactor (88). Cleavage of the ring is catalyzed by an extradiol ring-cleavage dioxygenase (reaction 2c) (89).

Toluene 4-monooxygenase of *Pseudomonas mendocina* KR1 catalyzes hydroxylation of the ring at the *para* position, producing *para*-cresol (Fig. 9, reaction 3a). The composition of the enzyme is similar to that of toluene 2-monooxygenase described earlier (90,91). Hydroxylation of the methyl group is catalyzed by *para*-cresol methyl hydroxylase, which is not an oxygenase as the hydroxyl group is derived from water. The second ring hydroxylation reaction of this pathway (reaction 3b) is likely catalyzed by 4-hydroxybenzoate 3-monooxygenase (cf. Fig. 7). This enzyme has been purified from a variety of bacteria. The enzyme of *Corynebacterium cyclohexanicum* is a

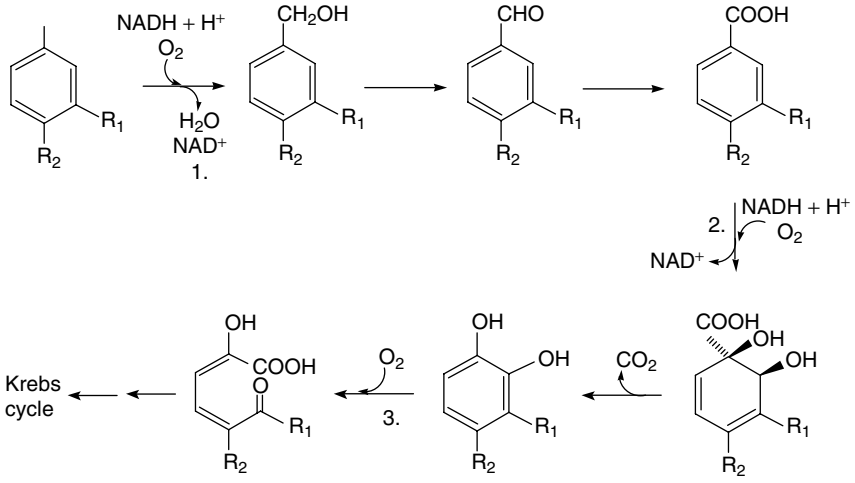

Figure 8. Involvement of oxygenases in the TOL plasmid–encoded pathway for biodegradation of toluene and xylenes. (*1*) Methyl group hydroxylation reaction catalyzed by xylene monooxygenase. (*2*) Ring dihydroxylation reaction catalyzed by benzoate dioxygenase. (*3*) Extradiol ring-cleavage reaction catalyzed by catechol 2,3-dioxygenase. Toluene, R_1 and $R_2 = H$; *meta*-xylene, $R_1 = CH_3$, $R_2 = H$; *para*-xylene, $R_1 = H$, $R_2 = CH_3$.

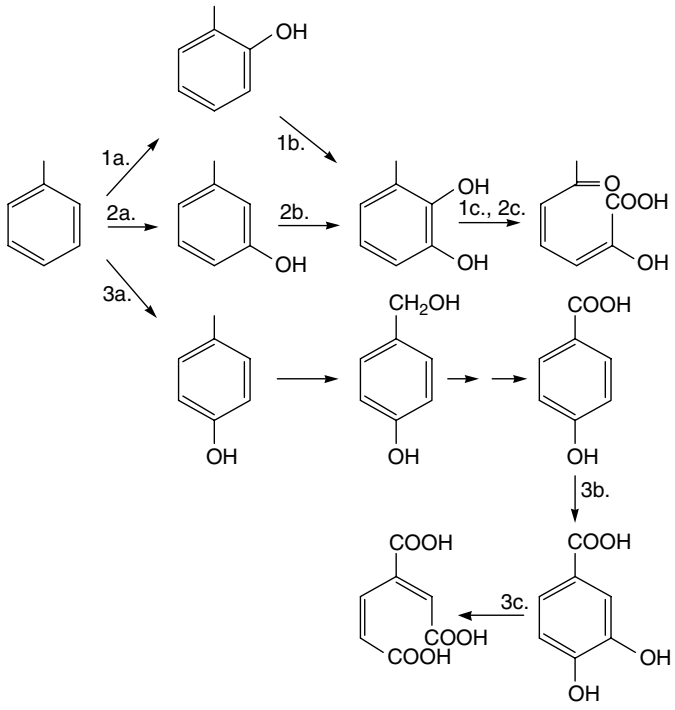

Figure 9. Three toluene biodegradation pathways that are initiated by monooxygenases that catalyze hydroxylation of the *ortho* (1a), *meta* (2a) and *para* (3a) ring carbons. The next reaction of pathways 1 and 2 involves a second monooxygenase-catalyzed ring hydroxylation reaction (1b and 2b), whereas pathway 3 involves methyl group hydroxylation with water rather than O_2 as the source of oxygen. The second ring hydroxylation of pathway 3 is catalyzed by a monooxygenase (3b). Ring-cleavage dioxygenases for pathways 1 and 2 catalyze extradiol cleavage of the ring (1c and 2c), whereas pathway 3 involves intradiol cleavage (3d). Molecular oxygen and other reactants have been omitted for clarity.

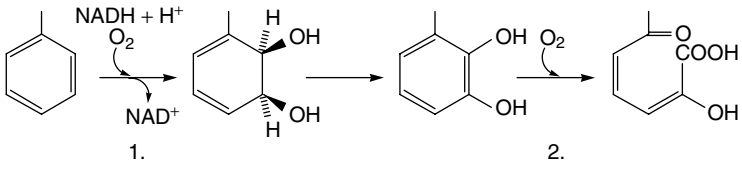

Figure 10. Involvement of dioxygenases in the pathway for the degradation of toluene. (*1*) Dihydroxylation reaction catalyzed by toluene dioxygenase. (*2*) Extradiol ring-cleavage reaction catalyzed by 3-methylcatechol 2,3-dioxygenase. (Absolute stereochemistry is not intended.)

monomeric flavoprotein (molecular weight of 47,000) that contains FAD as a cofactor and obtains reducing equivalents from NAD(P)H (92).

Monooxygenases participating in the biodegradation pathways of numerous other aromatic compounds, such as phenol, chlorophenols, nitrophenols and anthranilic acid are equally interesting, but will not be discussed here because of limited space.

DIOXYGENASES AND THEIR ROLES IN BIODEGRADATIVE PATHWAYS

Dioxygenases catalyze the addition of both atoms of molecular oxygen to their substrates, which are usually aromatic compounds. Two different types of dioxygenases participate in aromatic biodegradative pathways: those that catalyze dihydroxylation of aromatic rings of

compounds that lack two ring hydroxyl groups and those that catalyze cleavage of dihydroxylated aromatic rings. Examples of both reaction types are found in the toluene degradation pathways previously discussed (Figs. 8 and 9).

As previously discussed, aerobic degradation pathways for a large number of different aromatic compounds, especially for aromatic hydrocarbons, converge on a few common dihydroxylated intermediates that are substrates for ring-cleavage dioxygenases (Fig. 7b). The hydroxyl groups may be located on adjacent carbons (*ortho*), as for catechol and protocatechuic acid, or they may be located *para* to each other on the ring, as for gentisic acid. Ring-cleavage dioxygenases insert both atoms of molecular oxygen into the ring either between the hydroxylated carbon atoms (*ortho* or intradiol ring cleavage) or between a hydroxylated and a nonhydroxylated carbon atom (*meta* or extradiol ring cleavage). Many aromatic hydrocarbon biodegradation pathways involve extradiol ring cleavage as shown for three of the previously discussed toluene biodegradation pathways (Figs. 8 and 9). Extradiol ring-cleavage dioxygenases contain nonheme ferrous iron (Fe^{2+}) as a cofactor that binds and activates molecular oxygen during catalysis (4). Pathways involving intradiol ring cleavage are also common. These enzymes contain nonheme ferric iron (Fe^{3+}) as a cofactor that activates the aromatic substrate during catalysis. Both types of enzymes may be composed of one (α) or two different protein subunits ($\alpha\beta$) present in a variety of stoichiometries, depending on the organism in which they are found. For example, catechol 1,2-dioxygenase (intradiol cleavage) of *Alcaligenes eutrophus* CH34 is an α_2 homodimer with a molecular weight of 76,000 (93), whereas catechol 2,3-dioxygenase (extradiol cleavage) of *P. putida* mt-2 is an α_4 homotetramer with a molecular weight of 140,000 (37). Protocatechuate 3,4-dioxygenase of *Brevibacterium fuscum* (intradiol cleavage) is an $(\alpha\beta)_6$ heterododecamer with a molecular weight of 315,000 (94). Reactions catalyzed by aromatic ring-cleavage dioxygenases do not require externally supplied reducing equivalents, so reduced coenzymes and accessory electron transfer proteins are needed.

Aromatic ring hydroxylating dioxygenases initiate biodegradation pathways for aromatic hydrocarbons and many aromatic compounds that lack hydroxyl groups needed for ring cleavage. Hydroxylation of the ring is accompanied by the addition of two reducing equivalents, resulting in a product with both hydroxyl groups located on the same side of the ring (*cis*), as illustrated in the fifth known pathway for bacterial toluene degradation (Fig. 10). *cis*-Dihydrodiol dehydrogenases catalyze the second step of most pathways, which rearomatizes the ring producing substrates for ring-cleavage dioxygenases. Ring-cleavage products are then metabolized to intermediates that enter the central metabolic pathways.

Toluene dioxygenase catalyzes dihydroxylation of toluene in *P. putida* F1 (95). This enzyme system is composed of three protein components: a catalytic hydroxylase and two electron transfer proteins (ferredoxin and reductase) that supply reducing equivalents to the hydroxylase (Fig. 11). The reductase oxidizes NADH and transfers the electrons to the ferredoxin, which transfers the electrons to the hydroxylase. The reductase contains FAD as a redox cofactor, whereas both the ferredoxin and hydroxylase contain a 2Fe-2S iron-sulfur cluster as a redox cofactor. The hydroxylase also contains mononuclear ferrous iron, which has been proposed to bind and activate molecular oxygen as part of the catalytic mechanism of various hydroxylases (96). The reductase and ferredoxin components are monomers with molecular weights of 42,900 and 11,900, respectively. The hydroxylase is an $\alpha_2\beta_2$ heterotetramer with a native molecular weight of 151,000. This arrangement is typical of dioxygenases that act on benzene and biphenyl, though differences in the number of hydroxylase subunits have been reported (97,98). Dioxygenases that catalyze hydroxylation of the carboxylated aromatic compounds benzoic acid and phthalic acid lack a separate ferredoxin component, but the reductase contains a 2Fe-2S redox cluster, in addition to flavin, as a redox cofactor (97).

Additional examples of aromatic compounds that are dihydroxylated via multicomponent dioxygenases include biphenyl and PCBs, naphthalene, phenanthrene and other polynuclear aromatic hydrocarbons, heterocyclic aromatic compounds such as dibenzo-*para*-dioxin, dibenzothiophene, and carbazole, and 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (DDT). The dihydroxylated products of some of these substrates are illustrated in Figure 12. In some cases, the initial product of the hydroxylase is unstable and spontaneously rearomatizes without the aid of a dehydrogenase, as shown for the product formed from dihydroxylation of dibenzo-*para*-dioxin (Fig. 12d).

A final example of the roles of dioxygenases in biodegradation pathways is shown in Figure 13. 2-Aminobenzene sulfonic acid is oxidized to 3-sulfocatechol by 3-sulfocatechol 2,3-dioxygenase. The *cis*-dihydrodiol product

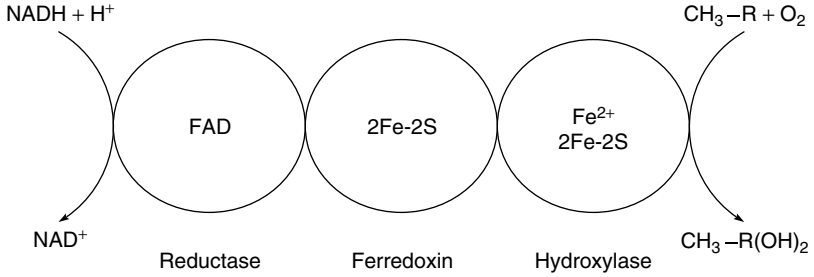

Figure 11. Proposed arrangement of the protein components of toluene dioxygenase of *P. putida* F1. R = C_6H_5 (phenyl).

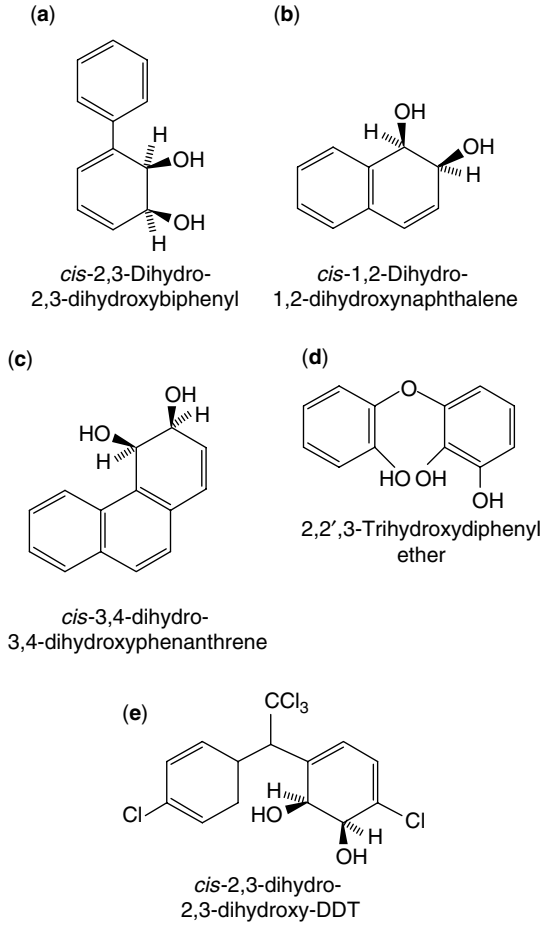

Figure 12. Products of dihydroxylation of some bi- and polycyclic aromatic compounds. The dioxygenase substrates are: (**a**) biphenyl, (**b**) naphthalene, (**c**) phenanthrene, (**d**) dibenzo-*para*-dioxin, and (**e**) DDT. (Absolute stereochemistry is not intended.)

of this reaction is unstable and spontaneously rearomatizes, eliminating the amino group as NH_3^+ and producing 3-sulfocatechol. The aromatic ring of 3-sulfocatechol is cleaved by 3-sulfocatechol 2,3-dioxygenase (intradiol ring cleavage), also producing an unstable intermediate that reacts with water producing 2-hydroxymuconic acid and bisulfite (99). This example illustrates that reactions catalyzed by a hydroxylase and a ring-cleavage dioxygenase can eliminate potentially inhibitory ring substituents and form a product (2-hydroxymuconic acid) that can be further metabolized via central metabolic pathways.

CONCLUSION

Over the past 50 years, a large body of evidence obtained from laboratory studies conducted with pure cultures and under optimized conditions indicates that the involvement of oxygenases in biodegradative pathways is common in nature. There is also evidence that these pathways are operational in the environment and contribute to the functioning of the global carbon cycle (100,101). It is likely that oxygenases play a crucial role in the survival of micooorganisms by allowing them to utilize chemically stable, and sometimes toxic, compounds as growth substrates. This probably accounts for the diversity of structure and catalytic mechanisms of these enzymes and promises that many oxygenases with novel properties and activities remain to be discovered.

The process of evolution has selected for oxygenases that play specific roles in sometimes complex biodegradative pathways involving the oxidation of naturally occurring organic compounds to carbon dioxide and water. Enzymes, including oxygenases, are usually very specific for their substrate(s) and the type of reaction they catalyze. Oxygenases are often tolerant of structural variation in the chemical structures of substrates, however, and may attack xenobiotic compounds that pose hazards to environmental health. Complete pathways for mineralization of xenobiotics may evolve in micooorganisms via the processes of mutation, rearrangement of genetic elements, recombination and genetic exchange among cells in the environment, as well as through genetic engineering and selection in the laboratory (102–104). Thus, biochemical constraints on the mineralization of pollutants may be circumvented and the potential exists for mineralization via biodegradative pathways that involve steps catalyzed by oxygenases.

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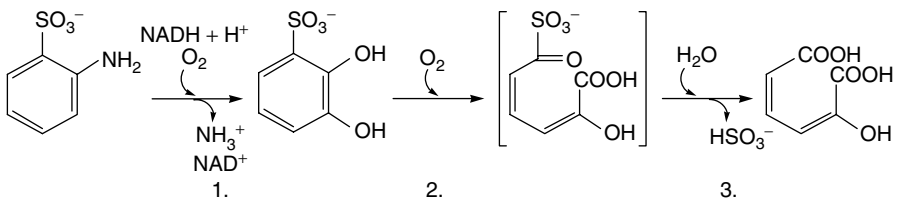

Figure 13. Involvement of oxygenases in the pathway for the degradation of 2-aminobenzenesulfonic acid. (*1*) Dihydroxylation reaction catalyzed by 2-aminobenzenesulfonate 2,3-dioxygenase. (*2*) Extradiol ring-cleavage reaction catalyzed by 3-sulfocatechol 2,3-dioxygenase. (*3*) Spontaneous reaction between the unstable ring-cleavage product and water.

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PALEOECOLOGY. See PALEOLIMNOLOGY: SUBFOSSIL ALGAE OTHER THAN DIATOMS AND CHRYSOPHYTES; PALEOLIMNOLOGY: USE OF SILICEOUS STRUCTURES OF CHRYSOPHYTES AS BIOLOGICAL INDICATORS IN FRESHWATER SYSTEMS

PALEOENVIRONMENTAL RECONSTRUCTION. See PERMAFROST

PALEOLIMONOLOGY. See PALEOLIMNOLOGY: USE OF ALGAL PIGMENTS AS INDICATORS

PALEOLIMNOLOGY: SUBFOSSIL ALGAE OTHER THAN DIATOMS AND CHRYSOPHYTES

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It is a standard practice in paleoecological studies to use biological remains to infer past environmental conditions. Chitinous animal remains (cladoceran exoskeletons and dipteran mouth parts), ostracods, and mollusks preserve well, can be numerous in lacustrine sediments, easily identifiable, and are sensitive to environmental fluctuations (1). Plant pollen is widely used for reconstruction of terrestrial plant communities. Aquatic plant macrofossils are commonly used both to reinforce pollen diagrams and to characterize past macrophyte community structure in littoral zones (2).

Phytoplankton assemblages are represented in the subfossil record by siliceous diatom tests (3) and chrysophyte cysts (4), which are often abundant and well preserved. A broader identification of the phytoplankton community is provided by algal pigments (chlorophylls and carotenoids) and other biochemical parameters in sediments (5–8). These indicators have been used to make statements about primary productivity, salinity, nutrient limitation, water level fluctuation, trophic-cascade dynamics, and watershed–lake interactions.

This article considers the application of other algal remains to paleolimnological studies. These groups, typically the Chlorococcales, Desmidaceae, and Cyanophyta, contain numerous taxa meeting criteria necessary to serve as effective biological paleoindicators (9). Their remains are preserved in sediments and can be identified to specific levels; documented ecological information exists for individual species, and they are sensitive to environmental change.

ANALYTICAL METHODS

There is no standardized technique for the preparation of lacustrine sediments for the analysis of algal remains. Usually, samples are prepared for pollen analysis, and algal microfossils are counted as a secondary component. Palynological (pollen) preparation is typified by the methods of Faegri and Iversen (2). Initially, a fresh sediment is inoculated with known quantities of an exotic pollen, typically *Eucalyptus* or *Lycopodium*, to estimate pollen and palynomorph concentrations. Samples are then treated with KOH or HF to break sediment aggregates and eliminate siliceous material (10). A milder bromoform alcohol solution can also be used instead with no discernible difference (11). An acetolysis step follows to remove cellulose and other refractory organic residues. If the samples are still coarse or clayey, they can then be passed through a fine sieve (12). To facilitate specimen identification, stains such as safranine (13) and gentian violet (9) are sometimes added before microscopy.

Unfortunately, the strong chemical treatments in this method probably dissolve the majority of siliceous and calcareous algal remains. Cronberg (4) recommended addition of *Lycopodium* spores for analytical purposes, but suggests a dilute NaOH treatment for the analysis of blue-green and green algal remains. In most studies targeting specific algal remains, sediments are left untreated (14) or diluted and preserved with glycerol (15) or ethanol (9).

For microscopy, silicone oil is the most common mounting medium (16–18), but glycerine is also used (19). Oil immersion and 1,000X stereomagnification are occasionally required (13,17), but 400X is usually the highest resolution at which routine identifications are made. Counting techniques vary widely. When performed as a component of palynological (pollen) analysis, taxa are enumerated until a significant number of pollen grains and spores is reached. This number typically ranges between 200 (13) and 900 (20). Some researchers highlight the importance of counting slides entirely to counterbalance nonrandom distributions of remains (20). Enumeration techniques are similar in algal-specific studies, but counts are based on numbers of algal specimens (9).

Strategies also vary in the expression of data. Most palynologists report algal remains either as a percentage of the total pollen sum (18) or as a percentage of both pollen and algal totals (21). Algal-specific studies employing this technique report microfossils as a percentage of the total algal sum (9). Most often, concentrations of algal remains are used (13–15). In such investigations, it is important to decide what constitutes an individual. Algal remains are first enumerated taxonomically, and then the counts are adjusted to account for different algal growth forms, especially the separation of single-celled, filamentous,

and colonial taxa. For instance, *Pediastrum* is reported as number of colonies, *Chara* and *Nitella* as number of oospores (13), and *Aphanizomenon* and *Anabaena* as number of akinetes (15).

PALEOECOLOGICAL APPLICATIONS OF ALGAL SUBFOSSIL TAXA

After diatoms and chrysophytes, green algae (Phylum Chlorophyta), especially genera within the Order Chlorococcales, are the most widely studied for their paleolimnological utility (Fig. 1). Oospores of macroalgae (Order Charales), however, are also enumerated. Species of the Family Desmidaceae (Phylum Conjugaphyta) are more resistant and numerous in sediments than the Family Zygnemataceae, and are more widely used (Fig. 2). Within the Cyanophyta, cell walls of *Gloeotrichia* and akinetes of *Anabaena* and *Aphanizomenon* species have been studied successfully (Fig. 3). A few genera of euglenoids, cryptophytes, and dinoflagellates have been of limited value in freshwater stratigraphic reconstructions (Fig. 4). Individual algal groups, their decay-resistant properties, and their application to paleolimnology are discussed later.

Phylum Chlorophyta

Order Chlorococcales. Of the algal groups discussed here, Chlorococcales have received the most attention. Species of Pediastrum, Botryococcus, and Scenedesmus contain sporopollenin, a decay-resistant protein, in their cell walls (22). Silica also is present in the cell walls of some Pediastrum species (23). Palynologists first noticed this durability, and mentioned the presence of green algal remains in their early studies (9).

Most quantitative studies with these algae have been in concert with pollen analyses. Cell counts of Chlorococcales, in conjunction with aquatic pollen, plant macrofossils, and other biological proxies, provide information about the lacustrine setting in which terrestrial pollen was deposited. The first significant studies included temporal trends for *Pediastrum* and *Botryoccocus* species since the last glacial period (16,24–27). Although some presented detailed taxonomic separation, most paleolimnological conclusions were based on total subfossil abundances of Chlorococcales (20). Combined with other paleoindicators, these early studies made inferences about lacustrine productivity, water depth, extent of aquatic macrophyte coverage, and cultural disturbance.

Meanwhile, other paleoecologists were seeking to obtain more precision in their interpretations of green algal remains. Reconstructed algal productivity strongly correlated with silt accumulation in Greenland glacial lakes (28) and those of central Europe (29). These interpretations were verified through limnological investigations and expanded to include nutrient

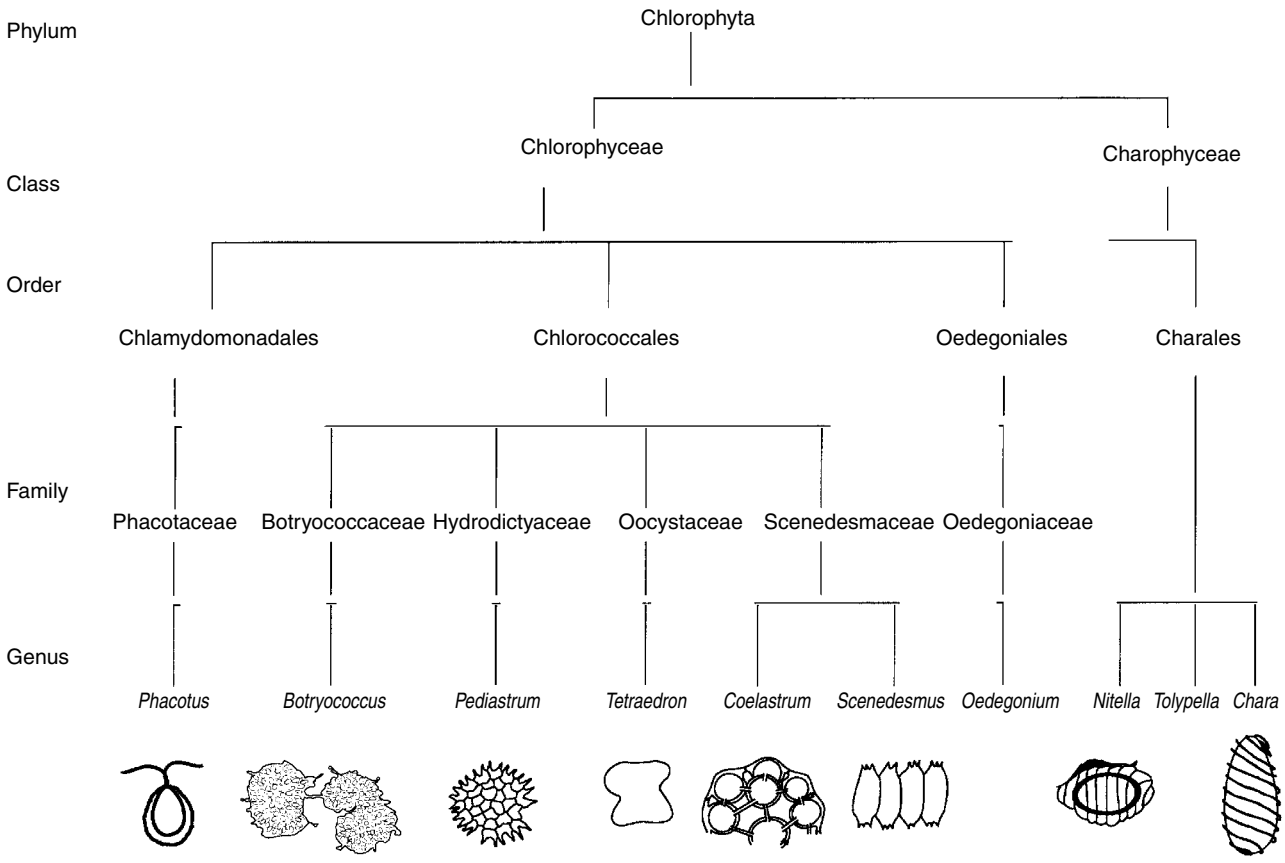

Figure 1. Decay-resistant genera of the Phylum Chlorophyta with representative species illustrated.

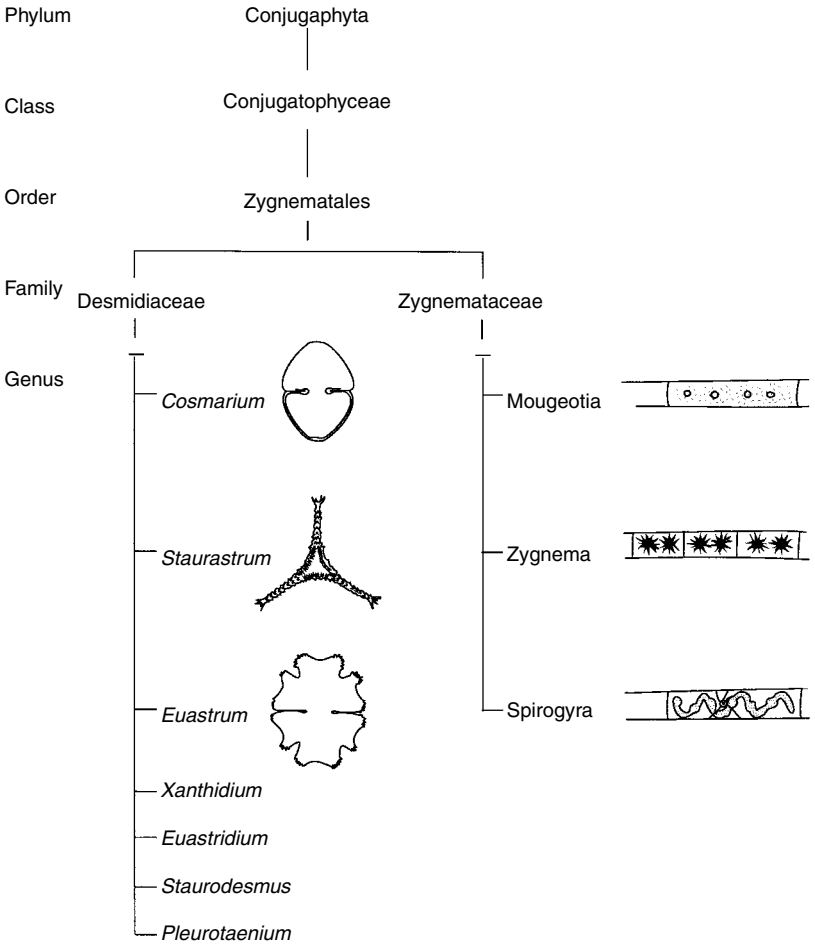

Figure 2. Decay-resistant genera of the Phylum Conjugaphyta with representative species illustrated.

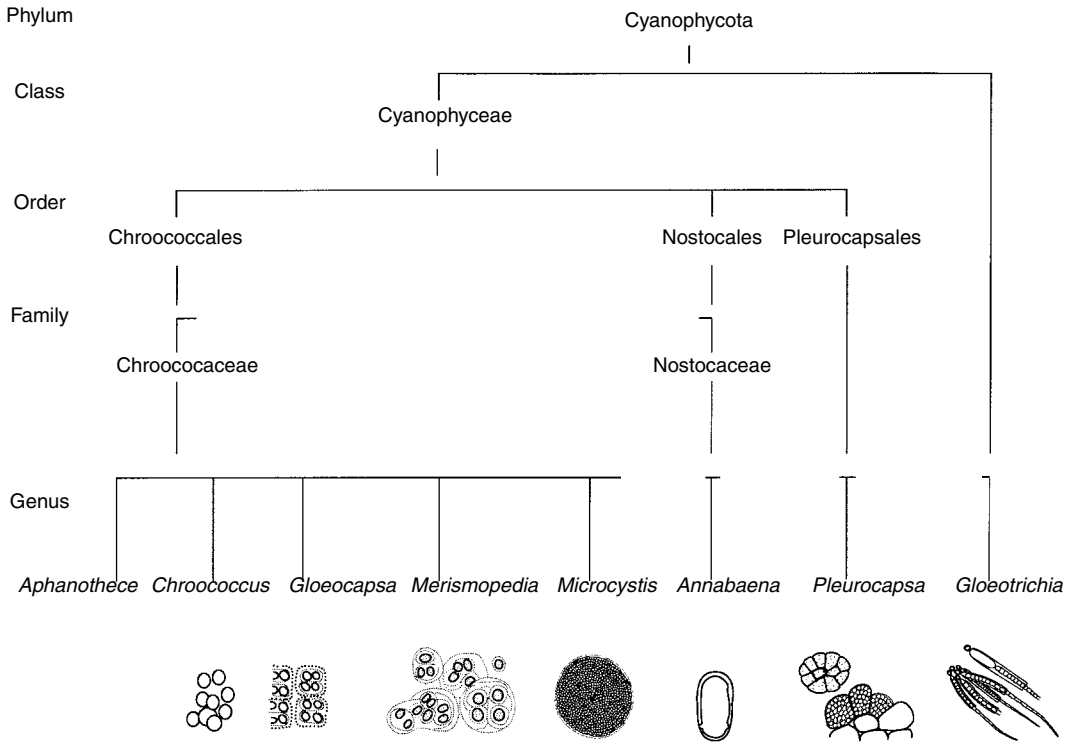

Figure 3. Decay-resistant genera of the Phylum Cyanophycota with representative species illustrated.

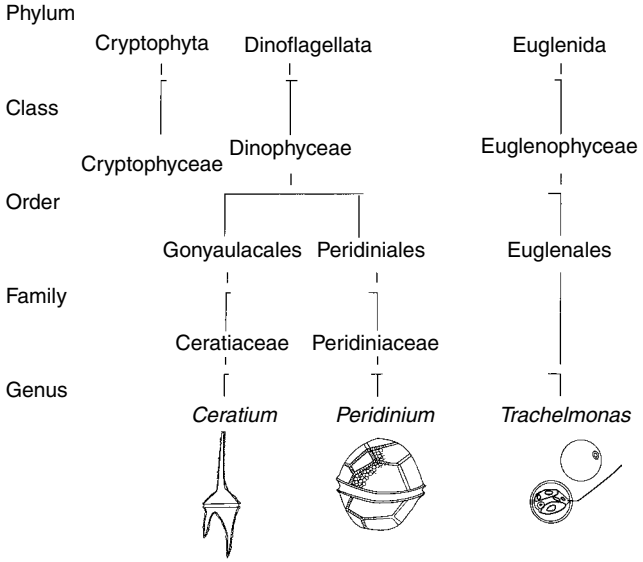

Figure 4. Decay-resistant taxa of the Phyla Cryptophyta, Dinoflagellata, and Euglenida with representative species illustrated.

availability in both subarctic (18,30,31) and temperate (32,33) lakes. Crisman (33) found correlations between subfossil green algae, desmid, and cyanophyte assemblages in lake sediments and terrestrial vegetation succession in Minnesota, implying temporal alterations in watershed-nutrient export. In a Nevada saline lake, Bradbury and coworkers (34) used the presence of *Botryococcus* to indicate past periods of freshwater conditions. *Pediastrum* abundances in the Great Lakes were used in conjunction with analyses of oxygen isotopes, silicate, and deposition of inorganic sediment to infer silt accumulation (13).

Unfortunately, broad geographic interpretations of these subfossil groups have been confounded by regional inconsistencies. For example, Anderson and Brubaker (17) attempted to explain water depth in northern Alaskan lakes as a function of *Pediastrum* frequency. Other researchers found the reverse relationship in Europe (35). Edwards and coworkers (21), however, found no relationship between *Pediastrum* and water depth, arguing that abundance was a function of multiple lacustrine factors and that *Pediastrum* subfossils in a sediment sample indicated, at best, the presence of standing water.

Order Charales. Often referred to as *macroalgae*, species of the order Charales are large, colonial taxa, associated with hardwater and secretion of marl. In sediment records, they are typically represented by oospores. *Chara* remains were noted in palynological studies as early as 1932 (36), and interpretations of their remains are usually conservative. For example, oospores of both *Nitella* and *Chara* species are often used to infer the presence of shallow marshy areas within a particular catchment (9,16). Some studies have equated these species with silty, lentic conditions (20). Eisner and coworkers (18) found abundant remains of *Chara*, *Nitella*, and *Tolypella* species in the sediments of Lake 31, Greenland. Although photomicrographs of the oospores were provided, paleoecological reconstructions were based on other data. In a departure, Yu (13) ran an oxygen isotopic analysis on *Chara* encrustations, marl, and mollusk shells to approximate paleotemperature in Twiss Marl Pond, Ontario. *Nitella* and *Chara* propagules were also enumerated, but, again, were not mentioned in the interpretation.

Phylum Conjugaphyta

Family Desmidaceae. Members of the Desmidaceae, the principal representatives of the order Zygnematales in the subfossil record, often contain pectin, silica, and iron compounds (37) in their cell walls. Their cells are generally thick and resistant to processing techniques used in pollen analysis, although they contain no sporopollenin (9). Taxonomic microfossil evaluations revealed abundant desmid subfossils in early palynological investigations (38–40), but quantitative paleoecological assessments were delayed. Limnological investigations found significant correlations between low-nutrient enrichment and desmid abundance (41–44), a relationship that paleoecologists have used to infer low productivity on the basis of both *Cosmarium* microfossil abundances in Ohio lake and peat bog sediments (9) and *Staurastrum manfeldtii* from the sediment record of Lake Gosciaz, central Poland (45). Conversely, enhanced productivity was inferred from sediments of a South Georgia Island lake on the basis of diatom abundances and sediment organic matter combined with the presence of three desmid species (46). A positive correlation was found between two species of the *S. manfeldtii* complex and species of the cyanophyte, *Anabaena* (49). This relationship was later used to imply nitrogen limitation in the Lake Gosciaz, Poland, and paleoreconstruction using *Staurastrum pingue* and *Staurastrum planctonicum* (19).

Family Zygnemataceae. To a lesser extent, taxa of the family Zygnemataceae have been used in lacustrine paleoreconstructions. Spores from species of *Mougeotia*, *Spirogyra*, and *Zygnema* have been reported in the sediments, but in insignificant numbers (33). Van Geel and coworkers (19) noted the presence of *Spirogyra* among other algal subfossil assemblages during a period of anthropogenic nutrient enrichment in Lake Gosciaz, Poland. Given their scarcity, conclusions were based on other, more numerous algal paleoindicators. Regardless of the relatively sparse literature, some researchers remain optimistic about the Zygnemataceae as paleoindicators, citing their sturdiness, ability to be identified to species, and sensitivity to environmental variables (11).

Phylum Cyanophycota

Also referred to as *blue-green algae* or *cyanobacteria*, the cyanophytes are prokaryotic photosynthetic organisms. They occur in unicellular, filamentous, and colonial forms. Cyanophytes enjoy several competitive advantages over their protist counterparts, including an ability to fix atmospheric nitrogen, control their position in the water column, and toxin production. These attributes have led to their proliferation in stressed environments.

Pectin-containing compounds are abundant in cyanophyte cell walls and sheaths (48), and have imparted a decay-resistant nature to some taxa (9). The species most commonly used in paleoecological studies are from the genus *Gloeotrichia*. On the basis of established positive relationships between cyanophyte abundance and lacustrine productivity (26,41,42,44,49), many studies have used the presence of these remains to infer eutrophication (9,19,50,51).

Other investigations noted that this genus can be seasonally epiphytic, planktonic, and epibenthic (52,53). Combined with other data for subfossil algal taxa, Birks and coworkers (16) used this cyanophyte to infer a past period of dense aquatic vegetation in Elk Lake, Minnesota. Also, given the heterocystic nature of *Gloeotrichia* (54), its heightened presence in late-glacial sediments has been associated with either early successional, nitrogen-poor conditions (55,56) or productivity-induced phosphorus limitation (53).

Cyanophytic akinetes, or spores, also have been used in paleoecological investigations. These decay-resistant structures can remain viable in lacustrine sediments for up to 64 years (57). Akinetes used in paleolimnological investigations typically belong to species of *Aphanizomenon* and *Anabaena*, known for their proliferation under eutrophic conditions, especially nitrogen limitation (58,59). In Lake Arendsee, Germany, akinetes in the sediment record were associated with such conditions (14). Extant *Aphanizomenon* populations proliferated in both light- and phosphorus-limited conditions (60), whereas *Anabaena* species only thrived under phosphorus limitation (61). Such differential responses were used to specify the nature of cultural eutrophication in a paleoecological study of Lake Gosciaz, Poland (19).

Other Algae

Other algal groups are known to preserve in sediments, but their scarcity has made it difficult for any significant conclusions to be drawn from the analysis of their subfossil remains. Euglenophyta are seldom used in studies of lacustrine sediments, in part because of the poor preservation of most taxa (9). However, *Phacotus lenticularis*, which has been associated with water hardness, preserves well because of the high calcium content of its cell wall (62). This species has been used in paleolimnological investigations to denote high alkalinity, and with the presence of other indicators, nutrient enrichment (14). Cryptophytes and dinoflagellates, also of limited abundance in lacustrine sediments, are rarely enumerated. Findlay and coworkers (59) did find relationships of these groups with light limitation and eutrophication in the Canadian Experimental Lakes Area. Both groups were enumerated in a follow-up study, but conclusions were limited because of low abundances (14). Dinoflagellate cysts were also counted by Huber (33) in a comprehensive study of algal remains in Gegoka Lake, Minnesota, but low numbers and identification problems inhibited their utility. Remains of the green alga, *Cladophora*, although numerous in some sediments, are counted as macrofossils when studied (18).

CONCLUSION

Of the subfossil algae surveyed, none have been used alone in paleolimnological reconstructions. Initial investigations combined species of chlorococcales and cyanophytes with the more widely used algal paleoindicators, diatoms, and desmids (23,62). Some investigations relied strongly on subfossil algae (9,19), but the data were mainly complementary to other biological indicators, such as pollen (18,21) or diatoms (14). A key problem associated with the use of these other algal groups is differential preservation of species in lacustrine sediments. Frederick (9) counteracted this through use of Nygaard's (41) compound quotient of lake trophic status, but this topic has not been addressed by later investigators.

Although currently of limited value, utilization of subfossil algae in paleoecological studies has great potential. Most investigations hesitate to draw conclusions unless other, more established proxies indicate similar conditions. However, there is a wide array of algal genera that are abundant, readily identifiable, and possess decay-resistant structures in lacustrine sediments. These genera display clearly defined responses to environmental variables including trophic state, nutrient limitation, and conductivity, but their use as single variable paleoindicators remains problematic until the issue of differential preservation of remains is addressed adequately.

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PALEOLIMNOLOGY: USE OF ALGAL PIGMENTS AS INDICATORS

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Algal and bacterial pigments (chlorophylls and carotenoids) are common components of lake sediments and have been under investigation for more than three decades. This article reviews the major pigments, mechanisms for their preservation in the sediment record, and their utility in paleolimnological reconstructions. Current interest in environmental history has focused attention on lake deposits as a medium within which natural (e.g., climate) and anthropogenic (e.g., eutrophication, atmospheric pollution) environmental changes are recorded in historical sequence. Pigments reach the surface of lake sediment both as particulate matter from autochthonous planktonic and littoral organisms, and as transported allochthonous plant matter from the catchment or shoreline.

Similar to a number of chemical and biological subfossil remains (e.g., diatoms), plant pigments can be used to infer environmental changes over different timescales (centuries to millennia). The validity of using sedimentary pigments as an index of past events or processes such as trophic condition and primary productivity depends mainly on the preservation of these relatively labile organic compounds, the extent of differential degradation during and after sedimentation, and the extent of allochthonous sources of sedimentary pigment products. When primary producers die, their remains decompose and are incorporated in the sediments. Physical, chemical, and biological factors are responsible for the sedimentary pool of organic matter (see later), and these are well reviewed

by Brown (1), Swain (2), Leavitt (3,4), and Cuddington and Leavitt (5).

However, rarely are pigments completely degraded and thus such *"biochemical fossils"* in lake sediment cores have proved useful for assessing past environmental changes in many lakes.

Finally, as demonstrated for many paleolimnological records, pigments are a useful tool for delineating historical lake development in which long-term limnological data are often lacking or very rare and the only way to obtain such data is through a paleolimnological perspective. Such an approach has value for lake management and monitoring (6).

PIGMENTS OF ALGAE AND PHOTOSYNTHETIC BACTERIA

Presence and Meaning

Higher plants, algae, and bacteria synthesize a variety of pigmented organic compounds that are used in photosynthetic reactions. Chlorophylls absorb solar energy more efficiently in the red portion of the solar spectrum at wavelengths that do not penetrate into deep water. For this reason, aquatic plants synthesize different carotenoids, which are used as accessory pigments, in so far as they absorb light in a wider range of the spectrum. Table 1 summarizes the main pigments (chlorophylls and carotenoids) and their taxonomic specificity.

Chlorophyll *a* is found in all photosynthetic aquatic organisms, with the exception of some species of autotrophic bacteria. As reported in the following text, its degradation products (phaeophytin *a*, phaeophorbide *a*, and chlorophyllide *a*) are relatively well preserved in lake sediments (11–13).

Carotenoids are also widely distributed in all photosynthetic organisms. Those commonly present in the sediment can be subdivided into two major categories according to their solubility in different solvents (e.g., methanol and petroleum ether): epiphasic (carotenes) and hypophasic (xanthophylls). The principal carotene is β-carotene, which is present in all algal taxa. Yellow xanthophylls display greater diversity than carotenes. The most abundant are lutein, neoxanthin, and zeaxanthin (8,14). In addition to these, there are specific xanthophylls, such as

Table 1. Principal Specific Pigments and Relative Taxa (4,7–10)

Pigment	Taxa
Chlorophylls	
Chlorophyll *a*	Common to all plants
Chlorophyll *b*	Chlorophytes, Euglenophytes, Trachaeophytes
Chlorophyll *c*	Chrysophytes, Pyrrophytes
Chlorophyll derivatives	
Phaeophorbide *a*	Chlorophyll *a* derivative (grazing)
Phaeophytin *a*	Chlorophyll *a* derivative common to all plants
Phaeophytin *b*	Chlorophyll *b* derivative common to all plants
Phaeophytin *c*	Chlorophyll *c* derivative (Chrysophytes, Pyrrophytes)
Carotenes	
α-carotene	Cryptophytes, Chlorophytes, Trachaeophytes
β-carotene	Common to all plants, some bacteria
Xanthophylls	
Alloxanthin	Cryptophytes
Astaxanthin	Invertebrate herbivores, some chlorophytes
Canthaxanthin	Invertebrate herbivores, some filamentous cyanobacteria
Echinenone	Total Cyanobacteria (formerly named blue-green algae)
Fucoxanthin	Siliceous algae
Lutein	Chlorophytes, Euglenophytes, Trachaeophytes
Myxoxanthophyll	Colonial and filamentous cyanobacteria
Oscillaxanthin	Oscillatoriaceae
Peridinin	Dinophyceae
Zeaxanthin	Cyanobacteria
Diadinoxanthin	Euglenophytes
Dinoxanthin	Pyrrophytes, Chrysophyceae
Myxol-2′-o-methyl-methylpentoside	*Oscillatoria limosa*
4-Keto-myxol-2′-methylpentoside	*Anabaena flos-aquae*
Sulfur photosynthetic bacteria	
Isorenieratene	Green sulfur bacteria (*Chlorobium* sp.)
Okenone	Red sulfur bacteria (*Chromatium* sp.)
Licopene	*Rhodospirillum* sp.
Spheroidene, spheroidenone	*Rhodopseudomonas sphaeroides*
Rhodopinal	*Lamprocystis, Rhodospirillum tenue*

fucoxanthin and diadinoxanthin, present in diatoms, myxoxanthophyll and echinenone, present in cyanobacteria, and oscillaxanthin present in filamentous cyanobacteria *Oscillatoria* spp.

In many meromictic lakes, two horizontally stratified populations of sulfur photosynthetic bacteria frequently occur, one near the chemocline and the other at greater depth. Each population contains pigments that are distinctive and group-specific and can be used in paleoecological studies to indicate, for example, changes in the physical and chemical environment.

PRESERVATION/DEGRADATION OF PIGMENTS AND ITS PALEOLIMNOLOGICAL SIGNIFICANCE

Sedimentary Processes and Physical-Chemical Factors

Pigments contain chromophorous groups that absorb visible light and give the molecules their characteristic colors. Double bonds and functional groups constitute attack sites for microorganisms, making the compounds highly sensitive to diagenetic changes (15). Despite this tendency, some pigments may become diagenetically more stable once they have arrived in the lake or sediments. The complete aromatization of the tetrapyrrolic ring of chlorophylls, which transforms them into porphyrines, and the hydrogenation of the C−C double bonds of carotenoids to form isoprenoidal alkanes, are the principal changes stabilizing pigments (16).

Regarding the important question of pigment preservation, many authors have discussed the different behavior of carotenoids and chlorophylls (2,17). First, pigments originating in the catchment usually do not arrive in the lake intact, that is, in their original form. Pigments of autochthonous origin are also rapidly degraded if they are not quickly incorporated into the sediment. For example, it was found that photo-degradation of chlorophyll derivatives in the photic zone of the lakes in Michigan was complete after only three days (18).

Carotenoids degrade more rapidly than chlorophylls in the water column, although once reaching the sediment, they are much more stable than chlorophylls. There is no evidence that carotenoids decompose significantly once buried in sediment; if decomposition does occur, it is similar to that of organic matter as a whole (13). Chlorophyll derivatives (phaeophytin, phaeophorbides, and chlorophyllides) are equally refractory to further decomposition and are always more abundant than nondegraded chlorophyll, which is usually present in high quantities only in newly deposited sediment (2,13).

Pigment degradation is particularly sensitive to the presence of oxygen and light (Fig. 1). In fact, decomposition rates in anoxic sediment are generally less than half of that observed at the water–sediment interface in well-oxygenated environments because of direct oxidation and increased activity of benthic organisms (19–21).

Photooxidation is the most important degradation process for pigments that are deposited in sediment; the half-life of carotenoids and chlorophylls is generally about 0.2 days, but may vary in relation to phytoplankton composition and presence of humic acids and light-absorbing compounds (18,22–24). Invertebrate activity in sediments of shallow lakes may also be important (25,26), as may the general physical-chemical and biological conditions of the water column (20,25).

Sediments of deep lakes usually receive less light or are completely aphotic, and oxygen may also be scarce. Thus, pigment degradation through photooxidation and that mediated by organisms (biological) is less than that of a shallow lake. Pigment conservation might therefore be better in sediments of deep lakes rather than shallow lakes (2,27–29). On the other hand, the longer residence time of the pigments in the water column of deep lakes can offset the favorable preservation environment of sediments.

Thus, under field conditions in which light and oxygen are high (e.g., unproductive holomictic lakes), carotenoids and chlorophylls are rapidly transformed into colorless compounds (30,31). However, preservation of fossil pigments is greater in meromictic than in holomictic lakes (3).

Light absorption by chlorophylls may result in formation of a highly reactive triplet excited-state molecule; this molecule may subsequently undergo redox reactions determining the production of radical species, which in turn cause pigment oxidation. Alternatively, excited chlorophyll may react with oxygen, itself a triplet molecule, to form ground-state chlorophyll and singlet oxygen. The latter reacts rapidly with most compounds containing double bonds (10).

Chlorophyll degradation may also be considerable during algal senescence and appears to result from loss of Mg^{2+} from the heme group (Fig. 2) (type I degradation) (32). Algal decomposition (33,34) produces rapid conversion of chlorophyll *a* (with Mg^{2+}) to phaeophytin *a* (without Mg^{2+}), but cell lysis or viral attack alone are insufficient to convert chlorophyll *a* to phaeophorbide *a*. On the other hand, the mechanism converting chlorophyll and phaeopigments into colorless compounds in the dark (type II degradation) is poorly known.

Biological Factors

Various biological factors and processes cause pigments to degrade before or during their deposition (Fig. 1). Among these are grazing by herbivores, and microbial degradation mediated by temperature associated with cell senescence (1–4,13,24,25,27,35–42).

Daley (33) and Daley & Brown (34) fed live cyanobacteria to *Daphnia* and observed that, at the same time as chlorophyll *a* was converted to phaeophytin and phaeophorbide, there was a 70% loss of total phorbins in 145 hours. Carotenoid degradation also occurs mainly during passage of pigments through the guts of herbivores. However, comparing the degradative ability of freshwater cladocerans and rotifers, Carpenter & Bergquist (43) suggested that grazing causes substantial but nonselective pigment degradation, and that the pigment content per unit of organic matter might increase after passing through the gut of herbivores. Leavitt and coworkers (44) considered grazing by large zooplankton the most important factor that determines the increase in the conservation of pigments and their derivatives in lake sediments.

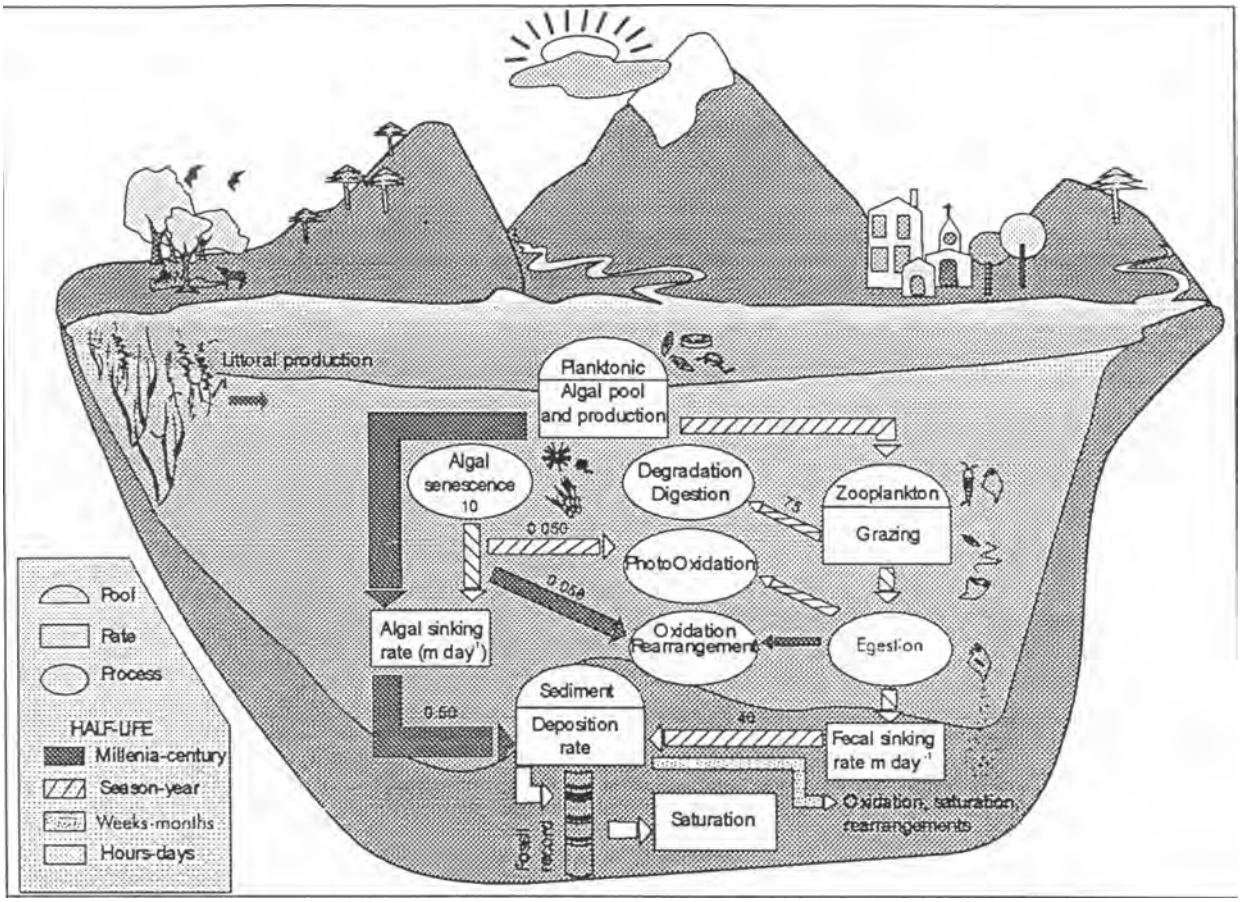

Figure 1. Major fluxes of autochthonous plant pigments in lakes. Sources of pigments: planktonic and benthic production, littoral macrophytes, allochthonous, and sediment resuspension. Model of individual algal cells (summerlike conditions) as they descended through a one-dimensional water column, underwent various loss process, and ultimately were deposited in the sediments. Hypothetical unproductive lake with the following simplified physical parameters: lake depth = 25 m; oxic zone depth = 20; light extinction coefficient = 0.30. Arrow width indicates importance of process (redrawn from P. R. Leavitt, *J. Paleolimnol.* **19**, 109–127 (1993), by permission). Approximate mean values are from K. Cuddington and P. R. Leavitt, *Can. J. Fish. Aquat. Sci.* **56**, 1,964–1,977 (1999). *Note*: Rate coefficients of algal recruitment and senescence are equal (10% day^{-1}). Initial algal abundance, 750 cells. Rate constants are characteristic of the carotenoid β-carotene: photoxidation in $E^{-1} \cdot day^{-1}$ (1 Einstein = 1 mol photons); oxidation in day^{-1}; herbivore grazing in % day^{-1}; pigment digestion (grazing degradation) in % $ingestion^{-1}$; algal sinking and fecal sinking in $m \cdot day^{-1}$. Percentage values implemented as probabilities.

To summarize degradation by herbivores: in general, there can be two concomitant, although different, situations that are not necessarily separable from each other. In one, consumption of algae by microherbivores causes severe pigment degradation (up to and exceeding 80% of initial content (33,34)); in the other, consumption of algal cells by large zooplankton (e.g., *Daphnia*) results in the pigments not undergoing the most effective photochemical degradation as they move through the water column. In the second case, the pigment is in fact excreted by the animal as fecal particles and rapidly sedimented without undergoing any great change (6,24).

Individual Pigment Stability

Chlorophyll *a* degradation is usually greater than that of carotenoids, and may change according to the structure of the food web of the water body (22–24,40,42). Undegraded chlorophylls are generally abundant only when algal remains are rapidly buried in an anoxic, cold environment. In some cases, it is a sensitive indicator of deep-water algal populations (45).

Each algal group has specific pigments with individual characteristics affecting the degree of conservation and degradation in the water column and sediments. Thus, because of selective degradation limits, pigments in sediments can be useful indicators of relative algal abundance (45).

Degradation rate constants of carotenoids, in particular, differ enormously; the ubiquitous β-carotene and phaeophytin *a*, and lutein, are the most stable carotenoids and thus recommended as sedimentary indicators of total algal abundance, whereas peridinin and fucoxanthin are among the most labile (20,24–26,46–48). β-Carotene and

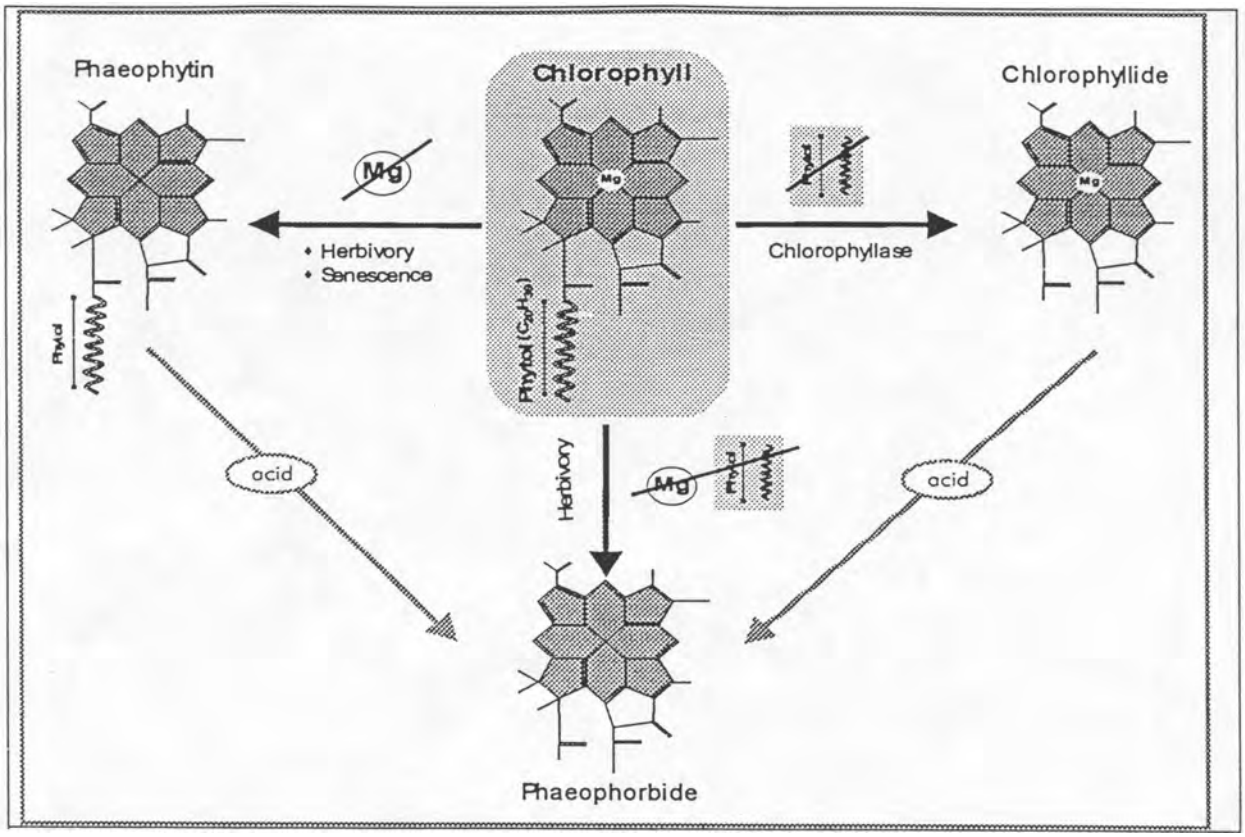

Figure 2. Processes of formation of chlorophyll derivatives (type I reactions). Chlorophyll *c* does not have a phytol chain. Allomers are formed from their respective chlorophylls in the presence of oxygen (from P. R. Leavitt, *J. Paleolimnol.* **19**, 109–127 (1993), by permission).

echinenone degrade less (about half) in anaerobic and aphotic conditions (69) than other pigments and organic compounds in cyanobacteria.

The specific pigments of the dinoflagellates (e.g., peridinin) are rarely conserved in sediments (20,24,25,44,46–48). Likewise, pigments of diatoms and chrysophytes (fucoxanthin and *c*-phorbin) are generally more labile than those from green algae (lutein and *b*-phorbin), Cyanobacteria (zeaxanthin and *α*-phorbin), and cryptophytes (alloxanthin, *β*-carotene and *α*-phorbin) (20,25,68). Lutein-zeaxanthin generally replaces fucoxanthin as the dominant carotenoids in saline lake sediments (50).

Algal Production and Its Relationship with the Sedimentary Pigments: The Importance of Temporal Scale in the Degradation Processes

Despite the various problems associated with conservation of photosynthetic compounds, sedimentary pigments and algal abundance, as well as production remain correlated through time, provided there is no change in basin size and depth, light penetration, thermal and chemical stratification, and deep-water oxygen content (4). Production/trophic state (at the broadest scale), morphometric features (at a narrow range of algal abundance) and foodweb effects (at the finest scale) are the three main hierarchical factors affecting algal production and deposition.

In agreement with Leavitt (4), the general consensus is that the observed relationship between algal biomass and subfossil concentration is site-specific and that the deposition process is regulated by the aforementioned hierarchy of control operating at three temporal phases:

1. rapid degradation in the water column (half-life, $T_{1/2}$ = days)
2. small postdepositional losses in surface sediments ($T_{1/2}$ = years)
3. very slow loss of double bonds in deep sediments ($T_{1/2}$ = centuries)

The fastest reduction in concentration therefore happens when cells die and sink to the sediment (point 1) when, under certain conditions, 99% of chlorophylls and carotenoids may be degraded (48,51). During the process of algal deposition, the principal degradation processes are photooxidation, digestion by herbivorous zooplankton, enzymatic catalysis during senescence, and oxidation either chemical or mediated by microorganisms (Fig. 1).

At the end of this stage, the matter deposited at the water–sediment interface (point 2) continue degrading at a slightly slower rate, which in turn depends on the quantity and type of pigments sedimented (20,46).

Thereafter, much slower degradation processes act at a century-millenium scale (point 3) (52,53). Swain (2) suggested that the long-term conservation of total

carotenoids was similar to that of chlorophylls. Variations in the relationship between these pigment groups thus reflect stratigraphic variation of a series of physical-chemical factors as well as phytoplankton community composition, presuming that there are no significant postdepositional changes.

The fact that individual pigments degrade at different rates according to steps in the settling process (water column, surface sediments, deep sediments) is very important and should always be kept in mind if pigment quantity is to be related to algal biomass. Studies on English lakes (54,55) highlighted the close correlation existing between chlorophyll derivatives and epiphasic carotenoids (carotenes) on the one hand and algal abundance on the other, and proposed an index of lake productivity. Züllig (56) demonstrated a strong correlation between myxoxanthophyll and lake primary production, and reconstructed the recent history of several Swiss lakes, using pigments isolated with a chromatographic technique that he developed. Later, others found a link between oscillaxanthin (the pigment of *Oscillatoria* spp.) and the trophic level of American and English lakes (57,58).

Subsequent studies in the 1970s and 1980s confirmed strong correlations between phytoplankton abundance and fossil pigments in many environments (38,59–67). Such relationships are especially strong for sediments below the first 5 cm of the sediment–water interface, which are not subject to continual processes of diagenesis. Excellent linear correlations between sedimentary carotenoids and algal biomass ($r = 0.90$) were developed for English lakes. Correlations between algal groups and sedimentary fossils were also very good. These results led to the conclusion that most organic matter and pigments were autochthonous in origin, and that pigments could be used as indicators of primary production. The existence of such relationships, especially those between concentrations of sedimentary pigments, algal biomass, and primary productivity rate should be strongest when lake morphometry and water chemistry are relatively constant (5).

Studies on lakes with basins differing in productivity also offer proof of the existence of strong correlations between phytoplankton and fossil pigment abundance. For example, in their examination of the surface sediments of Lake Mead (U.S.A.), Adams and Prentki (67) reported strong correlations between algal production and concentrations of total carotenoids and phaeopigments ($r = 0.97$, $r = 1.0$, respectively). Similar results were obtained by Brenner and Bindford (68) for 97 Florida lakes.

In a similar study of 12 Italian subalpine lakes using inter- and intralake comparison, Adams and coworkers (62), and Guilizzoni and coworkers (66,69) also found an excellent mathematical regression ($P < 0.01$) of surfacial lake sedimentary pigment concentration against current and recently derived measures of primary production. Other studies recorded increased pigment concentrations in sediment as a result of nutrient enrichment and thus increased productivity of the ecosystem (8,70,71).

Although these examples suggest that fossil pigment concentration increases as algal standing crop increases, it should be pointed out that pigment concentrations in sediments of oligotrophic lakes could be lower than expected for the following reasons (2): increased degradation before deposition; lower sedimentation rate in anoxic sediments; high dilution due to the input of allochthonous organic matter low in pigments; low-pigment production per unit of organic matter.

Swain (2) maintains that the correct stratigraphic interpretation of fossil pigments in lake sediments depends essentially on two factors that are highly correlated with trophic status: (*1*) degradation of autochthonous pigments and (*2*) dilution due to a load of allochthonous matter low in pigments.

Post-depositional degradation may obscure stratigraphic interpretations of pigments within a particular "degradation zone" (water-sediment interface), especially if events occur that normally favor increased pigment deposition, such as eutrophication and grazing.

Degradation and dilution processes are more evident in lakes with low productivity and a substantial allochthonous input. In addition, these environments usually have greater light intensity and high oxygen concentrations in the hypolimnion, which encourage photooxidative processes. On the contrary, conditions more favorable to pigment conservation are found in eutrophic lakes and particularly in meromictic environments in which the monimolimnion is invariably anoxic, does not mix and does not have bioturbation (13,37).

As for pigments, mineralization of most organic matter occurs during deposition (72–74). Therefore, if the losses of total organic matter are similar to those of carotenoids and chlorophylls, then concentrations of specific pigments could remain relatively unchanged despite considerable degradation of both fractions. In addition, input of low-pigment organic matter from the watershed would dilute the sedimentary pigment pool, whose temporal concentration variations may be determined by:

- significant inputs of allochthonous organic matter conveying low carotenoid concentrations yielding a high chlorophyll/carotenoid ratio (28);
- type of algal and macrophyte community;
- more or less favorable oxygen conditions;
- differential production of chlorophylls and carotenoids by plants (2).

The final important factor in pigment conservation is sedimentation rate of particulate matter present in the water column, because high sedimentation rate favors rapid segregation of pigments from the water-sediment interface, where physical-chemical conditions are highly favorable for decomposition (2,13,60,66,69,75).

Expression of Data

In all the cases in which phytoplankton and fossil pigments are correlated, researchers have used pigment concentrations (weight × unit of organic matter or moles per gram of organic matter) as a measure of their abundance. From the concentration of sedimentary pigments, then, it is possible to make a more precise

estimate of changes that occurred in phytoplankton abundance during the postglacial period, rather than using as a measure sedimentation flux or pigment accumulation (moles m^{-2} $year^{-1}$). In fact, pigment sedimentation rates (fluxes) are less suitable than fossil pigment concentrations in determining annual changes in phytoplankton community composition (4). However, expressing information as fluxes would have the advantage of being immune from errors of interpretation from decreased pigment concentrations caused by dilution effects of organic matter.

Carotenoids from Sulfur Photosynthetic Anaerobic Bacteria

In recent years, there has been growing interest in carotenoids of photosynthetic sulfur bacteria in sediment (8,9,72,76). The families of photosynthetic bacteria most commonly found in lakes are the chromatiaceans (red sulfur bacteria, e.g., *Chromatium okenii*) and chlorobiaceans (green sulfur bacteria, e.g., *Chlorobium* spp.). Because of the different light requirements and oxygen tolerance of these two groups they occupy different layers of the water column, often corresponding to the chemocline of meromictic lakes, with red bacteria vertically distributed immediately above the greens. McIntosh (77) believed that two bacterial carotenoids, okenone (red sulfur bacteria) and isorenieratene (green sulfur bacteria), were a valuable source of information on, for example, light penetration and the presence of sulfides in the water.

In many closed basins, under strictly anaerobic conditions it is quite common that intense populations of *R. sphaeroides* develop, and thus only yellow spheroidene is found in the sediment. The same bacteria produce red spheroidenone only when oxygen is present, even with severe hypoxin. Züllig (72) suggested that if only spheroidene is present and spheroidenone is absent from sediment, it is to be inferred that the lake was meromictic.

It is not safe to assume that decomposition rates among pigments are always similar. Although many stratigraphic studies were done on the relative abundance of pigments (8,71,72,78), this approach has been discouraged because of selective losses of carotenoids (4). On the contrary, the main changes in the historical record of microbial groups can be traced with success using within-core carotenoid analysis.

Indices Used for Evaluating Pigment Degradation

Various indices are used to highlight the conservation state of pigments. One is the ratio of 430 nm: 410 nm (ratio between the spectrophotometric absorbencies of an acetone extract at wavelengths of 430 nm and 410 nm) that represents the degree of conversion of chlorophyll to phaeopigments as a measure of the state of conservation of pigments in the sediment (79–82). Another common index of pigment degradation involves taking the spectrophotometric reading at 665 nm for the sediment sample before and after acidification with 0.2 M hydrochloric acid to yield the percentage of chlorophyll not degraded to phaeopigments (2).

In addition, it has been proposed (2,35,83) that the CD/TC ratio (chlorophyll derivatives/total carotenoids) is an index of decomposition and of trophic state. A low CD/TC ratio indicates optimum conditions for the preservation of these pigments in the sediment because of high carotenoid concentrations (29). Other authors (13,84,85) also believe that this index can be used in delineating the history of the lake as a measure of allochthonous loading of organic matter versus the autochthonous contribution. A high CD/TC ratio would indicate a major allochthonous load of organic matter. Because of contradictory results, probably linked to site-specific characteristics and the influence of physical-chemical, biological, and morphometric factors (4), the validity of this ratio as an index of pigment degradation is questionable.

RECONSTRUCTING LAKE ACIDITY AND TROPHIC STATUS

Acidification Studies

Studies on atmospheric chemical deposition and lake responses have been conducted since the early 1970s and have shown pronounced acidification throughout the world. Consequently, paleolimnological studies were undertaken to develop quantitative temporal reconstructions of lake pH. Most studies relied on subfossil remains of two algal groups to infer pH: diatoms and cysts and scales of chrysophytes. Such histories were then compared with trends in atmospheric deposition of substances derived from combustion of fossil fuels measured as carbonaceous particles. Some years ago, Guilizzoni and coworkers (80) proposed a new proxy record for lake pH reconstruction based on an easily measurable ratio of total plant pigments: the ratio of absorbance at wavelengths (430 nm and 410 nm) in acetone extract of lake sediments. A high significant linear correlation ($P < 0.001$; $r = 0.71$) between pH in 21 Alpine lakes and the 430 nm:410 nm ratio indicated that acidity greatly influenced the pigment ratio. Stratigraphic analysis of a sediment core from an alpine acid-sensitive lake in Italy (Fig. 3) showed a clear decreasing trend coincident with increased atmospheric pollution as inferred by the carbonaceous particle profile. These data were confirmed both by comparison with existing historical data on Italian thermal electricity production and by pH reconstructions based on diatoms and chrysophycean scale assemblages (80,86,87).

Eutrophication and Climate Change Studies

Paleolimnological studies have provided evidence of the long history of algal development and production in many lakes of Europe and North America based on extraction and identification of characteristic pigments from sediment cores. Many spectacular algal blooms were associated with natural conditions and not necessarily a result of human impact (88,89).

Fossil pigment stratigraphies from two crater lakes in central Italy (Albano and Nemi) served as a good example for tracing the postglacial history of phytoplankton development and primary production (Fig. 4). The two lakes displayed marked similar histories, and these were reflected in all biological (pigments, algal cells, invertebrates) and geochemical records (90,91).

Figure 3. Inferred pH from 430 nm:410 nm pigment ratios and Chrysophyte scales in sediment cores of a high-altitude lake in the Italian Central Alps (Lake Paione Superiore). Graphs of number of carbonaceous particles (No. g dry weight^{-1}) and of the Italian thermal electricity production (Million MWatt h^{-1}) are also indicated.

In general, the concentrations of pigments in Lake Nemi were much higher than those from Lake Albano. Some differences were associated with their distinct bathimetry, catchment size, and topography differences. The presence of the photosynthetic sulfur bacterial carotenoids (i.e., okenone and isorenieratene) in early and mid-Holocene (10,000–5,000 yy BP) was significant (Fig. 4). These pigments, however, were also abundant throughout the Holocene, except in the mid-Holocene where high water turbidity and consequently the low light availability very probably limited bacteria development.

Cyanobacterial pigments (a sum of specific algal carotenoids such as echinenone, oscillaxanthin, myxoxanthophyll, and zeaxanthin) mainly characterized the early phase of the Holocene and the recent past. These periods of high concentrations were phases of fairly high lake productivity with a seasonally anoxic water column separated by oligotrophic colder periods as inferred from diatoms cladoceran and chironomid remains (91).

These high levels of pigments and inferred total phosphorus concentrations are similar to those found in very eutrophic lakes today. The balance of evidence from Lake Albano sediments (e.g., presence of varves, high percentages of planktonic communities) points to substantially higher lake levels during these periods. In contrast, lake level was lower in Lake Albano between 3,000-ky BP and 3,500-yr BP, a period during which a human settlement was established on the shores of this lake and nearby lakes.

Cold episodes are defined by an abrupt decrease in pigment concentrations. Signs of marked changes detected in sediment records in the upper part are mostly anthropogenic; the human impact is superimposed on the sudden, general decrease of all biota during a global cold phase around 3,800 to 4,000 yr cal. BP. An oligotrophication phase and oxic sediments are evident from 3,500-yr cal. BP to 4,500-yr cal. BP.

NEW MARKERS AND SUMMARY OF THE PIGMENT PROXY RECORDS

From the aforementioned, it has been shown how environmental factors can control plant pigment deposition and how a strong theoretical background on in-lake processes is extremely important for paleolimnological interpretation. Indeed, fossil pigments from algae and sulfur photosynthetic bacteria are sensitive proxy-records for a number of environmental parameters such as oxygen concentration, variation in water chemistry and water level (Table 2) and are useful for reconstructing variations in community structure (Table 1).

Past studies on fossil pigments have been mainly focused on lake eutrophication and a few on lake acidification, and most of the advances and developments can be readily transferred to studies of past climate changes. In the context of paleoclimate studies, two pigment markers have been recently discovered: one, scytonemin, produced by benthic algae, can be used to infer past changes in UV radiation (92); The second, tetrapyrrole pigments and aromatic hydrocarbons, are diagenetic products of chlorophylls and carotenoids,

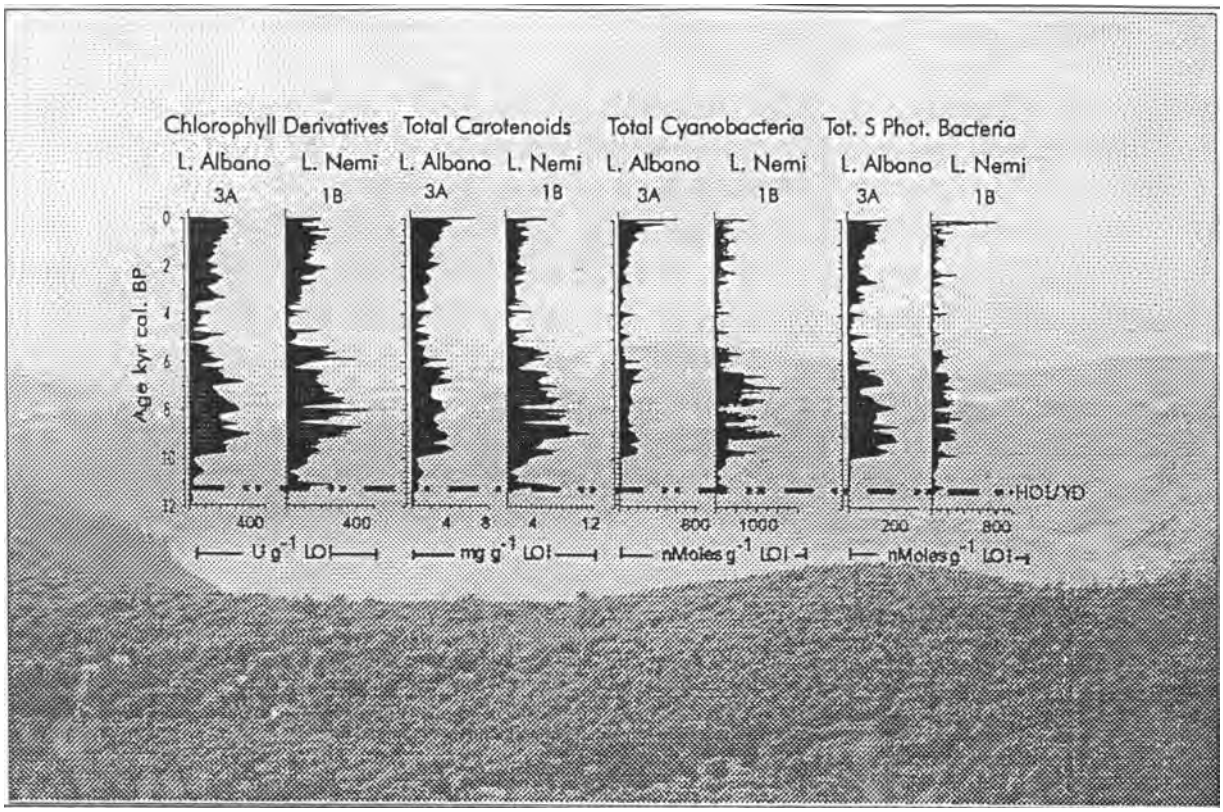

Figure 4. Holocene paleoenvironmental changes from Lakes Albano (*core 3A*) and Lake Nemi (core 1B) sediment cores based on pigments from algae and sulfur photosynthetic bacteria. Total plant photosynthetic pigments (chlorophyll derivatives, total carotenoids) and the group-specific HPLC measured carotenoids (total Cyanobacteria, total carotenoids from anaerobic, sulfur photosynthetic bacteria) are used as valuable indicators of past changes in aquatic productivity and past redox conditions. The evolution of Cyanobacteria populations is inferred from the sum of specific carotenoids (echinenone, oscillaxanthin, myxoxanthophyll, and zeaxanthin). The Holocene/Young Dryas boundary is also indicated.

respectively, may provide valuable information on oceanic circulation and climate (93).

Scytonemin is a pigment produced in the sheaths of certain cyanobacteria when they are exposed to high UV radiation; and two unique pigments (compound A and B) derived from the scytonemin are normally found in lake sediments. Leavitt and coworkers (92), in a study on midlatitude alpine lakes in Canada, found that past UV radiation was greater than that during the present period of anthropogenic ozone depletion of the stratosphere.

Rossel-Melé and coworkers (93), from their study on the so-called "Heinrich" events (cold periods probably resulting from freshwater released from massive melting of the Laurentide ice sheet) (94,95), demonstrated that autochthonous chlorine pigments and the chlorophyll and carotenoid derivatives of continental origin were observed only in this Heinrich sediment layers as a result of reduced bottom water oxygen content. The presence of these biomarkers of potential "source rocks," and their association with better preservation in anoxic conditions suggest that large meltwater input reduced or stopped deep-water formation in the North Atlantic.

Analyses of these pigments requires a technique, that is, the gas chromatography-mass spectrometry, often combined with high performance liquid chromatography (HPLC) to provide diagnostic information on their molecular structures. In turn, this can permit inferences to be made on the sedimentary environment and pathway of pigment degradation (96). This new approach will increase the number of biomarker molecules available for paleoenvironmental and paleoclimate reconstructions.

Because pigments laid down in lacustrine sediments reflect environmental conditions in the lake and its catchment at the time of their deposition, their records allow historical changes in lake trophic status, anthropogenic and natural (climate) changes to be determined, adding greatly to our understanding of development of lakes and associated processes. Such an understanding is necessary for a number of purposes including paleoenvironmental and paleoclimate data, which can be used for predictive models, and for enlightened lake management. It should be considered, however, that any interpretation of the early history of lakes based on pigments stratigraphy is enormously facilitated by any comparison with contemporaneous limnological and historical information. When

Table 2. Summary of the Main, Most Stable, and Most Informative Pigments: Sources, and Their Use as Proxy Records in Paleolimnology (see text)

Pigment	Taxa	Source	Proxy Record for:
Fossil pigments	All plants	Planktonic, littoral	Food-web changes, gross taxonomic composition
β-Carotene, Phaeophytins, and total carotenoids	Plantae, some bacteria	Planktonic, littoral, terrestrial (especially phaeophytin a)	Primary production, and total phototroph abundance
β-Carotene: Chl a	All plants	Planktonic, littoral	Deep algal bloom (low ratio)
Phaeophorbides	Chlorophyll derivatives	Planktonic	Grazing by zooplankton
Astaxanthin, astacene	Zooplankton, few algae	Planktonic	Zooplankton abundance
Vanadyl alkyl porphyrins	Diagenetic product of chlorophylls	Terrestrial	Ancient, organic rich sediments; anoxic conditions
Aryl isoprenoid hydrocarbons	Diagenetic product of carotenoids	Terrestrial	Ancient, organic rich sediments, anoxic conditions
Alloxanthin	Cryptophyta	Exclusively planktonic	Water level (?)
Two lipid-soluble pigments similar to Scytonemin	Certain benthic cyanobacteria	Alpine lake	Past aquatic UV radiation environments
Echinenone	Total cyanobacteria	Planktonic, littoral	Phosphate-eutrophication
Myxoxanthophyll, Oscillaxanthin	Filamentous cyanobacteria	Planktonic	Phosphate-eutrophication
Aphanizophyll	N_2-fixer cyanobacteria	Planktonic	Eutrophication
Okenone, Isorenieratene	Chromatiaceae (*Chromatium. okenii*) Chlorobiaceae (purple and green sulfur, photosynthetic bacteria, respectively)	Planktonic	Anoxia conditions and historical Secchi Disk transparency
Spheroidene, spheroidenone	*Rhodopseudomonas sphaeroides*	Planktonic	Meromictic/Holomictic conditions
430 nm: 410 nm index[1]	All algae	Planktonic, littoral, terrestrial	Acidification, pigment preservation
Chl a: Phaeophytin a; Chl a: β-carotene	All algae	Planktonic, littoral, terrestrial	Pigment preservation

[1]Acetone extract ratio of absorbance at two spectrophotometric wavelengths (430 nm and 410 nm).

these long-term data are available, it is possible to calibrate the pigment record as a paleolimnological indicator and thus accurately reconstruct ecosystem changes and food-web processes in lakes.

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PALEOLIMNOLOGY: USE OF SILICEOUS STRUCTURES OF CHRYSOPHYTES AS BIOLOGICAL INDICATORS IN FRESHWATER SYSTEMS

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Chrysophycean algae are a highly diverse assemblage of freshwater and marine organisms containing well over 1,000 described taxa (1). Although there are exclusively marine orders within the Chrysophyceae (2), as well as groups that have been split from the Chrysophyceae (e.g., silicoflagellates), the majority of the taxa are freshwater and most are euplanktonic in nature. Chrysophytes contain a vast array of morphological types, including unicellular, colonial, filamentous, and palmelloid forms, as well as both mixotrophic and strict photoautotrophic taxa. Even though chrysophytes are more common and can often form large concentrations in ponds and lakes, they can also form substantial populations in streams and rivers (3). As a group, chrysophytes often dominate the spring plankton flora in temperate waterbodies; however, individual species display a vast array of seasonal strategies (4). Chrysophytes can be found in waterbodies from the tropics to the arctic as well as across a wide trophic gradient, although they tend to decline in biomass in the most eutrophic habitats (5). They can form blooms, deep-water (metalimnetic) peaks (6), and are often responsible for taste and odor problems (7). Despite the wide diversity of morphological types and habitats supporting chrysophytes, it is a fact that at the species level many taxa are restricted along an array of environmental gradients (e.g., pH, dissolved salt, and trophic gradients) that make them especially useful as bioindicator organisms (4,8).

As reviewed by Preisig (2), the classification of chrysophycean algae is in a state of flux. Starmach (1) identified three subclasses within the class Chrysophyceae based largely on the number of flagella (none, one, or two) found on cells. Starmach's (1) system of classification was very similar to, and followed closely, that of Bourrelly (9,10). However, as reviewed by Preisig (2), many "uniflagellate" forms are now known to possess a second flagellum, and some species previously thought to be aflagellate can indeed produce flagella. Thus, Starmach's (1) classification scheme has given way to more modern concepts that include a wealth of new ultrastructural, biochemical, and molecular data. Several groups of organisms, including the Synurophyceae, silicoflagellates and pedinellids, have been moved from the Chrysophyceae as circumscribed by Starmach (1) and placed into new classes (11).

A group of chrysophycean organisms possess a covering of siliceous plates, called *scales*, and have historically been referred to as "scaled chrysophytes." Scaled chrysophytes were contained within the family Mallomonadaceae Diesing (10,12) or the family Synuraceae (1) within the class Chrysophyceae. In a classic study, Hibberd (12) recognized that ultrastructural features among some of the scaled chrysophyte genera differed substantially from others, and later Preisig and Hibberd (13,14) formed a new family, the Paraphysomonadaceae, to accommodate scaled chrysophytes with a cell ultrastructure similar to that of *Ochromonas* and *Chromulina*. Andersen (15) then moved the genera not in the Paraphysomonadaceae out of the class Chrysophyceae and placed them in a new class, the Synurophyceae. Thus, today scaled chrysophytes refers to taxa in two different algal classes.

The bulk of the use of chrysophycean algae as indicator organisms has been paleolimnological in nature and is based on siliceous remains of the organisms. Three distinctly different types of siliceous structures, cysts, scales, and bristles, are formed by the organisms and upon death ultimately become part of the sediment record. Because these siliceous structures are species specific they can be used effectively to reconstruct past floras. Cyst and scale remains have been incorporated most widely in paleolimnological studies. Cysts are globose resting stage structures (see later) presumably formed by all members of the Chrysophyceae and Synurophyceae. Scales are only produced by taxa in the family Paraphysomonadaceae (e.g., *Chrysosphaerella*, *Spiniferomonas*, and *Paraphysomonas*) and all members of the class Synurophyceae (e.g., *Mallomonas*, *Synura*, and *Chrysodidymus*). Bristles are specialized siliceous structures formed only by members of the genus *Mallomonas*.

The purpose of this article is to (*1*) introduce the reader to the anatomy and morphology of siliceous scales, bristles, and cysts; (*2*) provide an overview of the distribution of chrysophytes along environmental gradients; and (*3*) discuss how the group has been used in paleolimnological studies.

ANATOMY OF THE SILICEOUS CELL COVERING

All scaled chrysophytes possess a cell covering composed of siliceous plates referred to as scales (Figs. 1 and 2). The scales are sculptured with very specific designs and serve as the primary means of identifying taxa at the species and subspecific levels. Scales of taxa in the family Paraphysomonadaceae, class Chrysophyceae, are more or less radially symmetrical (Figs. 1d and 2d) and are overlapped on the cell membrane in a somewhat unorganized manner. With a few exceptions

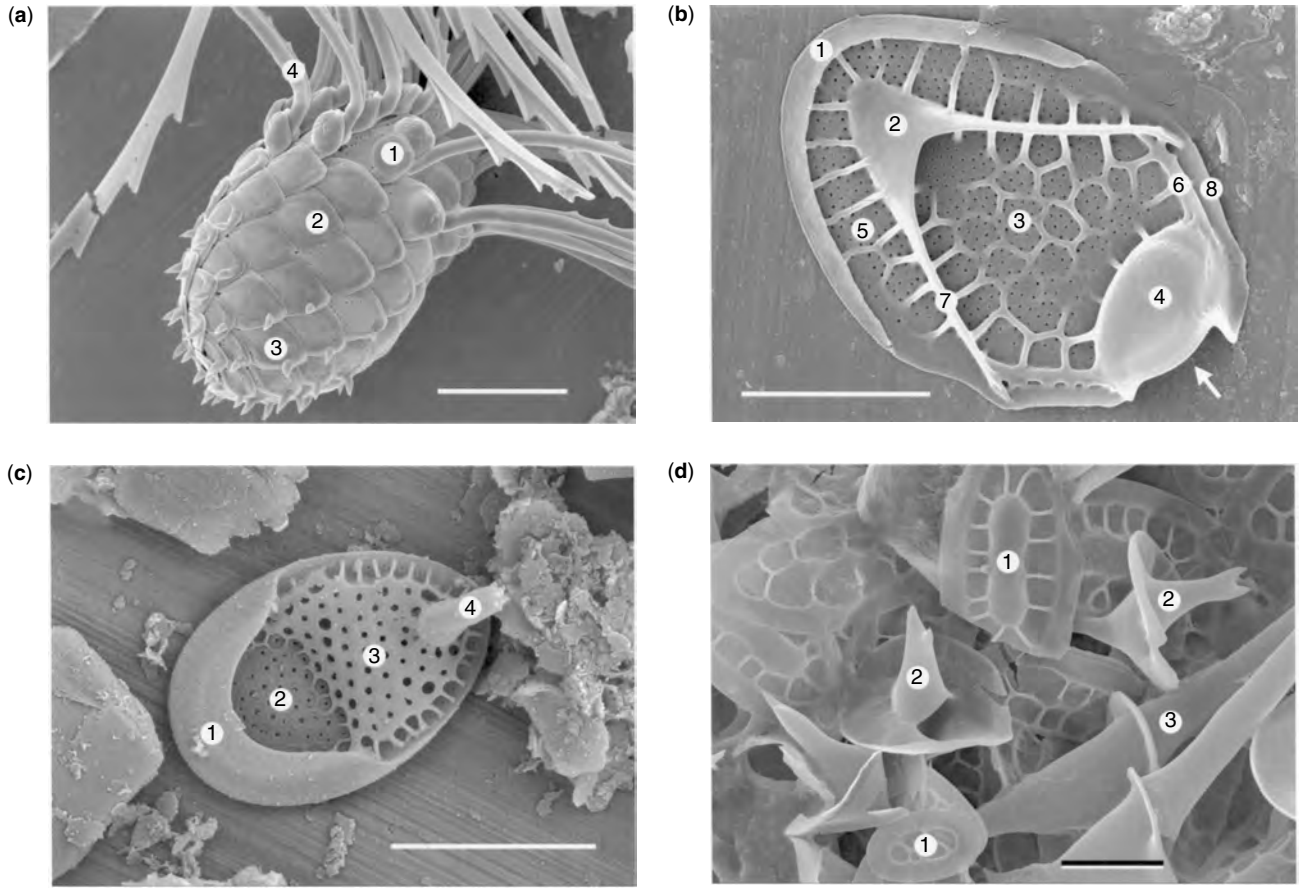

Figure 1. Cell and scale remains from scaled chrysophyte algae. (**a**) Whole cell of *Mallomonas tonsurata*. Note the arrangement of (1) domed, (2) domeless, (3) spined posterior scales, and (4) bristles. Scale bar = 5 μm. (**b**) A single domed scale of *Mallomonas crassisquama* denoting the (1) posterior rim, (2) base of the V-rib, (3) shield, (4) dome, (5) posterior flange, (6) anterior submarginal rib, (7) V-rib arm, and (8) anterior flange. Scale bar = 2 μm. (**c**) A spined scale of *Synura spinosa* denoting (1) posterior rim, (2) area of the scale lacking a secondary layer, (3) area of the scale with a secondary covering, and (4) the spine. Scale bar = 2 μm. (**d**) Plate and spine-scales of *Chrysosphaerella longispina*. Note (1) plate scales, (2) short spine scale, and (3) long spine scale. Scale bar = 2 μm.

the scales of synurophytes are bilaterally symmetrical and arranged on the cell surface in a highly organized manner (Fig. 1a) (16,17).

Scales of synurophyte taxa are overlapped in spiral rows. Each row starts with a scale that is part of a ring of scales surrounding the flagellar opening, includes a number of body scales, and ends with posterior or caudal scales. The shift in scale type along a given spiral row may be gradual or very abrupt (17,18). As a result, although the basic design or pattern of all scales of the same species remains similar, the shape and morphology of the scales can vary significantly. Within a given row scales are orientated with their distal ends pointed toward the anterior of the scale, perpendicular to the longitudinal axis of the cell, or in between these two orientations (16,19). The patterns that the scales are overlapped within a row, as well as the overlapping of neighboring rows, are discussed in detail in Siver and Glew (16) and Siver (17). The most notable exceptions to the patterns of scale design and position on the cell covering are two colonial taxa, *Synura lapponica* and *Tesselaria* (known only in Australia), whose scales are radially symmetrical and positioned in a layer around the colony and not around individual cells (20,21).

Regardless of the position and orientation of the scales on the cell, when the organism dies the cell covering disarticulates and the resultant siliceous pieces become archived in the sediments. Thus, the disarticulated components, primarily the scales, are used in paleolimnological studies (8). Despite the fact that paleolimnologists utilize scale remains, a basic appreciation of the morphology of the whole living cell is necessary to understand the range of scale morphologies possible for each species.

Taxa in the family Paraphysomonadaceae, such as *Chrysosphaerella*, *Spiniferomonas*, and *Paraphysomonas*, possess scales ranging from round or oval platelike to basketlike to ones with long projections known as spines. Most scales of taxa in this family have a relatively unpatterned under surface (the surface in association

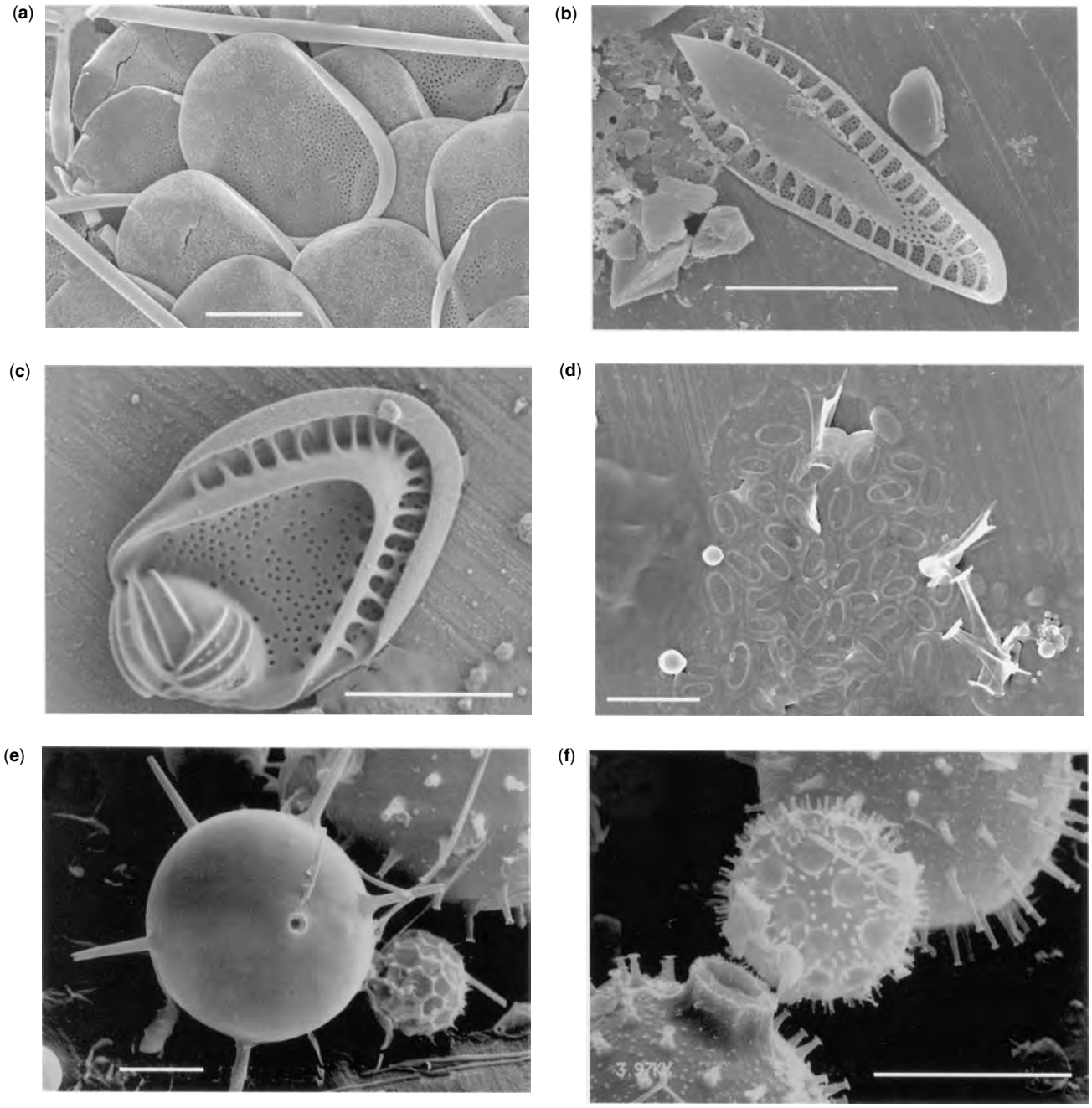

Figure 2. Scale and cyst remains of chrysophycean algae. (**a**) Scales of *Mallomonas caudata*. Note the simple nature of the scales. Scale bar = 2 μm. (**b**) Scale of *Synura petersenii* denoting the keellike spine positioned on top of the scale. Scale bar = 2 μm. (**c**) Domed scale of *Mallomonas acaroides* var. *muskokana*. Scale bar = 2 μm. (**d**) Scale remains of a cell of *Spiniferomonas takahashi*. Note the plate scales with a single lacuna and spine scales with short flared tips. Scale bar = 2 μm. (**e**) Chrysophycean cysts. Note the pore and surrounding collar on the centrally positioned cyst. Scale bar = 5 μm. (**f**) Chrysophycean cysts with surface ornamentation consisting of papillae, dimples, and spines. Scale bar = 5 μm.

with the cell membrane) and a slightly more patterned and sculptured exterior surface (13,22–24). Regardless, except for some species of *Paraphysomonas*, scales of taxa in the Paraphysomonadaceae are not as complex as those on taxa of Synurophyceae.

Platelike scales of *Chrysosphaerella* are oval in outline and range mostly from 2 to 4 μm in length (24). The two most important species, *Chrysosphaerella longispina* and *Chrysosphaerella brevispina*, differ in their scale morphology. The exterior surface of scales of *C. longispina* consists of a flat and smooth marginal rim, a raised elliptical-shaped smooth dome, called a *cupola*, and a series of radial ridges that connect the cupola to the marginal rim (Fig. 1d). The exterior design of scales of

C. brevispina is even simpler, consisting of a thickened oval-shaped and raised ring of silica that is positioned about 0.25 μm in from the scale margin. Except for the thickened ring that encircles a central lacuna, the external surface is unpatterned. Spine scales of *Chrysosphaerella* consist of two circular plates connected by a flared shaft (24). A long and hollow spine extends out from the surface of the second plate (Fig. 1d). The range in length of the spine varies between species.

Like *Chrysosphaerella*, scales of *Spiniferomonas* are of two primary types, oval platelike scales and spine scales originating from more circular-shaped plates (Fig. 2d) (22,23). All platelike scales of *Spiniferomonas* have either a single or two large depressions referred to as lacunae (Fig. 2d). With one known exception (25), scales with a single lacuna are larger than those with two lacunae. Other than the lacunae and the possible presence of one, two, or four rods, additional ornamentation is lacking on plate scales. The spined scales of *Spiniferomonas* taxa consist of a shaft that extends from either a flat or cone-shaped base. The shaft of the spine may be ribbed or smooth and the tip flared or tapered in design. Compared to other taxa of scaled chrysophytes most isolated scales of *Spiniferomonas* cannot always be identified to species. *Paraphysomonas* is a strictly heterotrophic genus whose members possess scales that range from being flat and quite simple to ones that are complex and highly three-dimensional in design (13,14). For most species of *Paraphysomonas* electron microscopy is needed for proper identification.

The basic design of synurophyte scales differs from silica-scale–bearing Chrysophyceae in having bilateral symmetry and in being typically more complex. All scales of synurophyte taxa consist of a base plate and an upturned rim (Fig. 2a). The base plate most often is perforated with small pores, and the upturned rim often encircles half of the scale and is referred to as the posterior rim. The end of the scale with the posterior rim is referred to as the proximal end, and the end that is typically aligned toward the flagellar portion of the cell the distal end.

The vast majority of synurophytes have additional siliceous structures, termed *secondary structures*, on the base plate (Figs. 1b, 1c, 2b, and 2c). The secondary layer on scales of *Synura* species usually consists of an additional layer of silica with pores and a series of ribs (26,27). In addition, scales, especially those on the anterior end of the cell, possess spines. For species within the Spinosae group of the genus *Synura*, the spine originates on the distal end of the scale and extends forward away from the base plate (Fig. 1c). For taxa within the section Peterseniae, the spine, also known as a keel or thorn, is positioned further back on the scale (Fig. 2b). In general, spines become reduced or are lacking on scales positioned on the posterior portion of the cell.

The most complicated types of scales are found in the genus *Mallomonas*, the largest genus within the scaled chrysophytes. Some species have highly ornate scales with specialized structures known as V-ribs, submarginal ribs, domes, and spines (17). A V-rib is a V-shaped to U-shaped ridge situated on the surface of the scale such that the base of the V or U lies near the posterior rim (Figs. 1b and 2c). The sides or arms of the V-rib extend toward the distal end of the scale and terminate at different areas of the scale, depending on the species. Many species have additional siliceous ridges known as anterior submarginal ribs (Fig. 1b). The anterior submarginal ribs are typically aligned parallel to the distal margins of the scale and extend from the ends of the V-rib arms to the distal end of the scale. A dome is a raised region of the distal end of the base plate that forms a cavity (Figs. 1b and 2c) from which one or more bristles emerge (see later). In some species domes are smooth and unornamented, whereas in others they are highly ornate. The overall structure of the dome is of taxonomic importance. Spines are siliceous structures that extend from the distal end of the scale. In species of *Mallomonas*, scales with spines, known as spined scales, are found exclusively on the posterior of the cell and are positioned such that the spines radiate out from the back of the cell.

The V-rib and anterior submarginal ribs, often referred to collectively as the submarginal rib, divide the scale into areas called the *shield* and *flanges*. The shield is that area of the scale bound by the submarginal rib, whereas the flanges are regions of the scale outside of the submarginal rib (Fig. 1b). The posterior flange is the portion of the scale situated between the posterior rim and the V-rib. The anterior flange is the region between the anterior submarginal ribs and the margin of the scale. The ornamentation of the flanges and shield may be very similar in nature or quite distinct from each other.

Bristles are elongated siliceous structures that are distinctly separate from scales and unique to the genus *Mallomonas* (Fig. 1a). Bristles are divided into a foot (proximal end) and a shaft (17). The foot of the bristle is tucked under the distal end of the scale within the cavity of the dome and the shaft radiates out from the cell. Because bristles are not physically fused with scales, their orientation changes with the swimming motion of the cell. Species often have two distinctly different types of bristles that are typically positioned on different parts of the cell. A wide degree of variation in the structure of the shaft and distal tip of the bristle is found between species. As with scale structure, because bristle morphology is distinct at the species level it is also of taxonomic significance. A wide degree of variation in the structure of the shaft and distal tip of the bristle is found between species.

CYST STRUCTURE AND MORPHOLOGY

All chrysophytes and synurophytes are capable of forming a siliceous resting stage known as a cyst, stomatocyst, or a statospore. Cysts are formed endogenously within a specialized vesicle called a *silica deposition vesicle* (SDV) as a result of either sexual or asexual reproduction (28). The formation of the cyst is a continuous process that, in most species, is believed to occur in two steps (29). The first step involves the formation of a primary or inner wall that usually results in a smooth unornamented structure that is presumably similar for many species (30). The second step results in the deposition of additional siliceous layers on the outside of the primary wall, including wall ornamentation, and eventually yields a mature cyst. The

mature cyst is often quite different and distinct from their immature counterparts (reviewed in 30).

Duff and coworkers (30) provided an excellent summary of cyst terminology and structure. A brief overview is provided here. Cysts are hollow, globose structures with a small opening referred to as the pore from which the protoplast will eventually emerge (Fig. 2e and 2f). The majority of cyst types range in diameter from 2 μm to just over 30 μm and come in a variety of shapes, including spherical, oval, pyramidal, and compressed or flattened (5,30). The outer surface of the cyst wall can range from being smooth (Fig. 2e) to highly ornamented with spines, papillae, nodules, and ridges (e.g., Fig. 2f). The pore is usually circular in shape and often surrounded by a siliceous ridge called a *collar*. Collars can be very simple or highly complex structures consisting of multiple concentric ridges surrounding the pore opening.

The shape, size, and ornamentation of the outer wall and collar form the basis for separating cysts into different morphotypes. Because each species is believed to produce a single type of fully mature cyst, shifts in the composition of cysts are believed to reflect species-level changes, making them especially attractive as paleolimnological indicators. The taxonomic identities are known for a relatively small percentage of the large number of cyst morphotypes that have been described (30). It is also inevitable that some cyst types are simply immature stages of more fully developed types, or perhaps represent different ends of the full range of cyst types possible for a given species. Despite these drawbacks cysts have been and will continue to be important bioindicators, especially in paleolimnological studies.

DISTRIBUTION OF CHRYSOPHYTES ALONG ENVIRONMENTAL GRADIENTS AND THEIR USE AS PALEOLIMNOLOGICAL INDICATORS

The remains of chrysophyte algae have been utilized successfully to study historical shifts in many different types of lake ecosystems, especially ones from North America and Europe. The increased use of chrysophytes to infer historical conditions over the past two decades is correlated closely with the rise in importance of paleolimnological studies as a tool for understanding environmental changes, especially anthropogenic-related ones (8). The remains of chrysophytes have now been used to examine changes in variables such as pH (31), specific conductivity (32), trophic status (33), salinity (34), and climate-related variables (35).

Many recent paleolimnological studies, including those incorporating chrysophytes, have used multivariate statistical methods to yield models that, in turn, are used to infer quantifiable changes in specific chemical variables. The statistical methods used to develop inference models are well established, and the reader is referred to Birks (36,37) and Birks and coworkers (38) and references therein, for details. Briefly, the following steps are used to develop and use an inference model. First, surface sediments (e.g., from the top 1 cm) are taken from a group of waterbodies in a given region. The suite of lakes, called the *calibration* or *training set*, is selected such that the individual lakes span the environmental gradient of interest. Present-day limnological data, most importantly for the parameter being inferred, is estimated for each lake in the calibration set and used to produce an environmental parameter-lake data matrix. The relative abundances of the inference organisms, in this case chrysophytes, are estimated from the surface sediments for each calibration lake and used to establish a species-lake data matrix. The environmental parameter-lake matrix is regressed on the species-lake matrix to estimate optima and tolerances for each species for the limnological variable of interest (Fig. 3). Once estimates of the species optima and tolerances are made, they can be used to infer the value of the environmental variable in a down core sample; this can be done using, for example, weighted averaging methods (36,37).

In addition to their utility in developing inference models, the species and environmental parameter data matrices can also be used to explore similarities and differences between taxa or sites (lakes), and to determine the relative importance of each limnological variable in controlling the distributions of the organisms in the suite of lakes. The most widely used statistical methods for these analyses include indirect and direct ordination techniques (39). Correspondence analysis (CA) and principal component analysis (PCA) are indirect ordination techniques that effectively arrange both species and sample scores in low-dimensional space along extracted (principal) axes. The axes are extracted such that they maximize the variance in the data, and scores can be correlated subsequently with environmental data (in an indirect manner). CA and PCA are effective methods for estimating the similarity (or dissimilarity) of samples in a quantitative manner on the basis of their floristic characteristics. Canonical correspondence analysis (CCA) and redundancy analysis, the canonical equivalents of CA and PCA, are direct ordination methods where the derived axes are constrained during the analysis to be linear combinations of the environmental variables. The reader is referred to Jongman and coworkers (39) for a discussion on ordination techniques. Ordination methods have been invaluable in our understanding of the distribution of chrysophytes along environmental gradients, including many of the studies referred to subsequently.

Acidic Deposition and Related Studies

Lakewater pH has been shown in many studies to be one of the main factors controlling the distribution of scaled chrysophytes (4,5,8). The importance of pH has been observed in floristic-based studies (22) as well as in ones using multivariate statistical analyses (40–42). In addition, pH has also been shown to be a primary factor responsible for the distribution of chrysophyte cysts (43–45). Most of the studies documenting the importance of pH have been from localities in North America, including Connecticut (40,46,47), the Adirondacks (41,44), Florida (48), Cape Cod (49), Iowa (50), Ontario, Canada, (43,51), and the northeastern United States (42).

Siver and Hamer (40) reported that the species diversity of scaled chrysophytes in Connecticut lakes and ponds was generally highest in slightly acidic waters,

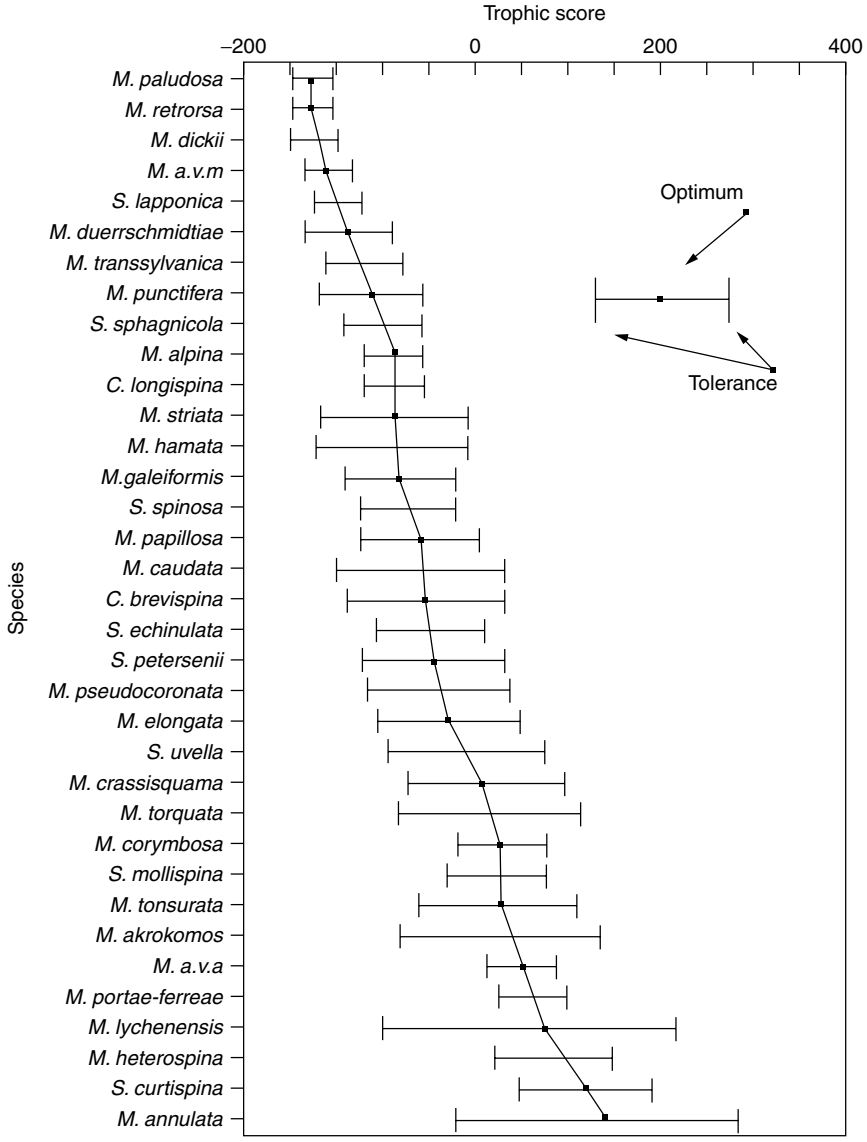

Figure 3. Optima and tolerances of 35 scaled chrysophyte taxa for trophic score. Lower and higher trophic scores are correlated with oligotrophic and eutrophic conditions, respectively. *Source:* P. A. Siver and L. J. Marsicano, *Beih. Nova Hedwigia* **114**, 233–246 (1996).

usually between pH 5.5 and 6.5, and decreased above and below this range. Nielson (52) observed a similar pattern in Danish lakes, and Siver and Smol (53) also reported a similar pattern based on the literature records suggesting that the effect of pH on species diversity was similar in many regions of the world. Although Dixit and coworkers (42) also reported the same general pattern for a large region of the northeastern United States, the maximum diversity was reported to be between pH 7 and 7.5.

Many species of scaled chrysophytes have well-defined distributions along a pH continuum (4,8,17,54) that often are similar in widely separated geographic regions (54). Specific distributions for many taxa are given elsewhere (17,22,41,53,54). It is clear that groups of species are consistently found at specific points along a pH gradient. For example, *Mallomonas canina*, *M. pugio*, *M. paludosa*, *S. sphagnicola*, and *Chrysodidymus synuroideus* are all taxa most often found below pH 6 and that have very low pH optima (4). At the other end of the pH gradient taxa, such as *Mallomonas acaroides* var. *acaroides*, *M. tonsurata*, *M. alpina*, and *M. portae-ferreae*, are more likely to be found. In addition, many cyst types are also differentially distributed along a pH gradient (44,55, and references therein). As a result of the rather narrow distributions of many taxa along the pH continuum, siliceous chrysophytes have been useful indicators of pH and have been instrumental in our understanding of how lake systems have responded to acidic deposition.

Scaled chrysophytes and cysts have been used to examine lake acidification in many regions of the world, especially lakes in the Adirondacks of New York, Connecticut, and the Sudbury, Ontario, region. Smol and coworkers (56) first suggested a strong relationship between scaled chrysophyte remains and pH in the Adirondacks, and quantitative inference work followed by Cumming and coworkers (41). In that work Cumming and coworkers (41) estimated that about 80% of waterbodies with a present-day pH of less than 6.5 had acidified

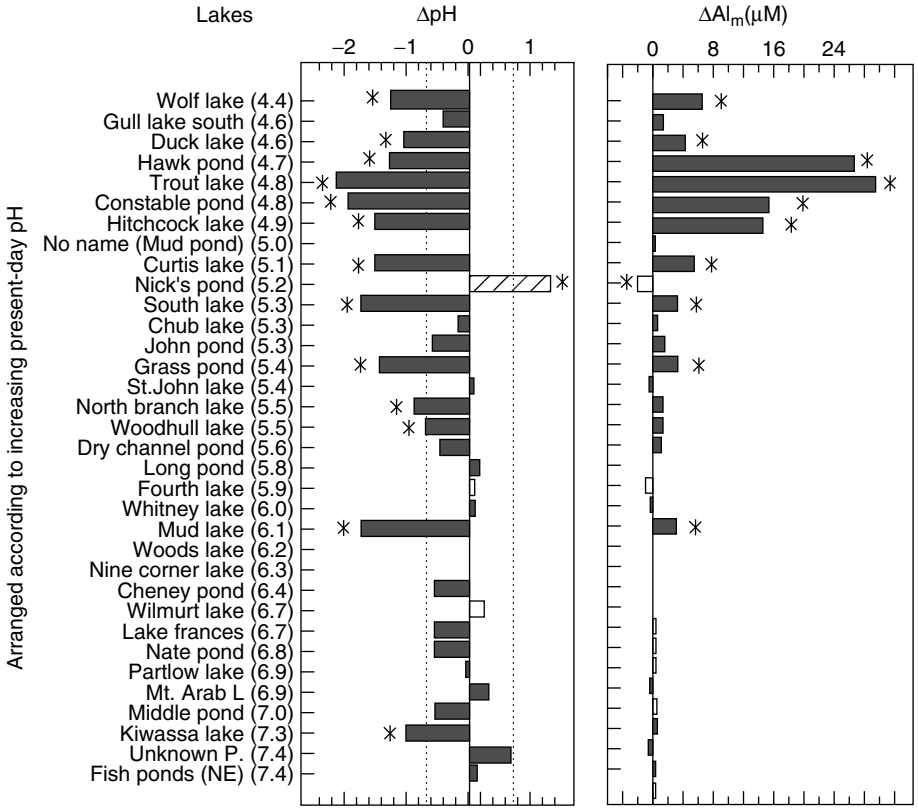

Figure 4. Chrysophyte-based estimates of historical changes (preindustrial to the present) in pH and total monomeric aluminum (Al_m) from 37 Adirondack study lakes. Values are presented as the differences in inferred water chemistry between the top (0 to 1 cm) and bottom (usually >25 cm) intervals of sediment cores. The dotted line represents $\pm RMSE_{boot}$ of prediction for pH. Asteriks denote significant changes. *Source:* B. F. Cumming, J. P. Smol, and H. J. B. Birks, *J. Phycol.* **28**, 162–178 (1992).

since the preindustrial period. Many of the waterbodies that had declined in pH had inferred increases in total monomeric aluminum concentrations (Fig. 4). In a subsequent study, Cumming and coworkers (57) reported that the Adirondack lakes with preindustrial pH values between 5 and 6 acidified first, starting at ca. 1900, whereas those that were slightly better buffered did not commence to acidify until about 1930 to 1950. On an average, the Adirondack lakes declined by 0.96 pH units since preindustrial times and many have not yet significantly begun to recover. Acidification of the Adirondack lakes was confirmed using cyst remains (44).

Using a similar approach Marsicano and Siver (31) began to explore acidification patterns of lakes in Connecticut that were situated in watersheds with thin and poorly buffered soils and on crystalline bedrock. They also reported that, despite the region being impacted by very acidic deposition, none of the lakes included in the study had acidified. In fact, several of the lakes appeared to have actually increased in pH. In a subsequent study of Connecticut lakes, Siver and coworkers (47) reported that none of the 23 lakes in the study had declined in pH, but that 20% had significantly increased in pH over the last century. Siver and coworkers (47) further concluded that despite receiving acidic deposition with pH values near 4.5, the Connecticut lakes were most likely being buffered via sources associated with increased residential development and through enhanced in-lake alkalinity production.

Scaled chrysophytes have also been used effectively to document changes in the pH, as well as other variables, in waterbodies near Sudbury, Ontario (51). Like lakes in the Adirondack region, lakes near Sudbury also dropped in pH by a mean of 0.9 units; however, some lakes had declined over 2 pH units (58). Scaled chrysophytes, along with diatoms, were also used to examine recovery of Sudbury lakes (51). Unlike the Adirondack lakes, Smol and coworkers (58) estimated that 40% of the waterbodies in the Sudbury region have increased in pH as emissions have declined, indicating that some recovery has occurred.

Inferring Dissolved Salt Conditions

Scaled chrysophytes are usually found in greater abundances and at higher species diversities in waterbodies with low specific conductance values (5,40,59), and are essentially absent in more saline lakes (60). Siver and Hamer (40) found that specific conductance was an important variable controlling the distribution of scaled chrysophytes, and further observed that species richness was also related to the dissolved salt content of the water. Both scale (41) and cyst (44) remains in lakes from the Adirondacks (New York) were found to be correlated significantly with specific conductance, and in a study of lakes from the northeastern United States (including the Adirondacks) Dixit and coworkers (42) reported this variable to significantly load onto the first axis of a CCA analysis. Zeeb and Smol (61) successfully used scaled chrysophyte remains to examine the effects of road salt on a waterbody in Michigan.

Siver (32) developed a scaled chrysophyte-based inference model for specific conductance that was subsequently used to trace changes related to deforestation (62) and

residential land use development (47). Siver and coworkers (47) found that 25% of lakes examined in Connecticut had significantly increased in specific conductance, some by more than 100%, over the last century, and that these waterbodies were primarily situated in watersheds that had become highly residential in nature.

Trophic Gradient Studies

Even though relatively high numbers of species can be found in eutrophic habitats (17,40,63–65), scaled chrysophytes have historically been shown to account for a larger percentage of the total phytoplankton biomass in oligotrophic and early mesotrophic lakes as opposed to more eutrophic waterbodies (4,5,7,66). As reviewed by Siver (4,5) chrysophytes have been reported as a dominant component of phytoplankton communities in oligotrophic lakes from many regions of the world, and Sandgren (59) reported that it was not uncommon for this algal group to account for between 10 and 75% of the biomass in these lake systems. The contribution of chrysophytes declined generally to less than 5%, with increased trophic status (59), and typically scaled chrysophyte taxa were less important in highly eutrophic habitats (17,63).

Despite their decreased importance in total biomass at more eutrophic conditions, nutrient-rich habitats can contain high numbers of species (67–69). For example, between 23 and 40 taxa have been reported from nutrient-enriched waters in China (70), Denmark (71), and Germany (64,72). Siver and Hamer (40) found no difference in species richness (number of species) in collections made over a total phosphorus gradient ranging from ca. 4 to 60 $\mu g\,L^{-1}$, supporting the hypothesis that large numbers of scaled chrysophytes can be found in oligotrophic as well as eutrophic sites (5). Dixit and coworkers (42) also reported little difference in species richness of scaled chrysophytes relative to nutrient gradients.

In addition to differences in biomass of scaled chrysophytes along a trophic gradient, there are also clear differences in how individual species are distributed along a trophic gradient (17,33). For example, in a study of waterbodies in Connecticut, Siver and Marsicano (33) reported *M. paludosa*, *M. retrorsa*, *M. dickii*, *M. acaroides* var. *muskokana*, and *Synura lapponica* as being almost exclusively found in very oligotrophic habitats, whereas other taxa such as *M. heterospina*, *M. portae-ferreae*, *M. acaroides* var. *acaroides*, and *Synura curtispina* were encountered in mostly eutrophic localities. Other taxa, such as *M. tonsurata* and *M. corymbosa*, may be found under slightly less eutrophic conditions, but hardly ever in oligotrophic lakes, whereas still other species, such as *M. elongata*, *M. pseudocoronata*, and *S. uvella* are more often observed in slightly eutrophic sites, but can also be found in oligotrophic habitats (17). Other studies by, for example, Siver and Vigna (73), Saha and Wujek (74), Santos and Leedale (75), and Gutowski (64,65) confirmed the fact that species are differentially distributed along trophic gradients. In addition, a number of studies (55,76) have reported that many chrysophyte cyst types are also related to trophic gradients. Because many species and cyst types are differentially distributed along trophic gradients they can be used to infer changes in trophic conditions.

Although qualitative in nature, the use of species-level changes to detect changes in lake eutrophication was initially reported by Munch (77), Battarbee and coworkers (78), and Smol (79). Munch (77,80) observed significant changes in scaled chrysophytes in Hall Lake, Washington, before and after the arrival of European settlement, and attributed the shifts to increased nutrient levels. Likewise, Battarbee and coworkers (78) noted distinct differences in the species diversity as related to different nutrient conditions in laminated sediments from a lake in Finland. Smol (79) also reported on the sensitivity of the scaled chrysophyte flora as a result of eutrophication of three lakes from Algonquin Park, Canada. In subsequent studies Haworth (81,82), Smol and Boucherle (83), Gutowski (65), and Zeeb and coworkers (76,84) related changes in chrysophytes to changes in trophic status. The use of scaled chrysophytes as bioindicators of trophic status is further discussed by Kristiansen (63).

Smol (85) proposed the use of the ratio of the number of cysts to the number of diatom frustules as an indicator of trophic status. The hypothesis was that the ratio of cysts to frustules would be high under oligotrophic conditions and decline as the water became more eutrophic. Although this ratio was later used to examine historical changes in the Great Lakes (86) and lakes in Yellowstone National Park (87), lumping all cyst types into a single group underutilizes their full potential use as bioindicators (8).

Because of the differential distribution of scaled chrysophyte species along a trophic gradient, Siver and Marsicano (33) were able to construct a paleolimnological model based on scaled chrysophytes to infer trophic condition of lakes in Connecticut. They reported a wide variability in the distribution of species along a trophic gradient (Fig. 3) and the resultant inference model, based on weighted averaging with tolerance downweighting, yielded an $r^2 = 0.74$ (Fig. 5). The model was subsequently used to evaluate changes in trophic status relative to alterations in land use over the last century and to make lake management recommendations (47). In a similar fashion, chrysophyte cysts have been used to quantify changes in the trophic status of lakes (55,76,84).

Studies Relating to Climate Change

Although chrysophytes have not been utilized as extensively as other indicator groups in paleoclimate work, the studies that have been undertaken indicate that they will certainly aid in our understanding of climate change (88). The value of the chrysophytes in understanding long-term climate change will most likely be related to the responses of species to temperature and dissolved salt concentrations (see earlier). Studies have related changes in species composition directly to lake water temperature (89), summer surface water temperature (90), and mean annual air temperature (35). Thus, as the temperature regime in a region shifts causing changes in lake water temperature and stratification characteristics, chrysophyte species composition would also be expected to change. As noted earlier, the composition of chrysophytes changes along a

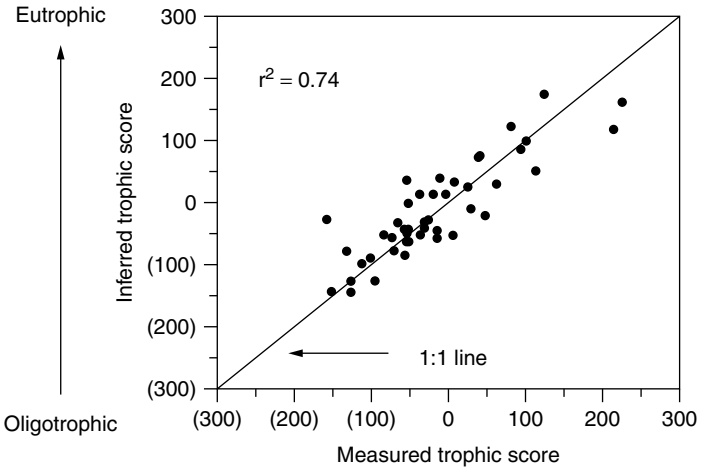

Figure 5. Relationship between the measured trophic score of 45 lakes in Connecticut, and values inferred from scaled chrysophytes. Lower and higher trophic scores are correlated with oligotrophic and eutrophic conditions, respectively. A 1 : 1 line is denoted. *Source:* P. A. Siver and L. J. Marsicano, *Beih. Nova Hedwigia* **114**, 233–246 (1996).

dissolved salt gradient (32). Thus, the hypothesis is as the ratio of precipitation to evaporation shifts in response to climate change the concentrations of dissolved salts in lake basins would also change resulting in shifts in chrysophyte species compositions and abundances.

Both Roijackers and Kessels (91) and Siver and Hamer (40) concluded that, although of lesser importance than other variables such as pH and specific conductance, water temperature was involved in determining how abundant and at what time of year populations of specific taxa would develop. Further work by Gutowski (64) and Siver and Hamer (89) showed that water temperature was an important variable controlling the seasonal succession of species and, as summarized by Siver (4), many taxa are clearly found to develop at different points along a temperature gradient. In addition to the fact that species are differentially distributed along a temperature gradient (17), absolute abundances are also known to be related, in part, to water temperature. In an excellent analysis of the literature, Sandgren (59) observed that the largest biomass of chrysophytes was between 10 and 20 °C, and that concentrations significantly declined at higher temperatures. Other studies collaborate the findings of Sandgren (59) and further indicate that maximal biomass levels of chrysophytes are often reported in temperate regions during spring mixing (5). As a result of these findings shifts in both species composition and total abundance may provide indirect evidence of climate change.

The use of algal remains, including studies based on chrysophytes, in tracking climate changes was recently reviewed by Smol and Cumming (88). Based on a 5-year study of scaled chrysophytes in a small pond in southern New England, Siver and Hamer (89) were able to develop a highly significant inference model for water temperature. Pla (35) was able to use sediment traps to determine differences in the seasonal occurrences of cyst morphotypes in the Pyrenees of Spain. Based on these studies, further development of models that directly infer water temperature may prove valuable in understanding climate change.

Other climate-related studies have incorporated the use chrysophyte cysts over both long and short time periods. For example, as part of a large-scale study of Elk Lake in Minnesota, Zeeb and Smol (92) noted a significant shift in the composition of cyst types at about 8,500 years ago when the region is believed to have shifted to prairie vegetation. Pla (35) reported that the proportion of warmer water cyst types had increased in a lake in the Spanish Pyrenees over the last several decades. Brown and coworkers (90) studied changes in the cyst morphotypes in lake basins that spanned treeline latitudes. Cumming and coworkers (34) and Zeeb and Smol (60) both discussed the use of cysts in inferring dissolved salt concentrations, especially as a potential indirect means of examining climate change (88). Based on these initial studies, chrysophycean algal remains will be useful in climate change studies.

CONCLUSION

Chrysophycean algae are a diverse group of organisms that exist in many different aquatic habitats. Three different types of siliceous structures, known as scales, cysts, and bristles, can be produced by chrysophyte algae and eventually become part of the sediment record. Because the siliceous structures are largely species specific their remains can be used to reconstruct effectively historical changes in the flora. This fact, coupled with the reality that many taxa are found to be rather restricted along different environmental gradients, make the chrysophytes an effective bioindicator group of organisms, especially from a paleolimnological point of view. Studies utilizing chrysophycean algal remains have been instrumental in understanding how waterbodies have responded to such anthropogenic disturbances as acidic deposition, cultural eutrophication, dissolved salt pollution, and land use changes. Undoubtedly, these organisms will play a role in understanding climate change in the years to come.

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PANSPERMIA THESIS.

See DESICCATION BY EXPOSURE TO SPACE VACUUM OR EXTREMELY DRY DESERTS: EFFECT ON MICROORGANISMS

PARALYTIC SHELLFISH POISONING.

See RED TIDES AND OTHER HARMFUL ALGAL BLOOMS

PARASITIC PROTOZOA: FATE IN WASTEWATER TREATMENT PLANTS

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Wastewater issue harbors large quantities of organic and mineral matter and is also loaded with many pathogenic microorganisms. Infected individuals (whether symptomatic or not) and certain water sources, such as those released from slaughterhouses, are major sources of microorganisms (viruses, bacteria, and parasites) with pathogenic potential for humans and animals. Parasites are found in the environment in their resistant forms: eggs for helminths and cysts or spores for protozoa.

WASTEWATER TREATMENT

Wastewater treatment leads to the production of treated wastewater and sludge (biosolids) resulting from the separation of the solid matter from the aqueous phase (Fig. 1).

The treated wastewater is generally released into surface waters, into soils, or directly into the sea. Whatever the final destination, this effluent must meet health standards because surface water is increasingly used as a source of potable water. Similarly, contaminated effluents released into the sea may have an impact on the health of swimmers and may affect the sanitary quality of seafood and fish. Finally, the search for improved water resource management has led to the reuse of treated wastewater for irrigation (irrigation of crops destined for human consumption or recreational areas). To avoid transferring pollution from wastewater to agricultural crops or recreational areas, wastewater effluents must comply with health standards.

Wastewater treatment involves three steps:

1. Pretreatment operations include screening, settling, and decanting procedures to remove coarse material and heavy (e.g., sand) or light (e.g., oil) matter.

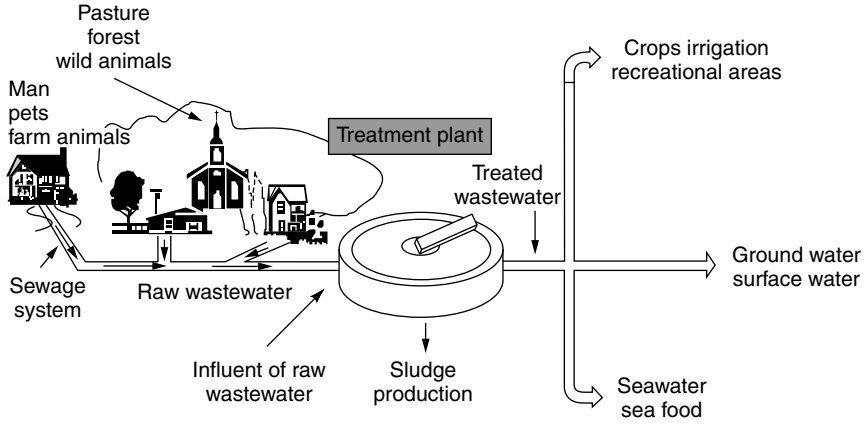

Figure 1. General outline of the wastewater circuit.

Table 1. Overview of Unit Processes and Operations Used in Wastewater Reclamation (1)

Process	Description	Application
	Solid/liquid separation	
Coagulation	Addition of chemicals to destabilize suspended and colloidal matter	Promote particle destabilization to improve flocculation and solids separation
Flocculation	Particle aggregation	Particle agglomeration upstream of liquid/solid separation processes
Sedimentation	Gravity sedimentation of particulate matter, chemical floc, and precipitates from suspension by gravity settling	Settleable solids removal
	Biological treatment	
Aerobic biological treatment	Biological metabolism of waste solids by bacteria in an aeration basin	Removal of organic matter from solution by synthesis into microbial cells
Oxidation pond	Ponds with 2–3 ft of water depth for aerated lagoons and sunlight penetration	Reduction of suspended solids, biochemical oxygen demand (BOD), fecal bacteria, parasites, and ammonia
Disinfection	The inactivation of pathogenic organisms using oxidizing chemicals, ultraviolet light, caustic chemicals, heat, or physical separation processes	Protection of public health coagulation enhancement
	Advanced treatment	
Activated carbon	Process by which contaminants are physically adsorbed onto the carbon surface	Removal of hydrophobic organic compounds
Reverse osmosis	Pressure membrane to separate ions from solution based on reversing osmotic pressure differentials	Removal of dissolved salts from solution and pathogens

2. Secondary treatment can be either biological (e.g., activated sludge, trickling filter, and oxidation pond) in which organic matter is digested by microorganisms or physicochemical in which chemical reagents (flocculants or coagulants) agglomerate solid particles that form decantable flocs. Sludge results from the sedimentation occurring during the preceding operations. These steps can be completed by a disinfection operation (e.g., chlorine and ozone).
3. An advanced treatment phase (e.g., activated carbon) aimed at improving the elimination of contaminants.

Table 1 presents some unit processes and operations used in wastewater treatment.

PROTOZOA FOUND IN WASTEWATER

Qualitative Aspects

Wastewater, surface water, and recreational water can carry a large quantity of protozoa released by infected or healthy carrier individuals and/or animals. The protozoa eliminated in fecal matter or urine are in the form of resistant cysts or oocysts (Table 2).

Table 2. Types of Protozoa found in Water: Qualitative Data

Protozoa Type	Protozoa Species	Presence in Waste Water	Surface Water	Recreational Water	Drinking Water	Reference
Flagellates	*Giardia intestinalis* or *lamblia*	+	+	+	+	(2–4)
	Naegleria fowleri		+	+		(5)
Amoebae	*Entamoeba histolytica*	+	+		+	(6,7)
	Acanthamoeba spp.	+[a]	+	+		(8–10)
Coccidia	*Cryptosporidium parvum*	+	+	+	+	(11–14)
	Cyclospora cayetenensis	+	+		+	(15–18)
	Isospora belli	+				(19,20)
Microsporidia	*Enterocytozoon bieneusi*	+[b]	+[c]			(21–23)
	Encephalitozoon intestinalis	+	+			(21–23)
	Vittaformae corneae	+[b]				(21,22)

Note: [a]Presence in sediment and soil contaminated by sewage.
[b]Detection by PCR in tertiary effluent.
[c]Detection by PCR in surface water and ground water.

Table 3. Parameters Influencing the Contamination Level of Water

	Entamoeba histolytica	*Giardia intestinalis*	*Cryptosporidium parvum*	Reference
Prevalence	10% World 20–80% Developing countries	7% World 2–15% Developed countries 20–30% Developing countries	1–16% Developed countries 5–40% Developing countries	(7,24,25)
Concentration in fecal matter	1.5×10^7 cysts/day	9×10^8 cysts/day	10^8–10^{10} oocysts/day	(26,27)
Animal reservoir	No	Yes	Yes	
Cross-infectivity between animals and humans	No cross-transmission has not been documented	Yes for instance, beavers implicated in water outbreaks	Yes associated with cattle and possibly other domestic and wild animal wastes	(28)
Resistance or survival in environment	Feces: 1 month Wastewater: 15–30 days Sludge: 15–30 days Surface water: 1 month	Surface water: 1–3 months Wastewater: 1 month Sludge: 3 months Soil: 2 months (−4° at 4 °C)	Feces: 4 months Surface water: 1–6 months Soil: 3 months (−4° at 4 °C)	(29)

Except for microsporidiae that have been demonstrated only by polymerase chain reaction (PCR), resistant forms (cysts and oocysts) of all the protozoa presented in Table 2 have been observed under the microscope.

Quantitative Aspects

Factors with a Possible Influence on Environmental Parasite Contamination. The concentration of the observed protozoa (*Entamoeba histolytica*, *Giardia intestinalis*, and *Cryptosporidium parvum*) depends on:

1. the prevalence in the environment,
2. the presence of an animal reservoir,
3. the quantity of cysts excreted and their resistance in the environment,
4. the social and economical conditions, and
5. the presence of effluents from slaughterhouse (Table 3).

Level of Contamination in Raw Wastewater. Various levels are reported in the literature; hence, mean concentrations cannot be given. Table 4 presents a few examples of data selected on the basis of geographic criteria.

It is important to emphasize the large variations observed in concentration levels. This could be explained, at least in part, by the wide range of methods used and their respective performances. Nevertheless, mean concentrations of *Giardia* cysts in raw wastewater are always higher than *Cryptosporidium* oocyst concentrations (59).

Globally, the concentration of protozoan cysts detected in raw wastewater is affected by

Table 4. Contamination of Raw Wastewater with Parasitic Protozoa

Type of Protozoa	Country	Concentration Cysts/L	Positive Samples	References
Giardia	North America			
	Canada	1.1×10^1–1.9×10^2	100%	(30)
		1×10^1–2.1×10^2		(31)
	U.S.	4.4×10^1–3.5×10^2		(32)
		4–1.4×10^4		(33)
		1–1.3×10^2		(34)
		2×10^3–2.2×10^4		(35)
	Central America			
	Mexico	7.1×10^2–1.3×10^3	100%	(36)
	Europe			
	U.K.	1×10^1–5.2×10^4	100%	(13)
		3×10^2–1.2×10^5	100%	(37)
	France	1×10^1–1.3×10^4	75–90%	(38)
		0.2–2.5×10^4	100%	(39,40)
	Spain	2.3×10^2–2.5×10^4	100%	(41)
		4.5×10^3		(42)
	Africa			
	South Africa	0–1.8×10^3	50%	(43)
	East Africa	1×10^3–2.5×10^4	37%	(41)
	North Africa	3×10^2–3×10^3		(44)
	Asia			
	Malaysia			
	Hong Kong	0–4.6×10^2		(46)
	Middle East			
	Israel	0–3×10^2	75%	(47)
Cryptosporidium	North America			
	Canada	8.3×10^2–1.2×10^4	100%	(31)
	U.S.	8.5×10^2–1.4×10^4	100%	(48)
		7–1.9×10^3	87%	(49)
		18–2×10^2	57%	(35)
	Europe			
		1–3.2×10^2	74%	(50)
	U.K.	5.6×10^1	65%	
		1×10^1–1.7×10^2	63%	(38)
		0–6×10^3		(13)
	Africa			
	South Africa	0–5×10^1	30%	(43)
	East Africa	1.2×10^1–7.3×10^1	54%	(51)
	Asia			
	Malaysia	0	0%	(45)
	Hong Kong	0–0.3	10%	(46)
	Middle East			
	Israel	0.2–2.7×10^2	100%	(47)
Entamoeba histolytica	North America			
	U.S.	No reported waterborne outbreaks since 1971		(52)
	India	9.1×10^1–6×10^2		(53)
	Middle East			
	Syria	4.6×10^2–7×10^3		(54)
	Israel	0–4		(55)

Table 4. (*Continued*)

Type of Protozoa	Country	Concentration Cysts/L	Positive Samples	References
Entamoeba histolytica	Africa			
	Burkina Faso	2.7×10^2		(56)
	Morocco	3.7×10^4		(57)
	South America			
	Argentina	0.9		(58)
	Middle America			
	Mexico	1.3×10^2–5.7×10^2	100%	(36)

1. the number of contributors (i.e., number of infected humans and animals in the community served by the sewage treatment works),
2. the quality of the cysts excreted and their resistance in the environment, and
3. the dilution by other waste discharging into the sewage treatment works.

Giardia cysts can thus account for up to 23% of all parasites in sewage (58). This high percentage of *Giardia* cysts in wastewater can be explained by the number of contributors (infected and/or healthy human carriers) and the large number of animals infected with *Giardia* (both *duodenalis* and other species), with both symptomatic and asymptomatic infections. Seasonal variations in cosmopolitan parasitic diseases have been described during the autumn and winter for *Giardia* (40) and during calving periods in feedlots for *Cryptosporidium*.

Entamoeba histolytica is found in geographical areas with a tropical (warm and humid) climate and poor sanitary conditions.

INFLUENCE OF WASTEWATER TREATMENT ON PROTOZOA CONCENTRATIONS

The treatment procedure varies from one station to another, but in general, wastewater treatment stations use biological and physicochemical processes. Each of these processes can be more or less effective with regard to the removal of protozoan parasites.

The capacity of treatment stations to remove and/or inactivate the largest possible percentage of protozoan cysts depends on the capacity of the different procedures to affect resistant forms.

Before reviewing the different procedures used, it is important to point out that data in the literature demonstrated the considerable variations observed both within and between sewage treatment works. It is also quite difficult to compare the different studies published because of variations in sampling and processing. Under these conditions, interpretations must be made with caution.

Removal of Protozoa by Sedimentation

Sedimentation is the fundamental operation for primary treatment, but is also involved in secondary and sometimes tertiary treatment. Primary and secondary sedimentation remove only a small proportion of cysts because cysts are small and have low density. The theoretical data in Table 5 summarizes density and settling velocities of freely suspended cysts as described by Stokes' law.

The main objective of primary treatment is to retain large particles that have much higher sedimentation velocities ranging from 500 to 150 cm/h.

The theoretical sedimentation velocity is given for isolated cysts, but when cysts are in contact with sewage particles, the sedimentation velocity increases with the size of the particle. The percentage of attachment of cysts and oocysts to wastewater particles appears to reach a maximum (75%) after approximately 24 hours, and prolonged incubation (48 hours) does not result in a higher percentage of attached cysts. This may be due to detachment of attached cysts caused by shear forces related to fluid movement (61).

Table 6 displays the percentage of cyst removal or reduction after primary treatment expressed in logarithm units.

Primary treatment is not very effective in removing protozoan cysts. Most rates are below 50%, with a maximum reduction of 90%. Studies aimed at determining the relationship between cyst removal and suspended solids removal during primary clarification have not been able to demonstrate any correlation (13).

Table 5. Theoretical Sedimentation Velocities of Cysts (7,60)

Parasite	Size	Density (gram per cubic centimeter)	Sedimentation Velocity (meters per hour)
Entamoeba histolytica	10–15 μm	1.06	0.11
Giardia intestinalis	9–15 μm	1.11	0.005
Cryptosporidium parvum	4–6 μm	1.05	0.001

Table 6. Calculated Removal Efficiencies of Primary Sewage Treatment for Protozoan Cysts

Parasite	Removal (% or reduction log 10 unit)	References
Cryptosporidium	44–90%	(61)
	80–84%	(62)
	52%	(63)
	4–30%	(13)
	No reduction	(31)
Giardia	18–80%	(32)
	0.1 log	(31)
	24–47%	(13)
	62–71%	(36)
E. histolytica	27–64%	(64)
	49%	(7)
	68–80%	(36)

Removal of Protozoa by Trickling Filters

Trickling filters alone do not appear to be efficient in removing protozoa from sewage. Reductions reported in the literature vary from 10 to 99%, with most results indicating 20 to 40% removal (Table 7). Many protozoan cysts will pass through trickling filters.

Casson and coworkers (32) and Robertson and coworkers (13) compared efficiency of trickling filters and activated sludge treatment at removing *Giardia* cysts. The trickling filters were found to be less efficient in removing cysts than activated sludge, but the difference was small (<5%).

Several studies on trickling filters have shown that a secondary sedimentation improves the system performance. The rotating biological contactors (RBC) treatment system may have a performance level equivalent to that of trickling filters.

Removal of Protozoa by Activated Sludge

The activated sludge treatment process has been studied in different countries, but the results are difficult to compare because of the differences in sampling (continuous or grab samples), identification methods, and the lack of simultaneous analysis of influent and effluent. Globally, the activated sludge process has little effect on protozoan cysts because the environment is wet and not hostile, temperatures are ambient, and retention times are short (6–12 hours). Cysts may become entrapped in the flocs, in which case they will be removed during secondary sedimentation.

Table 7. Calculated Removal Efficiencies of Protozoan Cysts by Trickling Filters

Parasite	Removal (%)	References
E. histolytica	25–74%	(7)
Giardia	10–99%	(40)
	30–80%	(32)
	74–85%	(13)
Cryptosporidium	5–38%	(13)
	93%	(38)

For *E. histolytica* cysts, removal rates of about 80% have been described (64). For *Giardia* cysts, Battigelli and coworkers report a 0.7 log reduction, whereas other authors have found higher levels of about 3 log (65). Robertson and coworkers (13) conducted a field study in which three sewage treatment works (two with primary treatment and one without primary treatment) were monitored over three years for concentrations of *Giardia* cysts and *Cryptosporidium* oocysts. At both sewage treatment works, calculated removal efficiency was significantly higher for *Giardia* cysts than for *Cryptosporidium* oocysts. Removal of *Giardia* cysts can be estimated to be 60 to 90% in sewage treatment works incorporating a primary process and a secondary process. Data analysis indicates that primary settlement alone is relatively inefficient at removing both parasites and that removal occurs during the secondary treatment stage.

For *Cryptosporidium* oocysts, the removal efficiency varies greatly from 15 to 98%, depending on the study. Robertson and coworkers (13) did, however, emphasize that variations in calculated removal efficiency were considerably greater for *Cryptosporidium* oocysts than for *Giardia* cysts in all the sewage treatment works studied.

The efficiency of activated sludge treatments would result from the large quantity of particles suspended during the aeration process. During the sedimentation stage, these particles flocculate and form a layer that acts like a filter; this layer would entrap the oocysts, holding them in the sludge (61). Laboratory studies have demonstrated that oocyst attachment to particles found in effluents of activated sludge is a rapid and widespread phenomenon (60). These data have not been investigated in the field, but they support the hypothesis that the presence of a large quantity of particles after activated sludge treatment favors oocysts removal during the secondary sedimentation stage.

Aerated lagoons and oxidative ditches can be considered as variants of the activated sludge process and have shown very similar removal rates for protozoan cysts. The removal efficiency of protozoan cysts and oocysts by the activated sludge process is summarized in Table 8.

Table 8. Impact of Activated Sludge Treatment on Protozoan Cysts

Parasite	Removal (% or reduction log 10 unit)	References
E. histolytica	83%	(64)
	0.7 log	(66)
	3 log	(34)
Giardia	88.5–93.8%	(38)
	60–90%	(13)
	97%	(32)
	98–99%	(67)
	80–84%	(62)
Cryptosporidium	90–98%	(61)
	84.5–98%	(66)
	79%	(48)
	46–54%	(38)
	15–91%	(13)

Removal of Protozoa by Waste Stabilization Ponds

Waste stabilization ponds can be used either as the principal treatment, which implies many successive ponds for sedimentation or as a secondary treatment of wastewater (40,51,68). Anaerobic and facultative ponds are theoretically designed for the removal of biochemical oxygen demand, whereas the function of maturation ponds is the removal of excreted pathogens. The efficiency of this treatment depends on the total theoretical retention time in the ponds to allow a good sedimentation of particles, although this is not the only parameter involved (39). Temperature, pH, and solar radiation also have an important effect. Table 9 summarizes the efficiency of stabilization ponds in removing protozoan parasites.

The results presented in Table 9 show that stabilization ponds are an efficient means of removing protozoan cysts if a certain number of conditions are met:

1. sufficient retention time (20 days to more than 30 days) (7,39,51) and
2. sufficient temperature and solar radiation (low radiation levels can occur depending on the season) (39).

The principal mechanism involved in protozoa removal probably is due to the adsorption of cysts and oocysts to suspended matter. Predation phenomena described for bacteria have not been reported with protozoa.

The occurrence and concentration of cysts and oocysts detected in pond effluent in series decreased sequentially, indicating that the removal of oocysts and cysts is also related to the cumulative effect of retention time.

Impact of Tertiary Treatments

Tertiary treatments are routinely used in many countries to improve the quality of effluents produced by conventional biological treatment.

Sand Filtration. Rapid filtration (5–15 m/h flow rate) and slow filtration (10–30 cm/h) on sand have been found to be the two effective tertiary treatment methods with different efficiencies for the removal of protozoa. Slow filtration provides complete cyst removal from secondary effluents (>3 log removal) (72,73), whereas rapid filtration can remove 70 to 80% of *Giardia* and *E. histolytica* cysts and 50% of *Cryptosporidium* oocysts (38,48,74).

Infiltration Percolation. There are few data on the performance of infiltration percolation on cysts removal from wastewater. Castillo and coworkers (75) reported that in Chile soils (sandy-lime and lime-gravel) can provide a total cyst removal with an infiltration rate of 20 to 30 cm/day.

Tertiary Lagoons. Conventional effluents can be further polished in tertiary lagoons. The mechanisms involved are similar to those described for waste stabilization ponds (76). A study conducted in the United Kingdom indicated a mean 0.6 log reduction of *Giardia* cysts (77).

Artificial Wetlands. Artificial wetlands are being increasingly used for the tertiary treatment of secondarily treated wastewater (activated sludge). Enhancement of water quality occurs due to the wetland's ability to remove nutrients, various chemical contaminants, and wastewater microorganisms. There are several types of wetlands: aquatic pond, subsurface flow, and surface flow.

Figure 2 shows a comparison of the mean efficiencies observed for *Giardia* cysts and *Cryptosporidium* oocysts removal in different types of wetland (78).

In all the wetland systems studied, the concentration of protozoan cysts decreased from the influent to the effluent. The aquatic pond system is the most efficient for removing protozoa. The primary mechanism of removal within the pond system is likely to be sedimentation, although other factors such as natural die-off and adsorption to detritus may also be involved to some degree (78).

High Rate Algae Ponds. This type of system has been efficiently used to remove *Cryptosporidium* oocysts (79) provided the retention time is sufficient. After three days retention, efficiency reaches 97% removal; 99.9% is reached after 10 days, and 100% after 12 days.

Disinfection

Chlorination. Chlorine is the most widely used disinfectant for municipal wastewater because it destroys target organisms by oxidizing cellular material. Chlorine can

Table 9. Removal of Protozoan Cysts in Waste Stabilization Ponds

Parasite	Removal (%)	Country	References
E. histolytica	30–100%	Mexico	(69)
	96%	Burkina faso	(56)
	100%	India	(64)
	100%	Kenya	(51)
	100%	Mexico	(70)
	100%	France	(39)
Giardia	(spring, summer, and autumn) 99,97% (winter)		
	100%	Cayman Islands	(68)
Cryptosporidium	90%	Brasil	(71)
	100%	Kenya	(51)

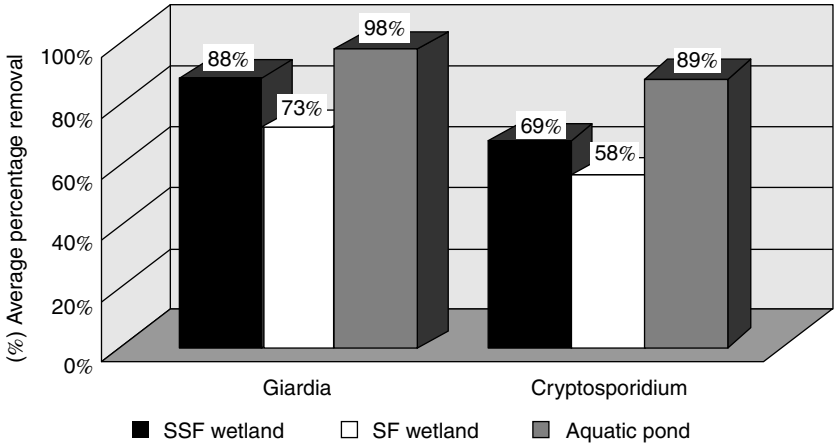

Figure 2. Comparison of average percentage removal of protozoa for an aquatic pond, surface flow (SF), and subsurface flow (SSF) wetland systems.

Table 10. Inactivation of Protozoan Cysts by Chlorination of Wastewater

Parasite	Chlorine Dose	Experimental Conditions	Inactivation Efficiency	References
Giardia	10 mg/L	pH 6.7 T° 22 °C T 3–4 h	82%	(80)
Entamoeba histolytica	12 mg/L	pH 6.7 T° 22 °C T 24 h	84%	(80)
		pH 6.8 T° 22 °C T 48 h	92%	(80)

be supplied in many forms, which include chlorine gas, hypochlorite solutions, and other chlorine compounds in solid or liquid form. It is a complex operation because of the difficulty in maintaining a constant chlorine residual level for effective disinfection. Cysts and oocysts resist quite well even at high chlorine concentrations (Table 10).

Table 11 describes some of the more common wastewater characteristics and their impact on chlorine.

Ozonation. Ozone is a very strong oxidant. The mechanisms of disinfection using ozone include direct oxidation/destruction of the cell wall, reactions with radical by-products of ozone decomposition, and damage to the constituents of the nucleic acids (purine and pyrimidine). Ozone is more effective than chlorine in destroying protozoan cysts, but low dosage may not effectively inactivate cysts (Table 12).

Ultraviolet (UV) Radiation. It is well known that UV radiation damages microbial RNA and DNA. For protozoan cysts, the exact target of UV radiation is not well known. The doses required to inactivate protozoan cysts are much higher than those currently used (Table 13).

UV radiation is a relatively simple method but is highly dependent on wastewater characteristics: turbidity, total suspended solids (TSS), and colloidal matter (83). For instance, UV disinfection with low presence lamps is not as effective for secondary effluent with TSS levels above 30 mg/L (81).

Table 11. Wastewater Characteristics affecting Chlorination Performance (81)

Wastewater Characteristic	Effects on Chlorine Disinfection
Ammonia	Forms chloramines when combined with chlorine
Biochemical oxygen demand (BOD)	The degree of interference depends on their functional groups and chemical structures
Hardness, iron, and nitrate	Minor effect, if any
Nitrite	Reduces effectiveness of chlorine and results in THMs
pH	Affects distribution between hypochlorous acid and hypochlorite ions and among the various chloramine species
Total suspended solids (TSS)	Shielding of embedded microorganisms and chlorine demand

Other Tertiary Treatment Processes. Several other treatment processes are in use or under experimentation, including membrane microfiltration or ultrafiltration. These systems provide excellent barriers against protozoan cysts and oocysts (84,85).

Table 12. Inactivation of Protozoan Cysts by Ozonation of Wastewater

Parasite	Ozone Residual Dose	Contact Time	Inactivation Efficiency	References
Giardia	0.4 mg/L	8–12 min	99%	(82)
Cryptosporidium	15 ppm	10 min	90%	(82)

Table 13. Inactivation of Protozoan Cysts by UV Radiation of Wastewater

Parasite	UV	Inactivation Efficiency	References
Giardia	100–160 mW s cm^2	80%	(82)
Cryptosporidium	100–160 mW s cm^2	80%	(82)

PROTOZOA FOUND IN SLUDGE AND THE IMPACT OF TREATMENTS

Treatment of wastewater leads to the production of large quantities of sludge where protozoa are concentrated. Quantitative data on *Giardia* cysts are given in Table 14.

The quantity of cysts removed is quite variable, and no correlation exists between *Giardia* cyst concentration in raw sludge and the size and/or geographic localization of the treatment station. The impact of sludge treatments on these cysts is difficult to assess.

Mesophilic anaerobic digestion would be moderately, or poorly efficient at cyst removal. Thermophilic aerobic digestion (55 °C) provides 100% inactivation. Storage techniques (>24 weeks) can remove protozoan cysts.

There are very few data concerning *Cryptosporidium* oocysts in sludge. Chauret and coworkers found in mixed sludge 5.3×10^2 oocysts/100 g wet sludge and indicated 0.3 log 10 reduction during the anaerobic sludge digestion (31).

CONCLUSION

Wastewater carries large quantities of protozoan cysts (*Giardia*, *Cryptosporidium*, *E. histolytica*, etc.). The concentrations observed depend on a certain number of parameters including the number of contributors (infected individuals and/or healthy carriers) excreting large quantities of cysts capable of surviving in the environment. Wastewater treatment leads to the production of treated wastewater, which flows into the receiving environment (surface water, sea, and soil), and which must meet sanitary standards.

Table 14. Occurrence of *Giardia* Cysts in Wastewater Sludge

Sludge Type	Initial Cysts Concentration	Treatment	Final Cysts Concentration	Removal	References
Raw sludge	7.7×10^4–3.3×10^6/Kg*	Mesophilic anaerobic digestion	1.0×10^5–4.1×10^6/Kg*	0–86.4%	(86)
Primary sludge	3.1×10^4–8.1×10^4/g*	Mesophilic anaerobic digestion dewatering	4.8×10^3–6.8×10^4/g* 3.8×10^4–1.4×10^5/g* 2.4×10^3–4.9×10^3/g*		(87)
Mixed sludge	1.1×10^3/100 g**	Mesophilic anaerobic digestion dewatering	2.5×10^1–2.8×10^3/100 g**	No reduction	(31)
Anaerobic-digested sludge and dewatered	4×10^3/g* 1.3×10^3–7×10^3/g*	Storage 24 weeks Compost	1×10^3/g* 5×10^2–3×10^3/g*		
Raw sludge	7.4×10^1–1.0×10^3/g**	Chemical thickening anaerobic digestion dewatering	1.50×10^2/g** 3.5×10^1/g**	97%	(34)
Dewatered sludge Anaerobically digested sludge	3×10^2–7×10^3/L**	Anaerobic digestion dewatering	1×10^2–3×10^3/L**		(33)
Primary sludge	4.4×10^3/g*	Thermophilic aerobic digestion	ND		(88)

*Dry Weight
**Wet Weight
ND: Not Detected

Protozoan cysts resist the different stages of conventional wastewater treatment quite well. They can be removed by adsorption to sewage particles and slow sedimentation, two operations that present a certain degree of efficiency in cyst removal.

For disinfection procedures, chemical compounds such as chlorine have little effect at commonly used doses. For other tertiary treatments, certain new techniques using different membranes capable of removing cysts are noteworthy.

Sludge also contains considerable quantities of cysts. If the sludge is reused, these cysts have to be eliminated. It would appear that radiation and/or destructive treatment of the cysts could be achieved by different methods using temperatures above 65 to 70 °C, long-term storage, and so forth.

Sanitary treatment of sludges opens a vast and emerging field of technological development.

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PATHOGEN, BACTERIAL.

See *MYCOBACTERIUM AVIUM* COMPLEX

PATHOGENIC *ESCHERICHIA COLI*

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Escherichia coli is a bacterial species that inhabits the gastrointestinal tract of humans and warm-blooded animals. Because of the ubiquity of this bacterium in the intestinal flora, it serves as an important indicator organism of fecal contamination (1). *Escherichia coli*, aside from serving as a sanitary indicator, has played an important role in the science of microbiology. It has been extensively utilized as a model organism in a variety of physiological and genetic research studies. The recent sequencing of the *E. coli* genome bears witness to the continued importance of this microorganism in scientific research.

Originally referred to as *Bacterium coli*, the initial isolation of this organism from feces was first reported in 1885 and was for many years considered a harmless commensal organism of the gut. It was not until the mid-1940s that it was determined that certain strains of this bacterial species could indeed serve as etiological agents of diarrheal illness (2). This finding was first associated only with illness in children in what was referred to as "summer diarrhea." At a later date, it was found that adults could also become infected. Today it has been well established that although generic strains of *E. coli* are nonpathogenic, many *E. coli* strains are capable of causing gastrointestinal disease. The disease-causing strains in general are very similar to the generic strains; however, some pathogenic strains differ in certain phenotypic traits such as carbohydrate fermentation and growth temperatures. The disease-causing strains have been categorized into pathogenic groups, on the basis of virulence characteristics, clinical manifestations, and epidemiological patterns. Individual serotypes have been found to be associated with specific pathogenic groups. Adhesion and attachment traits, as well as toxin and hemolysin production are the primary virulence factors used for distinguishing individual pathogenic groups. Virulence characteristics may be mediated by various genetic elements, such as plasmids (extra chromosomal genetic material) or bacteriophages (bacterial viruses). Molecular techniques have proven most useful for the detection of many of these traits. The primary pathogenic groups of interest in environmental microbiology are enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), and enteroinvasive *E. coli* (EIEC) (3). Other pathogenic groups such as enteroaggregative *E. coli* (EAEC) also exist but to date have not been definitely linked with transmission via an environmental source. Dissemination for all groups is by the fecal-oral route, with person-to-person or animal to person, as well as food and water being the primary means of transmission. Research studies on survival in aquatic environments have found that, under similar conditions of temperature and other water-quality parameters, pathogenic *E. coli* do not appear to persist longer than generic *E. coli*. The detection of pathogenic *E. coli* in the environment requires specialized techniques not normally employed in routine sanitary analysis; however, the presence of generic *E. coli* in a sample serves as an indication that pathogenic strains of *E. coli* and other enteric pathogens may be present (1).

The adherence to and invasion of the cells of the intestinal mucosa are important characteristics of the pathogenic groups. Adhesion is mediated by structures that project from the bacterial cell and are referred to as fimbrae or pili. Adherence patterns are determined by observation of strains in vitro on tissue culture cells.

Strains may adhere in a uniform manner or they may adhere in clumps or aggregates. Invasive strains are capable of penetrating the mucosal cells, and some strains have the ability to spread to adjacent cells after invasion. Certain strains also have the ability to efface the cells of the intestine. Microvilli, the small fingerlike projections of the intestinal cells, increase in length and eventually are destroyed during the process of effacement.

Toxin production is another major virulence factor used to characterize pathogenic *E. coli*. Not all pathogenic groups possess toxins and some toxins may be present in more than one specific group. Toxins are classified into two major types based on their response to heating. Heat-stable toxins can withstand heating for 30 minutes at 100 °C without losing their activity. Heat-labile toxins lose their activity under these conditions. Some *E. coli* strains produce potent toxins known as a verocytotoxins or shiga-like toxins, which are similar to the toxin produced by *Shigella*, the causative organism of bacterial dysentery.

Serological techniques are used to classify *E. coli* within pathogenic groups in reference to their antigenic structure. The O and H antigens are the primary phenotypic characters used in serotyping *E. coli*. The O antigens, referred to as somatic antigens, are thermostable and are the polysaccharide portion of the lipopolysaccharide complex attached to the bacterial cell membrane. Strains that are motile because of the presence of flagella contain H antigens. These antigens are derived from the flagellar structure.

ENTEROHEMORRHAGIC *E. COLI* (EHEC)

Escherichia coli O157 : H7 is one of the most frequently occurring serotypes in the EHEC group of pathogenic *E. coli*. Other serotypes such as O26 and O111 have also been associated with the EHEC group. *Escherichia coli* O157 : H7 attaches, invades, and effaces the intestinal mucosal cells. Strains of this group produce the toxins referred to as verocytotoxins or Shiga-like toxins. These toxins are mediated by the presence of bacteriophages. Strains may possess both a type I or type II toxin or may produce only one toxin type. The clinical symptoms of infection vary from nonbloody diarrhea to bloody diarrhea, which is generally referred to as hemorrhagic colitis. A life-threatening condition known as *hemolytic uremic syndrome* may develop from these infections and cause renal failure. This condition is often of greater risk in young children. *Escherichia coli* O157 : H7 is one of the serotypes of pathogenic *E. coli* that exhibit variations from the phenotypic characteristics associated with generic strains. Unlike generic *E. coli*, most strains of *E. coli* O157 : H7 do not readily ferment the carbohydrate sorbitol and also do not grow well at the elevated temperature (44.5–45 °C), which is used to detect the fecal indicator group of thermotolerant coliform bacteria. The infective dose for this organism is quite low, thus the ingestion of small numbers of the bacteria may be sufficient to cause disease. Other serotypes of EHEC include O26 : H11, O111 : H8 and O104 : H21. These serotypes may exhibit different virulence characteristics in terms of their attachment-effacement capabilities.

Escherichia coli O157 : H7 is widely known as a food-borne pathogen, but it has become increasingly recognized as the etiological agent in several waterborne outbreaks. Unlike other pathogenic *E. coli* groups, cattle and humans may serve as a reservoir for this organism. Thus, sources of contamination include runoff from cattle operations and human sewage. It has been reported that *E. coli* O157 : H7 can survive both in water (4,5) and manure (6). Several waterborne outbreaks have been reported relating to both drinking water and recreational water exposure (7). EHEC, and generic *E. coli* are sensitive to chlorination, and therefore proper chlorination of drinking water supplies should be sufficient for controlling these organisms (8). Many of the reported drinking water outbreaks have been linked to unchlorinated groundwater supplies. Contamination of wells via runoff and distribution system disturbances have been implicated in these outbreaks. One such outbreak in Cabool, Missouri in 1989 resulted in several deaths (9) as was also the case in a more recent outbreak in 2000 in Walkerton, Ontario. Lakes used for recreational water may also serve as a source of contamination and would not be subject to chlorination. Outbreaks have occurred in chlorinated swimming pools and water parks, where chlorine levels would not be sufficient to counteract an overt fecal accident.

ENTEROPATHOGENIC *E. COLI* (EPEC)

The EPEC was the first group of *E. coli* to be recognized as a cause of diarrheal illness and was initially reported as an etiological agent of infant diarrhea. Like EHEC, this group attaches, effaces, and invades the intestinal mucosa, but unlike EHEC does not produce toxins. Watery diarrhea, fever, and dehydration are the primary symptoms. In infants, these symptoms may be prolonged over an extended period (>14 days). In recent years, there has been a low incidence of EPEC infections in most industrialized nations, but this group remains an important cause of childhood diarrheal illness in developing countries. Although primarily a disease of children, adults can also become infected. The low occurrence rate in adults is thought to be due to the acquisition of immunity at an early age. The serotypes 018, 044, 055, 086, 0119, 0125, 0126, 0127 are several of the serotypes associated with EPEC. Serotype O111 has been implicated with this group (EPEC) and also with the EHEC group.

Person-to-person contact is a major source of contamination for EPEC, but water also serves as a means of transmission especially in areas with poor hygienic standards. A large waterborne outbreak occurred in 1971 at a conference center in Washington, D.C. The pathogen (serotype O111) was isolated both from fecal specimens and the unchlorinated drinking water supply (10). In 1969, an outbreak of gastroenteritis caused by serotype O126 was reported in a logging camp where the inhabitants had ingested sewage-contaminated water (11).

ENTEROTOXIGENIC *E. COLI* (ETEC)

The condition known as travelers' diarrhea is associated with the ETEC group. Travelers' diarrhea is most often seen in individuals traveling from industrialized countries to countries with poorer hygienic standards. Like EPEC, the ETEC group is also an important cause of infant diarrhea in less-developed countries. Adherence to intestinal mucosal cells in a uniform pattern is characteristic for the ETEC group; however, invasion does not occur. Toxin production does occur, and strains may produce a heat-labile toxin, a heat-stable toxin, or both types simultaneously. The heat-labile toxin is very similar to the toxin produced by toxigenic strains of *Vibrio cholerae*, the etiological agent of cholera. Infection often results in cholera-like watery diarrhea symptoms without blood or mucus and is accompanied by fever and vomiting. Serotypes in the ETEC group include O6, O8, O15, O20, O25, O27, O63, O78, O80, O114, O115, O128ac, O148, O153, O159, and O167.

ETEC was responsible for one of the largest waterborne outbreaks of pathogenic *E. coli* in the United States (12). In 1975 more than 2,000 individuals who had visited Crater Lake National Park in southwestern Oregon experienced gastroenteritis. ETEC serotype O6 was isolated from both fecal specimens and the park's water supply. Direct sewage contamination of the water supply coupled with inadequate chlorination procedures were cited as causative factors in this outbreak.

ENTEROINVASIVE *E. COLI* (EIEC)

EIEC cause an inflammatory disease of the gastrointestinal tract. These bacteria invade the intestinal mucosa, multiply within the epithelial cells, and may spread laterally to adjacent cells. While the disease process closely resembles that seen with *Shigella*, there have been no reports of toxin production from EIEC strains. Typical serotypes of EIEC include O28ac, O29, O124, O136, O143, O144, O152, O164, and O167. Endemic infections of EIEC appear to be limited to developing countries; however, outbreaks have been reported in industrialized nations. Strains of EIEC differ from generic *E. coli* in their inability to readily ferment the carbohydrate lactose.

Person-to-person spread appears to be the predominant mode of transmission. A waterborne outbreak of serotype O124 has been reported in a children's camp in Hungary. Drinking water contaminated from a leaking sewer was implicated and the organism was isolated from fecal specimens and a reservoir supplied by a spring water source (13).

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PATHOGENS, BACTERIAL.

See CHOLERA; LEPTOSPIROSIS; LYME BORRELIOSIS; *SALMONELLA* IN AQUATIC ENVIRONMENTS; *SHIGELLA*

PATHOGENS (BACTERIAL) IN WASTE STABILIZATION PONDS.

See WASTEWATER STABILIZATION PONDS

PATHOGENS IN ENVIRONMENTAL BIOFILMS

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The attachment of prokaryotic microorganisms to air–liquid, liquid–liquid, and solid–liquid interfaces in the aquatic environment (1), and their subsequent maturation into heterogeneous but highly ordered communities of biofilms predated by eukaryotic microorganisms, has represented an important step in the evolution of life on earth (2). Biofilms are ubiquitous in both the natural and built environments and exist despite, and frequently in response to, extremes of temperature, pH, redox potential, nutrients, and toxic molecules such as heavy metals, industrial organic pollutants, and biocides (3,4). Unlike the more commonly encountered monospecies cultures in vivo, environmental biofilms are usually comprised of complex consortia of microorganisms and can contain a high species diversity of aerobic and anaerobic bacteria, archaea, fungi, amoebae, protozoa, and nematodes (5). Of concern to modern society is the threat that microorganisms pathogenic to animals and/or man can survive and even flourish in such ecosystems. This is related to the sharp rise in the world population, itself causing bacterial, protozoal, and viral pathogen dissemination and amplification through person to person infective transmission, and the requirement of intensive agricultural practices to provide sufficient food, exacerbating propagation and transmission of zoonotic pathogens. The need

Table 1. Man and the Water Cycle

Location	Cause	Pollution
Land	Landfill leakage Sewage sludge disposal	Heavy metals, organics
River/ borehole	Cattle feces/manure Chicken faeces/manure Wild animals, beavers	*C. parvum, E. coli* 0157 *C. jejuni, Salmonella* spp. *G. lamblia, Leptospira* spp.
Lakes	Detergent (N, P) eutrophication	Cyanobacterial blooms/toxins
Distribution supplies	Ingress; poor treatment	Coliforms, *Ps. aeruginosa*; *Acinetobacter*, *Aeromonas* spp.
Buildings/cooling towers	Contaminated supplies	*Legionella*, *Mycobacterium* spp., amoebae
	Corrosion	Metal salts
Sea	Poor wastewater treatment	Coliforms, *Vibrio cholerae*, viruses

Source: From Keevil (2)

to dispose of increasing quantities of waste and wastewater from homes, industries, and intensive animal rearing facilities has strained the resources of farmers and many sewage treatment utilities worldwide. Poor sewage and animal waste disposal, and intensive stocking of animals in fields near abstraction points built for supplying potable water, has increased the transmission of microbial pathogens back into the natural environment and in water supplying agricultural, industrial, and domestic premises (Table 1). Consequently, the normal water cycle has been perturbed by the introduction of toxic organic compounds and heavy metals from disposal of waste to land and leaching into watercourses. Eutrophication of rivers and lakes has occurred owing to ingress of nitrogen and phosphorus resulting from excessive use of detergents, promoting biofilm formation, and production of toxic by-products from cyanobacterial biofilm blooms, such as microcystin hepatotoxins and anatoxin neurotoxins (6). According to World Health Organization statistics (7), developing countries no longer have a monopoly of waterborne diseases. Some 20 million people die each year from waterborne diseases, over one million of them in Europe. In addition to "traditional" microorganisms, new pathogens have emerged and are currently major concerns for industrialized countries. Their prevalence and relationship to persistence in aquatic biofilms will be featured in this article.

The complex physical and physiological interactions of the biofilm consortia, and survival of pathogens therein, has made their study difficult and several model systems have been advocated to reproducibly generate high species diversity biofilms in defined environments. Advances in microscopy, immunology, and molecular biology have also allowed the development of various light and epifluorescence (EF) techniques, utilizing antibody and molecular probes, to study biofilm physicochemistry and track individual species in situ. This article will focus, in particular, on the use of episcopic differential interference contrast (EDIC) and EF microscopies (8,9) to observe biofilms generated in a continuous culture ecological system in the laboratory to model biofilms in the environment. The development and use of chemostat models to study environmental biofilms and colonization by pathogens has been reviewed elsewhere (10) and, therefore, will only briefly be considered here. A primary seed vessel ensures the reproducible maintenance of complex microbial consortia and supplies subsequent growth vessels, each also receiving their own nutrient feed, in an open flow system for biofilm experimentation in defined environments. The chemostat vessels are constructed of nonferrous materials, typically titanium top plates with glass bodies and Teflon stirrers, to avoid leaching of iron, chromium, and manganese from the more conventional stainless steel fabrications which would perturb the chemistry of the nutrient supply (Fig. 1). Tiles or coupons of known physicochemistry can be inserted and removed aseptically after hours or months for microbiological and microscopic image-analysis. In contrast to field studies, the model is cheap, reproducible, and easily manipulated. It can be used to model natural water systems and potable water mains distribution and plumbing systems in buildings, and the effects

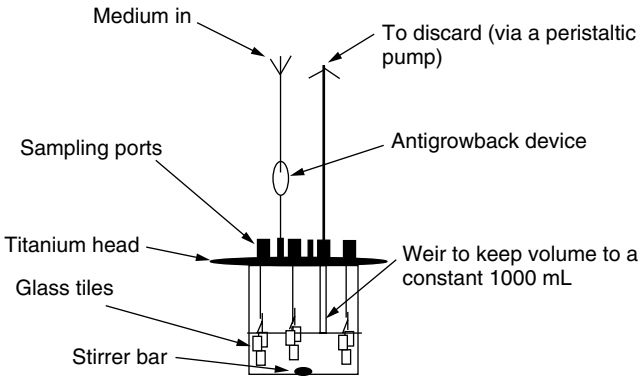

Figure 1. Diagram of the second stage model biofilm system with multiple assemblages of coupons suspended from rigid titanium wire inserted through silicone rubber bungs in the top ports. The weir system was used to maintain the volume at the required level. Temperature, oxygen and pH probes are not shown. *Source:* From Surman and coworkers (25).

of different growth rates, temperatures, environmental chemistry, and disinfectant concentration. The shear rate imposed on surfaces by water velocity can be determined by manipulation of the stirrer speed: typical water velocities of 0.2 to 3.0 m second^{-1} have been investigated. Importantly, the model has few working parts and can be assembled in Class III containment cabinets for the study of more infectious pathogens.

To date, this model and others have shown that biofilms consisting of aerobic heterotrophs and autotrophs provide an environmental reservoir or safe haven for the fecal indicator bacteria *Escherichia coli* (including verocytotoxigenic *E. coli* 0157), *Klebsiella oxytoca*, and *Klebsiella pneumoniae*, the opportunistic pathogens *Aeromonas hydrophila* and *Pseudomonas aeruginosa*, microaerophilic bacteria including *Legionella pneumophila*, *Campylobacter jejuni*, *Helicobacter pylori*, and *Mycobacterium avium*, pathogenic acanthamoebae and *Cryptosporidium parvum*, and possibly viruses such as Poliovirus-1.

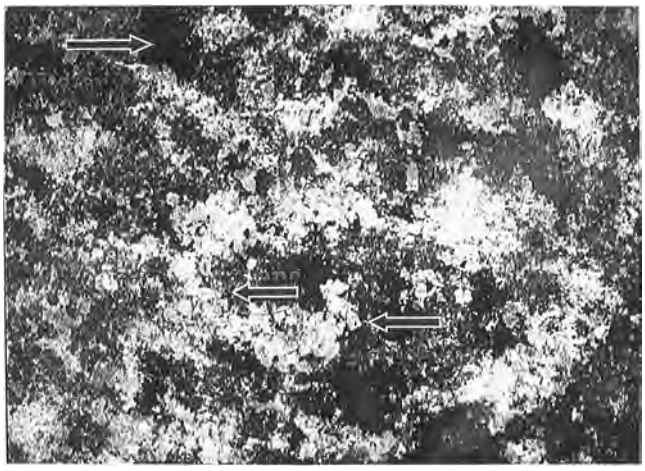

Figure 2. Heterogeneous biofilm showing stacks of microcolonies rising from the substratum and water channels. *Source:* From Keevil and coworkers (13). See color insert.

BIOFILM STRUCTURE

Biofilm formation comprises a dynamic flux of processes involving attachment, colonization and maturation, active detachment or passive sloughing, and predator grazing (1,3). A pseudo-steady state is reached for biofilms in potable and natural waters in which the numbers of recoverable, viable bacteria varies between 10^5 and 10^7 per cm^2, dependent on the nutrient availability, shear force, and physicochemistry of the substratum (5). By contrast, the numbers of viable bacteria recovered from the planktonic phase are typically only 10 to 10^3 per milliliter. Thus, considering the many kilometers of distribution main and plumbing pipe used to supply potable water from the treatment works to the tap, the biofilm represents a much more significant environmental reservoir of microorganisms than the water phase.

The chemostat biofilm model has facilitated important breakthroughs in our knowledge of biofilm structure, function, and ecology. The majority of the so-called *biofilms* are actually *extremely heterogeneous but highly ordered* in structure with many water channels penetrating to the substratum; these permit partial, convective ingress of nutrients and, presumably, antibiotics or disinfectants (11,12). The "biofilm" consists of a patchy basal layer, 5- to 10-μm thick, covered with stacks or fronds of microcolonies rising typically 100 to 200 μm above the substratum surface (Figs. 2 and 3). Variations in cell morphology and color within the microcolonies of the stacks suggest close physiological associations of consorting species (13). There are inevitably many water channels but the apparent spaces between the stacks is dependent on the physicochemistry of the substratum and its conditioning pellicle layer, the availability of nutrients, and the activity of grazing predators. Indeed, we have demonstrated that motile bacteria, protozoa, and nematodes can be seen passing through the channels. The convective flow of microscopic fluorescent beads through the channels has also been described (14,15). In nutrient-rich environments the biofilm may thicken and consolidate, with the water channels narrowing to

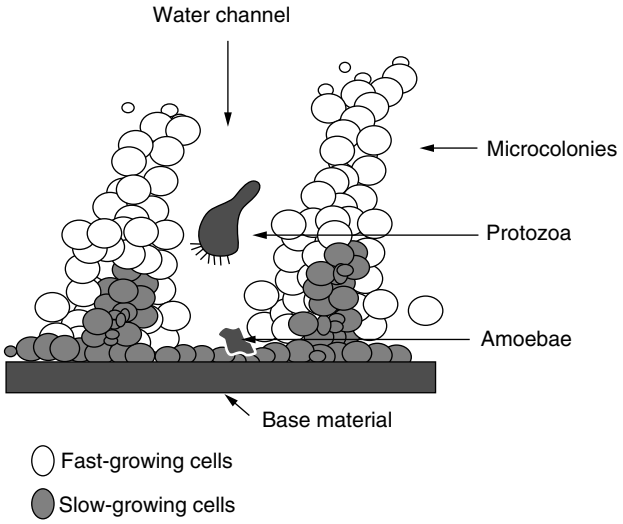

Figure 3. Open architecture structure of biofilm with fronds, water channels, and grazing eukaryotic predators. *Source:* From Keevil (157).

resemble a sponge. The structure can also appear confluent owing to the production of copious exopolysaccharide (EPS) gel within the channels. Any observation technique that requires even partial dehydration will result in shrinkage phenomena, generating artifacts. The structures described are still *largely microscopic* in scale. In the presence of sunlight, however, photosynthetic species can proliferate, producing secondary metabolites, EPS, and intracellular storage polymers to subsequently enrich the other members of the adherent consortium. This photosynthetic stimulation of biofilm metabolism can result in the proliferation of green macroblooms, obvious to the naked eye (13).

The surface of the so-called *biofilm* may scavenge reactive biocides such as chlorine, acting sacrificially to protect autochthonous and pathogen cells within. However, diffusion may not be rate limiting owing to

accessibility through the water channels that penetrate to the substratum. This penetrability might explain how less-reactive monochloramine appears to be a better residual disinfectant than chlorine to control biofilm microorganisms (16,17).

AUTOCHTHONOUS BIOFILM MICROFLORA

A diverse range of microorganisms are found in the planktonic phase of environmental waters and frequently also in sessile biofilm communities. These include autotrophic and heterotrophic gram-positive and gram-negative bacteria, yeasts, fungi, amoebae, ciliates, and rotifers (Table 2). Potable waters are treated to European Union and World Health Organization standards to prevent the spread of pathogens and indicator bacteria; however, this water is not intended to be sterile and, consequently, also contains a diverse range of microorganisms (18). Biofilms exist in all water distribution and plumbing systems at temperatures below 60 °C (19,20).

Very little is known about the presence of viruses in natural or potable waters, owing to lack of study, but it is presumed that bacteriophages are commonly present. It was widely believed that bacteriophages cannot infect surface-attached bacteria (biofilms) because such bacteria are protected by an exopolymeric matrix that binds macromolecules and prevents their diffusion into the biofilm. However, Doolittle and coworkers (21) were able to show that bacteriophage T4D+ can infect and multiply within *E. coli* cells growing as a biofilm. Subsequently, Hughes and coworkers (22) found that bacteriophages for three representative strains of gram-negative biofilm bacteria were of widespread occurrence. Lytic bacteriophage was isolated from local sewage for the bacterium 1.15, an EPS-producing pseudomonad found originally as a component of biofilms in a local river, and for two *Enterobacter agglomerans* strains from industrial biofilms. These bacteriophages are similar to other viruses for EPS-producing bacteria in inducing the synthesis of enzymes degrading the polymers that occlude the bacterial cell surface. The soluble phage enzymes each degrade their substrate by acting as endo-glycanohydrolases, and may serve as a detachment mechanism for nearby cells in biofilms. McLean and coworkers (23) have recently begun studies of bacterial biofilm–phage interactions. These authors anticipate that some phage are better adapted to growth in biofilms, some are adept to growing in mixed culture biofilms, and others are better adapted to infecting planktonic organisms. Whereas biofilms are now widely accepted as a fundamental aspect of microbial growth in nature, the field of phage ecology is quite new and an exciting challenge for the future.

Table 2. Microbiology of Water and Biofilms

Heterotrophic Organisms/ Genera	Other Organisms
Acinetobacter	*Nitrosomonas*, *Nitrobacter*
Aeromonas	Iron oxidizing bacteria
Alcaligenes	Sulfate reducing bacteria
Flavobacterium	
Methylobacterium	
Pseudomonas	Yeasts, *Actinomycetes*
Legionella	*Chladysporium, Aspergillus*
Enterobacter, *Klebsiella*, *Proteus*, etc.	
Micrococcus	*Acanthamoeba*, *Hartmanella*
Staphylococcus	*Paramecium*, *Tetrahymena*
Bacillus	*Lachrymaria*, *Vorticella*
Corynebacterium	

Source: From Keevil (2).

The significance of actinomycetes and filamentous fungi in biofilms is currently being investigated. Previous work found *Aspergillus* spp. in biofilms of large institutional buildings in Scotland (20). Wuertz and coworkers (24) investigated zinc uptake by activated sludge biofilm in a rotating biofilm annular reactor, which consisted of an external cylinder with removable slides and an internal solid drum. This work revealed a gradual change in zinc concentration associated with EPS, although the total zinc concentration in the biomass remained constant. Concurrently, the amount of extractable EPS decreased. This was a consequence of a microbial population shift, with bacterial counts decreasing and algal and fungal biomass increasing. This showed the adaptive response of fungal enrichment in biofilms for important processes such as heavy metal sorption: this may be important, for example, in biofilms on galvanized steel pipework. Importantly for public health, Surman and coworkers (25) reported the presence of *A. flavus* and *Acremonium strictum* in potable water biofilms generated in the two-stage chemostat model at 30 °C. These findings for the presence of fungi such as *A. flavus* in biofilms are disconcerting because Kelly and coworkers (2) not only found this species but also its associated carcinogenic toxic secondary metabolite, aflatoxin B2, at a concentration of 0.2 to 1.6 $\mu g\,L^{-1}$ from a cold-water storage tank. Therefore, we should not concentrate solely on the presence of bacteria in biofilms but be more aware of the potential for toxicants to be produced by autochthonous biofilms.

The predatory eukaryotes have evolved to graze environments in which there is a plentiful supply of food, that is, bacterial, yeast, and algal biofilm communities on marine and freshwater surfaces. This can be seen even using scanning electron microscopy in which amoebae sit on the substratum and sweep over and engulf the biofilm present (Figs. 3 and 4). Video microscopy has demonstrated how they swim or glide through the water channels in heterogeneous biofilm to find appropriate stacks or fronds of biofilm microcolonies on which to feed (Fig. 3). This feeding can be highly specific, perhaps involving chemotactic mechanisms sensing secondary metabolites excreted by desirable prey in the mosaic of microenvironments of the biofilm. Of importance, some of the grazing amoebae are opportunistic pathogens of man. Free-living amoebae cause three well-defined disease entities: a rapidly fatal primary meningoencephalitis, a chronic granulomatous amoebic encephalitis, and a chronic amoebic keratitis. For example, *Acanthamoeba* and *Hartmanella* spp. can cause keratitis of the cornea

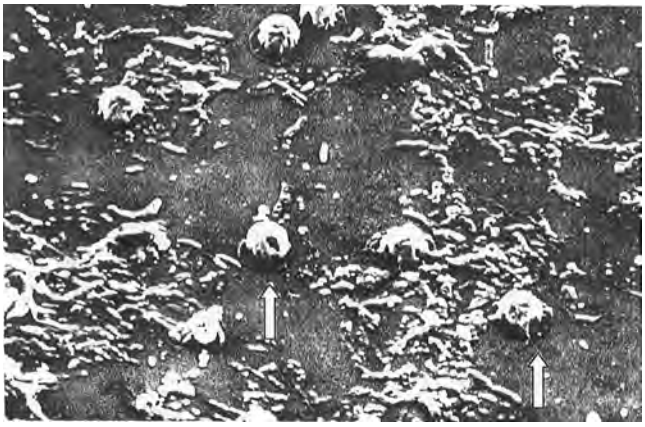

Figure 4. Scanning electron micrograph showing grazing of biofilm by *Acanthamoeba* sp. *Source:* From Rogers and coworkers (34). See color insert.

and subsequent blindness (26), whereas *Naegleria* spp. can invade the olfactory neuroepithelium and consequently migrate through olfactory lobes into the cerebrum and cause primary amoebic meningoencephalitis which is invariably fatal (27). The role of biofilm amoebae as carriers of bacterial pathogens will be discussed later.

AUTOCHTHONOUS BIOFILM PATHOGENS

Several studies have been undertaken to examine quantitatively the risks to human health posed by heterotrophic plate count (HPC) bacteria found naturally in ambient and potable waters (28–30). There was no convincing evidence that the HPC bacteria as a whole pose a public health risk. Only certain members are opportunistic pathogens. Using the four-tiered approach for risk assessment from the National Academy of Sciences, hazard identification, dose–response modeling, and exposure through ingestion of drinking water were evaluated to develop a risk characterization, which estimates the probability of infection for individuals consuming various levels of specific HPC bacteria (30). HPC bacteria in drinking water often include isolates from the genera *Pseudomonas*, *Acinetobacter*, *Moraxella*, *Aeromonas*, and *Xanthomonas*. Other bacteria that are commonly found are *Legionella* and *Mycobacterium* spp. All of these genera contain species that are opportunistic pathogens and may cause serious diseases. For example, the three nonfermentative gram-negative rods most frequently isolated in the clinical laboratory are (*1*) *P. aeruginosa*, (*2*) *Acinetobacter* spp., and (*3*) *Xanthamonas maltophilia*. Some of these species will now be reviewed further.

Pseudomonads

Pseudomonas aeruginosa is a major cause of hospital-acquired infections with a high mortality rate. It is also associated with otitis externa or folliculitis in people who have used swimming pools and spa pools (31), suggesting a possible link with aquatic biofilms. In addition, and of particular importance, *P. aeruginosa* biofilms occur in the lungs of chronically infected cystic fibrosis patients, in which they protect the bacteria against antibiotics and the immune response. The lung tissue damage is because of immune complex mediated chronic inflammation dominated by polymorphonuclear leukocytes releasing proteases and oxygen radicals (32). Accordingly, the majority of biofilm studies with this opportunistic pathogen have concentrated on its significant role in cystic fibrosis. Laboratory studies have modeled single strain cultures forming biofilm in rich nutrient media to ascertain the role of exopolymeric alginate formation, other attachment factors, expression of virulence determinants such as elastase and iron sequestering siderophores, and the influence of quorum-sensing *N*-acyl homoserine lactones (AHLs) on physiology and pathogenicity (discussed later). Little has been studied concerning its niche in the natural environment. One study showed that *P. aeruginosa* could form a biofilm in a PVC pipe containing sterile water and that viable bacteria could still be recovered after 7 days of exposure to 10 to 15 ppm chlorine (33). In another study, Rogers and coworkers (34,35) grew an aquatic biofilm consortium in drinking water and followed the initial attachment of pioneer organisms and the subsequent maturation of biofilm consortia on various metal and plastic substrata. Of note, *P. aeruginosa* was present in very low numbers in the original inoculum but was enriched in immature biofilms developing on polypropylene and polyethylene surfaces after one day (Table 3), and in more mature biofilms developing on stainless steel after 21 days (Table 4).

This study also showed that a range of HPC microorganisms could be isolated and cultured from the biofilms, including pseudomonads such as *P. acidovorans*, *P. diminuta*, *P. fluorescens*, *P. maltophila*, *P. mendocina*, *P. stutzeri*, *P. testosteroni*, *P. vesicularis*, *P. xylosoxidans*, and the related *Sphingomonas paucimobilis*.

Until recently, *Burkholderia cepacia* was classified as *Pseudomonas cepacia* because of phenotypic and morphological similarities to the *Pseudomonaceae*. It also is an opportunistic pathogen, similar to *P. aeruginosa*, and can cause biofilm-like infections in cystic fibrosis patients and in catheterized patients (36). It is commonly isolated from soil but is little studied in the aquatic environment. *Burkholderia cepacia* can be isolated from drinking water (37) and a survey of 85 public and private buildings in the province of Bologna (Italy) recovered it in low numbers from 3.5% (mean value = 1 cfu/100 mL) of the samples (38). Interestingly, *B. pseudomallei*, another opportunistic pathogen of man, which causes the fatal melioidosis in endemic areas of Southeast Asia and northern Australia (39), was recovered in high numbers (mean value = 578 cfu/100 mL) in about 7% of samples. *Burkholderia cepacia* has been isolated from the biofilm slime produced in paper mills, running typically at a temperature of 45 to 50 °C and at pH 4.5 to 5 (40), and in a subsequent study it was shown to form slime-producing biofilms on glass and stainless steel coupons (41). When inoculated into sterile water in a PVC pipe, it quickly formed a biofilm and viable bacteria could still be recovered after seven days of exposure to 10 to 15 ppm chlorine. These data suggest that the opportunistic *Burkholderia* spp. may therefore pose a waterborne risk to humans, possibly associated with the biofilm reservoir.

Table 3. Colonization of Plumbing Materials After 24 hours at 30 °C

	Steel	SS	Latex	E–P	PP	PE	uPVC	cPVC
L. pneumophila	4	0.1	15	9	1.5	0.5	2.9	1.5
P. aeruginosa					1.9	260		
P. acidovorans							37	880
P. diminuta				1,000	22			
P. fluorescens					26	380	1	
P. maltophila	80	10	8,000	100		20	3	
P. mendocina	60	25	4,000	3	3		4.7	210
P. stutzeri	90	1						
P. testosteroni	410	12	9,000		1	80	2	150
P. vesicularis						6.2	59	
P. xylosoxidans		36,000					310	
S. paucimobilis	100	10		1,000		0.2	1.2	300
Actinomycetes spp.	10	6	34,000		1		19	200
Aeromonas spp.			8,000					
Alcaligenes spp.	70			2,000	4	0.2		
Flavobacterium spp.	40	7	800	1,000	3	0.3	0.2	290
Methylobacterium spp.	110	4			3		5	
Klebsiella spp.	130							
Acinetobacter spp.	300	26	40,000	10,000	17	440	0.6	

Note: SS, stainless steel substratum; E–P, ethylene–propylene copolymer; PP, polypropylene; PE, polyethylene; uPVC, unpolymerized polyvinyl chloride; cPVC, chlorinated polyvinyl chloride.
Source: Data from Rogers and coworkers (34).

Table 4. Colonization of Plumbing Materials After 21 days at 30 °C

	Steel	SS	Latex	E–P	PP	PE	uPVC	cPVC
L. pneumophila	17	13	150	500	37	13	11	7.9
P. aeruginosa	30							
P. acidovorans						40		11
P. diminuta						2		
P. fluorescens				1,000				3
P. maltophila	10	11	3,000			10	10	
P. mendocina			13			40	0.01	
P. stutzeri	140	70	2,000					0.1
P. testosteroni		180				20		8
P. vesicularis	250							3
P. xylosoxidans						7	40	
S. paucimobilis	30	36	5,000	1,600	790	170	140	362
Actinomycetes spp.	130	2	7,000	8,000		9	0.01	2.8
Aeromonas spp.		6,000						
Alcaligenes spp.	10	10			320	80		30
Flavobacterium spp.	41		15,000	2,400		0.2	90	50
Methylobacterium spp.	20	150			140	30	60	60
Klebsiella spp.			31,000					
Acinetobacter spp.	70	39	22,000	3,100	400	180	40	60

Note: SS, stainless steel substratum; E–P, ethylene–propylene copolymer; PP, polypropylene; PE, polyethylene; uPVC, unpolymerized polyvinyl chloride; cPVC, chlorinated polyvinyl chloride.
Source: Data from Rogers and coworkers (34).

Legionellae

Legionella pneumophila was first recognized, and named accordingly, following an outbreak of a mysterious and deadly pneumonia that affected attendees of an American Legion convention in Philadelphia in 1976 (42,43). The source of the outbreak was *Legionella* bacteria in aerosolized water droplets originating from an air-conditioning system. Legionellosis is actually a group of respiratory diseases caused by legionellae, comprising: Legionnaires disease, an acute fulminating pneumonia with a low attack rate but causing approximately 12% fatalities; Pontiac fever, a mild, nonpneumonic fever with a high attack rate which was first recognized to have occurred at the County Health Department, Pontiac, in 1968 (44); and Lochgoilhead fever, a mild nonpneumonic,

atypical infection causing breathlessness and a long fever which was caused by *L. micdadei* at the Lochgoilhead leisure complex in Scotland in 1987 (45).

The *Legionella* genus includes 43 valid species, about half of which are linked to human illness. *Legionella pneumophila*, the principal etiological agent of Legionnaires' disease, causes 4 to 20% of cases of community-acquired pneumonia and has been ranked as the second or third most frequent cause of pneumonia requiring hospitalization. However, because of difficulties isolating this bacterium from infected individuals and of treatments that eradicate it before its presence can be verified, this figure may underestimate the pathogen's prevalence. The *L. pneumophila* species includes 16 serogroups, all of which are associated with disease, but serogroup 1 appears to be the most important, causing 50% of all *L. pneumophila* infections. *Legionella pneumophila* is a facultative intracellular parasite of both protozoa and human alveolar macrophages and is ubiquitous in aquatic environments. It typically infects man following inhalation of aerosols generated by devices such as air-conditioning systems, cooling towers, spas, showerheads, and grocery mist machines (46). *Legionella* does not transmit laterally in the human population, and is therefore considered to be an accidental or opportunistic pathogen of man and may be an occasional pathogen of other mammals, such as calves. Thus, legionellae may not have caused appreciable disease until after modern society invented such devices to provide the means for transmitting this natural parasite of amoebae into humans.

In terms of its ecophysiology, *L. pneumophila* is found in natural, industrial, domestic water systems and can survive high temperatures and low pH. It can be isolated from natural waters between 5 and 63 °C and multiplies between 20 and 45 °C (47). It is incapable of growth in sterile water and obtains its nutrient source from sludge and rust deposits, algae, amoebae, and bacteria in biofilms (9,48–50). Without question, legionellae are biofilm species. The engineering practices that have encouraged its dissemination include poor design and operation of calorifiers and water supplies with long runs and dead legs, inappropriate use of disinfectants such as chlorine, poor control of aerosol generation by cooling towers, showers, taps, recirculating spas, and humidifiers, and inappropriate selection of construction materials. The involvement of many of these parameters in proliferation of legionellae becomes clear from experiments using the chemostat biofilm model. It was demonstrated that biofilms of high species diversity could be generated reproducibly for many months on a range of plastic and metal materials immersed in medium hard potable water at 30 °C (Tables 3–5) (34,35). *Legionella pneumophila* was able to colonize these materials which are commonly used in cold or warm potable water supplies, including mild steel, stainless steel, polypropylene, polyethylene, unpolymerized polyvinyl chloride (uPVC), chlorinated polyvinyl chloride (cPVC), and the jointing compounds, latex and ethylene–propylene copolymer. The use of in situ immunogold labeling and EDIC microscopy clearly identified the presence and location of the legionellae in the biofilm (Fig. 5; 9). It is noticeable that they grow as distinct microcolonies within specific zones of the heterogeneous biofilm, the stacks or fonds, in which there are large numbers of respiring heterotrophs present. There is no evidence of amoebae having been present, again supporting several lines of data that indicate that legionellae can grow freely in biofilms. In the image shown, the biofilm was grown in a hard potable water with a high calcium carbonate content. The biofilm physicochemistry catalyzed the deposition of scale within the biofilm, thereby affecting its structure and probably resistance to heat and disinfection.

This colonization of a range of plumbing materials may be partly related to the fact that plastic and rubber materials can actively encourage the growth of microorganisms (51,52). Such materials can contain alkyl phthalates as plasticizers, butylated hydroxytoluene as antioxidant, stearates as lubricants, and thioethers as heat stabilizers. Indeed, *L. pneumophila* was also able to colonize the biofilm formed on bitumen-painted iron pipe used for distribution supplies at 25 °C, which may have provided a useful supply of organic nutrients

Table 5. Comparison of Plumbing Materials to Support Biofilm Formation and Colonization of *Legionella pneumophila* in Medium Hard Water at 30 °C

	Maximal Colonization[a]		Colonization Ratio	
Materials	Non-legionellae	*L. pneumophila*	Non-legionellae	*L. pneumophila*
Copper (aged)	70	0.7	1	1
Glass	150	1.5 (1%)	2.1	2.1
Polybutylene	180	2.0	2.6	2.9
Stainless steel	210	10 (5%)	3.0	14.3
Polyethylene	960	23	13.7	33
uPVC	1,070	11	15.3	15.7
cPVC	1,700	78.5 (5%)	24.3	112.1
Steel	4,900	450 (9%)	70	642
Ethylene–propylene	27,000	500 (2%)	386	714
Latex	89,000	550	1,271	785

[a]Colonization units are 10^3 cfu cm^{-2}. The colonization ratio is the cfu of the total microbial flora or legionellae recovered from each material referred to the copper data.

Note: Figures in parentheses represent the percentage of the total culturable microflora identified as *L. pneumophila*.

Source: Data compiled from Rogers and coworkers (34,35).

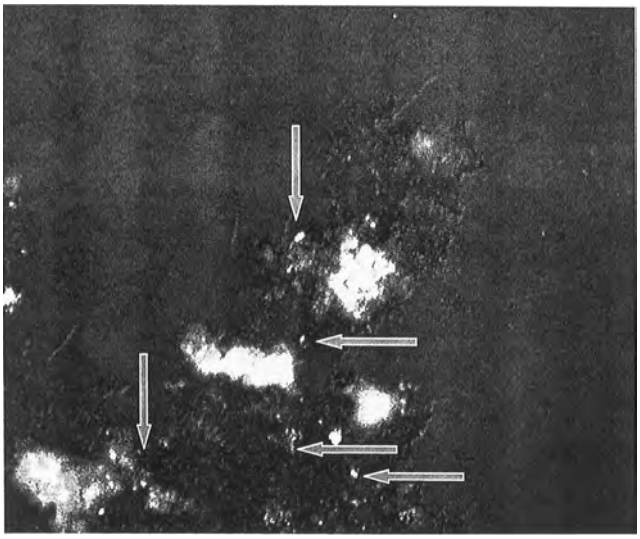

Figure 5. Episcopic differential interference contrast image of immunogold-labeled *L. pneumophila* in an autochthonous biofilm stack developed in hard potable water. The arrows mark microcolonies of cells. Large areas of lighter colored material are deposited calcium carbonate scale. *Source:* From Rogers and Keevil (9). See color insert.

Table 6. Colonization of Plumbing Materials by Aquatic Flora and *Legionella pneumophila*

Temperature	Material	Microflora	*L. pneumophila*
20 °C	Copper	2.2×10^5	0
	Polybutylene	5.7×10^5	665
	PVC	1.8×10^5	2,130 (2%)
40 °C	Copper	8.1×10^4	2,000 (2%)
	Polybutylene	1.2×10^6	112,000 (10%)
	cPVC	3.7×10^5	68,000 (18%)
50 °C	Copper	2.3×10^4	0
	Polybutylene	3.2×10^6	890
	cPVC	1.2×10^5	60
60 °C	Copper	4.5×10^2	0
	Polybutylene	4.3×10^4	0
	cPVC	5.2×10^3	0

Note: Figures in parentheses represent the percentage of the total culturable microflora identified as *L. pneumophila*.
Source: From Rogers and coworkers (35).

either directly to the legionellae or indirectly to the biofilm consortium which then produced secondary metabolites (2,53). Mild and stainless steels probably encourage proliferation of *L. pneumophila* in aquatic biofilms, in which its proportion of the total culturable flora increased from typically 1 to 9%, because the pathogen has a high requirement for iron (54,55). Fortunately, however, *L. pneumophila* is sensitive to copper and can only colonize biofilms on aged copper surfaces in low numbers (Table 6) (34,35). Although the total heterotrophic biofilm microflora on copper decreased by 70-fold compared to mild steel, the legionellae decreased over 600-fold. This effect of copper was also noticeable at different growth temperatures. Thus, there were no legionellae detected in biofilms on copper at 20 °C, whereas significant numbers were present on polybutylene and cPVC. Maximum colonization occurred at 40 °C when low numbers of legionellae were detected in biofilms on copper but very high numbers occurred on the two plastic materials. The proportion of the total culturable flora recovered as legionellae increased from less than 2% on copper to 18% on cPVC, a significant enrichment. Nevertheless, higher numbers of legionellae were recovered in biofilm on polybutylene because this material also supported threefold more biofilm than cPVC. Some codes of practice recommend that hot-water systems be maintained at temperatures greater than 50 °C to suppress survival of legionellae. This practice would seem to be acceptable where copper materials have been installed but significant numbers of legionellae were still recovered from biofilms on polybutylene and, to a lesser extent, cPVC. Consequently, it would seem more appropriate to operate hot-water systems at higher temperatures. In support of this conclusion, no legionellae were detectable in the scant biofilms grown on any of the three materials at 60 °C. These laboratory studies were subsequently supported by direct observations of plumbing pipework supplying water in German hospitals: only 2% of the copper surfaces recovered were found to contain any *L. pneumophila*, whereas 65 and 90% of polyethylene and iron surfaces, respectively, were positive for the pathogen (56). This again confirms the association of legionellae with iron surfaces and the inhibitory effects of copper surfaces, which would appear to be of benefit to the public health. The use of copper would be especially beneficial in the hospital environment in which many of the patients are immunocompromised and therefore more susceptible to infection by opportunistic pathogens.

Mycobacteria

Nosocomial outbreaks and pseudo-outbreaks caused by the nontuberculous mycobacteria (NTM), such as the *M. avium* complex, have been recognized for more than 20 years and continue to be a problem (57). The number of cases of pulmonary disease associated with *M. avium* is rapidly increasing and is approaching the incidence of *M. tuberculosis* in some areas. *Mycobacterium avium* is an environmental microorganism that is adapted to live both in the environment (mainly in water and soil) and in birds, fish, and mammal hosts. In humans, *M. avium* infection is seen in immunosuppressed patients, such as those with chronic lung disease and acquired immunodeficiency syndrome. For the latter, *M. avium* infection is a major cause of death (58,59). More recently, other populations were shown to be at risk of developing *M. avium* disease. For the majority of time, humans acquire *M. avium* through the intestinal tract in which the bacterium comes in contact with and translocates across the intestinal mucosa (60). However, some mycobacteria are easily aerosolized from water (61) and inhalation of aerosolized particles is also an important cause of pulmonary disease (62). Recently, five healthy people developed respiratory illnesses characterized by bronchitis, fever, and "flulike" symptoms after using a hot tub with water containing *M. avium* (63). The symptoms

and the results of investigations were more suggestive of a hypersensitivity pneumonitis than of an infection.

The reservoir for *M. avium* outbreaks is generally municipal and (often separate) hospital water supplies. For example, NTM were recovered from 12 (92%) of 13 reservoirs, 45 (82%) of 55 homes, 31 (100%) of 31 commercial buildings, and 15 (100%) of 15 hospitals in Los Angeles (64). The mycobacterial species involved are very hardy, able to grow in municipal and distilled water, thrive at temperatures of 45 °C or above, and resist the activity of organomercurials, chlorine, 2% concentrations of formaldehyde, and alkaline glutaraldehyde, and other commonly used disinfectants (57). The high number of *M. avium* isolates recovered from hospital water and their close relationship with clinical isolates suggests the potential threat of nosocomial spread. Indeed, it was concluded that potable water is an important source for the acquisition of *M. avium* infections (64). This has since been supported by research which showed that environmental and patient isolates of *M. avium* were resistant to chlorine, monochloramine, chlorine dioxide, and ozone in potable water (65). For chlorine, the product of the disinfectant concentration (in parts per million) and the time (in minutes) to 99.9% inactivation for five *M. avium* strains ranged from 51 to 204. Indeed, water-grown cells were 10-fold more resistant than nutrient-medium-grown cells.

Mycobacterium avium subspecies *paratuberculosis* (Map) is the causative organism of Johne's disease, a chronic enteritis of cattle that can also affect many animals, including rabbits, mice, rats, deer, antelope, mountain goats, camels, alpaca, and bison (66). Map has also been suggested as an etiological agent of Crohn's disease, a chronic inflammatory disease of the gastrointestinal tract of humans. Concern is growing that Crohn's disease may be associated with infection by Map ingested through not only improperly heat-treated milk but also contaminated water (67). However, the isolation of this pathogen is complex and time consuming, taking several months for culture, and there are little data about its distribution in the aquatic environment, treated water systems, or ability to colonize biofilms. In one study, the organism was believed to have been spread by standing water that was used by elk calves as a wallow and a source of drinking water (68). The water was believed to have been contaminated by an infected adult female elk introduced to the herd just before calving season.

There is scant evidence that nontuberculous bacteria form biofilms, but laboratory studies with the opportunistic pathogens, *M. fortuitum* and *M. chelonae* have shown that this is possible in high density polyethylene tubes used for water distribution (69). In a survey of water treatment plants and domestic water systems in Germany and France, mycobacterial species were found in 90% of the 50 biofilm samples taken (70). Their densities usually ranged between 10^3 and 10^4 cfu cm^{-2} (maximum density 5.6×10^6 cfu cm^{-2}). Organic substances such as plastics and rubber were usually colonized by larger numbers of mycobacteria than inorganic substances such as copper and glass. The authors suggested that mycobacteria are ubiquitous in biofilms and that solid–liquid interfaces, particularly plastic surfaces, may select for mycobacteria. In another study, *M. chelonae* was inoculated into PVC pipes containing sterile water and found to form biofilms after eight weeks (33). Of concern, viable organisms could still be recovered after seven days of treatment with 10 to 15 ppm chlorine. Fortunately, some mycobacteria are susceptible to dissolved copper salts (71), which might suggest that they will also be inhibited in biofilms on copper tube. Given the increasing threat of mycobacteria to public health, this possibility should now be investigated urgently.

ALLOCHTHONOUS BIOFILM PATHOGENS

Aeromonads and Indicators of Fecal Pollution

The presence in potable water of coliform bacteria in general, and *E. coli*, in particular, is a public health concern because it is assumed that these organisms are there as a result of fecal contamination of human or animal origin. Their presence is believed to be owing to (*1*) a loss of residual disinfectant (e.g., chlorine); (*2*) backsiphonage, cross-connections, line breaks, and/or repair of the distribution main; (*3*) survival and recovery of injured organisms; or (*4*) failure of the treatment plant (53). The presence of coliforms in drinking water, when there are no known breaches in treatment barriers, and the presence of these organisms in the absence of any evidence of fecal contamination, continues to be a major problem for the water industry, and has emerged as a critical regulatory issue. The chronic presence of coliform bacteria in drinking water supplies is characterized by: the absence of coliforms from water that leaves the treatment plant; the routine presence of coliforms in distribution supply samples at various points; the persistence of coliforms in the system despite the maintenance of a disinfectant residual that is assumed to be effective; and the persistence of the problem over a long time. *Escherichia coli* has attracted the greatest interest as an indicator of the microbiological quality of water, but other bacteria have recently been considered as waterborne pathogens. For example, aeromonads are ubiquitous in freshwater environments and *A. hydrophila* is being increasingly recognized as an etiological agent of gastrointestinal disease associated with chlorinated water supplies (72). Aeromonads are sometimes also associated with wound infections.

The possibility that coliforms and aeromonads can become part of the biofilm community, and show regrowth or aftergrowth, was investigated using the continuous culture model (53). Bitumen-painted mild steel is a material used for the construction of many potable water distribution supplies in the United Kingdom. Heterotrophic biofilms were developed on tiles of this material in potable water at 5, 15, and 25 °C and dilution rates of 0.1 and 0.2 $hour^{-1}$; these were challenged with *E. coli* and *A. hydrophila* isolated from distribution supplies. These organisms established in the planktonic population, become rapidly incorporated into the biofilms and survived many weeks at approximately 1% of the population (Table 7). Monochloramine has been advocated as a superior disinfectant for potable water systems rather than chlorine, owing to greater longevity in long distribution supplies with a high chlorine demand and

Table 7. Effect of Monochloramine on Colonization of a Heterotrophic Biofilm on Bitumen-Painted Mild Steel by *Aeromonas hydrophila* and *Escherichia coli*

Time (days)	Heterotrophs	*A. hydrophila*	*E. coli*
1	6.1 (4.9)	4.8 (3.3)	5.6 (2.8)
4	5.8 (5.9)	3.3 (3.0)	5.0 (5.1)
7	4.9 (5.3)	3.7 (4.3)	3.4 (4.6)
14	5.8 (5.6)	3.8 (4.2)	3.3 (4.2)
21	5.6 (5.3)	3.8 (4.5)	3.1 (4.3)

Note: The biofilm viable counts are expressed as $\log_{10}$ cfu cm^{-2}. Cultures were grown aerobically at 25 °C at a dilution rate of 0.2 h^{-1}. Values in parentheses indicate viable counts after addition of monochloramine to the culture medium at a concentration of 0.3 mg L^{-1}.
Source: From Mackerness and coworkers (53).

suggested ability to penetrate biofilms (16,17). When monochloramine was added to the model system at 0.3 mg L^{-1} (a concentration regularly used by water suppliers), there was no decrease in the biofilm viable counts although *E. coli* was eliminated from the aqueous planktonic phase. Rather, the numbers of *E. coli* and *A. hydrophila* recovered from the biofilms rose slightly. Higher concentrations of monochloramine were required for their eradication. Thus, *E. coli* and *A. hydrophila* can become part of the autochthonous heterotrophic biofilm and resist chemical disinfection. Subsequently, Block and coworkers (73) were able to show that their isolate of *E. coli* could grow slowly in an autochthonous biofilm on the pipework of a large-scale model distribution water supply operating at 20 °C and a residence time of 24 hours, producing an erratic transient colonization. The variable growth rate, initially 14 days but then decreasing, was related to the availability of biodegradable organic matter in the water and that provided by the biofilm.

Attempts to control dissemination and regrowth of pathogens and indicator bacteria in potable water through the use of oxidative disinfectants such as chlorine or ozone have led to subsequent biofilm problems. This is because these disinfectants oxidize refractory carbon to assimilable organic carbon (AOC), which fuels biofilm growth. Van der Kooij (74) has suggested that AOC concentrations of greater than 10 μg L^{-1} enable growth and biofilm formation in potable water systems. The implication of these findings is that it is essential to maintain low AOC concentrations for water in distribution to prevent regrowth and the multiplication of indicator bacteria and potential pathogens such as *Legionella*, *Aeromonas*, and *Pseudomonas* spp. With ozonation treatment of potable water becoming widespread, oxidizing refractory dissolved organic carbon to AOC, it is essential to install something like granular activated carbon and/or sand filtration to reduce the AOC postozonation.

Van der Kooij (75) demonstrated that aeromonads can utilize a wide range of low molecular weight compounds, including amino acids, carbohydrates, and long-chain fatty acids at a concentration of a few micrograms per liter. The concentration of substrates available in drinking water was usually below 10 μg Cl^{-1} and the autochthonous bacteria utilized these substrates more rapidly than the aeromonads. The multiplication of aeromonads in drinking water during distribution was therefore explained by their growth on biomass components in the biofilm and in sediments in the pipes.

Studies by Packer and coworkers (76) showed that *K. oxytoca* can also persist in the biofilm model of water distribution systems for several weeks at a temperature of 20 °C, irrespective of the chemistry of the source waters used for growth. Although there was a gradual decline in the numbers of klebsiellae which could be recovered from the model by culture, the decline was much less than that predicted by the theoretical wash-out rate of the chemostat system (for a review, see Keevil (10)). Thus, slow growth must have occurred. Similarly, a *Klebsiella* sp. was enriched in an autochthonous biofilm formed on latex substrata in potable water at 30 °C: yet this coliform was only present in very low numbers in the inoculum (34). Camper and coworkers (77) used staining with fluorescent antibody conjugates to show that *K. pneumoniae* could also exist as discrete microcolonies on the surface of biofilms generated in potable water. The inoculum growth rate had a dramatic effect on the ability of coliforms to remain on surfaces. The most slowly grown coliforms ($\mu = 0.05$ $hour^{-1}$) survived in the highest concentrations. Both coliforms and HPC bacteria were supported in larger numbers on a reactive substratum, mild steel, than on polycarbonate. Consequently, the occurrence of coliforms in a distribution supply or building plumbing system which has no fecal contamination might be because of the detachment of these organisms from the biofilm, from where they can be transported around the system and colonize new sites. The only course available for their control by the supplier is to use a residual disinfectant that persists and is nontoxic to man. The biofilm model system is ideal for deciding which disinfectant is the most appropriate under the conditions of use. For example, *A. hydrophila* is more susceptible to eradication with a low dose of monochloramine at 5 or 15 °C rather than warmer temperatures.

The ability of opportunistic pathogens such as *A. hydrophila* or *P. aeruginosa* to colonize and persist in high species diversity autochthonous biofilms may be related to the production of quorum-sensing AHLs (78–80). This population-dependent cell-to-cell communication was classically shown in the marine endosymbiont, *Vibrio fischeri*, to involve the LuxR/LuxI family of proteins, which are responsible for diffusible 3-oxohexanoyl-L-homoserine lactone signal response and synthesis for controlling light production in response to high cell density (81). In *P. aeruginosa*, two quorum-sensing regulons have been identified in which the LuxR homologs LasR and RhlR are activated by diffusible *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C_{12}-HSL) and *N*-butanoyl-L-homoserine lactone (C_4-HSL), respectively. The *lasR* and *rhlR* genes are linked to the *luxl* homologs *lasl* and *rhll*, which are responsible for synthesis of 3-oxo-C_{12}-HSL and C_4-HSL, respectively; *lasRl* and *rhlRl* are both involved in regulating the expression of several virulence factors, including elastase and rhamnolipid (82). In *A. Hydrophila*, the Lux homologs are AhyR/AhyI (80). The key signal is C_4-HSL, whereas *N*-hexanoyl-L-homoserine

lactone (C_6-HSL) plays a minor role. For coordinate gene expression at high cell density *ahyI* transcription requires AhyR and C_4-HSL (i.e., a positive feedback loop). Indeed, putative virulence determinants such as extracellular protease are regulated by C_4-HSL quorum sensing (83). The roles of AhyR, AhyI, C_4-HSL, and C_6-HSL in biofilm formation were defined in a study by Lynch and coworkers (80), which showed that wild type *A. hydrophila* formed normal biofilms on stainless steel substrata. However, an *ahyI* mutant was impaired in its ability to form biofilms but this ability was restored upon addition of exogenous C_4-HSL. Long-chain analogs, such as *N*-(3-oxodecanoyl)-L-homoserine lactone (3-oxo-C_{10}-HSL), induced the sloughing off of established biofilms. Therefore, *A. hydrophila* may respond to quorum-sensing cross-talk between different species by preferentially colonizing biofilm microenvironments in which there are appropriate concentrations of C_4-HSL but not 3-oxo-C_{10}-HSL.

Similarly, another marine *Vibrio*, *V. harveyi* possesses two parallel quorum-sensing systems, and each is composed of a sensor-autoinducer pair (81). *V. harveyi* reporter strains capable of detecting only autoinducer 1 (AI-1) (β-hydroxybutyl homoserine lactone) or autoinducer 2 (AI-2) have been constructed and used to show that many species of bacteria, including *E. coli* MG1655, *E. coli* 0157 : H7, *Salmonella typhimurium* 14028, and *S. typhimurium* LT2 produce autoinducers similar or identical to the *V. harveyi* system 2 autoinducer AI-2 (84). The genes responsible for AI-2 production in *V. harveyi*, *S. typhimurium*, and *E. coli*, called *luxSV.h.*, *luxSS.t.*, and *luxSE.c.*, respectively, are highly homologous to one another but not to any other identified gene. The *luxS* genes define a new family of autoinducer-production genes and that AI-2 is probably a furanone arising from secondary metabolism of *S*-adenosyl methionine. This again suggests that colonization of biofilms by a coliform is influenced by both its own physiological state and its response to that of the cell density controlled autochthonous microflora in a particular biofilm microenvironment, involving intraspecies and interspecies communication (81).

One approach to studying the physiology of *E. coli* in biofilms in situ utilized a strain containing a *lacZ* reporter linked to the *nirB* promoter (85). This promoter is susceptible to low oxygen concentrations and it was possible to see β-galactosidase expression identifying a mosaic of microenvironmental niches of low oxygen concentration in the biofilm in which the *E. coli* colonized (Fig. 6). The location of all of the *E. coli* bacteria was identified by measuring the glucuronidase activity, expressed by the *gusA* gene, with a fluorogenic substrate. The work confirmed the ability of *E. coli* to persist in waters of different chemistries and that the regions of lowest oxygen concentration and redox potential are in and closely adjacent to the biofilm stacks. This is presumably because of the concerted respiratory activity of the heterotrophic autochthonous microflora. It will be intriguing to ascertain the effects of lox oxygen concentration on the production by nontoxigenic and toxigenic *E. coli*, or possibly members of the autochthonous biofilm flora, of the important secondary metabolite involved in quorum sensing, AI-2, discussed earlier.

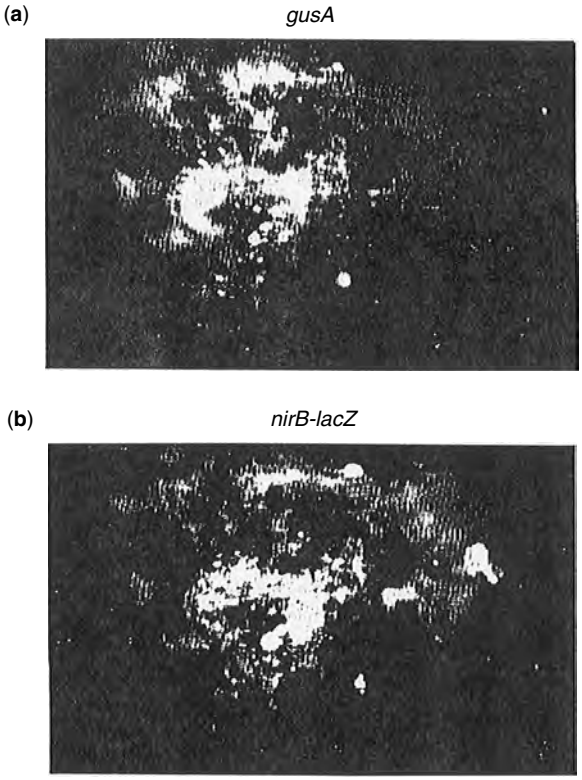

Figure 6. Colonization of *E. coli* in a potable water biofilm (**a**) and expression of the redox sensing nirB promoter linked to a lacZ reporter (**b**). *Source:* From Robinson and coworkers (85). See color insert.

Verocytotoxigenic *E. coli* 0157

Verocytotoxic *E. coli* (VTEC), most significantly serogroup 0157, is a particularly *nasty organism* (86). The bacterium is present as part of the normal flora of domesticated animals but is able to attach to enterocytes in the large intestine of man, colonize and efface the microvilli, and produce verocytotoxins (VT1 and/or VT2 and its variants) (87). In many patients it may cause hemorrhagic colitis, with a characteristic bloody diarrhea, and the toxins enter the bloodstream to target the kidneys. In 10% of cases, infection can lead to hemolytic uremic syndrome (HUS) or thrombotic thrombocytopenic purpura. These conditions may result in kidney failure in the young or stroke in the elderly. Indeed, the majority of cases of all renal failure in children are the result of HUS (88). The mortality rate is less than 10% in young children, but as high as 50% in elderly patients. Many patients require long-term kidney dialysis.

Outbreaks of have been associated with consumption of inadequately cooked minced beef, milk, yoghurt, cheese, cooked meats, meat pies, dry cured salami, raw vegetables, unpasteurized apple juice, and water (89). The waterborne outbreaks of *E. coli* 0157 include 20,000 cases in Swaziland in 1992 and 243 cases (four deaths) in Cabool, Missouri, in 1989 (90), 1,000 cases (two deaths) near Albany, New York,

in 1999 (91), and 2,000 cases (seven deaths) in Walkerton, Canada, in 2000 (92). This raises concern that the pathogen may survive long enough in feces-contaminated soil to wash into surface waters in which it could pollute potable water supplies, recreational waters, or water used for crop irrigation. Indeed, VTEC have been found to occur in significant numbers in rivers (93) and outbreaks have been described involving swimming in recreational lakes or pools in Europe and the United States (94,95). Recently, it has become clear from European and American studies that private and/or untreated drinking water supplies represent a significant risk for transmission of *E. coli* 0157 from fecal ingress, and several outbreaks have been described in Scotland and the United States (96–98).

Studies similar to the work described earlier with nontoxigenic coliforms have shown that *E. coli* 0157 : H7 is able to persist for many weeks in chemostat model systems receiving a continuous flow of a soft potable water at 10, 20, or 40 °C (99). This water type is typical of an upland catchment supply. The lower temperatures are representative of water supplied to buildings in moderate or hot seasons, the higher temperature reflecting poorly heated hot water supplies or those with poorly insulated pipes. Of note, *E. coli* 0157 was able to colonize biofilms of high species diversity and persist for several weeks at the lower temperatures (Table 8). Significantly higher numbers were detected in the biofilms on plastic pipework compared to copper or stainless steel. Similar results have now been obtained using moderately hard and hard potable waters. This suggests that the type of plumbing material used for supplying poorly treated water to the home or factory is important, and that use of copper pipe might confer some public health benefit by reducing pathogen persistence in biofilms. Another study has shown that *E. coli* 0157 will form a biofilm and grow on the walls of bottled water (100). These studies support the available epidemiological evidence that private water supplies with inadequate disinfection treatment, supplying tap or bottled water, may be at particular risk of harboring and transmitting *E. coli* 0157 (98).

Campylobacters

Current epidemiological evidence suggests that *C. jejuni* is the major cause of human gastroenteritis worldwide, responsible for 400 to 500 million cases of diarrhea each year (101). Of great significance, *Campylobacter* can also cause reactive arthritis and Guillain-Barre syndrome, which can result in paralysis and death. This important zoonosis is spread through contact with pets or consumption of raw milk and contaminated meat, particularly poultry. However, it also survives sufficiently well in untreated and inadequately treated aquatic environments to cause human disease. Survival in the water systems of animal husbandry facilities and animal processing units has also been suggested to promote infection in animals and cross-contamination of animal carcasses (102). Using the two-stage continuous culture biofilm model supplied with potable water, Buswell and coworkers (103) were able to show that several different isolates of the pathogen colonized the autochthonous high species diversity biofilm. These strains persisted, as measured by culture, for up to 4 weeks at 4 °C. Increasing the temperature to 30 °C reduced their persistence to less than 10 days, but this was considerably in excess of the 1 to 2 days observed in the absence of an autochthonous biofilm flora in preliminary sterile batch microcosm studies. Reducing the oxygen concentration generally improved the culturability of the strains. The influence of carbon concentration on the persistence of *C. jejuni* in biofilms was investigated by adding a low concentration of serine typical of reported lower and upper limits of carbon and amino acid load reported in surface water (5 nM and 5 μM). The addition of increasing concentrations of serine progressively reduced the persistence of the isolate by up to 50%. Conversely, the biomass of the autochthonous biofilm flora increased, indicating that the population had previously been carbon or possibly nitrogen limited. Buswell and coworkers (103) speculated that the balance of nutrients is likely to change the competitive interactions between the autochthonous flora, and intensify competition for other limiting nutrients that may be detrimental to the persistence of nutritionally fastidious campylobacters.

Initial surface colonization and structural development of either environmental or clinical biofilms is partly dependent on several types of cell–cell interaction between different pairs and groups of bacteria. For example, strong parallels exist between the early work of Kolenbrander (104) on coaggregation between dental plaque species and the autochthonous members of aquatic biofilms. Pairs of water isolates coaggregated to form visible flocs of various sizes (105). One strain coaggregated with every other strain tested, suggesting the possibility of it forming multigeneric coaggregates, and a role for this strain as a bridging organism, similar to that proposed for *Fusobacterium* and *Prevotella* in dental plaque. Indeed, Buswell and coworkers (105) were able to show varying degrees of coaggregation between *C. jejuni* and members of the autochthonous biofilm flora. Coaggregation scores were generally low with the pathogen but this is unlikely to reflect the strength of the interaction, just the size and density of the aggregates formed.

The potential for *C. jejuni* to coaggregate with members of the autochthonous biofilm flora was visualized directly using 16S rRNA probes. These have been used successfully to study biofilm structures, including cell–cell associations, and the detection of pathogens therein (106–108). Similarly, *C. jejuni* was tracked in the biofilm stacks using

Table 8. Colonization of *Escherichia coli* 0157 (Human Isolate PS 14) Survival in Potable Water Biofilms Formed on Different Materials at Temperatures Typical of Cold and Warm Water Supplies

Temperature (°C)	Glass	Copper	Stainless Steel	Polybutylene
10	+	+	++	+++
20	+	+	++	+++
40	+	+/–	+	+++

Note: +/– denotes little or no colonization; + denotes light colonization; ++ denotes good colonization; +++ denotes heavy colonization.
Source: From Keevil and coworkers (99).

a fluorescently labeled *Campylobacter*-specific 16S rRNA probe in conjunction with a differently labeled eubacterial probe to simultaneously visualize the rest of the biofilm flora. Use of fluorescence in situ hybridization with the 16S rRNA oligonucleotide probes demonstrated an extended association of the pathogen with the biofilm up to the time when the experiments were terminated, that is, 28 days at 30 °C and 42 days at 4 °C (103). This was long after the *C. jejuni* had ceased to be culturable. It was clearly associated with the microcolonies of the biofilm stacks, suggesting that it was incorporated within the biofilm matrix (Fig. 7). This location was probably preferred to obtain secondary metabolites from the biofilm consortium and also seek a lower oxygen, lower redox environments to suit its microaerophilic physiology. At 4 °C the *C. jejuni* were predominantly spiral shaped and contained an higher 16S rRNA content than the predominantly coccoid cells found at 30 °C. This may indicate that they became viable but nonculturable (VBNC) at the lower temperatures and persisted for many weeks in the biofilm.

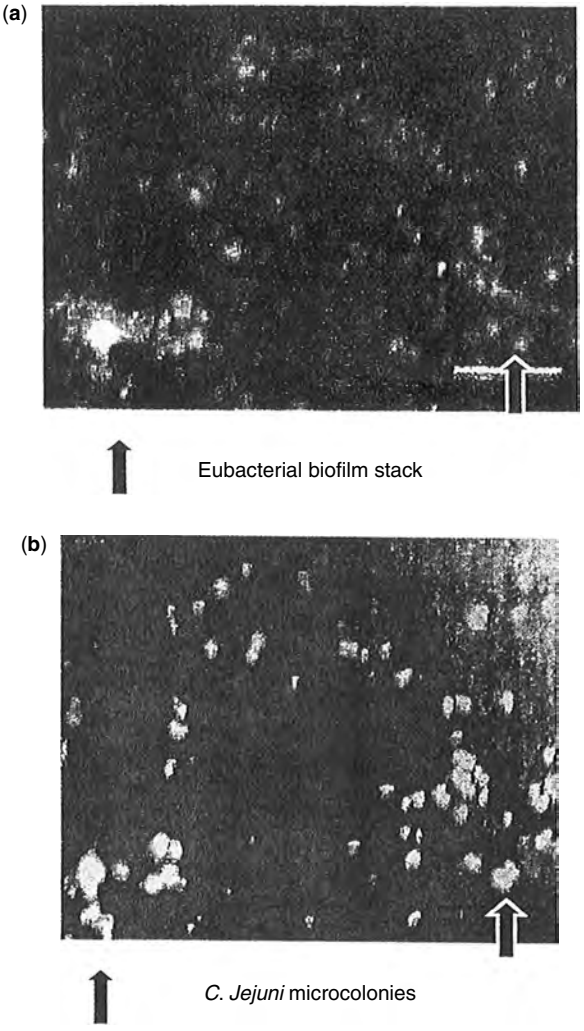

Figure 7. Detection of eubacteria (**a**) and *C. jejuni* (**b**) in biofilm stacks using fluorescence in situ hybridization with 16S rRNA oligonucleotide probes. *Source:* From Buswell and coworkers (103). See color insert.

Therefore, despite the sensitivity of *C. jejuni* to environmental stress, its persistence in a culturable form in aquatic biofilms can be sufficiently long for this to provide an important reservoir for transmission of the pathogen. The significance of a more extended persistence in a nonculturable form, with the possibility of an infectious VBNC state (109), has further consequences for long-term persistence, pathogenicity, and our ability to detect and control it easily.

Helicobacters

Campylobacter jejuni is closely related, both phylogenetically and physiologically, to the emerging pathogen, *H. pylori*, the causative agent of stomach ulcers. This microaerophilic pathogen is also involved with several gastrointestinal diseases including type B gastritis (110), gastric ulcer and mucosa-associated lymphoid tissue lymphoma, and gastric adenocarcinoma (111). In Western countries, rates of infection are as high as 60% by age 65, whereas in developing countries the prevalence of infection can be as high as 90% by 20 years of age (112). However, although the presence of *H. pylori* in the natural environment has also been demonstrated in a number of studies, similar to *C. jejuni*, its route of infection into humans is unknown. Recent research has found *H. pylori* in Peruvian drinking water (113) and the U.S. EPA have shown this pathogen to have a threefold greater resistance to chlorine than *E. Coli* (114). In a region of Japan with a high infection rate, *H. pylori*-specific DNA was detected in tap and well water, field soil, flies, and cow feces by nested polymerase chain reaction, and the *ureA* gene alignments were closely homologous to those of established clinical isolates (115). The detection of *H. pylori* in local water supplies in Canada may indicate a natural reservoir for the organism or possible contamination from human sewage (116). As *H. pylori* is an emerging pathogen associated not only with peptic ulcers but also gastric cancer (117), there is an urgency to understand its possible dissemination and methods of control in drinking water. Pure cultures of *H. pylori* appear able to form biofilms, particularly hydrophobic biofilms at the air–liquid interface of nutritious culture media (118) but little is known about colonization of aquatic biofilms. In one study, Mackay and coworkers (119) adapted a method originally described by Mackerness and coworkers (53) to study fecal pathogens in water and biofilms. This consisted of a single-stage chemostat, growing an autochthonous flora obtained from a water treatment works prechlorination, which supplied a modified Robbins device containing stainless steel stud substrata. When this model was inoculated with a culture of *H. pylori*, the pathogen could subsequently be detected in the biofilms for up to 192 hours using polymerase chain reaction (PCR) analysis of the 16S rDNA and specific primers to amplify a 500 bp product. If the cells were first heat-inactivated, then *H. pylori* could not be detected in the biofilm, suggesting that incorporation into autochthonous biofilms requires the organism to be in a viable state. The authors did not report isolating the pathogen from the biofilms using conventional agar culture. Clearly, more work is required to determine if it can actually persist in a viable state capable of subsequent

sloughing into raw or improperly treated water to cause waterborne disease.

In support of these preliminary studies, Park and coworkers (120) have analyzed a section of cast iron mains distribution pipe removed from an urban environment in the north-east of Scotland during routine maintenance work. Immediately upon removal of the pipe section, the interior lumen was swabbed to remove the biofilm layer. Subsequent analysis for the presence of *Helicobacter* DNA using a nested PCR approach produced a positive result. These authors claim that these data provide the first evidence for the existence of *Helicobacter* in biofilms found in water distribution systems anywhere in the world.

Cryptosporidia

Ten valid species of the coccidian protozoan parasites, *Cryptosporidium*, are currently recognized, and capable of causing infection in animals and/or man (121). Awareness of the importance of the human parasite, *C. parvum*, has increased since it was first identified as a cause of human enteritis in 1976. Molecular studies show that there are at least two genetic subgroups of *C. parvum*, one found almost exclusively in man (genotype 1) and the other present in man, cattle, and a range of small mammals (genotype 2). Cryptosporidiosis is particularly severe and protracted in people with impaired immunity, but can cause several weeks of debility and watery diarrhea in immunocompetent people, including abdominal pain, sometimes with anorexia, vomiting, and fever (122). Surveys of stools carried out in developed countries have shown the prevalence of infection to be between less than 1 and 4.5%, and many infections are subclinical. Children under two years of age (and their carers), animal handlers, travelers, and homosexual men are particularly likely to be infected. Outbreaks have been associated with contact with animals, consumption of inadequately pasteurized milk, use of swimming pools, and contaminated water supplies. Indeed, 400,000 people were affected in a single outbreak in Milwaukee, Wisconsin, in 1993 because of contaminated drinking water (123). However, only 6.7 to 13.2 oocysts per 100 L of water could be detected, suggesting a low infectious dose and the need for vigilance and rigorous water treatment. The low infectivity was confirmed for three *C. parvum* isolates (Iowa [calf], UCP [calf], and TAMU [horse]) of the genotype 2 subgroup ingested by healthy adults. The 50% infectious dose (ID_{50}) was found to differ among the isolates chosen: Iowa, 87; UCP, 1,042, and TAMU, 9 oocysts (124). *Cryptosporidium parvum* is an obligate parasite and has a complex life cycle that is completed in one host (122). The transmissible stage is the 5-μm diameter round oocyst (spore). After ingestion each oocyst releases four motile sporozoites into the gut, primarily in the small bowel. The sporozoites attach to the gut wall and develop by two asexual stages, producing merozoites and a sexual stage producing zygotes, which sporulate to produce two types of oocysts. These stages develop just inside the outer walls of the cells lining the gut; the infection is intracellular but it is extracytoplasmic, that is, the protozoa do not penetrate deeply into the host's cells. Oocysts mostly sporulate within the gut and two types are produced. The thin-walled type releases more sporozoites in the gut (auto-infection) and the thick-walled types are excreted in feces in a fully infective form. They do not need any further maturation, unlike many other coccidian protozoa. Oocysts can remain viable for about 18 months in a cool, damp, or wet environment. They are quite common in rivers and lakes, especially where there has been sewage or animal contamination. Consequently, the source of oocysts is usually either insufficiently treated surface water or contamination of drinking water with surface water. The complete removal of *C. parvum* from water supplies is difficult, even for modern water treatment plants. The pathogen's oocyst appears resistant to high concentrations of chlorine-based disinfectants but more susceptible to ozone (125). The oocysts' small size means that they may pass through the filtration systems. Control measures include not using water sources polluted by runoff from land contaminated by animals or otherwise contaminated with manure or sewage; good maintenance and design of the filtration systems (flocculation and slow sand filtration appear the most useful technologies), and careful control of the treatment facility and processes.

The chemostat model has been used to show that the 5-μm diameter oocysts can survive in potable water biofilms for many weeks at 20 °C in an infectious state (13,126). The *C. parvum* oocysts were labeled with FITC-conjugated antibody and observed by EF microscopy to attach to 16–day-old potable water biofilm stacks that were visualized with the nucleic acid fluorophore, propidium iodide (Fig. 8). After 24 hours contact with the biofilms, oocysts adhered at a concentration of 14,000 oocysts cm^{-2} and maintained a high concentration in the biofilms over several months. When the oocysts were recovered from the biofilms after several months they were found to be still capable of excystation, indicative of viability, and could cause an infection in an animal model. Similar attachment data were obtained by White and coworkers (127) using a tri-species biofilm (*P. aeruginosa*, a *Bacillus* sp., and an *Acidovorax* sp.) developed on stainless steel surfaces in a flow cell supplied with

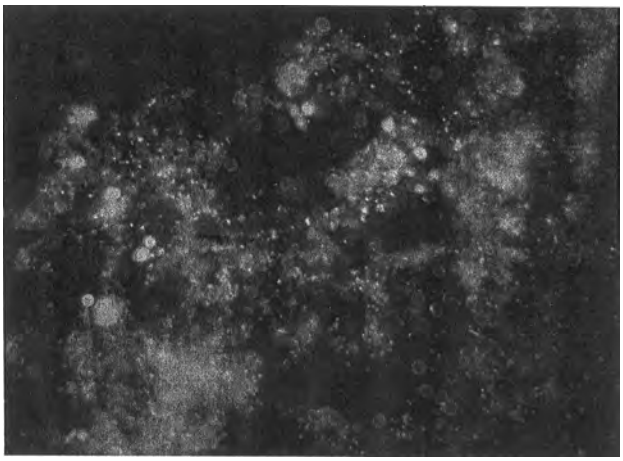

Figure 8. FITC-antibody-labeled *Cryptosporidium parvum* oocysts attached to potable water biofilm stacks stained with propidium iodide. *Source:* From Keevil and coworkers (13). See color insert.

potable water. When the system was challenged with *C. parvum*, the oocysts attached to the biofilm at twice the concentration compared to the bare surface without biofilm. The work of Rogers and Keevil (126) indicated that higher shear rates are required for removal of oocysts from the autochthonous biofilm and transient sloughing might explain the many sporadic cases of unknown origin, even long after previously contaminated source waters are considered safe. It was notable that biofilm-associated oocysts occurred in clusters; this would suggest that biofilm sloughing may release a small but effective dose of oocysts into the water system, which would also be difficult to detect by routine monitoring. The persistence of chlorine-resistant viable oocysts in the biofilm safe haven, therefore, presents an interesting challenge for the disinfectant industry and the safeguarding of the public health.

Enteroviruses

As discussed earlier, there has been little research on virus survival in biofilms. However, in one notable study, Quignon and coworkers (128) followed the survival of Poliovirus-1 pulsed into a 90-m-long pilot distribution system operating at a flow of 500 L hour^{-1}. Greater numbers of virus were recovered from the biofilm on the walls of the pipe than from the water flow; this was by a factor of 2 or 10 in the absence or presence of chlorine disinfectant, respectively. Consequently, this would suggest a tendency for virus accumulation within biofilms and protection of viral pathogens from disinfectants such as chlorine.

BIOFILM SAFE HAVEN

Extracellular Survival

It is now abundantly clear that biofilms provide an environment for autotrophic and heterotrophic microorganisms, to consort together, and grow in ordered communities. This is especially important in low nutrient aquatic environments and where carbon and nitrogen sources may be tied up in complex macromolecules such as humic and fulvic acids. Concerted metabolism provides an efficient breakdown of such complex, high molecular weight compounds and also releases secondary metabolites, which more fastidious species require (4,129,130). Indeed, we are still incapable of culturing many of the fastidious, unidentified species in biofilms now starting to be recognized through 16S rRNA analysis (131,132). Presumably, their nutritional and physiological requirements have evolved for life in the biofilm and we cannot yet provide these conditions for their growth as monocultures in the laboratory. For now they are considered viable but not culturable, but this should not be confused with the viable but unculturable state which describes a physiological transition to dormancy, perhaps akin to spore formation (109,133). Moreover, the heterogeneous mosaic structure of biofilms provides a safe haven, not only for essential nutrients but also to protect from extremes of physicochemistry, such as temperature, pH, and oxygen concentration, and antimicrobial substances such as heavy metals, disinfectants, and antibiotics. As discussed previously, this is not merely a physical protection but includes important physiological adaptations involving quorum sensing, oxidative stress OxyR and SoxR (134), and antimicrobial efflux MarA (135). Also of great importance is the general stress response with DNA supercoiling (136) and alternative RNA polymerase sigma factors coming into play (137) for upregulation of essential genes such as the heat shock molecular chaperones, DnaK, DnaJ and GrpE, and GroEL and GroES (138), the oxidative stress catalase, KatE, and osmotic shock, OsmY (139). The challenge will be to understand the complex interplay of all of these important regulators on biofilm physiology, which aids pathogen survival.

Intracellular Survival

It has been known for almost 20 years that *L. pneumophila*, the principal etiological agent of Legionnaires' disease, can survive intracellularly within cyanobacteria and a range of amoebae and other protozoa, including *Acanthamoeba*, *Naegleria*, *Hartmanella*, *Vahlkampfia*, and *Tetrahymena* spp. and *Dictyostelium discoideum* (49,140,141). Consequently, this provides another important mechanism which links survival of legionellae in the environment to biofilms through survival inside biofilm protozoal grazers. In some circumstances this endosymbiotic relationship not only affords protection against desiccation and disinfection, permitting widespread dissemination, but may also make the pathogen more virulent when inhaled by man (142,143). Following phagocytosis by amoebae, legionellae are able to multiply within the cytoplasm by evading the host lysosomal attack. After one or two days a single membrane vesicle of motile legionellae fills most of the trophozoite. The vacuole then lyses, liberating the pathogen back into the environment. Larger *Acanthamoeba* spp. can contain hundreds of legionellae in the infected vesicle before the lytic phase. The ability of *L. pneumophila* to grow in amoebae, other microorganisms, and lung macrophages, coupled with the early difficulties of first being able to isolate and grow this apparently fastidious pathogen, has led many to speculate that it is an obligate intracellular parasite. However, we have shown that this is not so: it is able to exploit the biofilm physicochemical heterogeneity to proliferate in the microaerophilic niches of the biofilm (85). In the absence of environmental hosts, it utilizes its considerable metabolic versatility (144) to grow on the consortium's secondary metabolites (9,34,35,145). This was finally confirmed by Surman and coworkers (25) who conclusively demonstrated that it is a facultative intracellular parasite when they grew the aquatic flora in the biofilm model in the presence of cycloheximide to eradicate all eukaryotic species. In these conditions, the numbers of legionellae actually increased by greater than 50-fold in the biofilms, indicating that classical predator/prey relationships might actually keep legionellae under control in a biofilm. Nevertheless, intracellular survival in amoebae is a useful evolutionary adaptation because it promotes extracellular survival by inducing a stress-resistant phenotype, characterized by altered morphology and envelope composition, increased resistance to antimicrobial agents, and induction of polyhydroxybutyrate as a carbon and

energy store for low nutrient environments (146,147). It may be no coincidence that in a survey of ground water, potable water, and whirlpools, 65% of the samples that contained legionellae also contained amoebae (148).

Mycobacterium avium is able to grow saprozoically on products secreted by the amoebae whereas *L. pneumophila* multiplied only in coculture (149). A comparison of amoebae infected with *L. pneumophila* and amoebae infected with *M. avium*, observed by electron microscopy, demonstrated that there were striking differences in the locations of the bacteria within amoebal cysts. Whereas *L. pneumophila* resided within the cysts, *M. avium* was found within the outer walls of the double-walled cysts of *Acanthamoeba polyphaga*. These locations may provide a reservoir for the bacteria when environmental conditions become unfavorable. Intra-amoebal survival and multiplication has been confirmed for ingested *P. aeruginosa*, *Burkholderia cepacea*, *Listeria monocytogenes*, *Vibrio cholerae*, *Edwardsiella tarda*, *Sarcobium lyticum*, and *Chlamydia* spp. (150–153). Intracellular survival, not necessarily with multiplication, has been shown with *Pseudomonas*, *Alcaligenes*, and *Bacillus* spp., *Aeromonas salmonicida*, *Yersinia enterolitica*, and *C. jejuini*, and coliforms including *E. coli*, *Citrobacter freundii*, *E. agglomerans*, *Enterobacter cloacae*, *K. pneumoniae*, *K. oxytoca*, *Shigella sonnei*, and *S. typhimurium* (149,154,155). Recently, Barker and coworkers have reported that *E. coli* 0157 is also capable of surviving ingestion by *Acanthamoeba* and will survive many weeks in intracellular vacuoles (156). As these amoebae are plentiful in the environment, both in soil and aquatic biofilms, this might therefore suggest that intracellular survival could provide another important mechanism for persistence of *E. coli* 0157 and other dangerous pathogens in the soil and aquatic environments.

In conclusion, the earlier description of quorum sensing showed how this is a powerful mechanism to rapidly switch the physiology and virulence of a pathogen when adapting to new environments. Endosymbiotic survival in amoebal cysts, particularly for pathogens capable of extensive intracellular multiplication such as legionellae, affords a microenvironment in which accumulation of autoinducing pheromone molecules such as AHLs, furanones, or peptides could readily occur. This will clearly merit further investigation.

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PATHOGEN SURVIVAL IN AQUATIC ENVIRONMNENTS.

See SOURCE WATER PROTECTION: MICROBIOLOGY OF SOURCE WATER

PERIPHYTON

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Periphyton is the algal community growing attached to all objects submerged in water. Growth on a substratum confers to periphyton peculiarities that distinguish it from phytoplankton, which is suspended in the water column, and make its sampling and measurement particularly challenging. Probably, because of these methodological difficulties, periphyton has been studied much less than other aquatic communities, despite mounting evidence of its crucial contribution to aquatic metabolism and food webs. Its distribution in rivers, lakes, and wetlands is regulated both by the availability and type of colonizable substratum and a balance between light, nutrients, current, and grazing. Periphyton responds to several anthropogenic disturbances and therefore can be used to monitor water quality.

DEFINITION, CHARACTERISTICS, AND DEVELOPMENT

The term periphyton, in the broadest sense, applies to all microflora (algae, bacteria, and fungi) growing attached to any underwater substratum. However, usually periphyton studies predominately address the algal component. Several synonyms for periphyton are used in the literature including Auwfuchs, biofilm, and benthic algae. Depending on the substratum on which the algae are growing, periphyton can be subdivided further into epiphyton (aquatic plants or filamentous algae), epilithon (rocks), epipsamnon (sand), epipelon (fine sediments), epidendron or epixilon (wood), and epizoon (aquatic animals). Finally, masses of algae floating among the substrata are called *metaphyton*.

On all substrata and environments, diatoms usually comprise the majority of species and often are numerically dominant. Cyanobacteria and Chlorophytes are always well represented and may also at times become dominant. Filamentous Xanthophyceans and Rhodophytes are less widespread, but they can occasionally be important. Other algal groups (Crysophyceans, Cryptophytes, and Dinophytes) that are prevalently planktonic can also be present but usually as minor components. In addition to taxonomic considerations, it is important to classify periphytic algae relative to growth-form or the degree of attachment to the substratum. Prostrate or adnate algae adhere their entire surface to the substratum. Other algae are attached only through a short mucilaginous pad or long stalk. Filamentous forms anchor through a holdfast cell or are freely floating. Some species are motile (especially pennate diatoms), and others are planktonic forms trapped within the periphyton matrix. Mucilaginous compounds secreted by algae and bacteria bond this matrix together. Algal sizes span several orders of magnitude from small blue-green Chroococcales and minute diatoms (e.g., *Achnanthes minutissima*), which are less than 10 μm in length, to macroscopic algae such as *Cladophora*, whose filaments can be few centimeters long.

Because of growth on a substratum, periphytic algae experience different challenges and advantages than that experienced by phytoplankton suspended in the water column. Self-shading and reduced water exchanges are a consequence of the vertical growth of the community on a horizontally limited substratum. At the same time, some refuge from grazing is offered by close proximity to a substratum. Although phytoplankton relies solely on the water column for nutrients, some attached communities such as epipelon (1) and epiphyton (2) can at least partially exploit elements released from their substratum. Nutrient retention and recycling is more effective in periphyton than in phytoplankton because of the closer aggregation of algae and bacteria within the complex matrix (3).

Because of its vertical stratification, periphyton resembles a forest where organisms compete for light and nutrients. Complex strategies related to growth-forms and sizes, like those observed in marine benthic communities, are encountered in periphyton (4). Small algae are fast-growing and apt to colonize any new empty substratum quickly. Their propensity to be easily grazed is partly counterbalanced by their proximity to the substratum. They must adapt to reduced light and water exchanges when the community becomes thick. Large algae are more often found in the overstory where resources are abundant but losses by grazing and sloughing are increased. They usually dominate mature communities. Operationally, periphyton is often separated in two fractions of loosely and tightly attached algae. The loose fraction, which is easily collected by shaking or brushing the substratum (see methods), is probably more metabolically active and readily available to grazers than the tight fraction, which is left relatively intact on the substratum by most sampling methods.

Consideration of the vertical structure of periphyton is crucial for understanding its dynamics, which are controlled principally by density-dependent factors. One other implication of periphyton structure is that it is generally less productive per unit of chlorophyll than phytoplankton. In fact, there is a systematic decline in the primary production of periphyton per unit of chlorophyll with increasing standing stock, whereas phytoplankton production and standing stock remain approximately proportional over a broad range of values (5).

Several studies have documented the typical development of periphyton from colonization of a bare substratum to establishment of a mature community and have delineated temporal changes in biomass and the importance of different growth-forms (Fig. 1). After a lag phase, biomass usually increases rapidly to reach the maximum value that can be supported by the substratum under given ecological conditions. At this point, self-shading and increasingly reduced water renewal at the base of the community leads to senescence of the basal layer. This weakens the

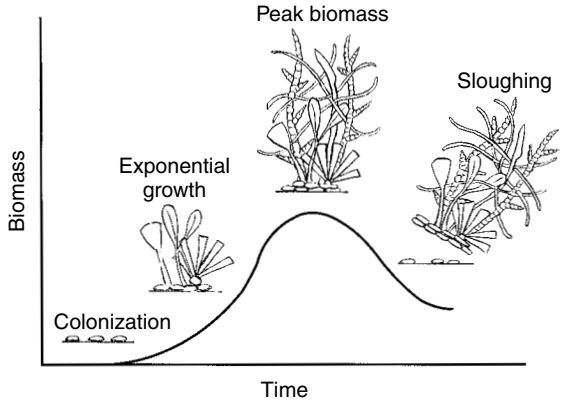

Figure 1. Changes in biomass and algal growth-forms during succession in a periphyton community.

attachment to the substratum and renders the entire mat vulnerable to sloughing. The speed with which peak biomass is reached varies from less than two weeks to around 100 days and largely depends on nutrient enrichment (6). Peak biomass increases relative to increasing nutrients, which result in better diffusion gradients across the mats and therefore development of thicker communities (7). Community composition also influences the peak biomass that can be supported by substratum. Diatom and cyanobacteria mats do not exceed chlorophyll concentrations of 400 mg/m^2 (8), whereas filamentous chlorophytes can reach 1,200 mg/m^2 of chlorophyll (9). The succession sequence of different taxa and growth-forms during periphyton development has been documented with scanning electron microscopy (10,11). A new substratum is first coated with an organic matrix and bacteria flora that form a favorable attachment site for early algal colonizers, usually small adnate diatoms. Afterwards, apically attached diatoms (rosette or stalked) appear, followed by filamentous forms.

METHODS USED TO STUDY PERIPHYTON

Sampling

Quantitative sampling of periphyton is difficult because of problems associated with detaching algae from the substratum and correctly estimating the area sampled. Different methods have been proposed relative to the substratum on which the periphyton grows. For epilithon, algae are detached from individual stones removed from the water by scraping with a scalpel (on smooth surfaces) or by brushing a known area of the substratum. When the substrata are too large or too deep to be lifted from the water, some form of enclosing device is used. The simplest is the sampler first devised by Stockner and Armstrong (12) consisting of a toothbrush connected to a syringe that can remove periphyton from a known surface underwater. This technique has been improved by adding another syringe to collect the scrapings, thereby improving sampling efficiency (13). Various modifications of this technique followed (14). Epipelon growing on fine sediments is mostly sampled with a small handheld core (15). Surface sediments of a few centimeters in depth from the top are retained for analysis. Separation of algae from the sediment remains a problem. One method based on algal phototrophism has been proposed in which algae are collected on lens paper or cover glasses placed over illuminated sediment (16). This technique is, however, selective for motile algae. Other methods take advantage of the differences in sedimentation between algae and sediments to separate epipelon (15). For epiphyton, detachment from the substratum is even more of a problem. Usually, the plant with its epiphytes is agitated vigorously in a jar filled partially with water. This procedure can last from 30 seconds to several minutes and be repeated several times. Effectiveness of the detachment is rarely complete (17). Sometimes, addition of chemicals that are able to solubilize the mucopolysaccharide matrix can lead to more complete sampling, but this is not suitable when epiphytes are used in analysis of photosynthetic pigments or primary production. Removal of epiphytes by a scalpel can contaminate the sample with macrophyte tissue.

A correct estimation of the sampled area is crucial to report quantitatively periphyton data, which are expressed per unit of surface. Rock surface area can be estimated by the weight of the aluminum foil necessary to wrap it (18) or alternatively by measuring the rock's three main diameters (19). Because plant surface measurements are laborious (20), epiphyton data are usually expressed per unit of dry weight of the host plant.

Artificial Substrata

To overcome problems of detachment from the substratum and determination of the sampled area, artificial substrata are being increasingly used. Since glass slides were first suspended in a lake in 1916, periphyton workers have experimented with many different anchoring devices and materials as varied as glass, tiles, styrofoam, nylon lines, plastic aquarium plants, and nutrient diffusing substrata (14). The U.S. Environmental Protection Agency suggests the use of frames anchored with rebars to the stream bottom and holding glass microslides or other suitable substratum (21). Artificial substrata are supposed to both decrease variability among replicates and to facilitate comparisons among sites by providing a uniform and standardized colonization substratum. However, there is still a controversy as to how well these substrata mimic natural communities. On the basis of a survey of the extensive literature on the subject, Cattaneo and Amireault (22) found that, in general, epilithon biomass was underestimated using artificial substrata, whereas epiphyton was somewhat overestimated. On stones, differences between natural and artificial substrata were smaller in warmer, richer sites and everywhere tended to decrease with longer colonization times (>30 days). For taxonomic comparisons, there was generally good agreement between diatom assemblages on natural and artificial substrata, whereas green and blue-green algae tended to be underestimated using artificial substrata. A careful evaluation of the goals of the study should precede the decision to use artificial substrata.

Biomass

The fastest method to estimate periphyton biomass is to measure it as a chlorophyll concentration. Various extractants, most commonly acetone and ethanol, have been used to extract pigments from the algae. Because periphyton can be associated with a significant amount of detritus and dead cells, correction for the presence of pheophytin (degraded chlorophyll) is advisable in order to correctly estimate living algae (23). The drawback of chlorophyll measurements is that pigment content is variable among different groups of algae and is influenced by light conditions. Values tend to be higher in algae growing in shade than under full light (24).

Measures of periphyton dry mass are unreliable because of the presence of inorganic sediments, but ash-free dry mass (after incineration at 550 °C) is a simple method to estimate periphyton total organic material. Alternatively, organic material can be estimated by carbon and nitrogen measurements with an elemental analyzer. All these measurements are not selective of the algal fraction but also include detritus, bacteria, and meiofauna.

Periphyton can also be quantified directly as algal biovolume by first counting and measuring algae under a microscope, and then calculating their volume by approximation to geometric solids. This method is time consuming but contemporaneously provides information on the taxonomic composition. There are some difficulties in estimating algal volume consistently because mucilage, vacuoles, and spines are sometimes present and are included or excluded from the volume calculation depending on the bias of the researcher or the goal of the study.

Composition

Taxonomic composition can be determined through microscopic examination of a periphyton subsample using a nanoplankton counting chamber or semipermanent glucose mounts (25). The number of cells to be counted depends on the goal of the study and the statistical analyses to be used. Most investigators enumerate from 300 to 500 organisms (26). Automated methods that use a computer interface to enter data can expedite enumeration and increase accuracy (27). Species composition is sometimes summarized in diversity indices that account for the number of species (richness) in the assemblage and the evenness of their abundance distribution among species (28).

A coarse assessment description of the taxonomic composition of the community can also be achieved by studying pigment composition using high-performance liquid chromatography (HPLC). This technique analyzes with great sensitivity individual chlorophylls and carotenoids allowing identification of different algal groups on the basis of their distinctive pigment composition. At the class level, good agreement exists between traditional taxonomical analyses based on microscopy and the faster delineation based on pigment composition (29).

Besides taxonomic composition, community structure can be described as size distribution by grouping algae in logarithmic size classes. Because metabolic rates are a function of body size (30), size distribution can be used to estimate community processes (31). Unlike phytoplankton in which size analysis can be automated with electronic particle counters, in periphyton, it must be done microscopically assisted by computer-based image analysis systems (32) because of contamination from inorganic and other biological particles.

Community composition also can be based on the concentration of different chemical constituents. Lipid and fatty acid compositions have been used to characterize algal communities with different taxonomic composition and to trace their usage in food webs (33). Cellular nutrient (carbon, nitrogen, phosphorus) ratios can serve as indicators of nutrient status for periphyton (34). Stoichiometry, as demonstrated in planktonic communities, might have important implications for periphyton usage by higher trophic levels (35).

Metabolic Rates: Production, Respiration, Grazing

Production and respiration of periphytic communities are usually measured by enclosing the periphyton on its substratum in test chambers (36) and measuring changes in oxygen or incorporation of ^{14}C during an incubation period. Measurements using chambers are relatively easy but suffer from several enclosure artifacts such as nonrepresentative sampling of heterogeneous stream vegetation and altered environmental conditions. The scaling-up of such results to an entire stream reach is particularly difficult when vegetation is not uniformly distributed. Measurement of gas exchange, oxygen or carbon dioxide, over an entire reach has the advantage of integrating metabolism over the whole heterogeneous community. This method, first proposed by Odum (37), requires accurate estimates of gas diffusion between air and water (38).

Estimates of periphyton grazing rates are usually obtained by excluding or adding grazers to periphyton communities and measuring, after a certain period, differences in biomass between treatments and controls (35). More rarely, grazing has been estimated by incorporation by the grazers of radioisotopically labeled periphyton (39).

DISTRIBUTION IN DIFFERENT HABITATS

Streams and Rivers

Periphyton is the most successful primary producer in streams. Along a longitudinal profile from the headwaters to large rivers, there are some general predictable patterns in periphyton distribution (40). Headwaters, at least in temperate North America, are usually heavily shaded by riparian vegetation, thus preventing significant development of periphyton. As the streams become larger (third, fourth order), more light can reach the bottom favoring algal growth. Periphyton importance is maximal in this midportion of the continuum. Further downstream, depth increases and light once again becomes limiting because of phytoplankton development and turbidity in the water column. Available substratum also changes longitudinally. Rocks and wooden debris are important in the upper reaches, whereas fine sediments and aquatic plants are the main substrata in the

downstream sections. Besides these large longitudinal changes, there are spatial differences also at a smaller scale between riffles that are shallow and with fast current and pools that are depressions in the stream bed with fine sediments and reduced current (41).

In running water, hydrology has an overwhelming effect in controlling the temporal distribution of periphyton. The time elapsed since the last flood is a good predictor of biomass, at least in disturbance-prone systems (42). Pronounced seasonality with spring and fall maxima, similar to those observed in lakes (see next section), is encountered only in streams with relatively stable water flow. The relative importance of biotic and abiotic control of periphyton is also dependent on the hydrologic regime because grazing effects are only detected during periods of stable flow (7).

Lakes

Lake morphometry dictates the extent of the littoral zone where periphyton can develop. Vertical profiles of epipelon biomass tend to show a maximum at intermediate water depths suggesting a balance between the effects of light and disturbance by wind and waves (15). Substrata available for periphyton change with depth. Rocks and macrophytes, especially in lakes with gentle slopes, prevail in the shallow littoral zone (eulittoral), whereas only fine sediments are found in deeper water. Diatoms and filamentous greens *(Cladophora*, *Ulothrix*, *and Oedogonium* in hard water and Zygnemataceae in soft water) are the dominant algae in the eulittoral. Epipelon of the deep littoral zone is usually dominated by mobile diatoms and blue greens (43) along with a significant presence of sedimented planktonic algae (15).

Temporal trends are less affected by meteorologic events in lakes than in streams. Typical patterns in temperate oligo and mesotrophic lakes resemble those observed for phytoplankton with spring and fall peaks in biomass, dominated by diatoms, and lower biomass in midsummer dominated by green and blue green algae (44,45). These trends could be related to higher nutrient availability and lower grazing at the beginning of the growing season. Higher grazing pressure in midsummer could lead to the predominance of larger, less palatable algae such as greens and blue greens. An alternative hypothesis for the wane of diatoms in midsummer could be silica limitation. Summer periphyton maxima are encountered in eutrophic sites following large growth of filamentous green algae (44).

Wetlands

Wetlands provide abundant colonizable substrata to periphyton because they are shallow with abundant macrophytes. The relative importance of the three main benthic communities (epipelon, epiphyton, and metaphyton) is regulated by hydrodynamics, stability of the water column, and nutrient supply. Macrophytes play a major role in these ecosystems by providing substratum for epiphytes and conversely reducing epipelon through shade and competition for sediment nutrients. Wind action can affect water turbidity and therefore the light regime. However, fluctuation in water levels is the overwhelming factor controlling algal distribution. On the basis of several years of research in Manitoba wetlands, Goldsborough and coworkers (46) generalized patterns generated by hydrodynamics. A dry state with epipelon dominance follows a period of drought. With increasing water level, an open state is reached in which development of emergent and submerged vegetation is important and accompanied by significant epiphyton growth. This state is the most typical in wetlands. Further increase in water level leads to the lake state in which macrophytes and epiphytes are reduced, whereas phytoplankton becomes prevalent. Nutrient supply also affects this succession by stimulating the massive development of metaphyton. The shading produced by these floating algae brings about the demise of plants and their epiphytes. At this stage, a switch between metaphyton or phytoplankton dominance is dictated by exposure to winds because turbulence reduces the persistence of floating algal masses.

IMPORTANCE OF PERIPHYTON IN THE METABOLISM AND FOOD WEB OF AQUATIC ECOSYSTEMS

To evaluate the importance of periphyton in the metabolism of aquatic ecosystems, it is necessary to compare its production with inputs of other sources of organic material. In streams, the importance of periphyton production (autochthonous carbon) has long been underestimated because early studies (47) in small, shaded reaches pointed out the overwhelming importance of organic material contributed by the surrounding riparian zone (allochthonous carbon). Later studies demonstrated that autotrophy can contribute a significant amount to the carbon budgets of stream ecosystems. Longitudinal trends in ecosystem processes were examined along a sub-Arctic river system (48). The importance of periphyton production was low in a first order station (4% of total) but increased with stream size to reach 29% in a fifth order reach. Biome type also affects the importance of autochthonous versus allochthonous contributions to river metabolism. Autotrophy is higher in arid regions where riparian vegetation is reduced relative to woodlands; deciduous forests promote more in-stream production than coniferous areas (49). A study of a New Zealand grassland river stressed the importance, besides longitudinal patterns, of hydrologic interannual variations. Autotrophy was prevalent at all stations during a year when water level was low and turbidity reduced, whereas respiration prevailed in the downstream stations during a year of high discharge (50).

Periphyton production can account for a significant portion of total lake production. The relative importance of periphyton compared with phytoplankton and macrophytes varies relative to basin morphology, shoreline development, depth of the euphotic zone, and nutrient loading. (51) Among the data summarized by Wetzel (52), the lowest percentage (1%) of the total production was measured in Canadian Shield lakes with an extremely reduced littoral zone, whereas up to a 70% contribution was noted in Lawrence Lake (Michigan) where epiphyton is highly developed on emergent and submersed vegetation. In a detailed study of primary production in a

Manitoba wetland (53), metaphyton was usually the dominant algal producer (mean 69.5%); the importance of epiphyton was maximal (25%) when the water level was low (7–28 cm), whereas the percentage of phytoplankton production peaked (12%) at high water level (67–88 cm).

Periphyton competes for available resources with other primary producers. Dense growth of epiphytes can limit production of host macrophytes by reducing carbon uptake and light intensity (54), whereas high phytoplankton biomass can similarly decrease light available to periphyton. Nutrients released from sediments can be incorporated by epipelon and prevent their utilization by phytoplankton (1). These interactions can explain patterns observed in several aquatic ecosystems consequent to enrichment. In shallow English lakes, it is likely that shading triggered a macrophyte decline by increasing epiphyton biomass during progressive enrichment leading to excessive development of phytoplankton and the demise of benthic communities (55). Along a productivity gradient of Antarctic and temperate and sub-Arctic Swedish lakes (Fig. 2), periphyton algal biomass was negatively correlated with phytoplankton biomass (measured as light extinction in the water column) when light extinction coefficient was greater than 1.5/m. Maximum periphyton biomass was reached at intermediate light extinction in which neither light nor nutrients were probably limiting. At low light extinction (<0.5/m) typical of oligotrophic lakes, periphyton and phytoplankton were probably limited by nutrients (56). Enrichment in oligotrophic lakes seems initially to favor periphyton over phytoplankton. Phosphorus enrichment following forest harvesting and wildfires stimulated periphyton biomass more than phytoplankton in oligotrophic Canadian Shield lakes (57).

The balance between primary producers can be changed by disturbances other than eutrophication. In an alpine lake, increased UV affected epilithon more than epipelon and phytoplankton. The differential response between epipelon and epiphyton was possibly related to the capacity of epipelic motile cells to find refuge in sediments (29).

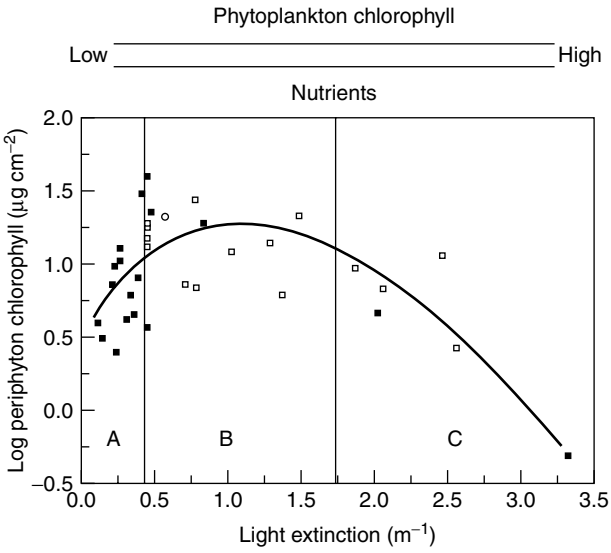

Figure 2. Variation in periphyton biomass along a productivity gradient expressed as light extinction in the water column. Samples are from Antarctic (closed symbols, n = 18) and Swedish lakes (open symbols, n = 16). Modified from L.-A. Hansson, *Limnol. Oceanogr.* **37**, 322–328 (1992) with permission.

Several studies suggest that periphyton is used by consumers in a disproportionate manner relative to its contribution to biomass and production of aquatic ecosystems. In Bear Brook (New Hampshire), where 99% of the energy input is allochthonous, an abundant caddisfly depended on periphyton for most of its growth (58). On the basis of analyses of diets of invertebrate (59), it has been traditionally thought that epiphyton was more available to consumers than the plant on which it grows. This hypothesis, sometimes challenged (60), has now been confirmed by a compilation of literature data on stable isotope ratios for several aquatic ecosystems. The consumer ratio more closely approximates epiphyte values than those of vascular plants (61). Similarly, a study of stable isotopes in fish in several tropical, temperate, and subarctic lakes indicated that the contribution of benthic algal productivity to food webs was substantial even in lakes where phytoplankton clearly dominated total carbon production. This discrepancy between the relative importance of periphyton and phytoplankton in production and food web contribution could be related to consistent methodological underestimation of benthic production. Possibly, the mismatch could indicate that periphyton concentrated on a substratum offers a more energy-efficient diet than dilute phytoplankton (62).

Not all periphyton production is directly used by grazers. Upon sloughing, a part of the detached algae can contribute to detritus or sometimes can remain in the water column and be used by filter feeders. Export rates have been rarely quantified and mostly in laboratory streams. In these systems, it has been demonstrated that grazers, by their somewhat wasteful feeding activity, dislodge algae and increase their export downstream compared with ungrazed controls (35).

CONTROL OF PERIPHYTON COMMUNITIES

Periphyton biomass present at any given time is the result of accrual through immigration and growth minus loss due to grazing and sloughing. Various variables are crucial in controlling these processes, with their importance changing according to environmental conditions. Geomorphology (i.e., stream order, lake morphometry) sets the primary control on periphyton, dictating the colonizable substratum and setting the context in which all other variables interact.

Temperature

Despite the importance that temperature exerts on all biological processes, its effect on periphyton communities has received less attention than that of other variables. Seasonal changes in biomass and composition cannot easily be ascribed directly to temperature because of the interactions with other factors such as nutrients, light, and grazing.

In an analysis of published studies of stream epilithon in situ and in the laboratory, maximum areal primary

production was a function of temperature up to 30 °C (63). Temperature sets the upper limit of production when other factors are not limiting.

Light

Because periphyton consists mainly of primary producers, light is a fundamental variable controlling its distribution and abundance. Relationships between available light and algal production are studied through the irradiance-photosynthesis curve (I/P). In most studies, photosaturation, or the light intensity at which photosynthesis is maximal, is encountered over a rather narrow range from 100 to 400 μ mol/m^2/s (64). Photoinhibition at high light intensity is more likely to be encountered in periphyton communities from shaded sites. These communities show shade adaptation and outperform communities from open sites at low irradiance (65). Photosynthesis-irradiance responses change with the thickness of the periphytic mat. The onset of photosynthetic saturation and photoinhibition occur at higher irradiance as periphytic mat thickness increases. With light microsensors, it is possible to demonstrate light attenuation across the mat, which varies not only with mat thickness but also with its composition (66).

In streams and small rivers, light availability is regulated by riparian canopy and, at least for streams flowing through deciduous forests, it changes seasonally. The effect of riparian canopy diminishes as the stream becomes wider. In large, deep rivers and in lakes, light decreases with depth differentially depending on water turbidity, color, and phytoplankton development. Periphyton growth can occur under ice cover or where light is severely limited by riparian vegetation or depth. This capacity to withstand extremely low light possibly could be linked to temporary heterotrophy (67).

Relationships between light and periphyton biomass are not always clear. In some instances (65,68), the positive effect of light is counterbalanced by grazing: increased illumination gets translated as increased grazer growth. Where grazing appears negligible, a poor relationship of light–biomass could be ascribed to other mechanisms. In streams, sun flecks can provide transient but intense illumination to shaded portions of the streambed (64). In lake littoral areas, at shallow depth where illumination is maximal, wave disturbance can counteract any positive light effect (15). There are some general patterns in the response of different algal classes to light. Chlorophytes, in particular filamentous green algae, are usually found only in well-illuminated sites, whereas diatoms, cyanobacteria, and rhodophytes seem better able to tolerate low light conditions (64).

Recently, because of the thinning of the ozone layer, UV radiation has increased and with it, interest in its effect on periphyton communities. In artificial stream channels in British Columbia, periphyton communities shielded from UV initially grew faster than unshielded controls. However, the effect on periphyton biomass was later reversed because chironomid grazers were reduced in UV-exposed channels (69). UV inhibition is likely to be severe particularly in extreme environments such as mountain lakes and streams (70,71). In these studies, periphyton biomass was reduced and taxonomic composition was altered. Diatoms were particularly sensitive to UV radiation, whereas cyanobacteria became dominant probably because of their ability to produce carotenoids as protection against UV damage (70). Production of mucopolysaccharides also conferred protection to some algae including *Hydrurus* and tube-dwelling diatoms (71).

Nutrients

The relationship between nutrients and periphyton has been examined in many experimental studies and through comparison of natural communities along nutrient gradients. In experiments, one or several nutrients are added to artificial channels or, more rarely, dripped directly into a stream. Increasingly, studies are using nutrient-diffusing substrata. Phosphorus and nitrogen are the most studied nutrients (72). Because most studies are from the temperate zone, in particular North America, findings cannot be generalized to tropical or arctic regions. Phosphorus is most often the nutrient-limiting periphyton growth, but, in regions where soils are phosphate rich, such as the southwestern United States, nitrogen can be limiting. Often colimitation by phosphorus (P) and nitrogen (N) is observed. As for other algal communities, limitation by phosphorus or nitrogen can be predicted from ambient N: P ratio. When this ratio is greater than 20, phosphorus limitation is expected, whereas a ratio less than 10 results in nitrogen limitation, and values of 10 to 20 reflect intermediate limitation.

Few studies have quantified the limiting concentrations for periphyton. For phosphorus, Bothwell (73) noticed in artificial channels that a concentration of 0.3 to 0.6 μg/L was sufficient to saturate growth of a thin diatom community. This growth-saturating concentration for benthic diatoms is of the same order of magnitude as observed for planktonic diatoms. Filamentous chlorophytes seem to require higher phosphorus concentration than diatoms to saturate growth. Because thick benthic mats impede nutrient diffusion, much higher phosphorus concentrations (25 to 50 μg/L) are needed to obtain maximum areal algal biomass (73).

Increased nutrients do not always translate into higher periphyton biomass because of the confounding effects of light, disturbance, and grazing. Variance in the relation between nutrient and biomass could also be linked to growth on a surface. Substratum saturation sets an upper limit to the amount of biomass that can develop before sloughing losses occur. Despite this noise in the response of periphyton to nutrients, it was possible to distinguish impoverished streams flowing through forested catchments from enriched streams in agricultural catchments on the basis of their periphyton biomass (7) (Fig. 3). The taxonomic response to nutrient status is more consistent than the response to biomass, and some general patterns are observed. Large algae, in particular filamentous greens (e.g., *Cladophora*, *Stigeoclonium*), tend to be more important in rich habitats. Epithemiaceae diatoms, which harbor cyanobacterial endosymbionts and probably fix atmospheric nitrogen, tend to become more important in N-limited habitats (74). In phytoplankton, phosphorus enrichment usually results in dominance

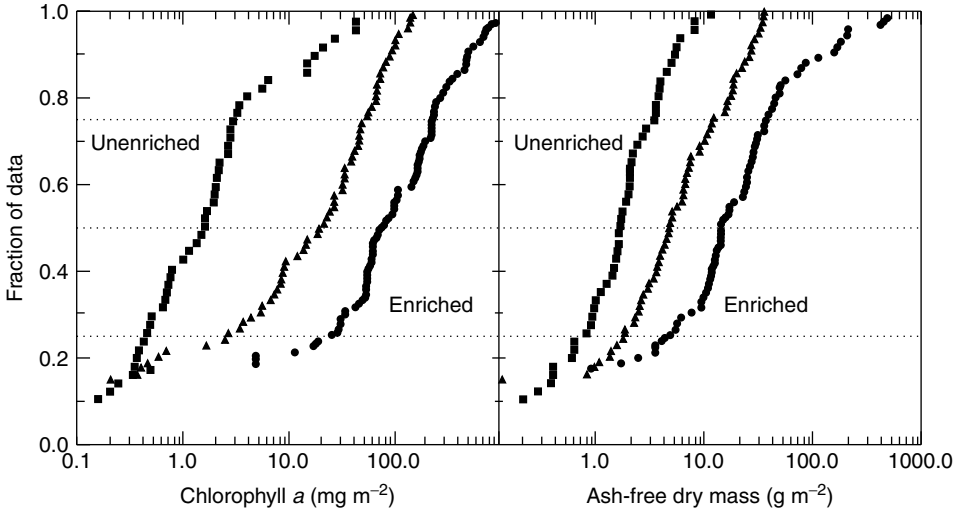

Figure 3. Cumulative frequency curves for periphyton chlorophyll and ash-free dry mass from unenriched (squares), moderately enriched (triangles), and enriched (dots) New Zealand streams, sampled monthly for a year. From B. J. F. Biggs, in R. J. Stevenson, M. L. Bothwell, and R. L. Lowe, eds., *Algal Ecology, Freshwater Benthic Ecosystems*, Academic Press, San Diego, Calif., 1996, pp. 31–56 with permission.

by cyanobacteria, whereas this is seldom the case in periphyton.

Hydrodynamics

Water currents are more obvious in streams and rivers where there is a rather striking alternation of riffles and pools. In the littoral zones of lakes and even in wetlands, however, there are currents generated by wind and waves. Water movement has potentially opposite effects on periphyton accumulation. Current favors nutrient exchanges by decreasing the thickness of the boundary layer around the algae. Increased phosphorus uptake, photosynthesis, and respiration have been observed with increasing current (75). This positive effect is counterbalanced by the negative action of shear drag produced by the current. Drag can detach part of the community from the substratum; filamentous, stalked, and apically attached algae are especially sensitive to such stress (41). Current also negatively affects algal immigration (76) while increasing algal export. Optimum current for different communities and environments is dictated by the equilibrium of these opposing effects of water motion. Maximum periphyton accrual is usually observed at intermediate velocities (10–50 cm/s). In general, saturating current velocity is higher for thick periphyton communities than for thinner ones (75). Evidence from both natural streams and artificial channels indicates that optimum current velocity decreases with nutrient concentration and declining light availability (75,77). Current has a destructive effect during a flood when it increases rapidly in orders of magnitude over baseline conditions. As discussed in the previous section on periphyton distribution, flood frequency is a crucial factor controlling stream periphyton (42).

In lakes, waves generate currents, which can have negative effects on periphyton accumulation. In a Nebraska reservoir, wind-induced turbulence led to a 61% decline in diatom density (78). However, a stimulating effect of wind-induced currents was observed when comparing periphyton biomass in exposed and protected sites along an island in a Quebec lake. This effect was significant at least during the early summer biomass peak (79). In both examples, motile algae were reduced in exposed sites, whereas stalked or rosette forms, which adhere to the substratum by mucilage secretion, were more resistant to wave turbulence.

Substratum

Because periphyton is by definition an attached community, substratum is an important variable in regulating its abundance and composition. Periphyton researchers have studied the effects of size and type of substratum. Size is important because it controls the stability of substrata both in streams and in lake littoral zones. Large rocks are not dislodged by storm events and favor colonization by filamentous and stalked algae. These growth forms are rarer on fine sediments in which motile algae that can escape burial are favored. Rocks are usually more exposed to current and associated water renewal. However, algae on fine sediments have access to groundwater and nutrients released from sediments. When communities on substrata of different sizes are compared, the data are somewhat contradictory. Periphyton is often better developed on large stable substrata (80), but sometimes the greatest biomass exists on fine substrata (81). Flood history before sampling probably explains these discrepancies (7). In a comparison of two different substratum types, periphyton production on fine sediments was 10 times more than that measured on wood in two Michigan lakes (82).

Complex and potentially important interactions with the substratum can be hypothesized for epiphytes that grow on living plants. Nutrient transfer between the host plant and its epiphytes is common (2). However, the importance of this transfer for epiphyton growth is still debated. In the mesotrophic Lake Memphremagog (Quebec), epiphyte biomass and production were similar on natural plants and plastic mimics (83). Interactions between plant and epiphytes seem more important in oligotrophic lakes where nutrients released by plants could provide a significant advantage to attached algae (45,84). Different

macrophytes can harbor epiphytic communities with differing biomass and composition. Allelopathic substances have been isolated from plant tissue (85) and could explain these differences. However, most available evidence suggests that differential colonization is related to the physical structure of the host plant. Macrophytes with dissected leaves tend to have a thicker coating of epiphytes probably because they provide better light and water renewal (86,87).

Grazing

Periphyton constitutes an important source of food for several aquatic animals. It is richer in proteins and lipids than detritus and more easily ingested than plant tissues. Taxa feeding mostly on periphyton are called *scrapers* and have specialized mandibles or bristles to detach the algae (e.g., some caddisflies and mayflies) or a rasping radula in snails. Less specialized invertebrates also are able to use periphytic algae easily dislodged from the substratum. Most studies on grazing focus on macroinvertebrates; however, meiofauna (protozoans, small oligochaetes, and chironomids) can also be important (88,89). Among the vertebrates, tadpoles (90) and fish (91), particularly in tropical streams (92), can feed on attached algae.

The large number of grazing experiments conducted in the laboratory and nature during the last 20 years has been recently reviewed (35,93). In experiments of grazer addition or exclusion, periphyton biomass was reduced by grazers in 70% of the cases. Grazing also decreased productivity but less consistently (60% of experiments). Grazing strongly affected the taxonomic composition of periphyton assemblages (81% of experiments). In particular, the physiognomy of the community changes with a decrease in percentage of overstory forms in response to grazing because they are more vulnerable to direct ingestion or dislodgment by grazers. From these reviews of grazing experiments, it appears that periphyton in lakes and streams is regulated by herbivory at least, if not more frequently than primary producers in other ecosystems (93).

RESPONSE OF PERIPHYTON TO ANTHROPOGENIC STRESSES

Eutrophication

Human activity, especially since the start of the industrial revolution, has resulted in increased inputs of nutrients to lakes and streams. In particular, nitrogen and phosphorus concentrations in inland waters have increased because of intensive agriculture, fossil fuel combustion, and wastewater disposal. There is a remarkable consistency in the response of planktonic algal biomass to nutrients. This has led to successful models to predict the effect of eutrophication on lake plankton (94). The response of periphyton to increased nutrients is less clear. Therefore, establishing guidelines for the management of stream and rivers where periphyton is the major primary producer is more problematic than for lakes.

As noted in the section on nutrients, in benthic communities, the effect of enrichment can be restricted by the importance of other factors such as available light, hydraulic perturbations, and grazing. For example, when the degree of eutrophication is relatively low (<50 μg total P/L), it can be translated into increased invertebrate biomass rather than periphyton proliferation (95). When enrichment progresses, it is accompanied by the appearance of nuisance algae that are aesthetically displeasing and interfere with recreational activities of fishermen, swimmers, and boaters. This nuisance level is usually reached when periphyton biomass exceeds 100 mg/m^2 of chlorophyll (96). Filamentous green algae, in particular *Cladophora*, are often associated with such nuisance growths in both streams (7) and lake littoral zones (97).

From a large comparative analysis of stream enrichment studies, Dodds and coworkers (98) suggested that target nutrient concentrations necessary to avoid proliferation of nuisance algae are 350 μg/L of total nitrogen and 30 μg/L of total phosphorus.

Acidification

In many areas of the world with poorly buffered soil, inland waters have been affected by acid rain. When water acidification is severe (pH$<$5.5), the whole food web is affected. Although this disturbance typically reduces the growth and abundance of aquatic organisms, periphyton biomass tends to increase in acidified lakes (99) and streams (100). Taxonomic composition is also strongly influenced by acidification with the appearance of dense growths of filamentous chlorophytes of the order Zygnematales. Shifts in diatom species are also observed, with the genus *Eunotia* consistently favored at low pH. The casual link between these changes and acidification has been confirmed by similar responses observed in experimentally acidified streams, artificial channels, and in whole-lake acid manipulations.

Abiotic and biotic mechanisms have been invoked to explain these responses in periphyton biomass and composition. Biomass stimulation is often ascribed to increased water transparency found in acidified systems because of phytoplankton reduction or changes in dissolved organic carbon concentration (101). Partial release from grazing pressure due to the sensitivity of many invertebrates to acidification has also been hypothesized (99) but not always confirmed (102). Alternatively, periphyton would benefit from reduced competition for phosphorus with bacteria, whose abundance is decreased by acidification (102). The growth of filamentous Zygnematales is possibly related to their capacity to obtain dissolved inorganic carbon at the low concentrations found in acid waters. This competitive advantage allows these algae to develop blooms and become a nuisance for swimmers and boaters. These algal proliferations are considered an early warning for lake or stream acidification because they usually appear when pH becomes less than 5.5 (103).

Metal Pollution and Other Contaminants

Mining activities in many areas of the world have led to high metal concentrations in some aquatic environments. If trace concentrations of metals are beneficial to algae, high concentrations found in disturbed sites interfere with

their metabolism. The effect of metals has been studied either by comparing impacted and control sites or by experimentally adding different metals to artificial channels. In general, metal stress affects species composition, more rarely community biomass and metabolism (104). Several studies have observed that green algae are particularly tolerant of high metal concentrations. In streams draining a mining region in Cornwall (105), filamentous green algae, particularly *Microspora*, were dominant. Changes in diatom communities are also observed; dominance of *A. minutissima* occurs in several polluted environments (106,107). The presence of deformed diatoms has been reported several times in the presence of metal pollution in both streams and lakes (108,109). These morphological abnormalities, probably linked to metal interference in silicic acid uptake and amino acid synthesis (110), could provide an easy tool to monitor metal pollution.

Organic toxicants enter lakes and rivers from agricultural (e.g., insecticides, pesticides) and industrial (e.g., detergents, dyes, resins) activities. The effects of these substances have been studied more on planktonic rather than on benthic algal communities. Organic toxicants, particularly herbicides, affect the composition of periphyton more than its biomass or production. Diatoms are generally more resistant to such contamination than other algal groups. Species diversity is often lowered in polluted sites. (111).

Climatic Changes

Because of global warming, significant climatic change is foreseen for some decades ahead. In addition to temperature changes that could influence the seasonality of periphyton and their grazers, increased variation in precipitation is predicted (112). This meteorologic instability could lead to more frequent floods and droughts even in regions where water flow has been rather predictable earlier. Hydrologic control would prevail over that from grazing or nutrients. For many systems, a drastic reduction in water level has been forecast. For example, a 1 m lowering of the base level of the St. Lawrence River has been forecasted for the next decade. This change is likely to favor the development of emergent vegetation compared with submersed plants (113). Such a vegetation shift could affect epiphyton development, because the simple stems of emergent plants offer less colonizable surface than submersed macrophytes. In marshes, severe drought, or conversely, periods of unusually high water would alter the balance between epipelon, epiphyton, and phytoplankton (46).

Biomonitoring

The ability of periphyton communities to respond to multiple anthropogenic stresses makes them useful for monitoring water quality. Biomass and production are usually less affected by disturbances than community composition. Therefore, most indices are based on analysis of taxonomic composition. Diversity indices are often calculated, but they do not always decrease in consequence of stress (114). Diatom analysis is the most used for routine monitoring, because these algae are often dominant, readily sampled, and relatively easy to identify. Moreover, information on the tolerance of many diatom taxa is available in the literature. Earlier indices were mainly based on the response of a limited number of indicator taxa to organic pollution. These indices have been subsequently refined both to include more taxa, scored according to their sensitivity and distribution, and to address different types of pollution. Their use has traditionally been more widespread in Europe (115). Numerical approaches using canonical correspondence analysis and weighted average regression are now gaining popularity because they use the entire assemblage to extract information on environmental conditions (116). Despite the traditionally greater popularity of biotic indices based on invertebrates, biological assessment programs in North America are starting to include diatom indices.

Besides taxonomy-based indices, other properties of periphyton appear promising for monitoring. Fatty acid composition has been used successfully to follow stream pollution (117). Size distribution responds to anthropogenic stresses related to nutrients and contaminants usually with shifts toward large algae (118).

Another use of periphytic algae in monitoring relies on their capacity to concentrate a range of substances in their cells. Most accumulation studies have focused on heavy metals, but accumulation of pesticides has also been examined. Coarse filamentous algae such as *Cladophora* have been especially used for this approach (115).

CONCLUSION

In streams, lakes, and wetlands, periphyton is an important primary producer, which is readily available to grazers. Despite periphyton importance in aquatic food webs, the complexity of this attached community has retarded its description and quantification. Further knowledge of periphyton response to anthropogenic stresses is crucial for setting guidelines for management and for effective monitoring of disturbance and recovery of inland waters.

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PERMAFROST

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Permafrost represents a stable environment that has allowed the prolonged survival of ancient microbial lineage at subzero temperatures. Significant numbers, 10^2 to 10^8 cells per one gram of dry soil (cells/gdw), of viable ancient microorganisms are known to be present within the permafrost. They have been isolated from the cores up to 400 m deep in the Canadian Arctic and at the lowest ground temperatures in Antarctica. The age of the cells corresponds to the longevity of the permanently frozen state of the sediments. The oldest viable cells date back to two to three million years (Myr) in northeastern Siberia, probably older in Antarctica. They are the only organisms known to have retained viability over geologic time periods and, upon thawing, renew their physiological activity. Therefore, the permafrost can be characterized as a unique physical-chemical complex, which can maintain life incomparably longer than any other known habitat.

Permafrost thawing as a result of anthropogenic or natural processes exposes ancient life to modern ecosystems.

The ability of microorganisms, the most ancient life-forms on the Earth, to live in a variety of natural environments has substantially modified our ideas about the viability of microorganisms and continually forces us to redefine the limits of both life and the biosphere. Microorganisms not only survive under conditions that seem unsuitable for life, but they also succeed in maintaining relatively large populations and a high diversity. The soil cover, where they are well studied, is two to three meters thick. Significant numbers of microorganisms also occur in subsoil horizons, which are much thicker than soil. In these subsurface layers microorganisms have been found at depths up to five kilometers (1–3). The discovery of microorganisms in these deep layers has caused the active development of the field of subsurface microbiology over the last two decades. However, outside the permafrost regions, even when working in the least permeable strata, one cannot completely exclude the penetration of viable bacteria from the surface. Therefore, it is impossible to determine the age of microorganisms isolated from cores from these regions, and the reports of viable microorganisms of Precambrian, Paleozoic, and Jurassic age (4–6) were not accepted with due seriousness. Moreover, in finely dispersed impervious strata viable aerobes and anaerobes only exist at depths of up to a few dozen meters (7).

The most fundamental aspect of any environment, the temperature regime, acts as a regulator of all of the physical-chemical reactions and forms the basis of all biological processes (8). More than 80% of the Earth's biosphere is both permanently cold and subjected to significant temperature fluctuations. Because we inhabit a cold planet, cold adaptation of microorganisms would appear to be an important trait. Because of their ability to cope with low temperatures, cold-adapted microbes, first isolated by Forster (9), can be regarded as Earth's most successful colonizers (10). They have adapted to the cold and populate now the all main ecological niches.

A significant volume of the Earth's cold environments consists of permafrost (perennially frozen ground) — a naturally occurring material with a mean annual temperature below 0 °C (11) and a portion of its moisture in the form of solid ice. Permafrost underlies about 20% of the Earth's land surface mostly in the northern reaches of North America and Eurasia (Alaska, Siberia, and Canada). In these regions, the permafrost reaches a thickness of more than 700 to 1,000 m in the north, thinning toward the south. It also occurs in the ice-free regions of Antarctica and Greenland and surrounding Arctic and Antarctica as offshore permafrost. Alpine permafrost can be found in the high mountains of Europe, western China, and both Americas. This considerable mass of frozen soil, up to several hundreds of meters deep, harbors a very large numbers of viable bacteria.

In the permafrost below the seasonally thawed layer, are numerous and various viable paleomicroorganisms (Fig. 1), prokaryotic, and eukaryotic, independent of the depth, with numbers ranging from 10^2 to 10^8 cells/gdw (12–15). This great mass of living matter, in quantities comparable to those of microbial populations in the soil cover, is peculiar to permafrost sediments. If we take into account the thickness of the deep permafrost layers, it is easy to conclude that they contain a total microbial biomass many times higher than that of the soil. Of all the natural environments, frozen fine-grained sediments, firmly bound together by ice, forms a unique system that seems to ensure the viability of microorganisms. In this isolated system, both the age of the microorganisms and the absence of an influx of alien microorganisms, are demonstrable.

The later was not fully realized until recently. The first data on the existence of bacteria in permafrost appeared at the end of the nineteenth century, in relation to the discovery of mammoths and studies of soils in Siberia (16,17). Five decades later, separately, and sometimes unrecognized by each other, microbes were discovered in Holocene and late Pleistocene permafrost of many Arctic regions (18–26). Viable cells in more ancient frozen sediments were found in association with the Antarctic Dry Valley Drilling Project (27). In all of these studies (3,15,28), the procedures and the application of drilling solutions did not guarantee the sterility of cores. Because of these methodological and technical difficulties, the earlier-mentioned reports were not considered with due attention, and the permafrost as a living stratum was not studied. Nevertheless, the authors of these early studies first raised the question of the possible preservation of viable cells in the permafrost. As early as 1975, Pewe (28) emphasized the need for further research in this field.

In recent investigations, new methods of sampling, storage, transportation, and control, as well as new specialized tests, have shown that the microorganisms seen in permafrost samples could not have penetrated from the outside, but are present in situ. The strict protocols for drilling and the subsequent handling of cores are designed to ensure uncontaminated material. These protocols (29,30) include the drilling techniques that do not require the use of drilling fluids and the aseptic methods to shave the cores before sampling.

The occurrence of a viable Cenozoic generation of microorganisms within the permafrost is intriguing because an analysis of their features may provide a window into microbial life as it was before the impact of humans. The recovered organisms may possess unique mechanisms that allow them to maintain viability for very long periods. They are easily involved anew in present-day ecological processes after instances of permafrost thawing, and may be vital in nutrient recycling and in the production and consumption of greenhouse gases over a large portion of the Earth's surface (15).

The viable organisms in the Earth's Cryosphere represent a significant part of Biosphere, the Cryobiosphere (Fig. 2), whose most inhabited part is the permafrost. The remoteness from anthropogenic impacts and the stability of the subzero temperature regime in the subsurface permafrost layers make it one of the more stable and balanced of the natural environments.

This environment provides life support and ensures the formation of microbial communities that realize unknown possibilities of physiological and biochemical adaptation to prolonged cold and remain virtually stable for millions of years. Evidently, the long-term impact of moderately subzero temperatures should be regarded not as the extreme and limiting but rather as a stabilizing factor supporting the viability of microorganisms adapted to these conditions. It is suggested that the mechanisms of such adaptation are universal and operate within the broad limits of modification for heterophasic natural systems.

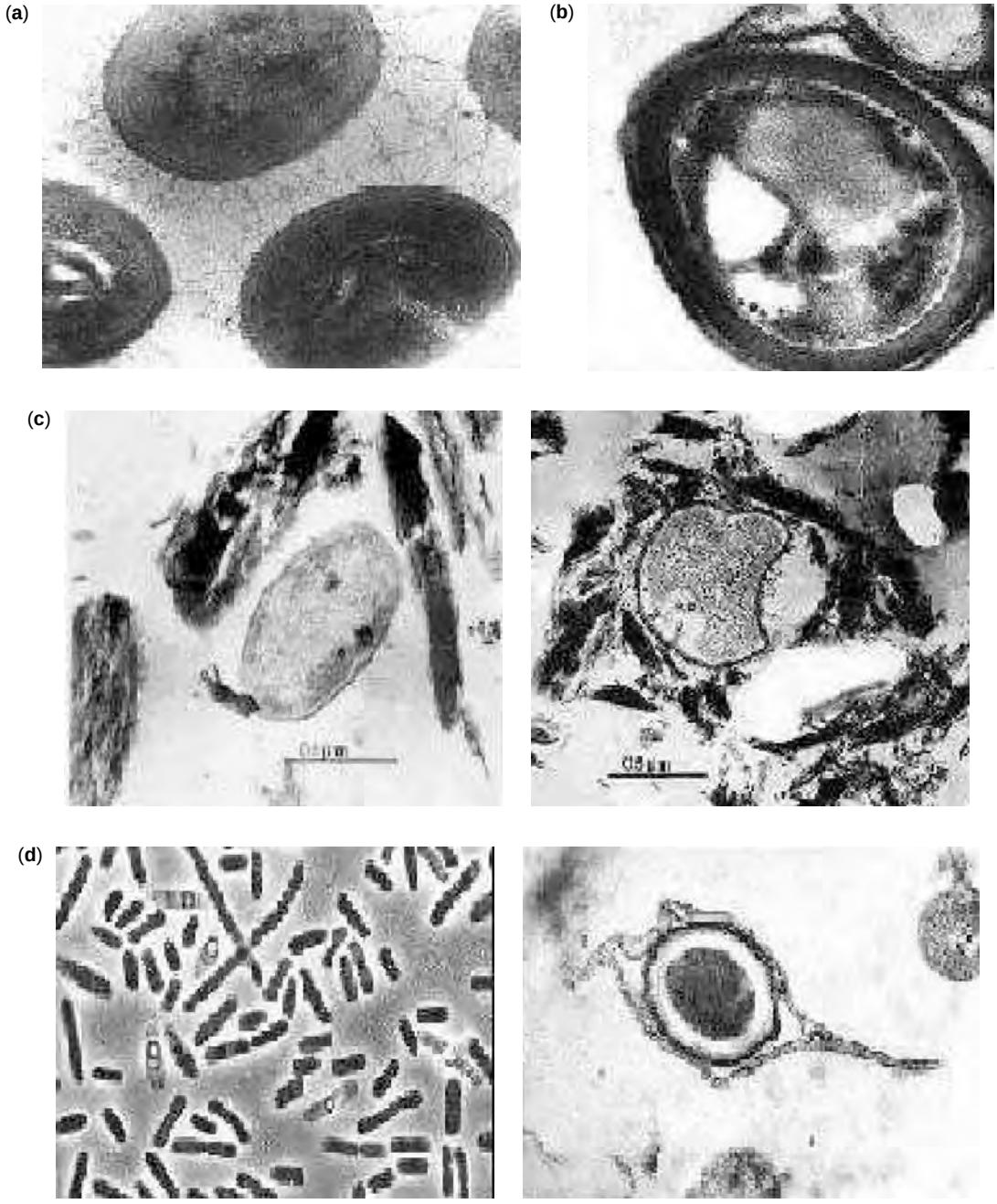

Figure 1. Permafrost biodiversity (examples). Viable aerobic bacteria: (**a)** Gram-positive; (**b**) Gram-negative (*Micrococcus* sp.); (**c**) rich culture of anaerobic microorganisms; (**d**) anaerobic psychrophyles from the lenses of supercooled water brines; ultrathin sections of isolated yeast: (**e**) *Sporobolomyces* sp.; (**f**) *Cryptococcus albidus*; Phototrophs: (**g**) green algae (*Clorococcus* sp.); (**h**) cyanobacteria (*Anabaena sp*.); Micromycetes: (**i**) *Penicillium brevicompactum*; (**k**) *Alternaria alternata* (Authors of isolates: R. Faisutdinova, N. Ivanushkina, G. Kochkina, S. Ozerskaya, E. Rivkina, A. Shatilovich, V. Shcherbakova, V. Soina, E. Spirina, N. Suzina, T. Vishnivetskaya).

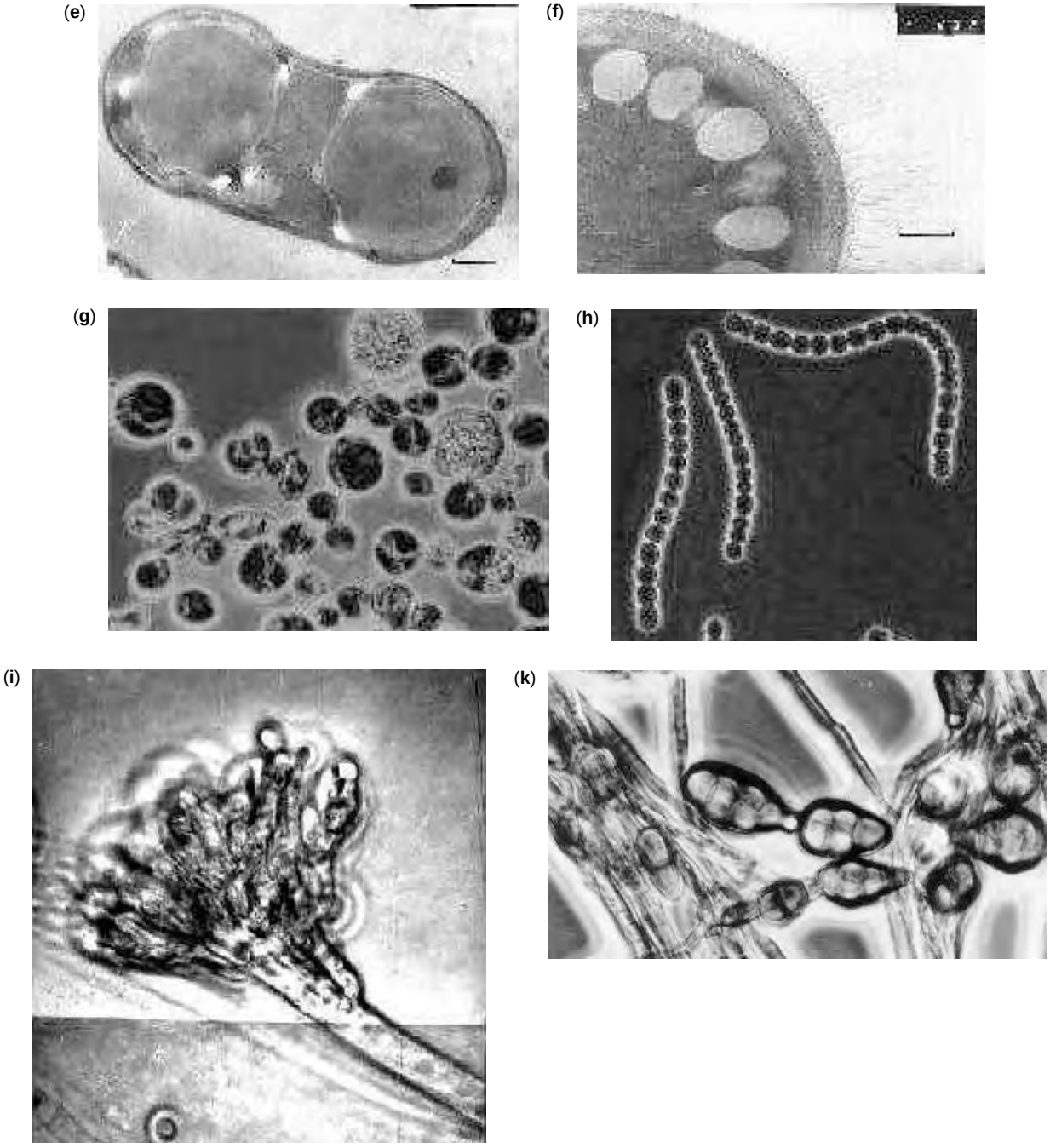

Figure 1. (*Continued*)

PARAMETERS OF PERMAFROST MICROBIAL HABITAT

Active Layer

In Arctic tundra, seasonal thawing starts in June and reaches its maximum (0.3 m in peat, 0.5 m in loam, and 1 m in sands) in mid-September. In forested tundra, the depth of the thaw in mineral soils increases by 20 to 70 cm, whereas in peat, it remains constant. Near the southern border of the permafrost, the maximal depth of the thaw reaches 2 to 3 m. The surface beneath the seasonal thaw layer is called the permafrost table. This is the physical and biogeochemical barrier that sharply restricts the influence of external factors and processes. The underlying cross section is firmly cemented, often totally filled in by ice. In such a closed system, there are no water-bearing horizons and no water infiltration. Thus, within the permafrost, because distinct from other layers, any viable microorganisms could not have been brought in from outside but exist in situ (29,30).

Temperature

The subzero temperature in the permafrost is favorable everywhere to the preservation of biological systems, and is the main factor contributing to the long-term

<table>
<tr><td colspan="5">Earth cryosphere</td></tr>
<tr><td colspan="5">Air in the upper atmosphere layers*</td></tr>
<tr><td>Glaciosphere</td><td colspan="4">Cryolithosphere</td></tr>
<tr><td colspan="5">Seasonally surface formations</td></tr>
<tr><td>Snow & ice cover</td><td colspan="4">Soil cover</td></tr>
<tr><td>10^1 cell/ml</td><td colspan="4">10^7-10^8 cell/g</td></tr>
<tr><td colspan="5">Long-term subsurface formations</td></tr>
<tr><td>Ice sheets & Glaciers</td><td>Ice veins</td><td>Rocky (dry) permafrost</td><td>Cryopegs</td><td>Permafrost</td></tr>
<tr><td></td><td></td><td>Overcooled rocks without ice</td><td>Free water brines within or below permafrost</td><td>Ice cemented soils with permanent temperature below freezing point</td></tr>
<tr><td>10^1 cell/ml</td><td>not found</td><td>not determined</td><td>10^2 cell/ml</td><td>10^3-10^8 cell/g</td></tr>
<tr><td colspan="5">Cryobiosphere</td></tr>
</table>

Figure 2. Components of the Earth's Cryobiosphere as a microbial habitat (*only atmosphere layers to constitute the medium of microbial transfer, but not a habitat).

survival of cells. In the Arctic, the mean annual permafrost temperature (T), as registered at high altitudes, is between −10 and −12 °C (Fig. 3a), rising toward the southern border of the permafrost to between −1 and −2 °C. In Antarctic polar desert, with its background of below freezing air temperatures even in summer, sometimes the temperature at the soil or rock surface is above 0 °C (31,32), but there is no layer of seasonal thaw. From our observations, the upper sandy layers are completely dry and uncemented because of the evaporation of the snow and the sublimation of surface ground ice. As a result, they are easily blown off by storm and spring winds, and a stable soil cover is not formed (similar to the situation on sandy dunes); the permafrost table coincides with the diurnal surface covered slightly by a dry frozen layer about 10 cm thick (33). As a result of this fact and the sharp temperature oscillation on the soil surface, the upper 1 to 2 cm often contains the minimum number of viable cells in comparison with underlying horizons (34,35). A similar distribution, but on a smaller scale, was found for cryptoendolithic microbial communities on and within the Antarctic sandstone (36–38).

The maximal (−18 °C) mean annual permafrost temperature in Antarctic free ice areas, such as the Dry Valleys, is registered at low levels near the coast. This temperature decreases in accordance with the drop in air temperatures when moving inland and toward higher altitudes, reaching the lowest permafrost temperatures on the Earth, between −24 and −27 °C (Fig. 3c). The Dry Valleys permafrost, then, differs from that in Arctic in the much lower temperatures of its sediments, temperatures at which there is an obvious absence of liquid water and water-bearing horizons, and the domination of the active processes of sublimation that excludes any downward infiltration. For this reason, in the Antarctic, even more than in the Arctic, the possibility of the penetration of modern microorganisms into the frozen strata is precluded.

Iciness and Unfrozen Water

The permafrost is characterized by a specific, multiphase state of water, which plays a dual role from the biological point of view (39). The solid phase (ice) makes up 92 to 97% of total water volume in permafrost and serve as a cryoconservant for biological objects. The iciness (W_i) of sandy-loam-based frozen ground in Arctic varies from minimal values of 12 to 20% to a wide range of maximal values, 40 to 50% (Fig. 3b), or 60 to 70%, depending on the presence of ice wedges. At the higher ice contents the pores are completely filled with ice, obviously excluding any migration inside the stratum. The cryogenic structure of these sediments is usually marked by the massive cryogenic texture (segregated ice-cement), typical of sand-based strata, or by the different orientation (reticular, layered) of ice streaks 1 to 2 mm to 2 cm thick in clay- and loam-based strata. The coarse-grained Dry Valleys sands, firmly cemented into a massive cryogenic structure, also contain an unexpectedly high (25–40% or more) iciness values (Fig. 3d).

Simultaneously with the solid water phase, the remaining 3 to 8% of the water is in an unfrozen state ($W_{unf.}$), most commonly as thin films enveloping organic and mineral particles and, possibly, as brine pockets in saline soils. The dependence of unfrozen water on the physical-chemical parameters of frozen strata has been studied by the many scientific schools (40–44). The quantity of the unfrozen water and thickness of its films is independent of the total ground iciness, but decreases (Fig. 4a,b) with temperature (45,46). At Arctic permafrost ($T = -9$ to -12 °C) the magnitude of $W_{unf.}$ in a loam is 3 to 5%. In sands, $W_{unf.}$ is minimal, tending to zero, although there a silt fraction in them that retains a measurable $W_{unf.}$ (only in Antarctic sands, because of the ultra low temperatures, the $W_{unf.}$ values there are so small that the instrumental methods fail to record them). The maximal values of $W_{unf.}$ (10%) are related to saline solutions in thinly dispersed marine facies. When discussing unfrozen water films, we can be sure that the thermal diffusion of

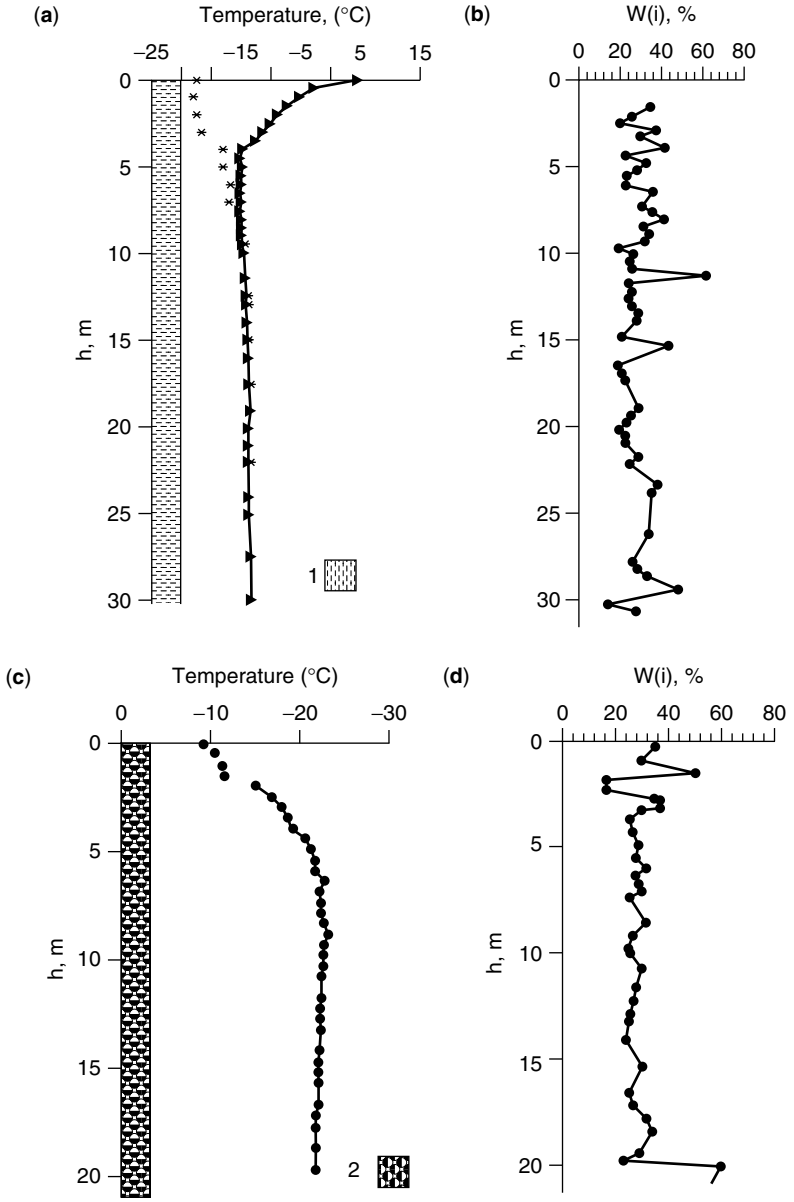

Figure 3. Ground temperatures and the iciness of permafrost sediments in high Arctic (**a**,**b**) and Antarctic Dry Valleys (**c**,**d**). Legend: 1 — sandy-loam; 2 — coarse-grained sands.

cells within the films is impossible because the thickness of these films (5–75 A°) is not comparable with the size of microorganisms, 0.5 to 1 μm (Fig. 4c), thereby precluding the migration of the cell within the unfrozen water.

In contrast to ice, the unfrozen water films serves as cryoprotectant and play a leading role in the preservation of microorganisms for the following reasons (39): these films, by coating the organomineral particles, protect the viable cells sorbed onto their surface from mechanical destruction by growing crystals of intrusive ice; these films make possible the mass transfer of microbial metabolic end-products within the permafrost, preventing the cell's biochemical death; these films may serve as a nutrient medium because the unfrozen water contains high concentrations of various ions and molecules; these films represent firmly bound "liquid" water with binding molecules, and this is why permafrost may be considered as a biologically dry environment.

The increasing content of chemical elements and salts in $W_{unf.}$ enhances their protective and transport role and their significance as a nutrient medium. Therefore, the unfrozen water might be considered as a main ecological niche where the microorganisms might survive. The validity of this assertion can be demonstrated by a comparison between the numbers of viable cells recovered from permafrost cores and the number of viable cells recovered from ice. In contrast to frozen soils with an abundance of microorganisms, the viable cells recovered from cores of the pure ice of the Antarctic Ice Sheet taken at the Vostok station (47) or Greenland Ice Sheets is on the order of a few dozens cells/ml of thawed water and increase (48) with increasing concentrations of dust

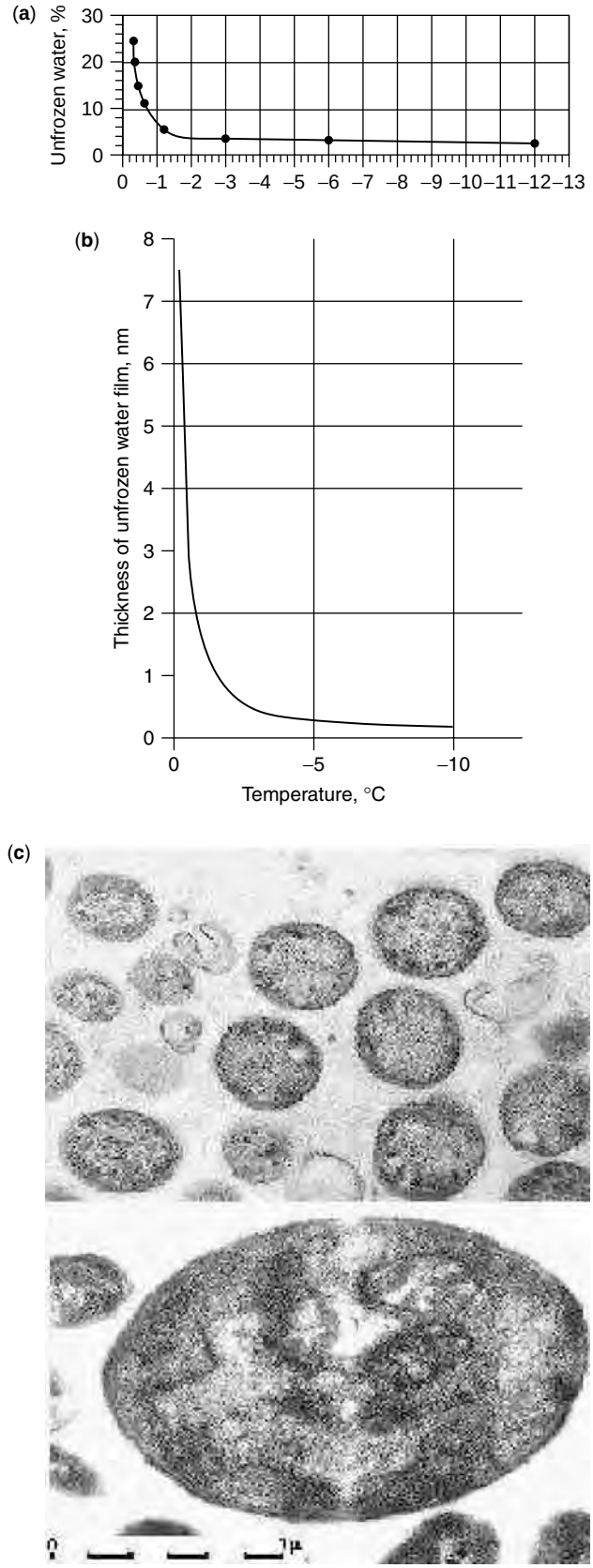

Figure 4. The size of viable microorganisms (**c**) and dependence of the (**a**) unfrozen water content ($W_{unf.}$) and (**b**) the thickness of unfrozen water films in nonsaline permafrost sediments from the temperature (40–46).

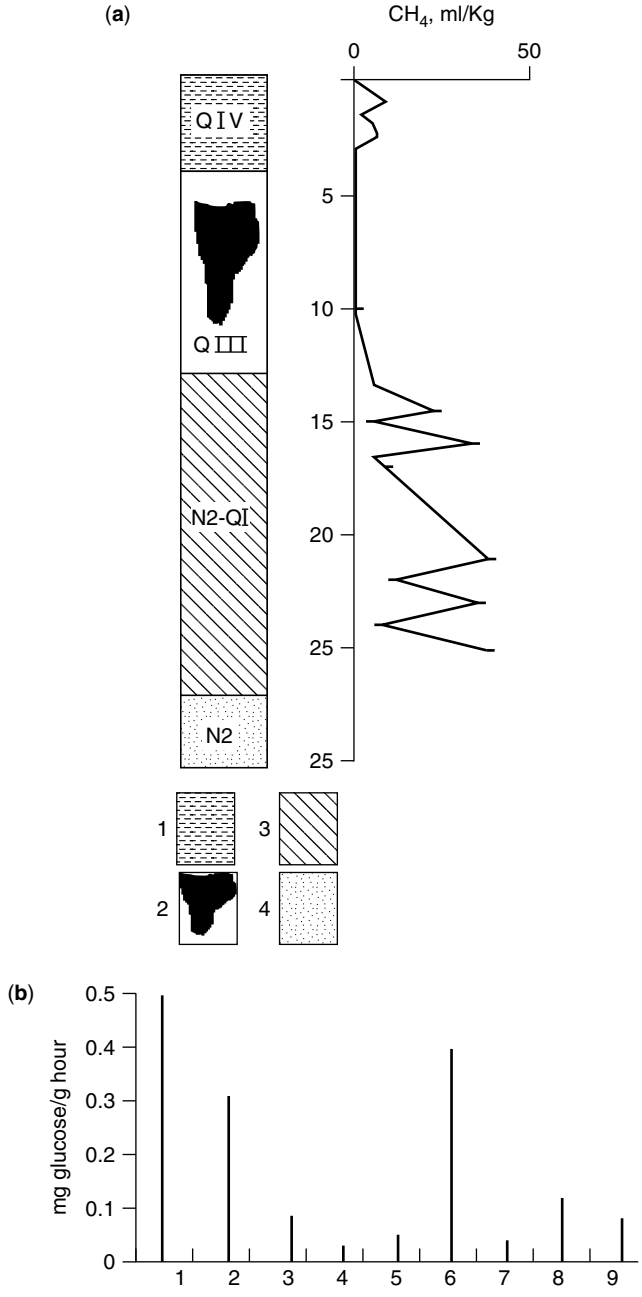

Figure 5. Biosignatures within Arctic permafrost: (**a**) the distribution and concentration of methane (51). Legend: 1 — Holocene lake-swamp deposits; 2 — late Pleistocene icy complex; 3 — late Pliocene-early Pleistocene lake-alluvial sediments; 4 — Pliocene sands; (**b**) the distribution of free extracellular enzymes (53): invertase and (**c**) pigments (54,55): chlorophyll. Permafrost age: 1 — modern tundra soil; 2 — Holocene; 3, 4 — late Pleistocene; 5, 6 — middle Pleistocene; 7 — early Pleistocene; 8 — late Pliocene-early Pleistocene; 9 — late Pliocene.

particles in the core. It should be noted that reliable data are available only for young, not older than Holocene ice layers. To date, no viable cells have been found in the fossil ices of Arctic — neither in intrusive ice nor in polygonal ice wedges (12,22). Both pure ice and permafrost

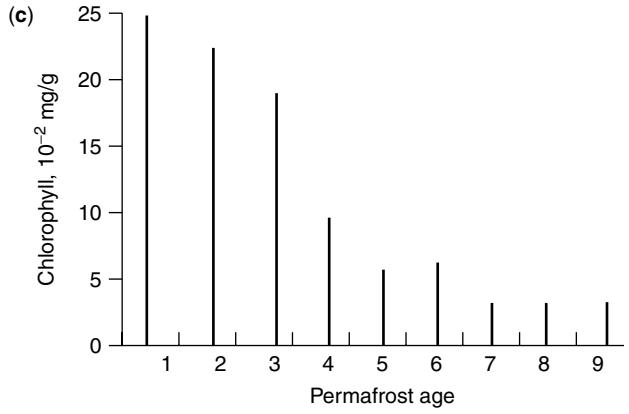

Figure 5. (*Continued*)

are at the same subzero temperatures. The only difference between the two is the amount of W_{unf} associated with suspended solids. In nature, pure ice, lacking suspended solids and their associated unfrozen water, has insufficient cryoprotection and transport abilities, and cells are destroyed mechanically by ice crystals. So, because the W_{unf} and pore space, fine dispersed frozen layers, with paleosols and peat buried in them, provides more favorable environment to microorganisms (49,50).

Gases

The gaseous phase of the permafrost is different from atmospheric air. The pore space of frozen strata is occupied by oxygen, nitrogen, methane, carbon dioxide, and other gases. The concentration of oxygen and nitrogen does not differ significantly from their values in the air, whereas the concentration of biogenic gases may be appreciably higher. The concentration of CH_4 (Fig. 5a) in Arctic permafrost varies from 2 to 40 mL/kg and CO_2 from 1 to 20 mL/kg. It should be noted that carbon dioxide is ubiquitous, whereas the occurrence of methane is determined by the age and genesis of the deposits and by the type of cryogenic stratum. In a generalized geologic cross section, methane-containing layers are sandwiched between layers free of methane (51). The fact that, for at least several hundreds of thousands years, the methane has not diffused from the methane-rich layers into adjacent layers devoid of methane implies that there is negligible diffusion of methane within the permafrost under both present and past conditions. The same conclusion can also be made at the distribution of carbon dioxide in permafrost layers. The facts suggest that these gases are held within the sediments in a clathrate form (52), making it impossible for cells to migrate with them.

The presence of methane, as well as the value of the redox potential ($Eh = +40$ to -250), indicate that conditions in Arctic permafrost are mostly anaerobic; the permafrost is composed of primarily lacustrine-alluvial and marine deposits. The Eh of investigated Antarctic eolian and glacial frozen sands vary from $+260$ to $+480$, indicating that the conditions here are not as anaerobic as in Arctic; this is confirmed by the absence of biogenic methane. In any case, it is the specific character of permafrost soils that, until the moment of transition to the permanently frozen state, they contain both aerobic and anaerobic microzones. This is why the ancient microbial communities consist of comparable numbers of anaerobic and aerobic species.

The ratio of anaerobes to aerobes is different in different geologic facies, that is, the part of the community that dominates depends on the origin of sediments. In one study, the number of anaerobes in swamp and lagoon soils was several orders of magnitude higher than that of aerobes (56), whereas in another study, based on alluvial deposits, aerobic groups dominated (12,50).

From a planetary science perspective, it is important to note that CO_2 and CH_4 do not prohibit long-term preservation of cells in permafrost. CO_2 is expected to be a major constituent of the gas phase in any permafrost on Mars, as is CH_4 in the permafrost associated with the satellites of gas giants (57). And what is more, the reduced state found in permafrost provides a model for possible life-forms on cryogenic planets without free oxygen. The finely-dispersed Martian dust appears favorable for the preservation of the maximum number of viable cells. Anaerobes should be dominant in Martian permafrost, even in the absence of organic carbon (58). Terrestrial anaerobic, chemolithotrophic, psychrotolerant bacteria, with their ability to assimilate CO_2 in an environment similar to that of Mars have been suggested as prospective analogs to the living forms that might be found in subsurface Martian layers (59). We note in closing that the formation of methane clathrate in frozen sediments at low pressures has implications beyond Arctic studies. Similar processes may represent important reservoirs of methane on other icy bodies in the solar system, in particular in the surface materials of comets (57).

CHEMICAL COMPOSITION AND ORGANIC MATTER

The typical acid-base characteristic (pH) of Arctic permafrost sediments is close to the neutral value (6,7), indicating faintly reduced conditions. The content of organic matter ($C_{org.}$) is 0.5 to 7%, a value that is independent of the depth of bedding and the age of soils. If one considers the thickness of the sedimentary cover, it has preserved a tremendous mass of organic carbon. For example, a one-square-meter section of the 100-m thick permafrost may contain 340 to 460 kg of $C_{org.}$ (60). The composition of the hydrocarbons and fatty acids in the permafrost indicates a primarily vegetative origin of the $C_{org.}$ (of the type of *Betula* sp.) in the sediments with a smaller portion of bacterial origin (61). This great mass of organic carbon (Table 1), which has not been processed by microorganisms, is still labile and, in the event of permafrost thawing, could become available for the ancient cells within the frozen soils to renew their physiological activity (53,62–64). In Antarctic cores, in contrast, the pH of medium is alkaline (8,9) and the total content of organic carbon is very low, often close to zero. It varies from 0 to 0.1% in eolian and glacial sands to 0.3 to 0.4% in lacustrine sediments.

Arctic sediments, rich in organic carbon and Antarctic soils, in which both the carbon and the unfrozen water content are close to zero and the temperatures is 10 °C

Table 1. The Content of Organic Matter ($C_{org.}$) and the Average Number of Viable Aerobic Microorganisms in Permafrost Depths

Kolyma Lowland			Mackenzie Delta	
Depth (m)	$C_{org.}$ (%)	cells/g	Depth (m)	cells/g
1.0	4.0	5×10^3	12.5	4×10^3
3.0	1.9	9×10^3	44.8	5.3×10^2
34.0	1.3	1.9×10^4	93.0	3.4×10^3
39.0	1.5	1×10^4	107.2	1.6×10^5
40.0	1.6	0	111.9	7.2×10^2
42.0	0.5	7×10^3	119.6	3×10^3
45.0	3.0	0	137.6	0
50.0	0.9	2×10^3	169.1	1.9×10^3
51.0	3.3	0	189.9	9.8×10^2
53.0	3.5	2.3×10^3	197.8	3.3×10^2
54.0	5.6	0	260.0	8.9×10^3
57.0	2.0	1.1×10^4	300.0	1.2×10^3
58.0	2.2	1×10^5	323.0	4.5×10^2
61.0	0.5	1.2×10^4	326.4	1.3×10^5
63.0	1.0	2.5×10^5	352.0	2.4×10^2

lower, contain about the same number of microorganisms (10^7–10^8 cell/gdw), as estimated from direct counts using epifluorescence microscopy and staining with acridine orange (50). Furthermore, this total quantity of microbial cells is relatively constant from modern up to 3-Myr-old sediments (Table 2) and independent of the sample origin, depth, physical/chemical composition, and temperature. In other words, there is a minimal constant level of microbial colonization that forms in natural systems in the presence of only mineral soil particles and short-term existence of free water. Possibly, this unexpected result shows that these minimal conditions are enough for the origin of life. For comparison, the total number of microorganisms recorded from depths of 1,500 to 2,750 m in the Antarctic Ice Sheet is also a constant, 10^3 cells/ml (48), less than the numbers from permafrost by several orders of magnitude.

Obviously, the chemical composition of permanently frozen ground differs in different areas and depths in accordance with their origin (Table 3 A). For example, in Antarctic Dry Valleys, Na^+ ions are dominant in water extracts, against a background of the equal presence of basic anions, and the dry residue reflects the freshwater genesis of the sediments. Water extracts from the main stratigraphic horizons in the Eurasian northeast are characterized by the hydrocarbonate-calcium and a freshwater composition. Arctic marine deposits are distinguished by the sodium-chloride salinity.

The results of culturing more than 5,000 frozen samples from different regions indicates that under similar natural conditions, the soil chemical composition is not a leading factor the determination of the structure of the viable microbial community in permafrost. Salinity plays an important, but not yet understood, role. Arctic freshwater terrestrial layers, formed under the same conditions as modern tundra soils, in contrast with their microbial communities, yielded bacterial colonies on media with high salt content, suggesting the existence of strongly halotolerant microbes in permafrost. It remains to be determined whether the salt tolerance of these strains may also be associated with their tolerance to freezing conditions (65). At the same time, the marine sediments did not contain halophylic microorganisms. The same is true of supercooled (up to $-10\,^{\circ}C$) saline groundwater lenses (cryopegs) found within the permafrost. Cryopegs are formed after the retreat of the sea and the freezing of

Table 2. The Total Number of Microorganisms (A), the Number of Viable Aerobic Cells and Percentage of Sterile Samples (B) in Permafrost Sediments of Different Age

Depth, m	Total Count (10^8 cells/g)	Depth, m	Total Count (10^8 cells/g)	
A	Antarctica (QII-QIY)*		Arctic (N2-QIY)**	
0.5	0.4	12.2	2.4	
4.5	0.4	18.8	0.5	
6.8	1.4	30.1	3.7	
8.2	0.6	32.2	1.5	
9.4	1.1	32.7	1.3	
10.2	0.1	34.4	1.4	
13.0	1.0	37.2	3.2	
15.3	2.5	43.5	0.4	
16.3	0.8	49.0	0.6	
	(>8.1 Myr)	54.8	0.2	
1.5	0.4	60.0	2.2	
2.7	0.3	64.3	0.2	
B				
Age (years)	Viable cells/g	Period, age (years)	Viable cells/g	Sterile Samples
modern soil	3×10^1–1×10^6	QIY (5–10×10^3)	1×10^5–2×10^7	0
(3×10^4)	8×10^2–1×10^4	QIII (1–4×10^4)	5×10^4–1×10^6	12%
(1.7×10^5)	1×10^2–1×10^3	QII (1–6×10^5)	1×10^3–3×10^4	33%
($> 8.1 \times 10^6$)	5×10^1–2×10^3	N2-QI (0.6–1.8×10^6)	8×10^2–7×10^3	50%
(2–15×10^6)	6×10^1–5×10^2	N2 (2–3×10^6)	2×10^2–4×10^3	

*lake, aeolian and glacial sands; **swamp, lake-alluvial and marine deposits.

Table 3. Chemical Composition of Permafrost Soils (A) and Supercooled Water (B) and the Number of Viable Cells

	PH	HCO_3^-	Cl^-	SO_4^{2-}	Ca^{2+}	Mg^{2+} K^+	Na^+		
(A) Valley, age	Antarctic Dry Valleys (sands) mM/100 g							Dry Residue g/l	cell/g
Meier, QIII	7.8–8.0	0.3	0.2–0.3	0.05–0.12	0.03–0.05	0.01–0.02	0.15–0.35	0.45–0.55	2.1×10^1–2.6×10^5
Taylor, QII	8.9–9.8	0.55–0.7	0.5–0.6	0.2–0.25	0.01–0.02	0.02–0.05	0.65–0.75	0.1–0.11	1.7×10^1–5.7×10^3
origin, age	Eurasian Northeast								
Lake swap,QIV	7.8–8.0	0.3	0.2–0.3	0.05–0.12	0.03–0.05	0.01–0.02	0.15–0.35	0.45–0.55	9×10^3–8×10^7
Lake alluvial ,QIII	6.3–7.3	0.6–0.9	0.4–1.2	0.2–0.25	0.01–0.02	0.02–0.05	0.65–0.75	0.10–0.11	2×10^3–1×10^5
Marine,QII	6.5–7.3	0.15–0.35	5.9–11.9	0.15–0.4	0.15–0.35	0.23–0.78	5.55–11.15	6.57–12.47	4×10^3–4.6×10^5
Lake alluvial, N2–QI	6.9–7.7	0.01–0.04	0.005–0.01	0.01–0.02	0.01–0.05	0.02–0.06	0.08–0.25	0.05–0.2	5×10^3–5×10^4
(B)									
hole, #	Water brine lenses within embedding frozen marine sediments								cells/ml
14/99	7.4	0.59	99.4	4.00	1.56	8.9	35.0	168.1	2.4×10^3
15/99	7.3	0.54	97.3	2.01	1.40	8.65	39.5	163.0	1.0×10^4
16/99	7.9	0.37	74.6	1.89	1.00	6.7	32.0	125.0	1.7×10^3
17/99	7.2	0.45	93.4	3.53	1.44	8.25	39.5	158.4	3.0×10^2

marine sediments. As a result of the freezing out of salts from the deposits, the concentration of salts in cryopegs, up to 170 to 300 g/l NaCl, is much higher (orders of magnitude) than in the embedding strata. And even in these environments viable cells were found, and were both anaerobic and aerobic and nonhalophylic (Table 3 B).

THE CORRELATION BETWEEN THE AGE OF PERMAFROST ENVIRONMENT AND THE AGE OF VIABLE MICROORGANISMS

There are no methods for the direct determination of the age of viable microorganisms. In general, the duration of cells cryopreservation does not necessarily coincide with the age of the embedding sediments. Instead, it always corresponds to the duration of the existence of permafrost (49). Two ways of permafrost origin are known: (*1*) syngenetically frozen layers, where sedimentation occurred concurrently with freezing from below, in which case the age of permafrost is taken to be equal to the age of sediments; (*2*) epigenetically frozen layers, where freezing occurred from the top after sedimentation, in which case the age of permafrost is taken to be younger than sediments. In syncryogenic horizons, where the age of permafrost is equal to the age of sediments, the age of cells also coincides with the age of sediments. Here, the higher the layer is in the profile, the younger it is with regard to its formation and to the duration of the frozen state. In other words, the upper strata of both the permafrost and the organisms populating it are younger. In the case of epicryogenesis, the duration of cell's preservation is again equal to the permafrost age, that is, to the time from the last freezing of sediments. However, because of the effects of thermal inertia on the penetration of the temperature wave, the deeper lie the sediments, the more recently they were frozen and the younger are their microorganisms (Fig. 6). Consequently, the most reliably proven are the ages of microorganisms in the syncryogenic strata. The northern Eurasia permafrost, in which traces of cryogenesis can be related to Mid-Pliocene shingles (66), is the most ancient in Arctic. The age of microorganisms here corresponds to the longevity of deposits frozen state; the most ancient are known date back to 3 Myr.

The analysis of numerous publications (67–71), best reviewed by Wilson (72) indicates that permafrost has existed under Antarctic climatic conditions for the last dozens of millions of years, greater than the duration of Arctic permafrost by a factor of ten (49,58). Viable bacteria (both aerobic and anaerobic), enzymes, pigments, and biogenic CH_4 were found in boreholes in one prospective site, Mt. Feather (73). The age of glacial syncryogenic soils of the Sirius Group dates back at least 2 Myr (74) or, probably, 15 Myr (75). $^{40}Ar/^{39}Ar$ dates on ash layers suggest that the most ancient ground ice below these layers may be more than 4.5 Myr old in Arena Valley (76) and 8.1 Myr old in Beacon Valley (77). Verifying these ages is critical because viable bacteria have been isolated in the frozen ice below this ash layer and if this age is correct, this is the oldest viable life on Earth.

PERMAFROST BIODIVERSITY

The permafrost microbial community has been described as "a community of survivors" (78). Survival is the result of the combination of cold temperature, desiccation, and starvation (3). Viable bacterial populations in permafrost soils can be viewed as the result of a continuous process of selection for forms that can withstand cell aging and prolonged exposure to subzero temperatures (79). The rates of metabolic activity at these temperatures, over periods of 2 to 3 Myr or longer, and in frozen soils would

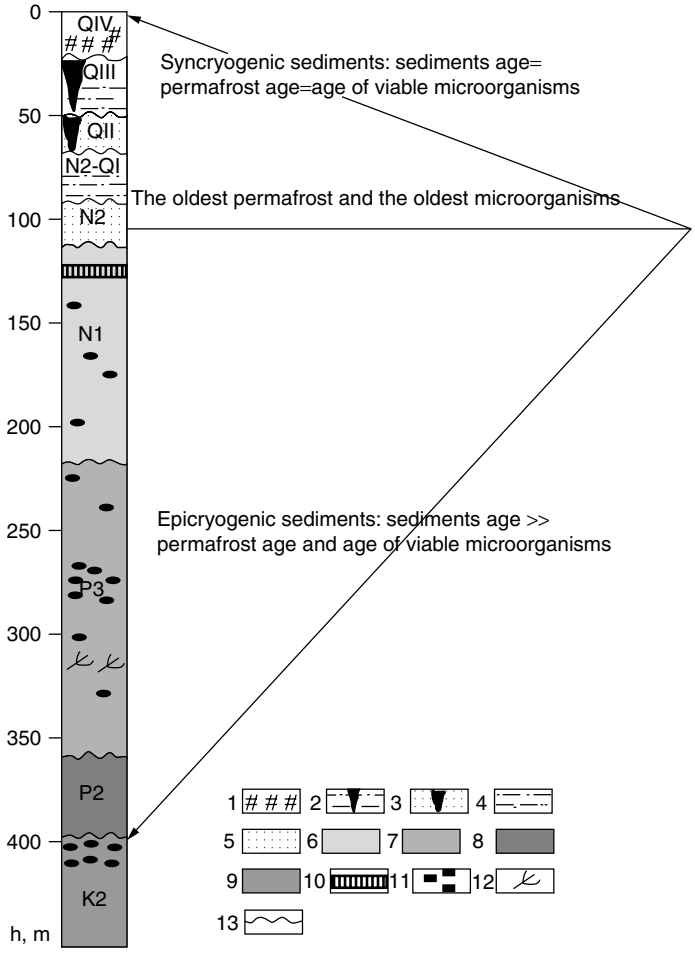

Figure 6. The correlation among age of sediments, the age of permafrost and the age of microorganisms in syncryogenic and epycryogenic thickness. (**a**) Holocene peaty soils; (**b**) late Pleistocene icy complex (loamy-sand with ice veins); (**c**) middle Pleistocene icy complex (sand with ice veins); (**d**) late Pliocene-early Pleistocene sandy-loam; (**e**) late Pliocene alluvial sands; (**f**) early Pliocene sandy soils; (**g**) late Paleogene (sandy soils); (**h**) middle Paleogene (sandy-loam and clay); late Cretaceous (sandy-loam and clay); (**i**) coal; (**j**) gravel and pebbles; (**k**) plant remains; (**l**) stratigraphic borders among geologic periods.

be expected to be extremely low. Therefore, permafrost may represent an example of an environment in which the survival of certain life-forms continues, and in which the stringency of the environmental conditions is such that the continuous evolution of successful adaptations is hindered. According to Morita (3) the permafrost may be considered as anoligotrophic environment in which the starvation-survival lifestyle is the normal physiological state.

After the prolonged exposure of the microorganisms to the permafrost environment, the ratio of readily culturable (hypometabolic) bacteria to those that may represent viable but nonculturable forms (deep resting cells) is determined by the extent and duration of exposure to subzero temperatures (50). It should also be kept in mind that some cells may have died when subjected to the stresses of thawing and exposure to oxygen because the samples were suddenly melted in the laboratory for microbial study at relatively high temperatures. Although these stresses are known to inhibit the recovery of a fraction of the community, strategies and techniques for the low-temperature recovery of bacteria from permafrost environments are only just beginning to be developed (65). Using the new techniques, it has been shown that permafrost bacteria, when isolated on rich media, were less numerous than those in diluted media, but had greater diversity. Similar results were also obtained during the isolation of bacteria from deep subsurface nonfrozen environment (80).

The ancient permafrost microbial community is predominantly bacterial, as is the community in the depths of Antarctic Ice Sheet (47,48). This is in contrast with modern soils in which the fungal mass is much greater than the bacterial mass. In Arctic permafrost, non–spore-formers predominate, whereas in Antarctic permafrost spore-formers dominate. In isolations of aerobic bacteria from Arctic permafrost at +20 °C, a significant fraction was found to be spore-formers (30). This contrasts sharply with isolations carried out at +4 °C, where spore-formers were only rarely obtained. Perhaps this reflects an intriguing paucity of spore-forming bacteria in frozen environments (65). Some isolated microorganisms carried mercury-resistant plasmids (81) and were more or less resistant to antibiotics (82).

Overall, a number of different morphological and physiological groups of microorganisms have been found, including spore-forming and sporeless, aerobic and anaerobic, bacteria, fungi, yeast, actinomycetes, representing more than 50 genera (Fig. 1). Morphologically, they are coccoid, coryne-like, nocardia-like, and rodlike gram-positive or gram-negative bacteria. Seen rather frequently are prokaryotes with thick cell walls and capsules, surrounded

by additional surface layers of low electron density. Eukaryotic cells, although present, seem less able to survive long-term cryopreservation (83). Even these data and well-known widespread existence of modern phototrophs in such environment as Antarctic Dry Valley, best described by Friedmann (36), it was a surprise to discover within the full permafrost darkness numerous viable phototrophs: cyanobacteria and green algae that had preserved their photosynthetic apparatus (84,85). During the last years, green algae were also isolated from Antarctic permafrost and mosses from the Arctic one. Until recently, these two groups of the lower plants represented the highest of known long-term surviving biological systems. Now protozoa were isolated from the buried Arctic soils. Viable seeds of higher plants were found within Siberian and Canadian late Pleistocene permafrost. It was shown that these seeds still were able to grow (86).

RESPONSE OF MICROBIAL COMMUNITIES TO PERMAFROST ENVIRONMENT

The data shows that during the long-term preservation at temperatures from −3 to −4 °C in the interior of Alaska, −5 to −6 °C in the Mackenzie delta and −9 to −12 °C in the Eurasian northeast, the number of viable microorganisms is independent of the permafrost temperature. At the same time near the southern permafrost boundary in western Siberia, with ground temperatures −1 to −2 °C, and in Antarctica, with ground temperatures as low as −18 to −25 °C, the number of viable cells decreases by several orders of magnitude (Table 4 A).

The microbial community, even after its long-term existence within the permafrost, according to their growth temperatures (87), are not psychrophilic bacteria (T_{min} at <0 °C, T_{opt} at <15 °C and T_{max} at <20 °C), known to be restricted to permanently cold habitats (88). Instead, they are predominantly psychrotrophic (T_{min} at <0 °C, T_{opt} at >15 °C and T_{max} at >20 °C). 95% of the isolated cells did not grow at temperatures higher than 30 °C (Table 4 B), but were often capable of grow at temperatures below 0 °C. In the presence of cryoprotectants such as glycerol, added to the nutrient medium in an attempt to simulate natural conditions in permafrost in which unfrozen water plays a cryoprotectant role, growth of ancient cells was observed on Petri dishes incubated at temperatures at least as low as −10 °C for one to one and a half years (39). According to Russell (10), this correlates with the lower temperature limit for growth of psychrophilic microorganisms. So, because these terms are not clearly delimited, one can define the isolated microbes as psychrotrophic-psychrophilic or psychrotolerant organisms.

The microorganisms isolated from the syngenetically frozen sediments, as well as from the modern tundra soils, are resistant to sharp temperature transitions through

Table 4. Viable Microorganisms in Permafrost

A. *The Number of Viable Aerobes in Permafrost at different Temperatures*

Kolyma Lowland, $T = (-10) - (-12)$ °C		Mackenzie Delta, $T = (-5) - (-6)$ °C		West Siberia, $T = (-1) - (-2)$ °C		Dry Valleys, $T = (-18) - (25)$ °C	
Depth, m	cells/g	Depth, m	cells/g	Depth, m	cells/g	Depth, m	cells/g
21.0–62.4	2.2×10^5–1.4×10^7	9.5–326.4	1.4×10^3–1.6×10^5	2.0–18.5	1.4×10^2–2.7×10^3	1.0–19.3	5.2×10^2–6.2×10^4

B. *The number of viable aerobic bacteria (cells/g) at the culturing temperatures*

	40 °C	30 °C	20 °C	4 °C
Antarctica, Tailor, and Maier Valleys, eolian, glacial, and lake bottom sands; T = −20 °C				
0.51–16.3	0–$<10^1$	0–1.1×10^3	$<10^2$–5.9×10^4	0–9.1×10^3
Antarctica, Mt. Feather (formation Sirius) and Beacon Valley (sands); $T = (-23) - (-27)$ °C				
1.0–3.0	0	0–1.5×10^2	7.6×10^1–6.3×10^4	0
Canadian Arctic, Mackenzie Delta, sands and loam				
9.5–41.2	0	6.4×10^1–1.9×10^3	1.4×10^3–1.6×10^5	0–6.5×10^3
Kolyma lowland, marine and alluvial sands and loam				
5.7–55.2	0	1.7×10^1–5.3×10^4	3.2×10^1–1.1×10^5	1.2×10^2–2.3×10^4

C. *The number of viable permafrost anaerobes (cells/g)*

Period (age, years)		Methanogens	Denitrifying	Sulfatreducers
QIV ($5–10 \times 10^3$)	1.0	2.0×10^7	2.0×10^7	2.0×10^2
QIII ($1–4 \times 10^4$)	4.4–17.0	2.5×10^7	$2.5 \times (10^5–10^6)$	0
QII ($1–6 \times 10^5$)	30.0–35.7	$(2.0–2.3) \times 10^7$	2.3×10^3–2.0×10^6	0–2.3×10^2
N_2-QI ($0.6–1.8 \times 10^6$)	37.2–64.3	$(2.0–2.5) \times 10^7$	2.5×10^3–2.5×10^7	0–2.0×10^2

0 °C and to freezing/thawing stress. Even after hundreds freeze per thaw cycles, the number and diversity of viable cells did not change, whereas samples from never frozen regions often become sterile after a dozen of these cycles. Microorganisms from epigenetically frozen marine sediments are somewhat intermediate; they are resistant to the long-term impact of subzero temperatures, but very sensitive to the phase exchange in surrounding microenvironment (79).

The number of culturable cells and their diversity is independent of the soil's origin and the depth of bedding, icing and cryogenic texture, chemical composition and organic matter content; microorganisms were isolated even in cases of extremely low levels of organic matter (Table 1). The number of culturable aerobic cells is dependent primarily on the duration of the frozen state, that is, by the permafrost (not sediments) age. The greater is the permafrost age, the lower are the number of viable cells, the lower their qualitative and morphological diversity, and the greater the number of sterile samples (Table 2 B). This suggests that freezing itself is a biodiagenetic factor, and that a long-term existence in the frozen state reduces an aerobe's viability.

These discussions are based on the culturable cells. Analysis of Arctic sediments showed that only 0.1 to 5% of the cells counted by epifluorescence microscopy grew on nutrient media, and in Antarctica, the percentage is reduced to 0.001 to 0.01% (50). Taking into account the temperatures and close-to-zero unfrozen water content of Antarctic layers, this low value could, to a certain extent, be explained by the deep resting state of the cells. At the same time the number of viable anaerobes: methanogens, denitrifying, sulfate- and iron-reducing bacteria (Table 4 C), if the community exists, is determined by the anaerobic origin of the frozen layer and is independent of permafrost age (56).

Because of the importance of unfrozen water, the number of viable cells under similar conditions is dependent on the textural composition of the permafrost, and increases with increasing soil dispersion. The finer the texture of the sediments, the larger the unfrozen water content in them, the thicker the water films, and the greater the number of viable cells. Evidently, good preservation of cell structures is determined by the equilibrium-state of the water inside and outside the cell (i. e., unfrozen water in cells and films). Violation of the equilibrium results in cell death. Consequently, ice formation in the experiments on Petri dishes results in cell death. In sands, with only minimal quantities of unfrozen water, the abundance and diversity of viable microorganisms is substantially lower than in loams, with unfrozen water contents of 5 to 8%. With this in mind, a series of experiments were done (89), and calculations showed that if enough solute had accumulated to achieve a total osmolality of 5.38 osm, then no exosmosis or cell shrinkage would be required for the cell to exist at −10 °C without danger of intracellular ice formation.

The phylogenetic diversity of the permafrost bacterial community has only recently begun to be addressed. For this reason we need to keep in mind that all above-mentioned discussions are based on the culturable cells, and, as is the case in other natural environments, a large portion of the permafrost microbial community remains uncultured. The data based on DNA extracted from modern Siberian tundra soils suggest that the clones are phylogenetically very diverse, and that many probably reflect new genera or families. Hence, most of this bacterial community has never been isolated and the physiology and function of its dominant members is unknown (90). The first molecular data have recently been obtained from ancient permafrost bacterial communities, using 16S rDNA sequencing. Phylogenetic trees derived from this data indicate that the Arctic isolates fall into four major groups, partly determined by the age of the permafrost. Most are high-GC gram-positive bacteria and β-proteobacteria. All γ-proteobacteria came from the late Pliocene-early Pleistocene samples, 2 to 3 Myr old. Most of the low-GC gram-positive bacteria came from the Holocene age, three to eight thousand years old (30). The communities also include members of Archaea, which have only recently been detected in the Siberian permafrost. These results, and as it was first shown in Greenland ice cores two to four thousand years old (91), demonstrated good preservation of ancient genomic DNA in both frozen environments, permafrost and ice.

Free extracellular enzymes were also found in permafrost sediments. Among these, invertase activity was found in all studied samples (Fig. 5b), whereas amylase, protease, and catalase activity were often detected (12,53,62). The association of immobilized microbial cells and organic-mineral complexes containing active enzymes that are resistant to the long-term effects of extremely low temperatures. This enables the enzymes and cells to restore their metabolic activity during permafrost thawing. Interaction with the heterogeneous medium becomes possible due to the availability of active immobilized enzymes. The distribution of enzyme activity in geologic sections differs from that of viable cells. It decreases sharply in the Holocene interval, then is approximately constant, independent of permafrost age (53). Invertase and some catalase activity were detected in permafrost samples from which viable cells were not isolated. This makes some enzymes attractive as a biomarkers. The same situation holds for chlorophyll pigments, which were often found in permafrost under dark conditions (Fig. 5c) (54,55). In Dry Valleys, chlorophyll is produced by green algae or cyanobacteria, which are often found in surface layers. Chlorophyll was found within the Antarctic permafrost samples, in which neither green algae nor cyanobacteria could be isolated. Because all data shows that DNA and microbial end-products can be preserved in permafrost even in the absence of viable cells, these studies may have wide implications.

METABOLIC ACTIVITY IN PERMAFROST ENVIRONMENT

The following features of permafrost microbial communities after their release from the frozen state have been detected: high viability and rate of cell proliferation; high rate of biochemical processes after thawing; intensive RNA synthesis immediately from the beginning of thawing;

and an extremely high thermostability in some microbial enzymes and an ability for them to become instantly activated (53).

A key question regarding the viable bacteria in permafrost is whether they are active in the permafrost environment or present in a suspended "dormant "state. Recent information suggests that permafrost microorganisms are not, as previously thought, in a frozen resting state. There are only a few data about metabolic activity of microorganisms below the freezing point, and only for recent microbial communities. Measurements show that Antarctic lichens may be active at temperature of $-17\,^{\circ}C$ (92). Recently, evidence has been found of low rates of bacterial DNA and protein synthesis in Antarctic snow, indicating that the organisms were metabolizing at ambient temperatures -12 to $-17\,^{\circ}C$ (93). The following facts provides indirect evidence that ancient microorganisms can be active in the permafrost nutritional-temperature regime and allows to speculate on the possibility of biogeochemical processes in situ: the ability of immobilized enzymes in permafrost to become instantly activated (53,62); the presence of usually metastable nitrites and ferrous sulfides (94,95); the simultaneous presence of methanogenic bacteria and methane (51,52,56).

For ancient microbial communities only the results of experiments with use of ^{14}C-acetate, shows that constructive metabolism is possible in permafrost of age 3 Myr down to $-10\,^{\circ}C$, resulting in the formation of bacterial lipids (64). However, the question about the metabolic state of microorganisms, microbiological and biogeochemical processes and life-forms within the permafrost remains open to debate. Recently, Rivkina and Laurinavichyus got a new data about the energetic metabolism–methane-formation in permafrost sediments using the ^{14}C-labeled substrates. The results of the experiment are the following: the activity of the CH_4-formation both from CO_2/H_2 and acetate was observed in native permafrost sediments at temperature down to $-17\,^{\circ}C$. One can suggest that the CH_4-formation below and above the freezing point are realized by different methanogenic community: psychrofilic and psychrotolerant, correspondingly. Remarkably, the incorporation of ^{14}C into methane above freezing point and directly below one just a little differed one each other. At lower subzero temperatures (down to $-17\,^{\circ}C$) CH_4-formation occur in the absence of liquid water, and even the unfrozen water in the sediments is lower than 1%. Realization of these chemical reactions still is not quite understandable. Nevertheless, one cannot exclude cellular activity (but not cell division) at a very low level. These results show that in natural deep frozen environment the metabolic redox reactions realized by ancient microorganisms represent an unknown form of the long-term preservation of viable ecosystems and the life maintenance over the aeons in permafrost.

If in an active state, even if at reduced level of activity, the bacteria should be able to repair cellular damage caused by racemization of amino acids and by radiation from natural radionuclides in the permafrost. Estimation of ground radiation has been made by McKay and Forman, using both elemental analysis of the radioactive elements in sandy and loam samples and direct measurements in the boreholes in the Kolyma lowland on the Eurasian northeast. The radiation dose received by the permafrost bacteria is about 2 mGy per year. Taking into account the age of bacteria, late Pliocene to late Pleistocene, the total dose received by cells would therefore range from 1 kGy in soils dozens of thousands years old to 0.6 kGy at over the 3 Myr age of microorganisms. Experiments with soils have shown that up to 30 kGy are required to sterilize soil at above-freezing temperatures and the results obtained by Japanese researchers, indicate that bacteria in soil have a much greater resistance to irradiation when frozen than when thawed. Our own results demonstrate that, at similar levels of ionizing radiation, a known quantity of viable cells and a total radiation dose of 5 to 50 kGy, most of the cells in frozen samples survived, whereas most of the cells in unfrozen samples died. The ability of cells to produce colonies after being exposed to total doses as high as 3 to 5 kGy shows that freezing increased the cell's resistance to radiation and raises the question of the uniqueness of permafrost as an environment in which microorganisms display a high resistance to radiation. From these data the dose from radionuclides diffused through the permafrost is far from sufficient for complete sterilization, that is, is not fatal for viable cells, but should be large enough to cause some selection effect and to destroy the DNA of ancient cells. Their viability and growth on media implies the capacity for DNA repair. Probably DNA repair is occurring in the frozen environment, that is, at the stable rate of damage accumulation, a comparable rate of repair also exists. From the biological point of view, it is important the permafrost preserves the cells from diffuse ground irradiation for thousands and millions of years. And what is more, in one series of experiments, the oldest, Pliocene cells, which had received an in situ maximal dose during the time of cryopreservation, were found to be more resistant to radiation than microorganisms from modern tundra soils.

PALEOENVIRONMENTAL RECONSTRUCTIONS

As the result of investigations into the microbiology of permafrost, it is now possible for the first time to use actual viable organisms for the purposes of reconstructing a past environment. In contrast to traditional methods using bioindicators, we can use not only of morphological indices of the fossil microflora, but functional indices as well. This highlights the permafrost as a natural biological database of Quaternary events. The microbial communities reflect the conditions at the time of syncryogenesis or the biogeochemical parameters of environment in the embedding strata at the moment of epicryogenesis. This makes possible different paleoecological and paleoclimatic reconstruction of the frozen, that is, most mobile, mantle of the Earth (29). For instance, we can reconstruct the vertical dynamics of the frozen thickness. Climatic oscillations in high latitudes are reflected in permafrost by its agradation in cryochrones, that is, cold periods, and its degradation (thawing) in thermochrones (warm periods). The cryochrones following thermochrones result in the refreezing of thawed thickness

and thus the whole section represents now one monolithic frozen sequence. Refrozen (epicryogenic) and never-thawed parts of this section have the similar physical-chemical and mineralogical characteristics, but differ in microbial and biogeochemical parameters. Therefore, permafrost dynamics in vertical cross sections, can be established by the distribution of viable microorganisms or their metabolic end-products. The depth of seasonal thawing of modern Arctic soils is marked by an accumulative horizon of viable microorganisms at the permafrost table (Fig. 7a), caused by their infiltration with melt-water (96). When the depth of thawing decreases, this horizon returns to the permanently frozen state. Just as the depth of modern seasonal thawing corresponds with present-day climate, the depth of thawing in the past corresponds with paleoclimatic conditions.

Microorganisms accumulate at the bottom of each thawing layer (on the permafrost table) formed during short- and long-term warming. In the Eurasian northeast, the Holocene Global Warming stimulated the local thawing of late Pleistocene sediments. Because of the new late Holocene cryochrone, the thawed thickness is refrozen again and merged with underlying horizons to form one cross section. Here the upper refrozen part, of "young" Holocene permafrost age and epicryogenic origin, contains many more viable microorganisms than the underlying never-thawed "ancient" syncryogenic late Pleistocene layer. The border between these two parts is marked by a horizon with increased numbers of viable cells (Fig. 7b). Thus, stratigraphic levels of thawing can be detected by microbiological data. The deeper the peak of microbial accumulation, the more ancient and stronger is the corresponding thermochrone. It is not important what factor, either climatic or geologic, caused the heat wave — microorganisms have also accumulated at the bottom of talik (nonfrozen) zones, below the sea, river, or lake water tables (15,29,79).

The methane distribution in permafrost can also be used as a biomarker. This gas is a product of the activity of methanogenic bacteria in anaerobic systems during

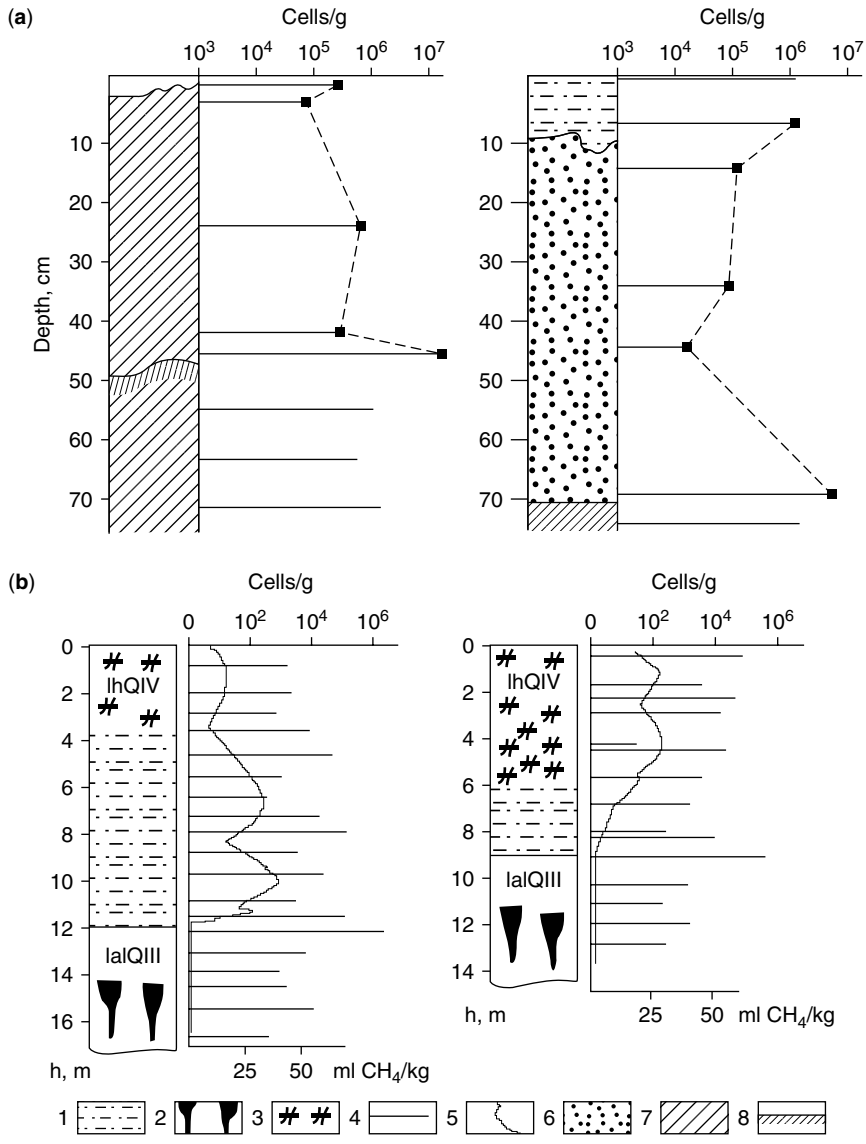

Figure 7. The distribution of viable microorganisms in the modern tundra cryosol profiles and underlying permafrost (97) and the accumulative layer of viable microorganisms on the top of permafrost table (**a**); the distribution of viable ancient aerobic microorganisms and biogenic methane in the refrozen, epicryogenic, and underlying syncryogenic, permafrost and the accumulative layer of viable microorganisms on their border (**b**). 1 — sandy-loam; 2 — late Pleistocene icy complex; 3 — peat; 4 — number of viable microorganisms; 5 — methane concentration; 6 — sands; 7 — loam; 8 — permafrost table.

thermochrones. The refreezing of thawed horizons from the top closes the system and preserves the methane as evidence of the past microbial activity. The depths of thawing detected by distribution of viable aerobic microorganisms correlate (Fig. 7b) with the depths of past activity indicated by anaerobic microorganisms (98). Thus, the same data have been received by two independent methods. The reconstruction, similar to the Holocene, may be used in deeper and more ancient Pleistocene layers, in which it may also be helpful to incorporate microbiological methods into an interdisciplinary study. The earlier-mentioned examples are simple but not the only possibilities for the use microbial data. For instance, using these data one can detect the processes that formed the buried soils, and from them the landscape conditions.

When considering paleoreconstructions, it should be remembered that permafrost not only contains more information because of the high microbial numbers and diversity, it is also much older than ice sheets and glaciers. The oldest glacial ice, 400,000 to 500,000 years old, is found at Vostok Station in Antarctica and in Gyliya ice cap in the Tibetan mountains (97,99). Although the permafrost ages can reach millions of years, stable ice thaws under the geostatic pressure or sunlight even at subzero temperatures.

THE PERSPECTIVES FOR FUTURE STUDY AND POSSIBLE APPLICATIONS

The presence of viable paleosystems within the permafrost is of interest from geologic and biological points of view. Unique to the permafrost environment is it possible to develop a microbiological timescale using a combination of available geologic data, viable paleocultures, and tools of molecular biology. It should be possible, using phylogenetic trees developed from the same organisms (based on RNA sequences) from the modern top layers to the several millions years old in the boreholes, to find out what are the differences among members of the same species as we go back in time. Then, if we compare those differences (mutation rate) with geologic time, we can speculate on how fast or slow evolution or adaptation takes place in that particular environment and for that particular type of organism. This would be a beginning of studies concerning the rate of evolution and biological clocks extending back the duration of the permanently frozen state in the soils, that is, the age of biota. Its prototype is based on the reduction of the microbial community with the increasing permafrost age and the disappearance of some indicator microbial groups in the reference points of the Pleistocene sections (29,49,54).

In biology, established phenomena form the basis for various scientific fields in cryo-, paleo-, and exobiology. Of special interest is the interaction of knowledge to our understanding of microbial evolution, geologic and planetary history, the spatial and temporal limits of the deep cold biosphere on the Earth and other bodies in space (15,49). Among these new scientific directions, one of the most general is the fundamental question of the duration of life preservation. Modeling, calculations, and experiments cannot solve this problem because the length of time cannot be reproduced. From this viewpoint, permafrost is a unique observatory of ancient viable systems. It makes possible the observation of the results of cryopreservation over geologic timescales. However, questions concerning the possibility of microbiological and biogeochemical processes below freezing point and the nature of life in permafrost remain open to debate. This is why it is so important to identify the boundary between inhibited metabolism and anabiosis and to determine how long microorganisms can preserve viability using these different mechanisms. It is also possible that other mechanisms for the long-term preservation of viable ecosystems and vital activity exist in permafrost. The freezing-resistance of the microbiocenoses may depend on the ability of individual microorganisms to enter the anabiotic or similar state upon the freezing of the soil. This is why we call them only viable, not living cells; because of their own cryoprotectant properties, they are not in a frozen state, but only in a cooled state. It has been shown that during freezing, the dehydration of macromolecules and the reorganization of membrane components lead to a considerable decrease of metabolic activity, which can be restored under favorable conditions (83). This mechanism might work for billions of years.

From an exobiological point of view, seven of the nine planets of the Solar System, as well as their satellites, comets, and asteroids are of a cryogenic nature (Fig. 8), that is, the permafrost is a common phenomenon in space. The terrestrial permafrost, which is inhabited by cold-adapted microbes and protects the cells against unfavorable conditions (radiation, gravity, damage by ice crystals, sharp temperature transitions, etc.), can be considered as an extraterrestrial model (100). The cells and their metabolic end-products (enzymes, pigments, and gases) found in the Earth's permafrost provide a range of analogs that could be used in the search for possible ecosystems and potential inhabitants on extraterrestrial

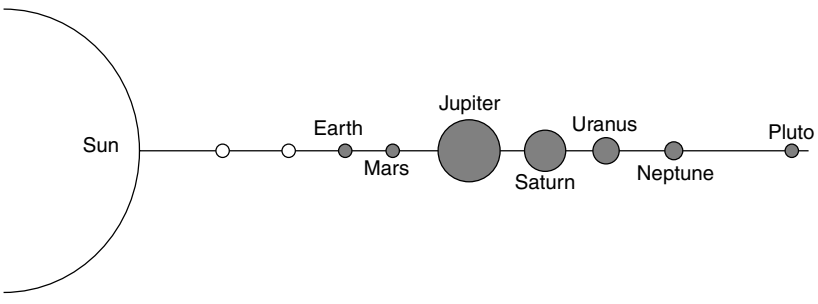

Figure 8. Planets of a cryogenic nature.

cryogenic bodies. If life ever existed on other planets during the early stages of development, then its traces may consist of primitive cryogenic forms at the cell level. By analogy with Earth, they might have been preserved and could be found at depths within the permafrost. Most intriguing is the possibility of traces of past life on Mars. Cameron and Morelli (27) first advanced theoretical concepts for solving the problems of Mars by using terrestrial models. For example, in the upper 500 m of Antarctica Ice Sheet viable cells were found at temperatures −50 °C (43), what is closed to Martian temperatures.

The main difference between Earth's permafrost and the outer planets is their age. In northeastern Eurasia, the oldest continuously frozen permafrost dates back about 3 Myr, whereas on Mars, the age of permafrost is about three billion years. This difference in timescale could have a significant impact on the possibility for life preservation. The Arctic frozen sediments inhabited by microbes are only approximate models for Mars. The Antarctic sediments may somewhat be closer. The limiting age, if one exists, within the Antarctic cores, in which the viable microorganisms were no longer present, could be established as the age limit for life preservation within the permafrost.

CONCLUSION

The permafrost is a reservoir of ancient microorganisms, many of which may remain viable. They are a model for resistance to freezing in both aerobic and anaerobic conditions, but, in contrast to the microorganisms in glacial regions, most of them are not psychrophilic. It was shown that in both Arctic and Antarctic permafrost, not only prokaryotic but also eukaryotic cells are preserved in a viable state and that the total biomass of microorganisms is comparable with the biomass of modern soil cover. Low temperatures should be considered as a stabilizing factor for sustaining life in the permafrost. Permafrost is a depository of ancient biomarkers, including biogenic gases, PAHs, biominerals, biological pigments, lipids, enzymes, proteins, nucleotides, RNA and DNA fragments and molecules, microfossils, and viable cells. The phylogenetic analysis under Tiedje leadership by sequencing their 16S rRNA genes shows that the majority of the isolates are members of eight genera. The gram-negatives are *Flavobacterium, Cytophaga, Sphingomonas*, and *Psychrobacter*. The gram-positives are *Arthrobacter, Rathayibacter, Exiguobacterium*, and *Planococcus*. Unfortunately, viable viruses of already beaten diseases as markers of past epidemics (smallpox, plague, and influenza) might be present there among them. Therefore, permafrost is of great significance for research in cryobiology, biotechnology, ecology, molecular paleontology, and the newly emerging field of exobiology. The psychrotolerant microbes have not received as much study as has been devoted to the thermophyles. Therefore, they represent a fertile area for scientific research. Special areas to be considered include studies of the *in situ* activity below the freezing point and after the thawing. The mechanisms for microbial survival during the long-term anabiosis or low metabolic state and during repeated freeze-thaw conditions must be studied in detail. Experiments with viable microorganisms at temperatures below freezing point suggest the need to develop of more sensitive equipment and methods for studies of this only open slightly unknown microbial world and measurements of their activity within natural frozen environment.

The strategy for the search for extraterrestrial life or its remnants must be based on studies of the most probable environments in which the life might be find, and the maximum period of time over which such life could be preserved. This is why the permafrost environments under the Martian surface may be the areas where the probability of finding life is the highest. The microorganisms of the permafrost may be considered as models for the types that might be encountered in the Solar System, if life should be found to have distribution in the Cosmos beyond the planet Earth. The features of permafrost bacteria suggest the need for development of improved protocols for their recovery from ancient frozen environment preparation for the analysis of life or its traces in extraterrestrial materials that should soon be returned from comets and Mars.

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PESTICIDE DEGRADATION IN SOILS

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Industrial countries have developed agricultural practices largely based on the use of synthetic organic pesticides, which since the 1950s have greatly enhanced the quantity and quality of food. However, because of the large amount applied since usage began, pesticides have been translocated throughout the entire environment. Pesticides are commonly found in surface waters and groundwater, leading to potential human exposure. Because pesticides are toxic by design, few public concerns of environmental significance are as fraught with emotion as is pesticide use. Soil is the ultimate receptacle of agricultural pesticides, and soil microorganisms are directly exposed to pesticides. Not only can many soil microorganisms tolerate contamination but many can also degrade pesticides through enzymatic reactions. Advances in the basic knowledge of the physiological, biochemical, and ecological processes involved in pesticide degradation, and their genetic determinants, make it possible to better predict the fate of pesticides in the environment and to design more realistic (bio)technologies for the resolution of problems of pesticide contamination.

The objectives of this review are, first, to present the main physiological mechanisms of pesticides degradation by soil microorganisms and to discuss some aspects of their genetic determinants as they can be deduced from the present knowledge on two families of chemicals, the phenoxy and the triazine herbicides. Then, we will examine how these mechanisms interact in natural environments and what conditions are conducive to the selection of strains or consortia showing increased degradation potential. In the final part, we will briefly describe the present state of the art of two practical applications: modeling of degradation, for improving the predictive capacity of commonly used transport models such as PRZM, GLEAMS, PELMO, PESTLA, ..., and bioremediation, which embraces a body of emerging biotechnologies for decontaminating soils from polluted areas.

HISTORICAL ASPECTS: THE HORMONE HERBICIDES

The Role of Microorganisms in Hormone Herbicide Degradation in Soils

As it was concluded that synthetic plant hormones fail to activate plant growth (1), two different groups of researchers, in the United States and the United Kingdom, were studying the differential inhibitory effect of these chemicals on weed growth. The discovery of the possible use of the 2,4-dichlorophenoxyacetic acid (2,4-D) (2) and the 2-methyl, 4-chlorophenoxyacetic acid (MCPA) (3) as selective herbicides was reported as "the greatest advance in the history of weed killing" (4). 2,4-D was preferred (5) because it was persistent enough to give a good and long-lasting protection compared to more naturally occurring β-indol- and α-naphthylacetic acid compounds.

Even with more stable synthetic compounds, it was observed that the herbicidal activity progressively decreased causing reductions in efficacy in the long term. An early discovery was that herbicidal activity of 2,4-D decreased rapidly in wet soils (6–11). Likewise, a decrease in soil temperature (8–12) or reasonable additions of readily available organic amendments (6,7,11) had similar inhibitory effects on herbicide effectiveness. From these preliminary investigations, soil microorganisms were suspected to be primarily involved in the persistence of the herbicidal activity.

More convincing evidence of microbial involvement in phenoxy herbicide degradation was deduced from the shape of the kinetics of hormone herbicide disappearance from soil. Sigmoidal dissipation curves suggested possible growth of a soil microbial population at the expense of the herbicide (13,14). Lag phases frequently observed in herbicide soil dissipation were assumed to reflect the necessary physiological adjustments required for soil microorganisms to adapt to and use the herbicide as a new substrate. Subsequent rapid dissipation was interpreted as resulting from an exponential-like proliferation of effective microorganisms.

Another evidence that soil microorganisms were responsible for hormone herbicide degradation was obtained from the comparison of the persistence of β-indolacetic acid and synthetic hormone herbicides

in sterile and nonsterile soils (5,8,10,11,15). Definitive evidence of microbial involvement in phenoxy herbicide degradation in soils was given when microbial species able to degrade these compounds were isolated from soils. Audus (16) was the first to isolate and identify a bacterial strain, *Bacterium globiforme*, able to use 2,4-D as the only carbon and energy source after perfusion of soil with a solution of the herbicide. Since these pioneering studies, a number of microbial species able to degrade phenoxyalcanoic acids have been isolated from agricultural soils or even from pristine soils, which had never been in contact with these compounds (17). For the most part, with the exception of *Aspergillus niger* (18), bacteria rather than fungi have been isolated. Achromobacteriaceae (*Flavobacterium*, *Achromobacter*, *Alcaligenes*) are well represented and *Pseudomonas* sp. strains, commonly recognized as ubiquitous and metabolically versatile, only reported in a few examples. Many of the isolated bacterial strains are only able to transform but not mineralize the phenoxy compounds. For instance, among 52 bacterial species isolated from sewage sludge, 41 were able to transform 2,4-D or 2,4,5-T without being able to use them as carbon and energy sources (19). It was later pointed out that conventional isolation procedures give a partial and biased view of the composition of the microbial communities involved in pesticide biodegradation in natural environments (20).

The Role of the Soil Matrix in Phenoxy Herbicide Degradation in Soils

An important aspect of preliminary studies was to show that the soil matrix also greatly influenced the persistence of the herbicidal effect. For instance, after two weeks of recirculation of water through six different treated soils, the solution from a peat soil had lost almost all herbicidal activity demonstrating the inactivating role of soil organic matter (21). By comparison, after six weeks of percolation through a sandy clay soil, the presence of 2,4-D could be detected. Other studies have shown that herbicide mobility and leaching, as affected by soil physicochemical properties, could partly explain the observed loss of efficacy (10,21,22). So, as early as the 1950s, the main microbial and physicochemical determinants of the fate of pesticides in soils had been identified.

BIOLOGICAL ASPECTS OF PESTICIDE DEGRADATION IN SOILS

As compared to the timescale necessary for microbial species to evolve new metabolic capabilities, it is reasonable to assume that most xenobiotic compounds will be biodegraded by "appropriation" or "recruitment" among the vast array of microbial enzymes, which have been acquired by microbes when using the numerous naturally occurring biogenic substrates found in very different ecological niches (23). For Leslie-Grady (24), such transformations, which collectively have been called either "fortuitous" (25) or "gratuitous" metabolism (26), are likely to be the primary processes for microbial degradation of xenobiotic compounds in natural environments. These processes are especially effective for chemicals that are structurally related to natural compounds. Yet a structural resemblance does not warrant biodegradability when the compounds cannot penetrate the cell, or at a rate high enough to induce the corresponding enzymes. Differential enzyme specificity may also generate accumulation of intermediates, such as chlorophenols, which may have deleterious effects, as in the case with phenoxyalcanoic acids. Convincing evidence has accumulated demonstrating that specific enzymes dealing with chlorinated intermediates are involved in their degradation. As stated by Dagley (27), increasing the xenobiotic character of a carbon compound by adding substituents such as halogens may not only modify mesomeric effects, especially important for aromatic structures, but also generate steric hindrance. Chemical analogy may be nonsensical from a biological point of view as far as enzyme specificity is concerned. For instance, chlorine elimination from the 2,4-D molecule is made via a lactonizing enzyme, which differs from its counterpart responsible for the same transformation of the nonsubstituted phenoxyacetic acid. Moreover, as reported by Dagley (27,28), cleavage of the C−Cl bond is not a biologically neutral transformation because it generates hydroxy compounds, which may have different subsequent biochemical pathways as compared to the nonhydroxylated template. In other words, at the enzyme level, benzoate degradation may have nothing to do with that of 4-chlorobenzoate.

Main Physiological Processes Involved in Pesticide Degradation by Pure Cultures

Degradation of the Pesticide as the Only Source of C and Energy: Mineralization. If a pure culture can mobilize enzymes to transform a xenobiotic compound into a form, in which it can feed into primary metabolism, it has the advantage of being able to satisfy its requirements for growth and energy from the "foreign" molecule. This degradation process, which has been described in the past for a limited number of pesticides, has beneficial environmental consequences because it does not generate pesticide residues other than "normal" end-products, carbon dioxide, and new microbial biomass. This enrichment has been successively called *soil adaptation* (15), *acclimation* (29) or, more recently, *accelerated degradation* (30). Initially reported for 2,4-D, it has also been shown for chemicals, which were traditionally thought to be relatively recalcitrant. For instance, atrazine is a moderately persistent herbicide, with half-life ranging from several days to several months (31–33). Recently, however, rapid mineralization of atrazine in soils has been observed (34,35) and several pure bacterial isolates or consortia have been described as having the capacity to use atrazine as carbon, and more frequently, nitrogen source (36–42).

Degradation of the Pesticide in the Presence of Other Carbon Substrate: Cometabolism. It has not been possible to isolate, from agricultural soils, microbial species able to use a large number of pesticides as sources of carbon and energy. Most of the isolated microbial strains can perform only limited transformations without deriving nutrients and energy for growth. These transformations

are collectively called cometabolism (43) because transformations are achieved while the culture is growing at the expense of a *cosubstrate* serving as a carbon and energy source. Different organic compounds, varying from the simple low molecular weight compounds (carbohydrates, easily biodegradable structural analogs), to more complex organic amendments (crop residues, farmyard manure, and sewage sludge) can be used as cosubstrates. Cometabolism does not result in extensive degradation of the pesticide and, in pure cultures, leads to the accumulation of intermediate products as demonstrated many years ago by the transformation of the herbicide 2,4,5-T into 3,5-dichlorocatechol by a *Brevibacterium* sp. (44). Some of these metabolites (e.g., chlorophenols, chloroanilines,. . .) may be more toxic than the parent compound and cause inhibition of the degradation process. There is some controversy about the biological mechanism(s) of cometabolism (45). The concept, initially coined *co-oxidation* by Leadbetter and Foster (46), was further named *cometabolism* by Jensen (47) to extend the concept to other transformations such as dehalogenations. Horvath was the first to try to give cometabolism a biochemical basis and a physiological definition. Cometabolism "describes oxidation of a nongrowth substrate during growth of a microorganism on a utilizable carbon and energy source,. . .. (it) does not infer presence or absence of growth substrate during the oxidation" (43). Hulbert and Krawiec (45) disagreed on the character of the physiological novelty of cometabolism and argued that it did not differ from ordinary catabolism or anabolism, and suggested "that use of the word cometabolism be abandoned." To reconcile the two points of view, Alexander (29) and Dalton and Stirling (48) proposed a practical approach: the term could be maintained for transformations exhibiting particular kinetic features, like a slow rate and pseudo first-order rate law indicating the absence of replication of the responsible microorganisms, and having ecotoxicological consequences as a result of the accumulation of intermediate compounds.

Genetic Aspects of Pesticide Degradation by Pure Cultures

Early Observations. In their experiments Audus (15,49) and Audus and Symonds (50) observed that the "archetype" of degrading strain was very unstable in its degrading capacity, which decreased after several transfers on solid 2,4-D medium and was lost after transfer on agar medium with sugars (glucose, saccharose) as only source of carbon and energy. Walker and Newman made identical observations (51) with a series of nine bacterial cultures isolated from different soils from which seven lost their 2,4-D degrading capacity after serial transfers. Fischer and coworkers (52) and Pemberton and coworkers (53) reported that different *Alcaligenes* sp. strains showed variable capacity to spontaneously lose the capacity to use 2,4-D as C source, and that the phenotype was better maintained in the presence of 2,4-D in the culture medium. By maintaining a *Pseudomonas cepacia* AC1100 strain for 18 generations on a glucose medium, Kilbane and coworkers (54) showed that only half the number of the colonies retained the capacity to degrade 2,4,5-T. Kilpi (55) observed the same instability for a dichlorprop and mecoprop degrading community when cultivated in the presence of benzoic acid. More recently, an atrazine-degrading strain, *Pseudomonas* sp. ADP, has been shown to have an unstable phenotype, especially in the presence of a nitrogen source (41).

By contrast, evidence has accumulated that once adapted, the soil microflora may retain its adaptation for months or even years. It has been assumed that the degrading capacity is maintained because of the presence in the soil of organic compounds that are the primary substrates for the enzyme system that can transform a pesticide through cometabolism (56). Waid (57) was the first to suggest the possible involvement of mobile extrachromosomal elements in maintaining the potential for pesticide degradation in soil communities.

Hormone Herbicides.

Evidence of Plasmid-Borne Degradation Genes. Soil adaptation to the degradation of a chemical such as 2,4-D was reported to result from the enrichment of effective microorganisms able to use the compound as a carbon and energy source. It was shown that soil adaptation could be detected even one year after initial treatment of the soil with the pesticide (14,58). Torstensson and coworkers (59) have suggested that under starvation conditions, adapted microorganisms are not subjected to much pressure to use alternative growth substrates, and could maintain their adaptation longer than under more nutrient-rich conditions. In 1976, Kaufman and Kearney (60) gave another explanation based on the presence in the soil of naturally occurring compounds, which could be alternative substrates of the enzyme systems acting upon the pesticide. To support this assumption, Sandman and Loos (61) have shown that catechol and homogentisic acid were oxidized more rapidly by an *Arthrobacter* sp. after cultivation on 2,4-D as compared to citrate. Waid (57) speculated that mobile extrachromosomal genetic elements described as "genetic transfer factors" were involved in an epidemic-like dispersion throughout the soil microflora of the gene sequences encoding for pesticide degradation. Experimental support for this assertion was given five years later by Pemberton and Fischer (62) who isolated a bacterial strain, *Pseudomonas* JMP116, later known as *Alcaligenes paradoxus*, shown to harbor a conjugative plasmid involved in 2,4-D degradation. This plasmid, pJP1 of the IncPI incompatibility group with a large host specificity, was further characterized as having a molecular weight of 58 Md (52) and also encoded for MCPA degradation. Segregants, which were cured, were no more able to degrade 2,4-D but maintained their capacity to degrade 2,4-dichlorophenol. This suggested either that 2,4-D degradation was entirely encoded by a set of genes present on the plasmid with a second set of genes responsible for 2,4-dichlorophenol degradation present on the chromosome or that only the 2,4-D monooxygenase encoding gene was plasmid-borne.

Localization and Regulation of the Degradative Genes on the Plasmid pJP4. Working with the canonical plasmid, pJP4, an 80-kb broad host range plasmid, very close to

pJP1 and isolated from a *Ralstonia eutropha* JMP134 strain (formerly *Alcaligenes eutrophus*), Don and Pemberton (63) localized five genes *tfd B*, *C*, *D*, *E*, and *F* encoding the transformation of 2,4-dichlorophenol into chloromaleylacetic acid. Kukor and coworkers (64) showed that the gene for maleylacetate reductase was chromosomally located and involved in tyrosine catabolism (Fig. 1).

Using Tn5 and Tn1771 transposon mutagenesis, Don and Pemberton (63) localized genes involved in mercury resistance, 2,4-D and 3-chlorobenzoate degradation.

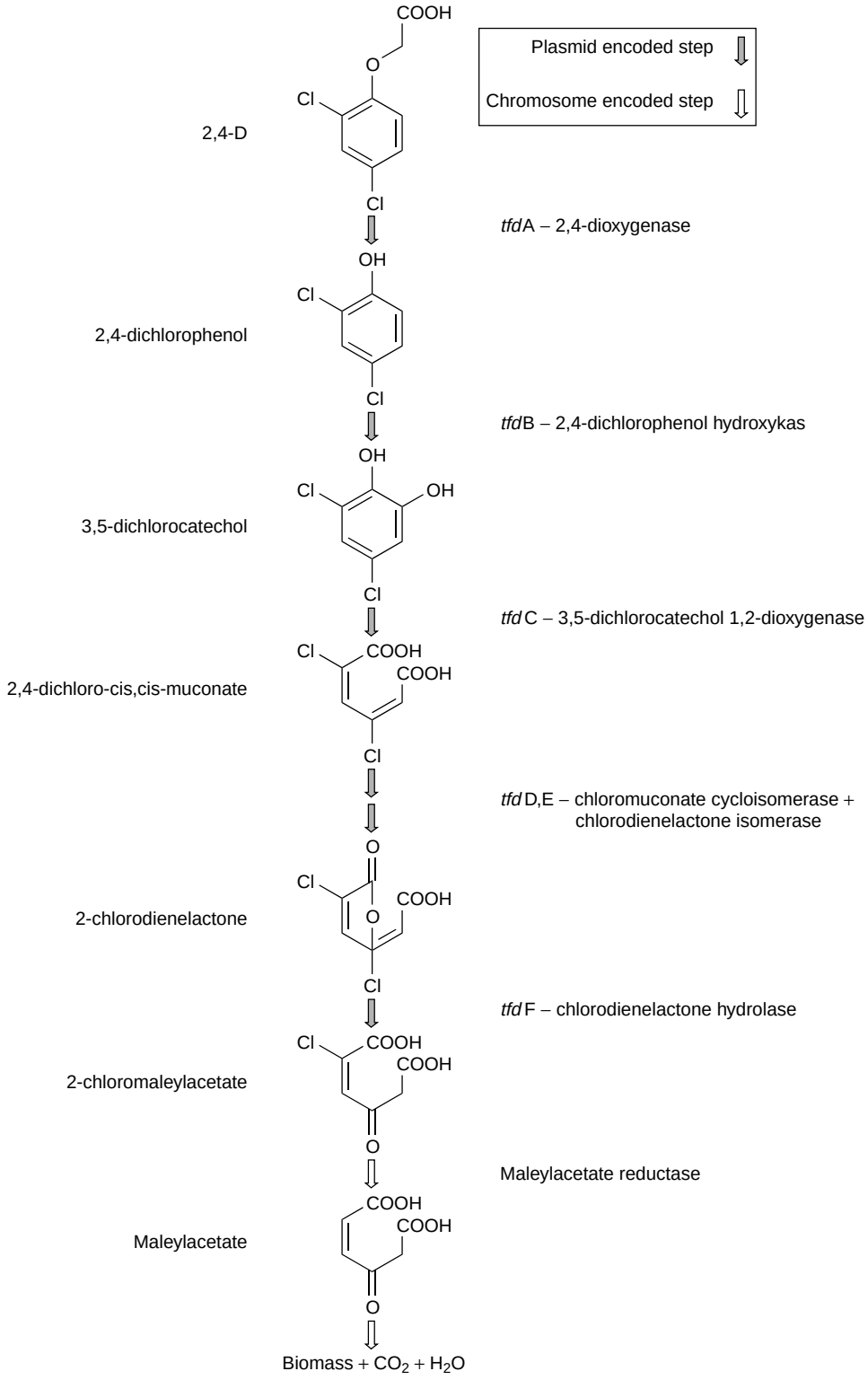

Figure 1. 2,4-D degradation pathway by *R. eutropha* JMP 134.

Locating the origin of the plasmid at the beginning of the fragment EcoRI A, the first genes are located at about 70 kb, whereas others are borne on EcoRI B fragment in between 28.8 and 43.3 kb. Four of the genes, which are also involved in 3-chlorobenzoate degradation, genes *tfdC*, *D*, *E*, and *F*, encoding respectively for dichlorocatechol 1,2-dioxygenase (or pyrocatechase), chloromuconate cycloisomerase, chlorodienelactone isomerase, and chlorodienelactone hydrolase are organized as one operon (65). Ghosal and You (66) also showed that the *tfdC* and *tfdD* genes were overlapping for four base pairs, with a possible coupling at the transcription level. The *tfdC* gene sequence has been elucidated. It is 765-bp long and corresponds to 255 amino acids and a molecular weight of the corresponding protein of 28,000 daltons. Later on, Streber and coworkers (67) located the *tfdA* gene for the 2,4-D oxygenase on the plasmid at a distance of 13 kb of the 2,4-D operon, and Fukumori and Hausinger (68) have shown that it encodes an α-cetoglutarate-dependent dioxygenase. It is largely distributed among gram-positive and gram-negative soil bacteria that do not degrade 2,4-D, suggesting that *tfdA* genes or closely related homologs are widespread among soil isolates, and likely to exist for a purpose other than the degradation of 2,4-D (69). Not all species able to degrade 2,4-D have the corresponding genes located on a plasmid. For instance, it was deduced from plasmid curing experiment that the 2,4-D catabolic genes of a *Burkholderia* sp. strain RASC were located on the chromosome (70).

Using complementation experiments with 3-chlorobenzoate, utilizing *Pseudomonas putida* strains, Ghosal and coworkers (71) suggested the existence on the fragments EcoRI E and F of pJP4 of genes suspected to play a positive regulatory role for the structural genes present on the fragment EcoRI B. The same authors observed that the 3-CB+ phenotype resulted from the constitutive expression of the structural genes borne on seven copies of the EcoRI B fragment. After having isolated a mutant of *A. eutrophus* JMP 134 expressing constitutively the 2,4-D degrading genes, Pieper and coworkers (72–74) made the assumption that a pJP4 negatively regulatory gene had been mutated. From studies with mutants deleted in the BamHI, F and E fragments of pJP4, evidence was given for the presence of a regulatory gene, *tfdR*, which repressed the expression of the *tfdA* gene and *tfdCDEF* operon (75). A second regulatory gene, *tfdS*, was also located on the same fragment and has been proposed to repress and activate the expression of the *tfdB* gene (76). Because *tfdS* and *tfdR* genes are in fact identical inverted repeats located at about 8 kb away from the *tfdCDEF* operon on plasmid pJP4, they should not have different regulatory effects. They are oriented divergently from respectively the *tfdA* gene and a *tfdD*-like gene (tfD_{II}) of a second $tfdC_{II}D_{II}E_{II}F_{II}$ operon. A third regulatory gene, *tfdT* (formerly *tfdX*, 77), highly homologous to the *tfdS* and *tfdR* genes has been located upstream of, and transcribed divergently from, the *tfdCDEF* operon. The presence of regulatory sequences upstream catabolic genes or catabolic operons has been interpreted as signifying that proximal pairs, *tfdA-tfdS* or *tfdR- tfdD*$_{II}$ . . ., and cis effects are perhaps important in the regulation of the TFD pathway (77). Yet, this is probably not true for the *tfdCDEF* operon. The putative regulatory gene, *tfdT*, is inactivated by the presence of an insertion sequence, which disrupts the ORF leading to the production of a dysfunctional protein. It is likely that the *tfdR* or *tfdS* genes can successfully take over the regulation of this operon (78). A new ORF was recently identified on plasmid pJP4, which was designated by *tfdK*. It was proposed to be responsible for the production of a transporter protein involved in an energy-dependent transport process (79). This is the first description of a gene involved in the facilitated transport of a pesticide molecule.

Ghosal and coworkers proposed a higher-level degree of regulation (71) after having observed that under extreme conditions of selection, gene expression is activated only after sequence rearrangement. No specific mechanisms for these rearrangements have been elucidated and nothing is known either about their dependency on host genome, their environmental constraints, or their actual functional significance. More recently, a self-transmissible 2,4-D degradative plasmid, pKA2, has been found either as a free state or integrated into the host chromosome without loss of the 2,4-D+ phenotype. This suggests that a chromosome–free plasmid cycle may occur to optimize fitness under conditions of resource fluctuations (80).

Polymorphism of the 2,4-D Degradative Genes. While the first *tfdA* gene coding for the 2,4-D monooxygenase was detected on pJP4 at a distance of 13 kb of the *tfdCDEF* locus (67), a second dissimilar locus on pJP4 was observed in the region of 2.5 kb adjacent to the *tfdC* gene. Hybridization experiments under low-stringency conditions demonstrated the presence of a related copy of a second gene coding for an active 2,4-D monooxygenase, $tfdA_{II}$ (81). Plasmid pJP4 was also shown to contain a second set of non identical chlorocatechol oxidation gene sequences physically separated by a 7 kb DNA region from the first (66). Later on, a putative ORF coding for a chloromuconate cycloisomerase was identified. This ORF was designated as an isofunctional gene, $tfdD_{II}$, and shared 57.6 % identity at the DNA level over 738 bp with the *tfdD* gene (77). Finally, a whole new additional set of *tfd* genes, that is, $tfdD_{II}C_{II}E_{II}F_{II}$, $tfdB_{II}$ was discovered on pJP4 (79).

A high degree of interspecies variation in 2,4-D degradative gene sequences has also been found. The 2,4-D dioxygenase gene *tfdA* and the 2,4-dichlorophenol hydroxylase gene *tfdB* have been described for different 2,4-D degrading soil isolates (82). It is likely to reflect different substrate specificity. An extensive study with 47 predominant 2,4-D degrading bacteria isolated from different 2,4-D treated or nontreated plots revealed four hybridization groups by using *tfdA*, *tfdB*, *tfdC*, and *tfdD* probes and a probe, *Spa* that detects a distinctly different 2,4-D degrading isolate, *Sphingomonas paucimobilis* (formerly *Pseudomonas paucimobilis*) (83). Hybridization group one strains are very diverse, contain plasmids and most of the plasmids exhibit hybridization to the *tfd* probes. Members of group two, which hybridize only with the *tfdA* probe, are a subgroup of group one. The highest number of isolates was found in group three. It

contained tightly clustered strains, which did not seem to contain small (<350 kb) plasmids and exhibited detectable hybridization only to the *Spa* probe. Group four was the "all other" group with very diverse isolates that did not hybridize to any of the probes. Probing thirty two 2,4-D-degrading isolates representing 19 genomic fingerprints from diverse locations with the *tfdA*, *-B* and *-C* sequences, Fulthorpe and coworkers (84) observed that the majority of the strains exhibited various degree of homology to the first 3 *tfd* genes of the 2,4-D degradative pathway. Moreover, most strains showed different combinations of high- and low-similarity *tfd*-like genes, suggesting that independent interspecies movement of these genes had occurred and that some strains had recruited individual genes from different sources.

Knowledge of the diversity of the genes coding for chlorocatechol dioxygenases (CCD) enzymes seems particularly useful for understanding how extended catabolic capacities have evolved from a few channeled metabolic steps involved in bacterial aerobic degradation of aromatic compounds. Dioxygenases catalyzing cleavage of the aromatic nucleus are an important class of enzymes for they control degradation of the aromatic material of the soil organic matter. It has been established that chlorocatechol dioxygenases of the modified ortho pathway (or type II dioxygenases) are a distinct subgroup of ring cleaving enzymes (85). Many of these enzymes are coded by plasmid located genes found in gram-negative strains, *clcA* on pAC27 (*P. putida*) (71), *tcbC* on pP51 (*Pseudomonas* sp. strain 51) (85), *tfdC* on pJP4 (*R. eutropha*, formerly *A. eutrophus* JMP 134) (65), pEML159 (*Alcaligenes* sp.) (86), pRC10 (*Flavobacterium* sp.) (87), pEST4011 (*P. putida* PaW85) (88) or pMAB1 (*Burkholderia cepacia*, formerly *P. cepacia* CSV90) (89). With the exception of the chlorocatechol 1,2 dioxygenase of *Rhodococcus erythropolis*, which exhibits only 15 to 22% identity to the catechol 1,2 dioxygenase and the chlorocatechol 1,2 dioxygenase (90), it seems that many of the type II pyrocatechases with different substrate specificity show some degree of homology in their amino acid sequences. For instance, it has been shown that some specialization among type II pyrocatechases has taken place: TcbC converts preferentially 3,4-dichlorocatechol, TfdC has a higher activity toward 3,5-dichlorocatechol and 3-chlorocatechol is more specifically transformed by ClcA (91). By comparison, the *R. erythropolis* enzyme has a distinct preference for the 4-substituted catechols (92). In the study of Fulthorpe and coworkers (84), many strains did not hybridize with *tfdC*, although they showed chlorocatechol dioxygenase activity. Diversification of the probes is needed to unravel the diversity of these CCD and other genes in environmental samples. An example of the recognition of the polymorphism of the *tfdC* genes by polymerase chain reaction (PCR) amplification and hybridization is illustrated by a study on 31 2,4-D degrading isolates, all in the α- or β-subdivision of the Proteobacteria (93). Hybridization has been performed with two specific probes produced from *R. eutropha* JMP 134 and a soil isolate, strain PLAE6, respectively. These probes are homologous to the *tfdC* genes of JMP 134 located on pJP4 and to the *tfdC* gene of *P. putida* PaW85 located on plasmid pEST4011. Most of the strains were found to contain *tfdC* genes on plasmids ranging from 78 to 532 kb, and two strains with no detectable plasmid were found to hybridize with the probes revealing chromosomal localization of catabolic genes. Strains and isolates, which carry a *tfdC* gene homologous to that of *P. putida* PaW85 have one copy of *tfdC* with similar restriction fragment length polymorphism (RFLP) patterns. On the other hand, strains carrying a *tfdC* gene homologous to the *tfdC* (pJP4) gene showed more variable RFLP patterns and carried two copies of *tfdC*, one homologous to the canonical tfdC of *R. eutropha* JMP 134 and the other, $tfdC_{II}$ as detected in *R. eutropha*, to *tfdC* of *P. putida* PaW85. This result illustrates the process of duplication of genes or of entire operons, which may occur and be selected in the presence of xenobiotics, recently introduced in the environment. In that case, *tfdC* of *P. putida* may be assumed to be the immediate ancestor of the canonical *tfdC* gene in *R. eutropha*.

Chlorocatechol 1,2 dioxygenase genes are also important from another point of view. They are often found as part of a relatively well-conserved gene cluster known as the chlorocatechol oxidative pathway (85). The cluster is an operational unit made of three or four structural genes, *tcbCDEF* (*Pseudomonas* sp. strain 51), *clc ABD* (*P. putida*) and *tfdCDEF* (*R. eutropha*), regulated by the *tcbR*, *clcR*, or *tfdR* (*tfdS*) genes coding for proteins, which are all members of the LysR family of transcriptional activators. Yet, individual genes have been found in other combinations, illustrating that evolution may proceed through different ways. For instance, a *tfdC*-like gene has been found associated with a *tfdB*-like gene in the degradative plasmid pEST4011. It was assumed that both genes form one operon, *tfdCB* (94), suggesting that isofunctional genes can be found at different levels of organization. This is another indication of independent movement of these genes and/or individual recruitment from different sources. Some of these genes are integrated in regulated polycistronic operons, which are likely to have evolved under a natural selective process that may have been operating before contamination of soils with chlorinated xenobiotic compounds. Others are present in more simple regulated structures, or even as proximal pairs with one structural gene being under the direct control of a regulatory gene.

Diversity of 2,4-D Degradative Plasmids. From 50 soil samples, Don and Pemberton (95) isolated 22 microbial species able to use 2,4-D as a sole source of carbon and energy. 17 of these strains lost the tfd+ character after 15 to 20 subcultures on peptone + yeast extract agar with no 2,4-D. Of the five strains showing a stable tfd+ phenotype, all were identified as *A. eutrophus*. Curing experiments demonstrated that the *tfd* genes are borne on conjugative plasmids, which belong to two different incompatibility groups. pJP2 and pJP9 are of the IncP3 group and show the capacity to degrade the non chlorinated phenoxyacetic acid (71); whereas, pJP3, 4 and 7 are of the IncP1 group. These plasmids encode for mercury resistance and for the degradation of MCPA and 3-chlorobenzoate.

Other 2,4-D degradative plasmids have been found in different bacterial species. The plasmid pEML159

has been detected in *Alcaligenes* sp. EML159 (86). Its size, 56 Md, and its restriction profile are very close to those of pJP4. The 45 kb plasmid pRC10 has been isolated from a *Flavobacterium* sp. (strain 50,001) (87), pEST4011 in a *P. putida* PaW85 (88), pMAB1 in a *B. cepacia* (formerly *P. cepacia* CSV90) (89), pTV1 in a *Variovorax paradoxus* (96), pKA2 in an *A. paradoxus* 2811P and pKA4 in a *Pseudomonas pickettii* 712 (81). The diversity of 2,4-D degradative plasmids has also been examined by complementation in soils that had been treated with 2,4-D for several years. Seven plasmids with different EcoRI restriction profiles, pEMT1 to pEMT7, have been detected. All but pEMT1 belong to the IncP1 incompatibility group (97). Another hybridization study with *tfdA*, *B*, and *C* probes to the DNA of 32 2,4-D degrading bacteria from different geographic locations has revealed a high diversity of hybridization patterns. The simple horizontal transfer of a large piece of DNA that contains all the *tfd* catabolic genes cannot explain this diversity. These results indicate that homologous or even identical genes may be found on different plasmid backbones in different order, suggesting that individual genes or groups of genes as the chlorocatechol oxidative pathway are moving independently to generate different associations as mosaic constructions (84).

There is evidence that plasmids involved in 2,4-D degradation can be exchanged between strains of different species or even different genera. For instance, Pemberton and coworkers (53) detected frequencies of transfer of the tfd+ phenotype varying from 10^{-3} to 5.10^{-2} per donor with six plasmids encoding for 2,4-D degradation. Friedrich and coworkers (98) obtained tfd+ transconjugants with different *A. eutrophus* at frequencies varying from 2.10^{-2} to 10^{-6} per donor cell after conjugation with the pJP4 containing *Escherichia Coli* JMP 397.

Horizontal transfer of a plasmid may result in modifications of its structure. Don and Pemberton (99) reported different molecular weights for the plasmid pJP2 in its natural host (150 Md) or in a transconjugant (37 Md). Friedrich and coworkers (98) observed a reduction in the size of the plasmid pJP4 in tfd+ *A. eutrophus* transconjugants, with the length of the deletion varying with the strain. Moreover, they detected plasmids, which may have resulted from multimeric associations of pJP4. After *E. coli* JMP397 was transformed with pJP4, Amy and coworkers (86) showed that the parent plasmid was present as a nonmodified copy together with a 66 Md plasmid. The formation of this plasmid was interpreted as resulting from the integration of chromosomal DNA. Taken together, these observations lead to the conclusion that spread of a plasmid in a composite microbial assemblage may result in structural diversification adapting the plasmid structure and functionality to the genetic environment of its different microbial hosts. We do not know exactly what are the mechanisms of recombination leading to these new genetic rearrangements in different bacterial strains, even if transposons have been suspected to play a role in the transfer of toluene and chlorobenzoate degradative genes (100). All the recipients cannot express the *tfd* genes. We do not know what are the species determinants, which govern gene expression and what are the functional consequences, which result from changes in gene sequence and organization. The observed gradual shifts in the distribution of the dominant species after long term 2,4-D applications revealed two different groups of successful competitors. The first group consisted of diverse strains of *Burkholderia*, *Alcaligenes*, or related species, all members of the β-subdivision of the proteobacteria class. These strains carry genes, which are highly homologous to *tfdA*, *-B*, *-C* of pJP4. Members of the second group were all included in the α-subdivision of the proteobacteria class. They were mainly related to the *Sphingomonas* genus and carry degradative genes that share no homology to *tfdA* and *−C* genes and only weak homology with *tfdB* of pJP4 (83).

The presence of an effective 2,4-D degrading community has been shown in pristine soils where one would not expect them to have a selective advantage (17). This is consistent with the assumption that *tfd* genes preexist in soils, where they are likely to determine degradation of naturally occurring compounds. Soil microorganisms may naturally produce halogenated and especially chlorinated compounds. For instance, chlorinated antibiotics are produced by Streptomycetes. 2,4-dichlorophenol is another natural compound, which is produced by *Penicillium* strains and suspected to have a hormonal (101) or an antibacterial activity (102). Many studies have demonstrated that the capacity to degrading chlorinated phenols is not uncommon in soils. (103–105). Chapman (106) made the assumption that the presence in soils of a microflora capable of degrading 2,4-D results from the combination of its joint capacity to degrade 2,4-dichlorophenol, for which a degradative pathway has been elaborated, and to cut ether bonds. Consistent with this assumption are the following observations *−i*: a well-organized and autonomously regulated operon of four genes involved in aromatic ring cleavage, the *tfdCDEF* operon in the case of 2,4-D, is commonly found in soil isolates indicating that evolution has already taken place before introduction of the herbicide, *−ii*: *tfdA*-like genes coding for an etherase are widely distributed among soil isolates, which do not degrade 2,4-D (69). Streber and coworkers (67), Harker and coworkers (75) and Pieper and coworkers (73) have shown that the product of the *tfdA*(pJP4) gene was an enzyme with a broad substrate specificity acting indistinctly upon 2,4-D, MCPA, but also on 4-chlorophenoxyacetic acid (4-CPA), 2-methylphenoxyacetic acid (2-MPA) and the nonsubstituted phenoxyacetic acid.

The Triazine Herbicides. Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) is the most widely used s-triazine herbicide to control a variety of broadleaf weeds infesting corn, sorghum, and certain other crops. Its widespread use has caused environmental concern because of frequent detection of atrazine in surface water (107,108), rainwater (109), tile drainage (110), and groundwater (111,112) at concentrations exceeding the EU regulatory limit of 0.1 $\mu g\,l^{-1}$ and the U.S. EPA maximum contaminant level of 3 $\mu g\,l^{-1}$ (113).

Atrazine is a moderately persistent herbicide, with half-lives ranging from several days to several months (31–33).

Up to 10 years ago, the most commonly accepted degradative pathway involved oxidative *N*-dealkylation with the formation of deethylatrazine (DEA), deisopropylatrazine (DIA), and deethyldeisopropylatrazine (DIEA) as major metabolites, and other heterocyclic compounds such as ammeline, ammelide, and cyanuric acid at trace concentrations (Fig. 2, left-hand side). Very little ring cleavage has been reported. It is now generally accepted that only alkyl side chains can provide carbon and energy to the degrading microflora (42).

A *Rhodococcus* strain TE1, which can metabolize the herbicide *S*-ethyl dipropyl tiocarbamate (EPTC) has been isolated from soil. This strain also transformed atrazine and other chlorotriazines, propazine, simazine, and cyanazine, under aerobic conditions to produce dealkylated derivatives, which were not degraded further in liquid culture. The atrazine-degrading phenotype was unstable and associated with it was a 77-kb plasmid also involved in EPTC degradation. The *atrA* gene responsible for the *N*-dealkylation reactions is borne on a 6.2-kb KpnI fragment also harboring the *eptA* gene for EPTC degradation (114). An inducible cytochrome P-450 enzyme system has been shown to be involved in the degradation of EPTC and atrazine by a *Rhodococcus* sp. strain NI86/21 (115). The current data provide evidence that in strain NI86/21 the *thcB* (coding for a novel type of cytochrome P-450 enzyme), the *thcC* (coding for rhodocoxin) and *thcD* (coding for rhodocoxin reductase) genes conferred the atrazine-degrading phenotype. Another *Rhodococcus* strain, *Rhodococcus corallinus*, was shown to dechlorinate and deaminate deethylsimazine. The gene responsible for the s-triazine hydrolase, *trzA*, was subcloned and transformed into different *Rhodococcus* strains, where it can be expressed. A plasmid carrying both *atrA* and *trzA* genes was constructed and introduced into three *atrA* and *trzA*-deficient *Rhodococcus* strains. Both genes were expressed in the recombinant cells (116,117). *Trz* genes coding for the s-triazine catabolism were also found in other microbial strains: *Pseudomonas* sp. strain NRRLB-12227, which metabolizes

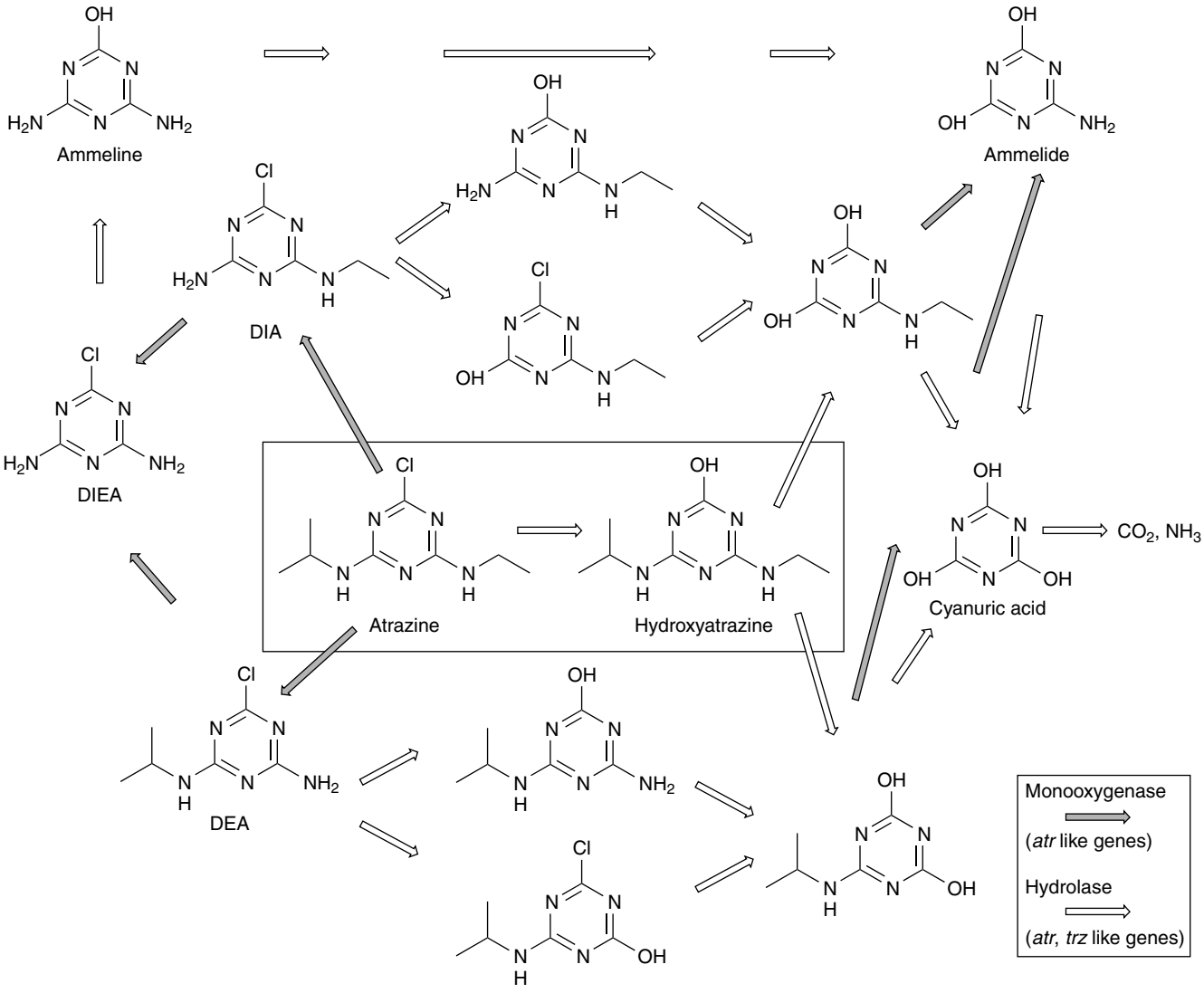

Figure 2. Suspected interconversions between atrazine transformation products in natural ecosystems (for abbreviations, see text).

melamine, *Pseudomonas* sp. strain NRRLB-12228, and *Klebsiella pneumoniae* 99 growing on ammelide and lacking the *trzB* gene coding for the ammeline aminohydrolase. Both *trzC* and *-D* genes share extensive sequence identity on the basis of their restriction enzyme cleavage maps. In *Pseudomonas* sp. strain NRRLB-12227 the *trzB* gene has been located close to the *trzC* gene, and the s-triazine catabolic genes seem to be present on a transposable element. More recently, it has been shown that the genes coding for the ammelide aminohydrolase (*trzC*) and the cyanuric acid amidohydrolase (*trzD*) are borne on an IncPI plasmid in *K. pneumoniae*. As demonstrated for 2,4-D, the presence of s-triazine catabolic genes on mobilizable DNA supports the idea that genetic exchange is an important process in the evolution of novel catabolic pathways (118,119).

Recently, there have been a number of reports on accelerated atrazine mineralization in soils under continuous maize cropping with yearly atrazine applications (34,35). Enrichment culture techniques have permitted isolation of consortia (36,40) and pure bacterial isolates (38,39,41,42) capable of complete mineralization of the chemical. With these strains, peripheral catabolism proceeds first through dechlorination, followed by hydrolytic side chain cleavage and opening of the ring structure. The biuret intermediate compound is further mineralized to CO_2 and NH_3 (Fig. 2, right-hand side). An atrazine-metabolizing *Pseudomonas* sp. ADP was isolated using the "clearing zone" technique on agar plates containing 1,000 mg/L atrazine (solubility 30 mg/L). The strain used atrazine as its sole source of nitrogen and carbon of the triazine ring was almost completely released as CO_2 (41). The genetic determinants behind these transformations have been partially elucidated. The first three genes have been cloned and sequenced. The first gene, *atzA*, codes for a chlorohydrolase and is involved in atrazine dechlorination with hydroxyatrazine being the first metabolite. Hybridization experiments using a probe containing the putative atrazine chlorohydrolase gene with the DNA of different atrazine degrading bacteria has indicated that the formation of hydroxyatrazine is widespread in natural systems (120,121). The protein AtzA shares 41% amino acid homology with the TrzA protein of *R. corallinus* NRRLB-15444R, involved in the dechlorination-deamination of deethylatrazine (CIAT), and deisopropylatrazine (CEAT), but not of atrazine. A second gene, *atzB*, codes for an amidohydrolase, which catalyzes hydroxyatrazine deamidation yielding *N*-isopropylammelide. It is located on the same 21.5 kb EcoRI genomic DNA fragment as *atzA* at a distance of 8.7 kb downstream. The AtzB protein has only 25% amino acid identity with TrzA (121). The *atzC* gene codes for an enzyme that catalyzes the hydrolytic deamidation of *N*-isopropylammelide to cyanuric acid and isopropylamine. This enzyme is also a member of the broad family of amidohydrolases. Sequence comparison of AtzA, -B and -C proteins in the most highly conserved *N*-terminal region containing a metal-coordinating histidine residue suggests that the corresponding genes diverged from a common ancestor (122). The *atz* genes are widespread and DNA sequence comparison of five other atrazine-degrading bacteria has revealed more than 99% identity indicating only limited evolutionary divergence (123). A *Rhizobium* sp. isolate, which was found to actively degrade atrazine with accumulation of hydroxyatrazine, has a chlorohydrolase sharing 92% identity to the chlorohydrolase of *Pseudomonas* sp. ADP (42). Moreover, in *Pseudomonas* sp. ADP, the *atzA*, *-B*, and *-C* genes are located on a 96-kb self-transmissible plasmid, pADP-1, conferring an unstable atrazine-clearing phenotype to *Pseudomonas* sp. ADP (123). Nothing is known on the mechanisms of regulation of the *atz* or *trz* genes except for a *Rhizobium* sp. isolate where the AtzA-like chlorohydrolase was constitutively expressed and not induced by atrazine (42).

PESTICIDE DEGRADATION IN NATURAL ENVIRONMENTS

Microbial Fallibility and Molecular Recalcitrance

In explaining the observed low degradability of some pesticides in soils and considering that soils naturally contain complex compounds difficult to mineralize, such as humic substances, Alexander (124) developed the concepts of "molecular recalcitrance" and "microbial fallibility." He criticized the paradigm of microbial omnipotence, which was speculated to result from the enormous metabolic diversity of the soil microflora together with its potential to adapt to new substrates. Several conditions must be fulfilled for a particular substance to be subject to biodegradation, such as the existence and presence of organisms having alone or in combination the potential for biodegradation, the possibility for the microbial population to proliferate in the environment, the possibility for the compound to be available and to penetrate the microbial cells, and the efficiency of appropriate enzymes. Alexander (125) has enumerated some of the main mechanisms of recalcitrance, which are reported on Table 1. Very often, molecular recalcitrance cannot be predicted from the molecular structure of the compound. Biodegradability is an experimental property, which results from the observation of the repeated failure to isolate degrading microorganisms and to find conditions where effective degradation takes place (24).

Environmental Factors Affecting Biodegradation of Pesticides

The soil is a complex and heterogeneous environment, which plays a crucial role in affecting growth, survival, and activity of microorganisms. Information about the effects of environmental factors on pesticide degradation is relevant from two practical points of view. First of all, a good mathematical description of the environmental factors, which govern microbial activity, is critical for obtaining mathematical models with a good predictive value. Secondly, some of these factors can be managed to optimize bioremediation procedures.

Moisture content and temperature are the most important factors regulating microbial activity. Water is a solvent for pesticide, and other nutrients and is essential to transport them inside the cell and for maintaining microbial activity. Biodegradation of pesticides in soil increases with water content up to a certain level, above which it declines as a consequence of poor oxygen transfer. A great majority of pesticides are better degraded

Table 1. The Main Mechanisms of Recalcitrance of Pesticides Biodegradation in Soils (After M. Alexander, *Biotechnol. Bioeng.* 15, 611–647 (1973))

Limitations due to intrinsic biological limitations
• the lack of the required novel array of enzymes
• the remote likelihood of finding a single mutant or an assortment of mutants with all the requisite enzymes
• a too narrow enzyme specificity
• the lack of sufficient carbon and energy for growth or of essential nutrients like oxygen and water, which control microbial activity,
• the production of toxic intermediates
Limitations due to environmental constraints
• inactivation of extracellular enzymes on the soil matrix
• inaccessibility of the substrate because of poor solubility
• localization in micropores too small for microbial penetration
• retention on soil organic and inorganic adsorbents
• complexing of the substrate with high molecular weight polymers
• formation of stabilized condensation products (126)

under aerobic conditions. However, under anaerobic conditions, other soil components such as nitrate ions are used as potential alternative electron acceptors. There is limited knowledge about anaerobic biodegradation processes. Some organochlorine chemicals (e.g., DDT) are better degraded in anaerobic conditions through reductive dehalogenation.

Soil temperature has a direct effect on the reaction rate of pesticide degradation, enzyme activity being optimum at mesophilic temperatures. Temperature is also important in modifying solute concentrations in the water phase. Under certain latitudes, some adaptation of the microflora to higher or lower temperatures has been described. Seasonally, soil temperature and moisture content do not vary independently. As a rule, in winter when the soil water content is optimum, temperature is the limiting factor. The reverse is true in summer, when water deficit limits microbial activity.

Other soil characteristics and properties are also of importance in regulating microbial activity. Soil pH may affect the ionic state of pesticides and hence their solubilization and sorptive properties. For instance, it has been demonstrated that accelerated degradation of atrazine in agricultural soils is dependent on pH (127). Organic matter and clays are crucial soil components and play a major role in the sorptive processes, which regulate availability of pesticides and protect them from microbial attack.

For pesticides that are cometabolized, the presence of easily available and biodegradable organic substances may increase the rate and extent of biodegradation. There is a rough positive correlation between the soil organic matter content and the rate of pesticide degradation (128). This is to be related to the higher level of microbial biomass combined with a higher biochemical diversity and a higher level of metabolic activity. Taken together, these biological characteristics increase the probability of finding effective microorganisms or the occurrence of evolutionary fitness with the possible emergence of an organism showing novel metabolic capacity.

Degradation by Microbial Assemblages Primary and Ultimate Degradations

Degradation of a pesticide in soil is a complex phenomenon involving the contributions of different kinds of microorganisms acting on the molecule at different rates, using different metabolic pathways, based on different physiological features. Again the example of 2,4-D gives an illustration of the actual biological nature of pesticide biodegradation. It has been observed that mineralization of 2,4-D in the soil follows a two-wave process. In the few days following its application, there is a first flush of mineralization achieved by a consortium of microorganisms, which are inhibited at high 2,4-D concentration (129) but respond positively to organic amendments (Soulas, unpublished). At this stage, the degradation process is interpreted as the result of the combination of cometabolic transformations by the opportunistic fraction of the soil microflora. The rate of mineralization increased again after one week to reach a maximum at about 15 days after application. The peak likely results from the taking over by a specific microbial population or community able to grow at the expense of the herbicide. At the phenomenological level, there is evidence that the same compound can be converted by both metabolism and cometabolism. An illustration of the inability of some microorganisms to completely mineralize 2,4-D is given by Bauer and coworkers (130), who isolated a *Pseudomonas* sp. strain able to cooxidize this herbicide in the presence of benzoate with the formation of monochlorophenol. There is no report of the accumulation of these phenolic intermediates in soils, as it is likely that degradation can proceed further through the action of other microbial strains.

Noting that degradation of 2,4,5-T by a *Brevibacterium* sp. resulted in the accumulation of 3,5-dichlorocatechol, and that this intermediate could be degraded by an *Arthrobacter* sp. (131), Horvath (43) suggested that an association of both organisms had the potential to completely mineralize 2,4,5-T. Chakrabarty and his collaborators have added experimental evidence in support of this assumption. In long-term chemostat co-cultures of a complex mixture of microorganisms from waste-dump sites, or chosen because of the presence of plasmids involved in the catabolism of aromatic compounds (plasmids TOL, SAL), Kellog and coworkers (132) isolated a mixed culture of three or four strains able to use 2,4,5-T as growth substrate after 8 to 10 months. A number of chemicals have been shown to be degraded by microbial consortia. This is the case with trichloroacetate (TCA) (133), parathion (134), silvex [2-(2,4,5-trichlorophenoxy) propionate] (135), mecoprop [2-(2-methyl, 4-chlorophenoxy) propionate] (57,136), and, more recently, atrazine (40).

For some chemicals, naturally occurring microbial communities fail to go to the point where transformation products can be channeled into central metabolic pathways, making carbon and energy available. These incomplete transformations have been collectively called

primary biodegradation (137). They generate the accumulation of intermediate compounds as demonstrated by DDT, which is converted into the more stable DDE and DDD in soils. By comparison, *ultimate biodegradation* denotes the process by which mixed cultures can account for complete mineralization (137). Probably, this situation is prevalent in soils. At the community level, primary and ultimate biodegradations are the functional equivalents of metabolism and cometabolism as defined for pure cultures. The possibility for a chemical to be used as a growth substrate causes the proliferation of microbes involved in the metabolic process. While maintaining a selective pressure by applying the chemical repeatedly on the same plot, long-term enrichment of responsible microorganisms may be enough to cause accelerated degradation of the pesticide as demonstrated in the 1950s for 2,4-D and other phenoxy herbicides (15). From a practical point of view, repeated applications of soil treatment chemicals may contribute to alter their efficiency against target organisms. Since the discovery of this phenomenon it has occurred with an increasing number of chemicals, carbofuran (138) and, more recently, atrazine (34) being two examples with practical significance.

Ecological and Evolutionary Advantages of Natural Microbial Communities

If microbial diversity is an important factor in metabolic complementation and efficiency, it may also help microbial communities to maintain activity under changing environmental conditions. Microbial communities have a greater fitness than pure cultures. This potential mainly results from enzymatic redundancy, which gives the community enough flexibility to tolerate small differences in substrate structure and to compensate for changes in environmental conditions regulating enzyme activity. Another reason for the ecological superiority of microbial communities is their potential for hosting alternative metabolic pathways, which may act as temporary by-pass routes that offer new possibilities for the flow of energy when adverse conditions prevent the main metabolic pathway to be fully operative. Moreover, the possibility for the microbial consortia to function on a partnership basis, for example, through enzyme complementation, exchange of growth factors,... contribute to give them additional metabolic capabilities that no single cell can exhibit separately.

It is also likely that, in soil, the different members of a functional community are in close proximity to one another, such as microbial aggregates. This spatial organization promotes efficient exchange and spread of mobile genetic elements such as plasmids, and increases the probability for the emergence of individuals endowed with a novel catabolic capacity. Within microbial communities, the potential for each individual species to adapt to new conditions depends not only on its genetic material but also on the genetic material it may receive from other species through horizontal transfer. For Reanney (139), spreading of some genes through horizontal transfer is a conservative process ensuring survival of the communities when conditions are no longer favorable for their maintenance.

In conclusion, if microorganisms are individually able to cope with xenobiotic organic compounds, their adaptive capabilities are enhanced when this potential is expressed within microbial communities. In communities, the genetic diversity is enhanced, as is the potential of occurrence of combinatorial events favorable to the emergence of new catabolic pathways.

Application 1: Bioremediation of Pesticide Contaminated Soils

Soil Bioremediation Techniques

Bioremediation technologies derive from the realization that microorganisms mainly mediate the destruction of pesticides in natural environments. The use of a microbial inoculant for the removal of a pesticide from soil was first suggested by Audus (15) for 2,4-D. Bioremediation techniques are called for in different circumstances (*1*) the lack or insufficient amount of cells (mostly microorganisms) or enzymes that can degrade a pesticide and (*2*) the insufficient level of metabolic activity of these cells or enzymes. Two different bioremediation technologies have been developed to remove or get rid of soil contaminants. They include *biostimulation* in which degrading activity of naturally occurring organisms is stimulated, and *bioaugmentation*, which consists of supplying the soil with selected strains of organisms. In situ technologies aim at stimulating degradation directly in the contaminated site. They are well suited for large-scale and non-point-source pollution, and stimulation of the naturally occurring microflora has been preferred to addition of potentially active microorganisms. Ex situ treatments are needed when conditions at a site are not conducive to microbial activity. In such cases, the soil is excavated and treated on specialized disposal areas or in bioreactors. Bioslurries are one type of treatment process, in which appropriate conditions for microbial growth are maintained in a bioreactor. Another process, composting, is conducted in piles where soil is humidified, mixed with degradable organic material such as straw and other inorganic nutrients, actively aerated, and, if necessary, enriched with selected microorganisms. Another large-scale method is land farming in which soil is spread out and tilled to promote aeration, homogenization of nutrients and inocula. Because of cost constraints, ex situ approaches are usually used for small volumes of soil with high levels of contamination.

Case Study: Bioremediation of Atrazine in Soil

Since its discovery in the 1950s, atrazine has been extensively applied as herbicide, especially in maize crops. It has long been recognized as a moderately persistent chemical with a half-life varying between a few weeks to a few months, and has become a major contaminant of soils and waters. For this reason, different approaches of soil bioremediation have been developed to treat atrazine.

The stimulation of atrazine biodegradation by the autochthonous microflora has been achieved in acidic soils that do not show accelerated degradation of atrazine. Liming of the soils has been proved sufficient to treat

two soils while two other soils responded positively only after inoculation with a selected atrazine-degrading strain (A. Yassir, personal communication). This result indicates that, in some soils, environmental conditions rather than the biological potential, limit biodegradation. This result was substantiated by another study indicating that dairy manure incorporation could stimulate rapid mineralization of atrazine in a maize-cropped soil (140). Enhancement of the degrading activity of the native microflora after addition of organic amendments may fail, however, especially when stabilization of pesticide residues by sorption to soil particles limits their bioavailability and rate of biodegradation, as has been demonstrated with soil-compost mixtures (141). Organic carbon can be added by growing plants in a contaminated area. Plants may secrete 10 to 20% of their photosynthates as root exudates, which can support the growth and metabolic activities of fungal and bacterial communities in the rhizosphere. Densities of rhizospheric bacteria can be as much as two to four orders of magnitude greater in rhizosphere than in bulk soils. It is not surprising, therefore, to find more microorganisms able to rapidly degrade in vegetated than in nonvegetated soils. Herbicide tolerant plants, such as *Kochia scoparia* (L) Roth, may enhance microbial degradation of atrazine in the presence of the other herbicide metolachlor and trifluraline, and at concentrations largely exceeding the field application rates (142). However, the effect of a crop on herbicide dissipation is not clear and may depend on other soil properties and environmental variables. In a field experiment, it was demonstrated that atrazine dissipation was more rapid in no-crop plots than in maize crop, and that reduction in the soil water content in the maize plot was speculated to be responsible for greater atrazine persistence (143).

Bioaugmentation has also been evaluated as a means to accelerate biodegradation of atrazine in soils. In laboratory experiments, it was demonstrated that a *Pseudomonas* strain YAYA6 isolated from a mixed microbial community could efficiently degrade atrazine, probably via *N*-dealkylation and dechlorination, when inoculated in soil. However, degradation rates were reduced in soils with a high organic matter content and with a low pH. Only 10% of initial activity was retained after a preincubation period of 42 days without atrazine (144). In another comparable study conducted with an *Agrobacterium radiobacter* strain J14a using atrazine as a sole nitrogen source, two soils were found to respond differently to J14a inoculation. This difference was probably related to differences in the indigenous atrazine-degrading communities and to differential competitiveness of the inoculated strain. The inoculant was unable to increase total atrazine mineralization in the most effective soil (145). *Pseudomonas* sp. strain ADP, capable of mineralizing atrazine with dechlorination as the first degradative step, is successful for bioaugmentation of subsurface soils where the indigenous microflora degrades atrazine, mainly via dealkylation. It was concluded from this study, that even if the potential for atrazine degradation exists in all soil strata, the most important factor is the presence of an atrazine-mineralizing community, and that bioaugmentation may be preferable to biostimulation for avoiding accumulation of undesirable metabolites (146).

White rot fungi are also good candidates for bioremediation. These organisms have a large array of exoenzymes with a high oxidative activity and a broad substrate specificity (peroxidases, laccases), which are involved in lignolytic activity. They tolerate a number of toxic compounds and can grow at the expense of low-cost organic substrates such as plant residues or industrial wastes, which can be used as carriers for soil inoculants. Among these fungi, *Phanerochaete chrysosporium* has been extensively studied for its capacity to transform atrazine into *N*-dealkylated metabolites in liquid culture. In one study, however, the observation of some fungal growth after inoculation of spores in a nonsterile soil, was not correlated with increased atrazine mineralization, not affected by the presence of the fungus (147).

Microbial consortia capable of complete mineralization of atrazine through ring fission in liquid culture and soil have been reported. For instance, in an uninoculated soil, 7% and 25% of applied atrazine was mineralized after 30 and 145 days, respectively, compared to 63% and 87% over the same periods of time in the same soil inoculated with a mixed microbial culture. Soil bound residues accounted for only 8% of applied atrazine in the inoculated soil and for 40% in the noninoculated control soil (148). It was also demonstrated that long-term survival of an inoculated microbial consortium was better in a rhizosphere soil (149).

Bioremediation: Unresolved Questions

There are several major issues concerning bioremediation that remain to be resolved. These issues include how to select an appropriate bioremediation technology, how to extrapole laboratory results to real situations, what are the long-term ecological consequences of introducing massive amounts of selected microorganisms into the soil, and what are future prospects of the use of genetically modified organisms.

Research on atrazine has illustrated the relative strengths and weaknesses of the different technologies now available to enhance pesticide degradation in soil. From an operational point of view, it would appear that bioaugmentation with mineralizing microbial strains or consortia, if available, is to be preferred to avoid accumulation of intermediates compounds, which may adversely affect the inoculum and other naturally occurring organisms. Nevertheless, there are limitations to the use of acclimated microbial cultures to degrade toxic chemicals. Strains selected in the laboratory in appropriate culture media may be subjected to stresses when they are reintroduced into the soil. Chemical shock, extremes of soil physicochemical and climatic conditions, starvation, competition, preferential use of other organic compounds, and predation, may destroy or harm the inoculum and suppress its degradative capability. Numerous studies have shown that not only do declines in the degrading activity of inocula result from a decrease in the number of microbial cells, but also from a decrease in the specific activity of surviving cells as a result of the loss of the corresponding encoding genetic

material. The maintenance of a successful detoxification potential also needs suitable environmental conditions, which may be achieved by using appropriate additives, substrates, or carriers, which may be added to the cell suspension for stimulation and protection. The demonstration of how survival of an inoculated mixed culture mineralizing atrazine is enhanced in a maize-planted soil (149) is a promising example. The question of whether microbial consortia are ecologically superior to pure cultures is critical especially when, as often happens, a mixture of pollutants is present in the contaminated environment. Most bioremediation research has been restricted to laboratory and microcosm studies under conditions that do not challenge a microbial community to compete and survive, as it would have to do under the fluctuating and heterogeneous soil conditions in the field. A more realistic approach of the efficacy and safety of a bioremediation application must be gained from studies at an appropriate scale and for a long enough period.

Enhancing evolution of new degradative pathways in microorganisms or even in plants may help in solving problems of chemical pollution. Not all pesticides have been found to be easily biodegradable and for many, extensive mineralization can be accomplished when the biochemical activities, present in mixed microbial communities, complement one other. The construction of a hybrid single organism can be achieved by taking advantage of natural or induced gene transfer to collect all the genes for the emergence of new metabolic pathways. Using chemostat selection, it has been possible to isolate from waste-dump sites and after several weeks continuous subculturing a genetically engineered variant, *B. cepacia* (formerly *P. cepacia*) AC1100, that rapidly uses 2,4,5-T as its sole source of carbon and energy (150). More recently, different recombinant strains have been constructed to degrade atrazine. Cloning on the same plasmid, the *atrA* and *trzA* genes, responsible for *N*-dealkylation and hydrolytic dechlorination respectively, and transferring the plasmid into an *atrA* and *trzA*-deficient *Rhodococcus* sp. strain resulted in full expression of the genes. The same procedure was unsuccessful when the plasmid was transferred into an *E. coli* strain (151). Several problems must be worked out with genetically engineered strains. Little is known on their competitiveness and stability in natural conditions, on the possibility for the constructed pathway to be disseminated in the indigenous microflora and possible consequences on the composition and structure of the indigenous microbial community. The last but not least difficulty to overcome, is the public acceptance of these emerging technologies based on the use of genetically manipulated organisms.

In conclusion, bioremediation appears to have a promising future. It has the potential to be an efficient and cost-effective strategy for reclamation of soil and aquatic environments. Further technical advances can be expected from a better understanding of soil microbial ecology, namely, how organisms interact with each other to perform new functions, generate new metabolic capabilities, and respond to environmental fluctuations.

Application 2: Modeling of Pesticide Degradation in Soil

Spreading of a pesticide in the environment depends on interactions among physical, chemical, and biological phenomena. Modeling is probably one of the most powerful approaches to obtain a comprehensive understanding of all these processes, which collectively contribute to the environmental fate of a pesticide. For these reasons, regulatory experts are asking for meaningful models they can rely on. Model calculations simulating pesticide behavior in the environment are increasingly used for research purposes and, in the last decade, for regulatory purposes. In the first case, models are used to validate mechanistic assumptions about the nature and rates of the different processes that govern the fate of pesticides in the environment. In using the second type of models, the objective is to obtain comparative estimates regarding accumulation, exposure, and leaching of pesticides to assess the ecotoxicological risk and the risk of their transport into the groundwater. These predictive and comparative aspects are especially important for evaluating new agrochemicals for which no monitoring data exist, which could provide more realistic exposure information.

A major challenge is to reduce uncertainty in the model outputs. More accurate and reliable predictions may be obtained in using better estimates of the parameters controlling the sorption and the transformation processes, to which soil persistence and leaching models are very sensitive (152–154). Since Hill and coworkers' early work (155), simple first-order rate reaction kinetics are commonly used to describe kinetics of pesticide degradation in soil, based on the assumption that only pesticide concentration limits degradation and microbial populations are present in excess (156). More recent research has amply demonstrated that degrading activity is often restricted to a small fraction of soil microorganisms, and the size of the degrading community may also limit the rate of degradation. As discussed earlier, some strains have the potential to grow at the expense of the pesticide, while others show increased degrading activity in response to organic amendments. All these changes of size and/or activity will dictate the rate at which a compound will be transformed in the environment. Considering these issues, one might expect that introducing more conceptual descriptions of microbial reactivity to pesticide and nutrients additions to soils may reduce the uncertainty of predictions of persistence and leaching models.

Metabolism and cometabolism have different implications for biodegradation kinetics of xenobiotic compounds because they correspond to different underlying biological processes. Metabolic degradation result in the formation of both transformation products and new active biomass. These reactions can be compared to autocatalytic chemical reactions, with a progressive increase of the rate in the early stage of the transformation. Moreover, a lag-phase of a few days or weeks often occurs before degradation starts. This phenomenon is often attributed to the limited number of active microorganisms that are commonly found in agricultural soils. It mainly reflects the time necessary

for those degrading microorganisms to reach a population level that is consistent with a rate of transformation that can be detected by common analytical procedures. In contrast, cometabolic transformations are performed by nongrowing microbial agents and follow simple enzymatic kinetics. The rate of the transformation starts at its maximum and decreases with the chemical concentration.

Basic Mathematical Formulations

It is expected that the rate of transformation directly reflects both the concentration of the chemical and the density of the active microflora. Paris and coworkers (157) proposed a second-order rate model based on the following equation:

$$-\frac{dC}{dt} = k_2\, CB$$

where k_2 is the second-order rate constant, C the concentration of the chemical and B the density of the active microbial population. In practice, pseudo first-order kinetics may be derived when soil microorganisms are in abundance and do not limit the degradation process and/or when they cannot grow at the expense of the chemical. In these circumstances, the transformation reaction is zero-order with respect to the concentration of active microorganisms (156). The pseudo first-order rate constant, k_1, is:

$$k_1 = k_2\, B$$

and the rate law:

$$v = -\frac{dC}{dt} = k_1\, C$$

The theoretical half-life, $t_{(1/2)}$, is the time necessary for obtaining a 50% decrease of any chemical concentration. It is related to the first-order rate constant by the equation:

$$t_{1/2} = \frac{\ln\, 2}{k_1}$$

and is approximated by the experimental parameter DT50, the 50% disappearance time of the initially added concentration. As stated by Hamaker (158), "for such a complex system as soil, it is somewhat unreasonable to expect that there will be a simple rate law" to fit observed disappearance curves that often deviate from first-order. Considerable modeling effort has been directed at obtaining more conceptual representations based on the consideration that soil degradation of pesticides depends on the contribution of different types of microorganisms and the effects of environmental parameters (temperature, water content, pH. . .) or soil properties (sorption).

In many cases, changes in population density resulting from using either the pesticide as carbon and energy source or any other easily mineralizable carbon substrates must be taken into consideration. For growth-linked or metabolic pesticide biodegradation, two additional equations must be written: one for the rate of growth of the degrading microflora:

$$\frac{dB}{dt} = \mu_{\mathrm{m}} \frac{SB}{(K_{\mathrm{s}} + S)}$$

with μ_{m} and K_{s} being respectively the maximum specific growth rate and the saturation constant, and the other for the mass balance:

$$B = B_0 + Y(S_0 - S)$$

where Y is the gross growth yield, B_{o} and S_{o} the initial biomass density and substrate concentration respectively (159). For cometabolic transformations, it is assumed that degrading microorganisms can grow at the expense of naturally occurring or added carbon substrates. Exponential growth is maintained until the microbial biomass approaches some limit value, B_{m}, known as the carrying capacity. With r being the maximum growth rate, the complete equation for these microbial dynamics is (160):

$$\frac{dB}{dt} = \frac{rB}{(1 - B/B_{\mathrm{m}})}$$

Several experiments have confirmed that temperature and water content are major soil parameters conditioning microbial activity and therefore pesticide degradation. Temperature effects have been described using two types of equations. A first series of equations is derived from the Arrhenius equation (161–163) and is based on the activation energy concept. Other equations make use of the Q_{10} coefficient, which is defined as the relative increase in the rate of transformation every 10 °C change in temperature. All these equations have in common the disadvantage of ignoring that for biological processes the activity-temperature relationship is a bell-shaped curve showing an optimum.

Likewise, the influence of soil moisture on transformation rates has almost exclusively been described using an empirical exponential equation initially proposed by Walker (161). Several examples of application (161,164) can be found in the literature.

The retention of pesticides on the soil matrix is a major process governing their fate in soils. It has been shown that sorption of pesticides on soil colloids controls their release into the soil solution, and it is commonly accepted that only the fraction in the soil solution is available for biodegradation (165). A diffusion-sorption-biodegradation (DSB) model has been developed to describe biodegradation under conditions of substrate limitation (166). The model correctly simulated the experimental data for biodegradation of glutamate and phenol diffusing out of synthetic aggregates of different sizes (167). Likewise, 2,4-D disappearance from soil has been adequately described by a model, which accounted for sorption to soil surfaces, diffusion into the internal matrix of soil organic matter or aggregates and microbial growth (168).

Practical Applications

Because leaching of pesticides to groundwater is of worldwide concern, there is a growing body of literature on the development of simulation models for quantitatively predicting the fate of chemicals in the root zone of agricultural crops and in the underlying unsaturated zone. Simulation models have been constructed for various

uses: research, management, regulatory, and screening purposes.

In the early 1970s, Walker (161) developed a mathematical model to simulate herbicide persistence in the top-soil layer under variable temperature and water content conditions that prevail in the field environment. The model combines first-order reaction rate law with equations describing the dependence of the rate constant, through the DT50 parameter, on environmental variables temperature and water content. Implementation of the model requires performing preliminary laboratory experiments to validate equations for soil temperature and moisture effects using inverse modeling. The predicted residual concentration of the herbicide in the field is then calculated with the model for actual values of moisture and temperature deduced from weather records.

Persistence and leaching management models are in their infancy and, except for particular situations, are unable to adequately simulate experimental data. Because biodegradation is the primary factor controlling persistence, this process deserves detailed attention. Descriptions in terms of overall first-order kinetics have been shown to have their limits. Consideration of the major microbiological phenomena underlying degradation may contribute to making management models more operational and of better value for the quantitative prediction of the contamination of ground waters.

CONCLUSION

As xenobiotic compounds, pesticides have revealed some aspects of microbial functioning in complex ecosystems, which were unknown from preliminary studies with pure cultures. We have learned that, when confronted by new chemical structures, which have not been previously registered in their genomic "memory," soil microorganisms respond collectively, taking advantage of metabolic capabilities, which are originally distributed among different strains and normally used for the biodegradation of naturally occurring compounds. Such a "cooperative metabolism" may be the starting point of a new evolving biochemical activity, which is further subjected to successive functional improvements through the selection of trial and error combinations of isolated genes or entire fully operative genetic structures. Horizontal transfer may contribute to the stepwise building of integrated mosaic gene assemblages in one microbial cell. We have to learn more about the determinism and processes governing exchange and stabilization of these emerging genetic structures and the timescale of this natural genetic engineering. This knowledge would be very fruitful from two points of view. Linking biodegradation rate to the biochemical pattern of natural microbial communities would help in elaborating the basis of predictive metabolism, which would be useful to predict and manage the persistence of a new chemical in the soil, to define improved agricultural practices for a better weed control, and to design new compounds compatible with sustainable agriculture. Basic knowledge of microbial ecology of pesticide biodegradation in natural complex ecosystems is also a prerequisite for elaborating more successful bioremediation technologies when bioaugmentation by inoculation with naturally occurring or genetically engineered microorganisms known to readily metabolize persistent chemicals has failed. Overcoming environmental constraints that limit survival or activity of introduced organisms or genes is one of the challenges for increasing effectiveness of bioremediation technologies.

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PESTICIDES, BIOREMEDIATION OF.

See BIOREMEDIATION: AN OVERVIEW OF HOW MICROBIOLOGICAL PROCESSES CAN BE APPLIED TO THE CLEANUP OF ORGANIC AND INORGANIC ENVIRONMENTAL POLLUTANTS

PEST MANGEMENT.

See BIOCONTROL, MICROBIAL AGENTS IN SOIL

PETROLEUM AND OTHER HYDROCARBONS, BIODEGRADATION OF

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Petroleum, literally rock oil, has been a part of the biosphere for millennia, but in the last hundred years it has become the fossil fuel that drives the modern world. It is used on an enormous scale; the global use of petroleum in the year 2000 was on the order of 1.2×10^{12} U.S. gallons crude oil per year [4.5×10^{12} liters/year; (1)], and this seems likely to increase. Crude oils are very complex mixtures of molecules, principally hydrocarbons, and although the major classes are well defined, we have no inventory of all the individual species in oil. Nevertheless, we do know that the vast majority of molecules in most crude oils are biodegradable. Hydrocarbon biodegradation can cause problem while the oil is under human control because it can lead to product deterioration, corrosion, and the potential accumulation of microbial biomass in critical locations. In the last decade or so, however, it has become clear that stimulating biodegradation (in a process known as *bioremediation*) offers an opportunity to reduce the environmental impact when crude oils and refined products escape into the environment. For many years, all biodegradation was thought to be aerobic, and indeed the very existence of petroleum reservoirs was taken as evidence that anaerobic biodegradation of hydrocarbons was a very limited phenomenon, if it occurred at all. More recently, it has become clear that hydrocarbon degradation can occur under a range of anaerobic conditions, including sulfate-reducing, nitrate-reducing, metal-reducing, and methanogenic conditions.

THE COMPOSITION OF CRUDE OIL

Crude oil is a fossil fuel, and the average age of commercially important crude oils is about 100 million years (71% between 180 and 85 million years ago (2)). It is generally accepted that aquatic algae, albeit usually with some terrestrial material, gave rise to petroleum, whereas terrestrial plants gave rise to the great coal reserves of the world. The oldest commercially valuable oils are from biomass generated in the Ordovician (486 million years b.p.), whereas others are as young as the late Tertiary (a few million years ago). Unusual circumstances such as those that prevail at the Guaymas hydrothermal vent site can even result in the formation of petroleum from biomass that is only approximately a thousand years old (3).

Petroleum has been a part of the biosphere for aeons because it has escaped through seeps on land and sea. Terrestrial seeps were the first locations to be drilled when oil production began in earnest in the nineteenth century. For example, the 1860 Drake well in Pennsylvania, the first U.S. well, was drilled on a seep site. Most of the known terrestrial seep sites had been drilled by the 1920s, but marine seeps continue to be explored today. It has been estimated that the annual input of crude oil into the sea from seeps is about 70 million gallons (about 300 million liters), accounting for approximately 8% of the total input (4). Some have even argued that the biodegradation of this oil contributes significantly to the local food web and to commercial fisheries (5).

Crude oils are principally hydrocarbons, with hydrogen to carbon ratio of between 1.5 and 2.0; the organic molecules are thus generally saturated molecules (i.e., the predominant form of carbon is $-CH_2-$). The convention in the oil industry is to call linear alkanes "paraffins," branched paraffins "isoparaffins," and cyclic alkanes "naphthenes" (Fig. 1). There are also significant amounts of aromatic carbon in all crude oils (Fig. 2) and polar molecules (Fig. 3) containing the heteroatoms oxygen, nitrogen, and sulfur. These latter compounds are known by a variety of names, including resins, NSOs, polars, and asphaltenes. Tissot and Welte (2) quote the average composition of 527 crude oil samples as 58.2% saturates, 28.6% aromatics, and 14.2% polar compounds, although the absolute values vary widely in different oils. On an average, there is rough parity among paraffins, naphthenes, and aromatics.

The paraffins (Fig. 1) span the range from a single carbon (methane) up to waxes with at least forty carbons and sometimes more. The largest alkane reported in petroleum is $C_{79}H_{160}$. Linear alkanes typically make up 15 to 20% of a crude oil, although their content can be essentially undetectable or as high as 35%, depending on source and reservoir conditions. There are

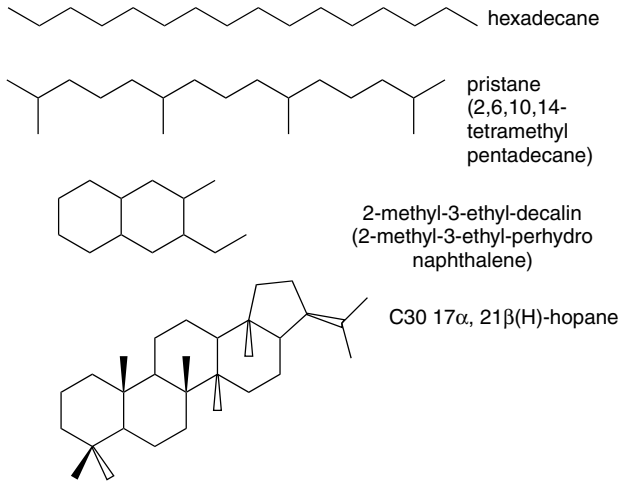

Figure 1. Saturated hydrocarbons. An alkane, an isoalkane, a small naphthene, and a naphthenic biomarker found in crude oils. As in all the figures in this article, hydrogen atoms are not shown unless on a heteroatom.

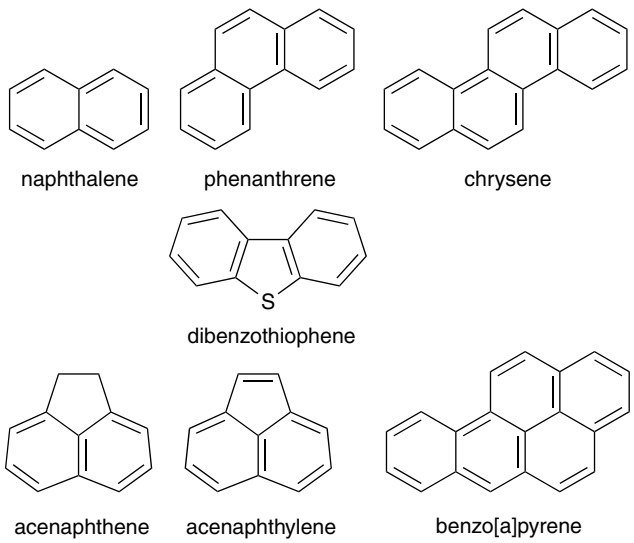

Figure 2. Aromatic hydrocarbons. A two-, three-, and four-ring polycyclic aromatic hydrocarbon found in crude oil, together with dibenzothiophene, an example of a sulfur-containing aromatic compound. The lower three compounds are typical pyrogenic polycyclic aromatic hydrocarbons that are only present at trace levels in most crude oils but are abundant in creosotes and coal tars.

also branched alkanes; most are found in the C_6 to C_8 range, but pristane ($C_{19}H_{40}$, Fig. 1) and phytane ($C_{20}H_{42}$), molecular relics of the phytol chains of chlorophylls, and perhaps other biomolecules are usually the most abundant individual branched alkanes. Pristane is thought to be the result of initial partial degradation of phytol in the presence of oxygen, whereas phytane is thought to be the result of initial degradation in the absence of oxygen. Alkenes, which are unsaturated alkanes, are present in only trace levels in crude oils, but they are produced during catalytic refinery processes

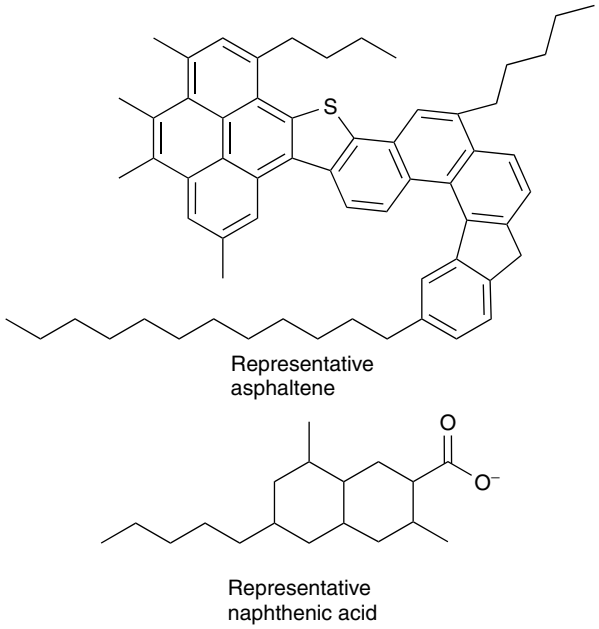

Figure 3. Polar oil components. Representation of what are thought to be a typical asphaltene and a typical naphthenic acid found in crude oils.

and can be abundant in refined products such as gasoline.

The naphthenes (Fig. 1) include parent compounds, such as cyclopentane, cyclohexane and decalin, together with their alkylated congeners. Tissot and Welte (2) quote the average composition of the naphthene fraction of 299 crude oils as 54.9% one- and two-ring naphthenes, 20.4% tricyclic naphthenes, and 24.0% tetra and pentacyclic naphthenes. These latter molecules are among the better-understood molecular biomarkers in crude oils, and they are used extensively in correlating reservoirs and source rocks (6), in assigning the depositional environment of source rocks (6), and more recently as conserved internal markers during biodegradation (7).

Because of the separation procedures used in the characterization of crude oils, any molecule containing at least one aromatic ring is included in the "aromatic" fraction, regardless of the presence of saturated rings and alkyl substituents. Sulfur aromatic heterocycles, such as thiophenes, benzothiophenes, and dibenzothiophenes (Fig. 2), are included in the aromatic category. Indoles and carbazoles, usually the most abundant nitrogen-containing species, and the less abundant basic nitrogen species such as quinolines are also included in the aromatic category but they are present at much lower concentrations. Alkylated aromatic species are usually more abundant than their parent compounds, with mono-, di-, and tri-methyl derivatives usually being most abundant. Nevertheless, the median aromatic structure probably has one or two methyl substituents together with a long-chain alkyl substituent (8).

The polar molecules are the most difficult to characterize because they are often unamenable to gas chromatography, which is the usual method of choice for the molecular characterization of petroleum. All are thought to contain

heteroatoms such as nitrogen, oxygen, and/or sulfur, and the category includes the porphyrins, usually nickel or vanadium species, naphthenic acids (Fig. 3), and large molecules known as asphaltenes. Some of these molecules have molecular weights that are into the thousands and even higher, and many are suspended in the oil rather than dissolved in it. Others are dissolved in the whole oil but are precipitated if more alkanes (e.g., pentane) are added. The polar fraction of the oil contains the majority of the color centers in crude oil, and in isolation these materials are difficult to distinguish from more recent biological residues such as the humic and fulvic acids that are common in soils.

Crude oils are classified commercially by several criteria, and among the most important is the specific gravity. The oil industry uses a unit known as American Petroleum Institute (API) gravity, which is defined as [142.5/(specific gravity)] — 131.5, and is expressed as degrees (°). Thus, water has an API gravity of 10°, and denser fluids have lower API gravities. Less dense fluids, for example, most hydrocarbons, have API gravities greater than 10°. For convenience, oils with API gravities greater than 40° are said to be light oils, whereas those with API gravities of less than about 16° are said to be heavy. Light oils have higher proportions of small molecules; heavy oils are rich in larger molecules. Viscosity is inversely proportional to API gravity, but it is also dependent on the physical state of the polar compounds and longer alkanes in the oil and is highly dependent on the temperature.

OTHER HYDROCARBONS

Petroleum is not the only source of hydrocarbons in the environment; it is not even the main one. Plants, especially conifers, emit large amounts of hydrocarbons (9), including isoprenes, that can lead to the generation of atmospheric haze (giving rise to names such as the Blue Mountains). Citrus fruits contain substantial amounts of terpenes such as limonene in their peels. Many beetles produce hydrocarbons (10). Some bacteria produce toluene (11) and termites produce naphthalene (12). Considerable amounts of polycyclic aromatic hydrocarbons and dioxins are generated during the partial combustion of biomass and solid waste (13). Similarly, coal and wood gasification, especially in the production of "town" or "manufactured gas," released substantial amounts of tar and polycyclic aromatic hydrocarbons into the environment (14). Creosote, which is made by high-temperature carbonization of wood or coal and widely used as a wood preservative, led to significant localized soil contamination with polycyclic aromatic hydrocarbons (15). These sources can be distinguished from petroleum by their composition. For example, the relative distribution of pyrogenic polycyclic aromatic hydrocarbons is different from its distribution in petroleum; anthracene is abundant in pyrogenic materials such as tars and creosotes but rare in petroleum, and four- and five-ring aromatics are abundant in coal tar but not in petroleum. Furthermore, the distribution of alkylated forms is different; unalkylated species are most abundant in creosotes and coal tars, while alkylated forms are most abundant in petroleum (16). Such differences are very important in determining the source of contamination at contaminated sites, and they are important at the basic microbiological level because the four- and five-ring aromatics are much more resistant to bacterial degradation than smaller aromatic compounds. Even more important in this regard is the potential presence of other contaminants; for example, creosote-contaminated sites are often cocontaminated with pentachlorophenol at potentially toxic levels. The focus of this entry is on the biodegradation of hydrocarbons from any source.

BIODEGRADATION OF HYDROCARBONS IN AEROBIC ENVIRONMENTS

Hydrocarbons have been a natural part of the biosphere for millennia, so it is no surprise that microbes take advantage of this food supply. Oil-degrading organisms have been found in almost all habitats in which they have been diligently searched for. They have been isolated from the bottom of the sea, including the Guaymas deep-sea site, the tops of mountains, soils, and shores everywhere, including the Arctic and Antarctic, and from the deserts. Extreme halophiles have been isolated, as have thermotolerant organisms. No extreme thermophiles have been isolated to date, but there is every reason to expect that they will be found. Table 1 lists the bacterial genera that contain species known to degrade hydrocarbons. Many can grow with hydrocarbons as the sole source of carbon and energy, although some such as the photosynthetic autotrophs only degrade aromatic hydrocarbons as an adjunct to their photosynthetic growth and cannot grow on

Table 1. Bacterial Genera That Contain Aerobic Oil-Degrading Species

Achromobacter	*Acidovorax*	*Acinetobacter*
Actinomyces	*Aeromonas*	*Agmenellum**
Agrobacterium	*Alcaligenes*	*Alteromonas*
*Anabaena**	*Aphanocapsa**	*Arthrobacter*
Aureobacterium	*Azospirillum*	*Azotobacter*
Bacillus	*Beijerinckia*	*Beneckea*
Brevibacterium	*Brevundimonas*	*Clavibacter*
Clostridium	*Comamonas*	*Corynebacterium*
Curtobacterium	*Cycloclasticus*	*Cytophaga*
Enterobacter	*Erwinia*	*Escherichia*
Flavobacterium	*Gordona*	*Klebsiella*
Lactobacillus	*Leucothrix*	*Marinobacter*
Micrococcus	*Microcoleus**	*Moraxella*
Mycobacterium	*Nocardia*	*Nostoc**
*Oscillatoria**	*Pasteurella*	*Peptococcus*
*Phormidium**	*Proteus*	*Pseudomonas*
Rhodococcus	*Sarcina*	*Serratia*
Spherotilus	*Sphingomonas*	*Spirillum*
Stenotrophomonas	*Streptomyces*	*Thermoleophilum*
Vibrio	*Xanthobacter*	*Xanthomonas*

Table 2. Archaeal Genera That Contain Aerobic Oil-Degrading Species

Halobacterium	*Haloferax*

Table 3. Fungal Genera That Contain Hydrocarbon-Degrading Species

Absidia	*Acremonium*	*Agrocybe*
Allescheriella	*Armillaria*	*Aspergillus*
Aureobasidium	*Basidiobolus*	*Beauveria*
Bjerkandera	*Botrytis*	*Candida*
Cephalosporiopsis	*Ceriporiopsis*	*Choanephora*
Circinella	*Cladosporium*	*Claviceps*
Cokeromyces	*Conidiobolus*	*Coriolopsis*
Corollospora	*Crinipellis*	*Cryphonectria*
Cryptococcus	*Cryptococcus*	*Cunninghamella*
Dendryphiella	*Drechslera*	*Emericellopsis*
Epicoccum	*Eupenicilium*	*Flammulina*
Fusarium	*Gilbertella*	*Gliocladium*
Gonytrichum	*Graphium*	*Gymnopilus*
Hansenula	*Helicostylum*	*Helminthosporium*
Hericium	*Humicola*	*Kuehneromyces*
Laetiporus	*Linderina*	*Lulworthia*
Marasmiellus	*Mortierella*	*Mortierella*
Mucor	*Nematoloma*	*Neurospora*
Oidiodendron	*Oxyspora*	*Paecilomyces*
Panaeolus	*Penicillium*	*Peronospora*
Pestalotia	*Pestalotia*	*Phanerochaete*
Phlyctochytrium	*Phycomyces*	*Phytophthora*
Pichia	*Pleurotus*	*Psilocybe*
Ramaria	*Rhizopus*	*Rhodosporidium*
Rhodotorula	*Saccharomyces*	*Saccharomycopsis*
Saprolegnia	*Scedosporium*	*Scopulariopsis*
Smittium	*Sordaria*	*Sporobolomyces*
Sporormiella	*Syncephalastrum*	*Talaromyces*
Tetracoccosporium	*Thamnidium*	*Torulopsiella*
Trametes	*Trichoderma*	*Trichosporon*
Varicosporina	*Verticillium*	*Zoophthora*
Zygorhyncus		

Table 4. Algal Genera That Contain Species That Are Able to Degrade Polycyclic Aromatic Hydrocarbons

Amphora	*Chlamydomonas*	*Chlorella*
Cylindrotheca	*Dunaliella*	*Petalonia*
Porphyridium	*Prototheca*	*Ulva*

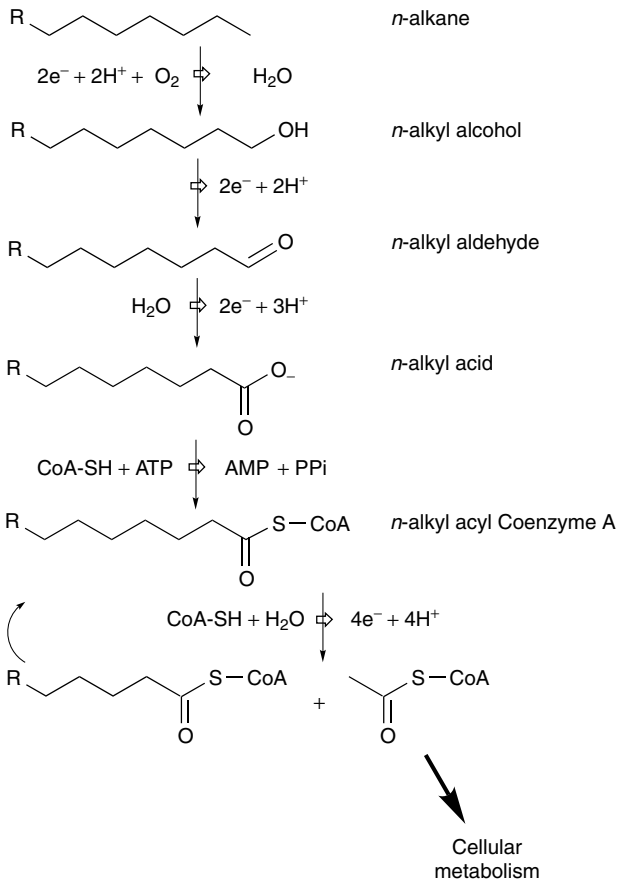

Figure 4. The pathway of alkane degradation exhibited by *Pseudomonas oleovorans* based on van Beilen and coworkers (17). The pathway, as all those in this article, is shown in an abbreviated form to give an overview of the energetic inputs and outputs of the pathway.

them alone. Table 2 shows the much shorter list of aerobic archaeal genera known to degrade hydrocarbons; Table 3 lists fungal genera; and Table 4 lists algal genera with species able to degrade polycyclic aromatic hydrocarbons, although again all are obligate photoautotrophs. There is no reason to believe that these lists are exhaustive, but they illustrate the diversity of organisms that are able to degrade hydrocarbons. This diversity of organisms is mirrored in the diversity of mechanisms for activating and degrading hydrocarbons shown as follows.

BIOCHEMICAL PATHWAYS OF AEROBIC HYDROCARBON DEGRADATION

Aerobic pathways of hydrocarbon degradation have been studied in considerable detail, and pathways are beginning to be clearly defined, at least for relatively simple hydrocarbons. It is the insertion of the initial oxygen atoms into hydrocarbons that initiates hydrocarbon degradation, and the discussion that follows will focus on these reactions. Subsequent oxidation is often through pathways with a much broader specificity, and often these pathways are essentially ubiquitous.

Linear Alkanes (Paraffins)

The biodegradation of alkanes has been elucidated in some detail, in particular in the organism *Pseudomonas oleovorans* (17). This organism oxidizes alkanes with 5 to 12 carbons, but similar systems seem to be involved in organisms that grow on alkanes with up to at least 20 carbon atoms. The initial reaction is the terminal oxidation of the alkane to the alcohol, the aldehyde, and the fatty acid, with subsequent "ß-oxidation" yielding acetyl fragments, attached to Coenzyme A, for cellular metabolism (Fig. 4). The initial hydroxylase is a monooxygenase, meaning that one atom of oxygen from a diatomic oxygen molecule is incorporated into the alkane, whereas the other is reduced to water. The electrons for this latter reduction come from NADH; thus, each hydroxylation represents a considerable energetic input by the organism. The subsequent oxidation to the aldehyde and the acid releases reducing equivalents, probably both to NAD^+; therefore, the NADH investment in the first step is amply repaid.

Subsequent ß-oxidation releases substantial amounts of energy for the organism.

In *P. oleovorans*, the genes for the alkane oxidation pathway are arranged as two clusters on a plasmid known as the OCT-plasmid (17). Interestingly, the nucleotide composition of the alkane oxidation genes is quite different from that of the chromosome, suggesting that the alkane oxidation genes may have come from quite a different organism, which in turn suggests that the genes may be quite widespread. At least nine genes are involved, and their function and sequences have been determined. Perhaps most interesting is the alkane monooxygenase catalyzing the initial oxidation. This enzyme is a 401 amino acid membrane-bound polypeptide, with six putative membrane-spanning regions. It contains ferrous iron in a nonheme environment, and contains no cysteines. The enzyme has quite a broad specificity; it is most active against *n*-alkanes with 5 to 12 carbon atoms, but it is also active against alkanes with 1 or 2 methyl substituents and a range of cyclic alkanes and substituted benzenes (17). This is reminiscent of the broad substrate range of soluble methane monooxygenase (18), albeit shifted to slightly larger compounds. Considerable progress is being made in understanding the catalytic cycle of this enzyme, which has a dinuclear iron center at its active site (19).

Some organisms seem to activate the oxidation of *n*-alkanes at both ends of the molecule. This is most often seen with larger alkanes, suggesting independent activation at the two ends of the molecule. As is discussed later, such diterminal attacks are apparently common for isoalkanes. Other organisms seem able to initiate the oxidation at a carbon other than the terminal one. This activity may be related to the systems that degrade cyclic alkanes and is discussed later.

A rather different pathway has been suggested by Finnerty (20). It involves the initial oxidation by a dioxygenase that puts both oxygen atoms into the alkane to form an alkyl hydroperoxide and the subsequent manipulation of this through the peroxy acid to the aldehyde (Fig. 5). The pathway has been most extensively studied in *Acinetobacter*, and the dioxygenase, which acts on *n*-alkanes from C_{12} to C_{20}, has been characterized as a flavoprotein that may contain a copper at the active site (21). The enzyme does not require a source of reductant beyond the substrate, as monooxygenases do, so the initial energetic investment in this pathway is much less than that described in Figure 4.

Eukaryotic oil-degrading organisms, for example, yeasts such as *Candida*, catalyze the hydroxylation of alkanes to primary alcohols with enzyme systems that involve Cytochrome P-450, a heme protein named for its prominent optical absorption feature generated by binding CO to the reduced form (22). P-450 systems are also found in some bacteria. Subterminal oxidation of *n*-alkanes to secondary alcohols has also been reported.

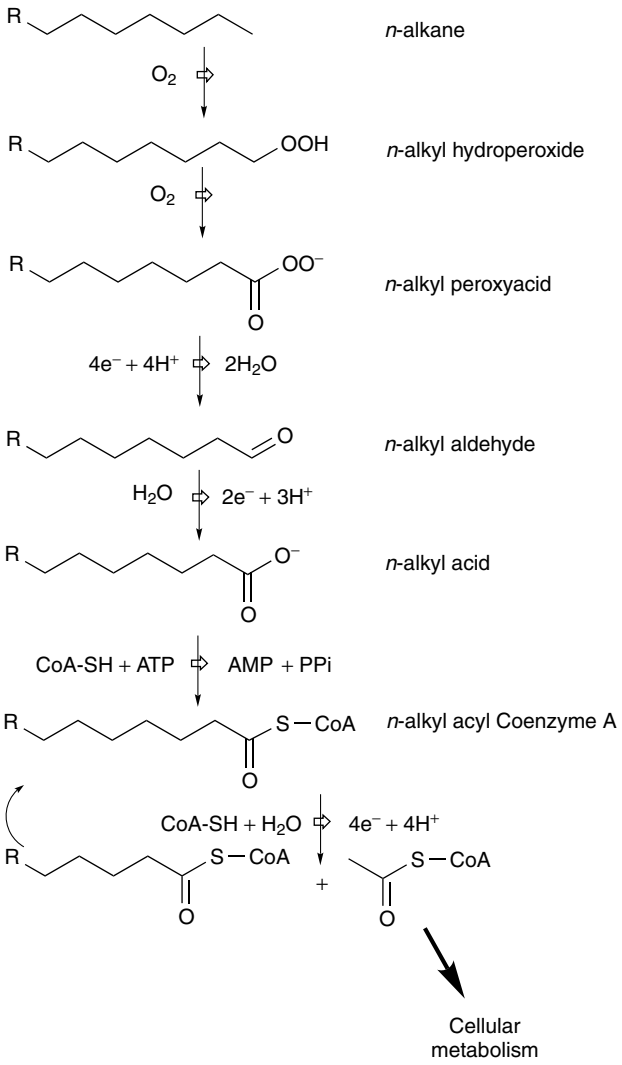

Figure 5. The pathway of alkane oxidation thought to occur in *Acinetobacter*, based on Finnerty (20), and Maeng and coworkers (21).

Branched Alkanes (Isoparaffins)

Linear alkanes are generally degraded preferentially to branched forms, so a decrease in the ratio of *n*-alkanes to branched forms (e.g., *n*-heptadecane: pristane) serves as an indicator of the onset of biodegradation in the environment. Nevertheless, simply branched alkanes such as pristane are reasonably readily degraded, for example, the pristane-degradation pathway has been worked out in a *Brevibacterium* (23); it involves diterminal oxidation to pristanedioic acid, with subsequent ß-oxidation, much as outlined in Figure 4 but potentially at both ends of the molecule. Interestingly, this work with *Brevibacterium erythrogenes* revealed a clear example of hydrocarbon diauxie; when provided with both *n*-hexadecane and pristane, no pristane degradation was seen until the *n*-hexadecane had been degraded to less than 5% of the total hydrocarbon (23). Other organisms seem completely unable to degrade branched alkanes, whereas they have a broad ability to degrade even very long *n*-alkanes (24).

Alkanes with more complicated branching, particularly those with tertiary carbon atoms, are generally much more resistant to biodegradation than pristane and phytane, but even compounds such as 2,2,4,4,6,8,8-heptamethylnonane are degraded by mixed consortia (25). Here, degradation

seems to involve the oxidation of an internal carbon to the ketone, further oxidation to the ester, and subsequent hydrolysis prior to ß-oxidation (Fig. 6). Presumably, the first reaction is catalyzed by a monooxygenase, but this has not been studied in detail. A similar pattern of degradation has also been seen for pristane. Adjacent quaternary carbons may pose a more difficult problem, and no pathway that can degrade this motif has been identified yet.

Cyclic Alkanes (Naphthenes)

The biodegradation of naphthenes has not received as much attention as that of the linear alkanes and aromatics, but degradation occurs quite readily. The octane hydroxylase has significant activity against cyclopentane and cyclohexane (17), and much degradation may be carried out by organisms that preferentially degrade linear alkanes. Some *Acinetobacters* can grow on cyclohexane as the sole source of carbon (26), and these organisms possess a cyclohexane monooxygenase that converts cyclohexane to cyclohexanol. Subsequent degradation of the cyclohexanol proceeds through the ketone, the cyclic acid lactone, and ring cleavage to the diterminal carboxylic acid (Fig. 7). The enzyme that converts the ketone to the lactone is another monooxygenase, and after the lactone is hydrolytically cleaved, there is probably another monooxygenase involved in the conversion to adipate. Because each monooxygenase turnover requires an NADH, there is a rather larger energetic investment in activating cyclic alkanes than in activating linear ones. Bacteria that are able to grow with decalin (perhydronaphthalene) as the sole carbon source have been isolated from Arctic oil seeps (28), but many organisms seem able to degrade naphthenes only cometabolically when supplied with a more degradable substrate such as a linear or simply branched alkane (29).

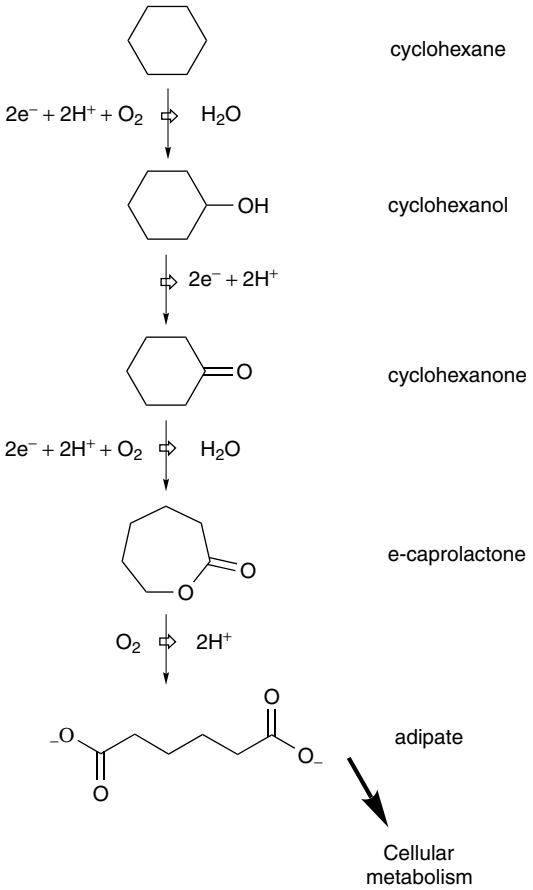

Figure 7. The pathway of cyclohexane biodegradation by a *Nocardia* species, based on Perry (26).

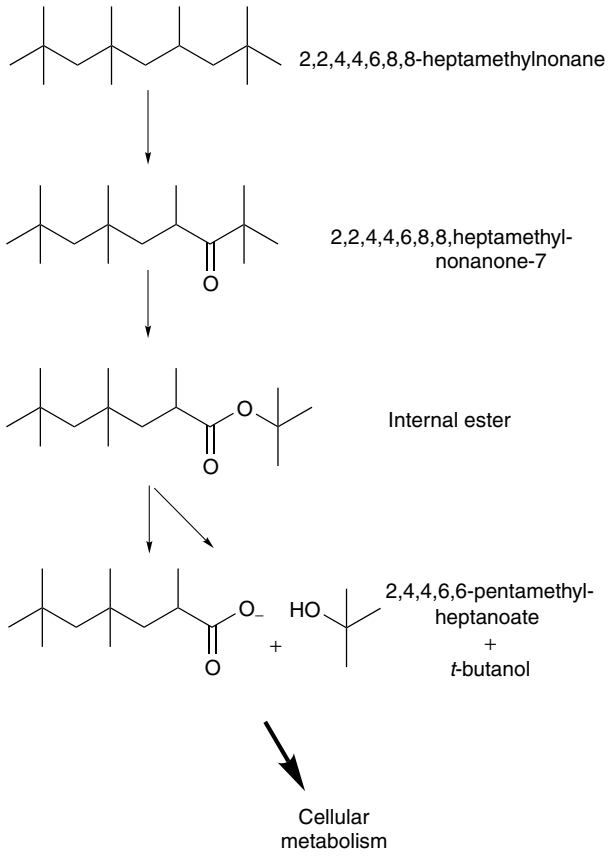

Figure 6. Proposed pathway of degradation of 2,2,4,4,6,8,8-heptamethylnonane, based on Rontani and Giusti (25). Presumably, both oxygen insertions are by monooxygenases, but this has not been shown.

Substituted polycyclic saturated hydrocarbons, such as the hopanes and steranes, are particularly resistant to biodegradation (30), and they are widely used in petroleum geochemistry to correlate oil reservoirs with their source rocks (6). They have also proven very useful as conserved internal markers for following oil biodegradation in the field (7).

Alkenes

Alkenes are essentially absent from crude oils and straight distillate fuels but can be quite abundant, that is, up to 20%, in modern gasolines. Some representative gasoline alkenes are shown in Figure 8. The best-studied biodegradation pathway is that of propylene by *Xanthobacter* strain py2 (30), which can grow with

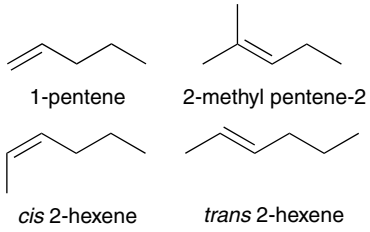

Figure 8. Some representative gasoline alkenes.

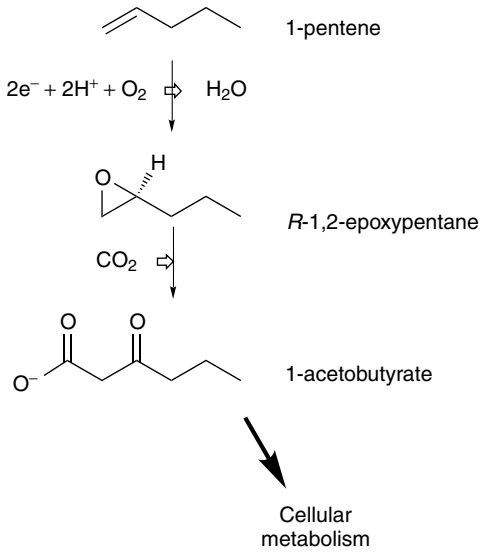

Figure 9. The pathway of alkene biodegradation. The figure shows the biodegradation of pentene; this is speculative, although based on the pathway elucidated for the biodegradation of propylene by Zhou and coworkers (30).

propylene as the sole carbon and energy source. The initial enzyme of the degradation pathway (Fig. 9), the alkene monooxygenase, has been extensively studied; it is an $\alpha_2\beta_2\gamma_2$-hexamer, containing two dinuclear iron active sites that seem rather similar to the active site of methane monooxygenase. Interestingly, although the enzyme catalyzes the epoxidation of a wide range of alkenes, it shows remarkable enantiomeric specificity in producing the *R*-enantiomer. It shows no activity toward alkanes, although it does attack benzene and toluene. Further metabolism of the epoxide involves carboxylation to generate an aceto-fatty acid (Fig. 9) that can then enter cellular metabolism.

Aromatic Hydrocarbons

The biodegradation of aromatic hydrocarbons, particularly under aerobic conditions, is a very active field of investigation and it is dealt with in detail elsewhere in this volume (31). Just as with alkane, isoalkane, and cycloalkane degradation, the initial reaction is the addition of oxygen, and just as with the alkanes, there is a marvelous diversity in the way this is achieved by different organisms. For example, as discussed by Zylstra (31), different organisms attack toluene by inserting oxygen into what appear to be all the possible positions on the

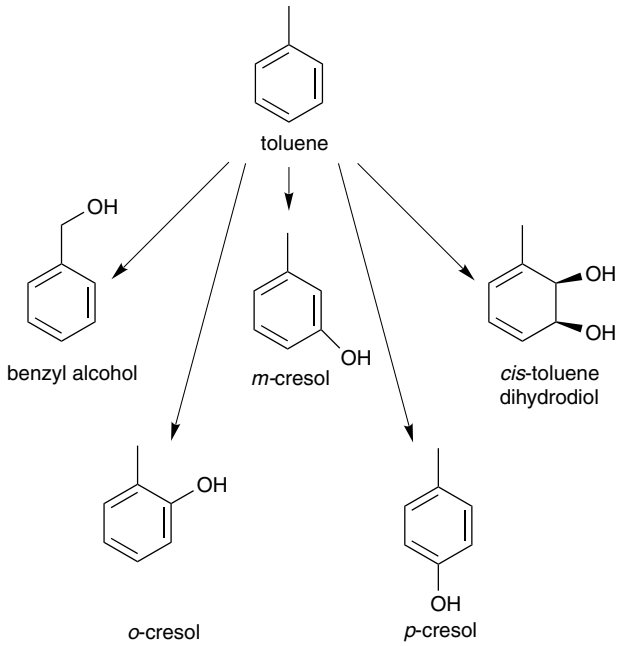

Figure 10. The diversity of toluene-activating systems found in different bacteria, based on Zylstra (31). All these reactions require NADH.

molecule (Fig. 10). This is true even within one genus, for example, *Pseudomonas*. Thus, *P. putida* mt-2 attacks the methyl substituent with a monooxygenase that converts toluene to benzyl alcohol, *P. cepacia* G4 attacks the ring at the *ortho* position to generate *o*-cresol, *P. pickettii* PK01 attacks the *meta* position to generate *m*-cresol, whereas *P. mendocina* KR attacks the *para* position to generate *p*-cresol; all these reactions are monooxygenases and use NADH to reduce the other atom of oxygen to water. *Pseudomonas putida* F1 uses a dioxygenase to generate *cis*-toluene dihydrodiol in a reaction similar to the initial oxidation of benzene that leads to the production of *cis*-benzene dihydrodiol. Again, there is substantial breadth in the substrate ranges of many of these enzymes, all of which require NADH as a source of reductant and all of which are usually plasmid-borne. Other organisms such as *Rhodococcus rhodocrous* seem to carry out several of the different reactions in one organism (32).

Subsequent oxidations convert the initially oxidized intermediates to substituted catechols and these are then cleaved to noncyclic structures. In *P. putida* mt-2, the benzyl alcohol is converted to benzaldehyde and then benzoic acid in reactions that are similar to those that convert *n*-alkanes to *n*-alkanoic acids. The acid is attacked by a dioxygenase to form a dihydrodiol, and this loses a carbon dioxide to become catechol. In the pathways that generate cresols and *cis*-toluene dihydrodiol, these are converted by various routes to methyl catechols (*o*- and *m*-cresols and *cis*-toluene dihydrodiol) or protocatechuate (3,4-dihydroxybenzoate) (31). The catechols and substituted catechols are cleaved by ring-cleaving dioxygenases either between the two alcohol functionalities (intradiol or *ortho*-cleavage) or adjacent to one of them (extradiol or *meta*-cleavage). The former have Fe^{3+} at

the active site and the latter have Fe^{2+} or Mn^{2+}, and crystal structures of representatives of the two types of iron-containing enzymes are available. Protocatechuate-3,4-dioxygenase, an intradiol cleaving enzyme, has Fe^{3+} coordinated in approximately trigonal pyramidal geometry by equatorial histidine, tyrosine and water, and axial histidine and tyrosine (33). 2,3-dihydroxybiphenyl-1,2-dioxygenase, an extradiol cleaving enzyme, has Fe^{2+} coordinated in square pyramidal geometry by two histidines, a glutamate, and two water molecules; one of the histidines is the axial ligand (34). Plausible models for catalysis in both enzymes have the catechol moiety displacing the water molecule(s) and the subsequent insertion of dioxygen cleaving the aromatic ring. The ring cleavage enzymes do not require NADH as a cosubstrate. Many intra- and extradiol enzymes have been partially characterized, and some have a very broad range of substrates, whereas others are very specific (35). Some even seem to catalyze both intra- and extradiol cleavage (36).

The degradation of longer-chain alkyl benzenes and alkyl polynuclear aromatics shows a similar diversity to that exhibited by toluene; the initial oxygen insertion can be either on the ring, usually to form *cis* dihydrodiols, or on the alkyl substituent, typically with the generation of the primary alcohol (37). The degradation of biphenyl proceeds by oxidation to the 2,3-dihydrodihydroxybiphenyl and then to the catechol with subsequent extradiol oxygenation to open the oxygenated ring. Dialkylated benzenes are readily degraded, sometimes as sole source of carbon (e.g., *Pseudomonas*) and sometimes cometabolically (e.g., *Nocardia*). Remarkably, organisms able to degrade *m*- and *p*-xylene seem unable to degrade *o*-xylene, and *vice versa*.

Bacterial degradation of naphthalene is usually by dioxygenase attack to yield the 1,2-cis-dihydrodiol, with subsequent reduction to 1,2-naphthohydroquinone. Subsequent extradiol cleavage yields 2-hydroxybenzalpyruvate, and the side chain is removed as pyruvate to eventually yield salicylate (Fig. 11,31). The genes that encode the enzymes for these reactions are usually plasmid-borne in Pseudomonads. Similar pathways degrade phenanthrene and anthracene, and the degradation of larger polynuclear aromatics may follow a similar route. Polycyclic aromatics with heteroatoms, such as dibenzothiophene and carbazole, can be attacked in the same way or by an angular dioxygenase attack, in which the carbon bonded to the heteroatom and the adjacent carbon in the aromatic ring are both oxidized, or by a direct oxidation of the heteroatom (38). This latter reaction is exploited in the biodesulfurization of fossil fuels (39). Similarly, some organisms degrade naphtheno-aromatics such as fluorene in a manner analogous to naphthalene, whereas others use monooxygenases to convert the naphthenic carbon to the alcohol and then to the ketone. This is followed by dioxygenation at the adjacent aromatic ring and cleavage of the naphthenic ring to yield, in the case of fluorene, a biphenyl derivative (38). As is the case with so many of the systems described here, the initial enzymes of this pathway have a very broad specificity and are able to cleave the naphthenic or aromatic rings of a wide range of substrates.

Alkylated polycyclic aromatic compounds are abundant in petroleum, usually being far more abundant than the parent polycyclic compound. Although the degradation of these compounds has not been studied in great detail, it is clear that they are readily degraded. The degradation of methyl naphthalenes by *Sphingomonas paucimobilis* has been studied in some detail (37), and it is clear that all mono-, di-, and triisomers are readily degraded and that this is initiated either on the methyl group or on the ring.

We have thus seen that aromatic molecules are degraded in several alternative ways by different organisms and that many organisms are able to degrade a wide range of compounds. It is noteworthy that this degradation is usually complete; radiolabeled experiments have demonstrated the complete mineralization of many polynuclear aromatic hydrocarbons by a diversity of bacteria. Nevertheless, bacterial degradation is generally most effective on compounds with four or fewer aromatic rings; molecules larger than this tend to be more resistant to bacterial degradation.

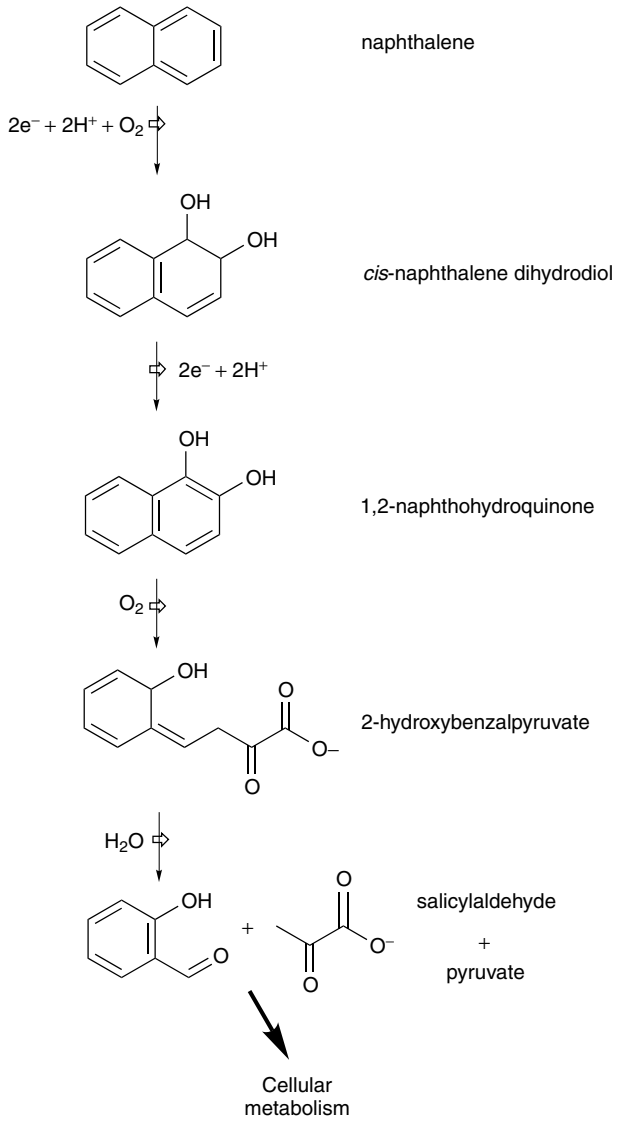

Figure 11. The pathway of naphthalene degradation in a *Pseudomonas*, based on Cerniglia (40) and Zylstra (31).

Fungal systems are also effective at oxidizing polynuclear aromatic hydrocarbons, but the products (intermediates) are often different from those produced by bacteria, and complete mineralization, although performed by some species, is not the norm. Perhaps the best-studied nonlignolytic fungus is *Cunninghamella elegans*. This filamentous organism uses a P-450 monooxygenase system to incorporate one atom of molecular oxygen into the polynuclear aromatic substrate while reducing the other oxygen atom to water. The polynuclear aromatic hydrocarbon is thereby converted to the arene oxide, which is hydrolyzed by an epoxide hydrolase to generate the *trans*-dihydrodiol. This in turn may be dehydrogenated to catechol. Alternatively, and apparently nonenzymatically, the arene oxide can rearrange to the phenol (Fig. 12). The phenol can then form conjugates with sugars or sulfate and can be excreted (40). P-450 monooxygenases seem to be widely spread in fungi, and they are known to attack larger polynuclear aromatic hydrocarbons with up to at least five and six rings.

A quite different degradation occurs if polynuclear aromatic hydrocarbons become substrates of the lignolytic system of white-rot fungi. This system, which has been extensively studied in the last decade, generates reactive species that oxidize lignin at some distance from the fungal hyphae. When confronted with polynuclear aromatic hydrocarbons, the lignin-degrading system can oxidize phenanthrene and anthracene to the respective quinones (41) and these are then mineralized, either by the fungus or by the bacteria, or become polymerized to other material in the system so that they can no longer be extracted. Early optimism that white-rot fungi might be broadly useful in degrading polycyclic aromatic compounds in contaminated soils seems to be fading (42).

Polar Components of Crude Oil

As discussed earlier, most crude oils contain significant quantities of polar, heteroatom-containing material; this material is difficult to analyze at the molecular level because it is essentially nonvolatile, and therefore unamenable to gas chromatography, although suggestions for typical molecules have been made (Fig. 3). Porphyrins in oil are very resistant to biodegradation, and like the steranes and hopanes, they are used in petroleum geochemistry to correlate oil reservoirs with potential source rocks (60). There is good evidence that naphthenic acids can be degraded by microorganisms (43), and if the structure suggested for a representative asphaltene in Figure 3 is reasonably accurate, it suggests that at least parts of the asphaltene fraction might be biodegradable. Some work suggests that this is indeed the case (44), but it seems likely that the majority of the polar materials in crude oil are not subject to significant biodegradation, and they may be as inert as the humic and fulvic acids of modern sediments.

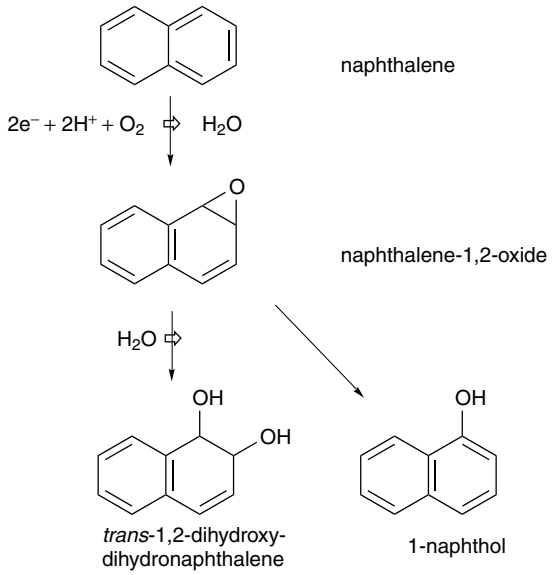

Figure 12. The pathway of naphthalene oxidation in fungi, based on Cerniglia (40).

Fuel Oxygenates

Although fuel oxygenates such as ethanol and MTBE (methyl *tert*-butyl ether) are not hydrocarbons because they contain oxygen, they may make up to 15% by volume of some fuels. Reviews of the current understanding of MTBE biodegradation are available (45,46).

OVERALL RATES OF AEROBIC BIODEGRADATION

Although the majority of compounds in crude oils and refined products are all biodegradable, their disappearance from the environment following a spill follows a well-defined order. Thus, the smaller linear alkanes (up to about 12 carbon atoms) and one- and two-ring aromatic molecules are degraded before branched alkanes such as pristane and phytane and polynuclear aromatic hydrocarbons with more rings. Three-ring aromatics, such as fluorene, phenanthrene, and dibenzothiophene are degraded at similar rates, and these are substantially degraded before much degradation of four-ring compounds is usually seen. Within each of these categories of aromatic compounds, the parent compound is degraded in preference to the alkylated forms, and the less alkylated species are degraded before the more alkylated ones (47).

This hierarchy of degradation has proven very useful in identifying the onset of biodegradation in the environment and in quantifying biodegradation. The ratio of the straight-chain alkane *n*-heptadecane to the branched pristane decreases during early stages of biodegradation and provides a qualitative measure of the process. Both heptadecane and pristane are, however, removed quite rapidly once biodegradation is well established. More resistant molecules, such as hopanes and steranes, can serve as internal standards once pristane biodegradation begins. Provided all the oil under consideration is from a single source, the conserved internal markers can provide a reference for the quantitation of biodegradation (7).

The overall pattern of biodegradation also provides a way of distinguishing oils from different sources in the environment. Alkylated phenanthrenes and dibenzothiophenes are degraded at very similar rates in the environment and the more alkylated forms are

among the more slowly degraded compounds. The ratio of trialkyl-dibenzothiophenes to trialkyl-phenanthrenes thus remains essentially constant as degradation proceeds. Because the absolute value of this ratio is often different in different oils, it can be quite useful for discriminating different sources. Furthermore, since alkylated dibenzothiophenes and phenanthrenes are usually degraded more rapidly in the environment than the corresponding alkylated chrysenes, the ratio of trialkyl-phenanthrenes to trialkyl-chrysenes provides a useful indicator of the extent of degradation when more conserved molecules, such as hopanes, are unavailable (48).

ROLE OF SURFACTANTS

Hydrocarbons present a particular challenge to biological systems because they are hydrophobic. Smaller hydrocarbons, particularly aromatic hydrocarbons, have a reasonable solubility (49), but larger alkanes are very poorly soluble. Different organisms seem to have developed different ways to get around this problem. Many produce surfactants that solubilize hydrocarbons, whereas others develop molecules in their cell membranes that adhere to the oil–water interface. Allen and coworkers (50) provide an example of how difficult it is to make broad generalizations; they examined the ability of *Pseudomonas* and *Sphingomonas* to degrade naphthalene and phenanthrene in the presence of a nonpolar, nondegradable (by these organisms) solvent (2,2,4,4,6,8,8-heptamethylnonane) or the nonionic surfactant Triton X-100. The heptamethylnonane increased the rate of degradation of both substrates for both microorganisms. The Triton X-100 increased the rate of degradation by the *Pseudomonas* but inhibited that by the *Sphingomonas*.

Nevertheless, the oil spill response community often recommends the use of dispersants to aid in the biodegradation of oils spills at sea (51), and although there has been controversy in the past, recent work demonstrates that some products clearly stimulate oil degradation in a safe and effective manner (52). Dispersant application was particularly successful in the recent *Sea Empress* incident off the southern coast of Wales (53).

ANAEROBIC DEGRADATION OF CRUDE OIL

For many years it was assumed that oil biodegradation could only occur under aerobic conditions. This was a logical view because any degradation must involve oxidation, and the very existence of oil reservoirs indicated that anaerobic degradative processes in such environments must be very slow. But in the last decade, it has become clear that at least some hydrocarbons are oxidized by bacteria under completely anaerobic conditions (54). Hydrocarbon biodegradation has now been shown under sulfate-, nitrate-, chlorate-, perchlorate-, carbon dioxide-, and ferric iron-reducing conditions. The number of organisms able to degrade hydrocarbons anaerobically that have been isolated in pure culture is still rather small (Table 5), and it is indeed likely that anaerobic hydrocarbon degradation under methanogenic and (per)chlorate-reducing conditions will require consortia of organisms, which will complicate isolation and characterization. Nevertheless, some progress has been made in understanding the basic biochemistry of the process under sulfate-reducing and nitrate-reducing conditions.

Table 5. Bacterial Genera That Contain Anaerobic Oil-Degrading Species

Azoarcus	*Desulfobacterium*	*Desulfosarcina*
Thauera		

BIOCHEMICAL PATHWAYS OF ANAEROBIC HYDROCARBON DEGRADATION

Since the phenomenon of anaerobic hydrocarbon degradation is so recently established, it is not surprising that pathways are only beginning to be understood. We can expect substantial progress in this area in the next few years.

Linear Alkanes (Paraffins)

The anaerobic degradation of *n*-alkanes has been demonstrated under nitrate-reducing (55), sulfate-reducing (56), and methanogenic (57) conditions, and microbes able to reduce sulfate and nitrate at the expense of alkanes have been isolated. Recent work (58) suggests that the initial step in the oxidation of *n*-alkanes is the addition of an internal carbon, probably C2, to the double bond of fumarate to produce an alkylsuccinate, as shown in Figure 13. This is probably followed by the addition of

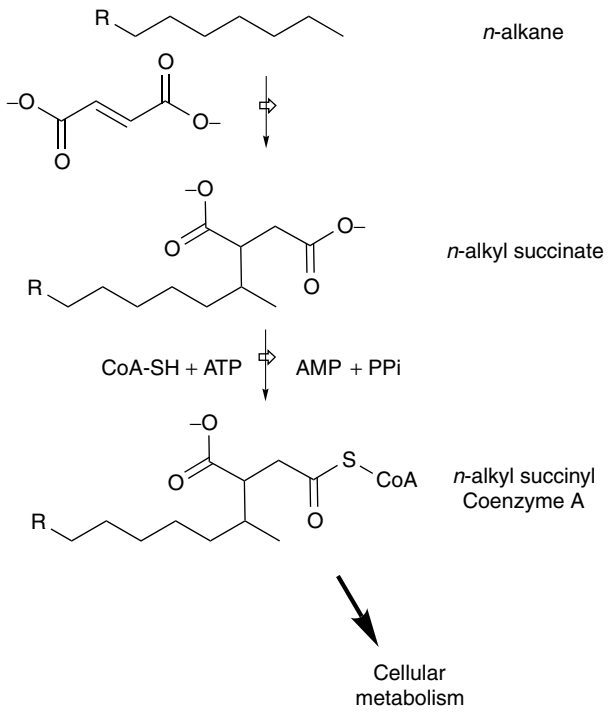

Figure 13. The pathway of anaerobic degradation of alkanes, based on Kropp and coworkers (58).

Coenzyme A to allow β-oxidation, as shown, although this has not yet been demonstrated.

Branched Alkanes (Isoparaffins)

Pristane and phytane undergo biodegradation under sulfate-reducing (56), nitrate-reducing (59), and methanogenic (60) conditions.

Aromatic Hydrocarbons. The biodegradation of aromatic hydrocarbons under anaerobic conditions is a very active field of investigation because it is the water-soluble benzene, toluene, ethylbenzene, and the xylenes (BTEX) fraction of gasoline that is often found as an environmental contaminant. Toluene is perhaps the most readily degraded of the group, and it has been shown to be degraded under sulfate-, nitrate-, chlorate-, perchlorate-, carbon dioxide-, and ferric iron-reducing conditions (61). Under nitrate-reducing conditions, the pathway of degradation again involves the addition of fumarate, as shown in Figure 14. The enzyme that catalyzes the first step, benzylsuccinate synthase, has been cloned and sequenced (61). It seems to be an $\alpha_2\beta_2\gamma_2$-complex with native mass of 220 kDa and shows strong similarities to glycyl radical enzymes such as anaerobic ribonucleotide reductase. Interestingly, the product is optically pure (R)-(+) benzylsuccinic acid, and the proton removed from the methyl substituent of the toluene is retained in the succinyl moiety of the product.

A similar activation has been seen with the xylenes, but an alternative pathway has been suggested for ethylbenzene. This involves hydration and carboxylation of the ethyl moiety to produce benzoylacetate, which is then added to Coenzyme A for further metabolism (61). All components of BTEX have been seen to undergo biodegradation under a variety of anaerobic conditions, although benzene seems the most recalcitrant. There are as yet no details of how benzene is activated anaerobically. Similarly, larger aromatic molecules, including naphthalene and phenanthrene, have also been seen to be biodegraded, but again there is less detail of how this occurs.

Although it thus seems that anaerobic degradation of hydrocarbons is a far more important process than was previously imagined, we are only beginning to understand the diversity of organisms that are able to carry out the reactions (Table 5), and are woefully ignorant of the range of substrates that can be degraded. All experiments to date suggest that anaerobic degradation in the environment is a much slower process than aerobic degradation, and as the vast reserves of ancient fossil fuels indicates, it must be severely inhibited in many environments.

We have seen that hydrocarbons serve as the carbon source for a broad diversity of microbes, bacterial, archaeal, and eukaryotic, in aerobic and anaerobic environments that span a range of temperatures and salinities. What seems to distinguish hydrocarbon-degrading microorganisms from their nondegrading relatives is their possession of enzymes to activate the relatively inert carbon-carbon bond in the substrate. In aerobic prokaryotes, this process of activation usually begins with a monooxygenase or a dioxygenase, whereas the anaerobes characterized to date either attach a fumarate moiety or hydrate and carboxylate an alkyl group. Although there

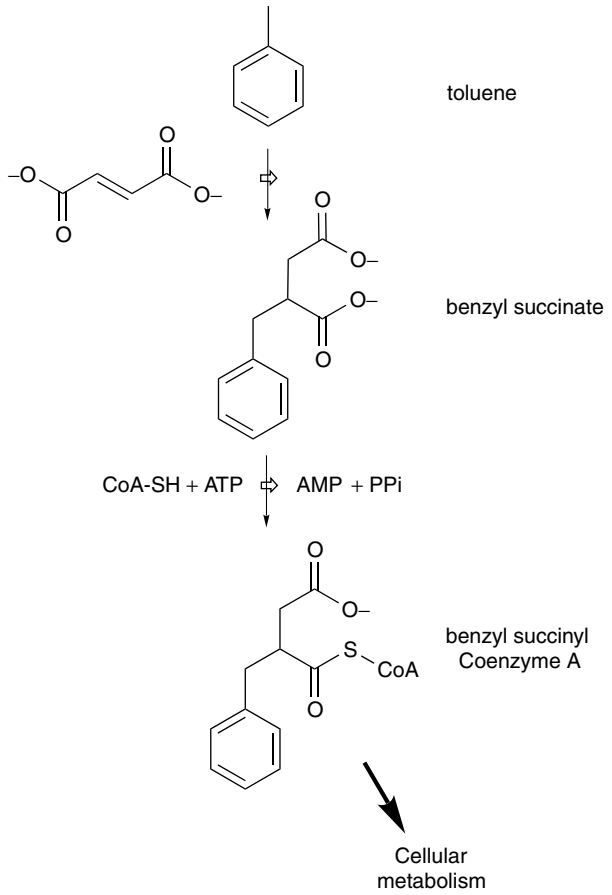

Figure 14. The pathway of anaerobic degradation of toluene, based on Heider and coworkers (61).

are still many areas in which our knowledge is minimal, such as how benzene and cyclic alkanes are activated anaerobically, it is apparent that the initial activation usually requires a substantial metabolic input into the molecule by the degrading organism. There is thus a strong selection pressure for the organism to get a return on this investment in subsequent metabolisms, and at least in aerobic environments, it is unlikely that metabolic intermediates are deliberately excreted into the environment. In anaerobic environments, the issue is complicated, and some environments, such as those in which methanogenesis is predominant, probably rely on symbiotic associations of hydrocarbon degraders and methanogens. Even here, however, there must be a strong selection pressure to maximize biological consumption, and if free energy is available, we may expect some organism to be exploiting it. Nevertheless, trace amounts of intermediates can sometimes be detected in the field (62), providing strong evidence that anaerobic degradation is indeed underway.

The situation in eukaryotic systems may be more complicated and is certainly less well understood. Very few fungi have been characterized in terms of their biodegradation ability, and it is likely that many of the organisms listed in Table 3 oxidize hydrocarbons only adventitiously and are not able to fully metabolize the molecules. Sometimes, this leads to the incorporation

of the majority of the hydrocarbon into inextractable soil material, and sometimes the activated molecules are consumed by bacteria that could not initiate the hydrocarbon degradation themselves.

FATE OF HYDROCARBONS IN THE ENVIRONMENT

Spills and Contaminated Sites

With the foregoing description of the biodegradation of hydrocarbons, with the final products being carbon dioxide, water, and microbial biomass, we are in a position to discuss the importance of this process in the biosphere. When hydrocarbons are released into the environment, the smallest molecules, those containing up to about 15 carbons, are likely to volatilize. This process can remove one- to two-thirds of surface spills of crude oils (4) and potentially all the gasoline spilled on impervious surfaces. Some terrestrial crude oil seeps produce so much volatile material that they may periodically ignite, but in the absence of ignition, it is thought that almost all the volatilized material is photochemically oxidized in the atmosphere or captured by rain and brought back to the ground where it is biodegraded. The residuum is much more viscous, and some seeps, for example, in Los Angeles, Azerbaijan, and Trinidad, give rise to large lakes of pitch (2). Some molecules are sufficiently soluble that they are washed out of exposed seeps or dissolve from slicks at sea or on freshwater (49), and this process also increases viscosity. Floating slicks will absorb large amounts of water (20 to 80%), and stable water-in-oil emulsions, known as *mousses*, often form (4). If enough energy is available, such as in a major storm, a floating oil spill will disperse as tiny drops of oil in water. This is what happened in the *Braer* spill off Shetland in 1993 (63). This process can be aided by the addition of dispersants (51,52,64). An alternative response to spilled oil is to burn it under controlled conditions (65).

Regardless of the initial fate of spilled or natural hydrocarbons, if they are not burned or photochemically oxidized, they will persist until they are biodegraded. As we have seen, the extent of biodegradation is dependent on many factors. It not only depends on the chemical composition of the oil but also on the environment in which the biodegradation is to occur. Gasoline and diesel fuels are very biodegradable, and it is not unusual for them to be essentially completely degraded under favorable conditions (66–68). Crude oils, and heavy fuel oils such as Bunker C used in ships, contain some molecules that are very resistant to biodegradation. McMillen and coworkers (69) have examined the short-term biodegradability of 17 crude oils in soil microcosms as a function of 78 different parameters that might determine the extent of biodegradation. These included 67 individual chemical species, percentage of sulfur, and so on. They found that the API gravity was the most useful predictor of biodegradability, at least for the most degradable fraction of the oils. At loadings of 0.5 wt% oil in a loam soil with appropriate nutrients, moisture, and aeration, more than 61% of the most degradable oil (API = 46°) was lost in four weeks, whereas only 10% of the least degradable oil (API = 15°) was consumed under the same conditions. Further degradation occurred on a longer timescale, and the literature reports biodegradation potentials as high as 97% for particularly light oils (70).

Many factors can conspire to hinder biodegradation in the environment. Temperature extremes are likely to inhibit degradation, especially low temperatures such as those found in Antarctic (71) and Arctic environments (72), although there are psychrotrophic isolates that are able to grow at subzero temperatures (73,74). On land, excess salinity, such as that found in some produced water brines, can have a strong inhibitory effect on oil biodegradation (69) until the salt is washed out by precipitation. Extreme exposure to sunlight may prevent all microbial growth.

Perhaps the most acute limiting factor is oxygen, for there are radical differences in the extent of biodegradation and its kinetics in oxic and anoxic environments. In the presence of adequate oxygen, nitrogen, phosphorus, and iron are likely to be the most limiting nutrients because they are not present in oils. The relief of these limitations has been the underlying strategy in the successful use of bioremediation in treating crude oil spills. Thus, the addition of oleophilic and slow-release nitrogen- and phosphorus-containing fertilizers was successful in substantially increasing the rate of biodegradation of oil after the *Exxon Valdez* spill in Alaska (7,75), and nutrient addition has been successful in several terrestrial applications as well (76).

In anaerobic environments, it seems that biodegradation is controlled by the presence of alternate electron acceptors. In general, the most energy-providing reactions occur most readily, and it is not unusual to see a succession of microbial processes as biodegradation proceeds. Thus, underground plumes of degradable hydrocarbons, such as dilute solutions of BTEX in groundwater, first undergo aerobic degradation until oxygen becomes limiting. If present, nitrate, (per)chlorate, or oxidized metal ions then serve as terminal electron acceptor until these are exhausted, in turn followed by sulfate, if it is present. Finally, methanogenesis takes over (77). There have been discussions about stimulating anaerobic biodegradation in the field by adding one or more of the limiting electron acceptors but this has not yet achieved widespread use.

Biodegradation of Oil and Refined Products During Use and Storage

Oil and refined products are frequently stored for some time before use, and several nations have strategic oil reserves in underground caverns. These caverns usually become contaminated with water, and oil-degrading microorganisms, whose activities potentially threaten oil integrity, are readily isolated from such environments (78). Diesel, kerosene, and jet fuel storage facilities, and even airplanes, can have similar problems, and this is a particular concern because some of the organisms can block fuel filters with potentially disastrous results (79). Other problems associated with fuel biodegradation include an increase in tank and fuel line corrosion and production of surfactants that encourage

water in oil emulsions. Proprietary biocides are often added to slow biodegradation in such situations (80).

Biodegradation in Oil Reservoirs

Oil reserves that have been "invaded" by aerobic surface waters have often been subject to substantial biodegradation. This is recognized by the absence of linear alkanes, the smaller aromatic hydrocarbons, and even some of the naphthenes. The Athabasca deposits of northern Alberta are thought to be one example of this phenomenon. Typically, biodegradation increases the specific gravity and viscosity of an oil, and indeed the Athabasca deposits are very viscous (2).

Although early production of oil from an oil field may occur owing to the inherent high pressure in the reservoir, later production is usually aided by pumping water into injection wells. Often this is accompanied by a "souring" of the production, an industry term for an increase in H_2S in the produced oil. Some of this may be the result of subterranean geochemistry; however, it is likely that the majority is the result of the activity of sulfate-reducing bacteria. These organisms typically grow by oxidizing small organic acids at the expense of reducing sulfate to sulfide (81), and they are widely implicated in metal corrosion. As discussed earlier, sulfate-reducing hydrocarbon degraders have now been found, and they may also play a role under reservoir conditions. To date, however, none of the reservoir sulfate-reducing isolates has been reported to degrade hydrocarbons. Thermophilic and mesophilic sulfate-reducing bacteria have been isolated from many wells (82–84) but it has usually been assumed that they have entered during drilling and production. More recently, it is becoming accepted that oil reservoirs may contain microorganisms even in the absence of substantial water incursions. Thus, L'Haridon and coworkers (85) have shown that anaerobic hyperthermophilic microorganisms are abundant even in previously untapped oil reservoirs, and may well be abundant throughout the deep crust of the earth. All of the organisms isolated to date have grown either autotrophically with hydrogen or sulfide as electron donor (84) or heterotrophically (82) as sulfate-reducers consuming small organic acids. As mentioned in the following text, there are now several examples of anaerobic hydrocarbon-degrading isolates and consortia isolated from surface environments. Whether such bacteria play any role in the maturation and/or degradation of crude oils remains to be seen.

APPLICATION

With the foregoing discussion in mind, we can ask how hydrocarbon biodegradation can be harnessed to beneficial uses for humankind. Three areas are worth brief discussion: bioremediation, bioprocessing, and biodesulfurization.

Bioremediation

Bioremediation is the term applied to a stimulation of biodegradation to minimize the environmental impact of a spill, whether recent or historical. As we have seen, the majority of hydrocarbons in refined products and most crude oils are biodegradable, but they do not provide a complete food for bacterial growth because they only provide carbon and hydrogen. In almost all aerobic environments, the biodegradation of significant concentrations of hydrocarbons is likely to be nutrient-limited. Ameliorating this limitation by adding fertilizers is the basis of most successful bioremediation strategies for soils and shorelines (7,75,76,86), and the technique is widely used. Nitrogen and phosphorus are the probable limiting nutrients in most marine environments, and care must be exercised that these are not added at high enough levels to cause problems such as eutrophication or toxicity. Adding oxygen, perhaps by sparging air, or adding a peroxide is often beneficial in stimulating hydrocarbon biodegradation in groundwater (86), where typically the concentrations of hydrocarbons are sufficiently low that nitrogen- and phosphorus-limitation is unlikely to be a significant problem. Early expectations that adding laboratory-selected or genetically engineered cultures of hydrocarbon-degrading bacteria onto spills or into aquifers would be a useful bioremediation technology have not been born out by subsequent experience. Rather, it is clear that indigenous hydrocarbon-degrading bacteria are ubiquitous and that adding microbes is usually unnecessary. One exception may be the inoculation of fungi to composting systems to degrade the heaviest polycyclic aromatic hydrocarbons (87).

Bioprocessing

Bioprocessing is the term applied to carrying out a desired chemical reaction biologically rather than chemically, and the expectation is that the biological process will have advantages in selectivity, environmental impact, or cost. Perhaps the best-studied hydrocarbon bioprocessing system is the alkane oxidation system of *P. oleovorans*, which has been proposed for the production of medium–chain length polyoxyalkanoate plastics (88) and octanoic acid (89). Biocatalysts for the production of (S)-styrene oxide (90) and optically pure aryl epoxides (91) have also been proposed. Bioprocessing of hydrocarbons is in its infancy, and we may expect significant developments in the near future.

Biodesulfurization

Biodesulfurization is the term applied to the microbial removal of sulfur from fuels. The principal sulfur-containing molecules in crude oils and refined products are the thiophenes and their congeners, with dibenzothiophene being particularly important. Current refinery practice is to reduce the sulfur to H_2S with hydrogen over a catalyst at high temperatures and pressures, and even so there are problems that some alkylated forms are sterically hindered and are only poorly removed. Biocatalysts, particularly those derived from *Rhodococcus* and related genera, offer the potential for removing sulfur from dibenzothiophenes at ambient temperature and pressure (39), although significant developments seem to be required before the process is ready for commercialization.

CONCLUSION

The majority of the molecules in crude oils and refined products are biodegradable under appropriate conditions. These hydrocarbons are in commerce; however, this biodegradation can be prevented by careful storage and by the addition of biocides. If the hydrocarbons get into the environment, they will be biodegraded, and this biodegradation can be stimulated by the alleviation of limiting factors. There has been well-documented success in this enterprise with the addition of fertilizers and oxygen, both on the land and on the shorelines.

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PETROLEUM RESERVOIRS, INFLUENCE, ACTIVITY AND GROWTH OF SUBSURFACE MICROFLORA IN

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Naturally occurring oil seeps have provided a source of fuel, particularly for lamps, for over 5,000 years and the 'perpetual fires' of Baba Gurgur in Iraq were lit on the site of escaping natural gas deposits. The petroleum industry, as we now recognize it, was founded on two principal events, which took place in the mid-nineteenth century. First was the filing of an English patent in 1850 by James Young who had devised a fractional distillation process to refine oil seeping from a coal deposit and an oil shale. Second, just nine years later in North West Pennsylvania, Edwin Drake set out to find the source of oil seeping from the ground in Oil Creek near Titusville. He correctly anticipated the possibility of finding a "rock oil" reservoir below the surface by directed drilling, hoping to provide a commercially significant source of oil. Finding that, initially he was drilling through gravel beds and suffering numerous well bore collapses, he came up with the idea of inserting a string of tubes into the bore, through which he could operate the drill at greater depth; the principal method used ever since to protect lengthening well bores. Thus, the two major activities of the petroleum industry, exploration and production (E&P) and postproduction refining, were born.

This review will briefly examine the nature of petroleum hydrocarbons and their generation and then describe the reservoir as a habitat for subsurface microorganisms, either indigenous or introduced. The consequences of exploitation of oilfield reservoirs have brought microbial problems to the industry, which with the benefit of the present knowledge of the subsurface biosphere, might have been avoided. The final section will address the potential benefits of oil recovery, enhanced microbially to increase yields and describe some of the emerging technologies.

ORIGINS OF HYDROCARBON RESERVOIRS

Naturally occurring petroleum ranges in appearance from an almost colorless mobile liquid to a green–black viscous substance mixed with sand and water. Within one oil field there may be a wide compositional variation, both horizontal and vertical. The main constituents of crude oil

are true hydrocarbons, comprising a mixture of aliphatic compounds (alkanes, general formula C_nH_{2n+2}), and cycloalkanes (C_nH_{2n}) with aromatics (C_nH_n). A number of nonhydrocarbon constituents are found in oils. These are commonly heterocompounds of sulfur, nitrogen, and oxygen and organo-compounds of vanadium and nickel. The lower molecular weight hydrocarbons that occur as natural gas may also contain hydrogen, helium, or argon.

There are two prevailing theories to account for the presence of the petroleum hydrocarbon reserves in the Earth's crust, which being separated by geological time and space, may not be mutually exclusive (1). The theory adhered to by Thomas Gold since the late 1970s is that natural gas and crude oil are derived not from biological debris but from the initial materials that formed the earth for more than 4.5 billion years ago. This is the abiogenic or deep-earth gas theory and is described in Gold's book *The Deep Hot Biosphere* (2). Hydrocarbon reserves are described as having been originally condensed as solids at great depth, 100 to 300 km below the Earth's surface, liquefied or vaporized by increasing internal heat and forced nearer to the surface. Some of the mobilized hydrocarbons have been temporarily trapped in porous rocks under impermeable caps at depths which are accessible to the oil operator's drill bit; others have burst violently to the surface and have been vaporized as methane, carbon dioxide, and water vapor in volcanic eruptions.

The greatest weight of evidence is currently on the side of the biogenic theorists, whose case is put authoritatively by North and Cornford (1,3). This theory holds that the association of oil and gas with thick sedimentary sequences, together with geological, isotopic, and chemical evidence from trapped organic remains buried in the sedimentary rocks is clear proof that hydrocarbons are ultimately derived from deposition and transformation of macro and microbiota. Examination of accessible ancient igneous rock extruded from the Earth's surface by vigorous geological activity, for example, the metamorphic shield areas of Scotland and Scandinavia bordering the North Sea sedimentary basin offers no evidence of significant hydrocarbons (3) to support Gold's igneous rock theory as a source for hydrocarbons.

The majority of petroleum hydrocarbons represent the result of organic deposition following global atmospheric change to an aerobic state, with an intact ozone layer (currently thought to have occurred at about 1200 Ma). This followed the evolution of primitive life, photosynthetic exchanges and establishment of the principal elemental cycles. The Cambrian era was a time of extraordinary growth and proliferation; the majority of petroleum deposits being exploited currently originate from organic material generated in late Cambrian through to Jurassic epochs (between 400 and 150 Ma). Figure 1 shows the principal geological horizons currently yielding oil and gas.

Source rocks result from sediments that have generated sufficient oil or gas, which are commercially significant. Such rocks are classified as immature (hydrocarbons not yet generated), mature (rocks, where HC generation has commenced), and postmature (those which have already generated and often expelled all hydrocarbons). The

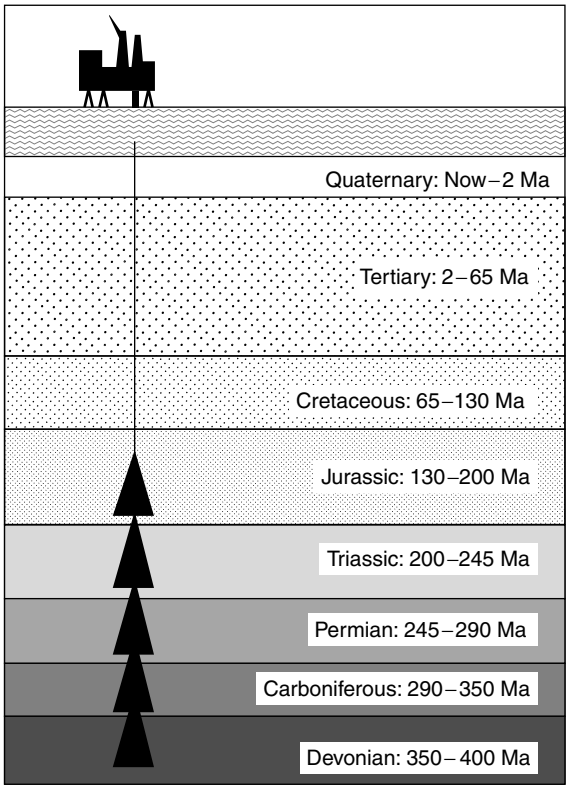

Figure 1. Schematic showing the geological horizons yielding subsurface hydrocarbon deposits.

indigenous organic matter in a source rock is called *kerogen* (solid or solvent insoluble), *bitumen* (liquid or solvent soluble), or gas (predominantly methane). These materials vary widely in character, depending on the source of the deposited organic material and the nature of deposition. By the time these hydrocarbon precursors have developed, the sediment may be several kilometers thick, and the deepest layers will have been irreversibly compacted into shales, sandstones, or limestones with pore spaces containing a mixture of the hydrocarbon precursors and water. Kerogen degenerates to bitumen and gas when temperatures increase during the slow burial process, releasing oil and gas. The achievement of a minimal temperature for this degeneration involves a minimum depth of burial, which is dependent on the immediate temperature gradient. The depth of burial itself is dependent on the time taken for sediments to accumulate.

The final steps in transformation to a crude oil are not fully understood, but result from the combinations of microbial, geothermal, geostatic, and temporal influences. Present day sediments in the deep ocean trenches yield important information on the diagenesis of organic matter into petroleum precursors. Claude ZoBell, at Scripps Oceanographic Institute, initiated the studies on bacterial activity in ocean sediments in the 1930s (4,5). During most of the 1940s and early 1950s, when actively involved in the petroleum industry, ZoBell tried to establish an incontrovertible case for production of

petroleum hydrocarbons by bacteria, particularly sulfate-reducing bacteria (6–8). Subsequent research has shown the presence and activity of bacteria at 80 meters below sea floor (mbsf) in the Pacific Peru margins (9) and 518 mbsf (age about 4.3 Ma) in the Japan Sea (10), which are capable of a wide range of functions in clearly separated zones of sediment cores. Any oxygen available in the uppermost layers of the sediment is rapidly utilized in ammonification processes, leaving an environment, in which bacterial distribution and activity are controlled by the availability of terminal electron acceptors (e.g., sulfate, nitrate), the bioavailability of organic carbon and methane gas.

Active bacterial migration through the porous sediments raises the possibility of preferential association in bacterial communities according to functionality, availability of electron donors and acceptors, and other parameters such as redox, temperature, hydrostatic pressure, and fluid incursion. All these contribute to the diagenetic processes. Cragg and coworkers (10) demonstrated the activity of nitrate and sulfate reducers, ammonifiers (aerobic and anaerobic), and acetogens with heterotrophs to depths of 518 mbsf with methane concentrations increasing with depth. Total counts of bacterial cells, dividing bacterial cells and total viable anaerobic bacterial counts showed the presence of an active population at the limit of their sample core. This indicated that bacteria continue to be present and active in still deeper sediments. Hence, by implication, these become the foundation of the indigenous rock *microflora* present in sedimentary core samples taken from many locations (11).

Maturation of the source rock is followed by expulsion of the hydrocarbons if sufficient quantities are generated to saturate the rock, allowing the excess to migrate. Migration occurs along the porous rock beds, diagenetic pathways (dissolution channels), and fracture pathways. Often the migration ends in a trap or reservoir either in domelike configurations, capped by impervious lithified rock or in deeper strata, confined by tectonic movement (folding and faulting) to zones on the shoulders of cap rock or the margins of anticlines or synclines (Fig. 2).

Deposition of mineral particles with decaying remains of macro- and microbiota between 400 Ma and 140 Ma became the source rock for the majority of petroleum hydrocarbons currently being exploited. About 30% of known reserves have already been produced in the past 150 years since the commercial extraction began, much of the remaining petroleum being less accessible than the lighter fractions first released from reservoir rock. Alternative strategies for exploiting these reserves include employment of microbially enhanced recovery techniques, which is discussed later in this chapter.

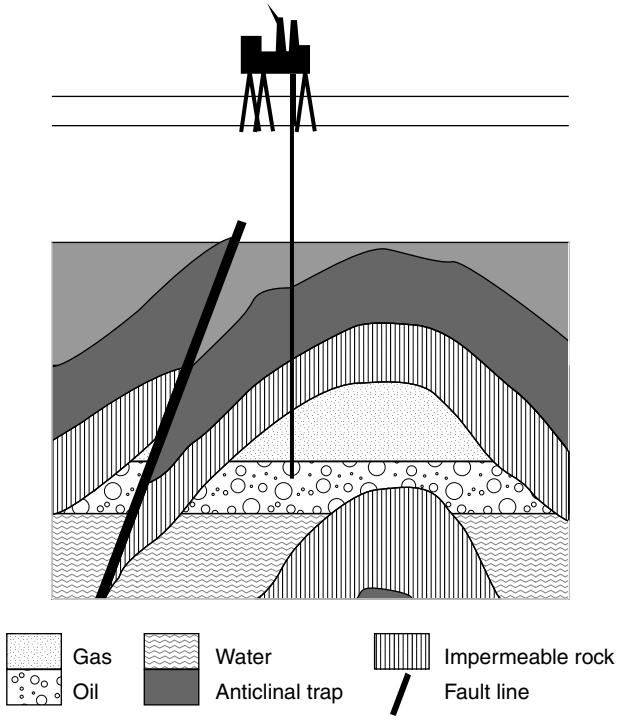

Figure 2. Cross section of porous rock, showing reservoirs of hydrocarbons in place following migration from source rock to trap.

OIL-BEARING ROCK FORMATIONS AS MICROBIAL HABITATS

Almost all sedimentary rocks have been formed under shallow and principally marine seas (3), resulting in sandstone, shales, and limestones either at depth in some areas or closer to the surface where glacial erosion has occurred (e.g., the Canadian Shield). Irrespective of the composition of a sediment during formation, water is an integral part of it. The nature of the available water has a very important influence on the activity of subsurface microorganisms. Water deposited with a sediment from the beginning is termed *connate water*. When the water-wet sediment is buried and increasingly compacted, some or all of its connate water is altered in composition: diluted, replaced, or even expelled by other waters. Water displaced by entry of oil or gas is called *free water*. Interstitial water resists displacement by invading hydrocarbons because it occupies pore spaces that are too fine for entry by the larger hydrocarbon molecules. The space occupied by interstitial water may be between 10 and 40% of the total pore space in a hydrocarbon-bearing rock, but does not necessarily constitute part of the general water column (which includes connate and free water) in the rest of the rock. The collective name for these three water types is *formation water*, with chemistry as complex as that of the hydrocarbons. They tend to be saline ranging from 1000 to 40,000 mgL^{-1} total dissolved solids (seawater contains approximately 3,500 mgL^{-1}) with the principal ions (sulfate, bicarbonate, chloride, sodium, magnesium, and calcium) distinguishing four main chemical types of oilfield brine (1,12). A comprehensive survey of formation water analyses has been compiled from 263 separate samples from 70 North Sea oil fields (12) identifying the distribution and abundance of ionic components. Additional data on depth, temperature, pH, pressure, total dissolved solids and potential bacterial carbon sources, the short-chain fatty acids acetate, propionate, and butyrate is also provided.

Reservoir rock can therefore be considered to be an intricate solid matrix, containing a complex mixture of hydrocarbons and waters in pore spaces and channels that vary in connectivity because of variations of cross section, transverse area and longitudinal shapes (13). The hydrocarbons and waters interact, depending on their relative abundance and specific constituents, resulting in matrix surfaces that are oil or water-wet and pores, which may contain water alone, oil alone, or mixtures of the two, creating fluid–fluid interfaces.

Temperatures in oilfield reservoirs are principally dependent on depth and latitude. Shallow land-based oil resources may lay only 50 mbs (meters below surface), similar to the fields of North America. Well bottom temperatures may be little more than 40 °C, yet in the deepest North Sea oilfields, at 1.2 to 6 km, the temperature may reach 200 °C with pressures between 15 and 80 Mpa (14,15).

MICROBIOLOGY OF OIL-BEARING ROCKS

For the purposes of this review we have confined our discussion principally to deep reservoirs in excess of 1000 mbs. Although there is an active microflora associated with shallow reservoirs, in both land and offshore formations, the intrusion of organisms from normal soil and water environments blurs the distinction between the "regular" soil–subsurface micro flora and that which could be considered to be intrinsic to a hydrocarbon-bearing sedimentary deposit. Recent studies throughout the world have revealed the presence of many organisms in waters from oil-bearing sediments. Both sandstone and carbonate formations, some over 3-km deep, have yielded bacteria from France and North Africa (16), the North Sea (17,18), Alaska (19), Indonesia (20), Russia (21), offshore California (22), Vietnam (23), and Japan (24). The physiology of these organisms is usually found to be consistent with the original habitat in terms of salinity and temperature that increases with depth (i.e., they are well adapted to their subsurface environments). Great emphasis has been placed on the presence or absence of sulfate-reducing bacteria (SRB) in most sampling programs because they are the most prolific members of the oilfield community and of economic importance to oilfield operators. SRB, including some spore-formers, have been recovered from deep (2–4 km, 60–120 °C) North Sea oil formations, derived from situations, which have no connectivity with injected fluids used to improve oil recovery (17,18).

In a study published in 1996 (15), the question was addressed as to whether some of the bacterial species that were observed to grow in production-tubings and facilities on oil-production platforms were derived from the reservoir source. Nilsen and coworkers (15) concluded that the thermophilic and hyperthermophilic sulfate reducers that they recovered were indigenous to the reservoir and belong to a deep subterranean biosphere. If organisms are associated only with the production facilities, then fluid samples taken from different wells, supplying one production platform, should yield only one strain of a particular species. Their sampling technique included thorough flushing of the common collection point on the production platform with each successive sample to avoid cross-contamination from the separate wells supplying the platform. Using a combination of enrichment culture and genus-specific fluorescent antibody staining for a range of previously identified members of the production platform population, it was possible to confirm that different strains were derived from waters produced by different wells and that their sampling technique did not promote cross-contamination.

Bernard and coworkers (25) carried out a survey of indigenous microorganisms in connate waters from 24 oilfields, using direct microscopic counts (fluorescent acridine orange) and enrichment culture in a range of selective media to reveal that most of the organisms selected were previously not known. Each field ecosystem offered a limited diversity of species, which consistently contained associations of anaerobic, fermentative (*Halobacterioides*, *Thermatoga*, and unknown spore-formers) sulfate-reducing bacteria (*Desulfovibrio*, *Thermodesulfobacterium* and *Desulfotomaculum*), methanogenic bacteria (*Methanohalophilus*, *Methanobacterium*), and unknown thermophiles. The conclusion drawn was that each ecosystem was in equilibrium, perpetuating itself through symbiosis of a limited number of genera and species. They also argued that because there was no injected fluid breakthrough in the wells sampled, the organisms were indigenous to the formation at sample depth.

This conclusion is supported by many recent publications, demonstrating the presence of previously unknown organisms, both bacteria and archaea. Methods used ranged from total count and culture techniques, followed by characterization on the basis of nutrient requirements, temperature range and optimum, antibiotic susceptibilities, and 16S rRNA or rDNA analysis (23–26).

Grassia and coworkers (27) conducted a systematic survey for thermophilic fermentative bacteria and archaea in production waters from 36 high-temperature (50–148 °C) reservoirs across the world, from Australia and New Zealand, the Middle East, United Kingdom, United States, and Venezuela. Salinities varied from 2,800 to 200,000 mgL^{-1} and pH varied from 5.5 to 8.5. The organisms recovered were assigned to three morphologically distinct groups: (*1*) nonsheathed rod-shaped bacteria, (*2*) sheathed rod-shaped bacteria, and (*3*) irregular coccoid archaea. The occurrence of these groups of organisms was compared in nonwaterflooded and waterflooded petroleum reservoirs (see next section for discussion of waterflooding).

Group 1 bacteria were glycolytic thermophiles that fermented glucose to one or more of lactate, acetate, ethanol, hydrogen, and carbon dioxide. All grew principally in low-salinity reservoirs, and on the basis of morphology and physiology were allied with the genera *Thermoanaerobacter* and *Thermoanaerobacterium*. Spore formation was not observed.

The Group 2 glycolytic thermophiles had very distinctive morphologies; some were similar to *Fervidobacterium*, a genus reported to occur in deep and hot subsurface environments but not otherwise associated with oilfields. Other members of this group were seen to be physiologically and

morphologically related to members of the Thermatogales, (*Thermotoga*, *Geotoga*, *Petrotoga*, and *Thermosipho*). All but the last of these have been isolated from petroleum reservoirs elsewhere (19,24). The G+C ratios were in the range 31 to 40%, typical of these genera. They produced similar end products from glucose and were resistant to almost 70 gL^{-1} rifampicin.

Group 3 contained a single strain of an extremely thermophilic archaea, which resembled members of the genus *Thermococcus*. A coccoid cell, this organism can grow to almost 91 °C, using complex nutrients, such as yeast extract and peptone. Carbohydrates do not support growth. Members of this genus have been found in other petroleum reservoir studies (19).

Reservoir salinity did affect the type of organisms recovered in the course of the work by Grassia and coworkers (27); few thermophiles that were able to grow in salinities over 40,000 mgL^{-1} were isolated. The global distribution of organisms with similar characteristics was well demonstrated in this work, and those recovered were capable of tolerating reservoir conditions that apparently determined the types of organisms present. Sheathed members of groups 1 and 2 were present irrespective of waterflooding activity by oil field operators, suggesting that these organisms were truly indigenous to the environment.

Several new genera of SRB and archaea, which thrive in North Sea reservoirs (28–30), have been described because research has focused on hot, petroleum reservoirs as a source of deep indigenous subsurface organisms. These have been isolated from produced water samples by enrichment in a variety of media. Characterization has been made by techniques such as morphology, tolerance of temperature, and salinity ranges, carbon source utilization in the presence of sulfate, ability to grow autotrophically with hydrogen, use of alternative electron acceptors, DNA base composition, and phylogenetic comparisons of 16s rRNA. Oligonucleotide probes have been produced to target specific members of sample populations for rapid assessment of community composition (31). Voordouw and coworkers (32) developed the reverse-sample genome probe method to compare community diversity in oilfield SRB populations by hybridization assays.

The studies mentioned in the preceding sections have focused on analysis of bore-hole fluids obtained at production well heads with precautions against cross-contamination of water samples at common collection points (15). Other workers have obtained fresh core samples from exploration oil well–drilling operations in pristine environments and observed the organisms in situ (33,34). Stringent techniques are required to prevent contamination of inner rock core with organisms from drilling equipment, drill fluids, and transporting containers. Samples of sandstone core from 1000 mbsf on the United Kingdom Continental Shelf were prepared for scanning electron microscopy by fracturing, following repeated solvent extraction to remove hydrocarbons from the pore spaces. Structures resembling bacterial cells were observed in every field of view (33) in close association with the rock grain surface. Other portions of the same core were crushed using aseptic techniques and distributed in a range of media selective for sulfate-reducing bacteria and other anaerobes yielding cultures, which proved the presence of active populations in the oil-bearing rock.

MICROBIAL CONSEQUENCES OF OIL RESERVOIR EXPLOITATION

The stages from initial exploration for a new oilfield and conversion of the field to full production may take years. It involves many progressive activities, all of which affect the reservoir environment with consequences for both microflora and operators. Many unforeseen problems have been encountered during the development and productive phases of oilfields, some of which may be attributed solely to the activity of microorganisms either from the formation or introduced from other sources inseparable from the process. A brief description of the processes employed may assist readers unfamiliar with production practice. Broadly, following production well drilling, the hydrocarbon recovery processes may be divided into three phases:

1. *Primary production* occurs under the influence of reservoir pressure alone, because reservoir fluids take the path of least resistance to the production well. Fluids produced at the wellhead, during this phase contain a mixture of varying ratios of oil and formation waters. Over a period of time, the reservoir pressure is gradually reduced so the rate of production decreases. Migration of the oil toward the production well must be improved to restore flow rates.
2. *Secondary recovery* is often achieved by drilling strategically placed injection wells into which water is pumped. The injected water is derived from whatever source is convenient; seawater, inland lake water, aquifer water from a suitable subsurface location or when it is increasingly used, re-injection of waters separated from previously produced fluids. As long as the injection well has been correctly sited to direct flow, where required, and the crude oil characteristics are suitable, the water-flood front displaces oil to the production well. Analysis of the waters produced with oil will indicate the time when the water breakthrough has occurred, that is, when the injected waterflood front has broken through into the production region of the formation. Improvement of the secondary recovery technique can be achieved by modifying the injected fluids with a variety of polymers to improve the mobility ratio between oil and water; residual oil may have been bypassed and left behind in disconnected oil ganglia or trapped by capillary forces. The viscous-to-capillary force balance between the water and oil phases may be changed by altering the viscosity of the water phase through addition of polymers (35).
3. Even after primary depletion and secondary recovery methods have been employed, it is possible that much oil still remains in the reservoir. Depending on the geology, as much as 70% can be left untouched in a heterogeneous reservoir system. *Tertiary recovery*

methods to exploit this oil are varied but are usually aimed at improving mobility of what are often heavier classes of hydrocarbons, with high viscosity and strong affinity to the rock surfaces. Methods include injection of chemical surfactants, caustic solutions or foam, gas injection (either carbon dioxide or reinjection of natural gas), or thermal methods (steam soak and drive, or in situ combustion) to mobilize the oil. It is in this area of oil recovery operations that microbially enhanced oil recovery (MEOR) finds a place, although this is as yet a relatively under-utilized technology.

Physicochemical Effects of Hydrocarbon Extraction on Reservoirs

Pressure. The drilling of an exploration well into a potential oil reservoir intrudes into an otherwise pristine environment, which has evolved in the absence of external influences from any source other than natural percolation of fluids in the porous rock matrices or exchange via aquifer sources. The immediate result of puncturing the surrounding impervious layers confining hydrocarbon reserves is a pressure release because gas, oil, and associated fluids seek to escape (primary production). In the early days of oil exploration this would often lead to rapid expulsion of some of the reservoir contents (a "gusher"), requiring considerable skill to control and cap off. Present day well-drilling equipment is fitted with blowout prevention valves. Nevertheless, the immediate effect in the near well borewell bore area is a perturbation of established equilibria because a pressure differential is created. The tendency will be for contents of the pore matrix to move toward the well bore when the advancing drill bit breaks and removes rock chips to the surface via circulating drill fluids.

Drilling fluids or mud are frequently highly saline and typically consist of water or organic phase emulsions, continuously circulated through the drill string where they transport drill cuttings to the surface. They also function as lubricating coolants for the drill bit. The pressure applied to inject the mud into the drill pipe contributes to controlling the down-hole pressure changes and assists in stabilizing the well bore. Drilling fluids return to the surface via the annular gap between well bore and drill pipe where they are separated from the cuttings before reuse, thus providing a continuous cycling of displaced material throughout the length of the well bore.

It is common for exploration wells to be drilled several years before any production commences at the site. In this case the well may be capped off before further work continues. Any fluids shut into the well are therefore in contact with bacteria indigenous to rock penetrated by the drill.

Temperature. Temperature gradients established in oil fields before exploitation are altered as a result of hydrocarbon extraction. Movement of the gaseous and fluid reserves from storage zones to producing wells initiates mixing in the reservoir. As primary production rates slacken and secondary recovery methods are applied, further changes are imposed. Cooling of the reservoir may come about as a result of injection of water to assist in secondary recovery. This is particularly evident in the deep oil reservoirs underlying the North Sea. The injected waters may be at 8 to 10 °C and enter a reservoir with a temperature of more than 100 °C. Thermal cracking is frequently caused by cold water injection into hot rocks. This results in substantial alterations in the sweep efficiency of the water flood, which may or may not be to the operator's advantage.

Temperature effects are less extreme in the reservoirs of the Gulf of Mexico as the differential between the reservoir and injected seawater is less marked. The rate of heat transfer in a reservoir is dependent on injection rate and rock characteristics, which vary from field to field. In general, however, cooling occurs at the injection well bore and the cooled zone extends gradually with the flood front. Where rock is highly fractured and connectivity between injection and production wells is established, a scenario emerges where the passage of injected water creates a cold finger through the reservoir.

Alongside the direct effect on reservoir temperatures, the cooling can also promote precipitation and deposition of scale in the reservoir. Mineral solubility in formation water is reduced as temperature and pressure decrease with increasing hydrocarbon production, resulting in precipitation of carbonates.

Water Chemistry. As formation of insoluble carbonate scale is principally a result of temperature and pressure reduction, sulfate scales are a frequent consequence of incompatibility between seawater and reservoir formation water chemistries (36). Seawater injection introduces a high level of sulfate ions (about 2,800 mgL^{-1}) with small amounts of calcium (Ca^{2+}, 420 mgL^{-1}) and traces of barium and strontium ions (Ba^{2+} and Sr^{2+}) to the reservoir. The original reservoir formation water may have traces of sulfate SO_4^{2-}, up to 3000 mgL^{-1} Ba^{2+} and or Sr^{2+} and tens of thousands mgL^{-1} of Ca^{2+}. The salinity of both waters classifies them as brines, and both are stable under normal oil field conditions when kept apart. Comingling of these brines in various locations of the reservoir and production systems results in deposition of sulfate scales of barite ($BaSO_4$), celestite ($SrSO_4$), or gypsum / anhydrite ($CaSO_4$) in North Sea reservoirs. Water chemistries vary from reservoir to reservoir and field to field, requiring constant monitoring and analysis by reservoir engineers to maintain efficient production.

Reservoir Chemical Treatments. Further chemical modification of the reservoir environment results from the injection of a comprehensive array of treatment chemicals such as chemical biocides, corrosion and scale inhibitors, oxygen scavengers, sulfide scavengers, emulsifiers, polymers, and surfactants. Some treatments are chemical combatants deployed to solve a chemical problem. For example, the majority of injected waters are deaerated before entry to the reservoir, as addition of oxygen to the otherwise hot, saline, and anaerobic system promotes anodic corrosion of the ferrous metals used in construction of the process equipment (37). Final traces of oxygen are eliminated by incorporation of reducing agents such as

ammonium bisulfite in the injected fluid to serve as an oxygen scavenger. However, the use of chemical biocides, and to a great extent hydrogen sulfide scavengers, is a direct response to the problems caused by active communities of bacteria throughout the system.

Reservoir Souring and Plugging

The combined effects of the physical and chemical changes imposed on hydrocarbon reservoirs are complex. All have an effect on the deep subsurface environment that was described earlier and are likely to affect the activity of both the indigenous microbial population and newly introduced organisms.

It is indisputable that microbial contamination by drilling operations occurs. For the purposes of microbiological sampling programs, lengthy methods are employed to distinguish native from introduced bacteria (38,39), which are not applicable in an industrial arena. Nonindigenous organisms are introduced to the environment as soon as the exploratory drill bit penetrates an oil-bearing horizon. Material from the overlying rock is carried with the advancing drill string, drill cuttings mixed with drilling fluids transport resident bacteria from one stratum to another. In the same way, subsequent drilling of production and injection wells contribute to further mixing of the contents of otherwise separate strata. Any fluids shut into the well following evaluation for production purposes are therefore in contact with a mixed population of bacteria.

Degradable components of drilling mud, particularly the diesel grade hydrocarbons used in oil-based mud are easily utilised by the microflora, in spite of the incorporation of preservative biocides. The biocides may be added only at a level to preserve the product until deployment in drilling operations. Dilution of the biocide will occur once the drilling fluid is in the rock formation, and thermal degradation may occur subsequently. The implications of this are that on reopening an exploration well or drilling in close proximity to an existing well there may be a detrimental and numerically enhanced microflora in place, which has already been exposed to typical oilfield biocides. These include oxidants, such as chlorine or ozone, cross-linking, nonoxidant aldehydes, or membrane-active quaternary ammonium compounds (40).

Alteration of the temperature profile within a reservoir has a profound effect on the activity of the microbial community, especially at the injection well bore. This is an area where microorganisms introduced from overlying strata and injected fluids, growing optimally at lower temperatures than the bulk of the reservoir may become well established in relatively narrow zones along the injection flow path. Similarly at the production wellhead, a microflora tolerant of intermediate temperatures becomes established.

Sulfate-Reducing Bacteria (SRB)

Discussion in the preceding sections has not dwelt on those members of the subsurface reservoir microbial community, which are acknowledged to be the principal problem for oilfield operators, namely the diverse assembly of sulfate-reducing bacteria. Postgate (41) describes SRB as having numerous roles in the context of oil and gas technology, both as indigenous inhabitants of waters in oil-bearing formations and colonizers of surfaces throughout the process. It has already been shown that these bacteria are prevalent in samples of reservoir fluids obtained from all depths, temperatures, and geologies.

SRB are opportunistic organisms implicated in economically deleterious activities throughout the industrial arena. Probably, the most important influence they have occurs throughout the oil and gas industry, where SRB biofilms are the tenacious and resilient focus for the corrosion of production and pipeline equipment, while the principal reservoir problems directly attributable to SRB are sulfide 'souring' and formation plugging. Almost every aspect of oil recovery can be hindered by the ubiquitous and opportunistic assemblage of sulfidogenic microorganisms (42).

The hydrogen sulfide generated by SRB is a serious problem for operators, whether from corroding biofilms in separator tanks or issuing from the wellhead in production gases and fluids. In the reservoir, hydrogen sulfide is soluble in crude oils, highly volatile and partitions into the natural gas phase. Concentrated or generated within the production facility, it poses a serious health risk to platform workers, being a highly toxic respiratory inhibitor. Crude oil and natural gas containing excessive amounts of sulfide must be treated before refining to reduce emissions of toxic sulfur products to the atmosphere. Regulatory mechanisms to ensure that no operator produces excessively "sour" products bring high cost implications to bear, for many operators.

A physical problem resulting from microbial activity in porous rock matrices is one of biomass. The influx of suitable nutrients and electron acceptors with injected waters and advancing flood fronts deeper in the reservoir results in active growth of bacteria attached to the rock surfaces. Biofilm development, eventually, bridges individual pore throats, blocking water access into the deeper reservoir and can ultimately result in loss of injectivity at the well bore and diversion of directed waterflood operations away from the desired zone. Extensive pore-blocking within one zone of a reservoir can result in substantial loss of production because mobile reserves of oil are protected from the waterflood front designed to sweep crude oil from the formation to the production well. Unchecked, the flow diversion could so reduce production efficiency of the reservoir that the operator is forced to abandon the field.

The link between water injection and reservoir souring has been demonstrated many times in the North Sea oil fields (43). Oilfields are occasionally sulfide sour from the start, in which case the sulfide may be thermochemical in origin, the result of earlier bacterial activity during maturation processes or thermal decomposition of sulfur compounds in the hydrocarbon reservoir (44). Following the commencement of water injection, sulfide souring can escalate rapidly because zones near well bore cool, allowing growth of a wider range of bacteria. Nutritional requirements of some organisms are satisfied by the influx of additional carbon sources, which are found in varying concentrations in injection waters. Away from

the immediate well bore area, temperatures are higher and here the indigenous micro flora is likely to be in a dormant condition until production operations promote the mobilization of hydrocarbons and associated waters, thus promoting microbial activity.

Survival of microorganisms in the natural environment is of great interest to the scientific community, particularly in relation to the subsurface (45). The deep subsurface is not accessible for in situ observation; therefore, any prediction of the activity of organisms must be based on simulated laboratory experimentation. Suspensions of dormant and actively growing thermophilic SRB (tSRB), derived from North Sea production fluids and exposed to pressures of 1000 ψ and 150 °C for up to 24 hours, were checked for metabolic activity and growth. A higher proportion of the dormant cells were capable of metabolic activity following this treatment (46). tSRB can survive conditions of temperature simulating both prolonged nutrient depletion in deep hot reservoirs and also cold aerated conditions for up to 21 months (47). These two different conditions are encountered in the North Sea oil industry when produced waters are either ejected overboard after separation from the oil production or mixed with seawater before reinjection into the reservoir for secondary recovery operations. Cells were seen to undergo a reduction in size when deprived of carbon sources. Following restoration of suitable electron acceptors and donors, it was possible to demonstrate metabolic activity in the tSRB populations and some growth.

Consortia of reservoir, derived thermophilic sulfidogens, inoculated into clear growth media in a series of vials containing different amounts of sand, produced significantly more sulfide, when there was more sand available for colonization (48). Planktonic tSRB passed through sand pod columns or sandstone cores attach to surfaces where they grow and develop biofilm under conditions, which simulate reservoir temperature, pressure, and water chemistry (49–51). It is reasonable to suppose therefore that there is active growth not only in the near well bore areas of oilfield reservoirs but also deeper in the formations when conditions are suitable. It is considered that most bacterial activity will occur in the mixing zone between the reservoir formation waters and the advancing waterflood front and subside once the waters have been depleted of utilizable components by the bacteria. Sulfide is more soluble in oil than the water phase and is often pushed ahead of the advancing waterflood to the production well head, some time in advance of injection water breakthrough.

The emphasis for oil field operators in recent years has been to learn new techniques for reducing deleterious bacterial activity. Most particularly, the early prediction of potential souring problems is important in order that suitable treatments can be applied in the near well bore area either before bacteria are forced deeper into the reservoir or before the water mixing zone contacts populations further from the injection well. One of the greatest problems facing reservoir engineers is how to predict the location, activity and potential for transmission of bacteria through reservoirs, given that it is unlikely to be possible to access the moving water mixing zone with biocides, once souring has become established. Laboratory core-flood experiments have provided useful information on the rate of transport of reservoir bacteria in porous matrices simulating the formation rocks. For example, it has been concluded that in rocks of moderate to high porosity, single planktonic cells of tSRB will spread at the same velocity as the advancing waterflood front (51). However, this does not aid prediction of the fate of large portions of biofilm which may detach from rock surfaces for a variety of reasons, including change in fluid flow, temperature, application of surface active agents or biocides. There is little information on the activity of cells in detached portions of biofilm. It is not known whether the cells remain in contact with one another following detachment, and if so, do constituent members of a detached agglomeration of cells enjoy a selective advantage over single cells shed to the planktonic phase from the parent biofilm.

Core flood experiments can give indirect information on the activity of the bacterial population in place by monitoring parameters relating to pressure changes, fluid flow rates and metabolic products, but to directly observe the biofilm attached to the rock, destructive sampling is necessary. New research techniques that use image analysis are evolving, and this enables direct observation of cell attachment, biofilm development and detachment. There are many factors influencing a range of control parameters that will contribute to a greater understanding of the behavior of bacterial populations in many environmental situations, including the porous matrices of oil field reservoirs (52).

Microbially Influenced Corrosion

We include a brief reference to microbially influenced corrosion (MIC) because it is directly related to the presence and activity of SRB, which are the principal cause of problems for oil field operators. In the oil and gas industry, corrosion of production "hardware," such as pipeline and other tubings, pump equipment, storage or separation tanks and hydrocyclones is invariably found to be associated with heavy surface colonization by SRB. Hundreds of millions of dollars every year are channelled by the oil and gas industry into efforts to reduce the effects of bacterially mediated corrosion of carbon steel (37,40). The problems arise not only in the "upstream" production equipment but also in "downstream" refined products storage and transport. In any setting where a water layer develops in contact with steels (inside or outside pipelines), either through condensation or washing processes, and most particularly when any period of stagnation and deaeration is intrinsic to a procedure, the risk of corrosion is high.

The sources of SRB contributing to the corrosion damage of oilfield-processing equipment are many. Although populations of SRB have been identified from many different deep subsurface environments (38,53) and also oil-bearing reservoirs (33,34,43,46–51), they are also ubiquitous throughout aqueous and surface environments (41,54). It is certain that many of the sulfate-reducing bacteria retrieved from corroding production

equipment on oil or gas production rigs will have been derived directly from the same deep subsurface environment as the production fluids themselves. Other SRBs will have contaminated the structure through general contact with the environment and established corrosive biofilms wherever conditions are conducive to their proliferation (55).

APPLICATION OF MICROBIAL TECHNOLOGY FOR EXPLOITATION OF PETROLEUM RESERVOIRS

Background

Petroleum microbiologists have endeavored for over 50 years to develop technology suitable for improving the recovery of hydrocarbon resources, using either microorganisms directly or the products of their activity. A variety of techniques have been attempted and with varying levels of success.

The microbial mechanisms with potential for improving oil recovery have probably been well defined in ZoBell's 1946 U.S. patent (56). He listed six different activities that have the potential to increase mobility of recalcitrant hydrocarbon deposits:

1. solubilization of calcareous material,
2. dissolution of sulfate minerals to increase permeability,
3. generation of gases to repressurize the reservoir and improve oil migration from rock pores,
4. growth of biofilm on rock pore surfaces to physically displace oil,
5. production of biosurfactants, and
6. reduction of oil viscosity by direct modification of oil or by solution of produced gases.

ZoBell had explored the idea of using sulfate-reducing bacteria because he saw that their activity fulfilled several of the qualifications in his list. Much of his research was based on results he obtained from shallow land wells, where injected water for secondary waterflooding was low in sulfate, and therefore the immediate problem of excessive sulfate reduction may not have been apparent in relatively small fields with low output (40). However the attendant disadvantages of hydrogen sulfide production coupled with corrosion, sour reservoirs, and iron sulfide plugging are now clearly recognized to outweigh the benefit of employing SRB. Nonetheless, ZoBell's original list of bacteriological functions as potentially valuable tools still deserves a place in any discussion of microbially enhanced oil recovery (MEOR).

A later patent, filed by Updegraff in 1957 (57) used the ability of the clostridial anaerobes and molasses to produce large amounts of acid and gas to aid oil recovery. Similar treatments using injection of cheap carbon sources, such as molasses alone, to stimulate indigenous populations were also tested. Field trials in Eastern European oil producing regions were applied to single wells, which were shut in after the treatment to allow growth, the cleaning effect of acids, and repressurization with gas to take effect. Improved oil recovery was generally observed, and this treatment is still applied in small-scale recovery operations.

Competitive inhibition of SRB or bacterial scavenging of hydrogen sulfide by injection of mixed communities of selected organisms is another approach that has been considered. Data were published from a small-scale field trial resulting in the replacement of the regular biocide and corrosion inhibitor dosing with a mixed community of naturally occurring select microorganisms and a suitable nutrient (58). Injectivity of the well was not compromised nor was oil production affected.

Microbial treatment of the larger fields that were developing throughout the world during the middle decades of the twentieth century was less easy to control. The direct benefits of similar treatments were difficult to justify, largely because the reservoir characterization was inadequate and the locations of waterflood fronts were difficult to identify with sufficient confidence.

Current Status

MEOR has still to find favored status in major oilfield operations. It has been applied on a small scale by comparison with more traditional reservoir treatment technologies, such as biocide dosing, sulfide scavenging, application of emulsifiers, scale inhibitors, and so on. However, these traditional methods are finding less favor with environmental control agencies whose global responsibility is to reduce the amount of potentially toxic and damaging pollutants in the environment.

The problem of pore plugging, loss of injectivity, and formation blockage during secondary recovery operations was recognized to be because of the detrimental bacterial activity. During the 1980s, researchers began to consider using the ability of bacterial cells and biofilm to divert flow as a tool to access residual and bypassed oil (59). This was seen as an important adjunct to secondary water- flooding techniques, which perform well until water breakthrough occurs. A water flood may leave behind oil, either because it is trapped by capillary forces in disconnected oil ganglia (residual oil) or because the flood front takes a path through the heterogeneous rock, which bypasses the oil. The watered route is called a "*thief*" *zone* and is of higher permeability than the surrounding rock, which still contains recoverable oil (35).

Conventional treatment for these problems is to employ additional chemicals in the water flood. Operators recover residual oil by use of surfactants to reduce interfacial tension between trapped oil and the aqueous phase. The remedy for recovering oil bypassed because of large-scale rock heterogeneity is addition of polymer to the water. This increases the viscosity of the water and effectively reduces the mobility ratio between the oil and water, thus remobilizing the oil by hydraulic pressure transfer in the pores between rock strata of different permeabilities (35). The polymers used are either synthetic polyacrylates or bacterially generated extracellular xanthans (this can be considered to be a form of MEOR). However, both these polymer types are degraded by a combination of reservoir conditions and metabolic activity of indigenous microflora and become ineffective. Data from sand pack and core flood experiments described the addition of bacteria to

a simulated waterflood, either in a starved state as ultra microbacteria, thus aiding deeper dispersion in the matrix, or as vegetative cells. Emplacement of the bacteria was followed by addition of nutrients, promoting growth and biofilm development of the injected bacteria (60,61). In a reservoir, diversion of the waterflood into less permeable zones of the surrounding matrix could be brought about and this would achieve the purpose of sweeping bypassed oil to the production well head.

An alternative approach has been suggested (62), which involves selection of bacteria from oilfield-produced waters. Following enrichment culture, starved cell suspensions would be prepared before reinjecting them to the reservoir. The starved bacteria would be dispersed with the waterflood to highly permeable reservoir zones, to await the arrival of a nutrient subsequently injected with the waterflood. The starved cells would then resuscitate, grow and plug the thief zones, thus promoting beneficial flow diversion.

Operators have been reluctant to attempt large field trials for flow diversion techniques, for the reason that a poorly located bacterial treatment could cause irreparable reservoir damage, costing lost production. There is still insufficient knowledge of the deeper reservoir geologies and flow patterns to be sure that the treatment will reach its intended destination. Computer models of reservoir flow characteristics have yet to include variables accounting for the movement and transport of bacteria in a reservoir.

Currently, the most promising route to the use of bacteria for improving oil recovery is the one that does not necessarily result in increased oil production. Returning to the detrimental effects of SRB in oil field reservoirs and their generation of hydrogen sulfide, more research effort is being devoted to competitive bacterial activity designed to suppress the effects of SRB growth. Pilot scale treatments of fields with *Thiobacillus denitrificans*, which oxidizes inorganic sulfides, have demonstrated a successful bioaugmentation technology for the treatment of sulfide-laden produced waters (63), particularly with nitrate addition (64). Sulfide levels were reduced by almost up to 92%, when the faster growing nitrate-reducing bacteria consumed available carbon sources more rapidly than SRB.

Future areas where MEOR techniques could develop include application of in situ anaerobic microbial desulfurization of heavy crude oils in South American oilfields alongside anaerobic degradation of bitumens and tars. These recalcitrant fractions of the global hydrocarbon complement are still largely in place in reservoirs throughout the world.

CONCLUSION

No deep subsurface environments have been more disturbed than those yielding hydrocarbons to fuel global energy requirements. The results of this exploitation have been instructive for oil field operators, who were unprepared for the consequences of their intrusion. The microbiological revelations, which have come to light, will surely yield new insights into the molecular origins of life and offer new technological solutions for the future.

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PETROLEUM RESERVOIRS, MICROBIAL DIVERSITY IN

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Because of the high temperatures and salinity gradients, oil reservoirs were long thought to be sterile environments, intolerable to life. However, soon after the advent of commercial oil production more than 140 years ago, workers noticed oil souring, that is, an increase in hydrogen sulfide. Hydrogen sulfide, which is toxic, damages production by reducing the value of the oil and degrading the drilling equipment. In an effort to explore whether these problems stemmed from biological processes, Bastin undertook the first microbiological study of oil wells. In 1926, Bastin reported the presence of sulfate-reducing microbes in oil reservoirs and questioned whether these microbes were autochthonous or introduced into the subsurface by drilling operations (1). This issue is still unresolved today. Within the last 25 years, numerous studies have been conducted to determine how microbes affect the quality of petroleum production, specifically their role in the biodegradation of oil and drilling equipment, as well as their potential to increase petroleum yields.

GEOPHYSICAL AND GEOCHEMICAL CHARACTERISTICS OF OIL RESERVOIRS

In commercial petroleum wells, water is usually extracted along with the oil. Microbes have been found in the aqueous phase of produced fluids and associated with oil (Fig. 1). The geochemistry of the reservoir, mineralogy, and the water determines the types of microbes found in reservoirs. Although the exact composition varies among petroleum reservoirs, the wells are generally anoxic. The gases usually found are carbon dioxide, methane, ethane,

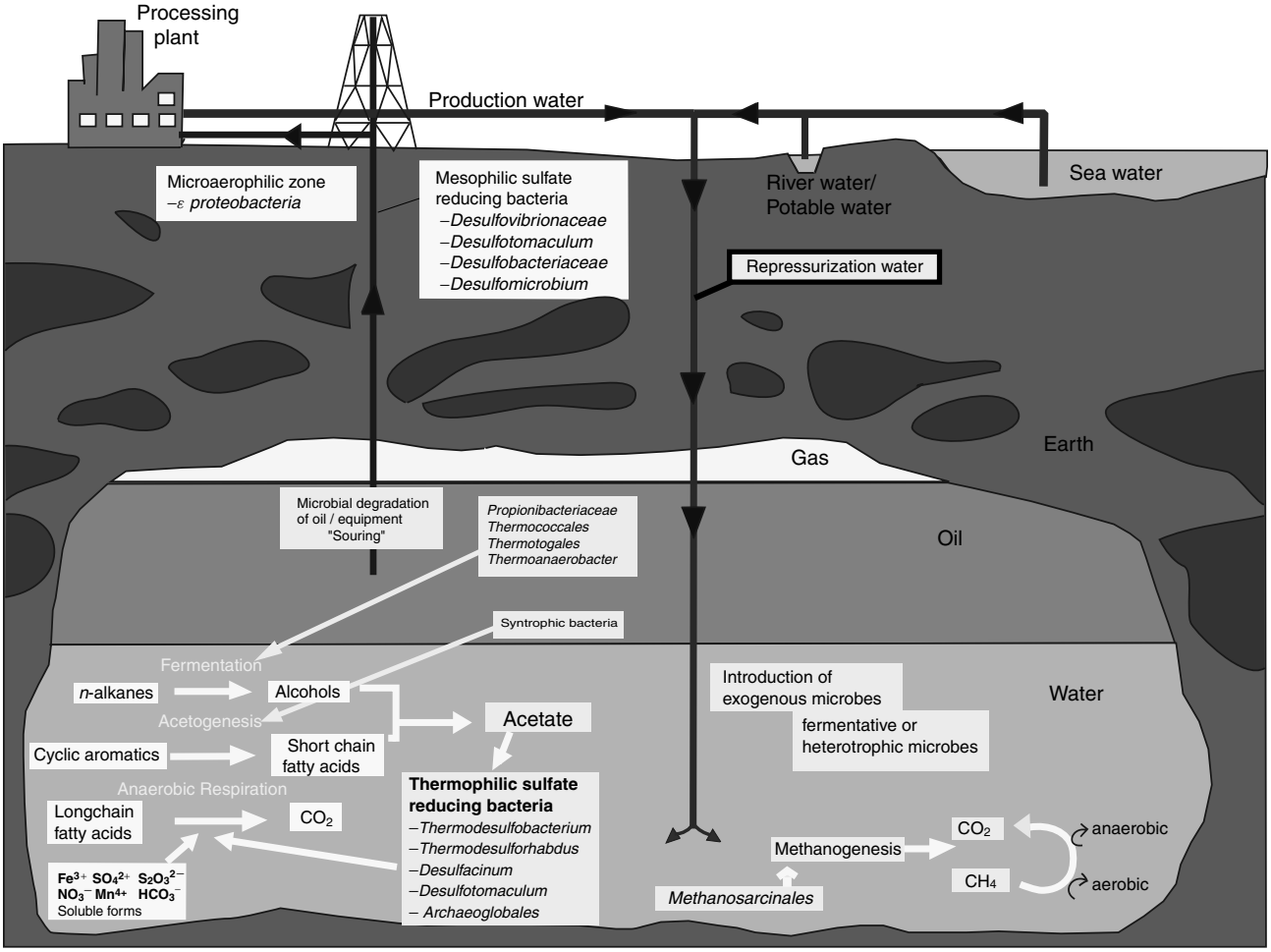

Figure 1. Cross section of a petroleum reservoir.

propane, butane, isobutane, and hydrogen sulfide. Oxygen may be introduced into the reservoirs by near-surface groundwater or via injection of seawater during secondary extraction of the oil. Oil companies do not measure the level of hydrogen; therefore, little information is available. The in situ pH ranges from 3 to 7.

In addition to pH, temperature and salinity of the reservoirs are considered the major factors that influence microbial activity. Temperature is a function of depth, with an average rate of increase of 3 °C per 100 m; the highest temperatures recorded from petroleum reservoirs are 130 to 150 °C. The salinity of the formation water is influenced by the surrounding rock layers and can range from freshwater concentrations to salt saturated. In addition, pressure also depends on depth and can reach up to 500 atm. Pressure may influence the physiological and metabolic properties of microbes in the reservoirs, but it is not believed to inhibit microbial activity in situ (2).

The chemical composition of reservoirs influences the types of metabolic processes that can occur, and hence the kinds of microbes that are able to grow in these environments. Routine chemical analyses performed by petroleum companies has revealed that the redox potential of the production waters is extremely low and that electron acceptors, such as oxygen and nitrate are generally absent. Sulfates, carbonates, and iron oxides, which are generally present in stratal waters, probably, support a diverse community of microorganisms. The primary electron donor is most likely H_2, derived from abiotic and biological sources. For example, H_2 is widespread in the deep subsurface and can serve as an electron donor for sulfate-reducers, methanogens, acetogens, and iron-reducers. Under certain environmental conditions, H_2 is produced through a chemical reaction between water and ferrous silicate minerals found in basalt (3). In addition, organic acids such as acetate, formate, propionate, and butyrate have been detected in many, but not all, petroleum reservoirs. In some wells, more complex organic acids have also been detected. Aerobic degradation of many of the organic molecules present in the petroleum reservoirs such as n-alkanes and alkylbenzenes has long been recognized; however, microbial, anaerobic degradation of complex organic acids has only been demonstrated within the last 20 years (4,5). Both aerobic and anaerobic microbial degradation of the hydrocarbons may contribute to oil decomposition (6,7).

Data concerning the presence of crucial biosynthetic elements, such as nitrogen and phosphorus, is either

limited or absent. If present, N_2 could be utilized by nitrogen-fixing bacteria. Also, resins and asphaltenes, significant components of crude oil, are made up of complex hydrocarbons that can contain nitrogen, sulfur, and oxygen (2), providing potential nitrogen, carbon, and energy sources for microbes.

MICROBIAL DIVERSITY OF PETROLEUM RESERVOIRS

Numerous mesophilic and thermophilic microbes have been isolated from oil reservoirs. However, it is estimated that as much as 99% of a microbial community is not easily cultivated; therefore, molecular phylogenetic-based approaches that do not rely on cultivating microbes have recently been used to assess the microbial diversity of oil reservoirs (8). By collecting samples from the environment, extracting total DNA, and then selectively amplifying the evolutionarily conserved small subunit (16S) ribosomal RNA (rRNA), environmental rRNA gene sequences can be obtained and compared to known sequences. These "phylotypes" can then be placed in a phylogenetic framework. Although sulfate-reducers have been the primary focus of microbial research at petroleum mines because of their damaging effects on industrial petroleum production, many other mesophilic and thermophilic microbes belonging to both domains *Archaea* and *Bacteria* have been classified by this approach.

Colonization of Allochthonous Microbes in Petroleum Reservoirs

To recover samples that would only contain truly indigenous microbes, the core material must be obtained aseptically; however, due to economical constraints, such samples are rarely available (9). To date, wellhead sampling is the sole means of sample collection. As a result, exogenous microbes can be introduced into the reservoirs through several channels and, consequently, take advantage of the energy sources present in (and introduced into) the reservoirs. For example, ground or surface water is often injected into the reservoirs to maintain pressure and improve oil recovery; this repressurized water can be of many origins such as seawater, river water, or production water. Allochthonous microbes can also be introduced into the reservoirs via drilling equipment. Moreover, the drilling equipment acts as a cathode that can generate H_2 which, in turn, can serve as an electron donor (2). Microbes multiply in the wells, and their metabolic by-products accelerate the degradation of the equipment.

Careful analysis of the data from petroleum reservoirs is crucial to distinguish indigenous microbes from exogenous ones. Several studies have developed useful criteria to aid in recognizing indigenous microbes. For instance, although temperatures in reservoirs have surpassed 100 °C, in situ oil biodegradation has not been observed above 82 °C (6). Furthermore, analysis of 87 water samples from North American oil reservoirs showed that microbial fatty acid concentrations were at a maximum at 80 °C in the reservoir (2,10,11); thus, the temperature limit for microbial life in petroleum reservoirs has been estimated to be between 80 and 90 °C. Thermophilic and hyperthermophilic microbes that grow at temperatures above 90 °C have been isolated from oil reservoirs (12,13); however, the ecology of subterranean hyperthermophiles is not completely understood, and these microbes may or may not be endemic to the reservoir environment. It is possible that these organisms originally are derived from hydrothermal vents and that they have been introduced into the reservoirs via injected seawater.

If the physiological characteristics of the recovered microbes correlate with the environment from which they were isolated, it is likely that they are autochthonous to the reservoir. Interpretation of the data is simpler when samples are obtained from high-temperature or high-salinity environments than it is when low-temperature, low-salt samples are retrieved. Because existing data implies that only strict anaerobes should be considered autochthonous to oilfields, the presence of aerobic microorganisms is used as an indicator of contamination.

MICROBES FROM OIL RESERVOIRS

Diversity of microbes in petroleum reservoirs has been gleaned through culturing and molecular phylogenetic approaches. Both mesophilic bacteria that grow optimally between 25 and 40 °C and thermophilic bacteria that grow best at temperatures between 45 and 80 °C have been cultivated from petroleum reservoirs. Within these physiological groups, aerobic, microaerophilic, and anaerobic organisms from reservoirs have been isolated or identified. The detection of closely related anaerobic bacteria in separate oil fields (14,15) supports the existence of a widespread anaerobic biosphere in these habitats.

The following sections provide an overview of distinct types of microbes identified and cultivated from petroleum reservoirs and their distinguishing metabolic processes. However, microbes do not exist as independent entities, rather their metabolic processes are intertwined and may be beneficial or inhibitory to the other members of the reservoir community. For example, the anaerobic breakdown of hydrocarbons may be the result of acetogens, methanogens, and sulfate-reducers. Each type of organism catalyzes a distinct reaction that ultimately leads to the formation of CH_4 and CO_2 (16).

Sulfate-Reducing Microbes

The majority of microbial research on petroleum reservoirs has focused on the identification and isolation of sulfate-reducers because of their detrimental effects on the economy of the oil industry (8). Moreover, hydrogen sulfide can be dangerous to the health of petroleum workers: at high concentrations, it can cause suffocation. Table 1 provides a compilation of sulfate-reducing microorganisms cultivated from petroleum reservoirs (2,17–24). These strict anaerobes, belonging to both the archaeal and bacterial domains, use sulfate as their primary electron acceptor, reducing it to hydrogen sulfide. Whether contaminants or indigenous, sulfate-reducers seem to be ubiquitous in oil wells, growing over a range of

Table 1. Microbes Isolated from Petroleum Reservoirs

Sulfate-reducers	Methanogens	Iron-reducers
Desulfovibrio sp.	*Methanobacterium* sp.$^{\Psi}$	*Deferribacter thermophilus**
Desulfotomaculum halophilum	*Methanocalculus halotolerans*$^{\Psi}$	*Thermotoga* sp.*
Desulfomicrobium apsheronum	*Methanohalophilus euhalobius*$^{\Psi}$	*Thermoanaerobacter* sp.*
Desulfobacter sp.	*Methanoplanus petrolearius*$^{\Psi}$	*Thermococcus* sp.$^{*\Psi}$
Desulfobulbus rhabdoformis	*Methanosarcina mazei*$^{\Psi}$	
Desulfobacterium cetonicum	*Methanosarcina siciliae*$^{\Psi}$	
Desulfotomaculum sp.*	*Methanoculleus* sp.$^{\Psi}$	
Desulfacinum infernum*	*Methanobacterium* sp.$^{*\Psi}$	
Thermodesulforabdus norvegicus*	*Methanococcus thermolithotrophicus*$^{*\Psi}$	
Thermodesulfobacterium mobile*		
Archaeoglobus sp.$^{*\Psi}$		

*denotes thermophilic microbes
$^{\Psi}$denotes members of the Archaea

temperatures and salt concentrations. The mesophilic sulfate-reducing microbes are involved in the corrosion of top well facilities, whereas the thermophilic sulfate-reducers appear to be involved in oil deterioration in situ (2).

Methanogens

Belonging exclusively to the domain *Archaea*, methanogens are strict anaerobes that produce methane through the concomitant reduction of CO_2 and oxidation of H_2. Certain methanogens are capable of utilizing other C1 compounds such as methylamine (CH_3NH_2), methanol (CH_3OH), and dimethyl sulfides ($(CH_3)_2S$) (25,26). Moreover, acetate (CH_3COO^-), which is present at very high concentrations in some reservoirs, may also serve as a substrate for acetoclastic methanogens, either directly or syntrophically from acetogenic organisms. Mesophilic and thermophilic methanogens that reside in slight to highly saline reservoir waters have only recently been reported (Table 1) (27–30). Hyperthermophilic methanogens — organisms that have a growth optimum of 80°C or higher — have not been cultivated from petroleum reservoirs; however, molecular phylogenetic analysis has detected phylotypes closely related to the hyperthermophilic *Methanococcus infernus* in a deep oil reservoir from Western Siberia (31).

In addition to SRB, methanogens may contribute to oil degradation. Recent research suggests that methanogens are involved in the anaerobic breakdown of long-chain hydrocarbons to methane. Whether methanogens catalyze this reaction alone or if they are part of a syntrophic community consisting of SRB and acetogens has not been fully assessed (16,32).

Fermentative Microbes

Fermentation is an anaerobic process through which microbes produce ATP. Organic compounds serve as both the primary electron donor and ultimate electron acceptor, and energy is produced by substrate-level phosphorylation (25). Organic acids that are present in oil reservoirs are potential substrates for both the mesophilic and thermophilic bacteria that are integral inhabitants of petroleum reservoirs (33).

Mesophilic Fermentative Bacteria. Mesophilic fermentative bacteria isolated from oil reservoirs are of industrial interest for microbially enhanced oil recovery (MEOR). These include anaerobic, moderate halophiles belonging to the genus *Haloanaerobium*. Increased petroleum yields are delivered as an indirect result of their metabolic products produced during growth (34). Other mesophilic, fermentative bacteria such as *Spirochaeta smaragdinae* (35), *Fusibacter paucivorans* (36), and *Dethiosulfovibrio* (37) have also been isolated. Molecular phylogenetic analysis of 16S rRNA genes from the enrichment samples have led to the detection of potentially strict anaerobic or facultative microbes associated with the genera *Clostridium, Eubacterium*, and *Synergistes* (38).

Thermophilic Fermentative Microbes. As most petroleum wells are characterized by high temperatures, thermophilic, fermentative bacteria have been isolated more often than their mesophilic counterparts (2). The thermophilic bacteria are generally of the order *Thermotogales*, namely, *Thermotoga*, *Thermosipho*, *Geotoga*, and *Petrotoga* (2,8,13,39,40). In addition to the sulfate-reducing microbes, *Thermotoga* may also play an important role in oil degradation by reducing sulfur and/or thiosulfate to sulfide (41,42). Sulfur reduction occurs among members of *Thermotoga* as a detoxification mechanism when the fermentation by-product, H_2, is oxidized. Evidence exists that may implicate thiosulfate-reducing microbes in the biodegradation of top well facilities (2,37), but the concentration of thiosulfate in oil reservoirs has not been studied extensively.

Other fermentative bacteria have also been isolated from petroleum reservoirs that may contribute to oil biodegradation. For example, the family *Thermoanaerobiaceae* includes members that are capable of reducing thiosulfate to elemental sulfur (*Thermoanaerobacterium*) or to sulfide (*Thermoanaerobacter*) (8,14). Additionally, the organism *Anaerobaculum thermoterrenum* is capable of reducing sulfur and thiosulfate to sulfide (33).

Fermentative microbes belonging to the domain *Archaea* have also been identified from petroleum reservoirs (2,8,12,13,43). They belong to the genera *Thermococcus* and *Pyrococcus*. That these organisms are endemic to the reservoirs has been questioned

(12,13,43), but their ability to reduce sulfur at elevated temperatures makes these organisms potential candidates in the biodegradation of oil (14,15,44). In fact, it has been postulated that these types of microbes may be the major source of hydrogen sulfide production, rather than the SRB (8).

Acetogenic Microbes. Acetogens produce acetate from CO_2 and H_2. In addition to the use of H_2 and CO_2, acetogens can utilize other C1 substrates or complex sugars as anabolic and catabolic substrates. At present, *Acetobacterium romashkowii* is the only acetogenic bacterium that has been identified in petroleum reservoirs (45).

Iron-Reducing Microbes. Ferric iron can exist in petroleum reservoirs in mineral form or bound in the layers of clay; some reservoirs contain ironstone belts that are tens of meters thick. Thermophilic iron-reducers that are able to couple H_2, organic acid, and hydrocarbon oxidation to iron reduction have also been isolated from petroleum reservoirs (Table 1) (46,47). Because of their ability to metabolize hydrocarbons, iron-reducers probably contribute to oil biodegradation. Iron reduction seems to be a conserved characteristic of thermophilic prokaryotes (48,49); however, iron reduction may not be the primary metabolism for these organisms. For example, *Deferribacter thermophilus* can take advantage of several electron acceptors, including manganese, and can also utilize many organic acids (2,46). Furthermore, species belonging to the genera *Thermotoga*, *Thermoanaerobacter*, and *Thermococcus* are able to utilize various sulfur compounds as electron acceptors (see section Fermentative microbes). *Shewanella putrefaciens*, a mesophilic iron-reducer detected from oil reservoirs (50) is able to reduce elemental sulfur, sulfite, and thiosulfate to sulfide (51), and thus, it may also play a part in oil souring.

CONCLUSION

Although the study of microbes in petroleum reservoirs began nearly a century ago, the bulk of existing knowledge has been obtained within the last 20 years. The degradation of oil and equipment continues to have a tremendous economical impact on petroleum companies. As neither the role that sulfate-reducers play in oil degradation nor the impact of introducing foreign bacteria and substrates via repressurization water is completely understood, further studies of reservoirs may reveal methods of inhibiting the biodegradation of oil. Conversely, increased awareness of oil-degrading microbes might aid in the cleanup of oil spills such as the *Exxon Valdez* disaster that occurred near Alaska in 1989. MEOR — the use of microbes to increase petroleum yield in situ — has been proposed, but many hurdles need to be overcome to make this process viable. Additionally, questions remain concerning whether exogenous bacteria are more effective for this process than indigenous ones and it is still not understood which metabolic types would best enhance oil recovery. Besides benefiting industry, the study of petroleum reservoirs may extend the perception of known life on this planet and elsewhere in the universe. Data obtained by microbiologists, geologists, and petroleum companies have begun to elucidate the biodiversity of oil reservoir ecosystems; however, further research involving interdisciplinary collaborations and continued molecular analyses is necessary for better comprehension of these ecosystems.

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PFIESTERIA: THE TOXIC PFIESTERIA COMPLEX

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The toxic *Pfiesteria* complex (TPC) is treated separately from other dinoflagellates (Division Pyrrhophyta) in this encyclopedia because *Pfiesteria* spp. have certain traits that, considered separately, are not unusual but that, considered collectively, changed several generalizations about toxic dinoflagellates (1). In addition to the fact that *Pfiesteria* significantly expanded scientific understanding about toxic dinoflagellates, *Pfiesteria* science — which is "young," having only begun in 1988 — rapidly was enmeshed with socioeconomic and environmental policy issues in the mid-Atlantic and southeastern United States, which led in turn to strengthened surveillance programs for harmful algae and fish disease in those regions (1–3). The TPC thus far includes two species, *Pfiesteria piscicida* Steidinger and Burkholder (4) (pronounced as fee-STEER-ee-uh pis-ki-SEED-uh) and *P. shumwayae* Glasgow and Burkholder (5) (species pronounced as shum-WAY-eye). Toxic strains (individual cells or populations derived from them) of these species have all of the following characteristics: (*1*) strong attraction to live fish; (*2*) toxic activity triggered by live fish or their fresh tissues and excreta; and (*3*) production of toxin(s) (6) that cause fish stress, disease, and death under ecologically relevant conditions [live cells tested with fish using the standardized fish bioassay procedure (1,6,7), at similar densities as those encountered during *Pfiesteria*-related fish kills (1)]. Additional species probably remain to be detected. Thus far, however, tested "pfiesteria lookalike" or "pfiesteria-like" species that physically resemble the two known toxic *Pfiesteria* species (e.g., various *Gymnodinium* spp. such as *G. puchellum*, various *Gyrodinium* or *Karlodinium* spp., *Cryptoperidiniopsis* (gen. ined.) spp., species informally named as "shepherd's crook") have not shown all three traits and, thus are not considered truly *Pfiesteria*-like species or members of the TPC (1). This writing will describe the presently understood biology, ecology, toxicity, and impacts of *Pfiesteria* spp.

GENERAL CHARACTERISTICS, SYSTEMATICS, AND LIFE CYCLE

Comparison with Other Toxic Dinoflagellates

Pfiesteria spp. technically are not algae (defined as primitive plantlike, photosynthetic organisms) but, rather, animal-like protozoans that do not have their own chloroplasts (8). Nevertheless, they are usually included within the broad group of organisms known as "harmful algae" (8), in part because they are close relatives of the so-called "red tide" toxic dinoflagellates that are nearly all plantlike (9). Although they cannot photosynthesize on their own, *Pfiesteria* species can be photosynthetic by consuming algal prey, retaining the prey chloroplasts and allowing the "kleptochloroplasts" to maintain function for hours to days to supplement their nutrition (5,10).

Pfiesteria spp. represent only two among many species of toxic dinoflagellates that have been newly described in the past 15 years (1). *Pfiesteria piscicida* was (*1*) the first toxic dinoflagellate demonstrated to have a strong attraction to live fish and their fresh tissues, secreta, and excreta (separated from the live animals for $\leq$3 h (11); (*2*) the first shown to be triggered to produce toxin by live fish (1,11); (*3*) the first shown to have a complex life cycle containing amoeboid as well as flagellated and cyst stages (1,12,13); (*4*) the first reported to be extremely diverse in its nutrition, able to take up diverse dissolved organic and inorganic nutrients, and also prey spanning trophic levels across the entire estuarine food web (11,13); and (*5*) the first linked to production of aerosols that promote serious neurocognitive impairment in humans (exposure to toxic *Pfiesteria* has been linked to other human health impacts as well; see below) (14,15). *Pfiesteria* additionally was shown to have a cyst stage unlike that of any other dinoflagellate but closely

resembling the cysts of a different division of organisms, the chrysophytes (5,12). Some of these characteristics do occur among other dinoflagellates (2,9,11,16), but had not previously been reported for toxic dinoflagellates (Table 1).

It is important to note that *Pfiesteria* spp. are similar to many other so-called "toxic algae" in that they have both toxic and benign naturally occurring strains that may be considered as one of three functional types with respect to toxicity status (1). Toxic strains can be induced to produce toxin in the presence of live fish (1,6,11), and can be characterized as weakly to highly toxic on the basis of the time and cell densities required for fish death. When zoospores of these strains are actively toxic, they are referred to as the TOX-A functional type; when separated from live fish so that they cease toxin production, they are referred to as the TOX-B functional type (1,6). TOX-B zoospores are regarded as nontoxic and appear to have negligible to very low toxicity, but they can engage in toxin production and become TOX-A zoospores when sufficient live fish are again detected (1,6,11). In contrast, "benign" strains are referred to as noninducible (NONIND functional type) because they cannot be induced to make toxin when fish are present (1,6). NONIND strains consistently fail to show toxicity (evidenced by a lack of distress, disease, or death in test fish), both when freshly isolated from field samples and after extended time in culture (1,6). Thus, the presence of *Pfiesteria* does not necessarily indicate a problem — in some years and in some locations, only NONIND *Pfiesteria* has been found (1). The three functional types of *Pfiesteria* respond differently to nutrients, algal prey, and fish (see following text). In other locations, the proportion of toxic versus benign strains has varied over time, from years when most collected strains have been tested as NONIND to years when most have been toxic to fish (1). The reasons for this variation in toxicity among populations of each *Pfiesteria* species, as for populations of other toxic algal species, are poorly understood. Expression of toxicity is probably controlled by environmental conditions (e.g., nutrition, history of recent feeding, availability of certain required organic substrates) as well as genetic traits, and is further complicated by aberrancies that can develop over time in culture (below).

In most toxic algae, the environmental signals that influence toxin production and the benefit derived from toxicity are poorly known. By contrast, toxicity in *Pfiesteria* spp. is stimulated by substances from live fish, and its toxin(s) narcotises, then kills fish that *Pfiesteria* then consumes as prey (Fig. 1; 1,11). *Pfiesteria* is chemosensorily attracted to live fish and their fresh excreta, secreta, and tissues (Fig. 2; 1,11). Most live fish stimulate *Pfiesteria* to complete its sexual cycle (1,5,11), and sexual reproduction is believed to be of general importance in strengthening the

Table 1. Comparison of Major Traits Between TPC Species and Other Dinoflagellates (including other toxic species), Indicating Features Shared in Common

Trait[a]	Freshwater	TPC Estuarine[b]	Other Estuarine	Marine	Other Toxic[b,c]
Attack behavior toward fish or other prey	+	+	+	+	(−)
Prey generalists (bacteria, algae, fish, etc.)	n.k.	+	+	+	(+)
Heterotrophy or mixotrophy[d]	+	+	+	+	+
Kleptochloroplastidy	n.k.	+	+	n.k.	−
Complex life cycle[e]	+	+	+	+	n.k.
Amoeboid stages	+	+	+	+	(−)
"Chrysophyte-like" cyst[e]	−	+	+	−	−
Number of newly reported toxic species (past 15 years)[b,f]	n.a.	2	n.a.	n.a.	>55
Environmental stimuli for toxicity	n.a.	live fish	n.a.	n.a.	?
Toxins characterized[g]	n.a.	−(purified)	n.a.	n.a.	+ (some)
Ichthyotoxins	n.a.	+	n.a.	n.a.	+
Aerosolized neurotoxins	n.a.	+	n.a.	n.a.	−
Aerosolized toxins that cause respiratory symptoms	n.a.	+	n.a.	n.a.	+

Source: From 1. J. M. Burkholder, H. B. Glasgow, and N. J. Deamer-Melia, Phycologia 40, 186–214 (2001), (in press).

[a]Traits are indicated as present (+); absent (−) or, for toxins, no; not known (n.k.); or not applicable (n.a.).

[b]Toxin producers are segregated into the "TPC" and "Other toxic" columns and are not also included in the "Freshwater," "Other Estuarine," and "Marine" categories.

[c]Includes more than 55 marine species (1,2).

[d]Although mixotrophy and heterotrophy have been reported in various toxic dinoflagellates, this is a poorly known, pioneer area of toxic dinoflagellate research (14).

[e]Before *P. piscicida* was discovered, complex life cycles had not been reported for estuarine dinoflagellates and were known in only a few species belonging to the Dinamoebales (6). Complex life cycles have been observed among two undescribed species within the nontoxic estuarine dinoflagellate taxon, "*Cryptoperidiniopsis*" *gen. ined.* (2,17). Similarly, chrysophyte-like cysts were not known in dinoflagellates before the discovery of *P. piscicida* (15), but are now known for *P. shumwayae* and *Cryptoperidiniopsis gen. ined.* (7,17). Note that complex life cycles may be more highly developed among shallow freshwater than marine species (18). However, many dinoflagellates have not been examined using techniques that would induce transformations to amoeboid stages if present. There is need for more thorough examination of dinoflagellate life cycles, especially in estuarine and shallow coastal species (19).

[f]Number adapted from published references 1. J. M. Burkholder, H. B. Glasgow, and N. J. Deamer-Melia, Phycologia, 40, 186–214 (2001). 2. J. M. Burkholder, Ecol. Appl. 8, S37–S62 (1998).

[g]While some dinoflagellate toxins are well characterized (e.g. brevetoxins, with more than 25 years of effort, and saxitoxins, okadaic acid, and some ciguatoxins), many are only partially characterized (*Pfiesteria*) or uncharacterized (19).

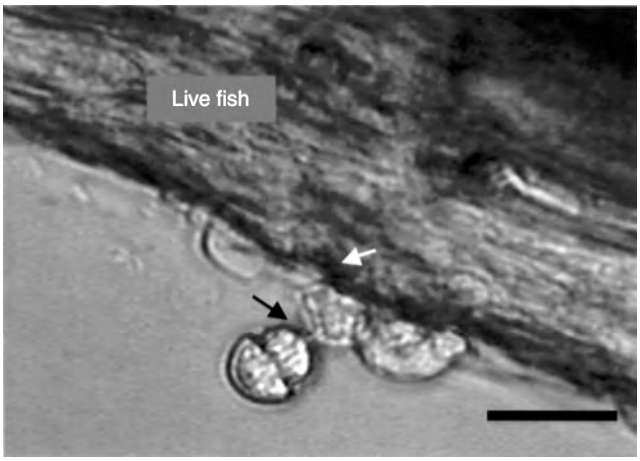

Figure 1. Light micrograph of a *Pfiesteria shumwayae* toxic zoospore (TOX-A functional type) with its peduncle (extended appendage used for attachment and feeding, arrow) attached to a larval sheepshead minnow (*Cyprinodon variegatus*), engaged in suctioning the contents from the tissue in active feeding (process called myzocytosis) (scale bar = 10 μm). (From 1. J. M. Burkholder, H. B. Glasgow, and N. J. Deamer-Melia, Phycologia 40, 186–214 (2001).) See color insert.

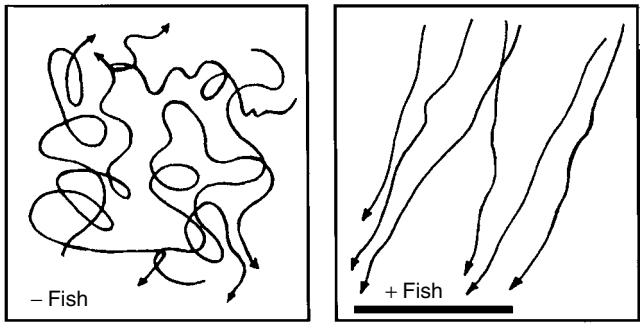

Figure 2. Chemosensory stimulation of *Pfiesteria* piscicida by fresh tissue indicated by motion analysis of six groups of toxic zoospores (TOX-A functional type) without fish (left panel) versus with a piece of epidermal tissue taken from a live tilapia (*Oreochromis mossambica*; right panel, with tissue position indicated by the black bar). Each arrow represents the mean of 10 cells per group. Directed movement is shown as net-to-gross displacement (NGD), a relative measure of path twistedness or convolution, in the absence versus the presence of fresh fish tissue (each trial = 120 seconds; a value of 1 describes a straight-line path of travel, whereas a value of 0.5 describes a random path at a 45° angle). (From 11. J. M. Burkholder and H. B. Glasgow, *Limnol. Oceanogr.* 42, 1,052–1,075 (1997).)

viability (fitness) and survival of species through genetic recombination.

SYSTEMATICS AND CELLULAR CHARACTERISTICS

The genus *Pfiesteria* was named in honor of the late Dr. Lois Pfiester, for her elegant research, which greatly strengthened general understanding about the biology of dinoflagellates, including species with complex life cycles (4). The species name in *P. piscicida* is Latin for "fish killer." The second known species, *P. shumwayae,* was named in honor of Dr. Sandra Shumway, for her research that has significantly advanced understanding about worldwide impacts of toxic dinoflagellates, especially on shellfish species and aquaculture (5). The two species have been placed within Division Pyrrhophyta, Class Dinophyceae, Order Dinamoebales, and Family Pfiesteriaceae (4,5). They can be identified (although the zoospore thecal plate structure is peridinoid), by the arrangement of the thin cellulose deposits (thecal plates) in the cell covering (amphiesma) of the zoospore stages (flagellated vegetative or asexual cells) (4,5,12).

The small, biflagellate zoospores of *Pfiesteria* spp. (diameter usually 7–14 μm, with known range of ca. 3–24 μm) closely resemble zoospores of various other estuarine dinoflagellates, and cannot be differentiated under light microscopy. Their identification requires swelling the sutures between the thecal plates (5), or stripping away the outer membranes that obscure the plates (4), so that the number, shape, and arrangement of the plates can be discerned under scanning electron microscopy (SEM; Fig. 3). *Pfiesteria piscicida* zoospores have plate tabulation Po, cp, X, 4′, 1a, 5″, 5c, 4s, 5‴, 2⁗, with a three-sided anterior intercalary plate (4; Fig. 3). *Pfiesteria shumwayae* zoospores have an additional precingular plate and this results in a four-sided anterior intercalary plate; thus, the tabulation is Po, cp, X, 4′, 1a, 6″, 6c, 4s, 5‴, 2⁗ (5). In addition to these differences in cellular structure, the two species also have been shown to have genetic differences (20). Although molecular probes, targeting the ribosomal DNA or other genetic material, have been developed for each *Pfiesteria* species and cross-corroborated by independent laboratories (17,20), there remains the possibility that the probes may also react with as-yet-undescribed estuarine dinoflagellates. Moreover, chemical substances, detritus, sediment, and other materials in natural samples or cultures with fish can sometimes cause spurious results with molecular probes. Therefore, SEM remains the "gold standard," that is, the most reliable technique for identification of *Pfiesteria* species (1,6).

Pfiesteria spp. have typical dinoflagellate organelles (1,4,5,9), including a "mesokaryotic" nucleus (with chromosomes remaining condensed during interphase) in zoospores but a typical eukaryote nucleus in amoebae (1). The chromosome number of *P. pisciaida* TOX-A zoospores recently isolated from estuarine habitat is 23 ± 2, as determined using flow cytometry, light microscopy, and transmission electron microscopy procedures on clonal populations (note, however, that chromosome number becomes variable and ploidy generally changes over time in culture) (1). This chromosome number is intermediate between the chromosome numbers of parasitic dinoflagellates (4–8 chromosomes) and some free-living species ($\geq$20), on the one hand, and certain photosynthetic, free-living dinoflagellates that have $\leq$270 chromosomes (18).

There is precedent for amoeboid stages in other dinoflagellate species from freshwater as well as estuarine and marine habitats (4,16,19). In addition to the direct observations reported by several independent laboratories

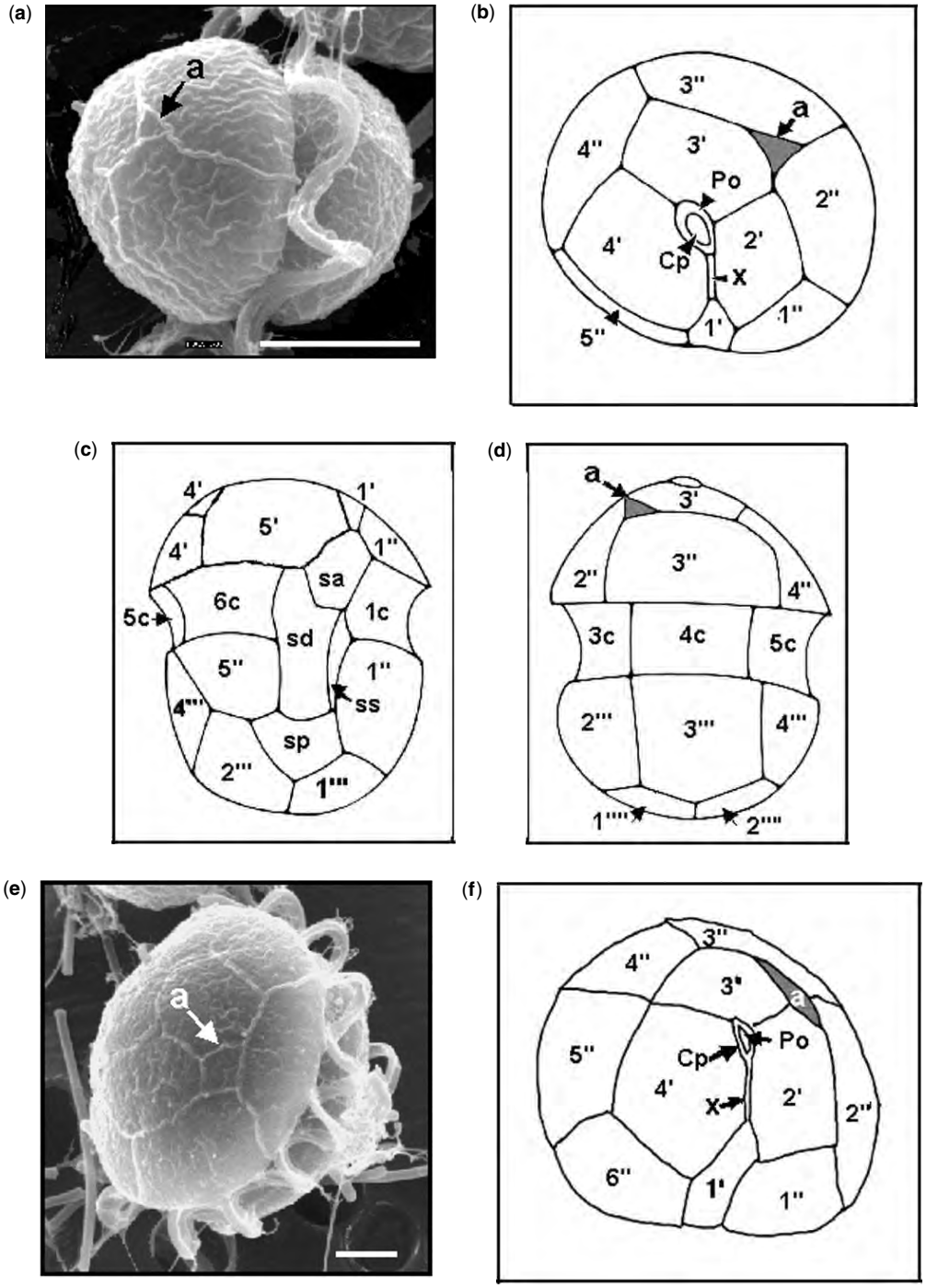

Figure 3. (**a**) Scanning electron micrograph of a suture-swollen zoospore of *Pfiesteria piscicida,* showing the three-sided anterior intercalary plate (**a**) (scale bar = 3μm; photographed by H. Glasgow, North Carolina State University); and (**b–d**) drawings of the plate structure of *P. piscicida* in (**b**) apical, (**c**) ventral, and (**d**) dorsal view (modified from 4. K. A. Steidinger et al., *J. Phycol.* **32**, 157–164 (1996).); (**e**) scanning electron micrograph of a suture-swollen zoospore of *Pfiesteria shumwayae*, showing the four-sided anterior intercalary plate (**a**) (scale bar = 1 μm; photograph by H. Glasgow, North Carolina State University); and (F-H) drawings of the plate structure traced from actual zoospores, including apical (**f**), ventral (**g**), and dorsal (**h**) views. (From 1. J. M. Burkholder, H. B. Glasgow, and N. J. Deamer-Melia, *Phycologia* **40**, 234–245 (2001).) See color insert.

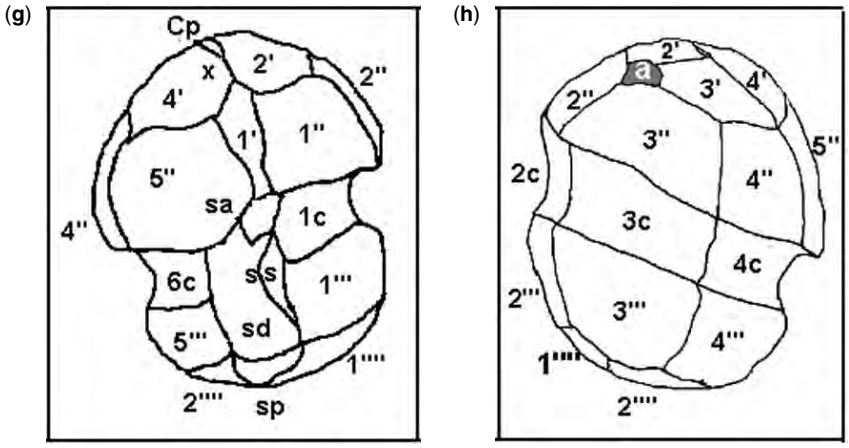

Figure 3. (*Continued*)

(1), polymerase chain reaction (PCR) and fluorescent in situ hybridization (FISH) molecular probe testing recently was performed on amoeba isolates to assess their identity (1,17). To retain pure cultures of amoebae without other life stages, and thereby avoid the potential for uncertainty in interpretation, we maintained the subcultures used for molecular probe analyses for long periods (months to years) without altering the prey source used in feeding to depress the potential for transformations from amoebae to other stages (1). Throughout the observations, no zoospores were noted in the amoeba cultures. The PCR analyses were positive for *Pfiesteria* spp., and were cross-corroborated by an independent laboratory (1).

Most *Pfiesteria* research has focused on zoospores because they are the most toxic known stage in the complex life cycles of *Pfiesteria* spp. The cell biology, behavior, and ecology of *Pfiesteria* amoebae are less well understood. Evidence of ichthyotoxic activity by a lobose amoeboid stage has been reported (11). On the basis of laboratory experiments with clonal *Pfiesteria* spp. cultures, most flagellated cells from TOX-A cultures are capable of transforming to amoebae (1,4,5,11–13). These stages rapidly settle out of the water column and remain active if abundant prey (bacteria, algae, fish remains) are available. Thus, amoeboid stages represent an important link between planktonic and benthic habitats in the life cycle (below), especially for toxic *Pfiesteria* strains.

COMPLEX LIFE CYCLES

The life cycles of most toxic dinoflagellates are incompletely known (21). Among these organisms, *Pfiesteria* spp. have been very intensively examined using multiple prey types and other food sources (1,11). Perhaps partly for that reason, *Pfiesteria* spp. have been confirmed by several independent laboratories to have complex life cycles with about 20 or more flagellated, amoeboid and cyst stages (Figs. 4,5; 1,11). Transformations among these stages are controlled by the type and abundance of available prey, especially finish (1,5,11–13). The *Pfiesteria* life cycle includes zoospore stages that can be produced by vegetative cysts and zoospore-derived palmelloid masses, and by at least one amoeboid stage (Fig. 4). The zoospores produce cysts that range from 6 to 25 μm in diameter (1). They can be chrysophyte-like with an outermost covering of organic and siliceous scales with bracts (4,5,11–13), or smooth with several wavelike markings on the thickened outer wall (Fig. 4; 11,12). Under sudden stressful conditions, within minutes zoospores can also form temporary cysts with a protective thick outer layer of mucilage (Fig. 4).

Sexual reproduction in *Pfiesteria*, with gamete fusion to form the sexual product or planozygote, occurs mostly in the presence of live fish, but can also occur in some strains fed algal prey (Fig. 4; 1,11,12). Gametes are produced by TOX-A zoospores, and may be of equal (isogamous, similar to the zoospores in size) or unequal size (anisogamous; the "−" gametes are 7 to 9 μm in diameter; the "+" gametes are 4 to 5 μm in diameter, with a very long longitudinal flagellum (12)) (1). Gamete fusion has been observed to take place via at least two mechanisms. In one mode, the gametes align so that horizontal groove (cingulum) of one cell is perpendicular to the cingulum of the other, with the longitudinal groove (sulcus) of the cells in direct contact. In the second mode, a conjugation tube forms between the two gametes and the protoplast of the (+) gamete moves through it to fuse with the (−) gamete. The resulting sexual product or motile planozygote (diameter 14–60 μm) is triflagellate, with one transverse and two longitudinal flagella (1,11). Planozygotes are usually ephemeral, lasting only for hours before producing four zoospores, if in cultures with fish; or under some conditions with algal prey. Alternatively, each forms a resistant, nonmotile, thick-walled sexual cyst (hypnozygote; Fig. 4; 1,26).

Amoebae in *Pfiesteria* spp. may be produced by zoospores, gametes, planozygotes, or cysts, resulting in considerable size variation (length ca. 5–120 μm for cultures recently isolated from estuarine habitat; Figs. 4,5) (1,5,11). They may be filose, lobose, or rhizopodial (the latter observed thus far in *P. piscicida*), with a smooth or rough outer covering (1,4,5,11). Amoebae are mostly produced in actively toxic, fish-killing cultures,

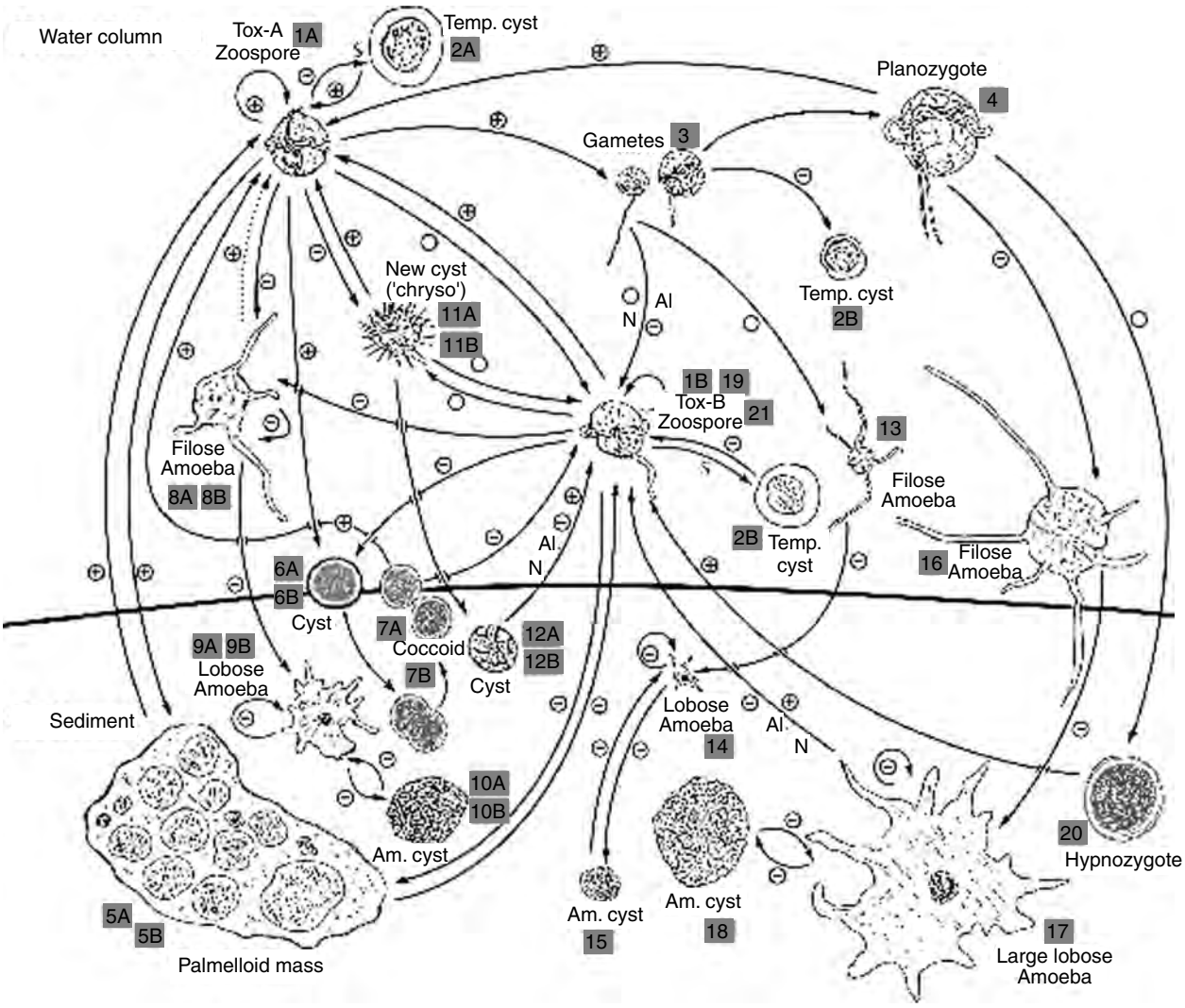

Figure 4. The complex life cycle of *Pfiesteria* showing stages and pathways that have been verified for both *P. shumwayae* and *P. piscicida* (note that *P. piscicida* has been under study for a longer period, and several additional stages and pathways have been determined for that species as well (14)). The pathways indicate the presence (+) versus the absence (−) of live finfish; A = presence of cryptomonads and certain other algal prey; N = nutrient enrichment as organic and/or inorganic N and P; S = environmental stressor such as sudden shift in temperature or salinity, physical disturbance, or prey depletion). Solid lines = verified pathways; dashed lines = hypothesized pathways. TOX-B zoospores (haploid; ploidy confirmed as in (1); temporarily nontoxic functional type in the absence of live fish prey, but capable of producing toxin when sufficient live fish are added (1,7); called nontoxic zoospores or NTZs in the *P. piscicida* schematic (14) are shown as one stage to simplify the schematic, but actually represent at least two stages in the life cycle because they are produced by both stages to which they can directly ("chrysophyte-like ["chryso"] cyst, temporary cyst, palmelloid mass — all haploid stages) or indirectly transform (from coccoid cells excysted from another cyst stage [both haploid] produced by TOX-B zoospores), and by large diploid lobose amoebae. TOX-B zoospores can also be formed from gametes through "reversion" or loss of sexual activity, and, importantly, by TOX-A zoospores (haploid; actively toxic functional type (1,7); called toxic zoospores or TZs in the *P. piscicida* schematic (14)) through cessation of toxin production when sufficient live fish are no longer available. TOX-B zoospores are similar to the larger or "–" anisogamous gametes (shown here) or to isogamous gametes (also possible in both *Pfiesteria* spp.) in appearance, although their size can vary fourfold depending on feeding activity. Zoospores can produce haploid filose, lobose, and rhizopodial amoebae. Cysts include stages with roughened or reticulate covering (from amoeboid stages, haploid except when derived from diploid amoebae); scaled covering (± bracts; from TOX-A and TOX-B zoospores; these cysts also can lose their scales and bracts over time so that they have a smooth covering (14) [not shown; and transitional forms to these cysts are not shown occur in *P. shumwayae* as in *P. piscicida* (14)), and hyaline covering (small cysts that can divide as in Spero and Morée (22), with darkened contents; and hypnozygote (*Caption Continued*)

although filose amoebae can also be produced by nontoxic cultures (1,4). *Pfiesteria* amoebae produce spherical to oval cysts with a roughened or reticulate outer covering (diameter ca. 4–25 μm) (1,4,5,11).

CULTURING *PFIESTERIA*

Pfiesteria spp. have been cultured with high cell production for sustained periods only by adding a prey source, and it has not been possible to induce strong toxin production unless live fish are added (1,11). *Pfiesteria* must be isolated (i.e., cloned as 1 axenic cell of *P. piscicida* or *P. shumwayae*) from natural estuarine water or sediment samples, and then maintained with prey. Therefore, a clonal culture of *Pfiesteria* is formally defined as consisting of a clonal population of *P. piscicida* or *P. shumwayae*, together with added prey (6). The most commonly used prey are algae, especially cryptomonads (13) and fish (11,12). Algal prey can be used to maintain toxic strains in nontoxic mode for short periods (up to 4–6 weeks is recommended), but live fish must be used to maintain toxic strains in actively toxic mode.

Addition of algal prey such as cryptomonad species (13) to natural estuarine water or sediment samples can support increased growth of dinoflagellate species that acquire nutrition through partial or complete reliance on heterotrophy or animal-like feeding on algae, following published procedures (1,13). Nontoxic strains of *Pfiesteria* spp. (TOX-B or NONIND functional types) can be cultured using this approach, among other dinoflagellates. However, algal assays commonly fail to detect toxic strains of *Pfiesteria* species in natural estuarine phytoplankton samples, whether they are actively toxic when collected or in a temporarily nontoxic mode (1,6). It is also important to note that although TOX-B and NONIND *Pfiesteria* can be grown indefinitely on algal prey, TOX-B strains can rapidly (within weeks) lose their ability to resume toxin production and become NONIND, unless they are periodically reexposed to live fish (1).

Maintenance of actively toxic (TOX-A) *Pfiesteria* cultures, and assessment of *Pfiesteria* involvement in fish kills, is accomplished using the standardized fish bioassay (7,27). The technique, which has been cross-corroborated with extensive quality control/assurance procedures, follows standardized steps while allowing flexibility to simulate the environmental conditions under which estuarine samples yielding the culture isolates were collected (1,7,27). Therefore, it is currently the "gold standard" for culturing actively toxic *Pfiesteria* spp., and also for evaluating their presence in estuarine water and sediments, given that a field-reliable assay for detecting *Pfiesteria* toxin(s) is not yet available (below; 1,7,28). The standardized procedure for this "fish bioassay" follows Henle-Kochs' postulates (29) modified for toxic rather than infectious agents. It has been relied upon since 1991 both to culture TOX-A *Pfiesteria* and to assess its involvement in estuarine fish kills (below) (1,11,12,30).

Actively toxic *Pfiesteria*, grown with live fish, should be held in biohazard III containment systems to protect laboratory workers from neurotoxic aerosols emitted by fish-killing cultures (1,7,11). Separate facilities are required for culture of healthy fish without *Pfiesteria* contamination, and with minimal associated microbial contaminants (11). Environmental conditions conducive for growth of toxic *Pfiesteria* spp. should be imposed (Table 2). Toxic *Pfiesteria* cultures with fish require high maintenance. The medium should remain clear and colorless to pale yellow, rather than clouded and/or discolored from high bacterial/fungal growth. The cultures also require rigorous sampling of environmental conditions and microflora/fauna to ensure that predatory microfauna, which inevitably are associated with fish, do not prevent or significantly reduce *Pfiesteria* cell production (7). Such steps are also necessary to ensure that fish health is not compromised by other factors (below). Even with these efforts, when given live fish consistently, and when cultures are regularly cleaned using serial dilution or other techniques, many toxic strains exposed to live fish have lost their ability to produce toxin and have become NONIND over time in culture. Toxicity can be sustained significantly longer (months to years) when *Pfiesteria* toxic strains are given live fish as opposed to other prey (10). The loss of toxicity in culture has also commonly occurred for various other toxic algal species, and appears to be an artifact of the highly artificial conditions (1). Thus far, techniques for long-term storage of viable cultures such as cryopreservation have not succeeded in maintaining toxin-producing strains (1).

ASSESSMENT OF TOXIC *PFIESTERIA* INVOLVEMENT IN FISH KILLS

Rationale

On the basis of numerous field investigations and laboratory experiments, the Center for Applied Aquatic Ecology at North Carolina State University has developed

Figure 4. (*Continued from previous page*) [diploid "cyst" at lower right]). Zoospores and gametes also form temporary cysts with thick mucus covering, which may settle out of the water column (arrows not shown). The triflagellated planozygote (diploid, from gamete fusion; with two longitudinal flagella and one transverse flagellum) can transform to diploid filose and lobose amoebae (right side of figure). Note that the life cycle stages and pathways shown here have been verified for both *P. piscicida* and *P. shumwayae*. *Pfiesteria piscicida* has been under study for a longer period, so other pathways in addition to those shown here have been determined for that species (14). Several stages in addition to TOX-A zoospores are believed to be toxic in *P. shumwayae* as in *P. piscicida* (14), and testing of these stages is underway. Other stages have been observed for both *P. piscicida* and *P. shumwayae*, but have not been included because of their as-yet-uncertain position in the life cycle.

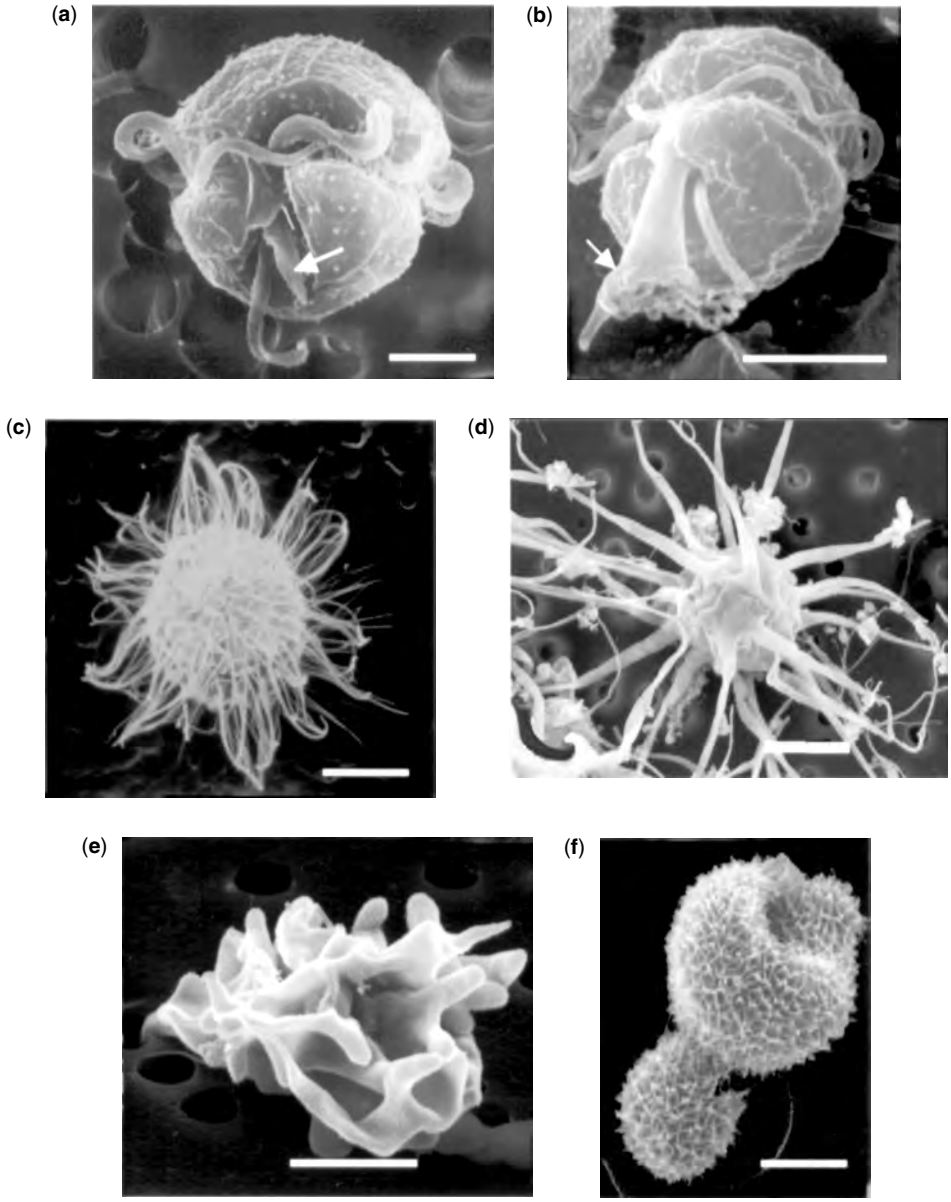

Figure 5. Scanning electron micrographs of *Pfiesteria* including (**a**) a *P. piscicida* actively toxic zoospore (biflagellated vegetative cell, toxic strain, TOX-A functional type), the most toxic stage in the life cycle, with retracted peduncle (arrow; organelle used to attach to and suction the contents from prey cells; scale bar = 3 μm; from 11. J. M. Burkholder and H. B. Glasgow, *Limnol. Oceanogr.* **42**, 1,052–1,075 (1997).); (**b**) a *P. piscicida* nontoxic zoospore (toxic strain, TOX-B functional type) showing the organelle called the peduncle (arrow) fully extended in feeding mode (scale bar = 5 μm; from 10. A. J. Lewitus, H. B. Glasgow and J. M. Burkholder, *J. Phycol.* **35**, 303–312 (1999).); (**c**) a cyst produced from a toxic zoospore (toxic strain, TOX-A functional type) of *P. piscicida* after fish death (scale bar = 3 μm; from J. M. Burkholder et al., *Nature* **358**, 407–410, (1992); J. M. Burkholder et al., *Nature* **360**, 768 (1992).); (**d**) a filose amoeba produced from a zoospore of *P. shumwayae* (scale bar = 20 μm); (**e**) a lobose amoeba of *P. shumwayae* (scale bar = 10 μm); and (**f**) two amoeboid cysts, with roughened or reticulate outer coverings (scale bar = 1 μm; figures d-f from H. B. Glasgow et al., *Phycologia* **40**, 234–245 (2001)).

a conservative protocol for assessing toxic *Pfiesteria* involvement in major fish kills (defined as affecting 1,000 or more fish (1,11)). The protocol depends on the standardized fish bioassay procedure as the presently available "gold standard" to diagnose whether an actively toxic population of *Pfiesteria* was present at a fish kill while the kill was in progress, that is, while fish were moribund but not yet dead (1). An assay to detect *Pfiesteria* toxin(s) would be more desirable because it would enable determination of whether the toxin(s) itself was present, which is the primary concern of public health officials as well as natural resource managers.

Table 2. Conducive Conditions for *Pfiesteria* Zoospore Production, Compiled from Experimental Laboratory and Field Data

Variable	Conducive to Cell Production	Negligible or Slow Cell Production
Temperature	>20-30 °C (>26 °C)	<20 °C, >30 °C
Salinity	>5-20 (10-15)	≤5, ≥20
Light (Quantity, Photoperiod)	0–300 μmol m^{-2} s^{-1}	>300 μmol m^{-2} s^{-1}
Nutrient Regime[b]	≫100 μgN_i,P$_i$ L^{-1}	<100 μgN_i,P$_i$ L^{-1}
pH	6.6-8.6 (>7.5)	<6.6, >8.6
Water Motion	low turbulence	mixed
Acclimation Period (after transport, for cell production, toxic activity)	>3-7 days	≤3-7 days
Algal Prey (zoospores, amoebae)	cryptomonads	cyanobacteria, other picoplankton
Finfish Prey (zoospores)	many species	*Gambusia*

Source: (Modified from 7. J. M. Burkholder et al.., Environ. Health Perspect. 109(Suppl. 5), 745–756 (2001).)
Note: Optima are indicated in parentheses, where known, with ranges indicating data for isolates from different geographic regions.

Such an assay has been developed for use in laboratory research (28,31). However, estuarine waters contain organic substances and other debris that are known to interfere with the assay (28,31), either by obscuring the presence of the toxin so that a "false negative" is reported, or by reacting with the assay reagents to give a "false positive" for the presence of the toxin. Sufficient quantity of purified toxin standard is not yet available to enable use of internal standards with estuarine water samples. Their addition in known quantity to replicate subsamples of the estuarine water sample of interest would enable detection of, and correction for, such interfering substances. Until enough purified toxin standard is produced to make that correction step possible, no laboratory assay for *Pfiesteria* toxin can be regarded as "field-reliable" to determine whether the toxin is present in natural estuarine samples (28,31).

In the meantime, and in recognition of the fact that causality of fish death by microbial pathogens or other factors can be inferred, but usually cannot be proven conclusively in a field setting, assessment of actively toxic *Pfiesteria* involvement follows a conservative approach. This assessment is restricted to in-progress kills in (7,11,30) consideration of the known behavior of *Pfiesteria* spp. These organisms commonly cease toxin production and leave the water column (by settling to the bottom sediment of the estuary, or by forming colorless amoebae that attach to the fish remains) shortly after fish death (1,11). Thus, the appropriate period to sample for actively toxic *Pfiesteria* is while fish are dying. The mere presence of *Pfiesteria* cells after fish death indicates nothing about whether *Pfiesteria* had actually been involved in the kill since, as stated, there are naturally benign as well as toxic *Pfiesteria* strains. Nor can detection techniques (e.g., molecular probes) discern between actively toxic *Pfiesteria* versus populations that were not engaged in toxic activity when collected (1). Even when purified toxin standard becomes available for use in a reliable field assay to detect *Pfiesteria* toxin, efforts probably will have to be restricted to sampling while fish kills are in progress or very shortly thereafter, because the toxin(s) is known to be highly unstable and commonly breaks down quickly (often within several hours) in water and aerosols (1,11,28,31).

In this conservative approach, *Pfiesteria* is ruled out as a primary cause of a major estuarine fish kill if other causative factor(s) can be detected (1,6,7,11). For example, about 90% of the fish in kills related to toxic Pfiesteria have been juvenile Atlantic menhaden (*Brevoortia tyrannus*), which are surface-schooling fish (1,30). In kills of surface-schooling fish, we have implicated low-oxygen stress as the primary cause, even when actively toxic *Pfiesteria* was verified, if anoxia (condition of acutely low dissolved oxygen [D.O.], ca 0 mg/L^{-1}) is present in more than the lower one-third of the water column of the affected area or immediately adjacent areas; or if hypoxia (D.O. < 4 mgL^{-1}) is present in more than the lower one-third of the water column over widespread areas within the kill zone, despite the fact that much of the upper water column would have remained available to provide oxygen-replete habitat (1,11,30). Thus, the protocol is biased in favor of low dissolved oxygen rather than *Pfiesteria* as a primary causative agent in fish kills.

Moreover, assessment of *Pfiesteria* involvement in fish kills is applied to areas with appropriate environmental conditions for toxic *Pfiesteria* activity (1,7,11,32). This range of focus includes in-progress, major estuarine fish kills that occur in quiet, warm, poorly flushed brackish waters (Table 2), especially kills involving large schools of menhaden or similar fish. In addition, waters that have been degraded by nutrient pollution from human sewage, animal wastes, cropland and lawn fertilizer runoff, urban runoff, and atmospheric sources appear to be preferred habitat (1,11,30,32). TPC species have broad temperature (active at 9–33 °C) and salinity tolerance (active at 2–35; active at 0–1 if calcium ion is present at ≥4 mg Ca^{+2} hardness/L (1,11)) (Table 2). Nutrient over-enrichment can stimulate *Pfiesteria* spp. by providing habitat with an abundance of other food resources to sustain these dinoflagellates in a nontoxic mode when fish are not available (below) (1,11,30,32). *Pfiesteria* does not become actively toxic under conditions of high volume of flow or high wave action or sudden decrease in salinity to near-freshwater or freshwater conditions (salinity <0.1 (33));

thus it would not be expected to be involved in fish kills following major storm events such as hurricanes (1,11,32).

The protocol to assess actively toxic *Pfiesteria* in estuaries has been focused on fish kills, rather than on epizootics (fish disease) in the absence of dying fish because the uncertainties inherent in attempting to diagnose the initial cause fish diseases — especially chronic diseases — are so much greater than those confronted in fish kills that often occur in response to an acute, more readily identified stressor that affects the fish in or near the area where they die (1,7). Nevertheless, it is of interest that nearly all *Pfiesteria*-related kills have involved a high percentage of menhaden with ulcerated lesions (referred to as fish kill/disease events (1,7,11,32)). Thus, within the context of appropriate environmental conditions, the occurrence of ulcerated lesions can also be helpful information. Fish lesions cannot be used as an 'absolute' indicator of *Pfiesteria*, since many stressors and microbial pathogens can be involved in chronic lesion development (1,11,32). Use of dying fish as sentinels, especially of dying menhaden with high incidence of disease, is in accord with renowned fish pathologists (29), while recognizing that few population indices (such as the existence of deep, bleeding lesions that are often chronic (34)) are disease-, disorder-, or condition-specific.

Sequence

Given appropriate conditions for toxic *Pfiesteria* activity, the procedure to determine whether it was involved in a major estuarine fish kill considers several points:

1. At least 300 pfiesteria-like zoospores/mL^{-1} must be present ("presumptive count" (7), light microscopy, 600x) where/while fish were dying. The basis is more than 1,000 laboratory experiments that have shown that actively toxic *Pfiesteria* can be lethal to fish at densities $\geq$300 TOX-A zoospores/mL^{-1} (1,11). Note that sampling must be conducted carefully to follow this caveat. In practice, it is difficult to arrive at the scene of a fish kill while fish are still dying but not yet dead, because fish often float just below the water surface when they are moribund and float at the surface only after death. Nevertheless, to implicate toxic *Pfiesteria*, fish kills should not be sampled hours or longer after the fish are all dead. Spatial as well as temporal mismatches between the fish kill and sampling must be avoided. By the time fish are sampled after capture, the boat may have drifted or the tide may have flushed out the water that was associated with the fish contained in, for example, a cast net held over the side. Commonly when *Pfiesteria* is involved in a kill, samples taken in the immediate location of the dying/diseased fish have contained $\geq$300 zoospores/mL^{-1}, but samples taken only about 70 meters from the site have contained little or no *Pfiesteria*. The stipulation that water samples must be sampled while dying/diseased but not yet dead, is highly conservative and probably underestimates toxic *Pfiesteria* activity. For example, water samples collected about 24 hours after fish death could contain about 200 *Pfiesteria* zoospores/mL^{-1}, representing a portion of the population that was actively toxic during the kill but which subsequently switched to other prey that were abundant in the area. Yet, by criterion #1, the kill technically could not be related to toxic *Pfiesteria*. A field-reliable assay for *Pfiesteria* toxin, applicable for use in water samples as well as fish tissue, will enable consideration of events detected and sampled post kill.
2. Active toxicity of *Pfiesteria* cells collected at the in-progress fish kill must be confirmed by standardized fish bioassays (7), wherein these cells are tested in the natural sample, then cloned and retested with fish (1,7,11);
3 Species identifications are made from scanning electron microscopy of suture-swollen (5) or membrane-stripped cells (4); and
4. Both toxicity and species identity are cross-confirmed by independent specialists with demonstrated expertise in dinoflagellate systematics and research with toxic *Pfiesteria* (7).

Standardized Fish Bioassays

The standardized fish bioassay procedure is used to culture actively toxic *Pfiesteria* as stated, and is also of critical importance in diagnosing the presence of toxic strains and their involvement in fish kills within estuaries and aquaculture facilities (1,6,7,11,32). Henle and Koch originally developed a set of standardized steps to assess whether a given infectious organism had caused disease (29). Application of this rigorous set of steps to assess actively toxic *Pfiesteria* involvement in fish kills required modifications because *Pfiesteria* is a toxic agent (note that there is no evidence, as yet, that *Pfiesteria* can act as an infectious organism; Fig. 6) (1,7,11).

In Henle-Kochs' first postulate, the infectious organism of interest must be present in the host (29). In the modification, *Pfiesteria* must be present at a fish kill or during disease, and must be detected in standardized fish bioassays (in association with fish death, and in the absence of other apparent causality) of samples taken where and while fish are dying (Fig. 6). *Pfiesteria* can be lethal to fish at densities $\geq$300 zoospores/mL^{-1} (11); thus, as a rapid screening technique with light microscopy ("presumptive count"), *Pfiesteria* can be eliminated as a potential cause of a fish kill if there were $<$300 pfiesteria-like zoospores/mL^{-1} present where and while fish were dying (1,6,7,11). In Henle-Kochs' second postulate, the infectious organism must be isolated from the host and grown in pure culture (29). In the modification, *Pfiesteria* must be isolated from fish-killing bioassays (1,6,7,11) and grown in clonal culture.

Henle-Kochs' third postulate states that the organism must be injected from pure culture into a healthy host, resulting in infection (disease) in the host (29). In the modification, clonal *Pfiesteria* must be added to healthy fish cultures (in a second set of fish bioassays, essential to confirm toxicity of a *Pfiesteria* isolate) and result in fish deaths (7); this often occurs after the fish develop lesions or hemorrhage, whether the *Pfiesteria* cultures are allowed

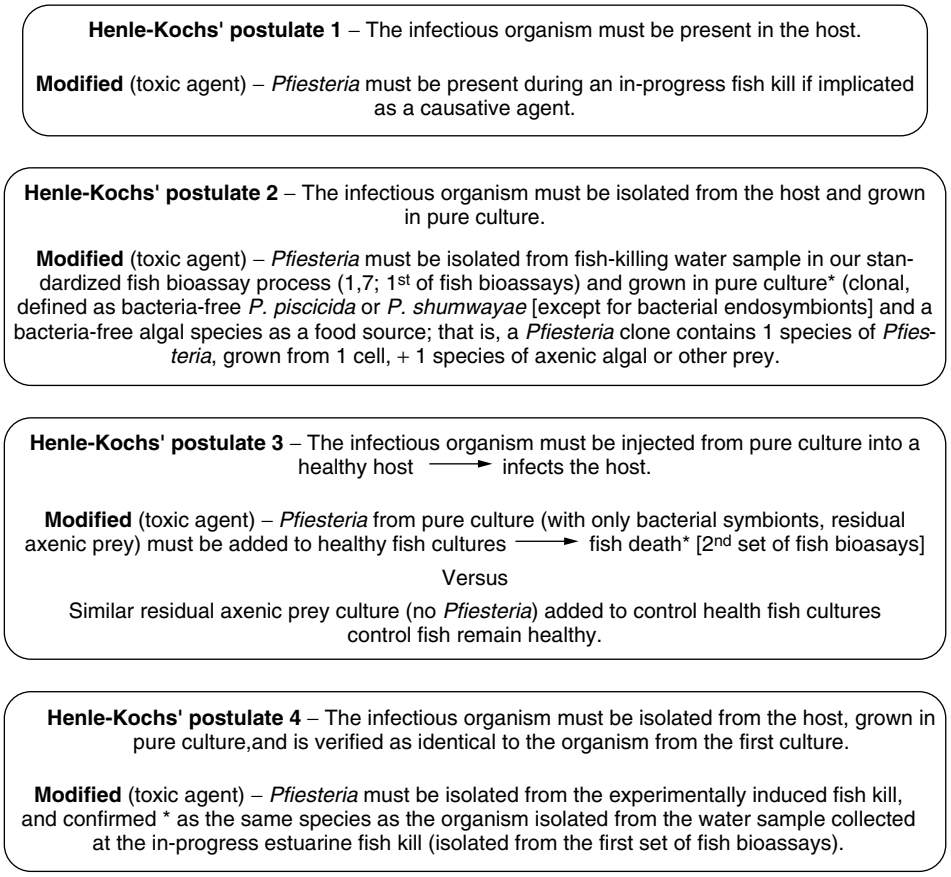

Figure 6. Schematic depicting use of the standardized fish bioassay procedure to diagnose whether actively toxic *Pfiesteria* was involved in a major estuarine fish kill, following Henle-Kochs' postulates modified for toxic rather than infectious agents (7,31). Asterisks (*) indicate the steps at which cross-corroboration should be complex by independent specialists with demonstrated expertise in *Pfiesteria* species identifications and toxicity (1). (From 32. H. B. Glasgow et al., *Environ. Health Perspect.* **109**(Suppl. 5), 715–730 (2001).)

direct contact with the fish, or are maintained within dialysis membranes (0.22 μm porosity) (11). By contrast, control fish remain healthy when residual cryptomonad culture (without *Pfiesteria*) is added (Fig. 6). In Henle-Kochs' fourth and final postulate, the organism must be reisolated from the experimentally infected host, grown in pure culture, and compared with the organism from the original culture (29). Here, *Pfiesteria* must be isolated from the second set of fish-killing bioassays, recloned, and the identification reconfirmed (7).

During the fish bioassay procedure, many environmental variables additionally are measured to compare test versus control fish cultures, to ensure that other causative factor(s) were not involved in fish death (7). Operating parameters are: temperature $\geq$20 °C (recommended); salinity >7; light (no established preference); pH $\geq$6.8–8.5; dissolved oxygen $\geq$5 mg/L (not supersaturated); nitrite $<0.7 mgL^{-1}$; and ammonium $<1 mgL^{-1}$ (7). Other nutrients are also measured. *Pfiesteria* and pfiesteria-like dinoflagellates are identified and enumerated, as well as a suite of other microorganisms, with focus especially on certain bacterial and fungal pathogens of fish, phytoplankton (including other species that can harm fish), and certain protozoans and copepods that can harm fish.

The Critical Importance of Timing

Pfiesteria spp. gradually become actively toxic when they detect live fish, but toxic stages are not attracted to dead fish and transform to nontoxic (TOX-B zoospores, nontoxic amoebae, or cysts) stages shortly after fish death (1,11,23). The biochemical pathways involved in toxic production apparently require time for activation if the population has not recently been in toxic mode, so that a previously inactive (encysted or temporarily nontoxic) population can require days to weeks to become active in producing toxin, whereas a toxic population that killed fish recently (hours) can be lethal to fish within a few hours (7,32).

Thus, *Pfiesteria* species have shown a biochemical and/or genetic predisposition for toxic activity stimulated by recent exposure to live fish (1,11,12), but actively toxic zoospores are highly sensitive to separation from live fish and rapidly encyst (usually within hours). Following such a separation period (<3 days), they typically excyst and resume lethal activity toward newly added test fish within 4 to 9 days (90% confidence interval, n = 20 fish bioassays with 10 fish per replicate; 95% confidence interval at less than 21 days; Fig. 7) (7). Populations that were not previously in actively toxic mode require considerably longer to kill fish in bioassays (typically 6–8 weeks or more) (7). These characteristics about *Pfiesteria* behavior were considered in designing an effective fish bioassay procedure to diagnose actively toxic *Pfiesteria* involvement in estuarine fish kills. Also note that field diagnostics are not available for many microbial pathogens which are considered harmful to aquatic life (7). Samples frequently are incubated to analyze for their presence, but the strains detected usually are not checked to determine whether

they were benign or virulent (7). Somewhat analogously, we "incubate" water samples with live fish to assess whether actively toxic *Pfiesteria* was present at estuarine fish kills (Fig. 7). However, the standardized fish bioassay is used to confirm, for every estuarine fish kill event that has been linked to *Pfiesteria*, that the population was not only present, but also actively toxic during the kill (analogous to a check for "virulence" or infectivity of an infectious agent) (7).

DISTRIBUTION, NUTRITIONAL ECOLOGY, AND PREDATION

Geographic Distribution

The distribution of toxic *Pfiesteria* outbreaks (TOX-A functional type) is restricted, thus far, to the largest and second largest estuaries on the U.S. mainland (Fig. 8; 1). The Albemarle-Pamlico Estuarine System of North Carolina has sustained 4–9 major fish kills (conservatively quantified, involving well over a billion fish) related to *Pfiesteria* as the primary cause (1,32). Several tributaries of Chesapeake Bay in Maryland have also been affected by actively toxic *Pfiesteria* (4 events involving ca. 50,000 fish) (1,2).

Nevertheless, toxic strains of *Pfiesteria* spp. in temporarily nontoxic mode (TOX-B functional type) are much more broadly distributed. Their known range extends from New York estuaries along the Atlantic Coast of the northeastern United States, south and west to Mobile Bay, Alabama on the Gulf Coast (1,11,35). They have also been documented from other geographic regions including the eastern Atlantic (Scandinavia) in the Northern Hemisphere, and New Zealand in the Southern Hemisphere (36). The recent availability of species-specific molecular probes to detect *Pfiesteria* species (17,20) has rapidly expanded the known distribution of these organisms (36). Areas testing positive for the presence of *Pfiesteria* are then tested with standardized fish bioassays to assess the potential for toxic activity of the detected

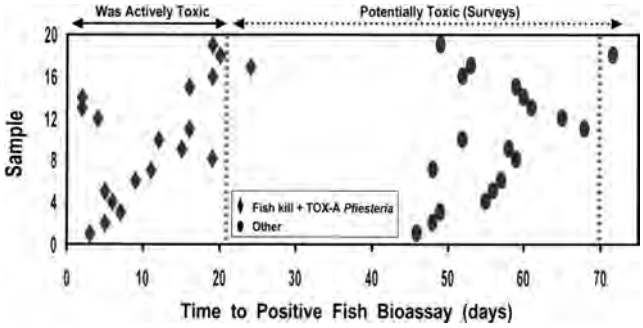

Figure 7. Comparison of the time interval required for positive fish bioassays (with fish-killing activity) for samples collected from estuarine fish kills in which actively toxic *Pfiesteria* spp. (TOX-A functional type) were implicated as primary causative agents (black diamonds; n = 20), versus samples that yielded toxic *Pfiesteria* in fish bioassays of samples taken during survey efforts in estuarine waters without diseased or dying fish (ovals; n = 20) (7). The latter estuarine areas were interpreted to contain potentially toxic populations of TPC species (TOX-B functional type). In all cases, sample transport and other delays extended for 1 to 2 days. Such handling is regarded as especially important for recently toxic populations, for example in efforts to implicate versus rule out involvement of actively toxic *Pfiesteria* from in-progress estuarine fish kills. Note that 19 of the 20 samples from events that were diagnosed as having involved actively toxic *Pfiesteria* tested positive for fish-killing activity within 20 days (longest lag period, 1 sample within 24 days). To err conservatively, we consider that samples appropriately handled (with <3 days' lapse, including transport, following collection during an in-progress fish kill) should produce fish-killing activity within ≤21 days for actively toxic *Pfiesteria* to be implicated as a causative agent involved in the kill. Also note that samples that contained potentially toxic *Pfiesteria* populations which had not been recently in actively toxic mode toward fish did not show fish-killing activity until incubated with live fish for more than 6 weeks; 19 of the 20 samples from that set were ichthyotoxic by 10 weeks, with 1 sample requiring slightly longer. On the basis of well over 1,000 fish bioassays with estuarine samples, thus far we have not obtained toxic isolates of TPC species that have required more than 10.5 weeks to exhibit fish-killing activity, with two exceptions that required more than 12 weeks (7). (From 7. J. M. Burkholder et al., *Environ. Health Perspect.* **109** (Suppl. 5). 745–756 (2001).)

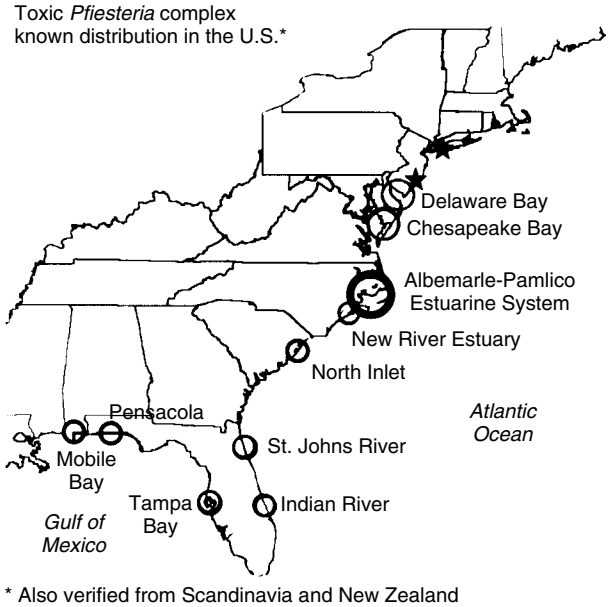

Figure 8. Known distribution of toxic strains of the toxic *Pfiesteria* complex (TPC) in U.S. estuaries. The circles indicate locations where potentially toxic strains (TOX-B functional type) of *Pfiesteria* spp. have been documented through the standardized fish bioassay procedure; and for the Albemarle-Pamlico Estuarine System, Chesapeake Bay, and the New River Estuary, locations where actively toxic *Pfiesteria* spp. have been confirmed during major fish kills, using the same procedure. The stars indicate locations where *Pfiesteria* has been detected using molecular probes (17), but these populations have not yet been tested to include toxic strains (thus, toxicity status is unknown). As indicated, toxic strains of *Pfiesteria* spp. have also been verified from Scandinavia (both species) and New Zealand (*P. shumwayae*). (From 35. H. B. Glasgow et al., in G. M. Hallegraeff et al., eds., *Proceedings of the Ninth Conference on Harmful Algal Blooms*, Intergovernmental Oceanographic Commission of UNESCO, Paris, France.

populations (6,7,11,36). Given that toxic strains of *Pfiesteria* have been confirmed to occur across wide gradients in environmental conditions (36), the data thus far indicate that toxic *Pfiesteria* will likely be found in many geographic regions throughout the world.

Nutritional Ecology

The nutritional ecology of *Pfiesteria* spp. is complex, and nutrient enrichment can stimulate these dinoflagellates through several general mechanisms. Nitrogen (N) and phosphorus (P), two major constituents of nutrient pollution from human activities, have been shown experimentally (as both organic and inorganic forms) to both directly and indirectly stimulate toxic *Pfiesteria* strains (1,11,23). Organic nutrient forms (e.g., glycerophosphate, amino acid mixtures, urea) can be taken up directly by actively toxic as well as nontoxic functional types of *Pfiesteria* zoospores, and by amoebae (1,11). Inorganic nutrient forms (nitrate, phosphate) can be taken up directly by kleptochloroplastidic *Pfiesteria*; or, inorganic nutrients can indirectly stimulate *Pfiesteria,* mediated through abundance of algal prey (1,10,11,23).

Pfiesteria spp. graze algal prey and bacteria, and zoospores have shown preference especially for cryptomonad algae (10,13). When cryptomonads and certain other flagellated algal prey are abundant, zoospores predominate among *Pfiesteria* stages, but if nonmotile prey are abundant (e.g., the cyanobacterium *Synechococcus Cyano the* or the diatom *Thalassiosira*), a higher proportion of the *Pfiesteria* population can consist of amoebae (11). Amoebae also consume cryptomonads and other flagellated algae; commonly in culture, individual lobose amoebae have been observed in the process of capturing up to eight cryptomonads at a time along the cell periphery. Accumulating evidence indicates that *Pfiesteria* thrives in nutrient over-enriched environments that characteristically have abundant food resources (e.g., 10^4–10^5 algal prey mL^{-1}) (1,11,23,32); and that, rather than competing for resources, when preferred prey in the water column begin to be depleted, it switches prey type or moves to the estuarine sediments where alternate prey are in abundance (1,23,32). On the basis of a decade of intensive field studies supported by many laboratory experiments, a conceptual model of the seasonal dynamics of toxic strains of *Pfiesteria* spp. has been constructed to serve as a framework for additional research to strengthen understanding about environmental controls on these dinoflagellates (32).

Pfiesteria spp. zoospores and amoebae can act as predators of macro- and microfauna including rotifers, ciliates, copepods (especially wounded individuals as known for other dinoflagellates such as *Katodinium fungiforme*) (11,13,37); shellfish and finfish (larvae, juveniles, adult stages); and mammalian tissues (e.g., red corpuscles and macrophages) (1,11). Zoospores show swarming activity toward shellfish (e.g., bay scallop *Argopecten irradians*, eastern oyster *Crassostrea virginica*) and finfish larvae (various species) under light microscopy, and appear to show similar attraction toward juveniles and adults (1,11,24). The dominant life cycle stages in the water column with live fish cultures are zoospores, periodically shifting to high gamete production. Under controlled laboratory conditions, TOX-A *Pfiesteria* zoospores have been lethal to all fish species and all individuals tested, including juvenile and/or adult stages of nine estuarine and seven exotic species of finfish, as well as juveniles or adults of four shellfish species (1,11). The only exception has been eastern oysters (24), wherein some adults have survived acute, long-term exposure (weeks; below). Zoospores consume bits of tissue and organic substrates from dying fish, and also attack and feed upon the live animals (1,5,11,13). After fish death, zoospores may remain in the water column if abundant algae and other prey are available; alternatively, if the dead fish are available, zoospores may transform to amoeboid stages that attach to and feed upon the fish remains (11,13,22).

The three functional types of *Pfiesteria* spp. show significant differences in response to nutrient enrichment, algal prey, and fish prey. In experiments with cryptomonad prey, NONIND zoospores attained highest cell production, TOX-A zoospores showed negligible cell production, and TOX-B zoospores were intermediate (38). A similar trend was shown among functional types in response to inorganic nitrogen (nitrate) or inorganic phosphorus (phosphate) enrichment (1). Test of short-term response to fresh fish mucus and excreta, and to shellfish and finfish larvae, usually has yielded the opposite trend, with highest chemosensory attraction shown by TOX-A zoospores of both *Pfiesteria* spp., intermediate response by TOX-B zoospores, and lowest attraction by NONIND zoospores (1,23,39).

Predators of Pfiesteria

Toxic *P. piscicida* zoospores (TOX-A and TOX-B functional types) can be grazed by various microfauna, including ciliates such as *Stylonichia* cf. *putrina*, rotifers (*Brachionus plicatilis* Müller, *Brachionus* sp.), and microcrustacean copepods (*Acartia tonsa* Dana) (11,13,40). Few ill effects on the grazers were apparent in early experiments, except for significant depression of rotifer fecundity after consumption of toxic *P. piscicida* zoospores for more than seven days (11,13,40). Microfauna such as tintinnids (*Tintinnopsis* spp., *Flavella* sp.), ciliates such as *Strombidium* sp. and *Mesodinium pulex*, and the colonial heterotrophic dinoflagellate *Polykrikos* sp. also were shown to graze *Pfiesteria piscicida* zoospores in natural estuarine plankton communities (41). Tintinnids and oligotrichous ciliates generally showed significantly lower grazing on recently TOX-A zoospores than on TOX-B and NONIND zoospores, although some ciliates grazed all three functional types similarly (1,41). Thus far, the data have indicated that although microfaunal grazing may be a significant source of mortality to "nontoxic" (NONIND and TOX-B) zoospores, grazing by these microfauna on toxic or recently toxic zoospores may be relatively low.

Shellfish macrofauna (northern quahog *Mercenaria mercenaria* juveniles; eastern oyster *C. virginica* pediveliger larvae, juveniles, sub-adults and adults; and bay scallop *A. irradians* pediveliger larvae and adults) have been shown experimentally to consume *Pfiesteria* zoospores, with varying consequences. High mortality occurred within minutes to hours for larval and juvenile stages, and for adult bay scallops fed TOX-A zoospores,

with no mortality of control animals that were not exposed to toxic *Pfiesteria* (11,24). In contrast, adult oysters were sometimes narcotized with depressed filtering activity, but were still alive after being fed TOX-A *P. piscicida* zoospores for three weeks (1,11,24). For scallop and oyster pediveliger larvae, mortality appeared to involve both physical and toxin effects. Larvae that had discarded their vela were physically attacked and devoured by the zoospores within minutes (Fig. 9; 24). In other experiments, oyster pediveliger larvae were maintained for 60-min trials with treatments as TOX-A zoospores, TOX-B zoospores, a mix of half TOX-B zoospores and half benign cryptomonad algae, and cryptomonad algae alone (controls) (24). The zoospores and/or cryptomonads were held within dialysis membrane tubing (0.22 μm porosity) to prevent direct contact with the larvae. Within 45 minutes, 100% of the larvae in containers with TOX-A zoospores were dead (n = 5 with 100 larvae per replicate); mortality was intermediate with TOX-B zoospores (ca. 50%) and with the mixture of TOX-B zoospores and cryptomonad algae (ca. 30%); and there was no mortality in the controls (36). These data indicate a toxin effect (24), since there was no direct contact between *Pfiesteria* and the larvae, and since the dialysis membrane would have allowed chemical communication (passage of *Pfiesteria* toxin) (1,11).

Grazing experiments indicated that juvenile oysters filtered significantly more NONIND than TOX-A zoospores over a 24-hour period, with intermediate filtering of TOX-B zoospores (24). Subadult oysters, in contrast, showed comparable filtering of NONIND and TOX-B zoospores, and significantly lower filtering of TOX-A zoospores. The data indicated that TOX-B zoospores retained residual toxicity that was detected by the sensitive larvae, but that the toxicity of that functional type/isolate was too low to affect filtering by the sub-adults (24).

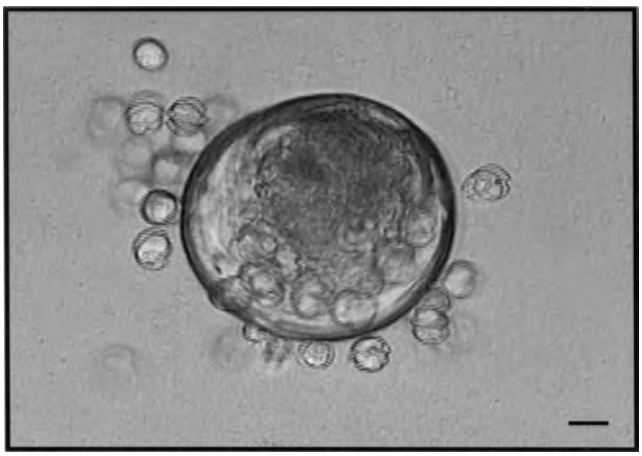

Figure 9. Actively toxic zoospores of *Pfiesteria piscicida* in the process of devouring an oyster pediveliger larva (scale bar = 10 μm). Note that many zoospores are swarming around the larva, and that some had used their peduncles to pry open the larva's shells to gain access to the soft tissues of the still-live organism which were being rapidly consumed. The tissues of this larva were completely consumed by the zoospores within less than 30 minutes, except for the tough adductor muscle which was not attacked. (Photo from 24. J. Springer, *Interactions Between Two Commercially Important Species of Bivalve Molluscs and the Toxic Estuarine Dinoflagellate*, Pfiesteria piscicida, MS Thesis, North Crolina State University, Raleigh, N.C., 2000.)

Given that subadult and adult oysters can remain viable after extended exposure to toxic *Pfiesteria*, the question remained: Is *Pfiesteria* killed and digested as it passes through the oyster gut tract? When feces from the subadult oysters used in the grazing experiment were examined, they were found to contain high numbers of *Pfiesteria* temporary cysts (24). These cysts were isolated and tested for excystment and zoospore motility over the next 24 hours (n = 5, 100 cysts per replicate). Within 20 hours after gut tract passage, about 90% of the previously TOX-A zoospores had excysted and regained motility, with lower (ca. 40-70%) survival by TOX-B and NONIND zoospores (24). These experiments collectively indicate that toxic *Pfiesteria* zoospores could potentially affect recruitment and survival of commercially important shellfish species, whereas adult oysters might be of value in trophic mitigation or control of toxic *Pfiesteria* (24). Shellfish that have consumed *Pfiesteria* zoospores before being transported for aquaculture could provide an important mechanism of dispersing toxic *Pfiesteria* to other geographical regions (24). These data also suggest the potential for certain shellfish species to concentrate viable, toxic *Pfiesteria* strains, supporting concerns about these toxic dinoflagellates in relation to seafood safety (24).

SUBLETHAL AND CHRONIC IMPACTS OF TOXIC *PFIESTERIA* ON FISH AND MAMMALIAN HEALTH

Finfish Disease

In controlled laboratory trials, toxic clonal *Pfiesteria* spp. have caused finfish disease in subacute exposure. Tested species have included juvenile tilapia (*Oreochromis mossambica*, *O. aureus*), striped bass (*Morone saxatilis*), and hybrid striped bass (*Morone saxatilis* x *M. chrysops*) (1). Toxic zoospore densities of ≥100 to 5,400 cells mL^{-1} have induced destruction of fish epithelium and skeletal muscle both as superficial, nonfocal lesions that destroy the osmoregulatory system over most of the fish body (42), and as deep focal lesions that often occur on the ventral surface of the fish where the epithelium is thinnest (Fig. 10; 1). Lesions typically develop within ≤2 to 12 hours, frequently involving hemorrhaging and exposure of viscera (1). The occurrence of lesions and the period required for their development from exposure to toxic *Pfiesteria* depend on the zoospore densities and the specific isolate used (1). Exposure to toxic *Pfiesteria* can also cause damage to finfish gill, hepatopancreas, cornea, kidney, liver, and brain (1,32). Control fish, treated similarly except without exposure to toxic *Pfiesteria*, have remained healthy without signs of disease except for occasional mild granulomas (1,32).

Recovery from sublethal exposure to toxic *Pfiesteria* has been tested by removing fish with mild to moderate lesion development from further exposure to toxic *Pfiesteria*, and tracking their health for six weeks. Control fish, treated similarly except for no prior exposure to toxic *Pfiesteria*, remained healthy without lesions (42). Although the test fish recovered from their earlier lesions, most developed infections from opportunistic bacteria and fungi and about

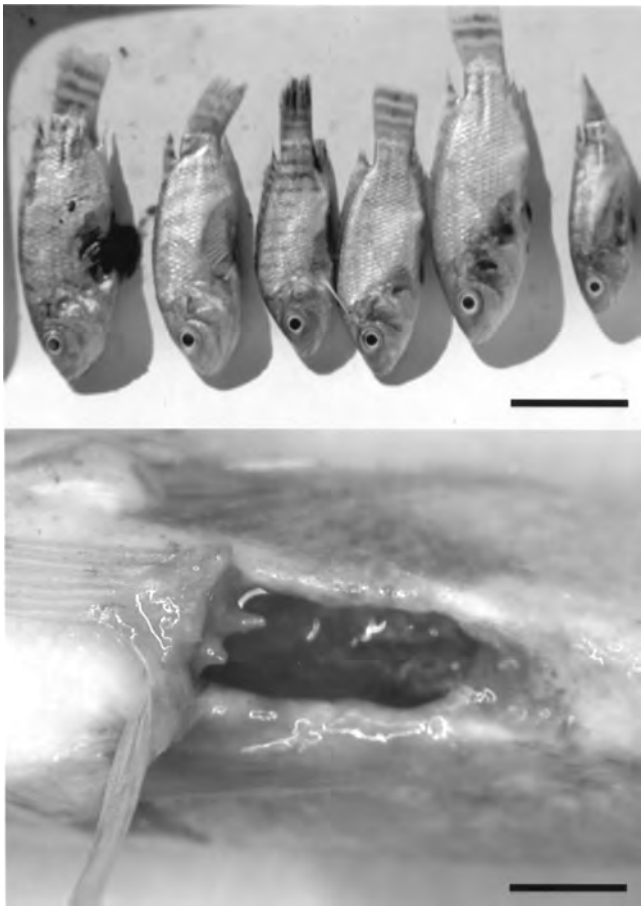

Figure 10. Focal lesion development resulting from exposure of tilapia (*Oreochromis mossambicus*, total length 5–7 cm) to actively toxic, clonal *Pfiesteria piscicida* in controlled laboratory trials, including (upper panel) Tilapia after 8 to 12 hr of exposure to 2.3 to 5.4×10^3 toxic zoospores/ mL (scale bar = 5 cm); and (lower panel) Oblique lateral view showing a deep, bleeding, ulcerated focal lesion posterior to the pectoral fin (scale bar = 1 cm). See color insert.

80% developed bleeding lesions (42). The data suggest that fish given sublethal exposure to toxic *Pfiesteria* may have a compromised immune system as a long-term impact. Experiments are underway to test this premise.

Pfiesteria has been implicated in some ulcerative disease events in juvenile Atlantic menhaden, and probably acts as a primary or secondary stressor in diseases of wild fish, depending on the specific circumstances (1). Two isolates of an *Aphanomyces* fungus (*A. invadans*) have been tested as being capable of acting as primary invaders with high virulence in causing menhaden lesions (43). Limited tests have been run to link cultured menhaden disease to the presence of invasive, highly virulent strains of *A. invadans* and weakly toxic strains of *P. shumwayae* (tested as incapable of causing deep focal lesions) (44). From such limited testing, the fungus has been assessed by some researchers as the primary initiator of menhaden lesions with toxic *Pfiesteria* involvement ruled unlikely or rejected (43,44), despite the fact that the fungus has been commonly described as a secondary opportunistic pathogen or a highly virulent invader, depending upon the strain (34,42). Virulence within a given pathogen species is known to vary widely, from benign to highly invasive (34). It is hypothesized here, instead, that toxic *Pfiestera* can interact (by physical attach and/or toxin effects) with his hind other pathogenic microbes to cause, promote, and/or advance ulcerated lesions and other estuarine fish disease (1,30). Field-reliable assays for purified *Pfiesteria* toxin will greatly advance efforts to clarify the role of *Pfiesteria* in estuarine fish epizootics (1,32).

Mammalian Health Impacts

Laboratory staff sustained respiratory, visual, and neurological impacts when they inhaled aerosols from fish-killing *Pfiesteria* cultures or had contact with culture water. The cultures had consisted of cell densities that were within the range of cell concentrations found at estuarine *Pfiesteria*-related fish kills (14). The most striking symptom was severe cognitive impairment involving learning disabilities, manifested as Alzheimer's-like short-term memory dysfunction, which required days to about three months for recovery to within normal range of function. Other symptoms indicated autonomic and peripheral nervous system dysfunction, asthma-like respiratory problems, skin lesions that did not respond to antibiotics, severe headaches that did not respond to normal treatment, joint and muscle pain, vomiting and stomach cramping, and problems with vision (14). Long-term effects, lasting for years and linked to the same exposures, have included autoimmune system dysfunction and chronic respiratory infections suggestive of immune system suppression. Experimental laboratory research repeatedly documented significantly reduced cognitive function and chronic learning disabilities in rats after they were exposed to toxic *Pfiesteria* culture (filtered, 0.2 μm porosity; or unfiltered) by subcutaneous injection (45).

Little information is available about human health impacts from environmental exposure to actively toxic *Pfiesteria*. In the absence of available purified toxin standard to enable field-reliable assays to detect *Pfiesteria* toxin (below), the most reliable symptom of toxic *Pfiesteria* exposure has been severe cognitive impairment with learning disabilities (14,15). The previously mentioned laboratory staff had recovered to within normal range of cognitive function within approximately three months or less, indicating that people suspected to have sustained environmental exposure to in-progress estuarine fish kills (only kills that were verified to have involved actively toxic *Pfiesteria*) should be evaluated for learning disabilities as soon as possible after exposure. However, thus far there has been only one clinical examination that was conducted shortly (within $\leq$3 weeks) after subjects had been exposed to major estuarine fish kills that were linked to actively toxic *Pfiesteria* (15) (it should be noted that the species identifications were confirmed as *Pfiesteria piscicida* at three of four events, and as *P. piscicida* with subdominant *P. shumwayae* at one event, but cross-corroboration of the identifications by three independent laboratories was not completed until after the clinical study (15) went to press (1)). That study described

"profound learning disabilities" in some subjects who were evaluated in the bottom 2% of the U.S. population in ability to learn or remember (data were normalized for age and education). These people required three to six months to recover to within normal range of learning ability (15).

Other efforts have been conducted to determine whether people sustain health problems after "exposure to estuaries," but in the absence of fish kills verified to have involved actively toxic *Pfiesteria* (46,47). Such efforts, logically, cannot be related to *Pfiesteria*, and would be analogous to an attempt to study burn victims without a fire. As mentioned sufficient purified toxin standard is not yet available to enable field-reliable diagnostic tests to verify whether people have sustained toxin exposure, and to quantify the exposure. Field-reliable assays for purified *Pfiesteria* toxin will greatly advance efforts to clarify the extent to which environmental exposure to actively toxic *Pfiesteria* causes human health impacts in estuaries (1).

Water-Soluble Pfiesteria Toxin

The mass-production of *Pfiesteria* toxin, an expensive undertaking in biohazard III containment systems, has remained severely restricted because the required funding has not been directed to support such efforts of specialists with demonstrated expertise and appropriately sized/equipped facilities (1,7). Despite this restriction, a water-soluble toxin from *Pfiesteria piscicida* has been purified and characterization of its chemical structure is nearly complete (48,49). A reporter gene assay has been developed to detect the toxin, using GH4 mammalian (rat) pituitary cells together with a c-fos-luciferase reporter gene (28,31). The c-fos promoter is ligated to a "reporter" — here, firefly luciferase — which, when activated, can be measured by light emission. The construct is stably expressed in rat pituitary tumor cells, so that the cells behave as a biosensor by generating light when they sense the *Pfiesteria* toxin. At low doses, the reporter gene is induced; at higher doses, a cytotoxic effect causes a decrease in the amount of light generated from the cells. This assay has been used to guide final purification of the toxin (28,48). Once sufficient purified toxin standard is available, the assay will be modified to make it "field-reliable" for use in detecting *Pfiesteria* toxin from estuarine water and fish tissue.

Kimm-Brinson and coworkers (28) presented peer-reviewed, published data on the mode of action of *Pfiesteria* toxin wherein clones from natural estuarine samples were tested in the standardized fish bioassay process and the clonal status, species identification, and ichthyotoxicity of the cultures were cross-corroborated by independent specialists in toxic *Pfiesteria* research. The water-soluble *Pfiesteria* toxin (described as putative because the toxin had not been purified at the time the paper was written) mimics an ATP neurotransmitter, and increases intracellular calcium membrane permeability in GH4C1 rat pituitary cells by a mechanism consistent with activation of $P2X_7$ purinoreceptors (24). $P2X_7$ receptors are mostly located on the surface of immune cells; they are well characterized in activated macrophages in peripheral tissues and microglial cells in the brain, and are key effectors of chronic inflammation (28). Microglial cells are specialized cells that act as the brain's immune system counterpart to macrophages in other organs. Targeting of $P2X_7$ receptors by *Pfiesteria* toxin presents a mechanism that could explain seemingly disparate impacts of toxic *Pfiesteria* exposure, such as lesions and inflammatory responses in finfish (e.g., following tissue damage by *Pfiesteria* cells), and central nervous system dysfunction as well as inflammatory responses such as asthma-like symptoms in humans (14,28).

CONCLUSION

Species of the toxic *Pfiesteria* complex thus far include *P. piscicida* and *P. shumwayae*. These species share morphological and genetic similarities, and have toxic strains that (*1*) show strong attraction to live fish; (*2*) exhibit toxicity that is triggered by live fish or their fresh tissues and excreta; and (*3*) produce toxin(s) that cause fish stress, disease and death under ecologically relevant conditions (the standardized fish bioassay process involves testing live *Pfiesteria* cells at similar densities as those encountered during *Pfiesteria*-related fish kill/disease events). Both *Pfiesteria* species also have a complex life cycle with multiple amoeboid, flagellated, and cyst stages, several of which are ichthyotoxic. TPC species have broad temperature and salinity tolerance. Their prey span the estuarine food web from bacteria to mammalian tissues, and they can be stimulated directly or indirectly by nutrient pollution. Zoospores of toxic strains can be either actively or potentially toxic (TOX-A and TOX-B functional types, respectively). Other naturally occurring populations can be benign (noninducible or NONIND functional type, apparently lacking the ability to produce toxins that cause fish disease or death). The three functional types of *Pfiesteria* spp. zoospores differ significantly in response to algal prey, predators, nutrients, and fish. Moreover, as an apparent artifact of culture conditions, toxic strains generally lose their ability to cause fish death and disease and become NONIND over time in culture. Toxic strains of *Pfiesteria* species have been confirmed from mid-Atlantic and Gulf Coast estuaries in the United States, and from northern Europe and New Zealand, indicating that these toxic dinoflagellates are cosmopolitan in distribution. At low cell densities, toxic strains can be causative agents of acute and/or chronic, diffuse and focal lesions, and of other fish diseases, as demonstrated in appropriately conducted fish bioassays. Respiratory, visual, and neurological impacts have been sustained by people exposed to aerosols from fish-killing *Pfiesteria* cultures, or to water and aerosols during estuarine fish kills associated with toxic *Pfiesteria*. Neurocognitive impacts from exposure to toxic *Pfiesteria* have been replicated experimentally in small mammals. A purified, water-soluble *Pfiesteria* toxin disrupts calcium metabolism in rat pituitary cells and mimics an ATP neurotransmitter that targets $P2X_7$ purinoreceptors found predominantly in fish and humans on microglial cells, the immune system cells that line the brain. Sufficient quantity of purified toxin standard is not

yet available, and is needed to develop field-reliable assays for use in early warning systems, and to resolve the role of toxic *Pfiesteria* in fish disease, seafood contamination, and human health impacts in affected estuaries.

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PHAGE: DETECTION METHODOLOGIES.

See BACTERIOPHAGE DETECTION METHODOLOGIES

PHOSPHORUS CYCLING IN AQUATIC ENVIRONMENTS: ROLE OF BACTERIA

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Phosphorus is an essential nutrient for life and often the limiting nutrient in aquatic ecosystems, especially freshwater ones. As the most abundant component of the aquatic biota and usually the component with the largest surface area, bacteria have an important role to play in the cycling of phosphorus in aquatic ecosystems, a role that has not always been recognized. They have high cellular phosphorus concentrations and provide an important source of phosphorus-rich particles for grazers. Bacteria also compete with phytoplankton for phosphorus uptake, and they regenerate inorganic phosphate from organic phosphate with enzymes near the cell surface, although this regenerated phosphate is often only available to the bacterial cell itself.

BACKGROUND

Definitions, Abbreviations, and the Aquatic Phosphorus Cycle

Varying definitions and abbreviations of environmental phosphate pools are a significant source of confusion for those new to phosphorus cycling studies. In this article, these will be limited to a few essential categories. Phosphorus (P) refers to any form of the element and may also be called phosphate (although strictly speaking this is the inorganic ion). Dissolved inorganic phosphorus (DIP) is the inorganic component of the dissolved pool; another commonly used term is soluble reactive phosphorus (SRP). DIP includes orthophosphate (P_i) and polyphosphate, composed of various polymers of orthophosphate, is also in this fraction. Dissolved organic phosphorus (DOP) is the organic component of the dissolved fraction; this has also been referred to as soluble nonreactive phosphorus (SNP). DIP and DOP together make up the total dissolved phosphorus (TDP). Particulate phosphorus (PP) may sometimes be separated into organic and inorganic fractions. Unlike the nitrogen cycle, the phosphorus cycle includes neither gaseous components nor oxidation state changes. Most of the transformations are from dissolved to particulate or the reverse, or from inorganic to organic or the reverse. Different phosphorus components dominate in different aquatic environments. In oligotrophic low-nutrient environments such as the open ocean and some lakes, the DOP is dominant. In many turbid rivers, the PP may dominate. Unlike the nitrogen cycle, the phosphorus cycle includes no gaseous components or oxidation state changes. Bacteria refers specifically to heterotrophic bacteria; cyanobacteria are discussed separately. Phytoplankton include all photosynthetic plankton, both eukaryotes and the prokaryotic photosynthetic cyanobacteria.

Figure 1 shows a simplified phosphorus cycle. The dissolved components are outlined in ovals, the particulate components in squares. The abbreviations are defined above. The arrows show the major interactions, although not every pathway is illustrated. For this article, the pathways to and from the bacteria are particularly important. Phosphorus may be sequestered or released by the sediments, depending on the redox state (see following section).

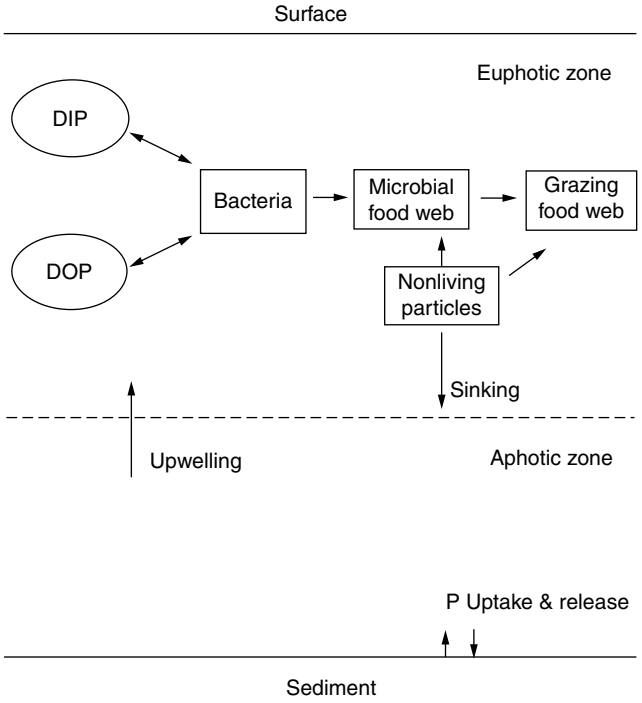

Figure 1. A simplified phosphorus cycle. Dissolved components are outlined in ovals, the particulate components in squares. Arrows indicate the major interactions, although not every pathway is illustrated. DIP is dissolved inorganic phosphorus, DOP is dissolved organic phosphorus, see text for more details. Modified from C. R. Benitez-Nelson, *Earth-Sci. Rev.* **51**, 109–135 (2000).

Pho Regulon of *Escherichia coli*

The molecular model used to describe bacterial transformations and assimilation of phosphorus has been the Pho Regulon of the gram-negative bacterium *E. coli*. This system has been studied for a long time and is the best described. Under P-sufficient conditions, *E. coli* cells use a low-affinity P_i transport system known as the Pit system, which is energized the cell's protonmotive force (2). At submicromolar concentrations this system becomes inefficient, and the Pho Regulon is turned on, as signaled by a rapid induction of alkaline phosphatase (AP) activity. A diagram of the Pho Regulon is shown in Figure 2. The Pho Regulon is a suite of genes that code for proteins that facilitate P-assimilation under low-P conditions, including the high-affinity Pst transport system for P_i. These include (examples only, not comprehensive) outer membrane porins (PhoE), periplasmic enzymes (AP or PhoA) and binding proteins (PstS), membrane-associated transport proteins (PstA, PstC), as well as cytoplasmic proteins with regulatory and other functions (PhoB, PhnO).

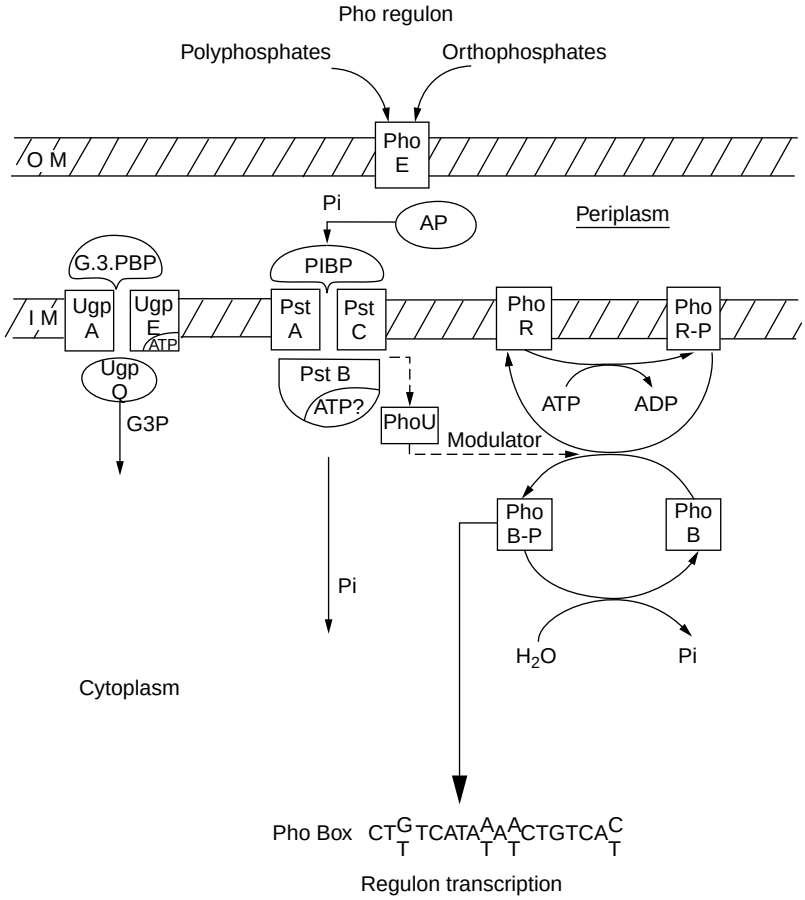

Figure 2. Illustrates the bacterial outer membrane (OM), periplasm, inner membrane (IM), and cytoplasm, working from the top down. The Pho Regulon is a suite of genes that code for proteins that facilitate phosphorus-assimilation under low-phosphorus conditions, including the high-affinity Pst transport system for P_i. Components of the Pst transport system are listed as PstA, PstC, and so on. PhoE is the outer membrane porin and AP is the periplasmic enzyme alkaline phosphatase. Proteins labeled Ugp are part of the transport system for glycerol-3-phosphate. Most of the other Pho proteins are involved in the regulation of the Pho Regulon expression. From A. Torrani-Gorini, in E. Yagil and S. Silver, eds., *Phosphate in Microorganisms: Cellular and molecular biology*, ASM Press, Washington, D.C., 1994, pp. 1–4.

The diagram of the Pho Regulon (Fig. 2) demonstrates that *E. coli* and most other bacteria transport phosphate largely in the inorganic form as orthophosphate (P_i), few organic phosphate compounds are taken up directly, most are hydrolyzed by alkaline phosphatase or other enzymes first. These enzymes remove the P_i group, allowing it and remaining organic component to be assimilated separately. Among the few exceptions to the rule that only P_i is directly transported, *E. coli* has a transport system for glycerol-3-phosphate, the Ugp system shown in Fig. 2, which is also considered a component of the Pho Regulon (2). *Escherichia coli* also has a UhpT system, which exchanges external glucose–6-phosphate for internal P_i (3). Only bacteria that are intracellular parasites, such as *Rickettsia* and others, which live in an environment with high organic phosphate concentrations, seem to gain a large fraction of their required phosphate through transport or exchange organic phosphate compounds (3).

Studies of the marine cyanobacteria *Synechococcus* and *Prochlorococcus*, which are important components of the marine foodweb, have shown that these organisms have a P_i-binding protein, homologous to the PstS gene product in *E. coli*. Expression of this protein was observed in lab cultures of *Synechococcus* when the P_i concentration declined below 50 nM (4). PstS expression by these organisms was also shown in field samples collected to a depth of 100 m at the Bermuda Time-series Study site (5), which is a microbially dominated, oligotrophic open-ocean station 82 km southeast of Bermuda. Others have reported evidence for phosphorus-limitation of heterotrophic bacterial growth at this site (6) where DIP concentrations in the euphotic zone can be as low as 1 to 10 nM (7) when measured with a highly sensitive method (8). Kinetics studies of Pi uptake at the same location show half-saturation constants in the same range (Ammerman, unpublished). The complete genome sequence of the freshwater cyanobacterium, *Synechocystis* sp., also reveals two genes that are highly homologous to the PstS gene in *E. coli* (9), as well as the rest of the Pst transport system. The draft genome sequences of both marine cyanobacteria discussed earlier, *Synechococcus* and *Prochlorococcus*, also clearly show the presence of the complete Pst system. These sequences are currently unpublished but are found on the website of the Institute for Genomic Research (*www.tigr.org*) under microbial genomes in progress.

Vibrio cholerae, the agent of cholera, is the first representative of this commonly isolated genus of heterotrophic marine bacteria to have its genome completely sequenced (10). It apparently has multiple high-affinity Pst phosphate transport systems on its two chromosomes, including two genes homologous to the *E. coli* PstS (10,11). Another component of the Pho Regulon in *V. cholerae* is the Ugp transport system for glycerol-3-phosphate. However, although *V. cholerae* is an enteric bacterium closely related to *E. coli*, it surprisingly has no homolog to PhoA,

the alkaline phosphatase gene. Alkaline phosphatase is an important component of the *E. coli* Pho Regulon and an enzyme activity commonly measured as an indicator of phosphorus limitation in aquatic environments (discussed later). It is apparent that the *E. coli* model of phosphorus assimilation will not be sufficient for all aquatic bacteria, even some of those closely related to it. Fortunately, however, the increasing availability of the genome sequences of aquatic microbial species will allow us to better address these questions.

BACTERIAL P CYCLING IN NATURE

Carbon to Phosphorus Ratios of Bacteria Cells

Bacteria have large phosphorus requirements and a relatively low carbon to phosphorus (C:P) ratio of about 50 (12). Interestingly, this ratio greatly exceeds the C:P ratio of phytoplankton; it is about double the well-established Redfield ratio of 106 : 1 (13). The bacterial C:P ratio in cultured cells is also extremely variable (varying by a factor of 50), much more than the C:N ratio, and reflects the C:P ratio of the growth media (12). The major phosphorus-rich components of bacterial cells are nucleic acids, membrane phospholipids, and sometimes the storage product polyphosphate, whereas protein is the major nitrogen-containing component. The fraction of bacterial biomass devoted to nucleic acids (particularly RNA) and phospholipids varies significantly with growth conditions, growth rate, and cell volume. The cell volume is also inversely related to growth rate. In contrast, the fraction of biomass that is protein, varies little with growth rate. This explains why bacterial C:P is more variable than C:N (12). The small size of heterotrophic bacteria relative to phytoplankton (including cyanobacteria) may also explain why bacteria are phosphorus-rich relative to phytoplankton. In smaller cells, the surface to volume ratio is greatly enhanced, and this means more phosphorus-rich membrane phospholipid (12).

Bacterial Role in Phosphorus Uptake and Regeneration

Phosphorus cycling has long been studied in lakes because of the importance of phosphorus as a limiting nutrient in freshwater environments. There have been fewer studies of phosphorus cycling in marine and estuarine environments because of the focus on nitrogen, not phosphorus, as the limiting nutrient. Most early studies of aquatic phosphorus cycling, at least those in the water column, emphasized the role of phytoplankton, rather than bacteria. Water column studies of phosphorus cycling by phytoplankton through the early 1980s have been extensively reviewed (14,15).

In the past, bacteria in aquatic ecosystems were considered to be important only in phosphorus regeneration, or mineralization of the organic to the inorganic form, followed by release to the environment. Only a few early papers suggested that they might be important in phosphorus uptake, see references in (16). However, since the development of the microbial food web paradigm in the early 1980s (17), many studies have examined the role of aquatic bacteria in phosphorus cycling, including the following reviews (16,18,19). Among other important roles in aquatic food webs, bacteria are very effective competitors with phytoplankton for P_i, as shown more than 15 years ago (20). Heterotrophic bacteria are now known to be responsible for about 60% of the inorganic phosphate uptake in various aquatic ecosystems (19), as determined by the radiolabeled-P_i taken up by the plankton fraction passing through 0.8 or 1.0 μm polycarbonate filter. Some of this uptake may be due to cyanobacteria or other small phytoplankton found in the same size fraction. Studies that were specifically corrected for phytoplankton found a somewhat lower fraction of uptake by the heterotrophic bacteria (19). Phosphorus-limited bacteria and cyanobacteria are also capable of "luxury uptake" of P_i beyond the immediate cellular requirements when additional P_i is provided (5,16).

In addition to competing directly with phytoplankton for uptake of DIP, heterotrophic bacteria can be important in the regeneration of phosphate and other nutrients (12), the role that they have been traditionally assigned. However, in P-limited systems, bacteria are probably not net mineralizers at all, but rather a sink for inorganic phosphorus (16). Any DOP regenerated to DIP by bacteria would be immediately taken up by the same cell. In this scenario, the bacteria would still be an important link in the regeneration process, as a major phosphorus-rich source of particles for grazers (particularly bacterivores) in aquatic ecosystems. Because bacterivory accounts for a large fraction of the bacterial production in many aquatic environments (21), this is an important connection. In non-P-limited systems, however, DIP that has been regenerated from DOP by bacterial "ectoenzyme" activity (discussed later) is made available for other organisms in the food web (22).

Bacteria have high-affinity transport systems, presumably because of the presence of the P_i -binding proteins discussed above. Vadstein (16) reviewed P_i uptake parameters for bacteria from his and other studies. In seven different bacterial strains or communities analyzed, the median half-saturation constant (K_m) for P_i uptake was 90 nM, with a range of 10 to 250 nM. This contributes to their effective competition with phytoplankton for P_i. However, they also have large cellular phosphorus requirements, as discussed earlier (12). Although bacteria are often considered more efficient at P_i uptake than phytoplankton (20), others suggest that this competitive ability varies with a uniform or pulsed nutrient supply (16). None-the-less, phosphorus-limitation of bacterial growth is common during summer stratification in lakes (16), and also occurs in marine systems (6,23,24).

Enzymatic Hydrolysis of DOP

Cell-surface enzymes of aquatic microbes together with "extracellular" enzymes that are dissolved or adsorbed to other than their original particles (25), are important catalysts in the decomposition of dissolved organic matter (DOM) and particulate organic matter (POM). In aquatic science these are sometimes collectively referred to as "ectoenzymes" to distinguish them from other types. Polymer hydrolysis can be the rate-limiting step in microbial DOM and POM utilization (26). Efficient hydrolysis and

utilization of these polymers is important to the rapid microbial growth seen in many aquatic ecosystems (27). In a review, Chrost (28) lists 18 different microbial ectoenzymes whose activities have been measured in natural waters and sediments. A major component of these are phosphatases, such as alkaline phosphatase, enzymes that hydrolyze phosphate groups from organic phosphate compounds (specifically phosphate esters) making the phosphate group available for assimilation.

Other ectoenzymes include those that hydrolyze polymeric carbohydrates or proteins, such as glucosidases and peptidases. Most of these ectoenzymes are found on the cell surface or in the periplasmic space of gram-negative heterotrophic bacteria (29), and appear to be ubiquitous in aquatic isolates (27). Certain ectoenzymes, such as alkaline phosphatase (AP) and leucine aminopeptidase, are also found in some species of cyanobacteria and eukaryotic phytoplankton (30–32). Alkaline phosphatase is typically induced by phosphorus deficiency, and in *E. coli* and some other prokaryotes, this enzyme is part of the Pho regulon discussed earlier. Alkaline phosphatase activity in eukaryotic phytoplankton is also a response to phosphorus-deficiency, as has been known for some time (14,33), however, phosphatase regulation in eukaryotic phytoplankton is not as well understood as in prokaryotes (5).

Marine field studies of phosphatases using high-sensitivity fluorescent substrates date back more than twenty-five years (34). In the last fifteen years there has also been increased interest in many different ectoenzymes in a variety of aquatic environments (28), particularly since the introduction of the fluorescent methylumbelliferyl and related derivatives (35). These derivatives offer the advantages of low background fluorescence and are available as substrates for many different enzymes. There have also been recent improvements in the analysis methods for the fluorescent hydrolysis products, such as the use of flow-injection (36) or of fluorescence microplate readers (37,38). Recently, alkaline phosphate activity has been measured by continuous flow methods aboard ship, allowing its surface distribution to be mapped in detail (39).

Alkaline phosphatase activity is easier to interpret than many other ectoenzyme activities, because it usually is a response to phosphorus-deficiency. Many of the early AP studies, mostly in phytoplankton, have been extensively reviewed (14,15). Although there is often a positive relationship between AP activity and biomass, it is probably not the controlling factor, as suggested by some (40). Very high AP activities were found in three different estuarine and coastal ecosystems only when DIP concentrations were less than 200 nM (18). Criteria for P-limitation based on AP activity normalized to chlorophyll *a* have been developed for freshwater, although not widely applied (41,42). The use of this or similar biomass-corrected criteria should eliminate the concern the AP activity is dominated by biomass effects.

Nonetheless, "alkaline phosphatase" activity, as commonly measured in the field, is a mix of different enzyme activities from different organisms that can hydrolyze the substrate, which is usually a fluorescent or chromogenic analog of natural substrates. These different enzymes probably also have different kinetic properties. Aggregate kinetic properties for AP activities measured in the field, typically range from 1 to 10 μM for the half-saturation (K_m), and 0.1 to 1.0 $\mu mol\, l^{-1}\, h^{-1}$ for the maximum activity (V_{max}) (38,39). Activity is also found in different size fractions, including phytoplankton greater than 2 μm, bacteria and cyanobacteria less than 2 μm (often referred to as "picoplankton"), and sometimes a "dissolved" fraction less than 2 μm. In a recent example from the oligotrophic Red Sea (42), most of the AP activity was associated with *Synechococcus* or dissolved. In contrast, in the eutrophic Mississippi River plume, about half of the AP activity is in the size fraction greater than 10 μm (Ammerman, unpublished). The function of dissolved activity is difficult to understand, but occurs regularly enough such that methodological or functional explanations are needed.

Clearly, new approaches are needed to better understand the sources of AP activity and its regulation. As discussed earlier, the genome sequence for *V. cholerae* does not even have a gene homologous to the *E. coli PhoA* gene for alkaline phosphatase, although we assume that most aquatic bacteria have this enzyme. An example of a novel approach to these issues is the use of the fluorescent ELF substrate to label the alkaline phosphatase of phosphate-stressed dinoflagellate, Prorocentrum minimum, in the lab and the field (43,44). ELF (Enzyme-Labeled Fluorescence; Molecular Probes, Eugene, Oregon) is an enzyme substrate that forms an insoluble precipitate at the site of the activity.

Another periplasmic ectoenzyme involved in phosphorus cycling, that has also been measured in aquatic ecosystems is 5′-nucleotidase, which is specific for 5′-nucleotides (45–48). This enzyme is involved in nucleotide and nucleoside salvage and metabolism, and is sometimes included in the Pho Regulon (2). However, at least in aquatic systems it apparently responds more to the demand for carbon rather than phosphorus (18), and is largely independent of the DIP concentration. Although it can regenerate significant amounts of DIP from nucleotides, its primary function may be to remove the Pi group from nucleotides, allowing further metabolism of the remaining nucleoside (18). Although apparently a prokaryotic enzyme, there is at least one report of 5′-nucleotidase activity in a eukaryotic phytoplankter (49). Both the *V. cholerae* and *Synechocystis* sp. genome sequences contain multiple proteins homologous to the 5′-nucleotidase gene (UshA) in *E. coli* (11).

Figure 3 shows the major steps in the breakdown of DOP polymers and monomers into P_i and an organic carbon moiety. Nucleases first hydrolyze nucleic acid polymers to monomers. AP and 5′-nucleotidase enzymes then hydrolyze these DOP monomers and those from other sources, including nucleotides and phosphate esters. Other unstudied enzymes may also be involved. The resulting P_i group and carbon moiety may then be taken up by separate transport systems in the same cell, or released to the environment in which they may be exploited by other cells. The fate of these groups apparently depends on the current nutritional status of the cell in terms of phosphorus and organic carbon.

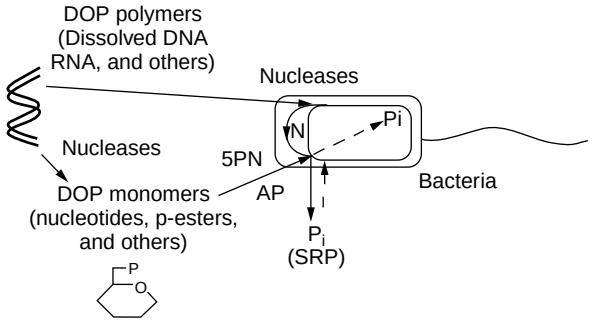

Figure 3. Diagrams the function of the bacterial ectoenzymes alkaline phosphatase (AP) and 5′-nucleotidase (5PN) in the hydrolysis of dissolved organic phosphorus (DOP). Other abbreviations include orthophosphate (Pi) and soluble reactive phosphorus (SRP). The "N" shows the path of a nucleotide after its production by nuclease and before hydrolysis by 5PN. From (47).

Sediments

Detailed discussion of bacterial phosphorus cycling in sediments is beyond the scope of this chapter, although a current review of the marine phosphorus cycle including the sedimentary component is available (1). Although most organic phosphorus that reaches the sediment is released into the water column, a small fraction is buried as organic matter, or through precipitation or other processes, including phosphorite burial in marine systems (1). If the deep water and surface sediment becomes anoxic, some of this sedimentary phosphorus may be released, a seasonal occurrence well known in the Chesapeake Bay and some other estuaries (50). Bacterial involvement is suspected in some of the processes that take up and release phosphorus from sediments, including phosphorite formation (1,51), but more study is needed.

CONCLUSION

The importance of bacteria to phosphorus cycling should be clear from the foregoing. Bacteria compete with phytoplankton for phosphorus uptake, hydrolyze organic phosphate compounds with ectoenzymes, and provide phosphorus-rich particles for grazers. Although there are many questions remaining to be answered, novel approaches are providing new opportunities for progress. The ongoing revolution in microbial genomics will allow us to describe the molecular mechanisms of phosphorus transformation and assimilation by aquatic bacteria. New enzyme substrates such as ELF (44), discussed above, and continuous enzyme assays (39), will improve the specificity and utility of enzyme data. New chemical and bioassay methods for P_i and other forms of phosphorus are pushing the detection limits and in situ concentrations ever lower, down to the nanomolar and picomolar levels (8,52). These are just a few examples of some of the exciting new developments in the field.

Various topics closely related to this subject have also recently been reviewed and the reader may want to consult them for further information. These include a general review of marine phosphorus cycling (1) and a review of marine DOP, including its properties and transformation processes (40). Also, there is a new monograph on marine microbial ecology (53), and a review of bacterial phosphorus cycling in lakes from a quantitative and modeling perspective (16). Finally, there is a review of the application of molecular methods to the assessment of P-limitation in marine bacteria and phytoplankton (5).

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PHOSPHORUS IN MARINE MICROORGANISMS.

See Inorganic Nutrient Use by Marine Microorganisms

PHOTOSYNTHETIC BACTERIA.

See Phototrophic Purple and Green Bacteria in Marine and Hypersaline Environments

PHOTOSYNTHETIC BACTERIA IN CAVES.

See Caves and Other Low-Light Environments: Aerophitic Photoautotrophic Microorganisms

PHOTOSYNTHETIC BACTERIA IN DRINKING WATER.

See Sulfur Bacteria in Drinking Water

PHOTOSYNTHETIC BACTERIA IN SOILS.

See Soil Bacteria

PHOTOSYNTHETIC BACTERIA IN WASTE STABILIZATION PONDS.

See Wastewater Stabilization Ponds

PHOTOSYNTHETIC PIGMENTS IN MARINE ALGAE AND BACTERIA

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Photosynthetic organisms have evolved a diversity of chemically distinct Chlorophylls (Chls) and bacteriochlorophylls (BChls). In most marine photoautotrophs, Chl *a* represents the major light-harvesting pigment and no plant without it is known to be able to trap light energy for photosynthesis. Recently, a new class of photoactive protein pigment compounds (rhodopsin family) has been discovered in marine bacteria, which may represent the basis of a new phototrophic pathway (1,2). It is not known at this time, however, whether rhodopsin-containing marine bacteria can fix CO_2.

Light-harvesting antenna pigments may also include Chl *b* and Chl *c* type pigments. Other types of Chl molecules observed in marine algae are more obscure (e.g., Chl *d* in Rhodophyta; (3)). In addition to Chls, photosynthetic organisms have evolved two main types of nonchlorophyllous pigments: carotenoids and phycobilins. Xanthophylls (the oxygenated derivatives of carotenoids) are present in all marine algae and have been used to chemotaxonomically identify major algal classes present in oceanic waters (4,5). Various xanthophylls can serve either a photoprotective function (e.g., diatoxanthin, zeaxanthin) or a light-harvesting function (e.g., fucoxanthin). Phycobiliproteins occur as the main light-harvesting pigments in cyanobacteria and some eukaryotic algae (Cryptophyta and Rhodophyta). They are important in harvesting energy in the low Chl-absorbing region of the spectrum and transferring this energy to

Chl *a* in the reaction center (6). This article describes the structure, general function, biochemistry, and ecology of marine bacterial and algal pigments.

BIOCHEMISTRY OF PIGMENTS IN ALGAE AND BACTERIA

Photochemistry of Pigments

When a pigment molecule in the ground state absorbs a photon of light, an electron in the molecule is excited to a higher energy level, by an amount equal to the energy contained in the photon. Multiple excited states are possible corresponding to the range of energy contained in photons, that the pigment can absorb. Pigment molecules can remain in an upper excited state for only a very short period before the electron loses energy, as heat, falling to the lowest excited state. This thermal relaxation from higher excited states is very rapid, occurring in less than 10^{-11} seconds, while the return to ground state is a slower decay process (10^{-8} to 10^{-10} sec). Therefore, the resulting energy state of the molecule is the same regardless of the energy contained in the absorbed photon (7). The amount of energy available for further photochemical processes is the same whether the molecule absorbed a photon of blue light or a photon of red light.

A pigment in the lowest excited state releases energy, thereby returning to ground state, in one of these three ways: thermal loss, fluorescence, or inductive resonant transfer to another molecule. Thermal loss is the slowest of these three processes, occurring in about 10^{-8} seconds for Chl. Fluorescence, the emission of a photon, is also a slow process (10^{-8} to 10^{-9} sec). Chl has a characteristically deep-red fluorescence, especially when illuminated with ultraviolet or blue light, which is the basis behind fluorometric determination of pigments. Fluorescence is prominent from intact cells exposed to intense light (in vivo fluorescence), overwhelming the faster resonant transfer of energy to other molecules; and also in solutions of Chl extracted in an organic solvent (e.g., 90% acetone) where resonant interactions are not possible. The fastest mode of return to the ground state (10^{-10} to 10^{-11} sec) is by inductive resonant transfer of the energy to another molecule. For this to occur, the two molecules must have an overlap in their resonant frequencies and be in a highly ordered arrangement. These ordered arrangements between pigments occur within the protein matrix of the light-harvesting complex and allow for the highly efficient (98%) transfer of light energy from the accessory pigments to the reaction center Chl for use in photosynthetic chemistry (8).

Chlorophylls

The basic structure of the Chls is a magnesium-containing porphyrin ring (Fig. 1). The variations in the composition of side groups attached to specific carbon members of the ring determine the different varieties of Chls (Table 1). A major difference among Chls is the presence of a long hydrocarbon chain attached to C7. The three varieties of Chl *c* and Mg DVP (see Table 2 for pigment name abbreviations used in this chapter) do not have this chain, making them much more polar than the other

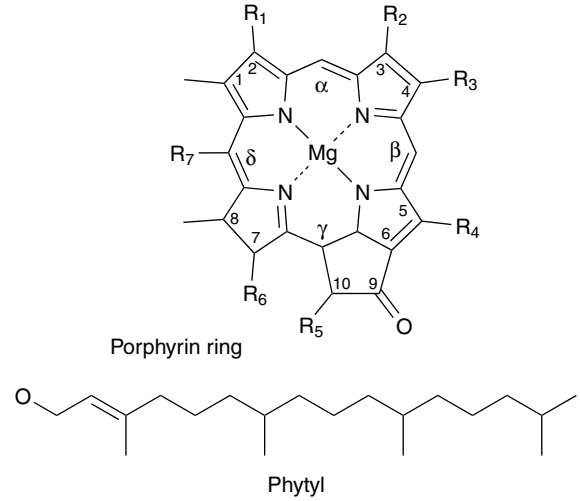

Figure 1. Basic porphyrin ring and Phytyl chain structures of Chlorophyll. Numbering system follows the Fischer system. Refer to Table 1 for the composition of each R-group.

Chls. Most Chls have the phytyl chain ($C_{20}H_{39}$), while BChls *a*, *c*, *d*, and *e* have a farnesyl chain ($C_{15}H_{25}$). These chains make them very nonpolar compounds. These chemical differences are manifested in the differing absorption spectra (Fig. 2a,b; Table 2). BChl *a* has two strong absorption peaks: one in the near ultraviolet between 300 and 400 nm and another in the infrared between 700 and 800 nm. By contrast, Chls *a*, *b*, and *c* have peak absorption bands in the blue (430–450 nm), the Soret band, and in the red (630–670 nm). About 50 chemically distinct and photosynthetically active Chls are known (9). All Chls absorb light poorly in the green part of the spectrum (480–550 nm). When extracted from cells into organic solvents Chls fluoresce strongly to release the light energy absorbed. The fluorescence spectrum is also indicative of the compound. Chl *a* has an excitation maximum at 430 nm and an emission maximum at 668 nm when dissolved in acetone.

The first step, of more than 25 reactions, in the biosynthesis of Chls begins with succinic acid, glycine, Coenzyme A, and the enzyme amino levulinic acid (ALA) synthetase to produce the precursor molecule ALA: 5-aminolevulinate ($NH_2CH_2COCH_2CH_2COOH$) (7). A full review of the biosynthesis of Chls can be found in Porra and coworkers (33). The next sequence of reactions produces protoporphyrin IX, a molecule containing the basic porphyrin ring structure common to all Chls. Insertion of an iron atom in the central ring creates the basic heme structure, the prosthetic structure of many enzymes including the cytochromes, which are important elements in the electron transport chain. Insertion of a magnesium atom into protoporphyrin IX, followed by four further steps, produces Mg DVP. This pigment has two names in common use in the scientific literature. Under the older Fischer nomenclature, the full name is Mg-2,4-divinyl phaeoporphyrin a_5 monomethyl ester. Under the newer IUPAC nomenclature the full name is Mg-3,8-divinyl phytoporphyrin-13^2-methylcarboxylate. Some authors have mistakenly combined these names.

Table 1. Comparison of Chemical Structures of Side Groups for the Various Chls in Algae and Bacteria

Pigment	R_1	R_2	R_3	R_4	R_5	R_6	R_7	Note
Chl *a*	$CH{=}CH_2$	CH_3	CH_2CH_3	CH_3	$COOCH_3$	CH_2CH_2COO-phytyl	H	
DV Chl *a*	$CH{=}CH_2$	CH_3	$CH{=}CH_2$	CH_3	$COOCH_3$	CH_2CH_2COO-phytyl	H	
Chl *b*	$CH{=}CH_2$	CHO	CH_2CH_3	CH_3	$COOCH_3$	CH_2CH_2COO-phytyl	H	
DV Chl *b*	$CH{=}CH_2$	CHO	$CH{=}CH_2$	CH_3	$COOCH_3$	CH_2CH_2COO-phytyl	H	
Chl c_1	$CH{=}CH_2$	CH_3	CH_2CH_3	CH_3	$COOCH_3$	$CH{=}CHCOOH$	H	1
Chl c_2	$CH{=}CH_2$	CH_3	$CH{=}CH_2$	CH_3	$COOCH_3$	$CH{=}CHCOOH$	H	1
Chl c_3	$CH{=}CH_2$	$COOCH_3$	$CH{=}CH_2$	CH_3	$COOCH_3$	$CH{=}CHCOOH$	H	1
Mg DVP	$CH{=}CH_2$	CH_3	$CH{=}CH_2$	CH_3	$COOCH_3$	CH_2CH_2COOH	H	1
Chl *d*	CHO	CH_3	CH_2CH_3	CH_3	$COOCH_3$	CH_2CH_2COO-phytyl	H	
BChl *a*	$COCH_3$	CH_3	CH_2CH_3	CH_3	$COOCH_3$	CH_2CH_2COO-farnesyl	H	2,3
BChl *b*	$COCH_3$	CH_3	$=CHCH_3$	CH_3	$COOCH_3$	CH_2CH_2COO-phytyl	H	2
BChl *c*	$CHOHCH_3$	CH_3	CH_2CH_3	CH_2CH_3	H	CH_2CH_2COO-farnesyl	CH_3	
BChl *d*	$CHOHCH_3$	CH_3	CH_2CH_3	CH_2CH_3	H	CH_2CH_2COO-farnesyl	H	
BChl *e*	$CHOHCH_3$	CHO	CH_2CH_3	CH_2CH_3	H	CH_2CH_2COO-farnesyl CH_3		

Notes: 1. For Chl *c* and MgDVP the $C_7{-}C_8$ bond is a double bond. 2. For BChl *a* and BChl *b* the $C_3{-}C_4$ bond is a single bond (10). 3. Various forms of BChl *a* have the phytyl, farnesyl, or geranyl-geranyl chain at R_6 (10,11). Refer to Figure 1 for the numbering system used to identify the carbon numbers and bonds. See Table 2 for pigment name abbreviations.

Two more chemical steps involving NADPH and light result in Chlorophyllide a (Chl-ide *a*), which is modified by the addition of the C_{20} phytyl chain (geranylgeraniol) to produce Chl *a*. Chl *b* is produced from Chl *a* by an oxygenase reaction on the methyl group attached to C #3, followed by a dehydrogenase reaction. BChl *a* is produced from Chl *a* via an oxygenase reaction on the ethyl group attached to C #2, and BChl *b* is derived from BChl *a*. The various Chl *c* compounds are produced by the modification of Mg DVP.

A Chl molecule resides in the photosynthetic reaction center at the beginning of the electron transport chain in all photosynthetic organisms, and is the final recipient of light energy transferred from the accessory pigments. The reaction center Chl absorbs light energy to attain an excited state. Upon transfer of an excited electron to the first electron receptor of the electron transport chain (Phtin *a*), Chl *a* oxidizes an electron donor to replace the lost electron. The green and purple sulfur bacteria use H_2S or other reduced sulfur compounds as the electron donor, with the resulting production of elemental sulfur. The nonsulfur bacteria can use a variety of organic compounds (e.g., succinate), H_2, or sulfides as the reductant. All other photoautotrophs use water as the electron donor with the resulting formation of molecular oxygen.

When associated with the reaction center apoproteins, the wavelength of maximum absorption shifts from the absorption measured while extracted in an organic solvent. This wavelength is used to identify the reaction center (e.g., P870, P700, or P680). Only three of the Chls listed in Table 1 actually reside in the photosynthetic reaction center. In the photosynthetic bacteria the reaction center Chl is BChl *a*. Purple bacteria (Rhodospirillaceae and Chromatiaceae) have only one photosystem (P870), which delivers electrons into a cyclical electron transport system (7). In a cyclic electron transport system there is no net oxidation of electron donors or reduction of acceptors, but a proton gradient across the membrane is still produced, which is used to synthesize ATP, and the electrons eventually return to P870 via cytochrome *c*. Green sulfur bacteria (Chlorobiaceae) have a noncyclic electron transport chain in which electrons from the oxidation of sulfide pass through cytochrome *c* and are excited within P840 by light to pass into the electron transport chain. Other photosynthetic prokaryotes and all photosynthetic eukaryotes contain two photosystems for the absorption of light energy and the excitation of electrons. Oxidation of water and the production of molecular oxygen occur in photosystem II (PS II), containing P680. Electrons excited by PS II pass into the first part of the electron transport chain containing plastoquinone, cytochrome *f*, and plastocyanin. Photosystem I (PS I) accepts electrons from plastocyanin, contains P700, and absorbs light energy to excite this electron for transport into the final part of the electron transport chain containing iron-sulfur proteins (ferredoxin). In prochlorophytes, the reaction center Chl is DVChl *a*, and in cyanobacteria and all eukaryotic algae the reaction center Chl is Chl *a*. Other Chls serve as accessory pigments to collect light energy and transfer the energy to the reaction center via resonant interaction.

Chlorophyll Degradation Products

Chls and carotenoids can degrade through a variety of processes both in natural and in laboratory settings. Numerous algae contain enzymes, which degrade Chl within cells. For this reason, 100% acetone (and not 90%) is recommended as an extractant to prevent the activity of the chloropyllase enzyme from degrading Chl *a* to Chl-ide *a* (34). Grazing processes (cell breakage and digestion of compounds) can degrade Chls and carotenoids in some specific ways (35,36). Recent evidence suggests that Chls are intrinsically more labile than carotenoids under protozoan grazing pressure (37). In addition, size appears to be an important determinant of carotenoid break down efficiency as it was observed that small protozoans ($<25\mu m$) degraded carotenoids with greater efficiency than did larger ($>80\mu m$) protozoans (37). During laboratory analyses, the actions of light, heat, and oxygen can degrade Chls into various breakdown products, such

Table 2. Chlorophylls and Carotenoids. Pigment Name, Abbreviation, HPLC Number for Figure 8, Molecular Weight, Wavelengths ($\Lambda - nm$) of Absorption Maxima, Extinction Coefficient (α) ($l\,g^{-1}\,cm^{-1}$), and references. Solvents are as Follows: (a) in vivo, (b) 90% Acetone, (c) 90% Acetone +1% Aqueous Pyridine, (d) Acetone, (e) Methanol, (f) Ethanol, (g) Diethyl Ether, (h) Petroleum Ether. an Asterisk (*) Denotes Estimated Values

Pigment	Abbrev.	No.	M.W.	Abs. Maxima λ (solvent)	Ext. Coeff. λ (solvent) α	Ref.
Chlorophyll c_3	Chl c_3		652.95	452.1, 626.3 (d)	452.9 (c) 346	12
Mg-2,4-divinyl phaeoporphyrin a_5 monomethyl ester	Mg DVP		610.94	437.7, 625.1 (d)	623 (e) 85.9	13
Chlorophyllide *a*	Chl-ide *a*		614.97	431, 664 (d)	664 (b) 127	14
Chl c_2	Chl c_2	1	608.94	444.6, 629.6 (d)	630.9 (c) 40.4	12
Chl c_1	Chl c_1	2	610.95	446.1, 629.1 (d)	630.6 (c) 44.8	12
Phaeophorbide *a*	Ph-ide *a*		592.67	409.5, 665.5 (d)	667 (b) 74.2	14
Peridinin	Perid	3	630.82	458, 474 (d)	466 (d) 134	15,16
19′-Butanoyloxy-fucoxanthin	19But	4	745.01	445, 471 (d)	445 (d) 147	17
Heteroxanthin	Hetero			419, 445, 476 (f)		18
Fucoxanthin	Fuco	5	658.92	446, 468 (d)	443 (d) 166	17
19′-Hexanoyloxy-fucoxanthin	19Hex	6	773.06	444.3, 470.3 (d)	445 (d) 142	15,17
Neoxanthin	Neox	7	600.88	415, 439, 467 (d)	438 (f) 227	19,20
Prasinoxanthin	Pras	8	600.88	450.9 (d)	446 (g) 250*	19,21
Violaxanthin	Viol	9	600.88	417, 440, 470 (d)	443 (f) 255	22,23
Diadinoxanthin	Diad	10	582.86	447.5, 478 (d)	447.5 (d) 223	24
Dinoxanthin	Dino		642.92	417, 441, 470 (f)	442 (d) 210*	19,24
Antheraxanthin	Anth		584.88	444, 472 (f)	446 (f) 235	25
Vaucheriaxathin (ester)	Vauch		616.88	421, 444, 472 (d)	444 (d) 250*	19,23
Alloxanthin	Allo	11	564.85	454.3, 483.5 (d)	454 (d) 250*	19,23
Diatoxanthin	Diat	12	566.87	454, 482 (d)	440 (f) 163.4	26
Monadoxanthin	Monad		566.87	428, 446, 476 (g)	446 (g) 250*	23,27
Lutein	Lut	13	568.88	425, 447.5, 476 (d)	445 (f) 255	28
Zeaxanthin	Zeax	14	568.88	454, 481 (d)	452 (d) 234	29
Parasiloxanthin	Para		571	N/D	N/D	
Canthaxanthin	Canth	15	564.85	472 (d)	466 (h) 220	19,23
Crocoxanthin	Croco		550.87	428, 445, 475 (g)	443 (f) 250*	19,27
Divinyl Chlorophyll *b*	DV Chl *b*	16	905.48	460, 664 (g)	N/D	30
Chlorophyll *b*	Chl *b*	17	907.49	470, 650 (a) 456.9, 645.5 (d)	646.8 (b) 51.36	7,15
Divinyl Chlorophyll *a*	DV Chl *a*	18	891.48	436, 661 (g)	660 (g) 112.2	30
Chlorophyll *a*	Chl *a*	19	893.50	430, 670–700 (a) 430.3, 662.1 (d)	664.3 (b) 87.67	7,15
BacterioChlorophyll *a*	BChl *a*		909.5	375, 800–890 (a)	775 (e) 38.7	7,11
BacterioChlorophyll *b*	BChl *b*		908.5	400, 1,020 (a)	N/D	7,11
BacterioChlorophyll *c*	BChl *c*		793.5	750–755 (a) 660 (d)	N/D	7
BacterioChlorophyll *d*	BChl *d*		779.5	725–755 (a) 654 (d)	N/D	10
BacterioChlorophyll *e*	BChl *e*		805.5	715–725 (a) 647 (d)	N/D	10
Chlorophyll *d*	Chl *d*		893.5	447, 688 (g)	688 (g) 110.4	31
Phaeophytin *b*	Ph-tin *b*		885.20	434.5, 653.5 (d)	657 (b) 31.8	19
Phaeophytin *a*	Ph-tin *a*		871.21	409.5, 665.5 (d)	667 (b) 51.2	14
Lycopene	Lycop		536.88	447, 474, 506 (d)	474 (d) 344.6	19,29
$\beta\varepsilon$-Carotene	α-Car		536.88	447, 475 (d)	448 (d) 270	19,32
$\beta\beta$-Carotene	β-Car		536.88	453.5, 479.9 (d)	454 (d) 250	(19,32)

as the 10-hydroxy lactone derivatives (38). For this reason pigment extractions and analyses are typically done under subdued light and at cold temperatures.

The primary categories of Chl degradation products are phaeophytins, chlorophyllides, phaeophorbides, and cyclic phaeophorbides. Ph-tins are created by the removal of the magnesium atom from the center of the porphyrin ring. In laboratory settings, this is accomplished by a simple acidification of the extract solution. Cleaving off the phytyl chain while leaving the remainder of the molecule intact creates Chl-ides. Ph-ides are created by a combination of these actions; removing both the Mg atom and the phytyl chain. Examples of typical Chl breakdown products are depicted in Figure 3.

A new class of degradation compounds (cyclic phaeophorbides) was recently discovered in marine sediments (39). It is believed that these compounds are formed via cyclization of either pyrophaeophorbide *a* or pyrophaeophytin *a* (39). High concentrations of the Chl *a* breakdown compound, CPP516, a cyclic phaeophorbide (40) were also detected in fecal matter collected in sediment traps in the Eastern Equatorial Pacific Ocean (41).

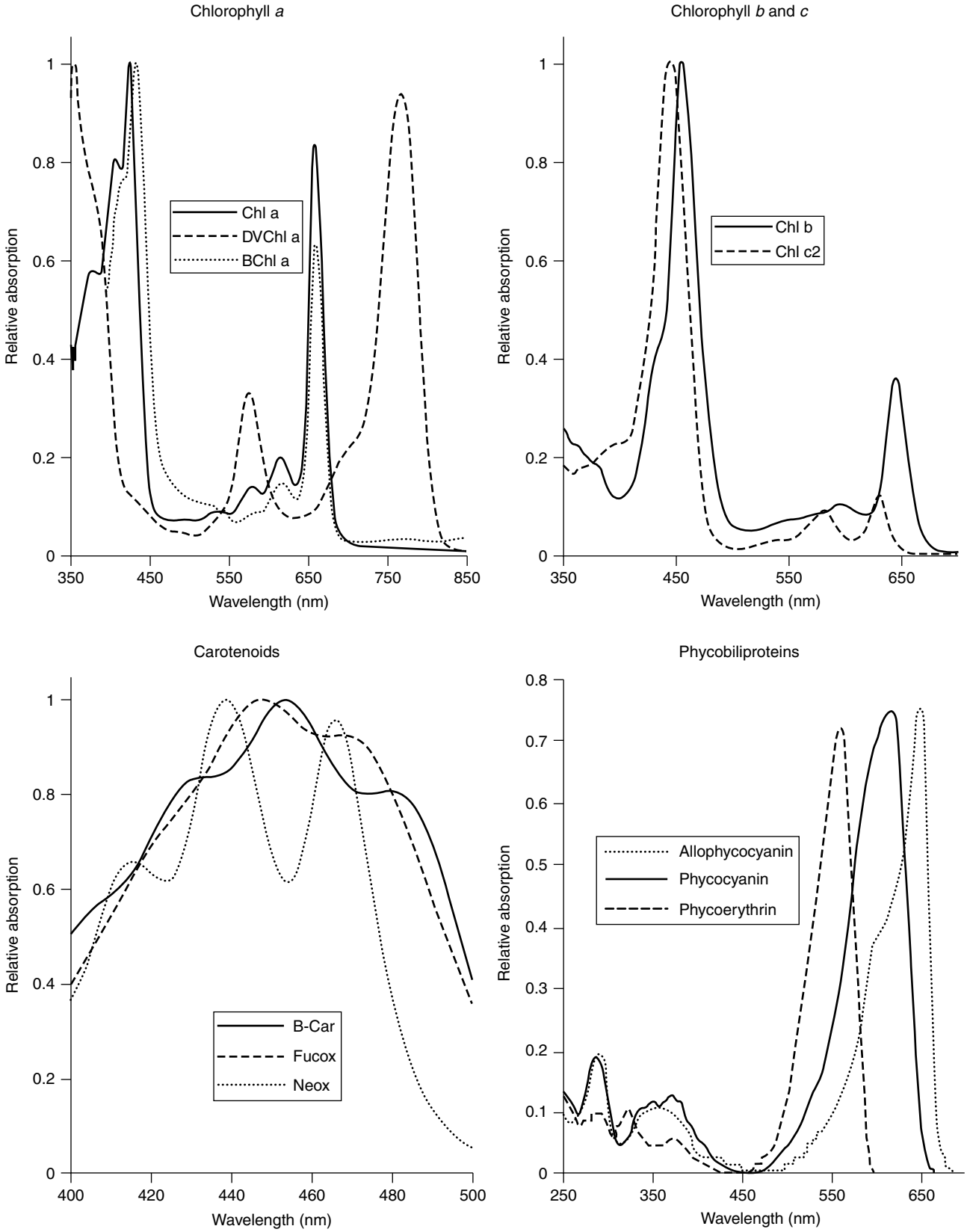

Figure 2. Absorption spectra of some representative (**a**) chlorophylls *a*, (**b**) chlorophyll *b* and *c*, (**c**) carotenoids, and (**d**) phycobilins. BChl *a* is from *Rhodopseudomonas sphaeroides*.

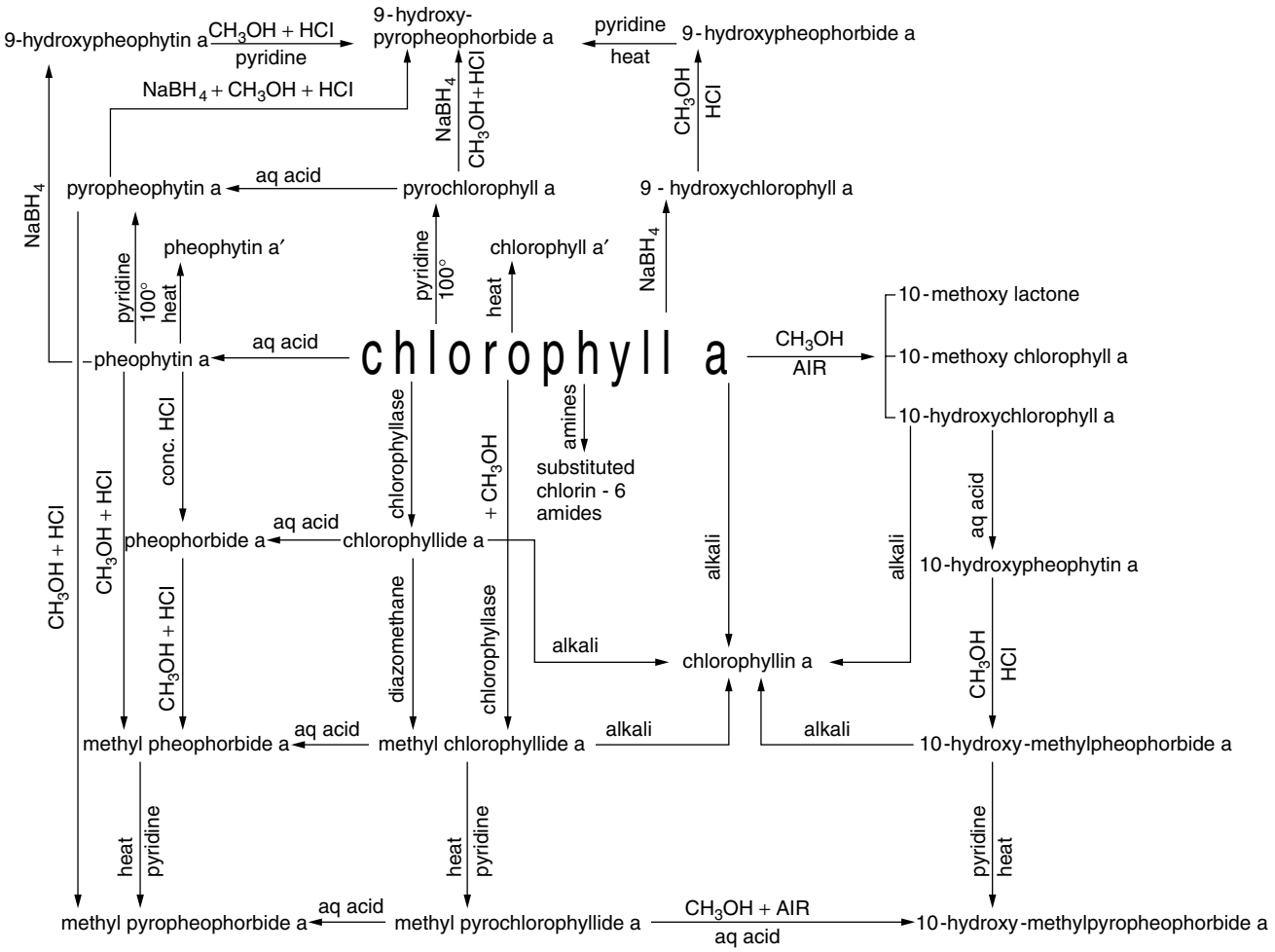

Figure 3. Pathways for the formation of chlorophyll degradation products (From Rowan, 1989).

The presence of cyclic phaeophorbides in fecal matter suggests that anaerobic sediment processes are not the only source of these compounds; macrozooplankton grazing process in the marine water column may also be a significant source of these cyclic phaeophorbide compounds (41). In one study of marine sediments, cyclic phaeophorbides contributed up to 70% of all Chl *a* degradation compounds (41). Further studies are warranted to determine the prevalence and abundance of these compounds in other marine environments.

Carotenoids

Carotenoids are present in bacteria and algae in a wide variety of forms. They are usually red, brown, yellow, or orange in color and have absorption peaks in the blue part of the spectrum (400–500 nm) (Fig. 2c; Table 2). Chemically, carotenoids have the basic structure of lycopene (Fig. 4) and are classified as hydrocarbons (the carotenes) or oxygenated derivatives (the xanthophylls) (8). While more than 600 carotenoids have been identified, the de novo synthesis occurs only in prokaryotes, fungi, algae, and higher plants. Carotenoids found in animals are the chemically modified products following ingestion (19). All carotenoids are derived from the basic structure of lycopene by any combination of the processes of hydrogenation, dehydrogenation, cyclization, or oxidation.

Photosynthetic bacteria synthesize a wide variety of carotenoids. The basic synthetic pathway is a sequence leading to the final product of spirilloxanthin (42) with variations of the biosynthetic pathway from intermediate compounds leading to other carotenoids. Five groups of carotenoids have been identified in photosynthetic bacteria (43,44); but, except for the green sulfur bacteria (Chlorobiaceae), there is little correlation between carotenoid composition and taxonomic categorization based on morphological aspects. Group 1 consists of the "normal" spirilloxanthin series of carotenoids produced by purple bacteria (Chromatiaceae and Rhodospirillaceae). In the case of the brown nonsulfur bacteria (Rhodospirillaceae), the final product is an earlier member of the series such as lycopene or rhodopene. Some facultatively aerobic purple nonsulfur bacteria make Group 2 carotenoids by a divergence of the "normal" synthetic pathway from the intermediate compound neurosporene. Under aerobic conditions these carotenoids are converted to the corresponding keto-carotenoids. Group 3 carotenoids are keto-carotenoids produced by anaerobic purple sulfur bacteria, the main carotenoid being okenone. Group

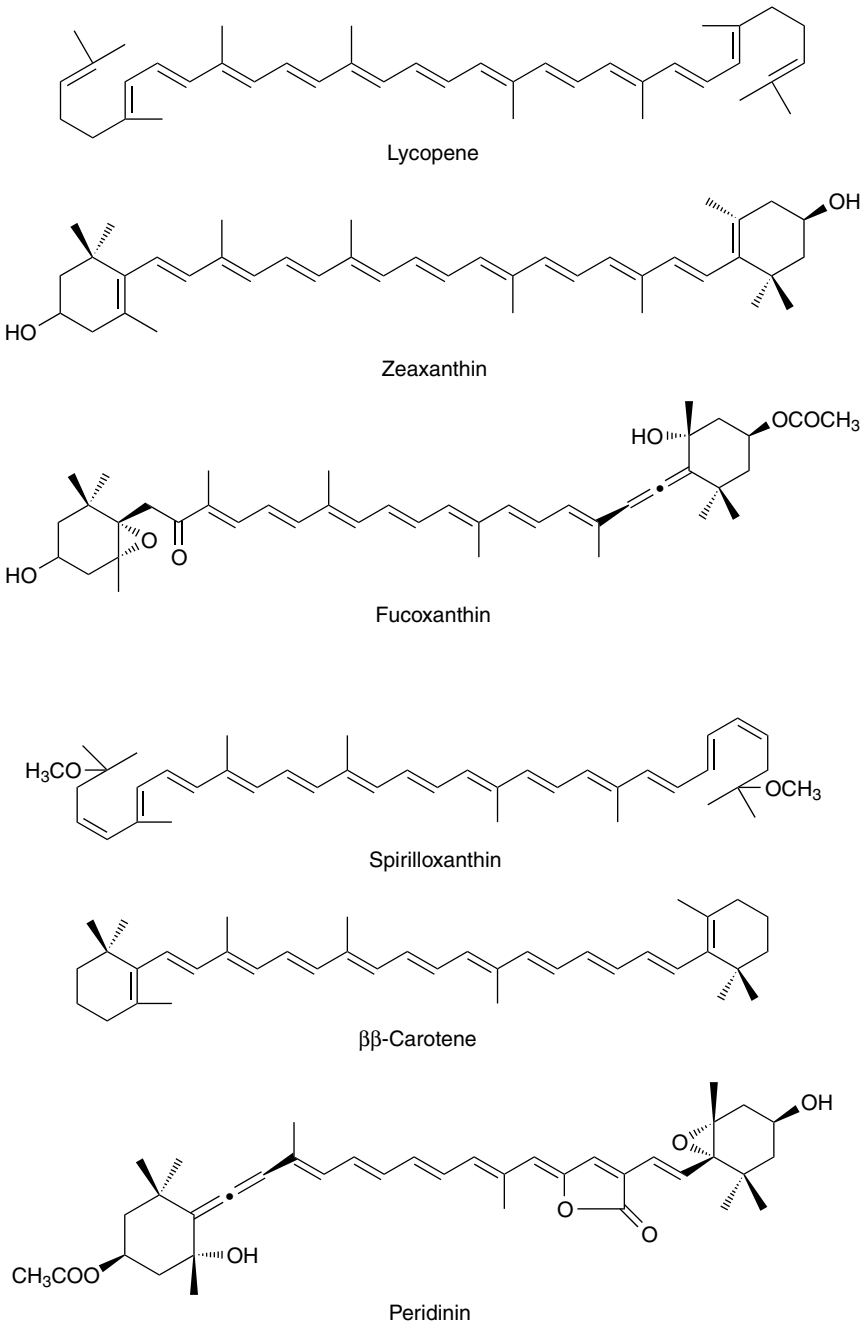

Figure 4. Chemical structures of some representative cartenoids.

4 carotenoids are produced by all purple sulfur bacteria and belong to the Warmingone-series, aliphatic carotenoids, which do not contain methoxyl groups. Rhodopene may be a precursor of these carotenoids. Group 5 carotenoids, chlorobactene and its hydroxy derivatives, are the major carotenoids in the green sulfur bacteria (Chlorobiaceae).

Algae contain more than 30 major ecologically relevant carotenoids (45). The biosynthesis of carotenes in algae is mediated by membrane-bound enzymes in the chloroplast (46). Whether the entire biosynthesis, from carbon fixation, through the production of acetyl-CoA and the subsequent production of *iso*pentenyl disphosphate, to the final carotenoid, occurs in the chloroplast is still in doubt. Some experimental evidence suggests that *iso*pentenyl disphosphate is formed in the cytoplasm and subsequently transported into the chloroplast for carotenoid synthesis (46). The enzymes for the desaturation of phytoene to create lycopene have been shown to reside primarily on the thylakoid membranes within the chloroplast (47). The epoxidation reactions converting Zeax to Viol also occur on the thylakoid membranes (48,49). Britton (50) reviewed the major biosynthetic pathways for carotenoid biosynthesis in algae. The xanthophylls Zeax and Lutein are formed by the enzymatically mediated hydroxylation of β-Car and α-Car, respectively (33).

The majority of carotenoids are noncovalently bound and highly ordered within the pigment-protein light

harvesting complex outside the photosynthetic reaction center. The pigments in this complex absorb light energy, often at wavelengths, that Chl cannot efficiently absorb, producing an excited state of the carotenoid (33). The transfer of energy to the reaction center Chl occurs by a resonant transfer of the energy from the excited state of one pigment to the ground state of another, requiring a spectral overlap between the pigments. Since the excited state of the carotenoids is extremely short-lived, highly ordered arrangements of the pigments are required within the protein matrix of the light harvesting complex. The pigments in the light harvesting complex consist of other Chls, carotenoids, or phycobiliproteins. It is the composition of this pool of pigments, that varies between taxonomic groups and can be used to identify groups of algae present in a mixed algal community.

Certain carotenoids do not act as accessory pigments to provide light energy for the reaction center Chl *a* molecule. Membrane-bound protein-carotenoid complexes found in cyanobacteria and prochlorophytes may play a role in ion-uptake processes (51). In eukaryotic algae, carotenoids are also involved in phototropic responses or as photoprotective agents in high-light environments to absorb and dissipate excess photons entering the cell (52,53). These carotenoids can exist free in the cellular cytoplasm, bound to protein complexes separate from the light harvesting complex, or bound as part of the light harvesting complex (33). In high-light conditions excited singlet state oxygen ($^1O_2$), a highly damaging molecule, can form from an energy transfer from excited state Chl. Carotenoids can reduce the formation of $^1O_2$ by quenching the excited state Chl or by directly deactivating $^1O_2$. After accepting the energy from the excited state molecule, carotenoids can dissipate the energy and return to ground state (33).

Xanthophyll Cycling

Protection against photoinhibition can also be achieved by the epoxidation/de-epoxidation reactions of xanthophyll cycling (54). In the dark-adapted state the xanthophylls exist in the epoxide form. In the presence of light and two H atoms, oxygen is removed to form the de-epoxide form and water. In phaeophytes, chlorophytes, and higher plants Viol is de-epoxidized to Anth, which is further de-epoxidized to Zeax (55). In chromophytic algae Diad is de-epoxidized to Diat in the light. The de-epoxidase enzyme has a pH optimum of about 5 (25) and is active in vivo only at low pH (<5.8) (56). Under high-light conditions the production of extra protons by photosynthesis could trigger the de-epoxidation reaction (25). Experimental results confirm that irradiance levels change the amount of Diad, as Diad is de-epoxidized to Diat (57). In addition, light intensity will affect the Diad + Diat pool size with larger Diad + Diat/Chl *a* pool sizes observed at higher irradiances (58). In nonchromophytic algae such as marine cyanobacteria, Zeax may serve a photoprotective function under conditions of high-light (59). Xanthophyll cycling, however, has not been documented in species of cyanobacteria, prochlorophytes, cryptophytes, or rhodophytes (60).

Phycobiliproteins

Phycobiliproteins are water-soluble photosynthetic antenna pigments found in cyanobacteria, rhodophytes, and cryptophytes (Fig. 5). These brilliantly colored (phycocyanins are blue and phycoerythrins are red) pigments were named after the organisms in which they were initially found. For instance, R-phycoerythrin (R-PE) and R-phycocyanin (R-PC) were first found in rhodophytes and C-PE and C-PC were first observed in cyanobacteria (61). The rationale for these designations is tenuous. For instance, C-PC occurs in both cyanobacteria and rhodophytes, but this nomenclature has persisted because the names relate to pigments with unique spectral characteristics. Most analytical determinations of oceanic phycobiliprotein concentrations have used either flow cytometric (62) or fluorescence spectrophotometric techniques (63), some of which utilize the in vivo glycerol decoupling method (64).

Phycobiliproteins harvest solar energy in regions where low chl *a* absorption occurs (500–650 nm) and function to transfer this energy directly to the reaction center Chl *a* molecule (Fig. 6). In addition, these pigments may also serve as cellular nitrogen storage compounds (65,66), although this function is somewhat controversial (67). Phycobilins are open-chained tetrapyrrole chromophores that are covalently attached to the biliproteins; the structure would be approximately similar to a Chl molecule that had lost its magnesium atom, been clipped, and been linearized (Fig. 5). There are five major phycobilin types, each with a unique spectral absorption curve (Fig. 2); phycocyanobilin (PCB), phycoerythrobilin (PEB), phycourobilin (PUB), phycobiliviolin (PXB), and cryptoviolin (CV). A few other minor phycobilin compounds have also been identified (68). In contrast to the Chl molecules, the phycobilins contain no metal ions.

Phycoerythrins carry one or two chromophore types; PEB and/or PUB. Some marine cyanobacteria species (e.g., WH 7803) have high ratios (ca. 4) of PEB:PUB (63,69)

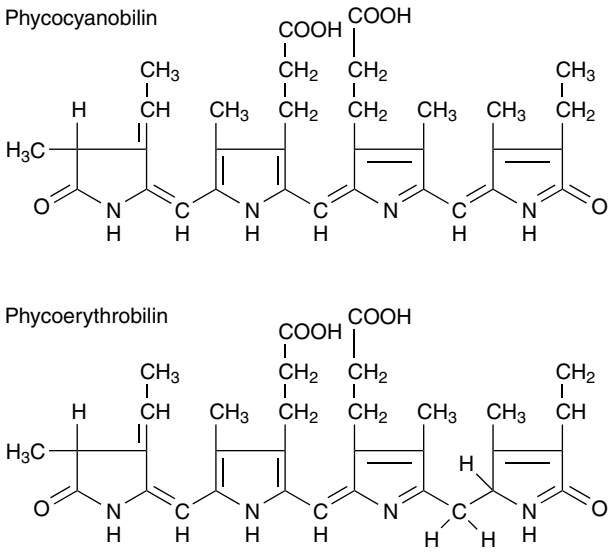

Figure 5. Chemical structures of some representative phycobilins.

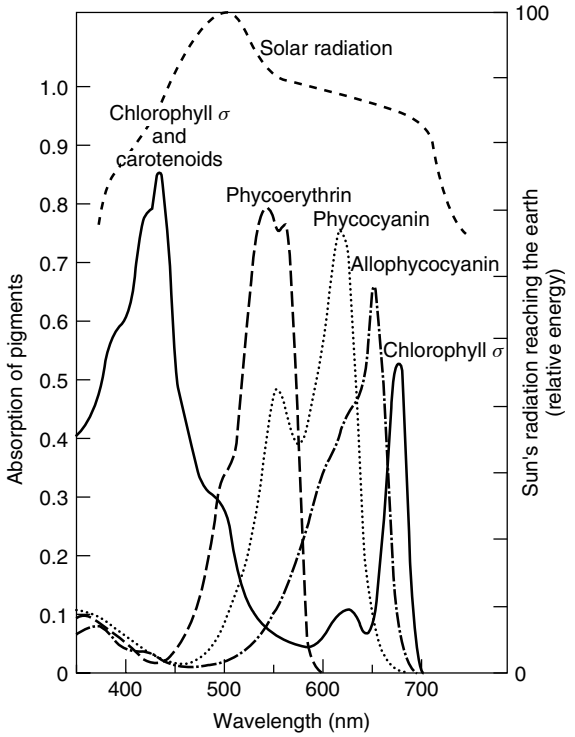

Figure 6. Absorption spectra of pigments and phycobiliproteins (From Gantt, 1990).

while other oceanic strains of *Synechococcus* spp. (e.g., WH 8103) have higher quantities of PUB with PEB:PUB ratios that are less than 1 (70). Both immunofluorescence techniques (71) and flow cytometric data (62) have revealed that oceanic *Synechococcus* spp. have very low ratios of PEB:PUB. Distinct PE spectral signatures were noted in oceanic and coastal species of *Synechococcus* in the Gulf of Maine and the North Atlantic Ocean (63). Open ocean species of *Synechococcus* in the North Atlantic were characterized as having a Type I PE spectral signature (fluorescence emission value of 555–565 nm) composed of both PEB and PUB (63). In contrast, the coastal *Synechococcus* had a Type II PE signature composed solely of PEB (determined from the 570 to 580 nm emission fluorescence). In the tropical and subtropical Pacific Ocean, PEB:PUB ratios of 0.50 to 0.67 were observed in *Synechococcus* dominated waters (72). A high PEB containing species was also observed in the Equatorial Pacific Ocean suggesting that PE compounds in oceanic waters may be more diverse than currently believed (72).

The idea that PE is only found in marine cyanobacteria, cryptophytes, and rhodophytes is no longer valid. A recent finding indicated that some marine dinoflagellate species (*Dinophysis* spp.) contained a unique PEB with a spectral signature, including a 545-nm maximum absorption peak (73). There was no evidence that endosymbionts were present and responsible for this spectral signature. It was also recently observed that the α- and β-subunits of PE were present in the genome of some species of marine *Prochlorococcus marinus* (74–76). The presence of PE is apparently associated with low-light acclimated strains of *P. marinus*, which reside at the bottom of the photic zone (76).

The biosynthesis of phycobilins follows the same pathway as Chls and BChls and branches off at the Protoporphyrin IX point. Ferrochelatase (protoheme ferrolyase, EC 4.99.1.1) catalyzes the last step of protoheme formation; the insertion of Fe^{+2} into protoporphyrin IX (68). Phycobilins are synthesized from protoheme via the synthesis of the intermediate compound, Biliverdin IXα (68). Heme oxygenase (EC 1.14.99.3) is the enzyme responsible for catalyzing the opening of the heme macrocycle to form biliverdin (68). Virtually all of the experimental work on phycobilin biosynthesis has been done using the rhodophyte *Cyanidium caldarium*.

Phycobilisomes

The phycobilisome is a supramolecular subcellular complex, which appears as tiny balls attached to the stromal side of the thylakoid membrane. The phycobilisome consists of chromophores, which are covalently bound to the apoprotein (via a thioether linkage) forming the phycobiliprotein unit. These proteins represent approximately 85% of the total protein in phycobilisomes. The phycobiliproteins are bound together by colorless linker polypeptides, which function to bind and stabilize the phycobiliproteins (77). The linker polypeptides are basic (with a pI of 6.0 to 8.5), whereas the phycobiliproteins are more acidic (with a pI of 4.3 to 6.3) (78). Phycobilisomes are either hemi-discoidal or hemi-ellipsoidal in shape (78). Most phycobilisomes are of the hemi-discoidal type (78) although, some are as broad as they are high (77). The actual size and shape of the phycobilisome is species dependent (79). Phycobilisomes are composed of two main structural elements; peripheral rods, and a core substructure (Fig. 7). The rods are entirely made up of phycoerythrin and phycocyanin. The core region consists of allophycocyanin (APC) perpendicularly attached to the terminal pigment, which transfers light energy from the phycobilisome to the photosystem II reaction center Chl *a* molecule.

The transfer of energy occurs via the anchor polypeptides progressively from the high-energy (500 nm) absorbing PE at the periphery to PC (620 nm) to APC (650 nm) and finally to the Chl *a* antenna in the PS II reaction center (Fig. 6) (also known as the terminal pigment). The energy transfer from the phycobilisomes to Chl *a* occurs rapidly,

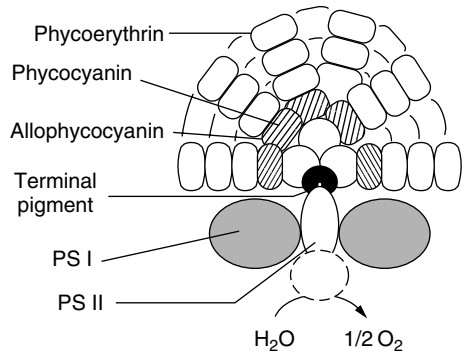

Figure 7. Arrangement of phycobilins, pigments, and photosystems in a phycobilisome (From Gantt, 1990).

on the order of nanoseconds (80). The radiation-less excitation energy transfer occurs with an efficiency greater than 95% (81). The excitation energy reaching the antenna Chl *a* in the PSII reaction center can then follow one of the two paths: the energy can be transferred to the PS II reaction center Chl *a* leading to the electron transport chain and PS I, or the energy can be transferred directly to the PS I antenna Chl *a*. The transfer of energy does not occur by emission of a photon followed by energy absorption by a neighboring molecule but rather by radiation-less, dipole-induced, dipole resonance energy transfer (82). In this process the energy is localized in a cluster of Chl molecules. The energy is then distributed throughout the Chl supermolecule and is not contained within any one individual Chl molecule. This process is similar to the system of localized electrons within a benzene ring.

The photosynthetic apparati of rhodophytes and cyanobacteria are unique here that they contain phycobilisomes as light-harvesting antenna and do not contain stacked or fused thylakoids. Cryptophytes, although containing phycobiliproteins, do not contain phycobilisomes.

Purified PE, PC, and APC are composed of at least two polypeptide subunits as revealed by sodium docecyl sulfate (SDS) gel electrophoresis (83). The lower molecular weight subunit is designated as α and the larger one as β, both having a size range of 15 to 22 kD (78). APC is regarded as the simplest phycobiliprotein containing one PCB per α and β-polypeptide. PC molecules generally consist of one PCB per α-polypeptide and two per β-polypeptide with a combined heteromere size (α_6, β_6) of ca. 220 kD (78). PE molecules have a larger number of chromophores than the other phycobiliproteins. In addition, some PE molecules also contain other subunits, although much less is known regarding these other subunits compared to the α- and β-subunits (83).

METHODS FOR IDENTIFICATION AND QUANTIFICATION OF PIGMENTS

Concentration and Extraction of Pigments

Algae samples collected from culture or a natural phytoplankton community are typically filtered onto GF/F filters. Cells collected on the filter are disrupted by grinding, sonication, or freezing, and the pigments are extracted by soaking in an organic solvent (e.g., methanol or acetone) at a low temperature ($<0\,^{\circ}$C) for up to 24 hours. While in solution, pigments are very susceptible to degradation, so care must be taken to keep the pigment extracts cold and away from bright light. For long-term storage of pigment extracts, placement in a $-80\,^{\circ}$C freezer or in liquid nitrogen is highly recommended. The sample should be centrifuged and/or filtered to remove particulate matter from the pigment extract solution.

Spectrophotometric Methods

The concentration of a purified pigment extract can be determined using a spectrophotometer. Purified pigments can be obtained from a number of commercial sources or by HPLC extraction, separation, and collection of the pigment as it elutes from the system. Since absorption spectra for each pigment vary considerably depending on the solvent the extinction coefficient (α) used for determining concentration (C) must be specific to the solvent and wavelength (λ). Table 2 lists extinction coefficients for a wide variety of pigments. To maintain the linearity of concentration versus absorption (A_λ), the measured absorption should not exceed 1.5. The equation for concentration (mg l^{-1}) using a 1 cm pathlength cell is:

$$C = 1,000^{*}\ A_{\lambda}{}^{*}\alpha^{-1} \tag{1}$$

The concentration of Chls and carotenoids in a sample extracted in 90% acetone containing a mixture of pigments can be determined using the equations of Jeffrey and Humphrey (15) and Parsons and coworkers (84). The absorption of the solution is measured at 480, 510, 630, 647, 664, and 750 nm. The absorbance readings at 630, 647, and 664 nm are then corrected by subtracting the absorption at 750 (known as the turbidity blank), which should be close to zero. For the absorptions at 510 and 480 nm, 2x and 3x, the 750 nm absorbance is subtracted, respectively. The concentrations (mg l^{-1}) of each of the Chls (*a*, *b*, and *c*) and the total carotenoids are determined from the following equations:

$$\text{Chl } a : (C_a) = 11.85\ A_{664} - 1.54\ A_{647} - 0.08\ A_{630} \tag{2}$$

$$\text{Chl } b : (C_b) = 21.03\ A_{647} - 5.43\ A_{664} - 2.66\ A_{630} \tag{3}$$

$$\text{Chl } c : (C_c) = 24.52\ A_{630} - 1.67\ A_{664} - 7.60\ A_{647} \tag{4}$$

Total Carotenoids:

$$(C_{Car}) = 7.6(A_{480} - 1.49\ A_{510}) \tag{5}$$

The concentration of Chl *c* is the sum of Chl c_1 + Chl c_2 + Chl c_3, and concentration for total carotenoids is only an estimate because of the wide variations in extinction coefficients among carotenoids. The pigment concentration (mg l^{-1}) in the initial sample can be calculated using the following equations incorporating the concentration determined above (C), the extract volume (V_e) and the filtered volume (V_f), both in ml:

$$\text{Conc} = C^{*}V_{e}{}^{*}V_{f}^{-1} \tag{6}$$

Fluorometric Methods

Chl *a*, in 90% acetone, fluoresces with a peak excitation about 435 nm and peak emission about 667 nm. The fluorometric method uses a Turner Designs fluorometer to measure Chl *a* concentration. Earlier models of the Turner Designs fluorometer required a two-step process to measure Chl *a* in a sample because of the additional fluorescence generated by Chl degradation products such as Ph-tin *a*. The fluorescence of the sample was measured (F_0), the sample was acidified with a couple drops of 10% HCl, and the fluorescence was measured again (F_a). The Chl *a* concentration (C_a) (μg l^{-1}) in the extracted sample is calculated:

$$C_a = K\frac{(F_0 - F_a)}{\text{Sens}} \tag{7}$$

Table 3. Measured Concentration and Fluorescence of Fluorescing Compounds (in 90% Acetone) Using a Turner Designs 10-AU Fluorometer

Pigment	Spec Conc. (μg l^{-1})	Fluor Conc. (μg l^{-1})	Percent Fluor:Spec
Chl *a*	366	372	102
Chl *b*	1,386	149	10
Chl $c_1 + c_2$	710	156	22
Chl c_3	58	8	14
Ph-ide	1,094	171	16
Ph-tin	1,103	138	13
Chl-ide	183	166	90

K is the calibration factor for the instrument and Sens is the sensitivity setting used for the measurement. Calibration of the instrument involves using successive dilutions of purified Chl *a* to establish a linear regression of $(F_0 - F_a)$ versus C_a, over the sensitivity range of the instrument.

The Turner Designs 10-AU does not require acidification of the sample because of the different light source and the narrow-band excitation and emission filters used (85). After a simple blank determination and one-point calibration using purified Chl *a*, the instrument reports Chl *a* concentration directly. Even in this setup, however, the concentration of Chl *a* can be overestimated due to the presence of secondary fluorescing compounds such as Chl *b*, Chl *c*, Chl-ide, Ph-tin, or Ph-ide. Table 3 gives the spectrophotometrically determined pigment concentration of pure standards, the fluorometric concentration reported as Chl *a* by the Turner 10-AU fluorometer, and the percent ratio of the two.

For other Chls, Ph-ide, and Ph-tin about 10 to 20% of the concentration will be reported as Chl *a*; but for Chl-ide, 90% will be reported as Chl *a*. In regions where these compounds contribute significantly to the total pigment present, the fluorometric method will greatly overestimate the concentration of Chl *a*.

HPLC Methods

The primary means to separate and identify specific pigments from cultures and natural communities involves concentrating cells by filtering the sample, extracting the pigments in an organic solvent, and separating the pigments using High Performance Liquid Chromatography (HPLC; 86). In order to reduce the solvent strength of the extract solution it is typically mixed with water or buffer solution before injection into an HPLC reverse phase system for separation and identification of the pigments.

Three main HPLC methods are generally employed to separate and identify pigments. All employ reverse phase (C_8 or C_{18}) columns and gradients containing mobile phases of aqueous and organic solvents. More polar pigments, such as Chl *c*, Mg DVP, and the Chl degradation compounds Chl-ide and Ph-ide, elute early in the gradient, followed by a series of xanthophylls, and finally the most nonpolar pigments such as Chl *b*, Chl *a*, and the carotenes. The first method (#1) (26) uses a gradient of methanol, acetone, and an aqueous solution containing the buffer ammonium acetate with or without the ion-pairing reagent tetrabutyl-ammonium acetate. A widely used method (#2) (87) uses a gradient of methanol, acetonitrile, ethyl acetate, and an aqueous buffer solution containing only ammonium acetate. The third method (#3) (88) uses a gradient of methanol, acetone, acetonitrile, and an aqueous solution containing pyridine as an ion-pairing reagent. The gradient profiles and mobile phase compositions for these methods are summarized in Table 4.

An in-line detector, such as a photodiode array or an ultraviolet/visible spectrophotometer, measures the absorption by pigments at a specific wavelength (typically about 440 nm) as well as recording the absorption across a spectrum at regular time intervals. A pigment can be identified based on its characteristic absorption spectrum and quantified on the basis of the detector response (peak area) at a specific wavelength. An in-line fluorescence detector can be very useful to measure fluorescing pigments, especially if present in very small amounts. If optimized for Chl *a* (i.e., excitation wavelength about 436 nm and emission wavelength about 666 nm) then a fluorescence detector is much more sensitive and accurate for the detection and quantification of most Chls and Chl degradation products (e.g., Chl-ide, Ph-ide, or Ph-tin).

The HPLC system is calibrated by repeated injections of pigment standards to establish a regression of detector response (peak area) to pigment amount (ng). A typical limit of detection for Chl *a* and other pigments is about 1 to 5 ng, and calibration amounts typically range from 1 to 200 ng. The exact amount will vary depending on the type of instrument. Coefficient of variation from replicate injections of standard pigments should be less than 3% on most HPLC systems.

For comparison, HPLC chromatograms of a pigment mixture that are separated by two different methods (#1 and #3) are presented in Figure 8. Elution order varies slightly for pigments 6 and 14. Method #3 provides near baseline separation between pigments 1 and 2 (Chl c_1 and c_2), provides for a better separation between xanthophylls 4 through 15, and even resolves pigments 18 and 19 (DV Chl *a* and Chl *a*). Separation between pigments 13 and 14 (Lutein and Zeax) is poor in both methods, but better in Method #3. Neither method separates pigments 16 and 17 (DV Chl *b* and Chl *b*). Method #3 does a better job of separating Chl-ide and Mg DVP from Chl c_1 and c_2 than Method #1 (not shown).

ECOLOGICAL DISTRIBUTIONS OF ALGAL AND BACTERIAL PIGMENTS

BChls are typically in very low abundance in marine oxygenated waters. Recent evidence, however, documents the prevalence of aerobic anoxygenic photoheterotrophs in surface ocean waters (89,90). These obligate aerobes that are closely related to purple photosynthetic bacteria have unusually high concentrations of carotenoids but low ratios of BChl *a*: cell (90). These facultative photoheterotrophs can metabolize organic carbon compounds when present but are also capable of photosynthetic light utilization when organic carbon sources are scarce (90).

Table 4. Summary of Buffer Solutions, Mobile Phases, and Gradient Profiles for the Three Main HPLC Methods

	Method #1 (26)				Method #2 (87)				Method #3 (88)		
HPLC Column	25 × 0.46 cm C_{18} 5 μm				25 × 0.46 cm C_{18} 5 μm				15 × 0.46 cm C_8 3.5 μm		
Aqueous Buffer Solution	0.5 M Ammonium Acetate Acetate pH 7.6				0.5 M Ammonium Acetate pH 7.2				0.25 M Pyridine (20 ml/L) Acetic Acid (10 ml/L) pH 5.0		
Mobile Phase A	85 : 15 Methanol: Buffer				80 : 20 Methanol: Buffer				50 : 25 : 25 Methanol: Acetonitrile: Buffer		
Mobile Phase B	Methanol				90 : 10 Acetonitrile: Water				20 : 60 : 20 Methanol: Acetonitrile: Acetone		
Mobile Phase C	Acetone				Ethyl Acetate				N/A		
Gradient Profile	Time	%A	%B	%C	Time	%A	%B	%C	Time	%A	%B
Time in minutes	0	100	0	0	0	100	0	0	0	100	0
Flow rate:	5	100	0	0	4	0	100	0	22	60	40
1 ml/min	10	7	93	0	18	0	20	80	28	5	95
	20	7	93	0	21	0	100	0	38	5	95
	28	0	100	0	24	100	0	0	40	100	0
	30	0	75	25	29	100	0	0			
	32	0	0	100							
	34	0	0	100							
	35	100	0	0							
	40	100	0	0							

Preliminary evidence suggests that these organisms are prevalent in the upper ocean and represent at least 11% of the total microbial community (90).

Bacteriorhodopsin, a retinal containing membrane protein that was recently discovered in the ocean, utilizes the in situ light energy in the marine photic zone to produce conformational changes in the pigment proteorhodopsin leading to light-driven proton pumping (via H^+-ATPase) (1,2). This pathway was formerly thought to exist only in halophilic Archaea but has recently been discovered in γ-proteobacterium in the marine environment and may represent a globally significant oceanic pathway for bacterially mediated light-driven energy production (1,2). Our understanding of the importance of bacterial photosynthesis in the ocean will undoubtedly undergo some major changes in the near future because of these recent discoveries.

In anoxic waters, such as, at the chemocline of the Black Sea, integral BChls in the anoxygenic zone can exceed total Chl *a* in the overlying oxygenated portion of the photic zone (91). Since the discovery of cyanobacteria (*Synechococcus* spp.; 92,93) and prochlorophytes (*P. marinus*; 94) in the open ocean, pigments have been used as diagnostic indicators for the presence of prokaryotic photoautotrophs. The abundance and distribution of marine prokaryotic algae are usually measured using flow cytometric techniques that utilize the presence of photosynthetic pigments (95). In the tropical and subtropical oceanic regimes of the world's oceans, the prokaryotic pigments, Zeax and DV Chl *a*, typically represent a significant fraction (20–60%) of the total algal pigments measured in surface waters (96–98).

Another recent technological development that has enabled researchers to utilize photosynthetic pigments to estimate the abundance of algal populations in the open ocean involves satellite remote sensing (99). Satellite imagery is being used not only to measure algal abundance but also to estimate productivity in the open ocean based on pigment concentrations in surface waters (100). New satellites such as Sea-viewing Wide Field-of-view Sensors (SeaWiFS) are now being used to assess phytoplankton Chl distributions and primary productivity in various remote oceanic realms (101). Other applications for pigment-based remote-sensing applications are also being explored. For instance, fourth derivative pigment analyses in conjunction with other optical measurements may prove useful in forecasting harmful algal blooms in coastal waters (102).

Macroalgae effectively absorb photosynthetically active radiation (400 to 750 nm) by producing various pigments to harness light energy in their specific ecological niche in the intertidal coastal zone. The rapid attenuation of red light in surface waters results in a vertical zonation of algal pigments observed among macroalgal species. The vertical distribution of seaweeds typically encountered are: the green algae residing in shallow water, the brown algae in the intertidal, and the red algae in the deeper subtidal portion of the water column.

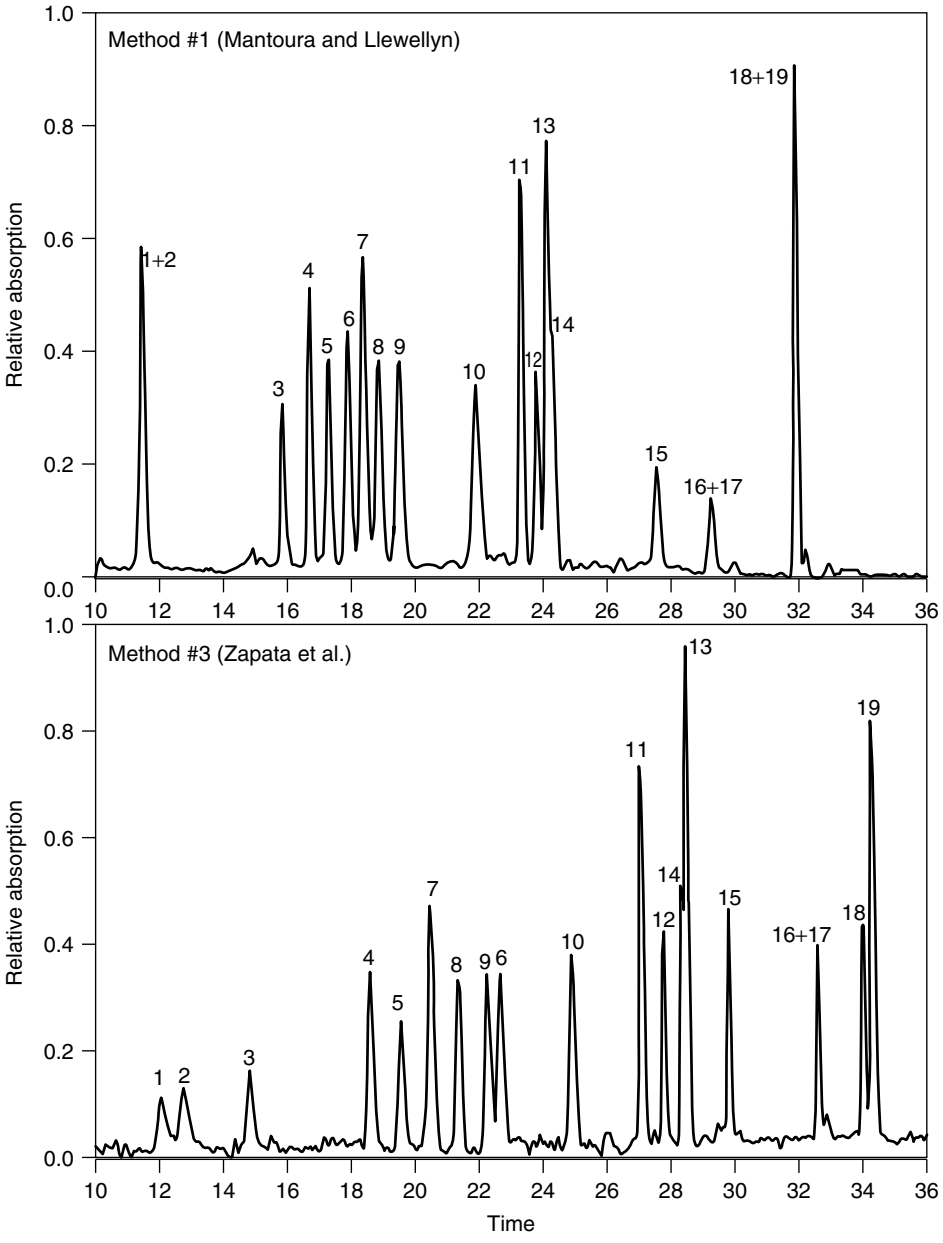

Figure 8. Comparison of HPLC chromatograms from Methods #1 and #3. Refer to Table 2 for identification of the numbered peaks.

PIGMENTS AND TAXONOMY

Photosynthetic pigments are increasingly used to identify the taxonomic composition of a sample collected from a community containing a mixture of taxa. Certain pigments are specific to single classes or a small number of classes, while other pigments are present across a wide variety of classes (Table 5). Distributions of pigments within the groups of photosynthetic bacteria have not been investigated as thoroughly as the distributions in algal groups. Within the eukaryotic algae, all classes that contain Chl *a*. Chl *b* are present in the Plantae algal classes (except the rhodophytes), the Euglenozoa, and the enigmatic protistan phylum Chlorarachniophyta. The remainders of the protistan and protozoan classes all contain one version or another of Chl *c*.

Photosynthetic Bacteria

While photosynthetic bacteria are classified on the basis of morphological and physiological aspects, rather than by pigment composition, some generalizations can be made. The two classes of purple bacteria (Rhodospirillaceae and Chromatiaceae) both contain BChl *a* as their primary photosynthetic pigment and BChl *b* as a secondary pigment. The suite of carotenoids produced by these classes varies widely within each group. The green sulfur bacteria (Chlorobiaceae) contain only minor amounts of BChl *a* in the reaction centers, and have BChl *c*, BChl *d*, and Bchl *e* as accessory pigments. The major carotenoids found in this class include chlorobactene and its hydroxy derivatives. The carotenoids (xanthophylls and carotenes) present in eukaryotic algae are the more diagnostic pigments, with some xanthophylls present in only one,

Table 5. Representative Groups of Photosynthetic Bacteria and Algae and Typical Pigment Compositions. Pigments in Bold Are Considered Diagnostic: Pigments in Parentheses Occur Only in Some Species or in Trace Amounts

Algal Group	Pigments	Refs
Prokaryotes		
Rhodospirillaceae	BChl *a* (BChl *b*) Lycopene, Neurosporene, Rhodopene, Spirilloxanthin	8,43
Chromatiaceae	BChl *a* (BChl *b*) Okenone, Spirilloxanthin, Warmingone — series	8,43
Chlorobiaceae	(BChl *a*) BChl *c*, BChl *d*, BChl *e*, Chlorobactene	8,43
Cyanobacteria	Chl *a*, **Zeax**, β-Car, **Phycobiliproteins**	14
Prochlorophyceae 1	**DV Chl *a***, **Zeax,** α-Car	14
Prochlorophyceae 2	**DV Chl *a***, **Mg DVP** (Zeax) **Para**, α-Car	14
Protozoa		
Euglenozoa	Chl *a*, Chl *b*, Diad (Diat) (Neox) β-Car	14
Dinoflagellatea	Chl *a*, Chl c_2, **Perid**, Diad, (β-Car)	14
Protista		
Chlorarachniophyta	Chl *a*, Chl *b*	103
Cryptophyta	Chl *a*, (Chl c_1) Chl c_2, **Allo**, Monad, α-Car, **Phycobiliproteins**	14,27
Diatomae	Chl *a*, Chl c_1, Chl c_2, **Fuco**, Diad (Diat) (β-Car)	14,104
Dictyochae	Chl *a*, Others?	
Haptophyta 1	Chl *a*, Chl c_1, Chl c_2, Fuco, Diad (Diat) (α-Car) (β-Car)	8
Haptophyta 2	Chl *a*, Chl c_1, Chl c_2, **Chl c_3**, Fuco, Diad (Diat) (α-Car) (β-Car)	8
Haptophyta 3	Chl *a*, Chl c_1, Chl c_2, **Chl c_3**, **19Hex**, Fuco, Diad (Diat) (α-Car) (β-Car)	8
Haptophyta 4	Chl *a*, Chl c_1, Chl c_2, **Chl c_3**, 19But, Fuco, **19Hex**, Diad (α-Car) (β-Car)	8
Raphidophyta	Chl *a*, Chl c_1, Chl c_2, Diad (Diat) Fuco, Zeax, β-Car	14,105
Phaeophyta		
Phaeophyceae	Chl *a*, Chl c_1, Chl c_2, **Fuco**, β-Car (α-Car)	31,106
Chrysophyceae	Chl *a*, (Chl c_1) Chl c_2, Fuco (Viol) (Neox) (Zeax) Diad (Diat) (β-Car)	14
Synurophyceae	Chl *a*, Chl c_1 (Viol) Fuco, (β-Car)	104,105,107
Eustigmatophyceae	Chl *a*, **Viol, Vauch** (Zeax) β-Car	14,108
Pelagophyceae	Chl *a*, Chl c_2, Chl c_3, **19But**, Fuco, (19Hex) Diad, (β-Car)	107
Phaeothamniophyceae	Chl *a*, Chl *c*, Hetero, Fuco, Diad, Diat, β-Car	109
Xanthophyceae	Chl *a*, Chl c_1, Chl c_2, Hetero, Diad (Diat)**Vauch**, β-Car	104,108
Plantae		
Chlorophyta	Chl *a*, **Chl *b***, Neox, Viol (Zeax) **Lut**, (α-Car) β-Car	14,106
Prasinophyta 1	Chl *a*, **Chl *b***, Neox, Viol (Zeax) **Lut**, (α-Car) β-Car	14,110
Prasinophyta 2	Chl *a*, **Chl *b***, **Mg DVP**, Neox, **Pras** (Zeax) **Lut**, (α-Car) β-Car	14,110
Prasinophyta 3	Chl *a*, Chl *c*, 19But, Fuco, (β-Car)	14,110
Rhodophyta	Chl *a* (Chl *d*) (Anth) (Lut) **Zeax**, β-Car, **Phycobiliproteins**	78,106

or a very few, classes. Some xanthophylls, such as Fuco and Diad, occur in many, if not most, classes.

Cyanobacteria (Cyanophyceae, Blue-Green Algae)

In addition to Chl *a*, cyanobacteria contain a suite of carotenoids, primarily Zeax, echinenone, myxoxanthophyll, and β-Car, but many species contain minor carotenoids such as Canth (111). Strains of *Synechococcus* contain Chl *a*, Zeax, and β-Car only (112,113).

Prochlorophyceae

Prochlorococcus contains the pigments DV Chl *a* and DV Chl *b*, which distinguish prochlorophytes from all other photosynthetic organisms (97,114,115). In addition, prochlorophytes contain Zeax and α-Car (116), and some strains contain phycoerythrin (74). Recently, a novel strain of *P. marinus* has been described from low-light, suboxic environments in the Arabian Sea and the Eastern Tropical Pacific (117). This strain, in addition to the normal prochlorophyte pigments, also contains Mg DVP and Parasiloxanthin. At higher light levels one strain of *P. marinus* also contained Chl *b* in small quantities (113).

Euglenozoa

Since euglenophytes contain Chl *a*, Chl *b*, Neox, Zeax, Siphonein, and β-Car they have been generally classified with the chlorophytes. However, they contain Diad as their major xanthophyll as well as Diat and Hetero, which are all characteristic of the Protistan algae. Combined with the consideration of ultrastructural characteristics there is sufficient cause to classify the euglenophytes as Protozoan algae (31,118).

Dinoflagellatea (Pyrrhophyta, Dinophyceae)

Dinoflagellates typically contain the pigments Chl *a*, Chl c_2, Diad, Diat, and β-Car, as well as Perid, which is uniquely associated with the dinoflagellates (119,120). The presence of endosymbionts can complicate the suite of pigments associated with dinoflagellates (121), with the addition of pigments typical of the endosymbiont. Some strains of *Gyrodinium* have been shown to contain Fuco or 19Hex, instead of Perid, as the main xanthophyll (122,123). Some dinoflagellates are reported to contain Chl c_1, in addition to Chl c_2 (124).

Chlorarachniophyta

Containing both Chl *a* and Chl *b*, the enigmatic alga *Chlorarachnion reptans* shows affinities to the Chlorophyceae; however, its ultrastructural characteristics more closely resemble the Cryptophyta (103). Ludwig and Gibbs (103) theorize that the chloroplasts in these species evolved from a chlorophyte endosymbiont, and, while they do not specify the other pigments present in these species, they are presumably the same as the Chlorophyceae.

Cryptophyta (Cryptomonada)

The primary Chl *c* pigment in cryptophytes is Chl c_2 (107); however, Chl c_1 and traces of Mg DVP have also been identified in the cryptophyte *Chroomonas* sp. (104). Alloxanthin is the primary carotenoid associated with cryptophytes (27,120,125), while crocoxanthin and monadoxanthin occur in most species but at lower concentrations (27,125). Cryptophytes are the one protistan algal group, that contains phycobiliproteins: phycocyanins and phycoerythrins.

Diatomae (Bacillariophyceae)

The amount of Fucoxanthin in a sample is usually correlated with the abundance of diatoms even though Fuco also occurs in other taxa, but in much smaller amounts (120).

Dictyochae (Silicoflagellates and Pedinellales)

No studies of the pigment composition of these algae have been reported to our knowledge.

Haptophyta (Prymnesiophyceae)

Some researchers have considered 19Hex as the diagnostic pigment for prymnesiophytes (126); however, in a survey of 29 species (50 strains) (127) the haptophytes were divided into four types based on their pigment composition. All 50 strains contained Chl *a*, Chl c_1 and/or c_2, Fuco, Diad, Diat, and β-Car. Type 1 contained no additional pigments; Type 2 contained Chl c_3; Type 3 contained Chl c_3 and 19Hex; and Type 4 contained Chl c_3, 19But, and 19Hex. Some strains also contained α-Car. Others (128) agree that 19Hex is not present in all haptophytes. This algal group has a diverse pigment composition and there is no clear correlation between pigment type and traditional Order or Familial classification.

Raphidophyta

Withers (105) report finding Fuco as the dominant pigment in raphidophytes, with lesser amounts of Zeax and trace amounts of β-Car in two strains of *Olisthodiscus luteus*. Fiksdahl and coworkers (129) report finding Fuco, Fucoxanthinol, and β-Car as the major pigments in two marine species (*Chattonella japonica* and *Fibrocapsa japonica*). Viol was a minor pigment (1 to 2%) of the total carotenoids and Zeax was also detected in one species. The pattern of pigment composition differed significantly from the freshwater species examined.

Phaeophyceae (Brown Algae)

In addition to Chl *a*, the phaeophytes contain chl c_1 and c_2, Fuco, and β-Car, with α-Car present in some species (106).

Chrysophyceae

Chrysophyte algae contain both Chl c_2 and c_1 (107). In an examination of the carotenoids contained in chrysophytes Withers and coworkers (105) found Fuco to be the major carotenoid in all species and β-Car was always present. A variety of other pigments, including Zeax, Diad, Diat, Neox, and Viol, have been found in some, but not all, species examined (105).

Synurophyceae

The Synurophyceae have recently been separated from the Crysophyceae, based on the absence of Chl c_2 and the presence of Chl c_1 in *Synura* and *Mallomonas* (107,130). Schimek and coworkers (104) also reported only Chl c_1 from *Synura petersenii*. Fuco is the predominant carotenoid in *Synura* with only trace amounts of Viol and β-Car (105).

Eustigmatophyceae

Eustigmatophytes contain Vauch and are differentiated from the xanthophytes by the absence of Chl *c* and the presence of Viol (108).

Pelagophyceae

The pelagophytes have been separated from the chrysophytes on the basis of a number of morphological, ultrastructural, and pigmentation features (131). *Pelagococcus subviridus* contains Chl c_3 and Chl c_2, but not Chl c_1 (107,132). Fuco and 19But are the major carotenoids, and Diad, Diat, and β-Car are minor carotenoids (133). The diagnostic pigment for Pelagophytes is 19But (120,132,134). Trace amounts of 19Hex have been found in another strain of *P. subviridus* (135).

Phaeothamniophyceae

Based partially on photosynthetic pigments, Bailey and coworkers (109) defined a new class of protistan algae containing genera formerly classified as Chrysophyceae or Xanthophyceae. The presence of both Hetero and Fuco is unique to this class.

Xanthophyceae (Tribophyceae)

In their survey of xanthophytes, Sullivan and coworkers (108) report finding that all of them have Chl *a*, Chl *c*, Diad, Diat, and β-Car. Most species also contained Hetero and Vauch, while none contained Mg DVP or Viol. *Pseudopleurochloris antarctica*, a new species described from Antarctic pack ice, contained Chl *a*, Chl c_2, Hetero, Diad, Diat, Vauch, and β-Car, but no Fuco or Chl c_1 (18).

Chlorophyta (Green Algae)

Chlorophytes contain the general suite of pigments as do the higher plants (42): Chl *a*, Chl *b*, Neox, Zeax, Lut, and

carotenes. Additional carotenoids found in some species of macroalgae include siphonaxanthin, siphonein, and astaxanthin (106).

Prasinophyta

On the basis of pigment composition, prasinophytes have been divided into three types (110). Type I exhibited the normal chlorophyte pigment suite (Chl *a*, Chl *b*, Neox, Viol, Zeax, Lut, and carotenes). Type II contained all of these pigments plus Prasinoxanthin, considered to be the diagnostic pigment for prasinophytes (21). Type III lacked both Pras and Chl *b*, and contained other carotenoids, which are present in chrysophytes (19Hex, Fuco, and Diad). Mg DVP has been reported in several species, including *Mantoneilla squamata* and *Pycnococcus provasolii*, Type II prasinophytes (104,110,136).

Rhodophyta (Red Algae)

In addition to Chl *a*, and the major carotenoids Zeax and β-Car, rhodophytes contain the phycobiliproteins: APC, PC, and PE (106). Some species contain the minor carotenoids Lutein or Anth (78,137). While Chl *d* has been identified, whether it plays a role in photosynthesis or is merely a degradation product remains to be determined (78).

CHEMTAX ANALYSIS OF PHYTOPLANKTON COMMUNITY COMPOSITION

CHEMTAX is a matrix software routine developed to determine the percentage of Chl *a* in a collection of samples allocated to different algal groups (138–143). CHEMTAX works within the MATLAB computing system to determine the class composition of a phytoplankton assemblage based on the photosynthetic pigments measured using HPLC separation techniques. CHEMTAX begins with an initial pigment ratio matrix containing the relative pigment content for each of the identified pigments within each taxonomic group. A second matrix is prepared with the pigment concentration for each sample in the study. CHEMTAX factors together the two matrices to produce a final matrix of the percent contribution of each taxonomic group to the total Chl *a* concentration in each sample. The preparation of an appropriate initial pigment ratio matrix is key to the proper analysis of pigments contained in an extracted phytoplankton sample. Mackey and coworkers (140) stressed the importance of calibrating CHEMTAX using major phytoplankton species native to the area where the data samples were obtained. Different regions will require different initial pigment matrices as a result of the different suites of phytoplankton present. Currently, researchers around the world are developing appropriate initial pigment ratio matrices for various oceanographic regimes, and then using these matrices to describe phytoplankton community assemblages and their impacts on biogeochemical cycles. It is important that future laboratory calibrations of the CHEMTAX method utilize ecologically relevant algal species and that oceanic studies corroborate the pigment-based algal distributions with microscopic identifications.

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PHOTOTROPHIC PURPLE AND GREEN BACTERIA IN MARINE AND HYPERSALINE ENVIRONMENTS

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The ability to transform light energy into biochemical energy is found in all major phylogenetic branches of life, the archaea, the eubacteria, and the eukarya. Two basically different biochemical mechanisms are known that transform light energy into biochemical forms of energy that can be used in metabolic processes, namely, retinal-containing rhodopsins and chlorophyll-containing protein complexes.

Retinal-containing proteins, the rhodopsins are well known as characteristic components of extremely halophilic archaea, the halobacteria. Rhodopsins have been intensively studied in these archaea in which they function in transmembrane proton transport (bacteriorhodopsins), in chloride transport (halorhodopsins), and as photosensory receptors (sensory rhodopsins) (1). A retinal-containing protein with characteristic features typical of archaeal rhodopsins was recently also found in γ-Proteobacteria of the SAR86 group (2), which is

widely distributed in marine environments. The proton export from intact cells by this protein, called *proteorhodopsin*, could be demonstrated (2). Although both bacteriorhodopsins and proteorhodopsins function as proton pumps and establish a light-driven transmembrane proton gradient, archaea and γ-Proteobacteria containing these proteins are basically chemoheterotrophic bacteria and depend on respiratory energy transformation for growth. They gain selective advantage in illuminated environments by their ability to use light-driven membrane energetization in addition. However, a phototrophic way of life based on light-driven energy transformation, as the principal source of metabolic energy, is not possible by these bacteria.

The chlorophyll-mediated energy transformation is widely distributed among eubacteria, but is not known to occur in archaea. On the basis of fundamental physiological differences, we distinguish among oxygenic phototrophic bacteria that use water as photosynthetic electron donors and produce molecular oxygen (cyanobacteria), anoxygenic phototrophic bacteria that use reduced substrates such as sulfide, hydrogen, ferrous iron, and a number of simple organic substrates as photosynthetic electron donors but do not produce oxygen during photosynthesis, and aerobic bacteriochlorophyll-containing bacteria ("ABC-bacteria"), which represent chemoheterotrophic bacteria with the potential to produce photosynthetic pigment-protein complexes and to perform light-mediated photosynthetic electron transport processes.

The aerobic bacteriochlorophyll-containing bacteria ("ABC-bacteria") are represented by a number of α-Proteobacteria, and at present by a single β-Proteobacterium. Although light-mediated energy-transfer apparently contributes to their energy budget (3,4), the principal process of their energy transformation is the oxidation of organic substrates by aerobic respiration. Photopigment biosynthesis and formation of the photosynthetic apparatus in these bacteria, in contrast to anoxygenic phototrophic bacteria, is not inhibited but depends on the presence of oxygen (5). These bacteria are widely distributed in the oxic marine environment (6). Indeed, fluorescence kinetic measurements demonstrated the widespread presence of bacterial photosynthesis, presumably by ABC-bacteria, in tropical surface waters of the eastern Pacific Ocean and also in coastal waters of the northwestern Atlantic Ocean (7). Phototrophy has not been demonstrated in these bacteria and their potential advantage in natural habitats, due to their photosynthetic potential, has not been unequivocally demonstrated. [Note that the distinction is made between *photosynthesis* (and *photosynthetic*) as the ability to perform light-mediated energy transduction and *phototrophy* (and *phototrophic*) as the ability to grow on the basis of light-mediated energy transformation as the principal source of energy.] From what is known about these bacteria, it is reasonable to assume that they grow just as other chemotrophic bacteria under oxic conditions in the dark (including the deep sea in general and — contradicting Yurkov and Beatty (8) — also in the vicinity of hot vent locations), but make use of their photosynthetic apparatus in illuminated oxic environments to gain additional metabolic energy (7).

Phototrophic bacteria are found in major eubacterial branches and have characteristic differences in the structure and function of the photosynthetic apparatus, in the pigment content and in important physiological properties. Characteristic properties to distinguish these major branches of phototrophic bacteria including *Chloroflexus* and relatives, Chlorobiaceae, cyanobacteria, *Heliobacterium* and relatives, and phototrophic purple bacteria (α-, β- and γ-Proteobacteria) are summarized in Table 1.

The Cyanobacteria (including those known as *prochlorophytes*, such as *Prochloron*, *Prochlorococcus*, and *Prochlorothrix* species) are phototrophic bacteria performing an oxygenic type of photosynthesis. In addition, some representatives are able to use reduced sulfur compounds as photosynthetic electron donors and under these conditions use only one photosystem in an anoxygenic type of photosynthesis. The most abundant phototrophic organism in the global ocean environment, most likely, is a very small oxygenic phototrophic prokaryote, *Prochlorococcus marinus* (9).

The Heliobacteria are anoxygenic phototrophic bacteria that contain bacteriochlorophyll *g* and carotenoids. They are highly sensitive to oxygen and some species form heat-resistant endospores. They are phylogenetically related to gram-positive bacteria and grow photoheterotrophically. Marine forms of these bacteria are so far not known.

The green nonsulfur bacteria (Chloroflexaceae), also known as *filamentous and gliding green bacteria* are anoxygenic phototrophic bacteria that contain bacteriochlorophyll *c* or *d* in light-harvesting complexes located in special light-harvesting organelles, the chlorosomes. They move by gliding, are tolerant to oxygen, and grow preferably as photoheterotrophs. These bacteria are adapted to hot freshwater environments. Although they have been observed microscopically in marine habitats, pure cultures of marine representative do not exist.

The green sulfur bacteria (Chlorobiaceae) are anoxygenic phototrophic bacteria that contain bacteriochlorophyll *c*, *d*, or *e* in light-harvesting complexes located in special light-harvesting organelles, the chlorosomes. They are obligately phototrophic, require strictly anoxic growth conditions, and have a low capacity to assimilate organic compounds. Depending on the pigment content, a number of green-colored and corresponding brown-colored species are known. Several species have been found in marine and also hypersaline environments, in which under appropriate conditions intensively colored, visible mass developments are formed.

The purple sulfur bacteria (Chromatiaceae and Ectothiorhodospiraceae) and the purple nonsulfur bacteria are anoxygenic phototrophic bacteria that contain bacteriochlorophyll *a* or *b* in the light-harvesting complexes located in the cytoplasmic membrane and various kinds of invaginations of it. Bacteria of this group are the most prominent and abundant anoxygenic of phototrophic bacteria in marine and hypersaline environments.

1. The *Chromatiaceae* are γ-Proteobacteria and grow well under photoautotrophic conditions and use

Table 1. Diagnostic Properties of Major Groups of Phototrophic Prokaryotes

	Chlorobiaceae	Chloroflexaceae	Purple bacteria	Heliobacteria	Cyanobacteria (including prochlorophytes)
Photosynthesis	anoxygenic	anoxygenic	anoxygenic	anoxygenic	oxygenic
Type of (bacterio) chlorophyll	bchl c, d, e (+ bchl a)	bchl c, d (+ bchl a)	bchl a, b	bchl g	chl a, chl b*
Phycobilins present	−	−	−	−	+/−
Type of reaction center	I	II	II	I	I + II
Reduction of NAD^+ by primary photosynthetic electron acceptor	+	−	−	+	+
Location of antenna	chlorosomes	chlorosomes	ICM	CM	ICM phycobilisomes**
Pathway of autotrophic CO_2-fixation	reductive TCA cycle	Hydroxypropionate-pathway	Calvin cycle	−	Calvin cycle
Preferred electron donor	H_2S, H_2	organic compounds (H_2S)	H_2S, H_2 organic compounds	organic compounds	H_2O
Chemotrophic growth	−	−	+/−	−	−/(+)

− characteristic absent;
+/− characteristic present or absent;
−/(+) characteristic absent or weak activity present;
*present in prochlorophytes;
**absent in prochlorophytes; CM cytoplasmic membrane; ICM internal membranes.

sulfide as photosynthetic electron donors, which are oxidized to sulfate via intermediate accumulation of elemental sulfur inside the cells. Photoheterotrophic, chemoautotrophic, and chemoheterotrophic growth has been demonstrated in several species.

2. The *Ectothiorhodospiraceae* are γ-Proteobacteria and can be distinguished from the *Chromatiaceae* by deposition of elemental sulfur outside or in the peripheral part of the cells, and their preference for alkaline and saline growth conditions. Some species may also grow chemotrophically under aerobic dark conditions.
3. The purple nonsulfur bacteria are α- and β-Proteobacteria and preferentially grow under photoheterotrophic conditions, though most of them also have the ability to grow photoautotrophically with either hydrogen or reduced sulfur compounds as electron donors. Some species can use sulfide as photosynthetic electron donors, but only a few are able to completely oxidize sulfide to sulfate.

Among those bacteria that perform a phototrophic way of life, as defined earlier, the most significant physiological and ecological differences are between oxygenic and anoxygenic phototrophic bacteria. Both of these groups have representatives living in the marine and also the hypersaline environment. Although individual species of phototrophic bacteria are well adapted to a particular habitat and mineral salts composition, qualitatively and quantitatively, as a group, phototrophic bacteria have adapted to the whole spectrum of salt concentrations from freshwater to saturated brines.

Depending on their different responses to oxygen, the oxygenic phototrophic bacteria and the anoxygenic phototrophic bacteria are found in different ecological niches. Cyanobacteria usually inhabit the upper part of the photic zone in lakes, on hard substrates and in the sea. Those cyanobacteria that can perform anoxygenic photosynthesis are found in the lower anoxic part of stratified environments such as the Solar Lake (10). The anoxygenic phototrophic bacteria inhabit the lower anoxic part of the photic zone. In addition to their ability to perform anoxygenic photosynthesis, many of them have the capacity to grow chemotrophically under microoxic to oxic conditions in the dark and have adapted to niches in the oxic environment and to unstable, not permanently anoxic habitats. In the marine intertidal zone, both oxygen-producing cyanobacteria and anoxygenic purple and green phototrophic bacteria often occur close together and may form colored blooms and microbial mats. This article deals with the biology of anoxygenic phototrophic green and purple bacteria that live in marine and hypersaline environments, but excludes the oxygenic phototrophic cyanobacteria. An overview on the biology of marine and halophilic cyanobacteria was given earlier (11).

GENERAL ECOLOGY OF PHOTOTROPHIC BACTERIA IN SALINE HABITATS

Anoxygenic phototrophic bacteria are widely distributed in shallow marine sediments and water and regularly form visible mass developments in marine environments of the coastal zone and at the chemocline of marine waters. Already Warming (12) reported on such massive developments of phototrophic purple bacteria at the coast of Denmark. Since then, similar observations have been made around the world's coastlines. Phototrophic bacteria occur at all kinds of shorelines and geographic regions and numerous examples have been described in the literature of colored blooms and mass accumulations of

phototrophic bacteria in the coastal zone (for reviews see 11,13–15). Environments suitable for the development of phototrophic bacteria include sand beaches, tidal flats, estuaries, salt marshes, closed bays, and coastal lagoons, but also stratified water bodies such as fjords, deeper bays, and saline lakes and salterns, and salt and soda lakes. They prefer the anoxic zones, but some of these bacteria live in anoxic niches of the bulk oxic marine environment, which is generally regarded as being devoid of anoxygenic phototrophic bacteria.

The intertidal environments range from permanently dry sediments (which receive water by capillary movements from the ground), over sediments falling dry during low tides, to sediments that are permanently covered with water, which is either partly exchanged with the tides or exchanged only occasionally with unusually high tides. The smaller the water exchange with the tides, the better the conditions for the establishment of an anoxic water body, by rise of sulfide into the water, and the better the conditions for the development of conspicuous blooms of phototrophic bacteria.

Environmental Conditions in Favor of Phototrophic Bacteria

The most important properties that determine the development and distribution of phototrophic bacteria in nature are the concentrations of sulfide and oxygen and the light intensity, which form countercurrent gradients. The depth at which phototrophic sulfur bacteria develop is largely restricted to the concomitant presence of light and sulfide. Some of the microbial activities that determine slope and position of these gradients, such as oxygen production by algae and cyanobacteria and sulfide oxidation by phototrophic bacteria, show diurnal, light-dependent fluctuations. Others, such as sulfide production by sulfate-reducing bacteria and oxygen consumption by respiratory bacteria, do not. As a result of these processes, the sulfide horizon rises during the night and goes down again during the day. The motile phototrophic purple bacteria are able to follow the moving sulfide horizon. Under favorable conditions, separate layers of green sulfur bacteria are formed underneath layers of phototrophic purple bacteria and of algae and cyanobacteria. Frequently, however, mixed communities (not well-separated layers) of purple and green sulfur bacteria are observed, and purple nonsulfur bacteria regularly accompany the mass development of phototrophic sulfur bacteria.

The Role of Hydrogen Sulfide. The ability of phototrophic sulfur bacteria to oxidize sulfide and other reduced sulfur compounds under anoxic conditions is one of their most characteristic and ecologically important properties. Sulfide is not only used as the electron donor and sulfur source, but also serves to maintain anoxic conditions. One of the possible final stages of anaerobic decomposition of organic matter is performed by sulfate-reducing bacteria. Whenever the activity of sulfate-reducing bacteria in sediments is high enough to raise the sulfide horizon into the photic zone, development of purple and green sulfur bacteria is possible.

Concentrations of sulfide and elemental sulfur and the relations of the different phototrophic bacteria to these compounds are significant factors in determining patterns of natural dominance and successful competition. Of particular importance are the affinities to these two sulfur compounds, their oxidation rates, the ability to use external elemental sulfur, and the ability to store elemental sulfur inside or outside the cells. The intracellular storage of elemental sulfur by Chromatiaceae gives these bacteria an additional advantage over those bacteria that store elemental sulfur outside the cells (green sulfur bacteria and purple nonsulfur bacteria). All elemental sulfur formed by Chromatiaceae is inaccessible to other bacteria, whereas the elemental sulfur formed by green sulfur bacteria is also available to Chromatiaceae. Therefore, intracellular stored elemental sulfur is of inestimable value for these bacteria: (*1*) under conditions of external sulfide depletion in the light, it serves as a reservoir of photosynthetic electron donors; (*2*) under dark conditions and in the presence of oxygen, it may support endogenous respiration (16,17); (*3*) under anoxic conditions in the dark, it may serve as an electron acceptor during endogenous fermentation of stored carbohydrates (18,19).

The Role of Light. Not only the quantity, but also the quality of light is of major importance for the development of phototrophic bacteria, and because of different pigment contents of the various phototrophic bacteria, light is also a selective environmental factor. The light quality required by a phototrophic bacterium is reflected in the absorption spectra of the photosynthetic pigment-protein complexes and is a characteristic property for a particular species and group of phototrophic bacteria.

Great variation exists with respect to the chemical structures and the light-absorbing properties of bacteriochlorophylls and carotenoids in different species. The light-harvesting organelles of the phototrophic green bacteria, the chlorosomes, contain either bacteriochlorophyll *c*, *d*, or *e*, all of which have their long-wavelength absorption maximum between 700 and 760 nm. Heliobacteria have a major absorption maximum of bacteriochlorophyll *g* at 770 to 790 nm. Most of the phototrophic purple bacteria have bacteriochlorophyll *a*, with long-wavelength absorption maximum between 800 and 900 nm. Quite a few species have bacteriochlorophyll *b*, with an absorption maximum at 1,015 to 1,035 nm, exceptionally also below 1,000 nm (e.g., at 986 nm in *Rhodospira trueperi*, 20). These spectral properties demonstrate that on the basis of (bacterio)chlorophyll absorption there is no competition among green bacteria, purple bacteria, and oxygenic phototrophic organisms. (The chlorophyll *a* of cyanobacteria and eukaryotic algae absorbs below 700 nm).

Within sediments, the availability of light severely limits the development of phototrophic bacteria. Light is strongly absorbed by sand. In fine sand containing 5% mud, about 10% of the surface light penetrates 1.5 mm, and only 1% is present in a 3-mm depth of sediment (21). In addition, sand acts as an infrared filter and allows longer wavelengths, a deeper penetration (22). As a consequence, only steep countercurrent gradients

of sulfide and oxygen below an oxic sediment surface will form suitable conditions for the development of phototrophic bacteria. Their development is therefore restricted to the uppermost few millimeters of the sediment, where they often form thin layers below a surface layer of cyanobacteria. Bacteriochlorophyll absorption is of major importance in sediments, because infrared radiation penetrates particularly deep into sandy sediments (22). In deeper layers of water, however, the use of bacteriochlorophylls for light harvesting is limited by the stronger absorption of infrared radiation by water, in particular above 800 nm. Therefore, bacteria with bacteriochlorophyll *b* are the least suited to develop in deeper layers of lakes. They appear to be well-adapted to shallow water and in particular to sediment habitats not permanently covered by water.

In aquatic marine habitats, where sulfide-containing layers receive only low light intensities, *Chlorobiaceae* have a general advantage over the phototrophic purple bacteria because of their more efficient light-harvesting machinery, the chlorosomes. This efficiency allows growth at very low light intensities (below 50 lux), not enabling phototrophic purple bacteria to grow. The phototrophic green sulfur bacterium, *Prosthecochloris aestuarii*, was grown at light intensities as low as 5 to 10 lux, if cocultured with the sulfur reducer, *Desulfuromonas acetoxidans* (23). In those cases in which detailed studies of the colored water layers have been undertaken, and in which these layers received only low light intensities, brown-colored *Chlorobiaceae* such as *Chlorobium phaeobacteroides* and *Prosthecochloris phaeoasteroidea* have been found as the dominant phototrophic bacteria. Light absorption by carotenoids is of major ecological significance in deeper layers of aquatic habitats, because radiation between 450 to 470 nm penetrates deepest into water (24,25). Light penetration of seawater parallels the absorption properties of the carotenoids of the brown-colored *Chlorobiaceae* (26), which thereby have a selective advantage. This has been taken as an explanation for their dominance in lakes, where the anoxic hypolimnia occupy deeper layers (26–28). By mixing pure cultures of *Thiocystis violacea*, *Chlorobium limicola*, and *C. phaeobacteroides* and incubation of these mixtures at various depths (down to 30 m) in marine coastal waters, it could be shown that, with increasing depth, sunlight selects the brown *C. phaeobacteroides* (29).

The Relations to Oxygen. Oxic parts of nature in principal are not considered to be environments for anaerobic bacteria, and the open ocean is generally regarded as being devoid of anoxygenic phototrophic sulfur bacteria. However, there are at least two strategies that enable growth, reproduction, and even successful competition of phototrophic purple bacteria under oxic conditions and are of importance in the marine environment: (*1*) true adaptation by metabolic flexibility in energy conservation, that is, the ability to readily use both photosynthetic and respiratory mechanisms and (*2*) the development in and effective use of anoxic niches in an apparently oxic environment.

The first strategy enables the facultative respiring phototrophic bacteria to develop under diurnal oscillating conditions as part time phototrophs and part time chemotrophs at the oxic/anoxic chemocline, in natural gradient systems. Respiration is common among purple nonsulfur bacteria, and also many purple sulfur bacteria are able to respire (30). In particular, *Thiocapsa roseopersicina* is of high metabolic flexibility, and this species is well equipped to take advantage of changing conditions from oxic/dark to anoxic/light conditions and even simultaneously drives respiration and photosynthesis (31–33). Several observations indicate that respiration is of selective advantage for this species in nature: (*1*) Elegant competitive experiments demonstrate the advantage of *T. roseopersicina* under dynamic changes of oxic to anoxic conditions and under diurnal changes of illumination (33). (*2*) It has been isolated from environmental samples by the agar dilution series kept in darkness over several transfers as a chemotrophic sulfur oxidizer (Imhoff, unpublished). (*3*) It has been found, though in low numbers, at the chemocline of the Gotland deep in the Baltic Sea (which was at 120 m depth in darkness) where light was absent or its availability critical. Chemolithotrophic growth is their likely way of life at such places. In conclusion, respiratory capabilities not only are of advantage in environments with steep chemical and physical gradients, in which during diurnal cycles changes of the light regime and of oxic to anoxic conditions occur and these bacteria may use both phototrophic and respiratory systems, but also in situations of prolonged darkness in which only respiration is possible.

An interesting example of the second strategy also is found among Chromatiaceae. Apparently, the strictly phototrophic *Marichromatium purpuratum* has adapted to anoxic niches in the bulk oxic environment and even succeeded to conquer niches in the open ocean. Originally, it was isolated from a strictly oxygen demanding marine sponge, which contained massive cell densities (34,35). Later, a *Didemnum* species revealed similar mass accumulations of this species (11), and more recently it was isolated from the stomach of marine copepods that have a translucent chitin mantle (36).

Habitats

Marine Shorelines and Sediments. The shorelines of marine environments with their numerous niches can be considered as the most important places for the development of phototrophic sulfur bacteria in recent times, although they are unstable and in a continuous change. Habitats of anoxygenic phototrophic bacteria at the shorelines occur at different geographic regions all over the world, from the polar regions and the sea ice to the equator, and are the most obvious and visible documentation of the presence of anoxygenic phototrophic bacteria in the marine environment. Most of our knowledge is from the moderate northern hemisphere, in particular the European and the American coast. Conditions that favor the development of anoxygenic phototrophic bacteria can be found wherever quiet water is present, for example, in small splash water ponds at rocky shores, in small puddles and large lagoons, and in various types of sediments. They develop in the sediments of sandy beaches, of salt marshes and tidal

flats, and in muddy masses of decaying biomass of algae or seaweeds (11,13,14,37).

It appears that artificial changes at the shorelines, in particular the removal of quiet-water zones and places of sedimentation due to the construction of dikes, have a serious impact on natural habitats of anoxygenic phototrophic bacteria. Certainly, such activities have destroyed or disabled many locations for the development of these bacteria. This is apparently the case at the German coasts of the Baltic Sea, where places for the mass development of phototrophic bacteria are not common. However, when new natural shorelines are formed outside the constructed dikes and situations are established leading to quiet water and accumulation of dead organic matter, massive blooms of phototrophic bacteria may develop. Interestingly, such locations often are found in natural reserves, although the development of anoxygenic phototrophic bacteria certainly was not a reason to preserve these areas. Examples from the German Baltic Sea shore are the "Bottsand" close to Kiel, the "Geltinger Birk" in northern Schleswig-Holstein, and the northern part of the island of Hiddensee.

The Wide Distribution of Phototrophic Bacteria in Marine Habitats. The obvious and massive developments that already can be seen with the naked eye naturally have attracted scientists and such places were selected for investigations and used as sources to isolate phototrophic bacteria. From these descriptions, the impression may result that phototrophic bacteria occur at a few restricted sites. However, their occurrence, distribution, and development in nature must be much wider. Much more frequently the number of phototrophic bacteria is expected to be below the level that causes colored spots and layers in sediments or in coastal waters. Obviously, the blooms form the tip of an iceberg and the invisible development is the much more widely occurring situation.

Anoxygenic phototrophic have been found, for example, in many coastal sediments, even if these appeared to be oxic at the surface and not provide conditions suitable for anaerobic phototrophic bacteria. It could be demonstrated, for instance, that purple sulfur bacteria are widely distributed in sediments of the Baltic Sea. Not only at the shoreline, but also in most of the sediment samples even below 20 m in water depth, purple sulfur bacteria were found in numbers from a few hundred to a few thousand per mL from the deepest to the lower sites. Numbers at selected sites of the shoreline were significantly higher. The numbers in sediments of the open Baltic Sea are far too high to be explained by introduction from shoreline regions where places of massive development are not common and numbers in total are not sufficiently high (Imhoff, unpublished results). Most common at these places were strains genetically related to *T. roseopersicina*. Also, green sulfur bacteria occur regularly in coastal sediments of the Baltic Sea, where viable counts were found approximately two orders of magnitude below those of purple sulfur bacteria (i.e., 10^3 to 10^4 cells /mL in a sediment at a pre-blooming state of purple bacteria with 10^5 to 10^6 cells /mL). This is a level that clearly escapes detection by the naked eye.

Obligately phototrophic green sulfur bacteria have even been found, though in very low numbers, in sediments of the subtidal zone from the German Waddensea, which were characterized by high bacterial activity, decomposition and sulfate reduction, and anoxic/sulfidic conditions close to the surface. However, because of the tidal movement of the water at these places, the sediments were dry and in contact with the air during the low water situation, and oxic water was introduced during high tides; therefore, the top layer always received oxygen and remained oxic or at least microoxic through most of the time. Anoxic niches could prevail or be established for a short period during low tides at the sediment surface of mud puddles when strong sulfate reduction occurred at the top layer of the sediment. Unlike places having microbial mats with a prominent contribution of phototrophic microorganisms that stabilize the sediment surface and a steep chemical gradient system, these locations were without surface stabilization and visible signs of phototrophic microorganisms (Imhoff, unpublished results). It is certainly difficult to consider these places as habitats of strictly anaerobic and obligately phototrophic green sulfur bacteria. However, anoxic microniches receiving some illumination may be sufficient to maintain small populations of these anoxygenic phototrophic bacteria. If the movement of the water is stopped and sulfide production causes prolonged anoxic and sulfidic conditions of sediment and water phase, in nature or artificially in the lab, green sulfur bacteria may develop and eventually bloom rapidly.

Great Sippewissett Salt Marsh. An outstanding example of a marine coastal habitat is found in the microbial mats of the Great Sippewissett Salt Marsh (Cape Cod, Massachusetts). A number of phototrophic bacteria were isolated and several new species were described from this habitat (20,37–39). Laminated microbial mats of unusual thickness regularly occur during summer at Great Sippewissett Salt Marsh, and Nicholson and coworkers (40) described one very well-developed mat. In sandy intertidal sediments of the marsh, the mats were about 10 mm thick and comprised four to five distinctly colored layers. Phototrophic purple sulfur bacteria of the central pink layer and the directly underlying peach-colored layer were identified by using both pigment analysis and fine structure as revealed in thin sections by transmission electron microscopy. The bacteria of the peach layer were recognized as *Thiococcus pfennigii* on the basis of the presence of bacteriochlorophyll *b* and the bundles of tubular intracellular membranes in the coccoid cells. Another bacterium with bacteriochlorophyll *b* was isolated from this habitat, which resembles *T. pfennigii* in many properties but is a motile coccus. It was described as *Thioflavicoccus mobilis* (39). In the peach-colored layer of mats from the same locality, also, small spirilloid cells were detected by scanning electron microscopy (about 1% of total cells). This bacterium was isolated and found to have bacteriochlorophyll *b* and to exhibit a number of unusual characteristics. It was described as a new species *R. trueperi* (20). Also, *Roseospirillum parvum* was isolated from this habitat (38).

Coastal Waters, Lagoons, Fjords. There are a number of reports on the development of phototrophic bacteria in shallow coastal waters, such as lagoons, but also in saline lakes and anoxic fjords (11,13,14,37). In the more stable estuarine habitats, such as closed bays and lagoons, besides purple sulfur bacteria, green-colored species of the *Chlorobiaceae* are frequently found in massive blooms. In these shallow waters, they may have an advantage over their brown-colored counterparts. Both green- and brown-colored *Chlorobiaceae* may, however, occur together in such environments as have been found in the Bietri Bay of Ebrie Lagoon (Ivory Coast) (41). In this shallow water, the layers of phototrophic purple bacteria and *Chlorobiaceae* were not clearly separated as, for example, in Solar Lake (10). A similar pattern, however, was found with the highest number of phototrophic purple bacteria in the uppermost part of the layer and the maximum cell density of *Chlorobiaceae* below. The brown-colored Chlorobiaceae became more dominant in the deepest part of the layer in this lagoon (41) and are considered to generally gain importance with the decline of the chemocline to deeper water layers as in lakes and fjords.

The Black Sea. A special case of a stratified marine habitat is the Black Sea. Although its anoxic layer did not reach the photic zone, and development of phototrophic bacteria seemed to be impossible, such bacteria have been isolated from the dark anoxic layers. Hashwa and Trüper (42) observed in enrichment and pure cultures from Black Sea bottom sediments at 600 and 2,240 m depth, respectively, *T. roseopersicina*, *Allochromatium warmingii*, and *C. phaeobacteroides*. These authors assumed survival of the phototrophic bacteria that may be washed away from their estuarine environment in the dark layers of the Black Sea, but excluded active growth. At about the same time of their study, there was a report on mass mortality of phototrophic bacteria in the Black Sea (43). These authors had only scanning-electron microscopic information of particle form, size, and number and therefore could not prove the existence of phototrophic sulfur bacteria, but claimed the mass mortality of bacteria such as *Thiocapsa*, that is, spherical purple sulfur bacteria. At the time of their investigation, there was no light at the chemocline, and phototrophic life was not possible. If the particles observed by Dickmann and Artuz (43) were bacteria, the development of a chemolithotrophically growing purple sulfur bacterium such as *T. roseopersicina* at the dark chemocline could be one possible explanation. The chemocline in the Black Sea had risen during the following years and the situation was much different approximately 10 years later, when it reached horizons receiving light of minor intensities and large amounts of bacteriochlorophyll *e* and brown-colored green sulfur bacteria were detected (44–46).

Sea Ice. A habitat generally regarded to be oxic but not anticipated to have anoxic niches is sea ice. In sea ice of the western Baltic Sea that formed a layer of 1 to 2 m thickness over several weeks in the late winter of 1996 (February/March), purple sulfur bacteria were found in significant proportions by using genetic approaches (47). Genetic sequence information obtained after PCR and DGGE separation gave evidence of a marine purple sulfur bacterium (three related sequences) together with other anaerobic bacteria in the interior of the ice cover (47). These data are the first strong hints on the possible existence of anoxic niches within the complex system of small brine channels within sea ice. Again, the oxygen tolerance, the potential of respiratory energy conservation, and the ability to grow both photo- and chemoorganotrophically are considered to be excellent prerequisites for an anoxygenic phototrophic bacterium to live in this habitat.

Salt and Soda Lakes. Although many habitats of phototrophic bacteria in the coastal zones contain brackish waters, others are more concentrated than seawater. Shallow waters in splash water ponds, coastal lagoons, closed basins, etc., of the coastal zone receiving intensive illumination from the sun are subject to evaporation. As a consequence, not only inorganic salts but also organic matter accumulates and chances for the occurrence of anoxic conditions and the development of anoxygenic phototrophic bacteria increase dramatically. These waters have higher fluctuations in salt concentrations and quite often the salinities are higher than in seawater. Phototrophic bacteria adapted to this type of habitat are expected to require or at least tolerate salt concentrations above seawater salinity. Many isolates from such places do so.

Hypersaline habitats show greatly varying ionic composition. Thalassohaline water occur as natural evaporation pools of marine water or as artificial evaporation ponds of marine salterns. A different type of hypersaline environments is found in various athallassohaline water of inland saltwater lakes. A few prominent examples of such lakes are the Great Salt Lake (Utah), the Dead Sea (Israel), and the soda lakes of the Wadi Natrun in Egypt. Halophilic phototrophic bacteria occur in visible masses and were isolated from marine salterns (48–50), alkaline soda lakes in the Egyptian Wadi Natrun (51–54), Russian soda lakes in Siberia and Mongolia (55,56), and the Solar Lake (10,57,58).

PHOTOTROPHIC BACTERIA ISOLATED FROM MARINE AND HYPERSALINE HABITATS

The most prominent groups of anoxygenic phototrophic bacteria in marine habitats are the purple and green sulfur bacteria. From the early literature, the impression is given that marine forms of purple and green sulfur bacteria are not well differentiated from their counterparts living in freshwater environments. Many of the isolates from marine habitats were identified or tentatively classified as species known from freshwater sources. Because, for many of the early isolates from marine environments, salt response has not been determined, it remains unclear whether they are true marine bacteria or not. Historically, the establishment of media and culture conditions for green and purple sulfur bacteria (59,60) stimulated the isolation and study of phototrophic sulfur bacteria mainly from freshwater sources. In the early 1970s, the majority of

known anoxygenic phototrophic bacteria was represented by isolates from freshwater sources, and only a few salt-dependent phototrophic purple sulfur bacteria had been described. The first salt-dependent and sulfide-tolerant marine purple nonsulfur bacterium described was *Rhodovulum sulfidophilum* (61). In particular, during the past two decades, the consequent application of salt concentrations relevant to marine and hypersaline environments, respectively, led to the isolation of a larger number of new, truly marine and halophilic, phototrophic bacteria specifically adapted to the salt concentrations of their habitats (Tables 2–4).

Chromatiaceae

Chromatiaceae are the most obvious and numerous phototrophic bacteria in many estuarine habitats. Purple sulfur bacteria, most frequently observed and also isolated from marine sediments and shallow waters, were assigned to *T. roseopersicina*, *T. violacea*, and

Table 2. Marine and Halophilic Purple Sulfur Bacteria

	Cell Size [um]	Salt Optimum	Salt Range	G + C Content	Osmotica	Salt Required	Salt Response	Habitat	Description
Lamprobacter modestohalophilus	2.0–2.5	1–4%	up to 9%	64.0		+	M	Salt lake	143
Halochromatium salexigens	2.0–2.5	8–11%	4–20%	64.6	suc, bet, ga	+	H	Saltern in Carmargue	70
Halochromatium glycolicum	0.8–1.0	4–6%	2–20%	66.1–66.5		+	M	Solar Lake	57
Thiohalocapsa halophila	1.5–2.5	4–8%	3–20%	65.9–66.6	suc, bet,ga	+	M/H	Saltern in Carmargue	71
Marichromatium gracile	1.0–1.3	2–3%	0.5–8.0%	68.9–70.4		o	B/M	Headley Harbor	144
Marichromatium purpuratum	1.2–1.7	5%	2–7%	68.4–68.9	suc, bet, ga	+	M	Marine sponge	35
Rhabdochromatium marinum	1.5–1.7	1.5–5%	1–6.5%	60.4		+	M	Sippewissett Salt Marsh	68
Isochromatium buderi	3.5–4.5	2–3%	1–5%	62.2–62.8		+	M	Salt marsh	62
Thiorhodovibrio winogradskyi	1.2–1.4	2–3%	0.2–7.2	61.0	sucrose	+	M	Salt lakes, marine microbial mat	69
Thiococcus pfennigii	1.2–1.5	0.5–2%	0–3%	69.4–69.9		o	B/M	Marine sediments, river mud	70
Thiorhodococcus minor	1.0–2.0	2%	0.5–9%	66.9		+	B/M	Brackish lagoons	71
Thioflavicoccus mobilis	0.8–1.0	2%	1–3%	66.5		+	B/M	Sippewissett Salt Marsh	39
Thioalkalicoccus limnaeus	1.3–1.8	1.0–6.0%	0.5–7.0%	63.8–64.8		+	B/M	Siberian soda lakes	56
Halorhodospira halophila	0.6–0.9	11–32%	7.5–35%	66.5–69.7	tre, bet, ect	+	H	Salt and soda lakes	72
Halorhodospira abdelmalekii	0.9–1.2	12–18%	8–22%	63.3–63.8	tre, bet, ect	+	H	Soda lakes	53
Halorhodospira halochloris	0.5–0.6	14–27%	10–35%	50.5–52.9	tre, bet, ect	+	H	Soda lakes	52
Thiorhodospira sibirica	3–4	1–3%	0.5–9%	56.0–57.4		+	B/M	Siberian soda lake	55
Ectothiorhodospira mobilis	0.7–1.0	2–3%	1–5%	67.3–68.4		+	M	Marine sediments	73,74
Ectothiorhodospira marismortui	0.9–1.3	3–8%	1–20%	65.0	suc, bet, cga	+	M/H	Dead Sea sulfur spring	75
Ectothiorhodospira marina	0.8–1.2	2–6%	0.5–10.0	62.8		+	M	Coastal sediment	76
Ectothiorhodospira haloalkaliphila	0.7–1.2	5%	2.5–15%	62.2–63.5	tre, bet, ect	+	M	Soda lake	76
Ectothiorhodospira shaposhnikovii	0.8–0.9	3%	0–7%	62.0–64.0		o	M	Salt flat	77
Ectothiorhodospira vacuolata	1.5	1–6%	0.5–10.0	61.4–63.6		+	M	Soda lake, salt swamp	78

B brackish, M marine, H halophilic salt response;
o not determined;
bet glycine betaine, ect ectoine, suc sucrose, tre trehalose, cg carbamoyl glutamine amide, ga N-acetyl-glutaminylglutamine amide.

Table 3. Marine and Halophilic Purple Nonsulfur Bacteria

	Cell Size [um]	Salt Optimum	Salt Range	G + C Content	Osmotica	Salt Required	Salt Response	Habitat	Description
Rhodothalassium salexigens	0.6–0.7	6–8%	5–20%	64.0 bd		+	M/H	Salterns, evaporated sea water pools	101
Rhodovibrio salinarum	0.8–0.9	4%	3–24%	67.4	bet	+	M	Marine salterns, salt lakes	102
Rhodovibrio sodomensis	0.6–0.7	12%	6–20%	66.2–66.6*	bet, ect	+	H	Dead Sea sediment	103
Roseospirillum parvum	0.4–0.6	1–2%	>7%	71.2		o	B/M	Great Sippewissett salt marsh	38
Rhodospira trueperi	0.6–0.8	2%	0.5–5%	65.7		+	B/M	Great Sippewissett salt marsh	20
Roseospira mediosalina	0.8–1.0	4–7%	0.5–15%	66.6*	sucrose	+	M	Saline sulfur spring Astara in Azebaijian	104
Rhodobium orientis	0.7–0.9	4–5%	2–8%	65.2–65.7		+	M	Tidal sea water pool	105
Rhodobium marinum	0.7–0.9	1–5%	0	61.5–63.8	tre, ga, bet	+	M	Tidal pools, tidal flats Solar Lake, common	58
Rhodovulum strictum	0.6–1.0	0.8	0.25–3	67.3–67.7		+	B	Tidal pools	106
Rhodovulum sulfidophilum	0.6–1.0	2.5	0–10	66.3–66.6	bet, gg, ect	+	M	Intertidal flat	61
Rhodovulum adriaticum	0.5–0.8	2.5–7.5	1–10	64.9–66.7		+	M	Marine lake, Malo Jezero	107
Rhodovulum euryhalinum	0.7–1.0	1–2	0.5–5	62.1–68.6		+	B	Common in sea waters	108
Rhodovulum iodosum	0.5–0.8	2.5–5.0	2–7	66		+	M	Intertidal flat	109
Rhodovulum robiginosum	0.5–0.8	2.5–5.0	1–7	69		+	M	Intertidal flat	109
Rhodobaca bogoriensis	0.8–1.0	1–3%	0–6%	58.8		–	B/M	African soda lakes	100

B brackish, M marine, H halophilic salt response;
o not determined;
bet glycine betaine, ect ectoine, suc sucrose, tre trehalose, gg glucosylglycerol, ga N-acetyl-glutaminylglutamine amide.

Allochromatium vinosum. Also, *A. warmingii*, *Thiocystis violascens*, *Allochromatium minutissimum*, *Thiocystis minor*, *Thiocapsa rosea*, *Marichromatium gracile*, and *Isochromatium buderi* were common (11,13,14,37). Of these bacteria, only *I. buderi* has been described as a marine and salt-dependent bacterium (62) and only this species and *M. gracile* belong to the phylogenetic branch of the truly marine and halophilic Chromatiaceae (63; Table 2). Other truly marine Chromatiaceae were described more recently and include *M. purpuratum*, *Rhabdochromatium marinum*, *Thiorhodococcus minor*, *Thiorhodovibrio winogradskyi*, and *Thioalkalicoccus limnaeus* (Table 2). Also, three genetically related species (*T. pfennigii*, *T. mobilis*, and *T. limnaeus*) that have spherical cells, containing bacteriochlorophyll *b* and tubular internal membrane systems, appear to be well-adapted to the lower salinity range of brackish and marine habitats. *Thioalkalicoccus limnaeus* lives in low salinity alkaline soda lakes at pH 9.0 (56).

Great metabolic versatility is an important selective advantage in particular in unstable marine coastal habitats. Interestingly, the most versatile phototrophic purple bacteria frequently are among the dominant species in highly dynamic coastal habitats (11,37,79–81). In particular, *A. vinosum*, *M. gracile*, *T. roseopersicina*, and *R. sulfidophilum* have frequently been observed and isolated from marine coastal habitats. The physiological versatility of these species is reflected (*1*) in the ability to use different possibilities of energy conservation and different photosynthetic electron donors, in particular sulfide and thiosulfate, (*2*) in the high potential of photoheterotrophic growth together with the ability to assimilate sulfate as sole sulfur source, and (*3*) in the ability to grow chemotrophically under oxic conditions in the dark, either autotrophically or heterotrophically.

The occurrence and dominance of *T. roseopersicina* in coastal marine environments deserves special attention. *Thiocapsa roseopersicina* is found in the phylogenetic branch of the freshwater Chromatiaceae (discussed later), and therefore part of the following discussion may be representative also for other species of this branch that live in the marine environment, but are not true marine bacteria. There are several reasons for *T. roseopersicina* to be very common in marine coastal habitats and among the most frequently isolated phototrophic purple sulfur bacteria. One of the most important reasons is its very high metabolic flexibility. This bacterium not only is tolerant to oxygen, but readily makes use of it by growing

Table 4. Salt-Dependent Green Sulfur Bacteria

Species and Strain	Strain: DSMZ No.	Strain: Other No.	Cell Size [µm]	G + C Content	Salt Optimum	Salt Required	Osmotica	Salt Response	Phylogenetic Group	Habitat	Description
	271^T		0.5–0.7	56.4	2–5%	+		M	1	Shallow saline waters, marine coastal sediments	117
Chlorobium vibrioforme	260^T	6,030	0.5–0.7	53.5	1–3%	+	Tre	B/M	1	Estuary	Pfennig*, 73
Chlorobium vibrioforme	262	6,132	0.5–0.7	57.1	1%	+		B	2a	Brackish pond	Pfennig*
Pelodictyon luteolum	273^T	2,530	0.6–0.9	58.1	2–5%	+		M	2a	Meromictic lake Polden	Pfennig*, 118
Chlorobium phaeovibrioides	269^T	2,631	0.3–0.4	53	1–2%	+		B	2b	Meromictic lake Langvikvann	119
Chlorobium vibrioforme f. thios.	265^T	1,930	0.5–0.7	53.5	1-2%	+		B	2b	Intertidal flat	Pfennig*, 110
Chlorobium vibrioforme	261	2,630	o	52	1%	+		B	2b	Meromictic lake Langvikvann	Pfennig*
Chlorobium limicola	263	NCIB 8327	o	56.6	1–2%	+		B	4a	o	Lascelles*
Chlorobium limicola		NCIB 8346	o	56.1	1%	+		B	4a	o	Lascelles*
Chlorobium chlorovibrioides	$1,377^T$		0.3–0.4	54.0	2–5%	+		M	4a	Saline meromictic lake	116
Prosthecochloris phaeoasteroidea	$1,370^T$		0.5–0.6	52.2	0.5–2.0	+		B	o	Saline meromictic lake	27
Pelodictyon phaeum	728^T		0.6–0.9	o	3%	+		B/M	o	Saline lake	120
Chloroherpeton thalassium		ATCC 35100^T	1.0	45–48.2	1–2%	+		B	1	Marine sediment	121

B brackish, M marine salt response; tre trehalose; o not determined Phylogenetic groups according to Alexander and Imhoff (unpubl.) Pfennig* strain specific informations from Pfennig (pers. comm.) Lascelles* strains isolated by Lascelles, not clearly systematically assigned.

by aerobic respiration; chemoorganotrophic and also chemolithotrophic growth in the dark is possible. As far as can be delineated from the ecological evidence and from preliminary experiments under enrichment conditions, *T. roseopersicina* can compete with chemolithotrophic sulfur bacteria and grow chemolithotrophically at the chemocline of sediments and marine waters. This versatility can be responsible for the wide distribution in marine environments, if these bacteria are able to cope with the prevailing salt concentrations. The salt relations of *T. roseopersicina* reveal no salt requirement but a tolerance and an indifferent response to the low concentrations found in brackish and marine habitats (37,82,83). The nature of osmotica accumulated by these bacteria upon salt stress, sucrose as sole component, reveal that they have limited capacity to adapt to saline conditions and are not fit for life at elevated salt concentrations. Sucrose is found in bacteria that are the least adapted to saline environments (84).

Halophilic Chromatiaceae species that optimally thrive at elevated salt concentrations beyond those of seawater are represented by a few specialized species such as *H. salexigens* (65), *H. glycolicum* (57) and *T. halophila* (66).

Ectothiorhodospiraceae

Most probably, early observations indicate the presence of bacteria that could be *Ectothiorhodospira* or *Halorhodospira* species in hypersaline environments long before the systematic position of these bacteria became known. Baas-Becking reported on a red halophilic spirillum from the Owens Lake, California (85), which apparently formed extracellular elemental sulfur, similar to the spiral and halophilic phototrophic bacteria found by Van Niel in some hypersaline lakes (86). Butlin and Postgate (87), during their work on the Ain-en- Zauia (Libya), observed many spiral bodies that might have been *Halorhodospira* cells. An isolate from the Owens Lake (California) was first described as a *Chromatium*, but fitted well with the properties of halophilic *Halorhodospira* species (88). It was recognized later as such (89,90). Post (91) in his work on the Great Salt Lake (Utah), did not mention the presence of phototrophic bacteria, but Brock observed a bacterium resembling *Hlr. halophila* in the more saline northern part of this lake and reported on the isolation of this bacterium (92). Jannasch (51) observed a red coloration in some alkaline salt lakes in the Wadi Natrun (Egypt), and attributed this coloration to the development of phototrophic bacteria. Microscopical observation showed that spiral forms were dominant, but attempts to isolate these bacteria were not successful. During our studies on these lakes, we found mass developments of both green- and red-colored species of the genus *Halorhodospira* that developed separately, but close together at some locations (54). Also, some representatives of *Ectothiorhodospira* species were found. A larger number of pure cultures were obtained from these lakes (11,52,53,93).

Ectothiorhodospira and *Halorhodospira* species are characterized by their distinct and obligate requirement for salt and alkaline pH (94), and by their deposition of elemental sulfur outside the cells, if grown with sulfide as photosynthetic electron donors (94–96). Species of these genera have been isolated from marine and hypersaline habitats, but not from freshwater sources. The two genera can be distinguished on the basis of their salt requirement. *Ectothiorhodospira* species have growth optima of 1 to 7% total salinity (tolerances up to 15% by *Ect. haloalkaliphila* and down to 0% of *Ect. shaposhnikovii*) and were isolated from marine environments and from hypersaline lakes. They include the slightly halophilic species *Ect. mobilis* (74), *Ect. vacuolata* (78), *Ect. shaposhnikovii* (77), and *Ect. marismortui* (75), as well as *Ect. marina* (76,82) and *Ect. haloalkaliphila* (76). Only strains of *Ect. haloalkaliphila* tolerate and grow up to 15% total salts, although their salt optima are much lower.

Ectothiorhodospira species are quite common in marine habitats typical for other purple bacteria (11,34,37,82,97), although never as numerous and dominant as representatives of the Chromatiaceae.

Halorhodospira species require very high salt concentrations and do not grow below 10% total salts. The extremely halophilic *Hlr. halophila*, *Hlr. halochloris*, and *Hlr. abdelmalekii* bloom in concentrated brines of alkaline salt and soda lakes at various places around the world (51,54,88,95,96), are well adapted to the high temperature, high pH levels, high light intensity, and high salinity of these environments and have not been isolated from marine habitats. *Halorhodospira halophila* is the most common of the extremely halophilic species and has been isolated from many hypersaline environments (11). *Halorhodospira halophila* strains show optimum growth at equal or even higher salinities than representatives of the known archaeobacterial "extreme halophiles." They develop in saturated brines of alkaline soda lakes.

The recently described *T. sibirica* is specifically adapted to low saline, alkaline soda lakes and phylogenetically belongs to the Ectothiorhodospiraceae (55).

Purple Nonsulfur Bacteria

Purple nonsulfur bacteria are regularly found together with purple and green sulfur bacteria in sediments and aquatic habitats, sometimes even in high numbers (11,79,98). Compared with the *Chromatiaceae*, which are easily recognized by elemental sulfur globules stored inside their cells, the purple nonsulfur bacteria are morphologically much less conspicuous. Most of the purple nonsulfur bacteria have been isolated from freshwater habitats and are very sensitive to even low concentrations of sulfide. For a long time, purple nonsulfur bacteria have been considered typically to inhabit freshwater habitats and to be unable to use sulfide as a photosynthetic electron donor (24,79,86). In marine environments, in which anoxic conditions normally correlate with the presence of sulfide, they would not find conditions for their development.

The first, truly marine purple nonsulfur bacterium isolated in pure culture was *R. sulfidophilum* (61), a bacterium that requires salt for optimum growth and is quite tolerant even to high sulfide concentrations (6 mM) and readily uses sulfide as a photosynthetic electron donor. Although *R. sulfidophilum* can grow at low salt concentrations, it has an optimum at 2.5% NaCl and apparently is restricted to the development in marine

waters. This habitat restriction is demonstrated by the fact that *R. sulfidophilum* has not been isolated from freshwater habitats and by the following observation. From two small puddles, a splash water puddle at the rocky Adriatic shoreline and a freshwater puddle not more than 10 m apart from the other but outside the splash water zone, the dominant purple nonsulfur bacteria were isolated. The isolates from the salty puddle yielded strains of *R. sulfidophilum*, whereas those from the freshwater yielded *Rhodobacter capsulatus* strains (11). *Rhodovulum sulfidophilum* is the most common marine purple nonsulfur bacterium and is even found in hypersaline environments. It has been found, sometimes in high numbers, in marine mud flats of the Dutch Waddensea, in marine sponges, in sandy sediments of the German Island Mellum, in salt swamps at Al-Azraq (Jordan), in various splash water pools, in marine lagoons and lakes (11). *Rhodovulum sulfidophilum* is well adapted to rapidly changing environmental conditions in marine tidal areas by its great metabolic versatility. The latter is reflected in the ability to grow photoheterotrophically with organic electron donors, photoautotrophically with hydrogen, sulfide, and thiosulfate as electron donors, and also chemotrophically under oxic conditions in the dark (61,99). In particular, its high tolerance toward sulfide and its ability to readily oxidize sulfide and thiosulfate to sulfate are prerequisites for a successful competition with purple sulfur bacteria in the natural environment. Such a successful competition could be demonstrated in enrichment cultures from tidal pools, where both *Marichromatium* and *Allochromatium* species together with *R. sulfidophilum* were present, and where the *Rhodovulum* rapidly outgrew the purple sulfur bacteria (11). All attempts to isolate *R. sulfidophilum* from freshwater sources were unsuccessful (11,61). The salt responses are slightly different, depending on the natural habitat of the isolate. Some strains did grow in the absence of salt; others were strictly salt dependent. Optimum growth of all strains was between 1 and 5% NaCl, and good growth of some strains was observed even at 10% salts (11).

Most of the purple nonsulfur bacteria from marine habitats known to date are typical marine bacteria that require NaCl and are not found in freshwater habitats (11, Table 3). These marine forms include the species of the genera *Rhodovulum* and *Rhodobium*. Another true marine bacterium from marine salt marshes is *R. trueperi* (20). Also, *R. parvum* is well adapted to marine salt concentrations (38). *Rhodobaca borogenensis* is a new isolate from an alkaline soda lake with low salt concentrations and adapted in its salt response to this habitat (100). True marine purple nonsulfur bacteria and some of their properties are shown in Table 3.

In addition, several halophilic purple nonsulfur α-Proteobacteria that live in hypersaline habitats are known. *Rhodothalassium salexigens* and *R. salinarum* are common to evaporated seawater pools and marine salterns (48,101,102), and *R. sodomensis* is well adapted to hypersaline environments (103). *Roseospira mediosalina*, although growing optimally between 5 to 7% NaCl, was isolated from a salty hot spring containing not more than 2% salts (104).

Chlorobiaceae

Chlorobiaceae are obligately phototrophic, strictly depend on anoxic conditions and on reduced sulfur compounds, do not grow under photoheterotrophic conditions, and can assimilate only a limited number of organic carbon sources while growing photolithotrophically (110,111). Therefore, they depend on quite stable environmental conditions to compete with the more versatile phototrophic purple bacteria. Nevertheless, Chlorobiaceae are common in marine habitats. Most obvious is their development in the more stable brackish and marine lagoons and ponds (37,78,112–114) and in the lower anoxic part of deeper aquatic marine habitats. In marine sediments, they occur in lower numbers and below the red layers of phototrophic purple bacteria (115).

Green sulfur bacteria that occur in brackish and marine water tolerate or require NaCl for growth and have a salt optimum between 2 to 5% (Table 4). Among these are the vibrioid green sulfur bacteria that were cultivated routinely at 1% NaCl (Pfennig, pers. comm.). *Prosthecochloris aestuarii* and *Chlorobium vibrioforme* are frequently observed in marine coastal habitats (37,82,115), but also *Pelodictyon luteolum* and *Chlorobium chlorovibrioides* were regularly found (115,116).

The most halophilic and also halo-tolerant species of the green sulfur bacteria is *P. aestuarii*. This species is widely distributed in estuarine environments and has been found in various lagoons and estuarine ponds (10,82,115,122,123). It does not grow in the absence of salt, has a salt optimum between 2 and 6% NaCl, and can tolerate more than 10% NaCl (82,115).

PHYLOGENY OF MARINE AND HALOPHILIC PHOTOTROPHIC BACTERIA

Many observations give rise to the assumption that the salt relations of phototrophic bacteria are an important selective environmental factor and that salt concentrations of natural habitats have determined in an exclusive way the development and evolution of a selected range of phototrophic bacteria. On the basis of 16S rDNA sequences, a detailed analysis of the genetic relationship of anoxygenic phototrophic bacteria has been made (63,76,83,124,125). These phylogenetic studies gave considerable support to the existence of truly marine and halophilic phototrophic bacteria and demonstrated evolutionary lines of these bacteria separate from their freshwater relatives (63,76).

Chromatiaceae

Major phylogenetic branches within the Chromatiaceae can be distinguished on the basis of their salt requirement, one exclusively containing marine and halophilic species, the other primarily freshwater bacteria (63). The marine branch includes the genera *Marichromatium*, *Halochromatium*, *Rhabdochromatium*, *Thiococcus*, *Thioflavicoccus*, *Thioalkalicoccus*, *Thiorhodovibrio*, *Thiohalocapsa*,

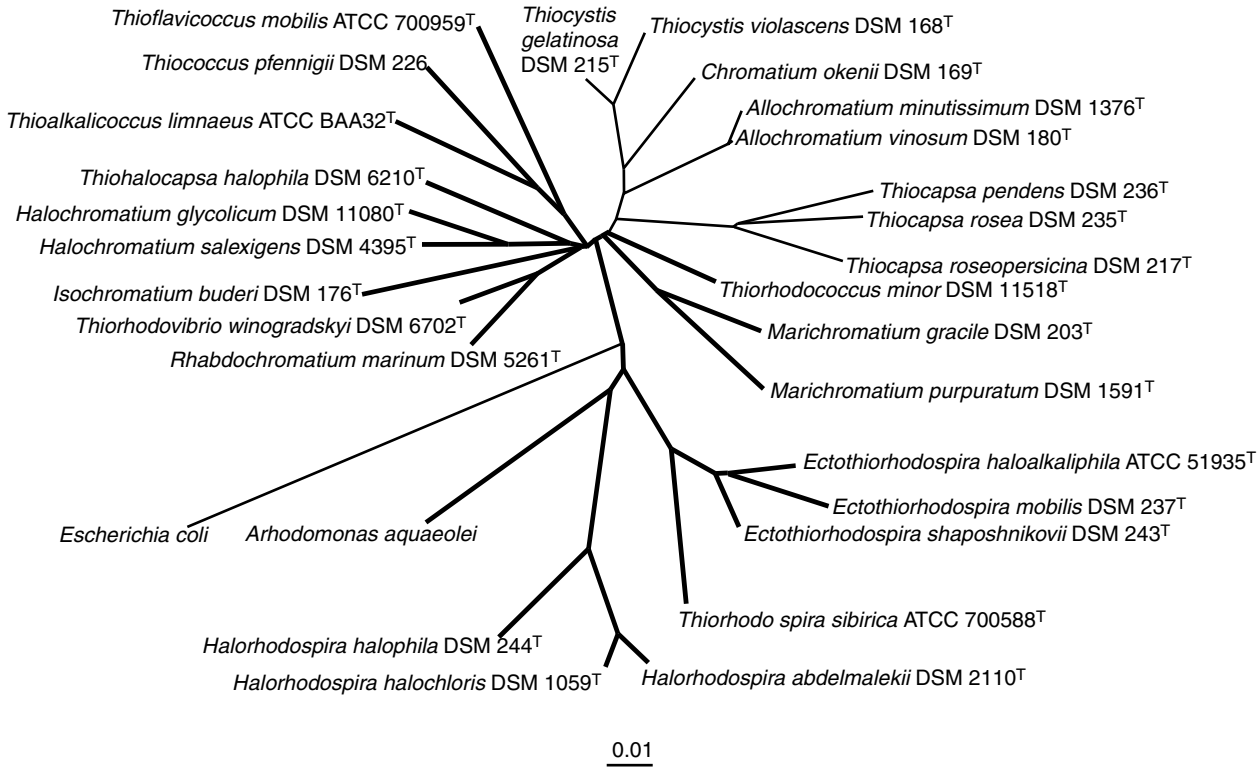

Figure 1. Phylogenetic tree of representative species of purple sulfur bacteria based on 16S rDNA sequence similarities and showing the phylogenetic lines of marine and halophilic species in comparison with those of freshwater species. Bold lines connect marine and halophilic strains. Sequence data used were published previously (63). In addition, 16S rDNA sequences of *Arhodomonas aquaeolei* (M26631), *T. sibirica* (AJ006530), *T. limnaeus* (AJ277023), and *T. mobilis* (AJ010125) were used. The distance bar indicates 1% sequence difference.

Thiorhodococcus, and *Isochromatium* (Fig. 1). Three of the marine species (*T. pfennigii*, *Thioalkalicoccus sibiricus*, and *T. mobilis*) that are adapted to the low range of salt concentrations of brackish and marine habitats contain bacteriochlorophyll *b*, have tubular internal membranes and are genetically related (20,39). Both the genetic relationship and the salt responses enable one to distinguish the halophilic *H. salexigens* and *H. glycolicum* and the marine *M. gracile* and *M. purpuratum* from each other and from freshwater species such as *A. vinosum* and relatives.

Another major branch of the purple sulfur bacteria represents the freshwater species (63). Several of these bacteria that do not have a specific requirement for salt and are routinely grown in freshwater media were frequently observed and also isolated from marine coastal habitats. Because they are tolerant to salt concentrations of brackish and marine waters and physiologically among the most versatile purple sulfur bacteria, they may well compete with the marine species of purple sulfur bacteria, in particular at low salt concentrations. Thus, although they do not require salt, their tolerance is sufficient to enable development and competition in numerous coastal habitats. Individual species of this group, such as *Thiocapsa litoralis* (126), even may grow better at minor salt concentrations than in its complete absence.

Ectothiorhodospiraceae

Two main phylogenetic subbranches of Ectothiorhodospiraceae represent the marine and moderately halophilic *Ectothiorhodospira* species on one and the extremely halophilic *Halorhodospira* species on the other hand (Fig. 1). Of particular importance in this context is the finding of phylogenetic relatedness of strictly chemotrophic halophilic bacteria without photosynthetic pigments, such as *Arhodomonas* and related species (127), which form a phylogenetic line of distant relationship to *Halorhodospira*. This is taken as support of the strong phylogenetic relevance of salt adaptation. Also, *T. sibirica* belongs to this group as a separate phylogenetic line related to *Ectothiorhodospira* (55).

Purple Nonsulfur Bacteria

The phototrophic purple nonsulfur bacteria have representatives in the α- and in the β-Proteobacteria. All known phototrophic β-Proteobacteria are freshwater bacteria, whereas a number of phototrophic α-Proteobacteria are represented by marine and halophilic species. According to the 16S rDNA sequence comparison, *Rhodobacter* species form two branches of marine and freshwater species. The marine species of *Rhodobacter* were removed from this genus and are now recognized as *Rhodovulum* species (128). Both genera represent closely related,

though phylogenetically separated, species of the α-3 Proteobacteria. *Rhodobium* species are genetically clearly distinct from all other α-2 Proteobacteria. Also, *R. trueperi*, *R. mediosalina*, and *R. parvum*, although related to *Rhodospirillum* species, form distinct phylogenetic lines of α-3 Proteobacteria. Not clearly affiliated to any of the other phototrophic α-Proteobacteria are the halophilic representatives, *R. salexigens*, and the two species of *Rhodovibrio*, *R. salinarum*, and *R. sodomensis* (Fig. 2).

Chlorobiaceae

Among the green sulfur bacteria, phylogenetic branches of bacteria having a salt requirement can be distinct from those of freshwater bacteria (Fig. 3). Those strains of green sulfur bacteria with 16S rDNA sequences similar to *P. aestuarii* have a definite salt requirement and are truly marine bacteria. This branch contains *P. aestuarii*, *C. vibrioforme* DSM 260, and *C. phaeovibrioides* DSM 1678 and is most distant to the other green sulfur bacteria (124,129, Alexander and Imhoff, unpublished). Genetically related to this group, though at a greater distance, is the marine *C. thalassium* (121). A second branch of salt-dependent green sulfur bacteria is formed by those strains known as *C. vibrioforme*, *C. phaeovibrioides*, and *P. luteolum*, all routinely grown at 1% NaCl (Pfennig, pers. comm.). *Chlorobium chlorovibrioides* together with two strains isolated by Lascelles represent a salt-dependent line within a further group of species (130, Alexander and Imhoff, unpublished). Salt-dependent green sulfur bacteria have the greatest phylogenetic distance among this group of bacteria and represent the deepest branching points. This gives support to the conclusion that the green sulfur bacteria have originated in the marine environment.

It should be noted here that the taxonomy of green sulfur bacteria is not reflecting the genetic relationship of these bacteria on the basis of 16S rDNA sequences, which strongly deviates from the current taxonomic system based on morphological and physiological properties (124,129).

SALT REQUIREMENT AND OSMOTIC ADAPTION

Common to typical marine species is a distinct salt requirement for optimum growth, which clearly distinguishes them from freshwater species growing best almost in the absence of salt. Although many of the marine and halophilic phototrophic bacteria can grow over a broad range of salt concentrations, most of them are strictly marine bacteria and have a lower limit below which no growth is possible. Physiological properties that define

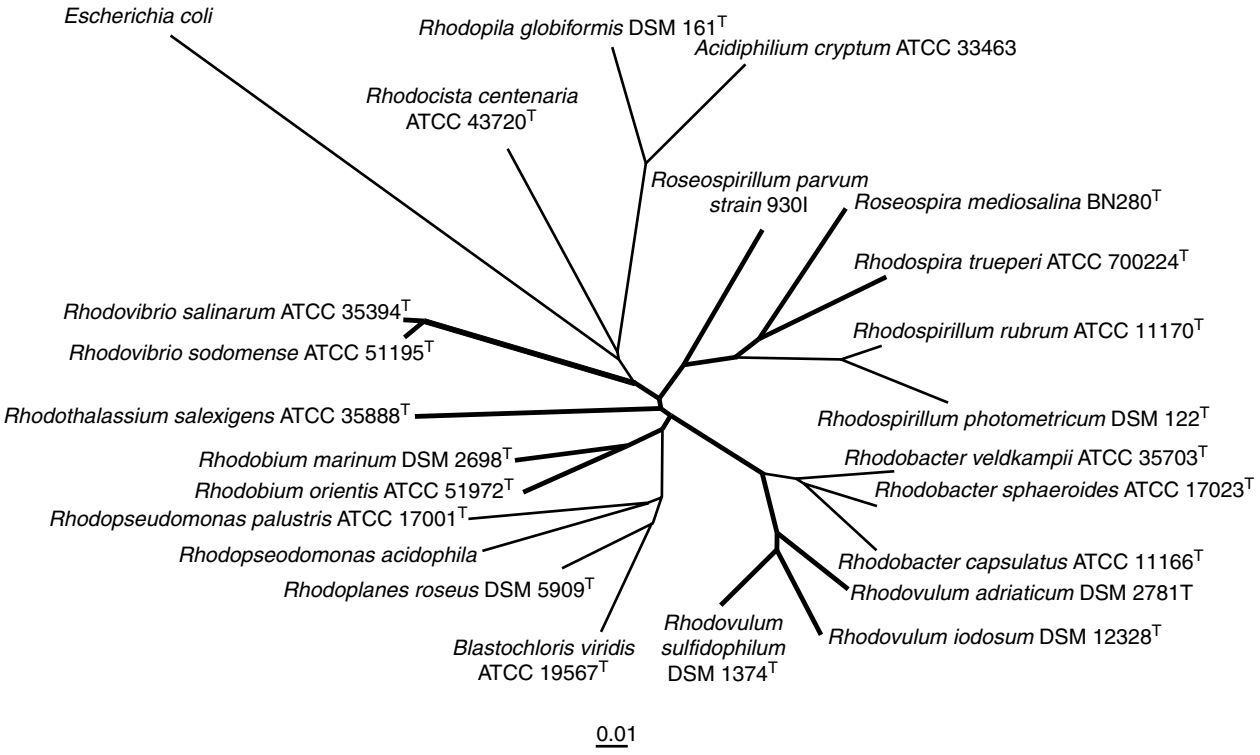

Figure 2. Phylogenetic tree of representative species of phototrophic α-Proteobacteria based on 16S rDNA sequence similarities and showing the phylogenetic lines of marine and halophilic species in comparison with those of freshwater species. Bold lines connect brackish water, marine, and halophilic strains. Accession numbers of the sequences used are D14431, D14432, M59072, D12701, D30773, D86513, AJ011919, AJ000989, X99671, D30778, D30777, D25312, M34128, D25313, D25314, D30790, D30792, D16418, D16423, Y15011, D16421, D16428, X53853. The distance bar indicates 1% sequence difference.

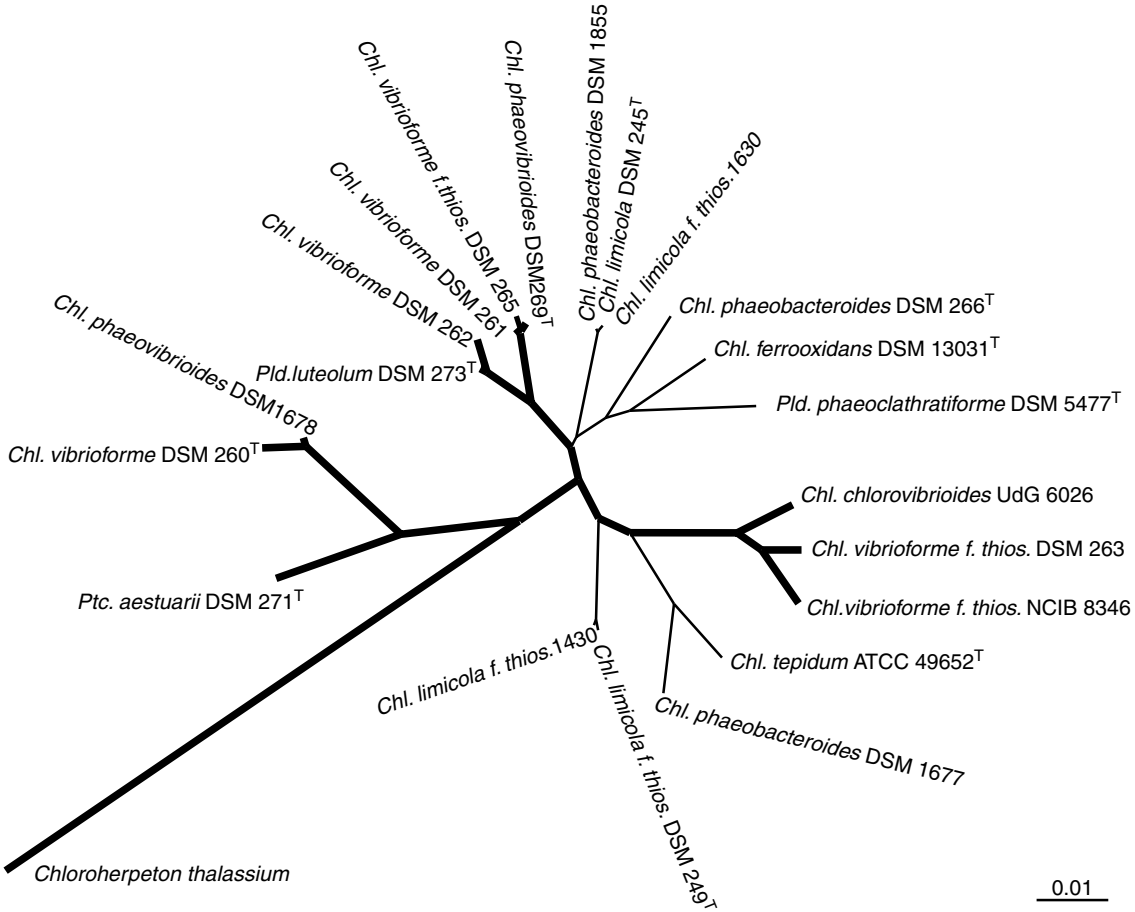

Figure 3. Phylogenetic tree of representative green sulfur bacteria based on 16S rDNA sequence similarities and showing the phylogenetic lines of marine and halophilic species in comparison with those of freshwater species. Bold lines connect brackish water and marine strains. Accession numbers of the sequences used are AF170103, Y07837, M62791, AJ290833, Y08107, Y08103, AJ290828, AJ290829, Y08105, AJ290826, Y08104, Y18253, Y081108, Y10649, Y10647, AJ290830, M58468, AJ290831, Y08102, AJ290825. The distance bar indicates 1% sequence difference.

the specific adaptation of bacteria to the marine environment are the requirement for salt and/or inorganic ions and are manifested in the accumulation of elevated concentrations of compatible solutes for osmotic adaptation (84).

How to Define Marine and Halophilic Bacteria?

The true marine nature of bacteria needs clear specification, but certainly cannot be defined as "growth in the presence of salt," because that could reflect a tolerance toward salt only, which is found to a different degree in a great number of freshwater bacteria. Freshwater species that do not require but are tolerant to salt concentrations in brackish and even marine habitats may develop there and even successfully compete with marine strains. An outstanding example of this last group of bacteria is *T. roseopersicina* (82,83).

The marine nature also cannot be defined as "no growth in the absence of salt" or inorganic ions below a threshold limit, because bacteria optimally adapted to the marine salt concentrations could be tolerant to low salt concentrations and grow in the absence of significant concentrations as, for example, *Ect. shaposhnikovii*. Although this does not appear to be common among true marine bacteria, those specifically adapted to the brackish water environment are also likely to grow in the absence of salt.

The only way to clearly define the need for and the degree of this requirement is the indication of the salt concentrations that define the growth optimum (84,131). Growth limits may be defined in addition. It should be noted that reliable results are obtained only if the growth response is tested over at least three consecutive subcultures at the same salt concentration.

Suggestions to define the ranges of the demand (132,133), and in addition the tolerances (84,131) have been made. Common natural environments to which bacteria have to be adapted represent freshwater, brackish water, marine habitats, slightly hypersaline water, and highly saline water including all possible transition stages. According to these natural situations and their growth optima, it has been proposed to classify bacteria as freshwater species (below 0.5%), brackish water species (0.5 to 2.0%), marine or slightly halophilic species (2.0

to 7%), moderately halophilic species (7 to 15%), and extremely halophilic species (more than 15%). Those bacteria that behave indifferent to salt concentrations from 0 to 3% NaCl or more, as *T. roseopersicina*, should be classified as slightly halo-tolerant freshwater bacteria.

Specific Sodium Requirement

Studies with halophilic *Halorhodospira* and *Ectothiorhodospira* strains indicate a distinct requirement for the sodium ion (134,135). Potassium ions were not required at elevated concentrations for growth of *Ect. haloalkaliphila* and could not effectively replace the high concentrations of sodium ions. The chloride ion could be replaced almost completely by sulfate and carbonate anions, and also elevated concentrations of sulfate were not required. Sodium and also chloride ions were actively excluded from the cytoplasm (134).

The question of a specific sodium requirement for *Ect. haloalkaliphila*, apart from any dependence on a specific external osmolarity, was approached by stepwise replacing NaCl in the medium by KCl or LiCl (11). A requirement of about 0.25 M NaCl was found. Below this concentration the growth rate decreased sharply (11). In similar experiments with *Hlr. halophila* and *Hlr. halochloris* by replacing NaCl with sucrose, a definite requirement for NaCl could also be shown for these species. NaCl could be substituted only partly by sucrose, indicating a specific requirement for NaCl by *Ectothiorhodospira* and *Halorhodospira* cells, which is, however, lower than the concentrations needed for optimum growth.

The structural and physiological basis for the increased dependence on NaCl in *Halorhodospira* cells is largely unknown. NaCl could be necessary for the integrity of the cell envelope or for some sodium-dependent processes within the membranes. It is, however, unlikely that variation in salt dependency of *Halorhodospira* species reflects the requirement for cell integrity. Cells of even the most halophilic strains do not lyse at 0.5 M NaCl. Sodium-dependent transport of glutamate and some other carboxylic acids has been found in *Hlr. halophila* and *Ect. shaposhnikovii* (136–138). Sodium-dependent transport systems per se do not, however, explain the high requirement for this ion. Maximal uptake rates of glutamate in *Hlr. halophila* were found well below the minimum growth-supporting concentration of NaCl (136).

Significant differences have been observed with regard to the requirement and growth responses toward various concentrations of the bivalent cations, magnesium and calcium. A specific cation requirement may be explained by the specific binding of cations to cell-surface structures. Isolates from alkaline soda lakes that almost are devoid of these cations demonstrate a requirement for minimum concentrations of these ions, whereas bacteria from thalassohaline salt lakes and from the Dead Sea with much higher concentrations of bivalent cations also have an increased requirement thereof (see 11,69,114). Thus, the concentrations of bivalent ions that favor optimum growth reflect the environmental concentrations and demonstrate that these bacteria are adapted to this aspect of their environment.

Osmotic Adaptation

To cope with elevated salt concentrations, transport systems for inorganic ions and uptake, respectively, synthesis of organic solutes to achieve osmotic adaptation is required. Whether or not it is the specific ionic requirement or the osmotic demand or both, osmotic adaptation is required in direct response to the actual external osmolarity (84).

It was the application of carbon-13 NMR spectroscopy that led to the recognition of organic solutes involved in osmotic adaptation of halophilic eubacteria. The biosynthesis of various organic compatible solutes was first demonstrated in a number of cyanobacteria. In a marine isolate of *Synechococcus*, O-a-D-glucopyranosyl-(1-2)-glycerol was found as principal osmoticum by application of NMR measurements (139). Later, a greater number of cyanobacteria have been analyzed and a correlation was found between the chemical nature of accumulated osmotica and the salt tolerance (140–142). It was demonstrated that the kind of accumulated solutes correlated well with the tolerated salt concentration. Typical freshwater cyanobacteria with a maximum salt tolerance of approximately 1 M NaCl accumulated sugars (sucrose, glucose, fructose, trehalose). Cyanobacteria from marine environments with maximum tolerance below 2 M NaCl accumulated glucosylglycerol, and more tolerant isolates from hypersaline environments accumulated glycine betaine or glutamate betaine (140). These findings suggested that a correlation exists between the upper limit of tolerated salt concentration and the solutes accumulated inside the cells, that is, the more compatible the accumulated solute the higher the salt or osmotic tolerance. The low compatibility of reducing sugars and the high compatibility of glycerol and glycine betaine have been demonstrated in many investigations (84,143–145). Structure, solubility, and accumulation in marine cyanobacteria indicate an intermediate compatibility of glucosylglycerol (142). In consequence, one of the reasons for the lack of salt or osmotic tolerance can be the inability to accumulate suitable compatible solutes by uptake or biosynthesis.

The situation is quite similar with anoxygenic phototrophic purple bacteria (84,146,147). Sucrose as the sole compatible solute is found only in species with low salt tolerance, including species frequently found in brackish and marine coastal waters including *T. roseopersicina*, *A. vinosum*, *T. violacea*, and *T. rosea* (147). Two green sulfur bacteria accumulated trehalose as the sole compound (147). Those species that are well adapted even to hypersaline salt concentrations accumulate a combination of glycine betaine, ectoine, N-acetyl-glutaminylglutamine amide (or other glutamine amide derivatives) with the one or the other sugar (trehalose or sucrose) in addition (Tables 2 to 4).

More elaborate studies have been made with *Hlr. halochloris*. The major compatible solute of this bacterium is glycine betaine, which is synthesized from acetate and carbonate in response to the external salinity and accumulated to more than 2 M inside the cells (148). Also, significant concentrations of ectoine and trehalose were found under certain growth conditions (149). The relative proportions of the three compatible solutes vary with the

growth conditions and the growth phase. Under nitrogen starvation, trehalose concentration increases up to 0.5 M in *Hlr. halochloris* and replaces, in particular, ectoine, which contains two nitrogen atoms per molecule. A trehalase has been characterized from this bacterium, which is specific for trehalose, has a high K_m of 0.5 M for trehalose, is activated by glycine betaine and inhibited by salts, most strongly by NaCl (150). These properties, in particular the inhibition by NaCl and the low K_m, are characteristic for an enzyme that degrades a compatible solute. Activation by betaine is suited to increase trehalose degradation if glycine betaine is accumulated, and under suitable conditions enables a rapid replacement of trehalose by glycine betaine. Also, the biosynthesis of glycine betaine and ectoine in *Hlr. halochloris* have been elucidated (151,152). All available evidence points to a comparatively low content of inorganic ions in the cytoplasm of *Halorhodospira* species. In agreement with a low ionic cell interior, is the strong inhibition by NaCl and KCl of several enzymes (isocitrate dehydrogenase, malate dehydrogenase, glutamate dehydrogenase, ribulose bisphosphate carboxylase, and ATPase) in *Hlr. halophila* and *Hlr. halochloris* (11,153,154). Light-dependent release of sodium and uptake of potassium ions indicates an energy-dependent maintenance of high-concentration gradients of these ions across the membranes.

Although ion transport and accumulation of compatible solutes in the cells is a necessary prerequisite to cope with increased osmotic pressures in the saline environment and to adapt to elevated salt concentrations, it appears that this ability defines the upper limit, that is, the tolerance rather than the requirement of a particular bacterium. The increase of internal concentrations is directly related to the external solute concentrations, respectively osmolarity, and not dependent on the optimum salt concentrations. A higher requirement for salt, however, in most cases also implicates a higher tolerance. Therefore, the kind and concentration of accumulated solutes is of importance for successful adaptation to the marine and hypersaline environment.

CONCLUSION

All available evidence from the ecological, physiological, and phylogenetic line is indicative of the existence of truly marine and halophilic phototrophic bacteria in the groups of green sulfur bacteria, purple sulfur bacteria, and also purple nonsulfur bacteria. Evidence for the heliobacteria and the green nonsulfur bacteria (*Chloroflexus* and relatives) is lacking or insufficient to draw clear conclusions. Major phylogenetic lines of purple and green sulfur bacteria represent branches of bacteria adapted to the saline and hypersaline environment. In addition, separate lines of evolution of the purple nonsulfur α-Proteobacteria are marine or halophilic bacteria. The phototrophic purple nonsulfur β-Proteobacteria are true freshwater species and no representative is known to occur in marine habitats.

Although the whole spectrum of salt concentrations is inhabited by phototrophic bacteria, individual species are adapted to a selected range according to the definitions given for freshwater, brackish water, marine, moderately halophilic, and extremely halophilic bacteria. Although the dependence on even low concentrations of salt prevents growth of strictly marine and halophilic species in freshwater habitats, the salt tolerance of several freshwater species gives a chance to these bacteria to develop in brackish and marine habitats. Similarly, salt responses of extremely halophilic species exclude their development in marine and brackish waters, whereas some of the marine species are tolerant to concentrations of the hypersaline range. The salt relations of the bacteria determine possible natural habitats and exclude others. If the environmental salt concentration is an exclusive factor for the development of bacterial species, habitat restriction for certain species should result and over evolutionary timescales give rise to different phylogenetic lines of bacteria. Thus, salt responses should, to the degree they are exclusive, show up in phylogenetic relationships of the (phototrophic) bacteria. Over evolutionary timescales this has resulted in a separation of species from freshwater habitats and saltwater habitats, allowing an overlap between freshwater and brackish water as far as common species in both kinds of habitats exist. This is exactly the picture that emerges from phylogenetic data, salt responses, and the growing knowledge on habitat ranges of the species.

Present day knowledge on the natural diversity is based on pure culture studies and depends on the success of our isolation and cultivation skills. New techniques of molecular genetic analysis that allow us the identification of bacteria in the natural environment without cultivation will enable us to determine the species composition of specific habitats and ecological niches. These methods will allow to prove the concept of habitat restriction and to determine the role of physicochemical factors for habitat selection and distribution of phototrophic bacteria. Quite interesting, the application of such molecular genetic approaches to determine the natural diversity of green sulfur bacteria in marine coastal habitats of geographically very distant places so far only yielded sequences belonging to the marine branches of the phylogenetic tree of the green sulfur bacteria (Alexander and Imhoff, unpublished results). These results not only proved the success of available culture techniques to isolate and cultivate representatives of important phylogenetic lines found in nature but also demonstrated the role of salt in habitat restriction.

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PHYCOTOXINS. See CYANOBACTERIA; CYANOBACTERIA-TOXINS IN DRINKING WATER; RED TIDES AND OTHER HARMFUL ALGAL BLOOMS

PHYLLOSPHERE. See PLANT–MICROBE INTERACTIONS IN THE MARINE ENVIRONMENT; SEAGRASSES COMMUNITIES; SUBAERIAL COMMUNITIES

PHYLOGENETICALLY BASED METHODS IN MICROBIAL ECOLOGY

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Molecular biological methods in microbial ecology are used to describe the composition and activity of complex communities by characterizing biomarker molecules, rather than by isolating and culturing microbial species in the laboratory. Commonly used biomarkers include pigments (1), lipids (2), quinones (3), proteins (4,5), and the nucleic acids, DNA and RNA, which are the focus of this chapter.

A serious limitation of traditional culture-based methods of studying natural microbial populations is that only species able to grow and outcompete other species, under particular growth conditions, will be detected (6–8). Traits conducive to growth in laboratory media do not necessarily confer competitiveness in the frequently harsher and more variable natural environment; it is generally impractical to test all possible permutations of temperature, carbon source, oxygen concentration, and so on. It may also be difficult to differentiate among closely related species on the basis of assayable phenotypic traits.

The limitations of molecular biological methods, on the other hand, include questions of extraction efficiency (9,10), differential molecular preservation (11), environmental heterogeneity and sampling scale, sample handling and contamination (12), and database completeness. Microbial activities in the environment are often best explored by a combination of traditional and molecular methods — time and money permitting.

A sometimes confusing proliferation of nucleic acid–based techniques has been developed in the past decade or so; many of them designated by acronyms (RT-PCR, T-RFLP, DGGE, etc.) that can seem quite mysterious to the uninitiated. Several currently popular methods are introduced here and have also been recently reviewed elsewhere (13–16). Further descriptions of RNA, DNA, and the enzymes that act on them can be found in microbiology, biochemistry, and molecular biology textbooks such as the excellent ones by Madigan (17), Stryer (18), and Alberts (19). An introduction to detailed experimental methods can be found in Sambrook and coworkers (20), and in the catalogs or on the World Wide Web sites of companies that supply oligonucleotide probes and primers.

BACKGROUND

Transcription and Translation: A Review

In the "central dogma" of biochemistry (21; for a historical perspective, 22), RNA polymerase transcribes DNA-encoded genes to RNA, and ribosomes then translate messenger RNA (mRNA) to protein (Fig. 1). DNA is a double-stranded polynucleic acid, with complementary "sense" and "nonsense" strands joined by adenine-thymine (A·T) and guanine-cytosine (G·C) base pairs. RNA is a single-stranded polynucleotide, transcribed from the "sense" strand, with uracil (U) replacing thymine. Proteins consist of amino acids joined by peptide bonds. Each of the 20 amino acids found in proteins is encoded by specific 3-nucleotide RNA sequences (codons). Transcription, translation, RNA and protein stability, and the enzymatic activity of proteins may all be regulated in response to environmental conditions. One goal of molecular microbial ecology is to measure the expression of specific genes in mixed populations, which might include

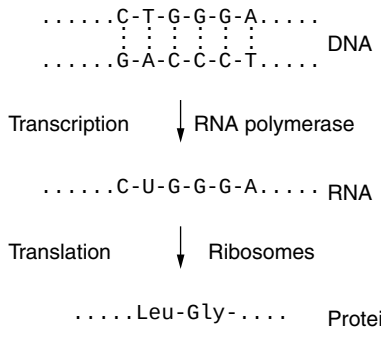


Figure 1. Transcription and translation: an overview.

thousands of species, and this, clearly, is an ambitious undertaking.

A more detailed view of transcription and translation is shown in Figure 2. RNA polymerase binds to specific DNA sequences (promoters) that precede genes or groups of genes (operons). Promoter recognition can be modulated by polymerase subunits (sigma factors) and regulatory proteins that may be produced or activated in response to environmental conditions such as heat shock or iron limitation. The polymerase moves along the DNA and produces an RNA molecule complementary to the coding strand. Transcription continues until the polymerase reaches a terminator sequence, at which point it dissociates from the DNA.

There are three major classes of RNA: ribosomal RNA (rRNA), transfer RNA (tRNA), and messenger RNA (mRNA). rRNA is a constituent of ribosomes, which are two-subunit rRNA/protein complexes. In prokaryotes (bacteria and archaea), the small subunit includes a 16S (SSU) rRNA, and the large subunit includes 23S and 5S rRNAs. Eukaryotes have 18S (SSU), 28S, 5S, and 5.8S rRNAs. Production and assembly of ribosomal components is tightly regulated (23–25). Several recent studies have given a detailed three-dimensional view of the complicated ribosome structure (26–28) showing how these components fit together.

tRNAs are small RNA molecules that carry specific amino acids on one end and have a 3-nucleotide RNA sequence (anticodon) complementary to the corresponding 3-nucleotide mRNA codon on the other end.

Ribosomes bind to specific ribosome-binding sequences in mRNA, complementary to a sequence in SSU rRNA, and begin translation at an initiation codon (most often AUG) encoding the amino acid methionine. The ribosome matches mRNA codons to tRNA anticodons and forms peptide bonds between sequential amino acids.

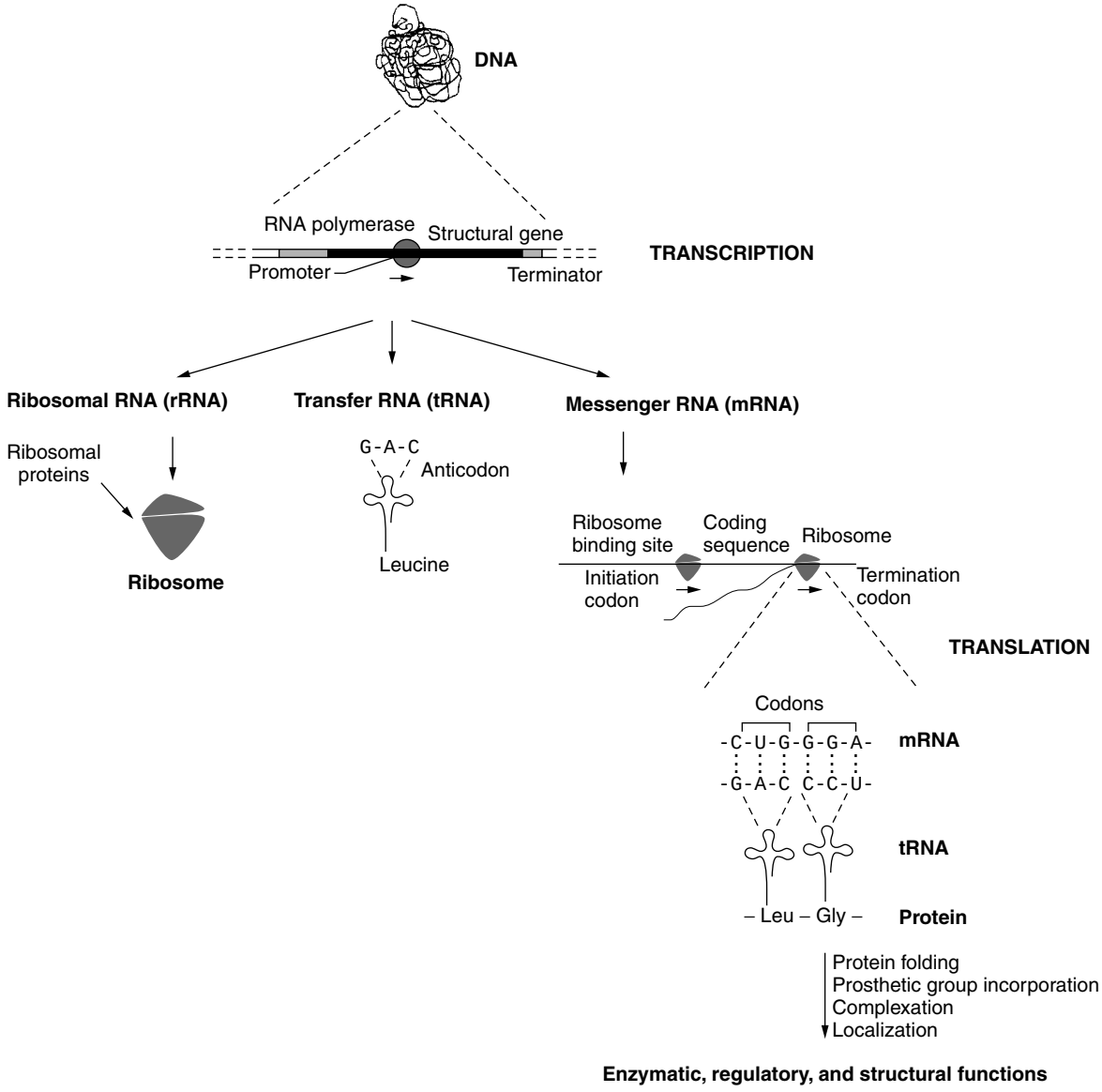


Figure 2. Transcription and translation: some details. See color insert.

Translation continues until the termination codon (UAG, UAA, or UGA) is reached. Newly produced proteins must then fold into their active configuration, and may require additional processing, cofactors, or localization to become functional.

From this brief overview, it can be seen that DNA-, RNA-, and protein-based methods measure different aspects of community activity. Transcription, translation, and protein maturation, as well as RNA and protein stability, may be regulated in response to environmental conditions. mRNA and protein stability may differ for a given gene (29), and proteins may have both active and inactive forms (e.g., 30). Both active and inactive microbes contain DNA, but active ones contain proportionally more RNA. To further complicate environmental studies, DNA can be stable extracellularly (31–33), whereas RNA is quickly degraded (a notorious problem in the laboratory), so amplified DNA sequences may not necessarily derive from the current population.

Phylogenetic Inference from Nucleic Acid Sequences

Nucleic acid sequences can be used to infer phylogenetic relationships and to identify unknown microbes by database comparisons. Steps in the construction of a phylogenetic tree are outlined in Figure 3. A collection of DNA sequences (in this case rDNA) is aligned. The dashes indicate positions in which other species (not shown) have additional bases. Mutations tend to occur less frequently in sequences encoding essential inter- or intramolecular interactions, such as substrate binding or secondary structure formation, so alignments generally reveal more- and less-conserved regions.

From the alignment, the number of base changes that would be required to change one sequence to another is computed for all pairs of species. Allowance is made for the presumed rate of back mutation. The resulting distance matrix is commonly shown as a tree, here in two different formats. In the radial tree, the evolutionary distance between species is represented as the sum of the lengths of the line segments connecting them. In the linear representation, distances are shown by the horizontal segments only. In both types of trees, nodes between branches represent inferred common ancestors.

The distance between two species is related to the time elapsed since divergence, but cannot be considered a direct measure of time. Mutation rates may vary among species, and between genes in the same species. For eukaryotic organisms, the molecular "clock" can be calibrated by comparison with the fossil record. For prokaryotes, with only a limited fossil record, calibration relies on evidence such as microbial molecules preserved in geologic formations.

Phylogenetic reconstruction is complicated by the possibility of gene transfer laterally between individuals (34,35) as well as vertically from an individual to its descendants (Fig. 4). This example shows the evolution of two genes in two species derived from a common ancestor. Gene A has been transmitted only vertically, whereas gene B was at some point transmitted horizontally from species 1 to species 2. Comparing the sequences of the genes in the present-day species, more differences will likely be seen between 1A and 2A than 1B and 2B. If only B were sequenced, a more recent common ancestor would be inferred.

As a result of gene transfers, therefore, different genes may yield different phylogenetic trees (36). It has been suggested that evolution might better be considered a network than a tree, with a combination of vertical and lateral exchanges, such that different genes in the same organism may have quite different histories (37). The relative importance of these two modes of inheritance should become clearer as the whole-genome sequences now being collected are interpreted (38).

The trees shown here were constructed with the neighbor-joining algorithm in the Arb sequence database program (*http://www.mikro.biologie.tumuenchen.de/pub/ARB*). A detailed discussion of the different algorithms available for phylogenetic analysis is beyond the scope of this article; an introduction may be found in Hillis and coworkers (39), and on-line resources have been collected by Felsenstein (*http://evolution.genetics.washington.edu/phylip/software.html*). DNA sequences obtained by researchers around the world are collected in a database maintained by the National Center for Biotechnology Information (*http://www.ncbi.nlm.nih.gov/*). For the most recent information on genome sequencing projects, The Institute for Genomic Research web site (*http://www.tigr.org*) is a good starting point.

Ribosomal RNA-Based Phylogeny

The SSU rRNA gene (the *rrn* operon or rDNA) is frequently used to construct evolutionary trees (40,41). It is an essential gene found in all known organisms, as well as in mitochondria and chloroplasts. It includes both quickly- and slowly-evolving regions, so that comparisons across both long and short evolutionary distances are possible. An extensive database of SSU rDNA sequences has accumulated, so newly acquired sequences may quickly be identified (see e.g., the Ribosomal Database Project web site, *http://www.cme.msu.edu/RDP/*). For determining the phylogeny of very closely related species or strains, however, longer or more quickly evolving sequences such as the 16S/23S spacer region (42) or the gyrase gene (43) may give better resolution than the SSU rRNA gene.

One complication for rRNA-based phylogeny is that many organisms have multiple *rrn* operons. Their sequences tend to be nearly identical, perhaps because of the requirement for interaction with ribosomal proteins (41,44), but some exceptions are known (e.g., 45,46).

It is generally considered that horizontal transfer of rDNA is unlikely, so that trees constructed using SSU rDNA sequences are predicted to accurately reflect the evolution of species. Ribosomes composed of a mixture of introduced and host-cell components might not work efficiently, so that cells containing them would grow slowly and be outcompeted in environments with limited resources. There is some recent evidence that this may not be entirely true. For example, an *Escherichia coli* strain with all seven of its chromosomal *rrn* operons deleted grew at near-normal rates when supplied with *rrn* operons from two of the three other bacterial species tried (47).

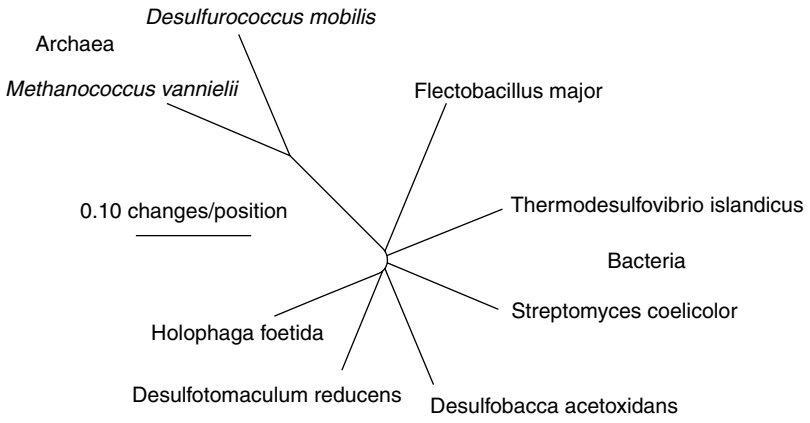
Sequence alignment

```
                                125     130     135  140     145     150    155
                                |       |       |    |       |       |      |
Holophage foetida              ..TA--GG-AGA-CCT-AC-CTTT--TTGT-GG---GGAAT-AA-CGTTC-C..
Desulfotomaculum reducens      ..TG--GA-TAA-CCT-GC-CTGA--TAGA-CC---GGGAT-AA-CAGCT-G..
Desulfobacca acetoxidans       ..TG--GG-TAA-TCT-AC-CTTC--GTTT-GG---GGGAT-AA-CCTAC-C..
Streptococcus coelicolor       ..TG--GG-CAA-TCT-GC-CCTT--CACT-CT---GGGAC-AA-GCCCT-G..
Thermodesulfovibrio islandicus ..TG--GG-TAA-CCT-GC-CCTT--AGGA-GG---AGGAT-AA-CTCGG-G..
Flectobacillus major           ..TA--TG-CAA-CCT-AC-CTAT--TATT-GG---GGGAT-AG-CCTTT-G..
Desulfurococcus mobilis        ..TG--GC-TAA-CCT-AC-CCTC--GGGA-GG---GGGAT-AA-CACCG-G..
Methanococcus vannielii        ..TG--GT-TAA-CTT-AA-CCTC--AGGT-GG---AGCAT-AA-CCTTG-G..
```

Distance matrix

Holophaga foetida							
Desulfotomaculum reducens	.195980						
Desulfobacca acetoxidans	.214141	.209104					
Streptococcus coelicolor	.222982	.197153	.224967				
Thermodesulfovibrio islandicus	.219530	.229107	.224000	.222707			
Flectobacillus major	.264645	.265557	.255349	.254470	.262537		
Desulfurococcus mobilis	.357243	.344189	.366644	.345017	.332329	.381429	
Methanococcus vannielii	.358665	.342500	.377095	.347376	.349735	.367742	.233516

Radial tree



Linear tree

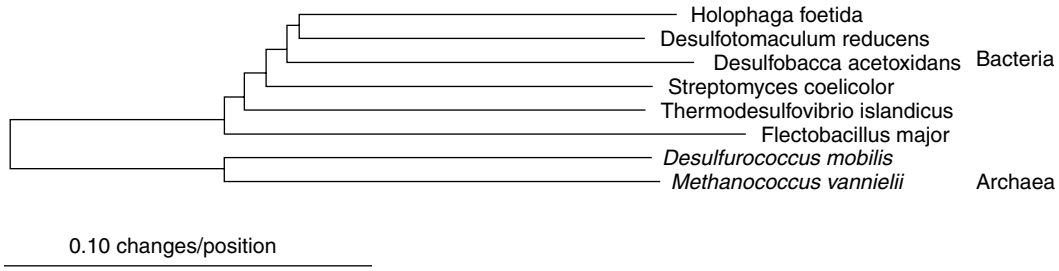


Figure 3. Construction of phylogenetic trees.

Many antibiotics kill cells or retard growth by interfering with ribosome function, and resistance can be conferred by ribosomal mutations. Gupta (48) has suggested that in environments in which antibiotic-producing bacteria are present, there might be a selection for acquisition of genes encoding antibiotic-resistant ribosomes, despite the possibly slower resultant growth rates. Evidence so far suggests that although such exchanges of rDNA may have occurred (49), they have been rare.

DNA-BASED METHODS

DNA can be considered the genetic potential of an individual or population. DNA isolated from environmental samples may be studied by direct hybridization with labeled probes that are cloned into plasmids or amplified by the polymerase chain reaction (PCR), with or without subsequent cloning.

Cloning and PCR increase rare sequences to assayable concentrations, but may introduce biases. Not all cloned

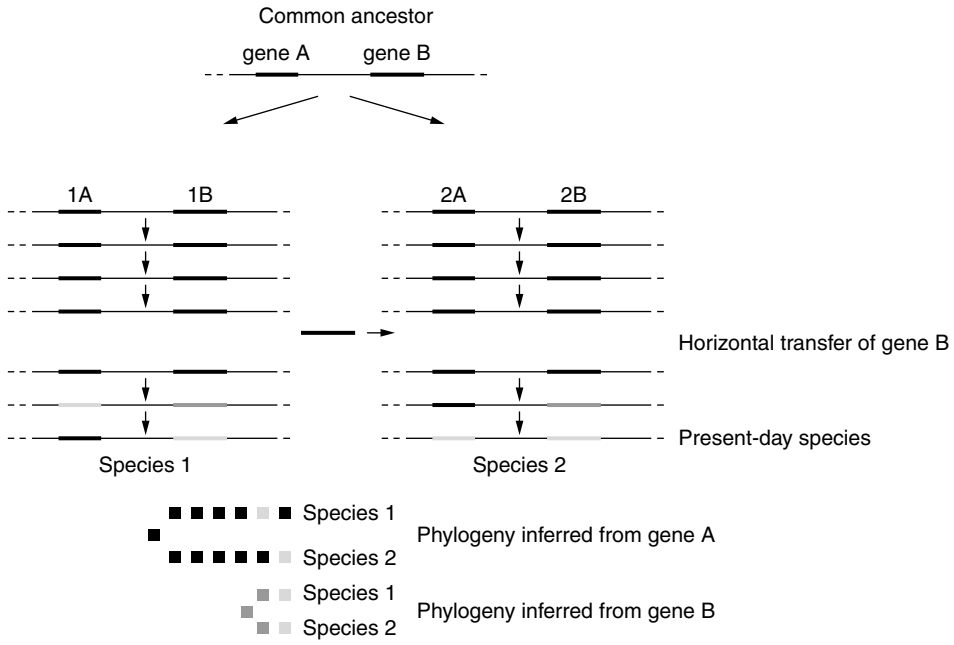


Figure 4. Lateral gene transfer. See color insert.

DNA sequences are stably maintained in host cells — for example, they may encode proteins that retard growth by competing for essential cofactors. Some DNA sequences are more readily amplified than others, for reasons not yet completely understood (50,51), hence not all sequences are necessarily amplified in direct proportion to their original abundance. It is also difficult to be sure that DNA (or any other biomarker) is being extracted with equal efficiency from all species in a sample. These caveats must be considered when designing experiments and interpreting results.

The Polymerase Chain Reaction (PCR)

PCR employs oligonucleotide primers and thermostable DNA polymerase to amplify target DNA sequences by temperature-controlled cycles of strand separation, primer annealing, and primer extension (Fig. 5). DNA isolated from an environmental sample is mixed with heat-stable DNA polymerase (e.g., Taq polymerase, isolated from the hyperthermophile *Thermus aquaticus*), dideoxy nucleotide triphosphates (dNTPs), and oligonucleotides complementary to the upstream and downstream ends of the gene to be amplified (primers). Reactions are performed in small volumes (typically 10–50 μL) in a thermal cycler capable of rapid cycles of heating and cooling. The reaction mixture is heated to separate it into single strands and then cooled to a temperature that allows stable but specific binding of the primers to their target sequences. DNA polymerase binds the resulting short double-stranded regions and elongates them. Repeated cycles result in numerous copies of the target sequence.

The primers used for PCR can be designed to target phylogenetic groups from the "universal" to the subspecies level. Annealing temperature, buffer composition, and number of cycles can be adjusted to control specificity. Optimum conditions must be determined empirically for each primer pair. This has become easier with the development of temperature-gradient PCR machines.

PCR amplification is prone to several types of artifacts. PCR bias was mentioned above. Chimeric sequences may be also produced, joining partial sequences amplified from two different templates (52,53), especially if short elongation times or too numerous cycles allow partially amplified sequences to accumulate (54). Computer algorithms are available to help detect these mixed sequences (55) by comparing different segments of amplified products to the database separately. If the two ends of a sequence have different closest relatives, the sequence is probably chimeric. Insertions, deletions, and base changes may also be introduced during elongation, but with a high-fidelity DNA polymerase, these should be found in only a very small proportion of product molecules and so are not likely to be detected by sequencing.

Cloning

Cloning is a method of separating the mixture of fragments obtained by PCR amplification, restriction enzyme digestion, or physical fragmentation (56,57) of a DNA sample (Fig. 6). The fragments are ligated with a plasmid, a small circular DNA molecule that can be maintained by bacteria as an extrachromosomal element. In the example shown, the plasmid encodes an antibiotic resistance enzyme and a lethal gene. The plasmid is linearized with a restriction enzyme that cuts it once, within the lethal gene, and ligated with PCR amplification products. Ligated molecules are introduced into *E. coli* cells made competent for direct DNA uptake by (for example) calcium chloride treatment (58–60), and the cells spread on antibiotic-containing agar plates. Only cells carrying active antibiotic resistance genes, but not expressing the lethal gene, will divide and give rise to colonies; the majority of such cells should contain plasmids with a lethal gene interrupted by a

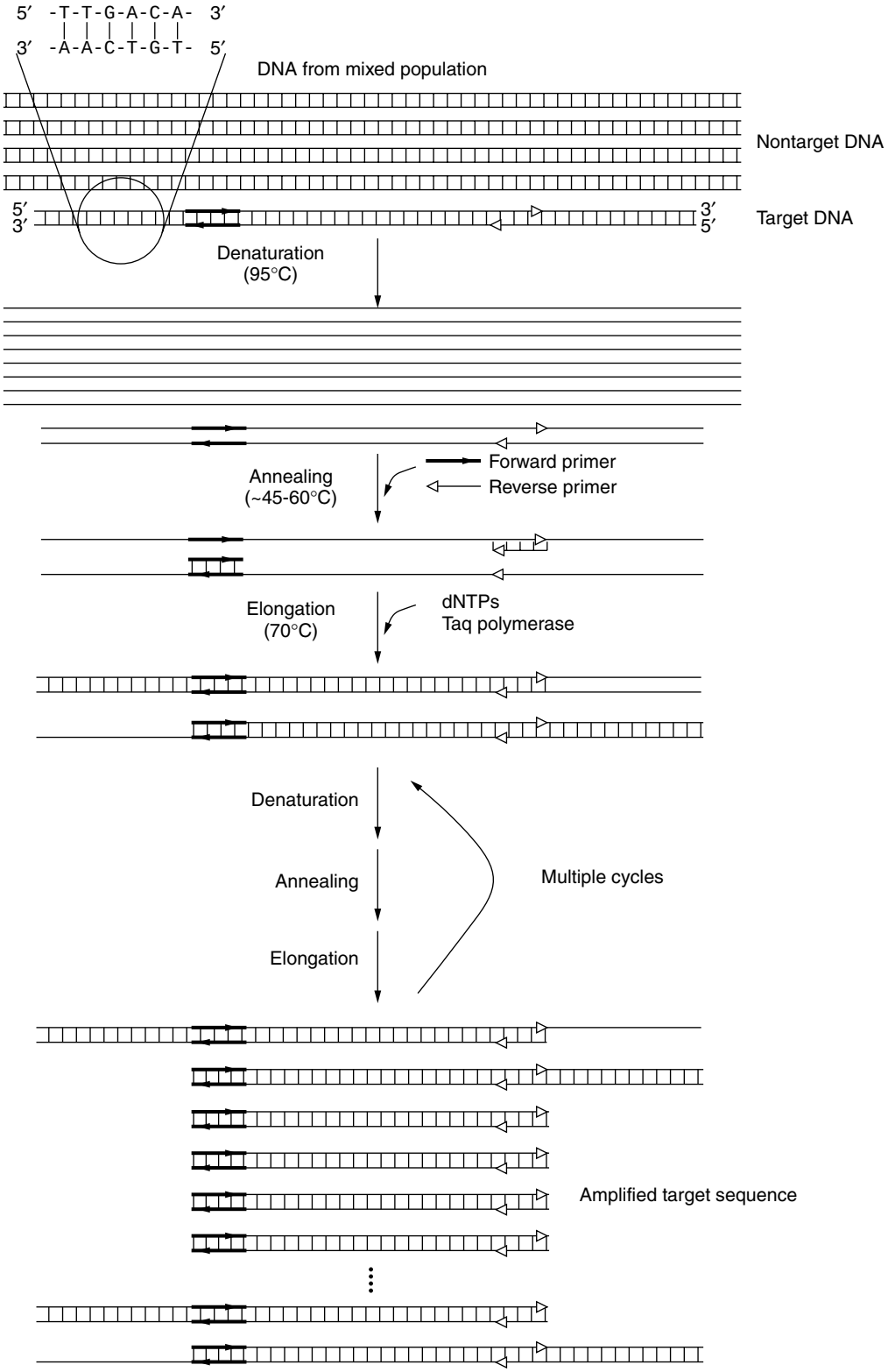


Figure 5. Polymerase chain reaction.

fragment of environmental DNA. Individual colonies are purified by repeated streaking on antibiotic-containing agar plates, and the cloned fragments are identified by DNA sequencing.

Denaturing Gradient Gel Electrophoresis (DGGE)

PCR products (amplificates) of similar length can be screened for sequence heterogeneity before sequencing by

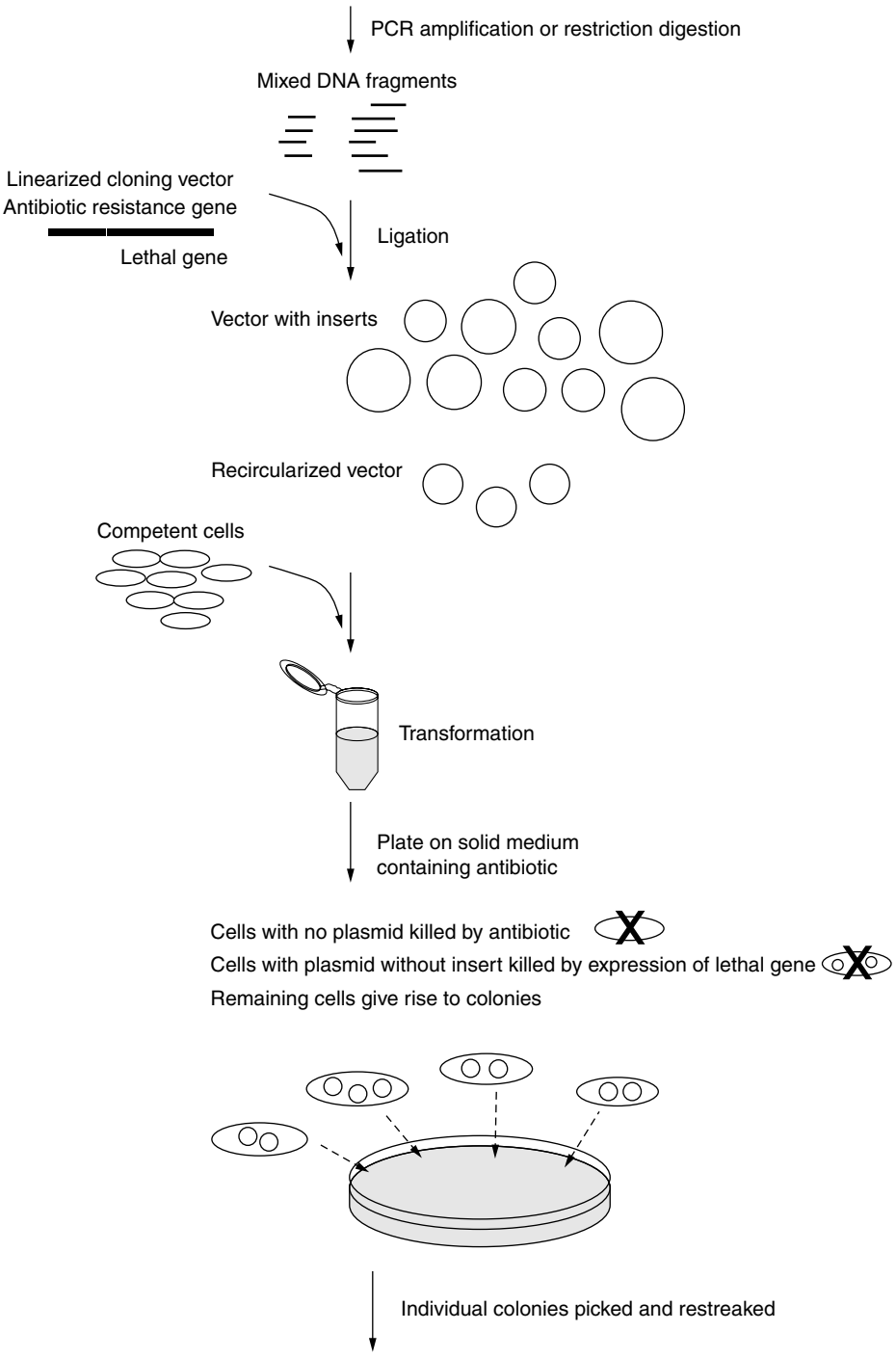


Figure 6. DNA cloning. See color insert.

several methods. Denaturing gradient gel electrophoresis (DGGE, Fig. 7) exploits the sequence dependence of DNA duplex denaturation (61) to separate molecules of similar length but different nucleotide composition. DNA from a mixed population is PCR-amplified with primers targeting the gene of interest (e.g., bacterial SSU rRNA). One of the primers has a GC-rich "tail," which is amplified as one end of the PCR product molecules. The mixture of product molecules is then electrophoresed on an acrylamide gel prepared with an increasing gradient of denaturant. DNA is negatively charged, because of the phosphate groups in its sugar backbone, and will move toward the positive electrode. The double-stranded molecules run through the gel until they reach a denaturant concentration that separates all but the high-GC clamp into single strands. The resulting Y-shaped molecule essentially stops migrating, forming a band that can later be visualized by staining with DNA-binding dyes such as ethidium

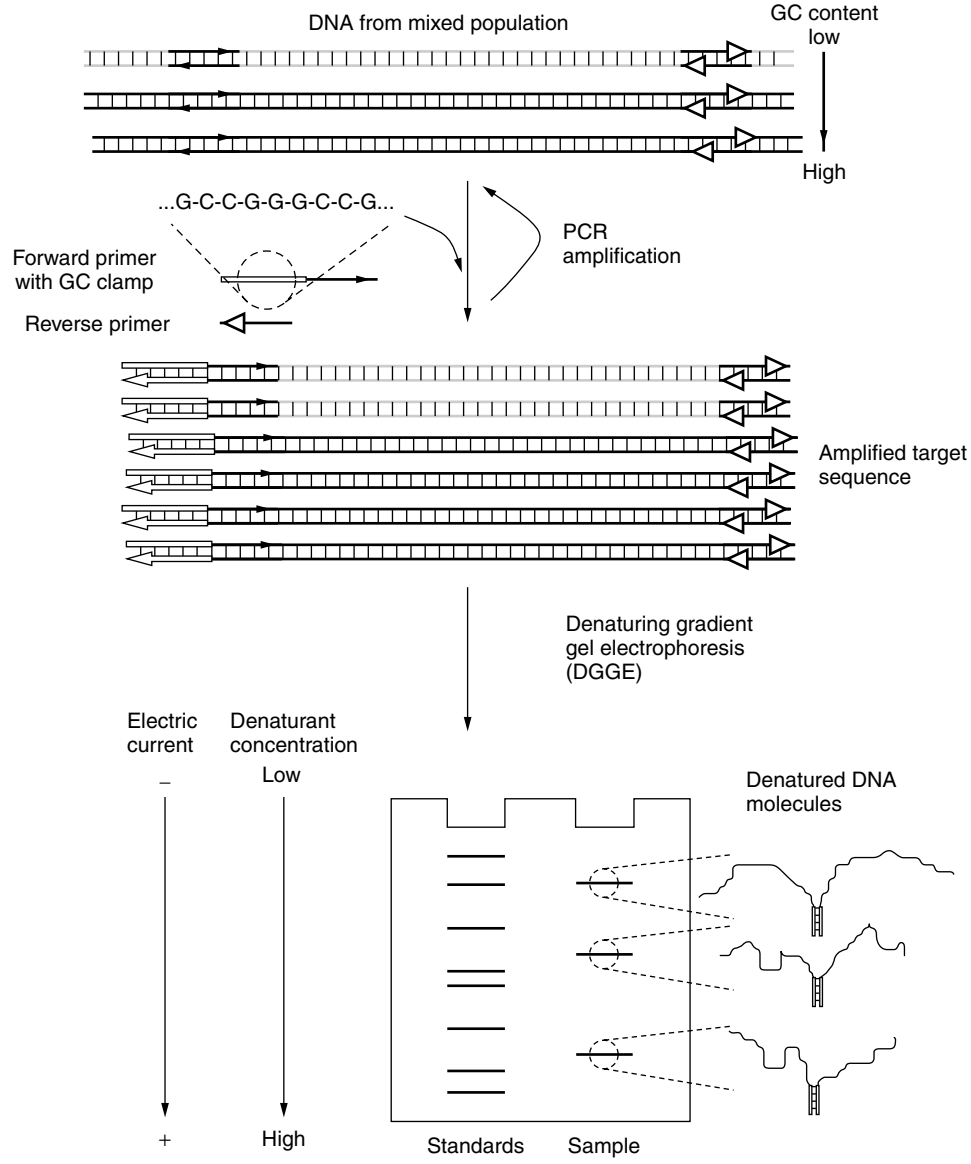


Figure 7. Denaturing gradient gel electrophoresis (DGGE).

bromide. Bands may be identified by comparison with known standards; hybridization to labeled oligonucleotide probes specific for particular groups; or DNA sequencing. The patterns of amplificates obtained from different environments, or from the same environment at different times, can be compared to detect changes in microbial community composition.

A variation of DGGE, TGGE, uses a heat gradient to separate molecules with different melting temperatures (62).

Terminal Restriction Fragment Length Polymorphism (T-RFLP)

T-RFLP is a method for estimating the number of different sequences in a mixture of PCR products (63). One of the two PCR primers is fluorescently labeled (Fig. 8). After amplification, the PCR products are digested with a restriction enzyme chosen to cut at different positions in different product sequences. This assumes that the sequences of all molecules likely to be in the sample are already known — this may not be the case for all samples. The digested DNA is then separated by gel electrophoresis on a DNA sequencing machine that can detect the fluorescent label. Fragment lengths are determined by comparison with fragments of known length. Because only the terminal fragments of each PCR product molecule are labeled, a unique band is ideally detected for each type of sequence. Bands may be identified by comparison with known sequences.

RNA-BASED METHODS

The advantage of RNA over DNA for community studies is that RNA reflects the active population more closely. Environmental studies have been based on both ribosomal

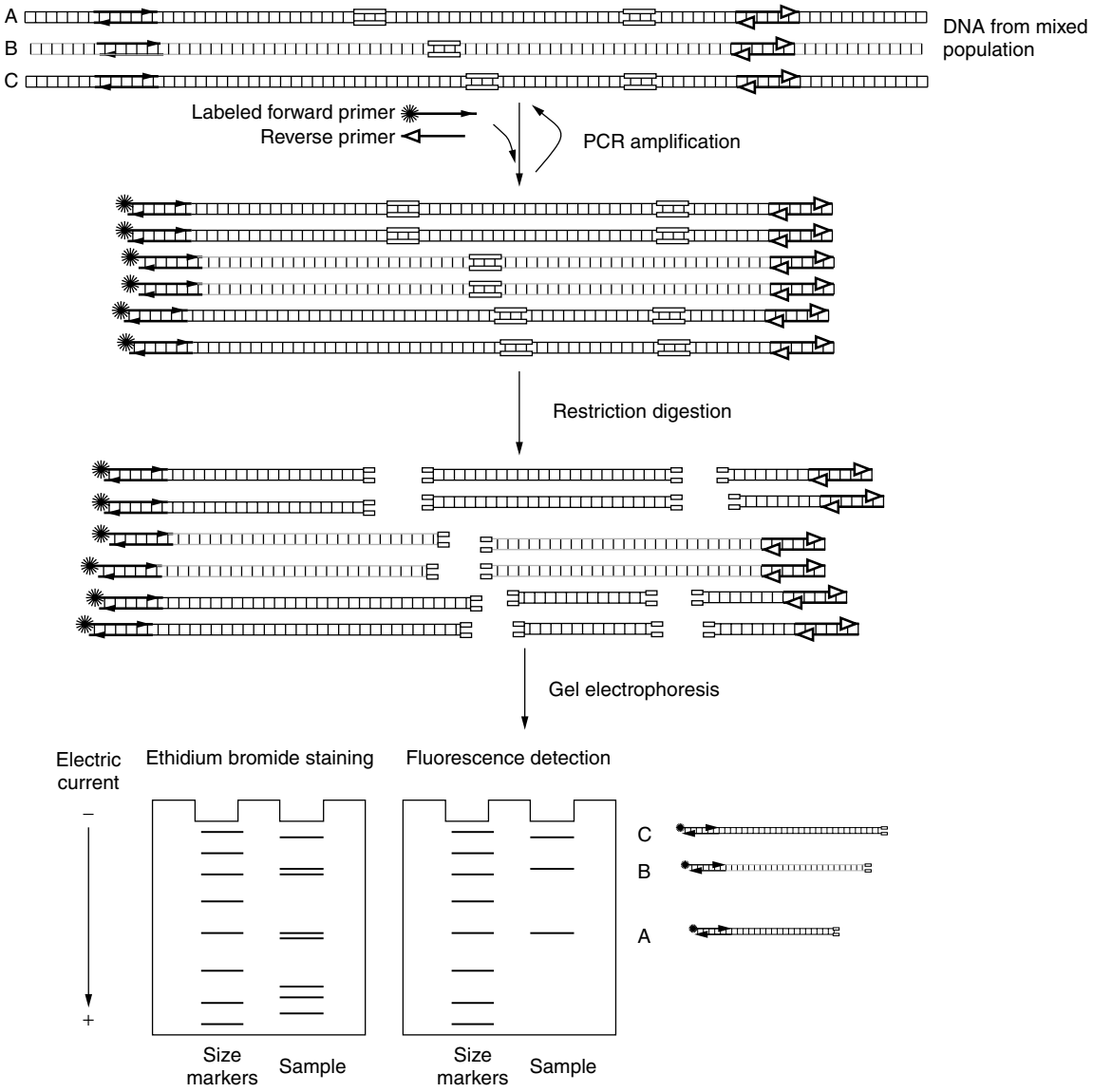


Figure 8. Terminal restriction fragment length polymorphism (T-RFLP).

and messenger RNA detection: rRNA as a measure of general metabolic activity and mRNA as a measure of specific functions.

SSU rRNA content generally increases with growth rate, so rRNA levels reflect population activity. Oligonucleotide probes can be designed to target the RNA of specific microbial groups, from the subspecies to the domain level, so that changes in their relative activities can be followed over time or compared between sites. It should be borne in mind, however, that the relationship between rRNA content and growth rate is not necessarily linear (64) and may differ among species and strains (65). Some microbial species may maintain a ribosomal reserve at very low growth rates, perhaps to allow quick response to improved conditions (66). Thus, the precise relationship between rRNA concentration and metabolic activity in a mixed population depends on community composition.

Ribosome production is regulated in macroscopic organisms as well as microbes. For example, RNA/DNA ratios are used as a measure of the condition of larval fish populations on the assumption that faster-growing individuals will posses more ribosomes and therefore more rRNA (67).

mRNA provides a more direct link to function than does rRNA. Ecologically important microbial activities such as nitrogen fixation are often scattered among distantly related species owing to independent evolution or interspecific gene transfer. This makes studying them by SSU rRNA sequences laborious, at best. However, the relatively low copy number per cell of mRNAs makes detection difficult. Some methods of signal amplification are discussed below.

Oligonucleotide Probe Hybridization

The rRNA contributed by different microbial populations may be measured following RNA extraction, by methods such as membrane hybridization, or within fixed

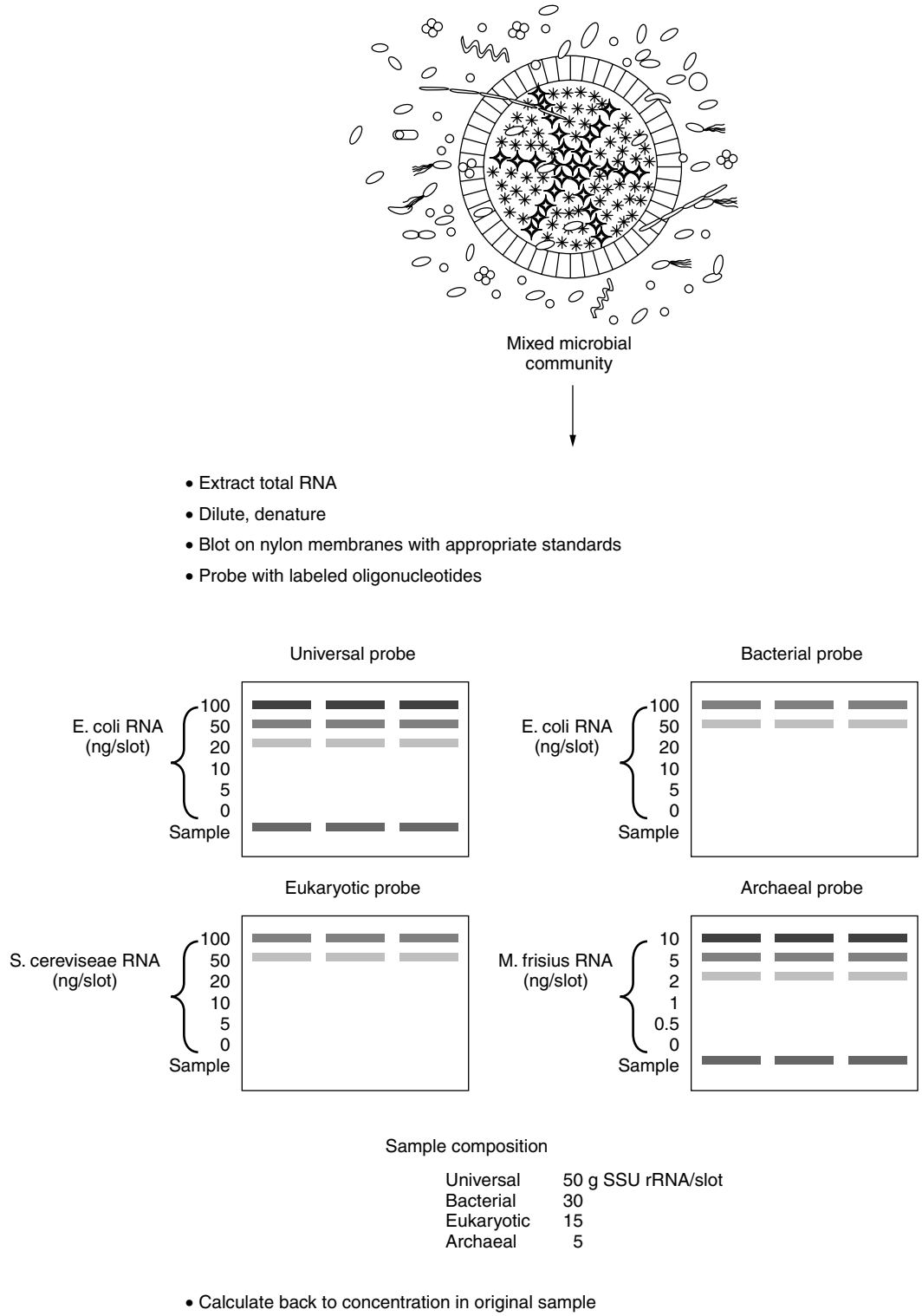


Figure 9. Membrane hybridization. See color insert.

cells, by in situ methods. Membrane hybridization, illustrated here for SSU rRNA (Fig. 9), is an example of an in vitro method. Total RNA is extracted and blotted in triplicate onto a positively-charged nylon membrane (RNA is negatively charged), along with a range of concentrations of a reference RNA. The membranes are hybridized with oligonucleotides targeting particular phylogenetic groups (68) — for example, archaeal or bacterial SSU rRNA. The concentration of target RNA in the samples is calculated by comparison with the reference RNA. To control for cross-hybridization, nested sets of probes are recommended — for example,

universal probe hybridization should ideally equal the sum of bacterial, archaeal, and eukaryotic probe hybridization.

In fluorescent in situ hybridization (FISH), fluorescently labeled oligonucleotides are hybridized to fixed microbial cells (69). SSU rRNA-targeted probes can be used for phylogenetic identification of organisms not yet obtained in pure culture, and multiple probes with different fluorescent labels can be used to visualize several species at once.

Laser confocal microscopy can give a three-dimensional picture of microbial associations (70,71). The development of automated image analysis systems for FISH should dramatically increase the quantitative information that can be extracted from a sample by this method.

mRNA Detection

There are exceptions to the central dogma: of particular relevance to this article, genetic information in retroviruses is carried by RNA rather than DNA, and transcribed to DNA by the viral enzyme reverse transcriptase. This enzyme has proven useful in molecular biological studies for the detection of mRNA, which is present at a much lower copy number per cell than is rRNA.

mRNA detection methods include in situ reverse transcription (RT) and in situ or in vitro RT-PCR (Fig. 10). RT-PCR is a method for detecting genes that are being actively transcribed, by amplifying RNA as DNA. It can be done either in vitro, which allows the amplified products to be identified by sequencing; or in situ, so that the spatial distribution of the expressed genes can be seen. In either case, RNA is hybridized with a specific oligonucleotide primer. Reverse transcriptase binds to the RNA/oligonucleotide hybrid and transcribes the RNA to DNA. The DNA may then be PCR-amplified, using labeled nucleotides to allow detection. Methods of mRNA detection in eukaryotic systems have recently been reviewed (72). Applications to microbial populations have been limited to data owing to the technical difficulty of specific detection of very low-abundance molecules.

FUTURE DIRECTIONS

Phylogenetically based molecular microbiological methods have greatly increased the number of detected microbial species; the next challenge will be to assign ecological roles to these many players. Several methods are currently being worked out in controlled environments for directly linking identifiable biomarkers to physiology.

For example, rRNA-targeted probes have been used to follow populations of sulfate-reducing bacteria in sediment slurries after addition of different carbon substrates to elucidate possible pathways of carbon flow in mixed populations (73). Fluorescent in situ hybridization has been combined with microautoradiography to directly identify cells incorporating radiolabeled substrates (74,75).

Isotopically heavy DNA was isolated from soil samples incubated with ^{13}C-labeled methanol (76), suggesting that the isotopic composition of nucleic acids might help directly identify natural populations using isotopically distinctive carbon or perhaps nitrogen sources such as biogenic methane or atmospheric N_2. However, this will depend in part on the degree to which cellular nucleic acids are synthesized de novo by a given species.

Judging from the rate at which new methods (and new acronyms) are appearing in environmental microbiology literature, the molecular approach is still in its early days. It will be interesting to see what new developments appear in the second edition of this Encyclopedia.

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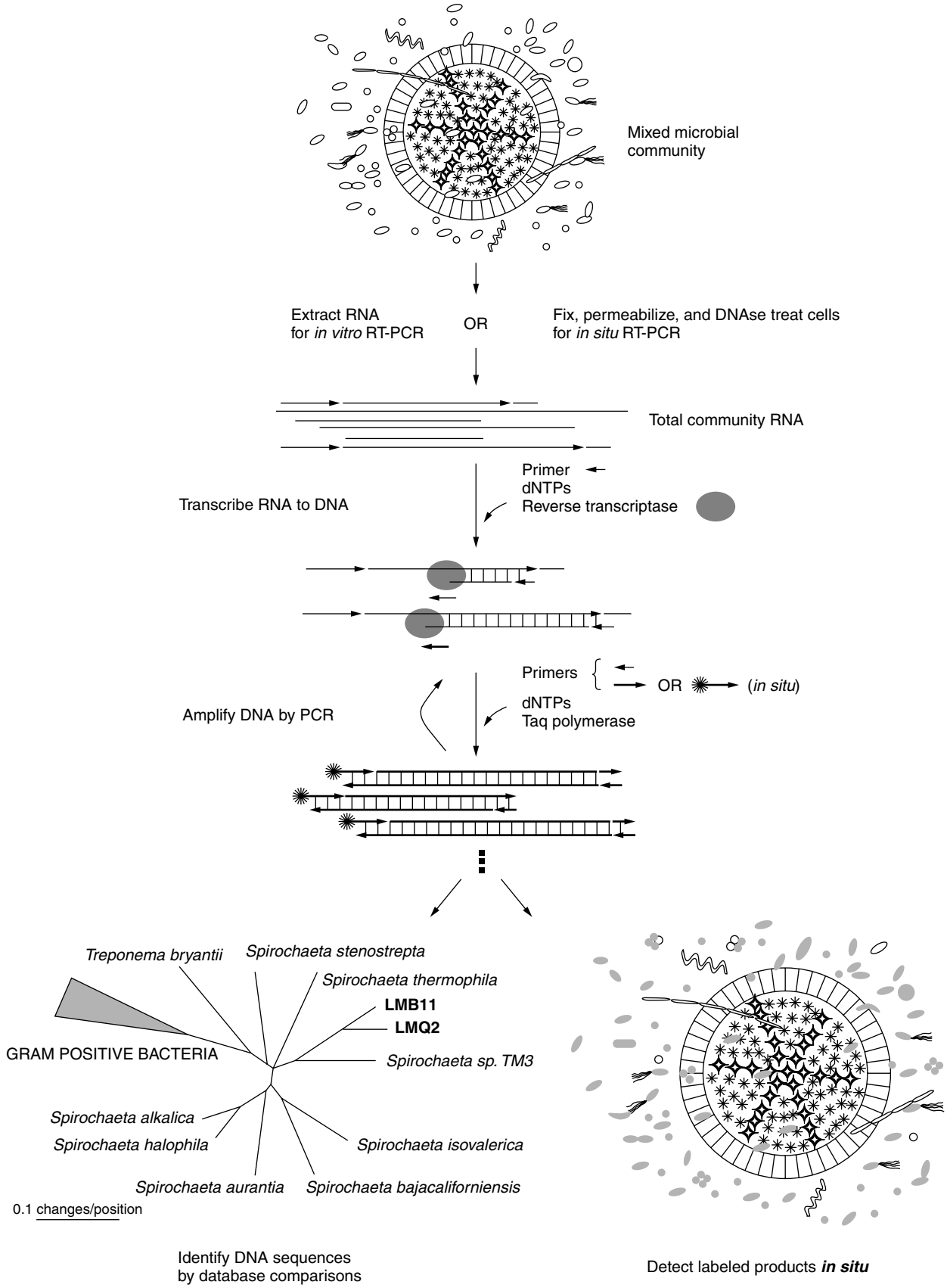

Figure 10. Reverse transcription-polymerase chain reaction (RT-PCR). See color insert.

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PHYLOGENETICS, ARCHAEA.

See ARCHAEA IN BIOTECHNOLOGY

PHYLOGENY.

See *GIARDIA*: DETECTION AND OCCURRENCE OF IN THE ENVIRONMENT

PHYLOGENY OF AQUIFER COMMUNITIES.

See IGNEOUS ROCK AQUIFERS MICROBIAL COMMUNITIES

PHYTONEUSTON.

See NEUSTON MICROBIOLOGY: LIFE AT THE AIR–WATER INTERFACE

PHYTOPLANKTON.

See MEROPLANKTON; PLANKTONIC ALGAE IN THE MARINE ENVIRONMENT; PRIMARY PRODUCTIVITY IN THE MARINE ENVIRONMENT

PHYTOPLANKTON, POLAR.

See Polar Marine Phytoplankton

PHYTOSTIMULATORS, BACTERIAL.

See Bacterial Phytostimulators in the Rhizosphere

PIGMENTS OF ALGAE AS PALEOLIMNOLOGICAL INDICATORS.

See Paleolimnology: Use of Algal Pigments as Indicators

PLANKTONIC ALGAE.

See Planktonic Algae in the Marine Environment

PLANKTONIC ALGAE IN THE MARINE ENVIRONMENT

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Planktonic algae (phytoplankton) are the base of the food web in the ocean, absorbing sunlight with chlorophyll *a* and accessory pigments for photosynthesis and providing most of the primary production of organic matter that ultimately feeds the rest of the organisms in the ocean. Only a small proportion of the ocean (<5%) is shallow enough for light to reach the bottom, allowing benthic plants such as macroalgae, seagrasses, and symbiotic algae inside animals such as corals to grow. Intertidal plants, such as marsh grasses and mangroves, also occupy a very small area of the ocean. Because benthic plants live in such a small area of the ocean, they contribute relatively little photosynthesis to the marine ecosystem, and planktonic algae provide over 95% of the primary productivity, upon which the entire ecosystem is dependent. An overview of the role of phytoplankton in the ocean can be found in references 1 and 2.

Among these planktonic algae, approximately half are prokaryotic cyanobacteria, which are discussed in another article. The other half is eukaryotic algae, belonging to around 10 different phyla within the kingdom of Protists. Table 1 lists these phyla, according to several different systematic schemes that have been proposed (3–6). The systematics of algae continue to be in a state of flux and will certainly change as molecular genetic data accumulate and replace the traditional systematic characteristics of pigments, chloroplast structure, types of flagella, cell wall structure, ultrastructure, storage products, modes of reproduction, and life cycles.

The distinction between algae and protozoa within the Protist kingdom is largely physiological–algae being autotrophic and protozoa being heterotrophic. Phylogenetically and morphologically, there is considerable overlap between algae and protozoa. For example, approximately half of all dinoflagellates are primarily or exclusively heterotrophic and the other half is primarily or exclusively autotrophic. Some species can be both autotrophic and heterotrophic (and both osmotrophic and phagotrophic) at the same time or at different times. Dinoflagellates such as *Pfiesteria* spp. can be photosynthetic in part of their life cycle, providing primary productivity at the base of the food web, and parasitic on fish at other times, essentially appearing as a predator near the top of the food web. A large number of euglenophytes and cryptophytes are also partially or totally heterotrophic.

The term *algae* is then used to characterize protists that are primarily photosynthetic. Although phylogenetically prokaryotic cyanobacteria are very different from the eukaryotic algae, physiologically and ecologically they are very similar in many ways, as they are both photosynthetic and live in essentially the same habitat. Therefore, they can be considered as prokaryotic algae. This article will focus on the eukaryotic algae.

HABITAT CHARACTERISTICS

A general discussion of the marine environment in which phytoplankton live can be found in references 1,7, and 8. Planktonic microalgae can be found everywhere in the ocean where there is enough light for photosynthesis, which typically requires at least 1% of full sunlight for growth. Generally, light intensity declines exponentially with depth and around 1% is left at around 100 m in clear open ocean water. The water column where there is enough light for photosynthesis in known as the photic zone. As one moves from the open ocean to coastal waters, the higher concentrations of particles and dissolved organic matter absorb more light and reduce the depth of the photic zone. All wavelengths of light are not absorbed equally. Infrared, red, and ultraviolet light are the most strongly absorbed (most within a few meters). In the open ocean, blue light (around 480 nm) travels the furthest, whereas in many coastal waters, the absorbance spectrum is shifted so that green light travels the furthest.

Because around 99% of the light is absorbed and converted to heat in the photic zone, the photic zone is often warmer, thus less dense than the water below. This often leads to a sharp temperature gradient (known as a thermocline) at some depth near the bottom of the photic zone. Near the coastline where large amounts of freshwater runoff occurs and near melting ice, the salinity of the surface water can be lowered, generating a halocline in which lighter, low-salinity water rests above denser, high-salinity water. Temperature and salinity are the two major factors that control the density of seawater, so their vertical gradients can generate a large vertical gradient in density, known as a pycnocline. This pycnocline greatly inhibits vertical movement and mixing of the warm surface waters with cold deep waters. There is a net flux of organisms and detritus from the surface through the

pycnocline. Therefore, most nutrients in the photic zone tend to be taken up by phytoplankton and eventually end up as particles that sink through the pycnocline into deep water. As a result, nutrients generated from the decomposition of these materials tend to accumulate in the deep waters. Because the pycnocline inhibits the upwelling of this dense, nutrient-rich water, a strong vertical gradient in nutrient concentrations, known as a nutricline, develops with low concentrations of nutrients in the photic zone and high concentrations below. This results in the fundamental dilemma that phytoplankton face in the ocean–the spatial separation of the two main resources they need: light and nutrients. In much of the photic zone, there is plenty of light for photosynthesis, but not enough nutrients to sustain much growth. Below the photic zone, there are plenty of nutrients but not enough light. Phytoplankton have many adaptations for dealing with this dilemma (to be discussed later). Phytoplankton grow best and tend to be most abundant where this spatial separation of light and nutrients is reduced. The pycnocline is strongest in the tropical open ocean and weakest in the polar and coastal regions. As a result, there are strong latitudinal and inshore-offshore gradients in nutrient concentrations and phytoplankton abundance, with the lowest levels in the tropics and central ocean. This accounts for the very clear water in the tropics and open ocean.

Most of the nutrients that support phytoplankton growth in the photic zone come from nutrient-rich upwelled water below. Near the coastline, land runoff can be a major source of nutrients. Atmospheric inputs may be of significance in some circumstances as well. Because of the insolubility of iron in seawater, upwelled water in the open ocean is depleted of iron relative to the other nutrients. As a result, atmospheric dust input appears to be an important source, leaving places far from land influences (such as the central Pacific and Southern oceans) iron limited. Agricultural activities and fossil fuel burning has increased the amount of bioavailable nitrogen in the atmosphere and it appears that the deposition of the atmospheric nitrogen could be of significance in some situations.

Close to 10% of the ocean area is over the continental shelves. This coastal ocean (regarded as the neritic habitat) has an average depth of around 100 m. Close to 90% of the ocean is around 4,000 m deep and is regarded as the open ocean or oceanic habitat. There is a relatively sharp transition from the continental shelf to the deep sea, so only a relatively small portion of the ocean is in this transitional zone. This neritic-oceanic boundary is also a relatively strong boundary between two very different ecosystems with very different environmental parameters and biological communities. For example, there are a great many more mechanisms that force nutrient-rich deep water up into the surface photic zone in the coastal ocean than in the open ocean. As a result, nutrient concentrations and phytoplankton abundance are typically much higher in coastal waters. Because of the large habitat differences, very few phytoplankton species live in both the coastal and the open oceans.

Because of the high specific heat content of seawater and the transparency of the water, which disperses the solar energy over many tens of meters, temperature does not fluctuate very much in the ocean compared with the land. The largest variation is seasonal in temperate regions. Ultimately, temperature is not a very important ecological factor compared to light and nutrients. Phytoplankton growth and biomass do show a negative correlation with temperature, however, because it reflects the strength of the pycnocline. Deep water, even in the tropics, is very cold. Therefore, in the tropics, warm surface water overlies cold water, generating a strong pycnocline that greatly reduces the upwelling of nutrients. In polar regions, both the surface and bottom waters are cold. In this case, the pycnocline is weak.

Most of the ocean has a salt content of around 3.5% (referred to as 35 parts per thousand by oceanographers). Because the major salts (sodium, chloride, sulfate, magnesium, calcium, and potassium) have residence times in the ocean of over a million years, they are well mixed and occur in very constant ratios relative to each other, allowing one to use total salt content (salinity) without referring to individual ions. Because the salinity of the ocean does not change much, it is not a major factor affecting phytoplankton in the ocean. Large changes occur only near areas of land runoff, shallow lagoons greatly influenced by rain and evaporation, and freezing and melting ice.

PHYLOGENETIC RELATIONSHIPS

There are two major phylogenetic lineages of algae: the chlorophytes, which have chlorophyll *b* as an accessory pigment, and the chromophytes, which have chlorophyll *c* as an accessory pigment (3–6) (Table 1). It is thought that each of these eukaryotic lineages evolved by endosymbiosis, with a prokaryotic cyanobacteria becoming the eukaryotic chloroplast. Some cyanobacteria have been found to be very similar to the chloroplast of chlorophyte algae. The chlorophyte lineage includes the phyla chlorophytes, euglenophytes, and prasinophytes. The green macroalgae appear to have evolved from the chlorophyte microalgae, and all higher plants (charophytes, bryophytes, and tracheophytes) ultimately evolved from them. Chlorophytes and euglenophytes tend to be quite abundant in freshwater ecosystems but rather sparse in the ocean. In marine ecosystems, they tend to be more important in brackish water ecosystems. Prasinophytes, on the other hand, appear to be in moderate abundance in the ocean. The chromophyte lineage includes dinoflagellates, diatoms, prymnesiophytes, chrysophytes, xanthophytes, and raphidophytes. Many of these phyla are predominately marine, although they can also be found in freshwater. As a broad generalization, however, the chlorophyte lineage tends to dominate freshwater ecosystems and the chromophyte lineage tends to dominate marine ecosystems. The evolution of the chlorophyte lineage ultimately led to the higher plants found on land. The evolution of the chromophyte lineage ultimately led to the macroalgal phaeophytes (brown algae). Some of the large kelps in the phaeophyte phylum begin to approach the morphological complexity of higher

Table 1. Several Systematic Schemes for Eukaryotic Algae

Textbook	Trainor (1978)	Bold and Wynne (1985)	Van den Hoek and Coworkers (1995)	Lee (1999)
Chlorophyte lineage	Chlorophyceae	Chlorophyta	Chlorophyta	Chlorophyta
			Chlorophyceae	Chlorophyceae
			Prasinophyceae	Micromonadophyceae
	Euglenophyceae	Euglenophyta	Euglenophyta	Euglenophyta
			Chlorarachniophyta	
Chromophyte lineage	Dinophyceae	Pyrrhophyta	Dinophyta	Dinophyta
		Chrysophyta	Heterokontophyta	Heterokontophyta
	Bacillariophyceae	Bacillariophyceae	Bacillariophyceae	Bacillariophyceae
	Chrysophyceae	Chrysophyceae	Chrysophyceae	Chrysophyceae
			Parmophyceae	Pelagophyceae
			Sarcinochrysidophyceae	Synurophyceae
				Dictyochophyceae
	Eustigmatophyceae	Eustigmatophyceae	Eustigmatophyceae	Eustigmatophyceae
	Xanthophyceae	Xanthophyceae	Xanthophyceae	Xanthophyceae
		Raphidophyceae	Raphidophyceae	Raphidophyceae
	Haptophyceae	Prymnesiophyceae	Haptophyta	Prymnesiophyta
Other groups	Cryptophyceae	Cryptophyta	Cryptophyta	Cryptophyta
	Rhodophyceae	Rhodophyta	Rhodophyta	Rhodophyta

plants, with their stems (capable of nutrient translocation), distinct leaves, and specialized sex organs. As the chromophytes tend to have high concentrations of accessory pigments such as the carotenoids fucoxanthin and peridinin, they tend to be yellow-brown in color, not green. In general then, terrestrial and freshwater plants tend to be green, whereas marine plants tend to be yellow-brown.

In addition to the two main lineages of algae, a few other phyla of algae exist with more obscure phylogenetic relationships to the rest of the algae. The rhodophytes (red algae) are mostly macroalgae, but some unicellular rhodophytes are found in the ocean, generally in low concentrations in brackish waters. They are different from the chlorophyte and chromophytes lineages by having phycoerythrin as an accessory pigment and not chlorophyll *b* or *c*. Cryptophytes are a phyla of microalgae that also have phycoerythrin and not chlorophyll *b* or *c* and tend to be found in relatively low concentrations in mostly brackish water.

At the present time, it is estimated that there are perhaps around 5,000 species of phytoplankton in the ocean, although the estimate could change substantially as new techniques are used to detect more species.

SYSTEMATICS

The identification of species of the large phytoplankton, such as many diatoms and dinoflagellates, is based primarily upon overall cell morphology and can be conducted by light microscopy. For species with hard parts (silica frustules of diatoms, calcium carbonate coccoliths of coccolithophores, cellulose plates of dinoflagellates) the detailed patterns on these structures, using scanning electron microscopy, can be used to distinguish species. For small cells with no hard parts, examination of ultrastructure is about the only way to distinguish species, and is undoubtedly not distinguishing many species because of the morphological similarities of species. New developments in molecular biology can be expected to provide better techniques for distinguishing species in the future.

The term species is often used in a somewhat arbitrary manner. Various morphological characteristics are used to define species; yet biologically and ecologically, one thinks of a species as a group of organisms with similar genetic composition (as a result of gene flow) and similar ecological requirements. In fact, there is considerable genetic variation within phytoplankton species, even among groups that clearly have significant gene flow (9). Cells of a species, all found within 1 L of water, can be shown to be genetically different. Populations of cells of the same species in different water masses can be genetically quite different from each other and have different ecological requirements. There is also evidence that because of the considerable genetic variability within populations, genetic changes occur in the timescale of seasonal changes, indicating how rapidly natural selection can occur on these organisms with a generation time on the order of a day or less.

EVOLUTIONARY HISTORY AND FOSSIL RECORD

An overview of the evolutionary history and fossil record of algae can be found in references 10–14. The first oxygenic photosynthesizers, the prokaryotic cyanobacteria, first appeared in the fossil record around 3.5 billion years ago. The eukaryotic algae did not appear in the fossil record until around a billion years ago, about when free molecular oxygen begins to appear on the earth. The earliest phylogenetic groups were probably the dinoflagellates and chlorophytes. As with any fossil record, there is a strong bias toward organisms with hard parts. In the

case of marine phytoplankton, the best preserved are the silica frustules of diatoms and calcium carbonate coccoliths of coccolithophores. Indeed, the fossil record of coccolithophores and diatoms is so good that their species distributions in cores taken around the world are used by paleoceanographers to estimate paleotemperatures, paleoclimate, paleocirculation, and so on. The ^{18}O and ^{13}C isotopic ratios in coccoliths are particularly important in paleoceanography. The fossil record of other groups is based primarily on the cellulose cell walls, with a bias in favor of the particularly thick and resistant cellulose walls of cysts.

Because of differential preservation, a complete evolutionary history of phytoplankton is impossible to discern, but the fossil record does show some interesting patterns. For example, coccolithophores first appear in the fossil record in large numbers in the Jurassic (145 to 210 million years ago), about the same time that calcareous foraminifera become planktonic. Diatoms first appear in the Cretaceous (65 to 145 million years ago), about the same time as a large increase in siliceous radiolarians appears. This leads to questions about the possible relationship between the changing biogeochemistry of the ocean and the evolution of phytoplankton with mineral hard parts.

By the end of the Cretaceous, coccolithophores were extremely diverse and cosmopolitan in distribution. Diatoms were still relatively sparse. At the time of the Cretaceous extinction around 65 million years ago, calcareous organisms were particularly hard hit by the extinction event (suggesting a drop in the pH of the surface ocean) and coccolithophores went almost completely extinct. The diatoms (and siliceous radiolarians) were affected very little by the Cretaceous extinction. As a result of the Cretaceous extinction event, diatoms appear to have been able to exploit the large decline in coccolithophores and occupy their resulting empty niches. Today diatoms, not coccolithophores dominate coastal and polar waters. Coccolithophores remain relatively important only in the temperate and tropical open ocean today.

MORPHOLOGICAL CHARACTERISTICS

Because all the different phyla of microalgae are subjected to similar selective environmental forces, there has been considerable convergent evolution in their sizes, shapes, and other morphological characteristics (15–17). Some of these can be seen in Figures 1, 2, and 3.

Cell Size

The eukaryotic phytoplankton range from around 2 to 2,000 μm in diameter. This translates to approximately a billion-fold range in cell volume. A common terminology for different size phytoplankton is shown in Table 2. Because phytoplankton are microscopic and thus outside the perception range of normal human experience, there is a tendency to think of phytoplankton as "little round green things" and to lump them all together. Translating that billion-fold size range to the human perceptual scales leads one to realize that this would be the same as lumping small weeds and redwood trees together as plants. In some situations this is appropriate, but in others, it is highly misleading. On this spatial scale, particles have Reynold's numbers well below 1 (generally 10^{-6} to 10^{-2}), resulting in viscous forces being much greater than inertial forces. As a result, the uptake of nutrients can be diffusion limited and the sinking rate increases with cell size. Both are important factors that influence the evolution of cell size in phytoplankton.

Phytoplankton are essentially surrounded by a sheath of stagnant water. Therefore, the transport of nutrients from the water to the surface of the algal cell is restricted by the rate of diffusion. In broad terms, the rate at which nutrients can diffuse to the cell surface is approximately proportional to the surface area, and the amount of nutrients needed for growth is approximately proportional to the volume of the cell. Therefore, the rate at which cells can accumulate nutrients and replicate their biomass is proportional to r^2/r^3 or $1/r$ (r is the cell radius). Small cells with a small radius clearly have an advantage over large cells with low Reynold's numbers where nutrients are a limiting factor (to be discussed in detail later).

Because planktonic algae are forced to drift with the water and thus are surrounded by stagnant water, the selective pressure of nutrient limitation leads to small cells. For this reason, the vast majority of phytoplankton in the ocean is only a few microns in diameter. Many are at the lower limits that are possible for a eukaryote, having only one chloroplast and one mitochondrion. An excellent discussion on the constraints and implications of minimum cell size in phytoplankton is given in reference 18. Phytoplankton are smallest in the subtropical waters of the open ocean in which nutrient concentrations are lowest, and are largest in upwelling areas in which nutrient concentrations are highest and sinking out of the photic zone is not a problem.

As there is enough light for photosynthesis in only the top 100 m or less of the ocean, it is important that phytoplankton have the ability to avoid excessive sinking. At such small Reynold's numbers, Stokes' law should predict the sinking rate of such particles in seawater (19,20). It would predict a sinking rate approximately proportional to the square of the radius. Actual experimental observations indicate a more linear relationship between cell size and sinking rate (19). In areas with strong thermoclines and fairly stagnant surface waters, such as the subtropical open ocean, sinking is potentially a major problem, so small cell size is advantageous. In upwelling areas, small cells drifting with the water will eventually be transported out of the nutrient-rich upwelling area. Upwelling tends to select for larger cells that sink at the same rate as the water

Table 2. Terminology for Cell Sizes (in μm)

Picoplankton	<2
Nanoplankton	2 to 20
Microplankton	20 to 200
Mesoplankton	200 to 2,000

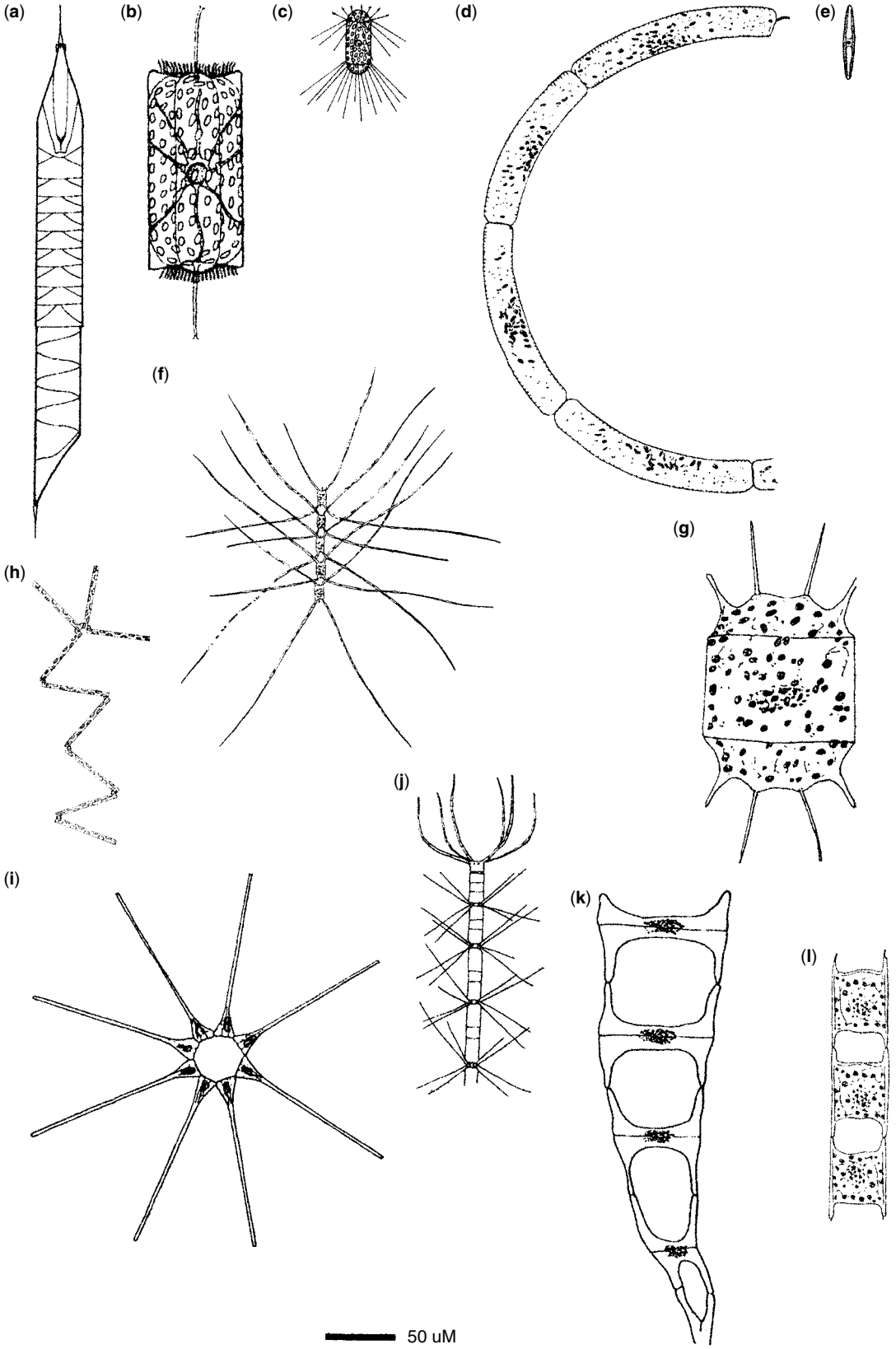

Figure 1. Diatoms. (**a**) *Rhizosolenia styliformis*; (**b**) *Ditylum brightwellii*; (**c**) *Corethron criophilum*; (**d**) *Guinardia striata*; (**e**) *Navicula directa*; (**f**) *Chaetoceros atlanticus*; (**g**) *Odentella mobiliensis*; (**h**) *Thalassionema nitzscioides*; (**i**) *Asterionellopsis glacialis*; (**j**) *Bacteriastrum elongatum*; (**k**) *Climacodium frauenfeldianum*; (**l**) *Hemiaulus sinensis*.

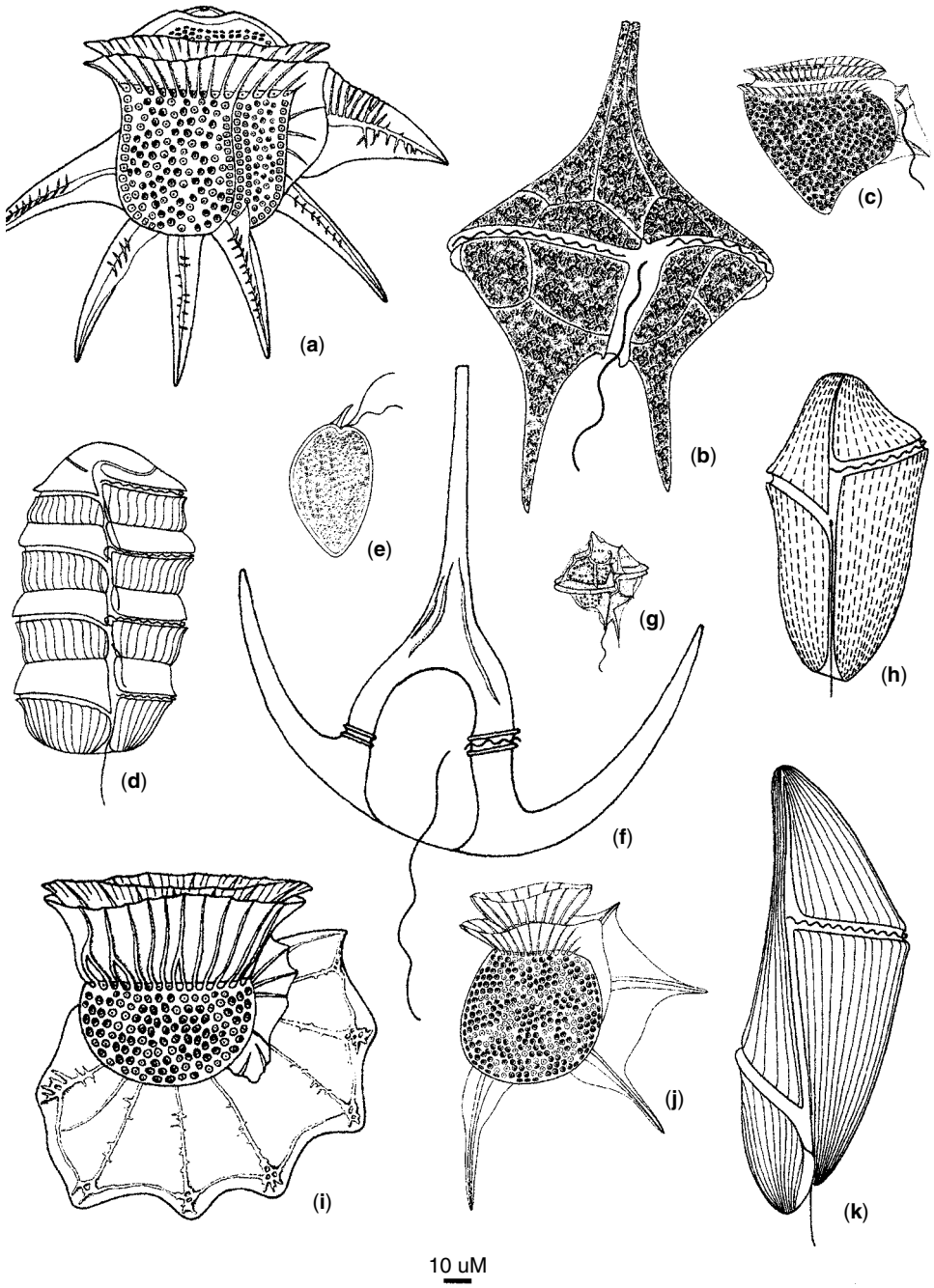

Figure 2. Dinoflagellates. (**a**) *Ceratocorys horrida*; (**b**) *Protoperidinium oceanicum*; (**c**) *Phalacroma rapa*; (**d**) *Polykrikos kofoidii*; (**e**) *Prorocentrum micans*; (**f**) *Ceratium tripos*; (**g**) *Pyrodinium bahamense*; (**h**) *Gymnodinium abbreviatum*; (**i**) *Ornithocercus steinii*; (**j**) *Dinophysis schuettii*; (**k**) *Gyrodinium spirale*.

upwells, leaving the cells in the nutrient-rich upwelling plume.

As a broad generalization then, phytoplankton communities are dominated by small species in areas with strong pycnoclines and low nutrient concentrations and by large species in turbulent water with high nutrient concentrations. As with most any generalization, there are significant exceptions. One can find very large diatoms and dinoflagellates in the open ocean and picoplankton in estuaries and upwelling areas. Their adaptations for surviving in these habitats despite their size will be discussed later. In most habitats, the entire range of phytoplankton cell sizes can be found, but there is a large shift toward a predominance of small cells in the nutrient-limited oceanic waters and toward a predominance of large cells in coastal waters and upwelling areas.

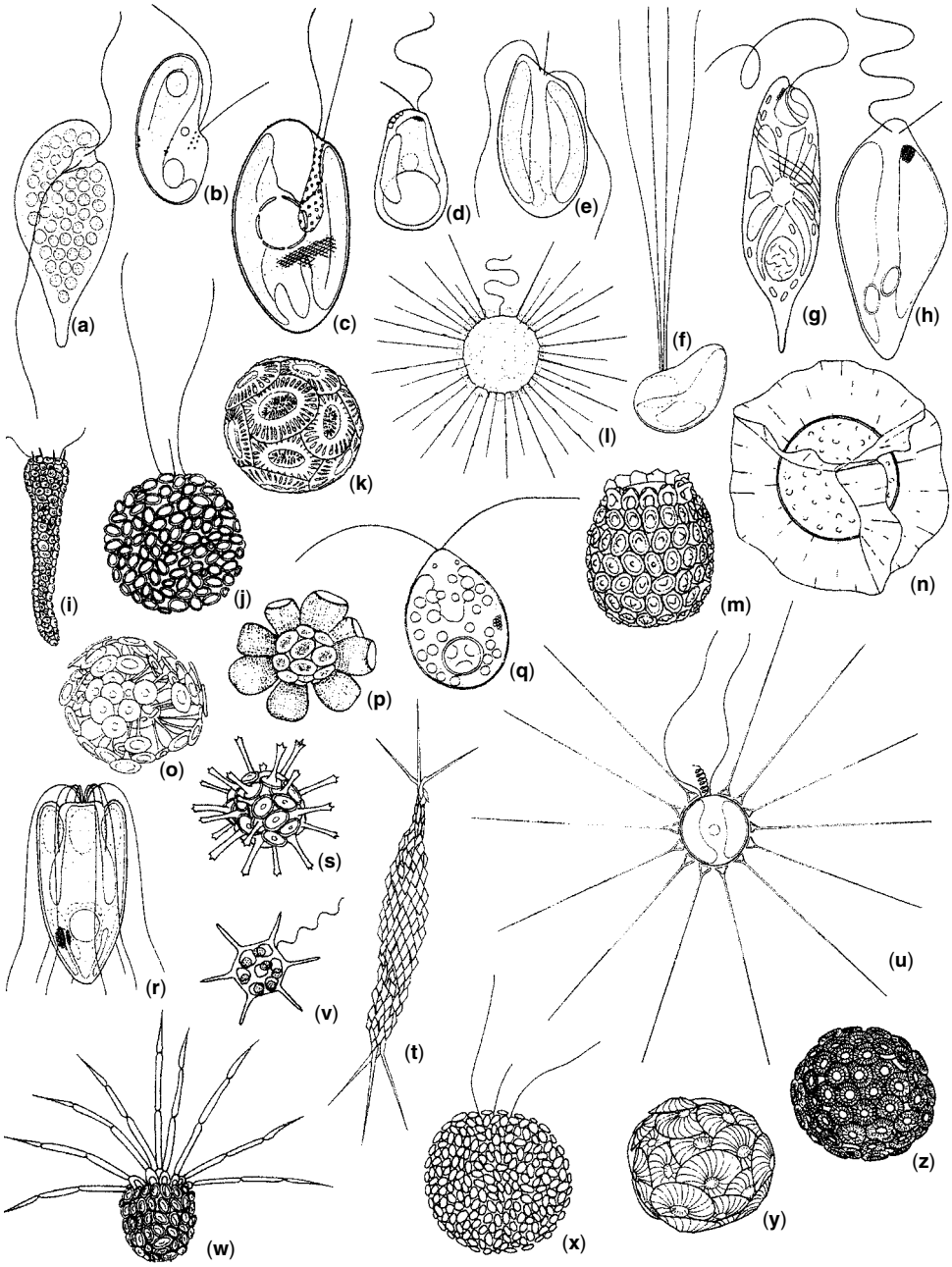

Figure 3. Flagellates. (**a**) *Chattonella subsalsa*; (**b**) *Hemiselmis virescens*; (**c**) *Rhodomonas salina*; (**d**) *Ochromonas bourrellyi*; (**e**) *Prymnesium parvum*; (**f**) *Pachyshaera pelagica*; (**g**) *Euglena viridis*; (**h**) *Pavlova gyrans*; (**i**) *Syracosphaera prologata*; (**j**) *Pleurochrysis carterae*; (**k**) *Emiliana huxleyi*; (**l**) *Parapedinella reticulata*; (**m**) *Zygosphaera hellenica*; (**n**) *Pterosperma vanhoeffenii*; (**o**) *Discosphaera tubifer*; (**p**) *Scyphosphaera apsteinii*; (**q**) *Dunaliella tertiolecta*; (**r**) *Pyramimonas octopus*; (**s**) *Rhabdosphaera claviger*; (**t**) *Calciosolenia murrayi*; (**u**) *Chrysochromulina hirta*; (**v**) *Dictyocha* sp.; (**w**) *Michaelsarsia elegans*; (**x**) *Crystallolithus hyalinus*; (**y**) *Calcidiscus leptoporus*; (**z**) *Umbilicosphaera sibogae*.

Cell Shape

Most phytoplankton are spherical or slightly oblong, particularly the smaller species. Species with flagella tend to be more oblong. A greater variety of shapes is observed in species with larger cell sizes, particularly diatoms and dinoflagellates. Diatoms can be shaped like petri dishes, cylinders, long rods, or rectangular plates. Many also have long spines protruding from them, which increase the surface/volume ratio and reduce the sinking rate. Dinoflagellates tend to have a variety of rather globular shapes, some with protuberances. Most are asymmetrical. Being the fastest swimming of the phytoplankton, they often have shapes that enhance swimming in one orientation and reduce sinking when in another orientation–thus the advantage of their asymmetrical shapes.

Colonies

Most phytoplankton exist as single cells, but some form colonies of various sizes. Among the smaller species, they often consist of typically two to eight cells, sometimes embedded in a gelatinous matrix, although in some cases, hundreds of cells can occur in one colony. Colonial diatoms and dinoflagellates tend to form linear chains.

Ultimately, it is thought that the sizes and shapes of various phytoplankton species have evolved primarily to solve the problems of diffusion-limited nutrient uptake, sinking, effective swimming, and grazing.

Cell Wall

Whereas some of the smallest phytoplankton have only a cell membrane and no cell wall, most have some type of cell wall. Most are made of various types of carbohydrates, particularly cellulose. It is usually a complex matrix of microfibrils. In some, such as dinoflagellates, it is composed of distinct plates of cellulose, the shapes of which can be used for taxonomic purposes. Many microalgae also surround themselves with a layer of gelatinous mucilage, the production of which often varies with environmental circumstances and physiological state. In the case of diatoms, the wall is heavily impregnated with silica, effectively providing a glass shell around the cell. The glass shell (known as a frustule) is in two halves that fit together somewhat like a petri dish (21). Upon cell division, each daughter cell gets half the frustule and synthesizes the other half. The distinctive patterns in these glass shells can be used for taxonomic purposes. Because these silica frustules preserve reasonably well in the fossil record, they have been very useful in paleoceanography for examining how the marine environment has changed over the history of the Earth. In the case of coccolithophores (a calcifying subgroup of prymnesiophytes), organic plates are covered with crystals of calcium carbonate in distinct patterns (called *coccoliths*). The distinctive patterns can be used to identify species, both in the water and in the fossil record. During cell division, each daughter cell gets approximately half the coccoliths. A number of different hypotheses have been proposed for the function of the coccoliths (22,23). Euglenophytes differ from most microalgae by having a very flexible cell wall composed of strips of protein. This allows them to constantly change shape.

A number of species, particularly dinoflagellates, chrysophytes, and raphidophytes, have structures called *trichocysts* embedded within their cell walls. They are used to inject mucus, "darts," or toxins into the environment or other organisms (15).

Flagella

Diatoms and rhodophytes do not have flagella (except the gametes of diatoms), but all the other phyla of microalgae have at least some species that have flagella. As a crude generalization, perhaps half of the microalgae in the ocean are capable of swimming, using flagella. Most have one or two flagella, but some can have more. When there are two or more, they usually emerge from the same part of the cell. A major exception to this is that many dinoflagellates have two flagella at right angles to each other. In many cases, one propels the cell through the water, while the other causes the cell to rotate. Most flagella are typically one to two cell diameters in length, but a wide range is found. Some flagella are simple and smooth, whereas others are covered with microfibrils called *mastigonemes*. These can be extended out from the flagella to increase surface area greatly (and thus friction and stickiness between the flagella and the water) or collapsed onto the surface of the flagella to reduce surface area. At the low Reynold's numbers of microalgae, friction between the cell surface and the water is the dominant factor, so complex interactions between mastigoneme movements and flagella movements are needed to move through the viscous media.

In addition to flagella, some prymnesiophytes have a flagellum-like structure called a *haptonema*, which can be very short or very long. It emerges from the cell between the two flagella. Long haptonema can form tight coils. In some species they are used for attaching to surfaces. In other cases, it is thought that the haptonema is used to capture particles for food.

Eyespot

A number of flagellates have an accumulation of pigments, often near the base of the flagella, known as an eyespot. Some can be quite complex in structure. It appears that the eyespot is used to determine the intensity and direction of light and then control phototactic behavior.

Vacuoles

The smallest phytoplankton may have only one small vacuole or even none at all. On the other hand, the largest diatoms usually have one very large central vacuole that occupies over 50% of the volume of the cell. Essentially, these diatoms have a thin layer of cytoplasm suspended on the inside of the glass shell, with the central core of the cell being one large vacuole. As a result, the ratio of surface area to cytoplasm volume is larger than in a cell without such a large central vacuole, thereby reducing the nutrient diffusion problem. In addition, these cells actively exclude heavy ions and accumulate light ions, so the vacuole ends up less dense than seawater. Because of the large volume of this less-dense vacuole relative to the whole cell, these large diatoms do not sink as fast as one might expect from Stokes' law and can even end up neutrally buoyant or even positively buoyant in seawater. High concentrations of nutrients can also be stored in the vacuoles, allowing cells to continue to grow and divide at times when external nutrients are not available.

Chloroplasts

Microalgal chloroplasts are found in a wide variety of sizes and shapes. Many of the smallest phytoplankton have only one chloroplast. Some large diatoms can have up to 50 or more chloroplasts spread throughout the cytoplasm on the inside of the frustule. Some large phytoplankton cells will have only two very large chloroplasts. Chloroplasts can be shaped as spheres, discs, bands, and stars. In many species, the chloroplasts change size and shape and can

migrate within the cells, depending on the light conditions. The pattern appears to expose more pigments under low-light conditions and expose less pigment under high-light conditions. Some diatoms even have chloroplasts that extend out into the their spines to avoid self-shading.

Storage Products

Distinct deposits of various carbohydrates, proteins, or oils can be found in many microalgae. These appear to be storage products that accumulate during unbalanced growth when there is a surplus of one resource and a deficit of another. These storage products can then be used at a later time when environmental conditions change.

Nucleus

In most cases, the nucleus of microalgae is similar to that of other eukaryotic cells. The euglenophytes and dinoflagellates differ in having their DNA organized in a rather primitive way. Because of the poor organization of the DNA in dinoflagellates, up to 50% of the volume of the cell can be occupied by the nucleus.

PHYSIOLOGICAL ADAPTATIONS

A large literature (24–27) on the physiology of marine phytoplankton exists.

Reproduction

Most of the time, microalgae reproduce by simple asexual binary fission. A wide range of division rates can be observed. As a broad generalization, dinoflagellates are the slowest, almost always doubling at less than once a day, and diatoms are the fastest with up to six divisions a day (a 64-fold increase in biomass in 1 day). Within a phylum, small cells, with their higher S/V ratio, tend to grow faster than larger cells.

A shift from asexual binary fission to sexual reproduction generally occurs when environmental conditions become unfavorable for growth. In general, it appears that the vegetative cells of diatoms are diploid, whereas most other phyla are haploid. In diatoms, meiosis precedes the production of gametes, whereas in the other microalgae, meiosis occurs after the fusion of two gametes have produced a diploid zygote. Some species are heterothallic, meaning that the gametes must come from different genotypes for the production of viable zygotes. Homothallic species have gametes that can fuse with gametes of the same genotype. Some species have isogametes, which appear identical to each other. Other species have anisogametes, the beginnings of the distinction between male and female gametes. In some cases, the anisogametes are of different sizes. Sometimes the male gamete has flagella and the female gamete does not.

Many species, particularly coastal species, have the ability to form resting stages, such as cysts. In some cases this simply involves the thickening of the cell wall and reduction of metabolism. In other cases, cyst formation is intimately involved in the life cycle and sexual reproduction. Germination of the cysts often requires quite specific environmental cues such as photoperiod or temperature changes. Because of the thick cell walls, cysts preserve better than vegetative cells and are better represented in the fossil record.

Control of Sinking

Typical sinking speeds of phytoplankton range from 0.1 to 100 m/day (19). Sinking is a double-edged sword for phytoplankton. Neutral buoyancy keeps phytoplankton in the photic zone indefinitely, and slow sinking rates keep them in the photic zone for long periods of time. Reduced or no cell movement relative to water, however, results in diffusion being the only mechanism for the transport of nutrients to the cell surface. If a cell sinks rapidly through the water, the diffusion boundary layer is reduced, enhancing nutrient uptake, but the cell is in danger of sinking out of the photic zone relatively quickly. Some species with large cells reduce this problem by having cell or colony shapes and orientations that cause them to spiral or zigzag horizontally through the water as they sink. This effectively diverts the inertial motion from vertical to more horizontal, thereby maintaining speed relative to the water and reducing diffusion limitation, but also reducing the net downward sinking out of the photic zone.

Sinking is not a major problem in upwelling areas in which the upwelling rate is greater than the sinking rate. Indeed, sinking can be advantageous, as it keeps the cells from being swept away as the upwelling waters reaching the surface then move out of the upwelling area and nutrients are depleted. Upwelling areas select for cells that sink at the same rates as the water moves upward and against neutrally buoyant cells that then get transported downstream. On a smaller scale, Langmuir circulation cells can also support and select for algae cells that sink rapidly. These vertical circulation cells are generated when persistent winds from one direction reach 3 to 6 m/seconds (28,29). Because cells spend more time in the upwelling plumes of Langmuir circulation cells than in downwelling plumes, large, rapidly sinking cells can be maintained in the photic zone indefinitely. In this situation, rapidly sinking cells are less affected by nutrient diffusion limitation than neutrally buoyant cells and end up with a competitive advantage. In these situations in which sinking is advantageous, the heavy silica frustules of diatoms and calcium carbonate coccoliths of coccolithophores are useful adaptations. When the winds die down or change direction, however, the Langmuir circulation cells collapse and large heavy cells begin sinking through the photic zone. As a result, competitive advantage shifts from rapidly sinking cells to neutrally buoyant cells. Therefore, variability in wind speed and direction can explain the relatively rapid changes in phytoplankton community composition and the wide distribution of cell sizes and sinking speeds in the ocean.

Phytoplankton cells do not sink at the rates predicted by Stokes' law. Large cells tend to have large vacuoles, which have a chemical composition different from that of seawater. Many large cells use energy to actually pump out heavy ions and accumulate light ions, resulting in a

cell vacuole in osmotic balance with seawater but having a lower density. Many large diatoms and dinoflagellates that would be expected to sink rapidly, based on Stokes' law, in fact can be neutrally buoyant or even positively buoyant as a result of this ion discrimination (30,31). It appears that some species use ion discrimination to change their density from positive to negative on a diel basis, allowing them to float up into the photic zone during the day for photosynthesis and sink down below the nutricline to take up nutrients at night.

Swimming

Many phytoplankton have flagella, which allow them to swim upward to counteract the results of sinking (32). Typical swimming speeds range from 0.01 to 3 cm/minute. They are able to detect the intensity and direction of light, resulting in phototactic behavior that appears to generate vertical distributions that, to some extent, optimize the ratio of light from above and nutrients from below (33,34). This phototactic behavior is thought to be one of the major reasons for the particularly high concentrations of phytoplankton near the bottom of the photic zone. Above this deep chlorophyll maximum, nutrients are limiting and light is in excess, whereas below, light is limiting and nutrients are in excess. This spatial separation of light and nutrients is a fundamental dilemma faced by phytoplankton in many parts of the ocean because the pycnocline tends to hold the colder, nutrient-rich water below the photic zone. Some large dinoflagellates solve this dilemma by swimming up into the photic zone during the day for photosynthesis and then down at night to take up nutrients. Some diatoms may use their vacuole to alter their buoyancy in the same way.

Adaptation to Temperature

Different species of algae can be found over the entire range of temperatures found in the surface ocean. Many polar species can grow at $-2\,^{\circ}C$, the lowest temperature at which seawater remains liquid. Eukaryotic algae have been found in hot springs up to $70\,^{\circ}C$ and are found in tidal pools that heat up considerably. Temperature affects which species live in various parts of the ocean, but does not restrict them from any parts of the photic zone (35–37).

Whereas the light reactions of photosynthesis are largely temperature independent, the dark reactions and most other biochemical processes are influenced by temperature. As a result, the enzyme and protein contents of algal cells tend to increase at lower temperatures to allow the biochemical processes to keep up with light harvesting. Conversely, the pigment contents of the cells tend to be greater at higher temperatures to be able to supply the higher demand of the dark reactions.

Overall growth of phytoplankton in response to temperature is similar to that of many organisms–a linear or exponential increase with increasing temperature up to an optimum, and then a dramatic decline with only a small further increase in temperature. Death often occurs only a few degrees above the temperature optimum. Most marine phytoplankton species do not survive above about $30\,^{\circ}C$. In polar waters, where the water never gets above around $2\,^{\circ}C$, many phytoplankton species die of excessive heat above $5\,^{\circ}C$. Conversely, many species survive quite well at temperatures far below their temperature optima.

Temperature and salinity determine the density of water, which in turn determines much of its movements in the ocean. Much of the ocean is characterized by fairly distinct water masses (with rather uniform temperature and salinity regimes within) with fairly sharp transitions at the boundaries between water masses. These boundaries tend to also be the major biogeographic (phytohydrographic) boundaries for phytoplankton species distributions, although primarily as a result of transport restrictions, not actual temperature restrictions.

Although phytoplankton grow faster at higher temperatures, on a global basis there is a negative correlation between temperature and phytoplankton photosynthesis and standing biomass. Phytoplankton biomass and productivity are lowest in the tropics and highest in polar regions, in general. The reason for this is that when surface temperatures are low, the thermocline separating nutrient-rich deep water and the photic zone is weak, allowing a large flux of nutrients into the photic zone to support high photosynthetic rates and algal biomass. In the tropics where surface temperatures are high, the thermocline is strong, thus restricting the flux of nutrients into the photic zone and greatly reducing photosynthesis and phytoplankton biomass.

Osmoregulation

Most of the surface ocean has a salinity between 33 and 37 parts per thousand (ppt), and local rainfall and evaporation does not cause it to fluctuate much. As a result, most marine phytoplankton tolerate only a relatively narrow range of salinities (are stenohaline), as they do not need adaptations to tolerate large changes in salinity. Close to the coastline, however, land runoff can cause a large decline in salinity, particularly in embayments. In estuaries, one can find the entire range from freshwater up to full strength seawater. In isolated or semi-isolated basins in arid areas, evaporation can generate hypersaline conditions. The most rapid changes in salinity can occur in shallow tidal pools. Obviously phytoplankton species that live in such habitats have to tolerate a wide range of salinities (are euryhaline) (38,39). The main method they use to maintain osmotic balance with seawater of varying salinity is to polymerize and depolymerize various polyol compounds such as glycerol, mannitol, sorbitol, or a number of other similar compounds (40,41).

Marine phytoplankton species that live in low salinity water generally are not stenohaline for low salinity, but rather are euryhaline and thus can tolerate high salinity as well. Down to around 5 ppt, the freshwater dilution of the seawater does not significantly change the ratios of ions found in seawater. Because the ratios of ions in freshwater are so different from those in seawater, the resulting ratios in water less than 5 ppt tend to be different from seawater and this apparently causes problems for most marine phytoplankton. Whereas a few species can grow as well in freshwater as in seawater, most grow very poorly or not at all in water less than around 5 ppt.

Adaptations to Light

A good overview of photosynthesis and phytoplankton adaptations to light can be found in references 2,42–48. Typically, the chlorophyll *a* content of phytoplankton cells is around 1% of the biomass of the cell, although this varies widely, depending on species and environmental conditions. Within the chloroplasts, photosystem II typically contains 200 to 300 chlorophyll molecules, along with various carotenoids (both carotenes and xanthophylls). It is here that photons are used to drive electrons off water, thereby generating molecular oxygen. The electrons are then transported to photosystem I, where photons are absorbed to boost the energy level of the electrons. Ultimately, these electrons are used to generate reduced nicotinamide adenine dinucleotide phosphate (NADPH) and adenosine triphosphate, which are then used in the dark reactions of photosynthesis to convert carbon dioxide to organic molecules. In addition to the standard C3 photosynthetic pathway, which starts with the carboxylating enzyme ribulose bisphosphate carboxylase, many algae use additional biochemical pathways for the conversion of inorganic carbon into organic compounds (49–51). For example, many phytoplankton have the enzyme phosphoenolpyruvate carboxylase, which uses the bicarbonate ion as its substrate. This pathway leads to the production of amino acids. Overall, the quantum yield of photosynthesis in phytoplankton is around 0.08 moles of carbon per mole of photons. This, of course, varies with species and environmental circumstances.

The relationship between photosynthetic rates and light intensity is typically shown as a "P versus I curve." As a broad generalization, net photosynthesis (total or gross photosynthesis minus respiration losses) is positive at light levels above approximately 1% of full sunlight. This of course varies with species and environmental conditions, and some polar microalgae have been found to grow at 0.01% of full sunlight. From this compensation point (where gross photosynthesis just compensates for respiratory losses), up to about 10 to 20% of full sunlight, the relationship between light intensity and photosynthetic rate is roughly linear, indicating light limitation. The slope of this line (designated as $\alpha = P/I$) is an index of photosynthetic efficiency. From 10 to 20% up to perhaps 50% of full sunlight, the P versus I curve is flat–increasing light does not increase the photosynthetic rate. At these light intensities, the biochemical reactions generating organic carbon (the dark reactions of photosynthesis) are the limiting step and photosynthesis is light saturated. Often above 50% of full sunlight, photosynthetic rates decline as a result of photochemical damage from UV radiation and the intense visible light (known as photoinhibition). The shape of the P versus I curves changes considerably among species as well as in response to other environmental factors such as nutrients and temperature.

Phytoplankton respond to changing light intensity with a variety of adaptations on various timescales. Some morphological and biochemical responses occur within seconds. The structure of the thylakoids can change within seconds. The size and shape of chloroplasts can change in minutes to maximize or minimize light absorption in dim or bright light (52). In many species, chloroplasts can migrate within the cell, dispersing in dim light to reduce self-shading and aggregating in bright light to increase shading (53,54). On longer timescales (hours), the amount of pigment changes and in some species the number of chloroplasts can change (55,56). In response to dim light, many algae increase the size of the photosynthetic unit, but some increase the number of photosynthetic units. Some carotenoids, such as violoxanthin and diadinoxanthin, are used to absorb light to protect against excessive light. Carotenoids and mycosporine-like amino acids are produced to protect against UV radiation.

Most algae have a variety of carotenoids (such as fucoxanthin or peridinin) which absorb light in the green and yellow region of the light spectrum, between the blue and red absorption peaks of chlorophyll *a* (57–59). These carotenoids absorb wavelengths of light that chlorophyll absorbs very poorly and transfer the energy to chlorophyll *a*. Chlorophylls *b* and *c* also absorb wavelengths longer than what chlorophyll *a* absorbs. These accessory pigments allow algae to absorb more light than they would with chlorophyll *a* alone. This is particularly important in dim light and at depth where, as a result of differential absorption of different wavelengths by water, the remaining light is of wavelengths that carotenoids and chlorophylls *b* and *c* can absorb better than chlorophyll *a*. As a result, the ratios of chlorophyll *b*/chlorophyll *a*, chlorophyll *c*/chlorophyll *a*, and carotenoid/chlorophyll *a* tend to be higher in dimmer light.

Many of the changes in algae in response to changes in light intensity are also observed on a diel basis (60). Chlorophyll content and capacity for photosynthesis are lower at night than during the day. This is not just a simple response to light intensity. Many of the photoadaptive responses are under the control of a biological clock (61). Because some of the adaptations take hours, this allows algae to prepare their photosynthetic apparatus ahead of time rather than simply responding to changes in light intensity and losing hours of light availability while adapting. This also keeps algae from responding to short-term fluctuations in light intensity. For example, it would not be advantageous to begin shutting down the photosynthetic apparatus in response to a brief drop in light intensity caused by a passing cloud or vertical mixing.

Nutrient Acquisition

As a broad generalization, the concentration of nutrients inside phytoplankton cells are typically about a million times greater than in the water in which they live. This indicates the volume of water out of which they must obtain nutrients to double their biomass for binary fission.

As nutrients are usually at very low concentrations in the ocean (62–65), most phytoplankton have uptake enzymes with very high affinities for the various nutrients (66–69). They have the capacity to store ten to a hundred times more nutrients in the cells than what they need for one cell division, a process known as luxury uptake (70,71). It is suspected that phytoplankton

experience a patchy environment on a microscopic scale, so excess stored nutrients from luxury uptake allows them to continue growing during times of limited access to external nutrients. For dinoflagellates that undergo daily vertical migration through the nutricline, luxury uptake allows them to take up and store nutrients while below the photic zone to utilize later after they have swum up into the photic zone. Growth rate has been found to then be related to the amount of stored nutrients in the cell.

Because bicarbonate is a major ion in seawater, marine phytoplankton are generally thought to not be carbon limited, although there is some evidence that carbon uptake could be diffusion limited (72). The two major nutrients that phytoplankton need that are usually in very low limiting concentrations are nitrogen (N) and phosphorus (P). Generally, phytoplankton have a molar elemental C/N/P composition of around 106 : 16 : 1, known as the Redfield Ratio (73). Upwelled deep water typically has inorganic C/N/P in ratios of around $1,100 : 15 : 1$ (74,75). A comparison of the two ratios indicates that the phytoplankton will run out of nitrogen first, and phosphorus soon after, but there is plenty of carbon. The conclusion is that nitrogen is the most important limiting nutrient in most parts of the ocean, with phosphate almost as limiting, and only about 10% of the carbon is taken up.

Upwelled water contains considerable amounts of nitrate but very little ammonia (64). Surface waters are depleted of nitrate by phytoplankton, causing a shift over to reliance upon ammonia as a nitrogen source. This ammonia comes from the decomposition of organic matter and excretion by animals, and is thus recycled nutrients within the photic zone. The organic matter that is produced by phytoplankton utilizing upwelled nutrients (primarily nitrate) is called *new production* because it is supported by nutrients new to the photic zone ecosystem (76,77). As these new nutrients come from outside the ecosystem, they lead to a net increase in biomass. The organic matter produced by phytoplankton using nutrients (mostly ammonia) recycled from heterotrophic activity in the photic zone is called *old production*, as it is supported by old nutrients that have already been in the photic zone ecosystem for awhile. This old production does not lead to an increase in biomass in the ecosystem because it relies upon old recycled nutrients that are derived from the decomposition of old biomass. Because phytoplankton must use NADPH to reduce nitrate to the redox level of ammonia for incorporation into amino acids, they preferentially take up ammonia until it is depleted before using nitrate. Whereas some phytoplankton can take up amino acids and other organic nitrogen compounds, it appears they are not very good at outcompeting heterotrophic bacteria for organic compounds at the low concentrations found in most seawater. The direct C–N bond in most complex organic molecules generally makes them unavailable to most phytoplankton as a nitrogen source.

Whereas eukaryotic algae are not capable of nitrogen fixation, some oceanic diatoms and dinoflagellates with large vacuoles have cyanobacteria inside. It is suspected that these cyanobacteria fix nitrogen and transfer some of the fixed nitrogen to the host diatom or dinoflagellate to help maintain a symbiotic relationship.

Both old (recycled) and new phosphorus are in the same redox state, PO_4^{-3}. In addition to inorganic phosphorus, however, many phytoplankton have phosphatase enzymes that can cleave the ester bond of organic phosphorus compounds (78,79). It appears that phytoplankton are capable of utilizing a large percentage of the organic phosphorus found in water.

Diatoms need as much silica (for their frustule) as nitrogen, whereas other groups of phytoplankton need little or no silica (80). This puts diatoms at a competitive disadvantage relative to other phytoplankton in areas that are low in silica. Silica is different from other nutrients in that it cannot be stored at high concentrations inside the cell to any great extent like the other nutrients because of autopolymerization problems. This forces diatoms to take up silica only at the time of frustule formation, which takes place primarily around the time of cell division.

Quantitatively, the next most important nutrient is iron, which is used in a wide variety of enzymes, particularly those involved in energy transfer and redox reactions (81,82). The amount (by moles) needed is around 1/100 to 1/10,000 that of phosphorus (83). It appears that iron is a primary limiting nutrient in some parts of the ocean. Other trace elements such as manganese, zinc, cobalt, copper, and selenium are needed in smaller amounts and may be limiting is some circumstances (84,85). In many situations, phytoplankton are faced with multiple nutrient limitation, as the ratios of nutrient requirements appear to be similar to availability ratios, as seen in the N/P ratios. It is less clear with trace metals, however, because many trace metals appear to be strongly complexed by organic molecules, making them unavailable to phytoplankton. At the same time, it appears that some trace metals can substitute for each other. It appears that in many parts of the ocean, a number of nutrients may be approximately equally limiting.

Coastal phytoplankton have higher requirements for NO_3, NH_4, PO_4, Si, Fe, Zn, and Mn than oceanic species, which corresponds to the higher concentrations of these nutrients found in coastal waters (84,86,87). This indicates that oceanic species have adapted to their low nutrient environment by increasing their efficiency of utilizing nutrients at low levels.

Approximately half of the marine phytoplankton species appear to need at least one vitamin (88). The one needed by the most species is vitamin B_{12}, although a number of species need thiamine or biotin. These vitamins are produced and excreted by various biota in the ocean, but it is not clear if their concentrations are low enough to limit the growth rate of some species. As many species do not require any vitamins, low vitamin concentrations in the ocean could only alter species competition and community structure, not overall primary productivity.

Paradox of the Plankton

One interesting aspect of marine phytoplankton communities is their high diversity (89). Unlike animal species, which have different diets, and terrestrial plants, which obtain nutrients from different patches of soil, millions

of phytoplankton cells representing hundreds of species can be found living in 1 L of water in the open ocean. Because all the species have essentially the same nutritional requirements and they are competing for the same nutrients in that liter of water, the question arises why does one species not eventually grow faster and outcompete all the other species for those nutrients. Considering the competitive exclusion hypothesis and the concept of niche partitioning, it is difficult to understand how so many species can all exist in a small, well-mixed parcel of water. This question is known as "The Paradox of the Plankton." Some hypotheses emphasize the variability of the environment as a mechanism for maintaining coexistence (90), but the highest diversity of marine phytoplankton is found in the tropical open ocean where environmental variability is lowest and the nutrients they are competing for are at their lowest concentrations. Phytoplankton species diversity is lowest in estuaries where nutrient concentrations are high and the environment is highly variable. This paradox of the plankton illustrates how much we probably do not know about the subtle relationships among marine phytoplankton species.

Avoidance of Grazing and Microbial Attack

Herbivores (insects, crustaceans, etc.) on large plants on land or macroalgae in the ocean generally are considerably smaller than their prey and take only small parts of the plant away. Because the plant survives to be able to propagate its genes in the future, there is a selective advantage for large plants to produce toxins or other noxious compounds that reduce grazing and thus biomass loss. In the case of microscopic unicellular algae, their grazers are much larger than them and actively consume large numbers of phytoplankton cells of a variety of species at a time. Any noxious or toxic compounds produced by one cell can hardly have much effect on a large herbivore. If it does have an effect, it will reduce grazing (on all the species, not just the toxic species) only after that toxin-producing cell has already been consumed and thus unable to propagate its genes. Therefore, there is no selective advantage for microalgae to produce toxins. Most of the toxins known to be produced by phytoplankton are toxic to fish, mammals or other organisms, not their herbivores. Those toxins appear to be produced for some other reason. The end result is that phytoplankton have relatively few options for deterring grazing. Their main strategy appears to be to outgrow their grazers.

Cell size can help avoid being grazed by some animals but not others (91,92). Crustaceans can typically filter cells larger than 5 to 10 μm, so smaller cells can avoid those grazers (93). Large cells can avoid being eaten by the small abundant protozoans. Any size cell may be able to avoid some groups of grazers but is vulnerable to another group.

Spines and long chains of cells may block the feeding mechanisms of some grazers. Some species, particularly those covered with mucilage may be able to survive gut passage, indeed profiting from being in such a nutrient-rich environment.

Some species of dinoflagellates are capable of producing flashes of bioluminescent light when disturbed. There is evidence that these flashes reduce predation (94,95).

Bacteria are known to attack and consume phytoplankton, but it is not known if they can attack healthy cells or only weakened or dying cells. Large numbers of viruses are found in seawater and some are known to be able to lyse algal cells. At the present time, however, not much is known about the quantitative role bacteria and viruses play in phytoplankton population dynamics. Bacteria and viruses can be found on the surface of cells, in their vacuoles, and in the cytoplasm, but their roles as symbionts, commensals, or parasites are not well known (96,97).

Toxins

Whereas the vast majority of marine phytoplankton are not toxic, a few species, primarily dinoflagellates, are known to produce toxins (98). Under certain circumstances, these species can produce large dense blooms, which can lead to the death of fish and others organisms in the food chain. In some cases, the toxin ends up in seafood, leading to human sickness and/or death (see RED TIDES AND OTHER HARMFUL ALGAL BLOOMS, this Encyclopedia).

ECOLOGICAL DISTRIBUTIONS

Biogeography

There is a large literature on the distribution of phytoplankton species, but references 99 to 104 provide an overview. Because the plankton are constantly being moved around by surface currents, and environmental conditions are reasonably uniform over large areas of the ocean, most marine phytoplankton have rather cosmopolitan distributions, much more so than benthic or terrestrial organisms. The four major biogeographic (or more properly, phytohydrographic) boundaries in the ocean that separate different phytoplankton communities are the coastal-oceanic boundary, the Antarctic Convergence, the latitudinal thermal gradients, and the north-south–trending continents.

The strongest and oldest phytohydrographic boundary is the coastal-oceanic one at approximately the shelf break where the continental shelf (typically around 100 m deep) ends and the bottom drops rapidly to the deep sea (typically around 4,000 m deep). This boundary is geologically formed, thus quite ancient. Very few phytoplankton species live in both coastal and oceanic waters. The oceanic habitat is characterized by a strong pycnocline, thus low nutrient concentrations in the photic zone. The water is also quite transparent, leading to a deep photic zone. A large number of mechanisms help weaken any pycnocline that develops in coastal waters, so they tend to have much higher concentrations of nutrients. Coastal waters also tend to have higher concentrations of dissolved organic matter, detritus, resuspended sediments, and planktonic biomass, leading to a much shallower photic zone. The much shallower water column and proximity to terrestrial weather systems also lead to environmental conditions in coastal waters changing much more rapidly. On a longer timescale, one has to consider that the continental shelves alternate between terrestrial and shallow marine ecosystems as sea level changes over time. Our present continental shelves have been flooded only for

the past 5,000 to 15,000 years. Species that populate the continental shelves have to be adapted to survive in other refuges when sea level is low and the shelves are dry land. Apparently, estuaries and not the oceanic habitat serve as refuges for coastal phytoplankton during times of low sea level. Many coastal species also live in estuaries but not in the open ocean.

The Antarctic Convergence is the second strongest phytohydrographic boundary separating phytoplankton communities. It developed around 30 million years ago as the Antarctic continent separated from the other continents and drifted to the South Pole. This allowed the Circumpolar Current to develop, which effectively isolates the Southern Ocean from the rest of the world. Over 50% of the species in the Southern Ocean are endemic. Relatively few species are found in both the Antarctic and Arctic because the two ecosystems are in fact quite different from each other in many ways.

Also important are the latitudinal thermal gradients found in the ocean today. The ocean today is divided into equatorial regions, subtropical gyres, subpolar gyres, and polar regions, each with their own distinctive phytoplankton communities. These latitudinal thermal gradients have been strengthening over the last few million years, making them even more important phytohydrographic boundaries.

Of lesser importance are the continents that separate the Atlantic from the Pacific and Indian oceans. In the past, a Tethyan Seaway connected all three oceans near the equator and phytoplankton species lived in all three. About 3 million years ago, tectonic activity generated the Isthmus of Panama, separating the Atlantic from the Pacific. Most phytoplankton species still live in all three oceans, but genetic divergence is developing as a result of the spatial separation today.

Invasions

Species distributions observed today cannot be regarded as being completely natural. Phytoplankton are transported around the world in the ballast tanks of ships. In a few cases, it has been documented that phytoplankton species had apparently never lived in a certain part of the world and then suddenly appeared and proliferated (105). It is suspected that transport in ballast tanks is the cause (106).

Seasonal Succession

One observes quite strong seasonal succession of species in temperate and polar waters (107). In polar waters, this is primarily the result of the large seasonal change in light availability–light intensity, day length, and winter winds and lack of a pycnocline causing mixing of the phytoplankton below the photic zone. In temperate regions, there are dramatic changes in species composition as the environment shifts between the winter and summer. In the winter, the pycnocline is weak or gone, thus nutrient concentrations are high, but the phytoplankton are getting mixed to great depths. In the summer, the pycnocline is strong, so nutrient concentrations are low, but the phytoplankton remain in the photic zone and light intensity is high and day length is long. This strong seasonal gradient between light limitation in the winter and nutrient limitation in the summer generates quite strong successional sequences of phytoplankton species.

EFFECTS ON BIOGEOCHEMISTRY

As marine phytoplankton are responsible for over 95% of the photosynthesis in the ocean as a whole, they are a major component of the biogeochemical cycles in the ocean and indeed the planet. They take up carbon dioxide, turn it into particulate carbon, which then sinks below the pycnocline into the deep sea, where it decomposes back into carbon dioxide (108). The residence time of this carbon dioxide–rich deep water is around a 1,000 years before it upwells back to the surface, releasing the excess carbon dioxide that has accumulated over this time span back to the atmosphere. The net effect of this biological pump (109,110) is to transfer large amounts of carbon dioxide from the atmosphere into the deep ocean, where it is stored for around 1,000 years. If this biological pump and resulting carbon dioxide disequilibrium, driven by phytoplankton, was stopped, atmospheric carbon dioxide would be around three times higher. As carbon dioxide is a greenhouse gas, the pumping of carbon dioxide out of the atmosphere by phytoplankton and storing it in the deep sea effectively cools off the Earth's climate.

Phytoplankton may also be affecting the Earth's climate in another way. Many species, particularly prymnesiophytes and dinoflagellates, produce dimethylsulfonium propionate, which through one of several mechanisms ends up as the gas dimethyl sulfide (111). This gas emerges from the ocean to ultimately form aerosol particles that enhance cloud formation over the ocean (112). Thus it appears that phytoplankton enhance the production of clouds, which reflect sunlight back out into space, thus cooling the earth's climate. Phytoplankton also produce a number of other gases that emerge from the ocean and affect the atmosphere's chemistry.

Phytoplankton modify their environment in other ways as well. Many phytoplankton produce organic compounds that specifically bind various trace metals, completely changing their chemical behavior (81,82). As one example, without this biological complexation by phytoplankton exudates, copper concentrations in the ocean would be high enough to be quite toxic to most phytoplankton. By producing specific compounds that tightly bind up this copper in the photic zone, phytoplankton have effectively detoxified their environment (113,114).

Phytoplankton take up not only carbon dioxide and nutrients, but also inadvertently many metals and other elements, along with pollutants. The same biological pump that transports carbon dioxide and nutrients into the deep ocean also transports these other materials as well (65). As a result, marine phytoplankton play a major role in most biogeochemical cycles in the ocean.

The production of silica frustules by diatoms and calcarious coccoliths by coccolithophores and the subsequent

sinking of the mineral hard parts and burial of a fraction of these materials in the sediments are important components of the biogeochemistry of these elements.

Eutrophication

As a result of the large increase in the human population and the associated runoff of sewage and agricultural fertilizer into coastal waters and atmospheric input of nitrogen from the combustion of fossil fuels, nutrient concentrations have increased in many coastal waters, particularly in the past century (115–117). This has led to increases in overall phytoplankton biomass and significant changes in phytoplankton species composition. In some cases, this also results in oxygen depletion, harmful algal blooms (including toxic species), alteration of the entire food web, and widespread death of fish and other animals. It is widely recognized that this problem is increasing over time.

PRODUCTS FROM MARINE MICROALGAE

One of the most widespread uses of marine microalgae is as food for animals in the aquaculture industry. Large amounts are fed to the larval stages of shrimp, clams, oysters, and other species in hatcheries (118,119). Wide variation exists among phytoplankton species as to which species are suitable for food. The industry has settled upon relatively few species that are widely used by many hatcheries. More research would surely identify more species that are suitable or perhaps even better foods.

Although marine microalgae produce quite a few useful biochemical compounds, they are not exploited much because of the difficulty and expense of harvesting microbes from water (120,121). One product from marine microalgae that is commercially viable is beta-carotene, which is produced in large quantities by certain green algae, and is used as a food coloring. Because of their long evolutionary history, phylogenetic diversity, and divergent biochemistry, one might expect microalgae to be a good source of antibiotics and other pharmaceuticals. This possibility remains relatively unexplored.

METHODS

Biomass

For the estimation of overall phytoplankton biomass in the ocean, the most widely used technique is to filter the water and measure the amount of chlorophyll *a* on the filter (122). Chlorophyll *a* concentrations are measured by extracting the pigment with a solvent (usually acetone or methanol) and measuring chlorophyll fluorescence with a fluorometer (the most sensitive method) or absorbance at a number of wavelengths using a spectrophotometer. The advantage of this method is that chlorophyll *a* is specific to algae (prokaryotic and eukaryotic). The disadvantage is that the ratio of phytoplankton biomass to chlorophyll *a* is variable, typically 25 to 150. Another approach is to actually count individual cells under a microscope and estimate the biomass from cell size. Not only is this laborious, but many cells cannot be easily seen by light microscopy and many cannot be easily distinguished as being either autotrophic (containing chlorophyll *a*) or heterotrophic. The presence of chlorophyll *a* readily and specifically identifies organisms as being photosynthetic. Low levels of chlorophyll cannot be easily detected microscopically, but can be measured fluorometrically.

Primary Productivity

The primary method of measuring primary productivity (rate of photosynthesis) in the ocean is to incubate a parcel of water with ^{14}C-labeled bicarbonate added (122). Because the concentration of bicarbonate in seawater is high, the added radioactive bicarbonate does not change the concentration or perturb the system or alter uptake kinetics. After a period of time, the water is filtered and the radioactivity remaining on the filter (presumed to be inside the phytoplankton cells as organic carbon, whereas unused bicarbonate has passed through the filter) is measured with a scintillation counter. An alternative, less-sensitive method of estimating photosynthetic rates is to compare oxygen concentration changes in incubated illuminated and dark bottles and/or observed diel changes in oxygen (see PRIMARY PRODUCTIVITY IN THE MARINE ENVIRONMENT).

Phytoplankton Cultures

Many phytoplankton species can be isolated from the ocean and grown in culture media as pure cultures (123). Because most reproduction by phytoplankton is by asexual binary fission, one can pick up a single cell in a micropipette and inoculate it into sterile culture media, and it will develop into a genetically pure clonal culture. This allows researchers to study the ecological requirements of individual species under controlled conditions as well as generate specific products produced by individual species. Cultures of many species can be obtained from various culture collections around the world.

CONCLUSIONS

Planktonic algae are found virtually everywhere in the surface layer of the ocean where at least 1% of sunlight reaches. Despite having similar (autotrophic) nutritional requirements, the planktonic algae are extremely diverse phylogenetically, distributed among ten or more different phyla. They also have diverse morphologies, with diameters ranging from one to a thousand microns. Phytoplankton provide almost all the organic carbon produced by photosynthesis in the ocean and thus provide the base of the entire marine food web. Being the dominant producers of oxygen and consumers of carbon dioxide and nutrients, planktonic algae have a major influence on the biogeochemical cycles of the earth and even the climate. Despite being the dominant autotrophs in the ocean, their standing biomass is low. The major factor limiting phytoplankton growth in the ocean is nutrients, primarily nitrogen and phosphorus, but also sometimes

iron. The combination of their growth being limited by sparse nutrients and strong grazing pressure by heterotrophs keeps the standing biomass of plankton algae low in the ocean, which is why most of the ocean is blue, not green. Despite this low biomass, planktonic algae harvest a large fraction of the solar energy used in the earth's ecosystems and they play a major role in the biogeochemical cycles and energy budget of the planet.

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PLANKTONIC MICROORGANISMS: BACTERIOPLANKTON

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Bacterioplankton are microscopic prokaryotic organisms occurring in the water column of aquatic ecosystems, including rivers, lakes, and the ocean. They are modern representatives of the oldest forms of life on Earth, dating back to as much as 3.5 billion years ago (1–3). As one of the most abundant types of organism on Earth (4–6), bacterioplankton occur wherever water exists, from geothermal springs to the polar ocean, in abundances that often exceed one billion cells per liter. As a group they are highly diverse in species, modes of energy acquisition, tolerance of oxygen and other chemicals, substrates consumed, and chemical compounds they make and release into the environment. The high abundance and ubiquity of bacterioplankton, combined with their metabolic diversity, make them critical to the movement and transformation of naturally occurring and anthropogenic materials, including many pollutants, on local, regional, and global scales. Their activities in the cycles of carbon, nitrogen, phosphorus, sulfur, and other elements directly influence the chemistry and water quality of oceans and lakes, and has a profound effect on the chemistry of the atmosphere, the temperature of the planet, and ultimately the overall state of the biosphere (7).

Especially in the last two decades, enormous progress has been made in understanding bacterioplankton, but there remain many questions regarding their phylogenetic diversity, population dynamics, adaptability to environmental change, interactions with each other and other organisms, orientation in microspace, physiological activities in nature, and how they structure the physical and chemical environment of aquatic ecosystems through their behavior and activities. With the imaginative application of a variety of new tools to measure bacterioplankton community composition and activities in natural environments, complemented by traditional approaches of studying microorganisms under controlled conditions, understanding of the nature of the bacterioplankton will surely continue to expand in the coming decade (8,9).

This article begins with a general description of bacterioplankton, followed by a discussion of their nutritional modes, and an overview of recent research regarding the taxonomic diversity of the bacterioplankton community. Next, the ecology of bacterioplankton is discussed in detail with an emphasis on factors controlling abundance, growth rates, and biomass production. Examples are provided from both marine and freshwater environments. After that, roles of the bacterioplankton in the cycles of carbon, nitrogen, and phosphorus are explained. The article concludes with a listing of current major research questions regarding bacterioplankton. Recent texts providing excellent overviews of various aspects of bacterioplankton biology include Ford (10), Fenchel and coworkers (11), Cooksey (12), Kirchman (13), and Wetzel (14).

PLANKTON

Plankton comprises a diverse group of organisms common to almost all surface-water ecosystems. Organisms are defined as being plankton according to their size and where they live, rather than taxonomically. Planktonic organisms generally are less than a few millimeters in length and live in open water. Although many species of plankton are capable of self-propelled mobility, because of their small size planktonic organisms are dispersed principally by water movement. Depending on the environment, the plankton community of an aquatic ecosystem may include phytoplankton (microscopic photosynthetic organisms, both prokaryotic and eukaryotic), zooplankton (animal plankton), protozooplankton (protozoan plankton), bacterioplankton (prokaryotic plankton), and viruses. In the photic region of most aquatic ecosystems, these types of organisms occur together.

In addition to a classification based on taxonomy, planktonic organisms may also be categorized according to size (15). Organisms larger than about 200 μm (1 μm is a millionth of a meter) in longest dimension belong to the macroplankton. Macroplankton are primarily zooplankton, and may be visible to the naked eye. But the vast majority of planktonic organisms are microscopic. Microplankton are organisms between 20 and 200 μm in length and consist of some animals, unicellular and colonial phytoplankton, and some protozoa. Nanoplankton are between 2 and 20 μm in length and consist mostly of small phytoplankton and protozoa. Organisms between 0.2 and 2 μm in length are called picoplankton, and consist mostly of bacterioplankton.

BACTERIOPLANKTON

The three main lineages, or domains, in which life is classified are Archaea, Bacteria, and Eukarya. Organisms are assigned to one of these three domains on the basis of gene sequences, cell structure, and in some cases, physiological properties. The Eukarya consist of organisms having a complex cell structure including a membrane-bound nucleus containing DNA in linear chromosomes, and other membrane-bound organelles such as mitochondria and chloroplasts. Archaea and Bacteria consist solely of prokaryotic organisms. Typical features of prokaryotes are a rigid cell wall, the absence of membrane-bound organelles, and a single unenclosed, usually circular, chromosome of DNA loosely bound in an aggregated mass called a *nucleoid*. Reproduction of prokaryotes is by *binary fission*, which is essentially the division of a single "parent" cell into two cells that are genetically copies of the parent cell. Besides having unique genetic properties, Archaea and Bacteria differ in the composition of cell membrane lipids, cell wall structure, and other features (3). The domain Bacteria consists of at least a dozen major divisions that, on an evolutionary basis, are more distant from each other than are animals and plants (3,16). The Archaea consist of fewer groups, although these are still widely distant.

The bacterioplankton consist of organisms in both the domains Archaea and Bacteria. They are the most

abundant organisms in the plankton (17). They occur wherever there is water, with abundances commonly between 0.5 and 5 billion cells per liter. Individual cells usually have the shape of straight or curved rods, spheres, or short filaments. Many bacterioplankton, perhaps most, are capable of motility by one or more flagella (18), allowing them directed movement toward or away from chemicals or other stimuli. They occur in the water column of aquatic environments as single free-living cells, attached to microscopic particles, or as colonies of attached cells. Most bacterioplankton are free-living, but where particulate material is in high concentration, as for example, following an algal bloom, bacterioplankton attached to small particles increase in number and their proportional contribution to the total bacterioplankton community (19). Individual free-living cells are typically less than 2 μm in diameter, with cell volumes between 0.02 to 0.1 μm^3. Attached cells can be as much as 10 times larger (11). Some colonies, especially those of the phototrophic cyanobacteria, may exceed a millimeter in the largest dimension. Many cells that appear to be "free-living" by microscopy may, in fact, be attached to a gel matrix of dissolved organic material, or to the cell surface mucous layers of other microbes (20).

Until the discovery that there are two distinct branches of prokaryotic life, all prokaryotes were referred to simply as *bacteria*. Often, the term *bacteria* is still used to refer prokaryotes as a group, but this usage can be confusing and will be avoided in this article. Instead, where prokaryotes are referred to generically, the term *prokaryote* will be used. Although it obviously is a holdover from prior usage, the term *bacterioplankton* makes no distinction between prokaryotes of the domains Bacteria and Archaea.

THE PHYSICOCHEMICAL ENVIRONMENT OF AQUATIC ECOSYSTEMS

The ecology, metabolic activity, and global significance of bacterioplankton must be considered in the context of their habitat. Bacterioplankton occur in all natural water bodies, including rivers, streams, lakes, and ocean. The ocean is by far their largest habitat, covering about 71% of the surface of the globe, and encompassing about 98% of all water in the biosphere (14). Inland water systems, including rivers, freshwater and saline lakes, comprise the other major habitats of bacterioplankton. Although they contain less than 0.01% of the water in the biosphere, these freshwater systems are major sources of water consumed by humans and other terrestrial animals, and their microbiology is of great interest.

As habitats for bacterioplankton, the ocean and inland water systems vary in substantial ways. The ocean has a salinity generally between 30 to 35 parts per thousand (ppt), and an average depth of 3,800 m, with a maximum depth of about 10,000 m. The surface mixed layer of ocean (the depth mixed by the wind) extends to perhaps 300 m, or about 2% of total ocean volume. At depths greater than about 1,000 m, the ocean is permanently cold, with a temperature between about −2 °C and 5 °C. This portion of the ocean, which is the deep ocean, is the largest habitat on earth. In the clearest parts of the ocean, light penetrates to about 100 m; below this depth it is aphotic (without light). At sea level, the pressure is 1 atmosphere (atm.), increasing by about 1 atm. for every 10 m depth. Because of deep mixing and downwelling currents, anoxic (without molecular oxygen) regions generally do not occur in most regions of the sea except in microenvironments such as within sedimenting particles or the guts of animals. However, in sediments, and in coastal environments receiving a high flux of organic matter, such as Chesapeake Bay or the Gulf of Mexico, hypoxia (low oxygen) or anoxia can develop in deeper portions of the water column. Most of the ocean away from coastal regions, that is, the "open ocean," is very low in dissolved organic matter, phosphorus, iron, and other nutrients, and considered oligotrophic (low production) in terms of biomass production. As discussed later, latitudinal variation in temperature, transparency, mixing depth, nutrient concentrations, and salinity may significantly influence spatial patterns in bacterioplankton community composition and activities in the sea.

There are many diverse inland water systems including freshwater lakes, saline lakes, temporary ponds, and running water systems of various kinds. Lakes and inland seas cover about 2% of the surface of the earth (14). Of these systems, approximately 45% of the inland surface waters of the world are saline, including the Black Sea and the Caspian Sea. On a volumetric basis, surface freshwater is concentrated in about 250 large lakes, which together contain about 75% of the volume of inland surface water; one of the most prominent of these large lakes is Lake Baikal of Siberian Russia, which alone contains approximately 20% of the worlds surface freshwater. However, the vast majority of lakes are small and relatively shallow, usually less than 20 m deep. Running water constitutes only about 0.1% of the land surface of the earth.

Across a latitudinal gradient, inland waters exhibit a wide range in temperature, mixing patterns, organic matter, nutrient concentrations, depth, and light penetration. Especially in temperate regions, seasonal changes in some of these physicochemical properties also occur. Depending on the geology of the watershed, and the hydrology of the basin, the salinity of freshwater lakes and running water ranges from very low (about 0.5 ppt) to higher than the ocean. Nutrient and dissolved organic matter concentrations also range widely, but because of the influence of the land, are generally higher than in the open ocean. Generally more turbid than the ocean, photosynthetically active radiation in lakes may penetrate to no more than about 10 to 20 m, sometimes much less, and thus a significant portion of the water column of many lakes is aphotic. Water temperatures of lakes vary with latitude, season, and depth from 0 °C, the temperature at which ice forms in freshwater, to higher than 40 °C in some areas. Because freshwater density decreases from 4 °C to 0°C, ice floats and, except where it is very shallow, lakes do not freeze to the bottom during winter. During summer, the water temperature of most lakes declines with depth, except in tropical and polar regions, where lake temperature may vary little with depth. This vertical temperature gradient,

Table 1. Terms to Describe Mechanisms of Energy Conservation and Carbon Metabolism of Living Things. As a Group, the Bacterioplankton Are Capable of All the Metabolic Modes Shown. Terms in Parentheses Are Widely Used Shorthand Versions of the More Cumbersome Full Descriptions

Metabolic Mode	Energy Source	Electron Source	C Source
Photolithoautotroph (Photoautotroph)	Light	Inorganic chemicals (e.g., H_2O, H_2S, S^o)	CO_2
Photoorganoheterotroph (Photoheterotroph)	Light	Organic chemicals	Organic C
Chemolithoautotroph (Chemoautotroph)	Chemicals	Inorganic chemicals (e.g., NH_4, H_2S, S^0, H_2)	CO_2
Chemoorganoheterotroph (Chemoheterotroph) (Heterotroph)	Chemicals	Organic chemicals	Organic C
Chemolithoheterotroph (Mixotroph)	Chemicals	Inorganic chemicals	Organic C

combined in some lakes with an increase in salinity with depth, may lead to at least temporary stratification of the lake into water layers that increase in density with depth. Consequently, depending on depth, surface area, salinity, and location, some lakes mix with the bottom continuously, some lakes mix for short periods only once or twice a year, and some lakes never fully mix to the bottom. In lakes that mix only occasionally or never mix fully with the bottom, a deep anoxic zone may occur, either permanently or transiently, where bacterioplankton having anaerobic modes of metabolism may flourish, but where few other organisms besides prokaryotes can survive. A prominent example is the Black Sea, where more than 87% of the volume is without molecular oxygen (14).

BACTERIOPLANKTON MODES OF ENERGY CONSERVATION AND CARBON METABOLISM

Metabolism refers to the sum of biochemical processes occurring in a cell. It is an indication of a common evolutionary link that prokaryotes share many properties of metabolism and cell structure with other life, for example, in assembling proteins and nucleic acids using essentially the same molecular building blocks as all other organisms on earth. Yet, prokaryotes as a group are remarkable for their metabolic diversity with respect to energy, carbon and other elements, and in other respects. This metabolic diversity, fully represented in the bacterioplankton, is the basis for much of the synthesis and degradation of organic matter, and patterns of nutrient cycling that occur on the planet. In this section, the metabolic diversity of the prokaryotes with respect to their mechanisms of energy conservation and carbon sources is outlined. Energy conservation refers to the mechanisms by which an organism obtains energy from the environment. Details of these nutritional modes can be found in most textbooks of microbiology.

All organisms must acquire from their environment, elements for biosynthesis (carbon, nitrogen, phosphorus, etc.), a source of electrons for construction of organic molecules and polymers, and a source of energy to build ATP molecules specifically (21,22). ATP (adenosine triphosphate) is the principal molecule used to drive energy-requiring chemical reactions in all cells. The terminology used to classify organisms nutritionally is based on their sources of energy, electrons, and carbon, and is summarized in Table 1. All terms used to describe these metabolic modes use the combining form troph as a suffix, derived from the Greek and meaning "to feed."

Organisms using organic molecules as carbon sources are called *heterotrophs*. Organisms that obtain carbon from carbon dioxide are called *autotrophs*. Autotrophs synthesize the organic biomolecules needed for maintenance or growth by first reducing carbon dioxide to sugar or other organic molecules, a process often referred to as *carbon fixation*. (Reduction is the addition of electrons or hydrogen atoms to a substance). As manufacturers of organic molecules from inorganic molecules, autotrophs provide the organic molecules on which heterotrophic organisms depend.

Autotrophs obtain the energy for synthesis of organic molecules and other purposes from either absorbed sunlight or by oxidation of consumed inorganic chemicals. Photoautotrophic organisms uses sunlight as an energy source, a process known as *photosynthesis*. Where a chemical is used by an autotroph, the organism is a *chemoautotrophi*. Chemicals used as energy sources by chemoautotrophs include reduced molecules of nitrogen (NH_4, NO_2^-), sulfur (H_2S or S^0), iron (Fe^{+2}), manganese (Mn^{+2}), or hydrogen (H_2), depending on the organism.

Most heterotrophic organisms are also chemotrophic, but unlike the chemoautotrophs, use consumed organic chemicals as sources of energy. There are two types of metabolic pathways used by heterotrophic organisms in catabolism (decomposition, with release of energy) of organic substrates, namely, respiration and fermentation. In respiration, an organic chemical substrate reacts chemically with an inorganic molecule such as O_2, NO_3, or SO_4 consumed from the environment. In this reaction, electrons stripped from the organic substrate (the electron donor) move, by a series of chemical intermediates, to the inorganic molecule (the electron acceptor). Energy released in this redox reaction (a chemical reaction involving the transfer of electrons from one molecule to another) can be harnessed by the cell to synthesize ATP. Respiration may be aerobic (using oxygen as an

electron acceptor) or anaerobic (using an inorganic electron acceptor other than oxygen). In either case, in respiration the organic substrate is catabolized fully to molecules of carbon dioxide and water.

Unlike respiration, which may be aerobic or anaerobic, fermentation is restricted to anaerobic environments. In fermentation, ATP is generated in the absence of an inorganic terminal electron acceptor. Instead, the same organic substrate is used as a source of energy, a source of electrons and, following partial decomposition, an electron acceptor. There are various kinds of fermentation pathways that differ in the type of substrate used, products formed, and number of ATP molecules made. In all fermentations, because catabolism of the substrate is incomplete, fewer molecules of ATP are synthesized per substrate molecule than in respiration. For this reason, fermentation is often described as a less efficient mode of energy metabolism than respiration. However, there are some organic molecules catabolized by fermentative organisms that cannot be used by at least some respiring organisms.

Finally, there is the requirement of electrons for the synthesis of organic molecules or polymers from more oxidized molecules. There are only two potential sources of electrons, namely, inorganic and organic chemicals. Lithotrophy refers to the use of electrons from inorganic molecules. Organotrophy refers to the use of electrons from organic molecules. Most autotrophs acquire electrons from inorganic chemicals. Aerobic photoautotrophs use water as the electron donor, whereas most anaerobic photoautotrophs use a reduced sulfur compound. For chemoautotrophic bacterioplankton, the same reduced inorganic chemicals described earlier as energy sources also serve as electron sources. Where water is used as an electron source in autotrophic metabolism, oxygen is generated, and the process is referred to as *oxygenic photoautotrophy*. Where one of the other reduced inorganic molecules is used, the process is anoxygenic. Unlike autotrophs, most heterotrophs acquire these electrons from the same organic chemicals used as a source of energy and carbon.

Putting the three prefixes of these italicized descriptors of metabolism together with the suffix troph (... troph), in the order, energy source-electron source-carbon source, the basic nutritional mode of any organism can be described (Table 1). For example, all plants and eukaryotic algae obtain energy from sunlight, electrons from water molecules, and carbon from carbon dioxide. This makes all plants and algae photolithoautotrophic (or photoautotrophic, for short). All animals and fungi obtain energy, electrons, and carbon from organic molecules, and are therefore chemoorganoheterotrophic (chemoheterotrophic, or simply heterotrophic, for short). Bacterioplankton, unlike animals and plants, as a group exhibit a highly varied array of nutritional modes.

Most bacterioplankton live in oxygenated water and are aerobic chemoheterotrophs (22). Modes of heterotrophic bacterioplankton respiratory metabolism in anoxic environments include nitrate reduction (denitrification), sulfate reduction, Fe^{+3} and Mn^{+4} reduction, and the reduction of carbon dioxide (a type of methanogenesis). There are also fermentative bacteria of various kinds in anoxic environments.

Autotrophic bacterioplankton also can be found in oxic and anoxic water. Aerobic photoautotrophic bacterioplankton primarily are in the division cyanobacteria. If there is sufficient sunlight, photoautotrophic bacterioplankton also may occur in anoxic water. Examples of photoautotrophic bacterioplankton in anoxic water include groups traditionally referred to as purple sulfur bacteria, purple nonsulfur bacteria, green sulfur bacteria, and green nonsulfur bacteria. Despite numerous genetic, morphological, and physiological differences among these groups (and between genera within these groups), they all have an anoxygenic, anaerobic photosynthetic metabolism, although some may be tolerant of oxygen. These organisms can be abundant in the water column of light-exposed anaerobic habitats having the appropriate electron donors such as clear, stratified sulfur-rich lakes (23), or as benthic bacteria in shallow sediments (24).

Chemolithoautotrophs (chemoautotrophs for short), use reduced inorganic chemicals as a source of both energy and electrons. There are numerous groups of chemolithoautotrophic bacterioplankton that vary in their sources of reduced inorganic compounds, but in most cases, the electron acceptor used by these organisms is oxygen. Examples of chemolithoautotrophy include the sulfur-oxidizing bacterioplankton (which use H_2S or S^o as energy/electron sources), nitrifying bacterioplankton (which use NH_3 or NO^-_2), and methanogenic bacterioplankton [which may use H_2 or acetate as energy/electron sources and produce methane (CH_4)]. Mixotrophic bacterioplankton are unusual in that they use an inorganic compound as an energy source and electron donor, but an organic compound as a carbon source.

Recently, it was discovered that cultivatable photoheterotrophic (actually photoorganoheterotrophic) bacterioplankton are active in the ocean (25). They metabolize dissolved organic matter (DOM) when available, using oxygen-dependent respiration. When DOM is in lower concentration, they are photosynthetic. In photosynthesis, they use DOM as an electron donor and so are anoxygenic, but in contrast to anoxygenic photoautotrophs, photoheterotrophic bacterioplankton live only in oxic environments. The photoheterotrophic genus *Erythrobacter* appears to be ubiquitous in the euphotic zone of the open ocean. In the northeastern Pacific Ocean, these anoxygenic photoheterotrophs may comprise at least 11% of the total bacterioplankton population in the upper 150 m of the water column, and it is possible that their contribution to the microbial community is even higher in more oligotrophic areas (25). Other species of photoheterotrophic bacteria occur in both marine (e.g., *Roseobacter*) and freshwater ecosystems (e.g., *Erythromicrobium*) (26), but little is known of their contribution to the productivity of these systems.

In summary, although a single species of bacterioplankton may be restricted in its metabolic mode, prokaryotes as a group exhibit a much broader range of capabilities in energy conservation and carbon metabolism than do plants and animals, which are either photoautotrophic or chemoheterotrophic, respectively. Prokaryotes, in contrast, are

capable of aerobic and anaerobic photoautotrophy, photoheterotrophy, multiple modes of chemolithoautotrophy, multiple modes of fermentation, and both aerobic and anaerobic chemoheterotrophy using a diverse array of organic substrates. The type of metabolism that is dominant at any one time or place depends on numerous environmental factors including salinity, oxygen concentration, availability of electron donors and acceptors, temperature, and organic matter concentration or composition. Their metabolic diversity, in combination with their abundance, make bacterioplankton extraordinarily important on a global scale for much of the cycling and transformation of materials that ultimately sustains all life on earth. Their role in global biogeochemical cycling is described in more detail in a later section.

BACTERIOPLANKTON TAXONOMIC DIVERSITY

A History of Methods

Until recently, identification of prokaryotes was based on phenotypic characters (morphological, biochemical, and physiological features) of cultivated isolates. From these methods, it was recognized that prokaryotic diversity is great, but as Stanier and coworkers (27) put it in a well-used text, "... the attempt to discern intergroup evolutionary relationships...seems doomed to failure." They go on to say, "The only attribute common to all these organisms is possession of the procaryotic cell as the unit of structure and function; although this certainly suggests a common primary evolutionary origin, the later divergences have been too great, and too remote, to permit reconstruction of evolutionary relationships on the basis of the properties of contemporary representatives." In other words, development of a trustworthy phylogeny of the prokaryotes that accurately represented evolutionary relationships seemed impossible in the late 1970s.

At about the same time, it became apparent that analysis of prokaryote diversity in the environment was deficient in another respect. Before the late 1970s, bacterioplankton were usually enumerated as colonies on nutrient-rich culture media. Typically, this technique involves spreading a small water sample of known volume across an agar plate enriched with nutrients. Cells reproducing in the medium form visible colonies that can be counted, each colony having descended from a single cell. A shortcoming of this approach is that some cell types may not grow in the culture medium used. This problem became most apparent with development of epifluorescence microscopy techniques, allowing direct counts of all bacterioplankton in a sample without cultivation (28). The method is straightforward. A dye that coats the bacterioplankton is added to a water sample, rendering them flourescent when illuminated with the appropriate wavelengths of light, and visible at 1000X.

Application of epifluorescence microscopy had two immediate consequences for our view of bacterioplankton ecology. First, it was discovered that most (more than 95%) bacterioplankton seen by this technique did not grow using traditional cultivation methods, and so estimates of bacterioplankton abundance and biomass went up dramatically, sometimes by two or three orders of magnitude. Secondly, microbiologists became increasingly concerned with the possibility that much naturally occurring bacterioplankton phylogenetic diversity was unknown. Obtaining organisms in culture has, until recently, been a prerequisite for taxonomic description. If only a small percentage of the bacterioplankton grew in commonly used culture media, perhaps many taxa of bacterioplankton had escaped detection. Without methods to analyze naturally occurring diversity, simple questions of bacterioplankton community ecology, for example, having to do with species composition or succession, could not be addressed (29).

In the early 1980s, the situation began to improve with the development of molecular methods of measuring microbial diversity. These approaches are based on sequencing of nucleotide or amino acid chains in nucleic acids and proteins, respectively. The molecule currently used most often as a phylogenetic marker in studies of the prokaryotes is the gene for the small subunit of prokaryotic ribosomes, the 16S rRNA gene. This gene is useful in the examination of diversity, and for the delineation of phylogenetic relationships, because it is contained by all organisms, is large enough to provide a sufficiently high amount of distinctive genetic information, and is highly conserved (unchanged over time) in some areas but less so in others. Furthermore, comparison between organisms of nucleotide sequences of genes allows a quantitative analysis of their relatedness by evolution (3).

Analysis and comparison of 16S rRNA genes of cultivated isolates, and other molecules, have been invaluable in constructing microbial phylogenetic relationships. In fact, the technique led to a revolution in the concept of evolutionary relationships of not just prokaryotes, but all life, and is the basis of the now widely adopted reorganization of life on earth into three main lineages, Bacteria, Archaea, and Eukarya (3).

Still, because many bacterioplankton taxa have not been isolated, the ability to examine natural microbial communities would remain poor if only cultivated bacterioplankton could be examined. Fortunately, a few years after molecular methods for phylogenetic analysis of cultivated isolates were introduced, a method was developed for the characterization of 16S rRNA genes of bacterioplankton from natural communities without cultivation (30). In the simplest variation of this approach, there are four main steps:

1. DNA is extracted from a natural bacterioplankton community. Contained in this DNA are a mixture of phylogenetically distinctive genes and gene fragments, such as the 16S rRNA genes, of specific organisms in the community.
2. Using the procedure *polymerase chain reaction* (PCR), the 16S rRNA genes of the community are preferentially amplified (repeatedly replicated), resulting in a mixture of DNA fragments, in which there are now large numbers of copies of each different 16S rRNA gene.
3. The mixture of different 16S rRNA genes are separated from each other by insertion into microbial

host organisms that easily can be kept as cultured isolates, such as the bacterium *Escherichia coli*. These foreign genes, carried by host organisms, are called *clones*. A collection of these genetically transformed host cells, containing cloned genes, is called a *clone library*. In the library, each host isolate contains a single type of 16S rRNA gene from the original plankton sample. Once the clone is inserted into the host, it can be further replicated within the host.

4. Following separation and cloning by insertion into host cells, the order of nucleotides of the different 16S rRNA genes can be sequenced. The diversity of the community can be evaluated on the basis of the different sequences obtained. Furthermore, by comparison of the different sequences, one can construct a phylogenetic tree illustrating the degree of relatedness of the organisms.

Using this approach, it is now possible to identify unique taxa of bacterioplankton on the basis of the genetic analysis of recovered genes, without the necessity of obtaining cultivated isolates (31,32). Furthermore, because comparisons of genes between organisms can be used to infer evolutionary relationships, it is now standard practice in studies of microbial communities to report not just the diversity present, but also a phylogenetic tree of the organisms found.

Another important recently developed tool for examination of taxonomic diversity of natural bacterioplankton communities are the nucleic acid hybridization probes (33). These probes consist of short nucleotide sequences (oligonucleotides) complementary to taxon-specific regions of RNA or DNA. They can be designed with various degrees of specificity, from universal probes that target bacterioplankton to probes that are group-specific or, in some cases, even species-specific. Where probes are marked with a fluorescent molecule, they can be used in a procedure called *fluorescent in situ hybridization* (FISH) (34). In this approach, a natural microbial community is exposed to one or more fluorescently labeled oligonucleotide probes. Hybridization of the probes to the targeted nucleic acids can be viewed by microscopy, and indicates the presence of particular taxa. These probes are now widely used to monitor spatial and temporal dynamics of particular groups of bacterioplankton in the natural environment (35).

Today, analyses of bacterioplankton community composition are based on molecular, combined with phenotypic, descriptions of cultivated isolates, sequencing of 16S rRNA (or other molecules) from natural communities, hybridization studies using targeted oligonucleotide probes, and other approaches (12,36). With this array of tools, there has been in the last decade a flood of new discoveries regarding the diversity, ecology, and evolutionary history of the prokaryotes.

A MODERN VIEW OF BACTERIOPLANKTON TAXONOMIC DIVERSITY

The systematic description of the prokaryotes is in progress, and will almost certainly undergo modification as additional information used in interpretation of phylogenetic relationships is obtained. Often, most of the organisms detected in modern studies of diversity, and included in phylogenetic reconstruction, have never been cultivated or even knowingly observed, and are known only from their genes, usually the 16S rRNA gene extracted from water. Terminology used for taxonomic classification in the following discussion of the bacterioplankton is partly from Garrity and coworkers (37) and Giovannoni and Rappé (26). There are several terms used in the following discussion that might be unfamiliar to the general reader. A distinct genetic sequence representing a phylogenetically identifiable taxon is referred to as a *phylotype*. A *clone*, as described earlier, is a number of copies of a gene (e.g., the 16S rRNA gene), or gene fragment, obtained by repeated replication starting with a single copy. Many phylotypes are known only from the distinctiveness of their cloned genes. A *gene cluster* is a group of DNA sequences from uncultivated organisms that are more closely related to each other than to any cultivated organism (38). A *clade* is a lineage of closely related (monophyletic) organisms, some of which may only be known from their genes.

BACTERIOPLANKTON OF THE DOMAIN BACTERIA

The domain Bacteria consists of at least 18 major lineages. Those having representatives regularly detected in aquatic environments include the *Proteobacteria*, green nonsulfur bacteria, Marine Group A, *Actinobacteria*, the *Cytophaga-Flavobacteria* group, and cyanobacteria.

The *Proteobacteria* are a diverse group of autotrophic and heterotrophic gram-negative bacteria that, according to gene cloning studies, are widespread and abundant in the ocean (26), and common in many lakes (39). They are separated into five subdivisions designated: α-*Proteobacteria*, β-*Proteobacteria*, δ-*Proteobacteria*, ε-*Proteobacteria*, and γ-*Proteobacteria*.

Important phylogenetically coherent groups within the α-*Proteobacteria* include the SAR11 gene cluster, the *Roseobacter* clade, and the SAR116 gene cluster. SAR11 is one of most commonly recovered gene clusters from the ocean and oligotrophic freshwater systems (26,40). Although not yet cultivated, on the basis of the use of flourescent probes for microscopic observation, members of this group are estimated to be about 0.25 to 0.7 μm in diameter. At present, little is known regarding its physiology or ecological role, but considering the ubiquity and abundance of SAR11, it is possible that these bacterioplankton are responsible for a high percentage of the nutrient cycling that occurs on Earth (26). Using a combination of oligonucleotide probes and radioactively labeled organic compounds to compare relative abundance of different taxonomic groups of bacterioplankton in coastal seawater samples with uptake of various substrates, it was found that α-*Proteobacteria* are particularly important in consumption of amino acids, but less so in the utilization of large biomolecules such as protein, chitin, and n-acetylglucosamine (41).

Bacterioplankton of the β-*Proteobacteria* subdivision have been detected in a wide variety of oxygenated

freshwater lakes (39,42), but are rare in open ocean marine systems (43). They do occur in coastal water, perhaps derived from freshwater runoff. It has been suggested that *β-Proteobacteria* have a low tolerance for marine salinity, but other selective factors may be at work in particular systems (44). For example, *β-Proteobacteria* were a minor proportion of the bacterioplankton community of oligotrophic freshwater Crater Lake, Oregon, perhaps because of limitation by trace metals, low dissolved organic carbon concentrations, high ultraviolet light exposure, an unusual phytoplankton community, or some combination of these factors (42).

SAR324 is a unique clade of the *δ-Proteobacteria*. It has been detected in the deep aphotic zone of both the Atlantic and Pacific Oceans, and is presumably adapted for life at high pressure, cold temperature, and the particular substrate content of the deep ocean.

Interestingly, among the cultivated isolates of the *Proteobacteria*, *γ-Proteobacteria* are most common. The implication is that the *α-*, *β-*, and *γ-Proteobacteria* differ in their nutritional requirements, with the *γ-Proteobacteria* favored where substrate concentrations are high (45,46). For example, in coastal marine waters, *γ-Proteobacteria* comprised approximately between 15 and 30% of the total bacterioplankton community and accounted for 19% and 29% of the cells consuming protein and amino acids, respectively (41). In deep water (1,200 m) of Lake Baikal, where sedimenting materials may accumulate, *γ-Proteobacteria* dominated the bacterioplankton (47). However, for reasons yet unknown, *γ-Proteobacteria* are generally uncommon in freshwater systems, perhaps because they are displaced by *β-Proteobacteria* (39,43).

In contrast to the organisms previously mentioned, which are all *Proteobacteria*, the SAR202 gene cluster has been classified with the green nonsulfur bacteria. SAR202 is intriguing in that it appears to be rare in marine surface water, but common in water of 200 m and deeper. The Marine Group A clade is a recently detected unique lineage of the bacteria. One of its uncultivated representatives, the SAR406 cluster, has been found in surface water and deep water of both the Atlantic and Pacific Oceans (26). Like most of the uncultivated bacterioplankton, little is known of the ecology of SAR324, SAR202, or SAR406, but through examination of their use of substrates, it should be possible to elucidate their roles in nutrient cycling (41).

Another important group of marine and freshwater bacterioplankton is the gram-positive *Actinobacteria* clade. In addition to a number of cultivated isolates, this group is represented by a cluster of closely related uncultivated gene clones. These bacterioplankton are commonly detected, especially in the photic zone, but do not appear to be as common as some of the other uncultivated bacterioplankton. Fewer studies have been conducted in freshwater environments, but at least in some lakes, the *Acintobacteria* can be abundant. In Lake Gossenköllesee, Austria, for example, they and the *β-Proteobacteria* were the dominant groups of bacterioplankton (48).

A broad group of bacterioplankton commonly encountered in plankton samples, and having both cultivated and uncultivated representatives, is the *Cytophaga-Flavobacteria* cluster. Members of this group are widespread and sometimes dominant in marine plankton, and common in many lakes (39,41,43,49). They are both free-living and attached to surfaces of detritus, algae, and animal bodies. In culture, representatives of these bacterioplankton produce hydrolytic exoenzymes that catalyze degradation of a variety of large biomolecules including chiton, agar, DNA, and cellulose; this suggests they are ecologically important in the degradation of particulate materials and dissolved biomacromolecules (41). For example, where protein was added experimentally to eutrophic seawater samples, bacterioplankton of the *Cytophaga-Flavobacteria* cluster dominated the community (41). In another study, their abundances increased with depth in the water column, suggesting that they may specialize in the degradation of recalcitrant macromolecules that might accumulate in deep water (43).

Cottrel and Kirchman (49) suggested that, perhaps because of poor PCR-amplification of *Cytophaga-Flavobacteria* genes, these bacteria have been severely underrepresented in diversity studies based on abundances of clones (i.e., clones of a particular type recovered from a sample). From the number of PCR-amplified cloned genes obtained in samples of coastal marine plankton, they found that representatives of the *Cytophaga-Flavobacteria* cluster comprised less than about 10% of the bacterioplankton community. However, using oligonucleotide probes (FISH) to measure their abundance in the same samples directly, they determined that the *Cytophaga-Flavobacteria* cluster were a numerically dominant component (up to 35%) of all bacterioplankton (49). As this study indicates, understanding of bacterioplankton community composition may need revision as limitations or possible biases of various molecular approaches for analysis of these communities are uncovered (50).

The most commonly recognized photoautotrophic bacterioplankton are of the cyanobacteria lineage (see CYANOBACTERIA, this Encyclopedia). The cyanobacteria are oxygenic photoautotrophs. They are common in both marine and freshwater systems, and consist of numerous species, many of which have distinct morphologies, are readily observable under the microscope, and have been studied for decades. The planktonic cyanobacteria occur as free-living single cells, or as colonies of cells in small bundles or filaments. Some species "fix" nitrogen, which means they are able to absorb and reduce molecular nitrogen (N_2) to ammonium, which is incorporated into proteins as an amino group. Nitrogen fixation requires the enzyme nitrogenase, which among the photoautotrophic plankton, is unique to cyanobacteria, and gives them an advantage in systems in which dissolved nitrogen compounds are in low concentration. The filamentous nitrogen-fixing cyanobacterium *Trichodesmium* spp. often forms visible aggregates in warm ocean water (51). Recently, it was discovered that abundant, single-celled, marine cyanobacteria in the size range of 3 to 10 μm express at least one of the genes for nitrogenase, which suggests that they may make a significant contribution to nitrogen fixation in the ocean (52).

In the late 1970s and 1980s, two new genera of cyanobacteria were identified in the ocean plankton, the genus *Synechococcus* and the prochlorophyte *Prochlorococcus* (53,54). Since then, *Synechococcus* has also been identified in many diverse freshwater environments, but *Prochlorococcus* may be restricted to marine environments (see PROCHLOROCOCCUS, this Encyclopedia). Organisms in both groups occur as single cells usually less than about 2 to 3 μm in diameter, and may be collectively referred to as *picocyanobacteria*. Besides their gene sequences, they are distinguished from each other by photosynthetic pigment composition. *Synechococcus* spp., like most cyanobacteria, have chlorophyll *a* and a group of accessory pigments called *phycobilisomes*. The prochlorophytes, in contrast, contain the unusual divinyl chlorophylls *a* and *b*, and lack phycobilisomes.

Depending on trophic conditions, *Synechococcus* and *Prochlorococcus* can occur in very high abundance, sometimes exceeding one million cells per milliliter, and in the vast oligotrophic areas of the oceans, these two genera dominate primary production (53,55). *Prochlorococcus* is probably the most abundant photosynthetic organism on earth (56). Nevertheless, until the development of the *flow cytometer*, these organisms were unknown because they could not be distinguished quantitatively from other bacterioplankton by microscopy. A flow cytometer can detect, and distinguish between, the fluorescence of various photosynthetic pigments at very low concentrations and thereby can be used to quantify individual phytoplanktons, including picocyanobacteria (57).

Remarkably, of the groups of bacterioplankton most often recovered from aquatic environments, only the cyanobacteria, the *Roseobacter* clade, and the *Cytophaga-Flavobacteria* cluster have representatives in culture collections (49,58). Various hypotheses have been suggested to explain the lack of success in cultivating many bacterioplankton phylotypes. One reason may be that it is difficult to reproduce precisely their nutritional or other growth requirements. A second possibility is that there is viral lysis of some bacterioplankton phylotypes on introduction to culture media. In any case, with additional effort, to successfully culture these presently "unculturable" species is likely (59).

BACTERIOPLANKTON OF THE DOMAIN ARCHAEA

(See ARCHAEA IN MARINE ENVIRONMENTS, this Encyclopedia)

The archaeal bacterioplankton consist of at least two major branches, or kingdoms, Crenarchaeota and Euryarchaeota. The Group I archaeal bacterioplankton are represented by a gene cluster of uncultivated organisms (but obtained as gene clones) in the Crenarchaeota. Group II archaeal bacterioplankton are a gene cluster of uncultivated clones in the Euryarchaeota. Recently, gene sequences recovered from marine deep-water plankton samples were classified as a distinct gene cluster of the Euryarchaeota, the Group III archaea (4).

Until recently, it was believed that archaea were restricted to "extreme" environments with very high salt content (such as the Great Salt Lake of Utah), high temperature, those that were very acidic (such as those that occur around some deep-ocean vents, and hot springs), or anoxic. Using molecular approaches to detect archaeal genes, it is now known that representatives of both the major archaeal branches are widely dispersed and common residents of the bacterioplankton communities of both freshwater and marine ecosystems (35,42,60–63). Compared with bacteria and eukarya, the genetic diversity of the archaeal bacterioplankton, at least in the ocean, appears to be low, with a few cosmopolitan phylotypes dominant in widely different ocean provinces (64). But archaea are abundant in the ocean; a recent estimate suggests that archaeal bacterioplankton are equivalent in number to bacterial bacterioplankton at ocean depths greater than 1,000 m, and constitute about 20% of the total marine picoplankton biomass worldwide (6).

The importance of archaea in bacterioplankton communities varies with depth, time, and trophic state. In a survey of eight different marine provinces, Massana and coworkers (64) found that while both Group I and Group II archaea occurred at the surface and below the photic zone in temperate regions of the ocean, Group I archaea predominated below the photic zone, and Group II archaea near the surface. In antarctic and subantarctic water, however, Group II archaea were practically absent (64). In the Gerlache Strait, a region west of the Antarctic Peninsula, archaea at all depths declined in relative abundance during repeated sampling over a two-week period of the austral spring (65). Similarly, Murray and coworkers (66) observed a marked decrease in archaeal rRNA from spring through the austral summer in Antarctic nearshore water. Archaea generally constituted a higher fraction (up to 50%) of the bacterioplankton community below the photic zone of both marine and freshwater systems (4,35,42,65). In the oligotrophic coastal water of California, archaea were less than 5% of the total bacterioplankton counted near the surface, but about 40% of the bacterioplankton at the 600 m depth (35). With increased algal biomass, archaea decline in their proportional representation in the bacterioplankton community (6,66). The latter observation suggested to Murray and coworkers (66) that bacteria may be competitively superior to archaea at times when nutrients are readily available, such as algal blooms.

The life histories and ecological roles of the archaeal bacterioplankton are still unknown. It has even been suggested that the Group II archaea, at least, may originate in the digestive tracts of zooplankton or fishes and not truly be metabolically active plankton (67). Further study of their population dynamics, growth rates, and metabolic activities is necessary for the elucidation of the ecological importance of the archaea in plankton environments. A similar statement could be made about much of the rest of the bacterioplankton.

SPATIAL AND TEMPORAL PATTERNS IN BACTERIOPLANKTON METABOLISM AND TAXONOMIC DIVERSITY

Spatial and temporal patterns in bacterioplankton communities can be described in terms of the metabolic properties of the communities, the taxonomic diversity of the communities, or, ideally, both. Here, general patterns

in space and time of dominant metabolic properties are described first. This is followed by a discussion of spatial and temporal patterns in taxonomic composition. Finally, there is a discussion of the link between metabolic properties and the taxonomic composition of bacterioplankton communities.

Spatial and Temporal Patterns in Bacterioplankton Community Metabolism

Spatial and temporal patterns in bacterioplankton metabolism occur in response to variation in environmental factors such as temperature, oxygen concentration, availability of electron donors and acceptors, light intensity and spectral composition, oxidation-reduction potential, and so on. Three examples illustrate how such patterns in bacterioplankton metabolic diversity can develop and be maintained.

The first example concerns spatial and temporal patterns in heterotrophic bacterioplankton metabolism in a system in which the availability of electron acceptors changes with time. The type of respiratory metabolism of heterotrophic bacterioplankton that dominates at any one time and place depends partly on the types of electron acceptors available in the environment. In general, the higher the reduction potential (tendency to be reduced) of an available electron acceptor, the more the energy that is released on oxidation of a particular substrate molecule, and the more ATP that can be synthesized. Because of its high reduction potential, more energy is acquired per substrate molecule with oxygen as an electron acceptor than with other electron acceptors. Thus, when oxygen is available, as in the ocean and the surface water of most lakes, aerobic heterotrophic bacterioplankton are usually the dominant prokaryotic heterotrophs. Depending on depth and mixing processes, however, oxygen may never be available or may seasonally become depleted in the deeper regions of the water column. Where oxygen is depleted, fermentative bacterioplankton and respiring bacterioplankton using other electron acceptors may thrive.

Consider a stratified lake in the temperate zone with depleted dissolved oxygen in the water at the bottom depleted. The order in which electron acceptors are used in anaerobic respiratory metabolism, and therefore the kinds of bacterioplankton that dominate at any one time and place, can be predicted by the energy yield for the reaction, and is typically (from high to lower energy yield) O_2, NO_3, MnO_2, FeOH, SO_4, and CO_2 (11,68). This sequence of electron acceptors suggests competition for organic substrates among bacterioplankton groups (69). In individual cases, however, energy yield alone does not explain the order in which microbial metabolic activities occur, and factors such as differential toxicity, substrate specificity, or growth rates may be important (70).

As a second example of spatial variation of bacterioplankton communities, consider the distribution of anaerobic phototrophic bacterioplankton in Mahoney Lake, British Columbia (23,71), maximum depth 14.5 m. Because of a pronounced vertical gradient in salinity, it is permanently anoxic below 6 m, and because of the geology of its watershed, it is high in sulfate. During the ice-free season, within a vertical 10-cm band located around 6.8 m, a very dense [up to 400 billion cells per liter (!)] population of the anaerobic phototrophic purple sulfur bacterioplankton *Amoebobacter purpureus* develops. In this narrow layer, oxygen is absent, but there is sufficient light and sulfide (used as an electron donor) to support anoxygenic photosynthesis by this sulfur-oxidizing organism. The sulfide that *A. purpureus* depends on is provided by the sulfate-respiring bacterioplankton within and below the layer.

Chemoautotrophic prokaryotes also exhibit distinct spatial patterns in abundance. In a stratified water column, these organisms may accumulate near the boundary between anoxic and oxic layers of the water column. Here they can catalyze energy-yielding oxidation-reduction reactions between upward-diffusing reduced inorganic compounds produced by anaerobic heterotrophic respiration in the anoxic zone and downward-diffusing molecular oxygen. This was documented in the water column of a Danish fjord, where chemoautotrophic bacteria using NH_4^+ (nitrification) and HS^- (sulfide oxidation) as energy sources were active just above the oxic–anoxic interface (72). Obviously, spatial patterns in microbial metabolism may be transient as conditions change with the season.

Spatial and Temporal Patterns in Bacterioplankton Community Taxonomic Composition

In samples of a particular water stratum from different oceans, there are bacterioplankton clones that are widespread, perhaps cosmopolitan and others that are less broadly distributed. Fuhrman and coworkers (73), for example, obtained identical clones in different marine samples, two pairs of which were between the Atlantic and Pacific Oceans. Nevertheless, they emphasized that marine bacterioplankton communities are diverse and not dominated by a particular clone sequence. In comparison, Mullins and coworkers (40) observed less diversity between the samples of bacterioplankton from the Atlantic and Pacific Oceans, a wider degree of overlap in composition between samples, and emphasized the similarity in bacterioplankton samples between the oceans. Similarly, DeLong (61) found identical archaea in both Pacific and Atlantic coastal water. Hagstrom and coworkers (59) obtained numerous isolates of bacterioplankton from the surface water of a wide range of marine areas. They observed that, although most genera of isolated bacterioplankton have closely related representatives in different oceans, the bacterioplankton community may be distinct, for example, in particular environments many of the isolates of bacterioplankton from the Baltic Sea were genera not obtained in other areas. They suggested that the low salinity of the Baltic Sea may select against many marine bacterioplankton.

Although some bacterioplankton may be cosmopolitan, at least within a particular water stratum, it is clear that along temporal and spatial gradients of some environmental factors, there are changes in the relative proportions of different components of the bacterioplankton community. Taxonomic variation in bacterioplankton communities occur along environmental gradients of salinity (74,75), oxygen (76), substrate composition (19,41,77),

depth (6,35), sunlight intensity and spectral composition (42), and grazing pressure (78). Discovery of these patterns in bacterioplankton community composition is essential for understanding bacterioplankton ecology, but at present the physiology of most components of the bacterioplankton has not provided a mechanistic explanation for them.

As an example, consider the importance of substrate composition on the taxonomic composition of the bacterioplankton community. In many aquatic ecosystems, the primary source of organic molecules used by heterotrophic bacterioplankton is phytoplankton production. At certain times of the year, usually because of an increase in inorganic nutrient availability, the phytoplankton population will temporarily increase dramatically in concentration, a phenomenon known as a *bloom*. Riemann and coworkers (19) followed the phylogenetic composition of a bacterioplankton community during and after an experimentally stimulated phytoplankton bloom. As the bloom progressed, there was a disappearance of three previously dominant, but uncharacterized, phylotypes of bacterioplankton, which they attributed to phylotype-specific grazing by protozoa or, possibly, viral mortality. Within a few days following the bloom, new phylotypes appeared, and bacterioplankton abundance, production, and enzyme activity became increasingly associated with small particles, suggesting microbial colonization of phytoplankton particulate debris. These presumably attached bacterioplankton had high growth rates and high cell-specific activities of protein, sugar, and lipid-degrading enzymes. Many of the attached bacterioplankton were from the *α-Proteobacteria* group, with some from the *Cytophaga-Flavobacterium* cluster (19). Other studies have found particle-associated bacterioplankton to be phylogenetically distinct from free-living cells (79,80).

The Link Between Diversity and Metabolism

It is evident from the discovery of previously unrecognized genes in samples of marine bacterioplankton that unrecognized metabolic activity of bacterioplankton of potentially great biogeochemical significance may exist. For instance, Zehr and coworkers (52,81) obtained the principal gene (and messenger RNA transcripts of the gene) for nitrogenase from apparently abundant, but in some cases uncultivated, unicellular, and filamentous, cyanobacteria, and *Proteobacteria*. These results suggest that nitrogen fixation probably occurs more broadly in the marine plankton than previously thought.

The tools of molecular biology also have been successful in discovering the potential for entirely novel modes of metabolism in bacterioplankton communities. For example, until recently, it was believed that most photosynthesis in the ocean was performed by cyanobacteria or eukaryotic algae. However, Béjè and coworkers (82) found genes for the protein bacteriorhodopsin in the uncultivated *γ-Proteobacterium* SAR 86. Bacteriorhodopsin enables a form of anoxygenic phototrophy in certain extremely halophilic archaea, but it had never been found in marine bacterioplankton. In another study, Kolber and coworkers (83) detected the presence of bacteriochlorophyll in marine bacterioplankton. This photosynthetic pigment of anoxygenic photoautotrophic bacteria had never been found in marine surface water samples. And, as mentioned earlier, recently discovered photoheterotrophic bacterioplankton may contribute substantially to the primary production in the ocean (25). These results suggest that in bacterioplankton communities, there are organisms with metabolic capabilities that are undiscovered, but have great biogeochemical significance.

Although it is now possible to describe bacterioplankton taxonomic diversity quantitatively, it remains a challenge for microbial ecologists to determine the relationship between diversity and community metabolic activity. Again, because few organisms commonly detected by molecular methods have been cultivated, relatively little is known of the metabolic activities of a substantial proportion of the bacterioplankton community. Increasingly, however, studies are being conducted to correlate phylogenetic and metabolic properties of the bacterioplankton community. For example, Ramsing and coworkers (84) examined relationships between the location and abundance of in situ sulfate-reducing bacteria quantified by oligonucleotide probes for sulfate-reducers, rates of sulfate reduction, and characteristics of the chemical environment along a vertical gradient in the water column of a stratified fjord in Denmark. Using fluorescent probes of varying specificity, from a general probe for all bacteria to genus-specific probes, they found pronounced spatial shifts in the bacterioplankton community within the water column associated with the chemical gradient.

Even small differences in genetic diversity (microdiversity) may be associated with significant differences in the metabolic activity or ecology. For example, Moore and coworkers (85) showed that co-occurring strains of the marine prochlorophyte *Prochlorococcus* had differences of only 3% in 16S rRNA sequence. These small genetic differences corresponded to ecologically significant differences in light-dependent physiology, with one population being able to grow at light intensities that inhibited growth of a co-occurring population. Similarly, Field and coworkers (86) hypothesized that genetic diversity of bacterioplankton within the SAR 11 cluster is related to niche partitioning along a depth gradient.

Clearly, there remains much to learn before predictive statements can be made regarding specific bacterioplankton abundance and distribution, the relationship of broad phylogenetic diversity (much less microdiversity) and the ecological function of naturally occurring microbial plankton. Experimental approaches using molecular techniques for the analysis of diversity can be used to address questions regarding the ecological determinants of bacterioplankton community composition and activity (19,41,77,87).

BACTERIOPLANKTON ABUNDANCE, BIOMASS, AND PRODUCTION

Total bacterioplankton abundance in aquatic ecosystems is generally between 0.05 and 5 billion per liter, although in particular systems, it can be less, or exceed this range,

by an order of magnitude (14). Spatial and seasonal variation in both heterotrophic bacterioplankton and picocyanobacteria are evident in some systems (88,89). In interecosystem comparisons, abundance tends to increase with algal biomass (90,91). In lakes, the lowest values are found in oligotrophic systems, and the highest values in eutrophic reservoirs and tropical alkaline saline lakes (14). In the ocean, bacterioplankton abundance averages about 0.5 billion per liter in continental shelf waters and above 200 m (including *Prochlorococcus* spp.), and about 0.05 billion per liter in the deep ocean (>200 m deep) (5). As a proportion of total microbial biomass, however, heterotrophic bacterioplankton biomass tends to increase with depth from approximately 55% of the total microbial biomass at 0 to 200 m, to virtually 100% of the microbial biomass from 200 to 1,000 m (92). Biddanda and Benner (93) estimated that approximately half of the bacterial biomass and metabolism of the open ocean occurs in the aphotic zone.

Bacterioplankton biomass in the ocean ranges approximately between 200 and 1,500 mg C m^{-2} (94). (The unit m^{-2} refer to the column of water under a squared meter of the surface). The lowest values reported by Ducklow (94) are from the Ross Sea, Antarctica. In nutrient-rich (eutrophic) coastal water, heterotrophic bacterioplankton biomass is generally much less than phytoplankton biomass, although exceptions exist where there are high concentrations of organic matter. In Chesapeake Bay, for example, where there are high levels of dissolved organic matter, heterotrophic bacterioplankton biomass is as much as 60% of the phytoplankton biomass (95). In a gradient from coastal areas to the nutrient-poor (oligotrophic) region of the ocean, phytoplankton abundance and biomass tends to decrease more than heterotrophic bacterioplankton abundance and biomass. As a consequence, in oligotrophic marine water, bacterioplankton biomass is commonly two to three times greater than photoautotrophic biomass (91,96).

As mentioned earlier, bacterioplankton consist of both free-living suspended cells and cells attached to particles in the water column. Although most of the bacterioplankton generally are free-living, size- and cell-specific metabolic activity are greater in the attached bacteria. The reason for these differences between free-living and attached bacteria is probably because of an increased concentration of substrates available at surfaces, possibly associated with reduced predation rates (79,97).

Besides the abundance and taxonomic composition, other important properties of a community are how fast and how much biomass is produced per unit of time. These properties are important both as fundamental measurements of the synthesis of organic matter, and because many other ecological processes are correlated with them. For example, the rate of biomass production of a microbial community is directly related to its consumption of carbon and other elements from the environment, its release of materials into the environment, and possibly the ways organisms in the community interact with each other (98).

With respect to bacterioplankton communities, there are several commonly used measurements that express amounts and rates of biomass production. The *production* of a bacterioplankton community is the amount of dry weight (or biomass, usually expressed in units of C) synthesized by the community per unit of volume (or per surface area) per unit of time. In bacterioplankton communities, biomass production could be due to increases in mean cell size with time, but is primarily because of reproduction of cells, in other words changes in population abundance. Production is, therefore, a product of both the biomass (or number) of organisms in the community and the instantaneous rate (specific growth rate) at which the biomass of the community is changing by reproduction. Theoretically, bacterioplankton production can be measured in either the absence (*gross production*) or presence (*net production*) of mortality (94). In the absence of mortality, bacterioplankton biomass will increase exponentially until there is a limitation by substrate shortage, space shortage, accumulation of toxic waste products, or other inhibiting factors. In natural environments, mortality of bacterioplankton generally keeps pace with bacterioplankton production such that bacterial biomass does not accumulate, and the net production is near zero. However, most techniques used to measure bacterioplankton production in nature are unaffected by mortality, and therefore measure gross production (98).

The *specific growth rate* of a bacterioplankton community is the rate of biomass production (e.g., in units: Cm^{-3} day^{-1}) divided by the mean amount of their biomass (in units: Cm^{-3}). Units for specific growth rate are inverse time (e.g., day^{-1}).

A final commonly used measurement of the rate of reproduction of a bacterioplankton community is *turnover time*, or *doubling time*. The turnover time of a community (or population) is the time required for the entire community to produce an equivalent amount of standing biomass, assuming no losses. It is calculated by dividing the mean biomass of a population by its rate of production; in other words, it is the inverse of specific growth rate.

According to Ducklow (94), gross bacterioplankton production in the ocean ranges approximately between 5 and 300 mg C m^{-2} day^{-1}, depending on the location and time of the year. Although rates of bacterial production by volume are typically higher in nutrient-rich water, as occurs along coastlines, oligotrophic regions of the ocean are generally much deeper. For this reason, oligotrophic regimes may have higher integrated (by surface area) bacterial production than coastal areas (94). In both marine and freshwater systems, rates of bacterioplankton community production by volume are often as large as the rate of zooplankton community production (99).

Mean specific growth rates of marine bacterioplankton range between 0.1 to 1 d^{-1} (94). Doubling times vary between 3.5 and 40 days, with the shorter time from the North Pacific during a bloom of phytoplankton, and the longest doubling time from the very cold Ross Sea (94). In most of the ocean, because rates of bacterioplankton production are extremely low but abundance is fairly

high, bacterioplankton growth rates are at the low of this range (100).

Commonly used techniques to measure each of the variables described earlier (production, specific growth rate, and doubling time) provide mean values for the bacterioplankton community. These may be of much use, but in a bacterioplankton community, individual organisms may vary widely in their rate of reproduction and metabolic activity. For example, under poor conditions, cells may stop growing and form resistant structures such as spores, or other quiescent states (101). Using epifluorescence microscopy alone, it can be difficult to distinguish between active, living cells and resting, or even dead cells. Clearly, interpretations regarding the community ecology of bacterioplankton would be very different if it were known that the population consisted of only a small fraction of actively growing cells, instead of consisting of cells growing at similar rates.

A variety of methods have been used to determine the percentage of actively growing cells in a bacterioplankton community. Some studies have suggested that most bacterioplankton are active and growing (102), but this may not always be the case (103). Small cells appear to be less active than large cells (104–106), and some bacterioplankton populations appear to consist of relatively few highly active cells in the presence of a greater number of dormant, or less-active, bacterioplankton (94,107). It is very likely that bacterioplankton constantly vary in metabolic activity, depending on nutrient availability or other factors, but the dynamics of bacterioplankton cell activity currently are poorly known.

THE FACTORS THAT CONTROL HOW FAST BACTERIOPLANKTON GROW

Depending on the composition of the community, rates of bacterioplankton production are affected by numerous environmental factors including organic substrate concentration, the concentration of inorganic nutrients, temperature, the presence of possibly inhibiting chemicals, sunlight intensity, oxygen concentration, pH, and so on. In this section, the influence of several important factors, such as mineral nutrient availability, substrate concentration, and temperature, is discussed.

Like other organisms, bacterioplankton biomass is mostly composed of the elements carbon, nitrogen, and phosphorus. Minor (or trace) elemental constituents include sulfur, iron, magnesium, and potassium. According to Liebig's law of the minimum (108), the element in the lowest concentration relative to the element ratios required by an organism for growth will limit, or regulate, that organism's growth rate. Probably, the element-limiting heterotrophic bacterioplankton growth rates varies geographically and temporally (109,110). For example, in coastal environments, heterotrophic bacterioplankton appear to be more often limited by organic carbon, whereas in offshore environments and many lakes, limitation of growth rate by mineral nutrients such as phosphorus or iron is more likely (110–113).

Heterotrophic bacterioplankton and phytoplankton (both prokaryotic and eukaryotic) depend largely on the same pool of dissolved nutrients. Their relative needs for these nutrients can be evaluated by their elemental biomass ratios (111). The C : N ratios of heterotrophic bacterioplankton and phytoplankton appear to be similar, between five and seven on a molar basis. In contrast, the C : P ratio of heterotrophic bacterioplankton is lower (average 53) than the C : P ratio of most phytoplankton (average 106) (114). In other words, relative to their requirement for carbon, most heterotrophic bacterioplankton have a higher demand for phosphorus than do phytoplankton. Consequently, where phosphorus is in low concentration, heterotrophic bacterioplankton growth may be limited by P availability, as demonstrated in numerous studies (109,115,116). Thus, there may be competition for this element between phytoplankton and heterotrophic bacterioplankton (111,116). The potential for such competition may be modified by the fact that phytoplankton, being autotrophic, are generally not limited by carbon, whereas heterotrophic bacterioplankton depend, at least partly, on phytoplankton production as a source of organic carbon. Little is known about the trace metal requirements for most naturally occurring bacterioplankton.

The mineral nutrients and organic substrates used by bacterioplankton have multiple sources. In small lakes or coastal environments, organic and inorganic molecules derived from aquatic plants or the watershed are important. In regions where there are high concentrations of animals (including humans) and in agricultural regions, organic and inorganic materials from these sources may stimulate vigorous growth of eukaryotic algae and bacterioplankton in adjacent aquatic ecosystems (117).

In the open ocean, and in pelagic areas of many large lakes, DOM and some mineral nutrients supporting bacterioplankton growth are in low concentration. In these environments, mineral nutrients may be derived largely from recycling processes occurring in the water column or, in some cases, from atmospheric dust. The primary source organic matter in these environments is production by autotrophic plankton, especially cyanobacteria and eukaryotic phytoplankton. The organic matter synthesized by these autotrophs is crucial to heterotrophic bacterioplankton production (118,119). In marine water, for example, about 50% of the primary production is processed daily by bacterioplankton (120).

There are several ways for the organic matter synthesized by phytoplankton to become utilizable by heterotrophic bacterioplankton. Phytoplankton produce both particulate cell structure and dissolved organic matter. The particulate matter becomes available for colonization and decomposition by bacterioplankton when the phytoplankton die, or as waste products of zooplankton that have consumed the phytoplankton. Upwards of 10% of the primary production leaches as dissolved organic molecules directly from the phytoplankton into the water (121). Although this source of DOM is important to bacterioplankton production, it is probably often insufficient to fulfill their demand. Perhaps more important ways that this photosynthetically derived DOM becomes available are in the waste products of protists and zooplankton, or by lysis of bacterioplankton cells by viral infection or other stresses (121,122). Nagata (121)

estimates that as much as 30% of the ingested organic matter of the prey is released as DOM by heterotrophic protozoa, and as much as 20% is released by zooplankton. Mechanisms of bacterioplankton mortality are discussed further in the following text.

In interecosystem comparisons, heterotrophic bacterial production is highly correlated with the production of phytoplankton biomass (net primary production), yielding a ratio of heterotrophic bacterial production to net primary production generally about 20 to 30% (99). Some exceptions to this relationship have been noted. For example, in the polar Gerlache Strait, Bird and Karl (123) reported a BP/PP ratio of 2 to 3%. They attributed the low bacterial production to the fact that bacterioplankton were kept in low numbers by protist grazers. Nevertheless, the correlation discovered by Cole and coworkers (99) suggests a dependency of heterotrophic bacterioplankton production on organic matter derived directly or indirectly from phytoplankton. This "coupling" is certainly the case in many systems, but in individual systems, especially where terrestrial sources of organic matter are significant, heterotrophic bacterioplankton growth may be less closely linked to phytoplankton production (124).

As it is for most organisms, temperature also is an important regulator of specific growth rates of bacterioplankton. Most cultivated bacterioplankton have a optimum range of temperature at which growth rate is at a maximum, growth rate declines with increasingly lower or higher temperature. The optimum temperature range varies between different isolates of bacterioplankton; some do better at cold temperatures, whereas others are best adapted to warmer, or even hot temperatures. In temperate ecosystems, bacterioplankton growth rates are positively correlated to temperature at least up to between 10 and 15 °C (88,125,126). But, as is evident from measurements of active bacterial metabolism in polar water, some bacterioplankton can grow at temperatures approaching the freezing point of seawater. These psychrotolerant (cold-tolerant) or psychrophilic (grow optimally at cold temperatures) bacterioplankton may play an important role in nutrient cycling both in polar water and the permanently cold deep sea (127).

Bacterioplankton are influenced by a multitude of interacting stimuli. Pomeroy and Wiebe (127), for instance, described how the relationship of the rate of bacterioplankton production and temperature can vary with substrate concentration. The basis of this interaction effect is probably that a reduction in temperature reduces substrate uptake and/or assimilation. Accordingly, an increase in substrate concentration, even at the low temperatures of polar water, can stimulate an increase in the rates of heterotrophic bacterioplankton activity. It is possible that the correlation observed between the temperature and bacterial productivity observed in some temperate lakes may be partly related to a reduction in substrate availability during colder months (128).

BACTERIOPLANKTON MORTALITY

Not long after the direct-count method of enumerating bacterioplankton was introduced, it was observed that their abundance does not vary more than about one order of magnitude. This is surprising because substrate concentrations, and bacterioplankton community production, can vary from one to several orders of magnitude between systems (129).

How can bacterioplankton exhibit turnover times as fast as a few days in eutrophic environments to as long as several weeks or more in oligotrophic environments while their, abundance is relatively uniform across trophic gradients? Most researchers now attribute this to density-dependent loss of bacterioplankton production. In other words, the faster bacterioplankton reproduce, the faster they die (129,130). This leads to the question of how bacterioplankton die.

Bacterioplankton die in various ways. One of the most important is by grazing of flagellated protists. In both the ocean and freshwater ecosystems, flagellated protists remove a large proportion of bacterioplankton production (131). These protists are mostly less than 20 μm in cell diameter and referred to as *nanoflagellates*. Depending on the species, they may be entirely heterotrophic, or combine photosynthesis with particle-feeding (132). Abundances of heterotrophic nanoflagellates may reach 10^6 cells per liter, or more (132). Other protist bacteriovores in the ocean include ciliates and sarcodines (foraminiferans and radiolarians) (133).

Ciliates can be significant consumers of bacterioplankton in freshwater and marine environments, but in most studies do not seem as important as nanoflagellates (134,135). For example, in a reservoir in South Bohemia, on an average about 70% of the bacterioplankton production was consumed by heterotrophic flagellates, and about 20% by ciliates (136). At least some protist grazers are selective in the bacterioplankton they eat based on prey size (137–139), activity (140), and species (141). They tend to remove more larger cells than smaller cells, and prefer motile over nonmotile bacterioplankton, although the latter effect could be a passive selection (142).

Animals also consume bacterioplankton. In marine environments, these include the larval and juvenile stages of some copepods, larvaceans, salps, and doliolods (131). In freshwater ecosystems, the major animal bacteriovores are cladoceran zooplankton, especially *Daphnia* spp., which when abundant can be even more significant as a source of bacterioplankton mortality than protist grazers (130,143,144).

Another important source of bacterioplankton mortality is lysis caused by viruses (bacteriophages) (145–147). Viruses are common in both freshwater and marine environments with abundances from 10^7 to more than 10^8 per liter, giving a ratio of viruses to bacteria of 2 to 50. Viral abundance, as is bacterioplankton abundance, is directly correlated with trophic conditions. Proctor and coworkers (148) observed that the percentage of visibly infected marine bacterioplankton ranged between 1 and 4%, but as infection is only visible in the last 10 to 20% of the infection cycle, they proposed that the actual percentage of infected bacterioplankton is probably closer to 5 to 40%. As viral infection rates increase with host abundance, it is likely that lysis by bacteriophages is a

more important cause of bacterioplankton mortality in eutrophic than oligotrophic environments (149,150).

Estimates of bacterial mortality due to bacteriophage infection range from less than 10 to more than 50% of the standing stock of bacterioplankton in the ocean (145,148,150,151). In Lake Constance, Germany, bacteriophage-induced mortality of bacterioplankton ranged between 1 and 24% of total mortality over the year (128). Seasonal patterns in bacteriophage activity have been examined in few other freshwater systems. In anoxic water, where nanoflagellates are not important, bacteriophage infection may be the primary cause of bacterioplankton mortality (151).

Theoretical analyses suggest that the taxonomic diversity of a bacterioplankton community will correspond both to the number of unique resources and the number of host-specific mechanisms of mortality (152). Both bacteriophages (151) and, as mentioned earlier, some protists show selectivity in bacterioplankton species they infect or consume. On the other hand, some bacteriophages may have a broad host range. For example, 7 of 85 bacteriophage isolates from the North Sea were capable of infecting 11 to 36 different hosts (153). At the same time, many bacterioplankton may be resistant to viral infection, as demonstrated for some strains of the cyanobacterium *Synechoccoccus* spp. (154). At present, the extent to which grazers and bacteriophages influence bacterioplankton community composition is still an open, fascinating question.

In addition to selective lysis of bacterioplankton, viruses may also influence microbial community structure via *transduction*. In transduction, segments of the host DNA incorporated by error into a bacteriophage during replication in one host cell are subsequently introduced into the genome of a second host cell on the movement of the phage from the first to the second host (155). In this way, transduction acts to transfer genetic information between similar or even distantly related hosts. Jiang and Paul (156) estimated transduction events in water off the coast of Florida at between 0 to 100 transductants per liter per day. It can also be an important mechanism of gene transfer in freshwater ecosystems (157,158). Although its ecological significance is not yet clear, transduction may act as a "genetic stabilizing mechanism" by reducing genetic differences between susceptible hosts in the community (147).

There remain many questions regarding the importance of lysis by bacteriophages compared with grazing with respect to bacterioplankton mortality in water of different trophic status. Especially in low productivity marine water, most studies show grazing rates by protists and zooplankton that exceed bacterioplankton production (131). But even in low productivity coastal water, bacteriophages can cause as much as 35% of bacterioplankton mortality (159,160). In more eutrophic water, in contrast, measured grazing rates are often less than measured rates of bacterioplankton production (129), suggesting a greater role for bacteriophages (131).

Grazers and bacteriophages can affect microbial community function as well as structure. By causing lysis of bacterioplankton and promoting nutrient release into the water, grazers and bacteriophages can stimulate cell-specific bacterioplankton and perhaps rates of phytoplankton production (161–165). Fuhrman (147) has hypothesized that lysis by viruses of bacterioplankton in the oligotrophic ocean may increase bacterioplankton production by about 30%; on a global scale, this could have important consequences for nutrient flux in the ocean.

Other mechanisms of bacterioplankton death have been considered, but their significance relative to grazing and viral lysis is not known. Some bacterioplankton may be lost from the water column by simply sinking; this is most probably true where cells are attached to a particle (92). Other potential sources of mortality are exposure to antibiotics produced by competing bacterioplankton (166), exposure to intense sunlight, or autolysis during times of nutrient starvation.

THE ECOLOGICAL SIGNIFICANCE OF THE BACTERIOPLANKTON

Significance in Nutrient Cycling

Bacterioplankton are critical to the cycling of carbon, nitrogen, phosphorus, sulfur, and numerous metals including iron, manganese, arsenic, antimony, mercury, copper, cobalt, selenium, cadmium, lead, and silver. Through their activities, bacterioplankton both respond to and help create the chemical environment of aquatic ecosystems, and even the atmosphere above aquatic ecosystems. Via consumption and/or regeneration of nutrients, heterotrophic bacterioplankton can both stimulate and inhibit the productivity of cyanobacteria and eukaryotic phytoplankton, depending on the environment. In this section, the activities of the bacterioplankton with respect to uptake, transformation, and regeneration of the macronutrients carbon, nitrogen, and phosphorus are discussed.

The role that bacterioplankton play in nutrient cycling depends on whether they are autotrophic or heterotrophic, whether they are in an environment with low nutrient and organic matter concentrations or a richer environment, whether they are attached or free-living, and on their species-specific ability to consume and/or metabolize a particular material. Like plants and eukaryotic algae, autotrophic bacterioplankton such as cyanobacteria and chemolithoautotrophs depend on inorganic compounds as sources of nutrients. Examples include CO_2, NH_4, NO_3, and PO_4. Heterotrophic bacterioplankton, on the other hand, can use either inorganic compounds or many types of small organic compounds as sources of nutrients. Decomposition of an organic compound, such as a peptide or carbohydrate, in addition to releasing energy, can make available various organic and inorganic components of the compound that subsequently may be assimilated into biomass or, if unrequired, released into the environment. Nutrient regeneration occurs when, in decomposition of an organic molecule, inorganic molecules are produced and released into the environment. The process in which inorganic compounds are produced by the decomposition of an organic compound is called *mineralization*. Inorganic molecules released into the environment by mineralization can be used by other microorganisms that depend on dissolved chemicals for nutrition.

Broadly speaking, bacterioplankton have dual roles in nutrient cycling through consumption and regeneration of dissolved nutrients (114). What determines whether a heterotrophic cell excretes or assimilates an element that it has consumed and is important in biomass synthesis? Where a consumed element is limiting bacterioplankton growth by being in short supply relative to the availability of other required elements, it is generally retained. Where it is in excess relative to the availability of other required elements, it is released or sometimes stored (11). For example, Tezuka (167,168) observed that above a freshwater algal substrate N : P ratio of 17 : 1, nitrogen was released during decomposition; below this ratio phosphorus was released. Similarly, Goldman and coworkers (169) found that nitrogen was released by a marine bacterioplankton assemblage only when the substrate C : N ratio was less than 10 : 1. In a study of three lakes in Ontario, Canada, Elser and coworkers (111) measured N : P biomass ratios in the bacterioplankton size fraction approximately between 8 and 35; the variance mostly was due to cell-specific phosphorus content rather than nitrogen content. At a N : P biomass ratio less than about 17 to 25 : 1, bacterioplankton retained the nitrogen. Above this ratio, where bacterioplankton presumably contained sufficient N to meet their needs, they released the nitrogen. These studies suggest that it is the stoichiometric relationship of substrate and the organism that has consumed the substrate that determines whether or not an element is released or not during decomposition (111).

Bacterioplankton and the Carbon Cycle

Bacterioplankton interact with the carbon cycle as both autotrophic synthesizers of organic molecules and heterotrophic consumers and decomposers of organic molecules. In the photic zone of the sea and lakes, cyanobacteria and eukaryotic phytoplankton provide much of the organic matter consumed by heterotrophic bacterioplankton and animals. In the absence of heterotrophic activity, organic matter would accumulate, and nutrients would not be regenerated in inorganic forms usable by primary producers. Where an environment has become polluted by sewage or even oil, consumption and degradation of these pollutants by heterotrophic bacterioplankton can be an important mechanism for the removal of these unwanted compounds. Wetzel (14) and Williams (170) provide comprehensive discussions of the various roles of bacterioplankton with respect to the decomposition of organic matter.

Rates of decomposition of organic matter depend, among other factors, on the temperature, availability of electron acceptors, and chemical structure of the substrate. As increased in temperature stimulates the rate of contact between enzyme and substrate, rates of decomposition tend to increase with a rise in temperature, at least up to the point at which the enzyme structure is disrupted (denatured). As discussed earlier, the higher the reduction potential of an electron acceptor, the more energy is released on oxidation of a substrate, and the faster the substrate will decompose. Finally, different kinds of organic matter decompose at various rates, depending on the state of the matter (dissolved or particulate), its molecular weight, and its accessibility to digestive enzymes. As is true for all cells, heterotrophic bacterioplankton can absorb only small dissolved organic molecules, such as amino acids, nucleotides, and simple carbohydrates. Larger dissolved molecules or particulate organic matter are first disassembled outside the cell by cell surface–attached hydrolytic enzymes (exoenzymes) (171). The small molecular subunits released by extracellular digestion can be subsequently consumed by the cell where they are used as an energy source by oxidation, or possibly directly incorporated into new cell biomass. The bacterioplankton-mediated turnover time for small organic compounds, such as simple sugars and amino acids, can be on the order of hours, whereas larger, polymeric organic compounds, such as complex polysaccharides (cellulose and hemicellulose) and lignin (complex structural polymer of wood), may persist in the environment for years.

In some circumstances, heterotrophic marine bacterioplankton, in addition of contributing to mineralization of organic matter, produce some dissolved organic compounds that are very slow to decompose, or refractory (172). The mechanism of how bacterioplankton produce refractory dissolved organic matter is unknown (172), but because these compounds degrade slowly, they can remain in the ocean for a long time. In this context, it is interesting to note that the mean age of dissolved organic matter in the ocean is greater than 2,000 years in surface water and 4,000 to 6,000 years in deep water (170).

Another important process in the cycling of carbon in aquatic environments is methanogenesis, the production of methane (CH_4). Methane is a product of metabolism by several different biochemical pathways, depending on the organism. All organisms producing methane are in the domain Archaea. They are obligate anaerobes and not likely to occur as free-living cells in the ocean, although they may occur in the guts of animals, anoxic bottom waters of lakes, and in sediments. The methane evolved may be used as an energy source by some bacterioplankton (methylotrophs), or may escape to the atmosphere where it acts as a greenhouse gas. Methanogenesis is more significant in freshwater environments than in the ocean both because freshwater environments are more likely to have an anoxic region of the water column and because methanogens are outcompeted for substrates by sulfate-reducing bacterioplankton in anoxic environments in which sulfate is abundant (173).

Bacterioplankton and the Nitrogen Cycle

Nitrogen, next to carbon, is the most quantitatively important constituent of microbial particulate biomass, and bacterioplankton are important in nitrogen cycling in a variety of ways. As mentioned earlier, many cyanobacteria fix nitrogen, which is a mechanism for the incorporation of inorganic molecular nitrogen into microbial biomass. In ammonification, bacterioplankton deaminate peptides with the liberation of NH_3. The NH_3 can be incorporated into cell biomass as, for example, an amino acid, or released from the cell into the environment. Some chemolithoautotrophic nitrifying bacteria use NH_3

as a source of energy for carbon fixation, oxidizing it ultimately to NO_3. In anoxic environments, denitrifying bacteria use NO_3 as an electron acceptor, reducing it to N_2. Finally, dissolved ammonium and nitrate are consumed directly from the water column by both autotrophic and heterotrophic bacterioplankton, and phytoplankton. Capone (174) provides a good overview of the marine microbial nitrogen cycle.

One way of quantifying the importance of bacterioplankton in nutrient flux is to calculate the percentage of an element consumed by heterotrophic bacteria relative to phytoplankton. Kirchman (114) notes that the uptake of ammonium by heterotrophic bacteria relative to the total uptake by the plankton varies from a low of 5% to as much as 78% in Georgia coastal waters. The overall median percentage is 40%. The energetic cost of nitrate uptake is higher than for ammonium; heterotrophic bacterioplankton account, on average, for about 16% of the nitrate uptake by the plankton, but this can be as much as 31% (114,175).

Bacterioplankton and the Phosphorus Cycle

Phosphorus is an essential element in nucleotides and nucleic acids (ATP, RNA, DNA) and phospholipid molecules, a major constituent of cell membranes. The primary source of phosphorus for bacterioplankton is dissolved orthophosphate (PO_4^{3-}), although dissolved organic phosphorus compounds may also be used, particularly at high substrate concentrations (14,176). Where an organic phosphorus-containing molecule is mineralized by an organism, and phosphorus in the molecule is in excess of the nutritional requirements of the organism, the phosphorus is released into the water column, primarily as PO_4^{3-}. In this way, heterotrophic bacterioplankton can regenerate phosphorus in a form that readily can be used by autotrophic organisms. However, because heterotrophic bacterioplankton growth is often limited by phosphorus, the bacterioplankton often can be more important as competitors with the phytoplankton for phosphorus, as opposed to providers (177–179). In these systems, the most important mechanisms for phosphorus regeneration may be death and lysis of bacterioplankton cells, or by the feeding and excretory activities of bacterivorous nanoflagellates and other grazers (180,181).

The median percentage of PO_4^{3-} uptake attributable to the size-fraction that is less than 1 μm when both freshwater and marine systems are considered is 60% (114). Where these measurements are corrected for uptake by cyanobacteria, between 24 and 46% of the PO_4^{3-} uptake is attributable to heterotrophic bacterioplankton.

The clear conclusion from these analyses is that bacterioplankton are exceedingly important in the turnover of growth-limiting elements in freshwater and marine systems. Their significance in plankton food webs is the subject of the next section.

IMPORTANCE IN THE FOOD WEB

The role of the bacterioplankton in the food webs of freshwater and marine systems is described by a conceptual model called the *microbial loop* (182,183). This model has been revised in various ways since it was first proposed, but remains useful as a means of illustrating the various roles that bacterioplankton have in the food web with respect to fluxes of energy and carbon. The essential features of the microbial loop are illustrated in Figure 1.

1. Solar energy captured by phytoplankton (cyanobacteria and eukaryotic phytoplankton) fuels primary production, the synthesis of new biomass from inorganic precursors including carbon dioxide.
2. By various mechanisms, much of this photosynthetically derived organic matter eventually enters the water in a dissolved state. Mechanisms cited as being important in this process include leaching directly from phytoplankton cells, lysis of bacterioplankton and phytoplankton by viruses or by other means, and excretion by heterotrophic protists and zooplankton.
3. Heterotrophic bacterioplankton consume this DOM from the water column, using it as an energy source and/or a source of new cell material, and respiring a portion of it as carbon dioxide.
4. Once converted into bacterioplankton biomass, this previously dissolved carbon is available to bacteriovores, and thereby enters (or reenters) the food web of particle-feeding organisms.

The model is referred to as a *loop* because DOM released from phytoplankton can, by incorporation into bacterial biomass, theoretically be returned to the food web (183). Following the discovery that bacterioplankton obtained much of their carbon from DOM, and that they had turnover times of as fast as a few days, it became of interest to know what proportion of DOM consumed by

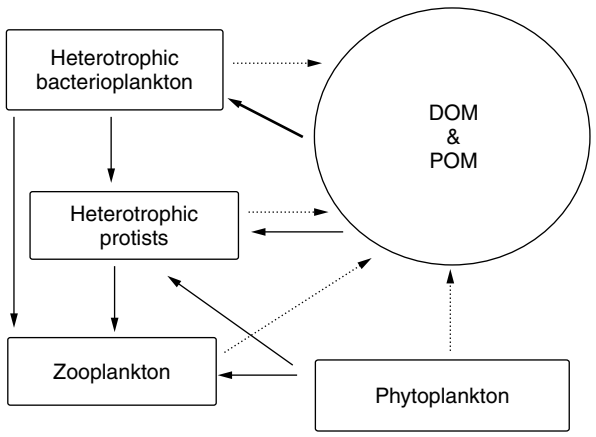

Figure 1. Main pathways of flux of organic matter through the microbial food web. Nonliving organic matter is represented by the compartment labeled DOM and POM (dissolved and particulate organic matter). All other compartments shown represent living organisms. Solid arrows show pathways of consumption of organic matter. Dashed arrows show pathways by which dissolved (DOM) and particulate (POM) organic matter is released from living organisms. This food web model is referred to as the *microbial loop* because DOM from all sources is partly channeled back into the food web by uptake by heterotrophic bacterioplankton, a pathway indicated by the large arrow.

bacterioplankton is ultimately realized in the secondary production of metazoan organisms, such as zooplankton and fish. The answer to the question depends on several factors. These include the number of links (i.e., the length of the food chain) between bacterioplankton and metazoan organisms, the growth efficiency at each step in the food chain (i.e., the proportion of consumed organic matter that is retained and converted to new biomass verses respired), and the extent that bacterioplankton are lysed by viruses as opposed to being grazed by protists or animals. By definition, the lower the growth efficiency at each trophic step, the higher the percentage of consumed organic carbon that is respired to carbon dioxide, and the less that is available for transfer to the next trophic link. Are bacterioplankton a link for the channeling of dissolved organic matter to metazoan consumers or are they a sink for this organic matter, such that most of the DOM they consume is never realized in metazoan production?

In an experiment to address this question directly, Ducklow and coworkers (184) introduced ^{14}C-labeled glucose into the plankton of an enclosed 300,000-L marine water column at Loch Ewe, Scotland. After 13 days, about 20% of the total label added was found in particulate matter, but only about 2% of the label ever passed into organisms greater than 1 μm in diameter. It was concluded that, at least in this environment, bacterioplankton acted primarily as a sink for organic carbon, such that it was mostly respired away by the microbial size fraction (184).

The link versus sink question was tested in a freshwater ecosystem by Wylie and Currie (185). Several of their conclusions are of interest. In agreement with Ducklow and coworkers (184), they found that bacterioplankton carbon is inefficiently transferred to zooplankton, but they also noted that algal carbon is transferred almost as inefficiently. For this reason, they suggested that it is misleading to single out the bacterioplankton as the only major sink for carbon in aquatic food webs; respiratory loss of carbon is high in both the autotrophic and heterotrophic components of the microbial food web. In terms of the total flux of carbon into zooplankton, however, they found that, depending on the types of zooplankton present, bacterioplankton could be important in their diet. Where copepods dominated the zooplankton community, insignificant amounts of carbon derived from bacterioplankton was measured in animal biomass. This can be explained by the fact that most copepods cannot feed on bacterioplankton directly. However, where the zooplankton community was dominated by cladocerans that can directly graze bacterioplankton, as much as 21% of the total flow of organic carbon to the zooplankton could be accounted for by bacterioplankton (including autotrophic picoplankton). They proposed that in systems in which cladocerans are abundant, and in which bacterioplankton biomass is high relative to algal biomass, as, for example, following the decline of an algal bloom or where terrestrial inputs of DOM are high, bacterioplankton can be important in directly supporting zooplankton productivity. As cladoceran zooplankton do not occur in the ocean, and as lake environments are more likely to receive loading of DOM from external sources than are marine environments, these results suggest a fundamental difference in the role of bacterioplankton as a food source in some freshwater plankton food webs compared with marine systems.

THE MAJOR QUESTIONS REGARDING BACTERIOPLANKTON

There are numerous questions regarding bacterioplankton that remain to be resolved. Some of these could not be adequately addressed until the recent development of molecular approaches for evaluating microbial diversity and in situ metabolic activity (8). As these questions have been at the heart of much of the ongoing research in microbial ecology in the last decade, and answers are beginning to emerge, the next decade promises to be an exciting and productive time for the study of aquatic microbial communities in general.

1. What is the taxonomic diversity of the bacterioplankton in different environments? What are the spatial and temporal patterns in taxonomic diversity?
2. What is the relationship between taxonomic composition and physiological function of bacterioplankton communities? How important is genetic microdiversity in niche partitioning of closely related organisms?
3. Are the newly discovered, many still uncultivated, organisms similar (or redundant) or distinct in physiological function? What is the significance of the physiological activities of the cultivated and uncultivated bacterioplankton to the patterns of energy and material flux through aquatic environments, in other words, ecosystem function?
4. What are the regulating factors that influence bacterioplankton population diversity and physiological activity? How are bacterioplankton community composition and function influenced by predators and parasites, or substrate composition, or light intensity or composition, or the interaction effects of these various environmental factors? Exactly what chemical compounds do bacterioplankton use, and in what ways?
5. Is there gene exchange between bacterioplankton, and to what degree? What are the mechanisms of gene exchange? What is the influence of gene exchange on bacterioplankton population composition, response to environmental change, and evolution?
6. What are bacterioplankton mortality factors? What is the relative importance of viruses, protozoa, and animal grazers in different environments? How does the kind of mortality bacterioplankton suffer shape the composition and metabolic activity of the bacterioplankton community?
7. What other kinds of interactions occur between different species of bacterioplankton, or between bacterioplankton and other organisms of the microbial community? Under what conditions do heterotrophic bacterioplankton and phytoplankton compete for nutrients, or support each other through excretion

of metabolites? What kinds of chemical communication, or chemical "warfare," symbioses or antagonisms, occur between microbial plankton and how do these behaviors influence nutrient cycling?

8. How might microbial biomass, or the physiological capabilities of the bacterioplankton, be used or modified for human application? What possibilities are there for bacterioplankton to be manipulated, perhaps engineered, to produce molecules of medical or industrial value, or as a source of food for humans or other animals, or in remediation of environmental damage?

Is microbiology entering a new golden age? Perhaps that question can only be answered in hindsight, but after many years of concerted effort, there is currently a strong feeling among microbiologists of finally being on the verge of a detailed understanding of the structure and function of natural bacterioplankton communities. Studies of bacterioplankton morphology, diversity, physiology, ecology, and genetic potential are certain to engage the interests of both basic and applied scientists from academia and industry for many years to come.

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PLANT–MICROBE INTERACTIONS IN THE MARINE ENVIRONMENT

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Intertidal and well-illuminated depths of subtidal zones support a wide variety of marine plant and algal species. The vascular plants are of particular importance because of their extensive growth in shallow marine environments and their great contributions to marine food webs and global element cycling. Marine vascular plants provide a variety of distinctive habitats for microorganisms and can greatly promote their growth and activity. Aboveground plant structures constitute the phyllosphere and typically support extensive growth of microorganisms. The phyllosphere is also a key location of plant biomass turnover for emergent plant species. Belowground plant structures and the sediments that they impact constitute the rhizosphere. The rhizosphere is heavily colonized by microorganisms and typically supports much higher levels of microbial biomass and activity than surrounding unvegetated sediments. Microorganisms can also be found within marine plant tissues and the endosphere constitutes a distinct and potentially important microbial habitat in marine vascular plants. Although the interactions between terrestrial plants and their microbiota have been intensively studied, those between microorganisms and marine plants have received substantially less attention. However, marine plant–microbe interactions are pervasive and have been shown to be extremely important to the productivity of marine ecosystems.

The major foci of this article are the microbiota associated with emergent and submerged marine grasses and the major interactions of the microbiota with their plant hosts. Much of the information available on marine plant–microbe interactions addresses nitrogen fixation (diazotrophy) and sulfate reduction. The information available on other microbial functional groups and their activities is also summarized.

PLANT-MICROBE INTERACTIONS OF EMERGENT MARINE PLANTS

Low energy intertidal zones in many parts of the world are dominated by emergent grasses, which occur in extensive and highly productive stands. Numerous species occur at various locations and one of the best studied is the smooth cordgrass, *Spartina alterniflora* (*Spartina* hereafter). *Spartina* is the dominant macrophyte in salt marshes along the temperate Atlantic coast of North America and the northern coast of the Gulf of Mexico. This plant grows extensively in the intertidal zone and can account for 75% of total primary production in some salt marsh systems (1). Because of its abundance and productivity and the extensive literature available on its microbiota, this species will be emphasized as an example emergent marine plant. Information on other plant species will be included as available and where appropriate.

Interactions in the Phyllosphere

The surfaces of the aboveground structures of *Spartina* are exposed to high levels of solar radiation and extremes of both temperature and desiccation, making them potentially hostile locations for many microorganisms. However, *Spartina* shoots and leaves become liberally coated with clays and other materials during tidal exchanges, particularly during spring tides. Epiphytic clay films are quite persistent and contain high levels of heterotrophic bacteria and *Cyanobacteria.* With the extremely high surface area of shoots in a *Spartina* marsh (as much as 6 m^2 of leaf surface for every m^2 of vegetated marsh), it is easy to see that this epiphytic microenvironment is potentially very important to microbially mediated nutrient dynamics and decomposition activities.

Most of the attention given to the epiphytic microenvironment of *Spartina* has focused on two key microbial activities, decomposition of standing dead plant biomass and diazotrophy. Both primary production (2,3) and decomposition (4,5) in many salt marshes are nitrogen-limited and diazotrophy is an important source of "new" nitrogen in these systems (6). In many instances heterotrophic diazotrophic bacteria are carbon- and energy-limited (7). Consequently, decomposition and diazotrophy in the phyllosphere can be viewed as somewhat interdependent. Standing dead biomass is abundant in *Spartina* marshes at all times of year and often occurs in about a 1 : 1 ratio with live plant shoots. The dead leaves and stalks are heavily colonized by fungi, particularly the ascomycetous fungus *Phaeosphaeria spartinicola* (8,9), which appear to be responsible for much of the decomposition activity. Decomposition rates for standing dead biomass are highly dependent on water availability, and when the stalks are wet the rates are comparable to those for submerged *Spartina* biomass and for other types of decaying vegetation (9,10). It is clear that initial decomposition of aboveground *Spartina* biomass begins in this standing dead phase before deposition of the dead leaves and stalks on the sediment surface or into tidal creeks.

Most of the diazotrophic activity in the *Spartina* phyllosphere is associated with standing dead shoots. Live shoots, even when heavily covered by epiphytic

clay layers, have only a fraction of the activity found in dead shoots (11). This virtual restriction of diazotrophy to standing dead shoots has been ascribed to a mutualistic interaction between the fungal decomposers and the diazotrophs although the specific organisms involved and the nature of the mutualism remain undetermined. The interactions of the various epiphytic and endophytic organisms in standing dead *Spartina* biomass support rates of diazotrophy equivalent to the highest rates reported for other decomposition processes (11). The key question of which organisms are responsible for diazotrophy in this phyllosphere system remains open.

Interactions in the Rhizosphere

The Rhizosphere Microenvironment. Emergent marine plants support extensive and highly active assemblages of microorganisms in close proximity to their roots. The rhizosphere can be defined to include the root surfaces (rhizoplane) and the surrounding sediments directly influenced by root products and their activities (12). In the case of some emergent marine plants, this zone of impact around roots and rhizomes can be fairly extensive and the rhizosphere sediment zones around different roots in dense root mats can overlap. This results in substantial microenvironmental heterogeneity within the root zone proper and makes precise delineation of where the zones of root impact begin or end difficult. The microbiota in this complex subsurface habitat include a substantial diversity of organisms having a broad range of growth requirements and physiological responses to plant-induced and edaphic (soil or sediment derived) variability in physicochemical conditions. Despite the substantial environmental variability that can occur over seasonal or shorter time frames, at least some of the residents of this habitat are persistent in that their signature nucleotide sequences can be recovered at different times of year (13).

Many of the organisms that can be isolated from root surfaces and surrounding soils utilize oxygen, which is provided in these otherwise anoxic soils and sediments through rhizosphere ventilation by the plants (Fig. 1). Marine plants grow in sediments that are typically water saturated and oxygen diffuses through water-filled pore spaces at only about 10^{-4} the rate in a similar unsaturated soil or sediment. The slow rates of oxygen diffusion and rapid consumption of oxygen by chemical and biological processes result in sediment anoxia at depths of only a few millimeters. In order to overcome the absence of oxygen and the presence of various phytotoxic compounds the plants ventilate their root systems (14–16). The importance of ventilation is clear because up to 60% of the biomass of salt marsh grasses such as *Spartina* is found below the sediment surface and this belowground biomass requires oxygen for respiration. A major adaptation of marine vascular plants is the development of a tissue containing interconnected intercellular gas-filled spaces called the aerenchyma, which can occupy as much as 70% of the plant tissue volume. Although the exact mechanisms of ventilation by many plants remain undetermined, diffusive and convective airflows through the aerenchyma of stems, rhizomes, and roots have been demonstrated for several species of emergent grasses (17), including *Spartina* (18). Most of these plants leak oxygen from root surfaces, thereby creating an oxidized rhizosphere (Fig. 2) that protects the plant from reduced toxins such as sulfides and reduced metal ions (19–21). This oxidized rhizosphere supports extensive populations of oxygen-utilizing bacteria. Anaerobic bacteria can also be found in reduced microzones around, on, and within the roots.

In addition to oxygen, emergent marine plants supply substantial amounts of labile carbon to the rhizosphere through exudation of soluble organic molecules, sloughing of mucigel, and decay of root tissues during senescence (12). Many rhizosphere microorganisms are intimately associated with the plant roots and their

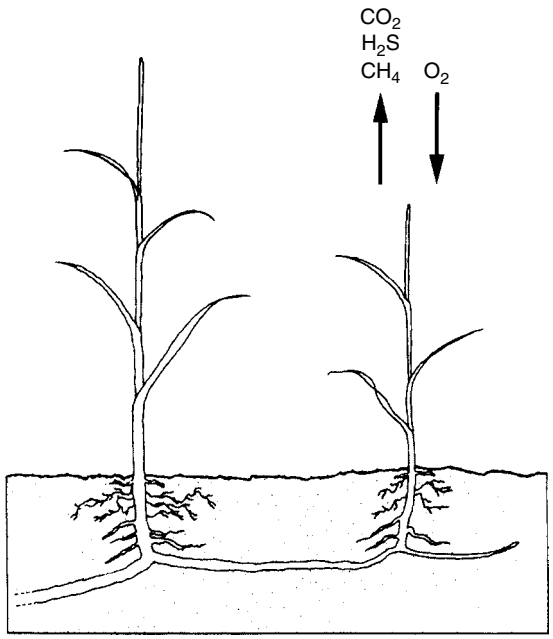

Figure 1. Mass transport of gases by the emergent marine grass, *S. alterniflora*. Gas exchange occurs through the aerenchyma and can result in substantial oxidation of sediments surrounding live roots. Modified from Dacey (14).

Figure 2. Sediment oxidation around live roots of the emergent marine grass, *S. alterniflora*. Note the dark strongly reduced sediments above the main root mass and the light-colored oxidized sediments associated with the root mass.

activities are strongly influenced by plant primary production (22–24). The activities of some rhizosphere microorganisms, particularly the diazotrophs, are strongly stimulated by soluble organic compounds from the plant tissues, including carbohydrates and carboxylic acids (24,25). The heterogeneity of the rhizosphere habitat in terms of organic carbon supply, provision of suitable terminal electron acceptors such as oxygen, and availability of presumed refugia from predation and/or chemical toxins provides niches for a great diversity of microorganisms.

Diazotrophic Bacteria and Diazotrophy in the *Spartina* Rhizosphere. Most of the nitrogen fixation activity in salt marshes is associated with plant roots (26,27). Consequently, the best-studied members of the rhizosphere microbiota of emergent salt marsh plants are the diazotrophic bacteria. Other rhizosphere bacterial groups that have received substantial study include the sulfate reducing bacteria (SRB) (28), some of which are also diazotrophs (29).

Numerous studies have employed pure culture isolation methods to recover diazotrophs from emergent grasses, including *Spartina* and the black needle rush *Juncus roemerianus* (*Juncus* hereafter). *Juncus* is an important plant found near the terrestrial fringe of many southeastern U.S. salt marshes and also occurs sporadically in small stands within the higher elevations of the *Spartina* zone. Other grasses, including other species of *Spartina* and *Juncus* are also found, but their rhizosphere microbiota have received less attention to date.

In addition to the distinct zonation in plant species observed in salt marshes (30), there is also a substantial gradation of plant morphology and productivity within the *Spartina* zone. Tall form plants ($\geq$1 m in height) grow along tidal creek banks. At higher elevations plant growth is stunted, resulting in short form plants ($\leq$30 cm in height). These differences in plant height and productivity are due to several pore water chemical parameters that form gradients from the tidal creeks to the upper marsh. The most important of these are soluble sulfide concentration, redox potential, and salinity (Fig. 3; 31). These edaphic factors, as well as temperature and ammonium concentration, also affect the activities of *Spartina* rhizosphere bacteria, particularly including the diazotrophs. Diazotrophic activity often differs between the tall and short form *Spartina* zones, with the tall form plants supporting higher levels of activity at most times of year (6,13,32).

A wide variety of diazotrophs have been recovered from the *Spartina* rhizosphere (Table 1). Obligately aerobic organisms including species belonging to the *Azotobacteriaceae* have been recovered from *Spartina*

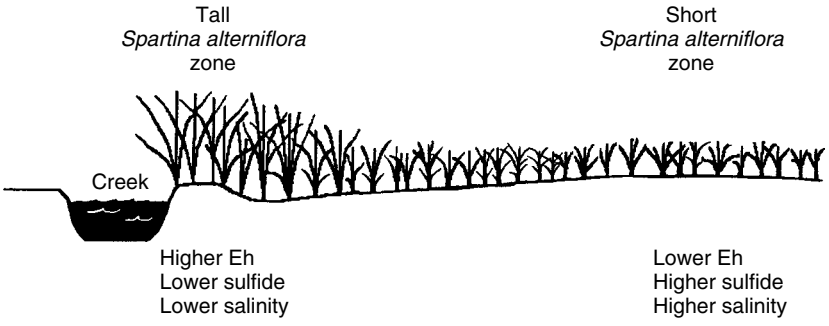

Figure 3. Diagram of the short and tall *S. alterniflora* growth zones showing key edaphic variables that influence plant height and productivity. Drawing by J. Pinckney.

Table 1. Examples of Diazotrophic Bacteria Isolated from the Rhizoplanes, Rhizospheres, or Vegetated Sediments Associated with the Emergent Marine Grass, *S. alterniflora*. These organisms reflect only a minor portion of rhizosphere diazotroph diversity, but provide examples of some key groups

Organism	Plant Source	Key Physiological Features	Reference
Arcobacter nitrofigilis	Rhizosphere	Microaerophilic, respiratory metabolism of organic and amino acids, anaerobic growth on aspartate and fumarate	38
Azotobacter sp.	Surface sediments Rhizoplane	Obligately aerobic, respiratory metabolism of carbohydrates, cyst formation	34 33
Clostridium sp.	Surface sediments	Obligately anaerobic, fermentative, endospore formation	34
Desulfovibrio sp.	Surface sediments	Obligately anaerobic, spiral shaped, sulfate reducing	34

rhizoplane (33) and from surface sediments in the *Spartina* growth zones (34). These are among the most abundant culturable diazotrophs in salt marsh vegetated soils and sediments, amounting to 10^7 cells (g dry wt)$^{-1}$ of sediments by most probable number (MPN) counts. It should be noted that the recovery of native bacteria from soils and sediments as well as other environments is subject to some important biases (35,36), including failure to recover species that are numerically significant and sometimes substantial underestimation of those species that are recovered. Numerical estimates based on laboratory cultivation should be viewed with caution, but cultivation does provide some insight into the types of organisms that can be recovered from natural samples and *Azotobacter*-like species are recovered frequently. Much lower numbers (around 10^3 cells g^{-1} sediment) of these organisms have also been recovered from unvegetated estuarine sediments (37). None of the 30 *Azotobacter*-like strains isolated by Herbert (37) would fix nitrogen in the presence of NaCl, which implies little participation by at least the isolated representatives of the *Azotobacteriaceae* in salt marsh nitrogen fixation.

Many microaerophilic and facultatively anaerobic organisms, mostly unidentified to date, have been recovered from *Spartina* rhizoplane (33) and from rhizosphere sediments (26). Approximately 10^5 heterotrophic diazotrophs per gram of rhizosphere sediment were recovered using semisolid media supplemented with glucose or malate (22,26). Microaerophiles were markedly enriched in the rhizosphere sediments relative to nearby nonvegetated sediments, demonstrating a pronounced "rhizosphere effect" for these organisms. Species likely belonging to the *Enterobacteriaceae* and the *Vibrionaceae* are among the microaerophilic and/or facultatively anaerobic organisms frequently isolated from *Spartina* rhizosphere (33) and from other estuarine sediment sources. Both *Klebsiella* spp. and *Enterobacter* spp. have been reported (37). Some *Klebsiella*- and *Enterobacter*-like isolates do not fix nitrogen in the presence of salt or seawater (37), whereas others are salt-tolerant (39). An obligately microaerophilic species, *A. nitrofigilis* (formerly *Campylobacter nitrofigilis*), has been isolated from *Spartina* roots and rhizosphere sediments from marshes as far apart as Georgia and Nova Scotia (38). This organism requires marine salinity for growth.

Obligately anaerobic diazotrophs, also mostly unidentified but apparently either fermentative (presumed *Clostridium* species) or sulfate reducing, have been amply demonstrated in the *Spartina* rhizosphere. MPN estimates of diazotrophic anaerobes were almost 10-fold higher than similar estimates of microaerophilic diazotrophs (26) and the majority of the organisms recovered seemed to be SRB. In contrast, nonrhizosphere surface sediments from a variety of salt marsh vegetation zones and from bare sediment locations, when inoculated into media suitable for *Clostridium* species or for *Desulfovibrio* species yielded only about 10^3 to 10^4 cells g^{-1} sediment for each group (34,37). Apparently there is a notable rhizosphere effect for at least some of these diazotrophic anaerobes. Studies employing sodium molybdate, a specific inhibitor of sulfate reduction, have demonstrated an important contribution of SRB to nitrogen fixation in shallow ($\leq$5 cm depth) short *Spartina* zone sediments (40). At greater depths, below the main mass of short *Spartina* roots and rhizomes, fermentative anaerobes contributed most of the diazotrophic activity (40). Both SRB and *Clostridia* isolated from estuarine sediments actively fixed nitrogen in the presence of NaCl and all 12 *Desulfovibrio*-like strains isolated by Herbert (37) required salt for diazotrophic activity. It is considered likely that diazotrophic SRB are significant contributors to rhizosphere diazotrophy in *Spartina* marshes although the specific organisms involved are not known (see following section).

In a recent study of oxygen-utilizing *Spartina* and *Juncus* rhizoplane diazotrophs, Bagwell and coworkers (33) inoculated fresh roots directly into semisolid media supplemented with different carbon or energy substrates but lacking combined nitrogen sources. Strains that grew out from the root surface and/or formed distinct bands of growth within the medium were recovered, isolated into pure culture, and characterized. A total of 339 strains were recovered; 67 from short *Spartina*, 103 from tall *Spartina*, and 169 from *Juncus*. All of these organisms were gram-negative and most were facultative anaerobes with a preference for microaerophilic growth conditions. The strains were grouped on the basis of physiological test results and tested for the presence of *nifH*, a key gene required for nitrogen fixation (see following section), yielding 54 groups of diazotroph strains (Table 2 and Fig. 4). Although nitrogen-efficient strains lacking detectable *nifH* were also isolated, 72% of the total strains recovered from *Spartina* and *Juncus* rhizoplanes were *nifH* positive. The majority of strain groups contained unidentified organisms probably belonging to the *Vibrionaceae* and *Enterobacteriaceae*. Some other strain groups contained unidentified organisms with characteristics similar to those of the *Rhizobiaceae*, *Spirillaceae*, *Azotobacteriaceae*, and *Pseudomonadaceae*. Although several strains were tentatively identified to genus or species level, the overwhelming majority of the isolates could not be easily identified on the basis of phenotypic traits. There is clearly an enormous diversity of oxygen-utilizing diazotrophs growing on the root surfaces of salt marsh grasses.

In addition to the great diversity of species and genera isolated from the rhizoplane of *Spartina*, there is a substantial range of physiological capabilities, such as substrate utilization, within many of the strain groups (41). This microdiversity is apparent even in strain groupings whose members are phylogenetically very closely related (i.e., strains within the same species), and probably contributes to physiological adaptation for the utilization of specific substrates or classes of substrates. Such specialization of ecotypes within closely related organism groups may reflect rhizosphere microenvironmental heterogeneity and a great variety of definable niches in the rhizoplane microenvironment. Extensive microenvironmental heterogeneity could contribute to the apparent stability of the rhizosphere microbial community (see following section), thereby preserving diversity. Microdiversity could also provide functional redundancy within the diazotroph assemblage, contributing to maintenance

Table 2. Key Physiological Features and Taxonomic Affiliations of Oxygen-Utilizing Diazotrophic Strains Isolated from the Rhizoplanes of Tall and Short Form *S. alterniflora* and *J. roemerianus*. Strains are designated by zone (S = Short Form *Spartina*, T = Tall Form *Spartina*, and J = *Juncus*), carbon source used for isolation (C = Citrate, M = Malate, G = Glucose, S = Sucrose), pH of the isolation medium (1 = pH 7.0, 2 = pH 7.5), and strain number. All listed strains were motile gram-negative rods. Physiological characteristics included in the table include oxygen requirements (A = Obligate Aerobe, M = Microaerophile, and F = Facultative Anaerobe), cytochrome oxidase production (OP = Oxidase Positive and ON = Oxidase Negative), and preferred substrate classes as determined from BIOLOG testing (C = Carbohydrates, CA = Carboxylic Acids, AA = Amino Acids, L = Low Utilization of all Substrate Classes, and B = Broad Substrate Range). The taxonomic groups closest in physiological characteristics to the unknown strains are provided, but these affiliations should be considered tentative. It should also be noted that the strains listed are representatives of strain groupings, some of which are not completely homogeneous. See Bagwell and Coworkers (33) for descriptions of isolation strategies and physiological and molecular biological testing methods. See Figure 4 for relationships among the various strains and to several known diazotroph species

Strain	Key Physiological Features	Closest Taxonomic Group
J-C2–37	F, OP, B	Unknown
T-C1–4	F, OP, C	Unknown
T-C2–3	F, OP, C, and CA	*Vibrionaceae*
T-C1–14	F, OP, C	*Vibrionaceae*
T-S2–12	A, OP, C, and AA	*Spirillaceae*
S-C2–8	F, ON, C, and AA	*Enterobacteriaceae*
J-M1–41	F, OP, B	*Enterobacteriaceae*
T-M1–5	F, OP, C, and AA	*Vibrionaceae*
J-M2–6	A, OP, C, and AA	*Spirillaceae*
J-M1–4	F, OP, C, and AA	*Vibrionaceae*
T-G2–15	F, OP, C, and AA	*Vibrionaceae*
S-C2–6	F, OP, C, and AA	*Vibrionaceae*
T-C2–8	M, OP, B	Unknown
T-C2–11	F, OP, B	*Vibrionaceae*
J-C1–1A	F, OP, C	*Vibrionaceae*
J-M2–1	F, OP, C, and AA	*Vibrionaceae*
S-M2–7	F, OP, AA	*Vibrionaceae*
T-G2–3	F, ON, C	Unknown
T-G2–12	F, ON, C	Unknown
S-C2–7	M, ON, B	*Vibrionaceae*
S-C1–11	F, OP, C	*Vibrionaceae*
J-C2–5	F, OP, C, and AA	Unknown
J-S1–23	F, ON, C	*Enterobacteriaceae*
S-M2–5	F, OP, B	*Vibrionaceae*
S-C1–9.2	F, OP, C	*Vibrionaceae*
S-C1–9.1	F, OP, C	*Vibrionaceae*
T-M2–29	F, OP, C	*Vibrionaceae*
J-S2–2	F, ON, C	*Enterobacteriaceae*
S-G2–1	M, ON, C	*Azotobacteriaceae*
S-G2–2	M, ON, C	*Pseudomonadaceae*
J-G1–1	M, ON, C	Unknown
J-S1–10	M, ON, C	Unknown
S-M1–12	F, OP, C, and CA	Unknown
S-M2–3RED	A, ON, B	Unknown
J-S2–16	M, ON, C, and AA	*Rhizobiaceae*
S-C1–1	A, ON, B	*Rhizobiaceae*
S-M2–6	F, OP, CA	*Vibrionaceae*
J-C1–10	M, OP, CA, and AA	Unknown
T-C1–1	F, ON, CA, and AA	Unknown
S-S1–1	M, ON, C, and AA	Unknown
S-M1–8	F, OP, C	*Enterobacteriaceae*
S-G2–4	M, OP, C	Unknown
T-G2–5	F, ON, C	*Enterobacteriaceae*
J-C1–25	F, OP, L	Unknown
S-M2–2	F, OP, L	*Vibrionaceae*
J-G1–8	M, ON, L	*Pseudomonadaceae*
J-C1–18	M, OP, L	*Spirillaceae*
T-M2–1	F, ON, L	*Enterobacteriaceae*
S-M1–1	M, ON, C	Unknown
J-S2–21	M, OP, L	Unknown
T-M2–14	M, ON, L	Unknown
T-G2–7	F, ON, CA	*Enterobacteriaceae*
S-C1–7	F, ON, L	Unknown
J-S2–28	M, ON, L	Unknown

(*continued overleaf*)

Table 2. (*Continued*)

of ecological function (diazotrophy) across a broad range of variation in environmental conditions.

Other recent studies have employed molecular biological methods to recover diazotroph signature nucleotide sequences from *Spartina* rhizosphere samples (13). Several of the genes encoding proteins required for nitrogen fixation are somewhat conservative in nucleotide sequence. The most conservative of these is *nifH*, the structural gene encoding the nitrogenase iron protein, which is found only in diazotrophic *Bacteria* and *Archaea*. Several researchers have devised polymerase chain reaction primers specific for *nifH* that greatly facilitate recovery of these diazotroph-specific sequences. The translated peptide sequences from these are suitable for phylogenetic analysis and have been used to examine the diversity of diazotrophic bacteria in various environments (42,43, and references therein). It should be noted that although molecular biological approaches of this kind avoid the biases encountered in pure culture isolation from natural samples, they also introduce certain biases of their own, affecting which sequences can be recovered. For a more complete discussion of these biases see Ward and coworkers (44) and Zehr and Capone (42). However, these molecular approaches appear to sample natural assemblages of diazotrophic bacteria more accurately than pure culture methods and permit the recovery of *nifH* sequences from novel, unknown, and currently uncultivatable diazotrophic organisms (42).

Piceno and coworkers (13) recovered *nifH* sequences from DNA extracted directly from short and tall *Spartina* rhizosphere core samples. Samples were collected from both tall and short *Spartina* zones at several different times of year to encompass the annual cycle of plant ontogeny and the range of edaphic variability that occurs during the year. The *nifH* sequences were resolved using denaturing gradient gel electrophoresis (DGGE), which

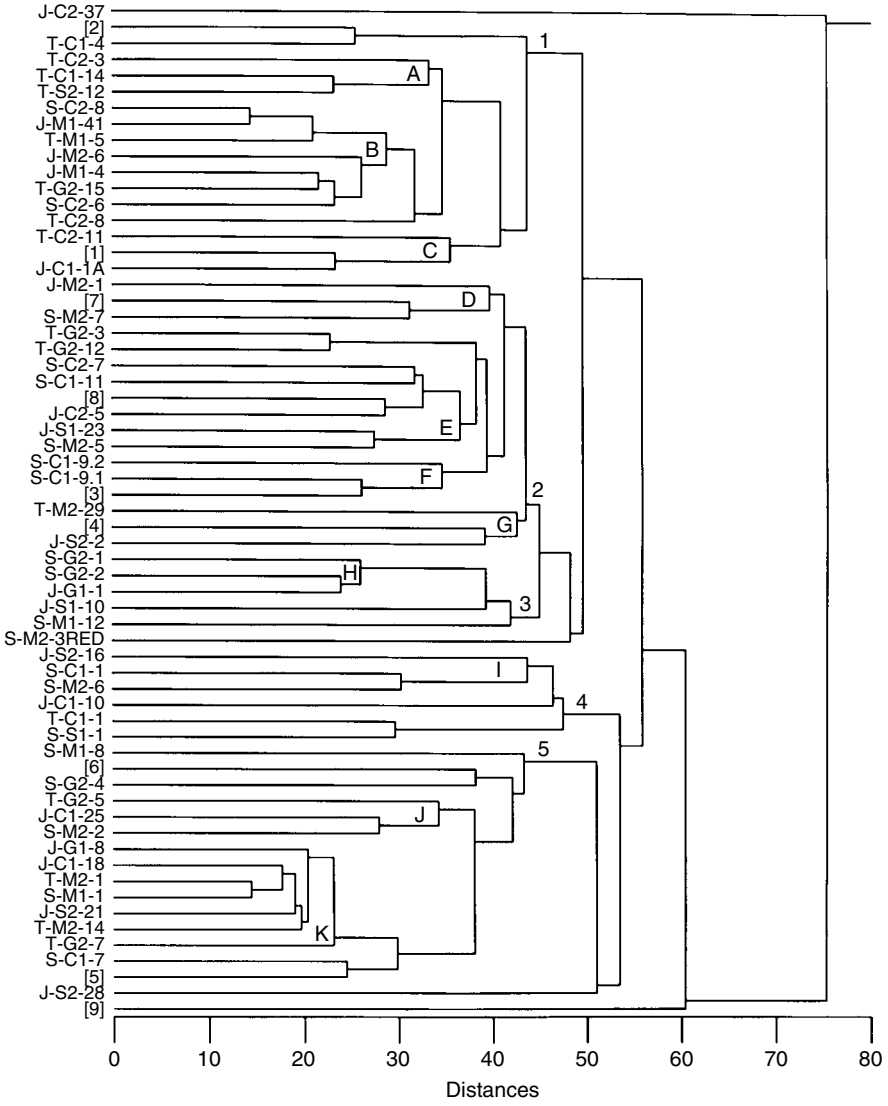

Figure 4. Dendrogram constructed from Euclidean distances of physiological test results for diazotroph strains from rhizoplanes of short and tall *S. alterniflora* and of *J. roemerianus*. Some selected known diazotroph species are included for comparison purposes. Strains designations are defined in the Table 2 caption. Known diazotrophs are as follows: [1] *Vibrio diazotrophicus*, [2] *Azospirillum brasilense*, [3] *Azotobacter chroococcum*, [4] *Klebsiella pneumoniae*, [5] *Azospirillum lipoferum*, [6] *Azotobacter diazotrophicus*, [7] *Rhizobium meliloti*, [8] *Rhizobium leguminosarum*, [9] *Azotobacter vinlandii*. Strain groupings and clusters are illustrated numerically and by letter at the appropriate node, respectively. See Bagwell and coworkers (33) for descriptions of isolation strategies and physiological and molecular testing methods.

separates different sequences on the basis of their melting temperatures (45). This approach yielded reproducible *nifH* sequence profiles that provided "fingerprints" for diazotroph diversity (Fig. 5). Quantitative comparison of band positions in these DGGE profiles revealed that the diazotroph assemblages associated with short and tall *Spartina* were quite similar in species composition, although diazotrophic activity was significantly higher and soluble sulfide levels were significantly lower in the tall *Spartina* zone. Also, the assemblages were quite stable, changing little during the year. Although methods involving PCR of a mixture of template sequences are inherently nonquantitative and no assessment of the quantitative representations of the different diazotroph species can be made from these data (i.e., band intensities do not reflect species abundances), it is clear that the diazotroph species composition in *Spartina* rhizosphere is quite stable (13).

The stability of the *Spartina* rhizosphere diazotroph assemblage was further tested through short-term manipulative experimentation. In some experiments, plots of short *Spartina* were fertilized with nitrogen, nitrogen plus phosphorus, or received no added nutrients (46). The intent of these experiments was to relieve nitrogen limitation. Diazotrophic bacteria have been considered to gain a significant competitive advantage in the rhizosphere through their unique capacity to fix nitrogen; and in salt marsh systems the diazotrophs appear to be carbon limited (32,47). Elimination of the advantage gained through diazotrophy was expected to result in the loss of species that competed poorly for available carbon (48). Fertilization treatments were maintained for eight weeks and rhizosphere core samples were collected after two weeks and eight weeks. Some additional samples were collected from plots that had been fertilized for over 12 years as part of a separate study. DGGE analysis of the rhizosphere diazotroph assemblage revealed that although short-term fertilization (up to eight weeks) increased nitrogen availability, it did not cause the loss of any detectable *nifH* sequences, and consequently, did not cause the loss of any detectable diazotroph species. One DGGE band was missing from the profiles from the long-term treatment

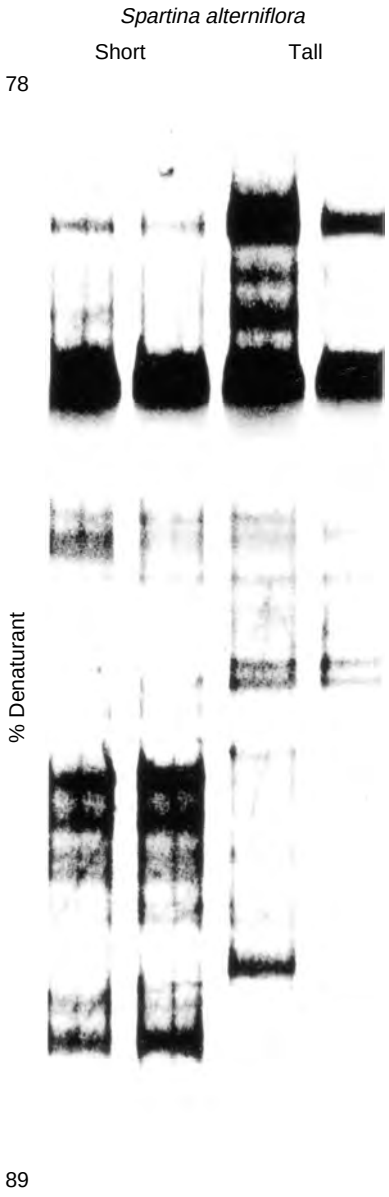

Figure 5. Denaturing gradient gel electrophoresis profiles of samples collected as part of a seasonal study of diazotroph species composition in the rhizosphere of short and tall form *S. alterniflora*. Note that only band positions are informative in defining diazotroph assemblages.

plots, indicating that species loss (or decrease in numbers to less than the detection limit of the method) is possible, but is certainly not rapid and seems to be restricted to a few taxa.

Other experiments altered carbon resource allocation by *Spartina* in order to limit carbon availability in the rhizosphere (49). The intent of these experiments was to further test the ability of the diazotrophs to compete for carbon against nondiazotrophic species. Plots in the short *Spartina* zone were either untreated or were shaded or clipped to cause the plants to reallocate resources away from the roots to compensate for reduced photosynthesis (50,51). Treatments were maintained for eight weeks and the diazotroph assemblage composition assessed as discussed earlier. The DGGE profiles were very similar across treatments, indicating that no detectable diazotroph species were displaced from the assemblage. These results imply that diazotrophs are able to compete successfully against nondiazotrophs for limiting carbon despite the high energy requirements of nitrogen fixation.

The outcomes of observational studies examining the *Spartina* diazotroph assemblage over an annual cycle (13) and experimental studies manipulating inorganic (46) and organic (49) resources indicate that this assemblage is very stable in species composition. This may be due in part to the very heterogeneous nature of the rhizosphere microenvironment, which may provide some "buffering effect" against short-term changes in host derived and edaphic environmental variables, and to the extensive microdiversity of diazotrophic organisms, which provides the capacity for interchange of strains having a high level of phylogenetic relatedness but substantial differences in physiological capabilities (41).

Although many different physiological types of diazotrophs have been isolated from the *Spartina* rhizoplane and rhizosphere, this approach provides an inadequate appraisal of diazotroph diversity. It is likely that only a fraction of the species present have been recovered and it is also entirely possible that many of the organisms that have been isolated, while able to grow rapidly on laboratory culture media, may be relatively unimportant in the rhizosphere environment (35). In order to obtain a more complete survey of the types of diazotrophs present in the *Spartina* rhizosphere, Lovell and coworkers (43) recovered the *nifH* DNA sequences from DGGE gels run by Piceno and coworkers (13) and Piceno and Lovell (46,49) and determined their nucleotide sequences. These were translated into amino acid (NifH) sequences and compared to NifH sequences from well-characterized diazotrophic organisms and to sequences recovered from other types of environments using *nifH* specific PCR primers. It should be noted that the diazotrophic bacteria are very diverse, covering a substantial range of genomic DNA mol% G + C contents. This results in a substantial bias in the mol% G + C content of the third nucleotide position in codons, which necessitates translation to amino acid sequences to facilitate meaningful sequence comparisons (52,53).

Phylogenetic trees constructed from the NifH sequences showed several large clusters representing important major groups of diazotrophs (Fig. 6). Three of these major sequence clusters contained *Spartina* rhizosphere NifH sequences. One major cluster contained numerous sequences from *Spartina* rhizosphere and sequences from known γ-*Proteobacteria* (and some β-*Proteobacteria*). This cluster was designated the γ-*Proteobacteria* cluster because of the prevalence of sequences deriving from known organisms in that group. Several of the *Spartina* sequences were very similar to those from *Pseudomonas stutzeri* and may represent species in the genus *Pseudomonas* or very closely related genera. Most of the *Spartina* sequences fell into distinct clades that contained no sequences from known species. One such sequence cluster had substantial similarity to the NifH sequence from

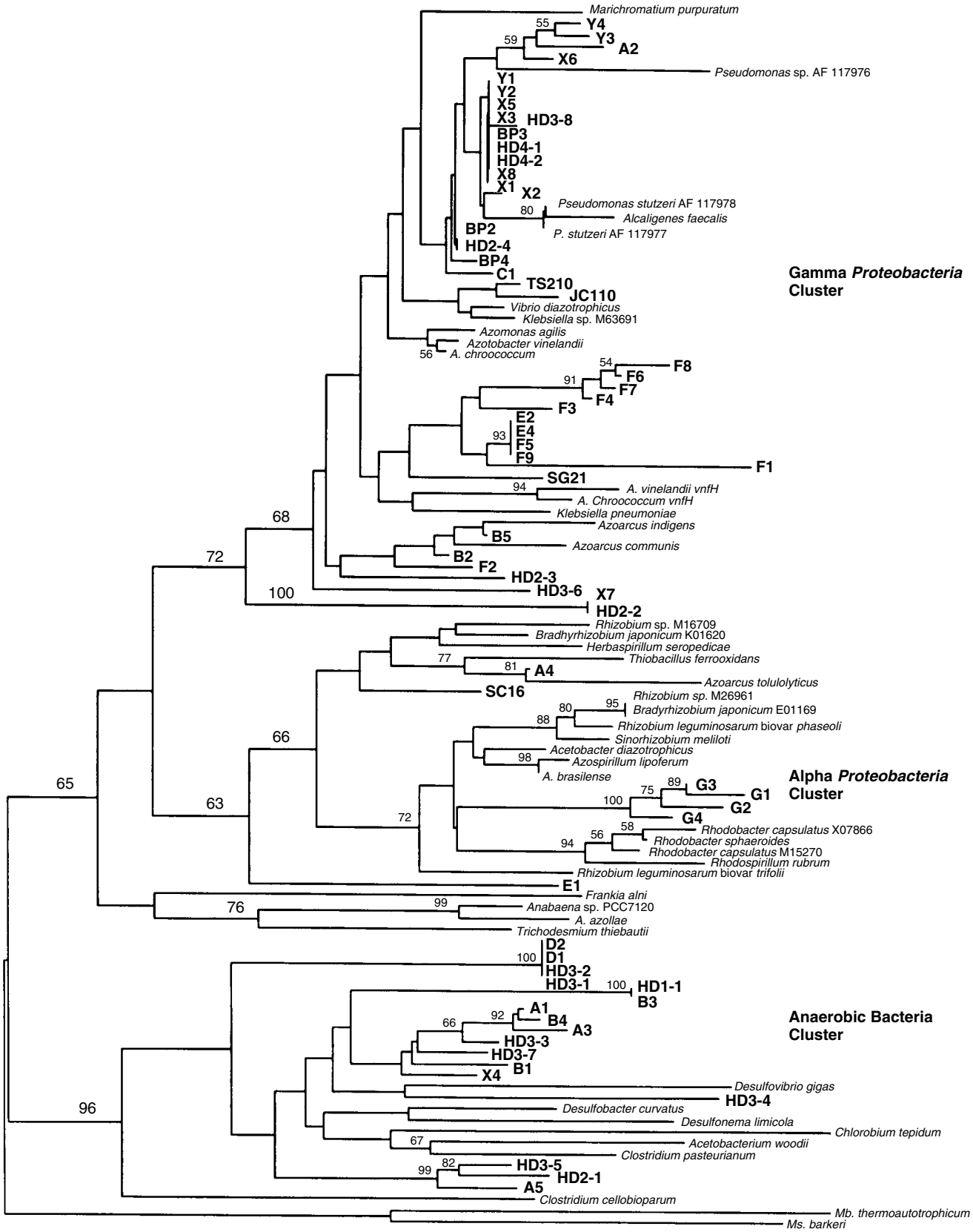

Figure 6. Phylogenetic analysis of *S. alterniflora* rhizosphere NifH amino acid sequences and sequences from selected known diazotrophs. The percentage of 1,000 bootstrap samples that supported each branch is shown. Capital letter designations in bold indicate the denaturing gradient gel bands from which the sequences were recovered (43). Bootstrap values below 50% are not shown.

a pure culture (SG21) isolated by Bagwell and coworkers (33) from the short form *Spartina* rhizoplane. Numerous NifH sequences from unknown bacteria inhabiting plant-associated and/or marine environments also fell into the *γ-Proteobacteria* cluster and a few showed significant similarities to *Spartina* rhizosphere sequences. However, none of the organisms from which these sequences were obtained have been characterized yet.

No *Spartina* rhizosphere sequences that were highly similar to NifH sequences from the *Azotobacteriaceae* or from *Klebsiella*-like organisms were recovered. It is possible that the biases inherent in PCR-based methods prevented recovery of such sequences; however, it is also possible that *Azotobacter*-like and *Klebsiella*-like organisms may not be as abundant in the *Spartina* rhizosphere as previously thought. The discrepancy between results of MPN counts using nonspecific media and the absence of NifH sequences from *Azotobacter*-like and *Klebsiella*-like organisms could also be due to misidentification of some of the organisms isolated from *Spartina* roots or associated sediments. Resolution of this issue will require quantitative determination of the abundance of authentic *Azotobacter* and *Klebsiella* species in the *Spartina* rhizosphere.

The second major cluster of NifH sequences includes those from the *α-Proteobacteria*. This cluster contains many familiar diazotrophs, including species of the *Rhizobiaceae* and the purple nonsulfur bacteria. Numerous sequences from unknown presumed *α-Proteobacteria* from various terrestrial and marine environments fall into this cluster, but few from the *Spartina* rhizosphere or other marine plant sources. It is noteworthy that NifH sequences from the rhizoplane of rice, a freshwater emergent grass that grows in anoxic sediments and significantly oxidizes its rhizosphere, also included few sequences belonging to the *α-Proteobacteria* cluster (54).

The third major cluster contained NifH sequences from known obligate anaerobes and many of the *Spartina* rhizosphere sequences. The reason(s) underlying the close association of NifH sequences from otherwise divergent anaerobes, such as the *Clostridia* and the SRB, is not known. However, anaerobe NifH sequences are more similar to each other than would be anticipated and the occurrence of unknown NifH sequences in this cluster provides good, although preliminary, evidence that these sequences are from anaerobic bacteria. Most of the *Spartina* rhizosphere anaerobe sequences formed well-supported monophyletic groups and none were closely related to any sequence from known diazotrophic anaerobes. Several sequences from rice rhizoplane and two from the rhizoplane of the sea grass *Halodule wrightii* (55) also fell into this cluster, but none were highly similar to sequences from the *Spartina* rhizosphere.

The finding of numerous NifH sequences from anaerobes is consistent with the known characteristics of the *Spartina* rhizosphere. Although the rhizosphere sediments in reasonably close proximity to live roots receive oxygen through the aerenchyma, sediments not impacted by plant inputs are likely to be anoxic (56). The rhizosphere core samples collected included live and dead roots and rhizomes, as well as surrounding sediment and organic matter (13,43). Results from inhibitor studies indicate a potentially significant role of diazotrophic SRB (40) and many of the sequences recovered from diazotrophic anaerobes show higher degrees of similarity to known diazotrophic SRB than to other anaerobic diazotrophs. The diazotrophic *Clostridia* appear to be poorly represented in the *Spartina* rhizosphere, consistent with the findings of Dicker and Smith (34) for surface sediments from various vegetated salt marsh zones. It is also possible that biases inherent in the molecular biological analyses prevented detection of *Clostridium*-like sequences. However, Ueda and coworkers (54) recovered few NifH sequences that were similar to clostridial sequences and they employed DNA extraction methods and PCR primers that were quite different from those of Piceno and coworkers (13). As was the case for the *Spartina* rhizosphere, NifH sequences from rice rhizoplane anaerobic diazotrophs that had substantial similarity to sulfate-reducer sequences were common. It should be noted that sequences within the anaerobe cluster are highly divergent, even within some well-defined clusters, and this might tend to obscure the membership of some *Spartina* anaerobe NifH sequences in those groups.

The exact identities of the *Spartina* rhizosphere diazotrophs are yet to be firmly established. NifH sequences are quite conservative, therefore similarity values for closely related species such as *A. chroococcum* and *A. vinelandii* (99.3%) or *A. brasilense* and *A. lipoferum* (99.3%) are very high. Consequently, the highest sequence similarity scores between *Spartina* rhizosphere NifH sequences and those from known diazotrophs are probably too low to reflect species-level relationships. In addition, many of the *Spartina* rhizosphere NifH sequences were found in distinct clades that were monophyletic with respect to known NifH sequences. This was also true when sequences from rhizoplanes of other wetlands plants were included in the analyses, with very few mixed clusters containing sequences from *Spartina* and rice or *H. wrightii* (43). At this time, the data available on diazotroph distributions and host affiliations in emergent marine grass systems are too sparse to allow firm conclusions regarding organism distributions or host specificity (or the lack of it) to be drawn. However, the prospect of host-specific diazotroph assemblages is certainly enticing and would have important implications for diazotroph diversity and interactions with emergent marine plants.

SRB and Sulfate Reduction in the *Spartina* Rhizosphere. As indicated by pure culture isolations, molecular biological analyses, and inhibitor experiments in the field, SRB constitute an important group of organisms in the *Spartina* rhizosphere. Numerous studies have measured rates of sulfate reduction in vegetated and unvegetated salt marsh sediments and their findings show the bulk of sulfate reducing activity and SRB to be located in shallow sediments and to be particularly associated with the roots of *Spartina*. Sulfate reduction rates in salt marsh sediments are extremely high (e.g., 57,58) and are strongly impacted by plant productivity (57). Sulfate reduction can be responsible for up to half of the total organic carbon turnover in these environments and SRB

are correspondingly numerous. SRB accounted for around 30% of bacterial rRNA in the *Spartina* rhizosphere and as much as 40% of bacterial rRNA in the *Spartina* rhizoplane (59). In contrast, only about 3% of bacterial rRNA from unvegetated marine sediments was from these organisms (60,61). Sulfate reduction and SRB are clearly responsive to the rhizosphere microenvironment and probably play an important role in the mineralization of *Spartina* biomass.

Pure culture isolation efforts have recovered SRB of the delta subdivision of the *Proteobacteria*, including species of *Desulfobacteriaceae* and *Desulfovibrionaceae* in the genera *Desulfoarculus, Desulfobacter, Desulfobacterium, Desulfobulbus*, and *Desulfovibrio* from *Spartina* rhizoplane or rhizosphere (62). Some of the isolates were somewhat divergent from representatives of known SRB genera and may represent new genera. PCR amplification of *Desulfobacteriaceae*-specific 16S rDNA sequences from *Spartina* rhizoplane material revealed abundant populations belonging to the genera *Desulfococcus* and *Desulfosarcina* (63). These organisms were not recovered as pure culture isolates (62) and were not especially abundant according to results from quantitative RNA-RNA hybridization (59), possibly indicating a PCR bias problem. The *Desulfococcus-Desulfosarcina*-like populations were responsive to plant growth dynamics, possibly because of alterations in organic carbon release at different stages in plant ontogeny, and are clearly quite tolerant of oxygen transported through the *Spartina* aerenchyma.

Other Microbial Activities and Associations Within the Rhizospheres of Emergent Plants. It is clear that the rhizospheres of aerenchymatous emergent plants, such as *Spartina*, are beneficial environments for the growth of many microorganisms. This oxic anoxic interface environment should favor the coupling of processes such as sulfate reduction and sulfide sulfur oxidation, denitrification, and nitrification, and iron manganese reduction and oxidation. In addition, chemoheterotrophic processes involved in organic matter turnover should be substantially enhanced in the *Spartina* rhizosphere. Unfortunately, few studies have been performed with regard to these processes, linkages among them, or the organisms involved in the *Spartina* rhizosphere. The information that is available from *Spartina* and some additional results from other systems are presented in brief here.

The aerenchymatous emergent freshwater macrophyte *Glyceria maxima* strongly stimulated activity and supported an abundance of nitrifying bacteria in the rhizosphere, particularly during periods of maximum growth and productivity (64,65). These oxygen-requiring bacteria were probably stimulated by oxygen introduction into the rhizosphere by the plant. The rhizosphere nitrifiers were also able to maintain both substantial numbers and their capacity to nitrify through periods of anoxia, which is often not the case for this group of organisms. There was also a positive relationship between *Glyceria* and denitrification activity in the rhizosphere. This relationship has been observed for other freshwater macrophytes, including *Littorella uniflora* (66), *Oryza sativa, Pontederia cordata*, and *Juncus effusus* (67). Under conditions of low nitrate availability, *Glyceria* strongly influenced the composition of the nitrate-reducing bacterial assemblage and shifted the predominant nitrate metabolism toward nitrate ammonification (68). When nitrate was abundant, the impact of *Glyceria* was negligible. *Spartina* also supports denitrification when grown under conditions of nitrate surplus, but competition between *Spartina* and the denitrifying bacteria for nitrate strongly restricts the extent of denitrification under nitrate-limited conditions (69). These effects of emergent macrophytes are viewed as significant because the flux of the gaseous products of denitrification from the rhizosphere through the aerenchyma to the overlying atmosphere can result in substantial nitrogen loss from vegetated sediments (67).

Roots of several emergent freshwater macrophytes including *Sparganium eurycarpum, Typha latifolia, P. cordata*, and *J. effusus* support dissimilatory reduction of ferric iron at quite high rates (70,71). This implies an important role of iron reduction in organic matter turnover in these mildly acidic, peat-rich freshwater systems. Roots of *Spartina* and of the sea grass *Zostera marina* also supported iron reduction, although at rates substantially lower than those of the freshwater plants (70). Iron reduction on roots of the latter two plants was not inhibited by molybdate implying that specialist iron-reducing bacteria, rather than sulfate-reducers, may be involved. Ferrous iron oxidation, through abiotic and/or biotic processes, is clearly feasible in the rhizospheres of plants that introduce significant levels of oxygen via aerenchymal transport. There is often visible evidence for this oxidation process in the form of iron plaque on the root surfaces (72). This material can include substantial levels of iron-oxidizing bacteria (73).

Rhizosphere ventilation also supports other oxygen requiring processes and organisms in the rhizospheres of aquatic plants. These include methane oxidation by methanotrophic bacteria growing on roots and rhizomes of various freshwater plants (74) and sulfur (thiosulfate) oxidation by various bacteria in the rhizosphere of rice (75). Although the specific organisms carrying out these activities have not been identified in most cases, it is clear that the coupling of various oxidative and reductive reactions in the carbon, nitrogen, iron, and sulfur cycles is supported in the rhizospheres of emergent marine plants. Such reactions could supply and regenerate oxidants essential for organic matter mineralization in anoxic vegetated marine soils and sediments.

Endosphere Microbiota

Although the majority of bacteria associated with emergent grasses are on the root and shoot surfaces, the interior intercellular spaces of many grasses are also well-known habitats for heterotrophic bacteria (76,77), including diazotrophic bacteria. *Spartina* is no exception (26,38) and unlike the roots of some terrestrial grasses (76), diazotrophy in *Spartina* roots is detectable immediately after excision, implying that this process occurs in roots of intact plants under natural conditions (78). Diazotrophic endophytes have also been found in rice (79,80), sugarcane (81), and Kallar grass (82). Endophytic bacteria appear to be

relatively common in grasses and although the nitrogen fixers have garnered the most attention, undoubtedly other types of organisms are also present (see (80)).

Bacteria have been observed intercellularly in the *Spartina* root outer cortex and also within visibly damaged root cortex cells (38). Most of these bacteria were in the lacunae and associated with cell walls. It is worthwhile to note that production of phytohormones by rhizosphere and endorhizosphere bacteria has been implicated in increased transport through roots, and altered patterns of root growth in some terrestrial grasses (83). However, until pure culture isolates are obtained and tested for phytohormone production, any discussion of the potential impacts of these bacteria on mass transport through the roots or on root development in *Spartina* must remain speculative.

PLANT-MICROBE INTERACTIONS OF SUBMERGED MARINE PLANTS

Shallow, well-illuminated subtidal locations can also support extensive stands of marine grasses. Sea grasses are quite diverse, with approximately 50 known species belonging to 12 genera (84). However, relatively few of these species have been examined for their interactions with microorganisms. As was the case for *Spartina* marshes, seagrass meadows are highly productive, often nitrogen-limited, and can support very high rates of diazotrophy relative to unvegetated sediments. In some seagrass flats as much as 50% of the plant nitrogen requirements can be met by diazotrophy in the rhizosphere sediments (85,86). Unlike the emergent grasses, living leaves of many seagrasses support extensive growth of complex and highly active epiphyte communities. Given the importance of diazotrophy in many seagrass systems it is no surprise that much of the information available on the interactions between the plants and their associated microbiota examines nitrogen fixation. Sulfate reduction rates are also elevated in seagrass meadow sediments and this process has been intensively studied.

Interactions in the Phyllosphere

Seagrass leaves are often heavily coated with epiphytic microorganisms including diatoms, *Cyanobacteria*, and heterotrophic bacteria and serve as both a solid support and a nutritional resource for these organisms. Bacterial numbers on surfaces of *Z. marina* leaves range from 10^6 to 2×10^7 cells cm^{-2} and increase with increasing distance from the leaf base (the youngest portion) toward the tip (87,88). In at least some cases, the mass of microbiota associated with leaf surfaces greatly exceeds that associated with root surfaces (89,90). The compositions and activities of these epiphytic communities are unknown for most seagrass species, but the epiphytes of a few species have undergone some study.

Culturable bacteria from leaves of *Halophila ovalis, Halophila stipulacea*, and *Halodule uninervis* collected at Aqaba (Jordan) showed highest densities in the summer months, when plant productivity was highest (90). Plate counts, averaged for the three species, ranged from 1.40×10^7 to 6.46×10^8 cells g^{-1} (wet wt). Numbers differed among the seagrass species and in general were higher on the *Halophila* species than on *H. uninervis*. Genera recovered from aboveground plant structures included *Arthrobacter, Actinomyces, Bacillus, Pseudomonas*, and *Vibrio*. Heterocystous *Cyanobacteria*, particularly *Calothrix* sp. are also common epiphytes on sea grasses, sometimes forming distinct colonies on mature leaves (89,91). These organisms can be significant contributors to phyllosphere diazotrophy (see following section).

The diversity of epiphytic bacteria associated with *H. stipulacea* was examined in detail using 16S rDNA sequence analysis (92). A total of 59 clones, belonging to 51 different amplified rDNA restriction analysis (ARDRA) groups, were sequenced. Most (62.6%) of the clone sequences belonged to the division *Proteobacteria*. The *γ-Proteobacteria* accounted for 27.3% of the clones and included sequences affiliated with the genera *Pseudomonas, Vibrio, Marinomonas, Oceanospirillum*, and other marine groups. Sequences affiliated with the *α-Proteobacteria* were also common, accounting for 24.2% of the total and including relatives of the genera *Hyphomonas, Roseobacter, Ruegeria*, and the family *Rhizobiaceae*. The remaining sequences included a small number affiliated with the *β-Proteobacteria*, several possibly belonging to the division *Proteobacteria*, but highly diverge from known sequences, and sequences affiliated with the *Verrucomicrobiales, Planctomycetales*, the high G + C *Firmicutes*, and the *Holophaga/Acidobacterium* division. In addition, sequences affiliated with diatom chloroplast rDNA sequences accounted for 20.2% of the total. Clearly, the diversity of seagrass phyllosphere bacteria, even on a single plant species, is very high and includes many organisms that are distantly related to any well-characterized bacterial species. This diversity likely encompasses numerous microbial activities, but the key functions of seagrass phyllosphere bacteria have received little study.

In addition to their high biomass and diversity, epiphytic bacteria inhabiting the phyllosphere of seagrasses are highly productive. Bacterial production rate estimates for the *Z. marina* phyllosphere are as high as 0.4 μg C h^{-1} cm^{-2} and largely due to increasing cell numbers, increase with increasing distance from the leaf base (87). The carbon and energy source supporting the extensive growth and productivity of epiphytic bacteria appears to be primarily exudation of organic carbon by intact *Z. marina* leaves. These exudates are efficiently assimilated by epiphytic bacteria (93), they amounted to about 10% of photosynthetically fixed carbon, and were sufficient to support most, if not all of the epiphytic bacterial production (87). Bacterial doubling times in the *Z. marina* phyllosphere are comparable to values estimated for water column bacteria, ranging from 1.5 to 17.6 hours (87,88).

Nitrogen fixation rates have been measured in the phyllospheres of several seagrasses and a broad range of values estimated. Tropical Australian seagrass meadows dominated by *Enhalis acoroides, Thalassia hemprichii*, and a mixture of *Syringodium isoetifolium* and *Cymodocea serrulata*, respectively, supported rates

of diazotrophy on aboveground plant surfaces of 4.2, 0.3, and 2.2 mg N m^{-2} d^{-1} (94). These values were about 13%, 5%, and 5% of total diazotrophy for seagrass meadows at the three sites. In a seasonal study of *Z. marina* in North Carolina, significant diazotrophy in phyllosphere portions of the plants was found only in the late fall through early winter (95). Phyllospheric diazotrophy was more significant than rhizospheric diazotrophy during this period and was ascribed to heavy colonization by heterocystous *Cyanobacteria*. Diazotrophy in the phyllosphere of subtropical *Thalassia testudinum* was also associated with heterocystous *Cyanobacteria* and amounted to 4 to 5 mg N m^{-2} d^{-1} at some sites during the summer (91). The varied microbiota of the seagrass phyllosphere are clearly capable of significant activities that can impact and contribute to seagrass success and productivity. The exact extent of such activities may be dependent on the seagrass species examined and the prevailing abiotic environmental parameters at the sites studied.

Interactions in the Rhizosphere

The Rhizosphere Microenvironment. Seagrasses grow in fully saturated, typically anoxic sediments and have the same potential problems with anoxia and accumulation of phytotoxins as the emergent plants (discussed earlier). The adaptations that help the seagrasses to solve these problems seem quite similar to those of the emergent plant species, namely, development of aerenchyma (96) and oxidation of the rhizosphere. Because seagrasses are completely submerged under most conditions and generally lack stomata, which limits transport of oxygen from outside the plants, photosynthesis appears to be the major source of oxygen (97). This oxygen can be transported through the aerenchyma from leaves to roots, with some leakage into the rhizosphere (20,98–100). Although measurement of the specific quantities of oxygen released is difficult and quantities vary substantially among seagrass species and meadow locations, free oxygen and detectable rhizosphere oxidation within a distance of a few millimeters from the roots has been demonstrated (99). Elevated oxygen levels were observed only when plants were illuminated and capable of supporting a favorable balance between photosynthetic oxygen production and root respiration. Rhizosphere oxygen concentrations declined exponentially when the plants were incubated in the dark. In addition, there was a pronounced seasonality of rhizosphere oxidation that differed between two seagrass species, *Potamogeton perfoliatus* and *Z. marina*, and also differed with depths of the sampling sites below the water surface for *Z. marina* (99). Oxygen release by the roots of seagrasses is clearly variable and a number of biotic and environmental factors control the degree of rhizosphere oxidation that can result. Oxidation of the seagrass rhizosphere should support growth of many oxygen-utilizing bacteria, as was seen for *Spartina*, but these organisms remain mostly uncharacterized at present.

In addition to oxygen, seagrasses are the primary source of organic carbon for their rhizosphere microbiota. The release of photosynthate from seagrass roots is substantial, approximately 10% of the carbon fixed in the case of tropical *H. wrightii* (101). A number of simple organic compounds have been tested for their stimulatory effect on diazotrophy in intact cores of *Zostera noltii* meadow sediments, with lactate and sucrose providing the greatest stimulation (84,102). Decomposition of belowground plant biomass and other sources of organic carbon must also be considered (103), but the tight coupling between bacterial activities and plant photosynthesis strongly implies that plant exudation is an important source of labile carbon for the rhizosphere microbiota.

The combination of carbon exudation and oxygen introduction has profound impacts on the rhizosphere microbiota, as illustrated by diel and seasonal variations in microbial biomass and activities (104,105). Seagrass rhizosphere bacterial growth rates, population sizes, and levels of activity are all closely coupled to plant primary productivity (84). The marked diel periodicity of bacterial activity and productivity in *Zostera capricorni* meadow sediments, with rates increasing from 5- to 10-fold during the morning hours, then declining in the afternoon (106), demonstrate this linkage. Direct microscopic counts of total bacterial cells in *Z. capricorni* rhizosphere sediments also increased by 50% during the summer months, when plant productivity was highest (105). However, it should be noted that different rhizosphere organisms can be affected differentially by plant productivity. The abundance of one *Z. capricorni* rhizosphere organism, *Alteromonas* sp., changed little with season (105). This may reflect nutritional versatility on the part of the *Alteromonas* species, or other adaptations (e.g., dormancy) that facilitate its persistence under resource limited conditions. Certainly plant productivity has major impacts on most rhizosphere species and on the gross activities of these organisms, though the dynamics of individual species are not well understood.

Diazotrophic Bacteria and Diazotrophy in Seagrass Rhizospheres. A few studies employing MPN and pure culture isolation methods have examined the numbers and types of culturable diazotrophs in sea grass meadow rhizospheres. Bulk rhizosphere sediments, sediments and bacteria tightly associated with roots, and root homogenates of *Z. marina* all showed quite high levels of diazotrophic bacteria (107). Under microaerobic incubation conditions cell numbers averaged 2.0×10^5, 4.5×10^6, and 3.4×10^5 cells g^{-1} (wet wt) for rhizosphere sediments, rhizoplane sediments, and root homogenates respectively. Anaerobic incubations yielded 1.4×10^5, 3.3×10^6, and 2.8×10^5 cells g^{-1} for the same sample types. A total of 127 strains of gram-negative, facultatively anaerobic, motile diazotrophs were isolated from these sources and their morphological and physiological features were consistent with their placement in the family *Vibrionaceae*. To date, molecular biological examinations of either the total rhizosphere microbiota or the diazotroph assemblage of seagrasses have been very limited. Kirshtein and coworkers (55) amplified *nifH* specific sequences from a salt-requiring *Klebsiella* strain originally isolated from *Ruppia maritima* roots and from DNA extracted from

H. wrightii root and rhizoplane material. The two *nifH* sequences from *Halodule* extracts were not very similar to those from known organisms, but fell into the anaerobe cluster and may represent SRB.

As is the case for other members of the rhizosphere microbiota, the diazotrophs are strongly influenced by plant primary productivity. Rates of diazotrophy, which range from 0.02 to 140 mg N m^{-2} d^{-1} (84), are clearly higher in seagrass rhizosphere sediments than in nearby unvegetated sediments (e.g., 85,108–110). The distribution of diazotrophic activity with depth in the sediments also corresponds to the distribution of belowground plant biomass (110), and the highest levels of activity are seen on the roots and rhizomes themselves (84). In meadows of *Z. nolti*, diazotrophic activity associated with belowground plant structures was up to 140-fold that recorded for rhizosphere sediments from which all roots and rhizomes were removed (84). Numbers of diazotrophic bacteria are also substantially higher in rhizosphere sediments than in unvegetated sediments (86) and microscopic examination has shown the roots of seagrasses to be heavily colonized by bacteria. In the case of the *Z. nolti*, rhizoplane bacterial assemblages have been estimated to be around 3×10^3 cells mm^{-2} (84,111).

Rapid transfer of nitrogen from rhizosphere diazotrophic bacteria to *Z. marina* and *Z. capricorni* has been demonstrated using ^{15}N labeling methods (7,85). In the case of tropical *Z. capricorni*, inputs from new nitrogen fixation into aboveground biomass alone were estimated to supply between 33 and 50% of plant nitrogen requirements. This tight association between rhizosphere diazotrophs and seagrasses can provide an important nitrogen source for the plants. However, it should be noted that the contribution of diazotrophy to plant nitrogen requirements varies among different seagrass species, geographic locations, and seasons, and is typically higher in tropical meadows than in temperate meadows.

SRB and Sulfate Reduction in Seagrass Rhizospheres. As seen in the emergent grasses, seagrass meadows support large populations of anaerobic bacteria, apparently dominated by the SRB. Very high rates of sulfate reduction that correlate strongly with plant distributions have been measured in several seagrass meadows (100,108,109,112–114) and the rates reported are often greatly in excess of those in unvegetated sediments. Sulfate reduction rates from isolated *Z. marina* roots were also much higher per gram dry weight than those in the meadow rhizosphere sediments (114,115). Rates of sulfate reduction (108,109,114) can be strongly influenced by plant photosynthetic activity, with substantially higher rates measured when the plants are incubated in the light versus in the dark. Very high numbers of SRB [10^7 to 10^9 cells (g dry wt)$^{-1}$] have also been estimated from the measured sulfate reduction rates and published cell-specific rates of sulfate reduction (114,115). These estimates are supported by results from a subtropical meadow of *H. wrightii*, where rhizosphere SRB numbers estimated by MPN and phospholipid fatty acid analyses were found to fall in the range of 10^7 to 10^8 cells (g dry wt)$^{-1}$ (116). Key SRB species in the *Halodule* rhizosphere appeared to belong to the genera *Desulfovibrio* and *Desulfobulbus*. In addition to their contributions to organic matter mineralization, inhibitor (molybdate) studies indicate that the SRB are an important group of heterotrophic diazotrophs in seagrass meadows. These organisms account for 25 to 95% of nitrogen fixation, with substantial variability between locations and seasons, even within the same seagrass species (109,110,117). Acetogenic anaerobes and acid fermenting *Clostridia*, although not as abundant as the SRB, were also detected in the *Halodule* rhizosphere (116). MPN estimates of acetogens were in the range of 10^5 cells g^{-1} of sediment, whereas butyrate-producing *Clostridia* amounted to about 10^4 cells g^{-1}. There was a notable rhizosphere effect for SRB and acetogens, but no significant enrichment of butyrate-producing *Clostridia* in the rhizosphere versus unvegetated sediments.

Other Microbial Activities and Associations in Seagrass Rhizospheres. Relatively few studies of other microbially mediated processes associated with seagrasses have been performed. Many of these have examined nitrogen cycle processes other than diazotrophy. Ammonia production (ammonification) from sediment organic matter occurs at high rates in seagrass meadow sediments relative to unvegetated sediments (118–122). This probably reflects higher levels of labile organic matter in seagrass meadow sediments. Nitrification and denitrification also tend to occur at high rates in these sediments. Sediments of a *Z. marina* meadow off western Alaska supported significant denitrification (98). Nitrate utilized in this process was proposed to arise from nitrification, an oxic process supported by oxygen transport to the rhizosphere by *Z. marina*. High rates of denitrification have also reported for a tropical *Halodule beaudetti* meadow (100).

Seasonal and other trends in nitrogen cycle processes vary among seagrass species and meadow locations. Ammonification and nitrification potentials tended to follow the annual cycle of productivity of temperate *P. perfoliatus*, peaking during periods of peak plant biomass, although denitrification potential did not (121). In contrast, although there were strong seasonal trends in ammonification, nitrification, and denitrification potentials in a temperate *Z. marina* meadow, all three processes displayed different trends (121). Given the small number of studies performed to date and the substantial differences in the methods employed, few broad conclusions regarding the interactions of seagrasses and the microbiota involved in nitrogen cycle processes other than nitrogen fixation can be drawn at present.

Sulfur cycle processes in seagrass meadows, with the exception of sulfate reduction, have also received relatively little attention. The chemocline microenvironment of the rhizoplane, with its overlapping gradients of oxygen (from the root) and hydrogen sulfide (from sulfate reduction) would certainly be expected to support sulfide- and sulfur-oxidizing bacteria. In addition, phototrophic green and purple sulfur bacteria are known to inhabit shallow marine sediments and could benefit from the organic enrichment of shallow seagrass meadow sediments. MPN estimates of the colorless sulfur bacteria

(CSB), phototrophic sulfur bacteria (PSB), and SRB in a temperate *Z. noltii* meadow showed that all three functional groups were present at fairly high abundances (123). The CSB were abundant from the sediment surface to depths of at least 5 cm, with highest numbers (around 10^9 cells mL^{-1}) in the upper 2 cm. PSB were much less common, with peak numbers less than 10^6 cells mL^{-1}, and were restricted to the upper 3 cm of the sediments. SRB were highly abundant at all depths examined, with more than 10^7 cells mL^{-1} in the upper two cm. As stated previously, MPN techniques can dramatically underestimate microbial numbers, so these functional groups are certainly abundant in seagrass meadow sediments and the sulfur cycle processes they catalyze are prominent features of seagrass meadow biogeochemistry. Tight coupling between sulfate reduction and sulfide oxidation processes has been invoked to explain the relatively low levels of sulfides found in some seagrass meadow sediments (123).

Endosphere Microbiota

Seagrasses contain a surprising abundance and diversity of bacteria within their root and rhizome structures. One endospheric *Klebsiella* strain has been identified in *H. wrightii* using classical cultivation and immunofluorescence methods (124). Using radioactively labeled probes specific for the clostridia, some species of acetogens, the low G + C content *Firmicutes*, species of *Desulfovibrio*, *α-Proteobacteria*, *Archaea*, and sulfate-reducing bacteria, Küsel and coworkers (116) observed high levels of colonization of the root epidermis and the intercellular root spaces of *H. wrightii*. *Bacteria* and *Archaea* hybridizing with all of the probes used were observed on the rhizoplane. Rhizoplane *Desulfovibrio* occurred primarily as single cells, whereas the acetogens formed large clusters. Clostridial, acetogen, and archaeal colonization extended into the root cortex and *Desulfovibrio* cells were seen deep in the root cortex, where 61% of the deepest cortical cells were colonized by these organisms. The identities and physiological properties of the cells hybridizing with the probes specific to *Archaea* are not known, but they may be methanogens. The prevalence of so many anaerobic bacterial groups within tissues that are oxygenated during periods of active photosynthesis is particularly interesting. Tolerance to oxygen exposure would seem to be a required trait for these organisms, many of which are typically regarded as completely oxygen intolerant. The extent and nature of the activities carried out by these endospheric anaerobes are not known, but it seems likely that they would be most active at night, when photosynthetic oxygen production cannot occur.

In addition to the endospheric bacterial flora, some seagrasses contain symbiotic fungi. *Thalassia testudinum* contains a common rhizomycelial chytrid, described as a *Nowakowskiella* or *Cladochytrium* species that occurs in living leaves (125). However, such fungal symbioses are not universal among seagrasses because no such symbionts were reported for *Z. marina* (126).

CONCLUSION

The similarities in the types of plant-microbe interactions observed in emergent and submerged marine plant systems are quite striking. In both types of systems, the plant clearly supplies important carbon resources to the bacteria in all of the plant-associated microenvironments. In the rhizospheres of both plant types, oxygen introduction by the plant roots is also of paramount importance and substantially impacts both the nature and the activities of the associated microbiota. Thus, some key processes, such as diazotrophy and sulfate reduction, and the regulation of these processes by photosynthetic carbon and oxygen supply to the rhizosphere have been identified. However, some key differences between emergent and submerged grasses are also apparent, particularly the prevalence of epiphytic bacteria on living leaves of the submerged plants. It is clear that these organisms are highly significant to some seagrass species, but their contributions and the constraints limiting their growth and activity are not well understood. The marine vascular plants are enormously productive and although under substantial pressure from development and anthropogenic pollution, provide significant carbon and nitrogen inputs to marine food webs. The interactions of these plants with their associated microbiota provide the foundation for this productivity and a better understanding of these interactions will be required to successfully manage these vital biotic resources.

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PLFA ANALYSIS.

See LIPID BIOMARKERS IN ENVIRONMENTAL MICROBIOLOGY

POINT-OF-USE TREATMENT DEVICES FOR DRINKING WATER.

See HETEROTROPHIC BACTERIA AS INDICATORS OF WATER QUALITY

POLAR MARINE PHYTOPLANKTON

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All life on earth is dependent on the ability of plants to use energy from solar radiation for the reduction of CO_2 and the synthesis of diverse organic compounds that support all animal life. Such primary production in the marine environment is accomplished primarily by unicellular phytoplankton, with relatively small contributions by multicellular algae that grow attached to the ocean bottom in shallow coastal regions, and is believed to exceed the magnitude of primary production occurring in all terrestrial environments. Oceanic phytoplankton are thus of vital importance in regard to global production of food materials in the marine environment as well as playing an

integral role in the sequestering of atmospheric CO_2 and the subsequent transport of organic particles to the deep sea. This assimilation of atmospheric CO_2 and subsequent transport of reduced carbon to the deep sea plays a vital role in the global carbon cycle and thus helps to minimize possible harmful consequences of the increase of CO_2 in the atmosphere (the "greenhouse effect"). The distribution of phytoplankton in the marine environment, however, is extremely variable because the biomass of phytoplankton that can be supported in the upper water column is dependent primarily on an adequate inorganic nutrient supply and sufficient solar radiation, and also on suitable physical factors such as water salinity and temperature. The availability of inorganic nutrients in surface waters is influenced mostly by the profile of water column stability and the physical upwelling of nutrient-rich deep water into the euphotic zone. Upwelling processes are found predominantly in the Southern Ocean and in subarctic Pacific waters, and to a lesser extent along the western coasts of Africa and the Americas (1), whereas vertical mixing is predominant in the Atlantic Arctic. Consequences of biological activity by phytoplankton in the polar regions thus must be viewed in the context of global processes, because both the world's atmosphere and ocean waters are in constant circulation, with mixing between the Northern and Southern Hemispheres (2–4). Because the biomass and rates of primary production by phytoplankton are limited by chemical, optical, and physical factors that are strongly influenced by dynamic physical processes and topographical characteristics of the sea floor and land masses, these latter aspects are briefly discussed later.

GENERAL CHARACTERISTICS OF PHYTOPLANKTON

The "phyto" prefix means that the cells have the physiological characteristics of green plants, whereas the "plankton" suffix signifies that the cells are freely suspended in the water column. Like all green plants, phytoplankton require only sunlight, CO_2, water, inorganic nutrients, and suitable physical conditions for growth (see section Major Factors Affecting Growth Rates of Phytoplankton). However, many types of phytoplankton have flagella or some gliding mechanism that gives them limited ability to move toward or away from external stimuli such as a change in light intensity or various chemicals. Such movements although are sufficiently slow that the distribution of cells in the water column is largely dependent on physical mixing processes. One exception to this although involves phytoplankton cells that grow either within the ice or attached to the underside of the ice in both polar regions. When the ice melts during spring and summer, these ice algae are liberated to the water column and are believed to initiate the growth of phytoplankton blooms commonly found near the receding ice edge. A brief description of the cellular appearance and major taxonomic groups of polar phytoplankton is given below.

Cellular Characteristics

Most phytoplankton occur as single cells, but in some species the cells may aggregate to form chains, clumps, or colonies. Single cells, which are microscopic and too small to be seen with the naked eye, are commonly categorized as picoplankton (<2.0 μm in size), nanoplankton (>2.0 μm but <20 μm), and microplankton (>20 μm, but usually <100 μm). The cellular structure and organelles of phytoplankton found in polar waters are eukaryotic (i.e., the nucleus and various plastids are enclosed within membranes) and basically similar to phytoplankton found in temperate or tropical waters. The gross chemical composition (i.e., cellular concentrations of protein, lipid, carbohydrates, nucleic acids, etc.) of polar phytoplankton does not differ appreciably from the composition of phytoplankton found in other waters. Some taxonomic groups of phytoplankton have thick cell walls that may be elaboratively sculptured and possess spines or appendages (e.g., the siliceous cell walls of diatoms and the cellulosic plates of dinoflagellates), while other groups may have no firm outer cell wall. Photoautotrophic procaryotic cells (nucleus and other organelles not membrane-bound), which include Cyanobacteria (also called blue-green algae in the division Cyanophyta), may be dominant components of the phytoplankton in tropical or temperate waters, but are found only occasionally in cold polar waters. Similarly, no filamentous nitrogen-fixing blue-green algae have been described in marine polar waters. Endosymbiotic associations among an autotrophic alga living within a protozoan are not common in polar waters, but occasionally such cells have been found to be a dominant component of the phytoplankton (5). Discussion in the following sections is restricted only to the dominant eukaryotic phytoplankton.

Adaptations to High Latitudes

The major environmental differences between polar waters and temperate or tropical waters that are important with regard to survival of phytoplankton are the low water temperatures and the seasonal cycle of solar radiation, which ranges from months of long summer days with high light to months of darkness during winter. Water temperatures in the polar regions generally range from $-1.8\,^{\circ}C$ (the freezing point of seawater) to approximately $+5\,^{\circ}C$, although water temperatures may approach $10\,^{\circ}C$ in some areas of the Bering Sea. Polar phytoplankton are physiologically adapted to grow at these low temperatures (see Section Major factors affecting growth rates of phytoplankton) and generally do not survive at temperatures exceeding approximately $10\,^{\circ}C$. The seasonal variation in solar radiation poses two problems for survival of phytoplankton: (*1*) during the winter months solar radiation is so low (there is no direct sunlight between the polar circle and the geographic pole at the time of the winter solstice) that most cells in the water column must survive by respiration of stored cellular substrates such as lipids or carbohydrates and (*2*) the combination of cooling of surface water and storm activity results in deep mixing of the upper water column in winter, so that phytoplankton cells are lost to deep waters and to the sediments. To survive such winter conditions, polar phytoplankton thus must have the ability to minimize respiratory requirements during periods of darkness and also to develop spores or other

reproductive cells that remain viable for many months or years. This ability to maintain cell viability during the long winter period requires physiological acclimation by the cells in response to slowly changing environmental conditions–sudden darkness would not result in a similar adaptive state.

Dominant Taxonomic Groups

Species from the following taxonomic divisions of phytoplankton are generally found in both polar regions. Although representatives from these groups are also represented in temperate and tropical waters, many of the species found in cold polar waters generally are not found in warmer waters. Similar species are often described from both the Arctic and the Antarctic, but because phytoplankton classification is still based largely on morphological characteristics and not on analysis of the genetic material deoxyribonucleic acid (DNA), it is not possible to say if these species are truly identical with regard to genetic constitution or if they merely have similar morphological appearance. Further information on phytoplankton taxonomy may be found in references (6–8).

Bacillariophyta. Diatoms are usually the dominant component of the phytoplankton in both polar regions. The cell wall consists of two overlapping siliceous frustrules, which are generally highly sculptured and often with spines. Cells may be single or connected to form long chains. Diatoms are a major food item for most zooplankton and krill.

Pyrrophyta. Dinoflagellates generally have two flagella for motility and may be either armored with thick, sculptured cellulosic plates (theca) or naked, without any theca.

Cryptophyta. Cyptophytes are naked, flagellated cells that have accessory photosynthetic blue (phycocyanin) and red (phycoerythrin) pigments in addition to chlorophylls *a* and *c*.

Haptophyta. Flagellated forms that may or may not have organic or elaborately sculptured outer plates of calcium carbonate (the Coccolithophorids). This group also includes *Phaeocystis* species, which are found either as solitary cells or organized into spherical colonies and at times is a dominant component of the phytoplankton in both the Arctic and the Antarctic. *Phaeocystis* species are known to produce the antibiotic acrylic acid. Bactericidal effects, however,m appear to be restricted to extremely acid environments such as penguin guts. Phaeocystis species also release demethylsulfide, which may, when released into the atmosphere, produce sulfuric acid droplets that act as condensation nuclei for cloud formation.

Chrysophyta. This varied group, also called golden brown algae, are flagellated and may have scales and needles composed of silica (the silicoflagellates). Other forms include *Dinobryon* and its close relatives, which are important in the sea ice flora.

Chlorophyta. This group of green algae, which contains chlorophyll *b* in addition to chlorophyll *a*, are represented in polar waters by small, unicellular *Chlorella*-like cells. Their biomass though, is generally small relative to that of other phytoplankton groups.

Cyanophyta. Small procaryotic unicells (also referred to as Cyanobacteria) are often dominant in tropical waters, but are rarely seen in cold polar waters. When they are found in the polar regions, it is usually in coastal habitats or in places where water temperature is greater than 5.0 °C.

PHYSICAL AND CHEMICAL ASPECTS OF THE ARCTIC AND THE ANTARCTIC

Although some environmental conditions in the Arctic and the Antarctic are similar (e.g., low water temperatures, seasonal cycle of solar radiation, and annual formation of sea ice), the two polar regions are also very different in regard to some factors that are important for the survival and growth of phytoplankton (9). The geographic areas to be included in the terms "Arctic" and "Antarctic" depend to a large extent on whether or not a strict, geographic criterion (e.g., the Polar Circles, which are located at approximately 66.5 °N and 66.5 °S) is most relevant, or if primary consideration should be given to the area where certain environmental conditions are found. In the latter case, the term "Arctic" is often delineated by the southernmost boundary where the air temperature isotherm during July approximates 10 °C. Such a boundary would be far south of the Arctic Circle over portions of northern Canada and would include portions of the Bering Sea, but it would be to the north of the Arctic Circle in the waters to the west of Norway as a result of the relatively warm northward-flowing Gulf Stream with the North Atlantic current. For simplicity, the discussion below will consider the Arctic marine ecosystem to include the High Arctic (the Arctic Ocean) as well as peripheral Low Arctic (also called sub-Arctic) waters, which include the Bering Seam Hudson Bay, Baffin Bay, Barents Sea, Greenland Sea, and the Norwegian Sea (Fig. 1a). The situation is far simpler in the Antarctic, where oceanographic conditions are fairly uniform from the coastal waters surrounding the continent northward to the Polar Front (formerly referred to as the Antarctic Convergence), which lies to the north of the Antarctic Circle (Fig. 1b). The accepted definition of the Southern Ocean thus is the oceanic area lying to the south of the Antarctic Polar Front, which lies between 47 and 60 °S and where the water temperature ranges from 4 to 5 °C. The major differences between the two polar regions are briefly discussed in the following sections.

Arctic

The geographic north pole (90 °N) is located in the relatively small Arctic Ocean, which is surrounded by the large land masses of North America, Europe, and Asia (Fig. 1a). As can be seen from the 1,000 meter depth isopleth, deep waters in the Arctic Ocean are limited primarily to the central deep basins; the relatively shallow (<300 m) continental shelf waters account for a large

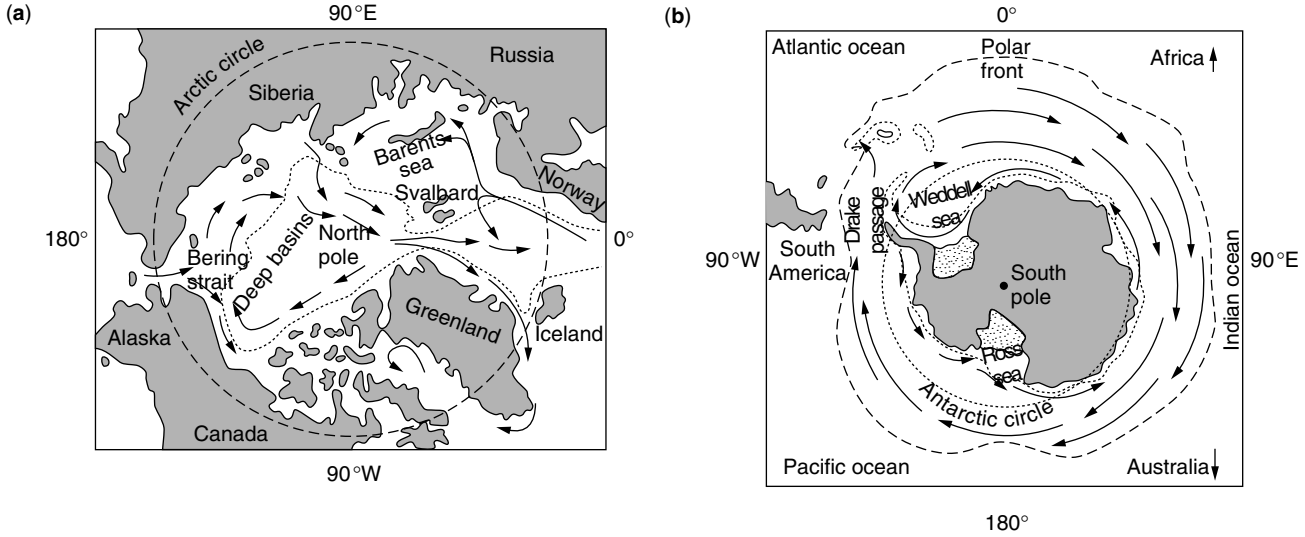

Figure 1. Maps of the Arctic (**a**) and Antarctic (**b**) to illustrate major differences in these two polar regions. Location of the Polar Circles are shown in addition to the 1,000 meter bottom contour (dotted lines). Note that different scaling factors have been used, as evidenced by the larger size of the polar circle in the Arctic as compared with that in the Antarctic. Arrows indicate directional flow of surface waters. See text for comments.

portion of the Arctic Ocean. Although the waters over the shelf areas occupy 37% of the Arctic Ocean area, they comprise only 2% of its volume. The permanent ice pack covers much of the area of the deep basins, where depths can exceed 4,000 m. Being largely enclosed by land masses, water flow into the Arctic basin is limited and is predominantly from the northeast Atlantic and subsequent flow to the northeast across the shelf region adjoining Siberia (10). There is also a substantial amount of fresh water flowing into the Arctic basin from northward flowing rivers, and also some periodic transport of water north through the Bering Strait. The river flow, however, is small compared to the flow from the Atlantic, but entrainment caused by the river waters helps create the transpolar flow. There is no large-scale upwelling on the Atlantic side; the area is known for downwelling or deep-water production. The extent and duration of sea ice in the Arctic is of much importance for the phytoplankton because sea ice scatters and absorbs solar radiation, resulting in light-limiting conditions in the underlying water column. Of the total ocean area of the Arctic Seas ($\sim 20 \times 10^6 km^2$), approximately 80% is generally covered with sea ice in winter and approximately 50% in summer (see also section Interannual Variability: Importance of Global Cyclic Climatic Events). Inorganic nutrient concentrations in Arctic waters are much lower than in the Antarctic. During spring-summer following the first bloom in ice-free waters, the concentrations of nitrate, phosphate, and silicic acid in surface waters are often close to zero, resulting in nutrient limitation of phytoplankton biomass and rates of primary production. During this low-nutrient summer period, nutrients derived from biological activity (e.g., ammonia, urea, dissolved organic nitrogen compounds) can be significant. The profiles of salinity, temperature, and water density during the summer growing season also differ appreciably between the two polar regions as indicated by representative data shown in Figure 2. It is seen that the upper water column of Arctic waters (Fig. 2a) is generally less saline, warmer, of lower water density, and with a much more stable upper mixed layer when compared with Antarctic waters.

Antarctic

In contrast to the Arctic, the South Pole (90 °S) is located on the high plateau of the Antarctic continent, which is a large land mass with mountains exceeding 5,000 m

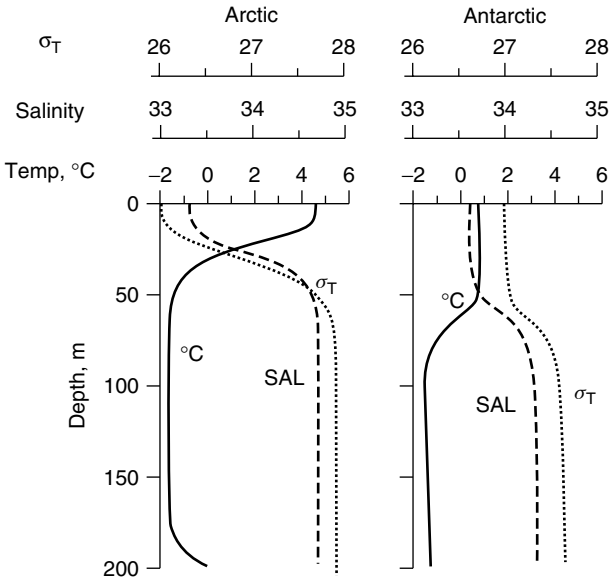

Figure 2. Typical mid-summer profiles of temperature, salinity, and water density in the upper water column of both polar regions. (**a**), data from the Barents Sea in the Arctic in July and August; (**b**), data from the Scotia Sea, Antarctica, in February.

in elevation. The continent is an isolated land mass, being surrounded by the Southern Ocean, which flows unimpeded through the southern regions of the Atlantic, Pacific, and Indian Oceans (Fig. 1b). As a result of its isolation and the vast ocean surrounding the continent, it is much colder in the Antarctic than in the Arctic. The northern boundary of the Southern Ocean is the Polar Front, which lies considerably to the north of the Antarctic Circle. The total area of the Southern Ocean is large ($\sim 36 \times 10^6$ km^2) and equal to about 12% of the world's ocean area. The mean depth of the Southern Ocean is greater than 4,000 m, with the deep (500–800 m) continental shelf areas being relatively small as compared with the area of pelagic waters. The continental shelf break as indicated by the 1,000 m depth contour (Fig. 1b) lies relatively close to the continent in sharp contrast to the situation seen in the Arctic. There is massive upwelling of deep, cold, and nutrient-rich water close to the continental shelf break around the continent, with the major source being southward-flowing North Atlantic Deep Water, which was produced mainly in the Greenland and Labrador Seas a century or more earlier. But with contributions also from the deep waters of the Pacific and Indian Oceans. These nutrient-rich waters upwell and flow in an east-northeast direction around the continent and is termed the Antarctic Circumpolar Current (ACC). Between the continent and the zone of divergence where the upwelling occurs, there is a westward flowing current called the West Wind Drift as indicated by the arrows in Fig. 1b. There is gradual warming of the surface waters of the ACC during this flow, so that there generally is a temperature gradient from close to zero near the continent to approximately 4 to 5 °C at the Polar Front. Within the Polar Front region these northeast-flowing waters of the ACC mix and submerge below the southward-flowing sub-Antarctic waters (8). Water temperatures north of the Polar front increase quite rapidly to about 10 °C at the Subtropical Convergence, which is the northern limit of sub-Antarctic waters northern limit of sub-Antarctic waters. The Polar Front region thus forms a natural boundary for many phytoplankton species that are found to the south in Antarctic waters, but are not present in waters to the north of the Polar Front.

Inorganic nutrient concentrations in pelagic Antarctic waters are very high, with the winter-time concentrations of nitrate, phosphate, and silicic acid being approximately 40 μM, 3.0 μM, and greater than 100 μM, respectively. Because of the low stability of the upper water column and the frequency of deep mixing by storm events that results in deep upper mixed layers, phytoplankton biomass in pelagic waters is generally low and the major inorganic nutrients are not depleted to the extent that the biomass of phytoplankton becomes limited by low concentrations of these elements. Data in Figure 2b show that the ranges of temperature, salinity, and water density in the upper 100 m of the water column in the Antarctic are much smaller than in the Arctic. Phytoplankton cells are generally fairly uniformly distributed by physical mixing processes throughout the depth of the upper mixed layer. This results in a low concentration of phytoplankton in the entire mixed layer, as the cells in the lower portion of the mixed layer will be limited by low light levels where the rate of photosynthesis approaches the rate of respiration. During the winter months the area of sea ice in the Antarctic is extensive and covers approximately 56% of the waters south of the Polar Front. This annual sea ice recedes fairly rapidly though in late winter and spring, so that by February or March ice covers only about 10% of the ocean area. Sea ice is of great importance for phytoplankton because (*1*) it attenuates solar radiation so that low light levels limit the photosynthetic rate in the water column below the ice, (*2*) it enables some species of phytoplankton to grow attached to or within the sea ice, with the result that large concentrations of phytoplankton may accumulate associated with the ice cover, and (*3*) with melting of the ice, the released algal cells may serve as an inoculum to initiate a bloom of phytoplankton in the ice-free waters.

INTERANNUAL VARIABILITY: IMPORTANCE OF GLOBAL CYCLIC CLIMATIC EVENTS

Marine climate is extremely variable at high latitudes, so there is no short-term "ecological balance," at least not in any meaningful steady state sense. Both polar regions are characterized by alternating "warm" and "cold" periods that may last for months or years, during which marine populations, including phytoplankton, may expand or contract. When the marine climate changes, primary producers respond within few days, and grazers within weeks to a few months. The impacts of such cyclic climate changes have been studied to a much greater extent in the Arctic regions than in the Antarctic. During the "warm" years in the Arctic, primary production may be up to 30 to 50% higher than in the "cold" years, primarily as a consequence of less ice cover. The high-productive years are characterized by greater frequency and intensity of summer storms that furnish nutrients after the conclusion of the spring and/or ice edge blooms. The warm and cold years in the Arctic Seas exhibit cyclicity, as evident from the longest existing arctic surface sea temperature (SST) time series, the Kola section in the Barents Sea. These data show that the 1930s were warm, as was the period 1946 to 1955, whereas the 1960s and the periods 1977 to 1981 and 1984 to 1988 were cold. However, because of the circulation pattern, the warm years in the Barents Sea are matched against cold anomalies off East Greenland, and thus the respective ecosystems cycle in counterphase. The causes of these cyclic climate changes are not well understood, although they appear to be related to atmospheric forcing related to changes in regional barometric pressure and wind systems, which in turn may have strong effects on ocean currents and upwelling processes. These cycles are generalized in a simple fashion by the North Atlantic, Arctic, and Pacific Decadal Oscillations, (NAO, AO, and PDO, respectively). Some cyclic climatic changes can have impacts on a global scale, such as the well-known El Nino and La Nina events, which often affected the entire marine food chain in both the Northern and Southern Hemispheres.

The impact on the marine environment caused by these cyclic climatic events may last for years or even decades

for long-lived and slow-reproducing organisms such as cod, some seabirds, and mammals. The impact of such cyclic events has not been documented in the Antarctic as well as it has for the Arctic, but it seems likely that the variability noted in the Antarctic for phytoplanktopn and higher trophic levels is related to the Southern Oscillation Index (SOI), which includes influences of the El Nino and La Nina events. All these cyclic climatic events mentioned above presumably embed the decadal Sunspot Cycles (11 and 22 years) and the Lunar Declination Cycle (18.6 years). The underlying mechanisms, however, are poorly understood, even though they ultimately have dramatic effects on marine productivieityt and to societal concerns as a whole.

RATES OF PRIMARY PRODUCTION

Both in the Southern Ocean and in the Arctic seas phytoplankton communities and photosynthetic rates are regulated by light received by the phytoplankton as they are carried up and down in more or less mixed water columns, and by nutrient supply. Light is regulated by surface irradiance, which depends on seasonality, latitude, and cloudiness, and the depth and intensity of vertical mixing, which in turn depends on wind speed and fetch and the resistance (stability) of the water column to mixing. Both polar regions have extensive cloud cover much of the time, often reducing incident solar irradiance to 15 to 40% of that occurring on a cloud free day. Mixing may also be forced by tides, shear forces between currents and island or bank effects. Mixing plays a dual role by also regulating nutrient supply to the surface layer; however, nutrient supplies can also be increased by upwelling that is wind-driven or by entrainment of deeper waters into brackish layers in near-coastal waters where there is ample freshwater supply, as in some of the Arctic shelf seas. The rate of primary production will be controlled primarily by the biomass of phytoplankton and the light intensity (solar irradiance) to which the cells are exposed. Some solar radiation is lost to the water by being reflected from the water surface; the light that enters the water column is attenuated with depth because of absorption and scattering by both particulate and dissolved materials. The depth to which 1% of the incident radiation penetrates the water column is referred to as the euphotic zone, and is commonly accepted as the lower depth at which net primary production can occur (i.e., where the rate of photosynthesis exceeds the rate of respiration). The euphotic zone may range from a meter or less in the case of rich phytoplankton blooms to greater than 100 meters in the clearest, nutrient-impoverished oceanic waters.

There is a fairly good relationship between the rate of primary production in surface waters (the upper few meters) and the magnitude of primary production when integrated for the depth of the entire euphotic zone. Availability of satellite imagery makes it possible to estimate chlorophyll-a concentrations on a synoptic basis over large geographic areas. Data in Figure 3 show the concentrations of chlorophyll-a in surface waters in both polar regions during the most productive spring-summer months. It is seen that most ice-free Arctic waters show a high biomass of phytoplankton, but that there is relatively little production within the Arctic basin because of persistent ice cover. Maximal chlorophyll-a concentrations in the Barents Sea are relatively low (range of 10 to 15 mg/cubic meter) because of depletion of nutrients. Because nutrients are higher over the Bering Sea shelf, chlorophyll-a concentrations of greater than 60 mg per cubic meter have been reported. Concentrations of chlorophyll-a in Antarctic waters are much lower than in the Arctic, except for a scattering of localized regions that show high biomass of phytoplankton. The areas of high phytoplankton biomass (usually 5 to 10 mg chl-a per cubic meter, but occasionally as high as 35 mg chl-a per cubic meter) are generally in coastal waters overlying the continental shelf (particularly in the Ross Sea), in waters close to the Antarctic Peninsula and Scotia Ridge, and in waters in proximity to receding ice edges. In contrast to these areas of high phytoplankton biomass (and associated primary production), most pelagic Antarctic waters have low phytoplankton concentrations (average of ~ 0.5 mg chl-a per cubic meter) throughout the entire summer period. However, there is considerable variability in surface chlorophyll-a concentrations in Antarctic pelagic waters as can be seen by the data in Figure 3b. The likely explanations for such low phytoplankton biomass despite high nutrient concentrations and ample solar radiation are discussed in Section Major factors affecting growth rates of phytoplankton.

The data presented in Figure 3 illustrate chlorophyll-a concentrations in the time period when phytoplankton are generally at their maximal levels. Because primary production supports all animal life in the sea, it is also important to consider rates of primary production throughout the entire year. In the Antarctic, rates of primary production in ice-free waters bear a fairly food relationship to the seasonal changes in solar radiation as essential macronutrients do not become limiting. Phytoplankton biomass starts to increase rapidly in spring (September–October), blooms develop in the late spring and summer period (November through February), after which the phytoplankton biomass declines with the progressively shorter days in March and April. The situation is slightly different in the Arctic, where the springtime blooms often assimilate nearly all the available nitrogen and silicic acid in the euphotic zone, resulting in a rapid decline in phytoplankton biomass and associated rates of primary production. Phytoplankton productivity during the remainder of the year depends largely on regeneration of nutrients in the upper water column by microbial processes (see section Phytoplankton and the Food Web). The timing sequences mentioned above may be modified in both polar regions by the presence of a receding ice edge, which will generally result in higher rates of primary production within 50 to 100 km of the ice edge. This enhancement of phytoplankton biomass is the result primarily of a shallower and more stable upper mixed layer induced by the freshwater ice-melt, but also by nutrient enrichment from materials contained in the melting ice. Such ice-edge blooms may occur as late as July in the highest latitudes of the Arctic.

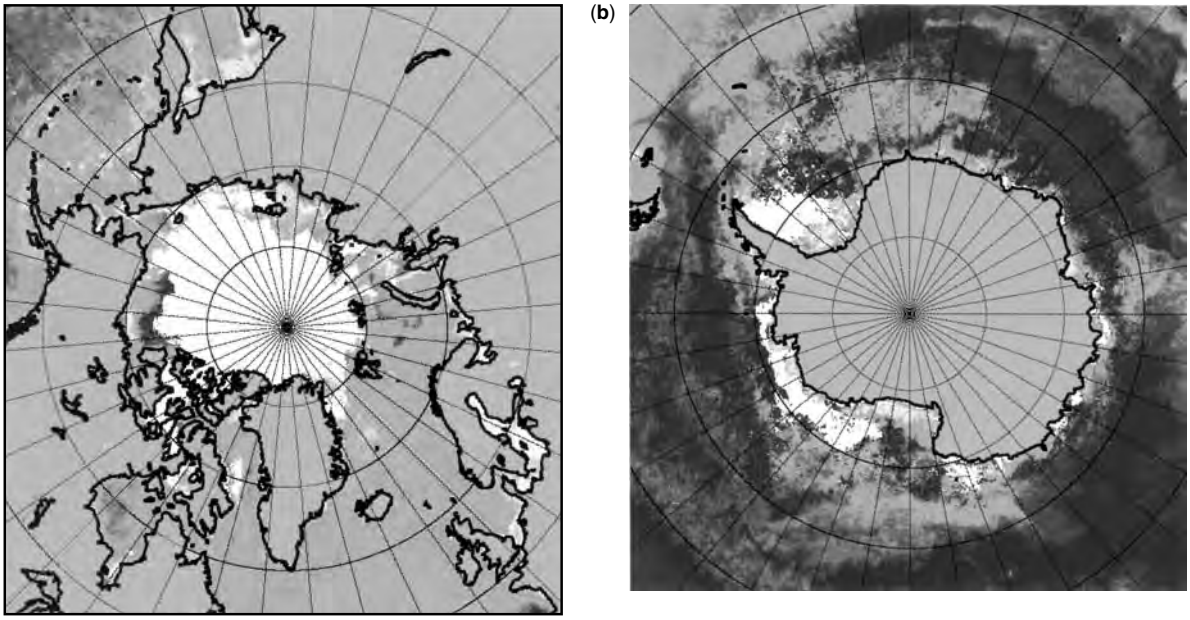

Figure 3. Satellite data images showing mean chlorophyll-a concentrations in surface waters during summer in the Arctic (**a**) and the Antarctic (**b**). In the Arctic, the data are composites for the months of May, June, and July for five years. In the Antarctic, the data are composites for the months of November, December, and January for three years. The same geographic and color scales have been used in both images. Highest chlorophyll-a concentrations are indicated by red (>30 mg chl-a per cubic meter), with decreasing concentrations as per the spectrum, with violet representing the lowest concentrations (<0.08 mg chl-a per cubic meter). White indicates areas where no data were obtained due to either ice or cloud cover. Images provided by the SeaWiFS Project, NASA/Goddard Space Flight Center, and ORBIMAGE, and the final figures were prepared by Mati Kahru.

As a result of the spatial and temporal variability in rates of primary production in both the Arctic and the Antarctic, it is difficult to give precise estimates for the total annual primary production in the polar regions. The rate of primary production in pelagic Antarctic waters is believed to be about 150 mg carbon per square meter per day during the growing season, whereas in coastal waters the rates are often between 0.5 and 3 grams carbon per square meter per day. Estimates for the mean annual primary production in the Southern Ocean range from a low of 20 to approximately 100 grams carbon per square meter per year. Estimates for rates of primary production in Arctic waters range from a low of about 15 grams carbon per square meter per year in the high Arctic, about 170 grams carbon per square meter per year in the ice-free Barents Sea, and between 200 and 800 grams carbon per square meter per year in waters over the Bering Shelf.

MAJOR FACTORS AFFECTING GROWTH RATES OF PHYTOPLANKTON

Solar Radiation

The portion of the solar electromagnetic spectrum incident upon the earth, which is of importance to phytoplankton, includes the ultraviolet wavelengths (UVR) from 285 to 400 nm and the visible wavelength radiation (400–700 nm), which are commonly referred to as photosynthetically available radiation (PAR). The absorption spectrum of chlorophyll extends into the UVR and hence the longer UVR wavelengths can also function to provide the energy needed to reduce CO_2 to carbohydrate, but this contribution by UVR is small compared with that of PAR. The deleterious impact of UVR on polar phytoplankton is discussed in the section Possible Impacts of Changing Climatic Conditions. This section thus deals only with photosynthetic rates as related to the intensity of visible radiation (PAR) or to the daily integrated flux of PAR. Phytoplankton photosynthetic rates as a function of solar irradiance varies considerably from species to species and also on the previous light history to which the cells have been exposed (11). The general relationship between the rate of photosynthesis and light intensity, however, is shown in Figure 4. The light compensation point (E_c) is where the rate of photosynthesis is equal to the rate of respiration, and hence there is no net gain in cellular carbon concentrations. The value for E_c is difficult to determine for natural phytoplankton assemblages, but it is usually in the range of 0.1 to 0.5% of the incident radiation ($\sim$1 to 5 μ Einsteins per square meter per second). At higher irradiances, photosynthetic rates increase in a hyperbolic fashion until the saturating light intensity (E_m) is reached. The value E_k, which resembles a half saturation constant to some degree is a useful indicator

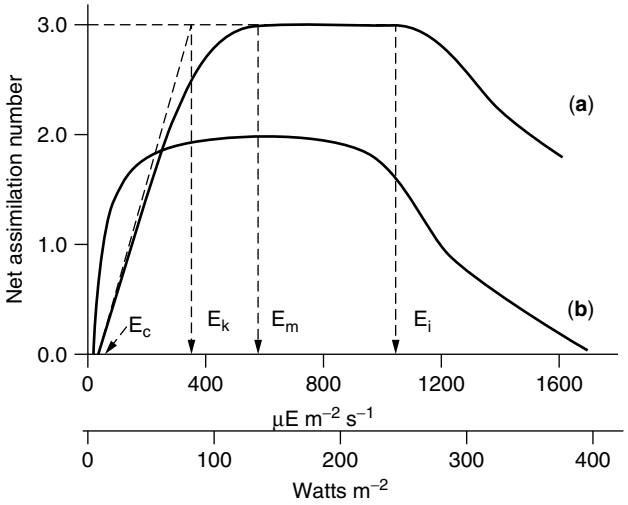

Figure 4. Generalized relationship between the net rate of primary production (mg carbon fixed per mg chl-a per hour) as a function of the solar irradiance to which the phytoplankton are exposed. Irradiance is expressed both as microEinsteins per square meter and also as Watts per square meter, as both these units are commonly used in the literature. The maximal values shown on the abscissa would be representative of maximal incident solar radiation on a clear day in the polar regions. Curve **a**, light-acclimated phytoplankton sampled from near-surface water; curve **b**, dark-acclimated cells sampled from close to the bottom of a deep upper mixed later. See text for details.

of the light-acclimation state of the phytoplankton. The higher the value of E_k, the more acclimated the phytoplankton are to high light conditions. Typical values of E_k for polar phytoplankton are generally in the range of 10 (shade acclimated cells) to 60 to 70 μ Einsteins per square meter per second, which is considerably lower than values for most phytoplankton in temperate or tropical waters. At E_m, the enzymatic capabilities of the photosynthetic mechanisms are functioning at their maximal rates. With increasing light intensity, the rate of photosynthesis remains fairly constant up to a certain point (E_i) where increasing irradiances will cause inhibition of CO_2 fixation. Such light inhibition of photosynthesis by PAR is not always found in polar phytoplankton assemblages, as the ability to utilize high light levels is dependent on the previous light history of the cells. Their nutritional status, and the seasonal variation in incident radiation (see data in Fig. 5).

Incident solar irradiance in the polar regions is much lower than at intemperate or tropical regions due to low sun angle to the horizon (Fig. 5a). However, as the day length is very long at high latitudes, the total solar radiation per day during the summer months can exceed that occurring near the equator (Fig. 5b). From the data shown in Figures 4 and 5, it is seen that irradiance will not limit rates of primary production in the upper portion of the euphotic zone of polar waters during the summer months. Light limitation does occur, however,

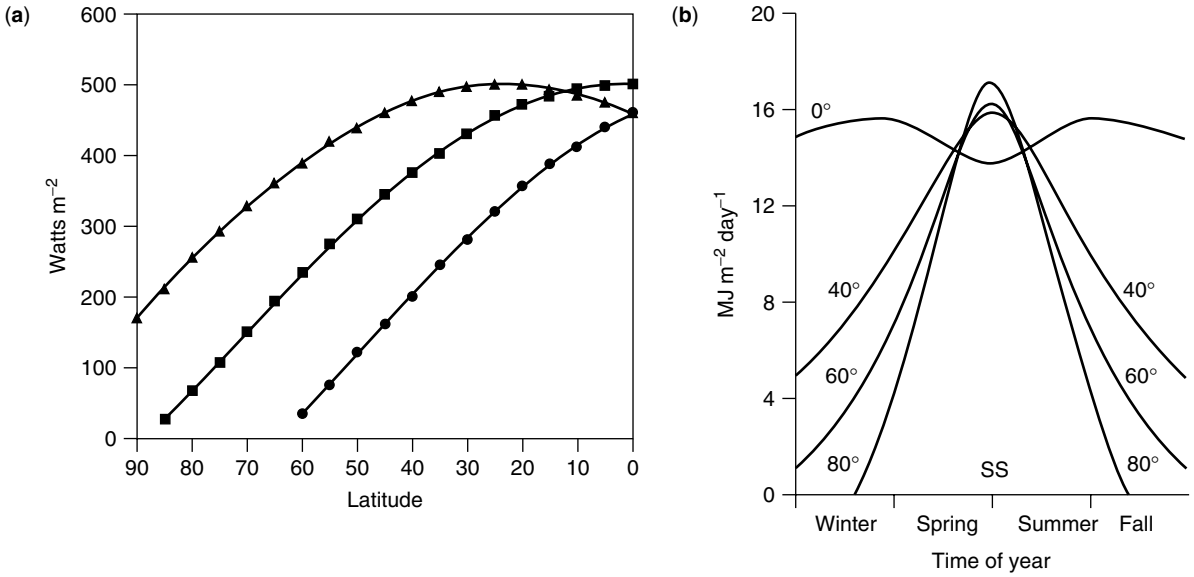

Figure 5. Solar radiation in the visible portion of the spectrum (400–700 nm) incident upon the Earth as a function of latitude and season; most of the Arctic marine region lies north of 60 degrees, while the Antarctic marine region lies sough of approximately 50 degrees. (**a**) Noontime solar irradiance at the times of the summer and winter solstice and the fall and spring equinox. The irradiance in the ultraviolet region (280–400 nm) shows a similar change with latitude, but the total energy in the UVR is only about 12 to 14% of that in the visible region. (**b**) Generalized values for total fluence of PAR (in megaJoules per square meter per day) as a function of latitude and time of year; SS is the time of summer solstice (June in the Northern Hemisphere and December in the Southern Hemisphere). Note that in the polar regions the daily fluence of PAR during the summer months can exceed the value in equatorial regions due to the long day length at high latitudes.

in surface waters during the winter months and also in the lower portions of the euphotic zone during summer. Spectral solar irradiance decreases in an exponential fashion with depth in the water column, with the shorter UVR and longer wavelengths in the visible portion of the spectrum being attenuated most rapidly, and the blue wavelengths in the visible portion penetrating the deepest into the water column. As chlorophyll-a shows maximal absorption of light in this same blue region of the spectrum, phytoplankton have efficient utilization of solar radiation in the lower portion of the euphotic zone. The photoresponse of polar phytoplankton to the changing spectral irradiance throughout the depth of the euphotic zone is illustrated by the data in Figure 6. Phytoplankton are generally fairly uniformly distributed throughout the upper mixed payer, which was at approximately 35 meter depth in Antarctic waters where the date for this figure were obtained. The photosynthetic rates due to PAR were saturated (and constant) throughout the depth of this upper mixed layer (from X to Y in Fig. 6), and declined rapidly in the lower portion of the euphotic zone (from Y to Z in Fig. 6) where light was limiting. The inhibition of photosynthesis in the upper portion of the water column is discussed in the section Possible Impacts of Changing Climatic Conditions.

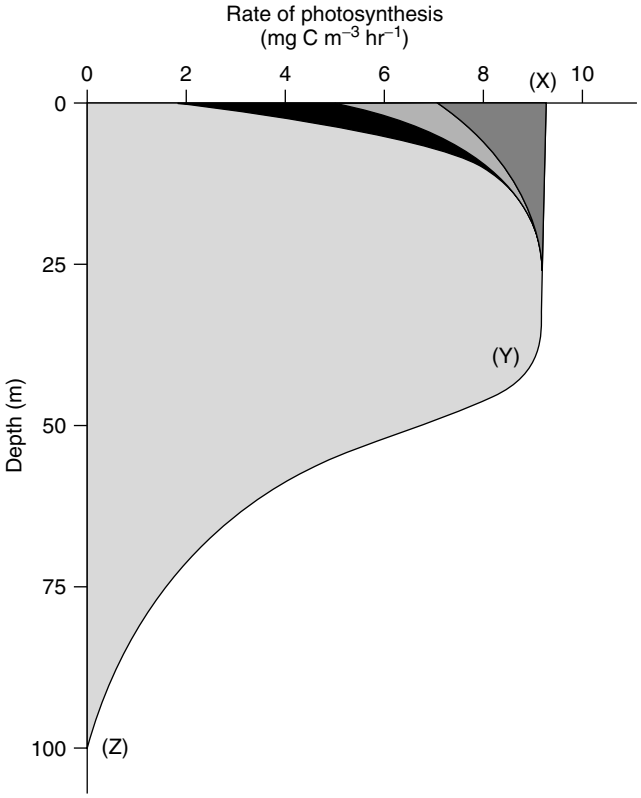

Figure 6. Diagram to illustrate the inhibitory effects of UV-A and UV-B radiation on rates of primary production under normal ozone conditions, and the impact of enhanced UV-B radiation under an extreme ozone hole in the Antarctic. The solid black area depicts the loss in primary production due to enhanced UV-B radiation. The lightly shaded area above that depicts the reduction in primary production due to normal levels of UV-B radiation, and the darker area above shows the loss due to UV-A radiation. The large lightly shaded area extending down to 100 m depth represents the profile of photosynthetic rates under conditions of depleted ozone concentrations. The depth of the upper mixed layer in the water column where these data were obtained was approximately 35 meters, and the 1% light level was at approximately 90 meters. From Lubin and Holm-Hansen (1995) with permission of the publisher, Academic Press, Inc.

Inorganic Nutrients

All plants, including phytoplankton, require the following essential macroelements for growth and viability: calcium, nitrogen, magnesium, sulfur, potassium, and phosphorus. Diatoms and silicoflagellates also require relatively high concentrations of silicon, which is a component of their outer cell walls. In addition to these elements, which are required in relatively high concentrations (half saturation constants are generally in the range of 0.1–0.5 μM), phytoplankton also require relatively low concentrations of the following essential microelements: manganese, molybdenum, copper, zinc, iron, boron, cobalt, and most likely selenium. Because the uptake of nutrients involves transport across the outer cell membrane, the ratio of the cell surface area to cellular volume is important and conveys some advantage to small cells when any essential nutrient element is present in limiting concentrations. One consequence of this is that when an element (e.g., Fe) is present in very low concentrations as in pelagic Antarctic waters, the growth rates of the small nanoplankton cells may not be limited by Fe availability, whereas the growth rates of the larger microplankton would be limited.

Macronutrients. Of the macroelements listed above, only nitrogen (N), phosphorus (P), and silicon (Si) have been found to limit phytoplankton growth in marine waters. These three elements are found in relatively high concentrations in surface Antarctic waters, with winter-time values (which are maximal because of upwelling of nutrient-rich deep water coupled with low phytoplankton assimilation due to low light levels) being approximately 40 μM nitrate (in addition to 0–2 μM ammonia), 3.0 μM phosphate, and more than 100 μM silicic acid. A few phytoplankton blooms have been reported in Antarctic waters where nitrate concentrations are less than 1.0 μM (but P and Si concentrations still sufficiently high that these elements are not limiting production), but concentrations of N, P, and Si in both coastal and pelagic waters generally are in excess (15 μM, 2.0 μM, and 40 μM, respectively) and not limiting phytoplankton growth rates. Because Si is a major constituent of diatom frustules and is not readily remineralized by microbial action in the upper water column when these cell walls settle to the sediments, the concentrations of silicic acid in surface waters close to the Polar Front may be relatively low (~10 μM) and possibly may be limiting phytoplankton biomass. The situation in the Arctic is very different, because the maximal winter-time concentrations of the three nutrients in surface waters of the Atlantic and Eurasian sides are 12 to 14 μM nitrate (in addition to 0–2 μM ammonia), 0-9 to 1.0 μM phosphate, and 6 to 8 μM silicic acid. On the Bering side the concentrations are

10 to 30 μM nitrate (+0–5 μM ammonia), 1 to 2 μM phosphate, and 25 to 50 μM silicate; these high levels are partly reflected in the Chukchi Sea and in the western Arctic at medium depths. Because of the shallower and more stable upper mixed layer in the Arctic, coupled with much less upwelling of deep water as compared with the Antarctic, these essential macroelements are often depleted to below detectable concentrations in the upper mixed layer during the phytoplankton bloom in spring. The relatively high winter nutrients and full depletion make large parts of the Bering Sea one of the most productive regions in the world. Following such nutrient depletion, phytoplankton primary production depends largely on regeneration of nutrients in the upper water column through the activity of grazing, heterotrophic protozoans, and zooplankton. Phytoplankton biomass and rates of primary production are low during such nutrient-limiting conditions. However, in the Nordic and Bering Seas, wind-induced mixing (with an input of nutrients into the euphotic zone) can be important also in the summer, as these waters are easier to mix than those farther north, which are stabilized by melt-water.

Micronutrients. Iron (Fe) is the only microelement for which there is convincing data showing that it is limiting phytoplankton biomass in pelagic Antarctic waters (1). Unlike most of the other essential elements, which are in high concentration in deep waters that upwell in both polar regions, the major source of Fe to surface waters is by aolian processes (transport and deposition of Fe via atmospheric fallout). As a result of its isolation and distance from the continental land masses, there is relatively little atmospheric input of Fe into the Southern Ocean. Recent experiments with adding large quantities of soluble iron salts to pelagic Antarctic waters have shown that such Fe additions result in rich phytoplankton blooms within 7 to 10 days and a change in the species composition of the phytoplankton from a nanoplankton-dominated assemblage to one dominated by microplanktonic diatoms. The variability in phytoplankton biomass in Antarctic pelagic waters (Fig. 3) is believed to be caused by variable Fe concentrations due to injection from coastal waters or upwelling associated with changes in bottom topography or with melting sea ice or icebergs. There are no data at present to indicate that Fe limits phytoplankton biomass in coastal Antarctic waters, but this possibility cannot be discounted. The Fe situation in the Arctic is very different because these waters are in close proximity of large land masses, and it is likely that substantial amounts of Fe are added by both aolian and river inputs into Arctic surface waters. It should be noted though that the chemical speciation of Fe in natural waters is complex, and the bulk of the iron input into Arctic surface waters may be complexed with particulates at the freshwater–seawater interface and then be lost to the euphotic zone by settling to the sediments. There are some preliminary data that suggest that Fe may at times limit phytoplankton production in Arctic waters, but because the macroelements N, P, and Si are also depleted during bloom formation, the addition of Fe to such waters would not be expected to have the same dramatic effect as found in Antarctic waters.

Temperature and Growth Rates. Temperature is a critical environmental variable for all living organisms because it affects enzymatic rates that control cellular metabolic processes. Compilation of literature data showing the relationship between temperature and growth rates of phytoplankton isolated from polar, temperate, and tropical waters shows that growth rates may be described by the van Hoff equation and predicts an exponential increase in reaction rates with an increase in temperature, with an approximate doubling of reaction rate with each 10 °C rise in temperature (12). Most polar phytoplankton are psychrophiles in that they are adapted to grow at low temperatures and do not survive at higher temperatures. The temperature range over which most Antarctic phytoplankton will grow and increase their photosynthetic rates is −1.8 °C to approximately 8 to 10 °C. Because water temperature at the Polar front is approximately 5 °C, Antarctic phytoplankton apparently have the metabolic capabilities to grow well even if ambient water temperatures should exceed that normally encountered by 3 to 4 °C. It is important to note that the effect of a change in temperature within the above range will not have the same effect on all species. Thus, temperature changes may have important consequences on species composition of the phytoplankton asemblages. Specific growth rates in polar phytoplankton generally range from 0.1 to 0.6, which is equivalent to doubling times of 0.15 to 0.9 doublings per day. The average specific growth rate in both polar regions is about at 0.5 per day, which is equivalent to 0.8 doublings per day.

Salinity. The salinity (a measure of the total salt content) of seawater is of importance to phytoplankton productivity in two ways. First, the density of seawater at the low temperatures occurring in the polar regions is largely determined by the salinity, and the profile of water density is of prime importance in affecting the distribution of planktonic cells in the water column. Second, the direct impact of salinity on phytoplankton plays a modest role for productivity unless it is less than 15 psu, which happens only in extreme cases. Such low salinities efficiently eliminate true marine species. Salinity, however, may play a role, albeit not a well known one, for the species composition of the community through effects on growth rate via osmotic regulation and nutrient uptake. A lowering of salinity may, for instance, shift the dominance to other species that originally made up a minority component. This may in turn make an impact on the quality of phytoplankton as food for grazers.

PHYTOPLANKTON AND THE FOOD WEB

The species composition and growth rates of natural phytoplankton assemblages must be considered in the context of interactions with all nonphotosynthetic organisms in water. Phytoplankton are being continually grazed (eaten) by heterotrophic organisms ranging in size from unicellular protozoans to large zooplankton and krill. There is thus a dynamic interaction between the producers (phytoplankton) and the consumers (all heterotrophic organisms), such that the biomass of phytoplankton tends to remain fairly

uniform from day to day, despite the daily fixation of carbon dioxide by the phytoplankton. As most zooplankton have the ability to graze preferentially (i.e., they select particles to be eaten rather than simply ingesting all particles) the zooplankton assemblages may thus have a strong impact on the species composition of the phytoplankton. Although diatoms are a major food item for most zooplankton, recent data suggest that they generally prefer small naked flagellates. This continual grazing of phytoplankton by zooplankton, coupled with the assimilation and breakdown of dissolved organic compounds by the bacterial populations, results in cycling of organic materials and the regeneration of inorganic nutrients within the euphotic zone. One result of these continual recycling processes is that all essential inorganic elements will always be present in the water column, albeit at very low concentrations. Because the assimilation of nutrients requires transport across the outer cell wall or membrane, the ratio of cell surface area to cellular volume will be important in regard to the ability of the organism to survive at very low concentrations of essential inorganic elements. Small nanoplankton thus have some advantage over the larger microplankton in regard to continued growth under nutrient-limiting conditions.

POSSIBLE IMPACTS OF CHANGING CLIMATIC CONDITIONS

Increase of Temperature. There is much concern that the increasing concentrations of CO_2 in the world's atmosphere will have a "greenhouse effect" and cause a rise in the world's temperature, with maximal effects in the polar regions. The projected increase for the polar regions is on the order of 2 to 5 °C. The impact of such a temperature rise in polar surface waters would be expected to have the following consequences in regard to phytoplankton. First, it would most likely increase the rate of primary production because of (*1*) Increasing cellular enzymatic rates, including the rate of photosynthesis. The projected increase in water temperature is well within the temperature range for growth by polar phytoplankton as discussed in the section Major Factors Affecting Growth Rates of Phytoplankton. (*2*) Heating of surface water would increase the stability of the upper mixed layer, which would help maintain phytoplankton at higher mean light intensities. Second, warming of polar environments would result in a dramatic decrease in the extent of annual sea ice. Such an increase in extent of ice-free waters would increase total primary production by phytoplankton. However, a decrease in extent and duration of annual sea ice would also have deleterious biological impacts as sea ice is very important for the marine biota because the variability in the formation and crystalline nature of sea ice provides excellent habitats for growth of a complex microbial food web, including unicellular algae (13). The algal layer on the underside of the ice may reach high biomass densities and persist throughout the dark winter periods. Sea ice and associated food resources provide an excellent habitat where juvenile krill find both protection from predation and sufficient food to meet their dietary requirements. This ice habitat is thus of much importance for the ability of many organisms, including juvenile krill, to have high overwintering survival rates. Because krill is a major food item for many higher trophic levels (e.g., birds, seals, whales) in the polar regions, such temperature-induced loss of sea ice might have serious consequences in the entire food chain in polar waters. Loss of sea ice would also have serious implications for polar bears, seals, and seabirds, which require annual sea ice to find sufficient sources of food.

Decrease of Ozone in the Stratosphere

British scientists at Halley Bay first detected the seasonal loss of ozone over Antarctica in the late 1950s, but the extent and severity of the ozone hole was not generally recognized until the mid-1980s. Because ozone absorbs the shorter wavelengths (UV-B radiation; 280–320 nm) of solar UVR, the biological significance of a decrease of ozone in the stratosphere is that there is an increase in the flux of UV-B radiation incident upon Earth (14). Ozone does not have any significant effect on the UV-A wavelengths (320–400 NM) of solar radiation. The energy content per quantum in the UV-B portion of the spectrum is high and capable of causing cellular damage, particularly to the genetic material (DNA). The total energy in the UV-B portion of the spectrum is relatively low ($\sim$ 0.5% of PAR). With the increasing size, depth, and duration of the ozone hole over Antarctica during the 1990s, much concern was expressed regarding the possibility of severe ecosystem damage in the Southern Ocean due to this enhanced UV-B radiation. The loss of ozone in the atmosphere is greatest in the polar regions and decreases in severity toward the equator. As a result of the unique atmospheric conditions prevailing over the Antarctic continent (particularly very low temperatures and the polar vortex with ice crystals), the depletion of ozone in Antarctica during spring (mainly October and November) is very severe, with the column ozone values decreasing to less then 100 Dobson units (DU) on occasion as compared with normal values of greater than 300 DU. The depletion of ozone in the Arctic is not as severe as in the Antarctic, as ozone levels generally do not fall below 300 DU. The impact of UV-B radiation as well as UV-A radiation (320–400 nm) on integrated primary production in the Antarctic water column during the period of the ozone hole is shown in Figure 6. Inhibition of photosynthesis by UV-B can only be detected in the upper 10 to 15 m of the water column, whereas that by enhanced UV-B radiation cannot be detected below approximately 10 m. Primary production, however, occurs down to at least the 1% light depth, which was at about 90 m where the experimental data for Figure 6 were obtained. In the worst case situation (<100 DU, sunny with no clouds), the loss of photosynthate due to enhanced UV-B radiation is less than 4% of the total production. If one calculates (taking into account ice and cloud cover) the annual loss in the magnitude of primary production due to enhanced UV-B radiation resulting from decreased ozone concentrations over the Southern Ocean, the value is less than 0.2% (15). This loss of primary production due to lowered ozone concentrations is small compared with normal variability in annual primary production in

the Antarctic (16), and hence it is not likely to result in serious consequences in regard to annual productivity in the polar regions.

GLOSSARY

Biomass

A value to express the total amount of live cells, which may be expressed in various terms such as cellular volume, wet or dry weight, or total cellular organic carbon. Chlorophyll-a concentrations are often used as a biomass indicator, but this is dependent on knowing the ratio of organic carbon to chlorophyll-a in the phytoplankton.

Dobson Units

A measure of the total amount of ozone in a unit column of the entire atmosphere. One Dobson Unit is equal to 1,000 times the thickness (in centimeters) of all the ozone if it were compressed to standard temperature and pressure.

Einstein

A unit commonly used to express solar irradiance in terms of quanta per unit area per unit time. One Einstein equals one Mole Quanta of solar radiation, which is equivalent to $602x10^{23}$ quanta.

Primary Production

The assimilation and reduction of carbon dioxide (CO_2) to organic compounds by chlorophyll-containing plants, with the energy required for these reactions being obtained by absorption of solar radiation, chiefly in the visible portion (400–700 nm) of the spectrum.

Photoautotrophic

The ability of chlorophyll-containing plants to synthesize all cellular organic materials from carbon dioxide and water, with the required energy for the reactions being provided by absorption of sunlight.

Phytoplankton Bloom

An expression to indicate that phytoplankton assemblages have increased in biomass so that chlorophyll-a concentrations exceed 1 mg chlorophyll-a per cubic meter.

Upper Mixed Layer

The depth of the upper surface layer of the water column where the water density remains close to constant and is characterized by a fairly uniform distribution of dissolved and plankton particles caused by vertical mixing processes.

Water Column Stability

A measure of the rate of change in water density with depth in the water column. With little or no change in water density with depth, the water column is easily mixed; when there is a large difference in water density with depth, the water column will be more stable and more difficult to mix vertically.

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POLYMERASE CHAIN REACTION (PCR).

See Field Release of Genetically Engineered Microorganisms (GEM); *Giardia*: Detection and Occurrence of in the Environment; Phylogenetically Based Methods in Microbial Ecology

POLYMERS IN ACTIVATED SLUDGE.

See Storage Polymers: Role in the Ecology of Activated Sludge

POPULATION GROWTH IN SOILS, KINETICS OF.

See Kinetics of Microbial Processes and Population Growth in Soil

PRESERVATION OF ALGAE FOR TOXICITY TESTING.

See USE OF MICROSCOPIC ALGAE IN TOXICITY TESTING

PRETREATMENT IN WATER TREATMENT PLANTS: PATHOGEN REMOVAL.

See MICROBIAL REMOVAL BY PRETREATMENT, COAGULATION AND ION EXCHANGE

PRIMARY PRODUCTIVITY.

See PLANKTONIC ALGAE IN THE MARINE ENVIRONMENT

PRIMARY PRODUCTIVITY IN THE MARINE ENVIRONMENT

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Marine primary productivity is generally defined as the photosynthetic reduction by procaryotic and eucaryotic organisms of inorganic carbon to organic matter using solar radiation as the energy source. Therefore, it is the amount of CO_2 fixed in a volume of water per unit time, and is essentially an instantaneous measurement. Productivity varies as a function of solar radiation, which changes over short timescales (e.g., due to clouds), as well as on a daily and seasonal basis. Because solar radiation penetrates to some finite depth in the ocean, primary productivity also occurs throughout that layer (generally called the euphotic zone). Because productivity varies both with depth and time, it is often integrated through both to give a daily, depth-integrated rate with units of $g\ C\ m^{-2}\ d^{-1}$. This is an essential variable to understand the structure and function of ocean systems because it is this rate that quantifies the flow of organic matter to the food web (1) and defines the biological influence on marine biogeochemical cycles (2).

HISTORICAL APPROACHES TO THE ASSESSMENT OF PRIMARY PRODUCTIVITY

Direct Measurement of Productivity

During the early 1900s, productivity was estimated by reductions in dissolved carbon dioxide (3), phosphate (4), and nitrate (5) (all are used in the formation of organic matter in relatively uniform removal ratios, now known as the Redfield ratios, which is the removal of carbon, nitrogen, and phosphorus in ratios of 106 : 16 : 1; ref. 6). Because oxygen is generated during photosynthesis, its production was used to estimate productivity as well (7). But as a result of the relative insensitivity of the analytical procedures used to measure the inorganic constituents, it was not until the introduction of the use of the radioactive tracer $^{14}CO_2$ that allowed for the sensitive measurement of the incorporation of carbon dioxide into organic matter in a variety of oceanic realms (8). This method involves the addition of radioactive bicarbonate to an enclosed sample, and after an incubation period in light, the sample is filtered. Any radioactive material retained on the filter must be in particulate form because productivity by definition is the production of organic matter $(CH_2O)_n$ via photosynthesis (Eq. 1)

$$nCO_2 + nH_2O \longrightarrow (CH_2O)_n + nO_2 \qquad (1)$$

The radioactivity is incorporated as a linear function of the ratio of $^{14}CO_2 : {}^{12}CO_2$ in seawater. Furthermore, because the reaction is carried out only using solar energy captured and converted to ATP by living cells, it represents biological fixation of carbon. ^{14}C-organic matter is relatively simple to detect accurately, especially with the improvement of liquid scintillation techniques, and therefore productivity can be measured relatively unambiguously using this technique. As a result, it became the method of choice to assess spatial and temporal changes of productivity and to experimentally determine the factors regulating oceanic productivity, and remains so today.

The method is not without uncertainties (9), as the length of incubation, the incubation conditions, the size of bottles, and the production of dissolved organic matter during photosynthesis make both measurement and interpretation problematic. Individual sources of error can be as much as 25% (e.g., DOC production; 10), and hence a single measurement of productivity can be difficult to interpret in the context of ecosystem dynamics. Such errors can be minimized by using consistent techniques, but variations among investigators remains a troublesome (and poorly addressed) problem in studies of marine productivity.

Biomass of phytoplankton correlates well with productivity (11,12), although in many specific instances it displays rather large deviations from the generally observed linear relationship. Many of these deviations are derived from the differences in the vertical distribution of irradiance in the water column (which follows an exponential reduction) with those of biomass (which is often distributed evenly in the upper mixed layer, and may increase or decrease below that depth). It is possible to collect discrete samples from the water column, measure the biomass within those samples and the photosynthetic response (the rate of photosynthesis over a wide range of irradiances during a short incubation) and in situ irradiance, and calculate the water column productivity based on these results (13). Productivity at a known depth has units of $mg\ C\ m^{-3}\ h^{-1}$, whereas integrated values generally are expressed in units of $mg\ C^{-2}\ d^{-1}$. Such estimates minimize uncertainties due to incubation length and provide realistic assessments of productivity at one location.

Based on these techniques, broad patterns of productivity of the ocean and its subsystems have been estimated (14,15; Fig. 1). In viewing this composite it must be understood that when this was produced, much of the ocean was (and still is) greatly undersampled, and no direct estimates were available for vast portions of the ocean. To generate the map, over 7,000 stations were used, most of which were collected by far-reaching expeditions of the former Soviet Union. Even with this number of stations, the productivity of the ocean in large part remained unknown. Furthermore, methodological inconsistencies were not assessed, and these differences undoubtedly contributed to some of the variations reported. Regardless, this estimate was extremely useful, in that it clearly delineated the regions where productivity was high and where it was low. It also provided a very useful contrast to a theoretical prediction of global productivity provided earlier by Sverdrup (16).

Koblentz-Mischke and coworkers (14) found that much of the ocean was highly unproductive, and that the major locations of greatly enhanced productivity were associated with coastal regions and physical features that brought nutrients to the surface layer. Specifically, productivity was very large in the following coastal regions: the Arabian Sea, northwest Africa, southwest Africa, the Indonesian archipelago, southern Greenland, the Barents Sea, coastal China and Russia, and coastal British Columbia (Fig. 1). Other isolated pockets of high productivity existed scattered around the globe. Similarly, very low productivity was associated with the vast oceanic gyres, such the North and South Pacific Gyres, the South Atlantic Gyre, the Sargasso Sea, and the Arctic Ocean.

It was also suggested by Koblentz-Mischke and coworkers (14) that the annual production of the global ocean was approximately 25 to 30 pg C y^{-1} (1 pg = 10^{15} g). This figure was widely accepted and used in attempts to understand the trophic transfer of photosynthetic material within the food web. One such example was the analysis by John Ryther (1). He divided the ocean into provinces with known areas, assigned (based on his own measurements) mean production rates to each area, and calculated the annual ocean production. His estimate of oceanic production was 20 pg C y^{-1}, a figure not unlike that of Koblentz-Mischke and coworkers (14). Ryther went on to predict that the fish yield based on that production was indeed finite, and although the scientific rationale for his arguments was widely criticized, his conclusions have in large part been verified by fishing statistics since 1970, as well as further theoretical work (17). Thus, despite the fact that highly productive, but temporally restricted, "events" were not included by either Ryther (1) or Koblentz-Mischke and coworkers (14), and their approaches were

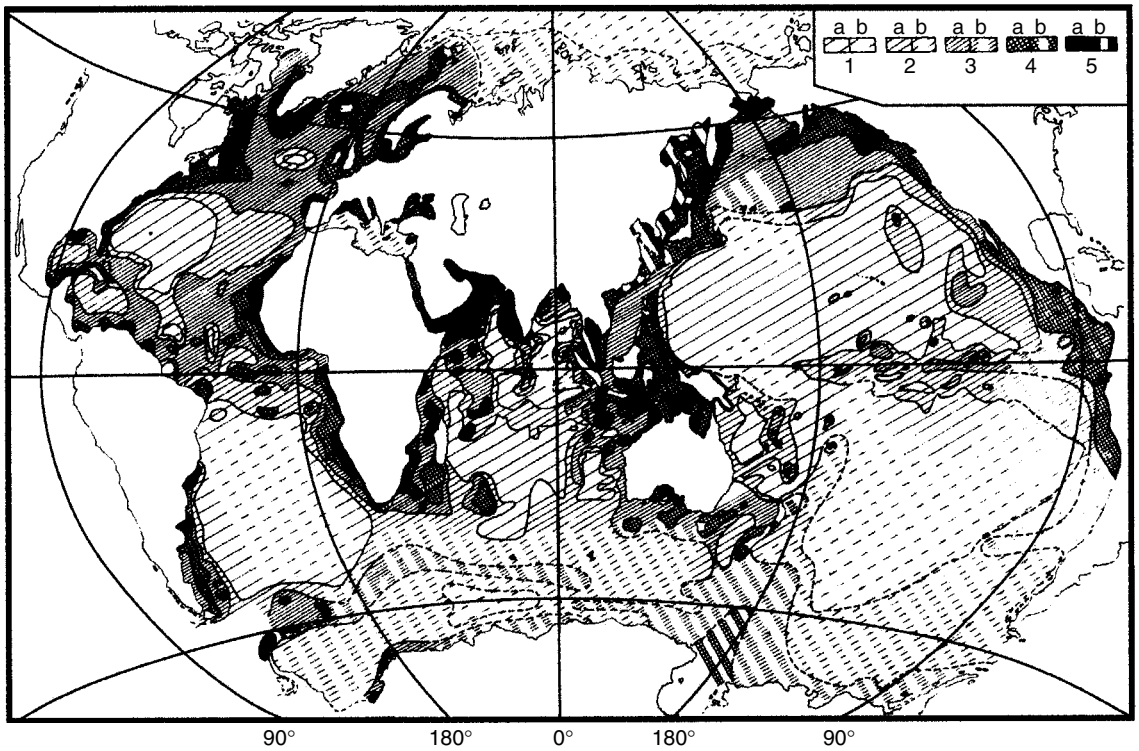

Figure 1. The distribution of primary productivity in the world's oceans as deduced from ^{14}C-primary productivity measurements (from O. J. Koblentz-Miscke, V. V. Volkovinsky, and J. G. Kabanova, in W. S. Wooster, ed., *Scientific Exploration of the South Pacific*, National Academy of Sciences, Washington, D.C., 1970, pp. 183–193.). Primary productivity is deduced from direct ^{14}C-primary productivity measurements (a) or indirect measurements of phytoplankton biomass, hydrogen, or oxygen saturation (b). Units are in mg of C per m^2 day^{-1}. (1) Less than 100; (2) 100–150; (3) 150–250; (4) 250–500, (5) more than 500. Courtesy of National Academy of Sciences, Washington, D.C.

very different, their estimate of the annual productivity of the ocean was largely equivalent.

Eppley and Peterson (19) compiled productivity data from a number of systems to understand the amount of productivity that can be exported from the euphotic zone. Their estimate of global productivity (and specifically, in offshore ocean areas with depths > 200 m) was between 19.1 and 23.7 pg C y^{-1} (based on independent compilations; 14,20), and they also suggested that neritic waters contributed another 3.9 pg C y^{-1}. The higher estimate specifically excluded productivity in polar systems in addition to that of coastal regimes. A later estimate using the Eppley and Peterson compilation suggested that by including Arctic and Antarctic waters global oceanic productivity would equal 27.2 pg C y^{-1} (Table 1; 21). DeVooys (21) estimated oceanic primary production to be about 43 Pg C y^{-1}, a figure which used corrections for release of dissolved organic matter during photosynthesis. Finally, the compilation of Pauly and Christensen (18) suggests that primary productivity of oceanic and coastal waters was 45.1 pg C y^{-1}, significantly above the earlier estimates. Their estimate was based on a theoretical division of the ocean into six ecosystem types. By using the known fisheries yield and by-catch and recalculating the transfer efficiencies, they estimated the primary production required to sustain the fisheries' yield. It is unclear why their value substantially exceeded other estimates.

MODERN APPROACHES TO THE ASSESSMENT OF PRIMARY PRODUCTIVITY

Indirect Measures of Phytoplankton Biomass and Productivity

Since 1980 tremendous advances have been made in the assessment of oceanic phytoplankton biomass using synoptic, whole-ocean assessments of pigment concentrations of the surface layer. These assessments have greatly altered our views of the ocean, in that we know more to fully appreciate the extent of the spatial and temporal changes in phytoplankton biomass, which occur in all areas of the ocean. Such an understanding was simply impossible using ship-based approaches to the determination of phytoplankton biomass. In addition to biomass measurement, a variety of models have taken the biomass and coupled that information with irradiance and predicted photosynthesis-irradiance relationships to produce either local (22–24) or global, synoptic estimates of primary productivity (25). Such estimates are invaluable because they incorporate the true variations in biomass and predict the temporal and/or spatial variations of productivity in the ocean. Although the prediction of productivity is highly dependent on the model used, they are by far the most powerful means to obtain synoptic, large-scale estimates of productivity. Furthermore, they then allow a means to test more refined hypotheses with either field programs or modeling.

Pigment concentrations (a proxy for phytoplankton biomass) can be ascertained by either moored deployments of fluorometers (26–28) or by sensors deployed on satellites. Moored fluorometers continuously measure the fluorescence of chlorophyll in the water at a known depth, and hence precisely assess the variations of phytoplankton in time at one location. Therefore, although they give no information on the spatial distribution of phytoplankton, they collect continuous measurements in time. When combined with ambient measurements of solar radiation and a model predicting photosynthesis as a function of irradiance and biomass, primary productivity can be estimated. This method was used in the Arabian Sea to give a continuous estimate of productivity (Fig. 2). It was found that whereas discrete measurements of productivity showed relatively little variation over the course of a year, predicted productivity ranged from approximately 0.35 to 1.3 g C m^{-2} d^{-1}, a fourfold difference (23). Thus, the ability to measure biomass and irradiance over daily intervals greatly improved the estimate of productivity in the region, as well as the understanding the processes influencing productivity. It has also been used in the western Sargasso Sea (22), and the annual estimate of productivity was 70% greater than historical estimates (29). This increase was probably due to the inclusion of short-term, hyperproductive events that were not sampled with ship-based measurements. As such, the ability of moored sensors greatly improved the precision of the productivity estimate. Furthermore, by providing an appreciation of the variability of these regions, it has facilitated a broad

Table 1. Oceanic Primary Production. Areas of Subregions Are Included in Total Area of Oceans, but Productivity Values Are Not [from W. O. Smith, *J. Mar. Chem.* 35, 245–258 (1991)]

Ocean	Offshore Area ($\times 10^{12}$ m^2)	Primary Production (g C m^{-2} y^{-1})	Total Primary Production ($\times 10^{15}$ g C y^{-1})
Indian	71.0	84	5.96
Atlantic	83.9	102	8.56
Pacific	167	55	9.14
Coastal	0.18	834	0.15
Equatorial	11.3	176	1.98
Antarctic	38.1	—	1.23
Ice edge	16.4	16	0.61
Open ocean	38.1	38	0.62
Arctic	13.1	1	0.13
Totals			27.2

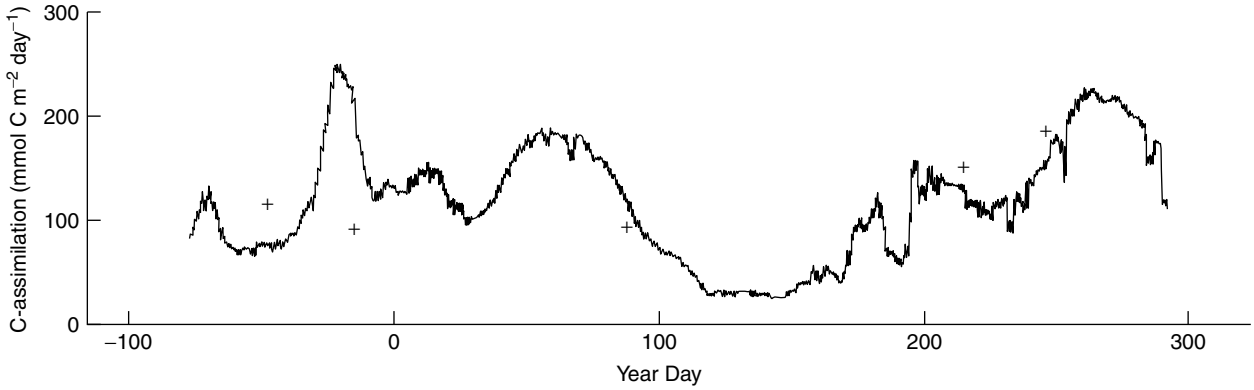

Figure 2. Estimates of primary production calculated from fluorometric estimates of chlorophyll, measurements of incident radiation, and a bio-optical model. Note the substantial variations in daily estimates, and the strong bias introduced by irregularly collected ^{14}C-estimates (+). From [J. Marra and coworkers, *Deep-Sea Res. II* **45**, 2,253–2,267 (1998)].

series of investigations into the productivity of oligotrophic regions and the mechanisms responsible for supporting it (30–32).

It is also possible to quantify pigments from space. The principle of operation is that solar radiation is reflected and spectrally modified according to the amount of pigment in the surface layer of the ocean. The resultant ocean color is quantifiable, and when calibrated with chlorophyll algorithms, relates directly to chlorophyll concentrations. Radiation penetrates to a depth of $1/k$ (where k is the attenuation coefficient in m^{-1} of the water), and this depth is known as an optical depth. Satellites can only see one optical depth, or approximately 22% of the euphotic zone. The attenuation of water is greatest when there is a large amount of particulate matter suspended in the water, and least when few particles and low concentrations of dissolved organic matter are present. Presently, there are two satellites with ocean color sensors operating, SeaWiFS (Sea-Viewing Wide Field of View Sensor) and MODIS (Moderate Resolution Imaging Spectroradiometer). They circle the Earth approximately once per day, and thereby provide ocean color nearly continuously. However, clouds interfere with the determination, and hence not all areas of the ocean are sampled as frequently as others. The resolution of the satellite sensor is approximately 1.1 km.

Generally, pigment data are binned over some time interval to produce a composite image, particularly if a global view of biomass is desired. Conversely, a single image of a cloud-free region can be mapped (Fig. 3). In this SeaWiFS image a fairly substantial phytoplankton bloom was observed in the upwelling region off northwest Africa, with chlorophyll concentrations exceeding 10 $\mu g\,L^{-1}$ in some locations, but with a steep gradient offshore to low values off the continental shelf. Similarly, the pigment concentration in the Gulf of Maine is shown in Figure 4. Not only is a large phytoplankton bloom off Cape Cod observed, but a mesoscale feature called a warm-core ring (33) is also shown off the coast. This feature is an oligotrophic patch of water that is physically trapped in an eddy-like feature, which transports warm water that originated on the eastern side of the Gulf Stream into the cooler coastal waters of New England, and has very low pigment levels. The variability of pigments within a relatively restricted area suggests that there are a number of factors simultaneously operating, which are responsible for controlling regional biomass.

These types of images can also be averaged over longer time periods (days to weeks) to elucidate seasonal trends of phytoplankton biomass. For example, pigment concentrations in the north Atlantic Ocean are known to increase rapidly in May of each year (26,34). Composite satellite images confirm this temporal trend, but also show that the increase is variable in space, producing different patterns at different locations, and also showing

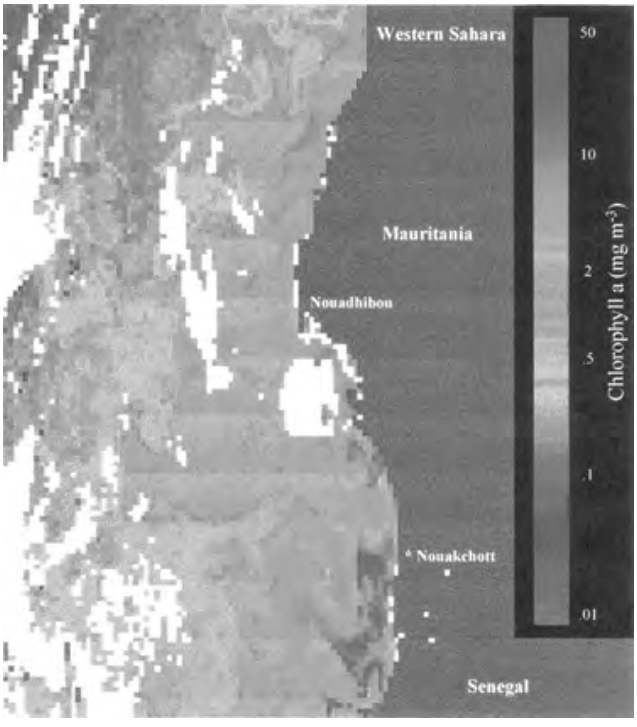

Figure 3. A SeaWiFS image of pigment concentrations off the coast of northwest Africa (March 22, 2001). Note the strong variations both offshore and along shore. See color insert.

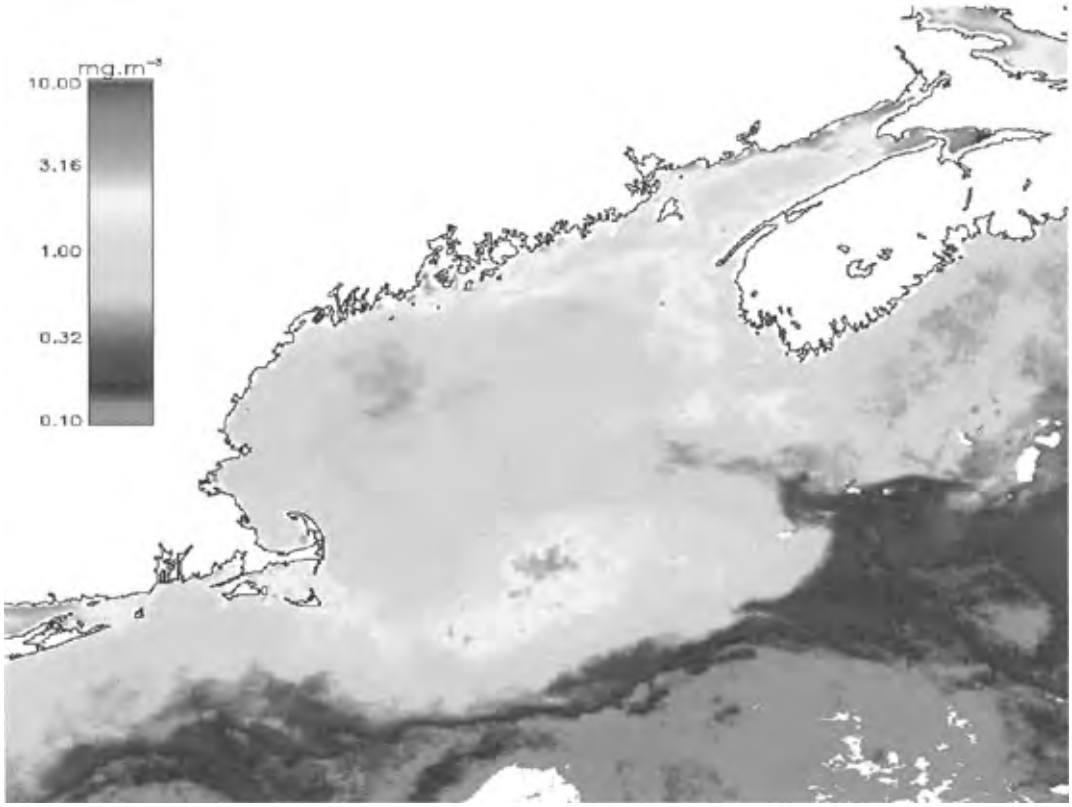

Figure 4. A SeaWiFS composite of pigment concentrations off the northeast coast of the United States (June 18–26, 1999). Note the presence of a large warm-core ring that substantially influences pigment concentrations and productivity. See color insert.

differences between years (Figs. 5a,b). Temporal trends as revealed from satellite images also show the expected south-north progression of the bloom in temperate waters (35,36).

If the biomass at the surface were measured each day and no vertical losses were to occur (via either ingestion or sinking), then the productivity could be estimated by the change in biomass at any location. However, such steady state conditions rarely if ever exist, and so productivity estimates are made in a different manner. In essence, the daily, depth-integrated productivity is modeled (25) using the following as input: (*1*) the pigment concentration of the surface layer (through one optical depth; C_{sat}), (*2*) daily incident solar radiation (photosynthetically active radiation; PAR in mol quanta $\mathrm{m}^{-2}\,\mathrm{d}^{-1}$), E_{o} (*3*) the length of the photoperiod in hours (D_{Irr}), (*4*) the depth of the euphotic zone [Z_{eu}, derived from the pigment concentration in the surface; (37)], and (*5*) the chlorophyll-specific optimum photosynthetic rate [$P^{\mathrm{B}}_{\mathrm{opt}}$ in units of mg C $(\mathrm{mg\ chl})^{-1}\,\mathrm{h}^{-1}$]. The equation relating these variables is as follows:

$$PP_{eu} = 0.66125 \times P^{\mathrm{B}}_{\mathrm{opt}} \frac{E_{\mathrm{o}}}{E_{\mathrm{o}} + 4.1} \times C_{\mathrm{sat}} \times Z_{\mathrm{eu}} \times D_{\mathrm{Irr}} \qquad (2)$$

The two variables in Equation 2 that are difficult to accurately estimate on a short-term basis are Z_{eu} and $P^{\mathrm{B}}_{\mathrm{opt}}$. The depth of the euphotic zone is difficult to predict because in many stratified regions of the ocean the maximum concentration of phytoplankton is found not at the surface but at depth (below the optical depth, which only includes the upper 22% of the euphotic zone). This deep-chlorophyll maximum has been repeatedly observed and occurs in nearly all areas of the ocean (38). Only in areas where vertical mixing is both vigorous and deep (so as to distribute phytoplankton evenly over the timescales of hours to days) is the distribution of pigments vertically uniform. To predict the total euphotic zone chlorophyll concentration from the amount in the upper portion of the layer requires a statistical analysis of a large number of chlorophyll profiles in a variety of regions.

A different approach to the same problem is to divide the ocean into a number of oceanic provinces, and to assume that the phytoplankton distribution within a province is similar throughout (39–41). This approach greatly simplifies the assumptions needed to assess chlorophyll vertical distributions, but also introduces a new set of assumptions (e.g., the spatial domain of oceanic provinces, their continuity in time, etc.). In general, more emphasis today is being placed on treating the ocean as a whole and reducing the variability in the estimates of vertical phytoplankton distribution via satellites.

Uncertainty is also introduced by the variable $P^{\mathrm{B}}_{\mathrm{opt}}$. Behrenfeld and Falkowski, 1997 (25) generated a seventh-order polynomial that related temperature with $P^{\mathrm{B}}_{\mathrm{opt}}$, and used temperature derived from satellite (AVHRR) images of the ocean to predict the optimum rate of photosynthesis.

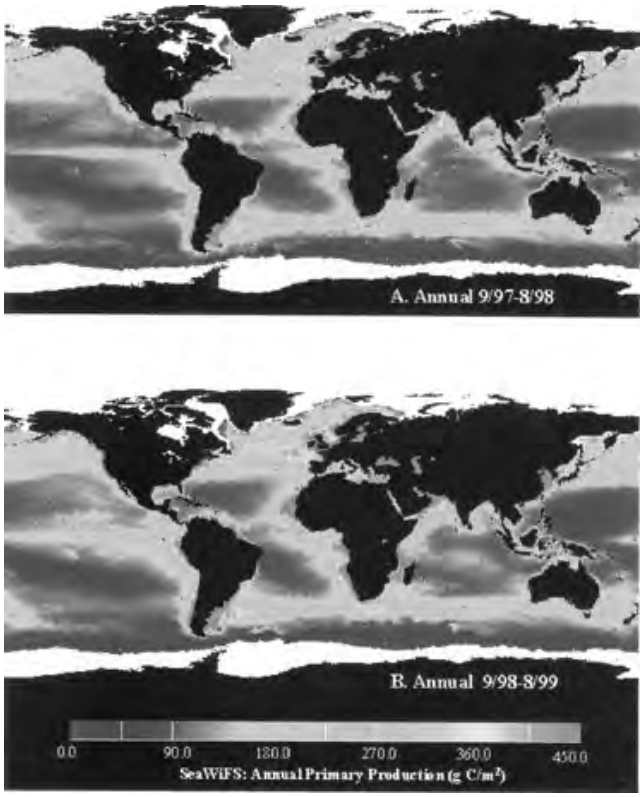

Figure 5. SeaWiFS annual composites of pigment concentrations throughout the ocean. (**a**) 1997–1998 and (**b**) 1998–1999. Strong spatial variations between years are observed, particularly in the equatorial pacific. See color insert.

It was also constrained at low and high temperatures, so that when T is less than $-1.0\,^{\circ}\mathrm{C}$, then $P^{\mathrm{B}}_{\mathrm{opt}} = 1.13\,\mathrm{mg\,C\,(mg\,chl)^{-1}\,h^{-1}}$ (42), and when T is greater than 28.5 $P^{\mathrm{B}}_{\mathrm{opt}} = 4.0\,\mathrm{(mg\,chl)^{-1}\,h^{-1}}$ (43).

Despite these uncertainties, global productivity can now be estimated using daily pigment data and photosynthetic models. Falkowski and colleagues estimate global productivity (2) to be approximately $47.5\,\mathrm{pg\,C\,m^{-2}\,y^{-1}}$ (Fig. 5a). This is substantially more than the analyses derived from discrete samples, and suggests that the productivity of the ocean is greater than thought a decade or more ago. They also investigated the sensitivity of the model to various parameters. For example, if $P^{\mathrm{B}}_{\mathrm{opt}}$ is held constant at a value of $4.54\,\mathrm{mg\,C\,(mg\,chl)^{-1}\,h^{-1}}$, the global productivity decreases by 2.3%. If $P^{\mathrm{B}}_{\mathrm{opt}}$ is generated from the Eppley, 1972 (44) temperature-growth relationship as modified for photosynthesis (43), global productivity decreases by 14.5%. Finally, if clear sky conditions are assumed and photosynthesis is modeled without photoinhibition (a decrease in photosynthesis at high light intensities), productivity increases by 6.1% (*http://marine.rutgers.edu/opp/Production/results/all2. html*). Such changes suggest that the estimates of productivity are relatively robust and are not overly dependent on the details of the model's formulation.

The seasonal changes in productivity can also be quantified using this approach. Global productivity in autumn is dominated by the widespread increase over much of the South Atlantic and South Indian Oceans (associated with the sub-Antarctic and subtropical waters), as well as the intense productivity of the Argentine coast, the coastal upwelling region of southern Africa, the Yellow Sea and the North Sea. The growth in the northern Atlantic and Pacific is also substantial (Fig. 6a). In winter (austral summer) the bloom in the south Atlantic and Indian Oceans intensifies, whereas that in the north Pacific and Atlantic all but disappears (Fig. 6b). Coastal productivity remains similar in the two seasons. In spring the opposite occurs — a large vernal bloom in the northern waters of the Atlantic and Pacific Oceans develops and that in the southern waters dissipates (Fig. 6c). Smaller features also change; for example, the extent of the high productivity region off the northwest African coast increases, whereas productivity in the Arabian Sea declines. In summer the northern bloom is the dominant productivity feature of the world's ocean, with productivity exceeding $50\,\mathrm{g\,C\,m^{-2}\,month^{-1}}$ over large areas (Fig. 6d). The productivity of the Arabian Sea also increased substantially, as did that of the Sea of Okhotsk, the Bering Sea, and the Barents Sea. The spring, summer, autumn, and winter global productivity estimates were 10.9, 13.0, 12.3 and $11.3\,\mathrm{pg\,C\,m^{-2}}$, respectively.

Regional estimates of productivity can also be made using the model (Table 2). The Pacific Ocean, by virtue of its size, has the greatest productivity, followed by the Atlantic, Indian, and Southern Oceans. The Mediterranean Sea contributes about 1% of the annual global production, and the Arctic Ocean (by virtue of the fact that it is largely ice-covered for significant portions of the year) less than 1% of the annual production.

A model similar to that of Behrenfeld and Falkowski (25) has been developed, but this model includes the effects of vertical mixing on the irradiance available to phytoplankton (45). In general, the two models reproduce the same general features, but the productivity derived from the Howard-Yoder model (45) is less than that from the Behrenfeld-Falkowski model due to greater irradiance limitation imposed by vertical mixing. It is presently uncertain which model reproduces the absolute productivity of a region more accurately.

Another method to estimate phytoplankton photosynthesis involves the evaluation of variable fluorescence to infer photochemical characteristics. For phytoplankton, a photon entering the cell can have one of three fates: utilized to drive photosynthesis, dissipated as heat, or reemitted as flyorescence. Thus, a change in active fluorescence can be indicative of a change in the efficiency of photochemistry. In recent years, a number of instruments have been designed primarily for this type of analysis such as Pump-and-Probe (PDP) (46,47,48) fast repetition rate fluorometry (FRRF) (49,50,51), and pulse amplitude modulated fluorescence (PAM) (52,53). These instruments (although they all accomplish this in a slightly different manner) evaluate the quantum yield of photochemistry to estimate phytoplankton photosynthesis. Although these methods are simple in practice, interpreting fluorescence signals in order to relate them to photosynthesis and productivity is complicated, and is still being developed and

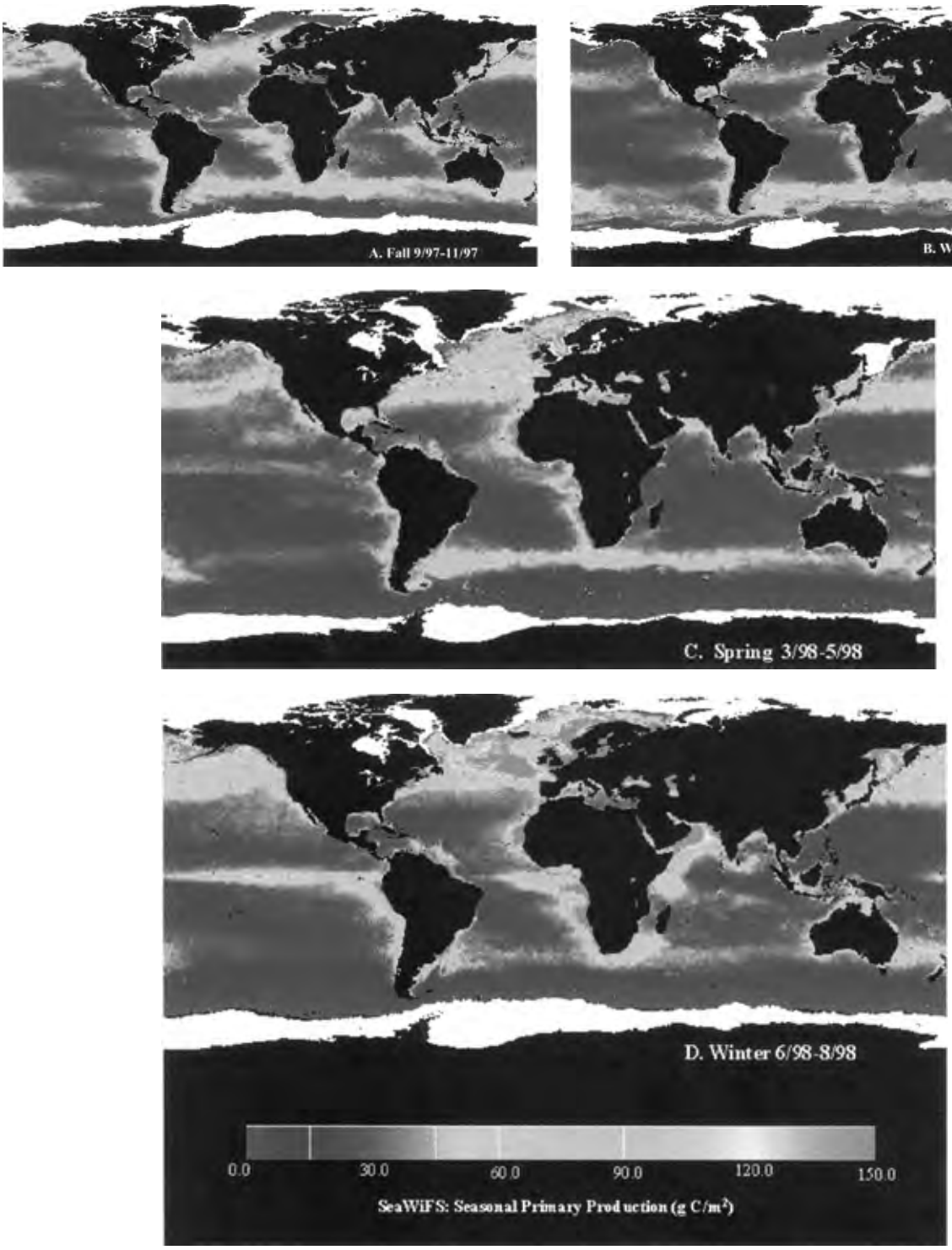

Figure 6. Estimates of primary productivity in (**a**) autumn, (**b**) winter, (**c**) spring, and (**d**) summer derived from satellite pigment estimates, incident irradiance, and the model of [M. Behrenfeld and P. G. Falkowski, *Limnol. Oceanogr.* **42**, 1–20 (1997)]. See color insert.

refined. One model by Kolber and Falkowski (49) estimates gross photosynthetic oxygen evolution normalized to chl a from the quantum yield of photochemistry, the functional absorption cross-section of PSII, the ratio of PSII reaction centers to chl a, the fraction of PSII reaction centers that are viable for oxygen evolution, the quantum yield of photochemistry at a particular light level, and that relative light level. All of these parameters, except for irradiance (which is measured directly) are estimated from fluorescence characteristics (for review, see 54). PAM based methods

Table 2. Regional Estimates of Primary Productivity from the Rutgers OPPT Model (see *http:/marine.rutgers.edu/opp/Production/results/all2.html*). Numbers in Parentheses Represent the Percentage of Global Productivity of Each Region

Region	Boundary	Productivity (pg C y^{-1})
Pacific Ocean	Pacific Ocean Basin, south of 70°N and north of 50 °S	19.7 (42.8)
Atlantic Ocean	Atlantic Ocean Basin, south of 70 °N and north of 50 °S	14.5 (31.5)
Indian Ocean	Indian Ocean Basin, north of 50 °S	8.0 (17.3)
Southern Ocean (Antarctic)	South of 50 °S	2.9 (6.3)
Mediterranean Sea	Mediterranean Sea	0.6 (1.2)
Arctic	North of 70 °N	0.4 (0.9)

can't directly measure the functional absorption cross-section of PSII directly, and therefore this parameter must be estimated spectrophotometrically (55). Because *in situ* fluorescence-based techniques are instantaneous and non-invasive, problems associated with "bottle effects" (when using ^{14}C or O_2 based methods) are eliminated. Another advantage of these types of instruments, especially FRRF, is that they can be towed underway to acquire real-time data for a broad area. Additionally, these types of instruments can be affixed to moorings for temporally refined measurements of phytoplankton photochemical efficiency.

Benthic-Associated Productivity

Benthic primary productivity, although generally limited to shallow shelf waters, can be a significant fraction of marine primary productivity at those locales. Thus, a substantial effort has been made to quantify total global benthic primary production. Bunt (56) hypothesized that benthic productivity equaled 0.65 to 6.5 pg C, which was equal to 2.8 to 28% of total global primary production. A revised estimate from Charpy-Roubaud and Sournia (57) suggested that productivity was closer to 2.89 pg C, or approximately 10% of estimates of total global primary productivity. Contributions to marine benthic primary productivity can be attributed to three broad groups: macroalgae (sea-weeds), including phaeophytes (brown algae), rhodophytes (red algae), and chlorophytes (green algae), microphytobenthic algae (such as diatoms living attached to the sediments), and algae in mutualistic associations, such as zooxanthellae. Excluded from this discussion are epiphytes, ice algae, seagrasses, and salt marshes.

The distribution of macroalgae is, in general, limited by light penetration, secure substrates for attachment, and sufficient physical energy to break boundary layer nutrient gradients (58). Daily carbon fixation rates are thought to be from 5 to 10 g C m^{-2} d^{-1} (59). Annual primary productivity of macroalgal communities was estimated at 1 pg C y^{-1} by Smith (60), while standing stocks were suggested to be approximately 400 times those of phytoplankton. Charpy-Roubard and Sournia (57) projected total global macroalgal production to be 2.55 pg C y^{-1}. These high production rates and limited distributions make sea-weed communities an integral and dynamic part of coastal ecosystems, allowing them to act as nursery grounds for fishes and invertebrates. Given their proximity to land, productivity of sea-weeds is particularly sensitive to anthropogenic eutrophication. Specifically, their productivity can be increased by direct input of nutrients or decreased by the stimulation of phytoplankton biomass through nutrient inputs, which in turn increases attenuation of PAR in the water column and decreasing the PAR available to macroalgal benthic producers.

Mutualistic algae or zooxanthellae — endosymbionts that fix carbon and transfer that reduced carbon to its host, and in return receives nutrients, maintained at a constant irradiance, and protection from predation — are dominated by the Dinophyceae. In general, these dinoflagellates have a biphasic life cycle; a free-swimming motile and a predominant coccoid symbiotic phase. Animal hosts include corals, sponges, radiolarians, foraminiferans, and mollusks. However, for models of primary productivity, high coral densities (10^6 cells cm^{-1}) of zooxanthellae provide considerable carbon fixation, with values close to 0.06 pg C y^{-1} (61). Muscatine and Weis (61) also suggest that if all zooxanthellae-invertebrate relationships were considered, then this number would effectively double.

Members of the microphytobenthos include any unicellular algal species living in or on benthic substrates, although diatoms tend to dominate this group. Microphytobenthic distribution is shallow and dependent on several factors, including size of sediment particles (62) and light and temperature interactions (63). Good global estimates of productivity for benthic diatoms are limited by several factors, including preferential sampling at midlatitude, shallow depths, and soft bottom conditions (57). Charpy-Roubaud and Sournia (57), after analyses of historical data, estimated global productivity to be 100 g C m^{-2} y^{-1}, although these rates varied from 5 to 300 g C m^{-2} y^{-1} from high to low latitudes.

NUTRIENT LIMITATION OF PRIMARY PRODUCTIVITY

Primary productivity can be reduced below maximum rates by a lack of a number of necessary metabolic needs, such as nutrients. Because carbon, nitrogen, and phosphorus assimilation occurs in ratios of 106 : 16 : 1 and

because nitrogen generally occurs (relative to phosphorus) in a slightly smaller ratio (6), it has long been considered that nitrogen limits productivity on short timescales (64). However, the presence of unused surface nutrients in a variety of environments (e.g., the Southern Ocean, the Equatorial Pacific, and Subarctic Pacific) have long perplexed oceanographers. These areas have been termed HNLC (high nutrient low chlorophyll) regions. Proposed explanations for lack of productivity in these areas have been trace metal limitation (65–67), intense grazing and phytoplankton removal (68,69), and advection (70). However, it was not until the development of ultra-clean analytical techniques that it became clear that iron could limit marine productivity. Martin (71) hypothesized that iron limited primary productivity in HNLCs, where the chief source of iron input was from atmospheric deposition. This was later confirmed through a series of in situ open ocean iron enrichments in the Equatorial Pacific (72,73). Since then, other expeditions, such as the Southern Ocean Iron Release Experiment (SOIREE) (74), have confirmed the overwhelming importance of iron in regulating marine productivity. Specifically, geographic variations in productivity (e.g., the contrast between the North Pacific and North Atlantic) are now largely attributed to variations in the influx of iron (both from below and from atmospheric sources).

Phytoplankton Size

The size of a phytoplankton cell can govern metabolic processes and primary productivity (75). This is largely due to the fact that as the size of a cell increases, its volume increases even more rapidly (i.e., smaller cells have a greater surface:volume ratio). If nutrients were supplied by diffusion (or any process linked to the relative surface area of the cell), then the rates of diffusion would be such that nutrients would be supplied more rapidly to smaller cells, thereby allowing them to grow faster than larger cells. Hence, if all other factors were equal, small cells should grow faster than large cells and dominate in the ocean. The fact that large cells do exist in nature, and at times are overwhelmingly important, signifies that larger cells have a variety of mechanisms, which allow them to exist and thrive in a variable environment. Conversely, small cells are apparently ingested rapidly by microzooplankton (such as ciliates and heterotrophic dinoflagellates) and thus can have larger loss rates than larger cells.

Further, the size classes and community composition can determine the quantity and quality of productivity. In the early years of discovery, phytoplankton were divided into two size classes: more than 22 μm (net plankton) and less than 22 μm (nanoplankton). Initial global estimates of primary productivity suggest that the contribution of nanoplankton is 88% (76). In contrast, temporally restricted high productivity events are dominated by net plankton (76,77). With technological advances such as flow cytometry, electron microscopy, and epifluorescence microscopy, a new size class has been introduced to account for very small (<2 μm) species, named *picoplankton*. Since the discovery of the relative high abundance of picoplankton, such as *Synechococcus* and *Prochlorococcus* (78), it is believed that nanoplankton modal size is much smaller (<5 μm). The difference in productivity for these two distinct groups is explained by complex biological and physical factors such as residence time due to upwelling, advection, and grazing, rather than inherent physiological efficiencies (79,80). For example, the nanoplankton size fraction tends to dominate physically stable environments and preferentially utilize ammonium (76,81), whereas the more than 5 μm fraction is most productive in nonsteady state environments (i.e., upwelling events) and primarily utilizes nitrate (76,79,80,82–84).

CONCLUSION

Studies of primary productivity have progressed dramatically in the twentieth century with advancements in technology. Ship-based studies demonstrated that broad patterns in productivity exist, as well as the locations of maxima and minima. However, satellite-based approaches improved the resolution in both space and time and afford us the capability of assessing productivity of regions and ocean basins accurately and repeatedly. As a result, insights into critical societal questions, such as the role of human impact on the global climate cycle, can now be investigated in detail. Understanding both the rate and fate of marine primary production is essential to the effective management of oceanic resources, and undoubtedly will be a focal point of future oceanic research.

Acknowledgments

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PROBES. See LASER SCANNING MICROSCOPY IN COMBINATION WITH FLUORESCENCE TECHNIQUES FOR BIOFILM STUDY

PROBES: RRNA-TARGETED PROBES FOR ACTIVATED SLUDGE.

See ACTIVATED SLUDGE — MOLECULAR TECHNIQUES FOR DETERMINING COMMUNITY COMPOSITION

PROCESSING OF SUBSURFACE SAMPLES.

See SUBSURFACE SAMPLES: COLLECTION AND PROCESSING

PROCHLOROCOCCUS

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The unicellular cyanobacterium *Prochlorococcus marinus* is the smallest known photosynthetic organism. We now recognize that *Prochlorococcus*, which lives in surface waters of tropical and subtropical regions of the open ocean, is probably the most abundant photosynthetic organism in the ocean. Why had oceanographers overlooked such an important component of the marine plankton? *Prochlorococcus* was not "discovered" until the late 1980s when improved sensitivity in flow cytometry permitted enumeration of these extremely small ($\sim$0.7 μm) cells. *Prochlorococcus* spp. are oxygenic photosynthetic prokaryotes that contain a unique suite of photosynthetic pigments, including divinyl Chlorophylls *a* and *b* (Chl a_2 and b_2), α-carotene, zeaxanthin and, in some cases, true Chlorophyll *b* (Chl b_1) and phycoerythrin. Studies over the past decade have revealed that *Prochlorococcus* dominates the picophytoplankton in tropical and subtropical regions of the ocean. At abundances of 10^5 cells mL^{-1}, they are frequently 10 to 100 times more abundant than their close relatives *Synechococcus*, the oceanic phycoerythrin-containing strains of cyanobacteria. Moreover, *Prochlorococcus* can contribute more than 50% of the total Chlorophyll *a* biomass. Growth rates of approximately one doubling per day are balanced by mortality due to grazing primarily by microzooplankton. *Prochlorococcus* populations make the largest contribution to primary production in the most oligotrophic regions of the ocean; in the Sargasso Sea they can account for an estimated 25% of total primary production, whereas in the North Pacific this percentage can reach 80%. They are found throughout the entire euphotic zone — from the surface to more than 100 m — which indicates *Prochlorococcus* is capable of growth over a broad range of light intensities. High ratios of Chl b_2 : a_2 permit *Prochlorococcus* to optimize growth at low light levels. The success of *Prochlorococcus* has been explained by the coexistence of multiple ecotypes, genetically similar strains (yet distinguishable by Chl *b* : *a* ratios and 16S rRNA and RNA polymerase gene sequences) that are physiologically diverse. *Prochlorococcus marinus* is the only known photosynthetic organism to contain both true chlorophyll *b* and phycoerythrin, so has recently been suggested to be the model for the ancestral photosynthetic bacterium of cyanobacteria and chloroplasts.

DISCOVERY

The unicellular organism that we now recognize as *Prochlorococcus* was first observed as an unknown "red-shifted" chlorophyll *a* derivative by Gieskes and Kraay in 1983 (1). Using high-performance liquid chromatography (HPLC) to characterize this unknown pigment, they detected 90 to 170 ng L^{-1} at stations in the oligotrophic eastern and western tropical Atlantic, but found none at stations in the North Sea (1). Moreover, it appeared that all of this novel pigment was associated with particles less than 1 μm, as was a large fraction of the carotenoid zeaxanthin, a photoprotective pigment. Small nonflagellated fluorescent cells were observed by epifluorescence microscopy that obviously were not *Synechococcus*. The unicellular *Synechococcus* cyanobacteria are easily identified by their characteristic bright orange phycoerythrin fluorescence, and at the time of this study *Synechococcus* were thought to be the dominant and ubiquitous photosynthetic picoplankton (2,3). Interestingly, three different cyanobacterial ultrastructural cell types had been described by Johnson and Sieburth in 1979 (3); however, the identity of the novel, small cells remained unknown.

In 1987, after flow cytometry was applied in oceanographic research and instruments were taken aboard research vessels for sea-going observations, Chisholm, Olson, and their coworkers reported finding numerous weakly fluorescing cells at the base of the euphotic zone (4). The discovery of this previously overlooked, abundant group of photosynthetic picoplankters "visible" only by flow cytometry was a major discovery in oceanography (4). The tiny cells were detected at depths corresponding to peaks in a divinyl-chlorophyll *a* pigment (Chl a_2) and chlorophyll *b* (Chl b_1); thus, it was proposed that these cells were free-living representatives of the prochlorophytes. The Prochlorophyta (5) had been proposed as a new algal division following the discovery of *Prochloron*, a photosynthetic prokaryote containing both true chlorophyll *a* (Chl a_1) and *b* (Chl b_1) that is found only as a symbiont in some tropical colonial ascidians (Didemnidae) (see section on "Phylogeny").

Other teams of oceanographers made similar observations of abundant red-fluorescing cells. Li and Wood (6) observed "very small red fluorescing (VSRF) bodies" in the central north Atlantic (31 to 34 °N) using both epifluorescence microscopy and flow cytometry. Although they could not positively identify cells by flow cytometry, they suspected the cells were not the small eukaryotes typically observed (7) because abundances (up to 10^4 cells mL^{-1}) of these weakly fluorescent cells were so much greater than the typical small eukaryotic algae. Similarly, Neveux and coworkers (8) observed abundant "green photosynthetic bacteria" (0.7×10^5 to 1.2×10^5 cells mL^{-1}) at the deep chlorophyll maximum layer ($\sim$100 m) in the Sargasso Sea (20 to 30 °N along 61 °W). The major pigments of these tiny cells were identified as divinyl chlorophyll *a* (Chl a_2) and a Chl *b*-like pigment. Because these free-living coccoid cells

differed considerably from other known prochlorophytes, specifically in their unusual pigment composition, these researchers suggested this novel cell type belonged to a new class of photosynthetic prokaryotes (8). Furthermore, given the high abundance of these prokaryotes, Neveux and coworkers speculated they were similar to cells identified by ultrastructural examination in 1979 by Johnson and Sieburth (Type II and Type III cells, (3)). In fact, Johnson and Sieburth stated that Types II and III appeared to be difficult to culture and probably did not contain phycoerythrin (3).

By 1990, two isolates of this novel organism had been brought into culture, and both were assigned to the genus *Prochlorococcus*. The first culture of *Prochlorococcus* was isolated by B. Palenik in May 1988 from a depth of 120 m in the Sargasso Sea and so is known as *SARG* (9). A clonal isolate of *Prochlorococcus* was obtained by serial dilution of the SARG culture and was added to the Culture Collection of Marine Plankton (CCMP) as CCMP1375 (also known as *SS120*). CCMP1375 was designated as the type strain for *Prochlorococcus marinus* in the first formal description of *P. marinus* published in 1992 (9). Note, however, this strain is not clonal in a strict microbiological sense, that is, growth from a single colony grown on agar plate, as growth on agar plates has not been successful, despite numerous attempts by several laboratories (9,10).

The second culture of *Prochlorococcus* was established in January 1989 from Mediterranean surface water near the Rhone estuary and was designated *MED* (11,12). A clonal strain, CCMP1378 (also known as *MED4*), was also obtained by serial dilution and documented in 1992 (9). These two strains represent distinct physiological genotypes, or ecotypes, whose differing physiological properties appear to be consistent with conditions from which each was isolated. Over the past decade, a considerable number of additional high- and low-light isolates have been obtained from the Atlantic, Pacific, and Indian oceans (12–14).

In 2000, the first axenic *Prochlorococcus* strain became available (10). This represents a major achievement. Success in obtaining a pure culture free of any contaminating bacteria was a long arduous process combining serial dilution to obtain a single-cell "clone" together with centrifugation to remove a larger, heterotrophic bacterial contaminant (10). The new strain was named *Prochlorococcus marinus* subsp. *pastoris* subsp. nov. (10). Interestingly, this *Prochlorococcus* strain, although derived from the original SARG culture, has turned out to be more similar to the CCMP1378 (MED4) clone. Thus, it may have been a minor component of the parent culture that was positively selected for in the cloning process (10). The considerable number of differences in the physiological and morphological properties between this new strain and the Type strain CCMP1375 justify its classification as a subspecies. After additional review, this subspecies may be reclassified as a second species of *Prochlorococcus* (10). The axenic strain is available through the Pasteur Culture Collection of Cyanobacteria (PCC9511) or the American Type Culture Collection (ATCC 700925) (10).

Rules of microbial nomenclature require that the formal description of a new genus must be published in the *International Journal of Systematic and Evolutionary Microbiology*. A "type strain" must be designated when a name is first published. Recognized names are then published in the "List of Bacterial Names with Standing in Nomenclature" (*http://www.bacterio.cict.fr/*). Both the genus *Prochlorococcus* and the species *Prochlorococcus marinus* are not yet valid because they have not been published under the Rules of the Bacteriological Code (1990 Revision), but recognition will undoubtedly soon follow the publication and validation of PCC9511.

METHODS OF DETECTION AND CULTIVATION

Beginning with the first discoveries, HPLC and flow cytometry have remained the standard and most reliable methods employed for detection and enumeration of *Prochlorococcus*. Electron microscopy has also been crucial to the identification of these prokaryotic cells (see section on Ultrastructure), but it is not practical for routine quantitative oceanographic studies.

High Performance Liquid Chromatography (HPLC)

Reverse-phase high performance liquid chromatography (HPLC) is a rapid method to separate the chlorophylls and carotenoids present in marine phytoplankton. The ternary gradient method of Wright and coworkers (15) is the standard method, but has been modified and improved over the past decade (16–20). Chromatography methods rely on differences in partitioning behavior between a flowing mobile phase and a stationary phase to separate components in a mixture. In reverse-phase HPLC, the more polar compounds pass through the column faster, whereas the less polar compounds (e.g., α-carotene, Chl *a* and Chl *b*) spend a greater amount of time in the column and so have longer retention times. As pigment components elute from the column, they can be quantified with an absorbance detector at 436 nm. Monovinyl and divinyl chlorophylls cannot be completely separated by the reverse-phase HPLC, so to quantify these two forms either the normal-phase method of Gieskes and Kraay (1) can be used (but this is not routinely used for oceanographic measurements) or an on-line diode array spectrophotometric method so that different wavelengths can be monitored (16,18).

In the field, samples for HPLC analysis are collected onto glass fiber filters (Whatman GF/F) which retain 98 to 99% of all *Prochlorococcus* cells (21,22). Filters must be stored frozen at $-80\,^{\circ}C$ until analysis to prevent pigment degradation. Using pigment characterization by HPLC, the composition of the phytoplankton community can be determined (23,24). CHEMTAX (CHEMical TAXonomy) is a popular program written for MATLAB™ that allows calculation of phytoplankton community assemblage composition using pigment concentrations and ratios of marker pigments (25). The investigator provides initial diagnostic pigment ratios, and by iteration the program will arrive at actual ratios which best fit the data. This way the composition of the phytoplankton community biomass (expressed as a percentage of total chlorophyll) can be calculated. Since the recognition of *Prochlorococcus*

as an important member of the microbial community, algorithms used to partition the chlorophyll biomass have been modified to accurately reflect both *Prochlorococcus* and *Synechococcus* (23,24).

Flow Cytometry

Because *Prochlorococcus* cells are extremely small they cannot easily be distinguished from other bacteria by light microscopy. The autofluorescence of *Prochlorococcus* is also extremely faint because of their size, so accurate enumeration by epifluorescence microscopy is difficult, if not impossible, except in samples from the deepest portion of the water column where cell fluorescence is brighter. Consequently, flow cytometry is necessary for positive identification and accurate quantification of *Prochlorococcus*. Flow cytometry has become a well-recognized, if not essential, tool in the study of marine microbial ecology since the early 1980s (26–32). In flow cytometric analysis, cells pass through a focused laser in single file. A pressurized sheath, or carrier fluid, ensures laminar flow to maintain alignment of the cells. For each particle, both light scatter (related to cell size) and fluorescence parameters can also be collected. In Figure 1, the optical layout for a typical benchtop instrument, such as the FACSCalibur flow cytometer (Becton Dickinson, San Jose, California) is shown to illustrate the partitioning of fluorescence signals. The "red fluorescence" signal (fluorescence >640 nm) is related to cellular chlorophyll content (see section on Vertical Profiles in the following text) and can be useful to compare populations during time-course or nutrient addition experiments, for example, reference (33), over multi-year time series, for example reference (34), and to distinguish subpopulations, or ecotypes, of *Prochlorococcus*, for example references (35,36). Data are plotted as two parameter histograms to identify populations. The small scatter and red fluorescence signals (Fig. 2) distinguish *Prochlorococcus* as a unique population. Green-fluorescing DNA-specific dyes can also be used to stain a sample to quantify cellular DNA content for cell cycle analyses (see section on Growth). Thus, in addition to enumerating cell abundances, it is possible to quantify cell "size" and chlorophyll or DNA content as well. Although there is not always a direct linear relationship between cellular fluorescence and pigment content, for instance, reference (37), the red fluorescence of *Prochlorococcus* can be used to indicate relative changes in pigment over time, for example reference (38). Using this parameter together with FALS, see reference (39), one can infer a physiological response to environmental change. For example, in the Equatorial Pacific increased cellular fluorescence was observed as a tropical instability wave passed through the study site stimulating phytoplankton growth (38).

The extremely dim fluorescence of many surface populations of *Prochlorococcus* can make it difficult to resolve these populations from the background noise. Olson and coworkers (40) improved the sensitivity of an older EPICS V instrument (Coulter) by replacing the beam shaping optics with a confocal lens to obtain a smaller beam spot, decreasing the sheath pressure to obtain a lower flow rate, running the laser in "all lines" mode to obtain up to 4 W of power, and using a new flow cell designed with a lens and mirrors to increase the signal detection. With this type of configuration, surface populations of *Prochlorococcus* could be resolved in the oligotrophic subtropical North Pacific (35). In many newer instruments, optimizations in the optics have been significant, but in moving to an air-cooled laser in bench top instruments, such as the FACScan or FACSCalibur (Becton Dickinson), the laser power is much reduced. To improve the sensitivity of the FACScan, Dusenberry and coworkers (41) also changed the optics to decrease the laser spot size at the interrogation point, but this necessitated decreasing the flow rate as well. The consequence of these changes is that the overall time of sample analysis increases. Sensitivity of commercial instruments remains a limitation for detecting dim surface populations (12). Samples for flow cytometric analyses can be examined live, or fixed and frozen for analysis on return to the laboratory (42,43). Frozen seawater samples must be stored at $-80\,^{\circ}C$ to preserve the chlorophyll autofluorescence.

Cultivation

Cultures of *Prochlorococcus* have been grown in both enriched seawater and artificial seawater-based media (9,10). The original strains were grown in a modified "K" medium (44) using both urea and ammonia as

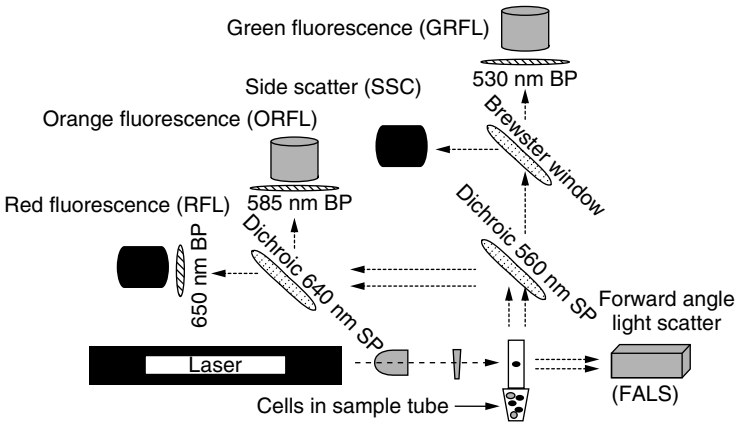

Figure 1. The optical configuration of the FACSCalibur (Becton Dickinson) flow cytometer used in analysis of photosynthetic picoplankton including *Prochlorococcus*. Laser provides blue light (488 nm) for excitation and as cells pass through focused laser, light is scattered. Detectors collect light scattered at small forward angles (FALS) and at a 90° angle, or "side scatter" (SSC). Fluorescence of cellular pigments is also collected. Using short pass (SP) dichroic mirrors — optical filters that pass all wavelengths of light shorter than stated cutoff and reflect all longer wavelengths — three different fluorescence signals 'colors' can be collected: red (chlorophyll), orange (phycoerythrin), and green fluorescence. Band pass filters (BP) are also used to further restrict wavelengths directed to each detector.

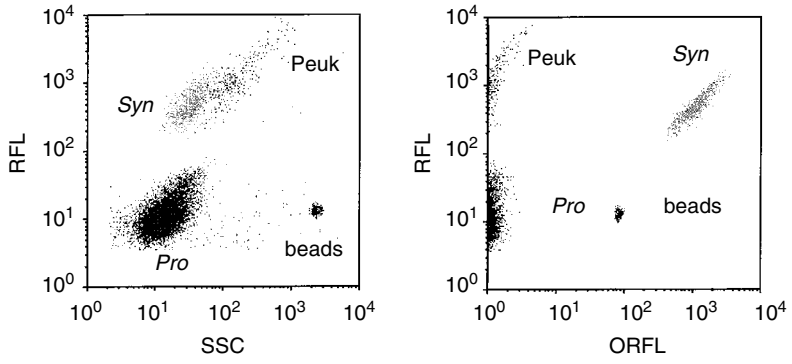

Figure 2. Cytograms for field samples of photosynthetic picoplankton collected in the eastern Gulf of Mexico at 27.50 °N 85.40 °W (August 1999). Data are plotted as 2-parameter histograms, "SSC versus red" and "orange versus red," to identify each population. *Prochlorococcus* (*Pro*) is distinguished from the larger picoeukaryotes (Peuk) by the smaller scatter (SSC) and chlorophyll (RFL) signals. *Prochlorococcus* is distinguished from the phycoerythrin-containing *Synechococcus* (*Syn*) by the absence of orange fluorescence (ORFL). Standard beads are added to all samples.

nitrogen sources, an organic phosphorus source, and eliminating copper from the trace metal mix (9). The "PC" medium (Bigelow Laboratory; *http://ccmp.bigelow.org/*) is similar (Table 1). The "Pro2" medium (13), which has been used successfully to isolate and maintain a variety of isolates, differs in that it utilizes inorganic phosphorus and lacks vitamins. The "PCR-S11" medium was designed during the task of achieving an axenic isolate. Modifications to provide greater maximum cell densities were investigated, including growth on ammonia as the sole nitrogen source and additional trace metals. These changes have been employed successfully in the new "PCR-S11" medium for the PCC9511 culture (Table 1) (10).

Table 1. Culture Media Used for Maintenance of *Prochlorococcus*

	PC[a]	Pro2 (13)	PCR-S11 (10)
Seawater (autoclaved) (mL)	1,000[b]	1,000[c]	1,000
Buffer:			
HEPES-NaOH (pH 7.5) (mM)			1
Nutrients:			
Urea (μM)	50	100	
NH_4Cl (μM)	50	50	
NH_4SO_4 (μM)			400
β-glycerophosphate (μM)	10		
NaH_2PO_4 (μM)		10	10
Trace metals:			
EDTA-Na_2 (μM)	11.7	1.2	8
$FeCl_36H_2O$ (μM)	1.2	1.2	8
$MnCl_24H_2O$ (nM)	90	90	
$MnSO_4H_2O$ (nM)			30
$ZnCl_2H_2O$ (nM)	8	8	
$ZnSO_47H_2O$ (nM)			3
$CoCl_2$ (nM)	5	5	
$Co(NO_3)_2$ (nM)			1.5
$Na_2MoO_42H_2O$ (nM)	3	3	
$(NH_4)_6Mo_7O_244H_2O$ (nM)			1.5
Na_2SeO_3 (nM)	10	10	
SeO_2 (nM)			1.5
$NiCl_26H_2O$ (nM)	10	10	1.5
$Na_2WO_42H_2O$ (nM)			0.3
KBr (nM)			3
KI (nM)			1.5
$Cd(NO_3)_26H_2O$ (nM)			1.5
$CuSO_45H_2O$ (nM)			1.5
$Cr(NO_3)_39H_2O$ (nM)			0.3
VSO_55H_2O (nM)			0.3
$KAl(SO_4)_212H_2O$ (nM)			3
H_3BO_3 (nM)			150
Vitamins:			
Thiamine-HCl (μM)	10		
Biotin (nM)	50		
B_{12} (nM)	50		7

[a] CCMP, Bigelow Laboratory.
[b] Microwave sterilize in Teflon bottles.
[c] Autoclave in Teflon bottle.

DESCRIPTION

Size

Prochlorococcus cells are extremely small. In the first formal description of *Prochlorococcus* by Chisholm and coworkers, the size of *Prochlorococcus* was estimated to be 0.6 to 0.8 μm in diameter and 1.2 to 1.6 μm in length (9). Initial field observations showed that most cells passed through a 0.8-μm Nucleopore filter but were retained on a 0.6-μm filter (4). Size measurements of cultures using the Coulter Multisizer ranged from 0.4 to 0.6 μm in width and 0.5 to 0.8 μm in length (39). For the axenic culture PCC9511, cell size was reported to range from 0.5 to 0.6 μm in width and 0.7 to 0.8 μm in length for newly divided cells, and up to 1.2 μm in length for cells ready to divide (10).

Ultrastructure

The prokaryotic nature of *Prochlorococcus* was established from transmission electron microscopy (TEM) images published in the initial report of their discovery (4). The cellular organization of these unicellular organisms shows a gram-negative-like cell wall and lack of a nucleus. Most of the DNA is concentrated in a central nucleoid area, a trait it shares with *Prochlorothrix*, but not Prochloron (45). The thylakoids, which house the light-harvesting and photosynthetic electron transport reactions, are arranged in closely spaced layers around the outer portion of the cell, parallel to the cell wall (Fig. 3). The fine structure of this internal membrane system differs between the two types of strains, or ecotypes. In the Type strain CCMP1375 and other low-light strains, the thylakoids are visible as concentric circles around the periphery of the cell (Fig. 3). In contrast, in high-light strains, such as CCMP1378 or PCC9511, the thylakoids are

arranged as a "horse-shoe" pattern, i.e., open at one end (10,46).

In cross-sectional views of *Prochlorococcus* CCMP 1375 cultures, the fine structure bears a strong resemblance to the "Type II" cyanobacterium described from TEM images of cells from the chlorophyll maximum depth (~100 m) reported by Johnson and Sieburth in their original description of cyanobacteria in the sea (3). It is now recognized that the low-light strains of *Prochlorococcus* are probably the "Type II" cyanobacteria, as reported by Chisholm and coworkers (9), whereas the high-light strains are probably the "Type III" cyanobacteria reported by Johnson and Sieburth (3,10).

One of the few additional distinguishing features is the presence of carboxysomes, the polyhedral bodies containing the enzyme ribulose bisphosphate carboxylase-oxygenase (RuBisCO), which is the primary CO_2-fixing enzyme of photosynthesis (Fig. 3). Immunolabeling *Prochlorococcus* cultures with probes targeting RuBisCO positively identified the presence of this enzyme (46).

Pigments of the Photosynthetic Apparatus

All *Prochlorococcus* are oxygenic phototrophs, which means they use the energy from sunlight to generate chemical energy and reducing power from water and obtain their cellular carbon requirement by fixing carbon dioxide. As in other phototrophs, light capture is accomplished by light harvesting centers, or antennae photosynthetic pigments. Light quanta absorbed by the light-harvesting complex are transferred to the reaction center, which is where the photochemical reactions occur. Reaction centers associated with antenna proteins are a common feature of photosynthetic systems to allow the organism to absorb a wider range of light energy.

The pigment composition of *Prochlorococcus* is unique, however, and unlike any other prokaryotic or eukaryotic photosynthetic organism (Table 2). Most notable is that all *Prochlorococcus* strains contain divinyl-chlorophyll *a* (Chl a_2), instead of the monovinyl or "true" chlorophyll *a* (Chl a_1) as their main photosynthetic pigment. In Chl a_2, an 8-vinyl group replaces the 8-ethyl group present in Chl a_1 (47). The structural change produces a red-shifted absorption spectrum, which means the maximum peak in absorption occurs at wavelengths that are 8 to 10 nm longer (towards the red end of the spectrum) than Chl a_1. As discussed in the preceding text, this "unknown chlorophyll *a*-like" pigment (1) has since been shown to be identical spectroscopically to Chl a_2 (21).

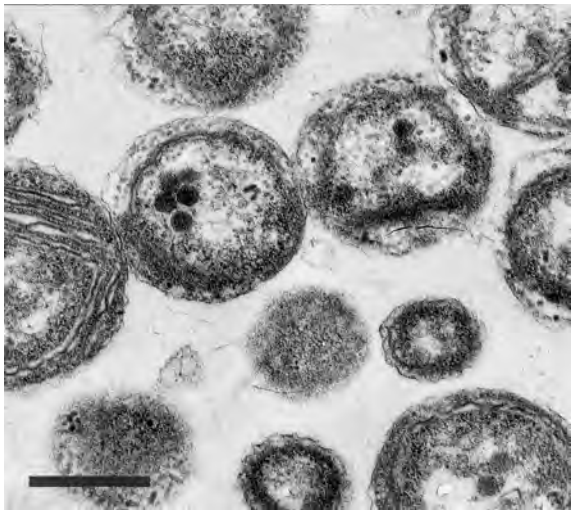

Figure 3. Transmission electron micrograph of *Prochlorococcus* culture, PAC1 (55). Scale bar = 0.5 μm. Sample TEM and photograph generously provided by Marcia Gowing (University of California, Santa Cruz).

The major accessory antenna pigment in *Prochlorococcus* is divinyl chlorophyll *b* (Chl b_2) for which the absorption peak is also red-shifted 8 to 10 nm (21). In addition to Chl b_2, several low-light ecotypes also synthesize monovinyl chlorophyll *b* (Chl b_1), but this only occurs under high-light conditions (Table 2; 11). Although most Chl b_2 in *Prochlorococcus* strains is associated with the light harvesting complexes of PSII, a significant amount is also associated with PSI. This is an unusual feature and is significantly different than the cyanobacteria, which do not have any antenna specific to PSI, and other phototrophs, which do not have Chl *b* associated with PSI (12).

All strains also possess small amounts of a chlorophyll *c*-like pigment. The identity of the Chl *c*-like pigment of *Prochlorococcus* was recently confirmed as Mg-3,8-divinylpheoporphyrin a(5) monomethylester (Mg 3,8 DVP a5) based on the characterization of a field sample from a deep layer of an almost pure "culture" of *Prochlorococcus* found in the suboxic portion of the water column in the Eastern Pacific (see section on Vertical Profiles) (51).

The carotenoids of *Prochlorococcus* include zeaxanthin and α-carotene. Carotenoids are generally considered to serve in a protective role; that is, they function to prevent or reduce photooxidation, the damage by excess visible light (58). Carotenoids are able to quench (inactivate) the excited states of molecules. In most cyanobacteria, zeaxanthin is bound to the phycobiliproteins. In the prochlorophyte *Prochlorothrix hollandia*, zeaxanthin is found between the plasma membrane and thylakoid membrane (59,60), but the location has not been identified *P. marinus*. Because photooxidation can be lethal, cells acclimated to high light generally have high zeaxanthin:Chl *a* ratios. For example, in North Atlantic field populations cellular levels of zeaxanthin in *Prochlorococcus* was constant at about 2 fg cell^{-1} (48). Consequently, the ratio of zeaxanthin:Chl a_2 increased for cells in the upper portion of the water column where light intensity was high. The ratio of zeaxanthin: Chl a_2 ranges from 0.4 to 1.2 in cultures (11) (Table 2).

In *Prochlorococcus*, α-carotene may serve a light-harvesting role as well. Evidence to support this function was seen in culture experiments (11) in which cellular concentrations of α-carotene and Chl a_2 co-vary over a range of light intensities. The exact function, however, has yet to be determined. The presence of α-carotene, typical of eukaryotic algae, is another unique characteristic of *Prochlorococcus* and distinguishes it from *Prochloron* and *Prochlorothrix*, which contain α-carotene (61).

The carotenoid complement of *Prochlorococcus* populations within the suboxic deep fluorescence maximum

Table 2. Comparison of High-Light and Low-Light *Prochlorococcus* Ecotypes Defined by Distinct 16S rRNA Sequences

	High-Light Strains	Low-Light Strains
Representative strains	CCMP1378 (MED4)	CCMP1375 (SS120)
(13)	MIT9302	MIT9303
Axenic strains	PCC9511 (10)	None yet available
Depth of isolation	5 m	120 m
Chl a_1	Absent	Absent
Chl a_2 (fg cell^{-1})	0.68 to 1.23 (10)	1.1 to 2.8 (11)
Chl b_1 (fg cell^{-1})	Absent	Present in some strains[a] 0.35[b] (11)
Chl b_2 (fg cell^{-1})	0.012 to 0.061 (10)	0.44 to 8.2 (48)
b : a ratio	Low ratio	High ratio
Cultures	0.017 to 0.050 (10)	1.0 (4)
	0.05 to 0.15 (16)	0.5 to 1.6 (11)
	0.05 to 0.65 (13)	0.47 to 2.6 (13)
Field samples	0.15 (16)	2.9 (16)
Zeaxanthin (fg cell^{-1})	0.65 to 74 (49)	0.53 to 0.83 (11)
	0.65 to 0.99 (11)	
Cultures	1 (50)	Less than 1 (50)
Field samples	2 (52)	0.69 (4)
α-Carotene (fg cell^{-1})	0.18 to 0.54 (11)	0.29 (4)
	0.29 (4)	0.24 to 0.99 (11)
Mg 3,8 DVP a5 (fg cell^{-1})	0.03 to 0.12 (11)	0.03 to 0.20 (11)
Prasiloxanthin		
Cultures	Not detected	Not detected
Field samples	Not detected	Present (51)
Temperature optimum (°C)		
Cultures	24 (50)	24 (50)
Temperature		
Maximum/minimum (°C)	27 / 12.5 (50)	27 / 15 (50)
Light saturated growth rate		
Type strain	0.63 ± 0.06 day^{-1} (86)	0.53 ± 0.06 day^{-1} (50)
Ecotype maximum growth	0.83 ± 0.03 day^{-1} (13)	0.63 ± 0.03 day^{-1} (13)
Growth irradiance optimum	45 ± 7 (13)	37 ± 8 (50)
μmol quanta m^{-2} s^{-1}	56 ± 13 (13)	22 ± 3 (13)
Photosynthetic efficiency, α_{ch1} (fg C fg Chl a^{-1} h^{-1} [μmol quanta m^{-2} s^{-1}]$^{-1}$)	0.05 (13)	0.11 (13)
Maximum photosynthetic rate, P^{ch1}_{max} (fg C g Chl a^{-1} h^{-1})	2 to 5 (13)	1.3 to 3.0 (13)
P^{cell}_{max} (fg C cell^{-1} h^{-1})	3 to 6 (13)	1.8 to 12 (13)
Maximum quantum yield[c] max (mol C mol Q^{-1})	0.06 to 0.09 (13)	0.07 to 0.11 (13)
Phycoerythrin	Absent	PE-III (52,53)
Thylakoid arrangement	Horse-shoe shaped (46)	Concentric (9,12)
LL adaptation	Single antenna *pcb* gene (54)	Multiple antenna *pcb* genes; a gene family of seven transcribed genes encoding different Chl a/b-binding proteins (54)
Phylogeny	"Newer" clade (55)	More closely related to PE-containing *Synechococcus* strains (55–57)

[a] Present in CCMP1375 (SS120), MIT9211, MIT9303, MIT9313 only (13).
[b] For culture acclimated to 133 μmol quanta m^{-2} s^{-1}.
[c] For culture grown at 9 μmol quanta m^{-2} s^{-1}.

layer (see section on Vertical Profiles) included high concentrations of an additional pigment: 7′,8′-dihydro-derivative of zeaxanthin (51). This pigment was identified as parasiloxanthin, a carotenoid that is found in prasinophytes. Intriguingly, both α-carotene and Mg 3,8 DVP a5 are also characteristic of prasinophytes.

A further example of the unusual pigment composition of *Prochlorococcus* is the recent discovery of a novel form of phycoerythrin (PE) in several strains (52,53). Algal biliproteins typically absorb light in the yellow-green regions, where chlorophylls generally absorb poorly or not at all, so extend the light harvesting capability of the cell. Following observations of orange, PE-like fluorescence in cells from below 100 m in the tropical Pacific Ocean, it was suggested *Prochlorococcus* may possess small amounts of a PE-like pigment (52). Subsequent examination of *Prochlorococcus* CCMP1375 identified functional genes for the α- and β-subunits of the PE pigment-protein

complex (52). Although similar to PE-II, the type found in *Synechococcus* which binds a phycourobilin-like pigment as the major chromophore, this novel PE-III differs significantly from previously known sequences of the α- and β- subunits and the presence of a single bilin attachment site on the α-subunit (52). Similar PE-III sequences have also been found in two strains from the North Pacific isolated from the chlorophyll maximum depth (100 m, PAC1 and PAC2) (53). All of these strains are characterized as low-light strains (55). These α- and β-subunit genes are not found in the high-light strain CCMP1378 or isolates from the surface waters of the Arabian Sea (53). The PE-III contains a unique phycourobilin chromophore that absorbs in the blue region (498 nm), which are the only wavelengths of light remaining at the base of the euphotic zone. Thus, this pigment complement would provide a selective advantage to the low-light strains inhabiting these depths.

Given the very low level of expression of the PE genes, the function of PE in *Prochlorococcus* was not immediately apparent in CCMP1375. Low-light *Prochlorococcus* strains lack not only phycobilisomes, but also the required pigments of light energy transfer that occur in cyanobacteria (i.e., the phycocyanin and allophycocyanin pathway to the reaction centers), so its role in light transfer was uncertain. Recently, however, it was demonstrated that in the low-light ecotype CCMP1375 PE-III may in fact serve a light-harvesting antenna function (62). The ability of PE-III to transfer of energy to divinyl chlorophylls was demonstrated by employing a spectrofluorometric assay, but the exact mechanism of energy transfer is still not known (62).

In summary, although *Prochlorococcus* was originally considered a 'prochlorophyte' based on the apparent presence of Chl *b* and absence of phycobiliproteins, the unique pigment composition (*Chl* a_2 and b_2, α-carotene, Mg 3,8 DVP a5, and, in some strains, PE-III) of this organism has distinguished it from the other two "prochlorophytes" and other cyanobacteria.

ECOLOGY

Geographic Distribution

Prochlorococcus can be found throughout the tropical and subtropical regions of the world's oceans. Initial studies of *Prochlorococcus* distribution and abundance focused principally on the North Atlantic (4,40,48). Since then, investigations have confirmed the overwhelming numerical dominance of *Prochlorococcus* worldwide (6,34,63–66). *Prochlorococcus* are most abundant between 40 °N and 40 °S, and their numbers diminish rapidly beyond this range (see Table 3). To date, the most northern latitude at which *Prochlorococcus* have been detected is 60 °N in the Atlantic (64). In the Southern Hemisphere, *Prochlorococcus* have been found at 48 °S in the Atlantic and at 40 °S in the southwestern Pacific, where they were found only at the base of the euphotic zone (67).

The geographical range of *Prochlorococcus* appears to be limited to regions where temperature is greater than 10 to 15 °C (12,64,71). Laboratory experiments have confirmed the temperature preferences of *Prochlorococcus* (see section on Growth); however, other factors may play a role as well. Generally, *Prochlorococcus* is most abundant in oligotrophic conditions, such as the subtropical central North Pacific (34) and the Arabian Sea (65), where nitrate concentrations are very low (<0.1 μM). The limitation of *Prochlorococcus* distribution with respect to nitrate has been reported numerous times and in a variety of oceanic regions (65,66,69,72). Surprisingly, *Prochlorococcus* are abundant in the equatorial upwelling regions of the Pacific and Atlantic (66,73). In the equatorial Pacific, however, abundances were greater during an El Niño-Southern Oscillation (ENSO) event than during normal conditions (73). *Prochlorococcus* are usually absent in

Table 3. Global Comparison of Maximum Densities of *Prochlorococcus* (cells $mL^{-1} \times 10^3$) and Depth Integrated Abundances for 200 m Water Column (cells $cm^{-2} \times 10^6$)

Location	Reference	Maximum Abundance (cells $mL^{-1} \times 10^3$)	200 m Integrated Abundance (cells $cm^{-2} \times 10^6$)
N. Atlantic subpolar 50 to 60 °N	64	8	15.9
N. Atlantic / Bermuda	64	180	1,300
(BATS) 27 to 45 °N	40	150	1,470
Eq. Atlantic (5 °S to 25 °N)	64	520	2,070
S. Atlantic 35 °N (absent below 38 °S)	63	280	2,200
N. Pacific subtropical 30 °N	68	36	Na
N. Pacific tropical (22 °N)	34	318	3,430
Eq. Pacific (0 to 5 °N)	38	181	1,360
S. Pacific tropical (10 to 20 °S)	67	344	3,275
	69	440	
S. Pacific (30 to 40 °S)	67	2	1.9
Arabian Sea (Intermonsoon) (10 to 20 °N)	65	700	2,320
Arabian Sea (Southwest monsoon) (10 to 20 °N)	65	506	2,660
Mediterranean Sea (Winter) (42 to 43 °N)	70	52	300

most shallow coastal waters and occurrence is generally negatively correlated with surface nitrate levels (65,74). Note, however, that the high-light strain MED4 was isolated from Mediterranean nearshore waters, just a few kilometers beyond the Rhône estuary. Absence of *Prochlorococcus* is also noted consistently in regions of well-mixed waters (75,76). Additional factors influencing *Prochlorococcus* distributions may be other limiting nutrients or perhaps inhibitory factors (see section on Physiology and Growth).

The occurrence of *Prochlorococcus* in atoll lagoons of the South Pacific was reported for the first time in 2000 (77). However, *Prochlorococcus* contributed only a small percentage (4%) of the biomass (77). Previously, the conspicuous absence of this organism was noted in coastal waters of tropical regions, such as Kaneohe Bay, Hawaii, which is a heavily impacted coastal subtropical bay (74).

"*Prochlorococcus*-like" cells, based on flow cytometric signature (small forward light scatter and chlorophyll fluorescence signals), have also been reported in some freshwater systems. For example, small cells in a eutrophic reservoir in southern Spain were characterized by flow cytometry, but the identity of the cells was not confirmed by HPLC pigment analyses (78). An additional example is the novel occurrence of small, red-fluorescing cells within the surface layer of a stratified saline lake on the island of Molokai, Hawaii. These cells were identified as *Prochlorococcus* based on their flow cytometric signature and the presence of Chl a_2, which was confirmed by HPLC (79).

Seasonal Patterns of Abundance

Given the apparent limitation by water temperature (see preceding text), the geographic distribution of *Prochlorococcus* may vary seasonally in some regions. For example, wintertime deep-mixing events appear to be responsible for seasonal minimum abundances observed in the North Atlantic at the Bermuda time-series station (BATS) (40,80). Conversely, summer/fall increases in abundance noted in a coastal bay in Japan evidently were linked to increased water temperatures, or were perhaps coincident with an intrusion of the Kuroshio (68).

Seasonal variations in abundance are observed for all three groups of the photosynthetic picoplankton community, although the magnitudes of the variations differ among regions. In the Sargasso Sea off Bermuda at 32° 15′N (North Atlantic), Olson and coworkers documented seasonal shifts in community structure that result in the alternation of *Prochlorococcus* dominance. In summer, the *Prochlorococcus*:*Synechococcus* ratio is 10 : 1, whereas in winter the ratio is close to 1 : 1 (40). In the Mediterranean Sea, winter and summer community structure is also considerably different (81). This contrasts with the observations in the North Pacific at 22 °N (34) where *Prochlorococcus* are always numerically dominant and the range in seasonal variation is much smaller. In the subtropical central North Pacific, small seasonal changes are evident for all three components of the picoplankton, not only for cell abundance but also for cellular parameters such as light scatter or pigment fluorescence (34). The difference between seasonal minimum and maximum values in 200-m integrated water column abundances was only twofold for *Prochlorococcus*, up to 10-fold for *Synechococcus* and up to 4-fold for the small eukaryotic algae (picoeukaryotes) (34). In addition, the timing of the seasonal cycles of the picoplankton is not in phase: abundance maxima of *Synechococcus* occur in winter, of picoeukaryotes in spring and of *Prochlorococcus* in summer or fall (34,40). In the Arabian Sea, strong seasonal forcing by monsoon winds produced much larger (up to 10-fold) seasonal and spatial variations in *Prochlorococcus* populations; however, highest abundances occurred during the Intermonsoon (65).

Vertical Profiles of Abundance

Throughout the majority of its range, *Prochlorococcus* abundance is uniform within the surface mixed layer (i.e., the well-homogenized uppermost layer of water characterized by constant temperature and density) and decreases rapidly below. Consequently, as the mixed-layer deepens, *Prochlorococcus* distribution extends deeper in the water column. A good example of the range in vertical distributions was seen in a transect in the eastern tropical Atlantic, where the *Prochlorococcus* uniform surface layer was 30 to 35 m at near shore eutrophic stations, deepened to 50 to 70 m in mesotrophic waters, and extended from 80 to 100 m depths at offshore oligotrophic stations (66). In some cases, there is a slight peak in *Prochlorococcus* abundance at the surface or near the chlorophyll maximum (35).

One of the most interesting features of the *Prochlorococcus* vertical distribution in the ocean is that it can exist successfully throughout the entire euphotic zone (surface lighted layer). In typical oligotrophic regions, where the euphotic zone extends to below 100 m, *Prochlorococcus* cells frequently are found down to 150 to 175 m below the surface (34,66). This represents an extremely broad range of light intensities to which *Prochlorococcus* can acclimate. The observed cellular chlorophyll fluorescence (Fig. 2) can increase up to 50-fold between the surface and deepest portion of its profile (35,38).

Another important feature of the vertical distribution of *Prochlorococcus* is the co-occurrence near the chlorophyll maximum depth of at least two distinct populations. Such "double" populations, each with distinct fluorescence properties, hence termed *dim* and *bright*, were first observed in the North Pacific (35) and in the Equatorial Pacific (38). Using the DNA-specific stain DAPI to investigate the DNA content of cell type, results confirmed they were two distinct populations with a slightly different DNA content (see section on Genome) (35). Subsequently, cultures of each cell type were established by flow cytometric sorting (36). The distinct fluorescence properties of each type have been maintained even after a number of years in culture. Physiological differences of these two cell types have also persisted in these strains and serve to distinguish the "dim" strains isolated from surface high-light and the "bright" strains isolated from the low-light depths. The physiological properties of these "ecotypes" (see section on Physiology and Growth) may

explain the vertical profiles observed and generally are consistent with their depth of isolation.

Suboxic Environments

Unusual vertical profiles have been observed within suboxic waters of the oxygen minimum zones in the Arabian Sea and eastern tropical North Pacific. Here, deep populations of *Prochlorococcus* were detected as discrete peaks or layers of almost "pure" cultures (51,82). The layers occurred between 80- and 140-m depths and were identified as "secondary fluorescence maxima" in profiles of in situ chlorophyll fluorescence. The "deep chlorophyll fluorescence maximum" is a common feature throughout the open ocean and most frequently seen at the base of the euphotic zone (where light is reduced to 1% of the surface irradiance, 1% I_0). These novel secondary fluorescence maxima occurred in suboxic waters at irradiances ranging from less than 0.1 to 2% of the surface irradiance. Initially, it was unknown if these secondary fluorescence populations could be growing at such low light intensities.

In the eastern tropical North Pacific, the biomass within the secondary fluorescence maximum made a significant contribution to standing stock of phytoplankton. Conversely, in the Arabian Sea this deep population apparently was only a small fraction of the biomass, and therefore thought to contribute little to primary production in this region (51). In situ experiments conducted in the Arabian Sea, however, suggested these populations may be more dynamic than predicted. Results from photosynthesis versus irradiance response curves indicated cells within this secondary fluorescence maximum are in fact an efficient population that is adapted to extremely low light (as low as 0.02 to 0.05% surface irradiance) because of their increased light absorption capabilities. Johnson and coworkers (82) suggested several possible hypotheses to explain these observations. The suboxic conditions could be inhibitory to microzooplankton grazers, thus the increased abundance of *Prochlorococcus* could be due to reduced grazing. Alternatively, the spatial variability or discontinuity in environmental conditions could lead to layering, suboxic conditions could produce enhanced growth, or physical mechanisms of aggregation might be operating to create the observed features.

Goericke and coworkers (51) suggested that as only *Prochlorococcus* thrive in this secondary fluorescence maximum layer, whereas both *Prochlorococcus* and eukaryotic picoplankton coexist in and below the primary chlorophyll maximum layer, the low oxygen concentrations of the secondary fluorescence maximum must be a crucial factor. However, they were not able to identify a particular physiological function that would be affected by low oxygen levels in eukaryotes but not in *Prochlorococcus*. There is no evidence for anoxygenic photosynthesis in *Prochlorococcus* (59); however, the capability for utilizing hydrogen sulfide as an electron donor for photosynthesis has been observed in other cyanobacteria species (83).

Contribution to Community Structure

Prochlorococcus are the numerically dominant members of the photosynthetic plankton community throughout most of the subtropical central gyres of the Pacific and Atlantic oceans. Abundance typically ranges from 100 to 500×10^3 cells mL^{-1} (Table 3), which greatly outnumbers all other picoplankton groups (34,40,84,85). Maximum abundances occur in nitrate-depleted, oligotrophic regions of the ocean. The highest cell abundance ever reported (700×10^3 cell mL^{-1}) was observed in the Arabian Sea during the Intermonsoon (65); the highest level of the *Prochlorococcus* marker Chl a_2 (up to 300 ng L^{-1}) was also measured during this season in the Arabian Sea (19).

In oligotrophic regions, the abundance of *Prochlorococcus* is usually 100-fold greater than *Synechococcus*; whereas in mesotrophic and coastal areas *Synechococcus* populations exceed *Prochlorococcus*. Thus, there appears to be an inverse relationship between *Prochlorococcus* and *Synechococcus* abundance (35). In fact, *Prochlorococcus* is frequently absent in coastal samples. The ratio of *Prochlorococcus:Synechococcus* abundance, therefore, can provide an index of community structure, with higher ratios indicative of more oligotrophic environments, see for example reference (65). This relationship appears to be consistent worldwide, with the exception of observations the Arabian Sea where monsoonal influences may affect the distributions (Fig. 4).

Total nonpigmented, heterotrophic bacteria typically occur at abundances of 10^6 cells mL^{-1} in open ocean regions. *Prochlorococcus* represents approximately 30% of the "total" bacteria throughout the euphotic zone of oligotrophic waters (43); however, this percentage is lower in the Equatorial Pacific (~20%) and at the Bermuda Atlantic Time Series (BATS) site (~10 to 20%) (38). Surface, dimly fluorescent populations of *Prochlorococcus* can be difficult to distinguish from the nonpigmented heterotrophic bacteria with standard epifluorescence microscopy and so may be counted as heterotrophic bacteria (43). Enumeration is more accurate using flow cytometry to analyze samples stained with a DNA-specific dye such as the UV-excitable dye Hoechst 33342 or the blue-light excited SYTO13 and SYBR dyes (86,87). In dual-beam analysis of Hoechst 33342-stained cells, the two lasers are tuned so that both DNA and chlorophyll fluorescence can be detected. Thus, heterotrophic bacterial cells (DNA-fluorescence signal only) can be distinguished from *Prochlorococcus* cells (both DNA-fluorescence and chlorophyll fluorescence signals) (43).

In terms of the total photosynthetic biomass, *Prochlorococcus* and cyanobacteria together account for more than half of the total Chl *a* biomass in many oligotrophic regions of the world's oceans (19,23). *Prochlorococcus* alone contributes an average of 67% of the estimated photosynthetic carbon biomass in the biomass in the subtropical North Pacific (65). The contribution by *Prochlorococcus* is the greatest in the most oligotrophic waters (33,88,89). In the Equatorial-Pacific *Prochlorococcus* contributed an estimated 41% of microbial C during an El Niño event (38), which greatly exceeded estimates of their contribution in the North Pacific (88). The contribution of *Prochlorococcus* to total gross primary production increases as the

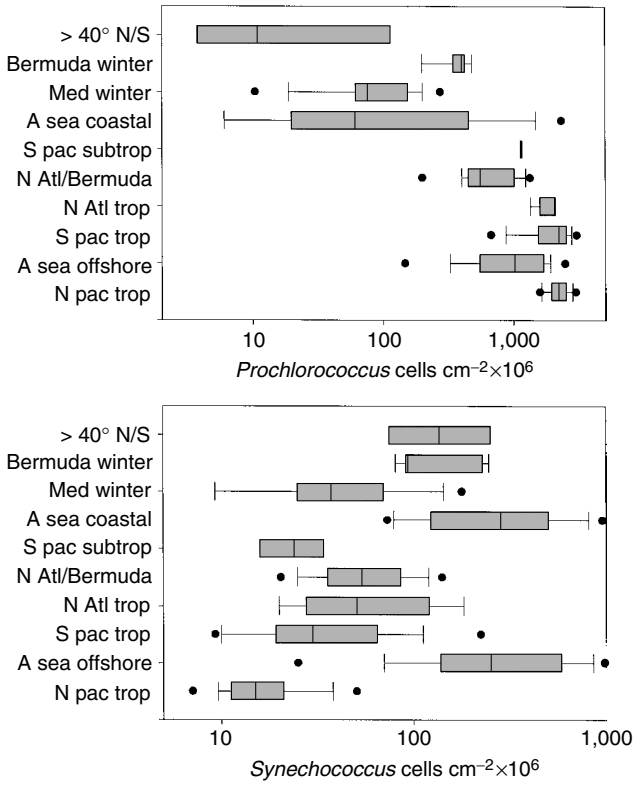

Figure 4. The inverse relationship between *Prochlorococcus* versus *Synechococcus* distributions is seen by comparing 200-m integrated abundances. Data are shown as a box-plot in which observations for each location are centered about the mean (indicated by the vertical line within box) and the 5th and 95th percentiles are shown as error bars, with data beyond these percentiles shown by filled circles. *Prochlorococcus* is more abundant in oligotrophic (tropical) regions of the open ocean, whereas *Synechococcus* exceeds *Prochlorococcus* abundance in mesotrophic waters and coastal areas. Data from selected sites in the Pacific (Pac), Atlantic (Atl), Mediterranean Sea (Med), and Arabian Sea (A Sea) as listed in Table 3 modified from reference (35).

euphotic zone of the water column becomes more oligotrophic. For instance, *Prochlorococcus* contribution was only 9% in the relatively nutrient-rich eastern Equatorial Pacific, but contributed 39% in the western Equatorial Pacific and 70 to 80% in the oligotrophic subtropical North Pacific (88). Similarly, the *Prochlorococcus* contribution was not appreciable in the Arabian Sea where nitrate was measurable in surface waters (90), but was 45 to greater than 90% in the Equatorial Northeastern Atlantic (66). Often, however, the small eukaryotic algae (picoeukaryotes) make the largest contribution to primary production (91).

A variety of conversion factors have been employed to estimate carbon biomass of each planktonic component and the contribution of each to total microbial biomass (89). Factors may vary under different growth conditions, so estimates from different studies may not always be directly comparable. Assumptions need to be carefully considered and viewed in terms of constraints by total particulate organic carbon and primary production.

PHYSIOLOGY AND GROWTH

Our knowledge concerning the factors that regulate the abundance, distribution, growth, and physiological status of *Prochlorococcus* in the open ocean remains limited. Here, known physiological and growth properties are discussed, and wherever possible the properties for the two ecotypes are contrasted (Table 2).

Temperature

One of the most significant factors to influence the large-scale distribution pattern of *Prochlorococcus* may be temperature, as discussed in the preceding text (see section on Distribution). Experimental work supports this hypothesis. In experiments designed to determine growth temperature optima, differences were observed in the temperature ranges of high- and low-light strains (50). Optimum growth occurred at 24 °C for both strains, however MED 4 was able to grow at 12.5 °C, whereas the minimum temperature for growth of SS120 was 15 °C (Table 3). Growth was observed up to 27 °C in culture; however, in field populations growth occurs at much higher temperatures. This suggests, again, that more than one ecotype of *Prochlorococcus* must exist among field populations.

Light

Growth of photosynthetic organisms is strongly linked to and limited by the availability of light (50). Growth of *Prochlorococcus* is tightly phased to the light-dark cycle (see section on Cell Cycle Analysis; Fig. 5), so that strong diel variations in abundance are observed (88,92). Cell division occurs after sunset, so that maximum cell abundances are observed around midnight, after which concentrations decrease until reaching a minimum the following afternoon. As division begins again prior to sunset (1,600 to 1,800 hours), cell abundance increases (88). Maximum growth rates have been observed at approximately 15% of the surface irradiance (88).

The success of *Prochlorococcus* throughout the entire euphotic zone suggests they have adapted to growth over a wide range of light intensities: from less than 1 μmol quanta $m^{-2}\,s^{-1}$ at the deepest occurrence up to near 2,000 μmol quanta $m^{-2}\,s^{-1}$ at the ocean surface. Several physiological properties of *Prochlorococcus* confer a definite selective advantage for growth under extreme low light levels at the base of the euphotic zone. The ability for photoacclimation is seen in the large increase in chlorophyll fluorescence per cell, often 50-fold, which suggests a significant increase in pigment per cell (35,40). In addition, the pigment complement of *Prochlorococcus* is consistent with growth at low light intensities because of the high Chl $b:a$ ratio. Strains with a high Chl $b:a$ ratio are optimized for absorption of blue light, which are the only wavelengths that penetrate to depth in the ocean (50). Flow cytometric measurements of *Prochlorococcus* populations reveal an extremely large

range in the ratio Chl b : Chl a_2. (note that Chl b measurements often include both b_1 and b_2). For example, ratios of Chl $b:a$ of 0.91 to 5.4 have been observed in the North Atlantic (48). This range vastly exceeds what is measured for any single culture grown in the laboratory under a wide range of light intensities (16,50) (see Table 2). Subsequent experiments with 10 different isolates have shown that strains can be distinguished based on their Chl $b:a$ pigment ratios and light responses. Light intensities that completely inhibit the low-light strains (high Chl $b:a$, high growth efficiencies) provide optimal growth conditions for some high-light strains (low Chl $b:a$) (13). It is interesting to note, however, that growth of all available *Prochlorococcus* strains under laboratory conditions is saturated at relatively low light intensities (18 to 87 μmol quanta $m^{-2}\,s^{-1}$) (13). So perhaps overall we may have only relatively low-light adapted strains in culture (12).

Photosynthesis

The two *Prochlorococcus* ecotypes have distinct photosynthetic characteristics that are consistent with their definitions as low or high Chl $b:a$ strains (11,13). Photosynthesis versus irradiance experiments were conducted with ten different strains to determine if photosynthetic parameters were consistent with pigment type (13). The parameter $_{chla}$, an index of the efficiency of photosynthesis, ranged from 0.03 to 0.125 fg C fg Chl $a^{-1}\,h^{-1}$ (μmol quanta $m^{-2}\,s^{-1})^{-1}$, but was twofold higher in low-light strains than in high-light strains (Table 2; 13). This increased efficiency apparently is because of an increased concentration of antenna complexes (11). Maximum rates of photosynthesis when normalized to Chl a_2, P^{chl}_{max}, ranged from 1.3 to 5.2 fg C fg Chl $a^{-1}\,h^{-1}$ and were higher for cells grown at high light (Table 2; 13). On a per cell basis, P^{cell}_{max} was highest for the low light strains (Table 2). All isolates of *Prochlorococcus* that have been investigated appear to be fairly sensitive to higher light irradiances, and most strains are photoinhibited above ~100 μmol quanta $m^{-2}\,s^{-1}$. The quantum yield of photosynthesis, $_{max}$, was relatively high for the strains examined and ranged from 0.063 to 0.107 mol C mol Q^{-1}. In experiments with MED4, the zeaxanthin/cell remained constant over varying light intensities (94), so absorption of light by zeaxanthin may explain some of the variation in $_{max}$ noted in the experiments of Chisholm and Moore (13).

The molecular basis for the physiological differences in photoacclimation between low- and high-light strains appears to result from the number of genes encoding Chl a/b-binding (*pcb*) proteins (54). The low-light-adapted *Prochlorococcus* strain SS120 possesses a gene family of seven transcribed genes, whereas the high-light-adapted strain MED4 possesses a single *pcb* gene. The presence of multiple antenna genes provides evidence to explain the capacity of low-light *Prochlorococcus* populations to survive at extremely low light levels (54) (Table 2). The pigment complement of *Prochlorococcus* (see preceding section on Light) also allows it to be very efficient at low light levels. Further information regarding photosynthetic performance and the photosynthetic apparatus itself is presented by Partensky and coworkers (12).

Regulation of photosynthetic activity has been investigated by measuring the transcription levels of the ribulose-1,5 diphosphate carobxylase/oxygenase (RuBisCO) large subunit (*rbcL*). RuBisCO is the essential, first enzyme in the photosynthetic fixation of carbon dioxide (95). A diel pattern in C-fixation and *rbcL* transcription (mRNA) was observed in field samples and in cultures, even when shifted to continuous light from a light/dark growth regime (96). Thus, in *Prochlorococcus* the expression of RuBisCO appears to be regulated, at least in part, by transcriptional control (96).

Nutrient Requirements for Growth

Throughout most of the oligotrophic subtropical gyres, where *Prochlorococcus* overwhelmingly outnumber other photosynthetic members of the plankton community, nutrient levels are extremely low, often below the limit of detection by standard methods (97). Therefore, investigation of the factors that may regulate growth of *Prochlorococcus* in this extremely oligotrophic environment is of considerable interest. Until now, definitive nutrient uptake experiments have not been possible because axenic cultures were unavailable. With results from the first axenic strain PCC9511, we now have a glimpse of some physiological properties of this novel group. Comparisons of

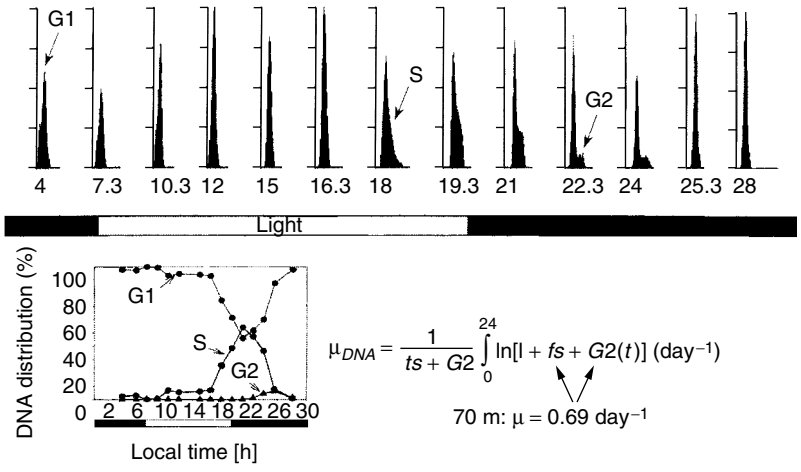

Figure 5. Flow cytometric analysis of *Prochlorococcus* cell cycle and growth rate calculation; following Carpenter and Chang (*Mar. Ecol. Prog. Ser.* **43**, 105–111 (1988)). The duration of the terminal event (S + G_2) is calculated as twice the period from maximum S to maximum G_2. Reproduced with permission, from Veldhuis and Kraay (*Sci. Mar.* **64**, 121–134 (2000)) (93).

nutrient requirements and preferences among strains will be determined as additional axenic strains become available.

Nitrogen

Growth of phytoplankton in the ocean is most often assumed to be nitrogen limited. From initial observations of *Prochlorococcus* abundance in the North Atlantic (40), it appeared that maximum population density tracked the nitracline (the depth at which a rapid increase in nitrate begins, often just below the base of the euphotic zone). Given the capability of *Prochlorococcus* for growth at low light levels (see preceding text) and the absence of detectable nitrate in surface waters of the oligotrophic subtropical gyres, this appeared to be a plausible explanation consistent with observed distributions. However, as more information detailing *Prochlorococcus* distributions was compiled (see earlier section on Ecology), it became apparent that *Prochlorococcus* was most often evenly distributed throughout the surface mixed layer (see preceding text), and not limited to a subsurface maximum near the nitracline.

Based on results from initial culturing attempts and recent development of the axenic high-light strain, it appears that urea and ammonia are the favored nitrogen sources. In fact, the high-light strain PCC9511 cannot grow on nitrate. This is not consistent with previous observations that *Prochlorococcus* appeared to track the nitracline and an increase in cell cycling noted with increased nitrate (40,98). Perhaps another factor covarying with nitrate may explain these results. Alternatively, there may be multiple strains with different nutrient preferences or capabilities. This would explain both situations: high-light strains typically are found in the surface mixed layer where nitrate is undetectable, and low-light strains in the lower portion of the euphotic zone capable of utilizing nitrate. Preliminary results have indicated a low-light strain is capable of nitrate uptake (10,99); however, definitive experiments will require axenic cultures of the different "ecotypes."

Phosphorus

In initial culturing attempts, an organic form of phosphorus appeared to be preferred, or at least led to greater success, when culturing *Prochlorococcus*. Further investigations with established cultures have shown growth to be equivalent in media with either inorganic orthophosphate or organic phosphate (NaH_2PO_4 or -glycerophosphate; see Table 1). Growth of PCC9511 was satisfactory with a number of alternative phosphorus sources (Na_2 -glycerophosphate, Na_4-pyrophosphate, glucose 6-phosphate or ATP) (10). Based on these results, it is hypothesized that *Prochlorococcus* may be capable of synthesizing ecto-phosphohydrolases, as both polyphosphate and organic phosphates require dephosphorylation before they can be taken up across the cell membrane (100). This capability would confer an advantage to *Prochlorococcus* because in much of its geographic range the supply of inorganic phosphate may be limiting (101–103). Experiments conducted in the North Pacific to examine the dynamics of phosphorus have shown that the microbial community appears to be P-limited (104). In this region, where *Prochlorococcus* numerically dominates the microbial community, dissolved organic sources of phosphorus may be important for growth.

Trace Metal Requirements

Iron is required by all photosynthetic organisms because it is an essential component of the electron transport system of photosynthesis. When iron is limiting, cells cannot synthesize functional electron transport components, which leads to loss of PSI and PSII reaction centers. Consequently, the quantum efficiency of photosynthesis is reduced. This means that when phytoplankton are iron-limited, the light absorbed by the antenna pigments is less likely to be trapped for photosynthesis and is more likely to be lost as fluorescence. Iron limitation of the larger phytoplankton (i.e., diatoms) was hypothesized to explain the low productivity observed in the equatorial Pacific where, in spite of high nutrient levels, chlorophyll biomass remains low. Experimental evidence suggests that iron may limit picoplankton as well. The cyanobacterium *Synechococcus*, a close relative of *Prochlorococcus*, has been shown to have a high iron demand (105).

Results from large-scale iron-enrichment studies conducted in situ in the equatorial Pacific (IRONEX I and IRONEX II) (106,107) showed that in experimental treatments with iron additions the quantum yield of photosynthesis of PSII increased rapidly in *Prochlorococcus* (108), as did cell division rates measured by cell cycle analysis (109) (see section on Growth). In spite of increased cell division, a concomitant increase in biomass was not observed because of the closely coupled grazer populations. Populations responding to nutrient additions in iron-enriched treatments were rapidly grazed, thus increased mortality balanced the growth of *Prochlorococcus*. Conversely, results in the subtropical North Pacific suggested *Prochlorococcus* were not iron limited (110).

The cobalt requirements of *Prochlorococcus* and *Synechococcus* have also been investigated. *Prochlorococcus* appears to have an absolute requirement for cobalt that exceeds typical concentrations found in the open ocean; thus, cobalt may be a limiting factor for *Prochlorococcus* in the oligotrophic ocean (111). Because total cobalt concentrations appear to be extremely low (20–100 pM) in the open ocean, an uptake mechanism utilizing cobalt-specific ligands has been proposed for *Prochlorococcus* (112). In the axenic high-light strain PCC9511, addition of vitamin B_{12} (cyanocobalamin) was not essential for growth (10).

The zinc requirement is low for most cyanobacteria, but is required for optimal growth by some strains (113). RNA polymerase is known to be a zinc-containing enzyme; thus, it is likely that zinc is an essential requirement for growth of cyanobacteria, including *Prochlorococcus*. The binding site is preserved among *rpoC1* sequences from eight cyanobacterial species, including *Prochlorococcus* (56), but the association of zinc with the binding pocket has yet to be demonstrated. For PCC9511, the addition of 1 μM zinc to a synthetic sea water medium had no effect on growth, but a 10 μM addition was toxic (10).

There are few known enzymes requiring copper in cyanobacteria, and a requirement of this element is not known in *Prochlorococcus* (10). Omission of copper from the medium apparently had no effect on growth. In fact, it has been proposed to have a toxic effect (4). The axenic high-light strain PCC9511 was tested for copper tolerance and growth was observed at 100 nM copper (10); however, growth at higher levels was not tested.

Nitrogen Fixation

The ability to fix atmospheric nitrogen (N_2-fixation) is an important property of some cyanobacteria, for example, *Trichodesmium* (114). No nitrogen fixation (*nif*) genes have been found in *Prochlorococcus* cultures (49,115); therefore, it seems likely that *Prochlorococcus* strains are not able to fix nitrogen. Nitrogen fixation has been observed in some unicellular *Synechococcus* cyanobacteria (116,117); however, these are benthic and freshwater strains rather than the marine phycoerythrin-containing *Synechococcus*, which are close relatives of *Prochlorococcus*. Reports of nitrogen fixation by *Prochloron* have been conflicting (118,119), but more recently evidence from the natural abundance of $^{15}N/^{14}N$ in isolated *Prochloron* cells indicated a significant percentage of the nitrogen was derived from nitrogen fixation. Thus, Kline and Lewin (120) suggest *Prochloron* may be capable of facultative nitrogen fixation. Given the tremendous diversity among the cyanobacteria, we cannot rule out the possibility that a nitrogen-fixing *Prochlorococcus* strain may yet be discovered.

pH

The effects of pH have not yet been examined specifically in *Prochlorococcus*. However, the marine *Synechococcus* cyanobacteria appear to prefer neutral, or slightly alkaline pH (121), which is typical of seawater throughout the geographic range of *Prochlorococcus*.

DYNAMICS OF *PROCHLOROCOCCUS* POPULATIONS

Estimates of *in situ* specific growth rates for *Prochlorococcus* are difficult to obtain in field samples using traditional oceanographic methods because it is not easy to separate *Prochlorococcus* from other picoplankton. Estimates of cell-specific growth rates for *Prochlorococcus* have been determined using the cell cycle method (88,92,122), ^{14}C-Chl a_2 labeling method (123), or zeaxanthin per cell (94). In addition, population dynamics have been examined using the dilution method (125,125) in combination with flow cytometric enumeration to obtain cell-specific growth and mortality rates. Some examples of these methods are discussed briefly in the following text; however, for full details and limitations of the methods the cited literature should be consulted.

Growth Rate Estimates Based on Cell Cycle Analysis

The cell cycle of *Prochlorococcus* is analogous to the eukaryotic cell cycle in that it has discrete periods of DNA synthesis following gap periods (98). Because the cell cycle of *Prochlorococcus* populations is highly synchronized with the light-dark cycle, DNA histograms display two peaks corresponding to G_1, the initial growth phase, and G_2, the late growth, separated by a DNA synthesis phase, S (Fig. 5). Using the model proposed by Carpenter and Chang (122), growth is computed from the fraction of cells in a terminal event (e.g., $S + G_2$). By measuring the DNA cell^{-1} distribution for a *Prochlorococcus* population frequently over a cell cycle and estimating the fraction (f) of cells in $S + G_2$, the growth rate can be calculated (see references (92,93,122)). An example of the cell cycle approach is shown in Figure 5 (93). Growth rates for *Prochlorococcus* in the equatorial Pacific were found to be 1 div day^{-1} in surface mixed layer and decreased to 0.2 div day^{-1} at 100 m. A comparative study in the eastern and western tropical Pacific revealed that *Prochlorococcus* grew equally well under mesotrophic and oligotrophic conditions, yet made a larger contribution to total primary production in the most oligotrophic regions of the North Pacific (88). In the Arabian Sea, growth rates computed by cell cycle analysis in some cases exceeded 1 div^{-1}, yet compared favorably with results of the dilution method (33) (see section on Ultradian Growth).

Because the growth rate of *Prochlorococcus* within the surface mixed layer appeared to be slower than for populations deeper in the water column, and the timing of cell division is often delayed as well, it was speculated that ultraviolet (UV) radiation exposure may have inhibitory effects on surface *Prochlorococcus* populations (92,126). The effect of UV radiation on *Prochlorococcus* has not been measured directly, however the picoplankton, such as *Prochlorococcus* are obviously subject to periodic high exposures of UV in clear oceanic waters. UV radiation is not limited to the immediate surface waters, but may penetrate fairly deep within euphotic zone. UV-B has been shown to penetrate to over 30 m and UV-A to 60 m (127). Experimental evidence suggests picoplankton, in general may be more sensitive to UV radiation. Boelen and coworkers (128) measured UV-B radiation vulnerability using the production of cyclobutane pyrimidine dimers (CPDs) as an index. In natural populations of plankton, they found production of CPDs to be size dependent; the DNA damage was greater in the 0.2 to 1.0 μm size fraction than in the 1 to 10 μm size fraction. A greater amount of damage was found at tropical latitudes, where *Prochlorococcus* is dominant, even though UV-B levels were higher elsewhere. In cases where CPD levels in field populations were low, it was speculated the communities received a low mean biologically effective dose because of wind-induced mixing, which would reduce overall exposure (128).

Phytoplankton class-specific growth rates can also be estimated using the pigment-labeling technique (129). Pigment synthesis rates are valid estimates of phytoplankton growth rates when growth is balanced. Because balanced growth is rarely the case in nature a precursor-product turnover model must be employed to accurately estimate growth rates from pigment labeling. Using this approach for *Prochlorococcus* in the Sargasso Sea, growth rates of 0.3 day^{-1} in surface waters and 0.1 day^{-1} at the base of the euphotic zone were obtained (123). Based on this

study, the average annual contribution to total primary production by *Prochlorococcus* populations was 25% (123).

Ultradian Growth and Rhythms

From most initial studies, it appeared that *Prochlorococcus* divided once per day (92). In many instances, however, *Prochlorococcus* exhibit ultradian growth (faster than 1 div day^{-1}), even though cell division is strictly phased to the light-dark cycle (14). Observations in both field populations and cultures showed that a second round of DNA replication and cell division proceeded immediately following the first division. This unique mode of ultradian growth suggests that control of the cell cycle is by a circadian clock or light-triggered timer rather than light intensity or duration. Most importantly, given *Prochlorococcus* are capable of division rates which exceed 1 div day^{-1}, these observations suggest *Prochlorococcus* could be nutrient-limited and so not growing at their maximal rate (14). If so, such results may have profound implications to our understanding of phytoplankton growth in the oligotrophic ocean.

Growth and Grazing Mortality

The dynamics of *Prochlorococcus* populations have also been investigated using the dilution method (124,125) combined with flow cytometric analysis to obtain specific growth rates (130–132). Maximal growth rates from 1.28 up to 2.2 day^{-1} have been reported (33,133). In almost all cases, the growth of *Prochlorococcus* is closely matched by losses due to mortality (e.g., 90% of growth in the Arabian Sea (33)). The coupling may become unbalanced if the environment changes, for example, because of strong environmental forcing. Although grazing by protozoans, such as heterotrophic flagellates and ciliates is the most significant fate of *Prochlorococcus* (74,130), losses owing to viral lysis could also be important (134,135). Grazing by the sponge, *Mycale lingua* was significant in the Gulf of Maine, where the efficiency of feeding on *Prochlorococcus* was 93% (136). Grazing by tropical appendicularians, which can effectively package *Prochlorococcus*, also contributes to mortality (137). In tropical coral reefs where the density of Gorgonian corals is high, the potential grazing impact on plankton communities is great (138). Thus, mortality because of coral grazing could be significant where *Prochlorococcus* are a large fraction of the community.

GENETIC DIVERSITY IN *PROCHLOROCOCCUS* POPULATIONS

Genetic Characterization

Prochlorococcus is thought to have the smallest known genome of all oxygenic photosynthetic bacteria (12). The genome size of MED4 is 1.6 Mbp based on results of genome sequencing (*http://spider.jgi-psf.org/JGI_microbial/html/prochlorococcus.med4.status.html*). Sequencing of a representative low-light strain, MIT9313, is underway (*http://spider.jgi-psf.org/JGI_microbial/html/prochlorococcus_homepage.html*); the preliminary size estimate is 1.9 Mbp (W. Hess, personal communication). This difference between high- and low-light strains in genome size is consistent with the 14% mean difference in DNA content reported earlier (35). The reported genome size for PCC9511 (2 Mbp) based on DNA renaturation kinetics and pulsed-field gel electrophoresis is slightly overestimated (10).

Overall, the cyanobacteria have a wide range in their DNA base pair composition, which is reported as percentage guanine + cytosine (mol% G + C). The marine *Synechococcus* cyanobacteria strains form a tight cluster within the range of 57 to 63% G + C (139). The mean DNA base composition in PCC9511 was reported to be 32.0 ± 0.1 mol% G + C (10). Thus, *Prochlorococcus* PCC9511 has a much lower mean DNA base composition than the typical marine *Synechococcus* cyanobacteria. Additional measurements will be possible as more axenic strains become available.

Diversity among Strains

The success of *Prochlorococcus* over such a wide range of light conditions has led to a number of investigations of *Prochlorococcus* diversity. Specific hypotheses related to the coexistence of genetically different populations adapted for growth at high- and low-light intensities have been examined using several different genes as markers. A high degree of genetic heterogeneity was observed within natural populations of *Prochlorococcus* based on sequences of a photosynthetic electron transport gene (*petB/D*) and intergenic region (140). Using flow cytometry to sort cells from depth profile samples, PCR amplification and cloning produced 6 to 21 different *petB/D* alleles. In samples from "double" populations (see earlier section on Vertical Profiles), overlapping sets of alleles were observed, which suggests that each population (genotype) was derived from a single gene pool. Sequences characteristic of the high-light *Prochlorococcus* clade were recovered at higher frequencies from cloned near-surface samples. This observation supports the hypothesis that *Prochlorococcus* populations are derived from a single gene pool; however, population shifts in response to environmental forcing (such as the surface nutrient-limited regime versus deeper light-limited regime) produce variations in genotype frequency (140).

Studies using the RNA polymerase ã subunit gene (*rpoC1*) examined diversity among both *Prochlorococcus* and *Synechococcus* sequences obtained by PCR amplification of field populations (141,142). From theses analyses, the strains could be divided into two clades, one genetically similar to the MED4 high-light strains and the second included low-light strains. The high-light clade could be further subdivided based on *rpoC1* sequences, however, physiological basis of this division is not known. Interestingly, none of the sequences matched the sequences of cultured isolates. An important result of these studies is that genetic diversity among *Prochlorococcus* is much greater than among *Synechococcus* clones, and may explain why *Prochlorococcus* are found over a greater range of depths (142). For example, in the North Pacific the *Prochlorococcus* distribution extends to a depth of 0.1% of surface light intensity, which is considerably deeper than *Synechococcus* (35).

Direct evidence to support the co-occurrence of multiple strains of *Prochlorococcus* was reported by Moore and coworkers (36). They found isolates established from the same water sample have very different light-dependent physiologies, for example, one growing maximally at light intensities that completely photoinhibit the other, yet their 16S ribosomal RNA sequences were remarkably similar (97%). Subsequently, Moore and Chisholm (13) have shown that there are at least two distinct physiological ecotypes. As discussed above, low-light strains have a higher concentration of Chl a_2 and b_2, a higher Chl $b_2 : a_2$ ratio, and higher photosynthetic efficiencies and photosynthetic capacities than strains adapted to high light intensities (see preceding section on Photosynthesis).

Diversity among *Prochlorococcus* strains is also demonstrated by analysis of the genes coding for the large subunit of RuBisCO (*rbcL*). Several forms of this enzyme (95) exist; Form IA is characteristic of the proteobacteria (e.g., nitrifying and manganese-oxidizing bacteria) and Form IB is characteristic of cyanobacteria (including most *Synechococcus*), green algae, and prasinophytes. However, *Prochlorococcus* strain GP2 and *Synechococcus* WH7803 possess a Form IA *rbcL* (143). A recent report from the Gulf of Mexico (144) provided the first description of Form IA *rbcL* transcriptional activity in the marine environment and suggested that Form IA *rbcL*-containing picoplankton (like *Prochlorococcus* GP2 and *Synechococcus* WH7803) may be important in the primary production of low salinity, surface water plumes of the Gulf of Mexico. It is not known how prevalent Form IA *rbcL*-containing picoplankton are worldwide. The other strains of *Prochlorococcus* examined to date (e.g., the MED and a North Pacific isolate) have a Form IB *rbcL* (145). There is no discernible relationship between the form of *rbcL* and the high- or low-light ecotypes defined by 16S rRNA sequences; however, based on only three sequences, it is probably premature to make any comparisons with 16S rRNA phylogeny (146). In field samples from the eastern Gulf of Mexico, six *Prochlorococcus* GP2-like Form IA *rbcL* sequences were obtained by PCR amplification, cloning, and sequencing (146). The similarity among these sequences (93–98%)—very similar, but not identical—again demonstrates that molecular microdiversity is commonly observed in marine microbial populations, for example see references (36,147). The coexistence of closely related, but physiologically distinct populations, may explain the microdiversity observed. As Moore and coworkers (36) conclude, "coexistence and distribution of multiple ecotypes permits the survival of the population as a whole over a broader range of environmental conditions than would be possible for a homogeneous population."

PHYLOGENY

When first discovered, *Prochlorococcus marinus* was described as a "prochlorophyte" because of the apparent similarity to *Prochloron didemni* (148) and *Prochlorothrix hollandica* (61), which are oxygenic prokaryotes containing Chl b_1 but lacking phycobilins. The pigment complement of *Prochlorococcus*, however, differs considerably from the other two prochlorophytes, specifically divinyl chlorophylls instead of true chlorophylls as their light-harvesting system (see earlier section on Pigments). Furthermore, analyses of the 16S ribosomal RNA (57) and RNA polymerase (*rpoC1*) genes (56) support a phylogenetic relationship between *Prochlorococcus* and the marine *Synechococcus* that is closer than with the other two prochlorophytes. Examination of two photosystem II genes (*psbA* and *psbB*) and photosynthetic electron transport genes (*petB* and *petD*) corroborate the close relationship of *Prochlorococcus* and *Synechococcus* as well (55,149). Lastly, the recent discovery of PE-containing *Prochlorococcus* strains further distinguishes *Prochlorococcus* and supports the close phylogenetic relationship with marine *Synechococcus* (52,53). Although data are available for only a limited number of strains, the presence of PE provides additional support for subdivision of *Prochlorococcus* clades: high-light adapted isolates, which lack PE genes, cluster together in a shallow recently arisen clade, whereas the low light adapted isolates, which have PE genes, are more basal (53,55).

Phylogenetic analysis of *Prochlorococcus* strains is of interest because of their role in the evolution of oxygenic photosynthetic organisms. Owing to their pigment complement, the "prochlorophytes" originally were thought to be ancestors of green algal and plant chloroplasts. But, based on molecular phylogeny of the 16S ribosomal RNA and RNA polymerase (*rpoC1*) genes they are now considered a polyphyletic group that is not any more closely related to chloroplasts than are the other cyanobacteria (56,57). In addition, examination of the Chl a/b binding proteins of all three prochlorophytes revealed a closer structural similarity to cyanobacterial Chl a binding proteins than to the Chl a/b binding proteins of green chloroplasts (150). Based on these studies, it was suggested that the prochlorophytes (*Prochloron didemni*, *Prochlorothrix hollandica*, and *Prochlorococcus marinus*) are not the specific ancestors of chloroplasts as originally proposed, but are diverged members of the cyanobacteria. Given the lack of phylogenetic unity among prochlorophytes, it was proposed that together with the cyanobacteria, they should be classified as Oxyphotobacteria (151).

One conclusion from these phylogenetic studies was that the ability to synthesize Chl b must have arisen independently several times in the prochlorophytes and in the chlorophyte ancestor (56,150). To test this hypothesis, Tomitani and coworkers (152) examined the genes responsible for Chl b synthesis, Chl a oxygenase (CAO), in two prochlorophytes and representative chlorophytes and higher plants. Phylogenetic analysis of the CAO sequences suggests a shared common evolutionary ancestor, and does not support the idea that Chl b arose independently in each of the prochlorophyte lines. Furthermore, they propose the hypothetical ancestral oxygenic photosynthetic bacterium was able to synthesize both Chl b and PE. *Prochlorococcus marinus* is the only oxygenic photosynthetic prokaryote known to contain Chl a_2 and Chl b_1 and b_2 as well as PE. Therefore, *Prochlorococcus* may be derived from this ancestral organism without changes in pigment complement, and thus may serve as a model for the ancestral oxygenic photosynthetic prokaryote (152).

GENOME SEQUENCING

Genome sequencing has been completed for two strains of *Prochlorococcus*: the high-light MED4 strain in 2000 and the low-light strain MIT9313 in 2001. Results from the Department of Energy Joint Genome Institute are available at: *http://www.jgi.doe.gov/tempweb/JGI_microbial/html/index.html*. MED4 is the smallest known photosynthetic organism, with a genome size of 1.67 Mbp, and MIT9313 has a genome size of 2.4 Mbp. Annotation of both genomes currently is underway. When analysis is complete, the results will complement the growing number of prokaryotic genomes that have been sequenced, including the first cyanobacterium *Synechocystis* PCC6308 (153) and a marine *Synechococcus* soon to be completed.

CONCLUSION

1. The novel complement of photosynthetic pigments of *Prochlorococcus* and improved HPLC techniques for separation of true chlorophyll a_1 and b_1 from the divinyl chlorophylls, Chl a_2 and b_2, has permitted estimates of the contribution of *Prochlorococcus* to the total chlorophyll biomass. Recognized differences in the ratio Chl b_2 : Chl a_2 are used to define the low- and high-light strains.
2. Improved sensitivity of flow cytometry was required to identify and enumerate *Prochlorococcus*. Flow cytometry remains an essential tool for the study of this organism.
3. Multiple ecotypes, clusters of genetically distinct strains with different physiological properties, coexist in the ocean. Consequently, *Prochlorococcus* are capable of growth at the surface to depths of approximately 200 m and so are thought to be the most abundant photosynthetic organisms in the ocean.
4. Throughout its range, *Prochlorococcus* growth rate is approximately 1 div day^{-1}. Maximal growth rates measured in cultures and in the field can exceed 1 div day^{-1}. Thus, the question of whether *Prochlorococcus* are in fact nutrient limited has been posed. As we learn more about the nutrition of the different ecotypes of *Prochlorococcus*, the factors limiting growth and distribution will be better understood.
5. In most regions, *Prochlorococcus* growth is closely balanced with losses owing to grazing by protozoans, such as heterotrophic flagellates. Additional grazers and viral lysis may be significant sources of mortality as well.
6. *Prochlorococcus* contribute a significant percentage of primary production; thus are important to the "biological pump" of carbon dioxide from the atmosphere to surface waters and ultimately to the ocean depths.
7. *Prochlorococcus* spp., with a genome size of approximately 2 Mbp, are the smallest recognized photosynthetic organisms. Until *Prochlorococcus* can be grown easily on agar plates, genetic studies will be difficult; however, tremendous progress has been made in just over a decade since their "discovery" by oceanographers. Most significant is their unique pigment complement that includes both chlorophyll b_2 and phycoerythrin. Phylogenetic analyses suggest that *Prochlorococcus* may serve as a model for the ancestral oxygenic photosynthetic bacteria, progenitor of cyanobacteria and chloroplasts.
8. Further development of PCR-based methods for rapid analysis of DNA sequences and probe development will provide a number of ways to address ecological, environmental, and evolutionary questions regarding this very important group of photosynthetic picoplankton.

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PRODUCTIVITY, BACTERIOPLANKTON.

See PLANKTONIC MICROORGANISMS: BACTERIOPLANKTON

PROTECTION OF SOURCE WATER.

See SOURCE WATER PROTECTION: MICROBIOLOGY OF SOURCE WATER

PROTISTAN COMMUNITIES IN GROUNDWATER

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Protozoa are ubiquitous, single-celled eukaryotic organisms that range in size from 2 μm to greater than 1 cm and are found in many environments (1). (Note: The terms protozoa and protozoan will be used in this review instead of protists and protistan to improve retrieval of the topic by readers.) Approximately 40,000 species of protozoa have been described, but these probably represent only a fraction of the species that exist. The seven phyla of Protista are classified by cell morphology and locomotion, but only two free-living, nonpathogenic phyla — the Ciliophora (ciliates) and Sarcomastigophora (flagellates and amoebae) — are associated with subsurface environments. There are particular problems with detecting flagellates and amoebae because they are so small, thereby making them hard to observe. In the case of amoebae, they are so slow moving that they are often hard to discriminate from sediment debris. Ciliates, the largest group of protozoa, are easier to find, but tend to be excluded from the subsurface because of their large size. The ecology of the free-living protozoa has been studied in marine and freshwaters, wastewater treatment systems, and soils. Protozoa in these environments are known to be phagotrophic, effectively preying upon bacteria and consuming particulate matter.

Until recently, very little was known about the incidence and ecology of protozoa in the subsurface (>30 cm below land surface). However, the existing data suggest that they may be important members of the subsurface food web. The purpose of this review is to discuss the protozoa that inhabit the subsurface, specifically what organisms are known to live there, how they move through the environment, and what roles they might play in pristine and contaminated subsurface environments. The review will only address free-living protozoa and will use current taxonomic nomenclature, though protozoan systematics and taxonomy are changing and evolving rapidly.

STUDIES OF PROTOZOA IN SOILS AND THE SUBSURFACE

Until the beginning of the twentieth century, microbiologists believed that protozoa were only found in marine and fresh waters. In 1909, Russell and Hutchinsen (2) discovered free-living protozoa in soil and postulated they were preying upon bacteria. However, the common belief at that time was that protozoa were primarily present in the soil as cysts (a resting state) capable of withstanding deleterious conditions. Cutler (3) and Sandon (4) described trophic protozoa as primarily being present in the upper 5 cm of soil. Since the 1920s, many papers (e.g., Refs. 5–8) have described soil protozoa and their ecology. Most of the early research suggested that protozoa were harmful to soil productivity by killing algae, fungi, and bacteria, and transporting "disease producers" to plants.

Studies of protozoa in subsurface environments have been much more limited. Munch and Petzold (9) reported finding them in groundwater from a sandy alluvial aquifer located between natural springs and the sea. They concluded that protozoan existence in the subsurface was possible to great depths, provided the groundwater flow was adequate and there was sufficient supply of oxygen and nutrients. Hirsh and Rades-Rohkohl (10) also reported finding viable protozoa in groundwater wells in significant numbers. One problem with the early studies was that protozoan abundance data were generated using groundwater collected from boreholes that were open to the surface and may not have been purged of standing water, thus being subjected to contamination with soil- and/or airborne species.

Since 1987, there has been an increasing body of literature on subsurface protozoa. Sinclair and coworkers (11–16) found protozoa in sediment cores collected from an array of pristine and contaminated sites. Their enumerations were performed using most probable number (MPN) culturing methods similar to those developed by Singh (17) for detecting amoebae in soil. Kinner and coworkers (18–20) have also reported finding protozoa in sediment cores from pristine and contaminated sites by using direct count methods. Travis and Rosenberg (21), in their model of in situ biodegradation of trichloroethylene (TCE) in an aquifer, concluded that protozoan predation provided the best explanation for the oscillations they observed in populations of methanotrophic bacteria. Zarda and coworkers (22) and Kota and coworkers (23) detected protozoa in aquifer sediments subjected to refinery and BTEX contamination, respectively. Hence, they have been detected in almost all cases where protozoa have been actively sought in subsurface sediments.

TYPES, ABUNDANCE, AND ENUMERATION METHODS FOR PROTOZOA

Most of the protozoa reported in the subsurface since 1987 have been heterotrophs, with the predominant type being small (nano) flagellates, along with some amoebae and very few ciliates. Taxonomic identifications are rare (18,24), often because the cells are small and difficult to isolate. In addition, few samples of protozoa from the subsurface are available to researchers; existing descriptions of species in the literature are often dated, and many of the species collected are ones that have not yet been described. Several genera of flagellates have been found in aquifers (18), including *Bodo*, *Cercomonas*, *Spumella*, *Goniomonas*, *Cryptaulax*, and *Cyathomonas*. Novarino and coworkers (25) categorized the flagellates they found on the basis of their swimming behavior — those that creep on solid surfaces (amoeboid), actively swim in pore waters (rarely attached), and swim and temporarily attach (mixed mode).

In most cases, amoebae have been reported without taxonomic identification because they are very difficult to detect and to describe. Novarino and coworkers (18) identified several naked amoebae in groundwater, including *Acanthamoeba*, *Hartmanella*, *Mayorella*, *Rosculus*,

Vahlkampifia, and *Vannella / Platyamoeba*. Very few ciliates have been found, most likely because their size (>10 μm) precludes their transport through aquifer sediments (18). Foissner (24) identified 38 species of ciliates in rapid sand filters that were being used to treat groundwater, including *Acineria*, *Aspidisca*, *Cinetochilum*, *Colpidium*, *Glaucoma*, and *Holosticha*. However, it is not clear if these ciliates were actually living in the aquifer or were a source of contamination in the well or filters.

A wide range of concentrations has been reported for protozoa in aquifers (Table 1). Because they may inhabit both pore water and grain surfaces, abundances may be more useful if expressed as numbers per cubic centimeter of aquifer, but to date most data have been expressed as the number of organisms per gram dry weight of aquifer material. Higher numbers are present at contaminated sites, probably because greater amounts of organic carbon result in more protozoan biomass production and growth. The bioavailability of the food source to the microenvironment inhabited by the protozoa probably also influences their abundance. However, the toxicity of some organic contaminants may inhibit them, as it does in other environments (27,28).

The concentration of protozoa observed in the subsurface may be affected by a number of factors, including sediment type, moisture content, organic content (including contaminant loading and type), ambient oxygen concentration, pH, Eh, and mineral content. Generally, protozoan abundance is lower in clay than in sand-based aquifers (Fig. 1), though populations in sandy sediment may sometimes be low. Whereas few samples have been collected from clay-laden aquifers, the lack of protozoa appears to be related to the lower hydraulic conductivity (a factor originally noted by Munch and Petzold (9)). Some clay-laden sediments also have low pH (<5), which may inhibit the protozoa (13). In addition, transport through clay-laden sediments may be difficult for cells that are typically greater than or equal to 2 μm.

Table 1. Protozoan Abundance Reported in Pristine and Contaminated Aquifers

Environment	#/gdw[a]	Reference/Site Location
Pristine aquifers	10^0 to 10^1	Sinclair and Ghiorse (11), Lula, Okla.; Beloin and coworkers (12); Lula, Okla.; Sinclair and coworkers (14); northeastern Kansas
Jet fuel plume	10^0 to 10^5	Sinclair and coworkers (15); Traverse City, Mich.
Coal tar site	10^1 to 10^4	Madsen and coworkers (16); New York
Abandoned hydrocarbon refinery	10^0 to 10^3	Zarda and coworkers (22); Hünxe, Germany
Sewage-contaminated aquifer	10^3 to 10^5	Kinner and coworkers (26); Cape Cod, Mass.

Note: () indicates location of site.
[a]gram dry weight.

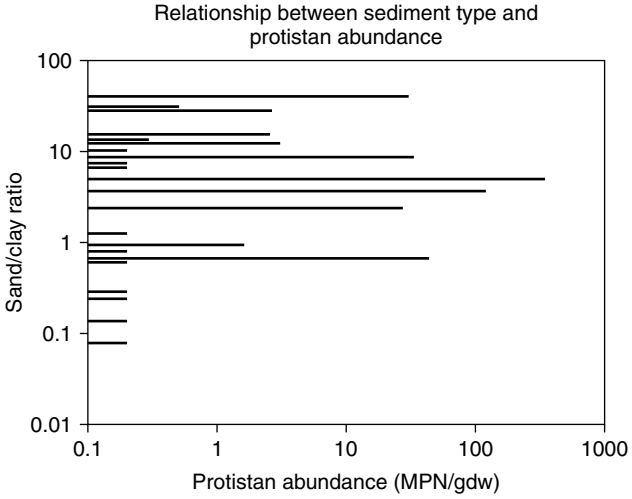

Figure 1. Relationship between sediment type and protozoan abundance in subsurface sediments from uncontaminated sites. Based on data from References 12 to 14.

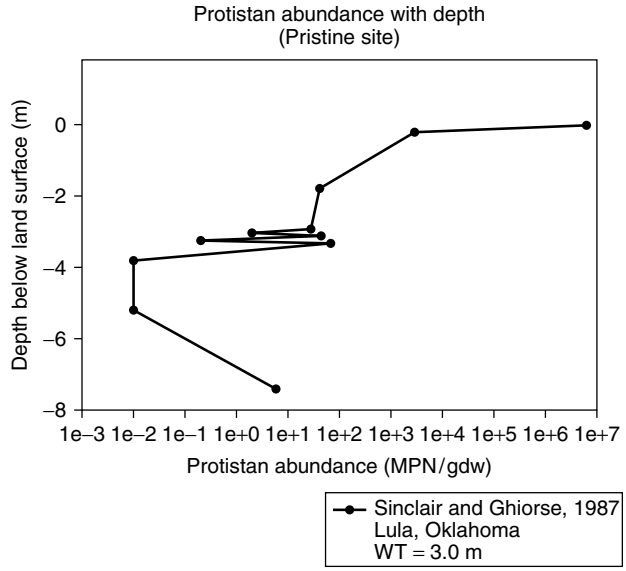

Figure 2. Protozoan abundance with depth in subsurface sediments from an uncontaminated site. The water table was located 3.0 m below the surface. Based on data from Reference 11.

Sinclair and Ghiorse (11) enumerated the protozoa from the surface to approximately 7.5-m below the surface in a pristine aquifer (Fig. 2) and observed the greatest numbers and diversity in the topsoil. Topsoils are organically rich, aerobic zones that provide favorable conditions for protozoan growth. The numbers decrease in the vadose (unsaturated) zone, most likely because the protozoa need moisture to live (29). In addition, there are fewer bacteria, a potential protozoan food source, in this zone. Many of the protozoa detected in the vadose zone are encysted, perhaps as a defense against desiccation. The only reported site where protozoan abundances were high in the vadose zone was in an area contaminated by jet fuel vapor (15), where bacteria were also abundant. In the saturated zone, protozoan abundance often increases above that detected

in the vadose zone or, occasionally, remains the same. Increases most often occur when bacterial abundance or organic content increases. Protozoan abundance within a given aquifer is usually lowest in pristine zones where organic content is generally minimal and the organic carbon available is often refractory (16,30). Kinner and coworkers (30) have shown that in a sandy aquifer, both the unattached bacterial and total protozoan abundances are related to the dissolved organic carbon (DOC) concentration. In the same aquifer, protozoan abundance decreases exponentially with the refractory nature of the DOC. The increase in protozoan abundance observed when DOC is elevated may result from a redistribution in bacteria from the attached to the unattached populations (31) because most protozoa graze on the unattached bacteria more readily than on those residing in a biofilm. There is little information on the toxicity of organic compounds, such as TCE and various petroleum hydrocarbons found at most contaminated sites, to subsurface protozoa.

Whereas protozoa were originally thought to inhabit only aerobic aquifers (32), more recent data suggest they can exist in suboxic and anoxic subsurface environments (22,30). Abundances of protozoa are generally higher in aerobic portions of aquifers (e.g., in contaminated aquifers where aerobic conditions are created to stimulate bioremediation (Fig. 3)). However, it is unclear whether greater protozoan abundance is related to increased bacterial activity and growth or the presence of oxygen.

There is little data on the effects of pH, Eh, and mineral content on protozoan abundance. Lower protozoan abundance has been documented at a pristine site with a pH less than 5 where clays were predominant (13). At that site, it was postulated that the lower pH could have caused a mobilization of metals, which inhibited the protozoa. Alternatively, the lower bacterial abundances at the site could have resulted in lower protozoan numbers. The pH in sandy aquifer sediments used to culture flagellates is known to affect their size (conditions of pH 6.0 and 7.0 resulted in 2 to 3 μm and 3 to 5 μm cells, respectively) (33).

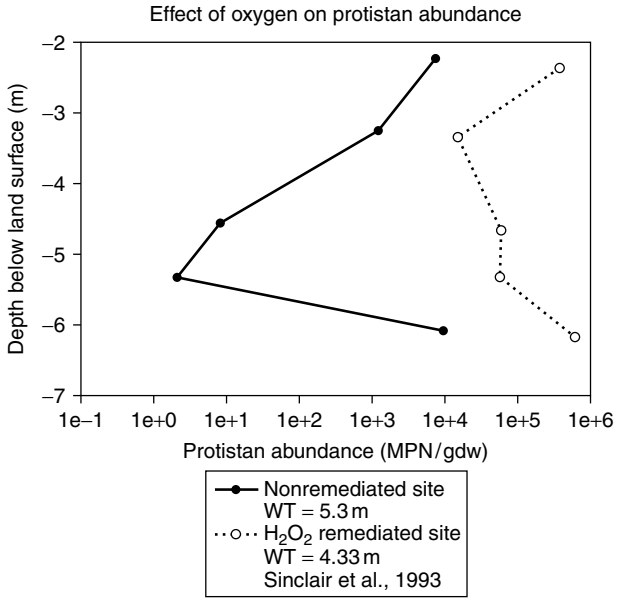

Figure 3. Protozoan abundance with depth in H_2O_2 remediated and unremediated subsurface sediments contaminated with jet fuel. The water tables were located at 4.3 and 5.3 m, respectively, for the two sites. Based on data from Reference 15.

Most of the recent studies reporting protozoan abundances (11–15,22,23) have used the MPN method of enumeration, originally developed by Singh (17) for amoebae in soil. In this method, a food source (i.e., bacteria) is provided to the protozoa in a series of dilutions of the sample. It is assumed that the protozoa consume the food source, grow, and reproduce during the incubation. Subsamples of the replicates at each dilution are usually examined microscopically for the presence of protozoa. An MPN estimate of the original population is made based on the amount of dilution necessary to eliminate the protozoa from the sample. Ronn and coworkers (34) optimized the MPN procedure for flagellates and amoebae in soil by comparing results obtained using different growth media and concentrations against *Enterobacter* as a standard. The MPN method is useful because it yields live material, so the types of protozoa present and their motility can be observed. As with all culturing-based enumeration methods, selectivity of the growth medium can affect the results. This problem is compounded with subsurface protozoa because their food source(s) is not usually known. Often *Enterobacter* is used as a model bacterial food source. In other cases, only the bacteria from the groundwater are used and diluted out simultaneously with the protozoa. Direct observation of the protozoa in the live samples from the MPN can be difficult because many of the cells are very small (2 to 3 μm) and hard to differentiate from bacteria and debris when using light microscopy. Overall, the MPN method can yield useful data, but it may underestimate protozoan abundance in situ.

Direct counts have been done on subsurface protozoa using methods developed for 4′,6-diamidino-2-phenylindole (DAPI) (19,20,30) and primulin stains (35,36). DAPI is problematic because it reacts with both prokaryotic and eukaryotic nucleic acids, so bacteria and protozoa are both stained. This means that the protozoa must be distinguished based on their morphology alone. Motility, which is often important in differentiating protozoa, is absent because the cells are fixed prior to DAPI staining. Primulin differentially stains eukaryotes and is, therefore, a preferred alternative to DAPI. However, primulin is also used on fixed material, so motility is absent. In addition, direct count methods are very tedious because the stain is often difficult to see in samples extracted from subsurface sediment where debris is present and there is background fluorescence.

Caron and coworkers (37) noted that differences between MPN- and direct count–derived abundances are a function of the trophic mode of the protozoa, their growth stage, and their ability to be cultured in the laboratory. Little is known about the trophic mode or growth stages of in situ protozoa in most subsurface environments. Novarino and coworkers (25) has noted that it is currently difficult to culture many subsurface protozoa because their food source(s) is not known.

Extraction procedures are used to remove the protozoa from sediment samples prior to direct counting and oftentimes before MPN dilutions. These protocols can consist of simply shaking the sediment in a buffer solution (35) or creating more complex density gradient separations that have 70 to 75% efficiency for extracting flagellates (25). Flagellates are easier to extract than amoebae because they are less-strongly surface associated. Hence, any enumeration method preceded by an extraction underestimates the abundances of amoebae.

Recently, gene probes have been used to enumerate the protozoa in the subsurface (22,25). Zarda and coworkers (22) have compared protistan abundances, at an abandoned oil refinery site, derived from an MPN method and a eukaryotic probe EUK516 (using an in situ hybridization technique). EUK516 yielded abundance estimates two to five orders of magnitude greater than the MPNs (Fig. 4),

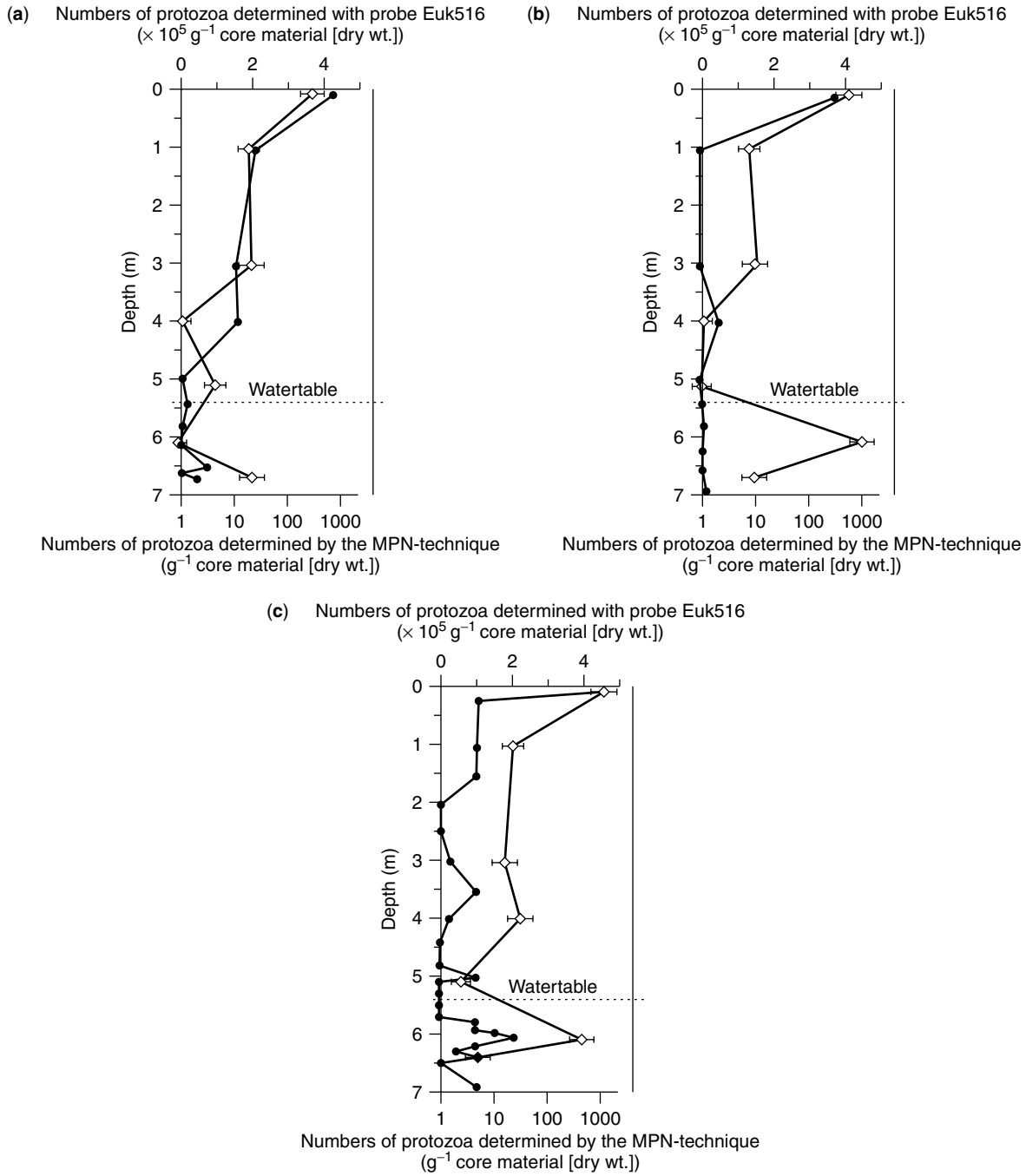

Figure 4. Total number of protozoa as determined by in situ hybridization with the Eukarya-specific probe EUK 516 (•) and by the MPN technique (◇) in cores A, B, and C from an aquifer located at an abandoned refinery for depths between 0.1 and 0.7 m. Reprinted from Reference 22.

but the trends in population density with depth were similar. Use of probes for enumeration of protozoa in the subsurface is very promising. Once issues concerning the lack of information on the protistan taxa present in the subsurface and contamination and interference issues are addressed, probes will probably be used widely to estimate in situ abundance.

Protozoa can exist in situ in a trophic (vegetative) form or an encysted (resting) stage where no reproduction or feeding occurs (1). Research on protozoa indicates that they are able to produce temporary cysts (38) to withstand unfavorable environmental conditions (e.g., presence of toxic chemicals and extremes of pH, temperature, or desiccation). The process and causes of encystment and excystment are not completely understood. Singh (17) developed an acid treatment, as part of his MPN enumeration procedure for soil amoebae, to differentiate between the numbers of trophic versus encysted cells present. Pretreatment with acid kills the trophic cells, whereas cysts survive. Subsequent neutralization of pH and MPN culturing causes the encysted protozoa to excyst, so that the abundance of encysted protozoa can be estimated. The effectiveness of the acid treatment as a method of calculating encystment is uncertain. Undoubtedly, direct observation of the samples with scanning electron microscopy (SEM) is the best method to discern the abundance of encysted cells, but this is impractical for most samples. Better methods that are more reliable than acidification and less tedious and expensive than SEM should be developed for characterizing the extent of encystment, which must be known for assessing the importance of protozoan activity in situ.

PROTOZOAN TRANSPORT IN THE SUBSURFACE

Harvey and his associates have conducted research on protozoan transport using laboratory columns of porous media and in situ injection and recovery experiments (39–42). Most of this work was conducted using the flagellate *Spumella guttula* cultured from sediment samples of an organically contaminated sandy aquifer located in Cape Cod, Massachusetts. *Spumella* can swim actively and also has the ability to attach temporarily to grain surfaces. Depending on the pH and organic content of the culturing medium used, its cells are 4 to 5 μm or 2 to 3 μm in diameter (33). Dimensionless concentration histories (P/P_0) of DAPI or hydroethidine-stained *Spumella* injected into the aquifer were compared to bromide recoveries downgradient, which were typically 80 to 100%. Both 2-μm, carboxylated, fluorescent microspheres and 2- to 3-μm, stained flagellates behaved similarly, the latter yielding a maximum abundance of 25% 1 m downgradient (Fig. 5), whereas 4- to 5-μm flagellates transported much more slowly (Fig. 6) with a maximum recovery of approximately 2%. All recoveries decreased with distance downgradient. Hydraulic perturbation caused remobilization of the 2- to 3-μm protozoa (Fig. 5).

The laboratory and field data indicate that cell size is critical in determining the extent of protozoan transport in the saturated subsurface. The Cape Cod

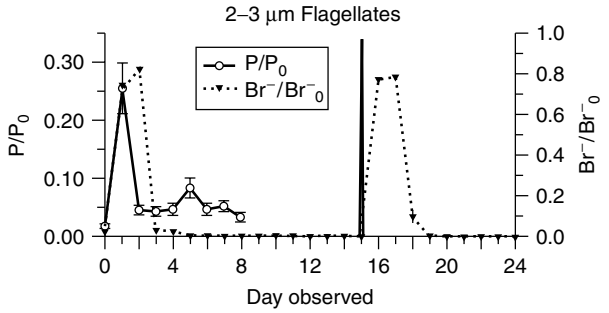

Figure 5. Dimensionless concentration histories of 2- to 3-μm diameter protozoa (P/P_0) and a bromide tracer (Br/Br_0) in a contaminated sandy aquifer. Data shown from a depth of 11 m below surface and 1.0 m downgradient from the injection point. No error bars are shown for $Br/Br_0 < 2\%$ RSD. Vertical line indicates time of hydraulic perturbation. Reprinted from Reference 33.

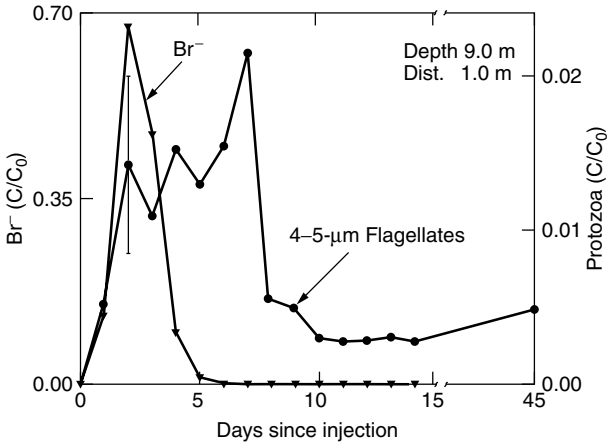

Figure 6. Dimensionless concentration histories (P/P_0) for 4- to 5-μm diameter flagellates and bromide in contaminated sandy aquifer sediments located 9.3 m below surface and 1.0 m downgradient from the injection point. The error bar at the first flagellate peak represents 1 standard deviation for replicate counts. Reprinted from Reference 48.

aquifer studies with microspheres (40) and the colloid-filtration theory (32) suggest that the 2- to 3-μm flagellates that are found in situ are approximately the optimum size for transport. Being highly mobile may be an effective strategy for a predator, allowing it to be rapidly advected downgradient when environmental conditions change (41). In addition, the low buoyant densities of the flagellates (1.02 to 1.03 g/cm^3) (42) further facilitate transport and are comparable to those of their potential food source, the unattached bacteria (41).

The effects of potential alteration of cell surface characteristics by staining and chemical fixation during laboratory and field studies need to be determined because these may change cell surface interactions. It is also likely that chemical, as well as hydraulic perturbations, in aquifers can affect protozoan transport because the cells are reversibly attached to grain surfaces (Fig. 5). Protozoan transport in the subsurface has ramifications for public health (e.g., movement of the pathogens such as *Cryptosporidium* and *Giardia*) and bioremediation

strategies. Further research is needed for understanding these effects.

PROTOZOAN ROLE IN THE SUBSURFACE

Most studies (Table 1) report higher protozoan abundance in contaminated aquifers where biodegradation is occurring than at pristine sites. An examination of the microbial ecology literature for aquatic and soil environments reveals several possible explanations for this relationship, including protozoan predation on bacteria and/or phage, protozoan an consumption of high molecular weight (MW) organics, and protozoan recycling of limiting nutrients (e.g., nitrogen, phosphorus).

Protozoan predation upon bacteria is well documented for marine and freshwaters, soil, and waste-treatment environments (43–47). Recent studies in an organically contaminated aquifer in Cape Cod, Massachusetts, have quantified flagellate predation on unattached bacteria (20,30,48). The first evidence of potential predation (20) was obtained from flow-through column studies where aquifer sediments containing bacteria and protozoa were injected with DAPI-stained bacteria. The effluent concentration of the DAPI-stained bacteria was monitored over time. Each column was then autoclaved to kill all microorganisms and the injection study was repeated. Bromide tracers were also injected into each column to ensure that they were hydraulically similar before and after sterilization. In the sterilized columns, approximately 80% of the influent DAPI-stained bacteria were detected in the effluent (C/C_0). When protozoa (primarily flagellates) were present, breakthrough was approximately 30% in one column and less than 5% in the other. The data suggested that the flagellates in the columns were preying on the DAPI-stained bacteria. Bacterial grazing and clearance rates (volume of water cleared of bacteria per protozoan cell per unit time) were calculated (Table 2). The difference in breakthrough for the two columns where protozoa were present was a function of the size of the microbes. Greater percentages of larger flagellates (3–5 μm) resulted in higher grazing and clearance rates. Because of the way these studies were designed, they did not provide direct evidence that the bacteria were ingested and degraded by the protozoa. In addition, the DAPI stain may have affected protozoan predation on the bacteria. It is likely the clearance rates were overestimated because the calculation ignored those protozoa residing in the sediments that were not being eluted.

In a second study (48), unattached bacteria from the aquifer were separated into four size classes and stained with DTAF. These bacteria were placed in flasks with 2- to 3-μm aquifer flagellates in time series grazing experiments, and the stained bacteria within the protozoa observed (Fig. 7). The protozoa preferentially grazed upon the 0.8- to 1.5-μm bacteria, the size class that exhibits the greatest productivity in the aquifer's organic contaminant plume. Clearance rates were similar to those in other environments (Table 3). The data suggested that the aquifer flagellates can consume, daily, as much as 12 to 74% of the unattached bacterial population.

These two studies showed that protozoa selectively prey on unattached bacteria and have the greatest impact on the most productive bacteria. Whereas attached bacteria constitute the greatest number and the most active bacteria in pristine aquifers, Murphy and coworkers (31) have shown that unattached bacteria play a greater role when organic contamination of an aquifer occurs, suggesting that protozoan predation on them could affect biodegradation rates. There are still no measurements of protozoan predation rates in situ and little is known about their effect on attached bacteria. Some of the attached bacteria are loosely attached to grain surfaces in the secondary minimum layer and these may be readily available to grazing protozoa. Novarino and coworkers (25) have suggested that the diverse group of protozoa present in aquifers feed in different ways and exhibit niche differentiation, perhaps based on their form of motility (creeping, swimming, temporarily attached).

Gonzalez and coworkers (54) reported that marine nanoflagellates can consume phages. There is data to suggest that phages are present in the subsurface (55), but the extent of protozoan grazing on them is unknown and needs to be explored.

Laboratory studies (56,57) with aquatic flagellates have shown that they can consume high MW organics (>40 K); however, this activity has not been documented in situ. In some aquifers, the organics have an MW of less than 30 K (30), suggesting that this potential protozoan food source is not abundant. It is possible that, under certain geochemical conditions, dissolved organic matter could be converted to colloidal (higher MW) material, but again this effect may be limited in most subsurface environments. Some evidence in aquatic environments indicates that protozoa may egest particles and reingest them (58,59), but the extent of this process in situ is also unknown.

Many studies (e.g., Refs. 60–62) have documented that protozoa in soil and aquatic environments recycle

Table 2. Grazing and Clearance Rates for Flagellates in Column Experiments with Sandy Aquifer Sediments (20)

Column	Grazing Rate (bact/protist•h)	Clearance Rate (nL/protist•h)	Bacterial Size (μm)	Flagellate Size (μm)
I	3	12	1.0 to 2.0	96% 2 to 3 μm 4% 3 to 5 μm
II	27	23	1.5 to 3.0	77% 2 to 3 μm 23% 3 to 5 μm

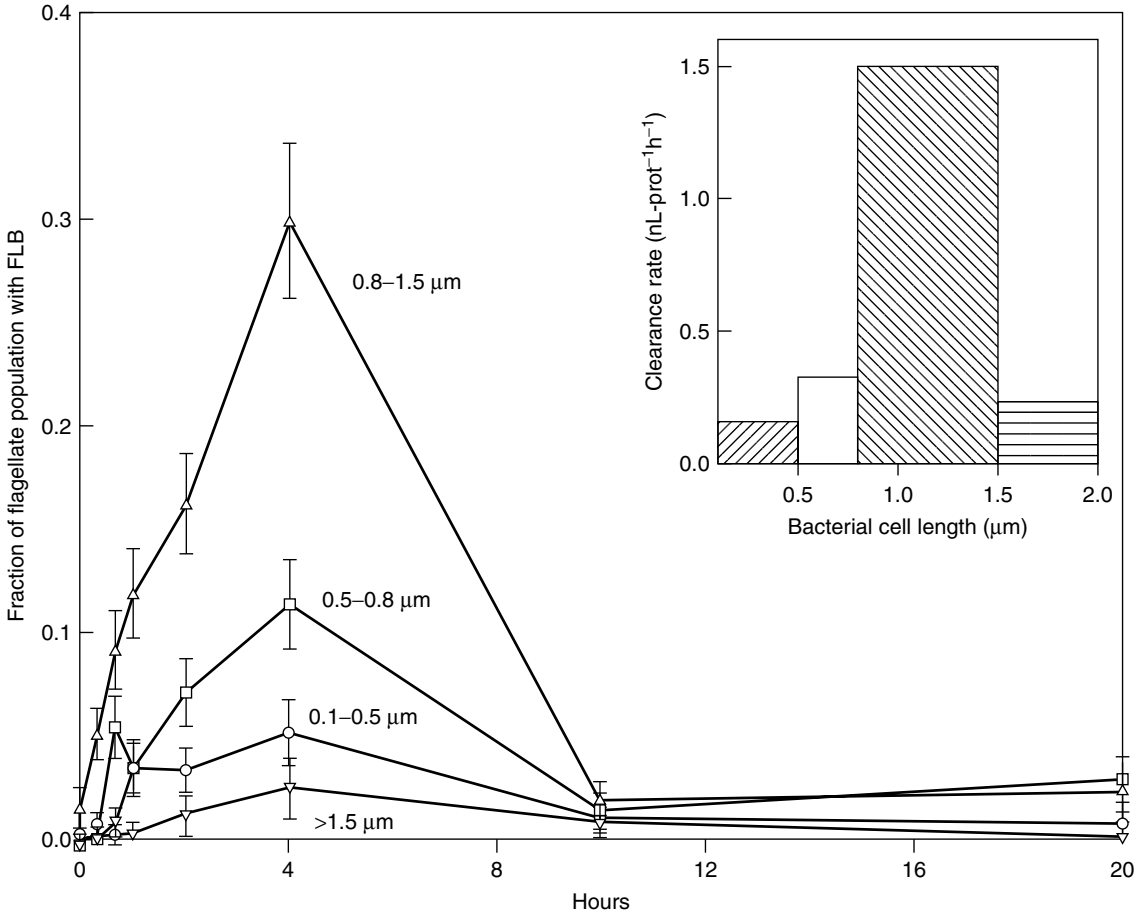

Figure 7. Uptake of fluorescently labeled bacteria by 2- to 3-μm diameter flagellates from a sandy aquifer during laboratory grazing experiments. Error bars represent standard errors of the mean and total experimental variability. The inset shows clearance rates for each size class of bacteria based on uptake rates observed during the first 4 hours of the grazing experiments. Reprinted from Reference 47.

Table 3. Grazing and Clearance Rates of Aquifer Flagellates for the Different Size Classes of Unattached DTAF-Stained Bacteria (48)

Environment (References)	FLB Cell Length (μm)	Bacterial Cell Volume (μm^3)[a]	Uptake Rate[b] (bacteria/protist•h)	Clearance Rate (nL/protist•h)
Contaminated aquifer (48)	0.1 to 0.5	0.06 ± 0.01	0.06	0.1
	0.5 to 0.8	0.14 ± 0.02	0.13	0.33
	0.8 to 1.5	0.21 ± 0.02	0.77	1.4
	>1.5	0.87 ± 0.05	0.04	0.2
Contaminated aquifer (columns) (20)	1 to 2		3	12
Rivers (43,49)[c]			1.1 to 90.4	0.2 to 8.9
Lakes (50,51,44)[c]			2 to 181	0.2 to 44
Marine (49,46)[c]			5.2 to 27.4	1.4 to 4.3
Estuarine (52,45,53)[c]				0.32 to 3.2

[a] Values are means ± standard errors.
[b] Calculated by using the clearance rate of bacteria and the total bacterial concentration of each size class present during grazing experiments.
[c] The values from these references represent a range of experiments conducted by several researchers at various temperatures with various sizes of fluorescently labeled bacteria and flagellates.

the nutrients that limit bacterial growth. For example, Goldman and coworkers (63) and Zwart and Darbyshire (64) reported that the ammonia excreted by protozoa regenerates the supply of nitrogen for use by bacteria. In pristine aquifers or those contaminated aquifers where bacteria are nutrient-limited, this could be the major role of protozoa and may directly stimulate bacterial biodegradation of organics. It is also possible that protozoa may excrete other substances, such as vitamins or amino acids, needed by bacteria (65).

IMPACT OF PROTOZOA ON CONTAMINANT BIODEGRADATION IN AQUIFERS

Data from most natural environments clearly show that when protozoa are present they have an impact on contaminant biodegradation rates as a result of their predation upon bacteria (38). It is likely that this is their function in the subsurface. Protozoan predation could either reduce bacterial bioclogging in aquifers or decrease or enhance bacterial degradation of organic contaminants in situ.

Sinclair and coworkers (15) and Zarda and coworkers (22) hypothesized that, by grazing upon bacteria, protozoa prevent bioclogging of aquifers and maintain hydraulic conductivity. This is especially important where organics and nutrients are present in substantial amounts, stimulating excessive bacterial growth. DeLeo and Baveye (66) conducted column studies with soil colonized by *Bacillus* and *Acanthamoeba*. The short-term effect (over the first days) of protozoan predation was a reduction in bacterial abundance and a decrease in clogging. However, over the longer term, columns with and without *Acanthamoeba* exhibited a similar degree of clogging. DeLeo and Baveye speculated that in the short term, either the bacterial aggregates were too large for protozoan predation, *Bacillus* inhabited crevices that were inaccessible to *Acanthemoeba*, or the bacterial migration and colonization along the columns was greater than the rate of amoeboid movement through the sediments. Most aquifers, whether pristine or contaminated, are carbon- or nutrient-limited (i.e., contaminants are only present at the ppb or low ppm level). Hence, bacterial abundances and growth rates are usually low, and bioclogging is not a problem except around injection wells. In these aquifers, the impact of protozoan predation is probably not prevention of bioclogging and maintenance of hydraulic conductivity.

In their model of the Savannah River aquifer, Travis and Rosenberg (21) found that protozoan predation on methanotrophic bacteria decreased the TCE degradation rate by 25%. These results suggest that protozoa may negatively impact biodegradation, which would be especially problematic at sites undergoing natural or enhanced bioremediation. One problem with such ecosystem modeling approaches may be that the bacterial kinetic constants used in the models are the same whether predators are present or absent. However, various studies (30,67,68) indicate that the constants change. For example, the K_s (half-saturation constant) of bacteria degrading organics can increase when they are being preyed upon by protozoa (30). Data from short-term, aerobic incubation of subsurface bacteria has shown that biodegradation rates of benzene, toluene, ethylbenzene, and the xylenes are higher when protozoa are absent (23). However, this negative impact could be a short-term effect, as shown by Wiggins and Alexander (69) for 2,4-dichlorophenoxyacetic acid (2,4-D) and PNP (Fig. 8) where protozoa lengthen the period of bacterial acclimation to the contaminants, but increase the overall extent of mineralization. Mattison and Harayama (70) observed an increase of as much as 7.5 times the rate of toluene degradation by bacteria when a flagellate was present.

Laboratory studies of the effect of protozoa on bacterial degradation rates are very difficult to conduct because it is hard to eliminate them from the ecosystem in controls. Organic inhibitors are often used to eliminate the protozoa (e.g., nystatin, cycloheximide) (69,71), but they may not be effective (46,72,73) or the inhibitors may become carbon sources for bacteria. In addition, sampling in these types of experiments must be frequent because the data must account for the effects of short-term cycling in the populations of the predator and prey, unless "steady state" has been established.

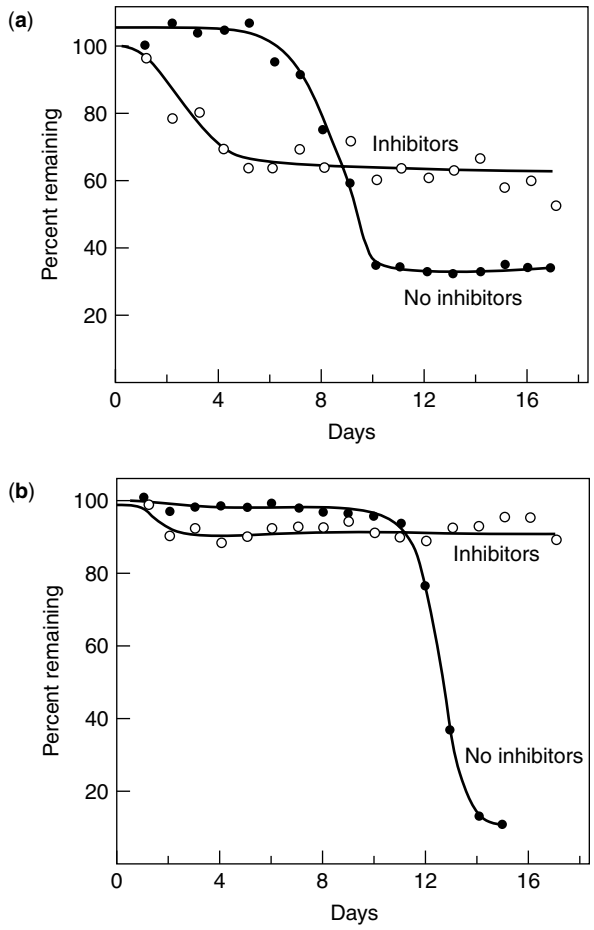

Figure 8. Effect of protozoa on the mineralization of 100 ng of 2,4-dichlorophenol (top) and 2,4-D (bottom) per milliliter of sewage. Cycloheximide and nystatin were used as inhibitors. Reprinted from Reference 69.

In many studies, starting with those by Cutler and coworkers (74) in soils, the observation that bacterial abundance is lower when protozoa are present, has led to the conclusion that bacteria, as a result, degrade less organic matter. The question that must be asked is whether the reduction in bacterial numbers caused by protozoan predation translates into less biodegradation of the organic contaminants by bacteria. This question relates directly to predator-prey dynamics.

Hunt and coworkers (67) and Kuikman and coworkers (68) have shown that protozoan predation decreases bacterial biomass. However, this results in more carbon, nitrogen, and phosphorus being available per bacterium. Both studies showed that increased carbon, nitrogen, and phosphorus stimulated the uptake and growth rates of the remaining bacteria and may have allowed the coexistence of bacterial species (75). Alexander (76) noted that the predator is "prudent" by not eliminating all prey. Rather than being an anthropogenic phenomenon, the prudent predator is simply exhibiting density-dependent feeding (i.e., the presence of fewer bacteria results in greater difficulty for the predator in locating the prey). As Sinclair and Alexander (71) have shown, slow-growing bacteria may be eliminated, whereas faster growing populations will not be. If the slow-growing bacteria in the subsurface are the only contaminant degraders or perform a critical step in the overall degradation process, then in situ degradation could be negatively impacted by protozoan predation. In addition, as Novarino and coworkers (25) have hypothesized, it is likely that because of niche differentiation (e.g., pore water swimmers versus surface-associated feeders), protozoa may not be able to consume all bacteria in a subsurface environment. Prey can also exhibit escape strategies, such as taking refuge in pores where protozoa cannot enter (77) or changing morphology to prevent ingestion (78).

There is preliminary laboratory evidence that unattached bacteria from a pristine sandy aquifer exhibit higher apparent growth rates and greater frequency of dividing cells when protozoa are present (Fig. 9) (30). Whereas this data suggests that protozoan predation may result in increased bacterial degradation of organic contaminants, experiments need to be conducted with more representative organic carbon sources (i.e., not just acetate) and in the field. It is also essential to have better estimates of the true bacterial growth rate (without protozoa present) to calculate more reliable bacteria kinetic constants and predation rates.

CONCLUSION

The last 15 years of the twentieth century saw a dramatic change in our fundamental knowledge of protozoa in the subsurface: from a realization that they are present in the environment to the understanding that they most likely impact in situ degradation of organic contaminants in some way. It has been shown that protozoan predation upon bacteria is occurring in the subsurface, as it does in other environments, and that it affects bacterial

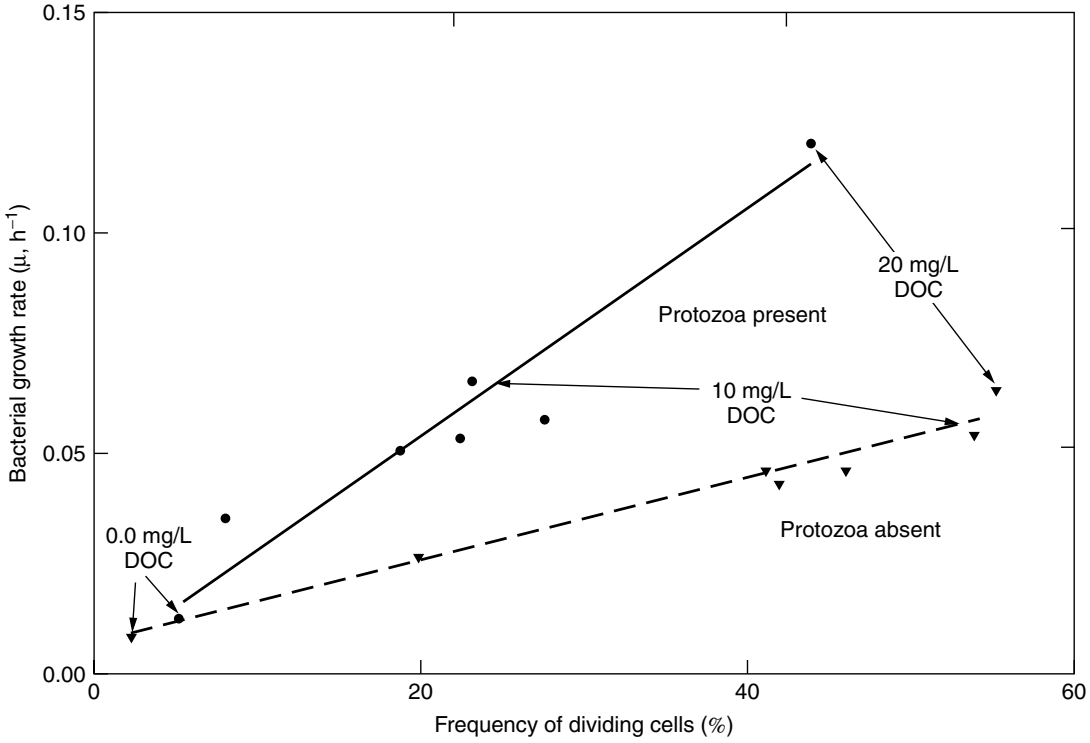

Figure 9. Measured (apparent) growth rates and the frequency of dividing cells for groundwater bacteria collected from an uncontaminated sandy aquifer with background dissolved organic carbon (DOC) of 0.9 mg/L at different concentrations of amended acetate (0 to 20 mg C/L) in 2-day closed bottle laboratory experiments. Source is Reference 30.

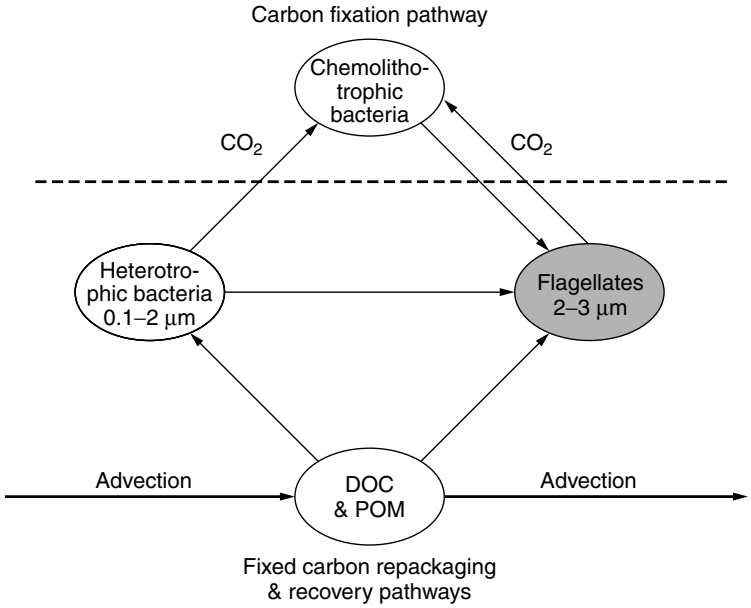

Figure 10. Carbon flow diagram for a microbial community inhabiting an organically contaminated sandy aquifer from Reference 30. On the basis of the classical carbon flow diagram for a microbial community in surface waters shown in Reference 81.

abundance. The question as to whether the predation aids or retards bacterial degradation of the organics remains unanswered and will be difficult to determine, primarily because contaminated aquifers are dynamic ecosystems. Fluctuations in bacterial and protozoan populations may be rapid (on the order of hours to days). This will necessitate laboratory experiments and field studies incorporating frequent sampling instead of using just a few "snapshots" of data to observe and determine the effects of predator-prey dynamics. For example, predator:prey ratios based on abundance or biomass have been used to determine possible relationships in aquifers, and these ratios vary widely in aquifers from $1 : 10^2$ to 10^6 (30) in both pristine and contaminated sites from the theoretical $1 : 10^3$ ratio (38). However, as Gasol and Vaqué (79) noted, such ratios may be misleading because they depend upon whether the predator:prey dynamics are at "steady state" and whether the correct prey (e.g., size class of bacteria, attached or unattached population) and predator (those that creep, swim, or exhibit a mixed mode of motility (25)) are used in the ratio.

The challenge ahead is to enumerate and characterize (e.g., niche differentiation, trophic versus encysted state) protozoa more carefully and to define their specific role(s) in pristine and contaminated subsurface environments (e.g., what are the protozoan food sources?, what is their impact on bacterial productivity and contaminant biodegradation?). In doing so, aquifer parameters and environmental geochemistry must be considered, as these may affect the ability of protozoa to survive and prey upon bacteria. It is possible that one explanation for reduced rates of contaminant biodegradation in some subsurface environments may be the result of inhibition or elimination of protozoa as a result of toxicity effects. Protozoa may be more susceptible to such inhibitions because they have a much less complex cell wall structure that separates them from the environment than do bacteria.

Madsen (80) concluded that the irony of studies of environmental microbiology is that laboratory experiments are simpler than fieldwork, but typically have less relevance. Hence, understanding the role and impact of protozoa in subsurface ecosystems will be difficult. Studies of predator-prey dynamics in other environments can provide direction and suggest approaches for laboratory and field research designed to study the ecology of subsurface protozoa. In addition, the food webs in the subsurface may be simpler to understand than those in most other natural environments because there are fewer trophic levels (Fig. 10): perhaps only one level of predator (protozoa) and prey (bacteria).

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PROTOZOA (FREE-LIVING) IN DRINKING WATER DISTRIBUTION SYSTEMS.

See Invertebrates and Protozoa (Free-Living) in Drinking Water Distribution Systems

PROTOZOA IN ACTIVATED SLUDGE

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The aim of the activated sludge process is to achieve a maximal reduction of the biological oxygen demand (BOD) of wastewater with a minimal production of biological solids. In this process, protozoa are important members of the communities involved in the removal of organic matter and dispersed bacteria. In fact, biological wastewater treatment processes rely on the natural self-purification capacity of aquatic environments, which is the result of the activity of microbial communities. Nevertheless, the activated sludge process differs from aquatic environments in the following characteristics:

- strong flow of organic matter into the system,
- accelerated decomposition processes,
- prevalence of heterotrophic organisms and then of the detritus food web,
- short biomass turnover time.

These characteristics deeply influence the organisms able to live in these artificial systems, so that among heterotrophic organisms only protozoa and small metazoa with life cycles shorter than the sludge retention time are able to compete in activated sludge process. It has long been known that protozoa are plentiful in activated sludge plants (1). It is common to find populations of protozoa on the order of more than 50,000 cells per mL in the mixed liquor, and this constitutes approximatively 9% of the dry weight of suspended solids in mixed liquor (2) (Fig. 1). The protozoa and small metazoa are able to feed on particulates, such as those coming in with the sewage or bacterial flocs. It is generally assumed that their primary role in the wastewater treatment is the clarification of the effluent (3,4).

PROTOZOAN OCCURRENCE AND DIVERSITY

Free-living Protozoa

Since the installation of the first activated sludge treatment facilities in 1922, many authors have noted the presence of free-living protozoa. Besides bacteria, the protozoan community is the most abundant and the species rich group within activated sludge. A total of more than 230 species of protozoa are encountered in activated sludge plants (5–7), and they are distributed among the following five classes or phyla: Phytomastigophora, Zoomastigophora, Rhizopoda, Actinopoda, and Ciliophora. Nevertheless, only a third of these species are regularly found in the mixed liquor, whereas the other forms drift into the aeration tank with the inflowing wastewater, and cannot establish themselves permanently within the protozoan community.

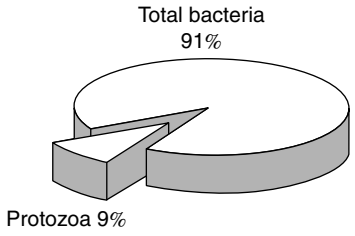

Figure 1. Proportion of protozoa biomass and sludge content in the activated sludge treatment process.

Flagellates. It is quite clear that although these protozoa from the two flagellate classes Phytomastigophora and Zoomastigophora are found in activated sludge plants, generally, they are relatively rarely seen because only 33 species have been identified. Small flagellates belonging to the class Zoomastigophora (*Bodo*, *Cercomonas*, *Trepomonas*) enter the plant in the influent wastewater, and their growth in the aeration tank is usually associated with poor operational parameters (poorly aerated sludge, overloaded plants, fermenting substances involved) (8,9). Large flagellates (Phytomastigophora) appear to occur less frequently in activated sludge and in smaller numbers than small flagellates, and their presence generally is associated with underloaded plants (10,11). Two genera are mainly found: *Euglena* and *Peranema*.

Amebae. A list of naked amebae observed in activated sludge plants is given in (6,12,13). Amebae occur in the raw, settled sewage and at the head of the aeration tank, but the greatest species diversity is found in the aeration tank, whereas the lowest number of species has been found in sedimentation tanks. The most common species observed in activated sludge plants belong to the genera *Vahlkampfia* (*V. avara*, *V. limax*), *Mayorella, Naegleria*, and *Hartmannella*. Among testate amebae, only few species (*Arcella*, *Euglypha*, *Centropyxis*, and *Pyxidicula*) are able to colonize activated sludge systems because of their high generation times that prevent them from growing in conventional plants with short sludge retention times. These protozoa develop high population densities mainly in extended aeration plants operating with high sludge age, high DO values and low sludge load that favor full nitrification (14,15,16). Among protozoa belonging to the phylum Actinopoda, only six species have been identified. These protozoa are infrequent in the activated sludge and their presence is associated with underloaded plants (15,17).

Ciliates. Of the 230 to 250 protozoan species that have been found so far in activated sludge plants, about 160 belong to the phylum Ciliophora, but less than half of them have been observed frequently (6,18,19). This number evidently is only a small part of the thousands of species of freshwater protista that could theoretically be observed in these environments. Table 1 shows the most important ciliates found in activated sludge. Ciliated protozoa are the most diverse group in terms of numbers of species in the protozoan community. Activated sludge mixed liquor of a plant treating domestic sewage normally contains up to 10 species of ciliates and numbers of individuals ranging from 10^6 to 10^7 L^{-1} (6,17). Although

Table 1. The Most Common Ciliated Protozoa Found in Activated Sludge Plants

Carnivorous or Omnivorous	Bacterivorous		
	Free-Swimming	Crawling	Attached
Acineria incurvata	*Colpidium colpoda*	*Acineria uncinata*	*Carchesium* spp.
Amphileptus spp.	*Cinetochilum margaritaceum*	*Aspidisca* spp.	*Epistylis* spp.
Coleps hirtus	*Cyclidium* spp.	*Chilodonella uncinata*	*Opercularia* spp.
Litonotus spp.	*Glaucoma scintillans*	*Drepanomonas revoluta*	*Pseudocarchesium* spp.
Suctoria:	*Paramecium* spp.	*Euplotes* spp.	*Vaginicola ingenita*
Acineta spp.	*Pseudocohnilembus pusillus*	*Stylonychia* spp.	*Vorticella* spp.
Podophrya spp.	*Spirostomum teres*	*Trithigmostoma* spp.	*Zoothamnium* spp.
Tokophrya spp.	*Tetrahymena* spp.	*Trochilia minuta*	

the rhizopods or the flagellates can dominate the protozoan community of activated sludge, this is infrequent and usually the ciliates hold the dominant position. Numerous species of carnivorous ciliates were found in activated sludge plants, nevertheless, the majority of ciliates present in the activated sludge are filter feeding on dispersed populations of bacteria. They are subdivided into three functional groups on the basis of their behavior (Fig. 2):

Free-swimming, those which swim in the bulk liquid phase and remain evenly in suspension in the sedimentation tank;

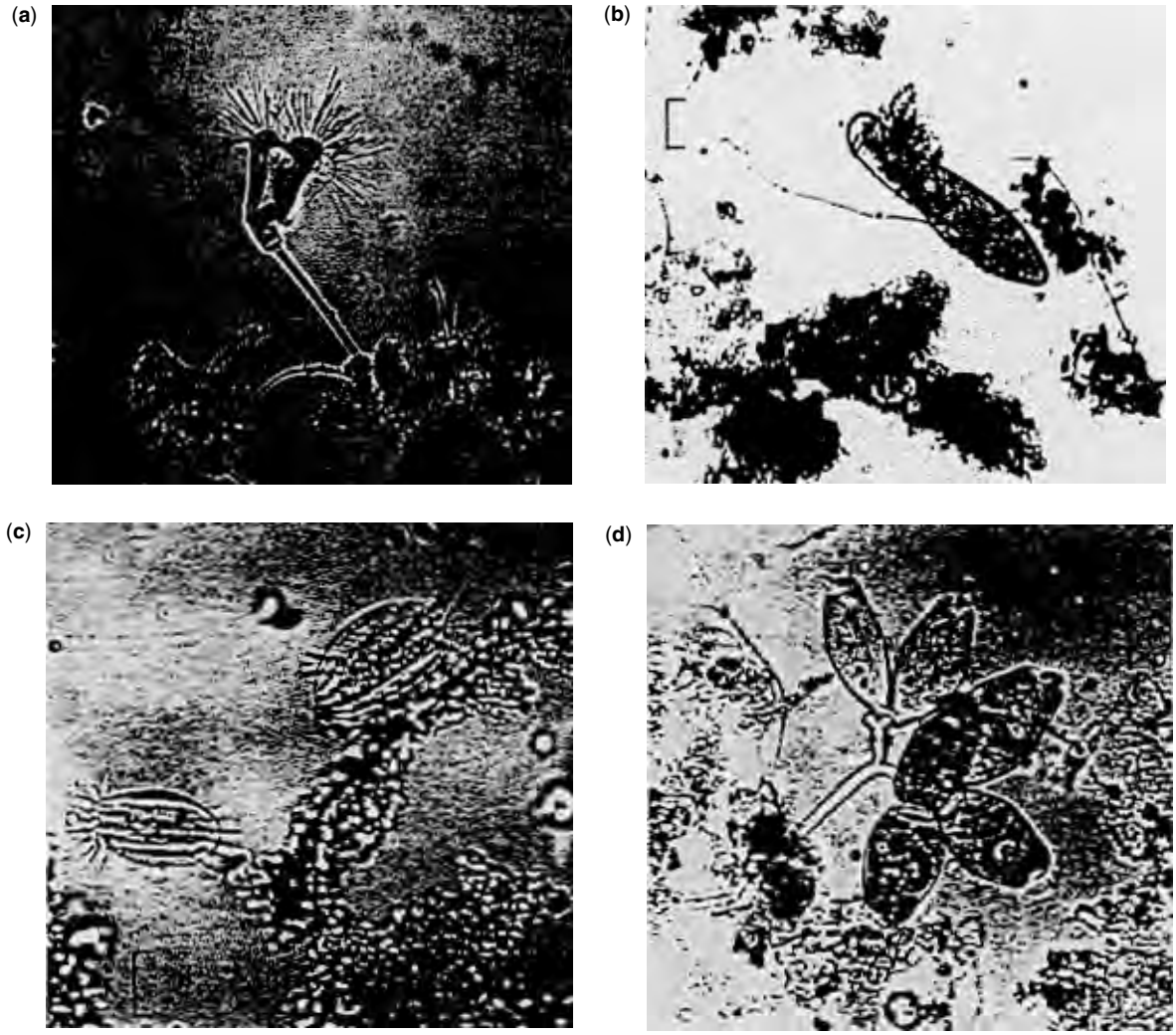

Figure 2. Micrographs showing the groups of ciliates found in activated sludge systems. (**a**) carnivorous ciliates (Suctoria); (**b**) free-swimming bacterivorous ciliates (*Paramecium*); (**c**) crawling bacterivorous ciliates (*Euplotes*); (**d**) Attached bacterivorous ciliates (*Opercularia*).

- *Crawling*, those that are not attached, but inhabit the surface of sludge flocs;
- *Attached*, those that are firmly fixed to the sludge floc by means of a stalk. They are strictly associated with flocs and thus settle during sedimentation.

All bacterivorous ciliates rely on ciliary currents to force suspended bacteria into their mouths (Fig. 3). Free-swimming and attached ciliates are in competition for bacteria and small flagellates dispersed in the liquid phase. Ciliates moving on the floc surface (crawling ciliates) feed on bacteria that lightly adhere to the floc, and to those that are dislodged by the ciliate feeding currents (4).

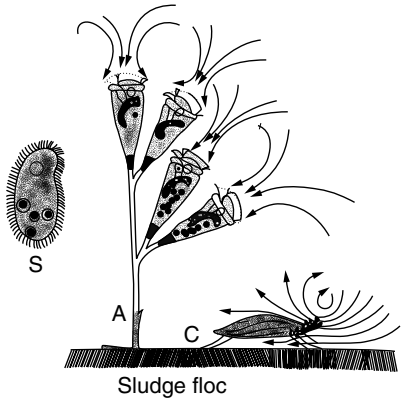

Figure 3. Feeding mechanisms of bacterivorous ciliates in the activated sludge system. Crawling ciliates (C) scrape bacteria from the surface of the flocs, while free-swimming (S) and attached (A) ciliates filter out dispersed bacteria in the mixed liquor (4).

Parasitic and Pathogenic Protozoa

Some protozoan parasites such as, *Giardia* and *Cryptosporidium* are recognized causes of diseases in humans and animals. *Cryptosporidium* is recognized worldwide as an important pathogen causing diarrheal illness in humans and animals. Routes of transmission involve contact with infected people and animals, and the waterborne route. However, only since 1985 has *Cryptosporidium* been recognized as the cause of waterborne disease (20). *Cryptosporidium* has many characteristics that enable its waterborne transmission, and common bacterial indicators of fecal pollution do not indicate its presence in water (21). *Giardia* is a protozoan capable of causing chronic diarrhea in humans. Fecally contaminated waters and consequent waterborne outbreaks of giardiasis have been widely reported (22). The cysts of the organism often remain viable for several months in water; furthermore, they are more resistant to disinfection than to fecal coliforms that are usually used as bacterial indicators of fecal pollution (23). For more details see PARASITIC PROTOZOA: FATE IN WASTEWATER TREATMENT PLANTS."

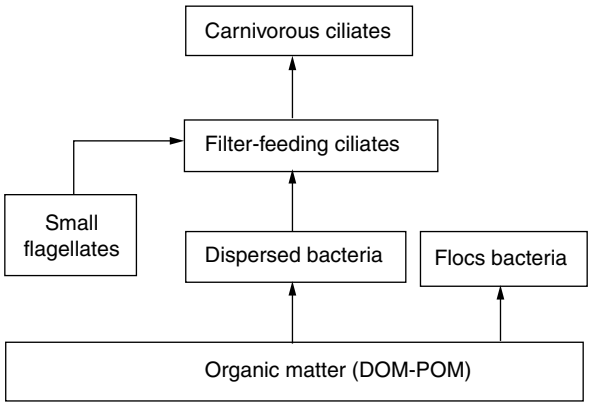

Figure 4. The microbial loop in the activated sludge of sewage treatment plants (27).

ROLE OF PROTOZOA IN PLANT OPERATION

Protozoa are known to have an important role in the activated sludge process, particularly in effluent clarification, and this in turn results in a reduced effluent BOD (24). In fact, in activated sludge, bacterivorous ciliates ingest large numbers of the dispersed bacteria, which are not associated with flocs, and whose growth would generate high turbidity of the effluent. In the absence of ciliated protozoa, in fact, effluents have a much higher BOD and are highly turbid because of the presence of many dispersed bacteria (3). Protozoa, moreover, feed on pathogenic and fecal bacteria contributing to their removal efficiency at 95% (25).

In aeration tanks of activated sludge plants a microbial loop is established (Fig. 4). The growth of the heterotrophic bacteria depends on quality and quantity of DOM. Conversely, for predator protozoa,, growth depends on the available prey. The dispersed bacteria serve as a food source for flagellates, rhizopods, and ciliates, whereas these in turn are prey for carnivorous protozoa.

The protozoan assemblage changes during the stabilization of activated sludge systems (Fig. 5). The plant start-up phase is characterized by the presence of species typical of raw sewage, such as small heterotrophic flagellates and free-swimming bacterivorous ciliates. These protozoa are not linked to the presence of flocs and cannot be considered typical components of the activated sludge microfauna. With the development of the biomass, they compete with protozoa better adapted to an aeration tank environment and rapidly decline in numbers. The following phase is characterized by the strong growth of protozoa typical of the aeration tank habitat, such as crawling and attached ciliates. The steady state phase is characterized by a protozoan community whose structure reflects the stable conditions in the aeration tank, with a balance between the organic loading and the mixed liquor suspended solids that are produced, removed and recycled (27). In a mature activated sludge, the species structure of the protozoan community is strictly dependent on plant management choices based on design characteristics aimed at guaranteeing optimum efficiency.

It should be emphasized that each of the three phases is characterized by a typical protozoan population structure. Free-swimming bacterivorous ciliates such as *Uronema*, *Cyclidium Colpidium* and small heterotrophic flagellates

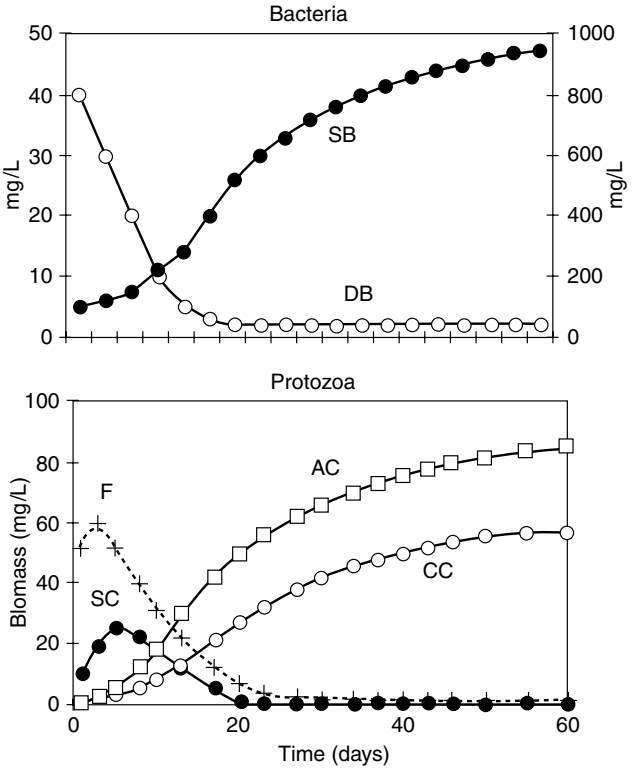

Figure 5. Dynamics of protozoa and bacteria biomass during the stabilization of activated sludge. (DB) Dispersed bacteria; (SB) floc bacteria; (F) small flagellates; (AC) attached ciliates; (CC) crawling ciliates; (SC) swimming ciliates.

such as *Bodo* and *Cercomonas*, are associated with the first phases of the colonization of the plant, whereas stalked ciliates such as *Epistylis* and *Vorticella*, and crawling forms such as *Aspidisca* are typical of the steady state phase (27). A fully functioning plant will not contain species characteristic of one of the colonization phases, unless dysfunctions cause instabilities in environmental conditions. These include changes in the amount of sludge, the degree of aeration, organic load, sludge retention time, and organic loading at input.

Nevertheless, there are some plant-operating conditions that heavily influence the microfauna assemblage. The dilution rate of the system is important since any species that cannot reproduce quickly enough to compensate for such a loss will be removed from the system. Thus, activated sludges with fast flow rates will tend to favor species with rapid rates of reproduction, usually the smaller protista such as heterotrophic flagellates or small ciliates (<30 μm). Activated sludge plants with slow flow rates host a greater diversity of organisms, including small metazoa, which, in contrast with protozoa, have slow rates of reproduction (10,17). Overloaded systems lead to a higher demand for dissolved oxygen, and low levels of free oxygen will favor those heterotrophic flagellates (9), amebae, and small ciliates (17) normally found in organically polluted habitats (Fig. 6). As the organic loading decreases, the diversity of organisms, which may colonize the mixed liquor, increases. In underloaded plants (i.e., extended aeration units), higher numbers of taxa are found but the number of individual population encountered is usually smaller (15,17).

PROTOZOA AS INDICATORS OF PLANT PERFORMANCE

The prevalent reactor operating conditions, in particular the sludge age, dissolved oxygen, and organic loading rate (F/M ratio), together with the composition of the influent wastewater, have a profound effect on the microbial populations inhabiting the aeration tank because these operating conditions dictate both the amount of time an organism has to reproduce in the reactor, and the amount of nutrients which are available to it (28). Changes in F/M ratio and sludge age, together with MLSS in the aeration tank, and the effluent BOD, correspond to quantitative and qualitative changes in the protozoan community. It has been demonstrated that the identification and enumeration of protozoa in activated sludge mixed liquor, may provide rapid information on plant operating conditions and performance (17,24,29).

Crawling ciliates (hypotrichs) decrease with increasing loading (no hypotrichs would be observed in sludge loaded above 0.6 kg BOD/kgMLSS/day), whereas stalked ciliates (peritrichs) are able to grow throughout a large range of sludge loadings (24). Nevertheless, at low loadings (0.1 e 0.3 kg BOD/kg MLSS/day) a wider diversity of ciliates, in terms of numbers of species with a more uniform distribution between the three functional groups, would be observed (24).

In field studies, the number of ciliated protists observed in a normally functioning plant ranged from 10^3 to 10^4 individuals/mL (17). Numbers of ciliates falling below 10^2/mL, corresponded to lower treatment efficiency (29). In this case, the low ciliate density was accompanied with increased BOD values in treated effluent, and a higher effluent turbidity caused by the presence of dispersed bacteria. Higher densities of ciliates ($>10^4$/mL) instead, were almost always associated with good purification and optimum plant performance (17,29). The microfauna of a well-operating activated sludge system is almost always

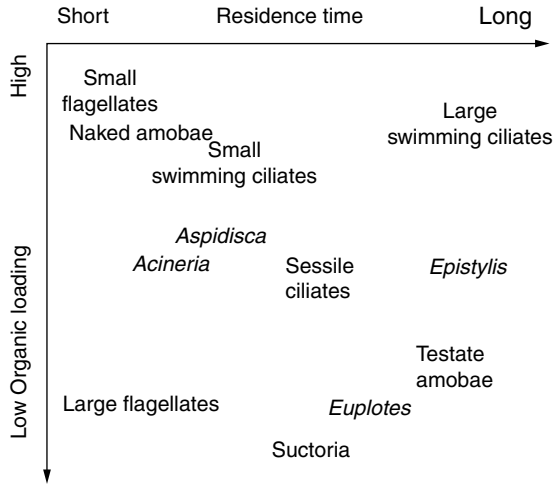

Figure 6. Influence of organic loading and sludge residence time on the protozoan assemblage in activated sludge.

highly diversified. More than 10 species of ciliated protozoa may be found in a sample of activated sludge mixed liquor, in which each species is never numerically dominant over the others, even though the ratios among species can differ (4). The sludge of plants affected by conditions such as shock load of toxic discharge, underloading or overloading, strong sludge extraction, lack of aeration, is often dominated by one species or functional group more tolerant to these conditions.

Thus, the structure of the microfauna is a valid indicator of plant performance and an efficient activated sludge plant possesses the following characteristics:

- High numbers of protozoa ($\geq 10^3$/mL).
- Microfauna composed chiefly of crawling and attached ciliates, with almost no small heterotrophic flagellates and free-swimming ciliates.
- High number of species in which none dominates numerically over the others by a factor greater than 10.

When the protozoan community lacks these characteristics, identification of the dominant group of the microfauna present allows a diagnosis of the problem with the plant (Table 2). In addition, identification of the protozoan species allows more complete information on the biological performance in the aeration tank to be obtained. In fact, if it is true that a protozoan community rich in attached and crawling ciliates indicates improved performance with respect to that found when free-swimming ciliates dominate, it also true that different species of crawling and attached ciliates can be associated with different operating conditions. Each species is able to provide additional information. This is the case for the attached ciliates *Vorticella convallaria* and *Vorticella microstoma*. These two peritrich ciliates are in direct competition in the activated sludge. *Vorticella microstoma* characterizes the first phase of colonization but it is then substituted by *V. convallaria*, which may reach high numbers during the steady state phase. In cases of lack of aeration in the mixed liquor, the importance of these two species may change due to their differing degree of tolerance to the lack of oxygen (30). Thus, dominance of *V. microstoma* indicates a poorly aerated sludge (24,30).

Table 2. Relationships Between Dominant Group of the Microfauna and Plant Performance (37)

Dominant Group	Performance	Possible Causes
Attached + crawling ciliates	Good	
Attached ciliates	Decreasing	Discontinuous load recent sludge extraction
Crawling ciliates	Good	
Free-swimming ciliates	Mediocre	Poor aerated sludge; overloading
Small heterotrophic flagellates	Low	Overloading; poorly aerated sludge; fermenting substances involved
Small naked amebae and Small heterotrophic flagellates	Poor	Very high load, not easily degradable
Testate amebae	Good	Very low sludge load; long sludge retention time; high DO in aeration tank; complete nitrification

THE SLUDGE BIOTIC INDEX (SBI)

It has been recognized that changes in the community structure and types of protozoa species in the mixed liquor of activated sludge may affect the food web of these artificial ecosystems, and, thus, may also influence the biological performance of plants. Results obtained from correlation analysis showed which physicochemical and operational parameters have the greatest influence on the functional groups of protozoa in the activated sludge (29). Small flagellates, free-swimming ciliates, and the peritrichs *V. microstoma* and *Opercularia* spp. are negatively correlated with DO, nitrifying ability of the plant, and the level of BOD removed, whereas these are positively correlated with the effluent turbidity. On the contrary, crawling ciliates and testate amebae appear to be positively correlate with DO, nitrifying ability, BOD removed, and negatively correlated with effluent turbidity.

Numerous biotic indices have been developed to assess the degree of pollution in freshwater environments. The performance of the plants has to be constantly monitored and is subjected to strict regulation. Nevertheless, malfunctions resulting in decreased purification efficacy are frequent. In the last decade the routine analysis of the microfauna as an indicator of activated sludge plant performance has become more common because it provides rapid and useful information on the biological activity of the sludge based on the community structure of the protozoa present (28,31). Some of these methods, however, are subjective indices based on the analyst's personal interpretation of the microfauna that colonize the activated sludge system under investigation. These indices cannot necessarily be applied directly to other similar plants. Objective indices have a great advantage over subjective ones, in that, index values assessed by different operators are comparable.

The SBI, an objective index based on protistan community, has been devised to monitor activated-sludge plant performance (29). This method is based on two principles:

- the dominance of protistan keygroups changes in relation to environmental and operational conditions of the plant,
- cell density and number of taxa diminish as the efficiency of the plant drops.

The SBI enables the operator to define the biological quality of the sludge by means of conventional numerical values (from 0 to 10) that are grouped into four quality classes (Table 3). An accurate identification of the various species of protista is important to obtain an accurate SBI value. However, some keys written

Table 3. Two-Way Table to Determine the Sludge Biotic Index (29)

Horizontal Entrance in the Table on the Basis of Both Keygroup and Density		Vertical Entrance in the Table. Total Number of Taxa of the Microfauna and Number of Small Flagellates F Counted Along the Fuchs-Rosenthal Chamber Diagonal (a = F < 10; b = 10 < F < 100)							
Dominant Keygroup	Density (ind./L)	>10		8–10		5–7		<5	
		a	b	a	b	a	b	a	b
Crawling + Attached Ciliates* and/or Testate Amebae	$\geq 10^6$	10	8	9	7	8	6	7	5
	$<10^6$	9	7	8	6	7	5	6	4
Attached Ciliates* >80%	$\geq 10^6$	9	7	8	6	7	5	6	4
	$<10^6$	8	6	7	5	6	4	5	3
Opercularia spp.	$\geq 10^6$	7	5	6	4	5	3	4	2
	$<10^6$	6	4	5	3	4	2	3	1
V. microstoma and/or *V. infusionum*	$\geq 10^6$	6	4	5	3	4	2	3	1
	$<10^6$	5	3	4	2	3	1	2	0
Swimming Ciliates	$\geq 10^6$	5	3	4	2	3	1	2	0
	$<10^6$	4	2	3	1	2	0	1	0
Small Flagellates (<100)†	$\geq 10^6$	4		3		2		1	
	$<10^6$	3		2		1		0	

*_Opercularia_ and _V. microstoma_ no dominant;
†along the Fuschs-Rosenthal chamber diagonal
Note: Conversion of SBI values into four quality classes

SBI value	Class	Judgment
8–10	I	Very well colonized and stable sludge; excellent biological activity; very good performance.
6–7	II	Well colonized and stable sludge; biological activity on decrease; good performance.
4–5	III	Insufficient biological purification in the aeration tank; mediocre performance.
0–3	IV	Poor biological purification in the aeration tank; low performance.

specifically for protista in sewage treatment processes and polluted waters are available (32–37). Organisms considered in the SBI method are: small and large flagellates, ciliates, testate amebae, and some small metazoa such as rotifers, nematodes and gastrotrichs. While all the species of ciliated protozoa and testate amebae contribute to the determination of the microfauna diversity, small flagellates and metazoa are very difficult to identify at species level and thus contribute each with one systematic unit only. Since carnivorous ciliates do not play an important role in the purification process, they contribute only to estimates of the total density and diversity of the microfauna.

The ecological characteristics of the seven keygroups used for to determine the SBI are the following:

Testate Amoebae

Testate amoebae are more abundant or dominant in sludges characterized by low loading, long sludge retention time, and high DO in aeration tanks that enable complete nitrification (14,15). Under these conditions, the quality of the effluent is excellent and a high biological performance of the plant is attained. Testate amebae grow only in plants with a long sludge age since these protists have low growth rates. Since their growth rate increases with temperature, testate amebae are often more common in summer. Nevertheless, when the sludge loading reaches high values (>1 kgBOD/kgMLSS · d) and the COD of the effluent is elevated, these protists are replaced by the peritrich ciliate *Opercularia* and by free-swimming ciliates (16).

Crawling and Attached Ciliates

These two functional groups normally codominate the protistan community in activated sludge plants. This is due to their different food habits, preventing competition. In fact, while attached ciliates feed on bacteria and small flagellates that are dispersed in the liquid phase, crawling ciliates are in close proximity to substrates of flocs and graze on bacteria loosely attached to them. Nevertheless, the ratio between the two functional groups tends to change with sludge loading. Crawling ciliates reduce their numbers as sludge loading increases so that above 0.6 kg BOD/kgMLSS · d most species of this group disappear (24).

Even if attached ciliates are normally codominant in the activated sludge, their numbers increase rapidly (over 80% of the whole microfauna) under transient conditions that reduce plant performance (17,26). Such transient conditions are:

- discontinuous input of organic load from the influent,
- quick increase of the sludge load due to loss of sludge

Attached ciliates are able to grow over a wide range of sludge loadings; nevertheless, at values ranging from 0.3 to 0.6 kg BOD/kgMLSS · d, these protists dominate, whereas for sludge loadings of 0.6–0.9 kg BOD/kgMLSS · d sessile ciliates and flagellates codominate (24).

Among stalked peritrich ciliates some species such as *V. microstoma* and *Opercularia* spp. are able to survive and grow in activated sludge systems under severe conditions (lack of oxygen, toxicants). When these forms are present as dominant or prevalent species, they must be considered separate key groups.

Opercularia spp.

Low numbers of *Opercularia* often occur in activated sludge, but their population density increases when the plant produces low-quality effluent. *Opercularia* spp. are associated with high final effluent BOD concentrations, and are among the most abundant forms at high plant loadings (24,31). These ciliates moreover can survive in stressed environments better than other protists. In fact, large numbers of *Opercularia* have been found in plants receiving industrial waste containing toxic substances (38). Moreover, these peritrich ciliates have been associated with high final effluent BOD and ammoniacal-N concentrations (14,15). *Opercularia* may be the only ciliate protozoan in sludges of plants treating industrial waste containing metal salts. High numbers of *Opercularia asymmetrica* have been found in activated sludge receiving heavily loaded organic waste from pharmaceutical companies (9). *Opercularia* spp. often are associated with *V. microstoma*.

Vorticella microstoma Complex and *Vorticella infusionum* Complex

The peritrich ciliates *V. microstoma* is a rare species, which often has been confused with the much more frequent *V. infusionum*. The main difference between *V. microstoma* and *V. infusionum* resides in the macronucleus: C-shaped or rod-shaped and in longitudinal axis of the cell (*V. microstoma*), semicircular and in transverse axis of cell (*V. infusionum*) (33). These two species are very similar ecologically and are commonly present in plants during the early phase of colonization but are soon substituted by other stalked ciliates during steady state conditions. When drastic and prolonged reductions in the dissolved oxygen concentration in the mixed liquor occur, dominance of *V. microstoma* or *V. infusionum* can be observed due to their high degree of tolerance to the lack of oxygen (30). In fact, these ciliate are considered as a polysaprobic species (33). Presence of these species thus, indicates a lack of dissolved oxygen in the aeration tank of activated sludge plants.

Free-swimming Bacterivorous Ciliates

Free-swimming bacterivorous ciliates together with small flagellates are the first to appear during the establishment of a sludge (39). They are soon replaced by attached peritrich ciliates owing to competition for bacteria dispersed in the mixed liquor. Attached forms, in fact, are filter feeders and more efficient than free-swimming ciliates in capturing suspended bacteria by means of ciliary currents. In fully functioning activated sludges, bacterivorous free-swimming ciliates sometimes dominate the protozoan communities of plants operating at sludge age too short or with both high sludge loading ($0.6-0.9$ kg BOD/kgMLSS $\cdot$ d) and lack of oxygen (40). These bacterivorous ciliates, in fact, require high concentrations of dispersed bacteria but survive better than other protists in the presence of toxic compounds in the influent and in conditions of inadequate of oxygen.

Small Flagellates

Small heterotrophic flagellates continuously enter the plant with influent where they are very numerous (39). These protozoans feed on dispersed bacteria and, in time, are substituted by bacterivorous ciliates. In a fully functioning activated sludge, in fact, these protists are outcompeted by bacterivorous ciliates and are subject to predaceous activity by other protists. These biotic pressures limit the presence of small flagellates in activated sludge to few individuals. By contrast, the presence of large numbers of these protists in a stable activated sludge system, is associated with poor performance in the aeration tank, caused by environmental or operating conditions, such as poorly aerated sludge, over loading, and fermenting substances entering the plant (17,26). In this case, the effluent from the system has an elevated BOD and is highly turbid as a result of the high density of these protists. Flagellates become the dominant protistan forms in over loaded sludges (>0.9 kg BOD/kgMLSS $\cdot$ d) (9,24).

Large flagellates greater than 20 μm (*Euglena, Peranema*) are infrequently found in the activated sludge systems. In contrast to the small flagellates, the presence of these protists is associated with very diluted organic matter entering the plant (10,11).

The values of SBI have been grouped into four quality classes representing the biological quality of the activated sludge through four ranges of judgments that are rather wide, and thus of reliable diagnostic value. The SBI method enables the operator to monitor the aeration tank performance. The strict applicability of the SBI was tested by investigating more than two hundred activated sludge plants, and results showed that the method gives correct analysis of the reactor performance in all the cases investigated. Although of proven accuracy, there is a limitation that restricts its application in practice. In fact, the technique relies on the identification of ciliate species, which can be time consuming and difficult, even for familiar genera. Species that may be difficult to separate taxonomically can exhibit quite different ecological characteristics, and the use of these species as the sole indicators of plants performance is therefore questionable. Moreover, since the SBI was set up specifically for the evaluation of the biological reactor performance, this index is unable to reveal any dysfunction in the final sedimentation tank (i.e., bulking, rising). The index was designed to be applied in all activated sludge plants including oxidation ditch, conventional aeration plants and extended aeration units. Nowadays, the SBI method is widely applied in Italy, Portugal and Spain, and to a less extent, in other countries.

CONCLUSION

The expanding role of biotechnology in the field of waste treatment, increased the interest in the application of biological indicators to better control the plant performance, and to improve the waste treatment in general. The biological reactors of waste treatment systems have been regarded as ecosystems made by humans and are subjected to extreme conditions. In activated sludge systems biotic components are represented by decomposers (floc-forming bacteria), which utilize the dissolved organic matter in the wastewater, and by consumers (heterotrophic flagellates, ciliates, amebae, and small metazoans) that feed on dispersed bacteria and other organisms. Activated sludge systems develop specific communities of protista, which are sustained by large populations of bacteria. Ciliates play an important role improving the quality of the effluent by filter-feeding bacteria dispersed in the mixed liquor. Since species and groups of protista depend on the environmental conditions in the aeration tank, the structure of the microfauna is a valid indicator of purification plant performance. The major part of situations regarding the plant performance are thus indicated by the dominant group of the microfauna. For these characteristics, the routine analysis of the microfauna as an indicator of activated sludge plant performance is becoming increasingly common. It quickly gives useful information on the biological activity of the flocs based on the community structure of the microorganisms present. The microfauna is also used to indicate changes in the performance of specific activated sludge plants (28,31), but these methods cannot necessarily be applied directly to other similar plants. On the contrary, the SBI, a objective index in which index values assessed by different operators are comparable, can be applied to any activated sludge plant to estimate the performance of the aeration tank (29). Potential gains from this technique include improved performance of BOD removal, nitrification, and the improvement of waste treatment in general.

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PROTOZOA IN MARINE AND ESTUARINE WATERS

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Protozoa have been defined historically as unicellular, eukaryotic organisms that obtain their nutrition heterotrophically. This mode of nutrition is typically accomplished by the ingestion of prey (phagocytosis), although the uptake of dissolved organic material also contributes to the nutrition of some species. The term "protozoa" originated as a means of distinguishing unicellular species exhibiting animal-like nutrition from those capable of photosynthesis (the microalgae). Since the 1970s, it has remained a primary distinction, albeit a problematic one, among taxa within the Kingdom Protista (1).

There are conspicuous problems with the term "protozoa." It is now widely accepted that this term does not indicate a true evolutionary lineage. One issue involves the grouping of organisms on the basis of nutritional mode, artificially separating unicellular organisms with animal nutrition from the photosynthetic unicellular algae. The phylogenetic inappropriateness of separating the algae from the classically defined protozoa has become clear as ecological, ultrastructural, and molecular studies of these species have progressed. Combined photosynthetic and heterotrophic ability (i.e., mixotrophy) has now been demonstrated for a large number of protistan species. More importantly, evolutionary relationships among protists derived from ultrastructural data (during the past few decades) and DNA sequencing studies (during the last decade) have indicated that the division of protists based on the presence or absence of chloroplasts does not reflect true phylogeny. It is noteworthy that use among ecologists of the term "protozoa" is slowly yielding to the term "phagotrophic protists," which recognizes the ability of unicells to consume prey irrespective of their ability to photosynthesize.

Another difficulty with the now-traditional classification of Whittaker arises from the rather arbitrary distinction between unicellular and multicellular species. For example, some groups such as water molds and slime molds that can form macroscopic, multicellular structures, are typically included within the protozoa, whereas macroscopic, multicellular algae are excluded. The latter distinction is inconsistent with the results of phylogenetic analyses that have demonstrated close affinities between micro- and macroalgae.

These deficiencies of past and present systematic schemes for unicellular eukaryotes have instigated a massive revision in the classification of these taxa (2). Modern hypotheses now favor highly revised (and diverse) taxa of unicellular eukaryotes grouped together with the plants, fungi, and animals within the Domain Eukarya. From these taxonomic building blocks, which are based largely on ultrastructural features (2), new hypotheses are emerging on the evolutionary relationships among these species.

Despite the polyphyletic nature of the traditional taxonomic groupings and the inconsistencies regarding nutritional modes, however, the term "protozoa" is still commonly employed today (3). In this article we will use the term synonymously with "phagotrophic protists," recognizing that the presence of a chloroplast would also classify many of these taxa as microalgae. Emphasis will also be placed on dominant, free-living forms in estuarine and marine ecosystems.

DIVERSITY OF PROTOZOAN FORM AND FUNCTION

Free-living protozoa inhabit nearly all aquatic (and terrestrial) ecosystems. Protozoa constitute a significant component of the living biomass of these ecosystems and represent a taxonomically diverse group of species. Tens of thousands of extant and extinct species (the latter based on fossil evidence) have been described and many more undoubtedly await discovery and description. Recent genetic studies of marine plankton assemblages have demonstrated previously undescribed species of protists (4,5). In addition, the existence of so-called "cryptic" species that are morphologically indistinguishable from one another has also been demonstrated by DNA sequencing (6,7). Discoveries similar to these will certainly increase in frequency as more molecular-based studies are conducted.

Nevertheless, a considerable controversy presently exists regarding the true species richness of protozoan assemblages and the degree to which these species are distributed globally. Some recent reports have claimed that many species of protozoa are cosmopolitan in their distribution, and thus global diversity might not be as great as previously suggested (8). These latter claims will continue to be tested as molecular and morphological data accumulate. Regardless of the final tally, however, protozoa will remain an enormously diverse assemblage of organisms.

Protozoa vary greatly in size, spanning several orders of magnitude. Most species are microscopic and range in size from some flagellated individuals that are less than 2 μm in cell diameter (9,10) to flagellated and ciliated protozoa that can attain a length of a few hundred micrometers in length (Fig. 1). A limited number are visible to the unaided eye. Some solitary radiolaria and foraminifera form structures that exceed a centimeter in size. Certain species of polycystine radiolaria that inhabit warm oceanic ecosystems form cylindrical, gelatinous colonies that can be more than a centimeter in diameter and several meters long. Collectively, protozoa form a conspicuous and important component of living biota over an immense size range.

Nutrition

Diversity in protozoan size and form is matched by diversity in diet. Osmotrophy (the absorption of organic

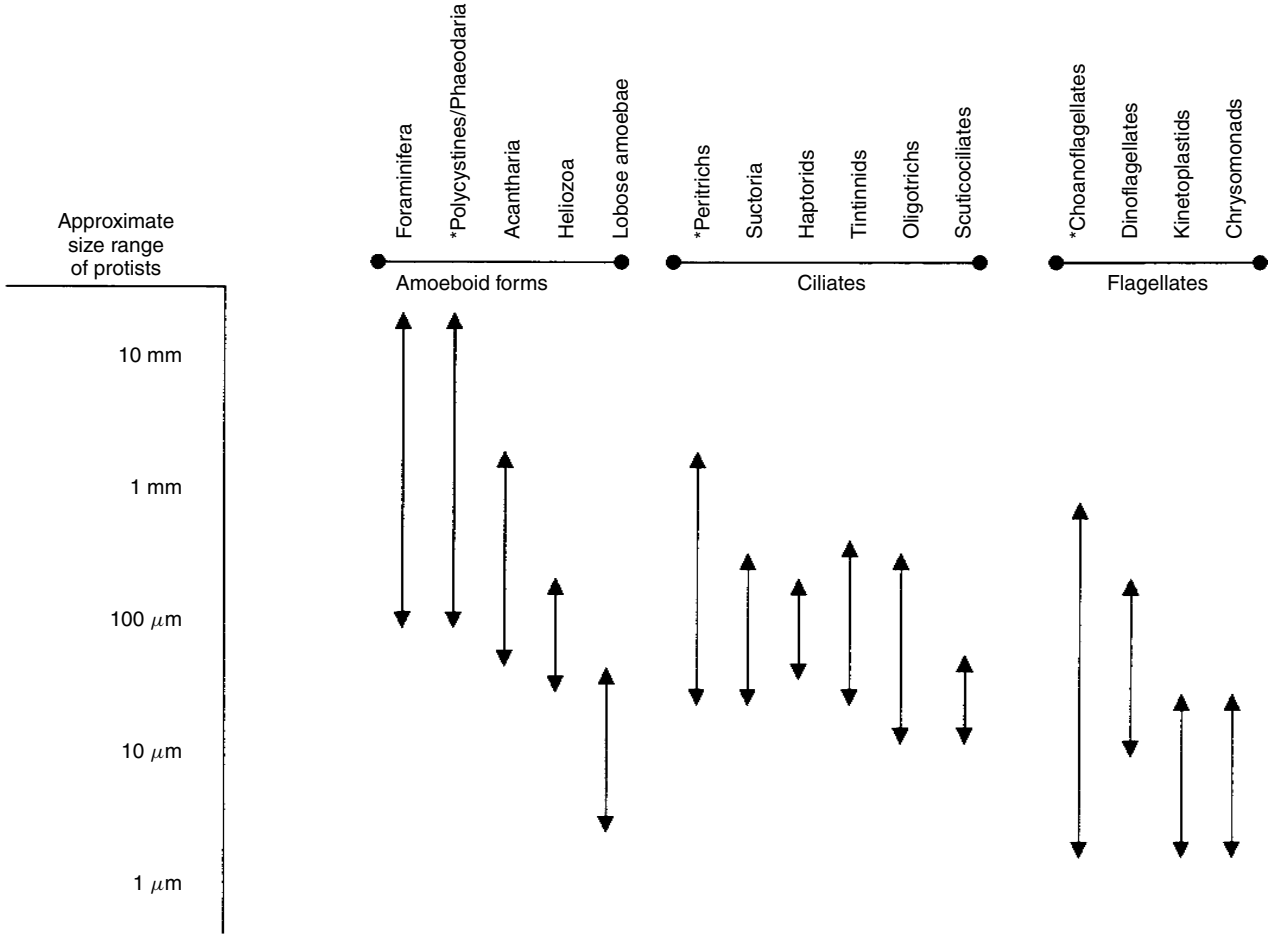

Figure 1. Approximate size ranges of some common marine protozoan taxa. Note that, collectively, protozoan sizes span more than four orders of magnitude. Most species, however, are generally between 2 and 200 μm in size.

compounds) and pinocytosis (the uptake of water and dissolved organic compounds into food vacuoles) are modes of nutrition exhibited by some protozoa, but phagocytosis (the capture and ingestion of particulate prey) is by far the most common means of protozoan food acquisition. Many species have developed specialized strategies and/or structures to facilitate phagocytosis. Most flagellated and ciliated species collect prey at the cell surface via the action of flagella or cilia or by water currents created by the action of those organelles. However, many variations on this theme exist. Some phagotrophic dinoflagellates possess a pseudopodial veil that can be extended from a pore in the thecal wall and used to surround and digest prey. Other phagotrophic dinoflagellates make use of a peduncle, which is a highly extensible, fingerlike projection of cytoplasm that attaches to prey and provides a portal for the extraction of the prey's cytoplasmic contents.

Amoeboid protozoa feed by using different types of pseudopodia, which are transient projections used in locomotion and to engulf prey. For small amoebae, this process is relatively straightforward. Suitable particles (usually bacteria and small protists) are ingested at the anterior edge of the cell as they are encountered during the movement of the amoebae along surfaces. Larger amoeboid forms (e.g., Foraminifera and actinopod protozoa) produce complex pseudopodial networks that are used to entangle and capture prey and then digest them. Pseudopodia can also be used to pierce the exoskeleton and remove tissues of prey such as copepods.

Diets among free-living phagotrophic protists differ among the various taxa and can be fairly specialized or quite broad depending on the species, its size, and its particular method of prey capture. For example, some minute flagellated protozoa are so small that they are capable of consuming only unicellular, prokaryotic prey (10). Although not limited by size, the planktonic ciliate *Didinium nasutum* also feeds rather selectively on other ciliates in the water column (11). In contrast, many of the larger amoeboid protozoa (Acantharia, Radiolaria, and Foraminifera) consume a wide array of microalgae, protozoa, and multicellular animals (12,13). Thus, protozoa may be bacterivorous, herbivorous, carnivorous, or omnivorous. Some are even cannibalistic.

Protozoan digestion differs from this process in multicellular animals because, as single cells, protozoa lack a digestive cavity. However, the process of digestion is analogous to that in higher organisms in that the prey undergoes acidification and then enzymatic

digestion in membrane-bound vacuoles formed around the prey at the time of ingestion. The digestive process can take 20 minutes to several hours, depending on the protozoan species, prey item, and environmental conditions. Utilizable compounds move from the food vacuoles into the cytoplasm of the protozoan during digestion, and the remaining material is then released to the external environment as a mixture of dissolved and particulate organic matter and remineralized compounds.

Growth, Reproduction, and Life Cycles

Protozoa (and protists in general) reproduce asexually, sexually, or by both methods. Asexual reproduction is usually accomplished by binary fission, multiple fission, or budding. By far, the most common form of reproduction among free-living protozoa is binary fission. This process involves mitotic division of an individual, with the duplication and distribution of organelles, to produce two more or less identical daughter cells. The relative simplicity of unicellularity and reproduction by binary fission allows protistan species to grow and reproduce rapidly when biological and environmental conditions are favorable. The fastest growing species can undergo several doublings per day under optimum conditions. Thus, most protists are capable of population growth rates that greatly exceed rates for most multicellular eukaryotes. This behavior explains the ability of microalgae and protozoa to grow rapidly and form "blooms" in many aquatic ecosystems.

Multiple fission and budding are less common modes of asexual reproduction among protozoa, but they are characteristic of some taxa. Some parasitic protozoa reproduce by multiple fission, presumably a mechanism for overwhelming the immunological defenses of the host. The nucleus divides repeatedly in this mode of reproduction, which is followed by cellular fission, giving rise to many daughter cells. Budding occurs in a number of free-living species, most notably in some groups of ciliated protozoa such as suctoria and peritrichs that have sessile adult forms. Ciliated swarmers provide a dispersal mechanism for these species.

Most protozoa increase in number by asexual cell division, and some flagellated and amoeboid taxa may use only this mode of reproduction. The life cycles of many protozoa, however, also involve sexual processes that can take several forms. Variations in the type of gamete produced include flagellated and amoeboid forms, as well as nuclei that simply migrate among attached cells. Three main modes of fertilization have been identified: autogamy, gametogamy, and gamontogamy. Autogamy is the process of fertilization in which both gametes come from the same parent. Cross-fertilization does not occur in this process and therefore all offspring are genetically identical. Gametogamy occurs when gametes are released into the water, and individuals of opposite mating types fuse to form zygotes. Gamontogamy occurs when two individuals (gamonts) physically unite to share gametes or gametogenic nuclei.

Some protozoa exhibit both asexual reproduction and sexual processes at different stages of their life cycles. Sexual processes in at least some of these species appear to serve the purpose of recombination, providing genetic variability that might allow the population to adapt to changing environmental conditions. For example, many ciliates reproduce by binary fission while growing under optimum conditions. Conjugation (a form of gamontogamy) initiates as food supply wanes or other conditions change. Conjugation in these species appears to be responsive to external environmental cues because the number of conjugating individuals in a population can be affected by changes in factors such as temperature, light, or population density. The process results in genetic recombination between paired individuals but it does not result in an increase in protozoan number (14). Although it does not increase the number of individuals in the population, conjugation may confer a competitive advantage on the species by maintaining genetic diversity within the population and thus allowing some segment of it to survive under a different set of growth conditions.

Other strategies for sexual processes exist. A number of protozoa alternate between generations of sexually and asexually reproducing life stages. This form of life cycle is common among foraminifera. In contrast to the rapid reproductive rates associated with species reproducing by binary fission, growth of an individual foraminifer to reproductive maturity can take weeks or months. When reproduction does take place, however, a great number of immature individuals or gametes are often produced simultaneously. Thus, slow reproductive rates in these species are compensated by large output at the time of reproduction.

Rapid reproduction rates allow protozoan populations to be highly responsive to favorable conditions. Additionally, these species also have life strategies for coping with unfavorable conditions such as adverse environmental factors (temperature, desiccation, etc.) or starvation. Many protozoa are capable of autophagocytosis of their own cellular constituents in the absence of food. These species greatly reduce their metabolic rates and shrink significantly in size as starvation progresses (15). Some species even divide one last time at the onset of starvation to form fast-swimming swarmer cells that presumably disperse in an attempt to find more favorable conditions. Other protozoa form resting stages called cysts that are highly resistant to changes in environmental parameters. The ability to form some type of cyst is common throughout many protozoan taxa. In some species, sexual processes are linked to cyst formation.

MAJOR PROTOZOAN TAXA, ABUNDANCES, AND DISTRIBUTIONS

As noted earlier, there is a tremendous diversity of protozoan morphology, motility, and diet. There are some common body forms, however, which have provided the rationale for the historical groupings of these taxa. Although there is a growing realization that some of these classifications are polyphyletic, in some cases they are still sensible on the basis of similarities regarding the ecological roles of the taxa.

On the basis of the older (and still commonly employed) taxonomic scheme, free-living protozoa are divided into

three large groups defined by their overall morphology and means of locomotion and feeding: amoeboid, flagellated, and ciliated forms. Many of the pathogenic or parasitic species display unique (or multiple) life stages and have been separated into their own taxa and thus constitute a few additional groups.

Amoeboid Protozoa

Free-living amoeboid protozoa have historically been grouped together into one subphylum on the basis of the presence of a single character during their life cycle, the pseudopodium. Pseudopodia are projections of the cytoplasm used for locomotion and food capture. Amoeboid protozoa lack rigid cell walls that give the cells great plasticity and thus the ability to form these projections. These structures vary in complexity among the different amoeboid taxa from relatively simple, lobed projections of the cell (e.g., in the lobose amoebae) to complex, anastomozing networks of pseudopodia (e.g., in the Foraminifera) to radiate structures stiffened by bundles of microtubules (e.g., in the Actinopoda). There is strong evidence that the presence of the pseudopodium is a result of convergent evolution. Thus, the amoeboid protozoa are actually a collection of a phylogenetically diverse group of taxa that exhibit outwardly similar cellular morphology and feeding behavior.

The amoeboid cell shape is highly amorphous, but many of these species possess mineralized skeletal structures that provide support for the pseudopodia and serve as taxonomic characters for identification. These skeletal structures can be elaborate, beautiful fabrications composed of silica, calcium carbonate, or strontium sulfate. Their presence allows many of these species to attain macroscopic size.

Lobose Amoebae. The simplest amoeboid forms are those bearing lobate, conic, or tubelike pseudopodia (Fig. 2a). This polyphyletic group contains the species that are most commonly encountered in introductory biology courses. Some lobose amoebae completely lack shells (the "naked" amoebae), whereas others bear simple scales, shells composed of secreted organic substances or agglutinated materials from the environment (sand grains, algal scales, etc.).

Lobose amoebae move by extending pseudopodia and drawing the remaining cytoplasm forward into the pseudopodia. Prey capture is accomplished by engulfing prey as the cell moves along a surface. Characteristic features for identification include cell size, shape, pseudopodial type, the presence or absence of cysts, and the form taken by the cell when it is dislodged from a surface (i.e., the "floating form"). Species of amoebae are typically identified from characters present in living specimens because preservation can distort cell and pseudopodial morphology. Thus, the taxonomic system is not very useful for ecological studies of natural protozoan assemblages in which many samples may be collected in a short period of time and preserved for later analysis. As a result, information on the distribution, abundance, and overall impact of these species in estuarine and marine ecosystems is meager relative to shelled amoeboid forms and other protozoa in general.

Free-living marine amoebae range in size from several micrometers to more than 100 μm. Most consume prokaryotes, microalgae, and other protozoa. Amoebae are most common on surfaces and in sediments in estuarine benthic ecosystems (16,17). Abundances of tens per cm^2 have been reported on macroalgal surfaces, although abundances in sediments range up to several thousand per cm^3. Amoebae are also present in the plankton in which they presumably colonize biotic and abiotic surfaces and the air-water interface (18–23).

Foraminifera. The foraminifera are a wide-ranging group of amoeboid protozoa possessing complex, branching networks of pseudopodia and shells (tests) composed of organic material, agglutinated particles, or calcium carbonate. The calcium carbonate tests of foraminifera preserve well, and the fossil record of these species is an important tool for paleoclimatological reconstruction (Fig. 2f). Foraminifera are conspicuous components of the marine benthos and the oceanic plankton (Fig. 2d). Benthic species are numerous, and specimens are abundant throughout all marine environments from salt marshes to the deep-sea, at abundances up to several hundred per cm^2 (24–26). Some species contribute significantly to the formation of coral reefs in tropical ecosystems. A small number of foraminiferal taxa have adapted to life in the water column. Planktonic forms are restricted to oceanic waters in which typical abundances are tens per liter for juvenile specimens to tens per m^3 for adults (27). One species even survives being frozen into polar ice (28).

Foraminifera form some of the largest individual structures of all protists. Disk-shaped benthic foraminiferal tests can exceed 1 cm in diameter (Fig. 2e), and some planktonic foraminifera form tests that are nearly a millimeter in diameter. Spines radiating from these latter tests produce an extraordinarily beautiful, delicate structure that can exceed a few centimeters in diameter. Large pseudopodial networks strewn upon these spines make these species highly conspicuous in the oceanic plankton.

Many of the foraminifera have lengthy life spans compared with other protozoa. The life cycles of relatively few species have been accurately described at this time, but those that have been characterized indicate complex life histories involving alternation of sexual and asexual life stages. Developmental times (from zygote to reproductively mature adult) can be weeks to months and perhaps longer. This can result in a wide spectrum of sizes for individual foraminiferal species in natural communities.

Large size for many species allows foraminifera to capture an enormous variety of prey ranging from bacteria to metazoan species (12,13,29). Virtually any organism that can be immobilized and infiltrated by the pseudopodial network will be consumed. Cannibalism has been observed in laboratory cultures.

Feeding rates on prey in these species are highly variable. This variability is due in part to differences in size and pseudopodial mass. It is also due to the

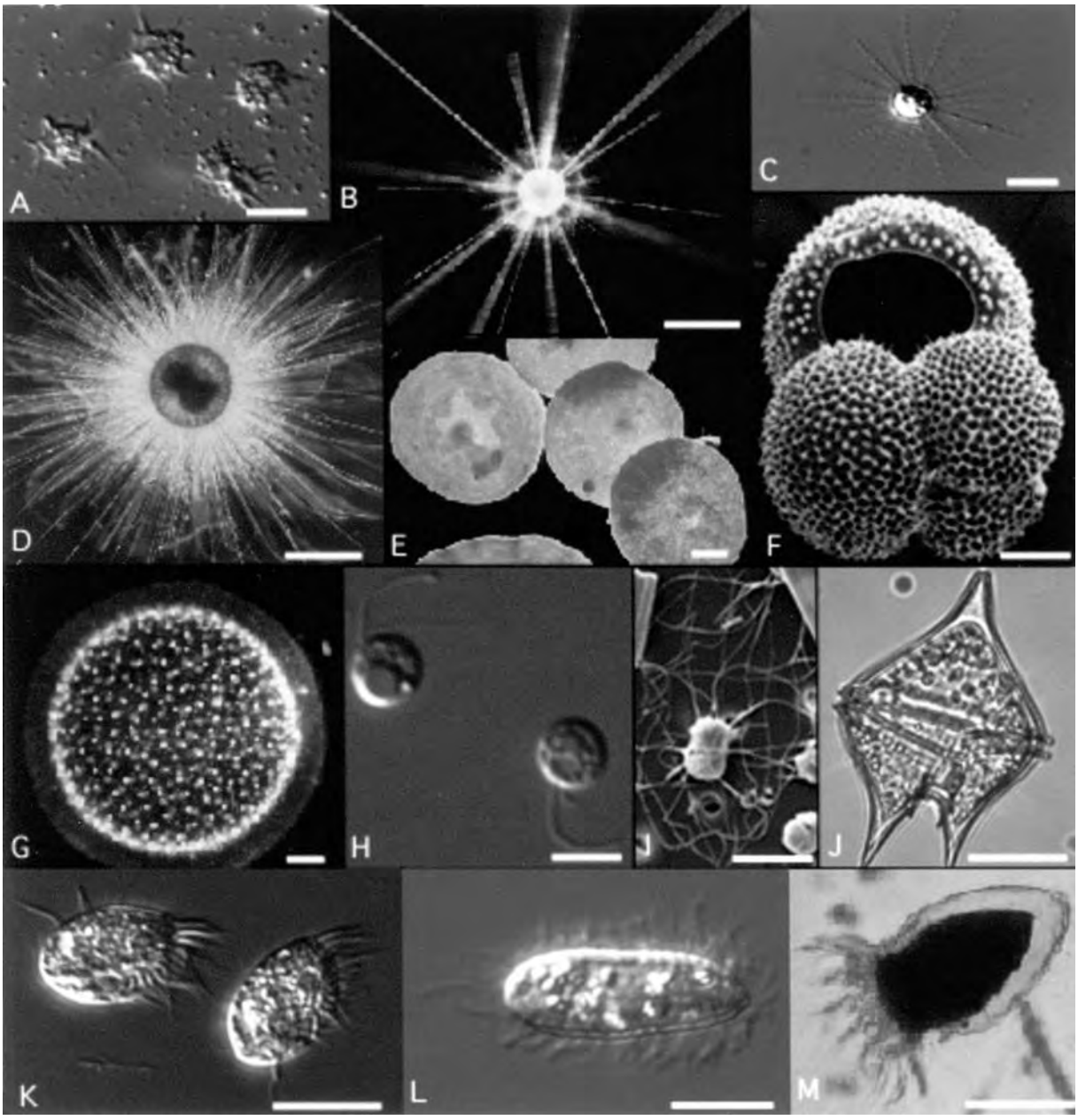

Figure 2. Micrographs of representative taxa of amoeboid, flagellated, and ciliated protozoa. (**a**) Four lobose amoebae enriched from the coastal plankton. (**b**) A live, oceanic acantharian with radiating spines of strontium sulfate. (**c**) An estuarine heliozoan. (**d**) A live planktonic foraminifer with radiating spines and a thick pseudopodial net. (**e**) Calcium carbonate tests of large, benthic, tropical foraminifera. (**f**) Calcium carbonate tests of a planktonic foraminifer. (**g**) A "small" gelatinous colony of living polycystine radiolaria. These colonies can reach meters in length. (**h**) Small chrysomonad flagellates. (**i**) Electron micrograph of a choanoflagellate. The dried basketlike structure is visible around the specimen. (**j**) A heterotrophic dinoflagellate. (**k**) Two hypotrichous ciliates showing azoral zones of membranelles (to the right) and cirri (upper left on lefthand specimen). (**l**) Scuticociliate showing relatively uniform ciliature on its surface. (**m**) Tintinnid ciliate. The transparent lorica is apparent around the opaque ciliate. Note similar body plans among the larger amoeboid forms (**b,c,d,g**). Also note that the overall size differential among these species is more than three orders of magnitude. Marker bars indicate 5 (**i**), 10 (**h**), 20 (**c,j,l**), 30 (**a**), 50 (**k,m**), 100 (**b,f**), 500 (**d**), and 1,000 (**e,g**) μm.

fact that many shallow-dwelling species derive nutrition from symbiotic algae that are present in high abundance within the cytoplasm. Symbiont photosynthesis in some foraminiferal species can provide sufficient nutrition to support normal vegetative growth without the need for particulate food, whereas other species require particulate food (30). Even among the latter species, symbiont nutrition can be important for survival in highly oligotrophic environments (31).

Actinopoda. The actinopod protozoa are a polyphyletic assemblage composed of amoeboid protozoa that form pseudopodia that are stiffened by microtubules. Traditionally this group has included the Acantharea, Polycystinea, Phaeodarea, and Heliozoea. The polycystines and the phaeodaria are still referred to, collectively, by the older term "radiolaria." The acantharia and radiolaria are unique in that most species are planktonic and largely restricted to oceanic environments. Similar to the foraminifera, these specimens can form complex networks of pseudopodia and (in some cases) mineralized skeletal components that are visible to the unaided eye. Some species of polycystines form colonies that are easily mistaken by divers for strings or clumps of minute eggs (the central capsules) embedded in a gelatinous matrix (Fig. 2g). Collectively, acantharia, radiolaria, and foraminifera are often the most conspicuous macroscopic organisms in tropical and subtropical pelagic ecosystems.

The actinopod taxa are distinguished from one another on the basis of details of cell morphology and ultrastructure as well as the geometry and composition of mineralized structures (when present). Acantharia produce strontium sulfate skeletal structures in some variation of either 10 or 20 radially oriented spikes (Fig. 2b). Radiolaria produce siliceous skeletons (when present) of diverse architecture, whereas members of the polyphyletic heliozoa produce organic or siliceous structures.

At a gross level of comparison, acantharia and radiolaria are functionally similar to planktonic foraminifera because of analogous cell designs (see Figs. 2b–d,g). This similarity results in a high degree of overlap in the types of prey captured by these species (12). Further ecological similarity is shown by the fact that many surface-dwelling acantharia and polycystines also possess intracytoplasmic symbiotic algae that contribute to the nutrition of the host. The morphological resemblance and trophic activities displayed by these species may represent convergent evolution to a body form and trophic activity that is particularly suitable for life in highly oligotrophic oceanic ecosystems.

In contrast to the acantharia and radiolaria, the heliozoa or "sun animalcules" are largely neritic where they are common in the plankton and benthos. These specimens also possess radially oriented pseudopodia (Fig. 2c). Although they have been historically classified as actinopods, the heliozoa appear to bear little phylogenetic relationship to polycystines, phaeodaria, or acantharia. Their shape is presumably an example of convergent evolution toward a comparable cell design.

Information on the distributions and abundances of polycystines and phaeodaria in the ocean are largely confined to skeleton-bearing forms that can be collected in plankton nets. As with the foraminifera, abundances vary greatly with location, depth, and whether or not one includes juvenile forms, but values of tens per m^3 to tens per liter are common (27). Acantharia can be considerably more abundant than radiolaria, but fewer data exist because their skeletons readily dissolve in most common preservatives. Acantharian abundances of tens per liter are typical. Heliozoan abundances are not well documented.

Flagellated Protozoa

This polyphyletic group of protozoa is composed of a wide assemblage of taxa that possess one to several eukaryotic flagella, flexible structures strengthened internally by a characteristic arrangement of microtubules. Flagella are responsible for locomotion in these species, and aid in feeding, either directly or via the creation of water currents that cause food particles to contact the cell surface where ingestion takes place.

Dinoflagellates are the largest (some $>100\ \mu m$) and probably the most conspicuous flagellated protozoa in the ocean (Fig. 2j). This group is usually claimed by botanists as a phytoplankton taxon, but nearly half of these species are heterotrophic and many chloroplast-bearing forms are actually mixotrophic (capable of prey ingestion as well as photosynthesis). Abundances of heterotrophic dinoflagellates can attain hundreds per ml even in oceanic plankton communities when diatoms (common dinoflagellate prey) are abundant (32), and photosynthetic species are well known as bloom formers. Dinoflagellates are biflagellate and usually possess a transverse furrow and longitudinal groove in which the flagella are situated. Their beating provides motility and gives most dinoflagellates a characteristic whirling or spiralling motion as they swim.

Dinoflagellates use a variety of mechanisms for obtaining nutrition (33). Some phagotrophic forms can feed by engulfing prey completely within the dinoflagellate cell. However, larger prey can also be attacked by means of a peduncle, or by extending a large feeding veil. The latter behaviors are used by some dinoflagellates to feed on animal prey or diatom chains that are often much larger than the dinoflagellate predator. Dinoflagellates can also be parasitic, invading appropriate hosts and multiplying within them. Finally, some heterotrophic dinoflagellates consume algae and digest most of the prey but retain the chloroplasts in a functional state for a limited period (34).

Another morphologically distinct group of flagellated protozoa is the Choanoflagellata. These uniflagellated species possess funnel-shaped or basketlike silicified structures or "collars" that aid in prey capture (Fig. 2i). They are common and abundant in the plankton. Abundances in coastal planktonic communities can reach thousands per ml (35,36), and these species appear to be particularly important in polar environments (37). Choanoflagellates are also common on surfaces to which they attach by means of a stalk. Individuals are mostly small ($<20\ \mu m$), but some attached and planktonic species can form large colonies. They feed primarily by phagocytosis of bacteria and cyanobacteria.

The remaining taxa of flagellated protozoa are less morphologically distinct (by light microscopy) than the dinoflagellates or choanoflagellates but no less important ecologically. Two groups of heterokont flagellates (cells bearing two flagella of unequal length and function) are particularly important; the chrysomonads and biocosoecids. Chrysomonads are small (generally <20 μm) flagellates often grouped with their chloroplast-containing relatives, the chrysophyte algae (Fig. 2h). Many chrysomonad species are devoid of chloroplasts, however, and feed by phagocytizing bacteria, small algae, and other small protozoa. These species are quite common in planktonic environments. The biocosoecids also possess heterokont flagellation but produce a lorica, a vaselike structure of chitin in which they live. These forms can be free-swimming or attached and, together with the chrysomonads and choanoflagellates, constitute an important source of mortality for prokaryotic organisms in the marine plankton.

The bodonids are a biflagellate taxon composed of small species (generally ≤ 10 μm) grouped together with the parasitic trypanosomes. Many bodonids are free-living, abundant in sediments, and are associated with suspended particulate material in the plankton in which they feed on attached and suspended bacteria (38,39). These species typically use one flagellum for maintaining contact with the substratum and the other for motility and prey capture.

In addition to these truly heterotrophic flagellates, a number of chloroplast-bearing protists (i.e., microalgae) also consume prey and can contribute to predation on bacteria, other algae, or protozoa in the ocean. These mixotrophic algae include numerous species of chrysophytes, prymnesiophytes, dinoflagellates, cryptophytes, and euglenophytes (40).

Collectively, the flagellated protozoa are probably the most numerous protozoa of both benthic and pelagic ecosystems. Typical abundances in the plankton range from hundreds per ml to tens of thousands per ml (41). Estimating the densities of minute flagellates in benthic ecosystems has proven more difficult because of the problems associated with distinguishing these small cells within the sediment matrix (42,43). As a consequence, less is known about the abundance or species composition of this community. Very little information is available for the deep-sea benthos (44–46).

Most studies of flagellate abundance in natural ecosystems have not reported the contributions of individual taxa of flagellates to total flagellate abundance because these communities are usually dominated by small species (<10 μm in size). The identifications of many of these small specimens are dubious when performed by light microscopy (the method of choice for enumerating these assemblages) because few morphological criteria can be distinguished at this level of examination. Electron microscopy, and sometimes observations of living specimens, is required for accurate identification of these taxa. Thus, most reports on the abundances of these species simply group them together as "colorless flagellates" or "nanoflagellates" (the latter term is a reference to their size; see Placing Protozoa into Models of Marine Food Webs, which is discussed later.)

Ciliated Protozoa

Ciliates appear to be the only monophyletic group of the three taxa of free-living protozoa thus described. These species are characterized by the presence of cilia (ultrastructurally similar to the eukaryotic flagellum) during some phase of their life cycle, two types of nuclei, and various aspects of cellular construction and reproduction. The ciliates have phylogenetic affinities with the dinoflagellates and the parasitic apicomplexans with which they share unique ultrastructural features (47). Recent systematic revisions group these three taxa into a single phylum, the Alveolata.

Ciliated protozoa tend to exhibit more structural complexity than the flagellates or amoeboid protozoa. Cilia are the fundamental character of the ciliates, but the nature and extent of ciliation can vary greatly among these species. For example, ciliature is totally lacking in the suctoria except for ciliated swarmers that are produced by budding from a parental cell. On the other end of the spectrum are species that have nearly uniform ciliation covering their surfaces. Other species form complex ciliary structures including an adoral zone of membranelles (rows of cilia fused to form membranelike veils that are used for locomotion and creating feeding currents) or cirri (groups of fused cilia that act as single appendages, usually for locomotion).

The site of phagocytosis on the surface of ciliates (the cytostome) tends to be more apparent in ciliates than in flagellates or amoeboid forms. It is often located in a furrow or depression and the cilia (when present) are typically oriented to create water currents that will deliver suspended particles to this area. The nonciliated suctoria possess tentacles that are used for grasping/holding prey and removing their cellular contents.

Ciliates are common and important protozoa in pelagic and benthic marine ecosystems. However, the taxonomic composition of the group varies considerably between these two environments. Marine planktonic ciliate communities are typically dominated by spirotrichous ciliates with a few notable exceptions. The spirotrichs include the tintinnids that live within loricae, and "naked" oligotrichs that lack loricae (Fig. 2m). These planktonic species play a major role in the consumption of small phytoplankton (and presumably small protozoa). Bacterivorous species of ciliates within the class Oligohymenphorea (e.g., *Uronema, Pleuronema*) occasionally become abundant in coastal and estuarine waters when prokaryote abundances are high (Fig. 2l). In addition, predatory ciliates within the subclass Haptoria often occur in the plankton. Abundances of planktonic ciliates range up to tens per ml for coastal ecosystems and approximately an order of magnitude less for oceanic ecosystems (48,49).

A common phenomenon among many planktonic marine ciliates is the ability to ingest algal prey and retain the chloroplasts of these prey in a functional state inside the ciliate for a limited period of time (generally less than a few days). This ability to retain functional chloroplasts provides these ciliates with additional nutrition relative to purely heterotrophic species. Not surprisingly, these mixotrophic species can constitute a very significant fraction of the total

assemblage of planktonic ciliates (50). One common "bloom-forming" genus (*Mesodinium*) is occasionally able to attain extremely high abundances (51).

The ciliate fauna of marine benthic environments is abundant, and typically much more diverse than planktonic assemblages. This tendency presumably reflects the greater diversity of physical microenvironments and generally higher abundances of prey in the benthos relative to pelagic ecosystems. Sediment grain size, organic loading, oxygen penetration, and other factors affect the ciliate community structure (52–54). Abundances of ciliates in coastal sediments can reach thousands per cm^2, with highest abundances in the oxygenated zone. Benthic ciliates often have elongated shapes and are highly flexible to allow penetration into and among sediment particles. The cilia of many of these species are often modified into fused clusters (cirri) that are used for crawling, and large membranelles for creating water currents to transport food to the cytostome (Fig. 2k).

Feeding of benthic ciliates has been addressed only sporadically. The definitive work on this topic, now more than 30 years old (55), demonstrated some degree of feeding specialization among benthic ciliates but also a fair degree of overlap in the type of prey consumed. This result is somewhat analogous to feeding preferences observed for planktonic foraminfera, acantharia, and radiolaria (12).

Biotic and abiotic surfaces are also important environments for ciliate colonization and growth. Species of vorticellids (*Vorticella, Zoothamnium*) and suctoria (*Acineta*) are commonly found in biofilms, or "Aufwuchs" communities, as are surface-associated forms of hypotrichous ciliates such as *Euplotes*.

ECOLOGICAL ROLES OF PROTOZOA

Most protozoan species are microscopic, but the magnitude of the ecological roles they play and their biogeochemical activities in marine ecosystems are anything but minute. Protozoa are the major consumers of microorganisms in the ocean, and are in turn prey for a wide variety of protistan and metazoan organisms. As such they constitute important trophic links between a variety of microbial taxa and larger consumers in marine food webs. In addition, protozoa remineralize and release what they do not incorporate, functioning as sources of dissolved and particulate organic matter in the ocean. Remineralization of organic material by protozoa is thus an important mechanism by which primary producers obtain essential nutrients.

Protozoa as Trophic Links in Food Webs

Protozoa constitute crucial links in marine food webs. With the exception of a few crustacea (mostly in freshwater), some marine tunicates, bivalves and sponges that feed on exceptionally small particles, they are the primary biological link between small (<2–3 μm) microorganisms and larger metazoa in marine food webs. Their grazing activities are essential for the conversion of the smallest marine microorganisms into particles large enough to serve as prey items for metazoan grazers such as copepods, fish larvae, and other suspension-feeding organisms in the plankton and benthos.

Protozoa are particularly important in habitats (such as oligotrophic gyres) where the primary producer assemblage is dominated by small algae and cyanobacteria (i.e., by species that are too small to be captured by most metazoa). They are also important where the production of living biomass is dominated by bacteria or detritus-based food webs (e.g., areas with high loading of labile organic material, and most oceanic ecosystems below a few hundred meters depth). In these locations protozoa may serve as the primary source of food for larger mesozooplankton or suspension-feeding benthic species.

Protozoa as Predators (Bacterivory, Herbivory, Carnivory). The rapid growth rates of protozoa in the ocean imply that significant amounts of prey biomass are consumed in marine food webs. Indeed, clearance rates (volume of water cleared of prey per unit time) and ingestion rates (number of prey organisms consumed per unit time) are much higher for protozoa on a "per weight" or "per volume" basis than for most metazoan species. These high weight-specific metabolic rates are consistent with well-known allometric relationships between the size of organisms and their rates of metabolism.

Gross growth efficiencies of protozoa (the percentage of prey biomass converted into protozoan biomass) are also high, relative to conversion efficiencies for most metazoa. Growth efficiencies reported in the literature vary widely, but a median value for most protozoa appears to be 30 to 40%, (56) that is, 30 to 40% of the prey biomass ingested by these species is converted into protozoan biomass. Thus, these species are efficient at "repackaging" small prey into particles that might be large enough to serve as food for species at higher trophic levels.

Marine protozoa are important consumers of prokaryote biomass (bacteria and cyanobacteria). Small flagellates appear to be the major bacterivores in many planktonic ecosystems (57), whereas ciliates and amoebae make a more significant contribution to bacterivory in benthic ecosystems. Lysis by bacteriophage is also an important source of mortality for marine bacteria, but the production of viral particles does not contribute directly to the nutrition of higher organisms. On a global basis, bacterial production must be matched by bacterial mortality because the overall abundance of these prokaryotes remains relatively constant over long timescales. Analysis of a large data set spanning observations in numerous marine and freshwater environments indicated a direct 1 : 1 relationship between bacterial production and bacterivory (41). This relationship implies a close match between production of bacteria on one hand and removal on the other hand. Protozoa play a fundamental role in the removal process.

Protistan herbivory of cyanobacteria and phytoplankton is also an important trophic process in the ocean. Ciliated protozoa alone probably remove more than half of the annual primary production in some marine environments. Ciliate herbivory can equal or even exceed production on short timescales (48,58). One can expect similar rates of consumption of small protozoa by ciliates,

but this form of protozoan predation is poorly quantified. Heterotrophic dinoflagellates play a significant role as herbivores when diatoms dominate the phytoplankton community (32,33).

Rates of ingestion among protozoa are highly variable. They are dependent on the protozoan species and its mode of prey capture, the type of prey being ingested, prey abundance, and the environmental conditions (particularly temperature). Maximal ingestion rates of bacteria by protozoa typically vary from a few per hour up to several tens per hour for small flagellated protozoa (i.e., 2–10 μm in size), to several thousand per hour for bacterivorous ciliates (20 to 50 μm in size). The former species rely on particle interception or weak feeding currents created by their flagella to capture prey. Many of these species handle and ingest bacteria individually. In contrast, most ciliate species use complex ciliary structures and rapid filtration rates to capture large numbers of bacterial prey.

Capture of animal prey (carnivory) by large amoeboid protozoa is at the opposite end of the ingestion rate spectrum. Some planktonic foraminifera that prey on copepods may capture only one prey organism every several days (29). This rate still constitutes a very significant source of nutrition because of the relatively large size of the prey. Ingestion rates of other free-living protozoa feeding on microalgae and protozoa are intermediate to the extremes just mentioned.

Protozoa feeding at maximal rates in laboratory cultures typically can consume their body weight in a short period (a few hours to a few days depending on the species), although these maximal rates may seldom be attained in nature. The ingestion rates of free-living phagotrophic protists in marine ecosystems are probably most affected by prey abundance. Suboptimum prey abundance results in a rapid decline in the ingestion rates for these species (59). Some species also exhibit "grazing thresholds"; prey concentrations below which feeding activities are either curtailed or are less effective at capturing prey (Fig. 3). In addition to prey abundance, ingestion rates are further modified by temperature, light, and other physical/chemical factors (60,61).

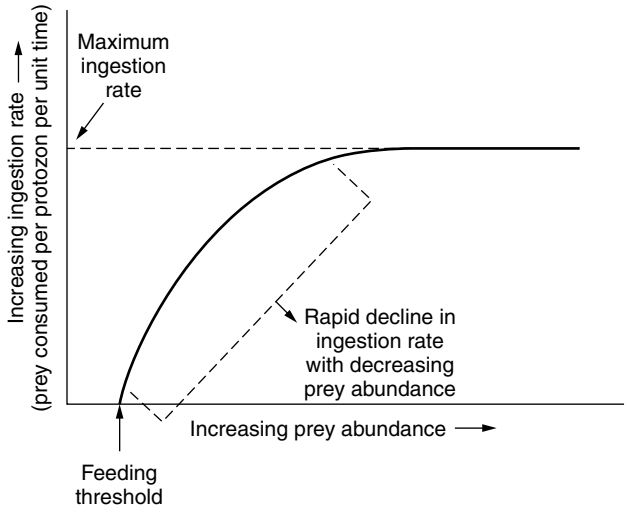

Figure 3. Idealized relationship between ingestion rate and prey abundance for phagotrophic protists. Feeding for some species does not take place below some nominal prey abundance (the feeding threshold; lower arrow). Above the threshold concentration, ingestion rate increases in direct proportion to prey abundance (bracketed area). At higher prey concentrations ingestion rate stabilizes at a maximum value (upper arrow). The maximum rate is dependent on the rate at which prey can be ingested, digested, and remains voided by the protozoa. The specific shape of this generalized curve varies with protozoan species and prey type.

Symbiosis and Mixotrophy. Most studies of protozoan trophic activity in marine ecosystems have focused on their roles as bacterivores, herbivores, and, to a lesser extent, as carnivores. However, protozoa participate in other types of interspecies interactions including various forms of symbiosis (used here in the general sense of "organisms living together"). These interactions are generally classified on the basis of whether they are beneficial, harmful, or neutral for the two partners.

Parasitic protozoa obtain nutrition from their respective hosts. This relationship is obviously beneficial to the protozoan but harmful to the host. Invasion of the host and evasion of the host's immunological defenses play important roles in obtaining nutrition. Protozoan parasitism in humans constitutes a major health issue in our society, and considerable effort has been expended to understand these infections. Much less is known regarding protozoan parasitism in the ocean. Parasitism of algae, other protozoa, and numerous multicellular organisms have been described, but extant knowledge indicates that parasitic forms are not a major component of the total abundance or biomass of protozoa in marine ecosystems. Nevertheless, there is a growing realization that these species can be an important factor in determining the population dynamics of their hosts (62–64).

A few protozoan classes are composed exclusively (or nearly so) of parasitic species. For example, the apicomplexans are obligately parasitic species whose free-living stages serve the purposes of dispersal and reinfection. Other taxa contain mixtures of free-living and parasitic species. A number of kinetoplastid flagellates are parasitic (e.g., trypanosomes that cause human and other animal diseases), whereas other species within this taxon are free-living and primarily bacterivorous (e.g., many bodonid flagellates). A significant number of dinoflagellates are parasites in other organisms, including a number of protists (e.g., in some dinoflagellates, radiolaria, and ciliates). One of these species, *Pfiesteria piscicida* has recently achieved infamy as a parasite and pathogen of fish, and for the amnesic effects on short-term memory in humans caused by exposure to this organism (65).

Mutualisms are associations between species in which both partners benefit from the association. Although the details of many of these relationships are not well characterized, many of these partners appear to trade nutrition for protection. Mutualistic relationships involving protists are numerous and diverse. They may involve two protistan

species (e.g., many foraminifera, radiolaria, and acantharia harbor photosynthetic dinoflagellates as intracellular symbionts), or they may involve a protistan species and a metazoan or prokaryotic species. Many well-documented mutualisms exist between photosynthetic dinoflagellate symbionts and cnidarian hosts (66). A number of mutualistic symbioses also exist between heterotrophic dinoflagellate hosts and photosynthetic cyanobacterial symbionts that colonize their outer surfaces (67). The photosynthetic symbionts in these relationships contribute to the nutrition of their hosts, presumably in exchange for the physical protection afforded by the host. These symbiotic associations appear to be excellent strategies for the host to cope with the highly oligotrophic conditions of oceanic ecosystems.

Commensalisms are associations between species in which one partner may benefit but the other is neither benefited nor harmed. Many protozoa exist as ectocommensals, attaching to or associating with the exterior surfaces of other protists, macroalgae, plants, or animals. An interesting example in the estuarine plankton is the attachment of vorticellid ciliates to the posterior end of some copepods (68). This relationship appears to have little effect on the copepod but presumably benefits the ciliate by creating water currents that bring prey to the ciliate, or perhaps by carrying the ciliate into an environment with high prey abundance.

Mixotrophy among protists is defined as the combination of photosynthetic and heterotrophic nutrition in a single individual. There are many variations on this theme among phagotrophic protists. One type of mixotrophy involves protozoa that are capable of ingesting and digesting microalgal prey but retaining the prey chloroplasts and maintaining them in a functional state (69). Several ciliate species, heterotrophic dinoflagellates, and at least one benthic foraminifer are capable of chloroplast retention. The chloroplasts retain photosynthetic ability in their new hosts for periods ranging from a few days to weeks but eventually lose function and are replaced with new chloroplasts from ingested prey. This ability enables these protozoa to function partially as photosynthetic organisms.

Another common form of mixotrophy involves the ingestion of particulate prey by photosynthetic flagellated protists. These species possess their own "true" chloroplasts but have also the ability to phagocytize prey (usually bacteria or other small protists). Numerous species of microalgae from a diverse array of taxa possess this form of mixotrophic ability (40). Prey ingestion in photosynthetic flagellates may serve several nutritional purposes including the acquisition of energy, major nutrients (nitrogen or phosphorus), or other specific growth factors such as vitamins or trace metals that might otherwise be difficult for them to obtain.

Protozoa as Prey. The biomass of phagotrophic protists in the marine plankton may be on the order of 10 to 20% of the total living biomass in these ecosystems and may comprise as much as 50% of the biomass available for consumption by planktonic metazoa. A global-scale synthesis has not yet been conducted but several regional surveys indicate that protozoa are a quantitatively important component of pelagic communities (32,49,70–72). Heterotrophic flagellates in the 2 to 20 μm size class of coastal and pelagic ecosystems can be as abundant as algae in the same size class, whereas ciliates and larger heterotrophic dinoflagellates constitute a significant fraction of total living biomass in the 20 to 200 μm size class. These heterotrophic assemblages are utilized as food by rotifers, cladocerans, some copepods, fish larvae, and many invertebrates (58,73). Unfortunately, the overall nutritional value of protozoan biomass to most zooplankton species is still not resolved.

In the benthos, recent work has shed light on the potential importance of protozoa as a food source for filter-feeding bivalves such as oysters (74). Bivalves efficiently retain particles over the entire size range of protozoan species. Protozoa act as a vital trophic link by repackaging very small primary producers and bacteria into sizes that can be effectively captured.

Protozoa as Trophic Sinks in Food Webs: Carbon and Nutrient Remineralization

Although biomass incorporated into protozoa is available as food for larger organisms capable of capturing them (i.e., a trophic "link" in food webs), unincorporated material is lost to the process of trophic transfer (i.e., a "sink" in these food webs) through respiration and the release of undigestable remains. These latter materials serve as substrates and nutrients for bacteria, phytoplankton, and other organisms capable of taking them up from seawater. Thus, what constitutes a "sink" for higher organisms actually provides a source of energy and/or nutrients for other biological components in marine food webs.

Early conceptualizations of the role of protozoa in marine food webs tended to argue that protozoa act rather exclusively as either links or sinks, but more recent depictions have recognized that they play a role in both functions simultaneously. Unincorporated material released by protozoa includes both organic and inorganic (i.e., remineralized) compounds. Respiration can oxidize food completely to carbon dioxide and inorganic nutrients, or digestion may be incomplete and result in the release of a variety of organic compounds.

On average, 60 to 70% of the prey biomass ingested by protozoa is released to their seawater environment. This release contributes significantly to the pool of inorganic nutrients and labile organic compounds utilized by bacteria and primary producers. Approximately half of the prey carbon that is not incorporated into protozoan biomass during digestion is released as particulate and dissolved organic material (75–79). These materials increase the concentration of labile dissolved organic compounds that are taken up by bacteria for energy and production. Thus, on one hand protozoan trophic activities act to reduce bacterial abundance by ingesting these prokaryotes, whereas on the other hand they stimulate bacterial growth by releasing organic matter that can serve as bacterial substrate.

The paucity of bioavailable forms of nitrogen and phosphorus is a major factor limiting primary production in most marine waters. Thus, the remineralization and

release of nitrogen and phosphorus by heterotrophic protists may constitute vital sources of these elements in the ocean (80). Most remineralized nitrogen and phosphorus is released by protozoa as ammonium and phosphate, respectively, and these compounds are readily taken up by most algae. Bacterivorous protozoa may play a particularly important role in this regard. By consuming bacteria and remineralizing a portion of the bacterial biomass into inorganic compounds, these protozoa serve a dual role of providing inorganic nitrogen and phosphorus to primary producers and removing bacteria that might compete for these materials with the primary producers (81,82). The importance of protozoa in remineralization processes has been recognized for many years and has been put to practical use in the biological treatment of sewage to reduce bacterial biomass, organic content, and biological oxygen demand.

Placing Protozoa into Models of Marine Food Webs

Aquatic ecologists have endeavored to place phagotrophic protists, and their biological significance, into the overall context of marine food webs since the inception of the concept of a "microbial food loop" in the 1970s (83). This has proven to be a daunting task because of the extremely high species diversity and huge range in size, form, and function encompassed by this group. Therefore, most formal modeling exercises have tended to aggregate protists into ecologically relevant groupings or "compartments" to reduce protistan complexity to a manageable number for mathematical models (59,84–86).

A fundamental manner of sorting organisms in most food web models continues to be the time-honored distinction between photosynthetic and heterotrophic modes of nutrition. Thus, protists in these models are typically split into "algae" and "protozoa" according to traditional descriptions of unicellular eukaryotes. There is generally no regard for mixotrophy or symbioses in these compartmental models.

Size is also an easy characteristic by which to classify organisms, and this criterion has been commonly applied to protistan assemblages in food web models. For example, plankton biologists typically group organisms according to cell size, with each class comprising approximately one order of magnitude. One of the systems most commonly used for planktonic microorganisms was described by Sieburth and coworkers (87). It designates organisms as "picoplankton" (0.2 to 2 μm in size), "nanoplankton" (2–20 μm), "microplankton" (20–200 μm), "mesoplankton" (200 μm–2 mm), and so forth. Protozoa fall largely into the nano- and microsize categories, although there are conspicuous exceptions to this rule. Size characterization of phagotrophic protists is generally combined with the expectation that "larger things eat smaller things," and thus trophic relationships in mathematical descriptions of microbial food webs usually represent each size category as food for the next largest heterotrophic category.

The food web descriptions resulting from these exercises typically reduce protistan diversity into a few manageable compartments, and depict carbon and energy flow in the microbial community in the direction from photosynthetic compartments toward heterotrophic compartments, and from smaller species toward larger consumers. In addition, some adjustments in the number of biological compartments has been made in recent years to accommodate protistan species that participate in both photosynthesis and heterotrophy simultaneously (via mixotrophy or symbiotic relationships) (Fig. 4).

There are some distinct disadvantages of this "box model" approach for representing protozoan activities in the ocean. These models obscure much of the biological diversity expressed within this enormous group of species by reducing the complex ecological strategies of thousands of species to a few functional groups. Also, they misrepresent some of the details of trophic interactions. For example, the ability of many heterotrophic dinoflagellates to feed on diatoms larger than themselves is not encompassed by the size-dependent grazing scheme that most of these models employ.

Nevertheless, these formal descriptions of protozoan activities in food webs allow the role of these unicellular eukaryotic species to be placed within the context of all marine life. This work has focused attention on how fundamental protozoa are in the overall function of marine food webs and the important roles that they play in biogeochemical cycles within the sea. More appropriate (and accurate) depictions will emerge as new information on protozoan diversity and activity becomes available and is incorporated into models of microbial food webs.

CONCLUSION

There is now wide recognition of the essential ecological roles played by protozoa in estuarine and marine environments. These species are phylogenetically, morphologically, and trophically diverse. They are, as a group, virtually ubiquitous in all marine habitats where they play pivotal roles in food webs as consumers of bacteria, cyanobacteria, microalgae, and protozoa, and as prey for a variety of multicellular species. In addition, many protozoan species participate in a variety of symbiotic associations ranging from parasitism to mutualism, and numerous species are capable of combining phototrophic and heterotrophic nutrition (mixotrophy). Collectively, these single-celled organisms constitute a significant portion of the energy utilization and elemental flow in the ocean.

Most free-living protozoan species have one of three basic body plans (amoeboid, flagellated, and ciliated forms). The abundances and trophic activities (herbivory, bacterivory) of these broad assemblages of protozoa have been characterized for many estuaries and for surface waters of much of the world ocean. In many instances, protozoan biomass and activities have been placed into context with these features for other groups of organisms. Thus, we now have a reasonably good appreciation of the relative importance of protozoan assemblages in many marine food webs and biogeochemical cycles. Recent mathematical models of energy and carbon flow have included protozoa as integral components of system function.

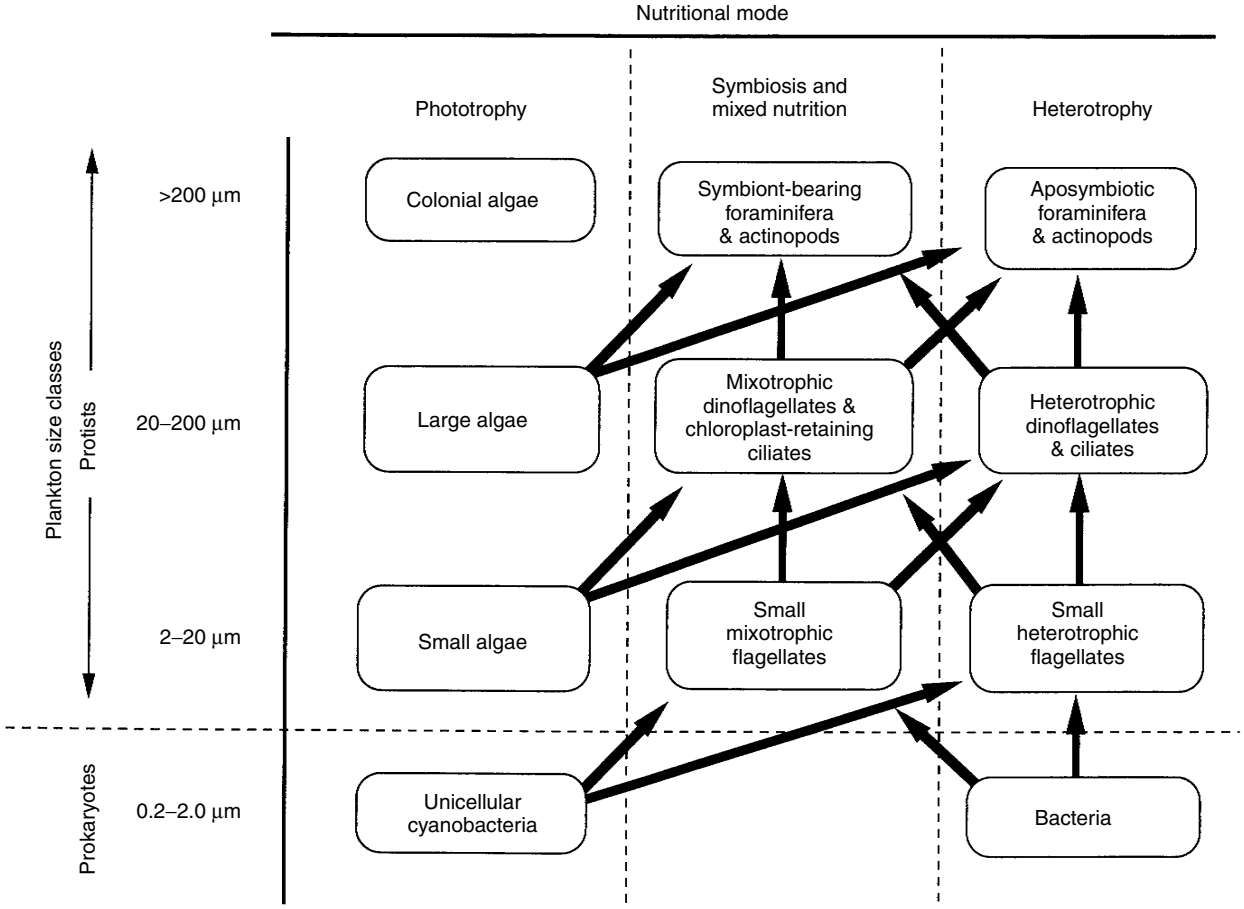

Figure 4. Box model of a generalized microbial food web indicating the roles of protozoa. Protists (and prokaryotes) are represented as aggregations of species of similar ecological function (i.e., protists with similar trophic status and activity). The species in this model have been aggregated according to nutritional mode (phototrophy, mixotrophy/symbiosis, heterotrophy) and by size. Energy is fixed into organic material by photosynthesis conducted by organisms in the two left-hand columns. Biomass is consumed by organisms in the two right-hand columns. Arrows indicate the presumed predator-prey interactions in the food web. In this particular model it is assumed that consumers eat all microorganisms one order of magnitude smaller than themselves. (modified from Caron and Finlay, 85).

Compared with significant progress in placing protozoa into proper ecological context, however, there is presently less consensus concerning the true species diversity within the group. Historically, these species have been defined morphologically, and it has been difficult to obtain a consensus regarding the validity of species distinctions based on minor differences in body size, shape, and ultrastructural features. This controversy regarding species diversity should diminish as new genetic methods of identification are intertwined with morphological criteria of species descriptions. In addition, as more physiological information is generated from cultured species of protozoa, this information should help define the species concept of these complex and fascinating creatures.

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PROTOZOA IN STREAMS. See STREAM MICROBIOLOGY

PROTOZOAN CILIATES IN FRESHWATER ECOSYSTEMS

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The term protozoa has traditionally been used as an all-inclusive designation for the most primitive unicellular and colonial animals (1). Recognizing the transitional nature of many of these forms and the possession of both plant and animal characteristics and functions by some, protozoans have now been divided into seven separate phyla. Most orders of the protozoan class Phytomastigophorea are considered plant phyla by phycologists because of their photosynthesizing ability. Likewise, four classes of the subphylum Sarcodina and the phylum Labyrinthomorpha are considered slime molds by mycologists. Even within the ciliated protozoans (phylum Ciliophora), there are mixotrophs, which possess both plant and animal characteristics.

Ciliates belong to the phylum Ciliophora, a group distinguished by the presence of cilia during at least one stage of their life cycle. There are eight classes of the phylum Ciliophora, of which five represent the dominant ciliates in freshwater lakes: Colpodea, Litostomatea, Nassophorea, Oligohymenophorea, and Spirotrichea. Colpodea possess resting cysts and are represented by the order Colpodida in freshwater. The Litostomatea are characterized by simple oral cilia and are represented in freshwater lakes mainly by the order Haptorida (representative genus *Mesodinium*). Many Nassophorea possess cirral polykinetids and are represented in fresh waters by the subclass Hypotrichia, order Euplotida (genus *Euplotes*). Perhaps the best-represented class of ciliates in lakes is Oligohymenophorea, a group characterized by oral structures in a ventral oral cavity and an oral apparatus, when present, which is distinct from body cilia. Of its two orders, Hymenostomatida (genus *Colpidium*) and Scuticociliatida (genus *Cyclidium*), the latter is dominant in most lakes, especially those of higher trophic state. Spirotrichea is the second most important class of ciliates in the plankton of freshwater lakes and is best represented in oligotrophic to mesotrophic lakes. These ciliates possess conspicuous right and left oral and/or preoral ciliature and are represented by two important orders in the freshwater plankton, Oligotrichida (genus *Strombidium*) and Choreotrichida (genus *Strobilidium*).

There are three additional minor classes of ciliates. Class Prostomatea possesses a cytostome that is apical to subapical and is represented mainly by the order Prorodontida (genus *Urotricha*). Karyorelictea are long, vermiform, and flattened, and are mainly components of the benthos. Phyllopharyngea have radially arranged microtubular ribbons.

Reproduction within the eight classes of Ciliophora is typically via transverse fission, but budding and multiple fission have also been reported (1). Sexuality involves conjugation, autogamy, and cytogamy. Under ideal conditions, populations can double in a matter of hours. Movement is achieved through synchronous beating of cilia that are arranged around the oral cavity and/or along the length of the organism, resulting in spiral movement through the water by many taxa. Ciliates range in size from smaller than 10 μm to larger than 3 mm, and taxonomy is based on features of the oral region and the positioning and patterning of cilia.

The vast majority of investigations of ciliates in freshwater ecosystems have focused on planktonic, and to a lesser degree benthic, assemblages in lakes. In addition to large-bodied predators feeding on smaller ciliates and rotifers, most ciliates are grazers on bacteria and algae that are funneled into their buccal cavity as they spiral through the water. The relative proportion of bacteria and algae in the diet is dictated by ciliate body size, with those taxa smaller than 30 μm feeding predominately on bacteria (2). The largest-bodied taxa are found at the sediment–water interface and only enter the water column during times of extreme hypoxia or anoxia in this zone (3). Within the water column, there is a dominance shift from large-bodied (>40 μm) to small-bodied (<30 μm) taxa with progressive eutrophication (4). Recently, increased attention has been given to mixotrophic taxa, those predominately large-bodied ciliates contributing to ecosystem primary productivity via either endosymbiotic algae (5) or organellar sequestering of plastids from ingested algae (6).

Traditionally, freshwater zooplankton research was limited to those forms retained by vertical tows through the water column with Wisconsin plankton nets equipped with either a 158-μm (#10 silk bolting cloth) or a 76-μm (#20 silk bolting cloth) filtering surface. The smaller mesh size was used in cases in which particular emphasis was to be placed on microzooplankton (rotifers and copepod nauplii) in addition to macrozooplankton (cladocerans and copepodite or adult copepods) (7,8). Protozoan ciliates were not components of routine zooplankton investigations until the 1970s because they were not retained by accepted net-sampling techniques, were severely damaged during water filtration, and/or were destroyed by the harsh preservatives used for zooplankton. Far too many early limnologists defined the biotic realm of the field narrowly to the exclusion of fish at the upper end of the food web and microorganisms at the lower end. Protozoans were considered a part of the latter. Fortunately, such omissions have been corrected, and protozoans are recognized as essential components of the structure and function of freshwater ecosystems.

SAMPLING AND LABORATORY APPROACHES

Recognizing problems associated with use of traditional plankton nets, most investigations of planktonic ciliates

in lakes rely on preserved ciliates settled out from discrete interval or integrated samplings of the water column. Such an approach produces statistically more reliable counts than those obtained from net or pump sampling of the water column (9). Cairns and his associates pioneered the use of artificial substrates, especially polyurethane foam, for temporally integrating ciliate composition in the sediments and water columns of streams, rivers, wetlands, and lakes (10–13). A subsequent investigation by Foissner and coworkers (14), however, demonstrated that such polyurethane foam substrates sampled significantly fewer (122 taxa) ciliates than were isolated from the natural substrate of a European river (174 taxa), whereas the use of litter bags as a substrate medium (150 taxa) was statistically comparable to the natural substrate values. To date, there is no standard methodology for sampling ciliates in freshwaters, but artificial substrates are still viewed favorably by many investigators because of the need to integrate assemblages in water quality studies temporally.

Delicate taxa of rotifers and ciliates can disintegrate in the 4% buffered formalin solution long used to preserve zooplankton samples (8). Instead, ciliate samples have been preserved either with mercuric chloride after staining with bromophenyl blue (4,15) or Lugol's solution, especially if samples are also being utilized for phytoplankton enumeration. James (9) noted that counts of most ciliate taxa were significantly higher in samples preserved with Lugol's solution and mercuric chloride rather than either formalin or glutaraldehyde. Concerns over mercury toxicity, however, favor the use of Lugol's solution for preservation.

Routine zooplankton enumeration is often done under a light microscope using Sedgwick-Rafter cells. Detailed taxonomy for rotifers, however, often requires slide preparation of mouthparts; and dissection of individual legs is needed for determination of adults of copepods and some copepodite stages. Because of their generally smaller body size and the need for close examination of cilia placement patterns, routine counts of protozoan ciliates are often based on settled samples in Utermohl chambers (16) examined at 400X, using an inverted microscope. Many taxa require even higher magnification and resolution for proper identification. Ciliate biomass is calculated by assigning a geometric shape to each taxon and estimating cell volume based on cell dimensions, and then applying a specific gravity of 1.025 and a carbon content of 7.5% (4).

SEASONALITY

Although best developed for the subtropics and tropics, a generalized pattern of planktonic-ciliate seasonality in lakes is beginning to emerge (Fig. 1). Oligotrophic lakes generally display maximum biomass of ciliates during fall (17–20) associated with expansion of oligotrich populations. Secondary components of the assemblages, Haptorida and Scuticociliatida, also peak during this period. Mesotrophic lakes often display bimodal peaks during late spring and early fall dominated by Scuticociliatida (17,21). Finally, eutrophic and hypertrophic lakes are characterized by peak planktonic ciliate biomass during summer dominated by small-bodied Scuticociliates (17). Ciliate abundance can display differences according to trophic

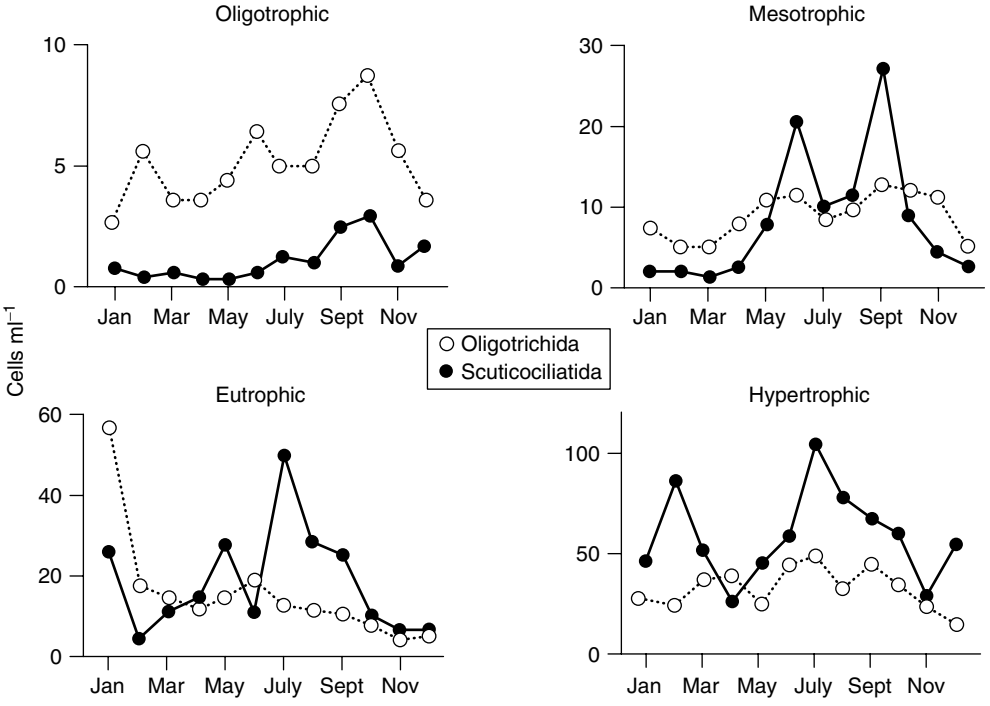

Figure 1. Seasonality of the protozoan orders Oligotrichida and Scuticociliatida in Florida. Values within trophic categories are means of multiple lakes. Figure adapted from J. R. Beaver and T. L. Crisman, *Hydrobiologia* **190**, 127–135 (1990).

state, but does not necessarily peak concurrently with biomass even within a particular trophic state category.

Large-bodied Oligotrichida are the dominant contributors to biomass and abundance peaks in oligotrophic lakes, whereas small-bodied Scuticociliatida dominate in eutrophic and hypertrophic lakes. It appears that seasonal patterns along trophic gradients are reflective of differences in food availability and lake mixing regimes (22). Small-bodied ciliates (i.e., scuticociliates) are principally bacterivorous and display maximum grazing efficiency on particles between 0.3 μm and 1.0 μm (23), and their biomass peaks in mesotrophic and eutrophic Florida lakes correspond well to bacterioplankton peaks (17). Large-bodied ciliates graze heavily on both picoplankton and nannoplankton in addition to bacterioplankton, and their seasonality would be expected to follow pulses in primary productivity with a strong contribution by these phytoplankton-size categories.

Although organically colored lakes can span the range from oligotrophic to hypertrophic, they differ from clear lakes of comparable trophic state by displaying an altered light regime, dissolved organic carbon (DOC) as an alternative biotic carbon pathway for the food web, and oxygen concentrations in the water column strongly negatively correlated with color levels and little influenced by lake trophic state (24). Beaver and coworkers (5) provided evidence that the seasonality of planktonic ciliates differs between clear and organically colored Florida lakes. Unlike oligotrophic lakes, it is the clear lakes that displayed peak biomass during fall and late winter, and the colored oligotrophic lakes that peaked during summer. Ciliate seasonality in mesotrophic and eutrophic lakes, however, resembled that of comparable clear lakes. The most pronounced difference across the trophic gradient was that colored lakes displayed significant numbers of mixotrophic ciliates during summer.

The vertical distribution of planktonic-ciliate abundance and biomass in the water column of lakes can change markedly seasonally (2). The vertical distribution of ciliate abundance in a Georgia (U.S.A.) reservoir was relatively uniform during winter, a period of mixing in this warm monomictic (water column mixing one time per year) lake, but after development of stratification in spring, abundance increased progressively in the epilimnion and decreased in the hypolimnion (25,26). A peak in scuticociliates developed just below the thermocline and was sustained from midsummer through early fall.

The vertical distribution of individual planktonic taxa in temperate and tropical lakes appears closely related to the development of thermal stratification, layering of phytoplankton, and bacterioplankton, and degree of hypoxia or anoxia in profundal waters (2,18,19,25). Large-bodied benthic ciliates (> 100 μm) in highly productive temperate lakes leave the sediment–water interface and ascend into the water column during summer as hypoxic or anoxic conditions develop at the sediment surface and move upward in the hypolimnion (3,27,28). A similar movement of large Hymenostomatida and Trichostomatida from sediments during peak productivity has been observed in eutrophic subtropical lakes (17).

RELATION OF CILIATE BIOMASS AND SIZE TO TROPHIC STATE

Several studies have noted a strong positive relationship between ciliate abundance and biomass and lake trophic state (4,26,29,30). For subtropical Florida lakes (4), the relationship between ciliate biomass and chlorophyll ($r^2 = 0.85$) was the strongest regression exhibited by any zooplankton component (31), and the values declined with increasing body size from rotifers ($r^2 = 0.54$) through cladocerans ($r^2 = 0.23$) to calanoid copepods ($r^2 = 0.07$). In a comparison with a similar suite of Canadian lakes, Beaver and Crisman (32) demonstrated that although the Florida lakes tend to have greater ciliate biomass for a given level of chlorophyll, there was not a significant difference between the biomass to chlorophyll regressions derived for the two lake regions (Fig. 2). Ciliate biomass does not appear to be significantly different between clear and organically colored lakes of a given trophic state in Florida (5).

In addition to biomass, ciliate assemblages change both structurally and functionally along trophic gradients. Small-bodied taxa (<30 μm) dominate the ciliate assemblages of most lakes, whereas larger-bodied ones (>40 to 50 μm) are replaced progressively with increasing phytoplankton production (4,32) reflecting a loss of Oligotrichida and an increase of Scuticociliatida (Fig. 3). This size shift is also evident within the Oligotrichida, and the largest-bodied euplanktonic ciliate found in Florida, *Stentor niger* (150 to 200 μm), is restricted to the most acidic ultraoligotrophic lakes, where it can contribute over 60% of total ciliate biomass (33).

Ciliates contribute approximately 10 to 60% of the total annual biomass of zooplankton in lakes, with values generally increasing as a function of trophic state (31,34–37). There is suggestive evidence that macrophyte-dominated eutrophic lakes have major structural, and presumably functional, differences in their ciliate assemblages (37) from phytoplankton-dominated

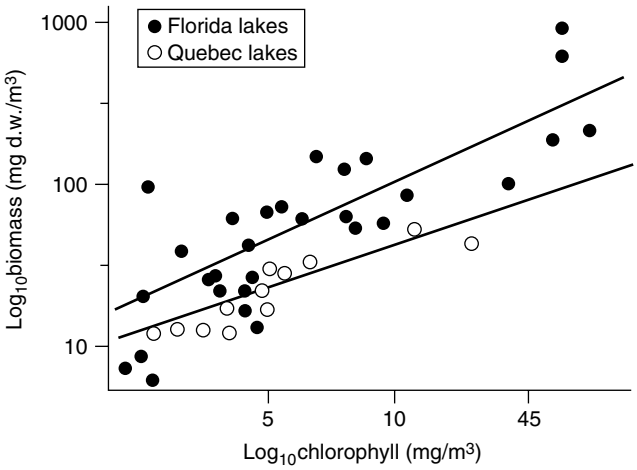

Figure 2. Relationships of annual mean biomass of ciliate protozoans versus chlorophyll for Florida and Quebec lakes. Figure adapted from J. R. Beaver and T. L. Crisman, *Hydrobiologia* **174**, 177–184 (1989).

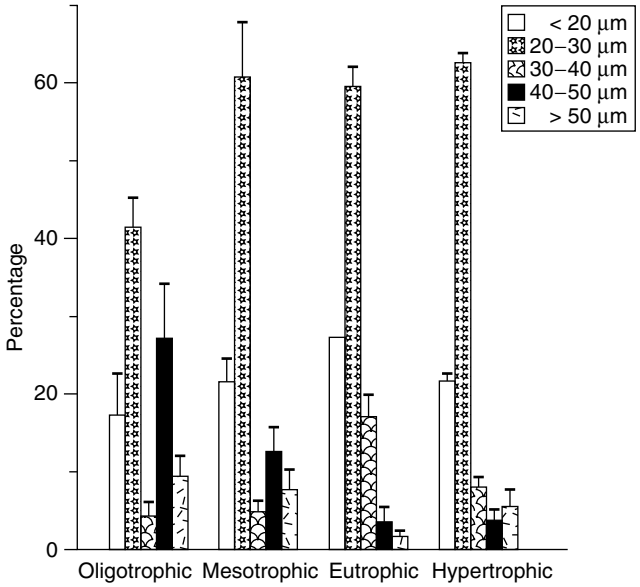

Figure 3. Contribution of ciliate size categories to annual mean ciliate biomass in Florida by trophic state. Figure adapted from J. R. Beaver and T. L. Crisman, *Limnol. Oceanogr.* **22**, 246–253 (1982).

ones, and this type of comparison is a promising research area for detailed investigation.

CONTROL OF CILIATE POPULATIONS

The structure of ciliate assemblages is controlled both by available food resources and predation and interference from higher trophic levels. Small-bodied ciliates (<30 µm) feed primarily on bacterioplankton and picoplanktonic algae, whereas the dominant food shifts from bacteria to progressively larger algal sizes with increasing ciliate body size (2,38). Heterotrophic microflagellates (39) and phagotrophic phytoflagellates (40) can compete with ciliates for food resources, especially bacterioplankton, but ciliate predation directly on such potential competitors can be significant seasonally in lakes (41). Ciliates and rotifers graze both heterotrophic nanoflagellates and bacterioplankton and thus assist carbon transfer to higher trophic levels (42).

While it has been suggested that ciliate populations can be severely food limited (43), numerous studies suggest that predation from larger ciliates and macrozooplankton (metazooplankton) is the principal regulator of ciliate abundance and structure (44–46). Although cladoceran predation is not insignificant, copepods, especially calanoids, appear to be the major zooplankton predators structuring the composition of ciliate assemblages (42,44,45). Experimental evidence suggests that although zooplanktivorus fish strongly control macrozooplankton composition and biomass, they have only weak control over ciliates, which can actually increase under high predation pressure (47). The trophic cascade effects on ciliates from piscivorous fish such as trout appears to be minor for lakes ranging from oligotrophic to slightly eutrophic (30). Burns and Schallenberg (44) caution researchers not to make broad generalizations on the role of individual trophic levels, especially zooplankton, regarding their control over ciliate composition and biomass, but instead to consider the importance of specific taxa.

NUTRIENT CYCLING

Zooplankton excretion of phosphorus can have a major influence over phytoplankton productivity (48), and release rates are inversely proportional to zooplankton body weight and size. Ciliates should display the highest excretion rates based on their small size and high metabolic rates (compared to crustacean zooplankton) (49–51). A ciliate population comprising as little as 1% of total zooplankton biomass could contribute approximately 50% of total phosphorus regeneration by zooplankton (52).

Both phytoflagellates and ciliates can be important regenerators of nitrogen in marine and freshwater ecosystems, but research for the latter systems is sparse (51,53–55). The role of ciliates for nitrogen and phosphorus regeneration is probably greatest when assimilable bacterial substrates have high carbon to limiting-nutrient ratios (e.g., nitrogen and phosphorus) (55).

MIXOTROPHY

Both phytoflagellates and ciliates can exhibit mixotrophy, a combination of phototrophy and phagotrophy in the same organism. In ciliates, mixotrophy is exhibited almost exclusively by select large-bodied taxa that contribute to ecosystem primary productivity via either endosymbiotic algae (5) or organellar sequestering of plastids from ingested algae (6). Mixotrophic ciliates are found in both marine and freshwater ecosystems, the latter spanning all trophic states and degree of organic color (5,56,57). Although a great deal is known about the role of phytoflagellates in freshwater, the literature on mixotrophic ciliates is surprisingly sparse.

Beaver and coworkers (5) reported that the large-bodied mixotrophic ciliate, *Strombidium* cf *oculatum*, dominated total ciliate biomass of several colored Florida lakes during summer and accounted for more than 50% of total annual biomass (Fig. 4). In addition, the proportion of total autotrophic biomass contributed by mixotrophic ciliates often exceeded that of phytoplankton during summer, approaching 96% on one occasion in a lake. Hecky and Kling (58) reported that mixotrophic ciliate biomass can exceed that of phytoplankton in Lake Tanganyika, and such mixotrophs contributed 4 to 69% of total primary production in two Australian lakes seasonally (59).

In nutrient-poor environments, possession of algal symbionts provides an additional food source, namely, algal metabolites, for the ciliate. The ciliate in turn would supply the algae with a dependable nutrient supply released during digestion of bacterial prey and would provide a predation refugium from zooplankton grazing. The importance of ciliate vertical migration patterns

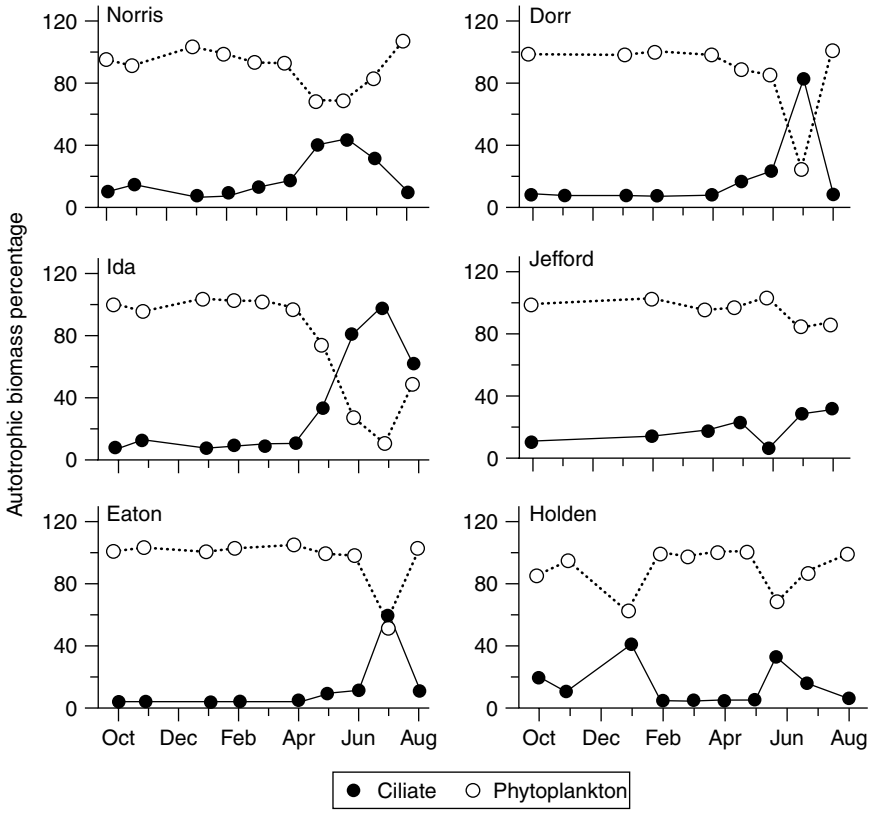

Figure 4. Percentage contribution of mixotrophic ciliates and phytoplankton to total autotrophic plankton biomass in six highly colored Florida lakes. Figure adapted from J. R. Beaver, T. L. Crisman, and R. W. Bienert, Jr., *Freshwater Biol.* **20**, 51–60 (1988).

for getting the algae to a more favorable light regime, especially in colored waters, is not well understood.

CILIATES AND THE MICROBIAL LOOP

The transfer and transformation of energy (carbon) from its production (photosynthesis) to its decomposition or deposition in the ecosystem memory (sediments) is fundamental to understanding the structure and function of aquatic ecosystems. Initially, ecologists viewed this as a unidirectional process (food chain) progressing through successive levels of heterotrophic consumers (trophic levels). With time came the realization that individual heterotrophs could function on multiple trophic levels as well as within a trophic level. Recognition of all such linkages led to the concept of the food web, with a feedback loop from all biotic components of the ecosystem to detrital and nutrient pools. The detrital and nutrient pools constituted both a short-term (recycled into the food web) and long-term (sealed from the food web in sediments) memory. Thus was developed a duality of energy transformation through food webs, primary production, and detritus.

Traditionally, limnologists focused on factors affecting phytoplankton production and its passage through zooplankton to planktivorous fish and finally to piscivorous fish. Rotifers, cladocerans, and copepods comprised most zooplankton investigations, and ciliates were excluded. It was not until the last quarter of the twentieth century that limnologists realized that direct utilization of picoplankton and bacterioplankton biomass by higher trophic levels in aquatic food webs depended on passage through phytoflagellates and ciliates. This alternative energy pathway was termed the microbial loop.

Ciliates play a key role in the microbial loop (Fig. 5). Small-bodied taxa are effective grazers on bacterioplankton and picoplankton, whereas larger-bodied taxa eat a wider array of foods. Some large taxa can be significant predators on small ciliates and phytoflagellates. Heterotrophic ciliates, in turn, pass energy to higher trophic levels via predation from macrozooplankton (metazooplankton) and fish.

The role of mixotrophic ciliates in the microbial loop is only beginning to be delineated. These tend to be the largest-bodied taxa in the ciliate assemblage and are probably beyond the size range preyed upon by zooplankton. Predation interactions with larval and adult fish are poorly known. Mixotrophic ciliates can contain a significant fraction of algal biomass, estimated from chlorophyll, especially in clear and dystrophic oligotrophic lakes (5,60). Currently, it is not known whether mixotrophic ciliates provide a shunting of energy directly to fish, thus bypassing zooplankton, or whether they are an energetic dead end in ecosystem carbon flow. Additional research is vitally needed to elucidate this pathway.

CILIATES AS EARLY WARNING BIOINDICATORS OF ECOSYSTEM CHANGE

Munawar and Weisse (61), relying on field data from the Laurentian Great Lakes, noted a dramatic decrease

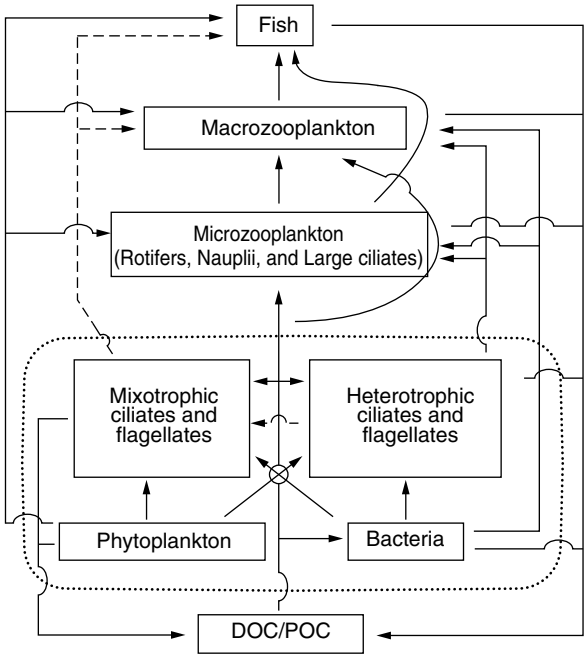

Figure 5. Conceptual model of the microbial loop in freshwater lakes and the role of heterotrophic and mixotrophic ciliates. The microbial loop is contained within the dotted circle.

of autotrophic picoplankton in eutrophic waters. They suggested that this important component of the microbial loop changed its structural and functional aspects along a trophic gradient and could possibly serve as an early warning indicator of pending environmental perturbation in lakes. Weisse (62) noted that the microbial loop operates in a similar manner in marine, estuarine, and freshwaters, but suggested that the overall importance of the microbial loop to ecosystem functioning decreases with increasing trophic state.

Beaver and Crisman (63) suggested that bacterioplankton should respond the quickest to cultural eutrophication because of their essential role in nutrient cycling. Bacterivorous ciliates should therefore respond rapidly to a shift in food availability and provide the first heterotrophic grazer documented change. In response to citizen complaints, an investigation was conducted between 1987 and 1988 for comparison with one conducted in 1979 to determine the extent of cultural eutrophication in Lake Weir, Florida. Although water clarity and nutrients did not change significantly during the period, microzooplankton in general, and ciliates in particular, increased significantly in response to increased food availability supporting concerns of the public and the need for pollution abatement.

Ciliates display a significant response to reduced pH, with a structural shift from a predominately small-bodied assemblage (20 to 30 μm) dominated by Oligotrichida, Scuticociliatida, and Haptorida in less acidic oligotrophic lakes to a large-bodied (40 to 50 μm) assemblage dominated almost exclusively by Oligotrichida in lakes at pH less than 5.2 (33). Both the impact of such changes on the structure and function of the microbial loop and its application to predicating ecosystem responses to acid rain are not clear. There is, however, great potential for utilization of ciliate assemblages as early warning indicators for climate change, heavy metal contamination, and eutrophication management.

CONCLUSION

Protozoan ciliates were largely forgotten from zooplankton investigations in freshwater lakes until the latter quarter of the twentieth century. Ciliates are well represented in freshwater and can be a major biomass contributor to the zooplankton community. Oligotrophic lakes are dominated by large-bodied taxa, which are progressively replaced by small-bodied taxa with increasing trophic state reflecting a dietary shift from predominately small phytoplankton to bacteria with increased biomass of the latter, especially in eutrophic and hypertrophic lakes. Ciliate biomass displays a strong positive relationship with increasing trophic state and is the strongest relationship noted for any zooplankton component. Both heterotrophic and mixotrophic ciliates play important roles in the microbial loop of lakes and affect energy transfer to higher trophic levels in the food web and nutrient cycling. It is proposed that ciliate biomass and taxonomic composition can be valuable early warning indicators of ecosystem change, including advancing cultural eutrophication and lake acidification.

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PROTOZOAN PARASITES. See AEROBIC ENDOSPORES; CYCLOSPORA: BASIC BIOLOGY, OCCURRENCE FATE AND METHODOLOGIES; DISINFECTION OF PROTOZOAN PARASITES; *ENTAMOEBA HISTOLYTICA*/*ENTAMOEBA DISPAR*; FREE-LIVING AMEBAS PRESENT IN THE ENVIRONMENT CAN CAUSE MENINGOENCEPHALITIS IN HUMANS AND OTHER ANIMALS; *GIARDIA*: BASIC BIOLOGY, GENETICS AND EPIDEMIOLOGY; *GIARDIA*: DETECTION AND OCCURRENCE OF IN THE ENVIRONMENT; ISOSPORA; MICROSPORIDIA: OCCURRENCE, FATE AND METHODOLOGIES; PARASITIC PROTOZOA: FATE IN WASTEWATER TREATMENT PLANTS; *TOXOPLASMA GONDII*; BIOLOGY OF CRYPTOSPORIDIUM

PROTOZOAN PARASITES, DISINFECTION OF. See DISINFECTION OF PROTOZOAN PARASITES

PROTOZOAN PARASITES IN FOOD. See VIRUSES AND PROTOZOAN PARASITES IN FOOD, INCLUDING METHODOLOGY

PROTOZOAN PARASITES IN SEDIMENTS. See FATE OF VIRUSES AND PROTOZOAN PARASITES IN AQUATIC SEDIMENTS

PSEUDOMONAS

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Members of the genus *Pseudomonas* are among the most commonly encountered microorganisms, in the environment. They are tremendously versatile microorganisms

that can survive under conditions that include nutrient-poor to toxic, abilities that have made it a favorite microorganism to study transformation of xenobiotic compounds. Several species are opportunistic pathogens that cause myriads of diseases. Their ability to attach to and colonize surfaces not only makes them important in biofouling, but also allows them to be protected against biocides.

The role of *Pseudomonas* spp. in the environment, especially as a biotechnological tool for the degradation of xenobiotic compounds, and all the clinical aspects has been thoroughly covered elsewhere. Although the genus *Pseudomonas*, in general, is covered in this article, the main focus is on *Pseudomonas aeruginosa*, its ecology and its role in public health.

GENERAL DESCRIPTION OF THE MICROORGANISM

Pseudomonas is a ubiquitous genus in nature found in association with a large variety of matrices such as soil, water, plants, and animals (1). They are gram-negative rods with one or several polar flagella, with a notable nonmotile exception, *Pseudomonas mallei*. These morphological characteristics are nonspecific and are used to describe a rather large group known as the "pseudomonads" (a nontaxonomic term). The genus *Pseudomonas* is composed of straight or slightly curved rods approximately 0.5 to 1.0 by 1.5 to 5.0 μm (2) and strict aerobic respirers. However, some species can also use nitrate as an alternate terminal electron acceptor and can thus also be found under anoxic conditions in the presence of this electron acceptor. One of the reasons for its ubiquity may be that these microoganisms do not require the presence of growth factors except for a few species such as *Pseudomonas diminuta* and *Pseudomonas vesicularis* (pantothenate, biotin, cyanocobalimin, methionine, or cystine) (2) and *Pseudomonas maltophilia* (some amino acids) (1). Although none of the pseudomonads can fix molecular nitrogen, at least one species has been reported to grow under nitrogen-limiting conditions.

Another reason for *Pseudomonas*' ubiquity may be the ability to use a large number of organic compounds as the source of carbon and energy. For example, some species may use more than a 100 different organic compounds (1), including some aromatic compounds. Additionally, members of this genus have the ability to grow and live under extremely low-nutrient conditions such as distilled water, and have a high tolerance to a variety of extreme environmental conditions such as high (43 °C) and low (4 °C) temperatures. Favero and coworkers (3) studied the prevalence and multiplication of *P. aeruginosa* in distilled waters and found that these bacteria can be present in apparently clear distilled water at concentrations up to 10^4 cells/ml.

Although it is well known that *Pseudomonas* can in fact grow under conditions that are nutrient limiting for most microorganisms, it is still not clear how *Pseudomonas* can multiply under these conditions. It has been suggested that they can use gaseous nutrients dissolved in distilled water, but this hypothesis is not entirely convincing (4).

Because *Pseudomonas* spp. can grow at such extremely low-nutrient concentrations and use such a variety of organic compounds, one species (*P. aeruginosa*, strain P1525), has been used to measure the Assimilable Organic Carbon (AOC) concentration in waters (5) as an indirect measure of the potential regrowth of microorganisms in potable water distribution systems (6).

ECOLOGY

Pseudomonas spp., and especially *P. aeruginosa* can be isolated from animal and inanimate environments (4) and can easily adapt to the existing environmental conditions. Studies by Favero and coworkers (3), showed that *P. aeruginosa* can initiate replication with a minimal lag phase of growth in such nutrient-poor environments as distilled waters.

In the hospital environment, they can be isolated easily from clinical specimens and the surrounding environment and can be readily grown in routine culture media. Humidity seems to be critical in the survival of these microorganisms in the environment, consequently *Pseudomonas* can be found in moist environments such as respiratory equipment, ventilators, endoscopes, pressure monitors, sinks, mops, vegetables, and in some humans they may be found as part of the normal microbiota in areas such as the axillae, perineum, and ears (7,8).

Ringen and Drake (9) were able to isolate *Pseudomonas* from soils that had been allowed to dry for several months at room temperature. Additionally, *P. aeruginosa* sprayed on soils and grasses could be recovered for up to 130 days, and it seems that the soil acts as a protective matrix for these microorganisms because their half-life was observed to be as short as 10.2 min under low humidity (10) conditions on metal surfaces (11).

A study by Correa and coworkers (12) in an oncology hospital in Brazil indicated that *P. aeruginosa* could be isolated from vegetable surfaces in spite of previous washing using a 1% hypochlorite solution as a disinfectant. Several vegetables were tested for the presence of this microorganism, including onions, green peppers, and tomatoes, although lettuce, chicory, and watercress seemed to be the most common sources of the bacteria. Some of the clinical isolates were found to be serotypically identical to the vegetable isolates, suggesting that the vegetables may be a source of these microorganisms in the hospital environment. A previous study by Kominos and coworkers (13), had already implicated vegetables as a possible source of *P. aeruginosa* in a general hospital. The latter study showed that *Pseudomonas* could be isolated from several vegetables in the hospital kitchen, and from cutting boards and the hands of kitchen personnel. From these data, Kominos and coworkers (13), also suggested that hospital patients could possibly ingest up to 5×10^3 of *P. aeruginosa* cells in an 80-g salad, which is considered to be the amount consumed in a typical meal. Similar to the study by Correa and coworkers (12), the vegetable isolates were similar (if not identical) to clinical isolates from the same hospital. It has also been suggested that flowers could be a possible source of *Pseudomonas* in the hospital environment.

The consumption of bottled water in North America has grown yearly by up to 25% (14). *Pseudomonas aeruginosa* in bottled water is regulated in the European Community but not in North America (15). Studies by Burge and Hunter (16) and Gonzalez and coworkers (17), showed that *P. aeruginosa* could survive in mineral water from 70 days to over one year depending, perhaps on the bacterial strain (18). These data indicate that bottled water may not necessarily be free of *P. aeruginosa*. However, the health implications of this for the general public remain in question (15). Bottled water could possibly serve as a source of these microorganisms if present in high concentrations.

Pseudomonas spp. are highly resistant to disinfectants, including those used during drinking water disinfection procedures. *Pseudomonas* were enumerated at different stages during water treatment processes by Grabow (19) who found concentrations ranging from 260 (in sand filtration), to 90,000 cells/100 mL in activated sludge effluents (19) at water reclamation plants as measured by the Most Probable Number method. They are an important part of the microbiota in wastewater treatment processes and have been isolated from fixed-film bed reactors, activated sludge, and stabilization ponds (20). They can also be readily isolated from soils, and are among the most important members of the denitrifying group of microorganisms (21) in biological processes as well as during wastewater treatment processes.

Denitrification is the bacterial-mediated reduction of nitrate or nitrite to gaseous nitrogen under low dissolved oxygen conditions (22). *Pseudomonas* spp. are among the most important species of denitrifiers in environments such as marine and fresh waters and sewage (2,23). Denitrification is rare in flowing waters as a result of the presence of dissolved oxygen, and nitrate reduction is possible only when oxygen is present at concentrations of 50% below the saturation level. However, the presence of high concentrations of organics in matrices such as sewage, would also affect the concentration of oxygen, and thus the rate of denitrification.

ISOLATION

Gilardi (24) indicated that peptone agar (that can be supplemented with 5% sheep or rabbit red blood cells) is used to isolate *Pseudomonas* in the clinical laboratory, although selective media such as MacConkey, eosin-methylene blue and Leifson deoxycholate are also useful. For the selective isolation of *P. aeruginosa* and some other species, cetrimide agar, Pseudomonas agar F, and Pseudosel agar can be successfully used. Although they can be grown readily in the above media, it is advised that during isolation the analyst use certain tests as a screening procedure, including (for example) a typical grapelike odor (*P. aeruginosa*) nonfermentation of carbohydrates, ability to grow at 42 °C (positive), gram reaction (negative), spore formation (negative), motility (positive, except for one species), production of hydrogen sulfide, and oxidation of glucose, xylose, lactose, and maltose (24). Some strains produce pigments that are also used as a means of screening isolates, for example, pyocyanin, a blue phenazine pigment (*P. aeruginosa*), and several other diffusible and nondiffusible fluorescent and nonfluorescent pigments (2).

Pseudomonas aeruginosa can be readily detected in waters using either the most probable number (MPN) method or membrane filtration (MF). Media used for MPN analyses include asparagine broth and acetamide broth. After incubation at 35 to 37 °C for 24 to 48 hours the tubes are observed under long-wavelength ultraviolet light. The presence of a greenish fluorescent pigment is considered positive. The medium M-PA is used for membrane filtration and results can be obtained after 72 hours (25), and the typical colonies are flat with light outer rims and centers that appear brownish- to- greenish-black. Positive samples for both the MPN and the MF need to be confirmed using the appropriate media such as acetamide broth or agar slants for the MPN and milk agar for MF. The reader is directed to APHA Standard Methods for the Examination of Water and Wastewater (26), or later editions, for details regarding all these procedures.

ENVIRONMENTAL SOURCES

As indicated previously, *Pseudomonas* spp. are ubiquitous, and thus, it is not clear as to what the source of these microorganisms is in a given matrix, or even in clinical specimens. They can be found associated with water, soil, vegetable material, animal feces, and fomites. However, it is not clear whether the microorganisms are intrinsically a part of the normal microbiota of those environments. It has been shown that new hospital sinks can be free of *Pseudomonas*, but are rapidly colonized (27). It is not entirely clear if *Pseudomonas* is part of the human or animal normal microbiota or if it is simply an allocthonous microorganism in these environments. Tannock (28) did not include *Pseudomonas* spp. as part of the normal microbiota of humans. Noble (29) included *Pseudomonas* as a microorganism that can be found on human skin, especially the feet, however, these are most likely transient microorganisms originating from environmental sources, because they are present primarily in those persons whose feet get wet at work. *Pseudomonas* can also be isolated from the oral cavity in which it is considered allocthonous and the source seems to be water, especially colonized dental units (30).

The presence of *Pseudomonas* in animal feces does not seem to be prevalent as shown by Ringen and Drake (9) in which only 11% of the fecal samples tested were positive for *P. aeruginosa*. Forty-seven percent of the sewage samples analyzed during this study were positive for *P. aeruginosa*. These data comparing fecal and sewage samples simultaneously indicate that the feces may not be the actual source of *Pseudomonas*, but rather that these microorganisms are multiplying under the conditions of the wastewater environment. These microorganisms are seldom isolated from the feces of healthy individuals (4). Between 3 and 6% of individuals in the nonhospital environment seem to harbor *Pseudomonas* in their feces, although the source may be the ingestion of food or water containing these microorganisms. This is in contrast to feces from hospitalized individuals

in which 18 to 20% are positive for *P. aeruginosa* (13) indicating that the hospital food, and perhaps water may be the main source of these microorganisms. *Pseudomonas* spp. are not listed as part of the normal microbiota of human and animal feces (31,32), although they are considered prominent members of domestic wastewater. Because of the presence of biodegradable hydrocarbons in wastewaters, high concentrations of *Pseudomonas* can be detected (23). One species, *Pseudomonas hirudinicola*, is found as part of the gut microbiota of the blood leech (*Hirudo medicinalis*). This microorganism actually lyses the erythrocytes in the gut of the leeches, allowing the host to use the nutrients (23); however, because of the specificity of its reservoir, this species is not likely to be found in most surface waters.

PSEUDOMONAS COLONIZATION

On the basis of previously published literature, Geldreich (33) suggested that the colonization of the gastrointestinal tract of healthy humans by *Pseudomonas* is not a common event. Work in the 1960s by Samish and Etinger-Tulczynska (34) suggested *P. aeruginosa* as an epiphytic microorganism capable of colonizing vegetables, such as tomatoes. Their work showed that the microorganism could be detected in the stem scar and the underlying core of tomatoes. They also indicated that the stem depression could be the point of entry of the microorganisms into the tomatoes. However, it has been difficult to determine if the true source of these microorganisms was the soil, water, or the fertilizers used (13). Other species such as *Pseudomonas fluorescens*, *Pseudomonas putida*, *Pseudomonas multivorans*, *P. maltophilia* and *Pseudomonas stutzeri* may be indigenous to the water environment (33).

If microorganisms attach to solid matrices, and the conditions are favorable for their replication, the attached cells may start the production of exopolysaccharides to form a thick layer of growth, referred to as the aufwuchs (growth upon, in German) (35) or biofilm (36). This structure, in the case of *Pseudomonas*, is composed of alginate, which are repeating units of D-mannuronic and L-glucuronic acids (10). The production of alginate can be observed in *Pseudomonas* by the ability to change from a nonmucoid to a mucoid phenotype, and this seems to be the key to colonize different environments (4). The mucoid phenotype produces large amounts of exopolysaccharide in the form of alginate. This mucoid phenotype may allow the microorganism to anchor to substrata in aqueous environments and it may also help prevent its dislodgement from the respiratory tract of infected individuals.

Davies and coworkers (10), showed there was activation of alginate production as a result of attachment. Using *P. aeruginosa* they demonstrated that planktonic cells produced less alginate than the attached cells, thus indicating that attachment may indeed lead to colonization of the substratum. In similar studies, Boyd and Chakrabarty (37) showed that the enzyme alginate lyase and its statement play a role in *Pseudomonas* detachment from biofilms.

Pseudomonas cells can also produce a biofilm when attached to epithelial cells and, as described by Pollack (7), colonization is preceded by attachment during infections. Alginate biofilm production is important in diseases such as cystic fibrosis (37,38) and urinary tract infections (39) because of the anchoring that prevents dislodgement, and perhaps because the alginate matrix may protect the underlying microorganisms from the action of antibacterial agents.

Sagripanti and Bonifacino (40) tested seven commonly used chemical disinfectants on *P. aeruginosa* anchored to metallic and plastic devices and it was noted that cell anchored to either matrix were 300-fold more resistant to the disinfectants than freely suspended cells.

Pseudomonas aeruginosa is also capable of colonizing potable water distribution systems, and can be found growing in a variety of materials (41). This microorganism has also been detected in swimming pools and whirlpools, and Rhame (42) has proposed that the high temperatures in these environments may help the growth and multiplication of these microorganisms. This is most probable, as supported by the disease outbreaks outlined in Table 1.

Pseudomonas aeruginosa cells seem to control the production of colonization factors via mechanisms of cell-to-cell signaling (48). These mechanisms referred to as quorum sensing, allow bacteria to behave as a community rather than as individual cells via the production of homoserine lactone-based molecules that control the expression of extracellular virulence factors, possibly including colonization factors. The reader is directed to Van Delden and Iglewski (48) for a thorough review of the quorum-sensing mechanisms. Work by Singh and coworkers (49) strongly suggested that *P. aeruginosa* cells in cystic fibrosis patients produce a biofilm and that most cells in the lungs of these patients are in the biofilm state. Additionally, the biofilm state may be the definite factor in the chronicity of *Pseudomonas* infections in cystic fibrosis patients.

PSEUDOMONAS AND DISEASE

Although *Pseudomonas* spp. were originally thought to be nonpathogens, their opportunistic pathogenicity was finally accepted in the mid-twentieth century (4), and were observed to be the etiological agents in a myriad of infections as opportunistic pathogens. *Pseudomonas* spp. have been implicated in cases of (*1*) melioidosis (*P. pseudomallei*), (*2*) glanders (*P. mallei*), (*3*) bacteremia, pseudobacteremia, endocarditis, pneumonia, keratitis, urinary tract infections (mainly *P. aeruginosa*, but also *Burkholderia (Pseudomonas) cepacia*, *P. pickettii* and *Xanthomonas (Pseudomonas) maltophilia*) (8). *Pseudomonas aeruginosa* has also been implicated in sclerokeratitis in contact and noncontact lens wearers (50).

Pseudomonas is an opportunistic pathogen; and thus, is rarely an infectious agent in the immunocompetent individual, even if it is sometimes found as part of the normal human microbiota. With the advent of antibiotic use, advances in intensive care as well as life-extending therapies, the number of *Pseudomonas* infections has

Table 1. Selected Outbreaks of Disease Associated with *Pseudomonas/Burkholderia* in Water (1990–Present)

Country	Source	Number of Cases	Disease
Australia (1990–1991)[a]	Treated Drinking Water	5	Melioidosis/Septicemia
Netherlands (1995)[b]	Freshwater Lake	98	Otitis Externa
United States (1995–1996)[c]			
Maine	Hot Tub	10	Dermatitis
Minnesota	Hot Tub	10	Dermatitis
New Mexico	Hot Tub	4	Dermatitis
Oregon	Lake	121	Dermatitis
Washington	Hot Tub	24	Dermatitis
United States (1997–1998)[d]			
Alaska	Spring	50	Dermatitis
Arkansas	Pool and Hot Tub	12	Dermatitis
Indiana	Pool	42	Dermatitis
Maine	Hot Tub	3	Dermatitis
Maryland	Hot Tub	7	Dermatitis
Wisconsin	Pool and Hot Tub	11	Dermatitis
United States (1999–2000)[e]			
Colorado	Pool and Hot Tub	19	Dermatitis/Folliculitis
Maine	Pool and Hot Tub	9	Dermatitis/Folliculitis/Otitis externa

After [a]Inglis and coworkers (43). The etiological agent in this outbreak was *Burkholderia pseudomallei*.
[b]van Asperen et al., (44).
[c]Levy et al., (45).
[d]Barwick et al., (46).
[e]Beckett et al., (47).

apparently increased. In the early twentieth century, severely burned patients as well as those with cystic fibrosis usually died because of the lack of treatment (4) that is currently commonplace. *Pseudomonas* infections in humans are usually caused by disruptions in the integrity of the physical barriers such as cuts and abrasions. In the hospital environment the circumvention of the barriers via catheters or parenteral drug injections (7) seems to be the most common route of infection. They are clearly opportunistic etiological agents of nosocomial infections commonly infecting burn patients as well as those undergoing chemotherapy. Data from 1980 to 1990 indicated that *P. aeruginosa* was the second most frequent gram-negative bacterium causing infections in hospitalized patients (4). They are in fact so common that the outer membrane proteins have been tested in vaccines in order to protect burn patients from *P. aeruginosa* bacteremia. A study by Kim and coworkers (51), concluded that the vaccine tested in patients with burns in 10% or greater of total body surface area was successful in protecting the patients (6.1% bacteremia in patients receiving the vaccine versus 40% in the patients receiving a placebo).

Pseudomonas endocarditis is common among illicit parenteral drug users, accounting for 58% of infections with gram-negative bacteria (52) and thus seems to be the most prevalent risk factor, however the authors indicated that there exist regional variations in its occurrence (7). Bacteremia seems to be an almost exclusively nosocomial infection common in hospitals and nursing homes (53), and the risk factors include antibiotic or corticosteroid therapy, prematurity, organ transplants, and traumatic injury. They are also common etiological agents in central nervous system infections that result from neurosurgery, head trauma, or bacteremic spread from other infection sites. *Pseudomonas aeruginosa* is the second most common isolate in cases of meningitis (7).

Cystic fibrosis patients typically died before the advent of antibiotic therapy was established. These patients can be infected with various microorganisms; however, mucoid strains of *P. aeruginosa* and *Burkholderia (Pseudomonas) cepacia* are among the most common. The reader is directed to Govan and Deretic (54) for a thorough review on cystic fibrosis and *Pseudomonas*.

Pseudomonas spp. are frequently the responsible agents in infections related to recreational water contact causing dermatitis, ear infections, and conjunctivitis (55). Although associated with infections in the immunocompromised, they have also been the etiological agents in community-acquired pneumonia in healthy patients (56). The latter study reported that previously healthy adults with a history of smoking are susceptible to community-acquired pneumonia. The risk factor seems to be exposure to aerosols of *Pseudomonas*-containing waters.

ENVIRONMENT AND PUBLIC HEALTH

It is clear that *Pseudomonas* spp. are an intrinsic part of certain environments, and as such it would be impossible to erradicate this microorganism. The mode of transmission may be via ingestion of food or water containing high concentrations of the microorganisms, although this may not necessarily lead to a diseased state in the host. In young infants and the immunocompromised, *Pseudomonas* may cause gastrointestinal infections such as necrotizing enterocolitis that can be fatal (57). However, gastrointestinal infections are perhaps underdiagnosed because they may be clinically inapparent (7).

Pseudomonas may result in skin and soft-tissue infections and there have been several outbreaks related to

the use of whirlpools, swimming pools, hot tubs, and spas (Table 1). Levine and coworkers (58), and Herwaldt and coworkers (59), reported several outbreaks of dermatitis associated with the use of these recreational waters. A more recent report by Beckett and coworkers (47), described two outbreaks of dermatitis/folliculitis associated with swimming pools and hot tubs that occurred between 1999 and 2000. In both outbreaks the levels of disinfectant had fallen below 2 mg/L and the pools and hot tubs were subjected to crowded conditions. A similar outbreak occurred in immunocompromised patients who had used a whirlpool bathtub (60). In this outbreak the source seemed to be a bathtub drain that was found to be positive for *P. aeruginosa* with a similar pulsed field electrophoregram as the patient isolates. Some of the outbreaks of dermatitis that have occurred in recent years are enumerated in Table 1.

A fatal septicemia outbreak occurred in Trinidad (61) affecting infants in a neonatal intensive care unit. A nurse, who failed to wash hands in between patients, may have been the source. Although no environmental sources of the *Pseudomonas* could be found, a strain with the same antibiotic resistance pattern was isolated from the only water tap at the intensive care unit.

All these outbreaks indicate that *Pseudomonas* is indeed an important opportunistic pathogen affecting particularly special populations. In almost every one of the outbreaks there seemed to be an environmental source that had been colonized by *Pseudomonas*. The fact that these microorganisms can form a biofilm and colonize pipes, faucets, drains, and other fomites is a clear indication that extreme care should be taken to try to keep colonization to a minimum. The study carried out by Sagripanti and Bonifacino (40) clearly indicates that disinfectants by themselves may not be enough to keep fomites free of *Pseudomonas*. This study showed that anchored cells are 300-fold more resistant to commonly used disinfectants than suspended cells. This may indicate that mechanical cleaning may be in order; together with disinfection procedures so as to better protect public health.

Although this chapter has mainly focused on *P. aeruginosa*, it is important to note that there have been reports of outbreaks of melioidosis associated with water treatment plants (43). *Burkholderia (Pseudomonas) pseudomallei* was isolated from environmental sources (Table 1) and the authors indicated that an aerator at a water treatment plant may have been the source of microorganisms that contaminated the potable water that served as a source of infection. The microorganisms can grow rapidly in well-aerated environments and can thus rapidly increase to concentrations that may present a public health problem. *Pseudomonas* spp. have been observed to grow well in the water-air interfaces of all water treatment processes, as well as on home water treatment devices. In the latter case, proper maintenance of the treatment devices is required to prevent colonization and growth of *Pseudomonas* as well as other microorganisms (25).

Burkholderia (Pseudomonas) pseudomallei and associated cases of meliodosis have been reported to occur mostly in the Far East (62) and Australia. However, a few cases have also been reported in Africa, Mexico, and South America (7), Oklahoma (63) and Georgia (64). Thus, this indicates that this microorganism is also present in the Western Hemisphere and thus may present a future public health problem as an emerging pathogen.

Hardalo and Edberg (15) proposed that perhaps the health risk posed by *P. aeruginosa* in drinking waters is overstated. In a thorough review of the literature they indicated that most of the outbreaks previously associated with drinking water and *Pseudomonas* were in fact linked to other sources of infection. Thus, in spite of the pathogenicity and omnipresence of this microorganism in the environment it would not be practical to try to eliminate it from drinking water, because it also has the ability to regrow in the distribution network, posttreatment. However, these are microorganisms that are important pathogens affecting primarily special populations, and therefore public health may be better served by public education and health advisories rather than by attempts to control or eliminate them from water and food.

CONCLUSION

Pseudomonas spp. are among the most commonly isolated microorganisms from the environment, although these data may be biased as a result of the media and methods currently used for their isolation, which may inhibit the growth of other microorganisms. Nonetheless, their importance in the environment is tremendous as is their role as opportunistic pathogens of the immunocompromised. Although the term "contamination" is often used when referring to the presence of *Pseudomonas* in recreational or other types of water, this is not the correct terminology. As indicated previously, *Pseudomonas* is omnipresent in the environment (e.g., soil, water, plants) as part of the resident microbiota, thus its presence in waters does not necessarily indicate contamination. The fact that these microorganisms are naturally present in the environment presents a true health problem as a result of the opportunistic nature of the pathogenicity. *Pseudomonas* may be present in tap water, bottled water, recreational waters, raw vegetables, and aerosols. Thus, it is advisable that special populations take special precautions because they are at the highest risk of infection.

It has also been mentioned that high concentrations of *Pseudomonas* can be found in treated and untreated sewage, thus it may be expected that in surface waters receiving sewage effluent *Pseudomonas* may be present in high numbers. In the latter case, the risk of exposure would be twofold, as a result of the possible presence of enteric pathogens and the opportunistic pathogen, *Pseudomonas*. Several epidemiological studies have been carried out in marine recreational waters (65,66) and freshwaters (67) in order to determine the public health risks of water exposure. These studies measured the concentrations of indicators of fecal contamination as well as the concentrations of *Pseudomonas*. However, these and other epidemiological studies to date have focused mostly on gastrointestinal diseases, thus it is currently difficult to

determine the true impact of the presence of *Pseudomonas* spp. in recreational waters on public health.

It is encouraging that new materials are being developed that may prevent or minimize biofilm formation in tubes used in catheters (68). Investigators are currently modifying polymers by incorporating the antibiotic ciprofloxacin. These materials act by slow release of the antibiotic, thus preventing colonization of the tubes and thus minimizing infection.

It is also encouraging that progress is being made to counteract the effects of colonization factors produced by *Pseudomonas*. The production of these colonization factors may be most important in terms of public health, first because of the increased resistance of the microorganisms to antimicrobial agents in infected patients and second because the ability to colonize the environment insures a constant reservoir of the microorganism. If all aspects of colonization are elucidated we will be well on the way to better protect public health from a very dangerous opportunist.

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PSYCHROPHILES. See EXTREMOPHILES: LIFE IN EXTREME ENVIRONMENTS

PSYCHROPHILIC BACTERIA. See COLD SHOCK

PSYCHROPHILIC BACTERIA: ISOLATION AND CHARACTERIZATION

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Life-forms proliferating in perpetually cold environments have developed ecophysiological and biochemical adaptations to optimize their activity at low temperatures. A broad spectrum of life is cold-adapted and includes bacteria, archaea, and simple and complex eukaryotes, ranging from algae to fish. Organisms that are cold-adapted are often referred to as *psychrophiles*, a word derived from ancient Greek and Latin, meaning literally cold-loving (psychros: cold, philus/phile: lover, loving). In the eponymous review on bacterial psychrophiles by Morita (1), a "true" psychrophile included any organism able to grow optimally at about 15–20 °C or less but was unable to grow at room temperature (20–25 °C) or higher. Research suggests true psychrophiles are comparatively uncommon as the majority of bacteria isolated in cold ecosystems are what have been termed psychrotrophic or facultative psychrophiles (2,3). These bacteria have temperature optima at 20 °C or more but are able to grow at 0 °C and the growth rates are usually equivalent to that of psychrophiles at low temperatures. These bacteria are most appropriately considered psychrotolerant (2). Psychrotolerant bacteria abound even in the coldest of environments, simply because many of them are ecophysiologically resilient and nutritionally versatile species.

The aim of this review is to provide an overview of various procedures useful for the isolation and characterization of psychrophilic bacteria. Most procedures detailed are general methods modified for cold-adapted bacteria. The review covers isolation, routine cultivation, maintenance, and general characterization of psychrophiles. Procedures to accurately determine cardinal growth temperatures using a temperature gradient incubator (TGI) are then detailed. Techniques for definitively identifying and quantifying fatty acids implicated in cold adaptation of cellular membranes, including polyunsaturated fatty acids (PUFA) and branched chain fatty acids, are explained.

ISOLATION OF PSYCHROPHILES

General Principles

Psychrophilic taxa are generally found only in permanently cold habitats, environments that have constant annual temperatures of less than 4 °C (1) and which have not been subject to warming over 25 °C even for a short duration. Psychrophilic species have been isolated from a variety of terrestrial environments, including vegetation and ornithogenic soils of Antarctica, boreal tundra soils, some chilled food products, surface snow and ice, and underlying sediments (Table 1). Other terrestrial environments have the potential to yield novel psychrophilic and psychrotolerant bacteria, including high alpine lakes (4) and subterranean karsts (5). As an example, Tasmanian karsts (Australia) have a constant annual average temperature of only 7 °C (D. Nichols, pers. comm.). Unusual

Table 1. *Psychrophilic taxa* That Have Been Well Characterized, Including Their Known Phylogenetic Affiliation, Habitat, Optimum Growth Temperature, and Isolation and Growth Medium

Species[a]	Phylogenetic Affiliation	Habitat	T_{OPT} (°C)[b]	Isolation and Growth Medium[c]	Reference
Colwellia demingiae	Gamma proteobacteria	Sea ice	10	Marine 2216	9
Colwellia hadaliensis	Gamma proteobacteria	Deep sea	10[d]	Marine 2216	10
Colwellia rossensis	Gamma proteobacteria	Sea ice	8–10	SWCm/marine 2216	9
Colwellia psychrerythreae	Gamma proteobacteria	Sea ice, surfaces of polar marine fauna, fish eggs	10–12	Marine 2216	9
Colwellia psychrotropica	Gamma proteobacteria	Seasonal marine basin	16–18	Marine 2216	9
Glaciecola punicea	Gamma proteobacteria	Sea ice	16–18	Marine 2216	11
Glaciecola pallidula	Gamma proteobacteria	Sea ice	13–14	Marine 2216	11
Methylosphaera hansonii	Gamma proteobacteria	Saline meromictic lake	10–15	NMS-seawater (1 : 1 CH_4 : air)	12
Moritella japonica	Gamma proteobacteria	Deep sea	10[d]	Marine 2216	13
Moritella marina	Gamma proteobacteria	Seawater	15–18	Marine 2216	14
Moritella yayanosii	Gamma proteobacteria	Deep sea	10[d]	Marine 2216	15
Psychromonas antarctica	Gamma proteobacteria	Ice shelf pond sediment, sea ice, marine sediment	10–12	Marine 2216	16,17
Shewanella benthica	Gamma proteobacteria	Deep sea	4[d]	Marine 2216	18
Shewanella violacea	Gamma proteobacteria	Deep sea sediment	8[d]	Marine 2216	18
Shewanella hanedai	Gamma proteobacteria	Polar marine sediment	14	Marine 2216	19
Shewanella gelidimarina	Gamma proteobacteria	Sea ice	17	Marine 2216	19
Polaromonas vacuolata	Beta proteobacteria	Under-ice seawater	4 (on agar)	SWCm/marine 2216	20
Octadecabacter antarcticus	Alpha proteobacteria	Sea ice	10–12	SWCm/marine 2216	21
Octadecabacter arcticus	Alpha proteobacteria	Sea ice	10–12	SWCm/marine 2216	21
Desulfotalea psychrophila	Delta proteobacteria	Arctic ocean sediment	10	DSMZ Medium 193	22
Desulfofrigus oceanense	Delta proteobacteria	Arctic ocean sediment	10	DSMZ Medium 193	22
Desulfofrigus fragile	Delta proteobacteria	Arctic ocean sediment	17	DSMZ Medium 193	22
Desulfofaba gelida	Delta proteobacteria	Arctic ocean sediment	7	DSMZ Medium 193	22
Gelidibacter algens	*Cytophagales*	Sea ice, under-ice seawater	14–18	Marine 2216	23
Psychroserpens burtonensis	*Cytophagales*	Seasonal marine basin	10–12	Marine 2216+0.5% Tween 80	23
Psychroflexus torquis	*Cytophagales*	Sea ice	8–10	Marine 2216	24
Polaribacter franzmannii	*Cytophagales*	Sea ice, seawater	10	SWCm	24
Polaribacter irgensii	*Cytophagales*	Sea ice, seawater	10	SWCm	25
Polaribacter filamentus	*Cytophagales*	Sea ice, seawater	10	SWCm	25
Polaribacter glomeratus	*Cytophagales*	Seasonal marine basin	15–17	Marine 2216	25
Wall-less spirochete	*Spirochaetales*	Marine and lake sediment	12	DSMZ Medium 689	26
Clostridium vincentii	*Bacillus*/*Clostridium* group	Ice pond sediments	12–14	DMSZ Medium 769	27
Methanogenium frigidum	*Methanomicrobiales*	Saline lake anoxic sediment and water column	10	MSH (7 : 3 N_2 : CO_2)	28
Terrestrial species:					
"*Methylobacter psychrophilum*"	Gamma proteobacteria	Tundra soil	~10	NMS (1 : 1 CH_4 : air)	29
Psychrobacter urativorans	Gamma proteobacteria	Ornithogenic soil, food	18	Trypticase soy + 1% NaCl	30
Psychrobacter frigidicola	Gamma proteobacteria	Ornithogenic soil	14–15	Trypticase soy + 1% NaCl	30
Aquaspirillum arcticum	Alpha proteobacteria	Sediment below snow and ice	10–15	Trypticase soy agar	31

Table 1. (*Continued*)

Species[a]	Phylogenetic Affiliation	Habitat	T_{OPT} (°C)[b]	Isolation and Growth Medium[c]	Reference
Flavobacterium psychrophilum	Cytophagales	Salmon with cold water disease; cold streams	~20	Anacker-Ordal medium	32
Flavobacterium xanthum	Cytophagales	Pond mud	~17	Nutrient agar	33
Clostridium estertheticum	Bacillus/Clostridium group	Frozen, chilled food	10–12	DMSZ Medium 642	34
Micrococcus antarcticum	Actinobacteria	Soil	16–17	Nutrient agar	35

[a]Only species with a growth temperature optima of up to 20 °C are included.
[b]T_{OPT} for several species are only approximate values.
[c]Media formulations are detailed in the indicated reference. DMSZ media are media from the DMSZ, German Collection of Microorganisms, Braunschweig, Germany (refer to website *www.dmsz.de* or the catalogue).
[d]*Colwellia hadaliensis* and *Moritella yayanosii* are extremely barophilic and require hydrostatic pressure at 70–90 MP for optimum growth (10,15); *Shewanella benthica*, *Shewanella violacea*, and *Moritella japonica* are moderately barophilic with optimum growth occurring at approximately 30 MPa of pressure (13,18).

sites, such as the subglacial Lake Vostok (78 °S 106 °E) buried about 3,700 m below the Antarctic ice sheet (6–8), may also offer opportunities for novel discoveries of unique and unusual cold-adapted bacteria.

The marine ecosystem appears to be the best place to find psychrophilic bacteria as the majority of the oceanic volume is cold (<5 °C) and insulated from periodic or intermittent solar insolation. Marine psychrophiles, almost without exception, are able to grow at temperatures down to the limit at which normal seawater stays liquid (−2 to −5 °C) and most known psychrophiles originate from the marine environment, isolated from deep waters, sea ice, and polar sediments (Table 1). Some anecdotal evidence suggests that ideal environments for isolating psychrophiles are both permanently cold and relatively eutrophic. For example Burton Lake, a moderately eutrophic seasonal marine basin located in Eastern Antarctica (Vestfold Hills, 68 °S 78 °E) has enriched psychrophilic bacterial populations throughout its waters (2), whereas adjacent coastal under-ice waters only have relatively low psychrophilic bacterial populations (3,16). The production of psychrophilic bacterial populations, as in other mostly heterotrophic bacteria is closely coupled to primary production (36) and the presence of enhanced levels of substrate, deriving from photosynthetic exudates and from lysed cells are thought to allow more efficient growth at low temperature (16,36,37). Studies by Pomeroy and Deibel (38) have suggested that at low temperatures, marine bacterial productivity becomes uncoupled from primary production. More recent evidence suggests that environmental spatio-temporal variability makes generalization in this respect difficult. It has been surmised that specific components of the marine bacterial community probably respond quite differently to the availability of substrate at low temperatures (39). On this basis, psychrophilic bacterial populations may undergo substantial changes in abundance and may have to be sampled over time at a given site to get a true indication of their presence or absence.

Psychrophiles may also abound in attached communities, on organic detritus or on the surfaces of aquatic biota in polar waters (Bowman, unpublished data). Data from sea ice communities and Antarctic seawater suggest that species of the order *Cytophagales* and gamma proteobacteria make up the majority of marine psychrophiles (16,40). Species of the order *Cytophagales* appear to be closely associated with phytoplankton, whereas gamma proteobacteria are more likely to be free-living. Many psychrophilic bacteria, all grouping in the gamma subclass of *Proteobacteria*, have been isolated from the abyssal ocean and are usually barophilic or barotolerant. Specialized methods are required for their study (41–44). As isolation efforts today have not been particularly extensive in most cases, there still appears to be a significant scope for the isolation of novel psychrophiles from the natural environment.

Sample Treatment

The samples to be investigated for psychrophiles should be stored at all times at 0 to 4 °C until used in experiments; exposure to higher temperatures (<25 °C for a few hours at most) should be avoided when possible; in any case short exposures will usually only have a modest deleterious effect on the psychrophilic populations present. Samples should never be frozen, particularly if the samples are marine in origin, as many psychrophiles are easily lysed by freeze-thawing in the absence of cyroprotectants. For example, sea ice samples must be thawed in seawater at 0 to 4 °C to prevent hypotonic shock to the microorganisms present.

Samples can be added directly to or spread onto isolation media as is usual for traditional isolation. The receiving media, and if dilutions are needed the diluent medium, should be chilled before use (on ice or in the refrigerator). Primary incubation of isolation plates, enrichments cultures and the like should proceed at temperatures of 0 to 5 °C. Some psychrophiles may not grow on agar at temperatures of 10 °C or greater. However, virtually all known psychrophiles can grow at 10 °C in liquid media. Incubation at very low temperatures (<0 °C) has little apparent benefit in selective isolation of psychrophiles as psychrotolerant bacteria can grow almost as fast as psychrophiles at these temperatures. Incubation times required for the development of visible

growth are dependent on the initial populations present in the samples.

Media, Enrichment, and Enumeration

Essentially, most existing isolation protocol for bacterial isolation can be directly adapted to the isolation of psychrophilic bacteria. The only critical criterion is that the source material must have a history of permanently low temperature (1). A useful way to enhance the isolation of psychrophiles is to briefly enrich the sample in the liquid isolation media at in situ temperature (1). This has been found to work quite well for the isolation of psychrophiles from sea ice diatom assemblages (16) in which enrichments were performed at about 0 to 1 °C for 24 hours. The enrichment should be carried out for up to only 48 hours and the sample immediately plated or transferred to fresh media.

The direct enumeration of psychrophiles is only practical if their populations are greater than that of psychrotolerant bacteria, for example, in sea ice algal assemblages (2,3,36; Bowman, unpublished data). By determining most probable number (MPN) counts (45) at 0 to 2 °C and at 25 °C for a given sample, the proportion of psychrophilic versus psychrotolerant bacteria can be revealed.

Routine Maintenance and Preservation

Many chemoheterotrophic psychrophiles can be maintained on agar plates or slants for long periods at 1 to 2 °C in a frost-free cooler or refrigerator. Care must be taken to avoid freezing of the media due to ice-nucleation events, as it will result in complete loss of viability of the cultures. Media should be supplemented with suitable antifungal agents, such as cycloheximide (at 100 μg ml^{-1}, add from a filter-sterilized 10% ethanol stock) and nystatin (at 250 U ml^{-1}, add from a 25,000 U ml^{-1} filter-sterilized methanol stock). If agar plates are used, they need to be reasonably dry to avoid bacterial contamination. Most psychrophiles that have been isolated should be subcultured every four to six months when stored at 2 °C. Storage at higher temperatures (4 to 10 °C) requires more frequent transfer (once every 1–3 months) as viability is lost at a higher rate. Some psychrophiles, such as the species *Colwellia psychrerythreae* and some members of the *Cytophagales*, are quite delicate and will die on plates in only a few days. These species can be preserved as dense suspensions in sterile seawater and stored at 2 °C for several months.

For longer-term storage, including preservation of delicate strains, a dense suspension of cells is prepared in about 2 to 5 ml of growth media that has been supplemented with 20 to 30% glycerol or dimethyl sulfoxide. The suspensions should then be frozen initially at −20 °C and then stored at −70 to −80 °C. For continued recovery of cells from the frozen suspension, repeated thawing should be kept to a minimum. For most psychrophiles, direct inoculation of frozen cryopreserved culture directly to plates or liquid media is usually sufficient. Large numbers of small aliquots of the cryopreserved culture(s) may also be a convenient safeguard as they are thawed only once, used, and then discarded. Special tubes and boxes for cryopreservation storage are available from a number of laboratory suppliers.

Characterization

Characterization data has a variety of uses; firstly, the data is required for taxonomic analysis, particularly if the objective is to describe psychrophilic strains as a novel species or genus. Beyond the purposes of taxonomy, many "traditional" phenotypic tests can also used for the prescreening of psychrophiles for cold-adapted enzymes. Methods for the quantitative analysis of cold-adapted enzymes are much the same as for normal enzymes. The only safeguard to be taken is to prevent denaturation of enzymes due to their generally greater heat lability. The characterization, mechanistic characteristics, and features of cold-adapted enzymes have been covered in numerous reviews (46,47) and are covered in the other chapter in this book on psychrophiles (see PSYCHROPHILIC BACTERIA: ISOLATION AND CHARACTERIZATION, this Encyclopedia). There are no phenotypic tests specifically dedicated to the characterization of psychrophilic bacteria. Tests that should be used depend on the bacterial group in which the strain falls among, whether it is a chemoheterotroph or a more specialized group such as a methanotroph. Most other techniques required for bacterial characterization, including chemotaxonomic and genotypic methods, follow the same approach as that for other prokaryotes. The methods for detailed fatty acid analysis are given later in this chapter because psychrophiles may have unusual fatty acid components.

DETERMINATION OF CARDINAL TEMPERATURE VALUES

Principal and Applications

The square root growth model (48) has been implemented to accurately determine cardinal growth temperatures of a variety of psychrophilic and psychrotolerant bacteria (2,9,49–50). This model is based on the principle that the square root of the growth rate is linearly related to temperature and can predict growth rates across the entire biokinetic range (48). The model can be defined as follows:

$$\sqrt{r} = b(T - T_{MIN})(1 - \exp c(T - T_{MAX})) \qquad (1)$$

where r is the growth rate at temperature T, T_{MIN} is the notional minimum growth temperature (where $\sqrt{r} = 0$), T_{MAX} is the notional maximum growth temperature (where $\sqrt{r} = 0$), b is the slope of the regression line and c is the coefficient to be estimated experimentally. Together with the optimum growth temperature (T_{OPT}), T_{MIN} and T_{MAX} are cardinal temperatures for the biokinetic range of a given organism. All cardinal temperatures occur over a continuum range, including T_{MIN}. For marine psychrophilic and psychrotolerant bacteria, T_{MIN} values are usually in the range of −5 °C to −22 °C. TGI-based analysis of cardinal temperatures provides a useful set of autoecological data for psychrophilic bacteria, can be

used for physiological and environmental comparisons and provides useful data for strain characterization.

To accurately obtain growth rate data over a temperature range, a temperature gradient incubator (TGI) (available from Advantec or Terratec Pacific Ltd.) is employed. Alternatively, several temperature-adjustable water baths can be used. The TGI has a bar shaped block that is fitted at each end with thermal and cooling units that can contain 24 to 30 test tubes and can also be oscillated to keep cell suspensions evenly mixed and to avoid oxygen gradients. The test tubes used are custom-made L-shaped tubes (18-cm stem, 7-cm side arm) made of optical quality glass, designed such that spillage does not occur when the TGI is operating and so that they can be placed directly in a spectrophotometer (e.g. Spectronic 20D series made by Spectronic Instruments is very suitable for the task) to read optical densities.

Inocula used in the experiment should be grown to the late logarithmic or stationary growth phase in a suitable growth medium and at a temperature that ensures rapid growth. L-tubes, containing 10 ml of the growth medium, are placed in the TGI for at least one hour to allow for temperature equilibration with the TGI, set with a minimum temperature of about 0 °C and a maximum temperature of 30 °C or so (for psychrophiles); for psychrotolerant bacteria, the maximum temperature end of the TGI should be set to about 45 to 50 °C. The L-tubes are inoculated with sufficient growth to achieve an absorbance at 540 nm of about 0.1 (if the initial cell concentration is about 10^{10} cells ml^{-1} the amount would be 200–300 µl). Optical density readings are then taken just following inoculation. L-tubes are agitated at about 40 oscillations per minute. At periodic intervals after inoculation, optical density values at 540 nm and the time since inoculation are recorded. Between optical densities of 0.01 and 1.5, turbidity increases linearly (51). The experiment is effectively complete for an L-tube once its optical density at 540 nm exceeds about 1.5, because only the exponential growth rate area of the growth curve is needed and recordings into the stationary growth phase are not necessary. In any case, a minimum of 15 readings should be recorded for each tube. At approximately one-generation intervals and following growth cessation, the temperature is recorded using an electronic thermometer fitted with a thermocouple from each L-tube (e.g. Fluke Inc). Optical density values plotted against time should form a sigmoid curve from which maximum specific growth rate (μ_{max}) and doubling time (t_d) can be determined from the steepest tangent to the fitted curve as follows:

$$\mu_{max} = \frac{Ln2}{t_d} = \frac{Ln(10)B}{e^1} \qquad (2)$$

where B is the slope of the steepest tangent. The doubling time can be thus determined by fitting the linear section of the growth curve (exponential growth region) with a regression line and then by determining the time interval (in minutes) required for the optical density to double in this region. To determine cardinal temperatures, the reciprocal square roots of either growth rates or generation times are taken and plotted against their respective temperatures. A nonlinear regression is then fitted using appropriate software (e.g., SigmaPlot, UltraFit) as shown in Figure 1.

It should be noted that a detectable decrease in growth yield occurs at the extremes of the supra- and suboptimum temperature range. This has the effect of potentially skewing growth rate information. Thus, T_{MIN} and T_{MAX} values are subject to a potential level of error (i.e. ±1–2 °C). To counter this, viable count data derived by serially diluting cultures from high and low end of the TGI and plating onto agar (incubated at the T_{OPT}) can also be used to help pinpoint the temperature of the growth–no growth interface.

Determination of the T_{OPT} of Cold-Adapted Enzymes

TGI technology can also be conveniently used for assaying enzymes over a range of temperatures to determine T_{OPT}. Colorimetric procedures are useful because they can be measured directly from L-tubes spectrophotometrically, following termination of the reaction. A simple Arrhenius-type model can be used to predict enzyme T_{OPT}, T_{MAX} and T_{MIN} and can be shown in logarithmic form (52):

$$\ln k = \ln \mathrm{A} \exp(-E_a/\mathrm{RT}) \qquad (3)$$

k = the enzyme rate.
A = the collision factor (a constant equal to the number of collisions between reactants per unit time).
E_a = the "activation energy" (related to the enthalpy of activation).
R = the gas constant (1.987×10^{-3} kcal mol^{-1} K^{-1} = 8.314 J mol^{-1} K^{-1}).
T = the absolute temperature (°K).

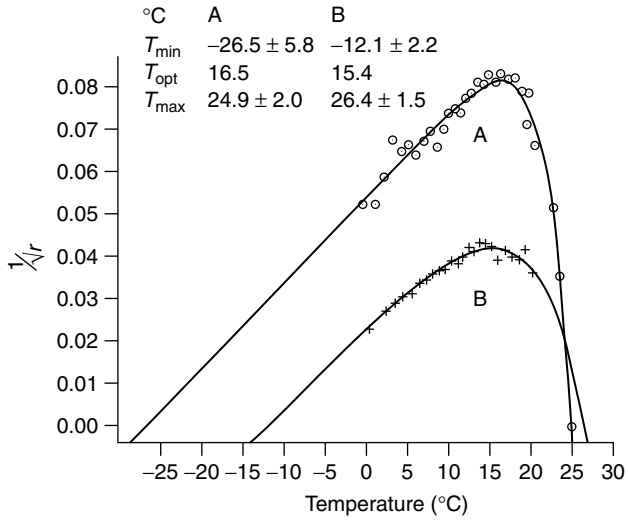

Figure 1. Square root growth rate–temperature plots of the type strains of A) *Shewanella gelidimarina* and B) *Glaciecola punicea*. The cardinal temperatures ($T_{MIN}/T_{OPT}/T_{MAX}$) for *Shewanella gelidimarina* and *Glaciecola punicea* are −26.5±5.8/16.5/24.9±0.2 and −12.1±2.2/15.4/26.4±1.5, respectively. The plotlines are nonlinear regressions based on the square root-growth model (48), using the Macintosh program UltraFit (v 3.0). The data shown was adapted from references (49) and (50).

From the TGI enzyme data, the natural logarithm of the rate is plotted versus the reciprocal of the absolute temperature (°K). A linear fit should be obtained for temperatures in the suboptimum range, the slope of which is equal to the activation energy (E_a) for the enzyme reaction. This model is only intended to describe enzyme function in the suboptimum temperature range.

It has been hypothesized that bacterial growth response to temperature is dictated by a rate-limiting enzyme-catalyzed "master reaction" and thus protein synthesis and ribosomal integrity govern the limits of growth. Models developed to test this are based on the changes of protein conformation with relation to temperature. A more exacting mechanistic model for determining the effects of temperature on enzyme catalysis rates, throughout the entire kinetic range, has been synthesized by combining the Arrhenius-based models of Brandts (53), Murphy and coworkers (52,54):

$$\text{rate} = \frac{CT\exp(\Delta H^{\ddagger}/RT)}{1+\exp(-n(\Delta H^{*}-T\Delta S^{*})+\Delta C_{\text{p}}[(T-T^{*}_{\text{H}})-T\ln(T/T^{*}_{\text{S}})])/RT} \quad (4),$$

C = an experimentally derived value, encompassing enzyme concentration; substrate concentration; a proportionality constant to account for measurement units, and the factor kT/h where k = the Boltzman constant and h = the Planck constant.

$\Delta H^{\ddagger}$ = activation enthalpy of the reaction catalyzed by the enzyme.

ΔC_{p} = difference in heat capacity (mol^{-1} amino acid residue) between the native and denatured-state of the enzyme.

T^{*}_{H} = temperature (K) at which the ΔC_{p} contribution to enthalpy is 0.

T^{*}_{S} = temperature (K) at which the ΔC_{p} contribution to entropy is 0.

ΔH^{*} = value of enthalpy at mol^{-1} amino acid residue of the enzyme.

ΔS^{*} = value of entropy at mol^{-1} amino acid residue of the enzyme.

n = number of amino acid residues in the enzyme.

For this model, the values C, the number of amino acids, the heat capacity change (ΔC_{p}) involved in denaturing the enzyme, and the activation energy ($\Delta H^{\ddagger}$) need to be estimated from the available data. Values for T^{*}_{H}, T^{*}_{S}, ΔH^{*} and ΔS^{*} have been previously reported to be constant for all globular proteins (54). An example of the application of this method is shown in Figure 2.

Analysis of Fatty Acids Associated with Psychrophiles

The ability to maintain cellular membranes in a homeoviscous state is an important adaptation of psychrophilic bacteria to cold (2). In this respect, psychrophilic bacteria often express high levels of anteiso- and iso-branched fatty acids and unsaturated fatty acids, depending on the taxonomic group (2,56). Several psychrophiles have the ability to form polyunsaturated fatty acids (PUFA), a trait unusual among bacteria (57). PUFAs produced by psychrophiles include eicosapentaenoic acid ($20:5\omega3$), docosahexaenoic acid ($22:6\omega3$) and arachidonic acid ($20:4\omega6$); fatty acids that are important "nutriceuticals" (58).

For the analysis of these fatty acids, a modified Bligh and Dyer procedure (59,60) is used to obtain an extract of whole-cell fatty acids and of neutral lipids (hydrocarbons, sterols, waxes etc.). Fatty acids are then transesterified to form methyl esters (fatty acid methyl esters, FAME) and analyzed by GC-MS techniques (61). GC-only systems that allow for rapid fatty acid analysis, such as the MIDI system, identify FAME components by retention times alone and are not able to definitively identify all fatty acids, including many monounsaturated fatty acids and unusual fatty acids

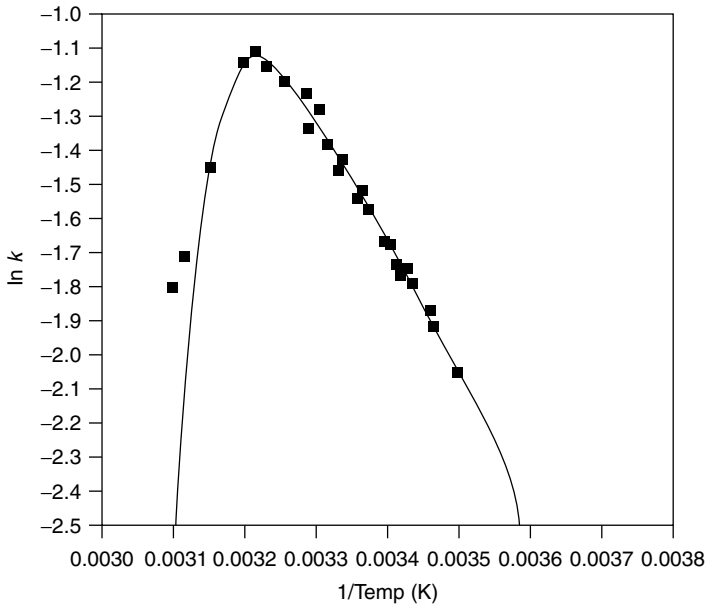

Figure 2. The effect of temperature on the catalytic rate of β-galactosidase from the psychrotolerant Antarctic bacterium *Flavobacterium hibernum*. The data (55) was fitted with an Arrhenius mechanistic model (52), which in this case assumes the constant C to be 85.1, the parameter $\Delta H^{\ddagger}$ (activation energy) was equivalent to 2.88×10^4 kJ, Cp (heat capacity) was equal to 67.2 kJ mol^{-1} amino acid residues and the number of amino acids (n) was equal to 167.

such as PUFAs. Thus, a high proportion of the fatty acid profile can be left unidentified or even misidentified. Using GC-MS based procedures, accurate and definitive identification of fatty acids can be achieved, including those with unusual or novel structures. Thus, if it is possible, GC-MS methods should be used when describing the fatty acids of psychrophiles. Identification of the position of double bonds in the monounsaturated fatty acid FAME is possible by dimethyldisulphide (DMDS) derivatization (62). In this method, DMDS in a chemical reaction catalyzed by iodine and attacks the fatty acid at the double bond, resulting in CH3S adducts that can be identified by GC-MS. For more complex PUFAs, such as those indicated in the preceding section, the number of mass fragments derived from DMDS derivatization makes mass spectra too complicated to be interpreted. Instead, PUFA FAME can be reacted with 2-amino-2-methylpropanol to create 2-alkenyl-4,4-dimethyloxazoline (DMOX) derivatives (63). DMOX derivatives have the advantages of having high volatility and allowing direct GC analysis, and their mass spectra are easily recognizable, allowing unambiguous determination of the positions of unsaturation.

CONCLUSION

Continued investigation of the biodiversity, ecology, physiology, and genetics of psychrophilic prokaryotes is clearly needed. There are opportunities to increase our understanding of adaptation of life to extreme environments and learning new concepts in how biological mechanisms have adapted to operate at the lowest temperatures. Further isolation of novel psychrophilic taxa, covering all types of microbial processes, will obviously aid in this learning process and provide opportunities in the biotechnological arena. The study of psychrophiles should continue to advance our knowledge for some time at both basic and applied research levels.

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PSYCHROPHILIC MICROORGANISMS.

See Cold-Adapted Microorganisms: Adaptation Strategies and Biotechnological Potential; Use of Cold-Adapted Microorganisms in Biotechnology

PSYCHROTOLERANT MICROORGANISMS.

See Extremophiles: Life in Extreme Environments; Use of Cold-Adapted Microorganisms in Biotechnology

PSYCHROTROPHIC BACTERIA.

See Cold Shock

PSYCHROTROPHIC MICROORGANISMS.

See Cold-Adapted Microorganisms: Adaptation Strategies and Biotechnological Potential

PULP AND PAPER INDUSTRY: MICROBIOLOGICAL ASPECTS OF

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Pulp is a fibrous material produced either by mechanical or chemical means, or a combination of the two, that separate the cells of woody plants into lignin, cellulose, and hemicellulose. This pulp is then used to form a homogenous sheet of interwoven cellulose fibers that can be made into a variety of paper products. Pulp and paper products are an important part of economies and the lives of individuals throughout the world, and natural resources, including trees and water, are consumed in the production process. Paper provides a means of transferring and documenting information through newspapers, journals, books, and for writing and computers. It is estimated that 155 million tons of wood pulp are produced each year and this number is expected to increase by 67% by 2010 (1). Also, nonwoody plants are an important source of fiber for the production of pulp and paper in many countries where wood fiber is limited (2,3). Microorganisms are involved in many aspects of the pulp and paper making processes and can have a profound effect on the quality of the final product and the effects on the environment. Several negative aspects of microorganisms are the degradation and discoloration of wood fibers that reduce pulp yields, brightness and paper quality, and the formation of biofilms during the pulp and paper making process that can reduce the quality of the final products by causing deposits on the paper. Several positive aspects of microorganisms are the use of lignin-degrading fungi to produce pulp and paper, the use of microorganisms and enzymes to bleach pulp, and the use of fungi and bacteria to reduce pitch problems and associated toxic levels of resin acids in mill effluents.

WOOD DEGRADATION AND BIOLOGICAL PULPING AND BLEACHING

Fungal Wood Degradation

Wood fibers are made of lignin, cellulose, and hemicellulose. The percentage of each component varies between tree species (4). Cellulose is composed of repeating glucose anhydride units, whereas hemicellulose is a polymer of five sugars, including glucose, mannose, galactose, xylose, and arabinose. Lignin is a complex phenolic polymer that can be found between wood fibers in the middle lamella and is incorporated with cellulose and hemicellulose within the cell walls of the fibers (4). Lignin imparts the rigidity and stiffness to the wood and is a difficult polymer to break down by most microorganisms except fungi.

White and brown rot fungi are found in the Basidiomycete family and are most commonly associated with substantial loss of wood fiber in trees and wood stored as chips or whole logs. Decayed wood fibers used in the pulp and paper process can cause reduced pulp yields, reduced paper strength, and discoloration of the pulp. Fungi are composed of filamentous cells called *hyphae* that make up the mass of mycelium. Fungi utilize the available sugars and nutrients in wood, and the hyphae penetrate and degrade cell walls and pit membranes by excreting different enzymes that can break down a variety of cell wall components in wood cells. Some species of white rot fungi simultaneously degrade lignin, cellulose, and hemicellulose. Some white rot fungi can selectively degrade substantially more lignin and hemicellulose than cellulose. Some examples of important white rot decay fungi are *Heterobasidion annosum*, *Inonotus circinatus*,

Inonotus tomentosus, and *Phellinus weirii*, in conifers and *Ganoderma* sp. *Tremetes versicolor*, *Phellinus termulae*, and *Fomes fomentarius* in hardwoods (5). It has been estimated that decay in coniferous trees from *P. weirii* causes 4 million cubic meters of lost wood each year (5). Brown rot fungi degrade the carbohydrate component of the cell walls and leave the lignin. Some examples of important brown rot decay fungi are *Phaeolus schweinitzii* in conifers, *Fomitopsis pinicola* and *Laetiporus sulphureus* found in both conifers and hardwoods, and *Piptoporus betulinus* in hardwoods (5). Other microbial pathogens can cause substantial loss or reduced growth of trees that can have an effect on the availability and quality of wood fiber used for the pulp and paper industry.

Biological Pulping

In the pulp and paper making process, cellulose is the most important component that must be separated from lignin by chemical, mechanical, or both chemical and mechanical methods. Recent studies have looked at pretreating wood chips with lignin-degrading fungi prior to pulping to reduce the amount of chemicals and energy needed during the pulping process (6–9). Several white rot fungi have been screened and found to substantially remove more lignin than cellulose (6,7) (Table 1).

From laboratory studies of these and other fungi *Ceriporiopsis subvermispora* was selected for further study because it grew equally well on aspen and loblolly pine chips, substantially reduced the amount of energy needed during the mechanical pulping process, increased the strength of the paper, and enhanced brightness of the pulp (6). A large-scale study was done at the Forest Products laboratory in Madison, Wisconsin, with 40 tons of spruce chips inoculated with *C. subvermispora* and incubated for two weeks. Chips were mechanically pulped and it was determined that a 30% savings in energy was achieved versus nontreated chips under the same conditions. The strength and brightness properties of the pulp were also significantly enhanced compared to nontreated chips (10). Pretreatment of wood chips with *C. subvermispora* before chemical pulping was also found to reduce residual lignin contents by 30% over control chips. However, the brightness of the pulp from this treatment was lower because of the production of chromophores by the fungus (11).

Pulp from nonwoody plant materials constitute 10.6% of the fiber used to make paper products in many developing countries and could be a source of fiber to help supply the growing demand for pulp and paper (3). Jute is a nonwoody plant grown in Bangladesh, India, China, and Thailand. Mechanically pulped jute fibers pretreated with *C. subvermispora* and incubated for two weeks had a 33% reduction in the amount of energy needed for pulping versus control fibers not treated and incubated under the same conditions (2). Also, the strength of the paper was increased by 33% compared to control jute fibers not treated with *C. subvermispora* (2). Other nonwoody fiber material are available throughout the world that could benefit from pretreatment with microorganisms to produce pulp and paper with less energy and enhanced paper quality.

Substantial amount of research has been done on the mechanisms and selection of lignin-degrading fungi, and the engineering process needed to produce pulp and paper using fungi to pretreat wood or pulp. The production of pulp and paper using fungi is a promising environmentally friendly approach that could reduce dependency on chemicals and also maintain the quality of paper products needed for today's market. Some problems still remain, as it is difficult and expensive to provide a hospitable environment for the targeted fungi to grow because the temperature, moisture, and competition from other microorganisms need to be regulated. Also, the cost of producing the inoculum and engineering and converting the individual mills for a biological pulping process may be costly as each mill is unique in that they use different processes, wood species, and are located in different climates.

Biological Bleaching

After the wood has been pulped, its color is too dark to be used for printing grade and tissue paper because of the high residual lignin content. Further treatments are needed to bleach the pulp, which may include the use of chlorine dioxide or chlorine-free bleaching using oxygen, ozone, hydrogen peroxide, and peracids (12). Delignifing white rot fungi such as *T. versicolor*, *Phanerochaete chrysosporium* and *P. sordida* have been used as a pretreatment to reduce the amount of residual lignin in the pulp. Pulp treated with *T. versicolor* showed increased brightness, with substantial decrease in the residual lignin of the pulp, and reduction of the amount of chlorine needed for bleaching (12). Several important enzymes, including laccases, manganese peroxidases, and lignin peroxidases, derived from fungi are responsible for the degradation of the lignin and the biological bleaching

Table 1. Results of Lignin and Wood Sugar Analyses of White Pine Decayed for 12 Weeks by Several White Rot Fungi[a]

	Loss (%)				
Fungi	Biomass	Lignin	Glucan	Xylan	Mannan
Ceriporiopsis subvermispora	25	50	3	48	13
Phanerochaete chrysosporium	20	31	4	44	0
Phlebia brevispora	27	49	11	60	16
Phellinus pini	27	45	8	55	40

[a] Composition of sound *Pinus strobus* = 33% lignin, 41% glucose, 8% xylose, and 11 % mannose. Data based on information from reference 6.

effect. Attempts have been made to use these individual lignin-degrading enzymes extracted from fungi in pulp for biological bleaching with limited success.

Xylanase enzymes from bacteria and fungi have been successively used to treat pulp before bleaching in both laboratory and mill trials (13). It was demonstrated that xylanase-treated hardwood and softwood pulps had higher pulp yields, increased brightness, and reduced amounts of bleaching chemicals needed by 20% in hardwoods and 15% in softwoods (13). The mechanism of how xylanase works to improve the properties of pulp is still not fully understood.

Microbial Degradation of Pitch and Staining of Wood

Pitch is a term used to describe resin and fatty acids, sterols, steryl esters, waxes, terpenes, and glycerol esters found in woody plants that are not part of the cell structure and are soluble in neutral, nonpolar organic solvents (14,15). These pitch components can be problematic in the pulp and paper manufacturing process by causing deposits on machines and paper, resulting in reduced paper quality and increase costs to mills (15,16). It has been estimated that a 1,000-ton pulp per day mill could lose more than two million dollars per year to problems associated with pitch (17). High resin acid concentration in effluents produced by the pulp and paper making process has been demonstrated to be toxic to fish and other aquatic organisms (18). The amount and types of resins and fatty acids differ greatly between hardwoods and softwoods and within these two groups (15). Pitch in softwoods is located in resin canals and parenchyma cells, whereas in hardwoods it is located in ray parenchyma cells (14). Current methods employed to reduce pitch include the use of additives such as talc, dispersants, enzymes added to pulp, and seasoning of logs and chips before pulping (16).

Seasoning of wood has long been used as a method by paper mills to reduce pitch and the problems associated with deposits on paper (14). The degradation of resin and fatty acids in seasoned wood is the result of oxidation, volatilization, enzymatic hydrolysis, and activities of microorganisms. A detrimental effect of long-term wood storage is the increased loss of fiber caused by fungi that decay the wood and reduce fiber yield and the proliferation of staining fungi in the Ascomycete family such as *Ophiostoma* and *Ceratocystis* species. These staining fungi are pioneering microorganisms that can quickly colonize and discolor wood, resulting in increased use of bleaching chemicals being added to the pulping process. Hyphae from stain fungi invade the surface of the wood and penetrate the ray parenchyma cells and resin canals of pine species and vessel elements and longitudinal and ray parenchyma cells in hardwoods (Fig. 1) (19).

Stain fungi also have the beneficial effect of degrading substantial amounts of resin and fatty acids with little or no destruction of wood fibers (19). The stain fungus *Ophiostoma piliferum* was selected for further study because of its fast growth and its ability to degrade pitch in southern yellow pine (19). Since *O. piliferum* stains wood, an albino strain was developed by an ascospore mating of different isolates of *O. piliferum*. This albino strain, called Cartapip™ was developed by Sandoz Chemical Corporation of Charlotte, North Carolina (now Agrasol, Inc., Raleigh, North Carolina), and demonstrated rapid surface and internal growth of mycelium, and substantial degradation of pitch. It also controlled the growth of wild-type stain fungi naturally found growing in the wood (19,20). This can greatly reduce the time that chips need to be stored with the same or better results as long-term storage with minimal loss of wood fiber, reduced wood staining, and a decrease in the use of chemicals during the pulping process. When logs are cut for use in pulp and paper production, they are sometimes stored for varying lengths of time. Cartapip™ has been used in the field to inoculate freshly cut pine logs. It has been demonstrated to control the growth of wild-type blue staining fungi (21). In mill trials, loblolly pine chips treated with Cartapip™

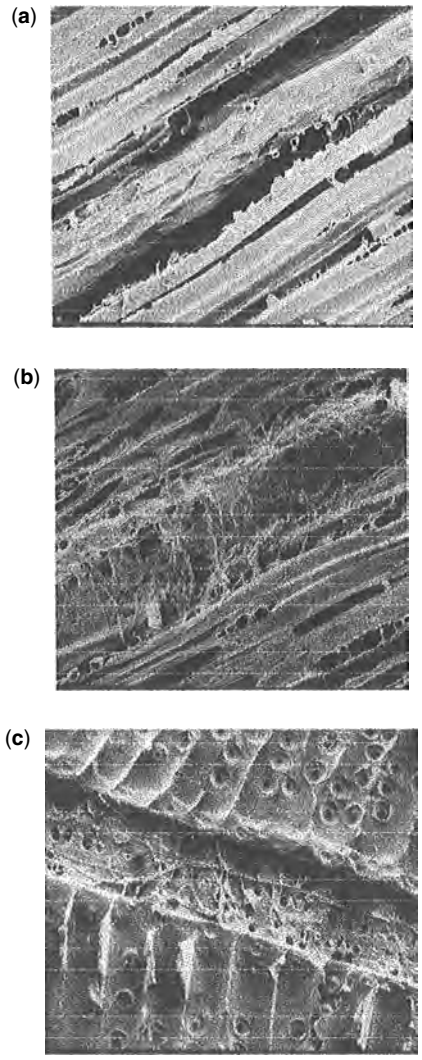

Figure 1. Scanning electron microscope of mycelium from the albino strain of *Ophiostoma piliferum* (Cartapip™) pretreated loblolly pine chips two weeks after inoculation. (**a**) Resin canal of loblolly pine before pretreatment. (**b**) Resin canal two weeks after pretreatment showing mycelium and removal of pitch. (**c**) Ray parenchyma cell two weeks after pretreatment showing mycelium.

degraded 45% of the pitch over a 12-day period versus chips at the beginning of the trial. Individual resin and fatty acids were also reduced (19,20). Cartapip™ can also grow and degrade pitch in hemlock, fir, jack, radiata and southern yellow pines, birch, cottonwood, aspen, and maple (22) (Table 2).

Loblolly pine chips pretreated with the albino strain of *O. piliferum* (Cartapip™) in the mill increased the production of paper by 17%, improved paper strength and quality, and reduced the amount of sodium hydrosulfide by 28% during the bleaching process, as compared with chips that were not pretreated (22).

Other fungi have also been found to degrade pitch in pine, including *Ophiostoma piceae*, *O. ainoae*, and *Lecythophora* species (23). A variety of microorganisms can easily degrade fatty acids and triglycerides in pitch, but the degradation of steryl esters and waxes mostly found in hardwoods is more difficult for fungi to degrade (24). Several fungi were screened and found to degrade these steryl esters and waxes extracted from aspen, including Basidiomycetes (*Bjerkandera adusta*, *P. chrysosporium*, *T. versicolor*, *Peniophora polygonia*), Zygomycetes (*Cunninghamella elegans*), and imperfect fungi (*Aspergillus luchuensis*, *A. awamori*, *A. flavus*, *Aureobasidium pullulans*, and *Gliocladium roseum*) (25). Other important wood species used for pulp and paper are eucalyptus in which fungi (*Phlebia radiata*, *Funalia trogii*, *Bjerkandera adjust*, and *Poria subvermispora*) have been shown to degrade 75 to 100% of the esterified sterols and other pitch components in *Eucalyptus globulus* (26).

Bacteria are ubiquitous and a variety of species were isolated from wood in which several demonstrated the ability to degrade pitch in pretreated southern yellow pine chips over a two-week incubation period (27) (Fig. 2a). The species *Pseudomonas fluorescens* NRRL B21432 degraded substantial amounts of individual resin and fatty acids found in the wood (27) (Table 3). In U.S. Pat. 5,766,926, Blanchette and coworkers demonstrated that pitch was also reduced in other woods, including aspen, spruce, maple, and mixed hardwoods pretreated with bacteria (28).

It was also demonstrated that the bacteria could degrade the pit membranes inside pretreated loblolly pine chips incubated for 14 days, which could facilitate the penetration of pulping chemicals and reduce cooking times (27) (Fig. 2b).

Lipase enzymes have also been used in the pulp and paper industry to reduce pitch and problems associated with wood resins. In Japan, laboratory and mill trials used a lipase enzyme produced from *Candida cylindracea* that was applied to ground Japanese Red pine pulp. This enzyme hydrolyzed 70% of the triglycerides (29). Triglycerides are a major component of pitch and their reduction could decrease the use of chemicals to control the pitch content in pulp and increase the quality of the paper (29). In a study by Fisher and Messner (1992), unbleached softwoods treated with a lipase called Resinase A (provided by NOVO Enzyme Process Division Bagsvaerd, Denmark) for two hours demonstrated a 80% reduction in triglycerides. Lipase, however, does not affect the fatty acid component, which can also cause pitch problems. Further treatment with sodium hydroxide solution was needed to remove the remaining fatty acids (30).

Table 2. Reduction of Pitch in a Variety of Woods[a] Pretreated with the Albino Strain of the Staining Fungus *Ophiostoma piliferum* (Cartapip™) for Two Weeks. The Percent Reduction is in Comparison with Control Wood Chips Incubated in the Same Conditions as the Wood Inoculated with Cartapip™

Wood Species	% Reduction
Hemlock-Fir mix	6.5
Jack Pine	22.2
Radiata Pine	21.5
Southern Yellow Pine	18.1
Aspen	10.0
Birch	10.7
Cottonwood	14.4
Maple	22.5

[a]Wood was chipped before pretreatment. Data based on information from reference 22.

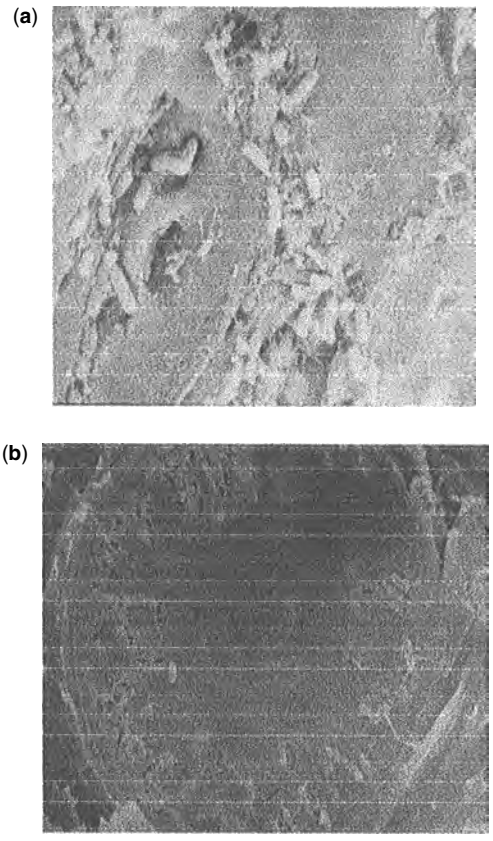

Figure 2. Loblolly pine chips inoculated with *Pseudomonas fluorescens* NRRL B21432. (**a**) Bacteria in pitch deposits on the surface of the wood 7 days after inoculation. (**b**) Bacteria degrading the margo region of a pit membrane 14 days after inoculation.

Table 3. Percentage of Pitch Removed From Bacteria Treated and Nontreated Southern Yellow Pine Chips Incubated For 14 Days

Treatment	% Pitch[a]	Comparison Groups(s)[b]	% Reduction
Fresh[c]	4.4±0.78	A	
Control with water	3.9±1.10	A, B	11.4
Serratia marcescens (NRRL B21430)	3.4±0.33	B	22.7
Xanthomonas campestris (NRRL B21429)	3.2±0.16	B	27.3
Pseudomonas sp. (Strain UM-18)	3.2±0.25	B, C	27.2
Pseudomonas sp. (Strain UM-74)	2.9±0.31	C	34.1
Pseudomonas fluorescens (NRRL B21432)	2.6±0.49	C	40.9

[a]Mean ± standard deviation from four replicates.
[b]Means within groups designated by the same letter were not significantly different at the 0.05% level using Ducan's new multiple-range test.
[c]Represents the amount of pitch at the beginning of the experiment.
Data based on information from reference 27.

Another important role of biological treatment of wood or pulp with pitch degrading microorganisms and enzymes is the reduction of toxic levels of resin acids released into the environment from pulp and paper mills, which can account for up to 70% of the effluent toxicity and a major source of fish toxicity (18). Current methods to treat effluents that are derived from pulp and paper manufacturing is to remove the solids by sedimentation and clarification, followed by secondary or biological treatments using aerobic or anaerobic conditions (31–33). The aerobic biological treatment of pulp and paper effluents is the most widely used method to remove dissolved organic molecules by indigenous microorganisms. These include oxidation basins, stabilization ponds, lagoons, sludge areas, or batch reactors (31). The mixed microflora of these aerobic treatments include bacteria, fungi, protozoa, rotifers, yeast and viruses, which are responsible for oxidation of organic molecules (32). Bacteria, including several *Pseudomonas* species, such as *Pseudomonas abietaniphila* BKME-9, and *Zoogloea resinophila* Dha-35 were isolated from pulp and paper biological treatment processes and have demonstrated the ability to degrade different resin acids (34,35). A polymerase chain reaction assay has been developed to screen for resin acid–degrading bacteria. The use of these bacteria could greatly reduce toxic levels of resin acids in the effluent systems and their release into the environment (35).

The treatment of wood and/or pulp with fungi, bacteria or enzymes could be a more environmentally safe way to treat wood and pulp to remove harmful pitch in the pulp and paper making process; however, it has its limitations. Microorganisms must be selected for the best growth on the particular wood species used in mills, environmental conditions need to be modified to enhance the growth of the microorganisms and enzymes in the wood or pulp, and the cost to the mills to implement and set up a process for the use of biological treatments may not be economically viable.

Associated Problems with Biofilms

During the pulp and paper making process, biofilms or slime can build up in machines in the mill and cause deposits on the final product and economic loss to the mill (36). Slime is the accumulation of microorganisms, their by-products, and organic and inorganic materials that can take on strange shapes and sizes (36). Water is the main source of microorganism contamination, along with the raw materials and preexisting biofilms. Bacteria, fungi, and yeast are the predominant microorganisms present in these biofilms and biocides are the main control mechanism (36). The type of biocide that is selected for use in controlling biofilms is also important and may affect the quality of the paper products (37,38) (see BIOFOULING OF INDUSTRIAL SYSTEMS).

CONCLUSION

The production of pulp and paper will continue to play a vital role in the economies of many countries and in the advancement of our society. Microorganisms are involved in many aspects of this process. Research in microbiology will continue to provide information on understanding the role microorganisms have in fiber and pitch degradation, staining of wood, biofilm production, bioremediation of effluents from mills, and other aspects relating to pulp and paper production. Understanding these processes will help us select microorganisms that will improve pulp and paper production and reduce the impact it may have on our environment.

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QUALITY ASSURANCE/QUALITY CONTROL IN SUBSURFACE SAMPLING AND PROCESSING.

See SUBSURFACE SAMPLES: COLLECTION AND PROCESSING

QUALITY, SOIL QUALITY.

See SOIL QUALITY: THE ROLE OF MICROORGANISMS

QUANTIFICATION OF MICROBIAL BIOMASS

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Recent estimates of global microbial biomass indicate that prokaryotic cellular carbon ranges from 330 to 550 Pg (1 Pg = 10^{15} g). This is more than half the estimated carbon found in plants. Bacteria are also rich in nitrogen and phosphorous and prokaryotic biomass may represent the largest living reservoir of these essential elements (1). One of the greatest challenges of microbial ecology has been the development of quantitative and nonselective methods for the study of microorganisms in their natural environments. Microbial biomass is one of many parameters that help examine the roles microorganisms play within natural systems. On a global scale, the quantification of living microbial biomass is important in understanding biogeochemical cycling, global carbon budgets, ecosystem energy flow, food-web dynamics, and carbon turnover and sequestration. On smaller spatial and temporal scales, it can be used to detect disturbance and perturbation in both natural and engineered systems. Biomass is defined as the mass of living biological material in a defined area. In most environments, it cannot be measured directly because of the difficulty in distinguishing viable carbon biomass from other organic components in the sample matrix. Historically, researchers have used abundance measurements or activity-based assays to estimate microbial biomass (Fig. 1). In this review, the advantages and disadvantages of some of these procedures are discussed and specific methods for the determination of viable biomass using cell

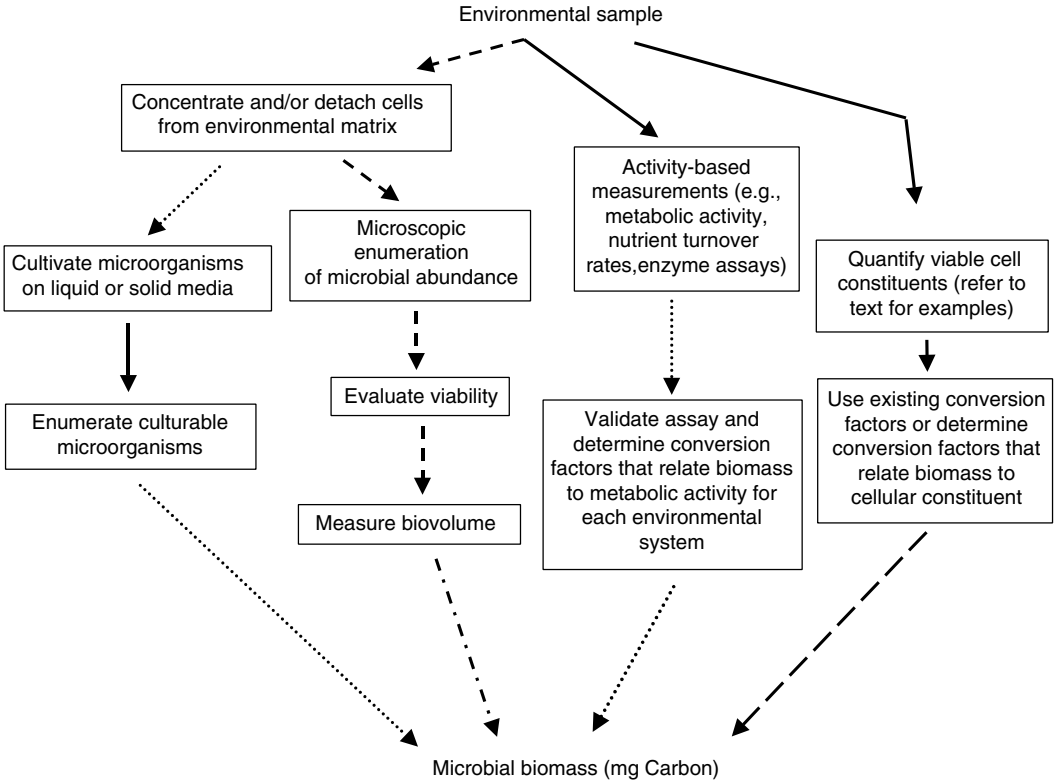

Figure 1. Experimental approaches commonly used for estimating microbial biomass. Dashed arrows indicate steps in the procedures in which current methods may introduce bias or uncertainty to the final biomass estimate. The extent of bias or uncertainty is indicated by the size of the dash where finely dashed lines indicate that current methods for measurement or calculation of these parameters may be indeterminate.

constituents as biological index molecules are elaborated (Fig. 1). Assays specifically designed for the quantification of microeukaryote or archaeal biomass are discussed separately.

CLASSICAL METHODS OF BIOMASS ESTIMATION

Culture-based methods such as the direct viable count or the most probable number procedure have proven to be inadequate for estimating total viable microbial biomass. These techniques are often selective and only permit the growth of organisms that are able to flourish under certain defined laboratory conditions. Typically, these techniques detect only 0.1 to 10% of the microbial community when compared to direct measurements of abundance (2–6).

The quantification of microbial abundance is another approach commonly used for the indirect calculation of microbial biomass. Epifluorescence microscopy coupled with the use of nucleic acid stains such as acridine orange and 4′,6-diamidino-2-phenylindol (DAPI) is commonly used for the enumeration of total number of bacteria in aquatic samples. Scanning electron microscopy and transmission electron microscopy provide higher resolution and have also been used for enumeration and sizing of microbial populations (7). Phytoplankton biomass has been estimated in a similar manner using fluorochrome-induced fluorescence and autofluorescence of plant pigments (8). The use of cell sorting techniques and high-resolution flow cytometry is an improvement on traditional methods of bacterial enumeration and can help monitor the dynamics of specific microbial populations (9,10) when combined with specific fluorescent probes (either antibodies or oligonucleotides).

The use of direct microscopic count techniques for biomass estimation presents many challenges. Most bacteria live an attached lifestyle (11); therefore, in many systems microorganisms need to be quantitatively removed from surfaces, particles, host tissues, or each other before enumeration. Methods of sonication, homogenization, or dispersion are not uniformly effective for all populations (12–15). In some matrices, visualization of organisms may be obscured by detrital particles, sediment granules, or neighboring organisms (7,16,17). There are significant concerns associated with the choice of stain used in enumeration techniques. Although DAPI is considered to be more DNA specific than acridine orange, it binds poorly to DNA at high salt concentrations. In marine microbiology cells are often fixed with formaldehyde before enumeration. Recent evidence suggests that under these conditions DAPI stains reactive bacterial surfaces and is not specific to nucleoid containing cells (18). Therefore, total counts may overestimate bacterial abundance by 68 to 98% (18). Once microorganisms are collected and enumerated, there are uncertainties in using a per-cell carbon conversion factor for the determination of biomass (19–22). The range of dry weights for bacterial isolates from soil and litter varies between 1.66 and 67.5×10^{-13} g cell^{-1} (23). Assuming that 50% of cell mass is carbon, dry weight estimates for natural assemblages of aquatic bacterial cells are in the range of 0.4 to 0.5×10^{-13} g cell^{-1} (19). The use of inappropriate conversion factors will lead to corresponding errors in biomass estimates. Alternatively, many researchers have sought to measure biovolume and generate conversion factors for the determination of viable microbial biomass. Determination of size class distributions of microbial populations are achieved directly by using an eyepiece graticule or indirectly by taking photomicrographs or by using an image analysis system (7,8,19). Apparent size measurements are affected by sample preparation and sizing method (20,24–26) and artifactual errors can be significant (19,20).

There are several methods for calculating biomass from cell volume and their respective merits are still under debate (21). Studies indicate that for both bacteria and algae, smaller cells have more biomass per unit volume than large cells (27–30). For bacteria, the carbon to volume ratios can vary by a factor of 500 depending on the size classification (21). These variations are likely due to both real biotic variations (either in community composition or in physiological state) and cell dimension measurement errors (20).

Soil microbiologists have used chloroform fumigation incubation (CFI) and extraction (CFE) techniques for the indirect determination of biomass. The details of these techniques are described elsewhere (31). In each technique, soil samples are flooded with chloroform vapor to kill any living biomass. In the CFI technique, fumigated soils are reinoculated with a small amount of original soil. Mineralization of the freshly killed biomass is measured over a 10-day period and the resultant CO_2 and NH_4^+ are measured and used to calculate carbon and nitrogen biomass. The analysis of appropriate controls is essential and results should be calibrated by measuring carbon dioxide evolution derived from the addition of cells with known carbon and nitrogen contents (31,32). Some soils display background respiration from degradation of soil organic matter that is equal to the mineralization rates following fumigation, suggesting very low or even negative biomass (31). Although questions about these experimental problems are still being debated in the literature (33,34), investigators have continued to revise the equations for biomass calculation to account for the discrepancies (35). One of the major problems with this technique is that it requires the assumption that the respiratory activity of the reinoculated population is directly proportional to the quantity of newly killed biomass in the fumigated soil. These two parameters are frequently not correlated (36–39) and metabolic activity is often not a good predictor of biomass.

The CFE technique is not dependent on physiological measurements and requires no further incubation of soil samples. Killed biomass and other soil organic matter components are extracted from fumigated soils with 0.5 M K_2SO_4. Carbon content is determined from elemental analysis. Unfumigated soils are analyzed in the same way and used as background controls. These estimates have a high degree of uncertainty since biomass carbon extraction efficiencies can be variable depending on soil pH and soil type (40,41).

BIOCHEMICAL CONSTITUENTS

Technical problems and experimental biases associated with traditional biomass measures prompted microbial ecologists to use a biochemical approach for studying microorganisms in the environment. In 1981, Jenkinson and Ladd (42) put forth several useful criteria for the selection of a biochemical component that could be used for the indirect measurement of microbial biomass in soils. Through the years researchers have adapted these criteria (3,43–47) and facilitated the development of sensitive, quantitative, and unbiased methods that can be applied in any environment. The biochemical index molecule should (*1*) be common to all cells, (*2*) be present in a constant proportion in all microbial cells regardless of physiological state, (*3*) be present only in viable cells and be rapidly degraded on cell death, and (*4*) be present in sufficient quantity to be readily extracted from environmental samples. The quantity of this component should be easily related to biomass. Lastly, methods should be readily available for quantitative extraction and sensitive detection of the specific component within environmental samples. A number of different biochemical components have been used for the estimation of microbial biomass including cellular protein, ATP, phospholipid fatty acids, and nucleic acids. Cell constituents such as muramic acid, lipopolysaccharide, photosynthetic pigments, ergosterol, and glycerol ether lipids are phylogenetically restricted and are useful when a specific group is to be targeted. Each technique has certain advantages in the study of microbial communities, but none of these completely satisfy the described criteria as a perfect metric for total microbial biomass.

Cellular Protein and Adenine Nucleotides

Total protein can be easily quantified and is a useful measurement for characterizing bacterial populations in the laboratory. However, intracellular protein content varies with organism and growth conditions and, as a biomass measure, its use is limited. In addition, detrital protein pools are often high in environmental samples. For many years it was assumed that biomass production was proportional to ATP production. As this cell constituent is rapidly degraded on cell death and is present in all organisms, it is considered a reliable method for the indirect measurement of biomass. It is now well known that intracellular ATP concentrations change with growth rate, metabolic state, and nutrient limitation (48–51). Reported ATP contents per cell vary more than 1,000-fold (52). Total intracellular adenine nucleotide pools ($A_T = ATP + ADP + AMP$) are less sensitive to changes in specific growth rate (53–55), but are still somewhat variable with metabolic status (51,52). Much less is known about these relationships, and the utility of A_T as a surrogate for biomass is still not clear (52).

Phospholipid Analysis

All living cells contain polar lipids and in most microorganisms these are dominated by glycerophospholipids. Phospholipid content is relatively stable as a proportion of dry cell mass (56,57) although the actual fraction varies with different groups of microorganisms (47,58). In most environments, intact phospholipids are quickly hydrolyzed after cell death (within minutes to hours), releasing soluble phosphate (59). Consequently, phospholipid-bound phosphate and phospholipid fatty acids are unique to living organisms and meet many of the described criteria as appropriate cell constituents for indirect biomass estimation (46,59). These membrane components can be quantitatively recovered by organic solvent extraction, and sensitive detection methods are readily available. Balkwill and coworkers (16) compared these techniques to other biomass measures in a carefully designed experiment and found that these biochemical constituents were reliable measures of viable biomass, and under certain conditions, could be related to bacterial abundance. The following sections detail the experimental methods used for microbial phospholipid analysis and the subsequent determination of viable biomass.

Phospholipid fatty acid analysis has been effectively used for the determination of microbial biomass and community structure in almost every environment on Earth (60), including plant litter, ice cover, surface soils, aquatic sediments, biofilms, deep subsurface sediments and aquifers, planktonic communities, subterranean microbial mat communities, the Antarctic desert, hydrocarbon-contaminated sediments, filtered indoor air samples, sewage sludge, and rhizospheres. To obtain sufficient biomass for detection, sample concentration may be required in some environmental matrices (e.g., open water, indoor air, and industrial metalworking fluids). It is best to avoid using plastics either in sample preparation or as artificial substrates for growth when lipid analysis is to be applied. Lipid-soluble contaminants are often released from these materials during organic solvent extraction and can interfere with the detection of target molecules. Whenever possible, samples should be transferred directly to the solvent extraction mixture immediately after collection. This provides the best opportunity to obtain a snapshot of total viable biomass and community structure. If sample preservation is necessary, the best option is to immediately place the samples on dry ice and keep frozen at $-70\,^{\circ}C$ until lipid extraction can be performed. Up to 50% of the biomass may be lost if samples are allowed to thaw before lipid extraction (61). Storing samples at $4\,^{\circ}C$ can lead to biomass losses (61) and shifts in community composition (62). Laboratory-grown cultures should be centrifuged and rinsed with an appropriate buffer solution before extraction.

There are two general approaches to biomass estimation by means of phospholipid analysis (Fig. 2). The most convenient and inexpensive assay quantifies phospholipid-bound phosphate (PLP) as a principal component of living cells. The second approach is an extension of the first and involves the measurement of ester-linked phospholipid fatty acids (PLFA). The latter technique provides increased sensitivity and additional information on community composition (47,60,63). Unfortunately, PLFA techniques only allow the detection of bacteria and microeukaryotes and overlook the archaeal community

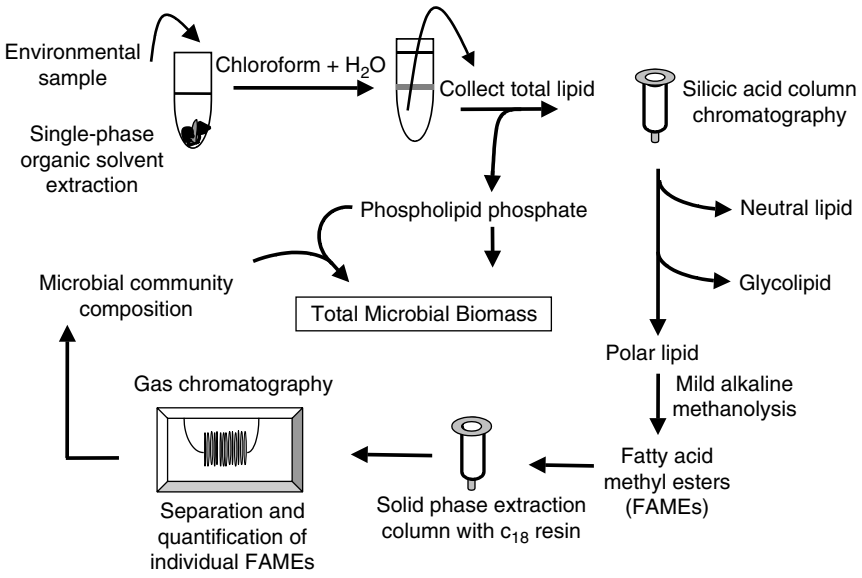

Figure 2. Experimental approaches for the determination of total microbial biomass using phospholipid analysis. Sub-samples of the total lipid extract may be removed for the determination of phospholipid phosphate and subsequent estimation of total microbial biomass. Alternatively, the determination of microbial community composition by quantification of PLFA may be used in conjunction with phospholipid phosphate data to estimate viable carbon biomass for individual groups (e.g., phototrophic microeukaryotes or prokaryotes).

component. The PLP technique allows detection of *Archaea* although few species have been characterized in this way, and conversion factors are not well established. Some researchers are using alternative biochemical approaches to better quantify this group of organisms and these methods are discussed in a separate section.

The general procedures for phospholipid analysis are outlined in the following segment but the reader should consult other references for more detailed protocol descriptions (47,56–60,63). When performing any type of quantitative lipid biochemistry, the researcher must take special precautions to minimize sample contamination. Procedural blanks should be processed in parallel with all sample sets to monitor contamination from reagents or solvents. Interfering compounds can originate from a multitude of other sources including dust, hair, fingers, plastics, soaps, adhesives, and oil lubricants. Laboratory glassware should be washed in hot soapy water (phosphate-free detergent is recommended) and vigorously rinsed at least 10 times with both tap water and deionized water. Once dry, all glassware should be baked for at least four hours at 450 °C to remove all organic contaminants. Use glass for sample and reagent handling whenever possible since organic solvents can dissolve interfering compounds from some plastics. High-purity organic solvents of gas chromatography/mass spectrometry (GC/MS) grade should be used when samples are destined for PLFA analysis. All screw caps for test tubes and reagent bottles should be teflon-lined.

The initial steps of the lipid extraction were adapted from Bligh and Dyer (64) and consist of a single-phase organic solvent extraction containing methanol, chloroform, and phosphate buffer (2 : 1 : 0.8) (56,59). Dichloromethane can be substituted for chloroform and gives equal recovery after an overnight extraction (47). Frostegard and coworkers showed greater extraction efficiency using citrate buffer in soils with high organic matter content (65). After a suitable period (typically 2–16 hours depending on the specific protocol), the single-phase extraction is split into aqueous and organic phases by addition of chloroform and water to obtain a final solvent ratio of 1 : 1 : 0.9 (methanol:chloroform:buffer/water). During the second step of the lipid extraction, samples are shaken vigorously and stored at 4 °C overnight in the dark to allow for phase separation. Lipid-soluble components are recovered from the lower organic phase. During recovery, the sample may be passed through a fluted Whatman 2V filter to remove residual water and extracted debris. When soils or sediments are used it may be difficult to collect the entire organic fraction, therefore it is best to measure the exact volume of chloroform recovered and normalize final biomass estimates accordingly. Samples are concentrated by nitrogen evaporation.

Total viable biomass is quantified by measuring PLP according to the procedures described by Findlay and coworkers (46). Briefly, replicate subsamples of the total lipid fraction are transferred to glass ampules and dried under nitrogen. The dried lipid is suspended in a potassium persulfate solution (made in saturated sulfuric acid, 0.36 N). Ampules are heat-sealed and the lipid-bound phosphate is liberated after overnight acid digestion at 95 °C. Orthophosphate is then detected using a colorimetric assay with ammonium molybdate and malachite green (46). A standard curve constructed using glycerol phosphate (ranging from 0 to 15 nmol per ampule) should be analyzed in parallel with all experimental samples.

The PLP method of biomass estimation is both quantitative and reproducible (coefficients of variation less than 5%) for environmental samples (46). When working in environments with high amounts of microbial biomass (e.g., surface soils, sediments, and some aquatic environments), the technique is convenient and inexpensive. Its use is limited when analyzing low-biomass samples. Lower limits of detection are around 0.1 nmol lipid-bound phosphate corresponding to approximately 3.4×10^6 cells per ampule (46). Therefore, if only PLP analysis is to be performed, the sample should contain at least 1×10^7 cells.

Microbial biomass and community structure can also be estimated from the quantification of individual PLFA. Extensive details of lipid biomarker analysis are described by Tunlid in LIPID BIOMARKERS IN ENVIRONMENTAL MICROBIOLOGY, this Encyclopedia. Briefly, the polar lipids are recovered from the total lipid extract by silicic acid column chromatography. Ester-linked PLFA are derivatized to form fatty acid methyl esters (FAMEs) by mild alkaline methanolysis (66). Resultant FAMEs can be purified on solid phase extraction columns packed with C_{18} resin (63), and separated and quantified by gas chromatography (GC) with flame ionization detection. Structural identification is verified by GC/MS. When using these techniques for biomass determinations, it is important to add a recovery standard such as phosphatidyl choline nonadecanoate (a 19 carbon PLFA present in very small amounts in natural samples) before lipid extraction. Phospholipid recovery from silicic acid columns can vary between laboratories and silica gel manufacturer; therefore, it is important to account for potential losses.

The quantification of individual PLFA provides greater sensitivity than the PLP method with detection limits at approximately 10^5 bacterial cells (67). Mass spectral characterization of membrane components has further been refined with the application of soft ionization techniques for the characterization of intact phospholipids (68). These molecules can be separated by liquid chromatography and subsequently quantified and characterized by electrospray ionization mass spectrometry (ESI/MS). These analyses increase the sensitivity of the assay by an order of magnitude and provide more specific information by discerning phospholipid classes as well as fatty acid profiles (68). This type of structural characterization has been performed with only a small number of bacteria, but expanding this database will undoubtedly improve our ability to detect and identify specific groups of microorganisms. Unfortunately, these techniques require sophisticated analytical equipment that are very expensive. Even routine PLFA analysis requires the use of high-purity reagents and specialized equipment such as gas chromatographs equipped with flame ionization and mass selective detectors.

The assay has been modified in a variety of ways to minimize extraction volume (46), enhance extraction efficiency (65,69), increase sample throughput (46,69), and enhance corecovery of other lipid biomarkers (70,71). For example, Macnaughton and coworkers developed a pressurized hot solvent extraction procedure that requires smaller extraction volumes and is complete in 35 to 40 minutes (69). When compared to the modified Bligh and Dyer procedure, the accelerated solvent extraction system gave similar yields for vegetative biomass and enhanced PLFA recovery for spores and airborne biomass. Other adaptations have been made to obtain other types of biological and physical information from a single sample. These include simultaneous extraction and purification of nucleic acids for molecular analyses (72), separation and specific derivitization of phoshpo-ether lipids for archaeal community structure determination (73), and simultaneous recovery of organic pollutants for additional chemical characterization (74).

Quantifying phospholipid content in environmental samples is relatively straightforward, but relating those values to a meaningful biomass estimate is much more difficult. If the experimental question is confined to determining differences among treatments for a single study or a few similar studies, it may be just as convenient to report biomass as nmol PLP per gram dry weight or volume of material. However, if interstudy comparisons are necessary, it might be useful to report biomass in units that are more easily interpreted.

Like cell size, cellular phospholipid content can vary with the physiological status of the cells. For instance, *Vibrio cholerae* cells had a 99.8% decrease in cellular phospholipid content after only seven days of starvation (75). Similarly, different groups of microorganisms have varying amounts of phospholipid in their cytoplasmic membranes (47). Consequently, phospholipid content cannot easily be related to cell abundance in natural microbial communities. In contrast, the relationship between phospholipid content and bacterial carbon appears to be less sensitive to changes in growth state and substrate concentration (76,77). Brinch-Iversen and King (76) determined conversion factors between phospholipid-bound phosphate and bacterial carbon for a variety of bacterial isolates and enrichment cultures. R. H. Findlay has summarized these results and comparable data for other types of microorganisms (47). The suggested conversion factors to calculate biomass in grams carbon for each group are 190 μmol P g^{-1} C for aerobic bacteria, 100 μmol P g^{-1} C for anaerobic bacteria, and 50 μmol P g^{-1} C for eukaryotes. To effectively apply these conversion factors, the researcher should have some information about the microbial community composition of the target environment. If the community is dominated by bacteria and the environmental conditions are well defined, then the choice of conversion factor is straightforward. When microeukaryotes or *Archaea* are prevalent, PLFA analysis can provide community structure information. For communities dominated by bacteria or microeukaryotes, the ratio of nmols PLFA to nmols PLP ranges between 1.7 and 2. Very low PLFA:PLP ratios (less than 0.8) may indicate a significant archaeal component. Similarly, the phospholipid biomarker analysis allows the researcher to quantify the relative dominance of prokaryotes versus microeukaryotes. Partitioning phospholipid biomass into prokaryote and microeukaryote components allows the more judicious application of conversion factors and more precise estimation of microbial carbon.

Nucleic Acid–Based Techniques

The development of nucleic acid–based techniques has drastically expanded our view of microbial diversity and has allowed the study of microbial community dynamics at the population level. Microbial ecologists have applied these tools through several different approaches that are thoroughly discussed elsewhere (78–82). If nucleic acid–based techniques are to be used as quantitative descriptors and to compare communities from diverse environments, at least two criteria must be met. Target nucleic acids should be extracted equally well from various environments and from various types of microorganisms (78).

These technical difficulties have not been fully resolved but investigators are making strides to quantify and account for relative extraction efficiencies of nucleic acids in various environmental matrices (78,83–85). There is also significant concern about biases associated with PCR amplification of community DNA and the generation of rDNA clones (86–88).

The use of rRNA for microbial community analysis is advantageous since it represents viable populations and can be used to discern community structure. In situ hybridization techniques that allow the identification of individual cells have proven to be extremely useful for studying population dynamics and physiological states of organisms in certain environments (89). Interpretation and extrapolation of these types of data is not clear-cut (90). Kerkhof and Kemp quantified per-cell rRNA content of nine strains of marine Proteobacteria during non-steady-state growth conditions (90). Cellular rRNA contents increased approximately 2- to 100-fold during the growth cycle, and there was a 2- to 15-fold difference in peak per-cell rRNA content between strains. Therefore, in a given environment, two populations may be equal in size and in a similar physiological state but still exhibit a 15-fold difference in total 16S rRNA content. In this situation, analysis of community RNA with phylogenetic probes would erroneously suggest that one population was numerically dominant. At present, nucleic acid–based techniques have utility as qualitative descriptors of microbial diversity and community structure but do not provide a quantitative description of microbial abundance or biomass. More research relating nucleic acid–based community assessment results to independent biomass or community structure measures (e.g., phospholipid fatty acid analysis) will help evaluate the influence of potential experimental bias imposed by some molecular techniques.

Bacterial Cell Wall Components

Muramic acid, lipopolysaccharide, and teichoic acid have all been proposed as biochemical index molecules for indirect biomass estimation. These cell wall components are only found in bacteria and can be recovered from the residual interface of a two-phase lipid extraction (45,91). Muramic acid is one of the amino sugars that constitute the peptidoglycan layer. Standard conversion factors have been put forth to relate muramic acid content to bacterial carbon for gram-positive bacteria, gram-negative bacteria, and cyanobacteria, but in reality this component can be highly variable for all groups. Lipopolysaccharide, a component of the outer membrane of gram-negative bacteria and cyanobacteria, has been used for both taxonomic characterization (92) and as a general biomass marker (93). Similarly, teichoic acids can be extracted and quantified from the cell walls of gram-positive bacteria (94). Because of their restricted distribution, they are of little use as biomass markers in most environments.

Photosynthetic Pigments

Satellite-derived measurements of ocean color can now be effectively used to calculate phytoplankton biomass in most areas of the open ocean (95–97). Primary production models incorporate a variety of parameters including chlorophyll concentrations derived from remote sensing, sea surface temperatures, incident solar irradiance, and knowledge of other physiological parameters that effect photosynthesis and pigment concentration. Model estimates indicate that oceanic phytoplankton biomass (approximately 1 Pg C) is turned over every week and is responsible for the global oceanic net primary productivity of 45 to 50 Pg C annually (95). Similarly, satellite observations have been used to revise estimates for terrestrial inputs to global primary production (98). On a slightly smaller scale, phytoplankton biomass and population structure can be determined by means of enumeration by flow cytometry and chlorophyllous pigment analysis with spectrofluorometry. Partensky and coworkers (99) combined these techniques to provide a detailed description of the vertical structure of picophytoplankton populations in the northeastern Atlantic Ocean. Similar to lipid analysis, photosynthetic pigments can be extracted from sediment or water samples with organic solvents such as acetone or methanol for the determination of phytoplankton biomass. Historically, these types of methods have been criticized because chlorophyll *a* contents (as a percentage of cell biomass) can vary significantly with light intensity and nutrient availability (96). In sediments, chlorophyll *a* concentrations are often overestimated because of the co-measurement of degraded pigments (100). Chromatographic methods for the quantification and identification of chloropigments and carotenoids (100) allows the investigator to account for the presence of pigment degradation products and can reveal important information about algal community structure.

FUNGAL BIOMASS

Fungi play an extremely important role as decomposers in both terrestrial and aquatic systems. These organisms often grow on or within opaque substrates such as rocks or plant surfaces. Traditional methods of community analysis (microscopy or culture-based techniques) are considered inadequate since in many environments hyphal structures are not clearly visible or easily separated from their associated substratum (101).

Ergosterol is a membrane component and with few exceptions is restricted to eumycotic fungi (101). As a constituent of intact membranes, its abundance should reflect the amount of living fungal biomass in an environment. This membrane component has been related to biomass by a number of investigators and the values range from 1.9 to 11.5 mg ergosterol g^{-1} mycelium (102). These conversion factors yield very high values for fungal biomass, and seem unrealistic (102) when compared with independent measures of bacterial biomass. It is likely that the ergosterol assay detects nonliving hyphae and these measures may overestimate viable fungal biomass.

Similarly, chitin is a dominant cell wall component in most fungi and has been proposed as a unique marker for total fungal biomass. Ekblad and coworkers (103) recently investigated the utility of these methods as indirect biomass measures. They monitored the variability of ergosterol and chitin content with respect to fungal species, growth conditions, and mycorrhizal age. Results

suggested that when the two are measured in combination they provide a reasonable estimate of total and living fungal biomass (103). Gessner and Newell offer a thorough discussion of the ergosterol assay and the various quantitative extraction techniques commonly employed for fungal biomass determinations (101). Standard techniques for lipid biomarker analysis may also reveal the presence of fungi through the detection of the polyenoic PLFA 18 : 2w6 and sterols in the neutral lipid fraction (Fig. 2; 60,102). There are uncertainties with conversion factors and relative extraction efficiencies. Therefore, these data should be applied only as relative measures of fungal biomass (102).

ARCHAEAL BIOMASS

Among the three domains of life, we know the least about the relative distribution and ecological role of the *Archaea*. Before the 1990s, it was assumed that they were only found in extreme physical environments (halophiles and thermophiles) or highly specialized niches (methanogens). Recent ecological surveys of bacterial phylotypes suggest that these organisms are more ubiquitous than previously thought (104–108). Most of these organisms are yet to be cultivated, but their molecular signatures are commonly found in a variety of aquatic and terrestrial habitats (104,109,110). We have very little quantitative information about their relative contribution to microbiota in most environments and better biomass estimates would be useful for determining their ecological roles.

Archaeal membranes are composed of phytanyl-based ether-linked lipids and thus the determination of ester-linked PLFA overlooks these organisms. It appears that most genera of this domain have glycerophospholipids (111). Although rarely applied to this group, the PLP method appears to be equally effective for the quantification of phosphorus-containing ether lipids of the archaea. For example, pure cultures of *Thermoplasma acidophilum* contain approximately 21 μmol PLP $g\,C^{-1}$ (112). This value is consistent with the report by Langworthy and coworkers (113) that phospholipids represent 1.75% of the cell's dry weight. Still, there is little information about archaeal phospholipid content as a proportion of cell mass and the application of general conversion factors for carbon biomass estimates is imprecise. Nevertheless, as a gross estimate, we may assume that lipids comprise 2.2–4% of the mass of an archaeal cell and 22–94% of these are polar lipids (111). With a few exceptions, these are dominated by phospholipids (111). Therefore, if we assume that the average molecular mass of archaeal phospholipids is 900 (including both diether and tetraether lipids) and 50% of a cell's dry mass is carbon, the projected phospholipid content ranges between 10 and 83 μmol P per gram bacterial carbon. These values are comparable to the range of values reported for microorganisms in the other two domains (14–520 $\mu mol\,P\,g\,C^{-1}$) (47). Ether lipid analysis has already been applied toward archaeal community structure determinations (73,104,109,110) and the further application of quantitative lipid biochemistry for archaeal biomass determinations would complement nucleic acid–based measurements that estimate archaeal diversity. Together these techniques will expand our understanding of microbial community dynamics in many environments.

CONCLUSION

During the last two decades there has been a rapid development of new techniques for in situ detection of microorganisms. Our increased ability to detect and identify these organisms has led to a new understanding of the vital role they play in the biosphere. The preceding discussion focused on evaluating the appropriateness of current detection methods for determining the quantitative importance of microorganisms in the environment. Several of the techniques described here may be useful for detecting organisms that are of quantitative significance. In turn, these methods may not be sensitive enough to detect certain organisms that are few in number but are functionally important. Each of the described methods has certain advantages and the researcher should carefully consider the experimental question and available resources while choosing the experimental technique. Similarly, results should be interpreted in the light of the inevitable shortcomings of each method.

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QUORUM SENSING IN BIOFILMS.

See BIOFILMS IN NATURAL AND DRINKING WATER SYSTEMS; EXTRACELLULAR ENZYMES IN BIOFILMS; PATHOGENS IN ENVIRONMENTAL BIOFILMS

R

RADIATION EFFECT ON BACTERIA. See NUCLEAR WASTE RESPOSITORY IN YUCCA MOUNTAIN: MICROBIOLOGICAL ASPECTS

RADIATION EFFECTS ON MICROORGANISMS. See SPACE MICROBIOLOGY: EFFECTS OF IONIZING RADIATION ON MICROORGANISMS IN SPACE

RADIOACTIVE WASTE DISPOSAL, GEOMICROBIOLOGY OF

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Wastes containing significant quantities of radionuclides are produced not only by processes associated with nuclear power generation and the production of nuclear weapons but also by many other industrial, medical, and research activities. In common with other potentially hazardous wastes, the disposal of radioactive materials was somewhat haphazard in the past; either because their potential toxicity was not recognized or because of the pressures of bomb production during the Cold War period. However, over the last 20 years or so, a much more responsible approach to the disposal of such waste has evolved due both to generally increasing environmental awareness and to an increasing concern by the general public of all things nuclear. The growth of the new field of radioactive waste geomicrobiology has been paralleled by the development of rigorous methods to evaluate the performance and demonstrate the safety of special radioactive waste repositories.

Research and development (R&D) efforts in the 1970s focused on what was considered to be the more problematic waste — high-level waste (HLW) — which has such high concentrations of radioactivity that radiogenic heat output is significant. An example showing the diversity of radioactive waste is provided in Table 1. A consensus developed that deep disposal in stable geologic formations was the safest option for such waste. However, it was recognized that extensive investigations would be needed to demonstrate convincingly that no risk to the environment would arise over the very long timescales over which the contained radionuclides are potentially hazardous. This could be on the order of a million years. It was at this time that the evidence for the existence of microbial life in deep geologic formations was beginning to accumulate and some seminal studies of the potential influence of microorganisms on an HLW repository were carried out (2,3). Much to the surprise of many in the radioactive waste field, this early work showed clearly that microbial processes could be significant and led to nuclear waste management organizations being significant supporters of applied geomicrobiology.

Over the last 20 years, radioactive waste geomicrobiology has expanded to include analyses of disposal options for other types of waste. Projects have covered a wide range of areas including

- fundamental microbiology of deep geologic formations,
- microbial tolerance to extreme conditions,
- biodegradation of repository materials,
- interaction of microbes with radionuclides and their by-products, and
- microbial influences on the geochemistry of the repository environment.

The work involved has included field sampling, laboratory studies, and the development of mathematical models. All will be discussed from the viewpoint of those charged with developing concepts for nuclear waste disposal and assessing the long-term safety of existing and planned repositories.

FUNDAMENTALS OF RADIOACTIVE WASTE DISPOSAL

This article focuses on current concepts for the disposal of radioactive wastes in deep geologic formations. It does not directly include evaluation of "dilute and disperse" options for radioactive waste, which involves direct input of gaseous nuclides into the atmosphere or of liquid (occasionally solid) wastes into rivers, seas, or oceans. The role of microorganisms in the biogeochemical cycling of radionuclides after geologic disposal is, in principle, similar to that for other trace elements as considered elsewhere in this encyclopedia. There is also no direct consideration of near-surface disposal options; although many of the general principles discussed are equally applicable to surface repositories, in terms of microbiology, they tend to be more similar to disposal facilities for conventional (e.g., domestic) or chemotoxic wastes.

Deep geologic disposal (at least several tens if not hundreds of meters below surface) has been implemented, or is being planned, in many countries for many types of waste (e.g., 4). As yet, however, no repository for the most active HLW is operational, although significant steps in this direction have been taken in Finland and Sweden, and extensive site-characterization efforts are ongoing in the United States of America. Background principles of radioactive waste disposal can be found in a number of textbooks (5,6).

Table 1. An Extract from the Swiss National Inventory Providing Some Properties for HLW (WA-1) and Various Types of L/ILW–Average Values for Single Containers (1)

Waste Sort	Raw Waste	Material of Conditioning	α-Activity Bq/Container	β-γ Activity Bq/Container	Surface Dose Rate (Sv/h)	Heat Output (W)
			WA (Reprocessing Waste)			
WA-1	High-level vitrified residues	Glass	1.1×10^{14}	2.8×10^{16}	3.4×10^{3}	2.8×10^{3}
WA-2	Precipitates and sludges or $BaCO_3$ and MEB crud	Bitumen or cement	3.6×10^{8}–1.8×10^{10}	3.5×10^{11}–4.3×10^{12}	1.2×10^{-1}–5.2×10^{-1}	8.2×10^{-2}–4.5×10^{-1}
WA-4	Hulls and ends	Cement	1.4×10^{11}–2.8×10^{11}	1.4×10^{14}–8.2×10^{14}	2.4×10^{1}–3.9×10^{1}	2.0×10^{1}–1.2×10^{2}
WA-5	Technological low-level waste	Compacted	2.6×10^{6}–1.2×10^{8}	8.0×10^{7}–3.8×10^{9}	2.1×10^{-5}–1.0×10^{-4}	4.5×10^{-6}–2.1×10^{-4}
WA-6	α-emitting technological waste	Cement	7.4×10^{10}	7.3×10^{11}	7.0×10^{-3}	8.0×10^{-2}
WA-7	Centrifuge cake slurry	Cement	3.8×10^{11}	4.0×10^{13}	2.0	3.5
			MIF (Waste from medicine, industry, and research)			
MIF-1	β, γ-emitting waste	Cement	0.0	3.7×10^{10}	9.5×10^{-3}	3.1×10^{-3}
MIF-2	Tritium-bearing waste	Cement	0.0	1.2×10^{13}	3.0×10^{-5}	1.1×10^{-2}
MIF-3	"α" waste	Cement	1.2×10^{8}–1.3×10^{10}	0.0–1.1×10^{9}	3.0×10^{-9}–2.5×10^{-6}	1.0×10^{-4}–1.2×10^{-2}
MIF-4	Radium-bearing waste	Cement	2.1×10^{9}	0.0	5.4×10^{-6}	1.6×10^{-3}
MIF-5	"α, β, γ" waste	Cement	7.6×10^{8}–1.9×10^{9}	5.9×10^{10}–1.7×10^{11}	1.7×10^{-2}–3.2×10^{-2}	7.7×10^{-3}–1.5×10^{-2}

Note: Dose is dominated by γ-radiation; therefore absorbed dose (in Gray-Gy) is approximately equal to individual dose equivalent (in Sieverts–Sv).

Geologic repositories are based on the "multiple-barrier principle," in which long-term safety is assured by a series of engineered and natural barriers (Fig. 1). Waste is generally solidified in some kind of stable matrix, encapsulated in a container or some other type of package, and placed into underground tunnels, caverns, or silos. This packaged waste, along with any backfilling used to seal void spaces and other structural materials such as tunnel linings or plugs, is termed the *engineered barrier system* (EBS). Surrounding these engineered barriers is the rock itself, termed the *geosphere*. The "near-field" consists of the waste itself, the EBS, and disturbed rock surrounding the repository. The "far-field" comprises the undisturbed geosphere and extends into the biosphere.

Very many different combinations of engineered and geologic barriers have been designed to provide safe repository concepts for particular combinations of waste type(s) and geographic-geologic setting. Low- and intermediate-level waste (L/ILW) may be produced in a wide range of solidification matrices (e.g., cement, bitumen, resins, and metals), but is usually contained in steel drums or concrete packages with disposal caverns backfilled with cement-based grout (Fig. 2). Although such waste is very heterogeneous, the large amounts of cement and concrete used in construction tend to dominate the "near-field" in and around the EBS.

HLW usually consists of spent fuel — if this is considered as a waste for "direct disposal" or the solidified, high-activity wastes from the reprocessing of such fuel. In the latter case, the solidification matrix is usually a borosilicate glass or some kind of ceramic [e.g., "SYNROC" (7)]. A range of corrosion-resistant canisters has been developed for HLW, using steel, copper (Cu), titanium (Ti), or special alloys. The backfill used is either purified clay (e.g., bentonite) or clay–sand mixtures in many concepts (Fig. 3).

The simplified picture outlined holds for most national programs in which waste is disposed of in crystalline or sedimentary rocks below the water table. In these cases, the EBS plays a powerful role of delaying the access of liquid water to the waste and reducing the rate of subsequent release of radionuclides to surrounding groundwater. In many analyses, it is calculated that most nuclides decay to complete insignificance within the EBS.

The situation may be somewhat different in the case of "dry" evaporitic rocks such as salt. In such a case, the main barrier is the impermeable rock. Here, concern in the design of the EBS may focus on processes with the potential to perturb this function (e.g., production of gas). Crushed rock salt may be the ideal backfill in this case. Similarly, for deep repositories in desert areas, which are above the water table, low water availability is a key factor contributing to safety. In this case the EBS may be designed to take maximum credit for this factor (e.g., by ensuring temperatures stay above 100 °C for very long periods of time). Either no backfill at all or simple crushed rock for mechanical stability have been suggested for this case.

Despite the great diversity in the details of individual repository systems, a common concern is the quantification of their behavior over very long periods of time. The formalized procedures used to carry out such a

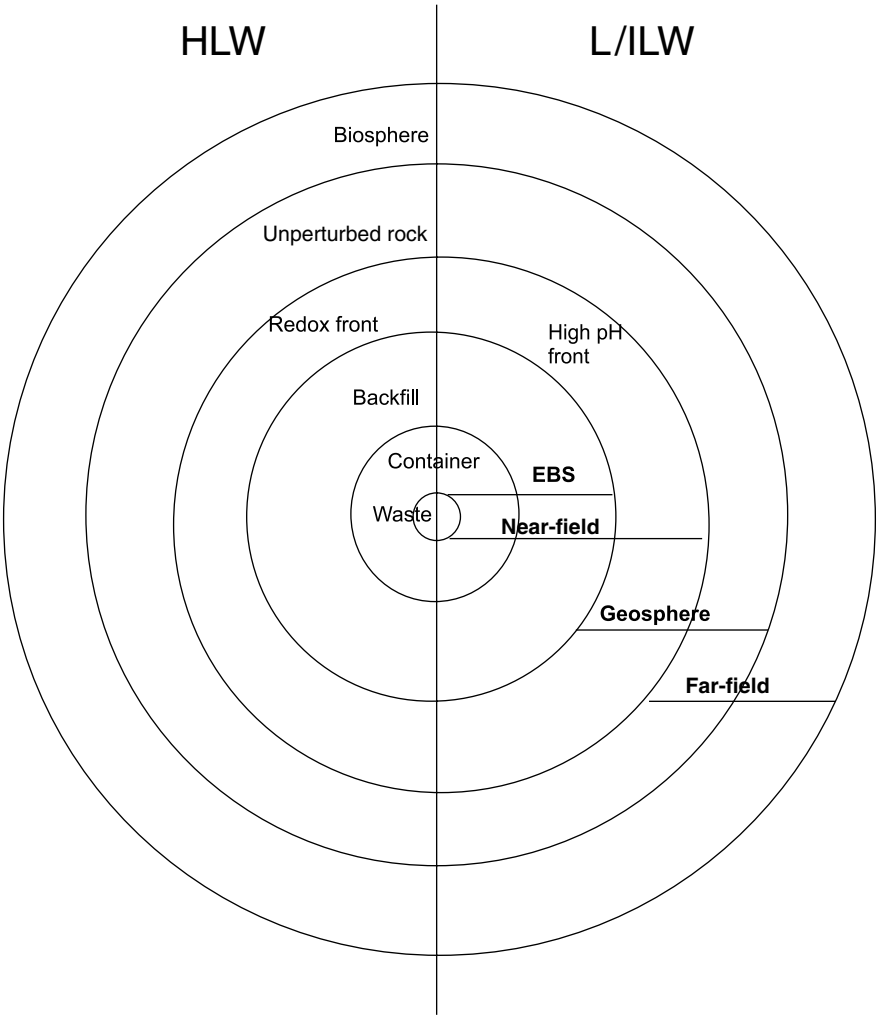

Figure 1. Radioactive waste–disposal concepts. EBS = engineered barrier system.

"performance assessment" (PA) utilize a database of the characteristics of the waste, the EBS, the surrounding geologic barrier, and the surface "biosphere." This provides input and boundary conditions for a series of models to represent slow EBS degradation, the release of radionuclides, and their transport through the geosphere to the accessible environment. Because it is not possible to predict the future, such analyses are carried out for a range of possible futures, termed scenarios. Given the requirements for PA it is important to consider the effects of microbial activity in this context. The following sections consider, in turn, the presence of microorganisms within the repository system, their possible influence on the performance of the engineered and natural barriers and, finally, the more challenging questions of how such influences can be quantified, especially in view of the enormously long timespans involved.

MICROBIOLOGY OF RELEVANT GEOLOGIC FORMATIONS

Following initial literature searches, an early part of several national geomicrobiology programs was to establish the presence of microbes in geologic formations being considered as host rocks for repositories; the rationale being that if no indigenous populations are present then microbes may not be viable in the environment selected and hence not be a problem. Studies have taken place in granites in Canada (8), Japan (9), Sweden (10–12), Switzerland (13), and the United Kingdom (14,15). Analyses have also been performed in sedimentary rocks in Belgium, Germany (10), the United Kingdom (15), Italy (16), and Japan. Evaporites (gypsum and salt) were sampled in Switzerland and Germany (17,18). In the United States, work has been undertaken on volcanic tuff (19,20). General reviews of microbial populations in a range of potentially relevant formations have also been presented (21,22). Analyses have mostly concentrated on groundwaters, although solid materials have been investigated for microbial content (14,15). In addition, studies on biofilms found on fracture surface in granitic environments have also been performed (23). All these studies have confirmed the presence of microbes in every sampled environment. Numbers vary from 10^2 colony-forming units (CFU) per milliliter to 10^5 CFU per milliliter in some groundwaters. A single exception is the salt site at Asse in Germany. Here quantification was impossible, although there were indications of the presence of small populations of extremely oligotrophic organisms

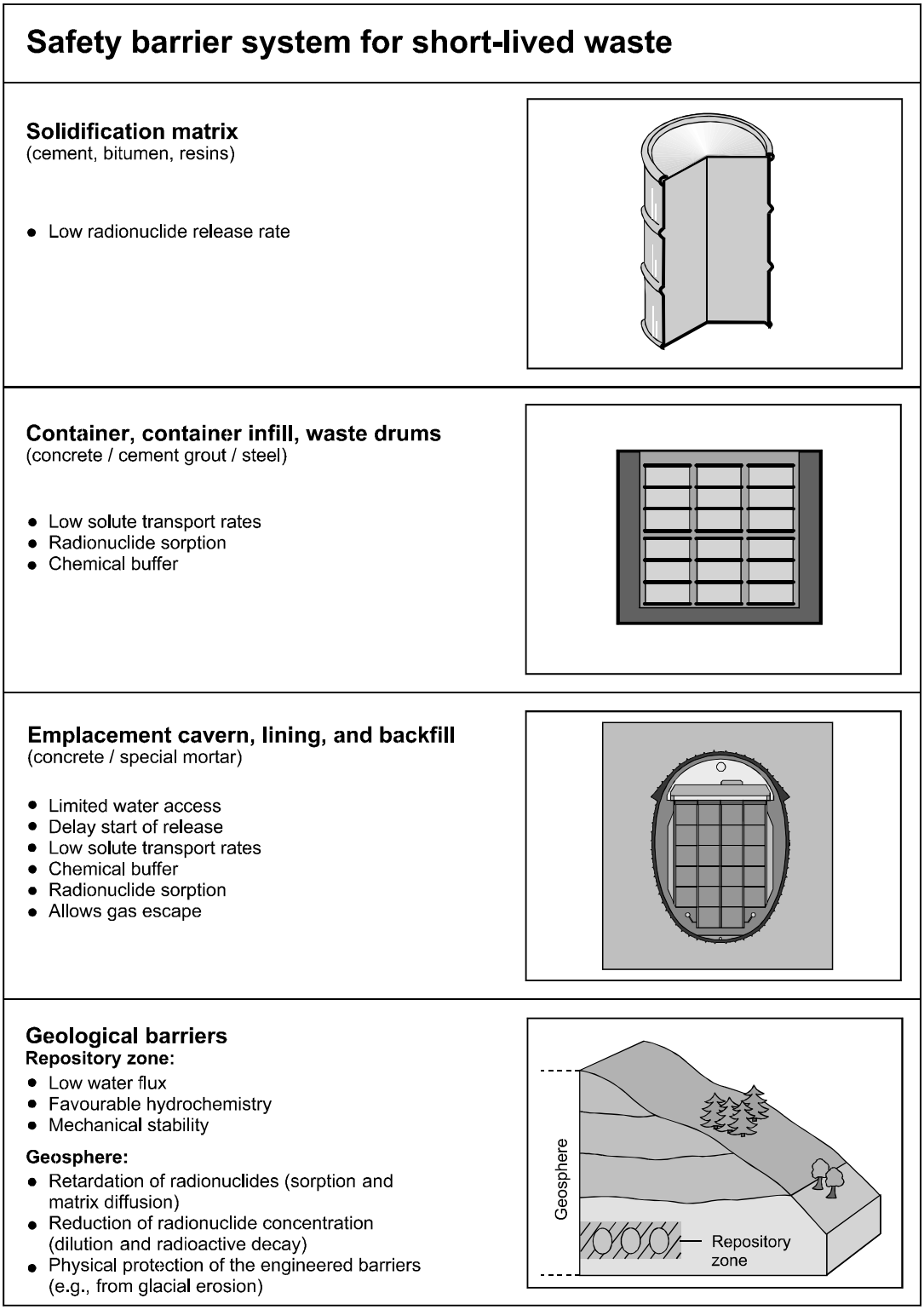

Figure 2. Example of a sequence of engineered barriers for L/ILW.

adapted to hypersaline conditions. A summary of findings at selected sites is given in Table 2.

The composition of microbial populations varies with site (24). It is, however, increasingly recognized that the analysis of ambient microbial populations is part of site characterization (25) and may be more critical to understanding the hydrogeochemistry of the site than to assessing the role of microbes on repository performance. For example, the oxidizing-reducing (redox) conditions of a site are critically important in assessing

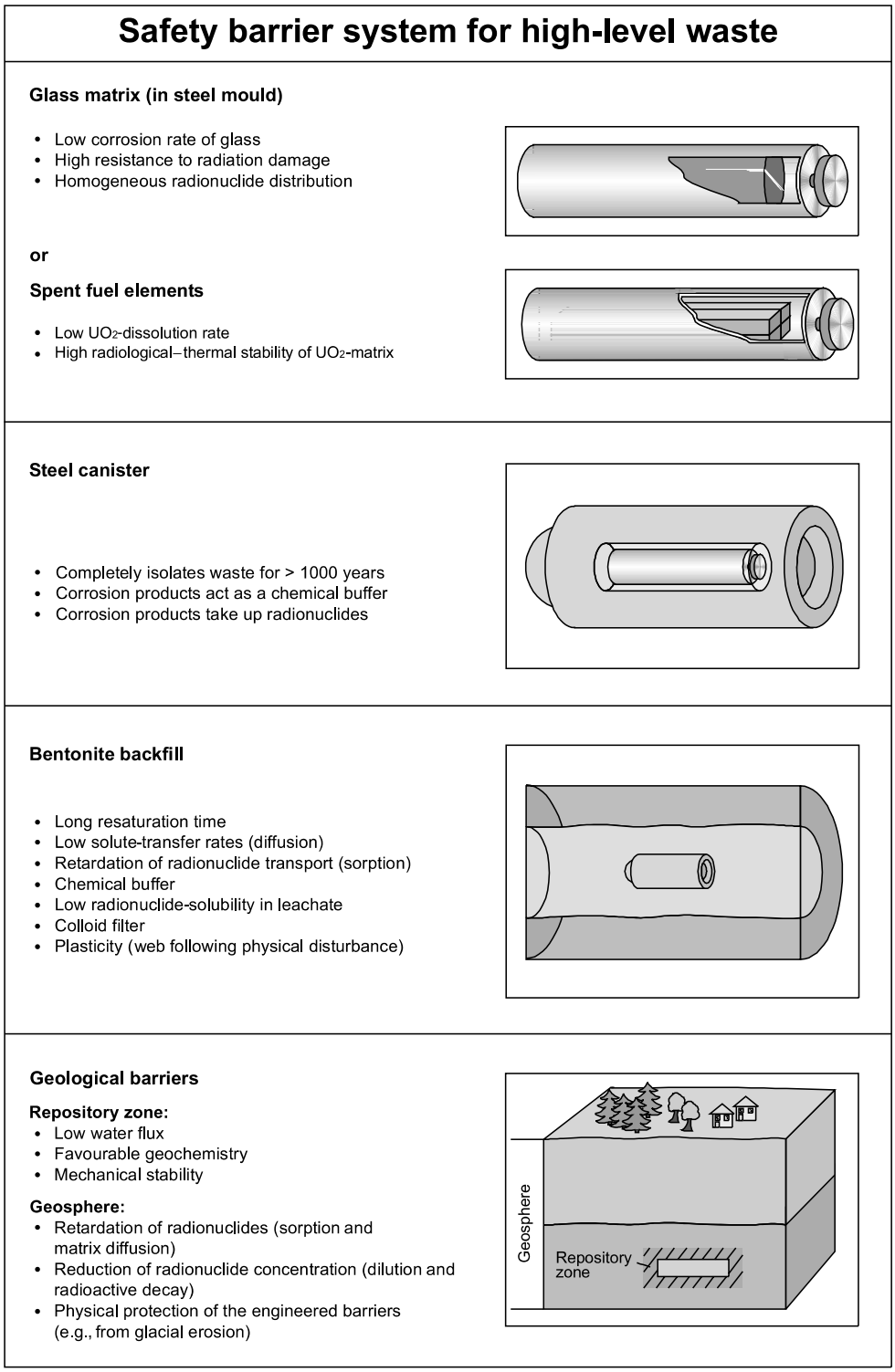

Figure 3. Example of a sequence of engineered barriers for and high-level waste/spent fuel.

site suitability. However, many of the redox complexes used to characterize "Eh" are multielectron transfers of sulfur (S), nitrogen (N), and carbon (C) species, which are kinetically slow at relevant temperatures and will proceed only with microbial catalysis (26). In experiments with rock and groundwater from a granitic environment, sulfate and iron-reducing bacteria appeared to catalyze secondary smectite formation (27). It is important, therefore, to focus on the activity levels of relevant microbial groups, such as sulfate-reducing bacteria (SRB), sulfur oxidizers, and methanogens, rather than counting numbers of cells or "stamp collecting" microbial families or species.

Table 2. Bacterial Populations in Deep Groundwater Environments (24)

Location	Geology	Depth (mbgl)	Bacterial Count
Canada	Granite	350–400	Total counts 10^3–10^5 cells ml^{-1}
Japan	Granite	Approx. 400–790	Total counts 10^2–10^7 bacteria ml^{-1}
Stripa, Sweden	Granite	799–1,240	Total counts 2.0×10^1–1.3×10^5 cells ml^{-1}
Aspo, Sweden	Granite	129–1,078	Total counts 1.5×10^4–1.8×10^6 cells ml^{-1}
Grimsel, Switzerland	Granite	Approx. 350 m	9.5×10^1–9.0×10^4 CFU ml^{-1}*
Altnabreac, U.K.	Granite	10–281	9.4×10^5 CFU ml^{-1}*
Mol, Belgium	Boom clay	190–223	1.2×10^3 CFU ml^{-1}*
Harwell, U.K.	Oxford clay	165–331	8.6×10^3–3.5×10^5 CFU ml^{-1}*
Asse, Germany	Salt	750	ND
Yucca	Volcanic	60	10^2–10^3 bacteria g^{-1}dry weight
Mountain, U.S.A.	Tuff		NB Above water table

*= aerobic heterotrophs.
ND = Not detected.

THE NEAR-FIELD

A range of microbes, potential energy, and nutrient sources are added to the subsurface environment during repository excavation, construction, and waste emplacement. These changes may convert the environment from a low nutrient system to one that, at least temporarily, could support substantial microbial growth and metabolic activity. Work on the disposal of spent fuel in granitic environments, for example, has shown that a significant amount of nutrients may be introduced from explosive residues associated with excavated rock, which is reused in backfill material. Also organic matter in backfill clays has been shown to increase microbial viability when treated with heat and radiation (23). For assessing microbial effects on the EBS, measurements on microbial populations in emplaced materials are probably more relevant than studies of the host rock. Alternatively, studies of natural alkaline environments could be relevant in which cement and concrete are of interest in the waste-disposal concept (28,29).

Tolerances to Repository Conditions

Conditions in a repository vary with waste type and with materials used in its construction (Table 1). For HLW, conditions are very radioactive (hundreds to thousands of Sieverts at the surface of the waste) and hot (in most cases ~50–100 °C). In the early phases of some concepts for the Yucca Mountain proposed site in Nevada, United States of America, the temperatures could be up to 300–400 °C. There is also be considerable pressure generated from the overlying water column and rock burden (up to ~10 and 25 MPa, respectively) and, for some concepts, high salinity up to concentrated brines. Heat and radiation are much less in an L/ILW repository, although the concretes used generate a hyperalkaline environment.

Such extreme conditions were initially thought to preclude life and hence most early analyses considered that a repository would be a completely sterile environment. However, this encyclopedia is full of examples of individual microbial species tolerating specific extreme environments. Some of those relevant to a repository are given in Table 3. However, it is difficult to find reported work on environments where all the conditions generated by a repository are produced (30).

Table 3. Tolerance of Microbes to Extreme Environments (24)

Condition	Example of Organism	Limit of Growth
High temperature	"Black smoker" bacteria	Reported to 113 °C.
Low temperature	*Sporotrichum carnis*	−20 °C
High pH	Nitrifying bacteria	12
Low pH	*Thiobacillus ferrooxidans*	0
High salinity	*Halobacterium halobium*	50% salt by weight
Low salinity	*Salmonella oranienburg*	70 ppb dissolved salts
High pressure	*Desulfovibrio desulfuricans*	180 MPa
Radiation	*Deinococcus radiodurans*	Single dose 5,000 Gy
Chemical toxins, e.g., $PbCl_2$	*Aspergillus niger*	67 mg ml^{-1}

The ability of microbes to simultaneously tolerate high-radiation doses and high temperatures is of particular interest to HLW programs. Studies in Canada suggest that radiation and desiccation effects could create a zone of depleted or reduced microbial activity extending a few tens of centimeter into a buffer material (23). Work on indigenous microbes from the Yucca Mountain, Nevada Test Site demonstrate that they are capable of surviving gamma radiation up to 10^4 Gy (at 1.63 Gy min^{-1}) in a viable but nonculturable form and can be resuscitated to a culturable form (20,31). Other radiation work with sulfate-reducing bacteria found a joint tolerance up to 80 °C and 310 bars and a separate tolerance of up to 10^3 Gy over 40 hours (24). Observations of the reactor core at Three Mile Island showed that microbes were present and were receiving doses of 10 Gy hr^{-1} despite the hydrogen peroxide used regularly as a biocide (32). All these figures are compatible with dose estimates of HLW disposal.

The effects of moisture content on microbial presence were investigated further in an in situ experiment with a

full-scale nuclear fuel waste disposal container in which a heater (simulating a nuclear fuel waste container) was surrounded by sand and bentonite backfill material (33). Microbes could only be isolated and were viable from the backfill material in which the moisture content was above 15%. This suggests that buffer material is populated by viable microorganisms only where the moisture content is above a value where free water is available for active life. The effects of temperature on microbe survival and migration were studied for the Yucca Mountain project. Here a block of tuff was heated to a maximum temperature of 142 °C. Two test isolates were found to tolerate the conditions and to migrate through the tuff itself to a distance of 1.5 m from their injection point (34).

Tolerance to alkaline conditions has been shown in a study of alkaline groundwaters in Jordan in which a range of microbes tolerated pH 12 and above (29). Work in France has shown also that fungi, likely to be important in LLW, grow when in contact with cement and reduces the water's pH (35). In experiments in which sulfate-reducing bacteria have been grown over a range of pH and Eh, activity at pH 8–10 was found to be enhanced by decreasing Eh (36).

In summary, microbial tolerance to a whole range of extreme environmental conditions demonstrate that a repository, even for HLW or for a repository backfilled with cement, cannot be assumed to be sterile for its entire lifetime. Given this fact, it then becomes clear that an assessment must be made of the likely impacts of microbial activity on the waste itself and on the containment materials.

Biodegradation of Repository Materials

Considerable work has been performed on the biodegradation of a number of repository structural materials and on materials used for encapsulation and wasteforms. These include bitumen, ion-exchange resins, organic components of L/ILW (e.g., cellulose), concretes, backfill material, and metals.

Much work has been undertaken on bitumen degradation because it is extensively used in nuclear waste solidification, for example, for encapsulation of ion-exchange resins. Laboratory studies have confirmed that biodegradation can be expected of both the bitumen and the resins themselves under aerobic and anaerobic conditions (37,38). In an operational repository, biodegradation is likely to be more rapid because consortia of microbes are present that work together to degrade such materials more efficiently than under experimental conditions. However, recalcitrant organic fractions are likely to remain (39), which are unavailable for microbial use. Much work has been performed on cellulose degradation because this is a major component of some LLW (40). A variety of organic acids are produced, iso-saccharinic acid being particularly significant. The impact of this acid production in relation to an alkaline repository is being intensively studied and modeling approaches have been developed (41).

Degradation of cements and concretes is commonly observed under aerobic conditions (42) and could occur in the early phase of a repository. Sulfur-oxidizing bacteria such as *Thiobacillus* sp. oxidize sulfur, sulfides, and thiosulfates producing sulfuric acid under aerobic conditions. Nitrifying organisms use ammonia and produce nitric acid in the same conditions. These acids can then attack the concrete matrix by dissolving calcium silicate hydrates (CSH) gel and $Ca(OH)_2$. Direct anaerobic corrosion of concretes is not known, although organic acids produced from microbial attack on organic materials could be a significant factor (35). Biofilms can also develop on concrete surfaces (40,43) although the organisms may be utilizing the organic plasticizers added to concrete to increase their workability. The local alkaline conditions produced by the concretes seem unlikely to preclude potential growth.

Microbially influenced corrosion (MIC) may affect the integrity of metals and alloys, such as steel, Cu, and Ti, which may be used as encapsulation materials (8,44–46). In addition, gas may be generated by such corrosion and this could exert pressure on containment materials, again affecting integrity. MIC can be either direct, microbes using the metals as energy sources, or indirect where microbes change the local conditions to enhance chemical corrosion to take place. In both cases the corrosion effects will be controlled by the amounts of nutrients and energy sources available to the organisms. In laboratory experiments simulating an L/ILW disposal regime, sulfate-reducing bacteria enhanced carbon steel corrosion by three times the rate of a control (42). Other experiments in realistic conditions have also shown potentially more important localized and deep pitting of steels, which was directly attributable to microbial action (44).

Ti and Cu alloys are candidate encapsulation materials in several HLW-disposal concepts. The use of such expensive material is justified by their corrosion resistance over very long periods of time and hence the possible role of microbes has been a significant area of study. There are no reports on MIC of Ti (45).

Within an HLW repository, the environmental conditions will change with time. Upon emplacement of the waste, the conditions will be warm and oxidizing. As the radionuclides contained in the waste decay and the initially trapped O_2 is consumed (by corrosion of the container and microbial activity), conditions will change to be cool and anoxic. Consequently, the nature of the chemical-corrosion reactions will also change with time. In general, localized corrosion and fast uniform corrosion are only expected to occur in the oxidizing phase when sufficient oxidant is present (8). For HLW such a timescale would correspond when conditions are most hostile and in which lack of moisture might inhibit microbial activity (33). However, later repopulation is possible — although this will depend on whether organisms can move through the pore space in the backfill material. Experiments to investigate buffer material repopulation with viable *Pseudomonas stutzeri* after a "sterilization" period suggested that movement could not take place within a compacted bentonite matrix (23). However, movement was shown to occur at interfaces between the backfill and other experimental materials. This suggests that fractures or discontinuities within the EBS may be a preferred pathway for microbial migration, for example, cracks in the backfill resulting

from desiccation. Resealing will occur as groundwater resaturates the buffer, but resealing may be slower than microbial movement. This remains to be investigated. If a zone of depleted microbial activity is created during the high-heat and desiccation period and repopulation is limited by the pore size of the buffer material then microbial activity would be limited to regions outside this depleted zone. Only anaerobic corrosion, probably involving sulfate-reducing bacteria, could occur because the repository would no longer be in the oxidizing phase. In this case, the only microbial impact on the encapsulation materials will result from the diffusion of microbially reduced S species to the container surface. Modeling studies have predicted the extent of sulfate reduction in such a situation and the consequent effects on copper corrosion are likely to be minimal (46). Biogenic organic acid production is likely to be very limited in an HLW environment (33).

Influence on Radionuclide Release

In addition to degrading the materials used to immobilize radionuclides, as considered earlier, microbial activity can influence the release of radionuclides in a number of ways (47):

- Alteration of bulk pore water chemistry (especially pH and redox),
- production of organic complexant by-products, and
- direct uptake onto or into cells.

The alteration of bulk chemistry predominantly occurs due to microbial catalysis of reactions, which produce or consume either protons or oxidants. A good example would be pyrite oxidation (consuming oxidants and producing protons), which may directly lead to acid conditions under which many radionuclides are much more soluble than in a more neutral environment. Even if the initial acid production is buffered by, for example, calcite dissolution, the resultant increase in carbonate concentration in solution could well increase the solubility of many key radionuclides (e.g., actinides).

The catalysis of redox reactions is not, however, necessarily detrimental to near-field performance. Radiolysis of water can give rise to production of equal quantities of oxidants (e.g., H_2O_2) and reductants [e.g., hydrogen (H_2)]. Because H_2 is kinetically inert, this gives an effectively oxidizing environment that tends to increase the solubility of many key nuclides. The presence of microbes that can obtain energy from the recombination of H_2 and H_2O_2 would, however, minimize the potential for this "oxidizing" front or "redox front" to form (Fig. 1).

The catalysis of redox reactions may directly involve radionuclides. Elements such as selenium (Se) may be present in water in an oxidized form (SeO_3^{2-} or SeO_4^{2-}, in this case) that, although thermodynamically unstable in reducing repository conditions, is kinetically hindered from undergoing reduction. It is known that common groups of microbes often "carry" such trace nuclide in their catalysis of reactions of major elements [e.g., sulfate-reducing bacteria producing selleno-sulfides (29)].

The production of organic by-products that can mobilize trace elements is well known for a wide range of relevant organisms. The most extreme examples are organic molecules that are directly utilized for this purpose (e.g., siderophores), which can complex strongly (effectively irreversibly) with a range of relevant elements, notably actinides (48). Nevertheless, a very wide range of by-products ranging from simple organics (e.g., formate, acetate, and oxalate), larger biodegradation products (e.g., iso-saccharinic acid from the breakdown of cellulose), and large macromolecules (fulvic and humic acids) can be of importance. This has been reviewed in detail elsewhere (48).

The third key process considered is radionuclide sorption onto or uptake into microorganisms (49). The net effect of such sorption depends on the extent of its reversibility and the mobility of the organisms involved. Irreversible uptake is of most significance but its net effect is negative only if the organisms are mobile. Uptake into microorganisms may, superficially, be difficult to distinguish from sorption onto outer membranes. The difference becomes particularly significant for the cases in which internal mineralization occurs — which is well known for many microbial groups (50). In the extreme case, this can result in the immobilization of radionuclides in mineral forms, as can occur in the formation of ores of elements such as uranium (U). The same process can be detrimental, however, if the microbes containing the concentrated radionuclide can migrate through the engineered or natural barriers or if the mineralized form is released as a mobile colloid.

A general conclusion from these observations is that, if microbial activities are significant, there are a number of processes that could influence the rate (and form) of release of radionuclides from the EBS. The processes may, however, be either positive or negative in terms of their effect on the containment of radionuclides. In performance assessment of repositories, the worst case is normally assumed but, as seen from observations of natural concentration of radionuclides in areas of reasonably intense microbial activity (1,52a,b), this may be overly pessimistic.

Gas Production

Gas production is of considerable concern in repositories where the waste has a high organic content or contains large quantities of metals. This is because of both the potential for gas pressurization and the inherent properties of gas (radio or chemotoxicity, flammability, and chemical reactivity). Gases can be formed by microbial activity in three ways:

- Direct biodegradation of organic materials, which act as a nutrient source for biogenic gas formation,
- direct catalysis of anaerobic corrosion of metals, and
- indirectly, by producing chemical environments, which cause production of gases, for example, acid conditions that enhance corrosion of metals.

Microbes can also act as consumers of gases (53). For example, carbon dioxide (CO_2) is utilized as an electron

acceptor for methanogens and acetogens, whereas H_2 can be used as an electron donor.

Under the aerobic conditions encountered in a repository soon after waste emplacement carbon dioxide is likely to be the main gas produced by direct microbial action. As the redox conditions change, a range of other gases are produced. Sulfate-reducing bacteria generate sulfide that may form toxic hydrogen sulfide gas and, under very reducing conditions, methane and H_2 may be formed by methanogens. The net effect of microbial activity is difficult to quantify because gas may also be produced by inorganic mechanisms. Studies on degradation of cellulosic waste under alkaline conditions have showed that carbon dioxide is the major gas produced[1] but once the redox potential dropped, methane is also generated and becomes the principal component of the produced gas (40). Little net H_2 was produced — probably being used as a substrate by some species. For HLW, biogenic gas production is not expected to be very significant because the amount of organic material is generally much lower. Studies of gas production from laboratory systems containing backfill and granitic groundwater have shown that the backfill may have a suppressing effect on methane production, which may be attributable to competing sulfate-reduction processes (23).

THE FAR-FIELD

The main aim of a repository is to contain radionuclides and thus any process causing their transport is of concern. In deep geologic systems, advective groundwater flow is the main process mediating such transport. Some engineered barrier materials, such as bentonite and good quality concrete, have such low hydraulic conductivities that advective transport is minimal. Hence diffusion may dominate solute transport. Host rocks with similar properties would be very desirable because it can be shown that diffusive transport is extremely slow for distances of even tens, if not hundreds, of meters. In natural systems such rocks normally contain discontinuities (fractures, faults, etc.), which can serve as potential advective pathways. Repository construction may also produce possible advective short circuits.

The key questions are thus whether microbial processes could influence the rate of transport of radionuclides in the undisturbed geosphere and if the presence of the repository could influence the geosphere-barrier role.

Radionuclide Migration in Undisturbed Systems

The radionuclide transport properties of any medium depend on both the processes moving solute in solution (advection, diffusion, and dispersion) and those transferring solute from dissolved to a solid phase (sorption and precipitation).

The physical processes of advection, diffusion, and dispersion can be influenced by microbes only if they can increase (because of dissolution of solid phase) or close (clogging and formation of biofilms) fluid-filled pore space. If very intense microbial activity occurred in an EBS in which porosity was very small, an influence might well be expected. Significant effects for macroporous EBS systems or for the geosphere would require either very high concentrations of biomass or biofilms/secondary-alteration products. Laboratory experiments have, rather unexpectedly, observed blocking of flow cells within two days owing to intense microbial growth–alteration of crushed rock (27), but the extent to which this is relevant to real systems is not known. Another exception to this generalization would be in cases in which advective flow occurs in fractures with diffusion into nonflowing porosity of the surrounding rock. Biofilms on fracture surfaces could certainly limit access to the matrix and thus greatly reduce the retardation of many key radionuclides.

Sorption is a bulk term for a range of processes causing uptake of solute onto surfaces. Such sorption is generally assessed in laboratory-uptake experiments and, in cases in which microbiology is considered at all, it is assumed that the experimental systems already include microbial effects because no attempts are made to limit microbial growth. In some cases, the agreement between the predicted rate of retardation based on laboratory-sorption measurements with that observed in in situ tracer tests argues that this assumption is justifiable. Experiments suggesting that microbial activity plays a role (54) do, however, suggest that care must be taken when attempting to develop mechanistic sorption models based entirely on physicochemical mechanisms.

The effects of microbes on radionuclide migration can be positive (if sorbed onto attached organisms or onto biofilms) or negative (if sorbed onto mobile or motile organisms). Biofilms have been observed forming on surfaces in deep subsurface environments (55). In many low nutrient environments, microbes are starved; becoming small and mobile and capable of penetrating deeply into geologic formations (56). Experiments in which microbes are in solution suggest that they have varying effects in conventional radionuclide-sorption experiments. For example, in experiments with ^{137}Cs, microbes appear to compete for sites on rock materials with the radionuclide (54). Indeed at the Yucca Mountain repository site there is a potential risk of the subsurface microbial population promoting plutonium transport, based on the high potential for biogenic chelation and carbon dioxide production to accelerate transport (49).

Perturbations Caused by the Repository

Physical damage to the host rock caused by construction processes will not be considered further here, although it is noted that microbial processes can contribute to such perturbation effects [e.g., oxidation of pyrite in the "excavation-damaged zone" (57)].

Two major chemical perturbations that can result from the presence of the repository have been introduced previously in the "near-field" section — the oxidizing redox front resulting from radiolysis caused by HLW (especially important for directly disposed spent fuel) and the high pH plume resulting from hyperalkaline leachates from repositories. The extent to which these reaction fronts can be locations of enhanced microbial activity and the

[1] under alkaline conditions this will be quickly consumed by carbonation reactions

consequences of such activity have been reviewed (58). It was concluded that microbial processes probably play a critical role in the development and movement of redox fronts as commonly observed in nature (e.g., roll-front ore deposits). A microbial role in the high pH front was shown to be theoretically possible, but was identified as an area in which relevant experimental data and field observations were lacking.

EVALUATING THE RISK OF MICROBIOLOGICAL EFFECTS

The greatest challenge in nuclear waste management is demonstrating the safety of repositories over the period for which the radionuclides remain harmful; up to hundreds of thousands or millions of years. Although it is clearly impossible to predict repository evolution in detail over such long periods, the deep geologic environment has the great advantage of very low fluxes of energy or materials that makes simple, scoping assessments feasible. Procedures for quantifying very slow physical (e.g., water flow and rock deformation), chemical (e.g., corrosion and radionuclide sorption), and geologic (e.g., uplift/erosion and fault movement) processes have been established. Quantifying the influences of the microbial processes discussed in the previous sections is less well developed.

Model Development

The clear aim of microbiological studies in the nuclear waste field is to provide information for the safety assessment of repositories. Two main modeling approaches have been examined to date. The first is based on models that use energy and nutrient inventories to calculate maximum microbial biomass and thus quantify perturbing effects in a particular scenario. The second uses models that describe microbial communities and their interactions in organic degradation processes in a more explicit manner.

The nutrient and energy inventory calculations approach was first examined in work performed for the Swiss HLW repository (59) and has subsequently been used for their L/ILW concept (60).

The fundamental assumption is that the maximum possible biomass for the extremely oligotrophic environments found deep underground will be set either by the flux of utilizable energy or by the supply rates of essential elements such as C, N, S, or phosphorus (P) (Fig. 4). Of course, both these constraints are difficult to quantify rigorously and require discussion of questions such as the following:

- What exoenergetic reactions can be used by the chemolithotrophic organisms supporting any microbial communities present, what is their efficiency of utilization of such reactions, and what are the energy requirements to maintain very slowly growing biomass?
- What inorganic sources of essential trace elements can be utilized, what is the rule of utilization of these sources, and what are the energy requirements involved?

Such questions are fundamental to quantitative geomicrobiology and although they cannot yet be answered in a rigorous manner, bounding values can be set, which should represent a worst case (which maximizes possible consequences; "conservative" in the performance-assessment jargon). Such calculations have shown that microbial influences in a Swiss HLW repository are not so great as to perturb the anticipated "sterile" worst case.

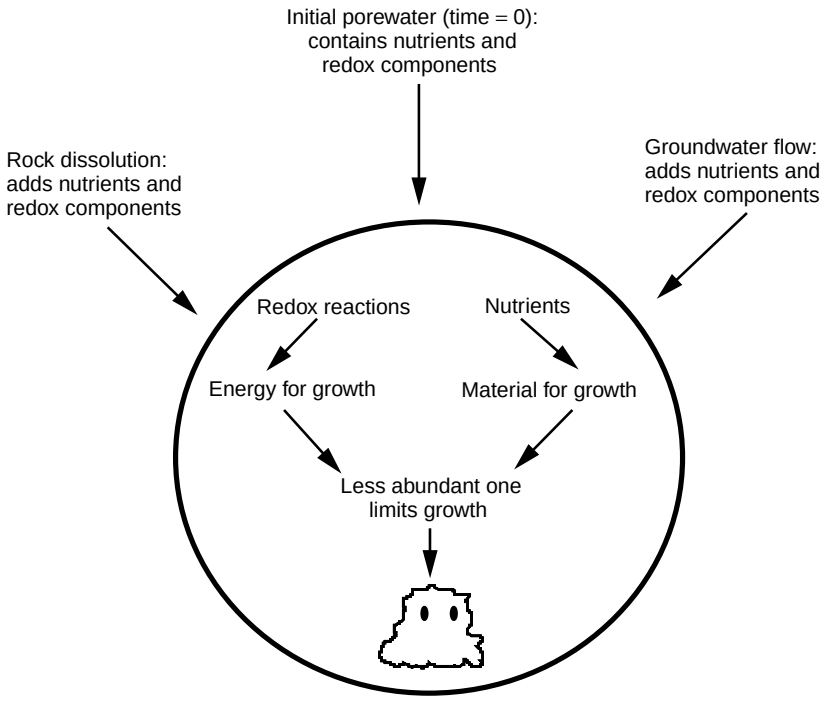

Figure 4. Diagrammatic representation of nutrient/energy computer models.

They also identified the low energy flux, set by the limited availability of electron acceptors (oxidants) as the main constraint on microbial growth. A similar approach was taken for a reference British HLW case (61) that came to the same conclusions, as did the Canadians (62). In the L/ILW situation, by contrast, energy supply was less critical and phosphorus was predicted to be the limiting nutrient. This modeling approach, which determines maximum available energy from a list of all potential redox half-reactions, has been formalized in the generic computer code BGSE (63), which can be used for a wide variety of waste types or subsurface environments.

The alternative approach has been developed that views the LLW repository as a complex system of microbially catalyzed organic-breakdown reactions (64). A generalized kinetic model of organic breakdown to generate biomass (active and inactive), soluble metabolic products, chemical intermediates, and gases has been developed (40). The rate of appearance and disappearance of these products can be modeled over the lifetime of a repository by solving the set of coupled kinetic equations subject to the boundary conditions defined by materials inventories and fluxes in the repository. Microbial activity in a British LLW repository has been modeled over the first 400 years of the repository lifetime, although the model does not yet take into account evolution of some key environmental variables such as pH and temperature.

The models described focus on looking at the "near-field" of various types of repository and quantifying the maximum extent of some potentially perturbing processes (e.g., gas production, metal corrosion, and production of organic by-products). Efforts to quantify effects in the far-field have been more limited. However, it has been demonstrated that the energy/nutrient-constraint approach can be applied to the particularly relevant question of the extent to which chemical perturbation from the EBS can penetrate the geosphere (58).

Verification and Validation

The microbiology models were all developed based on a range of observations and data from laboratory and natural system studies. The key questions to be addressed thereafter are the following:

- Do the models correctly solve the equations describing the system (verification)?
- Do the models adequately represent reality (validation)?

The former question is the easier and can be addressed either by comparison of computer code results and direct analytical solutions for simple test cases or by intercomparison between different models. Validation is much more challenging and requires testing model predictions against observations of systems with appropriate levels of complexity and that operate over appropriate timescales.

To address processes occurring over timescales of thousands or millions of years, conventional laboratory studies are inappropriate and hence similar geologic or archaeological systems must be sought; the "natural analog" approach (51,52a,b).

Natural analogs can be used to first confirm that long-term microbial influences are significant in particular environments. These have included U movement in sites in the United Kingdom (65), at the Poços de Caldas uranium mine in Brazil (66), and at hyperalkaline sites in Oman and Jordan (29). These studies have shown that microbes are ubiquitous in environments resembling those found in a repository and that they play a role in U-mobilization and deposition. For example, at Poços de Caldas, the microbes found around redox fronts, where the U was being mobilized and then reconcentrated, were supported by nutrients and energy supplied by the rock-alteration processes. It was suggested that the complex S-cycling around the redox front was microbially catalyzed. Moreover, this would explain the formation of nodular pitchblende concretions associated with secondary pyrite at the redox front (66).

The energy/nutrient-limit modeling approach has been tested by comparing predictions with observations from both laboratory and natural analog studies. In experiments examining the effects of microbial activity on a suite of repository materials (44), populations of $\sim 10^6$ CFU per cubic centimeter were found, which were much lower than that predicted by modeling. At Maqarin natural analog site in Jordan the modeling approach suggested a population of up to 100 million organisms per cubic centimeter. The observed populations are factors of between 1,000 and 10,000 smaller than this (29). These differences between observed and predicted populations are not unexpected because plate counts always underestimate actual populations. However, at Poços de Caldas uranium mine, the maximum annual production of 0.01–0.1 gram biomass (dry)/m^2 at the redox front was in good agreement with observed standing populations (66). Taking all these results together it appears that microbes may not be able to use all the available nutrients in a repository environment but the modeling approach appears to be conservative in all cases (possibly overly pessimistic).

Analogs may also be used to check models of individual processes, such as cement degradation, bitumen leaching, metal corrosion,.and so forth, but, to date, attempts to quantify the role of microbes in such processes has been very limited. Studies at existing repositories (for radioactive and/or toxic wastes; usually shallow sites with a poorly defined composition) can also be used, to some extent, as analogs of deep disposal sites. Results must be treated with caution because, in such sites, water movement is usually rapid and pH varies from neutral to acid (67). General trends can, however, be clearly observed. For example, leachates rich in organics are produced whose concentrations are reduced by microbial activity; recalcitrants such as the chelating agent EDTA do, nevertheless, remain.

Treatment in Formal Performance Assessments

Until the late 1970s, microbial processes were not even mentioned in safety assessments of potential deep repositories for radioactive waste. Even in the mid 1980s, when the potential for microbial perturbations was acknowledged, microbes (together with organics and colloids) were usually considered simply as "problem

areas" or "open questions" (e.g., 68). For wastes containing large quantities of organic materials, the potential was assessed quantitatively, even if only by very simple scoping calculations.

At the turn of the century, the position is improved with attempts made to quantify microbial processes in most performance assessments, even if under the heading of a "perturbation scenario" rather than in the main "expected evolution" or reference case. As more projects move toward site-specific studies and implementation, the pressure to carry out a more rigorous quantitative assessment of all "open questions" will increase and this can be expected as a key development over the next decade.

INTO THE FUTURE

Although the basic concepts of nuclear waste management remain constant, several recent trends are apparent, which are influencing the development of disposal options. For example, there are increasing pressures to delay final disposal, monitoring waste at special surface storage facilities or in deep repositories that are kept open for periods of up to 100 years or more. If any problems are detected during this period (in terms of containment), then the waste can be easily retrieved and problems rectified before final disposal. There is also time to reevaluate the choice of final disposal of the waste, if future improved technological solutions present themselves. Such extended storage has, however, implications for microbiological processes. Extended storage, whether at the surface or at depth, will ensure prolonged exposure of the waste to both aerobic conditions and to microbial contamination. As a result, the rates of biodegradation of materials, gas production and so forth will increase, with consequences that have not been assessed as yet. Increasingly, as national programs move toward implementation, countries are seeking to optimize somewhat simple initial designs by taking into account decades of experience in R&D and the actual practicalities of waste disposal. These optimized designs seek to reduce long-term uncertainties by strengthening the EBS while also easing the practicality of construction/quality assurance and reducing costs (69). As yet, none of the published studies includes a determination of the significance of microbiological effects for these changing repository designs but this will clearly be another focus for future work.

Public confidence in the safe disposal of radioactive waste has been identified as one of the most important factors to be addressed before a repository can be implemented. All possible perturbing effects must be shown to have been examined based on knowledge that is state of the art. It is therefore essential that, as advances are made in relevant areas of microbiology, they are assessed in the context of waste disposal. For example, the recent discovery of nanoorganisms (70) in extreme environments could well have implications for radionuclide migration. Such organisms may well be more mobile than other larger microbes and could transport nuclides more rapidly away from the repository.

Conversely, the simple models developed for assessing microbiological effects in a repository, which were described earlier could be modified for use in other environments. Such an application could be enhancing the bioremediation of contaminated land, by identifying and controlling the rate-limiting microbial process, or examining the potential for microbial life on other planets using geochemical data only. As such models are utilized more widely, they will tend to improve and the resultant second-generation models can then be reapplied to repository studies. The potential for cross-fertilization of ideas beyond radioactive waste disposal is immense and movements in this direction represent one of the few predictions of the future, which can be made with confidence.

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RAINWATER ROOF CATCHMENT SYSTEMS, MICROBIAL QUALITY OF

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Clean and safe water piped to homes by a reliable supplier (water utility) and available at the turn of a tap is taken for granted by most people in developed countries. However, in developing countries, large populations of people are not supplied with clean water piped into their homes. Even in developed countries, many houses do not receive piped water from a public water utility because of practical or economical conditions related to houses being too far away from a public water utility, houses being located at too high an elevation, or because the houses in rural areas are too few and too scattered. Under these conditions, homeowners will rely on the most feasible source of water for their household needs, such as surface waters (rivers, streams, lakes) or groundwater sources (boreholes, wells). However, under some conditions these sources of water are not readily available, and harvesting (collecting and storing) rainwater may be the most feasible way to obtain water for household use. In its simplest form, open tanks can serve the dual purpose of collecting and storing rainwater. However, this method is applicable only to few places where rainfall is frequent and the volume of water required is low. In most situations, a designated surface area or catchment is required to collect sufficient volumes of rainwater. Pipes or channels are then required to transport this water to a storage tank or cistern for future use. In some situations, the ground (cemented area, lined cliff-side, roads, airport runways) is used as a rainwater catchment area. Ground catchment systems will be excluded from discussion in this paper because they are generally designed for community use and are vulnerable to many sources of contamination, and will generally require some treatment, depending on the expected sources of contamination. Also excluded are catchment systems, which utilize large surfaces of public buildings such as stadiums because these systems are generally highly sophisticated and the water collected is not for household use. The objective of this review is to assess the microbial and hygienic quality of rainwater collected from roof catchment systems from private homes and stored in cisterns or tanks for household use. This class of systems will henceforth be called rainwater roof catchment systems (RRCS).

USE AND STATUS OF RAINWATER ROOF CATCHMENT SYSTEMS

Historically, RRCS are most commonly used in developing countries and in rural areas of developed countries where water is not provided by a public or a private purveyor. For these homeowners, the primary motivation to build RRCS is to obtain sufficient quantity of water for household use at the least cost. Homeowners have long assumed that the quality of water collected by RRCS is good because rainwater is a known source of purified water. However, during the process of collecting and storing rainwater, all RRCS contribute to contaminating the collected rainwater. Multiple factors, such as the design, materials used, maintenance of the RRCS, surrounding environmental conditions, and the practices of the homeowner, contribute to the degradation of the quality of collected rainwater. These factors are in turn controlled by practical considerations such as the wealth and education of the homeowner, as well as the availability of power supply, building materials, and technical support in that area. In most cases, homeowners will follow the recommendations of people, agencies, or local construction companies in the immediate area to build RRCS and will choose the most economical system. This has led to great variations in the construction materials used and in the design of RRCS used in different areas of the world.

The status of RRCS is that each system is considered a private source of water because it serves only a limited number of people (<25). This status of RRCS has led to several consequences. First, the quality of water from RRCS has never been regulated, and as a result the quality of water from RRCS has generally not been determined. Second, since government agencies are not responsible for the quality of water collected by RRCS, no government agency has been identified or tasked to support the needs of homeowners who utilize RRCS. Third, construction and design of RRCS have not been optimized based on water quality. Fourth, even in developed countries where building codes are required for the construction of RRCS, the codes focus on structural stability rather than water quality. Fifth, research publications that discuss problems and solutions for RRCS are not readily available to homeowners or those who build RRCS throughout the world.

TYPES OF RRCS USED THROUGHOUT THE WORLD

Although there are many different designs of RRCS used in different parts of the world, the quality of water collected by RRCS is primarily affected by the design of the RRCS, the materials used, the sanitary conditions of the country, and the resources (wealth, education, supplies, technical support) available to the homeowner.

Minimally Designed RRCS

Figure 1 illustrates the design of a minimally designed RRCS. This type of RRCS is characteristically found only

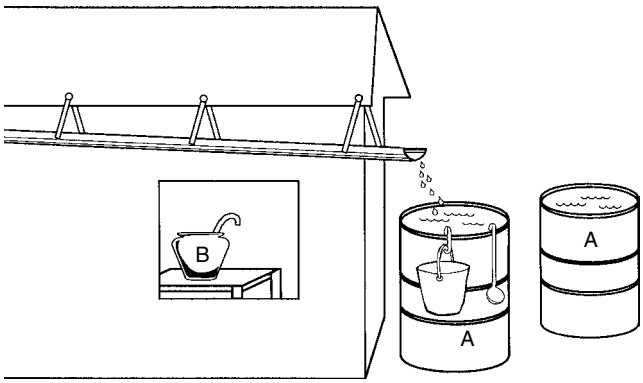

Figure 1. Example of a minimally designed RRCS showing oildrums with open tops A, used as cisterns, with ladles and buckets to collect water from the cistern and a household container B of water.

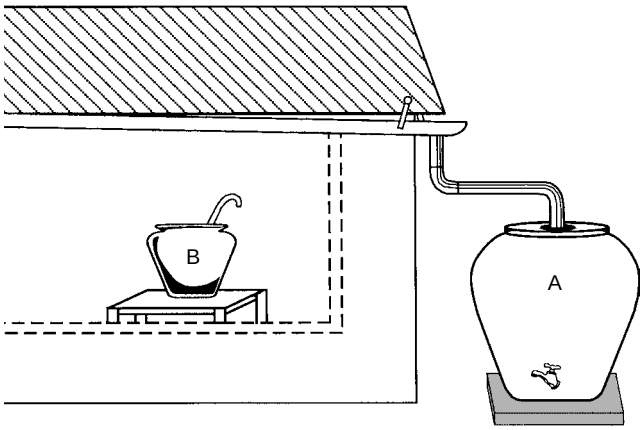

Figure 2. Example of practically designed RRCS showing closed Thai jar cistern A) with water collecting spigot and a household container B) of water.

in remote areas of developing countries where homeowners have minimal resources such as building materials and where technical support from agencies is not available. Therefore, homeowners use material available in the area to design RRCS to simply collect rainwater. Under these conditions, the roof of the house may be plant material (thatched roof), lumber, or corrugated iron. A split bamboo suspended at the ends of the rooflines is often used as gutters. The rainwater is collected into any available and sturdy tank, which is generally small enough to be carried or moved by the homeowner. Empty, used, and uncovered oildrums (200-l capacity) are often used because this kind of tank is available even in remote areas throughout the world. The top of these oildrums is often uncovered and therefore, this source of collected water is highly vulnerable to contamination by all the elements including dust as well as by animals that will seek this source of water. Moreover, the homeowners themselves will contaminate this source of water by using unclean buckets, ladles, or hands to obtain water from this source. Because minimally designed RRCS are used primarily in isolated areas and are not designed to maintain good water quality, this kind of RRCS will be excluded from further discussion.

Practically Designed RRCS

Figure 2 illustrates the popular Thai jar model used extensively in Thailand as an example of a practically designed RRCS. Practically designed RRCS generally result from previous experience. They are characterized as systems, which can be economically built on site, using materials readily available in that area and using a relatively simple construction technique. As most practically designed RRCS are used in developing countries where water is collected primarily for essential needs (drinking, cooking, washing), the water usage rate is low (5–20 liters/person/day) and the size of the cistern is relatively small. For example, the most popular Thai jar model has a volume capacity of 2,000–3,000 l and water programs in that country have promoted its use, its construction using ferro-cement, and design features to maintain water quality (1). For example, the tops of the jars are covered to prevent external contamination and a spigot is installed near the bottom of the cistern so water can be collected without contaminating the water in the cistern (see Fig. 2). The advantages of small-sized cisterns include their ease of construction, the ease by which they can be visually checked to determine when the jar is contaminated, and the ease by which these cisterns can be washed. The main disadvantage of these small tanks is that not enough water can be stored to maintain hygienic conditions in the home. Some households use multiple tanks to store larger volumes of water. Larger sized cisterns (10,000 l) are constructed in other parts of Thailand and in other parts of the world, but they increase the complexity in the construction techniques and increase cost. In summary, these practically designed RRCS represent the majority of the RRCS used throughout the world, especially in developing countries but also in developed countries as well.

Homeowners that use practically designed RRCS often lack many modern resources, such as electricity, to pump the water from the tank back to the house. Thus, the water must be collected from the storage tank outside of the house and brought back into the house where a household container of water is stored for household use (see Fig. 2). Because the total volume of stored water is of limited supply, all household utensils and hands of homeowners cannot be adequately washed. As a result, the container used to collect the water from the reservoir tank (cistern) is usually contaminated and will further contaminate the water before it is brought into the house. Within the house, use of unclean ladles, spoon, cups, and hands are some of the common ways by which post contamination of the water collected by RRCS occurs (2). This secondary contamination of water collected by RRCS is a common and serious problem but is not specifically related to the collection of rainwater by RRCS. This problem persists in all households that must transport water to their homes from an external source (rivers, wells), and then store water in the house for domestic uses.

Commercially Designed RRCS

Figure 3 illustrates a commercially designed RRCS that can be built on site or prefabricated by a private company.

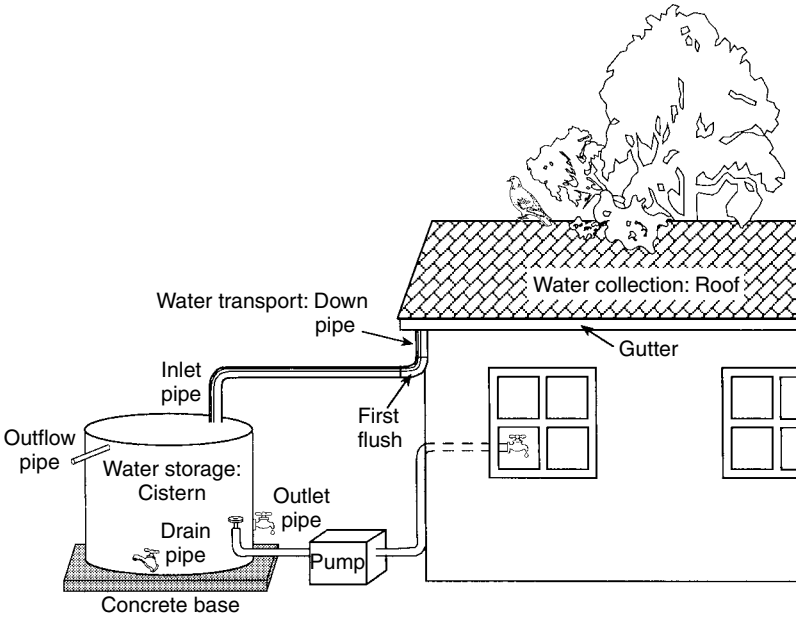

Figure 3. Example of commercially designed RRCS, showing the three major components (water collection, water transport, water storage) of RRCS, and subcomponents, including a pump to transport the water from the cistern back into the house via pipes and faucets.

These companies can manufacture RRCS using traditional and newer building materials (fiberglass). Because the services, materials, and transportation for commercially designed RRCS are expensive, these systems are primarily used by relatively wealthy homeowners. Even in developed countries, homeowners who use RRCS are generally not wealthy and will choose materials and design based on minimal costs. There are several advantages for homeowners in developed countries. The first advantage is the availability of commercially designed RRCS, which are built with good quality control and can be sized for the needs of the homeowner. In this regard, homeowners in developed countries are accustomed to using more water per capita (>200 liters/day/person) and therefore, they generally need to build homes with larger roofs to collect more rainwater and larger cisterns to store more water (e.g., 38,000 to 114,000 l). Some homeowners choose to have more than one cistern to store more water for household use and as a source to put out fire. The second advantage is that when cisterns run dry, private water purveyors are available to transport water to fill cisterns, although this is usually a costly option. The third and very important advantage is the availability of power supply and pumps so water collected in the cistern can be pumped back into the house via piping similar to houses receiving piped water (see Fig. 3). This option eliminates the serious problem of secondary contamination of the collected rainwater. Moreover, it enables homeowners to use many of the available technology such as in-line filters, point of use, and point of entry devices (3) that have been developed to improve the quality of water for homeowners with piped water. In summary, commercially designed RRCS have the potential of providing homeowners with a reliable source of relatively clean household water. However, the rainwater collection system of these commercially built RRCS is still susceptible to contamination by many of the same factors that contaminate rainwater collected by practically designed RRCS.

COMMON COMPONENTS OF RRCS AND THEIR IMPACT ON WATER QUALITY

All RRCS comprise common components and the design, the materials used, the maintenance, and the practices of the homeowners can affect the quality of the collected rainwater. The functional roles of each RRCS component and its susceptibility to contamination, especially with fecal matter, is discussed. The commercialized RRCS as shown in Figure 3 will be used as the model to illustrate each of the components.

The Water Collection Area: The Roof

For individual homes, the roof is the catchment or surface area, which is large enough and strategically located to collect rainwater (Fig. 3). However, because the roof is exposed, it tends to accumulate contaminants such as dirt, leaves, and fecal droppings of animals, especially birds. Trees, which overhang the roof, create additional problems because they are constant sources of leaves and branches, they provide a habitat for birds and insects, and they also provide a means for other animals (rodents) and insects to gain access to the roof. These animals often contaminate roofs with their urine, their fecal droppings, and occasionally their dead bodies. Deteriorating roof material and lead in paint are other known sources of contaminants from the roof. Thus, when it rains, all of the contaminants on the roof will pollute the collected rainwater. Of these contaminants, waterborne microbial pathogens are especially significant because they may be present in animal fecal droppings on the roof, they contaminate and survive well in water, and they may cause disease when ingested. Roofing materials made of smooth and nonwater absorbing materials (tiles, galvanized iron) reduce the accumulation of contaminants, whereas roofing materials made of rough and water-absorbing materials (wood, shingles, gravel) tend to trap more contaminants. Periodic washing of the roof can wash

away the contaminants but is usually not feasible because of insufficient supply of water and the difficulty of washing the entire roof.

The Water Transport System: Gutters and Downpipes

Gutters and downpipes serve to transport the rainwater collected from the roof to a storage tank (Fig. 3). Light-weight, smooth, nonporous, and nondegrading materials (plastics, aluminum, galvanized iron) are suitable. A particular problem with gutters is that all the contaminants on the roof are washed into the gutters and because gutters have only a slight slope, heavy debris such as dirt and leaves tend to accumulate in the gutters. This results in a habitat, which is conducive to the growth of insects, microorganisms, plants and favors the survival of fecal-borne pathogens. Thus, gutters often serve as the site for the concentration of contaminants. However, gutters are accessible to homeowners and periodic cleaning can greatly reduce the transport of contaminants to the cistern. Another means to reduce large-sized contaminants such as leaves from entering the cistern is to install filters, screens, or diverters on the downpipes. However, the actual use of these screens and filters is limited because they must be cleaned regularly to be effective and they can reduce the volume of water to be collected.

Recognizing that the initial volume of rainwater falling on the roof will be the most contaminated, a first-flush mechanism has been developed to collect and void the initial volume of the most contaminated rainwater before directing the subsequent volumes of cleaner water to the cistern. There are many designs of this first-flush mechanism and they are usually installed as part of the downpipe design (see Fig. 3). Mechanical, first-flush mechanisms are simple to operate but homeowners must be there during the first rainfall to void the collected water and to direct the subsequent flow of water into the cistern. There are two practical limitations to this device. First, homeowners cannot be relied on to be present to operate this first-flush device during the initial rainfall. Second, if this device is set to void, there is a danger that it will void most of water. Automated first-flush devices are more feasible because they are not dependent on timely operation by the homeowners. Although the use of first-flush systems is highly recommended, Gould and Nissen-Petersen (1) have reported that their actual use is limited.

The Water Storage Tank: Cistern

Because rainfall is not continuous, a water storage tank or cistern is required to store sufficient rainwater for future household use, especially during the long, dry periods. These cisterns must be large enough to store sufficient volumes of water based on expected household needs and frequency of rainfall to replenish the supply in the tank. Cisterns are usually built as a separate structure next to and below the roofline of the house. Although cisterns can be placed below ground level, most cisterns are built above ground as shown in Figure 3. To maintain the quality of the collected water, the top of the cistern must be sealed from external contamination. It should be noted, however, that many cisterns do not have a tightly sealed cover and many are covered with only a wire mesh material. These kinds of cisterns are highly susceptible to external contamination.

An example of an above-the-ground cistern with a sealed top and the four basic subcomponents is shown in Figure 3. The first component is the water inlet line, which is generally located at the top of the tank and serves as the port where the collected water enters the tank. To prevent external contamination at this point, this water inlet line should be sealed to allow only water to enter. However, in many cisterns, this intake line is not well sealed and any opening allows insects, animals, dust, microorganisms, and sunlight to enter the tank at this site. If significant sunlight penetrates this tank, it will induce the multiplication of cyanobacteria and algae, which can affect the esthetic qualities (discoloration, bad taste, bad odor) of the stored water. The second component is the overflow pipe that is placed near the top of the tank. This pipe is required to allow excess water to flow out of the tank and therefore this pipe is usually left open to the environment. However, this opening serves as an opening for insects, animals, and dust to enter the tank. Fine meshed screen can be made to cover this pipe opening, to allow water to pass, but prevent the entrance of insects and animals. The third component is the drainpipe that is placed near the bottom of the tank for the purpose of draining out the sediments. This pipe is usually closed and is only opened when the cistern is cleaned. The fourth component is the water outlet pipe that is usually closed but is opened to collect water for household use. This outlet pipe is located several centimeters above the drainpipe to prevent sediments in the cistern from being drained out with the water used by the homeowner. However, two conditions have been identified to cause sediment in the tank to become resuspended and to impair the quality of the water drawn from the cistern. First, when the volume of the water in the tank drops to such a low level that turbulence is caused by suction of water out of the tank and this causes resuspension of the sediment in the cistern. Second, when sediment accumulation in the tank reaches a level that is so close to the outlet pipe that the sediment is resuspended as water is drawn from the tank.

Water entering the cistern is already contaminated with particulate materials and microorganisms. Besides serving to store water, cisterns serve as settling tanks and self-purifying systems. Thus, most of the particulate contaminants that enter the tank will eventually settle to the bottom of the tank to form a layer of sediment. This process will clarify the stored water, which is used by the homeowner. Microbial contaminants such as fecal-borne pathogens are so small that it takes time for them to settle into the sediment and they often remain suspended in the water for long periods of time. If the homeowner ingests water contaminated with sewage-borne pathogens, there is a chance that the homeowner will become infected and diseased. However, most of the microbial pathogens are incapable of growing in the water stored in the cistern, and with time the pathogens will die due to physical, chemical and biological activities within the cistern. Thus, with time, the cistern serves as a self-purifying system.

In houses where power supply is not available, water is collected into buckets from the outlet pipe and carried into the house. This creates the problem of secondary contamination, which was discussed earlier. In houses where power supply is available, extension pipes are attached to this outlet pipe, and electrical pumps are used to pump the water from the outlet pipe to a piping system throughout the house (see Fig. 3). As stated earlier, piping the water back into the house avoids the serious problem of secondary contamination and enables homeowners to use many of the point of use and point of entry devices that have been developed to purify water for piped systems (3).

PROFESSIONAL ORGANIZATIONS AND GUIDELINES FOR RRCS

The International Rainwater Catchment Systems Association (IRCSA) is the professional organization that has been officially addressing all problems related to harvesting rainwater throughout the world (4). This professional organization held its first international conference in Honolulu in 1982 and has continued to hold biannual international conferences and to cosponsor other regional conferences on this subject. The most recent, the 9th International Rainwater Catchment System Conference, was held in Brazil in 1999. Proceedings from all of these conferences have been published and contain a wealth of information. More recently, another professional organization called GARNET (Global Applied Research Network) has initiated worldwide email conferences on specific problems associated with rainwater harvesting (5).

Anyone involved in harvesting rainwater should seek the assistance of IRCSA and GARNET. It is recommended that homeowners obtain examples of relevant research articles (2–8), booklets (9,10) and books (1,11) that specifically address how construction, design and maintenance of RRCS affect the quality of the collected water. Unfortunately, these publications are often too theoretical, too specific or too academic to be useful for most homeowners. The recent (1999) publication of a book entitled "Rainwater Catchment Systems for Domestic Supply" by John Gould and Erik Nissen-Petersen (1) is suitable for homeowners, builders of RRCS, and researchers because it is comprehensive, up-to-date and highly descriptive. Some of the recommended construction guidelines to improve the quality of the cistern waters are summarized as follows:

- Use nondeteriorating material characterized by smooth, nontoxic surfaces to construct roof, gutter, pipe, and storage tank. Rough surfaces will trap contaminants, and degradation of building material will contribute to water contamination. Aluminum, galvanized iron, clay, and other smooth, nonwater absorbing materials are recommended for roof material. Aluminum, galvanized iron, plastics, and copper are some of the materials recommended for gutters and pipes. Stronger and more durable materials, such as steel, concrete, ferro-cement, polyethylene and fiberglass, are recommended to build the storage tank or cistern. To prevent leaching of building materials and to retard biofilm growth, the inner walls of some tanks may be lined with vinyl or plastic material.
- Use lead-free construction materials (paint, nail, solder) because lead in drinking water is a definite health hazard, and water collected by RRCS is highly susceptible to lead contamination.
- Establish routine cleaning and maintenance of roof, gutters, and inlets to cisterns because these areas can be expected to become contaminated with dust, leaves from trees and droppings from birds, insects, geckos, and occasionally, the entire bodies of dead animals (birds, cats, rats).
- Use a first-flush mechanism near the downspout area to divert and void the initial volume of rain that contains most of the contaminants washed from the roof and gutters.
- Install screens, diverters, or coarse filters in the downpipes or at the inlet pipe to remove large debris, such as leaves, from being transported into the cistern. Be sure the cistern is covered and does not contain openings as ports for contamination. A wire mesh is recommended to cover the overflow pipe because this pipe is usually left open and this opening allows insects and small animals to enter the tank.
- Install filters to remove particulates and suspended matter from water drawn from the cistern because contaminants (heavy metals, pathogens, nutrients) are concentrated on particulate matter. By removing these particulates and suspended solids from the cistern water, the health risk to homeowners will be reduced. Moreover, this procedure will optimize the water for any subsequent disinfection procedures. In developing countries, a simple sand and gravel filter can be used to filter the water drawn from the outlet pipe. In developed countries, a ceramic filter can be placed within pipes leading from the tank back to the house. However, since these kinds of filters must allow free flow of water, their porosity will not remove all of the contaminating microorganisms, metals, and organic matter in the collected water.
- Because RRCS are vulnerable to contamination with microorganisms, disinfection is the only reliable means to consistently produce water that is microbiologically safe for household use. It should be noted that the obvious method of chlorinating water stored in a cistern has not been widely used and accepted because the homeowner must determine the dose of chlorine to add, which is dependent on both the quantity and quality of water in the tank. Moreover, the simple addition of chlorine to the tank does not ensure effective disinfection because mixing the chlorine in the water is a prerequisite for effective chlorination and this is not practical in a large cistern tank. If the water in the tank is mixed well, it will resuspend the sediment, which will decrease the quality of the water and react with the chlorine. Another problem is the input of additional contaminated water that can only be effectively disinfected by another treatment with chlorine. Homeowners generally feel that these requirements are too demanding. Moreover, the

strong taste of chlorine in chlorinated cistern water becomes unacceptable to most homeowners. Thus, many homeowners do not disinfect water stored in a cistern. There is a need for a more feasible means to disinfect water.

VULNERABILITY OF RRCS FOR MICROBIAL CONTAMINATION

A reasonable assessment can be made regarding the vulnerability of RRCS to microbial contamination of water stored in cisterns. Although rainwater is generally considered of excellent quality and suitable for drinking, anthropogenic practices related to agricultural, industrial, and urban activities have been shown to be responsible for air pollution in the form of particulates, sulfur dioxide, carbon monoxide, nitrogen oxides, hydrocarbons including pesticides, and lead (12,13). Thus rain falling in these areas of activities may also become contaminated with these atmospheric pollutants before it reaches the roof. Of these air pollutants, formation of acid rain is a serious problem. Acid rain is often produced by coal-fired electric plants (12), and is caused by the chemical reaction of rainwater and sulfur dioxide. However, some atmospheric pollution is due to natural causes. For example, the emissions from Kilauea Volcano on the island of Hawaii, state of Hawaii, has increased its venting of sulfur dioxide from approximately 80,000 m tons annually during periods of low activity to approximately 370,000 m tons of sulfur dioxide annually during high activity, which has covered the period from 1983 to the present (14,15). This increased level of atmospheric sulfur dioxide has exacerbated the problem of acid rain on the island of Hawaii. Acid rain causes lead to leach from building materials (paint, nail, solder) used in the construction of many of the 7,000 RRCS located on that island. This has resulted in increased levels of lead in cistern waters impacted by acid rain, and has raised concerns that the most susceptible population (children) who drink cistern water may be at risk. Under nonacid rain conditions, the leaching of heavy metals from roof materials is generally below health levels in the cistern waters, but remains a potential problem (16,17).

In contrast to chemicals, there is little evidence to show that microbial pollution of the atmosphere is a significant factor. Microbial contamination occurs during the process of collecting and storing water. Wind carries dirt and leaves onto the roof. Some animals (birds, gecko, rats, cats, insects) can gain access to the roof and will leave fecal droppings that are potential sources of pathogens to infect humans. Occasionally, these animals die on the roof and their bodies deteriorate on the roof or in the gutters. These materials, which accumulate on the roof and gutters, are resuspended by rain and transported to the cistern. If the cistern tanks are not completely sealed they become vulnerable to contamination, even during dry days. In summary, there does not appear to be any practical means to prevent the contamination of rainwater collected by the RRCS. Therefore, water collected by RRCS will be contaminated with microorganisms and nutrients. The degree of contamination will vary with each RRCS and will depend on multiple factors related to the design, the material used, the surrounding environment and the maintenance and treatment procedures used for each system.

Microbial contamination of cistern waters can have two detrimental effects. The first detrimental effect is to alter the esthetic quality of the water such as adding color, turbidity, taste, or odor. Although these changes may not be related to health effects, homeowners can easily detect this type of change in their water quality and will usually initiate action to address this problem. The microorganisms responsible for these changes in water quality are primarily those that grow under ambient temperature conditions such as in soil, water, plants, insects, reptiles, and worms. Microorganisms that can grow in these habitats represent numerous species and are generally nonpathogenic to humans. Many of these microorganisms can be cultured using general microbiological growth media and they are called heterotrophic bacteria. The concentrations of heterotrophic bacteria in cistern water can be used to estimate the overall quality of that water source. The second detrimental effect is when pathogenic microorganisms contaminate cistern water and affect its hygienic quality. Cistern waters contaminated by pathogens can transmit diseases to humans. Fecal-borne pathogens are the most likely to be transmitted by cistern waters because they represent a large and heterogeneous group (protozoa, bacteria, virus), survive for extended periods in cistern water, and infect humans as a result of drinking contaminated water. However, there are other kinds of potential pathogens, which can be transmitted by cistern waters via other means. For example, legionella bacteria are transmitted by aerosolized contaminated water, whereas acanthamoeba protozoa, adenovirus, and staphylococcus bacteria can cause infections of eyes and skin by direct contact with water, and leptospira bacteria in water infect humans by actively burrowing into broken skin or mucous membranes. Homeowners usually cannot detect that their cistern waters are contaminated with pathogens and will continue to use this water for most household and consumptive uses. There are numerous microorganisms that are pathogenic to humans, but only a few are likely to contaminate cistern waters at a given time. Moreover, they will not be able to multiply in the cistern water environment and will tend to die with time. Because many of these pathogens may survive for weeks in cistern waters, cistern waters contaminated with pathogens represent a definite health risk to homeowners.

AN ASSESSMENT OF THE MICROBIAL AND HYGIENIC QUALITY OF CISTERN WATERS

Because RRCS have been classified as private sources of water, no agency is responsible for the quality of cistern waters used for household purposes. As a result, most of the available data on the quality of cistern waters are based on studies conducted by research organizations such as those at universities rather than agencies associated with federal, state or county regulatory agencies. The available microbial monitoring studies for cistern waters

can be characterized as being conducted on a sporadic basis from many different countries, mostly from developing countries, because RRCS are used more extensively in developing countries. The reported studies from developing countries often represent studies conducted under very particular conditions, such as the surrounding environment, the building materials used, the design of RRCS, and the economical and hygienic status of the cistern owners. For studies assessing the microbial or hygienic quality of cistern waters, cistern water samples have primarily been analyzed for total coliform and/or fecal coliform bacteria because United States Environmental Protection Agency (U.S. EPA) (18) and WHO (19) have adopted hygienic standards for drinking water based on 0 total coliform per 100 ml and 0 fecal coliform per 100 ml. The microbial monitoring data show that although some cistern water samples can actually meet the coliform standard (20,21), most cistern water samples cannot meet the total coliform standard (17–25) and often contain very high concentrations of total coliform, fecal coliform and *Escherichia coli*. In addition to the coliform standard, U.S. EPA and WHO have recommended that drinking water should not contain more than 500 per ml of the total heterotrophic bacteria because higher levels of total heterotrophic bacteria can interfere with the enumeration of total coliform bacteria. In addition, concentrations of heterotrophic bacteria in drinking water reflect the nutrient level in that source of water and are used as an independent measurement of the overall quality of that water source. The available monitoring data show that concentrations of total heterotrophic bacteria in cistern waters often exceed 500 per ml (17–25) and provide additional data that cistern waters are susceptible to contamination, and that the quality of cistern waters does not meet the expectation of a good drinking water source.

Gould (26) recently reviewed the available monitoring and epidemiological data for cistern users and reported that although most cistern waters cannot meet the coliform drinking water standards, these results do not necessarily mean that this source of water is unsafe to drink. Gould points out that millions of people in rural areas around the world depend on rainwater for household use and the number of reported cases of serious health problems related to drinking cistern waters is very few. Gould (26) reports that the best documented study is the report by Koplan and associates, (27) of a salmonellosis outbreak at camp in rural Trinidad, West Indies, where 63 of 83 people developed gastrointestinal illness. In that study, epidemiological and monitoring evidence indicated that the collected rainwater was heavily contaminated by dried and fresh feces of birds that had accumulated on the roof of that house. The feces from these birds were concluded to be the most likely source of the salmonella causing the outbreak. However, this was not a typical RRCS used by a homeowner. Rather it was a system used only occasionally for a camp facility. It is unlikely that homeowners would allow the roof of their homes to become fully contaminated with feces of birds or to allow overhanging trees as a roosting place for birds. Thus, the results of this study do not apply to most RRCS used by homeowners. Less convincing evidence was reported to link drinking cistern waters with infections with other pathogens such as salmonella, campylobacter, giardia, and cryptosporidium (28).

Although the epidemiological method is the traditional approach to show that water is the cause of an outbreak, this approach is effective when large numbers of people are exposed to a common source. It is less effective in determining the risk of using cistern waters because the users represent a small number of people who are exposed to different sources of cistern waters, and these sources of water cannot be assumed to be of the same quality. Moreover, incidences of individual cases of illness among a small number of people are not reported to officials. Finally, cistern users often live away from urban centers, and medical services are not easily accessible. Moreover, many cistern users are susceptible to other sources of infections such as contaminated food. In the absence of good epidemiological data, Simmons and Heyworth (28) proposed that the microbial risk assessment (MRA) method be applied to predict the microbial health risks to people who consume cistern waters. This method estimates risk based on expected concentrations of pathogens, the likelihood of exposure to these pathogens in cistern waters, the vulnerability of the population exposed, and characteristics of the specific pathogen such as infectious dose and disease outcome. The rationale for this approach has been reports that several pathogens have been recovered from cistern water samples obtained from different countries. For example, salmonella bacteria and *Clostridium perfringens* have been recovered from cistern waters in Hawaii, (29), *Cryptosporidium* and *Giardia* from Virgin Islands (30) *Legionella* from Puerto Rico (31) and Kentucky (32), and *Leptospira* from Hawaii (33).

Using the microbial risk-assessment approach, Sasaki and associates (34) reported an association between the use of cistern water for household use and infections with leptospirosis in Hawaii. Using a similar approach, Eberhart-Phillips and associates (35) reported an association with using rainwater as a source of household water and incidence of campylobacter infections in New Zealand. These risk assessments studies are effective in linking drinking cistern waters with the likelihood of contracting specific diseases. The results of these kinds of studies are useful in establishing management decisions in the absence of other kinds of data. However, these kinds of data fail to address specific questions related to microbial water quality in specific RRCS and whether outbreaks of diseases occurred among users of this source of water.

RECOMMENDATIONS FOR MICROBIAL CONTAMINATION IN RRCS

Historically, the number of studies that have measured the microbial quality of cistern water samples have been limited and represent sporadic studies from various parts of the world. The results of these specific studies cannot be easily applied to predict water quality of RRCS used at other parts of the world because many of the conditions at a given study site differ significantly from other study sites. The cumulative results of all these studies show that RRCS

throughout the world are vulnerable to contamination with microorganisms and the water collected in cisterns cannot be expected to consistently meet the drinking water standards based on absence of coliform bacteria. Moreover, pathogens have also been recovered from cistern waters. However, there is little supporting evidence for serious disease transmission to people who consume cistern water, and most cistern owners generally consider their source of water to be of satisfactory quality for household and consumptive uses.

In summary, the problem of the quality of water collected by RRCS can be defined by the three most common questions. First, is the quality of most cistern waters safe for drinking and therefore, acceptable as a potable source of water? Second, if cistern waters cannot meet the established drinking water standard based on concentrations of coliform bacteria, should this source of water be declared as unsuitable for potable use? Third, is there a reliable, feasible, and inexpensive method to treat and disinfect all cistern waters to meet the coliform standard? Fourth, should a less stringent or alternative water quality standard be applied to assess the hygienic quality of cistern waters?

The results of most of the previously conducted studies could not adequately address the above questions because the studies collected only minimal monitoring data, and the studies evaluated RRCS from various sites around the world. Moreover, in many of these studies, questions regarding personal hygiene and secondary contamination of cistern water could not be adequately resolved. To adequately address these questions, researchers at the University of Hawaii have completed a series of comprehensive studies to assess the microbial quality of cistern waters in a community that offered several advantages. The first advantage is that the location of the study is in a country (State of Hawaii, U.S.) with high hygienic standards and effective environmental and health regulations to maintain a sanitary environment. Thus, many of the problems related to unhygienic conditions and contamination of food will play a minor role in assessing the microbial quality of cistern waters in this study. The second advantage is that the community is characterized as comprising relatively wealthy residents with large, expensive homes. The houses are provided with all modern conveniences except piped water from a public utility. Thus, most of the houses use commercialized RRCS with covered cisterns to obtain household water. Moreover, the water from the cisterns is piped into the house using pumps so water can be drawn from faucets in the houses, similar to houses that received piped water from a water utility. This eliminates the problem of secondary contamination. The third advantage is that this population of cistern users (approximately 110 houses) is stable, highly educated, and belongs to an active community association in which common problems are discussed. As a result, communication between the residents and with the researchers is good. The fourth advantage is that there was sufficient variety in the building materials used, the design used and the maintenance and treatment of the RRCS. The fifth advantage is that the quality of water from the cisterns of this community has been studied for more than 16 years. The sixth advantage is that the community is located only four to six kilometers from the University of Hawaii and therefore sampling and analysis of the samples could be completed expediently. The seventh advantage is that the microbial monitoring studies were comprehensive in that several different microorganisms were always measured along with other relevant parameters. Thus, the hygienic quality of cistern water was better defined than in most other studies. In summary, the conditions at this community were optimized for RRCS to collect high-quality rainwater. Using cistern waters from this community, experiments were designed to address the problems related to the hygienic quality of cistern users in both developing and developed countries throughout the world. The major conclusions and recommendations from these studies are summarized below.

Appropriate Microbial Water Quality for RRCS

The worldwide microbial drinking water standard for potable water provided by a water utility is 0 total coliform per 100 ml and 0 fecal coliform per 100 ml. This is a very stringent standard developed specifically for water utilities that have the resources and supporting regulations to use the most protected water source and the best technology to provide this drinking water product. These standards were not established for rainwater catchment systems and other private sources of water. Because water to be used for drinking should be safe, it is reasonable to expect that the same hygienic water quality standard should be applied to all sources of drinking water. However, there are large populations of people that are not provided piped water, and they must obtain water for their household use from alternative sources (wells, streams, rivers, lakes, rainwater). These populations are generally not provided funds, incentives and technical support to properly treat their water source. Recognizing that these populations must obtain water from an alternative source and that these sources of water will not be used by large populations, the WHO classified this alternative source of drinking water as a nonpiped source, and recommended a more practical bacterial water quality standard of not more than 10 total coliform per 100 ml (19).

Technically, RRCS are grouped with other nonpiped and generally unprotected sources of water. However, the vulnerability of RRCS to contamination by fecal matter differs significantly from that of surface (streams, rivers, lakes, wells) and groundwater sources that are highly susceptible to contamination by human feces (sewage) and animal waste. Historically, epidemics of waterborne diseases have been documented from contaminated surface and groundwater sources. In contrast, it is unlikely that the roof of houses will become contaminated with human feces and with the feces of most large land-dwelling animals. It should be noted that contamination of water by human feces results in the highest risk to transmit more diseases to humans because this source of feces may contain all fecal-borne pathogens, including human enteric viruses, enteric bacteria, and enteric protozoa. The contamination of water by feces with the larger, land-dwelling animals (cattle, pigs, sheep) results in moderate risk to transmit diseases to humans because these animals

produce large volumes of waste and may contain most of the fecal-borne bacterial and protozoan pathogens but not the human enteric viruses as these viruses infect only humans. In reality, relatively few kinds of animals (birds, rodents, insects) can gain access to the roof of houses and will contaminate this area with their feces. The feces of these animals will not contain human enteric viruses, but will contain fewer kinds of human pathogenic bacteria and protozoa. Moreover, these animals will generally produce relatively small volumes of fecal matter. Finally, soil and dust are common contaminants of roofs. In this regard, coliform bacteria, including *E. coli*, have been reported to multiply in the soil environment of tropical areas, and these coliform bacteria have a very low association with fecal-borne pathogens (36).

Based on the assessment of fecal contamination of RRCS, it can be reasonably assumed that the source of the coliform bacteria recovered from RRCS will not be from feces of human (high risk) or large ground dwelling animals (moderate risk), but from the limited number of animals (low risk) that can gain access to the roof or soil (very low risk). These assessments were supported by monitoring data from RRCS that showed elevated levels of total and fecal coliform bacteria from cistern water samples, but the general absence of two other fecal indicators (*Clostridium perfringens*, FRNA coliphages), which are always found with fecal coliform bacteria in sewage (37). Since concentrations of total heterotrophic bacteria are elevated in cistern waters, the assay for total coliform bacteria may be interfered with, whereas the assay for fecal coliform will not. On the basis of specific assessment for RRCS, Fujioka (38) proposed a more appropriate potable drinking water standard for RRCS based on 10-fecal coliform per 100 ml. This proposed standard takes into consideration the actual risk associated with concentrations of coliform bacteria in cistern waters and the need for homeowners with RRCS to have a reasonable chance of implementing improvements and disinfection procedure so rainwater can be used as a source of drinking water.

A Practical Approach to Disinfect Cistern Water for Household use

Recognizing that all RRCS are vulnerable to contamination with fecal bacteria, Fujioka (38) recommended that this source of water should be disinfected before it is used for drinking. Boiling the water is an acceptable method to disinfect the water, but this approach has been recognized as being too energy demanding. Thus, there is a need to find a practical, economical and reliable method to disinfect sufficient volumes of cistern water to meet the daily household needs. To address this need, Rijal and Fujioka (39) determined that the highest risk for disease transmission is related to consumptive use of cistern waters, such as drinking, and in the preparation of food. In comparison, there is a low risk associated with using cistern water for other household uses, such as bathing, toilet flushing, general cleaning purposes, watering of plants, and even washing of dishes if they are dried before use. In this regard, the volume of water used for consumptive use represents a minor fraction of the total daily needs.

A practical approach is to selectively disinfect the water to be used for consumptive purposes. In this regard, point of use devices such as small UV units can be placed under the sink to disinfect water flowing from the kitchen faucet. These under-the-sink UV units are readily available, are economical in cost, and can be easily installed. The advantage of UV is that overdosing results in better disinfection without affecting the taste and chemical properties of water. Moreover, UV treatment requires a short contact period and UV-treated water can be obtained directly from the kitchen tap. However, to overcome the electrical and maintenance cost of UV, one can selectively treat a set volume of water to be stored in bottles for daily use. Using this approach, the UV unit need only be used for an hour or less per day to treat the volume of water required for consumptive uses such as drinking, brushing teeth, and preparing food. This will greatly reduce the energy and maintenance cost and will extend the life of the UV system. Two of these, under-the-sink and UV units, were installed in two homes, and the cistern waters before and after UV treatment were assayed for various bacteria (total coliform, fecal coliform, *E. coli*, fecal streptococci, total heterotrophic bacteria). The results showed that these UV units were able to disinfect at least 99.9% of the fecal indicator bacteria in cistern waters. After UV treatment, total coliform and fecal coliform bacteria were no longer detected and therefore the treated cistern water was able to meet current drinking water standards (39).

Use of Solar Power to Disinfect Cistern Water

In developing countries, large populations of people live in places where electricity and piped water are not available. Under these conditions, RRCS are often used to obtain water. These communities require a reliable and economical source of renewable energy source. Fortunately, ample sunlight is generally available in places where people use RRCS. Sunlight is a readily available and renewable energy source. Several technologies have been used to harness this energy source for use by rural communities. Sunlight or solar energy can be used to disinfect water using three technologies. The first and simplest technology is to use the rays of sunlight to disinfect cistern water designated for drinking. If the cistern water is turbid, it should be clarified by settling or by filtration to remove the large particles. The water is then added to transparent bottles and the bottles are exposed to sunlight for several hours or an entire day. Several studies (29,40) have shown that this method effectively inactivates most pathogens, especially bacteria such as salmonella and fecal indicator bacteria (fecal coliform, *E. coli*, fecal streptococci).

The second technology is to use sunlight to heat the water to approximately 60 °C to attain pasteurization conditions. There are many economical solar-pasteurization units designed for rural use, and when properly used, solar-pasteurized water will effectively inactivate most pathogens and microorganisms. Fujioka and Rijal (41) evaluated the effectiveness of an inexpensive (approximately $20) solar pasteurization unit that is not dependent

on electricity and was constructed of durable material to treat 19 liters (5 gallons) of cistern water, stream water and stream water contaminated with sewage. Under sunny conditions in Hawaii, the water in this solar-pasteurizing unit reached pasteurizing temperature within three to five hours of exposure to sunlight and effectively disinfected the fecal indicator in all three types of water. Although practical and easy to use, the major disadvantage of solar-pasteurization units is the small volume of water that they can disinfect per day.

The third technology is to utilize solar energy to charge a battery that can then be used to power a UV system to disinfect water. The advantage of this system is that it can treat large volumes of water without the need for pumps or external sources of electricity. The disadvantage of this system is that its component parts are expensive and sophisticated and moreover, the system is fragile. However, the operation and maintenance of this system is relatively simple, and a trained person can maintain this system for a long period of time. A likely scenario is to train members of the family who live next to the well, from which the entire village obtains its household water, to operate and maintain this solar UV unit. Villagers will obtain water from this well and will be instructed by this family on whether the water needs to be prefiltered before it is poured into a vessel established at a predetermined height so the water will flow by gravity through the UV unit at a specified rate to be sufficiently dosed with UV. As time for disinfection is very short, the villagers can promptly take home their disinfected water, which should be suitable for consumptive uses. A small prototype of this solar-powered UV system that was able to process 1.5 l of water per minute was assembled at the top of a building at the University of Hawaii. Natural cistern water samples were then allowed to flow by gravity through the UV unit. Analysis of the water before and after this treatment with UV showed that 99.9% of fecal indicator bacteria (fecal coliform, *E. coli*, enterococci) and up to 99.999% of total heterotrophic bacteria (37) were inactivated. Thus, the treated cistern water was able to meet the current coliform drinking water standard. In summary, where electricity is not available, these solar power technologies can be used to disinfect cistern water.

A Homeowner's Test to Measure the Hygienic Quality of Cistern Waters

The hygienic quality of water is based on determining the concentrations of fecal indicator bacteria. All approved tests for fecal indicator bacteria require specialized laboratory equipment and trained laboratory personnel. Thus, the only way to determine whether the homeowner's cistern water meets drinking water standards is to send samples of this water to a certified water quality laboratory. This option is not possible for many owners of RRCS. Thus, there is a need for a simple test that homeowners can use to determine the hygienic quality of their water and the degree to which the water is contaminated with fecal bacteria. The test should be reliable in showing that treatment has disinfected the bacteria in the cistern water sample and rendered the water safe to drink. Of all the available methods, the presence or absence test for hydrogen sulfide–producing bacteria is the only simple and reliable test that can be conducted by the homeowner because this test does not require any special equipment such as a laboratory incubator. Moreover, the concentrations of hydrogen sulfide producing bacteria were shown to correlate with concentrations of total coliform bacteria before and after treatment of the water (37,41,42). The test can be carried out at a wide temperature range (20–35 °C) under ambient conditions.

To conduct this test, the homeowner will be provided with sterile and capped vessels containing filter papers with dried bacterial growth medium. These test vessels can be stored at room temperature for a year. To initiate this test, the homeowner opens the caps to two vessels and fills one vessel with 10 ml of cistern water and another vessel with 100 ml of that same water. Each of these vessels will have an etched mark to easily determine the level of sample to be added. The vessels are then capped and held at room temperature and read after 24–48 hours. The test is positive for hydrogen sulfide–producing bacteria when the filter paper and water turn black, an end point that is easy to read. When the 10 and 100 ml samples of water are determined to be negative, the quality of the water is assumed to meet the U.S. EPA drinking water standard of 0 coliform per 100 ml and the proposed RRCS water standard of 10 fecal coliform per 100 ml. When both 10 ml and 100 ml samples are positive, the quality of the water is assumed to exceed the two standards. However, if the 100 ml sample is positive but the 10 ml sample is negative, the water is assumed to exceed the current EPA drinking water standard of 0 total coliform per 100 ml but to meet the proposed cistern water quality standard of <10 fecal coliform per 100 ml. This test is recommended for homeowners because it will empower them to determine when the quality of their water is satisfactory and when it is not and how their own actions and treatment methods can affect the quality of their water. The feasibility of this test for homeowners and the reliability of this hydrogen sulfide test as a surrogate for coliform test have been previously determined (37,39,41,42).

CONCLUSION

Rainwater is a naturally clean source of water. However, much of it falls onto the ground, becomes contaminated, and flows out to sea without being used as a source of drinking water. As the supply of available freshwater for potable use becomes limited, the use of more rainwater for potable use should be encouraged. For many years, homeowners who live in remote areas where water is not provided by water utilities, have determined that collection of rainwater is the most feasible way to obtain water for household use. For some, no other reliable source of water is available. For others, alternative sources of surface and groundwaters are so polluted that the health risk of drinking these sources of water is extremely high. Although water collected by RRCS often contains elevated concentrations of coliform bacteria, this source of water is much safer than alternative sources of surface or groundwater sources in the area with equivalent

concentrations of coliform bacteria. This source of water has historically been used for all household uses, including drinking, and most homeowners accept this source of water as satisfactory and safe.

Government agencies have not addressed the water needs of homeowners who use RRCS because this source of water has been determined to be private and therefore outside the regulations and responsibilities of any government agency. When questions are raised regarding the quality of water collected by RRCS, the approach has always been to apply the same assumptions on water quality and the same expectation that this source of water should also meet the same microbial standard of zero coliform per 100 ml as has been established for drinking water provided by a water utility. This approach has discouraged the use of RRCS and the development of technology to improve the quality of water collected by RRCS. However, a realistic assessment of rainwater collected by roof catchment systems shows that the expected ratio of coliform bacteria to potential sewage-borne pathogens is low because only a limited number of animals can contaminate the roof with their feces and it is highly unlikely that sewage will contaminate the roof of houses. Thus, the potential of disease transmission in water collected by RRCS differs from that from surface or groundwater sources, which are susceptible to contamination by sewage and feces of man and many animals. A more realistic water quality standard for water collected by RRCS should be developed to encourage the use of this source of water. It is time for the government to implement a policy to encourage more effective use of all sources of water, including rainwater, and to ensure that all populations within a country obtain sufficient water of good quality. One of the steps to implement this goal is to designate a government agency to provide more education and assistance to homeowners who use RRCS and to encourage the development of better technology to improve the quality of rainwater collected by homeowners.

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RED TIDES AND OTHER HARMFUL ALGAL BLOOMS

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Harmful algal blooms occur around the world from New Zealand to Norway and from China to Europe in almost all seas, coastal waters, bays, and freshwater. In the marine environment, they have been known as red tides, although they are not always red or visible to the human eye, and not always associated with tides. So the term "red tide" has been a misnomer and its replacement "harmful algal blooms" (HABs) is more descriptive of the events that occur (1). The word "harmful" means that during toxic events, animals and humans can become sick or even die. In nontoxic events, there still can be impacts that affect community structure or even cause mortalities due to other factors, such as hypoxia. The second word "algal" means that algae, typically microscopic algae including phytoplankton, cause the harmful effect(s). The last word "bloom" means that the microalgae are at high enough concentrations in seawater to cause an impact, whether it is a fish or bird kill, toxic shellfish, sick marine mammals, or altered habitat and community structure. This concentration may be high enough to discolor the seawater red, brown, green, yellow, white, or other colors. In 1993, Sournia (2) estimated that there were 60 to 78 toxic species, or about 2% of the world's phytoplankton. Of the 100 or so toxic microalgae known today, over 70% dinoflagellates. The economic impacts from HABs are often difficult to determine, but a recent report (3) estimated that between 1987 and 1992, the average loss of revenue for a HAB in the United States was 49 million dollars (using Yr 2000 dollars). This is a conservative estimate. A breakdown of costs revealed that 45% was for public health impacts, and about 37% for commercial fisheries, 13% for tourism, and 4% for monitoring/management.

THE CAUSATIVE ORGANISMS (ALGAE)

Most harmful microalgal species range from several micrometers to 120 μm or so, and although many can be identified with a light microscope, some require the magnification of a scanning electron microscope or special processing in order to visualize specific characters. HAB species occur in the following botanical classes: Dinophyceae (dinoflagellates), Diatomophyceae (diatoms), Rhapidophyceae (flagellate), Prymnesiophyceae (flagellate), and others (4,5). There are several classification schemes available, and the use of one over the other is a matter of choice by the specialist.

Sample Preparation and Identification

Fixation and preservation techniques can be critical because some of the species are fragile and distort easily, for example, flagellates and some dinoflagellates (Fig. 1). Typically, field samples are collected and either handled live back at a central facility or fixed and preserved in 5 to 10% acidic or nonacidic Lugol's solution of iodine and potassium iodate or fixed and preserved in buffered 2% glutaraldehyde (GTA) (6–9). Nonacidified Lugols can keep many dinoflagellates in good shape for several years if the sample is refrigerated. If left to stand at ambient room temperature, the sample will only last several months and much of the material will clump. Once the solution loses its color and is clear, the sample is no longer preserved and will deteriorate rapidly. This can also happen if sodium thiosulfate is added to the sample to destain specimens in order to use other stains or see certain characters. Again, it is best to add Lugol's to the sample in order to preserve it, or, to preserve it in buffered formalin. Buffered GTA is a good fix for some flagellates and dinoflagellates, but there can be distortion and samples have to be refrigerated. For scanning electron microscopy, Lugol's fixed material can be post fixed with paraformaldehyde and processed following standard techniques (Paula Scott, FMRI, personal communication). This technique has been applied to both dinoflagellates and diatoms. For toxic pennate diatoms, it is best to clean the frustules for light microscopy or SEM (10). The best techniques allow the processing of live material, for example, for unarmored dinoflagellates such as *Karenia*, the best fixation protocol is a combined GTA-OsO4 fix in the cold at 4 C, but the sample has to be adjusted for osmolality (9). This cannot be done in the field, so live samples are collected

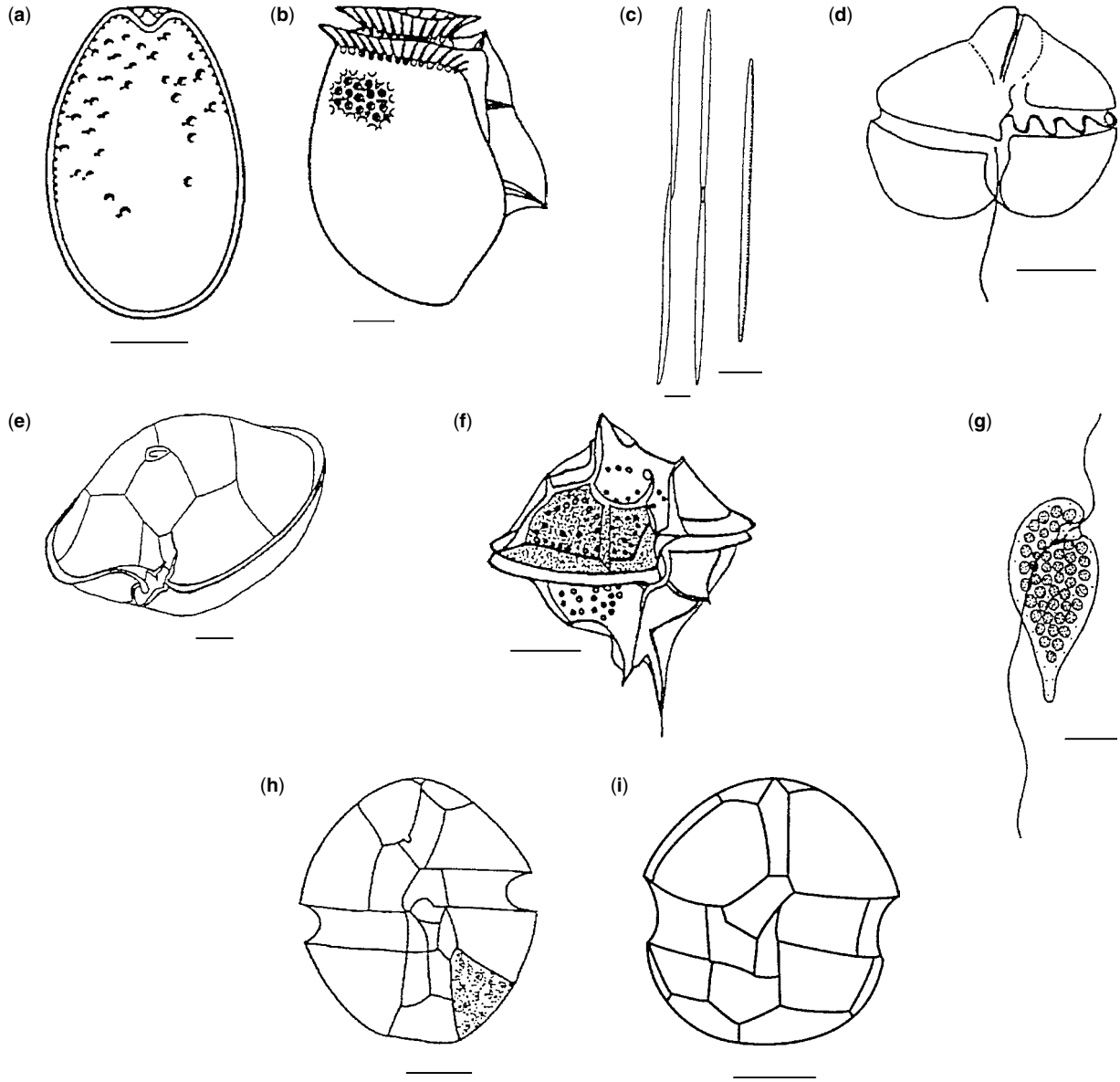

Figure 1. All scale markers = 10 m. Figures after K. A. Steidinger and K. Tangen, *Identifying Marine Phytoplankton*, Academic Press, San Diego, Calif., **1997**, pp. 387–584. (13) unless otherwise noted. (**a**) *Prorocentrum lima*, (**b**) *Dinophysis acuta*, (**c**) *Pseudo-nitzschia pseudodelicatissima* after Hasle and Syvertsen (14), (**d**) *Karenia brevis*, (**e**) *Gambierdiscus toxicus*, (**f**) *Pyrodinium bahamense*, (**g**) *Chattonella subsalsa* after Throndsen (15), (**h**) *Alexandrium minutum*, (**i**) *Pfiesteria piscicida*, original.

in containers, which are wrapped in wet paper to keep them at ambient temperature. If samples are shipped by overnight express, HAB species are usually alive and well and ready for fixation. The added advantage to live samples is that the investigator can see cytological, and often some morphological features clearly, for example, color, nucleus, and shape, as well as swimming behavior of a flagellate or dinoflagellate. These can be critical observations.

Knowing the identity of the organism can often (*Gymnodinium breve*) pinpoint what toxins are involved. For example, *K. brevis* and *K. brevisulcata*, both produce brevetoxins as do other to be named *Karenia* species and these toxins have been found in edible bivalves. Another (*Karenia* species) produces gymnodimine, a nonpolyether toxin. Several rhapidophytes in the genus *Chattonella* also produce brevetoxins. In Florida, the *Karenia* are typically neritic species, while *Chattonella* are typically estuarine (11); however, their distribution can be wider. Toxic *Chattonella* occur in high numbers where there are edible shellfish; however, no human illness has been associated with these blooms to date. Other rhapidophytes such as *Fibrocapsa* and *Heterosigma* reportedly produce brevetoxins as well. In addition, there can be toxic and nontoxic isolates of *C. subsalsa* and probably other *Chattonella*. To date, there have been no

nontoxic isolates of *K. brevis*, but that is not true for other dinoflagellates. *Gambierdiscus toxicus* has toxic and nontoxic strains as does *Alexandrium tamarense*, both armored dinoflagellates. In the case of *G. toxicus*, which was a monospecific genus until recently (12), differences in toxicity can be found in the same geographic locale.

Pfiesteria piscicida, a thinly armored dinoflagellate that is a heterotroph capable of phagocytozing other plankton and even attaching to fish, has been associated with fish kills in the mid-Atlantic states (13–15, see also *Pfiesteria*: The Toxic Pfiesteria Complex, this Encyclopedia). It produces a water-soluble ichythotoxin that is as yet uncharacterized (16). There are several other non-*Pfiesteria* lookalikes, which can easily be confused with the fish-killing *P. piscicida* at the light microscope level, but they can be differentiated at the SEM level (17). This becomes important if cell counts of undifferentiated *Pfiesteria* species and *Pfiesteria*-like species, commonly called PLO counts, are being used as a screen for processing and treating with further protocol. In addition, there are molecular probes that can identify and quantify *P. piscicida*'s presence in water or sediment samples, even preserved samples (18–19). To produce toxins, *P. piscicida* needs exposure to fish as a stimulant; however, not all isolates become toxic. So again, there are toxic and nontoxic strains (20). The age of the culture is also important. Burkholder and Glasgow have found that isolates can lose their toxicity after six months to a year. So, although specific toxins or toxin activity has been associated with known species of dinoflagellates, flagellates, and diatoms, the presence of a specific species does not automatically mean it is toxic, rather it means there is the potential for toxicity and harmful effects to living resources and even to humans. All surveillance programs should have the capability of isolating HAB species, testing them for toxins or toxin activity, and looking at any variability in toxin production and potency, geographically, providing the toxins are characterized.

Molecular Techniques for Identification

As more HABs occur, as more surveillance programs are implemented in shelf and bay waters, and as fewer taxonomists emerge to face this challenge, the efficacy of rapid assays or probes is being realized. This can be inferred by available protocols, kits, and the increased number of DNA gene sequences for HAB species on GenBank (*www.ncbi.nim.nih.gov/web/Genbank*). New molecular technology in the form of nucleic acid or other biochemical probes is making identification of HAB species less complicated and less time consuming. Microscopic examination is typically lengthy and can require an expert for the final species identification, while a DNA probe designed for that specific species can accurately detect very low quantities of a specific species, or even a specific strain. The potential for the new probes is tremendous; not only can they be used in the laboratory, but also they can be adapted and configured for automated instrument packages at sea (21). Some probes have been shown to cross react with other species within a group and are less specific, but often this can be resolved. The most promising aspects of this new technology are accuracy without an "expert," rapid results, and *in situ* results from a buoy at sea or a platform in an estuary. The different probes use different approaches, such as antibody-antigen reactions and different ways to visualize the results, for example, chemiluminescence or epifluorescence. The technology is advancing on a daily basis using a variety of nucleic acid methods, for example, polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP), and sandwich hybridization for detecting specific nucleic acid sequences from cell homogenates (22,23). In addition, cell surface recognition enzyme-linked probes targeted for proteins such as lectins, have worked with whole cells where there can be a colorimetric or chemiluminescent reaction.

EFFECTS AND IMPACTS (HARMFUL)

Many HABs are associated with the shellfish poisoning of humans (24–26). If shellfish, such as oysters and clams, are exposed to a HAB, they can filter out the microalgal cells/particles with toxins and become toxic to humans that consume them. The most noted shellfish poisoning is Paralytic Shellfish Poisoning, and it occurs worldwide with a historic human mortality rate of about 20% of those affected. Today, with good surveillance and control programs, shellfish poisoning incidents are lower, except where they are new events, and resource and public health managers are not prepared. The United States Food and Drug Administration requires that states that ship shellfish interstate have a biotoxin control program that involves surveillance and testing protocol that would monitor shellfish growing areas for HABs and their toxins (27, see also Cyanobacteria-Toxins in Drinking Water, this Encyclopedia). Paralytic Shellfish Poisoning (PSP) has specific causative organisms, most of which are dinoflagellates, which produce the toxins saxitoxin, neosaxitoxin, and/or gonyautoxins. Cyanobacteria or blue-green algae are also known to have saxitoxin, as are nonpigmented bacteria. PSP occurs in the waters of the United States, Canada, Mexico, Central America, South America, Africa, Europe, Scandinavia, the Mediterranean, and the Pacific, Malaysia, Philippines, Japan, China, India, and elsewhere (25,28). Of all the HABs known, PSP is associated with the most human mortalities and is the most widely distributed. However, it is not alone. There are other shellfish poisonings such as Neurotoxic Shellfish Poisoning (NSP) caused by brevetoxins produced by gymnodinioid dinoflagellates such as *Karenia* (fish kills, bird kills, marine mammal mortalities, airborne respiratory irritant, and human illness, but not human mortalities) (29,30). Another type of poisoning is diarrheic shellfish poisoning (DSP) which is also caused by dinoflagellates, for example, some species of *Prorocentrum* and *Dinophysis* (Fig. 1a and 1b). The toxins associated with these dinoflagellates are also thought to work in synergy with viruses or other agents to promote tumors in vertebrates and mammals. A fourth type of shellfish poisoning is known as Amnesic Shellfish Poisoning (ASP) and is caused by another microalgal group known as diatoms; the most notable species are in the genus *Pseudo-nitzschia* (Fig. 1c). This type of poisoning has caused

severe effects in humans, such as permanent or short-term memory loss and death. The most famous cases occurred at Prince Edward Island in 1987 and represented the first recognized Amnesic Shellfish-Poisoning event. Although this poisoning has had its most notoriety by causing human amnesia, the toxin domoic acid has been associated with marine mammal and bird kills in California (31,32), again produced by the diatom *Pseudo-nitzschia*.

Toxins associated with shellfish toxicity, such as saxitoxin, brevetoxins, domoic acid, and okadaic acid, have also been implicated in living aquatic resource impacts. The death of pelicans and seals off California due to domoic acid are just two examples which illustrate one path of intoxication. Typically, illness and death are due to the transfer of toxins through the food chain. In the case of the pelican deaths, pelicans ate anchovy, which had eaten and accumulated the toxins from *Pseudo-nitzschia*. Toxins such as saxitoxin, which is best known for bioaccumulation in shellfish (and consequently impacting humans), can accumulate in zooplankton and ultimately affect larval fishes and larger animals (33). If in any of the "who eats who" scenarios there are toxic microalgae, there is the likelihood of bioaccumulation and effects further up the food chain. There are other effects less well known but certainly experienced by beachgoers when there is a Florida red tide caused by *K. brevis* (Fig. 1d). With blooms of this organism and some other gymnodinioids, the toxin adsorbed to particles can become airborne in sea spray (34) and create respiratory irritation. Beachgoers can be affected for the time of exposure; however, the symptoms of coughing and tearing usually disappear when people are no longer exposed to the irritation. Occasionally, some people will experience contact dermatitis from being in the water. The question being asked today is whether there are any long-term chronic effects of exposure, and this is being investigated by an interdisciplinary team headed by D. Baden of the University of North Carolina at Wilmington (personal communication). Very few epidemiological studies have been done on people exposed to HAB toxins over long periods, and therefore little is known about chronic effects over a lifetime.

Ciguatera, known as tropical fish poisoning, occurs in tropical and subtropical areas of the world (25), and is caused by dinoflagellates such as *Gambierdiscus* sp., notably *G. toxicus* (Fig. 1e). There are several aspects of ciguatera, which make it different from the other types of poisoning. Although ciguatoxin is a polyether compound related to brevetoxins, the ciguatoxin does not kill fish (that we know of), rather it accumulates in the muscle tissue as well as organs. In most of the other cases of bioaccumulation, for example, saxitoxin, brevetoxins, and domoic acid, the toxin is found in the digestive tract and organs like the liver or kidneys but not in the muscle or flesh of fish. That is why there are few effects on humans from eating fish exposed to the other toxins. In many parts of the world, fishermen fillet their catch and discard the entrails. In countries where fish are eaten whole and there are events like PSP, (for example, southeast Asia) (35) there have been human mortalities associated with eating whole fish exposed to toxic *P. bahamense var. compressum* (Fig. 1f). But with ciguatera, the toxin ciguatoxin and other toxins accumulate in the muscle tissue so that filleting the catch is not a protective procedure. There are more than 175 reported human symptoms (36,37) associated with ciguatera around the world. It is thought that this is an indication that more than one toxin is involved (38) and there has been speculation on okadaic acid, palyotoxins, prorocentriles, histamines, and other types of bioactive compounds contributing to the symptomology. The confounding aspect of ciguatera is that victims can have a recurrence of the illness years after the first insult, and the illness can be very debilitating. Of course ASP also has long-term effects. Typically with PSP, NSP, and DSP, the effects are over within several days to a week.

Toxins and Their Detection

Phycotoxins or biotoxins can be neurotoxic, cytotoxic, hemolytic, or cause immunosuppression (25). With many of the neurotoxins, activity is at the sodium or calcium channel of nerve and muscle membranes (39). These toxins can close or open channels and cause an imbalance that interferes with nerve transmission or muscle relaxation/contraction. There are specific sites in the channels where the toxin molecule binds, and this known activity has been used in designing analytical tests for the biological activity of the toxin. For example, with brevetoxins that cause marine animal mortalities, a receptor-binding assay with a tritiated toxin analog is used. The natural toxin actively competes with the analog for a specific site on sodium channels in rat synaptosomes (40–42). There are a variety of toxin assays, like an ELISA, (enzyme-linked immuno sorbent assay), which can detect the toxin itself (not its activity) in the nanogram range, and quantify the concentration with a color reaction that is read on a plate reader that interprets colorimetric density. Other detection methods utilize high performance liquid chromatography (HPLC) and variations of HPLC to enhance its capabilities as well as LC- mass spectrometry. More recently, a very sensitive test (micellar electrokinetic capillary electrophoresis with laser-induced fluorescence) with detection limits of picograms has been developed and used in looking at the transfer of toxins through the food web (43).

Most toxins are considered secondary metabolites (44) that may have had or still have a function. For example, toxins could protect their host from UV light, or actually be an intracellular nutrient store. Or, they could inhibit other species by being toxic to predators, or being alleopathic to competitors. More recently, their role as a siderophore is being reevaluated. Domoic acid in toxic *Pseudo-nitzschia* species has recently been shown to act as a siderphore and bind trace metals such as iron and copper. Through binding, it can make trace metals available for cell metabolism, or remove trace metals which may be toxic (45). Toxin production in HAB species can vary depending on environmental cues, such as stress, including phosphorus limitation (46), light and salinity (47), or cell cycle stage and differences in genetic strains.

A recent review, "Seafood and Freshwater Toxins", (39) gives an excellent description of toxins produced by

harmful algae. HAB toxins typically have gastrointestinal (diarrhea, nausea, vomiting), neurological (lethargy, disorientation, paralysis, loss of short-term memory), and/or hemolytic effects on animals. The most potent of the marine nitrogen-containing toxins (there are also non-nitrogen-containing low molecular weight toxins) is palyotoxin, produced by the dinoflagellate *Ostreopsis siamensis*, with an LD50 (μg/kg) of 0.5 compared to gymnodimine, produced by *Karenia* species, which has an LD50 of 450 μg/kg. Many HAB species produce multiple toxins, such as the dinoflagellate *K. brevis* that has nine brevetoxins. Brevetoxin B has an LD50 of 60 μg/kg, compared to brevetoxin B2 which has an LD50 of 300 μg/kg. Brevetoxin B blocks neuromuscular transmission, and when this happens in the diaphragm muscle of an animal, the animal can die from respiratory failure. *Karenia brevis* and related brevetoxin-producing dinoflagellates are not the only HAB organisms that produce brevetoxins, *Chattonella* (Fig. 1g), *Fibrocapsa*, and *Heterosigma* species do as well (6,11). Not only do different toxins produced by the same species have varying potencies but also different isolates of the same species can have different proportions of toxins and different potencies. One recent finding that has a public health implication is that shellfish, and other animals that take up HAB species and their toxins, can actually metabolize the toxin(s) and alter the toxin structure and activity, which can make detection difficult depending on the method (48). The mouse bioassay method is the accepted "gold" standard for public health issues (6,27), but there are a variety of methods for chemical detection which involve very sensitive methods. Kits for several toxins are now available that can be used in screening protocol (6). Scientists are seeking a rapid, effective alternative to the mouse bioassay for certain toxins. These alternative methods will have to be compared against the mouse bioassay results to insure adequacy in protecting public health.

In some cases, it is debated as to whether the HAB species itself or endo- or exocellular bacteria produce the toxins (49,50). Bacteria within residual or accumulation bodies, such as in some mixotrophic *Alexandrium* (Fig. 1h), may represent a symbiotic association, whereby the intermediate products in bacterial metabolism might produce the precursors for saxitoxins. However, dinoflagellates have been shown to have toxin genes and are able to produce toxins on their own without bacteria.

BLOOMS

Although this review deals specifically with microalgal blooms, there are macroalgal blooms of *Codium*, *Gracilaria*, *Ulva*, and other green, red, and brown algae that can be nuisance blooms (1). They may impact living resources through creating turbidity, high organic loads and low dissolved oxygen, stranding organisms in wrack lines, and altering food webs; however, they have not been associated with public health risks or known illnesses. Most of these large biomass events occur in bays or lagoons, but can occur in open shelf areas, like the seasonal accummulation of "June Grass," on the northwest Florida shelf. Other nuisance blooms can be ascribed to microalgae as well, where the species does not produce a toxin but its abundance can cause the same effects of the macroalgal blooms, for example, turbidity, altering food webs, and even affecting reproductive success in marine animals. One such bloom is known as "brown tide." In Texas, the causative organism is *Aureoumbra lagunenis* while in the northeast United States, it is a related species known as *Aureococcus anophagefferens*. Both are pelagophytes, which have adapted to changing environments of reduced flushing, elevated salinities, and are efficient at nutrient uptake (51–55). For these blooms to continue as they do, there has to be reduced grazing pressure.

Microalgal Bloom Detection

Microalgal blooms can occur in two marine realms, the pelagic and benthic realms. Pelagic covers most of the vertical water column, whereas, benthic means the bottom, whether it be a hard bottom such as reefs or outcroppings, or a soft bottom such as sand. These organisms can be free in the water or attached by mucilaginous strands or sheaths to a substrate such as a macroalgal frond or seagrass blade. In the pelagic realm, the microalgae are typically known as phytoplankton because they contain photosynthetic pigments and are planktonic. The blooms can often be visualized when cell concentrations are high because of the chlorophyll and accessory pigments. For Florida red tides caused by *K. brevis*, cell concentrations in the millions can be detected by surface discoloration, whereas a satellite over 400 miles above earth can detect concentrations at 10^5 cells per liter of seawater. In this case, the color sensors aboard the satellite are more sensitive than the human eye (56). Detection of bloom concentrations is a forewarning of potential harmful effects. There are several methods of detection currently available. Sentinel stations provide a monitoring program with the ability to monitor the water as well as resources for HAB species or their toxins. States impacted by HABs typically have monitoring programs which test shellfish meats for toxins, and then those growing areas are regulated based on a threshold level of toxin in meats. Or, monitoring programs test the water using microscopic or molecular probe techniques. These programs, if sampling is frequent enough, provide adequate warning. As was discussed earlier, sensors aboard satellites can detect chlorophyll concentrations at certain levels. Usually, this means that a pelagic bloom, in order to be detected, is already established in surface waters. With this technology, the bloom can be tracked and, in concert with other parameters like wind direction and intensity, its movement may be predicted. Satellites also have sensors that can detect sea surface height, sea temperature, and other physical variables that can be used in predicting the physical and meteorologic setup of bloom initiation and initial growth (57–60). Blooms of benthic species are typically not obvious without collecting substrate such as macroalgae and determining the abundance of the attached microflora. Occasionally, benthic species can become pelagic with disturbance of the bottom sediments, dislodging and floating of substrate, transport of cells by gas bubbles from the bottom, or just migration into the

water column. This happens with *G. toxicus* and *P. lima*, two well known benthic species. With many HAB species, there is coupling between the pelagic and benthic realms beyond the species habitat preference discussed earlier. In this scenario, a pelagic dinoflagellate or other HAB species can produce a benthic resting stage known as a cyst or zygote. These resting stages can lay dormant for months or years and still be viable, unless they are buried too deep in the sediment by sedimentation or reworking.

Sequential Bloom Development Phases

Steidinger (61,62) introduced the concept of sequential bloom development stages: initiation, growth, maintenance, and termination. Although this applies to the sea, it is mimicked in the laboratory with the culturing of microalgae. In an artificial culture, populations of species are studied in an enriched artificial growth medium. First, there is the inoculation of cells into the medium. This is followed by a lag stage in which the population adjusts to its new environment and can even condition the water for its growth. After the lag phase comes a log phase, where there is exponential growth until the population peaks. This plateau can be maintained for some time until the culture goes into senescence. This initiation phase (63) in nature requires that there be an inoculum of the HAB species. This could be from benthic resting stages where cells excyst and come out of this stage and become planktonic in the pelagic realm. Or, the inoculum of cells can come from another area, in essence be introduced. This can happen via water mass movement such as oceanic intrusions, eddies, even ballast water and sediment discharge. Once there is an inoculum, then the ambient conditions have to be such to support growth of the initial population and this will require a certain level of major and minor nutrients, including organics. The growth phase (63) of a bloom involves competition with other species and outcompeting them, particularly if the bloom is near monospecific, for example, 80 to 98% of the microalgal population being dominated by one species (64). In this phase, population growth has to exceed loss factors such as advection, cell death, predation, et cetera. Once the gains exceed the losses, there is a developing bloom. When the bloom reaches high concentrations, for example, Florida red tides in the millions of cells per liter of seawater, then the maintenance phase (63) supports high biomass through physical concentration by winds and currents, as well as adequate available nutrients to support the higher biomass volume. During the initiation to growth to maintenance transitions, the *K. brevis* blooms are often being moved across the shelf from the mid-to-inner regions. Recently, it has been speculated that, at least for Florida red tides, there is a chemical-biological-physical coupling, which promotes large events. Walsh and colleagues (65,66) have shown by an analysis of historical data that wet deposition of Saharan dust precedes cyanophyte blooms of *Trichodesmium erythraeum* on the west Florida shelf. It is known that *Trichodesmium* (a N_2 fixer) precedes and co-occurs with *K. brevis* blooms, and there was speculation that this cyanophyte conditioned the water for such dinoflagellate blooms (67,68). Now it is a matter of establishing whether the dissolved organics released by this nitrogen fixer support growth of *K. brevis* directly or indirectly. It has been established that inorganic nitrogen is not at a supporting level on the midshelf, but that organic nitrogen is high enough to support bloom concentrations during blooms (69,70). Vargo and colleagues speculated that phosphorus, caused by the abundance of nitrogen, was the limiting nutrient inshore where blooms have their most significant harmful effects. *Trichodesmium* blooms in the open sea can be larger than *K. brevis* coastal blooms and over 50% of the nitrogen fixed daily by this cyanophyte is excreted as dissolved organic nitrogen (71,72). Such a large amount of new nitrogen can be used by the associated heterotrophic community as well as by successional species (73). The last phase, termination phase (63) has also been known as the dispersal phase and is really not well understood. Why do blooms disappear? Where do they go? Are they advected out of an area by currents, winds, or other physical means? Do they just form resting stages and settle out of the water column? Do marine viruses or bacteria attack and kill them? Different HABs may have different termination mechanisms, for example, some of the *Alexandrium* species that cause PSP have vegetative cells, which undergo sexual reproduction and produce zygotes that settle out of the water column into the sediments. These zygotes excyst sometime later when conditions (light, temperature, D.O.) are suitable (74,75). In many cases, cyst dormancy is entrained, and to have encystment out of seasonal cycles means that the entrainment has to be broken.

CONCLUSION

Harmful algal blooms occur worldwide and are principally caused by microalgae, which produce bioactive compounds such as neuro- or cytolytic toxins that can cause illness and death in animals and humans. The intent of this review is to give the reader an introduction to these events, so they can seek further information if they desire. Today, because of Internet access, information on HABs is readily available and updated. In some cases, abstracts, papers, and even manuals can be downloaded from web sites. Key words would be red tide, brown tide, harmful algal blooms, ciguatera, paralytic shellfish poisoning, diarrhetic shellfish poisoning, amnesic shellfish poisoning, neurotoxic shellfish poisoning, fish kills, mass mortalities, phycotoxins, biotoxins, *Gymnodinium*, *Karenia*, *Alexandrium*, *Pyrodinium*, *Gambierdiscus*, *Dinophysis*, *Prorocentrum*, *Pfiesteria*, *Psuedo-nitzschia*, *Heterosigma*, *Chattonella*, *Prymnesium*, *Aureococcus*, and *Aureoumbra* as a start. For web sites, two are listed that have many links to other HAB sites, *http://www.floridamarine.org* and *http://www.whoi.edu/redtide*. One of the questions that will appear on these sites is whether harmful algal blooms are increasing in frequency. The answer is not as straightforward as might be expected. It is a split verdict—yes and no. Yes, new areas are being affected, it seems, every year. In 1972, a Canadian *Alexandrium* bloom was transported down to the northeast states and advected inshore where it established resident populations through cysts on the bottom. Was this species there before this inoculation? Probably, but it did not bloom

and did not cause obvious shellfish poisoning, maybe because of low cyst density. In the 1970s, the Pacific experienced *P. bahamense* var. *compressum* blooms that spread from Papua New Guinea to other areas such as Malaysia and the Philippines (35). Did currents and winds and upwelling/downwelling events cause this? What was the origin of the Central America *P. bahamense var. compressum* blooms in the Pacific? This armored species, as well as unarmored gymnodinioids found in New Zealand, and new benthic armored species associated with ciguatera endemic areas, account for many of the new areas affected by planktonic HAB species. Most of these species were "discovered" as new, although they probably existed there before, and were never studied and differentiated. *Pseudo-nitzschia* blooms in the Pacific and Atlantic are now causing concern, where several years ago no one knew they were toxic. There are established species that were not known to be toxic, and there are "new species" that were not known to be toxic and cause risk to natural resources and public health. Smayda (76) referred to the "new species," (which had not caused problems) as the hidden flora meaning that they were there all the time, just not stimulated to reach bloom concentrations and cause an impact. Today in some areas actual frequency of blooms is lessening or in the down cycle, but clearly in other areas HABs are increasing in frequency, as in New Zealand. Or in some areas, one HAB species may not be blooming whereas another has taken its place. It would appear that each geographic area needs to be considered separately in relation to the historical record, if there is one, and in relation to transport and inoculation mechanisms. Earlier, ballast water was mentioned as a mechanism. It is documented that ballast water (and sediment) can transport resting cysts of *Alexandrium*, and that Australian and Japanese populations of *Alexandrium catenella* and *A. minutum* are the same genetically (6,22,77–79) The inference being that toxic microalgae, in a resting stage, can be transported via ships and if receiving waters are suitable, they can survive and grow. Introductions of exotic species, and in some cases pathogens, show that if receiving waters are suitable, a toxic or nuisance alga can become established and cause resource, economic, and public health risks.

The introduction of harmful algal bloom species whether by natural processes, for example, currents and winds, or human related events, such as ballast water, shellfish stock transplanting or aquaculture product movement, is a concern. There are surveillance programs that can be expanded, integrated, and enhanced to accomplish detection and quantification of HAB species or their toxins. In time, hopefully, autonomous instruments that can measure environmental variables and HAB species will be aboard buoys, platforms, ships of opportunity, autonomous submersible vehicles and gliders. Near real-time reception of telemetered data is a reality and data can even be displayed on web sites. All of this makes forecasting a possibility in the near future. With forecasting comes the question of mitigation and control (80). In one example, clays are being either used in Asian countries (8) or tested for use in North America (81) as flocculants and adsorbents for HAB species and their toxins at aquaculture sites or in small contained areas. Certainly forecasting and prediction using coupled biological-physical models are part of mitigation and one further step toward management (82). Often, harmful algal blooms are looked at as imbalances in nature, but some HABs could be natural perturbations, which actually build stability and resistance into a system, and if control were applied, it might lessen productivity (62).

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REEDBEDS FOR WASTEWATER TREATMENT.

See WETLANDS AND READBEDS FOR WASTEWATER TREATMENT

REGROWTH OF BACTERIA IN WATER DISTRIBUTION SYSTEMS.

See BIODEGRADABLE DISSOLVED ORGANIC CARBON IN DRINKING WATER

REGULATION OF BOTTLED WATER.

See BOTTLED WATER, MICROBIOLOGY OF

REGULATION OF THE COMMERCIAL USES OF MICROORGANISMS

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A large variety of microorganisms, including many genetically engineered microorganisms (GEMs), are used commercially because of advances in modern biology. However, many of these commercial applications, particularly those intended for use in the environment, are subject to government health and safety regulations. These regulations have largely been established in response to the scientific debate that followed the first proposals to use engineered microorganisms in open environment field–testing. Although these regulations are not as stringent as they were when first introduced, they may potentially affect both research and commercial uses of certain microorganisms, with the need to gain regulatory approvals adding time and expense to certain projects or programs.

COMMERCIAL USES OF MICROORGANISMS

Microorganisms have been used for the benefit of mankind for centuries and have had significant commercial utility for most of the twentieth century. Processes like wine and beer fermentation, now known to be microbiological in nature, have been used for centuries and were in many cases known to our early ancestors. In recent decades, mankind has discovered and learned how to harness responsible microbial agents, and a number of naturally occurring microorganisms have been used in fermentation throughout the twentieth century for production of commercial products such as antibiotics, food-processing and other industrial enzymes, and other food additives. Natural characteristics of microorganisms have been used in agriculture for more than 100 years — the first commercial products incorporating nitrogen-fixing rhizobia to promote growth of legumes were introduced in 1898, and the first microbial pest control agents were approved for commercial sale in the 1940s. Microbial cultures are also used in wastewater treatment, hazardous waste bioremediation, enhanced oil recovery, and many other industrial processes (1,2).

HISTORY OF BIOTECHNOLOGY REGULATION

Early Years: The Recombinant DNA Debates

Microorganisms, as natural products with a long history of safe use, are considered by many to represent safe and beneficial products when used correctly for agricultural purposes in the environment or for the manufacture of commercial products through fermentation. In fact, microbial products such as *Bacillus thuringiensis* and nitrogen-fixing rhizobia have long been favored by the organic farming community and are often considered an important part of sustainable programs such as integrated pest management.

Microorganisms (as well as plants and animals) have been genetically manipulated for many years using classical techniques, and the strains resulting from such manipulations and their wild-type parents were subject to little or no government regulation for much of the last century. However, this situation changed due to the advent of the powerful new technologies of modern molecular biology. When the techniques of recombinant DNA (rDNA) were invented in 1973, public and media reaction to this discovery led to an unprecedented public debate over the safety and ethical implications of recombining genes from different sources, and leading scientists in the field convened an international symposium to review the available scientific evidence about the technology's possible risks (3). This conference ultimately led, in 1976, to the promulgation, by the U.S. National Institutes of Health (NIH), of guidelines for the safe conduct of rDNA experiments. These guidelines assigned, to many possible rDNA experiments, physical and biological

controls appropriate for the perceived levels of risk to ensure as much as possible that recombinant organisms would be contained within laboratories. The guidelines originally prohibited the deliberate outdoor release of any recombinant organism. Although the guidelines were binding only on institutions receiving federal funds, they have served as a safety standard for industry and academic researchers alike.

At first, the major safety concern involved potential public health hazards that might occur if altered organisms were to accidentally leave the laboratory, but this lessened over time. As the NIH Guidelines were progressively relaxed, deliberate releases of engineered organisms into the environment became possible on a case-by-case basis with the express permission of the NIH and its outside scientific advisory committee, the Recombinant DNA Advisory Committee (RAC). NIH's 1983 approvals of the first field tests of recombinant microorganisms shifted the attention of the public and the scientific community to the possible adverse environmental effects of the use of engineered plants and microorganisms outside the laboratory. The resulting public controversy and litigation delayed these early field tests and ultimately led to the adoption of a comprehensive federal regulatory framework that ensured review of all early field test proposals (described in a following section).

The NIH Guidelines remain in effect to this day, with the most recent version effective June 24, 1994 (59 Federal Register 34496). The Guidelines were progressively relaxed as more experience was gained with rDNA techniques and as specific risk assessment experiments showed initial concerns to be largely unfounded. Today very few classes of experiments require any kind of prior approval and most rDNA experiments are conducted at safety level one or lower. In fact it was estimated that by the early 1980s, 80–90% of the rDNA experiments conducted across the country were exempt from the Guidelines (4). The NIH Guidelines have not adversely affected the conduct of microbiology research in the United States, due to their widespread acceptance and their ongoing modification to accommodate evolving concepts of risk.

The Federal Coordinated Regulatory Framework and its History

In the early years of the biotechnology era, the NIH Guidelines were the only federal government oversight over this technology; however, they did not have the power of actual government regulation, which concerned many observers. Congress briefly considered passing legislation to make compliance mandatory, but ultimately took no action (3). This led to the passage, beginning in the late 1970s, of several local and state laws that required compliance within that jurisdiction. The most famous of these was adopted in Cambridge, Massachusetts, in 1977, after a frenzied series of City Council debates. These first ordinances did little more than mandate compliance with the Guidelines, but, beginning in 1981, a number of communities began adopting ordinances requiring institutions to obtain permits for rDNA research and large-scale manufacturing. These laws were designed to give local governments better control over biotechnology research in the industrial laboratories that were by then beginning to appear. Cambridge was again the first to pass such a law and several other towns and cities, all in Massachusetts, followed suit throughout the 1980s (3). By the late 1980s, about 20 communities and four states had adopted such laws (5).

The combination of voluntary research guidelines and local ordinances served as the only regulation directed specifically at biotechnology for some time. Commercial firms using biotechnology to develop new drugs and diagnostics were approaching the U.S. Food and Drug Administration (FDA) about the regulations for such products. Aside from requesting information about the genetic changes introduced into the production organism, FDA created no new requirements for rDNA products or genetically modified organisms used in drug production. However, beginning in 1983, academic and commercial biotechnology research programs began to progress from the laboratory into the environment. This development triggered a concern over possible environmental effects of biotechnology and over the adequacy of voluntary guidelines to regulate the diverse areas where biotechnology would be used commercially.

Concerns first arose in the area of agricultural applications of rDNA technology. Agricultural organisms developed by any technology and tested in laboratories or greenhouses must also be tested in the field under conditions approximating actual farming use before they can be accepted and purchased by farmers. The first proposals in 1982 to 1983 for field tests of engineered organisms triggered new discussions and debates within the scientific community over the possible environmental risks of GEMs released into the environment and the ability to predict or monitor such environmental behavior. These discussions also showed that guidelines adopted to cover indoor biomedical laboratory research were not well-suited to apply to outdoor agricultural experimentation.

The first few of these proposals all arose from academic laboratories, which were subject to the NIH Guidelines. So-called "deliberate releases to the environment" were among those experiments originally prohibited under the Guidelines, but which were later put into a class where they could be allowed on approval by both the RAC and the Director of the NIH. In 1982 and 1983, three experiments involving genetically engineered plants were approved by NIH, and in 1983, NIH issued an approval for the first field test of an engineered microorganism (6,7). This was a proposed field test by Stephen Lindow and colleagues at the University of California, Berkeley, of an organism later dubbed the "Ice-Minus" bacterium (another test of a similar microbe was later proposed and carried out by a commercial firm, Advanced Genetic Sciences). This microorganism was a variant of a strain of *Pseudomonas syringae* that plays a crucial role in ice nucleation on plant leaves; the wild-type microorganism contains a protein that promotes ice crystal formation, and the engineered variant had the gene encoding this protein deleted. Lindow and his colleagues wanted to field-test the variant, to test its ability to provide potato plants with the ability

to withstand freezing temperatures up to a few degrees lower than normally possible.

The NIH approved this experiment in June 1983, following a review by the RAC over the course of several months. Shortly before the experiment was to begin, it was stopped by a lawsuit filed by a coalition of activist groups led by author Jeremy Rifkin, alleging that such tests posed unacceptable environmental risks and that the NIH had not followed proper federal procedures for assessing such risks in approving the experiment. Rifkin alleged that NIH should have prepared an environmental assessment under the terms of the National Environmental Policy Act and made that assessment available for public comment before issuing a final approval. Rifkin eventually won the suit (although a final decision took several years), and NIH was forced to prepare environmental assessments for any deliberate release experiments it intended to approve (6).

Although the court case took so long to resolve, its impact on commercial biotechnology was more immediate because it exposed the unsuitability of the NIH Guidelines as commercial regulation. Commercial companies could not live with the possibility that proposed field tests could be continually delayed by the time-consuming NIH review process, the need for public comment periods, and the threat of litigation by activists opposed to the technology.

At the same time, the federal government was beginning to realize that it needed a regulatory process more stringent than the NIH Guidelines to oversee the growing biotechnology industry, and agencies other than FDA began contemplating how they might use their existing statutes and regulations to apply to biotechnology. The most prominent of these was the U.S. Environmental Protection Agency (EPA), which, in the summer of 1983, announced that it was considering regulations under the Toxic Substances Control Act (TSCA) that could have potentially covered most, if not all, aspects of commercial biotechnology, even those that were already regulated elsewhere in the federal government. The political reaction to this announcement and the aftermath of the Rifkin lawsuits were among the factors that led to the Reagan Administration's decision to take a more comprehensive approach to biotechnology regulation.

In April 1984, the Administration convened an interagency working group to develop a unified approach to the regulation of commercial biotechnology. The group included representatives from seventeen federal agencies and cabinet departments and published the results of its work in a Federal Register notice on December 31, 1984 (49 Federal Register 50856). This notice, entitled "Proposal for a Coordinated Framework for Regulation of Biotechnology," had three components: (*1*) a "matrix" of existing laws and regulations applicable to biotechnology products; (*2*) policy statements by the FDA, EPA, and the U.S. Department of Agriculture (USDA) describing their approaches to regulate biotechnology products; (*3*) a proposal for the oversight of biotechnology regulation by two interagency committees: one to oversee scientific issues and one to cover policy issues. A public comment period on this notice followed.

The regulatory matrix established what would become a cornerstone of federal regulatory policy: that existing laws and regulations would suffice for the regulation of biotechnology products. The agency policy statements were in all cases preliminary; FDA and USDA made no new proposals beyond their existing regulations, whereas EPA put forward an initial proposal to apply TSCA and the pesticide law FIFRA (Federal Insecticide, Fungicide and Rodenticide Act) to regulate biotechnology products. The EPA statement was controversial, primarily because EPA proposed a "process-based" approach, choosing to regulate only those organisms produced by recombinant DNA and related techniques rather than a "risk-based" approach focusing on the inherent traits of the final microorganism, regardless of how it was constructed.

This Federal Register notice generated a great number of comments from the public, and the working group then changed many of its basic assumptions. Its next public pronouncement came in November 1985, when the Biotechnology Science Coordinating Committee (BSCC) was created as the new interagency committee. Established under the provisions of the Federal Coordinating Council for Science, Engineering and Technology, the BSCC consisted of senior policy officials from those agencies primarily involved in oversight of biotechnology research and commercialization.

The BSCC assumed responsibility for continuing development of the Coordinated Framework and published an updated version on June 26, 1986 (51 Federal Register 23302). This notice largely had the same thrust as the 1984 version, although both USDA's and EPA's proposed regulatory approaches were modified somewhat. The 1986 Coordinated Framework provided the basis for the first round of biotechnology rulemaking, which began taking place in 1987, with the adoption of regulations for transgenic plant field–testing by the USDA. The BSCC is no longer in existence, having been displaced by other working groups more closely controlled by the White House. Although aspects of this framework have been relaxed over the years, for the most part it remains in effect as of this writing.

The Coordinated Framework and Its Philosophy

The philosophy behind federal regulation under the Coordinated Framework is that biotechnology products can be regulated on a product-by-product basis under existing statutory authority. This is sensible because biotechnology is a tool being applied to many industries, resulting in products of many different types. It might have been difficult to abandon the product-specific regulatory structure that had existed for decades to create a new system for a small class of dissimilar products whose only similarity is their method of manufacture.

Hence, biotechnology products are regulated under a scheme that follows from the product categories traditionally regulated by three federal agencies: FDA, EPA, and USDA. The FDA, with existing authority over human drugs and biologics, animal drugs, medical devices, and human and animal foods, food additives, and their manufacturing processes, regulates the lion's share of biotechnology products, all under preexisting statutory and regulatory authority. Many uses of microorganisms in commercial fermentation are regulated by the FDA under

these statutes. EPA uses existing authority to regulate new chemicals produced by biotechnology (under TSCA) and pesticides derived from or containing microorganisms (under FIFRA). Products regulated under traditional USDA authority are primarily animal biologics (e.g., vaccines) and certain microbes derived from plant or animal pathogens.

The Coordinated Framework primarily relied on existing laws and regulations and spawned new rules only for a small number of biotechnology products: primarily, engineered organisms intended for use in the environment. Thus, USDA began using the Plant Pest Act to regulate environmental introductions of transgenic plants and certain agricultural microorganisms. EPA is using TSCA to oversee certain commercial uses and "deliberate releases" of microorganisms not regulated elsewhere in the federal government. Outdoor rDNA experiments conducted by academic or government laboratories are to be regulated by the agency supervising or funding the work, such as the NIH, the USDA, or the National Science Foundation.

In the early years, under the framework, every proposed field test of an engineered microorganism or plant was subjected to a thorough risk assessment, focusing on the potential environmental effects of the introduced microorganism. Such assessments examined generally applicable questions such as the toxicity, infectivity, persistence, competitiveness, and possible dispersal of GEM (see a following section). Early field tests were often required to include extensive testing to monitor the persistence of GEM in the environment and to detect its possible transfer beyond the experimental field. In later years, agency rulemaking relaxed the standards so that fewer plants and microorganisms were subject to such thorough scrutiny.

The Scope Policy and the Finalization of the Framework

The intent of the Coordinated Framework has always been to provide a risk-based (and science-based) approach to biotechnology regulation. Politically this has not always been possible because genetically engineered (i.e., recombinant) organisms were singled out by public interest groups as allegedly posing unique risks, and in the early years of regulation, this led to federal oversight of any proposed outdoor use of an engineered plant or microbe, regardless of the size or scale of the use and even if used for research purposes. However, beginning in the late 1980s, the risk-based approach became the focus of a federal interagency committee, whose formal task it was to define a presumably narrow class of microorganisms judged to be risky enough to warrant regulatory oversight even for small-scale field testing. This committee published its initial recommendations known as the Scope Policy in July 1990 (55 Federal Register 31118–31121). The Scope Policy gave individual agencies guidance to define categories of those "organisms deliberately modified in hereditary traits" that could be excluded from oversight at the level of small-scale R&D by creating several categories of exclusion based on the method used in their creation.

However, over time, the federal government reopened the Scope negotiations, partially because the categories of exclusion were seen as process-based (i.e., they were defined by the types of modifications imparted to the organism in question). The interagency group eventually abandoned the July 1990 document in favor of a revised Scope Policy that was published in February 1992 in the Federal Register (57 Federal Register 6753–6762). This final statement on scope read as follows:

> Within the scope of authority provided by statute, federal agencies shall exercise oversight of planned introductions of biotechnology products into the environment only upon evidence that the risk posed by the introduction is unreasonable.

The policy also stated that "where oversight is warranted, the extent and type of oversight measure(s) must be commensurate with the gravity and type of risk being addressed. . ." The Federal Register notice stressed that the new Scope Policy was a risk-based approach: that is, it is totally blind to "process" (i.e., how an organism is created) and is solely based on the inherent risks of the organism and the environment into which it will be placed. The thrust of the policy was to ensure that genetically altered organisms received no greater oversight than would be warranted by similar nonaltered organisms. Furthermore, the 1992 policy recognized that the risk-based scope principles could only be interpreted in the context of the different statutes administered by the agencies.

The publication of this policy opened the door to new biotechnology rules substantially less rigid than those that were adopted earlier: the FDA's 1992 food biotechnology policy (allowing transgenic plants to be used in food without labeling or special testing) and the USDA's 1993 revised regulations for agricultural field tests (further revised in 1997 to provide greater deregulation, see below). The Scope Policy also allowed EPA to continue its rulemaking efforts under both FIFRA and TSCA, which had been stalled by the six-plus years of interagency wrangling. The history and status of rules relevant to genetically engineered microorganisms are discussed in the following section.

Current Status of U.S. Regulation of Commercial and Environmental Uses of Microorganisms

Under the Coordinated Framework, microorganisms used in commerce might be regulated as shown in Table 1 and as summarized below. The regulatory programs relevant to environmental uses of microbes are explained in more detail in the following sections.

Microorganisms, including GEMs, used in commercial manufacturing would be regulated by the agency having jurisdiction over the end product. For example, FDA would regulate microorganisms used to produce drugs, biologicals, or food additives. Many microbes used to manufacture products not regulated by FDA would generally be regulated by EPA under TSCA;

- Microorganisms, including GEMs, designed to act as pesticides or that are deemed to act through killing or displacing an agricultural pest would be regulated as pesticides by EPA under FIFRA;

Table 1. Major U.S. Laws and Regulations Affecting Genetically Engineered Microorganisms

Law	Agency	Legislative Citation	Biotechnology Regulations	Microbial Products Covered
Federal Food, Drug and Cosmetic Act	Food and Drug Administration	21 USC 301	Many parts of 21 CFR	Microorganisms used to manufacture drugs, biologicals, foods or food additives, animal feed or feed additives.
Federal Insecticide, Fungicide and Rodenticide Act	Environmental Protection Agency	7 USC 136	40 CFR 152–186, especially Parts 158 and 172	Microbial pesticides.
Toxic Substance Control Act	Environmental Protection Agency	15 USC 2601	40 CFR 725	Microorganisms for industrial bioprocesses, nonpesticidal agricultural uses, bioremediation
Federal Plant Pest Act	Department of Agriculture	7 USC 150aa	7 CFR 340	Engineered microorganisms for agriculture that may be plant pests.

- Modified microorganisms intended for environmental use other than as a pesticide may be subject to EPA regulation under TSCA. Certain plant growth — promoting bacteria (e.g., for nitrogen fixation), microorganisms for bioremediation, enhanced oil recovery, and similar uses, are included in this category;
- Microorganisms that are, or are derived from, a plant pest, may be subject to regulation by the U.S. Department of Agriculture (although USDA would generally defer to EPA authority for any organisms in this category that also fall into one of the previous two categories).

THE POTENTIAL ENVIRONMENTAL IMPACTS OF INTRODUCING ENGINEERED MICROORGANISMS INTO THE ENVIRONMENT

The regulatory framework described in the preceding section was largely driven by scientific debate over the possible risks of introducing microorganisms, particularly genetically modified microbes, into the environment. Although magnified and possibly distorted by the concerns of the general public and environmental activists, several legitimate scientific issues underlie this concern. There are numerous historical instances where exotic or nonindigenous plants or microbial species dramatically outcompeted native flora or fauna after intentional or accidental introductions into a new environment, with resulting adverse effects. Although not an ideal model for the introduction of microorganisms possessing only a few genetic differences from wild type, this paradigm shaped the early debate. Accordingly, among the issues identified as important to assess in proposed uses of GEMs in the environment were (*1*) the toxicity, infectivity, or other risks inherent to the GEM itself; (*2*) the ability of the GEM to persist or become established in the environment; (*3*) the ability of the GEM to compete with or displace natural microflora at the release site; (*4*) the possibility that the GEM could spread or be dispersed from the release site; and (*5*) the possibility that genes introduced into the GEM could themselves spread through horizontal gene transfer to be taken up by and expressed in different microbial species (1).

This scientific debate continued for much of the 1980s and a detailed discussion thereof is beyond the scope of this article. Although many scientific issues remain for investigation, for the purposes of the development of regulations, much of the debate was settled in the late 1980s by the appearance of peer-reviewed reports (8,9) that generally concluded that the behavior of GEMs in the environment would be similar to that of nonengineered strains introduced into new environments and that such behavior could be predicted and monitored using appropriate risk assessment tools. The results of many of the earliest GEM field tests and the monitoring programs that accompanied them have generally borne out the predictability and low risks of many uses of GEMs in agriculture (1).

EPA REGULATION OF MICROBIAL PESTICIDES UNDER THE FEDERAL INSECTICIDE, FUNGICIDE AND RODENTICIDE ACT

Background

Most of the genetically engineered microorganisms that are used in the environment are pesticides that are regulated by EPA, using existing regulations under the pesticide law FIFRA (7 U.S. Code 136). A number of naturally occurring microorganisms have been used for decades as pesticides because their natural properties give such strains the ability to selectively kill or inhibit growth of certain agricultural pests. More recently, genetically modified or engineered microorganisms have also been used or proposed as pesticides.

All pesticide products (e.g., insecticides, herbicides, and fungicides) must be registered by the EPA before they can be sold commercially, and microbial pesticide products are generally subject to the same registration requirements as

are chemical pesticides (described in a following section). It is necessary to conduct several years of field tests first to identify commercially viable pesticides and to gather the data on efficacy and safety that are needed to support EPA registration. EPA allows most pesticide products to be field-tested at any level up to 10 terrestrial acres without agency oversight, in recognition of the limited risks posed by such small-scale experimental uses. However, to conduct field tests of greater acreage, companies must first obtain an experimental use permit (EUP), which requires the applicant to conduct much of the testing that would later be required for product registration, including product characterization, short-term toxicological testing, and a certain amount of environmental effects testing, including effects on nontarget organisms.

Under EPA's biotechnology regulations, however, the ten-acre exemption has been removed for certain genetically altered microbial pesticides, including ones of recombinant origin. For such products, applicants must first notify EPA of any proposed field test, regardless of size, and possibly obtain an EUP to conduct the test. To obtain EUPs for microbial products, applicants must carry out standard safety testing along with testing to assess the potential environmental effects of the product, often relying on results of monitoring studies conducted during small-scale tests. The scope of this oversight was narrowed in the final biotechnology rules that took effect in September 1994, but many biotechnology-derived microbial pesticides are subject to this extra level of regulation.

History

The first microbial pesticide, *Bacillus popilliae*, was registered for agricultural use in 1948, and since then interest in microbial pesticides has grown. Beginning in 1974, EPA began to investigate the scientific basis for risk assessment of microorganisms used as pest control agents, and in 1983 the agency issued testing guidelines for microbial pesticides and biochemical pesticides (pesticides based on natural chemicals that act through biochemical pathways rather than through sheer toxicity) as Subpart M of the Pesticide Assessment Guidelines. Using these guidelines and existing regulations, EPA registered about a dozen naturally occurring microorganisms for use as pesticides in the years before the biotechnology era.

In 1984, even before the earliest proposal for the Coordinated Framework, EPA established an interim policy for regulation of genetically modified microbial pesticides ("Microbial Pesticides: Interim Policy on Small-Scale Field Testing," October 17, 1984, 49 Federal Register 40659). This main feature of this policy was that the ten-acre (terrestrial) exemption under the then-existing EUP regulations (40 CFR 172.3) would not automatically apply to tests using genetically altered and nonindigenous microbial pesticide products and that EPA should be notified before initiation of any such testing. Since that time, the Agency has used this notification scheme to evaluate small-scale tests, involving genetically altered and nonindigenous microbial pesticides, for possible risk to human health or the environment and to determine whether EUPs would be required before the tests could be initiated.

EPA's final statement of policy, incorporating this interim policy, was published with the Coordinated Framework on June 26, 1986, and the Agency stated its intention to codify the major elements of the notification procedure in the revised EUP regulations. EPA published its first formal proposal to amend its EUP regulations on January 22, 1993 (58 Federal Register 5878) and this Proposed Rule relied heavily on public comments on the 1986 policy statement and the Scope Policy described in the preceding section. After consideration of public comment on the Proposed Rule, EPA issued a Final Rule entitled "Microbial Pesticides; Experimental Use Permits and Notifications" on September 1, 1994 (59 Federal Register 45600), which took effect on October 31, 1994, and which is described below.

Microbial Pesticide Regulations

Using principles established for the earliest (nonengineered) microbial pest control agents, microbial pesticides are regulated by EPA under the same regulations used for chemical pesticides. Some of the required testing data is different for living biological agents, but for genetically modified biopesticides the key issue became the identification of those modified microorganisms that required additional oversight at the level of small-scale field–testing.

Large-scale tests under 40 CFR Part 172 are defined as any terrestrial application on a cumulative total of more than 10 acres of land or any aquatic application on more than one surface acre of water. Although EPA has generally presumed that testing on up to 10 acres of land or one surface acre of water would not require EUPs, the Agency decided that small-scale tests in the environment with some microbial pesticides may pose sufficiently different risk considerations from conventional chemical pesticides to require a closer evaluation at the small-scale testing stage. Therefore 40 CFR Part 172 was amended to require agency notification before initiation of small-scale testing in the environment of certain microbial pesticides so that EPA may determine whether these tests should be conducted under an EUP.

Under the 1984 interim policy, all genetically modified microbial pesticides and all microorganisms not indigenous to the United States required EPA notification before field-testing at any level, and many of these products were judged to require an EUP before tests could be carried out. The 1994 microbial pesticide regulations relaxed this requirement through three important provisions. First, the notification requirement was lifted for many genetically modified microbial pesticides: the only organisms now requiring notification are those where the pesticidal property is imparted or enhanced by the genetic manipulation, and notifications are specifically not required for genetic modifications that are solely deletions or rearrangements of a single genome. Second, notifications are no longer required for nonindigenous microbial pesticides, except in the rare cases where such organisms would not be covered by the USDA's importation regulations (described in a following section). Finally, the regulation gave EPA the authority to exempt specific classes of microorganisms

from the need for notifications at small-scale testing. This has allowed EPA to exempt categories such as many genetically modified *B. thuringiensis* strains from the need for notification at the early stages of R&D.

The rule proposed in 1993 had requested comment on two options for defining which microbial pesticides would be subject to the notification requirement. A third option was also discussed but only for illustrative and comparative purposes. In the proposed rule, EPA indicated its preference for Option 1, under which notification would be required before small-scale testing of microbial pesticides "whose pesticidal properties have been enhanced or imparted by the introduction of genetic material that has been deliberately modified." This option was selected in the final rule as the one that best addresses potential risks presented by certain categories of microbial pesticides by focusing the notification requirement on tests involving microbial pesticides with the potential for presenting new and different hazards or exposures to humans or the environment on the basis of simple and directly addressable criteria.

In the proposed rule, EPA no longer required notifications for any naturally occurring nonindigenous microbial pesticides, based on its experience since 1984 with the assessment of numerous nonindigenous microbial pesticide products for which notifications were submitted under the interim policy. This provision was included in the final rule but, in response to public comment, the rule retained a provision that EPA requires notifications at the small-scale testing stage for nonindigenous microbial pesticides that have not been reviewed by USDA.

The final regulations added a Subpart C, "Notification for Certain Genetically Modified Microbial Pesticides," to the existing EUP regulations and specified the data that is required to accompany notifications. EPA's review of a notification must be completed within 90 days and at the conclusion of the review, the Agency could either approve the test without requiring an EUP; approve the test without requiring an EUP if certain modifications in the proposed test plan are incorporated; require additional information; require an EUP for the test; or disapprove the test because of the potential for unreasonable adverse effects.

The final rule also provides that testing conducted within a facility with adequate containment and inactivation controls would not be subject to the notification requirement. Responsibility for selection and use of adequate containment and inactivation controls would lie with the researcher or institution conducting the test.

Data Requirements

Registration of biopesticides follows existing EPA regulatory procedures for all pesticides, with specific data requirements enumerated in Subparagraph M of 40 CFR Part 172. These requirements include

- product analysis to identify the active ingredient and any inert substances added thereto;
- toxicology testing, including short-term tests to evaluate the potential for toxicity, infectivity, or pathogenicity (Tier I), as well as more specific and sensitive tests in Tiers II and III;
- residue data to quantitate the amount of the pest control agent or its toxins that might appear on food or feed crops, although this is only required if toxicology testing leads to human health concerns;
- ecological effects and environmental expression testing, also grouped in tiers, to assess possible effects of the agent on nontarget organisms (Tier I), with further testing to address more specific concerns in Tiers II, III, and IV.

EPA has stated that most microbial pesticides will not require testing beyond Tier I, but some genetically engineered products might be judged to need some Tier II tests. In addition to safety testing, pesticide registration applications must also include proof of the product's efficacy, generally obtained in field tests.

EPA is also responsible under the Food, Drug and Cosmetic Act to establish tolerances (maximum allowable concentrations) of pesticides in food products. Applicants, to register pesticides for use on specific crops, must petition the Agency, with the necessary data to establish a tolerance level or to obtain an exemption from tolerance, based on a lack of human toxicity of the product. Many biological products are toxic only to the target pest (*B. thuringiensis* being a good example) and many have had little trouble obtaining exemptions from tolerance.

Products Approved Under FIFRA

Table 2 shows the microbial pesticide products that EPA has approved to date under FIFRA and the interim and final biotechnology regulations. This table groups these products according to taxonomic criteria (e.g., bacteria, fungi, viruses), but the products can also be grouped according to their end use. Most of the approved products are insecticides, with the bulk of these being wild type or mutant strains of *B. thuringiensis*, an organism producing a delta-endotoxin that is toxic to specific orders of insects but that is generally nontoxic to nontarget species. More recently, GEMs expressing *B. thuringiensis* endotoxins in other host species have also come onto the market. Other bacteria and fungi are used for insect control, as are a large number of natural and modified insect viruses. In all cases, the biological agent has high selectivity for the targeted insect pest.

Another class of product is fungicides, where a number of bacterial and fungal strains are used as agents against specific fungal diseases of plants, roots, or stored grain. *Trichoderma* species have been the most frequently investigated and registered in this category. There are also biological herbicides, generally fungal strains that can selectively kill weedy plants without harming crop species. Smaller classes of products include nematicides and bactericides.

EPA BIOTECHNOLOGY REGULATION UNDER THE TOXIC SUBSTANCES CONTROL ACT

Background

EPA is using TSCA to regulate the microbial production of certain chemicals or enzymes not regulated elsewhere in

Table 2. EPA-Approved Microbial Pesticides under FIFRA

Microbial Pesticide	Type of Pesticide: Target Pest
Bacteria	
Agrobacterium radiobacter K84	Bactericide: Crown Gall Disease
Agrobacterium radiobacter K1026	Bactericide: Crown Gall Disease
Bacillus cereus Strain BP01	Microbial plant growth regulator
Bacillus popilliae & Bacillus lentimorbus	Insecticide: Japanese beetle larvae
Bacillus sphaericus	Insecticide: Mosquito larvae
Bacillus sphaericus Serotype H5a5b strain 2362	Insecticide: Mosquito larvae
Bacillus subtilis GBO3	Fungicide: Rhizoctonia, Fusarium, other diseases
Bacillus subtilis MBI 600	Fungicide: Rhizoctonia, Fusarium, other diseases
Bacillus subtilis var. *amyloliquefaciens* strain FZB24	Fungicide: fungal diseases in greenhouse, indoor plants
Bacillus thuringiensis Berliner	Insecticide
Bacillus thuringiensis CryIA(c) & Cry I(c) delta-endotoxin in killed *Pseudomonas fluorescens*	Insecticide: larvae of many moth species
Bacillus thuringiensis subsp. *aizawai*	Insecticide: Wax moth larvae
Bacillus thuringiensis subsp. *aizawai* GC-91	Insecticide: Wax moth larvae
Bacillus thuringiensis subsp. *israelensis*	Insecticide: Mosquito larvae
Bacillus thuringiensis subsp. *israelensis* EG2215	Insecticide: Mosquito larvae
Bacillus thuringiensis subsp. *kurstaki*	Insecticide: Lepidopteran larvae
Bacillus thuringiensis subsp. *kurstaki* BMP123	Insecticide: Lepidopteran larvae
Bacillus thuringiensis subsp. *kurstaki* EG2348	Insecticide: Lepidopteran larvae
Bacillus thuringiensis subsp. *kurstaki* EG2371	Insecticide: Lepidopteran larvae
Bacillus thuringiensis subsp. *kurstaki* EG2424	Insecticide: Lepidopteran and Coleopteran larvae
Bacillus thuringiensis subsp. *kurstaki* EG7673 Coleoptera	Insecticide: Coleopteran larvae
Toxin	
Bacillus thuringiensis subsp. *kurstaki* EG7673	Insecticide: Lepidopteran larvae
Lepidoptera Toxin	
Bacillus thuringiensis subsp. *kurstaki* EG7826	Insecticide: Lepidopteran larvae
Bacillus thuringiensis subsp. *kurstaki* EG7841	Insecticide: Lepidopteran larvae
Bacillus thuringiensis subsp. *kurstaki* M200	Insecticide: Lepidopteran larvae
Bacillus thuringiensis subsp. *tenebrionis*	Insecticide: Coleopteran larvae (Colorado potato beetle)
Burkkholderia cepacia type Wisconsin IsoJ82	Fungicide/Nematicide
Burkkholderia cepacia type Wisconsin M36	Fungicide/Nematicide
Pseudomonas aureofaciens strain Tx-1	Fungicide: Turf grass diseases
Pseudomonas fluorescens 1629RS	Bactericide: Frost-forming bacteria
Pseudomonas fluorescens A506	Bactericide: Frost-forming bacteria
Pseudomonas syringae 742RS	Bactericide: Frost-forming bacteria
Pseudomonas syringae ESC 10	Fungicide: Postharvest decay on fruits
Pseudomonas syringae ESC 11	Fungicide: Postharvest decay on fruits
Streptomyces griseoviridis K61	Fungicide: Seed rot, root and stem rot, damping-off disease
Fungi	
Ampelomyces quisqualis M10	Fungicide: Powdery mildew on grapes, tomatoes, strawberries, ornamentals
Beauveria bassiana ATCC 74040	Insecticide: Whitefly, grasshoppers, locusts, crickets
Beauveria bassiana GHA	Insecticide: Whitefly, grasshoppers, locusts, crickets
Candida oleophila isolate I-182	Fungicide: Rot disease on fruits and vegetables
Colletotrichum gloeosporioides f.sp. *aeschynomene* ATCC 20358	Herbicide: Northern joint vetch
Gliocladium catenulatum Strain J1446	Fungicide: Pythium, Rhizoctonia
Gliocladium virens G-21	Fungicide: Pythium, Rhizoctonia
Lagenidium giganteum	Insecticide: Mosquito Larvae
Metarhizium anisopliae ESF1	Insecticide: Cockroaches, flies
Paecilomyces fumorsoroseus Apopka strain 97	Insecticide: Whitefly, thirps, aphids, spider mites
Puccinia canaliculate (Schweinitz) Langerheim ATCC 40199	Herbicide: Nutsedge weeds
Trichoderma harzianum ATCC 20476	Fungicide: Damping-off diseases
Trichoderma harzianum Rifai T-22 (KRL-AG2)	Fungicide: Pythium, Rhizoctonia
Trichoderma harzianum Rifai T-39	Fungicide: Gray mold
Trichoderma polysporum ATCC 20475	Fungicide: Wood rot microorganisms
Viruses	
Anagrapha falcifera Nucleopolyhedrosis Virus (NPV)	Insecticide: Lepidopterans
Autographa californica NPV	Insecticide: Alfalfa looper
Cydia pomonella Granulosis virus	Insecticide: Codling moth
Douglas fir tussock moth NPV	Insecticide: Douglas fir tossack moth
Gypsy moth NPV	Insecticide: Gypsy Moth
Helicoverpa zea NPV	Insecticide: Corn Earworm

(*continued overleaf*)

Table 2. (*Continued*)

Microbial Pesticide	Type of Pesticide: Target Pest
Mamestra configurata NPV	Insecticide: Bertha Army Worm (approval pending)
Spodoptera exigua NPV	Insecticide: Beet Army Worm
Miscellaneous Microbials	
Nosema locustae	Insecticide: Grasshoppers
Nonviable Microbials	
Bacillus thuringiensis CryIA(c) & Cry I(c) delta-endotoxin in killed *Pseudomonas fluorescens*	Insecticide: Larvae of many moth species
Bacillus thuringiensis K CryIC in killed *Pseudomonas*	Insecticide
Bacillus thuringiensis subsp *kurstaki* delta-endotoxin in killed *Pseudomonas fluorescens*	Insecticide: Caterpillars
Bacillus thuringiensis subsp San Diego delta-endotoxin in killed *Pseudomonas fluorescens*	Insecticide: Colorado potato beetle
Myrothecium verrucaria, Dried fermentation solids & solubles of	Nematicide: Parasitic Nematodes

Source: U.S. Environmental Protection Agency (*http://www.epa.gov/oppbppd1/biopesticides/*).

the government, and planned introductions of microorganisms into the environment that are not regulated under other federal statutes. TSCA (15 U.S. Code 2601) is a law requiring manufacturers to notify EPA at least 90 days before commencing manufacture of any "new" chemical, that is, one that is not already in commerce for purposes not subject to regulation as a pesticide or under the food and drug laws. In the Coordinated Framework, EPA decided to use TSCA in this same "gap-filling" way to capture those microorganisms that were not regulated by other federal agencies. The primary areas that therefore became subject to the TSCA biotechnology regulations were (*1*) microorganisms used for production of non-food-additive industrial enzymes, other specialty chemicals, and in other bioprocesses; (*2*) microorganisms used as, or considered to be, pesticide intermediates; (*3*) microorganisms used for nonpesticidal agricultural purposes; and (*4*) microorganisms used for other purposes in the environment, such as bioremediation.

The key to the historical development of this regulation was the definition of organisms that are "new," and which therefore are subject to reporting requirements under TSCA. Another feature of the regulations is a two-tiered review structure, under which all commercial uses of new microorganisms (within the scope of TSCA's applicability) would require agency review, and research uses of new microorganisms in the open environment ("uncontained uses") would also require some agency oversight, regardless of scale (although most indoor research is exempted). The regulations adopted in final form in 1997 instituted reporting requirements specific for microorganisms (but which paralleled the commercial notifications used for traditional chemicals), while also creating new requirements to provide suitable oversight over outdoor uses of genetically modified microorganisms.

Procedures under the TSCA biotechnology regulations are similar to existing practice for new chemical compounds. Manufacturers of chemicals new to commerce must file premanufacture notices (PMNs) with EPA at least 90 days prior to the first intended commercial sale, use, or importation. Manufacturers must submit all relevant health and safety data in their possession, and although EPA has published guidance documents, specifying the types of data that it wants to see in PMN submissions, there are no formal data or testing requirements under TSCA. As applied to new chemical entities, TSCA is a "screening" statute: the 90-day review period is sufficient only to allow EPA to identify chemicals that might pose an environmental or public health risk, and in such case it can take any of several actions to extend the time period and keep the product off the market until suitable data is submitted to show its safety. The large majority of PMNs are approved within the 90-day period after only brief agency review.

History

TSCA biotechnology policy originated in EPA's 1986 Coordinated Framework statement that the agency intended to use this law to regulate certain commercial uses of "new" microorganisms (it was in this 1986 statement that the policy was first restricted to microorganisms and excluded plants and animals). This statement introduced the concept that EPA would consider any "intergeneric" organism (i.e., one containing deliberate combinations of genetic material from more than one taxonomic genus) as "new" under TSCA. The rationale for this definition (that the agency acknowledged was somewhat arbitrary) was that *intra*generic organisms were likely to occur naturally via normal mechanisms of genetic exchange but that organisms in different genera were unlikely to exchange DNA via natural mechanisms. Intergeneric microorganisms not regulated by other federal agencies were proposed to be subject to mandatory PMN reporting if they were to be used for commercial purposes and voluntary PMN reporting if used for research outside of contained facilities, in the latter case because of the potential for microorganisms to multiply and disperse beyond the site of application.

Effective with the June 26, 1986 publication of the Coordinated Framework, EPA established this scheme as an interim policy. This required intergeneric microorganisms used in indoor manufacturing (i.e., fermentation) to be subject to premanufacture notification 90 days or more before commercial use. The interim policy also subjected all outdoor research uses of "intergeneric" organisms to agency review under "voluntarily submitted" PMNs. With this 1986 publication, EPA also began the process of drafting and adopting formal regulations under TSCA for certain commercial uses of microorganisms.

In late 1988, EPA informally circulated for public comment a controversial advance draft of a proposed regulation, that would have subjected many naturally occurring microorganisms to EPA oversight, in addition to broadly capturing most engineered microbes. These draft

rules were withdrawn and never formally proposed, in part because of the negative public and industry reaction and in part because the process was superseded by the Scope Policy discussions discussed in the preceding sections.

In June 1991, EPA formally announced the availability for public comment of draft proposed regulations for biotechnology products under TSCA. The draft-proposed rule and its governing definitions were based on guidance from the first federal Scope Policy. For example, in this proposal, EPA abandoned the definition of "new" organisms as "intergeneric" and instead used a definition arising from the first Scope Policy: "organisms with deliberately modified hereditary traits," except for those falling under certain exclusion categories. EPA solicited public comments on this draft and intended to use the comments to produce actual proposed regulations within one year.

However, the publication of the revised Scope Policy in February 1992 rendered obsolete many of EPA's 1991 assumptions and definitions, so the agency had to revise its rulemaking efforts. EPA was finally able to publish proposed regulations in September 1994. These proposed rules returned to the definition of "new organism" as an "intergeneric organism" and were otherwise in conformance with the second Scope Policy. After a public comment period and much internal discussion and revision, the rules were finalized in April 1997 and took effect 60 days later in June 1997.

Overview of EPA TSCA Biotechnology Regulation

EPA's final TSCA biotechnology rule was published in the Federal Register on April 11, 1997 (62 Federal Register 17910–17958). These rules amended the existing TSCA PMN regulations by creating a new 40 CFR Part 725, that specifies the procedures for EPA oversight over commercial use and research activities involving microorganisms subject to TSCA. The rule also amends other existing parts of the TSCA regulations, where necessary, to be consistent with the new microbial sections.

The final rule defines a "new organism" as an "intergeneric organism" as instituted in the Coordinated Framework and used continuously since then in the interim policy. Organisms that are not new, including naturally occurring and classically mutated or selected microbes, are exempt from reporting requirements under TSCA.

New microorganisms used for commercial purposes not regulated under any of several other specific federal statutes require premanufacture reporting 90 days in advance of the commercial activity, using a new procedure called a Microbial Commercial Activity Notification (MCAN) that is analogous to the previous biotechnology PMN procedures under the interim policies and to long-existing PMN practice for chemical entities. However, several exemptions from MCAN reporting are possible for specific organisms that qualify. The most important exemption categories involve the use, as the recipient organism, of certain of the microbial species having the longest history of safe commercial use in fermentations, including *Acetobacter aceti*, *Aspergillus niger*, *Aspergillus oryzae*, *Bacillus licheniformis*, *Bacillus subtilis*, *Clostridium acetobutylicum*, *Escherichia. coli* K12, *Penicillium roqueforti*, *Saccharomyces cerevisiae*, and *Saccharomyces uvarum*. A procedure was also put into place for EPA to create new exemption categories based on appropriate scientific evidence.

Generally speaking, research activities involving new microbes are exempt from reporting if used only in "contained structures." The rule specifically contemplates that this exemption would apply broadly to many types of structures, including greenhouses, fermenters, and process stream bioreactors. Outdoor experimentation remains potentially subject to some sort of reporting, although the preexisting requirements were eased in several ways: certain exemption categories are created, although at this writing these exemption categories apply only to certain strains of *Rhizobium* and *Bradyrhizobium* engineered in specific ways. Field tests that do not qualify for an exemption can be conducted under a reduced-reporting requirement known as TSCA Environmental Release Application (TERA). The TERA process replaced the previous (voluntary) policy under which all outdoor uses of intergeneric microorganisms were reviewed under PMN reporting, regardless of the scale or potential risks of the field experiment. The regulations specify that TERAs would be reviewed by EPA within 60 days, although the agency could extend the review period by an additional 60 days. In approving TERAs, EPA has the authority to impose conditions or restrictions on the proposed outdoor use of GEMs.

Interestingly, because TSCA is a statute covering "commercial" introductions of new chemicals (i.e., into commerce), EPA in the final rule decided that noncommercial research would be exempt from TSCA, meaning that many academic research activities, unless clearly supported by or done for the benefit of a for-profit entity, would be exempt from TSCA reporting.

Data Requirements

The technical information that is required for each MCAN submission includes the following:

- Description of the recipient ("host") microorganism and the new microorganism resulting from the engineering.
- Genetic construction of the new microorganism.
- Phenotypic and ecological characteristics of the microorganism.
- By-products of the production process.
- Total production volume.
- Worker exposure and environmental release.
- Health effects information.

For submissions of TERAs, the following technical information is needed beyond that listed in the preceding section for MCANs:

- Phenotypic and ecological characteristics of the subject microorganism as they relate to the conditions of the proposed research and development activity (e.g., habitat, geographical distribution, and source of the recipient).

- Survival, dissemination, and detection of the subject microorganism.
- Anticipated adverse ecological effects.
- A detailed description of the proposed research and development activity.
- Methods of application or release.
- Characteristics of the test site.
- Information on monitoring, confinement, mitigation, and emergency termination procedures.

These data requirements are described in more detail in a document entitled "Points to Consider in the Preparation of TSCA Biotechnology Submissions for Microorganisms" (available at *www.epa.gov/opptintr/biotech/biorule.htm*), but the agency has a fair amount of leeway in determining which data are needed for each GEM based on its characteristics and intended use.

Microorganisms Approved Under TSCA

EPA has been receiving PMNs and other notifications of biotechnology products under TSCA since 1987. As listed in Table 3, most of the notifications received were for contained applications: uses of intergeneric microorganisms for manufacturing products for commercial purposes not regulated by other federal agencies. The bulk of these have been for manufacture of industrial enzymes but several others have been for manufacture of pesticide intermediates (which, under the statutes, are covered by TSCA,

Table 3. Submissions to EPA for Microorganisms Under TSCA

Host Microorganism	Modification
	PMNs for contained applications of microorganisms
Escherichia coli	Containing human gene for insulin-like growth hormone
Bacillus subtilis	Engineered for protease production
Bacillus licheniformis	Engineered for enhanced hydrolase production
Bacillus, (alcalophilic strain)	Engineered for enhanced production of protease
Aspergillus oryzae	Engineered for enhanced lipase production
Bacillus subtilis	Engineered for enhanced alpha amylase production
Bacillus licheniformis	Self-cloned for enhanced alpha amylase production
Bacillus subtilis (asporogenic strain)	Containing *S. aureus* antibiotic resistance gene and lipase gene
Bacillus licheniformis	Modified to contain *B. stearothermophilus* alpha-amylase gene and antibiotic resistance genes
Bacillus (sporulation deficient)	Containing genes enhancing subtilisin production
Aspergillus oryzae	Containing cellulase gene
Bacillus licheniformis	Containing xylanase gene
Bacillus licheniformis	Containing pullulanase gene
Escherichia coli K12	Containing gene for indigo production pathway
Escherichia coli K12	Containing *P. putida* naphthalene dioxygenase gene cluster
	Test marketing exemptions for microorganisms
Pseudomonas fluorescens	Containing delta endotoxin gene from *B. thuringiensis*
	MCANs for contained applications of microorganisms
Bacillus amyloliquefaciens	Engineered for commercial biosynthesis of an alpha-amylase enzyme
Bacillus lentus	Engineered for commercial biosynthesis of subtilisin
Pseudomonas fluorescens	Engineered for commercial biosynthesis of an enzyme for secondary oil recovery
	PMNs for environmental applications of microorganisms (all are research uses unless specified)
Rhizobium meliloti	Engineered for yield enhancement in alfalfa
Pseudomonas aureofaciens	Engineered to contain genes from *E. coli*
Rhizobium meliloti	Engineered to contain antibiotic resistance genes
Bradyrhizobium japonicum	Engineered to contain antibiotic resistance genes
Rhizobium meliloti	Engineered to contain antibiotic resistance genes and genes to enhance nitrogen-fixing ability (certain strains also allowed for commercial sale)
Bradyrhizobium japonicum	Engineered to contain antibiotic resistance genes and genes to enhance nitrogen-fixing ability
Psuedomonas fluorescens HK44	Containing naphthalene degradation gene and bioluminescent reporter gene
	TERAs for environmental applications of microorganisms (all are research uses)
Bradyrhizobium japonicum	Engineered to enhance competitiveness and nitrogen-fixation abilities in soybean plants
Pseuodomonas putida	Modified to emit detectable light in presence of TNT

Source: U.S. Environmental Protection Agency (*www.epa.gov/opptintr/biotech/biorule.htm*).

not FIFRA). Since the adoption of the final rules in 1997, several MCANs have been received for such products.

There have also been numerous PMNs (and more recently, TERAs) received for environmental introductions of altered microorganisms. Most of these have been for genetically altered nitrogen-fixing bacteria (*Rhizobium* or *Bradyrhizobium*), and in fact several strains of engineered *Rhizobium meliloti* for improved nitrogen fixation are the only recombinant microorganisms used in the open environment approved for commercial sale under TSCA. In addition to these agricultural tests, there have been two notifications relating to bioremediation, both for R&D projects, involving bacteria modified to contain marker genes to facilitate environmental monitoring of introduced strains. There have been no PMNs or MCANs submitted to EPA for uses of microorganisms in bioremediation or industrial processes other than production of enzymes.

U.S. DEPARTMENT OF AGRICULTURE BIOTECHNOLOGY REGULATIONS

Background

The third major regulatory program that might affect microorganisms, including GEMs, used in research and in the environment are the plant pest regulations of the USDA. Historically, USDA has maintained regulations under the Federal Plant Pest Act (7 U.S. Code 150 aa) that are intended to prevent the entry of plant pathogens and pests into the United States or to regulate the movement within the United States of plant pests that have been introduced for research or other legitimate purposes. The goal of these regulations is to prevent American agriculture from infestation from pest species not native to North America, and under these regulations, permits are needed for the importation or interstate shipment of certain listed plant pest species.

In 1987, USDA promulgated regulations under the Plant Pest Act to allow the agency to regulate certain uses of genetically engineered plants and microorganisms that were derived from, or contained genetic material from, plant pests. These regulations became the major U.S. government oversight over the field–testing of transgenic plants (because most transgenic plants are constructed using sequences derived from *Agrobacterium tumefaciens* or other microorganisms subject to this regulation) but this rule has also been used to regulate importation, interstate transport, and field-testing of certain GEMs derived from species having plant pest characteristics.

History and Regulatory Requirements

USDA has regulated the movement of plant pests since the passage of the Plant Pest Act in 1957. Plant pests are defined as "any living stage of any insects, mites, nematodes, slugs, snails, protozoa, or other invertebrate animals, bacteria, fungi, other parasitic plants or reproductive parts thereof, viruses, or any organisms similar to or allied with any of the foregoing, or any infectious substances that can directly or indirectly injure or cause disease or damage in any plants or parts thereof, or any processed, manufactured, or other products of plants." Under regulations in 7 CFR Part 330, it is necessary to obtain a permit in advance for the importation or interstate shipment of any organism meeting this definition.

In the Coordinated Framework, USDA through its Animal and Plant Health Inspection Service (APHIS) proposed new regulations, 7 CFR Part 340, under the Plant Pest Act that would allow it to review in advance proposed environmental uses or interstate shipments of genetically engineered plants or agricultural microorganisms. The title of the rule, "Introduction of Organisms or Products Altered or Produced through Genetic Engineering which are Plant Pests or which there is Reason to Believe are Plant Pests" states the philosophy behind the regulation: to cast a broad net to regulate many categories of engineered organism by defining a long list of genera, families, and orders that "are or contain plant pests." A genus or family was included on the list if even one species therein was known or believed to be a plant pest, with the result that many organisms not necessarily having plant pest characteristics would fall under these regulations. Anyone proposing to "introduce" (import, move interstate, or release into the environment) an organism derived by genetic engineering from a species on the list, needed to first obtain a permit from USDA APHIS. This permit scheme parallels the permits that have long been needed for movement of known plant pests under 7 CFR Part 330.

These regulations were finalized with some revisions in June 1987 (52 Federal Register 22892–22915). Among the more important revisions were the imposition of time frames for USDA review of 120 days for an environmental release permit and 60 days for a movement permit, the addition of a petition procedure to request additions or deletions in the list of potential plant pests, and a provision for notification of the state in which a field test was proposed to take place. Under the final regulations, a person or institution wishing to conduct a field test of a regulated organism needed to consult APHIS and possibly apply for a permit to conduct the test. For permit applications involving field tests, the submission was to include a description of the modifications made to the organism, data characterizing the stability of these changes, a description of the proposed field test, and the procedures to be used to "contain" the organisms in the test plot. Submitters needed also to assess potential environmental effects.

APHIS review of permit applications was straightforward and was generally completed well within the 120 days allowed by the regulations. From 1986 to 1990, APHIS approved about 100 field tests of transgenic plants under these regulations and the program was largely considered successful by government and industry alike. Because of the experience gained in its review of transgenic plant field tests, APHIS issued a revised final rule on March 31, 1993 (58 Federal Register 17044–17059), establishing a less-stringent scheme, whereby most transgenic varieties of six specific crop species (the ones most frequently tested up to that date) could be field tested merely on advance notification to APHIS (i.e., without a permit), and also establishing procedures for specific

varieties to be delisted so that permits for use of such plants would no longer be required. In a further relaxation, on May 2, 1997 (62 Federal Register 23945–23958), the notification scheme was extended even further so that virtually all transgenic plants could be field tested merely upon agency notification without need for a permit, except certain plants derived from species known to be noxious weeds. Genetically engineered microorganisms, however, were not affected by these revisions and for the GEMs covered by these regulations, most proposed uses in the environment still require a permit rather than qualifying for the notification procedure.

Although primarily used to regulate field-testing of transgenic plants, the Part 340 regulations have been used to regulate introductions of a relatively small group of GEMs, as shown in Table 4. Many of these are engineered versions of organisms having pesticidal or other agriculturally useful properties, and although some of these permits were issued to persons or institutions that were also subject to EPA regulation (e.g., under FIFRA), several of these were for research uses that were not regulated by other federal agencies.

Table 4. Genetically Modified Microorganisms Approved by USDA for Environmental Use Under the Plant Pest Act

Microorganism	End Use or Application
Aspergillus flavus	Not available
Cephalosporium gramineum	Not available
Clavibacter xyli	Endophyte of corn, engineered to express *B. thuringiensis* endotoxin.
Cryphonectria parasitica	Virulence-attenuated pathogen (chestnut blight)
Fusarium graminearum	Virulence-attenuated pathogen (wheat head scab disease), also reduced mycotoxin production
Fusarium moniliforme	Virulence-attenuated pathogen (corn ear rot), also reduced mycotoxin production
Fusarium sporotrichioides	Not available
Heterorhabditis bacteriophora Pseudomonas	Not available
Pseudomonas putida	Engineered to express luciferase gene as marker.
Pseudomonas syringae	Virulence-attenuated pathogen (bacterial speck disease)
Pseudomonas syringae	Reduced epiphytic survival
Rhizobium etli	Nitrogen fixation
Rhizobium fredii	Nitrogen fixation
Rhizobium leguminosarum	Nitrogen fixation
Rhizobium meliloti	Nitrogen fixation
Xanthomonas campestris	Virulence-attenuated pathogen (bacterial spot disease)
Xanthomonas campestris	Crucifer black rot pathogen, engineered to express luciferase gene as marker.

Note: The microbial species mentioned, genetically modified in various ways, have received permits or have otherwise been allowed to be field-tested or imported into the United States by the U.S. Department of Agriculture.

Source: U.S. Department of Agriculture. (*http://www.aphis.usda.gov/bbep/bp/* and *http://www.nbiap.vt.edu/cfdocs/fieldtests1.cfm*)

Data Requirements

Among the data required to be submitted to USDA to support an application for a permit under Part 340, is the following scientific information:

- All scientific, common, and trade names, and all designations necessary to identify the donor organism(s), recipient organism(s), vector or vector agent(s).
- A description of the anticipated or actual expression of the altered genetic material in the regulated article and how that expression differs from the expression in the nonmodified parental organism.
- A detailed description of the molecular biology of the system (e.g., donor-recipient-vector) used to produce the regulated article.
- A detailed description of the purpose for the introduction of the regulated article, including a detailed description of the proposed experimental and production design.
- A detailed description of the processes, procedures, and safeguards that have been used to prevent contamination, release, and dissemination in the production of the donor organism, recipient organism, vector or vector agent.
- A detailed description of the proposed procedures, processes, and safeguards that will be used to prevent escape and dissemination of the regulated article at each of the intended destinations.
- A detailed description of the proposed method of final disposition of the regulated article.

OTHER U.S. BIOTECHNOLOGY REGULATION

Uses of microorganisms in commerce or in the environment are potentially subject to other regulations as well. Two types of regulation where rules specific to microbiology exist are federal export controls and state regulation of biotechnology activities.

The U.S. Commerce Department's Bureau of Export Administration regulates exports of all commodities, requiring that permits be obtained in advance for export of commodities to certain politically sensitive countries. The Department maintains regulations specific for biological materials based on concern over spread of biological weapons. Regulations that took effect in early 1989 created a situation where almost all exports of recombinant organisms would require an advance permit from the Commerce Department. In response to public comments that these regulations were overly broad, in March 1990 the Department published revised rules, requiring permits only for exports of potentially pathogenic organisms (15 CFR 742.2; 55 Federal Register 11361–11362), thus limiting the impact of these regulations.

In the late 1980s, there was a flurry of activity in state legislatures to enact biotechnology laws. Many of these covered possible field tests of GEMs or transgenic plants and by the time this activity peaked in 1991, there were approximately nine state laws that would impact planned environmental introductions of GEMs. Some of these laws required separate state permits for planned environmental uses of GEMs or transgenic plants, whereas others were simply notification schemes to ensure that the relevant state government agency was informed of proposed field tests (5,10). USDA's decision to require formal notification of the states in which field tests were proposed helped in part to stem the need for further state regulation, however, it is possible that environmental uses of GEMs in certain states would trigger additional regulatory requirements.

BIOTECHNOLOGY REGULATION OUTSIDE THE UNITED STATES

Several nations or jurisdictions outside the United States have adopted biotechnology regulations. Two of these regulatory schemes are briefly described in the following section, as they relate to the possible environmental use of genetically engineered microorganisms.

Canada

Canada's regulatory approach resembles that of the United States, in that existing laws and regulations are used to regulate biotechnology in a product-specific way. Therefore many products of biotechnology would be regulated in Canada under federal laws, such as the Pest Control Products Act (pesticides), the Seeds Act (plants), and the Fertilizers Act (nitrogen-fixing microbes and inorganic fertilizers). In November 1997, after several years of deliberation, Environment Canada issued biotechnology regulations under the Canadian Environmental Protection Act (CEPA) that are similar in scope and approach to the U.S.EPA's TSCA regulations. Environment Canada will use CEPA to conduct risk assessments of certain biotechnology products that are new to commerce in Canada and are not regulated by other federal agencies. Among products that would fall within the scope of this law would be microbial cultures used for bioremediation.

Environment Canada considers microorganisms as being potentially subject to these "New Substance Notification" (NSN) regulations if they meet the definition of "new substance." Unlike the U.S. EPA, however, Environment Canada did not create a specific definition of "new microorganism" based on scientific criteria but instead is relying on the definition in the law that a "new substance" is one intended for introduction into commerce that is not on the domestic substance list as having been used in commerce between January 1, 1984 and December 31, 1986. Thus, if a microorganism was used in commerce in this time period in a way such that "its entry into the environment was unrestricted," it is exempt from reporting; but all other microorganisms, regardless of make-up, are subject to reporting. In this way the Canadian CEPA regulations are broader than those of the U.S. EPA, in subjecting a larger class of microorganisms to regulation, including naturally occurring or classically mutated strains.

Under the NSN regulations, any person who manufactures or imports substances subject to notification must provide a notification package to Environment Canada, which contains certain information specified in the regulations. Environment Canada uses this information to conduct a risk assessment prior to entry into commerce. These requirements are generally similar to those used by the U.S. EPA. Information on the Canadian biotechnology rule is available at *www.ec.gc.ca/cceb1/eng/biohome.html* and the regulations themselves can be found at *www.ec.gc.ca/cceb1/eng/nsnregejan1597a.html*. A Guidelines document that is similar to the EPA "Points to Consider document" can be accessed at *www.ec.gc.ca/cceb1/eng/97gle2.html*.

European Union

Many countries now in the European Union have adopted biotechnology regulatory policies following the same historical pattern as that seen in the United States: early concerns over public health issues, leading to controls over contained uses, followed by commercial regulation triggered largely by environmental concerns. For example, many European countries have adopted laboratory and manufacturing guidelines similar to the U.S. NIH Guidelines. Most of these laws were initially aimed at laboratory research but some covered commercial activities as well.

Within the European Union these individual country laws have been subsumed by two Union-wide biotechnology directives adopted by the European Commission's (EC's) Environmental Directorate, DGXI, in April 1990 (EC directives are not themselves laws or regulations but require each EU member state to adopt its own implementing laws to achieve the result specified in the directive). The first is a directive, covering contained uses of genetically modified organisms. This directive is similar to the NIH Guidelines in establishing appropriate procedures, both at small-scale and large-scale, for the use of engineered organisms in contained systems. Regulatory authorities in the member states must be notified or must actually approve certain biotechnology activities.

The other directive DGXI adopted was aimed at outdoor uses of genetically manipulated organisms. This directive required each member state to appoint a regulatory agency with the authority to review and approve environmental introductions within its jurisdiction. Proposals for R&D field tests must address similar environmental issues as are considered in the United States. Member states have sole authority to approve R&D uses within their jurisdiction, after EC notification and a 90-day review period. Commercial use proposals are to be made to a single member state. The competent authority would review the application and forward its recommendation to the Commission within 90 days. For products recommended for approval, other EC member states then have 60 days to lodge objections and if none are raised, the manufacturer could sell the product throughout the Union. Any state, however, may prohibit sale of a product if it had

reason to believe that the product presented unreasonable risks.

The approach adopted by the EU for implementation by its member states differs from the product-specific approach adopted by the United States and Canada, by creating a single biotechnology law to govern all field releases of engineered organisms regardless of end use. In some countries this has created the situation where a GEM might be subject to dual regulation, under the biotechnology law and the product-specific law applying to the use for which the GEM is intended (e.g., a pesticide law). This was among the early criticisms levied against the DGXI directive by industry spokespersons.

However, during the years this directive has been in effect it has worked reasonably well to allow for research field tests of genetically modified plants and microorganisms, although certain countries within the EU have been less than hospitable to outdoor uses of GEMs. More recently, however, this directive has allowed individual nations to ban commercial use or shipment of transgenic plants intended for food use, leading to significant uncertainties and consumer concerns throughout Europe about engineered plants in foods. These concerns seem not to be directed to environmental uses of GEMs or plants for the most part.

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REGULATIONS FOR DRINKING WATER.

See SOURCE WATER PROTECTION: MICROBIOLOGY OF SOURCE WATER

REMOVAL OF PATHOGENIC MICROBES BY GRANULAR HIGH-RATE FILTRATION

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With proper design and operation, filtration can act as a consistent and effective barrier for microbial pathogens. The most commonly used filtration processes in potable water treatment and the pore size of filter medium are listed in Figure 1. The sizes of different microbial particles are also included. These size spectra provide insight into understanding the removal mechanisms and efficiencies and into developing strategies to remove microbes by different filtration processes.

Granular filtration is the most widely used filtration process in drinking water treatment. A comprehensive review of granular filtration process is available (1). High-rate granular filtration is the focus of this chapter. Slow sand filtration, which is operated under a very low filtration rate (less than 0.4 m/h), is described in another chapter. Note that the design/operating criteria and mechanisms to remove microbes by slow sand filtration are considerably different from high-rate granular filtration processes. Membrane filtration, another effective barrier for microbial pathogens, is also covered in another chapter.

PROCESS DESCRIPTION

In granular filtration, water passes through a filter consisting of a packed bed of granular materials. Removal of microbes is achieved when microbes or microbe-associated particles deposit on the filter medium. This removal is within the granular medium (depth filtration) rather than on the top layer of the filter medium only (cake filtration). After a period of operation, the head loss through the filter increases or the effluent quality deteriorates to an unacceptable level; the filter has to be cleaned by backwashing. Initially, after backwashing, filters exhibit a poor performance followed by a period with improvement in filtrate quality until they reach a stable level of performance. Passage of microbial pathogens during this so-called ripening period can be formidable. Different methods are used to minimize this impact, such as filter-to-waste (wasting the initially filtered water) or slow start (limiting initially filtration rate until the filtrate quality is acceptable).

Granular filters can be constructed as monomedium (typically with silica sand), dual media (usually with anthracite coal and sand), and trimedia (commonly with coal, sand, and garnet). A schematic representation of these configurations is shown in Figure 2. Granular-activated carbon is used when both filtration of particles and adsorption of organic compounds are considered. Depending on raw water quality, granular filtration can be operated in three different modes: conventional, direct,

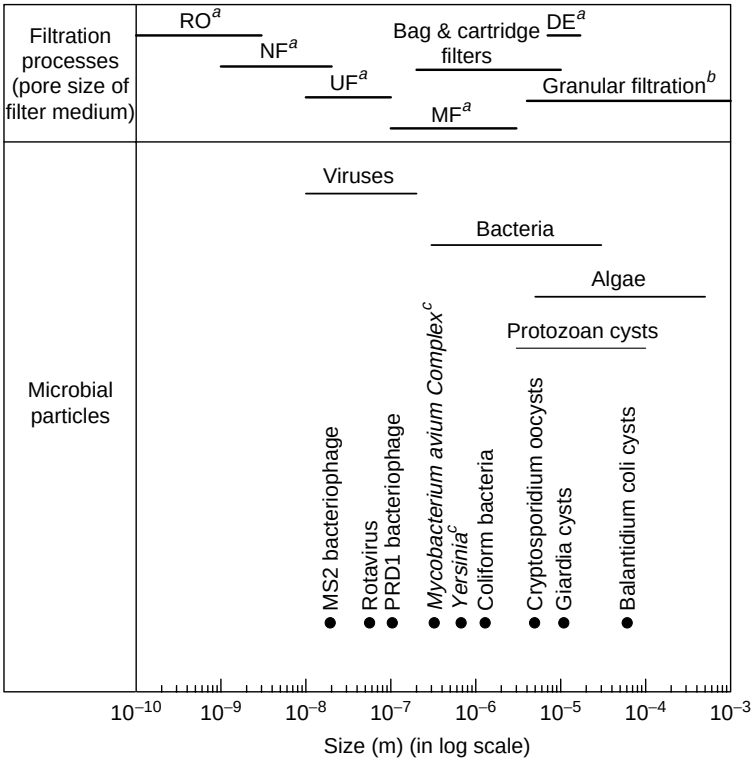

Figure 1. Pore size of filter medium and size of microbial particles.
[a]RO: reverse osmosis; NF: nanofiltration; UF: ultrafiltration; MF: microfiltration; DE: diatomaceous earth filtration.
[b]Including slow sand filtration. Slow sand filter has a lower pore size than rapid-rate filter.
[c]These bacteria are rod-shape. The sizes shown represent the smallest dimension.

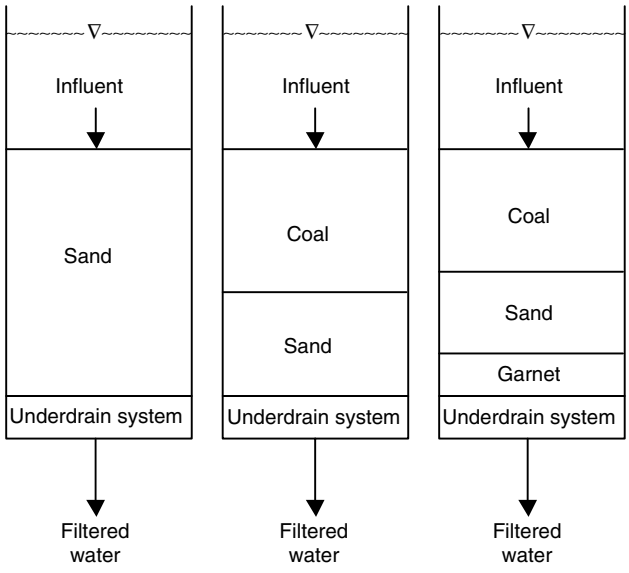

Figure 2. Schematic representation of filter configurations.

and in-line filtrations. The operation of conventional treatment includes addition of coagulants (rapid mixing), flocculation (slow mixing), sedimentation, and filtration. When sedimentation is omitted, the process is termed *direct filtration*. If both flocculation and sedimentation tanks are omitted, it is termed *in-line filtration*. Conventional treatment is appropriate for raw waters with a variety of water quality. Direct and/or in-line filtrations are used for raw water with good quality (low turbidity and color).

Removal of microbial pathogens by granular filtration is not a purely physical process. By comparing the pore size of granular filters with the size of most microbes shown in Figure 1, one can interpret that effective removal of microbes by granular filtration cannot rely on physical straining, at least at the initial stage of a filter run. The removal of particles by granular filtration is considered to involve two sequential steps: transport of particles from suspension to filter medium followed by the attachment of particles to the medium (2). Transport step is determined by the physical hydrodynamic properties of the system. Important transport mechanisms include diffusion, interception, and sedimentation (see below). Process variables such as the size and density of microbes, size and depth of filter medium, and filtration rate affect transport efficiency. For transport of motile microorganisms, an additional mechanism is the active movement of the cell (3). Attachment is determined by the surface and solution chemistry of the system (4). Unfavorable interaction among particles and filter medium must be avoided so that particles can attach to the filter medium. Proper chemical pretreatment is used to achieve this purpose and is the single most important factor in determining filtration efficiency. Without proper chemical pretreatment, rapid rate filtration probably works as a simple strainer and cannot be an effective barrier to microbial pathogens. Traditionally, chemical coagulation before filtration is used to destabilize particles and thus promote their adherence to the filter medium. Recently, mechanistic study has been performed and showed that applying oxidants such as chlorine and/or ozone prior to filters can also change the surface properties of particles and, in particular, destabilize particles (5). This result has been used

to explain the positive effects of preoxidation on filtration performance.

FILTER PERFORMANCE

The importance of chemical coagulation pretreatment on removal of microbes by granular filtration has been emphasized by numerous studies. Al-Ani and coworkers (6) conducted a pilot-scale filtration study for low-turbidity waters (less than 1 ntu). Without chemical pretreatment, poor filtration performance was observed. The removal by filtration averaged 69% (0.51 logs) for *Giardia* and 28% (0.14 logs) for turbidity. When optimum coagulation conditions were achieved by adding alum and polymeric filter aids, the removal efficiency increased to exceed 95% (1.30 logs) for *Giardia*, 99% (2 logs) for total coliform bacteria, and 70% (0.52 logs) for turbidity. Other process variables such as filtration mode (direct and in-line filtrations), filter media (monomedia with sand and dual media with sand and anthracite), and temperature (5 °C and 18 °C) did not exhibit significant effects on filtered water quality in this particular study. Varying filtration rate from 5 to about 20 m/h showed little effect on the removal of *Giardia*, total coliform bacteria, and standard plate-count bacteria but increased turbidity in filtered water.

Robeck and coworkers (7) reported that when alum was used as coagulant, the removal of poliovirus type 1 by a pilot-scale dual-media filter was more than 98% (1.70 logs). Ongerth (8) conducted pilot studies for conventional and in-line filtrations. Without chemical pretreatment, removal of Giarida cysts averaged 75% (0.60 logs) for conventional treatment and 64% (0.44 logs) for in-line filtration. With optimum chemical pretreatment by alum and polyelectrolytic filter aid, the removal increased to 98% (1.70 logs) for conventional treatment and 93.6% (1.19 logs) for in-line filtration.

Nieminski and Ongerth (9) performed a two-year evaluation of the removal of Cryptosporidium oocysts and Giardia cysts at pilot-scale and full-scale filtration plants, both operated under direct filtration and conventional treatment modes. Dual-media filters with anthracite and sand were used. The results indicated that maintaining optimum coagulation conditions to produce low turbidity (0.1 to 0.2 ntu) filtered water provided effective removal of *Giardia* and *Cryptosporidium*. Under optimum coagulation conditions by polymers together with either alum or polyaluminum chloride, the average removal of *Giardia* was 3.3 logs or better and the average removal of *Cryptosporidium* was 2.3 logs or better in both pilot-scale and full-scale plants, regardless of the treatment modes (direct filtration or conventional treatment). Correlation between the removal of cysts and of surrogate parameters were also investigated. A high correlation was found between removal of cysts and of the respective size particles (4–7 μm and 7–11 μm), with an R^2 value of 0.79 or higher. A low correlation was established between the removal of *Giardia* and *Cryptosporidium* and the removal of turbidity (R^2 value 0.64 or lower). This indicated that particle counting could serve as a better indicator of cyst and oocyst removal than turbidity, at least for this particular study. No correlation was found between log removal of seeded cysts and log removal of heterotrophic plate counts (R^2 value 0.08 or lower), meaning that heterotrophic plate counts were probably not a good surrogate in the evaluation of cyst removal.

Swertfeger and coworkers (10) evaluated the effects of filter media design on cyst and oocyst removal. Pilot-scale filters with three different media designs were used. These included monomedia (sand with a depth of 750 mm), fine dual media (anthracite and sand with a depth of 900 mm and 300 mm, respectively), and deep dual media (anthracite and sand with a depth of 1,500 mm and 300 mm, respectively). The feed water to the pilot systems was taken from the effluent of a sedimentation unit in a full-scale water treatment plant and was in optimum coagulation condition. They reported that there was no statistically significant difference in the filtration performance for the different media. Removal of *Giardia* was 4.4 logs or better, with greater removal efficiency in the summer than in the winter. Removal of *Cryptosporidium* during the summer was similar to that in winter and averaged 2.7 logs or better.

Payment and coworkers (11) reported the water quality monitoring results for a full-scale conventional water treatment plant using dual media filters. Coagulation was provided using alum and activated silica. Prechlorination was applied at a dosage of 1 mg/L. The results confirmed that a properly operated conventional treatment plant provided a substantial barrier to microbial pathogens. Giardia cysts were detected in only 1 of the 32 filtered water samples, with a mean removal of 3.6 logs after filtration (including removal by coagulation and sedimentation). Removal of Cryptosporidium oocysts was lower than that for *Giardia*. Oocysts were detected in 7 of the 32 filtered water samples with a mean removal of two logs. *Clostridium perfringens* were detected in 9 of the 33 filtered water samples with a mean removal of 4.4 logs. No human enteric virus was detected in 32 filtered water samples with a mean removal of 3.1 logs (assuming that the concentration of human enteric virus in filtered water was equal to the detection limit). *Somatic coliphages* were detected in 24 of the 32 filtered water samples with a mean removal of 3.5 logs.

FILTRATION MODEL

In this section, a mechanistic performance model for removal of particles by granular filtration is used to predict the impact of process variables on removal of microbial pathogens. This model was first developed and applied in water filtration by O'Melia and coworkers (2,12). Substantial modifications have been made by Fitzpatrick and Spielman (13), Rajagopalan and Tien (14), and others. An extensive review of these theoretical models is available (15). The version by Rajagopalan and Tien is used in this report. Model equations used in these calculations are shown in Table 1.

In this model, particle removal by granular filters is considered to involve two steps: transport and attachment. Particles are transported from suspension to the close distance of a media grain. The transport step is

Table 1. Equations Used in Model Calculations for Figure 4

(1)	$\frac{C}{C_0} = \exp\left(-\frac{3}{2}\frac{(1-f)\alpha\eta_0 L}{d_c}\right)$	C: influent concentration; C_0: effluent concentration; f: porosity of the packed bed; α: attachment efficiency; η_0: overall single collector efficiency; L: bed depth; d_c: diameter of the filter media (collector)
(2)	$\eta_0 = \eta_D + \eta_I + \eta_G$	η_D: single collector efficiency due to diffusion; η_I: single collector efficiency due to interception; η_G: single collector efficiency due to gravity
(3)	$\eta_D = 4.0A_s^{1/3}\left(\frac{D}{Ud_c}\right)^{2/3}$	A_s: flow parameter to account for the effects of neighboring collectors (see equation (6)); D: diffusion coefficient of particles (see equation (8)); U: filtration rate
(4)	$\eta_I = A_s N_{LO}^{1/8} R^{15/8}$	N_{LO}: van der Waals number (see equation (9)); R: size ratio (see equation (11))
(5)	$\eta_G = 3.38 \times 10^{-3} A_s N_G^{1.2} R^{-0.4}$	N_G: gravitational force number (see equation (10))
(6)	$A_s = \frac{2(1-\rho^5)}{2-3\rho+3\rho^5-2\rho^6}$	k: Boltzmann constant; T: water temperature; d_p: diameter of particle; μ: water viscosity; A: Hamaker constant; ρ_p: mass density of particle; ρ: mass density of water; g: acceleration due to gravity
(7)	$\rho = (1-f)^{1/3}$	
(8)	$D = \frac{kT}{3\pi d_p \mu}$	
(9)	$N_{LO} = \frac{4A}{9\pi\mu d_p^2 U}$	
(10)	$N_G = \frac{(\rho_p - \rho) g d_p^2}{18\mu U}$	
(11)	$R = \frac{d_p}{d_c}$	

Note: Equation (1) is the master equation used to calculate the removal efficiency of a clean bed.

physical hydrodynamic in nature. Three transport mechanisms are important for water filtration: interception, sedimentation, and diffusion. Interception refers to the mechanism by which particles following the streamline of fluid flow come into contact with a media grain by their own sizes. Sedimentation refers to the mechanism by which particles with density greater than that of water deviate from the streamline of fluid flow by gravity and come into contact with a media grain. Diffusion refers to the mechanism by which particles subjected to random motion by their thermal energy come into contact with a media grain. A schematic representation of these transport mechanisms is illustrated in Figure 3. Single collector efficiencies have been well developed to describe these transport mechanisms.

Once particles come into contact with a media grain, removal is achieved only if the particles attach to the media. The attachment step is dominated by various physicochemical interactions between particles and media grain at a short distance. Because of these interactions, it is not necessary that all the contacts between particles and media lead to attachment. An attachment efficiency (α) is used to represent the fraction of successful contact. The value of α varies from 1.0 (all contact result in attachment) to 0.0 (no contact results in attachment). In drinking water treatment, α is promoted by chemical coagulation pretreatment, with a higher value of α provided by a better coagulation condition. A combination of the single collector efficiency, attachment efficiency, and the total number of media grains in a filter results in a predictive equation for removal efficiency.

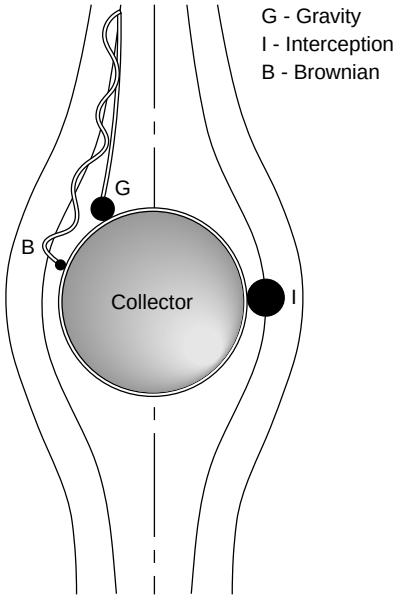

Figure 3. Particle transport mechanisms toward a single collector in water filtration. The thick line represents the particle trajectory. The thin line represents the fluid streamlines (after Yao and coworkers (2)).

The effects of process variables on removal efficiency of particles by granular filtration are presented in Figure 4 as functions of particle size. Process variables include coagulation condition, filtration rate, medium diameter, filter depth, and water temperature. Input parameters

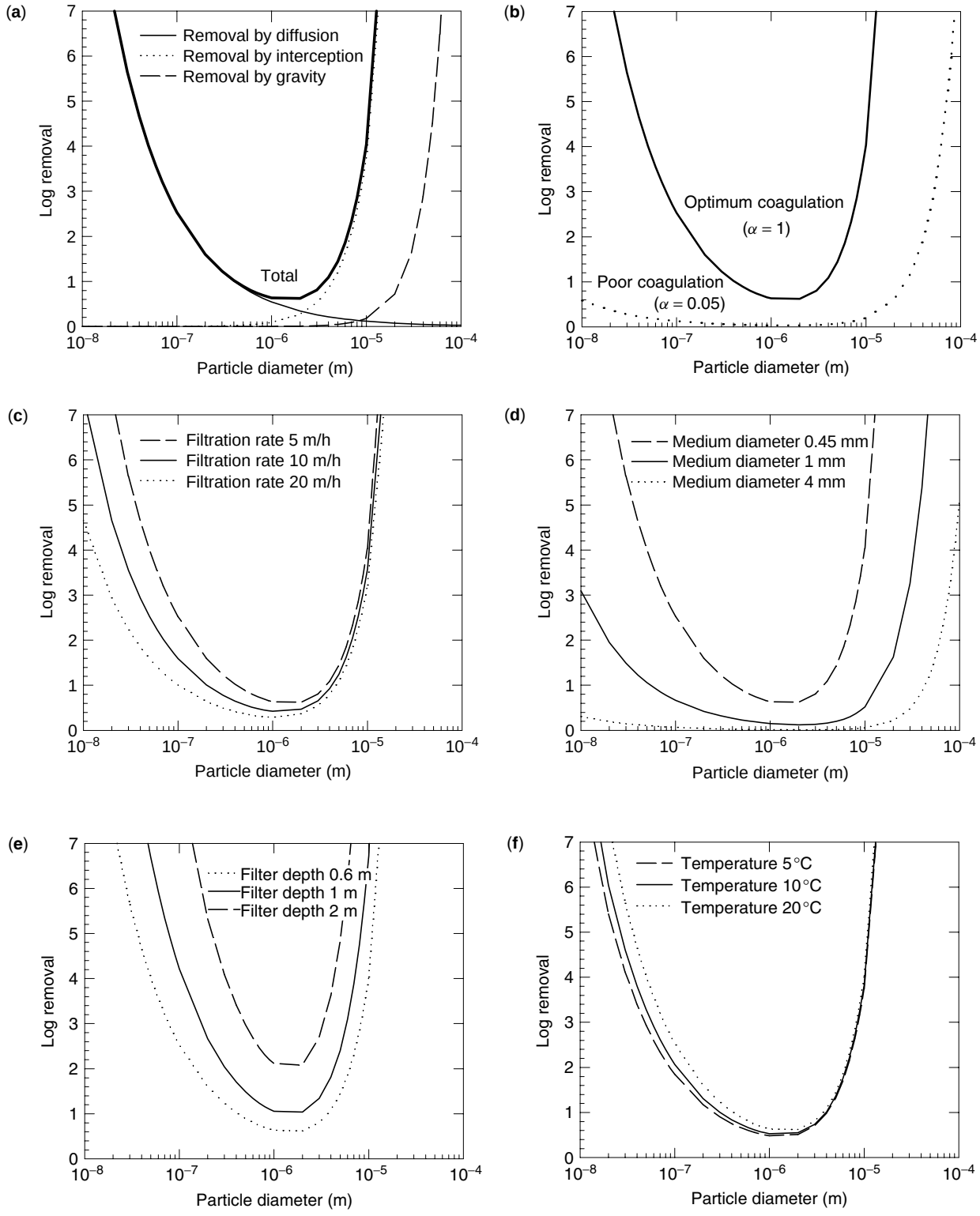

Figure 4. Effects of process variables on removal efficiency of granular filtration (simulation parameters are shown in Table 2).

used in these simulations are tabulated in Table 2. These theoretical results are interpreted to discuss the removal of microbial pathogens by granular filtration. However, limitations of the model should be realized. The model is developed for a clean bed and for a monodisperse suspension, meaning that temporal variation in filter performance is not considered. Furthermore, the model is developed for passive (nonmotile) particles. Some microbes, such as some species of coliform bacteria, are motile. Cell motility may result in different transport

Table 2. Parameters Used in Model Calculations for Figure 4

Process Variables	Values
Coagulation condition (α)	1.0[a]
Filtration rate	5 (m/h)[b]
Medium diameter	0.45 (mm)[c]
Filter depth	0.6 m[d]
Water temperature	20 °C[e]
Media configuration	Monomedia
Particle density	1.05 (g/cm^3)
Hamaker constant	10^{-20} •(Joule)
Filter porosity	0.4

Note: [a]1.0 and 0.05 in Figure 4b.
[b]5,10, and 20 in Figure 4c.
[c]0.45,1, and 4 mm in Figure 4d.
[d]0.6,1, and 2 m in Figure 4e.
[e]5,10, and 20 °C in Figure 4f.

mechanisms and removal efficiencies. The effects of cell motility on filter performance are not well known and are not considered by the model. This model has been tested experimentally for nonmicrobial particles, with great success. However, there is no systematic experimental testing of the model for microbes.

Model calculations indicate that removal mechanisms and efficiency are determined dramatically by particle diameter (Fig. 4a). Microbes in submicron sizes such as viruses are removed (transport) by Brownian diffusion. Removal efficiency of these small particles decreases with increasing particle size as smaller particles diffuse faster than larger particles. With an optimum coagulation condition ($\alpha = 1.0$) and the assumption that the sizes of microbes do not change before entering the filter, this filter can theoretically remove 6.38 logs of MS2 bacteriophage (2.5×10^{-8} m), 3.21 logs of rotavirus (7.0×10^{-8} m), and 2.53 logs of PRD1 bacteriophage (10^{-7} m).

Microbes with a diameter larger than about a few microns, such as protozoan cysts, algae, and some bacteria, are removed by interception (Fig. 4a). Removal efficiency of these larger particles increases with increasing microbial size because larger particles can be easily intercepted by the filter medium. When the filter is operated under an optimum coagulation pretreatment condition, the theoretical removal efficiencies are 1.44 logs for Cryptosporidium oocysts (5×10^{-6} m) and 4 logs for Giardia cysts (10×10^{-6} m). Higher removal efficiency of Giardia cysts when compared with that of Cryptosporidium oocysts has been observed by numerous experimental studies (9,10). Removal by gravity is never a dominant mechanism in these calculations, even for large microbes such as *Balantidium coli* cysts (6×10^{-5} m). This is because particle density (1.05 g/cm^3) is very close to the density of water. The insignificance of gravity effects is probably true for most microorganisms influent to filters, unless they are associated with dense particles.

A minimum in the theoretical removal efficiency is observed for microbes with size about 1 μm (Fig. 4a). Particles in this size range are too large for diffusion to be effective and too small for interception to be effective. For the same optimum coagulation condition, the model predicts that the filter only removes 0.64 logs of coliform bacteria (1.0×10^{-6} m). Although the sizes of some bacteria are in this minimum-removal size range, it should be emphasized again that the model does not consider cell motility.

The significance of chemical coagulation pretreatment on filtration performance is indicated obviously by the calculations (Fig. 4b). When coagulation conditions change from optimum ($\alpha = 1.0$) to poor ($\alpha = 0.05$), theoretical log removals deteriorate from 6.38 to 0.20 for MS2 bacteriophage, from 0.64 to 0.03 for coliform bacteria, from 1.44 to 0.07 for Cryptosporidium oocysts, and from 4 to 0.20 for Giardia cysts. These predicted effects of chemical coagulation on filtration performance are qualitatively consistent with many experimental results (6,8).

The effects of filtration rate on filter performance are either significant or slight, depending on particle size (Fig. 4c). For microbes with size greater than about a few microns and with a mass density close to water, increasing filtration rate from 5 m/h to 20 m/h only slightly decreases removal efficiency. A similar behavior has been observed experimentally by Al-Ani and coworkers (6). In these theoretical calculations, log removal of Giardia cysts reduces from 4.03 to 3.58 and to 3.22 when the filtration rate increases from 5 to 10 and to 20 m/h. This is because particle removal by interception is not strongly affected by filtration rate. The effects of filtration rate on removal efficiency are much more pronounced for submicron microbes. Theoretical log removal of rotavirus decreases from 3.21 to 1.27 when the filtration rate varies from 5 to 20 m/h. This is because particle removal by diffusion is a combination of convective diffusion process and is strongly affected by filtration rate.

Medium size (Fig. 4d) and filter depth (Fig. 4e) exhibit considerable effects on filtration performance for microbes of all sizes. Decreasing medium size and/or increasing filter depth increase removal efficiency. This is, in part, because the number of filter media grains increases with decreasing medium size and/or with increasing filter depth and thus favors the capture of particles. Decreasing medium size also enhances contact opportunity between particles and media grain by diffusion and interception. Temperature had some effects on the removal of submicron microbes and had almost no effect on the removal of microbes larger than 1 μm (Fig. 4f). When the temperature varies from 20 to 5 °C, theoretical removal efficiency of the MS2 bacteriophage decreases notably from 6.38 logs to 4.66 logs, whereas the removal of Cryptosporidium oocysts reduces from 1.44 log to only 1.31 logs. This is because particle removal by diffusion is a strong function of temperature. A higher temperature corresponds to a low water viscosity and to a higher diffusion. Particle removal by interception is, on the other hand, not affected by temperature.

CONCLUSION

A critical review of removal of microbial pathogens by high-rate granular filtration is provided in this document. Included are the operation principle, the

removal (transport) mechanisms and efficiency, and the process parameters affecting the performance. A clean-bed filtration model is used to elucidate the transport mechanisms of different microbial pathogens in granular filters and to predict the effects of process variables on treatment efficiency.

Under proper design and operation conditions, granular filtration is a consistent and effective barrier to pathogenic microbes. Providing proper chemical pretreatment to promote adherence of particles to the filter medium is the single most important factor in determining filtration performance. Proper engineering design establishes good physics, but it is difficult and expensive to remove microbes effectively if the particle surface is not properly adjusted by chemical pretreatment.

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RESIDENTIAL ENVIRONMENTS. See Bacterial Contaminants in Residential Environments

RETENTION OF MICROORGANISMS ON SOLID SUBSTRATA. See Activity and Carbon Transformations in Biofilms; Adhesion, Immobilization and Retention of Microorganisms on Solid Substrata

RHIZOSPHERE. See Plant–Microbe Interactions in the Marine Environment; Seagrasses Communities

RHIZOSPHERE, ARCHAEA IN. See Archaea in Soil Habitats

RHIZOSPHERE MICROBIOLOGY

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The rhizosphere is a concept that describes the physical location in soil where plants and soil microorganisms interact. From the perspective of soil microorganisms, the rhizosphere is an oasis in what is by comparison the barren desert of the bulk soil. The "rhizosphere effect" is the stimulation of microbial growth in the soil surrounding the root due to the release (exudation and secretion) of organic compounds from the root. As a result of these releases, intense microbial activity and larger microbial populations occur in this zone in comparison to the bulk soil, with microbial populations reaching up to 1×10^9 cells per cm^3, 10 to 100 times larger than the population in the bulk soil. Rhizosphere microorganisms include bacteria, fungi, flagellates, amoebae, mites, and viruses. An interacting trinity of the soil, plant and the soil organisms associated with the root system determines the rhizosphere environmental status (Fig. 1). Rhizosphere research can be separated out into three broad but distinct interrelated positions, (*1*) the influence of roots on microorganisms in the soil, and the influence of rhizosphere organisms on (*2*) plant growth, and (*3*) soil borne pathogens and plant disease (1).

THE RHIZOSPHERE; A DEFINITION

Lorenz Hiltner first defined the term *rhizosphere* in 1904 (3) as the volume of soil contiguous to and influenced by the plant root. Hiltner's initial use of the term was in reference to the zone of intense microbial activity around the roots of Leguminoseae, with an emphasis on the critical role of microbial activities in the rhizosphere, in the nutrition and general health of plants. He postulated that plant nutrition in general dependended on the composition of the soil flora in the rhizosphere. He also recognized that the attraction of beneficial bacteria by root exudates would be accompanied by the attraction of other less beneficial organisms that were also able to adapt to specific root secretions. This observation summarizes the focus of rhizosphere science research because of the influence of the rhizosphere to general plant nutrition, growth and development, and its role in plant pathogenesis.

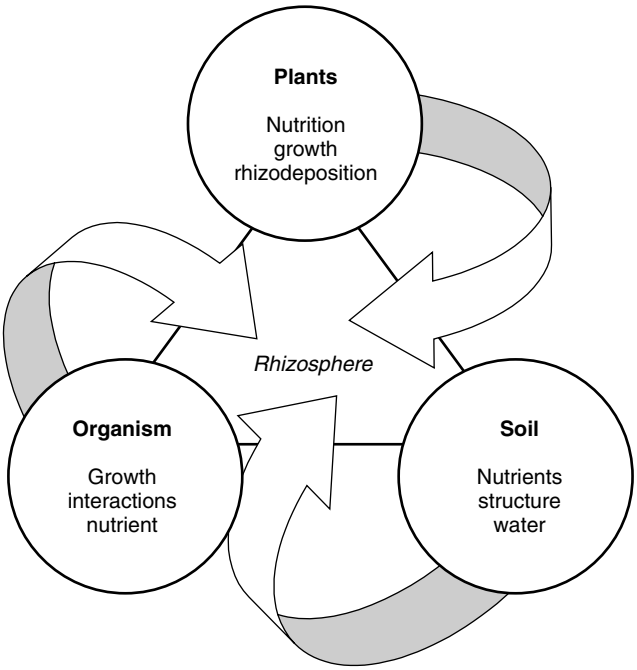

Figure 1. The rhizosphere trinity: the interacting factors that determine the environmental conditions of the rhizosphere. (After J. M. Lynch, in J. M Lynch, ed., *The Rhizosphere*, John Wiley & Sons, Chichester, U.K., 1990, 1–10.)

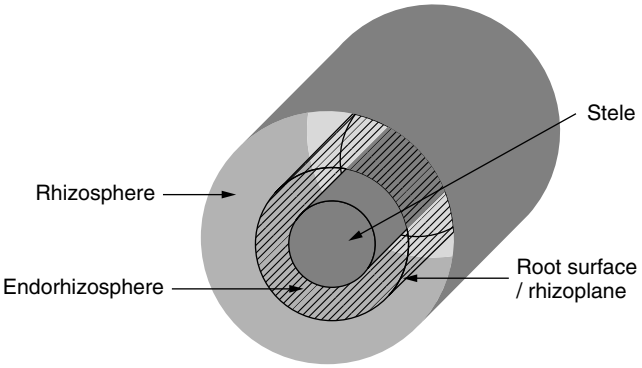

Figure 2. Schematic diagram of the rhizosphere (not to scale) showing the three spheres of microbial activity around the root.

The term "rhizosphere" is derived from the Greek word for root (*rhizo* or *rhiza*) and "sphere" meaning one's field of action, influence, or existence. While the rhizosphere is defined as the volume of soil contained within the sphere of influence around the plant root (Fig. 2), there is a lack of a precise physical delimitation. This is because the extent of the rhizosphere will by definition depend on factors such as the rate of exudation, and the mobility and consequent utilization impact that the released substrates have on the microbial population. This said it is generally acknowledged that the rhizosphere effect can be to a distance of 1 to 2 mm from the root surface, but can extend up to 5 mm. Subsequent research divided the rhizosphere into more defined spatial regions such as the outer rhizosphere, the inner rhizosphere (*endorhizosphere*), and the root surface (*rhizoplane*). Further definitions have been coined for more specialized regions such as the *mycorrhizosphere* where mycorrhizal fungi are in association with the roots and *actinorhizosphere* or *actinorhiza* when actinomycetes are associated with root nodules. Although these regions are theoretically defined, it is in practise difficult, and probably inappropriate, to study them apart.

The Rhizosphere Effect

The rhizosphere effect occurs because in most mineral soils, the activity and growth of the microbial biomass is limited by the availability of substrate, especially carbon. As such the entry of substrate into the soil around the root system, is a key factor governing the size and activity of the microbial biomass (4). Yet, while the extent to which the quantity and composition of exudates affect microbial community structure are largely unknown (4), it is suggested that the rhizosphere effect can be beneficial, harmful, or neutral (2) (Fig. 3).

An extensive variety of organic compounds originating from the plant have been found in the rhizosphere and are known as rhizodeposition products. These compounds have been classified under standard terms to help readily identify their organic nature and source. Rovira and colleagues (5) classified the compounds as follows:

Exudates. Chemically diverse low molecular weight compounds (i.e., sugars, organic & amino acids as well as more complex compounds such as vitamins and plant hormones) that leak from intact cells and into the soil, either directly through epidermal cell walls or into intercellular spaces and then into soil via cell junctions.

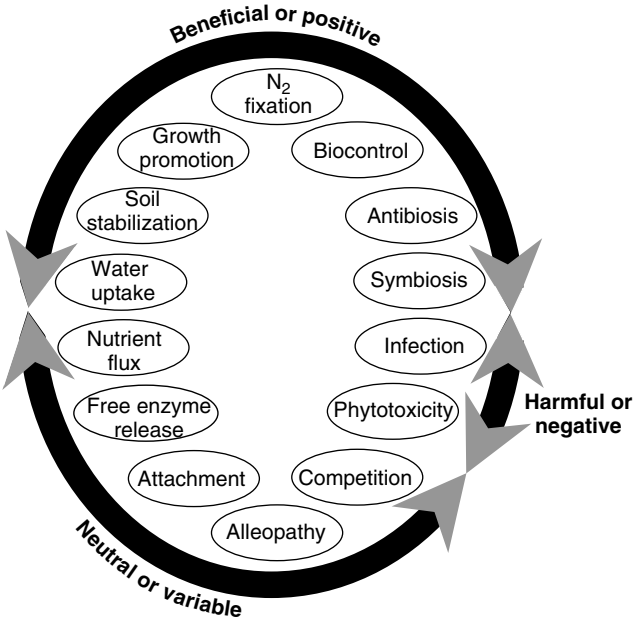

Figure 3. Process values of rhizosphere organisms. (After J. M. Lynch, in J. M Lynch, ed., *The Rhizosphere*, John Wiley & Sons, Chichester, U.K., 1990, 1–10.)

Secretions. Compounds that root cells actively release to the rhizosphere

Plant mucilage. From four sources from various parts of the root including

- Golgi bodies secretions predominately by the root cap cells
- Hydrolysates of the primary cell wall located between the root cap and the epidermis
- Secretions from epidermal cells and root hairs with primary walls, often augmented by the physical rupturing of the cells by soil minerals
- Compounds released as a result of dead epidermal cells undergoing microbial modification and degradation

Mucigel. A layer of gelatinous material on the root surface comprising natural and modified plant mucilages, bacterial cells and their metabolic products such as capsules and slimes, and also colloidal soil minerals and organic matter

Lysates. Material released through the lysis of older epidermal cells

Given the difficulties in distinguishing experimentally the source of these organic substances, often the concept of categorizing them in terms of their subsequent utilization as microbial substrate is used. For example, they may be classified as low molecular weight compounds that can be readily assimilated by the microbial biomass, or more complex compounds such as the polysaccharides, polypeptides nucleic acids, pigments and so on, which require extracellular enzymic activity before assimilation. Also, there are structural materials such as cell wall components that require saprophytic degradation activities before the carbon can be utilized by the general soil biomass. This latter approach to categorizing exudates has been suggested to be more appropriate to the study of rhizosphere microbial population dynamics (6).

Rhizosphere products are not liberated evenly over the root surface, their presence in the rhizosphere is dependent on the developmental stages of the root. The changes in root development gives rise to a longitudinal gradient of exudation that may reflect the gradient in microbial catabolism of root exudates and differences in root cell activity. The temporal and physical locations of their release are elaborated on in Figure 4. The heterogeneous nature of this release of organic material has implications for the bacterial populations that benefit from it, causing both numbers and types of organisms to fluctuate as the root grows. Work by Griffiths and coworkers (4) has shown that when compounds characteristic of root exudates are added to a soil at a range of concentrations, a consistent trend can be seen within the soil community structure with access to these exudates. Essentially, as the substrate quantity increased, both bacterial biomass and fungal biomass increased, with gram-negative bacteria responding differently from gram-positive bacteria, and fungi dominating over bacteria at high rates of release. Further examination of commonly accepted biomarkers indicated that the proportion of gram-positive bacteria remained more constant at lower substrate loading and declined as loadings increased, while gram-negative bacteria, increased with increasing substrate loadings only declining at the very highest inputs.

As the root cap zone develops there is a massive excretion of a carbohydrate rich gel that serves to lubricate the root tips development through the soil, and causing the outer cap cells to break off and become sloughed into the soil. From their development from root cap meristematic cells, they differentiate progressively through specialized stages before eventually becoming detached from the root cap (7). Consequently by the time they become external to the root, they have served several functions such as secretion and the sensing of gravity and other environmental signals. These sloughed root cap cells contain starch, and remain viable in the soil for up to three weeks, during which time they continue to secrete mucilage (8) and possibly perform other functions.

More recent work on root cap cells has allocated a further and more defensive function to these sloughed cells and renamed them 'root border cells' in recognition of their bacterial border role. The previously accepted wisdom was the constant shedding of these cells was to prevent physical damage to the root cap per se. However, Hawes and coworkers argue that there is no experimental data to support the role of these cells as lubricants (9,10). Further, it is suggested that border cell production is turned off and on at the plant's will, without hampering the roots ability to penetrate solid media (9,11). Hawes and colleagues postulated that border cells serve the plant by enabling the root to define its own ecology, with their best-characterized properties involving specific recognition and responsiveness to soilborne microflora. They can in a genotype-dependent manner attract zoospores (12), synthesize defensive structures in response to fungal attack (13), repel or bind pathogenic bacteria, and control growth and gene expression in symbiotic bacteria. Such chemotactic attraction and recognition might create 'biased rhizospheres' by dictating which populations can colonize the plant root system, with direct and indirect influences for virtually all of the consequent physical and chemical parameters (7). Border cells have also been implicated in acting as decoys to attract both nematodes and fungi away from the advancing root tip, until the zone of elongation has passed thus reducing their chances of infecting the root system (7).

Behind the cell cap in the zone of elongation the epidermal cells emerge and begin to elongate. At first these cells have only the primary wall, although once elongated they develop a secondary wall. Root hairs develop in the region behind this and they function for a relatively short period before autolysis, with an accompanying proliferation of bacterial cells that utilize the products. These microorganisms also invade the epidermis and cortex, and while at stage no physiological damage occurs, the outer cortex ceases to function. This final stage in rhizosphere development represents the state of many crop species just before harvest with the root material left in the soil becoming part of the soil organic matter on harvest. A more detailed account of the stages in the life

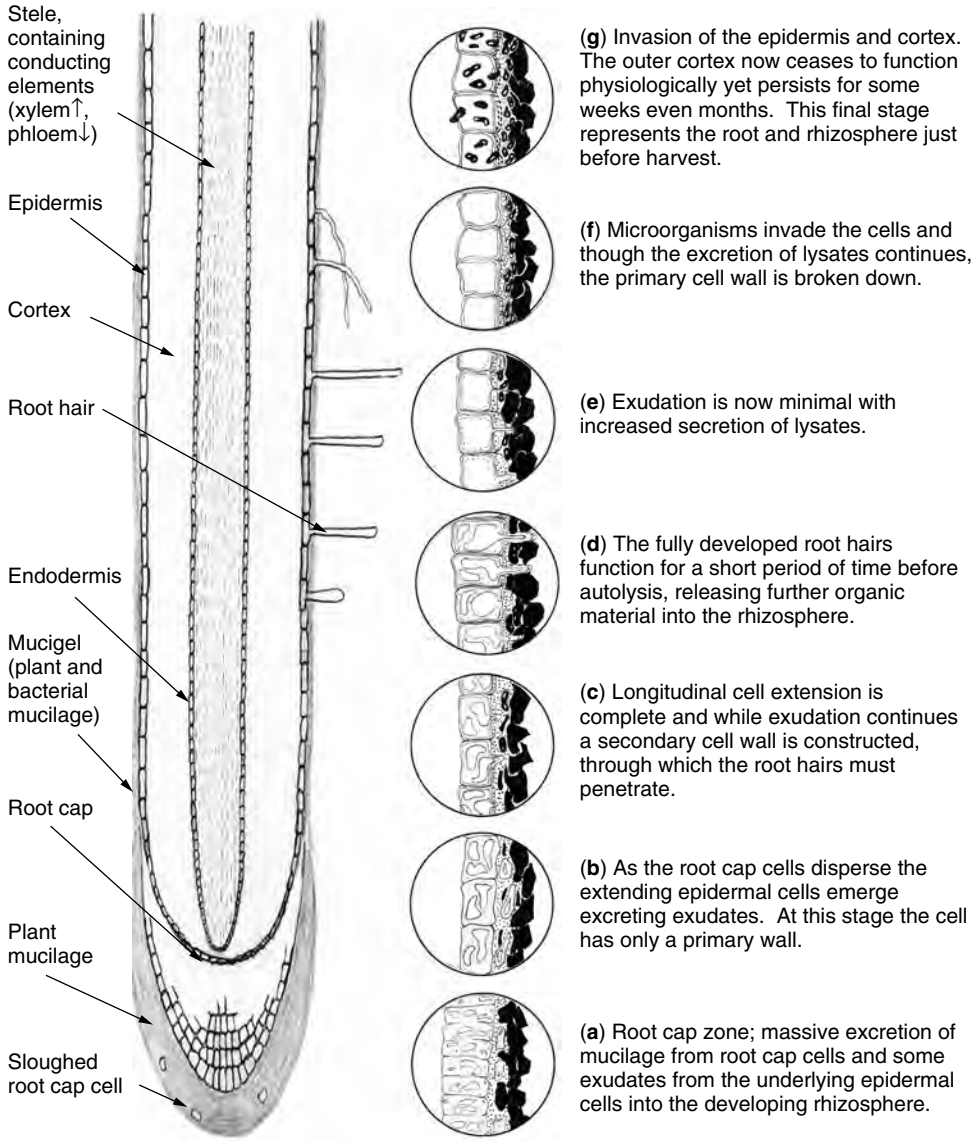

Figure 4. Temporal and physical locations of release of root exudates (Drawing not to scale) (Adapted from A. D. Rovira, R. C. Foster, and J. K. Martin, in J. L Harley and R. S Russell, eds., *The Soil Root Interface*, Academic Press, New York, 1979, 1–4.)

cycle of a root surface cell is given by Foster, Rovira, and Cook (8).

The Nature of Rhizosphere Exudates and Secretions

Root exudates include carbohydrates, amino acids and other organic acids, vitamins, nucleic acid derivatives, and various miscellaneous compounds. Among the carbohydrates, at least ten sugars have been identified as being exuded from a wide range of plants species, with hexoses glucose and frucose as being the most abundant (14). Others include the pentoses; xylose, ribose, and arabinose and the disaccharides sucrose and maltose. This wide range of sugars found in exudates suggests that the exudation of sugars is a general phenomenon with little if any effect in determining the composition of the microbial population it attracts to the rhizosphere (14).

In contrast to the exudation of carbohydrates, plants differ widely in the range and amounts of amino acids that are exuded. A total of 22 amino acids have been found in pea root exudates compared with up to fourteen in oat-root exudates. The most abundant amino acids produced by pea (10- and 21-day old) are homoserine, threonine, and glutamine and by oats, lysine, serine, and glycine. In addition to this, the total amount exuded by peas far exceeds that produced by oats. Such qualitative and quantitative differences between the composition of exudates from different plant species when grown under the same conditions are likely to have far reaching effects in the composition and density of the microbial population

that inhabit the rhizosphere (14). Other organic acids including members of the tricarboxylic acid cycle occur in the exudates of several plant species. These acids are suggested to not only provide the rhizosphere microflora with substrate, but to also have important secondary effects in altering the rhizosphere environment, such as changing the pH and chelating metal ions.

The chelation of metal ions is considered an important function of root exudates, and the organic ligands secreted, termed *phytometallophores* with a subclass called *phytosiderophores* specializing in iron chelation, particularly so. These compounds serve to enhance the uptake by the plant of essential metals. Research into the production of *phytometallophores* has shown that the interaction between the plant and the root environment can change the composition of the exudate. Fan and coworkers showed that as iron deficiency increased, the total quantity of exudate per gram of root remained unchanged, but the relative quantity of carbon allocated to phytosiderophores increased to approximately 50% of the total exudate (15).

Of the vitamins, the most common vitamins found in the exudates of roots are biotin and thiamine, although the levels recorded are not deemed as sufficient to meet the requirements of all vitamin-requiring microbes within the rhizosphere. This said the B-group vitamins, are produced at what is considered biologically active levels by most bacteria and fungi isolated from soil or plant root systems (16,17). Their production by rhizosphere organisms has been shown to be related to the potential of organisms such as *Pseudomonas* and *Azotobacter* to enhance nitrogen-fixation and the growth of legumes nodulated by *Rhizobium* (18). It has also been shown that the application of the herbicide simazine considerably affects the production of this vitamin group by *Azotobacter* (19). The nucleic acids adenine, guanine, uridine, and cytidine are also present in root exudates as well as exoenzymes such as phosphatase and invertase. Recent work shows that inoculation of pea rhizosphere with *P. fluorescens* F113 significantly increases alkaline phosphatase, sulfatase, and urease activities (20).

Among the great variety of other compounds found in root exudates, are compounds such as phenol derivatives and other substances toxic to microorganisms, emphasizing the important point that not all plant exudates are necessarily beneficial to microorganisms. The rhizosphere is colonized by plant growth promoting rhizobacteria (PGRB) and deleterious rhizobacteria (DRB), which may serve to inhibit plant growth. The detrimental action of these bacteria is primarily through the production of phytotoxins (21), but may also be through phytohormone production, competition for nutrients and the inhibition of mycorrhizal function. Furthermore, it is suggested that the function of rhizobacteria may alternate from that of plant growth promotion to inhibition, depending on their interaction with the plant, mycorrhizal fungi, and the changing environmental condition of the soil. As such, the influence, that rhizobacteria have on plant growth should be assessed on the net consequences after both positive and negative effects have been considered (21). There is also evidence that DRB are often associated with yield declines in continuous monocultures (22,23), and in growth depression in minimum tillage systems (23).

The composition of the exudates will be subjected to change as the plant itself experiences changes, whether this is due to the changes that occur throughout the plants life cycle or from physical changes in it's environment such as drought. For example, recent work (24) utilized HPLC to identify and quantify exudates from tomato plants, and has shown that the total amount of sugar carbon considerably increases during development from seedling to plant root. While sugars were being exuded during all stages of development, the composition of the sugars changed. The sugars glucose, maltose, and xylose were the major sugars in seed exudate, with fructose and glucose in seedling exudate, and glucose and xylose in root.

In terms of external influences, it is known that factors that alter the rate of production and translocation of photosynthate have an indirect effect on the release of organic substances from the root (14). For example, defoliation of sugar maples causes changes in the amount of sugar, amino compounds, and organic acids exuded (25) and water stress to pine seedlings results in diminished exudation (26). In contrast to this, an increase in the amount of mucilage exuded from roots under increasing soil water stress has been shown in wheat plants (27). Additionally, if field soils are allowed to approach permanent wilting point and subsequently rewetted, a rapid release of amino acids from roots has been shown as the plants regain turgor (28).

Rhizosphere Bacterial Population

The bacterial population of the rhizosphere differs from that in the bulk soil both in terms of population numbers and composition. It contains large numbers of gram-negative non-spore-formers with a higher proportion of chromogenic and motile forms, and more ammonifiers, nitrifiers, denitrifiers and aerobic cellulose decomposers. However, it sustains a lower number of gram-positive cocci, and organisms capable of utilizing aromatic acids than found in the bulk soil (29). Short gram-negative rods respond most to rhizosphere conditions and invariably constitute a greater percentage of the rhizosphere than bulk soil communities. The three genera *Pseudomonas*, *Achromobacter*, and *Agrobacterium* are examples of gram-negative rods that are greatly stimulated within the rhizosphere (14).

Given that the rhizosphere exudations and secretions serve to enhance and maintain a higher microbial population than that found in the bulk soil it must also be assumed that there is a high level of microbial competition within the rhizosphere. This in turn enhances selective pressure for those microorganisms most suited to the rhizosphere environment. Most favored are rapidly growing and biochemically versatile organisms over slower growing less diverse organisms. Such selection was shown by Wallace and Lochhead (30), who utilized an empirical scheme to recognize seven nutritional groups of bacteria as determined by their ability to grow on media of varying complexity. The media contents ranged from only glucose and mineral salts to preformed amino acids and vitamins or unidentified growth factors contained in soil and yeast

Table 1. Incidence of Bacterial Nutritional Groups, Comparison of Oat Rhizosphere and Bulk Soil

Requirements for Maximum Growth	Rhizosphere/Bulk Soil (R : S) Ratio	
	% of Whole Population $\times 10^{-6}$	Total Count Soil g^{-1} $\times 10^{-6}$
Sugar and inorganic salts only	2.5	21.6
Amino acids	5.5	46.0
B vitamins	1.2	10.5
Amino acids & B vitamins	0.5	4.2
Yeast extract	0.7	6.0
Soil extract	0.8	6.7
Yeast and soil extract	0.3	2.5

Increasing complexity of medai

Data from R. H. Wallace and A. G. Lochhead, *Soil Sci.* **67**, 63–69 (1949). Adapted from B. N. Richards, *The Microbiology of Terre strial Ecosystems*, Longman Scientific and Technical, Harlow, U.K., 1987.

extracts. Organisms requiring no special growth factors or requiring amino acids only were enhanced in the rhizosphere (Table 1). Selection of amino acid utilizing bacteria reflects the larger amount of amino acids in the rhizosphere than bulk soil. In general terms, bacterial populations within the rhizosphere are considered less fastidious than those found outside. More recent work by Fan and coworkers (15) has demonstrated that barley roots grown in iron-limited nutrient solution produce more amino acids, suggesting that amino acids play some role in Fe acquisition.

The R:S ratios indicate consistent, preferential enhancement within the rhizosphere of bacterial groups requiring either no special growth factors or amino acids only.

In contrast to the quantitative effect that the rhizosphere has been demonstrated to have on bacteria, the rhizosphere has little effect on fungal numbers but does enhance certain genera of fungi. For example, imperfect fungi *Fusarium* spp. are prominent members of the rhizosphere and a common example of this phenomenon, but numerous other genera such as *Trichoderma* and *Aspergillus* are represented. There are also ecological links between fungal and bacterial members of the rhizosphere community. For example, different microbial inocula can affect the colonization of pea roots by mycorrhizae (32). The mycorrhizal component of the soil and its mutualistic symbiosis with the plant root system was first critically documented by Harley (33), but there have been many subsequent reviews (34–37).

Root Colonization. Organisms associated with seeds, such as the common fungi (Penicillium spp., Aspergillus spp.) and bacteria, contribute very little to the established rhizosphere (1). The young seedling is first colonized by chance by bacteria that first encounter the influence of the developing root. A more species rich community characteristic of the rhizosphere establishes as the root matures. The developing root tip and root hairs are commonly free of microorganisms, with bacteria colonizing at sites of lateral root emergence from the main root stem. The rhizosphere effect then increases with the age of the plant, reaching a peak at the height of vegetation and declining as senescence approaches. This succession varies among plant species with the timing at alternative periods during their life cycles, with crop species generally exhibiting a greater rhizosphere effect than trees. The most pronounced population increases involve the amino acid requiring bacteria and other organisms with simple nutritional requirements and rapid growth rates (1). The dominance of specific bacteria with certain plants is also a phenomenon that has been noted.

As previously noted, the nature of the secretions changes through the root life cycle, and consequently the root associated microbial populations undergoes changes. Most root colonization is triggered as the root tip advances through the soil, with the root tip releasing substrate that stimulates the growth and activity of the dormant bacteria within the sphere of its influence. The process of root colonization can be conceptually divided into a series of steps: migration toward plant roots, attachment, distribution along the root, and growth and establishment of the microbial population (38). A number of microbial traits involved in these steps have been postulated, including chemotaxis, agglutination potential, the use of flagella, and the ability to utilize complex carbohydrates. Which of these characteristics is most important in root colonization was determined by Hozore and Alexander (39) working with bacteria isolated from both bulk soil and the rhizosphere of soybean. They found that the rhizosphere and soil isolates differed significantly in their ability to move along soybean roots, with rhizosphere isolates better able to colonize root segments further from the point of inoculation than the soil isolates. They suggested that there were no significant differences in adhesion to root tips, or in chemotactic response to exudates, tolerance of low osmotic potentials or the agglutination of cells by exudates. While concluding that mobility along the root

is important to successful colonization of the rhizosphere, none of the other characteristics is individually sufficient to ensure rhizosphere competence.

In a study of the colonization of barley roots grown in sterile soil, Hansen and coworkers (40) used two *P. fluorescens* strains (DF57 and Ag1) and a confocal laser scanning microscope with the ability to detect single bacteria of both strains on the roots. They demonstrated that seed-inoculated bacteria rapidly colonized the root surface (rhizoplane) by active migration. After one day, the anterior part of the root was densely covered by the inoculated bacteria, occupying the crevices between epidermal root cells. As the roots elongated, the rhizoplane bacterial population formed long strings of closely associated cells. After seven days, however, these string-forming cells became partially detached, developing a patchy distribution along the root.

THE BENEFITS OF THE RHIZOSPHERE

Nutrient Cycling

The rhizosphere is an environment created and maintained by the plant at cost to the plant, but the plant receives considerable benefits from this energy input. The acquisition of nutrients is the preliminary goal of the investment that the plants make in modifying their environment modifications. The interactions with the rhizosphere microbial populations enhances the availability and uptake of nutrients that might otherwise have been unavailable to the plant root system. This happens in a number of ways and for a number of nutrients, but probably the most important nutrient is nitrogen, the discussion of which can be generalized to other nutrients.

Plant roots and the microbial populations that inhabit them are integral to the nitrogen cycle (Fig. 5). Although the microbial population of the bulk soil is involved in the conversion of nitrogen through its various forms in the nitrogen cycle, it is the enhanced populations within the rhizosphere that provide the majority of the readily available forms of nitrogen to the host plant. Nitrogen, that is subsequently released when the plant dies or is consumed.

Nutrient cycling in soil is extremely dependent on the supply of energy to the soil biota. Given that the source of this energy is carbon from organic compounds, it can be seen that the nitrogen and carbon cycles are inextricably linked with carbon as the impetus. This carbon and nitrogen link is often referred to as the carbon nitrogen ratio.

Mineralization and Immobilization. The decomposition of plant material, with the accompanying mineralization and immobilization of the organic nitrogen it contains, are key processes in the soil nitrogen cycle. Once incorporated into the soil matrix, plant residues bring about a rapid increase in microbial biomass as the material is colonized. The progressive decomposition of nitrogen-containing compounds by heterotrophic microorganisms using organic material as a source of combined carbon for both respiration and cell synthesis results in ammonium for use by the organism itself, with the excess released as waste. If insufficient nitrogen is contained within the substrate then the organisms will utilize soil mineral nitrogen, resulting in nitrogen immobilization. The balance between the release of nitrogen and immobilization depends on the carbon-to-nitrogen ratio

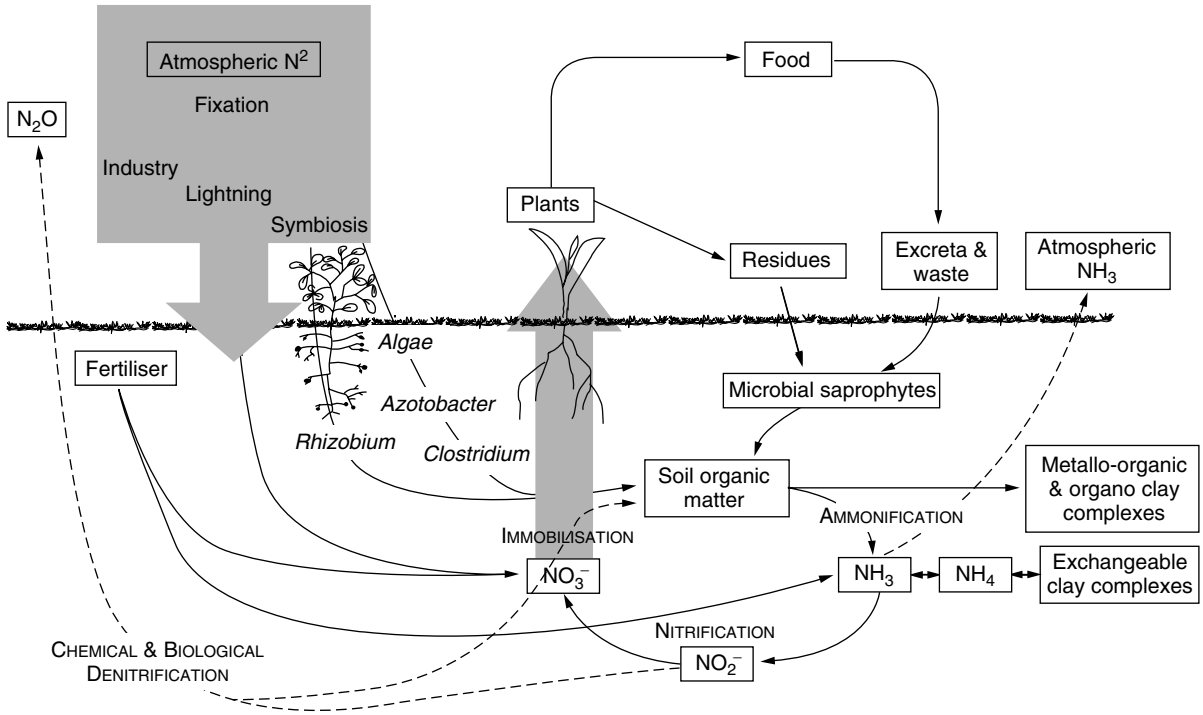

Figure 5. Ecology of the nitrogen cycle. (After J. M. Lynch, *Soil Biotechnology, Microbiological Factors in Crop Productivity*, Blackwell Scientific Publications, London, U.K., 1983.)

of the material being decomposed, the energy efficiency of the organism and the carbon-to-nitrogen ratio of the cells being synthesized. The carbon-to-nitrogen ratio of the substrate is the most influential of these factors, with ratios varying from 5:1 for animal wastes to 100:1 for cereal straws. The ratio at which nitrogen undergoes neither net mineralization nor immobilization is approximately 35:1. Thus this microbial-mediated process is important, because it supplies nitrogen to plants in a preferred form, which is less susceptible to leaching, and provides the raw material for nitrification, the next step in the nitrogen cycle.

Given that ammonifying bacteria have a high R:S ratio (in excess of 50:1) (31) one would expect a greater mineralization of organic matter within the rhizosphere than bulk soil. In the field situation, however, less mineralization occurs under crops than in a fallow soil, even when correcting for the nitrogen taken up by the plants. This anomaly is explained by ^{15}N studies (42) that show that although the net mineralization rate in cropped soil is only half of that in fallow soil, the total quantity mineralized is greater under the crop. The difference is due to rapid assimilation of the mineralized matter by the microbiota within the rhizosphere of the crop.

The aforementioned illustrates that the availability of nitrogen to the plant is a result of the opposing processes of mineralization and immobilization and the plant's ability to compete for available nitrogen. This suggests that the overall microbial effect in the rhizosphere is detrimental as often as it is beneficial, with nitrifying bacteria being suppressed by root exudation (43). However, experimental comparisons of rhizosphere and nonrhizosphere soils has demonstrated that the rhizosphere effect on net N mineralization from indigenous soil organic matter is positive in many cases but not in all (44). The actual amount of microbial nitrogen immobilized by root-derived carbon seems to be counterbalanced by stimulation of nitrogen mineralization by promoted grazing of protozoa, the consequential release of ammonium by assimilation, as well as by higher microbial turnover of soil organic matter. It is hypothesized that the effect can also be beneficial, as a consequence of the nitrogen form involved (nitrate), and because the continual cycling of the nitrogen compounds within the microbial community prevents their loss from the system through leaching, thus providing a more constant supply to the plant (14).

Dinitrogen Fixation. Biological dinitrogen fixation is the enzymatic reduction of dinitrogen (N_2) from the atmosphere to ammonia. Nitrogen fixing bacteria have the capacity to produce the enzyme system nitrogenase, an enzyme system that confers the ability to fix N_2. The nitrogenase system consists of two proteins, the iron (Fe-) protein and the molybdenum-iron (MoFe-) protein that together catalyze the ATP-dependent reduction of atmospheric N_2.

The biological fixation of molecular nitrogen is carried out by several "free-living" bacterial genera, some of which are rhizosphere-associated, and by several other genera that are mutualistically associated with plants. In terrestrial systems, however, the largest contribution of combined nitrogen comes from the symbiotic fixation of dinitrogen by rhizobia, with fixation rates often two to three orders of magnitude higher than those exhibited by free-living bulk soil dinitrogen fixers (See Nitrogen Fixation in Soils (Free-Living Microorganisms), Nitrogen Fixation in Soils — Free-Living Microbes this Encyclopedia.). Estimates of the amount of dinitrogen fixed biologically range from 139 to 170 million tonnes N_2 annually, (45,46) with the symbiotic associations of arable land accounting for 25 to 30% and permanent pasture for another 30%. Such increases in plant-available nitrogen are a consistent consequence of growing both crop and pasture legumes, resulting from the combination of conserved soil nitrogen, greater mineralization potential and the return of the fixed nitrogen (47). Consequently the benefits of crop legumes can be considerable.

The best studied examples of symbiotic nitrogen fixation are the soil bacteria of the genera *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium*, collectively referred to as rhizobia. These organisms have the ability to induce the formation of nodules on the roots of leguminous plants. In these nodules the host plant provides the bacteria with their nutrient requirements and an environment conducive to nitrogen fixation. In return the bacteria fix dinitrogen in excess of their requirements, which is subsequently released as ammonia and taken up by the plant. (See Nitrogen Fixation in Soils (Symbiotic), this Encyclopedia.)

The nodule development begins with the attachment of rhizobia to the host root hairs causing deformation of the hair, resulting in its curling (Fig. 6). Then infection threads form within the curled hair and allow the bacteria passage into the root. Accompanying the infection process is the inducement of cell divisions in the root cortex leading to primordium formation. The infection threads are then attracted to centers of increased mitotic activity of the primordium cells and release bacteria into these cells by endocytotic processes. The now infected cells differentiate and mature into nodules thus providing the environment for dinitrogen fixation (48). Nodulation is, however, host specific, with each rhizobia species restricted to a subset of plant species.

While the rates of nitrogen fixation for free-living bacteria are relatively low in comparison to those in a symbiotic or mutualistic relationship, the bacteria that conduct fixation under such conditions are widespread in soils and do contribute to soil nitrogen inputs. The rate of free-living bacteria such as *Azotobacter* and *Azospirillum* are increased under rhizosphere conditions, because of their increased efficiency as a result of more readily available organic compounds exuded from roots (49). Consequently, it can be seen that the symbiotic fixing of dinitrogen in the rhizosphere causes a substantial benefit to the plant system's ability to acquire and utilize nitrogen. The large biological significance of nitrogen fixation and the importance of the process for agriculture continue to prompt major fundamental research in this area. Research on the mechanisms of fixation and the regulation of its activity, is motivated by the aim of developing more effective systems and to optimize the use of nitrogen fixation in agriculture.

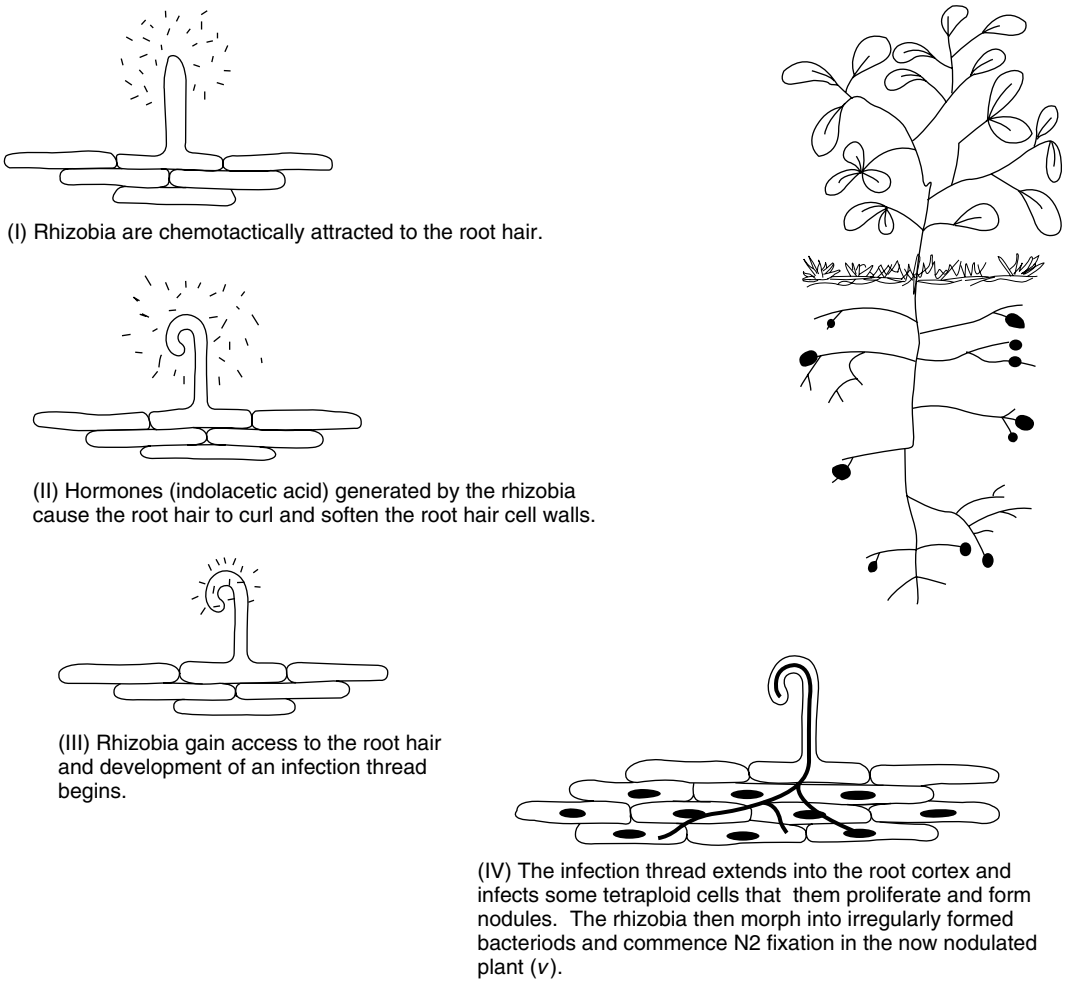

Figure 6. Infection process of a leguminous plant root system.

Global Warming. There is intensifying interest in the effect of rising global temperatures, due to global warming, on mineral cycling. The concern, involving the linked cycling of carbon and nitrogen, is that the predicted increases in the ambient temperature will lead to decreases in the organic matter content of soils (50). Furthermore, as the products of organic matter decomposition, such as carbon dioxide, are implicated in generating global warming, the process will induce a positive feedback effect on global warming. Increased levels of carbon dioxide and temperature are predicted to affect both the activity and the community structure of the rhizosphere microbial community. It is generally accepted that plants grown in atmospherically elevated carbon dioxide levels substantially increase their carbon input into the rhizosphere. And although it is known that elevated carbon dioxide enhances rhizosphere respiration more than root biomass, the fate and functions of this extra carbon input to the rhizosphere are not clear (51). Cheng and Johnson (52), using carbon isotope techniques, found that elevated atmospheric carbon dioxide in a wheat-soil system significantly increased substrate input to the rhizosphere due to both increased root growth and increased root activities per unit of roots. They also found that nitrogen treatments changed the effect of elevated carbon dioxide on soil organic matter decomposition, thus indicating that soil nitrogen status was important in determining the effect of elevated carbon dioxide on soil organic matter decomposition (52). Similar results were found by Elhottova (53), who also noted that there was an accompanying change in the rhizosphere bacterial community toward nutritional groups utilizing more complex organic material.

Nutrient uptake by plants may also be increased because of modifications to root architecture, physiology, and or mycorrhizal symbiosis (54). The relationship between enhanced nitrogen uptake and biomass production under elevated carbon dioxide is highly significant (55). Such a relationship indicates that plants have the ability to enhance their nitrogen uptake through modifications in their physiology, morphology, architecture, or mycorrhizal associations. Such changes could be a determining influence in how individual species respond to increased carbon dioxide levels, especially with regard to increased biomass production.

Carbon dioxide concentration may also influence exudation by roots (54). In microcosms with yellow birch (*Betula alleghaniensis* Britt.), Berntson and Bazzaz (56) showed

that elevated atmospheric carbon dioxide significantly altered the cycling of nitrogen plant carbon-to-nitrogen ratios were significantly increased, gross mineralization and NH_4^+ consumption rates were decreased, and relative microbial uptake of NH_4^+ was increased. Such changes represent a suite of nitrogen cycling negative feedback on nitrogen availability due to increased atmospheric carbon dioxide levels. Enzyme activities in the rhizosphere of wheat have also shown changes under elevated carbon dioxide levels, with 3- to 24-fold increases in potential denitrification activity in roots grown under elevated carbon dioxide compared with ambient levels. This indicates that atmospheric carbon dioxide concentration can influence rhizosphere denitrifier activity (57). Elevated atmospheric carbon dioxide may also increase infection by both ectomycorrhizal and arbuscular mycorrhizal fungi (58).

The primary negative feedback of increased carbon dioxide levels generally reduce the amount of nutrients available to plants. Such negative feedback include increased C/N ratios in litter, and subsequently reduced mineralization rates, increased immobilization of available nutrients by a larger soil microbial pool, and increased storage of nutrients in plant biomass and detritus due to increases in net primary productivity. The principal positive feedback, however, are mostly plant-mediated, with the exception of the hypothesis that increased microbial biomass will have a concurrent increase in mineralization rates. For a review of impacts of increased carbon dioxide levels on microbial communities, see Berntson (55).

These rhizosphere-based changes have enormous implications for the wider environment. For example, it has been demonstrated that the single largest and most dominant species within a plant community was reduced in biomass and nitrogen content by elevated carbon dioxide levels, whereas several smaller less dominant species showed enhancement (55). Such evidence suggests that in time, elevated atmospheric carbon dioxide levels may have significant consequence on community composition, leading to ensuing changes in ecosystem functionality. For example, if elevated atmospheric carbon dioxide levels cause reductions in nitrogen cycling because of the accumulation of nitrogen in plant biomass or reductions in mineralization rates, long-term forest productivity may be constrained (59).

Crop Production and Disease Resistance

The acquisition of nitrogen and other nutrients is not the only way in which the rhizosphere benefits the plant. Plant productivity can also be enhanced by other direct and indirect effects stimulated within the rhizosphere. The most notable of these effects is termed biocontrol. The subject matter of biocontrol is founded on the principles of microbial competition, amensalism, parasitism, and predation, or a combination of two or more of these interactions. Although these interactions are clearly important in rhizosphere ecology, they are among the least well understood of rhizosphere processes (60). This is partly because microbial community dynamics in soils seldom mirror simple positive or negative interactions. Consequently, conditions at any point reflects the aggregate total of numerous interactions set in motion by a supply of an exploitable nutrient source and the absence of environmental extremes. In this case, the environment is provided by the plant root system.

Early studies on the potential of siderophores to mediate bacterial control of root disease led to the use of the term "plant growth promoting rhizobacteria" (PGPR), or "plant growth promoting bacteria" (PGPB) (41). Another term in common usage is that of deleterious rhizobacteria (DRB). The effects of DRB on field grown plants are often subtle, so consequently have attracted less scientific interest than have PGPR (21). PGPR control the damage inflicted on plants by phytopathogens via a number of mechanisms. These include outcompeting the pathogen for resources or habitat space, physical displacement, synthesis of antibiotics or other small molecules that inhibit pathogenic growth, production of enzymes that inhibit pathogen growth and stimulation of plant systemic resistance (61,62)

The increasing need for a steady and healthy food supply begets a compelling need for the control of plant diseases. Current methods for disease suppression are dominated by synthetic pesticides, breeding programs, and management of the plant's environment. With growing concerns over the environmental fate of synthetic pesticides and the development of pathogenic resistance to such controls, there is a need for novel solutions that can provide effective disease suppression while minimizing negative effects on human health and the environment.

The rich diversity within the microbial world offers many opportunities to modify the rhizosphere microbial communities and thus provide powerful biological alternatives to the use of synthetic pesticides in disease control. Use of such biological control agents may be more robust than synthetic methods because of the complexity of the microbial interactions involved and the sheer number of mechanisms utilized by a single organism (63). Many adaptations of the organisms suited to the rhizosphere environment, refined over many millions of generations, maybe useful in biocontrol. Consequently the commercial exploitation of PGPR has, and continues to be, a goal of considerable microbiological research.

Pseudomonas species, particularly the fluorescent pseudomonads, are the most studied biocontrol rhizosphere competent bacteria (23). Beneficial pseudomonads suppress pathogens through their capacity to produce a diverse array of microbial metabolites including antibiotics, siderophores, and volatile compounds (2). For example, under Fe (III) starved conditions, fluorescent pseudomonads produce the iron-sequestering siderophore pyoverdine, or utilize plant produced phytosiderophores (12). These capabilities allow the bacteria to usurp iron from within the immediate rhizosphere environment, reducing levels and depriving other deleterious and plant pathogenic organisms of this essential element. Of the range of antibiotics excreted by *P. fluorescens* 2,4-diacetylphloroglucinol has received the greatest attention (20,64–68). The metabolite 2,4-diacetylphloroglucinol (2,4-DAPG) is produced by many strains of fluorescent *Pseudomonas* spp. and has biocontrol activity against soilborne fungal plant pathogens. It has been shown to enhance root nodulation of pea roots and

produce larger and healthier (pink) nodules (32). Also, the bacterium increases mineralization rates and enhances nitrogen uptake from mineralized organic residues, whilst reducing soil microbial activity (69) and enhancing antagonism toward *Pseudomonas ultimum*, thus significantly increasing sugar beet seed emergence (67). However, such enhanced activities are species dependent (70). It has also been shown to be effective against a number of diseases, such as that caused by the potato soft rot agent, *Erwinia carotovora* subsp. *atroseptica* (66), the preemergence damping-off disease experienced by sugar beet (71). Fluorescent pseudomonads are also implicated in inducing systemic resistance in beans against halo blight disease (72) and viral diseases in cucumber, tomato (73) and tobacco (74–76), and in the suppression of "take all" of wheat with the antibiotic phenazine-1-carboxylic acid (77), or through modifications of soil populations and enzymes through DAPG-production (78).

Unfortunately, the use in agriculture of PGPR can be problematic. The transfer of results from the laboratory to the field has not realized initial expectations in agriculture. For example, it has proven difficult to optimize cost-effective mass production and application of *P. fluorescens* (77). So even with increasing interest in PGPR, (79) the success of microbial biocontrol is quite variable among trail results and are often unpredictable. Possible reasons for these failures include a lack of knowledge of the active biocontrol mechanisms and difficulties in preparing successful application formulas that remain viable through long-term storage (80). With regard to the latter problem, one solution is the use of sporulating gram-positive organisms that form spores and survive the heat and desiccation processes involved in preparing seed coatings. There is a substantial knowledge base and a wealth of experience in organisms such as *Bacillus* and *Streptomycetes*, both of which have been sources of industrial enzymes and antibiotics, and useful for insect control, for many years. In fact, *Bacillus thuringiensis* (Bt) now accounts for more than 90% of all marketed biopesticides (for foliar application not root pests), representing a worldwide market of $110,000,000 annually (81). The organism *Bacillus polymxya* (renamed *Paenibacillus polymyxa*) has a range of plant growth promoting properties, including dinitrogen fixation, soil phosphorus solubilization, production of antibiotics, chitinase, and other hydrolytic enzymes as well as the enhancement of soil porosity (82). The gram-positive bacteria have received less attention than the fluorescent pseudomonads, in part because they are less amenable for genetic research and in part because less is known about their mechanisms of disease suppression (83). For a review of this topic see Emmert and Handelsman (63).

FACTORS INFLUENCING THE RHIZOSPHERE

Physical Factors

The rhizosphere is a dynamic environment, always in a state of change. The changes can be brought about by a number of events mediated by plants or by the soil organisms that the roots encounter. However, the rhizosphere is also subject to changes imposed by the wider environment in which the plant root system resides. These physical factors determine the rates and reactions of biochemical processes within the plant as well as interactions between roots and microorganisms, and within the microbial community. These factors include temperature, water availability and movement, oxygen availability, indigenous pH, light intensity (as encountered by the plant), salinity and pollution levels, as well as the influence of the soil structure itself.

pH. The rhizosphere exhibits pronounced changes in rhizosphere pH, often in excess of one or two units. Such differences can be seen by the use of pH-sensitive dyes and demonstrate that plants of different species grown in the same soil can create quite different pH environments. It has also been shown that the pH varies dramatically at different points along the root system (84). These effects are due in part to organic acids, low molecular weight carbon-, hydrogen-, and oxygen-containing compounds, distinguished by their retention of one or more carboxyl groups and detected in all organisms. Depending on the dissociation properties and number of carboxylic groups, these organic acids can carry varying negative charge, allowing the complexation of metal cations in solution and the displacement of anions from the soil matrix. Consequently these compounds are proposed to be involved in a number of soil processes ranging from the mobilization and uptake of nutrients such as P (81,85,86) and Fe, the detoxification of metals (e.g. Al 87,88), microbial proliferation in the rhizosphere and the dissolution of soil minerals leading to pedogenesis (89). It is also suggested that pH mediated weathering rates are significantly faster in the rhizosphere than in bulk soil (90) and are enhanced by ectomycorrhizal associations.

Increasing pH levels may also render the rhizosphere environment unsuitable to certain pathogenic organisms. A 30% increase in growth of *Asparagus officinalis* under greenhouse conditions was observed with seedlings inoculated with *Pseudomonas putida*, an organism known to be antagonistic to the crown rot pathogen *Fusarium moniliforme*. The increased growth was suggested to occur as a result of the plant growth–promoting effect of organic acids secretion, such as succinic and lactic acids, and creating conditions in which the populations of pathogens are reduced (91).

Changes in pH alter the solubility and diffusion coefficients of a number of compounds essential to biological health, including compounds containing micronutrients such as zinc, chromium, nickel, manganese, selenium, cobalt, copper, and molybdenum (92). Consequently, plant or microbial populations can utilize nutrients previously unavailable to them by actively adjusting the surrounding pH. However, the release of such compounds are generally in response to specific environmental stresses (e.g. Fe, Al, and P or anoxia) and are species-specific. pH changes will also affect the efficacy of biocontrol organisms, as has been shown for the production of DAPG by *P. fluorescens* (93). For a review on the influence of pH on soil microorganisms see Lynch (94), and for a more specific review on organic acids in the rhizosphere see Jones (89).

Temperature. Temperature affects all chemical and biochemical processes in the rhizosphere, whether directly in its effect on chemical reaction rates of enzymes, or indirectly through effects on growth or exudation rates of the plant itself. Plants are adapted to optimum temperature ranges, above and below which growth is inhibited or permanently halted. Similar restrictions apply to microbial populations. For example, the optimum temperature for the production of chitinase enzyme by *Streptomyces lydicus* occurs at 25 to 30 °C (95).

Although it is not clear how temperature affects root exudation it is influenced by both increases and decreases in temperature, with sudden changes causing increased exudation (96). Composition and exudate quantity, are affected by temperature changes. (97) This was demonstrated in, *Zea mays* seedlings in which a seven fold increase in exudation was noted, with a release of three new oligosaccharides and sucrose, when cooled from 19 to 5 °C (98). It is hypothesized that a decrease in temperature affects the permeability of the root cells, giving rise to increased exudation.

In general, PGPR colonize the rhizosphere and root system more efficiently at higher root zone temperatures (99) and suboptimal root zone temperature has been shown to adversely affect growth, development, dry matter production, and grain yield (100). Infection and early nodule development processes are most sensitive to temperature. In fact nitrifiers are thought to be more susceptible than common heterotrophs to low temperatures, because of the inefficient nature of their metabolism (101). Increased temperatures also retard nodulation, (102) delaying the development of lateral roots and root hairs and disintegrating the infection threads of *Sinorhizobium* sp. at 42 °C. Temperature changes have also been shown to affect carbon allocation with leaves favored above storage organs at higher temperatures (103).

Freezing of soil pore water also affect water and nutrient uptake, locking nutrients up in a solid medium. However, certain PGPR can survive freezing temperatures, (i.e., −20 and −50 °C) and retain the ability to grow and promote root elongation of both spring and winter canola at 5 °C. To determine the mechanistic basis for this behavior, it was discovered that *P. putida* GR12-2 synthesized and secreted to the growth medium a protein with antifreeze activity (104). At temperatures below 0 °C, freezing injury will also occur. This is a consequence of the formation of ice particles physically destroying the surrounding structures. The effect is irreversible and on thawing a considerable leakage of metabolites will occur in addition to cell death. To induce frost damage and benefit from the discharged metabolites, some organisms, however, have evolved ice-nucleation mechanisms, that can increase the temperature at which ice particles form inside plant and root systems. Water in plant tissues can be supercooled to around −5 °C without causing damage. However, in the presence of ice nucleating bacteria, such as *Pseudomonas syringae*, which produce a protein complex in its outer membrane, which serves as a nucleus for ice formation, ice formation can begin at −1 °C (105). While this phenomena occurs in phyllosphere bacteria; there may be undiscovered parallels in the rhizosphere.

Light Intensity. Given that light through photosynthesis is the source of energy for plants it is of no surprise that changes to light levels and leaf cover have concurrent changes in the exudation patterns of the root system. Investigations have shown that under high light intensity, there is a significant increase in the partitioning of ^{14}C labeled material into the root system but that incorporation of recently assimilated carbon into the microbial biomass is not altered. Under higher light intensity, there was preferential allotment of carbon into the root system and a reduction in specific leaf area, without an increase in total plant mass (106). It has also been shown that under zinc deficiency, increases in light intensity markedly enhances the release of phytosiderophores from Triticum aestivum L. (cv. Aroona) (107). The same work showed that iron deficient barley (Hordeum vulgare L. Europe), when first grown at low light intensity followed by exposure to high intensity for 24 and 48 hours, enhances the release rate of phytosiderophores by about four and seven fold, respectively. The results demonstrate a particular role of light intensity in phytosiderophore release from roots under both zinc and iron deficiency.

Changes in received light levels as a result of aboveground herbivore grazing can also affect carbon fluxes. Grazing increases belowground carbon fluxes (roots, root exudates, and rhizosphere respiration), thus increasing resources (e.g., root exudates) available to soil organisms, especially microbial populations (108). This work also suggested that although herbivory increases respiration, it does not affect patterns of translocation. There is an increasing body of information attesting to the importance of plant physiological responses to herbivory in regulating soil organisms and, consequently, key soil processes such as decomposition and nutrient mineralization. For a review of the influence of herbivory effects on soil organisms, see Bardgett 1998 (109).

Water and Oxygen. Water is essential for all life. It makes up a large percentage of the biomass of all life-forms retaining cell turgidity and, in the case of soil, serves as a carrier of water-soluble minerals and other compounds as well as facilitates the dispersal of metabolites and waste products. Accordingly, changes in the soil water content have substantial effects on the rhizosphere microbial population. As moisture content drops, competition for this essential resource increases, the supply of utilizable substrates declines, and dispersal of toxic by-products is slowed. These processes result in reduced microbial numbers and activity. As plants and microbes differ in their abilities to capture and retain water, the community structure of the rhizosphere will also change. Decreasing soil water may indirectly affect microorganisms by altering the types and quantity of exudates released into the rhizosphere (110).

Oxygen also plays a vital role in sustaining biological life of organisms adapted for its use, that is aerobic organisms. Oxygen and water content are closely linked because oxygen has a low solubility in water. Consequently, as the water content of soils increases, a greater portion of the soil pore volume becomes saturated, creating anoxic zones.

The small amount of oxygen that is able to diffuse into these anoxic zones is under intense competition from aerobes. Soil oxic-anoxic interfaces arise within the plant root system, separating aerobic from anaerobic processes. Such interfaces arise in virtually all environments. An explanation of the basic principles involved and specific examples can be found in Brune (106).

Certain plants, such as rice, have evolved to modify the oxygen content of their rhizosphere by bringing oxygen down from the leaf surface. This is performed via aerenchyma, which is a porous tissue connected with the aboveground parts of the plant. Such root aeration can be performed by efficient pressure driven ventilation (111–113) or, as in the case of rice (*Oryza sativa*), by simple molecular diffusion (114). While the primary function of this root aeration is to provide the subsurface plant tissue with oxygen for aerobic metabolism, it has repeatedly been shown that oxygen is also exuded from their root systems, (115) thus creating aerobic zones in a water saturated environment (116).

The rhizosphere microbial population has also been implicated in inducing modifications to the rhizosphere that affect water dynamics (107). The production of exopolysaccharides by bacteria can help protect them from drought stresses by enhancing water retention (117). The exuded polysaccharides increase aggregate stability of the soil, which helps maintain soil porosity (108), which in turn provides hydraulic conductivity allowing water to penetrate the soil and fill soil pore spaces. In contrast, rhizosphere organisms have been shown to produce water-repellent exudates, which alter the hydraulic characteristics of the soil, with consequential effects on water runoff, the disruption of soil aggregates on rapid wetting and increases in the flow of water between the aggregates (118).

THE FUTURE OF RHIZOSPHERE SCIENCE

Rhizosphere research generally concerns three major but interrelated areas; the influence of roots on soil microorganisms, the influence of rhizosphere organisms on plant growth, and soil borne pathogens and plant disease. Many questions remain in these areas, however, recent advances in technologies and knowledge may advance our achievement of these objectives.

The main obstruction to obtaining reliable and pertinent data from the rhizosphere is the difficulty in conducting in situ sampling. Even recording essential information such as rhizosphere pH is fraught with problems regarding the purity of sample and temporal variations. Consequently, there is a pressing need to further develop nondestructive techniques to sample from the rhizosphere soil without contamination from the bulk soil and using methods to sample on a microscale. Noninvasive techniques have been developed, such as image analysis, utilizing X-rays and nuclear magnetic resonance to elucidate root structure at a greater spatial resolution and to potentially calculate water fluxes (119). Such techniques combined with chemical and fluorescent dyes could also be used to investigate a wide range of root induced changes in chemical conditions at the root-soil interface. There is also a recognized need for more quantitative data on rhizosphere dynamics measured in more natural experimental systems (119), especially with regard to nutrient movement.

Ten years ago predictions regarding the future of biocontrol looked forward to "research undoubtedly showing biocontrol to be safe, effective, and economically feasible" (120). However, scientific endeavors have yet to achieve this, in part due to public concern about genetic modifications, including public disquiet in Europe and elsewhere over the release of genetically modified organisms to the wider environment and their inclusion in food production.

Genetic manipulation of plants and microorganisms is generally seen as contributing greatly to the advance of rhizosphere research (120,121). Again, the fluorescent pseudomonads have been the focus of much of this research (69,78,122–128). "Marking" the organism in a way that allows easy detection facilitates tracking of organisms in the rhizosphere and under field conditions. Such marking is often carried out by conferring the chosen organism with the ability to resist certain antibiotics to which the indigenous population are susceptible. It is this practise of developing resistant organisms that is the foundation of public unease about genetic manipulation of microorganisms. The use of organisms modified in this way is unsuitable for field release or greenhouse trials given the risks of transfer of this ability to indigenous organisms and the resulting consequences. Consequently, alternative methods of marking inoculants have been developed. For example, the organism *P. fluorescens* F113 and its DAPG negative mutant contain what has been described as the most utilized genetic marker system, that of *lac* ZY (129). This marker confers the ability to utilize lactose as a sole carbon source. In the case of *P. fluorescens*, this allows the organism to be distinguished from indigenous populations. This marking system is further enhanced with the use of a chromogenic dye (5-chloro-4-bromo-3-indolyl-β-D-galactopyranoside (X-gal)) that confers a blue color into the organisms carrying the *lac* ZY gene cassette.

The system described earlier has been used successfully in European Community funded field trials by the IMPACT consortium on biocontrol organisms, which investigated interactions between microbial inoculants and resident populations in the rhizosphere of agronomically important crops. (For further information see the web site *http://www.ucc.ie/impact/index.html*) Such research is needed because the ecological effects of releasing genetically modified organisms (GMOs) into the environment are not known. For example, modifications to the rhizosphere brought about by the introduction of a modified *Pseudomonas* with enhanced biocontrol abilities could interfere with nutrient cycles (69,70).

An example of an inoculant, modifying nutrient cycling was reported by Brimecombe and coworkers who studied how inoculation of pea (69) and wheat (70) seeds with *P. fluorescens* F113, modified as described earlier, influenced nitrogen uptake. Their findings indicated that the cycling of nitrogen was affected by the DAPG positive inoculant, which enhanced nitrogen uptake from

mineralized residues, despite its reduced activity in the soil. In contrast, nitrogen uptake by wheat seeds in a subsequent experiment was decreased by inoculation. Further work utilizing the same organisms sought to explain the apparent contradiction by examining the effect of plant-microbe interactions on bacterial-feeding nematodes and protozoa (130). It was found that pea seeds inoculated with either *P. fluorescens* F113 or the DAPG negative strain gave rise to an increase in the number of nematodes and protozoa in comparison to noninoculated seeds. Introducing the bacteriophagous *Caenorhabditis elegans* into noninoculated germinating pea seeds exerted a nematicidal effect on *C. elegans*. Inoculation with *P. fluorescens* arrested this effect, which suggested that the active nematicides were being metabolized by the inoculant. Inoculated wheat seeds, however, displayed lower nematode populations and unaffected protozoan numbers, with no nematicidal effects detected in either inoculated or noninoculated systems detected. Given the significant role attributed to the soil microfauna in nitrogen cycling, any disturbances to this part of the soil biota brought about by inoculation with *P. fluorescens* become very pertinent.

Such work highlights the need for further investigations, especially given the importance of nitrogen in agroecosystems and the globally increasing nitrogen inputs such systems receive, with possible implications for nitrogen leaching.

Despite the vast amount of research already conducted on rhizospheres, the new techniques mentioned earlier and the rapidly expanding knowledge presently being unveiled make it unequivocally clear that there is an extensive amount of work yet to be conducted, with many opportunities remaining. One particular realm of opportunity, is that concerned with bridging laboratory work with field based experimentation. Augmenting laboratory-based findings, achieved in microcosms, with field based work is difficult. Often the original findings are unsupported by work carried out in the more complex and less manageable environment. The use of a "halfway" approach is often required, using larger systems (macrocosms) to replicate the field environment whilst still maintaining some control over the experimental conditions. Rhizosphere microbiology and rhizosphere science in general remains incomplete, with much exciting work to be accomplished.

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RHIZOSPHERIC EFFECT.

See RHIZOSPHERE MICROBIOLOGY

RIBOSOMAL RNA-TARGETED PROBES.

See FLUORESCENT PROBES FOR IN SITU ANALYSES OF MICROBIAL COMMUNITIES

RIBOTYPING.

See CAPILLARY ELECTROPHORESIS IN GENETIC ANALYSIS AND RIBOTYPING OF MICROBIOTA IN THE ENVIRONMENT

RIBOTYPING METHODS FOR ASSESSMENT OF IN SITU MICROBIAL COMMUNITY STRUCTURE

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The structural ribosomal RNA genes and gene products have become the targets of choice for microbial ecologists trying to uncover novel variations in uncultured microbial species, relate microbial community structures to environmental processes, and monitor anthropogenic effects on microbial diversity and community structure. As the term suggests, ribotyping refers to a number of established methods that rely on the manipulation and analysis of ribosomal nucleic acids to characterize microbial isolates or communities. Although several ribotyping techniques are also useful in classification and taxonomy of microbial isolates, this discussion is confined to the characterization of unknown microbial communities, and the manipulation of nucleic acids extracted directly from environmental samples. These methods form part of a suite of methods designed to avoid the biases inherent in culture-based analyses of microbial communities (1) and attempt to take into account the fact that the microorganisms most relevant to environmental processes are commonly unknown and resistant to laboratory cultivation (2–4). Ribotyping strategies differ widely in their complexity and demand on human and material resources. They also differ in the amount and type of information they can provide, as well as their potential for introducing artifacts and biases.

These properties are summarized in Table 1. Thus, the choice of approach adopted is not trivial, and utilization of more than one approach to analyze a given sample is generally guaranteed to provide more information and reliability than any single method. The methods described here are ideally combined with those described in the chapters by Macnaughton and Sharp and Sutton, and are summarized in Figure 1. Factors influencing choice of technique(s) include the taxonomic level of focus (domain, phylum, genus or species), the amount of information available a priori for this taxon, the number of samples to be analyzed, the type of environmental sample, and the biomass and diversity of target and nontarget organisms in the sample.

Ribotyping methods generally target the small subunit ribosomal RNA or RNA gene (SSU rRNA or SSU rDNA). These are typically termed 16S in Bacteria and Archaea, and 17S or 18S in Eukarya. These terms, which refer to sedimentation rates, are rather inaccurate, as variation in the sizes of these molecules between species can

Table 1. Summary of Ribotyping Methods

Method	Potential for Artifacts	Technical Difficulty	Recovery of Sequence Data	Reproducibility	Suitability for Large Sample Sets	Recovery of Phylogenetic Information
Forward array	Medium	High	No	High	High	Yes, can be quantitative
Reverse array	Medium	High	No	High	High	As above
Shotgun clone analysis	Low	Medium	Yes	Low	Low	Excellent
PCR-clone Analysis	High	Medium	Yes (dependent on primer choice)	Medium	Low	Excellent, but prone to artifacts
DGGE/TGGE/TTGE	Medium	Medium	Yes (dependent on primer choice)	Medium-High	Medium	Good, but only short sequences from most abundant target organisms
SSCP	Medium	Medium	Yes (dependent on primer choice)	Medium-High	Medium	As above
RISA	Low	Medium	Yes (dependent on primer choice)	High	Medium	Poor
ARDRA	Medium	Low	No	High	Medium	Poor
T-RFLP	Medium	High	No	High	High	Fair, with good future potential

DGGE = denaturing gradient gel electrophoresis; TGGE = temperature gradient gel electrophoresis; TTGE = temporal thermal gradient electrophoresis; SSCP = single strand conformational polymorphism; RISA = ribosomal intergenic spacer analysis; ARDRA = amplified ribosomal DNA restriction analysis; T-RFLP = terminal restriction fragment length polymorphism.

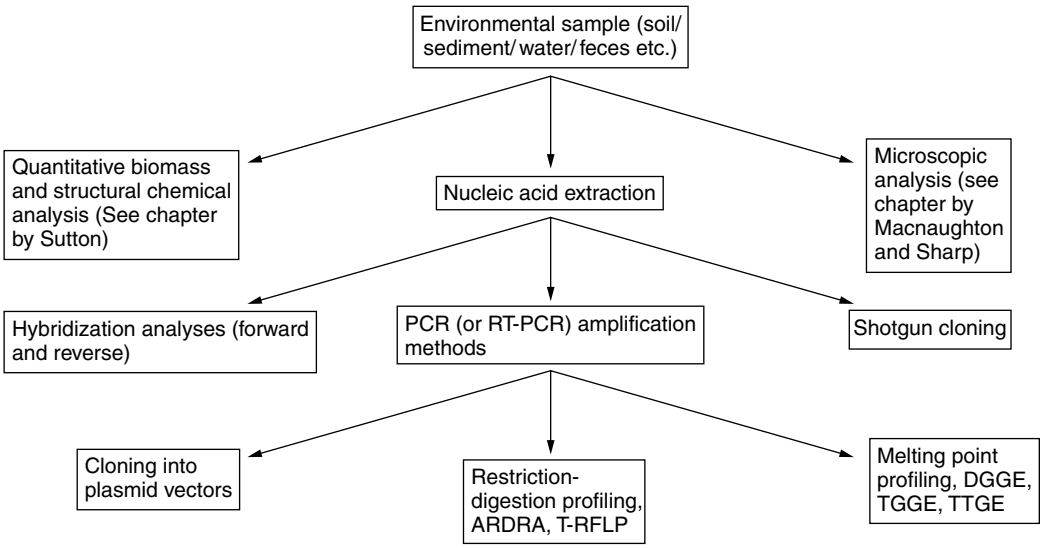

Figure 1. Schema representing the major subgroups of culture independent microbial community assessment procedures. Using methods from more than one category in analysis of a sample will almost certainly improve confidence in the quality of the data recovered.

be quite considerable, but are used here as common designations and terms of convenience. The use of small submit ribosomal RNA markers as targets for the analysis of microbial communities carries a number of important advantages that include the following:

- It is a ubiquitous marker, with a homologous function across all organisms.
- Conserved secondary structure facilitates the alignment of nucleotide sequences.
- The presence of both highly conserved and variable domains allows targeting of both broad and narrow taxonomic groups, respectively.
- The relatively high target number (multiple gene copies in many organisms, and high copy number or 16S rRNA per cell) makes detection relatively easy.
- 16S and 18S sequences carry sufficient information to allow phylogenetic inference.
- Phylogenetic inferences based on this molecule commonly match well with other taxonomic methods, such as morphology, phage sensitivity, chemotaxis, and so on.
- There is a large and expanding comparative sequence database (5).
- The relationship between rRNA production and protein synthesis rates means that rRNA levels can provide a first-order approximation of cell activity (6,7).

The use of large subunit rRNA or rDNA offers many of the same advantages, and this target actually possesses a greater degree of phylogenetically useful information. It is, however, less widely used because of the difficulty of amplifying and sequencing this larger target, and the ever-increasing advantages provided by the vast SSU database. The final product-encoding portion of the ribosomal operon is the 5S rRNA gene, whose small product was used in some of the earliest nucleic acid sequence comparisons between microorganisms (8–10). Although this target can be used for some ribotyping strategies, its scant phylogenetic content has limited its use. The portions of the ribosomal operon that do not code for structural RNAs, (the internal transcribed spacers [ITS] I and II) are highly variable and thus offer the possibility to discriminate between even very closely related species or strains (11,12). A given cell containing more than one ribosomal RNA operon is likely to contain appreciable variation in these regions (13). However, the lack of structural homology in ITS regions across taxa makes broad comparison of sequence information difficult or impossible. The relative use of different ribosomal operon targets will be reflected in the amount of attention they receive in this chapter, that will primarily address SSU-targeted strategies.

Currently, popular ribotyping methods can be divided into two subgroups, PCR-independent, and PCR-dependent. Quantitative PCR-independent ribotyping strategies depend on the hybridization of defined probes to membrane-bound environmentally derived nucleic acids, or vice versa, and are also covered in the chapter by MacGregor. The remaining PCR-independent method, analysis of shotgun-clone libraries, is now rarely employed, but has some unique advantages. PCR has revolutionized the ability to recover genes from the environment, but the amplification of complex mixtures of nucleic acids involves the generation of potential biases and artifacts. An in-depth discussion of the advantages and risks of PCR-mediated methods falls outside the scope of this chapter (14,14a), but certain potential limitations and artifacts are discussed in the context of the specific approaches presented in later sections. Community ribotyping methods either use rRNA molecules or the genes that encode them (rDNA) as the target of analysis. The targeting of either RNA or DNA has important implications not only in the interpretation of results, but also in terms of methodological constraints. RNA is less stable than DNA, and rRNA levels may therefore provide information concerning general levels of cell activity (15,16), although it is not yet known precisely how well rRNA levels per cell correlate with metabolic activity in natural samples. RNA is also less stable than DNA during laboratory manipulations, so extra care must be taken when analyzing rRNA samples (17). Furthermore, rRNA templates must first be reverse-transcribed into DNA for methods that require enzymatic amplification.

The stability of DNA in environmental samples is not fully known. Nonactive, dead or even partially destroyed cells may still have some DNA intact, and the longevity of free DNA in natural samples is highly influenced by the nature of the environmental matrix. Trevors (18) reviewed the stabilizing effect of the soil matrix on free DNA. Thus, it must be remembered that rDNA can be recovered from organisms that do not have ecological significance in the sample at the time of sampling. Further, rDNA copy number per genome varies between different microorganisms, and these differences must be taken into account when trying to extrapolate rDNA levels to cell numbers. These differences in gene copy number also influence the capacity for community ribotyping methods to detect a given cell type (19,20).

The majority of ribotyping techniques rely on the use of previously recovered sequence information. Thus, one must bear in mind that all primers and probes used in ribotyping studies are based on extant sequence information. The accuracy of such oligonucleotides in identifying their desired targets within uncharacterized environmental samples is only as good as the comprehensiveness of the database used to design them, and must be kept under constant revision (21,22,22a). Therefore, all ribotyping studies utilizing so-called "signature sequences" are liable to both detect nontarget organisms and fail to detect target organisms when applied to samples containing an unknown mixture of species. This highlights the importance of acquiring novel sequence information from both pure cultures and environmental samples, especially within phylogenetic lineages that are poorly represented at present.

PCR-INDEPENDENT METHODS

PCR-independent ribotyping methods may be quantitative or nonquantitative. The quantitative methods are based

on hybridization of genetically informative labeled probes to environmental nucleic acid targets. As hybridization of probes to targets within intact cells (in situ hybridization) is covered elsewhere in this Encyclopedia (FLUORESCENT PROBES FOR IN SITU ANALYSES OF MICROBIAL COMMUNITIES), this discussion is limited to methods requiring hybridization to nucleic acids extracted from environmental samples.

Perhaps the greatest advantage of PCR-independent ribotyping approaches is that no in vitro amplification of nucleic acid targets is necessary, thus circumventing any potential biases or artifacts that such amplifications may introduce. Nucleic acids extracted from the environment are analyzed directly. One of the major limitations, however, can be in the amount of sample necessary to carry out the ribotyping analysis, which can be especially problematic when targeting microorganisms that are low in numbers or only represent minor components of the community. Secondly, the quality of hybridization support is critical, as variations within a given membrane may seriously affect the quality of quantitative interpretations from the results (23).

Forward Sample Array Hybridization

This ribotyping method generally involves the spotting of extracted nucleic acids onto a membrane, which is subsequently hybridized with a battery of oligonucleotide probes that are specific to different taxonomic groups under highly defined conditions of stringency. Protocols differ in regard to how the probes are labeled, the type of membrane used, and the detection of hybridization products. The strategy usually involves the use of a hierarchical set of hybridization probes, which allows definition of the total amount of extracted 16S rRNA or rDNA to be defined and divided among the probe-targeting taxonomic groupings (Fig. 2). The decision as to which taxonomic groupings are to be included depends on the discretion of the researcher, and the availability of suitable probes. The taxonomic level of the study also influences the number of hybridization probes used. Repeat-probings of a membrane or set of replicate membranes can vary between just a few, if one is interested only in the relative amounts of eukaryotic, bacterial and archaeal target, for instance, to several hundred in theory, if one were interested in subdividing to fine taxonomic levels. In practice, most analyses focus on major microbial groups and a small number of subgroupings, functionally important in the samples under investigation (24).

As mentioned earlier, the accuracy with which probes detect their target group is difficult to predict when examining environmental samples. The level of uncertainty

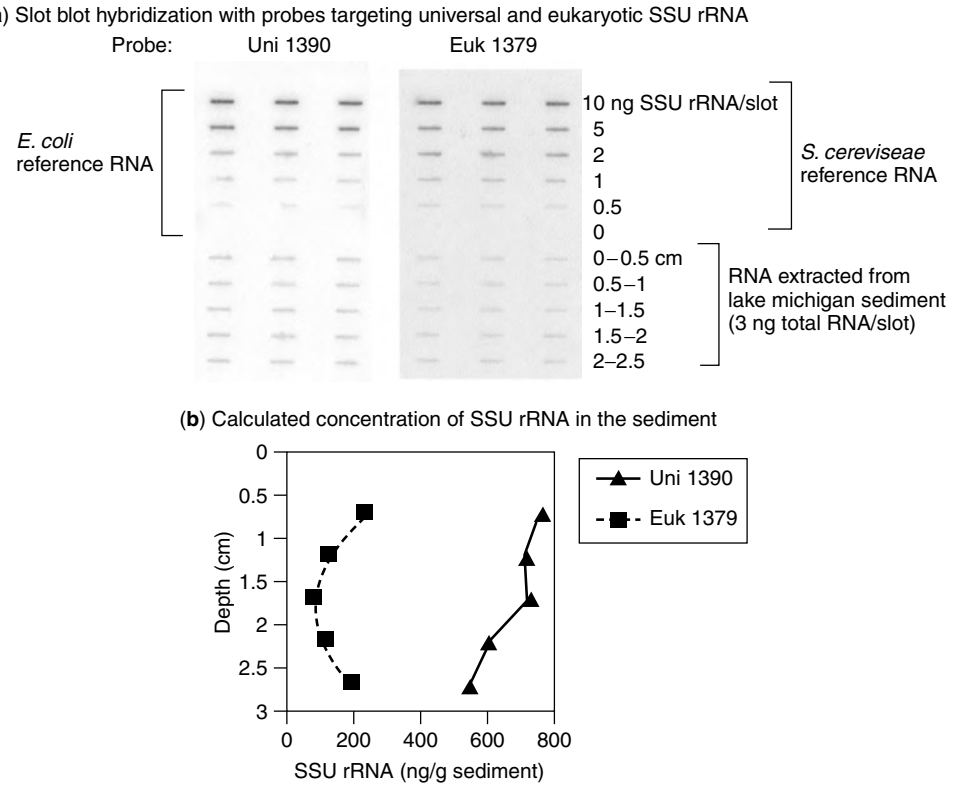

Figure 2. Quantitative membrane forward-array hybridization. (a) Raw data; (b) quantified data. This experiment quantified the numbers of all microorganisms (Bacterial, Archaeal, and Eukaryotic) using a universal probe, and the numbers of eukaryotic microorganisms with depth in a freshwater lake sediment. The amount of probe bound was compared to the level of probe bound to standard quantities of Eukaryotic (*Saccharomyces cerevisiae*) and prokaryotic (*Escherichia coli*) SSU RNA. The quality of both the RNA and hybridization membrane are of crucial importance in such studies (17,23). Courtesy of Barbara J. MacGregor, Max Planck Institute for Marine Microbiology, Germany. See color insert.

decreases as more information is gathered on a taxon, but such limitations are inherent to all techniques that use oligonucleotide probes or primers to examine incompletely defined environmental samples. Furthermore, the determination of optimum hybridization conditions can be a difficult task, and cross-hybridization with closely related target groups can affect the accuracy of results, even when using optimum hybridization stringencies. Thus, although results may allow quantitative interpretation of nucleic acid abundance, the accuracy of the analysis is still limited to the reliability of the probes used. The use of an hierarchical approach, however, can provide an indication of the coverage of some probes. Where all known subgroups within a taxonomic unit are represented in an analysis, the summed hybridization results of the subgroups can be compared to the result of the group-specific hybridization. The use of such internal checks usually reveals that not all organisms can be recognized by a defined set of probes, resulting in a certain amount of "undefined" rRNA or rDNA signal. Conversely, use of partially degraded RNA can lead to quantification difficulties, which falsely suggest that subgroups are in fact far more abundant than the major taxonomic groups of which they are a part (17). Such an artifact occurs when the probe site defining a given subgroup is protected from degradation relative to the probe site defining the parent taxon. The accuracy of such analyses will continue to increase with continued DNA–RNA-extraction and probe development. Such development not only increases the accuracy with which currently screened groups can be quantified, but also will expand analysis to include additional, currently not known, microbial groups.

The number of times that such a membrane can be hybridized can be limiting, and sequential hybridization can be time-consuming. In practice, several replicate membranes are therefore usually made for concurrent analysis. Membranes typically contain several samples to be compared, and spots containing known concentrations of nucleic acids derived from control organisms of known phylogenetic affiliation. Such controls allow for standardization of hybridization signals and comparison across duplicate membranes.

This approach has typically been used for the broad-scale characterization of microbial communities (25), although it can also be applied to detect shifts between closely related taxa in some cases (26). Forward array hybridization experiments have also been highly illustrative in examining the discrepancies between direct ribotyping surveys and those relying on the culturing of bacterial colonies on solid media (3).

Forward sample array hybridization has also been carried out after the introduction of a PCR step and, for example, denaturing gradient gell electrophoresis (DGGE) analysis (27). Although this is usually not desirable because of the possible introduction of PCR artifacts, and the loss of quantitative data inherent in this, it may be necessary in cases involving very small sample volumes or low target numbers.

Reverse Sample Array Hybridization

Typical forward dot blot hybridization studies fix the sample of interest to a membrane and use specific oligonucleotides to probe the sample for signature sequences of interest. However, as the number of microbial groups to be identified increases, it becomes unwieldy to strip membranes and reprobe for each target group. Reverse sample array hybridization allows for the detection of multiple bacterial groups, at various taxonomic levels, within a single analysis.

As the name suggests, reverse sample array hybridization switches the placement of sample and probe with respect to forward array probing. In this case, the environmental sample is typically labeled in some way, and allowed to pass over a surface to which probes have been fixed. Labeled DNA (RNA or cDNA, if the starting material is rRNA) fragments that hybridize with the attached probe sequences remain in association with the surface, whereas nonmatching sequences are washed away. The depth and breadth of the analysis depends on the number and the taxonomic level of probes spotted on the hybridization surface. When using a battery of species-specific probes, such arrays have typically been used for strain identification (28,29). Alternatively, nucleic acid extracts from mixed microbial communities can be used to detect and quantify specific community members (30–32) or characterize a microbial community across several taxonomic levels.

The number of taxon-specific probes can vary from a few to potentially hundreds or even thousands. The nature of these "reverse probes" can also vary from specific oligonucleotides to total DNA extracts from pure cultures. The specificity of the probes used depends on the desired breadth and depth of the analysis. Reverse array hybridization analysis has been applied to detect shifts both between domain-level groups (33) and within specific bacterial groups (32), as well as to detect the presence and changing distribution of specific microbial populations (34).

Nucleic acids acting as reverse probes can be pipetted to a membrane (35) or, in a rapidly developing new technology, applied to fixed supports such as DNA chips (see Biochip-Based Devices and Methods in Microbial Community Ribotyping, this Encyclopedia). The method used will depend on the number of reverse probes to be used and the facilities available. In the near future, it is likely that reverse probing of oligo- and polynucleotides bound to microchips will become the dominant form of nucleic acid community monitoring, although currently the capital costs of such systems are prohibitive for their widespread use.

Shotgun Cloning

Some of the first 16S rDNA sequences recovered from uncultured organisms were derived from shotgun cloning experiments. In this technique, total environmental DNA or reverse-transcribed RNA is cloned into a phage or plasmid vector to create a library. The library is then hybridized to a suitable probe using plaque or colony lifts to select clones of interest, followed by

subcloning and/or sequence analysis (36). Such cloning strategies have revealed novel microbial diversity, and sequence information so recovered may be more reliable than sequences recovered after PCR (14). However, few laboratories can afford to carry out the laborious screening required for the examination of direct environmental clone libraries. The increasing sensitivity of screening procedures may make this strategy a more viable option for some environments in the future.

PCR-DEPENDENT METHODS

The chief advantages of PCR-mediated methods over those not employing amplification steps are provided by the specificity of PCR methods and the added sensitivity afforded by target amplification. Thus, such techniques enable analysis of microbial communities or certain parts thereof, using only small portions of sample. The goals of a particular study and the available target group(s) determine the choice of PCR primers used, and this choice is critical. The use of primers targeting broad taxonomic groups can be employed to track a wide variety of microbial populations, but results may become unwieldy for highly complex communities. On the other hand, primer combinations specific for groups at lower taxonomic levels can be used to study particular monophyletic subsets of a community (37). Such approaches can be very useful in reducing complexity and focussing on certain groups of interest. However, phylogenetic groupings do not always correspond to functional groupings (e.g., nitrate-reducing bacteria, or traits carried by plasmids), and such approaches cannot reveal information about community members falling outside the specific group. Also, not all primer combinations can be used equally well for all methods because of technical constraints. In principle, all PCR-dependent techniques can also be applied to the analysis of RNA molecules by the addition of a reverse transcriptase step before enzymatic amplification.

The major drawback of PCR-based approaches is the potential introduction of artifacts during the enzymatic amplification of target molecules. Even assuming a perfect match between the primers chosen and the target group of interest, it is seldom reasonable to assume that the ratio of recovered sequences faithfully represents the ratios of the source organisms in the environmental sample tested. For example, two of the most conserved regions in the bacterial 16S rRNA gene were targeted by Muyzer and coworkers in the seminal use of PCR-DGGE to profile bacterial communities (20), yet heterogeneities exist within these sites (22,38). Figure 3 shows the consequence of trying to amplify a 16S gene fragment from a γ-proteobacterium with mismatches to one of these primers in the presence of sequences with perfect matches. This demonstrates that even when present at 20% of the starting templates, the product from this organism is negligible in competition with sequences with perfect matches.

Replication errors may also occur during PCR, and further errors may be introduced during cloning, such that recovered sequences may differ substantially from the original target molecules. The relative seriousness of potential artifacts depends on the technique and

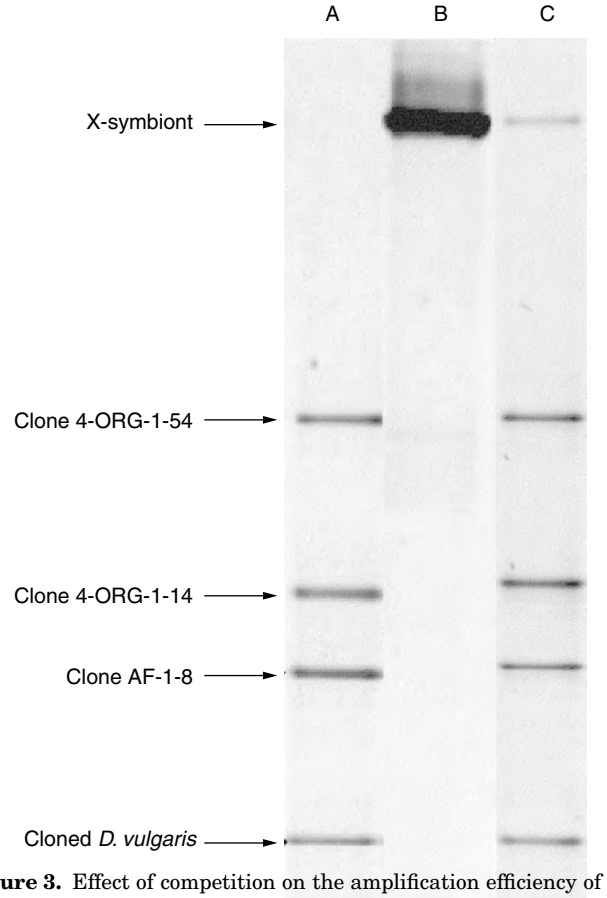

Figure 3. Effect of competition on the amplification efficiency of tempates with imperfect primer matching on PCR-DGGE profiles. The 16S rDNA sequence of the X-symbiont of *Anomoneura mori* has mismatches to the forward PCR-DGGE primer described by (20). The other sequences used have perfect matches. When mixed in equal proportions prior to PCR, the X-symbiont 16S rDNA fragment was not detected (A), although it amplified efficiently in reactions containing no other templates (B). When the priming site was modified to encode a perfect match to this primer, it coamplified with competitor templates with an efficiency approaching that of the competitors (C). From experiments described in (38).

methodology involved, and the limitations of PCR must always be kept in mind when interpreting the results of the ribotyping techniques discussed in the following sections. More detailed descriptions of potential PCR artifacts can be found elsewhere (14,39–42b), and this chapter only focusses on specific advantages and disadvantages of the presented techniques.

In Situ PCR and In Situ RT-PCR

Structural information and localization of bacterial cells and activities in situ is lost using the destructive methods of most ribotyping strategies. In fact, the ability to detect structural aspects of microbial communities is perhaps the most interesting feature of the in situ hybridization techniques described in the entry Fluorescent Probes for In Situ Analyses of Microbial Communities, this Encyclopedia. One problem with some in situ hybridization protocols is the lack of sufficient

signal for accurate detection. Recent technical advances have made it possible to combine the structural resolution of in situ hybridization with the detection sensitivity of PCR techniques. In some cases, it is possible to perform amplification reactions, targeting either DNA or RNA templates, without the disruption of structural features of the sample (43–45). Amplified target nucleic acid is then detected by subsequent use of internal hybridization probes. While such techniques show promise for revealing structural aspects within certain microbial communities, the procedures can be technically demanding and difficult to reproduce. It remains to be seen if these in situ amplification strategies will become viable ribotyping methods for widespread use.

Clone Library Screening

Ribotyping of bacterial communities by cloning near-full-length rDNA fragments has the potential to generate more novel information than any other ribotyping method (46). Compared with the gel-based profiling methods described later, a greater length of sequence can be retrieved from clone libraries, providing stronger phylogenetic inference. Compared with hybridization analyses that do not generate novel sequence information, the information recovered is more additive and easily accessed by other researchers from public sequence databases. Cloning-based approaches typically address two issues, (*1*) the identity of community members, and (*2*) the diversity within the targeted community.

The first of these issues is addressed by phylogenetic analysis of recovered sequences. Such analyses can usually provide satisfactory information as to the identity of the microorganism from which the sequence was recovered, but as mentioned previously, it is difficult to correlate the frequency of sequence recovery with cell numbers. Furthermore, sequence anomalies introduced during PCR, cloning, or sequence determination can affect the fidelity of results. In addition to polymerase errors, the creation of chimeric DNA fragments and heteroduplex DNA molecules can result in novel sequences not present in the original target pool. Chimeric molecules are PCR artifacts in which the 5′ and 3′ ends are derived from different source organisms. These might be interpreted incorrectly as being derived from novel microbial lineages, and may contribute to overestimations of diversity (39). Although careful analysis may detect such artifacts (e.g., the "Check Chimera" program, 5), many such sequence anomalies no doubt escape detection, and the introduction of artifactual sequences in clone libraries has in some cases been estimated to be quite high (47–47b). Resolution of the problem of cloning heteroduplices may be overcome by digestion of PCR products with T7 endonuclease I before ligation (47c).

The issue of community diversity can be addressed by comparing the redundancy within rDNA libraries obtained from different samples. Ecological theories such as "collectors' curves" used to estimate "coverage" can be employed in such comparisons. Coverage is calculated by compiling the sequences generated, then calculating the likelihood that the next clone sequenced already exists within the database. In a simple mixture of two species, it will quickly become apparent that each sequence derived is identical to one previously recovered. However, for environments such as soil, where the species diversity has been estimated at 5,000 to 10,000 distinct species per gram (48), the number of clones that need to be analyzed to provide a good picture of community structure is enormous and prohibitive. Where such attempts have been made, coverage has generally been assessed by the method described (49–51), and diversity using the Shannon index (52).

It is possible to define two samples as different using a cloning-based strategy by demonstrating that the slope of the Collector's Curves are different (different diversities, Fig. 4), or that the distribution of sequences is different (different community structures). However, given the number of clones that must be analyzed to achieve a reasonable estimate of community structure in a highly complex system such as surface soil (50), comparisons between samples can become highly impractical using such a strategy. Where only the recovery of novel sequence information is sought, it is possible to reduce the number of clones to be sequenced by screening clones by restriction analysis (53) or denaturing gradient gel electrophoresis (54) to avoid the sequencing of duplicate or highly related clones. Also, clones above a given level of identity can be grouped into operational taxonomic units (OTUs) to reduce the complexity of data for the Collector's Curve analysis (50). Nevertheless, cloning strategies are most often limited to a mere glimpse into some of the types of microorganisms present in a sample, except when applied to relatively simple communities such as laboratory enrichment cultures (55). Comparisons can become more manageable and meaningful when the cloning strategy employs specific primers to target a specific microbial group of interest (37,56).

Community Fingerprinting Techniques

Any given stretch of rDNA between conserved PCR priming sites is likely to differ in length between certain groups of organisms, but separation of rDNA fragments

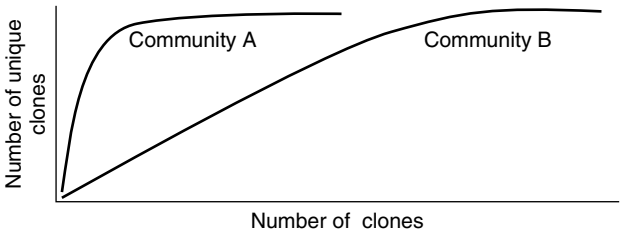

Figure 4. Schematic "Collector's Curves" of cloned rDNA fragments OTUs. Community A is less diverse than community B. Defining this relies on the repeated recovery of operational taxonomic units (OTUs) and statistical proof of difference between the curves. An OTU may be considered as a unique sequence, or a group of sequences within a limit of sequence identity (generally 97%). Alternatively, OTUs have been defined as clones with identical restriction–digestion patterns, DGGE or SSCP migration behavior, which may not equal sequence identity. Defining diversity by this type of analysis is as strongly influenced by community evenness as DGGE/SSCP/T-RFLP and RISA analyses. None of these methods are ideal for the measurement of species richness, an equally important aspect of diversity.

on the basis of length is too crude an approach to provide details regarding differences between environmental communities. The following section describes the generation of community "fingerprints" based on exploitation of sequence differences between the rDNA fragments of different species. The following rule of chromatography should be kept in mind while reading the technique descriptions mentioned in the following sections: Two samples generating different patterns are different, but two samples generating identical patterns are not necessarily identical. This rule applies to any single-dimension chromatographic procedure; there are no exceptions. It is therefore imperative that assumptions of community or band identity are supported by additional data.

One of the major advantages of community fingerprinting approaches is that entire populations of nucleic acids are analyzed together, instead of individually, as in clone library analysis. This allows for a far more rapid comparison of multiple communities. As with other approaches, analyses can target either rRNA or rDNA templates by RT-PCR or PCR respectively, and RNA- and DNA-based community profiles can be readily compared.

As only the most dominant DNA molecule types will be detected (those comprising at least 1–2% of the entire target population; 20), individual sequence anomalies introduced during the PCR amplification of complex samples are typically present in concentrations that are too low to affect the analysis. However, this level of detection can also be considered as a major drawback of community profiling methods because some ecologically significant microbial populations may constitute only a small fraction of the total rRNA or rDNA of a sample and therefore escape detection.

Profiling Amplified rDNA Fragments by Separation on the Basis of Melting Point: DGGE, TGGE, TTGE

DNA fragments differing in sequence by as little as a single base pair can have easily identifiable differences in melting point. Denaturing gradient gel electrophoresis (DGGE), termperature gradient gel electrophoresis (TGGE), and temporal thermal gradient electrophoresis (TTGE) all operate on this principle and are considered together here. The mixed (RT-) PCR products generated by 16S rRNA- or rDNA-specific amplification reactions typically contain populations of DNA fragments that contain differences in nucleotide sequence. However, because of nearly identical lengths, such fragments cannot be separated based on size as in a standard agarose or acrylamide gel analysis. DGGE, TGGE, and TTGE all rely on the sequence-specific differences in melting behavior between DNA fragments and separate such mixed (RT-) PCR products into bands containing the constituent populations of DNA sequences. When DNA fragments become partially single-stranded (ss), mobility is drastically impaired with respect to double-stranded (ds) DNA. This partial denaturation can be induced by elevated temperature or denaturing chemicals such as urea and formamide, and is dependent on the G : C content of the fragment and other structural aspects determined by the nucleotide sequence. In a gradient of denaturing potential, DNA fragments with low melting points will effectively stop moving earlier than fragments with a higher melting point, thus leading to the separation of these DNA species. Gradient gel methods usually employ the use of a denaturation-resistant clamp to anchor one end of the PCR product and to help stabilize its melting behavior. The clamp can contain a string of G:C base pairs introduced at the 5′ end of one of the primers (57) or a photoreactive cross-linking group (psoralen), which is activated after PCR (58).

The differences between DGGE, TGGE, and TTGE reside in the manner in which double-stranded DNA fragments are induced to melt. DGGE employs a gradient of chemicals, usually a mixture of urea and formamide, which interfere with DNA base-pairing, held at a constant temperature (usually 60 °C) submerged in a buffer-filled waterbath throughout the procedure. As double-stranded DNA passes through the gel during the course of electrophoresis, it comes to a concentration of denaturants that, in combination with the elevated temperature, induces the amplified fragment to denature. TGGE employs a more specialized apparatus, in which the gel is cast with a constant concentration of these chemicals, and is run horizontally against a hot plate. The hot plate increases in temperature toward the end of the gel, with the effect that double-stranded DNA passing through the gel is exposed to a linearly increasing gradient of heat, until it denatures and is retarded within the matrix. TTGE is a similar concept. The gel is again made with a constant concentration of denaturant and submerged in buffer. As the gel-run proceeds, the buffer tank increases in temperature in a linear fashion, such that DNA fragments of increasing melting point migrate longer before denaturation and retardation.

These are rapid and powerful methods for monitoring changes in community structure over time or space. Multiple samples can be compared side by side and across gels, provided that migration standards are included in each run (20,59). Comparison of banding patterns can give some measure of similarity among different samples analyzed, often expressed as dendrograms based on band sharing or total lane similarity (60–62). Sequence information can be obtained from individual DGGE bands by band excision, DNA elution, reamplification, and sequence determination. The partial 16S rDNA sequences obtained can be subjected to phylogenetic analysis, thus providing clues to the identity of dominantly present or active community members (63–66). Alternatively, phylogenetic information can be derived from DGGE banding patterns by hybridization analysis. In this procedure, DNA from the DGGE gel is blotted onto a support membrane, and hybridization follows with a number of phylogenetically informative probes, in a fashion similar to forward sample array hybridization (20,27,67).

However, some severe limitations exist. Not all DNA fragments are suitable for denaturing gradient analysis, because of unstable melting behavior or size limitations. The typical maximum size of DNA fragments used in such analyses is 500 to 600 base pairs, although adaptations allowing analysis of fragment sizes of up to 1.5 kb have been reported (68). Also, not all DNA fragments exhibit stable melting behavior, potentially leading to fuzzy bands or multiple-banding patterns. Many PCR strategies use

degenerate primers, which can lead to multiple bands from a single-template sequence, especially when degeneracies are located in the primer not containing the GC-clamp (66). Furthermore, base pair differences that do not affect fragment melting behavior (and thereby mobility) may go undetected, and multiple sequence differences that have opposing affects on fragment mobility may serve to cancel each other. Thus, identical band migration does not necessarily indicate sequence identity, and distance between bands cannot be used as an indicator of phylogenetic relatedness. In all cases, it is essential that the parameters for optimum band separation be determined empirically for each set of primers to be used.

A common mistake made when interpreting DGGE banding patterns is to assume that the number of bands recovered accurately provides a measure of microbial diversity. This grossly oversimplifies the issue of diversity, which has been controversial among ecologists for many years. Diversity seems an intuitively obvious concept, but is difficult to quantify. It consists of two parameters, species richness and the evenness of the distribution of species in a given sample. A sample containing 100 species of even distribution is considered more diverse than one containing numerically 99% of one species, and an even distribution of the remaining 99. Gel patterns, as currently interpreted, are not adequate to assess microbial diversity. First of all, only the most common community members are represented in the banding pattern (ribotypes that represent at least 1 to 2% of the entire target community). DNA fragments that are represented in low numbers will fall into the background. The most diverse communities (i.e., with many evenly distributed species) will produce so many faint bands that the end result will be a smear across a range of denaturant concentrations. Figure 5 shows an example of a community approaching this state. The bacterial community of a sample site highly contaminated with chromium was analyzed at three depths by PCR-DGGE. The surface community was too diverse for any dominant ribotypes to be identified by band excision. The pattern simplified with depth, such that at the greatest depth, the most abundant organisms were easily identified by band excision and sequence analysis. Counting the visible bands alone would suggest that the community became less diverse with depth. However, the richness of the species may have remained equal, or even have increased. DGGE cannot assess this. Such methods assess only the evenness of species distribution (69). Øvreas and coworkers (70) provide a good example of how the issue of diversity can be approached by combining methods, during a study of the effects of methane on microbial communities in soil. In this case, the native microbial community, whose diversity was determined to be high by total DNA reassociation (a gram of soil containing about 8,000 fold the complexity of an *E. coli* genome), produced a smear on PCR-DGGE with bacterial specific primers. Methane treatment reduced the diversity of the soil community by about 20 fold, resulting in some distinct bands by DGGE. More sensitive detection systems, such as those employing fluorescent dyes and digitized imagers (71), may help to increase the resolution of DGGE gels, but such problems are difficult to solve for highly complex systems.

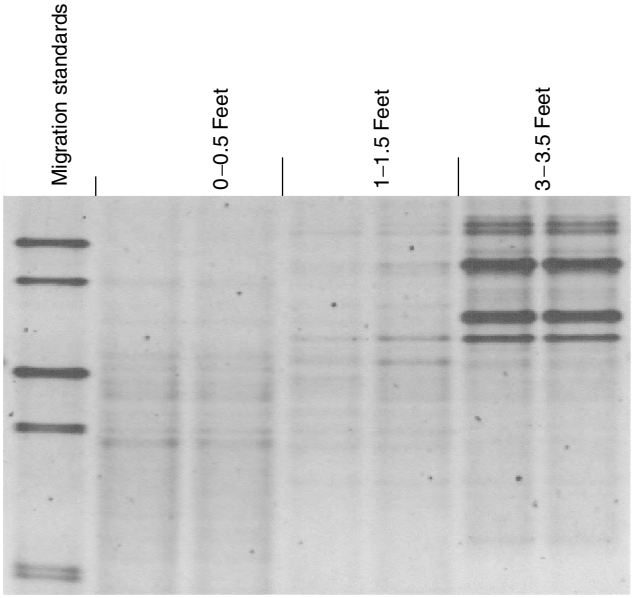

Figure 5. PCR-DGGE profiles from a chromium contaminated soil by depth. The complexity of the banding pattern decreased with depth, indicating a decrease in the evenness of distribution of the species present. All species indicated by bands in the surface sample may have been present at equal numerical abundance in all samples, but their signal was lost due to the increasing relative abundance of a few organisms. The "usefulness' of the method may have been considered to have increased with depth, as the remaining bands could be excised and sequenced to provide information on the phylogenetic positions of the source organisms, that were mostly ε-proteobacteria and members of the *Bacteroides-Flexibacter-Cytophaga* phylum. GenBank accession numbers AF188401-AF188413.

One way of reducing the complexity of community analyses to a manageable level is to target only certain monophyletic groupings within a particular PCR-DGGE analysis. Specific PCR primers have been used in combination with DGGE to study the distribution and diversity of a variety of bacterial groups (66,72,73). Such analyses can also be designed to target eukaryotic microorganisms (61,65).

An additional complicating factor is the possible presence of rDNA sequence heterogeneity within a single bacterial cell. Slight sequence differences between rRNA operons can lead to the production of multiple DGGE bands from a single pure culture (74). Furthermore, the presence of heteroduplex molecules may lead to additional artifactual DGGE bands. Such molecules occur when single-stranded DNAs of less than 100% complementary anneal to each other instead of to their exact complements during the PCR stage. This can easily occur within mixed PCR products, especially if amplification conditions are less than optimum and complementarity between DNA fragments is high. Such heteroduplex molecules exhibit different melting behavior than their homoduplex counterparts, leading to the detection of spurious bands. The heteroduplex nature of DGGE bands can be examined by cutting out the band in question, reamplifying it, and subjecting the subsequent product to another round of DGGE. Homoduplex bands produce a single band, whereas

heteroduplex bands form two to four bands (75). Thus, one must exert caution when interpreting DGGE gels with respect to diversity assessments. Similar warnings also apply to the use of other community fingerprinting techniques, such as those described below.

Single Strand Conformational Polymorphism (SSCP)

Like the techniques described earlier, single strand conformational polymorphism (SSCP) analysis uses sequence-dependent mobility differences to separate mixed PCR products into their constituent DNA populations. In this technique, all PCR products are first denatured. The resulting single-stranded DNA (ss-DNA) molecules are then allowed to fold upon themselves, thus producing a sequence-dependent secondary and tertiary structure, that is determined by the potential for intramolecular base pairing. The mobility of these folded ss-DNA molecules in a nondenaturing polyacrylamide gel is determined by the shape and size of each molecule. Thus, each population of DNA molecules of a given sequence should in theory produce two bands on a gel, one for each DNA strand (76,77). Clearly, the analysis of ss-DNA eliminates the problem of heteroduplex bands present in other profiling methods.

Visualization of ss-DNA can be performed by a number of methods. Ethidium bromide detection is less efficient for ss-DNA than ds-DNA. Silver staining or stains that bind equally well to ss-DNA and ds-DNA are therefore most often used. Alternatively, radioactive or nonradioactive label can be incorporated during the PCR for DNA detection. One of the major advantages of SSCP is that each ds-DNA population produces two markers that can be used for comparative identification. For example, this may be helpful in using SSCP analysis to track an organism added to an environment. Although the sharing of two bands between samples by no means proves identity, the predictive value of two bands is far better than one. In practice, banding patterns can become highly complex, to the point where it becomes impossible to determine the bands that should be analyzed as pairs. The complexity of SSCP patterns can be reduced by labeling only one of the primers used in the PCR (78). This can simplify banding patterns significantly, but detects only one of the two ss-DNA molecules per DNA species. With the advent of automatic gel systems that can recognize multiple fluorescent dyes in a single gel lane, it should become possible to label both primers with different dyes to maximize the clarity and information of SSCP banding patterns.

As with DGGE, it is not valid to deduce biodiversity from the number of SSCP bands. In addition to the limitations addressed for DGGE, artifactual bands can be produced by competing single-strand formations of some molecules. Such additional bands have been referred to as unstable conformers (79) and can lead to multiple bands derived from a single DNA sequence. Also, the changes induced by sequence differences can range from minor (or none) to major, depending on how the overall base-pairing of a ss-DNA molecule is affected. Phylogenetic distances therefore cannot be inferred from differences in SSCP mobility.

The sensitivity of SSCP for the detection of sequence differences is thought to be comparable or slightly less than that of DGGE (80). Because SSCP relies on changes in intramolecular base pairing to detect sequence differences, base changes that do not affect the secondary and tertiary structure of the rDNA being analyzed may go undetected. This is a very real possibility given the conserved secondary structure of ribosomal gene products, and care must be taken to avoid targeting of regions with high levels of conserved complementary. In practice, ITS regions are often targeted by SSCP, where such de facto intramolecular complementarity is not present.

Like DGGE, SCCP analysis can also be coupled with the use of primers that are specific for microbial groups of interest and eukaryotes (81). In practice, SSCP is most useful in the description of either simple communities or a narrow subset of the total community. Although it can be used as an effective tool to detect and track known isolates (by comparison to standards), SSCP results can become very difficult to interpret when applied to more complex issues of community structure.

Community Profiling Based on Restriction Digestion: ARDRA and T-RFLP

Amplified ribosomal DNA restriction analysis (ARDRA) is based on cleavage of PCR amplified rDNA products with a suite of Type-II restriction endonuclease enzymes chosen to cleave rDNA in the less well-conserved regions of the molecule (82–84). The cleavage products are separated by size using gel electrophoresis. Like the other fingerprinting methods described here, ARDRA generates an "at a glance" view of how similar or different multiple bacterial communities are, without prior knowledge of the organisms involved. This method, in its simplest form, does not require any specialized equipment over that which is normally found in a molecular biology laboratory, which is its primary advantage. Its primary disadvantage is that, although it conveys useful information on whether large differences between communities exist, the nature of those differences in terms of the species or phylogenetic groups effected is not readily available. Carrying out hybridization analyses with probes specific for conserved rDNA regions before excising bands from a duplicate gel may help overcome this, but the complexity of these operations removes the advantage of speed from ARDRA as an analytical approach. Some of these objections can be overcome by discarding the majority of ARDRA products and analyzing only those corresponding to either the 5′ or 3′ end of the molecule, a technique termed *terminal-restriction fragment length polymorphism* (T-RFLP).

(T-RFLP) analysis of rDNA amplified from bacterial communities provides the same "at a glance" evaluation of community differences as those methods described earlier (Fig. 6). However, the potential to derive some phylogenetic information directly from the banding pattern is currently unique to T-RFLP. Its growth in popularity is due to the ready availability of the necessary equipment, the generation of patterns that are easily compared between gels, and the ability to make phylogenetic predictions from the fingerprint generated. This area has recently been reviewed by T. L. Marsh (85),

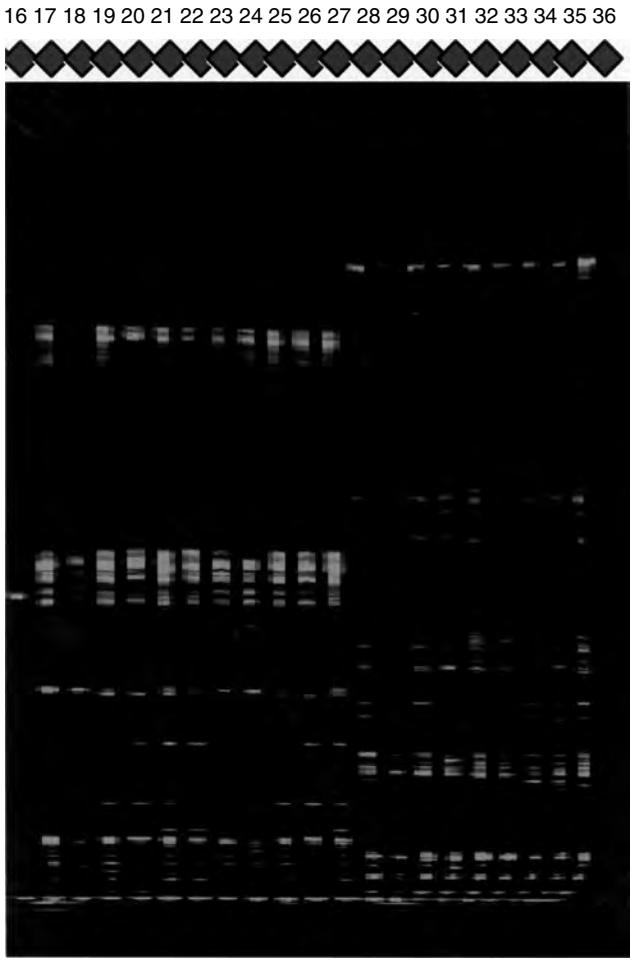

Figure 6. T-RFLP analysis of bacterial communities. This digital image was generated by an ABI377 automatic sequencing apparatus and shows lanes 16–36 of a T-RFLP gel. Lane 16, the single band was produced from a pure culture of *Alcaligenes eutrophus*. Lanes 17–27 are profiles from multiple DNA extractions from subsamples of a control soil, and lanes 28–36 are from a contaminated plot. The profiles of the control and contaminated soils are clearly different, demonstrating that the bacterial community has responded to the presence of the contaminant. Courtesy of Terrence L. Marsh, Department of Microbiology, Michigan State University, U.S.A. See color insert.

who largely developed the method (86). Of the gel-based PCR-ribotyping methods, this shows the greatest potential for generating additive information allowing researchers to compare their results to those of others. A facility allowing phylogenetic inference from T-RFLP banding patterns by comparison to those predicted from published data has been created by Michigan State University at *http://www.cme.msu.edu/RDP/html/analyses.html* (5).

T-RFLP begins in the same manner as ARDRA, with the exception that one of the PCR primers is 5′ end labeled with a fluorescent tag. Restriction endonuclease digestion is carried out with a single enzyme for each gel run. The choice of enzyme is usually based on a priori knowledge of the first occurrence of a recognition site for a given enzyme in a given taxonomic group. This is 3′ to the 5′ end of the molecule if the forward PCR primer was labeled, and 5′ to the 3′ end of the molecule if the reverse PCR primer was labeled. rDNA fragments amplified from a given community are usually digested with several enzymes to recover information pertaining to a range of taxonomic groups. Accurate sizing of the terminal restriction fragments is essential for taxonomic purposes, and cannot be achieved by conventional agarose gel electrophoresis. Rather, the digested products are mixed with a molecular weight ladder prestained with a different fluor, then separated using an automated DNA-sequencing apparatus. The apparatus detects both fluors as they pass a laser-detection system, and custom software can determine the length of T-RFLP fragments to within 1 to 2 bases by comparison to the molecular weight standards. It is this level of sizing accuracy that provides strong advantages of T-RFLP over the other PCR-based ribotyping methods described in this article.

A major criticism of this approach is the dependence on published data for phylogenetic interpretation. Currently, environmental samples are usually dominated by organisms that have no close relatives in the sequence databases, and therefore placement within this frame of reference based on T-RFLP patterns may be seriously flawed. Phylogenetic conclusions based on T-RFLP data must, at present, be complemented by the use of another community profiling method capable of recovering sequence information directly.

None of the methods mentioned earlier are capable of discriminating between very closely related organisms within a sample–an unavoidable consequence of the slow rate of mutation of rDNA genes. Most mutations in the regions between rDNA genes, however, including large insertions and deletions, have no effect on the fitness of the cell. Such approaches have a number of acronyms, but the term RISA is preferred.

Ribosomal Intergenic Spacer Analysis (RISA)

Ribosomal intergenic spacer analysis (RISA) is another PCR-based community profiling method, and exploits conserved sequences within the 16S and 23S rRNA genes as priming sites to elucidate the sequence of the region separating them. This region is so variable that even a single organism with multiple rRNA operons may show considerable sequence differences in the spacer regions separating its 16S and 23S molecules (12,13,87). Several suitable sites exist in the 16S rDNA for the design of PCR primers facing the 23S rRNA gene; the 23S rRNA gene encodes a single conserved site at its 5′ end suitable for a PCR primer oriented toward the 16S rDNA. Thus, for a given isolate, several fragments can be amplified by RISA, containing varying amounts of the 3′ end of the 16S rDNA, the entire intergenic spacer, and a short fragment of the 5′ end of the 23S rDNA. The intergenic spacer may vary considerably in length between even closely related species. When analyzing a complex mixture of species therefore, as in an environmental sample, a simple size fractionation of the PCR amplification product by polyacrylamide gel electrophoresis is sufficient to generate a fingerprint of the dominant species within the target group. When considering unknown bacterial communities, the sequence of the highly variable intergenic spacer is

not likely to be useful in determining the phylogenetic placement of the source organism. However, the 16S rDNA portion of the product can be used for this purpose, and the intergenic spacer then provides a fine-scale marker for the dominant organisms found in the sample. This subject has been reviewed recently (11).

Rather than relying solely on size separation, this approach can be combined with other fingerprinting techniques such as DGGE, although fragment size restraints may be a limiting factor (88).

Quantitative Interpretation in PCR-Based Community Fingerprinting

Making quantitative statements relating a gel band to cells per gram of environmental matrix is complex, contentious, and has not been frequently attempted. Even the assumption that the source organism of a strong band was more abundant in the environmental sample than one generating a weaker band is open to considerable criticism, and must be experimentally justified (20,66,89). Reasons for this include uncertainties over lysis efficiency, DNA–RNA recovery and the relative efficiency of the PCR–RT-PCR in recovery of different sequences (14,90). Such difficulties are by no means restricted to the environmental microbiology laboratory, and have spawned lively debates in many branches of the life sciences (91). The reader is directed to the following articles as an introduction to this subject (14,39–42,92). An obvious concern is the level of match between primers and the target sites in the 16S rDNA of a largely unknown and complex template. Figure 3 demonstrates that while a given primer pair may amplify a single target in isolation efficiently, amplification of that target may be completely abolished in competition with sequences that have a better match to the primers used. Compared to questions of lysis efficiency of unknown organisms during nucleic acid extraction from environmental samples, investigation of the dynamics of the PCR step is a relatively simple area of study. Recently attempts have been made to introduce a quantitative aspect to this using defined mixtures of templates (40), "Real-Time" PCR (93), and the inclusion of a specially designed internal standard (38). All studies agree that the use of more PCR cycles than are necessary to generate a useful product damages the fidelity of the ratio of templates within the reaction. Few laboratories currently have the facilities to optimize the number of cycles used in the PCR routinely. This is not a trivial operation when handling large numbers of environmental samples from diverse sample types, as the amount of template DNA used in the reaction is hard to standardize, and the extent of inhibition of the polymerase by sample-derived contaminants is not uniform between samples. This problem can be overcome by using real-time PCR equipment, which can monitor the point at which log-linear amplification ceases; however, such equipment is not yet standard even in the best-funded laboratories. The use of internal standards may have a more widespread applicability, as no additional equipment is required over that necessary for standard PCR-based ribotyping. However, the extent of PCR optimization and characterization of amplification efficiencies required is difficult and time consuming, and therefore eliminates many of the advantages that these methods hold over non-PCR-based ribotyping attempts. These problems are compounded by the variety of subtle differences in procedure between laboratories, that include differing nucleic acid extraction protocols, thermocyclers, enzymes, enzyme enhancers, and gel visualization equipment. In the absence of standard procedures, quantitative interpretation of ribotype banding patterns is not likely to become a common procedure, and investigators must word their findings carefully to reflect these concerns.

CONCLUSION

From the foregoing, it should be clear that no one ribotyping method can be guaranteed to provide a complete overview of a bacterial community. These methods have evolved rapidly over the last decade, and refinement continues to be rapid. The power of gel-based methods has not been fully realized as yet because of the need to develop mathematical frameworks to compile profiles such that they can be used in descriptive modeling of field-scale microbial communities (94,95). It is likely that in the near future, PCR-based methods may be entirely superseded by hybridization-array technologies, which avoid the uncertainties and biases of the amplification step. However, it is clear that the understanding of microbial diversity, even at the rRNA gene sequence level, is still far from complete. As the design of hybridization arrays depends entirely on the available sequence information, the continued use of PCR-based methods to generate sequence data and complete our view of microbial diversity is a prerequisite to the development of comprehensive arrays. Ribotyping information also provides an invaluable insight into the function of unculturable organisms, providing direct links to their functional genomics (96). A major technological constraint to the generation of sequence data from the gel-based ribotyping methods is recovery of bands, which usually requires manual excision. Related sections in this volume describe the application of capillary electrophoresis as an improvement to the use of polyacrylamide gels (see Capillary Electrophoresis in Genetic Analysis and Ribotyping of Microbiota in the Environment, this Encyclopedia), and development of microchip hybridization arrays for automated analysis (see Microarrays: Applications in Environmental Microbiology, this Encyclopedia).

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RIBOTYPING OF MICROBIAL COMMUNITIES.

See Biochip-Based Devices and Methods in Microbial Community Ribotyping

RICE AGRICULTURE.

See Flooded Soils

RICE SOILS.

See Flooded Soils

RISK ASSESSMENT.

See Field Release of Genetically Engineered Microorganisms (GEM)

RISK ASSESSMENT OF ENVIRONMENTAL EXPOSURE TO VIRUSES

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Risk is the possibility or likelihood of an adverse effect occurring due to consequences set forth from a particular hazard or hazards. A hazard may be a chemical, a landfill, a person's behavior, or a microorganism such as a virus. Such hazards may affect the survival of an ecosystem or the quality of human life. Potential hazards may be defined and evaluated using a risk assessment methodology to determine associated risks. A risk assessment paradigm has been developed considering chemical hazards (1,2), but this approach has been modified and implemented to address the human health risks associated with exposure to microorganisms such as bacteria, protozoa, and viruses found in the environment (3–7). Viruses, in particular, are of interest because of their role as waterborne agents of disease and to the wide range of human health outcomes associated with viral infections. Information from virus risk assessments can be used to help policy makers make informed decisions concerning the risks that such hazards pose.

THE RISK-ASSESSMENT APPROACH

The Development of the Risk-Assessment Paradigm

Risk assessment is a component of the risk analysis process that involves risk assessment, risk management, and risk communication (8). Risk management is a decision-making process that applies the information learned from a risk assessment and incorporates judgment in interpreting and evaluating the results. Risk managers then attempt to convey this information to the public (risk communication) in a way that all interested persons understand.

The science of risk assessment involves evaluating the risks posed to a society or the environment for a better understanding of the scope of the problems that may result from exposure to such hazards. The National Research Council (NRC) has attempted to improve the policy-making process of the U.S. government by developing the methodology necessary to evaluate risks posed by various hazards. The risk assessment paradigm was initially developed in the early 1970s to form a partnership between science and government that would help educate the government on current scientific issues. This was in response to the need for improved hazard identification to meet the mandates of the Safe Drinking Water Act and Clean Air Act Amendments. This framework was developed considering chemical hazards and has been used to assess chemicals in the environment for more than 25 years (1).

MICROBIAL RISK ASSESSMENT: ITS DESIGN AND APPLICATION FOR VIRUSES

The risk-assessment paradigm initially developed by the NRC to address human exposure to chemical hazards has been used to evaluate the human health impact of exposure to microorganisms in food and various water supplies (4–7,9–17). Human health outcomes from exposure to microorganisms can be evaluated quantitatively through a formal risk-assessment approach using dose-response information for specific microorganisms.

There are four fundamental steps in the risk-assessment framework: (*1*) hazard identification, (*2*) dose-response assessment, (*3*) exposure assessment, and (*4*) risk characterization (1). Each of the first three steps is an integral component of the fourth step, risk characterization. The original (chemical) NRC risk-assessment paradigm provides the foundation for the risk-assessment methodology for microorganisms; however, there are various factors pertaining to microorganisms that need to be considered when conducting a risk assessment. The following sections discuss each of the four steps of the risk-assessment process, the issues that should be addressed while considering risks associated with microorganisms (e.g., viruses), and associated human health information obtained from such assessments of viruses.

Hazard Identification

A substance is determined as harmful or otherwise on the basis of laboratory and field data as well as information obtained from epidemiological studies. In the original (chemical) risk-assessment approach, hazard identification defines a hazard by presenting evidence of a causative relationship between the agent and the adverse effect. However, in microbial risk assessment, a broader scope of information is used. A complete description of the resulting human illnesses are identified (both chronic and acute), including severity ranges and the immune status of the populations affected. Morbidity, mortality, hospitalization ratios, and any information on endemic and epidemic diseases are also included here. It is important to present this information when conducting risk characterizations to provide a more complete description of the hazard. Completing the hazard identification step of the paradigm can be challenging if there is no available epidemiological surveillance information. This is often the case for viruses in terms of fully understanding associated endemic and epidemic disease. Waterborne outbreaks are not reportable in the United States, so the available information of such events probably represents only a small fraction of what actually occurs.

Human Health Outcomes Associated With Viral Infections. Human exposure to enteric and respiratory viruses can cause a wide range of health outcomes. "Infection" is the colonization of growth of a microorganism in the body. Infection does not ensure that clinical illness will result from that infection; often infections with enteric viruses are associated with asymptomatic infections, particularly infections with some of the enteroviruses. Whether clinical illness (morbidity) will manifest from a viral infection will depend on the type, strain, and infectivity of the virus, its route of transmission, and the immune status and age of the host (9,10,18). Morbidity ratios (which represent the number of individuals who become ill from all those infected) for enteric viruses of 10% (for poliovirus) and of as high as 97% (observed with hepatitis A virus) have been documented (Table 1) (19–21). In children less than five years of age, hepatitis A virus infections are usually asymptomatic but the morbidity ratio among adults is 75%. Conversely, children infected with rotavirus are more likely to show signs of symptomatic illness (gastroenteritis) than adults (22). The amount of exposure (level of dose) has not demonstrated a role in illness development when

Table 1. Occurrence of Clinical Illness for Viral Infections (19–26)

Virus	Morbidity Ratio (%)
Astrovirus	12.5
Coxsackievirus type A16	50
Coxsackievirus type B	5–96
Echovirus	50
Hepatitis A (adults)	75
Hepatitis A (children)	5
Poliovirus type 1	0.1–1*
Rotavirus (adults)	40–56
Rotavirus (children)	72

*paralysis

considering a highly infective virus such as rotavirus (23). Therefore, it is assumed that developing a clinical illness from an infection is a "conditional probability" that is independent of exposure level (6).

Depending on the particular virus and the serotype, a wide range of health outcomes can result from a viral infection. There are two types of coxsackievirus (types A and B) and 29 serotypes, and this virus in particular is associated with a greater number of serious illnesses when compared with any waterborne virus, including aseptic meningitis, respiratory illness, myocarditis, pericarditis, pleurodynia, encephalitis, insulin-dependent diabetes mellitus, and gastroenteritis (27–33). While considering the other enteroviruses, echovirus infections also cause gastroenteritis, respiratory infection, myocarditis, pericarditis, and aseptic meningitis, but outcomes are not as severe as those associated with the coxsackieviruses (18,32). Poliovirus infections can result in paralysis but has not been a serious public health concern since the introduction of the poliovirus vaccine in the 1950s.

Rotavirus is the most common cause of viral gastroenteritis worldwide and is considered the most infectious waterborne agent known (4). Adenovirus is considered only second to rotavirus in terms of its significance as a pathogen of childhood gastroenteritis (34,35). There are 49 types of adenoviruses and human health outcomes resulting from infections include acute respiratory disease (ARD), conjunctivitis, urethritis, cervicitis, and hemorrhagic cystitis. Norwalk and Norwalk-like viruses are the primary agents responsible for adult gastroenteritis (36,37) and astrovirus-related diarrhea has been observed throughout the world in children and the elderly (38–43). Infections with hepatitis A virus cause liver damage and jaundice. Hepatitis E virus (formally known as enterically transmitted non-A, non-B hepatitis) is responsible for more than 50% of the acute cases of hepatitis occurring in developing countries (44). Typical clinical signs of infection include abdominal pain, nausea and/or vomiting, fever, and jaundice with the highest percentage of symptomatic cases occurring in young-to-middle-aged adults (20–40 years of age) (45).

Viruses causing respiratory-related illnesses include the influenza and parainfluenza viruses, rhinovirus, coronavirus, adenovirus, and respiratory syncytial virus (RSV), RSV being a leading cause of lower respiratory tract illness in children (18). RSV may also be responsible for as many as 40% of cases of childhood pneumonia and as much as 90% of bronchiolitis cases (46). RSV infections may result in bronchitis, otitis media, and upper respiratory disease. The parainfluenza viruses contribute significantly to illnesses of the lower respiratory tract in children (47). The different types of parainfluenza viruses can cause a variety of health outcomes in children including croup, pneumonia, and bronchiolitis. The influenza viruses account for worldwide epidemics of febrile respiratory disease that result in persons seeking medical attention and hospitalization. Health outcomes of infection can range from asymptomatic infections to flulike symptoms (fever, chills, myalgia, and sore throat) to death. Influenza illnesses can lead to secondary bacterial infections that result in myocarditis, pericarditis, pneumonia, meningitis, encephalitis, and Guillain-Barré syndrome. There are more than 100 serotypes of rhinovirus with most infections causing symptomatic respiratory illness (18). Common symptoms include cough, sneezing, sore throat, and nasal congestion. Children are more prone to infections, particularly children under one year (48). It has been suggested that as many as 35% of common colds are caused by coronaviruses (49,50). Many of the 49 adenovirus serotypes cause respiratory-related illnesses including acute respiratory disease (ARD), acute febrile pharyngitis, and pneumonia (51). Adenoviruses are attributable to 2 to 7% of all lower respiratory tract illnesses (52) and as many as 10% of the cases of childhood pneumonia (53).

Some viral illnesses may require hospitalization and thus the severity of specific viral infections is measured as a "hospitalization ratio." Hepatitis A virus, for example, can cause severe damage to the liver and is associated with the highest hospitalization ratios when compared with other waterborne viruses (18). Diarrhea caused by rotavirus has been shown to be the most common cause of hospitalization for childhood diarrhea in the United States (54) with an estimated hospitalization ratio of 0.5% per year for rotavirus-related illness in children below than five (55). Hospitalizations for coxsackievirus aseptic meningitis have occurred during common source outbreaks involving as many as one of every three with the illness (56). A hospitalization ratio of 2.2% has been documented during a food-borne outbreak of Norwalk virus affecting students on a college campus (almost half of persons ill sought some medical attention) (57). Hospitalizations are also observed during epidemics of influenza in the United States in which 16.9 per 10,000 cases were hospitalized during the 1980 to 1981 outbreak (58).

Death can result from viral illnesses and mortality (case/fatality) ratios from 0.0001% to as high as 0.94% have been observed for the waterborne viruses (Table 2). It is estimated that 300 deaths caused by diarrheal illnesses occur each year in the United States among children less than five years of age and approximately 20 to 40 of these deaths can be attributed to rotavirus diarrhea (62–65). Mortality ratios greater than 50% have been observed in transplant patients with rotavirus illnesses (66). It is important to realize that information on mortality for many of the viruses such as the enteroviruses actually refers to hospitalized cases because of the lack of a

Table 2. Mortality Ratios for Viruses (9,26,59–61)

Virus	Mortality Ratio (%)
Adenovirus	0.01
Coxsackievirus type A	0.12–0.5
Coxsackievirus type B	0.59–0.94
Echovirus type 6	0.29
Echovirus type 9	0.27
Hepatitis A	0.60
Norwalk	0.0001
Poliovirus type 1	0.90
Rotavirus	0.01

reporting system in the United States for these viruses (except for poliovirus); thus *mortality ratios* reflect those who died from an illness severe enough to require hospitalization. Death due to hepatitis A virus is twice as high among the hospitalized cases than among the nonhospitalized cases (26).

Age and immune status of the host as well as the virus characteristics that determine whether illness will manifest from an infection also influence the probability of mortality from that illness. Persons above 40 years of age, for example, have a greater chance of dying from a hepatitis A infection than younger cases (67). The elderly (persons above 65) have a greater risk of dying from enteric and respiratory virus infections (36,61) and as many as 90% of deaths caused by influenza occur in this population (58). Mortality is also higher among other immunocompromised populations, such as AIDS patients, transplant recipients, and pregnant women. Mortality because of adenovirus infections has been observed to be as high as 60% among transplant patients (68,69). Hepatitis E virus infections are associated with an unusual high case-fatality ratio among pregnant women (17 to 33%) (70,71) with death resulting from hemorrhage.

Transmission. Viruses may be transmitted from host to host through person-to-person (direct) contact or through transmission routes that involve more than one medium (e.g., water, food, and fomites) (18). The main routes of entry into the host include ingestion or inhalation, with some viruses (e.g., the enteroviruses) being able to enter the host via both the respiratory tract and through a fecal-oral transmission route (gastrointestinal tract). Ingestion of microorganisms (e.g., viruses) may be achieved through contaminated drinking water, recreational water, food, soil, and fomites. Fomites, in particular, may contribute to the complex transmission routes of some viruses. Depending on the type of virus and its medium, the type of inanimate surface, and the temperature and humidity, enteric viruses are capable of surviving on inanimate objects for a few hours to as long as many days (72). It is important to understand the transmission route of a particular virus while conducting a human health risk assessment because the infectivity of a microorganism may differ from one route to another. Specifically, the number of microorganisms required to infect the respiratory tract is usually less than that required for infection when transmittance is by ingestion.

Secondary transmission, subsequent person-to-person transmission, magnifies the impact of exposure to microorganisms (73–75) and is not a consideration while assessing risks associated with chemical exposures. Secondary attack rates for enteric viruses have been reported to range from 30% for Norwalk virus to greater than 90% for poliovirus (10). Person-to-person transmission is not commonly observed in the case of some viruses that are transmitted fecal-orally such as hepatitis E virus.

Disease Outbreaks. Both waterborne and food-borne outbreaks of viruses have been documented in the United States and throughout the world. In most outbreak situations, the etiological agent of disease is not identified. It has been estimated that as many as 10,000 cases of microbial waterborne disease occur each year in the United States with three to five outbreaks associated with community drinking water systems documented annually (18). Outbreaks involving water systems that meet microbial quality standards have occurred (76–79). From 1946 to 1980, the responsible agent was not identified in more than 50% of the documented waterborne outbreaks in the United States (76); enteric viruses were identified as the cause of 12% of these outbreaks. Although waterborne disease outbreaks have been documented for some of the enteric viruses, the occurrence of these outbreaks are underestimated due to the underreporting of outbreaks involving water. When an outbreak investigation is conducted, however, it may be particularly difficult to identify the causative agent because of the lapse of time that may have occurred between exposure of a population to contaminated water and the onset of the water analyses. In many cases when the causative agent of an outbreak is not identified, enteric viruses are suspected to be the cause of the outbreak because of the nature of the resulting disease and the attack rates observed (36).

Waterborne outbreaks have been associated with Norwalk and Norwalk-like viruses (36,37,80), rotavirus (81–84), adenovirus (85–88), coxsackievirus (89–90), hepatitis A and E viruses (90–93), echovirus (94), and astrovirus (95). Norwalk viruses are the primary agents responsible for adult gastroenteritis and it has been estimated that 33 to 65% of the nonbacterial gastroenteritis outbreaks in the United States are due to Norwalk viruses (36,37,96). Norwalk has also been implicated in outbreaks involving contaminated commercial ice (97). Outbreaks of waterborne rotavirus have been associated with either direct fecal contamination of a water supply or improper treatment. Hepatitis A and E viruses have been responsible for numerous waterborne outbreaks worldwide with hepatitis E outbreaks occurring in countries with poor sanitation (98). Disease outbreaks involving recreational water caused by adenovirus, coxsackievirus, and echovirus result in cases of gastroenteritis, conjunctivitis, and/or pharyngoconjunctival fever.

As with waterborne outbreaks, the etiological agent in nearly half of the documented food-borne outbreaks in the United States is never identified (7) (see VIRUSES AND PROTOZOAN PARASITES IN FOOD, INCLUDING METHODOLOGY, this Encyclopedia). Although the majority of the food-borne outbreaks are caused by bacterial pathogens, viruses have also been associated with food-borne outbreaks, particularly Norwalk and Norwalk-like viruses, hepatitis A virus, and astrovirus (95,99–105). Consumption of contaminated shellfish and the unhygienic practices of food handlers have contributed to their occurrence. Norwalk-like viruses are considered the most common food-borne pathogens (106) and Norwalk/Norwalk-like viruses are identified in 90% of the food-borne outbreaks of viral origin (107). These viruses have been implicated in outbreaks involving lettuce and bakery goods that possibly resulted from the contamination of food by food handlers (108–110). In one documented outbreak of Norwalk virus involving salad, the lettuce had been contaminated by raw shellfish that had been prepared in

the same sink (108). In England, gastroenteritis is commonly associated with the caliciviruses (which include Norwalk) that result from consumption of contaminated shellfish (100). Norwalk virus has also been implicated in outbreaks involving airborne transmission (111,112). Food-borne outbreaks of hepatitis A virus and astrovirus involving shellfish consumption have also been documented (95,99,103). Hepatitis A has frequently been associated with contaminated salad items in the United States (113) and has recently been implicated in outbreaks in Finland (114); it is only rarely connected with food-borne outbreaks in the United Kingdom (115).

The risk assessment process may stop with "hazard identification" if no adverse effect is found or if it is decided, based on current information, that a regulatory action take place. It is pertinent that the assessor should be able to recognize fallacies or biases in the available information on a particular hazard and be selective about what information is used. The ability to accurately interpret the information presented in the scientific literature is critical.

Dose-Response Assessment

The dose-response assessment involves describing the relationship between the dose of the virus and the incidence, or the extent of the adverse human health effect. The goal of this step is to determine the probability or likelihood of a human health consequence occurring from exposure to different levels of microorganisms. The human health consequence may be infection, illness, or death. As mentioned earlier, infection is the colonization or growth of the microorganism in the host, which may or may not lead to clinical illness. The probability of illness depends on several factors related to the particular virus and the host. The type and strain of the virus can influence whether clinical illness will develop but there does not seem to be a correlation with the level of dose a host receives through ingestion, as shown with a highly infectious virus such as rotavirus (23). Host factors influencing disease outcome include age, nonspecific host factors (such as preexisting state of nutrition), and preexisting immunity (10).

Dose-response studies using human volunteers have been conducted for several viruses including rotavirus (23), coxsackievirus (116,117), hepatitis A virus (118), poliovirus (119–122), echovirus (123), and adenovirus (124). This is unlike the dose-response assessment of chemical risk, which involves the extrapolation of animal data to humans. This has not been a primary issue in microbial risk assessments because dose-response models have been developed based on studies involving humans. In human dose-response studies, volunteers are exposed (via ingestion or inhalation) to variant doses of a microorganism and the fraction of volunteers that become infected are observed and recorded. It is necessary to use high concentrations of pathogenic microorganisms in order to observe high frequencies of infections with a minimum number of volunteers. Although the application of lower doses of microorganisms would reflect a more realistic picture of exposures to a population, studies involving greater numbers of volunteers (i.e., increasing the costs) would be required to demonstrate a dose-response relationship. Mathematical models must therefore be used to extrapolate the available data from human-feeding studies to demonstrate the effects of exposure to low doses of microorganisms.

Mathematical models have been evaluated for their plausibility in representing or simulating the microorganism–host interaction; in particular, a quantitative evaluation of risks of waterborne microorganisms has been conducted (3). A model is considered biologically plausible in describing the microorganism–host interaction if it considers two factors (18). A model must first consider that a human exposure contains a random distribution of microorganisms; therefore, an exposed population would experience a distribution of doses. The second factor to be considered is the ability of microorganisms in an exposure to initiate infection at a particular site within the host. This is dependent on the host ingesting at least one pathogenic microorganism and that a proportion of the consumed microorganisms will overcome the host's defenses and initiate infection (3). Infection (as opposed to illness or death) is the human health end point of interest in the dose-response assessment, which leads to a more conservative assessment of human health risks. Risks associated with illness and death can be assessed by incorporating morbidity and mortality ratios in the models.

The formulation of mathematical models that may represent a microorganism–host interaction has been described elsewhere (18). Humans may be exposed to pathogenic viruses through the consumption of water or food or through inhalation of aerosols. A proportion of individuals exposed to the microorganism, P_i, will become infected. Two mathematical models in particular have been shown to adequately describe the infection process demonstrated in dose-response studies of pathogenic microorganisms, including viruses. The exponential model

$$P_i = 1 - \exp(-rd) \tag{1}$$

(where P_i = the probability of infection, r = the number of microorganisms that survive and are capable of initiating an infection, and d = the number of microorganisms ingested or inhaled) assumes a random distribution of microorganisms in the exposure and a constant microorganism–host interaction. The parameter r is further defined as:

$$-r = \ln(0.5)/N_{50} \tag{2}$$

with N_{50} = the median infectious dose. The exponential model can therefore be rewritten as

$$P_i = 1 - \exp[\ln(0.5)(d/N_{50})] \tag{3}$$

to represent a dose-response relationship. If the microorganism–host interaction is a heterogeneous interaction where parameter r is not a constant but is defined by a beta-probability distribution (3,125,126), the infection process can be represented by the beta-Poisson model (a modified exponential model):

$$P_i = 1 - (1 + d/\beta)^{-\alpha}, \tag{4}$$

Table 3. Dose-Response Parameters for Specific Viruses*

Virus	Model	Parameters	Reference
Adenovirus	Exponential	$r = 0.4172$	124
Coxsackievirus type B4	Exponential	$r = 0.007752$	116
Echovirus type 12	beta-Poisson	$\alpha = 0.374$	123
		$\beta = 186.69$	
Echovirus type 12	Exponential	$r = 0.012771$	128
Hepatitis A virus	Exponential	$r = 0.548576$	118
Poliovirus type 1	beta-Poisson	$\alpha = 0.1097$	120
		$\beta = 1524$	
Poliovirus type 1	Exponential	$r = 0.009102$	122
Poliovirus type 3	beta-Poisson	$\alpha = 0.409$	121
		$\beta = 0.788$	
Rotavirus	beta-Poisson	$\alpha = 0.26$	23
		$\beta = 0.42$	

*α, β, and r are parameters of the dose-response interaction.

where P_i = the probability of infection, d = the number of microorganisms ingested or inhaled, and α and β represent parameters of the microorganism–host interaction. β can be further defined as:

$$\beta = N_{50}/(2^{1/\alpha} - 1), \tag{5}$$

which results in the following equation for the beta-Poisson model:

$$P_i = 1 - [1 + (d/N_{50})(2^{1/\alpha} - 1)]^{-\alpha}. \tag{6}$$

The exponential and beta-Poisson models are evaluated using the method of maximum likelihood (6) to determine which best fits the data obtained from dose-response studies. Parameters of the best-fitting model are then determined for each microorganism as well as upper and lower confidence limits. Such parameters and confidence limits for specific viruses have been reviewed elsewhere (127). Table 3 lists the best-fitting model and model parameters for specific viruses.

The beta-Poisson model is shallower than the exponential model but approaches the exponential model as α increases (and as the number of microorganisms required to initiate an infection increases), which represents a decrease in the variability of the microorganism-host interaction (Fig. 1). The beta-Poisson model represents a more gradual host response to exposure and this model best fits the dose-response data sets for many of the viruses (Table 3). Both the exponential and beta-Poisson models assume that only one pathogenic microorganism can cause infection in a host; threshold models have not been shown to reflect the dose-response relationship observed in experimental studies (18). Annual and lifetime risks of infection can be determined using the following equations:

$$P_{\text{year}} = 1 - (1 - P_i)^{365} \tag{7}$$

$$P_{\text{lifetime}} = 1 - (1 - P_i)^{25550} \tag{8}$$

Risk estimates of illness and death can also be computed from these models by incorporating morbidity and mortality ratios of that particular microorganism (6). The probability of developing clinical illness from an infection ($P_{D:I}$) is a conditional probability that an individual displays disease, given that they were infected. The development of symptoms depends on the type and strain of virus and host age. Morbidity ratios for viruses are listed in Table 1 again depending on the virus and host age. A morbidity ratio of 50% (the midpoint value) has often been incorporated in risk modeling for the enteric viruses (6). The risk of illness is determined by multiplying the risk of infection (P_i) by the morbidity ratio.

As discussed previously, mortality can result from illnesses caused by viruses with the conditional probability of such an occurrence ($P_{M:D}$) depending on the same factors connected with the development of clinical illness. Immunocompromised populations (which include the elderly, the very young, transplant recipients, and HIV-infected individuals) have a greater chance of dying from illnesses associated with enteric and respiratory viruses. Mortality ratios ranging from 0.0001% to 0.94% have been observed (Table 2). Host age, in particular, accounts for the observed range of mortality ratios for some of the viruses. For example, the probability of dying from a hepatitis A

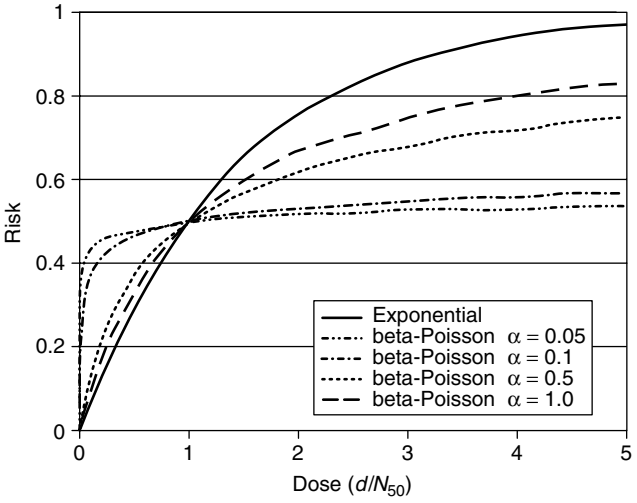

Figure 1. Exponential and beta-Poisson models on an arithmetic scale.

infection increases for individuals above 40 years of age (from 0.3% to 1.0%) (67). For viruses associated with nonreportable diseases in the United States such as some of the enteroviruses, the mortality ratios are based on hospitalized cases. The risk of death is calculated by multiplying the risk of illness ($P_{D:I}$) by the mortality ratio.

Multiple Exposures and Immunity. The effect of multiple exposures is not completely understood. Both infection and disease have been used as an end point in human-feeding and human-inhalation studies; however, the effects of multiple exposures have not. In microbial risk assessment, it is assumed that each exposure is statistically independent of another, however, the development of temporary or permanent immunity may alter this assumption. The immune status of the host also influences the human health outcome associated with most microbial exposures as has been observed among immunocompromised populations. The dose-response models described earlier reflect data formulated for a single exposure of microorganisms, however, there is the obvious circumstance of multiple exposures. Data obtained from human dose-response studies represent a single dose but do not address the issue of multiple exposures, which is a potential occurrence when dealing with microorganisms transmitted through the environment.

Haas and coworkers (1999) addressed the possible effects of multiple exposures using two approaches (18). The first approach assumes that the risk posed by an exposure is statistically independent of a risk associated with another exposure. This traditionally has been an assumption made in microbial risk assessments to date because of the lack of data that address such issues (6). This means that the risk of a health outcome (infection, illness, or death) from an exposure is not related to a prior exposure. A second approach is to sum individual doses (to represent the multiple exposures) and incorporate that dose into the appropriate dose-response equation to determine the overall risk. For both these approaches, additional information is needed to address the issue of immunity or the development of hypersensitivity resulting from an initial exposure. Dose interactions (of similar and dissimilar microorganisms) as well as the dose-host relationship need to be assessed.

Exposure Assessment

Exposure assessment determines the intensity and frequency of human exposure to the hazard. The concentration of viruses in the dose (e.g., food, water, and aerosol), the volume of the dose, and the number of times a person is exposed to that dose are assessed. Routes of exposure and issues characterizing the exposed population are also addressed. Other factors that should be included (which are not applicable to chemical hazards) are the survivability of the virus in the environment and its susceptibility to inactivation by treatment or disinfection.

One objective of the exposure assessment is to determine the average concentration of viruses in or distribution of viruses throughout an exposure. Data are often lacking on the occurrence of viruses in the environment although epidemiological investigations of waterborne and food-borne outbreaks caused by viruses can provide some occurrence information. Detection methods for viruses are not 100% efficient (129) and therefore the concentration data available may be an underestimation of the true concentration levels. The implementation of sensitive laboratory techniques that can detect the low levels of viruses capable of initiating infection can be a useful tool in developing a virus database during surveillance studies. The characterization of concentration distributions of microorganisms in water and food samples has been previously described (18).

Many of the enteric viruses have been isolated from water supplies, including groundwater, surface water, and drinking water. Often, however, specific viruses are not identified; most studies have evaluated water supplies for the presence of enteroviruses as a group. Surface waters exposed to sewage can become contaminated with viruses but the types and concentration levels of viruses present in the contaminated water depend on the source of contamination (raw sewage, treated effluent, or natural reservoir), the time of year of sampling, the amount of rainfall, and the incidence of enteric viral disease among the source population (130). The unhygienic practices of food handlers and sewage contamination of shellfish-harvesting areas greatly contribute to the likelihood of an exposure to contaminated food (115).

The outcome of an exposure assessment depends on the capability of the virus to withstand environmental stressors and its susceptibility to inactivation by treatment. Specific viruses exhibit unique capabilities to survive in the environment and resist inactivation by water treatment or disinfection. Rotavirus is capable of surviving on inanimate surfaces for more than one hour (131). Coxsackieviruses are very stable in the environment (132,133) and are more resistant to chlorine and ultraviolet light disinfection than most of the other enteroviruses (134,135). Adenoviruses are the most resistant of the enteric viruses to ultraviolet light disinfection and the enteric adenoviruses appear to be the longest surviving enteric viruses in water (136). Hepatitis A virus is reported to be a thermally stable enteric virus and studies have shown hepatitis A to survive for extended periods in groundwater (137). Viruses spread via aerosols can also overcome environmental stressors. Respiratory syncytial virus can remain viable for up to six hours on household surfaces and on skin for as long as 20 minutes (138,139). Influenza A and B viruses can survive on inanimate surfaces for hours and are more readily transmitted through aerosols during conditions of low temperature and humidity (140–143).

Not only is the concentration of the virus an important component of the exposure assessment step but also understanding the amount of dose and the frequency of exposure is also essential. Both of these may be defined as point estimates or distributions. Information is available on consumption or contact distribution for media containing microorganisms and this database has been reviewed elsewhere (18). The specific transmission route of the microorganism is first identified (e.g., ingestion of fish, inhalation during showering, ingestion of drinking water, etc.) and then the dose and exposure frequency are

determined. The U.S. EPA uses an exposure of 2 L/person per day for risk estimates of drinking water that is based on a survey conducted in the United States on water ingestion of defined age groups (144). An exposure of 113 g/meal may be used to assess risks associated with ingestion of fish, for example, or an exposure of 130 ml/swim may represent the amount of surface water ingested during swimming (145).

Characterizing the exposed population is also important. Roseberry and Burmaster (1992) observed an increase in tap water intake by persons above 65 years of age; an exposure of 4 L/person per day (as opposed to 2 L/person per day) is used in risk assessments for this subpopulation, which was given as the water intake for the 97.5th percentile of the elderly (144). Pregnant women have also demonstrated higher tap water consumption than the general population (146). Shellfish consumption, for example, is higher among some populations than others (147). Inhalation rates of potential virus-contaminated aerosols range from 15 m^3/day for children to 20 m^3/day for adults (145).

Risk Characterization

The objective of the risk characterization step (which can be qualitative or quantitative) is to estimate the risk of an adverse health effect occurring on the basis of exposures determined in the exposure assessment. Using a mathematical model that reflects the dose-response relationship of the microorganism–host interaction and best estimates of exposure, a quantitative measure of the human health risks can be achieved. Point estimates of risks or risk distributions can be determined. It is important to state any assumptions and uncertainties made during the first three steps.

Ideally, human health risks should be characterized for specific viruses (e.g., coxsackievirus) rather than groups of viruses (e.g., the enteroviruses). As is described throughout this chapter, the human health consequences of exposure to different viruses vary greatly. This necessitates the desire for more information on the occurrence of specific viruses in the environment. Risk assessments for coxsackievirus type B4 (148) and rotavirus (15), for example, have been conducted. By applying the appropriate dose-response model and assuming a 2 L/person per day exposure of drinking water, risks of infection, illness, and death for various concentrations of coxsackievirus and rotavirus can be estimated (Table 4). The estimated daily risk of infection from exposure to one coxsackievirus and one rotavirus in 1,000 liters of drinking water is approximately $1 : 100,000$ and $1 : 1,000$, respectively. Annual risks of infection for the same concentrations would increase to $5.6 : 1,000$ for coxsackievirus and $3.6 : 10$ for rotavirus. These annual risk estimates for both viruses do not meet the U.S. EPA's recommendation that microbial risks of infection should not exceed $1 : 10,000 (10^{-4})$ per year (149). These exposures result in lifetime risks of infection of $3.3 : 10$ for coxsackievirus and $9.9 : 10$ for rotavirus. At the same concentrations, risks of clinical illness for both coxsackievirus and rotavirus are comparable to those for infection. Lifetime risks of death range from approximately $5 : 100,000$ to $4 : 1,000$ for both viruses at all concentrations in Table 4.

A goal in any risk assessment is to avoid overestimating or underestimating the risks imposed by a hazard. The assumption of exposure levels (e.g., 2 L/person per day) may provide a degree of conservatism for some populations and employing consumption distributions (as opposed to point estimates) in the exposure assessment may address exposure variability. The U.S. EPA's focus on infection (as opposed to illness or death) in the recommendation that microbial risks of infection should not exceed $1 : 10,000 (10^{-4})$ per year (149) introduces a level of conservatism in the risk analysis of microorganisms

Table 4. Risks of Infection, Illness, and Death for Coxsackievirus Type B4 and Rotavirus*

Virus Concentration per 1,000 L	Coxsackievirus			Rotavirus		
	Daily	Annual Infection	Lifetime	Daily	Annual Infection	Lifetime
0.1	1.6×10^{-6}	5.7×10^{-4}	3.9×10^{-2}	1.2×10^{-4}	4.4×10^{-2}	9.6×10^{-1}
1	1.6×10^{-5}	5.6×10^{-3}	3.3×10^{-1}	1.2×10^{-3}	3.6×10^{-1}	9.9×10^{-1}
10	1.6×10^{-4}	5.5×10^{-2}	9.8×10^{-1}	1.2×10^{-2}	9.9×10^{-1}	9.9×10^{-1}
100	1.5×10^{-3}	4.3×10^{-1}	9.9×10^{-1}	9.6×10^{-2}	9.9×10^{-1}	9.9×10^{-1}
		Illness			Illness	
0.1	1.2×10^{-6}	4.2×10^{-4}	2.9×10^{-2}	6.9×10^{-5}	2.5×10^{-2}	5.4×10^{-1}
1	1.2×10^{-5}	4.2×10^{-3}	2.5×10^{-1}	6.9×10^{-4}	2.0×10^{-1}	5.5×10^{-1}
10	1.2×10^{-4}	4.1×10^{-2}	7.4×10^{-1}	6.7×10^{-3}	5.5×10^{-1}	5.5×10^{-1}
100	1.2×10^{-3}	3.2×10^{-1}	7.4×10^{-1}	5.4×10^{-2}	5.5×10^{-1}	5.5×10^{-1}
		Death			Death	
0.1	6.9×10^{-9}	2.5×10^{-6}	1.7×10^{-4}	6.9×10^{-9}	2.5×10^{-6}	5.4×10^{-5}
1	6.9×10^{-8}	2.5×10^{-5}	1.4×10^{-3}	6.9×10^{-8}	2.0×10^{-5}	5.5×10^{-5}
10	6.9×10^{-7}	2.4×10^{-4}	4.3×10^{-3}	6.7×10^{-7}	5.5×10^{-5}	5.5×10^{-5}
100	6.9×10^{-6}	1.9×10^{-3}	4.4×10^{-3}	5.4×10^{-6}	5.5×10^{-5}	5.5×10^{-5}

*Assuming 2 L/person per day exposure, morbidity ratios of 0.75 (coxsackievirus) and 0.56 (rotavirus), and mortality ratios of 0.0059 (coxsackievirus), and 0.0001 (rotavirus).

but provides protection for the immunocompromised populations who may be more likely to develop severe health consequences from the microbial exposure. Human health risks may also be an overestimation for those with preexisting immunity to that particular virus. The estimated risks, however, do not reflect the phenomenon of secondary and tertiary transmission, which greatly contribute to the likelihood of exposure to microorganisms.

A degree of uncertainty exists in all risk assessments. An analysis has been conducted to measure the uncertainty in the estimation of risks using the microbial risk assessment models. A Monte-Carlo analysis was performed to compare the uncertainty that is attributed to exposure variability to the uncertainty associated with virus infectivity and morbidity and mortality ratios (6). It was concluded that the greatest degree of uncertainty is due to the variability in exposure and not because of the dose-response parameters used in the assessment. Surveillance studies are needed to create a database of virus occurrence in the environment to improve estimate host exposures. Such data obtained from virus monitoring would greatly enhance the exposure assessment step of the risk assessment paradigm, but the accuracy of the concentration values used for host exposure is dependent on the accuracy and frequency of the sampling.

Risk estimates have been compared to epidemiological data obtained from waterborne and food-borne disease outbreaks and from an intervention study to determine the accuracy of the predicted estimates in an attempt to validate the developed microbial risk models (7,18,150). Attack rates and duration of exposures from outbreak data were used to estimate the probable dosage that occurred to produce the observed attack rate. These exposure estimates were then incorporated in the appropriate microbial risk assessment model and the risk of infection (P_i) was calculated. The calculated P_i was compared with the attack rates observed from the outbreak. Such comparisons have suggested that the models can successfully predict outcomes from exposure to microorganisms. (It should be noted that P_i refers to the human health end point of infection and attack rates reflect illness among a population.) Model predictions of daily and annual risks of clinical disease of rotavirus have been found to be comparable with such risks observed in an epidemiological study (18,151).

Risk characterization was initially designed to be a summary of the information compiled from the first three steps of the risk assessment framework with the idea that the process of this step would be conducted without the influence of risk management. An attempt has been made to integrate these two processes with the belief that this would help include public participation, which would therefore increase the chance of reaching decisions that would generally be acceptable (152).

MICROBIAL RISK ASSESSMENT APPLICATIONS

A primary objective in developing a risk assessment approach for estimating the human health risks associated with exposure to microorganisms is to provide a framework that can be used by regulatory agencies to evaluate microbial water quality and to determine an appropriate level of treatment that will achieve an acceptable level of risk. This approach can also be used in the food safety arena to address human health issues of exposure to food-borne pathogens. Microbial risk assessment can be applied to provide information that can be utilized in setting standards and formulating regulations. This tool is the link between what is known and the action to be taken (Fig. 2). Estimated risk levels associated with waterborne or food-borne viruses can be used to determine what specific action should be taken (e.g., what levels of water treatment should be administered to a water supply) to achieve an acceptable level of risk or to evaluate the efficacy of current treatment practices. As mentioned in the hazard identification section, rotavirus has been identified as the most infectious virus based on available dose-response data. Rotavirus has been used in the development of standards for surface water treatment in the United States (4).

Microbial risk assessment provides a comparison for risks associated with other environmental hazards. A concern for the long-term health effects from exposure to disinfectants (e.g., chlorine) and disinfection by-products in water has tempted some experts to suggest altering doses of chlorine and chlorine compounds during disinfection. A decrease in the amount of disinfection administered could greatly hinder the microbial quality of water. This relationship was demonstrated in the reduced number of microbial outbreaks and deaths when disinfection was first implemented in water treatment. A comparison of microbial and chemical risks may be necessary before other forms of treatment are considered. Without an estimation of the microbial risks involved, the options and consequences surrounding this issue could never be fully addressed.

Results of risk assessments of specific microorganisms can also identify potential indicator organisms that can be used to target microbially unsafe potable waters. Current standards utilize coliform bacteria as indicators of contamination, but studies have demonstrated that there is no correlation between the prevalence of bacteria and the prevalence of viruses and parasites (153–155). By reviewing the data on the occurrence of viruses in water supplies and their ability to resist environmental influences and conventional water treatment practices, these microorganisms may be introduced to this role.

Perhaps the most useful aspect of microbial risk assessment is its application for all types of water and food sources and populations. The dose-response models can be used for populations in developing countries and in the industrialized areas and calculated risk estimates can be used to assess microbial water and food quality for different sources in all parts of the world. Most of the exposure data that is obtainable for viruses are from studies conducted in developing countries. However, this information can be incorporated in a risk assessment in which the risk estimates could also be useful in an industrialized setting. By including the nature of the population under consideration during the risk characterization, the estimated human health risks can be appropriately interpreted. Another outcome

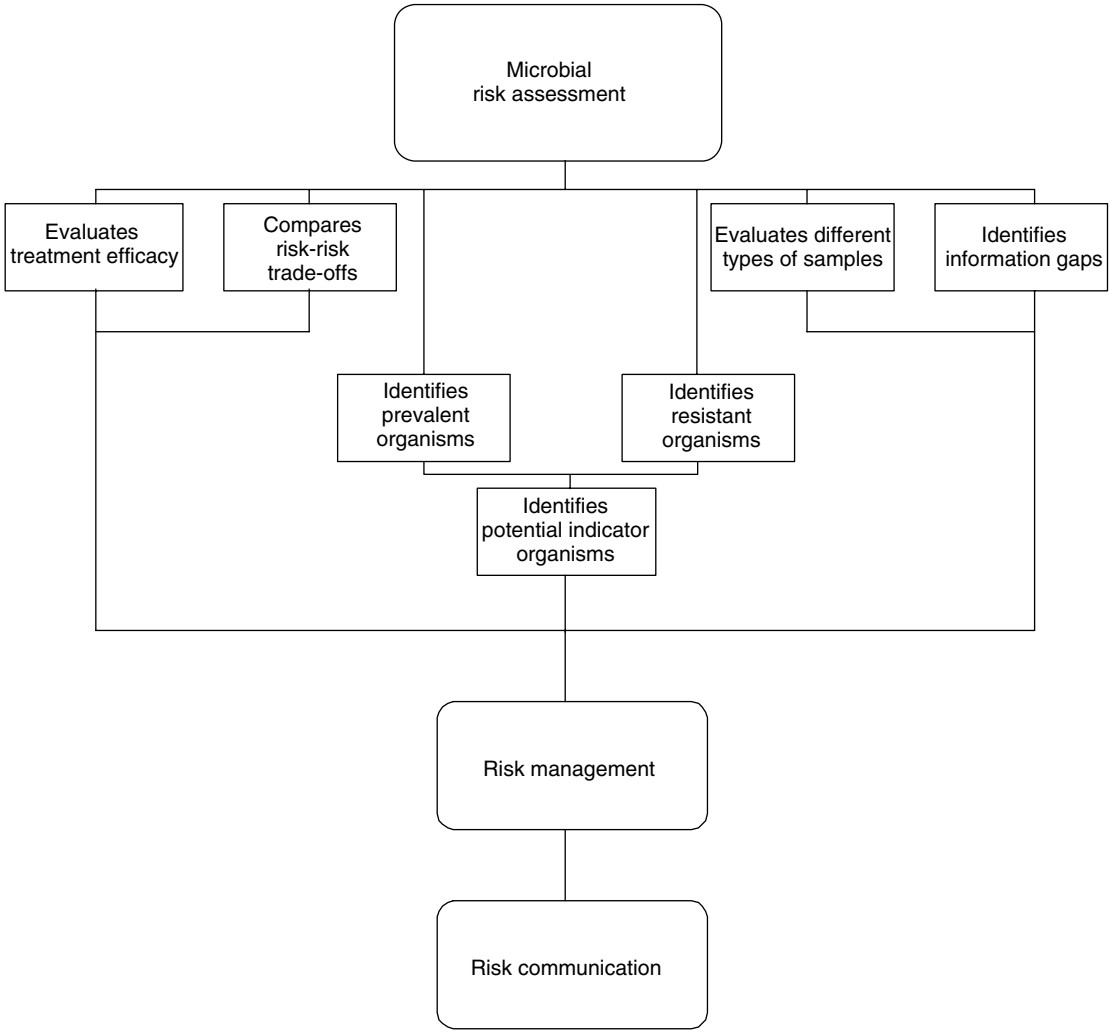

Figure 2. Microbial risk-assessment applications.

of a risk assessment is the identification of information gaps. Identifying what is not known about a microbial hazard may be just as important as the information that is obtained. These gaps highlight the areas in which more research and study are needed for a better understanding of the scope of the hazard.

Incorporating science with policy is a complex challenge. Because of the great variability of virus infectivity, pathogenicity, occurrence, and populations most affected, conducting risk assessments for specific viruses would provide invaluable information on the members of this diverse group of pathogens.

CONCLUSION

The microbial risk assessment framework can be used to identify and evaluate viruses as human pathogens; a task that traditionally relies on epidemiological data obtained during disease outbreaks. This approach helps to quantitatively define the human health significance of virus occurrence in the environment. The potential hazards associated with environmental exposure to different viruses indicate the need to evaluate specific viruses while considering human exposures. More data are needed on the occurrence and transmission of viruses in the environment to improve exposure assessment and thereby enhance risk characterization. Such assessments provide the necessary component for risk analysis of microorganisms. As an integration of science and policy, microbial risk assessment can offer risk managers a means of interpreting environmental quality data.

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RIVERINE BIOFILMS. See BIOFILMS IN NATURAL AND DRINKING WATER SYSTEMS

RIVERS, PERIPHYTON IN. See PERIPHYTON

ROOT EXUDATES. See NITROGEN FIXATION IN SOILS — FREE-LIVING MICROBES

ROOT SECRETIONS. See RHIZOSPHERE MICROBIOLOGY

ROTAVIRUSES

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Rotavirus is the principal etiologic agent of viral gastroenteritis in humans and animals. Rotavirus infects all children throughout the world and causes a life-threatening disease in children under five; however, the outcome of infection varies in different countries. In developing countries, rotavirus infections alone are responsible for approximately one million deaths annually, and death due to rotavirus infection is significant in Asia, Africa, and South America. In the United States, rotavirus infections account for 100 to 200 deaths annually, approximately 2% of infected children are hospitalized, and hospital costs exceed $1 billion each year (1,2). After replicating in the gastrointestinal tract, rotaviruses are excreted in large quantities and may be dispersed in water and soil (1–8). The stability of rotaviruses in the environment, and their resistance to physicochemical treatment processes in water and wastewater treatment plants facilitate their transmission. Rotaviruses can be found in freshwater, seawater, estuaries, groundwater, sewage and in effluents (advanced and waste treatment plant waters), drinking and tap water, water columns (as suspended or fluffy or compact sediments), shellfish, soils, sediments, sludges, flocs, vegetables, hospital equipment and toys (1–8). This widespread distribution may play a role in outbreaks of rotavirus gastroenteritis.

ROTAVIRUS CLASSIFICATION

Rotaviruses are classified in one of nine distinct genera in the Reoviridae family. This family contains the following genera: *Orthoreovirus, Orbivirus, Rotavirus, Coltivirus, Aquareovirus, Cypovirus, Fijivirus, Phytoreovirus, and Oryzavirus*. Viruses in the Reoviridae are icosahedral in structure (1,2,9). Rotaviruses have a distinct morphological appearance by negative-stain electron microscopy (EM). The name of the virus is derived from the Latin word "rota," meaning wheel because the virus particle resembles the rim of a wheel connected to spokes. Complete infectious virus particles are triple-layered nonenveloped icosahedral particles measuring approximately 100 nm in diameter when viewed by transmission EM (Fig. 1a); incomplete 70 nm in diameter double-layered nonenveloped icosahedral particles are not infectious (Fig. 1b). Animal rotaviruses were first identified in 1963 by EM in stool specimens from mice and vervet monkeys. In 1969, 70-nm virus particles were identified in diarrheal stools from calves, and the successful cultivation of the virus in primary cell cultures was first reported in 1971. Australian scientists discovered human rotaviruses in 1973 when they visualized the virus by EM in duodenal biopsies obtained from acutely ill infants and young children with diarrhea. Within a relatively short time, a clear association between gastrointestinal illness and the observed virus particles was established, and antigenic relatedness among animal and human rotaviruses was recognized (1,2).

The rotavirus genus is divided into seven antigenically distinct groups or serogroups (A to G) that infect mammalian or avian species based on serologic tests such as immunofluorescence (IF), enzyme-linked immunosorbent assay (ELISA), or immunoelectron microscopy (IEM) (2). Group A rotaviruses are the most important because of their high prevalence and pathogenicity in both humans and a variety of animals, including monkeys, cows, pigs, horses, sheep, goats, dogs, cats, rabbits, mice, turkeys, chickens, pigeons, parrots, as well as several zoo animals. Group B and C rotaviruses have been found in humans, pigs, and cows; group B rotaviruses have additionally been found in sheep, ferrets, goats, and rats. Group E rotavirus has only been identified in pigs, whereas group D, F, and G rotaviruses have been found in both chickens and turkeys (1,2,10). Unless otherwise noted, group A rotaviruses are the focus of this review.

Historically, the inner capsid protein VP6 was the first target of rotavirus classification. VP6 is the most immunogenic protein of the virion (after infection antibodies to VP6 are easily detected), and the most sensitive immunologic diagnostic assays are based on detection of this protein. VP6 bears the epitopes of the subgroup (SG) specificities that allow antigenic classification of rotaviruses into SG I, SG II, both SG I and II, or into neither SG according to reactivities with two monoclonal antibodies (MAbs) (1,2). A binary classification

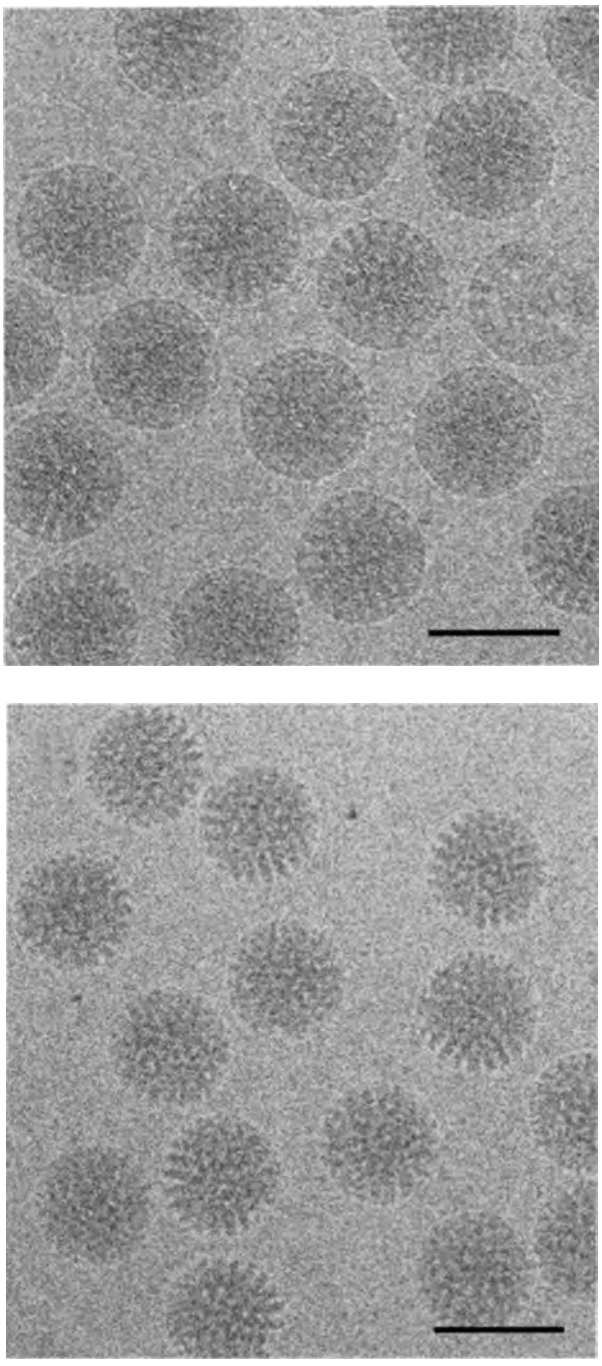


Figure 1. Electron microphotograph of triple-layered (**a**) or double-layered (**b**) simian SA11 rotavirus embedded in vitreous ice as examined by cryoelectron microscopy. Bar = 100 nm.

system reminiscent of the one used to classify influenza viruses has been established to characterize the two outer capsid proteins, VP4 and VP7 that independently elicit neutralizing antibodies (1,11). Thus, rotavirus strains are classified into VP4 or P-serotypes (for protease-sensitive) and VP7 or G- (for glycoprotein) serotypes. Classification of rotaviruses into VP4 or VP7 serotypes is performed by cross-neutralization assays using hyperimmune sera against VP4 alone or the whole virus, respectively. Traditionally, a distinct G serotype is defined as a reciprocal 20-fold or greater difference between the homologous and heterologous neutralization titer when a new strain is tested against antisera raised to prototype strains of known G serotypes. Alternatively, anti-VP7 serotype-specific monoclonal antibodies can be used to determine the G serotype of a particular strain. To date, 14 G serotypes have been identified and shown to belong to distinct G genotypes (defined by sequence analysis and/or nucleic acid hybridization data). G serotype designations coincide with G genotype designations (11). Rotavirus G serotype can be predicted with considerable accuracy by nucleotide sequence analysis of the VP7 gene because there is a high degree of conservation of sequence in variable regions among rotaviruses belonging to the same serotype. Similarly, rotaviruses are classified by P-serotypes or P-genotypes. P-genotypes are used more frequently because of the speed of sequencing and difficulties in producing good antibodies to distinguish among P-serotypes. However, not all P-genotypes correspond to a distinct P-serotype, and out of 20 different P-genotypes, only 13 P-serotypes have been identified with available antisera or anti-VP4 monoclonal antibodies that recognize specific VP4 neutralization specificities. Classification into VP4 serotypes is performed using the same criterion of the 20-fold difference in antibody titer (11).

Until all P-serotypes corresponding to all P-genotypes identified are determined, the following nomenclature is being used to identify the characteristics of rotavirus strains: The P-serotype (when known) is denoted by its number and the P-genotype is denoted immediately after the P-serotype number within square brackets. P-serotype and/or genotype must precede the G-serotype. For example, the human rotavirus Wa strain belongs to the P-serotype 1A, P-genotype 8, and G-serotype 1. Therefore, the serotype designation of the Wa strain is P1A[8],G1. In the case in which the P-serotype is not known, the P-genotype precedes the G-serotype. Rotavirus strains belonging to 10 G-serotypes (G1-G6, G8-G10, G12) and 9 P-serotypes (P1A[8], P1B[4], P2A[6], P2C[6], P3[9], P4[10], P5A[3], P8[11],and P11[14]) have been isolated from humans (1,2,11). Table 1 denotes examples of described human rotavirus strains with the indicated P/G serotype characteristics. When a particular "human serotype" is also commonly found in animal rotavirus strains, the specific animal silhouette is indicated next to the respective P-or G-serotype. Although not depicted, serotype G3 includes rotavirus strains isolated not only from humans but also from monkeys, horses, pigs, dogs, cats, mice, rabbits, and sheep (1,2,11).

Classification of a rotavirus strain can be described by using a cryptogram that contains the following information: serogroup or species of origin or place of origin or strain designation or year of isolation/P-serotype[P-genotype], G-serotype/SG specificity. Thus, the complete classification of the prototype rotavirus simian SA11 strain is A/Si/SouthAfrica/SA11/1958/P5B[2],G3/SGI. In the future, this cryptogram may be modified to include

Table 1. G- and P-Serotype Combinations of Human Rotavirus[a]

P Serotype [Genotype]	G Serotype 1	2	3 Multiple[b]	4	5	6	7	8	9	10	11	12	13	14
6[1]														
5B[2]														
5A[3]			Ro1845											
5B[3]														
1B[4]		DS-1	CHW17									L26		
7[5]														
2A[6]	M37			RV-3										
2B[6]														
2C[6]	AU-19													
9[7]														
1A[8]	Wa		YO	VA70	BR1054				WI61					
3[9]	K8		AU-1			PA151								
4[10]				57M				69M						
8[11]										116E				
4[12]														
13[13]														
11[14]	GR475/87					PA169		HAL1166		Mc35				
[15]														
10[16]														
[17]														
12[18]														
[19]														
[20]														

[a]Table shows names of prototype or representative human rotavirus strains of the indicated P and G serotypes. Viruses with P or G serotypes predominantly associated with infection in humans and in certain animals species are indicated by the corresponding silhouette of the most prevalent species. Some G and P serotypes (shaded) have only been isolated from animals.

[b]The VP7 serotype G3 has been found in viruses from many species; it has been recovered from monkeys, dogs, cats, rabbits, pigs, horses, mice, and sheep. Detailed description of human and animal viruses are given in the 4th edition of Fields Virology (2).

other characteristics of gene products important in immunity or virulence (2,11,12).

ROTAVIRUS STRUCTURE

The rotavirus particle is composed of three concentric protein layers surrounding the 11 segments of double-stranded (ds) RNA that encode the six structural viral proteins (VP), VP1-VP4, VP6, and VP7, and six nonstructural proteins (NSP), NSP1-NSP6 (1,2,9). Each genome segment, with the exception of gene 11 that encodes two viral proteins (NSP5 and NSP6), codes for a single viral protein. The innermost core layer is formed by 120 molecules of VP2 and encloses a less well-defined subcore that contains the genomic dsRNA. The VP2 inner core also contains the proteins VP1 (the RNA-dependent RNA polymerase) and VP3 (a guanylyltransferase and methylase) that are organized as a complex inside particles at the icosahedral fivefold vertices (9). The middle layer is made up of 780 molecules of the rotavirus protein, VP6 (9). The outer layer consists of 780 molecules of the glycoprotein VP7 and 60 dimers of spikes of VP4, the hemagglutinin, and cell attachment protein. Complete infectious rotavirus particles that contain the outer capsid proteins VP7 and VP4 are referred to as triple-layered particles (TLPs) and are approximately 1,000 Å

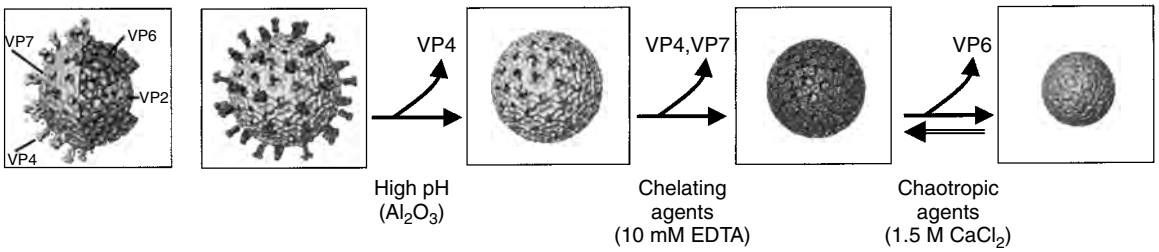
in diameter (9). Double-layered particles (DLPs), which are noninfectious, lack the outer capsid proteins and are approximately 705 Å in diameter. Rotavirus particles composed of VP2 (and VP1 and VP3) are called single-layered particles (SLPs) (2,9).

Electron microscopy easily distinguishes the three forms of particles, TLPs, DLPs, and SLPs that can be separated by centrifugation in gradients of cesium chloride (CsCl) (1,2,9). The physical property of virions used most often for purification is virion density. In CsCl gradients, virions are relatively stable and viral infectivity is stable to extremes of pH (3.5–9.0). Virion stability is strain-dependent and some strains, particularly human virus strains, may be less stable than viruses isolated from animals. Virion stability during purification and storage at 4 °C probably is determined by a particular gene 4 or its encoded protein VP4, and by specific interactions of VP4 with VP7 (1,2). The VP4 spikes can be removed by treatment of virions at pH 11.2 with ammonium hydroxide; removal of VP4 may also occur during other physical or chemical manipulations of particles such as treatment with organic solvents. The outer capsid proteins VP4 and VP7 of TLPs are removed by treatment of virions with chelating agents such as 10 mm EDTA, or with ethanol, resulting in replication-competent, but noninfectious, DLPs. Treatment of DLPs with chaotropic agents, such as 1.5 M $CaCl_2$, removes the VP6 protein and generates replication-incompetent SLPs (1,2). The structural and biological properties of rotavirus particles are summarized in Figure 2.

The existence of the different protein layers has been documented by studying properties of the rotavirus structural proteins VP2, VP6, VP4, and VP7 that have been cloned into recombinant baculovirus vectors, and the recombinant rotavirus proteins have been produced or coexpressed in insect cells infected with baculovirus vectors (2). Stable virus-like particles (VLPs) self-assemble following expression of VP2 alone. Coexpression of VP2 and VP6 alone or with VP4 results in the production of double-layered 2/6- or 2/4/6-VLPs, respectively. Coexpression of VP2, VP6, and VP7, with or without VP4, results in triple-layered 2/6/7- or 2/4/6/7-VLPs. All VLPs maintain the structural and functional characteristics of native particles (2). The production of rotavirus VLPs has represented a breakthrough to facilitate determining protein function and protein–protein interactions among the different viral capsid proteins. VLPs could be useful as noninfectious particles to monitor biophysical characteristics of virus migration in water or soils, as have VLPs of Norwalk virus, another gastroenteritis virus.

ROTAVIRUS GENES AND GENETICS

The sequences of all 11 genome segments of two rotavirus strains, human KU and simian SA11 have been determined (2). The simian rotavirus SA11 strain is considered the prototype rotavirus strain, and its genome segments range in size from 3,302 (segment 1) to 667 (segment 11) base pairs (bp). Sequences from different rotavirus strains show that each RNA segment starts with a 5′ guanylate followed by the 5′ end noncoding sequences, an open reading frame (or two in the case of genome segment 11), the 3′ noncoding sequences, and ends with a 3′ terminal cytidine. In addition, the plus-stranded



Structure:	Triple-layered	Triple-layered	Double-layered	Single-layered
Proteins:	VP 1, 2, 3, 4, 6, 7	VP 1, 2, 3, 6, 7	VP 1, 2, 3, 6	VP 1, 2, 3
Outermost protein:	VP4 and VP7	VP7	VP6	VP2
Infectious:	Yes	No	No	No
Active polymerase:	No	No	Yes	No

Figure 2. Structural and biological properties of rotavirus particles. Three-dimensional structures of a rotavirus, obtained by cryoelectron microscopy, show triple-layered, double-layered, and single-layered (core) particles. The cutaway diagram on the left shows the outer capsid composed of the VP7 shell and VP4 spikes, the intermediate VP6 layer, and the core VP2 layer that contains the subcore proteins VP1 and VP3 and the dsRNA genome. The double-layered and single-layered particles can be produced by sequential degradation of infectious triple-layered particles as depicted. (Reconstructions courtesy of B. V. V. Prasad, Baylor College of Medicine, Houston, Texas.)

RNA is capped at the 5′ end with m7GpppG$^{(m)}$Gpy, but there are no polyadenylated sequences at the 3′ end (2).

The rotavirus dsRNA genome segments can be extracted from viral particles and separated by polyacrylamide gel electrophoresis (PAGE) into eleven distinct bands (Fig. 3) (1,2,10,13,14). These 11 genome segments can be grouped in four size classes according to their migration in polyacrylamide gels: group I (segments 1–4), 1.5 to 2.2×10^6 Dalton (d); group II (segments 5–6), 0.8 to 1×10^6 d; group III (segments 7–9, a distinctive triplet), 0.5×10^6 d; and group IV (segments 10–11), 0.2 to 0.3×10^6 d. In total, the rotavirus genome possesses a molecular weight of 11 to 12×10^6 d (2,10,13). Each group A rotavirus strain has a characteristic RNA profile (or electropherotype), and for this reason, PAGE has been employed extensively in epidemiological studies of these viruses (1,2,10,13,14). PAGE is a simple and rapid method to determine if rotaviruses are present in a sample, provided that 10^5 particles/ml are present. In some human strains, the cognate genome segment 11 migrates between the cognate genome segments 9 and 10; these strains are said to possess a "short" electropherotype pattern of RNA migration, as opposed to those with a "long" (normal) electropherotype. In addition, the cognate genome segment 11 of several human strains migrates even more slowly than that of the "short" electropherotype strains, and these are referred to as "super-short" electropherotype strains (1,2,10).

Some rotaviruses do not display the distinctive RNA migration pattern, and these viruses have been identified as either nongroup A rotaviruses (formerly referred to as pararotaviruses) or group A rotaviruses with rearranged genome segments (Fig. 3) (2,10,13). In viruses with genome rearrangements, normal RNA segments are missing in an electrophoretic profile, and additional, slower migrating bands of RNA replace these. These new bands usually represent concatemeric forms of dsRNA containing sequences specific for the missing RNA segments (2,13). Genome rearrangements have been found in viruses from animals (pigs, cows, and rabbits) and from both normal and immunodeficient children. Rotavirus genome segments also undergo rearrangements in tissue culture, particularly when virus is passed sequentially at high multiplicity of infection (2,10,13).

Table 2 summarizes the gene-coding assignments and briefly lists the properties of the rotavirus proteins. The rotavirus proteins can be divided into structural proteins (found in virus particles) and nonstructural proteins (found only in infected cells and not in mature virus particles) (2). Among the rotavirus structural proteins, the inner capsid protein VP6 and the outer capsid proteins VP4 and VP7 are the ones that have been studied in detail, both molecularly and antigenically. In general, the nonstructural proteins play a role in

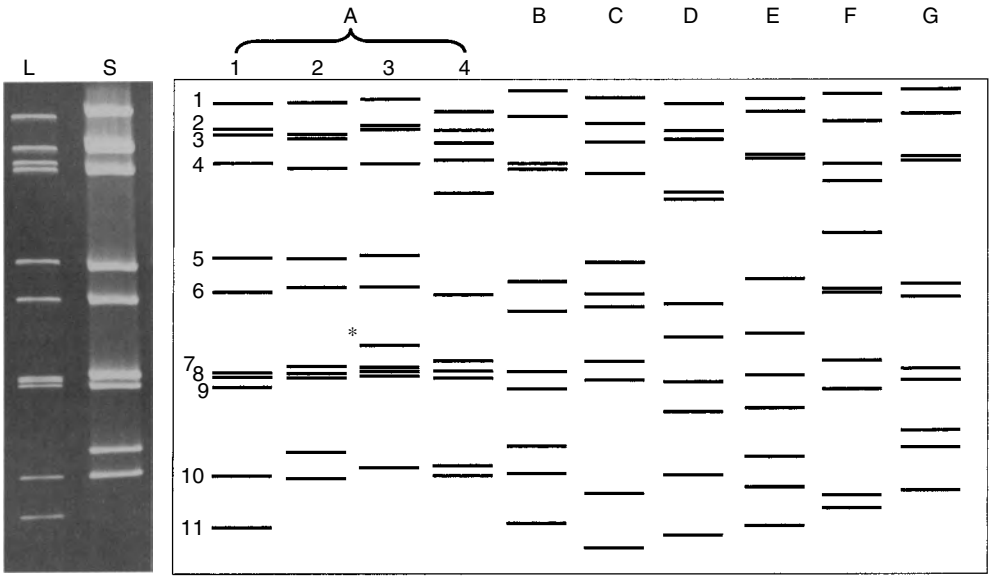


Figure 3. Schematic representation of the patterns of migration of the 11 segments of dsRNA of rotavirus serogroups A to G. The left panel shows the dsRNA electrophoretic patterns of group A human rotaviruses with either a long electropherotype (L) or short electropherotype (S). The right panel shows lanes with schematic representations of the dsRNA electrophoretic patterns of group A human rotavirus with long electropherotype (lane A1); human rotavirus with short electropherotype (lane A2); porcine rotavirus with rearranged (*) genome (lane A3); group A avian (turkey) rotavirus (lane A4); human group B rotavirus (lane B); human group C rotavirus (lane C); avian (turkey) group D rotavirus (lane D); porcine group E rotavirus (lane E); avian (chicken) group F rotavirus (lane F); and avian (chicken) group G rotavirus (lane G). The illustrated patterns are not exact, as all these antigenically-distinct rotaviruses have not yet been analyzed on a single gel to allow direct comparisons.

Table 2. Protein Coding Assignments[a] and Location and Functions or Properties Associated with Each Rotavirus Genome RNA Segment

Genome Segment	No. of Base Pairs[b]	Protein Product[c]	Molecular Weight[d]	No. of Amino Acids[e]	Properties or Functions of Encoded Protein
1	3,302	VP1	125 K	1,088	Subcore protein, single-stranded RNA-binding, slightly basic, RNA-dependent RNA polymerase
2	2,690	VP2	94 K	880	Core protein of SLPs, RNA binding, leucine-zipper, essential for polymerase activity of VP1
3	2,591	VP3	88 K	835	Subcore protein, ssRNA-binding, basic, guanylyltransferase, methyltransferase
4	2,362	VP4	88 K	776	Outer capsid spike protein, dimer, hemagglutinin, neutralization antigen (P), cell attachment protein, contains putative fusion region, virulence, protease-enhanced infectivity, cleaved into VP5* and VP8* protein products, VP5* permeabilizes membranes
5	1,611	NSP1	53 K	495	Slightly basic, zinc fingers, RNA-binding, host-specific (?)
6	1,356	VP6	41 K	396	Inner capsid protein, trimer, subgroup antigen, major diagnostic target
7	1,104	NSP3	34 K	315	Slightly acidic, RNA-binding, interacts with translation initiation factor eIF4G1, inhibits host translation, cytoskeleton associated
8	1,059	NSP2	35 K	317	Basic, RNA-binding, NTPase activity, binds VP1 and NSP5, involved in viroplasm formation
9	1,062	VP7	38 K	326	Major outer capsid protein, trimer, neutralization antigen (G)
10	751	NSP4	28 K	175	Rough endoplasmic reticulum glycoprotein, Ca^{+2} binding, role in morphogenesis, intracellular receptor, enterotoxin
11	667	NSP5	26 K	198	Phosphorylated, RNA-binding, O-glycosylated, autocatalytic kinase, interacts with NSP6 and NSP2
		NSP6	11 K	92	Product of second open reading frame, interacts with NSP5, hyperphosphorylated

[a]Coding assignments for group A simian rotavirus SA11 strain.
[b]Calculated from nucleotide sequence data.
[c]Determined by biochemical and genetic approaches. VP, viral protein; NSP, nonstructural protein.
[d]Estimated for the simian SA11 proteins from analyses on SDS-polyacrylamide gels.
[e]Calculated from the deduced amino acid sequences from nucleotide data and from the longest potential open reading frame.

genome replication and virion assembly but the precise role played in such processes has not been fully elucidated (2).

Genetic analyses of rotaviruses have contributed significantly to the understanding of the biology and function of the proteins of these viruses. Reassortment is the major distinguishing feature of rotavirus genetics (2,13). Genome reassortment occurs frequently among viruses with segmented genomes, and this property is responsible for the antigenic shifts of influenza virus strains, contributing to the evolution of pandemic strains. Reassortment occurs when genetically related viruses with segmented genomes exchange segments after coinfection. Dual infection of cells with more than one rotavirus strain occurs commonly in animals or humans and allows for the reassortment of genome segments. Mixed infections between two rotavirus strains lead to the generation of reassortant rotaviruses that contain specific combinations of genome segments derived from each of the parental rotavirus strains (2,10,13,14). Reassortment, a classical genetic technique, has been exploited as a powerful tool to map various phenotypes and viral mutations to specific genome segments, or to identify the protein product encoded by a particular genome segment because reassortant viruses (progeny) can be isolated by plaque assay (2,10,13). However, more detailed genetic analysis of rotaviruses is limited by the lack of a reverse genetics system in which synthetic transcripts derived from cloned DNA, corresponding to the entire genome of a segmented dsRNA virus, give rise to a replicating virus.

The overall genetic relatedness among each genome segment can be assayed by RNA-RNA hybridizations performed under high stringency conditions (2). RNA-RNA hybridization has provided molecular evidence to show close interspecies relationships between human and animal strains, or confirm the existence of naturally occurring rotavirus reassortant strains. Currently, there are three human genogroups, represented by the Wa, DS-1, and AU-1 prototype strains. Several animal genogroups have also been identified but the extent of their relationship among each other and to human strains has not been completely elucidated (15).

ROTAVIRUS BIOLOGY

In vivo, rotaviruses infect only the differentiated (mature) columnar epithelial cells covering the villi of the small intestine (1,2,16). Undifferentiated, replicating enterocytes in the crypts and lower portions of the villi are not susceptible to infection. The study of the virus–cell interactions was long hampered by the difficulty of growing

rotaviruses in vitro. Fortuitously, improved cultivation of these viruses came about when primary kidney cell cultures and cell lines derived from monkey, bovine, or canine kidney were identified to be highly permissive for rotavirus entry and efficient replication (1,16). The African green monkey kidney (MA104) cells are the best and most commonly cell line used to cultivate rotaviruses. Also, rotavirus infectivity is enhanced by proteolytic cleavage of the spike protein VP4 into two cleavage products, VP8* and VP5*, which remain associated with the particle (1,2). The spike protein VP4 is also involved in the early interaction of the virus with the cell and is the attachment protein (1,2,17–19). Other important biological functions of VP4 include the ability to agglutinate erythrocytes (hemagglutination) — mapped to a region in VP8*, restricted growth in cell culture, protease sensitivity associated with plaque formation, and virulence (1,2). The glycoprotein VP7 may be involved in the early events of rotavirus entry into cells, and possibly internalization (2).

The tissue- and cell type-specific tropisms displayed by rotaviruses indicate that a specific host cell surface receptor (or receptors) mediates virus recognition (2,17–19). Numerous reports provide strong evidence supporting the existence of specific rotavirus receptors. Most of these studies have implicated glycoconjugates (glycoproteins, glycolipids, and glycosphingolipids) as the putative rotavirus receptor(s) (2,17–19). Early studies suggested that animal rotaviruses require the presence of N-acetyl-neuraminic (sialic) acid (SA) residues on the cell surface for efficient binding and infectivity, whereas human rotaviruses do not (2,17). However, only a limited number of both human and animal strains had been tested, and recent analysis of a larger number of rotavirus strains for their dependence on SA for infectivity revealed that most animal rotavirus strains, such as the human rotavirus strains, are SA-independent (17). In addition, the isolation of variants of SA-dependent animal rotavirus strains that no longer depend on the presence of SA to bind and infect cells efficiently, indicating that binding to SA is not an essential step for rotavirus infection (17–19). Rotavirus entry into cells may occur by direct plasma membrane penetration, by receptor-mediated endocytosis, or by both mechanisms (2).

Rotavirus replication occurs entirely in the cytoplasm of infected cells (2). The replication cycle has been studied in cell lines that support rotavirus replication, and rotavirus infection can easily be detected by immunofluorescence using antibodies to either structural (Fig. 4a) or nonstructural proteins (Fig. 4b). Detection of rotavirus nonstructural proteins indicates that rotavirus replication has occurred because these proteins are not found in mature virions. Although the entire rotavirus replication cycle has not been completely elucidated, some steps of the replication cycle are known (2,20):

(*1*) Complete infectious virions bind to an unidentified cellular receptor(s) through interaction of the viral attachment protein VP4. (*2*) Following entry (by direct penetration or by receptor-mediated endocytosis), the outer capsid proteins VP4 and VP7 are removed by a mechanism that may involve low intracellular Ca^{+2} concentration ($[Ca^{+2}]_i$) that is required for the outer capsid stability. (*3*) Removal of VP4 and VP7 activates the virion's RNA-dependent RNA polymerase and the 11 mRNAs transcribed from the RNA genome segments are extruded from the channels on DLPs. (*4*) The mRNAs are translated by the cellular translation machinery and the rotavirus NSP3 protein regulates the switch from translation of cellular poly(A)$^+$ mRNAs to viral mRNAs. (*5*) Once all the viral proteins have been synthesized, the mRNAs interact with both viral structural (presumably VP1, VP2, and VP3) and nonstructural proteins in a complex, in an incompletely understood manner to form nascent (immature) virus particles. This process occurs at electron dense sites, called viroplasms, adjacent to the nucleus and the membrane of the endoplasmic reticulum (ER). (*6*) In the nascent particles, the mRNA is replicated to synthesize the 11 dsRNA genome segments. (*7*) The inner capsid protein VP6 is added to form DLPs that are formed

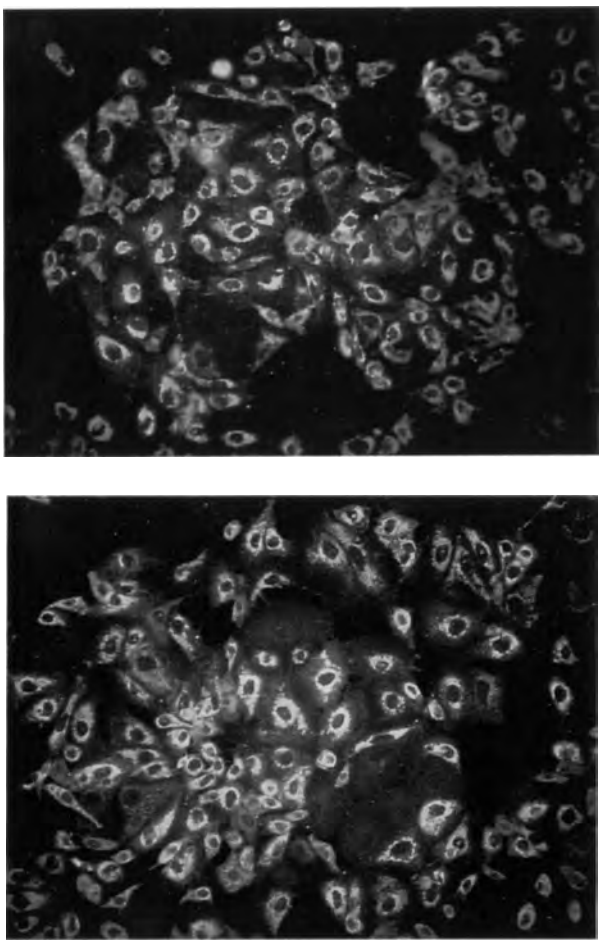


Figure 4. Embryonic African green monkey kidney (MA104) cells infected with simian rotavirus SA11 strain. Rotavirus-infected cells were fixed with cold (20 °C) methanol and (**a**) purified rotavirus-specific rabbit IgG (1 : 300) or (**b**) purified rotavirus NSP4-specific rabbit IgG (1 : 300) was used to detect viral antigen. A 1 : 150 dilution of a goat anti-rabbit IgG conjugated to FITC was used to develop the assay. Rotavirus replication is cytoplasmic. The detection of the nonstructural protein 4 (NSP4; B) shows that virus is replicating within cells.

at the periphery of the viroplasm and bud through the adjacent membranes of the ER. A cytoplasmic tail of the ER-transmembrane rotavirus NSP4 protein acts as an intracellular receptor for the newly formed DLPs. (*8*) During budding, the outer capsid glycoprotein VP7 is added along with VP4 to produce immature TLPs that contain a transient envelope that is later lost as the TLP matures in the lumen of the ER. (*9*) Infectious TLPs are released by cell lysis and by a newly described nonclassical vesicular transport mechanism (21).

ROTAVIRUS EPIDEMIOLOGY AND VACCINES

Rotavirus has a worldwide distribution. Data from epidemiological studies in the developed and developing countries have revealed that rotavirus is the major etiologic agent of serious diarrheal illness in infants and young children under two years of age throughout the world. All children in the world are infected with rotavirus (1,2). In general, symptomatic rotavirus disease predominantly affects young children, with the peak attack rate for severe diarrhea between 6 and 24 months of age. In some developing countries, the peak attack rate may be as low as three months of age. The incidence of hospital admission is greatest in children below the age of two, and then declines rapidly (1,2,16). In developed countries, mortality from these infections is low although approximately 2% of children are hospitalized. In developing countries, rotaviruses are the leading cause of life-threatening diarrhea in infants and young children (1,2,16). The burden of rotavirus diarrheal disease in infants and young children less than 5 years of age in developing countries has been estimated to be more than 125 million cases, and approximately one million infants and young children less than 5 years of age die from rotavirus diarrheal illness each year. The goal of rotavirus vaccination strategies is not the eradication of the disease, but the prevention of the severe, dehydrating diarrhea that leads to hospitalization or death (1,2,16).

The widespread distribution of rotaviruses in the community is supported by the universal acquisition of serum antibodies to these viruses at an early age (1,2,16). A high prevalence of rotavirus antibodies is maintained into adult life indicating that subclinical (mild or asymptomatic) rotavirus reinfection occurs throughout life. Frequent asymptomatic adult infections may be important in maintaining the transmission of infection in the community. For example, secondary infection in parents occurs from contact with young children actively infected with rotavirus, as evidenced by a high level of serum antibodies and seroconversion in parents. In addition, rotavirus infections and diarrhea illness have been reported in closed adult communities, such as geriatric hospitals or homes for the elderly. Primary rotavirus infections are usually symptomatic and are detected infrequently in individuals who are not ill (1,2,16). Usually, the peak of rotavirus infections occur during the winter months (along with the peak of respiratory diseases) in temperate climate regions, whereas rotavirus infections occur more prominently during the rainy season in tropical regions. However, regardless of geographic region, symptoms usually occur with the primary infection, which is followed by induction of protection against subsequent symptomatic infection. For this reason, the ratio of symptomatic to asymptomatic infection decreases with age. In newborns, rotavirus infection is common but results in either asymptomatic or mild disease due to protection provided by passively acquired maternal antibody (1,2,16).

Surveillance information is extremely important for planning, implementation, and assessment of disease control and prevention (1,2,16,22). In preparation for the release of rotavirus vaccines, molecular epidemiology studies characterized the circulating rotavirus strains in humans. Such studies have shown that temporal and geographic differences exist in the types of strains that circulate in the human population. In developed countries, more than 80% of rotavirus strains circulating in humans belong to G serotypes 1 to 4 in combination with P-serotypes 1A or 1B. However, other G-serotypes (G5, G6, G8-G10, and G12) and P-serotypes (P2A, P2C, P3, P4, P5A, P8, and P11) have been described, particularly in developing countries. In the developing world, G5, G9, and G10 strains in combination with P2A or P8 serotypes are becoming more prevalent. Currently, unusual G9 strains in the United States, France, Italy, and Australia indicate the appearance of more diverse G serotypes in the developed world as well (22).

After more than 15 years of testing and clinical trials, in August 1998, a three dose, live-attenuated tetravalent RRV vaccine, Rotashield^{TM}, was licensed in the United States (23). The vaccine was shown to be 80% effective in preventing severe life-threatening disease although the vaccine did not prevent rotavirus infection. Unfortunately, a possible association with intussusception (a bowel obstruction in which one segment of bowel becomes enfolded within another segment) following vaccination was noted postlicensure and the vaccine was withdrawn from the market in October 1999 (23). This was considered a major setback in the rotavirology field. Vaccination efforts using subunit (VLPs) and live-attenuated human or bovine-human reassortant vaccines continue to be pursued.

ROTAVIRUS CLINICAL SYMPTOMS AND PATHOPHYSIOLOGY

Although subclinical (asymptomatic) infections may occur, rotavirus infection causes a range of symptoms with diarrhea being the common denominator. Diarrhea may be mild to severe and may cause fatal dehydrating illness. The incubation period of rotavirus diarrhea is approximately 48 to 96 hours. In general, the clinical manifestations of patients hospitalized with rotavirus diarrhea include fever, dehydration, and vomiting (1,16).

Symptomatic rotavirus infection with severe dehydrating diarrhea is mainly observed in children between 6 to 24 months of age, in domestic animals between one to two months of age, and in mice, rats, and rabbits within the first 15 days of life (1,16). Several host-related factors may contribute to the age distribution of rotavirus–induced diarrhea: (*1*) Although the unidentified cellular rotavirus

Table 3. Environmental Samples in Which Rotavirus Has Been Detected

Type of Environmental Sample Tested	Method of Detection[a]		
	Genome	Infectious Particles	Antigen
Fresh (lake, river, creek) water	RT-PCR, IMC/RT-PCR	Cell culture	ELISA
Seawater	RT-PCR, IMC/RT-PCR		
Estuaries	RT-PCR	Cell culture	ELISA
Groundwater	RT-PCR	Cell culture	
Sewage	RT-PCR, RFLP	Cell culture, FFA	ELISA, EM, IEM
Waste treatment plant (secondary effluent)	RT-PCR, EM	Cell culture, FFA	ELISA, EM
Advanced waste treatment plant (tertiary effluent)	RT-PCR, EM	Cell culture, FFA	ELISA, EM
Drinking water	RT-PCR	Cell culture, FFA	EM
Distilled water	RT-PCR		ELISA
Tap water	RT-PCR		ELISA
Soil, sediments, sludges, flocs	RT-PCR	Cell culture	ELISA
Shellfish	RT-PCR	Cell culture	ELISA
Vegetables			ELISA
Moist surfaces, hospital equipment, toys	RT-PCR		ELISA

[a]Includes most common detection methods used, but does not exclude other detection methods.
RT-PCR, reverse transcription-polymerase chain reaction; *IMC/RT-PCR*, immunocapture/RT-PCR; *ELISA*, enzyme-linked immunosorbent assay; *FFA*, fluorescent focus assay; *RFLP*, restriction fragment length polymorphism; *EM*, electron microscopy; *IEM*, immune electron microscopy.

receptor is expressed throughout life, as evidenced by rotavirus infection in all age groups, a secondary cellular receptor involved in disease induction may be age-dependent and may account for the severity of disease in children and in the young of animal species. (*2*) The regulation and concentration of proteolytic enzymes, required for efficient virus entry into enterocytes, in the intestinal tract vary with age. (*3*) The composition of mucins in the intestine at the time of infection may play a role in determining the severity of rotavirus infection because the functional properties of mucins may also vary with age. Mucins in the intestine and in milk inhibit rotavirus replication in vitro and may inhibit replication and reduce duration of diarrhea in mice. (*4*) The rate of replacement of intestinal epithelial cells of the small intestine and fluid absorption in the large intestine increase with age. (*5*) Gut maturation is important in the pathophysiology of rotavirus infection and may be influenced in part by acquired immunity (1,16).

Rotavirus infection in the young is characterized by watery diarrhea, impaired D-xylose absorption, and abnormal gastric motor function, decreased levels of disaccharidases (maltase, sucrase, and lactase), and destruction of enterocytes consisting of shortening and atrophy of the intestinal villi, mononuclear cell infiltration in the lamina propria, distended cisternae of the endoplasmic reticulum, mitochondrial swelling, and sparse, denudation of microvilli (1,16). Several mechanisms by which rotavirus induces diarrhea have been proposed, and it is likely that a combination of these mechanisms contribute to diarrhea. The most prominent idea is that malabsorption occurs secondary to cell death (1,16). Although in some studies malabsorption correlates with histopathologic changes in the intestine, this does not explain the early watery diarrhea that occurs before villus blunting and other histopathologic changes. Moreover, mice infected with group A rotaviruses exhibit diarrhea in the absence of clearly defined changes in the intestine (1,16). Thus, lack of a clear association of mucosal damage and diarrhea supports other mechanisms of pathogenesis such as villus ischemia secondary to release of vasoactive agents from infected enterocytes, enterotoxigenic activity of the rotavirus NSP4 protein (24–27), and activation of the enteric nervous system (28) that result in or enhance Cl^- secretion (25).

Rotavirus deaths are usually associated with dehydration, electrolyte imbalance, and aspiration of vomitus although other preexisting conditions may contribute to death (1,16). It is likely that malnutrition plays an important role in increasing the severity of clinical manifestations of rotavirus infection. Rotaviruses can produce a chronic symptomatic infection or serious illness in immunodeficient children associated with prolonged shedding of rotavirus. During chronic infection in immunocompromised children, rotavirus can undergo marked changes in its genome, as indicated by abnormal electrophoretic migration patterns of rotavirus RNAs (1,10,14,16). Rotavirus infections have been associated with other syndromes, including necrotizing enterocolitis and haemorrhagic gastroenteritis in neonates, and pneumatosis intestinalis and hyperphosphatasemia in infants. However, these associations are very rare and further studies are needed to confirm the significance of these findings (1). The live-attenuated tetravalent RRV vaccine was withdrawn from the market because of a possible association with intussusception, but the possibility that intussusception is associated with natural rotavirus infections remains to be documented (23).

MODES OF TRANSMISSION OF ROTAVIRUSES

Rotavirus is mainly spread by the fecal-oral route (1,2,16). The primary source of infection of infants is contact with other infants with gastroenteritis in homes, hospitals, or daycare centers. Nosocomial infections play an important

role in transmission of rotaviruses (1,16). Another source of infection is contact with an infected sibling or parent with clinical or subclinical disease. The large number of viral particles shed in feces (10^{10-12} particles per gram of feces) and the resistance to physical inactivation may contribute to the persistence and easy spread of these viruses (1,2,16). Large quantities of rotavirus can be shed from the intestinal tract before and after cessation of diarrhea and consequently, infants may acquire the infection easily. Certain epidemiological features (the seasonal excess in cooler months and the rapid spread in both temperate and tropical zones; see previous section) have led to the suggestion that rotavirus may also be spread by the respiratory route (1).

The precise role of contaminated water in transmission of rotaviruses is uncertain and rotaviruses detected in water samples and raw or treated sewage have been suspected but not confirmed in rotavirus gastroenteritis outbreaks in several instances (Table 3) (1,3–8). Thus, rotavirus may be transmissible by contaminated water. Of special significance is the fact that wastewater treatment operations reduce the amount of rotavirus, but do not eliminate the virus, from the effluent (6,29,30). Therefore, infectious rotavirus may survive and come into contact with humans or animals via food, water, or aerosols generated by spray irrigation of wastewater. The mechanism of rotavirus transmission and the outcome of the viral infection depend on physicochemical or biological factors (1,6,7,31). Rotavirus transmission by water is often difficult to trace because of the lack of availability of routine laboratory services to confirm the viral etiology. However, the presence of rotaviruses in freshwater, sewage effluent waters, well- or groundwater, seawater, or sewage- or sludge-amended soils cannot be ignored. Routine use of epidemiologic criteria as an alternative to laboratory confirmation could allow better assessment of the importance of the presence of rotavirus in water samples and link its presence to outbreaks of rotavirus gastroenteritis until effective laboratory methods are widely implemented (6,7,32,33). Outbreaks of rotavirus gastroenteritis may be propagated by contamination of water supplies, and controlling such outbreaks depends on identifying and removing the source of contamination (32–34). Whether rotavirus spreads by contaminated waters or soils, the key question is how important is this spread in relation to other means of spread. In a few cases, rotavirus contamination of water has been implicated as the source of rotavirus epidemics. However, the importance of rotavirus spread by water can only be established if the presence of rotavirus polluted waters can be associated with the common and seasonal occurrence of rotavirus strains in the community (32–34). Currently, it is difficult to identify how rotavirus contamination of the environment plays a role in the distribution and occurrence of the rotavirus strains that circulate normally in a community or in outbreaks. Therefore, epidemiologists and environmentalists must work together to directly assess the role of rotavirus spread by water (or soil) relative to other mechanisms of rotavirus spread in the community.

ROTAVIRUS HOST RANGE AND RESERVOIRS

There has been speculation on the role of animals as a source of rotavirus infection of humans. Although rotaviruses do cause infection in a wide variety of animals, rotavirus infection does not appear to be predominantly a zoonosis (a disease or infection that is naturally transmitted between vertebrate animals and humans). Although certain naturally occurring animal rotavirus strains may infect humans, this type of interspecies transmission appears to be a rare event (1,2,15). Recent and widespread seroepidemiological data using improved assays to detect and classify rotavirus in field samples suggest that interspecies transmission does occur in nature, as exemplified by reports from India (cows to humans) and Brazil (pigs to humans) (15). Also, the possibility of reassortment occurring between animal and human strains has been suggested and evidence of transmission between humans and animals is accumulating. Examples of rotavirus interspecies transmission may include human Ro1845 (dogs to humans), feline FRV-1 (humans to cats), equine H-1 (pigs to horses), and the naturally occurring reassortant human/feline Cat2 rotavirus strains (15). However, no direct proof of interspecies transmission is available yet.

DETECTION, OCCURRENCE, AND PERSISTENCE OF ROTAVIRUSES

Rotaviruses have been detected throughout the world. Moreover, rotaviruses are the main etiologic agents of severe acute infantile gastroenteritis in every geographic region studied. In temperate-climate countries, rotavirus infection exhibits a seasonal pattern, with epidemic peaks occurring in the winter months every year (1,2). Rotavirus infections are almost absent during the summer months. The usual seasonal pattern of rotavirus infection observed in the temperate-climate countries is not necessarily observed in countries with less-marked seasons than climate-temperate countries. In tropical countries, rotavirus infection occurs predominantly during the slightly cooler or the dry season although rotavirus infection can also occur throughout the year or during the rainy season. The cause for the seasonal pattern of rotavirus infection is not known, but the low relative humidity during certain months has been suggested as a factor that enhances the survival probability of rotaviruses in the environment (1,2). However, it is important to point out that a correlation with the temporal pattern of infection and humidity has not been found in every epidemiological setting studied. Rotavirus infection may appear in one place in the fall and spread progressively until late winter or early spring (1). Rotavirus does not establish persistent infections unless the individual is immunodeficient, in which case a chronic infection is often established (1,2,10,13,14).

As a result of human activities, rotaviruses may enter the aquatic environment, soils, and sediments and threaten human health (1,6). However, before their entry into natural ecosystems, rotaviruses from humans or animals must first survive exposure to a variety

of adverse environmental factors of physicochemical or biological nature and the relatively harsh environment in wastewater treatment plants (6–8,31–34). The tightly organized structural protein coat protects the virion from the adverse action of temperature, sunlight and ultraviolet (UV) light, pH, divalent ions, or sewage microflora. Temperature probably is the most important factor involved in the destruction of rotaviruses outside their host cell. Although a temperature slightly above 60 °C may be adequate for protein denaturation and lead to rotavirus inactivation, rotavirus populations are heterogeneous and may include thermoresistant virions. Although it has been reported that 50 mJ of UV irradiation is sufficient to reduce viral titer to by more than 2.5 logs, data on the virucidal action of UV light on rotaviruses are limited. Nevertheless, the use of UV radiation has been proposed for the disinfection of water supplies and sewage effluents. Rotaviruses are stable at pH values encountered in natural waters (pH 5 to 9). Rotaviruses are not susceptible to acid in general, and they may survive in acidic waters (pH 3 to 5). In contrast, high pH exposure may result in virus inactivation due to loss of the spike protein from particles. Rotaviruses released into the environment may also be subjected to inactivation following the production of antiviral substances by the biota living in natural waters and wastewaters (6,7,31).

In wastewater treatment plants, rotaviruses are subjected to physical, biological, and chemical treatments that greatly help in their removal or inactivation, but still in certain treatment plants, infectious rotavirus can survive (6–8, 32–38). The survival of rotavirus both in fresh and marine waters depends on factors mentioned earlier. Rotavirus can survive for weeks in drinking and recreational waters and for a few hours on human hands (39). In air and on nonporous solid surfaces, the survival of rotaviruses benefits from a relative humidity of 50% and viral infectivity can be retained from several days to a few weeks. In natural waters, rotavirus particles may become associated with suspended solids and settle in the sediments and that can result in prolonged survival rates. In soil systems, the survival of rotaviruses has not been studied in sufficient detail to know the relative persistence of these viruses in this complex ecosystem. Rotaviruses have not been associated with shellfish-transmitted illness; this may reflect some property of the stability of these viruses (40,41). It is possible that shellfish take up rotaviruses but their infectivity is lost because the spikes are removed (40).

Rotaviruses are stable at ambient temperatures for a prolonged period (up to one year). Therefore, in hospitals and daycare centers, disinfection of contaminated material and hand washing are crucial to contain rotavirus infection. Among all disinfectants tested, ethanol (70–80%) is more effective than formaldehyde (10%) or sodium hypochlorite solutions at inactivating rotaviruses. Because rotaviruses are relatively resistant to chlorine, contamination of drinking water with low levels of rotaviruses may occur repeatedly, leading to sporadic cases of infection that may remain unreported (1,6,31–33). The fact that rotavirus is able to survive for prolonged periods on various surfaces under different conditions is likely to contribute to the rapid spread of these viruses. This is why rotavirus infections persist in newborn nurseries and an elevated number of nosocomial rotavirus infections occur in hospitals. The persistence of asymptomatic neonatal rotavirus strains over a long period plays an important role in the dissemination of the infection in neonates (1,2,16).

RECOVERY AND CONCENTRATION OF ROTAVIRUSES IN ENVIRONMENTAL SAMPLES

The number of rotavirus particles in raw sewage can vary greatly depending on several factors such as level of hygiene of the population, incidence of disease in the community, socioeconomic level, and the season of the year. A number of methods are available to recover rotaviruses in water and soil (6–8,29–31,34–38,42,43), but most of them suffer from one to several limitations. For example, no single method can be used to detect rotaviruses in all types of waters, whether natural, waste, or treated waters, and rotavirus recovery depends greatly on the water quality and is influenced by particulates, organics, and salts. The best method for rotavirus concentration from water should be sensitive enough to concentrate rotaviruses present in both water and wastewater, be capable of processing large volumes of a variety of waters in the least possible time, be straightforward to perform, be economical, and capable of detecting viral aggregates and virus adsorbed to suspended solids in water samples (6–8,29–31,34–38,42,43).

An important consideration is that rotaviruses, as all viruses, adsorb readily to a number of substances, including soils, sediments, fabrics, celite, starch, alumina gel, tricalcium phosphate, and various resins (6–8). Traditional methods to detect viruses in water samples generally use adsorption or entrapment, ultracentrifugation, adsorption to inorganic salts and clay minerals, adsorption to polyelectrolytes, soluble ultrafilters, hydroextraction, electroosmosis, and two-phase polymer separation to initially concentrate viruses (6–8). However, these methods have limited application to rotaviruses because they cannot be used in highly turbid waters or waters containing a high concentration of organics, and they can only be applied to small volumes of waters ($\leq$3 liters). With the advent of the microporous filter adsorption-elution technique, the detection of rotaviruses from a variety of water samples of different quality has improved. The main advantage of this technology is that it can be scaled up to test large quantities of water and can recover even low amounts of infectious rotavirus particles (6–8).

Because infectious rotavirus may not be entirely removed from wastewater by current sewage treatment processes, a method to recover and detect infectious rotavirus in any kind of water is critical to establish the effectiveness of water treatment processes and the public health significance of rotavirus in water (6–8,29–31,34–38,42,43). Concentration or recovery of rotavirus from a water source involves three basic steps: (*1*) Adsorption (concentration), (*2*) elution, and (*3*) reconcentration (recovery). The actual procedure varies depending on the source or type of water, but certain

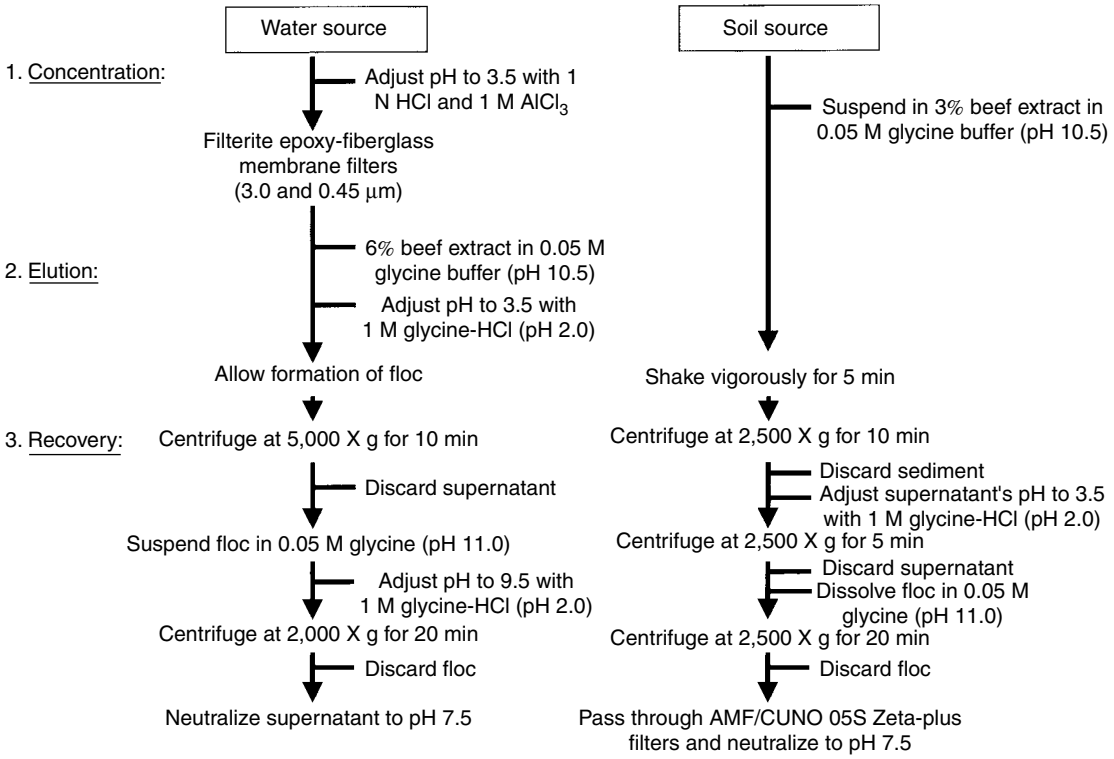


Figure 5. General procedures to recover and concentrate rotavirus from water or soil environmental samples.

general guidelines can be followed (Fig. 5) (6–8). In the adsorption step, the water sample is concentrated by passing the water through a combination of 3.0 and 0.45 μm Filterite epoxy-fiberglass membrane filters. Filterite filters are recommended because these clog less easily than other types of membrane filters such as acrylonitrile polyvinyl chloride copolymer, Cox epoxy-fiberglass-asbestos, or Millipore nitrocellulose filters (6–8). To enhance rotavirus adsorption to the membrane filters, the pH of the water sample must initially be adjusted to pH 3.5 with 1 N HCl and 1 M $AlCl_3$ to a final concentration of 1 mM or 0.5 mM $AlCl_3$ for seawater or others, respectively. In some cases, concentration of rotaviruses from sewage by microporous filters is often difficult because the high turbidities of these waters can rapidly clog the filters; therefore, during clarification, the native pH and salt concentration of sewage must be unaltered to prevent virus adsorption to suspended solids. Adsorbed rotavirus is then eluted from the filter by passing a small amount (2 to 4 ml) of 6% beef extract in 0.05 M glycine buffer (pH 10.5) for about one minute. This primary eluate is acidified to approximately pH 3.5 with 1 M glycine-HCl (pH 2.0) buffer to allow formation of the floc. Once the floc is recovered, it is neutralized prior to be assayed or stored at −80 °C. To recover rotavirus from sewage-contaminated seawater, the floc can be reconcentrated by centrifuging at 5,000 X g for 10 minutes. The supernatant is discarded and the floc is suspended in 0.05 M glycine (pH 11.0) to further concentrate to a smaller volume. Because the pH of the glycine will drop on contact with the floc, the pH must be adjusted to more than or equal to 9.0 before centrifuging a second time at 2,000 X g for 20 minutes. This time the floc is discarded and the supernatant is neutralized to pH 7.5 to avoid precipitates that may interfere with the rotavirus detection assays (6–8). The efficiency of recovery of rotaviruses from tap water, raw sewage, treated sewage, seawater, and drinking water is more than or equal to 30%.

Rotavirus concentration technology has made it possible to concentrate rotaviruses from sewage, tap water, seawater, and wastewater. However, the binding of these viruses to solids indicates that sampling of the water alone may not give a true indication of the potential disease hazard. Rotavirus present in sewage discharges can also be associated with solids, and rotavirus adsorbed to solids is known to remain infectious (6–8,29–31,34–38,42,43). Rotavirus can also adsorb specifically to soil particles after wastewater or sewage sludge is treated by recycling water through crop irrigation. Generally, viruses are removed using this method by percolation through the soil. The amount of rotavirus adsorbed to soil depends on the nature of soil (sand, clay, silt, etc.), the level of the water in which the viruses are suspended, and the flow rate of the virus suspension through the soil. The survival time of rotaviruses in soils is not known and this is an important concern in crop irrigation with wastewater and land management. Survival in the soils depends on factors such as temperature, soil moisture, pH, availability of nutrients, and the deleterious effect of the microflora present in the soil (6–8,29–31,34–38,42,43). The particulate matter to which rotaviruses can be associated may settle and accumulate in the bottom sediments of both freshwater and marine environments. Near marine sewage outfalls, rotaviruses and other viruses may settle into the sandy or slimy sediments and reach high concentrations. Because

rotaviruses can persist in soils and in sediments, these may represent a reservoir for rotaviruses. Therefore, the land disposal of wastewaters poses a potential risk that could lead to possible public health problems for humans and animals. The increasing demand for drinking water in many countries faces two main problems, that is, scarcity of drinking water and the proper processing and disposal of increasing amounts of wastewaters. The purification of wastewater has been and continues to be a prime environmental issue. Fortunately, groundwater, if contaminated, has the tendency to regenerate itself due to the adsorption processes in aquifers and to the metabolism of their microflora (6–8). This makes an underground recharge of tertiary wastewater an ideal last step before the wastewater is reused.

To recover rotaviruses from sediments or soils (Fig. 5), these are suspended in 3 volumes of 3% beef extract (in 0.05 M glycine buffer, pH 10.5) to elute the virus from the sediment or soil (filters cannot be used because of easy clogging), shaken well for five minutes and centrifuged at 2,500 X g for 10 minutes. The sediment is discarded, the pH of the supernatant is adjusted to 3.5 with 1 M glycine-HCl (pH 2.0), and it is centrifuged at 2,500 X g for 5 minutes. The supernatant is discarded and the organic floc is dissolved in 5 to 10 mL of 0.05 m glycine buffer (pH 11.0) to make a mixture whose final pH is around 9.5 to 10.0. The dissolved floc is centrifuged again at 2,500 X g for 20 minutes, and the sediment is discarded. Because toxicity to cell culture monolayers is a problem often encountered in the assay of eluents from sediments, the final supernatant is passed through AMF/CUNO 05S Zeta-plus filters at neutral pH (to avoid rotavirus adsorption to the filters) (6–8).

METHODS OF DETECTION OF ROTAVIRUSES

The clinical symptoms of rotavirus illnesses are not sufficiently specific to allow diagnosis on this basis alone. Therefore, laboratory confirmation is required to distinguish between rotavirus infection and other causes of gastroenteritis. Diagnosis of rotavirus requires detection of virus, viral genome or viral antigen, and/or demonstration of a serologic response. Several assays have been developed for the detection of rotaviruses in stools. Stool specimens, collected between one and four days of illness and stored at 4 °C, are ideal for virus detection because the peak of and amount of viral shedding usually coincide with clinical disease (virus shedding can continue once symptoms have stopped). If stool samples are not available, rectal swabs or diapers can also be used.

Detection of rotavirus is also possible in environmental samples such as freshwater, groundwater, seawater, wastewater (i. e., sewage), and soil. New methods capable of detecting rotaviruses in large liquid volumes are now available, but the precise concentration of rotaviruses (or other viruses) in water that can produce disease in humans has yet to be determined although it is thought to be low (6–8,29–31,34–38). In animals, the minimal amount of virus necessary to produce disease or infection in germ-free gnotobiotic piglets is one plaque-forming unit. Therefore, it is difficult to set standards of permissible rotavirus content of waters. In the meantime, waters continue to be monitored for the presence of bacteria according to certain bacteriological standards. Methods to detect rotaviruses in shellfish have also been developed, but as mentioned previously, shellfish have not been shown to play a role in the spread of rotavirus to humans (41,44). The advantage of being able to detect and type rotaviruses in environmental samples is that the information obtained is useful in molecular epidemiology studies monitoring the transmission of a particular rotavirus strain. The ability to detect rotaviruses in water provides a means to identify suspected sources for rotavirus contamination in outbreaks of gastroenteritis. Although numerous efforts have been made to detect rotaviruses from contaminated streams, in sewage, and in effluents from sewage treatment plants, rotavirus surveillance in water is not routinely monitored. Because rotavirus infections are seasonal in areas with a temperate climate, such changes are likely to be reflected in a given community's sewage.

There are several assays or laboratory procedures available for the detection of rotaviruses in stool or environmental (water or soil) samples (Table 4), and the most common are: cell culture (cultivation and isolation), electron microscopy (EM), immune electron microscopy (IEM), enzyme-linked immunosorbent assay (ELISA), polyacrylamide gel electrophoresis (PAGE), RNA-RNA-hybridization, and reverse transcription-polymerase chain reaction (RT-PCR) (6–8,29–31,34–38,42,43). These assays are discussed in details in the section that follows. Cell culture is specific and sensitive but impractical for many settings. The method of choice in many laboratories is ELISA because it does not require specialized equipment and it is easy to control for nonspecific reactivity. However, unless rotaviruses can effectively be concentrated from the environmental samples, ELISA is not sensitive enough (10^4 to 10^5 particles/mL are required). Conversely, RT-PCR is the most sensitive of all available assays (32), and the detected viral sequences can be characterized by analysis of the restriction fragment length polymorphism (RFLP) of the amplification products to detect the strain diversity in the samples without actual sequencing of any genes (33). However, it is important to keep in mind that detection of viral sequences cannot be correlated with virus infectivity because the presence of viral sequences in a particular sample does not mean the viruses are infectious. Currently, laboratory or commercial diagnostic tests (ELISA and RT-PCR) are available for rotaviruses of groups A, B, and C.

ASSAYS TO DETECT ROTAVIRUS

There are numerous assays available to detect rotavirus or immune responses to rotavirus. Among all the assays available to detect rotaviruses, only a few have been (or could be) used to detect the presence of rotavirus in environmental samples. Therefore, this section focuses on those assays that have been used or have potential to detect rotavirus in environmental samples.

Table 4. Methods Available for Detection of Rotaviruses[a] in Environmental Samples

Method	Sensitivity	Detection of Infectious Virus	Availability
1. Recovery in cell culture	≥1 IPP	Yes	Not routinely used
2. Fluorescent focus assay	≥50–100 IPP	Yes	Not routinely used
3. Electron microscopy	10^5–10^6 PP	No	Not routinely used
4. Immunoelectron microscopy	10^4–10^5 PP	No	Not routinely used
5. Immunologic:			
A. Enzyme-linked Immunosorbent assay	10^4–10^5 PP	No	Routinely used
6. Molecular:			
A. Polyacrylamide gel electrophoresis	10^5–10^6 PP	No	Rarely used
B. RNA hybridization	10^5–10^6 PP	No	Not routinely used
C. Reverse transcription — polymerase chain reaction	10^1–10^2 PP	No	Routinely used
D. Immunomagnetic capture reverse transcription polymerase chain reaction	5–10^1 PP	No	Not routinely used[b]
E. Restriction fragment length polymorphism	5–10^1 PP	No	Not routinely used[c]

[a] Probably strains belonging to all serotypes may be transmitted via water or soil.
[b] *IPP*, infectious physical particles; *PP*, physical particles.
[c] New assays that should be used in the future.

Recovery in Cell Culture

Rotaviruses can be recovered from stool specimens or environmental samples directly in cell culture with acceptable efficiency. However, cultivation of rotaviruses is not routinely performed because isolation of rotaviruses is very laborious, and four to seven blind passages may be needed to succeed. Some animal strains grow efficiently in cell culture, in contrast to human strains, and pretreatment of inocula with trypsin (always present in the normal habitat of rotaviruses, the small intestine), inclusion of a lower concentration of trypsin (and no serum because it contains high levels of trypsin inhibitors) in the culture maintenance medium, and rolling of cell cultures during incubation assist in primary isolation of rotaviruses in cell culture. RNA analysis to rule out the possibility of "re-isolation" of a laboratory cultivable strain is necessary, as cell cultures are susceptible to cross-contamination by "airborne" rotaviruses routinely cultivated in the laboratory. When dealing with large numbers of samples, it is very useful to obtain the electropherotypes first and then choose a few representatives of selected RNA pattern for further study. Although infectious rotavirus particles present in environmental samples can be unequivocally detected (and amplified) in cell culture, this method is not practical with most environmental samples.

Although many cell lines support the growth of group A rotaviruses, primary or continuous monkey kidney cell cultures are the most commonly used for their propagation. The most widely used cell line to propagate rotaviruses is the African green monkey (MA104) cell line. Other commonly used cell lines include rhesus (RhMK, LLC-MK_2), African green (AGMK, BSC-1, Vero), and Buffalo green monkey (BGM) kidney cells (1,2,16). Also, rotaviruses can grow efficiently in human adenocarcinoma cell lines, such as Caco-2, HT-29, or T-84, which despite their colonic origin, spontaneously differentiate into cells displaying many of the morphologic and biochemical properties of mature enterocytes. These characteristics include cellular polarization with the appearance of brush border and tight junctions, as well as the expression of intestinal hydrolases such as sucrase isomaltase, neutral aminopeptidase, and dipeptidyl dipeptidase IV (2,21). These intestinal cell lines are currently being used to cultivate other enteric viruses and therefore are useful to detect a variety of potential pathogens (21).

Fluorescent Focus Assay (FFA)

FFA is reliable, inexpensive, and practical for testing large numbers of stool or environmental samples (1,35,40,41). FFA provides an excellent method to detect infectious virus particles in stool or concentrated environmental samples. FFA permits for one round of virus replication without further virus amplification in cell culture. Fecal or environmental samples are trypsin-activated before inoculating confluent MA104 cell monolayers. The inoculum is removed and cells are incubated overnight in medium containing no serum or trypsin. On the next day, the cells are fixed with cold methanol (−20 °C) and a diluted anti-rotavirus hyperimmune serum is added to the cells for 2 hours at 37 °C. Appropriately diluted antispecies specific immunoglobulins conjugated to fluorescein isothiocyanate (FITC) are then added and incubated for 2 hours at 37 °C. The fluorescent foci are counted in an inverted fluorescence microscope, and the infectious virus titer is determined by averaging the number of foci in duplicate wells of each sample (11). Virus replication is exclusively in the cytoplasm (Fig. 4). The number of foci are then adjusted according to the volume and dilution of the inoculum to allow determination of the titer as focus-forming units (FFU) per milliliter (FFU/mL). The sensitivity of FFA is approximately greater than or equal to 50 to 100 FFU/mL, but sensitivity may decrease or be hampered because toxic inhibitors may be present in the test sample. The main disadvantage of using FFA to detect rotaviruses in stool or environmental samples is that many clinical virus strains may have limited ability to replicate in vitro without adaptation, and the amount of infectious virus may be underestimated. FFA requires

assaying large volumes of concentrates to detect low levels of virus contamination. This technique is not widely used but is a powerful technique to detect infectious rotavirus particles in environmental samples.

Electron Microscopy (EM)

EM of stool suspensions was the first routine method for diagnosis of rotavirus infections and allows for direct visualization of the virus in stool specimens (1,40). Usually a 10% suspension of feces is made in distilled water or phosphate buffer saline (PBS), clarified by centrifugation, stained with phosphotungstic acid (PTA) or uranyl acetate, and examined directly by EM (1). EM has the advantage of high specificity because rotaviruses have a distinctive morphological appearance, but 10^5 to 10^6 particles per milliliter are necessary to detect the virus. Direct EM examination of stools permits detection of rotavirus in 80% to 90% of the virus-positive specimens because rotavirus is excreted in such large numbers. The main advantage of using EM is that it detects rotaviruses of all serogroups (A to G) and other enteric viruses that would not show up in regular serological rotavirus tests. One important consideration when performing EM is that detection of intact particles requires the pH of the PTA or uranyl acetate to be less than 5.0. A pH greater than or equal to 5.0 may result in the loss of the outer capsid of group A, B, or C rotaviruses or in the disappearance of particles altogether (1,2). It is recommended that 1% uranyl acetate (pH 4.3) or 2% PTA (pH 4.5) be used for negative staining of rotaviruses. Currently, EM is used mostly as a confirmatory assay and is not frequently used as a primary method for rotavirus diagnosis unless EM is performed as part of a comparison of several diagnostic tests. In this case, EM is used as a reference standard to identify false-positive results. Although EM is rarely used to detect rotavirus in environmental samples, it has been used to detect rotavirus from sewage concentrates. However, the number of rotavirus particles present in the concentrate of sewage samples has to be amplified in cell culture before detection by EM. Therefore, the applicability of EM to detect rotavirus in environmental samples is limited and requires concentration or cultivation of the virus present in the sample.

Immune Electron Microscopy (IEM)

IEM, based on aggregation of virus-antibody complexes in solution followed by centrifugation and negative staining of the pellet increases the sensitivity of rotavirus detection to approximately that of standard enzyme immunoassays (1,2,40). Although IEM is not necessary for the detection of rotaviruses, it was used in early studies to demonstrate the relationship between different rotaviruses and the location of group antigens. For IEM studies, PTA (pH 4.5) is superior to uranyl acetate because it allows a defined visualization of antibody and virus. More recently, solid-phase IEM for specific detection of group A rotaviruses has been developed. Grids are first coated with protein A, then with an antirotavirus antibody by floating the grid on a diluted hyperimmune antiserum; later, the grids are rinsed and floated on stool or the test specimen containing virus before negative staining. Compared with direct negative staining, IEM enhances trapping of rotavirus particles on the grids and decreases adsorption of unrelated debris (1). IEM has been rated as 30 times more sensitive than direct EM and 10 times more sensitive than enzyme immunoassays (see the section that follows), and has the potential use to identify these viruses in water samples. By changing the antiserum placed onto the grid, IEM has been extended to allow for subgrouping and serotyping of rotaviruses, and can be used to differentiate among the morphologically identical group A, B, and C rotaviruses. However, IEM is labor-intensive when used for a number of specimens and is not widely used for environmental samples.

Immunological Assays

A variety of immunological assays have been successfully used to detect rotaviruses in fecal or concentrated environmental samples.

Enzyme-Linked Immunosorbent Assay (ELISA). Many varieties of ELISAs for rotavirus antigen detection have been developed, and this is now the most commonly used technique. Early tests were not sensitive enough to detect rotaviruses in environmental samples (1,6,30,32). However, modifications have allowed the use of ELISA to detect rotaviruses in environmental samples. Enzyme-linked immunosorbent assay frequently uses broadly reactive antibodies or monoclonal antibodies to the inner capsid protein VP6 to detect rotaviruses. The basic design of the assay is simple and does not vary considerably from laboratory to laboratory. First, a solid phase, usually a polystyrene microtiter plate is coated with antibodies against rotavirus. Then, the sample (stool specimen or environmental sample containing the antigen) is added and the unbound material is washed away. Bound antigen is then detected and quantitated by adding either enzyme-conjugated antirotavirus antibody (direct test) or a second unlabeled antirotavirus antibody (that must be from a species different from the one in which the coating antibody was made) followed by an enzyme-labeled antiimmunoglobulin specific for the second antibody (indirect test) (1). In either case, a chromogenic substrate for the enzyme is finally added and the bound enzyme (indicating bound antigen underneath) is detected by a colorimetric reaction that is quantitated on a commercial ELISA plate reader.

Direct ELISAs are commercially available and have the advantage that only one antiserum is required (part of it must be enzyme-conjugated), and that the test is faster than an indirect ELISA test. Indirect ELISAs have the advantage that the second antibody has an amplifying effect, thus rendering these more sensitive than direct ELISAs. Eventually, the choice of procedure will depend on whether the aim of the test is a field diagnosis or a definitive epidemiological study. The type and quality of plastic affects binding of the coating antibody and thus the sensitivity and reproducibility of the whole assay. Gamma-irradiated polystyrene appears to give the best results, and it is no longer necessary to avoid using the outside row of wells of microtiter plates that was not economical.

The quality of the coating antibodies is also very important. High-titered hyperimmune antisera are prepared by immunizing rabbits, mice, or guinea pigs that are devoid of antibody to rotavirus with purified virus particles. Immunization of hens followed by extraction of immunoglobulins from the yolks of their eggs, is also efficient. Group A rotavirus-specific monoclonal antibodies, which for general diagnostic purposes must be cross-reactive, offer increased sensitivity and reproducibility. Horseradish peroxidase is the most popular enzyme for antibody conjugates. Peroxidase is inexpensive and easy to conjugate to immunoglobulins. A variety of commercial antiimmunoglobulin conjugates are available and antirotavirus conjugates are available too. A variety of substrates are available: 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), o-phenylene diamine (OPD), and 3,3′,5,5′-tetramethyl benzidine (TMB). If necessary, results can be read visually, but spectrophotometers (ELISA plate readers) provide precise results. Figure 6 shows a diagram illustrating the indirect ELISA protocol for rotavirus antigen detection.

Each series of tests should include known positive and negative samples as controls. To determine cutoff values for rotavirus-positive samples, it is common practice to average a number of negative control optical density (OD) readings and to take the mean plus two or three standard deviations as the cutoff value. This value is then subtracted from test readings. Alternatively, each test reading can be divided by the mean of the negative (N) control OD readings; the sample is considered positive (P) if this P/N ratio greater than or equal to 2. False-positive reactions can occur in rotavirus ELISAs, owing either to particular reagents, rheumatoid factor or similar substances in stools, or the presence of interfering contaminants in environmental samples. To control for this problem, at the coating stage, alternate rows of wells in a microtiter plate can be coated with the usual capture antibody and, ideally, a preimmunization serum from the same animal at the same dilution. Each sample is tested in each row, and samples giving a color reaction in both rows are considered to be false-positives. Most commercially available rotavirus detection test kits are direct immunoassays, and numerous comparisons between commercial ELISA tests, previously published ELISAs and EM (see the section that precedes) have been reported in the literature. One major obstacle faced by environmental virologists is that the virus concentrations in water samples rarely reach the detection level of ELISA. Therefore, concentration of water samples is necessary prior to testing. In addition to detecting rotavirus antigen in fecal, water, soil, or tissue culture-medium samples, ELISA can easily determine the G serotype, P-serotype, or SG of a particular rotavirus strain.

Serotyping. Rotavirus serotyping involves the detection of specific epitopes on VP7 and on VP4. G- and P-serotypes are defined by the reactivity of antibodies to each of the two outer capsid proteins, VP7 and VP4, respectively. With rotavirus strains that are cell culture-adapted and grow well, G-serotyping can be carried out using conventional plaque reduction neutralization assays (PRNAs), or fluorescent focus neutralization assays (FFNAs) (1,2,11). The neutralization titer is taken as the reciprocal of the greatest dilution of antiserum that reduces the fluorescent cell focus count by at least 66% (11). The test can also be applied directly to fecal or environmental (water or soil) samples but there are practical limitations. Successful G serotyping is possible only if the water, soil, or fecal sample has an infectivity titer of at least 10^3

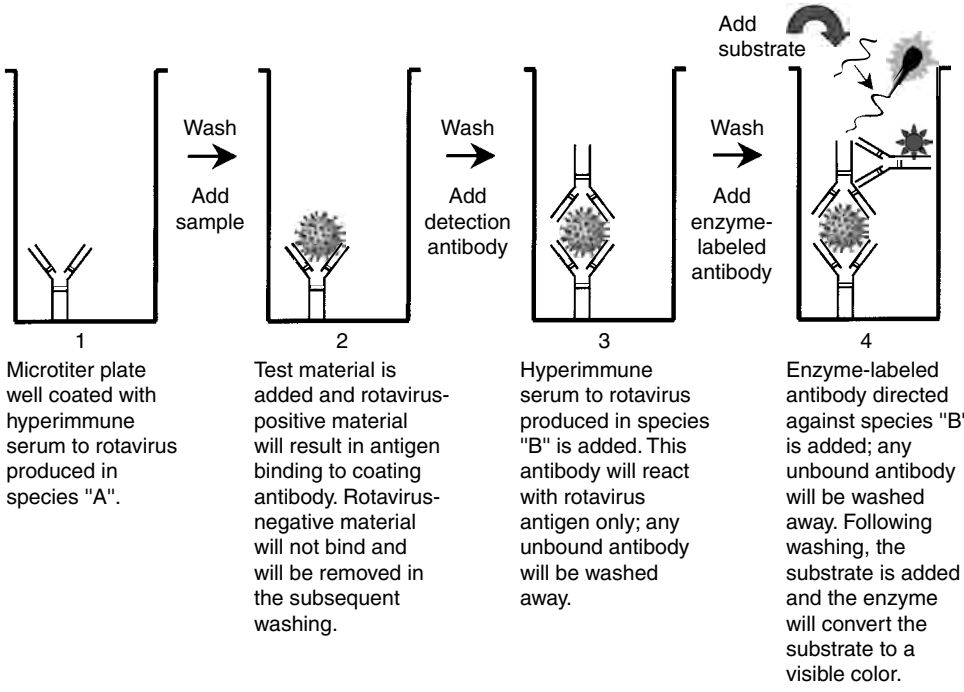


Figure 6. General procedure for indirect ELISA to detect rotavirus antigen.

focus-forming rotavirus units per milliliter. Therefore, water and soil samples must be concentrated prior to testing (6–8). Furthermore, many strains are untypable because of marked cross-reactions with different sera, some of which may be due to shared VP4 types. Twenty-fold homologous/heterologous titer ratios are required for distinguishing rotavirus serotypes with hyperimmune sera. An ELISA procedure for serotyping is the ideal test because of the limitations mentioned earlier regarding application of the PRNA and FFNA to environmental or fecal samples and the amount of work required for cell culture adaptation of human rotaviruses as well as the amount of work involved in the neutralization tests themselves. Currently, MAbs specific for both G- and P-serotypes are available for characterizing rotavirus directly in concentrated environmental or fecal specimens by ELISAs (1,2). However, ELISA is less sensitive than molecular assays (see the text that follows).

MAbs to almost all G serotypes are now available (1). MAbs may be used as coating or detection antibodies because serotype-specific antigens are located on the outer capsid of rotavirus particles, in serotyping ELISAs, EDTA, or EGTA must not be included in the sample diluent because these chelating agents will remove the outer capsid proteins (Fig. 2). Similarly, care must be exercised not to include EDTA or EGTA during the concentration of environmental samples if serotyping information is thought by using ELISAs (1,9). More frequently because of the low concentration of virus in environmental concentrates, epidemiological surveys of rotaviruses in sewage, marine waters, and drinking waters will use molecular assays (see text that follows) to determine the G type of the rotaviruses present in the environmental samples. Approximately 65 to 85% of rotaviruses present in stool samples are typable by G serotyping ELISA. Because of the existence of intraserotypic variation among strains of a particular G serotype, it is important to use a panel of MAbs to different neutralization epitopes to detect all rotavirus strains of a G serotype. For the same reason, if hyperimmune sera are used in the test, they must contain neutralizing antibodies against each of the serotypes to be detected (1,11). In all sample types, the amount of group-specific VP6 greatly exceeds that of VP7, therefore, serotyping ELISAs are never as sensitive as ELISAs to detect the group antigen. Nevertheless, serotyping ELISAs are already a great improvement over the infectivity/neutralization-based assays.

MAbs to VP4 are not yet available for all VP4 serotypes (1,2). VP4 serotype classification is based on neutralization assays using hyperimmune sera raised to reassortant rotavirus strains. Also, many of the MAbs to VP4 are only reactive to a subset of strains within a P-serotype, can cross-react with other P-serotypes, or give high background readings. MAbs to VP8* are better immunological reagents for P-serotyping than those directed to VP5* the other proteolytic cleavage product of VP4 (1). ELISAs to determine the P-serotype of a rotavirus strain requires the presence of intact infectious TLPs. It is likely that the spike protein VP4 may be lost during the procedures used to concentrate viruses from environmental samples. This may explain why P-serotyping of rotaviruses present in environmental samples has not been performed although P-genotyping (see below) has been employed (32).

Subgrouping. Rotavirus subgrouping detects specific antigenic epitopes expressed on the major inner capsid protein VP6 that are distinct from shared common (group A) epitopes. SG-specific MAbs are available, and ELISAs similar to that described above for antigen detection are the method of choice for subgrouping (1,2). Some MAbs can be used for coating plates, whereas others function better as detection antibodies. Polyclonal group-specific antibodies are used as the other part of the sandwich. Fecal or concentrated environmental (water or soil) samples are tested in parallel in assays involving SG I and SG II MAbs, results are read by spectrophotometer, and the ratio of reactivities is determined. The minimum ELISA ratio required for SG assignment will depend on the antibodies employed and has to be determined by testing a number of rotaviruses of known subgroup, but it is usually about two-fold. Rotavirus SG typing is not routinely performed in epidemiological studies, and much less in environmental samples, but when performed, it can provide interesting information about the distribution of antigenic combinations on rotavirus strains circulating at any given time.

Molecular Assays

The advent of molecular assays, that are sensitive and do not require cultivation of rotaviruses for their detection, is facilitating molecular epidemiological studies of rotaviruses. It is important to stress that although very sensitive, molecular assays do not detect infectious rotavirus particles (40). The presence or detection of the rotavirus genome or particular genes does not prove the virus is infectious (although it is potentially infectious).

Polyacrylamide Gel Electrophoresis (PAGE). Analysis of the dsRNA segments of rotaviruses by PAGE results in characteristic migration patterns of the 11 genome segments (Fig. 3). These patterns (or electropherotypes), which are easily recognized and reproducible for individual strains, cannot be directly correlated with serotype, but electropherotyping provides a rapid discrimination between rotavirus strains in an epidemiological study (1,2,10,13,14,16). This technique is not sensitive enough to be applied to environmental samples unless the samples are concentrated; therefore, its use is limited and is not recommended to detect rotavirus in environmental samples.

RNA Hybridization. RNA hybridization was first applied at a research level for investigating the sequence relatedness of genome segments among various rotaviruses, using electrophoresis and Northern blotting (40,41,44–45). For diagnostic purposes, the most interest is currently being taken in dot (or spot) hybridization assays ("dot blots") in which heat-denatured viral nucleic acid is immobilized on a solid support (nitrocellulose membrane) and probed with labeled complementary RNA or DNA. For general rotavirus detection, probes based on

genome segments encoding nonstructural proteins are the most useful because these generally show maximal sequence homology between strains. Although the specificity of rotavirus detection by RNA hybridization is good, the sensitivity is similar to that of electropherotyping with silver staining, EM, and ELISA. In a comparative study of ELISA and dot hybridization for the detection of rotavirus in various dilutions of fecal specimens, the dot hybridization method was 10- to 100-fold more sensitive than the ELISA (40,45). The applicability of this technique to detect rotaviruses in environmental samples depends on sample concentration and it is not used as routinely as it could be, but the assay can easily be modified for field assays.

The nucleic acid in rotavirus concentrated from large volumes of environmental samples can be detected using rotavirus-specific cDNA or mRNA probes. These probes are radiolabeled or labeled with nonradioactively with biotin. The nucleic acid extracted from each test sample is denatured and immobilized on filters, and the hybridization reaction is performed in sealed plastic bags containing filters and the probe in a solution that promotes association of complementary sequences. The plastic bags are floated in a water bath during the hybridization step, and after washing, the filters are exposed to X-ray films (if detected using radioactivity) or developed with streptoavidin (if detected using biotin) (40,45). Usually, a minimum of 10^5 particles can be detected using these assays.

G- or P-genotypes can be determined by hybridization with single gene substitution (VP7 or VP4) reassortants used as probes, or hybridization with oligonucleotide probes prepared from the VP7 or VP4 sequence of prototype rotaviruses of different G- or P- serotypes. However, among the molecular assays used to detect and classify rotaviruses, oligoprobe hybridization requires additional expertise. Although not widely used, this technique has successfully detected rotaviruses present in various types of water samples and shellfish.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR). The rotavirus dsRNA genome can also be extracted from stool or concentrated environmental specimens and can be used for group-specific RT-PCR (32,33,44). This technique makes use of the PCR basic methodology by first extracting the dsRNA genome of rotavirus. After the dsRNA is extracted, it is used as a template in a reverse transcriptase-mediated PCR. The reaction mixture that contains the necessary buffer, dimethyl sulfoxide (to separate the strands of dsRNA), proper amount of deoxynucleoside triphosphates, and primers corresponding to both the 5′ and 3′ ends of the gene to be amplified, is heated at 95 °C for five minutes. Usually, RT from avian myeloblastosis virus is used initially to make the cDNA (for one hour at 42 °C) to be amplified for 40 cycles of denaturation, annealing, and extension using *Taq I* polymerase. RT-PCR assays are the most sensitive detection methods currently available for detection of rotavirus. Because nonspecific amplicons that are the same approximate size as virus-specific amplicons may be generated from clinical and environmental samples, a confirmatory test (dot or Southern blotting) must be performed following the RT-PCR reaction. The use of RT-PCR allows G- or P-genotyping of rotavirus-positive specimens (32,33).

The biggest challenge in the processing of environmental samples is the elimination of interfering substances (inhibitors) that may decrease the sensitivity of the assay or inhibit detection of rotavirus in rotavirus-positive samples. Therefore, the use of appropriate internal RNA standards as controls is essential to properly perform the assays. The addition of low concentrations of an internal standard RNA control allows the detection of inhibitory substances without compromising the sensitivity of the RT-PCR assay, and permits the identification of false negative results. The internal standard has the advantage of allowing differential detection between wild-type viral RNA and standard using internal oligoprobe hybridization. Although not yet widely used, this powerful technique has been used for the detection of rotaviruses in treated and untreated sewage, fresh and marine waters, and shellfish. The applicability of RT-PCR may permit the acquisition of clear and important epidemiological data on the predominant rotavirus G- or P-types in the environment and provide information on the occurrence of asymptomatic rotavirus infections in the community. Significant progress toward diminishing the possibility of waterborne rotavirus infections could be achieved by establishing correlations between the prevalence of certain rotavirus P- and G-genotypes in the environment and the incidence of the different serotypes/genotypes causing clinical infections.

Since RT-PCR is extremely sensitive, careful laboratory and experimental design must be upheld to avoid false-positive results that can be obtained due to laboratory and sample contamination. To avoid carryover contamination, it is important to physically separate the pre- and post-PCR working areas, and use separate reagents and equipment for pre-PCR sample processing. This problem cannot be overemphasized enough and all assays must contain several controls to monitor for possible contamination.

G Genotyping. RT-PCR of the cognate gene segment coding for VP7 (RNA segment 7, 8, or 9, depending on the strain) is used to detect and classify (G serotype) group A rotaviruses since G genotypes are conserved among strains of the same G genotype. In brief, G genotyping involves the amplification of fragments of the gene encoding VP7 using oligonucleotide primers complementary to the 5′- and 3′-ends of the variable regions that are very distinct between different G-serotypes, but highly conserved within each G-serotype. The determination of the G-genotype by PCR can be performed directly from the cDNA obtained after random priming-RT, either as a single round PCR or as a seminested PCR (32,33). In the nested PCR, the first round amplifies the whole length of the VP7 gene and the second round is a reaction that includes oligonucleotide primers that are specific for a certain G-serotype and a primer common to all G-serotypes. RT-seminested PCR generally is chosen for its specificity and sensitivity in detecting a low viral load in environmental or fecal samples. Highly

contaminated waters do not require virus concentration, and the inhibitors present in sewage extracts can be eliminated by nucleic acid purification onto granular cellulose — a purification technique also used successfully for detection of rotavirus in shellfish. G genotyping by RT-PCR have been employed to detect rotaviruses present in sewage and drinking water. Interestingly, amino acid sequences of a region of VP7 that predicts G serotype identified rotavirus strains of human, bovine or porcine origin in drinking water obtained from homes in France. These results question the efficiency of some drinking water treatments to remove rotaviruses. In addition, the detection of the rotaviruses in drinking water might be correlated with the occurrence of rotavirus infections in children from the same homes. The presence of viral sequences of animal origin in water intended for human consumption leads to the question of whether such water may transmit animal rotaviruses (32,33). Although not proven, animal rotaviruses in drinking water could participate in the occurrence of reassortant viruses in humans or interspecies transmission of rotaviruses from animals to humans.

P Genotyping. As for G-genotype determination, RT-PCR can be performed for P-genotyping using oligonucleotide primers complementary to the variable regions in the gene segment 4 (encoding VP4) that are highly conserved within each P serotype but distinct among different P-genotypes (32,33). Also, P-genotyping can be performed as a single-round PCR or as a seminested PCR. In the seminested PCR, the first-round consensus oligonucleotide primers amplify a fragment of the genome segment 4 common to all P-types. The second round PCR includes the 3′-end consensus primer and different oligonucleotide primers complementary to variable regions that are specific for each P-type.

Immunomagnetic Capture (IMC) Reverse Transcription-Polymerase Chain Reaction (RT-PCR). The use of immunomagnetic capture (IMC) RT-PCR (IMC/RT-PCR) has been used for concentration of rotaviruses from clinical and environmental samples (46,47). In this type of assay, paramagnetic beads are coupled to MAbs against the group A-specific inner capsid VP6 protein and used to "capture" and purify rotaviruses present in water samples. Following capture, the dsRNA genome is made available for RT by disrupting the particles with heat. Potential inhibitory substances are removed effectively from freshwater but not seawater; samples and rotaviruses are concentrated into a small volume during a single step (47). A single or nested PCR is capable of detecting low amounts of rotaviruses. This assay is rapid, specific for group A rotaviruses, and displays increased sensitivity over ELISA methods alone. IMC/RT-PCR is capable of detecting 0.005 PFU (approximately five particles as indicated by EM) in a sample provided the amplicon is less than 200 bp (47).

Restriction Fragment Length Polymorphism (RFLP). Rapid comparison of a large number of rotavirus isolates and establishment of relationships between some restriction sites on particular rotavirus genes can be made by RFLP (33). Presence of rotavirus is first detected in fecal or environmental samples (raw sewage or treated effluent) by RT-PCR to confirm rotavirus contamination, and then the viral sequences detected can be further analyzed genetically by RFLP. RFLP is extremely convenient for strain differentiation and classification of bacteria and DNA viruses (mostly adenoviruses), and has been used for further characterization of the VP7 gene and the G-serotype or species of origin of rotavirus strains rotavirus strains present in stool or environmental samples. The amplified cDNA copies of the rotavirus genes are digested with selected endonucleases (restriction enzymes). Differentiation and classification of rotavirus strains depends on the fact that small differences in DNA sequence can alter restriction-enzyme cutting patterns. A single base pair difference in a particular gene position, for example, may eliminate a restriction-enzyme cutting site, giving rise to a large difference in the lengths of certain restriction fragments produced from the DNA at that position. The small differences of the digested DNA fragments between different rotavirus strains are known as RFLP. RFLP is a fast and relatively inexpensive method to detect rotavirus strain diversity in the environment. The identity of the RFLP profiles can be used to establish a correlation between rotavirus contamination in water samples and the viral sequences obtained from clinical samples (33). Therefore, this technique is useful for studying epidemiological relationships between strains involved in human infections and those in rotavirus-contaminated waters. Because more than one rotavirus strain can be found in contaminated raw sewage and treated effluents, several RFLP are not unexpected. In one study in France, the RFLP profiles obtained from raw sewage and treated effluents were compared with those obtained from human rotavirus isolates involved in infections in children from the same area (33). Identical sequences were detected in both environmental and clinical samples suggesting that rotavirus contamination of environmental samples may be significant enough to warrant public health consideration. Also, this study revealed that the diversity of RFLP profiles was greater during the winter and spring months correlating with the seasonal variations in rotavirus infections (33). Combination of RT-PCR and RFLP provides a novel approach to compare the presence of rotaviruses in environmental samples for molecular epidemiological studies. In addition, direct sequencing of amplicons can identify rotaviruses of specific molecular characteristics.

CONCLUSION

In developing and developed countries, rotavirus diarrhea is the leading cause of diarrhea-associated deaths and hospitalizations of young children, respectively. In temperate regions, institutional outbreaks of the disease occur mainly in cold dry weather, whereas in tropical settings, the seasonality is less well defined. Transmission is by the oral-fecal route. Rotaviruses belong to the *Reoviridae* family, are segmented dsRNA viruses, they exhibit antigenic and genetic variability,

and can cause mixed infections. These nonenveloped viruses are resistant to the outside environment such as waters and soils that can serve as reservoirs for the spread of rotaviruses in the environment. Rotaviruses are found in several surface waters, groundwater, sewage and its effluents, shellfish, and soils. Sewage treatment methods are not efficient to eliminate rotaviruses and these viruses are able to persist and are discharged into fresh and marine environments with treated effluents. Rotaviruses are relatively resistant to commonly used hard-surface disinfectants and hygienic hand-washing agents. Many of the methods for rotavirus detection and quantification are time-consuming and labor-intensive. Molecular assays such as gene probes and RT-PCR methods detect rotavirus, but they cannot determine if the viruses are infectious and, therefore, whether they pose a definite risk to exposed individuals. RT-PCR-based methods for rotavirus genotyping have proven to be a useful tool for epidemiological investigations. The advent of new detection methods to identify rotavirus contamination of environmental samples should enable epidemiologists to demonstrate if rotaviruses circulating in environmental water or soil samples at any given time can be responsible, at least in part, for outbreaks of rotavirus diseases in some communities. Further optimization of molecular assays and their commercial availability and worldwide implementation is needed. In the future, use of microarrays to detect and genotype rotavirus in environmental samples will enable fast and comprehensive identification of rotavirus waterborne outbreaks. A better understanding of modes of transmission will be needed to properly direct public health policy about the significance of detection of genome sequences in environmental samples. Rotavirus outbreaks need to be linked to rotavirus-contaminated environmental samples, and existing surveillance systems need improvement. Efforts should focus on establishing a database or an electronic reporting network to exchange information regarding outbreaks that is to be made available to both epidemiologists and environmentalists so that comparison of clinical and environmental samples and geographic distribution of human and animal rotavirus strains can be known. The routine use of standardized techniques to detect rotaviruses in environmental water samples could identify seasonal variability, risk periods, virus strain variability, and possible sources of rotavirus outbreaks. Water is a precious resource and further work is required to build a strong scientific understanding of the importance of monitoring water quality and keeping this resource safe.

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RURAL WATER SUPPLIES. See HETEROTROPHIC BACTERIA AS INDICATORS OF WATER QUALITY

S

SALINITY EFFECTS ON THE PHYSIOLOGY OF SOIL MICROORGANISMS

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The surface layer of the earth supporting plant life, the soil, is the product of two processes: the decomposition of rock and the decay of plant and animal life. Soils are solid matrices permeated to some degree by aqueous solutions and gaseous components. Materials existing in a solid state, soil minerals, and organic matter constitute the soil proper. The liquid and gaseous components, that is, water and air, in the interstices between the soil materials fluctuate continually and frequently with conditions independent of the soil itself. In addition, the organic fraction varies widely but seldom exceeds five percent by weight, except in soils formed mainly from plant remains. The soil compartment is dynamic and, usually, shows intense biological activity, chiefly in the soil zone influenced by plant roots, the rhizosphere. However, the extent and the properties of the soil microorganism population are permanently affected by various soil parameters such as temperature, pressure capillarity of soil matrix, pH, availability and the nature of its mineral composition (calcium, phosphorus, . . .) or organic requirement, presence of toxic metals (aluminum, cadmium, mercury, . . .), salinity and water content. Large variations in these parameters depend on the weather perturbations, the modifications of physical and chemical soil properties, the metabolic activities of microorganisms and plant roots, as well as human activities. Soil moisture is often the chief limiting factor for crop production and enormous efforts are made to increase and conserve the supply of available water. The moisture content of soils represents the balance between additions and removals of water. The main source of soil water, natural precipitations, is supplemented in certain areas by irrigation and by water brought up from deeper soil layers by capillarity. However, the latter process is generally too slow to be of real significance in supplying the water needs of plants. Removal of water from soils result from evaporation at the soil surface, transpiration from plants, percolation downward due to gravity, and surface runoff, which decreases the potential supply of soil water. Consequently, soil moisture shows marked differences in its movement and availability to microorganisms and plants.

Besides drought, salinity is a major problem in arid and semiarid areas, and nearly 40% of the world's land surface can be categorized as having potential salinity problems (1). The current estimates of the agricultural land affected by salt range from 400 to 950 million ha (2). Saline soils are those soils that contain less absorbed or exchangeable sodium and those that are high in nonalkaline salts such as sodium chloride and sodium sulfate. Another type of saline soils are those that contain enough salts such as sodium chloride to maintain flocculation and repress hydrolysis of the clay portion of the soil, which is itself the insoluble salt of a strong base and a weak acid. The soil habitat is inherently heterogeneous, and it can be expected that a wide range of salinities might be present in any one saline soil (3). Saline soils appear to harbor mostly halotolerant rather than halophilic microorganisms, presumably reflecting adaptation to periodic episodes of relatively high dilution (4). In addition to naturally occurring in soils, an excess of salts can also derive from intensive irrigation. In regions of low rainfall, salts accumulate where drainage is poor and are derived from percolating moisture insufficient to wash out soluble salts added in the irrigation water. Many irrigation projects have failed because of salinization caused by lack of proper drainage facilities or failure to use enough water to move excess salts down or out of the soil profile.

In the presence of excess salts in the soil solution, that portion of the soil in which the microorganisms and the plant roots obtain nutrients, the uptake of water by living cells is limited and yields are strongly reduced. Although definite limits cannot be set for the amount of soluble salts required to reduce the yield of plants, less than 0.2% of salts in a soil is usually harmless to agricultural salt-tolerant crops, such as sugar beets, alfalfa, cotton, tomatoes, and barley. However, such values or lower percentages are harmful for less tolerant plants including beans, peas, soybeans, vetchs, and oats. In the case of microorganisms, the range of salt concentration allowing growth in saline soils vary from 0.5% NaCl to almost 20%, reflecting the heterogeneity of this environment. It should be emphasized that the salinity of the soil from which the microorganisms are isolated does not correlate with the salt concentration, allowing growth of these organisms in vitro (5). The salt most dominant in soils are not only the carbonates, chlorides and sulfates of sodium but also of magnesium and calcium, or mixtures of two or more of these salts. However, salinity mainly refers to an excess of sodium chloride.

In this entry, the focus is mainly on the physiological responses of soil bacteria to increased salinity. The mechanisms that have been developed by soil bacteria to survive and proliferate in saline environments, and the molecular characteristics of various systems involved in these responses are covered.

SOIL MICROBIOLOGY AND STRESS CONDITIONS

Soil Microorganisms

As mentioned previously, it is obvious that the soil is not an inert material and one gram of soil may contain billions of living microorganisms. The organic

matter that is already present in the soil, and that being constantly added by higher plants, animals, and microorganisms, provides carbon and energy for soil microorganisms. In general, highly productive soils contain an abundance of microorganisms, whereas less productive soils support small populations. Although these facts do not prove that soils are productive because of their high microbial populations, or vice versa, there is much experimental evidence showing that microorganisms contribute to the productivity of soils through their various activities. Evidently, one of these activities is the decomposition of organic matter added to the soil through the use of green manures, animal manures, crop residues, and microorganisms. Among other activities associated with microorganisms in the soil, their contribution to cycles of matter is particularly important, both quantitatively and qualitatively. For example, certain steps in the nitrogen cycle (ammonification, nitrification, denitrification, nitrogen fixation) are exclusively carried out by microorganisms.

The soil microorganisms that have received the most attention are bacteria, including actinomycetes, algae, fungi, and protozoa. In fact, soil algae and protozoa occur in relatively small numbers in soils when compared with bacteria. Their numbers have been estimated at 2.7×10^4 and 1×10^3 per gram of soil for algae and protozoa, respectively, in comparison with 5.3×10^7 and 1×10^5 per gram of soil for bacteria and fungi, respectively (6). Fungi exist in soils in many different forms (microscopic or macroscopic), and their numbers and kinds depend on moisture, temperature, composition of the soil, and amount of organic matter present (see "SOIL FUNGI: NATURE'S NUTRITIONAL NETWORK," this Encyclopedia). Some fungi are capable of living in association with higher plants, particularly trees, forming the mycorhiza, which benefit the host plant by increasing the capacity of the roots to absorb nutrients. Within the soil, the size of the population of fungi is greatly enriched (12-fold) in the rhizosphere, but a much more dramatic effect (23-fold increase) is observed on the bacterial population (6). Clearly, the rhizosphere soil is a zone of intense microbial activity not only for symbiotic bacteria (rhizobia) but also for free-living bacteria. The importance of organic exudates from plant roots as an energy substrate for microbial colonization has long been known. Roots exudates are complex mixtures of many compounds that vary with plant species, plant age, and environmental conditions. However, they are not simply building blocks for microbial cells or common growth cofactors, which can be used by nearly all microorganisms. In fact, some root exudates can be degraded only by some, but not other, soil bacteria and are considered as nutritional mediators, which contribute to specificity in the rhizosphere (7).

Usually, the predominant bacteria found in the rhizosphere of most plants are gram-negative nonsporulating cells, which include the symbiotic rhizobia (*Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Allorhizobium*, and *Azorhizobium*), the associative bacteria (*Azospirillum*), the free-living bacteria (*Pseudomonas*, *Azotobacter*, *Xanthomonas*, *Erwinia*), and the plant pathogen (*Agrobacterium*). On the contrary, the populations of gram-positive soil bacteria are typically higher in bulk soil than in the rhizosphere, with the exception of some *Bacillus*, such as *B. polymyxa*, *B. megaterium*, *B. circulans*, and *B. brevis* (8). Because the effects of salinity have been examined only in a small number of soil genera, the section on the molecular aspects of bacteria adaptation to salinity will focus mainly on rhizobia (*Sinorhizobium meliloti*) as models for rhizosphere bacteria, and on *B. subtilis* as model for bacteria from the bulk soil. As pointed out previously, microorganisms can be subjected to salinity problems within the soil ecosystem, and increasing salt concentrations may have a detrimental effect on soil microbial populations as a result of direct toxicity, and through osmotic stress. Bacteria have developed mechanisms for adaptation to high osmolarity to survive and proliferate in saline soils or rhizospheres. A brief overview of the osmolarity and osmotic pressure of the soil is provided here, and the next section examines the osmoadaptative mechanisms in soil bacteria. Effects of osmotic stress on soil fungi has received little attention so far.

General Features of Soil Osmolality and Osmotic Pressure

Water is an essential component of the soil system, and soil water is intimately involved in controlling physical parameters of the soil (temperature, aeration, ...). Water with its dissolved salts constitutes the soil solution. A typical soil has an osmolality (= osmotic pressure of a solution expressed as moles of solute per kilogram of solvent or osmolal) estimated at less than 50 milliOsm/kg of water (9). This osmolality is equivalent to the solute concentration that would yield its osmotic pressure, and for a particular solute depends on the degree of its dissociation in water. Instead of being measured with osmometers, osmotic pressures are often approximated by calculated osmolarities as the sum of the concentrations of osmotically active solutes in the solution (expressed as moles of solute per liter of solutions or osmolar). The osmotic pressure is directly related to the water activity (a_w), which is simply the thermodynamic activity of the solvent water, and represents an index of the amount of water that is free to react. More definitions about these parameters have been discussed in some previous reviews on bacterial osmoregulation (8,10,11) and desiccation tolerance (12). In the presence of an excess of sodium chloride, the soil solution has a high solute content, which contributes simultaneously to both osmolality and ionic strength. In addition, Na^+ and Cl^- may also have an ion-specific effect on bacterial physiology. Such a soil solution restricts the movement of water through the bacterial membrane, and results in decrease in the water available to the bacteria. Because biological membranes are permeable to water but not to most other metabolites, the decrease in the external water causes an instantaneous efflux of water along the osmotic gradient. This process results in a partial dehydration of the cytoplasm and a decrease in the cytoplasmic water activity, and consequently leads to a decrease in the cytoplasmic volume, a phenomenon known as plasmolysis (Fig. 1). Ultimately, such plasmolysis affects metabolic

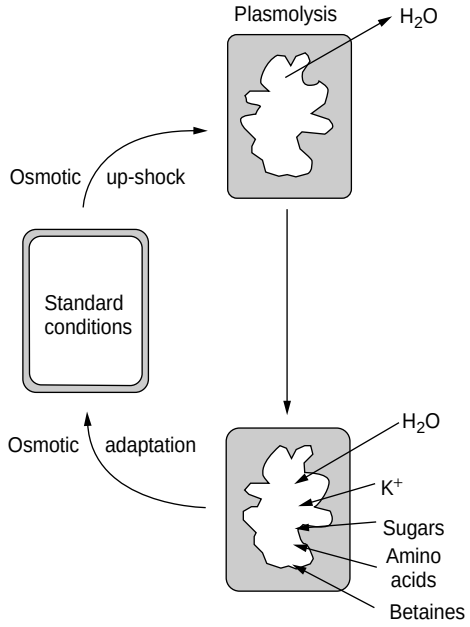

Figure 1. Scenarios following osmotic upshock and osmotic adaptation. When exposed to increased salt concentration, bacteria will primarily experience plasmolysis followed by osmotic adjustment because of accumulation of compatible solutes via uptake or biosynthesis.

systems and functions of cytoplasmic macromolecules and results in the cessation of growth.

Microbial Strategies for Adaptation to High Osmolality

To survive and proliferate in environments of high salinity, cells must respond by modulating their cytoplasmic osmolality, and as a result of intracellular osmotic adjustment, the initial cytoplasmic volume is restored (Fig. 1). The turgor pressure, which is the hydrostatic pressure exerted by the cytoplasmic membrane on the peptidoglycan, is also restored. This mechanical force is critical for the expansion of the cell wall during growth. Turgor pressures have been estimated in bacteria and are much higher in gram-positive bacteria (15 to 20 atmospheres) than in gram-negative bacteria (0.8 to 5 atmospheres) (13).

To deal with environments of increased salinity, bacteria have evolved two different strategies: (*1*) the intracellular accumulation of inorganic ions such as K^+; the most conspicuous representatives of bacteria adopting this strategy (salt in cytoplasm) are extremely halophilic *Archaea* (*Halobacteriales*), which will not be considered here, (*2*) the accumulation of selected organic molecules, the so-called compatible solutes; this response to osmotic stress (salt-out or organic-osmolyte type) is prevalent not only in *Bacteria* and *Archea* but also in fungi, plants, and animals (14,15)

COMPATIBLE SOLUTES AND OSMOPROTECTANTS

Properties and Mechanism of Action

Compatible solutes can be accumulated to high levels (several moles per liter) in the cytoplasm of stressed cells by de novo synthesis or transport without interfering with vital cellular processes. In many bacteria, most compatible solutes are metabolic dead-end molecules not subjected to catabolism or incorporation into macromolecules. In general, compatible solutes are polar, highly soluble molecules, and they usually do not carry a net electrical charge at physiological pH. Because of their accumulation, the water activity of the cytoplasm is reduced, and thus, cell volume and turgor can be restored (Fig. 1). As a result, an important stimulation of the growth rate of the cell is observed. Some of these solutes that alleviate the inhibitory effect of increased osmotic stress on microorganisms when they are added to the growth medium are also called osmoprotectants. A typical example is given in Figure 2: addition of low concentration of glycine betaine (1 mM) in a medium of high osmotic pressure restores the growth of *Sinorhizobium meliloti* which is inhibited by an excess of NaCl (14). In addition to growth, the presence of intracellular glycine betaine also stimulates specific enzyme activity such as nitrogenase. For instance, nitrogen fixation by the soil bacterium *Azospirillum brasilense* is totally inhibited by the presence of 0.3 M NaCl, and glycine betaine greatly stimulates this activity (Fig. 3), whereas this compound does not affect the same activity in cells grown in minimal medium (16).

Although there is generally a good correlation between intracellular accumulation of the supplied osmoprotectant

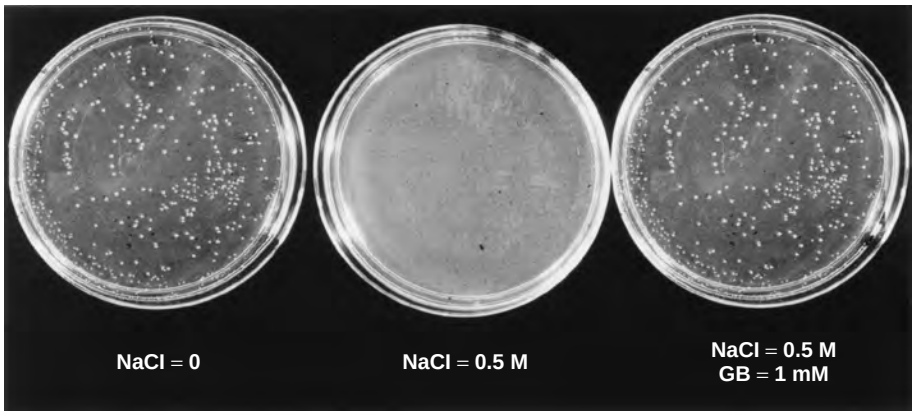

Figure 2. Glycine betaine as an osmoprotectant for *S. meliloti*. The presence of glycine betaine (GB) in a medium containing inhibitory level of salt (NaCl 0.5 M) allows normal bacterial growth as in the absence of salt (NaCl 0).

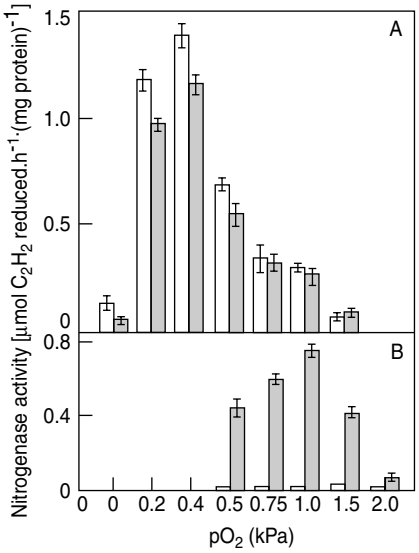

Figure 3. Stimulation of specific nitrogenase activity of whole cells of *A. brasilense* sp7 by glycine betaine. Cells are grown in the absence of salt (a) or in the presence of 0.3 M NaCl (b) without glycine betaine (open bars) or with glycine betaine (shaded bars). Nitrogenase activity is measured as acetylene reduction at various pO_2 (16).

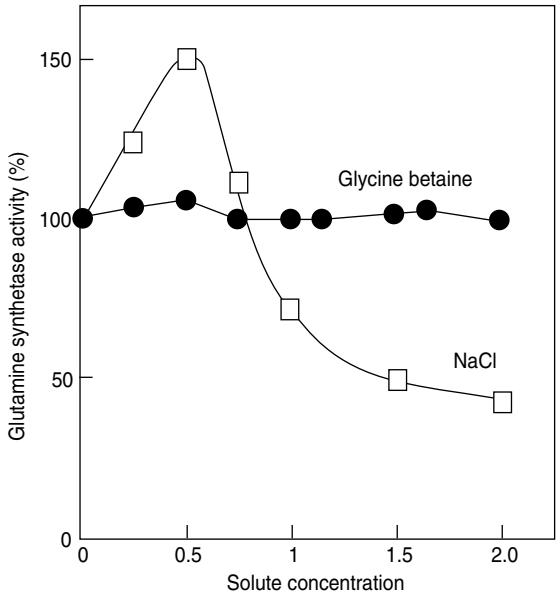

Figure 4. Glycine betaine as a compatible solute preventing protein denaturation. Specific glutamine synthetase activity is measured in vitro in the presence of increased NaCl or glycine betaine concentrations. Taken from S. R. C. Warr, R. H. Reed, and W. D. P. Stewart, J. Gen. Microbiol. 130, 2,169–2,175 (1984) with permission.

and growth restoration under osmotic stress conditions, this does not hold true for a few compounds such as ectoine and sucrose, at least in the case of *S. meliloti*. None of these compounds are accumulated by this bacterium, whereas both improve the growth at high osmolality (17,18). However, it is noteworthy that ectoine, for example, is accumulated in the halophilic bacteria, *Ectothiorhodospira halochloris* (19).

In addition to the obvious predominant role compatible solutes play in cellular osmotic adjustment, some of them serve as stabilizers of proteins and cellular components against the denaturing effects of high osmotic strength. The first suggestion of such property was made by Schobert (20), who had proposed that the aliphatic portion of the proline ring binds to the nonpolar residues on protein via hydrophobic interactions. Such an interaction would result in coating the protein with a hydrophilic shell that would enhance its solubility. In fact, the protective and stabilizing effects of compatible solutes on in vitro protein denaturation have been reported (21), and is illustrated in Figure 4. In this example, glutamine synthetase activity is strongly inhibited by increased concentrations of NaCl, whereas glycine betaine, even at 2 M, is totally compatible with full enzyme activity. Recently, it was demonstrated that glycine betaine plays an important role in the behavior of cellular macromolecules, and may actively assist in vivo protein folding in a chaperone-manner (22). Compatible solutes also display a general stabilizing effect by preventing the denaturation of proteins also caused by heating, freezing, and drying (23).

Spectrum of Compatible Solutes

Theoretically, a vast number of compounds could serve the function of osmotic balancing agents. However, an organism may use only a few compounds to fill this need. Figure 5 presents the structure of the main compatible solutes used by soil bacteria and Table 1 shows a list of soil bacteria together with the compatible solutes used by each bacteria. It should be noted that two major techniques, high-performance liquid chromatography (HPLC) and natural abundance ^{13}C-nuclear magnetic resonance spectroscopy (NMR), have been used to characterize these compounds, and to demonstrate their accumulation in cells. The prominent compatible solutes or osmoprotectants found in soil bacteria are potassium ions, few amino acids (glutamate, proline), sugars (trehalose, sucrose, and mannosucrose), polyols (glucosylglycerol and mannitol), quaternary ammonium compounds (glycine betaine, proline betaine, carnitine, γ-butyrobetaine, crotonobetaine, choline-*O*-sulfate), sulfonium compounds (dimethylsulfoniopropionate, DMSP, and dimethylsulfonioacetate, DMSA), ectoine, and a small peptide (N-acetylglutaminylglutamine amide, NAGGN).

Two important points should be emphasized:

1. Under salt-stress conditions, a given bacterium can accumulate a selection of several compatible solutes, and the importance and the composition of its compatible solute pool can vary in response to growth phase and growth conditions such as salt concentration in the medium. For example, in *S. meliloti* cells subjected to moderate salt stress (0.15 M NaCl) and growing in the absence of osmoprotectant, only biosynthetic accumulation of glutamate is detected (Fig. 6). As the NaCl concentration is increased, the presence of NAGGN (35) is also observed, but at a lower concentration than glutamate. At the highest

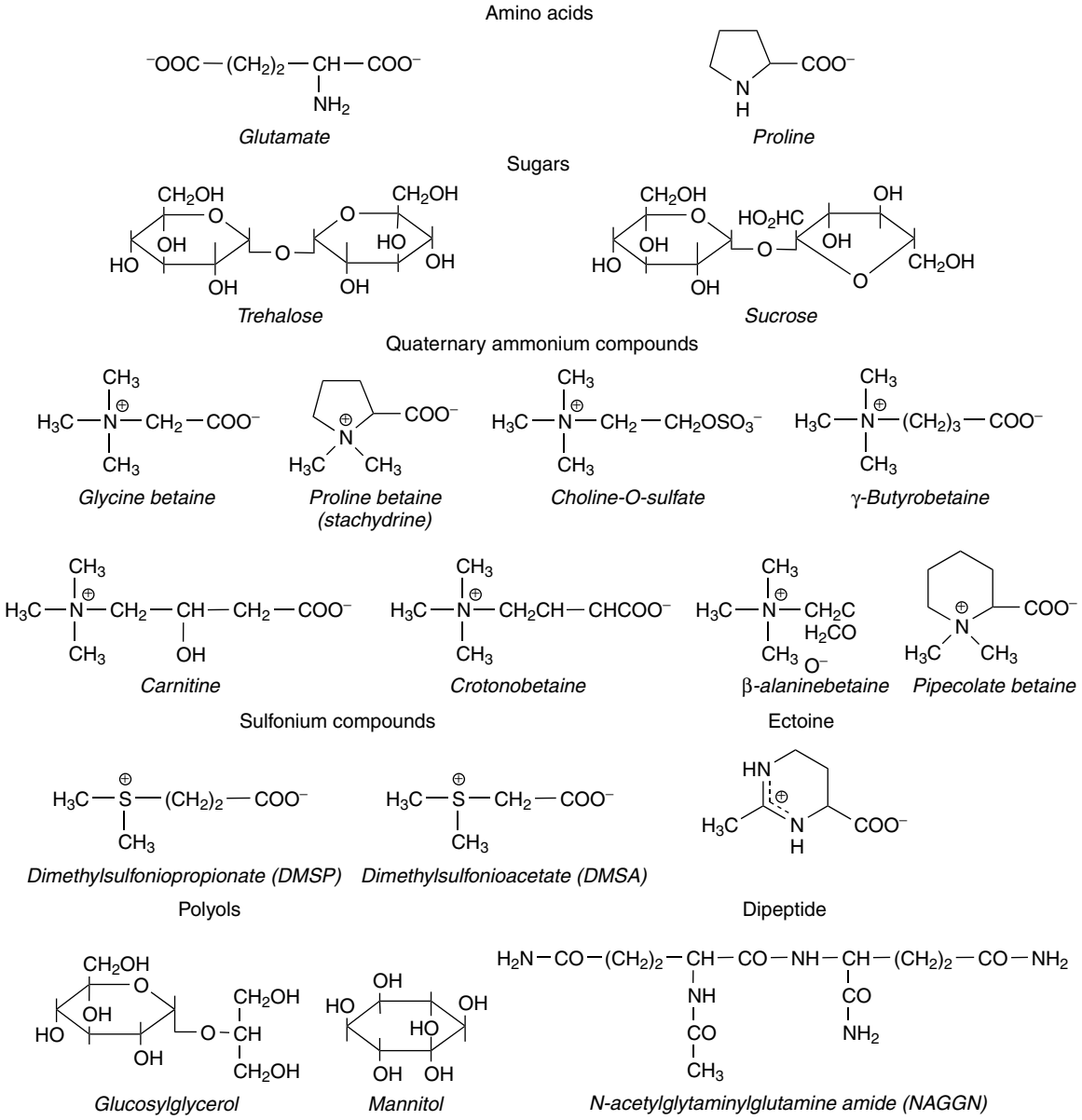

Figure 5. Compatible solutes and osmoprotectants in soil bacteria.

salt concentration tested (0.6 M NaCl), the glutamate level falls, whereas that of NAGGN continues to rise, so that NAGGN dominates the spectrum, and a third osmolyte, trehalose is detected (29). Thus, each of these osmolytes is elicited at a characteristic osmotic threshold. In the presence of an exogenous osmoprotectant such as glycine betaine, the buildup of the previous osmolytes (glutamate, NAGGN, and trehalose) is suppressed, and high cytoplasmic levels of glycine betaine are accumulated through the action of osmoregulatory transporters. Such shifts in osmolyte preference from de novo biosynthetic osmolytes to exogenous osmoprotectants can be related to the energy cost in terms of the number of ATP molecules required for both mechanisms, biosynthesis or uptake. The bioenergetic aspects of osmotic adaptation to high salt concentration have been the subject of a recent review (46).

2. A hierarchy exists among the compatible solutes, not all of them are equal. Potassium and glutamate inhibit many enzymes at high concentrations, and they serve to regulate the cytoplasmic osmolality only at relatively low external osmolality. At high salt concentration, the cells preferentially accumulate neutral compatible solutes, and one of the most commonly encountered is glycine betaine, which can be accumulated at very high concentration, 1 or 2 M. The advantage of being able to accumulate "cocktails" of compatible solutes is obvious, and the regulation of the synthesis or the uptake of each of the solutes is optimized according to the external conditions and the needs of the cells.

Table 1. Solutes Used by Soil Bacteria Subjected to Salinity

Compatible Solutes	Bacteria	Reference
Ions		
Potassium	*Sinorhizobium meliloti*	24
	Sinorhizobium fredii	25
	Bradyrhizobium japonicum	26
Amino acids		
Glutamate	*Azotobacter chroococcum*	27
	Agrobacterium tumefaciens	28
	Sinorhizobium meliloti	29
	Pseudomonas mendocina	30
	Azospirillum brasilense	27
	Pseudomonas pseudoalcaligenes	30
Proline	*Azospirillum brasilense*	27
	Bacillus subtilis	31
	Corynebacterium glutamicum	32
Sugars		
Trehalose	*Pseudomonas aeruginosa*	29
	Azotobacter chroococcum	27
	Azospirillum brasilense	33
	Azospirillum halopraeferens	33
	Bradyrhizobium japonicum	34
	Rhizobium leguminosarum bv. *trifolii*	35
	Sinorhizobium meliloti	29
Sucrose	*Sinorhizobium meliloti*	18
	Rhizobium leguminosarum bv. *phaseoli*	18
Mannosucrose	*Agrobacterium tumefaciens*	28
Quaternary ammonium compounds		
Glycine betaine	*Sinorhizobium meliloti*	36
	Azospirillum brasilense	16
	Pseudomonas mendocina	30
	Bacillus subtilis	37
	Corynebacterium glutamicum	32
	Pseudomonas aeruginosa	38
	Pseudomonas putida	38
	Pseudomonas fluorescens	38
Proline betaine (stachydrine)	*Sinorhizobium meliloti*	39
	Bacillus subtilis	31
Choline-*O*-sulfate	*Bacillus subtilis*	40
γ-butyrobetaine	*Bacillus subtilis*	41
Carnitine	*Bacillus subtilis*	41
Crotonobetaine	*Bacillus subtilis*	41
Sulfonium compounds		
Dimethylsulfoniopropionate (DMSP)	*Sinorhizobium meliloti*	42
	Bacillus subtilis	43
Dimethylsulfonioacetate (DMSA)	*Bacillus subtilis*	43
Ectoine	*Pseudomonas halosaccharolytica*	19
	Pseudomonas halophila	19
	Sinorhizobium meliloti	17
	Bradyrhizobium japonicum	17
	Corynebacterium glutamicum	32
	Bacillus subtilis	44
	Rhizobium leguminosarum bv. *viciae*	17
	Rhizobium leguminosarum bv. *trifolii*	17
Polyols		
Glucosylglycerol	*Pseudomonas mendocina*	30
	Pseudomonas pseudoalcaligenes	30
Mannitol	*Pseudomonas putida*	45
Dipeptide		
N-acetylglutaminylglutamine amide (NAGGN)	*Sinorhizobium meliloti*	35
	Pseudomonas mendocina	30
	Pseudomonas pseudoalcaligenes	30
	Pseudomonas aeruginosa	38
	Pseudomonas fluorescence	38
	Pseudomonas putida	38

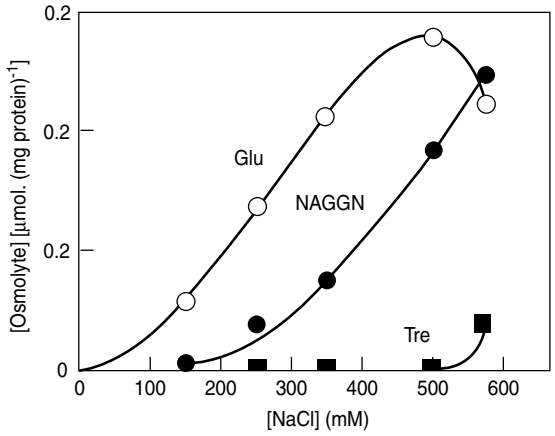

Figure 6. Intracellular compatible-solute composition of *S. meliloti* as a function of NaCl concentration in the growth medium. Cells were grown in the absence of exogenous osmoprotectant and accumulate glutamate (Glu), N-acetylglutaminylglutamine amide (NAGGN), and trehalose (Tre). Taken from L. T. Smith et al., J. Exp. Zool. 268, 162–165 (1994) with permission.

Phases of Osmotic Adjustment

The mechanisms by which bacteria sense osmotic variations, osmosensory mechanisms, are still poorly defined. As defined recently by J. M. Wood (11), "an osmosensor is a device that detects changes in extracellular water activity (direct osmosensing) or the resulting changes in cell structure (indirect osmosensing)." There is already good evidence that osmosensors are mainly integral cytoplasmic membrane proteins, which may detect and control a temporal cascade of cellular changes and osmoregulatory responses. Because a detailed overview of osmosensing based mainly on the intensively studied enteric bacteria has been proposed very recently by Poolman and Glaasker (47), Wood (11), and Csonka and Epstein (48), only a brief illustration of the timescales of well coordinated physiological responses to elevated osmolality is presented here (Fig. 7). The prime signal is turgor pressure, which either directly or probably through its effects on membrane proteins or on protein-lipid interactions, controls the movement of potassium. Thus, the primary response to an osmotic upshock is a massive uptake of K^+, such as observed in the enteric bacteria, mediated by the Kdp and Trk transport systems. This response is considered to occur within milliseconds to minutes of the osmotic shift. To maintain electroneutrality of the cytoplasm, the glutamate pool is increased by de novo synthesis, but other processes such as putrescine and proton efflux may also contribute. It has been proposed that K^+, and its counterion glutamate, act as a second messenger, which can inform the cytoplasm about the external osmolality (48). Although this concept presents a unified explanation of the responses of osmotic regulation, it may be difficult to test. The second phase, which may occur shortly after the osmotic upshock, is characterized by the accumulation of compatible solutes, allowing displacement of K^+ and counterions through specific and nonspecific efflux systems. In the absence of exogenous osmoprotectants, such accumulation depends on full expression of enzymes involved in biosynthetic pathways of various compatible solutes (proline, glycine betaine, trehalose, NAGGN, ...), whereas in the presence of osmoprotectants, this accumulation is mediated by direct uptake as a consequence of induction of genes encoding specific transporters.

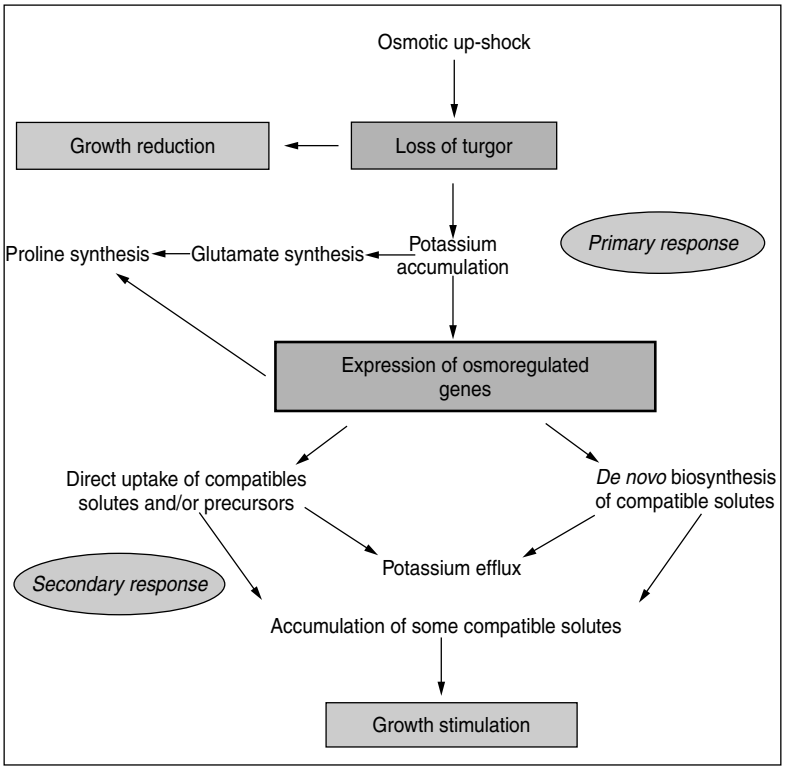

Figure 7. Phases of osmotic stress responses in bacteria.

Besides increased salt concentration, rain, and flooding/irrigation in soil subject bacteria to osmotic downshocks, which invoke a substantial remodeling of the cells. These changes include alterations in membrane and/or periplasmic protein composition, lipid composition, periplasmic glucan levels, and extracellular or capsular polysaccharide biosynthesis (8). In addition, the increase in turgor, produced by downshocks, and because of sudden water influx, requires the cell to rapidly reduce its pool of compatible solutes. Because most of these compatible solutes are metabolically inert in the majority of soil bacteria, and thus cannot be degraded, efflux systems should respond to high turgor. In severe downshock, mechanosensitive channels play a primary role in massive compatible solute expulsion; however, in smaller increases in turgor, specific export systems allow moderate rates of efflux (31,48).

Accumulation Versus Nonaccumulation

The concept of durable compatible solute accumulation within the cells exposed to osmotic upshock was established on the basis of studies of enteric bacteria, along with other gram-negative bacteria and several gram-positive bacteria. However, it is not entirely applicable to the symbiotic soil bacterium, *S. meliloti*, which has the capability to catabolize most of the osmoprotectants known to date, including glycine betaine, proline betaine, and ectoine. When this bacterium is subjected to salt stress both betaines are accumulated at high concentrations. This is not the case for ectoine, which is however, very protective against osmotic stress (17). When cells are grown under high-osmolarity conditions (0.5 M NaCl), in the presence of ectoine, this compound is not accumulated and its intracellular concentration remains too low (12 mM) to make any significant contribution to cell osmotic equilibrium. Ectoine, unlike glycine betaine, does not repress the synthesis of endogenous compatible solutes such as glutamate, NAGGN, and trehalose. Moreover, the intracellular glutamate level is strongly increased during the growth of the cells, and glutamate itself is very significantly involved in the osmotic balance restoration. Thus, it was proposed that ectoine may belong to a new class of nonaccumulated osmoprotectants, which act as chemical mediators (17). It is also noteworthy that ectoine is also capable of protecting other rhizobia insensitive to glycine betaine. Sucrose also acts as a powerful osmoprotectant for *S. meliloti* without being accumulated by osmotically stressed cells (18). On the contrary, sucrose is actively catabolized, and, like ectoine, does not suppress the accumulation of the major endogenous compatible solutes, and increases the level of cytosolic glutamate. How sucrose and ectoine stimulate the accumulation of glutamate in stressed cells of *S. meliloti* is not known, but it has been shown that both osmoprotectants do not act as close precursors to glutamate.

As previously mentioned, the accumulation of compatible solutes in soil bacteria cells subjected to increased salinity is dependent either on direct uptake from the soil solution (or the rhizosphere) or on biosynthesis, either de novo synthesis or production via the uptake of a direct precursor. Both mechanisms occur simultaneously in a majority of bacteria. In the following two sections, the focus (*1*) is on salinity tolerance in the gram-positive bacteria, mainly *B. subtilis* as an example of very well studied bulk soil bacteria and (*2*) on osmoprotection in the gram-negative bacteria, chiefly *S. meliloti* a typical representative of the rhizosphere and the microsymbiont of alfalfa root nodules. The biosynthesis and catabolism of glycine betaine have been examined in great detail in this bacterium. During the last decade, much attention has been focused on the molecular mechanisms of osmotic adaptation in these two soil bacteria.

SALINITY TOLERANCE IN GRAM-POSITIVE SOIL BACTERIA

Bacillus subtilis, A Typical Bulk Soil Bacterium

In addition to de novo biosynthesis of compatible solutes, most bacteria have developed transport systems used to acquire osmoprotectants. These compounds are naturally found in various soils because of natural decay of microbial, plant and animal cells, and also in the rhizosphere because of root exudates. However, these compounds are always present at very low concentrations. Thus, bacteria have evolved uptake systems with very high affinities (K_m values in the micromolar range), to allow the accumulation of nanomoles or micromoles of exogenous osmoprotectants to molar concentrations in the intracellular compartment. To deal with soils showing different compatible solute contents, bacteria are able to accumulate a large variety of osmoprotectants or precursors of compatible solutes, either because of a large spectrum of substrates transported by a given transporter or the presence of several highly specific transporters.

Bacterial transporters involved in osmotic regulation belong to two groups, depending on their structural complexity and the driving force used for substrate uptake:

*symports are composed of a single membrane-spanning protein that is highly hydrophobic and transports a substrate together with an ion, either H^+ or Na^+.

*ABC (ATP Binding Cassette) transporters are multicomponent systems, which usually consist of three different proteins: a binding protein located either in the periplasm of gram-negative bacteria or linked by a lipid anchor to the outside face of the cytoplasmic membrane of gram-positive bacteria, a dimeric hydrophobic protein spanning the inner membrane, and a peripheral ATPase (dimeric) located on the cytoplasmic side. Substrate translocation across the cell membrane is dependent on direct ATP hydrolysis.

These two types of osmoregulated uptake systems were first described in the enteric bacteria, *Escherichia coli*, in which a H^+ symport (ProP), and an ABC transporter (ProU) were shown to be involved in the uptake of proline and glycine betaine under hyperosmotic conditions (48,49). In soil bacteria, transporters for osmoprotectants or their precursors have been studied at the molecular level only in *C. glutamicum* (50) and *B. subtilis* (31). Because of the large quantity of available data, *B. subtilis* is chosen here for further description. This bacterium, which is widespread in nature, is a low G-C content, facultative anaerobic endospore forming rod-shaped cell. The

natural habitats of *B. subtilis* are mainly the upper layers of various soils in which frequent fluctuations of water availability lead to large osmolality variations.

Molecular Characteristics of Uptake Systems. After a sudden increase in external osmolality, *B. subtilis* initially responds by a rapid uptake of K^+, followed by de novo synthesis of the compatible solute proline. This primary response contributes to the recovery of turgor and the resumption of bacterial cell growth. When the osmotic constraint remains, *B. subtilis* can accumulate various osmolytes, and develop five elegant osmoprotectant uptake systems (called Opu) for transport of a large variety of osmolytes or their precursors (choline). Single and multicomponent systems used by *B. subtilis* are shown in Figure 8. OpuD and OpuE are symport systems, highly substrate specific, that allow the uptake of the main osmoprotectants, glycine betaine (OpuD) and proline (OpuE) together with Na^+. In contrast, OpuA, OpuB, and OpuC belong to the ABC superfamily of transporters composed of an extracellular substrate-binding lipoprotein anchored to the membrane by the lipid group. When bound to its specific protein, the substrate is delivered to the integral membrane component, either composed of a unique protein for OpuA or two distinct proteins for OpuB, and OpuC. The substrate translocation across the inner membrane depends on direct ATP hydrolysis by associated ATPase present on the cytoplasmic side of the membrane. These transporters share different substrate specificities (Fig. 8). On osmotic shock, the transport of betaines is mainly assumed by the high specific OpuA system (narrow range of substrates) and to a less extent by OpuC (wide range of substrates) and OpuD, which are mostly devoted to glycine betaine uptake. The OpuC system plays an essential role in *B. subtilis* osmoprotection because it is the sole transporter carrying various osmoprotectants, which cannot be taken up by the other Opu systems. This includes ectoine transported with a low affinity, and compounds, such as carnitine, crotonobetaine, γ-butyrobetaine, choline-*O*-sulfate, and proline betaine, transported by a high-affinity system (40,41,44). The OpuA, OpuC, and OpuD transporters also play a major role in the uptake of 2-dimethylsulfonioacetate (DMSA), the sulfonium analog of glycine betaine, and of 3-dimethylsulfoniopropionate (DMSP), a dominant osmolyte in marine algae (43).

Depending on the availability of choline in the soil, *B. subtilis* is also able to synthesize glycine betaine. This synthesis occurs by a two-step reaction that first converts choline into glycine betaine aldehyde using a metal containing type III alcohol dehydrogenase (GbsB). The aldehyde is subsequently converted into glycine betaine by a glycine betaine aldehyde dehydrogenase (GbsA) (37). Like most microorganisms, *B. subtilis* does not synthesize choline that might be exogenously provided. Two different ABC systems allow high-affinity uptake of choline in *B. subtilis*: one is strictly specific for choline uptake (OpuB), whereas the other one (OpuC) is the less specific osmoprotectant transporter. It is noteworthy that, although they exhibit a striking difference in substrate specificity, the OpuA and OpuC proteins share high sequence identity (70 to 85%). As their corresponding genes, organized in operon, are located close to each other on the *B. subtilis* chromosome, it was suggested that these two systems have evolved from a duplication event of a primordial gene cluster (51).

Regulation of Uptake Systems. To allow quick intracellular accumulation of osmoprotectants after an increase in external osmolality, corresponding transporters display both a very high affinity (K_m) for their substrates and a high velocity of uptake (V_{max}) in cells grown at elevated salinity. This is typically the case for OpuA-mediated

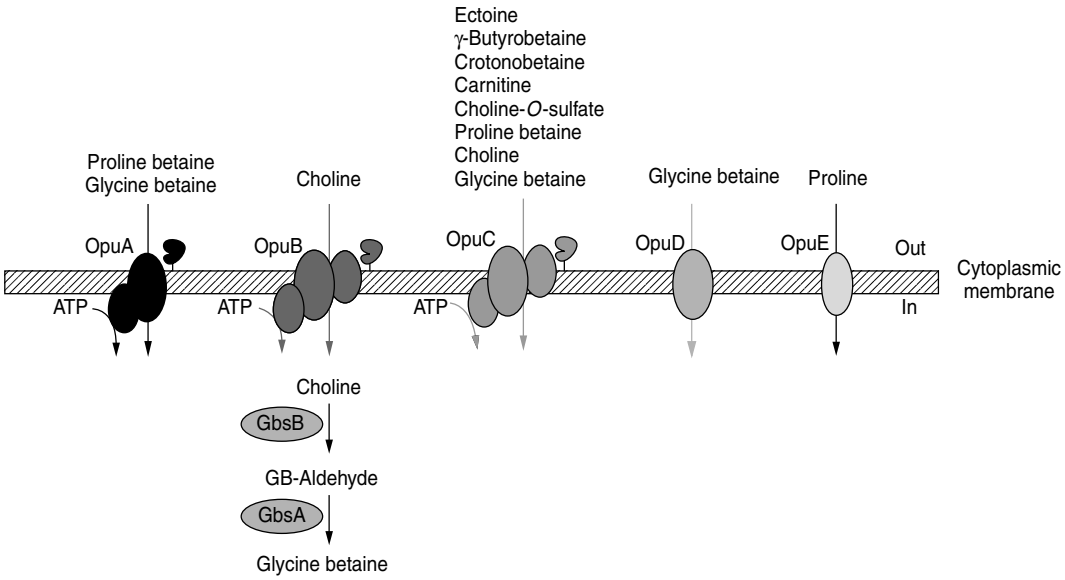

Figure 8. Transport systems involved in response to salt stress in *B. subtilis*. Taken from B. Kempf and E. Bremer, Arch. Microbiol. 170, 319–330 (1998) with permission.

glycine betaine transport in which initial uptake activity for [^{14}C]-glycine betaine is stimulated about fourfold in cells grown at high osmolality compared with control cells (52). The activation of transport depends on the degree of osmotolerance of the microorganisms considered, and the level of compatible solutes accumulated is adjusted on the intensity of the salt stress imposed. Salt stimulation could be due to either allosteric activation of preexisting proteins or to increased expression of transporter genes. It has been shown that, in a high-salt environment, glycine betaine transport through OpuD results from both de novo synthesis and allosteric activation, whose mechanism is still unknown (53). The other Opu transporters are also upregulated at the level of transcription in response to increased salinity. Expression analysis of *opuA* and *opuE* genes show that they are both under the control of two promoters: one strongly induced by salinity, whereas the other is constitutively expressed for *opuA* or transiently stimulated by salt for *opuE*. The nature of the osmosensor that coordinates transcription of these genes remains unclear in *B. subtilis*, as in the well-studied operon *proU* of *E. coli*. In this latter host, it has been suggested that supercoiling of DNA in the promoter region might contribute to the 100-fold induced expression observed in saline stress condition. Depending on the availability of osmoprotectants in the environment, transcription of the osmoregulated genes could also be turned off. For example, salt stimulation of the proline transporter OpuE is strongly reduced when the major osmoprotectant glycine betaine is present in growth medium. This reflects a preference based on the nature of the compatible solute used for osmoprotection.

Efflux of Compatible Solutes. In the soil or rhizosphere, bacteria are also exposed to hypoosmotic shocks mainly caused by rain or irrigation. The consequence is an influx of water into the cell followed by a quick reduction of the intracellular osmolyte pool to avoid a dramatic increase in turgor. In contrast to *S. meliloti*, which can use some osmoprotectants as carbon or nitrogen sources (see Osmoprotection in gram-negative rhizosphere bacteria), *B. subtilis*, like most microorganisms, does not catabolize these compatible solutes. They are instead rapidly released to adjust cell turgor to the new extracellular osmolality. In *E. coli*, it has been shown that the opening of mechanosensitive channels (Msc) allows the efflux of osmolytes in a few seconds. These channels display little ion or solute specificity but are crucial for managing hypoosmotic stress, even if they are not the only means of removing compatible solutes from the cell (see 31 for review). In soil bacteria, none of these channels have yet been characterized at a molecular level.

Other Gram-Positive Bacteria. *Corynebacterium glutamicum*, a soil bacterium used for industrial amino acid production, also accumulates a large variety of osmolytes by uptake from the environment. Compared with *B. subtilis* in which three of the five osmoregulated Opu transporters belong to the ABC superfamily, *C. glutamicum* is equipped with three secondary carriers composed of a single transmembrane protein, which plays a direct role in osmotic adaptation. EctP allows the translocation of ectoine, proline, and glycine betaine, ProP is a proline/ectoine carrier, and BetP is a high-affinity glycine betaine uptake system (50,54). This bacterium uses an additional proline transporter (PutP), but only for anabolic purposes, and not for osmoadaptation (32). As for *B. subtilis*, the capacity to use a large spectrum of compatible solutes may help the bacterium to adapt in soils of different salinities and compositions.

OSMOPROTECTION IN GRAM-NEGATIVE RHIZOSPHERE BACTERIA

Sinorhizobium meliloti, A Nitrogen-Fixing Root Endosymbiont

Betaines are among the most potent osmoprotectants for *S. meliloti*. Glycine betaine can be taken up from the external medium or synthesized from choline and its derivatives, choline-O-sulfate and phosphorylcholine. Proline betaine, which occurs widely in *Medicago* species, is also actively transported but cannot be synthesized by *S. meliloti*.

Transport of Betaine and Precursors. Glycine betaine uptake is strongly stimulated when *S. meliloti* is grown in the presence of NaCl or subjected to an osmotic upshock (36). In the latter case, a high-affinity uptake activity is increased fourfold within one minute, and this activation may involve a constitutively expressed transport system. Obviously, different betaine transport systems are present in this bacterium, but only one has recently been characterized at the molecular level (55). This system (Hut), an ABC-type transporter, is primary involved in histidine uptake, transcriptionally regulated by this amino acid, independently of the osmolality of the medium. However, Hut is also able to transport glycine betaine at low affinity, and proline betaine at high affinity. It seems mainly required for the transport of these three compounds when they are used as carbon or nitrogen sources. Previous studies have suggested that glycine betaine uptake in *S. meliloti* is mediated by a high-affinity system (K_m of 5 μM) probably related to the well-characterized ProU system of *E. coli*. A highly specific glycine betaine binding protein (GBBP) has been identified in periplasmic fractions of *S. meliloti* cells grown in the presence of added NaCl. This protein shows a mass of 32 kDa, similar to the GBBP ProX from *E. coli* (56). This second glycine betaine uptake system might be transcriptionally regulated because the GBBP is barely detectable in periplasmic fractions obtained from cells grown in the absence of NaCl, whereas it is abundant in salt-stressed cells.

High-affinity uptake of proline betaine (K_m of 10 μM) is also strongly stimulated by the addition of NaCl to the growth medium (39). Such uptake is strongly inhibited in the presence of an excess of glycine betaine in the assay, and it is more likely that proline betaine enters into the cells via an osmoregulated glycine betaine transporter. However, the presence of a periplasmic proline betaine binding protein could not be detected and, this betaine does not compete with glycine betaine for binding to the GBBP, even in the presence of a 100-fold excess of proline betaine.

Choline, the precursor of glycine betaine, is also imported from the external medium via high-affinity systems. Three kinetically distinct choline transport activities have been identified in *S. meliloti* (57). Two activities are constitutive, one of low affinity (K_m of 100 μM), and one of high affinity (K_m of 6.5 μM), which is inhibited at high salt concentration, and may be involved in the utilization of choline as carbon and/or nitrogen sources. A third choline transport activity has also high substrate affinity (K_m of 0.4 μM), and is most probably transcriptionally regulated by the substrate. Such a system is believed to play an important role in osmoregulation because it retains full activity under high osmolality. These three systems have not yet been characterized at the molecular level, but one could be an ABC-type transport system. The identification of a choline-binding protein in periplasmic fractions of *S. meliloti* grown under an excess of NaCl in the medium is consistent with this possibility (56).

Biosynthesis of Glycine Betaine. *Sinorhizobium meliloti*, as most microorganisms, is unable to de novo synthesize glycine betaine, and uses a two-step oxidation of choline to produce this compatible solute (58). Moreover, *S. meliloti* displays the unusual capability to transform choline derivatives, choline-*O*-sulfate and phosphorylcholine, into choline (59). Choline is a common constituent of the eukaryotic membranes in the form of phosphatidyl choline, and therefore, is widespread in different environments, including the soil and the rhizosphere. Thus, choline is available for the bacteria and can be taken up. The glycine betaine biosynthetic pathway from choline or its derivatives has been characterized at the molecular level in *S. meliloti*. Four genes, *betICBA*, most probably organized in one operon are involved (59,60). The *betA* and *betB* gene products are implicated in the conversion of choline into glycine betaine, and are well conserved with their homologs BetA and BetB of *E. coli* (60). The first step of choline transformation is performed by a FAD-containing membrane-bound choline dehydrogenase (BetA), which can also oxidize glycine betaine aldehyde (GBA) into glycine betaine. The second enzyme, BetB, is soluble and catalyzes the conversion of the toxic intermediate, GBA, into glycine betaine (Fig. 9). BetB is highly specific and presents a high salt-tolerant activity. Choline-*O*-sulfate and phosphorylcholine are hydrolized into choline via BetC, a choline sulfatase, which does not have any counterpart in *E. coli* (59). In contrast to *E. coli* and *B. subtilis*, *S. meliloti* does not use choline-*O*-sulfate or phosphorylcholine as osmoprotectant per se, because these compounds cannot restore growth of a salt-stressed *betC* mutant. It is tempting to argue that the presence of BetC in *S. meliloti* confers to this bacterium a selective advantage in the rhizosphere, as *S. meliloti* has the unique capacity to use choline-*O*-sulfate as an energy source, whereas *B. subtilis*, for example, can use this compound only to cope effectively with high salinity (40).

The expression of *betA* and *betB* genes is strongly induced by choline or choline-*O*-sulfate, independently of the osmolarity of the medium or of the presence of other carbon and nitrogen sources (61). The transcriptional

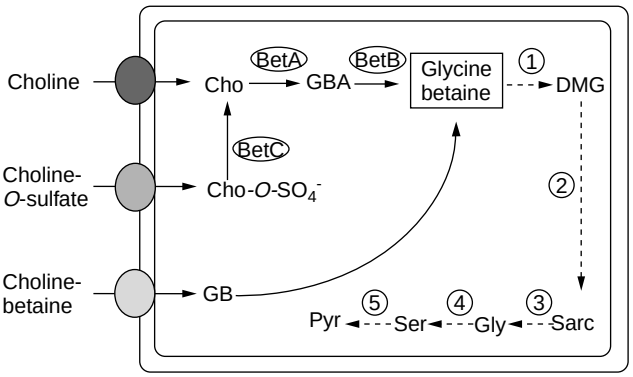

Figure 9. Biosynthetic and catabolic pathways of glycine betaine in *S. meliloti*. Symbols: GB, glycine betaine; Cho-*O*-SO_4, Choline-*O*-sulfate; Cho, choline; GBA, glycine betaine aldehyde; BetC, choline sulfatase; BetA, choline dehydrogenase; BetB, glycine betaine aldehyde dehydrogenase; 1, glycine betaine transmethylase; 2, dimethylglycine dehydrogenase; 3, monomethylglycine dehydrogenase; 4, serine transhydroxymethylase; 5, serine dehydratase.

regulation of the *bet* genes may involve the BetI protein, which displays 33% identity with BetI from *E. coli*; in this bacterium BetI is a repressor of the *bet* genes. Both BetI polypeptides are characterized by a conserved N-terminal region that contains an helice-turn-helice motif involved in binding to their DNA target sequence (62). In addition, the *betA* and *betB* genes are transcribed at all stages of nodule development in *Medicago sativa*, from the initiation of the infection thread to the mature nodule. Such finding is in good agreement with the BADH and CDH activities measured in bacteroids (63). This suggests that choline, and/or its precursor choline-*O*-sulfate, are available for the bacteria during the symbiotic interaction with alfalfa.

Catabolism of Betaines. A remarkable feature of *S. meliloti* is its ability to use glycine betaine and proline betaine as carbon and nitrogen sources when grown under low osmolality medium or following an osmotic downshock (36,58). The catabolism of glycine betaine involves successive demethylations of the quaternary ammonium, which are catalyzed by glycine betaine transmethylase, dimethylglycine dehydrogenase, and monomethylglycine dehydrogenase, before subsequent degradation into serine and pyruvate via a serine transhydroxymethylase and a serine dehydratase (Fig. 9). The specific activities of these enzymes are reduced when cells are grown at high salt concentration, thus permitting cells to maintain accumulated glycine betaine as a compatible solute. However, when the cells are grown in the presence of a high choline concentration (7 mM), glycine betaine degradation is still efficient, even in bacteria cultivated at high salinity (58). Consequently, intracellular glycine betaine levels in stressed cells begin to decrease during the second-half of the exponential phase especially if exogenous glycine betaine is no longer available (64). It should be pointed out that, in natural soils or in the rhizosphere, such high choline concentrations may never occur.

Proline betaine catabolism proceeds through two N-demethylations that generate monomethylproline and

proline, which enters the general metabolism (39). This pathway is also strongly inhibited in salt-stressed cells of *S. meliloti*. Two loci of the *S. meliloti* genome are essential for the degradation of proline betaine. The first locus, located on the symbiotic plasmid to the right of the *nod* regulator *nodD2*, contains four open-reading frames with functional homology to known proteins, including a putative Rieske monooxygenase (Stc2) and a putative NADPH-FMN-reductase (Stc4), which are necessary for the first demethylation of proline betaine (65). These enzymes may form a multienzyme complex that resembles the oxygenase complex involved in xenobiotic degradation in some gram-negative bacteria. Expression of the *stc2* gene is specifically induced by proline betaine, but not by other betaines, monomethylproline, or proline (66). The second locus *stcD*, located on the chromosome, is required for the conversion of monomethylproline to proline. The *stcD* gene encodes a putative demethylase, which belongs to the family of flavoproteins characterized as α/β-barrel oxidoreductase (67). The expression of this gene is also strongly enhanced by the presence of proline betaine in the growth medium. Because mutants incapable of growing on proline betaine as the sole carbon and nitrogen sources are less efficient at forming root nodules on *Medicago*, and less competitive against isogenic wild-type strains, it has been suggested that proline betaine catabolism contributes to rhizobial colonization of alfalfa seedling roots (67).

Others betaines including trigonelline, a secondary plant metabolite, carnitine, or γ-butyrobetaine are used by *S. meliloti* as carbon and nitrogen sources, and may act as osmoprotectants (36). The genetic loci responsible for the catabolism of trigonelline and carnitine, are also located on the symbiotic plasmid (66).

Other Osmoprotectants in *Sinorhizobium meliloti*. As in other bacteria, K^+ and glutamate accumulation are initiated immediately after an osmotic upshock (24). Glutamate synthesis does not implicate de novo protein synthesis. However, neither glutamate synthase (GOGAT) nor glutamate dehydrogenase (GDH) activities appear stimulated by salt. Attempts to show allosteric induction of GDH activity by salt or activation by K^+ have been unsuccessful. It was suggested that transaminases mediate glutamate accumulation during salt stress through the transfer of nitrogen from other amino acids to α-ketoglutarate. In addition, GOGAT activity does not contribute to the excess of glutamate made by *S. meliloti* in response to osmotic stress, because a mutant deficient in GOGAT retains full glutamate production under salt-stress conditions. As already mentioned, at increased salt concentration, *S. meliloti* also synthesize NAGGN and trehalose (Fig. 6).

The sulfonium compound, dimethylsulfoniopropionate (DMSP), acts as another powerful osmoprotectant in *S. meliloti*, whereas dimethylsulfonioacetate (DMSA) and dimethylthioethanol (DMSE) are toxic to stressed and unstressed cells as a consequence of their catabolism (42). In presence of exogenous DMSP, stressed cells accumulate this osmolyte as a dominant cytosolic compatible solute, and produce minor amounts of the other synthesized osmolytes (glutamate, NAGGN, and trehalose). Uptake of DMSP is osmoregulated, and a 2.5-fold stimulation is observed in cells grown at high salt concentration. This uptake is strongly inhibited by glycine betaine and DMSA, which suggests that the three compounds are apparently taken up via the same transporter. In contrast to glycine betaine and DMSA, DMSP is the only nonmetabolizable osmolyte found in *S. meliloti* (42).

The tetrahydropyrimidine ectoine appears almost as effective as glycine betaine in improving the growth of salt-stressed *S. meliloti* cells, although this compound is not accumulated (see section, Accumulation Versus Nonaccumulation). Ectoine transport displays a K_m of 80 μM, is highly specific, inducible by the substrate, periplasmic protein-dependent, and slightly enhanced by salt (17). Endogenous ectoine is catabolized and assimilated as carbon and nitrogen sources through a pathway, which is distinct from that described for glycine betaine.

The last nonaccumulated osmoprotectant identified in *S. meliloti* is sucrose, which is particularly efficient in promoting the recovery of cytoplasmic volume after plasmolysis as a result of high osmolality (18). Under osmotic stress conditions sucrose is catabolized but does not serve as precursor for the main osmolytes (glutamate, NAGGN, and trehalose). The uptake of sucrose is strongly reduced during the lag phase following osmotic upshock, but is progressively restored to the level of unstressed cells when the cultures enter the exponential phase.

Other Gram-Negative Bacteria

Besides *S. meliloti*, the osmoadaptative responses of other rhizobial species have not been thoroughly studied, and most of the studies have been conducted with isolates poorly represented in most culture collections. Recently, the taxonomy of the legume root-nodulating bacteria has undergone major revision and improvements, and still is in a state of transition. Based largely on sequencing of the 16S and 23S rRNAgenes, six genera have been recognized: *Rhizobium*, *Mesorhizobium*, *Sinorhizobium*, *Azorhizobium*, *Allorhizobium*, and *Bradyrhizobium* (68). Although bacteria of the genus *Bradyrhizobium* are more salt sensitive than other genera, marked variations in salt tolerance are observed among rhizobia. A number of strains of *B. japonicum* are growth inhibited by NaCl concentration lower than 100 mM, whereas growth of various strains of *S. meliloti* and *R. leguminosarum* still occurs at salt concentrations of more than 300 mM (69). Rhizobia isolated from woody legumes also show substantial salt tolerance: strains from nodules of *Hedysarum*, *Acacia*, *Prosopis*, and *Leucaena* plants can tolerate up to 500 to 800 mM NaCl (70). Nevertheless, many species of rhizobia adapt to saline conditions by the intracellular accumulation of compatible solutes. Thus, when grown in media of elevated osmotic strength or immediately after an osmotic upshock, a variety of rhizobia accumulate potassium via uptake (25,26), and glutamate via biosynthesis (29). Until now, K^+ transporters have not been characterized at a molecular level in rhizobia.

As in *S. meliloti*, exogenous supplies of glycine betaine and choline, enhance the growth of various rhizobia (*Rhizobium tropici*, *S. fredii*, *Rhizobium galegae*, *Mesorhizobium loti*, and *Mesorhizobium huakuii*)

subjected to salt stress (69). However, both compounds are inefficient for very salt-sensitive strains, such as *R. leguminosarum*, *Rhizobium etli*, and *B. japonicum*. Except for *B. japonicum*, all tested strains exhibit transport activity for glycine betaine and choline. Nevertheless, the main physiological role of both compounds in the family *Rhizobiaceae* seems to be as an energy source through catabolism, whereas their contribution to osmoprotection is restricted to certain strains. Because the capacity to use glycine betaine as a growth substrate has been reported only in a few soil species, including *Azospirillum lipoferum*, (71) and *P. aeruginosa* (72), it is tempting to speculate that the ability to catabolize glycine betaine could increase competitiveness of the rhizobia in the rhizosphere (69). Trehalose is also accumulated by various rhizobia (Table 1) grown in media of high salinity, whereas proline is not. This amino acid does not play a role in osmoregulation in rhizobia, and in contrast, is actively catabolized through the *putA* gene product (73). Ectoine, also improves the growth of *B. japonicum* and *R. leguminosarum* bv *viciae* and bv *trifolii* in high-salt medium (17) without being accumulated, and sucrose has a similar effect on *R. leguminosarum* bv *phaseoli* (18).

All the previous compatible solutes already mentioned in rhizobia have been observed in several other gram-negative bacteria (Table 1) including *A. chroococcum* (27), *A. tumefaciens* (28), various *Pseudomonas* (19,30,38,45), *A. brasilense*, and *A. halopraeferens* (16,27,45).

The other compatible solutes not yet examined here are represented by the polyols, which are accumulated in *Pseudomonas*, and the mannosucrose identified in *A. tumefaciens* (Table 1). In response to salt stress, *P. mendocina* accumulates O-α-D-glucopyranosyl-α-(1 $\rightarrow$ 2)-glycerol, also called glucosylglycerol (30). The intracellular concentration of this osmolyte increases with the quantity of NaCl in the growth medium, and its accumulation relies on de novo biosynthesis. Such a compound is not detected in rhizobia, and previous to that study, the pivotal role of glucosylglycerol in osmoadaptation had been shown only in photosynthetic bacteria. Another pseudomonad, *P. putida*, accumulates mannitol as a compatible solute (45) when subjected to osmotic stress induced by ionic and nonionic compounds. Accumulation of mannitol is a typical feature of salt-stressed *P. putida* strains, as this polyol is not detected in *P. aeruginosa*, *Pseudomonas cepacia*, and *P. fluorescens*. The mannosucrose (β-fructofuranosyl-α-mannopyranoside) has been identified as the major osmolyte in salt-tolerant strains of *A. tumefaciens* (28). The mannosucrose accumulation, which depends on biosynthesis, is osmoregulated and not observed in less tolerant biotypes. In the case of *A. tumefaciens*, as in the case of *P. putida*, addition of glycine betaine to the growth medium results in accumulation of the betaine with parallel depletion of mannosucrose and mannitol, respectively. This finding is in accordance with results already mentioned for other bacteria. Glycine betaine, when available, seems to be a better compatible solute than others because it reaches higher levels than does the sum of those solutes it displaces. In addition, the uptake of glycine betaine is probably less energy consuming than de novo synthesis of mannosucrose or mannitol.

PHYSIOLOGICAL EFFECTS OF SALINITY ON RHIZOBIUM-LEGUME SYMBIOSIS

Within the soil, a wide range of bacteria have the ability to fix nitrogen, that is, convert the stable nitrogen gas in the atmosphere into ammonia, via the aid of an enzyme complex, nitrogenase which catalyzes the following reaction:

$$N_2 + 8H^+ + 8e^- + 16\ ATP \rightarrow 2\ NH_3 + H_2 + 16ADP + 16Pi$$

The nitrogenase is expressed in free-living diazotrophs soil bacteria, such as *Azotobacter*, *Clostridium*, or *Azospirillum*, and in bacteroids, which are the symbiotic form of rhizobia. These rhizobia elicit on the roots or in some cases on the stems of their legume hosts, the formation of specialized organs called nodules. These nodules constitute an ecological niche in which the necessary conditions for the reduction of atmospheric nitrogen are provided, particularly concerning oxygen and energy availability for bacteroids. In infected cells of nodules, that is, cells invaded by the microsymbiont, free dissolved oxygen is supplied to bacteroids at very low concentrations (5 to 40 nM) by the presence of large amounts (3 mM in soybean nodules) of leghemoglobin. This hemoprotein binds free oxygen and avoids complete inactivation of the nitrogenase, which is extremely sensitive to oxygen. Inside nodules, bacteroids are surrounded by a peribacteroid membrane (PBM) of plant origin that effectively segregates the bacteroids from the plant cytoplasm, which contains leghemoglobin, and determines the type and quantity of compounds that are transferred between the two partners of the symbiotic association. Therefore, rhizobia are totally dependent on their plant hosts for nutrients and oxygen when living within the nodule. The organelle-like structure consisting of bacteroids, the peribacteroid space (PBS) and the PBM (Fig. 10) is referred to as the symbiosome (74). Although the principal exchanges between the symbiotic partners are reduced carbon from the plant host to the bacteroid and ammonia from the bacteroid to the plant, other important exchanges also occur (75).

Soil salinity is one of the major factors limiting legume production, particularly in arid and semiarid regions. Increasing salt concentrations in the soil affect the *Rhizobium*-legume symbiosis by osmotic stress (drought) and nonosmotic effects (toxicity and ion imbalance). This symbiosis and nodule formation on legumes are more sensitive to salt or osmotic stress than are the free-living bacteria (76). Both salinity and drought inhibit the expansion and curling of root hairs, the proportion of root hairs containing infection threads, and reduce the number and the size of nodules on *Vicia faba* roots (77). The inhibitory effect of salinity on soybean nodulation has been attributed to a decrease in rhizobial colonization, and a shrinkage of root hairs (78). When salt stress is applied to mature nodulated soybean, nitrogen fixation, determined by the reduction of acetylene into ethylene, rapidly decreased. This inhibition has been attributed to a direct effect on nitrogenase, or an indirect effect through decreases in leghemoglobin content, bacteroid respiration

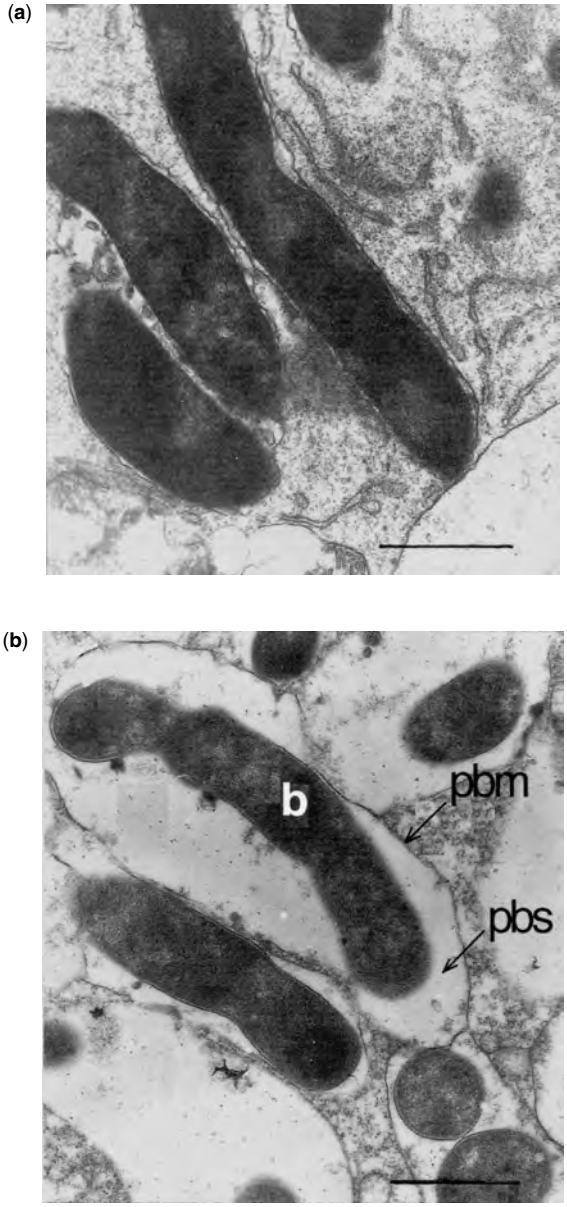

Figure 10. Electron micrographs of the nitrogen-fixing zone of a five-weeks-old alfalfa nodule. (**a**) control plants not subjected to salt stress; (**b**) plants submitted to 0.1 M NaCl during two weeks. Bars indicate 1 μm. Symbols: b, bacteroid; pbm, peribacteroid membrane; pbs, peribacteroid space.

rate, and malate concentration in nodules (79). Under salt-stress conditions, changes in structure occur in the nodule cortex, particularly a decrease in the surface of the inner cortex cells in parallel with the occlusion of intracellular spaces by glycoproteins (80). Consequently, the oxygen diffusion resistance in the nodule increases, and thus limits bacteroid respiration and energy production, which is crucial for nitrogenase activity. Such inhibition can be partly restored by increasing the partial oxygen pressure around nodulated roots from 20 to 60 kPa. Moreover, these structural changes also induced ions exclusion (Na^+ and Cl^-) from the central zone of the nodule, possibly through a K^+/Na^+ interchange within the cortex, especially in legumes known to be tolerant to salinity (81).

Exogenous glycine betaine application partially restores nitrogen fixation of salt-stressed alfalfa seedlings nodulated by *S. meliloti* (82). This betaine is rapidly taken up, and slowly translocated to the shoots. In unstressed plants, glycine betaine catabolism is extremely slow in shoots, but significant in roots, and even more important in nodules. Under salt stress, the rate of glycine betaine catabolism is significantly reduced, mainly in nodules. Experiments conducted with bacteroids isolated from alfalfa nodules demonstrate that the microsymbiont is able to actively take up glycine betaine, choline, and proline betaine (83). Moreover, these transport activities are stimulated in the presence of salt, and choline, which did not appear as an osmolyte per se for bacteroids. Free-living cells of *S. meliloti* further metabolized choline into glycine betaine, following the pathway described previously (63). Because the plant host has the capacity to produce choline and proline betaine, these compounds are probably available for the bacteroids during symbiosis. All these results are consistent with the in planta expression of *betA* and *betB* gene already mentioned (see section Biosynthesis of Glycine Betaine).

Besides quaternary ammonium compounds, the accumulation of various amino acids, particularly proline, is also a general response of plants to environmental stress. In legume nodules, proline is the most widely distributed osmolyte accumulated under salt stress (84,85). Proline is partly responsible for the osmotic adjustment in the nitrogen-fixing zone of nodules after its accumulation inside symbiosomes. As a consequence, the PBS volume increases two- to threefold (Fig. 10). In *V. faba*, an amide-producing legume, this accumulation results both in a passive diffusion through the PBM, and the very low rate of uptake by bacteroids. Thus, proline is not used as an energy-yielding substrate for fababean bacteroids, but may play a role in osmotic stress adaptation (85). The situation seems different in ureide-exporting nodules like soybean, in which nitrogen fixation by bacteroids isolated from stressed nodules could be enhanced by eight fold when exogenous proline is provided. In this case, proline catabolism may supply a significant fraction of the energy requirement for nitrogen fixation (86). In addition to proline, asparagine concentration is greatly increased in salt-stressed alfalfa nodules, whereas the level of other amino acids is very limited. Among carbohydrates, pinitol concentration is significantly enhanced, especially in bacteroids, and pinitol may contribute to the tolerance to salt stress. However, trehalose concentration remains low in salt-stressed nodules, and its role in osmoregulation appears unlikely in alfalfa (84).

CONCLUSION

The ability of soil bacteria to adapt to increased salinity is essential for their survival and proliferation. Clearly, the main physiological response to salinity is the accumulation of compatible solutes. Such accumulation depends either on de novo synthesis or uptake of osmoprotectants. However, the utilization of exogenous

osmoprotectants is preferred over biosynthesis of endogenous compounds, when available in the environment. The prominent compatible solutes used by soil bacteria are potassium ions, a few amino acids (glutamate and proline), sugars (trehalose, sucrose, and mannosucrose), polyols (glucosylglycerol and mannitol), quaternary ammonium compounds (glycine betaine and other betaines), sulfonium compounds (dimethylsulfoniopropionate and dimethylsulfonioacetate), ectoine, and a small peptide (N-acetylglutaminylglutamine amide). In most soil bacteria, glycine betaine is the preferred compatible solute and generally provides the highest level of salt stress tolerance.

The remarkable property of compatible solutes to overcome the inhibitory effects of salinity is the result of a dual function. These compounds are accumulated in the cytoplasm of the cells up to molar concentrations without affecting the physiological function of the living cell. Thus, they are used to restore and maintain cell turgor in media of high osmolality, a key condition for cell growth. Compatible solutes also have special interactions with proteins and serve as stabilizers, protecting proteins from denaturation at high ionic strength. Although it has been proposed recently that glycine betaine, for example, may actively assist in vivo protein folding in a chaperone manner (22), the protective property of compatible solutes on proteins is not fully understood.

The mechanisms by which soil bacteria sense osmotic variations in the environment are still poorly defined. However, it is more likely that the molecular mechanisms of osmosensing will vary among various osmoprotectant uptake systems, and full characterization of these mechanisms remains an exciting unknown area in the field of osmotic regulation. Studying this largely unexplored basic scientific area will provide a better insight not only into physiological, biochemical, and genetic mechanisms used in nature to maintain a functional cell under osmotic stress conditions but may also be exploited for biotechnological and agricultural purposes. Protection of enzymes against denaturation processes (heat, freezing, drying and inactivation) with compatible solutes has been already observed many times, and it is reasonable to assume that this protective effect has a wide range of potential applications in bio- and enzyme technology. On another line, there is currently a need to develop salt- and drought-tolerant plants, because increases in the salinity of soils or in water used for irrigation result in decreased productivity of most crop plants. Construction of salt-resistant plants carrying bacterial genes, which encode enzymes for the synthesis of glycine betaine, has been already employed with success (87). Henceforward, the metabolic engineering of biosynthetic pathways for osmoprotective compounds into agronomically important crop plants is a promising approach to enhance salinity tolerance in those plants.

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SALINITY TOLERANCE.

See SALINITY EFFECTS ON THE PHYSIOLOGY OF SOIL MICROORGANISMS

SALMONELLA IN AQUATIC ENVIRONMENTS

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TAXONOMIC POSITION OF *SALMONELLA*

Salmonella are facultatively anaerobic gram-negative straight rods, usually motile by means of peritrichous

flagella, included in the family *Enterobacteriaceae* (1). According to Bergey's Manual the genus includes two species, *S. bongori,* containing less than 10 extremely rare serovars or types, and *S. choleraesuis*, embracing more than 2,500 serovars, although the number of serovars continues to increase (1,2). The latter species is divided, on the basis of phenotypic and genetic characters, into six subspecies: *arizonae*, *choleraesuis*, *diarizonae*, *houtenae*, *indica*, and *salamae*. Serological studies of these organisms have resulted in the establishment of an identification procedure known as the Kauffmann-White scheme (3). This system arranges the organisms into serovars, on the basis of the nature and combinations of cell surface and flagellar antigens, and the organisms are designated by numbers and letters given to different O, Vi, or H antigens (3,4).

The names given to the salmonellae do not follow the usual rules of nomenclature for bacteria. The organisms were originally named by indicating the disease and the animal from which the organisms were isolated. Some of these names continue to be used, such as *S. choleraesuis* and *S. typhimurium*. More recently, this type of nomenclature has been abandoned because the older names imply a more limited pathogenicity than it is actually found. Currently, names are assigned indicating the city, state, or even the country where the organism was first isolated (e.g., *S. london*, *S. montevideo*, *S. panama*). It has been recommended that diagnostic laboratories report their isolates as named *Salmonella* serovars or, in the case of unnamed serovars, by their antigenic formulae and subspecies. Examples include *Salmonella typhi* or *Salmonella* serovar *typhi*; *Salmonella choleraesuis* subsp. *salamae* serovar 56:z_{10}:e,n,x (this antigenic formula represents the O antigens: the phase 1 H antigens and the phase 2 H antigens, respectively) (3,5). The taxonomy of the genus is under discussion because some authors consider that the genus should be formed by four species, *S. typhimurium*, *S. enteritidis*, *S. choleraesuis* (including six subspecies) and *S. bongori*, whereas others propose a reduction of the genus to a single neotype species *S. enterica* (6,7).

THE ROLE OF *SALMONELLA* IN HUMAN INFECTION

Members of the genus *Salmonella* are major international food-borne pathogens, approximately 30,000 cases being reported each year in the United Kingdom (8), and estimated cases in the United States range between 800,000 and four million each year, with approximately 500 fatalities (9). There are several reviews on the control of food-borne outbreaks of *Salmonella* and their prevention (4,10–12). Three major types of clinical symptomatology can be recognized: acute gastroenteritis, septicemia, and typhoid fever. Salmonellosis, or salmonella gastroenteritis is a widespread infection that often begins with a sudden headache, abdominal distress, diarrhea, nausea, and sometimes vomiting (4). Usually, the disease is self-limited, with recovery in a couple of days, although it can persist for a couple of weeks or more (12). Dehydration can occasionally be life threatening, particularly in very young or elderly people. Antibiotic treatment is necessary in less than 2% of clinical cases (13). Although the infection may initially be only a gastroenteritis, on occasions it may progress to an enteric fever that manifests itself as a systemic infection (4,12). Transient bacteremia occurs in less than 5% of adults presenting gastroenteritis, but at a somewhat higher rate in children and immunocompromised patients (10,14). AIDS patients are 19.2 times more likely to develop salmonellosis than others. The serotypes commonly isolated in these patients are *S. typhimurium*, *S. dublin*, *S. newport* and *S. enteritidis* (10). Salmonellosis is primarily acquired through the consumption of raw or undercooked contaminated food of animal origin (mainly meat, poultry, eggs, and milk). *Salmonella* are among the bacteria that are most commonly known to produce travellers' diarrhea (15,16). An important preventive measure in hot tropical climates is the avoidance of raw salads, food, and drink that are cold or have been cooled with ice cubes (16). The fecal-oral route principally spreads infection produced by *Salmonella*, either from animal to animal or animal to human. Although in developed countries human to human transmission is uncommon, it can occur in special care units and residential homes for the elderly (13). The incidence of salmonellosis has increased 20-fold over the last 10 to 15 years in some countries in Europe, with *S. enteritidis* and *S. typhimurium* being the most common serotypes (13). However, there is a possibility that this increase is merely due to the improvements related to detection and reporting. It is alarming that since the early 1990s, the presence of multiple drug resistant strains of *S. typhimurium* has increased considerably (13,17).

Typhoid and paratyphoid fevers are acute systemic diseases caused by *S. typhi* and *S. paratyphi*, respectively, which are exclusively human pathogens. *Salmonella typhi*, *S. paratyphi* A, and *S. paratyphi* B can invade tissues and cause septicemia (18) in patients suffering from high temperature rather than diarrhea. This is known as enteric fever. The other common symptoms being headaches, central nervous system disruptions, malaises, anorexia, splenomegaly, and rose spots on the trunk (12,19). The World Health Organization (WHO) (20) estimated that 17 million people worldwide are affected by typhoid fever every year, with approximately 600,000 deaths. Approximately 10% of untreated patients will discharge bacteria for up to three months and 2 to 5% will become permanent carriers. The mortality rate among untreated patients can be as high as 15%, but falls to around 1% after antibiotic treatment (12). It has been reported that no more than 20% of those exposed to *S. typhi* become infected with typhoid fever. The disease is mainly transmitted by food and water contaminated by the feces and urine of patients and carriers. Although infections produced by *S. typhi* have considerably decreased in the industrialized world, sporadic cases still appear, sometimes linked to visits to developing countries where typhoid fever is still common (15,21).

IMPORTANCE OF *SALMONELLA* IN WATER ENVIRONMENTS

Humans and animals excrete a great number of potential pathogenic microorganisms in their feces that can

contaminate the receiving waters, including bacteria, viruses, fungi, and parasitic protozoa (22). The microbiological quality of natural waters and the potential health risk has been reviewed (22–24). Ingestion of contaminated drinking water or the accidental ingestion of bath water contaminated by feces is the main cause of waterborne diseases. The use of contaminated water in any stage of food processing presents a serious risk to human health because food can provide an ideal growth medium.

As with many other enteropathogens, the importance of *Salmonella* in environmental waters, resides in its capacity to contaminate food products (e.g., shellfish, vegetables). However, *Salmonella* has to be present in sufficient concentrations to initiate infection and to develop the disease. In addition, the susceptible host has to come into contact with the pathogen at the minimum critical dose. Infective dose levels may vary from 10 cells (*S. typhi*) to more than 10^5 (*S. typhimurium*) (25) although greater or lesser doses have been indicated by other authors (4,12,26). Although feces of infected humans who either manifest clinical symptoms or are asymptomatic carriers are the major source of *Salmonella*, farm animals, animal pets, and wildlife serve as very significant reservoirs of this microorganism (16,23). The percentage of infected individuals within a given mammalian group (man included) ranges from less than 1 to 25% (23). The quantity of *Salmonella* excreted by infected individuals has been estimated as 10^6 per gram of feces (23), and the number of these organisms found in a liter of raw sewage ranges from 20 to 8×10^4 (27).

There are a great number of studies from a range of countries that have investigated the incidence of *Salmonella* in rivers, lakes, sediments, coastal waters, and beaches. In these environments some strains of *Salmonella* are predisposed to pathogenicity, whereas others are not. The factors that govern this behavior are not clear but are probably linked to the host and to the presence and activation of virulence mechanisms in the bacteria (28). Pathogenic strains can quickly become opportunistic, invading the human body under conditions of stress, weakened immunity, or general physical deterioration associated with advancing age. The severity of the disease relates to the serotype of the organism, the number of bacteria ingested, and the host susceptibility. The incidence of *Salmonella* in water environments varies worldwide because of its different prevalence in the human and animal population, and the level of sanitation and development of sewage treatments. In industrialized areas there has been an increase in the number of sewage treatment plants, some including disinfection, and a reduction in the number of carriers due to the introduction of antibiotic therapy (29). In developing countries with poor sanitary conditions, the disease remains frequent or endemic (30,31). As *S. typhi* and *S. paratyphi* exclusively colonize humans, their ecology is closely related to the distribution of human sewage, and *S. typhi* has been isolated from water and sewage when cases of enteric fever have been reported from the nearby populations (12).

Relationship Between Stormwater Runoff and *Salmonella*

Stormwater is often the major cause of water quality deterioration in receiving waters, especially in bathing areas. During heavy rains, animal wastes are washed down from pasture and forest, and from manure applied as fertilizer, often causing city sewerage systems to overflow. Sewerage systems and wastewater treatment plants have a finite capacity. Following prolonged or heavy rainfall, the capacity of either system may be exceeded and the excess flow will be diverted to an adjacent watercourse (32,33). Stormwater runoff can contain high densities of microorganisms of either human or nonhuman origin. The incidence of human source microorganisms is influenced by cross-connection between stormwater and urban sanitary sewers. Groundwater systems and shallow wells can be contaminated by surface-runoff. The overflow from old and/or poorly maintained sewerage systems after heavy rainfall with subsequent contamination of drinking water sources was the cause of an outbreak of typhoid fever in Tajikistan in 1997 (34). Remedial actions proposed in some studies that quantify the elevation in bacterial numbers caused by wet-weather discharges in recreational areas, include disinfection at the outlet or redirection of storm outfalls to separate them from the sewerage systems (35). Preventive measures based on model systems and planned location of discharge effluents have been proposed in order to minimize the impact of rainfall on the microbiological quality of the receiving waters, and thus avoid receiving waters posing a health risk (36,37). The health risk associated with stormwater contact in bathing areas has recently been investigated in Santa Monica Bay, California. Results indicate an increasing risk of adverse health outcomes associated with stormwater contact, including gastroenteritis (38). It is important therefore that the bathing population is aware of the fact that water quality can deteriorate considerably after heavy rain and this knowledge may influence their decision to bathe or not to bathe on rainy days (33). Stormwater runoff may also wash out fertilizers and food, which if prepared from animal products, may be highly contaminated with *Salmonella* (18).

Salmonella has frequently been isolated in receiving waters following wet-weather events (39,40,41,42,43,44). Identical serotypes have been detected in sediments at several locations within an estuary after heavy rainfall (42). For example, 4,500 *S. thompson* per 100 ml were recorded from stormwater (23), whereas concentrations ranged from 1 to 10^4 per liter in other urban runoffs (23,45,46). *Salmonella* transported by stormwater through a wastewater drain at the University of Wisconsin experimental farm were isolated regularly at a bathing beach 800 meters downstream (39).

The Role of Seagulls in the Spread of *Salmonella*

Salmonella have been commonly isolated in the feces of birds, particularly the gull species (16,47,48). Importantly, these birds have been involved in outbreaks of salmonellosis in humans and animals (46,49). Seagulls are scavengers that frequent open garbage dumps, eat contaminated food wastes, and contribute *Salmonella* in

their feces to overnight roosting sites on lakes, open water reservoirs, and coastal waters (23,48,50). Gull feces have been shown to contain a range of 1.5×10^2 to 1.2×10^4 *Salmonella* per gram and can therefore have an impact on the microbiological quality of freshwater reservoirs and beaches. Of special importance are freshwater reservoirs situated in upland areas where water is of high quality and treatment is therefore minimal (51,52,53). Levresque and coworkers (48) demonstrated that the deterioration of the microbiological quality of water through the presence of gulls at a lake-bathing site was directly proportional to the number of birds recorded. They were able to detect a decrease in the microbiological quality of the water when as few as 30 birds were present. Preventive measures proposed to lower the risk to bathers from contact with *Salmonella*, include the following: keeping the beaches free from litter, use of closed rubbish bins to minimize attraction of birds, and alerts to the population not to feed the gulls (48). Waterborne outbreaks of salmonellosis associated with bird droppings that are washed out from roofs leading to contamination of the water supplies have been described (25,50,54).

WATER TREATMENT AND *SALMONELLA*

The distribution of *Salmonella* in raw human sewage largely reflects its incidence in the nearby population. The same would apply for wastewater originating from farms or livestock (55). It has been indicated that a sewage collection network of 50 to 100 homes is the minimum size required to have a reasonable chance of successfully detecting salmonellae in the wastewater (56).

Wastewater Treatment

In small communities, sewage containing *Salmonella* may be discharged directly to the ground in septic tanks or be conducted to wastewater treatment plants (WWTP). Septic tanks are banned in many areas because sewage may contaminate groundwaters. Modern sewage treatment in WWTP involves three steps: *(1)* primary treatment, a physical process that implicates the separation of large debris, followed by sedimentation. *(2)* Secondary treatment is a biological process carried out by microorganisms. *(3)* Finally, the tertiary treatment is a physicochemical process that removes turbidity by removing nutrients, organic matter, metals, or pathogens (57). Microbial pathogens are not eliminated after the primary process unless disinfection is included. Secondary and tertiary treatments of wastewater are applied for additional environmental protection when the water is ultimately discharged into rivers and lakes. Sewage from large urban communities requires at least secondary or tertiary treatment with disinfection (such as chlorination, UV, ozone) for onshore or near-shore discharges in order to protect the receiving waters from pathogenic organisms such as *Salmonella*. Such measures are particularly necessary when the waters are used for recreation or shellfish farming (37).

Large bodies of water, particularly open coastal waters, offer a greater opportunity for effective dilution of wastewater outfalls when discharged through long sea outfalls (58). Sewage does not require chlorine disinfection if outfalls are well designed (length, depth, and diffusion). This is important because chlorine and chlorine derivatives can be hazardous to marine organisms and to the people who consume them (58). Discharge of either treated or untreated sewage through properly designed long sea outfalls may pose a very low risk to human health because of the low probability of the sewage plume reaching beach areas (37). The dilution of *Salmonella* in seawater together with the environmental factors affecting its survival in natural waters may considerably lower its concentration (59).

The removal of *Salmonella* after each step of sewage treatment is detailed in Table 1 (27,37,60). Mandatory requirements for processing municipal sewage to secondary treatment level have produced significant improvements in the quality of surface waters (23). Unfortunately, wastewater treatment is not always consistent because of processing difficulties and the occasional by-pass of raw waste inputs following major storm events (23).

It has been reported that several *Salmonella* serotypes showed improved adaptation to seawater stress after a period in wastewater (61), and that they may even be able to grow (62). Treated industrial waste effluents may contain considerably higher numbers of *Salmonella* than municipal sewage. Thus, 4.4% of viable *Salmonella* was detected in waste effluents compared with 2.1% in municipal sewage (63).

Although sewage can be an important resource for agricultural purposes, such as crop irrigation, it can pose an important health risk if inadequately treated. Cordano and Virgilio (64) reported that the serotypes isolated from water used for crop irrigation, including *S. enteritids, S. newport*, and *S. typhimurium*, coincided with those

Table 1. Removal of *Salmonella* During Sewage Treatment[1]

Treatment	Percent Removed	Organisms per Liter
Raw sewage (no treatment)	0	$5.0 \times 10^3 - 8.0 \times 10^4$
Primary treatment[2]	95.5–99.8	$1.6 \times 10^2 - 3.4 \times 10^3$
Secondary treatment[3]	98.65–99.996	$3.0 \times 10^0 - 1.1 \times 10^3$
Tertiary treatment[4]	99.99–99.9999995	$4.0 \times 10^{-6} - 7.0 \times 10^0$

[1]According to (27,37,60).
[2]Physical sedimentation.
[3]Primary sedimentation, tricking filter/activated sludge and disinfection.
[4]Primary sedimentation, tricking filter/activated sludge, disinfection, coagulation, sand filtration and disinfection.

isolated from vegetables. There are indications that the *Salmonella* present in salad crops is a major contributor to the endemic nature of the pathogen (23,64). Monitoring of pathogenic microorganisms in raw municipal sewage may give an indication of their prevalence in the general population. This measure is recommended in case of outbreaks or epidemics in order to control their evolution in the population (37).

During municipal sewage treatment, sludge is produced. Sludge, or biosolids, is a by-product of physical (primary treatment), biological (activated sludge) and physicochemical (precipitation of suspended solids by chemicals) treatment processes (65). Recorded numbers of *Salmonella* spp. in primary and secondary sludge are 10^2 to 10^3 (dry weight) (66) and 9×10^2 (per gram), respectively. These high numbers would pose a health hazard when sludge is scheduled for agricultural reuse (67).

Drinking Water Treatment

Rivers, streams, lakes, and underground aquifers are all used as sources of drinking water. The important factors in choosing the best available raw source water for drinking water treatment supply include *(1)* adequate quantity throughout the year; *(2)* water quality that is amenable to treatment; and *(3)* measures of watershed protection from domestic, industrial, and agricultural pollution (46). Fluctuating microbiological characteristics of the raw water may compromise the water supply (23). Drinking water treatment of raw water sources includes pre- and postchlorination combined with other conventional processes such as flocculation, sedimentation, and filtration (67). Several serotypes of *Salmonella* (*S. enteritidis*, *S. anatum*, *S. infantis*, *S. montevideo*, *S. newport*, *S. give*, *S. infantis*, and *S. poona*) were isolated from the Missouri river at the intakes the of water treatment plant (46). Groundwater has long been considered to be of excellent quality because the soil barrier is considered effective in isolating the water source from surface pollutants (46). As a consequence, treatment is frequently nonexistent or limited to control water hardness, taste and odor removal, or simply disinfection (46). A variety of waterborne outbreaks have been attributed to untreated or poorly treated groundwater contaminated with pathogens, including *Salmonella* (12,46). As indicated previously, the concentration of salmonellae and the prevailing serotypes will be related to the disease prevalent in upstream human and farm animal populations at each specific community. Therefore, the quality of raw water at intakes of drinking water treatment plants may differ considerably (46). Water treatment technology can successfully process poor quality source water containing in excess of 2,000 fecal coliforms per 100 ml, producing potable water that meets drinking water standards, although any break in the chain of treatment could allow substantial levels of microorganisms to enter the finished product (46). Treated water, which is microbiologically safe when it enters the distribution system, may suffer deterioration before it reaches the consumer. Protection of drinking water from contamination by human or animal excreta, food processing water and stormwater runoff is of great importance for public health protection (46). Routine monitoring of drinking water for fecal indicator organisms is carried out according to the different regulations that apply in each country or region (53). According to Geldreich (23), although monitoring water quality is an important aspect, data gathering alone cannot protect public health. It provides a warning system that should trigger appropriate responses for the protection of the public from the potential presence of waterborne pathogens. Another way of anticipating pathogenic exposure is through increased national surveillance for waterborne outbreaks. In each situation it is important to characterize the etiological agent, to establish its occurrence and geographic distribution, and to identify control measures (46).

The Centers for Disease Control (CDC) in the United States has been recording data on waterborne outbreaks for many years. Since 1971, the CDC and the U.S. Environmental Protection Agency (EPA) have maintained joint surveillance systems for collecting and periodically reporting data on the occurrence and cause of waterborne disease outbreaks. Similar systems have been developed in the United Kingdom and in most other European countries.

Salmonella in Biofilms

Biofilm formation (the buildup of microbial communities on surfaces, such as the growth of microorganisms on the inner surfaces of pipes) is of special concern in the drinking water industry because pathogenic microorganisms can be protected from inactivation by chlorine disinfection due to the complex nature of the biofilm (53,68). The growth of bacteria on pipe surfaces is limited by the concentration of essential nutrients in the water, in particular assimilable organic carbon. WHO Guidelines (18) indicate that drinking water must be of low turbidity after treatment if adequate chlorination is to be achieved. In addition, a low load of assimilable organic carbon in treated water is considered to be an important factor in reducing survival time and preventing the regrowth of salmonellae within the distribution system. Reported survival times range from a few days to more than 100 days (18). It has been demonstrated that salmonellae are able to develop biofilms (69–71). Biofilms can detach from the inner surface of drinking water pipes and contaminate the drinking water. Biofilms have gained considerable importance in the context of water and food hygiene (68). Barker and Bloomfield (71) demonstrated that *Salmonella* could survive in biofilms associated with domestic toilets when family members had recently suffered an attack of salmonellosis. *Salmonella* could survive in toilet biofilm from 28 to 50 days after diarrhea had stopped or after experimental seeding.

SURVIVAL OF *SALMONELLA* IN AQUATIC ENVIRONMENTS

Allochthonous pathogenic bacteria are not well adapted to grow and survive in aquatic environments, mainly because of the lack of nutrients and to the exposure to biotic and abiotic factors of these ecosystems (72). The number of microorganisms discharged by sewage and industrial

wastewater into natural water environments are therefore rapidly reduced in the receiving waters as a result of different mechanisms, the two most important ones being physical dilution and microbial inactivation (73,74). These processes depend on various physicochemical and biological factors, such as water temperature (75,76), adsorption and sedimentation processes (77), toxic effects of heavy metals (78), action of sunlight (79–83), predation by bacteria, viruses, and protozoa (84–87), lack of nutrients (83,88), competition with autochthonous microbiota and antibiosis (80,85), and osmotic stress (89,90). Several of these factors provoke stress and sublethal injuries that prevent the growth of the microorganisms in the selective media used for their detection. In fact, detection of *Salmonella* is less efficient when a proportion of the bacterial population is physiologically injured (91). According to Singh and McFeters (92) microorganisms maintain their virulence and pathogenic characteristics even if they are stressed or injured. However, others authors indicate that changes produced in *S. typhimurium* after residence in seawater for a considerable time (32 days) decrease its capability to withstand the gastric barrier (93).

A number of studies have demonstrated that several pathogenic microorganisms can survive for longer periods than indicators because they may be less affected by environmental stress (94,95). For this reason, pathogenic microorganisms have been detected in waterborne outbreaks even when the level of indicator organisms have been low (96,97).

Environmental stress and sublethal injuries provoke the following effects on the allochthonous pathogenic bacteria of aquatic environments, including *Salmonella* (98):

1. Cellular damage, including a decrease in size and activity of the bacterial cell; changes in the cellular volume and shape; damage to the cellular membranes; alteration of superficial appendages, such as flagella and pili; loss of chemical cellular components (lipids, carbohydrates, proteins, RNA, etc.); and alteration of the LPS-carbohydrates on the outer layer wall.
2. Physiological alterations, including loss of intracellular ATP molecules, permeability of nutrients, reduced production of toxins, and an inability to grow in culture media.
3. Reduction of virulence.

Dutka and Kwan (99) compared the survival of *S. thompson* and two indicator organisms in Hamilton Bay (Canada) using membrane filter chambers. The results indicated that these three microorganisms could survive for at least 28 days. Using the same methodology, Moriñigo and coworkers (100) demonstrated that there were no significant differences ($p < 0.001$) in the survival rates in seawater among five serotypes of *Salmonella* tested (*S. enteritidis*, *S. infantis*, *S. potsdam*, *S. typhimurium* and *S. london*). In addition, low survival capability (less than 11%) was achieved after 48 hours of exposure to seawater, with an increase in sublethal injury on exposure to the seawater. Borrego and coworkers (59) reported similar survival characteristics of *Salmonella* and *Shigella* in a marine environment to those of fecal pollution indicators; however, it was observed that these pathogens do not have the ability to adapt well to seawater. Bakhrouf and coworkers (90) explained these results by the fact that *Salmonella* is very sensitive to osmotic down-shock, and the transit of this microorganism by wastewater can modify its behavior in the marine environment. It has been indicated that suspended matter drastically impairs the bactericidal effect of sunlight on pathogenic enteric bacteria (101,102). Cornax and coworkers (103) demonstrated that *S. paratyphi* showed a similar or even lower inactivation rate than that obtained by indicator microorganisms in seawater, the visible light and biotic components of seawater being the most important inactivating factors. Similarly, Rhodes and Kator (75) obtained a die-off of about 3 log and noted sublethal stress of *Salmonella* spp. in filtered estuarine water. These authors concluded that a reduction in *Salmonella* titers was associated with an increase in the number of microflagellates and plaque-forming microorganisms.

Xu and coworkers (104) and Grimes and coworkers (105) reported that indicators and enteric pathogenic bacteria, including *S. enteritidis*, exposed to salt-water microcosms could be recovered after a nonculturable state. Recovery was accomplished by harvesting viable but nonculturable (VBNC) cells from the seawater microcosm. Later, several authors demonstrated experimentally the existence of indicator and pathogenic microorganisms in the VBNC state in different aquatic environments (106–108). The VBNC bacteria remain viable in seawater for a long time (106,109,110) and maintain their infective capability and pathogenic potential (95,111,112). Therefore, the VBNC state of pathogenic microorganisms may constitute a serious hazard to public health because of the fact that these VBNC bacteria are not detected in water samples using conventional methods of detection and enumeration.

WATERBORNE OUTBREAKS CAUSED BY *SALMONELLA*

Waterborne transmission (especially when drinking water is involved) is a highly effective way of spreading infectious agents to a large population (53,113). At the beginning of the twentieth century, modern conventional drinking water treatment, involving filtration and disinfection, was shown to be highly effective in the control of important enteric diseases such as cholera and typhoid fever. Waterborne outbreaks have been closely associated with *S. typhi* and, much less frequently, with *S. paratyphi* B or other *Salmonella* serotypes (12,18). When attributed to heavy contamination, the onset is explosive and the majority of cases develop over a period of just a few days, and may be followed by a secondary peak of contact cases (18). In the United States, 486 outbreaks of typhoid fever were recorded between 1920 and 1991, however no further cases were recorded from 1991 to 1996. The majority of cases (466) occurred between 1920 and 1990 (114–116). In 1994, Galbraith (117) reviewed the reported outbreaks in the UK from 1911 to 1986.

Today waterborne outbreaks of typhoid fever (Table 2) and of other non-typhoid salmonellosis in industrialized

Table 2. Waterborne Outbreaks of Typhoid Fever (from 1970 to 2000)

Place	Cases	Year	Possible Source	Reference
British liner sailing into Vancouver	53	1970	Fecal contamination of drinking tanks	122
Washington (USA)	11	1972	Fecal contamination of a groundwater supply	123
Mexico	83	1972	Drinking from a sewage contaminated canal	124
Florida (USA)	225	1973	Failure of chlorinating of the water supply	125
Saugli (India)	9,000	1975–1976	Endemic situation, sewage contamination of wells and poor chlorination	119
Chile	—	1977–86	Endemic situation	120
Taiwan	52	1983	Drinking from a river or well	126
Krayot (Israel)	77	1985–1989	Drinking non-chlorinated well-water	127, 128
Baramullah (India)	230	1988	Fecal contamination of a groundwater supply	129
Kieng Giang (South Vietnam)	9,179	1990–1992	Endemic situation and lack of clean water supply	121
Saudi Arabia	81	1992	Fecal contaminated aquifer/poor maintenance of filters and lack of chlorination	130
Catalonia (Spain)	9	1994	Sewage contaminated drinking fountain	118
Dushanbe (Tajiskistan)	8,900	1997	Contaminated drinking water after treatment	34

parts of the world are relatively rare and their occurrence is associated with breakdown of water treatment processes (118), when water is contaminated after treatment (34) or when nondisinfected drinking water was consumed (57). Table 2 also records the outbreaks of typhoid fever in different parts of the world from 1970 to 2000 (12). In some of these typhoid outbreaks (Chile, India, Israel) the disease was endemic in the population or had produced intermittent outbreaks for several years (119,120). Some outbreaks went on to become real epidemics, as in South Vietnam, where between 1990 and 1993 a total of 12,228 cases were recorded with 33 mortalities (121).

Salmonellosis has been a reportable disease in the United States since 1942, and the CDC through its *Salmonella* surveillance system has collected data since 1963. The incidence of reported cases was 8 per 100,000 in 1963, whereas in 1983, 19 cases per 100,000 were reported (131). Waterborne cases of salmonellosis were not recorded from 1920 to 1940, probably obscured by the *S. typhi* outbreaks, but from 1941 up to 1994, 26 cases were reported (12). No drinking water outbreaks attributed to *Salmonella* were recorded during 1995 to 1996. It is likely, however, those reported cases represent only a small fraction of the total number of infections that actually occur. It has been estimated from outbreak data that only one in 75 to 100 cases of salmonellosis in the United States is reported (132,133). However, the increase in reported cases is also likely to be unrepresentative because of the improvement in reporting and recognition of salmonellosis. Studies in developed countries indicate that more than 80% of all salmonellosis occurs individually rather than as outbreak (18). In recent years, an increased number of outbreaks have been observed in small community and noncommunity systems (114). In the United States the proportion of reported outbreaks associated with community drinking water systems in which the cause was attributed to problems at water treatment plants has steadily declined since 1989 (e.g., 72.7% in 1989 to 1990; 62.5% in 1991 to 1992; 57.1% in 1993 to 1994 and 30% in 1995 to 1996) (134). This decrease most likely reflects improvements in the operation of treatment plants; however, the CDC recommends that efforts should be increased to prevent cross-connection, in particular by installing and monitoring backflow prevention devices (134).

Outbreaks in Recreational Waters

At the beginning of the twentieth century, swimming-associated outbreaks of *Salmonella* were quite common, but incidents declined progressively. According to Dufour (29), this decline was due to a steady increase in the number of sewage treatment plants with a disinfection step, especially in large population centers, and a widespread use of newly discovered antibiotics that helped to limit the spread of disease and, thereby the number of infected individuals in the discharging population. Although there is a broad spectrum of illnesses that have been associated with swimming in marine and fresh recreational waters, *Salmonella* does not appear in the list of disease outbreaks in the United States between 1985 and 1994 (12). Of the 37 outbreaks associated with recreational water (1995–1996) only one, with 3 cases, was attributed to *S. java* and was due to inadequate chlorination of a swimming pool.

Swimming and other recreational activities in which the accidental ingestion of water can occur are known to

increase the risk of gastrointestinal illness, even in non-outbreak settings (135–137). According to Geldreich (23), waterborne pathogens are a worldwide problem that require urgent control through environmental protection to avoid further escalation of their occurrence. The release of pathogens is, and will be, a constant threat because it is unavoidable for people or animals to be, to a greater or lesser extent, infected by pathogenic microorganisms. The introduction of multiple barriers to provide maximum public health protection is, therefore, essential. This can be achieved by the management and maintenance of wastewater treatment, control of runoff and the adequate treatment of water supply to prevent pathogens infecting increasing numbers of people and thus becoming more prevalent in the population and the environment (23).

The improvement in the sanitation systems of developing countries, plus improvements in surveillance systems on an international scale and information derived from previous outbreaks will help reduce the incidence of waterborne outbreaks of *Salmonella* in the new century (138).

SALMONELLA IN SHELLFISH

Bacterial and viral disease outbreaks have been associated with all major types of edible bivalve mollusks, and human enteric pathogens have been isolated from shellfish collected from both natural waters and harvesting areas (19,139–144). Bivalve mollusk shellfish are filter feeders and use siphoning organelles and mucous membranes to sieve suspended food particles from the aquatic environment as a source of food. If bacteria or viruses contaminate their surrounding water, these mucous membranes may entrap the pathogens and transfer them to the digestive tract. Shellfish, because they are usually consumed whole and raw or only partly cooked, may act as passive carriers of human pathogens (145).

The presence of *Salmonella* spp. in shellfish is predominantly due to fecal contamination of harvesting sites or cross-contamination by human carriers, particularly food handlers (146). In the United States, most outbreaks of salmonellosis are traced to contaminated products of terrestrial animals. However, a frequently identified vehicle for *Salmonella* is shellfish, although current data are inadequate to make any attempt at estimating attributable risk (147). The annual food-borne disease incidence data taken from the epidemiology-based food-borne disease outbreak surveillance system for the 14-year period from 1973 to 1986, documents an average of 55 food-borne outbreaks of non-typhoidal *Salmonella* infections affecting a total of 3,944 people per year. In the same time frame, only six shellfish-borne outbreaks involving 147 cases were reported (148).

The NETSU database that attempts to document all occurrences of shellfish-borne disease outbreaks has, since 1894, reported only two shellfish-associated outbreaks of confirmed non-typhoidal salmonellosis between 1894 and 1973 (149). A 100-case outbreak occurred in Florida in 1947, traced to contaminated oysters. The other outbreak, with 22 cases, occurred in New York in 1967 and was associated with oysters imported from England (150). However, several sporadic cases of salmonellosis associated with shellfish occurred in 1989 and 1990. In 1989, three cases of salmonellosis were associated with mussels harvested in Maine and consumed in Connecticut, *S. infantis* being isolated in two of the cases. In October and December 1989, and later in 1990, oyster-associated cases were reported in Florida (149). Outbreaks of salmonellosis have been associated with shellfish in other countries. Thus, an outbreak of salmonellosis that involved 50 persons in Italy was associated with the clam *Venus verrucosa* (151), and the causative agents were *S. typhimurium* and *S. mbandaka*. Although the NETSU database on shellfish-associated outbreaks reports only a few outbreaks caused by *Salmonella*, this microorganism has been detected frequently in contaminated shellfish (152,153,154,155,156).

On the contrary, the historical association between shellfish and *S. typhi* was identified early in the nineteenth century. During the first quarter of the twentieth century, 22 typhoid fever outbreaks were reported in the United States (157). In 1925, there was a large outbreak of typhoid fever associated with the consumption of raw oysters, resulting in a 10% fatality rate and the establishment of the first national program on shellfish sanitation (157). However, several typhoid fever outbreaks have been reported involving consumption of raw mollusks before 1980 (150,158).

Mollusks may acquire *S. typhi* from sewage-polluted water. However, in the United States, seafood-related typhoid fever is unlikely because there are few cases or carriers to discharge *S. typhi* into sewage or estuaries (19). The risk associated with imported seafood may vary, depending upon the rates of infection in the country of origin and the degree of pollution of the harvest waters. Sporadic cases have been reported in consumers of raw shellfish in the Mediterranean area (159) and, on the basis of the incidence of typhoid fever in local populations, the risk associated with products imported from developing countries would appear to be elevated (160).

In addition to the risk posed by contaminated harvest waters, carriers can also introduce *S. typhi* and other non-typhoid salmonellae into seafood during processing or preparation. Available data suggest that at least 10^5 *S. typhi* in foods is necessary to cause disease (161). Therefore, only shellfish from grossly polluted waters and ready-to-eat seafood contaminated during preparation and then affected by temperatures high enough to allow bacterial growth are likely to transmit typhoid fever.

DETECTION AND ENUMERATION METHODS

Many regulations for different kinds of water have been developed at national or state level all over the world with the aim of protecting human health and reducing the impact of wastewater in water environments (18,37). Drinking water regulations stipulate that water should be supplied to the consumers free of any risk to human health (free of any toxic substance and/or pathogenic microorganism).

Because the analysis and detection of all potential pathogenic microorganisms present in water is impractical

on a routine basis, surrogate indicators are used. These are indicators of fecal pollution (total coliforms, fecal coliforms, *Escherichia coli* and fecal streptococci or intestinal enterococci) that, if present indicate a risk of the presence of pathogenic microorganisms. Regulations rely on analysis of one or more of these indicators to evaluate the microbiological quality of the water (18,162).

The advantages and disadvantages of indicators of fecal pollution as surrogates of pathogenic organisms are broadly reviewed in the literature (24,53,163). However, there is not always a direct correlation between the presence of fecal indicators and that of pathogens. In fact, *Salmonella* had been proven to be present under circumstances in which indicators were not detected or were at very low numbers (23,43,44,100,164–169). The direct detection and enumeration of *Salmonella* will be necessary when there is a suspicion of a potential health risk attributable to this microorganism.

The detection and enumeration of *Salmonella* from water samples is a difficult process, mainly because this genus includes a variety of serovars that have different physiological and cultural characteristics. In addition, *Salmonella* is usually present in small numbers compared with coliforms and it may be sublethally injured reducing efficiency of isolation. Therefore, an optimum methodology that overcomes all these difficulties and that can be useful for all types of water samples is still not available. Conventional methods for the detection (presence or absence or nondetection) generally consist of the following steps: *(1)* concentration, *(2)* preenrichment in nonselective media, *(3)* selective enrichment, *(4)* isolation, and *(5)* identification.

Concentration: *Salmonella* is usually present in water samples in small numbers, therefore it is necessary to examine a relatively large volume of sample (usually one liter) to isolate the organism. Several concentration procedures have been described, including the swab technique, the diatomaceous earth technique, use of borosilicate glass microfibres bonded with epoxy resin, and a membrane filtration technique, the latter being the most commonly employed.

Preenrichment: Preenrichment in nonselective broth medium for 16 to 24 hours at 35 to 37 °C facilitates the recovery of stressed and sublethally injured *Salmonella*, and increases its low numbers with concurrent growth of competitive microbiota. Quarter-strength Ringer's solution, buffered peptone water (BPW), nutrient broth and lactose broth are among the most common preenrichment media (170–172).

Selective enrichment: This step promotes the growth of *Salmonella* with a concomitant suppression of background organisms (173). No single enrichment medium can be recommended that allows optimum growth of all *Salmonella* serotypes. The use of two or more selective enrichment media in parallel is advised for optimum detection; the most commonly used media are selenite broths, tetrathionate broths, and Rappaport broths. Growth of interfering organisms is reduced by the high selectivity of these types of media and incubation at elevated temperatures (41 to 43 °C for 16 to 24 hours) (171). Tetrathionate and Rappaport broths will inhibit the growth of *S. typhi* and therefore, when this serotype is targeted, selenite broth will have to be used. Several semisolid media, including Diagnostic semisolid *Salmonella* agar (DIASALM) (174), Semisolid Rappaport-Vassiliadis (SRVA) (175), and Modified semisolid Rappaport-Vassiliadis (MSRV) (176) have been used in the selective enrichment step. On the other hand, the addition of nitrofurantoin to semisolid media resulted in higher isolation rates of the *S. enteritidis* serovar (174,177).

Isolation in plating media: Solid selective media are used after initial isolation steps to further inhibit the competitive microorganisms and to allow presumptive distinction of *Salmonella* colonies by cultural characteristics. Plating media commonly used for *Salmonella* detection may classified into three groups: *(1)* differential media with little or no inhibition toward nonpathogenic bacteria, such as eosin methylene blue agar (EMB); *(2)* selective media containing bile salts or sodium deoxycholate as inhibitors, such as MacConkey agar, deoxycholate agar, xylose lysine deoxycholate agar (XLD), Hektoen agar (HE) or *Salmonella-Shigella* agar (SS); and *(3)* selective media containing brilliant green dye, such as brilliant green agar (BG), or bismuth sulfite agar (BS), and novobiocin brilliant green-glucose agar. Some serotypes such as *typhi* and *dublin* are sensitive to media that contain malachite green, and therefore, the use of at least two selective media in parallel is recommended (178).

Identification: The identification of *Salmonella* by colony characteristics on selective plating media has limitations inherent in the biological variations of certain organisms and cannot be relied on even for tentative identification. Suspected colonies grown on selective solid media must be subcultured and further characterized by biochemical confirmation. Final verification is based on serological identification (3).

The procedures described earlier are those used to analyze for the presence or absence of *Salmonella* in a water sample. However, if the concentration of the organism has to be estimated, a quantitative procedure is required such as the one described in the Standard Methods (179). This procedure involves concentrating of the water sample by the membrane filtration technique. After blending the membrane in peptone water, a quantitative 3- or 5-tube Most Probable Number (MPN) method with selective enrichment broth is used. After incubation, an amount from each tube is streaked onto BG and XLD agars. Colonies suspected of being *Salmonella* are biochemically identified from each plate and serologically verified (3). From the combination of *Salmonella* negative and positive tubes, the MPN of *Salmonella* of the original sample is calculated.

Several modifications have been performed on some of the steps of this standard methodology. Moriñigo and coworkers (172) proposed a method for the specific detection and enumeration of *Salmonella* from polluted natural waters. The method consisted of preenrichment of the filtered sample in buffered peptone water (BPW), selective enrichment in Rappaport-Vassialidis (RV) broth and Selenite cystine broth, and plating on XLD and TSBG agars. Weber and Rackelmann (180) evaluated

the 1-2 Test demonstrating that it had higher specificity (97.7%) and sensitivity (94.7%) than the classical method. Later, Cherrington and Huis-in't-Veld (181) proposed a new method named 24-hour Screen that involved an overnight enrichment of the sample in Muller-Kauffmann tetrathionate broth, subcultured for 4 hours in M broth with novobiocin, and detection of *Salmonella* by Bactrace and Salmonella-tek ELISA. Bernagozzi and coworkers (182) used two different procedures for detecting *Salmonella* spp. in environmental water samples: a rapid method (enrichment in Salmosyst broth and plating in Rambach agar) and a longer assay (preenrichment in BPW, enrichment in selenite cystine and plating in BG agar). The results obtained demonstrated that the rapid method seemed more effective. Recently, Hoorfar and Baggesen (183) compared the performance of the internationally accepted standard method to two new (1-day) culture methods, Salmonella Enrichment Broth (SEB) and Revive, and an alternative preenrichment broth, named Universal preenrichment Broth (UB). The classical protocol was more efficient, but the preenrichment of the samples in UB may substantially increase its sensitivity.

Several improvements have been proposed in the different steps of the quantitative determination of *Salmonella*. In the preenrichment step, the supplementation of BPW with ferrioxamine E or G or ammonium-iron (III) citrate significantly increased the motility of *Salmonella* and allowed its detection after six hours (177,184).

Moriñigo and coworkers (178,185) compared the selectivity and efficiency of several enrichment broths for the recovery of *Salmonella* spp. from polluted natural waters. The results indicated that several *Salmonella* serovars grew slowly in tetrathionate broth and in media containing brilliant green dye, and *S. typhi* growth was completely inhibited by these media. The enrichment medium that resulted in a higher detection of *Salmonella* from freshwater and seawater was RV broth incubated at 43 °C and its modifications, to which sodium novobiocin had been added.

Several comparative studies have been conducted on the efficiency and differential ability of the selective media used for the *Salmonella* isolation after the enrichment step (172, 175,186,187). Moriñigo and coworkers (186) concluded that XLD and brilliant green-phenol red-lactose-sucrose agars were the most selective media for all *Salmonella* serotypes. Semisolid plating media were used to isolate *Salmonella* spp. from different sources, including feces (187), food (175) and water samples (172) and appeared, in general, to be more effective than broth media. These media have been used in the selective enrichment step, allowing the detection of *Salmonella* in one day (184) or the specific recovery of *S. enteritidis* by the addition of nitrofuratoin (174,177,188). New plating media have been designed, such as B × LH (189) and XLT-4 (190).

Traditional culture-based methods for analysis of *Salmonella* in food and water sources are relatively slow and cumbersome, and results can be ambiguous owing to the poor sensitivity with samples contaminated with low levels of this organism (191). A number of rapid methods have been developed in past few years to overcome this problem. These rapid methods fall into three groups: (*1*) immunofluorescence antibody technique; (*2*) enzyme-linked immunosorbent assay (ELISA); and (*3*) DNA probes.

The direct fluorescent antibody (FA) technique is a rapid and effective means of detecting salmonellae in fresh and seawater samples. It may be used as a screening technique to provide rapid results from large numbers of samples, but because of potential cross-reactivity of antibodies, positive FA results should be confirmed by other methods. Modifications of this technique have recently been applied to detect *Salmonella* spp. in environmental water systems (192,193).

The Salmonella-Tek ELISA test system was compared to conventional bacteriological isolation for specific detection of *S. enteritidis* and *S. gallinarum* (194,195). This screen system provides a promising test for the detection of *Salmonella* antigens in samples, even when they are present at low concentrations (196).

DNA probe methods are based on the detection of specific nucleic acid sequences using labeled DNA-fragments (300–1,500 base pairs) or short oligonucleotides (15–30 bp) to probe for the presence of salmonellae in mixed cultures. The preparation of polynucleotide probes is laborious because it involves isolation, purification, and labeling of a DNA fragment from recombinant bacteria. By contrast, oligonucleotides are easily synthesized and have highly reproducible quality. In addition, the hybridization time can be reduced with these short probe molecules because of a higher concentration of DNA probe. Several sets of oligonucleotide primers (phoP, Hin, H-1, sef A, iroB, ST1 and ST15) in conjunction with the polymerase chain reaction (PCR) assay have been applied to detect *Salmonella* from food and water (197–201). The results demonstrate that the PCR-based methods can favorably replace the culture-based methods because they are quicker, less labor-intensive, reproducible, and provide results that match perfectly those obtained with the standard culture methods (202,203).

CONCLUSION

Members of the genus *Salmonella* are major food-borne and waterborne pathogens; several serotypes are usually involved in salmonellosis or salmonella gastroenteritis. Serovars *typhi* and *paratyphi* are the etiological agents of enteric fevers (typhoid and paratyphoid fevers), affecting exclusively to human population. Outbreaks of enteric fevers are generally associated with inadequate drinking water treatment processes.

Salmonella in water environments is considered as the main source for human infection, following ingestion of contaminated water or food products. Although feces of infected humans (either diseased or asymptomatic carriers) are the major source of *Salmonella*, farm animals, animal pets, and wildlife serve as significant reservoirs of these microorganisms. Sewage from human populations or wastewater originating form farms or livestock, along with stormwater runoff and gull feces, are the main contamination sources of natural waters. A matter of special concern is the fecal contamination of bivalve

mollusks harvesting areas because shellfish has been frequently identified as a vehicle for *Salmonella*.

Secondary and tertiary treatments of wastewater are required for discharges into aquatic natural environments in order to protect the receiving waters from *Salmonella*. However, special efforts must be made to avoid biofilm formation in drinking water distribution systems.

The survival of *Salmonella* in aquatic environments is also reviewed and compared to that of indicator microorganisms. Although *Salmonella*, as other allochtonous pathogenic bacteria, is not well adapted to grow and survive in aquatic environments, several studies demonstrated that *Salmonella* presents similar or even lower inactivation rate than those obtained by indicators in water ecosystems. The recovery of *Salmonella* in a viable but nonculturable state that maintains its pathogenic potential is also of concern.

Finally, current *Salmonella* detection and enumeration methods are examined. The specific detection and enumeration of *Salmonella* from polluted natural waters can be achieved by preenrichment of filtered sample in buffered peptone water, selective enrichment in Rappaport-Vassiliadis broth and selenite cysteine broth, and plating on selective and differential solid media. Enzyme-linked immunosorbent assays and PCR-based methods have been applied for the specific detection of *Salmonella* in samples, even when they are present at low concentrations. These techniques will probably replace the culture-based methods because they are quicker, less labor-intensive and highly reproducible.

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SALT PRODUCTION

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HISTORICAL SKETCH

Sea salt can be produced from the ocean by evaporating seawater to dryness in shallow ponds or containers. The product, mainly sodium chloride, will be gritty with gypsum and bitter with compounds of magnesium, potassium, chloride, and sulfate. Seaside pools carved into rocks along the Mediterranean coasts and remnants of ancient cauldrons for boiling seawater attest to the use of this method to produce salt by civilizations in antiquity. Prior to the Middle Ages, saltmakers improved the method by using large ponds or natural lagoons in which seawater was evaporated by sun and wind (1). Use of shallow connected concentrating ponds through which seawater flowed, evaporated, became increasingly salty, and deposited salt in crystallizing ponds was introduced in the early Middle Ages (2) and constituted a significant technological advance for salt production. (This innovation resulted in today's solar saltworks or salinas.) Early saltmakers (briners) advanced the process further by relying on indicator organisms and communities in the ponds for critical aspects of management, particularly brine (concentrated seawater) clearing, storage and transfer, harvest facilitation, and seepage control. For example, the saltmakers used brine shrimp (*Artemia*) to clear brine of undesirable suspended substances (3,4) prior to salt deposition. The death of brine shrimp and the appearance of pink colors caused by bacteria provided clues that brine had become highly saline and was ready for transfer to crystallizing ponds (crystallizers) where the salt would precipitate (5). However, prior to placing concentrated brine in crystallizers, the briners had allowed microorganisms to grow an organic layer on the floors to reduce water loss through seepage (6) and to facilitate harvest of the salt without contamination by the native black muds (5,7,8). Other events of biological origin recorded for early solar saltworks and their products include excessive accumulation of sediments in ponds (9) that resulted in abandonment of salinas, unpleasant odors arising from the ponds (10), damage to fish preserved with sea salt (11), and use of black sediments for medicinal purposes (12).

MODERN METHODS

The twentieth century has seen few fundamental innovations for producing sea salt in salinas. However, mechanical equipment has replaced manual labor formerly used to pump water, prepare crystallizer floors after harvest for a subsequent season, and to harvest, wash, and stockpile salt (Fig. 1). Also, modern instrumentation has replaced biological clues to determine salinity, move and store brine, calculate evaporation, monitor weather, and check product quality. In some salinas, this data is managed in computer programs (13) to optimize depths, facilitate brine management, and improve the efficiency of the salt harvest and washing processes. Modern salinas and their production methods are largely responsible for decreasing the once highly expensive price of salt to the current level of about twenty dollars per ton.

The size and production capacity of solar saltworks and the competitiveness of salt from seawater have increased significantly during the twentieth century. Salinas that manufacture from 100,000 to more than 1 million tons

Figure 1. A modern salt harvesting machine. (With permission of Shark Bay Salt Joint Venture.)

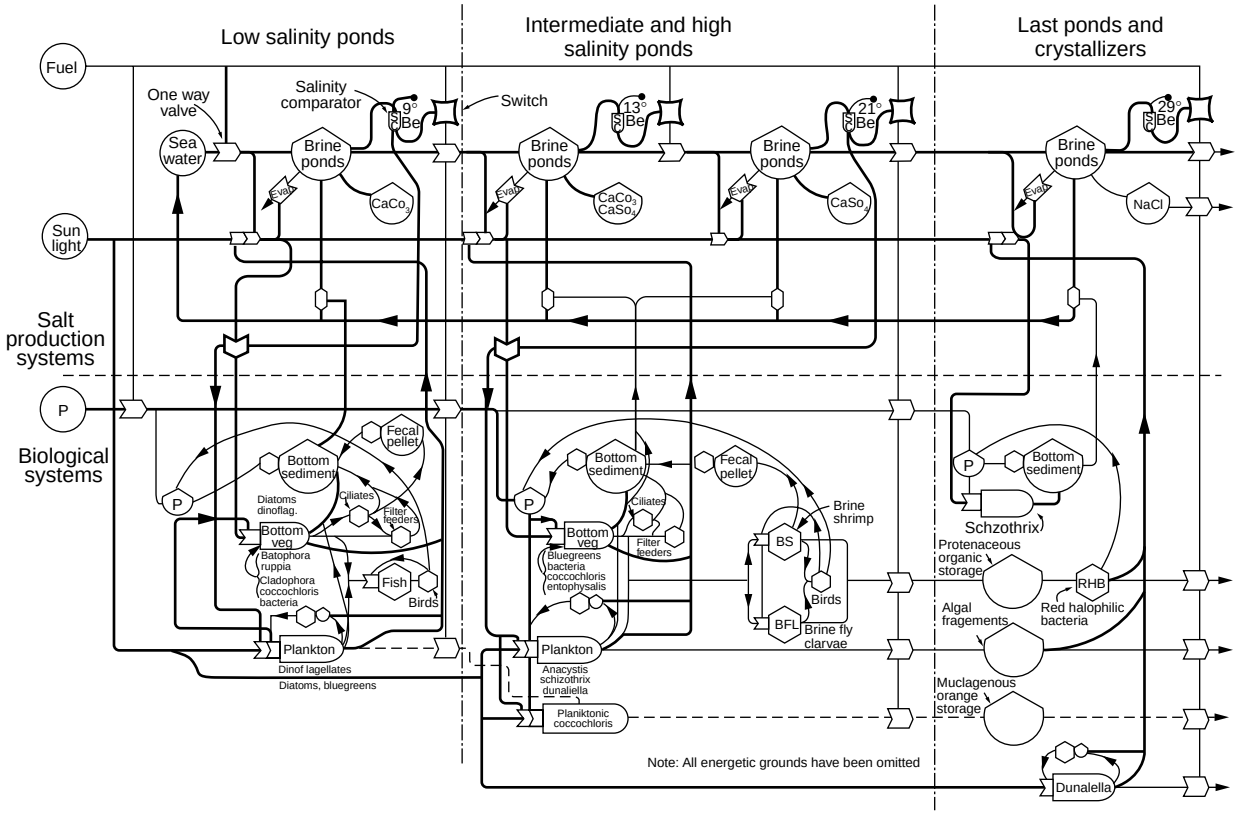

Figure 2. A conceptual model depicting close proximity and interaction of the physical and biological systems (18). (With permission of *Introduction to Applied Phycology*, 1990.)

yearly are common, and their salt now accounts for about one-third the annual worldwide production (about 200 million tons) of sodium chloride. Expansion to increase production is underway in many saltworks, and several large, new salinas are currently under construction or planned for the near future. Because the energy for evaporation of water is without cost, the reserves (seawater) inexhaustible, and water transport of the product from seaside locations relatively inexpensive, solar salt is more economical to produce and distribute than mined salt. In addition to producing salt, the ponds and adjacent dry properties provide habitat free of human disturbance for terrestrial and aquatic plants, microorganisms, and animals (2). Liquid wastes or bittern (the supernatant solution above the salt in crystallizers) of many salinas are processed to produce fertilizers or magnesium compounds, used to seal new crystallizers against seepage, or retained permanently in storage ponds on salina property. Solid wastes (salt and insoluble substances produced by the salt-washing process) are placed in special ponds where the salt is dissolved, recrystallized, and harvested; gypsum on floors of high salinity ponds is harvested on a regular basis in specially designed salinas.

Physical aspects of saltworks (e.g., brine flows, water depths, salinity management, and harvest processes) are generally well understood (13–16), but biological systems essential to salt production are less well appreciated. Because organisms living in the brine ponds strongly influence water evaporation (16), precipitation of calcium carbonate and gypsum ($CaSO_4 \cdot 2H_20$) along the salinity gradient, and salt quality and quantity (15,17), knowledge of the interaction and relationship of the physical and biological systems (Fig. 2) are essential for the salt producer. This article reviews selected physical processes and systems of commercial salinas, describes practical aspects of the macroscopic and microscopic life in brine ponds and their involvement in salt production, offers information to manage the biological systems to enable continuous and economical production of high-quality salt at design capacity, and provides proactive methods to deal with disturbances and other problems common to saltworks.

THE PHYSICAL SYSTEM OF SOLAR SALTWORKS

Modern solar saltworks consist of one or more series of connected concentrating ponds (40 to 70 cm deep) through which seawater flows, evaporates, becomes increasingly salty, and deposits sodium chloride in crystallizer ponds (30 to 50 cm deep). In the downstream flow of water in the concentrating ponds, salts of low solubility (in respect to sodium chloride) precipitate, with calcium carbonate first dropping out near three times (Specific Gravity 1.060) and calcium sulfate (gypsum) first appearing near four times seawater salinity (S.G. 1.107). Shortly before saturation with sodium chloride (S.G. 1.210), brine is repeatedly pumped (or continuously flows) into crystallizer ponds and

allowed to evaporate, and the supernatant liquid (bittern) above the salt is periodically (or continuously) removed after the brine reaches S.G. 1.252. As the salt is deposited, small amounts of calcium and magnesium sulfate also precipitate, but the highly soluble compounds (with respect to sodium chloride) remain in the bittern solution. After 5 to 20 cm of salt has accumulated, the bittern is removed, and the crop is harvested, washed, and stockpiled for a time. The wash machinery moves the salt on a woven steel belt or along a perforated screen sprayed with seawater or brine. Washing removes much of the calcium, magnesium, sulfate and insoluble substances; about 12 to 15% of the salt is lost during the process. On the stockpile, salt increases in quality as the water and soluble contaminants continue to drain away. Salt is reclaimed from the stockpile with bulldozers or other equipment that push the crystals onto continuous belts, which load the product into ships or trucks.

In many solar saltworks, this physical process results in continuous and economic production of 1,000 metric tons of high-quality salt per hectare of crystallizer surface per year. On a dry basis, the purity of the washed salt may exceed 99.7%. [High quality salt (= world standards) maintains contaminants and water in the following ranges: Ca = 0.03 to 0.05%, Mg = 0.02 to 0.04%, sulfate = 0.11 to 0.16%, insolubles = 0.01 to 0.02%, and H_2O = 2.5 to 3%. A typical sample of the salt contains a high percentage of solid, large crystals, and a low percentage of small, hollow, or pyramidal crystals.]

TYPES OF SOLAR SALT WORKS

Most commercial solar saltworks are either seasonally or continuously operated. Most seasonal saltworks operate in temperate or tropical climates with hot, dry summers where 65 to 80% of the annual precipitation occurs during the winters (e.g., shores of Atlantic Europe, the Black Sea, East Africa, Mediterranean, San Francisco Bay in California, U.S.A.). A desired salinity gradient (from seawater density to saturation with sodium chloride) is maintained throughout the system of ponds only in summer, and the crop deposited on floors of soil or marl is harvested at the end of the summer. After harvest, concentrated brines remaining in the salina are stored in deep ponds where they will be used to reestablish the salinity gradient at the beginning of the next salt-producing season (19). (The reader is also referred to the entire set of articles in Volume 9, number 4, 1982, of Géologie Méditerranéene.).

Continuously operated saltworks, common in the dry tropics with low annual rainfall (Western Australia, Southern Africa, Central and South America), maintain a desired salinity gradient throughout the year, and the crop is deposited above salt floors. The salt floor (pavement) is a 5- to 10-cm layer of salt deposited above the native bottom substance of the crystallizers. Salt floors are not harvested; they provide structural strength to support heavy equipment; and they permit harvest of the crop free of mud and pebbles. Harvest may be continuous or confined to the summer season.

In both types of saltworks, tides and pumps may assist entry of seawater at the intake; concentrating and crystallizer ponds are placed along the contours of the land to allow gravity to assist flow of water and brines; and pumps are used in the circuit where flow by gravity cannot occur. For most saltworks, the ratio of surface area of concentrating to crystallizer ponds is about 10 to 1, but ratios of 15 to 1 are common.

THE BIOLOGICAL SYSTEM

Plants, fish, small animals, and microscopic organisms inevitably develop a biological system composed of planktonic (floating) and benthic (bottom-dwelling) communities in the ponds of every solar saltworks. Both communities include organisms (e.g., plants, algae, bacteria) able to manufacture organic matter from carbon dioxide, water, inorganic nutrients, and sunlight, and organisms that consume organic substances (e.g., bacteria, protozoa, and crustaceans).

Kinds of organisms and concentrations of dissolved substances occurring from the seawater intake to brine saturated with sodium chloride divide a solar saltworks into low, intermediate, and high salinity ponds. Species diversity decreases as salinity increases in each successive pond of the three groups. Although notable differences exist among the seaweeds, seagrasses, fish, and some crustaceans of solar saltworks in the various continents, the microorganisms composing biological systems show little variation in species from place to place.

The biological system in solar salt works can either aid or harm salt production. The biological system can help salt production (*1*) by manufacturing quantities of organic matter sufficient only to aid evaporation, to reduce water loss through seepage, and to furnish nutrients for all ponds at desired levels, (*2*) by maintaining bottom-dwelling (mat) communities at desired and well-functioning conditions (e.g., desired species diversity and thickness, and ability to sequester inorganic and organic nutrients), and (*3*) by allowing calcium carbonate and calcium sulfate to precipitate and remain at appropriate places along the salinity gradient. These biological systems (favorable systems) consist largely of organisms adapted to narrow salinity ranges, and they are associated with saltworks that continuously and economically produce high-quality salt at design capacity.

The biological system can harm salt production by producing and accumulating excessive quantities of organic matter. The organisms of these unfavorable systems are adapted to wide salinity ranges; their activities result in salt production at less than design capacity; and they necessitate increased expense and effort (e.g., for pond maintenance, and during salt harvest, transport, washing, and stockpiling) to bring the quality of the product to world standards.

Characteristics of Biological Systems in Low Salinity Ponds

Of the ponds in a saltworks, those of low salinity (S.G. 1.025 to S.G. 1.074) have the most surface area, contain the greatest volume of water, and produce the largest biomass and variety of organisms. More organic substances are produced than retained or consumed in these ponds.

The planktonic community consists of low concentrations of many species of well-represented microorganisms (e.g., bacteria, blue-green algae, chrysophyceans, cryptomonads, diatoms, dinoflagellates, crustaceans, and protozoa; see references 19 and 20 for lists and pictures of organisms at the different salinity ranges) that aid evaporation and furnish nutrients for the entire salina. In biological systems favorable to salt production, concentrations and composition of species remain unchanged over time, and water clarity allows sufficient light to reach the pond floors and maintain the benthic community in a functioning condition.

In biological systems unfavorable to salt production, the planktonic community may become dominated by a single species of algae, for example, *Ochromonas*, *Synechococcus*, *Gymnodinium*, or nanosize blue-green algae. When highly concentrated, these organisms deplete dissolved oxygen at night, decimate the small animals and protozoa, and exclude light from the benthic community.

The several-layered benthic community, interspersed with attached seaweeds, seagrasses, and scattered tufts of filamentous green algae (*Cladophora*), completely covers the pond floors. The upper layer (1 to 5 mm, yellow to orange) contains microorganisms similar to those of the plankton as well as unicellular, colonial, and filamentous blue-green algae (e.g., *Synechocystis*, *Gomphosphaeria*, *Microcoleus chthonoplastes*, *Oscillatoria*, *Phormidium*), unicellular green algae, protozoa, dinoflagellates, small animals (copepods, molluscs, nematodes, ostracods), bacteria, loose flocculents of organic matter and fecal pellets. The second layer (1 to 2 mm, green) consists largely of unicellular and filamentous blue-green algae, diatoms, nematodes, protozoa, and bacteria. The third layer (1 to 2 mm, pink to bright red), is dominated by photosynthetic bacteria. The lower layers (20- to 50-mm thick or more, black) include filamentous and photosynthetic bacteria (*Beggiatoa, Chromatium*) and organic matter. Small animals, protozoa, and bacteria — above and within the upper layers — oxidize important quantities of organic materials, contribute flocculents (mainly fecal pellet fragments) to the water and help maintain the benthic community at desired biomass. During strong winds, part of the uppermost layer becomes suspended and imparts additional color to the water.

In biological systems favorable to salt production, the benthic community maintains desired thickness and controls seepage, permanently sequesters important quantities of combined nitrogen and phosphate from the water and locks them in the black lower layers (17,19,21), remains firmly attached to pond floors, and sustains a diverse assemblage of well-represented organisms. In unfavorable systems, the community may grow excessively to decrease pond surface areas and volumes or become dominated by a few species, detach from floors, and float downstream. In these systems, *Cladophora* may grow from tufts to floating rafts that completely cover the surface of ponds, decrease evaporation, and exclude light from the benthic community.

When inorganic and organic substances, organisms, and floating segments of detached benthic communities are exported into the intermediate salinity ponds, they disintegrate and become nutrients for the biota in the more saline environment. Exports from favorable systems power the downstream biota at desired levels; those from unfavorable systems result in communities unfavorable to salt production and gradual silting up of ponds.

Characteristics of Biological Systems in Intermediate Salinity Ponds

Planktonic organisms produced in these ponds (S.G. 1.075 to S.G. 1.160) include blue-green algae (*A. halophytica*, *Dactylococcopsis*, *Synechococcus*), green algae (*Dunaliella salina*, *Dunaliella viridis*, *Tetraselmis*), diatoms (*Amphora*, *Mastogloia*, *Navicula*, *Nitzschia*), brine fly larvae (*Ephydra*), brine shrimp (*Artemia*), dinoflagellates (e.g., *Amphidinium*, *Gymnodinium*), several ciliate and flagellate protozoans (e.g., *Fabrea*), and numerous bacteria. Because fish and crustacean predators are largely excluded by salinity, *Artemia* may establish large populations. In biological systems favorable to salt production, the microplankton consists of many well-represented species, most of which are food for *Artemia*. The plankton color the brine and aid evaporation, but grazing by *Artemia* maintains suspended organisms and particulates produced in these ponds and imported from the low salinity system at levels that allow light to reach the pond floors. Appropriate concentrations of nutrients in the water, proper management of depths and flows, and competition for resources among the species prevents excessive growth of *A. halophytica*, *D. salina*, and *F. salina*. The composition and concentration of nutrients and plankton flowing out of the intermediate salinity ponds maintain downstream systems at desired conditions.

In unfavorable systems of intermediate salinity ponds, development of large populations of *A. halophytica*, *D. salina*, and *F. salina*, and associated mucilage increase brine viscosity, decimate the *Artemia* population, and shade out the benthic community. Exports of large populations of these organisms and organic substances create systems unfavorable to salt production in the downstream system.

The benthic community is a several-layered mat whose uppermost layer (1- to 3-mm thick, orange-brown) includes fecal pellets, sun-bleached blue-green algae and green algae, protozoa, and bacteria. The second layer (2- to 3-mm thick, green) contains blue-green algae and green algae (*A. halophytica*, *D. salina*, *D. viridis*, *Oscillatoria*, *Phormidium*, *Spirulina*), diatoms, protozoa, and bacteria. A middle layer (1-mm thick, red) is dominated by *Chromatium*. The lowermost layer (5 to 10-mm or more, black) consists primarily of *Beggiatoa*, *Chromatium*, *Thiovulum*, and other bacteria situated above and within organic deposits. In the most saline ponds of the intermediate salinity range, the uppermost layers of the benthic community may be interspersed with calcium sulfate crystals.

In benthic systems favorable to salt production, the communities completely cover pond floors, maintain desired thicknesses, house a diverse group of well-represented microorganisms, remain permanently attached to pond floors, and lock away in their lowermost layers important quantities of combined nitrogen and

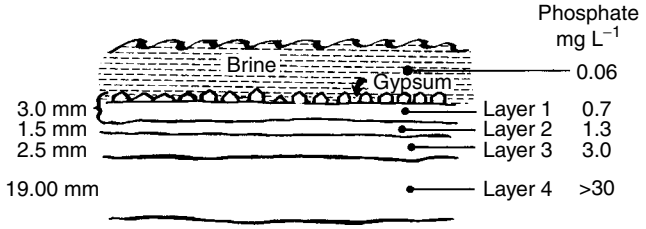

Figure 3. Diagram showing a mat segment with phosphate concentrations of each layer. (*International Journal of Salt Lake Research*, Vol 4, 1996, p. 338, Biological and physical events involved in the origin, effects, and control of organic matter in solar saltworks, Joseph S. Davis and Mario Giordano, Fig. 1, Copyright 1996 Kluwer Academic Publishers, with kind permission from Kluwer Academic Publishers.)

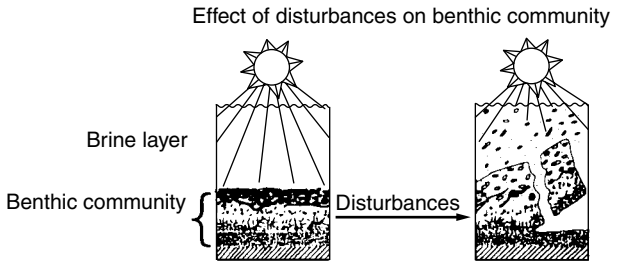

Figure 4. Diagram showing detachment of mat from floor in unfavorable conditions. (With permission of Dampier Salt Limited.)

phosphate from the overlying water (Fig. 3). In systems of intermediate salinity ponds unfavorable to salt production, benthic communities become dominated by *A. halophytica* and associated mucilage, and they accumulate excessively to decrease pond volumes and surface areas; in severe situations large segments (10 to 1,000 mm^2, 50- to 100-mm thick) break free of the bottom and float (Fig. 4) to the surface (21). As the mucilage in the rafts dissolves, the brine becomes viscous, many organisms are liberated into the brine, and the massive flow of organic substances becomes highly damaging to salt production in the downstream ponds.

Characteristics of Biological Systems in High Salinity Ponds

These ponds include the most saline concentrating ponds (S.G. 1.161 to S.G. 1.214) and the crystallizers (S.G. 1.215 to S.G. 1.260); their surface areas, volumes, and variety of living organisms are the smallest in the circuit of ponds. Most of the organic matter that provides food for the communities is imported from upstream. Planktonic organisms produced in these ponds include the aerobic red halophilic bacteria (*Halobacterium salinarium*), anaerobic bacteria, protozoans, and *D. salina*. In biological systems favorable to salt production, the red halophilic bacteria dominate the planktonic community, and concentrations of *D. salina* remain low. The red halophilic bacteria population oxidizes (permanently removes) much of the imported organic matter and multiplies to provide red colors that aid solar energy absorption and brine evaporation. In systems unfavorable to salt production, excessive imports from upstream and subsequent release of organic substances by large populations of *D. salina* create anaerobic conditions during darkness, color the brine black, decrease concentrations of the red halophilic bacteria, deteriorate salt quality and quantity, and lessen the integrity of the crop and salt floors (22).

At floor areas of concentrating ponds without gypsum, a benthic community develops whose upper layer (1 to 2 mm thick, yellow-green) contains filamentous and unicellular blue-green algae, green algae, protozoa, and bacteria, and whose lower layer (5- to 30-mm thick, black) contains bacteria and organic substances. At floor areas covered with gypsum, blue-green algae and bacteria live within the deposit, and organic substances form a lowermost black layer (5- to 20-mm thick) on the native bottom soils. At areas covered with salt, filamentous blue-green algae, bacteria, and organic substances accumulate below the sodium chloride.

In systems favorable to salt production, the benthic community controls seepage, maintains desired thickness, and sequesters nutrients from the overlying brine. Mat microorganisms may control gypsum accumulation by converting the lowermost crystals to fine particles (23), part of which is volatilized to sulfide (24). In crystallizers, the salt floors and/or the current crop remain firm and able to support heavy machinery; and most of the harvested crystals are large and solid. In the wash process, about 12 to 15% of the salt is lost.

In the concentrating ponds of systems unfavorable to salt production, benthic communities may grow excessively and decrease surface areas and volumes, and *A. halophytica* above the gypsum develops mucilaginous mats which break free and float downstream (Fig. 4). In deeper areas of the ponds, mucilage results in viscous brine and causes gypsum to precipitate as microscopic, single crystals rather than in firm, compact deposits. Pond floors become slimy and white with mucilage and gypsum, and the viscous brine transports the single crystals to the downstream ponds. In the crystallizer ponds of unfavorable systems, excessive organic substances interfere with crystal formation and cause salt floors and the crop to lose their structural integrity and become unable to support heavy machinery. The harvested crystals are mostly small, hollow, pyramidal "hoppers" that retain contaminants in their cavities, between layers, and on their surfaces (25,26). When washed, losses of salt are considerably more than 20%.

Characteristics and Management of Key Organisms

Artemia. The largest populations of these crustaceans occur in intermediate salinities; however, when sufficient nutrition is available, low concentrations of brine shrimp may survive throughout the high-salinity concentrating ponds. The well-known essentiality of *Artemia* to salt production lies in the ability of the animals to ingest suspended particles up to 50 micrometers (27), consume large quantities of organic matter (e.g., particulates, algae, bacteria, protozoa), sufficiently clear the brine and enable light to reach the benthic community, and deposit wastes in compact fecal pellets (28) that drop to the pond floors and become incorporated in the benthic community. When

most *Artemia* reach the high salinity ponds, they die and furnish highly suitable food for the aerobic red halophilic bacteria (29) in the downstream ponds. In many saltworks, the local *Artemia* strain is self maintaining, sufficiently vigorous to sustain suspended particles and organisms at desired levels, and able to quickly develop functioning populations. However, reintroduction of *Artemia* may be required when numbers of the animals are too small to control the plankton and to remove suspended organic substances. New introductions are necessary when numbers of the local strain remain too small or are unable to adequately clear the brine (30).

Fast establishment and maintenance of functioning *Artemia* populations can be aided by introducing cysts, freshly hatched nauplii, and adults of the animals at the lowest densities in the brine circuit where salinity excludes predation by copepods and fish (30). Further practices to aid *Artemia* populations include increasing brine depths in the intermediate salinity ponds for higher plankton production and prevention of brine temperatures from exceeding 35 °C (30), maintaining salinities within and between ponds in narrow ranges, modifying ponds to suppress wave formation and eliminate large accumulations of the animals and their cysts in windward corners, and restraining excessive growth of planktonic microorganisms in ponds of low salinity.

Aphanothece halophytica. These algae exist as individual planktonic cells that release dissolved mucilage into the water, as colonies of loosely aggregated cells within mucilage suspended in the water (Fig. 5), or as densely packed cells embedded within firm mucilage on pond floors or adherent to the top of the gypsum deposits. Colors of the algae range from bright green to blue-green or golden yellow to pale yellow. Although *A. halophytica* reproduce best in intermediate salinities, the algae survive and continue to produce mucilage well into high salinities (e.g., S.G. 1.180). When part of diverse planktonic and benthic communities, *A. halophytica* are not harmful to the biological system. However, with fluctuating salinities and/or high concentrations of nutrients, the organisms grow, dominate their communities, and release mucilage. Growth of *A. halophytica* can be suppressed by maintaining communities favorable to salt production in the upstream ponds, by management practices and design features that favor growth of benthic communities (shallow brine depths, "finger dikes" that partially traverse ponds to decrease wave fetch, and appropriate numbers and sizes of ponds) (21), by reintroduction of *Artemia*, and by devices and procedures that keep salinities within and between each concentrating pond in narrow and unchanging ranges.

Dunaliella salina. These motile algae reproduce best in intermediate salinities, but they survive and divide slowly in the high salinity and crystallizer ponds (Fig. 6). Colors of the algae range from green to orange to red. With fluctuating salinities and/or high concentrations of nutrients (21), large populations of the organisms develop, which color the brine bright green to yellow-orange to brick red, release organic substances (31), increase costs of harvest and washing, and decrease the quality of salt. In addition to the management procedures listed to control *A. halophytica*, *D. salina* populations and the released organic substances can be decreased by allowing salt to precipitate on floors of the high salinity concentrating ponds.

Fabrea salina. These ciliate protozoa, confined almost entirely to the intermediate salinities, are too large to be consumed by *Artemia*. In fluctuating salinities and in high concentrations of combined nitrogen and phosphate, the ciliates develop dense populations, consume almost all the plankton, and starve the *Artemia* population. The ciliates can be controlled by maintaining specific gravities in narrow and unchanging ranges within each pond of the low and intermediate salinity ranges.

Halobacterium salinarium. At high concentrations (10^7 to 10^9 per ml), these red halophilic bacteria color the high salinity brines pink to red and maintain organic substances at safe levels. These motile bacteria are highly sensitive to petroleum spills (32) and to ionic concentrations of the brine; with sufficient dilution, the bacteria burst and die (33). Desired concentrations of these

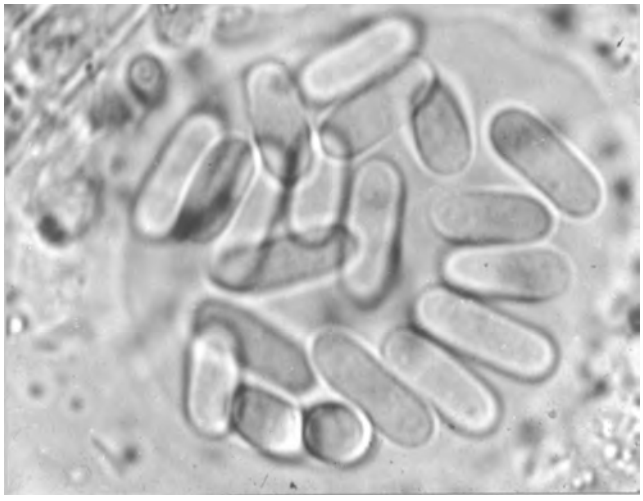

Figure 5. *A. halophytica* within its mucilagenous envelope.

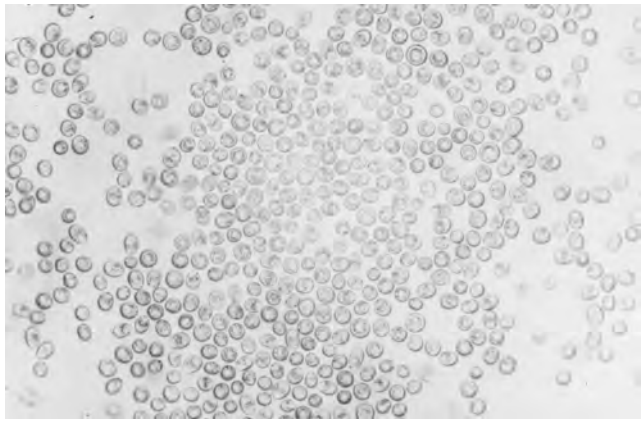

Figure 6. *D. salina* dominant in high salinity brine.

bacteria can be obtained by maintaining in the upstream ponds a diverse assemblage of microorganisms (e.g., *Dunaliella*, *Artemia*, *Dactylococcopsis*, *Synechococcus*, protozoa, brine fly larvae, bacteria) and by maintaining brine largely free of mucilage and cells of *A. halophytica*. Conditions that maximize the bacteria include shallow depths (21), long residence of the brine in the high salinity concentrating ponds, and deposition of salt on the floors of concentrating ponds to stabilize salinities.

PROACTIVE MANAGEMENT

Effects of high velocity winds, excessive rainfall, nutrient-poor or nutrient-rich intake water, and spills of petroleum or other deleterious substances into the water can be minimized with appropriate planning, special construction details, and proper brine management. Data gathering, data interpretation, and periodic inspections can indicate the development of undesirable conditions and allow adjustments and timely repairs before disasters occur.

High Velocity Winds and Rainfall. Saltworks at hurricane- and cyclone-prone locations require reinforced dikes armored with rip-rap, ponds whose long dimensions dampen wave effects of the prevailing winds, and "finger" dikes to decrease wave fetch and prevent back-mixing of brines of differing salinities within ponds. After rainfall, restoration of desired salinities can be aided by decanters at appropriate locations, canals, gates, and pumps for increased salinity control and to channel decanted water away from the ponds, and by appropriate adjustment of flow rates until the desired salinity gradient is restored (21).

Insufficient Nutrients in the Intake Water. This condition results in clear water in all ponds, insufficient evaporation, and leakage of water through pond floors and dikes. Davis (1978) applied several kinds of fertilizers to the most-downstream ponds of a new saltworks with nutrient-poor intake water until desired planktonic and benthic systems developed. Fertilization was then moved upstream until the entire system of ponds developed benthic systems that completely covered pond floors and controlled leakage. Small amounts of fertilizers were periodically placed in the intake water to maintain the system at desired levels (20). Other methods for controlling leakage include transfer of concentrated brines to ponds that leak (34), mixing "leaky" soil with clay (35), and construction of ponds on relatively impervious soil (5).

Excessive Nutrients in the Intake Water. Effects of high concentrations (18) of nutrients in the intake water and from other sources (birds, land runoff) are insidious, and they result in gradually developing unfavorable biological systems, often unobserved during the tenure of a single management official. The paucity of published data precludes meaningful assignment of values to the category labelled excessive nutrients.

Excessive concentrations of nutrients in the intake water (as combined nitrogen and phosphate) can be lowered to safe levels by use of a biofilter (36), harvest of biological products, and by appropriate pond design (21). For small saltworks, a biofilter 3 m by 12 m containing water at 10- to 20-cm depth removed about 60% of the combined nitrogen and phosphorus from the intake seawater (36).

Salinas may be designed for increased efficiency of nutrient sequestration and removal. Davis (1993) recommended five or more ponds in each salinity range (21), ability to operate the saltworks at depths that allow light to reach pond floors, harvest of biological products (*Artemia*, *D. salina*, fish, shellfish), and periodic removal of gypsum, bottom muds, and sediments. Although accumulated organic substances on the floors of crystallizers of seasonal saltworks are removed with the salt during harvest, periodic removal and replacement of salt floors are essential to control accumulations of organic substances in continuously operated saltworks.

Large numbers of birds may roost or nest on the dikes, feed in the water, contribute damaging quantities of nutrients to the brine, and predate excessively on *Artemia*. Control measures include use of noise cannons, bird-scaring devices, and adjustment of pond depths to exclude wading birds.

Release of Petroleum Products and Maintenance of Dikes and Equipment. Detrimental effects of petroleum products to the red halophilic bacteria (32) can be minimized by careful inspection of pumps and harvest vehicles, routine replacement of hoses carrying hydraulic fluids, and removal of all vehicles from crystallizers at night are essential. Immediate removal of spills of petroleum in crystallizers during harvest or postharvest grading minimize damage to marl or soil floors, the crop, and salt floors.

Proactive care includes periodic surveilance and fast repair of damaged dikes, proper maintenance of pumps, gates and weirs; and observations on the condition, careful inspection, and repair of salt floors. Appropriate inventory of spare parts and availability of portable pumps and other machinery are particularly important during harvest times.

Data Gathering and Utilization

Data routinely obtained by most saltworks (evaporation rate, salinity, rainfall, depths and flow rates, concentrations of contaminants in raw and washed salt, range of crystal sizes in representative salt samples, efficiency of the salt harvest and wash processes) appropriately displayed over time provide valuable information on the physical systems and indirectly indicate the effectiveness of the biological system. Value of this information for management of biological systems can be increased by routinely determining and displaying concentrations of combined nitrogen and phosphorus, organic content of the intake water, condition and composition of the benthic communities, and concentrations and conditions of key organisms in the water.

CONCLUSION

Production of high-quality sea salt at the design capacity of a solar saltworks requires integration of biological and

physical processes in the ponds. Proper management of the processes enables salt production under both favorable and unfavorable conditions. The latter include high concentrations of nutrients in the water, excessive rainfall and high velocity winds, and damage to the brine shrimp and red halophilic bacteria populations.

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SALT PRODUCTION, INVOLVEMENT OF MICROORGANISMS IN.

See SALT PRODUCTION

SAMPLING IN SUBSURFACE ENVIRONMENTS.

See SUBSURFACE SAMPLES: COLLECTION AND PROCESSING

SAMPLING OF BIOAEROSOLS.

See BIOAEROSOL SAMPLING AND ANALYSIS; SAMPLING TECHNIQUES FOR ENVIRONMENTAL MICROBIOLOGY

SAMPLING TECHNIQUES FOR ENVIRONMENTAL MICROBIOLOGY

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Sample collection methodology is an important consideration for any microbiology endeavor because the data obtained is directly related to the way samples are collected and handled before analysis. The precise strategy used will be dictated to some extent by the analytical method chosen, but general guidelines can be stated for several environmental matrices. This review will present currently used sampling techniques for water, soil or sediment, and air samples, emphasizing considerations for molecular or chemical analyses rather than activity or culture-based analyses, in part to conserve space. Activity measurements and culture-based analyses are important (1) in combination with community structure information, but sampling issues for such analysis can be involved and may need to be tailored to each environment. Clinical (tissue) specimen sampling and food sampling will not be covered in this article. Where sampling strategies may deviate substantially for a matrix within each of the general categories covered, the methods will be presented separately. Given the breadth of the topic at

hand, the descriptions will be necessarily brief. For more detailed information, consult the appropriate sections of this Encyclopedia and the references cited herein.

SAMPLING STRATEGY

The project or study goals will determine the sampling strategy, especially regarding sample distribution across a site or matrix and subsampling or pooling samples before analysis. Surveys of new areas should take into consideration the natural variability of environmental parameters, allowing patterns to be assessed. Preliminary studies to assess variability of a variety of factors across boundaries or gradients expected to influence microbes are a necessity for designing meaningful studies and experiments. The spatial resolution necessary to accurately characterize microbial assemblages and communities will be determined by physical, chemical, and biological factors. Reviewing basic principles of geostatistics as discussed in References 2 and 3 will improve sampling designs for a variety of environments.

Often, it is not feasible or even possible to sample at a resolution truly meaningful to the microbes of interest. In this respect, biofilms provide a valuable opportunity to study microbial growth patterns and the chemical parameters that both influence them and are influenced by them. Microelectrode and scanning confocal laser microscopy studies of biofilms provide the closest approximation to relevant physicochemical measures currently available. Unfortunately for many important environmental matrices, physical structures, such as coarse soil particles, can preclude or severely restrict the use of microelectrodes or microscopic techniques. Also, even when a variety of approaches are possible for an environmental matrix, time and monetary constraints may necessitate a circumscribed sampling approach.

When sampling, it is important to either sample in a truly random manner or to sample in such a manner as to accommodate the established structure, such as environmental gradients or macroscale environmental patchiness. If the purpose of an experiment is to assess the effect of a treatment, then minimizing natural variability as much as possible is advisable. When sampling in an environment where unavoidable patches are much larger than the sampling unit, a randomized block design may be most useful. When sampling in an environment where the patches are much smaller than the sampling unit, a simple random sampling strategy may be sufficient. In this context, a simple (or completely) randomized design would be the equivalent of placing a sectioned grid over a sampling area, assigning each section a number, and using a random number generator to assign each treatment (including all replicates) to each numbered section. Conversely, a randomized block design would section an area by physicochemical boundaries and then assign equal numbers of replicates of each treatment to each section. Of course, the placement of each replicate within a section could be assigned randomly. Several design schemes are depicted in Figure 1 (as taken from Ref. 4) and also are depicted in Ref. 2. Regardless of the sampling design, it should go without saying that a nonmanipulated (i.e., control) sample should be included in every experiment.

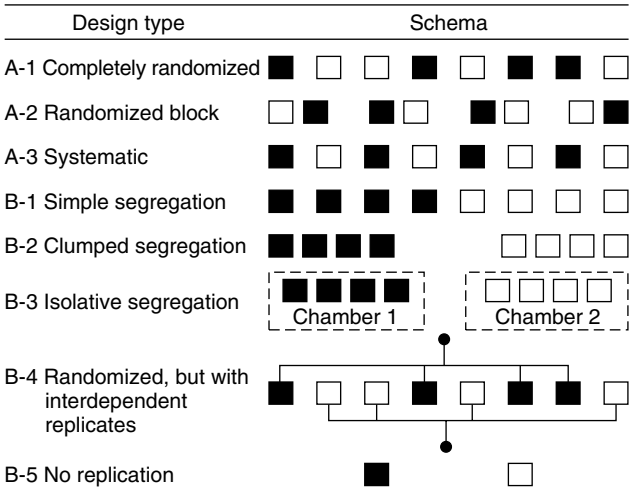

Figure 1. Schematic representation of various acceptable sampling designs (A) of interspersed replicates of two treatments (shaded, unshaded) and various ways (B) in which the principle of interspersion can be violated. Redrawn from Figure 8.2, p. 273 from *Ecological Methodology* by Charles J. Krebs. Copyright © 1989 by Charles J. Krebs. Reprinted by permission of Pearson Education, Inc.

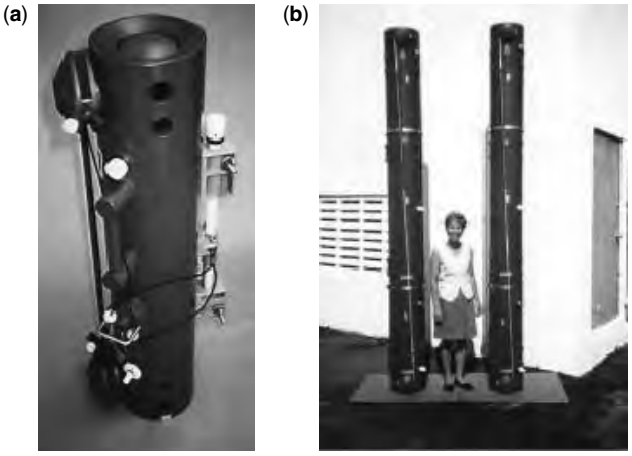

Figure 2. Go-Flo sample bottle with closed top valve. Ball valves at each end are opened when the bottle reaches 1 atm pressure (about 30 ft) and are closed by a messenger (or electronically) when the bottle reaches the desired depth. Bottles typically range from 2.5 to 30 L (**a**) but can be made as large as 200 L (**b**). Images and information generously provided by General Oceanics, Inc. (*http://www.GeneralOceanics.com*). See color insert.

It is always advisable to know how the data will be analyzed statistically prior to sampling. Several texts can be consulted (4–6), and consulting a statistician can be an excellent way to assure the method chosen makes sense. van Elsas and Smalla (2) discussed sampling strategies and presented a table of questions to help guide soil sampling, and these considerations are equally applicable to other matrices. Krebs (4) provided more detailed explanations and examples of sampling strategies, using ecological data to familiarize the reader with equations.

Once the basic statistical parameters are understood, one can consult more recent literature to investigate methods for analyzing complex data (e.g., phospholipid fatty acid or denaturing gradient gel electrophoresis profiles) (7–11).

As with most sampling issues, whether to pool (or composite) samples will depend on the question being addressed (12). Pooling samples will probably cause one to lose information (13), which, if it was known, might affect future experimental designs or even direct testable hypotheses. On the other hand, pooling samples for surveys of large areas may be the only feasible way to characterize communities. For homogeneous environments, using a sample taken from a composite sample may be sufficient (13). However, in other environments, maintaining spatial resolution may be very important (14). Riemann and coworkers (15) collected depth-integrated samples from the deep sea and discussed how this may have affected the apparent diversity of DNA sequences retrieved. The decision to pool samples also may be directed by practical considerations, such as labor or equipment availability. van Elsas and Smalla discussed these issues in greater detail (2).

SAMPLE QUANTITY

The amount of sample that is required will depend on the type and number of analyses performed. Thirty grams of soil or sediment commonly are extracted for phospholipid fatty acid (PLFA) analysis, and 10 to 20 g of material is usually more than sufficient for nucleic acid extractions. As one example of a culture-based analysis, Fredrickson and Balkwill (16) suggested using 10 g soil in 95 ml buffer (e.g., 0.1% sodium pyrophosphate, pH 7.0) with subsequent 1 : 10 dilutions for MPN assessments of (sub-) surface soils. Therefore, collecting 75 to 100 g of material per sample location is generally sufficient to conduct several analyses.

The amount of water required for these same analyses will depend on the concentration of biomass present. One to two liters of water is required for PLFA analysis of moderately clear water (low biomass), whereas the amount of water analyzed may be restricted to a few hundred milliliters or less in very turbid water samples. For other analyses, several hundred to 1000 liters may be needed [e.g., for viral or pathogenic cyst detection from natural waters (17)]. However, if a full chemical constituent analysis is required and numerous analyses (macromolecular, culturing, etc.) are to be performed on a sample, then larger amounts of sample or numerous representative samples may need to be collected. Several chemical and routine culturing analyses are detailed in the Standard Methods for the Examination of Water and Wastewater (12) (e.g., Table 1060:I summarizes information for sample volume, container matrix, preservation, and storage recommendation for several measurable physical and chemical water parameters).

An important point to consider when collecting sample material is the limit of detection (LOD) or quantitation (LOQ) for the analyses being performed. Sufficient material must be collected to yield detectable or quantifiable product. For PLFA, at least 10^5 cells are required for detection and 10^{7-9} cells (*Escherichia coli* cell equivalents) or a total of approximately 200 pmol PLFA per sample are required for meaningful data analysis of pure cultures and complex communities, respectively (18). For nucleic acid-based analyses, the limit of detection is harder to define because of the uncertainties in DNA extraction and coextraction of inhibitors for various analyses, many of which are PCR-based analyses (see RIBOTYPING METHODS FOR ASSESSMENT OF IN SITU MICROBIAL COMMUNITY STRUCTURE). Felske and Akkermans (13) demonstrated that one gram of soil was sufficient to characterize the dominant bacteria in grassland surface soils over a fairly large area using nucleic acid techniques. For several matrices, this quantity of material is sufficient for nucleic acid extractions when samples are well mixed or composited. This observation will probably not hold true in other environments, particularly for low biomass environments (19).

SAMPLE SIZE (NUMBER OF SAMPLES)

The number of samples required to accurately define a parameter for a system will depend on the natural variability of that parameter. For a "quick and dirty" microbial community characterization survey, a minimum of three to four samples from a relatively homogeneous matrix may suffice. However, in spatially heterogeneous environments, a greater number of samples may be required to gain a representative perspective (19). Some investigators pool samples to reduce the number of samples processed in surveys but this should be done only with good reason (as discussed previously) because of the potential loss of useful information. It is beyond the scope of this article to discuss whether subsampling from a very large sample is pseudoreplication or an acceptable form of replication, given the physical scale of influential parameters on microbes. It is, however, a point to consider before sampling, and Krebs (4) discussed three kinds of pseudoreplication (including temporal pseudoreplication that could be analyzed as a repeated measures analysis of variance) to help differentiate them from truly replicated sampling designs.

Determining an adequate sample size for experiments is trickier than that for surveys and may require data from a pilot study. Motulsky (5), in *Intuitive Biostatistics*, explained simply the important parameters for testing hypotheses, including α, β, and power indices. Krebs (4) provided a more detailed explanation of these parameters and the equations required to estimate sample size in relation to a desired level of power.

SAMPLE TRANSPORT AND STORAGE

The most common practice is to transport samples on ice (or blue ice) if cell components (e.g., lipids or DNA) will be analyzed within a few days. If samples must be stored (see the following text), then it is common practice to freeze the samples immediately (e.g., putting soil or filtered samples on dry ice) and transport them frozen

until they can be transferred to a freezer, preferably stored at −80 °C. When collected for culturing or physiological measurements, samples can be kept as close to in situ temperature as possible if they will be processed quickly (within a few hours); otherwise, it is better to keep samples cool than to let them become too warm before culturing. Generally, samples should not be stored unless it is absolutely necessary. There can be remarkable increases in culturable heterotrophs during storage (19,20), even when stored below 10 °C, and changes in phospholipids have been documented as soon as several hours after disturbance (21). van Elsas and Smalla (2) noted that storing samples for up to three weeks at 4 °C in the dark may be acceptable for some analyses but that control measures or assessments should be included whenever possible to check for changes.

Maintaining in situ aerobic or anaerobic conditions after sampling also is important when activity or culture-based analyses will be performed. Collecting and transporting samples under anaerobic conditions is discussed in References 16 and 22. Cragg and coworkers described an inexpensive, field-portable bag for anaerobic sample transport and storage. Wine bags, consisting of nylon, aluminum, and polyethylene material, were used in conjunction with an activated Anaerocult-A sachet and a heat sealer to generate and maintain anaerobic (CO_2) conditions for at least three years. The bags were sterilizable by autoclaving or gamma irradiation and can accommodate moderately large samples. If these or similar containers are not used, then samples should be collected in containers that are not readily gas permeable and should be transported under N_2 or Ar when possible.

Water can be transported and stored in presterilized containers or filtered, even in the field, and placed in presterilized filter holders (e.g., Petri dishes). Several filter materials are used for molecular analyses [e.g., polycarbonate (23,24), Durapore (25), Sterivex (26)]. Anodized aluminum filters (Anodiscs, manufactured by Whatman) are used routinely for collecting material from water samples for subsequent lipid or DNA extraction. An advantage of using Anodiscs for DNA extraction is that they can be crushed after filtration and placed into 2-ml screw cap tubes for subsequent bead-beating cell lysis. Alternatively, once filtered, samples can be stored at 4 °C for several days or at −80 °C for long-term storage (23,26). If RNA instead of DNA will be extracted, it is best to freeze the filters immediately. Filtering material onto Anodiscs is useful for medium to high biomass samples, but other filter materials may be needed for low or very low biomass samples. Fuhrman and Davis (25) used Durapore filters for analyzing deep-sea samples, whereas Riemann and coworkers (15) used capsule filters.

Basic handling criteria for soils are similar to those for water. It is best to process soils as soon as possible, given that microbial community changes can occur rapidly (27). Soils can be stored at 2 to 4 °C for short periods (several days) for certain analyses but freezing is commonly used for long-term storage. Freezing for some matrices may adversely affect some cellular components, potentially reducing DNA recovery because freezing happens slowly in soils and sediments. If long-term storage is the only option prior to analysis, then freezing at −80 °C, often after shaking or sieving, is the option that is most widely used currently (28). Air-drying may severely decrease or alter the cellular component to be analyzed and should be avoided (2,3,29). Lyophilization, however, is used successfully for long-term sample storage for lipid analysis (18) and may also be acceptable for ergosterol analysis (29).

Obviously, samples collected for physiological measurements (especially activity measurements) should be held at a temperature close to in situ temperature and processed as soon as possible; this has been discussed further by van Elsas and Smalla (2). Freezer packs should be used to keep samples cool during sample transport unless tightly sealed (and, for shipping, preferably plastic) containers are used. If regular ice is used to keep samples cool before processing, the ice should be double-bagged. It is very important that soil or sediment samples are not subjected to excessive wetting during transport, which can result from leaking ice containers or damaged sample containers. Presterilized 50-ml screw-cap tubes are excellent short-term storage containers, especially for small soil cores, when wet ice is used for sample transport. Self-sealing (30) or Whirl-pak® plastic bags also are very handy.

Gessner and Newell addressed sample storage issues for determining fungal biomass in plant litter via ergosterol analysis (29). The storage methods presented may be useful for soil analysis too, although this was not stated explicitly. The authors emphasized the importance of not air-drying the samples and suggested that methanol immersion followed by storage at 4 °C is the best procedure for preserving ergosterol content. Freezing followed by lyophilization (just prior to sample analysis) and homogenization may be acceptable, but preliminary studies should compare ergosterol content from such samples to those that have been methanol-preserved.

Caron (31) briefly discussed sample preservation for various protozoa, including algae. Because various protozoa require different preservation solutions [e.g., an alkaline preservative must be used for foraminifera so as not to dissolve the tests, whereas acidic Lugol's solution is used for ciliates (31)], multiple aliquots of a sample may need to be preserved in these various ways to provide the most complete assessment possible. Several detailed procedures are presented in Reference 32; these procedures allow simultaneous sample fixation and preparation for later microscopic examination for activated sludge, freshwater, and marine water samples.

FURTHER CONSIDERATIONS BY SAMPLE MATRIX

Water (Fresh and Marine)

Grab samples are used commonly for collecting surface water samples. Grab samples are collected by immersing a bottle or bag into the water to a desired depth (33) or drawing water from a specified depth using a pipette (34) or tubing (35). A device designed to sample subsurface water is described in Reference 12, Section 9060 A.g. Other samplers to note for shallow- or moderate-depth water environments are those discussed in References 36 and

37. The flow-through samplers (e.g., the Friedinger bottle or the Go-Flo Niskin bottle; Fig. 2) are particularly good samplers for both their simplicity and ability to collect moderate to large amounts of sample (>250 ml to 60 L, General Oceanics has even made a 200-L sampler) (36,37). Several of these samplers also are used for deep-water sampling (see the following text).

Usually, samples are transported in bottles and then processed after returning to a lab (23); although, for very large samples, the water may be passed through filters in the field (see the following text). Sometimes, glass fiber filters or other large pore filters are used to remove large particulate material from samples before filtering onto a 0.45 or 0.22-μm pore size filter (24–26) for sample analysis. Prefiltering should be done only when necessary because it may affect the results, given that microbes are known to attach to particles.

For some analyses, very large amounts (several hundred liters) of sample are required and so water is filtered in the field (38–40). This is often the case when the goal is to detect pathogens or viruses from surface water, particularly for viable assays. Payment and coworkers (40) described a cartridge filtration and backflushing method [modified by Kaucner and Stinear (39)] for detecting several pathogens [*Giardia*, *Legionella*, and *Clostridium* (and *Cryptosporidium* by Kaucner and Steiner)], and Hurst (17) discussed cartridge filtration for viral collection. Hurst also detailed sampling and retrieval protocols for sampling viruses from drinking waters and wastewaters. Sampling flow rates ranged from 4 to 40 L/min for the various large-volume investigations.

As a special area of surface waters, the air–water interface is an important component, and several devices have been used to collect surface films. Several samplers were discussed in References 36 and 37. Screen samplers are widely applicable, and Nucleopore membrane filters are used commonly to sample the neuston. Screens can be made of stainless steel or Nytex mesh, are inserted into a water column vertically, and then are withdrawn horizontally (parallel to the air–water interface) for sample collection. Surface material from approximately 150 to 300 μm is collected. For additional information concerning air–water interfaces, see NEUSTON MICROBIOLOGY: LIFE AT THE AIR–WATER INTERFACE, this Encyclopedia.

When sampling water columns for protozoa (including algae), water can be passed through a series of sieves as described in Reference 37. Delicate organisms may be damaged when collected by sieving or by filtration (31). Alternatively, one of the several types of plankton nets (Fig. 3) or the Hardy Plankton Recorder (a continuous-flow apparatus) can be used (36,37). Samples collected by the Hardy Plankton Recorder are automatically formalin-fixed as the sample is collected.

Drinking water treatment and distribution systems and wastewater treatment facilities are other commonly sampled aqueous environments. Grab samples and biofilm samples are used to assess microbiological parameters in various parts of drinking water treatment and distribution systems (12,41). Grab samples are collected after flushing pipes thoroughly and then collecting samples in bottles or by lowering bottles into open reservoirs (12, Section 9,060 A.3). The samples are kept cool until analyzed (within 24 hours for culturing analyses) (12, Sections 9,060 B). For large-volume water sampling, Hurst noted that cartridge filters could be stored at −70 °C when the samples could not be processed for viral elution within two days but that some viruses lost viability when they were frozen (17). The effect of long-term or frozen storage on other organisms was not mentioned, although Schaefer (42) stated that filtered samples should not be frozen if they will be assessed for *Giardia* or *Cryptosporidium* because ice crystals disrupt the cysts and oocysts, thereby affecting detection. Also, when the water is chlorinated, it is necessary to dechlorinate the water before sample transport and storage. Sodium thiosulfate should be added to a final concentration of 100 mg/L (12, Section 9,060 A.2.). Dechlorination is especially important when samples are concentrated (17,42). A sampling apparatus design and detailed sampling protocol are presented in Reference 42.

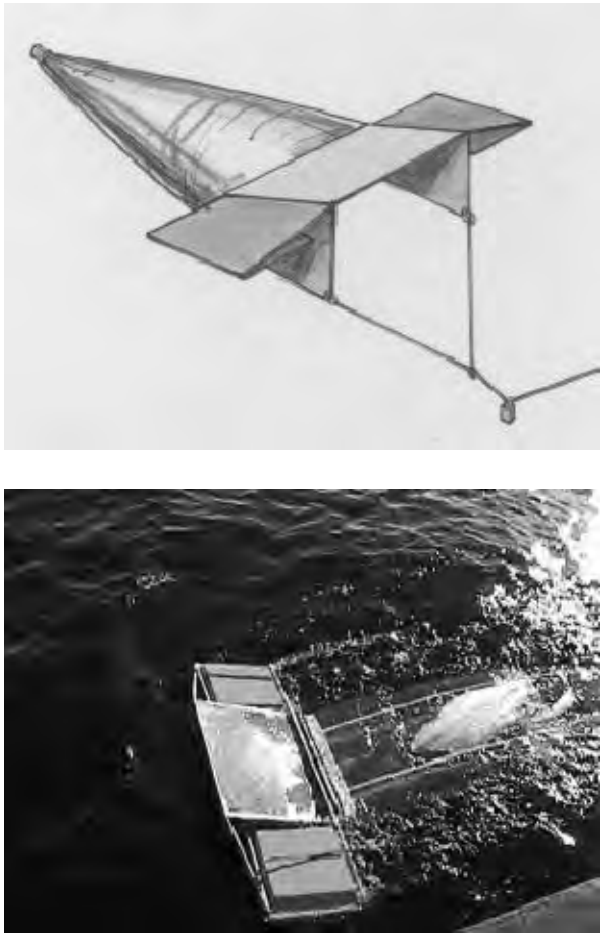

Figure 3. Manta Net plankton sampler. (**a**) Diagrammatic representation. (**b**) Image of net in use. Other net designs are available for vertical hauls through the water column. Images courtesy of Ocean Instruments, Inc., San Diego, California (*http://www.oceaninstruments.com*).

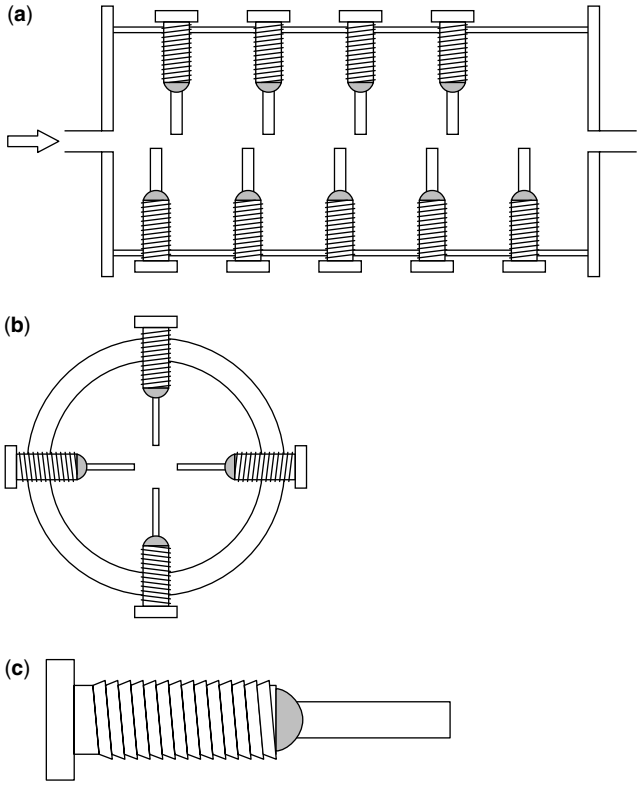

Figure 4. Schematic diagram of a Robbins device that can be installed in water distribution systems. (**a**) Longitudinal section of the device showing the inflow and outflow of water. (**b**) Cross section demonstrating the arrangement of bolts. (**c**) Single bolt with mounted glass slide. Redrawn from Figure 1, p. 2,294 from Manz and coworkers, *Appl. Environ. Microbiol.* **59** (7), 2,293–2,298. Copyright © 1993 by ASM Journals. Reprinted by permission of American Society for Microbiology Journals.

Given that organisms in drinking water may well come from bacteria released from biofilms, it is important to analyze biofilms as well as bulk water samples from these sources. An excellent way to monitor biofilms in pipes is to use a Robbins device (43,44) [or a modification thereof (41,45)]. This device has slides (or "coupons") attached to screws or bolts that are positioned around the circumference of a pipe or material housing (Fig. 4). Water flows across the coupons and exits the device without stagnating around the coupons. The coupons can be made from any material of interest that can be attached to the holders or painted onto a suitable substrate. Various configurations for Robbins devices are depicted in the aforementioned articles.

Biofilms are of concern in many other places too, such as industrial processes and wastewater treatment facilities, where characterizing the organisms present can help solve problems. Biofilms collected from such facilities may well be amenable to microscopic analysis if debris is not much of a problem. Scanning confocal laser microscopy (SCLM) is widely used in conjunction with fluorescent dyes and labeled probes to study biofilms (46). Ladd and Costerton (44) gave detailed procedures for studying biofilms (e.g., measuring activity) and briefly stated ways to grow and collect biofilms. Kalmbach and coworkers (45) also described planktonic cell collection via filtration and biofilm handling for in situ hybridization. For culture-based assessments (e.g., sulfate-reducing bacteria in oil-production facilities), recovered biofilms should be mechanically disrupted, for example, by shaking them with glass beads in a buffer containing a nontoxic, nonionic detergent to facilitate quantitation (46,47).

Biofilms can be obtained by suspending coupons in an environment and then retrieving the coupon for analysis (as described previously) or by using biofilms produced in situ if the support material is amenable to handling (48). In either case, biofilms can be transported in sterile liquid [e.g., buffer or water drawn from the collection site (47)] or fixed immediately in buffered formalin (43,48). Immediate fixation in formalin or another fixative is especially useful when microscopic examination is planned. If destructive analytical methods are used (e.g., PLFA or nucleic acid extraction), then scrapping and collecting the biofilm into bags or bottles may be sufficient. Standard collection and transportation recommendations then apply. However, glass or metal coupons resistant to the chemicals used in an analysis can be processed directly (i.e., without removing the biofilm first).

The deep sea is being studied more frequently as better sampling equipment is designed and as more nonculture-based analytical methods become available. While culture-dependent studies were still the primary means of studying this environment, several sampling devices were developed (and are still being developed) to maintain in situ conditions during sampling. One such device was described and illustrated in Reference 49 and a diagram of a similar multisampler is presented in Figure 5. This sampler allows up to 12 samples to be collected, either in high-pressure sampling units or in Niskin bottles. The high-pressure sampling unit [designed by Bianchi and coworkers (49; Fig. 6)] allowed subsample collection without sample decompression and had an insulating coat to alleviate temperature changes until the unit could be properly incubated after retrieval (49). In addition, the entire high-pressure sampling unit could be autoclaved. Bianchi and coworkers demonstrated the importance of maintaining in situ pressures during activity measurements using this device (49). Another example of equipment used to maintain high hydrostatic pressures for ex situ experiments was illustrated elsewhere (50).

The importance of maintaining in situ conditions will depend on the use of collected material and the organisms themselves. Activity-based analyses provide the greatest challenge; sampling for other analyses is less demanding. Deming stated that maintaining in situ temperature was more important than maintaining hydrostatic pressure for recovering culturable psychrophiles (50). It was also noted that the temperature sensitivity seemed less important for recovering hyperthermophiles than for recovering psychrophiles. For molecular studies, Niskin bottles (15) on Rosette samplers (25) have been used for sampling deep-sea environments. It was not necessary to maintain in situ temperatures or pressures for these studies.

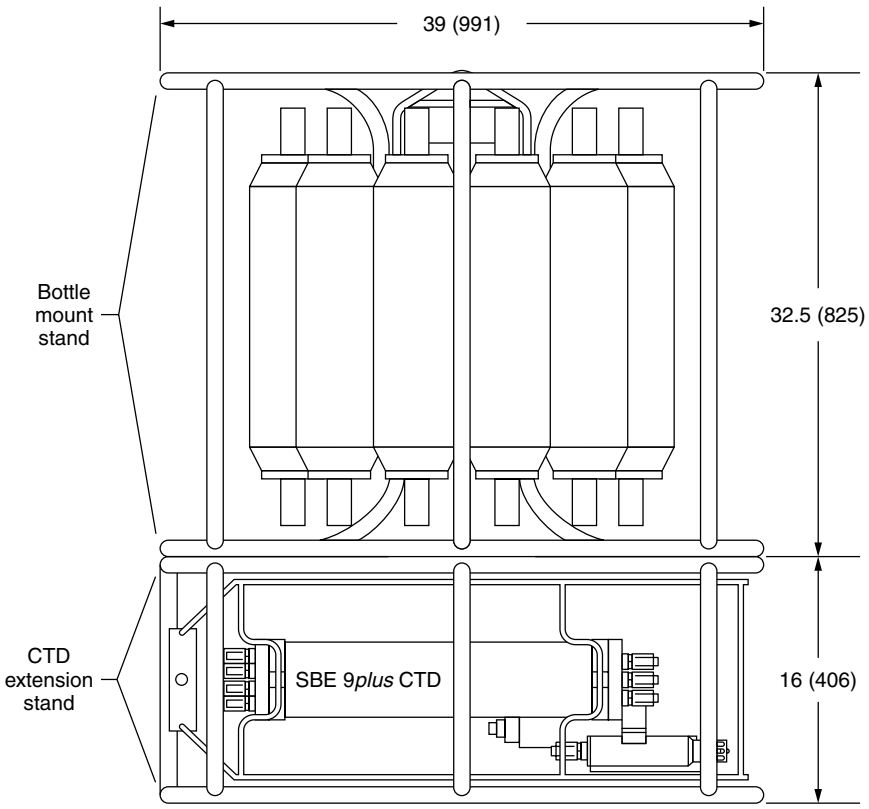

Figure 5. Diagram of a multibottle water sampler (SBE 32 Carousel Water Sampler) showing bottle stand and an optional 9*plus* CTD unit used to close bottles at preprogramed depths. Additional equipment, such as a data logger, can be mounted as well. Diagram and information kindly provided by Sea-Bird Electronics, Inc. (*http://www.seabird.com*).

The one aqueous environment other than the deep sea that poses significant sampling challenges is groundwater. Thankfully, there is an extensive literature base from which to draw advice. In particular, sampling considerations for remediation sites are presented in great detail in Reference 51. Details about samplers and monitoring wells for soil water and groundwater sampling are provided in Reference 52. Briefly, soil water or groundwater collections from the vadose zone (generally 15 m or less in depth) can be made with pressure or vacuum water samplers. Vacuum samplers from permanently installed samplers collect interstitial water and water from adjacent macropores. When using permanent samplers, it is best not to sample within one year of well installation. Various problems are associated with vacuum samplers, a very important one being that it may be difficult to obtain sufficient sample for analysis (52). Pan or wick-type samplers collect water from macropores, especially following wetting events (precipitation or irrigation) (52). The amount of water collected will depend on the system, although the samplers should generally be emptied after each wetting (e.g., rain) event to prevent cross-contamination.

Bailers or various kinds of pumps can be used to collect water from groundwater monitoring wells (16,52). This equipment and any tubing should be sterilized or disinfected before samplers are deployed. Bailers equipped with check valves are the least expensive samplers but may yield the least representative sample because of mixing and aeration commonly experienced with these samplers (52). Bladder or submersible pumps that can be used to collect water from depth are described in Reference 52 and are best when used as dedicated pumps (i.e., one pump per well). Whenever possible, groundwater should be pumped until a stable chemical (Eh, pH) composition is measured before a sample is collected (53,54). Pumping or purging at least two to three well volumes is common.

When the groundwater is known or suspected to be anaerobic, precautions should be taken to maintain the anaerobicity, at least until the physicochemical parameters are measured. This can be a problem with tubing material, such as that used with a peristaltic pump, because some of the tubing is gas-permeable. Low or non–gas-permeable tubing should be used for everything except the area around the pump head, where flexible tubing is necessary. A compromise between low gas permeability and flexibility around a pump head is C-Flex™ tubing (Consolidated Polymer Technologies, Cole-Parmer), which can be sterilized and which works under vacuum. A relatively new sampler, presented in Reference 55 and shown below in Figure 7, was designed to minimize pressure differences during groundwater sampling. This sampler purportedly maintains in situ gas concentrations better than other samplers, thereby yielding more representative groundwater samples.

Soils and Sediments

Collecting intact cores is the best method for sampling soils or sediments. Shovels, trowels, or other scoops are

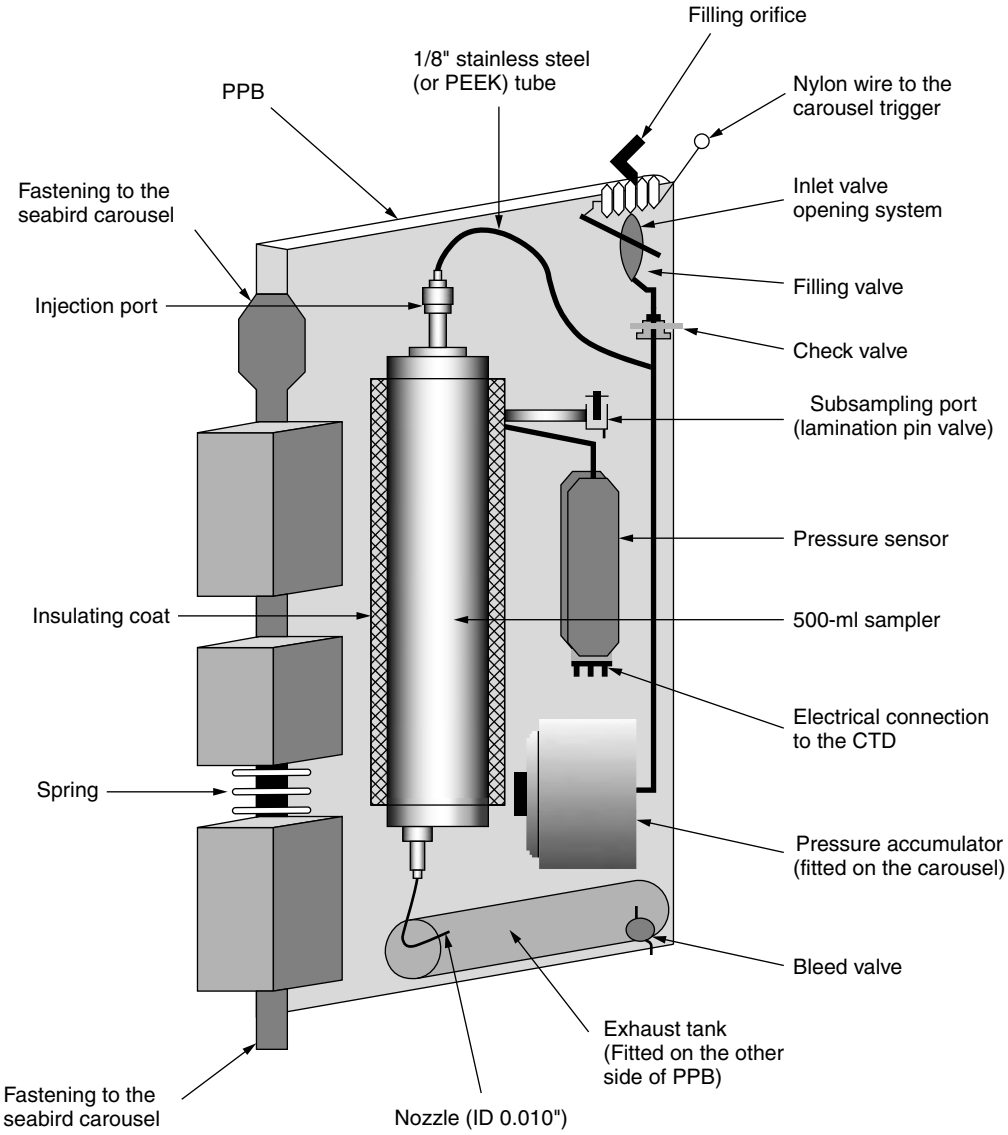

Figure 6. Diagrammatic representation of the high-pressure sampler unit (HPSU) designed by Bianchi and coworkers that was mounted onto an SBE 32 Carousel Water Sampler. The exhaust tank is fitted on the hidden side of the polypropylene board (PPB). The sampling unit's specifications and operating information are detailed in Bianchi and coworkers, 1999 (49). Redrawn and reprinted from A. Bianchi, J. Garcin, and O. Tholosan, *Deep-Sea Research, Part 1* **46**, 2,129–2,142 (Fig. 2, p. 2,132), Copyright © 1999, with permission from Elsevier Science.

easy to use, but may overestimate the contribution of surface inhabitants in relation to the deeper residents unless one is careful to sample to a predetermined depth. Coring provides an even representation of organisms at various depths and causes the least disturbance before sample processing. Shallow surface samples can be collected by hand using cut-off syringes (50) or hand augers (2). Additionally, cores with silicon-sealed injection ports (Fig. 8) can be used for activity measurements of various types (56) but have disadvantages (e.g., diffusion limitations) as well as advantages (e.g., minimal disturbance of natural physicochemical properties) as discussed by Deming (50).

Submerged surface sediments have been sampled with dredges, trawls, and box corers (36,37). Diagrams of various grab samplers and corers are presented in References 36 and 37 along with discussions of the advantages and disadvantages of each. The Jenkins, Craib, and Barnett frame-mounted corers provide the least disturbance, regarding both vertical core compression and water-sediment surface layer disturbance, of the samplers discussed (36). These corers have hydraulic pistons to slowly drive cores into the sediment, and the last two have ball valve end seals to better retain sandy sediments. Additionally, the Barnett sampler has a 12-core sampling capacity. Examples of a box corer and a multicorer are shown in Figure 9. Herbert (36) also discussed several ways to remove cores with minimal disturbance.

Terrestrial subsurface coring requires more sophisticated devices (coring tools), a variety of which are described

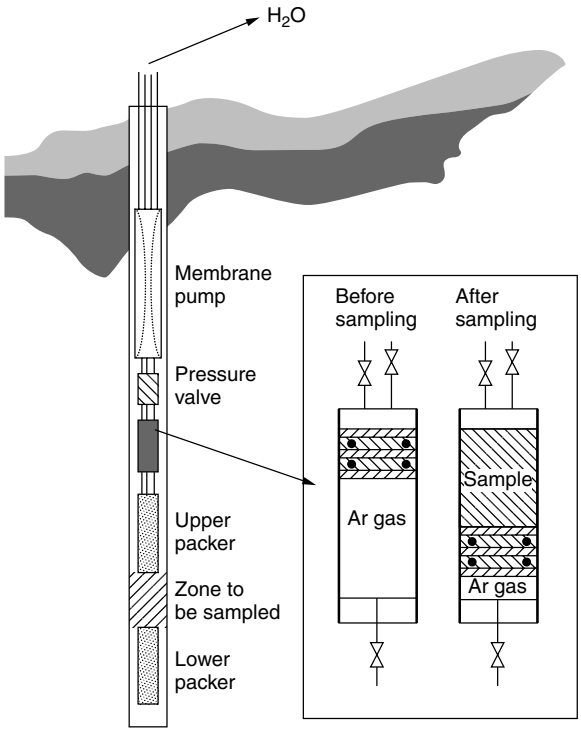

Figure 7. Diagram showing a pressurized groundwater sampling system (called PAVE) deployed in a borehole. The sampler can be opened and closed from the surface, and multiple sample vessels can be used simultaneously. This unit was specifically designed to improve the accuracy of gas and microbiological measurements in retrieved samples. Copyright © 1999 from S. A. Haveman, K. Pedersen, and P. Ruotsalainen, *Geomicrobiol. J.* **16**, 277–294. Reproduced by permission of Taylor & Francis, Inc., *http://www.routledge-ny.com*.

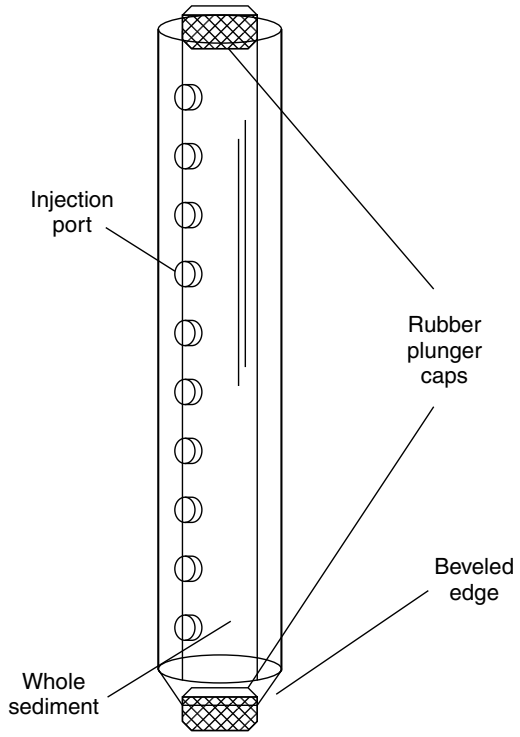

Figure 8. Diagram of a sediment corer with injection ports (which can be sealed with silicone) commonly used for activity measurements. Modified (cut-off) syringes work well; beveled edges may be necessary for some sediments. Adapted from Figure 2 (Chapter 39), p. 372 from J. W. Deming, in Unusual or extreme high-pressure marine environments, C. J. Hurst and coworkers, eds., *Manual of Environmental Microbiology*, ASM Press, Washington, 1997, pp. 366–376. Copyright © 1997 by ASM Press. Reprinted by permission of ASM Press.

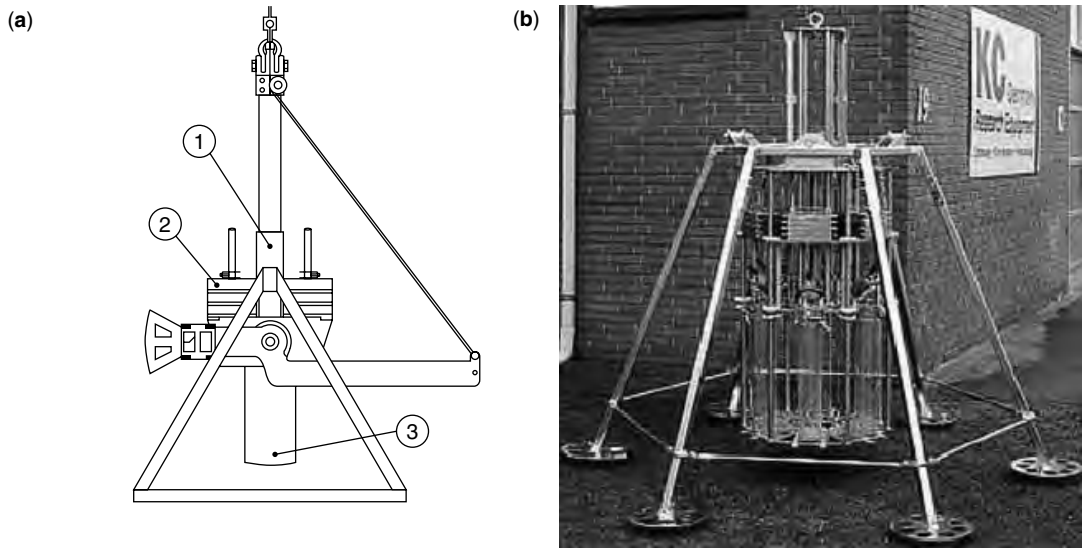

Figure 9. Examples of sediment corers. (**a**) Diagram of a box corer with the frame, weights, and sample tube enumerated, respectively. (**b**) Image of a multicorer with detachable legs, gyro-suspension, and polycarbonate sampling tubes. Both images were provided courtesy of KC Denmark (*http://www.kc-denmark.dk/*). See color insert.

in References 53 and 57. This topic is presented in greater detail in a subsurface drilling and sampling chapter in this Encyclopedia. A brief presentation of the most basic considerations follows. Presterilized (foil wrapped and autoclaved) liners can be used with some subsurface corers to aid with short-term storage and transport (19,53). Such liners are used for coring unconsolidated subsurface soils (53). Liner material should be selected carefully regarding the soil matrix and the possibility that bacteria could use the material as substrate (e.g., acetate liners), particularly if long-term storage is necessary. When sampling subsurface sediments that may be anaerobic, it is important to minimize oxygen exposure. Coy (Ann Arbor, MI) anaerobic chambers can be set up at makeshift field labs and samples can be processed as described in Reference 53. Cragg and coworkers (22) also described a method for maintaining anaerobic conditions in bags for years. As stated previously, retrieved cores can be capped immediately (16) and sealed with bee's wax or, when the sediment is not flush with the core liner, the sediment can be sealed with wax and then capped before being transported. This precaution is important to prohibit sloughing or mixing of unconsolidated sediments during transport and can help maintain field sediment moisture content. When sampling the subsurface, it is also necessary to decontaminate (e.g., steam clean) all the equipment that might contact a sample (16,58).

A detailed procedure for subsurface coring is described in Reference 16. Briefly, clean and disinfect all equipment that will come in contact with sampling material. Prepare the borehole or corer with tracers (such as by placing a plastic bag of tracer compound in the shoe of the core barrel so that it ruptures on impact, see Fig. 10), place an appropriate core liner (e.g., Lexan, PVC, stainless steel) into the core barrel, collect the sample, cap the core liner ends, and place the liner in a glove bag containing Ar or N_2 for transportation. When removing material for analysis, outer core material should be pared (53,59) to reduce the chance of including contaminated material and inner and outer material should be examined by tracer analysis (16). A variety of tracers can be used to account for possible contamination from drilling muds or circulating fluids, for example, chemical tracers (Br) (58) or (pseudo-) biological tracers [fluorescent microspheres (58) or nonindigenous bacteria (60)]. Chemical tracers can be added to drilling muds but microspheres are often too costly for this and so usually they are used only as described above. Detailed procedures for handling samples and sampling equipment are presented in References 19,57,58, and 61.

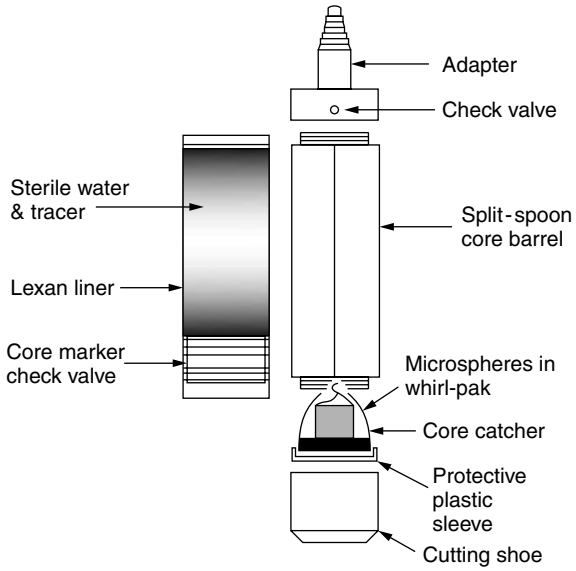

Figure 10. Diagram of a split spoon core barrel containing tracers and core liner for sampling saturated subsurface sediments by cable tool, hollow-stem auger, or rotary drilling methods. Redrawn from Figure 1 (Chapter 59) from J. K. Fredrickson and T. J. Phelps, in Subsurface drilling and sampling, C. J. Hurst and coworkers, eds., *Manual of Environmental Microbiology*, ASM Press, Washington, p. 528 Copyright © 1997 by ASM Press. Reprinted by permission of ASM Press.

The last soil habitat discussed will be the rhizosphere. This is a cursory treatment; a more detailed examination is presented in RHIZOSPHERE MICROBIOLOGY, this Encyclopedia. The rhizosphere is loosely defined as the area of soil or sediment influenced by plant roots (3), mainly root exudates or cell lysates. Rhizosphere studies, therefore, vary in the distance from the plant from which material is collected. Whipps and Lynch (1990) differentiated the endorhizosphere, ectorhizosphere, rhizoplane, and rhizosphere from one another; thus, one can envision different ways of studying these zones: TEM or other microscopy for the endorhizosphere (62), SCLM for the ectorhizoplane or rhizoplane (63), culturing (64,65), or direct extraction (11,66,67) for some or all locations. Campbell and Greaves (68) reviewed a variety of methods for studying rhizospheres, including sampling considerations for culture-based investigations. Several examples of culture-based sampling methods are presented in the literature. For example, Smit and coworkers (69) plated serial dilutions of slurries consisting of 10 g of soil in 100 ml $MgSO_4$ (10 mM) to recover culturable soil fungi. Fredrickson and Balkwill (16) suggested using 0.1% sodium pyrophosphate to recover culturable bacteria from soils or sediments. Bagwell and coworkers (70) and McClung and coworkers (71) used a "rhizoplane" inoculant method to retrieve culturable organisms by rinsing (sterile buffer is probably best for this purpose) soil from the root and inserting the root into semisolid nutrient agar. This culture method recovered both aerophiles and microaerophiles (70).

Slightly different sampling and processing methods are used for molecular-based studies than for culture-based studies. Smit and coworkers (69) presented a method for sampling rhizosphere fungi. The material adhering to the roots after gentle shaking was transferred to tubes containing gravel and phosphate buffer (120 mM, pH 8). Samples were vortexed for 30 s and then DNA was extracted from the supernatant via bead-beating. This method and others (67) work well for molecular studies.

Air

Air sampling is usually conducted to determine if a health risk is likely given the number and/or types of bacteria or fungi present in a specified environment. There is growing concern about health risks associated

with organisms in indoor air, but the best sampling and analytical methods have not been established. Various samplers are used routinely to recover culturable bacteria and fungi (via impingement, impaction onto agar, swabbing, or settling), but culture-based analyses are known to greatly underestimate the number of organisms present (72,73). Furthermore, viable (or culture-based) analyses are expected to be as biased in assessing species composition as the methods that have been used for soil and water samples (74). One must keep in mind, however, that the purpose for air sampling must guide the sampling protocol used and that culture-based analyses may be the most appropriate choice in some instances. Other analytical methods are being pursued, however, to address the suspected shortcomings of current sampling methods in the majority of indoor environments. Nonviable or direct analytical methods are gaining popularity as a result of their success in numerous other settings (75–79), although much work is needed in this area before the findings are rendered meaningful and useful. The most appropriate bioaerosol sampling method for molecular analyses is still being developed.

It is also important to consider a more complete sampling strategy for indoor air quality assessments than merely sampling the air. It may be important to sample possible source material (HVAC units, carpeting, wall board, ceiling tiles, humidifiers, etc.). Sampling considerations and guidelines for such investigations are presented elsewhere (80–84). Of course, consulting a certified industrial hygienist to assist with sampling strategies is advisable if one does not have expertise himself. Additionally, the reader is directed to BIOAEROSOL SAMPLING AND ANALYSIS, this Encyclopedia, for a more extensive discussion of most of the topics addressed herein and for further information concerning bioaerosol assessments.

There are a variety of samplers available for bioaerosol assessment (85,86). Willeke and Macher (87) compiled a table listing the most widely used air samplers and their pertinent information for sample collection (an abbreviated list is presented in Reference 77). It is important to choose a sampler with a d_{50} cut point [the point at which 50% of the particles of that size are retained by the sampler (87)] sufficient to sample the organisms of interest. A table of calculated and reported d_{50} values for various samplers is presented in Reference 77 and in BIOAEROSOL SAMPLING AND ANALYSIS. No one sampler will provide a complete assessment of all components of indoor air, but several samplers are used routinely for culturable microorganisms. The most commonly used collection methods for sampling biological agents (including allergens, bacteria, fungi, dust mites, viruses, and volatile compounds) are compiled in Reference 88, Table 6.1, and readers are referred to this table for specific comparative information. It is important to familiarize yourself with basic air sampling concerns before sampling. For instance, samplers need to be calibrated before and after sample collection, and they need to be oriented correctly when sampling in air streams (87,89,90).

Although there are no safety threshold limit values (TLVs) for bacterial or fungal concentrations in air currently (80), it is important to determine the concentration of these organisms in indoor and outdoor air samples to determine if the cells are multiplying indoors. Usually, colony-forming units are counted from plated samples and the total CFUs are compared among indoor and outdoor locations. In this instance, the comparison yields the important information. If accuracy is more important, then a nonculture-based assessment tool may be necessary. Macnaughton and coworkers (72) demonstrated that phospholipid fatty acid analysis gives more accurate biomass measurements than do culture-based methods. Regardless of the sampling method, sampling equipment needs to be calibrated against a primary standard before and after a sampling event for the data to be meaningful. If the calibration measurements differ by more than 10%, the samples should not be used (77,80). Equipment calibration is discussed in greater detail elsewhere (87,91).

Bioaerosol counts differ throughout a 24-hour period and can be affected by the number of building occupants, occupant activity, ventilation operation, and other factors. Unfortunately, the number of samples needed to thoroughly characterize a building would probably be cost-prohibitive for a fully replicated sampling scheme. A good guideline is to collect at least duplicate plates from each site (i.e., each place in a building) at least twice in a 24-hour period (with the first set of duplicate samples taken at least two hours before the second set of samples) on the basis of guidelines in Reference 80, Chapter 5. A recent suggestion (80) is to collect duplicate samples at least three times a day on three consecutive days (representative of standard building conditions) to estimate average inhalation exposure. Guidelines for times of the day to sample, length of sample collection, sample location within a room, and other considerations are given in Reference 80.

Agar impactors are easy to use and require the least amount of processing after sample collection (see Fig. 2 in BIOAEROSOL SAMPLING AND ANALYSIS). Slit-to-agar and multiple-hole impactors are used often for culturable microbes, although they are expensive. Many labs offering bacterial and fungal counts from such plates also rent these units precalibrated, thus making sampling even easier. For sampling both bacteria and fungi, the culture media used will depend on the organisms one expects to recover. Media used for culturing bacteria can be trypticase soy agar (TSA) or blood agar (incubated at 35–37 °C) when culturing human commensal bacteria or R2A (incubated between 20 and 30 °C) when culturing general environmental organisms. Fungal media are generally malt extract agar (MEA) or 2% MEA, which lacks glucose and peptone (incubated at 18–22 °C), but DG-18 and rose bengal (MEA base) also are used. Wu and coworkers found that DG-18 yielded a greater number of taxa and organisms (colony-forming units) than MEA in a hospital study, ostensibly because of the slower growth of normally rapidly growing fungi, thus allowing growth of less competitive organisms (92). It is important to note also that rose bengal becomes fungicidal when exposed to light (93). In general, media plates should not be exposed to temperature extremes.

Liquid impingers also are used to collect culturable microorganisms (see Fig. 4 in BIOAEROSOL SAMPLING AND ANALYSIS). The collection fluid can be sterile water, buffer, or media, and should be chosen to optimize recovery of the target organisms. Mineral oil is a suitable collection fluid and is less problematic than buffer or water for long collection times because it is less prone to evaporative losses. The fluid used should be compatible with sample processing and minimize added stress on collected microbes. Liquid impingers can be useful for sampling in environments where the microbial concentration is unknown because the liquid can be serially diluted to optimize colony counts. Also, several analytical methods can be used to examine one sample from a liquid impinger. The sample can be used for particle counting methods, concentrated and used for microscopy, or plated onto a variety of media for culturing different microbes. Biochemical and immunological assays (e.g., of endotoxins or fungal toxins) are also possible from collection fluid. Liquid impinger samples also can be used for PCR-based analyses, although they may have to be filtered to concentrate the organisms of interest. For pros, cons, and caveats associated with impinger samplers, and especially for information about particle bounce and reaerosolization with improper use, see BIOAEROSOL SAMPLING AND ANALYSIS (87).

Molecular-based detection methods for bioaerosols have proven successful in various settings (75,76,79,94,95), and sampling procedures often rely on membrane filtration. Indeed, the greatest benefit of using a PCR detection approach is that viability is not a concern and therefore filtering becomes a reasonable collection method. Teflon (PTFE) (76) and polyethersulfone (dissolvable in chloroform) (95) membranes have been used to collect bioaerosols, as have glass fiber filters (for PLFA analysis) (72,73).

Samples should be analyzed within 24 hours for viable analyses. When samples are collected for chemical and molecular analyses but will not be processed within a few days, the samples can be stored frozen (such as samples collected onto filters or filtered liquids). For example, samples collected on glass fiber filters for PLFA analysis should be stored frozen, preferably at $-80\,^{\circ}C$, for long-term storage. One exception to this generalization is endotoxin analysis. Endotoxin is a component of interest in indoor air quality assessment because it is produced as the lipid-A portion of the lipopolysaccharide (LPS) component of the outer membrane of gram-negative bacteria. The bacteria do not have to be viable, nor does the cell wall have to be intact for this allergen to cause problems. There is a discrepancy about the best sample storage method for endotoxin analysis (96). However, it appears that dry samples can be frozen or held desiccated at $4\,^{\circ}C$, whereas bulk liquid samples should not be frozen. Formaldehyde preservation is not recommended if endotoxin concentrations are expected to be low (96).

CONCLUSION

As should be evident at this point, no one sampling method or unit (equipment) will serve even one environmental matrix completely. The best sampling design will accommodate not only the environmental matrix but also the analysis (analyses) to be performed. The foregoing information serves only as a guideline for sampling issues, and the readers are referred to the material referenced herein for more detailed information required before sampling in any environment. Additionally, a vast amount of information about many samplers is available through websites, and readers are strongly encouraged to take advantage of this information when appropriate. Viable analyses require the greatest effort in maintaining sample integrity (especially regarding temperature and O_2/gas concentrations), whereas molecular and/or chemical analyses may require one to "freeze" the sample to reduce losses and great changes over time. The preservation method depends on the parameters measured.

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SCREENING CHEMICAL TOXICITY IN SOILS.

See TOXICITY TESTING IN SOIL, USE OF MICROBIAL AND ENZYMATIC TESTS

SEAGRASSES COMMUNITIES

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Seagrasses are a group of rooted aquatic vascular plants with worldwide distribution (1). Seagrasses form productive ecosystems in coastal and estuarine environments, and are valued for several ecosystem functions, including filtering of nutrients and suspended sediments, erosion control, providing food and refuge for fish, and habitat for several endangered species of turtles and marine mammals (2,3). Microorganisms play a key role in several of these processes, including nutrient cycling and the trophic transfer of seagrass primary production. Microbially mediated processes are of vital importance to the carbon and nitrogen cycles supporting seagrass production and ecosystem function (4). In particular, microbes may enhance seagrass growth via nitrogen fixation, and are responsible for the decomposition and remineralization of seagrass detritus (5–7). Autotrophic and heterotrophic bacteria, cyanobacteria, fungi, and eukaryotic microalgae are all found in association with seagrasses. This article will review the distribution of microorganisms within the components of the seagrass ecosystem and describe the most important microbially mediated processes within those systems.

SEAGRASS ECOLOGY

Seagrasses occur in temperate and tropical environments along a depth gradient from the intertidal to approximately 50-m depth (1). The lower depth limit is controlled either by the penetration of light through the water column or by hydrostatic pressure. Seagrasses can grow in low

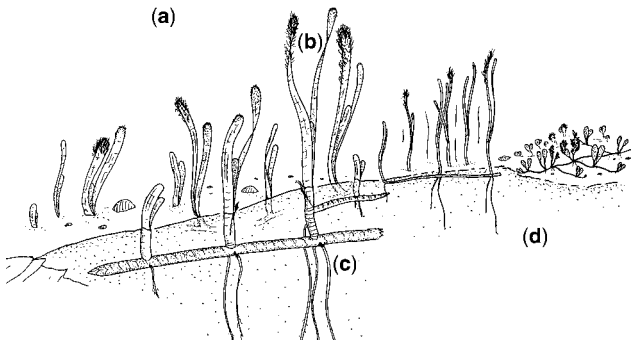

Figure 1. A mixed-species seagrass bed, illustrating placement of the (**a**) water column, including leaf canopy, (**b**) phyllopshere, (**c**) rhizosphere, and (**d**) sediments. From left to right, the growth forms of *Thalassia testudinum*, *Halodule wrightii*, and *Halophila decipiens* are shown.

Table 1. Distribution and Physical Characteristics of Several Common Seagrass Species (1,9)

Species	Geographic Distribution	Average Aboveground Biomass ($gdw\,m^{-2}$)	Average Belowground Biomass ($gdw\,m^{-2}$)
Zostera marina	North Atlantic and Pacific	298.4	159.7
Zostera noltii	Northeast Atlantic, Mediterranean, Black Sea	82.5	66.1
Zostera capricornii	Australia	191.4	176.0
Halodule wrightii	Caribbean, Gulf of Mexico, Indo-Pacific	253.5	193.3
Thalassia testudinum	Caribbean, Gulf of Mexico, Western Atlantic	519.0	582.5
Thalassia hemprichii	Indo-Pacific	86.9	209.9
Posidonia oceanica	Mediterranean	501.0	1,610.7
Cymodocea nodosa	Mediterranean, east Atlantic	146.7	285.0
Enhalus acoroides	Indo-west Pacific	72.0	392.4
Halophila decipiens	Pantropical	77.5	66.0
Halophila ovalis	Indo-west Pacific, Australia	54.8	21.1
Halophila stipulacea	Western Indian Ocean	2.3	2.6

organic–matter siliceous or carbonate sands, and in high organic–matter content muds (8). They can form extensive meadows in quiescent lagoons and embayments, or occur in smaller patches in high-energy environments with strong currents or wave action (8). The structure and life history of seagrasses is also variable, and affects associated microbial populations. Long-lived seagrasses (e.g., *Thalassia testudinum*, *Posidonia* sp.) have a higher percentage of structural carbohydrates and fiber and a greater proportion of belowground root/rhizome biomass extending deeper into the sediment than do seagrass species that are smaller in stature and shorter-lived (9) (see Fig. 1). A list of seagrass species mentioned in this article, along with their geographic distribution and average aboveground and belowground biomass, is provided in Table 1.

Seagrasses are in decline in many developed coastal regions because of shoreline development, fishing activities, and increased water column turbidity and nutrient concentration (10). A marine protist, *Labyrinthula*, has been implicated as being responsible for the seagrass wasting disease that decimated *Zostera marina* populations in the 1930s and 1940s and may have contributed to a seagrass die-off documented in Florida Bay in the 1990s (11,12).

DISTRIBUTION OF MICROORGANSIMS IN SEAGRASS ECOSYSTEMS

Four distinct microhabitats supporting microbial populations and activities can be identified within a seagrass ecosystem: (*1*) water column overlying the seagrass bed, including water in the seagrass canopy; (*2*) phyllosphere containing epibiota on seagrass leaves; (*3*) rhizosphere in sediments, including microorganisms intimately associated with seagrass roots and rhizomes; and (*4*) aerobic surficial and anaerobic subsurface sediments in the seagrass bed (see Fig. 1).

Water Column

The water column overlying seagrass beds contains heterotrophic and autotrophic bacteria, cyanobacteria, and eukaryotic microalgae. The bacterial population is about evenly divided between free-living and particle-attached forms, though the per-cell activity of the latter is usually greater (13). Studies in both temperate and tropical systems have found a positive correlation between measures of seagrass productivity and the water column bacterial community, suggesting a direct utilization of seagrass production by heterotrophic bacteria in the overlying water. Examples include a positive correlation between the diel rates of seagrass photosynthesis and water column bacterial production, increased bacterial abundance and production in the water column over seagrass beds than over adjacent unvegetated sediments (13,14), and evidence for the microbial utilization of dissolved organic carbon (DOC) excreted by seagrasses into the water column (15). Calculations of carbon needed to support bacterial production in the water column have also indicated that

Table 2. Abundance and Growth Parameters of Bacteria in Three Microhabitats (Water Column, Phyllosphere, Sediments) within the Seagrass Ecosystem

Habitat and Seagrass Species	Location	Bacterial Cell Density	Bacterial (Carbon) Production	Doubling Time	Reference
Water column					
Thalassia testudinum	Texas	0.9 to 2.7 $\times 10^9$ L^{-1}	22 to 109 mg $C m^{-2} day^{-1}$		(15)
T. hemprichii	Australia	1.6 to 2.5 $\times 10^9$ L^{-1}	18 mg $C m^{-2} day^{-1}$	8 to 14 hours	(13)
Zostera capricornii	Australia	1.1 to 1.7 $\times 10^9$ L^{-1}	13 to 32 mg $C m^{-2} day^{-1}$	1 to 8 days	(14)
Posidonia oceanica	Mediterranean	0.05 to 1.15 $\times 10^9$ L^{-1}	16 to 95 mg $C m^{-2} day^{-1}$	5 to 23 hours	(16)
Halodule wrightii	Texas	4.3 to 10.6 $\times 10^9$ L^{-1}			(17)
Phyllosphere					
Halophila ovalis	Aqaba	0.2 to 3.7 $\times 10^8$ g wet wt^{-1}			(18)
Halophila stiplacea	Aqaba	0.1 to 10.6 $\times 10^8$ g wet wt^{-1}			(18)
Halodule universis	Aqaba	0.1 to 5.1 $\times 10^8$ g wet wt^{-1}			(18)
Zostera capricorni	Australia	1.2 to 9.0 $\times 10^6$ cm^{-2}	0.2 to 2.1 mg $C m^{-2} day^{-1}$	1 to 31 days	(14)
Zostera marina	Massachusetts	1.0 to 8.5 $\times 10^6$ cm^{-2}	0.4 μg C $hour^{-1}$ cm^{-2}	1.5 hours	(19)
Zostera marina	Chesapeake Bay	1.0 to 7.0 $\times 10^7$ cm^{-2}		17 hours	(20)
Posidonia oceanica	Mediterranean	0.01 to 2.0 $\times 10^7$ cm^{-2}			(29)
Sediments					
Zostera capricorni	Australia	1.2 to 1.7 $\times 10^9$ g^{-1} dry wt	3 to 45 mg $C m^{-2} day^{-1}$	1 to 19 days	(13,14)
Posidonia oceanica	Mediterranean	0.1 to 0.6 $\times 10^9$ g^{-1} dry wt			(21)
Mixed species	Australia		3 to 13 mg $C m^{-2}$ day^{-1}		(22)
Halodule wrightii	Gulf of Mexico		185 mg $C m^{-2} day^{-1}$		(23)
Zostera marina	North Carolina	0.42 $\times 10^9$ g^{-1} dry wt			(24)

seagrass production plays an important role in supporting the production of heterotrophic bacteria in the water column (15–17).

Bacterial density in the water column overlying seagrass beds exhibits a range over several orders of magnitude (Table 2). Estimates of bacterial production in the water column range from 13 to 95 mg C $m^{-2} day^{-1}$. These growth rates indicate that bacteria in the water column can process a substantial portion of seagrass primary production, with estimates for specific seagrass systems ranging from 7 to over 50% (15–17). Bacterial production in the water column can be an effective means of transferring seagrass primary production into marine food webs, as bacteria are grazed by a variety of zooplankton and filter feeders, which in turn become prey to larger organisms.

Much less is known about the relationship between seagrasses and photosynthetic microalgae or cyanobacteria in the water column. Phytoplankton are competitors with seagrasses for water column nutrients, and when water column nutrients increase, phytoplankton biomass increases (25). Increased phytoplankton biomass leads to decreased penetration of light through the water column, and seagrass growth can become light-limited. A frequent result of estuarine eutrophication is the replacement of benthic seagrass production with water column phytoplankton production (10,26).

Seagrass canopies reduce water current and turbulence, and can create a gradient within the water column in living and detrital particles, with greater particle and microbial abundance within the canopy (26,27). In a *Posidonia oceanica* bed, bacteria dominate the water column heterotrophic biomass, whereas picoplankton (cyanobacteria *Synechococcus*) dominate the autotrophic biomass (26). Living and nonliving particles are of approximately equal biomass in the water column over the seagrass canopy, whereas detrital particles are fourfold greater than living particles within the seagrass canopy.

Phyllosphere

Seagrass leaves support a diverse and abundant epibiota, including autotrophic and heterotrophic bacteria, cyanobacteria, fungi, and eukaryotic algae, in addition to a diverse group of macroalgal epiphytes and epifauna (20,28,29). Seagrasses with a longer leaf turnover time generally have larger leaves and support a greater epibiota than those with short (<12 days) turnover times. A gradient in abundance of microorganisms can be found along a single leaf, and among leaves in a seagrass shoot. Outer leaves, and the tips of leaves, are relatively older and harbor a denser microbial assemblage than younger leaves and the basal portions of leaves (19,29).

Production by heterotrophic bacterial populations on seagrass leaves has been linked to the utilization of organic carbon, phosphorus, and nitrogenous compounds excreted by the leaves (19,30,31). An estimated 1 to 30% of daily seagrass net productivity may be incorporated into bacterial biomass on leaves, and the amount of organic carbon excreted by seagrass may be sufficient to provide nearly all the carbon required for epibacterial growth (19,23).

Colonization of seagrass leaves by organisms follows a successional pattern, which can be seen along a single leaf or in a gradient from youngest to oldest leaf on a shoot. Heterotrophic bacteria are abundant early in the succession of

the epibiotic community, with homogenous patches of rod and coccoid-shaped cells occupying the leaf surface (29,32). Diatoms quickly follow bacteria, and often dominate the epiphytic community (32–34). The epiphytic diatom community on co-occurring seagrasses is similar, suggesting that site-specific rather than substrate-specific characteristics dictate the epiphytic diatom community composition (34,35). On long-lived seagrasses, the bacterial-diatom community may be followed by encrusting algae, filamentous macroalgae, and an abundant community of protozoans, nematodes, and copepods (28).

There are several factors that influence the biomass and community composition of microorganisms occupying the seagrass phyllosphere. Seagrasses and microorganisms compete for light and nutrients (33,36), although there may be mutualistic relations as well, with seagrasses providing organic carbon to bacteria and nitrogen-fixing microorganisms providing nitrogen that ultimately gets incorporated by the seagrass (see later section). Seagrasses with longer leaf turnover times, or with higher concentrations of plant sugars, may support higher densities of epibacteria (18). Seagrass leaves also contain phenolic compounds, which can inhibit microbial growth (37), and are at least partially responsible for the frequent observation of increased epiphytic biomass on older leaves, which have a lower concentration of phenolics. Differences in the epiphytic communities found on seagrass leaves and structural mimics have been documented, (38,39) suggesting that there may be a direct interaction between leaf chemistry and development of the epiphytic community.

In several studies, the epiphytic communities have been demonstrated to have no deleterious effect on seagrass carbon fixation or growth (39,40). However, an increase in water column nutrient supply can result in the increased growth of seagrass epiphytes and decrease in seagrass biomass (33,36,41). Shading by increased epiphytic biomass has been suggested as a cause of seagrass decline in eutrophic estuaries (42,43). Nutrient addition does not always result in increased epiphyte growth, however, and other parameters, including water residence time, water column depth and physical mixing properties, as well as the presence of microalgal grazers, affect the relationship between nutrient supply, epiphytic growth, and seagrass production (25,41,44).

Epiphytic photosynthetic bacteria, cyanobacteria, and eukaryotic microalgae, in addition to macroalgae, can account for a significant amount of primary production within the seagrass ecosystem, and may also contribute to the pool of DOC utilized by epibacteria. In tropical and semitropical systems, epiphytic primary production has been estimated to equal or exceed seagrass primary production (40,45,46). In temperate systems, epiphytic primary production is estimated at between 8 and 50% of seagrass primary production (39,47,48). Numerous studies have demonstrated that epiphytes growing on seagrass leaves are the primary food source for many animals utilizing seagrass habitats, particularly in tropical ecosystems (49–53).

Species of the family Labyrinthulaceae, fungus-like protists, have been isolated from the leaves of 11 seagrass species collected from around the world (54). *Labyrinthula* is associated with seagrass "wasting disease," an outbreak of which in the 1930s caused massive destruction of *Z. marina* beds along the Atlantic coasts of Europe and North America (55). In the 1980s, *Labryinthula* was also implicated in a *T. testudinum* seagrass "die-off" which occurred in the Florida Keys (11). Further studies have revealed that *Labyrinthula* is commonly found in the older leaf tissue of many seagrass species, and may play a functional role in natural plant senescence (54) This finding, and the occurrence of *Labyrinthula* in many lush, productive stands of seagrass, suggests that there are causative factors in addition to the presence of *Labyrinthula* that result in outbreaks of wasting disease or seagrass die-offs.

Seagrass Rhizosphere

The seagrass rhizosphere, including rhizomes, roots, root hairs, and associated sediments, harbors an active heterotrophic bacterial population. The diffusion of oxygen from the roots into the rhizosphere and the exudation of organic carbon and amino acids from roots are features of the rhizosphere that differentiate the activity and composition of the rhizospheric microbial community from the sedimentary microbial community (23,56–58). The highest density of microorganisms and highest rates of microbial activity are generally found in two areas of the rhizosphere: the zone of maximal root/rhizome biomass and the root growing tips. The depth of the maximal root/rhizome biomass zone is variable, ranging from 0 to 1 cm in *Halophila* spp. to 20 to 30 cm depth in *T. testudinum* or *P. oceanica* beds (Fig. 1). The dominant microbial processes in the rhizosphere include sulfate reduction and nitrogen fixation. Nitrogen fixation will be discussed in a separate section later. Methanogenesis, nitrification-denitrification, and ferric iron reduction also occur in the rhizosphere. These processes, and the relationship between seagrass photosynthesis and microbial activity in the rhizosphere, are summarized later.

Aquatic plants living in reduced sediments rely on the diffusion of oxygen to root and rhizome tissue to preserve aerobic metabolism in belowground tissue, a necessity for plant survival (58). Oxygen can diffuse out of the plant tissue into the rhizosphere, creating a thin aerobic zone and potentially permitting aerobic microbial activity in the seagrass rhizosphere. Direct measures of the aerobic zone surrounding seagrass root/rhizomes are few, but a thin (<100 μm), oxygenated layer has been documented in several seagrass species (57,59) and a relatively low concentration of sulfide in the rhizosphere can also be attributed to oxidation via O_2 excreted by roots (56,60). Steep oxygen gradients and the excretion of organic carbon from plant tissue create a rhizosphere capable of supporting a diverse population of microorganisms.

Nitrification-denitrification and ferric iron reduction, two processes associated with oxic–anoxic interfaces, occur in the rhizosphere of *Z. marina,* although at much lower rates than are typical for the rhizospheres of freshwater vascular plants (61,62). Ferrous iron accumulates on root/rhizome surfaces, and iron-reducing bacteria have

been isolated from root surfaces, although it is not yet known whether dissimilatory iron reduction or the activity of sulfate-reducing bacteria is responsible for the observed activity (61) The activity of these bacteria may be important in controlling the availability of iron for plant uptake. Similarly, coupled nitrification-denitrification in the rhizosphere could also play an important role in controlling the concentration and speciation of dissolved inorganic nitrogen available for plant uptake. Denitrifying bacteria have been isolated from the rhizosphere of several seagrass species (see later).

A strong link between seagrass photosynthesis and rhizospheric microbial activity is evident in *Zostera capricornii* beds, where 90% of the root/rhizome biomass is located in the 0- to 5 cm-depth layer. Whole plants receiving decreased irradiance support lower rates of sulfate reduction and nitrogen fixation in the rhizosphere, and microbial activity in the surrounding sediment is less than that found in the rhizosphere (Fig. 2). Rates of sulfate reduction in the *Z. capricornii* rhizosphere average between 4.5 and 8.2 μMol SO_4^{2-} gdw^{-1} day^{-1}, and a substantial portion of the daily carbon fixed by *Z. capricornii*, 8 to 18%, is required to fuel the activity of rhizospheric bacteria (63). Nitrogen fixation may be more reliant on the organic carbon substrate available in the rhizosphere than sulfate reduction. Microbial activity in the rhizosphere accounted for only 4% of the total, depth-integrated sulfate-reduction rates measured in *Z. capricornii* beds, whereas rhizospheric microbial activity accounted for 39% of the depth-integrated nitrogen fixation (63). A similar result was reported from intertidal *Z. noltii* beds, where rhizospheric sulfate reduction accounted for 11% or less of the sedimentary total, whereas rhizospheric nitrogen fixation accounted for 31% of the total sedimentary nitrogen fixation (64).

Sulfate-reducing bacteria represent a large component of the bacteria associated with the *Z. marina* rhizosphere, and are primarily associated with the root/rhizome surface (65). Surface sterilization of the roots results in a 80 to 95% decrease in both sulfate-reducing and nitrogen-fixing activity, and it is likely that a substantial number of the sulfate-reducing bacteria in the seagrass rhizosphere are capable of nitrogen fixation. Inhibition of sulfate reduction in the *Z. nolti* rhizosphere results in a significant (50 to 80%) reduction of nitrogen-fixation activity, similar to results obtained in experiments with the rhizospheric microbial community associated with *Z. marina* and *T. testudinum* (64,66–68). The population of sulfate-reducers occupying the *Z. marina* rhizosphere from a seagrass bed in Denmark exhibit a high tolerance to O_2 exposure, which may be an inherent feature of the microorganisms or indicative of a consortial relationship with aerobic bacteria occupying the rhizosphere (65). Sulfate-reduction rates in the rhizosphere are substantially higher than those in adjacent sediments, ranging from 20 to 50 μMol SO_4^{2-} gdw^{-1} day^{-1}. Rhizospheric sulfate reduction in *Z. marina* occurs faster in the light than in the dark, and is enhanced by the addition of organic substrates, again demonstrating the reliance of rhizospheric sulfate-reducing bacteria on the products of seagrass photosynthesis (69).

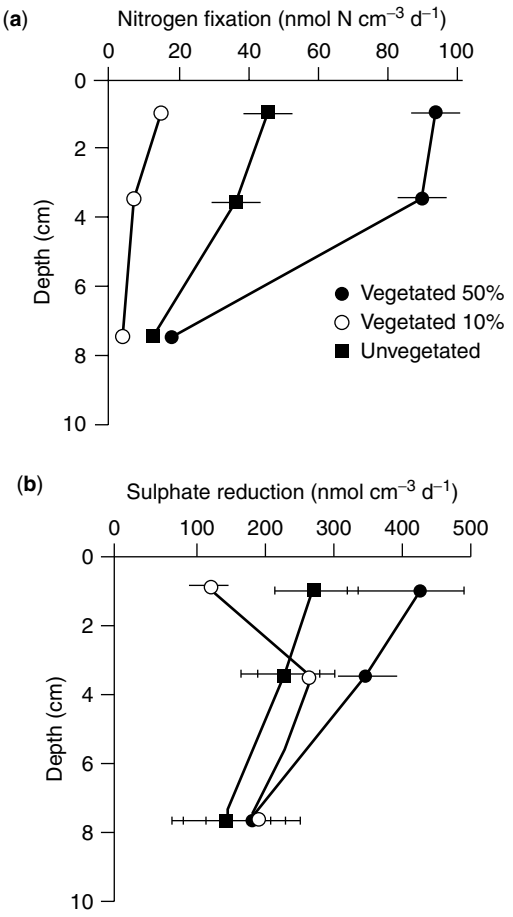

Figure 2. Effect of reduced surface irradiance on rhizospheric (**a**) nitrogen fixation and (**b**) sulfate reduction in a *Z. capricornii* seagrass bed. Depth distribution of nitrogen fixation and sulfate-reduction rates in adjacent seagrass bed sediments are also illustrated. Redrawn from (63).

Utilization of root exudates by the sulfate-reducing community does not always result in a diurnal variation in sulfate-reduction rates, however. In both an intertidal *Z. noltii* bed and mixed-species stands of tropical seagrasses, researchers found no diurnal variation in rates of rhizospheric sulfate reduction (22,70). However, in the tropical seagrass bed in Australia, other measures of microbial activity, including nitrogen fixation and incorporation of tritiated thymidine, did exhibit a diurnal pattern indicative of direct utilization of seagrass photosynthate. This discrepancy may be because sulfate-reducing bacteria do not utilize the photosynthates directly, but rather rely on products resulting from bacterial hydrolytic and fermentative activities (22).

A variety of facultative and strict anaerobes have been isolated from the seagrass rhizosphere. Acetogenic (*Acetobacterium*) and sulfate-reducing (*Desulfovibrio*) bacteria were the primary components of the microbial community occupying both root epidermal and deeper cortex layers of *Halodule wrightii* (71). Bacteria were detected in 100% of the epidermal cells, over 90% of the exterior cortex cells, and up to 60% of the interior cortex cells. Nitrogen-fixing *Klebsiella* species have also been isolated from

intracellular root tissue of *H. wrightii* (72). Many of the bacteria isolated from *H. wrightii* roots are gram-positive clostridia, in contrast to the gram-negative population that typically dominates seagrass bed sediments (71,73). Methanogenic bacteria (Archea) and denitrifying bacteria have also been identified from *Halodule* spp. root samples (71,74).

Seven groups of denitrifying bacteria have been isolated from the rhizosphere and surrounding sediment of *H. universis* and *T. hemprichii*. The isolates are primarily gram-negative rods, and included strains that could be identified as *Pseudomonas*, *Alcaligenes*, and *Vibrio*. Several strains that were halophilic, facultatively anaerobic, and fermentative also demonstrated denitrifying activity (74).

Isolates from *Z. marina* are primarily associated with root/rhizome surfaces, rather than intracellular or root cortex tissue. However, a novel sulfate-reducing bacterium, designated *Desulfovibrio zosterae*, has recently been isolated from surface-sterilized root/rhizome tissue (75). Gram-negative rods capable of exopolymer production and possessing a respiratory metabolism represented the majority of strains isolated from the sediment and rhizosphere of *Z. capricornii*. An *Alternomas* spp. represents 1.5 to 2.0% of the bacterial population in *Z. capricornii* and adjacent sediments, although it is found in highest numbers in association with root/rhizome tissue (76).

Sedimentary Bacteria

Seagrass bed sediments exhibit a broad range in physicochemical parameters, from fine-grained, organic rich sediments to coarse-grained, organic-poor sediments. In temperate areas, sediments are usually a combination of siliceous sands and finer silt-clay particles, whereas in many tropical environments carbonate sands and silts predominate. Regardless of the sediment matrix, however, sediments within seagrass beds are generally finer-grained and have a higher organic matter content than adjacent unvegetated sediments, because of the trapping of particles by the seagrass canopy and the accumulation of seagrass detritus within the sediments (8). Sources of primary production to support microbial activity in seagrass beds are not limited to the seagrasses. Carbon production by phytoplankton, macroalgae, and epibenthic and epiphytic microalgae can equal or exceed seagrass primary production, and all provide organic substrate to support heterotrophic bacterial activity in seagrass bed sediments (21,46,77). Rates of microbial activity in nonrhizospheric seagrass sediments are generally lower than those in the rhizosphere, and higher than microbial activity rates measured in unvegetated sediments. This trend in microbial activity is correlated with the gradient in available organic matter that occurs from the rhizosphere to adjacent sediments.

Seagrass bed sediments are primarily anoxic, although there may be a shallow oxidized zone at the sediment surface and around live seagrass roots. In the aerobic zone, bacteria oxidize organic substrates completely to carbon dioxide. A zone of denitrification may exist at the aerobic–anaerobic interface, and denitrifiers also oxidize organic material to carbon dioxide, converting nitrate to ammonia and N_2 in the process. In the anaerobic zone, fermentative bacteria convert organic matter to short-chained organic molecules and carbon dioxide. These end products are then used by sulfate-reducing and methanogenic bacteria, resulting in the end products of carbon dioxide and methane, respectively. In marine sediments, sulfate reduction rates far exceed rates of methanogenesis. Differences in belowground plant biomass, annual primary production between seagrass species, and differences in nutrient status and temperature between sites, prevent generalizations about the amount of seagrass carbon remineralized by sediment bacteria, and the role of sediment bacteria in nutrient recycling.

The microbial community inhabiting seagrass sediments includes aerobic and photosynthetic microorganisms in surficial sediments. The photic zone in seagrass bed sediments is very shallow because of the fine-grained nature of the sediments and shading by the seagrass canopy. Nonetheless, in some tropical systems the sand microflora can account for a greater proportion of system primary production than the seagrasses themselves (46,78), and algal carbon has been identified as a major source of organic carbon supporting microbial activity in seagrass bed sediments (21,77). The aerobic zone in seagrass sediments is usually shallow, extending from a few millimeters in depth to at most several centimeters.

The abundance of bacteria in seagrass sediments ranges from 10^8 to 10^{10} cells gdw^{-1}, which falls on the upper end of the range for marine sediments (Table 2). The major factors controlling bacterial abundance in marine sediments are particle size (sediment surface area) and organic matter content. These factors, in addition to seasonal changes in the availability of detrital carbon, are likely correlated with bacterial abundance in seagrass bed sediments, although few comparative studies are available (21,79).

A variety of techniques, including tritiated thymidine incorporation, leucine incorporation, and measures of O_2 uptake or CO_2 production, have been utilized to examine factors affecting the activity of bacteria in seagrass sediments. Published estimates of sedimentary bacterial carbon production exhibit a range of over two orders of magnitude (Table 2), and it should be noted that these estimates include the activity of rhizospheric bacteria. It is therefore not surprising that diel variation in the activity of sedimentary bacteria has been observed in several cases, and the utilization of organic carbon from root exudates has been postulated to support a significant fraction of sedimentary bacterial activity (60). The relationship between sedimentary bacterial activity and seagrass production may vary along different spatial and temporal scales, as demonstrated in an examination of *P. oceanica* beds in the Mediterranean. Bacterial activity was positively correlated with annual seagrass production, but on seasonal timescales, bacteria and seagrass production were inversely correlated. This is attributed to competition for inorganic nutrients between the microbial and macrophyte communities (79,80) The role of nutrients in microbial decomposition of detritus is discussed in more detail later.

Microbial communities at the interface between the aerobic and anaerobic sediments resemble those that occur in the seagrass rhizosphere. Depending on the availability of electron donors and organic matter, manganese reduction, dissimilatory nitrate reduction, and ferric iron reduction may occur near the oxic–anoxic interface. Denitrification rates are higher in seagrass than in adjacent unvegetated sediments, and may also exhibit a diel cycle, with peaks at dawn (56,81)

Sulfate reduction is the dominant anaerobic process in seagrass bed sediments, and because of the shallow depth of the aerobic zone in seagrass bed sediments, sulfate-reducing bacteria remineralize the major portion of seagrass productivity, after initial utilization by hydrolytic and fermentative bacteria (4,22). Rates of sulfate reduction in seagrass bed sediments are reportedly 5 to 50% lower than rates associated with the rhizosphere, but generally higher than sulfate-reduction rates in unvegetated sediments (4,60,64,69).

NITROGEN FIXATION IN SEAGRASS ECOSYSTEMS

Nitrogen is often the limiting nutrient for marine primary production, and so there has been considerable interest in measuring rates of nitrogen fixation in marine ecosystems. Nitrogen fixation is a microbially mediated process that converts N_2 into biologically available nitrogen. Some of the highest rates of nitrogen fixation measured in marine environments are associated with seagrasses (82,83). Factors controlling nitrogen-fixation rates and spatial and temporal patterns of nitrogen fixation vary among seagrass systems, and the following discussion will describe nitrogen fixation in several seagrass species. In most studies, the acetylene-reduction technique is used to estimate rates of nitrogen fixation, whereas in a few instances the incorporation of ^{15}N-labeled N_2 has been measured (6,7).

Nitrogen fixation has been measured in the phyllosphere, rhizosphere, and sediments associated with seagrasses of the genus *Zostera*. The highest rates occur in the rhizosphere, are correlated with the zone of highest root/rhizome biomass, and are usually, but not always, associated with roots rather than rhizomes (63,67,84–86). Rhizospheric nitrogen fixation is between 25 and 200% higher in light than in dark incubations, and is stimulated by the addition of organic substrate, suggesting a tight coupling between seagrass photosynthesis and rhizospheric nitrogen fixation (6,63,68,85,87,88). Figure 2a illustrates the depth distribution of nitrogen fixation in the rhizosphere and surrounding sediments of *Z. capricornii*, and the deleterious effect of lower surface irradiance on rhizospheric nitrogen-fixation rates (63). The seasonal pattern of rhizospheric nitrogen fixation in *Zostera* spp. also reflects the dependence of nitrogen-fixing bacteria on seagrass photosynthate, as nitrogen-fixation rates are higher during the plant's major growing season (85,88). The physical location of nitrogen-fixing bacteria associated with *Zostera* spp appears to be on the root/rhizome surface, as opposed to intracellular or cortex tissue (86).

Both dissolved inorganic nitrogen and oxygen concentrations can affect rates of nitrogen fixation in the *Zostera* rhizosphere. Reduction of N_2 by the nitrogenase enzyme is an energetically expensive reaction, and the nitrogenase enzyme is rendered inoperable upon exposure to oxygen. Accordingly, many microorganisms only fix nitrogen under nitrogen-limiting conditions, and must protect the enzyme from oxygen using physiological, spatial, or temporal separation of enzyme activity from oxygen. Rhizospheric nitrogen fixation in a *Z. marina* bed in Denmark showed no inhibition of acetylene-reduction activity with the addition of between 200 and 800 $\mu M\ NH_4$ (84), whereas the addition of 100 to 200 $\mu M\ NH_4$ or NO_3 reduced rhizospheric nitrogen fixation by 20 to 40% in a *Z. marina* bed off Long Island, New York (67), and 10 to 50 $\mu M\ NH_4$ inhibited 50% of the acetylene-reduction activity in a *Z. nolti* rhizosphere (89). These data suggest rhizospheric nitrogen fixation will persist even in sediments with fairly large concentrations of dissolved inorganic nitrogen, as DOC excreted by the seagrass provides ample organic substrate to fuel the process. Although the rhizosphere is generally found in anaerobic sediments, oxygen can diffuse through the rhizomes to the roots, creating microaerobic conditions at the root tips. Results comparing acetylene-reduction rates in aerobic and anaerobic conditions often, but not always, indicate higher rates under anaerobic conditions, particularly for whole-core incubations (63). The deleterious effect of O_2 on rhizospheric nitrogen fixation is consistent with reports that sulfate-reducing bacteria are responsible for between 25 and 80% of rhizospheric nitrogen fixation (64,68,84,85). Over a hundred strains of microaerophilic and facultative anaerobic nitrogen-fixing bacteria have been isolated from *Z. marina* roots collected in Japan (87), all belonging to the family Vibrionaceae. Of these, the majority were in the genus *Vibrio*, and a group of *Photobacterium* were also identified.

Nitrogen-fixation rates in the phyllosphere and adjacent sediments are much lower than those in the rhizosphere of *Zostera* beds. Estimated rates of rhizospheric nitrogen-fixation range from approximately 1 to 7 $mg\,N\,m^{-2}\,day^{-1}$ for *Z. marina* and *Z. noltii* to between 10 and 40 $mg\,N\,m^{-2}\,day^{-1}$ for *Z. capricorni* (6,67,68,85). Nitrogen-fixation rates in the phyllosphere of *Zostera* spp are usually reported as absent or in trace amounts (67,88). The exception is a report of epiphytic cyanobacteria colonizing *Z. marina* leaves in the fall, which exhibited acetylene-reduction rates higher than the rhizosphere (88). Nitrogen fixation associated with *Z. marina* rhizome detritus has been reported from North Carolina, and could contribute another 1.5 $mg\,N\,m^{-2}\,day^{-1}$ (24).

Experiments using ^{15}N-labeled N_2 demonstrate that N_2 fixed in the rhizosphere is taken up by the plant and approximately 50% translocated to the leaves (6). In that experiment it was demonstrated that nitrogen fixation could provide between 33 and more than 50% of the annual nitrogen demands of *Z. capricorni*, similar to estimates based on acetylene-reduction activity (63). Estimates of the contribution nitrogen fixation makes to the annual nitrogen demands of the temperate species *Z. marina* and *Z. noltii* are much lower, ranging from 3 to 28% (67,68,85).

Halodule wrightii, a seagrass species with a wide distribution in subtropical and tropical latitudes, harbors endophytic nitrogen-fixing bacteria in root and rhizome

tissue (72). The bacteria, a *Klebsiella* sp., lines cortical cells intracellularly, particularly in root tips and in actively growing rhizome tissue. The bacteria are able to fix nitrogen under aerobic conditions, presumably by compartmentalizing nitrogenase in the cell and thus protecting it from oxygen inactivation. In addition to the endophytic bacteria, substantial numbers of nitrogen-fixing bacteria are also associated with the root/rhizome surface (72,90). As sometimes observed in *Zostera* spp., rhizospheric acetylene-reduction rates are higher in aerobic than anaerobic incubations in *H. wrightii* (90). Substantial rates of nitrogen fixation are also associated with the microbial community on *H. wrightii* leaves, with much lower rates associated with sediments and the water column (90,91).

In general, measured rates of nitrogen fixation are higher in tropical than in temperate seagrass species. Several species of tropical seagrasses support nitrogen-fixing epiphytic populations, including *H. wrightii*, *T. testudinum*, and *Halophila stipulacea* (82,91,92). In *H. stipulacea* meadows in the Red Sea (Gulf of Elat), phyllospheric nitrogen-fixation rates were 21 times higher in the light than in the dark, and were not affected by treatment with DCMU, a photosystem II inhibitor, suggesting that photosynthetic bacteria or cyanobacteria were the primary nitrogen fixers (92). Phyllospheric nitrogen fixation represents 89% of total nitrogen fixation associated with *H. stipulacea*. In contrast, nitrogen fixation associated with the phyllosphere of *Thalassia* is less than that which occurs in the rhizosphere, and is associated primarily with the colonization of leaves by cyanobacteria (82).

Estimates of rhizospheric nitrogen fixation associated with *T. testudinum* range from 5 to 140 $mg\,N\,m^{-2}\,day^{-1}$, with an average value of about 20 $mg\,N\,m^{-2}\,day^{-1}$ (93,94). Rhizospheric nitrogen fixation rates for other species of tropical seagrasses fall within the same range, and are approximately 10 to 20 times higher than estimates for temperate seagrasses (7,85,95). Tropical seagrasses often exhibit phosphorus limitation, rather than the nitrogen limitation often noted for temperate species (96), but also see Reference (97). Although this is largely caused by the presence of carbonate sediments, which bind phosphorus more tightly than the siliceous or clastic sediments found in temperate areas, the alleviation of nitrogen limitation may also be a result of the higher rates on nitrogen fixation found in tropical seagrasses.

MICROBIAL DECOMPOSITION OF SEAGRASS DETRITUS

There are few grazers that directly consume seagrass plants, so the large majority of seagrass primary production is subject to microbial decomposition prior to entering the food web or ultimately being remineralized. Heterotrophic bacteria are the microorganisms primarily responsible for the decomposition of seagrass detritus, although fungi may play a role in the decomposition of seagrass detritus deposited in the intertidal (98). Bacteria colonize detrital seagrass rapidly, and decomposition is often conceptualized as having three phases: an initial leaching and utilization of soluble material, microbial decomposition of relatively labile particulate detritus, and a slower decomposition of more refractory material. Thus, exponential decay rates are often calculated, although the results of many decomposition experiments can also be fitted with a linear function. The rate and efficiency with which microbes decompose seagrass detritus contribute to the overall extent to which a seagrass ecosystem may support secondary production and/or export organic matter to adjacent ecosystems.

Both free-living and attached microorganisms are able to rapidly utilize the dissolved organic matter released by seagrasses in the initial phase of decomposition. The proportion of detrital carbon released as DOC during a three-day abiotic leaching period has been estimated at between 13 and 27.6% for *T. testudinum*, *Syringodium filiform*, and *Z. marina* leaves and root/rhizomes (99,100). A lower estimate of approximately 2.6% of total detrital carbon was made for the proportion of monomeric carbohydrates leached from *Cymodocea nodosa* leaves over 2 days (101). In flask incubations, heterotrophic bacteria grow quickly on DOC leached from seagrass detritus, and include both free-living and aggregate forms (100,101). Flagellates quickly graze the free-living bacteria and exhibit a sharp increase in density that coincides with a significant reduction in bacterial cell density (100,101). Dissolved organic matter (DOM) is released from detritus both via abiotic leaching and as a result of hydrolysis of particulate organic matter by microbes attached to detrital particles. In the later stages of decomposition, the activity of attached bacteria may be supplemented by DOM released from fresher detritus, in addition to DOM utilized via a coupled hydrolysis uptake (101).

The rate at which seagrass detritus decomposes is dependent on a number of factors, including age and species of origin, temperature, and environmental setting (i.e., buried versus aboveground). Most investigators have utilized litterbags to estimate decay rates for detritus in a variety of settings (Table 3). Decay rates can be calculated as:

$$k = \ln(W_o/W_t)\left(\frac{1}{t}\right) \quad (1)$$

where W_o = original dry weight, W_t = dry weight at end of period, and t = time (101). Decomposition in the initial 3 to 14 days results in the loss of between 25 and 55% dry weight of *Halophila decipiens* plants, *T. testudinum* roots, and *Z. marina* whole plants and rhizomes, and estimated decay rates (k) range between 0.012 and 0.126 day^{-1} (Table 3) (5). Similarly, *H. wrightii* leaves in litterbags suspended in the water column lost 35.5% of the initial ash-free dry weight in 24 days (102). During this initial stage, there is evidence that leaves and roots decompose faster than rhizomes, and *Thalassia* rhizomes in particular are resistant to decay (Table 3) (99). Decomposition rates slow considerably after the first few weeks, and litterbag studies show that over a period of between 120 and 240 days, seagrass detritus decays at rates ranging from 0.003 to 0.018 day^{-1} (Table 3). Very little decay may occur after the first 4 to 6 months of decomposition (101,102). It should also be noted that decay rates as estimated by weight loss in litterbags may be an overestimate, caused by the loss of very fine particulate matter from the litterbag. A direct comparison

Table 3. Decomposition Rate of Several Seagrass Species as Determined by Litterbag Experiments. Incubation Time is Expressed in Days. The Variable k Expresses Weight Loss per Day, and is Calculated as $k = \ln(W_o/W_t)(1/t)$

Seagrass Species and Plant Part	Location	Incubation (Days)	Time % Weight Loss	k	Reference
Halophila decipiens whole plant	Caribbean-surface	6.5	26	0.048	(104)
	Caribbean-buried	6.5	62	0.126	
Zostera marina whole plant	Virginia (mid-Atlantic)	3	25	0.096	(105)
		42	70	0.029	
Zostera marina roots	North Carolina (mid-Atlantic)	17	28	0.019	(99)
		170	50	0.004	
Zostera marina rhizomes		17	18	0.012	
		170	60	0.005	
Thalassia testudinum roots	Florida (Gulf of Mexico)	14	55	0.057	(99)
		247	79	0.006	
Thalassia testudinum rhizomes		14	8	0.006	
		247	20	0.0009	
Thalassia testudinum leaves	Florida	30	38	0.016	(106)
		160	100	nd	
Cymodocea nodosa leaves	Mediterranean	14	12	0.009	(101)
		231	51	0.003	
Halodule wrightii leaves	Texas (Gulf of Mexico)	24	35	0.018	(102)
		419	76	0.003	
Posidonia oceanica leaves	Mediterranean	64	25	0.013	(103)
		117	62	0.003	
Zostera marina leaves	Mediterranean	120	89	0.018	(107)
Cymodocea nodosa leaves	Mediterranean	120	87	0.017	(107)
Zostera noltii leaves	Mediterranean	120	79	0.013	(107)
Posidonia oceanica leaves	Mediterranean	150	72	0.008	(107)
Posidonia australis leaves	Australia (Shark Bay)	120	35	0.003	(108)
		160	85	0.012	(108)
Amphibolis antartica leaves	Australia (Shark Bay)	120	62	0.008	(108)
		160	58	0.005	(108)

of *P. oceanica* decomposition rate using O_2 uptake and litterbag weight loss showed that only about 40% of the weight loss from litterbags could be attributed to microbial respiration (103). Others have noted that decomposition in litterbags may be slower than in unconfined detritus because of the exclusion of shredders (5).

Estimates of the efficiency with which bacteria are able to convert detrital carbon into biomass exhibit a wide range, although in general they fall within the 10 to 30% range typical of estimates for bacterial growth on vascular plants. Not surprisingly, the highest estimates are generally associated with the utilization of DOC or the early stages of particulate decomposition, and lower efficiencies occur in the later stages of decomposition (100,101,105,109).

The concentration of lignin, structural carbohydrates, and phenolic compounds varies between species of seagrass, and seagrasses with relatively higher concentrations of these compounds will decompose more slowly (5,99,110). Other factors, including temperature, depth, oxygen concentration, and sediment type, have also been shown to affect seagrass decomposition rates, although not in a consistent matter (5,103). In several instances, studies have noted that anaerobic or buried detritus decomposes faster than detritus suspended in the water column or on the sediment surface, suggesting that an anaerobic microbial community is necessary for the remineralization of the more refractory organic matter (5,99).

Inorganic nutrients, particularly nitrogen and phosphorus, are likely a controlling factor in the microbial decomposition of seagrass detritus. The C/N and C/P ratio of seagrasses is two to five times higher than that of bacteria biomass, and additions of inorganic nitrogen and phosphorus to seagrass sediments result in increases in microbial activity and rates of organic matter loss (111). A correlation between detrital C/N content and decay rate has been found when comparing decomposition of different plant parts (leaves versus rhizomes), and comparing senescent versus detrital material (99,103). However, a broad analysis of published seagrass decomposition data failed to find a positive correlation between C/N ratio and decomposition rate, suggesting that nitrogen content is but one of several factors affecting microbial decomposition rates of seagrass detritus (5). Although microbial decomposition of many vascular plants results in a significant increase in the detrital C/N ratio, this has been rarely observed in studies of seagrass decomposition (5,101,110).

Microscopic examination of the microbial community responsible for the decomposition of seagrasses reveals a diverse community dominated by bacteria. Bacteria quickly colonize surfaces of detrital particles, and initial communities dominated by only a few bacterial types soon give way to diverse communities composed of coccoid, rod-shaped, stalked, and filamentous bacteria (24,32,101,105). Some studies have noted the presence of particularly large bacterial cells, greater than 0.6 μm^3, indicative of a sapotrophic environment (24,105). Rod-shaped and filamentous bacteria have been noted to occupy crevices on leaf surfaces and tunnels within cell walls (101,112). These features are often surrounded by halos, suggesting extracellular lysis of cellular material. Both microscopic observations and biochemical analysis of seagrass detritus suggest that the cuticle is particularly resistant to microbial decay, as is fibrous detrital material (102,112). Filamentous bacteria may be particularly important in the degradation of structural carbohydrates, and the diversity of microbes in close proximity to one another suggests an active microbial consortium, such as has been described for the degradation of organic matter in other aquatic environments (24,112,113).

The role of bacteria in transferring seagrass primary production to grazers has been examined by estimating the amount of seagrass carbon converted into microbial biomass associated with detritus. In each instance, attached bacteria have been found to constitute a minor amount of detrital carbon, and several studies have concluded that bacteria attached to detrital particles are not an important food item for benthic feeding organisms. Moreover, estimates of the amount of detrital carbon converted to microbial biomass are also very low, with most estimates well under 10% (104,105). These estimates do not include microbial growth on DOC released by both living and detrital seagrass, nor do they include an accurate assessment of extracellular microbial production. Most of these calculations are based on carbon, and as noted, bacteria have a lower C/N ratio than detritus and so make a greater contribution to the nitrogen requirements of consumer organisms. Although bacteria attached to detrital particles appear to play a minor role in transferring secondary production to consumer organisms, bacteria are part of other trophic pathways that may transfer seagrass primary production to secondary production (114,115).

CONCLUSION

Seagrasses form productive ecosystems in shallow coastal areas worldwide. The vast majority of this primary production is initially utilized by microorganisms either in the water column, attached to living plant surfaces, on detrital particles, or in adjacent sediments. Aboveground plant surfaces also provide structure for a rich epibiotic community, including bacteria, cyanobacteria, microalgae, and other protists. Members of the *Labyrinthulae* family have been identified to play a role in seagrass senescence, and certain strains are capable of initiating cell death. No other microorganisms have been identified as seagrass pathogens. Because seagrasses are in decline in many parts of the world, due to coastal development and eutrophication, a closer look at the relationship between microbial activity and the productivity associated with seagrass ecosystems is warranted.

Nitrogen-fixing bacteria and cyanobacteria occur throughout the seagrass ecosystem, and nitrogen-fixation rates often show a diel or seasonal pattern consistent with the direct utilization of seagrass photosynthate. Nitrogen-fixing microorganisms can provide a significant amount of the nitrogen required for plant growth, and plant uptake of fixed nitrogen has been documented. However, bacteria can also remove nitrogen from the ecosystem via coupled nitrification-denitrification, which may be facilitated by the organic matter and O_2 excreted by seagrasses. The availability of other essential plant nutrients, including iron and phosphorus, can also be controlled directly via microbial activity. Bacterial sulfate reduction, the dominant process in seagrass bed sediments, produces sulfide, which is deleterious for plant growth. Future efforts should be directed toward understanding how changes in water quality, nutrient concentration, and salinity can alter the relationship between seagrasses and their associated microbial communities.

The degree to which the detrital food chain succeeds in transferring seagrass production to consumers is still poorly understood, and appears to be highly variable. In some instances, highly productive seagrass beds appear to contribute little to the food chain supporting fish and shellfish, whereas other reports document a significant connection. Very little is known about how the composition and activity of the microbial community in seagrass ecosystems may affect the efficiency of this trophic transfer, and how environmental variables may alter this relationship.

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SEA ICE MICROORGANISMS

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Organisms living in polar regions are exposed to harsh environmental conditions. In contrast to the restricted development of life on the exposed land, enormous biomass of bacteria, protists, and metazoans are found in the more sheltered marine environments of the Arctic and Antarctic. One key factor in the polar marine ecosystems is sea ice. Already during the first expeditions to the poles, scientists observed discoloration of the sea ice that was due to accumulations of millions of unicellular algae per liter of sea ice. Combined efforts in biological, physical, and chemical studies elucidated the processes, allowing for life inside the frozen seawater. This contribution reports on the diversity, biomass, and productivity of algae and bacteria in polar sea ice with a regional focus on the Arctic.

DIFFERENT TYPES OF ICE

Ice occurs in cold climate regions as freshwater and as marine ice that are distinctively different in their physical and chemical properties (1). Freshwater ice is formed on lakes or rivers by freezing of freshwater or by accumulation of snow on glaciers. Icebergs are large floating masses of ice, which detached from coastal glaciers and are transported by wind and currents out to sea. Freshwater ice consists of freshwater ice crystals, incorporated sediment, and gas bubbles. Marine ice, which is formed by the freezing of seawater, is basically a two-phase system consisting of solid ice and liquid brine (plus intrusions of particles and gas bubbles). Three major types of sea ice can be distinguished depending on location and ice age. Fast ice is attached to land and forms ice sheets in coastal areas where it may grow either annually or for several years. Pack ice (annual pack ice or multi-year pack ice) consists of separate ice floes (floating flat pieces of ice with varying sizes ranging from a centimeter to a kilometer scale), drifting on the surface of the ocean with wind and currents. The structure and the dynamics of the sea ice are complex. The seasonal and interannual variation of its extent (Fig. 1) was studied by satellite analysis of passive-microwave data with a spatial resolution of app. 25×25 km grid size (2). On an average (period 1979 to 1987), minimum Arctic ice extent ($9.3 \cdot 10^6$ km^2) is reached in September and maximum in March ($15.7 \cdot 10^6$ km^2). This seasonal change is mainly (86%) caused by ice formation and melt in the marginal Arctic seas on the Eurasian and Canadian shelves (e.g., Barents Sea, East Siberian Sea, Kara Sea, and Laptev Sea), whereas the central Arctic ice cover is rather stable (14% of total variation of ice extent). New ice formation starts (depending on latitude) in August/September and commences throughout the winter until March with little interannual variability of the timing. Snow accumulates on the ice surface during winter to a thickness of about 40 cm (3). In May/June, snow melt starts and low saline ponds form on the surface of the ice floes (4,5).

In winter, sea ice extents from the Antarctic continent up to 2,200 km and covers on average $19 \cdot 10^6$ km^2 in September. From October onward, ice retreats to the coast ending with the minimum average ice extent of $4 \cdot 10^6$ km^2 with an interannual variability of 11% (2). The formation of sea ice occurs over areas of approximately $7 \cdot 10^6$ km^2 in the Arctic, and $16 \cdot 10^6$ km^2 in the Antarctic (1). The physical processes during early ice growth in a wave zone such as an achieving ice edge are basically understood and described as following the "pancake cycle" (6,7): the

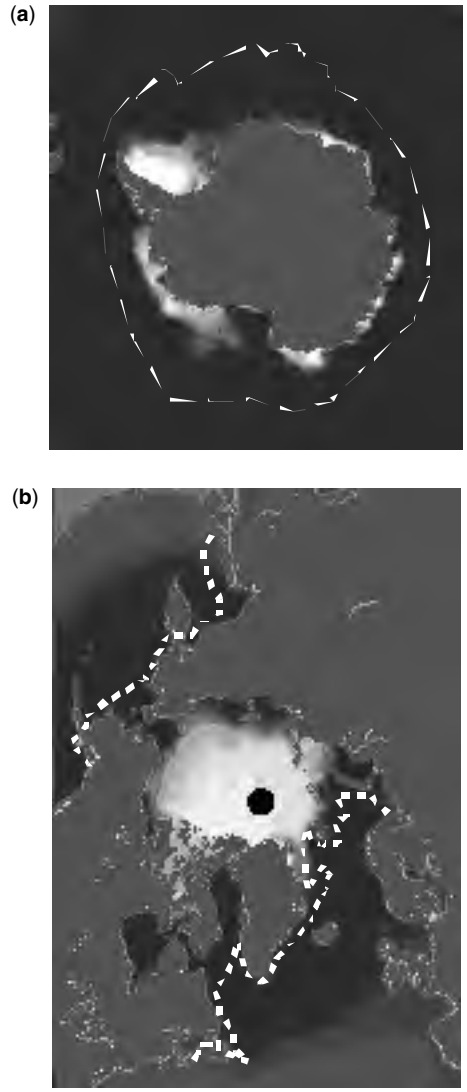

Figure 1. Example for the seasonal variation between minimum and maximum (dashed line) sea ice extent in the Antarctic (**a**) and Arctic (**b**). See color insert.

freezing of seawater leads to an accumulation of ice crystals (frazil ice) in a surface layer a few centimeters thick called grease ice. Further freezing results in the consolidation of the grease ice in the form of either small pancakelike structures or under calm conditions, in uniform ice sheets called nilas ice. During ice formation, a fraction of the incorporated brine solution (high saline water between ice crystals) drains out from the ice, thus transporting salts, dissolved nutrients, and particles from the ice into the water column (8,9). Subsurface formation of very large frazil ice crystals (discs of 10 to 15 cm in diameter and about 0.5 cm thick), so-called platelet ice, caused by upward flow of supercooled water along the floating Antarctic ice shelves, is only known from Antarctica (10,11).

Ice thickness data for the Arctic were compiled from upward looking sonar data of twelve submarine cruises for the period 1958 to 1992 (12). The mean draft of Arctic pack ice varied interannually between 2.8 m (in 1986) and 4.4 m (in 1970) with a mean of 3.6 m over the entire investigation period. Ice thickness increases from areas with mainly first-year ice (e.g., Russian Arctic: thickness <2 m) to areas with multi-year ice cover (central Arctic, region north of Greenland: thickness 7 to 8 m) (13,14). Overall, ice thickness changes seasonally by about 0.5 to 1.0 m (14). The dominant first-year ice in Antarctica reaches maximum thicknesses of 1.5 to 2 m (15).

The sea ice drifts with wind and ocean currents were studied in detail in the Arctic by the Arctic Buoy Program (16). In the Beaufort Sea, ice drifts in a large anticyclonic gyre, the so-called Beaufort Gyre, with speeds from close to 0 in its center to 3 $cm\,s^{-1}$ in the marginal zones. Ice floes need about 5 to 10 years to complete a cycle. Seasonal fluctuations in the wind stress cause reversal of the current and the formation of polynyas (areas of open water surrounded by sea ice). From the Russian shelves, the Transpolar Drift transports pack ice across the central Arctic to the major export area, the Greenland Sea, with speeds of 2 to 10 $cm\,s^{-1}$ (3). The ice movement from the East Siberian Sea to the Fram Strait requires approximately three to four years. About 10% of the sea ice of the central Arctic is exported each year through Fram Strait into the Greenland Sea (1) where it melts.

The three-dimensional structure of the brine system varies with ice texture. In granular ice, an irregular network of brine channels and pockets develops, whereas channels are more vertically oriented in columnar ice (17). In the bottom 1 to 3 cm of growing and stable sea ice, a so-called "skeletal layer" of parallel oriented ice platelets with brine filled spacing of about 0.5 to 1.0 mm within each ice crystal is formed. The brine salinity and the volume of the brine channels are dependent on the temperature and the salt content of the ice (18–20). A decrease in the ice temperature from $-4\,^{\circ}C$ to $-10\,^{\circ}C$, for example, leads to an increase in brine salinity from 70 to 144. The minimum observed temperature of newly formed Arctic sea ice in autumn 1995 ($-6.9\,^{\circ}C$) corresponds to a brine salinity of 110 representing an approximately threefold increase of ambient salinity compared to seawater. Within first-year and multi-year ice floes seasonally fluctuating gradients of temperature, bulk salinity, and brine properties evolve (4,5) and, for example, brine salinity may vary between 0 and above 120 at the ice surface with season, whereas parameters at the ice–water interface are relatively constant.

The seasonality of light is remarkable in polar areas and consequently, organisms experience day lengths from 0 to 24 hours (Fig. 2). Under-ice photon flux is controlled by the thickness and characteristics of the snow and ice, and the enclosed sediment and biota (1). The albedo (= ratio of reflected to incident shortwave radiation) of ice floes covered with cold snow varies between 0.80 and 0.85, decreasing to 0.70–0.75 with the onset of snowmelt. Snow-free multi-year ice floes have albedo values ranging from 0.55 to 0.75 depending on surface temperature. First-year sea ice and melt ponds exhibit lowest values (0.15 to 0.60). The bulk shortwave extinction coefficient k_z varies with depth (21) and has in deeper layers a mean value of 1.5 m^{-1}.

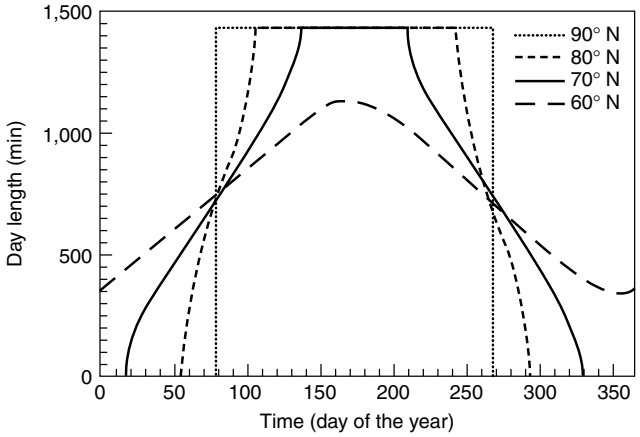

Figure 2. Day length (min) in Arctic latitudes.

The quality of the light is altered by the wavelength dependent spectral absorption coefficient k_λ which has relatively constant values between 400 and 500 nm, and increases for longer wavelengths. Red wavelengths are more quickly absorbed, and the spectrum changes with increasing ice depth with maximum transmission between 450 and 550 nm. Algal accumulations may have considerable impact on the spectrum and absorption of the shortwave radiation (22–25). As a consequence of the above mentioned factors, shortwave radiation intensities are reduced by orders of magnitude under sea ice compared with surface irradiance.

PRIMARY PRODUCERS WITHIN THE ICE

Life at the Surface of Ice Floes

Historically, the snow and melt pond habitats associated with the surface of sea ice received little attention (26,27), although snow and glacial fields on land were known to be colonized by algae, bacteria, fungi, and protozoa (28). There, algae occurring in concentrations of more than $5{\cdot}10^5$ cells ml^{-1} can stain snow and glaciers, as has been shown for many parts of the world (29). Coloration of the surface of Arctic pack ice floes occurs for at least two reasons. Grey to brownish coloration due to lithogenic sediments (so-called "dirty ice") has received increasing interest from marine geologists (30). First evidence for the occurrence of algae on the surface of ice floes was provided in 1828 (31) when red snow patches were seen on sea ice close to Svalbard at 82° N. A detailed study showed that a red snow patch community on an Arctic ice floe belonged to the so-called "nivalis cryobiont"-complex with the chlorophyte *Chlamydomonas nivalis* as the dominant species (32). *Chlamydomonas nivalis* is the typical red snow alga for the northern hemisphere (29) and probably the most common and widely distributed snow algal species of all (33). Several other chlorophytes (*Ancylonema* sp., and *Chlorella* sp.) and one cyanophyte species (*Stigonema ocellatum f. paniformes*) were found in addition to *C. nivalis* during the Russian ice drift studies "North Pole 22" in 1975 and "North Pole 23" in 1979 in the central Arctic Ocean, whereas diatoms were absent (34). The lack of living diatoms is typical for all temporary snowfields (also on land), which are mostly dominated by *Chlamydomonadaceae* such as *C. nivalis* (35). The occurrence of empty diatom frustules on the surface of sea ice (36) is not the result of active growth in the snow–ice interface but from passive accumulation due to ice melt. No observation of surface ice or snow coloration due to typical snow algae have been published for Antarctic sea ice.

Chlamydomonas nivalis is a psychrophilic species and survives temperatures of −35 °C (37), which makes winter survival on ice floes temperature independent. *Chlamydomonas nivalis* is introduced into the central Arctic Ocean from land by northward blowing winds (38,39) like pollutants emitted in North America or Europe (40) Besides growth, high concentrations can be achieved by passive accumulation. The snow melting in spring leads to an increase in particle concentrations at the remaining snow or ice surface (by freshwater flushing) and to particle transport into melt ponds and the upper layers of the sea ice (32). Here, *C. nivalis* is able to grow during the entire Arctic summer, partly because of its protection against ultraviolet (UV) radiation by the red carotenoid pigments (29,41). Primary production estimates for algae on the surface of Arctic ice floes are rather rare but the few obtained rates (e.g., 87 mg C m^{-2} d^{-1}) of a *C. nivalis* patch may even exceed those of bottom ice algae (42). Lower surface algal production rates of 0.2–4 mg C m^{-2} d^{-1} were probably not determined within colored snow patches (34). On the basis of volume specific incorporation rates (43,44) and cell counts (32), potential primary production may range from 3 to 1,200 mg C m^{-3} snow d^{-1} leading to an integrated value of 12 to 480 mg C m^{-2} d^{-1}. The formation of mucilaginous aggregates by snow algae in association with nonliving material (32,38) allows the cells to trap lithogenic material for an improvement of the growth conditions in the vicinity of the cell. The mucilaginous aggregates formed by *C. nivalis* may furthermore provide an agent for fast vertical transport of the generally fine-grained ice sediments, which can occur in extremely high concentrations of up to 51 g l^{-1} (30), after ice melt.

Melt ponds cover up to 60% of the surface of Arctic ice floes (1), however, knowledge on biological properties is rare (5,32,34,45). In the central Arctic two types of melt ponds can be visually distinguished by their color (45). Blue ponds turned out to be freshwater habitats with a medium salinity (Psu) of 0.1. Green ponds had salinities of about 29 and were connected to seawater either by holes in the ice floes or via channels to the leads. Besides differences in pH and nutrient concentrations, the composition of the community also differed. In both types considerable concentrations of bacteria, photo- and heterotrophic protists including *C. nivalis* were described from this habitat (26–47). Besides flagellated algae (e.g., *Chlamydomonas* spp., *Carteria* sp., *Pyramimonas* sp., *Chrysolykos* sp., and *Dinobryon* sp.), heterotrophic protists (e.g., several dinoflagellate species, *Cryothecomonas* sp., and *Lachrymaria* sp.) also live in the ponds (47). Higher concentrations of pennate diatoms (e.g., *Nitzschia frigida* and *Nitzschia grunowii*) occur in the salty green ponds compared to the freshwater ponds (27,46,47), probably inoculated by wave flushing or migration from the sea

ice into the ponds (48–50). The latter process is supported by the development of typical ice algal communities at the bottom of green ponds (47,50). In the freshwater ponds inoculation by wind transport (51), river inflow (52), and by surviving cysts on the ice is likely.

The Bottom and Ice Interior

Distribution of organisms within the Antarctic and Arctic sea ice are very different (Fig. 3; 53). Four major habitats in Antarctic sea ice can be distinguished on the basis of the vertical distribution of sympagic-ice associated organisms in individual ice floes (54). Biomass maxima may occur (*1*) at flooded ice surfaces, (*2*) at the freeboard layer, (*3*) in interior ice parts, and (*4*) in the bottom layer. In Arctic sea ice, distribution patterns seemed to be less complex with only two regions of high algal standing stock, both associated with the bottom of the ice floes: (*1*) bottom communities (53), and (*2*) sub-ice communities (5). Consequently, biological research focused on the bottom centimeters of Arctic sea ice neglecting the biological properties of the inner parts of the ice. However, recent studies on the vertical distribution of bacteria in pack ice floes from the Barents and Laptev Seas and ice algae in the central Arctic have already demonstrated that the paradigm of the dominance of bottom communities in Arctic sea ice has to be revised (55,56).

The algal pigment concentration in Arctic pack ice floes (55) vary vertically over entire ice thicknesses of up to 6.7 m between individual ice segments of about

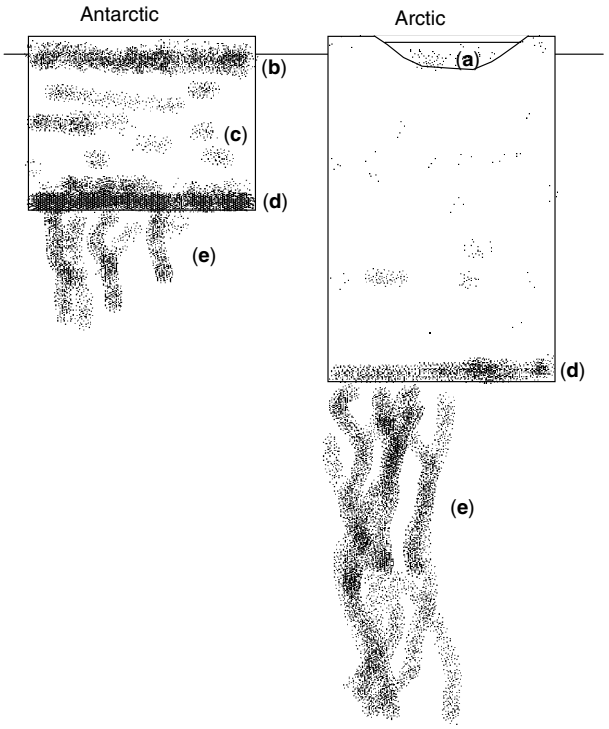

Figure 3. Regions of major algal biomass accumulation in Arctic and Antarctic sea ice: (**a**) melt ponds on Arctic summer ice, (**b**) freeboard and infiltration layers, (**c**) internal layers, (**d**) bottom layers and (**e**) attached under-ice algae. Ice thickness in Antarctica <2 m, in the Arctic 2–4 m.

10 cm thickness between 0.0 and 109.2 μg chl *a* l^{-1} melted ice. In most cores (85%), biomass maxima occur in the bottom 30 cm of the ice floes; only 35% harbor at least one internal maximum. The integrated algal abundances range from 320 to $14,100 \cdot 10^6$ algal cells m^{-2}, with the calculated ice algal carbon (IAC) from 13 to 457 mg C m^{-2} with phototrophic flagellates (including dinoflagellates) contributing on average 48% to total abundance and 20% to IAC. Pennate diatoms contributed 52% of total abundance and 72% of IAC. Although *Synechococcus* -like picocyanobacteria are incorporated into newly forming sea ice (57), they are nearly absent in older first-year and multi-year ice floes. Toward the bottom of the ice floes, the algal abundances increase from about 390 cells ml^{-1} to 3,850 cells ml^{-1}, and the relative composition changes with a decrease in the contribution of phototrophic flagellates from 97 to 32% (median values) and an increasing fraction (from 0% to 68%) of pennate diatoms. The IAC varies between 28 and 2,160 mg C m^{-3} (median = 180 mg C m^{-3}) in the lowermost segments, with a share of 46 to 100% (median = 91%) by pennate diatoms. The seasonal variation (spring to autumn) of integrated algal pigment concentration in Greenland Sea pack ice was negligible. These observations from Arctic pack ice differ from observations from annual coastal fast (58,59) or pack ice (23,60,61). In the coastal areas of Canada, ice algae grow exponentially until the ice melts in May reaching final integrated chl *a* concentrations that are two orders of magnitude higher than the pack ice data (Table 1). Pennate diatoms are the major primary producers in these regions and algal biomass is concentrated in the bottom segments (62,63). Episodically, accumulations of centric diatoms instead of pennate forms were reported (50,64).

So far over 200 diatom species (mainly pennate taxa) and over 70 species of flagellates from Arctic sea ice have been described. Approximately the same amount of diatom species were identified from the southern ocean (79). Although the knowledge about the diatom diversity is rather complete, little information is still available about the diversity of photo- and heterotrophic flagellated taxa, which are susceptible to handling artifacts. The melting process of sea ice samples have a severe impact on the observed communities, and direct ice melt tends to underestimate the diversity and biomass of the more vulnerable flagellates compared to diatoms (55,75).

In the Antarctic sea ice, algal biomass that is indicated by pigment concentration can exceed values of that of the Arctic at the snow–ice interface (infiltration community), in the interior parts and at the bottom (Fig. 3, Table 1). Under coastal fast ice, loose aggregations of platelet ice crystals may accumulate forming a specific habitat allowing for extensive algal growth (11,80). Algal growth in Antarctic sea ice follows the seasonal trend as described for Arctic fast ice (81). Two distinct algal blooms form in the bottom layer of the sea ice of which one forms in autumn after ice formation with maximum values up to 829 mg chl m^{-3}. After decrease during winter, spring bloom values exceed 1,000 mg chl m^{-3}. In the pack ice of the Weddell Sea, biomass seem to be lower with 4.5 mg m^{-3} ice (73). Integrated algal biomass in Antarctic sea ice ranges with season and location from about

Table 1. Integrated chl *a* Concentrations (mg m^{-2}) in Sea Ice from Various Arctic and Antarctic Locations

Region	Ice Layer Studied (cm)	Algal Biomass (mg chl *a* m^{-2})	Reference
Arctic			
Hudson Bay	Bottom 3–4	max. app. 170	65
	Bottom 3	26–100	66
	Ice–water interface	max. >300	67
Resolute Bay	Bottom 3	max. >120	23
	Bottom 3	max. >250	23
	Bottom 6	max. >300	60
	Bottom 5	max. 80–100	61
Frobisher Bay	Total core	>100	68
Barrow Strait	?	max. 79.8 (mean 18.3)	69
	?	max. 52.3 (mean 7.6)	69
Central Arctic	Bottom 2–4	0.1–14	70
Central Arctic	Total core	<0.01–7.09 (median = 1.4)	55
Greenland Sea (spring)	Total core	0.73–11.25 (median = 2.22)	71
Greenland Sea (autumn)	Total core	1.04–18.40 (median = 2.12	71
Barents and Laptev Sea	Total core	0.02–4.14 (median = 0.62)	71
Antarctic			
Lutzow-Holm Bay		97	49
Peninsula		122	72
Weddell Sea (summer)	Interior	1.4	73
Weddell Sea (summer)	Interior	9.6	74
Weddell Sea (spring)	Interior	51	75
McMurdo	Bottom	309	76
Syowa	Bottom	30	77
Davis	Bottom	15	48
Davis	Platelet ice	164	78

1.4 mg chl m^{-2} (74) to above 2,900 mg m^{-2} (82). Beside diatoms, the haptophyte *Phaeocystis antarctica* may contribute a large fraction to the biomass in infiltration and freeboard layers (e.g., Kristiansen and coworkers 1998).

Under-Ice Habitat

In summer, melt of sea ice leads to reduction of salinities of the upper meters of the water column. Apollonio defined the so-called "halocline flora" (83), which is characteristic for the upper 2 m of Arctic waters in summer and occurs at a constant biomass level of 1 to 2 mg Chl *a* m^{-3} for periods of six to eight weeks. The vertical stabilization allows the increased biomass in this layer, which also caused a *Chrysochromulina* bloom under Baltic Sea fast ice near Tvärminne, Finland (84). The halocline flora is dominated by phototrophic flagellates (Chlorophyceae and Chrysophyceae) with *Chlorella* sp., *Oocystis* sp., *Scenedesmus bijugatus* and *Ochromonas* sp. as major taxa (27).

Melt water can also accumulate under the ice floes in depressions and influences the exchange rates between ice and water column by enhanced desalination, sealing, and topographic smoothing of the ice bottom. Such under-ice ponds cover at least 5% of the total ice area of the Arctic (85). Biological activity within such ponds can be high: in one brackish water under-ice pond an algal bloom had formed within an algal pigment concentration of 29.6 mg m^{-3} with *Pyramimonas* sp. (20,320 cells ml^{-1}) as dominant species (86). In addition, phototrophic euglenophytes (<1 cell ml^{-1}), heterotrophic flagellates (4,360 cells ml^{-1}), and bacteria ($2.21 \cdot 10^6$ cells ml^{-1}) were identified.

Certain algae in the Arctic and Antarctic are able to use the underside of ice floes as a suitable surface for attachment. The often meter-long huge bands of diatom *Melosira arctica* hang from Arctic ice into the water column (87), mainly below young ice and first-year ice (70,88). These curtainlike structures consist of drilled chains, wrapped in a mucilaginous envelope. Co-occurring with *M. arctica*, several pennate diatom species (e.g., *Licmophora* sp.), heterotrophic flagellates, dinoflagellates, and ciliates, which were either attached to, gliding on, or moving between the chains were found. In Antarctica certain diatom taxa like *Berkeleya rutilan* form mat strand assemblages, which either float directly below the ice or are also attached to the ice bottom (48,89).

NUTRIENT CONCENTRATIONS

Nutrient concentrations in the ice are generally about one order of magnitude below winter concentrations in

Arctic surface waters. However the accumulated algal biomass in sea ice does not only reflect the initial nutrient concentrations but also the nutrient supply during ice algal growth (69). Initial bulk concentrations of inorganic nutrients in newly formed Arctic sea ice are below surface values because of brine drainage (e.g., nitrate concentrations below 4 mmol m^{-3}; (57) and decrease further with time. In coastal areas, nitrate is accumulated by ice algae and bulk concentrations (4 to 123 mmol NO_3 m^{-3}) in the bottom 3 cm of the ice exceed values of Arctic surface water and concentrations in the brine of pack ice floes by far (90,91). Only during spring brine nutrient concentrations in the bottom ice layer of Arctic sea ice are above typical k_s values (half saturation constant for nutrient uptake) for marine microalgae (92); limitation is likely in summer and autumn (93). However, measurements of nutrient uptake kinetics of Arctic sea ice algae under suboptimum growth conditions are still lacking. Growth limitation by nutrients is also expected for Antarctic ice algae (94,95) although initial concentrations in Antarctic surface waters are among the highest in the ocean of the world.

Resupply of inorganic nutrients may occur by either molecular diffusion across the viscous sublayer (mainly in the skeletal layer) from the water column or by biological regeneration of nutrients (96). The potential contribution of desalination of the ice sheet to the nutrient flux is predicted to be small because internal concentrations are low (69). Several studies in coastal sites pointed out the strong coupling between the strength of tidal currents and the induced supply of nutrients from the water into the ice, and the algal growth (69,91,97,98). In the central Arctic the coupling mechanisms are considerably different (55). Velocity of tidal currents varies regionally with lowest values (<10 cm s^{-1}) in the central Arctic (99), and values >50 cm s^{-1} in the Canadian Arctic or on the Russian shelves. The free drift of ice floes (in comparison to fast ice) and low current velocities can lead to a reduction of the current shear at the ice–water interface and consequently nutrient exchange rates between water and ice. The described pycnocline formation under the sea ice by freshwater accumulation below the pack ice additionally hinders exchange between ice and water in summer.

BACTERIA IN SEA ICE

A large fraction of total biomass in Arctic and Antarctic sea ice is contributed by bacteria (56). With the onset of algal growth, a favorable habitat for bacteria is created within the ice matrix and the increase in bacterial abundance and activity follows the increase in algal biomass (100). In Arctic fast ice, bacterial production is only a minor fraction of the ice-based carbon cycle when compared with primary production estimates (100). For other Arctic and also Antarctic locations, bacterial production can exceed those of algae for certain periods of the year (101,102). Information about the abundance and characteristics of ice bacterial communities of pack ice floes in the Eurasian sector of the Arctic is sparse in comparison with the knowledge on fast-ice systems (103). The volume of individual fast-ice bacteria ranges between 0.47 μm^3 (100,104) and 0.65 μm^3 (105). Those from drifting pack ice were lower with a median value of 0.09 μm^3, which resembles the median for bacterial volume from drifting Antarctic ice floes (0.07 μm^3, (101)). The bacterial cell volume is influenced by environmental parameters like food availability or grazing pressure. Dissolved organic material (DOM) concentrations in the ice are mostly higher than in the underlying water column (105–108) and the bacteria in the DOM-rich fast ice habitat are larger than the pelagic cells (105). A direct relation between DOM concentration and bacterial cell size was also observed in marine benthic habitats (109). The different DOM concentrations in fast ice and pack ice are one factor responsible for the observed bacterial cell size differences also because the algal biomass in Arctic fast ice is significantly higher than in the pack ice floes (110). Grazing processes have been already proposed by (101) as another size-controlling factor. This idea is further supported as heterotrophic flagellates are feeding on bacteria in Arctic fast ice as effectively as in other aquatic environments (104). However, the observed size differences between Arctic and Antarctic ice bacteria may also be because of the different techniques applied (100). The ratio of bacterial to algal biomass is not constant along vertical profiles within sea ice. The algal biomass exceeds the bacterial biomass in Antarctic (111) and Arctic sea ice (56,100) within the algal maxima at the bottom of the ice floes. Comparison based on the integrated values revealed that the integrated bacterial biomass was up to 10 times higher than the algal biomass. The relation of bacterial to algal biomass for Antarctic pack ice decreases from, on average, 55.1% in spring to 21.2% in autumn (112). Real estimates of bacterial abundances and biomass are biased by varying integration depths, ranging from 0.04 m (100) to 2.71 m (56). Vertical patchiness in the distribution of bacteria occurs on different scales inside the ice, with 78% of the bacterial biomass in the lowermost 5 mm of a 4-cm-long bottom section (104). To resolve spatial relationships between ice algae and bacterial biomass, small-scale sampling strategies with vertical resolution below 1 cm must be used, as have been successfully applied to the bottom parts of the fast ice in the Canadian Arctic (104,113).

The dominant heterotrophic bacterial taxa include proteobacteria, many pigmented marine *Cytophaga*-like and *Flavobacterium* rod-shaped and filamentous bacteria (CFG group), and gram-positive micrococci (114). *Archaea* are also known to inhabit Antarctic sea ice (115). A large fraction of bacterial genera and species are yet to be discovered, as demonstrated for Antarctic samples (116,117). The bacteria in the ice are not only exposed to grazing by flagellates but also lysis by bacteriophages, which have been described for Arctic sea ice (113). The mean ratio of viruses to bacteria in ice samples is high compared with the water column data. High virus activity could explain the discrepancy between primary and secondary production in Antarctic ice floes observed by Grossman and Dieckmann (101).

ADAPTION TO THE ENVIRONMENT

The microorganisms living within the sea ice are adapted both morphologically and physiologically to the unique temperature, salinity, light, and spatial characteristics of their habitat.

Temperature

All physiological processes are related to temperature, however, the rate at which metabolism decreases with varying temperatures, varies. Incorporation of radioactive tracers by Antarctic bacteria and algae are significantly higher at temperatures well above the in situ conditions (−1.9 °C) with optima between 2 and 15 °C (118). Both ice algae and bacteria can be called psychrophilic (119) because their growth optimum was below 15 °C. For Arctic cold regions, a higher relative decrease in bacterial versus algal production rate was observed, with decreasing temperature, causing an uncoupling of their activities in cold water conditions, which could explain higher DOC concentrations in these waters (120,121). However, studies from both Arctic and Antarctic provide also contradictory evidence.

Salinity

The salinity in the sea ice brine channels covaries with temperature as described earlier. Sea ice algae have evolved enormous osmoregulation potential, which allows them to grow at salinities (PSU) as high as 100. Four Arctic diatom species, isolated from Chukchi and Beaufort sea ice, were relatively euryhaline and maintained growth rates of 0.6 to 0.8 divisions per day over a salinity range of 10 to 50 (122). The growth of bottom ice algal communities of the Antarctic pack ice was observed at salinities from 11.5 to 34, and growth rates increased with increasing salinity (123). Growth of ice microalgae from the Weddell Sea (Antarctica) occurred down to −5.5 °C and a corresponding salinity of 95 (124). Lower temperatures and higher salinities were also tolerated. Greenland Sea ice algae exhibited positive growth over a salinity range of 4.0 to 74.0, with growth rates of 0.12 to 0.19 day^{-1} of the algal community and 0.32 to 0.39 day^{-1} for individual species (125). Antarctic sea ice algal communities, dominated by *Nitzschia stellate* and *Amphiprora* sp., tolerated higher salinities (30 to 50 PSU) better than lower ones (123,126), whereas assemblages dominated by other species such as *Pleurosigma* sp., *Amphiprora kufferathii*, and *Pinnularia quadratarea* exhibited maximal growth rates at 6 to 10 PSU (127,128). Acclimation to changing salinities is achieved in two ways, either extracellularly and anisoosmotically with inorganic ions, or intracellularly using organic compounds. Arctic and Antarctic unicellular ice algae, for example, are known to synthesize osmolytes like prolin or DMSP

Table 2. Comparison of Photosynthetic Parameters and Growth Rates of Bottom and Interior Ice Algae

Ice Habitat	Alpha Alpha*	$P^B{}_m$	P^B P^{Chl}	Primary Production	Growth Rate	Area	Method	Reference
Bottom			0.6–7.5			Antarctic	simulated	76
	0.003–0.091	0.04–0.38			0.13	Antarctic	simulated	150
					0.01–0.09	Antarctic	in situ	162
				140		Antarctic	in situ	163
				16.8–184		Arctic	in situ	164
					0.07–0.37	Arctic	—	69
	0.02–0.045	1.4–2.8		57.5		Arctic	simulated	64
					0.15–0.8	Arctic	—	165
	0.0005–0.05	0.08–0.44		20–157		Arctic	in situ	166
				<24–360		Arctic	in situ	167
			<0.015			Arctic	in situ	168
	0.002–0.033	0.03–0.08				Arctic	simulated	155
	0.002–0.063	0.01–0.33				Arctic	simulated	169
			0.1–0.2			Arctic	in situ	170
	0.03–0.125	0.6–0.13		21–463		Arctic	simulated	23
	0.004–0.41	0.01–4.9			0.08–0.23	Arctic	simulated	96
				14		Arctic	simulated	171
				5.62	0.26	Arctic	in situ	172
			1.86		0.13–0.16	Arctic	in situ	173
					0.06–0.15	Arctic	—	174
			<0.05–0.6			Arctic	in situ	154
	0.11–0.32	1.8–5.2				Arctic	simulated	175
				0.5–310		Arctic	simulated	70
	<0.01–0.15*		0.02–0.31 (Chl)	0.25–2.56	0.01–0.33	Arctic	in situ	93
Interior				45		Antarctic	modeled	176
	<0.01–0.230*		0.01–0.90 (Chl)	0.25–7.11	0.01–0.501	Arctic	in situ	93

Note: Photosynthetic efficiency (Alpha/Alpha*, defined in text) in (μg C μg Chl a^{-1} h^{-1}) (μmol m^{-2} s^{-1})$^{-1}$; maximum photosynthetic rate under light saturation (P^B_m) in (μg C μg Chl a^{-1} h^{-1}); biomass/chlorophyll *a*-specific photosynthetic rate (P^B or P^{Chl}) in (μg C μg Chl a^{-1} h^{-1}); Integrated primary production (mg C m^{-2} d^{-1}); growth rate (d^{-1}). Thickness of bottom and interior layers vary for the different authors; values represent means or ranges.

(dimethylsulfoniopropionate) (129) to remain isoosmotic with the surrounding medium if salinity increases. Similar experiments on salinity tolerance on Antarctic sea ice bacteria revealed upper thresholds for active growth of about 90 (128).

Light or Dark Adaptation

Incident irradiance in polar areas varies primarily with seasons, over diel cycles, and on even shorter timescales with cloud cover. During the polar winter, microalgae faces total darkness for a period of up to six months (130). A natural Arctic pack ice algal community, incubated for a dark period of 161 days, remained at 40% of the initially determined algal concentration and 64% of the initially observed species level (131). Many experimental studies on the impact of the light regime were conducted with cultures of Arctic and Antarctic diatoms (130,132). To survive long periods of darkness, marine microalgae have at least three survival strategies: facultative heterotrophy, storage, and utilization of energy reserves at a reduced metabolic rate, and resting spore formation. These strategies are not exclusive and vary in importance between species (130). Studies on Arctic, temperate, and Antarctic diatoms demonstrated their ability to utilize DOM for nutrition (133–136). Other studies (137) consider this process to be insignificant. The storage of energy in the form of lipid droplets or carbohydrates has been observed in many diatoms (138–140). Analysis of lipid composition of Arctic ice algae showed a predominance of neutral lipids over glycolipids and phospholipids (141,142). The high level of neutral lipids (37 to 94% of total fatty acids), independent of season, points toward the assumption that neutral lipid production is a basic characteristic of ice algae regardless of season (142). The production of other lipids may vary with season (143,144). Production of glycolipids in Arctic ice algal cultures may significantly increase after exhaustion of inorganic nutrients (145). Combined with energy storage, reduction of cellular metabolism (134,146) enables the survival of algal cells during the dark polar winters.

Many algal groups (e.g., diatoms and dinoflagellates) have evolved specific cell types and stages to survive unfavorable environmental conditions (147). In studies on Antarctic (130,146) and Arctic (50,131) ice communities spore formation was however rarely reported. Consequently, resting spores do not play an important role in winter survival of most ice protists except for specialists like dinoflagellates living in the upper part of Antarctic fast ice (148,149).

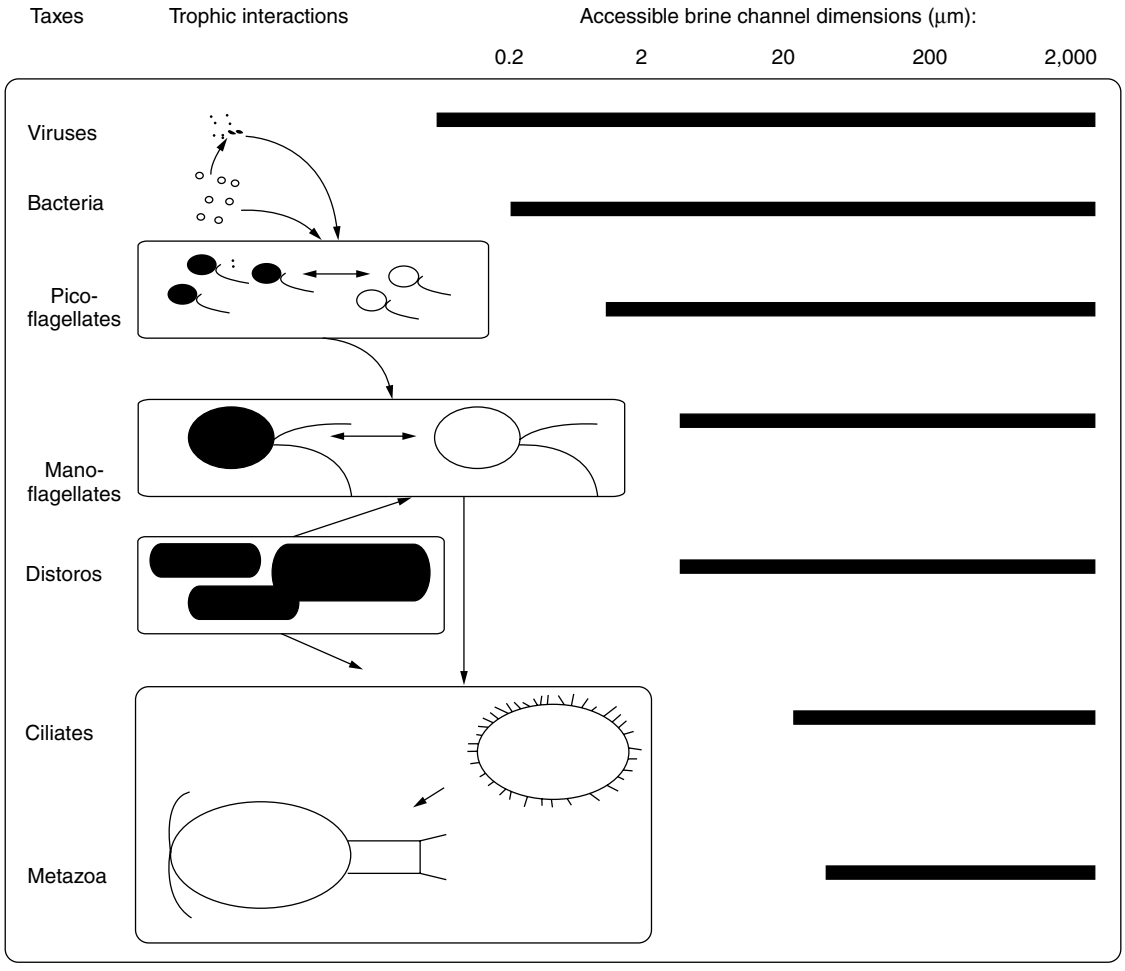

Figure 4. Schematic representation of the influence of brine channel diameter on the structure of the sympagic food web.

The high photoacclimation potential of polar phytoplankton and sea ice algal assemblages allows survival and rapid growth in low-light conditions (150–153). Chlorophyll specific algal production rates (P^{Chl}-ratios) of bottom ice algal communities from Arctic first-year pack ice ranged between 0.04 and 0.31 $\mu g\,C\,\mu g$ Chl $a^{-1}\,h^{-1}$ (93), similar to data from first-year bottom ice algae collected near Resolute (Table 2; 93,154). P^{Chl}-ratios of the interior communities in first-year segments are higher and ranged between 0.05 and 0.90 $\mu g\,C\,\mu g$ Chl $a^{-1}\,h^{-1}$. The P^{Chl}-ratios of 0.02 to 0.05 $\mu g\,C\,\mu g$ Chl $a^{-1}\,h^{-1}$ for bottom communities and 0.01 to 0.13 $\mu g\,C\,\mu g$ Chl $a^{-1}\,h^{-1}$ for interior communities of multi-year sea ice are typical for photosynthetic responses to very low irradiance measured in situ (0.4 to 3.3 $\mu mol\,m^{-2}\,s^{-1}$ (93)) compared with saturation intensities around 5 to 10 $\mu mol\,m^{-2}\,s^{-1}$ (23,130,150,155–157). Positive net photosynthesis of Arctic pack ice algae occurred even at a mean irradiance level of 0.36 ± 0.05 $\mu mol\,m^{-2}\,s^{-1}$ (93), which is well below threshold values from other field observations (1–9.3 $\mu mol\,m^{-2}\,s^{-1}$; (157–160)), but coincide with laboratory values of 0.2–2 $\mu mol\,m^{-2}\,s^{-1}$ (155,161).

Habitat Structure

The diameters of the brine channels span from few micrometers to several centimeters (17). The accessible pore space in sea ice plays a similar role for the geochemical cycling and biological interaction as shown for other marine and terrestrial habitats (177). At least pennate diatoms, many ice flagellates (e.g., *Anisonema* sp., *Thaumatomastix* spp., and *Protaspis* spp.), ciliates, turbellarians, and crustaceans have a behavioral preference for moving and feeding along the ice crystal surfaces, although a quantification of the surface area in relation to the concentration of attached or gliding biota is still missing. Many ice inhabiting organisms (e.g., bacteria, snow algae, diatoms, and metazoans) produce extracellular organic substances (32,48,83) and could alter the ice surface by coating it with transparent exopolymer particles (TEP) or via release of antifreeze chemicals. The spatial and temporal variability of brine channel geometry will cause changes in the structure of the sympagic food web (Fig. 4), excluding larger taxa from narrower brine channels. The brine volume itself is affected by changes of ice temperature and bulk salinity (19,20). Reduction of brine volume by, for example, cooling increases the concentrations of dissolved and particulate matter in the brine network, thereby exposing the biota to higher levels of nutrients, changes osmotic stress and increases grazing pressure as long as grazing is density-dependent.

GRAZING ON THE SEA ICE ALGAL BIOMASS

Precise measurements of ice algal production are difficult because of the type of habitat. Conservative estimates (178) calculated that about 20 to 25% of the entire primary production in polar seas is contributed by ice algae. A certain fraction of ice algal production is ingested by heterotrophic protists (179) and sea ice metazoans (180–182). Additionally, larvae of benthic animals (polychaetes and mollusks) migrate in shallow coastal areas into the brine channel network of first-year ice (183). The knowledge on the potential contribution of algal grazers to the carbon flux within polar sea ice is rudimentary and was partially ignored (184,185). Calculated grazing pressure by ice fauna (181) is by one order of magnitude lower than mean primary production estimates of 57 $mg\,C\,m^{-2}\,d^{-1}$ (70) and 83 $mg\,C\,m^{-2}\,d^{-1}$ (186) of Arctic sympagic algae.

CONCLUSION

Research over the last decades has considerably increased our understanding concerning the diversity, biomass, and activity of microorganisms in the unique sea ice environment. The strong linkages between physical and chemical properties and the seasonal development of the ice microbial communities were studied in detail. New endemic species of ice bacteria and protists were described, and others will be added. Focus of sea ice research will extend the current issues but also proceed to new topics. Many sea ice covered regions like the Canadian Basins in the Arctic and the western Weddell Sea in Antarctica have hardly been studied ever, offering opportunities for future exploration. Future investigations will focus on the ecophysiology of ice-associated organisms and their adaptations to the extreme conditions (e.g., the fluidity of membranes or the activity of enzymes at low temperatures). Additionally, effects of the shrinking ice extent on biological processes in Arctic waters will be an important issue over the next years to decades.

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SEDIMENTS.

See Denitrification in the Marine Environment; Fate of Viruses and Protozoan Parasites in Aquatic Sediments; Plant–Microbe Interactions in the Marine Environment; Seagrasses Communities

SEDIMENT SAMPLING.

See Sampling Techniques for Environmental Microbiology

SEDIMENTS AND PLFA ANALYSIS.

See Lipid Biomarkers in Environmental Microbiology

SEDIMENTS, ARCHAEA IN.

See Archaea in Marine Environments

SEDIMENTS, BIOREMEDIATION OF.

See Bioremediation: An Overview of How Microbiological Processes Can Be Applied to the Cleanup of Organic and Inorganic Environmental Pollutants

SEDIMENTS: NITRIFICATION IN.

See Nitrification in Aquatic Systems

SEDIMENTS, NITROGEN FIXATION IN.

See Nitrogen Fixation in the Marine Environment

SEDIMENTS: SULFATE REDUCTION IN MARINE SEDIMENTS

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Biological sulfate reduction is an important component of the sulfur and carbon cycles in marine sediments. Sulfate-reducing bacteria are anaerobes that are situated at the terminus of the anaerobic food web where they liberate inorganic carbon and sulfide as the primary end products of organic matter mineralization. Dissimilatory sulfate reduction may account for half or more of the total organic carbon mineralization in many sediments, especially those close to shore where sulfate reduction is the dominant anaerobic process (1,2). Oxygen is usually consumed rapidly near the surface in coastal sediments and alternate electron acceptors such as nitrate and metal oxides may be depleted within a few millimeters to centimeters of the surface as well. Because seawater is rich in sulfate, its use in marine sediments as a bacterial terminal electron acceptor during organic matter oxidation can occupy several vertical centimeters. It has been estimated that approximately 5×10^{12} g yr^{-1} of sulfate-(S) is reduced by bacteria globally with greater than 95% occurring in the ocean (3). Most of this sulfate reduction occurs in coastal regions that receive high inputs of organic material. Besides its importance in the degradation of organic compounds, the reduced sulfur produced during sulfate reduction is important geochemically, because it is highly reactive, and involved in the precipitation of highly insoluble metal sulfides and the accumulation of dissolved sulfide. The reduced products can be readily reoxidized under oxidizing conditions and can act as substrates for autotrophic bacteria, thus completing the sulfur cycle.

In this article, the focus is mainly on the role of sulfate reduction in the mineralization of organic matter, including its importance in both vegetated and unvegetated sediments and coastal marshes. In addition, characteristics of sulfate-reducing bacteria, their abundance and diversity, aspects of their biochemistry, interactions with other bacterial groups, and geochemical considerations are addressed.

BIOGEOCHEMICAL CYCLING

Anaerobic Food Web

The anaerobic bacterial food web is more complex than the aerobic web because many anaerobic systems require microbial consortia to supply intermediate compounds via fermentation reactions (Fig. 1). As will be discussed in more detail later, the primary role of sulfate-reducers

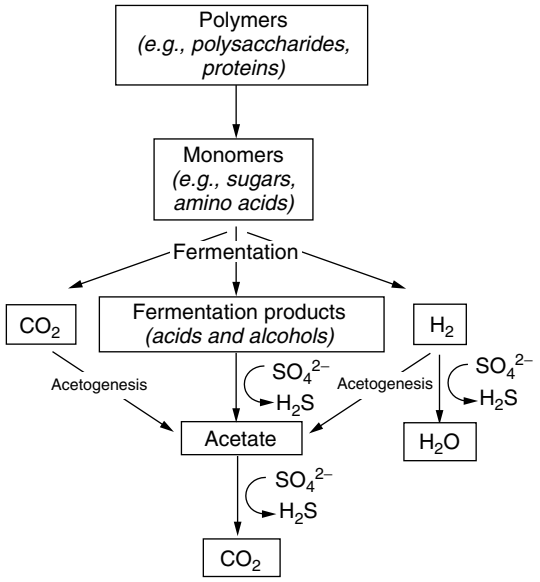

Figure 1. The anaerobic microbial food web displaying the role of sulfate reduction in the utilization of organic fermentation products and hydrogen and the production of acetate.

is to complete degradation by mineralizing relatively small organic substrates to carbon dioxide. Although the group as a whole contains a diverse array of metabolic capabilities, sulfate-reducers are not involved in polymer breakdown and few seem to be capable of degrading compounds more complicated than simple monomers or fermentation products.

Biogeochemical Zonation and Bacterial Competition

Rates of carbon mineralization in sediments generally decrease exponentially with depth and usually display a seasonal cycle that follows temperature changes. The pathway of decomposition depends primarily on which terminal electron acceptors are utilized, with oxygen utilization (aerobic respiration) occurring first, followed by nitrate, manganese, iron, sulfate, and carbon dioxide (methanogenesis). The order of electron acceptor use is governed by thermodynamics (the energy yield), substrate affinity (the ability of a physiological group to lower substrate concentrations to a level which cannot be used by other groups), and aspects of the biochemistry of the respective bacterial groups (e.g., anaerobes restricted to anoxic sediments). In most cases, the terminal processes in each zone are mutually exclusive, although overlaps between zones and the co-occurrence of processes have been noted in certain situations. As can be seen in Fig. 2, sulfate reduction is restricted to near the bottom of biogeochemical hierarchy. However, when organic loading is rapid and/or organic material is relatively labile, rapid use of electron acceptors results is thin vertical zones in which the sulfate-reducing region can be within millimeters to a few centimeters of the surface. Sulfate reduction can also occur within reduced microniches in oxidized sediments (4), especially when these niches are relatively large, that is, $<200\ \mu m$. Because seawater is rich in sulfate, the vertical extent of the sulfate-reduction zone can be several centimeters to tens of centimeters or more. Hence, sulfate reduction can be a dominant process in organic matter mineralization in marine sediments.

The most reliable method to determine the extent of the sulfate-reduction zone is to measure sulfate in sedimentary pore waters (Fig. 2). The vertical profile of sulfate is also an indication of the rate of sulfate reduction and the sedimentation rate; a steeper decline in sulfate signifying a more rapid rate of sulfate reduction (5). However, sulfate profiles can be greatly disrupted by the activities of burrowing benthic fauna or vascular plants; effects that will be addressed further in the following text.

The sulfate concentration in sediments affects sulfate reduction only when concentrations are quite low. The reduction of sulfate in marine sediments appears to be zero-order with respect to sulfate down to concentrations of about 2 mM (5,6). In freshwaters, sulfate concentrations must be much lower before they limit sulfate reduction. Because freshwater contains little sulfate compared with seawater, the importance of sulfate reduction in sediments increases in an estuary as the salinity increases (7). Therefore, the vertical extent of the sulfate-reduction zone increases substantially as more sulfate becomes available, whereas the methanogenic zone is "pushed" deeper into the sediment and its contribution to carbon mineralization decreases in importance.

The competition among terminal bacterial groups is easily demonstrated by amending sediments with alternate electron acceptors and observing changes in bacterial activities. For example, sediments receiving amendments of nitrate or freshly precipitated iron or manganese oxides display a rapid decrease in sulfate-reduction activity that is accompanied by an increase in nitrate reduction or metal oxide reduction, respectively. These changes in electron acceptor use are often accompanied by decreases in the concentrations of substrates used by both physiological groups, underscoring the ability of bacteria within vertically higher zones

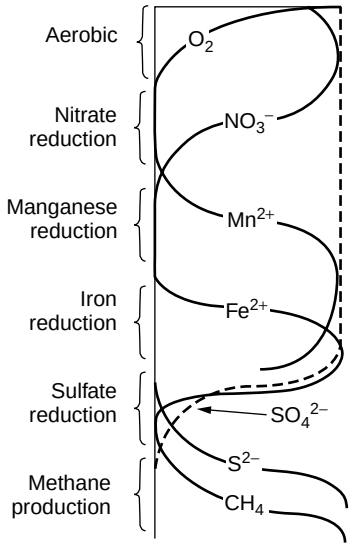

Figure 2. Vertical biogeochemical zones in sediments. Plots on the right represent the chemical profiles most widely used to delineate the vertical extent of each zone.

to lower substrate concentrations to levels below the minimum level required for other groups of bacteria (8,9). The minimum threshold concentrations of hydrogen for nitrate-reducing bacteria, for example, are several fold lower than those required for sulfate-reducers, which in turn are several fold lower than the minimum concentration that can be used by methanogens. Therefore, the terminal bacterial group in each biogeochemical zone can effectively preclude the growth of those from the deeper zone by maintaining low substrate concentrations. In the case of the sulfate-reducing bacteria, they are excluding methanogenic bacteria.

Sulfate Reduction versus Methanogenesis. Considerable controversy exists regarding the interaction between sulfate reduction and the methanogenesis in sediments. Geochemical evidence indicates that methane is consumed within the methanogenic zone suggesting that sulfate-reducing bacteria are capable of utilizing methane that diffuses from below (10,11). Radiotracer studies have shown that labeled methane is oxidized to carbon dioxide at the interface between the zones (12). Early work suggested that a sulfate-reducing isolate was able to oxidize methane, but this was not confirmed. It was suggested that methane oxidation at the interface between the methanogenic and sulfate-reduction zones may be because of a syntrophic relationship in which methanogenic bacteria act in reverse by converting methane to hydrogen and carbon dioxide, while sulfate-reducing bacteria consume hydrogen (13). Further evidence exists that a unique archaeal group is present at this interface (14,15) and biogeochemical and molecular data seem to indicate the presence of a phylogenetically distinct group of sulfate-reducers in this region as well (16).

Some electron donors are more readily metabolized by methanogens than sulfate-reducers and these "noncompetitive" substrates can allow methanogenesis to occur in the presence of active sulfate reduction (17). Examples of these types of substrates include C_1 compounds such as methylated amines, methylated sulfur compounds (e.g., dimethyl sulfide and methane thiol), and methanol. Methylated nitrogen and sulfur compounds are common degradation products of osmoregulating compounds found in certain marine algae and salt marsh grasses, and their use by methanogens partially explains the occurrence of significant concentrations of methane in sulfate–containing estuarine sediments and salt marsh sediments. In fact, the degradation of the osmoregulant glycine betaine in marine sediments produces acetate and trimethylamine, the former of which is consumed by sulfate-reducing bacteria whereas the latter is consumed methanogenically (Fig. 3; 18). Methanol is a degradation product of plant structural components such as pectin.

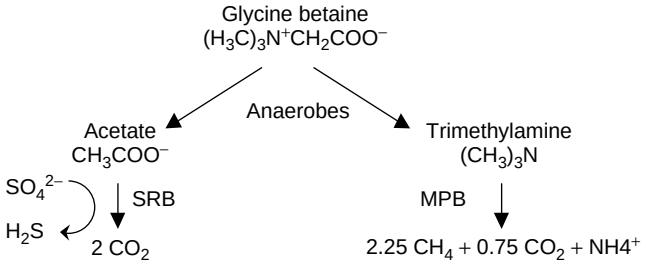

Figure 3. An example of the formation of a "noncompetitive substrate" (trimethylamine). Glycine betaine, an osmoregulant in marine organisms, is degraded to acetate and trimethylamine, which are consumed by sulfate-reducers and methanogens, respectively (18).

Geochemical Considerations

Sulfate reduction greatly influences the biogeochemical cycling of elements in sediments. The cycle of sulfur can be quite complex and involves several diverse groups of bacteria. The sulfide end product is highly reactive chemically and also serves as a significant electron donor for sulfur oxidizing bacteria. The oxidation of sulfide can produce intermediate redox phases of sulfur such as elemental sulfur (S^0) and thiosulfate ($S_2O_3^{2-}$) that can serve as both electron donors and acceptors for various bacteria. Sulfide is also a primary source of sulfur for assimilation by anaerobic bacteria such as methanogens. Sulfide is a strong reductant that easily reduces metals in sediments, and it rapidly precipitates metal ions as highly insoluble metal sulfides, the most notable being iron monosulfide (FeS) that imparts the black color to anoxic sediments. The cycling of iron is controlled to a large extent by sulfate reduction because sulfide can reduce iron oxides and precipitate iron (Fig. 4). The accumulation of precipitated metal sulfides and pyrite in marine sediments has produced a large reservoir of minerals over geologic time. In addition, large mineral deposits like the copper sulfide ores in Germany are thought to have formed from the reaction of bacterially produced sulfide with copper-rich solutions in marine microbial mats. Sulfate-reducers are also capable of producing magnetic minerals (19).

Sulfate-Reduction Activity

Knowledge of the role of sulfate reduction in sediments has increased greatly since the introduction of the use of ^{35}S as a tracer for determining sulfate-reduction rates (20). At first, rates were estimated from the incorporation of ^{35}S into dissolved sulfide (H_2S) and acid volatile sulfides (iron monosulfides, FeS), which were thought to be the only significant products. However, it was determined

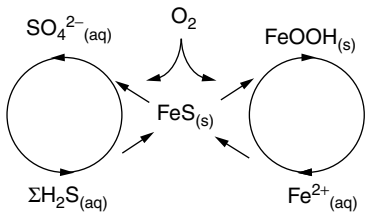

Figure 4. Interactions between the cycles of sulfur and iron in sediments. Processes moving from top to bottom represent reduction reactions whereas those moving from bottom to top are oxidation reactions. Iron monosulfide (FeS) is precipitated from sulfide and reduced iron, and FeS, Fe^{2+} and H_2S are able to be oxidized biologically and chemically. FeOOH reduction can occur biologically and chemically as well, but SO_4^{2-} reduction is only biological.

that in some habitats, such as salt marsh sediments and near the oxic/anoxic boundary, a significant fraction of the reduced sulfur produced during sulfate reduction is rapidly converted to pyrite (FeS_2), a compound that was previously thought to be produced at rates on the order of several years (21). Methods now routinely utilize a reduction step using reduced chromium under acidic conditions to incorporate all major reduced inorganic sulfur species that may form during sulfate reduction (22,23). Sulfate-reduction rates can also be determined from losses of sulfate in incubated sediments and from mathematical models that describe the loss of sulfate with sediment depth in terms of sedimentation and diffusion (24–26). Depth increases in reduced sulfur compounds and the abundance of sulfate-reducing bacteria are useful as comparative indicators of the sulfate-reduction process, but they are poor indicators of actual rates of activity.

The rate of organic matter input (i.e., sedimentation) and the availability of sulfate control the rate of sulfate reduction in sediments. Sulfate is rarely limiting in marine systems except in brackish estuarine waters and within depth in sediments in which sulfate has been depleted. Organic matter deposition is highest nearshore where primary production or sediment transport is high. Sulfate-reduction rates in sediments can span several orders of magnitude, but typical nearshore rates in the upper 5 to 10 cm are often approximately 50 to 500 nmol ml^{-1} day^{-1} (3). Rates in sediment with unusually rapid sedimentation rates can reach 2,000 nmol ml^{-1} day^{-1} (27). Sulfate reduction in salt marshes and microbial mats can reach rates as high as 4,000 and 14,000 nmol ml^{-1} day^{-1}, respectively (28,29).

Stoichiometry

The mineralization of nutrients from marine organic matter during sulfate reduction follows a stoichiometry similar to that present in typical marine organic material. In general, two moles of carbon are mineralized per mole of sulfate reduced. Because marine organic matter exhibits a C:N:P ratio of approximately 106:16:1 (30), the mineralization of nitrogen and phosphorus during sulfate reduction obeys this stoichiometry as follows (31):

$$(CH_2O)_{106}(NH_3)_{16}H_3PO_4 + 53\ SO_4^{2-} \longrightarrow 106\ CO_2 + 16\ NH_3 + H_3PO_4 + 53\ S^{2-} \quad (1)$$

Because sulfate reduction is a relatively inefficient process and the growth of sulfate-reducers per mole of substrate is low, their nitrogen and phosphorus demand is also low and ammonium and phosphate accumulate in sulfate-reducing sediments at ratios like those mentioned in the preceding text (32). Inorganic carbon produced during sulfate reduction is in the form of the bicarbonate ion ($HCO3^-$) rather than as carbon dioxide and this bicarbonate is a major contributor to the ability of sediments to neutralize acid. In addition, using the Richards stoichiometry (eq. 1), it is possible to use rates of sulfate reduction in marine sediments to determine the turnover or residence time of nitrogen and phosphorus in sediments and to predict the flux of nutrients into overlaying waters from sulfate-reducing sediments (33).

Seasonal Effects and Oxygen Consumption

The dissolved and solid phase products of sulfate reduction are responsible for consuming a significant portion of the oxygen that diffuses into sediments, and this oxygen sink can be equal to or greater than the amount of oxygen consumed by aerobic bacterial respiration (34). In typical coastal marine sediments, it is possible that 90% of the reduced sulfur produced during sulfate reduction each year is recycled back to sulfate (Fig. 5). The remaining sulfide is buried as a metal sulfide and eventually transformed into pyrite (FeS_2). The amount of energy that is acquired by bacteria during sulfate reduction is quite low compared with aerobic bacteria, in part because the sulfide end product retains a significant portion of the original energy. However, this sulfide can act as an energy source for other bacteria, especially photoautotrophic and chemoautotrophic bacteria in aerobic surface sediments.

Sulfate reduction activity varies greatly throughout the year, primarily in response to changes in temperature, and this seasonality affects the annual cycling of iron minerals and oxygen. During summer when microbial activities are rapid, alternate electron acceptors including oxygen are consumed near the sediment water interface and maximum rates of sulfate reduction may occur near the sediment surface (Fig. 6). During winter when microbial activity is slow, oxygen diffuses further into sediments and the sulfate-reduction maximum is not only slow, but situated deeper in the sediments (Fig. 6). In addition, any solid phase reduced sulfur (e.g., FeS) that accumulated in surficial sediments from sulfate reduction during summer is gradually oxidized in winter by the deepening front of oxygen. Therefore, a strong seasonal

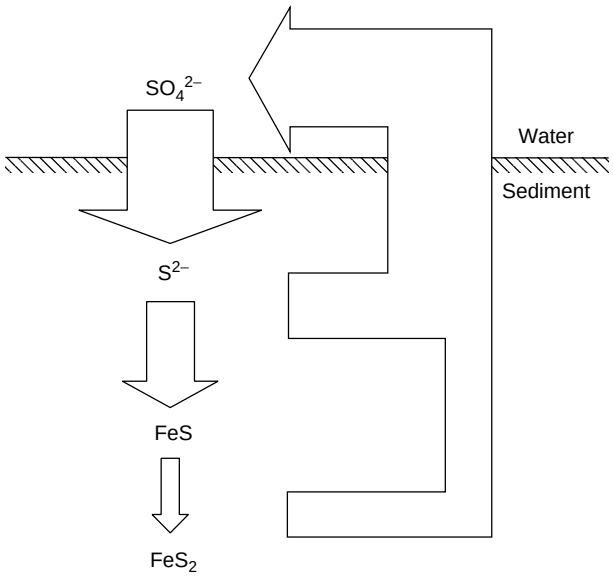

Figure 5. The recycling and reoxidation of reduced sulfur in marine sediments. The bulk of sulfur reduced is recycled back to SO_4^{2-}, including a large portion of the FeS. A small fraction of the FeS is converted to pyrite each year.

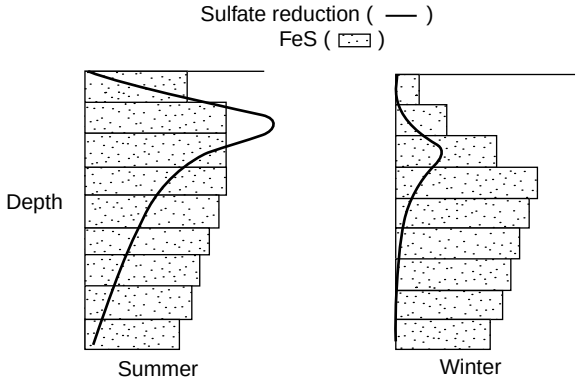

Figure 6. Vertical sulfate-reduction rates and concentrations of iron monosulfide (FeS) in hypothetical marine sediments in summer and winter. Rates in winter are slower with a deeper maximum, and FeS in winter is oxidized back to SO_4^{2-}.

cycle of inorganic sulfur is common in nearshore sediments with net accumulation in summer and net oxidation in winter (33,34). Seasonal sulfate-reduction activity is also influenced by changes in rates of organic matter inputs. For example, periodic plankton blooms are often followed by significant increases in sulfate-reduction activity as planktonic material is degraded. In addition, sulfate-reduction rates increase when the growth of seagrass or salt marsh grasses increase.

METABOLIC DIVERSITY

Unlike their aerobic counterparts, sulfate-reducing bacteria often require microbial consortia to supply appropriate substrates for energy and growth. It is generally accepted that sulfate-reducing bacteria oxidize products of fermentative bacteria such as fatty acids, alcohols, some aromatic acids, selected amino acids, and hydrogen. However, this list is incomplete and is continually growing as new metabolisms are discovered. Sulfate-reducers can also metabolize chemolithotrophically when utilizing hydrogen as an electron donor. Because most of the substrates used by sulfate-reducers are provided by a variety of other bacteria, these compounds represent rapidly recycled intermediates that accumulate quickly when sulfate-reducing bacteria are inhibited (35). Sulfate-reducing activity can be specifically inhibited through the use of sulfate analogs such as group VI oxyanions like molybdate and selenate. Amendments of these inhibitors to sediments results in the accumulation of volatile fatty acids and hydrogen and show that acetate is a primary electron donor utilized by sulfate-reducers (Fig. 7).

Although the reduction of SO_4^{2-} is considered to be the classic role of sulfate-reducing bacteria in the environment, these bacteria are capable of diverse metabolisms, including (*1*) nitrate/nitrite reduction; (*2*) iron and manganese reduction; (*3*) arsenate reduction; (*4*) metal methylation and demethylation; (*5*) organic fermentation; (*6*) use of xenobiotics and petroleum by-products; (*7*) sulfur disproportionation; (*8*) the utilization of various intermediate redox states of sulfur, and; (*9*) the production of intracellular magnetite. Oxygen reduction has been

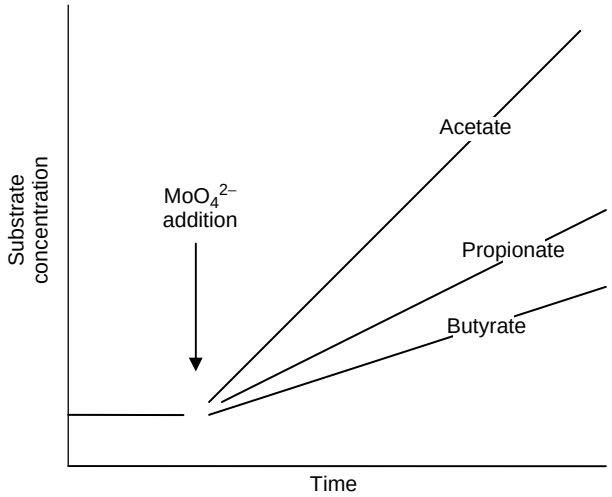

Figure 7. Use of a sulfate-reduction inhibitor (molybdate) to elucidate substrates used by sulfate-reducing bacteria. On inhibitor addition (arrow), those compounds that were being consumed during sulfate reduction accumulate at a rate equal to that at which they were consumed (35).

demonstrated under microaerophilic conditions and can be quite rapid (36). Oxygen-dependent growth has also been determined (37).

Physiologically, sulfate reduction is divided between complete and incomplete oxidation processes. Incomplete oxidizers utilize a variety of substrates, many of which are incompletely degraded to acetate. For example:

$$2\ \text{lactate} + SO_4^{2-} \rightarrow 2\ \text{acetate} + 2\ CO_2 + 2\ H_2O + S^{2-} \quad (2)$$

or

$$4\ \text{propionate} + 3\ SO_4^{2-} \rightarrow 4\ \text{acetate} + 4\ CO_2 + 2\ H_2O + 3\ S^{2-} \quad (3)$$

Some incomplete oxidizers are also capable of fermenting substrates without sulfate respiration, for example:

$$3\ \text{lactate} \rightarrow 2\ \text{propionate} + \text{acetate} + CO_2 \quad (4)$$

However, as will be pointed in the following text, some strains of sulfate-reducers are capable of fermentations that yield hydrogen (H_2) as one of the end products, but this H_2 must be removed quickly by a syntrophic partner. All sulfate-reducers isolated before 1977 were incomplete oxidizers including mostly members of the genus *Desulfovibrio*, the first of which was isolated more than 100 years ago (38). Although no isolates existed before the late seventies, it was postulated for many years that sulfate-reducing bacteria were capable of utilizing acetate as an electron donor.

The first acetate utilizer, and therefore complete oxidizing sulfate-reducer, isolated was a gram-positive sporulating bacterium of the genus *Desulfotomaculum* (39). Since then, several species of acetate-utilizing gram-negative bacteria have been recovered representing a suite of

metabolic capabilities and representing new genera. Complete oxidation entails the production of carbon dioxide as a major end product and includes the use of acetate, for example:

$$\text{acetate} + SO_4^{2-} \rightarrow 2\ CO_2 + H_2O + S^{2-} \quad (5)$$

Although physiologically distinct in many ways, the incomplete and complete oxidizing sulfate-reducing bacteria can coexist with the former supplying acetate to the latter in some instances.

Sulfate-reducing bacteria also can be involved in interspecies hydrogen transfer, in which hydrogen equivalents are transferred to other bacteria in lieu of sulfate respiration, for example:

Desulfovibrio

$$2\ \text{lactate} + 4\ H_2O \rightarrow 2\ \text{acetate} + 2\ CO_2 + 4\ H_2 \quad (6)$$

H_2-utilizing methanogen

$$CO_2 + 4\ H_2 \rightarrow CH_4 + 2\ H_2O \quad (7)$$

In essence, sulfate-reducers act as fermenting bacteria and the path to H_2 formation is favorable because of the maintenance of low H_2 partial pressure by the H_2-consuming methanogen. In addition, methanogenic bacteria can also use the acetate produced.

Disproportionation

During the past 15 years it was demonstrated that sulfate-reducing bacteria have the ability to conduct inorganic fermentation of sulfur compounds, known more correctly as sulfur disproportionation (40). Thiosulfate, sulfite, and elemental sulfur can be disproportionated and this ability has been found in many sulfate-reducing genera (41) and occurs as follows:

Thiosulfate

$$S_2O_3^{2-} + H_2O \rightarrow SO_4^{2-} + HS^- + H^+ \quad (8)$$

Elemental sulfur

$$4\ S^0 + 4\ H_2O \rightarrow SO_4^{2-} + 3\ HS^- + 5H^+ \quad (9)$$

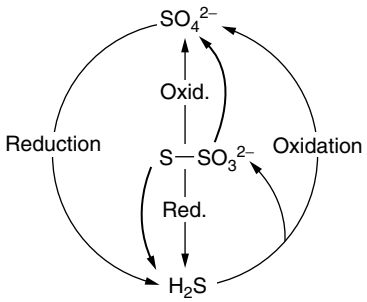

Figure 8. Thiosulfate shunt in marine sediments. Typical oxidation and reduction reactions are labeled. The heavy lines indicate the process of thiosulfate disproportionation (44).

Sulfite

$$4\ SO_3^{2-} + H^+ \rightarrow 3\ SO_4^{2-} + HS^- \quad (10)$$

Pure cultures have been isolated that are capable of all these reactions including an isolate that is an obligate disproportionator that does not reduce sulfate (42).

Disproportionation reactions are important in sediments because they provide a mechanism for anaerobic sulfur cycling (43). Thiosulfate appears to be a key component of the sulfur cycle in sediments because it can be oxidized, reduced, and disproportionated. Sulfate-reducing bacteria are key components of this cycling because they are capable of reducing sulfate and thiosulfate and they are able to disproportionate thiosulfate. A "thiosulfate shunt" seems to exist in sediments in which thiosulfate couples both reductive and oxidative pathways of the sulfur cycle [Fig. 8; (44]. Thiosulfate can be the main product of sulfide oxidation in reducing sediments, and much of this can be disproportionated with the remainder either oxidized to sulfate or reduced to sulfide. Hence, most of the sulfide produced during sulfate reduction can be ultimately oxidized to sulfate after passing through a thiosulfate intermediate. Elemental sulfur disproportionation is not energetically favorable under standard conditions. However, bacteria are able to grow via this process when metal oxides are present to rapidly remove sulfide (45). Although disproportionation is important in the sulfur cycle, it has been argued that it is not a major mode of metabolism in terms of bacterial energetics or growth (46). However, sulfide scavenging by sedimentary metal oxides may allow the process to act as a common metabolic process (42).

DIVERSITY OF SULFATE-REDUCING BACTERIA

Sulfate-reducing bacteria are a complex physiological group and classifying them has traditionally required the consideration of several properties, the most important of which are motility, cell shape, G + C content of DNA, the presence of desulfovirin and cytochromes, growth temperature, use of various electron donors, and the ability to conduct complete or incomplete oxidation of organic substrates. However, the recent analysis of rRNA sequences has allowed for a more thorough organization of the sulfate-reducing bacteria into four major groups: (*1*) gram-negative mesophiles; (*2*) gram-positive spore formers; (*3*) thermophilic *Bacteria* spp.; and (*4*) thermophilic *Archaea* spp. The latter two groups will not be addressed here. The gram-negative mesophilic group of sulfate-reducers is placed within the delta (δ) subdivision of the Proteobacteria and includes two families: the Desulfovibrionaceae and *Desulfobacteriaceae*. The *Desulfovibrionaceae* includes the genera *Desulfovibrio* and *Desulfomicrobium*, and this family appears to be rather phylogenetically distinct. The *Desulfobacteriaceae* family is much less distinct and includes several genera (perhaps > 20) including many of the complete oxidizing species (47). The gram-positive spore-forming sulfate-reducers constitute primarily members of the genus *Desulfotomaculum*.

Before the late 1970s, only two genera of sulfate-reducing bacteria were known, *Desulfovibrio* and *Desulfotomaculum* species. The desulfovibrios have received the most attention, primarily because they are relatively easily isolated from the environment and not difficult to maintain in laboratory culture. They were originally described as gram-negative sulfate-reducing bacteria that are curved rods, do not produce spores, and utilize primarily hydrogen and lactate as electron donors. However, the group has been expanded to include members that are capable of using several other electron donors and electron acceptors, the latter including nitrate, oxygen, and metal oxides. They are the classic examples of sulfate-reducers that conduct incomplete metabolism with acetate as an important end-product. Phylogenetic analyses have shown the desulfovibrios to be sufficiently diverse and distinct to warrant placement within their own family, the *Desulfovibrionaceae* (48). *Desulfovibrio* species are routinely isolated from marine sediments and probably are an important component of sulfate reduction in the sea.

Desulfotomaculum species are gram-positive, rod shaped, spore-forming sulfate-reducing bacteria that as a group display wide phylogenetic diversity. They are also quite phylogenetically distinct from all other sulfate-reducing bacteria. *Desulfotomaculum* species are not considered to be particularly important in marine sediments compared to the nonspore-forming sulfate-reducers. However, they are easily isolated from organic-rich sediments and they possess metabolic capabilities that are known to be important in sediments. Their ability to form spores does give them a competitive advantage in some habitats and they are found to dominate environments like rice paddy sediments that undergo wetting-drying cycles that could harm nonsporing cells. *Dtm. acetoxidans* was the first sulfate-reducer isolated that was capable of acetate oxidation.

Knowledge of the breadth of the sulfate-reducing bacteria has expanding greatly in the past 20 years. Many new lineages of sulfate-reducers have been isolated and described. The phylogeny of sulfate-reducers has expanded to include the family *Desulfobacteriacae* (49), which includes many new genera that are capable of complete and/or incomplete oxidation; the genera *Desulfobacter*, *Desulfobacterium*, and *Desulfococcus* to name a few. Members of the filamentous, gliding sulfate-reducing genus *Desulfonema* also appear to be common inhabitants of organic-rich sediments, especially within redox gradients (50). The recent explosion in information on sulfate-reducing bacteria clearly demonstrates the ability of the group to completely oxidize organic substrates including acetate, and their importance in decomposition processes in sediments. Sulfate-reducers as a whole are phylogenetically distinct from other bacteria, which have lead to the discovery of signature sequences of ribosomal subunit genes that have been used as oligonucleotide probes and PCR primers for the detection, determination of relative abundance, and microscopic visualization of members of the group.

Sulfate-Reducing Populations in Sediments

The population composition of sulfate-reducing bacteria has been investigated using culturing, biochemical, and genetic methods. In general, estimations of abundance using plating or most-probable number (MPN) methods are 10^2-10^5 ml^{-1}, which appear low when considered in terms of the rate of in situ sulfate reduction and culture estimates of rates per cell. This discrepancy is undoubtedly because of the inability of viable counting techniques to recover the majority of bacteria present. MPN techniques yield more realistic estimate of sulfate-reducer abundance (10^6-10^8 ml^{-1}) when applied to marine microbial mats (51) and salt march sediments (28).

Because of the complex nature of the anaerobic bacterial food web, it is generally thought that the sulfate-reducers account for only about 5% of the total bacteria present despite their important role at the end of the food web. In situ hybridization techniques that use fluorescent oligonucleotide probes to visualize individual cells of specific bacteria groups have yielded sulfate-reducing bacteria counts in marine sediments as high as 3×10^7 ml^{-1}, which represented up to 6% of the total *Bacteria* (52). Hybridizations of bulk sedimentary RNA with probes also demonstrated that about 1 to 6% of the total bacteria present in sediments were sulfate-reducing (53). Sulfate-reducers in salt marsh sediments can account for more than 30% of the total (eu)bacteria (28). This high percentage is probably due to the fact that marsh grasses exude organic substrates from roots that can be used directly by sulfate-reducers, thus circumventing the need for fermenting bacteria.

A wide variety of sulfate-reducing bacteria are found within sediments. Enumerations of bacteria using MPNs supplemented with specific substrates for sulfate-reducing groups have shown the presence of bacteria able to use many substrates including lactate, ethanol, acetate, malate, and propionate (54). Lactate and acetate utilizers often outnumber other groups illustrating the importance of both incomplete and complete oxidizing sulfate-reducers in sediments. Additions of various electron donors illustrate the importance of both general types of sulfate-reducers. For example, propionate amendments cause a temporary increase in acetate concentrations, whereas lactate amendments result in a temporary increase in propionate and acetate (55). Sulfate-reducing marine sediments also display an abundance of even chain bacterial phospholipid fatty acids indicative of the presence of acetate-utilizing bacteria of the genus *Desulfobacter* (55). Using MPN methods, acetate-utilizing sulfate-reducers were most abundant in a marine microbial mat (51), whereas ethanol utilizers greatly outnumbered acetate utilizers in salt marsh sediments (28).

Molecular analyses have furthered the description of sulfate-reducing groups in marine sediments. Both whole-cell in situ hybridizations and hybridizations using RNA extracted from sediments have been employed to investigate the diversity of sulfate-reducers. In addition, diversity has been investigated by sequencing rDNA clones and rDNA bands excised from denaturing gradient gel electrophoresis (DGGE). Incomplete oxidizing groups of sulfate-reducers seem to dominate in marine sediments,

primarily members of the *Desulfovibrionaceae*, but also *Desulfobulbus* species are abundant. However, the distribution of groups varies with depth and among habitats. For example, members of the *Desulfobacteriaceae* and *Desulfovibrionaceae* were equally abundant in the upper 2.0 cm of sediments, but the incomplete oxidizers (*Desulfovibrionaceae* species) dominated at depth (53). Complete oxidizers were essentially absent from Arctic Ocean sediments (56), yet they dominated sediments inhabited by salt marsh grasses (28). Complete oxidizers, that is, *Desulfobacteriaceae* species accounted for more than 20% of the total *Bacteria* RNA in some instances in the marsh, whereas *Desulfovibrionaceae* species were a small fraction in all cases. Members of the *Desulfobacteriaceae* are metabolically diverse and may be well-suited for environments that undergo widely changing seasonal redox changes like those encountered in salt marsh sediments and at the sediment surface. The presence high numbers of *Desulfobulbus* species in marsh sediments (28) may reflect the ability of members of the genus to conduct sulfur disproportionation reactions (see the following text), which would be stimulated by plant-mediated redox cycling within the rhizosphere and the production of intermediate redox states of sulfur.

Many desulfovibrios are capable of aerobic respiration, are able to withstand several hours of full aeration, and are common inhabitants of oxic regions of microbial mats (57). Their rapid motility allows them to position themselves in the microaerophilic gradient where they can benefit from organic compounds released by photosynthetic bacteria. The complete oxidizing gliding sulfate-reducer *Desulfonema* is a common inhabitant of the oxic–anoxic interface in microbial mats (50,51,58), and is also abundant within the partially oxygenated rhizosphere of salt marsh plants (59). Filamentous morphology, aggregate formation, and diurnal migration are all adaptations that allow *Desulfonema* species to thrive in the rapidly changing conditions of a microbial mat. Although, conflicting results are apparent, it is clear that sulfate-reducing bacteria populations in marine depositional environments are highly dynamic, metabolically versatile, and important contributors to elemental cycling.

INTERACTIONS WITH MACROORGANISMS

Plants and animals inhabit many sedimentary sites, and their presence greatly affects the distribution and activity of sulfate-reducing bacteria. Although the physiology of plants differs greatly from that of animals, the ultimate effect of their presence is similar such that each is capable of introducing organic matter and oxidants into sulfate-reducing regions. Hence, macroorganisms are responsible for stimulating subsurface redox cycling of elements. Figure 9 is an example of how a salt marsh plant and a deposit-feeding polychaete cause such a subsurface cycle with the plant introducing dissolved organic carbon and oxygen, while the worm introduces particulate organics and iron oxide as the oxidant. Although both organisms stimulate the cycle of sulfur and iron, the plant causes dissolved iron to cycle more rapidly than the dissolved sulfide, whereas the worm causes the opposite to occur.

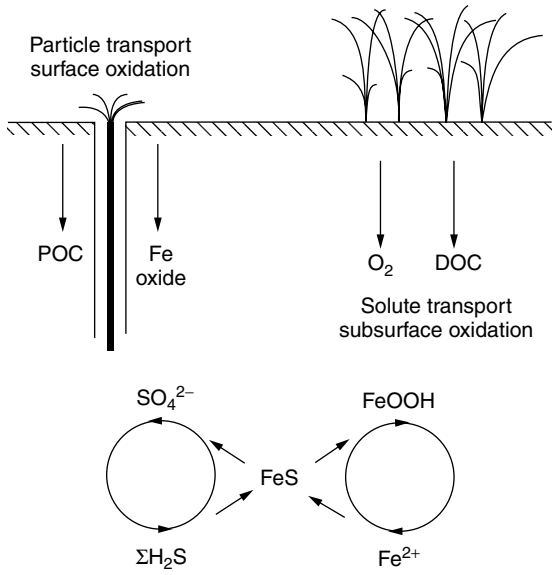

Figure 9. Effect of a deposit-feeding worm (left) and rooted aquatic plants (right) on the sulfur and iron cycles in nearshore marine sediments. Worms transport particulate carbon (POC) and sold phase iron oxides to depth by depositing fecal pellets at the surface. Plants transport oxygen and dissolved organic carbon (DOC) to depth by exuding these materials directly from roots (60).

Salt Marshes

Sulfate reduction is a key component of bacterial metabolism in marine sediments inhabited by vascular plants including salt marsh grasses that inhabit the intertidal zone and submerged sea grasses. Sulfate reduction in marsh sediments can account for most of the decomposition that occurs (61,62). Sulfate-reduction rates are among the highest ever measured in marine sediments and populations of sulfate-reducers are abundant. Vascular plants not only provide particulate organic material to sediments, but they also alter sediments by releasing dissolved organic materials and oxygen from roots. The release of root exudates fuels microbial activity belowground whereas oxygen release stimulates subsurface redox cycling of elements and provides a redox gradient capable of supporting an active bacterial community.

Spartina alterniflora, the common cordgrass prevalent along much of the East Coast of the United States, releases dissolved organic matter and oxygen from roots only when plants are elongating aboveground. Exudation ceases on flowering in midsummer. Sulfate-reduction activity responds to this release by increasing rapidly when exudation begins in the spring and decreasing abruptly at the onset of reproductive growth (62). Roots washed free of sediment support an active sulfate-reducing community that responds to these changes in plant growth stage. For example, the relative abundance of *Desulfobacteriaceae* species increases drastically when plants are growing aboveground, which may be because of the ability of this family to thrive and compete under conditions of rapid changes in redox conditions (28,59). As mentioned previously, members of the *Desulfovibrionaceae* represent only a small portion of the sulfate-reducing community

in these marsh sediments. *Spartina alterniflora* releases ethanol from roots during anaerobic metabolism and it appears that *Desulfobulbus* species, which are abundant in the rhizosphere, consume this material rather than *Desulfovibrionaceae* species

Sea Grasses

The sea grass rhizosphere also supports a sulfate-reducing community that is much more active than in unvegetated sediments nearby. In some plant species, rhizosphere sulfate reduction displays a diel cycle in which activity increases during the day because of photosynthesis-driven exudation of organic matter from roots (63). Similar to salt marshes, many sulfate-reducers are attached to seagrass roots, and these communities are unusually tolerant to oxygen exposure (64). Sulfate-reducing bacteria have been identified as the key group involved in nitrogen fixation in the rhizosphere and they may be responsible for satisfying a significant portion of the plant nitrogen demand (65,66). Hence, sulfate-reducers can have a mutualistic symbiosis with marine plants. Although sulfate-reducers are rather loosely attached to seagrass roots, active *Desulfovibrio* species have been found within roots and even deep within root cortex cells (67), which suggests a highly developed mutualism.

Benthic Fauna

Sedimentary sulfate reduction is also influenced by the presence of burrowing macrofauna. Benthic worms and bivalves pump oxygenated water into reducing sediments and fauna introduce organic matter to depth by reworking and turning over surficial sediments and by stimulating organic accumulation in burrows. Sediments inhabited by reworking fauna support rapid rates of sulfate reduction throughout the reworking zone whereas uninhabited sediments display rapid decreases in rates with depth (33). Sulfate-reducers degrade organic matter originally destined for aerobic decomposition at the sediment surface after it is prematurely buried by infauna. These effects are also influenced by the seasonality of animal activity in which sulfate-reduction activity increases sharply when infaunal reworking activity commences in late spring (68). Walls of oxygenated burrows have steep redox gradients that support complete elemental cycles within the burrow wall, and sulfate reduction is an important component of this cycling (69).

CONCLUSION

Sulfate reduction is the most important anaerobic bacterial process in nearshore marine sediments in terms of the mineralization of carbon and nutrients, the support of autotrophic processes near the sediment surface through recycled sulfur, and the formation of sedimentary minerals. The abundance of sulfate in seawater allows sulfate reduction to occur over wide depth-intervals in sediments and sulfate reduction consumes more oxygen than aerobic bacteria in may instances. During the last 20 years, our knowledge of the phylogenetic and physiological diversity of sulfate-reducing bacteria has increased dramatically and has elucidated the wide impact of these bacteria on marine deposits. Although sulfate-reducers that inhabit marine sediments generally fall into one of three phylogenetic families, the metabolic diversity within each family is great, and it is highly likely that many new members with even more amazing capabilities will be discovered soon. Sulfate-reducers are key players at redox boundaries, are capable of migrating within these boundaries, and demonstrate an ability to interact syntrophically with other aerobic and anaerobic bacteria. They are also important contributors to sediments inhabited by plants and animals and they have acquired the ability to live symbiotically within tissues. As molecular, microelectrode, and high technology methods continue to advance, this important group of bacteria will certainly be shown to be even more important in the biogeochemistry of marine sediments.

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S GASES.

See TRACE GASES SOIL

S-LAYER, ARCHAEAL.

See ARCHAEA IN BIOTECHNOLOGY

SEPTIC TANK SYSTEMS

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Septic tank systems are the most popular type of small-scale processes for on-site treatment of domestic wastewater in unsewered urban and rural areas, and can provide a practicable and cost-effective solution to wastewater treatment in these situations. In the United States, for example, some 24% of households are connected to tank systems (1). In England and Wales only 4% of properties are unsewered, but this still amounts to more than 750,000 septic tanks in use (2).

Septic tank systems consist of a tank and a drainage field. The tank is a watertight, underground vessel that provides conditions suitable for the settlement, storage, and partial decomposition of sludge (settled waste solids). Raw wastewater is fed to the tank from the dwelling served and the settled wastewater is discharged to the drainage field through the tank outlet. Sludge accumulates at the bottom of the tank and has to be removed periodically.

Three zones are normally present in the septic tank:

1. a scum layer that forms a crust on the surface of the tank liquor,

Table 1. Advantages and Disadvantages of Septic Tank Systems

Advantages	Disadvantages
Low capital cost	High space requirement
Low energy requirement	Tank effluent is unsuitable for open discharge
Low maintenance	Potential for local nuisance and health hazard
Potential for groundwater recharge	Potential for groundwater pollution
Low sludge production	Requires some owner education and participation

2. the wastewater from which solids are deposited, and
3. the bottom sludge layer of deposited material.

The organic matter in the tank may undergo anaerobic digestion, and the extent of stabilization depends on the size of the tank, frequency of sludge removal, and temperature.

The drainage field functions both as an effluent-disposal facility and a treatment zone (see section "Drain Field"), typically consisting of a system of subsurface irrigation pipes or a soakaway, which drains the effluent into the surrounding soil. Proper design and installation of both the tank and drainage field are essential for the efficient operation of the system as a method of wastewater treatment.

The main advantages and disadvantages of septic tank systems for on-site treatment of wastewater are summarized in Table 1.

DESIGN

Tank Size

The main function of a septic tank is to provide primary treatment by separating and retaining solids from the incoming flow. Tanks should be designed to maximize retention, because high levels of solids in the outlet flow block soil pores and reduce the life of the drainage field. The principal factors to be considered in deciding on tank capacity are

- average daily flow of wastewater,
- adequate hydraulic retention time, and
- adequate sludge- and scum-storage space.

The average daily flow of wastewater to the tank depends on the average water consumption and the number of people served. Per capita water consumption depends on a number of factors including climate, demography, and socioeconomic levels (3,4).

An appropriate period of time is required for the retention of wastewater in the tank to allow the settlement of heavier solids and flotation of lighter material, such as fats and grease, present in the wastewater. Hydraulic retention times (HRT) recommended by the United States Public Health Service, for example, range from 12 to 24 hours (5).

The size of tank should provide sufficient capacity for solids capture, and space is also needed for solids retention. The amount of storage required depends on the planned frequency of sludge removal from the tank and the expected rate of anaerobic decomposition of the sludge.

Sludge (septage) is usually removed from the tank at predetermined intervals ranging from six months to three years. Longer desludging intervals are appropriate in warm climates because of the greater extent of sludge stabilization and volatile solids (VS) loss that occurs at high ambient temperatures. Typically, cleaning is necessary when sludge and scum occupy more than one quarter of the tank volume or exceed approximately 500-mm depth (6).

As an example, these two design requirements are brought together in the relevant British Standard (7). The minimum total capacity of a septic tank, where desludging is carried out at not more than 12-monthly intervals, is given by:

$$C = 180P + 2{,}000 \quad (1)$$

where

C = capacity of the tank (liters)
P = the design population with a minimum of 4

The value of 180 in Equation (1) is based on the required storage capacity for wastewater and solids and provides for proportionately higher retention times at lower populations to allow for surges in flow expected from small systems (8).

Tank Configuration

Septic tanks can be of various shapes, the most common being rectangular, square, and circular in plan (Fig. 1). Single- or two-chamber tanks are in common use. Where two-chamber tanks are used, it is recommended that the main sludge-retention chamber should contain at least two-thirds of the total tank capacity (7).

The design and location of inlets, outlets, and inter-compartment connections can have considerable influence on tank operation (9). The main design requirement is to minimize turbulence as the wastewater enters the tank to avoid the disturbance of settled sludge. Short-circuiting can be minimized by providing small baffle walls at the tank inlet and outlet.

Tanks are ventilated to allow digestion gases to escape. This is usually by the house drain vent stack, provided the incoming house drain is not trapped before entry to the tank. Alternatively, ventilation may be provided at the

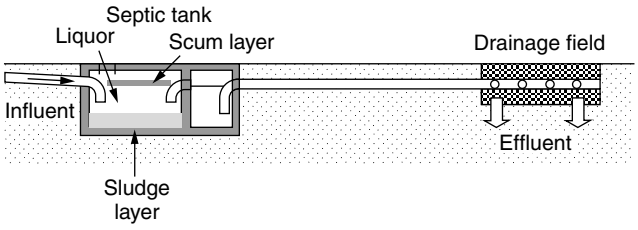

Figure 1. Typical septic tank system.

tank itself, either by special vent pipes or by ventilating manhole covers.

Prefabricated septic tanks are widely available constructed from glass fiber, thermoplastics or precast concrete sections. The main design requirements are that they must be watertight, structurally sound, and durable. Inspection manholes reach up to ground level and precautions are normally taken to prevent the entrance of storm runoff into the tank.

Tank Location

A septic tank is located so as to permit straightforward collection of wastewater from the dwelling and discharge to the drainage field. Standard "setback" distances have been commonly applied to various structures or natural features to minimize potential problems from leakage, particularly around the inlet and outlet pipes (Table 2).

Risk assessment and geostatistical investigations by the U.S. EPA (11) suggest that septic tank systems should be located at distances of 80–325 m away from abstraction wells, depending on aquifer and source characteristics, based on allowing natural inactivation of viruses to take place (i.e., an 11-order magnitude reduction in virus concentrations in groundwater compared with the source). However, the U.S. EPA does not currently advocate a specific setback or reliance on natural viral inactivation to protect groundwater sources. Current proposals are for on-site inspections for all groundwater systems supplemented by microbial monitoring at priority sites to identify high-risk facilities that are required to take corrective action. This aspect will be further explored in the section on pathogen removal and potential health implications.

Size of the Drain Field

The effluent of a properly designed and efficient septic tank is slightly turbid, owing to finely divided solids in suspension, and has a relatively low biochemical oxygen demand (BOD). However, it remains offensive and malodorous and contains almost no dissolved oxygen. In addition, the effluent is potentially harmful to health, because it may contain enteric pathogenic bacteria and viruses, as well as cysts and eggs of parasitic protozoa and helminths that pass through the tank (3). It is not normally suitable for direct discharge to a watercourse or other open channels or for irrigation of crops. A secondary-treatment stage is required to remove much of the dissolved polluting matter and the disease-causing agents, and this is usually provided by the drainage field. The objectives of the drainage field are thus to

- absorb the tank effluent,
- provide aerobic treatment to the effluent, and
- protect water resources and prevent nuisance.

A number of drainage field options are available and the selection and design will depend particularly on the outcome of a site assessment, based on factors such as (2)

- subsoil (nature, depth, percolation value),
- location of water table and watercourses,
- site (size, location, slope),
- adjacent habitats,
- surrounding topography, and
- number and effectiveness of other septic tank systems in the area.

Table 2. Setback Requirements for Septic Tanks and Drainage Fields (10)

Minimum Distance from (m)	Septic Tank	Drainage Field
Buildings	1.5	3.0
Property boundaries	1.5	1.5
Wells	30	30
Streams	7.5	30
Embankments	7.5	30
Pools	3.0	7.5
Water pipes	3.0	3.0
Paths	1.5	1.5
Large trees	3.0	3.0

Subsurface Irrigation

Subsurface irrigation systems are the preferred method of field drainage in most situations. They consist of a network of perforated pipes laid in trenches filled with gravel (12). They allow the effluent to be widely distributed through a large volume of soil and minimize the risk of soil saturation. The pipes are laid with a slight gradient (typically 1 in 200) at a depth not exceeding 750 mm. A typical subsurface irrigation system is shown in Figure 1. The drainage field is located downslope of the tank and property, and away from stormwater soakaways, wells, and the site boundary (Table 2).

There are three key aspects to consider in the rational design of subsurface irrigation systems (13):

- *Soil Hydraulic Characteristics*. The permeability of the receiving soil will influence the size of irrigation system required and this can be measured on site. For low-permeability soils (coefficient of permeability $<5 \times 10^{-5}$ cm s^{-1}), this may be the limiting factor in design.
- *Biological Mat*. The permeability of biological mat (biocrust) that forms at the trench–soil interface may become limiting in permeable soil. Higher loading rate systems can be designed by increasing the HRT in the septic tank to improve solids/BOD removal.
- *Protecting Groundwater Quality*. The principal factor is the type of subsurface soil and distance to the water table from the subsurface irrigation system.

Water-infiltration rate after partial clogging determines the length of pipe required for subsurface irrigation. For practical purposes the infiltration rate is regarded as being broadly similar for most soil types and is conservatively taken as 10 l m^{-2} d^{-1} (13). The required length of trench, L, can be estimated from the following equation:

$$L = \frac{Pw}{2di} \qquad (2)$$

where

P = design population
w = water used (l capita^{-1} d^{-1})
d = effective depth (m)
i = infiltration rate (l m^{-2} d^{-1})

A factor of two is used in the denominator of Equation (2) to account for the dispersion of effluent on both sides of the trench. The effective depth is measured from the base of the trench to the water table. The design can be adapted to local site and soil conditions using information obtained from in situ percolation tests (14,15).

Subsurface irrigation is not appropriate for sites with low soil permeability, high groundwater conditions, shallow depth to bedrock, or excessive land slope (16).

Soakaways

A soakaway is a point drainage field consisting of a gravel-filled pit excavated to a depth of 2 to 5 m and with a diameter of 1 to 2.5 m. The soakaway should be as large as practicable, and never smaller than the tank itself (16). Soakaways take up less space than subsurface irrigation systems, but distribute the effluent less efficiently and are only appropriate in areas of low groundwater level.

Other Methods

Alternative "ground-based" options for effluent treatment are possible on sites with high groundwater or low-permeability soils. One approach, for example, is a mound system in which tank effluent is pumped into a gravel bed or trench over a sandy soil mound. The wastewater is treated as it percolates into the indigenous soil (5).

Evapotranspiration systems utilize rapid-growing grass species to reduce the water content of the effluent by transpiration to the atmosphere. Effluent is distributed in open joint pipes below the evapotranspiration bed, which is constructed from a layer of gravel, 200–500-mm deep, underlying topsoil (100 mm) and is planted with an appropriate high biomass production grass species. A retaining earth bank is usually constructed around the bed. Nutrients supplied in the effluent act as a fertilizer and are taken up into the plant biomass that is periodically cut and removed. A disadvantage with evapotranspiration systems is that they require intensive management input to operate efficiently (5).

OPERATION

Tank Performance

Treatment Efficiency. The five-day biochemical oxygen demand (BOD_5) and total suspended solids (TSS) removal efficiencies of septic tanks in temperate climates are typically in the range 25 to 45% and 50 to 70%, respectively (17–19). These are similar to values usually achieved by plain sedimentation. Approximately 20% of the total nitrogen (TN) and 30% of the total phosphorus (TP) is removed in the retained sludge (20). Septic tank effluent typically contains 75 mg/l TSS, 140 mg l^{-1} BOD_5, 300 mg l^{-1} chemical oxygen demand (COD), 40 mg l^{-1} TN, and 15 mg l^{-1} TP (21), although larger TN concentrations of 100 mg l^{-1} have also been reported (22). The N and P contained in the effluent are predominantly in soluble inorganic forms.

A comparison of septic tank installations showing high and low rates of sludge accumulation (23), reflecting the effectiveness of anaerobic digestion in the tank, shows that TS, VS, COD, and concentrations of volatile fatty acids are significantly greater in sludge from systems with high sludge production and low rates of microbial activity (Table 3). Accumulation rates greater than 0.225 l capita^{-1} d (82 l capita^{-1} y) may be indicative of septic tank failure in terms of microbial activity and organic matter stabilization.

The major processes influencing pathogen removal from wastewater in septic tanks are settlement of solids and hydraulic retention time. Bacteria and viruses attached to the solids settle to the sludge layer, and eggs of helminths and protozoa will also settle if sufficiently dense (24). Pathogens that do not settle to the sludge layer remain suspended and will pass out of the septic tank in the effluent. For these organisms, hydraulic retention time is the key parameter that controls the loss of viability because many pathogens are susceptible to environmental stress outside the host environment and naturally decay

Table 3. Physicochemical Properties of Sludge from Septic Tanks with Low or High Sludge Accumulation Rates (23)

Parameter	Low Rate (<0.18 l capita^{-1} d^{-1})	High Rate (>0.225 l capita^{-1} d^{-1})
pH	6.7	6.6
Eh (mV)	−310	−339
TS (g l^{-1})	37	46
VS (g l^{-1})	26	32
CH_4 (ml l^{-1} d^{-1})	26	16
COD (mg l^{-1})	2502	3873
Acetic (mg l^{-1})	194	340
Propionic (mg l^{-1})	300	547
Isobutyric (mg l^{-1})	22	46
Butyric (mg l^{-1})	42	36
Isovaleric (mg l^{-1})	45	92
Valeric (mg l^{-1})	15	24

with time. Septic tanks, by virtue of their limited retention capacity and operation at ambient temperature, do not significantly stabilize the sludge and provide little or no reduction in the infectivity of enteric pathogens or parasites discharged to them (24). The accumulation of sludge in the tank reduces retention times and the benefit of this operational feature in pathogen elimination is severely compromised when desludging is not carried out on a regular basis or when the tank capacity is inadequate.

The maximum removal achieved by septic tanks is of the order of a 2-log decrease in the pathogen content of the effluent compared with the influent and, as a consequence, significant concentrations of all the pathogen types remain. Typical decreases in fecal coliform (FC) bacteria of about 1 to 2 times 106 FC 100 ml^{-1} are usually observed in efficient septic tank systems (25). However, 7 to 27 times 106 FC 100 ml^{-1} were detected in the effluent from overloaded septic tanks in Cyprus, whereas untreated household wastewater contained approximately 30 times 106 FC 100 ml^{-1} (26). These data emphasize the potentially significant passage of enteric pathogens through septic tanks that are inappropriately designed and managed in terms of capacity and desludging regime. Other research (27) shows that the numbers of indicator bacteria may increase as wastewater passes through the tank. Viruses are not significantly removed by settlement in the tank. However, viral counts and numbers of other enteric pathogens in tank effluents are usually high only if infections have occurred (21).

Hydraulic Retention Time (HRT). HRT is the volume of the available space between the settled sludge and scum divided by the design flow rate. This is a key design parameter influencing tank performance and the extent of wastewater stabilization and pathogen removal. For example, TSS removal was increased by a further 28% in a study in which the HRT was extended from 20 to 35 hours (28). An increase in HRT from 22.5 to 90 hours resulted in increases of BOD and TSS removal from 60 and 53% to 85 and 91%, respectively, as well as small increases in nutrient removal (29).

Shape and Compartmentalization. Tank shape influences the velocity of the wastewater through the septic tank, the depth of sludge accumulation, and the possible development of stagnant zones. If the tank is too deep, then the reduction in surface area lowers the efficiency of sedimentation. Conversely, a tank that is too shallow has inadequate space available for sludge and scum storage (30). However, shape has relatively little effect on overall treatment efficiency for tanks of comparable volume. For example, a comparison of circular and rectangular tanks showed that both forms achieved similar average BOD_5 removals of 43 and 47%, respectively (31). Generally, a rectangular shape is considered to be the most efficient form of single-compartment tank with an optimum length that is three times the width and the recommended depth of the tank is in the range 1.2–1.8 m (31).

A two-compartment arrangement may improve the removal of BOD and suspended solids compared with a single compartment of similar total capacity (6,14), due principally to the capture in the second compartment of solids that pass through the first compartment. No additional improvement in performance is gained from more than two compartments.

Effect of Temperature. Ambient temperature conditions have an important effect on the performance and biological processes within the tank including sludge-accumulation rate, which is indicative of the extent of microbial activity and stabilization in a septic tank (23). Increasing temperature has little overall effect on TSS, but significantly improves BOD_5 removal, and a high degree of wastewater stabilization is possible in warm climates. For example, BOD_5 and TSS removal efficiencies in excess of 80% were obtained in a septic tank system in Bombay, India (32). In northeastern Brazil, an overall retention time of 11 h was sufficient to remove 67 and 78% of the influent BOD_5 and SS, respectively, entering a communal septic tank (33). This level of performance is comparable to that achieved by anaerobic ponds in warm climates, which effectively remove 60 to 80% of influent BOD_5 and TSS, respectively (34,35). Little reduction in BOD_5 occurs below 10 °C in anaerobic ponds, but, as temperature increases the BOD_5 is typically reduced by 40 to 60% at 20 °C and by more than 80% at $\geq$25 °C (36).

Anaerobic digestion of organic matter proceeds slowly at temperatures below 15 °C (Table 4). The production of methane gas (CH_4), which is indicative of active anaerobic digestion, usually only occurs at temperatures of $\geq$15 °C (17) and the observed increase in sludge VS content also reflects a reduction in the extent of microbial degradation and stabilization of organic matter as ambient temperatures decline (Table 5). Consequently, appreciable solids accumulation occurs in cool climates and must be accommodated by the provision of greater tank capacities. In cold climates twice the tank volume is required compared with regions where wastewater temperatures are atleast 12 to 15 °C (37) for sludge storage. The HRT also becomes more critical as ambient temperatures decline (38) and longer retention times are required at low temperatures to achieve even a modest-level organic matter stabilization by anaerobic digestion processes.

Sludge (Septage) Production. The volume of sludge (or septage) produced in a septic tank depends on the following criteria:

- concentration of suspended solids in the influent,

Table 4. Effect of Temperature on Anaerobic Treatment (37)

Temperature (°C)	[a]Rate of Methane Formation Relative to That at 35 °C	[a]Retention Time Required for Treatment Relative to That at 35 °C (days)
5	0.1	10.0
15	0.4	2.5
25	0.8	1.2

[a]35 °C = 1.0.

Table 5. Effect of Septic Tank Liquid Temperature on Sludge Volatile Solids (VS) Content (33)

Tank Liquid Temperature (°C)	VS in Sludge (%)
0.6	79.0
4.4	77.4
15.0	74.6
26.0	52.7
28.5	37–47

- concentration of suspended solids in the effluent,
- extent to which the solids are liquefied or converted into gas, and
- moisture content of the sludge after differing periods of retention.

However, these factors are potentially highly variable so that sludge-accumulation rates are difficult to predict in practice. In the United States, sludge-accumulation rates are typically in the range 0.82–1.6 l capita^{-1} d^{-1} (300–600 l capita^{-1} y^{-1}) (3) and in Europe a value of 0.25 l capita^{-1} d^{-1} (93 l capita^{-1} y^{-1}) is reported (39). However, much lower accumulation rates are possible in warmer climates because of the greater extent of sludge stabilization. In Zambia, for example, sludge-accumulation rates are as low as 0.08 to 0.11 l capita^{-1} d^{-1} (30–40 l capita^{-1} y^{-1}) and an average value reported for South Africa is 0.09 l capita^{-1} d^{-1} (32 l capita^{-1} y^{-1}) (40).

Septage represents a mixture of aged solids combined with fresh material and contains potentially significant numbers of pathogenic bacteria, viruses, protozoal cysts, and helminth eggs that settle from the liquid phase (3). It is only partially stabilized, is potentially malodorous, and is 50 times stronger than municipal wastewater in relation to its BOD_5 and COD content (41). Therefore, sludge from septic tanks presents a significant microbiological and environmental risk and requires careful and responsible disposal. Septage may be disposed of by discharging to wastewater treatment works, where conventional wastewater and sludge-treatment processes reduce the risk of infectivity and the residual biosolids are disposed of by landfilling, incineration, or are reused on land as a fertilizer-replacement and soil-conditioning product. Aerobic digestion of septage and mesophilic anaerobic codigestion with landfill leachate also has potential in mitigating environmental problems caused by the disposal of poorly treated septic tank sludges (42,43). However, the sludge may be recycled directly on land, where it is managed in the same way as untreated urban wastewater biosolids by the provision of barriers to disease transmission and minimization of odor nuisance. These measures have a well-founded scientific basis (44) and include incorporation of the sludge into the soil by cultivation immediately after application, or subsurface injection, coupled with restrictions on the sowing, harvesting, and types of crops that can be grown (45,46). There are no reported cases of disease outbreak where these practices have been followed for untreated sludge (47). Nevertheless, in some countries, direct land application of untreated wastewater sludge, including the sludge from septic tanks, is not considered favorably because of uncertainty over the potential risks of disease transmission.

Drainage Field Performance

Biocrust/Biological Mat. The biological mat or biocrust that develops at the trench–soil interface penetrates the soil to a limited extent but it forms predominantly near the soil surface to a thickness of 5–30 mm (48). The crust consists of captured wastewater solids, mineral precipitates, and a high concentration of microorganisms consisting mainly of aerobic bacteria but also some protozoa and nematodes. The extent of the crust layer is also linked with the accumulation of microbial polysaccharides (49). A mature crust is regarded as an essential feature for efficient biological treatment and is also potentially an effective barrier to bacteria and viruses (3,41)

Treatment Efficiency. The efficiency of wastewater treatment by the drainage field is often assessed in terms of the improvements in water-quality parameters achieved between the tank outlet relative to various depths in the soil profile below the drainage field. For example, BOD, COD, and TSS removals of 75 to 95%, a decrease in ammonium-N content of 80 to 90%, and reductions in phosphate concentration of 25 to 50% were recorded at a depth of 1.5 m for a drainage field on sandy clay soil and treatment efficiency increased with the depth of unsaturated soil (50). The degree of bacterial removal is also strongly influenced by the extent of soil saturation (41) (the transport and attenuation of microbial pathogens in septic tank systems are discussed in more detail later). In another example (51), more than 90% removal of BOD and COD and more than 99% removal of coliform bacteria were reported for septic tank effluent flowing through 2 m of chalk and, again, treatment efficiency was shown to increase with depth (51).

Operational Problems

Septic tank systems fail to operate efficiently owing to a number of common faults. The problems are mainly associated with backing-up or surface-flooding with wastewater, and with pollution of surface or ground waters. Most operational problems are related to poor location or lack of maintenance (2). Inadequate design and installation are also important factors exacerbating the poor performance of septic tank systems.

In North America, septic systems contribute the largest volume and most numerous sources of all effluents discharged to groundwater, representing approximately 20 million potential point sources of groundwater pollution (52). During the period 1971 to 1978, overflow or seepage of wastewater primarily from septic tanks or cesspools was responsible for 41% of the outbreaks of disease and 665 of the illnesses caused by contaminated untreated groundwater in the United States (53). This aspect is discussed again later in the chapter.

PROCESS MICROBIOLOGY

As discussed, there are two physically separate steps in the treatment of wastewater by septic tank systems including

1. a primary stage for the settlement of solids accompanied by varying degrees of anaerobic digestion in the tank and
2. a secondary stage consisting (normally) of aerobic oxidation and (occasionally) anoxic denitrification in the drain field/soil.

Both stages have important roles in the attenuation and transformation of pollutants and for reducing enteric pathogens and parasites. The stabilization of organic matter and transformations of soluble mineral N, which is the principal inorganic environmental contaminant of concern in domestic wastewater, are mediated by microbial processes in the tank and drain field. These processes have been well researched and are summarized below. The biogeochemical evolution of domestic wastewater in conventional on-site septic tank systems can be described by simple, microbially catalyzed redox reactions involving organic C and N in wastewater in three different redox zones in the system (52). Anaerobic digestion of organic matter and production of CO_2, CH_4, and NH_4^+ predominate in the first zone (tank). In the second zone, gaseous diffusion through the unsaturated soil of the drain field supplies O_2 for aerobic oxidation of organic C and NH_4^+. It is also possible for a third redox zone to occur in which NO_3^- is reduced to N_2 gas by anaerobic denitrification. More details of the specific microbial activities involved are outlined in the following text.

Tank

Retained solids in a septic tank are digested anaerobically by bacteria reducing the volume of accumulated sludge. The dissolved and suspended colloidal organic components of the wastewater are also degraded by microbial action. Together, these processes remove the settleable matter from the wastewater and reduce its BOD. Anaerobic bacteria have slow growth rates so that although a small proportion of the BOD is removed by conversion to cellular material, the contribution to sludge production is minimal and the predominant mechanism of biodegradation is fermentation to CH_4 (54).

Drain Field

Sites of Microbial Activity and Mineralization. The secondary treatment of septic tank effluent is based on microbial activity in the biocrust and the upper layers of soil in the drainage field. Soluble and colloidal organic compounds and simple inorganic nutrient salts, particularly ammoniacal N and phosphate compounds including detergent residues, are applied to the soil in the effluent. Biodegradable organic constituents are mineralized by many types of aerobic soil bacteria to simple end products. Nitrogen in septic tank effluent is typically in the ratio 80% NH_4–N and 20% organic N and there is virtually no NO_3–N present (55). Inorganic NH_4–N ions are liberated during the mineralization of organically bound N in the septic tank and drainage field (56).

Importance of Gas Exchange for Microbial Activity. Biochemical nutrient transformations occur within the top 500 mm of soil in the drainage field where diffusion processes maintain adequate gas exchange and the oxygen status of the soil atmosphere to sustain aerobic biological activities (52). If O_2 supply is limiting in the drainage field, however, aerobic mineralization and nitrification processes are arrested and the accumulation of organic matter carried by the effluent will block the soil pores causing failure of the septic system. Soil porosity controls permeability and gas exchange processes and is critical for the correct biological functioning of the drainage field. A continuum of air-filled pore space is essential for effective gas diffusion and this requires an air-filled porosity of approximately 10% in soil (52). Soil types with the textural characteristics similar to fine-grained sand provide the optimum physical environment for maximizing gas exchange in the drainage field. By contrast, impervious or clay soils are unsuitable as a drainage field for purification of septic tank effluents (5).

Nitrification. Active nitrification (57) processes have been recorded in drainage fields with an average atmospheric composition of 19.6% O_2, some 5 cm from the clogged or crust zone (55). In sandy soils, however, nitrification of NH_4–N to NO_3–N is essentially complete within about 2 cm of the biocrust (55). Nitrification activity is most prevalent in the zone of aeration between the drainage field and the capillary zone, but is suppressed by anaerobic conditions where groundwater is near the base of the field. Under these circumstances, NH_4^+ can leach to the groundwater, although concentrations decline with distance from the drain system as a result of absorption to soil colloids (3,55).

Nitrate formation by NH_4^+ oxidation in the drainage field and leaching to groundwater is one of the primary impacts of septic systems at most sites and is generally an unavoidable consequence of the proper functioning of conventional septic systems (52,58). Estimated average N-loadings to the drainage field and inputs to groundwater in septic tank effluent are in the range 4–8 kg N capita^{-1} y^{-1} (22,59). Complete nitrification in the soil may contribute approximately 33 kg NO_3–N to the groundwater per year for an average family of four persons (59). Consequently there is a significant risk of NO_3^- concentrations exceeding drinking water standards in the effluent plume generated from septic tank systems (60). Nitrate may be transported significant distances from the site before physical dilution and dispersion processes reduce concentrations in groundwater to a level that may be acceptable for potable abstraction. The potential environmental significance and extent of N-loadings from septic tanks have been demonstrated in a modeling study of N-flows in a 360-ha mixed land use catchment in north-central Connecticut (61). In that example, septic tanks represented 25% of the N exported from the catchment.

Denitrification. Denitrification reduces NO_3^- principally to N_2 gas under anoxic soil conditions and is performed by a range of bacteria (57) that are commonly found in the subsurface environment. Organic carbon is usually the principal electron donor in denitrification reactions and 70–75% of the NO_3^- lost from a septic tank plume in an unconfined medium sand aquifer was due to carbon oxidation (62). The remainder was associated with sulfide oxidation, which also plays an important role. Oxygen is the preferred electron acceptor to NO_3^- in metabolism and oxic conditions are thought to inhibit denitrification activity. However, denitrification increases when gas exchange to the atmosphere is restricted and may be observed in alluvial soil as the air-filled porosity decreases below 11–14% (63). In practice, denitrification is rarely observed in the drainage field of septic tank systems because the availability of organic carbon (OC) is limited because of microbial mineralization processes. The reported cases of significant denitrification activity are associated with certain localized and site-specific factors and can be generally regarded as exceptional rather than the rule. For example, NO_3^- attenuation from septic systems by heterotrophic denitrification occurs in certain aquifers (62,64) with elevated OC concentrations (0.15% by weight; the OC content is more usually approximately 0.03% by weight). Denitrification is also possible where hydrogeological conditions in the drainage field cause partial anaerobiosis of the soil. In one case (65), more than 80% of the NO_3^- in recharge from a drainage field was lost by denitrification caused by a fluctuating water table and relatively warm soil temperatures (18 °C).

Phosphate. The extensive use of phosphate builders in detergent products in industrialized countries means that significant amounts of polyphosphate are transferred to the wastewater from clothes' washing and bathing. Nevertheless, approximately half the P-loading of septic tank effluents is derived from urine and feces (22). Typically, the P-loading to the drainage field in septic tank effluent is 0.6 kg capita^{-1} y^{-1}. The hydrolysis of polyphosphate compounds to potentially labile orthophosphate is enhanced by bacterial activity in soil and residual organic forms of P present in septic tank effluent are also mineralized to this inorganic form. However, P in septic tank effluent is almost entirely present as orthophosphate (22). Conventional opinion has been that orthophosphate ions are bound in immobile minerals in soil and little significance was attached to the potential consequences of P-accumulation. However, this view has changed in the light of recent research demonstrating that soils eventually reach P-saturation, causing leaching losses that contribute to diffuse pollution of surface waters and subsequent eutrophication (66). Soils attenuate septic system phosphate through mineral-precipitation reactions (67), but this capacity also becomes saturated with time, allowing phosphate to advance at a slow but potentially significant rate (60). Phosphate migration in the saturated zone appears to be controlled by chemical adsorption processes that allow slow but progressive movement. Consequently, the gradual accumulation of P in the vadose zone of the drainage field and the mobility of the phosphate plume in groundwater indicate that septic tank systems can be significant contributors of phosphate to nearby surface waters with potentially long-term implications for surface water eutrophication.

Biodegradation of Household Chemicals

Detergents, solvent cleaners, disinfectants, and other types of chemical products are widely used in households in developed, industrialized countries and are discharged in the wastewater. The fate and attenuation of household chemicals in septic tank systems have important implications for the environment and in particular for groundwater contamination particularly in countries such as the United States, where both household chemicals and septic tanks are commonly used (1). Chemicals present in laundry detergents represent the largest proportion of substances discharged in wastewater from households.

Studies of the chemical fate and transport of detergent residues in septic tank systems emphasize the importance of biodegradation and chemical-sorption processes in attenuating these household chemicals. Some biodegradation is possible in the septic tank itself, although the main process is associated with sorption onto the settling solids and partitioning into the sludge that is retained in the tank (68). Organic chemicals can be categorized according to three principal types of behavior when applied to soil in waste (69):

1. volatile compounds that are quickly lost to the atmosphere,
2. compounds that are rapidly mineralized by microorganisms and have little or no persistence, and
3. persistent compounds that are strongly adsorbed onto organic matter in waste and soil.

The main types of household chemicals fall within the first two categories. The use of persistent potentially toxic organic compounds has largely been phased out and they are not likely to be significant constituents of modern household products.

The fate and attenuation of detergent residues is of primary interest in assessing the potential environmental implications of domestic wastewater disposal by septic tanks. For example, linear alkyl benzene sulfonate (LAS) and nitrilotracetric acid (NTA) are important by-products from detergent use, but these chemicals are quickly biodegraded with mineralization half-lives in soil and groundwater of 9 to 17 days and 1 to 3 days, respectively (70). The maximum average LAS concentration measured in domestic septic tank effluents was 6.5 mg/l (22). Degradation rates are most rapid in soil of the drainage field and proceed at a slower rate in soil sampled further away from the discharge, emphasizing the importance of acclimation by the microbial community to the types of chemical present. Properly functioning septic tank systems are effective at removing synthetic organic chemicals from domestic wastewater and this is principally achieved through biodegradation processes (70). However, physico-chemical attenuation may also play a role. For example, the sorption of LAS by the soil matrix is controlled by a hydrophobic

mechanism and is enhanced with increasing organic matter and clay contents in soil (71).

Bleaching agents and organic solvents are potentially more potent environmental chemicals than those originating from detergents and are also discharged to septic tanks. The processes influencing the attenuation and transport of dichlorobenzene (DCB) in a septic tank groundwater plume have been examined by injecting the chemical and a tracer (NaBr) into the septic system (72). The tracer arrived at the groundwater table after 10 days, whereas DCB arrived after 57 days, indicating significant attenuation and retardation during migration through the aerobic unsaturated zone at the test site. There was evidence of significant biodegradation of DCB and the estimated half-life was 15 days. Increased attenuation was observed after a 7-day lag period for biological acclimation, further substantiating the importance of biodegradation processes.

The formation of adsorbable organic halid (AOX) from the use of bleaching agents containing sodium hypochlorite contributes significantly to the discharge of these potentially toxic organic compounds in domestic wastewater. Adsorbable organo-halogens may be removed in the septic tank by sorption onto the sludge solids and biodegradation when there are only background traces of AOX present in the domestic wastewater (0.1–0.2 mg l^{-1}) (73). However, significant removal rates of 87–94% are also reported (73) when AOX concentrations are elevated from the use of sodium hypochlorite in bleached laundry water. Approximately 30% of the generated AOX may be removed in the tank and the remainder is attenuated by the drainage field. Adsorbable organo-halogen compounds have minimal persistence and do not accumulate in the sludge or in the soil, and are rapidly degraded in the septic system. Biodegradation is probably the main mechanism of attenuation because the performance of the drainage field at removing AOX is enhanced by the use of hypochlorite, triggering the possible microbial adaptation to the presence of AOX.

These examples illustrate that household chemicals are usually effectively removed by properly functioning septic tank systems by sorption and biodegradation processes in the tank and drainage field. There are no reported examples in the scientific literature in which significant groundwater contamination has occurred with anthropogenic organic chemicals originating from domestic septic tank systems. The attenuation processes operating within the septic system and the intrinsic biodegradability of household chemicals minimize the potential risk to groundwater.

Pathogen Removal and Potential Health Implications

Septic tank effluents are unlikely to achieve the microbiological quality required for re-use as irrigation water (74) and are unsuitable for this purpose without further treatment. It is also inadvisable to grow vegetables or other food crops on or adjacent to the drainage field because of the risk of infection from consuming contaminated produce. The closure of water and nutrient cycles by reusing domestic sewage is advocated for reasons of community sustainability, but this depends on providing appropriate posttreatment for the removal of pathogens (75–77). Under most circumstances the effluent is not recycled and disposal to a drainage field is the usual method of treating the effluent to inactivate the pathogen load. The processes responsible for pathogen removal in the drainage field include death rate, flow rate, filtration, adsorption, microbial competition and antagonism, moisture, pH, sunlight, and temperature (78,79).

With proper hydraulic functioning of the drainage field, the populations of total coliforms, fecal coliforms, and enterococci are reduced to background levels within relatively short distances (60 cm) below the percolation trench in unsaturated soil (79). The most abrupt reductions in bacterial populations occur in the biocrust at the interface of the drainage field trench and the soil. The biocrust zone is only several centimeters in thickness but is effective in trapping and retaining bacteria. It is critical to the purifying action of the septic tank drainage field and represents the primary barrier to subsurface escape of fecal microorganisms (79). A soil-transition zone is usually observed from a high bacterial population in the biocrust region, that may be equal to or greater than the levels found in the effluent, to reduced populations in deeper zones equivalent to uncontaminated soil. Usually, 30–90 cm of permeable soil beneath the base of the drain field trench is adequate for complete bacterial removal and a more restrictive region is necessary to form the clogged biocrust zone. This is in line with the recommended practice for the installation of septic tank systems in the United States that require 120 to 150 cm of "suitable" soil as an adequate zone for the protection of groundwater (79). However, the transition zone may not effectively retain all pathogen types. In particular, owing to their small size, viruses can travel significant distances from the drainage field into the subsurface rock (51).

There are many reports of environmental problems caused by the contamination of ground and surface waters with enteric pathogens from septic tank installations. The potential risk of disease transmission from potable or recreational use of water contaminated by septic tank systems is a major concern in many different regions of the world (80–85).

The contamination of water used for drinking from private supplies is often the main cause of human exposure to enteric pathogens originating from septic tanks (81). Bacteria can migrate long distances of up to 600 m in certain aquifer types such as sandy subsurface rock, placing nearby potable abstractions potentially at risk (86). In a cohort study of 181 rural families (87) the numbers of *Escherichia coli* indicator bacteria in well water were significantly associated with gastrointestinal illness as a direct consequence of groundwater contamination with enteric pathogens from septic tanks. Increasing the distance between the septic tank system (i.e., the drainage field) and abstraction well above 20 m significantly reduced the likelihood and extent of contamination with the fecal indicator bacteria. Although these results provide assurance that risks to health can be mitigated by adopting appropriate setback distances, bacteria are poor indicators of other enteric diseases, including viruses (84) or parasites, such as Giardia oocysts (82). To assess the potential for infections

from these organisms requires the direct quantification of protozoan oocysts and the isolation of an appropriate indicator virus. A recent investigation (84) of the survival and transport of an enterovirus indicator and fecal coliform bacteria from septic systems located on sandy soil types showed that the main issues relating to viral and bacterial contamination of groundwater from septic tanks were that

- system design is less important than soil properties and the depth of the vadose zone,
- under suboptimal conditions, septic tank systems on sandy soils with seasonally high water tables can cause groundwater contamination with enteric pathogens,
- risks of viral contamination are highest in coarse textured (sand) soils when water tables are shallow (e.g., small vadose zone or saturated soil conditions) and in winter when temperatures are low, and
- large reductions in enteric pathogens are possible if the clay content of the drainage field is at least 15%, if the vadose zone is at least 1 m deep and if the drainage field distribution lines do not become submerged in the groundwater.

Although retention of enteric microorganisms by drainage fields may be effective in unsaturated soil conditions, a common fault is the siting of tank installations on land that has a shallow water table or where the soil may be seasonally waterlogged. Under saturated flow conditions, transport of microorganisms is markedly increased compared to unsaturated flow. In particular, the small size and surface electrical properties of viruses facilitate transport over potentially long distances by saturated flow and migrations of 1,000 to 1,600 m in channeled limestones, and 250 to 410 m in glacial silt-sand aquifers have been recorded (11). For example, enteroviruses were detected in a shallow, sandy soil aquifer receiving tank effluent (87), and poliovirus or bacterial phage tracers were also isolated in the vicinity of a septic tank (88). However, other research (89), using coliphages as indicators of pathogenic human viruses, showed that coliphage concentrations in groundwater in an unconfined sand and gravel aquifer were close to the limit of detection (1 coliphage per 1,000 l) at a typical setback distance of 30.5 m.

The oocysts of parasitic protozoa, Cryptosporidium and Giardia are more than 100 times larger than viruses, which restricts their movement by intergranular flow. Oocysts of these parasites are rarely detected in groundwater. However, their occasional presence (90) indicates that removal by filtration in the unsaturated and saturated zones may not necessarily guarantee the protection of water quality from contamination with these types of organism, particularly in fractured or channeled aquifer systems or in very coarse gravel aquifers.

PERFORMANCE ENHANCEMENT

The problems associated with the discharge of pollutants from septic tank systems and the subsequent contamination of ground and surface water resources' nutrients has prompted the development of a variety of methods that improve performance to mitigate the environmental impacts. Most of the alternative approaches or enhancements are microbiologically mediated, as discussed in the following sections.

Seeding

The benefits of seeding an active population of anaerobic bacteria are well known for enhancing the treatment efficiency of anaerobic wastewater stabilization ponds and improving effluent quality (34). For example, consistently higher BOD_5 removals are observed from the enhanced seeding of supernatant in ponds that retain a layer (e.g., 400 mm) of aged (2 y) sludge compared to ponds where all the sludge is periodically removed (34). The provision of an inoculum source of anaerobic bacteria to accelerate the establishment of effective anaerobic digestion can also markedly improve the efficiency of septic tank systems for wastewater stabilization. This is done by collecting sludge from an existing tank, with an established anaerobic microbial population, and adding this material to a new installation or by leaving a small amount of sludge (e.g., equivalent to 20% of the tank volume (8) behind in the tank after routine desludging operations.

Microbial Additives

There is a growing market in products that aim at improving the extent of biodegradation in septic tanks and reduce sludge accumulation, odor, and drainage field blockage. These products are usually in the form of dried bacterial cultures and may also include enzymes. The general consensus, however, is that such products are ineffective (15). Additives based on hydrolytic yeasts, enzymes, and bacteria are ineffective because the rate-limiting step in sludge stabilization in septic tanks is usually CH_4 formation (23). The microorganisms responsible for biotransformation and stabilization processes in septic tanks and the drain field are ubiquitous and well adapted to the environment, and inoculated bacteria are usually ineffective competitors compared to indigenous groups. However, there is some evidence supporting the use of additives to improve biodegradation efficiency of septic tank systems (23). For example, a formulation of "mineral insoluble additives" stimulated the different stages of anaerobic digestion and reduced sludge production in septic tanks by as much as 60%, reducing the frequency of desludging up to 15 to 20 years (23). There are also claims that additive formulations could be developed to ultimately reduce the amount of sludge produced by as much as 90%.

External Organic Carbon Substrate

The introduction of a carbon source to support heterotrophic denitrification in the drainage field as solid phase substrate or by liquid injection is highly effective for NO_3^- removal. In the injection system, for example, methanol is introduced at the base of a specially constructed drainage field above an impermeable layer to intercept and denitrify the NO_3–N (91). Demonstration

trials showed that this technique significantly reduced the inorganic N-content of drainage to 0.1 mg N l^{-1} within 1 m of travel through the impermeable pan beneath the drain field from an initial value of 41 mg N l^{-1} (as NH_4^+) in the effluent (91). An arguably more innovative approach is to install porous barriers constructed from reactive solid organic carbon material (sawdust) to intercept water flow within the drainage field for passive in situ NO_3^- attenuation (92). Reactive porous media barriers in field trials have achieved significant attenuation of NO_3–N of 60–100% of input concentrations up to 125 mg N l^{-1}.

CONCLUSION

Septic tank systems provide a relatively simple and practicable method of treating wastewater from dwellings in unsewered, remote, or rural locations, and they can be effective in reducing the risk of enteric disease transmission when properly functioning. The basic engineering principles for designing a suitable tank are well defined and there is also general guidance for constructing the drainage field. However, it is the operation of the drainage field where most potential environmental problems arise because of the wide variability in soil conditions and subsurface hydrogeology that affects system performance. A conflict emerges between the basic requirement for a porous soil structure to facilitate aerobic biodegradation and nitrification processes, whereas pathogen retention is more effective in fine-textured soils.

Nitrate production is an inevitable consequence of a correctly operating septic system and this is probably the most significant contaminant of groundwater impacted by septic tank discharge. Biological denitrification is rarely present in drainage fields. Phosphate is also a major constituent of septic tank effluent and presents a potential risk of surface water eutrophication. However, P is usually effectively retained in the drainage field by chemical precipitation reactions in most types of soil. Common household chemicals rapidly biodegrade in the drainage field and there is no evidence for their accumulation in soil or sludge.

Very little pathogen removal occurs during sedimentation of solids in the septic tank and the majority of the pathogen load is eliminated from the wastewater by the drainage field. The occurrence of pathogenic organisms in the wastewater depends on the extent of infections in the individuals using the septic system. Although the drainage field can be effective at reducing pathogens, there are many examples in which this barrier breaks down and significant groundwater contamination is reported with important implications for human health.

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SEQUENCING BATCH BIOFILM REACTOR (SBBR).

See ACTIVATED SLUDGE — SEQUENCING BATCH REACTORS

SEQUENCING BATCH REACTOR (SBR) TECHNOLOGY.

See ACTIVATED SLUDGE — SEQUENCING BATCH REACTORS

SHELF STORAGE OF BOTTLED WATER.

See BOTTLED WATER, MICROBIOLOGY OF

SHELLFISH, SALMONELLA IN.

See *SALMONELLA* IN AQUATIC ENVIRONMENTS

SHIGELLA

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Shigella are gram-negative, non-spore-forming, facultative anaerobic bacilli closely related biochemically and antigenically to *Escherichia coli*. Most fail to ferment lactose or to produce gas when fermenting glucose, and all are nonmotile (1). They cause a disease called dysentery (bacillary dysentery or shigellosis), an infection of the large bowel characterized by abdominal cramps, diarrhea, and fever. Initially, the diarrhea may be copious and the liquid stools often contain blood and mucous. It is sometimes accompanied by other symptoms such as vomiting and headache, and the infection rarely involves other parts of the body (2).

Shigella spp. are usually acquired by drinking water that is contaminated with human feces or by eating food washed with contaminated water. The organisms invade the cells lining the colonic mucosa and multiply there, killing the cell; this is the cause of the symptoms produced. However, it occasionally invades the bowel beyond the surface lining. At least one species, *Shigella dysenteriae*, also secretes a toxin that most likely plays a role in tissue destruction and more serious systemic disease.

Shigella spp. continue to have an important global impact, causing an estimated one million deaths and 163 million cases of dysentery annually. The organisms have demonstrated extraordinary competence for acquiring plasmid-encoded multiantibiotic resistance previously used as first-line therapy (4). This finding, in addition to the low infectivity and potential complications with complex or often unexplained pathogenesis, have led several laboratories to try to understand the pathogenesis of shigellosis, with the aim of developing a vaccine against the disease.

Complete overviews on the pathogenesis, epidemiology, clinical significance, detection, and diagnosis of *Shigella* strains have been published elsewhere (3,5–8). This article focuses on the recent progress made to understand the genetic and molecular basis of shigellosis.

THE GENUS *SHIGELLA*

There are four different species of *Shigella*, divided on the basis of differences in O-antigen of their lipopolysaccharide and differences in some biochemical reactions. These are named *Shigella dysenteriae* (13 serotypes), *Shigella flexneri* (15 serotypes), *Shigella boydii* (18 serotypes), and *Shigella sonnei* (1 serotype) (1). In general, *S. dysenteriae* accounts for deadly epidemics in developing countries, *S. flexneri* and *S. sonnei* are responsible for endemic disease, the former being prevalent in the developing world, the latter in developed countries, and *S. boydii* accounts for most cases of infection in India and neighboring countries. From these species, the most extensively studied is *S. flexneri*.

OVERVIEW OF PATHOGENESIS

The fundamental event in the pathogenesis of *Shigella* is the ability to invade and colonize the human intestinal epithelium. This triggers an intense acute inflammatory response with infiltration by polymorphonuclear leukocytes. The pathogenesis of *Shigella* is a multistep process that depends on the capacity of the bacteria to cross the colonic mucosa via M cells associated with gastrointestinal-associated lymphoid tissue (GALT). The bacteria then invade epithelial cells resulting in an inflammatory response caused by cellular components, which facilitate further bacterial invasion. Most of the virulence determinants responsible for invasion of epithelial cells are encoded on a large plasmid that is unique to virulent *Shigella* and enteroinvasive *E. coli* (EIEC) strains.

Virulence Plasmid

The initial observation that established the essential role of plasmids in *Shigella* virulence was done in *S. sonnei* and shortly thereafter in *S. flexneri* (9,10). Noninvasive *S. sonnei* isolates obtained on subculture in the laboratory recovered their invasive abilities when a large plasmid, normally found in clinical isolates of *Shigella*, was reintroduced (9). Since this initial observation, the so-called virulence plasmid (Fig. 1) has been shown to encode genes for (*1*) the production of invasion plasmid antigens (Ipas); (*2*) the synthesis of a type III secretion apparatus, a flagella-like structure able to deliver *Shigella* effector proteins into the eukaryotic cell; (*3*) the induction of endocytic uptake of the bacteria and disruption of endocytic vacuoles; (*4*) the intra- and intercellular spreading phenotype, and (*5*) the regulation of plasmid-encoded virulence genes.

Two independent groups have recently reported the complete sequence analysis of the large virulence plasmid in *S. flexneri* serotype 5a (11,12). The DNA sequence indicated that the genes necessary for entry of bacteria into epithelial cells are clustered within a 31 kilobases (kb) region of the 213-kb plasmid. The genes within this region have been extensively characterized (13). The entry region

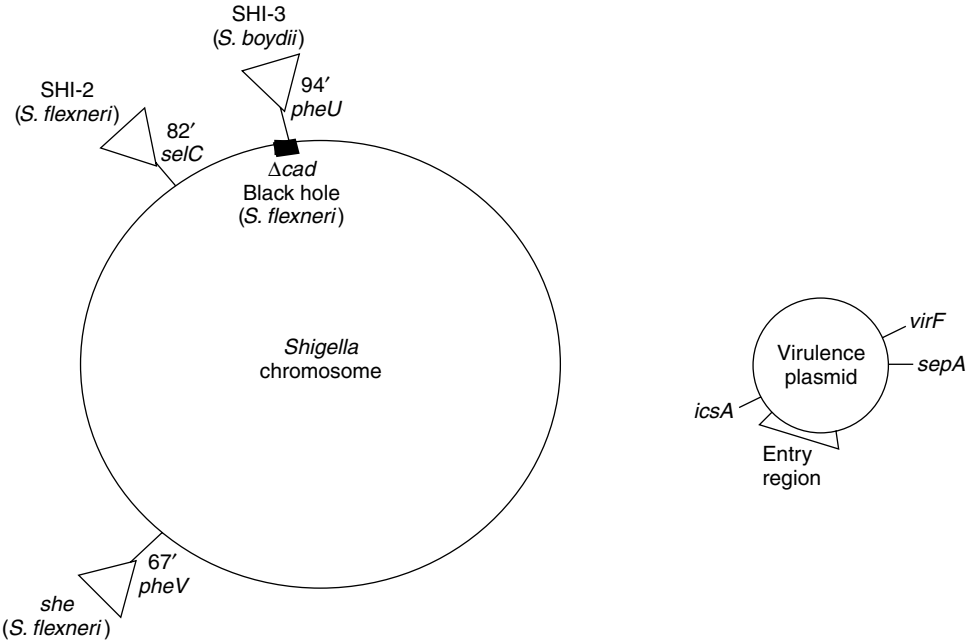

Figure 1. Location of selected pathogenicity islands and selected plasmid-encoded genes of *Shigella*. The location in the chromosome of the pathogenicity islands (PAIs) is indicated by minutes on the basis of the chromosomal map of *E. coli* K-12, and the genes adjacent or disrupted by the insertion of the PAI. The approximate location of the black hole is indicated with a closed square. The region implicated in the entry into epithelial cells and genes associated with pathogenesis are indicated in the virulence plasmid.

is a pathogenicity island-like cluster (see later) that contains (*1*) the *mxi* and *spa* genes encoding components of a type III secretion apparatus; (*2*) the *ipaA*, *B*, *C*, and *D* and *ipgD* genes encoding proteins secreted by this machinery; (*3*) the *ipgC* and *ipgE* genes encoding cytoplasmic chaperones required for stability of IpaB and IpaC, and IpgD, respectively; (*4*) the *virB* gene encoding a protein required for transcription of the *mxi*, *spa* and *ipa* genes; and (*5*) additional genes of unknown function.

Outside of the entry region, other genes associated with virulence have been identified. They include (*1*) the *icsA* (*virG*) gene encoding an outer membrane protein that is directly responsible for the ability of the bacteria to move within the cytoplasm of infected cells; (*2*) the *virF* gene encoding a transcriptional activator that controls expression of *icsA* and *virB*; and (*3*) the *sepA* gene, which encodes a secreted serine protease of the autotransporter family.

In addition, the virulence plasmid contains two copies of the *shet2* gene encoding a putative enterotoxin, and genes encoding several secreted proteins, which include *virA*, *ipaH4.5*, *ipaH7.8*, *ipaH9.8* and six uncharacterized genes designated (outer *Shigella* proteins): *ospB*, *ospC1*, *ospD1*, *ospE1*, *ospF*, and *ospG*. The proteins encoded in this plasmid are directly involved in the entry into epithelial cells and invasive phenotypes observed in the pathogenesis of *Shigella* strains.

Entry into Epithelial Cells and Type III Secretion System. In order for *Shigella* to enter an epithelial cell, the bacterium must adhere to its target cell. Entry into the host cell appears to be receptor-mediated, but the specific adherence factor has not been defined (3). The bacterium crosses the epithelium in selected areas corresponding to M cells of the follicle-associated epithelium (FAE) (Fig. 2; 14). More than 25 different genes are essential for *Shigella* entry and most of them are located in two adjacent operons located in an area called the entry region (Fig. 1; 7). One of these operons, the *mxi-spa* operon encodes a type III secretion apparatus that allows bacterial proteins to be secreted or translocated into the host cell cytosol (6). There are at least 25 proteins secreted through the Mxi-Spa secretion apparatus (11). The Ipa proteins (IpaA-D), which are encoded in the adjacent operon, are critical for *Shigella* entry and are among the major products secreted through the type III secretion apparatus (6). The secretion of Ipa proteins is induced by the epithelial cell and controlled by IpaB and IpaD, which prevent secretion of proteins before the bacterium-host cell contact occurs. The type III secretion apparatus allows the insertion of a pore into the host epithelial cell membrane. This pore contains a complex of the IpaB and IpaC proteins that induce a cascade of cellular signals, which result in actin polymerization and internalization of the bacterium. Among the other secreted proteins, IpaA and IpgD are required for the formation of a focal adhesion–like structure at the bacterium-cell membrane contact site (15), whereas the function of the other secreted proteins is unknown. *Shigella* are then internalized by a process caused by the formation of fingerlike extensions of the plasma membrane known as filopods that are quickly remodeled in lamellipods, resulting in an endocytic vacuole that entraps the microorganism (7).

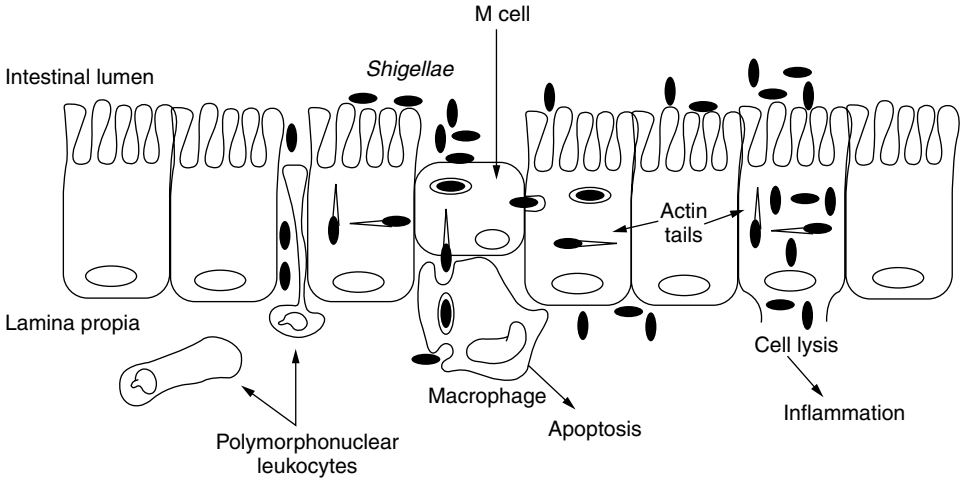

Figure 2. Entry and dissemination of *Shigella* in epithelial cells. The bacteria do not invade the intestinal epithelium directly. Instead, *Shigella* enter the M cells by inducing their own endocytosis (see text for details). *Shigella* escapes the endocytic vacuole and uses the cell cytoskeleton actin to spread from cell to cell. Then, they invade the epithelial cells and macrophages. *Shigella* invasion induced programmed cell death in macrophages, which triggers the initial stages of inflammation. *Shigella* multiply in the cytoplasm and cause the subsequent death of the infected epithelial cell inducing an intense response of inflammatory cells (polymorphonuclear leukocytes), bleeding, and abscess formation.

Intercellular and Intracellular Spread. After engulfment, *Shigella* is surrounded by a membrane-bound vacuole within the host cell (Fig. 2). *Shigella* rapidly lyses the surrounding vacuole and is released into the cytosol, where it grows and divides. Once the microorganism has escaped from the vacuole, it quickly becomes coated with filamentous actin and ultimately forms an actin tail at one pole of the bacterium (16). *Shigella* actin-based motility is mediated exclusively by the IcsA/VirG outer membrane protein (17). IcsA/VirG recruits host cytosolic components to induce actin nucleation. The neural Wiskoff-Aldrich syndrome protein (N-WASP), vinculin, the Arp 2/3 complex and several other host cytoskeletal proteins interact directly or indirectly with IcsA/VirG to cause actin tail formation, which propels the bacterium through the cytoplasm (7). When the pathogen reaches the plasma membrane of the cell, it forms a fingerlike projection from the surface of an infected cell to the surface of an uninfected cell. The tip of the protrusion penetrates the surface membrane of the adjacent cell with the subsequent internalization of the microbe. *Shigella* break out of the double membrane of the vacuole and are released into the cytoplasm, thereby starting a new cycle of infection in a new host cell.

Following host cell invasion and penetration, degeneration of the epithelium and inflammation of the lamina propia characterize *Shigella* infection. After crossing M cells, *Shigella* are found in an area essentially populated by macrophages and dendritic cells. *Shigella* expressing the invasion phenotype have another useful role, which is to cause the death of phagocytic macrophages by activating normal programmed cell death (apoptosis) (18). Macrophage apoptosis caused by the bacterial IpaB protein not only permits bacterial survival following the crossing of the FAE but is also central to the early triggering of inflammation (19). It is likely that this apoptotic process participates in the inflammatory rupture of the epithelial barrier and facilitation of bacterial dissemination.

Invasion of epithelial cells by *Shigella* stimulates the release of proinflammatory cytokines and chemokines, such as IL-8. This event accounts for the recruitment of polymorphonuclear leukocytes (PMN) to the site of infection and their transmigration through the epithelium, which causes major tissue destruction (13). It has been shown that IL-8 production by invaded epithelial cell results in the containment of *Shigella* infection at the epithelial level, but at the cost of massive epithelial destruction, particularly by PMNs (20).

Shiga Toxin

Shigella dysenteriae serotype 1 is unique among *Shigella* species in the production of a potent toxin known as the Shiga toxin (Stx) (21,22). Stx is a bipartite molecule composed of a single enzymatic A-subunit and a pentamer of receptor-binding B-subunits. The toxin binds to a glycolipid receptor found in target cells, globotriaosylceramide (Gb_3: Galα1–4-Galβ1–4-glucosylceramide), and it is internalized by endocytosis. The A-subunit is proteolytically cleaved and reduced, generating A_1 and A_2 peptides. The A_1 peptide inhibits mammalian protein synthesis by cleaving the N-glycosidic bond at adenine residue 4,324 in the 28S RNA of the 60S host cell ribosome. The importance of this toxin is that infections with Stx-producing bacteria may lead to hemolytic uremic syndrome (HUS), an often fatal kidney failure condition, particularly in children. In addition to *S. dysenteriae* type 1, related Shiga toxins are secreted by enterohemorrhagic *E. coli* (EHEC) strains and other bacteria that are associated with cases of HUS on a worldwide basis.

Pathogenicity Islands and "Black Holes"

In general, pathogenicity islands (PAIs) are large and unstable genetic elements, acquired by lateral gene transfer, with different G + C content, often associated with tRNA genes, which contribute to the virulence of bacterial pathogens. The concept of PAIs was developed on the basis of data on genome structure and pathogenicity of enteric organisms, especially pathogenic *E. coli*. However, this concept is now used broadly in other gram-negative and gram-positive pathogens. In this section, the recent progress in the identification and characterization of different PAIs in *Shigella* species will be discussed and the new concept known as "black holes" introduced.

Pathogenicity Islands. In *Shigella* spp. four distinct PAIs have been identified (Fig. 1). These include the plasmid-encoded *ipa/mxi-spa* locus described previously; the *she* PAI, encoding an enterotoxin and several autotransporter proteins; and the SHI-2 and SHI-3 PAIs, encoding aerobactin iron-uptake systems.

The *she* PAI is 46-kb unstable chromosomal locus inserted next to the *pheV* tRNA gene in *S. flexneri* serotype 2a (23,24). This PAI carries the *set1A* and *set1B* genes encoding the two subunits of ShET1 enterotoxin and the genes encoding the autotransporter proteins SigA, Pic, and Sap. In addition, this PAI includes genes commonly found in PAIs of pathogenic *E. coli* strains, and several open reading frames (ORFs) of unknown function. ShET1 production results in fluid accumulation in a rabbit ileal loop model. Pic, is a secreted protease implicated in mucinase activity, serum resistance, and hemagglutination. Finally, SigA, is a cytopathic protease that contributes to intestinal fluid accumulation. The precise contribution in pathogenesis and epidemiology of the *she* PAI is under further investigation.

The siderophore aerobactin is a low molecular weight iron chelator with high affinity for iron frequently found among *Shigella* and enteroinvasive *E. coli* (EIEC) strains that has been associated with increased virulence (25). The genes involved in synthesis and transport of aerobactin, which are often plasmid-encoded, were recently found within a pathogenicity island in two *S. flexneri* strains from serotypes 2a and 5 (26,27). This island, designated SHI-2 (*Shigella* pathogenicity island 2), is located downstream of *selC*, and occupy 23.8-kb in *S. flexneri* serotype 5 and 30-kb in *S. flexneri* serotype 2a. The G+C content of the island is slightly lower than that of the rest of the *Shigella* chromosome (51%) and varies from 48.5% in serotype 5 to 46% in serotype 2. In addition to the aerobactin transport and synthesis genes, SHI-2 encodes other genes found in the PAI of enterohemorrhagic *E. coli* O157 : H7, and genes encoding proteins, which confer immunity to colicins I and V.

A fourth island has been recently characterized in *S. boydii* strain O-1392 (28). SHI-3 is located within a 21-kb region between the *lysU* and *pheU* genes. The island contains the aerobactin operon flanked by genes found in mobile genetic elements. The fact that SHI-3 was found in other locations among closely related species, suggests that this PAI is an unstable and mobile genetic element associated with virulent *Shigella* species, although its role in virulence has not been established.

Black Holes. One common biochemical property of *Shigella* and EIEC strains is their lack of lysine decarboxylase (LDC) activity. The LDC^- phenotype has been proposed to be associated with virulence and Maurelli and coworkers found that introduction of *cadA* (the gene for LDC) in *S. flexneri* 2a causes attenuation in the virulence and inhibition of enterotoxin production (29). Failure to transduce *cadA* into the chromosome of *Shigella* led to the analysis of the chromosomal region flanking this gene in *S. flexneri* and EIEC. Comparison of the chromosomal region flanking *cadA* in *S. flexneri* and EIEC with *E. coli* K-12 revealed a large chromosomal deletion up to 90 kb in the vicinity of the *cadA* gene (Fig. 1). This novel concept called "black holes" suggests that deleting genes that are detrimental to the pathogenesis of the organism, provides an evolutionary advantage that enables *S. flexneri* and EIEC to enhance virulence.

Other Potential Virulence Factors

Several chromosomal genes have been implicated in *Shigella* virulence. These include the *sodB* gene, the lipopolysaccharide (LPS) synthesis genes and in *S. dysenteriae*, the *shu* locus (3,30). *sodB* encodes the superoxide dismutase protein, and $sodB^+$ *Shigella* are resistant to cell-mediated bactericidal activity, which seems to be a decisive factor in the development of colitis (31).

The O-specific polysaccharide domain of LPS is both an essential virulence factor and a serotype-specific protective antigen (32). Its importance in virulence was initially inferred from the avirulence phenotype of rough *Shigella* in animal models and the inability of these strains to spread from cell to cell in tissue culture monolayers (3,33). Recently, it has been shown that both the length distribution and the number of LPS O-antigen molecules on cell surface are important for *S. flexneri* invasion and full virulence (34,35).

Finally, in *S. dysenteriae* type 1, genes encoding the heme iron transport system are contained on a region found in two distantly related lineage of pathogenic *E. coli* and *S. dysenteriae* strains, but not in other *Shigell*a species, suggesting acquisition by horizontal gene transfer. In *S. dysenteriae*, however, a mutation in the heme uptake system did not reduce the virulence, suggesting that the presence of additional iron transport systems in *S. dysenteriae* complement the function of the heme uptake system (36). The role of the multiple *S. dysenteriae* iron transport systems in virulence is currently under investigation.

SHIGELLA AND THE ENVIRONMENT

Regulation of *Shigella* virulence is subject to tight control by several mechanisms involving many proteins and an array of environmental signals (Fig. 3). It is thought that the tight control by environmental factors prevents inappropriate expression of the virulence genes in or

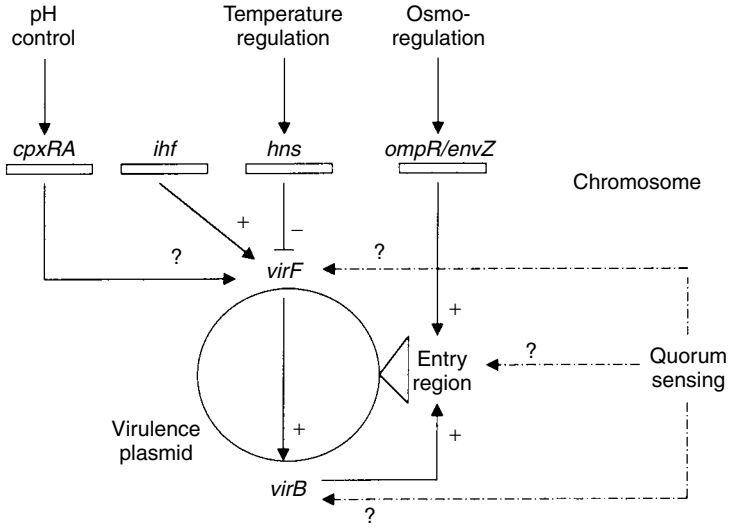

Figure 3. Schematic representation of the key elements in the *Shigella* virulence gene regulatory cascade. For simplicity, only the entry region is shown in this figure as target of regulation. The environmental factors are shown controlling the expression of chromosomal genes. The proteins encoded in these genes activated or repressed the plasmid-encoded virulence genes. The question marks refer to those mechanisms of regulation of virulence genes that has not been totally defined (+, positive regulation; –, negative regulation).

outside the host. The expression of *S. flexneri* virulence genes is activated when bacteria are shifted from 30 to 37 °C, in medium of moderate level of osmotic stress (similar to that of physiological saline) and a pH of 7.4 (37). Temperature is a key environmental cue exploited by *S. flexneri* to sense passage into the human gut (38). The factors controlling the thermal regulation have been identified and include chromosomally encoded H-NS, which repress virulence gene expression at 30 °C, and the virulence plasmid-encoded VirF and VirB proteins, which are transcription factors required for the expression of the invasive phenotype at 37 °C (37).

A second global regulator, the integration host factor (IHF), also contributes to the expression of virulence genes of *S. flexneri*. IHF is required for the positive activation of *virF* in both exponential and stationary phase cultures and for the activation of *virB* in stationary phase (37).

Transcription of *virF* is also under pH control. In *S. sonnei*, the pH control is dependent on the chromosomally located *cpxRA* genes, a member of the two-component regulatory system (39). The control by *cpxRA* can be overruled by thermal regulation and so far, the direct binding of CpxR to the promoter region of *virF* has not been demonstrated. A second two-component regulatory system has been implicated in the control of *S. flexneri* virulence (40). The osmotic stress sensor protein EnvZ and the response regulator OmpR have been shown to control the expression of plasmid-located virulence genes, but direct evidence of this regulation has not been obtained.

It has been proposed that an additional environmental factor, the cell density, is also involved in the control of virulence gene expression in *Shigella* (41). Bahrani and coworkers showed that the expression and secretion of Ipa proteins is increased with cell density, peaking in stationary phase cultures. This data suggests that the quorum sensing molecules present in stationary phase cultures (known as autoinducers), influence *Shigella* virulence gene expression. Recently, Day and coworkers demonstrated that the expression of *ipa*, *mxi*, and *spa* invasion operons is maximum in stationary phase bacteria and that conditioned media derived from stationary phase cultures enhance the expression of these loci (42). This report also showed that expression of *virB*, a gene encoding the transcriptional factor essential for the expression of the invasion loci, peaks in late log phase. Their data showed that the quorum sensing autoinducer molecule active in late log phase, does not influence the expression of the invasion operon and is not required for *Shigella* virulence (42). More data is required to define the role of quorum sensing in the expression of virulence factors in *Shigella* strains.

Epidemiology

Shigella are highly host-specific, causing natural infections in humans and only occasionally in other higher primates. The target susceptible populations are infants, the elderly, and the very ill that suffer the most severe symptoms of the disease, but all humans are susceptible to the disease to some degree. *Shigella* are not easily killed by stomach acid, so an infectious dose of as few as 10 organisms can cause the infection. Since production of shigellosis requires ingestion of such small numbers of the organism, it is extremely difficult to control the spread of the disease. Transmission occurs most quickly in overcrowded populations with poor sanitation where it more easily spreads from person to person. Although transmission via food is less common, contamination of food with human sewage (either directly or via contaminated water) has led to outbreaks, especially with cold, uncooked foods such as salads and raw vegetables.

In developing countries, *Shigella* is a common endemic infection because of inadequate sewage disposal and lack of effectively treated water supplies. Ninety-nine percent of the estimated *Shigella* cases worldwide occur in developing countries (163.2 million from 164.7 million total) (4). The majority of *Shigella* isolates in developing countries correspond to *S. flexneri*, with *S. sonnei* being the next most prevalent. However, when pandemic dysentery caused by *S. dysenteriae* serotype 1 appears, the rate of infection increases and often becomes a leading cause of

death. In developed countries, most cases of shigellosis are seen in young children with *S. sonnei* as the predominant serotype (4). In addition, family members frequently acquire infection from infected individuals in the same household, so that infection can spread from children to adults and vice versa.

Outbreaks of infection have been described in other groups of people in close contact, such as in military bases, refugee camps, among cruise ship passengers or following natural disasters. Severe forms of infection can occur, particularly in travellers to developing countries. In these cases, the patient may become very ill and dehydrated, and in the absence of medical treatment, dysentery can be fatal.

VACCINE DEVELOPMENT

Although *Shigella* species are important agents of diarrheal epidemics worldwide, causing an estimated 164.7 million cases of dysentery and approximately 1.5 million deaths annually (4), there is no current licensed vaccine to prevent shigellosis. There are three factors that have restricted the development of such a vaccine, including (*1*) the inability to raise serum antibodies, which confer immunity after parentally injected inactivated whole-cell vaccines; (*2*) the lack of a good animal model; and (*3*) only indirect evidence of the immune mechanism(s) in humans activated after infection (43).

Despite these factors, a new generation of candidate vaccines had shown great promise for the prevention of shigellosis (44–46). The primary role of a *Shigella* vaccine would be to protect against clinical disease. An additional benefit would be to interfere with infection and colonization. The most important *Shigella* strains to be targeted for vaccine development are *S. flexneri 2a*, *S. dysenteriae* type 1, and *S. sonnei*. However, the possible emergence of new serotypes has been emphasized. The emergence of *S. flexneri* serotypes 1, 3, 4, and 6 in several countries and a switch toward a predominance of *S. sonnei* in developing countries has been observed (4).

Several approaches to the development of *Shigella* vaccines have been tested. Two of the most promising approaches in development of candidate vaccines include the use of live attenuated strains and acellular vaccines based on lipopolysaccharide/polysaccharide antigens. Several attenuated vaccine candidates have been derived from *S. flexneri* 2a. Recently, CVD 1207, a derivative from the wild-type *S. flexneri* 2a strain, was tested in a phase one clinical human trial. This live attenuated vaccine candidate retains the ability to invade epithelial cells but cannot effectively spread intercellularly after invasion, does not produce enterotoxin, and has limited proliferation in vivo. CVD 1207 was shown to be highly attenuated and well tolerated at dosage levels that were markedly reactogenic with earlier invasive *S. flexneri* 2a vaccine candidates, but showed modest serum antibody responses and was insufficiently immunogenic after a single dose (46). Another attenuated *S. flexneri* 2a vaccine candidate has been successfully tested in human trials. Strain SC602 carries deletions of the plasmid-borne virulence gene *icsA* and the chromosomal aerobactin locus *iuc*, which resulted in a double attenuation of its capacity to move intra- and intercellularly, and of its survival within tissues. Double-blind, placebo-controlled studies on the safety and the immunogenicity of this candidate vaccine indicated that SC602 is the first attenuated candidate vaccine that provides protection against shigellosis in a stringent, challenge model (45).

Shigella polysaccharide-protein conjugates are vaccine candidates consisting of proteins that elicit enhanced antibody responses against the poorly immunogenic polysaccharide (47). A phase II (double-blind, randomized, and vaccine-controlled) study of a conjugate vaccine composed of *S. sonnei* O-specific polysaccharide bound to *Pseudomonas aeruginosa* recombinant exoprotein A (*S. sonnei*-rEPA) has been conducted in Israel (44). The study revealed that the vaccine was safe and highly immunogenic and indicated that one injection of *S. sonnei*-rEPA confers type-specific protection against *S. sonnei* shigellosis (44). Recently, the safety and immunogenicity of *S. sonnei and S. flexneri* 2a conjugates were evaluated in a clinical trial using different carrier proteins (43). The data suggested that these conjugates were safe and immunogenic and they can be used to induce a more complete and long-lasting immunity against shigellosis (43).

The essential requirement for the development of a *Shigella* vaccine in the near future is the clear understanding of the host innate immune response to the organism and how this affects the adaptive immune response throughout the disease. An ideal vaccine should be easy to administer, preferably orally, although parental vaccines should not be discarded if all the following requirements are met: well-tolerated; able to induce a high-level, long-term protection after a single dose; multivalent, and easy to manufacture. In addition, it is essential that the new strategies for vaccine development take into consideration most of the antigenically distinct serotypes of *Shigella* causing endemic and epidemic infections. It has been estimated by Kotloff and coworkers that if a vaccine had 70% efficacy, up to 91 million cases and more than half million deaths might be prevented each year (4).

CONCLUSION

Recent progress to better understand *Shigella* infection has been possible by establishing the interactions between the bacterial secreted products and their target host cellular components. The completion of the *S. flexneri* virulence plasmid sequence and the fast progress in the elucidation of the genomes of other intestinal pathogenic strains, including the sequence of the *Shigella* chromosome, will allow the analysis and identification of other putative virulence genes implicated in the pathogenesis of the microorganism. This approach has become particularly important in determining the real contribution of other chromosomal-encoded proteins in addition to the already identified virulence factors. More studies are needed to determine, for example, the regulatory networks controlling the expression of the genes in the chromosomal PAIs, and to define the

global scheme of signaling pathways at the bacterial or the cellular level that coordinate the expression of the full range of virulence factors implicated in the disease.

The global understanding at the molecular and cellular levels of the pathogenesis of *Shigella* will enable the development of the ideal vaccine that covers most of the major *Shigella* serotypes and effectively provides protection against the disease. This vaccine is obviously needed since there is a high disease burden mostly in developing countries and a tendency of resistance to current antibiotics, but the major challenge to overcome in the assessment of *Shigella* vaccine efficacy is the absence of previous candidate vaccines that develop full protection.

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SIDEROPHORES.

See METALS: MICROBIAL PROCESSES AFFECTING METALS

SIDEROPHORES IN MARINE BACTERIA.

See INORGANIC NUTRIENT USE BY MARINE MICROORGANISMS

SILICEOUS STRUCTURES OF CHRYSOPHYTES.

See PALEOLIMNOLOGY: USE OF SILICEOUS STRUCTURES OF CHRYSOPHYTES AS BIOLOGICAL INDICATORS IN FRESHWATER SYSTEMS

SILICON IN MARINE MICROORGANISMS.

See INORGANIC NUTRIENT USE BY MARINE MICROORGANISMS

SLUDGE. See BIOSOLIDS: ANAEROBIC DIGESTION OF; WASTEWATER AND BIOSOLIDS AS SOURCES OF AIRBORNE MICROORGANISMS

SLUDGE, GRANULES IN. See ANAEROBIC GRANULES AND GRANULATION PROCESSES

SNOW AND ICE ENVIRONMENTS

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Average present-day temperatures at Earth's poles are well below 0 °C and water is readily found in its solid form as ice and snow within both the terrestrial and marine environments throughout the year. At lower latitudes, ice and snow are also prevalent features of terrestrial and aquatic systems at high elevations (e.g., alpine environments) and at lower elevations during winter. Ice and snow are only present at extremely high altitudes near the equator. Hence, the distribution of ice and snow environments is discontinuous, yet ubiquitous in time throughout the globe. The physical binding of H_2O into a solid hexagonal matrix has profound affects on the physical and chemical habitats of organisms (microbial or otherwise) to the extent that the abilities of organisms to persist are tested. As a result, there is a general trend for decreasing biological diversity along the gradient of environments with ice and snow persistence (1). At the extreme end of the spectrum, where ice and snow persist at temperatures that are continually below 0 °C, prokaryotic microbiota are the primary life forms present (2,3), although spores and pollen of higher plants may persist (4).

Microbiota within ice and snow experience a heterogeneous microenvironment in which the transition of water between the liquid and solid phases determines the activity of liquid H_2O, the transport of life-sustaining solutes, and the transfer of radiant, thermal, and chemical energy. All of these processes critically influence the ability of microbes to remain viable, adjust their metabolic processes, and generate macromolecules and cells. How ice environments have been manifested during the evolution of Earth's climate and biosphere (5) and whether or not these environments have helped determine the evolution of life throughout Earth's dynamic ecosystems are highly speculative at this time (6,7). However, it is apparent that an array of microbes possesses the ability to survive freezing conditions, to grow, and form functioning microbial communities throughout a range of ice and snow environments. Noteworthy are the microbial communities in (*1*) temperate snow fields, (*2*) sea ice, (*3*) ponds and holes on glaciers, and (*4*) ice covers on lakes. These communities, nominally consisting of autotrophic primary producers and heterotrophic bacteria, function to provide the basis for new energy acquisition and internal material cycling that support ice-based ecosystems. Ice environments such as permafrost (4), the snow and ice in the polar ice caps (3), and accretion ice formed from lakes covered by the Antarctic ice cap (2,8,9) are still being investigated to determine if these environments support microbial aggregates to the extent that they can be considered ice communities/ecosystems or whether these ice and snow environments merely provide a cold refuge for cells capable of survival therein.

The major freshwater ice and snow microbial habitats are reviewed within this contribution to provide information regarding the physical and chemical attributes of ice and snow microenvironments that either allow or preclude microbial colonization, survival, growth, succession, and adaptation. That is, the environments reviewed within this article are those within (*1*) snow (including snowfields in temperate alpine regions and on polar ice caps), (*2*) glacial ice (including polar ice sheets and temperate glaciers), and (*3*) accretion ice on lakes. In many instances, the fundamental characteristics of the microhabitats may be similar, yet the formation processes, dynamics, and microbial populations therein can differ greatly. Sea ice and permafrost are the two additional extensive ice biomes, yet present knowledge regarding these environments as ecosystems warrants full and separate discourses that are presented in other contributions within this Encyclopedia (see sections on Permafrost and Sea Ice). Similarities and contrasts between the aforementioned ice environments and the attributes of the microbiota within ice and snow environments are discussed throughout this article.

SNOW

The availability, timing, and duration of liquid water are primary determinants of when and where microbial communities develop in snow and ice environments. Within snow fields in temperate regions, the development of microbial communities is determined primarily by the balance between seasonal warming and melting that allows liquid water to become available within the snow's ice matrices. A cool climate is required that allows snow fields to accumulate enough mass to allow sufficient time during the melting seasons for microbial excystment, microbial migration, and growth processes that are integral parts of the life histories

of snow microbiota. Because of the "race" between a snowbank's dissipation and biological growth and succession processes, not all snow fields are capable of developing productive communities of snow-ice microbes. Snowfields in high alpine environments or in areas with high winter precipitation are more likely to meet this "window" and develop dense blooms of microalgae. Snow banks that are transient or are only saturated with liquid water for a few days before they completely melt are not likely to provide a viable habitat for microalgal growth or microbial succession. On the other extreme, snowfields that continually remain below the freezing point of water do not generate measurable liquid contents, and luxuriant growth of both heterotrophic and autotrophic microbiota can be extremely inhibited (3).

Snow communities have been known since the time of Aristotle (10) and early work and reviews have emphasized the taxonomy and the physiology of snow microalgae (11–13). More recently, studies have advanced to include investigations of the relationships between algae, bacteria, protists, and metazoans in temperate snowfields to the extent that these environments are now being studied as distinct microbial ecosystems inhabiting transient snow environments (14,15).

Pink snow or red snow is often caused by *Chlamydomonas nivalis* and is perhaps the most commonly recognized of the snow algae. The red coloration is caused by the carotenoid pigmentation of aplanospores (nonmotile algal spores; 16). Although this life-stage of *C. nivalis* may be the most recognized and *C. nivalis* is the most studied of microbiota in temperate snowfields, it is by no means the only photoautotroph present in snow. Many additional species of green algae (Chlorophyta, e.g., *Chloromonas*, 17), diatoms (18), and Cyanobacteria (13,19) are also commonly found in temperate and polar snow environments, although diatoms are only reported on occasion (19). The life histories of the diatoms and Cyanobacteria that may be coupled to snow dynamics are relatively unknown compared to those of the Chlorophyte algae, namely, *Chlamydomonas* (20) and *Chloromonas* (17) species.

The life history of *Chlamydomonas* and *Chloromonas* in snow environments includes the processes of (*1*) deposition of spore or zygotes on the soil surface in the summer, (*2*) spore burial by cold-dry snow during the winter, (*3*) excystment when snow banks begin melting in the spring, (*4*) migration of the excysted flagellated stages up to the snow surface where mating may or may not occur (5), and (*5*) spore or zygote formation induced by environmental cues such as UV radiation or nitrogen deficiency (14,16).

The in situ production of reduced carbon compounds and growth of snow algae is supported by nutrients deposited with the snow as dissolved matter, atmospheric dust, and forest litter (21). The forest litter and atmospheric dust also provide energy for heterotrophic bacteria such that a microbial-based food web often develops during a snowbank's melting period consisting of heterotrophic flagellates, ciliates, fungi, tartigrades, and worms (10,21). The energy supporting the transient food web is derived from both allochthonous materials and that from resident photoautotrophs. Sattler and coworkers (85) have reported evidence of microbial growth within cloud droplets below 0 °C, which implies that the microbial cycling of materials in snowfields may actually begin before snow crystals are deposited.

Bacteria associated with snow algal blooms are often attached to the aplanopsores and algae, suggesting the microenvironments surrounding these cells are probable zones of enriched organic matter and nutrition (21). Similar biofilms with polysaccharide matrices may confer freezing protection (22,23) through the depression of the freezing temperature of water and the stabilization of macromolecules (proteins and lipids) during desiccation that is induced by freezing (23).

Microbial survival and production within permanently cold snow (i.e., less than 0 °C) such as that found at the South Pole or at extremely high altitudes do not have substantial periods when liquid water is available. Hence, snow algae are not typically present in such environments; rather, desiccant resistant strains of bacteria (e.g., *Deinoccous*, *Bacillus*) and fungi are more typically reported (2,3). Carpenter and coworkers (3) reported significant uptake of tritiated thymidine in South Pole snow at temperatures below −12 °C during a 24-hour incubation. If active in situ, these bacterial populations must rely on allochthonous input of reduced organic matter because photoautotrophic populations are virtually nonexistent (3).

GLACIAL ICE

Glaciers and ice sheets contain several distinct environments in which microbes can colonize, grow, and develop extensive communities (Fig. 1). The accumulation zones of glaciers and ice sheets are areas where the mass balance leads to the net accumulation ice mass due to precipitation in the form of snow. Air temperatures average below 0 °C and liquid water availability in the snow is limited to short seasonal melting periods or along ice crystal boundaries. The stratigraphy of the glaciers and polar ice sheets in accumulation zones consists of fresh snow, compacted snow or firn, glacial ice with visible air inclusions, and ice with no visible air inclusions. The thickness of the snow and firn layers is determined by the weight of the snow compressing the ice and melding the ice crystals through recrystallization processes. Below a depth of approximately 1,000 meters, the weight of the overlying ice provides sufficient pressure to convert the air in the bubbles into gas clathrates. Hence, ice below approximately 1,000 meters appears clear because gas clathrates have similar refractive indices as ice water. Because alpine glaciers are typically less than 1,000-m thick, they rarely contain clear ice formed from pressure recrystallization processes.

Liquid water available to support metabolism within the glacial ice of polar ice caps is held to be a primary constraint on microbial processes. With average and maximum air temperatures well below freezing point throughout the year in accumulation zones, bulk liquid water is not created through thermally induced melting processes. Rather, liquid water availability is more likely limited to microhabitats at snow crystal boundaries and

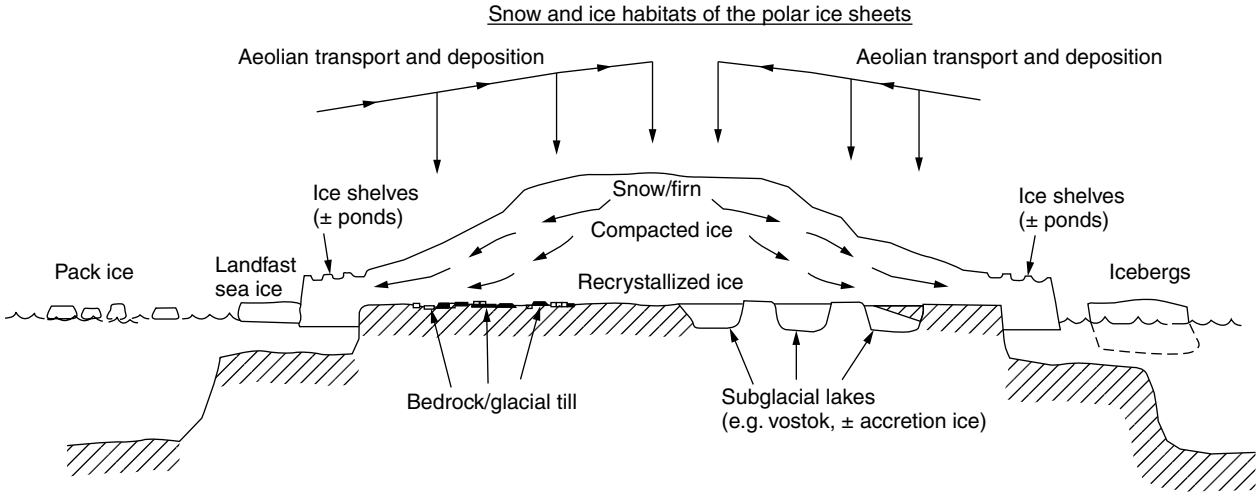

Figure 1. Schematic of snow and ice environments in Antarctic and Greenland ice caps. Arrows denote the movement of materials (ice/snow, particulates, etc.).

in microhabitats with high solute concentrations created through reprocessing of the ice habit (25). Trophic structures that would include primary (either photoautotrophic or chemoautotrophic), heterotrophic secondary production and material cycling within these ice sheets have yet to be demonstrated. Rather, studies thus far have indicated more of an abundance of heterotrophic microbes (spore-forming and non-spore-forming bacteria, and fungi), although at low concentrations ($>10^4$ cells ml^{-1}; 24) relative to concentrations (10^5 cells ml^{-1}) typically found in aquatic ecosystems. Because of the apparent lack of active autotrophs, metabolism of allochthonous materials deposited during the ice sheet accrual appears to be the primary source of energy for these systems (3,25). The extent to which in situ processing of these materials occurs remains to be determined.

Surveys of polar ice cores and snow indicate that desiccation-resistant strains of microbiota appear to be most prevalent in these environments. Specifically, gram-positive non-spore-forming bacteria, spore-forming bacteria, planctomycetes, fungi, and the radiation/desiccation resistant *Dineococcus* (3) are common among the microbes recovered from ice cores and polar snow. Christner and coworkers (24), in surveys of polar ice and temperate glacial ice (e.g., Taylor Dome of Antarctica, Guliya ice cap, Sajama ice), also found more cultivable bacteria in nonpolar ice cores and implied that the distance to source materials was a primary factor controlling the abundance of biota in these settings. Taken a step further, their work further implies that the production of cells in ice sheets is not substantial, relative to the mass of cells initially colonizing these ice habitats; rather, cell abundance is more a function of transport and deposition processes. Diatom frustules, unicellular algae, and dust are also found within the Antarctic ice cap, and bacterial cell have been higher in sections of ice with dust (2). Higher bacterial numbers associated with dust particles implies either increased deposition of microbes along with the dust or microbial proliferation in the microenvironment associated with the dust. Existing information on cell identities and abundances alone does not provide evidence that the accumulation zones of polar ice sheets provides an environment whereby in situ growth or cell proliferation occurs. The observations that microbiota can be recovered from glacial ice aged more than 10,000 years and that measurable metabolic activity is rapid implies that in situ basal metabolic activity should be occurring to preserve viability against lethal processes such as cold denaturation of proteins, amino acid racemization, and radiation-induced destruction of macromolecules.

The ablation zones of glaciers and ice sheets are the zones where sublimation, evaporation, and direct liquid water runoff lead to the net loss of ice mass. The surfaces of glaciers and ice sheets in these zones are often riddled with pockets of sedimentary material and organic matter that have melted and formed cylindrical holes in the ice surface (26–28). Such cryoconites holes, once started, enhance the localized collection of particulate materials, which creates positive-feedback processes, leading to further augmentation of the deepening and widening of the cryoconite holes. However, the opacity of the surrounding glacial ice (which attenuates radiation penetration into these holes), in conjunction with the seasonal freezing of the melt water and surface sublimation, provides a negative feedback that prevents cryoconite holes from deepening much beyond a half of a meter in depth (29,30). Bulk liquid water becomes available in cryoconite holes during the summer melting periods and allows these cryoconite microenvironments to support biota including diatoms (31), desmids (29), Cyanobacteria (29), bacteria (31), protists, and metazoans such as tartigrades (32). Although it is now known that cryoconites support microbial populations, relatively little is known about the structure and dynamics of the bacteria therein. The primary emphasis of studies thus far has been on the physical properties of the habitat (30,33,34) and the identities of the resident photoautotrophs and protists (31,32). Studies having a bacteriological emphasis on these cryo environments seem highly warranted.

Bedrock, mud, or subglacial lakes lie below the glacial ice of major ice caps or glaciers. The extent of the subglacial liquid water throughout the polar regions is still being explored. However, several subglacial lakes lying beneath the Antarctic ice cap have been detected (35). Studies on the origin and dynamics of these lakes are in their infancy because they were only discovered in the past decade. One of the largest of these lakes, Lake Vostok, lies beneath 3,700 meters of ice in East Antarctica. The overlying ice on this lake contains snow and glacial ice and accretion ice that formed from freezing of lake water onto the base of the overlying glacial ice (36). Both the glacial ice and the accretion ice in the Vostok ice core contain heterotrophic microbial cells (2,37,38). The cells in the accretion ice, however, are most likely to have originated from the subglacial lake rather than via direct deposition in the overlying snow and subsequent burial, which is the presumed source of the materials and cells in the glacial ice (24). Algae or protists have not been recovered from either ice type.

Extensive floating and grounded ice shelves form where glacial ice sheets float over the coastal ocean along continental or island margins (Fig. 1). The major ice shelves of the Antarctic are the Filschner-Ronne, which flows into the Weddell sea, and the Ross ice shelf, which flows into the Ross sea although lesser ice shelves (e.g., Amery, Larsson, etc.) are found throughout the coastal margin. In a few coastal areas of the Antarctic (most notably McMurdo Sound near Ross Island), coastal ice shelves exist in a climate in which the average summer air temperatures in conjunction with sediment laden glacial ice allow the formation of surface melt features (ponds and cryoconites). Glacially derived ice shelves are also present along the margins of Greenland and northern islands in the Arctic (39). Lesser Arctic ice shelves are also found in regions surrounding northern islands (e.g., Ellsmer and Ward Hunt) that have formed by the formation and persistence of land-fast sea ice that may reach thicknesses exceeding 5 to 10 meters (e.g., the Ward Hunt ice shelf) (39,40). The origin and dynamics of these ice shelves differ from those derived from glacial ice such that sea ice accretes on the bottom of the ice shelf whereas the upper surface experiences ablation and evaporative losses. In this regard these sea ice derived-ice shelves are similar to perennial ice covers on polar lakes (see following text), whereby an "ice-conveyor" is created by adding "new" ice on the bottom and ablating "old" ice from the upper surface.

The surfaces of ablating Arctic and Antarctic ice shelves appear similar, both having undulating surfaces consisting of ridges and troughs running approximately parallel (39,40). Within the troughs, extensive ponds and streams form that undergo annual melting and freezing. It is within these annual melt features that viable, productive, and extensive microbial systems are found (40–42). Ice shelf microbial ecosystems have been studied most extensively in the McMurdo Sound region of Antarctica, yet recent studies in the Arctic indicate the microbiota are similar in that they too are primarily composed of mat-forming Cyanobacterial consortia. Ice shelf mat-forming microbial consortia are most often dominated by filamentous Cyanobacteria (Oscillatoriales and Nostocales) that form the cohesive macroscopic structure that also harbors viruses, bacteria, diatoms, and protists (41,43). Microbial mats on ice shelves may develop distinct vertical zones with changes in pigmentation and biota induced by strong vertical gradients in radiant energy and oxidation-reduction potentials (43,44). Although these mats may become vertically deliminated, the continual movement and freeze-thaw cycles of the ice environment probably precludes the layering to the extent of those on the benthos of ice-covered lakes that have been termed modern-stromatolites (45). Although anaerobic sediments can develop below Cyanobacterial mats in the Antarctic ice shelves and sulfate reduction and methanogenesis has been demonstrated (46), the uniqueness of these ice-anerobic environments that are caused by ice movement and freezing and thawing are not understood at this time. Most probably annual disruptions of the chemical and physical gradients often necessary to develop layered microbial communities have large effects on the microbial dynamics within the distinct zones of sulfate oxidation/reduction, nitrate oxidation/reduction, carbon dioxide oxidation/reduction typical of mats, and associated anaerobic sediments.

ICE COVERS ON LAKES

Sea ice supports active microbial communities and their composition, productivity, dynamics, and role in polar ecosystems have been studied in the Arctic (47) and Antarctic oceans for many decades (48–51). During the 1990s, researchers have also realized that ice covers on freshwater lakes around the world also support active microbial communities/consortia (52–54). Specifically, it is now known that perennial ice covers on Antarctic lakes and seasonal ice covers on high mountain lakes contain active microbial communities that produce and cycle materials within the interior of the ice matrices.

A common feature of lake ice covers that develop and support microbial communities is the presence of substantial liquid water (>10% by volume) within the ice interior — either transiently or during prolonged summer melting. The same can be said of sea ice, yet a liquid water environment on the order of 5% by volume is maintained throughout the year within sea ice in brine pockets (55). Freshwater ice covers are unlike sea ice in that they do not contain enough dissolved salts to create brine pockets and channel networks that would provide a substantial volume of liquid water within the ice sheet year-round. Rather, liquid water within freshwater ice covers is present as films of water along ice crystal or particle boundaries when temperatures are below 0 °C or in bulk quantities in association with melting or dynamic flooding processes.

Flooding and infiltration of liquid water within the snow cover occurs when the weight of the snow cover depresses the surface of the ice below the hydrostatic water level and is the primary mechanism creating a liquid water environment in the ice covers on high mountain lakes. When flooding occurs during colder winter months, the layer of flooded snow can refreeze (or at least partially refreeze), which creates a layered structure to the ice

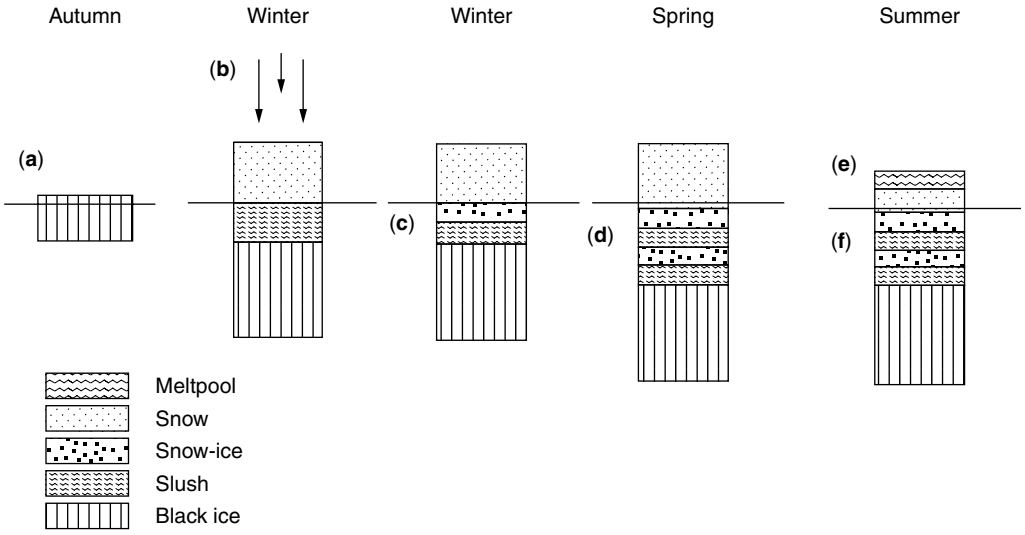

Figure 2. Dynamics of temperate ice covers. (**a**) Black ice forms from freezing the lake water during the autumn. (**b**) Snowfall and accumulation on black ice. When the accumulating snow cannot be supported by the buoyancy force of the black ice, the surface of the consolidated ice is depressed below the surface of the lake and the ice floods with lake water, creating slush (lake water mixed with snow crystals). (**c**) During winter the slush may entirely or partially refreeze, thus creating a layered ice stratigraphy with liquid water between consolidated snow ice and black ice layers. (**d**) Through multiple flood and freeze cycles high mountain lake ice covers can generate several layers of slush and consolidated ice. In these slush layers, bacterial activity and growth occurs through the winter (58). (**e**) Meltponds form during the summer melting. (**f**) Slush layers remain during melting phase. Both the meltponds and the slush layers in the spring and summer are "hotspots" for bacterial production and protist activity that is linked to new growth of photoautotrophs (54).

cover consisting of dry snow, consolidated ice, slush (snow crystals plus lake water) and consolidated ice (Fig. 2). Growth of microbiota and succession stages consisting of autotrophic algae, bacteria, heterotrophic flagellates, and ciliates have been documented within slush layers in ice covers in the Austrian Alps and the Pyrenees Mountains of Spain (53,54). The slush habitats created through flooding and the subsequent growth and succession in these lakes is not unlike the processes that create near-surface habitats in sea ice in the Antarctic pack ice (50,52,56,57). Similar microbial successions are likely to occur in high mountain lakés throughout the world, yet documentation of their distribution is limited.

Snow algae (*Chlamydomonas* sp.) inhabit surface melt pools (Fig. 2) that develop late in the melt cycle of high mountain lake's ice covers (53). Within the ice cover's layered structure, autotrophic species can be similar to those in the underlying water column, yet in some years the slush layers develop distinct autotrophic community compositions (53,54). Bacterial populations within the ice covers have been shown to be distinct from those in the under ice water column when comparing the relative percentages of α-, β- and γ-subclasses in these environments (58). The activity of bacteria in slush habitats covers the range from that of oligotrophic to eutrophic production rates (53) and are probably responsible for observed nitrate and nitrite chemistries indicative of denitrification (59,60). The growth and succession and release of microbes in the melting ice covers affect the lake's water column dynamics in a manner similar to that proposed for marginal ice edge zones within the Arctic and the Antarctic (61).

Liquid water habitats are generated through surface and internal melting during the austral summer within several perennial ice covers on freshwater lakes in the Antarctic (Fig. 3; 62). Local microscale (micrometers to meters) melting is facilitated by the absorption of solar radiation by dark material (sediments, pigmented algae, organic matter) during the periods when the ice covers warm to 0 °C and remain isothermal (Fig. 3; 62). Because there is no temperature gradient in the ice covers during summer, the absorbed radiant energy is not conducted away from the place where it is absorbed. Hence, local melting ensues and creates pockets of liquid water that facilitate the accumulation of particles and creates aggregates of materials that melt and migrate downward through the ice covers (63). Several lakes in the McMurdo Dry Valleys of the Antarctic (e.g., Hoare, Fryxell, Miers, Bonney, Vida) have the sediment accumulations at 0.5 to 3 meters depth (63,64) and it is within these sediment-aggregates that microbial communities, nominally consisting of Cyanobacteria and heterotrophic bacteria, have been found to persist and grow (52,64).

Microbial communities in perennial Antarctic ice covers contain microbiota similar to those found in surrounding terrestrial environments (65). Cyanobacteria of the genus *Phormidium, Nostoc* and *Leptolyngbya* are common within the mats or flakes of matlike aggregates in these ice covers (52,64,65). Similar Cyanobacteria dominants are

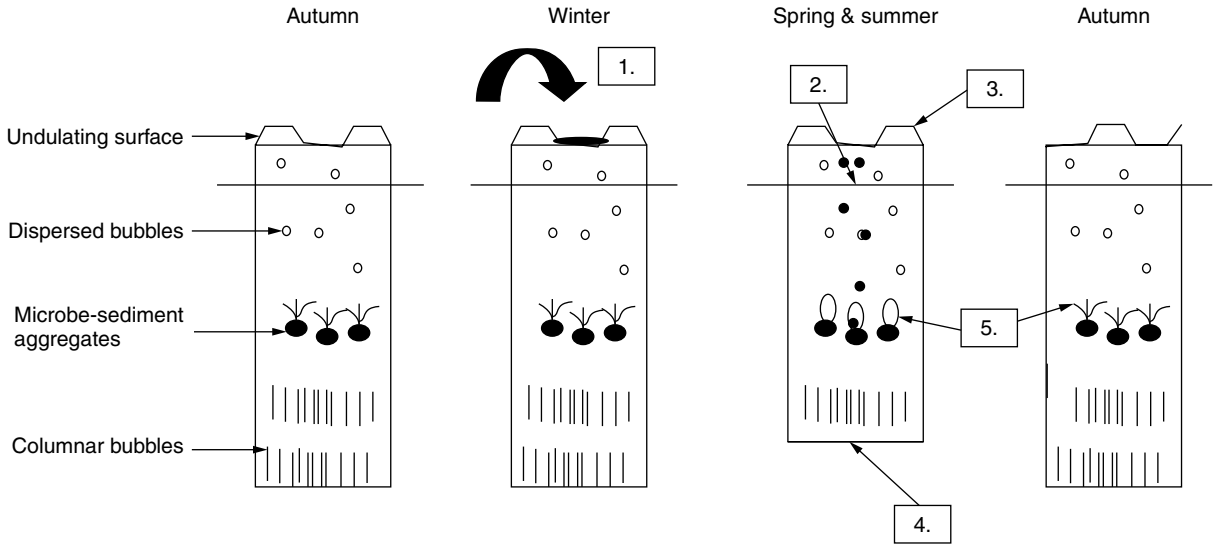

Figure 3. Dynamics of temperate perennial ice covers. Microbe sediment aggregates are located within the ice covers interior in a frozen state during the autumn. 1. Sediment and microbial aeolian deposition on the ice surface — primarily during winter storm events, basal freezing occurs during this time as well. 2. Surface deposited sediments migrate downward during spring and summer because of absorption of solar radiation and localized melting. 3. Sublimation of ice evaporation of meltwater from the upper surface. 4. Basal melting reduces thickness of ice cover during summer. 5. Cyanobacterial consortia in sediment aggregates exposed to liquid water grow during summer (64), additional migrating materials collect onto aggregates during this time as well. During autumn and winter these habitats refreeze, producing arched bubbles indicative of remnant water pockets (63).

found in the soils (65), streams (66,67), glaciers (29), and benthic mats in the lakes (68). The genera of Cyanobacteria in ice or the aforementioned habitats are not typical dominants of phytoplankton in lakes. Rather the lake's phytoplankton are more typically composed of autotrophic eukaryotic algae, although populations of Oscillatorian Cyanobacteria have been found in the plankton of some Dry Valley lakes (69). Bacterial 16S rRNA recovered from the ice-sediment aggregates included clusters grouping with the *Planctomycetales*, *Roseococcus*, and *Rhodoferax* species.

Oligonucleotide probe hybridization assays indicate that these sequences are also found in the McMurdo Dry Valley soils (65). Bud-forming bacteria (e.g., *Planctomycetales*) are common members of soil environments, and their presence in the ice-sediment environment may suggest that this habitat is most readily colonized by terrestrial microbiota rather than truly aquatic forms.

Bacterial and algal cells present below the sediment layers in perennial ice covers have been shown to be active in ice meltwater. These cells are present at relatively low concentrations (bacteria, 2-9e 11 cells m^{-3}) compared with concentrations in association with sediment aggregates (bacteria, 2e12 to 3e13 cells m^{-3}). The composition of these bacterial and algal populations has not been determined via molecular techniques or compared with the sediment aggregates or the water column biota. However, it is probable that they are derived solely from the water column because the sediment aggregates reside above this ice, and the ice structure is not indicative of any ice-sediment interactions that would be evident as melt features (63). Because these microbial cells are active and most probably move upward with the ice on an annual basis they encounter the sediment aggregate zones. When these water column derived biota mix with the sediment-aggregate communities it is unknown if they grow, survive, and contribute to the cycling of material in this new environment. The similarities between the 16S rRNA in the sediment aggregates and those within the terrestrial habitats and the dominant algae comprising mat-forming algae suggest that the terrestrial biota dominate within these perennial ice environments, and the water column derived microbiota are either outcompeted by the terrestrial microbiota in this solid-liquid environment or supplied at a much lower flux than the flux of biota from terrestrial habitats. The distinction between these two possibilities is not known at this time, and it is probable that the community structure in these habitats is determined by a balance of these two processes.

A diverse assemblage of diazatrophs, detected via recovery, amplification, and sequencing of *nifH*, resides within the sediment aggregates in the perennial ice (70), and low rates of N-fixation have been demonstrated (71,72). Hence, diazatrophic bacteria and Cyanobacteria may help these communities meet a portion of their annual nutrient demands, although the flux of nutrients via the flux of new ice from below supplies some nutrients as well. However, the autotrophic growth rates and production compared to the supply of nutrients from the flux of ice meltwater and diazatrophy are not likely to maintain new production by photoautotrophs throughout the year (64). Hence,

the ratio of new production to that based on recycled materials is likely to be low, and net biomass accumulation within the microbial community is also likely to be slow. Because these habitats are extremely heterogeneous over small scales (10- to 1,000-fold variations in sediment concentrations over 1–20 centimeters), relative to the instruments used to sample them (typically 7-cm diameter SIPRE core barrels), and biomass accumulation is slow, it will be difficult to document seasonal or annual changes in biomass within these environments. New techniques for monitoring microscale (<1 centimeter) changes in biomass within ice are perhaps ways of monitoring such processes. Viewing of microscale environments within sea ice has been accomplished on samples brought back into cold rooms using standard brightfield, phase, and epifluorescence microscopy (73). Yet, the application of microscale imaging has yet to be accomplished in situ. New probes being developed for surveying ice environments down boreholes (74) offer the potential for in situ microbiota detection and monitoring.

ECOPHYSIOLOGY OF ICE AND SNOW ENVIRONMENTS

The apparent dominance of microbes from glacial and ice sheet habitats desiccation-tolerant (see discussion in the preceding text) suggests these phenotypes confer a capacity for survival in the frozen condition and proliferation in ice. However, because aeolian deposition is the primary transport process into glaciers and ice sheets, it follows that the organisms surviving the dehydration process associated with aerosol transport are most likely to be the ones that are recovered from these ice environments regardless of their ability to survive once in ice. Hence, atmospheric transport may serve as a physiological barrier to colonization of glaciers and ice sheets.

Once in ice sheets, the ability of organisms to tolerate removal of water from cells and rewetting is likely to be a trait that predisposes the organisms to survive the rigors of a snow or ice environment in which freezing of liquid water leads to the concentration of extracellular solutes and lowering of extracellular water activity. The production of compatible solutes as osmoprotectants under such conditions also offers cryoprotection during freeze thawing (23,75). Hence, the snow and ice environments in which freezing of water is a prominent process (as opposed to melting) are likely to promote the preferential growth of desiccation-tolerant microbiota. These environments include the glacial ice of glaciers and polar ice caps, cryoconite holes, the perennial ice covers of lakes, and snowfields that experience freeze-thaw cycles. The slush habitats of ice covers on alpine lakes and the snowfields experiencing the melting process are environments that would not be expected to impose a requirement for surviving desiccation or freeze-thaw cycles. Specifically, these environments can be colonized by biota that may never experience a freezing environment. Interestingly, these environments are those that contain an abundance of eukaryotic algae, protists, and metazoa with life stages that are not generally considered to be desiccation-resistant or desiccation-tolerant. Many of the microhabitats in sea ice (e.g., the ice water interface, summer slush habitats; 56,57) are also examples of ice and snow environments in which freeze-thaw resistance may not be critical for colonization and proliferation.

Besides the production of compatible solutes, which leads to desiccation and protection against intercellular ice crystal formation (23), microbes are known to possess the ability to synthesize macromolecules affecting the ice crystal habit (76). Such molecules, broadly defined as Ice Active Substances (IASs) may nucleate ice crystal growth, depress the freezing point of water, or inhibit crystal growth or recrystallization. All of these actions may act as cryoprotection because they directly or indirectly deter the intercellular ice crystal formation that leads to mechanical loss of membrane potential and cellular mortality.

Knowledge regarding IASs comes primarily from studies on cultured microbes or microbial consortia in association with agriculture. IAS production by microbes within the ice and snow environments discussed in this review are highly limited. IASs have been detected in association with photosynthetic freshwater and terrestrial consortia in habitats experiencing partial freezing and partial dehydration (77). Interestingly, IASs have not yet been detected within the major freshwater ice or snow ice habitats, yet they have been found in bacterial isolates from polar aquatic environments (78).

Besides having to cope with changes in water between solid and liquid form, snow and ice communities continually experience temperatures of 0 °C or below. In accordance with evolutionary dogma, such environments may be expected to contain psychrophilic microbes. Surprisingly, many of the ice and snow environments reviewed herein lack the characteristics of psychrophilic microbiota (43,79). Notably, the dominance of Cyanobacteria exhibiting slow maximum growth rates and optimum growth rates above 15 °C in the Antarctic's perennial ice covers (64), cryconites, streams, and ice shelf ecosystems (43) suggest that other environmental parameters besides low temperatures provide the selection criteria for survival within these environments. Furthermore, the residence time of biota in these continually cold environments may not be of a sufficient time relative to mutation and growth rates for selective processes to be effective in producing microbes optimally adapted to low temperatures.

Environments containing communities of biota that are not specifically adapted for growth and persistence therein have been called "absolute extreme environments" (E. I. Friedmann) to delineate them from environments that may seem extreme from an anthropocentric point-of-view and yet are occupied by microbiota that are extremely well adapted to live therein. The perennial ice environments, ice shelves, glaciers, and the polar ice caps may indeed be absolute extreme environments whereby the proliferation of microbes occurs and yet may be approaching the limits to which life can persist and function.

CONCLUSION

It would be remiss not to mention that the studies of these extreme environments (absolute and not-so-absolute) and

the abilities of microbiota to survive, grow, and form microbial consortia capable of synergistic survival and growth have direct and indirect implications on assessing the possibility of microbial growth and survival in space and on other planets. Most pertinent to the studies of ice and snow environments on Earth as extraterrestrial analogs are the potential microbial habitats on the surface of Mars that are thought to have existed over 3.5 billion years ago (80), the polar ice caps of Mars of the present (81), and the *contemporaneous* ice habitats existing on Europa (82–84). In our present-day search for habitats on other worlds, we are indeed learning about the distribution and characteristics of life in the snow and ice environments on Earth. It is hoped that we will understand these cold environments on Earth to the extent necessary to define their role in the origin and evolution of life on Earth and their responses to changes in global environments in the past and in the future.

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SOIL ARCHAEA.

See ARCHAEA IN SOIL HABITATS

SOIL BACTERIA

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Bacteria are small, predominantly single-celled, prokaryotic microbes that inhabit soils throughout the world (Fig. 1). Population sizes vary with nutrient availability, temperature, aeration, and other abiotic and biotic factors, but bacteria are generally the most numerous of all soil microbes, often attaining populations of 10^8 to 10^9 colony-forming units (CFU) per gram of soil (1). Microbial growth in soils is usually limited by the availability of organic carbon, so bacterial populations are typically highest in the organic-rich surface layers of soils and in the immediate vicinity of plant roots (a distinctive habitat known as the rhizosphere). Most species grow optimally at moderate temperatures and neutral pH, but collectively, bacteria tolerate a greater range of environmental conditions than any other group of soil microbes. Bacteria also metabolize a greater variety of organic and inorganic substrates than any other group of soil organisms. Through their diverse metabolic activities, bacteria play crucial roles in soil formation, organic matter decomposition, remediation of contaminated soils, biological transformations of mineral nutrients, mutualistic interactions with plants, animals, and other soil microbes, municipal waste treatment, and plant and animal diseases.

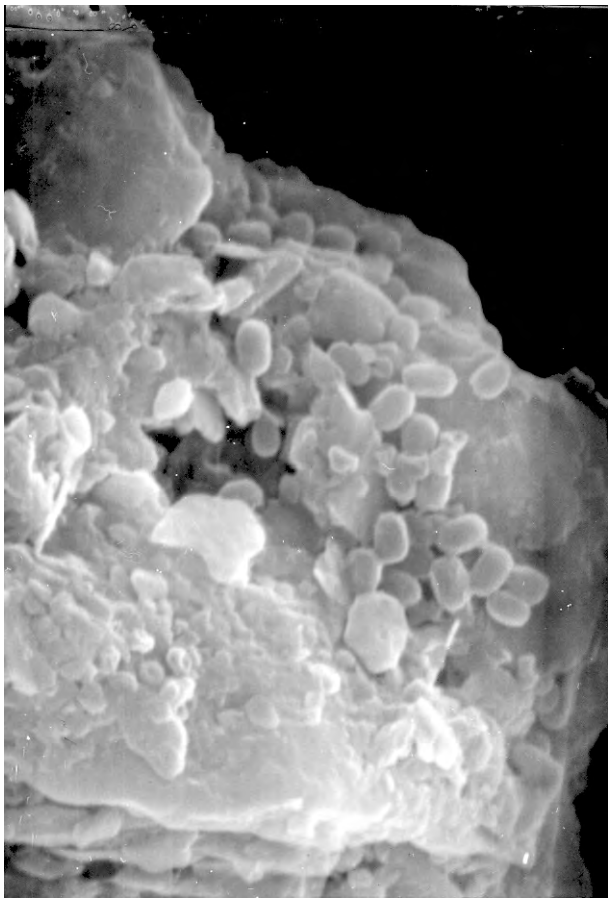

Figure 1. Scanning electron micrograph of a microcolony of short, rod-shaped bacterial cells on the surface of a soil aggregate. *Source:* Dr. E. Florance, Lewis & Clark College. Used with permission.

BACTERIAL CELL STRUCTURE AND FUNCTION

Phylogenetic schemes based on 16S ribosomal RNA (rRNA) sequences distinguish three major domains of life — Bacteria, Archaea, and Eucarya (2,3). Bacteria and Archaea are single-celled prokaryotic microbes, whereas the domain Eucarya includes single-celled and multicellular organisms with a eukaryotic cell structure. Although Bacteria and Archaea resemble one another

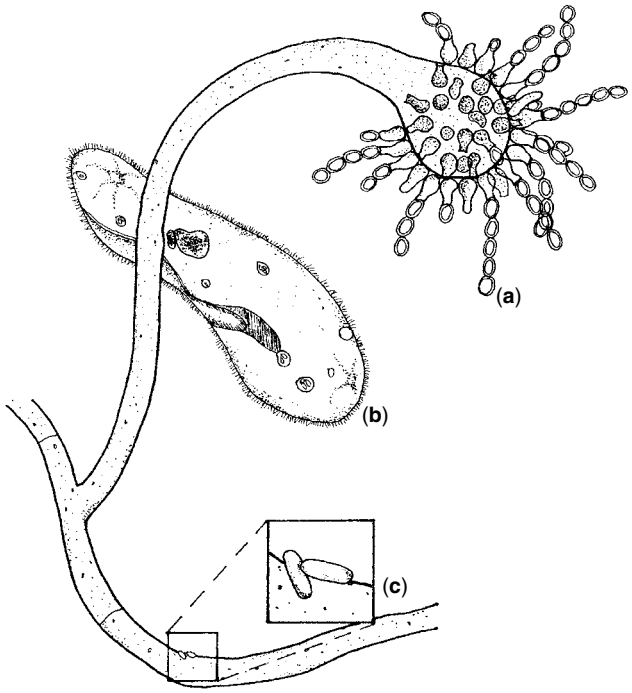

Figure 2. Relative sizes of common soil microbes. (**a**) an asexual fungal fruiting body (*Aspergillus*), (**b**) a protozoan (*Paramecium*), (**c**) a bacterium. *Source:* Original drawing by Kim Luoma. Used with permission. Drawing of fungal fruiting body adapted from J. Webster, *Introduction to Fungi*, Figure 114A, p. 201, © 1970. Reprinted with the permission of Cambridge University Press.

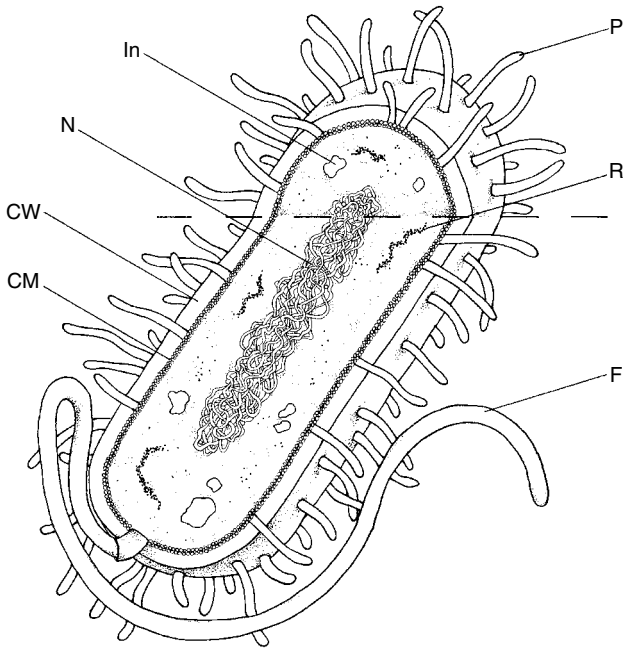

Figure 3. Generalized structure of a bacterial cell. CM = cytoplasmic membrane, CW = cell wall, N = nucleoid, In = inclusion, R = ribosomes (occurring in the form of a polysome), P = pilus, F = flagellum. *Source:* Original drawing by Kim Luoma. Used with permission.

morphologically, they differ significantly in biochemical composition and in certain aspects of their physiology and genetics.

Prokaryotic cells are distinguished by their small size and simple intracellular organization. Most bacterial cells range from 0.2 to 2 μm in diameter and from 1 to 10 μm in length. The small size of bacterial cells enables these microbes to grow and adapt to changing environmental conditions much more rapidly than larger, more complex eukaryotic organisms (Fig. 2). Small cells have a relatively large surface-to-volume ratio, which enables bacteria to rapidly absorb and distribute nutrients throughout the cytoplasm, while minimizing the amount of cellular material that they must synthesize.

Bacterial cells are composed of cytoplasm enclosed within a cell envelope, which, in most species, includes a rigid cell wall (Fig. 3). Many species produce surface appendages that function in movement or attachment to surfaces, and some form characteristic intracellular structures that serve to store nutrients or enhance survival under adverse conditions. The nuclear material of the cell consists of a single DNA molecule (and associated proteins), which is not separated from the cytoplasm by a nuclear envelope.

Cell Envelope

The envelope of a bacterial cell consists of one to three layers — the cytoplasmic membrane, cell wall, and outer membrane. The cytoplasmic membrane is the innermost layer of the cell envelope and is present in all bacteria. In most species, the cytoplasmic membrane is enclosed by a cell wall composed of a rigid, meshlike polymer known as *murein* or *peptidoglycan*. Many soil bacteria have an additional membrane, known as the outer membrane, which lies outside the cell wall. Microbiologists often refer to the combination of the peptidoglycan layer and the outer membrane as the cell wall of these bacteria.

Four groups of bacteria are distinguished by the composition and complexity of the cell envelope (Fig. 4):

- Gram-positive bacteria have a thick cell wall and no outer membrane
- Gram-negative bacteria have a thin cell wall surrounded by an outer membrane composed of phospholipids and lipopolysaccharide
- Acid-fast bacteria have a thin cell wall surrounded by an outer membrane composed of long-chain fatty acids known as *mycolic acids* (4)
- Mycoplasmas have no cell wall or outer membrane

Gram-negative and gram-positive bacteria are the most common types of bacteria in most soil habitats. Only two genera of bacteria, *Mycobacterium* and *Nocardia*, are known to have an acid-fast envelope (5). *Mycobacterium* and *Nocardia* are present in many soils, and several species are pathogenic to animals or humans. Both groups are classified with the actinomycetes, a distinctive group of soil bacteria that are discussed in more detail later in this chapter. Mycoplasmas occur strictly as plant, animal, or human parasites.

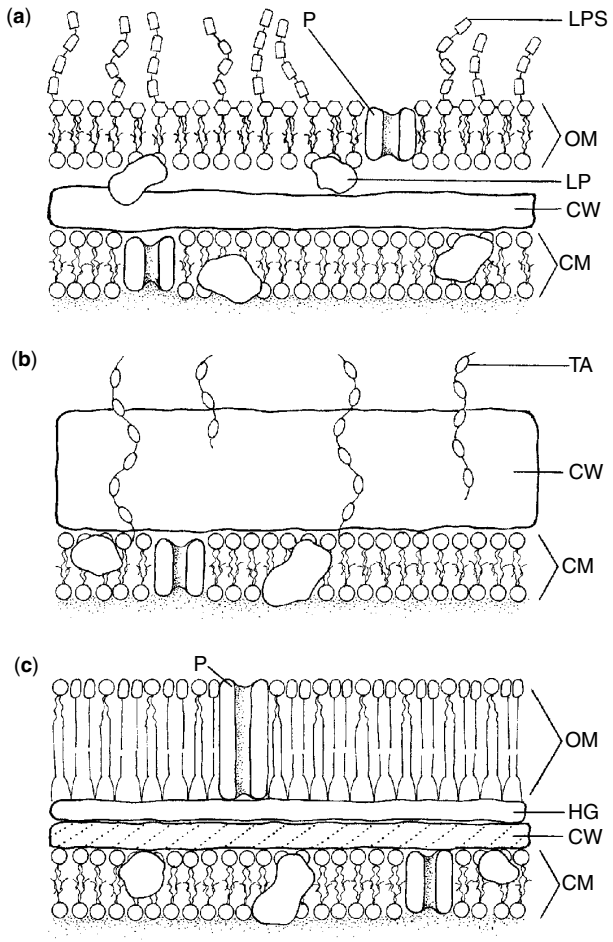

Figure 4. Structure of (**a**) gram-negative, (**b**) gram-positive, and (**c**) acid-fast bacterial cell envelopes. CM = cytoplasmic membrane, CW = cell wall (peptidoglycan layer), OM = outer membrane, LP = lipoprotein, LPS = lipopolysaccharide, P = porin, TA = teichoic acid, HG = heteroglycan. *Source:* Original drawing by Kim Luoma. Used with permission. Art adapted with permission from H. Nikaido, 1994, *Science* **264**, 382–388, Figure 1, © 1994 by the American Association for the Advancement of Science.

Cell Wall. The cell wall is the most distinctive structure of bacterial cells. Virtually all bacteria are enclosed by a wall composed of peptidoglycan, a polymer that is not found in any other type of organism. Peptidoglycan consists of long strands of alternating amino sugars, *N*-acetylglucosamine and *N*-acetylmuramic acid, with tetrapeptide side chains covalently linked to each *N*-acetylmuramic acid residue. The side chains in adjacent strands are cross-linked by short peptide chains, forming a strong, meshlike framework that encloses the cell.

The thick cell walls of gram-positive bacteria are composed of multiple layers of peptidoglycan. Gram-positive cell walls also contain teichoic acids (anionic polysaccharides composed of repeating subunits of glycerol phosphate, ribitol phosphate, or glucosyl phosphate) and lipoteichoic acids, which extend outward from the cell surface into the surrounding medium. The functions of teichoic acids and lipoteichoic acids are uncertain, but they may serve as recognition and binding sites in gram-positive bacteria and provide a negatively charged cell surface for binding cationic nutrients (6,7).

The thin cell walls of gram-negative bacteria consist of fewer layers of peptidoglycan, in some cases only a single layer, and they do not contain teichoic acids. With fewer layers of peptidoglycan and less extensive cross-linkage than typically occurs in gram-positive walls, gram-negative cell walls are generally more flexible than gram-positive cell walls.

In both gram-positive and gram-negative bacteria, the primary functions of the cell wall are to protect the cell from osmotic lysis and to determine the shape of the cell (8). The solute concentration in the cytoplasm of a bacterial cell is usually much higher than the solute concentration outside the cell. As a result, water diffuses into the cells, generating internal pressure (turgor) that would cause the cells to burst if they were not enclosed by a rigid wall. Gram-negative cells can withstand turgor pressures of up to 0.3 MPa, and gram-positive cells can withstand pressures 5 to 10 times higher (9,10).

The shape and arrangement of cells are important morphological characteristics of soil bacteria. Bacterial cells exhibit a wide variety of shapes (Fig. 5), but the shapes most commonly observed among soil bacteria are rod-shaped cells, called *rods* or *bacilli* (singular, bacillus); spherical cells, called *cocci* (singular, coccus); spiral-shaped cells, called *spirilla* (singular, spirillum); and long, branching filamentous forms, called *actinomycetes*. Some species are intermediate in shape. The more common among these are short rods, called *coccobacilli*, and short, comma-shaped spirilla, called *vibrios*.

In some species of bacteria, particularly among the cocci, individual cells often do not separate after undergoing cell division. Instead, the cells remain attached in characteristic arrangements. Cocci that divide longitudinally form chains of cells called *streptococci* (singular, streptococcus). Those that divide randomly form irregular clusters of cells called *staphylococci* (singular, staphylococcus). A few species of cocci form planar packets of four cells or cuboidal packets of eight or more cells. Rods that remain attached end-to-end following cell division form chains of cells called *streptobacilli* (singular, streptobacillus), whereas those that align side-by-side, rather than end-to-end, form an arrangement called *palisade*.

Outer Membrane. The outer membrane of gram-negative bacteria is a bilayer composed of two different types of lipid molecules. The outer layer is composed primarily of lipopolysaccharide (LPS), interspersed with phospholipid, whereas the inner layer is composed almost entirely of phospholipid. The polysaccharide portion of the LPS extends outward from the surface of the cell and is highly anionic at neutral pH. The negatively charged surface of gram-negative cells interacts strongly with cations in the surrounding solution and may facilitate the development of soil minerals (11). LPS may also play an important role in the colonization of plant roots by gram-negative bacteria (12), and the lipid portion of the polymer is toxic to animals and humans when gram-negative pathogens are attacked by host cells (13).

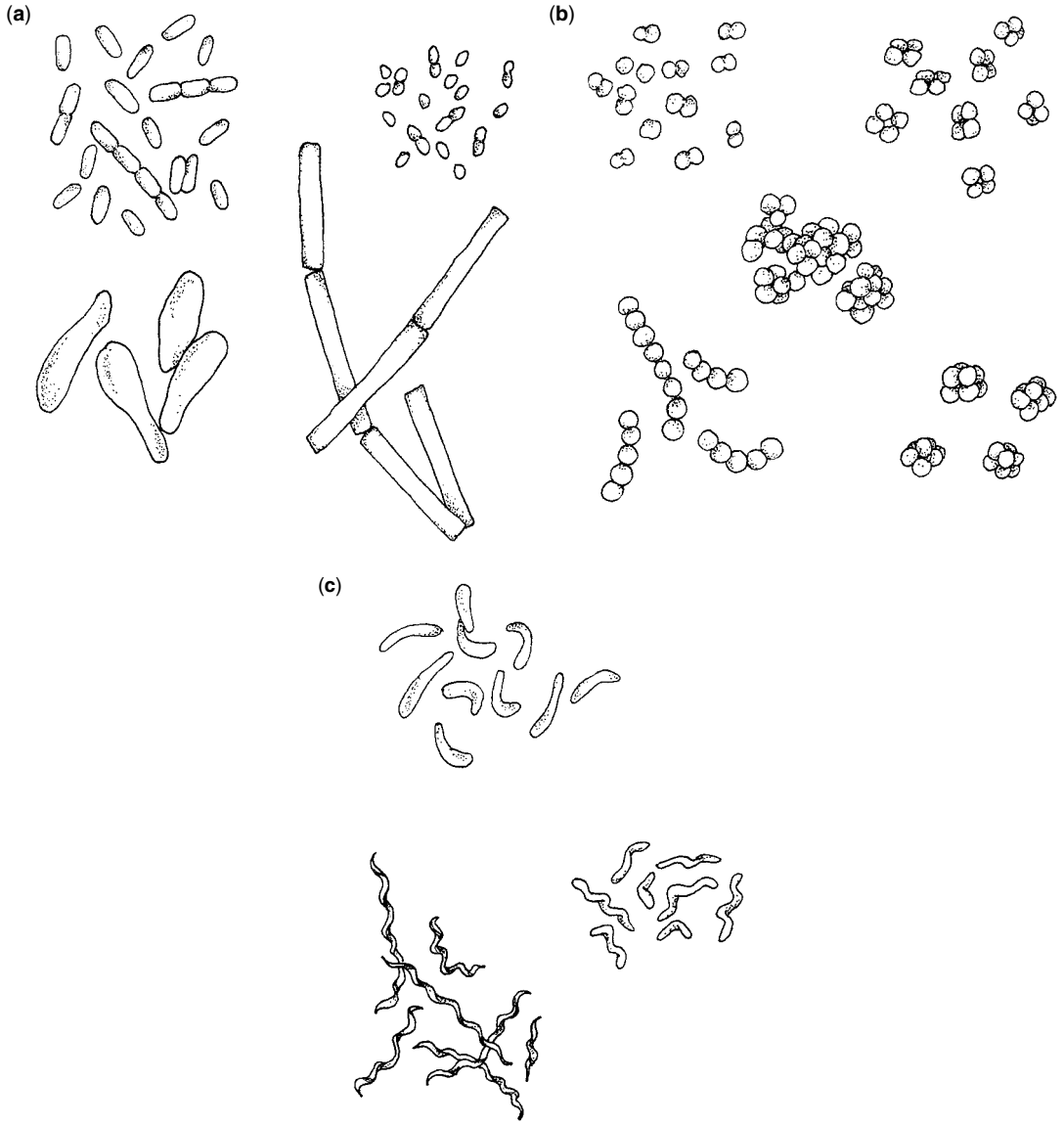

Figure 5. Common morphologies of soil bacteria. (**a**) bacilli, occurring as single rods, coccobacilli (short rods), pleomorphic (irregularly shaped) rods, and streptobacilli (chains); (**b**) cocci, occurring as diplococci (pairs), streptococci (chains), staphylococci (irregular clusters), tetrads, and sarcina (cuboidal packets); (**c**) spirilla, vibrios (comma-shaped), and spirochetes (corkscrew shaped). *Source:* Original drawing by Kim Luoma. Used with permission.

The outer membrane also contains numerous protein channels, called *porins*, which allow water and small dissolved molecules and ions to pass through the outer membrane and the porous cell wall to the cytoplasmic membrane. Larger molecules cannot pass through the porin channels and, therefore, gram-negative bacteria tend to be more resistant than gram-positive bacteria to toxic substances in the environment. Lipoproteins covalently link the outer membrane to the cell wall.

Cytoplasmic Membrane. The cytoplasmic membrane is a phospholipid bilayer studded with numerous *transmembrane* and *peripheral* proteins. Transmembrane proteins extend through the lipid bilayer and have distinct domains on either side of the membrane. Peripheral proteins attach to the inner or outer surface of the membrane by associating with the membrane lipids or by binding to the internal or external domains of the transmembrane proteins.

The phospholipid bilayer forms an effective barrier between the interior of the cell and the external environment. Only water and a few small, nonpolar molecules, such as O_2, CO_2, and N_2, can diffuse directly through this portion of the membrane. The movement of other substances into or out of the cell is mediated by transport proteins in the membrane. Some of these proteins, known as *permeases*, transport substances passively (without expending metabolic energy) in response to energetically favorable electrochemical gradients. Other proteins actively transport substances across the membrane by coupling transport with the hydrolysis of ATP (ATPases)

or by coupling the movement of one substance down an electrochemical gradient with the movement of another substance against an electrochemical gradient (cotransporters).

Electron transport proteins and quinones in the cytoplasmic membrane catalyze oxidative phosphorylation, the final stage of respiration during which bacteria synthesize most of the ATP required for growth and generate the proton (H^+) gradient, which provides energy for the uptake of many essential nutrients. Similar proteins and lipids in the membranes of photosynthetic bacteria catalyze photophosphorylation, the light-activated process by which these bacteria synthesize the ATP and reduced coenzymes needed to assimilate carbon dioxide into carbohydrates.

Sensor proteins in the cytoplasmic membrane play a key role in two-component regulatory systems, which enable bacteria to respond rapidly to changing conditions in their environment (14). When activated by changes in temperature, pH, or reduction potential, binding of external signal molecules, or other environmental stimuli, sensor proteins activate regulatory proteins in the cytoplasm (the second component of the two-component system), which control a variety of cellular activities including cellular movement, metabolism, osmoregulation, transport, competence (the ability to take up DNA from solution and become genetically transformed), and sporulation (15).

Periplasm. The portion of a gram-negative cell that lies between the cytoplasmic membrane and the outer membrane is known as the periplasm. This unique compartment, which is present only in gram-negative bacteria, contains many proteins that perform essential functions for the cell (16). Included among these are hydrolytic enzymes, which catalyze the initial steps in catabolism; detoxifying enzymes, binding proteins that facilitate the movement of substances from porin channels to specific transporters in the cytoplasmic membrane; and some enzymes involved in peptidoglycan synthesis.

Capsules. Many soil bacteria secrete polysaccharides or glycoproteins that form a distinct layer covering the surface of the cell (17). The consistency of this material varies from a thin slime layer to a thick, gelatinous capsule, depending on the species of bacteria and the type and availability of organic nutrients in the soil. The capsule or slime layer enables the bacteria to avoid desiccation as the soil dries and protects them from phagocytosis by larger soil microbes. Thick, sticky capsules may also play a role in the attachment of bacterial cells to surfaces and in the formation of biofilms. These and other extracellular polysaccharides also play an important role, along with fungal and actinomycete hyphae, in cementing sand, silt, and clay particles into stable aggregates that improve soil structure.

Cytoplasm

Nucleoid. The cytoplasm of a bacterial cell does not contain the extensive array of membrane-bound organelles that permeate the cytoplasm of larger, more complex eukaryotic cells. The most distinctive structure visible within the cytoplasm of a bacterial cell is the nucleoid (Fig. 3). This is the region within the cytoplasm that contains the bacterial chromosome, a large, double-stranded DNA molecule that contains most or all of the genetic information of the cell. The chromosomal DNA of a bacterial cell is usually circular and is closely associated with structural proteins and catalytic proteins involved in DNA replication, repair, and transcription (18). Unlike the nucleus of a eukaryotic cell, the nucleoid is not enclosed by a nuclear envelope.

The bacterial chromosome contains all of the information needed for cell growth and reproduction. Most bacteria also contain one or more additional DNA molecules, known as *plasmids*. Plasmids are usually circular, like chromosomal DNA, but they are much smaller and contain much less information. Most contain only a few genes that confer specific, nonessential functions, such as resistance to an antibiotic or toxic substance, or the ability to break down a specific substrate. Plasmids are dispersed throughout the cytoplasm and can replicate independently. As a result, bacterial cells often contain multiple copies of one or more plasmids. Plasmids are passed from generation to generation as a population of cells grow, and some contain genes that enable them to transfer copies of themselves from cell to cell, sometimes between different species of bacteria, by a process known as conjugation. Although conjugative transfer of plasmids has been shown to occur in soils (19), the extent to which this type of genetic transfer occurs in native soil populations remains uncertain.

Ribosomes. The cytoplasm surrounding the nucleoid has a uniform, grainy appearance, largely due to the presence of thousands of ribosomes actively engaged in protein synthesis (20). Bacterial ribosomes are smaller than eukaryotic ribosomes, and they are sensitive to different chemical agents; this property makes ribosomes a common target for antibacterial drugs. The difference in size is expressed in Svedburg (S) units based on the rate at which the particles sediment when centrifuged. Bacteria contain 70S ribosomes, whereas eukaryotic cells contain larger 80S ribosomes in the cytoplasm and 70S ribosomes in mitochondria and chloroplasts. Prokaryotic (70S) and eukaryotic (80S) ribosomes consist of two subunits, a small subunit (30S in prokaryotes, 40S in eukaryotes) and a large subunit (50S in prokaryotes, 60S in eukaryotes), both of which are composed of rRNA and protein. Current phylogenetic schemes designed to represent the evolutionary relatedness among living organisms are based on the degree of similarity in the nucleotide sequences of the rRNA in the small subunits of prokaryotic ribosomes (16S rRNA) and eukaryotic ribosomes (18S rRNA).

Inclusions. Other visible structures within the cytoplasm are collectively referred to as *inclusions*. The most common types of inclusions are storage granules, which are produced when an excess supply of certain nutrients is available in the soil environment. Several species of bacteria store excess carbon in the form of poly-β-hydroxybutyrate, a lipidlike substance, or glycogen, a glucose polymer. Some store excess phosphorus as polyphosphate granules, also known as metachromatic

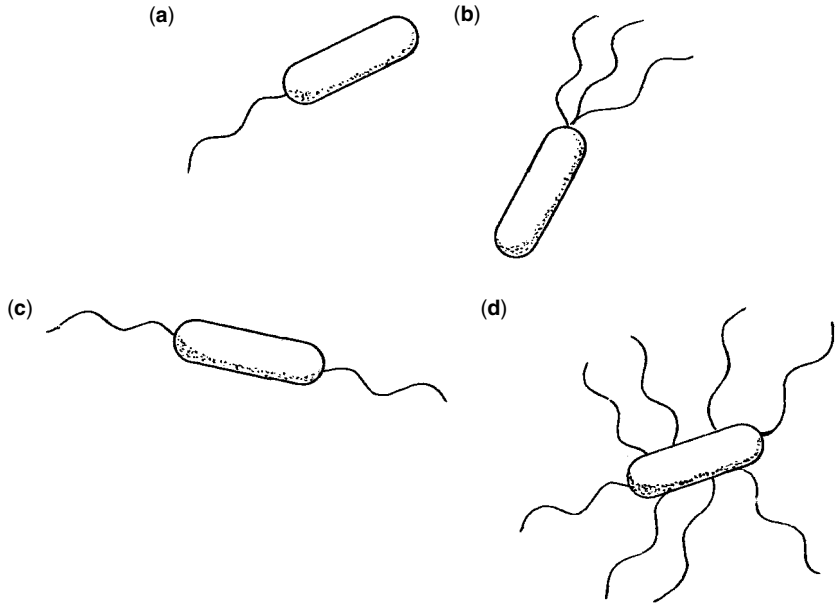

Figure 6. Common arrangements of bacterial flagella. (**a**) monotrichous (single flagellum at one pole of the cell), (**b**) lophotrichous (tuft of flagella at one or both poles of the cell), (**c**) amphitrichous (single flagellum or tuft of flagella at both poles of a cell), (**d**) peritrichous (flagella all around the cell surface). *Source:* Original drawing by Kim Luoma. Used with permission.

granules because they appear red under a light microscope when stained with methylene blue. Some sulfur-oxidizing bacteria produce elemental sulfur granules, which they can use as a source of energy when external supplies of reduced sulfur become depleted.

Appendages

Many bacteria produce slender, hairlike appendages that are anchored in the cell envelope and extend outward from the surface of the cell. The two most common types of appendages — pili (singular, pilus) and flagella (singular, flagellum) — have important functions in the soil environment.

Pili. Survival in soil often depends on the ability of bacterial species to adhere to soil particles, attach to plant roots or to other soil organisms with which they form a symbiotic association, or to form biofilms (aggregates of microbial cells attached to one another, encased in extracellular secretions, and bound to a surface) on pipelines, pilings, or other objects buried in soil. These types of attachment are mediated by pili (also known as *fimbriae*)–short, hairlike appendages that cover the surface of the cell (21,22). Many gram-positive bacteria and virtually all gram-negative bacteria are capable of producing pili. Some plasmids encode a special type of pilus, known as a *sex pilus*, which enables a host cell to attach to a recipient cell and transfer a copy of the plasmid to the recipient by conjugation.

Flagella. Many soil bacteria produce longer appendages, called *flagella*, which enable them to swim in the soil solution when the moisture content of a soil is sufficiently high. Some species form a single flagellum, whereas others produce two or more flagella arranged in characteristic ways (Fig. 6). Reversible motor proteins associated with the cytoplasmic membrane rotate the flagella to generate movement (23). Some microbiologists have questioned whether the moisture content of soils is generally high enough for this to be an important type of motility (24), but it seems unlikely that many species would have retained the complex machinery and regulatory mechanisms involved in flagellar movement if it did not provide a useful function. Flagellar motility depends largely on the thickness of water films on soil particles and on the corresponding matric potential of the soil solution. Motility is negligible at matric potentials lower than -0.1 MPa, but flagellar movement can be significant when continuous water films larger than the size of the bacterial cells are present in the soil (25).

A unique group of gram-negative bacteria known as *spirochetes* have flagella that do not extend outward from the cell envelope. In these bacteria, the flagella are arranged in a bundle, known as an *axial filament*, which wraps around the cell in the space between the cell wall and the outer membrane. Rotation of the axial filament produces a corkscrew type of movement that enables spirochetes to swim through viscous liquids, such as the aquatic sediments that they typically inhabit.

Other types of motility enable bacteria to move on surfaces, such as soil particles or aggregates that are covered by a thin film of moisture. Some species produce lateral flagella, which enable them to swarm on these surfaces, whereas others exhibit alternate forms of motility described as gliding or twitching. Several models have been proposed to explain these types of movement, but none has been established conclusively (24). It is likely that many different mechanisms operate in bacterial motility.

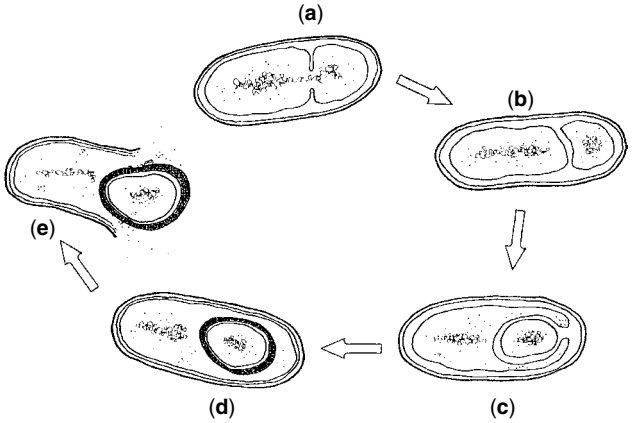

Figure 7. Formation of a bacterial endospore. (**a**) A sporulating cell replicates its nucleoid and undergoes unequal division of the cytoplasm, (**b**) a forespore (FS) develops at one pole of the cell, (**c**) the remainder of the cytoplasm surrounds the forespore, enclosing it within a double layer of membrane, (**d**) the forespore matures into an endospore by synthesizing a thick, multilayered wall around a dehydrated core, (**e**) the cell lyses and releases the mature endospore. *Source:* Original drawing by Kim Luoma. Used with permission.

Endospores

A few soil bacteria, including members of the gram-positive genera *Bacillus* and *Clostridium*, produce *endospores* in response to nutrient depletion or other environmental stresses (26). Endospores are highly resistant survival structures that enable a bacterial population to withstand high temperatures, desiccation, radiation, and exposure to toxic chemicals. As nutrients become depleted, or when cell density reaches a critical level, vegetative (actively growing) cells transform themselves into metabolically inert endospores (Fig. 7). When favorable conditions for growth return, the endospores germinate to form a new population of vegetative cells. Unlike the reproductive spores produced by fungi and actinomycetes, the primary function of bacterial endospores is to enhance survival under unfavorable conditions, rather than increasing the number of individuals in a population. Each bacterial cell in a population of sporeformers transforms itself into a single endospore, which subsequently germinates to form a single vegetative cell, so there is no increase in the number of individuals as a result of sporulation.

Endospores are among the most resistant forms of life known, and the bacteria that produce them are ubiquitous. Any method designed to sterilize materials must be sufficiently harsh to destroy these structures to ensure sterility. Standard conditions to destroy endospores in small volumes of materials with moist heat are 121 °C at 15 psi for 15 to 20 minutes. Larger volumes or dense materials, such as soil samples, require much longer exposure times.

ARCHAEA

Archaea resemble bacteria in many respects. They are prokaryotic, predominantly single-celled microbes, which display a similar variety of cellular morphologies and a similar range of metabolic capabilities. Despite these similarities, archaea appear to be distantly related to bacteria phylogenetically (2,27). Archaeal cells have a strikingly different biochemical composition, they exhibit unique metabolic capabilities not found in bacteria or eukaryotes, and they often inhabit extreme environments that are lethal to other types of organisms.

One of the major differences between archaea and bacteria is the structure and composition of the cell wall. Archaea are classified as gram-positive or gram-negative based on staining properties that parallel those observed in bacteria, but neither group of archaea contains peptidoglycan in its cell wall (28,29). Gram-positive archaea have a thick, homogeneous cell wall composed of *pseudomurein* or heteropolysaccharides. Pseudomurein resembles the peptidoglycan in bacterial cell walls, but does not contain muramic acid or the D-amino acids found in the bacterial polymer. Gram-negative archaea have a thin wall composed of protein or glycoprotein. There is no outer membrane or periplasm in these archaea, unlike their bacterial counterparts.

The unique feature of archaeal cells is the chemical nature of their membrane lipids (30). The lipids in archaeal membranes consist of branched-chain hydrocarbons linked to glycerol by ether bonds, whereas the phospholipids in bacterial and eukaryotic membranes are composed of straight-chain fatty acids linked to glycerol by ester bonds. In some archaea, diether lipids form a bilayer similar to that found in other organisms. In others, long hydrocarbon chains linked to glycerol at both ends (tetraethers) form a less-fluid monolayer membrane. The unique structure and composition of archaeal membranes is thought to play a key role in their ability to survive in extreme environments (31).

Archaea have circular chromosomes like bacteria; but a different class of structural proteins maintain archaeal chromosomes in a different physical conformation that more closely resembles the structure of eukaryotic chromosomes (32). Several key enzymes, including archaeal RNA and DNA polymerases, are also more similar to their eukaryotic counterparts than they are to the corresponding bacterial enzymes (33,34). Archaea are sensitive to many of the same antimicrobial agents as eukaryotic microbes, and are resistant to many antibacterial agents. These observations support the hypothesis based on 16S rRNA sequence analysis, that archaea share a more recent common ancestor with eukaryotes than with bacteria (2).

Archaea are often regarded as remnants of ancient microbes whose range of habitats is limited to extreme environments that are inhospitable to other types of organisms. Archaea are frequently isolated from hypersaline terrestrial or aquatic habitats, and from hot or acidic environments such as hydrothermal vents, hot springs, and anaerobic bioreactors. Recent observations indicate, however, that archaea may constitute as much as one-third of oceanic plankton (35,36). As microbiologists learn more about these microbes, perceptions regarding the distribution and ecological niches of archaea may change.

PHYSIOLOGICAL ECOLOGY OF SOIL BACTERIA

Soils teem with diverse populations of bacteria engaged in a multitude of chemical transformations that are crucial to soil fertility and soil structure, and for maintaining the intricate web of life in terrestrial ecosystems. The primary niche of soil bacteria is to function as decomposers. By feeding on nonliving organic matter in soils, saprophytic bacteria convert the complex organic compounds in plant and animal residues to carbon dioxide, water, NH_4^+, phosphate, and other simple inorganic nutrients, thereby returning these nutrients to the soil in a form that plants and other soil microbes can use. Bacteria abound in a limitless variety of habitats, play major roles in soil formation and in the cycling of several key inorganic nutrients, and participate in a broad range of interactions with one another, with other soil microbes, and with higher organisms.

Nutritional Requirements

Macronutrients. The nutrients that bacteria require in the greatest amounts are carbon, hydrogen, oxygen, nitrogen, phosphorus, and sulfur. These elements are structural components of the biological molecules (carbohydrates, proteins, lipids, and nucleic acids) that constitute most of the dry mass of bacterial cells. Potassium, sodium, calcium, and magnesium are also required in substantial amounts. These "secondary" macronutrients function as cofactors for cytoplasmic enzymes and maintain ionic balance in bacterial cells.

Soil organic matter and water are the primary sources of carbon, hydrogen, and oxygen for most soil bacteria. Some species obtain carbon and oxygen from carbon dioxide. Nitrogen and sulfur may be obtained from soil organic matter or assimilated from soluble ions (NO_3^-, NH_4^+, or SO_4^{2-}) in the soil solution. Some bacteria can use N_2 as a source of nitrogen when other forms are not available. These bacteria reduce N_2 to NH_3 by a process known as *nitrogen fixation*, sometimes in symbiotic associations with plants. Phosphorus is almost always taken up in the form of phosphate ions because bacteria are unable to transport most phosphorylated organic compounds.

The large, diverse populations of bacteria and other microbes in soils rapidly convert organic substrates into microbial biomass. Competition for these substrates is intense, and the availability of organic carbon often limits microbial growth. The availability of other macronutrients for plants and higher trophic levels depends, in part, on the concentration of these nutrients in organic residues relative to the amount of carbon that is available for microbial growth. When the concentration of nitrogen, phosphorus, or sulfur in soil organic matter exceeds that which is needed for microbial growth using the available carbon, the excess is released into the soil in a form that plants can take up. This process is termed *mineralization*. When the concentration of these nutrients is at or below the levels needed for microbial growth, the nutrients are assimilated into microbial biomass and rendered unavailable to plants, a phenomenon known as *immobilization*.

Micronutrients. Several nutrients are required in lesser amounts. These elements often serve as structural components or activators of specific enzymes in bacterial cells. Iron is the micronutrient that is usually required in the greatest amount. Others include cobalt, zinc, molybdenum, copper, and manganese.

Iron is required as a cofactor for many bacterial enzymes, including several of the electron transport proteins that function in bacterial energy metabolism. Although it is one of the most abundant elements in Earth's crust, iron often exists primarily in the form of insoluble ferric hydroxides that bacteria cannot take up. As a result, most soil bacteria produce siderophores to scavenge trace iron from the soil solution. Siderophores are low molecular weight organic chelators that have a very high affinity for Fe^{3+} ions (37). Bacteria take up the chelated iron through specific receptors in the cytoplasmic membrane. Competition for iron plays a significant role in the interactions among rhizosphere bacteria, and the production of siderophores by some pseudomonads has been suggested as a potential mechanism for the suppression of plant pathogens by these bacteria (38).

Sources of Carbon, Energy, and Reducing Power

Like all living organisms, bacteria require carbon in greater amounts than any other nutrient, and they obtain it from a variety of sources. Heterotrophic bacteria obtain carbon from soil organic matter. Most are saprophytes that feed on nonliving plant and animal residues or humus. Several species are symbionts or pathogens that invade the tissues of other living organisms. Autotrophic bacteria obtain most or all of their carbon from carbon dioxide. These bacteria assimilate carbon dioxide into carbohydrates or other organic compounds that can be metabolized to support cell growth.

In addition to carbon and other essential nutrients, bacteria must obtain energy from their surroundings and generate reduced coenzymes to fuel the biosynthetic pathways that sustain cell growth. Chemotrophic bacteria obtain energy by oxidizing organic or inorganic compounds. Phototrophic bacteria obtain energy from sunlight (or other sources of light energy). Both types of bacteria generate reducing power by coupling the oxidation of an organic or inorganic substrate with the reduction of coenzymes such as NAD^+ (nicotinamide adenine dinucleotide) or $NADP^+$ (nicotinamide adenine dinucleotide phosphate), small organic molecules that function as soluble electron carriers in bacterial cells. Those that oxidize an organic substrate are classified as organotrophs, whereas those that oxidize inorganic compounds are classified as lithotrophs. Table 1 lists the most common nutritional classes of soil bacteria, based on sources of carbon, energy, and reducing power.

Oxygen Requirements

Oxygen (O_2) concentrations vary widely in the infinite variety of microsites in soils. Large pore spaces filled with air provide a relatively high concentration of O_2, which favors aerobic metabolism, whereas smaller pore spaces filled with water can lead to the formation of strictly

Table 1. Common Nutritional Classes of Soil Bacteria

Nutritional Class	Source of Carbon	Source of Energy (ATP Synthesis)	Source of Reducing Power
Chemoorganotrophic heterotrophs[a]	Organic compounds	Chemical oxidation	Organic compounds
Photoorganotrophic heterotrophs[b]	Organic compounds	Light energy	Organic compounds
Chemolithotrophic autotrophs[c]	CO_2	Chemical oxidation	Inorganic compounds
Photolithotrophic autotrophs[d]	CO_2	Light energy	Inorganic compounds

[a] Commonly referred to simply as heterotrophs, with the understanding that organic substrates provide energy and reducing power as well as carbon.
[b] Commonly referred to as photoheterotrophs.
[c] Commonly referred to as chemoautotrophs or chemolithotrophs.
[d] Commonly referred to as photoautotrophs.

Table 2. Oxygen Requirements and Energy Metabolism of Bacteria and Archaea

Oxygen Requirement	Type of Energy Metabolism	Final Electron Acceptor	Reduced Product of Energy Metabolism
Aerobes	Aerobic respiration	O_2	H_2O
Facultative anaerobes	Anaerobic respiration[a]	Nitrate (NO_3^-) or Nitrite (NO_2^-)	N_2, N_2O, NO[b]
		Nitrate (NO_3^-)	Nitrite (NO_2^-)[c]
		Nitrate (NO_3^-)	Ammonium (NH_4^+)[d]
		Fumarate	Succinate
	Fermentation	Organic compound	Organic acids, alcohols
Obligate anaerobes	Anaerobic respiration	Sulfate (SO_4^{2-})	Hydrogen sulfide (H_2S)[e]
		CO_2	Acetic acid (CH_3COOH)[f]
		CO_2	Methane (CH_4)[g]
	Fermentation	Organic compound	Organic acids, alcohols
Aerotolerant anaerobes	Fermentation[h]	Organic compound	Organic acids, alcohols

[a] Facultative anaerobes respire aerobically when O_2 is available.
[b] Denitrifying bacteria.
[c] Nitrate-respiring bacteria.
[d] Dissimilatory nitrate reduction to ammonium.
[e] Sulfate-reducing bacteria.
[f] Acetogenic bacteria.
[g] Methanogenic archaea.
[h] Aerotolerant anaerobes are incapable of respiring aerobically or anaerobically.

anaerobic microenvironments. Oxygen concentrations can also vary widely with time, as soils undergo periods of saturation with water, which may lead to temporary hypoxic or anoxic conditions, and periods of dryness. It is not surprising then that soil bacteria exhibit a full range of adaptation to different O_2 concentrations. Table 2 summarizes the oxygen requirements of soil bacteria and the types of energy metabolism exhibited within each group.

Many soil bacteria are obligate aerobes that grow only in microsites containing relatively high concentrations of O_2. These bacteria obtain energy exclusively by aerobic respiration, so they cannot grow in the absence of O_2. Obligate anaerobes cannot survive in aerobic microsites because they lack the enzymes needed to eliminate the toxic products (hydrogen peroxide and superoxide) that are formed when O_2 serves as an electron acceptor. These bacteria reside in anaerobic microsites and generate energy by fermentation (using pyruvate or a derivative of pyruvate as final electron acceptor) or by anaerobic respiration (using an inorganic compound as final electron acceptor).

Many soil bacteria can grow in the presence or absence of O_2. Facultative anaerobes respire aerobically when O_2 is available, but can alter their metabolism to grow anaerobically in the absence of O_2. Some facultative anaerobes shift to fermentative metabolism under anaerobic conditions, whereas others shift to anaerobic respiration. In either case, aerobic respiration is the preferred mode of metabolism because the high reduction potential of O_2 makes this type of metabolism more energy efficient than fermentation or anaerobic respiration.

Habitats

Bacteria thrive in a virtually limitless variety of habitats. They are abundant in aerobic and anaerobic environments, and they tolerate an exceptionally wide range of temperatures. Most soil bacteria are mesophiles,which grow optimally at temperatures in the range of 15–35 °C. Several species, known as *thermophiles*, grow at temperatures of 45 to 80 °C, and some extreme thermophiles can grow at temperatures approaching 100 °*C*. Thermophilic bacteria typically produce heat-stable proteins that do not denature at elevated temperatures and incorporate increased amounts of saturated fatty acids in their membranes (39).

Other species of bacteria have adapted to grow at temperatures below 15 °C. Psychrophilic bacteria grow optimally at these temperatures, whereas psychrotrophic bacteria are capable of growing at 15 °C or below, but grow optimally at slightly higher temperatures. These cold-tolerant bacteria produce "cold-active" enzymes and synthesize large quantities of unsaturated fatty acids to maintain the fluidity of the cytoplasmic membrane at low temperatures (39).

Many bacteria have adapted to the acidic soils characteristic of regions with high precipitation, and some can tolerate the extremely low pH of acid springs or other specialized habitats. Acidophilic bacteria grow optimally at pH below 5, and extreme acidophiles (such as *Thiobacillus thiooxidans*, a sulfur-oxidizing bacterium) can grow at pH as low as 1. Acidophiles often incorporate acid-resistant fatty acids into their cytoplasmic membranes and maintain a neutral pH in the cytoplasm by actively transporting H^+ out of the cell (40). Alkalophilic bacteria grow at pH as high as 10.5 in arid and semiarid regions.

Poor drainage and rapid surface evaporation in arid regions often results in the formation of salt-affected or saline soils. Many bacteria have adapted to the high salt concentrations (saturation extract electrical conductivity >4 dS m^{-1}) of these soils by producing membranes and enzymes that function in solutions of high ionic strength or by accumulating solutes in the cytoplasm to compensate for the high solute concentration of the soil solution (41). Bacteria that tolerate high salt concentrations are said to be osmotolerant. Some archaea have adapted so completely to saline environments that they cannot grow without high concentrations of sodium. These salt-dependent archaea are called *halophiles*. Bacteria and archaea that tolerate dry habitats, but not necessarily high salt concentrations, are called *xerophiles*. In most soils, matric potential has a much greater influence on the availability of water for plants and soil microbes than osmotic potential.

Aerobic Habitats

Heterotrophic Bacteria. Heterotrophic bacteria play a major role in organic matter decomposition and bioremediation of soils contaminated with organic wastes. The primary products of organic matter decomposition are carbon dioxide, microbial biomass, and complex organic compounds that resist further decomposition. Aerobic or facultatively anaerobic bacteria degrade organic substrates most efficiently when a soil, compost heap, or bioreactor is well aerated. Under these conditions, the bacteria respire aerobically and a major portion of the carbon is oxidized to carbon dioxide or assimilated into microbial biomass. Carbon dioxide generated by aerobic respiration serves as a source of carbon and oxygen for autotrophic microbes and plants.

Bacteria rapidly metabolize the carbohydrates, proteins, fats, and nucleic acids in soil organic matter, but decompose other substances, such as the lignins, waxes, oils, and resins in plant residues, much more slowly. The unaltered remains of plant and animal residues, along with polyaromatic compounds that are formed during decomposition, become part of the stable organic fraction of soils known as *humus* (42). Humus improves soil structure by binding clay particles together to form stable aggregates and enhances soil fertility by improving the retention of nutrient ions and water. Nutrients are slowly released from this fraction of soil organic matter as specific groups of bacteria gradually break down its complex constituents.

Actinomycetes. A unique group of aerobic, heterotrophic bacteria known as actinomycetes deserve particular attention because of its distinctive morphology and metabolic capabilities. Actinomycetes resemble fungi morphologically. They form long, slender, branching filaments called *hyphae* and produce asexual reproductive spores, called *sporangiospores* when enclosed within a sac or *conidia* when not enclosed in a sac. They are distinguished from fungi by their small size, prokaryotic cell structure (including a gram-positive cell wall, 70S ribosomes, and the absence of a nuclear envelope), and sensitivity to antibacterial agents. Actinomycete hyphae are similar in diameter to rod-shaped bacterial cells (1–2 μm), and are considerably smaller than fungal hyphae (10 to 50 μm diameter).

Actinomycetes grow slowly and compete poorly with other soil microbes for readily available organic substrates, but they fill an important niche in soil communities by breaking down an enormous variety of organic compounds (43). Actinomycetes break down chitin, lignin, hemicelluloses, keratin, and other plant, fungal, and animal polymers that many other soil microbes do not decompose. They also tolerate alkalinity and moisture stress better than other soil microbes, which gives them a competitive advantage in arid soils.

Another distinguishing characteristic of actinomycetes is their ability to produce antibiotics. The ecological significance of antibiosis in the soil environment remains largely unknown, but the impact on human health has been enormous. More than 50 of the antibiotics currently in use are produced by actinomycetes of the genus *Streptomyces* (39). Streptomycetes are also notable for the production of *geosmins*, sesquiterpenoid compounds that give soils their characteristic earthy odor.

Chemoautotrophic Bacteria. Chemoautotrophic bacteria generate energy and reducing power by oxidizing inorganic nitrogen or sulfur compounds, iron, or hydrogen (H_2) (Table 3). The ATP and reduced coenzymes derived from these chemical oxidations are used to assimilate carbon dioxide into simple organic compounds that serve as precursors for cell growth. Synthesis of ATP is coupled to electron transport, with O_2 as the sole or preferred final electron acceptor, as in aerobic respiration of organic substrates.

Nitrifying Bacteria. Two groups of chemoautotrophs, collectively referred to as *nitrifying bacteria*, function synergistically in soils by oxidizing different forms of inorganic nitrogen (44). The ammonia-oxidizing bacteria (*Nitrosomonas* and related genera) oxidize ammonia (NH_3) to nitrite (NO_2^-), whereas the nitrite-oxidizing bacteria

Table 3. Energy Sources of Chemoautotrophic Soil Bacteria

Chemoautotrophs	Substance Oxidized to Generate ATP	Oxidized Product of Energy Metabolism	Representative Genera
Ammonia-oxidizing bacteria	Ammonia (NH_3)	Nitrite (NO_2^-)	*Nitrosomonas*
Nitrite-oxidizing bacteria	Nitrite (NO_2^-)	Nitrate (NO_3^-)	*Nitrobacter*
Sulfur-oxidizing bacteria	Hydrogen sulfide (H_2S), metal sulfides, elemental sulfur (S^0), thiosulfate ($S_2O_3^{2-}$)	Sulfate (SO_4^{2-})	*Thiobacillus*
Hydrogen-oxidizing bacteria	Hydrogen gas (H_2)	Hydrogen ions (H^+)	*Pseudomonas*, *Alcaligenes*
Iron-oxidizing bacteria	Ferrous iron (Fe^{2+})	Ferric iron (Fe^{3+})	*Thiobacillus ferrooxidans*

(*Nitrobacter* and related genera) oxidize nitrite to nitrate (NO_3^-). Both groups of nitrifying bacteria consist almost exclusively of obligate aerobes.

In well-aerated soils at neutral to alkaline pH, the NH_3 released by mineralization of organic nitrogen is rapidly converted to NO_3^-, the primary form of inorganic nitrogen used by plants. Nitrate is very soluble in water and does not adsorb to clay particles, which makes it highly susceptible to losses by leaching, runoff, and denitrification. These processes reduce soil fertility and contaminate ground and surface water. Excess NO_3^- in drinking water is harmful to wildlife and humans, especially infants.

Sulfur-Oxidizing Bacteria. Several genera of soil bacteria oxidize sulfides (H_2S or metal sulfides), elemental sulfur, or thiosulfate ($S_2O_3^{2-}$) (45). Complete oxidation of these substrates yields sulfate (SO_4^{2-}), the form of sulfur that is most commonly used as a nutrient by plants and soil microbes. Most sulfur-oxidizing bacteria are obligate aerobes or microaerophiles (aerobic organisms that grow best at low concentrations of O_2), though at least one species (*Thiobacillus denitrificans*) is a facultative anaerobe that can use nitrate as a final electron acceptor. Many are also obligate chemoautotrophs. Some sulfur-oxidizing bacteria are facultative autotrophs that oxidize organic substrates when they are available.

Sulfur-oxidizing bacteria are widely distributed in nature and highly active in well-aerated soils. Mineralization of sulfur-containing organic compounds in soils provides a large portion of the H_2S and metal sulfides that these bacteria oxidize. Sulfides may also be produced by sulfate-reducing bacteria in anaerobic microsites.

Complete oxidation of reduced sulfur compounds yields substantial amounts of H^+, in addition to sulfate, which significantly lowers the pH of the microenvironments where sulfur-oxidizing bacteria reside. Acidification of the soil solubilizes phosphates and other mineral nutrients, which generally enhances soil fertility. Several sulfur-oxidizing bacteria are obligate acidophiles that not only tolerate the acid that they produce, but grow optimally at pH less than 4. Some species are less acid-tolerant and deposit elemental sulfur rather than oxidizing sulfides to sulfate.

Surface mining operations may unearth large deposits of metal sulfides, such as iron pyrite, creating a situation that can lead to large-scale acid production by sulfur-oxidizing bacteria (46). Acid runoff from surface mines kills aquatic plants and animals and renders polluted waterways unusable as a source of water for human consumption or for industrial purposes. Microbial oxidation of metal sulfides, especially in copper and uranium ores, can also be used beneficially in mining. Sulfur-oxidizing bacteria can be used to solubilize metals when the metal content of an ore is too low for recovery by smelting by a process known as *microbial leaching* (47).

Iron-Oxidizing Bacteria. A few species of soil bacteria can obtain energy for growth by oxidizing ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}). The energy yield from this oxidation is low, so these bacteria grow slowly when using iron as a source of energy. Ferrous iron spontaneously oxidizes to Fe^{3+} under aerobic conditions at neutral or alkaline pH, but it is relatively stable at acidic pH. As a result, Fe^{2+} is most abundant in acidic or waterlogged soils and aquatic sediments. Favorable conditions for iron oxidation occur when an acidic soil is well aerated or when groundwater containing substantial amounts of dissolved Fe^{2+} seeps into an aerobic zone. The Fe^{3+} produced by microbial or chemical oxidation of iron is much less soluble than Fe^{2+}, and generally precipitates in the form of ferric hydroxides with characteristic reddish-orange colors.

Hydrogen-Oxidizing Bacteria. The chemoautotrophic bacteria that oxidize H_2 are facultative lithotrophs that normally grow heterotrophically when organic substrates are available in the soil. In the absence of an oxidizable organic substrate, they can oxidize H_2 for energy and use carbon dioxide as their source of carbon. All hydrogen-oxidizing bacteria have hydrogenases associated with the cytoplasmic membrane. Some species also have cytoplasmic hydrogenases that directly couple the oxidation of H_2 with the reduction of NAD^+.

Photoautotrophic Bacteria. Many soil and aquatic bacteria, collectively known as *cyanobacteria*, grow photoautotrophically in aerobic environments. The metabolism of these bacteria is very similar to that of eukaryotic algae and plants. Their photosynthetic pigments and electron transport proteins are organized into two photosystems, and their primary photosynthetic pigment is chlorophyll *a*. Cyanobacteria use water as the primary electron donor for

photophosphorylation and generate O_2 as a product of their metabolism. Carbon dioxide is assimilated by the Calvin-Benson cycle, as in most autotrophic organisms.

This large and diverse group of prokaryotes derives its name from the blue-green pigmentation produced by the combination of the green chlorophyll a and the blue accessory pigment phycocyanin. Some species produce phycoerythrin as an accessory pigment and, as a result, are red or brown in color. All cyanobacteria are gram-negative, but they vary widely in microscopic morphology. They may be single-celled or colonial, or form branched or unbranched filaments. Many of the filamentous cyanobacteria fix atmospheric nitrogen (N_2), usually within specialized cells called *heterocysts* which protect nitrogenase (the oxygen-sensitive enzyme that catalyzes N_2 fixation) from O_2.

Cyanobacteria thrive in many different types of soils and aquatic habitats. They are often among the most abundant primary producers in marine and freshwater environments, and in hot or saline soils. After periods of rainfall, they may form crusts on the surface of desert soils that help to retain moisture and add organic matter to the soil. Several species form symbiotic associations, known as *lichens*, with fungi on rock surfaces, and some form symbiotic associations with ferns, liverworts, or other nonvascular plants.

Anaerobic Habitats

Heterotrophic Bacteria. Organic matter decomposes slowly in anaerobic soils or sediments because anaerobic metabolism is less energy efficient than aerobic respiration. Anaerobic bacteria must oxidize more substrate to obtain comparable amounts of energy, so less is available for cell growth. Less organic carbon is converted to carbon dioxide and microbial biomass, and more accumulates as foul-smelling, acidic waste products. Carbohydrates and lipids are converted to organic acids and alcohols, whereas proteins are converted to organic acids, polyamines, and mercaptans.

In the absence of oxygen, bacteria obtain energy for growth by respiring anaerobically or by fermenting organic substrates. Anaerobic respiration involves many of the same metabolic pathways as aerobic respiration, but with different final electron acceptors. Bacteria can respire anaerobically using nitrate, sulfate, carbon dioxide, fumarate, or other inorganic or organic compounds as the final electron acceptor. Energy yields vary widely depending on the reduction potential of the final electron acceptor. Electron acceptors with higher (more positive) reduction potentials yield more energy than those with lower reduction potentials.

Table 4. Products of Common Bacterial Fermentations

Type of Fermentation	Products of Fermentative Metabolism
Ethanolic	Ethanol + CO_2
Homolactic	Lactic acid
Heterolactic	Lactic acid + ethanol + CO_2
Mixed acid	Ethanol + succinic acid + lactic acid + acetic acid + formic acid + H_2 + CO_2
Propionic acid	Propionic acid + acetic acid + CO_2
Butyric acid	Butyric acid + Acetic acid + H_2 + CO_2
Butanediol	2,3-Butanediol + lactic acid + acetic acid+ formic acid + H_2 + CO_2
Homoacetogenic	Acetic acid
Methanogenic	Methane

Fermentation involves a variety of metabolic pathways that convert pyruvate, a key intermediate in several catabolic processes, or other metabolites to various organic acids and alcohols. The energy yields of these pathways are lower than those obtained with aerobic or anaerobic respiration. Fermentative bacteria are often grouped on the basis of the products that they form under anaerobic conditions (Table 4).

Denitrifying Bacteria. Many soil bacteria can respire anaerobically using nitrate as their final electron acceptor. Most are facultative anaerobes that reduce nitrate only when O_2 is not available for aerobic respiration. In the absence of O_2, or at low O_2 concentrations, these bacteria reduce nitrate or nitrite to dinitrogen gas (N_2). Variable amounts of nitrous oxide (N_2O) and nitric oxide (NO), intermediates in the reduction pathway, are also produced depending on the pH and the concentrations of O_2 and nitrate in the soil (48). The resulting loss of available nitrogen from the soil is termed *denitrification*.

Denitrifying bacteria fill an essential niche in the nitrogen cycle, but their activity is often viewed as harmful because of its consequences in agriculture. Substantial portions of the nitrogen in fertilizers applied to agricultural crops can be lost through denitrification (42), and the nitrous oxide released into the atmosphere harms the environment by contributing to global warming and ozone depletion (49). On the other hand, denitrification is beneficial in municipal water treatment because it provides an inexpensive means to remove nitrate from wastewater and possibly even groundwater.

Sulfate-Reducing Bacteria. The ability to respire anaerobically using sulfate as the final electron acceptor is limited to a few genera of obligately anaerobic bacteria, but these bacteria are widely distributed in nature (50). Sulfate-reducing bacteria convert sulfate to hydrogen sulfide (H_2S), a gaseous end product, but H_2S is not lost from the soil to the same extent as the gaseous products of denitrification. Hydrogen sulfide reacts with metals in the soil to form insoluble metal sulfides, and it can be reoxidized to sulfate or elemental sulfur by chemoautotrophic or photoautotrophic bacteria.

Chemoautotrophic Bacteria. Two groups of prokaryotic microbes use carbon dioxide as their final electron acceptor for anaerobic respiration. Both are obligately anaerobic and both can grow chemoautotrophically, using H_2 as an electron donor, or heterotrophically. The two groups also use the same pathway to assimilate carbon dioxide when growing autotrophically; despite these similarities, they are distantly related phylogenetically and they reduce carbon dioxide to different end products.

Acetogens. Acetogens are bacteria that reduce carbon dioxide to acetate when they use carbon dioxide as the final electron acceptor for anaerobic respiration (51). When growing chemoautotrophically, they obtain energy by oxidizing H_2 and use CO_2 as the final electron acceptor for respiration and as the source of carbon. Unlike most autotrophic organisms, which assimilate carbon dioxide into sugars by the Calvin-Benson cycle, acetogenic bacteria assimilate carbon dioxide into acetyl-CoA by the acetyl-CoA pathway. Acetyl-CoA is then used as a precursor for the biosynthesis of cellular materials. Acetogens can also grow heterotrophically in anaerobic habitats, using carbon dioxide as the final electron acceptor for anaerobic respiration, and they can ferment a variety of organic substrates. Acetate is excreted as a waste product of fermentative metabolism.

Acetogenic metabolism is not associated with a particular phylogenetic group of bacteria, but is scattered among several different groups which commonly inhabit anaerobic environments. Some maintain a symbiotic association with termites. Acetogens live in the hindgut of the insect, where they covert the CO_2 and H_2 produced by cellulose fermentation into acetate, which can be absorbed and oxidized by termites (52).

Methanogens. Methanogens are archaea that reduce carbon dioxide to methane when they use carbon dioxide as the final electron acceptor for anaerobic respiration (53). Like the acetogenic bacteria, these archaea often grow chemoautotrophically, using H_2 as an electron donor and CO_2 as an electron acceptor and carbon source, and they assimilate CO_2 by the acetyl-CoA pathway. Methanogens can also produce methane heterotrophically from a few simple organic substrates, including acetate, methanol, and formate.

Methanogens thrive in anaerobic soil microsites and aquatic sediments, and in the rumen of cattle and other ruminant animals. Since they metabolize only a few simple organic substrates, they typically are members of a complex food web in these environments. Methanogens feed on the H_2, CO_2, acetate, and other products of anaerobic metabolism generated by microbes that decompose more complex organic materials. For many years, sewage treatment facilities have taken advantage of these microbial communities in anaerobic digesters, using the methane produced by methanogenic archaea as a source of energy for heat and electricity (46).

Photoautotrophic Bacteria. Two types of photosynthetic bacteria commonly inhabit anaerobic environments that have sufficient light to support phototrophic metabolism. The two groups, known as *green bacteria* and *purple bacteria*, produce different photosynthetic pigments, assimilate carbon dioxide by different biosynthetic pathways, and differ in the arrangement of photosynthetic membranes inside the cell (54). They are usually found in riparian soils, mud flats, shallow lakes or ponds, or deep, clear lakes in which light can penetrate to anaerobic depths. Unlike the cyanobacteria, they do not use water as an electron donor for photophosphorylation and they do not evolve O_2. As a result, the green bacteria and purple bacteria are often described as *anoxygenic* photoautotrophs.

Green bacteria and purple bacteria grow photoautotrophically only under anaerobic conditions, and most species in both groups are obligately anaerobic. Most purple nonsulfur bacteria, and some of the green bacteria, can grow as photoheterotrophs under anaerobic conditions, using light energy to synthesize ATP while assimilating organic compounds as their primary source of carbon. Most purple nonsulfur bacteria and some green bacteria can also grow heterotrophically under aerobic conditions. Most of the purple bacteria can also fix N_2, and thereby play an important role in the nitrogen cycle in the environments that they inhabit. Table 5 contrasts some of the distinguishing characteristics of the major groups of photosynthetic bacteria.

Green Bacteria. The primary photosynthetic pigments of the green bacteria are bacteriochlorophylls c, d, and e, and a variety of carotenoid pigments that give the bacteria a green to greenish brown color. These pigments are concentrated in cytoplasmic vesicles called *chlorosomes*.

Table 5. Characteristics of Photosynthetic Bacteria

Group	Oxygen Requirement	Photosynthetic Electron Donors	Oxidized Products	CO_2 Fixation Pathway
Cyanobacteria	Aerobes	H_2O	O_2	Calvin-Benson cycle
Green sulfur bacteria	Obligate anaerobes	H_2S, S^0, H_2	S^0, SO_4^{2-}, H^+	Reverse TCA cycle
Green nonsulfur bacteria	Usually anaerobic[a]	Usually organic compounds[b]; can use H_2 or H_2S	H^+, SO_4^{2-}[c]	Hydroxy-propionate pathway
Purple sulfur bacteria	Obligate anaerobes	H_2S, S^0, H_2	S^0, SO_4^{2-}, H^+	Calvin-Benson cycle
Purple nonsulfur bacteria	Usually anaerobic[a]	Usually organic compounds[b]; can use H_2 or H_2S	H^+, SO_4^{2-}[c]	Calvin-Benson cycle

[a] Green nonsulfur and purple nonsulfur bacteria can grow chemoheterotrophically under aerobic conditions in the dark.
[b] Green nonsulfur and purple nonsulfur bacteria preferably grow photoheterotrophically under anaerobic conditions.
[c] Products of photoautotrophic growth with H_2 or H_2S as electron donors.

Chlorosomes are cylindrical or ellipsoidal in shape and are closely associated with the inner surface of the cytoplasmic membrane. The electron transport proteins and reaction center pigments that catalyze photophosphorylation are organized into a single type of photosystem in the cytoplasmic membrane. Green bacteria use reduced sulfur compounds (H_2S, thiosulfate, or elemental sulfur) or H_2 as electron donors for photoautotrophic growth, and assimilate carbon dioxide by the reverse tricarboxylic acid (TCA) cycle or by the hydroxypropionate pathway. They generally oxidize reduced sulfides to sulfate, but in environments containing excess sulfide they form sulfur-storage granules. Interestingly, the sulfur granules are formed outside the cell, but they often remain bound to the cell surface where they can still be oxidized when needed.

Purple Bacteria. Purple bacteria produce bacteriochlorophylls a and b and various carotenoid pigments which give different species an array of different of colors — red purple, purple-violet, brown, orange, rust, or pink. They typically have an extensive system of photosynthetic membranes in the cytoplasm containing a single type of photosystem. In some species, the photosynthetic membranes form layers of flattened sheets called *lamellae*, whereas in others they form spherical vesicles. In either case, the photosynthetic membranes are closely associated with the cytoplasmic membrane.

Two types of purple bacteria are distinguished by the electron donors used for photophosphorylation. Purple sulfur bacteria oxidize reduced sulfur compounds (H_2S, thiosulfate, or elemental sulfur) or H_2, whereas purple nonsulfur bacteria oxidize organic compounds or H_2. Purple nonsulfur bacteria are capable of oxidizing reduced sulfur compounds, but the concentrations of these substrates that support vigorous growth of purple sulfur bacteria are toxic to the nonsulfur bacteria (39). Both groups assimilate carbon dioxide by the Calvin-Benson pathway (the same pathway used by cyanobacteria, algae, and plants). Purple sulfur bacteria form cytoplasmic granules of elemental sulfur when excess sulfide is available in the environment.

CONCLUSION

Bacteria are among the most numerous and ubiquitous of all soil organisms. They are single-celled, prokaryotic microbes whose small size and simple cell structure enable them to grow and adapt rapidly to changing environmental conditions. Bacterial cells consist of a cytoplasmic membrane, nucleoid, ribosomes, and a cell envelope that usually includes a cell wall composed of peptidoglycan. Two major groups of bacteria (gram-negative and gram-positive) and two smaller groups (acid-fast and mycoplasmas) are distinguished by the structure and composition of the cell envelope. Most bacteria may also produce a variety of other structures, such as capsules, pili, flagella, plasmids, inclusions, or endospores, which perform specialized functions and enhance their survival in soils.

Bacteria thrive in all types of environments and derive nutrients and energy from many different sources. Most soil bacteria are heterotrophs that feed on nonliving organic matter in soils or form symbiotic associations with plants, insects, or other soil microbes. Many are autotrophs that use carbon dioxide as their sole or primary source of carbon. Some autotrophic bacteria use light energy to synthesize the ATP and reduced coenzymes needed for carbon dioxide fixation, whereas others oxidize inorganic compounds such as ammonia, nitrite, sulfides, or H_2. Bacteria flourish in aerobic and anaerobic habitats, and many species can shift their metabolism from aerobic respiration to anaerobic respiration or fermentation in response to changing O_2 concentrations. Collectively, they tolerate exceptionally wide ranges of temperature, pH, and soil moisture (water activity).

Through their diverse metabolic activities, bacteria play major roles in soil formation, organic matter decomposition, and remediation of contaminated soils. They mineralize and immobilize inorganic nutrients, mediate all of the oxidative and reductive transformations of nitrogen and sulfur in the biogeochemical cycles of these elements, and form commensal, mutualistic, and parasitic interactions with plants, animals, humans, and other soil microbes. No other group of soil organisms has a greater impact on soil fertility, water and air quality, and on the quality of plant, animal, and human life.

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SOIL BIOREMEDIATION (PESTICIDES).

See PESTICIDE DEGRADATION IN SOILS

SOILBORNE DISEASES, BIOCONTROL OF.

See BIOCONTROL, MICROBIAL AGENTS IN SOIL

SOIL CRUSTS, BIOLOGICAL.

See DESERT ENVIRONMENTS: BIOLOGICAL SOIL CRUSTS

SOIL DISTRIBUTION OF MICROORGANISMS

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Microorganisms have significant roles in the processes of biodegradation and nutrient transformation in terrestrial ecosystems. Microorganisms are abundant in soil and sediments, and active populations are even found in geologic materials far below the earth's surface. In general, population distributions are controlled by substrate inputs. Thus, substrate-rich environments such as the plant rhizosphere or the forest-litter layer have higher population densities than do root-free surface soil. Surface

soil have far larger populations of microorganisms than deeper sediments, which receive organic substrates only through leaching of materials from the surface.

The distribution and activity of microorganisms is rarely uniformly distributed in soil and sediments. Microbial activities are often localized in "hot spots" and, conversely, greatly reduced in other areas (1). Quantification of these heterogeneous distributions can provide insights into the ecological controls that govern microbial population. Techniques for quantifying spatial variability of populations or processes that have been used in the fields of plant ecology, geology, and soil sciences are now being applied to microbiological investigations (2–4). Spatial variability or spatial distribution is the variation in a population or process across a defined space, which is, generally, defined by the context of the investigation. Variation in space may arise from gradients in environmental variables, such as temperature or substrate concentrations, or from unknown factors. A major objective of microbial ecologists working with soil, plant roots, or sediments is to estimate microbial population densities or process rates. This article briefly describes several types of random and geostatistical sampling methods, and respective data analysis procedures. These descriptions are conceptual in nature, and the reader should consult the cited literature for more in-depth descriptions.

DISTRIBUTIONS AT DIFFERENT SCALES

Selection of appropriate techniques and sampling strategy is dependent on the scale of variation. The potentially overwhelming number of different scales relevant to microbial ecology can be simplified to include the microscale, the stratum scale, and the regional or ecosystem scale (1,2). Regional or ecosystem scale studies covers the quantity and distribution of microorganisms within one or more large geographic units. The definition of a region or ecosystem is often based on criteria with little immediate meaning for the microbiologist, such as geographic or edaphic areas, agronomic-use areas, vegetation zones, or political boundaries. Sampling techniques at these scales generally use volumetric sampling and extraction of cells or their components to estimate population or processes. Total microbial biomass in surface soil is estimated to range between 110 to 1,940 kg C ha^{-1} in arable soil, 500 to 2,100 kg C ha^{-1} in forest soil, and 280 to 2,200 kg C ha^{-1} in grassland soil across a wide variety of regions (5). Even within a geographic region, microbial biomass can vary significantly, as shown in Table 1, with much of the variation attributable to different land uses.

In the Central and Southern High Plains (MLRA 67 and samples shown in Table 1), only a single soil series was sampled, but multiple soil series were sampled in the Northern Mississippi Valley Loess Hills and the Palouse Prairie MLRA. However, variability, as estimated by the standard deviation, was as great within the two MLRAs where sampling was restricted to a single soil series as in the two MLRAs where different soils were sampled as well.

The particular scale addressed in this study estimates the microbial biomass for different land uses within a region, but does not provide an estimate of the spatial distribution of the total biomass within the region or specific land uses.

An example of an assessment of spatial variational scale is the mapping of downy mildew (*Bremia lactuca* Regel) disease incidence in lettuce fields of the Salinas Valley of California (9). Surveys of 32 to 55 fields were conducted during the growing season to determine the mildew severity (by measuring the percentage of infected plants). Global positioning systems (GPS) and geographic information systems (GIS) were used to construct maps showing the spread of disease across the valley during the growing season. It should be recognized that results are often scale-dependent. Although spatial patterns present across small areas are inherently included in studies encompassing larger areas, small-area patterns may be obscured or averaged by the lower resolution required by large-area studies or by large-area patterns.

Table 1. Effect of Land Use on Mean Microbial Biomass (mg C kg^{-1} Soil) in Four Major Land Resource Areas (MLRA) in the United States. Adapted from J. J. Brejda and coworkers, *Soil Sci. Soc. Am. J.* 64, 974–982 (2000); J. J. Brejda, T. B. Moorman, D. L. Karlen, and T. H. Dao, *Soil Sci. Soc. Am. J.* 64, 2,115–2,124 (2000); and J. J. Brejda, D. L. Karlen, J. L. Smith, and D. L. Allan, *Soil Sci. Soc. Am. J.* 64, 2,125–2,135 (2000)

MLRA	All[a]	Land Use			
		Cropland	CRP[b]	Forage Pasture	Forest or Range[c]
Central High Plains	450 ± 410	310	420	560	740
Southern High Plains	180 ± 150	170	160	NA	220
Northern Mississippi Valley Loess Hills	600 ± 430	370	450	750	840
Palouse Prairie	380 ± 335	280	330	440	700

[a] Mean and standard deviation for all land uses.
[b] Conservation Reserve Program; formerly cropped lands vegetated with perennial grasses and legumes.
[c] Range land in the Central and Southern High Plains. Forestland in the Palouse Prairie and Northern Mississippi Valley MLRA.

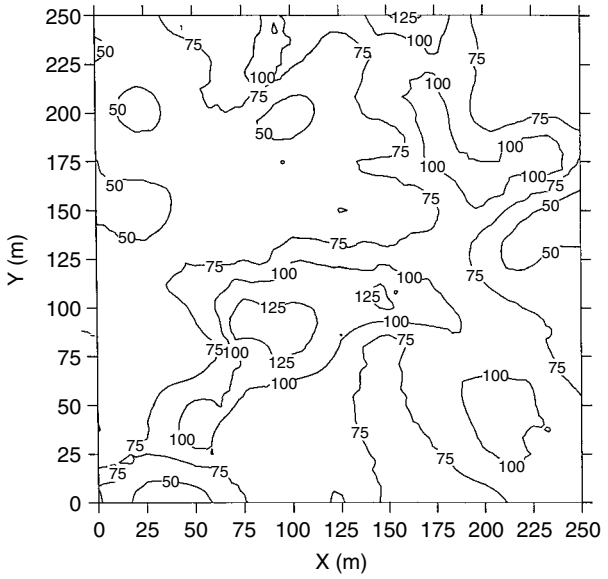

Figure 1. Microbial biomass ($g\,C/m^2$) at a 15 cm depth from the surface in an agricultural soil in central Iowa (3) estimated by kriging.

Spatial variability at the stratum scale considers variation within a single natural body, such as a particular unit of soil or sediment, a layer of water, or a plant canopy; the distributions of total microbial biomass, microbial activities (e.g., methane production); or of individual metabolic groups (e.g., cellulose degraders) in soils, subsurface sediments, or water (10,11). These studies were accomplished using a variety of sampling and enumeration methods and they address variability at the scale of a few meters to hundreds of meters in distance. The distribution of the total microbial biomass at the field scale is mapped in Figure 1.

The microscale encompasses spatial distances ranging from microns to centimeters. Direct microscopic counts of bacteria on root surfaces showed that the distribution of cells was aggregated (12). In this study, the distribution of bacteria was measured using techniques that visualize bacterial cells in situ with minimal disturbance of their natural position on the root surface. In contrast, another study followed the colonization of root systems by an *Enterobacter* strain containing *lux* genes at the centimeter scale (13). Individual cells were not counted, but the light generated by the *lux* gene was used as an indicator of *Enterobacter* colonization.

SAMPLING FOR SPATIAL DISTRIBUTIONS

Understanding the distribution of a parameter (microbial population or process) in space requires a sampling design that relates to units of soil or sediment that can be described in space. Two fundamental considerations that apply after the scale of the study is defined are estimation of the mean and the variability associated with the estimation at different points within the study space. Conventional and geostatistical sampling strategies and their respective statistical analyses of the data allow the estimation of means and variances in space, but the assumptions associated with these approaches differ sharply.

Three types of random sampling are most often used in environmental microbiology. The objective of the study may be to compare populations of microorganisms in the soil that have different histories or treatments imposed on them by an experiment, or different natural histories or gradients. The first approach is random sampling of defined areas or depths of soils at one or more locations. Microbial populations in the different areas of soil or sediment can be compared statistically. This approach assumes that rational criteria can be provided to define the sampling areas. For example, microbial populations in soils under different vegetation types could be compared. An assumption in random sampling is that the individual samples are independent of each other. An example of this approach is the measurement of population and diversity of cellulolytic bacteria in different landscape positions in an 8-ha agricultural field (11). Three soil cores were taken from each landscape position and microbiological analyses were performed. The results were reported as mean densities of culturable cellulolytic bacteria with standard errors.

Random sampling can also be applied to transect sampling, random sampling within blocks, and to stratified random sampling. Stratified sampling refers to the random sampling of individual strata (units of soil, roots, water). Individual means for each strata can be calculated, and then combined to estimate the population of a larger area, with the grand mean weighted by area, strata variance, or other appropriate factors (14). Stratified random sampling was used [Brejda and coworkers (6–8)] to measure the effects of land use on total microbial biomass in the surface soil (Table 1). Sample sites were stratified according to land use and geographic distribution within each MLRA to assure that the sampling strategy would produce accurate, unbiased measurements of microbial biomass in each land use. Morris and Boerner (15) used stratification to obtain samples from different moisture regimes in forested watersheds.

There are often situations in which effective criteria for stratification or other delineation of sampling areas are not known at the start of the investigation. Sampling of soil, sediment, or water using grids or transects can be effective in providing information on the spatial distribution of the microbial community under these circumstances. Quantitative information about the spatial distribution is often displayed through a graph or map. An example of this is the result of Cambardella and coworkers (3) shown in Figure 1. Samples were taken at 25 m intervals in a 250 ± 250 m grid, with additional sampling points at 2, 5, and 10 m intervals for a total of 241 sampling points.

ANALYSIS AND INTERPRETATION OF SPATIAL DATA

Campbell and Noe (16) described three steps in the analysis of spatial data, that is, mapping of populations, analysis of data distributions to determine randomness

or aggregation, and statistical analysis of dispersion and/or autocorrelation. There are a variety of procedures that can produce maps from spatially distributed data, irrespective of whether the data are produced from grid sampling or random sampling. Mapping requires interpolation of measured values to unmeasured areas. Contour mapping typically uses inverse distance weighted average of the nearest four to eight data points (nearest-neighbor interpolation) to estimate the value of a parameter, but alternative methods (minimum curvature, natural neighbors, kriging, radial basis function) are also available in commercially available software. These procedures produce estimated values in a defined space (a distribution), but do not estimate the variability associated with the estimate, except for kriging. An initial map of the data will help guide the course of statistical analyses.

Microbiological data are often abnormal in their statistical distribution (3,11,17), but many data are approximately log-normal in their distribution (15,16,18). Conventional statistics calculated using log-transformed data often analyzed log-normal data sets, but bias can be introduced through this approach. For instance, the geometric mean (mean of log-transformed data) is actually an estimator of the population median. Additional information on statistical procedures for abnormal distributions, including log-normal tests and nonparametric tests, are provided in References 14 and 18. Although log-normal distributions are often encountered, other distributions, such as the Weibull, gamma, and beta, should also be considered if log-transformation provides only marginal improvement in normality. The beta-binomial distribution has been applied to the data on plant disease, and the parameters derived from this distribution can be used to test the aggregation or dispersion of the pathogen's incidence in a plot or field. These tests were used in conjunction with geostatistical procedures by Xaio and coworkers (19) in their study of the soilborne pathogen, *Verticillium dahliae*, causing the wilting of cauliflower. Still, analyses based on statistical distributions generally assume that the data are independent from one another and not correlated in space.

Geostatistical sampling and analysis provide a method for estimating the distribution of a parameter over a space with an estimate of the variance associated with the estimates that describe the distribution. Geostatistical procedures are particularly useful for determining if data are autocorrelated in space and for the analysis of such data. Autocorrelation is simply the tendency of samples located near each other to be more similar than samples taken farther apart. The semivariogram relates variance in a parameter (z) to the distance between the samples (h):

$$\gamma(h) = \left[\frac{1}{2N(h)}\right] \sum_{(i=1\ldots N)} [z(x_i) - z(x_i + h)]^2$$

where $\gamma(h)$ is the summation of the semivariance in z between points $(x_i, x_i + h)$ separated by the lag distance (h). The lag distance (h) is often called the *separation distance*. N(h) is the number of pairs of sampling points separated by the separation distance (20). The semivariance $\gamma(h)$ is calculated for all combinations of x_i and $x_i + h$, as the lag distance between pairs of points (h) increases. Geostatistical sampling may be accomplished using a systematic grid sampling, random sampling, or randomized sampling within blocks or grid cells. Care must be taken to create a sampling design that leads to more than 25 pairs of points in each lag distance class (h), if the information about the variance structure is to be obtained at that distance. After the semivariance is calculated, the semivariogram is fitted to a model by nonlinear least-squares regression procedures (20). In some data sets, a single semivariogram may describe the semivariance pattern in all directions, which is termed an *isotropic semivariance*. In other data sets, there is a directional bias to the data and different semivariograms will be required for different directions (anisotropic semivariance). Directional bias may be caused by external factors, such as plant rows, or by unknown intrinsic factors. In practice, the computation of semivariance and model fitting is performed using one of a variety of software packages.

The semivariogram provides information on the variability in space. The example of a semivariogram shown in Figure 2 was produced from the 241 measurements of microbial biomass described by Cambardella and coworkers (3). The data was log-transformed before analysis and the semivariance (γ) was described by an isotropic, spherical semivariogram. The general formula for a spherical semivariogram is illustrated in Figure 2.

$$\gamma(h) = C_0 + C\left[1.5\left(\frac{h}{A_0}\right) - 0.5\left(\frac{h}{A_0}\right)^3\right] \quad \text{for } h \le A_0$$

$$\gamma(h) = C_0 + C \quad \text{for } h \ge A_0$$

where (h) is the lag distance, C_0 is the nugget variance, C is the structural variance, and A_0 is the range. In this example, the semivariance increases from a minimum value (the nugget, C_0) of approximately 0.05 to a maximum, called the *sill* ($C_0 + C$), of 0.15. The nugget accounts for approximately 31% of the total semivariance. The nugget variance represents the variation in microbial biomass that is present at the lowest value of (h), that is, 2.5 m in this study, and infers

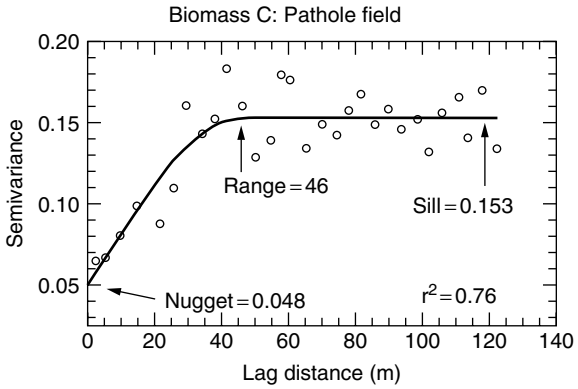

Figure 2. Spherical semivariogram for microbial biomass in a central Iowa agricultural soil.

Table 2. Range (A_0), Nugget (C_0) and Sill ($C_0 + C$) for Selected Semivariograms Describing Spatial Variability of Microorganisms in Soil. Adapted from C. A. Cambardella and coworkers, *Soil Sci. Soc. Am. J.* 58, 1,501–1,511 (1994); G. P. Roberton and coworkers, *Ecol. Appl.* 7, 158–170 (1997); L. M. Dandurand, D. J. Schotzko, and G. R. Knudsen, *Appl. Environ. Microbiol.* 63, 3,211–3,217 (1997); M. Möttönen and coworkers, *Soil Biol. Biochem.* 31, 503–516 (1999); S. J. Morris, *Soil Biol. Biochem.* 31, 1,375–1,386 (1999)

Parameter	Matrix	Semivariogram	A_0, h_{max}(m)[a]	C_0(%)[b]	$C_0 + C$
Ergosterol[c]	Forest soil	Spherical, isotropic	4, 10	0.037 (4)	0.90
Total microbial biomass	Arable soil	Exponential, isotropic	21, 200	0.067 (46)	0.146
Total microbial biomass (PHF)[d]	Arable soil	Spherical, isotropic	46, 125	0.048 (31)	0.153
Total microbial biomass (NT)[d]	Arable soil	Spherical, isotropic	190, 200	0.032 (27)	0.119
Pseudomonas fluorescens	Root surface	Spherical, anisotropic (90°)	10 to 41 $\times 10^{-6}$, 60	0.18–0.92	0.88–1.05
Fungal biomass	Forest soil	Linear sill	0.36, 2.0	2.0 (66)	3.01
Bacterial biomass	Forest soil	Linear sill	1.96, 2.0	1.88 (63)	3.04

[a] h_{max} indicates the maximum separation distance reported.
[b] Value in parentheses is the nugget expressed as a percentage of the sill.
[c] Ergosterol is a biochemical measure of fungal biomass.
[d] PHF and NT indicate two nearby fields described in Reference 4.

that substantial variation is present at smaller scales. In some semivariograms, the nugget accounts for nearly all the semivariance, which indicates that the data are randomly distributed. The range in this example is the lag distance (h = 46 m) where the semivariance reaches the sill (maximum semivariance). Samples separated by distances exceeding the range are truly independent, whereas samples separated by distances smaller than the range will tend to be increasingly autocorrelated as h becomes smaller. The data in this example were described by a spherical semivariogram, but linear, exponential, or Gaussian models may describe other data sets. Most of the commercially available softwares offer a variety of fitting options.

Although the semivariogram provides information on the variability in relation to scale (distance), it provides no information on the distribution of the measured parameter in space. Kriging is a nearest-neighbor interpolation procedure that also estimates the standard error of the kriged mean using the semiovariogram. The kriged map of microbial biomass in Figure 1 was produced using the semivariogram described in Figure 2. Kriging is an interpolation procedure, which is applicable to spatial data that is autocorrelated, which distinguishes kriging from other interpolation procedures, and which makes no assumptions about the spatial relatedness of the data.

A summary of different results obtained from studies of spatial variability of microorganisms in soil is presented in Table 2. These few data reveal several key points. First, the estimates of range (A_0) are scale dependent, which probably reflects the hierarchical nature of spatial data for microorganisms. The range of the maximum lag distance (h_{max}) in these different studies was from 60 μm to 200 m. Second, even across the different scales, the nugget accounts for a large proportion of the total variability, indicating that these measures are not strongly autocorrelated in space. Finally, even when similar measurements are made in closely adjoining physical areas, such as the two fields studied by Cambardella and coworkers (3), the parameters from the semivariograms describing the spatial variability vary considerably.

Spatial information on biological processes can provide clues about environmental controls and the interrelation of microorganisms to other organisms in the ecosystem. Rossi and coworkers (20) provide an interesting account of the application of geostatistics and other techniques to a wide variety of ecological data. Clearly, establishing some quantitative estimate of the parameter of interest (usually a mean or median) and mapping by kriging or other techniques establishes the nature of spatial distribution. The next step is interpretation, and although there are no clear guidelines to the interpretation of spatial data, the work of previous investigators offers some insights into the potential uses of spatial data.

Spatial data are often used in combination with other data obtained at the site of study. The microbial biomass distribution in Figure 1 provides an example. Although organic carbon and microbial biomass are correlated, the points deviating from this correlation trend line shows patterns. For instance, the area of the field with high biomass in the area around the coordinates of (X = 100, Y = 100) is well above the biomass predicted by regression against the organic carbon content of the soil. The relationship between two variables can be approached by conventional regression or geostatistical techniques (covariograms, correlograms), which are described in detail by Rossi and coworkers (20). The difference between conventional regression and geostatistical procedures resides in the assumptions of independence of samples, or in the autocorrelation of samples in space. Finally, geostatistics can be combined with more complex measures of the microbial community, such as the index describing genetic diversity of *Pseudomonas* strains in relation to the spatial scale reported by Cho and Tiedje (23).

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SOIL ENZYMES

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Soil may be looked upon as a biological entity (i.e, a living tissue with complex biochemical reactions). Soil contains free enzymes; immobilized extracellular enzymes stabilized by a three-dimensional network of macromolecules and enzyme proteins within microbial cells (1). Each of the organic and mineral fractions in both bulk soil and rhizosphere has a special influence on the total enzymatic activity of that soil (Fig. 1) (2).

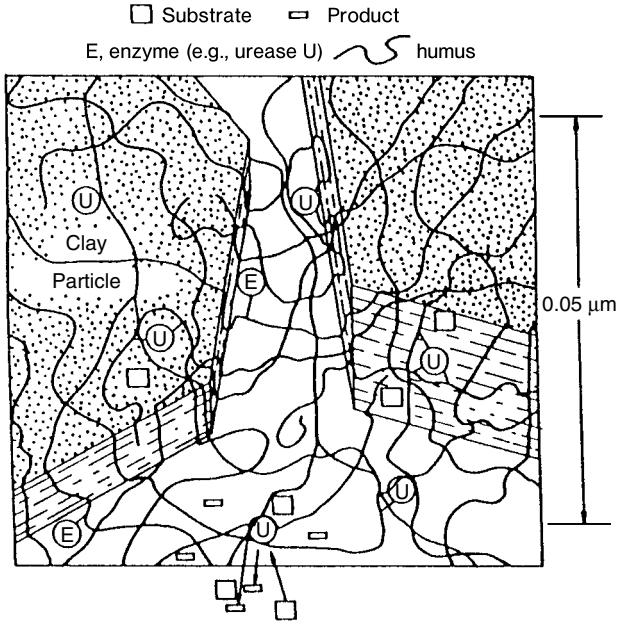

Figure 1. This model for soil enzyme location and activity consists of enzyme embedded in, and perhaps chemically attached to, a humus polymer network in contact with clay particles. Substrates, such as urea, can reach the enzyme by diffusion through pores too small for enzyme to penetrate (2).

Reactions in the soil environment involve chemical, biochemical, and physical processes. Soil contains a large number of biochemical reactions mediated by enzymes, especially those involved in nutrient (e.g., carbon, nitrogen, sulfur, and phosphorus) cycling. Most biochemical reactions are catalyzed by enzymes, which are proteins with catalytic properties. Catalysts are substances that, without undergoing permanent alteration, cause chemical reactions to proceed at faster rates. The reaction is expressed by the following equation:

$$[\mathrm{E}] + [\mathrm{S}] \underset{k_2}{\overset{k_1}{\rightleftharpoons}} [\mathrm{ES}] \xrightarrow{k_3} [\mathrm{E}] + [\mathrm{P}] \qquad (1)$$

where E refers to enzyme, S to substrate, ES is the enzyme-substrate complex, P is the product released, [] indicates concentration, and k_1, k_2, and k_3 are the rate constants.

In addition, most enzymes are specific for the type of chemical reactions in which they participate. Enzyme specificity is often dictated by the nature of the groups attached to the susceptible bonds. For example, α-chymotrypsin and trypsin are proteolytic enzymes capable of hydrolyzing certain peptide bonds in protein. α-Chymotrypsin will hydrolyze peptide bonds in which the carbonyl group (−C=O) of that bond is supplied by the amino acid L-tyrosin, L-phenylalanine, or L-tryptophan. Trypsin will hydrolyze peptide bonds in which the carbonyl group of the peptide bonds is supplied by the amino acid L-arginine or L-lysine. Another example of the specificity of enzymes involves maltase and cellubiase. Maltase hydrolyzes maltose to glucose, whereas cellobiase hydrolyzes cellobiose to glucose but not vice versa. Differences between the two substrates seem slight in that

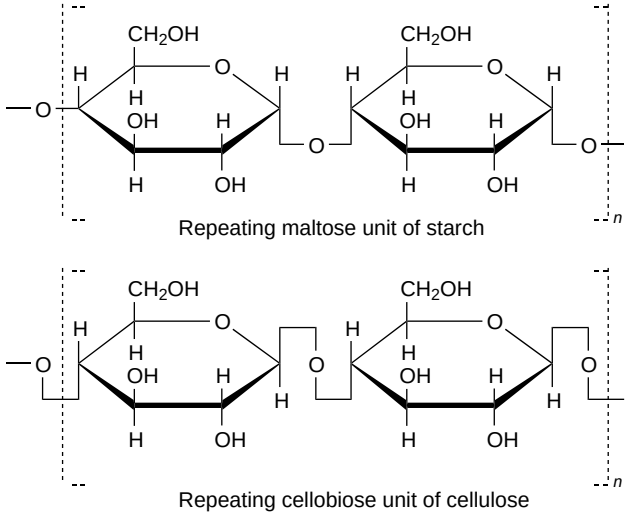

Figure 2. Repeating units of starch and cellulose.

maltose (repeating unit of starch) is an α-glucoside and cellobiose (repeating unit of cellulose) is a β-glucoside (Fig. 2).

All living systems, ranging from bacteria to the members of the animal kingdom, from algae and molds to the higher members of the plant kingdom contain myriad of enzymes catalyzing complex network of chemical reactions. Enzymes also are found in ponds, lakes, rivers, water-treatment plants, and animal manures. They exist either in extracellular forms separate from any living cell or intracellular forms as part of the living biomass. These enzymes are involved in the synthesis of proteins, carbohydrates, nucleic acids, and other components of living systems and also in the degradation of, and the cycling of, nutrients.

The study of enzymes, in general, is a subject of interest to many disciplines in science ranging from biology to physical sciences. Enzymes have a significant place in biology. Life on this planet depends on the complex of chemical reactions facilitated by specific enzymes, and any alterations in the enzyme protein structure that affect activity might have far-reaching consequences for living organisms. It is safe to say that soil would remain lifeless and basically unaltered without enzymatic reactions. Within the past five decades, enzymology, the science of studying enzymes, has developed rapidly. Scientists may now specialize in this specific field of biochemistry and often this specialty can be focused on a group of enzymes or on even a single enzyme.

FACTORS AFFECTING ENZYME REACTIONS

Temperature

The activity of any chemical reaction increases with temperature, approximately doubling for every increase of 10 °C. The rate of enzyme-catalyzed reaction increases as the temperature increases until some high temperature is reached at which the rate begins to decrease because of enzyme inactivation. Enzyme-catalyzed reactions, however, are less sensitive to temperature changes than their uncatalyzed counterparts. Whereas the uncatalyzed reaction rate may double with every 10 °C elevation of temperature, the enzyme-catalyzed reaction rate will increase by a factor of less than 2. The temperature dependence of the rate constant can be described by the Arrhenius equation:

$$k = A \exp\left(-\frac{E_a}{RT}\right), \tag{2}$$

where A is the preexponential factor, E_a is the energy of activation, R is the gas constant, and T is the absolute temperature in degree Kelvin. The Arrenhius equation can be expressed in log form

$$\log k = \left(-\frac{E_a}{2.303\,RT}\right) + \log A, \tag{3}$$

where $\log A$ and E_a can be determined from the intercept and slope, respectively, of a linear plot of the dependence of $\log k$ on $1/T$ at lower temperatures, generally between 10 and 50 °C. When the value of k is not available, apparent values or any parameter that is proportional to the rate constants, can be plotted.

pH

Changes in hydrogen ion activity influence enzymes, substrates, and cofactors by altering their ionization and solubility. Variations in such properties as ionization or solubility influences the rate of catalyzed reactions. Enzymes, being proteinaceous, exhibit marked changes in ionization with fluctuations in pH. Characteristically, each enzyme has a pH value at which the rate is optimum, and at each side of this optimum the rate is lower; thus, the catalytic action of enzymes operates in a somewhat restricted pH range. Enzymes are usually most stable in the vicinity of the optimum pH, and they are irreversibly denatured with extremes in acidity or alkalinity. Therefore, it is essential to assay (measure the reaction rate) enzyme-catalyzed reactions at, or close to, the optimum pH of activity by using a buffer. In addition to controlling the ionization of enzymes and substrates, the buffer used maintains the optimum pH for the duration of the reaction. This is especially important in enzyme assays involving substrates or products with acidic or alkaline properties (e.g., production of ammonium, NH_4^+) from hydrolysis of amides, urea, L-asparagine, and L-glutamine in soils). Also, because ionic species of the buffer might have a marked effect on enzyme activity, it is normally recommended that only one buffer system be used for determination of the pH profile.

Cofactors and Ionic Environment

Many enzymes are not catalytically active except when in combination with a nonprotein component. Generally, these nonprotein cofactors are heat-stable, dialyzable substances of low molecular weights. Cofactors are often described as activators, coenzymes, and prosthetic groups, and it is often difficult to distinguish between the meaning assigned to these terms. Prosthetic groups are usually defined as substances that are bound firmly to the enzyme, whereas, coenzymes are usually

classified as organic substances that freely dissociate from enzymes. Most coenzymes and prosthetic groups are transformed during the sequence of the catalytic reaction and need to be generated before they can again participate. Some enzymes are activated by inorganic ions furnished by mineral salts. Usually, these inorganic ions are not changed during the catalyzed process but are required before the enzyme can carry out the reaction.

Substrate Concentration

When the enzyme concentration is held constant and the substrate concentration is allowed to vary over a wide range, the velocity of the enzyme-catalyzed reaction may be described by a rectangular hyperbola (Fig. 3). At a low substrate concentration, the velocity of the reaction follows a first-order reaction equation. That is, the rate of product formation is proportional to the substrate concentration. At higher substrate concentrations, a maximum velocity is obtained, which is independent of increased concentrations of substrate, and a zero-order reaction equation applies. The magnitude of the maximum velocity is proportional to the enzyme concentration; the higher the enzyme concentration, the higher the maximum velocity. A mathematical equation describing the rate equation for the effect of substrate concentration on the velocity of enzyme-catalyzed reaction was first introduced by Michaelis and Menten (3). Derivation of their equation is presented by Neilands and Stumpf (4) and White and coworkers (5).

The Michaelis-Menten equation

$$v = \frac{V_{\max}[\mathrm{S}]}{(K_{\mathrm{m}} + [\mathrm{S}])} \tag{4}$$

satisfactorily describes the rectangular hyperbola curve shown in Figure 3. It agrees with the theory that enzymes act by forming an unstable complex of enzyme and substrate before forming a product. The symbol in the equation are as follows: v is the reaction velocity, $V_{\max}$ is the maximum reaction velocity, S is the substrate, [] is the concentration expressed in mole/liter, and K_{m} is the Michaelis constant. Equation [4] can be written in three linear transformations:

$$\frac{1}{v} = \frac{1}{V_{\max}} + \left(\frac{K_{\mathrm{m}}}{V_{\max}}\right)\left(\frac{1}{[\mathrm{S}]}\right) \tag{5}$$

Linweaver-Burk transformation

$$\frac{[\mathrm{S}]}{v} = \frac{K_{\mathrm{m}}}{V_{\max}} + \left(\frac{1}{V_{\max}}\right)[\mathrm{S}] \tag{6}$$

Hanes-Wolf transformation

$$v = V_{\max} - K_{\mathrm{m}}\left(\frac{v}{[\mathrm{S}]}\right) \tag{7}$$

Eadie-Hofstee transformation.

Plots of the variables of those relationships normally give straight lines. The values of the slopes and intercepts are commonly used for calculation of the constants from a set of experimental data. Once the K_{m} and $V_{\max}$ are known for a particular enzyme reaction under given set of conditions, the reaction velocity, v, can be calculated for any substrate concentration. The Michaelis constant (K_{m}) is the substrate concentration at which the reaction velocity is one-half of $V_{\max}$ (Fig. 3), and it is by far the most fundamental constant in enzyme chemistry. It has dimensions of concentration (mole/liter), and is constant for the enzyme under rigidly specified conditions. In addition to providing an index of the affinity of the enzyme to its substrate (the lower the K_{m} values, the greater the affinity), this constant is useful for estimating the substrate concentration necessary to give a maximum velocity. The form of the Michaelis-Menten equation is such that approximately 10 and 90% of the $V_{\max}$ is achieved at substrate concentration corresponding to $K_{\mathrm{m}} \times 0.1$ and $K_{\mathrm{m}} \times 10$, respectively (4).

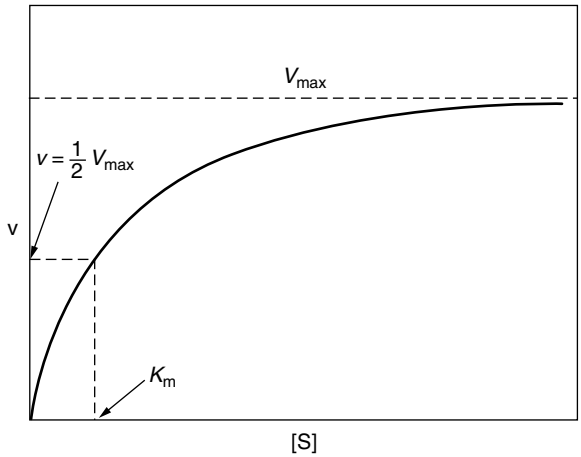

Figure 3. The rectangular hyperbola describing the effect of substrate concentration [S] on reaction velocity (v).

ENZYME INHIBITION

The subject of enzyme inhibition requires special consideration because soils receive a variety of organic and inorganic chemicals. Some of the compounds, such as fertilizers, pesticides, and animal, municipal, and industrial wastes, are added in practices of soil and crop management. Other compounds, such as salts or trace elements, are added to soils as impurities in lime and fertilizers and as industrial pollutants. Also, inhibition of soil enzymes for controlling the release of fertilizer nutrients has received attention within the past 30 years. Most of this research, however, has been concerned with the use of enzyme inhibitors for controlling urea hydrolysis in soils because urea-N is one of the most widely used nitrogen fertilizers. Unfortunately, most of the compounds tested for inhibition of urease in soils are general enzyme inhibitors, affecting not just urease activity but a wide range of soil enzymes. With the exception of the mechanisms of inhibition of soil enzymes by trace elements, generally, little information is available on the mechanisms of inhibition of soil enzymes by various inhibitors.

An inhibitor may produce an irreversible or reversible decrease in the enzyme rate constant or reaction velocity. An irreversible inhibitor forms a stable compound, often by

the formation of a covalent bond with a particular amino acid residue in the enzyme structure, which is essential for the catalytic activity. The phenomenon of reversible inhibition is characterized by equilibrium between the enzyme and inhibitor defined by an equilibrium constant (*Ki*), which is a measure of the affinity of the enzyme for the inhibitor.

Although the kinetics of enzyme inhibition are complex and varies with the enzyme, substrate, and inhibitor, three main groups of reversible inhibition can be distinguished by their characteristic effects on plots of $1/v$ vs. 1/[S] (Lineweaver-Burk plot). The kinetics of most enzyme reactions in the presence of varying concentrations of inhibitors are such that the linear double reciprocal plots of $1/v$ vs. 1/[S] show lines similar to those in the absence of inhibitor except that (*1*) the slope, (*2*) the intercept, or (*3*) both are altered. The first case is called competitive (Fig. 4a), the second uncompetitive, and the third noncompetitive inhibition (Fig. 4b). The kinetic diagnostic for uncompetitive inhibition (case 2) is that in the double reciprocal plot the slopes remain constant (i.e., the lines are parallel), but the intercepts vary. This form of inhibition is rare with single substrates. One possible, but not likely, mechanism entails combination of the inhibitors exclusively with the ES complex. This type of mechanism, however, is more common in more complex cases. The other two cases are more common with single substrates and are likely the mechanisms of inhibition of most soil enzymes (Figs. 4a and b).

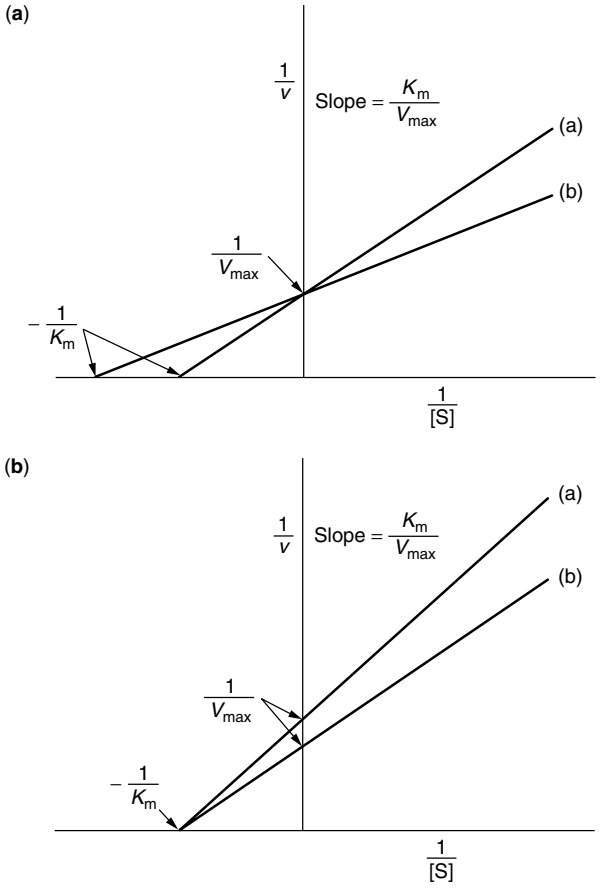

Figure 4. Linweaver-Burk plots of the Michaelis-Menten equation describing competitive inhibition; (**a**) in the presence of inhibitor and (**b**) in the absence of inhibitor. A, competitive inhibition; B, noncompetitive inhibition.

ENZYMES IN SOILS

The first known report on enzymes in soils is the paper presented by Woods at the 1899 Annual Meeting of the American Association for the advancement of Science in Columbus, Ohio (6). Little significant progress was made, however, in the area of soil enzymology until the late 1950s. This lack of progress was mainly due to lack of appropriate methods and procedures for enzyme activity measurement, especially in soils, and the lack of understanding the true chemical nature of enzymes. Although Sumner first isolated urease in crystalline form from jack bean (*Canavalia ensiformis*) meal in 1926, for which he received a Nobel Prize, it took several decades for this field of biochemistry to mature. Questions asked during the early years of soil enzyme research dealt with the origin, stabilization, importance in plant nutrition, and the role of soil enzymes in organic matter turnover. After several decades of research, many of these questions still remain to be answered.

The history of abiontic (free extracellular enzymes, enzymes bound to inert soil components, active enzymes within dead microbial cells, and others associated with the dead cell fragments collectively called abiontic enzymes) soil enzyme research has been elegantly prepared by Skujins (6), and reviews of recent advances and state of knowledge in this field are presented in a book by Burns (7) and in book articles by Kiss and coworkers (8) Skujins (9,10), Ladd (11), Dick and Tabatabai (12), Tabatabai (13), Gianfreda and Bollag (14), and Ruggiero and coworkers (15). Many of the enzymes detected in soils are hydrolases, but others fall in the class of oxidoreductases, transferases, and lyases (Table 1).

Sources of Enzymes in Soils

Microorganisms, soil animals, and plants release enzymes into the soil environment. A conceptual scheme of the composition of soil enzyme activities is illustrated by Skujins (6). It has long been known that ribonucleases and alkaline phosphatase, for example, are excreted by *Bacillus subtilis* under certain conditions, and pyrophosphatase and acid phosphatase may exist extracellularly on the surface of cell walls of *Saccharomyces mellis*. Microbial production of useful enzymes is a well-developed process. The enzymes produced include protease, amylase, glucose isomerase, pictinase, and lipase. Therefore, it is not surprising that microorganisms seem the logical choice for supplying most of the soil enzyme activity. This is because of their large biomass, high metabolic activity and short lifetimes, which allow them to produce and release relatively larger amounts of extracellular enzymes than can plants or animals.

State of Enzymes in Soils

The term *state of enzymes in soils* has been used to describe the phenomenon whereby enzymes exist in the

Table 1. Enzymes Assayed in Soils

Class	EC Number[a]	Recommended Name	Substrate
Oxidoreductases	1.1.3.4	Glucose oxidase	Glucose
	1.7.3.3	Urate oxidase	Uric Acid
	1.10.3.1	Catechol oxidase	Catechol
	1.10.3.2	Laccase	Phenylenediamine
	1.11.1.6	Catalase	Hydrogen peroxide
	1.11.1.7	Peroxidase	Pyrogallol, chloroanilines
	1.14.18.1	Monophenol monooxygenase	Catechol, pyrogallol, hydroquinone
Transferases	2.4.1.5	Dextransucrase	Sucrose
	2.4.1.10	Levansucrase	Sucrose
	2.8.1.1	Thiosulfate *S*-transferase (rhodanese)	Thiosulfate + cyanide
Hydrolases	3.1.1.1	Carboxylesterase	Hydroxymethylcoumarin butyrate, malathion
	3.1.1.2	Arylesterase	Phenyl acetate
	3.1.1.3	Triacylglycerol lipase	4-Methylumbelliferone nonanoate
	3.1.3.1	Alkaline phosphatase	*p*-Nitrophenyl phosphate
	3.1.3.2	Acid phosphatase	*p*-Nitrophenyl phosphate
	3.1.4.1	Phosphodiesterase	*Bis-p*-nitrophenyl phosphate
	3.1.6.1	Arylsulfatase	*p*-Nitrophenyl sulfate
	3.2.1.1	α-Amylase	Starch
	3.2.1.2	β-Amylase	Starch
	3.2.1.4	Cellulase	Cellulose, carboxymethylcellulose
	3.2.1.6	Endo-1,3(4)-β-D-glucanase	Laminarin
	3.2.1.8	Xylanase	Xylan
	3.2.1.20	α-D-Glucosidase	*p*-Nitrophenyl-α-D-glucoside, maltose
	3.2.1.21	β-D-Glucosidase	*p*-Nitrophenyl-β-D-glucoside, cellobiose
	3.2.1.22	α-D-Galactosidase	*p*-Nitrophenyl-α-D-galactoside, melibiose
	3.2.1.23	β-D-Galactosidase	*p*-Nitrophenyl-β-D-galactoside, lactose
		Peptidases	*N*-benzoyl-L-arginine amide, benzyloxycarbonyl
		Proteinases	Casein, gelatine
	3.5.1.1	Asparaginase	Asparagine
	3.5.1.2	Glutaminase	Glutamine
	3.5.1.4	Amidase	Formamide, acetamide
	3.5.1.5	Urease	Urea
	3.5.1.13	Arylacylamidase	Propanil
	3.6.1.1	Pyrophosphatase	Na-pyrophosphate
Lyases	4.1.1.15	Glutamate decarboxylase	Aspartic acid
	4.1.1.25	Tyrosine decarboxylase	Tyrosine
	4.1.1.28	Aromatic-L-amino acid decarboxylase	Tryptophan, DOPA
	4.3.1.3	L-Histidine ammonia lyase	L-Histidine

[a]EC number, Enzyme Commission number authorized by the International Union of Biochemistry.

soil. Describing the state of an enzyme in soils is an attempt to describe the location and microenvironment in which it functions and how the enzyme is bound or stabilized within that microhabitat. Several theories have been proposed to explain the protective influence of soil on extracellular enzyme activity. The dominant mechanisms of enzyme immobilization and stabilization that have been proposed (16) are summarized in Fig. 5. These are most likely mechanisms of enzyme immobilization in soils.

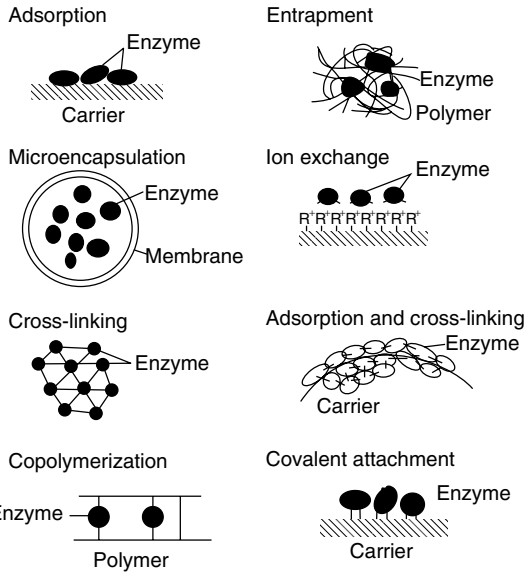

Figure 5. Schematic representation of methods of immobilizing enzymes (16). Most likely that enzymes are present in soils in more than one of those forms.

Soil Enzyme Assays

Anyone who studies enzymes is concerned with the general area of catalysis, and anyone who works with catalysis is most certainly concerned with the velocity of chemical reactions (chemical kinetics). Therefore, to understand the kinetics of an enzyme reaction in soils, it is fundamental that the meaning of the results obtained for a given sample be understood.

At the simplest level, the development of an assay procedure is necessary when investigating enzyme reactions in soils. This involves adding a known amount of soil

to a solution containing a known concentration of substrate and determining the rate at which the substrate is converted to products, under carefully controlled conditions of temperature, pH, and ionic strength. To design an adequate assay, one must know (*1*) the stoichiometry of the reaction catalyzed; (*2*) chemical species that must be present in addition to the substrate (e.g., Mg^{2+} is required for the reaction catalyzed by pyrophosphatase; the enzyme that catalyzes the hydrolysis of pyrophosphate fertilizer to the plant-available form, orthophosphate); (*3*) the kinetic dependence of the reaction on such required species; (*4*) the optimum conditions of temperature, pH, and ionic strength; and (*5*) a suitable means for monitoring product appearance or substrate disappearance. Methods used for assay of several enzymes are summarized in Table 2.

Concentrations of Active Enzyme Protein Equivalents in Soils

One of the most difficult problems currently facing soil biochemists is the separation of extracellular enzymatic activity from activity associated with living organisms

Table 2. Methods Used for Assay of Enzyme Activities in Soils

Class/EC Number	Recommended Name[a]	Reaction	Assay Conditions	
			Substrate[b]	Optimum pH
		Glycosidases		
3.2.1.20	α-Glucosidase	Glucoside-R + H_2O → Glucose + R−OH	*p*-Nitrophenyl-α-D glucopyranoside (10 m*M*)	6.0
3.2.1.21	β-Glucosidase	Glucoside-R + H_2O → Glucose + R−OH	*p*-Nitrophenyl-β-D glucopyranoside (10 m*M*)	6.0
3.2.1.22	α-Galactosidase	Galactoside-R + H_2O → Galactose + R−OH	*p*-Nitrophenyl-α-D galactopyranoside (10 m*M*)	6.0
3.2.1.23	β-Galactosidase	Galactoside-R + H_2O → Galactose + R−OH	*p*-Nitrophenyl-β-D galactopyranoside (10 m*M*)	6.0
		Amidohydrolases and arylamidase		
3.4.11.2	Arylamidase[c]	L-leucine β-naphthylamide → L-leucine + β-naphthylamide	L-leucine β-naphthylamide (2.0 m*M*)	8.0
3.5.1.1	L-Asparaginase	L-Asparagine + H_2O → L-aspartate + NH_3	L-Asparagine (50 m*M*)	10.0
3.5.1.2	L-Glutaminase	L-Glutamine + H_2O → L-glutamate + NH_3	L-Glutamine (50 m*M*)	10.0
3.5.1.4	Amidase	R−$CONH_2$ + H_2O → NH_3 + R−COOH	Formamide (50 m*M*)	8.5
3.5.1.5	Urease	Urea + H_2O → CO_2 + $2NH_3$	Urea (20 m*M*)	9.0
4.3.1.1	L-Aspartase[d]	L-Aspartate + H_2O → Fumarate + NH_3	L-Aspartate (200 m*M*)	8.5
		Phosphatases		
3.1.3.1	Alkaline Phosphatase	RNa_2PO_4 + H_2O → R−OH + Na_2HPO_4	*p*-Nitrophenyl phosphate (10 m*M*)	11.0
3.1.3.2	Acid Phosphatase	RNa_2PO_4 + H_2O → R−OH + Na_2HPO_4	*p*-Nitrophenyl phosphate (10 m*M*)	6.5
3.1.4.1	Phosphodiesterase	R_2NaPO_4 + H_2O → R−OH + $RNaHPO_4$	*Bis*-*p*-Nitrophenyl phosphate (10 m*M*)	8.0
		Sulfatase		
3.1.6.1	Arylsulfatase	$ROSO_3^-$ + H_2O → R−OH + H^+ + SO_4^{2-}	*p*-Nitrophenyl sulfate (10 m*M*)	5.8

[a] For the methods used, see Tabatabai (13).
[b] Figures in parentheses are the substrate concentrations under assay conditions.
[c] Acosta-Martínez and Tabatabai (17).
[d] Senwo and Tabatabai (18).

in soils. Although antiseptic agents have been used to retard or minimize microbial metabolism during the assay, especially in assay involving long incubation time (several hours), it is essential to evaluate the effect of the antiseptic agent on the enzyme-catalyzed reaction rate before it is used in soil assays. Methods that are based on sterilization of soil samples with high-energy irradiation or with use of antibiotics have also been evaluated, but such methods also alter enzyme reaction rates.

Recent work at Iowa State University showed that a chloroform fumigation technique, coupled with the use of a reference protein preparation could be used to estimate the enzyme activity associated with the microbial population and the extracellular enzyme activity in soils. Because enzyme proteins are present at different locations in the microbial cells, this method was useful in estimation of activities of urease (the enzyme involved in hydrolysis of urea fertilizer in soils) and arylsulfatase (the enzyme involved in hydrolysis of ester sulfate of soil organic matter to plant-available form, sulfate). Results with 10 Iowa surface soils (0 to 15 cm) showed that, expressed as percentages of total activity, the urease activity of the microbial biomass ranged from 37 to 73% (average = 54%). The remaining activity (27 to 63%, average 46%) was extracellular (Table 3) (19). The arylsulfatase activity of the microbial biomass ranged from 40 to 73% (avearge = 58%). The remaining activity (27-60, average = 43%) was extracellular (Table 4) (20).

Table 3. Urease Activity of Various Pools in Selected Iowa Surface Soils

	Urease Activity[a]			
Soil	Total mg NH_4^+-N kg^{-1}2 h^{-1}	Extracellular mg NH_4^+-N kg^{-1} 2 h^{-1}	Microbial Biomass	Specific Activity μg NH_4^+-N mg^{-1} C_{mic} $2h^{-1}$
Weller	23	12 (52.2)	11 (47.8)	95
Grundy	56	22 (39.3)	34 (60.7)	187
Luther	35	22 (62.9)	13 (37.1)	419
Pershing	38	14 (36.8)	24 (63.2)	85
Clinton	103	36 (35.0)	67 (65.0)	243
Gosport	57	31 (54.4)	26 (45.6)	110
Grundy	35	17 (48.6)	18 (51.4)	101
Muscatine	38	22 (57.9)	16 (42.1)	80
Okoboji	104	28 (26.9)	76 (73.1)	217
Harps	146	67 (45.9)	79 (54.1)	101
Average	64.3	27.1 (46.0)	36.5 (54.0)	164

Source: Ref. (19). S. Klose and M. A. Tabatabai, *Soil Biol. Biochem.* **31**, 205–211 (1999).
[a] Total urease activity is that obtained for the chloroform-fumigated sample without toluene. Extracellular is the activity obtained for the nonfumigated sample without toluene. Specific activity is urease activity of the microbial biomass divided by C_{mic} (organic carbon of the microbial biomass). Figures in parentheses are the values expressed as percentages of the total activity.

Table 4. Arylsulfatase Activity of Various Pools in Selected Iowa Surface Soils

	Arylsulfatase Activity[a]			
Soil	Total mg PN	Extracellular kg^{-1} soil h^{-1}	Microbial Biomass mg PN kg^{-1} soil h^{-1}	Specific Activity μg PN mg^{-1} C_{mic} h^{-1}
Weller	69	27 (39.1)	45 (65.2)	388
Grundy	133	58 (43.6)	75 (56.4)	412
Luther	4	2 (50.0)	2 (50.0)	65
Pershing	128	48 (37.5)	80 (62.5)	497
Clinton	231	109 (47.2)	122 (52.8)	442
Gosport	187	92 (49.2)	95 (50.8)	403
Grundy	170	65 (38.2)	105 (61.8)	490
Muscatine	295	105 (35.6)	190 (64.4)	955
Okoboji	301	81 (26.9)	220 (73.1)	613
Harps	361	218 (60.4)	143 (39.6)	182
Average	188	80.5 (42.8)	107 (57.7)	455

Source: (20) S. Klose and M. A. Tabatabai, *Soil Sci. Soc. Am. J.* **63**, 569–574 (1999).
[a]Total arylsulfatase activity is that obtained after chloroform fumigation without toluene treatment. Extracellular is the activity obtained from total–nonfumigated without toluene. Specific activity is arylsulfatase activity of the microbial biomass divided by C_{mic} (organic carbon of the microbial biomass). Figures in parentheses are the values expressed as percentages of the total activity.

Table 5. Estimated Active Enzyme Proteins in Selected Iowa Surface Soils

	Enzyme Protein Equivalent (mg Protein kg^{-1} soil)[a]												
	Glycosidases[b]				Amidohydrolases[c]					Phosphatases[d]		Arylsulfatase[e]	
Soil	α-Gal	β-Gal	α-Glu	β-Glu	L-Asg	L-Glu	Amid	Urea	L-Asp	Acid-P	Alk-P	A	B
Harps	0.031	1.65	4.78	0.021	2.38	1.18	4.25	3.60	8.52	12.2	5.2	9.0	37.6
Okoboji	0.038	2.12	4.78	0.018	0.82	0.69	4.27	2.60	3.28	21.5	3.2	7.5	31.4
Muscatine	0.018	1.18	3.58	0.014	0.84	0.58	3.60	0.95	2.81	16.3	3.3	7.3	30.7
Grundy	0.022	1.06	3.58	0.014	0.56	0.45	2.80	0.87	1.84	25.7	1.8	4.2	17.7
Gosport	0.035	2.53	3.88	0.014	0.59	0.57	3.46	1.42	2.81	29.2	1.8	4.6	19.5
Clinton	0.033	2.76	4.18	0.019	0.80	0.71	7.30	2.60	2.88	33.5	2.1	5.7	24.1
Pershing	0.028	1.41	3.88	0.013	0.35	0.22	2.12	0.95	1.09	34.5	0.97	3.2	13.3
Luther	0.010	0.29	1.64	0.005	0.41	0.10	0.65	0.87	0.40	8.8	0.36	0.10	0.42
Grundy	0.033	1.47	3.28	0.012	0.37	0.38	3.32	1.39	1.65	21.0	1.1	3.3	13.9
Weller	0.020	1.18	3.13	0.011	0.19	0.14	1.98	0.57	0.78	22.7	0.97	1.7	7.2
Average	0.027	1.56	3.67	0.014	0.73	0.50	3.38	1.58	2.61	22.5	2.1	4.7	19.6

source: (21) S. Klose and M. A. Tabatabai, *Agron. Abstr.* p. 211 (1998).

[a] Calculated for the nonfumigated soils (except for urease and arylsulfatase that were based on fumigated samples) based on their activity values and specific activities of the purified reference enzyme proteins.

[b] α-Gal: α-Galactosidase, β-Gal: β-Galactosidase, α-Glu: α-Glucosidase; β-Glu: β-Glucosidase.

[c] L-Asg: L-Asparaginase; L-Glu: L-Glutaminase; Amid: Amidase; Urea: Urease; L-Asp: L-Aspartase.

[d] Acid-P: Acid phosphatase; Alk-P: Alkaline phosphatase.

[e] A: source: *H. pomatia*; B: source: *P. vulgata*.

Other recent studies estimated the concentrations of 12 enzyme proteins in soils (21). The averages in 10 Iowa surface soils ranged from 0.014 mg active protein kg^{-1} soil for β-glucosidase to 22.5 mg active protein kg^{-1} soil for acids phosphatase (Table 5). These calculations were not intended to give accurate concentration of enzyme proteins in soils but instead provide some quantitation of enzyme protein equivalent. Actual concentrations of enzyme proteins in soils are undoubtedly much greater than those calculated because many soil components can inhibit activity, and structural stabilization also leads to decreases in the activity. The calculation do illustrate one reason for the difficulty encountered in the extraction and purification of enzymes from soils (22). From the results reported in Table 5, it is clear that the small concentrations of enzyme proteins in soils are either denatured during extraction or bound tightly with the soluble carbohydrates in soils that makes their separation very difficult.

FACTORS AFFECTING ENZYME ACTIVITIES IN SOILS

Among the many factors that may affect enzyme activities in soils, cropping history, soil amendments, and some environmental factors have the most influence. Seasonal variation in urease and arylsulfatase activities has been documented (23). Waterlogging (flooding) of soil produces changes in the redox potential and pH, and consequently, in the activity of many enzymes (24). Other studies on the extracellular enzyme activities in ecosystems have shown that vegetation cover, agricultural chemicals, and industrial pollutants have a marked influence on soil enzymes (25). Generally, the effect observed differ markedly and depend on many factors. Because of the importance of urea as a fertilizer in world agriculture and the potential of soil urease to cause volatilization of NH_3 produced from urea hydrolysis in soils, especially from calcareous soils, several workers have investigated various enzyme inhibitors for agricultural application. Discussion of the inhibitors tested on urease activity in soils and enzyme interactions with various agricultural and

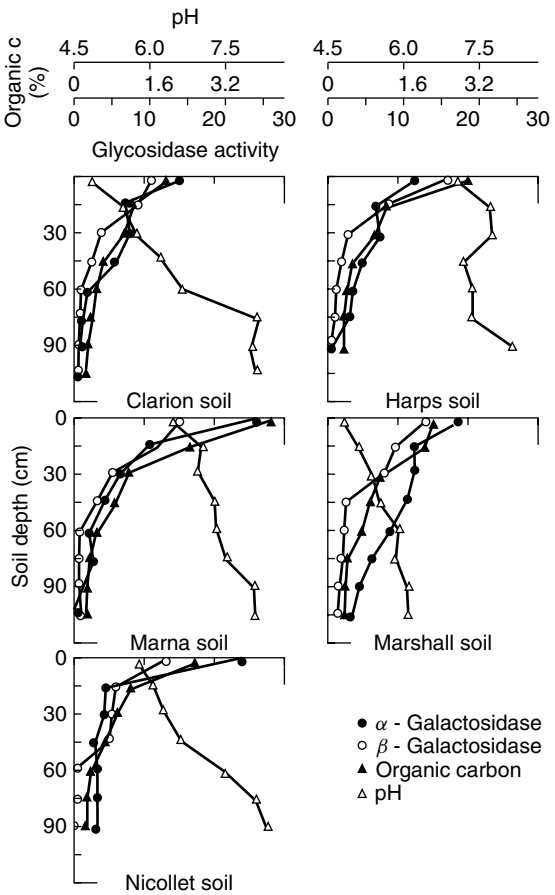

Figure 6. Distribution of organic C, α- and β-gactosidase activities and trends of pH with depth in soil profile samples. Galactosidase activity is expressed in mg of *p*-nitrophenol released kg^{-1} soil h^{-1} at 37 °C (29).

industrial chemicals, including pesticides, is presented by Kiss and coworkers (8) and Bremner and Mulvaney (25).

Distribution in Soil Profiles

Although the patterns of distribution of the activities of several enzymes in soil profiles have been studied by many workers, the relative factors that affect the activities through the profile have not been clearly established. As with other biochemical reactions in soils, however, enzyme activities are associated with organic matter distribution profile and generally decrease with depth (Fig. 6). This has been demonstrated for arylamidase (26), arylsulfatase (27), urease (28), glycosidases (29), amidase (30), L-asparginase (31), and L-glutaminase (32).

Cropping Systems

Sustainable management practices in agriculture, including crop rotations and fertilization systems to maintain soil quality and productivity and minimize the negative effects of agricultural production on the environment, have significant effects on soil biochemical processes. Long-term cropping systems and fertilization can influence important soil properties such as soil structure and density, pH, the quantity, quality, and distribution of soil organic matter and nutrient cycles within the soil profile. Most of those effects are derived from changes in microbial biomass carbon and nitrogen. In general, the highest microbial biomass carbon and nitrogen contents are found in multicropping systems involving oats or meadow, and the lowest in continuous corn and soybean systems (33). Similarly, the highest enzyme activities are found in systems that contain oats or meadow in the rotation and the lowest in soils under continuous corn or soybean system, as shown for total, activity of the microbial biomass, and extracellular activity of arylsulfatase in Fig. 7. This is mainly derived from the fact that the fine root systems of oats and meadow (alfalfa) have much greater surface area and rhizosphere than the roots systems of corn and soybean.

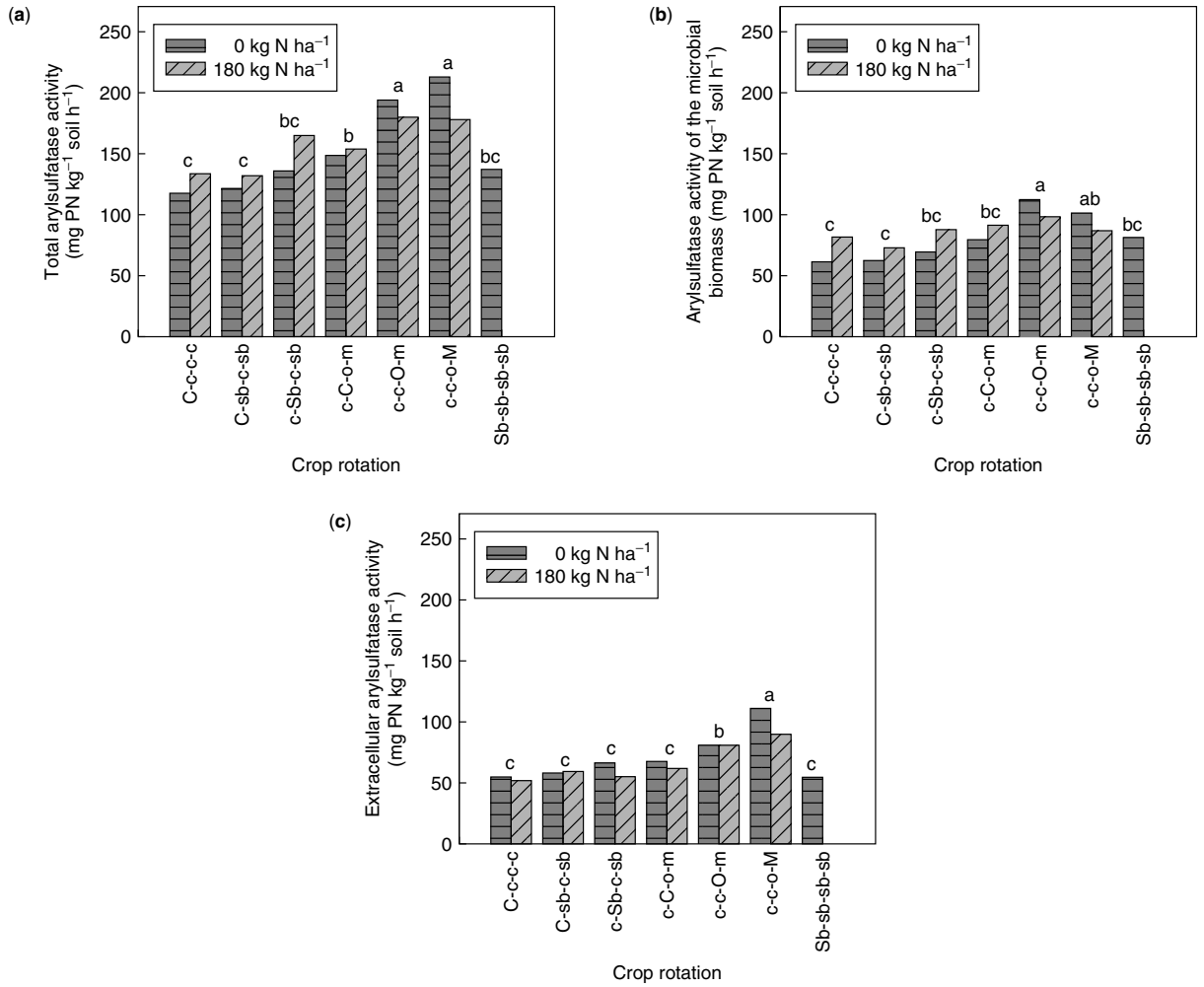

Figure 7. Effect of crop rotation on total (A), microbial biomass (B), and extracellular (C) arylsulfatase activity in soils at the Northeast Research Center, Iowa in 1996. *Different letters* indicate significantly different means at $P < 0.05$ according to the least significant difference test. For crop rotations, *capital letter* indicates crop in which samples were taken. *C* corn, *Sb* soybean, *O* oats, *M* meadow, *PN* *p*-nitrophenol (40).

Tillage and Residue Management

Tillage and crop-residue management practices may lead to significant changes in biological, chemical, and biochemical properties of soils and alter the composition, distribution, and activities of soil microbial community and enzymes. Organic carbon and nitrogen accumulate at the surface soil (0–2.5 cm) under reduced tillage involving crop-residue placement. Accumulation of inorganic nitrogen, sulfur, and phosphorus at the surface soils may also occur under no-till systems with residue placement. In addition, fungal densities are affected by long-term tillage practice, with higher densities of fungal hyphae but lower colony-forming units in buried residues of conventional tillage than in surface residues on no-till. The difference in microbial dynamics and population, due to soil management practices, may also be reflected in the differences in enzyme activities of soils, as have been shown for the activities of glycosidases, amidohydrolases, phosphatases, and arylsulfatase by Deng and Tabatabai (34–36).

pH and Lime Treatment

Soil acidity or alkalinity under field conditions affect numerous biochemical reactions. Among the many enzymes studied in soils, the activities of phosphomonoestearses are the most interesting because there are two such enzymes (acid and alkaline phosphatases). Studies have shown that acid phosphatase is predominant in acid soils and that alkaline phosphatase is predominant in alkaline soils (37–39). There are two reasons for this phenomenon. The first reason is that acid phosphatase is produced by fungi, which are predominant in acid soils, and that alkaline phosphatase is produced by bacteria, which are predominant in alkaline soils. The second reason is that these enzymes are induced in the microbial population and this induction is affected by the soil environment; to overcome the environmental limitations the organisms produce more enzyme proteins to release inorganic phosphorus for microbial nutrition. The effect of soil pH on the distribution of acid and alkaline phosphatases is shown in Fig. 8.

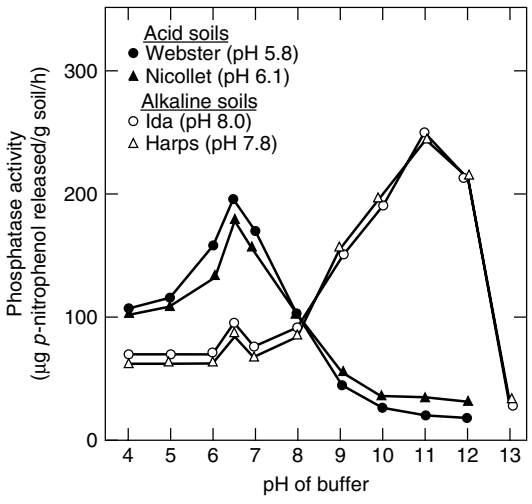

Figure 8. Phosphatase activity in acid and alkaline soils (38).

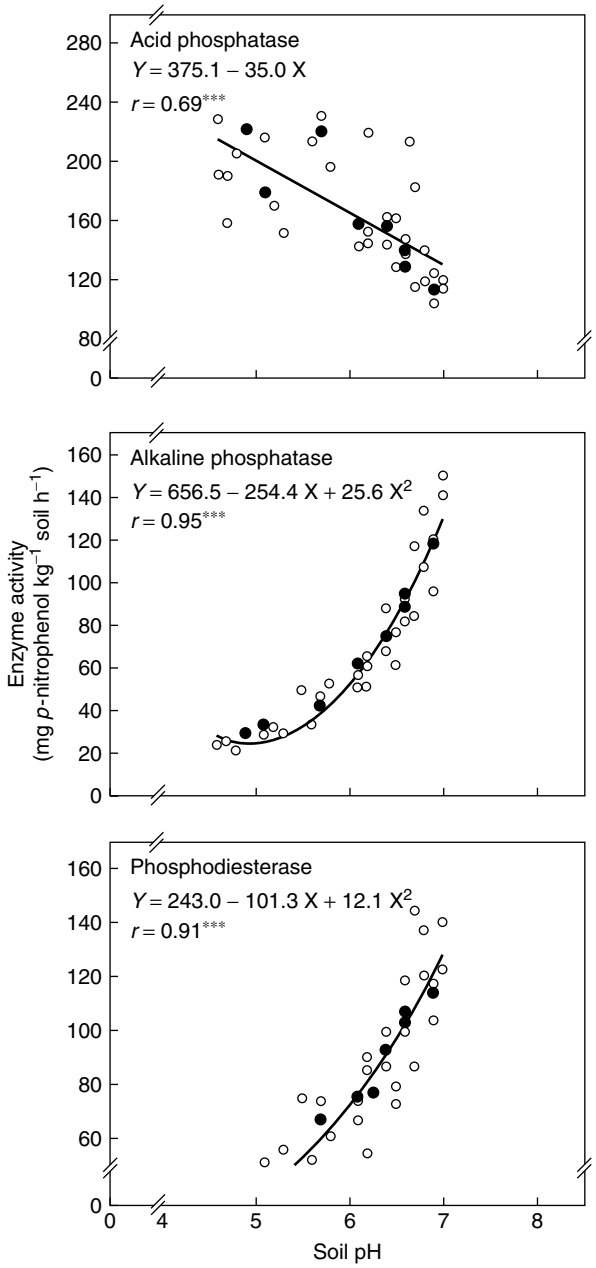

Figure 9. Effect of soil pH on the activities of phosphatases. Open symbols are results obtained for individual soil samples and solid symbols are averages obtained for four field replicates (41).

Application of lime to acid soils normally leads to significant increases in pH and, thus, in the chemical and biochemical reactions, and microbiological processes. Such treatment results in changes in the solubility of many chemical compounds and improvement in the environment of plant roots, increasing soil microbial biomass, with impacts on dynamics and diversity and, therefore causing significant changes in enzyme activities. With the exception of acid phosphatase activity, which decreases with increasing soil pH, the activities of all other enzymes increase with increasing pH (Fig. 9).

Table 6. Correlation Coefficients (r) and Slopes of Linear Relationships Between Soil pH and Enzyme Activities

Enzyme	Enzyme Activity vs. Soil pH[a] r	Δ Enzyme Activity[b]/ Δ Soil pH
Glycosidases		
α-Glucosidase	0.56***	4.4
β-Glucosidase	0.87***	38.5
α-Galactosidase	0.53**	4.5
β-Galactosidase	0.81***	8.8
Amidohydrolases and arylamidase		
Arylamidase	0.74***	9.0
L-Asparaginase	0.84***	15.8
L-Glutaminase	0.73***	107
Amidase	0.61***	14.4
Urease	0.71***	6.2
L-Aspartase	0.80***	1.0
Phosphatases		
Alkaline Phosphatase	0.89***[c] (0.95***)[d]	97.0[c](25.6)[d]
Acid Phosphatase	−0.69***	−35.0
Phosphodiesterase	0.89***[c] (0.91***)[d]	39.4[c](12.1)[d]
Sulfatase		
Arylsulfatase	0.66***	11.2

Source: (41) V. Acosta-Matinez and M. A. Tabatabai, *Biol. Fertil. Soils* **31**, 85–91 (2000).

[a] $^{**}p < 0.01$, $^{***}p < 0.001$.

[b] All activities are expressed in mg product released kg^{-1} soil h^{-1} (change in enzyme activity per a unit change in pH).

[c] Linear regression for soils with pH > 6.0.

[d] Second linear regression values.

The Δ activity/Δ soil pH ratios (change in enzyme activity per one unit change in soil pH) for L-glutaminase is the highest, followed by those of L-asparaginase, amidase, arylamidase, urease, and L-aspartase. This ratio for L-glutaminase is not only the most sensitive to soil pH changes among those involved in N cycling, but also the most sensitive among the 14 enzymes listed in Table 6. The activity of L-glutaminase could be used as an early and sensitive indicator of soil stress (41).

Toxic metals

The activities of many enzymes are inhibited by trace elements. The enzyme affected include α- and β-glucosidases and α- and β-galactosidases (29,42), urease (28), phosphatases (43), arylsulfatase (44), nitrate reductase (45), L-asparaginase (31), L-glutaminase (32), L-aspartase (46), and cellulase (47). Trace elements are added to soils as impurities in fertilizers, as components of industrial wastes or sewage sludges (48,49), or present in fuel oils and gasoline (e.g., Pb, V) (50,51). The degree of enzyme inhibition varies with the concentration and form of trace element, the soil investigated, and the type of enzyme assayed (11,52). Several soil properties may influence the toxic effect of trace elements, including organic matter content, type and amount of clay, and soil pH. In general, Ag (II) and Hg (II) are the most effective enzyme inhibitors. These elements react with the sulfydryl groups (−SH) of the active enzyme sites and effectively inhibit the catalytic property of the enzyme. The sulfhydryl groups are present in the active sites of most enzymes.

Pesticides

Pesticides have significant direct and indirect effects on the activity of various enzymes. The outcomes depend on the complex reactions between the compound and the microbial population, the enzymes and or the soil colloids (31,52,53).

CONCLUSION

Soils as biological systems contain numerous enzymes derived from the microbial community and soil animals, and plant residues, and roots systems. Methods are available for assay of activities of many enzymes in soils. Human activities such as cultivation, cropping, fertilization, lime application, tillage and residue management, and waste disposal affect the levels and distribution of enzyme activities in soils. The activities of some enzymes can be used as a monitoring tool to assess the effect of pollutants on ecosystem stress, health, and quality.

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SOIL FORMATION, ROLE OF MICROORGANISMS.

See WEATHERING: MINERAL WEATHERING AND MICROBIAL METABOLISM

SOIL FUNGI: NATURE'S NUTRITIONAL NETWORK

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Fungi are among the most important, numerous, and diverse denizens of soil. Mycologists and microbiologists have frequently treated soil as a single, uniform habitat for fungi and other microbes. In fact, soil is a complex ecosystem comprising myriad minuscule habitats and niches for microorganisms. Soil is a three-dimensional matrix of organic and mineral particles and their decomposition products (1). It is interlaced with plant roots and has islands of resources swarming with invertebrates and microbes. On top of this complexity is the fourth dimension of time, as organic additions from above and mineral substrates from below are broken down and subsequently moved through the soil by the action of earthworms and other invertebrates (2). Different soils, including the soils of different biomes, have widely differing physical and biotic characteristics (3). As a result, different soils may have different fungal populations and activity. Fungi are dominant both numerically and by biomass in many soil ecosystems, particularly in temperate, boreal, and arctic-alpine regions and in other areas where soils have high organic matter contents (4). The incredibly important individual roles of fungi, such as decomposition and mycorrhizal

symbioses are now well recognized. However, these represent just part of the connections made by soil fungi among members of all trophic levels and organic and inorganic nutrient sources (5). The functioning of this belowground nutritional network may have significant impacts on the aboveground community (6–9) and on important environmental variables such as global levels of atmospheric carbon dioxide (10).

As will be discussed later, it is difficult to create a definition of a fungus that is applicable to all fungi. However, one of the characteristic features of most fungi is filamentous growth, and it is this feature that allows fungi to make connections within the soil ecosystem. The microscopic, filamentous cells of fungi are called hyphae (singular hypha), and the fungal individual often consists of a highly branched network of millions of hyphae, together called a mycelium. The mycelium is the original Internet, capable of exploring the multidimensional space of soil and of rapid, long-distance transport of energy (carbon compounds), resources (nutrients), and information (proteins and nucleic acids, including mitochondria, nuclei, and viruses) (11–13).

A brief article such as this one can barely touch on the wealth of research on soil fungi, and so I have chosen to provide an overview with references to major literature, and a discussion of interesting topics about which we still know very little.

DECOMPOSITION, MYCORRHIZAE, AND BEYOND

Fungi are heterotrophic eukaryotes. As such, they must obtain their metabolic carbon either by symbiosis or by absorption from their environment, often preceded by enzymatic digestion of insoluble organic compounds. Thus, there are two fundamental (but not necessarily separate) modes of nutrition among the fungi: as decomposers of dead organic matter or as symbionts (parasites, commensals, or mutualists) of other living organisms ranging from bacteria to other fungi, plants, and animals. Whereas bacteria are the primary decomposers of dead animal matter, fungi are the decomposers par excellence of the other 90% of the organic wastes in terrestrial ecosystems. This is because the fungi, especially the Homobasidiomycetes, are the best equipped with enzymes such as laccases, lignin, peroxidases, manganese peroxidases, and cellulases for degrading lignocellulosic plant wastes (14–16). In addition, their hyphal growth form allows them to penetrate solid substrates such as wood (17) and to communicate among distant resource units (18,19). Decomposer fungi lacking enzymes to degrade intact lignin chew away at the cellulose exposed as the lignin is removed by other fungi, and on the breakdown products of lignin, until much of the original plant biomass is respired as carbon dioxide (17,20,21). The remaining recalcitrant phenolic fractions become complexed with fungal and microbial cell wall or sheath materials and subsequently add to the humic fraction of soil (3). During the decomposition process, many or most fungi act in consortium, either as a result of coevolved symbioses or by circumstance of physical proximity, with other fungi, protists, and prokaryotic microbes. In addition, some ectomycorrhizal (and possibly also decomposer) fungi may etch micropores in feldspar, hornblende, and other soil minerals by release of organic acids, and subsequently use and transport these nutrients to their mycorrhizal host plants (22).

Fungi are also well known as parasites and pathogens of plants, but the range of organisms that fungi attack and consume in soil spans the range of organisms that occur there. Probably no soil organism, from bacterium to macroinvertebrate, is immune to attack by fungi, and some examples of these relationships are described in the following sections. Many symbioses involving fungi are more mutually beneficial, and the most important of these relationships are the mycorrhizae (from the Greek, meaning fungus roots) formed with plants (23–28) (see also MYCORRHIZAE: ARBUSCULAR MYCORRHIZAE and MYCORRHIZAE: ECTOMYCORRHIZAL FUNGI, this Encyclopedia). There are several morphological types of mycorrhizae, and many terms for these types of associations. The simplest summary is to divide mycorrhizal types into those formed by members of the Glomales (Zygomycota) with about 90% of the world's vascular plants, and those formed by members of the Ascomycota and Basidiomycota with selected families and genera of trees and woody perennials. In the former, variously called endomycorrhizae, arbuscular mycorrhizae (AM) or vesicular-arbuscular mycorrhizae (VAM), a coenocytic (multinucleate) fungus forms shrubby structures called arbuscules for nutrient transfer within root cortical cells. Swollen cells called vesicles may also be formed, either within or between root cortical cells. An extensive mycelium explores the soil for nutrients and water, which are exchanged via arbuscules for plant photosynthate. Spores are usually formed on the external mycelium and, although large for fungal spores (up to 1 mm in diameter), are not produced in the massive fruiting bodies that characterize the other group of mycorrhizal fungi. The other group of mycorrhizae includes some associations in which the fungal cells remain outside of root cortical cells (called ectomycorrhizae or ECM), and also mycorrhizae formed by the same fungi in orchids, ericaceous shrubs, herbs, and even liverworts in which there is cellular penetration by the fungus (26,29–31). In both cases, plants benefit most from mycorrhizal associations when growing under conditions of nutrient or drought stress — in other words, when growing under most natural conditions (26). A number of tree genera, including *Populus* (Salicaceae) and *Eucayptus* (Myrtaceae) are known to form both vesicular-arbuscular mycorrhizae and ectomycorrhizae (32,33) and, in both cases, the proportion of VAM-infected roots declines as the trees mature. The effects of the different (or combined) mycorrhizae on the host plants may differ, particularly under different nutrient regimes (33,34). Mycorrhizae may be maintained whether they are cost-efficient or cost-inefficient in terms of carbon cost per unit of acquired mineral nutrient, so long as photosynthesis or growth are strongly nutrient limited (35).

There has been a tendency to treat the mycorrhizal fungi as solely symbiotic and without saprotrophic capabilities. Among plant pathologists, the ability of many fungi to form a continuum from decomposer to plant parasitic symbiont has been recognized for many years, and

known to aid in establishment and persistence in the absence of susceptible plant hosts (36). A similar continuum from decomposers to mutualist ectomycorrhizal symbionts helps to explain the tremendous connections that may be made by such fungi within the soil ecosystem, from recalcitrant organic or inorganic sources of mineral nutrients to one or more plant hosts (26). At the base of this saprotroph-mutualist continuum may be the evolutionary switches that have evidently occurred from saprotroph to ectomycorrhizal habit (and perhaps back again) in multiple lineages (37). I will return to these community-level and ecosystem-level connections after introducing the major groups of fungi and funguslike organisms.

GROUPS OF FUNGI AND FUNGUSLIKE ORGANISMS

Over the years, mycologists have studied fungi and organisms that have been thought to be fungi, united by possession of one or more of the following "fungal" characteristics: extracellular digestion, absorptive nutrition, filamentous growth, reproduction by spores, or causing disease in plants or animals. For more than a century, morphological evidence has been used to exclude some of these organisms from the Fungi, and now modern evidence based on molecular phylogenetic analyses has provided support for these decisions. The fungi and funguslike organisms belong in two Domains (Bacteria and Eukarya) (38,39) and at least four Kingdoms of the Eukarya (Mycetozoa, Stramenopila, or Heterokonta, Heterolobosea, and Fungi) (40). McLaughlin and coworkers (41) provide a recent synthesis of the classification and ecology of fungi and funguslike organisms excluding actinomycetes and slime moulds. This brief review is based on that synthesis, and landmark papers in the systematics of prokaryotes (42–46) and protistan eukaryotes (40,47–51).

Nonfungi

Actinomycetes. The Actinomycetes (or "Ray Fungi") are not fungi, nor even eukaryotes. Actinomycetes resemble fungi in their filamentous growth and reproduction by spores. In addition, actinomycetes have the very fungal characteristic of producing extracellular digestive enzymes and subsequently absorbing selected breakdown products as food. However, actinomycetes are best classified among the G+C-rich gram-positive Bacteria (43,44). Many, particularly in the genera *Streptomyces* and *Streptoverticillium*, are important soil organisms, which contribute to the breakdown of lignocellulosic plant residues (52,53) and produce geosmin, a secondary metabolite responsible for the characteristic odor of freshly tilled soil (54). Members of the genus *Frankia* form N-fixing symbioses with the roots of *Alnus*, *Myrica*, and other shrubs and trees (55). It was recently discovered that the fungus-gardening ants make use of an antibiotic-producing streptomycete to control weed molds in their fungus gardens (56). For reviews on the biology and identification of actinomycetes, see (57–60).

"Water Molds" (Oomycota, Hyphochytriomycota). These two groups are united by the possession of motile, flagellated spores: the zoospores of Hyphochytriomycota ("hyphochytrids") have one anterior flagellum; those of Oomycota ("water molds") have two flagella — one posterior and one anterior. The anterior flagella in both groups have two rows of fine, tubular hairs and are called tinsel flagella or stramenopile flagella. The phylogenetic relations of both groups appear to be with the golden-brown (chromophyte) algae in what is sometimes called the Kingdom Stramenopila (47,61,62) or Heterokonta (40). Because members of both groups are dependent on free water for zoospore dispersal, they are found most commonly in consistently moist soils and freshwater ecosystems (63,64), although many oomycotans also produce drought resistant resting spores. Soil-inhabiting Oomycota include the important plant pathogens *Pythium* and *Phytophthora*, which cause damping off and root rots that affect nearly all vascular plants, including many crops (65–68). Other genera of Oomycota in soil are saprotrophic, or parasitic on algae, rotifers, nematodes, and other minute soil fauna (63,69,70). The hyphochytrids of soil are poorly known, but generally regarded as relatively unimportant saprotrophs or parasites of soil algae and fungi (64). The Oomyota comprise approximately 700 (71) to 1100 species (63) and the Hyphochytriomycota 24 or 25 species (63,71). The Labyrinthulomycota ("net slime molds") of marine environments, which possess zoospores similar to those of the Oomyota, are related to the Stramenopila and not to other slime molds (72,73).

"Slime Molds" (Myxomycota and Dictyostelids, Acrasiomycota, and Plasmodiophoromycota). This is a polyphyletic group of "slime molds," with the most species-rich group being the Myxomycota (74) and the best-studied being *Dictyostelium* (75,76). At one stage of their life cycle, all of these organisms have an amoeboid motile stage, and the amoeboid forms of dictyostelids, acrasids, and myxomycetes may be abundant in rhizosphere soil (77). Individual amoebae aggregate to form multicellular pseudoplasmodia (dictyostelids) or multinucleate, acellular plasmodia (myxomycetes). This "slime stage" is motile and continues to feed on small organic particles and microbes before climbing to fruit, often on the surfaces of grass, wood, or bark (78). Plasmodia and fruiting bodies of certain myxomycetes may be conspicuous on lawns or gardens, but (should the homeowner not feel blessed by their presence) can readily be washed away with a blast from the garden hose. Evidence from morphology and the few taxa included in molecular phylogenetic analyses suggest that the Myxomycota (with approximately 720 species; 71) and Dictyosteliomycota (with 46 species; 71) form a monophyletic group that is basal to the clade containing true fungi and metazoan animals (40,50). The name Mycetozoa ("fungus animals") has been revived and suggested as the kingdom name of this group by Baldauf and coworkers (40). The dozen or so known species of Acrasiomycota (71), although morphologically and ecologically similar to the dictyostelids, are very distantly related and are members of the protistan kingdom Heterolobosea (40). The Plasmodiophoromycota, whose members are primarily known as root pathogens of vascular plants, are the exception within this otherwise peaceable group, and in fact they

too are not apparently related to the Mycetozoa. *Plasmodiophora brassicae* is the cause of club root in crops of the cabbage family and *Spongospora subterranea* causes powdery scab of potatoes (65,79). Sequences of small-subunit ribosomal DNA place *Plasmodiophora* in a clade with the alveolate and heterokont protists (49). Available rDNA sequences support the relatedness of *Spongospora* and *Polymyxa* but do not provide sufficient evidence to place this clade with any certainty (80).

True Fungi

"Chytrids" (Chytridiomycota). Although the Chytrids and Zygomycetes as currently defined appear to be paraphyletic with respect to one another (48,81–83), each group shares certain morphological and ecological characteristics that make it worth continuing to recognize these phyla for the present. Chytrids are aquatic fungi in the sense that they, such as the Oomycota, possess flagellate zoospores that require free water for motility. However, they may frequently be isolated from soils, including soils of very dry environments such as deserts (84,85). There are approximately 800 to 1,000 described species (71,85). Soil-inhabiting chytrids include saprotrophs with the ability to decompose cellulose, chitin, and keratin, as well as species that are parasites of soil algae, nematodes, tardigrades, and vascular plants (69,85). The chytrid *Synchytrium endobioticum*, the causal agent of potato wart disease (86), recently made international news when it was discovered in Prince Edward Island, and the United States government temporarily halted import of all potatoes from this important potato-growing region of Canada. Zoospores of most chytrids have one posterior flagellum, but members of the order Neocallimastigales are multiflagellate. The latter are an important group found in the rumen of herbivorous mammals, where they contribute to the digestion of cellulosic plant cell walls in the animals' food (87). The rumen fungi may have a reservoir (or an unknown part of their life cycle) in soil or dung (85).

Zygomycota. The Zygomycota is a diverse group of fungi in terms of morphology and ecology, but with approximately 1,056 accepted species (71), a relatively small group in number. Among the Zygomycota are some groups that are mycorrhizal with green plants (Glomales forming arbuscular or vesicular-arbuscular mycorrhizae, and Endogonales forming ectomycorrhizae), and these are arguably among the world's most important fungi. Glomalean mycorrhizal symbioses enable approximately 90% of the vascular plants in the world, including many crops grown in low-input cropping systems, to survive drought and nutrient stress (26). The Zygomycota also includes many groups that are soil saprobes, others that are parasites of other soil fungi or algae, and still others that are parasitoids of insects (Entomophthorales) or minute soil invertebrates (Zoopagales) (88–90). It has been suggested that the Glomales evolved in symbiosis with the emergence of land plants (91–93), and that they are the most ancient of the Zygomycota, with saprotrophic, mycoparasitic, and other lifestyles having evolved subsequently. This idea is not supported by current phylogenetic analyses (82–83,90) but there is unfortunately little or no bootstrap support for the basal topology indicated in these studies. Resolution of the relationships among deeply diverging lineages of fungi (and other organisms) will require the combined force of additional sequences from proteins or other gene regions. The arbuscular mycorrhizal Glomales, such as most Zygomycota, are coenocytic and have up to 2,000 nuclei per spore. These nuclei show intraspecific levels of genetic variation (94) but more widely divergent sequences recovered from glomalean spores should be viewed with caution as possible contaminants (95).

Ascomycota (including most "Deuteromycota," "Lichenomycota" and Yeasts). The Ascomycota are the largest group of fungi, with approximately 33,000 described species (71). Inclusion of organisms in the Ascomycota has traditionally been based on their production of sexual spores (meiospores) in a sac-shaped cell called an ascus (plural asci). For this reason, a separate class (Deuteromycetes) or phylum (Deuteromycota) has been used for fungi that lack known sexual structures. The majority of these are asexual relatives of Ascomycota (96). Most fungal systematists have abandoned the Deuteromycota, and many are now trying to unify the classification of these fungi (97–99). Asexually reproducing or nonsporulating fungi may be identified as members of the Ascomycota or Basidiomycota by various morphological or chemotaxonomic methods (reviewed in 100), as well as by phylogenetic analyses of their DNA sequences (101–103). Among the filamentous Ascomycota are many of the most important soilborne pathogens of crop plants, including wilts caused by *Fusarium* and *Verticillium* and root and stem rots caused by *Cochliobolus, Giberella, Gaeumannomyces, Phymatotrichopsis*, and *Sclerotinia* (36,67,104). Other Ascomycota, particularly in the Clavicipitaceae, grow as endophytes within plant tissues, usually without causing disease symptoms (105–107). When growing in the leaves and stems of grasses, they may decrease flowering, and by production of toxic secondary metabolites, reduce the palatability of the grass to grazers or cause physiological harm to herbivores that consume infected plants (105,106,108). Other Ascomycota, ranging from the cultivated or wild-harvested truffles (Tuberaceae) to inconspicuous cup-fungi (e.g., *Hymenoscyphus ericae*), form mutually beneficial mycorrhizal relationships with the roots of vascular plants (26). Still others are obligately or facultatively parasitic on other soil fungi, and offer the possibility of biological control of soilborne fungal diseases of crop plants (109–114). The Ascomycota includes nematode-destroying fungi such as *Arthrobotrys* (69,115), and fungal parasitoids of insects (e.g., *Cordyceps*, 116–118) and other soil invertebrates (119).

Yeasts are fungi adapted to life in aqueous environments through growth of separate, usually elliptical cells that divide by budding or fission (120,121). Their growth form makes them more suitable for study by microbiologists trained in bacteriological techniques than by classical mycologists used to dealing with filamentous fungi (122). A majority of yeasts, including the most economically important ones such as *Saccharomyces cerevisiae*, belong

in the Ascomycota (123). Although many yeasts are found in association with fruits, flowers, and other sources of readily assimilated sugars and other carbohydrates, some are more oligotrophic in their growth requirements and are found in soil, where they may be important members of the decomposer consortium breaking down plant and animal wastes (122,124).

Lichens are symbiotic associations between fungi and green algae or cyanobacteria, in which the fungal partner (myobiont) forms a characteristic structure (what is seen as a lichen) that encloses and protects the alga or cyanobacterium (photobiont) (125–128). Just as with mycorrhizal symbioses, lichens evolved independently in the Zygomycota, Ascomycota, and Basiomycota (129), but by far the majority of lichen species (more than 20,000 described species) are in the Ascomycota (71). Lichens form the dominant ground cover over large areas of arctic and alpine environments and, together with "independent" fungi, algae, and cyanobacteria (or more likely consortia of these), form soil crusts that protect soils in arid environments such as the southwestern United States (130–132) (see DESERT ENVIRONMENTS: BIOLOGICAL SOIL CRUSTS, this Encyclopedia).

Basidiomycota. The Basidiomycota are the second-largest group of true fungi, with more than 22,000 described species (71). The Basidiomycota may be divided into four major groups, the Ustilaginomycetes (smuts), Urediniomycetes (rusts), Heterobasidiomycetes (jelly fungi) and Homobasidiomycetes (mushrooms and relatives). Only the latter group is important in soil, although various stages of the life cycles of smuts and jelly fungi may occur in soil and be important to their survival. The Homobasidiomycetes, with approximately 13,000 described species, include gilled mushrooms, boletes, polypores, coral fungi, stinkhorns, and crust fungi, many with their mycelial phase occurring in soil (133). This great diversity has mostly been overlooked in surveys of soil fungi. Within the Homobasidiomycetes are some very important plant pathogens, including *Rhizoctonia* (sexual state *Thanatephorus*, 134–136) and timber pathogens *Armillaria*, *Phellinus*, and *Ganoderma* (137,138). The majority of Homobasidiomycetes (perhaps 8,500 described species) are saprotrophic leaf and wood-decomposing fungi (6–8,37). Some of these same fungi are also important within soil, breaking down recalcitrant components of plant cell walls and also forming recalcitrant compounds themselves, which become part of the humic fraction in soil (3). Approximately 4,500 described species of Homobasidiomycetes form ectomycorrhizal relations with woody vascular plants in 30 families (26,31,37). Forests of ectomycorrhizal trees are dominant over much of the temperate, boreal, and alpine regions of the world, whereas ectomycorrhizal trees may be locally dominant or widely dispersed in tropical forests (26). The Homobasidiomycetes also include two groups of nematode-destroying fungi, in *Hyphoderma* (139) and the Pleurotaceae (140). Homobasidiomycetes related to *Leucocoprinus* and *Crinipellis* are the fungi cultivated by leaf-cutting ants and their relatives (141). In addition, a great many saprotrophic Homobasidiomycetes are bacterivorous, attacking and consuming live colonies of bacteria in their nitrogen-limited environments (101,142–144). And, both saprotrophic (e.g., *Tephrocybe*) and ectomycorrhizal (e.g., *Hebeloma*) Homobasidiomycetes are included in a group known as ammonia fungi or corpse-finder fungi (145–147), which fruit near animal corpses or accumulations of dung or urine. Given the recent discovery of nitrogen transfer from living or dead collembolans to a host tree by the ectomycorrhizal Homobasidiomycete *Laccaria bicolor* (148), the idea of linking nutrients in a dead rabbit directly to a pine tree through an ectomycorrhizal *Hebeloma* no longer seems so far-fetched.

FUNGI AS LINKS BETWEEN RESOURCES AND TROPHIC GROUPS, OVER DISTANCE AND TIME

Since Trappe and Luoma (5) wrote their stimulating chapter entitled *The ties that bind: fungi in ecosystems* there has been a growing awareness among mycologists and ecologists of the abilities of fungi to link different individuals and species of the plant community with each other and with nutrients and other resources (22). There is now an ample literature showing connections made by glomalean mycorrhizae between herbs and trees (149), or by ectomycorrhizal fungi between different forest tree species (150,151), tree age classes (152), from trees to understory ericoid shrubs (29) or herbaceous plants including achlorophyllous species (153–154), and even thalloid liverworts (30). This sharing of carbon, nutrients, and water within ecosystems via the fungal network has great implications for ecological paradigms such as competition, succession, and patterns of diversity. In this section I will briefly review some of these findings and try to extend the concept of fungal nutrient networking to include members of all trophic levels and the abiotic nutrient resources in soil.

In many soils, there is more fungal biomass than that of all other organisms, excepting plant roots, combined. However, soil is also teeming with bacteria, a diversity of microscopically exquisite protists including amoeboid and ciliate forms, and micro- and macroinvertebrates including nematodes, mites, collembolans, larval and adult insects, and earthworms (155). Among these, the most numerically abundant include the bacteria (approximately 10^9 or more cells per gram of dry soil) and nematodes (often 10^6 or more per square meter). Various fungi have evolved to make use of this entire gastronomic smorgasbord. Although some are specialists that feed on just one or a few species of nematodes, rotifers, or amoebae (156), others are more generalist and also have enzymatic abilities to decompose lignocellulosic plant wastes (143)—in other words, to tap into both biotic and abiotic sources of nutrients and energy and make these available across all trophic levels.

Pioneers in the study of fungi that attack soil microfauna were Charles Drechsler (157–159) and George Barron (69,120). Their wonderful illustrations gave glimpses into a world that few others have seen: amoebae, mites, nematodes, tardigrades, and even springtails (Collembola; 160) falling prey to fungi armed with adhesive knobs or nets, constricting or nonconstricting rings, or even miniature ballistic missiles (161). Although most of the

fungi attacking small animals are microfungi, members of the Ascomycota, Zygomycota, and Chytridiomycota, a few mushrooms from the Basidiomycota are also known to attack nematodes (139,140,162). The study of the oyster mushroom (*Pleurotus ostreatus*) as a nematode-destroying fungus led to the discovery that it and several other lignin-degrading mushrooms attack and consume living colonies of bacteria (142), including gram-positive, gram-negative, and nitrogen-fixing species (163). The list of known bacterivorous fungi includes most saprotrophic species of Homobasidiomycetes that have been tested (144), and the list of potential prey has been extended to include yeasts (164), plant pollen (165) and algae (166). Ectomycorrhizal Homobasidiomycetes that were tested for this ability in vitro all proved unsuccessful, but the results might have been different had the tests been done while the fungi were in association with a photosynthetic host.

Perhaps most interesting to ecologists is the possibility of a short-circuit in nutrient cycles, whereby mycorrhizal fungi tap into nutrients in plant litter, other fungi, fauna, and microbes in soil, and pass these nutrients on to their plant hosts. Evidence for this possibility is certainly accumulating. David Read and coworkers have published numerous studies on the nutritional capabilities of *Hymenoscyphus ericae*, the mycorrhizal associate of most ericoid shrubs, which typically grow in low pH, nitrogen-starved environments (167,168). This fungus has the ability to utilize nutrients in lignin (169), chitin (170), and dead plant and fungal tissues (171), and can transport nitrogen from plant litter to mycorrhizal seedlings (172). It was subsequently discovered that this same fungus also forms mycorrhizal associations with the dominant tree species (29) and thalloid liverworts (30), opening the possibility for community-wide nutrient exchange. Lindahl and coworkers (173) provided a very nice demonstration of the potential for a short-circuit in nutrient cycling by the ectomycorrhizal fungi *Suillus* and *Paxillus*, which captured radiolabeled phosphorus from a saprotrophic mushroom (*Hypholoma*) and transferred this phosphorus to mycorrhizal pine seedlings. Koide and Kabir (174) found that the effects on nitrogen and phosphorus contents of red pine seedlings grown with both the ectomycorrhizal fungus *Pisolithus* and saprotrophic soil microbes were additive or synergistic, depending on soil nitrogen content. The mechanism of the effects seen, whether based in exploitative or interference competition, was not determined.

The interactions between collembolans and soil fungi, including those forming arbuscular mycorrhizae and ectomycorrhizae, have been a hot topic for many years (175,176). Approximately 50% of all studies on the effects of collembolan grazing on mycorrhizal symbioses have used *Folsomia candida* (177), which, however, feeds preferentially on nematodes, not fungi (178). More ecologically realistic studies are needed, including collembolan densities similar to those in nature and sensible combinations of collembolan and fungal taxa (177). Nonetheless, there is a general consensus that grazing by collembolans and other arthropods regulates the activities, biomass, and numbers of spores of soil fungi (175,179,180). However, it is important to remember those fungi that attack and may be regulating the numbers of soil fauna. Among these, it seems, is an ectomycorrhizal mushroom, *Laccaria bicolor*, which was found to reduce numbers of collembolans living in microcosms where the fungal mycelium was present and to transfer nitrogen from living or dead collembolans to the mycorrhizal plant, in exchange for photosynthetic carbon (148). The mechanism of the capture or attack of collembolans by *Laccaria* remains unknown — many marvelous mysteries remain among the soil fungi.

CONCLUSION

The importance of fungi in terrestrial soil ecosystems is becoming more widely recognized by ecologists, agronomists, and microbiologists. Fungi are physically dominant in terms of biomass in many soils, particularly in boreal, arctic, and alpine areas. Their abilities to enzymatically degrade complex plant polymers and xenobiotic compounds and to connect members of many different trophic levels within the soil system make them a force to reckon with, whether you are a nematode or an ecosystem ecologist. Molecular methods are increasingly making it possible to determine the diversity and activities of fungi in selected soil microhabitats and will be critical in finally opening up the most pressing and interesting questions in soil biology.

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SOIL GENETIC ECOLOGY

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The term *soil genetic ecology* is broadly defined as the study of nucleic acids (RNA and DNA) in an ecological context. Soil genetic ecology encompasses a diverse range of topics, including methodologies (1), genetic exchange (2,3), and biodiversity studies (4), although, it commonly implies a methodological approach that uses nucleic acids as indicators of microbial community composition and activity. This entry will focus on the latter topic.

The introduction into soil ecology of methods derived from molecular genetics greatly advanced scientists' understanding of the functioning and diversity of soil microbial communities. These communities are among the most diverse and complex in nature, and molecular genetic approaches allow insights into these complex systems, which were previously inaccessible. Genetic methods are now commonly employed by soil microbiologists and are routinely used in a range of applications. A few examples of these applications include:

- evaluation of biodegradative potential in a soil contaminated with organic pollutants (5,6),
- characterization of nitrogen-fixing bacteria in agricultural soils (7,8),
- discovery of new groups of bacteria and characterization of their distribution in the environment (9,10),
- insight into the evolution and spread of microbial genes in nature (11,12), and
- discovery of novel enzymes and antibiotics that may be of use in industrial and pharmaceutical applications (13,14).

The primary advantage of using nucleic acids to study soil microbial community requirements for laboratory cultivation are bypassed, thereby avoiding biases resulting from limitations in current abilities to cultivate many microorganisms in the laboratory. Soils are complex systems that provide diverse and spatially separated bacterial habitats, and support a great diversity of soil bacteria. A recent molecular study estimated that more than 10,000 bacterial species were present in a gram of forest soil, and a significant number of these species may not be included in standard cultivation-based laboratory studies due to unknown growth requirements (15). Direct

microscopic total bacterial counts in soils typically range from 10^9 to 10^{10} bacteria per gram of soil, although numbers based on cultivable methods may be two to three orders of magnitude lower, suggesting an inability to cultivate more than 0.01% of soil bacteria.

Analysis of nucleic acids extracted directly from environmental samples allows information encoded in DNA and RNA to be accessed directly without laboratory cultivation. Depending on the application, DNA or RNA is directly extracted from the sample without initial separation of microbial cells from the samples. Different types of information are available depending on whether DNA or RNA is analyzed. The "blueprint" of an individual organism is encoded in its DNA, and information regarding the composition of a community is contained in the community genome. RNA is transcribed (produced) from DNA when a particular gene is active, such that RNA provides information on the activity of a gene of interest.

As with all methods, those used in genetic ecology are subject to limitations and biases. At this time, most soil genetic ecology studies focus solely on assessing or comparing microbial species present in different environments. This type of study does not take into consideration the physiology or ecological role of the target species. Questions related to physiology and functional role in a community are difficult to address, and most methods currently available are not appropriate for addressing these questions (16). Physiology and ecology can, in some cases, be tentatively inferred from the phylogenetic placement of the target groups (e.g., most photosynthetic bacteria fall into a closely related phylogenetic group). This type of estimation is not generally applicable, however.

Most applications of molecular methods currently in use are based on characterization of ribosomal RNA (rRNA) or the genes, which encode rRNA (rDNA). Ribosomes are the sites of protein synthesis in all cells and are composed of specific ribosomal proteins and ribosomal RNAs. Ribosomes are composed of two subunits, the large (LSU) and small subunit (SSU). In prokaryotes, the LSU is composed of specific ribosomal proteins and two types of RNA (5S and 23S). The SSU is composed of ribosomal proteins and 16S rRNA (17). Most environmental and phylogenetic analyses focus on 16S rRNA and its corresponding gene (16S rDNA).

The genetic sequence, or order of nucleotides, in rDNA and the corresponding rRNA is characteristic of an individual phylogenetic group, and knowledge of the genetic sequence may be used to infer the phylogenetic affiliation of a bacterial strain. rDNA sequence alone cannot, however, be used to determine affiliation to the level of species. Information regarding the species composition of a community may be obtained by characterization of rDNA present in DNA extracted from the sample. Concentrations of ribosomes, and hence of rRNA, present in a cell are directly related to the growth rate of the cell, such that concentrations of rRNA in soil are characteristic of the activity of a given phylogenetic group of bacteria (18).

rRNA and rDNA analyses are particularly powerful approaches toward characterization of soil microbial communities because individual groups of bacteria may be targeted for analysis. Most 16S rRNA genes are approximately 1,500 base pairs in length, and certain regions of these genes are characteristic of broad phylogenetic groups such as domains (Archaea, Eucarya, and Bacteria), while other regions are characteristic of more narrow groups such as phyla, families, and genera (19). The most commonly used approach toward targeting individual regions of rDNA is via the polymerase chain reaction (PCR), as will be discussed in the following section on methods (20).

METHODS

As is true for all soil ecology studies, the first step must be to establish an appropriate sampling design and to thoroughly mix the soil samples after collection. Most soil genetic ecology studies follow a general structure as outlined in Figure 1. The first step is typically to isolate the desired nucleic acids (DNA, RNA, or both) from the sample, followed by amplification of the target genetic segments by the polymerase chain reaction, and subsequent analysis of the target sequences by any of a number of approaches.

Isolation of Nucleic Acids from Soil Samples

Purification of nucleic acids from soils can be problematic due to copurification of humic contaminants that may inhibit further enzymatic manipulation of the nucleic acids (e.g., polymerase chain reaction) (21), although significant advances have been made in this regard in recent years. Commercial kits for isolation of DNA and RNA are available, and work quite well for many applications and many soil samples. These kits may not be satisfactory for some soils, however, and a general description of principles involved in nucleic acid isolation is presented in the following paragraphs.

In most studies, microbial cells are lysed in the presence of the soil particles to release RNA and DNA. A preliminary wash with phosphate buffer is occasionally conducted prior to lysis in order to remove extracellular DNA, which may interfere with subsequent analysis. Cell lysis can be achieved by physical or chemical means or by a combination of both. The most efficient method is a combination of physical disruption ("bead-beating," in which the sample is mixed with glass beads and shaken) and treatment with detergents and enzymes, which disrupt microbial cell walls. This general lysis approach is very efficient for lysing most gram-negative bacteria, and to a lesser extent efficient for gram-positives and fungi. After nucleic acids are released from the cells, they are extracted from the soil particles by washing with a phosphate or salt buffer. Humic compounds typically coextract with the nucleic acids and must be removed prior to analysis. Many methods have been devised to remove humic compounds, but the purification method of choice may depend on the soil sample. These methods include selective binding and elution to chromatographic resins and selective precipitations (22).

Polymerase Chain Reaction (PCR)

PCR is used to synthesize many copies of a particular region of a gene from a DNA sample, thereby increasing (or

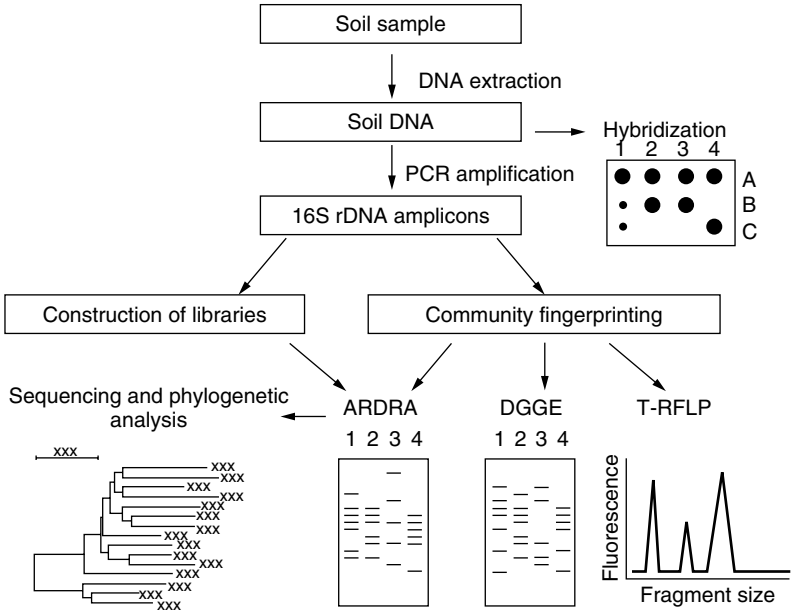

Figure 1. General methodological scheme for most soil genetic ecology studies. The individual method chosen depends on the application.

amplifying) the concentrations of the gene of interest over a million-fold. This allows genes of interest to be produced in such high quantities relative to other DNA in the sample. Nontarget DNA may be considered background and will not enter into analysis. Briefly, PCR is accomplished by using small synthetic pieces of DNA ("primers") that are complimentary to DNA, which flanks the gene of interest (e.g., 16S rDNA). These primer DNAs bind to their complimentary regions within the sample, or template, DNA. DNA polymerase (the enzyme that synthesizes DNA) begins copying DNA between the primers. Once the DNA between the primers has been copied, the DNA is split apart by heat, and the reaction begins again (23).

The PCR reaction mixture consists of DNA template (soil DNA), deoxyribonucleotides (dATP, dCTP, dGTP, and TTP), primers, $MgCl_2$, and DNA polymerase. This mixture is incubated in a thermal cycler that precisely controls the temperature of the individual wells in which reactions are conducted. Three steps compose a PCR cycle: (*1*) denaturation, in which the strands of DNA are separated at high temperature (approximately 92 °C); (*2*) annealing, when the temperature is lowered to allow the primers to anneal to the complimentary regions in the template DNA (typically between 50 and 60 °C, depending on the primers); and (*3*) extension, during which most of the synthesis of DNA occurs (72 °C). This process is repeated between 20 and 40 cycles (23).

The specificity of the PCR is based to a large degree on the selection of primers. Regions of 16S rDNA that are specific to a phylogenetic group of interest, such as the Proteobacteria, may be targeted such that only 16S rDNA from Proteobacteria will be amplified. Conversely, if one is interested in characterizing most of the bacterial groups in a sample, primers that target regions of the gene that are common to all bacteria may be used. In the latter case, 16S rDNA from many of the thousands of bacterial species present in a soil sample may be amplified. Genes other than rDNA may be analyzed in this fashion depending on the type of information desired. For example, if one is interested in nitrogen cycling, primers directed toward regions of genes controlling nitrogen fixation, nitrification, or denitrification may be targeted (23).

PCR amplification of a set of genes from a soil sample may result in amplification from hundreds or thousands of different strains. Genes from these different species must be separated, so that the individual genes may be analyzed. Several approaches for separation and analysis of these genetic sequences are in common usage, although they vary with regard to resolution and expense (Fig. 1).

Cloning and Sequence Analysis

The approach with the greatest resolution for separation and analysis of genes from mixtures of organisms is cloning the individual genes and determining their genetic sequence. In this approach, PCR amplification products (amplicons) are inserted into an appropriate plasmid-cloning vector through the action of the enzyme ligase. These recombinant molecules are introduced into bacterial cells (typically, a specific strain of *E. coli*) by transformation, a process in which cells are induced to take up DNA. Transformed colonies are grown on petri plates, and individual colonies are assumed to have arisen from a single cell that has taken up one of the original recombinant plasmids. Recombinant plasmids are extracted from selected colonies, and the distribution of different 16S rDNA molecules may be estimated by Amplified Ribosomal DNA Restriction Analysis (ARDRA) (24).

ARDRA employs enzymes (restriction endonucleases) that cut DNA at specific recognition sites based on different genetic sequences. This results in the production of fragments of different sizes and numbers that are approximately characteristic of the 16S rDNA. When these digestion products are electrophoresed on an agarose gel, a pattern of different DNA fragments are observed that are similar to a merchandizing bar code. These "bar codes"

may be regarded as operational taxonomic units (OTUs) that estimate the richness of genotypes in the clone library. They are also used to select unique clones to be sequenced, such that similar clones are not sequenced and efforts duplicated.

Once unique clones are selected, the genetic sequence of the inserted 16S rDNA is typically determined by an automatic sequencer. The sequences are compared with known sequences from one of a number of databases available on the Internet, such as the Ribosomal Database Project (*http://rdp.cme.msu.edu/html/*) or Genbank (*http://www.ncbi.nlm.nih.gov/GenBank/*). The phylogenetic affiliation of the different sequences recovered can be determined by placing them into a phylogenetic tree using one of a variety of software packages designed for this purpose (25). Not surprisingly, most DNA sequences analyzed in this manner belong to individual strains that have not been studied previously. In a recent analysis of this type of data, Pace and colleagues concluded that 12 of 35 total divisions of bacteria have not been cultivated in the laboratory (26).

Denaturing Gradient Gel Electrophoresis (DGGE)

Cloning and sequence analysis of PCR amplification products is time consuming and expensive, and is rarely conducted on more than a few samples. Fingerprinting methods, such as Denaturing Gradient Gel Electrophoresis (DGGE) (27) and Terminal Restriction Fragment Length Polymorphism (T-RFLP) (28), are commonly employed for rapidly comparing compositions of multiple samples. These methods provide information on the relative compositions of communities, but do not directly provide information on the nature of individual species in the samples. DGGE is based on PCR amplification of relatively short segments (approximately 500 base pairs) of DNA from the gene of interest. If segments of this gene from different strains differ slightly in their DNA composition, they may be separated on an electrophoresis gel that has increasing concentrations of a DNA denaturant (e.g., urea or formamide) along the direction of DNA travel through the gel. PCR primers used in DGGE are designed such that one primer has a very long stretch of the nucleotides G and C (the "GC clamp") that will prevent the product from completely denaturing. Instead, the rDNA portion of the amplification product will denature while the clamp remains closed. This results in a "butterfly" type of conformation that stops the fragment from moving in the gel. DNAs of different sequences denature at different points along the denaturant gradient, and under these conditions DNA that has been denatured stops moving through the electrophoresis gel. DNA from different strains denature at different positions in the denaturing gradient gel and stop migrating, resulting in the separation of DNAs from different strains in the sample. The resulting electrophoresis gel is stained with ethidium bromide so that the DNA is visible under ultraviolet light, and a banding pattern, or fingerprint, characteristic of the different forms of the gene of interest present in the sample is observed. Each band of DNA represents an individual form of the gene. Individual bands may be compared to determine its presence or absence between samples. These bands may be excised from the gel and the genetic sequence determined, or they may be transferred to a DNA-binding membrane and hybridized to specific gene probes to yield specific information on the origin of the bands (27).

A limitation of DGGE is that it should be used for narrow phylogenetic groups rather than larger ones (such as all bacteria). When broad phylogenetic groups are targeted, resolution of individual bands is difficult, if not impossible, and a smear rather than discrete bands is observed.

Terminal Restriction Fragment Length Polymorphism (t-RFLP)

T-RFLP is also a PCR-based approach used to compare the composition of different samples, but which may have a lower degree of resolution than DGGE. In T-RFLP, target genes are amplified by PCR such that one end of the amplification products are labeled with a fluorescent molecule. These labeled amplification products are digested with one or more enzymes that cut DNA at specific locations, which may vary between different sequences (28,29). The lengths of the individual digestion products are characteristic of the individual gene form (or strain). The lengths of the end, or terminal, fragments may be accurately determined by an automatic sequencer, and are characteristic of given strains. Limitations of this approach include the possibility that terminal fragment lengths are not unique to an individual strain or phylogenetic group of interest. In addition, the limit of detection of individual fragment sizes may be rather low, and will underestimate the number of phylotypes present in the sample. This is particularly true when using PCR primers that are directed toward broad phylogenetic groups, such as the bacteria. This limitation is alleviated somewhat by using primers that are directed toward narrow phylogenetic groups.

Limitations of PCR-Based Methods

Most genetic ecology approaches in use at this time are based on PCR, which has a specific set of considerations that should be understood to prevent misinterpretation of data. Among these considerations are

- misamplification caused by improper reaction conditions. This may lead to analysis of genes that differ from the desired targets;
- diversity that may be masked as a result of focusing solely on a single gene. Most analyses are based on variability within the 16S rDNA of different phylogenetic groups, and relatively little variability exists between strains with regard to their 16S rDNA. Considerable diversity may exist between the genomes of strains that have very similar 16S rDNA. Horizontal gene exchange over many thousands or millions of years may have resulted in one strain having several important genes that may be lacking in another strain, although they may share 99% similar 16S rDNA (30).
- chimera formation. Chimeras are PCR artifacts that result in one end of the amplification product arising

from one gene, and the other end arising from another gene (31). This type of PCR artifact can only be detected by determining the genetic sequence and statistical analysis of the sequence (32).

- PCR that is not inherently quantitative. The concentrations of individual PCR amplification products may not be used to estimate concentrations of the gene in the sample without appropriate controls (33).

Hybridization

One of the most commonly used approaches for analysis of community composition is hybridization. This approach offers a lower degree of resolution than those based on PCR, but has the advantage that it may be quantitative and relatively simple. Quantitative hybridization is frequently used when concentrations of rRNA from various phylogenetic groups are measured, such that information regarding relative activities of specific functional groups is obtained (34). When DNA is the target, information regarding the relative abundances of specific genotypes is obtained.

In this analysis, the primary requirement is that a gene probe be available for the genetic sequence of interest (the target sequence). The gene probe is usually either a cloned version of the target gene or a synthetic oligonucleotide (short piece of DNA), that is complimentary to the target sequence. Probes can be directed toward specific functions, such as enzymes encoding the degradation of an organic contaminant or nitrogen fixation, or toward specific phylogenetic groups in which rRNA or rDNA would be the target. The gene probe is labeled either radioactively (usually with ^{32}P) or nonradioactively (with a fluorescent molecule). Target nucleic acids (either DNA or RNA) from the sample are spotted on a nucleic acid–binding membrane through a vacuum filtration manifold. Alternatively, whole colonies may be fixed to the membrane and lysed to release their nucleic acids. Nucleic acids bound to the membrane are denatured (the strands of DNA are separated) by alkali and then neutralized. The labeled probe is added to a salt solution with the membrane and the probe allowed to hybridize, or combine with, its complimentary sequences within the target nucleic acids. Excess probe is washed off and the amount of label remaining on the filter is usually detected by exposure to X-ray film. The amount of exposure of the X-ray film is directly proportional to the amount of label, and hence target, on the filter (35).

The greatest limitation of hybridization is the current lack of knowledge concerning the molecular genetics of soil microorganisms. In order to apply gene probe analysis to soil microorganisms, the molecular genetics of both the gene probe and the target sequences must be known. The gene of interest must have been cloned, and the nucleotide sequence of the target gene must be similar enough to that of the gene probe for hybridization to occur. If there is more than one type of gene responsible for a given function, hybridization may underestimate the total numbers of colonies capable of performing the function of interest. Conversely, if nontarget genes share enough sequence similarity with the gene probe for hybridization, overestimation of the numbers of target cells will occur. Underestimation due to the presence of alternative genes coding for similar functions is the most common source of error, and it is impossible to control without a detailed knowledge of the genetics of soil microorganisms. Overestimation of target colonies by hybridization with similar genes can be controlled to some extent by including closely related genes as negative controls during hybridization.

ENVIRONMENTAL GENOMICS AND THE FUTURE OF SOIL GENETIC ECOLOGY

Genetic methods are developing at a very rapid rate, thanks in large part to recent initiatives to determine the DNA sequence of the human genome. Many of these innovations are being transferred to soil microbiology and are forming the next generation of methodological advances in soil ecology (16). A general theme of many of these novel approaches is a movement away from the current reliance on PCR due to problems with PCR as previously discussed.

Among the most promising innovations transferred from genomics is the development of microarrays, or gene chips. Microarrays are small pieces of glass that may contain thousands of unique pieces of DNA that represent a genome of interest, such as a human genome or a soil microbial "community genome." DNA or RNA from soil samples may be hybridized to a microarray containing 16S rDNA sequences representative of a suite of phylogenetic groups of interest and/or specific functional genes (e.g., genes involved in biodegradation of a given pollutant) to rapidly determine the presence or relative activities of these groups. Application of microarrays to microbial ecology is still in the developmental phase, but the approach is very promising and may become routine in the near future (36). (See MICROARRAYS: APPLICATIONS IN ENVIRONMENTAL MICROBIOLOGY, this Encyclopedia.)

Tremendous genetic potential is present in a gram of common garden soil, and genomic tools are opening this potential to industrial applications. Advances in the ability to clone large fragments of DNA and rapidly screen these clones for specific functions are making possible exploration of soil DNA for genes encoding novel biosynthetic and catalytic enzymes and antibiotics for medical and industrial applications. This approach is also in the development stages, but discovery of novel antibiotics has demonstrated the promise of this approach (13,14).

As mentioned earlier, one of the major limitations of 16S rDNA analysis is that it may mask microbial diversity. Sequence analysis of 16S rDNA may indicate the general phylogenetic placement of a strain, but little else. Novel cloning strategies are currently being applied in marine microbial ecology that clones large segments of DNA, including 16S rDNA and adjacent genes. This strategy may allow more information regarding the ecological role of the strain to be inferred, and is likely to be applied to soil microbial ecology soon.

Historically, one of the most troublesome aspects of soil genetic ecology has been isolation of DNA and RNA of sufficient purity for the desired application. Automated

purification of soil DNA is currently under development, and will be a great advance for all genetic applications when completely optimized (37).

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SOIL MICROBIAL BIOMASS.

See BIOMASS: SOIL MICROBIAL BIOMASS

SOIL MICROORGANISMS: ROLE IN BIODEGRADATION

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While the production of food and fiber is directly dependent on soil, the importance of microorganism to the formation and stability of soil has only been fully realized in the last 100 years. The contributions of the soil organisms to the function of soil can be viewed as being the biochemical engine that drives most of the important soil processes. The microorganisms are responsible for the breakdown of dead plant and animal materials, effectively ridding the earth of millions of tons of organic materials each year. It is during this process that the microorganisms provide the organic building blocks that are used to establish soil structure, helping to form a stable land surface. A well-structured soil contributes to good plant growth and water infiltration, which diminish the potential for runoff events and protects water quality. It is during the plant and animal degradation process that microorganisms begin a recycling process, which converts organic forms of nutrients to inorganic and plant available forms. Microorganisms have been playing an even more important role. It is now clearly established that microorganisms not only degrade natural organic materials but they are responsible for degradation

of many materials of human origin. These materials can include oils, pesticides, gasoline, and organic solvents. The soil microbial populations have been shown to be responsible for the conversion of contaminants to less dangerous materials, although toxic products are possible.

This article's focus is on the factors that affect the functioning of the microbial component of soil. We will address this by providing information describing the resident status of organisms in soil, and then describing the factors that control their activity and finally, describe how microbial population responds to pressures from humans and how we harness their inherent abilities. In this regard, we will show how inputs from humans can both suppress and maximize the functioning of soil microbial populations. A clear understanding of these factors is needed if we are to successfully manage soil to maintain both its inherent functions and to meet secondary goals such as allowing environmental cleanup.

THE SOIL STRUCTURE

Defining the Microbial Habitat

Soil particles, sand, silt, clay (i.e., the components of soil texture) are the base units of soil structure and it is the arrangement of these particles that results in soil that are typically viewed in the landscape. Soil particles range in size from sand particles as large as 2 mm to tiny clay particles (i.e., very coarse sand 2.0 to 1.0 mm, coarse sand 1.0 to 0.5 mm, medium sand 0.5 to 0.25 mm, fine sand 0.25 to 0.10 mm, very fine sand 0.10 to 0.05 mm, silt 0.05 to 0.002 mm, clay less than 0.002 mm). These particles rarely exist as independent units. Soil particles are more typically found bound together as aggregates with the linkage between particles resulting from organic bridges and binding agents often of microbial origin. In turn, it is the size and stability of all of the aggregates that determine the size and arrangement of soil pores. It is the soil pores that control the flow of air and water in soil (Fig. 1).

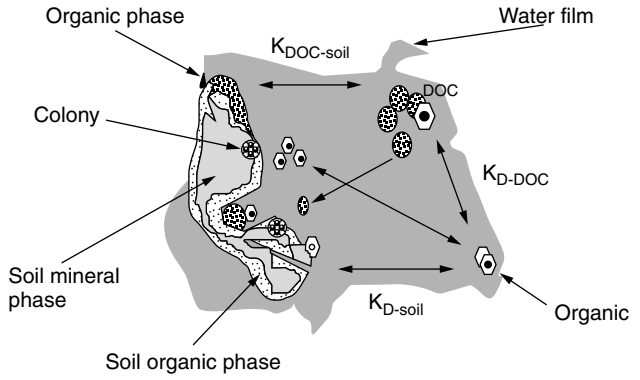

Figure 1. Diagram depicting the relationship between organic contaminants and the microbial populations. The distribution of contaminants between the soil surface and the soil solution is indicated by the K_D. The value K_{DOC} indicates the distribution of soil organic matter between surface and solution, and K_{D-DOC} indicates the reaction between contaminants and the dissolved organic matter in soil. In the case of K_D and K_{DOC}, a lager value indicates more solid-phase retention and a smaller dissolved solution content for the organic matter. In the case of K_{D-DOC}, a larger value indicates more interaction between the dissolved organic matter than the contaminant and less contaminant in solution. The shaded areas represent water films.

Although it was pointed out in 1927 (1), we are only now fully appreciating the importance of the soil aggregate as a habitat for soil microorganisms. Because of the microorganism's size (typically 1 μM or less), a tendency toward sessile growth, the soil's low intrinsic nutrient status (2), and their resulting slow growth rates, soil microorganisms are generally required to function under multiple limitations. In response to these multiple stresses, a typical terrestrial bacterium is found to colonize mineral surfaces often within a soil aggregate. In turn, they become indirectly responsible for improving the stability of the soil aggregates as the microcolony of cells grows and produces polysaccharides and other agents that bind the cells to the surface and also bind smaller soil particles together.

Aggregate Formation

The exact process of aggregate formation is speculative but is thought to follow a sequence in which a few particles become bound into a microaggregate. The microaggregates are then bridged or combined with other microaggregates to form larger aggregates (3). Both biological and physical processes aid in the formation of soil aggregates. Soil freeze and thawing cycles, soil drying, and fungal hyphae growth tend to push particles together. Microbial decomposition of organic residues (plant materials) releases many organic materials that act as bridge and then stabilize the adjoining particles. These materials become the core of what will be soil organic matter. Recent work has shown the organic materials released by the microorganisms to be biophysically complex with molecular weights exceeding 7,000 $gmol^{-1}$ (4).

It is generally thought that stable aggregates are created largely by microbial processes as the binding agents, which hold the aggregate together, are the materials released by cells during the degradation of plant materials or resident soil organic matter. Burrowing soil animals such as worms and insects and the growth of plant roots also push the smaller aggregates together and the reoriented surfaces act as locations for microbial colonization and additional fungal growth. Fungi have been shown to release compounds such as glomalin (a glycoprotein), which further stabilizes the matrix (5,6). Singh and Singh (7) demonstrated differences in the portion of microaggregates and macroaggregates as a function of soil use. Forest soil, with little disturbance, showed the greatest number of macroaggregates, whereas cropland soils were formed primarily from microaggregates. During aggregate formation, soil organic matter begins to buildup, as it was made unavailable within the newly formed microaggregates. Soils that have high levels of contamination (metals or organics) tend to be reduced in structure (8), possibly associated with lower microbial activity and impact from antropogenic actions. Clearly, significant changes in microbial biomass and metabolism along with soil management will alter the soil structure (9).

The distribution of aggregate sizes within a unit of soil will alter the resulting pore size distribution in that soil.

Tillage or other disturbance greatly reduces the aggregate size and liberates entrained (trapped) carbon that became resident in the formation process (10). These changes can affect subsequent plant growth (11). A high-quality soil will have a range of aggregate sizes from small to large; correlated to this is a range of pore space sizes. From the microbial perspective, aggregate geometry and size along with the resulting pore spaces are critical to their function both directly from the perspective of water and air infiltration and indirectly from the perspective of the improved plant growth, which becomes a source of carbon (11,12).

The net effect is that biological processes form the aggregates and the arrangement of both the sizes of aggregates will create pore spaces because pore spaces are found within and between both small and large aggregates. The pore spaces inside the aggregates are sometimes referred to as occurring within the soil matrix because they occur inside a soil structural unit (13). These matrix pores may connect to the larger pore spaces that occur between the stable soil aggregates. However, matrix pores may occur within an aggregate without being connected to the larger aggregate pore spaces. As a result, a typical soil is intermixed with small and large holes and connected and unconnected channels.

The soil microbial community is associated with both types of pore spaces as the population is found both in and outside the aggregate. However, most microbial populations tend to be found on the inside of pore spaces within aggregates. Transport of air and water within soil is governed by the three-dimensional arrangement of pores. Thus, the proximity to pores strongly influences functioning of the soil microorganisms inside the aggregate. Therefore, soil aggregate stability is a key soil property affecting the functioning of the soil (14). The stability of the aggregate reflects both microbial and nonmicrobial processes. At many contaminated or deteriorated sites, the soil structure has been lost, which then limits the soil's ability to function.

Soil Microorganism — Living in an Aquatic World

Even in what seems to be a dry soil, soil bacteria function best in an aquatic environment. They function best when covered by a water film. This water film fills small soil pores and acting as transport system for nutrients and dissolved oxygen. Soil water is described using units of megapascals (MPa). These are tension values and indicate the amount of energy needed to extract free water. Different soil types (i.e., clay, silt, or sand) will have different contents (percentage moisture) at the same water potential. For example, at 20% moisture, a clay soil has a greater ability to hold water (lower water potential) than a sandy soil. A saturated soil has a water content equivalent to 0 mPa, whereas at a water potential of -1.5 mPa, little water is available and the soil is dry. Soil microbial populations function best at water contents (%) equivalent to -0.01 MPa of water potential pressure (15). At the -0.01 MPa the water potential–content combination, the soil tends to have an optimum arrangement in terms of both water film thickness and open space for the exchange of oxygen from the air (Fig. 1). Soil microbial activity will decline as the soil dries (moves toward more negative water potentials) because without water, the bacteria will dehydrate and lose function and they are unable to receive nutrients. Conversely, too much water will suppress aerobic metabolic activity because the soil water near the cells cannot be replenished with oxygen at a rate corresponding to microbial consumption.

As a result, interactions between oxygen and water within the soil are complex because the gas that enters the soil in the open portion of the pore must dissolve in water before the bacteria use it. The solubility of oxygen in water is 0.028 mL O_2 mL^{-1} H_2O atm^{-1} (16) or as the more common expression 8 mg O_2 L^{-1} (70 °C). The situation is further complicated by the diffusion rate of O_2 in water which is about 1×10^4 of the diffusion of oxygen in air [values of diffusion coefficients are 2.5×10^5 cm^2 sec^{-1} in water versus 0.189 cm^2 sec^{-1} for air (17)]. As a result, the level of oxygen tends to decrease with soil depth as both poor water solubility and low diffusion rates limit oxygen transport. The decline in oxygen levels down the soil profile reflects both respiration by organisms near the soil surface and restriction on flow related to decreasing pore size and increasing water content with depth in the profile. However, the larger pore spaces near the soil surface generally allow for oxygen diffusion for the upper portions of the profile keeping it aerobic.

A somewhat analogous situation occurs at a smaller scale within soil aggregates. The population near the aggregate surface first uses the available oxygen. As was found with the soil profile, free water in the soil pores limits the inward flow of oxygen. However, in the case of the aggregate, the restrictions on the flow of oxygen into the aggregate are even more significant because the oxygen must diffuse down very small pore spaces. As a result, an aggregate within an otherwise aerobic soil can develop an anaerobic core. Smith and Arah in 1986 (18) and others (19) demonstrated the presence of anaerobic microsites or conditions in otherwise aerobic soil.

MICROORGANISMS IN SOIL

Diversity and Cell Density in Soil

Trevors (20) has described soil as a "virtually limitless pool of genetic information contained in bacteria." This conclusion reflects the fact that surface soil can contain some 4,000 different microbial genotypes with as many as 10^9 (one billion) cells in 1 g (21,22). Others have suggested the number could be as high as 40,000 bacterial species in 1 g. Subsurface soils tend to have lower population levels but can exceed 10^7 cells g^{-1} (23,24). Surprisingly, given the sheer numbers of microorganisms, the microbial population comprises less than 3% of the soils organic carbon and occupy only 0.001% of the soil's volume (25). Therefore, microorganisms are not densely packed but rather live as "islands" or microcolonies on soil surfaces in aggregates or embedded in decaying organic matter (26). Hissett and Gray (27) have shown that microorganisms reside on less than 0.17% of the surface of the organic matter of the soil and less than 0.02% of the mineral surfaces of the soil. This pattern of distribution reflects

the lager surface area of the soil. For example, the specific surface area of soil clay can range from 5 to more than 750 $m^2 g^{-1}$.

Movement of Cells in Soil

Microorganisms generally adhere to soil surfaces by electrostatic interactions, London-van der Waals forces, and hydrophobic interactions (28,29). The overall movement of bacteria in soil is controlled by their tendency to undergo sorption onto soil particles or their transport and trapping in small soil pores (30). Most movement of bacteria in soil occurs by passive transport where the cells are moved with flowing water. On the other hand, active transport that is facilitated through the expenditure of energy is fairly limited (31–33). Tan and coworkers (34) found that passive movement was retarded by the adsorption of bacteria onto surfaces. Generally, passive movement will carry bacteria greater distances, over several centimeters, than active movement (35), which carries then over a few millimeters.

The predominant factors affecting bacterial transport are the ionic strength of the suspending solutions, which affects the charge properties of the cell and the surface, the soil surface properties, and the flux rate of water through the system. Trevors and coworkers (36) found that the movement of a genetically engineered strain of *Pseudomonas* through a soil column was a function of water flow rate and the number of times the column was flushed. This is supported by the work of Gagliardi and Karns (37) who showed for *Escherichia coli* that high levels of bacterial penetration into the subsurface could be seen when high rates of water input occurred along with the application of the bacteria. Cho and Kim (38) demonstrated using *Salmonella typhi* that bacteria introduced with manure could survive in both viable and viable but nonculturable (resting) states. Longer-term survival was not addressed in either study but Gagliardi and Karns (37) showed that significant growth could be seen even in the short periods (18 days) following application of bacteria and manure. They reported a 15-fold increase in the number of bacteria going in and leaving the system, when compared with the numbers inoculated into the system.

Cell Retention on Surfaces

Cell retention in soil is a function of the type of bacterium and the soil surfaces. It has been reported that the migration of bacteria with a hydrophobic exterior was two to three times slower compared with a similar strain with hydrophilic surface properties (31); authors were able to correlate cell retention with the adhesion of the hydrophobic strains. Other work has shown that the contribution of flagella to movement declines as the soil dries and the water film thickness decreases. As opposed to increasing the potential for movement, it is suggested that presence of flagella may enhance cell sorption onto soil surfaces (32). The movement of motile bacteria, especially ones with large numbers of flagella, may be impaired as the flagella increase the cell's overall volume preventing its movement through small pore necks (32,33).

Surface and Pore Neck Colonization

The location of bacterial colonies within soil is influenced by soil structure, the location of nutrients, and pore size (39–42). The exact factors that lead to colonization of a given pore or surface region of a pore space are unresolved. However, detailed analysis of soil structure has shown that greater than 80% of the cells found in soil are located within the smaller aggregate fractions of the soil (43) rather than on outer surfaces. Work by Fisk and coworkers (44) has shown that for introduced bacteria, a prime location for colonization is along the intergrain area where mineral and organic materials meet. They also observed that most of the resident bacteria are located along the same interfacial areas.

At the microscale, the arrangement of cells in soil aggregates is the predominant factor influencing cell behavior. Soil bacteria are sessile and become physically resident on soil surface. The size of the actual pore space in which bacteria reside governs the amount of available oxygen and nutrients. Pores between areas colonized by bacteria and areas colonized by predators such as protozoa also affect the long-term survival of the bacteria. They are most often found within pore spaces connected to adjoining interaggregate spaces through a channel with a pore neck sized between 0.25 and 6 μm in diameter (45,46) (Figs. 1 and 2). A pore with a 0.25 μm diameter limits entry of bacteria, whereas sizes in excess of 6 μm allows entry of protozoa. By colonizing pore spaces connected with a pore neck size of less than 6 μm, the bacteria can be protected from protozoa, which require a larger pore diameter to invade the space (40,46–49). It has also been shown that protozoan activity and predation often coincide with high bacterial numbers and activity (50). Therefore, the feeding activities of protozoa are effective in controlling the size of active population of soil bacteria and may control the establishment of inoculated species sometimes used in remediation situations or as seed inoculants (46,51).

Microorganisms colonize the soil matrix as the soil is forming and have also been reported to occur in the microaggregate soil fractions between 2 and 53 μM in size (50). Recent work has suggested soil bacteria to be mainly associated with the clay and silt fractions and

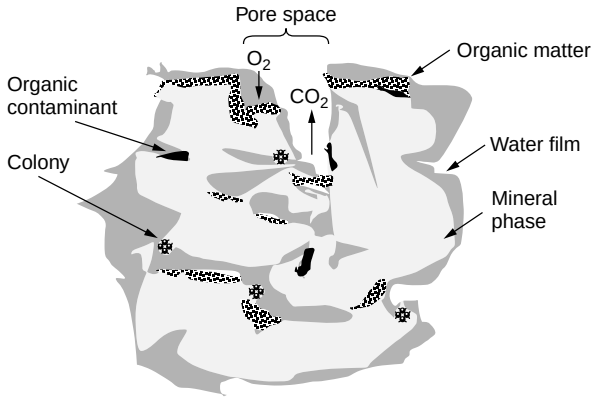

Figure 2. Diagram depicting the relationship of a resident soil microbial populations to the open pore space, organic matter, and organic pollution. Water film thickness is variable as is the distribution of both natural organic matter and the contaminant.

fungi to be associated with the organic materials and coarse sand fractions (52).

A ready supply of carbon and electron acceptors (oxygen, Mn^{4+}, Fe^{3+}, SO_4^{2-}) is thought to be a controlling factor for the active colonization of an area (53,54). Gram-positive bacteria are often found on the outside surface. In contrast, gram-negative bacteria are generally found within the aggregates and pore spaces (47). This distribution may reflect a selective pressure applied by changes in water availability because gram-positive bacteria are generally more resistant to drying than are gram-negative bacteria (15). The internal regions of the aggregate will have more stable water content because the small pores spaces are slower to dry when compared with the outside that is exposed to the pore space air. The slower drying rate reflects the smaller pore necks and a lower flux of water. On the other hand, the wetter conditions can also lead to anaerobic conditions. These findings support the conclusion that the soil microbial population is discontinuous, not forming a biofilm as is found in the typical wastewater biotreatment facilities (44).

Feeding the Microbial Community

Once attached to soil, soil microorganisms remain relatively immobile for the majority of their existence. As a result, organic materials must be transported to the microorganisms to be used. Therefore, the arrangement of micropores, which controls the flow of water in turn controls, the flow of nutrients, will influence the locations where colonies develop. The discontinuous arrangement of microbial population structure in and out of the pore spaces is a key factor affecting transformations of organic compounds. While the residency of the bacterial population inside small pore necks protects them from predation, small pore neck sizes also limit access to nutrients, which must diffuse inside the soil aggregate to be used. This diffusion-linked pattern of growth accounts for the microorganism's occurrence along interfacial areas between regions of soil organic matter and mineral surfaces. The mineral surface gives the bacteria a point of attachment, access to potential electron acceptors other than oxygen, whereas the adjacent organic materials provide a long-term source of nutrients over a short diffusion pathway (Fig. 2).

The active portion of the soil microbial community makes use of carbon and nitrogen derived from/during plant decomposition or the mineralization of native soil organic matter (55). A strong correlation between plant productivity and active soil microbial population has been shown (56). Others have shown a strong correlation between microbial biomass and available soil carbon (57). Subsurface microorganisms are dependent on carbon leached from surface materials or materials delivered from adjacent aquifers and the capillary rise of groundwater. Patterns of microbial activity reflect the pattern of available carbon. In particular, long-term undisturbed sites tend to have higher carbon and activity levels (enzymes, respiration) deeper in the soil profile than do sites that are routinely tilled (58). Mixing of the active soil population with residues and the inclusion of air and water that enhances the degradation processes. Of particular note is the finding that manure, which is high in dissolved organic carbon (DOC), can significantly increase the size of soil microbial biomass (59) and can influence subsequent degradation rates of chemicals (60). Work has shown that soil microbial diversity is also affected during the composting of manure in the soil (61). These findings suggest that the microorganisms in soil and the subsurface are generally faced with a poor nutrient availability and that increasing the nutrient availability will alter the composition of the population.

Because of the lack of available nutrients, the majority of the organisms in soil are in a "resting stage"; they are alive but are maintaining themselves in a low level of activity (25,62). In fact, less than 1% of the soil bacteria are typically recovered using typical laboratory isolation procedures (63). Studies to compare soils have confirmed this finding. Direct isolation of DNA from soil shows a higher phylogenetic diversity than does isolation of individual colonies (64). Indeed, the inactive biomass constitutes the major portion of the "limitless pool of genetic resource" described by Trevors (20). Environmental stress or other situations (e.g., an input of nutrients or chemicals) can cause part of these inactive members to respond and become active, whereas other parts of the active population may become inactive. This switching allows a staged response to an outside perturbation and avoids the need for all members of the population to maintain themselves at high levels of activity under all conditions. This is a critical consideration because most soil and subsurface material has limited available nutrients. Shift ability is most clearly demonstrated in an example of microbial response between acttive and inactive states in a special region near the roots called the *rhizosphere*. This rhizosphere region forms near a growing root (approximately 1 mm away) when microbial populations are consuming carbon, and other materials, released from the root. Recent studies (65) have confirmed earlier reports and shown that the types of active microorganisms near the root are responding to the types of available nutrients in the rhizosphere. Before these studies, most rhizosphere work indicated the number of cells increased in the rhizosphere, but a clear correlation to changes in the population structure (types of organisms) had not been made (66).

Like the situation occurring for surface soil, the subsurface is composed of diverse microbial population capable of many biochemical processes. The subsurface tend to have smaller pore spaces as the geologic materials are more tightly packed and as a result, the size of the biomass and its activity tends to decrease with increasing depth. Small pore neck size, high bulk densities [low nutrients, poor water availability, and few dissolved electron acceptors (especially oxygen)]. The literature on the subsurface has shown it to be both complex and heterogeneous (67,68).

SOIL MICROBIOLOGY AND CONTAMINATION

Transport and Delivery of Contamination in Soil Influences Biodegradation

Contaminant metabolism (or any metabolism) is governed by transport and the availability of substrates. As

Andersson and Henrysson (69) point out, contaminants such as polycyclic aromatic hydrocarbons (PAH) are limited in their availability to indigenous microflora and this makes degradation or remediation difficult. Malina and coworkers (70) and Welp and Brümmer 1999 (71) describe three factors as limiting hydrocarbon biodegradation in the field. These are the inherent toxic nature of the material and metabolites that may form, the net availability of the chemicals as modulated by surface sorption, solubility, and speciation, and changes that may occur in the microbial population in response to the chemical. Ogram and coworkers (72) showed the organic molecule 2,4-D once sorbed to soil was recalcitrant to biodegradation and Shelton and Parkin (73) reported that desorption of carbofuran in soil limited biodegradation. Moreover, Scribner and coworkers (74) reported that the biodegradation of simazine was limited by desorption. These studies indicate that sorption-desorption processes play a major role in biodegradation by affecting bioavailability of contaminants in the soil. This is because soil is a surface-dominated environment and sorption is the main mechanism controlling the level of the chemical in solution. In general, the level of organic carbon and pH of the system, which affects both the chemical and the surface chemistry, control sorption processes. The toxicity of some materials is modulated by the interactions with a surface that retains some fraction of the material lessening the solution concentration.

The majority of studies investigating sorption and transformation of chemicals in soil have been conducted at relatively low chemical concentrations. The dominant mechanisms responsible for the retention of chemicals in soil and subsurface environments are hydrophobic partitioning (in organic matter), hydrogen bonding, and dipolar interactions. Soil organic matter has been consistently implicated as a major component in controlling the sorption in soil. The early work of Talbert and Fletchall (75) showed strong positive correlation between the chemical retention by soil and organic matter. Others have confirmed this (76–78). In studies of sorption using Atlantic coastal plain soils, Johnson and Sims (76) observed that retention of several chemicals correlated strongly with organic matter content and exchangeable acidity. These data suggest that although hydrophobic partitioning of chemicals into soil organic matter is an important mechanism, electrostatic and pH effects are also important (79,78). These mechanisms do not act independently, however, and the relative contribution of one mechanism over another will depend strongly on the amount of soil organic matter, pH, and clay content. The sorption of dissolved, nonpolar organic compounds in soil and sediment is a widely studied phenomenon (80,81). This process is described as an equilibrium distribution of the nonpolar organic solute between the aqueous phase and the organic matter in the soil. The mechanism of retention is thought to be a partitioning phenomenon, similar to the partitioning of hydrophobic organic compounds between an organic solvent phase and aqueous phase in a biphasic solvent system. This process is often characterized by linear sorption isotherms in which the sorbed concentration is directly proportional to the solution phase concentration. However, there have been a number of other studies to show that surface hydrolysis, hydrogen bonding, and surface-mediated chemisorption can occur and will affect the retention of the material (82–84).

High concentrations of chemicals pose a significant nonpoint source pollutant contamination potential at contaminated sites (24,85). Sorption as identified by studies at dilute aqueous solution may not be a predominant mechanism controlling the retention of concentrated chemicals typically encountered at contaminated sites. This is because at contaminated sites, bulk chemical trapping in pore spaces and matrix creates a chemical "source zone" because organic compounds in pore spaces diffuse into soil aggregates (86). Bulk chemical trapping retains a higher fraction of the total load. Whereas sorption and trapping in the smallest pores may hold a fraction of the material that is more difficult to degrade. Microbial degradation of source zones tends to be from the outside of the zone inward. This reflects the fact that chemicals are often toxic in the source zone and that microorganisms may be limited by the supply of oxygen or secondary nutrients (Fig. 3).

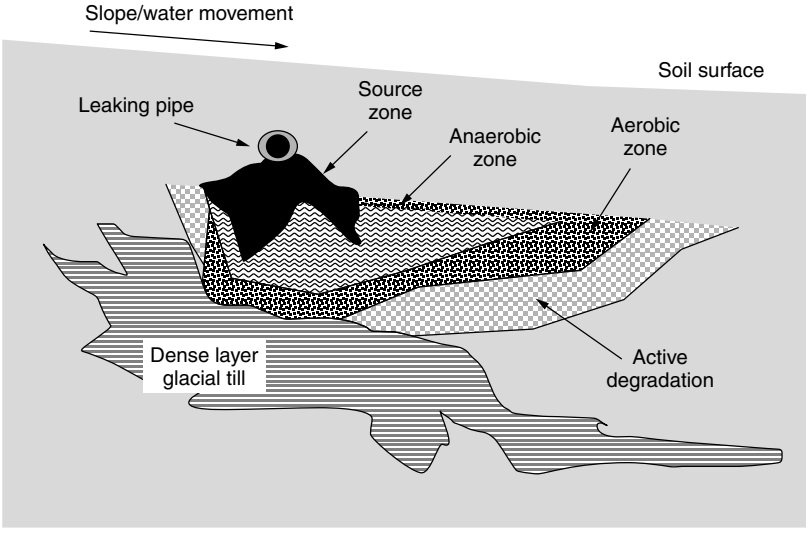

Figure 3. The zones typically encountered near a hydrocarbon leak or spill. The source zone is surrounded by a zone of oxygen depletion, an aerobic zone, and zone of active degradation. The zone of active aerobic degradation is influenced by both the availability of oxygen and the availability of secondary nutrients, primarily nitrogen, and phosphorus. Movement of the hydrocarbon is often influenced by the presence of dense subsurface layers.

Overall distribution and transport of chemicals to a location in the profile is limited by the flux of water through the system and retention. Vapor transport of chemicals may also occur. The retention reactions for either can include surface sorption and trapping in small pores and spaces. In terms of biodegradation of introduced materials, Sawhney and coworkers (87) and Steinberg and coworkers (88) showed that 1,2-dibromoethane (EDB), a water-soluble, biodegradable, and weakly sorbed organic molecule, persisted in soil for long periods. This is in contrast to controlled laboratory studies in which it was shown that the material could be rapidly degraded in culture. In soil, the chemical appears to become entrapped in the soil matrix passing down soil pores (possibly smaller than 0.25 μm) where it is protected from degradation. Others, Borchers and Perry (89) and Powlson (90), have shown that in soil, there are pools of physically protected nitrogen and carbon, which act as a slow-release source of the nutrient. Again, it seems the pore neck size may limit diffusion of nutrients to microorganisms and the soil solution. Roberston and coworkers (168) found that there was a significant patterning of NO_3^- release across a landscape indicating localized differences in microbial ability and the formation of NO_3^-.

Studies to address the temporal and spatial variability associated with the field-scale distribution of microorganism and their abilities to degrade recalcitrant organic compounds are limited (91). Evaluation of field-scale variability in a soil microbial populations has found that major differences in a *Rhizobium* population are evident at sampling distances of less than 0.2 μm (92) and that there is a spatial variability in the degradation of pesticides in soil (41). Others have shown similar results and confirm that the distribution of bacteria in soil is not uniform, but that the distribution reflects both the soil structure and available nutrient supply (42). Coresuil and Weber (93) suggest that a correlation between the size of the standing biomass and the onset of hydrocarbon degradation exists and as a result, a critical population size is needed before rapid degradation becomes possible. This has also been shown for the degradation of propylene glycol (85).

PHYSIOLOGICAL PRINCIPLES AND THE UNDERLYING BIODEGRADATION REACTIONS

Using Soil Organisms for Biodegradation

Biodegradation describes the processes microorganisms use to catabolize (i.e., break down and simplify) a variety of compounds (chemicals, soil organic matter, and plant and animal residues) that would otherwise persist in the environment. In general, these simplification processes are used by the resident soil microflora as a means of obtaining both nutrients (carbon, nitrogen, phosphorus) and energy (ATP). Biodegradation describes a fundamental set of processes in which microorganisms are converting materials to recover energy and materials for building new cells. Madsen (94) has defined bioremediation as "a managed or spontaneous process in which biological, especially microbial, catalysis acts on pollutant compounds, thereby remedying or eliminating environmental contamination." Therefore, bioremediation is a direct utilization of inherent microbial abilities and can take many forms (Fig. 4).

Three factors, namely, the microorganisms, the contaminant, and the environmental setting function to modulate the field-scale biodegradation and bioremediation process (94–98). The contaminant's chemical structure (i.e., the number of carbon rings, side chains, halogens, and bonding arrangement), concentration (ngg^{-1} to gg^{-1}), physical placement (either the soil surface or in solution), the presence of electron donors or acceptors, and the physiological abilities of the microorganisms, either resident or introduced, interact to control the success of biodegradation or bioremediation. If any of these components are less than optimum, microbial-mediated degradation will be slow or not take place. In fact, it has been suggested that the long-term residency of contaminants in soil and the subsurface indicate the presence of poor or suboptimal conditions and the suboptimal condition has caused the contaminated site to form in the first place. The operational goal of a successful bioremediation program is to overcome the suboptimal conditions at the site by maximizing the functioning of the resident or applied microbial population to achieve a biodegradation of the materials. Removing the rate-limiting factors and promoting the activity of the microbial populations should allow

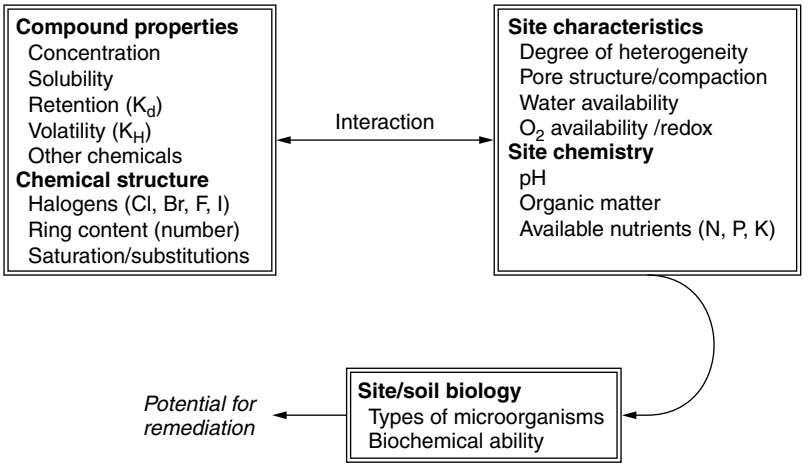

Figure 4. A diagram relating the three major components in site remediation, the compounds chemistry, the site properties, and the abilities of the microbial populations.

the community to destroy the pollutant. In essence, the key question in bioremediation becomes: what chemical or biological feature of the site has prevented the microbial population from removing the pollutant?

Successful bioremediation of surface and subsurface environments can result from a manipulation of the contaminated system to encourage the destruction of the contaminant by the microorganisms. This approach is bolstered by the fact that many laboratory studies have shown that biodegradation of pollutant chemicals can occur. However, the transformation rates estimated in the laboratory studies tend to be much higher than that found in the field. It is suggested that this difference reflects a spatial variability that hampers the microbial population. Therefore, the microbial community's ability to respond to the chemical is lessened in the field, as compared with the response at the laboratory scale (69).

Microbial Processing of Contamination

Long and coworkers (24) showed that exposure to high-level ($mg\,kg^{-1}$) petroleum contamination alters the characteristics of a subsurface microbial community. These results indicate that high levels of petroleum contaminants can exert toxic effects on microorganisms, but at low concentrations it is possible to enrich specific degraders. Contamination of a forest soil with pentachlorophenol was shown to reduce both soil microbial biomass and the numbers of collembolans, enchytraeids, and fungal-feeding nematodes. Therefore, it was felt that the diversity of soil fauna was reduced with the high-level contamination (99).

Low levels of contaminants (a few $\mu g\,kg^{-1}$) can result in the development of chemical-adapted microbial communities in the contaminated samples. This illustrates an outcome of Trevors (20) concept of "limitless genetic resource" as portions of the population are able to adapt to the situation. However, in highly contaminated materials, containing metals, or organics, or both, total microbial biomass is generally lower (52,24) indicating that a toxic effect has occurred. It has been shown that the chemical 1,2-dicholorobenzene reduces the fungal population size and can selectively reduce bacterial numbers (100). Function can also be impacted. Contamination of soil with copper has been shown to alter nitrification rates (101). These findings, in part, explain why some contamination can persist as it impacts basic microbial functions.

Soil microbial populations have been shown to adopt to materials such as aircraft deicing fluids (ADF) — Propylene Glycol, provided the population is not exposed to extremely high levels at the outset (85). With exposure to low levels of ADF (5 to 10%) the onset of degradation was delayed, but the overall rate of degradation was high. They found that high levels of ADF (40%) inhibited all degradation, a situation that is analogous to that described by Long and coworkers (24). It is also clear that mixed contamination can have differential effects on the response of the population. Gasoline, which is composed of many organic molecules, will degrade in soil. However, the presence of high levels of 2-ethyltoluene and trimethylbenzene will inhibit the degradation of other fractions of the gasoline, again showing the interrelatedness of the response (102).

Assimilatory and Dissimilatory Reactions and the Removal of Contamination — Aerobic

What is clear from the available wealth of reaction data is that bioremediation can make use of the four features of an active population, that is, their need for assimilatory nutrients such as carbon, nitrogen, sulfur, phosphorus, their need for a source of electrons, their enzymatic nonspecificity (co-oxidation), and their need for terminal electron acceptors. A detailed assessment of the redox and energy needs of microorganisms set in the context of biodegradation of contaminants in soil and water can be found in Harris and Arnold (103). They state that growth of an organism is a function of both assimilatory and dissimilatory steps, whereas maintenance of the organisms is primarily dissimilatory. Assimilatory reactions gather the required carbon, nitrogen, sulfur, and phosphorus for biomass (cell) production. Dissimilatory reactions provide the energy for assimilatory reactions and any subsequent maintenance processes. In general, the utilization of nitrogen and phosphorus is to satisfy the assimilatory aspects of the degradation process and the oxidation of material is to release reducing power from the substrate to generate ATP. Most efforts at bioremediation presumed that microbial populations respond to the targeted material as a source of carbon under aerobic conditions. Only recently has the potential of anaerobic processes been exploited.

For assimilatory processes the ratio of carbon to nitrogen should approach 30 : 1 (104) if optimum microbial biomass production is to occur. However, Dibble and Bartha (105) have shown oil degradation to be most rapid at C : N rations of 60 : 1, whereas others (106) have shown that degradation of propane and butane quickly becomes nitrogen-limited and that in response, the population may begin to fix gaseous nitrogen. The application of nitrogen sources should be done with care. Wrenn and coworkers (107) showed that in poorly buffered sea salt solutions, application of NH_4^+ to aid in the degradation of crude oil reduced the pH and degradation rates as compared with other nitrogen sources. O'Connor and Young (108) showed that nitrogen additions to phenol-contaminated sites would enhance degradation of the organic as much as twofold. They pointed out that the effectiveness of the nitrogen source was related to the type and position of the substitution on the phenol. Zhou and Crawford (109) provided an evaluation of nutrient application and the kinetics of BTEX degradation. They showed that all types of nitrogen (NH_4^+ as vapor, NH_4NO_3) were equal in their effectiveness as nitrogen source for BTEX degradation. One of the interesting findings of their work was that very low C : N ratios (1.8 : 1) suppressed degradation as compared with a ratio of 50 : 1. They also showed that in subsurface samples, an optimum oxygen addition giving a 10% enrichment was the most effective in stimulating degradation. The importance of adding phosphorous to the bioremediation system was also noted. Mills and Frankenberger (110) have shown that the addition of K_2HPO_4 at up to $500\,mg\,kg^{-1}$ would enhance degradation of diesel fuel. Rasiah and coworkers (111) have also indicated the

importance of added phosphorous sources in oil degradation. They found an effect from the type of nitrogen source added. In general, they rated the effectiveness of the nitrogen sources as $Ca(NO_3)_2 > NaNO_3 > KNO_3 > NH_4NO_3 > NH_4Cl$. Others have indicated that for bioremediation, the ratio should be in the range of 120 : 10 : 1, C : N : P, respectively (112).

Pope and Matthews (113) point out that nutrient requirements for enhancing biodegradation in the field have not been thoroughly studied. Finklea and Fontenot (114) showed that typical field cultivation (to enhance air and water entry) combined with an addition of nitrogen, phosphorus, and potassium (13-13-13) was effective in stimulating the degradation of atrazine in contaminated soil. Applications of manures were shown to stimulate the degradation of atrazine and 2,4-D in soil. It is suggested that the DOC in the manure stimulated the general soil growth of the population. However, other work has shown atrazine to be a ready source of nitrogen (115) and the addition of soluble carbon could have stimulated a demand for nitrogen resulting in the degradation of the chemical. A large-scale use of fertilizer was made in efforts to remediate the Exxon Valdez oil spill in Prince William Sound, Alaska (116). Work by Ilyinsky and coworkers (117) had indicated that fertilizer materials coated in oil or an oil membrane would have improved penetration into oils in the environment. The fertilizer addition gave higher hexadecane and phenanthrene mineralization than was found in the untreated soil (118).

Mineral nutrients have been effective in stimulating the degradation of petroleum wastes in surface soil (111) and *p*-nitrophenol in aquifer solids (119). In contrast, addition of nitrogen of up to 80 mg kg^{-1} did not affect the mineralization of added atrazine in a surface soil (115). Pothuluri and coworkers (120) were able to increase the degradation of alachlor in a subsurface soil 15 to 35% by adding a mixture of glucose and hydrolyzed casein. In contrast, glucose reduced the degradation of several xenobiotic compounds (119,121,122) in lake water and aquifer solids. A consideration of the importance of minor or trace elements is lacking for bioremediation systems.

Semprini and coworkers (123–126) have shown that it is possible to use substrate co-oxidation to degrade groundwater resident contaminants, in particular, chlorinated aliphatic hydrocarbons (CAH). The system is based on the co-oxidation, or the fortuitous transformation, of an organic compound by microorganisms. The microorganism receives no direct benefit from the fortuitous transformation of the CAH material. In the case of CAHs, few with more than two chlorine substitutions have been shown to support aerobic microbial growth as a carbon source. Semprini (123–126) has shown that methane monooxygenase used to oxidize methane by methanotrophic bacteria is able to concurrently oxidize CAHs. When the contaminated site is flushed with methane and oxygen to stimulate the methanogens, the co-oxidation of the CAHs may also occur. In other studies, they have shown a similar approach using the co-oxidation CAHs with phenol as the primary substrate in a cometabolizing system (127). A co-oxidation approach has been used by Aziz and coworkers (128) in the construction of a hollow-fiber flow-through system that couples a CH_4 oxidation by a methanotrophic bacteria with the co-oxidation of a CAH — tricloroethlyene (TCE) — in contaminated groundwater. They were able to show a significant reduction in TCE with co-oxidation.

Regardless of the types of available carbon and nitrogen, microorganisms must use an electron donor/acceptor couple to capture energy derived from dissimilatory reactions. This approach is universal and used in fermentative, anaerobic or aerobic reactions. For aerobic processes, the cell's biochemical system is routed to capture the generated energy (electrons released) during oxidation by reducing NAD+ (nicotinamide adenine dinucleotide) to NADH. ATP is generated while the NADH is being reoxidized to NAD+. In aerobic systems, the electrons are passed down the electron transport chain, across the cell membrane, and this creates an energy gradient. Under aerobic conditions, the electron acceptor is oxygen. However, other substances such as NO_3^- and SO_4^- are also selectively used. ATP synthase enzymes capture the electrical and chemical potential of the gradient and the overall process is referred to as *oxidative phosphorylation* as it results in the formation of ATP.

In aerobic systems, the combined dissimilatory and assimilatory reactions using oxygen as an electron acceptor are described by the following reaction:

$$\text{Substrate} + \text{Nutrients} + O_2 \rightarrow \text{Biomass} + CO_2 + H_2O + \text{Metabolites} + \text{Energy}$$

An example reaction (disregarding biomass production) to illustrate the process is the oxidation of glucose:

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O + \text{energy}.$$

This translates to two moles of oxygen per mole of carbon utilized. The stoichiometry for toluene oxidation is:

$$C_6H_5 - CH_3 + 9O_2 \rightarrow 7CO_2 + 4H_2O + \text{energy}.$$

This translates to 2.57 moles oxygen per mole of carbon oxidized to carbon dioxide. If we consider benzene (and disregard biomass production), we find the following reaction:

$$C_6H_6 + 7.5O_2 \rightarrow 6CO_2 + 3H_2O$$

The transfer of electrons demands 7.5 moles or 240 g of oxygen per mole of benzene when an aerobic system is in place. This translates to 3.15 g of oxygen per gram of benzene. A 10,000 kg hydrocarbon plume contained in soil would require 3.15×10^4 kg of oxygen for complete mineralization. Air contains 21% oxygen by volume; to complete the degradation, a total of about 1.5×10^5 kg of air would be required. Assuming the contaminated soil has a bulk density of 1.33 Mg m^{-3}, it would contain 50% pore space and 50% solids. Assuming half of the pore space is filled with water, we would have an available air supply of about 0.33 Mg or 3,300 kg of air. Under these conditions it would take some 45 replacement volumes of

air to supply enough oxygen to allow mineralization of the plume. This assumes that all of the material is available for degradation, the material is evenly distributed within the site, the bulk density is uniform throughout the profile, the oxygen that is available instantaneously and other nutrients (nitrogen and potassium) that are not limiting, and that the chemical levels are not toxic. Few of the constraints aforementioned are commonly found to occur at a contaminated site. For example, the level of oxygen within the soil profile will tend to decrease with depth and the secondary nutrients tend to be limited. Moreover, as the biomass builds up, it will require a significant level of oxygen to maintain itself. When the concentration of oxygen in the profile is decreased by 5%, reflecting oxygen utilization at the surface, the volume of air needed to resupply the degradation processes in the subsurface is increased by 150% to 3.8×10^5 kg.

It stands to reason that the aerobic degradation rate may become a function of oxygen resupply to the regions undergoing degradation processes. The resupply is a function of transfer into and through both the soil profile and the water films. Some have suggested the use of hydrogen peroxide (47% oxygen), and not soil, as a soluble form of oxygen for groundwater supplementation. Oxygen is rapidly released from the hydrogen peroxide as the liquid encounters enzymes and metals. However, hydrogen peroxide is toxic to microorganisms at concentrations above 100 mgL^{-1} and successful use has been limited (129,130). Prosen and coworkers (131) suggested a system in which pure oxygen is generated on-site and this, instead of air, is pumped into the subsurface. Because air is 21% oxygen, use of pure oxygen would increase the efficiency fivefold. In all cases, the introduction of air or oxygen into anoxic subsurface conditions can be problematic because the oxygen can undergo abiotic reactions with reduced mineral surfaces and can be removed from the solution before it can serve as a terminal electron acceptor for microbial processes (132).

Assimilatory and Dissimilatory Reactions and the Removal of Contamination — Anaerobic

Modeling efforts for subsurface systems support the finding that for xenobiotic compounds, lack of oxygen is a limitation for degradation (133). Under aerobic conditions, pyridine and hydroxylated pyridines were more rapidly transformed than under anaerobic conditions (134). They tested surface and subsurface soil that had been exposed to these compounds for several decades. All the pyridine derivatives tested were degraded within two weeks in the presence of oxygen. Under anaerobic conditions, however, longer time periods were required. Jet fuel in the subsurface environments can have a long-term residence as a result of a lack of available oxygen (135). A similar situation is suggested for propylene glycol because oxidative degradation was enhanced by the presence of oxygen (85). Remediation sites can have a further complication because of the presence of a second material such as oil that can inhibit the flow of air or water. Oil perched on water changes the diffusion profile and acts as a barrier for oxygen transfer into water (136) providing a diffusivity of about 2×10^3 $cm^2\,s^{-1}$ for oxygen moving in oil (137). Moreover, the film thickness will change the transfer rate; films thicker than 100 μm will diminish the overall transfer velocity by as much as half.

Under oxygen-limited conditions, strict and facultative anaerobic bacteria are able to metabolize and grow if supplied with one of a number of alternative electron acceptors. The use of anaerobic organisms offers possibilities in bioremediation applications (95,138). In contrast to aerobic respiration, anaerobic organisms make use of a number of electron acceptors. A general reaction scheme for various electron acceptors typically used by microorganism is given by:

$$\text{Substrate} + \{NO_3^-, Mn^{4+}, Fe^{3+}, SO_4^{2-}, CO_2\}$$

$$\downarrow$$

$$\text{Biomass} + CO_2 + \{N_2, Mn^{2+}\ Fe^{2+}\ S^{2-}\ CH_4\}$$

The type of electron acceptor favored will reflect the redox status of the system. Therefore, the sequence of reduction, NO_3^-, Mn^{4+}, Fe^{3+}, SO_4^{2-}, CO_2 reflects the oxidizing capacity of the chemical half-reaction (139). Kazumi and coworkers (140) showed that 3-chlorobenzoate could be degraded with NO_3^-, Fe^{3+}, or SO_4^{2-} acting as the electron acceptor. McFarland and Sims (141) have developed a conceptual framework for interpreting the thermodynamics of PAH degradation in the environment. They extended their model to not only oxygen as an electron acceptor, but also NO_3^-, Mn^{4+}, Fe^{3+}, SO_4^{2-}, CO_2. They pointed out that thermodynamically the reduction of Mn^{4+} may be favored in groundwater and subsurface systems given its widespread availability and that the ΔG° for the reduction of Mn^{4+} gives a free energy change similar to that found with oxygen. However, the usefulness of Mn^{+4} as an electron acceptor is limited by the low solubility of the mineral (141).

At the center of a subsurface hydrocarbon plume, the conditions may be anoxic and highly reduced (142). At the edges of the plume, the conditions are more aerobic and better oxygenated because the contaminated water mixes with noncontaminated water. Others (143) have used the subsurface formation of methane, an indicator of extremely reduced conditions, to map plume migration in soil and aquifers. More recently, Lovely and coworkers (144) have demonstrated the use of dissolved hydrogen to describe the predominant terminal electron accepting processes (TEAPs) occurring in an aquifer. This approach allows a better description of the redox chemistry and contaminant transformation processes occurring in anoxic groundwater systems. The TEAPs method is more robust than other signature processes because it relies on the occurrence of characteristic concentrations of hydrogen. Hydrogen values are less ambiguous than CH_4 or SO_4 because hydrogen is quickly cycled, is poorly reactive with mineral surfaces, and has a short half-life (145). Chapelle and coworkers (145) used the TEAPs concept to describe the distribution of redox states within a hydrocarbon-contaminated aquifer.

Nitrogen as NO_3^- can be introduced to serve as an electron acceptor in moderately reduced or hypoxic (low oxygen) conditions. For example, it has been shown that

for toluene, the following reaction is possible:

$$C_6H_5 - C_6 + 6NO_3^- \rightarrow 7CO_2 + 4H_2O + 3N_2 + \text{energy}$$

The advantage of NO_3^- as an electron acceptor is that it is more soluble than oxygen (660 g NO_3^- $L^{-1}H_2O$), allowing it to be distributed throughout a contaminated aquifer or subsurface material. Dissolved NO_3^- will move with water through flow paths and into micropores and spaces. NO_3^- will serve as an electron acceptor in the remediation of contaminated aquifers in which BTEX is the primary contaminant (132,146,147). Gersberg and coworkers (148) used NO_3^- as an electron acceptor in the remediation of BTEX in an oxygen-poor aquifer. This NO_3^- approach was also used by Burland and Edwards to degrade benzene under anaerobic conditions and supported other studies that had shown that anaerobic removal of benzene was possible (149). Kazumi and coworkers (140) showed that the utilization of monochlorobenzoate isomers (2-,3-, and 4-chlorobenzoate) by microbial consortia in river sediments was possible under denitrifying conditions. They were also able to show that a loss of 3-chlorobenzoate would occur under iron- and sulfate-reducing conditions and under methanogenic conditions. For a given hydrocarbon, the NO_3/N_2 couple is energetically similar to the O_2/H_2O couple. The standard-state reduction potential for oxygen is 1.22 volts, whereas NO_3^- reduction to nitrogen gas an E_h^0 of 1.24 volts. This shows the relative energy available in the redox couples is quite similar.

Not all bacteria capable of aerobic degradation of a given aromatic contaminant and capable of NO_3^- reduction are automatically capable of degradation of the same contaminant when using NO_3^- as the electron acceptor. It is unclear as to the exact pathway used by facultative microorganisms when degrading aromatic compounds under NO_3^--reduction conditions. Downs and coworkers (150) has used NO_3^- in an approach similar to that of Hutchins and coworkers (132) to degrade BTEX, but they implicated that aerobic step was needed in the degradation of benzene. Others have reported a similar finding (132,152). Rate constants developed for the comparative studies of BEXT removal under either aerobic or denitrifying conditions indicated that reactions occurring under denitrifying conditions are somewhat slower than that reported for aerobic processes (132). Leahy and coworkers (152) demonstrated the potential of toluene-oxidizing bacteria to degrade trichloroethylene (TCE) under hypoxic conditions when NO_3^- was present. They also demonstrated that TCE could act as its own inducer suggesting the possibility that concurrent introductions of toluene into TCE-contaminated sites is not necessary to achieve degradation.

Anaerobic processes are particularly effective in removing halogens from haloorganic compounds; however, the rates are relatively slow. Nozawa and Maruyama (153) demonstrated that the anaerobic metabolism of phthalate and other aromatic compounds can be conducted by the denitrifying soil bacterium *Pseudomonas* sp. strain P136. In addition, dinoseb (an insecticide) that is not degraded in contaminated soil under aerobic conditions can be degraded when anaerobic conditions are established (155,156). In this study, anaerobiosis was developed by treating the soil with starchy potato-processing waste materials and allowing the aerobic population to deplete the available oxygen. The anaerobic microbial consortium degraded dinoseb completely, and the formation of polymerization products produced aerobically was avoided. This approach has been applied to the removal of munition-contaminated soil. Funk and coworkers (157) reported that a wide spectrum of explosives materials, including 2,4,6-trinitrotoluene, could be rapidly reduced and then degraded in soil systems when anaerobic conditions were established. The anaerobic transformation of polychlorinated biphenyls, DDT, and percholoroethylene has also been reported (158). Moreover, the nematicide 1,2-dibromo-3-chloropropane (DBCP), which persists in groundwater and soil, has been shown to be converted to organic products and carbon dioxide when soil suspensions are placed under anaerobic conditions (159).

CONCLUSION

Over the last 100 years, the study of soil microbiology has revealed that a wide array of inherent biochemical abilities exist in each gram of soil. Trevors' (20) statement describing soil as a virtual limitless pool of genetic information contained in bacteria can be coupled to other work that has shown microorganisms to be distributed in almost every type of soil and terrestrial location. The importance of the soil organisms to the ecosystem stability is clear. In fact, the study of soil microorganisms was hampered for years by our inability to separate the cells from their growth environment. This makes the study of soil ecology unique because few other areas of ecological investigation have such difficulty in separating the studied organisms from their habitat.

Therefore, the structure and function of soil reflects the interrelatedness of the organism and their habitat. This habitat is constantly changing in response to the actions of the population of cells that are present. This is somewhat surprising, given the wide distribution of cells across a volume of soil, but illustrates the importance of their biochemical processing abilities. Our use of soil is dependent on the microbial population and their ability to reduce the waste and recycle these stored nutrients. In recent years, our dependence on soil microorganisms has expanded as we now rely on them to remove pollutant materials.

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SOIL NITROGEN CYCLE

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Nitrogen is an essential element for life. It is found in key biological compounds, such as nucleic acids and proteins. Microorganisms and their activities are crucial to supplying biologically available nitrogen; in fact, only certain prokaryotes (and more recently, humans) can produce such nitrogen. In general, microorganisms play a key role in the transformation of nitrogen into its many available forms and thereby influence primary productivity in terrestrial and aquatic ecosystems.

Nitrogen in its various naturally occurring organic and inorganic forms is usually benign, at least at concentrations typically encountered by living organisms. Indeed, nitrogen is very often the nutrient most limiting productivity in many terrestrial and some aquatic systems. Increased exogenous inputs of nitrogen from chemical nitrogen fertilization or other anthropogenic activities have in some cases altered the natural balance, thereby contributing to environmental problems such as soil acidification, surface water eutrophication, groundwater contamination, and atmospheric pollution.

An understanding of nitrogen cycling in soils is critical to sustain the productivity of natural and managed ecosystems without environmental degradation.

FORMS OF SOIL NITROGEN

Although we tend to think of soil as solid, composed only of mineral particles and organic substances, it also has varying fractions of liquid, the soil solution, and gas, the soil atmosphere. Nitrogenous compounds exist in each of these phases (Table 1).

It is perhaps surprising that such a large fraction of soil nitrogen is composed of the relatively inert gaseous N_2. With the exception of the N_2-fixing prokaryotes that possess the remarkable ability to convert N_2 into biologically available forms, no other organisms can utilize N_2. Dinitrogen is returned to the atmosphere by denitrifying bacteria, as the metabolic end-product of NO_3^- reduction. Although N_2 is environmentally benign, the same is not true for other nitrogenous gases. Nitrous oxide (N_2O) is found at low concentrations and, on release to the atmosphere, it acts as a greenhouse gas and is implicated in the destruction of stratospheric ozone. Other nitrogenous gases present in soil at low levels include NH_3, which is in chemical equilibrium with NH_4^+ in the soil solution, and NO, an intermediate or by-product of several nitrogen cycle processes. Elevated NH_3 concentrations volatilizing from animal manures, in combination with the release of anthropogenically produced NO_x compounds, have increased the input of nitrogen in precipitation in many parts of the world with unintended effects on nitrogen cycling.

Soil solution contains organic and inorganic forms of nitrogen. Ammonium (NH_4^+) and nitrate (NO_3^-) are commonly considered the most soluble nitrogen forms; however, in some soils, soluble organic nitrogen is present

Table 1. Amounts of Important Forms of Soil Nitrogen (1). For Comparison, a Typical Agronomic Crop Might Contain About 25 g N m^{-2}

Nitrogen Form	Concentration	Content[a] (g N m^{-2})	Relative Fraction (%)
N_2	78 kPa	230	20.6
N_2O	≥31 mPa	90×10^{-6}	~0
NH_4^+	<0.05 to 5 mM	2.2	0.2
NO_3^-	<0.05 to 20 mM	4.4	0.4
Organic N	<0.1 to 5 g N kg^{-1} soil	880	78.8

[a]Based on median concentrations for 1 m^3 of soil at a bulk density of 1.25 Mg m^{-3} with a pore space fraction of 0.5 that is filled half with air and half with soil solution.

in greater concentrations (2). Soluble organic nitrogen may be a more important pool of available nitrogen than previously thought. Recent studies have shown that it can be a major source of nitrogen assimilated by microorganisms and plants (3,4). Just as different soils contain different proportions of soluble organic and inorganic nitrogen, the relative amounts of NH_4^+ and NO_3^- also vary, primarily because of the activity of nitrifying bacteria. In many undisturbed systems, especially temperate and boreal forests and heathlands, NH_4^+ is the dominant form. In most agricultural systems, native grasslands, and many tropical forests, NO_3^- is present in higher concentrations. In some aridic soils with high pH (>8), there may be measurable concentrations of NO_2^-, an intermediate in several nitrogen cycle processes.

Nitrogen associated with the solid matrix of the soil is largely insoluble organic matter, although soluble forms can be held on cation and anion exchange sites of the solid matrix and NH_4^+ can be sequestered, or fixed, within the interlayers of certain 2 : 1 clay minerals, such as the illites. Soil organic nitrogen typically comprises most (>90%) of the total soil nitrogen and exists in numerous forms, many of which defy clear chemical characterization. Those forms of organic nitrogen that can be identified are termed nonhumic substances, and include fragments of nucleic acids, peptides, and oligomers of amino sugars found in microbial cell walls. The bulk of soil organic nitrogen is made up of humic substances, nitrogenous compounds of varying size and complexity, which make up the amorphous organic material in soil. Historically, several chemical-fractionation schemes, on the basis of solubility in organic solvents or inorganic acids and bases, have been used to characterize soil organic nitrogen and assess its availability to plants. These methods, which have been greatly enhanced by modern spectroscopic methods, have provided some insight into the chemical nature of soil organic nitrogen but have not been particularly useful in explaining the biological availability of soil organic nitrogen. It should be noted that generally 2 to 5% of soil organic nitrogen is found in the living microbial biomass, which represents a source of organic nitrogen that is biologically active and turns over relatively quickly as will be discussed later.

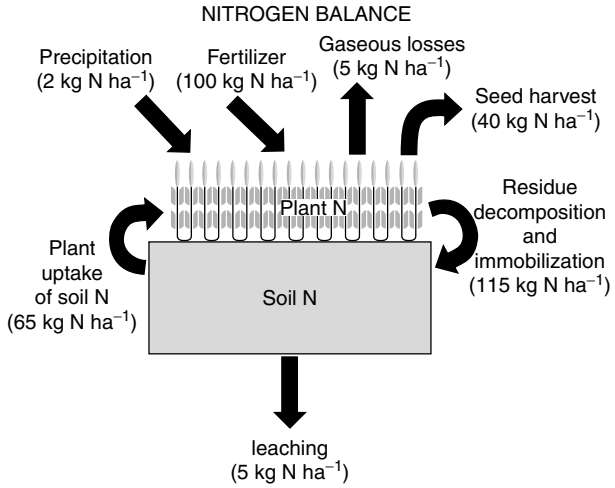

Figure 1. A nitrogen balance for grass seed production in the Willamette Valley of Oregon. Major inputs and outputs of the plant-soil system are shown along with the internal transfers between plant and soil (5–9).

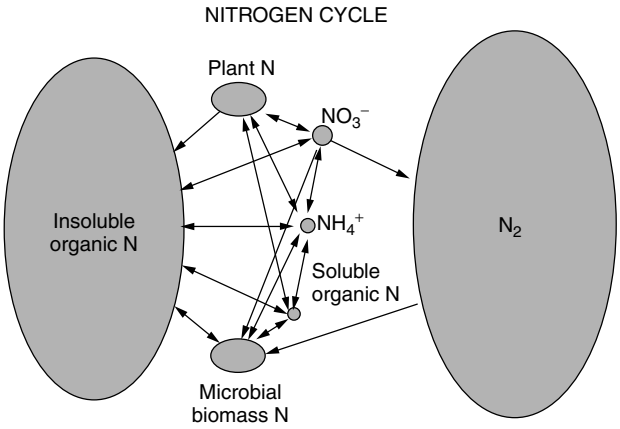

Figure 2. Diagram of a soil nitrogen cycle. Sizes of the pools are proportional to typical nitrogen concentrations. Arrows indicate nitrogen transformations that are described throughout this article.

THE NITROGEN CYCLE AND NITROGEN BALANCES

The conversions of nitrogen from one form to another are generally depicted in a diagram consisting of boxes, which represent the several pools of nitrogen, connected by arrows, which represent the various biological processes that transform nitrogen into its different forms. Such abstractions of the nitrogen cycle can be quite simple, showing only the major pools and transformations, or can be exceedingly intricate, showing all pools and known reactions.

A relatively simple nitrogen cycle model often suffices to represent the nitrogen balance for a given system, whether that is an individual field or the globe. The example shown in Figure 1 represents the nitrogen balance for a perennial grass seed crop. Such diagrams often emphasize the inputs and outputs and are less concerned with the transformations of nitrogen that occur within the system.

A reasonably detailed nitrogen cycle is depicted in Figure 2 and serves to illustrate several important principles. As mentioned previously, nitrogen exists in many chemical forms and these pools differ in size. Although the relative rates of the processes in Figure 2 are not depicted, it is generally true that the turnover rates of the nitrogen pools are inversely proportional to their size. The nitrogen in plants and microorganisms turn over in the range of 0.5 to 5 years; soluble nitrogen pools turn over much faster, perhaps daily in some systems (10); the nitrogen in insoluble soil organic matter turns over at the scale of decades to centuries; and the N_2 pool of the atmosphere is so large that it may not turnover completely in a million years. Except for N_2, all nitrogen pools are affected by at least four or five processes. This clearly lends

complexity to the nitrogen cycle but also emphasizes the importance of competition among different fates of each nitrogen pool. Most often, this competition is between different types of organisms and the outcome of the competition is a key to regulating nitrogen cycle processes.

DINITROGEN FIXATION

The production of biologically useful 'fixed' nitrogen from the large, relatively inert pool of N_2 gas in the atmosphere is dominated by biological N_2 fixation. About 175 Tg (10^{12} g) of nitrogen are fixed annually in terrestrial ecosystems by N_2-fixing prokaryotic microorganisms whereas only about 10 Tg are fixed by atmospheric chemical processes, such as the production of nitrogen oxides by lightning (11–13). In the history of the Earth, biological N_2 fixation has been the major input of nitrogen into terrestrial and aquatic ecosystems. During the last century human activities, such as fossil fuel consumption, biomass burning, and industrial production of chemical fertilizers, have increased inputs of nitrogen to the point that anthropogenic inputs are now comparable to natural processes. Enhanced utilization of symbiotic, N_2-fixing plants–particularly legumes–for agricultural production has also been an additional input locally and globally. In fact, enhancement of N_2 fixation has been a major impetus for research in an effort to meet the food and fiber needs of mankind.

Biochemical Process

The reduction of N_2 to NH_3 that requires breaking a triple bond, is an energy intensive, reductive process performed by the nitrogenase enzyme complex. The nitrogenase complex is composed of two proteins: Dinitrogenase, an enzyme with a MoFe cofactor, which reduces N_2 to NH_3, and dinitrogenase reductase, an Fe-containing enzyme, which provides the reducing equivalents for the reaction. In addition to reducing N_2, nitrogenase also reduces H^+ to H_2 and a variety of other alternate substrates, including acetylene. The ability to reduce acetylene to ethylene has been widely used as an assay for nitrogenase activity and a means of estimating N_2-fixation rates (14).

The highly reducing conditions required for N_2 fixation can only occur in the absence of oxygen. Nitrogenase itself is completely inactivated by oxygen. Several strategies have been developed by N_2-fixing prokaryotes to deal with oxygen, including: avoidance by living in anoxic environments, high respiratory activity, and the production of specialized structures that are relatively impermeable to oxygen.

Under optimum conditions, the balanced reaction requires 16 ATPs for each N_2 fixed, but under normal conditions perhaps 50% more energy is used. This high-energy cost has a direct impact on the amounts of N_2 fixed by different types of N_2-fixing prokaryotes and is also a likely reason why N_2 fixation is under tight genetic and biochemical regulation.

N_2-Fixing Organisms

Dinitrogen fixation is limited to prokaryotic microorganisms, mainly bacteria plus some archaea. This capability is quite widespread among the bacteria, possibly because of lateral transfer of key genes involved in N_2 fixation among taxonomic groups. From an ecological perspective, however, a phylogenetic classification is not especially useful. It is more helpful to group N_2-fixing microorganisms as either free-living or as more or less closely associated with other organisms, typically plants. Such a categorization is useful because there is a general relationship between the type of association and the amount, or rate, of N_2 fixation (Table 2).

Free-living N_2-fixers, such as *Azotobacter*, an aerobe, and *Clostridium*, an anaerobe, normally fix less than 1 kg N ha^{-1} yr^{-1} because they must compete with other heterotrophic microorganisms for available carbon. Dinitrogen fixation rates are higher for free-living cyanobacteria because they can derive energy directly from sunlight.

Dinitrogen-fixing bacteria are commonly found associated with the surfaces of plant leaves or roots. These habitats are relatively rich in available carbon compared with soils and tend to have higher rates of N_2 fixation. *Azospirillum* associated with the rhizospheres of tropical grasses, can fix up to 25 kg N ha^{-1} yr^{-1}, but even higher rates have been found for *Acetobacter diazotrophicus*, which lives in the internal tissues of sugarcane. Some of the cyanobacterial associations, such as the one with *Azolla*, can fix very large amounts of N_2.

Table 2. Estimated Average Rates of Biological N_2 Fixation for Specific Organisms and Associations (14)

Organism or System	N_2 Fixed (kg ha^{-1} y^{-1})
Free-living microorganisms	
Cyanobacteria	25
Azotobacter	0.3
Clostridium pasteurianum	0.1–0.5
Grass-bacteria associative symbioses	
Azospirillum	5–25
Cyanobacterial associations	
Gunnera	10–20
Azolla	300
Lichens	40–80
Leguminous plant symbioses with rhizobia	
Grain legumes (*Glycine, Vigna, Lespedeza, Phaseolus*)	50–100
Pasture legumes (*Trifolium, Medicago, Lupinus*)	100–600
Actinorhizal plant symbioses with Frankia	
Alnus	40–300
Hippophaë	1–150
Ceanothus	1–50
Coriaria	50–150
Casuarina	50

Mutualistic symbioses between root-nodulating bacteria and plants allow for the highest rates of N_2 fixation. These tightly-coupled associations can fix more than 100 kg N ha^{-1} yr^{-1} and are probably responsible for the bulk of the N_2 fixed biologically each year. The legume-rhizobia symbiosis is the most well known and characterized, in part because legumes have been used in agriculture for thousands of years. In general, grain legumes fix less N_2 than small-seeded leguminous plants used for pasture (Table 2). The actinorhizal symbiosis (so-called because the N_2-fixing partner, *Frankia*, is an actinomycete) can fix N_2 at rates comparable to legumes and represent a major input of nitrogen during primary and secondary succession in many ecosystems. In general, the amounts of N_2 fixed by these root-nodulating symbioses are proportional to the primary production of the host plant.

AMMONIFICATION

Ammonification, the production of NH_4^+ from organic nitrogen compounds, is sometimes loosely referred to as nitrogen mineralization, although the latter term sometimes refers to the production of both NH_4^+ and NO_3^-. Because organic nitrogen compounds in soil range widely in their diversity and complexity, the production of NH_4^+ is an amalgamation of several enzymatic processes. The general pattern is the depolymerization of large organic nitrogen compounds by extracellular enzymes followed by microbial uptake of the monomers and subsequent release of NH_4^+ by the activity of intracellular enzymes. These pathways are understood quite well for individual biopolymers, such as the proteins, aminopolysaccharides, and nucleic acids, which comprise living cells (15). However, the organic nitrogen that has been stabilized in humic substances may be more protected from enzymatic attack. It is important to note that predation of bacteria and fungi by the soil fauna can contribute to ammonification, partly through enzymatic processes in their guts and also as a result of the subsequent degradation of their nitrogen-containing waste products, such as urea. Urea is rapidly broken down to NH_4^+ and CO_2 by extracellular ureases in most soils.

Ammonification is not restricted to a narrow group of organisms, rather it is carried out by all heterotrophic microorganisms. In many cases, NH_4^+ production is probably a by-product from the search for available carbon, although it is possible that the production of the various extracellular enzymes and regulation of intracellular enzymes is under regulatory control, which is dependent on the relative availability of carbon and nitrogen. Consequently, there is a balance between the production of NH_4^+ by ammonification and its consumption by assimilation.

ASSIMILATION

Assimilation is the incorporation of nitrogen compounds into microbial or plant biomass. Microbial assimilation of nitrogen is often used interchangeably with the term, nitrogen immobilization. Most often assimilation refers to the uptake of inorganic nitrogen, NH_4^+ and NO_3^-, and subsequent conversion to organic nitrogen; however, organisms are also capable of directly assimilating small, soluble organic nitrogen compounds.

Biological Assimilation of Nitrogen

It is generally held that NH_4^+ assimilation is the dominant nitrogen assimilation mechanism in soils. This principle is based in part on empirical studies and partly on the theoretical preference for NH_4^+ by heterotrophic microorganisms, which are limited by carbon. When present in high concentrations, NH_4^+ is assimilated by glutamate dehydrogenase; however, at the low NH_4^+ concentrations (Table 1) typically found in soils, the glutamine synthetase-glutamate synthase pathway, which requires energy, is used (11,14).

Although many microorganisms are known to take up and utilize small organic nitrogen molecules, such as amino acids, the importance of this nitrogen assimilation pathway is not well established. Traditionally, it is assumed that organic nitrogen compounds are first mineralized, releasing NH_4^+ as a by-product, which is subsequently assimilated as microorganisms build biomass. This has been termed the MIT (mineralization-immobilization turnover) process. Several recent studies, however, have suggested that the direct uptake and assimilation of amino acids can be important in soils (16).

Assimilatory NO_3^- reduction has long been viewed as a minor pathway of biological nitrogen immobilization in soils. Extrapolating from studies of bacteria growing in pure culture, NH_4^+ concentrations in most soils are high enough to inhibit NO_3^- assimilation, and this has been shown in some studies with soil (17). Furthermore, because NO_3^- reduction to NH_4^+ requires energy, assimilatory NO_3^- reduction is more costly for heterotrophic soil organisms, which are often carbon limited. However, immobilization of NO_3^- has been observed after the addition of fresh residues, and more recent studies of forest soils high in organic matter have shown that NO_3^- assimilation may be more common than previously thought (10).

Chemical Immobilization of Inorganic Nitrogen

Not all immobilization of inorganic nitrogen into organic forms is biological in nature. Studies using ^{15}N-labeled NH_4^+ or NO_3^- often show rapid incorporation of the label into soil organic matter at rates that appear to be too quick to be biologically mediated (18,19). The exact mechanisms of this abiotic fixation of inorganic nitrogen are not clearly known, but probably involve reactions with active phenol or quinone moieties of the soil organic matter (20).

NET PRODUCTION AND CONSUMPTION OF NH_4^+

The balance between ammonification and NH_4^+ assimilation is of practical importance. A soil poised to immobilize NH_4^+ will be nitrogen deficient in terms of plant productivity. However, a soil with high rates of ammonification relative to NH_4^+ assimilation is likely to lose a significant

amount of nitrogen to the environment through leaching or gaseous losses, which result from nitrification and denitrification, as will be discussed later.

Effect of Substrate C : N Ratio

During decomposition, several factors affect the balance between NH_4^+ production and consumption. These factors include: the 'quality' of the substrate, the types of organisms involved in decomposition, and their growth efficiency and nitrogen content. Substrate quality refers to how easily it can be degraded, which in turn is related to its chemical composition, e.g., the relative amounts of nitrogen, lignin, soluble organics, etc. A good general index of substrate quality is its C : N ratio, at least during the initial phases of decomposition. The major decomposing organisms, fungi, and bacteria, differ in their C : N ratios and how efficiently they incorporate substrate carbon into biomass. In general, fungi have a wider C : N ratio (perhaps 10 vs. 4) and are slightly more efficient (50 vs. 40%) than bacteria. When these factors are combined (Fig. 3), one can determine the C : N ratio of the substrate, which just balances the decomposers' needs for carbon and nitrogen. As a rule of thumb, net nitrogen immobilization occurs when the substrate has a C : N ratio wider than about 20 or 25 and net nitrogen mineralization occurs when it is narrower. A more efficient use of carbon and/or a lower decomposer C : N ratio causes this 'critical' substrate C : N ratio to decrease.

Measuring Gross Production and Consumption Rates

Because changes in the size of the NH_4^+, or NO_3^-, pool provide information only about the net processes of production and consumption, alternative methods must be used to determine the gross rates of production and of consumption. In some cases this may be done using

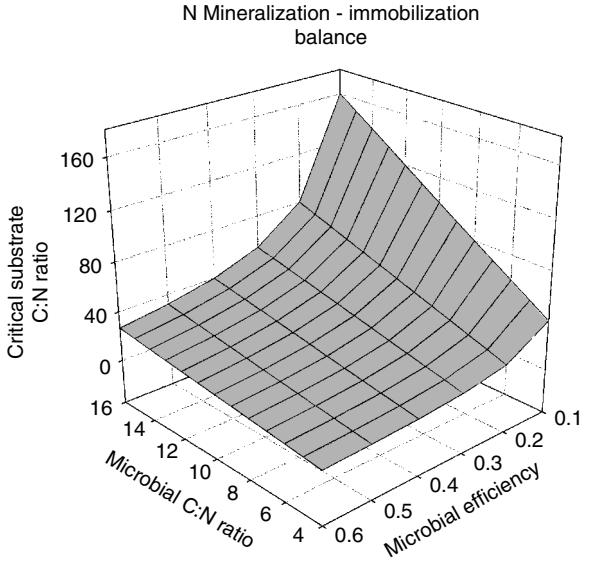

Figure 3. The critical substrate C : N ratio–the ratio in which ammonification and nitrogen immobilization are balanced–as a function of microbial efficiency and C : N ratio. When substrate C : N ratio exceeds the critical value, nitrogen is immobilized.

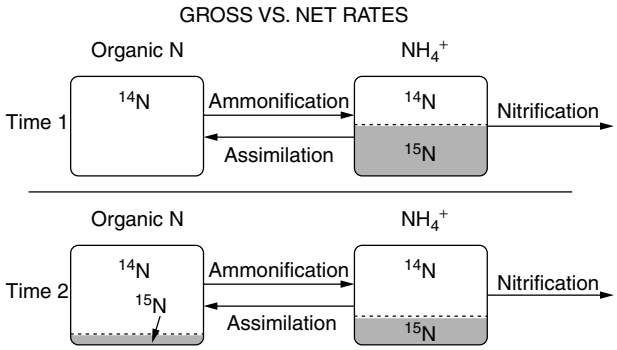

Figure 4. A diagrammatic example of how ^{15}N can be used to determine the gross rates of NH_4^+ production and consumption. Gross rates of NO_3^- production and consumption can be obtained in an analogous fashion.

a process-specific inhibitor or alternative substrate. For example, acetylene can be used to specifically block NH_3 oxidation and the difference between the NO_3^- produced in the presence and absence of acetylene is equal to the gross nitrification, or NO_3^- production rate. This assumes that the inhibitor does not influence any other processes related to the availability of substrate or the consumption of NO_3^-.

The potential limitations involved in using inhibitors can be circumvented by using ^{15}N-labeled substrates or products and measuring the changes in the ^{15}N-abundance as well as total pool size of the product pool (21). The basic principle of this approach can be illustrated by taking the simple case where the production and consumption of NH_4^+ are equal (Fig. 4). In this case it is obvious that the size of the NH_4^+ pool does not change with time; however, if some $^{15}NH_4^+$ was added to this pool, its ^{15}N abundance would decline. Such data can be analyzed to determine the individual rates of NH_4^+ production and consumption (21,22).

NITRIFICATION

Nitrification is the production of NO_3^- by microorganisms. It is of particular practical interest because NO_3^- is the form of inorganic nitrogen that is most susceptible to loss from the soil, either through leaching or subsequent transformation to gaseous endproducts via dentrification. These losses are of concern because they reduce the amount of nitrogen available for plant production, which is often the major nutrient limitation, and because of undesirable environmental consequences of NO_3^- in surface and groundwaters.

The Autotrophic Nitrification Process

Typically nitrification refers to the sequential oxidation of NH_3, which is in equilibrium with NH_4^+, to NO_2^- and then to NO_3^- by two groups of autotrophic bacteria. The conversion of NH_3 to NO_2^- involves several steps and results in the production of energy:

$$2NH_3 + 3O_2 \rightarrow NO_2^- + H^+ + H_2O$$

$$\Delta G_0' = -45\ \text{kJ mol}^{-1}\ e^-$$

Ammonia monooxygenase converts NH_3 to NH_2OH (hydroxylamine), which is subsequently oxidized to NO_2^- by hydroxylamine oxidoreductase. In addition to NO_2^-, H^+ is produced, and NO and N_2O can be produced as by-products. Thus, NH_3 oxidation has the potential to acidify soils and also release radiatively active greenhouse gases. There are several closely related genera of autotrophic NH_3-oxidizing bacteria. *Nitrosomonas* are often the most easily isolated NH_3-oxidizers but often *Nitrosospira* are numerically dominant in soils (11,14).

The oxidation of NO_2^- to NO_3^- is a one-step process carried out by NO_2^--oxidizing bacteria via the enzyme nitrite oxidoreductase:

$$2NO_2^- + O_2 \rightarrow 2NO_3^-$$

$$\Delta G_0' = -39\ \text{kJ mol}^{-1}\, e^-$$

This reaction is less energetically favorable than that for NH_3 oxidation. The NO_2^--oxidizing bacteria also form a cohesive taxonomic group, with *Nitrobacter* being the representative genera. Because NO_2^--oxidizers utilize the NO_2^- produced by NH_3-oxidizers, autotrophic nitrification can be thought of as a commensalistic relationship, in which both partners benefit. In fact, studies in other environments, such as sewage treatment plants and aquatic systems, have found nitrifying bacteria growing in association in dense aggregates of cells (23).

The Heterotrophic Nitrification Process

It is recognized that NO_2^-, and in some cases NO_3^-, can be produced by the activities of heterotrophic microorganisms or nonspecific monooxygenase enzymes. Several heterotrophic bacteria and fungi can oxidize NH_4^+ or organic nitrogen compounds to NO_2^- or NO_3^- without gaining energy from the process. Furthermore, the low specificity of monooxygenase enzymes, e.g., methane monooxygenase, can result in NH_3 oxidation to NH_2OH (24). The rates of heterotrophic nitrification are generally thought to be low relative to the autotrophic process and the ecological importance of heterotrophic nitrification is unclear. Nevertheless, the heterotrophic process has been found to dominate in some habitats, such as acidic forest litters and soils (25).

Factors Affecting Nitrification

Nitrification obviously requires that microorganisms capable of producing NO_3^- are present. With rare exceptions, such as following soil fumigation, this is the case. Another requirement that is usually met is the availability of O_2. Because nitrification is dependent on molecular oxygen, aerobic conditions are required for the production of NO_3^-. This is true for most soils, except when fully saturated.

As with most microbial processes, the most common limitation to growth or activity is the supply of the energy-supplying substrate. In the case of nitrification, this is NH_4^+, or more precisely NH_3. Consequently, soils with high NH_4^+ concentrations, or high rates of NH_4^+ production, generally have higher rates of nitrification. Typical examples are arable soils, which often receive relatively large inputs of nitrogenous fertilizers, and are mixed and otherwise managed to promote good fertility and water availability. After an initial lag period, nitrification rates are often elevated following a physical disturbance and/or the removal of vegetation. This is thought to be largely a response of increased NH_4^+ availability resulting from enhanced ammonification and the reduction of plant nitrogen uptake, which allows an increased population of nitrifying bacteria and concomitant net production of NO_3^-.

Additional constraints on autotrophic nitrification include a variety of environmental and edaphic factors; however, pH and specific inhibitors require special mention. It is generally observed that nitrification rates are very sensitive to low pH. This may be because nitrifying bacteria are relatively sensitive to pH but may also be caused by the relatively high pK_a (9.5) of the NH_4^+-NH_3 equilibrium, which means that at pH 5.5, the ratio of NH_3 to NH_4^+ is 1 : 10, 000. At low pH, NO_2^- is present primarily as HNO_2, which is toxic to nitrifying bacteria.

The observation that NO_3^- concentrations or net production of NO_3^- are often low in soils under late successional or climax vegetation has led to the speculation that there may be specific chemicals associated with, or produced from, the plant litter than inhibit nitrifying bacteria (26). Furthermore, the wide substrate range of the ammonia monooxygenase enzyme makes it susceptible to inhibition by a range of substrates, which can compete with NH_3, such as methane and other hydrocarbons (27,28). Although this potential is real, and may occur in some cases, most tests of specific inhibitors thought to be associated with late successional plants (e.g., phenolics, tannins, etc.) have not been found to be inhibitory (29). Therefore, it is more likely that the lack of observed nitrification is caused by intense competition among nitrifying bacteria, heterotrophic microorganisms, and plants for available NH_4^+.

Although naturally occurring inhibitors of nitrification may be of minor importance, several effective chemical inhibitors have been developed for use in agriculture. nitrogen-serve (nitrapyrin) was one of the first commercial nitrification inhibitors but many others are used world-wide, including DCD (dicyandiamide) and Dwell (etridiazole). The use of these inhibitors slows the nitrification of fertilizer nitrogen, which can enhance plant nitrogen uptake and limit losses to the environment (11,14).

DENITRIFICATION

Denitrification involves anaerobic reduction of NO_3^- to gaseous end-products, dominantly N_2 (30). Through denitrification nitrogen is recycled into the atmosphere, which represents the largest annual export of nitrogen from terrestrial ecosystems at about 140 Tg (11,12). Normally NO_3^- reduction is associated with bacterial respiration in which the NO_3^- is used as an alternate electron acceptor in place of O_2; however, gaseous nitrogen oxides can be produced by other biotic and abiotic (chemodenitrification) processes. Furthermore, some bacteria can reduce NO_3^- to NO_2^- (nitrate

respiration) or to NH_4^+ (DNRA–dissimilatory nitrate reduction to ammonium).

The reduction of NO_3^- to N_2 is a multistep process, which involves four enzymes, which function sequentially to carry out the following reaction:

$$2NO_3^- + 12H^+ + 10e^- \rightarrow N_2 + 6H_2O;$$
$$\Delta G_0' = -112\ \text{kJ mol}^{-1}\ e^-$$

Nitrate is reduced to NO_2^- by dissimilatory nitrate reductase, an enzyme that is in common with all bacteria, which dissimilate NO_3^-. Generation of ATP is linked with this reaction. Nitrite is reduced to NO by nitrite reductase, of which there are two types. Heme-containing nitrite reductases are more common, occurring in about two-thirds of all denitrifiers, which have been tested; the remainder have a Cu-containing nitrite reductase. Currently, it is not clear whether there are major differences in the kinetics or regulation of these two types of nitrite reductase, which thereby confers some type of competitive advantage in certain environments. Nitric oxide is a freely diffusible intermediate, which is reduced to N_2O by nitric oxide reductase and is the step during which the N–N bond is formed. The final reduction of N_2O to N_2 is done by nitrous oxide reductase, a Cu-containing enzyme, which is inhibited by acetylene. These last two steps also appear to be linked to ATP production. It should be noted that the inhibition of nitrous oxide reductase by acetylene, the so-called acetylene block technique, allows denitrification to be measured with great sensitivity using gas chromatography.

A relatively large fraction of soil bacteria, between 0.1 and 5%, can denitrify, and denitrifiers represent a diverse group of bacteria. In this respect, denitrification is more like ammonification than nitrification. Some of the more common denitrifying genera include *Alcaligenes*, *Bacillus*, and *Pseudomonas*.

Factors Affecting Denitrification

As with nitrification, a heirarchy of factors influence the denitrification rate. Oxygen availability, or rather its absence, exerts the greatest effect on denitrification in soil because denitrification occurs anaerobically. Oxygen acts by regulating denitrifier enzyme synthesis and directly affecting enzyme activity. Nevertheless, soils do not have to be completely free of O_2 for denitrification to be detected because even well-aerated soils can have microsites devoid of O_2, such as the interior of soil aggregates (31). These anaerobic microsites occur because the supply of O_2 by diffusion that is greatly slowed by water-filled pores, can be insufficient to meet the respiratory needs of heterotrophic microorganisms, resulting in zones of anaerobiosis. This microscale heterogeneity is one reason for the high variability in denitrification rates often observed in the field.

The next most limiting factor to denitrification is either the supply of carbon, the source of reducing equivalents for most denitrifiers, or NO_3^-, the alternate electron acceptor. The relative balance of available carbon to NO_3^- varies by management and soil type. Because of inputs of fertilizer nitrogen and loss of soil carbon as a result of tillage, denitrification in agricultural soils tends to be most often limited by carbon. In most unmanaged systems NO_3^- tends to be more limiting, with the exception of soils that receive high anthropogenic inputs of atmospheric nitrogen or some native tropical soils that often have high concentrations of NO_3^- and low organic matter content (32).

Additional soil characteristics, such as temperature and pH tend to be of less importance in determining the denitrification rates, except at extremes of their range. Soil pH does, however, exert an effect on the relative proportions of gaseous products, with relatively higher ratios of N_2O to N_2 occurring at lower soil pH. The proportion of N_2O produced also tends to be greater in soils that have an excess of NO_3^- relative to available carbon.

Dissimilatory Reduction of NO_3^- to NH_4^+

Many genera of bacteria can reduce NO_3^- to NH_4^+ under anaerobic conditions. Although less energy is gained in this dissimilatory process compared to denitrification, more reducing equivalents per NO_3^- are used:

$$NO_3^- + 10H^+ + 8e^- \rightarrow NH_4^+ + 3H_2O;$$
$$\Delta G_0' = -75\ \text{kJ mol}^{-1}\ e^-$$

This has led to the concept that DNRA is most important in energy-rich (high carbon), low NO_3^- environments, such as sediments, and would therefore be minor compared with denitrification in most soils (30). Recent investigations have suggested that DNRA may be more prevalent in soils than previously thought (33).

ENVIRONMENTAL CONSEQUENCES OF NITROGEN CYCLING

It is well known that nitrogen is often the primary nutrient limiting primary productivity in most terrestrial and some aquatic ecosystems. But too much nitrogen, especially in the forms of NO_3^-, NO, and N_2O, can create problems for environmental and human health, whether these excesses are of natural origin or the result of human activity (34).

Acute Toxicity

The greatest direct human health concern related to excess nitrogen is high concentrations of NO_3^- in drinking water. Federal drinking water standards require less than 10 mg NO_3^--N L^{-1}. Ingested NO_3^- that is converted to NO_2^- interferes with O_2 transport by hemoglobin, causing a condition known as methemoglobinemia. Infants are especially sensitive, hence the common name of "blue-baby syndrome" for this condition. Fortunately, this affliction is uncommon. High NO_3^- concentrations in water may also enhance formation of carcinogenic nitrosoamines.

Eutrophication and Acidification

Because ecosystem productivity is often limited by nitrogen, excess nitrogen in any forms (organic, NH_4^+, or

NO_3^-) and delivered by any means (direct fertilization, leaching or overland flow, atmospheric deposition) can potentially alter the biomass and composition of ecosystems. Eutrophication was first observed in aquatic systems, where enhanced biomass production resulted in higher decomposition, which in turn created anaerobic conditions. Aquatic eutrophication as a result of nutrient loading remains a concern (35). The large areas of hypoxia or anoxia in the Gulf of Mexico are one contemporary example.

In some industrialized regions of the world, increased inputs of nitrogen into forested ecosystems has led to the phenomenon of nitrogen saturation. When terrestrial ecosystems become saturated with nitrogen, it results not only in larger inputs of nitrogen into associated aquatic ecosystems but also can directly affect the productivity and species composition of the terrestrial system. Acidification as a result of increased fertilizer nitrogen inputs has long been observed in agricultural systems as well as in natural systems that have active N_2-fixation, such as stands of red alder (36). Therefore it is not surprising that atmospheric deposition of nitrogen oxides that are accompanied by H^+, and NH_3 deposition, which can result in enhanced nitrification, result in the acidification of water and soil. Nitrogen saturated forests can display signs of decline, such as the loss of productivity or an increase in mortality. In some cases this has been attributed to nutrient imbalances resulting from acidification and leaching of base cations. Changes in vegetative composition have also been observed in terrestrial systems receiving elevated inputs of nitrogen (37).

Ozone Dynamics and Climate Change

Nitrogen oxides emitted into the atmosphere influence ozone concentrations in several ways. Nitric oxide is a precursor of tropospheric ozone and contributes to photochemical smog, a phenomenon associated with high density of motor vehicles. Conversely, N_2O catalyzes the destruction of ozone in the stratosphere, reducing the effectiveness of this layer in blocking incoming UV radiation.

Concentrations of tropospheric N_2O are increasing at a rate of about 0.25% per year (38). Most of this increase results from increased losses of N_2O during the biological processes of nitrification and denitrification, which in turn are enhanced by anthropogenic nitrogen inputs into ecosystems. Although concentrations of N_2O in the atmosphere are smaller than those of CO_2 by a factor of 1,000, N_2O is 150-fold more efficient than CO_2 at absorbing infrared radiation. Furthermore, N_2O absorbs radiation in a different spectral region than CO_2. Therefore, a similar increase in N_2O has a disproportionately large effect on climate change.

CONCLUSION

It is clear that much is known about the nitrogen cycle — the organisms involved, the transformations that occur, and their interactions with environmental factors. It is also clear that new information continues to be discovered, both about the basic properties of the nitrogen cycle and about its practical implications. Some of the key areas that require additional work include:

- Better quantitation of global flux estimates. Although not as publicized as the "missing C," the errors in estimating global fluxes of nitrogen are still large.
- More data on the importance of soluble organic nitrogen in different terrestrial ecosystems. This pool may represent a significant amount of nitrogen that is taken up by plants and microorganisms, and may also represent a larger loss to ground and surface waters than expected.
- Better understanding of the gross production and consumption of NO_3^- in soil and the controls on these processes. The processes of NO_3^- assimilation by microorganisms and of DNRA are particular voids in our understanding.
- Determining relationships between the composition and activity of the microbial communities that cycle nitrogen. For example, we know that there are many species of denitrifying bacteria in soil but we do not know if they vary in their activities or respond differently to environmental changes. Potential commensalistic associations among nitrogen cycling microorganisms are also unexplored.

It will be important to incorporate what we learn from such new areas of research with our current knowledge of the nitrogen cycle in order to balance the needs for increased supplies of food and fiber, with those of ensuring a clean and safe environment for the future.

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SOIL QUALITY. See TOXICITY TESTING IN SOIL, USE OF MICROBIAL AND ENZYMATIC TESTS

SOIL QUALITY: THE ROLE OF MICROORGANISMS

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Soil quality is important because of the unique role soil plays in the human environment. From foundations for buildings to contamination buffers and a water regulator to a medium for the production of food and fiber, soils are important in everyday life. Soil quality or the capacity of the soil to function is dependent on its intended use, its inherent properties, and its current condition. Society should equate soil quality with air and water quality for sustainability. Thus, the concern is not only the state or condition of soil quality but also its direction and rate of change as measured by sensitive soil indicators with quantifiable limits and interpretations. Soil microorganisms (termed in general microbial biomass) and their physiological functioning have been promoted as essential soil quality indicators. If microbial biomass is indicative of the health of the soil and its functioning capacity, the microbial biomass dynamics (state, mass, and activity) should be apparent in both aggrading and degrading soil systems. This paper describes microbial biomass as it is related to soil quality. Evaluation of microbial biomass as a soil quality indicator is explored in some aggrading and degrading systems. The use of microbial biomass and its metabolic functions for the monitoring and analysis of soil quality is examined.

SOIL QUALITY

In the last 10 years, there has been an increased interest in the concept of soil quality because it relates to the health of the global biosphere. In a global context, soil quality affects not only soil productivity but also is a significant factor governing environmental quality, human and animal health, and food safety and quality (1). Soil quality is of equal importance to humankind as is air and water quality, thus it is apparent that simply protecting soil quality by slowing soil degradation or maintaining the current level of soil health will not provide the soil quality that will be needed for future generations. Soil quality must be improved as well.

Soil quality per se is not a new concept, having for centuries been related to soil degradation. The degradation of soil or soil quality from human activities has affected many civilizations over the last 7,000 years (2). Good soil quality means different things to different people, influenced by our relationship to the land, what we use it for, and how much of it is available. Evaluation of soil quality is thus context sensitive, a fact appreciated early by soil scientists such as Hilgard (3), who advised "The observer's field of vision should be broad enough to embrace concurrently the several points of view... must be conjointly considered in forming one's judgment of

land." Recent recognition of the importance of soil quality is evidenced by the worldwide activity to identify and quantify land degradation. Programs of the United nations Environment Programme (4), the Food and Agriculture Organization (5) and the International Soil Reference and Information Center (6) have attempted to map human-induced soil degradation worldwide.

The recent interest and activity in soil quality research can easily be put in perspective by a quick search of the Internet. In addition, it is well known that professional soil science and ecological societies have championed this concept and produced official definitions and terminology. Whether we define soil quality (or soil health) as "the soil's fitness to support crop growth without resulting in soil degradation or otherwise harming the environment" (7), or as "the continued capacity of soil to function as a vital living system, within ecosystem and land-use boundaries, to sustain biological productivity, promote the quality of air and water environments, and maintain plant, animal, and human health" (8), we seem to all be speaking the same language. Soil provides for human sustainability by functioning as a medium for plant growth and as an environmental buffer and filter for cycling water, altering chemicals, and cleaning air.

Unfortunately, our ability to quantitatively assess soil quality has lagged far behind our ability to produce articles and speculation on the subject of soil quality. Developing a soil quality index cannot simply be an academic exercise because it will be called on not only to provide a basis for land capability classification (e.g., for productivity, health, or environmental quality) but also to assess the impact of management practices, to quantify the value of land as a base for taxation or land credit, to establish regulatory compliance, and to provide information for monitoring or modeling environmental changes.

Because soil systems are complex, evaluating soil quality will require the integration of biological, chemical, and physical soil parameters as well as human influences (1,9,10). Much effort has been directed toward identifying the soil variables that can be used to estimate soil quality because several different assessments may be derived from the same set of data (Table 1). Measuring, monitoring, and evaluating soil's biological characteristics for relationships with changes in soil quality is very promising because these properties are believed to change most rapidly with changing environmental conditions. Thus, a great deal of effort has been given to identifying key biological indicators and interpreting their characteristics and fluctuations with respect to soil quality (see *Biological Indicators of Soil Health* (8), *Methods for Assessing Soil Quality* (11) and (12)).

Two biological parameters that may be potential indicators of soil quality are soil respiration (CO_2) and microbial biomass. These parameters have extensively been examined (5,13,14) for their ability to predict changes in soil properties and processes under laboratory and field conditions. The calculation of an active soil carbon fraction from respiration that turns over rapidly in short-term soil incubation studies may provide a uniform entity that may be more widely comparable among soils (16).

Table 1. Soil Chemical, Physical, and Biological Indicators of Soil Quality[+]

Chemical Indicators
pH
Total C and N (SOM)
Inorganic nutrient (N, K, P)
Electrical conductivity
Physical Indicators
Bulk density
Texture
Rooting depth
Water infiltration
Resistance
Biological Indicators
Microbial biomass C and N
Mineralizable N
Soil respiration
Respiration/biomass
Enzymes
Nitrification potential

[+]Doran and Jones (11)

Soil Organic Matter (SOM)

Soil organic matter plays a major role in terrestrial ecosystem development and functioning. In both undisturbed and cultivated systems, potential productivity is directly related to SOM concentrations. Organic matter contents range from less than 0.2% in desert soils to more than 80% in peat soils. In temperate regions, SOM ranges between 0.4 and 10.0%, with humid region soils averaging 3 to 4% and semiarid soils 1 to 3%. Although it is only a small fraction of the soil, components of SOM control air and water relationships for root growth and provide resistance to wind and water erosion.

Soil organic matter is a complex mixture of living, dead and decomposing material, and inorganic compounds. Most of the SOM is derived from plant tissue decomposition but some is formed from decomposing microfauna and microorganisms. This mixture of decomposing entities makes the chemical composition of SOM difficult to determine. About 15% of SOM is identified as polysaccharides, polypeptides, and phenols (17). This includes 20% carbohydrates, 20% amino acids and amino sugars, and 10 to 20% aliphatic fatty acids (18). The rest of SOM is humic material, which is a dark amorphous substance derived from the transformation of organic residues.

The dynamic nature and complex chemistry of SOM makes it a major source of plant nutrients. With 95% of soil nitrogen, 40% of soil phosphorus, and 90% of soil sulfur being associated with the SOM fraction, decomposition and turnover can supply the majority of macronutrients required for plant growth. The organic nitrogen content of soils ranges from 0.02 to 1.0%. Soil organic nitrogen is dominated by amino acids and amino sugars, which constitute 20% of the soil carbon but 30 to 40% of the soil nitrogen. Other constituents identified after acid hydrolysis of SOM are ammonia (NH_3) and an acid insoluble

Table 2. General Properties of Soil Organic Matter (SOM) and Associated Effects on Soil Properties

Property	Remarks	Effect on Soil
Color	Organic matter causes typical dark color	Facilitates warming
Water Retention	SOM holds up to 20 times its weight	Prevents drying, significantly improves moisture retaining properties
SOM and Clay	Forms structural aggregates	Facilitates gas exchange, increases permeability
Chelation	Forms stable complexes with polyvalent cations	Enhances micronutrient availability
Buffer Action	Exhibits buffering from changes in pH	Helps to maintain uniform reaction in soils
Cation Exchange	Increases holding capacity for cations in soil	SOM can increase cation exchange capacity by 20 to 70%
Mineralization	Decomposition of SOM yields NH_4, PO_4 and SO_4	A source of nutrients for plant growth

fraction and hydrolyzable unknown nitrogen. The amino acids and sugars are the major source of inorganic nitrogen from SOM via the decomposition process.

It is the biological interaction between SOM and microorganisms that promotes aggregation and good soil structure. In long-term field studies in which residue is conserved or added water stable aggregates increase in mass and stability (19). The biological metabolism of organic residues by microorganisms produces binding agents such as polysaccharides that "glue" mineral particles into aggregates. The benefit of aggregation, which is influenced by crop rotation and microorganisms (20), is increased porosity that increases water infiltration and provides proper aeration for microbial and plant root activity. More detailed analysis of the role of microorganisms in soil aggregation can be found in Tate (21) and Paul and Clark (18). A summary of the effects of SOM on soil properties is presented in Table 2.

Soil Microorganisms

The soil microorganisms are predominately bacteria and fungi existing in different proportions, depending on the soil system. Soil microorganisms constitute the dynamic fraction of soils and play a major role in soil functioning because of interactions with SOM and the cycling of nutrients. Microorganisms also interact chemically and physically with soil mineral particles and soil water. In general, soil organisms take up and release nutrients, decompose organic substrates, produce soil gases, and promote soil aggregation.

Figure 1 depicts the cycling of organic substrates and nutrients in soil by microorganisms or microbial biomass. The mineralization process produces nutrients, and the oxidation and reduction processes can decrease nutrients. This process is controlled by temperature, moisture, soil disturbance, and the quality of SOM. These factors, together with the size and activity of the microbial population regulate the rate of decomposition and release of macro and micronutrients essential for plant growth.

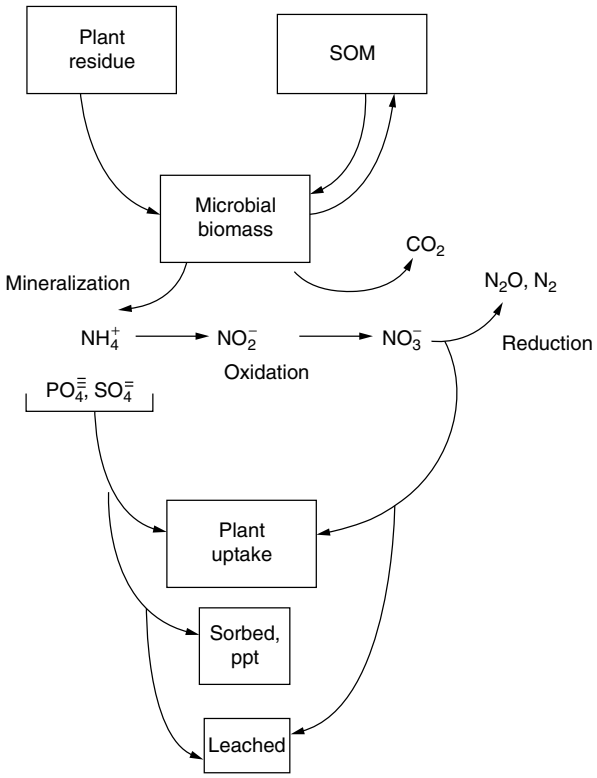

Figure 1. The cycling of nutrients and residues by soil microorganisms.

The biological mineralization of organic residues will produce inorganic NH_4^+, PO_4^{-3}, and SO_4^{-2} in ratios similar to the material being decomposed. Thus, it is evident that the inorganic nutrient cycles are linked to each other and are driven by microbial utilization of carbon for energy, which also influences the microbial diversity and community composition (22).

In the absence of externally applied nutrients, the limiting factor for plant production will be the mineralization

rate of macronutrients and the quality and quantity of SOM. It has been shown that for a winter wheat crop producing 16 tons/ha of dry matter, 302, 36, and 32 kg/ha of nitrogen, phosphorus and sulfur, respectively, are needed for plant uptake. The average nitrogen, phosphorus, and sulfur in the readily available (labile) SOM pool is 180, 17 and 9 kg/ha, respectively, which is 60, 47, and 28% of the nitrogen, phosphorus and sulfur requirements for the crop (23). With the turnover of the labile pool being quite rapid, the natural cycle of these elements possibly could supply the majority of the crop nutrient requirement.

Nutrient cycles in natural ecosystems have slower transformation rates but are more efficient, decreasing the chance for losses. In agricultural systems, with the addition of fertilizers, losses can be substantial if not highly managed. Because the active soil nitrogen is related to the microbial biomass (24) the addition of inorganic fertilizers, especially anhydrous ammonia, can adversely affect the pH of the soil, and eventually the microflora, decreasing the soil quality (25).

Microorganisms' Influence on Soil Quality

Being closely associated with SOM, soil microorganisms can have both positive and negative effects on soil quality. Table 3 gives some beneficial and detrimental effects of microorganisms in relation to soil quality attributes. As discussed previously, the mineralization of plant nutrients is critical for healthy plant growth and development. The processing of plant litter and residues into SOM increases soil aggregation and thus water infiltration and aeration. This process increases soil structure and decreases bulk density. Microorganisms detoxify soil by degrading toxic compounds, both natural and man made. Organisms also consume greenhouse gases such as methane and in some cases, nitrous oxide.

On the negative side, soil microorganisms can decrease soil quality by producing leachable compounds that may escape plant uptake and may contaminate surface or groundwater. Microbial metabolism of SOM decreases the positive effects of SOM over time and produces more recalcitrant compounds with less fertility. Soil organisms will also compete with plants for nutrients, decreasing plant production and quality. Soil microorganisms produce greenhouse gases through the decomposition of SOM and the reduction of inorganic nutrients.

These beneficial and detrimental effects of soil microorganisms on soil quality are somewhat offset by each other but in some ecosystems or agricultural management systems the balance can be pushed more toward a positive or negative. This will result in systems aggrading or degrading in soil quality.

Table 3. Beneficial and Detrimental Effects of Microorganisms on Soil Quality

Beneficial Effects
Mineralization of nutrients for plants
Processing residues into organic matter
Increasing soil structure
Degrading toxic compounds
Consume greenhouse gases
Detrimental Effects
Producing leachable compounds
Metabolizing SOM
Compete with plants for nutrients
Produce greenhouse gases
Pathogens on plants

Biological Soil Quality Measurements

As discussed before, biological indicators are thought to be more sensitive to change than chemical and physical parameters because of the rapid turnover of the active biological fraction. The rapid turnover of this fraction, including microbial biomass, makes it sensitive to changes in climate, crop rotations, tillage, and other management practices in agricultural systems (26). In natural systems, the active fraction would be sensitive to disturbance, species invasion, and atmospheric pollution inputs.

Soil organic matter is often included with biological indicators because of its close relationship with soil microorganisms and nutrient cycling. The soil microbial biomass constitutes 1 to 4% of the total soil organic carbon (23) and is associated with light density fractions of SOM (27). It is difficult to measure small changes in SOM even after several years of alternative management. Thus, surrogate measurements that have been developed to reflect changes in SOM may also be used to describe changes in soil organisms. Some of these measurements are microbial biomass, soil respiration, soil enzymes, and measurements such as nitrification or mineralization potential (12).

Microbial indicators of soil quality can be used at three levels of organization. The hierarchical stratification of microbial populations, microbial communities, and process level measurements can be of use in determining the direction and change in soil quality. The population or species level would serve as a bioindicator of perturbation or change. Because there are approximately 10,000 different species of organisms in a gram of soil (17), the form and function of the bioindicator species needs to be rigorously defined and interpreted. In addition, environmental effects on the population dynamics of these bioindicator species needs to be considered (28). Microbial community analysis describes species diversity and composition, for example, fungal: bacterial ratio. Fungal to bacteria ratios can be used as a measure of balance of nutrient cycling and decomposition (18,21). These types of studies have been successful in evaluating degraded soil, however, their usefulness for routine soil quality monitoring has been questioned because different systems respond differently to management or change (28,29). Process level studies describe the cycling of nutrients and organic matter in soil and the transformations of elements and ions. Soil processes are fundamentally easier to measure than microbial species or diversity because it is usually a product formation or a substrate disappearance that is measured. These chemical constants can be analytically quantified whereas many species and diversity measurements are qualitatively interpreted.

Thus in general, microbiological indicators of soil quality are better suited for describing natural and managed systems than are chemical and physical indicators. The microbial parameters or indicators integrate the physical and chemical environment into their function in soil systems. Microbial indicators reflect system decomposition and nutrient regulation and respond quickly to changes in the soil environment. This is not necessarily true of physical and chemical soil properties that may change slowly or become buffered to the extent that change is difficult to measure until the soil is highly degraded.

Microbial Biomass

Several procedures have been developed for estimating soil microbial biomass carbon and other cell constituents. A widely used method is the chloroform fumigation incubation method (CFIM) (30). The method is based on killing the soil organisms with chloroform and measuring the decomposition of the dead organisms as carbon dioxide during a subsequent ten-day incubation. Because less than 100% of a dead organism will be released as carbon dioxide, a conversion factor (k_c) is used to relate the carbon dioxide evolved to the total mass of organisms. The conversion factor is estimated to be 0.41. During the incubation, native organic carbon is also mineralized to some extent and thus the biomass calculation becomes

$$\text{Microbial biomass} - \text{C} = \frac{\text{CO}_2(\text{f}) - \text{CO}_2(\text{c})}{k_c}$$

where f is the fumigated sample and c is the unfumigated control sample. There has been significant debate over the proper control because nonbiomass carbon may be mineralized at different rates in fumigated and unfumigated samples (31,32).

Direct extraction methods for carbon and nitrogen that will alleviate some of the problems with incubation after fumigation and provide for a more rapid analysis, have been developed (33,34). The fumigation extraction method (FE) involves fumigating a soil sample, removing the chloroform, and extracting the sample with 0.5 M K_2SO_4. The filtered K_2SO_4 extract is analyzed carbon for and nitrogen (35,21). This method appears to be affected by moisture content of the sample (36–38) but, unlike the CFIM, is suitable for acid forest soils (39,40).

The substrate induced respiration (SIR) method involves inducement of respiration by adding glucose to a soil sample in which the increase in respiration before microbial growth is related to microbial biomass carbon (41). This method was initially developed to distinguish bacterial and fungal biomass and has been calibrated to the CFIM. This method is rapid and conducive to analyzing numerous soil samples at one time, although it cannot be modified to include biomass nitrogen. In addition, the moisture and nutrient status of the soil may make interpretation of the biomass values between ecosystems difficult (42).

Another method relies on measurement of adenosine triphosphate (ATP) extracted from a soil sample (43,44). The extraction of ATP from soils may vary depending on SOM concentration and texture, which can create problems in calculating total biomass. In addition, available phosphorus and other amendments can effect the results of ATP analysis. This method has been used for studying microbial energetics but has been used less often for biomass estimations and appears to be more related to biomass activity than microbial biomass (45). However, recently Contin and coworkers (46) found that ATP varied insignificantly (9–12 umole ATP g^{-1} biomass carbon) over a variety of soils and suggested that the measurement was a valid estimate of microbial biomass.

Of the methods discussed only the CFIM and FE can provide biomass nitrogen values by extracting the inorganic nitrogen after fumigation (47,48). As with the biomass carbon method, the nitrogen method also requires a factor to relate nitrogen mineralized to total biomass nitrogen. This factor (k_n) has been determined by a number of procedures, and ranges from 0.32 to 0.68 (49–51). There is little agreement on the correct k_n value, and it has been suggested that investigators simply report the nitrogen flush after incubation (23).

The method used to measure microbial biomass is investigator-driven, with ease of analysis, soil type, and management information being some of the criteria for choice. The three methods have been correlated in a number of studies, though the correlation can be highly variable across different scales (52,53). Beck and coworkers (54) reported microbial biomass data on 20 different soils analyzed by seven different laboratories using 10 variant methods of the SIR, FE, and CFIM. They found all of the methods to give almost identical rankings for microbial biomass in the soil samples, however, there were soil to soil variations and systematic calibration errors that hampered detailed comparison of the data. They also found that vegetation and land use affected the conversion factors to total microbial biomass among the methods as did the changes in the microbial community structure and sample prehandling. Thus, close attention to the literature on microbial biomass methodology is required to produce and interpret microbial biomass data.

Respiration

Soil or microbial respiration is measured as carbon dioxide, either in laboratory incubations or in situ field measurements and is a measure of microbial activity and substrate quality. Respiration measurements can be coupled with biomass measurements and process measurements to produce ratios that may be more applicable for comparing management or ecosystem differences. For example, Anderson and Domsch (55,56) found the ratio of respiration to microbial biomass to be a sensitive indicator of cropping systems and temperature regimes. Smith (16,57) used the nitrogen mineralized per unit of microbial biomass (qN) to describe differences between forest clearcuts and within a semiarid shrub-steppe ecosystem.

Respiration under laboratory conditions usually is quantified in two ways. First, incubating a soil sample in a container closed by a septa and analysis of the headspace by a gas chromatograph (GC) with a thermal conductivity detector (TCD). This method is rapid (2 min/sample) and accurate even with inexpensive GCs. The second method is the incubation of soil in a closed container containing

a vial of strong base to trap the carbon dioxide. After the incubation period the trap is removed and the excess base titrated with acid and the carbon dioxide is calculated from the base used to trap carbon dioxide (58).

Field measurements of respiration can range from simple to complex with varying degrees of precision. The simplest measurement of in situ soil respiration is the closed chamber method. The chamber can be fitted with a septum for headspace gas extraction or a vial of base can be placed on the soil surface and covered with the chamber. Once the chamber is placed on the soil, or pushed slightly into the soil, the carbon dioxide evolving from the soil is allowed to accumulate for a certain period of time. Several measurements should be made during a one-hour period. Other measurement options such as Draeger gas detection tubes and the use of flow though infrared spectrometers are available (15).

In situ soil respiration is sensitive to the abiotic influences of temperature and moisture and thus will vary diurnally and seasonally. If systems are to be compared, respiration measurements should be conducted throughout the year and twice during a sampling day, preferably morning and afternoon. The addition of fresh organic residues or other perturbations to the soil will significantly affect respiration measurements. For comparing substrate availability and microbial activity between systems, the laboratory incubation for potential respiration is recommended.

Enzymes

Microbial enzymes exist as both intercellular and extracellular proteins in soil. More than 50 soil enzymes have been identified but only a few have received much attention because of the ease of detection or measurement and their role in nutrient cycling. The enzymes amylase, lipase, and glucosidases are involved in the carbon cycle; proteases, deaminases, and ureases in the nitrogen cycle; phosphatases in the phosphorus cycle; and arylsulfatase in the sulfur cycle. The oxidoreductase, dehydrogenase, cycles carbon from SOM and has been widely studied as an indicator of microbial activity. Dehydrogenase is an intracellular enzyme that is thought to reflect only viable microorganisms in soil, though it has not always been reflective of numbers of viable organisms by plate count or O_2 consumption (59,60). However, other studies found a significant correlation between dehydrogenase and other microbial parameters (61). Long-term studies also show that dehydrogenase and other enzymes are sensitive to changes in management systems (62,63), levels of soil nutrients (64), and organic matter additions (65).

Enzyme activities are attractive as indicators of soil quality because they are related to SOM and microbial biomass, are sensitive and change more quickly than chemical or physical properties, can indicate past management conditions, and are relatively simple and rapid to measure (66–68). Most enzymes correlate positively with pH, which is important in soil quality and plant growth (69). Literature studies indicate that dehydrogenase is the enzyme that correlates most with microbial biomass, respiration, and organic matter. The analytical method for dehydrogenase is simple enough to process numerous samples per day and is a good check on respiration and biomass measurements.

The method for dehydrogenase activity is a reaction of the enzyme with 2,3,5 triphenyltetrazolium chloride (TTC) for 24 hours to form triphenyl formazan (TPF) that is detected colorimetrically using a spectrophotometer at 485 nm. A standard curve is prepared from a standard TPF solution. The results are reported as mg TPF kg^{-1} soil 24 h^{-1} (66).

For a comprehensive treatment on enzymes and soil processes see the special issue of the international conference on "Enzymes in the Environment" (70).

Microbial Processes

There are a number of measurements that can provide information on how a system is functioning, the presence or absence of communities of organisms, and even the cycling of nutrients in the system. These measurements are termed *potentials* because they usually are carried out under ideal conditions in the laboratory. Some potential measurements are nitrogen mineralization potential (N_{min}), nitrification potential (N_{nit}), denitrification potential (DNP), phosphorus mineralization potential (P_{min}), sulfur mineralization potential (S_{min}), and substrate utilization potential (C_{min}). These process potentials usually are laboratory-based measurements, however, theoretically they could be completed in the field.

The mineralization potential measurements for nitrogen, phosphorus, and sulfur can be measured with carbon dioxide evolved during soil incubation if no carbon was added. Incubation times range from a few days to weeks depending on the objectives. A seven-day incubation will give the amount of nutrient mineralized during that time. A three-week incubation in which the nutrient is measured several times (destructive sampling) can provide a kinetic analysis of mineralization. The respiration measurement can be conducted by the methods outlined previously, and the soil extracted for the nutrient(s) at the time of interest, (see *Methods of Soil Analysis* (71) for further information on extracting nitrogen, phosphorus, and sulfur).

Some of the measurements such as nitrification, denitrification, and substrate utilization require the addition of substrates and the measurement of an end product. In the case of nitrification, the soil is supplemented with ammonium (NH_4^+) and incubated. At the end of the incubation, the soil is extracted and the extract analyzed for nitrate (NO_3^-), the product of nitrification. Similarly, NO_3^- is added to soil under anaerobic conditions for analysis of denitrification potential. In this assay, the disappearance NO_3^- can be considered to be the denitrification potential, or the formation of an intermediate nitrous oxide (N_2O) can be measured. The substrate utilization assay is similar to N_{nit} and DP assays with the addition of different carbon substrates and the measurement of product formation, in this case carbon dioxide. Consult *Methods of Soil Analysis* (71) for more details on process potential measurements.

Soil Sampling Strategy and Pretreatment

The temporal and spatial variability of soil attributes and processes is substantial (9,15,53,59). Biological parameters can have a coefficient of variation (CV%) of 50 to

70% over small areas, whereas physical attributes may range from 25 to 40% CV and chemical properties from 5 to 30% (53). It is important to be consistent in sampling from the field to landscape scales both for spatial and temporal considerations (53). Some simple considerations for soil sampling are discussed later, for a more exhaustive analysis of sampling strategies consult these cited articles (1,7–9,11,12,71).

The two most important aspects of soil sampling are spatial representation and landscape topography. In a geostatistic sampling of a 60×60 km area it was found that slope position was the dominate factor in the variability of soil parameters, even exceeding rainfall zones (author, unpublished data). Thus when soil sampling, particular attention should be given to incorporate topography into the scheme. Within topographic position transects of samples should be taken (even if composited) to address the spatial variability of the landscape.

Temporal variability of soil properties, especially biological attributes, can also be large even on a small scale (53). Because temperature and moisture are regulating factors of biological activity, the more stable these parameters are when the soil is sampled, the more consistent are the data, both temporally and spatially. Sampling in spring or in fall provides soil with more uniform soil moisture and average temperature.

Sample pretreatment can have a great effect on many soil attributes, particularly biological parameters. In general, for biomass measurements it is desirable to preincubate the soil sample under ideal moisture conditions. To avoid a drastic soil perturbation, such as wetting up a dry soil, sampling should be conducted when field soils are at optimum moisture content, mostly in spring and fall. In many soil process studies, such as soil respiration, soils are not preincubated and measurements are started the day after the incubation begins. In process studies in which potentials are measured, it is not necessary to preincubate the soil sample.

Interpretation of Biological Indicators of Soil Quality

The interpretation of biological indicators of soil quality is difficult because of several factors: (*1*) temporal and spatial variability of soil biological parameters, (*2*) the high variability of in vivo laboratory analysis for incubations and potential measurements, (*3*) the interaction of chemical and physical properties on biological variables, and (*4*) the lack of knowledge of the magnitude of the biological indicator in relationship to soil quality.

Because nutrient cycling, residue and litter decomposition, and SOM transformations are catalyzed by soil microorganisms some measure of biological activity is necessary to evaluate soil quality. Changes in microbial biomass with changes in management and methodology can be detected (63,72,73). However, microbial biomass is highly variable in both time and space and can be affected by simple agricultural management operations. Thus, long-term monitoring of biomass numbers is necessary to establish a baseline for changes in management. In natural ecosystems this monitoring is also important to monitor trends in biomass to determine if the system is aggrading or degrading.

As previously mentioned microbial biomass measurements may be useful in soil quality analysis when expressed as a ratio. The most common ratio is the microbial carbon/total soil carbon (C_{mic}/C_{tot}) that ranges between 1 and 4% (74,23). Deviations from this ratio could indicate soil degradation, or perhaps soil aggradation. It is not known why this ratio is so narrow for soil throughout the world, however, it is possible that the factors controlling this ratio are similar across a range of soils.

Biological activity may be best indicated by respiration that may be high or low, which may be good or bad. Because respiration means SOM turnover and litter decomposition accompanied by nutrient release, from a plants perspective the higher the activity the better. However, this activity also increases SOM loss from the system that if not retarded, will decrease soil quality. These conclusions by themselves are contradictory, although if we consider respiration activity and total carbon inputs, we may come to a different conclusion. Thus, carbon loss must be considered relative to carbon inputs in which a high respiration rate may be beneficial in releasing plant nutrients, yet not decrease SOM because of high organic inputs. The C_{loss}/C_{inputs} depicts the microbial yield or efficiency ratio for carbon metabolism; if this ratio is high the system will sequester more carbon per unit input. These types of calculations can be useful in determining the change in SOM with time.

As is discussed in detail the next section, the metabolic quotient (qCO_2) expressed as basal respiration per unit of microbial biomass, is useful in describing aggrading and degrading soils. It also appears to be a useful index to compare cropping systems, chronosequences, and different ecosystems. Although the interpretation may be difficult, this property, if measured properly, is a fundamental characteristic of soil and soil development.

Process measurements, as discussed earlier, usually are interpreted as indicators of nutrient supplying power or potential nutrient loss. Because these complex biochemical processes are highly sensitive to stress, they may be useful for evaluating soil quality and the direction of change in soil quality (75). Nitrogen mineralization data is usually thought of as plant available nitrogen, however, coupled with high nitrification rates (N_{nit}) this process becomes less beneficial because of the potential for gaseous loss and nitrate leaching. The biogeochemistry of sulfur and phosphorus is more stable with respect to gaseous and leaching losses (Fig. 2), thus high rates of S_{min} and P_{min} are more likely positive processes involving soil microorganisms. Process measurements have been coupled with field measurements to evaluate soil quality relationships (76).

The interpretation of any indicator, chemical, physical, or biological, for soil quality evaluation, is complicated by external factors such abiotic factors of temperature and precipitation, and management factors such as tillage, cropping systems, residue quality fertilization, forest clearing, grass harvesting, and grazing.

Many studies have compared tillage and soil quality (77,78). Most have shown that no-tillage increases

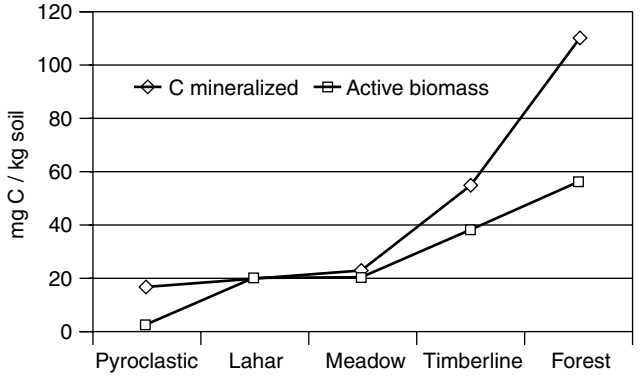

Figure 2. Carbon mineralized and the amount of active microbial biomass (MB) from lupine rhizosphere soil from the disturbed areas of Mount Saint Helens (92).

SOM, infiltration, soil structure and crop yields while decreasing soil erosion (77,79). Some of the most sensitive indicators to conservation management (no-tillage) practices are fractions of SOM, microbial biomas, and respiratory activity (80,81). No-tillage systems often conserve carbon and support greater microbial activity and biomass than tillage systems (82–84). However, because of some compaction and lack of residue incorporation, no-tillage may increase nitrous oxide (N_2O) production from soils and decrease methane (CH_4) consumption (85).

Crop rotations maintain higher levels of soil biochemical activity than monocropping and rotations with a fallow period (86–88). Also forage systems and grasslands maintain greater microbial activity and biomass than do arable cropping systems (89–91). Much of this phenomenon can be attributed to greater residue inputs; however, the litter or residue quality also plays an important role in the decomposition and sequestration of carbon and nitrogen through microbial action.

THE METABOLIC STATE OF MICROORGANISMS AND SOIL QUALITY

Because of the interest in using microbial biomass to assess soil quality, in the following section data from aggrading and degrading systems are analyzed to find out to what extent measurements of microbial biomass are useful were in characterizing these systems. In addition, an analysis of the potential and limitations of the metabolic quotient (qCO_2) is presented. The overall objective is not to provide an exhaustive review of the subject but to provide the reader with a flavor for the potential use of microbial biomass and related parameters in soil quality analysis and assessment.

Aggrading Systems

Systems recovering from various degrees of disturbance rely on vegetation for initial carbon inputs to induce microbial activity and the subsequent buildup of SOM. If microbial biomass or the active SOM fraction is indicative of the health of a soil and its functioning as a quality medium, then its dynamics (state, mass, and activity) should be discernible in aggrading soil systems.

Mount Saint Helens. In 1980, the volcano Mount Saint Helens erupted in the state of Washington in the United States. This eruption produced massive destruction of a mixed high meadow-forest ecosystem. The aftermath produced five distinct systems including areas of pyroclastic volcanic substrate, mudflow material, tephra-covered meadow, and relatively undisturbed timberline and forest. These systems formed a disturbance gradient that was inversely related to soil carbon and nitrogen concentrations from the pyroclastic material to the areas of original forest. By 1987, the most disturbed sites had been recolonized by nitrogen-fixing lupines.

Seven years after these areas were disturbed by the eruption lupine root zone soil (LR) showed greater increases in carbon and nitrogen concentration compared with non-lupine root zone soil (NR) (92). Figure 2 depicts the quantity of carbon mineralized during laboratory incubation and the amount of active microbial biomass, determined by substrate induced respiration (SIR), from LR soil in each of the five disturbed areas of Mount Saint Helens (92). These trends are similar to the total carbon and nitrogen concentrations of the soils and are significantly greater than for NR soil (not shown). This suggests that there is a significant relationship between the recolonization of disturbed areas by lupines and the advent of significant microbial activity.

Nitrogen fixation was highest at the more disturbed low nitrogen sites (93) totaling 10.3 $kg\,ha^{-1}\,y^{-1}$ for LR soil and 3.3 $kg\,ha^{-1}\,y^{-1}$ for NR soil. Thus an increase of 7 $kg\,ha^{-1}\,y^{-1}$ could be attributed to lupines. The retention of fixed nitrogen over these aggrading sites is related to the increasing microbial biomass and activity in these systems.

This data showed that ecosystem development was correlated with increased soil carbon and nitrogen, microbial activity and respiration efficiencies. The increase in microbial biomass, activity and carbon and nitrogen cycling is a positive feedback to the further development of soil that can support ecosystem succession from a lupine to a forest system.

Conservation Reserve Program. The Conservation Reserve Program was initiated in the United States in 1985 to retire highly erodable farmland from crop production to reduce soil erosion and to increase soil quality. By 1993, 14.6 Mha had been planted to grass or trees with a contract period of 10 years. Much research has been devoted to the degrading effects of cultivation on soil; however, little information is available on the recovery of cultivated land after the cessation of farming. Two studies have recently investigated the process of aggrading soils converted from agriculture to grasslands over periods of 50 and 7 years.

Burke and coworkers (94) sampled 12 sites in northeastern Colorado, that had been abandoned from cultivation for 50 years. Total and active carbon and nitrogen pools for the native, cultivated, and abandoned sites were determined. As expected the degradation of SOM from 50 yrs of cultivation resulted in a loss of approximately 50% of the total carbon and nitrogen from the system (Table 4). After 50 years of recovery and the establishment of perennial grass, soil total carbon and nitrogen

Table 4. Carbon and Nitrogen Pools and Mineralization Rates from Native, Abandoned from Cultivation and Cultivated Shortgrass Steppe Soils (94)

	Native	Abandoned	Cultivated
Total C (g/m^2)	1,479	987	823
Total N (g/m^2)	86	62	46
N min ($g/m^2/d$)	0.12	0.12	0.10
C min ($g/m^2/d$)	2.33	2.54	1.86
Microbial C (g/m^2)	93.9	91.3	54.3
Microbial nitrogen (g/m^2)	10.2	10.5	8.8

increased by about 20 and 35%, respectively, compared to the cultivated soils. Soil microbial biomass, measured by CFIM, and microbial activity had totally recovered to levels found in native grasslands that had never been cultivated (Table 4). The carbon and nitrogen mineralization rates were about 30% and 20% greater for soils of the native and abandoned sites, respectively, compared with the cultivated sites. Microbial biomass carbon in the native and abandoned sites was about double that of the cultivated site, however, the biomass nitrogen was only about 20% greater, resulting in a curious biomass carbon/nitrogen ratio for the cultivated soil of 6 compared to about 9 for the native and abandoned sites. It is most likely this result is due to the fertilizer nitrogen inputs to the cultivated system because all the sites have a similar total soil carbon/nitrogen ratio of about 17.

This data shows that after cultivation ceases, the nutrient and active SOM pools increase fairly rapidly, although the total SOM recovers slowly. This slow recovery for total SOM is likely because of the greater levels and activity of microbial biomass and to the slow buildup of stable organic matter pools that were lost during the cultivation period.

In a study of soil quality in CRP and wheat-fallow (W-F) soils, Staben and coworkers (72) found that after 4 to 7 years in CRP, the soils did not increase significantly in total carbon but had more total nitrogen. The study on 20 paired sites in eastern Washington showed few significant differences in soil biota measurements, however, the trends in the activity data suggest that the active SOM fraction is increasing. Figure 3 shows the cumulative carbon evolved and the rate of carbon evolved from the CRP and W-F soils during a 60-day incubation. The CRP soil continued to rapidly mineralize carbon after the initial two-week period and mineralized 25% more carbon than the W-F soil (Fig. 3a). The higher decomposition rate in the CRP soil after the initial flush of carbon dioxide indicates shows that this "extra" carbon being mineralized is from a secondary carbon pool, mainly cellulose and hemi-cellulose compounds (Fig. 3b). Secondary carbon pools are important in soils because they serve as energy and nutrient reserves for microorganisms. The building of these pools and reserves is an indicator that the soil is increasing in soil quality.

Although these are "young" recovering soils, the increase in pH with the CRP treatment (6.4 CRP versus 5.7 wheat fallow) will have a positive effect on nutrients, plant growth, and microbial activity. The buildup of a

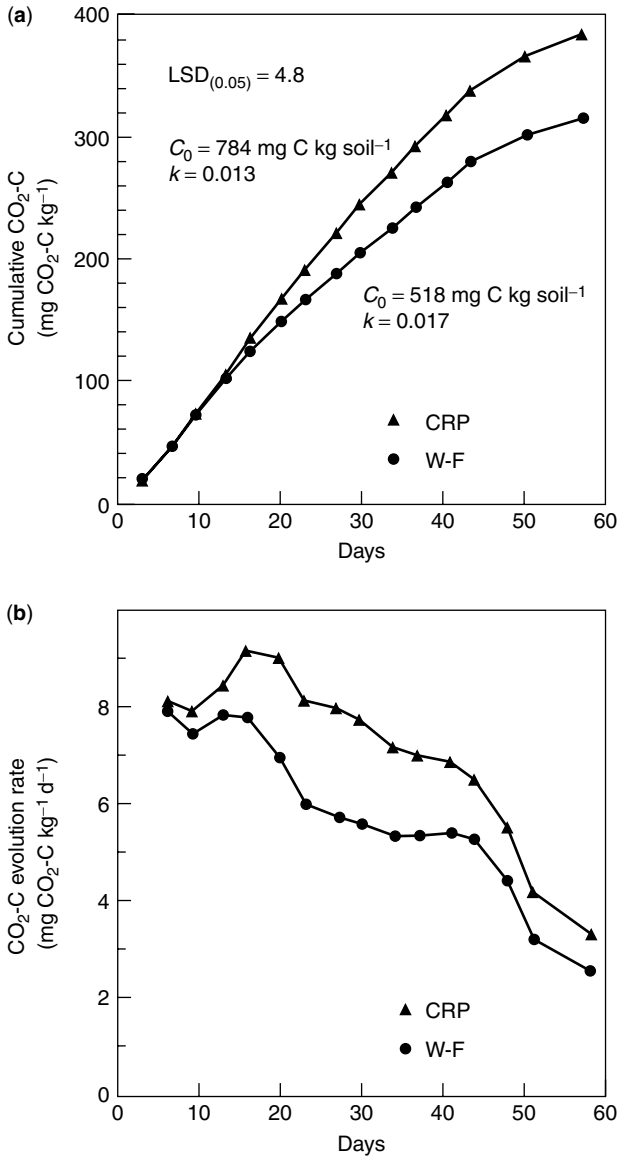

Figure 3. Cumulative carbon evolved and the rate of carbon evolved from the conservation reserve program (CRP) and wheat-fallow (W-F) soils during a 60-day incubation (72).

secondary carbon pool is important for soil structure, microbial activity, and as a precursor to more resistant and stable carbon pools. From the mineralization studies on these soils it was apparent that the W-F soils were carbon limited and that in moving to a carbon-rich system such as CRP, the soils were conserving nitrogen, thus preventing nitrogen loss through leaching and denitrification. These very subtle changes in the soil chemistry and biota indicate that the CRP soils are increasing in soil quality and that differences will become more evident with time since cultivation increases

Degrading Systems

Of even greater interest on a global basis is the development of sensitive biomarkers that would indicate if

soils were degrading or potentially could be susceptible to desertification. Significant efforts have already been made to evaluate the effects of tillage, management, crop rotation, soil erosion, and toxicology on microbial biomass (77,81,82,86). However, there has been little use of these measures to explain entire systems and whether they may be aggrading or degrading. An important question in this analysis is whether to measure the microbial biomass concentration, its activity, or its response.

Bioindicators. Gallardo and Schlesinger (95) hypothesized that as organic matter is lost because of desertification of semiarid grasslands and that the soil microbial biomass will shift from being nitrogen limited to carbon limited. If this were true, metabolic activity or response to substrates could potentially be used as a sensitive indicator of degrading soils subject to desertification. On a global basis these bioindicator methods are important because an estimated two billion hectares of soil have been degraded, either chemically, physically, or biologically (96).

Using a desertification sequence of grassland, mesquite, creosotebush, and tarbush vegetation in the Chihuahuan Desert, Gallardo and Schlesinger (97) measured microbial biomass nitrogen by fumigation-extraction (FE), in carbon and nitrogen amended soil. Figure 4 shows the response of the biomass-nitrogen to carbon and nitrogen amendments. The grassland soil showed a significant increase in biomass nitrogen with nitrogen and C + N additions as compared with the water-only control. Statistical interaction between the carbon and nitrogen treatments indicated a response to carbon addition only when nitrogen was also applied. In the mesquite soil there was an independent response to carbon and nitrogen with no interaction indicating the soil was mainly nitrogen limited. The soil with creosotebush exhibited a significant and comparable response to carbon and C + N additions. Similar responses were observed with the soil with tarbush in which a significant response to carbon and C + N was observed and not to nitrogen fertilization alone.

In the nitrogen-limited soils, supporting grassland and mesquite, the microbial biomass nitrogen of the water control averaged 10.7 and 11.3 ug/g soil, respectively, whereas the carbon-limited systems with creosotebush and tarbush had biomass nitrogen levels of 18.2 and 16.7 ug/g soil, respectively. The shifting of nutrient limitations from nitrogen to carbon during desertification or shrub invasion is supported by this data. However, the higher levels of microbial biomass nitrogen in the carbon-limited soils suggest possibly a higher retention of nitrogen and a tighter nitrogen cycling in these soils. This hypothesis is supported by the relative response of the systems in which the biomass nitrogen of the grassland and mesquite soils increased by 100% with the addition of C + N and the creosotebush and tarbush soils only increased about 30% to 50%.

Garcia and Henandez (98) sampled 21 degraded soils from the semiarid Spanish Mediterranean to determine their biological characteristics and to develop bioindicators for these soils. The soils were grouped by texture and compared with undisturbed and reforested soils. A suite of biological and enzyme activity measurements was conducted and principal component analysis was used to determine the most relevant indicators of degradation.

Table 5 gives three properties of the 21 degraded soils grouped by texture. The average decrease in organic matter was 67%, which correlates well with the observed decrease in microbial biomass (FE) and basal respiration (Table 5). Dehydrogenase concentration, an indicator of microbial activity, was lowest in the more clay soils and was reduced by 50 to 80% in all soils compared with the undisturbed soils. The loss of SOM was most closely related to changes in the microbial biomass, B-glucosidase, and arylsulphatase as modeled by stepwise variable analysis.

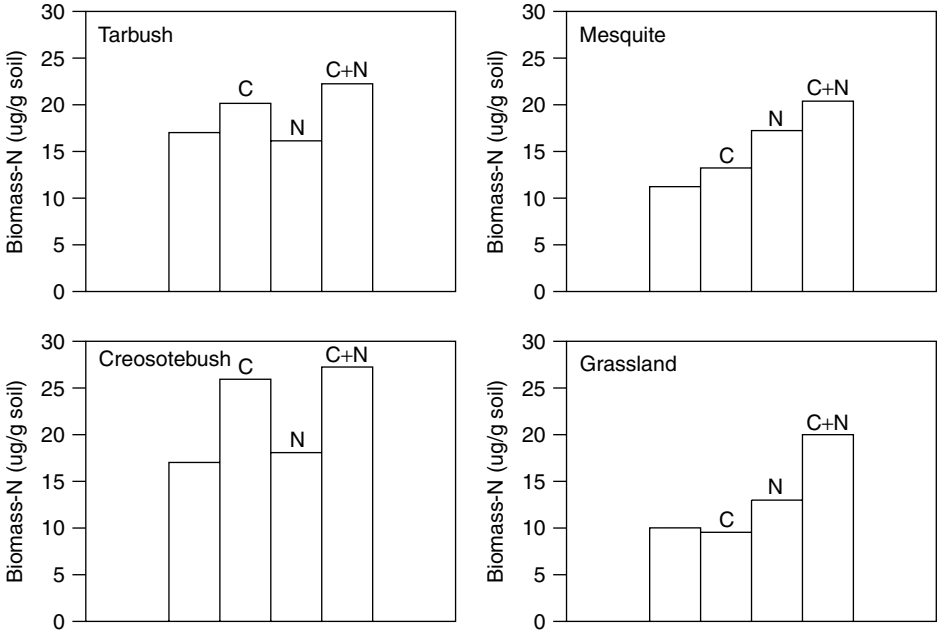

Figure 4. The response of biomass nitrogen to carbon and nitrogen amendment for the desertification sequence of grassland, mesquite, creosotebush, tarbush vegetation in the Chihuahuan Desert (97).

Table 5. Biological Analysis of Intensely Degraded Soils Subject to Desertification in Spain (98)

	Microbial Biomass ug-C/g-soil	Dehydrogenase ug INTF*/g-soil	Basal Respiration ug-C/g-soil/d
Clay loam soils	340	50	22
Silty clays soils	101	17	17
Clay soils	162	16	17
Undisturbed	450	90	81
Reforested (50 y)	1,236	141	206

*p-iodo-nitro tetrazolium formazan

The two studies mentioned in the preceding section demonstrate the potential usefulness of biological parameters for evaluating changing systems and degraded soils. In the Gallardo and Schlesinger (97) study, changes in system direction from nitrogen-limited to carbon-limited could now possibly be inferred by the simple measurement of biomass nitrogen. Whereas, in the Garica and Henandez (98) study, a broader suite of parameters was needed to show the reduced soil health because some of the key activity measurements did not follow the overall trend. The interacting and complex factors controlling microbial activity make it difficult to choose a few simple biological measurements that can be universally used to evaluate soil quality.

The Case for qCO_2 and Soil Quality

The objective for developing soil quality methodology is to provide sensitive measures that will detect the rate and direction of change in the quality of a given soil. However, defining a "silver bullet" soil quality indicator is proving to be elusive because of the previously stated complex factors controlling biological parameters in soils. Soils are buffers, and as with any good buffer, absorption or change is slow until the end point. The end point, however, is of little use in soil quality analysis.

A measure of microbial activity that has received significant attention as a sensitive indicator of soil development and quality is the metabolic quotient or basal respiration to biomass ratio. The metabolic quotient has been used to evaluate microbial physiological conditions (55), plant ecosystem succession (99,100), soils with different cropping histories (56), and effects of environmental conditions on soil microbial biomass (101,102). The history and application of qCO_2 are also detailed in Anderson (103) and Wardle and Ghani (104).

Besides the effects of disturbance on microbial metabolism, qCO_2 may be useful as an indicator of systems under stress (104). The current popular interpretation of qCO_2 is that under ecosystem development from young to mature, qCO_2 values will decrease due to increased efficiency of microorganisms (see following). In contrast, in disturbed or stressed ecosystems, qCO_2 will increase due to reduced efficiency of microbial biomass.

The metabolic quotient and its relationship to ecosystem development is an interpretation of ecosystem energetics as put forth by Odum (105,106) in which ecosystems are hypothesized to develop toward a lower primary production to respiration ratio. In the early stages of ecosystem development, primary production exceeds system respiration and organic matter and microbial biomass will accumulate. As the system matures this ratio decreases until the system enters the climax stage. Insam and Haselwandter (99) proposed that the primary decomposer level could be substituted for the system level and thus microbial respiration per unit of microbial biomass (R_{micr}/B_{micr}) should decrease with ecosystem maturity.

The hypothesis mentioned in the preceding section was tested by Insam and Haselwandter (99) on two primary

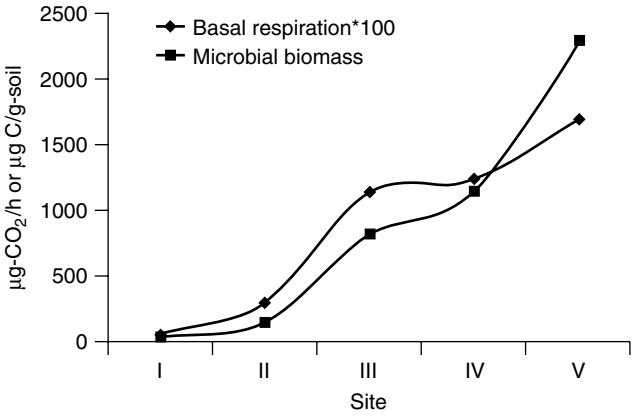

Figure 5. Soil microbial biomass (MB) and basal respiration (BR) from the Rotmoos moriane succession in Austria. Sites I to V have development ages of 1, 5, 65, 135, and 1,000 years, respectively (99).

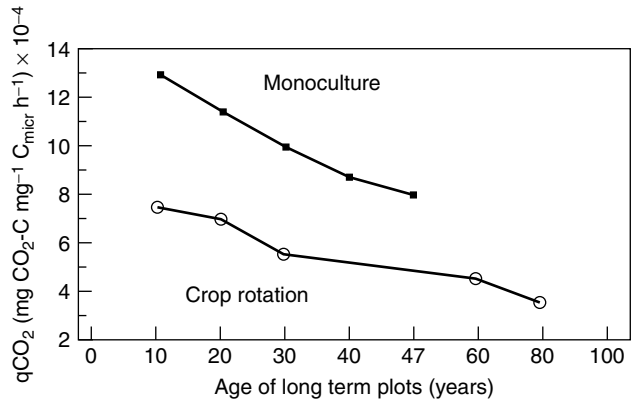

Figure 6. Metabolic quotients (qCO_2) of soils under crop rotation and monoculture for varying lengths of time (56).

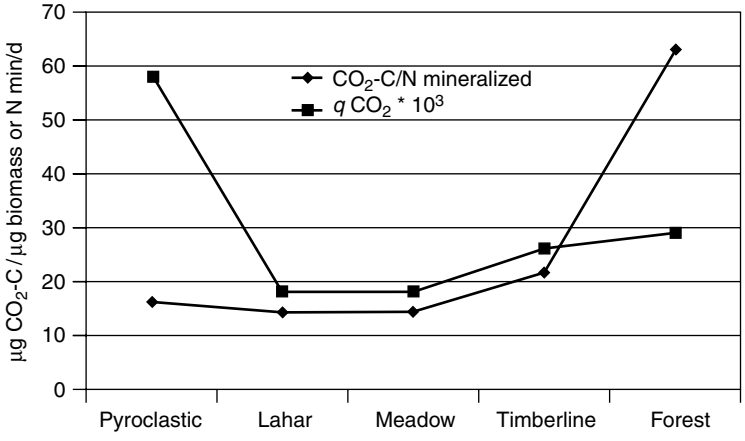

Figure 7. Metabolic quotient (qCO_2) and mineralization ratio (CO_2/N_{min}) for five sites at Mount Saint Helens ranging in disturbance from the pyroclastic site (volcanic tephra) to relatively undisturbed forest sites (92).

successions on receding moraines in Austria and Canada. The age of the soils formed on the Rotmoos moriane succession in Austria range from one and three years to over 1,000 years. Figure 5 shows the data for microbial biomass (SIR) and basal respiration (original Table 2) for sites I to V with development ages of 1, 5, 65, 135, and 1,000 years, respectively. For sites I to III the biomass and basal respiration increased exponentially; however, site IV had significantly greater biomass than site III but the same basal respiration. The significant increase in biomass from site I to V with a less rapid increase in basal respiration resulted in an exponential decrease in qCO_2 over the succession.

Anderson and Domsch (56) used the concept of qCO_2 to evaluate soils with different cropping histories. When comparing soils with different crop rotations, they found that the metabolic quotient decreased with the length of time the soil had been in rotation (i.e., age of plot, Fig. 6). In addition, they found the same trend for soils under monoculture, but the monoculture soils had significantly greater respiration per unit of SIR than did soils in crop rotation. Statistical analysis of the 20 monoculture plots versus the 21 crop rotation plots showed a significant difference for qCO_2 but not for other variables such as pH, organic matter, percentage of clay, or fertilizer. The observed differences in qCO_2 may be due to differences in complexity of organic substrates and the soil enzymatic structure.

The relationships of qCO_2 and CO_2/N mineralized were used to determine the substrate quality relationships of sites devastated by the 1980 eruption of Mt. St. Helens volcano in U.S.A. (70). Figure 7 shows the qCO_2 and mineralization ratio for five sites ranging in disturbance from the pyroclastic site (volcanic tephra) to the relatively undisturbed forest site. qCO_2 was highest for the most disturbed site, decreased dramatically for all other sites, then increased slightly from the moderately disturbed lahar site to the forest site. The CO_2/N mineralized relationship showed an opposite trend possibly indicating rapid cycling of nitrogen at the disturbed sites and greater immobilization at the less disturbed sites.

These seemingly contradictory trends for physiological attributes of qCO_2 have not gone unnoticed. Wardle and Ghani (104) reviewed the literature concerning the use of qCO_2 as an indicator of disturbance and ecosystem development. They evaluated studies of primary and secondary succession, a 10,000-year chronosequence, and the Mt. St. Helens data presented earlier to show the inconsistency of qCO_2 to decrease with succession. They used data from numerous studies to show that although qCO_2 was generally negatively correlated with soil properties such as biomass, percentage of carbon, percentage of nitrogen, pH, and clay, there was very little statistical significance. They also evaluated 16 studies of environmental disturbance such as chemical and manure additions and cultivation and concluded that qCO_2 did not consistently increase with disturbance.

Wardle and Ghani (104) concluded that qCO_2 was not useful for describing ecosystem development because it responded unpredictably and did not always decline along successional gradients. In addition, qCO_2 can be insensitive to disturbance and unable to distinguish between the effects of disturbance and stress. However, the utility of the metabolic quotient may be in its sensitivity to environmental degradation and its use as a measure of substrate quality and microbial efficiency.

Although qCO_2 may not be the best ecosystem development indicator, it may be used to investigate changes in soil systems to establish metabolic efficiencies, energy flow, and potential stress caused by nutrient limitations. Other relationships such as microbial biomass carbon to total carbon ratio (C_{mic}/C_{org}) may be useful in conjunction with qCO_2. This relationship has been useful in studies of SOM and carbon turnover (107,108), and in studying chronosequences (109) and the impact of climate and temperature on microbial activity (55,110). The C_{mic}/C_{org} ratio can also be an indicator for changes in soil texture (111,112), aggregation and structure (113,114), toxicity (115) and tillage (116). Other useful ratios, which have not received much attention, are respiration to mineralized nitrogen (CO_2/N_{min}) discussed in this paper and the ratio of nitrogen mineralized to microbial biomass (qN) discussed in Smith (16).

Soil quality changes are not likely to be quantified with a single measure. Most soil scientists agree that a suite of biological, chemical, and physical properties will be required to quantify the direction and rate of soil quality change. However, attention should be focused on

microbial physiological parameters and their relationships to each other to detect small changes in the quality of soils. It is evident from the forgoing discussion, that whatever parameters are chosen, the measurement and understanding of the state and function of the soil microbial biomass will play an important role in soil quality analysis.

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SOIL SAMPLING. See SAMPLING TECHNIQUES FOR ENVIRONMENTAL MICROBIOLOGY

SOIL SLURRY SEQUENCING BATCH REACTOR (SS-SBR). See Activated Sludge — Sequencing Batch Reactors

SOILS, BIODEGRADATION IN. See Wetlands: Biodegradation of Organic Pollutants

SOILS, BIODIVERSITY IN. See Biodiversity in Soils: Use of Molecular Methods for its Characterization

SOILS, DISTRIBUTION OF MICROORGANISMS IN. See Soil Distribution of Microorganisms

SOILS, KINETICS OF MICROBIAL PROCESSES. See Kinetics of Microbial Processes and Population Growth in Soil

SOIL TOXICITY TESTS. See Toxicity Testing in Soil, Use of Microbial and Enzymatic Tests

SOILS, VIRUS SURVIVAL IN. See Virus Survival in Soils

SOLAR POWER: DISINFECTION OF CISTERN WATER. See Rainwater Roof Catchment Systems, Microbial Quality of

SOLID WASTES. See Compost: Biodegradation of Toxic Organic Compounds

SOLID WASTES MICROBIOLOGY. See Landfilling of Municipal Solid Wastes: Microbiological Processes and Environmental Impacts

SOLVENTS, EFFECTS ON MICROORGANISMS. See Toxicity of Organic Solvents to Microorganisms

SOLVENT-TOLERANT BACTERIA. See Toxicity of Organic Solvents to Microorganisms

SORPTION PROPERTIES OF BIOFILMS

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Microbial aggregates such as films, microbial mats, flocs, and sludge are commonly subsumed under the expression "biofilms" (1). All of these aggregates represent three-dimensional, spongelike hydrogels with a considerable sorption capacity. From an ecological point of view, this facilitates the sequestering of organic molecules from the water phase, providing nutrients for the immobilized organisms (2). This is considered as part of the survival strategy for biofilm organisms in oligotrophic environments. Also, biofilms influence the distribution and fate of pollutants in soils, sediments, and waters. Sorption to biofilms represents a significant aspect of the general phenomenon of "biosorption." Material sorbed to biofilms is biodegradable and can be metabolized or otherwise chemically altered, or it can remain unchanged, with a potential to be remobilized when the biofilm decomposes. Thus, biofilms represent both a sink and a source for sorbed substances. These can be: inorganic ions, organic substances, precipitates, particulate matter and, last but not least, the water that is retained in the biofilm matrix. This is schematically depicted in Figure 1 (after Ref. 3).

The reason that the term "sorption" has been chosen for this article is that both *ad*sorption and *ab*sorption can occur in biofilms. The polymer matrix allows the incorporation of abiotic substances by various retention mechanisms, which are not yet known in all cases. There are different sites within a biofilm in which the interaction may take place, such as cell walls, membranes, and interior and matrix molecules. Also, sequestering of matter from the bulk aqueous phase occurs not only by adhesion to biofilm components but also by complexation, redox reactions, or by precipitation caused by microbial metabolites or owing to entrapment in pores and channels of the biofilm matrix. In all of these cases, the sequestered substances accumulate within the biofilm and, thus, are regarded as "sorbed." This interpretation may be considerably broader than allowed by a definition that is based solely on thermodynamic properties. However, all of these kinds of mechanisms are the basis for many published and patented attempts to purify water from pollutants by "biosorption." In most of these cases, "biomass" and the actual retention mechanisms are treated as black boxes, with emphasis being placed on the final outcome of the many interactions (e.g., percentage removal of a pollutant), rather than the mechanisms. The objective of this article is to shed some light on these black boxes.

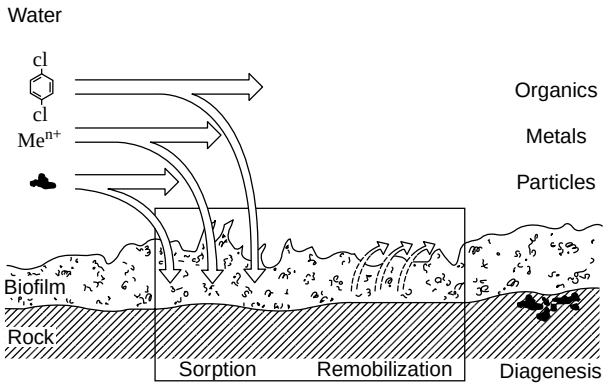

Figure 1. Interaction of dissolved and suspended material at interfaces covered with biofilms. (After H.-C. Flemming, J. Schmitt, and K. C. Marshall, in W. Calmano and U. Förstner, eds., *Environmental Behavior of Sediments*, Lewis Publishers, Chelsea, Mass., 1996, pp. 115–157.)

SORPTION SITES IN BIOFILMS

When a dissolved or particulate substance transported by the water phase meets a biofilm, it encounters a highly heterogeneous hydrogel with very different sorption sites. These include:

- Extracellular polymer substances (EPS), mainly consisting of polysaccharides and proteins (see Refs. 1 and 4);
  - Charged groups, for example, $-COO^{-1}$, $-SH^{-}$, $-SO_4^{2-}$, $-H_2PO_4^{-}$, $-NH_4^{+}$, $-NRH_2^{+}$, etc.
  - Apolar groups, for example, aromatics, aliphatics such as found in proteins; also, hydrophobic regions in polysaccharides.
- Cell walls (see Ref. 5)
  - Outer membrane of gram-negative cells (lipids)
  - Murein or teichoic acid layer of gram-negative and gram-positive bacteria, respectively.
- Cytoplasmic membrane (lipids).
- Cytoplasm.

Figure 2 shows the complex structure in a transmission electron micrograph of a bacterial monolayer on a synthetic polymer after exposure to river water (courtesy of G. Geesey). The organisms can be clearly seen. The fibrillar structures represent the EPS matrix after desiccation. The black spots are particles entrapped by the matrix. In Figure 3, the sorption sites in biofilms are schematically depicted.

Extracellular Polymeric Substances

EPS consist of polysaccharides, proteins, and lipids. In gram-negative cells, the EPS are composed of lipopolysaccharides, capsule polysaccharides, and other excreted polysaccharides and proteins that are less firmly bound to the cell surface (1,7). In gram-positive cells, lipoteichoic acids as well as polysaccharides and proteins, which are not anchored in the cell wall, contribute to the

Figure 2. Transmission electron micrograph of bacteria attached to a synthetic polymer exposed to river water (courtesy of G. Geesey and L. Jang, in H. C. Ehrlich and C. L. Brierley, eds., *Microbial Mineral Recovery*, McGraw-Hill, New York, 1990, pp. 223–247).

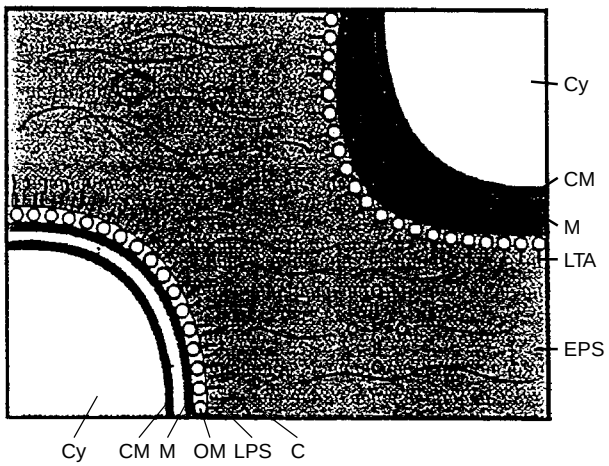

Figure 3. Different sorption sites in a biofilm, including a gram-negative (left) and a gram-positive organism (right). CY, cytoplasm; CM, cytoplasmic membrane; M, murein; OM, outer membrane; LPS, lipopolysaccharide; C, capsule; LTA, lipoteichoic acid. (After H.-C. Flemming, *Water Sci. Technol.* **32**, 27–33 (1995). J. Wingender, T. Neu, and H.-C. Flemming, eds., *Microbial Extracellular Polymeric Substances: Characterization, Structure and Function*, Springer, Berlin, Germany, 1999 (6).

Figure 4. EPS microdomains of different density. (After A. W. Decho, in R. E. Riding, and S. M. Awramik, eds., *Microbial Sediments*, Springer, Heidelberg, Germany, 2000, pp. 9–15).

EPS. Many microorganisms produce EPS, whether grown in suspended cultures or in biofilms. A considerable part of the protein moiety of the EPS may be extracellular enzymes. It seems that the composition of EPS is not constant but is influenced by growth conditions and environmental stress (8). Many strains form more than one specific type of EPS, and the composition of the EPS may change during the life cycle of such organisms (9). The EPS molecules provide the forces responsible for cohesion of the biofilm and adhesion to the substratum. This is performed by weak interactions such as van der Waals forces, electrostatic interactions, and hydrogen bonds (10). The density of the EPS matrix seems to vary considerably as can be demonstrated by various staining techniques. Decho (11) has introduced a concept of "exopolymer microdomains" in order to describe these inhomogeneities (Fig. 4).

Cell Walls

Gram-positive. The cell wall of gram-positive cells (see Fig. 3) is composed of teichoic acids. The structure

contains both positively and negatively charged sites. The teichoic acid portion may contain a D-alanine residue in place of the β-D-glucose as shown. Anionic sites include carboxylate (from peptidoglycan) and phosphate (from teichoic acid). Positively charged sites are exclusively ammonium, from D-alanine (teichoic acid), amino sugar (glycan), and diaminopimelic acid (peptide portion of peptidoglycan).

Gram-negative. The envelopes of gram-negative cells (see Fig. 3) display a lower overall charge density and are chemically and structurally very different from gram-positive cell walls (12). As in the case of gram-positive bacteria such as *Bacillus subtilis*, purified peptidoglycan from the gram-negative organism *Escherichia coli* binds metals quite efficiently. The overall contribution of peptidoglycan to metal binding in gram-negative organisms is quite small because of the limited quantity of this substance in the cell envelope and its shielding from the environment by the overlying outer membrane.

Lipopolysaccharides and Lipid Membranes

Definite hydrophobic compartments in a biofilm are represented by the lipopolysaccharide layer of gram-negative cells and the lipid membranes. The membranes are composed of phospholipids with the hydrophobic groups directed inward and the hydrophilic groups toward the outside, where they associate with water. Embedded in the matrix are hydrophobic proteins. Hydrophilic proteins and other charged substances, such as metal ions, are attached to the hydrophilic surface. The membranes make up about 2% of the overall dry biomass, thus representing only a small capacity for the sorption of hydrophobic substances.

Cytoplasm

The interior of bacteria also represents a possible location for the accumulation of dissolved substances (13). It represents a water phase separated from the environment by the lipid membranes and controlled by selective transfer mechanisms across these membranes.

SORPTION OF WATER, METALS, ORGANIC SUBSTANCES, AND PARTICLES

Water

Biofilms are highly hydrated, containing 85 to 98% water. As water is the main component of biofilms, effective mechanisms must be present to retain the water. Most of the water is associated with the EPS matrix as the following assessment demonstrates: the cell density in a biofilm ranges between 10^{10} and 10^{11} cells mL^{-1} of biofilm mass, and one cell has an estimated mass of 10^{-12} g. The water content of a cell is about 85%; thus, only about 1 to 10% of the water in a biofilm is cytoplasmic whereas 90 to 99% is retained by the EPS matrix. The mobility and viscosity of this water is crucial for the biological processes in biofilms as these factors have a strong impact on the transport of substrates and products, although very little research on the role of water has been performed. More than 99% of the water molecules in biofilms seem to be as mobile as in the free (bulk) water phase, as investigated by NMR spectroscopy (14); however, another investigation by ATR-FTIR spectroscopy indicates discrete steps in the exchange kinetics of H_2O and D_2O (15). From this study, it was concluded that the water in the EPS matrix is organized in a fine structure, which influences its properties in the biofilm.

In air-exposed habitats, water retention is considered to be an important mechanism for resistance to desiccation (16,17). The importance of the water structure in microbial aggregates has been acknowledged in the context of sludge dewatering, in which the immobilization of water by biofilms has significant economical and ecological implications. Here, pore and channel water is classified as *free* and behaves thermodynamically in the same way as pure water. It can be efficiently removed by mechanical pressure, but with considerable energy cost. *Bound* water has been defined as the proportion of the overall water content that is removed only by thermal drying processes and is retained by mechanisms other than entrapment; it makes up 5 to 7% of the overall water content (18). However, the exact binding mechanisms are still not clear, and the definitions of strongly bound and weakly bound water are hypothetical (19). If the EPS structures could be changed in such a way that they release the water, this would represent substantial progress in sewage sludge dewatering. Attempts have been made to destroy the hydrated structure by enzymatic attack (20,21). Such processes have been patented (22,23), but they have not yet proven to be effective in practice. Water plays a key role in the corrosion of microbially colonized surfaces. The local accumulation of water at interfaces provides an important prerequisite for the dissolution processes occurring during microbially influenced corrosion and weathering. These processes are influenced by microbial products dissolved in the EPS matrix and interacting with the interfaces.

Metals

The deposition of metals in sedimentary biofilms represents a process of geological dimensions (24). This phenomenon is technically used for the removal of pollutants from wastewater and it can cause considerable problems when metals sorb to and thereby contaminate wastewater sludge, preventing its safe disposal. Metal binding in biofilms can occur in different ways, for example, by (*1*) ion exchange reactions, (*2*) complexation, and (*3*) precipitation, including redox reactions. The mechanisms can be both active and passive. Active binding occurs through the excretion of binding, chelating, or precipitating cell products in response to the presence of the dissolved substance. These mechanisms are known from studies on detoxification, mainly investigated in terms of heavy metal resistance (25). In addition, active transport systems may allow the uptake of, for example, metal ions into the cytoplasm. This might be because of the inability of the cell to discriminate between essential metal ions such as calcium and poisonous metal ions such as cadmium. Methylation and demethylation as well as oxidation and reduction are active processes altering the sorbed species and contributing to the overall sorption

capacity of a biofilm (26). Metal binding by bacterial surfaces is considered largely as a passive phenomenon within the process of electrostatic interaction between cationic metals and anionic cell surface groups. Consequently, it is not necessary that the organisms be viable, only that their surfaces remain intact (27). Passive binding mechanisms occur through ion exchange, mainly of the EPS and the cell wall, or through precipitation and/or complexation by biofilm components *already present* and not formed in response to the bound species. Passive binding can be performed by dead biomass as well as by living biomass through partitioning and nonmetabolic processes. In some instances, the sorption capacity of the dead biomass can be greater, which is utilized in metal recovery from wastewater streams (26).

Extensively studied for their metal-binding action are the EPS produced by bacteria in sewage treatment facilities (see Ref. 3). EPS bind a wide variety of metal ions such as Pb^{2+}, Sr^{2+}, Zn^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+}, Mg^{2+}, Fe^{2+}, Ni^{2+}, and Ag^{+}. Theoretical predictions of EPS-binding capacities, based on estimated numbers of available carboxyl and hydroxyl groups, suggest a very high capacity, especially for acidic polysaccharides. This means that a small amount of EPS could theoretically bind a large amount of a given metal. As EPS are sometimes composed of a matrix of fibrils with high molecular weight, the binding capacities of EPS cannot easily be expressed in terms of surface area, but rather must be expressed in terms of weight-specific binding (4,28).

Most empirical measurements so far have indicated high weight-specific binding capacities of EPS for many metals. The affinities of EPS for metals vary depending on the specific metal and microorganisms involved (4). Harvey and Luoma (28) found a binding capacity for lead to be 0.13 μmol of Pb per milligram EPS. They calculated that if EPS represented only a very small portion (ca. 3%) of the organic matter in sediments, the EPS could still complex all available Pb^{2+} in the surface-layer sediments of a Palo Alto salt marsh. As there exists a competition between H^{+} and metal ions, the stability constants strongly depend on the pH. Some EPS have a high affinity for particular metal ions (29,30). The stability constant of Cu^{2+} complexes with the EPS of bacterial isolates from river sediments has been reported to be 10^9; the stability of Cu^{2+}-complexes with humic substances is in the same range (31). Not all association of metals with EPS may, however, be because of adsorption. Because the solubility of the metal itself depends on pH, it is probable that the metals associated with EPS under natural conditions are the result of both ion-exchange binding processes and the precipitation and later physical entrapment of the metal by the polymer (32).

EPS binding processes can be important in the downward transport of metals in ocean environments. Close associations between bacterial EPS and a variety of metals have been found in both sediments (33) and the water column (34). Open-ocean bacterial aggregates called *bacterial snow* below 100 m often have been found with EPS capsules containing metal precipitates. Their frequency increased with depth and implies the downward accumulation of metals on EPS aggregates. Similar associations have been found in freshwater lakes (29).

Microbial Mineralization. Numerous bacteria in aquatic sediments encounter and bind a wide variety of metals in their environment. As the sediments accumulate, all of the microbial and mineral components become subject to geological forces that eventually result in rock formation. During this time, chemical and physical changes occur within the sediments as diagenetic processes. The cell walls of bacteria, present in these sediments, make suitable biological templates for the concentration of metals and the nucleation of crystals (35,36) and can often greatly influence the initial mineralization process (37). It has been demonstrated that Cd^{2+} in activated sludge was preferably bound to the cell walls and not to the EPS (38). Bacteria and their remains in metal-rich waters (39) and sediments around geothermal sites (40,41) have been shown by transmission electron microscopy (TEM), analytical electron microscopy, and electron diffraction to act as nucleation sites for authigenic minerals. Figure 5 is a scanning electron micrograph showing microorganisms as templates for the deposition of iron oxides.

Iron-silica precipitating species were associated with bacteria in geothermal sediments, whereas the bacteria in metal-rich waters were associated with Fe-Al silicate (clay) polymorphs, millerite (NiS), and mackinawite (FeS_{1-x}). In the latter environment, bacteria are thought to initially bind and concentrate iron and nickel. These concentrated metals then react with bacterially produced sulfide from the sediments or soluble silica to produce the corresponding mineral. Similarly, todokorite (MnO_6, 41) and Mg-calcite (marine peloids) deposits have also been shown to be nucleated by bacteria (27,42). A common example of bacterial metal binding is the slimy, rust-colored coating of Fe(III)-oxyhydrates in water pipes and in ponds and creeks. This is because of the binding of iron by the microbial biofilms that grow on these surfaces (43). Manganese(IV) oxide is found in similar environments, precipitated by biofilms (44,45).

There are many examples in which biotechnology uses the sorption capacity of biofilms. Chromium removal from synthetic waste streams by biofilms on disks of a laboratory-scale rotating biodisc contactor was about 98% effective during steady state addition of Cr(VI) at

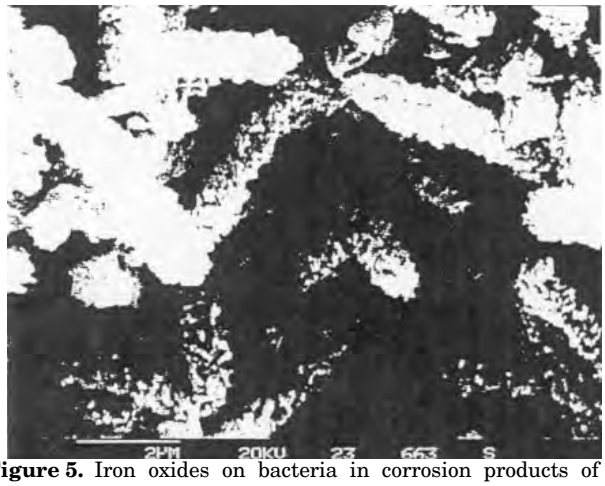

Figure 5. Iron oxides on bacteria in corrosion products of a drinking-water pipe.

concentrations of 100 to 200 mg L^{-1}. Analysis of the soluble chromium ions demonstrated that Cr(VI) was first reduced to Cr(III), which then appeared to be adsorbed by the biofilm. Chromium levels associated with the biofilm were found by chemical analysis to constitute 6.5 to 7.0% of its dry weight (46). Deans and Dixon (47) reported the biotechnological use of the uptake of Pb^{2+} and Cu^{2+} by EPS. The mechanisms of active and passive metal immobilization have been extensively reviewed (see Ref. 3).

The external environment and internal metabolism of living bacteria often exert a profound influence on the chemistry of bound metals. These influences include changes in oxidation state, formation of organometallic compounds, and formation of precipitates owing to detoxification or energy-yielding mechanisms of the bacterial protoplast. Alternatively, the metals may be affected indirectly by the production of metabolic end-products such as SO_4^{2-} and S^{2-} or an alteration in the local pH and/or E_h. The results of microbial activity may ultimately lead to metal immobilization, remobilization, and/or the formation of metal aggregates (27).

Microbial Precipitation. This results when living bacteria produce and excrete a substance that chemically reacts with metals present in solution to produce an insoluble metal compound. Next to carbonate deposition, the production of hydrogen sulfide by sulfate-reducing bacteria (SRB) and the subsequent formation of insoluble metal sulfides represent an important example. SRB, of which the most prevalent genera are *Desulfovibrio* and *Desulfotomaculum*, live in anaerobic environments such as lake sediments, swamps, and anoxic soils, but also in aerobic systems that contain biofilms above 50 to 200 μm of thickness. In biofilms of this thickness, the base region is mostly anaerobic and provides a habitat for anaerobic microorganisms such as SRB. Recent investigations, however, combining microelectrode measurements and scanning laser confocal microscopy reveal that there may exist anaerobic clusters of microconsortia rather than completely anaerobic layers, whereas channels penetrate the biofilms, implying that localized aerobic conditions may still prevail (48). The SRB oxidize organic matter and use sulfate as electron acceptor, reducing it to sulfide:

$$SO_4^{2-} + 10(H) \longrightarrow H_2S + 4H_2O$$

The hydrogen sulfide reacts with metals to form water-insoluble metal sulfide compounds. Precipitation of metals by the activity of SRB has been documented to occur in the natural environment leading to the removal of metals from streams and lakes (49). This naturally occurring activity has been reproduced in purpose-built impoundments to remove soluble metals emanating from mining operations (50). From calculations based on primary productivity and sulfate reduction rates in microbial mat communities, it has been estimated that sufficient sulfide could be produced in order for ore-grade iron and heavy metal sulfides to form (51).

Metals can also be precipitated from solution by HPO_4^{2-} derived from a phosphatase located on the bacterial cell surface. The phosphatase enzyme cleaves glycerol-2-phosphate liberating HPO_4^{2-}. Macaskie and Dean (52), working with *Citrobacter* sp., have demonstrated the cell-bound precipitation of Cd^{2+}, Pb^{2+}, and U^{4+} ions by HPO_4^{2-}. *Citrobacter* sp., immobilized as a biofilm on reticulated foam, was found to be effective in removing 90% of the uranium (1 mM uranyl acetate, pH 6.9) provided at a flow rate of 1.25 mL min^{-1}. After passage of 40 L of uranyl nitrate, the amount of uranium precipitated by the phosphatase activity was 9 g uranium per gram bacterial dry weight. Polyacrylamide gel-immobilized cells were comparably effective in removing uranium.

Bacterial calcification is another example for the precipitation of inorganic material by biofilms. The formation of calcium carbonate particles in microbial mats and other aggregates was suggested to be, at least in part, because of heterotrophic bacteria (53). On a global scale, bacterially induced precipitates of calcium carbonate can be the dominant component in thick sequences of traverine deposited from hot spring waters (54); thus, precipitation represents a biogeochemical mechanism of a geological dimension. From laboratory experiments, van Knorre and Krumbein (55) concluded that heterotrophic bacteria do not precipitate carbonate particles by a specific mechanism. An increase in carbonate alkalinity was sufficient to explain calcium carbonate precipitation. Increased carbonate alkalinity, if induced by bacteria, is usually a direct effect of physiological activities. Bacterial calcification is apparently only a side effect of bacterial metabolic activity and also largely depends on abiotic conditions; but, without bacteria, the formation of carbonate particles from precipitated calcium carbonate would not occur in some environments. Bacteria and other microorganisms influence the abiotic parameters of their environment considerably.

Oxidation and Reduction. Although oxidation and reduction of metals require the function of living organisms in most cases, some metal ion reductions can occur passively via chemically reactive sites on the cell walls of microorganisms. Depending on the metal species, both oxidation and reduction result in metal immobilization. Microgradients of oxygen and carbon dioxide produced by respiration play an important role in the oxidation and reduction potential within biofilms. The oxidation of manganese is performed by a limited number of bacterial species (see detailed review in Refs. 45,56) and mature spores, such as those of marine *Bacillus* species. The oxidation by bacterial cells and *Bacillus* spores results in the formation of insoluble manganese oxide:

$$Mn^{2+} + \tfrac{1}{2}O_2 + H_2O \longrightarrow MnO_2 + 2H^+$$

Microbially mediated oxidation of Mn^{2+} has been demonstrated to actively occur on a geological scale. Even under arctic conditions, rates were found to be sufficient to oxidize all of the Mn^{2+} in a lake under study (57).

Under anaerobic conditions, an acidothermophilic archaebacterium of the genus *Sulfolobolus* has been demonstrated to reduce Mo^{6+} to Mo^{5+}. This metal-ion reduction requires actively metabolizing bacteria because the reaction appears to be coupled to the oxidation of S° to SO_4^{2-} (50).

Methylation and Demethylation. Methylation and demethylation reactions are mechanisms that living bacteria can use to transform metals in their environment (58). Methylation reactions of tin, for example, are environmentally significant because methylation increases the volatility and the toxicity of tin, as well as altering its mobility in the environment. The same is true for mercury (59). Butylmethyltins have been reported in the sediments of several harbors. As antifouling paints from ship hulls were the principle source of tin in those harbors, it was assumed that butyltins could be partially degraded and then methylated in situ. Although 95% of the tributyltin in the water column is associated with particles, environmental concentrations are about three orders of magnitude greater in the microlayer of sediments than in the water column (60). Brinckman and coworkers (61) suggested that adsorption and concentration of tin compounds and microorganisms in surface slicks and on suspended particles could provide sites for biomethylation in aquatic systems. Dimethyltin and trimethyltin were detected by GC–MS when sediments of Chesapeake Bay were incubated with $SnCl_4$. Methylated tins were not detected in poisoned or in sterile controls (60). The production of monomethyltin was correlated positively with the number of sulfate-reducers and of sulfur oxidizers. *Desulfovibrio* spp. isolated from the sediments were able to carry out the methylation (62). Potential mechanisms for methylation were reviewed by Thayer and Brinckman (63).

A practical example may illustrate the importance of the sorption potential of biofilms, as it is exploited for the identification of emitters of pollutants into sewage lining systems. For this purpose, the metal content of the biofilms on the surfaces of the sewers is analyzed systematically upstream (64). As soon as the concentration of heavy metals decreases, the site of emission has been intersected and crossed. Thus, the emitter must be between the sampling points with the last high and the first low metal concentration. The "memory effect" of this biofilm is based on the accumulation of heavy metals from the wastewater stream. The system, based directly on the sorption properties of biofilms, has improved the effectiveness of the monitoring authorities and reputedly helped to maintain lower pollutant concentrations in sewage sludge (65).

Organic Substances

The sorption of organic solutes to biofilms is a well-known phenomenon. Biofilms sequester them as nutrients from the water stream and, thus, manage to maintain a complex habitat (2). The sorption is a prerequisite for biodegradation and as such, is part of any technology using biofilms for water purification. Only the less biodegradable substances accumulate. This is true for some pesticides and aromatic chlorinated hydrocarbons that occur in high concentrations in digested sewage sludge. The unwanted sorption of organic pollutants by biofilms is vastly documented, especially in sewage treatment plants (see Ref. 3).

The potential for enhanced mobility of hydrophobic pollutants by cotransport with bacteria in saturated soils was evaluated from measurements of biosorption of ^{14}C-labeled hexachlorobenzene and dichlorodiphenyltrichlorethane (DDT) to five strains of soil and sewage bacteria (66). The sorption process could be described by a linear partition equation and appeared to be reversible, but desorption kinetics were slow and/or partially irreversible. The DDT partition coefficients varied with equilibrium time, possibly reflecting DDT-induced changes in the physiology of the bacteria. The partition coefficients, normalized in the masses of bacteria, ranged from 250 to 14,000 for live cells, but the largest coefficients were associated with autoclaved cells of a *Pseudomonas* sp. This is an example of passive sorption. In a further experiment on the sorption capacity of the bacterial biomass, the sorption capacity was greater for DDT than for hexachlorobenzene, but was not correlated to overall bacterial hydrophobicity, measured by hydrophobic interaction chromatography. The 1.2×10^9 cells of a *Bacillus* sp. strain per milliliter enhanced DDT transport by about eightfold, whereas an advective–dispersive–sorptive equilibrium model for two mobile phases, waste and free-living bacteria, suggested a 14-fold enhancement, based on the DDT partition coefficient. Model calculations based on literature data covering a wide range of organisms and compounds suggested that 10^6 cells/mL would increase the mobility of very hydrophobic compounds (octanol-water partition coefficient $K_{ow} > 6$) whereas higher densities of bacteria (10^8 cells/mL) would have a significant impact on compounds with a $\log K_{ow} > 4$.

Investigations on the uptake of nutrients support considerations on the sorption properties of biofilms. Stream sediment with a natural biofilm generally adsorbed more glycine, aspartic acid, and lysine than sterile sediments (67). The adsorption of glycine, aspartic acid and lysine to glass-beads was measured at three pH values. The positively charged lysine molecules were generally adsorbed more readily than the others. Beads coated with sterile leaf leachate as opposed to sterile glass beads adsorbed more lysine and aspartic acid, but no glycine. The same pattern was found when beads coated with leachate and colonized by bacteria were compared with beads coated with sterile leachate. Killing the bacteria on the beads with glutaraldehyde did not affect adsorption rates. Increasing Ca^{2+} concentration from 5 to 50 mg L^{-1} decreased adsorption of lysine and increased adsorption of aspartic acid. In natural habitats, low molecular weight substrates are more likely to be used as substrates for bacteria suspended in the water phase. Macromolecules, however, are more likely to be sorbed at surfaces and in biofilms (68).

Sorption data gained in sterile systems may differ profoundly from those gained in the presence of microorganisms and colonized surfaces. It has been found that pesticides such as chlortolurone are not sorbed on clean clay surfaces and not degraded by a suspended mixed soil microbiota. However, in the presence of clay, significant sorption was observed and the microbiota were able to degrade the pesticide, as indicated by the occurrence of metabolites (3). Similar effects of clay surfaces on microbial activity were reported earlier (69,70). Higher concentrations of montmorillonite (10 g L^{-1}), however, might inhibit bacterial growth, as results from Marshman and

Marshall (71) indicate. The effect of surfaces on bacterial activity has certainly to be taken into account. Thus, organic substances in water, which may have appeared to be nonbiodegradable in suspended biodegradation tests, may under certain instances be degraded in the presence of surface-rich material. The most likely explanation for this effect is that the cells settle on the surfaces and then exhibit different physiological properties (72). This occurrence has been demonstrated by the experiments of Dagostino and coworkers (73).

As explained earlier, the EPS can contain significant amounts of proteins. These may well provide apolar sections that interact and sorb apolar organic molecules. However, the polysaccharide moiety, although hydrophilic by nature, must interact with hydrophobic molecules as well. This consideration is supported by the colonization of hydrophobic surfaces by means of the EPS of hydrophilic bacteria and by the performance of biofilm reactors that degrade hydrophobic organics. It is known that many EPS have surface-active properties (74) which must play a central role in such processes. It is especially the lipid moiety of the cell walls, such as the outer and inner membrane, which are predestined as sorption sites for hydrophobic pollutants. However, the sorption capacity of these must be considered as very limited. The amount of a hydrophobic compound that can be maximally bound to the lipid membranes of biofilm cells can be estimated from bioconcentration and K_{ow} factors. Especially for some pesticides and PCB, bioaccumulation in microorganisms in activated sludge has been reported. Accumulation rates are similar for dead and living cells, indicating a sorption process independent of physiological activity.

Bacterial sorption was characterized by the partition coefficient between biomass and medium (75); however, it has to be pointed out that many other authors could not confirm the correlation between K_{ow} and K_B and vast differences in the bioaccumulation by different bacteria have been observed in the meantime. Furthermore, such investigations do not differentiate between different sorption sites. Detailed research on the sorption site for benzene, toluene, and xylene (BTX) as examples for hydrophobic substances in an activated sludge system revealed that most of the BTX was accumulated in the EPS, whereas only a minor part was associated with the cells and their membranes (Fig. 6). This result was surprising because of the hydrophilic nature of the EPS matrix. However, obviously a substantial amount of hydrophobic substances (up to 8% w/w dry weight) such as pentachlorophenol can be accumulated in biofilms (38).

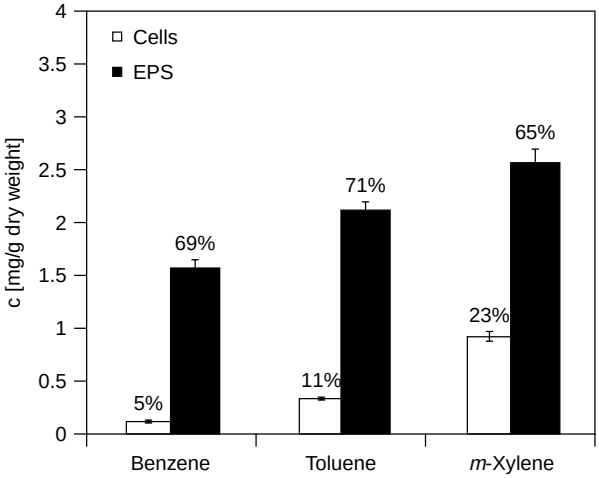

Figure 6. Partition of benzene, toluene, and xylene (BTX) between EPS and cells in activated sludge. (After R. Späth, H.-C. Flemming, and S. Wuertz, *Water Sci. Technol.* **37**, 207–210 (1998)).

Colloids

Colloids are solid or semi-solid particles of approximately 10 nm to 1 μm in diameter (76). They are ubiquitous and numerous components of natural soil and water environments (77) are present, which inevitably contact and permeate biofilms. The role of colloidal particles in natural environments has long been acknowledged as paramount to biogeochemical cycles (78). The importance of colloids in microbial ecology lies in their large sorption capacities, resulting from a collectively large surface area, and their ability to be transported through much of the biofilm matrix via pores and channels, thus acting as transport vectors for nutrients and contaminants. Some colloidal particles act as nucleation sites for the formation of particulate aggregates, which constitute the suspended "snow" in rivers, lakes, and oceans. In natural environments, colloids are represented by inorganic and organic particles of iron and aluminum oxides, and hydrophobic aggregates of macromolecules such as globular proteins and humic substances. Smaller bacteria and other microorganisms fall within the size range of colloidal particles and are thus regarded as living colloids biocolloily if they have no means of self-motility. Colloids and organic macromolecules including lignin and humic substances are responsible for binding and transporting organic and inorganic matter, including contaminants (79). Iron, manganese, and aluminum oxides, for example, strongly bind transition metals (80). As much of the dissolved organic matter possesses an affinity for solid surfaces (81), most of the suspended colloidal particles are associations of minerals and natural organic matter, such as organic acids. The behavior of colloids is strongly influenced by pH and electrostatic charge, and these properties influence the binding of other matter or flocculation. Therefore, colloidal particles, which become deposited on or within biofilms, have already sorbed significant quantities of organic and inorganic matter, and potentially constitute one of the major nutrient sources for biofilm organisms. Figure 7 shows two examples of particulate matter localized within biofilms.

The mechanisms for particle transport into biofilms were reviewed by Bouwer (82). This phenomenon can be most readily visualized using fluorescent microbeads and assessing their locations throughout the biofilm matrix using confocal laser microscopy or a combination of cryo-sectioning and epifluorescence microscopy (83). Small, chemically functionalized microspheres allow an assessment of interstitial binding sites, analogous to the labeling of cell surfaces with colloidal gold or ionic probes. Particles with a diameter of 10 μm can traverse thick biofilms by advection and convection owing to the pore and channel biofilm morphology (83). The deposition of Fe(III)

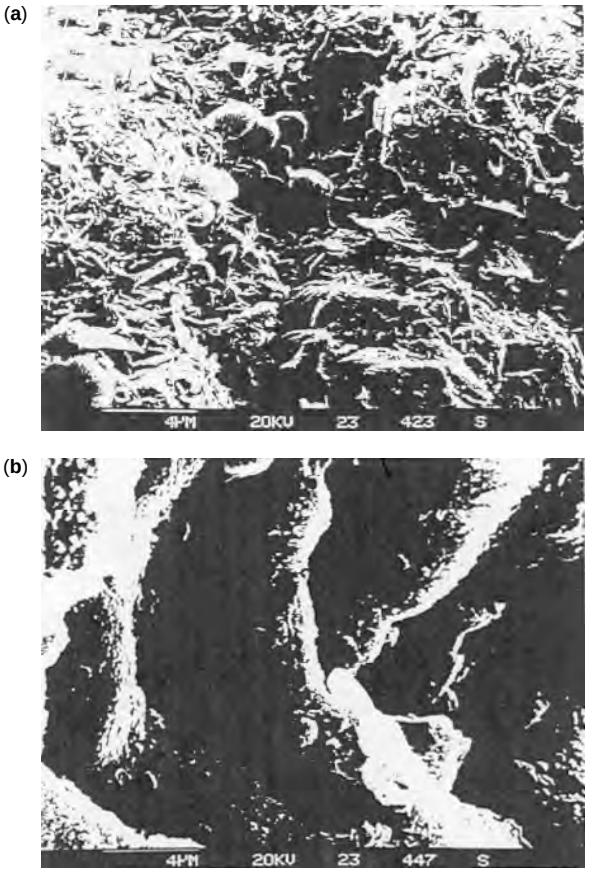

Figure 7. Particulate matter in microbial biofilms: (**a**) mixed consortium and (**b**) fungal hyphae with adsorbed particulates.

colloids within a biofilm of *Burkholderia cepacia* was shown to be only slightly higher than control surfaces (80); however, incorporation of clay particles by a *Pseudomonas fluorescens* biofilm was found to have a substantial impact on a number of parameters, including mass transfer and activity (84). Biofilms formed in the presence of kaolin particles had greater quantities of biomass, and a greater capacity to withstand substrate limitation. Another study showed the enhanced nitrification capacity of activated sludge biofilms in the presence of powdered clay (85). The inputs of biodegradable matter from colloids and their own sorbed components relative to other nutrient sources (cell turnover, macromolecular adsorption) is unknown, but in some systems, this may be a dominant mechanism sustaining the productivity of biofilm organisms.

REMOBILIZATION

From the preceding examples, it is clear that bacterial surfaces and biofilms are not inert chemical structures. They represent a dynamic system in which the various components are synthesized, assembled, modified, and finally broken down by autolysins and sloughed off into the environment. Thus, they may contribute to the remobilization of the sorbed substances. McLean and Beveridge (27) pointed out: "When the effects of pH, E_h, interfacial nature, and chemical attributes of the metal (heat of hydration, charge density, electronic shell, etc.) and the presence of competing ions and other biological surfaces are also taken into account, we can begin to grasp the true complexity of bacterial metal binding." Obviously, some or all of these parameters are applicable to all sorbed substances in natural environments, but the ability to accurately measure metal species provides one of the best examples to enable an assessment of remobilization events. By nature, the immobilization of metal ions in biomass cannot be irreversible. The biological binding sites sooner or later will be degraded. The fate and transport of the metal is directly related to the fate and transport of the bacterial cell. In some instances, this process will lead to mineral formation (86) and is responsible for the deposition of large ores. However, in other cases the sorbed metal ions will return to their more soluble form and be remobilized.

Experiments with cell walls of *B. subtilis* and *E. coli* envelopes adsorbed to kaolinite and smectite clays and with the corresponding organic material–clay aggregates showed the complexity of remobilization processes (87). Bound to these substances were Ag(I), Cu(II), and Cr(III). The sorbed metals were then leached with HNO_3, $Ca(NO_3)_2$, EDTA, fulvic acids, and lysozyme at several concentrations. The findings on remobilization of the sorbed metals, in general showed the order $Cr^{2+} < Ag^{+} < Cu^{2+}$. In the cell wall, clay, and composite systems, Cr^{3+} was very stable; at pH 3, 500 mm EDTA, 120 ppm fulvic acid, and 160 ppm Ca^{2+} released less than 32% (wet weight) of the sorbed chromium. Silver (45 to 87%) and copper (up to 100%) were readily removed by these agents. The organic chelators were in general less effective at mobilizing certain metals than elevated Ca^{2+} or low (acidic) pH values. Lysozyme digestion of *Bacillus* walls remobilized Cu^{2+} from walls and Cu–wall–kaolinite composites. Ag^{+} and Cr^{3+} smectite inhibited enzyme activity to some extent, and the metals remained insoluble. The concentration dependency of the leaching agents was not always given; for example, the Ag^{+} mobility decreased with some clays and composites treated with high fulvic acid, EDTA, and lysozyme concentrations. It was assumed that the particle size of the deposited metal may account for some stability changes. The large and compact aggregates of Cr^{3+} and Ag^{+} as seen in TEM were less likely to be remobilized. Interesting is the comparison of the stability of metal complexes of low molecular weight complexing agents with the stability of metal—EPS complexes. The EDTA complexes have stability constants of up to 10^{18}, and the complex of Cu^{2+} with 1-hydroxyethane-1, 1-diphosphonic acid (HEDP) is about 10^{19} (88). If these complexing agents occur in the water phase, it should be expected that eventually sorbed metal ions are rapidly complexed. However, the remobilization in this case is unexpectedly low. Gutekunst (64) carried out elution experiments with EDTA and HEDP, which showed only marginal remobilization of biofilm-bound metal ions. These observations indicate that separate mechanisms in addition to complex formation must be effective in metal binding by biofilms. Considering organic pollutants bound in lipid membranes, a rapid release after death of a cell is probable as the phospholipids belong to the first cell components to degrade when a cell dies (89).

CONCLUSION

In the complex system of sorbing species in soils and sediments, biofilms are a part of the "organic matter." In this lumped parameter, the biofilm organisms and their polymer matrix represent a dynamic sink and source for sorbed substances. The biofilm forms a gel phase between the solid mineral surface and the liquid phase. Sorption and desorption occur passively and actively. Both biotic and abiotic processes can be involved in the sorption process. The sorption capacity of biofilms has not yet been assessed. A prerequisite will be that biomass can be discriminated from other soil or sediment components, in particular humic acids. It is possible that the overall sorption capacity of biofilms is not impressively high compared to abiotic sorbents; however, as the biofilm can influence all parameters involved in the sorption process such as pH, E_h, oxygen content, and ionic strength, abiotic sorption will be influenced by biofilm processes as well. This is the indirect effect of the physiological activity of biofilm organisms, and this effect depends not on the amount of biomass but rather on its activity at specific sites. This aspect will have to be addressed in further research. Although it is possible that the sorption capacity of biofilms does not exceed that of abiotic organic components such as humic acids, the dynamic nature of biofilms gives them a particular role in the sorption process. This is why they have to be considered in the assessment of the distribution of pollutants in surface and groundwater, if models of the distribution of pollutants in the aquatic or soil environment are to be realistic. The existing data on adsorption must be questioned if these are obtained under microbiologically undefined conditions. This review reveals a substantial research need in many aspects. Usually, biomass is still taken as a black box; and the specific binding sites for different sorbents are evaluated only in very few cases. The binding mechanisms for lipophilic molecules by EPS in particular are not well understood. The overall binding capacity of biofilms remains unclear as well as the remobilization potential.

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SORTING OF MICROBIAL CELLS.

See METHODS FOR FLOW CYTOMETRY AND CELL SORTING

SOURCE WATER PROTECTION: MICROBIOLOGY OF SOURCE WATER

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The control of waterborne diseases depends on a multiple barrier approach: source water protection, adequate and

reliable water treatment, measures to prevent a breach in the integrity of the oftentimes vast network of pipes known as the distribution system that carry the treated water to the customer, and the use of a disinfectant such as chlorine to control any waterborne pathogens that do manage to enter the distribution system. A breach in any one of these barriers undermines consumer protection.

Source waters, as used in this overview, are defined as those surface waters and ground waters used by drinking water systems. Some of these source waters may also be used for swimming, bathing, and other recreational activities, and waterborne disease associated with such activities occurs with some frequency.

The overview begins with a survey of the sources of disease-causing organisms (pathogens) that might contaminate water, the factors that affect whether the pathogens reach a water body (surface or ground), and the environmental factors that affect waterborne pathogen survival in the water. This is followed by a short review of commonly used indicators of source water quality. A brief summary of governmental requirements that protect source waters closes the overview.

PATHOGEN SOURCES

Human pathogens in source waters may come from point sources, non-point sources, and recreational water activities, or they may be indigenous to the source water. Point sources are those in which the source can be clearly identified, such as wastewater treatment plants, wastewater and sludge disposal sites on land, septic tanks, and sewage spills. Usually, the greatest concentration of waterborne fecal pathogens infective for humans originate from point sources (1). Point sources may include wastes of human origin as well as livestock origin. The United States has about 45,000 animal feedlot operations (2), and fecal waste from these operations is often stored in waste ponds or as solid manure piles. Heavy rains can cause pond overflow and manure pile dissolution, resulting in severe contamination of water sources (3). Such animal waste may also contain human pathogens of significant public health importance, including *Cryptosporidium parvum*, *Salmonella*, and *Escherichia coli* O157:H7. Additionally, use of antibiotics in livestock and the resultant release of antibiotic-resistant pathogens into the environment, as well as zoonotic transfer of resistant strains, are also of concern (4).

Nonpoint sources are those in which the source of contamination is not clearly defined or is unknown. Thus, control of these sources is much more difficult to achieve. Examples of nonpoint sources associated with human pathogens include urban runoff, water recreation activities, and wildlife. Urban runoff and stormwaters are transported to source waters either directly via storm drains or, in older cities, to wastewater treatment plants (combined sewers), which during a heavy rainfall may exceed their capacity and cause the release of untreated sewage into the source water. The pathogens in urban runoff can originate from soil, street runoff, animal wastes, and other sources. The fecal bacteria density is much higher in sewered than nonsewered areas and is directly related to housing density, urban development, and domestic animal density (5).

Table 1. Waterborne Disease Associated with Recreational Water in U.S.: 1986–1998[a,b]

Disease	Outbreaks	Cases
AGI[c]	23	2,111
Shigellosis	19	1,716
Amoebic meningoencephalitis	18	18
E. coli O157 gastroenteritis	9	293
Dermatitis c/w[d] schistosoma	7	203
Giardiasis	4	65
Norwalk-like gastroenteritis	3	89
Leptospirosis	3	389
Cryptosporidiosis	3	429
Aseptic meningitis	1	4
Pseudomonas aeruginosa dermatitis	1	50
Pharyngitis (adenovirus 3)	1	595

[a]Includes water types that might be used as sources for drinking water, such as lakes, streams, creeks, ponds, and canals. Excludes swimming pools, hottubs, hot springs, puddles, water slides, and fountains.
[b]Data are adapted from Refs. 6–11.
[c]AGI is acute gastroenteritis of unknown etiology.
[d]c/w = consistent with.

Water recreation activities such as swimming, bathing, and boating may be a major nonpoint source of pathogens in ambient waters. A number of outbreaks associated with recreational waters have been attributed to fecal discharges during such activities (Table 1). Wildlife sources may also carry human pathogens such as *Cryptosporidium parvum*, *Giardia lamblia*, and *Salmonella* species. Animals often defecate near water sources and thus their fecal material is easily washed into these waters, especially during a rainfall. Table 2 illustrates the variety of animals that are infected by pathogens that also infect humans. Other animals may carry human pathogens, but are not infected by them. For example, *Cryptosporidium parvum* is transported in aquatic birds, but does not infect and grow in the birds (34).

Some pathogens are normal inhabitants of ambient waters and soil. Most are opportunistic bacterial pathogens such as *Pseudomonas aeruginosa*, *Mycobacterium avium intracellulare*, *Legionella pneumophila*, and several strains of aeromonads, flavobacteria, and *Klebsiella*. (Opportunistic pathogens generally only infect those individuals who have a weakened immune system or who are particularly sensitive for other reasons.) In addition to the opportunistic pathogens, at least two protozoan species indigenous to water, *Naegleria fowleri* and *Acanthamoeba* spp., can cause illness in healthy individuals. Several species of cyanobacteria (blue-green algae) are also free-living pathogens. Wastewater and agricultural runoff, even in the absence of fecal pathogens, increase the nitrogen and phosphorous levels in a water source, which may increase the levels of these free-living pathogens.

FACTORS AFFECTING THE TRANSPORT OF FECAL PATHOGENS TO SURFACE WATER

Some sources discharge fecal pathogens directly into surface water. Among these sources are many sewage

Table 2. Natural Animal Reservoirs for Waterborne Disease Pathogens of Humans

Organism	Major Disease	Animal
Bacteria		
Aeromonas hydrophila	Gastroenteritis	None (free-living)
Campylobacter jejuni	Gastroenteritis	Poultry, pigs, cattle, sheep, dogs, cats, and many others (12,13)
Escherichia coli O157:H7	Hemorrhagic colitis, hemolytic-uremic syndrome	Cattle and other ruminants (12,14)
Other pathogenic *E. coli*	Gastroenteritis	Cattle and pigs (uncommon) (15–17)
Helicobacter pylori	Peptic ulcers, stomach cancer	None known (18)
Legionella pneumophila	Legionnaires Disease, Pontiac fever	None (free-living)
Mycobacterium avian intracellulare	Pulmonary disease, gastroenteritis	None (free-living) (19)
Pseudomonas aeruginosa	Dermatitis	None (free-living)
Salmonella typhi	Typhoid fever	None known (12)
Salmonella paratyphi	Paratyphoid fever	Rarely domestic animals (12)
Other *Salmonella* spp.	Gastroenteritis (salmonellosis)	Poultry, pigs, cattle, rodents, reptiles, and many other domestic and wild animals (12,20)
Shigella spp.	Bacillary dysentery	Nonhuman primates (12)
Vibrio cholerae	Cholera	None (free-living in brackish and estuary waters)
Yersinia enterocolitica	Gastroenteritis	Primarily pigs, rarely other domestic and wild animals (12,21,22)
Enteric viruses (human strains)		
Norwalk and other caliciviruses	Gastroenteritis	Humans only (12)
Rotaviruses	Gastroenteritis	Humans only (12)
Enteric adenoviruses	Gastroenteritis	Humans only (12)
Astroviruses	Gastroenteritis	Humans only (23)
Polioviruses	Polio	Humans only (23)
Coxsackieviruses	Upper respiratory disease	Humans only (23)
Echoviruses	Upper respiratory disease	Humans only (24)
Hepatitis A virus	Infectious hepatitis	Nonhuman primates rarely (12)
Hepatitis E virus	Hepatitis	Unknown, but evidence exists for pigs, rats, and other animals (12,24,25)
Protozoa		
Acanthamoeba spp.	Eye infection	None (free-living)
Cyclospora cayetanesis	Gastroenteritis	None known (26,27)
Cryptosporidium parvum	Cryptosporidiosis (gastroenteritis)	Many mammals, especially calves (28)
Entamoeba histolytica	Amoebic dysentery	Nonhuman primates (29)
Giardia lamblia	Giardiasis (gastroenteritis)	Muskrats, beavers, small rodents, and many other domestic and wild animals (30)
Enterocytozoon bieneusi	Gastroenteritis	Nonhuman primates, pigs, several other mammals (31)
Encephalitozoon intestinalis (a microsporidium)	Gastroenteritis	Pigs, cattle, goats, dogs, donkeys (32)
Naegleria fowleri	Primary amebic meningoencephalitis	None (free-living)
Toxoplasma gondii	Flu-like symptoms	Cats (33)
Blue-green algae (Cyanobacteria)	Gastroenteritis, liver damage, nerve damage	None (free-living)

Note: spp. means species.

treatment facilities and water recreational activities. Sewage treatment facilities provide secondary treatment that usually includes disinfection to kill pathogens before discharging the effluent into the receiving water, but some level of pathogens survive. Also, water supply plants need to clean their filters occasionally by reversing the flow of water. This filter backwash water may contain high levels of pathogens, including *Cryptosporidium* and *Giardia*, that contaminate the source water.

For pathogens not directly discharged into surface water, the likelihood of a fecal pathogen reaching a surface water body from its point of origin depends on the climate, watershed terrain, soil properties, filtering capacity of the vegetation cover, nature of the pathogen, and proximity of the fecal source to the water body. Pathogens may even reach surface water via groundwater discharge if the aquifer is unconfined and highly transmissible (35,36).

Stormwater is the most obvious vehicle for transporting fecal pathogens from animal feedlots and other fecal sources in the watershed that are not deliberately discharged directly into the water. Major storms can result in rapid movement of pathogens over the watershed and into receiving streams and lakes. Depending on soil characteristics, fecal pathogens may move into the

subsurface and contaminate groundwater. Air and water temperature play a role in pathogen survival; colder temperatures increase fecal pathogen survival.

Generally, fecal pathogens in the environment are bound. They are adsorbed to organic or inorganic particles or embedded in fecal matter, and are often aggregated with other microbes. This association usually greatly enhances the survival of fecal pathogens in the environment. *Salmonella*, for example, can be excreted by cattle in levels up to 10^7 per gram of feces (37) and can survive for between 8 (poultry slurry) (38) and 286 days (cattle slurry) (39). (Slurry is a watery mixture of animal feces, urine, and small amounts of bedding.) *E. coli* O157:H7 can survive in sheep manure in the field for 21 months, for 47 days in cattle manure (40) and for eight days in cattle slurry (41) at room temperature. At lower temperatures (4 °C), this organism can survive for 38 days in cattle slurry, whereas *Salmonella typhimurium* can survive for 66 days under the same conditions (41). Viruses such as picornaviruses, rotaviruses, parvoviruses, adenoviruses, and herpesviruses have been found to survive for many weeks (greater than six months for rotaviruses) in manure under nonaerated, dry conditions (42).

ENVIRONMENTAL FACTORS IN PATHOGEN SURVIVAL IN AMBIENT WATERS

The survival of human fecal pathogens in a water body is affected by a number of physical, chemical, and physiological factors. The more important ones are indicated next.

Water Temperature

Generally, fecal pathogens can survive longer in colder waters because metabolic processes slow down (43). Some may also survive longer in very warm waters with a high organic load. In tropical and subtropical regions, the higher temperatures and organic loading of water is more similar to that of the gut of humans and other warm-blooded animals. In these water, *E. coli* can survive and even grow (see section on Indicators of source water quality). The density of *Salmonella typhimurium* declined by 90% in 28.8 hours in a temperate source water as against 131 hours in a tropical source water (44). Other enteric bacterial pathogens may show a similar temperature effect. Nonfecal pathogens indigenous to water (e.g., *Pseudomonas aeruginosa*, *Legionella pneumophila*) also increase in density with warmer waters. In contrast, the survival of enteroviruses, *Cryptosporidium* oocysts, and *Giardia* cysts decreases with increasing water temperatures (45,46,47).

Competition

The enteric bacterial pathogens discharged into ambient water face stiff competition for nutrients by indigenous aquatic bacteria. Moreover, the aquatic bacteria may produce substances that inhibit the enteric pathogens (43,48). Thus, the aquatic bacteria place the enteric pathogens at a selective disadvantage and reduce their survival time.

Protozoan grazing is a major factor in the decrease of enteric bacteria in natural water (49,50). However, a few waterborne pathogens such as *Legionella pneumophila*, *Mycobacterium avium*, *Vibrio cholerae*, and *E. coli* O157:H7 survive and even grow within certain common amoeba (51–55). A few other bacteria may form filaments as a defense strategy against grazing by protozoa (56), but it is not known whether enteric pathogens have this capability. Bacteria can also be protected from predation by adsorption onto clay particles (57).

Available Nutrients

Opportunistic bacterial pathogens indigenous to water, especially *Pseudomonas aeruginosa*, quickly increase in density upon the introduction of organic matter into the water body. In contrast, the effect of organic load on enteric pathogens is variable. In one study, higher turbidity and suspended solids protected enteroviruses at 1 °C, but the reverse was found at 22 °C, possibly because the warmer temperature increased total bacterial growth (58). Survival of enteric viruses is greatly enhanced in sewage sludge disposal sites in marine waters (59). Enteric bacteria, as noted in the previous section, including pathogens, may survive longer in very warm waters with a high organic load. Higher levels of organic material (diluted sewage) increased the survival time of *Salmonella enteritidis* at 37 °C (60), but decreased survival at 20 °C (61). At cold temperatures, *Salmonella* survival increased with higher organic levels (62). Survival may be significantly enhanced if the enteric bacteria sink into the freshwater sediment, especially an organically rich one (63,64). Because enteric protozoa and viruses do not grow outside a host animal, changes in the nutrient level should not directly affect their numbers or survival in the water environment.

Sunlight

The deleterious effect of ultraviolet (UV) light on genetic material of microorganisms is well known (43). Published studies on the effect of sunlight, which includes UV light, have focused on the inactivation of microbial indicators (see section on Indicators of source water quality) rather than on waterborne pathogens (65,66). Waterborne pathogens shown to be inactivated by UV light include the hepatitis A virus (67), *E. coli* O157 (68), *Legionella* (69), *Cryptosporidium parvum* oocysts (70), cyanobacteria (71), and poliovirus (72). In waste stabilization ponds, it has been shown that sunlight, as well as high dissolved oxygen concentrations and high pH, is required for significant reductions of fecal coliform densities (73).

Means of Protection

Survival of fecal pathogens in ambient water is enhanced by adsorption onto organic matter. However, some pathogens have other means for protecting themselves in the water environment. *Cryptosporidium parvum*, *Giardia lamblia*, and other pathogenic protozoa can form resistant cysts, oocysts, or spores that protect them from environmental stresses. In addition, when nutrients are low, some bacteria can activate genes to become more

efficient scavengers and can acquire a spore-like state (74). It is not known whether any fecal pathogens can do this.

Some fecal bacterial pathogens enter a viable but nonculturable (VNC) state in water as a result of environmental stress. In this condition, the bacteria apparently remain viable but lose their ability to grow on either nonselective media or on culture media normally used for their recovery. There is a strong debate about cell viability in the VNC state. Some believe that these bacteria remain viable and infective but have become dormant as a survival measure against environmental stress (75). Others contend that these organisms are dead or are dying and that successful attempts to resuscitate them merely indicate that a few bacterial cells remained alive (76). Regardless, it is known that some pathogens in the VNC state retain their pathogenicity (77). A VNC state has been reported for most waterborne pathogens (Table 3).

PATHOGEN OCCURRENCE IN SURFACE WATERS

Few large surveys have been conducted on any waterborne pathogen in ambient water. In one study, the source waters for 72 water treatment plants in 15 States (United States) and two Canadian provinces were analyzed for *Giardia* and *Cryptosporidium*. *Giardia* and *Cryptosporidium* cysts or oocysts were detected in 53.9% and 60.2% of the 347 samples, respectively (average sample volume was 499 L, with a range of 87 L to 3,394 L). For the last 262 water samples, the geometric mean for *Giardia* was 2.0 cysts/L, whereas that for *Cryptosporidium* was 2.4 oocysts/L (91). A large percentage of the cysts or oocysts were considered dead because of the absence of observable internal structures.

In another survey, 29 surface source waters used by large water supply systems were tested for the presence of astroviruses, enteroviruses, and adenovirus types 40 and 41 (92). Testing was accomplished by an integrated cell culture-reverse transcription nested polymerase chain reaction procedure. Of the 29 samples concentrated from large water volumes (generally 10 L–100 L), astroviruses were detected in 51.7%, enteroviruses in 58.6%, and adenoviruses in 48.3%.

In a third survey, source water samples were collected on a monthly basis from 350 large surface water systems for 18 months and the samples were tested for *Cryptosporidium* and other organisms. Only 44% of the source waters were *Cryptosporidium*-positive in at least one of the 18 samples (median volume three liters). About 83% of the 350 systems had observed mean *Cryptosporidium* concentrations below 0.1 oocyst/L (93).

Table 3. Waterborne Pathogens and Fecal Indicators Known to Enter Viable but Nonculturable State

Organism	Medium	Reference
Campylobacter jejuni	Filter sterilized surface water	78
Shigella dysenteriae	Pond, river, drain, lake water	79
Salmonella enteriditis	Sterile river water	80
Salmonella typhimurium	Marine	81
Salmonella spp.	River	82
E. coli O157:H7	Sterile municipal water, lake, reservoir	83
Vibrio cholerae	Artificial seawater	84
Helicobacter pylori	Sterile river water, distilled water	85
Legionella pneumophila	Sterile tap water	86
Aeromonas hydrophila		87
Pseudomonas fluorescens	Soil	88
Enterococci	Freshwater, seawaters	89
Escherichia coli	Seawater, estuarine	90

GROUNDWATER

In the United States, 92% of the 168,000 water systems serving at least 25 people use groundwater, and more than 100 million people obtain most or all of their drinking water from these groundwater supplies (94). The majority of these systems (61%) serve fewer than 100 people. In addition, there are about 15 million private wells that serve fewer than 25 people. A well draws water from an aquifer, which is an underground water-bearing permeable material that contains significant amounts of water. An aquifer may be unconfined or confined. Confined aquifers possess an overlying boundary layer of low permeability rock or clay that acts as a barrier to prevent or slow the flow of water into, or out of, the underlying aquifer. As a result, pathogens from sources above the confining layer are prevented, or greatly impeded, from reaching the aquifer. Typically, groundwater percolates through confining layers very slowly; it may take over 100 years for the water to flow one meter.

In contrast, an unconfined aquifer does not have an overlying rock layer of low permeability and may thus be vulnerable to contamination from a fecal source at the surface or in the shallow subsurface (e.g., septic tank). Shallow wells in an unconfined aquifer are often characterized by significant and relatively rapid shifts in water characteristics such as turbidity, temperature, conductivity, or pH that correlate to climatological or surface water conditions. In addition, the pumping of shallow wells in an unconfined aquifer may pull in water from nearby lakes and other bodies of surface water. As a result, the well water may contain material normally associated with the surface, such as diatoms, insect parts, plant debris, pollen, crustaceans, or large-diameter pathogens such as *Giardia lamblia* and *Cryptosporidium parvum* that do not reproduce outside a mammalian host (95).

Wells may become fecally contaminated via infiltration from the surface or from underground sources of contamination. Surface sources include land application of wastewaters, seepage from waste lagoons, infiltration of polluted water from lakes and other surface water bodies, waste from livestock operations, and improperly constructed sanitary landfills (96). Contaminated surface water sources may also enter an improperly constructed, protected, or maintained well directly, either along the

casing or through cracks in the sanitary seal around the casing. Several groundwater systems have experienced a disease outbreak, including cryptosporidiosis, in this manner (97). Fecal contamination can also reach a drinking water well intake from underground sources such as septic systems, leach fields, cesspools, or leaking underground sewer lines. About 25 million septic tanks exist in the U.S. (98). The volume of septic tank waste released into the subsurface has been estimated at one trillion gallons per year (99).

Enteric pathogens, including viruses, are usually retained near the surface (or source in the case of a septic tank). However, a small fraction of the pathogens are carried along with the water flow through the surrounding soil and rock to collect within the water-saturated zone beneath the water table. Whether an infectious enteric pathogen reaches the area of a well intake depends on the rate of transport through the subsurface and how long the pathogen survives. Transport depends on a complex and site-specific interaction between the soil (and bedrock) characteristics, environmental factors, and the nature of the organism. The factors that affect the movement of enteric pathogens through the soil include the type of soil, pH, cation levels, amount of soluble organic matter, flow rate, degree of water saturation, and pathogen attributes (100).

As a pathogen moves downward and laterally with the water flow, it may become attached (adsorbed) to soil and rock particles. An adsorbed organism may detach (desorb), especially after a rainstorm, and readsorb to soil particles further along the flow path. Besides adsorption, larger microbes or microbial aggregates may be filtered out by tight pore spaces. Enteric pathogens may either slowly percolate through the soil and aquifer material or seep more rapidly through natural pathways in some types of bedrock. These pathways include (*1*) soil macropores, (*2*) large fractures in a fractured bedrock hydrogeology, (*3*) fractures in karst hydrogeology that enlarge through the dissolution of limestone, dolomite, or other soluble rock, and (*4*) large pore spaces characteristic of gravel bed hydrogeology. Karst land, which is often characterized by caves, caverns, and sinkholes, constitutes about 40% of the land in the United States east of Tulsa, Oklahoma (101). These natural pathways provide a direct and efficient link to and through the groundwater, and pathogens may reach the groundwater in as little as a few minutes or hours after release (102) with little loss in number from removal or inactivation.

Pathogen survival depends upon the characteristics of the soil, including moisture content, moisture-holding capacity, temperature, pH, and amount of organic matter (100). At the soil surface, sunlight reduces survival. The fate and transport of a particular species may be strain-specific. A factor in pathogen survival is the level and activity of the indigenous subsurface flora. Indigenous organisms, fully adapted to their environment, will normally outcompete the enteric pathogens for nutrients (103) and inactivate them with their toxins. The predominant group of soil bacteria is the gram-negative rods, especially the pseudomonads and flavobacteria. However, gram-positive rods and cocci are also common (104). Protozoa that graze on bacteria will thrive at fecally contaminated sites (104). Fungi and even algae are common in shallow groundwaters with sufficient oxygen (105), and may also compete with the enteric pathogens for nutrients. Because a pumped well draws in both aerobic and anaerobic waters, some strict anaerobes such as *Clostridium* and the methanogens may also be present in well water, although their effect on enteric pathogens in the water is not known.

With these inhibitions, it is unlikely that more than a small fraction of the original enteric pathogen densities would reach the well unless a rapid channel existed. Despite this, most documented waterborne disease outbreaks in the United States each year are associated with groundwater systems. Between 1971 and 1998, 59% of the 661 reported outbreaks, with more than 93,000 cases of illness, were associated with such systems (106,10,11). The actual incidence of waterborne disease is undoubtedly much higher than those documented.

INDICATORS OF SOURCE WATER QUALITY

A number of tools have been used to assess the quality of freshwaters, including source waters used by drinking water systems. Oxygen consumption is used in several ways to measure pollution in natural waters and wastewaters. Biochemical oxygen demand (BOD) is a measure of the biodegradable organics in water, chemical oxygen demand (COD) is the amount of an oxidant such as dichromate that reacts with the water sample, total oxygen demand (TOD) is a measure of the amount of oxygen required to completely oxidize all material in a sample, and dissolved oxygen (DO) is exactly that. The total organic carbon (TOC) and assimilable organic carbon (AOC) are also measures of pollutants in water, although the AOC is used primarily to assess the potential for biofilm growth in the pipes of the distribution system. Microbial types and diversity are also used to characterize water quality. For example, certain algae are associated with clean waters, other algae with polluted waters. Toxicity testing of freshwater using ciliates, mollusks, larvae, and many other organisms indigenous to the water is also a common procedure to assess water quality. Turbidity (opacity) is also used as an indicator of source water quality (107,108).

For groundwaters, the microscopic particulate analysis (MPA) is often used as an indicator of surface pollution. The MPA involves direct microscopic examination of material normally associated with the surface, such as diatoms, insect parts, plant debris, pollen, crustaceans, *Giardia lamblia* cysts and *Cryptosporidium parvum* oocysts (95).

To determine the potential public health risk associated with a source water, one could monitor pathogens directly. However, this is impractical because the variety of potential waterborne pathogens makes a search for all pathogens extremely difficult, time-consuming, and expensive. Moreover, a number of methodological problems exist, including the large sample volume often needed to recover a pathogen, the low efficiency of current techniques for recovering and detecting known waterborne pathogens in water, the lack of suitable culture media for

many pathogens, and the sparsity of laboratories with trained technicians needed to carry out the analyses.

Because of the problems with trying to detect specific enteric pathogens, indicators of fecal contamination are used instead of pathogens as indices of the potential health risk associated with source water. The presumption is that enteric pathogens, whose normal habitat is the gastrointestinal tract of warm-blooded animals, are always associated with fecal contamination. Thus, in theory, the absence of fecal contamination denotes the absence of enteric pathogens. An ideal indicator of fecal contamination in source waters (surface or ground waters) should possess the following characteristics.

- Always present in fecally contaminated water
- Always absent in water that is not fecally contaminated
- Present at a density related to the level of fecal contamination
- Present in polluted waters at much higher densities than fecal pathogens
- Suitable for all types of source water, both ground and surface
- Does not reproduce in water
- Rapid detection by simple, inexpensive, and reliable laboratory tests
- Stable and nonpathogenic

Extensive literature reviews of indicators in source waters have been published (103,109–112). The most widely used microbial indicators of fecal contamination are *E. coli*, fecal coliforms, fecal streptococci, and enterococci. These and several others used less frequently are discussed next. The densities of several indicators in human and animal feces are presented in Table 4.

Escherichia coli

Escherichia coli is common in the intestines of all warm-blooded animals, but does not normally survive for long in water. However, several studies have suggested that this bacterium can grow in waters in tropical and semitropical areas (120–123). *E. coli* is a member of both the fecal coliform group and the total coliform group (these two groups are defined next). Recent reports suggest that antibiotic resistance patterns and DNA fingerprinting may be useful in distinguishing among animal and human sources in fecally contaminated waters (124).

Fecal Coliforms

Fecal coliforms are members of the coliform group that can ferment lactose at 44°–45°C. The majority are *E. coli*, although some *Klebsiella* strains and more rarely *Enterobacter* strains are also fecal coliform-positive (123,125). One objection to the use of this group as a fecal indicator is that *Klebsiella* can proliferate outside a living host and are especially common in wastewaters from paper plants. However, clinical strains of *Klebsiella* are more likely to be fecal coliform-positive than environmental strains (85% vs. 16%) (126). Some fish, snakes, insects, and vegetation harbor fecal coliforms in low numbers (125,127). Fecal coliforms are a subset of the total coliform group.

Total Coliforms

Total coliforms are a group of related bacteria in the family *Enterobacteriaceae*. The group is not defined in precise

Table 4. Fecal Indicators: Density in Animal Feces (per *gram* × 1, 000)[a,b,c]

Animal	Fecal Coliforms	Fecal Streptococci	*C. perfringens*	*Bacteroides*	Male-specific Coliphage	Somatic Coliphage
Farm animals						
Cow	230	1,300	0.2	<0.001	0.084	400–22,000
Pig	3,300	84,000	4	500	4.137	3,400
Sheep	16,000	38,000	199	<0.001	0.0015	3,000
Horse	12.6	6,300	<0.001	<0.001	0.950	22
Duck	33,000	54,000	–	–	0.0131	–
Chicken	1,300	3,400	0.25	<0.001	1.867	11,000
Turkey	290	2,800	–	–	–	–
Pets						
Cat	7,900	27,000	25,100	795,000	–	–
Dog	23,000	980,000	251,000	500,000	0.0021	41
Wild animals						
Mouse	330	7,700	<0.001	795,000		–
Rabbit	0.020	47	<0.001	396,000		–
Chipmunk	148	6,000	–	–		–
Human[d]	13,000 (100%)	3,000 (100%)	1.6 (25–35%)	5,000,000 (100%?)	<0.01 (<10%)	<0.1–100 (23–77%)

[a] i.e., multiply the above values by 1,000.
[b] Adapted from Refs. 109–115.
[c] Considerable variation in the values have been reported among investigators in the literature.
[d] Values in parentheses in the "human" row are the percentages of healthy adults not on antibiotics that carry the specified fecal indicators in their gut (bacteria from Ref. 116, viruses from Refs. 115, 117–119).

taxonomic terms, but by whether they produce the enzyme β-galactosidase, which allows lactose metabolism. Total coliforms include most species of the genera *Enterobacter*, *Klebsiella*, *Citrobacter*, and *Escherichia*, as well as some species of *Serratia* and other genera. Total coliforms are not often used as a source water quality indicator because most species are natural and common inhabitants of the soil and ambient waters (e.g., lakes, rivers, and estuaries) as well as the gastrointestinal tract. However, they are useful as a drinking water indicator for assessing water treatment efficiency and for denoting possible breaches of distribution system integrity.

Fecal Streptococci/Enterococci

Fecal streptococci are members of the genus *Streptococcus* that are Lancefield Group D (one of several immunological groups based on the presence of specific carbohydrate antigens). Their normal habitat is the gastrointestinal tract of warm-blooded animals (107). The enterococci group (genus *Enterococcus*) is a subset of the fecal streptococci group that is able to grow in a medium containing 6.5% sodium chloride at pH 9.6 at both 10 °C and 45 °C (107). The two groups are almost identical, but the fecal streptococci group includes *S. bovis* and *S. equinus*, neither of which are enterococci (107). Preliminary evidence suggests that antibiotic resistance patterns in fecal streptococci can be used to determine the animal source of fecal contamination in water (128). Recent reports suggest that antibiotic resistance patterns, restriction enzyme analysis of chromosomal DNA, and species identification may be useful in distinguishing among animal and human sources in fecally contaminated waters (128–131). Data suggest that the fecal streptococci or enterococci groups do not proliferate to any extent in the environment, although they may be found occasionally on plants, probably as a result of insects (121,125,131).

Coliphage

Coliphage are viruses that infect *E. coli*, although a few may infect other bacteria (132). There are two major groups of coliphages: somatic and male-specific (also called F-specific). The somatic coliphages are a heterogenous group that enter the cell wall of *E. coli*. The male-specific coliphages are those that only enter tiny hair-like appendages (pili) found on some *E. coli*. Both groups have been found in ambient waters (133,134) and groundwaters (135). Subgrouping the male-specific RNA coliphages by serological and physicochemical properties helps distinguish among animal sources in fecally contaminated waters (136).

Clostridium perfringens

Clostridium perfringens is a bacterium that is consistently associated with human fecal wastes (137). The organism is a strict anaerobe that forms an endospore that is highly resistant to environmental stresses. Some question exists about the extent to which *C. perfringens* proliferates in the environment (123), perhaps because of its persistence in soil. The organism may be a more useful fecal indicator in raw surface waters (138–140) than in groundwater (141). Europeans use a somewhat broader indicator, the sulfite-reducing clostridia, the most common of which is *C. perfringens* (142).

NONMICROBIAL INDICATORS OF FECAL CONTAMINATION

Chemical substances such fecal sterols, caffeine, metals, and nitrate have also been proposed as fecal indicators. Fecal sterols have generated the most attention, and data suggest that it may be possible to differentiate human fecal contamination from that of herbivores by comparing the level of coprostanol, the principal human fecal sterol, with that of 24-ethylcoprostanol, which is most common in herbivores (143).

LAWS AND REGULATIONS PROTECTING DRINKING WATER SOURCES

The European Union requires secondary or equivalent treatment, before discharge, for urban wastewater that enters a collection system (EU document 391L0271). The requirement is less stringent for some wastewater facilities in high mountain regions where cold temperatures undermine biological treatment. The treatment standard is more stringent where wastewater is discharged into a sensitive water body, including those used by drinking water systems because a high nitrate concentration is of concern. To verify compliance, the wastewater facility must monitor BOD, and, for a sensitive water body, total phosphorus and/or total nitrogen in the wastewater discharge.

The World Health Organization (WHO) is currently preparing microbiology guidelines for the quality of recreational waters. Guideline values for coastal waters are to be based on the density of fecal streptococci (144). However, according to WHO (144), insufficient epidemiology information is available for deriving a separate guideline value for freshwater. Also, WHO has not established guideline values for wastewater discharge into water bodies.

In the Unites States, Congress has passed several environmental laws that affect discharges into ambient water or groundwater. The two key laws, with respect to the protection of drinking water sources, are the Safe Drinking Water Act (42 U.S.C 300f to 330j-26) and the Clean Water Act (33 U.S.C. 1251 to 1387). These two laws require the United States Environmental Protection Agency (EPA) to develop and implement regulations and programs that, among other provisions, protect waters used by drinking water systems. These regulations and programs are briefly described next.

Safe Drinking Water Act (SDWA)

The objective of the SDWA is to protect drinking water supplies from biological, chemical, and physical contamination. One SDWA provision (Section 1453) requires each State to develop and implement an EPA-approved source

water assessment program (SWAP) to determine the susceptibility of each public water system to potential threats of contamination from the source water. A public water system is one that regularly serves at least 25 people at least 60 days per year or has at least 15 service connections.

Another SDWA provision (Part C) requires EPA to regulate underground injection practices that may endanger drinking water sources. These practices involve the disposal of fluids into injection wells. Class V wells are the only type that pose a potential public health threat to groundwater systems and include large-capacity septic tanks, leach fields and cesspools associated solely with sanitary wastewater disposal. Currently, EPA regulations on Class V wells ban the use of large-capacity cesspools (used by 20 or more people) in which the waste has not received primary treatment.

As required by a third SDWA provision (Section 1412), EPA has published and subsequently strengthened a drinking water regulation that controls the levels of *Cryptosporidium*, *Giardia*, and viruses in drinking water systems that use surface water or groundwaters under the direct influence of surface water. Under this regulation (known as the Surface Water Treatment Rule), such a system must filter its source water unless the system can demonstrate that the source water is not subject to significant fecal contamination. Among the requirements for avoiding filtration, the system must collect source water samples one to five times per week, depending on the number of people served, and determine the density of either total coliforms or fecal coliforms. To avoid filtration, the source water must contain no greater than 100 total coliforms/100 mL or 20 fecal coliforms/100 mL. Also, the turbidity may not exceed 5 Nephelometric Units.

Clean Water Act (CWA)

The objective of the CWA is to restore and maintain the chemical, physical, and biological integrity of the Nation's waters. Under the CWA, a state must develop water quality standards for all surface waters, including wetlands, within its boundaries. A state water quality standard for a particular water body must (*1*) designate a beneficial use(s) for the water body (or for a segment of the water body), (*2*) identify the water quality criteria necessary to meet the designated use, and (*3*) protect existing uses and prevent the degradation of water quality. Types of designated beneficial uses include source waters for a drinking water supply, swimming and other recreational activities, shellfish harvesting, agriculture, industrial processes, and protection and propagation of desirable fish, shellfish, and wildlife.

EPA has not set mandatory microbial limits for any of these designated uses. However, EPA recommends that states use the following limits for primary contact recreational waters (i.e., swimming waters): for freshwaters, 126/100 mL *E. coli* or 33/100 mL enterococci; for marine waters, 35/100 mL enterococci. Many states use EPA's dated fecal coliforms (FC) criteria for such waters (a running geometric mean of 200 fecal coliforms/100 mL with no more than 10% of samples exceeding 400 fecal coliforms/100 mL). For shellfish growing waters the Food and Drug Administration recommends several fecal coliform limits (145); in the United States such limits have minimal affect on drinking water sources since few freshwater commercial shellfish harvesting areas exist.

As part of the state's program for implementing water quality standards, the state must issue permits for all point sources of pollution that discharge into water bodies. If the state determines that a water body fails to meet a water quality standard, it must establish a total maximum daily load for the specific pollutant(s) that causes the impairment. To meet this load limit, a state may prescribe a more stringent permit for a point source that discharges this specific pollutant(s) into the water body. For example, a discharge permit typically would require a wastewater treatment facility to practice secondary sewage treatment. However, for an impaired water body, the state may also set a maximum limit for the density of fecal coliforms, *E. coli*, or enterococci in secondary-treated sewage.

The implementation of state water quality standards should protect drinking water sources by minimizing the degradation of, and possibly even improving, the quality of surface waters used by drinking water systems.

CONCLUSION

Drinking water in developed countries is normally safe. Yet in the glow of technological wizardry that presently mesmerizes our society, we must not be lulled into a false complacency that our water systems are infallible. Over the course of the last century, developed countries have seen a dramatic decrease in waterborne diseases, especially in serious diseases. Yet we must not assume that lack of detection necessarily signifies its absence. Our institutional capability for recognizing a waterborne disease outbreak, much less the normal background (endemic) levels of waterborne disease, is poor. Even after an investigation of a documented outbreak, the source of the causative agent often remains speculative. Entry of pathogens into the distribution system can be insidious. And often, seemingly innocuous microbes are either later found to be pathogenic or evolve into pathogens by the incorporation of plasmids or by some other change in their genetic material.

To safeguard against this threat, we must rely on a multibarrier approach for the control of pathogens: protection of the source waters, provision of adequate and reliable water treatment, and protection of the integrity of the distribution system. Source water protection assumes proper control of point and nonpoint discharges into the water. The quality of source water can change quickly. A heavy rain will wash fecal contamination from the land into the water body or downward into the groundwater. The contamination load can be especially acute when a heavy rain occurs after a prolonged drought. Yet a healthy appreciation by drinking water system managers for the limits of mechanical and chemical processes and the extent of human fallibility will go a long way in guarding against any eventuality that might pose a serious health risk.

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SOURCES OF FECAL CONTAMINATION.

See FECAL CONTAMINATION, SOURCES OF

SPA AND HOT TUB MICROBIOLOGY

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Historically, the practice of bathing in hot water has been practiced by many cultures since ancient times. Bathing in mineral and thermal water can be traced back to as early as 500 B.C. in Greece (1). Greek baths, frequently located near volcanoes, were extremely popular and were considered useful in the cure of physical ailments. The word "spa" originally derived from the name of a town located in eastern Belgium, which at the time was part of the Roman Empire (1). The therapeutic qualities of mineral baths located in this city became popular with the Romans, who eventually constructed elaborate public baths throughout their empire. Native American communities, including the Mohawks, Pawnees, and Lakotas, were also reported to have used heated mineral springs (1).

In the 1700s, physicians sent patients to resorts built around natural hot springs with bubbling mineral water. The physicians believed that bathing in or even consuming mineral water imparted medicinal advantages to their patients, including cures for rheumatism, skin infections, and poor digestion. Indeed, bathing in water that ranges from 98 to 112 °F (37 to 44 °C) is known to relax muscles, enlarge blood vessels near the surface of the skin, and improve circulation (2).

Over the past 30 years, spas have become extremely popular in the United States for relaxation and pleasure. The term "hot tubs" is sometimes used synonymously with "spas." Spas are similar to hot tubs except that they are constructed of plastic, concrete, or metal, whereas hot tubs have generally been constructed of wood. Moreover, spas are equipped with air jets, but many hot tubs are not (3).

Finally, it is important to distinguish whirlpool baths from either spas or hot tubs. Whirlpool baths are essentially bathtubs with water jets; they are commonly located in bathrooms, are drained after each use, and typically do not have separate heating devices.

OPERATIONAL PARAMETERS OF SPAS AND HOT TUBS

Hot tub or spa capacity varies considerably. The models found at public or commercial facilities, of course, typically accommodate greater numbers of people than do those in use at private homes. Resort spas developed from natural mineral springs are often the size of swimming pools. The temperature settings of spas vary according to preference, typically ranging up to 108 °F (4). However, professional trade associations, such as the National Spa and Pool Institute, as well as most operators of public hot tubs and

spas, recommend 104 °F as the maximum temperature. The water may be agitated by high-velocity pumps that can be adjusted to maximize bubbling. Water is recirculated for filtration and heating purposes.

Filtration is achieved by diatomaceous earth (DE), sand, or cartridge filters. DE and sand filters — referred to as granular filters — require backwashing to maintain proper performance and to minimize colonization of microorganisms (3). Cartridge filters are not backwashed, but they must be routinely serviced to ensure sanitary conditions and proper performance.

Disinfection is typically accomplished by chlorine- or bromine-based compounds (3,5). Chlorine is commonly applied as sodium hypochlorite or lithium hypochlorite, at concentrations ranging from 2 to 4 mg/L (as free chlorine). Chlorine gas can be used to produce the desired residual, but this is more commonly performed at commercial-size facilities. Levels of chlorine can dissipate rapidly, depending on bather loading, and frequent monitoring is required. Deposition of organic material from the bathers can build up a substrate for microbial growth and may form chloramines, limiting the effectiveness of chlorine as a disinfectant (6).

Manufacturers and health agencies recommend "shock dosing" (i.e., adding high levels of chlorine) to oxidize accumulated organic matter. If a chlorine residual of 0.5 mg/L cannot be achieved after shock dosing, it is recommended that the water be replaced (5). Bromine is also used as a disinfectant, but it must be applied at levels twice that of chlorine to achieve the same results. Bromine can be added as either sodium or potassium bromide in conjunction with sodium hypochlorite or potassium persulfate (1-bromo-3-chloro-4,4-dimethylhydantoin [BCDMH]).

Although ozone is known to be an effective disinfectant, it does not produce a stable residual and consequently must be supplemented with a secondary disinfectant, such as chlorine or bromine, when used in spas. Ozone is applied "off-pool" during the circulation process to enhance maximum mixing. Ultraviolet (UV) light is also used for spa disinfection, but, like ozone, it must be applied in an "off-pool" manner. UV light does not produce a residual and is commonly supplemented with hydrogen peroxide as part of the disinfection process. The concentration of free hydrogen peroxide in the spa should be at least 40 mg/L (5).

Table 1. Concentration of Enteric Pathogens Excreted by Infected Individuals

Organism	Concentration in Feces (per gram)	Reference Source
Giardia	$1 \text{ to } 5 \times 10^6$	10
Cryptosporidium	10^6 to 10^7	11
Poliovirus	10^2 to $10^{6.5}$	12
Coxsackie and echovirus	10^2 to $10^{7.2}$	12
Hepatitis A	10^8	13
Rotavirus	10^{10} to 10^{12}	14
Salmonella	10^{10}	15
Shigella	10^5 to 10^9	15

MICROBIAL LOADING IN HOT TUB AND SPA WATER

Microorganisms may be added to the spa indirectly, as a result of the shedding of organisms from the skin surfaces of bathers in the spa, or directly, from accidental fecal releases. Early studies by Hanes and Fossa (7) indicated that coliform release by bathers reached maximum levels within 15 minutes. Further work by Rose and Gerba (8) suggests that coliforms released by children aged 18 months to 9 years (10^5 to 10^6 coliforms per 100 mL) were much higher than those from adults (10^1 to 10^2 coliforms per 100 mL). Based on these observations, it has been estimated that the average amount of fecal material released during bathing in recreational waters is 0.14 g per person, with a worse case of 10 g (9). The amount of pathogens associated with this fecal material varies appreciably and is dependent on the infection status, age, and personal hygiene practices of the individual. Table 1 (10–15) summarizes concentrations of pathogens excreted by infected individuals. *Giardia* and *Cryptosporidium* concentrations can range from 10^6 to 10^7 per gram of feces, whereas viral pathogens, such as rotavirus, can be as high as 10^{12} per gram.

Studies directly assessing the release of pathogens by recreators are limited. Rose and coworkers (16), however, did evaluate enterovirus and rotavirus levels associated with bather activity in a small creek. In samples collected downstream from the recreators, these researchers noted that the average levels of enterovirus and rotavirus were 0.045 and 0.67 per 100 L, respectively, per individual. Given the inefficiency of virus detection methodology and the fact that the water was collected under flowing stream conditions, it is reasonable to assume that these values are far lower than the true values for what was actually excreted.

Although most studies focus on indirect pathogen loading associated with fecal material in the perineum area, it is important to note that fecal releases can contribute to significant pathogen loading of recreational waters. Bell and colleagues (17) reported that fecal releases do occur during recreational bathing activity, resulting in numerous outbreaks of disease. It has been calculated that such activity can result in the dissemination of up to 10^{14} enteric pathogens into the water column (9). Collectively, this information can be used to estimate pathogen loading under various recreational scenarios, including use of spas or hot tubs.

WATERBORNE ILLNESS ASSOCIATED WITH SPAS AND HOT TUBS

Spas may contain a wide range of microorganisms, including algae, protozoa, bacteria, viruses, fungi, and yeasts (5). Table 2 (18) lists human pathogens that may be present in aquatic environments as a result of human activity. Although the individuals using the spa introduce most of these organisms, some may also be introduced from the source water used to fill the spa or from contamination by surrounding environmental sources. Illnesses associated with spa usage include those caused by bacterial, viral, and protozoan organisms,

Table 2. Human Pathogens That May Be Present in Water as a Result of Human Contact

Virus	Bacteria	Protozoa
Adenovirus	*Salmonella*	*Entamoeba histolytica*
Astrovirus	*Shigella*	*Giardia lamblia*
Calicivirus	Enteropathogenic *E. coli*	*Balantidium coli*
Coronavirus	*Yersinia enterocolitica*	*Cryptosporidium*
Enteroviruses	*Campylobacter*	
Poliovirus	*Vibio cholerae*	
Coxsackie A, B	*Leptospira*	
Echovirus		
Hepatitis A, E		
Norwalk		
Reovirus		
Rotavirus		
Small round viruses		

Source: Yates et al. (18).

such as *Pseudomonas aeruginosa*, *Pseudomonas cepacia*, *Mycobacterium marinum*, *Legionella*, *Klebsiella*, *Yersinia*, *Acanthamoeba*, *Giardia*, *Cryptosporidium*, and papilloma viruses (5). Other pathogens that may be transmitted from one person to another via spa or hot tub usage include *Naegleria fowleri*, *Escherichia coli*, *Staphylococcus* species, *Streptococcus* species, *Salmonella*, and enteroviruses (5).

Skin diseases (folliculitis) are probably the most common ailments reported following hot tub use (6). Indeed, the term "hot tub burns" — commonly used in the hot tub and spa industry to describe skin rashes caused by the bacterium *Pseudomonas aeruginosa* — was first recognized in the 1970s (6). Zacherle and Silver (19) noted that with increasing use of hot tubs, there was a concomitant increase in cases of a distinct clinical syndrome appearing several hours or days after hot tub exposure. The syndrome consisted of a maculovesicular, often pruritic, rash, commonly associated with symptoms that included fever, upper respiratory tract complaints, axial adenopathy, and breast tenderness. Culturing of pustules from a patient with this syndrome revealed *P. aeruginosa*. The authors noted that serotypes of this organism are known to produce exotoxins that may be responsible for the folliculitis. In particular, serotype 0 : 11 has been isolated from patients' lesions and from whirlpool water in previous folliculitis outbreaks (20–22).

In a review of whirlpool-associated cases of folliculitis, Berger and Seifert (6) noted that the prevalence of *P. aeruginosa* suggests that this organism has developed a tolerance to relatively high temperatures and high levels of chlorine. They also pointed out that serotype 0 : 9 is a concern in folliculitis cases. These authors suggested three primary environmental conditions known to be associated with outbreaks of folliculitis: prolonged exposure to the water, excessive numbers of bathers, and inadequate pool care. Elevated temperatures, coupled with entry via hair follicles or breaks in the skin, were considered key factors in *P. aeruginosa* infection. In addition to causing folliculitis, *P. aeruginosa* was also implicated as the causative agent in four cases of severe hemorrhagic external otitis in a group of university students following hot tub exposure (4). In this episode, among 24 members of a university coeducational fraternity who used a hot tub, two had simple dermatitis, and four developed severe external otitis. Follow-up inspection and culturing of the hot tub revealed the presence of *P. aeruginosa* serotype 0 : 10. Two previous cases of pool-associated *P. aeruginosa* otitis were cited in the present study (20,21). In 1997 and 1998, more than 75 cases of *P. aeruginosa* dermatitis and otitis externa were reported with swimming pool and hot tub use, and from 1999 to 2000, 28 additional cases were described (23). These recent outbreaks were largely attributed to inadequate disinfection procedures (See PSEUDOMONAS, this Encyclopedia.).

Legionella infections have also been associated with hot tub usage. A compilation of these reports can be found on-line at:

http://www.q-net.net.au/~legion/Legionnaires_Disease_Spas_Hot_Tubs.htm

This site also summarizes several reports on spa-contracted folliculitis caused by *P. aeruginosa.* Miller and associates (24) reported that 34 resort guests experienced an illness that met a symptom-based case definition of Pontiac fever. Each of the ill guests either had used the indoor hot tub or had been within 3 m of it. Samples collected from the hot tub were positive for *Legionella pneumophilia* serogroup 6. Testing of convalescent-phase serum indicated that 64% of the ill guests had a fourfold or greater rise in antibody titer against *L. pneumophilia* serogroup 6. Maintenance records for the hot tub indicated that the temperature during the incident ranged from 38.9 to 40 °C, with a free bromine residual of 2 to 3 mg/L. This incident suggested that even properly operated hot tubs could be responsible for transmission of *Legionella*. Moreover, because of the aerosol produced by the hot tubs, even those in close proximity were found to have significant exposure to this organism.

Guerrero and coworkers (25) reported that 14 passengers contracted Legionnaires' disease through use of whirlpool water baths aboard a cruise ship. Cultures taken from a sand filter used in recirculation of the whirlpool water were positive for the presence of the same *L. pneumophilia* species (based on monoclonal antibody subtyping patterns) as found in clinical samples taken from the patients. In Virginia, 23 cases of Legionnaires' disease, resulting in two fatalities, were reported among customers visiting a large home-improvement center (26). Investigation of this outbreak indicated that these customers were in close proximity to a hot tub display, which illustrates the importance of aerosolization in transmission of these organisms. Testing of the hot tub filters revealed *L. pneumophilia* serotype 01 isolates that were identical to those obtained from the sputum of the patients. Miyamoto and coworkers (27) reported isolating *L. pneumophilia* serogroup 3 from a patient who had nearly drowned in a hot spring spa. Molecular-based testing revealed that the organism was associated with this spa. Significantly, this report also noted that in a survey of 135 hot springs in Japan, 62% were positive for the presence of legionellae.

Embil and associates (28) reported five cases of pulmonary illness associated with exposure to *Mycobacterium avium* complex in hot tub water. In this study, five healthy people developed respiratory illnesses characterized by bronchitis, fever, and "flulike" symptoms after using a hot tub. Sputum and lung biopsy samples collected from the patients, as well as water samples collected from the hot tub, were all positive for the presence of *M. avium* complex. The authors noted the ubiquitous nature of *Mycobacterium* in aquatic environments, aerosolization conditions in the hot tub, and resistance to disinfectants as predisposing conditions for contracting this illness. (see *MYCOBACTERIUM AVIUM* COMPLEX, this Encyclopedia.)

Nerurkar and coworkers (29) reported on the transmission of herpes simplex virus (HSV) in commercial spas in the Washington, D.C., area. Several of these businesses were closed when a large number of clients reported contracting genital HSV infections following use of the facilities. Water samples were collected from two of these health spas and examined for the presence of HSV; however, no HSV was recovered from the samples. Additional studies were conducted to assess the survival characteristics of HSV on plastic-coated benches and seats in spa facilities. It was noted that HSV could survive for up to 4.5 hours on these surfaces. The authors concluded that survival of HSV on plastic surfaces commonly associated with spas could be a factor in the nonvenereal spread of this virus.

Tallis and Gregory (30) reported an outbreak of hepatitis A associated with a spa. In this episode, six males (ages 8 to 15 years) contracted hepatitis A after recreating in a spa. Investigation of the case indicated that the infected individuals had been spitting mouthfuls of water at each other. The authors concluded that the infection was probably caused by the shedding of an infective individual, with subsequent ingestion by others using the spa. The spa was customarily disinfected with hydrogen peroxide and UV light. The investigators suggested that this disinfection protocol may not have been adequate to control the virus, as previous evaluations of hydrogen peroxide and UV light disinfection in Victoria had shown poor results.

Samples and colleagues (31) reported an instance of *Acanthamoeba keratitis* associated with the use of a hot tub. In this study, a 42-year-old man developed a severe case of optical keratitis. Samples from the subject's hot tub and from his eye revealed the presence of *Acanthamoeba castellani*. This organism was also isolated from the surrounding garden soil. The authors noted that amoebae can commonly be isolated from aquatic environments, including swimming pools, and have been shown to grow well in temperatures up to 36 °C. Indeed, the authors cited a report from northern Bohemia indicating that 16 fatal cases of primary amoebic meningoencephalitis were associated with recreation in a swimming pool. Although *Naegleria fowleri* was noted to be the agent responsible for the fatalities in this outbreak, several species of *Acanthamoeba* were also recovered from the swimming pool water. In the current study, the authors suggested that infection by *A. castellani* could be a public health concern and that further studies would be needed to establish the prevalence of this organism in hot tubs.

MICROBIAL PERSISTENCE IN AQUATIC ENVIRONMENTS

Elevated temperatures, filtration, and disinfectants are used to control microorganisms in spas and hot tubs. Disinfectants are probably the single most important factor in controlling contact with pathogenic microorganisms. Many investigators have reported that microorganisms may persist in aquatic environments in spite of the presence of a disinfectant agent (32–35).

Microbial resistance to disinfectants may be a function of: (*1*) the presence of physical or chemical agents that impair the ability of the disinfectant to inactivate the organism, (*2*) the presence of particulate matter that can provide physical protection from disinfection, and (*3*) association with nematodes or other vectors (e.g., amoebae) that can provide a physical barrier from the disinfectant (36). Microbial resistance to disinfectants may also result from factors that can be classified as cell-mediated processes. This form of resistance is physiological in nature and is based on the organism's ability to develop adaptive cellular features to survive under adverse environmental conditions. Examples of cell-mediated processes include polymer or capsule production, which may act to limit the penetration of disinfectant agents into the cell; cellular aggregation, which provides physical protection for internal organisms; and alteration of the cell envelope structures, which restricts the entrance of disinfectants into the cell (36). It is important to note that organic loading from bathers, high water temperatures, and aeration and agitation of the water can quickly deplete disinfectant residuals in the spa (4,6).

Microbial attachment to particulate material (e.g., organic compounds, plankton, microscopic organisms) has been observed to aid in survival against disinfectant agents (37). Under optimal chemical and physical conditions, maximum disinfectant efficiency is achieved when the disinfectant agent has unhindered access to the target organism. Particulate matter may interfere with this process, either by creating a disinfectant demand — thus neutralizing the action of the disinfectant — or by physically shielding the organisms from the disinfectant (38). Disinfectant protection is enhanced with decreasing organism size and increasing particle volume (39). Herson and associates (40) demonstrated that attachment of bacteria to particulate material aided survival in the presence of chlorine. These authors noted that levels of bacteria were higher in relation to increased turbidity. Scanning electron micrographs revealed the presence of particles colonized by bacteria.

The introduction of bacteria into potable water systems by nematodes or crustaceans was studied as early as 1911 by Hoerhammer (41), who found that the crustacean *Cyclops* could ingest large quantities of *Salmonella* and thus act as a reservoir for this pathogen. Chang and coworkers (42) observed that pathogenic bacteria and viruses ingested by nematodes were protected from high levels of chlorine (e.g., 80 to 90 mg/L). King and colleagues (43) found that coliforms as well as bacterial pathogens (*Salmonella*, *Yersinia*, *Shigella*, *Legionella*, *Camplyobacter*), when contained in either *A. castellanii*

or *Tetrahymena pyriformis*, were more resistant (e.g., 30- to 120-fold) to chlorine disinfection. Rowbotham (44) indicated that amoebae of the genera *Acanthamoeba* and *Naegleria* could harbor legionellae and act as infectious reservoirs for this organism. Kilvington and Price (45) observed that viable *Legionella* could be recovered from *Acanthamoeba polyphaga* exposed to 50 mg/L chlorine. Clearly, the presence of amoebae in aquatic environments may be a significant factor in *Legionella*-associated illnesses contracted in spas.

Many bacterial species are capable of producing an external layer composed predominantly of polysaccharides and, to a lesser extent, polypeptides or polysaccharide-protein complexes (46). Such a structure, commonly referred to as the *capsule* or *slime* layer, provides nutrient storage, protection from phagocytosis, and adhesion to surfaces to enhance survival in the environment (47,48). Several groups of investigators have indicated that bacteria associated with biofilms and capable of producing capsules are more resistant to disinfection. LeChevallier and coworkers (49) evaluated the role of biofilm formation (*Pseudomonas pickettii*, *Moraxella*, and *Pseudomonas pacumobilis*) in promoting resistance to chlorine, chloramines, and chlorine dioxide. They observed that resistance to chlorine or chlorine dioxide increased 150- to 3,000-fold compared to the resistance of unattached cells; resistance to chloramines, however, increased only twofold. Seyfried and Fraser (50) also noted that isolates of *P. aeruginosa* from swimming pools with chlorine residuals of 1.5 mg/L had more extensive capsule layers than those isolated under lower chlorine concentrations (0.4 mg/L), suggesting that this structure conferred enhanced resistance to disinfectants. It has been reported that such microorganisms as *Legionella* and *P. aeruginosa* have colonized filter material used for spas and hot tubs (5). This colonization, and the subsequent resistance to disinfection, was most likely facilitated by production of capsular material and formation of a biofilm layer. Moreover, Broadbent (5) reported that this situation has led to fatal cases of Legionnaires' disease.

Many investigators have studied antecedent growth conditions in relation to enhanced disinfection resistance (36). Favero and Drake (32) reported the isolation of *Staphylococcus*, *Streptococcus*, *Pseudomonas*, and *Alcaligenes* from swimming pools disinfected with chlorine and iodine. They observed that growth in swimming pool water conferred 10 times more resistance to iodine than did growth in trypticase soy broth.

Pyle and McFeters (51) noted that *P. aeruginosa* grown under low-nutrient conditions resulted in greater resistance to iodine disinfection. Cargill and coworkers (52) observed that growth under low-nutrient conditions increased the resistance of *L. pneumophilia* to iodine. The Cargill study concluded that the increased resistance resulted from possible increased aggregation, protective extracellular material, and/or other physiological changes under low-nutrient conditions. Stewart and Olson (53) observed that the resistance of *Klebsiella pneumoniae* to chloramines was increased when cells were grown under low-nutrient conditions. They attributed this enhanced resistance to (*1*) increased aggregation, (*2*) increased capsule material, and (*3*) changes in the cellular envelope structure.

The typical spa or hot tub environment, characterized by low-nutrient conditions, may foster development of the protective mechanisms that assist microorganisms to survive in the presence of the disinfectant agents most commonly used in spas and hot tubs. Frequent reports of *P. aeruginosa*-related folliculitis suggest that these organisms, in particular, have indeed developed survival mechanisms.

HEALTH GUIDELINES FOR SPAS AND HOT TUBS

Proper maintenance of spas and hot tubs is essential in the control of microbial disease. Specific microbial standards and monitoring frequencies for public spas and hot tubs vary with local and state regulatory agencies. South Australia's National Environmental Health Forum has published a guideline on water quality for heated spas (5). This document provides specific recommendations for disinfection, water chemistry, and general operation of spa pools. Monitoring recommendations for microbial agents contained in this publication include heterotrophic-plate-count bacteria, less than 1 in 100 mL; *P. aeruginosa*, not detected in 100 mL; *Legionella*, not detected in 1,000 mL; and pathogenic amoebae, not detected in 1,000 mL. This organization also recommends chlorine residuals of at least 2 to 4 mg/L. The U.S. Centers for Disease Control and Prevention (CDC) recommend a free residual of 3 mg/L for chlorine and 4 mg/L for bromine (54).

General recommendations to reduce microbial illness associated with spas and hot tubs (55) include the following:

- Adhere to pool and hot tub recommendations and regulatory requirements for pH and disinfectant levels.
- Have a thorough knowledge of basic aquatic facility operation.
- Provide training for maintenance staff on system capabilities, maintenance, and emergency monitoring systems.
- Monitor pool and hot tub disinfectant residuals frequently, especially during periods of heavy bather loading.
- Understand appropriate use of cyanurates in disinfection and testing.

Additional detailed information on guidelines for proper operation of spas and hot tubs can be obtained on-line from the National Spa and Pool Institute (*www.nspi.org*), from the CDC (*www.cdc.gov*), or from various local and state public health agency websites. Comprehensive reviews by Broadbent (5) and Freije (3) also provide excellent information on this topic.

CONCLUSION

Hot tubs and spas have become a popular means of recreation. Indeed, it has been reported that over a

million Americans have purchased spas and hot tubs since 1990 (56). Human contact with aquatic environments can clearly introduce pathogenic and nonpathogenic microorganisms, including bacterial, viral, and protozoan parasites. The concentration and persistence of these organisms in spas and hot tubs, as discussed in this current review, are subject to a number of complex factors. When spas and hot tubs are properly operated and maintained, the associated incidence of microbial illnesses is relatively low. However, numerous reports of illnesses related to spa and hot tub use suggest the necessity for continued vigilance over maintenance practices.

It is also important to note that even under proper maintenance conditions, cases of microbial illness have been reported. Contamination of spa and hot tub water during high pathogen loading events (e.g., excessive bather loading, or an accidental fecal release) can temporarily compromise the ability of the system to remove pathogenic organisms. Contact with this contaminated water can result in skin, ear, or eye infections and, if the water is ingested, can lead to gastrointestinal illness. As discussed in this current review, numerous studies have also indicated the unique adaptive ability of microorganisms to persist in treated spa and hot tub water. Microorganisms can colonize filtration devices and develop adaptive characteristics to enhance their resistance to disinfecting agents.

In recognition of potential illnesses associated with the use of spas, hot tubs, or pools, many regulatory agencies have developed guidelines to minimize the risk. Although most illnesses are generally self-limiting for immunocompetent individuals, the CDC has indicated that children, pregnant women, and people with weakened immune systems (e.g., persons infected with the human immunodeficiency virus [HIV], organ transplant recipients, or those receiving certain types of chemotherapy) are at greater risk of developing severe or life-threatening disease (57). The CDC admonishes these individuals to take additional precautionary measures, including consultation with health care providers or avoidance of high-risk environments.

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SPACE MICROBIOLOGY: EFFECTS OF IONIZING RADIATION ON MICROORGANISMS IN SPACE

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Cosmic radiation, of solar and of galactic origin, is one of the external sources of energy of significant impact on our biosphere. During the first billion years of Earth's history, solar ultra-violet (UV) radiation of wavelengths below 290 nm did penetrate the atmosphere and reach the surface of the Earth. This is the time span when the decisive processes of abiotic organic chemical evolution, appearance of life, and diversification of microorganisms took place on Earth (1). In these processes, solar UV radiation played a major role. The role of the heavy ions of cosmic radiation in the evolution of our biosphere is less clear because the surface of the Earth is largely spared from the cosmic radiation due to the deflecting effect of the geomagnetic field and the huge shield of 1,000 g/m^2 provided by the atmosphere. However, in space, the natural radiation encountered is a complex mixture of charged particles of galactic and solar origin and particles trapped by the geomagnetic field.

Since the advent of space flight and the establishment of long-duration space stations in Earth's orbit, such as Skylab, Salyut, MIR, and the International Space Station, the upper boundary of our biosphere has extended into space. Such space missions expose humans and any other biological system to a radiation environment of a composition and intensity not encountered on Earth. To prevent detrimental health effects caused by the radiation environment of space, radiation protection guidelines have been elaborated for humans in space. The guidelines are based on (*1*) dosimetry and modeling of the radiation field in space, (*2*) studies on the biological effects of the heavy ions of cosmic radiation encountered in space or produced at heavy ion accelerators on ground, and (*3*) studies on potential interactions of cosmic radiation and other parameters of space flight, above all microgravity. Among other test systems, microorganisms have been used in space experiments and on ground to determine the biological effectiveness of this particulate radiation in space (2). Furthermore, any habitat in space will inevitably be contaminated by a specific cabin microflora. Bacteria, fungi, and algae will grow on surfaces of the spacecraft and on the human body if there is sufficient supply of nutrients and water. If plants or animals are present, either as test systems or for bioregenerative life support purposes, their microflora has also to be considered. Knowledge of the effects of space radiation on this microflora inside the spacecraft is of utmost importance to safeguard the health and well-being of the crew and the liability of bioregenerative life support systems.

It might also be possible for life to be confronted with radiation in space by natural processes. Our atmosphere teems with viruses, bacteria, algae, microfungi, fungal spores, spores of mosses and ferns, pollen, minute seeds, and protozoan cysts. These are found at concentrations of possibly hundreds to thousands per cubic meter. Viable microorganisms, predominantly black conidia and fungal spores, have even been found at altitudes as high as 77 kilometers; it is assumed that in these instances, pigmentation offers a selective advantage by protecting against the intense solar UV radiation prevalent at these high altitudes (3).

Since the discovery of a certain group of meteorites of probable Martian origin, it has become obvious that matter can be exchanged between the planets of our solar system, for example, from Mars to Earth. Especially during the early phase of heavy bombardment, which lasted until approximately 3.8 billion years ago, impactors up to a kilometer in size have struck the planets of our solar system. Such gigantic impacts lead to the ejection of a considerable amount of soil and rocks that are thrown up at high velocities, some fractions reaching escape velocity (4). These ejecta leave the planet and orbit around the sun, usually for timescales of a few hundred thousand or several million years until they either impact another celestial body or are expelled out of the solar system. The question arises whether such rock or soil ejecta could also be the vehicle for life to leave its planet of origin. Soil microorganisms or endolithic microbial communities are candidate terrestrial microbial systems that might be ejected by such large impacts. If so, they will be exposed to space radiation during their interplanetary journey. Radiation effects and potential protection and repair mechanisms could have profoundly affected the chances for a viable transfer of microbes within our solar system.

Among the planets of our solar system, Mars and probably the Jovian moon Europa are considered the best candidates for providing the prerequisites for the support of life, either in the past or the present. However, a putative Martian biota would be exposed to much higher radiation dose levels than life on Earth. This is the consequence of the fact that Mars does not possess either an effective magnetic field or a thick atmosphere. Therefore, the biologically effective dose at the surface of Mars caused by ionizing radiation from space is about 100 times higher than that at the surface of the Earth.

After a short description of the general effects of radiation on microorganisms and their responses in order to cope with the radiation stress, specific biological problems resulting from exposure of microorganisms to the space radiation environment are examined. This will include the effects of the heavy ions of cosmic radiation on microorganisms as cellular model systems for radiation protection purposes or as part of the cabin microflora, the role of radiation in affecting interplanetary transfer of microorganisms as in the case of microbes inside meteorites, and the impact of the radiation climate on a putative extraterrestrial biota, as on Mars.

BIOLOGICAL WEIGHTING OF RADIATION

Ionizing radiation is measured in the S.I. unit of absorbed dose per mass unit, the Gray (Gy), with 1 Gy equal to

the net absorption of 1 J in 1 kg of material (water). However, the biological effectiveness of radiation depends largely on the local energy distribution, the Linear Energy Transfer (LET). Therefore, different qualities of radiation can have different biological effectiveness even at the same physical dose. The relative biological effectiveness (RBE) describes this dependence of the biological effectiveness on LET. RBE is the ratio of the physical doses of the test radiation and, for example, X-rays, leading to the same biological effect. The RBE value can be different for different biological systems, depending on their stage in the growth cycle and other environmental factors such as oxygen content. To assess the effectiveness posed by radiation to humans and to the whole biosphere, estimates must be made of both the amount and type of radiation under consideration and the radiobiological effectiveness of the different components of the radiation. For this purpose, the quality factor Q has been introduced. Q is the biological weighting function of ionizing radiation and has been obtained by averaging over a variety of RBE values for the same LET value. Its relation to the LET of the radiation is shown in Figure 1 (5). For X-rays and γ rays, Q is equal to 1. For a given dose of high-LET radiation, the dose equivalent, H, is the product of Q and the absorbed dose:

$$H = QD \tag{1}$$

with H = the dose equivalent, Q = the quality factor and D = the absorbed dose. The S.I. unit for the dose equivalent is Sievert (Sv). For a mixed-radiation field composed of ionizing radiations of different radiation qualities, i (as encountered in space), the dose equivalent, H, is given by

$$H = \sum N_i \, Q_i \, D_i \text{ (Sv)} \tag{2}$$

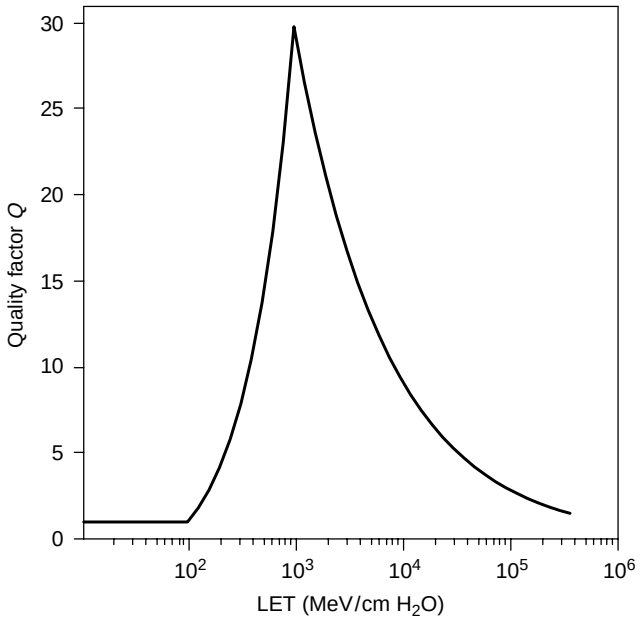

Figure 1. The quality factor Q is the biological weighting function of ionizing radiation and is dependent on the linear energy transfer (LET) of the radiation under consideration (5).

with D_i = absorbed dose, deposited in biological matter by the radiation i (Gy), Q_i = radiation quality that is described as a function of LET and N_i = a special factor that accounts for specific exposure conditions (e.g., dose rate, fractionated exposure, microgravity) or special physiological properties. Q has originally been developed for radiation protection purposes. Therefore, it is mainly based on radiation risks for cancer induction in mammals.

ENVIRONMENTAL RADIATION AND LIFE

Life on Earth, throughout its almost four billion years history, has been shaped by interactions of organisms with their environment and by numerous adaptive responses to environmental stressors. Among these, radiation, both of terrestrial and of cosmic origin, is a persistent stress factor that life has to cope with. Radiation interacts with matter, primarily through the ionization and excitation of electrons in atoms and molecules. These matter–energy interactions have been decisively involved in the creation and maintenance of living systems on Earth. Because it is a strong mutagen, radiation is considered a powerful promoter of biological evolution on the one hand and an account of deleterious consequences to individual cells and organisms (e.g., by causing inactivation or mutation induction) on the other. In response to the harmful effects of environmental radiation, life has developed a variety of defense mechanisms, including the increase in the production of stress proteins, the activation of the immune defense system, and a variety of efficient repair systems for radiation-induced DNA injury.

As a reactive chemical species, DNA is the target of numerous physical and chemical agents. These can induce a broad spectrum of DNA lesions (Fig. 2, Table 1), including damage to nucleotide bases, cross-linking, and DNA single- and double-strand breaks. Despite all of these lesions, the DNA is functionally more stable than the two other cellular macromolecules, RNA and protein. This stability can be attributed to the following three factors: (*1*) the primary structure of DNA is all that is needed for transfer of information; (*2*) because of the double-helical structure, DNA carries the information in duplicate; and (*3*) there are molecular mechanisms of different complexity to undo the DNA damage, thus maintaining cellular survival and genetic integrity (6,7).

DNA repair encompasses the molecular reactions that eliminate damaged or mismatched nucleotides from DNA. There is a variety of repair mechanisms, each catalyzed by a different set of enzymes. Nearly all of these mechanisms depend on the existence of two copies of the genetic information, one in each strand of the DNA double helix: if the sequence in one strand is accidentally changed, information is not lost irretrievably because a complementary copy of the altered strand remains in the sequence of nucleotides in the other strand. Examples are given below for different DNA repair mechanisms in microorganisms: (*1*) direct repair, (*2*) mismatch repair, (*3*) base excision repair, (*4*) nucleotide excision repair, (*5*) recombinational repair, and (*6*) the SOS response.

Direct repair mechanisms simply revert the damaged bases to their unmodified form. The ability of photolyase,

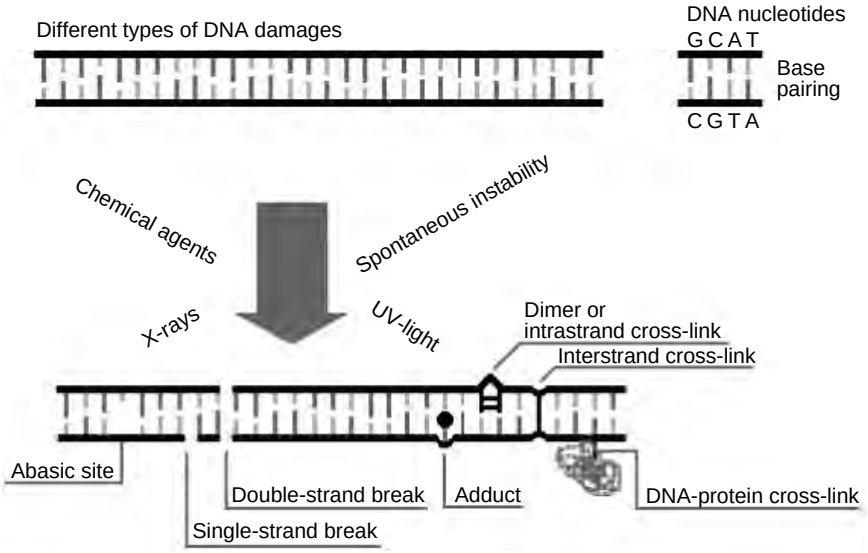

Figure 2. Different types of DNA damage.

Table 1. DNA Lesions Produced by Different Internal and External Conditions and the Pathways Used by Bacteria for their Repair

DNA Lesion	Cause	Repair Mechanism
Base mismatches, loops, bubble structure	Replication errors, recombination	Base excision, general mismatch repair
Deamination and depurination	Heat	Base excision
Oxidative damage	Oxidative metabolism, anoxia and hypoxia, ionizing radiation	Base excision
Alkylation and alkyl adducts (nonbulky and bulky adducts)	Nitrogen mustard, polyaromatic compounds	Direct repair, base excision, nucleotide excision
Intrastrand cross-links, (bulky adducts)	UV, cisplatin	Direct repair, nucleotide excision
Interstrand cross-links	Psoralen, malphalan	Nucleotide excision, recombination
Single-strand break	Ionizing radiation, oxidative stress	Ligation
Double-strand break	Ionizing radiation	Ligation, recombination

in the presence of light (photoreactivation), to remove UV-induced lesions in DNA is the best-studied repair reaction of this type (8). Genes coding for photolyase activities (PHR genes) have been found in many, but not all, prokaryotic and eukaryotic organisms. For example, among the prokaryotes, *E. coli* and *Synechocystis* sp. possess photolyase, but *Bacillus subtilis* and *Deinococcus radiodurans* do not. Among the eukaryotes, *Neurospora crassa* and *Saccharomyces cerevisiae* possess photolyases but *Schizosaccharomyces pombe* does not. Another example of direct damage reversal is repair of O^6-methylguanine by the transfer of the alkyl group from the DNA to a cysteine in a protein via an O^6-methylguanine-DNA methyltransferase (9). The enzyme removes other alkyl groups from the O^6 position of guanine and from the O^4 position of thymine with varying efficiencies. It appears to be present in all living organisms. A final example of direct damage reversal is the sealing of a subset of nicks in DNA by DNA ligase. Of course, DNA ligase can only seal nicks having 5′-phosphates and 3′-hydroxyls. Nicks with other configurations or nicks accompanied by additional backbone or base damage require more complicated repair mechanisms.

The mismatch repair pathway corrects DNA damages arising from replication mistakes (10). The distorted double helix is recognized by the MutS protein. Additional binding of the MutL stabilizes the complex. The MutS–MutL complex activates MutH, which locates a nearby methyl group and nicks the newly synthesized strand opposite the methyl group. The fact that the newly synthesized strand is not immediately methylated at GATC sequences in bacterial DNA, although the old strand carries methyl groups in proximity to the replication fork, allows *E. coli* cells to distinguish the presumably correct (old strand) from the presumably incorrect (newly synthesized) strand. This use of methylation to distinguish the parental strand is probably peculiar to bacteria. Data from yeast in vitro mismatch repair experiments suggest that single-strand nicks, which are present in nascent DNA strands, between Okazaki fragments (short stretches of 1,000–2,000 bases of the lagging (3′–>5′) strand of DNA, synthesized in the 5′–>3′ direction during discontinuous replication that are later joined by DNA ligase into a covalently intact strand), provide a signal for strand-specificity in these organisms. Excision is accomplished by

cooperation between the UvrD (Helicase II) protein, which unwinds from the nick in the direction of the mismatch, and a single-strand specific exonuclease of appropriate polarity (one of several in *E. coli*), followed by resynthesis (Polymerase III) and ligation (DNA ligase).

The base excision repair system removes the damaged base by means of a glycosylase. There are many glycosylases with narrow substrate ranges. Following removal of the damaged base by a glycosylase, the abasic sugar (AP site) is removed by cleavage of the 3′-phosphodiester bond by an AP lyase that is often associated with the glycolyase activity. Then the 5′ bond of the AP site is hydrolyzed by AP endonuclease. The resulting single nucleotide gap is filled in by DNA Pol I and then ligated. AP sites are also generated by spontaneous hydrolysis, by oxidative stress and by ionizing radiation, and hence are frequent lesions in DNA.

The nucleotide excision repair pathway is an elaborate repair system that removes bulky lesions from DNA (11–14). In *E. coli*, three proteins, the products of the *uvrA*, *uvrB*, and *uvrC* genes, are responsible for damage recognition and DNA nicking. The current model is that, in an ATP-dependent reaction, a dimer of the UvrA protein forms a complex with a single UvrB molecule. Next, the UvrC protein binds to the UvrB protein, thereby activating it to nick the DNA approximately four nucleotides 3′ to the damaged site. This, in turn, activates UvrC to nick the DNA approximately seven nucleotides 5′ to the damage. It is possible that activation of UvrC is a consequence of a conformational change in the DNA after nicking by UvrB. All these steps require ATP binding but not ATP hydrolysis. The fourth protein, the *uvrD* gene product known as DNA helicase II, displaces the damage-containing oligonucleotide. At the same time, the UvrC protein is released and afterwards the UvrB protein is displaced. Following the release of the excised oligonucleotide, the single-stranded gap is filled in by DNA Polymerase I in *E. coli* and then the newly synthesized DNA is ligated to the old one. The enzyme system that is responsible for the dual incision, which is also referred to as excision nuclease, is the sole repair system for removing bulky lesions from DNA and also plays a back-up role for other repair systems because it is capable of removing nonbulky lesions, such as O6-MeGua and AP sites.

Interstrand cross-links pose a unique problem for the cellular repair machinery. Both base excision and nucleotide excision rely on the redundant information in the undamaged strand to correctly fill in the excision gap, resulting from damage removal. In cross-linked DNA, both strands are damaged, and hence the genetic information in both strands has changed. The cell overcomes this dilemma by combining excision repair with recombinational repair (15–17). First, dual incisions are made in one strand by the excision nuclease; second, through homologous pairing of the incised duplex with the intact sister chromosome, a segment of the intact duplex is paired with the gap to generate a three-strand noncovalent intermediate and the transferred strand is ligated in to form a three-stranded covalent intermediate; third, the excised oligomer, remaining linked to the duplex through the cross-link is recognized as a bulky adduct and is excised by dual incisions. The gaps generated by repair and recombination in the damaged and sister duplex, respectively, are filled in by DNA polymerases and the newly synthesized DNA is ligated.

The best-studied example of an inducible repair pathway is the SOS response in *E. coli* and other bacteria (18,19). In these bacteria, any block to DNA replication caused by DNA damage produces a signal (thought to be an excess of single-stranded DNA) that induces an increase in the transcription of more than 15 genes, many of which code for proteins that function in DNA repair. The signal first activates the *E. coli* RecA protein, which then destroys a negatively acting gene regulatory protein (a repressor) that normally suppresses the transcription of the entire set of SOS response genes. Studies of mutant bacteria, deficient in different parts of the SOS response, indicate that the newly synthesized proteins have two effects. Firstly, as would be expected, the induction of new DNA repair enzymes increases cell survival. When the mutants deficient in this part of the SOS response are treated with a DNA-damaging agent such as ultraviolet radiation, an unusually high proportion of them die. Secondly, several of the induced-proteins transiently increase the mutation rate by greatly increasing the number of errors made in copying DNA sequences. Although this has little effect on short-term survival, it is presumably advantageous in the long term because it produces a burst of genetic variability in the bacterial population and hence increases the chance that a mutant cell with increased fitness will arise. The DNA repair system activated in the SOS response is not the only inducible DNA repair system known. Bacteria have another system that is activated specifically by the presence of methylated nucleotides in DNA and there is at least one inducible DNA repair system in yeast cells.

In addition to repair systems, several other feedback controls operate to restrain the cell cycle control in eukaryotic systems until particular conditions are satisfied. A well-characterized feedback control operates at the mitotic entry checkpoint to prevent cells with damaged DNA from entering mitosis until the damage is repaired (20,21). The response to DNA damage is usually studied using experimentally induced lesions in DNA, such as those created by X-rays. Many radiation-sensitive (rad) mutations have been isolated in budding yeast and at least one, called rad9, has been shown to code for an essential component of the feedback control mechanism. Mutants lacking rad9 still possess the machinery for DNA repair but fail to delay in G2 when they have been irradiated. Therefore, they proceed into mitosis with damaged chromosomes; not surprisingly, they are killed by doses of radiation that normal cells would survive.

The most radiation-resistant bacterium known is *D. radiodurans*. It was originally isolated from samples of canned meat that were thought to be sterilized by high doses of γ-radiation. Typically, it is found in locations where most other bacteria have died under extreme conditions, ranging from the shielding pond of a radioactive cesium source to the surfaces of Arctic rocks. Researchers examined the bacterium's cellular

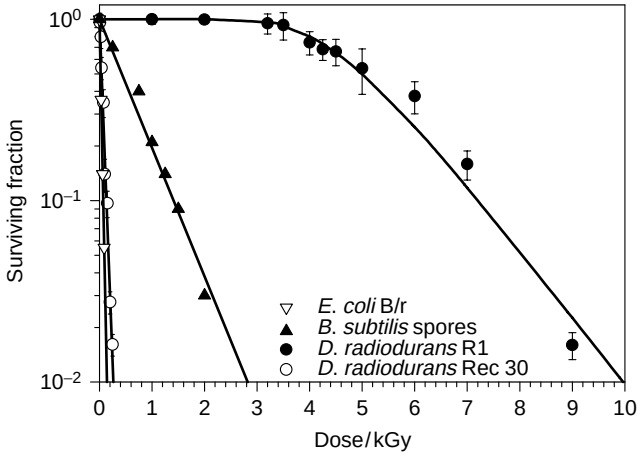

Figure 3. Representative survival curves for *D. radiodurans* R1 (closed circles) and its recombination deficient mutant Rec30 (open circles), compared to survival curves for spores of *Bacillus subtilis* (closed triangles) and *E. coli* B/r (open triangles), following exposure to X-rays (23).

repair genes and discovered that although *D. radiodurans* contained the usual complement of repair genes found in other radiation-sensitive bacteria, it has an unusually large redundancy of repair functions (22). Furthermore, the regulation of its repair activities is highly coordinated with the cell cycle. In Figure 3, the radiation sensitivity of the highly radiation resistant bacterium *D. radiodurans* R1 is compared with that of a mutant, *D. radiodurans* Rec30, that is deficient in DNA repair and two other bacterial representatives, that is, spores of *B. subtilis* and cells of *E. coli*. Each major designation on the *y*-axis represents a reduction in viability by a factor of 10. The D_{37} dose (i.e., the dose of radiation required to reduce the population by 37% of its original size, or e^{-1}; this is interpreted as the average dose required to inactivate a single colony-forming unit) for the *E. coli* culture is 30 Gy, which is approximately 200 times lower than that of *D. radiodurans*. *D. radiodurans* has a characteristic shoulder of resistance to approximately 3,000 Gy; at doses below that value there is no loss of viability, although up to about 100 double-strand breaks have been induced in the chromosome of each cell (22). Above 5,000 Gy, there is an exponential decline in viability and a D_{37} dose of between 6,000 Gy and 7,000 Gy for cultures in exponential phase. It is remarkable that in *D. radiodurans* Rec30 that has lost its repair capacities, the radiation sensitivity reaches similar values as in *E. coli* (23).

TERRESTRIAL RADIATION ENVIRONMENT

On Earth, the ionizing component of the radiation environment originates from primordial radionuclides of the Earth's crust, from radionuclides produced by cosmic rays in the Earth atmosphere, and for the last 100 years, from anthropogenic components of different sources, including nuclear weapons, nuclear power technology, increased radon exposure inside buildings, and medical applications of radiation and radiomimetric pharmaceuticals. In addition, our planet is continuously bombarded by charged particles coming from the sun and from sources outside our solar system. However, the surface of the Earth is largely spared from the cosmic radiation source because of the deflecting effect of the geomagnetic field and the huge shield provided by the atmosphere.

The dominant portion of energy loss in matter is contributed by the ionization of orbital electrons in the shielding material via Coulomb interaction. For protons and heavy ions, continuous deceleration can be assumed. With electrons, slowing is not continuous; straggling effects occur. Bremsstrahlung because of deceleration is important only for electrons. For higher particle energies, collisions between the primary ions and the nuclei of the shielding material represent a significant interaction. Through fragmentation of the incoming particles and disintegration of the target nucleus, new particles such as mesons, pions, secondary protons, neutrons, alpha particles, and some heavier components, which again can interact with the shielding material, are produced.

Collisions of cosmic ray particles with the nuclei of molecules of the atmosphere generate secondary radiation, creating and increasing a cascade that reaches a maximum radiation dose at an altitude of 25 km and then decreases to sea level. Mass shielding of the Earth's atmosphere amounts to 1,000 g/cm^2, thereby attenuating the cosmic radiation dose rate received at sea level by a factor of nearly 1,000, to 0.35 mSv/a (24). Hence, on Earth, the cosmogenic component contributes about one-sixth to one-tenth to the average annual terrestrial dose rate of natural ionizing radiation, which on most parts of the Earth amounts to 2.4–4.1 mSv/a. (However, in areas with a high concentration of radioactive rock components (e.g., in Kerala, India), the annual terrestrial dose rate can reach values up to 13 mSv/a). This dose rate increases with altitude; at an altitude of 10 km, the cosmic radiation rate is approximately 100 times higher than that at sea level (25). At an altitude of 25 km, the dose equivalent rates per hour are of the same order of magnitude as in Earth orbit at an altitude of about 300 km (Fig. 4).

THE RADIATION ENVIRONMENT OF SPACE

Radiation conditions in space are quite different from those on Earth. In interplanetary space, the radiation field is composed mainly of two groups: (*1*) the solar cosmic radiation (SCR) and (*2*) the galactic cosmic radiation (GCR) (26,27). Near the Earth, a third radiation component is present: the radiation trapped by the Earth's magnetosphere, the so-called radiation belts. Typical integral energy spectra for these components are shown in Figure 5.

Solar cosmic radiation (SCR) consists of the low-energy solar wind particles that flow constantly from the sun and the so-called solar particles events (SPEs) that originate from magnetically disturbed regions of the sun that sporadically emit bursts of charged particles with high energies (28). These events are composed primarily of

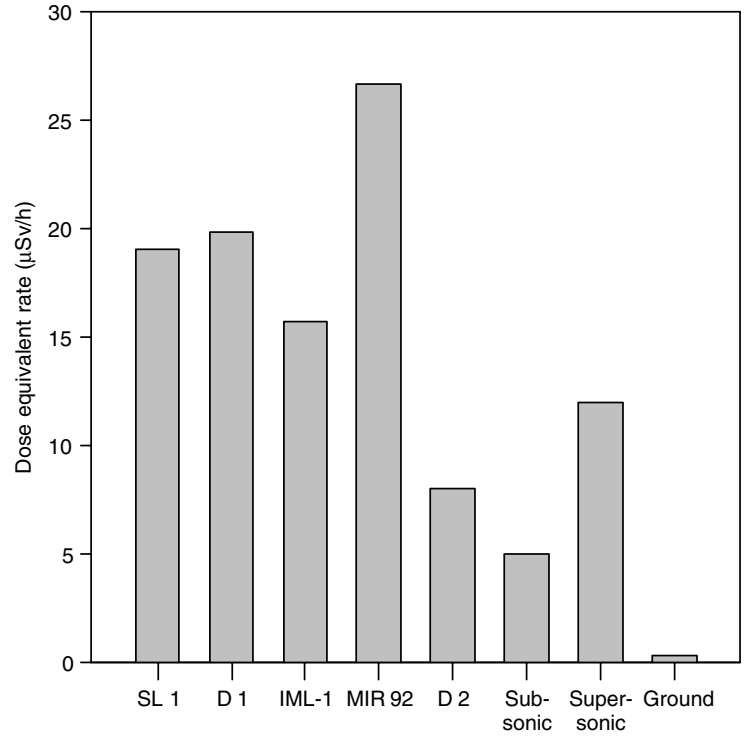

Figure 4. Dose equivalent rates (μSv/h) caused by the components of cosmic radiation in Earth orbit at altitudes of about 300 km as measured on board of Spacelab 1 (SL 1), Spacelab D1 (D 1), International Microgravity Laboratory (IML-1), the MIR station (MIR 92), and Spacelab D2 (D 2), in air traffic for subsonic conditions (about 10-km altitude) and supersonic conditions (about 25-km altitude), and at sea level on Earth.

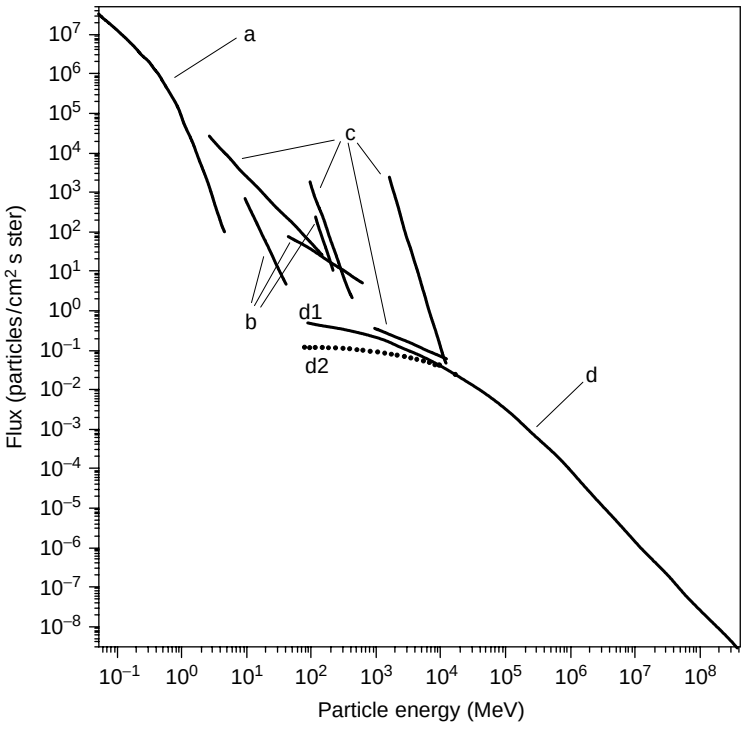

Figure 5. The energy spectra of the components of the radiation field in space near the Earth: a) electrons (belts); b) protons (belts); c) SPEs; d) heavy ions of galactic cosmic radiation; d1) during solar minimum; d1) during solar maximum.

protons, with a minor component (5–10%) being helium nuclei (alpha particles) and an even smaller part (1%) being heavy ions and electrons. SPEs develop rapidly and generally last for no more than some hours. However, some proton events observed near the Earth may continue over several days. The emitted particles can reach energies up to several GeV. Doses as high as 10 Gy could be received in a worst-case scenario within a short time. Such strong events are very rare, typically less than three events during the 11-year solar cycle. For low Earth orbit (LEO), the Earth's magnetic field provides a latitude-dependent shielding against SPE particles. Only in high-inclination orbits, SPEs create a hazard to man in space, especially during extravehicular activities.

Galactic cosmic radiation (GCR) originates outside the solar system. They have their origin in previous cataclysmic astronomical events such as supernova explosions. Detected particles consist of 98% baryons and 2% electrons. The baryonic component is composed of 85% protons (hydrogen nuclei), with the remainder being alpha particles (helium nuclei) (14%) and heavier nuclei (about 1%). The fluence of GCR is isotropic and energies up to 10^{20} eV can be present. Although iron ions are one-tenth as abundant as carbon or oxygen, their contribution to the GCR dose is substantial, as dose is proportional to the square of the charge. With increasing solar activity the interplanetary magnetic field increases, resulting in a decrease in the intensity of GCR of low energies. This modulation is effective for particles below some GeV per nucleon. The fluxes are further modified by the geomagnetic field. Only particles of very high energy have access to low-inclination orbits. Toward higher inclination, particles of lower energies are allowed. At the pole, particles of all energies can impinge in the direction of the magnetic field axes. Because of this inclination-dependent shielding, the number of particles increases from lower to higher inclination.

The radiation belts are a result of the interaction of GCR and SCR with the Earth's magnetic field and the atmosphere. Two belts of radiation are formed, comprising electrons and protons and some heavier particles trapped in closed orbits by the Earth's magnetic field. The main production process for the inner belt particles is the decay of neutrons produced in cosmic particle interactions with the atmosphere. The outer belt consists mainly of trapped solar particles. In each zone the charged particles spiral around the geomagnetic field lines and are reflected back between the magnetic poles acting as mirrors. Electrons reach energies of up to 7 MeV and protons up to 600 MeV. The energy of trapped heavy ions is less than 50 MeV. The trapped radiation is modulated by the solar cycle; proton intensity decreases with high solar activity, whereas electron intensity increases and vice versa.

The different types of radiation in space cause different amounts and kinds of biological damage per unit of absorbed dose. Therefore, the different types of radiation received in space have to be known and weighted using the quality factor Q. Figure 6 shows the contribution to the absorbed dose rate (Gy/d) and the dose equivalent rate (Sv/d) attributed to the various components of cosmic radiation. It is clearly seen that the total dose rate is about twice as high for MIR 92 than for IML-1 and the contributions of the different components of radiation to the total dose are quite different for the two missions. The reason for this difference is the higher altitude of the MIR orbit when compared to that of the IML-1 mission and a different period in the solar cycle.

While converting the radiation exposure of all different groups of radiation (however, in the absence of SPEs) to absorbed dose, the exposure during solar minimum conditions as example for the STS84 mission (orbit of the International Space Station) arrives at 132 μGy/d for the GCR and at 233 μGy/d for the radiation belt portion, in total at 365 μGy/d. For interplanetary space, slightly lower daily doses are expected behind comparable shielding thicknesses. The reason for this is the absence of the dose contribution of the radiation belt particles and an increase of the GCR part, due to the absence of geomagnetic shielding.

Absorbed dose, however, is an average quantity that does not precisely reflect the characteristics of energy deposition by a heavy ion of cosmic radiation. Heavy ions induce extremely high ionization densities in highly localized cylindrical volumes around their trajectory through matter, and therefore are capable of damaging a number of contiguous cells located along their trajectory. It is therefore necessary to determine the effect probability of the transversal of a heavy ion in dependence of its energy and charge. Some results are given later in this paper. The probability, within a three-year space mission, of a cell of the human retina (180 μm^2) or the human brain (60 μm^2) being hit by a heavy ion of $Z > 15$ is up to 0.24 or 0.08, respectively; and for ions of $Z = 26$ (iron) the numbers arrive at 0.07 and 0.02, respectively.

EFFECTS OF COSMIC RADIATION ON MICROORGANISMS

Inactivation

In space, microorganisms are exposed to the highly energetic type of radiation that may have severe consequences on their viability and the integrity of

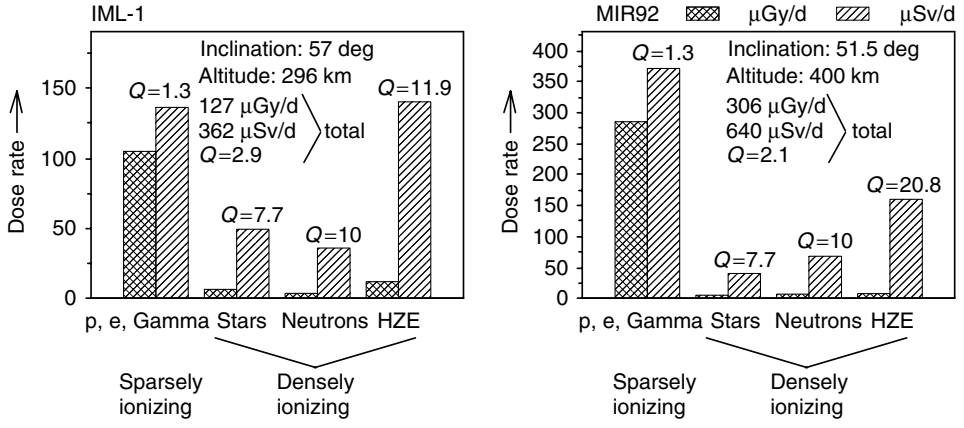

Figure 6. Absorbed dose rates (μGy/d) and equivalent dose rates (μSv/d) of the sparsely ionizing and the three densely ionizing components of the radiation field in LEO, determined from dosimetric measurements during the Spacelab mission IML-1 and the MIR'92 mission (29).

genetic information stored in their DNA. Among the ionizing components of radiation in space, the heavy primaries, the so-called HZE particles, are the biologically most effective species (30,31). To investigate the effects of single heavy ions on biological objects in a resting state (e.g., *Bacillus subtilis* spores), the Biostack concept was developed (32). In this system, biological systems are sandwiched between layers of nuclear track detectors and then exposed to the source radiation. This method helps (*1*) localize each heavy ion's trajectory in relation to the biological specimens (Fig. 7); (*2*) investigate the responses of each biological individual hit separately, with regard to its radiation effects; (*3*) measure the impact parameter (i.e., the distance between the particle track and the sensitive target); (*4*) determine the physical parameters (charge Z, energy E, and linear energy transfer LET); and finally, (*5*) correlate the biological effect with each heavy ion parameter. These studies provide important basic information on the attempts to assess the radiation risks for humans in space.

Bacterial spores, having a cytoplasmic core with a geometric cross section of 0.2 to 0.3 μm^2 are suitable test systems in HZE particle research, especially for sounding the biological effectiveness in dependence of the impact parameter for each particle's trajectory when applying the Biostack concept (Fig. 7). For this purpose, spores of *B. subtilis* (wild-type with regard to DNA repair) in suspension of 0.001% aqueous polyvinyl alcohol solution (PVA) were spread onto cellulose nitrate (CN) track detector sheets. After drying, a fixed contact of the spore monolayer with the track detector was achieved. These sheets, carrying the biological layer, were stacked between pure plastic detector sheets (32). The complete Biostack experiment package (about $10 \times 10 \times 10$ cm^3 in size) accommodated in a hermetically sealed aluminum container was stowed inside the spacecraft (Apollo or Spacelab) and exposed to cosmic radiation. Total absorption by the spacecraft walls was approximately 2.4 g/cm^2. The flux of heavy ion particles with LET $\geq$ 170 keV/μm was 2 to 3 particles/cm^2d.

After retrieval and disassembly, CN foils carrying a spore layer were etched (6N NaOH, 30 °C) only on the CN-side, resulting in an etch cone at the side opposite to the spore layer along the trajectory of an HZE particle. The procedure was followed by individual etching of each etch cone under microscopic control (pinpoint etching), until the tip of the etch cone approached the spore layer up to a few microns. By extrapolation of the etch cone, the target area in the spore layer could be determined. The target area was defined as an area of a radius of 5 μm off the point of intersection of an HZE particle with the spore layer. Spores of the target area were individually transferred onto nutrient agar by micromanipulation. During the subsequent incubation, germination, outgrowth, and growth were followed until the microcolony was formed. The original position of each spore relative to the HZE particle's trajectory (i.e., the impact parameter) was obtained from micrographs and after break through etching of the etch cone (33). The accuracy in determining the impact parameter — depending on the dip angle of the trajectory — was up to 0.2 μm, which is well below the size of a single spore. The physical parameters of the respective HZE particle, such as Z and LET, were obtained from the adjacent physical detector foils. Figure 8 shows the frequency of inactivated spores as a function of the impact parameter b. About 1,000 individual spores were analyzed. Spores within $b \leq 0.2$ μm were inactivated by 73%. The frequency of inactivated spores dropped abruptly at $b > 0.2$ μm. However, 15 to 30% of spores located within $0.2 < b < 3.8$ μm were still inactivated. Hence, spores were inactivated well beyond 1 μm, a distance that would approximately correspond to the dimensions of a spore. At a distance of 1 μm, the mean δ-ray dose ranges between 0.1 Gy and 1 Gy depending on the particle, and declines rapidly with increasing b (34). This value of 0.1 to 1 Gy is by several orders of magnitude below the D_{37} (dose reducing survival by e^{-1}) of electrons that amounts to 800 Gy. Therefore, the radial long-ranging effect around the trajectory of an HZE particle (up to $b = 3.8$ μm) cannot merely be explained by the δ-ray dose.

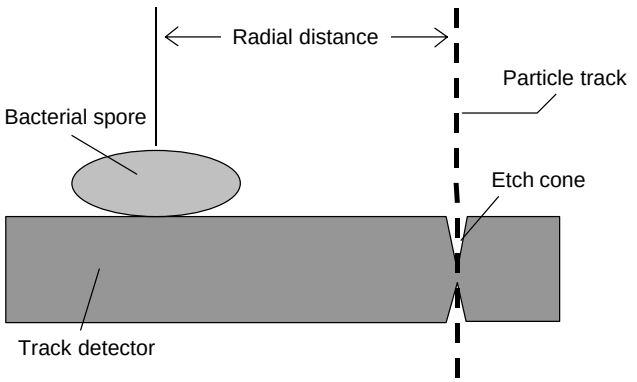

Figure 7. Biostack method to localize the trajectory of single particles (HZE particles) of cosmic radiation relative to individual bacterial spores in a spore monolayer (32).

Taking these results from the experiments in space, one can draw the following general conclusion: the inactivation probability for spores, centrally hit, is always substantially less than one. This means that heavy ions of even high LET might not necessarily kill a spore, even by a direct hit of the spore core. The effective range of inactivation extends far beyond the range of impact parameter, where inactivation of spores by δ-rays can be expected. This far-reaching inactivation of spores might reflect the so-called thindown effect at the end of the ion's path. The term is based on nuclear track detector analysis in which the track first increases in width to a maximum value and then thins down like a sharpened pencil (35), close to the stopping end of the path of a heavy ion. In this track regime, energetic δ-rays play a dominant role in the production of the cross section.

The dependence of inactivated spores from impact parameter points to a superposition of two different inactivation mechanisms: a short-ranged component reaching up to 1 μm may be traced back to the δ-ray dose and a long-ranged one that extends at least to somewhere between 4 and 5 μm off the particle's trajectory, for which additional mechanisms, such as shock waves, UV radiation, or thermophysical event (34), are conjectured.

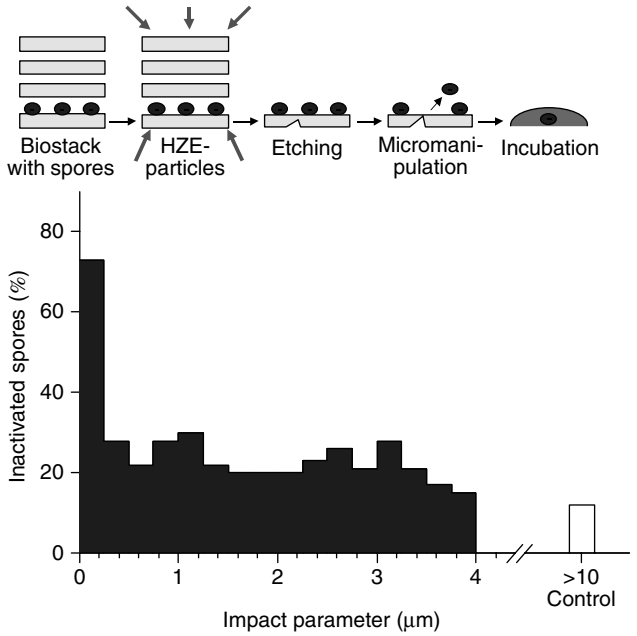

Figure 8. Results from Biostack experiments in space: inactivation probability of spores of *B. subtilis* as a function of the distance from the particles' trajectory (30). See color insert.

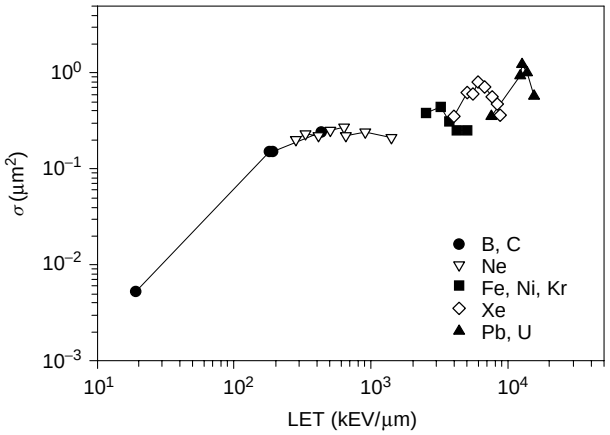

Figure 9. Inactivation cross-section σ of *B. subtilis* spores as a function of LET after irradiation of the spores with heavy ions from accelerators (36).

These results from space experiments were largely confirmed by experiments at heavy ion accelerators (36–38). The dependence of the inactivation cross-section σ of *B. subtilis* spores on the LET of the particles (Fig. 9) shows an increase of σ with increasing LET at LET $\leq$ 100 keV/μm, a saturation of σ at LET of 200–1,000 keV/μm and so-called σ-LET hooks at LET $>$ 2, 000 keV/μm. The saturation value of σ of 0.25 μm^2 that is identical for the different ions used is equivalent to the projected area of the spore core. This innermost part of the spore, containing the dehydrated cytoplasm and DNA, is most likely the sensitive site of the spore. The σ-LET hooks that occur at very high LET values are separate for each ion used and reflect the so-called thindown effect (see the preceding section for explanation). A comparable dependence of the inactivation cross-section on LET has been found for other microorganisms, such as cells of *E. coli* (38), of *D. radiodurans* (39), and of *S. cerevisiae* (40), as well as for mammalian cells (31). Double-strand breaks of the DNA (41) and bulk DNA lesions were the main cause of inactivation.

Mutation Induction

Mutagenesis is another important radiobiological end point because it directly reflects radiation damage to the DNA with the potential of severe consequences to biological diversity. Because cosmic radiation comprises heavy ions of a wide spectral range in mass and energy (Fig. 5), their mutagenic potential may vary with the physical properties of the ion under consideration. Experiments with microorganisms at heavy ion accelerations have shown that the cross-sections for mutation induction also show the "thindown" phenomenon (Fig. 10). The data are best interpreted by suggesting a "mutagenic belt" inside the track of a heavy ion that is restricted to an area, where the density of departed energy is low enough not to kill the cell (spore), but high enough to induce mutations (42,43). In this connection, due to their high inactivation potential, ions at very high LET values do not induce mutations (Fig. 10).

Repair of DNA Damage Caused by Heavy Ions of Cosmic Radiation

The involvement of DNA repair after irradiation with heavy ions reflects the cellular response to a radiation-induced DNA damage associated with the capacity to restore its DNA. It may lead to an enhanced survival of the affected microorganisms relative to those cells that are blocked in such a response. Comparing the inactivation cross-sections of spores of *B. subtilis* strains of different repair capacity, it was found that in most cases DNA repair enhanced survival after heavy ion irradiation (36). Repair based on recombination events was more efficient than repair based on repair replication. Repair is especially efficient in the extremely radiation-resistant cells of *D. radiodurans* (22,44), which therefore

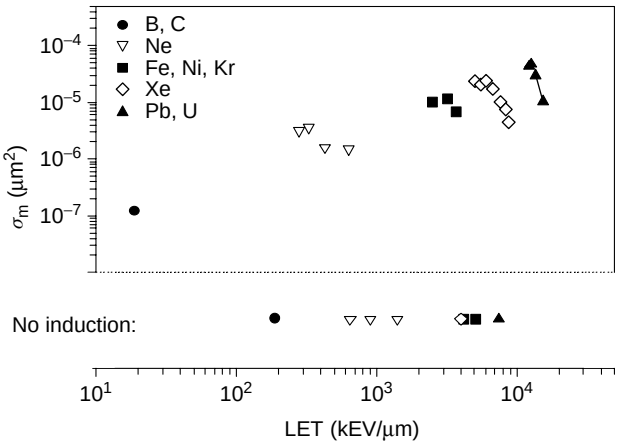

Figure 10. Mutation induction cross section into histidine prototrophy of *B. subtilis* spores as a function of LET after irradiation of the spores with heavy ions from accelerators (36).

exhibit an extraordinary ability to withstand damage induced by heavy ions (23,39).

RESPONSES TO COMBINED ACTION OF SPACE RADIATION AND MICROGRAVITY

Besides the unique radiation field in space, the cessation of the gravity stimulus that life on Earth has adapted to is another important source of potentially detrimental effects inevitably present during space flight. In response to microgravity, several essential cellular functions, such as signal transduction and gene transfer (45), are impaired [see also SPACE MICROBIOLOGY: EFFECTS OF IONIZING RADIATION ON MICROORGANISMS IN SPACE, this Encyclopedia]. In addition, synergistic interactions of radiation and microgravity have been observed in disturbances of the development of insect and plant embryos (2).

In attempts to explain these reported synergistic interactions of radiation and microgravity, it has been conjectured that microgravity might interfere with the operation of some cellular repair processes (46,47) that will result in an augmentation of the radiation response. Experimental support in favor of this hypothesis has been provided in a space experiment, utilizing a temperature-conditional repair mutant of *S. cerevisiae*, in which the extent of repair of DNA double-strand breaks (DSBs) was reduced by approximately a factor of two compared to the ground control at Earth's gravity (48). However, this observation could not be confirmed in a follow-up experiment (49). Examining several different repair pathways in different bacterial systems that were irradiated prior to the space mission, Horneck and coworkers provided evidence that in the microgravity environment, cells were able to repair radiation-induced DNA damage almost normally (50). Using the cell cultivation facility BIORACK of the European Space Agency ESA during the Spacelab mission IML-2, the following repair functions were investigated: (*1*) the kinetics of rejoining of radiation-induced DNA strand breaks in *E. coli* cells; (*2*) the induction of the SOS response in cells of *E. coli*; and (*3*) the efficiency of repair in spores of *B. subtilis* of different repair capacity. A comparison of cells that were allowed to repair in microgravity to those under gravity (one gravity reference centrifuge on board or corresponding ground controls) did not show any significant influence of microgravity on the enzymatic repair reactions studied (Fig. 11). The data suggest that the synergistic effects of microgravity and radiation in biological systems, which has been observed in several instances, probably cannot be explained by a disturbance of intracellular DNA repair in microgravity.

Therefore, other mechanisms have to be postulated for the synergistic action of microgravity and radiation: (*1*) at the molecular level, the consequences of a convection-free environment, (*2*) at the cellular level, an impact on signal transduction, on receptors, on the metabolic–physiological state, on the chromatin, or on the membrane structure, and (*3*) at the tissue and organ level, modification of self-assembly, intercellular communication, cell migration, pattern formation, or differentiation.

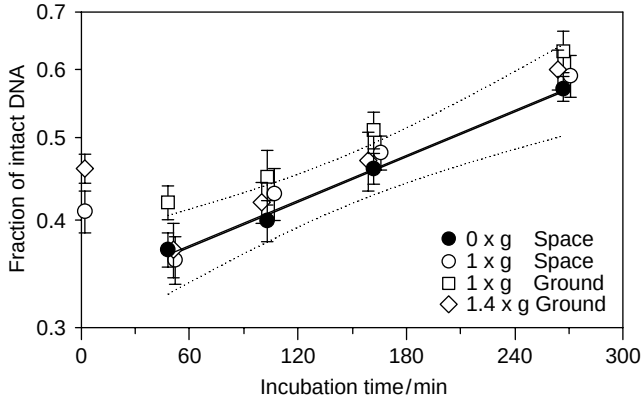

Figure 11. Repair of radiation-induced DNA damage under microgravity conditions: Rejoining of DNA-strand breaks in cells of *E. coli* B/r that have been irradiated by X-rays prior to the spaceflight at low temperatures, and incubated when in space [modified from (50)].

CHANCES OF INTERPLANETARY TRANSFER OF LIFE

The question of the chances of interplanetary transfer of life, initially termed "Panspermia" by S. Arrhenius (51), has recently received increased attention, especially since the detection of meteorites that originate from Mars. As shown earlier, *B. subtilis* spores can survive even a central hit of a heavy ion of cosmic radiation. Such heavy ions of cosmic radiation are conjectured to set the ultimate limit on the survival of spores in space because they penetrate even thick shielding. A spore's maximum time to escape a hit by an HZE particle (e.g., iron of LET > 100 keV/μm) has been estimated to be several hundred thousands up to a million years. This time span complies with estimates for boulder-sized rocks to travel from one planet of our solar system to another (e.g., from Mars to Earth) by random motion (4). However, it has been estimated that a few months are sufficient for an interplanetary transfer of microscopic particles (52).

Concerning shielding against radiation in space, a few micrometers of meteorite material are sufficient to give efficient protection against extraterrestrial UV, if the material is without cracks. Less than 0.5 g/cm^2 shielding is required to protect against the diffuse X-rays. Both the high-energy particles in SPEs and the GCR particles are more difficult to shield against because of their high penetration depth in material and because of the creation of secondary particles in nucleus–nucleus interactions. In fact, the dose increases with shielding depth before absorption becomes the dominant process.

On the basis of experimental data from accelerator experiments with *B. subtilis* spores (36), an estimated density of 3 g/m^3 for meteorite material (taken from data of Martian meteorites) and a NASA model on the HZE component of cosmic radiation (53), Mileikowsky and coworkers (54) have calculated the dose rate behind different thicknesses of shielding. The dose rate reaches its maximum value behind a shielding layer of about 10 cm; behind 130 cm it is about the same as that obtained without any shielding. The dose rate is significantly reduced only for higher shielding thicknesses (Fig. 12). The

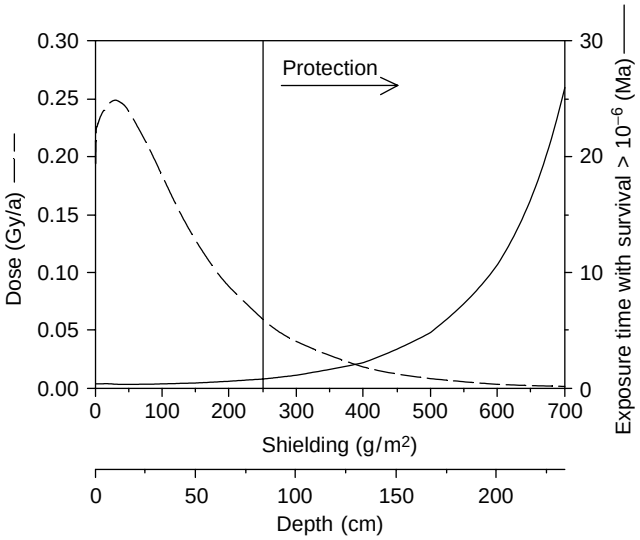

Figure 12. Shielding of bacterial spores against galactic cosmic radiation by meteorite material (54).

calculations also show that even after 25 million years in space, a substantial fraction of a bacterial spore population (10^{-6}) would survive the exposure to cosmic radiation if shielded by two to three meters of meteorite material. The same fraction would survive after about 500 thousand years without any shielding, and after 350 thousand years after one meter of shielding. However, one has to bear in mind that cosmic radiation is only one of the parameters of the hostile space environment. For a successful viable transfer throughout our solar system, microorganisms have to cope with the full matrix of all environmental stressors governing the space milieu.

ENVIRONMENTAL RADIATION AND PUTATIVE HABITATS ON MARS

There is growing evidence that the physical and chemical surface properties of early Earth and Mars were very similar (55) and that before 3.5 billion years ago and even sporadically in more recent time periods, the climate on Mars was wet and more temperate, allowing the presence of large quantities of water on its surface (56). At that time, life had already started on Earth. Under the assumption that life emerges at a certain stage of planetary evolution if the right environmental physical and chemical requirements are provided, it is legitimate to assume that conditions on early Mars were as favorable for life to emerge as on early Earth. A closer look at the radiation climate of Mars and its effectiveness on terrestrial model systems may provide clues for estimating the habitability of Mars, in early epochs or even today.

The radiation climate on the surface of Mars is governed by the following components: (*1*) solar UV radiation, including the UVC and UVB range ($\lambda >$ 200 nm); (*2*) charged particles of galactic and solar origin, as well as neutral secondaries; and (*3*) emissions from radionuclides of Mars itself. The biological effects caused by solar UV-radiation are considered elsewhere (57); this paper will concentrate on the ionizing components. Because of low shielding by the Martian atmosphere, 5 to 16 g/cm^2 depending on altitude, and the lack of a magnetic field, a substantial portion of cosmic radiation reaches the surface of Mars. In addition, highly energetic (up to several GeV) solar particle events consisting of protons, helium nuclei, and a small portion of heavier ions occur sporadically above all at the end of the solar maximum. Therefore, the biologically effective annual dose rate amounts to 0.1 to 0.2 Sv/a, depending on the altitude (58). The intensity of cosmic radiation is also a function of the direction of the incident particles: with increasing zenith angle, the amount of protection increases. With increasing depth, the regolith provides a certain radiation protection, although one has to consider the secondary radiation produced.

Although the radiation dose rate on the surface of Mars is about 100 times higher than that on Earth, it cannot be considered a limiting factor for microbial life on Mars. A terrestrial example is the most radiation resistant bacterium *D. radiodurans*. It can tolerate radiation doses up to 3,000 Gy without any significant inactivation (39,44) (Fig. 3). Thus, the annual dose rate on Mars is three to four orders of magnitudes lower and would not impair growth of microorganisms possessing potent DNA repair systems. Likewise, *B. subtilis* spores with a D_{37} of 600 Gy would be able to survive the radiation exposure on the Martian surface for extended periods. If microbial life once existed on Mars, spores could be suitable candidates to survive dry interim periods between two wet epochs that have been suggested to have occurred during Martian history (59).

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SPACE MICROBIOLOGY: MICROGRAVITY AND MICROORGANISMS

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Both the opportunity and the need to characterize the effects of reduced gravity on living organisms accompanied the advent of manned spaceflight. In addition to studying how cosmonauts and astronauts fared, microbiological experiments were "piggybacked" on many of the early Soviet and U.S. missions. Zhukov-Verezhnikov and coworkers summarized the results of the "first microbiological experiments in space" by concluding that, "on flights similar to the orbit of the spaceship Vostok I, there is practically no effect from factors capable of primary action on isolated cells" (1). In fact, based on early theoretical analyses, many scientists did not expect to find any effect of microgravity on single cells smaller than about 10 microns (2). Despite this prediction, a general synopsis of the research over the past few decades reveals that many differences do occur in microbial growth and behavior as a result of spaceflight, presumably being due primarily to weightlessness. An overview of the literature also reveals inconsistencies in the findings reported by different investigators and in the interpretation of the data. Infrequent spaceflight opportunities, complicated by varying experimental conditions, add to the difficulty of establishing reproducible results and drawing consistent conclusions. As a result, the regulating mechanisms responsible for the changes observed in microbes caused by spaceflight remain largely unknown. The expanding volume of characterization data, however, is now paving the way toward hypothesis-driven research aimed at isolating variables and identifying causal factors.

Numerous microbial spaceflight experiments have been performed since the 1960s onward. Much of the earlier research has been previously documented in the following articles, cited here for reference (3–14). This current

Table 1. Synopsis of Findings Reported in the Primary Literature Reviewed in this Article. Differences, where Noted, Represent Effects Induced by Spaceflight Relative to Ground Controls

	References
Bacterial Suspension Cultures	
Shorter lag phase	19,21–24
No change in lag phase	20,24[a]
Increase in growth rate, shorter doubling time	19–21
No change in growth rate	22,23
Increase in duration of growth phase	23
Higher final populations, increased proliferation, increase in biomass	16,17,19,23
"Stimulating effect on growth" reported as a secondary observation	6,27–31
No change in growth yield, cell mass or SOS expression	35
Genetic transfer efficiency (conjugation) enhanced	29
Antibiotic effectiveness decreased	6,22,27,28,30,31
Variable antibiotic resistance noted after long duration mission	52
Cell wall thickness after flight — variable results	6,20,22,27,30,53
Spore formation — variable results	19,20
Bacterial Surface Growth Cultures	
Shorter lag phase	37,39
Slower growth rate	37
No change in growth rate	39
Larger cells with decrease in length-to-width ratio	37
No change in final population	39,40
Reduced final population	37
Biofilm growth	61,62
Eukaryotic Cells	
Increased growth rate and cell volume — Paramecium	43
Gravity sensing thresholds observed — various	44,49
Gene expression altered — human cortical renal cells	50
Increased secondary metabolite productivity (antibiotics)	64
Human–Microbe Interactions	
Altered immune system function	54
Increase in clinically significant bacterial isolates	55
Occurrence of infection	56
Dermatology issues	57
Microflora exchange (in vivo)	58,59

[a] specifically for a motile strain

summary focuses primarily on describing findings from literature published from the mid-1980s through the 1990s, and a short synopsis of the results is provided in Table 1.

GROWTH KINETICS AND MOTILITY

Microorganisms are highly dependent on intracellular and environmental parameters. Cellular movement within a liquid medium becomes important if one considers a microbe to be a point source, excreting metabolic waste products and consuming nutrients with a resulting sphere of molecular concentration gradients extending radially outward from the cell. The availability of nutrients and the removal of metabolic by-products in suspension cultures are, in turn, affected by various forces acting on the cell (sedimentation, diffusion, motility, etc.) as well as on the bulk fluid (diffusion, convection, etc.). To fully characterize the influence of gravity on living systems, it must be isolated and treated as an independent variable.

Gravity results in a constant sedimenting force on particles, dependent on density differences with the surrounding environment. Brownian motion, or diffusion, imparts random movement to particles due to thermal energy, and is independent of gravity. Brownian movement has been theoretically described as a counterbalancing force to sedimentation in the case of particles less than about 0.5 microns in diameter and below a density difference of about 0.03 g/cm^3 (15). On the basis of these criteria, nonmotile bacteria suspended in an aqueous medium fall just over the threshold into being capable of being influenced by gravity. Although random Brownian movement of the cells is still greater than that due to gravity at any given instant, the cumulative and unidirectional force of gravity progressively moves the cells toward the bottom of the container. Stirring the culture will, of course, also serve to maintain the cells

in a state of suspension as occurs in weightlessness, but as opposed to the quiescent environment encountered in space, stirring imparts additional motion to the cells and mixes the medium; thus consequentially adding variables, rather than helping to isolate them.

Theoretical prediction of the overall outcome of weightlessness becomes uncertain at the level of single cells on the threshold of gravity's influence. To attribute differences in cellular behavior to weightlessness, all aspects of gravity-driven phenomena must be considered. Gravity produces weight and/or sedimentation of the cell and/or its internal components. Gravity also causes changes in the external fluid environment (liquid or gas) surrounding the cells. The effects can largely be characterized as internal or external phenomena and are commonly referred to as *direct* and *indirect*, respectively. Delineation by primary (weight), secondary (sedimentation), and tertiary (environmental) effects is also sometimes used to describe various responses to gravity.

The majority of the investigations discussed later in this chapter deal with prokaryotes. The principal gravity-dependent factors governing the extracellular environment of bacterial cells can be expected to similarly affect eukaryotic microbes, but these larger and more complex organisms introduce added complexity associated with gravity-driven responses (including sensing) of intracellular components. Discerning the so-called internal (direct) from the external (indirect or environmental) variables presents unique challenges that are not fully explored in this review.

Early Spaceflight Bacterial Growth Experiments

Mattoni was the first to report that *Escherchia coli* growth in a liquid medium produced "significantly denser populations as a result of spaceflight than identical cultures on earth" (16). Speculation into what caused the increase was suggested to be related to altered nutrient transfer into and waste transport out of the cell as a result of the reduced gravity environment. Elaboration on this theory continues into the present era. This and other research, conducted during the 1968 U.S. Biosatellite II mission, were reported in a dedicated issue of BioScience (June 1968, Vol. 18, No. 6). Mesland summarized the results from the STS 61-A (Spacelab D-1) BIORACK experiments by noting that, "microgravity increases proliferation in bacteria and unicellular organisms" (17). This conclusion was termed "quite unexpected," with the underlying mechanisms remaining basically unknown.

Alpatov and coworkers discussed the results from several experiments performed on the biosatellite COSMOS-1887 and summarized the cellular biology goals as including "searching for gravity-dependent processes in the cells and nonspecific gravity sensors and identifying adaptive changes in the cell, occurring in weightlessness" (9). In the same journal volume, Parfenov presented the general conclusion that "individual one-celled organisms showed no signs of any gravity-dependent processes" (18). Seemingly, contrasting interpretations of data such as these exemplify the controversy currently surrounding the specific role(s) that gravity plays at the cellular level.

Regardless of the various primary experimental objectives and subsequent conclusions drawn from the data, the majority of investigations to date have indicated that weightlessness does appear to influence microbial growth and behavior. In an attempt to characterize the specific effects of spaceflight on microbial growth kinetics, each stage of growth — lag, exponential, and stationary — can be individually examined. Other factors, such as suspension versus agar cultures and motile versus nonmotile cells, should also be considered separately. Care should be taken to avoid interpreting reports of a higher "final population density" being reached to imply that a faster "growth rate" occurred, or vice versa. Ultimately, however, when attempting to establish cause-and-effect relationships, the common underlying biophysical parameters associated with reduced gravity must be consistently held accountable in governing all aspects of observed altered responses.

Transition from Lag to Exponential Phase in Suspension Cultures

Several investigations have indicated that spaceflight results in a shortened period of lag following on-orbit inoculation. A shortened lag phase on orbit (based on the observation that the maximal rate of increase in optical density occurred about five hours earlier in the flight unit compared to the ground control for *Bacillus subtilis* suspension cultures) was reported by Mennigmann and Lange (19), although a follow-on experiment failed to confirm the results. Mennigmann and Heise (20) later reported that the earlier finding may have been an artifact of specific test conditions.

Although not specifically reported as such, interpretation of the growth curve data presented for two other experiments using *E. coli* also indicated the possibility of a shorter lag phase having occurred on orbit (21,22). Through the use of on-orbit optical density recording, Klaus and coworkers observed the lag phase for suspended cultures of nonmotile *E. coli* to end four to eight hours sooner than for synchronized and comparably maintained ground controls (under conditions where the transition from lag to growth typically occurred 24 hours following inoculation) (23). Thévenet and coworkers also reported a shorter lag phase for suspended bacterial cultures on orbit, using a nonmotile strain of *E. coli* (24). In contrast, however, it was shown that lag phase was not affected in a complementary experiment using a motile strain of *E. coli*. This presents a significant observation, indicating that motile bacteria may be less susceptible to the indirect effects of sedimentation because of the overriding swimming motion.

Berg and Turner (25) showed that wild-type motile *E. coli* swim randomly at velocities of ~30 μm/sec, whereas the concurrent velocities driven by sedimentation and diffusion are calculated to be ~0.1 and ~0.5 μm/sec, respectively (23). As the movement of the cell due to sedimentation is estimated to be two orders of magnitude less than that of motility, it is presumed that the absence of gravity is unlikely to create noticeable

environmental (indirect) effects on a swimming organism. For a nonmotile bacterium, however, the absence of gravity limits cell motion to the forcing function of diffusion only, resulting in a less mixed extracellular fluid environment. Over time, this more quiescent state is suggested to alter the chemical gradients surrounding the organism and induce a corresponding physiological response. For example, one mechanism theorized to bring about the end of the lag phase is that excreted cofactors, such as enzymes, must reach a requisite concentration in the extracellular medium before the cells begin doubling (26). A decrease in net mass transport in the medium may allow these compounds to build up within a quasi-stable microenvironment surrounding the cell, given the absence of convection and sedimentation in weightlessness, thus signaling that the requisite concentration has been reached sooner than if the compounds were being further diluted throughout the bulk medium in normal gravity. This physical phenomenon is suggested to be the cause of the shortened lag phase duration in space (23). Thévenet and coworkers proposed that it is possible that the absence of gravity allows a small "cloud" of dissolved carbon dioxide to accumulate around the bacterial cell, which consequently allows it to come out of lag phase sooner than ground controls (24). This is consistent with laboratory studies that have shown that a lack of dissolved carbon dioxide in the medium can increase the lag phase (26). The proposed cascade of events described earlier in this section (removal of physical force due to gravity → altered chemical gradient around the cell → unique physiological response) exemplifies what are typically referred to as indirect effects of gravity.

Growth Rate and Final Population of Bacterial Suspension Cultures

The first observations of increased bacterial growth rate on orbit were made during the STS 61-A (Spacelab D-1) shuttle mission. Mennigmann and Lange reported an increase in both the rate of growth and in total final biomass yield of *B. subtilus* in space (19). In a follow-on experiment, Mennigmann and Heise reported the growth rate for *B. subtilis* on orbit was initially similar to that of ground controls, but later increased to about twice the growth rate observed in the controls (20). Other investigations have found the growth rate for *E. coli* on orbit to be similar to the ground controls (22) or statistically unaffected (but exhibiting a slightly slower trend) with the duration of the exponential phase on orbit, being two to five hours longer than the matched ground controls (23). Higher final cell population densities have been reported to occur for space grown bacterial suspension cultures reaching the stationary phase (16,19,23). Additional mention has also been made of a "stimulating effect on bacterial multiplication in space conditions" in other experiments not intended specifically to evaluate growth kinetics or population density per se (6,27–31).

Interestingly, similarities can be observed between the enhanced growth kinetics of *E. coli* batch cultures grown in space and specific bioprocessing characteristics achieved terrestrially through the use of a fed-batch glucose method. It has been demonstrated that using a controlled feeding technique (on earth) to limit the availability of glucose to the cells results in a slower, prolonged growth phase and a subsequent increase in final cell concentration (32,33). It is suggested that a reduction of extracellular mixing in the quiescent fluid environment experienced in the weightless environment of space may give rise to similarly reduced levels of glucose molecules being present in the (nonmotile) cell's immediate surroundings, albeit under normal batch conditions (34).

Data reported from an experiment performed on Biocosmos-2044, however, contradict the findings of increased growth rate and final yield. Bouloc and D'Ari found no differences between spaceflight samples and ground cultures in the growth yield per gram of carbon, in mean cell mass, or in the level of expression of the SOS response (35). Growth yield was not measured directly, but was determined using the relationship that mean cell volume increases proportionally to growth rates for *E. coli* (36). In drawing definite conclusions from these data, consideration needs to be given to several additional factors. Although growth rates were not directly reported here, it was stated that a minimal medium was used for this flight experiment. For growth rates less than one doubling per hour (as might possibly be expected using a minimal medium), the average cell mass tends to become constant (36). Therefore, if the flight and ground culture growth rates were different, but they were both undergoing near or less than one doubling per hour, that difference would presumably not be detectable based on cell volume alone. In addition to the potential of whether this may or may not have been taken into account, Manko and coworkers reported that *Proteus vulgaris* cells were shown to exhibit a slower growth rate on orbit (albeit on agar, further described later in the chapter), but the flight cells tended to be larger and showed a decrease in length-to-width ratio compared to the ground controls (37). This suggests that the normal ratio of cell volume to growth rate may be altered by other factors associated with spaceflight that could skew the otherwise predictable relationship referred to earlier in the chapter. Finally, gravity-dependent factors associated with hardware used to contain the samples may also influence growth kinetics, particularly of the ground controls. For example, bacteria grown in tall, vertical test tubes have further to sediment relative to otherwise comparably treated cultures grown in short, flat containers. The differing distances that cells are allowed to "fall" before reaching the container bottom may play a role in whether or not a cumulative response to gravity is realized. As such, a thorough description of the hardware used is integral to interpreting results from space research, but unfortunately, is not always provided in sufficient detail.

In a later study intended to examine the effects of microgravity on the microenvironment and membrane transducing pathways of the cell, Gasset and coworkers reported observing a doubling time of 46 minutes for *E. coli* on orbit versus 59 minutes on the ground (21). However, it was concluded that microgravity had no effect on the growth rate as the flight cells were not correspondingly larger in size as might be expected for

faster growing cells (36). Again, the final conclusion that no differences occurred may be debatable, depending on the factors described earlier in the chapter. Considering the potential for cell dimension changes, the doubling time measurement, which indicated that a significantly higher growth rate occurred on orbit, possibly provides a more accurate metric.

Bacterial Cultures Grown on Solid Substrate

Bacterial cultures grown on semisolid agar have similarly been reported to exhibit a shortened lag phase on orbit. In an experiment performed on Salyut-7, Manko and coworkers reported that all flight samples of the bacterium *Proteus vulgaris* cultivated on semisolid agar exhibited a considerably shorter lag phase, as well as a higher level of metabolic activity (37). Because the cells were grown on a solid substrate, the environmental effects of bulk fluid behavior were mitigated; therefore, it is presumed that the cells must have been "directly" affected by gravity. Swarming behavior was studied by inoculating cells onto meat peptone and monitoring the rate of ring-shaped colony formation with an increase in activity noted for the flight samples. As previously referred to, the authors also reported an increase in size of the flight cells, along with a gradual reduction in the ratio of length-to-diameter not seen on the ground. Interestingly, in the same journal volume it was concluded that although a number of experiments have shown that spaceflight does affect microorganisms, theoretical analysis maintains that gravity does not have a direct influence on individual cells smaller than 10 μm (38). Kacena and Todd also reported a shortened lag period, but no difference in growth rate on orbit for *E. coli* or *B. subtilis* when grown in space on an agar medium (39). Final population density for bacterial cultures grown on agar, however, does not appear to be increased by spaceflight (39,40), and may even be reduced (37).

Simulating Weightlessness by Clinorotation

A device called a clinostat can conditionally provide a state referred to as "simulated weightlessness" (41). For suspension cell cultures, this is achieved by rotating a cylinder completely filled with liquid medium (i.e., no air space) containing the cells at a constant velocity. After a brief start-up period, the rotational velocity of the container wall is transferred radially inward until no relative fluid motion exists. Therefore, although gravity remains present, the g-vector is continually being reoriented when the fluid and cells rotate together like a rigid body. Within mathematically defined constraints, the suspended cells can theoretically remain "motionless" with respect to their (rotating) fluid environment as they do in actual microgravity (42). However, the force of gravity still acts on cells in a clinostat, so the total effects of convection and sedimentation are reduced but not eliminated. The "direct" effects of gravity, presumably, causing events such as stress on the cellular membrane or internal organelle displacement, are still present, although in a constantly changing direction, which may, in fact, add additional stress on the organism.

Clinostat data pertaining to effects on bacterial growth generally appear similar to those from actual spaceflight (16,20,39,42), but this may not always be true for larger organisms having internal components with varying densities. Factors including gravity-threshold phenomena, nonlinear relationships and biophysical parameters must all be carefully considered in assessing the applicability of clinorotation to simulate weightlessness for a given system.

Eukaryotes

Not surprisingly, larger, more complex eukaryotic microbes have been shown to exhibit various responses that are perhaps more directly influenced by gravity. The ciliates *Paramecium* and *Loxodes* use gravity as an environmental stimulus governing orientation (gravitaxis) and swimming velocity (gravikinesis). Although these phenomena are well characterized in terms of their occurrence, the cellular mechanisms responsible for sensing gravity are not fully understood.

Planel and coworkers first reported an increase in the growth rate and cell volume of *Paramecium tetraurelia* cultivated onboard Salyut-6 (43). Factors such as temperature, launch acceleration and vibration, and cosmic radiation were ruled out through measurement and controls. Similar to the bacterial research, it was hypothesized that weightlessness would result in randomly distributed waste and nutrients that may indirectly serve to enhance the growing conditions of *Paramecia* in space. A second hypothesis was posed that the weightless condition may also play a more direct role by reducing the energy normally required for "upward" motility, thus allowing the metabolism to be directed more toward reproduction.

Using an onboard centrifuge (NIZEMI) to observe the swimming orientation of *Paramecia*, Hemmersbach and coworkers showed that a threshold for negative gravitaxis exists somewhere between 0.16 g and 0.3 g (44). In contrast to the *Paramecia*, *Loxodes* did not react to accelerations in space, although they exhibited typical graviresponses at hypergravity and normal gravity before and after flight. It was not clear if other factors affected the outcome. No indication of the microorganisms having adapted to the space environment was observed in these experiments, further corroborating previous "nonadaptive" findings for *Paramecia* (45,46), *Loxodes* (47), and *Euglena* (48). Studies of an acceleration sensitive contraction period exhibited by the *Myxomycete* (acellular slime mold) *Physarum polycephalum* indicated that a threshold of sensitivity exists for this organism at as low as 0.1 g, and that any acceleration above the threshold can induce the complete response-regulation process (49).

The indication of response thresholds further suggests that gravity-dependent processes are likely to be nonlinear, thereby complicating direct extrapolation of results among hypergravity, normal gravity, and hypogravity studies. Through the analysis of viscosity and density parameters of cellular components, clues may be found to help identify candidate gravisensors within the organism as functions of the specific levels of acceleration found to elicit the observed responses. The exact underlying mechanisms by which the reported gravity perception is brought

about, however, remain largely undetermined. Ultimately, other factors, such as launch acceleration, on-orbit vibration, and radiation, must also be taken into account to fully discern the influence of gravity on living organisms. Onboard centrifuge controls can also be useful in isolating the effects caused by these variables.

GENETICS

The effects of spaceflight on three basic mechanisms for genetic information exchange — conjugation (cell-to-cell), transduction (bacteriophage-to-cell), and transformation (free DNA fragment-to-cell) — have also been investigated by using *E. coli* on the STS 61-A (Spacelab D-1) shuttle mission (29). In the case of conjugation, although no differences were found in the rate of transmission of early markers (ile 0.8 min), later markers (arg 27.1 min, and pur 38.6 min) appeared to be transmitted more efficiently under microgravity conditions, with three to four times as many recombinants compared with ground controls and flight controls in an onboard 1 g centrifuge. The hypothesized explanation was that mating pairs of aggregates were less likely to be disrupted in the quiescent microgravity environment, thus giving rise to higher recombination frequencies for the later markers. Because transfer of early markers did not appear to be enhanced, it was assumed that the actual formation of mating pairs or aggregates was not impacted. No significant difference was noted for transduction and it was suggested that the bond between the cell and the phage is more stable and less sensitive to stresses. No conclusion was drawn from the transformation experiment because of the unexplained variations in viable counts and in the number of transformants. In addition to the specific reported test findings, an "increase in cell number" was observed under microgravity. Even taking into account the higher cell concentration, a difference in the transmission of the later characters was evident.

Although not directly within the purview of a microbial review, an interesting observation regarding gene expression in a steady state culture of human renal cortical cells has recently been reported (50). It was noted that more than 1,632 genes were changed in the microgravity environment. Whether or not similarly induced alterations might extend to microbial systems remains to be determined.

ANTIBIOTIC EFFECTIVENESS

Other investigations have shown that (in vitro) bacterial cultures grown in space are able to proliferate in the presence of normally inhibitory concentrations of antibiotics.

The Cytos-2 experiment flown on Salyut-7 in 1982 revealed that a fourfold increase in Minimal Inhibitory Concentrations (MIC) of colistin and kanamycin was necessary to stop the growth of in-flight *E. coli* samples compared to ground controls (6,27). The MIC for chloramphenicol and erythromycin was also higher in cultures of *Staphylococcus aureus* on orbit. No retention of the increased resistance was noted in postflight tests, however. A follow-on experiment was flown aboard the Spacelab D-1 mission in 1985. The MIC of colistin was twofold higher for the flight cultures (28,30,31). A 100-fold increase in the number of viable cells was also observed in the flight samples, which were given sub-inhibitory levels of antibiotics, compared with similar ground controls. In a third experiment conducted in 1992 on the Spacelab IML-1 mission, a higher MIC was again observed using dihydrostreptomycin for in-flight of *E. coli* cultures, and a higher growth rate was reported in cultures using a sub-inhibitory level of antibiotics (22). However, no difference was found in either experiment between onboard samples centrifuged at 1 g and those exposed to microgravity. Although this finding may be unique in suggesting that differences may be attributable to spaceflight factors other than gravity, it is important to point out that the 1 g flight controls were not placed on the centrifuge until five days (31) or eight days (22) after launch. Using the same equipment, Gasset and coworkers noted that because the "controls were not placed on the centrifuge during the long storage period they could have been [prematurely] influenced by possible microgravity effects" (21). Complementary ground-based studies of antibiotic resistance at three sub-inhibitory levels of hypergravity (2,5, and 10 g) showed no significant difference in growth rates of bacteria cultivated with or without sub-inhibitory concentrations of dihydrostreptomycin when compared to one gram controls (51). These findings suggest that differences observed in reduced-g are not likely to be linearly extrapolated into hyper-g regimes.

Juergensmeyer and coworkers exposed four species of bacteria to spaceflight for a four-month duration aboard the Mir space station and then tested postflight susceptibility to a suite of 12 antibiotics (52). The data indicated that each species responded differently to the antibiotics, frequently becoming less resistant, but occasionally more, with no discernible pattern identified.

Different theories have been proposed to explain the reduction of antibiotic effectiveness on orbit. Perhaps the simplest explanation is that the reduced antibiotic effectiveness observed on orbit is not so much a result of increased resistance by the microbe, but more a consequence of enhanced growth (30,31). Observations that in-flight cultures of *S. aureus* had "a very marked increase of the thickness of the cell wall" led to the theory that some antibiotics may be rendered less effective in space by the modifications in the cellular envelope (thickness or permeability); however, no change was found in the cell wall thickness of *E. coli* (6,27,30,53). More recent experiments have further indicated that no change occurred in the cell wall thickness of *E. coli* (22) or *B. subtilis* (20) in flight. A definitive explanation of how bacteria are able to proliferate in normally inhibitory levels of a drug on orbit has yet to be established.

CREW HEALTH AND LIFE SUPPORT

In addition to the in vitro antibiotic effectiveness studies, changes in the human–microbe interaction in space represent another critical, yet not well-understood phenomenon. Data exist to suggest that the human

immune system, both cell mediated and humoral, may be altered by spaceflight (54). Pierson and coworkers showed that the incidence and total counts of clinically significant bacterial isolates from urine samples collected from 144 astronauts, spanning 25 space shuttle missions, were somewhat greater postflight when compared with that in preflight baselines (55). Outbreaks of diarrheal illness have been reported onboard the U.S. shuttle and at least two life threatening infections, one Soviet and one United States, have occurred during space travel, with one case resulting in a long duration mission being terminated early because of an "acute inflammatory disease" (56). Dermatology concerns in space may also arise from the difficulties of bathing combined with the potential for facilitated microbial growth, and little is known about percutaneous delivery of pharmaceutical agents during spaceflight (57).

Isolation of individuals in an airtight environment presents a separate factor that must be considered in the spacecraft environment when studying drug resistance acquired via in vivo microflora exchange. Taylor and Zaloguev conducted an experiment during the Apollo-Soyuz Test Project to assess possible bacterial transfer in the confined capsule environment (58). The results of postflight incubation showed that microflora samples from crew members collected during the mission presented a higher resistance to antibiotics than the pre- and postflight samples. Il'lin reported that the majority of coliform bacterial cultures sampled postflight from the Salyut-7 crew were resistant to tetracycline, yet there had been no resistant strains in the preflight samples, suggesting that those found later had arisen from microbial and/or plasmid exchange with visiting crew members (59).

Finally, advanced human life support systems planned for long duration space habitation will probably utilize regenerative biological organisms to recycle air, water, and food. These systems will need to be monitored for functionality and pathogenicity. Wastewater treatment represents one such critical application that will potentially be highly dependent on microbial processes (60). Biofilm formation presents a related microbiological concern that has begun to be analyzed for effects arising from spaceflight. One experiment indicated that biofilms formed on orbit appeared to accumulate somewhat, but not statistically significant, higher bacterial areal densities than ground controls (61). It has also been reported that *Burkholderia cepacia* (61) and *Pseudomonas aeruginosa* (62) were able to grow and form biofilms in space in the presence of low concentrations of chemical disinfectants.

The unique environment encountered during spaceflight may significantly alter the normally stable, symbiotic relationship between humans and microbes, and also affect the likelihood, spread and treatment of pathogens throughout a closed spacecraft. Understanding and controlling these phenomena represent critical steps toward maintaining a healthy and sustainable habitat for long duration human exploration of space.

SPORULATION AND SECONDARY METABOLITES

Although most investigations into microbial behavior in space to date have sought to characterize the observed microbial responses as they pertain to fundamental gravitational biology questions and immunological concerns, applied research encompassing space biotechnology is beginning to gain momentum. In addition to the changes in general microbial growth and behavior reported to occur in space, altered sporulation patterns and specific productivity of secondary metabolites have also been observed. In theory, sporulation and secondary metabolite formation compete for common nutrients (63); therefore, unique or related changes observed in either process may give insight into if and how gravity alters the cellular metabolic pathways.

Mennigmann and Lange reported substantially reduced spore formation on orbit by *B. subtilis* when compared with ground controls (19). The number of spore-producing cells was also reportedly much lower on orbit. A follow-on experiment carried out by Mennigmann and Heise produced conflicting results, however, with 60% of the *B. subtilis* flight cells producing spores compared to only 15% in the ground controls (20).

A pilot study aimed at quantitatively assessing secondary metabolite production in space was carried out onboard STS-77 using the eukaryotic fungus *Humicola fuscoatra* to produce an antibiotic called *monorden* (64). Two different agar-based media, defined and complex, were used to grow the fungus. In both media, an equivalent amount of fungal biomass produced up to 190% higher specific yields of the antibiotic when cultured on-orbit than in controls maintained under similar conditions on Earth. As the fungus was grown on a semisolid agar medium, the increased antibiotic production in space could not be attributed to a lack of cellular sedimentation. Therefore, a direct effect of reduced gravity on the organism itself or physical alterations affecting other subcellular level processes pose more likely explanations (e.g., altered fluid uptake from the agar or convective currents at the surface). In addition, a disparity of results noted between the different types of media used (defined versus complex) further corroborates the theory that microbial response to spaceflight may be medium dependent (20).

Related antibiotic production investigations have been performed using a High-Aspect Rotating Vessel (HARV) bioreactor on the ground. This special bioreactor is similar to a clinostat in attempting to simulate certain facets of the microgravity environment through rotation. Fang and coworkers investigated the production of three antibiotics β-lactam, Microcin B17 and Gramicidin S, using *Streptomyces clavuligerus*, *E. coli* and *Bacillus brevis*, respectively, under simulated microgravity conditions using the rotating wall bioreactor (65–67). The production of β-lactam was inhibited in the rotating environment and Microcin B17 was found to accrue in the medium as opposed to its normal intercellular accumulation, but Gramicidin S production was unaffected. As with clinorotation, the ultimate ability of this system to accurately simulate spaceflight depends on multiple experimental parameters.

The mixed results reported thus far on sporulation and antibiotic production suggest that although the altered inertial environment experienced in space or with the HARV can affect cellular secondary metabolism, it does not

necessarily produce consistent responses between various microbial species and/or specific test parameters.

FUTURE OPPORTUNITIES

In summary, the majority of evidence to date indicates that the reduced gravity experienced during spaceflight affects microbes through mechanisms related to changes in the surrounding fluid environment, as well as or in addition to, and to responses driven by internal gravity-dependent actions in larger organisms. Specific test conditions, growth medium, and assay methods must be carefully taken into account when attempting to draw conclusions from the results. Hardware design and operational methods usually vary significantly between different space flown experiments. These in turn can affect both the independent and dependent variables, thereby complicating the process of generalizing findings and conclusions. In addition, it does not yet appear feasible to directly extrapolate findings between species, further limiting the availability of existing comparable studies. Reproducible and systematic evaluations are necessary to confirm the reported findings and to begin conclusively identifying the underlying causal factors. Ground-based studies utilizing clinostats, HARV bioreactors and centrifuges will certainly add critical insight into how gravity affects microorganisms, but the International Space Station (ISS) currently being assembled on orbit will ultimately provide the continuous presence of a space laboratory needed to fully enable the emerging field of gravitational biology.

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SPACE: SURVIVAL IN SPACE.

See Desiccation by Exposure to Space Vacuum or Extremely Dry Deserts: Effect on Microorganisms

SPACE VACUUM.

See Desiccation by Exposure to Space Vacuum or Extremely Dry Deserts: Effect on Microorganisms

SPATIAL DISTRIBUTION OF MICROORGANISMS IN SOILS.

See Soil Distribution of Microorganisms

SPORES AS INDICATORS.

See Aerobic Endospores; *Clostridium*

STALK-FORMING BACTERIA.

See *Gallionella ferruginea*: An Iron-Oxidizing and Stalk-Forming Groundwater Bacterium

STARVATION OF MICROORGANISMS IN SUBSURFACE SEDIMENTS.

See Microbial Starvation Survival in Subsurface Environments

STATISTICAL ANALYSIS.

See Data Analysis and Modeling

STORAGE OF ENVIRONMENTAL SAMPLES.

See Sampling Techniques for Environmental Microbiology

STORAGE POLYMERS: ROLE IN THE ECOLOGY OF ACTIVATED SLUDGE

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Activated sludge is a complex consortium of several populations of microorganisms, whose ecology is strictly dependent on wastewater composition and process conditions. In turn, process performance strictly depends on activated sludge metabolic behavior and composition (e.g., the presence of bacteria promoting bulking and/or foaming phenomena, hindering good separation of activated sludge in the secondary settler). (See Filamentous Bulking in Activated Sludge, Control of; Activated Sludge — Foaming)

Many microorganisms, even unrelated from a phylogenetic point of view, have the ability to transform substrates into polymeric materials (storage compounds), some being able to store more than one compound. The ability to store has a strong competitive advantage, and is a key factor in determining the ecology (i.e., the population dynamics) of activated sludge. According to the original definition (1), storage compounds have an energy-storage function: these compounds usually are produced when the supply of energy from external substrates is in excess of that required for cell growth and related processes. Once the supply of energy from external substrates is no longer sufficient for optimal growth or maintenance of the cell, the stored compounds can be used as internal substrates and this is advantageous in the struggle for existence over microorganisms that do not have a similar mechanism. The ability to store internal polymers has been reported for several microorganisms (1) including bacteria that are the most numerous and the most important population of activated sludge microorganisms (2).

The storage process basically consists of an imbalance between the time and extent to which the substrates are available in the medium and the time and extent to which the cells are able to use them for growth. These unbalanced growth conditions are often produced in activated sludge processes by deliberate process design and operation in order to exploit their potential in determining the activated sludge ecology. The aim is to select or enrich the activated sludge for microorganisms that are more favorable to the required process performance. (see ACTIVATED SLUDGE — THE MICROBIAL COMMUNITY.)

STORAGE POLYMERS

Cellular material is mainly composed of complex chemical components of polymeric nature (proteins, RNAs, and DNA) and most can be used by the cell as an endogenous source of energy for maintenance, when there is a lack of external substrates. So although most of these polymers can act as reserve materials, the definition of storage polymers has to be restricted to the specialized ones, that is, biopolymers specifically synthesized as carbon and/or energy reserves that are not basal components usually produced by the cells for growth.

In unbalanced growth conditions in which storage is likely, the substrate can also be removed quickly by accumulation (3), that is, the substrate is transported into the cell and maintained in an almost unchanged form or transformed into low-molecular-weight metabolic intermediates. Although accumulation is another mechanism for synthesis of reserve materials, this will cause unfavorable concentration gradients in the transport and the need for high osmotic pressure (2). Thus, accumulation is a less efficient mechanism than is storage in which the molar concentration of substrates or intermediates is reduced by their polymerization. In other words, storage acts also as a mechanism for preventing the accumulation of internal metabolites at toxic levels during the periods of nongrowth (4).

As an additional mechanism for preventing too high an accumulation, the release of solutes or extracellular polymers has also been observed. The production of extracellular polymers, mainly polysaccharide in nature, is frequently seen in bacteria, offering them other advantages including the formation of a protective slime or promoting floc formation (see ACTIVATED SLUDGE — THE FLOC). However, there is no evidence that these compounds are synthesized specifically as reserve materials.

According to the earlier definitions, the term *storage polymers* has to be used for a few particular compounds, namely, certain polysaccharides, lipids (polyhydroxyalkanoates), and polyphosphates (1). All these compounds are of relevance in activated sludge processes and are briefly described in the following text.

A more limited range of microorganisms can store other compounds, such as nitrogen (N) (the cyanophycins in cyanobacteria) (5) or sulfur (S) (as energy reserve in sulfur bacteria relevant to the activated sludge, in that it occurs in some microorganisms causing filamentous bulking, such as in *Thiothrix*) (6,7).

Polyhydroxyalkanoates (PHAs)

PHAs are linear polyesters of 3-hydroxyl fatty acid monomers, the carboxylic group of one monomer forming an ester bond with the hydroxyl group of another monomer. The most common PHA in activated sludge is poly-3-hydroxybutyrate (PHB, Fig. 1a), but other PHAs have also been found in activated sludge (e.g., 3-hydroxyvalerate, 3-hydroxymethylvalerate, etc.). PHB is also used for the production of biodegradable plastics (8) and is present in the cells as various-sized granules, with a high affinity for fat-soluble dyes such as Sudan black, that can be seen in bright field microscopy, or Nile blue, under epifluorescence microscopy (9).

Polyphosphates

Polyphosphates are known to be present in the cells as cyclic or linear forms of different lengths (Fig. 1b). The interest in this compound increased dramatically since the end of the 1970s when polyphosphate was suggested to play a key role as an anaerobic cellular energy source in activated sludge plants designed to stimulate enhanced biological phosphorus removal (EBPR) from wastewater. (see ACTIVATED SLUDGE — THE PROCESS; ACTIVATED SLUDGE — MICROBIOLOGY OF NITROGEN REMOVAL)

Polyphosphates are present as large reserve granules inside cells and stain by many basic dyes such as toluidine blue, changing color from blue to red (metachromasy). In the activated sludge that performs EBPR, the polyphosphates are usually shown by Neisser staining, appearing as dark granules in the cells (6,10).

Glycogen

Glycogen is a polysaccharide of glucose units linked between carbon 1 and 4 in the α-orientation (Fig. 1c) with occasional α-(1 $\rightarrow$ 6) branch linkages (5). Glycogen normally is present in cells as granules smaller than PHAs and they are harder to observe by optical microscopy, giving only a weak reaction with diluted iodine.

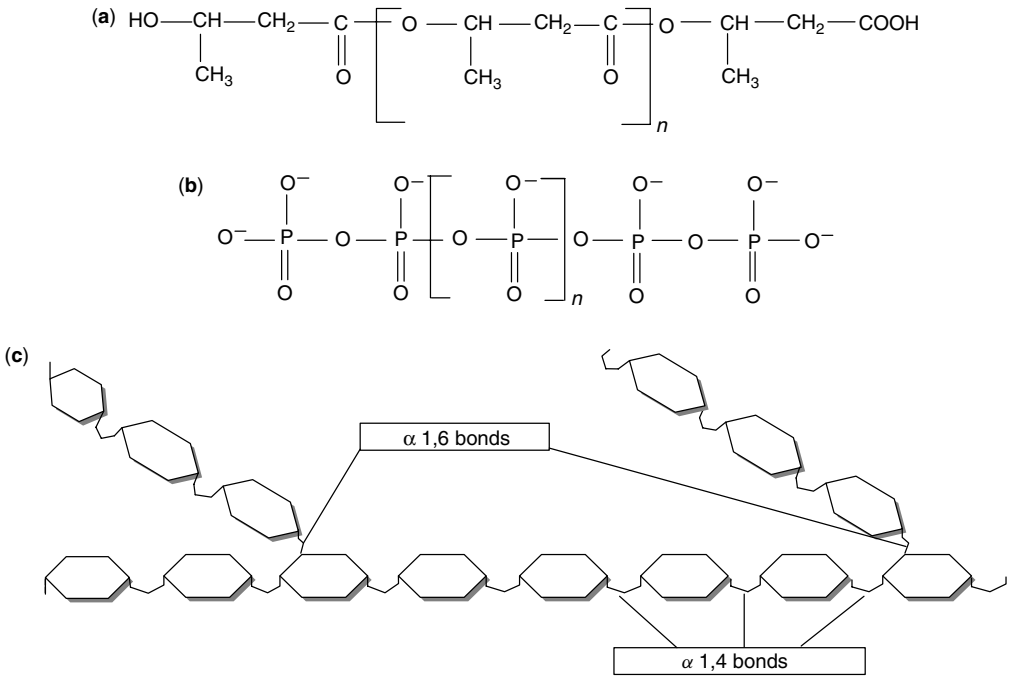

Figure 1. Structure of the main storage polymers: polyhydroxybutyrate (**a**), polyphosphate (**b**), glycogen (**c**).

STORAGE PROCESSES UNDER UNBALANCED GROWTH CONDITIONS

Although storage can occur under balanced growth conditions (11), it is observed more frequently under unbalanced growth conditions (12). As already stated, storage most often is present when some growth limitation prevents the cells from using the substrates for growth at the same rate at which they are transported into the cell and partially used to produce energy and reducing power and excess carbon (C) and energy are diverted into synthesis of storage polymers (1). Typically, this growth limitation can be caused by two different conditions that have been referred to (4) as metabolic (or external) induction and kinetic (or internal) induction.

Metabolic (External) Induction

In the case of metabolic induction, an excess of a readily usable carbon and energy source is available in a culture that is prevented from growing at its maximum rate by the absence or lack in the medium of some other essential component for cellular synthesis. A typical example is a deficiency of nitrogen with respect to carbon in the medium. Because most growth-associated components contain this element, overall growth is hindered, whereas carbon uptake and related energy production are not. Hence the energy available is used to transform excess carbon into storage compounds with no nitrogen content.

This metabolic (external) limitation is exploited for the production of commercial polymers (8,13,14) such as copolymers of 3-hydroxybutyric and 3-hydroxyvaleric acids (PHB/HV). The growth medium is typically designed to contain an excess carbon with respect to nitrogen, and the process is typically operated in two steps. First, the bacteria, usually *Ralstonia eutropha* (formerly known as *Alcaligenes eutrophus*), grow while the medium remains nutritionally well-balanced. Once the medium becomes nitrogen-deficient, the residual excess of carbon is stored until the cells almost totally consist of polymer (up to 80% as dry weight). Most of the available knowledge on physiology and biochemistry of PHA storage has been reviewed in this context (8,13). Similarly, polyphosphate can be stored in cells following sulfur or nitrogen starvation: again growth is hindered and the cells accumulate polyphosphate (15).

However, these conditions of nutrient deficiency rarely are reported for activated sludge processes treating municipal wastewater, although they might occur in the presence of wastes of industrial origin and eventually cause bulking problems (2,16).

The most frequent case of storage caused by external nutrition limitation in activated sludge processes is the storage of carbon source in the anaerobic phase of alternating anaerobic/aerobic processes (e.g., in processes for EBPR). Here, the limiting component is not an element to be used by the cell for growth but is the external electron acceptor necessary for energy production [molecular oxygen (O_2) or nitrate]. Apparently, in this situation, some microorganisms have the ability to use alternative sources of energy and reducing power in order to take up the substrate (usually acetate) and store it (as PHB) at rates and extent less than those when O_2 or nitrate is used in respiration. These include hydrolysis of polyphosphate as an energy source (17) and glycolysis of glycogen as both source of energy and reducing power (18–20). This anaerobic storage differs from that previously described, in that it seems to occur in

energy-poor instead of in energy-rich conditions. However, it is not clear whether cell growth is hindered due to such energy limitation or whether storage is the faster process in assimilating available substrates in the highly dynamic anaerobic/aerobic environment (see the following text). It is also noteworthy that during subsequent aerobic growth, internal reserves (polyphosphate and glycogen) are replenished, although no external or internal nutrition limitation is evident. Thus, this is an example of simultaneous growth and storage.

PHB accumulation can also be induced by oxygen limitation, in which PHB acts as a sink for excessively generated intracellular reducing power of electrons (1).

Kinetic (Internal) Induction

In the case of kinetic induction, no external limitation exists, that is, the medium contains all the substrates to allow cells to grow at their maximum rate. Nevertheless, the cells cannot immediately increase their growth rate to the maximum level because they have previously been under conditions of slow growth or maintenance, that is, they are deficient in the sufficient "synthetic machinery" needed for high growth rates (4). In other words, as a result of its previous growth and physiological conditions, the cell suffers an internal limitation. This is the case that is most likely to occur in activated sludge processes because of their highly dynamic conditions (21).

This internal limitation depends on the physiological adaptation of cells. When microbes are transferred to a different environment, fundamental changes occur in their composition (mainly their macromolecular composition, i.e., relative concentration of proteins, RNA, DNA, etc.) as it adapts to new conditions. This change in their macromolecular composition is known as *physiological adaptation*, and the macromolecular composition at a given time is referred to as the *physiological state* (22). When the environment is relatively steady, physiological adaptation produces a final physiological state that is optimum for cell growth in that environment, and no further adaptation is necessary. Growth of a well-adapted cell with no change in composition is also referred to as *balanced growth*, and growth in the presence of physiological adaptation (23,24) is referred to as *unbalanced growth*.

Bacterial cells in balanced growth synthesize all components (DNA, RNA, proteins, etc.) at the same relative rates. On transfer to different environmental conditions (e.g., higher carbon source concentration), the rate of RNA synthesis rapidly changes, whereas the rates of DNA, protein, and other compound synthesis and the rate of cell division only later follow the changes in RNA content (25,26).

When growth restriction is removed, the cell's ability to immediately increase substrate-removal rate is determined by its physiological state. Sometimes, particularly in activated sludge process, cells are not growing in a steady state but in fluctuating conditions, possessing an "extra synthetic machinery" and a limited ability to immediately increase their specific growth rate (available reaction potential, ARP, 27). However, the RNA usually is not sufficient to allow the culture to grow at its maximum specific rate and therefore the "synthetic machinery" level must be increased first (28). Hence, this will increase gradually with time, the specific growth rate will also increase and eventually reach the maximum value possible under the new conditions (the so-called growth response).

Consequently, when the culture experiences a sudden increase in available substrate and/or energy, it can adapt itself to the new conditions by storing the available substrate (storage response, phase I in Fig. 2a). Storage as well as growth, involves transport of substrates, oxidation of a portion for energy production, and use of another portion for synthesis. However, synthesis of storage polymers is simpler and faster than synthesis of all cellular components. In that sense, storage is a mechanism to maintain a more balanced situation inside the cell between energy production and energy utilization (21,29).

After being formed at high substrate concentrations, stored polymers are consumed as internal substrates for growth when external ones are at limiting concentration (phase II in Fig. 2a). It has been reported for *Paracoccus pantotrophus* (30), that the growth yield on acetate through formation and consumption of PHB is only 6% less than yield from direct growth on acetate under balanced conditions. This indicates that the storage response is quite efficient from an energetic point of view. Hence, microorganisms able to store substrates and reuse them for growth could have a competitive advantage, which is relevant when considering the selection of different populations in activated sludge.

For these reasons, storage response often prevails on the growth response during transient feeding conditions in activated sludge (12). Such prevalence is stronger when more of the substrate is in a form readily available to

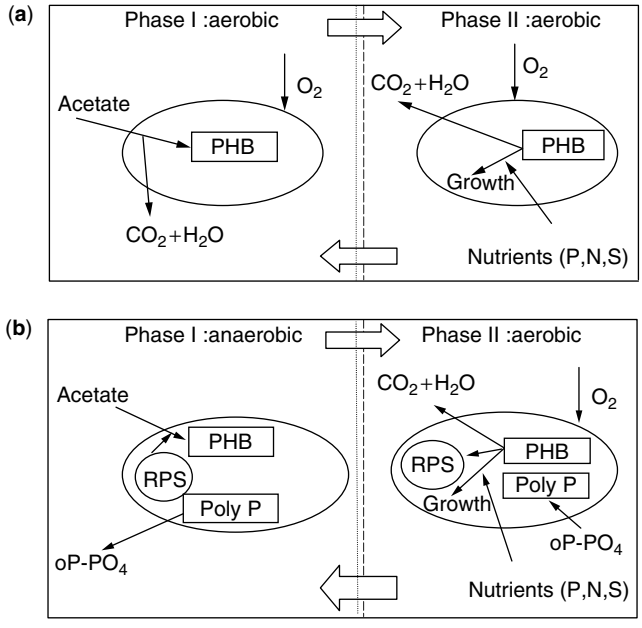

Figure 2. Storage response under different conditions. (**a**) Aerobic storage (phase I: storage phase, phase II: growth phase); (**b**) anaerobic/aerobic storage (phase I: anaerobic storage, phase II: aerobic growth and storage). RPS: reducing power source.

the cells (i.e., soluble, low-molecular-weight, and simple substrates such as volatile fatty acids and carbohydrates). On the contrary, for slowly biodegradable substrates, it is assumed that the storage is less important in transient conditions (4) as there is a need for their hydrolysis before they can become available to the cell.

AEROBIC STORAGE IN BULKING AND FOAMING CONTROL

Satisfactory performance of activated sludge processes implies that good separation and thickening of the sludge are obtained in the secondary settler. Unfortunately, activated sludge processes are often disturbed by numerous separation problems that include dispersed growth, viscous or filamentous bulking, pinpoint flocs, rising sludge, and foaming or scum formation. (see FILAMENTOUS BULKING IN ACTIVATED SLUDGE, CONTROL OF; ACTIVATED SLUDGE — FOAMING).

Usually, most of the problems are related to the presence of large amounts of filamentous microorganisms in the activated sludge; worldwide, 30–40% of traditional activated sludge plants suffer bulking and foaming problems. In particular, filamentous bulking is caused by the overgrowth of filamentous bacteria, which usually protrude from the flocs and interfere with sludge settling. Although more than 30 different filamentous microorganisms have been reported (10) in bulking sludges, their overgrowth has been explained according to some limited general physiological differences between the groups of so-called floc formers and filaments (2) (see FILAMENTOUS BACTERIA IN ACTIVATED SLUDGE: CURRENT TAXONOMIC STATUS AND ECOLOGY).

Storage is involved in controlling bulking phenomena in process configurations in which a substrate concentration gradient is created (corresponding to an F/M gradient). Figure 3 shows examples such as plug-flow configuration of the aeration tank (no longitudinal mixing because of the tank shape or compartmentalization, Fig. 3a), presence of a selector (a small tank receiving both the return sludge and the influent before the main one, Fig. 3b), and contact-stabilization configuration (similar to the previous one but with intermediate settling, Fig. 3c).

On the basis of empirical evidence that activated sludge from these configurations usually has better settling properties, two theories have been used to explain why the floc-forming bacteria are favored in the presence of a gradient (2), one based on balanced growth and the other based on unbalanced growth (i.e., storage) (see FILAMENTOUS BULKING IN ACTIVATED SLUDGE, CONTROL OF). The first approach assumes that floc formers usually have both higher specific maximum rate (μ_{max}) and semisaturation constant (K_s) than filaments. Hence, floc-formers have a μ_{max}-based strategy of competition, that is, they grow faster than filaments when substrate concentration is high, as in the presence of a concentration gradient, whereas filaments have a K_s-based strategy, that is, they grow faster than floc formers when substrate concentration is low, as in completely mixed reactors.

The second approach is based on the observation that in the presence of a substrate gradient, bacteria alternately

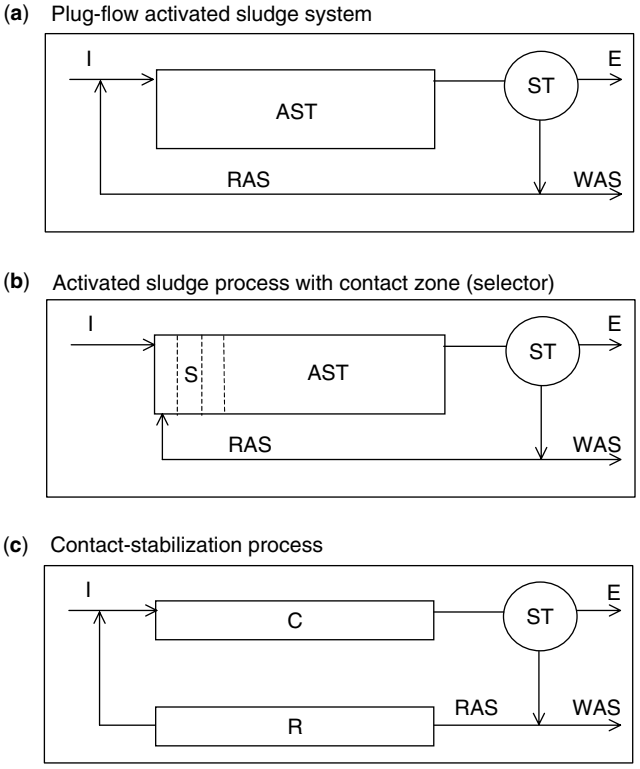

Figure 3. Activated sludge process configurations in which a substrate concentration gradient is created and storage is more likely to occur. I: influent, E: effluent, RAS: return activated sludge, WAS: waste activated sludge, AST: activated sludge tank, ST: settling tank, S: selector area, C: contact zone, R: regeneration zone.

experience high and low substrate concentration because they are circulated continuously through the plant. Because of this unbalanced condition (also called a feast and famine regimen), storage and accumulation are the main mechanisms for enabling fast substrate uptake when they arrive in the substrate-rich area coming from a substrate-limiting situation (31,32). Thus, selection is based not only on an ability to quickly accumulate or store the substrate but also on an ability to subsequently reuse it for growth. According to the accumulation-regeneration theory (3), the use of accumulated or stored compounds during growth is also necessary to regenerate their limited accumulation or storage capacity. The better settling performance obtained in configurations with substrate concentration gradients seems to indicate that floc formers have this type of competitive advantage over filaments (2).

Although these approaches are not mutually exclusive, it is widely accepted that storage plays a relevant role in competition between floc formers and filaments, at least in highly dynamic processes and in the presence of readily biodegradable substrates (3,12,21). Nevertheless, very little experimental confirmation has been published on full-scale plants or at least with real activated sludges and wastewater. Most of the knowledge comes from pure or mixed culture studies under aerobic conditions (12), as briefly summarized in the following section.

Pure Culture Studies

Studies have been carried out on floc formers such as *Arthrobacter globiformis* (33,34) and *Zoogloea ramigera* (11); on typical filaments such as *Sphaerotilus natans* (33,34), Type 021N (11), and *Gordona amarae* (35), and on other bacteria such as *Pseudomonas putida* (22,36), *Aquaspirillum autotrophicum* (37), and *Paracoccus pantotrophus* (38).

Although not all these studies explicitly consider the storage, they all investigate the transient response of a biomass (harvested from a continuous steady state culture) to a pulse addition of an excess of substrate (short-term batch tests). As already mentioned, once substrate limitation is removed from earlier culture conditions, a rapid increase of substrate-uptake rate (SUR) usually occurs and the related "observed yield" is often high (here, observed yield has to refer to the formation of biological suspended solids, independently from their composition, so also including stored compounds). In the absence of direct determination of storage polymers, the high rates and "observed yields" have often been taken as evidence of storage. In some cases (11,33), SUR and "observed yield" were higher in batch tests than under unrestricted balanced growth (i.e., maximum values obtained in the parent chemostat). This trend could only be due to growth and confirms the presence of storage as a fast and high-yield response. When directly determined, the observed amount of polymer formed per unit of substrate removed ranges from 0.32 to 0.79 COD of formed polymers/COD of removed substrate (12), often representing most of the overall "observed yield."

Figures 4 and 5 summarize pure culture studies performed using acetate as the sole carbon source (11,35,38). Specific SUR (Fig. 4a) and observed yield (Fig. 5a) in batch tests are reported as functions of dilution rates D (growth rate) in the parent chemostat. It is quite evident that both specific SUR and "observed yield" depend on the previous D and that in a "theoretical" situation, different microorganisms could dominate at different D. This indicates that the previous physiological state of the biomass can be the key factor in the kinetic selection of different microorganisms in mixed cultures. Figures 4b and 5b report the same data shown in Figures 4a and 5a, except that D has been normalized with respect to the maximum growth rate of each microorganism. A general trend can be observed for the different bacteria. The transient response seems to decrease as D either becomes very similar or very low with respect to the μ_{max}. In all studies, the transient response at D near μ_{max} tends to correspond to the steady state behavior. For microorganisms in which transient SUR presents a maximum as a function of D, the authors (11) assume that this corresponds to the maximum storage capacity. Inconsistent to the SUR, the observed yield seems to show a constant trend: it slightly decreases as D increases, which seems to indicate that storage also decreases; accordingly, direct measurement of storage yield and storage capacity confirmed that both tend to increase as D decreases (30).

Little attention has been given to substrates other than acetate. *Arthrobacter* sp. and *Sphaerotilus natans*, grown at one D ($1\ d^{-1}$) in glucose-limited conditions, have storage responses under transient conditions (33,34). However, glucose-uptake rate for *Arthrobacter* sp. was more than twice that for *S. natans*, and the ratios of stored polymer to the substrate taken up were 0.7 and 0.3 COD/COD, respectively, thus showing that the floc former has a higher storage response than the filament. A higher storage response is clearly advantageous to the floc former *Arthrobacter* sp., with respect to the filament *S. natans*, when competing under transient conditions. A storage response has also been observed for *A. autotrophicum* on pyruvate (37) although only 12% of removed substrate was recovered as stored polymer. In experiments performed on *P. putida* cultivated in an L-lysine-limited feed (22) at a dilution rate less than $0.3\ \mu_{max}$, the oxygen-uptake rate (OUR) under transient conditions was faster than

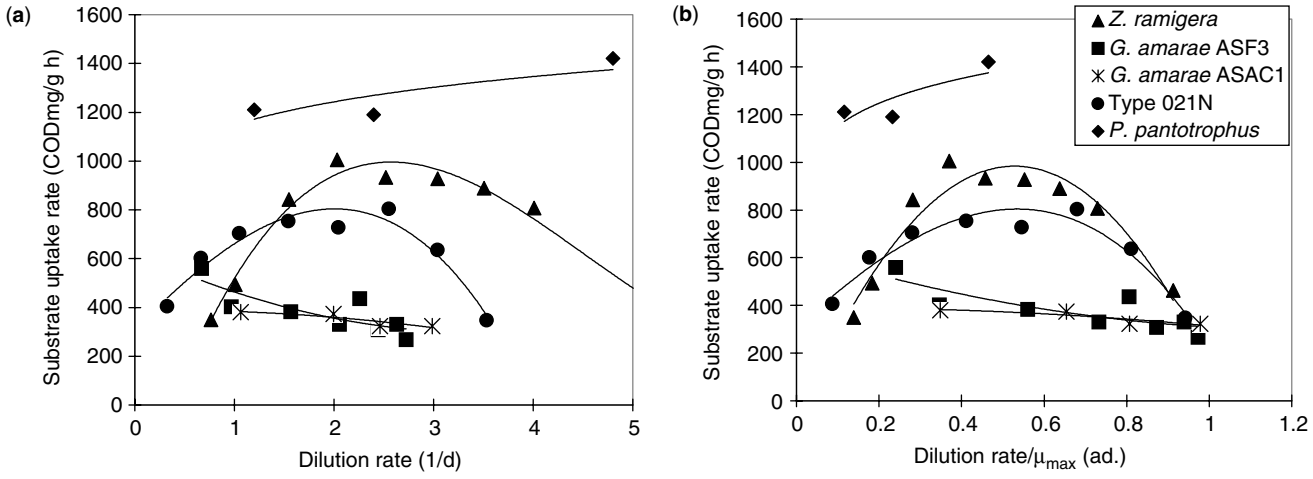

Figure 4. Maximum substrate uptake rate in transient conditions (batch tests in the presence of an excess of acetate) for different microorganisms, as a function of the previous steady state growth rate, expressed as

a) dilution rate in the chemostat

b) ratio of dilution rate and maximum specific growth rate for each microorganism.

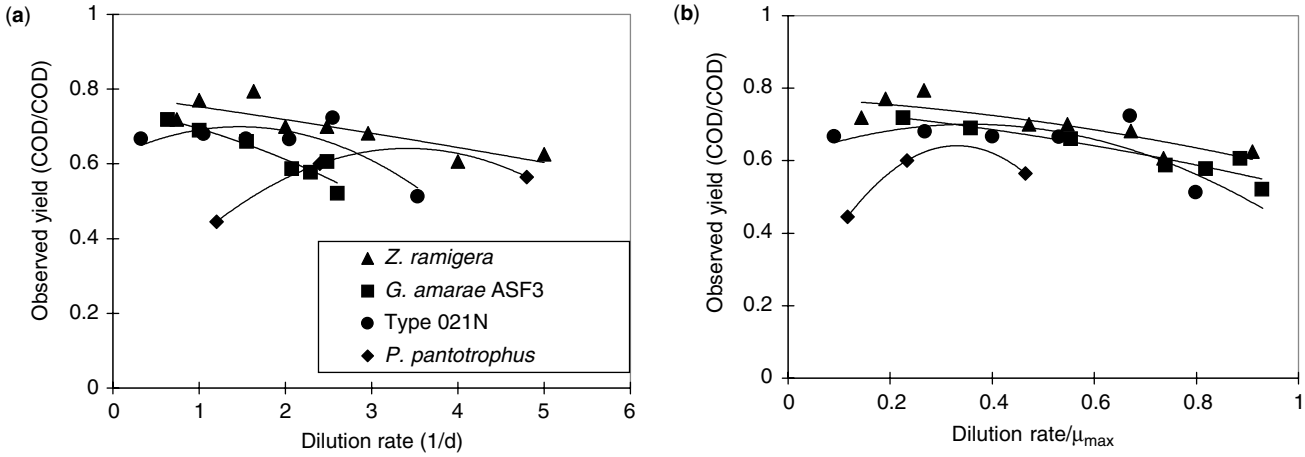

Figure 5. Observed yield in transient conditions (batch tests in the presence of an excess of acetate) for different microorganisms, as a function of the previous steady state growth rate, expressed as

c) dilution rate in the chemostat

d) ratio of dilution rate and maximum specific growth rate for each microorganism.

Note: Original units of most data from authors of this entry.

that under steady state conditions, whereas the rate of synthesis of growth-associated macromolecules remained unchanged. Hence, the available surplus of energy under transient conditions could probably be used for synthesis and accumulation of intermediary metabolites, whereas storage polymers were not identified.

Very few studies have been performed on pure cultures grown under periodic feeding conditions. A comparison was made of the transient responses of both *Arthrobacter* sp. and *S. natans* grown under continuous versus intermittent feed of glucose (34). For both microorganisms, growth under periodic conditions increased storage response with respect to steady state culture.

It has to be stressed that the range of D investigated so far (usually $>1\ d^{-1}$, corresponding to culture age <1 d) does not correspond to growth conditions that are most typical of activated sludge processes (in which the sludge age is usually much higher). Hence, there is still a lack of knowledge on the dynamic response of pure cultures growing at high sludge age (low growth rate).

Mixed Culture Studies

Studies with mixed cultures (31,34,39–44) have been related more directly to bulking control. These studies have been typically performed by comparing the transient response of sludges exposed to continuous feeding (usually bulking sludges) with that of sludges with intermittent feeding regimens or with the presence of a selector (usually well-settling sludges). It has to be stressed that in the case of mixed cultures, the effects of different growth conditions include significant change of both the physiological state of microorganisms and the distribution of microbial population in the sludge.

Well-settling sludges typically presented faster substrate uptake and higher "observed yields," which have been usually interpreted as indirect evidence for more relevant storage response. The importance of a storage response has been confirmed by direct determination of polymers in sludges storing glucose mainly as glycogen (34,42) or storing acetate as PHB (29,43), and confirms that the more the bacteria are able to store substrates during imposed transients and subsequently reuse them for growth, the more they are likely to have a competitive advantage. Predominance of such microorganisms will increase the transient response of the sludge.

As an exception, it has also been shown (44) that although intermittent feeding always causes a storage response, a bulking sludge can also be selected. The authors showed that this was due to high storage response of an unusual filamentous bacterium that eventually became dominant in the sludge. They also investigated the effects of different dilution rates in an intermittently fed reactor and showed (45) that increase in D from 0.33 to 2.64 d^{-1} caused an increase and then a decrease in the transient response (because of the corresponding trend in the storage response). This behavior is in qualitative agreement with studies reported earlier on pure cultures (11).

By studying the effects of temperature (15–35 °C) on the settleability of sludges, storage contribution to external substrate removal (acetate) appeared to decrease as temperature increased (29). Accordingly, the sludge settleability decreased (i.e., the sludge volume index) as the temperature increased.

Activated Sludge Studies

Lack of detailed studies on real wastewater and sludges is due to practical difficulties and simultaneous presence of many different phenomena such as sorption, accumulation, storage, and growth on soluble substrates and enmeshment and hydrolysis of particulate. Some data are available from respirometric batch experiments in which typically a known amount of a selected substrate is added to a sample of activated sludge. In these conditions, it

has been shown that the respirometric curve (i.e., OUR as function of time) presents two different phases, at high and low OUR (both higher than the endogenous OUR). Because a single and readily biodegradable substrate is used, the presence of the second phase at low OUR has been attributed to the consumption of the stored product, indicating the presence of storage during the first phase (46). Recently, it was confirmed experimentally (47) that PHB was formed when mixing domestic wastewater and activated sludge. Moreover, more PHB was formed than that possibly coming from acetate present in the wastewater, thus suggesting that PHB storage also was due to other organic compounds present in the wastewater.

ANOXIC STORAGE IN NITROGEN REMOVAL PROCESSES

Activated sludge processes for biological nitrogen removal are characterized by the presence of an "anoxic" tank (here anoxic refers to the absence of oxygen in the presence of nitrate) where the nitrate reduction (denitrification) takes place. (see ACTIVATED SLUDGE — THE PROCESS; ACTIVATED SLUDGE — MICROBIOLOGY OF NITROGEN REMOVAL). Storage under anoxic conditions has been studied much less than that under aerobic or anaerobic conditions, although it is likely that it also is important in nitrogen-removal processes (21,48) in which the removal of the major fraction of organic substance occurs when nitrate is present as the only electron acceptor.

Storage can be relevant when, in a system with pre-denitrification, the anoxic tank has a plug-flow configuration or contains a selector (so introducing a feast and famine regimen). Similar to aerobic selectors, storage under anoxic conditions can be a key factor of microbial competition for the substrate. Literature evidences of a positive effect of storage phenomena on settling properties of sludge has been reported widely under aerobic conditions (see previous section) and the same selection of floc-forming biomass therefore is expected under anoxic conditions. Moreover, such selection also should be stronger under anoxic conditions because most of the reported filaments are not able to denitrify (2). However, a recent study showed that, although filaments are less able than floc formers to store substrate into PHA, they nevertheless can grow and proliferate in an anoxic-aerobic SBR fed with a mixture of readily and slowly biodegradable substrates (49).

The presence of storage can be enhanced when an external source of readily biodegradable COD (RBCOD) has to be added to increase the denitrification rate. In this regard, it is important to observe that the external substrate often is the effluent of a fermentative process (50,51) and therefore is rich in volatile fatty acids, the favorite substrate for PHA storage. Besides storage under anoxic conditions, denitrification on the stored products also can be important in increasing "endogenous" denitrification rate. As an example, an aerobic contact-anoxic stabilization process has been proposed (52) in which a higher "endogenous" denitrification rate is achieved in the anoxic tank as a result of previous biosorption and storage of COD in the aerobic one.

The formation and/or consumption of storage products in the anoxic zone will affect the overall "observed yield" and therefore the COD/N ratio (i.e., the amount of COD that is needed to reduce 1 unit of nitrate nitrogen to molecular nitrogen). As already stated, storage requires less energy than growth that corresponds to a lower amount of oxidized substrate per unit of removed substrate. Theoretical calculations, considering acetate as substrate (48), show that maximum biomass yield is 0.58 COD/COD when acetate is only stored into PHB, whereas it is 0.36 COD/COD when it is used directly for growth. These different values correspond to 6.8 and 4.5 mg COD/mg reduced N-NO_3, respectively. On the other hand, growth through formation and consumption of PHB will require more energy (maximum yield 0.31 COD/COD corresponding to 4.1 mg COD/mg reduced N-NO_3) than direct growth on acetate. So the overall "observed yield" and related COD/N ratio will vary depending on relative extent of storage of external substrates and consumption of internally stored ones in the anoxic tank.

With regard to experimental evidences of anoxic storage, recent studies (48,49,53,54) have shown that anoxic storage of PHB occurs under transient conditions by using acetate or ethanol as carbon source. From the reported studies, it is clear that storage mechanisms under anoxic conditions are basically the same as those under aerobic conditions: storage response is induced by feast and famine conditions. Kinetics of PHB storage under anoxic or aerobic conditions also has been compared. By using biomass cultivated under fully anoxic conditions, different evidences were found: some authors (54) showed that acetate uptake rates were almost similar when switching from anoxic to aerobic conditions; on the contrary, other authors (48) reported a three times higher acetate uptake rate under aerobic conditions with respect to anoxic ones. However, in both studies the amount of stored polymer per amount of removed acetate was quite similar and almost independent of the type of electron acceptor present.

More limited are the studies of glycogen storage under anoxic conditions. Storage of carbohydrates (probably glycogen) under anoxic conditions has been shown when glucose was the carbon source (49): in that case, formation of internal carbohydrates accounted for the whole "observed yield" (overall formation of biological solids).

ANAEROBIC/AEROBIC STORAGE IN EBPR PROCESSES

During the 1970s an important observation was made in activated sludge plants: sometimes in treatment plants with an anaerobic zone at the head of the plant, phosphorus (P) was removed to an increased extent in the aerobic zone. The aerobic sludge presented a higher phosphorus content and consequently the effluent a lower phosphorus level, (<1 mg/L). Many large treatment plants began to be built on the basis of this new process (17), named EBPR (see ACTIVATED SLUDGE — THE PROCESS).

Intense research (55,56) since then has tried to elucidate the microbial basis of the phenomenon. The common information obtained from both full-scale and pilot plants are as follows:

- In the anaerobic zone there is a P-PO_4 release, (see , corresponding to an uptake of the carbon source.
- Carbon sources are not equally efficient and the best are simple molecules and monomers, such as short-chain fatty acids (SCFA), and particularly acetate, widely selected for research studies as the preferred anaerobic substrate. In real plants the role of other classes of compounds has to be considered.
- Substrate assimilated is converted simultaneously into polymers reserve material, including PHAs, such as not only PHB but also PHV′3 methyl PHV′, etc., when SCFA different from acetate are fed.
- In the aerobic zone, oxidation of stored PHAs occurs with growth of the biomass and reconstitution of the polyphosphate reserves. The final result is that a sludge with a higher phosphorus content is produced, and can be disposed of (17).
- The EBPR sludges microscopically look quite different from ordinary activated sludge, with large clamps of bacteria, cocci in shape, which, if sampled in the aerobic reactor, strongly stain with Neisser stain because the cells are full of polyphosphate (57).

To explain these observations, the existence of bacteria possessing a peculiar metabolism was proposed. These bacteria would have the capacity to anaerobically use their polyphosphate as energy source, allowing them to transport substrates and synthesize PHA. These bacteria would have a mixed metabolism (Fig. 2b) involving anaerobic storage of the carbon source using energy from polyphosphate (Phase I in Fig. 2b) and aerobic growth and storage of polyphosphate (Phase II in Fig. 2b). Crucial is the involvement of internal-storage carbohydrate reserves for production of reducing power ($NADH_2$ probably) necessary for the anaerobic synthesis of PHB from acetate. The term *poly P bacteria* was used to describe organisms with this ability.

Twenty-five years have not been sufficient to clarify the metabolism of these poly P bacteria and identify them (55,58). A pure culture of a poly P bacterium performing the chemical transformation depicted in Figure 2b has never been obtained. Nevertheless, the existence of such poly P bacteria is the only way to explain adequately the observations repeatedly made with EBPR.

EBPR represents one of the most intriguing cases of storage because all the three main classes of storage compounds are involved and mutually interconnected: PHAs, polyphosphates, and carbohydrates polymers. Full-scale EBPR plants currently are used in many countries but uncertainties still exist with the process. Sometimes the plants fail to reach expected performances and the required low phosphorus level in the effluent; this explains why many of them have a tertiary chemical-physical treatment for phosphorus removal, to cope with unexpected failures.

Some of the reasons given to explain the failures include

- The unsuitability of the organic carbon sources in the anaerobic zone. Basically, SCFA are thought to promote EBPR. Carbohydrates can be fermented to SCFA by other heterotrophs, so acting in favor of the metabolism shown in Figure 2b. However, the energy derived from carbohydrate fermentation can also be used for directly storing carbohydrates (55). As outlined in the following, this is a competing metabolism with respect to the one shown in Figure 2b. Very little is known about the role of proteins as substrates for poly P bacteria under anaerobic conditions.
- Use of suitable carbon sources by denitrifying bacteria can occur if too much nitrate and nitrite are circulated back into the anaerobic zone from the aerobic one.
- The existence of bacteria competing in the anaerobic zone for SCFA, without involving polyphosphate. Basically, these would use the internal stored carbohydrates as energy source. Also, the existence of these bacteria has been supposed; they have been indicated as GAOs (56). Even though G-bacteria (60) were formerly assumed to behave as GAOs, this has not been demonstrated yet (see ACTIVATED SLUDGE — THE "G-BACTERIA").

EBPR certainly is a complex process in which the bacteria alternate under different conditions: the microbiology of these processes is not explained and a proper study of these systems represents a good challenge for the future.

It is important to underline that the phenomenon also is complicated by the involvement of chemical and physical phenomena, because of the presence of cations such as calcium and magnesium in the wastewater. Calcium and magnesium phosphates have a very low solubility product and it is possible that precipitate formation and dissolution can occur in different environments (61). EBPR in some cases may be a biologically mediated physical-chemical phenomenon.

Detailed characterization of the storage processes in EBPR sludges are available mainly on pilot plants; complete sets of data concerning the substrates and polymers involved (acetate, phosphate, polyphosphate, PHB, and carbohydrates) are available.

An activated sludge in a traditional aerobic plant shows neither appreciable anaerobic acetate uptake nor PHB synthesis. Sludges grown in alternating anaerobic/aerobic conditions on wastewater containing appropriate amounts of phosphate (high enough to allow storage of polyphosphate) and suitable carbon sources (containing or suitable for producing SCFA) show the typical turnover of the polymers depicted in Figure 2b.

The following points can be highlighted (62–64):

- The values for anaerobic acetate uptakes are variable (12.2–73.8 mg/g SS h) but are lower compared with aerobic processes (at least 1 order of magnitude less).
- The amount of PHB formed per acetate taken up depends on the experimental conditions and the reducing power source (e.g., 1.3 Carbon mole PHB/Carbon mole acetate at pH 7.4).
- The anaerobic phosphate releases are quite variable, between 3.7 and 60.9 mg P/g SS h and also are strongly influenced by the pH value.

- Total carbohydrates usually decrease in the anaerobic conditions and increase again during the aerobic phase. The amount used is variable in comparison to polyphosphate turnover.
- Aerobic uptakes rates of phosphate are lower than the release rates.
- The phosphate released/acetate uptake ratio ranges from 0.24 to 0.80 P-mole/C-mole, and is strongly influenced by pH.
- EBPR sludges may contain up to 10% P and only part of it is released anaerobically.

It may be concluded that anaerobic storage involving PHAs, polyphosphates, and probably glycogen, is a process occurring slower than under aerobic conditions, as far as the PHAs synthesis is concerned (12). The key point is to allow the poly P bacteria cells to use properly their storage materials to accumulate more phosphate than they need, leaving a minimum level in the final effluent.

Moreover, there is evidence that microorganisms other than poly P bacteria and GAOs can take up soluble organic compounds in the anaerobic step of EBPR processes, by using different energy sources and by producing different storage compounds. In particular, the fermentation of carbohydrates (namely to lactic acid, 59,65), has been shown to be an alternative way for facultative heterotrophic bacteria to obtain the energy necessary for the storage of organic substrates (namely as glycogen) and possibly for growth itself. Provided fermentable substrates are present, storage is quite likely to occur because bacteria undergo both metabolic and kinetic limitations (alternating anaerobic and aerobic conditions and alternating high and low substrate levels, respectively).

CONCLUSION

Present knowledge on the storage of carbon source by bacteria under aerobic conditions is based mainly on using synthetic media with a limited range of carbon sources (practically only acetate and glucose) and using empirically selected cultured bacteria and sludges approaching the conditions under which real plants operate. In these cases, when the process imposes a kinetic pressure on the biomass (i.e., the concentration gradient of the carbon sources), a storage response usually is established without any necessity of other external limitations (e.g., lack of nutrients or electron acceptors). Under such conditions, storage often is the main mechanism for substrate removal, although other mechanisms also can contribute, depending on the previous physiological state of the biomass. There is no evidence that storage is restricted to just a few particular microorganisms and both filamentous and floc former ones have been reported to be able to store carbon sources.

Floc formers appear to have a higher ability to store than filamentous microorganisms, that is, they have a competitive advantage under conditions of unbalanced growth. In general, laboratory-scale studies on pure and mixed cultures seem to confirm the importance of storage on population dynamics in activated sludge. However, there still is a lack of knowledge both at a basic and applied level. At a basic level, pure culture studies on transient response have been based mainly on bacteria that have been grown

- Under balanced growth (steady state) conditions.
- At low solid retention time (high D).
- On synthetic media with a single carbon source.
- Using once a single disturbance.
- By focusing separately on a single transient mechanism (usually growth or storage response).

To improve knowledge on how different process conditions can affect the type and extent of the transient response, more representative conditions and a wider range of phenomena should be investigated. For example, studies should look at using

- Solid retention times typical for activated sludge processes.
- Periodic conditions of cultivation (that also means more operating variables have to be considered, namely cycle and feed length).
- Substrates other than acetate and glucose, and mixtures of them.
- Focusing on several phenomena (sorption, accumulation, and release).

Moreover, process conditions and transient responses should be linked to each other through the description of the physiological state (internal composition) of the microorganisms, by also using advanced experimental techniques such as labeled substrates and nuclear magnetic resonance (NMR) studies (20,66,67).

Similarly, there is a lack of data about storage processes under conditions of nitrate respiration in anoxic reactors (see ACTIVATED SLUDGE — MICROBIOLOGY OF NITROGEN REMOVAL). Systematic studies need to address the identity and kinetics of the bacteria mainly responsible.

The microbiology of EBPR is complex with several bacterial populations, some not yet identified, carrying out different reactions; it is hoped that soon the definitive clarification of the groups able to synthesize anaerobically PHAs from acetate, either poly P bacteria or their competitors will be accomplished. Then it will be possible to elucidate their identity, physiology, and biochemistry. Particular attention has to be focused also on the fermentative bacteria because their activities in anaerobic conditions is crucial for directing the fermentation of wastewater in the anaerobic reactor to supply suitable substrates for the poly P bacteria in EBPR.

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STREAM MICROBIOLOGY

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Streams differ from other environments in several ways, such as the presence of a constant flow of water. These differences influence the nature of the stream microbiota and the role of microorganisms in ecosystem function. Generally, microbial processes in streams are dominated by benthic processes (those in biofilms and sediments) and microorganisms play a central role in the food web. Specifically, bacteria and fungi act on the detrital pool, making this major pool of organic carbon available to higher trophic levels. Streams are also vital to humans as sources of drinking water and conduits for carrying away wastes. Such activities are associated with the occurrence of specific types of viruses, bacteria, and protozoa that are detrimental to human health and adversely affect water quality. In addition, streams are greatly influenced by the land around them and microorganisms from the terrestrial surroundings may enter into streams, especially after flooding. Overall, streams are important to the function of human societies, and microorganisms play an underappreciated part in stream ecology

Streams present their microbial inhabitants with a unique set of physical and chemical features unlike other aquatic ecosystems. These environmental conditions influence the ecology of the microorganisms and their roles in the food web. Moreover, stream microorganisms tend to be less well studied than microorganisms in other types of aquatic ecosystems (1,2).

PHYSICAL FEATURES OF STREAMS

Perhaps the most obvious feature of streams is the flow of water. On the one hand, this flow ensures that microorganisms are continuously supplied with dissolved nutrients and organic carbon (3,4). On the other hand, this limits their ability to form stable planktonic (meaning "free floating" organisms in the water) communities. These two different effects of water flow combine to ultimately create an environment for microorganisms unlike other aquatic ecosystems, such as lakes.

The two effects of flow, provision of dissolved compounds and the need to maintain position (and not be swept away by the flowing water), makes streams benthos (from the Greek word for "bottom") dominated systems (3,4). Unlike lakes, where the water column features a diverse and important planktonic community (including bacterio-, phyto- and zooplankton), stream microorganisms seldom can sustain self-supporting, planktonic communities except in large rivers. Only in rivers with suitable discharge, light levels, and other environmental features, do planktonic communities and associated food webs develop (5). Thus, for example, bacterioplankton (i.e., bacteria in the water column) do not contribute as much in terms of secondary production to a stream as benthic bacteria (6). In these streams with limited microbial production in the water column, it is thought that microorganisms present in the water are primarily derived from the benthos or other substrata, washed in from the surrounding drainage basin, or derived from reservoirs and backwaters (6,7,8).

Occupancy of benthic biofilms by microorganisms can minimize the loss of cells because water moves through the stream and can help concentrate dissolved materials (9). Thus, biofilms coat the surfaces or substrata in streams and play a major role in microbial ecology. These biofilms consist of an intermingled array of bacteria, algae, protozoa, and fungi embedded in an extracellular polysaccharide matrix.

The connection between the stream water and the biofilms extends through the top layer of sediment into the hyporheos (3). The hyporheos (meaning under the flow) lies beneath the surface sediments where the surface water mixes with groundwater. This provides a unique (but rather unstudied) habitat for microorganisms and a potent site for diverse biogeochemical processes because of differences in oxygen concentrations, redox potentials, the presence of various inorganic compounds containing N, Fe, or S, and patterns of water movement.

STREAMS AND THEIR WATERSHED

Streams are intimately and directly connected to the terrestrial world, thus inputs of inorganic and organic compounds and microorganisms from the surrounding terrestrial environment (or catchment, watershed) are important (3). This has important water quality implications because undesirable microorganisms can be washed into the streams from septic systems, feedlots, and combined sewer overflows or carried into streams by animals. This also means that the microbial community in a stream naturally contains both autochthonous (from inside the stream) and allochthonous (from outside the stream) cells that are intermingled and may play very different roles. However, distinguishing between allochthonous and autochthonous-derived cells is problematic.

The strong connection between streams and their surrounding catchment highlights another manner in which streams differ from most other ecosystems: they are an important natural resource commonly used as sources of drinking water and that also fill the role of carrying away wastes from human activities (10). Some wastes are directly discharged into streams, such as sewage effluents, which may harbor allochthonous microorganisms that can be detrimental to human health. In addition, various nonpoint sources of pollution, such as runoff from agricultural and residential areas, add dissolved chemicals (such as nutrients from fertilizer, pesticides, etc.) to streams that can affect the microbiota. Allochthonous microorganisms originating in surrounding regions can also be carried into the stream water, particularly during times of high rainfall.

STREAM FOOD WEBS

Because of the influence of the catchment and the impact of the flowing water, the food webs of streams and the

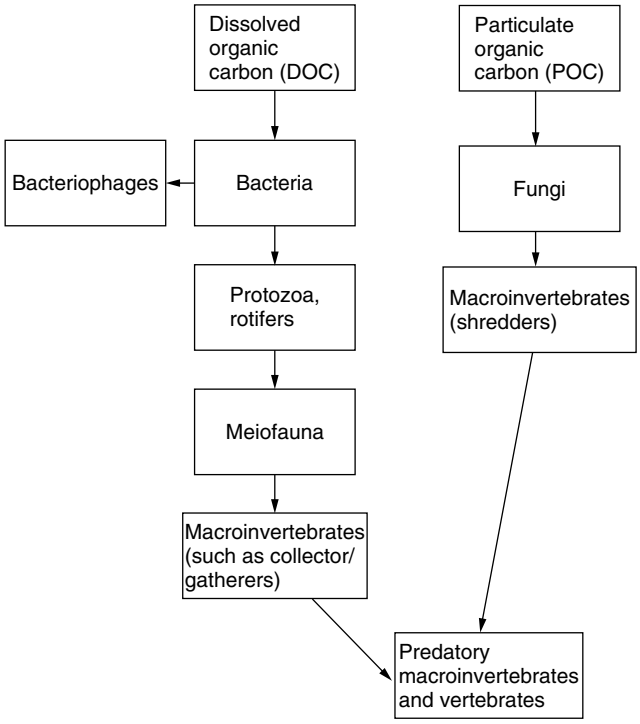

Figure 1. Simplified detritus-based stream food web.

roles of microorganisms differ from those found in other systems (11; Fig. 1). Typical streams that are encountered and studied throughout much of the world are shaded by vegetation (called riparian vegetation) that grows along their banks. This means that light is limited and thus primary production within the stream channel is also limited. Exceptions to this tendency are found in places where the growth of trees in the riparian zone is limited by climatic conditions, such as in desert streams that have minimal shading and abundant primary production in the stream channel (12). Also, reservoirs, lakes, and backwater areas may contribute algae directly to streams and rivers.

In shaded, small, headwater streams (also called low order streams; a first-order stream has no tributaries, a second-order stream occurs when two first-order streams join, and so on) input of leaf litter represents a main source of organic carbon (13). As described in the river continuum concept, the features of a stream change as it grows larger and draws from a progressively larger drainage area (14). As the channel broadens and the order of the stream increases, the riparian vegetation no longer fully shades the stream providing an opportunity for in-stream primary production. This pattern of change in primary production with increasing order is complicated by nutrient inputs, temperature, and hydrology (12). As the lotic (flowing water) ecosystem grows to be a river or joins a river, turbidity, and depth ultimately may limit light and primary production.

In streams, throughout their lengths, dissolved organic carbon (DOC) is a dominant form of organic carbon (15,16). The various forms of detritus, such as dissolved organic matter, and fine particulate organic matter, coarse particulate organic matter, are utilized by stream microorganisms that help make this resource available to higher trophic levels. Furthermore, often (based on the in-stream primary production) streams are heterotrophic, meaning that they rely on organic compounds from outside the system (17). Another way of describing the base of the food web is that streams tend to be detritus-based (detritus refers to all the organic matter not tied up in living organisms or biomass). The importance of DOC and the reliance on detritus means that, in streams, microorganisms play a very central role in the food web (Fig. 1; 11).

The various components of the microbial community in streams are discussed below and each play a different role. These roles are complementary and the depth of knowledge about a particular component is quite variable. In addition, to the basic ecology of microorganisms in streams, microbial aspects of stream water quality are also discussed.

VIRUSES

Aquatic microbial ecologists have in the past few years placed a new emphasis on the role of viruses in aquatic systems (18). Studies of planktonic viruses suggest that in lakes and oceans they are a major source of bacterial mortality. It is quite possible that viruses are also important in streams but the number of stream viral ecology studies is extremely limited (19). In one such study, Lemke and coworkers (19) found that viral numbers in stream water (determined based on staining with the Molecular Probes stain YO PRO and examination with epifluorescence microscopy; 20) averaged 2×10^7 ml^{-1} and greatly exceeded the number of bacteria. However, the number of bacteria was similar to or greater than viral numbers in sediments and leaf biofilms. Viral numbers in streams seem to be comparable to those found in other systems but there have been few studies so generalities cannot be drawn.

Advances in staining and detection of viruses have made enumeration of the total number of viruses in streams a more simple undertaking compared with earlier methods involving plaque assays or electron microscopy (Table 1; 20,21). Counts obtained via staining of nucleic acids (YO PRO is commonly used) provide accurate numbers based on comparisons to electron microscopy-based enumeration. However, information about total viral numbers is not particularly useful, in some ways, because the identities of the viruses are unknown. In streams, specifically, the presence of certain types of viruses is a significant health concern, so efforts have focused on detecting specific kinds of viruses.

Because of the importance of viruses to microbiological aspects of water quality, more information is available for streams concerning specific kinds of viruses (compared with viral ecology studies) that are not naturally produced in water but that can be found in streams (Table 2). For example, viral human pathogens in stream water can be an important water quality issue. Streams can be contaminated with a variety of viral pathogens through the introduction of untreated fecal waste (22). Initial efforts concerning aquatic environmental virology

Table 1. Commonly Used Methods in Stream Microbial Ecology

Microorganism	Total Number	Biomass	Production
Viruses	EM, EFM with nucleic acid stains (such as YO PRO)	image analysis	tritiated thymidine incorporation into DNA
Bacteria	EFM with nucleic acid stains (such as DAPI/Acridine Orange)	image analysis	tritiated thymidine incorporation into DNA, tritiated leucine incorporation into protein
Protozoa	EFM with stains such as DAPI or primulin, light microscopy of live specimens or progartol staining	image analysis	—
Fungi	indirect, conidia enumeration	ergosterol content	^{14}C acetate incorporation into ergosterol
Algae	light microscopy	chlorophyll *a* content	^{14}C bicarbonate uptake

Note: See text for references and more details; EM stands for electron microscopy and EFM stands for epifluorescent microscopy.

Table 2. Selected Human Pathogens Encountered in Water (for references and details, see text)

Group	Types
Viruses	Hepatitis A and E viruses, enteroviruses, caliciviruses, rotaviruses, astroviruses, and Norwalk-like viruses
Bacteria	*Campylobacter jejuni*, *Salmonella, Shigella*, some strains of *Escherichia coli*, *Yersinia, enterocolitica*, misc. opportunistic pathogens
Protozoa	*Giardia*, *Cryptospordium*, *Entamoeba histolytica*
Fungi	None
Algae	None

focused on polioviruses; today enteric viruses are a major concern and the most common consequence of infection is gastroenteritis (23). Viruses of concern include hepatitis A and E viruses, enteroviruses, caliciviruses, rotaviruses, and astroviruses (24). Norwalk-type viruses are among the major causes of waterborne illnesses (22) and present special methodological concerns.

Traditionally, viral pathogens in water have been detected/enumerated using cell culture-based methods (24). However, viruses isolated from the aquatic environment may not readily grow under such conditions and thus must be detected using other means (note: this same type of phenomenon also is widespread for bacteria and is discussed in a subsequent section). Another limitation is that the number of viruses of a given type in water can be quite low (22). Often, polymerase chain reaction (PCR)–based methods (in which viral nucleic acids are amplified) can be used to detect specific types of viruses, including those that are not amenable to growth in the lab or that are present in low numbers (23,24). The Norwalk-like viruses illustrate the advantages of using molecular biology techniques: these small, fastidious viruses defy laboratory cultivation and thus must be detected and identified using other methods (25).

Freshwater ecosystems can also play important, indirect roles in the ecology of viral pathogens by serving as hosts for viral vectors, such as mosquito and blackfly larvae (22). This same role is important for some bacterial and protozoan parasites, as well. Streams are generally not suitable for mosquito larvae because of the flowing water but backwater areas and reservoirs can be habitats for the growth of the juvenile mosquitoes (26). Thus, the role of streams as hosts for viral disease vectors is somewhat limited. However, other stream residents (i.e., blackfly larvae) are vectors for significant parasitic (nonviral) diseases including river blindness (Onchocerciasis caused by a filarial parasite; 27).

Viruses in streams can also be used as indicators; specifically, examination of coliphages has been used as an indicator of fecal contamination (28). Bacteriophages used as indicators of water quality do not multiply in the aquatic environment and may be more abundant in wastewater discharges than viral pathogens of humans (22). Traditionally, the abundances of specific bacteriophages are determined using plaque assays in which phages are detected by the formation of plaques (cleared areas) on lawns of bacteria on agar plates. It seems likely that such culture-based methods suffer from the same limitations that are encountered when enumerating human pathogens.

BACTERIA

Bacteria play a particularly important role in streams because they convert the dominant organic carbon source (DOC) into bacterial biomass; this results in an increase in the size of the organic carbon resource making it available for consumption by higher trophic levels (such as protozoa and small invertebrates; Fig. 1; 11). Because of this role, bacteria are more well studied, in some ways, than are some of the other microbial residents of streams. However, bacteria are typically lumped together as a single unit (or "black box") and most studies typically report values relevant to the full bacterial community but ignore the underlying populations.

Bacterial populations in streams, in many ways, can transcend "normal" habitat boundaries (29). In other words, bacteria are likely to readily immigrate between the benthos and water column, between the stream channel and the riparian zone, and between the hyporheos and the surface water. Thus, boundaries, such as the banks of the stream, may not represent the limits or edges of a stream bacterial population.

Bacteria are thought to move through a stream in a spiral fashion (29,30) in a manner analogous to the movement of nutrients (31). Spiraling of materials through streams is described by the nutrient spiraling concept that models release of nutrients, transport downstream, and subsequent uptake (31). In other words, nutrients "cycle" between the benthos and the water column as in any aquatic ecosystem but in streams the flow of water displaces these cycles resulting in a spiral. Thus, nutrients travel some distance (the spiraling length) downstream before they are taken up again. Leff and coworkers (29) speculated that bacteria exhibit this same tendency; in that, they are released from biofilms by a variety of different mechanisms, and are then transported downstream some distance before they reattach to a surface. This process was termed information spiraling and subsequently researchers have attempted to measure the information spiraling length (32). It is thought that the information spiraling length is influenced by factors such as: the presence of bacterivorous invertebrates, substrate type, and retentive structures in the stream (such as debris dams).

Bacterial community level measurements commonly reported in streams include total numbers, number of active cells, biomass, and production (Table 1). Numbers are typically based on treating cells with nucleic acid–specific stains (such as Acridine Orange and DAPI, 4′-6-diamidino-2-phenylindole) and viewing them under epifluorescence microscopy (reviewed by 33). Biomass determinations are often made after staining, based on sizes determined manually or by using an image analysis system. Production is typically measured based on incorporation of tritiated thymidine into DNA or tritiated leucine into protein (34).

Like many other environments, a small percentage (typically less than 1%) of bacteria in streams that can be detected by microscopy are culturable (2). Because of this low culturability, widely used methods rely on molecular biology–based approaches. Some of these methods are as simple as determining total numbers based on staining with nucleic acid-specific stains, whereas others that are aimed at examining bacterial species composition or diversity are more involved.

Examination of the bacterial community as a whole has revealed several general trends. The number of bacteria in stream water ranges over several orders of magnitude from 0.01 to 100×10^6 ml^{-1} (8,35–49). Within a given stream, total numbers typically do not vary more than one order of magnitude over the course of a year (41,42,50–53). Changes within a stream are often correlated with physical variables, such as turbidity, discharge, and rainfall (8,41,42,54), although a variety of other relationships have also been detected (39,55). Overall, bacterial numbers in water are often correlated with discharge or precipitation, suggesting that inputs of allochthonous cells during rain events and scouring of cells from the benthos are important (8). In other cases, are correlated with temperature bacterial numbers in the water (36,44).

Bacterial numbers in stream sediment also vary greatly among streams (ranging from 0.002–7.21x 10^9 g^{-1}; 8,47,56,57). Organic matter content tends to be highly correlated with bacterial number in the sediments.

Leaves in streams often represent a major source of detritus, which is readily colonized by bacteria and fungi (as discussed in a subsequent section). Microorganisms use these leaves both as a surface and as a source of organic matter, making the leaves, as described in the section on fungi, more palatable for invertebrates. One focus of studies on bacterial responses to leaves in streams is the use of leaf leachate by stream bacteria (58,59,60). Collectively, these studies have demonstrated that the response of bacteria to leachate depends on the nature of the leachate (concentration, source) and the composition of the bacterial community.

Epifluorescent examination of stream bacteria has been coupled with activity determination in some studies. One commonly used approach is the INT (2-[p-iodo-phenyl]-3-[p-nitrophenyl]-5-phenyl tetrazolium) method. With this method, respiring cells are detected by reduction of INT by the electron transport chain that causes the formation of a red deposit in active cells (61). In Australian rivers, Boon (55) found that typically less than 25% of planktonic bacteria were active. In a stream in Alabama (U.S.A.), Johnson and Ward (62) found that 15 to 26% of benthic bacteria were active, whereas only 2 to 7% of planktonic bacteria were active. Higher activity within benthic habitats suggests that these locations are sources of planktonic bacteria that then become inactive when they enter the water column. An alternative explanation is that the water column contains higher numbers of inactive cells derived from allochthonous sources.

Other methods have been used to detect activity; fluorescein diacetate (FDA) hydrolysis has been used in some freshwater environments (63) but has not been applied frequently in stream studies. Nalidixic acid, an antibiotic that inhibits cell division, is also used in activity determinations; active cells become abnormally large because cell division does not occur (64). Buchanan-Mappin and coworkers (35) found using this approach that, in stream water, 34.6% of cells were active. Results obtained with the INT method have been shown to be similar to those from the nalidixic acid method (65).

Bacterial production in streams is less commonly measured than bacterial number and is also quite variable among streams. In the water column, production ranges from 0.002 to 70 μg C l^{-1} h^{-1} (39,55,66–69) and water column production is typically lower than benthic production, which ranges from 0.04 to 140 mg C m^{-2} h^{-1} (6,39,67,70–74) and varies among sediment types. Differences are most likely related to the organic matter content of the sediment (65,69,70). For example, Findlay and coworkers (70) found that production in backwater sediments was more than 20 times higher than production

in the sandy mid-channel of the Ogeechee River (Georgia, U.S.A.). Also, Kaplan and Bott (75) found diel variation in benthic bacterial production that was related to temperature and concentrations of algal exudates. There is also considerable intrasite variability in production perhaps resulting from spatial heterogeneity (76).

Examinations of "natural" bacterial populations of specific species or higher taxa in streams are less common in the ecological literature than those dealing with whole bacterial communities. However, a large body of research has been conducted on the occurrence of indicator species (most notably *E. coli*) and pathogens (such as *Salmonella* and *Shigella*) that are not necessarily present naturally and often cannot reproduce in streams (22). The presence of such organisms is a significant water quality issue.

Perhaps the greatest effort aimed at enumerating bacteria in stream water has been on determination of the numbers of *E. coli*, fecal coliforms, or fecal streptococci as indicators of fecal contamination. Such bacteria are transients in the stream and are generally present because of inputs attributable to human activities (77). Typically, such bacterial indicators of fecal contamination are monitored based on enumeration following specific cultivation protocols.

Widely used protocols for enumeration of bacterial indicators are primarily based on cultivation of the bacteria. One commonly used approach is to concentrate bacteria from water samples by filtration (membrane filtration or MF) and to then incubate the filters on various types of selective media (such as Endo medium; 77). Most probable number (MPN) techniques are also used. Some methods that do not rely on culturing, such as nucleic acid-based detection or fluorescent antibodies, are also suitable and have the advantage of detecting cells that are not culturable.

Survival of *E. coli* has been studied in freshwater because it is a widely used indicator species and some are pathogenic. Several studies have examined survival of *E. coli* in lakes and found that mortality rates are generally high but survival is enhanced in the presence of sediment or other particles (78,79). In streams and rivers, survival of *E .coli* is also limited and is related to light and predation by protozoa (80,81).

Native populations of stream bacteria have rarely been examined and our knowledge of bacterial population ecology in streams is quite limited compared with what is known about lakes and oceans (2). Approaches that have been used to characterize the composition of the stream bacteria community (without attempting to cultivate the bacteria) include fluorescent in situ hybridization (FISH) (43,51,82) and amplification of 16S rDNA from DNA extracts (these PCR products can then be examined using, for example, denaturing gradient gel electrophoresis, 83).

Perhaps the most significant trends to emerge from these studies of stream bacteria population ecology are that (*1*) bacterial population dynamics are not reflected in the overall bacterial number, and (*2*) bacterial populations exhibit pronounced seasonal changes (43,51,53,82). In some cases, protozoan grazing, DOC pool composition, and anthropogenic disturbance appear to potentially be related to spatial changes in population sizes of particular species (43,49,51,53,82). The effect of these factors appears to vary among bacterial species; for example, *Acinetobacter calcoaceticus* populations vary spatially in a South Carolina (U.S.A.) stream in a manner that appears to be related to changes in anthropogenic disturbance; other bacterial species examined in this stream do not exhibit this same tendency (49,51).

Other studies of stream bacteria have focused on processes mediated by bacteria, such as denitrification and nitrification. Denitrification, a process mediated by heterotrophic bacteria under anaerobic conditions, has been documented in lotic ecosystems (84). The importance of denitrification varies among streams, depending on nitrate concentrations and the extent of anaerobic zones in the sediment (85). Rates of denitrification in stream sediments range from 0 to 345 $\mu mol\,N\,m^{-2}$ and are highest in anthropogenically disturbed systems (84). Although some estimates suggest that 7 to 35% of nitrogen input into rivers is lost by denitrification (84), Cooper (86) found that denitrification accounted for less than 15% of nitrate depletion in a New Zealand headwater stream. Nitrification also occurs in streams with some biofilms providing suitable environments for the ammonia-oxidizing bacteria, *Nitrosomonas* (87).

Bacteria are made available to higher trophic levels through consumption by protozoa as described in the next section. In contrast, the direct role of bacteria as food for macrofauna, specifically macroinvertebrates, is less well established and less widespread among different invertebrate taxa. The feeding method and mouth parts of the macroinvertebrate greatly influences their ability to eat bacteria.

Filter feeders, such as blackfly larvae, are capable of directly consuming planktonic bacteria and bacteria attached to small particles (54). Blackflies are able to rapidly digest bacteria (88); however, planktonic stream bacteria are smaller than cultivated bacteria so they cannot be captured as efficiently (54). In a field experiment, Hall and coworkers (32) demonstrated that blackflies accounted for 91% of bacterial ingestion in a small stream.

Bivalves are another important filter feeder that can consume bacteria. Laboratory studies have shown that both native unionid mussels and exotic species, such as *Corbicula*, can clear water of bacteria (89,90). In addition, *Corbicula* can use pedal-feeding to supplement filter-feeding and directly affect benthic bacterial abundance (91). In the laboratory, disruption of the sediments by *Corbicula* may shift bacterial population distribution (92). However, McEwen and Leff (93) found that unlike other types of invertebrates studied, guts of *Corbicula* were not readily colonized by ingested bacteria.

Other macroinvertebrates, called collector/gatherers, ingest bacteria associated with benthic particles. Austin and Baker (94) examined the gut flora of the mayfly nymph, *Ephemera*, and found it to be dominated by gram-negative rods, including *Acinetobacter* and *Flavobacterium /Flexibacter*. Mayfly nymphs were not selective in consumption of bacteria of different species but different bacteria were digested to different extents. In contrast, Baker and Bradnam (88) found that two other mayfly

nymphs, *Baetis,* and *Ephemerella*, were not able to digest bacteria.

Shredders are a functional group of macroinvertebrates that consume bacterial and fungal cells on leaf particles yet bacterial carbon appears not to be important in meeting their energy needs (95). Specifically, bacterial biomass was not a major source of carbon for the isopod *Lirceus* (96) and accounted for less than 1% of the carbon respired by a stonefly nymph, *Peltoperla*, and a cranefly larvae,*Tipula* (97). Although direct consumption of bacteria may have limited importance for shredders, the activity of microbial fermenters in the hindguts provides an alternative source of organic carbon and aids in digestion (98).

Another pathway of carbon transfer from bacteria to higher trophic levels is through meiofauna (including interstitial animals), such as rotifers, copepods, and nematodes. It has been found that copepods consumed 22% of bacterial production per day (99). Also, chironomid larvae (the aquatic larvae of a group of small flies) and copepods preferentially assimilate bacterial carbon compared with fine particulate organic matter (95). However, the extent of predation by meiofauna may not have the intensity to alter bacterial standing stocks unless certain taxa (i.e., rotifers) are particularly abundant (100).

Bacterial carbon transfer to higher trophic levels also occurs by the consumption of extracellular materials, particularly polysaccharides (101). Rounick and Winterbourn (102) found that stream invertebrates were able to readily assimilate heterotroph-dominated biofilms (including the living cells and extracellular matrix). In addition, blackfly larvae assimilated extracellular polysaccharide from a bacterium with 80 to 90% efficiency and the carbon content of the extracellular material was nine times greater than the bacterial biomass (103). Within the stream sediments, hyporheic biofilms may provide food for interstitial meiofauna (104).

Macroinvertebrates can also have indirect, more subtle effects on bacteria in streams (29). Specifically, macroinvertebrates can enhance the detachment of bacteria from stream biofilms because they feed and move across surfaces (92,105). Also, macroinvertebrates are rapidly colonized by a wide variety of bacteria, and bacteria are abundant on exoskeletons, cases of caddisfly larvae, and in the invertebrate guts (93,105). Some bacteria appear to survive gut passage and are deposited in the feces of the invertebrates (93). Colonization of invertebrates by bacteria enhances bacterial dispersal and can affect the distribution of bacteria among different habitats (93,105,106). Studies have shown that invertebrates are potent vectors of bacterial transport (93,105) and that bacteria ingested by stream insect larvae can persist in the gut into adulthood (93).

PROTOZOA

In streams, like other aquatic environments, protozoa are viewed as a major contributor to bacterial mortality and serve as a link to higher trophic levels (11). However, much more information is available about the microbial loop in marine systems and freshwater lakes (106–108). In these other ecosystems, the role of protozoa as a link between bacteria and higher trophic levels is more clearly illustrated (106) compared with streams. In the food web of streams, protozoan biomass that is derived from bacterial consumption is available for consumption by animals. This process acts to increase the size of the available carbon resources allowing a greater opportunity for carbon transfer to higher trophic levels; for example, few macroinvertebrates can effectively feed on bacterial-sized particles (3).

Protozoans are a major source of bacterial mortality and this role has been investigated in streams primarily through the use of fluorescently labeled bacteria (FLB; 109). In the Ogeechee River of Georgia (U.S.A.), an average of 15.6% of the water column was cleared of bacteria by protozoans per day (110). Protozoans were then consumed by filter-feeding macroinvertebrates (111). Estimates from the Butron River (Spain) suggested that flagellates grazed 43 to 65% of bacterial production in the water column depending on the season (112). Ciliates had higher consumption rates than flagellates but because ciliates typically have much lower abundances in rivers, they were less significant sources of bacterial mortality (113).

Benthic bacteria are also heavily grazed by protozoans (114). In White Clay Creek (Pennsylvania), protozoans grazed about 80% of benthic bacterial annual production. Ciliate grazing rates were considerably higher than flagellate grazing rates (46 to 176% higher) but ciliates were less abundant (9.1×10^3 versus 1.3×10^6 cm^{-2}) and the investigators concluded the flagellates were the most significant grazers (114).

Protozoa are commonly enumerated based on microscopic examination of live samples or after staining of preserved samples with DAPI or primulin (Table 1). The most abundant types are small flagellates and ciliates; generally ciliates are much less common than flagellates (43,110,112). Few studies have examined spatiotemporal changes in protozoan number, but in one such study, the number of planktonic protozoa in the Ogeechee River did vary greatly among sites (43). Benthic protozoan number also varies among locations in a stream; Schmid-Araya (115) found that the number of ciliates and flagellates was highest in the hyporheos compared with the surface of the benthos. Total numbers of benthic protozoa range from less than 1 to 68×10^5 cm^{-2} (75,115–117).

Identification of protozoa is typically based on morphological appearances but identification of stream protozoa (especially the small and abundant flagellates) is quite difficult (118). The size of the organisms coupled with a lack of distinguishing features and changes in appearance induced by exposure to preservatives, suggests that as for bacteria, molecular-based methods may be needed for identification. Techniques used for bacteria, such as in situ hybridization with fluorescently labeled probes, can be used to identify and enumerate protozoa (118). However, this approach has to be applied yet with much frequency in any environment.

Like algae (as discussed in a subsequent section), the presence or absence of certain protozoan taxa or changes in community composition may be a useful indicator of

ecosystem health (119). Biological indicators and indices are widely used to evaluate the ecological "health" of different streams and are often based on examination of fish and invertebrate communities. Protozoa could also potentially be used in such indices because they are widespread, diverse, and responsive to environmental changes (119).

Some pathogenic protozoa, most notably *Giardia* and *Cryptosporidium*, can occur in streams as the result of human activities (Table 2). In the same way as bacteria that are introduced into streams in feces, many protozoan parasites do not reproduce in streams but have a negative impact on water quality (22). *Giardia lamblia* is perhaps the most common cause of waterborne disease in the United States; this organism can enter streams in the feces of wildlife independent of human activities. *Cryptosporidium parvum* is another major concern; oocysts of this organism can enter water from human and domestic animal feces. Protozoan pathogens are particularly problematic because unlike bacterial pathogens and indicators, there are few simple established methods for detection and enumeration (77).

FUNGI

Fungi are a major contributor to the decomposition of leaf litter or, more generally, particulate organic carbon (POC). The most important and well-studied component of the stream fungal community are the Hyphomycota (3,120). Other nonfungal eukaryotes, like the Oomycota, potentially are important but are not well studied.

In streams, the hyphomycetes decompose POC through the production of extracellular enzymes (3,121). These enzymes are maintained in the biofilms and intermingle with extracellular enzymes produced by bacteria and algae. The biofilm enhances the retention of these enzymes and facilitates overall enzyme activity and degradation of POC (especially complex, recalcitrant compounds such as lignocellulose). The activity of these enzymes has been measured in a variety of studies.

Seven classes of extracellular enzymes have commonly been measured in streams (122–126). The enzyme classes examined include those capable of hydrolyzing: (*1*) organic phosphates (phosphatase), (*2*) organic sulfates (sulfatase), (*3*) carbohydrates (chitobiase, glucosidases, xylosidase), (*4*) cellulose (endo- and exocellulase, cellobiase) (*5*) proteins (protease, peptidase), (*6*) lipids (lipase and esterase), and (*7*) lignin (phenol oxidase and peroxidase). Enzyme activity is commonly determined by measuring the concentration of label hydrolyzed from a representative substrate. Substrates are typically linked to either a fluorochromic label, 4-methylumbelliferyl, or a colorimetric label, paranitrophenol, and enzyme activity is measured.

Enzyme activity is positively correlated with several environmental factors. Biofilms generally have greater activity than the water column (127) and, among particles, higher activity is associated with high surface area to volume ratios (121,122). Relationships between activity and microbial biomass and productivity (123), phytoplankton abundance (124), and water quality (125) have also been reported. Enzyme activities appear to be unrelated to cell number (124,128).

Fungi in leaf biofilms are consumed along with the leaves by leaf-eating invertebrates (or shredders). The fungi (and other microorganisms) contribute directly to the nutrition of the invertebrates and also act to make the leaf more digestible (120). Thus, microbial conditioning of leaves acts to make leaves more palatable and increases their nutritional value (129). Different fungal species differ in their ability to make leaves more palatable and this is reflected in the feeding preferences of the invertebrates (130). Also, different types of macroinvertebrates differ in their use of fungi on leaves; for some (such as the isopod, *Asellus*) fungi themselves provide an important food source, whereas for others (such as the amphipod, *Gammarus*) the action of the fungi in making the leaves more palatable is more significant (131).

Fungi can also provide another benefit to leaf-shredding invertebrates: extracellular enzymes consumed by the invertebrates may remain functional in their gut (129,132). Because aquatic insects do not generally exhibit much cellulase activity, acquisition of enzymes from fungi may help in leaf digestion (3).

The composition of the fungal community in streams has been determined to some degree but direct quantification of population sizes has not been accomplished. This is because of the mycelium-style bodies of the fungi; definition and delineation of individuals is difficult and hyphae may persist that do not contain cytoplasm (34). Thus, typically, conidia are recovered, identified based on their morphology, and enumerated. In one such study, Chauvet (133) discovered that the species composition of the hyphomycetes community differed among streams perhaps based on differences in environmental conditions. In addition, there is a documented succession of changes in species composition on leaves (134).

Community level studies of fungi have focused largely on the examination of extracellular enzymes. However, in the past few years it has become possible to measure fungal biomass and production (Table 1). To estimate biomass, the amount of ergosterol, a membrane sterol unique to fungi, can be determined (135). Similarly, fungal production can be estimated by amending samples with radiolabeled acetate and subsequent detection of the label in ergosterol. In one such study, Suberkropp (136) found that the annual production of fungi on decomposing leaves in a stream was 34 $g\,m^{-2}$. Using this same method, Weyers and Suberkropp (137) showed that fungal production exceeded bacterial production on leaves and that fungal production varied among streams.

ALGAE

Algae are abundant in streams where light is not limiting (3). Typically, they are found in biofilms or periphyton and thus are intermingled with a variety of other microorganisms. Periphyton is often described based on the nature of substrata on which it is formed; for example, epiphyton is on plants, epilithon is on rocks, epipelon is on soft sediments, and epixylon is on wood. (See also PERIPHYTON, this Encyclopedia.)

Diatoms are typically the most diverse algae in streams; cyanobacteria, green and red algae are also abundant (3). The contributions of these various taxa to the community biomass varies among streams. In addition, certain groups of species seem to frequently co-occur and can be placed into named assemblages that are characteristic of particular conditions (138). In addition, to these larger scale differences, periphyton is distributed in a patchy manner at a given location.

Perhaps the most common approach for examining algal abundance is to determine the amount of chlorophyll in a sample and use this to estimate total algal biomass (Table 1; 3). Many algae are amenable to identification based on morphology; for diatoms, in particular, the features of the silica tests are quite useful taxonomically. One difficulty encountered in the study of algal abundance in streams are the problems associated with collecting quantitative samples and removing algae from surfaces. For these reasons, some studies employ artificial substrates, such as ceramic tiles or glass slides, which are placed in the streams and recovered after algal colonization.

Factors that influence the amount and type of periphyton include light, temperature, flow rate, substrate type, water chemistry, and herbivory (3). Many studies have been conducted concerning the role of these factors in determining algal biomass, production, and community composition but no clear generalities have emerged. Some studies have focused on the effects of disturbance, namely, flooding, on standing stocks of periphyton and have determined that frequency of floods and the extent to which they cause movement of the sediments are important (139). Other studies have shown that the presence of grazers (including the number and type) is important (140) but that the effects of grazers are somewhat inconsistent among different studies (141). Nutrient limitation (142) and light limitation (143) can also be important. Overall, a multitude of factors can influence algal ecology in streams and it is likely that the role of these factors depends on environmental conditions and features of the algal community (144).

As a result of the great diversity in the diatom component of the algal community, some studies have suggested that examination of diatom assemblages may be useful in understanding the "health" or "quality" of a stream (145). Diatoms of different species differ in their responses to stream conditions and thus examination of the composition of the diatom assemblage can be used to calculate indicators of biotic integrity.

CONCLUSION

Microorganisms are important in streams for several reasons: they play a central role in the food web, contribute greatly to the nitrogen and carbon cycles, and some are human pathogens that have detrimental effects on water quality. Benthic biofilms play a major role in microbial ecology of streams because they allow organisms to maintain their position in the flowing water and facilitate concentration of dissolved organic carbon and nutrients from the overlying water. Because streams tend to be detritus-based, bacteria and fungi play central roles in the food web. Bacteria are the primary utilizers of the DOC, whereas fungi play a major part in the degradation of POC through the use of extracellular enzymes. The action of these microorganisms makes the carbon and energy in the detrital pool available to higher trophic levels. In the case of bacteria, this connection to other trophic levels is accomplished by protozoa, which are major sources of bacterial mortality in streams.

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STREAMS, PERIPHYTON IN.

See Periphyton

STREPTOCOCCI, FECAL.

See Fecal Streptococci/Enterococci in Aquatic Environments

STRESS.

See Metal Stressed Environments, Bacteria in; Stress Response in Archaea; Stress Response in Bacteria: Heat Shock

STRESS GENES.

See Stress Response in Archaea; Stress Response in Bacteria: Heat Shock

STRESS PROTEINS.

See Stress Response in Archaea; Stress Response in Bacteria: Heat Shock

STRESS RESPONSE IN ARCHAEA

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Salient aspects of the stress response in archaea are covered in this article. The stress response, also called heat-shock response, in the other prokaryotes, the bacteria is treated in the entry Stress Response in Bacteria: Heat Shock, this Encyclopedia. In that entry, basic concepts are introduced and explained. Consequently, they will not be repeated here but frequent reference to the Stress Response in Bacteria: Heat Shock, entry will be made to complement this entry and enhance its understanding.

Several reviews are available in print and electronically on various aspects of the archaeal stress genes, proteins, molecular chaperones, chaperonins, and related topics such as implications for general biology, medicine, environmental microbiology, and biotechnology/industry. These publications will be quoted as often as is convenient to provide the reader with the opportunity to expand on what is presented in condensed and simplified fashion in this article. In these reference publications, there is extensive bibliography covering original work, microreviews, and general articles, which the reader may also consult for experimental details, hard data, hypotheses, and predictions. The aim of this article is to provide an overview of the field — so hopefully the reader will become acquainted with the basics — highlighting areas that are still unexplored and exciting roads that the curious mind might follow in the pursuit of new knowledge.

TERMINOLOGY

Most of the terminology used in discussing stress response and related topics has been clarified in the entry Stress Response in Bacteria: Heat Shock. However, there are specific terms that apply more often to archaea than to other organisms and require explanation here. Organisms grow at various temperatures within a range that is characteristic for each species/strain, but grow faster and better at a certain temperature within that range, and this temperature is called the *optimum temperature for growth* (OTG). Organisms can be classified, considering the OTG, into several groups: psychrophiles, psychrotolerants, mesophiles, thermophiles, and extreme thermophiles or hyperthermophiles, with OTG (in degrees centigrade or C for Celsius) of 15 or less, 20 to 30, 35 to 40, 50 to 70, and 80 or higher, respectively (1). Temperatures higher and lower than the optimum may cause cell stress, which would be more marked as the difference with the optimum increases, up to the point at which there will be no stress response but cell paralysis followed by death. Another parameter to consider is the duration of the exposure to the stressful temperature, the longer the exposure and the higher the heat-shocking temperature the greater the impact on the cell, which will result in a more pronounced stress response or in death, as mentioned earlier. The same applies to any other stressor, such as environmental salt concentration, pH, barometric pressure, and so on.

It follows from the classification of organisms based on the OTG that a temperature that is optimum for a species may be stressful for another. Thus, while describing stress caused by heat shock one refers to a particular organism and a defined set of conditions pertinent to that organism and considering primarily its OTG. The same principle applies to stress caused by stressors other than heat shock (see list of stressors in the entry Stress Response in Bacteria: Heat Shock).

THE THREE DOMAINS OF LIFE

A significant revolution within biology was started by the discovery of the archaea, formerly named archaebacteria (2). These organisms were first distinguished from other prokaryotes, the bacteria, by comparative analysis of 16S rRNA sequences (2), antigenic fingerprinting (3) and cell-wall chemistry (4). The proposal was made to classify all living cells into three main evolutionary lines of descent or phylogenetic domains, Bacteria, Archaea, and Eucarya (5–7). Although this classification has been disputed and may not be perfect, it has been and still is useful to understand the diversity of life. It has been taken into account in this article, and as it will become apparent to the reader, the classification does indeed help organize knowledge on the stress response and antistress molecules and mechanisms. Stress genes and proteins can be grouped into "bacterial" and "archaeal" sets considering their similarity in sequence and other properties, such as the promoters and transcription patterns in the case of the genes. However, instances of a "bacterial" gene existing in an archaeal genome complicate the evolutionary schemes inferred from comparative analyses of sequences (8); one must invoke lateral (horizontal) gene transfer as a probable mechanism that would explain the presence of a "foreign" gene in an archaeon. A case in point is the stress, molecular chaperone gene *hsp70(dnaK)* (9), as it is explained in the following section. Despite some of the arguments and observations against the subdivision of all life into the three domains mentioned earlier, it is clear that archaea can be distinguished from bacteria by some important features such as the basal transcription and translation machineries, and the DNA repair mechanism (10–16). These machineries and mechanism in archaea are more similar to those operating in eukaryotic cells than to those of bacteria. Pertinent to this article, also some components of the chaperoning systems in archaea are more similar to their counterparts in eucarya than they are to the bacterial homologs (17). This is illustrated by the chaperonins that belong to the group II in archaea and also in eucarya, whereas bacterial chaperonins are different and belong to group I. As more and more genomes are sequenced, the "contamination" of archaeal genomes with bacterial genes grows in magnitude, and the separation between the two domains becomes increasingly fuzzier. This state of affairs, which is reflected in the data presented in some of the tables of this article, must be borne in mind by the reader. It is likely that in the not too distant future many of the today's clear-cut differences among the three domains will no longer be evident. Consequently, it is expected that while going over this article the reader will see new territories for exploration, discover critical questions that still need an answer, and think of experiments to perform for obtaining the answers and of theories for explaining what is now obscure.

THE BEGINNINGS

Initial studies of the stress response in archaea were done in the classical fashion with the means available at the time: mid-1980s and early 1990s (18). Archaeal cells were submitted to heat stress, that is, they were heat-shocked by abruptly rising the temperature of the culture several degrees above the OTG. The cells were kept at the elevated temperature for variable periods from about 10 minutes to

Table 1. Examples of Heat-Shock Response in Archaeal Species: Main Features[a]

Organism		Heat Shock		Heat-Shock Proteins (Hsp)		
Archaeal Group *Species*	OTG (°C)[b]	Temp.(°C)[c]	Duration (min)	Number[d]	MM Range (kDa)[e]	Hsp70
Extreme halophiles						
Halobacterium volcanii	45	60	75	4–6	21–105	No[f]
Methanogens						
Methanococcus voltae	30	45	9–12	11	18–90	No
Extreme thermophiles						
Sulfolobus acidocaldarius	70	85	90	5	22–86	No[g]
Sulfolobus shibatae	70	88,92	60	1	55	No
Pyrodictium occultum	102	108	240	1	56;59[h]	No
Strain ES4	77–99	102	120	1	98	No

[a]Reproduced from E. Conway de Macario and A. J. L. Macario, *Trends Biotechnol.* **12**, 512–518 (1994), in which additional information may be found, with permission from the copyright owner; [b]OTG, optimum temperature for growth; [c]Heat-shocking temperature; [d]Number of protein bands determined by one-dimensional sodium-dodecyl sulfate-polyacrylmide gel electrophoresis followed by autoradiography (*H. volcanii*, *M. voltae*, *S. acidocaldarius*, and *S. shibatae*) or densitometry (*P. occultum* and strain ES4); [e]MM, molecular mass estimated from migration distance in the gel by comparison with a standard ladder made of proteins with known masses; [f]*hsp70(dnaK)* genes have been demonstrated more recently in this and other species of this group by direct cloning of the gene, or by genome sequencing (see also Table 2(I)); [g]Absence of the *hsp70(dnaK)* gene has been confirmed in every extreme thermophilic archaeon whose genomic sequence has been determined up to this time; see examples in Table 2 (II); [h]Described as an ATPase complex with two polypeptides of 56 and 59 kDa (19).

about 4 hours, and subsequently lysed to examine protein patterns. These were compared to those of lysates from nonstressed cells. A sample of the reported data is shown in Table 1.

Several features emerge from examining the data. One of them is the tendency by the investigators to apply the stressor for long periods by comparison with similar experiments performed with bacteria (see the entry STRESS RESPONSE IN BACTERIA: HEAT SHOCK). This trend might be because of a requirement of archaeal species, at least some of them, for a longer exposure to stressors as compared with bacteria for mounting a detectable stress response.

Another noteworthy feature is that, contrary to the typical bacterial response to heat shock that is manifested by an increase in many proteins, some archaeal species show an increase in just one protein.

Last but not least, these early experiments already indicated that the Hsp70(DnaK) molecular chaperone is not present in some archaeal species. This is an important topic that will be treated in some detail in a subsequent section.

GENE CLONING

In the late 1980s, efforts were made in a few laboratories to clone the genes producing some of the Hsp observed in archaea responding to stressors. In 1991, the gene encoding the chaperonin subunit TF55 was cloned from the thermophile *Sulfolobus shibatae*, and sequenced (20). The deduced protein was found to be the homolog of the eukaryotic molecule t-complex polypeptide-1 (TCP-1), and was subsequently established to be the same type of molecule as that of a component of an ATPase complex found earlier in the cytoplasm of the hyperthermophile *Pyrodictium occultum* (19). Afterward it was also established that TCP-1 is the typical chaperonin of the eukaryotic cytosol; subsequent studies have advanced considerably our knowledge of the chaperonin family in the three domains of life (see section The Chaperonins).

Also in 1991, the occurrence of the *hsp70(dnaK)* gene in an organism of the domain Archaea was demonstrated for the first time by cloning and sequencing (21). At the time, this finding seemed simply to confirm the general belief that the *hsp70(dnaK)* gene is highly conserved and universally distributed — it is present in every bacterial and eukaryotic species tested, therefore, it seemed only natural that the gene will also exist in archaea without exceptions. However, the discovery of the gene in an archaeon acquired a new dimension when it was observed that some archaeal species do not have it. As mentioned in the preceding text (see Table 1), early studies had indicated that the product of the gene, the molecular chaperone Hsp70(DnaK), did not show up in the protein patterns detected in heat-shocked cells, in contrast to the rule in bacteria and eukaryotes, in which an increase in Hsp70(DnaK) is observed in response to heat shock (18). The search for the *hsp70(dnaK)* gene among archaea using indirect means such as blottings (Northern, Southern, Western) and protein pattern determination by electrophoresis produced negative results in several instances — suggesting absence of the gene — but the results could not be considered definitive. This had to wait until the genomes of archaeal organisms were sequenced, and the situation is now quite clear, as explained later.

DISCONTINUOUS DISTRIBUTION OF THE *hsp70(dnaK)* GENE WITHIN THE DOMAIN ARCHAEA

One of the major conceptual revisions within the field of stress and antistress mechanisms was fueled in the last five years or so by the cumulative evidence demonstrating the discontinuous occurrence of the *hsp70(dnaK)* gene among archaea (9,18,22). In contrast, the gene has been found in bacteria and eukaryotes with no exception. This is one example showing a difference between the Archaea and the other two domains of life. A sample of the data is displayed in Table 2. The situation pertinent to one

Table 2(I). Occurrence, or Lack Thereof, of the *hsp70(dnaK)* Gene Among Prokaryotes. I. Methanogenic Archaea[a]

Species, Strain	OTG (°C)[b]	*hsp70(dnaK)*	Genome Size (Mb)[c]	Demonstrated by:
Methanosarcina mazeii S-6	37	Yes	n.d.[d]	S, N, W, seq.[e]
mazeii JC3	37	Yes	n.d.	N
mazeii LYC	37	Yes	n.d.	N
sp. JVC	37	Yes	n.d.	N
acetivorans C2A	37	Yes	5.7	N
barkeri	37	Yes	5.7	S
thermophila TM-1	50	Yes	5.7	S, N, seq.
Methanospirillum hungateii	37	No	n.d.	S
Methanobacterium				
thermoautotrophicum ΔH	65	Yes	1.8	seq.
Methanococcus voltae	37	No	n.d.	S, W
vannielii	37	No	n.d.	S, P
jannaschii	85	No	1.7	S, seq.
Methanothermus fervidus	85	No	n.d.	S, P
Methanopyrus kandleri	100	No	n.d.	S, P

[a]Modified from A. J. L. Macario, M. Lange, B. K. Ahring, and E. Conway de Macario, *Microbiol. Mol. Biol. Rev.* **63**, 923–967 (1999), in which additional information may be found, with permission from the copyright owner; [b]OTG, optimum temperature for growth; [c]Mb, millions of base pairs; [d]n.d., not determined; [e]Abbreviations are: S, N, and W, Southern, Northern, and Western blotting, respectively; seq., sequencing of gene or genome; and P, polymerase-chain reaction (PCR).

Table 2(II). Occurrence, or Lack Thereof, of the *hsp70(dnaK)* Gene Among Prokaryotes. II. Extreme Halophilic and Extreme Thermophilic Archaea[a]

Species, Strain	OTG (°C)[b]	*hsp70(dnaK)*	Genome Size (Mb)[c]	Demonstrated by:
Haloarcula marismortui	45	Yes	n.d.[d]	seq.[e]
Halobacterium cutirubrum	45	Yes	n.d.	seq.
halobium	45	Yes	n.d.	S, P
sp. NRC-1	45	Yes	2.6	seq.
Thermoplasma acidophilum	55	Yes	1.7	seq., P
Sulfolobus solfataricus	70	No	3.0	S, P
Sulfolobus sp.	70	No	n.d.	S
Archaeoglobus fulgidus	83	No	2.2	seq., P
Desulfurococcus mobilis	85	No	n.d.	S, P
Thermococcus tenax	88	No	n.d.	S, P
Pyrococcus furiosus	100	No	2.0	seq.
horikoshii	100	No	1.7	seq.
woesei	100	No	n.d.	S, P
abyssi	100	No	1.8	seq.
Pyrobaculum aerophilum	100	No	2.2	seq.
Aeropyrum pernix K1	100	No	1.7	seq.

[a]Modified from A. J. L. Macario, M. Lange, B. K. Ahring, and E. Conway de Macario, *Microbiol. Mol. Biol. Rev.* **63**, 923–967 (1999), in which additional information may be found, with permission from the copyright owner; [b]OTG, optimum temperature for growth; [c]Mb, millions of base pairs; [d]n.d., not determined; [e]Abbreviations are: seq., sequencing of gene or genome; S, Southern blotting; and P, polymerase-chain reaction (PCR).

Table 2(III). Occurrence, or Lack Thereof, of the *hsp70(dnaK)* Gene Among Prokaryotes. III. Hyperthermophilic Bacteria[a]

Species	OTG (°C)[b]	*hsp70(dnaK)*	Genome Size (Mb)[c]	Demonstrated By:
Thermus thermophilus	70	Yes	n.d.[d]	seq.[e]
Thermomicrobium				
roseum	70	Yes	n.d.	seq.
Thermotoga maritima	80	Yes	n.d.	seq.
Aquifex aeolicus	83	Yes	n.d.	seq.
pyrophilus	83	Yes	n.d.	seq.

[a]Modified from A. J. L. Macario, M. Lange, B. K. Ahring, and E. Conway de Macario, *Microbiol. Mol. Biol. Rev.* **63**, 923–967 (1999), in which additional information may be found, with permission from the copyright owner; [b]OTG, optimum temperature for growth; [c]Mb, millions of base pairs; [d]n.d., not determined; [e]seq., sequencing of gene or genome.

of the archaeal groups, the methanogens, is depicted in Table 2(I). Different lineages of methanogens either have or do not have the gene. Interestingly, among the species that do not have the gene are the hyperthermophilic methanogens. Table 2(II) presents information on the other two archaeal groups, the extreme halophiles and the hyperthermophiles. All the extreme halophiles studied thus far have the gene but none of the hyperthermophiles do. Remarkably, all the hyperthermophilic bacterial species examined do have the gene, as illustrated in Table 2(III). Thus, one may conclude that the lack of the *hsp70(dnaK)* gene is a trait present only in archaea and is more generalized among hyperthermophiles. Also noteworthy, is the absence of the genes encoding the other two components of the molecular chaperone machine (see pertinent section in the following text), the proteins Hsp40(DnaJ) and GrpE, whenever the *hsp70(dnaK)* gene is missing (9). The three genes seemed to have evolved together, as a group.

STRESS PROTEINS IN ARCHAEA

A comprehensive list of stress proteins is available in the entry Stress Response in Bacteria: Heat Shock, this Encyclopedia. Examples of stress proteins and molecular chaperones in archaea are provided in Table 3. Bacterial equivalents are also shown for comparison. Once again, differences between these two prokaryotic groups are apparent. However, some archaeal proteins resemble more the homologs from bacteria than those from eucarya, for example, the components of the molecular chaperone machine (17,21,23–25). In contrast, other archaeal proteins are closer to the eukaryotic than to the bacterial homologs, for instance, the chaperonins ((17,26–28); and see following section). Another important group encompasses comparatively small molecules, the small heat-shock proteins also known as sHsp (17). These are currently being studied and their role in protein folding and refolding, and in the stress response is being assessed.

THE MOLECULAR CHAPERONE MACHINE

This machine is made of three major components, Hsp70(DnaK), Hsp40(DnaJ), and GrpE or nucleotide exchange factor and, depending on the circumstances, it also includes one or more cochaperones (28–31). The three main proteins associate and interact while performing the tasks of assisting nascent polypeptides to fold correctly or partially denatured proteins to refold, and newly made proteins to translocate to the cell's locale where they reside and function, for example, the periplasmic space in prokaryotes and the organelles in eukaryotes.

As discussed in a preceding section, a number of archaeal species do not have the machine whereas others do. A sample of the latter is displayed in Table 4. It is apparent that the organisms possessing the machine are mesophiles or moderately thermophilic; no hyperthermophilic species that has the machine has been found yet.

The functions and mechanism of action of the machine have been extensively studied in bacteria, particularly in *Escherichia coli* (29–31), and also in eukaryotes (32–34), but very little is known about the archaeal counterpart (17). One may assume that the machine works in archaea under the same circumstances and by the same mechanisms known for bacteria, but this inference would be unwarranted. Although the components of the machine are very similar in sequence to the homologs from bacteria, particularly gram-positives, such as *Bacillus subtilis* and others (17,21,23–25), and one may even hypothesize they were received during evolution from bacteria by lateral gene transfer (9,22), they may not follow the same rules that dictate the behavior of the bacterial machine. In the archaeal cell, the intracellular milieu is different as compared with that of bacteria and, most importantly, the genes are within a DNA with promoter regions different from those of bacteria (10,14–16,35). Thus, at least the mechanisms controlling the production of the machine components must be expected to be different in archaea by comparison with bacteria. This is suggested by the transcription patterns of the *hsp70(dnaK)*, *hsp40(dnaJ)*, and *grpE* genes in the only archaeal species studied up to this time, *M. mazeii* (36,37). In this organism, the genes are clustered together like in several gram-positive bacteria (35), but are not transcribed as a unit or operon, as they are in bacteria (see entry Stress Response in Bacteria: Heat Shock). In *M. mazeii* the genes are transcribed separately, each from its own promoter, and consequently the transcripts are monocistronic. In contrast, the genes function as an operon in bacteria and the transcript is polycistronic. These data indicate that the regulatory mechanisms in archaea differ from those of bacteria and, therefore, one might expect that the biochemical interactions of the chaperone machine components among themselves, and with the substrate and ATP are also somewhat unique for archaea. This idea is also encouraged by the realization that the cochaperones present in archaea are not the same as those found in bacteria (see section on Cochaperones). In any case, this is an extremely interesting topic that deserves investigation.

THE CHAPERONINS

Bacteria and archaea differ in their stock of chaperonins: the former have the bacterial or group I and archaea have the eucaryal or group II molecules (17). The typical example of group I chaperonin is GroEL, an Hsp of the Hsp60 family, Tables 3 and 5 (see also entry Stress Response in Bacteria: Heat Shock in this Encyclopedia). Group II chaperonins, exemplified by the TF55 molecule characterized a decade ago, are the homologs of the eukaryotic cytosolic chaperonin TCP-1 (see Section The Beginnings, in the preceding text; 38). The structural features, biochemistry, evolution, and functions of archaeal and eukaryotic chaperonins have been extensively investigated and reviewed (19,26,27,39–43). Suffice to say here that depending on the species, archaea possess one, two, or three different chaperonins or subunits of the chaperonin complex, sometimes called thermosome. The subunits associate to form homo- or hetero-oligomeric rings, which in turn associate with each other to build a cylindrical

Table 3. A Sample of Stress Proteins and Chaperones in Prokaryotes[a]

Family Name(s)	MM (kDa)[b]	Examples	
		Bacteria	Archaea
Heavy, High MM, Hsp100	100 or higher	ClpE	No[c]
Hsp90	81–99	HtpG	No
Hsp70, DnaK, Chaperones	65–80	DnaK; Hsc66	Hsp70(DnaK)
Hsp60, Chaperonins	55–64	GroEL	TF55; Chaperonin subunits (thermosome subunits)[d]
Hsp40, DnaJ	35–54	DnaJ; Trigger Factor (TF)	Hsp40(DnaJ)
Small Hsp, sHsp	34 or lower	GroES; Hsc20; GrpE; PPIase; PDIase (DsbA-D, G); IbpA and IbpB; SecB; Hsp15; other	GrpE; PPIase; PDIase; Prefoldin (GimC); other sHsp[d]

[a]Modified from A. J. L. Macario and E. Conway de Macario, *Frontiers Biosci.* **6**, d262–d283 (2001). *http://www.bioscience.org/2001/v6/d/macario/fulltext.htm* with permission from the copyright owner; for additional references see (17); [b]MM, molecular mass; [c]No, not yet investigated, or investigated but not yet found, or found but incompletely characterized; [d]Some archaeal species whose genomes are currently being sequenced appear to have *groEL* and *groES* gene homologs.

Table 4. A Sample of Molecular Chaperone-Machine Genes Found in Archaea[a]

Gene	Organism	OTG °C[b]
hsp70(dnaK)	*Methanosarcina mazeii* S-6	37
	thermophila TM-1	50
	Methanobacterium thermoautotrophicum ΔH	65
	Haloarcula marismortui	45
	Halobacterium cutirubrum	45
	Halobacterium Sp. NRC-1	45
	Thermoplasma acidophilum	55
hsp40(dnaJ)	*Methanosarcina mazeii* S-6	37
	thermophila TM-1	50
	Methanobacterium thermoautotrophicum ΔH	65
	Halobacterium cutirubrum	45
	Halobacterium Sp. NRC-1	45
	Thermoplasma acidophilum	55
GrpE	*Methanosarcina mazeii* S-6	37
	thermophila TM-1	50
	Methanobacterium thermoautotrophicum ΔH	65
	Halobacterium Sp. NRC-1	45
	Thermoplasma acidophilum	55

[a]Modified from A. J. L. Macario and E. Conway de Macario, *Frontiers Biosci.* **6**, d262–d283 (2001). *http://www.bioscience.org/2001/v6/d/macario/fulltext.htm* with permission from the copyright owner; the genes have been demonstrated by direct cloning and sequencing of individual genes or by genome sequencing; [b]OTG, optimum temperature for growth.

body with a central cavity. The latter is called the folding chamber because it is where polypeptides are folded into a functional shape. Interaction of the chaperonin complex with the chaperone machine in eukaryotes and in bacteria is under active investigation. In bacteria, GroEL also forms polymers, rings, and a cylinder with a cavity whose lid is provided by GroES (41,44,45). In some instances it has been proposed that the molecular chaperone machine "presents" the nascent polypeptide in a "foldable" state to the GroEL complex and promotes its entry into the folding chamber from which the protein emerges with a functional shape (29). If the folding process was unfinished, then the partially folded polypeptide reenters the cylinder's cavity for a second round of assisted folding. These cycles continue until a fully folded protein emerges. An ATP cycle accompanies these folding steps which has been studied in bacteria and in archaea in relation to subunit and ring interactions, and in relation to allosteric changes in the conformation of the multiring complex (40,45).

A crucial question that remains unanswered is whether the molecular chaperone machine of archaeal species that have it interacts with the group II chaperonin complex that coexists in the same cell (9,17). This is an important theme not only within the field of archaea but also for the entire field of protein folding that deserves investigation, and archaea, such as *M. mazeii*, which has the chaperone machine and the group II chaperonins, offer unique experimental models for this investigation.

THE COCHAPERONES

Recent studies have revealed that the chaperoning of nascent polypeptides can occur cotranslationally, that is, as the amino acid chain is being assembled on the ribosome (29), and that it involves ribosome-associated chaperones (28). These are named cochaperones or chaperone cofactors, as are other molecules that interact with the three major components of the chaperone machine, as explained in a preceding section, Table 5. A bacterial cochaperone is the trigger factor (TF), and eucaryal cochaperones are BAG-1, Hop, Hip, and NAC, all described in detail in a recent publication, which contains extensive referencing to the original work (28).

A search for genes that would encode cochaperones in fully sequenced genomes from five archaeal species with various OTG, including one species possessing the molecular chaperone machine, did not reveal any clear, highly conserved example of such genes (28). However, it must be said that the search provided some evidence favoring the existence of archaeal representatives of the eucaryal Hop and alpha-NAC subunit, a point that deserves further investigation.

PREFOLDIN

Prefoldin was found in the cytosol of eukaryotic cells and is composed of six subunits (46,47). The subunits associate in a multimeric complex, GimC, which assists protein folding. In archaeal genomes, a similar system has been found, but it includes only two genes that encode only two different subunits, alpha and beta. The archaeal alpha subunit would be the equivalent of the eukaryotic subunits Gim2 and Gim5, whereas beta would be the homolog of the eukaryotic Gim 1, 3, 4, and 6 subunits.

The molecules from *Methanobacterium thermoautotrophicum*, named MtGimC alpha and beta subunits, were studied experimentally (48,49). The archaeal complex is a heterohexamer (two alpha and four beta subunits) with a total mass of 87 kDa. The functions of the archaeal prefoldin and its role in the stress response have not yet been fully elucidated. The genes are not stress inducible, which raises questions about whether prefoldin does belong in the category of stress proteins, although it certainly seems to act as a chaperone. If this were the case, prefoldin would be one example of molecular chaperone that is not a stress protein. Future experiments ought to aim at answering the questions of whether prefoldin interacts with the molecular chaperone machine and/or with the chaperonins in the process of assisting protein folding and refolding in vivo, and whether prefoldin plays a role in cell survival in the face of stress and in cell recovery after stress. Other topics that deserve investigation are the mechanism of action of archaeal prefoldin, and preferred intracellular substrates under physiological conditions and during the stress response.

OTHER STRESS PROTEINS AND ANTISTRESS MECHANISMS IN ARCHAEA

Archaeal organisms are widespread in nature and occupy a diversity of ecosystems from the mild (i.e., suitable to human and many other warm-blooded animals) to the extreme: very cold or hot, very acid or alkaline, high barometric pressure, and so on. One, therefore, expects that archaea have evolved an array of molecules, and mechanisms to cope with these, sometimes unusual by human standards, environments that constitute their habitats. Also, a number of different stressors have been used experimentally to study the stress response in archaea,

Table 5. Examples of Molecular Chaperones and Chaperonins in the Three Phylogenetic Domains[a]

Bacteria	Archaea	Eucarya[b]
GroEL (Hsp60)	No[c]	mt, chl: Hsp60 (Rubisco subunit-binding protein); ct, ER: No
GroES (Hsp10)	No	mt, chl: Yes; ct, ER: No
No	TF55; thermosome subunits	ct: TRiC (CCT; TCP-1) subunits; mt, chl, ER: No
G+, DnaK(Hsp70)[d]	Hsp70(DnaK)[e]; No-hyp.[f]	No
G−, DnaK(Hsp70)[d]	No	mt, chl: Hsp70; ct, ER: Hsp70 paralogous
DnaJ(Hsp40)	Hsp40(DnaJ)[e]; No-hyp.	ct, mt, chl, ER: Yes
GrpE	GrpE; No-hyp	mt, chl: Yes; ct, ER: No
G−, HptG	No	ct, ER: Hsp90 paralogous; mt, chl: No
TF (trigger factor)	No	No
No	No[g]	Cochaperones: BAG-1, Hop, Hip, NAC, in the ct

[a] Modified from A. J. L. Macario, M. Lange, B. K. Ahring, and E. Conway de Macario, *Microbiol. Mol. Biol. Rev.* **63**, 923–967 (1999) with permission from the copyright owner; [b] Abbreviations are: mt, mitochondria; chl, chloroplast; ct, cytosol; ER, endoplasmic reticulum; [c] No, not yet investigated or not well characterized, or investigated but not yet found — some archaeal species whose genomes are currently being sequenced appear to have *groEL* and *groES* gene homologs; [d] G+ and G−, gram-positive and- negative bacterial type of DnaK, respectively; [e] Protein similar to gram-positive bacterial homologs but, for those studied up to this time, transcription-initiation mechanism similar to that of eucarya; [f] No-hyp., not yet investigated, or investigated but not yet found in hyperthermophiles; [g] A survey of five genomes suggests that archaea possess equivalents of Hop and the alpha subunit of NAC (28).

Table 6. Examples of Stressors, Other Than Heat, Tested with Archaeal Cells[a]

Stressor	Organism
Hyperosmolarity	*Pyrococcus furiosus*, *M. thermophila* TM-1, *M. mazeii* S-6, *Haloferax volcanii*, *Methanococcus igneus*
Hypoosmolarity	*Haloferax mediterranei*, *H. volcanii*
Pressure	*Pyrococcus* ES4, *Pyrococcus* ES1, *Methanococcus thermolithotrophicus*, *M. jannaschii*, *Thermococcus peptonophilus*
Ethanol	*Methanococcus voltae*
UV light	*Sulfolobus acidocaldarius*, *P. furiosus*
Copper	*Methanobacterium bryantii*
Heavy metals	*Methanosarcina mazeii* S-6
H_2O_2	*Methanococcus voltae*
Casaminoacid/Fe^{2+} starvation	*Metallosphaera sedula*
PH	*Pyrococcus furiosus*
Ammonia	*Methanosarcina mazeii* S-6, *M. thermophila* TM-1[a]
Phosphorus starvation	*Sulfolobus acidocaldarius*

[a]Reproduced from A. J. L. Macario, M. Lange, B. K. Ahring, and E. Conway de Macario, *Microbiol. Mol. Biol. Rev.* **63**, 923–967 (1999), in which additional information may be found, with permission from the copyright owner.

a sample of which is shown in Table 6. The archaeal antistress mechanisms are indeed varied and comprise the genes and molecules described in the preceding section, and others, which are briefly discussed later.

Small Heat-Shock Proteins and Nonproteinic Antistress Molecules. In addition to the better known components of the molecular chaperone machine and the chaperonins, there are other proteins and nonproteinic compounds that play a role in protein folding and protection from the consequences of stress, and in the stress response, which also deserve attention. A comprehensive list of stress proteins is provided in the entry Stress Response in Bacteria: Heat Shock, this Encyclopedia, and some of them are listed in Table 3 (small Hsp). These have recently been reviewed and an extensive bibliography is available in the review articles (17,50).

Antioxidants. Oxygen is necessary for life to many aerobic organisms, including humans. However, it can give rise to toxic radicals that the cell must eliminate or neutralize, otherwise they will damage many molecules especially lipids, proteins, and DNA. The antioxidant defense system is constituted of superoxide dismutases (SOD) that scavenge toxic oxygen derivatives and thus it is considered part of the cell's armament against oxidative stress. SODs have recently been found in archaea but their precise role and evolution have not yet been elucidated (51).

Built-in Protein Stability. Those archaeal species with OTG above 50 °C, particularly the hyperthermophiles with OTG around or above 80 °C, present a challenge to common knowledge about protein stability, which is mostly grounded on the study of enzymes and other proteins from mesophilic organisms. It is well known that heating denatures many proteins, and one wonders how the enzymes and other molecules in extreme thermophiles manage to maintain a functional shape. Several studies have looked into this problem, and the conclusions are somewhat surprising (52,53). No specific structural feature has been identified that would be distinctive of proteins from hyperthermophilic archaea and that would per se endow them with extra stability in the face of high temperature (52). Different proteins have evolved different strategies for stability but they all resort to the same weak electrostatic forces and hydrophobic interactions among the same 20 amino acids that are found in the proteins from mesophilic organisms. Other observations indicate that the surface of hyperthermophilic proteins have a larger proportion of solvent-accessible charged residues paralleled by decrease of polar (noncharged) amino acids (54), and these proteins have more salt bridges than the mesophilic molecules (55).

Resistance to Changes in the Environmental Salt. Cells exposed to a change in external salt concentration mount short- and long-term responses (56). Osmoregulation and osmoadaptation mechanisms are set in motion. Water moves in or out of the cell in the direction dictated by the salt gradient. Organic solutes (osmolytes) accumulate inside the cell either by uptake from the surrounding medium or by de novo synthesis. Archaea have unique osmolytes that contribute to stabilize intracellular proteins in the face of altered osmolarity, and do not interfere with other cellular functions. These osmolytes along with the synthesis of chaperonins and molecular chaperones seem to cooperate in the stress responses elicited by hypo- and hyperosmotic shocks. However, more information is needed to reveal all the mechanisms involved in these responses.

Cell Membrane. Many archaeal species inhabit extreme environments, very cold or hot, very acid or alkaline, or very salty. Many hyperthermophiles are also extreme acidophiles and live at very low pH as compared with the pH that is optimum for human cells. These extremophiles have unique membrane-spanning tetraether lipids (57). A compromise must be reached by the molecules of the extremophiles between the rigidity necessary to withstand potent stressors and the flexibility needed to perform functional intramolecular and intermolelcular interactions. Archaea offer a uniquely rich set of organisms and molecules for studying how the latter manage to perform their physiological functions, very much like those of mesophilic homologs, but under very different conditions of pH, temperature, and salinity.

THE PROTEASOME AND PROTEOLYSIS

The proteasome is a proteolytic multisubunit assembly that occurs in the cytosol and nucleus of eukaryotic cells, and in the prokaryotes gram-positive actinomycetes and archaea (58,59).

Proteolysis, its role in maintaining the intracellular protein balance together with the chaperoning systems, and its participation in the stress response and its regulation in bacteria have been explained in the entry Stress Response in Bacteria: Heat Shock, this Encyclopedia. Moreover, the archaeal proteasome has been discussed in detail in a recent publication (58).

Proteases such as FtsH (HflB), Lon, ClpAP, ClpXP, HslUV (ClpYQ), and proteasome complexes are energy dependent and their participation in the stress response is suggested by the fact that when they are inhibited, or are present at low levels in the cell, the stress response is deficient. Furthermore, the genes coding for some of these proteolytic enzymes (e.g., subunits of the proteasome, Lon, ClpXP, FtsH, and HslUV) are stress inducible.

The amino acid sequences of these proteases are not conserved, and therefore are not very similar to one another, but they all form multimeric complexes and ring structures with a central cavity, like the chaperonin complex discussed in a preceding section. The central chamber is the place where degradation of polypeptides occurs after they have gained access into the central cavity through narrow windows. The size of these windows allows the passage of thin structures, which suggests that only denatured polypeptides can enter the proteolytic chamber.

The archaeal 20S proteasome, like its bacterial equivalent, has a simple composition by comparison with the eukaryotic homolog, including only two to four different subunits — in contrast the eukaryotic 20S proteasome is made of 14 different subunits.

There are also proteasome-associate AAA ATPases (ATPases associated with various cellular activities) that cooperate to unfold misfolded polypeptides and to dissolve aggregates of proteins and thus allow their introduction into the proteolytic chamber of the proteasome. In addition, there is an archaeal proteasome-activating nucleotidase (PAN) that, as its name suggests, activates the proteasome and promotes proteolysis. All these molecules are currently under investigation to determine their role in vitro and, most importantly, in vivo, in normal cellular physiology, in the cell's response to stress, and in recovery after stress.

MULTICELLULAR STRUCTURES

Some archaeal species undergo morphological conversions and form multicellular structures (60). *Methanosarcina* species are the most remarkable in this regard, and *M. mazeii* has been studied in some detail. This species forms three main morphotypes: single cell, packet, and lamina in laboratory cultures (61), which differ not only in macro- and microscopic anatomies, but also in composition (62). The argument can be made that these morphotypes are part of a life cycle that reflects a differentiation-development process that might be set in motion by environmental changes and stress (28). The three morphotypes also occur in complex ecosystems, such as methanogenic bioreactors (63,64), as illustrated in Figure 1. The single cell morphotype consists of cells (3 to 5 micrometer in diameter) separated from one another that grow in suspension in liquid medium. They are fragile and sensitive to different stressors: mechanical shearing, pressure, chemicals, temperature, and antibiotics. In contrast, the packet morphotype is considerably resistant to all these stressors. A packet

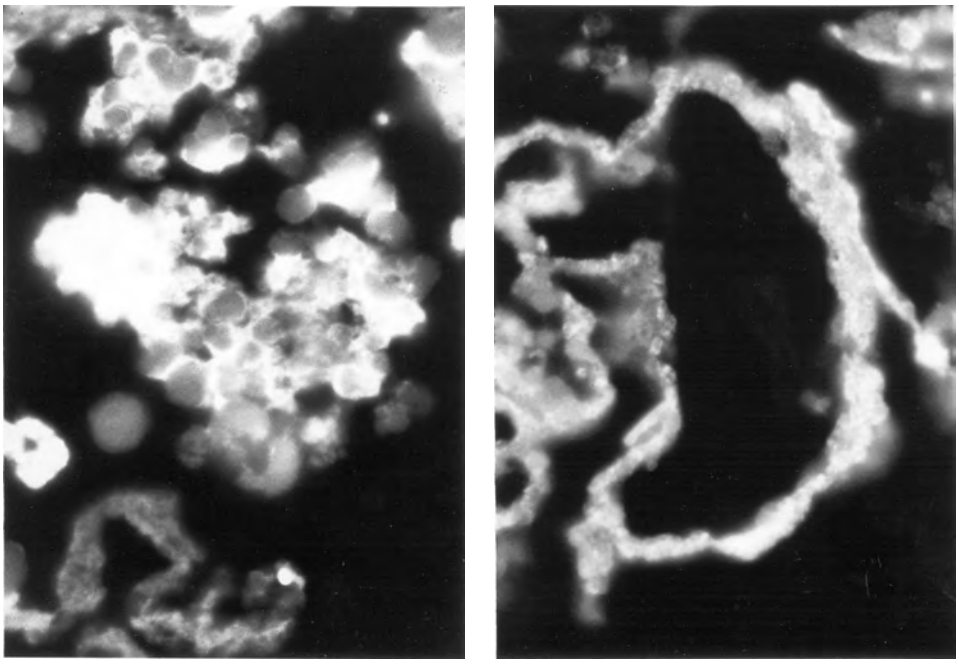

Figure 1. Microscopic view of two thin histological sections of a granular consortium from a methanogenic bioreactor showing *Methanosarcina* packets (left) and lamina (right); the latter has been torn apart from the body of the granule by the sectioning blade. The lamina folds onto itself, sometimes several times, and forms cylindroids that appear in sagital sections, such as the one shown on the right, as an irregular ring or more or less concentric rings, respectively. In this preparation, the packets and lamina are bright with fluorescence because the sections were submitted to a method, indirect immunofluorescence, which uses a calibrated antibody probe of predetermined specificity spectrum and specifically stains *Methanosarcina* cells. Scale: 1 cm = 11.2 micrometers for both, left and right panels. Reproduced from J. E. Schmidt, A. J. L. Macario, B. K. Ahring, and E. Conway de Macario, *Appl. Environ. Microbiol.* **58**, 862–868 (1992) (65), with permission from the copyright owner.

is formed by many cells held together by intercellular connective material, which also surrounds the whole structure, Figure 1, left panel. The latter is globular in shape, and can reach large sizes with diameters measurable in millimeters rather than in micrometers. The third main morphotype of *M. mazeii* is also a multicellular structure, but in contrast to the packet, which is spheroidal, it is flat, see Figure 1, right panel. Its thickness rarely surpasses three cell diameters, that is, the flat structure is typically a cell monolayer. Because of its shape, this morphotype is called lamina. It grows in suspension — it does not stick to the bottom or the walls of the culture vessel — and reaches several centimeters in every horizontal direction. Lamina is more resistant to stressors than single cells but not as nearly as resistant than the packets.

It may be hypothesized that formation of multicellular structures by bacteria, archaea, or eukaryotes is, at least in part, an antistress mechanism, and that the capacity to form such structures conferred evolutionary advantage to primitive organisms (28). Likewise, today's organisms may be able to resist stressors more efficiently by forming multicellular structures. Experimental observations with *M. mazeii* suggest the following chain of events: a stressor impacts on single cells, these mount a stress response including the synthesis and extrusion of the substance that will form the intercellular connective material, and as the cells multiply they remain together enmeshed in that material. Cells continue to thrive and multiply inside the newly-formed multicellular structure protected from the environment and its potentially stressful changes.

FUTURE PROSPECTS

A number of aspects of the stress response in archaea deserve study either because they are not yet as well understood as their bacterial and eucaryal counterparts, or because they do not have a known equivalent in the other two life domains and are interesting for this very reason. Some of the topics open for investigation have been mentioned in the course of this article. Others pertain, for example, to the potential applications of the knowledge gained studying the archaeal stress response and antistress mechanisms in areas at first sight unrelated to these organisms, for example, medicine (66). It must be borne in mind that archaeal organisms might be suitable experimental models to study specific aspects of cell and molecular biology that are directly pertinent to eukaryotic biology, including human's. A case in point is represented by the basal transcription factors and the transcription-initiation mechanism, which involve molecules very reminiscent of the eukaryotic counterparts (12). In this regard, it is also important to realize that very little is known on the control and regulation of the stress genes in archaea. Regulatory factors, pertinent regulatory DNA sites, and DNA-factor(s) and factor–factor interactions have not yet been elucidated. Recent work in *M. mazeii* showed that stress-gene promoters induce conformational changes on a promoter-binding transcription-initiation factor, the TATA-binding protein or TBP (67). These changes were not induced by a promoter for a nonstress gene, and were different for each promoter tested, that is, those for *hsp70(dnaK)*, *hsp40(dnaJ)*, and *grpE*. It may be speculated that the TBP conformational changes are a mechanism for differential gene expression. Each gene's level of induction would be determined by the type and magnitude of the TBP conformational change. Similarly, the mechanism would be involved in determining the levels of induction required by the prevailing environmental conditions, physiological or stressful, and thus regulate the transition from basal to stress-induced transcription.

Furthermore, it is safe to say that the entire complement of chaperones, chaperonins, and cochaperones of archaeal cells has not yet been unveiled. New members of these families of molecules are still being discovered in bacteria (68) and in eukaryotes (69,70), and one can predict that many more will be discovered not only in these organisms but also in archaea. The time is ripe to search for archaeal homologs of eukaryotic, and possibly also bacterial, stress and chaperoning molecules and systems, and to discover new ones, unique to archaea. Studies on these topics in archaea will certainly shed light on similar phenomena that might also occur in eukaryotes, and will provide the means for developing strategies and tools to manipulate stress genes and their products. One may envisage in the near future the engineering of cells that are highly resistant to stressors of importance in environmental microbiology and related biotechnology industries.

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STRESS RESPONSE IN BACTERIA

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Stress is a fact of life in nature. Bacteria and other microbes inhabiting the natural environments are constantly subjected to deleterious and fluctuating conditions that can be harmful. The pH may shift abruptly to dangerous levels, and so can a host of other conditions, namely, temperature, salinity, osmotic pressure, oxygen, reactive oxygen species concentration, and the nutrient supply. The specter of starvation is nearly always present (1). Oceans are estimated to have 0.8-mg carbon/L, most of which is

nonavailable for bacterial growth, and in freshwater the concentration of individual carbon compounds can be as low as 6 to 10 μg/L. In soils also nutrients are scarce because most of its 0.8 to 2% carbon is recalcitrant humus. For the disease-causing bacteria too life is a constant struggle. To establish infection in a mouse, the pathogen *Salmonella typhimurium*, for example, must first survive passage through in the stomach, where the average pH over a 24-hour period is as low as 1.5. It is then eaten up by the host macrophages, in which it encounters not only the pH stress but is also attacked by reactive oxygen species, and suffers nutrient deprivation. A bacterium's challenge is thus to be ever on guard to prevent damage to its vital components that stresses might cause. This challenge is met in two fundamental ways: first, by increasing the ability to neutralize stress in the face of that stress; and second, to increase the ability to repair the damage that the stress might cause. Because the damage that different stresses inflict has common ingredients, the second response often results in elevated general resistance, that is, what does not kill makes a bacterium stronger overall.

Both the ability to escape stresses and to resist them is achieved by the synthesis of a special class of proteins termed the stress proteins, which in turn is attained by the activation under stress of complex regulatory mechanisms involving transcriptional, translational, and posttranslational control. At the onset of starvation, for example, a large number of proteins are synthesized, which fall in different temporal classes: early, in-between, and late (Fig. 1; 2). Our understanding of the

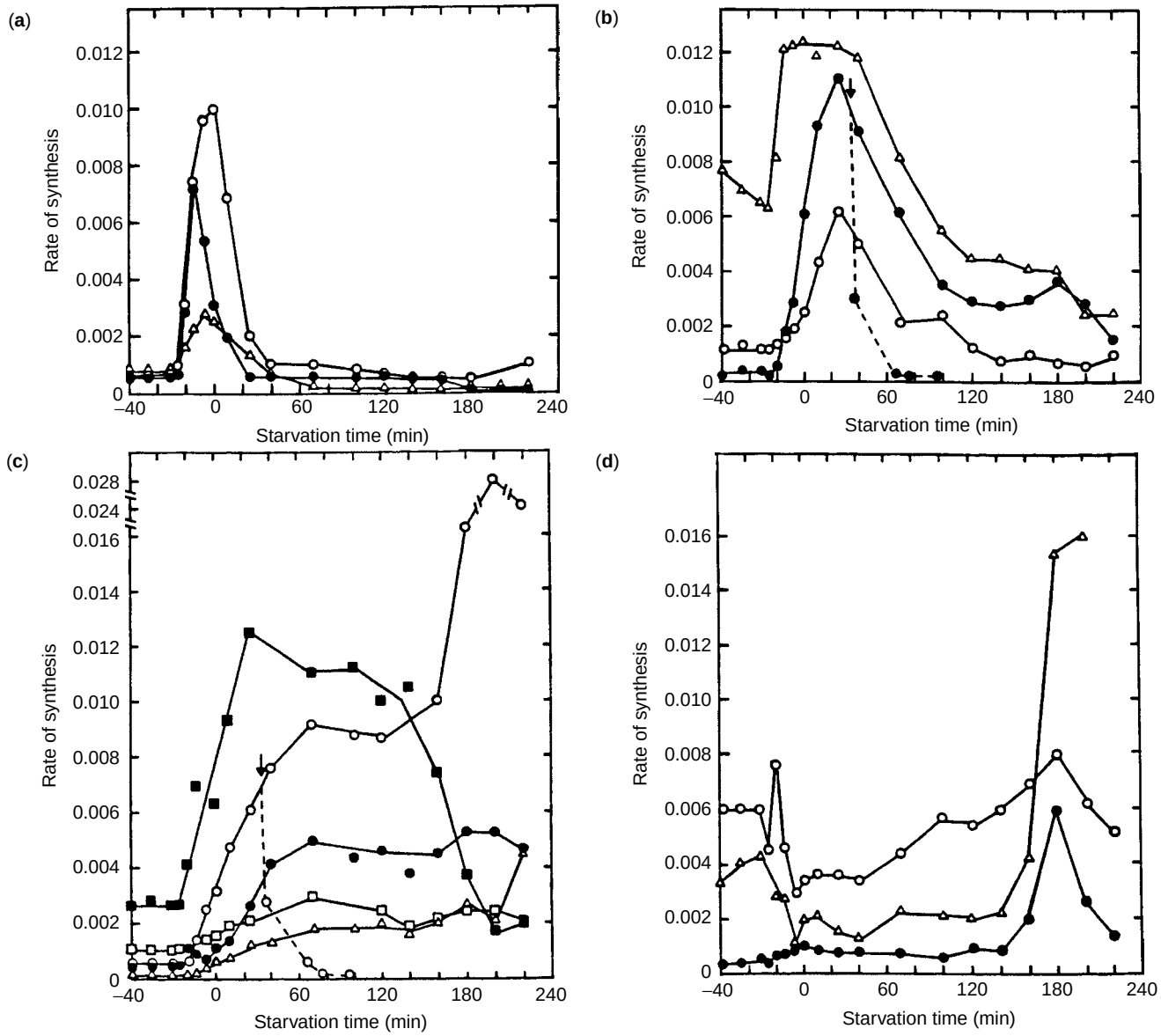

Figure 1. Rate of synthesis of selected polypeptides in *E. coli* entering starvation due to glucose exhaustion. Dashed lines show rate of synthesis of certain polypeptides after readdition of glucose (indicated by the arrow). A similar temporal pattern was observed upon succinate starvation. Reproduced with permission from R. G. Groat et al., *J. Bacteriol.* **168**, 486–493 (1986).

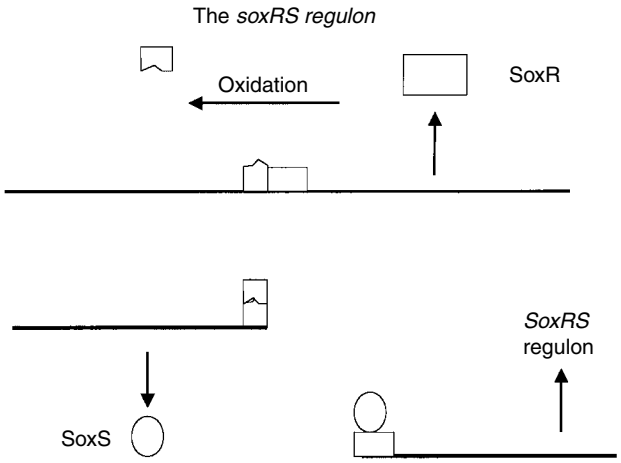

Figure 2. SoxRS regulation of the genes involved in defense against the $O^{\cdot -}$ radical. The change in the configuration of the SoxR protein upon oxidation by $O^{\cdot -}$ is schematically represented to show that in its altered configuration it can activate SoxS transcription, which in turn activates the individual genes of the SoxRS regulon.

mechanisms responsible for activating the synthesis of stress proteins and how these proteins enable the cell to survive stresses has increased significantly in recent years. These insights have potential practical benefits. For example, the molecular mechanisms responsible for the expression of genes during starvation conditions (involving the starvation promoters) offer the possibility of improving in situ bioremediation. Further, inasmuch as the stress genes and proteins are concerned with survival, their manipulation opens up the prospect of enhancing or diminishing bacterial life. Control of the former will improve bioremediation and ecosystem management, and of the latter, disease containment. The stress response, moreover, appears to be involved in the elevated antibiotic resistance of bacterial biofilms that cause diseases that are notoriously difficult to treat, and is therefore a suitable target for addressing this problem also.

THE STRESS PROTEINS PROTECT AT TWO LEVELS

As stated earlier, bacteria deal with stresses using two strategies, one is to attempt to neutralize and escape the stress and the other is to increase their capacity to repair the damage caused by that stress. I will consider these two strategies separately under the headings of "escape" and "robustness" responses. For example, when subjected to starvation, bacteria not only acquire additional skills to obtain food but also prepare themselves to better withstand the consequences of starvation. The same is true, by and large, of other stresses. The measures taken to escape a stress tend to be specific to that stress. Thus, escaping starvation necessitates synthesis of a set of proteins different from those required to escape oxidative or osmotic stress. However, the measures required to withstand the consequences of different stresses have a greater degree of commonality. This is because all stresses lead to a similar outcome, namely, damage to cellular macromolecules, such as proteins, nucleic acids, membranes, and disruption of the cell's ionic composition. Thus, although the escape response tends to be unique to different stresses, the robustness response shares many common ingredients. The fact that all stresses evoke a core robustness response is a relatively recent recognition (5).

Stress Proteins and Escape from Stress

This aspect of the stress response in the context of starvation and the oxidative stresses will be illustrated in the text that follows.

Starvation. Many proteins synthesized either de-novo or whose levels increase during starvation are concerned with alleviating the dearth of the limiting nutrients (Table 1). These proteins may act at all metabolic levels: transport, substrate capture, flux through the central pathways, and the capacity to use additional compounds. Phosphate-limited cells increase the amount of a porin in their outer membrane that facilitates the passage of phosphate compounds into the periplasm where a high affinity binding protein, also induced under phosphate limitation, promotes high affinity uptake; in the periplasm the phosphate compounds are hydrolyzed by bacterial alkaline phosphatase, which also increases under phosphate starvation. Concomitantly, a new transport system in the cytoplasmic membrane capable of transporting inorganic phosphate (P_i) with a high affinity, the Pit system, takes over in place of the low affinity Pst system (6). A similar change occurs during starvation for other nutrients. Under carbon starvation, high affinity periplasmic transport proteins enhance carbon scavenging (7), and under potassium limitation, Kdp a high affinity transport system, replaces the low affinity Trk system. Another example of shifting to a high affinity system relates to glycerol utilization. When glycerol becomes limiting, cells utilize a different pathway for its catabolism. This involves glycerol kinase rather than glycerol dehydrogenase as the first step in glycerol catabolism; because the kinase is a higher affinity enzyme, metabolism of low glycerol levels is facilitated (8).

Increase in the concentration of substrate-capturing enzymes further augments the scavenging capacity. For example, hexokinase and lactate dehydrogenase levels go up in glucose and lactate limited cells, respectively; and glutamine synthetase (GS) levels increase in ammonium-limited cells. (GS is the first enzyme in ammonium assimilation, catalyzing conversion of glutamate to glutamine.) A general increase in enzymes of central catabolic pathways occurs, which promotes channeling of low level catabolites through them. Thus, a combination of synthesis of high affinity enzyme and a general increase in enzyme levels are among the measures a cell adopts to maintain metabolism at rates consistent with survival in low nutrient environments (9,9a,10).

The starving bacterial cells also induce the synthesis of transport and catabolic enzymes for substrates that may not be present in the environment but that may serve as alternate sources of the missing nutrient. Thus, cells starved for glucose not only increase their ability to scavenge glucose but also, for example, scavenge lactose

Table 1. Enzyme/Transport Systems Induced in Nutrient-Limited Cells to Escape Starvation

Enzyme	Nature of Change	Function	Limiting Nutrient
Periplasmic binding proteins	Increase in level	Enhance transport (Maltose, ribose histidine)	Carbon substrates
Pst	Increased affinity	Enhance Pi transport	Phosphate
PhoE	Increased levels	Facilitate Pi passage through the outer membrane	Phosphate
Kdp	Increased affinity	Enhance K^+ transport	Potassium
Hexokinase	Increase in level	Substrate capture	Glucose
Lactate dehydrogenase	Increase in level	Substrate capture	Lactate
Glycerol kinase	Increased affinity	Substrate capture	Glycerol
Bacterial alkaline phosphatase	Increase in level	Substrate capture	Phosphate
Glutamine synthetase	Increase in level	Substrate capture	Ammonium
Glucose-6-phosphate dehydrogenase	Increase in level	Enhance flux through catabolic pathways	Carbon substrates
Phosphofructokinase	Increase in level	Enhance flux through catabolic pathways	Carbon substrates
Pyruvate kinase	Increase in level	Enhance flux through catabolic pathways	Carbon substrates
Aconitase	Increase in level	Enhance flux through catabolic pathways	Carbon substrates
Isocitrate dehydrogenase	Increase in level	Enhance flux through catabolic pathways	Carbon substrates
Malate dehydrogenase	Increase in level	Enhance flux through catabolic pathways	Carbon substrates
β-galactosidase	Increase in level	Amplify metabolic potential	Carbon substrates
CstA	Increase in level	Amplify metabolic potential	Carbon substrates

and peptides (11): the former by increased synthesis of β-galactosidase, the latter by that of the CstA protein, which is involved in peptide transport. Thus, even if the measures to better scavenge the dwindling supply of an existing substrate fail, the chances are improved that other substrates that may appear in the environment would be readily used, thereby relieving starvation.

For motile bacteria, an additional means of escape from stress is to make optimum use of their capacity to move away from a stressful environment: if all else fails, flight to potentially greener pastures might help! Toward this end, maximal flagellar synthesis and its optimum functioning are also a part of the stress response in bacteria (12).

Oxidative stress. Oxygen possesses two unpaired parallel electron spins and is electronegative, which give it a tendency for univalent reduction that generates highly toxic reactive oxygen species (ROS). The presence of certain compounds in the environment stimulates ROS generation and bacteria synthesize special enzymes to mitigate this problem. Compounds such as paraquat, nitrocompounds, chromate, quinones, and several dyes are subject to univalent reduction, whose products, being autooxidizable, set up a redox cycle, generating the ROS, $O_2^{\cdot -}$ (13). When compounds predisposed to univalent reduction are present, bacteria synthesize enzymes such as nitro-, chromate, and quinone reductases that catalyze their divalent reduction. These enzymes make nitro compounds, chromate, and others unavailable for univalent reduction and as the products of their divalent reduction are stable, ROS generation is minimized.

The bacterial cell is alerted to the presence of compounds predisposed to univalent reduction via the protein encoded by the *soxR* gene, which senses $O_2^{\cdot -}$ (Fig. 2; 14). In the presence of this radical, the SoxR protein, which is constitutively synthesized, becomes activated by direct oxidation by $O^{\cdot -}$. This protein is a homodimer with two [2Fe-2S] centers per dimer, which is where the redox changes occur, involving $[2\text{Fe-2S}]^{1+} \leftrightarrow [2Fe-2S]^{2+}$ conversion. The oxidized SoxR activates the transcription of the *soxS* gene, whose product in turn induces transcription of the *soxRS* regulon. The products of this regulon include the reductases mentioned earlier, which prevent $O_2^{\cdot -}$ formation, as well as manganese specific superoxide dismutase, and other enzymes that can decompose this radical. An additional enzyme is concerned with repairing the damage that $O_2^{\cdot -}$ can cause; this will be considered under the robustness response. At the end of the stress, SoxR is reduced by an NADPH-dependent reductase.

It may be an indication of the danger posed by the oxidative stress that bacteria employ another protein, OxyR, to sense the presence of H_2O_2. H_2O_2 directly oxidizes OxyR, and the resulting conformational change, which has been documented by crystal structure, enables OxyR to activate transcription of genes whose products decompose H_2O_2. In the absence of the H_2O_2 stress, OxyR is reduced by an enzyme called glutaredoxin 1 (14).

Stress Proteins and Cell Robustness

As various classes of stress proteins are synthesized (Fig. 1), cells acquire increasing resistance to stresses in general; this is termed the general stress resistance (GSR) response. Figure 3A illustrate this phenomenon for heat: cells starved for increasing duration acquire increasing resistance, but this occurs only if stress protein synthesis is allowed. Inclusion of chloramphenicol, or inhibition of protein synthesis by other means, prevents the resistance development (15). What is true of heat applies also to other stresses: starved cells show increased resistance to the stresses listed in Table 2. Figure 3A and Table 2 deal with this phenomenon in the context of starved cells, but cells subjected to other stresses also give the GSR response, as is illustrated for resistance to H_2O_2 developed by cells subjected to the individual stresses shown in Figure 3B; 16,17). Partial starvation, such as that experienced by bacteria in a low dilution rate chemostat culture, also elicits the GSR response; this is illustrated for ClO_2 resistance of *E. coli* (Fig. 3c). When grown at a dilution rate of 0.05 h^{-1} (14-hour doubling time) because of partial

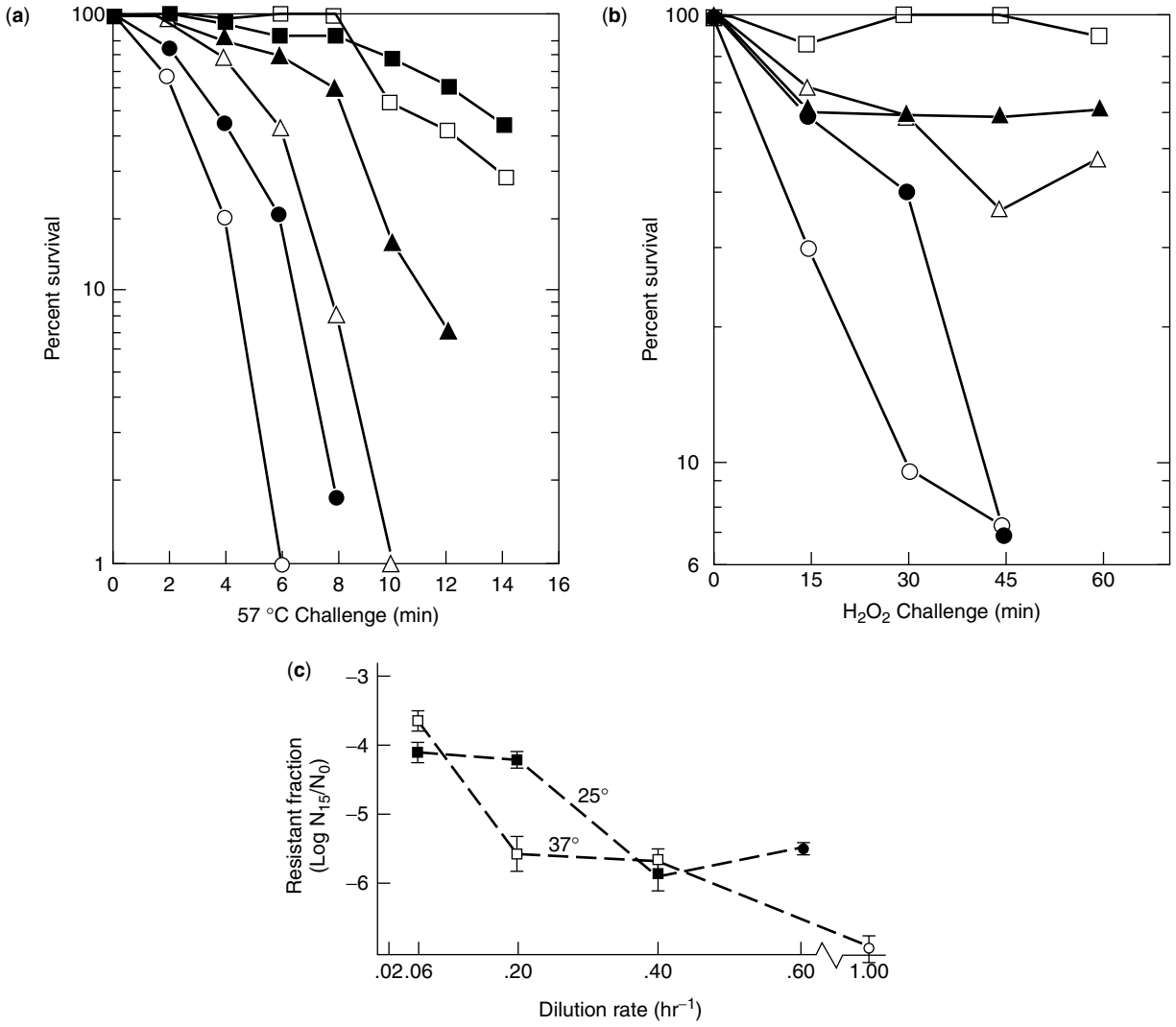

Figure 3. (**a**) Induction of thermal resistance in *E. coli*. Cells grown at 37 °C were exposed to 57 °C during exponential growth (O), or at 1 h (△), 2 h (▲), 4 h (□), or 24 h (■) after glucose exhaustion from the medium. (●) represents culture starved in the presence of chloramphenicol. Reproduced with permission from D. E. Jenkins, J. E. Schultz, and A. Matin, J. Bacteriol. 170, 3910–3914 (1988). (**b**) Comparison of the H_2O_2 resistance of exponential phase (o), or glucose-starved(□) *E. coli* cultures to growing cultures stressed by heat (△), H_2O_2 (▲), or ethanol (●). [Reproduced with permission from D. E. Jenkins, J. E. Schultz, and A. Matin, *J. Bacteriol.* **170**, 3910–3914 (1988)]. (**c**) Effect of growth rate as controlled by carbon limitation in a chemostat on the $C1O_2$ sensitivity of *E. coli*. N_{15}/N_0 represents the survival ratio after 15 min exposure to $C1O_2$. Note that the maximal growth rates (O, ●) were obtained using cells grown in batch cultures. Sensitivity of cells grown at two temperatures (25 °C, and 37 °C) is shown. Reproduced with permission J. D. Berg, A. Matin, and P. V. Robert, *Appl. Environ. Microbiol.* **44**, 814–819 (1982).

Table 2. Stress–Induced Resistances

• Starvation	• ClO_2
• Heat	• Ethanol
• Cold	• Acetone
• pH extremes	• Deoxycholate
• Oxidation	• Toluene
• Hyperosmosis	• Irradiation
• Cl_2	• Antibiotics and other antimicrobials

starvation, this bacterium becomes several orders of magnitude more resistant to this stress than when grown at its maximal rate (18).

THE BIOCHEMICAL BASIS OF GSR

While the detailed mechanistic basis of how starvation proteins confer GSR remains to be elucidated, ongoing research continues to contribute to our knowledge. Clues are provided by the findings that the stress proteins associated with GSR can decompose ROS, repair cell proteins and DNA, and strengthen the cell envelope. ROS decomposition has been touched upon earlier; other processes will be briefly discussed in the following text.

Protein Repair. This is brought about by molecular chaperones, which constitute a large and diverse class of proteins with an indispensable role in cell physiology under all conditions. For example, during protein synthesis, they bind to nascent polypeptides to ensure proper folding of the mature proteins, and are necessary for the translocation of certain proteins through the cell membrane (19). Their protective role in stresses arises from the fact that they can minimize damage and/or renature proteins damaged by stress. The chaperones are slow ATPases, which, when bound to ADP, have a high affinity for unfolded proteins but a low affinity for them when bound to ATP. These characteristics determine the duration of their action on an unfolded part of a protein, and ensure the continuation of the process until renaturation is completed. The chaperone, DnaK, interacts with the proteins DnaJ and GrpE in this process (Fig. 4). DnaJ binds to the denatured protein and presents it to the DnaK-ATP complex. Repair proceeds while ATP of the DnaK is hydrolyzed, and results in a stable complex consisting of the substrate protein, DnaJ and DnaK-ADP. The substrate protein is recovered by a process in which GrpE plays a role, and involves the release of DnaJ from the complex, and conversion of DnaK-ADP to DnaK-ATP (20).

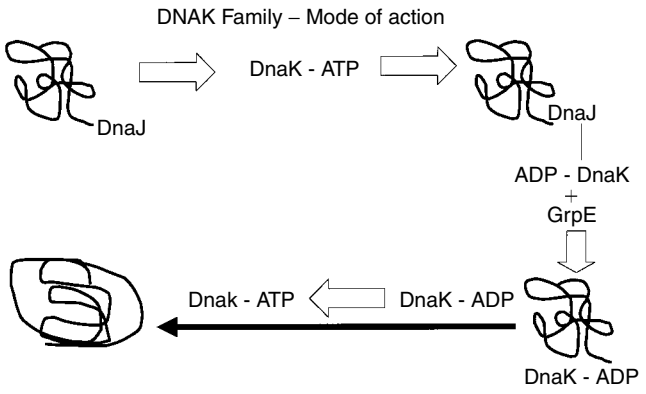

Figure 4. Repair of a denatured protein by the DnaK family of chaperones. Based on F. U. Hartl, *Nature* **381**, 571–580 (1996).

DNA Repair. Endonuclease IV is a part of the *soxRS* regulon discussed earlier: it participates in the repair of damage to DNA caused by oxidative and other stresses. Similarly, the stress proteins exonuclease III and AidB repair damaged DNA (20a), the latter by reversing DNA methylation. A major system for DNA repair is the so-called SOS response (21), which is activated by several stresses, namely, starvation, UV irradiation, and others that result in DNA damage. The proteins whose synthesis is activated by this response include those that promote DNA synthesis, inhibit DNA degradation as well as affect various kinds of DNA repair, such as long-patch repair, daughter-strand gap repair, and double-stranded break repair.

Envelope Reinforcement. The stress protein D-alanine carboxypeptidase increases peptidoglycan cross-linkage and thus accounts, at least in part, for the increased strength of the cell wall. The *otsBA* (*pexA*) operon-encoded proteins, by stimulating trehalose biosynthesis, increase the strength of cell membranes (22).

Molecular Regulation of the Stress Response

Synthesis of individual stress proteins is the result of regulation at all three levels of control, that is, transcriptional, translational, and posttranslational levels.

Transcriptional Control of Stress Genes. This occurs as a result of changes in the cellular concentration of sigma and transcriptional factors. Sigma factors are small proteins that, on association with the RNA polymerase *core* enzyme (abbreviated as E), determine if a promoter is recognized by the resulting RNA polymerase *holoenzyme*. Four sigma factors, σ^{70}, σ^{32}, σ^{s}, and σ^{54} have a role in the transcription of stress genes; their holoenzymes recognize sequences located, as a rule, in a region (called the promoter) that is 10 and 35 nucleotides upstream of the transcriptional start site. Different holoenzymes recognize different promoters.

Role of Eσ^{70}. The stress promoters transcribed by Eσ^{70} are generally weak promoters, that is, they deviate from the consensus promoter sequence for this holoenzyme. As a result, their transcription depends on the availability of ancillary transcriptional factors. This is the case with a large number of starvation genes (24); these are transcribed by Eσ^{70} if cyclic AMP (cAMP) is available. cAMP binds a protein called CRP and together they bind to the cAMP / CRP consensus sequence present upstream of the promoters of such genes. cAMP concentration is kept low in nutrient-sufficient cells but goes up as the cells experience nutrient scarcity, thus fulfilling the requirement for the transcription of these genes by Eσ^{70}. It is noteworthy that the starvation genes whose

transcription is cAMP dependent play no role in the GSR response because mutants deficient in cAMP, and therefore unable to induce transcription of these genes, are normal in the GSR response. What then is the role of cAMP-dependent stress genes? This question has been investigated only in the context of the starvation stress, and here their role is entirely concerned with providing escape from starvation. These genes encode proteins (such as those listed in Table 1) concerned with uptake and catabolic functions and which thereby enhance the scavenging capacity of the cell for the substrate whose supply begins to dwindle, as well as the cell's capacity to use alternate sources for the limiting nutrient.

Role of Other Sigma Factors. Most of the genes that encode stress proteins concerned with the GSR response require RNA polymerase holoenzymes that contain the stress sigma factors, primarily σ^s, and σ^{32} (25). These genes have a different promoter region than $E\sigma^{70}$-transcribed promoters, and they are either not transcribed, or transcribed at a low level in unstressed cells because the concentration of the stress sigma factors is low in such cells. In stressed cells, however, the levels of these "alternate" sigma factors increase, thereby elevating the $E\sigma^s$ and $E\sigma^{32}$ levels to a point as to permit high-level transcription of stress genes. Some of the increase in the concentration of these sigma factors under stress could be due to the increased transcription of their genes in stressed cells. However, the bulk of this increase occurs due to translational and posttranslational regulation, as is discussed later.

Stress genes transcribed by $E\sigma^{32}$ generally contain consensus sequences both for $E\sigma^{70}$, and $E\sigma^{32}$ in their promoters and many therefore are transcribed also in unstressed cells, but the transcription increases during stress because of the additional availability of $E\sigma^{32}$ (26). This makes sense because the products of many of these genes are molecular chaperones (also referred to as the heat shock proteins), which as we have seen, play essential roles in cell physiology under all conditions.

The $E\sigma^s$-transcribed genes possess a variation of a $E\sigma^{70}$-type promoter, and structurally σ^s closely resembles σ^{70}. Several promoters can be recognized by both $E\sigma^{70}$ and $E\sigma^s$ (27,28), depending on the presence of ancillary transcriptional factors. Thus, the increased expression of *pexB* (also called *dps*), which is involved in protection against oxidative stress, is dependent on $E\sigma^s$ under some stresses, but on $E\sigma^{70}$ under others, depending on the availability of the ancillary transcriptional factors (29,30). Changes in the core RNA polymerase or the cytoplasmic ionic composition can also effect this shift. These findings suggest that the regulatory apparatus of $E\sigma^s$-transcribed genes evolved from those transcribed by $E\sigma^{70}$, possibly to ensure flexibility in the transcription of these genes, permitting absence of transcription under unstressed conditions in which $E\sigma^{70}$ alone is primarily present, but nevertheless allowing transcription without $E\sigma^s$, under appropriate conditions.

The role of σ^{54} in stress response is also important. Genes regulated by this sigma factor play a role in nitrogen and carbon starvation and formation of pili (31a,32), which enable stressed cell to attach to appropriate surfaces, promoting survival. Transcription activation by $E\sigma^{54}$ does not generally involve an increase in the level of the sigma protein. Rather, it is the phosphorylation of a protein called NtrC that activates it. NtrC is a member of two-component systems, which are considered in the following text.

Posttranslational Regulation of Stress Gene Expression

These mechanisms are the major factor in regulating σ^{32} and σ^s levels and have been studied extensively in this context.

σ^{32}. The gene that codes for σ^{32} is called *rpoH*. Computer analysis shows that the 153–247 region of the *rpoH* messenger RNA (mRNA) can base pair with its "translational apparatus." The translational apparatus consists of the initiation codon, and sequences upstream to it, including the Shine-Dalgarno sequence, which are complementary to the ribosomal RNA. For the translation of an mRNA to occur, it is necessary that the translational apparatus is available for correct binding to the ribosomes. However, the 153–247 segment of the *rpoH* coding region can base pair with this apparatus, leading to its sequestration, thereby inhibiting translation (Fig. 5). It is thought that the secondary structure is transiently disrupted when cells are exposed to heat shock, thereby accounting for increased translation of the *rpoH* mRNA during this shock. Indeed, removal of the 153–247 region of the gene results in high-level expression of the (truncated) gene product, even without the heat shock. In essence the region 153 to 247 of the intact gene acts as an antisense element, resulting in the formation of a secondary structure of the *rpoH* mRNA that prevents translation under normal conditions. Heat shock breaks the hydrogen bonds, relaxing the mRNA secondary structure, and permitting translation (26).

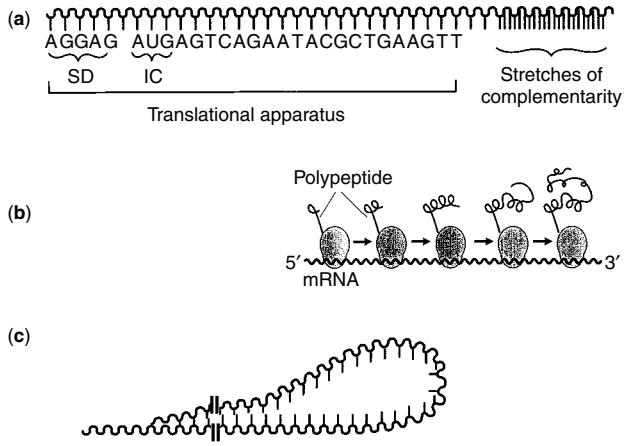

Figure 5. Schematic representation of sequestration of the translational apparatus of *rpoH* mRNA by the 153–247 complementary sequences in its coding region. (**a**) Representation of the *rpoH* gene sequence; (**b**) Translation of an mRNA with unsequestered translational apparatus; (**c**) *rpoH* mRNA with sequestered translational apparatus. Based on T. Yura, H. Nagai, and H. Mori, *Annu. Rev. Microbiol.* **47**, 321–350 (1993) and A. Matin et al., Microbial Ecology of Infectious Disease, American Society for Microbiology, Washington, D.C., 1999, pp. 30–48.

Another mechanism that contributes to σ^{32} increase under stress is the stabilization of the sigma protein. In unstressed cells, σ^{32} associates with the DnaK family of chaperones. This association keeps it in a conformation that is readily attacked by a variety of proteases, such as Lon, FtsH, ClpQY. The chaperones, however, have a higher affinity for denatured proteins. Thus, as the concentration of the latter increases during a stress, they attract the chaperones to themselves and away from σ^{32}. Free σ^{32} acquires a different configuration in which it is relatively immune to proteolysis. This may be the mechanism by which σ^{32} levels increase under starvation and other stresses.

$\sigma^s \cdot \sigma^s$ levels, which are about 140 pmoles per mg protein in the unstressed cells, increase by some fourfold to 520 pmole per mg protein in stressed cells. However, the σ^s synthesis rate decreases by some 50% in the stressed cells due both to diminished transcription of the *rpoS* gene (that codes for this sigma factor), as well as decreased translation of the *rpoS* mRNA. So why do σ^s levels increase in the stressed cells? The sigma protein becomes some seven times more stable in the stressed cells. The half-life of σ^s protein is approximately four minutes in unstressed cells, but increases to 27 minutes under stress (Fig. 6), thus accounting for the fourfold increase in the sigma factor concentration despite its reduced synthesis (33,34,35).

How σ^s becomes more stable in stressed cells is not known, but some interesting aspects of this phenomenon have come to light. We know, for instance, that a specific protease in the cell degrades this sigma factor and accounts for its instability in the unstressed cells (34). This protease is called the ClpXP protease and is made up of two proteins, ClpX and ClpP. Interestingly, ClpX by itself is a molecular chaperone and performs the role of a protein protector. But when combined with one of several partners, such as ClpP, ClpA, ClpC, and so on, it becomes a protease that can target certain proteins specifically (36). Thus, σ^s is attacked by the ClpXP complex and not by, for instance, by ClpXA.

Other proteases present in the cell also do not attack σ^s. Why this sigma factor is the target of a specific protease, and not others, is also not known, but investigations into this aspect of σ^s regulation are ongoing and promising. Bacterial and other cells are selective in what proteins they degrade under different environmental exigencies. How they are able to make this selection is not known. Therefore, what we learn about the mechanism of the ClpXP protease recognition of σ^s as its target, while leaving the nontarget proteins unscathed, will have much significance. An interesting finding in this regard is that the 173–188 amino acid region of σ^s is essential for its sensitivity to the ClpXP protease (Fig. 7). If this region is deleted, or certain amino acids in this region are altered, the resulting protein is not attacked by the protease (34,37).

Sensing Stresses. Heat stress is probably sensed via the melting of hydrogen bonds in the *rpoH* mRNA. This, as we have seen earlier, is believed to be the mechanism of increased *rpoH* mRNA translation leading to increased cellular σ^{32} levels and the heat shock gene transcription. An additional mechanism involves the DnaK chaperone family. As discussed earlier, this family of proteins dissociates from σ^{32} in the presence of denatured proteins leading to the stabilization of the sigma protein and the consequent triggering of the heat shock response. Thus, any stress that causes increase in cellular denatured proteins will activate the heat shock response.

The Two-Component Systems. Starvation for certain nutrients and other stresses are sensed and appropriate transcriptional activations affected via systems that involve two proteins, a sensor protein (HPK) and a response regulator protein (RR). HPK has the property of autophosphorylation at a histidine residue. P-HPK is unstable in unstressed cells, but becomes stable in the presence of a specific stress. It then phosphorylates

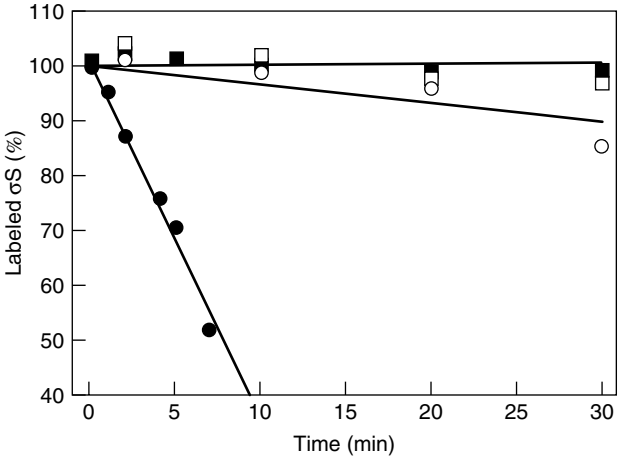

Figure 6. Comparison of half-life of σ^s in exponential phase (solid symbols) and stationary phase (open symbols) cultures in cells with (circles) and without (squares) ClpXP protease. (Reproduced with permission from T. Schweder, K. Lee, O. Lomovskaya, and A. Matin, *J. Bacteriol.* **178**, 470–476 (1996).

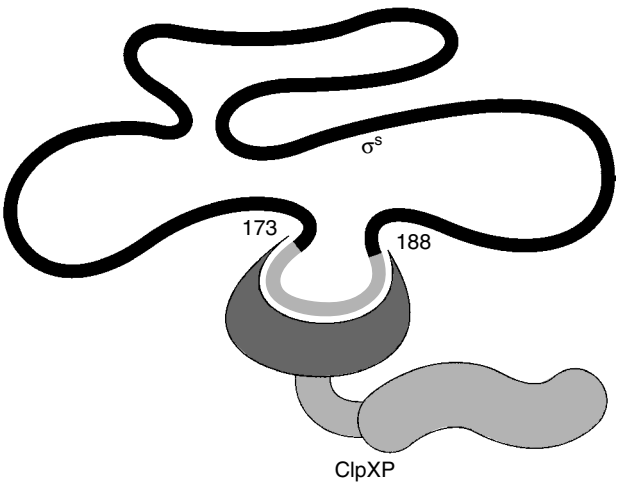

Figure 7. Schematic representation of the σ^s protein, showing that the 173–188 stretch of amino acids of the protein is targeted by the ClpXP protease. Based on Ref. 34.

RR at an aspartic residue, which enables it to activate the transcription of a number of genes. Several pairs of HPK and RR proteins, each involved in sensing a specific aspect of the environment, have been found. The HPKs of different systems share homology of about 100 amino acids in their C-terminal end; the RRs share homology in the 130 amino acid segments of their N-terminal end.

Phosphate and nitrogen starvations are sensed through such systems (6,31). The sensor protein for phosphate sensing is a membrane protein called PhoR. Its phosphorylated form is stabilized when the cell experiences phosphate scarcity. It then phosphorylates its cognate RR, which is called PhoB. Several genes concerned with increasing phosphate-scavenging capacity (refer to the earlier text) contain in their promoter region a specific sequence, called the phosphate box. P-PhoB (but not PhoB) can bind to this sequence and activate the transcription of such genes. The two-component system involved in sensing nitrogen scarcity consists of NtrB (HPK) and NtrC (RR). During ammonium scarcity, P-NtrC that is generated binds to a specific sequence activating the transcription of the gene *glnA*, which codes for glutamine synthetase.

A two-component system might operate also in sensing carbon starvation. A protein called SprE (or RssB) (37,38), which bears homology to the RR component of the two-component sensing system, has a role in regulating σ^s levels. In its absence the ClpXP protease is unable to degrade this sigma factor. The cognate HPK protein for SprE has not yet been found, but it has been shown that SprE gets phosphorylated under starvation conditions. P-SprE specifically makes σ^s sensitive to the ClpXP protease because other substrates for this protease are not affected (39). It is possible that P-SprE (but not SprE) alters the σ^s conformation in a way so as to make it susceptible to the ClpXP action, as is illustrated in Figure 8. If SprE in fact directly acts on σ^s, it would be an unusual RR in interacting with a protein instead of DNA.

Recently evidence has been obtained suggesting the involvement of a GTP-binding (G-) protein, called FlhF, in sensing stresses (40). This protein is present at one of the poles of the *Pseudomonas putida* cell very likely on the cell membrane. Its absence robs the cell of the ability of mounting the GSR response. Conditions such as starvation that result in GSR protection in the wild type, fail to afford this protection to the mutant. This is illustrated in Table 3. Although the starved wild-type strain fully resists exposure to different stresses, the mutant is overwhelmed by a similar exposure. Return of the gene encoding FlhF to the mutant restores the GSR mounting ability. Interestingly, besides its potential role in enabling *P. putida* to initiate a stress response, FlhF is also involved in correct placement of flagella on the cell. Although the wild type has flagella at one of the poles, the mutant shows a random distribution (Fig. 9). Restoration of the gene in multicopy or a single copy restores the polar location—in the former case with increased flagellar number. The mutant is incapable of directional motility. Because flagellar formation is also a part of the stress response (see earlier text), it is possible that FlhF has a sensor role and in its activated form coordinates gene expression concerned with the GSR response as well as correct flagellar assembly (Fig. 10).

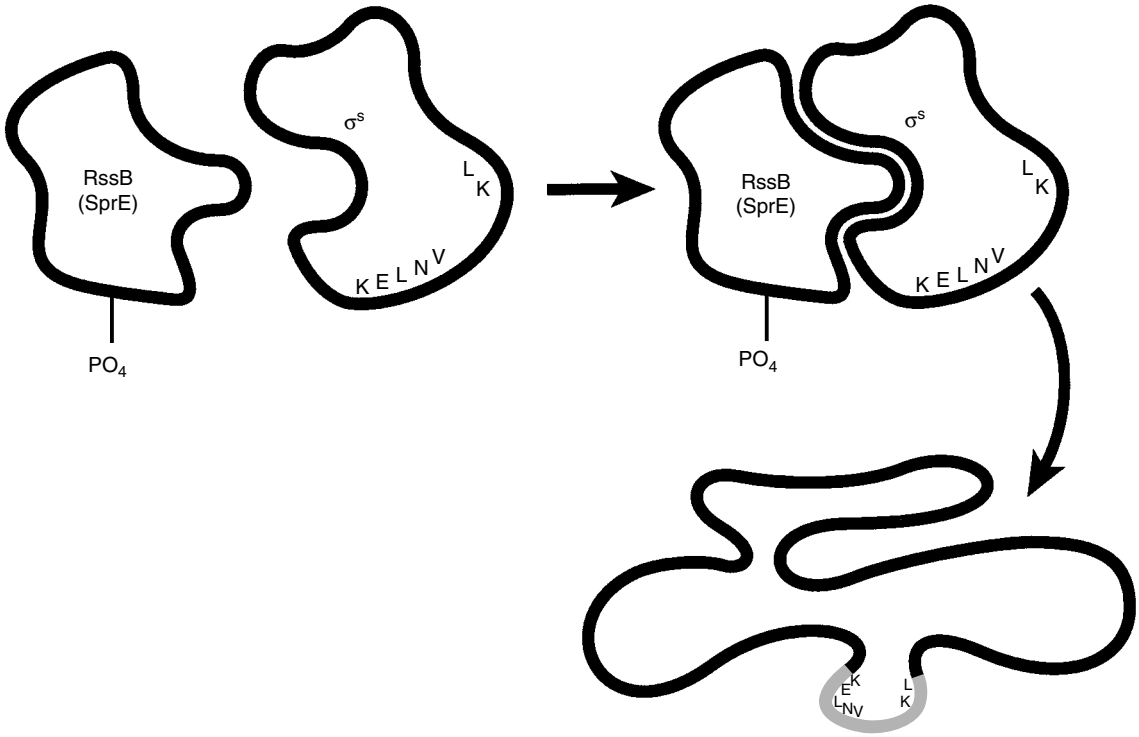

Figure 8. Schematic representation of the possible mechanism whereby phosphorylated SprE alters σ^s configuration in which it becomes susceptible to the ClpXP action.

Table 3. Effect Resistance of Mutation in *flhF* Gene on General Resistance

Strain	Percentage Remaining Viability After Exposure to:			
	Heat	H_2O_2	Ethanol	Starvation
MK1 (W.T.)	100	100	100	100
MK107 (Mutant)	8	43	28	20
MK202 (Compl.)	95	92	90	102

APPLICATIONS OF THE STRESS RESPONSE

Use of Starvation Promoters in Bioremediation. Environmental pollution is a serious problem as numerous Superfund sites lie scattered across the country and noxious chemicals from the Department of Energy (DOE) sites threaten to contaminate drinking water sources. Bacteria can degrade such chemicals and bacterial bioremediation is therefore a promising approach to address this problem. A hurdle that must be overcome

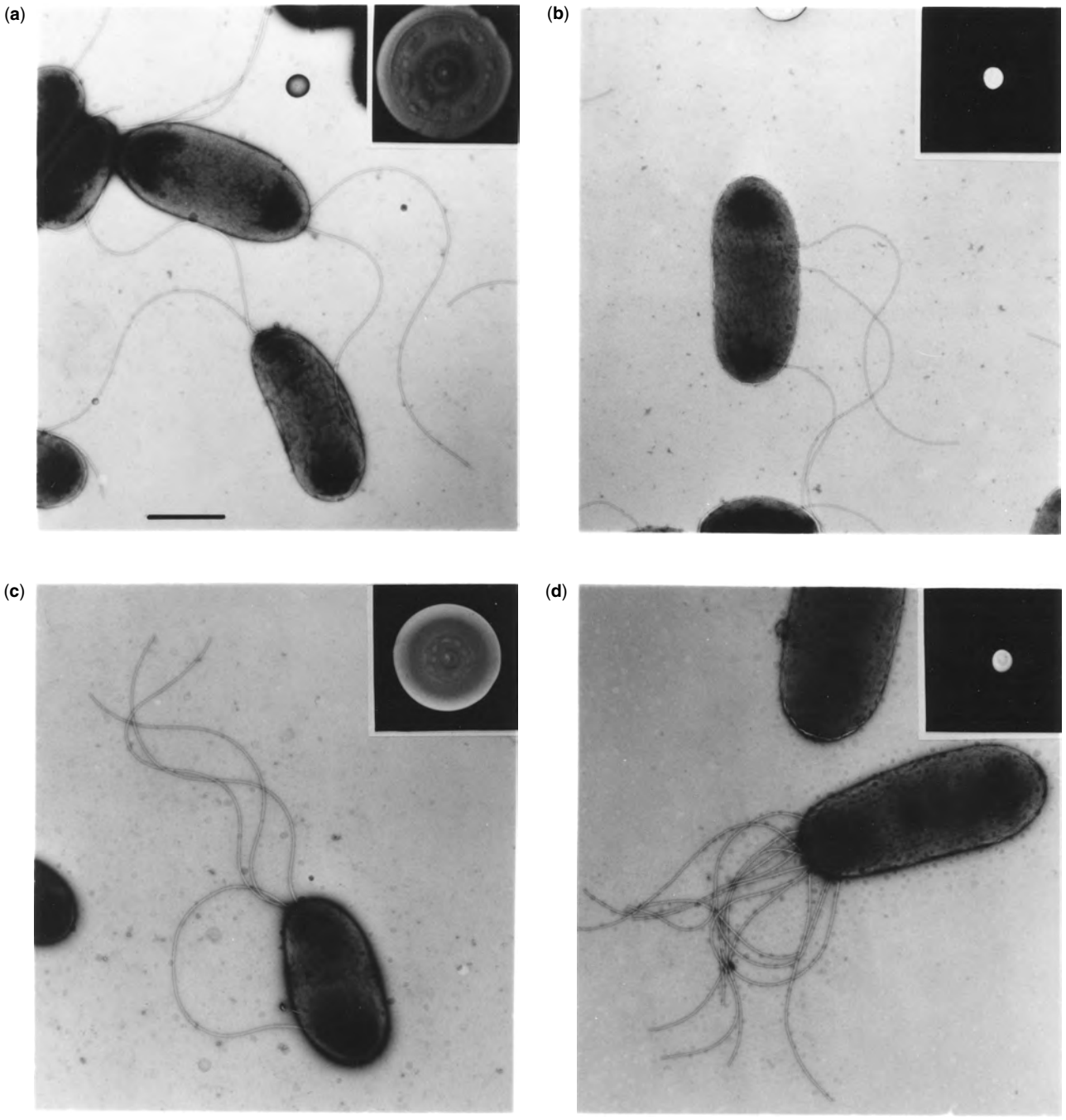

Figure 9. Flagellar arrangement of wild-type *P. putida* (**a**), *flhF* mutant (**b**), *flhF* mutant complemented with a single copy (**c**) or multicopies of the *flhF* gene (**d**).

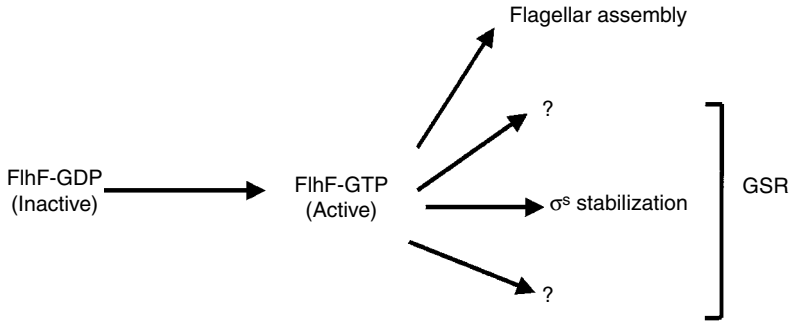

Figure 10. Hypothetical scheme for the sensing of stress and coordination of flagellar placement and GSR by FlhF.

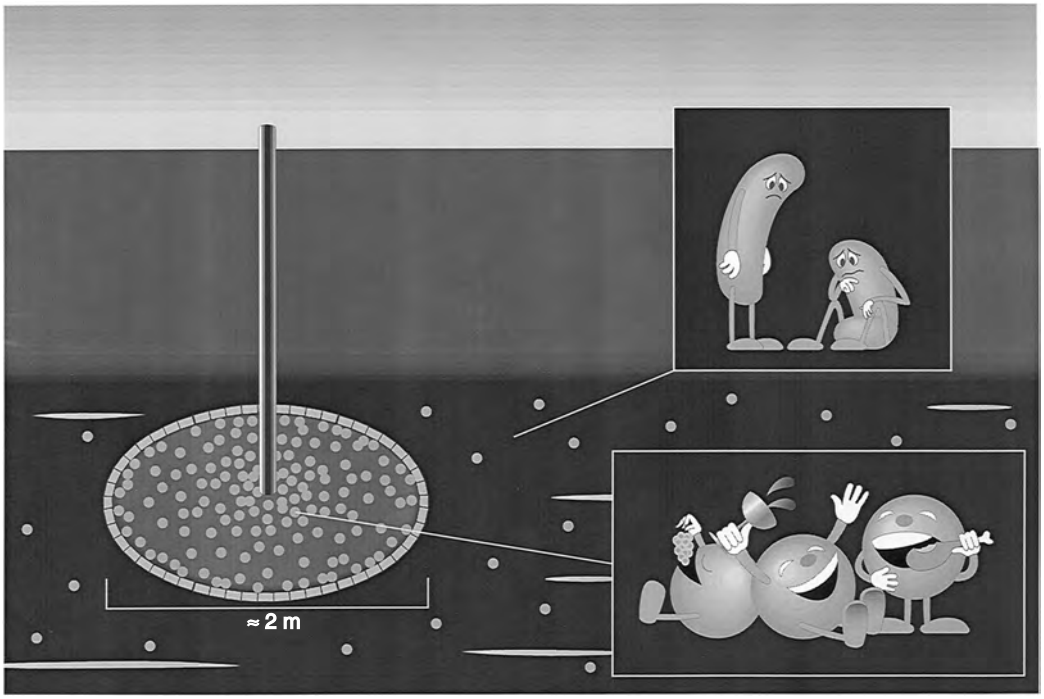

Figure 11. The effect of biostimulation on bacterial growth around the feeding port, resulting in the confinement of effective remediation to a narrow zone.

to make this workable is the low activity of bacteria in nature. Given that nutrient scarcity and other stresses are commonplace, bacterial growth in nature is very slow, and so is the expression of their activities, including those needed in bioremediation. One approach to address this problem is through biostimulation, wherein nutrients are added to polluted environments to stimulate the growth of indigenous bacteria. By this method, significant removal of the carcinogenic pollutant, trichloroethylene (TCE) has been achieved from contaminated field sites (41).

Biostimulation, however, has problems also. The growth of the indigenous flora around the feeding port can be so vigorous that the introduced nutrients are effective only in a narrow zone surrounding the port: a wall of biomass forms near the entry port that consumes the nutrients and physically prevents their further spread (Fig. 11). In a seminal study, significant remediation occurred in only a two-meter zone surrounding the feeding port (41). The problem of biomass formation is exacerbated by the fact that many reactions catalyzed by bacteria that are useful in bioremediation are cometabolic processes. This means that the bacterium catalyzing them does not benefit from these reactions, and thus spends minimal resources in bringing them about. As the bacteria would rather grow than remediate, by far the bulk of nutrients added to the environment is utilized in fueling biomass production (42). Thus, if a way could be found to dissociate expression of bioremediating reactions from the need for active growth, it would improve bioremediation.

This is where the starvation promoters may be useful (43). These are promoters that, as we have seen, are activated primarily in slow growing bacteria and thus afford a means of expressing a desired activity at a high level in such bacteria with minimal biomass formation. A starvation promoter-driven system for TCE bioremediation has been constructed in *E. coli* and *P. putida*. This was done by placing the *tmo* gene complex [which codes for toluene monooxygenase (TMO)] under the control of a starvation promoter. TMO, whose natural role is to enable bacteria to grow on toluene, can degrade

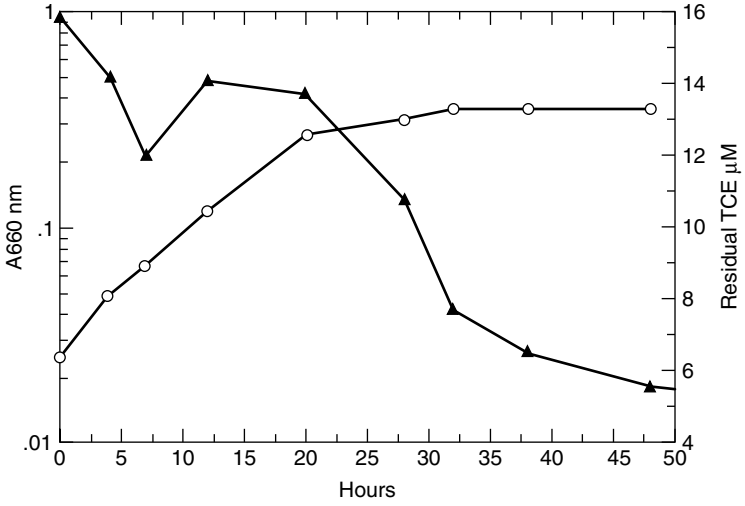

Figure 12. TCE utilization by engineered *E. coli* in different growth phases. [Reproduced with permission from A. Matin, C. D. Little, C. D. Fraley, and M. Keyhan, *Appl. Environ. Microbiol.* **61** 3,323–3,328 (1995).]

TCE cometabolically. Bacteria containing such genetic constructs degrade very little TCE during rapid growth, but show a high rate for this degradation when growth slows down (Fig. 12). Using this approach, it has been possible to reduce biomass formation per unit TCE removed by a hundredfold (44).

Metal and Radionuclide Bioremediation. Heavy metal and radionuclide contamination is among the more serious environmental problems. Chromate, for example, is carcinogenic and is a widespread contaminant of drinking water supplies; this and other heavy metals along with radiation-emitting radionuclides are among the most prevalent pollutants at DOE sites. The soluble form of these contaminants cannot be contained at the contaminated sites and threatens to seep into rivers and other sources of drinking water. An approach to address this problem is to convert, through microbial activity, the soluble salts of these pollutants into less soluble ones, to minimize the possibility of seepage. Cr(VI) and U(VI), for example, are very soluble but Cr(III) and U(III) are much less so.

Recent findings indicate that several enzymes that bacteria utilize for confronting the oxidative stress (see earlier text) can also reduce metals. As mentioned previously, nitroreductase can reduce Cr(VI) to Cr(III) and several other enzymes characterized as quinone reductases can also bring about this reaction. The genes coding for these different reductases bear a significant degree of homology. This makes it possible to use them as raw material in DNA shuffling technology for evolving superefficient reductases. DNA shuffling has proven to be highly effective in producing desired properties in a protein, and enzymes improved by several orders of magnitude have been engineered through this approach (45). Using the gene homologs mentioned previously of the reductases, it should be possible to engineer general-purpose reductases that are capable of reducing several metals with high efficiency and that are resistant to the inhibitory action of other contaminants present in a polluted site.

Manipulating Robustness. Through a study of the stress response, we have now an understanding of several gene products that protect bacteria and can, in principle, make them more robust. Altering the mix of molecular chaperones, the DNA repair enzymes, and those dealing with the management of ROS, and evolving proteins with a superior capacity to perform these tasks holds the possibility of generating bacteria that are better able to function in hostile environments. Conversely, by purposefully targeting the proteins critical for survival, it should be possible to eliminate bacteria when that is desirable. The two contexts in which having more resistant bacteria can help are discussed here. The first is bioremediation. Bacteria that can help address environmental problems can be made more effective if their ability to survive and function in the highly challenging environment of contaminated sites could be improved. In addition to the usual stresses of nutrient dearth and unpredictable deleterious changes, the contaminated sites often contain a mixture of toxic compounds. Maintaining robustness and activity in such environments is difficult and hampers the capacity of the resident bacteria to effect remediation. The second example in this context is the National Aeronautics and Space Administration (NASA) space program. NASA envisages colonization of space and generating self-sustained ecosystems in space stations and in human colonies on other planets. For these tasks, bacteria resistant to the hostile conditions of such locations would be needed.

Exploitation of the stress response can also help us meet the challenges of bacterial disease better. These are on the rise as bacteria have acquired resistance to many of the antimicrobial agents that had, over the past half a century, kept these diseases at bay. New therapeutic agents are therefore needed to again establish control over these diseases. Effective targets for such agents are the proteins that orchestrate the resistance response. Suitable candidates in this connection are the stress sigma factors, the sensing mechanisms and other critical molecules involved in conferring GSR.

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STRESS RESPONSE IN BACTERIA: HEAT SHOCK

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This article deals with heat shock in a general way blending the old with the new, that is, traditional knowledge from the classics are discussed along with novel, more recent information mostly obtained by means of new methods. This trend will be apparent in the bibliography, which encompasses publications from the 1960s to the present.

Other articles elsewhere cover the same field but facets that are yet to be published are discussed here; or similar

themes already treated in the past are viewed from new standpoints. The expected result is an article accessible to all readers that will cover the main subjects of heat shock and stress response studies whether they are initiated in the field of heat shock or not.

Although an attempt is made to present a comprehensive picture of microbial stress, emphasis is placed on regulatory mechanisms and proteolysis because these are two of the areas in which considerable progress has been made in the last decade or so. In addition, the focus will be stress as it pertains to the prokaryotes bacteria because they are most relevant to environmental microbiology. The other prokaryotes that are also directly pertinent to environmental microbiology are the archaea, but they are treated in another entry of this Encyclopedia (STRESS RESPONSE IN ARCHAEA).

Each topic is explained with the aim of providing the general principles while unveiling the connections among themes in the same article, and with the aim of showing the connections between stress and its consequences (the stress response) with cell biology and environmental microbiology. Thus, critical reviews, overviews with novel viewpoints, short mini- and microreviews, and original articles will be quoted. The reader will thereby have the opportunity to consult various types of published literature depending on his or her interest or need, the general picture, the hypotheses, the hard evidence, the new data, the experimental details, the future prospects, or the possible ways toward the acquisition of more knowledge.

TERMINOLOGY

Heat shock is a classic term that indicates a sudden elevation of temperature affecting an object. The object may be inanimate (e.g., a piece of glass) or animate (e.g., a living cell). Here we are concerned with the latter.

Heat shock is a stressor that induces a heat-shock response (henceforth called stress response) in living cells, provided it is not of such strength that kills the cells immediately.

There are many stressors that induce a stress response in living cells and more complex organisms, Table 1 (1,2). A sudden temperature elevation, heat shock, is but one of the many stressors known.

The term heat shock is often used as synonym of stressor, and consequently heat- shock response is used as synonym of stress response (3). This usage of the terms heat shock and heat-shock response is due to several reasons. For example, the experiment usually cited as the seminal contribution to the literature on cell stress involved a "temperature shock" applied to the fly *Drosophila buschi* (4). This treatment induced a puffing pattern on the salivary gland chromosomes of the fly. The pattern was determined to be the reflection of gene activity unchained by the heat shock, and was accompanied by increased production of messenger RNA (mRNA). It was later demonstrated that the mRNA molecules encoded proteins that were named heat-shock proteins (Hsp). For this reason, to this day, stress genes and proteins are abbreviated *hsp* and Hsp, respectively, even when stressors other than heat shock are involved. However, this state of affairs is gradually changing and the more general terms stress genes and stress proteins are being used more and more often (5). The words heat-shock response, heat-shock gene, and heat-shock protein are reserved for cases in which the stressor heat shock is the protagonist. The abbreviations *hsp* and Hs, however, are still in widespread use to denote stress genes and proteins, regardless of the organism or stressor being considered.

RETROSPECTIVE AND DEFINITIONS

The study of cell stress in eukaryotes preceded its investigation in prokaryotes, as it may be inferred from reading the preceding section.

Table 1. Cell Stressors Relevant to Environmental Microbiology[a]

Type	Name Description
Physical	Heat; cold, several types of irradiation, including ultraviolet light
Oxygen	Oxygen-derived free radicals (ROS), hydrogen peroxide, anaerobiosis to aerobiosis shift, and hypoxia-anoxia.
pH	pH shift, alkalinity, and acidity.
Biological	Host reaction against an invading microbe.
Osmotic	Changes in the concentration of salt, sugars, other osmolytes (hyper- or hypoosmotic shock).
Nutritional	Starvation: multiple; specific (carbon, glucose, nitrogen, phosphate, and nitrate).
Antibiotics	Puromycin, tetracycline, and nalidixic acid.
Alcohols	Ethanol, methanol, butanol, propanol, and octanol.
Metals	Cadmium, copper, chromium, zinc, tin, aluminum, mercury, lead, and nickel.
Insecticides and pesticides	Lindane, diazinon, paraquat, thiram, and tributyltin.
Mechanical	Compression and shearing.
Other	Benzene and derivatives, phenol and derivatives, mutagens, arsenite, arsenate, amino acid analogs, and desiccation.

[a]Modified from A. J. L. Macario and E. Conway de Macario, in G. Fink ed., The Encyclopedia of Stress, vol. 2, Academic Press, San Diego, California, 2000, pp. 350–357 (3), with permission from the copyright owner. ROS, reactive oxygen species. These agents cause stress in cells from the three phylogenetic domains, Bacteria, Archaea (both prokaryotes), and Eucarya (eukaryotes).

Noteworthy landmarks in the progress of the science dealing with cell stress span many years of research. For instance, in 1962 it was reported that a "temperature shock" induced chromosomal puffing with increase in mRNA synthesis in the fruit fly, as described in the preceding text (4). Twelve years later, it was established that heat shock induced the production of a special group of proteins, the Hsp (6). By 1981, the heat-shock response of a bacterium, the archetypal experimental model for the gram-negatives, *Escherichia coli*, had been analyzed in some detail (7). This work pioneered the study of stress in the prokaryotes. Research with gram-positives such as *Bacillus subtilis*, the typical experimental model for this group of bacteria, showed that these organisms' heat-shock response shares some but no all the characteristics of the response of *E. coli*. *Bacillus subtilis* (8–15) and other gram-positives such as *Bradyrizobium japonicum* (11,16–18), *Agrobacterium tumefaciens* (19), *Caulobacter crescentus* (20), and *Streptomyces coelicolor* (21–24) show distinctive characteristics as compared with *E. coli*.

Another milestone was the development of the molecular chaperone concept to describe a special group of proteins that assist other proteins in the folding process (25,26). This process leads to the final, functional configuration of a protein, called the native configuration or state. Many Hsp are chaperones. This promoted the study of the stress response and stress proteins because they were found to participate in important cellular events, above and beyond the response to stress.

The understanding of the molecular chaperones progressed considerably with the identification of the chaperonins in bacteria (see later) and in archaea and eukaryotes and the complexes they form to assist in protein folding (27); see also this Encyclopedia, entry STRESS RESPONSE IN ARCHAEA. Also considerable progress has been achieved in the analysis, mostly *in vitro*, of the biochemical events that accompany the protein folding process (28–39).

One of the first molecules, if not the first, to be identified as playing a role in protein biogenesis was GroEL ("Gro," phage growth; "E," a phage-growth defect correctable by a mutation in the E phage-head gene; "L," large subunit). This nomenclature arose because it was found that GroEL is necessary for the assembly of the oligomer that joins the phage lambda head to its tail (40,41). This function of GroEL was discovered in the early 1970s. The name chaperonin was coined in the late 1980s to encompass GroEL and related proteins with a molecular mass of, or near to, 60 kDa. Thus chaperonins constitute a family of structurally similar and phylogenetically related proteins named the Hsp60 family (42–44). Members of this family discovered since the 1970s are involved in protein folding. In fact, it was the discovery of one its members' function that set the stage for the development of the molecular chaperone concept. Chloroplasts, organelles present in plant cells, contain a protein, the ribulose-biphosphate carboxylase-oxygenase (Rubisco) enzyme, which is a complex assembly of subunits. It was discovered in the 1990s that another protein was necessary for the assembling of Rubisco, but this assistant molecule did not become part of the final complex (25). In other words, the assistant molecule was performing a function somewhat akin to that of ladies that chaperoned young maidens in the old days so they would not go astray in their social relations. The molecular chaperones guide nascent ("young") polypeptides to avoid entanglement and reach a mature functional shape (29,30,45). The Rubisco-binding protein was later found to have an amino acid sequence about 50% identical to that of GroEL, which placed it within the chaperonin or Hsp60 family (46).

Similarly, the description of the molecular chaperone machine, and the elucidation of many of its biochemical reactions during protein folding and other cellular functions (28–39), helped the advancement of the study of the stress response and the elucidation of its intricate molecular mechanisms, including its own regulation.

The fields of heat shock, stress response, and chaperones were enriched by the discovery of the archaea, and the proposal that all living cells can be grouped into three main lines of evolutionary descent or phylogenetic domains: Bacteria (eubacteria), Archaea, and Eucarya (eukaryotes) (47). Bacteria and Archaea are prokaryotes; they do not have a nucleus that contains the main genome wrapped within a membrane — the nuclear membrane — as eukaryotes do.

Molecular chaperones and chaperonins were found to be present also in archaea, but their characteristics were thought to be somewhat puzzling in view of what was known for bacteria and eucarya. For example, the archaeal chaperonins resemble those of the eukaryotic cytosol despite the fact that archaea are prokaryotes (27,42,43). In contrast, the molecular chaperone-machine components Hsp70(DnaK), Hsp40(DnaJ), and GrpE in archaea are of bacterial type (27,48–50). Furthermore, several archaeal species do not have these chaperones, in sharp contrast to bacteria, which have these three molecules with no known exception (51). The same is true for eukaryotes; they all have in the cytosol the eucaryal homologs of the molecular chaperone-machine components (27). Also interesting is the fact that the chaperones and chaperonins present in the organelles (e.g., mitochondria) of the eukaryotic cells are of bacterial type, which constitutes a strong argument in favor of the bacterial origin of these organelles (43,46,49,50,52).

The findings discussed earlier in the three phylogenetic domains clearly show that in discussing the stress response, its molecular protagonists and their interactions and regulatory mechanisms, one must distinguish which species and cellular compartment are under consideration to be sufficiently precise so the data and ideas are properly understood. This basic tenet is of critical importance in the search for the role of stress and molecular chaperones in disease as pathogenetic or antidisease mechanisms (53,54).

BEYOND CELLS

A discussion of heat shock, and therefore stress, ought to be accompanied by clarifications on the true extent of the field. Stress has been studied since time immemorial, particularly in what concerns complex organisms, namely, humans. In modern times, in the 1950s there was a boom

in the recognition of stress as a cause of many bodily alterations via the neuro-endocrine system (55,56). Hans Selye studied the response of whole organisms, organs and tissues to stressors, mostly from the endocrinologic viewpoint. One of his definitions runs "stress is the nonspecific response of the body to any demand made upon it" (56). Although this portion of the larger field encompassed by stress will not be dealt with here, it is pertinent to say that in the last analysis microbial stress, cell stress, organismal stress, human stress, are all examples of stress due to environmental stressors, and they all share essential characteristics. All stressors cause in the end similar lesions. They alter the internal conditions of the cell (a microbial cell, or a cell from a uni- or a multicelled organism) in such a way that proteins are affected and tend to lose their native, functional configuration. It follows that molecular chaperones are key elements in normal and pathologic physiologies of any cell in as much as they promote protein folding, and refolding. Survival in the face of stress depends on the mechanisms that maintain the array of cellular proteins in functional shape. This is why the stress response involves mainly molecular chaperones and other stress molecules that protect the essential cell components from stressor-induced damage.

PROPERTIES

The impact of heat shock on a living organism, uni- or multicelled, is detectable at various levels: supracellular and intracellular, molecular, and supramolecular, and genetic (3,57). It follows that the methods for studying the stress response vary depending on the level targeted for analysis: the whole cell, a multicellular structure, a cell membrane, a molecule, or a gene or group of genes (3,27,58,59).

The typical property of the stress response is an increase in the Hsp (6,60–62). This is due to one or more mechanisms acting in parallel, such as the increase in the rate of: (*1*) *hsp*-gene transcription; (*2*) mRNA translation; (*3*) protein synthesis-maturation beyond translation and (*4*) increase in the life span of mRNA.

The increase in the Hsp levels is immediate and transient. For example, in bacteria, it starts very rapidly (seconds) after the stressor hits the cell, reaches the peak in a few minutes, and usually returns to near normal levels within 30 minutes. The mRNA is polycistronic (20,63–65). Whole clusters of Hsp genes that are arranged one after the other, in functional units called operons, are transcribed in series from one promoter upstream of the gene that lies at the beginning of the cluster, namely, at the 5′-end of the operon. A long mRNA is synthesized that is processed into shorter pieces representing individual genes, which are translated immediately.

Another typical property of the heat-shock response is that Hsp participate in their regulation as they are key players in regulating their own synthesis (21,66–68). This self-regulatory circuits will be discussed under Regulation, later.

The properties of the stress responses of archaea and eukaryotes are different from that of bacteria. Archaea and eukaryotes respond to heat shock with sustained increases of Hsp, the genes are induced (and probably also regulated) individually rather than in series or operons, which results in monocystronic mRNAs (69,70). The stress response of eukaryotes will not be treated here in any detail, and that of archaea is described elsewhere in this Encyclopedia, under the entry Stress Response in Archaea.

HEAT SHOCK AND OTHER STRESSORS

A stress response may be caused by any one of a wide array of agents in the environment in which an individual cell or organism lives. A list of stressors, including heat, salt, and alkali shocks, directly pertinent to environmental microbiology is displayed in Table 1 (58).

HEAT-SHOCK PROTEINS

The stress response is characterized by an increase in a variety of proteins. Some of them are molecular chaperones as explained in a preceding section, but many others are not; they are involved in different roles in a concerted effort to save the proteins and other cellular components from the damaging effects of stressors. These effects are usually caused by stressors via a pH change, or oxidation, or accumulation of a metal when the stressor is such.

A list of stress proteins is shown in Table 2, and a sample of Hsp in bacteria is displayed in Table 3 (3,58,71).

REGULATION

The stress response and the synthesis of Hsp are examples of self-regulatory systems via circuits of molecular interactions amenable to biochemical and genetic dissections. The whole event from the sudden action of the stressor, heat shock for instance, to the full fledged cellular response and its aftermath, includes several steps: sensing the stressor's impact and transducing the stress signal from the periphery to the DNA through membranes and cytosol, inducing the stress genes and the synthesis of stress proteins accompanied by downregulation of house keeping genes, sensing the abatement of the stressor's impact followed by a decline in the stress-induced molecular damages, and downregulation of the stress response with a return to normality or near normality at the end. The last two or three stages require not only a decrease in the Hsp production but also a return to normal levels of the proteins whose synthesis had been diminished as a consequence of the stress from the beginning of the stress response.

Stressors, whose damaging effects are protein and DNA injury, cause stress. Damaged proteins and DNA induce a series of repair mechanisms among which the molecular chaperoning system is paramount. The signal that a stressor generates at the cell's surface must be recognized — sensed — at this surface, after which sensing the signal must traverse the cell's outer structures and gain the cytosol and eventually reach the DNA to modify gene behavior. Signal transduction is then a major component of the stress response, or perhaps

Table 2. Stress Proteins[a]

Group	Examples
1	Molecular chaperones and chaperonins in organisms of the three phylogenetic domains: Bacteria, Archaea, and Eucarya. These proteins are classified into families according to their molecular mass, form the small to the large and very large heat-shock proteins (see Table 3).
2	Regulators of stress (heat-shock) genes: positive regulators or activators (e.g., heat-shock factor or HSF, in eukaryotes); sigma factors (e.g., sigma-32, sigma-E, sigma-B) in bacteria; negative regulators (e.g., HSF4 in eukaryotes; HrcA and HspR in bacteria); (See Table 4.)
3	Proteases: Clp family; Lon/La; HtrA; FtsH; in bacteria. Proteasome components and ubiquitin in archaea and eukaryotes.
4	Proteins in the intercellular connective material of multicellular structures formed as a response to stress (e.g., in the archaeon *Methanosarcina mazeii*).
5	Transport proteins (e.g., TrkA in the archaeon *M. mazeii*).
6	Enzymes and cofactors involved in the synthesis of other stress proteins listed above in 1–5), and other molecules such as thermoprotectants or "chemical" chaperones (e.g., trehalose in the yeast *Saccharomyces cerevisiae*; di-*myo*-inositol phosphate and cyclic diphosphoglycerate in some archaea).
7	All proteins, enzymes, cofactors, and gene-regulatory factors involved in sporulation (e.g., in the bacterium *B. subtilis*).

[a]Modified from A. J. L. Macario and E. Conway de Macario, in G. Fink ed., *The Encyclopedia of Stress*, Vol. 2, Academic Press, San Diego, California, 2000, pp. 350–357 (3), with permission from the copyright owner. Stress proteins are also called heat-shock proteins and are abbreviate Hsp (the genes are denoted *hsp*). See also reference (58).

Table 3. Classification of Stress Proteins (Hsp) and Examples of Bacterial Representatives[a]

Family Name(s)	Mass (kDa)	Bacterial Example
Heavy; high molecular weight; Hsp100	100 or higher	ClpE
Hsp90	81–99	HtpG
Hsp70(DnaK); Hsp70; DnaK; chaperones	65–80	DnaK; Hsc66
Hsp60; chaperonins	55–64	GroEL
Hsp40(DnaJ); Hsp40; DnaJ	35–54	DnaJ
Small Hsp; sHsp	34 or lower	GroES; Hsc20; GrpE; PPIase; other

[a]Modified from A. J. L. Macario and E. Conway de Macario, *Frontiers Biosci*. 6, d262–d283 (2001). *http://www.bioscience.org/2001/v6/d/macario/fulltext.htm* (58), with permission from the copyright owner. Archaeal and eukaryotic counterparts can be found in references (27,53,54). See also the chapter STRESS RESPONSE IN ARCHAEA, this Encyclopedia.

more accurately of the initial phase of the response. Signal sensing and transduction precede the start of the antistress mechanisms per se. Immersed in these series of stages involving signal transduction and the initiation and action of the antistress mechanisms are regulatory circuits. These circuits monitor the levels of stress and of the stress response, and shut the latter down when the danger has passed.

REGULATORY CIRCUITS AND NETWORKS

Research on the regulation of the stress response has been going on for years. The focus has been more often than not on the response to heat shock and the regulation of the genes that encode Hsp, particularly the components of the molecular chaperone machine (*hsp70(dnaK)*, *hsp40(dnaJ)*, and *grpE*), and the chaperonins (the *groEL/S* system in bacteria).

Relatively little is known about stress signal recognition and transduction, but considerable progress has been made in the understanding of chaperone and chaperonin gene regulation especially in *E. coli* and *B. subtilis*.

In *E. coli*, one of the components of the RNA polymerase, a protein called heat-shock factor or sigma-32, probably recognizes a change in the secondary structure of mRNA caused by stress, and thereby proceeds to induce transcription of molecular chaperone and chaperonin genes by binding to the heat-shock promoter (67,68,79–81). Other sensing devices detect DNA supercoiling and melting, and protein unfolding (denaturation) (81).

At the other end, the molecular chaperone machine genes are downregulated by a process involving degradation of the heat-shock factors sigma-32 and sigma-E with participation of molecular chaperones (66,79,82,83). For example, in the absence of a distress signal due to the presence of denatured polypeptides, the machine is

partially idle: it only performs the basal chores of assisting in the folding of nascent polypeptides and promotes sigma-32 degradation via interaction with a protein called FtsH (a protease). However, when a stressor impacts on a cell, and stress occurs, many proteins become denatured to various degrees (those most severely damaged beyond repair by the chaperones are removed by proteases, as will be described later) and require immediate attention. The chaperones are called into action to restore the configuration of many proteins. As a result, sigma-32 is freed from the prodegradation action of the chaperones and thus becomes available to perform its function, which is to induce stress genes, the molecular chaperone genes included. Hsp increase and the stress response proceeds. When most denatured polypeptides have been restored to their normal configuration, chaperones are no longer needed and return to the task of sigma-32 degradation. This factor diminishes and the chaperone genes are down regulated.

In addition to this circuitry, others add to the increased production of Hsp during the stress response. Examples are an increase in the translation rate of the mRNA for sigma-32, which is produced by a gene named *rpoH* (19).

Also, in *E. coli* other stress genes are controlled by sigma-E (72,84,85). This factor belongs to a group that responds to extracytoplasmic stimuli, for example, denatured proteins in the periplasm.

A third group of genes in *E. coli* is controlled by sigma-54, which acts in conjunction with an activator (86).

In *B. subtilis*, a representative of the gram-positive bacteria, regulatory mechanisms differ from those described in the preceding text for *E. coli* — a representative of gram-negative bacteria. In *B. subtilis* there seems to be at least three different regulatory systems that sense the presence of a stressor such as heat shock, in different ways (8,9,11).

Class I stress genes, among which are those that code for the components of the chaperone machine and the chaperonins GroEL and GroES, depend for induction by stress on a factor called sigma-A, a *cis*-acting DNA site (element) named CIRCE (controlling inverted repeat chaperone expression) and a repressor (HrcA) that binds the CIRCE element. Class II stress genes are induced by another factor, sigma-B, whereas Class III stress genes are induced via other mechanisms that do not involve sigma-B or CIRCE-HrcA, Table 4.

In summary, stress gene regulation may be mediated by negative or positive mechanisms. Example of a positive regulation is the use of an alternative sigma factor, as is the case of sigma-32 in *E. coli*. Stress sets in motion a mechanism that activates genes that were "dormant" or functioning at very low rates (basal or constitutive levels of activity) prior to the impact of the stressor. Negative mechanisms involve repressors, which bind a DNA element (e.g., CIRCE) and keep the pertinent gene inactive. Stress causes the repressor (e.g., HrcA) to abandon the DNA site to which it is bound in the absence of stress and, thus, the pertinent gene can be transcribed by RNA polymerase. Posttranscriptional regulation also occurs (87).

Last and most important, negative and positive mechanisms coexist in many organisms and are used to regulate different groups of genes as required by the circumstances dictated by the presence of stressors in the environment. The result is that the cell has a network of interlaced mechanisms whose crisscrossing pathways are used as required. The network insures that a multitude of antistress mechanisms are ready to defend the cell against stressors on a very short notice and with the flexibility necessary to deal with a wide range of stressors.

Table 4. Examples of Stress-Gene Regulators in Bacteria[a]

Mechanism	Organism	Factor [DNA Element]	Regulated Gene(s) and Operons
Positive control	*Escherichia coli*	Sigma-32 (RpoH)	*dnaK*, *dnaJ*, *grpE*, *groEL/S*, other
		Sigma-E (sigma-24; RpoE)	*htrA*, *rpoH*, *rpoH* operon, other
		Sigma-54 (sigma-N; RpoN) + PspF	*PspABCDE*
	Bacillus subtilis	Sigma-B (SigB)	*gspA*, *csbA*, *katE*, other (Class II stress genes)
Negative control	*Bacillus subtilis*	HrcA [CIRCE]	*groEL/S*, *dnaK* operon (Class I stress genes)
		CtsR	*clpC*, *clpP*, other (Class III stress genes)
		Not yet identified	*htpG*, *fthsH*, *lon*, other (Class IV stress genes)
	Bradyrhizobium japonicum	[ROSE]	*hspA*, *rpoH1*, other
	Clostridium acetobutylicum	CtsR (?)	*hsp18*
	Leuconostoc oenos	CtsR (?)	*hsp18*
	Streptomyces albus	HspR [HAIR]	*dnaK* operon, *clpB*
		OrfY (RheA)	*hsp18*
	Streptomyces coelicolor	HspR	Not yet identified

[a] Modified from F. Narberhaus, *Mol. Microbiol.* **31**, 1–8 (1999) (11), with permission from the copyright owner. See also references (17,18,21,72–78).

PROTEOLYSIS

Stress causes protein denaturation, that is, unfolding. This may be extensive and irreversible for some or most molecules in a cell. In the latter case, cell death is likely to occur because of formation of precipitates by the damaged protein molecules (53,54). If polypeptide denaturation is not extensive enough to cause precipitate formation and cell death, the antistress mechanisms will promote protein refolding and restoration (38,39,88,89). In this process molecular chaperones are key instruments. In addition, the cell possesses the means to eliminate polypeptides beyond repair, and thus avoid precipitate formation when protein denaturation is not massive. Proteases are proteolytic molecules, that is, enzymes that digest polypeptides, which actively participate in the elimination of severely damaged polypeptides and incipient precipitates (75,90–96). Proteases associate with themselves and other auxiliary molecules including molecular chaperones to build proteolytic machines (95,97–101).

A sample of proteases is presented in Table 2 (group 3), and more information is available in the cited publications.

The interplay between chaperones and proteases is another example of a cellular mechanism in which molecules with seemingly opposing functions help each other in maintaining normal cell physiology and cell survival in the face of stress.

The interplay of chaperones and proteases in protein folding, refolding, and degradation has been studied in a few microbes, but most progress has been made using *E. coli* as the experimental model. The advantages of this organism as compared to many other gram-negative and gram-positive bacteria, is that it can be readily manipulated genetically. Genes can be introduced with ease into *E. coli* by means of vectors, after which it is possible to determine whether the transformed bacterium acquired properties derived from the functioning of the introduced gene. For example, a stress gene can be introduced and then its response to stressors can be assessed in the transformed host by measuring the levels of the gene's mRNA, or by evaluating the protein-folding capacity of the host. Furthermore, there are many, well-characterized *E. coli* mutants lacking one gene or another. These mutants can be examined in terms of stress responses and survival properties in the face of different types of stress. By correlating these properties with the presence and absence of a gene, one may be able to infer the gene's function and overall role.

CHAPERONES AND PROTEASES IN REGULATION

In *E. coli*, the chaperones trigger factor (TF), Hsp70 (DnaK), and Hsp40(DnaJ) participate in the folding of nascent polypeptides of nearly 350 different proteins ranging in size from 16 to 167 kDa (59). Long multidomain polypeptides are the most prone to misfolding and entanglement as they emerge from the ribosome, and therefore are those most helped by chaperones (45). In the absence of the chaperoning systems, these complex proteins would not reach their functional configuration. The GroEL/S chaperonins assist newly made polypeptides so these can fold correctly. Five to 15% of the *E. coli* proteins with sizes in the range 20 to 60 kDa are assisted by chaperonins (59). All these chaperoning functions are a quality control system, so to speak, for the cell. In addition, as described in a previous section, proteases also participate in maintaining a "clean" intracellular environment by removing damaged proteins and precipitates. Thus, the chaperoning and proteolytic systems cooperate with one another to maintain the cellular proteins within a functional range of shapes and concentrations in a cellular milieu devoid of debris and precipitates. Both systems also interact as they participate in the regulation of their own components and in the regulation of the stress response. This interaction may follow at least two pathways. In one, chaperones would present damaged polypeptides to proteases in a manner that favors the recognition of the polypeptide by the proteases and promotes the proteolytic activities of the latter. In this pathway chaperones and proteases cooperate with one another.

In the other pathway, competition rather than cooperation between chaperones and proteases is the central process. The pool of polypeptides would be composed of a series of populations exhibiting different degrees of denaturation from the native state to the irreversible, precipitation-prone extreme. This pool would be in a constant flow or kinetic partition between the chaperoning and the proteolytic systems. The more damaged a peptide, the more it will be taken up by the proteases, whereas the less damaged molecules will be refolded and recovered by the chaperones.

It is likely that the cooperative and competitive pathways coexist in the same cell, and that they are used as the cellular and environmental conditions dictate.

NEW LOOKS OF AN OLD THEME

Several novel ideas concerning the stress response, its regulation, and the role of stress and stress genes in life have been put forward recently. These ideas are either reinterpretations of earlier data, revisions of preexistent hypotheses and proposals, or fresh outlooks based on novel information provided by newly developed technologies such as genome sequencing and molecular phylogenetic analyses using complex software unavailable before.

The role of stress in evolution is being investigated (102,103) but the data are still incomplete and conclusions are not warranted. However, it is tempting to speculate that stress genes did play a role in positive selection during evolution, and that they play a similar role today in the face of stress. One can envisage that thousands of millions of year ago, when life was still in its earliest stages, the environment must have been loaded with stressors such as extreme acidity or alkalinity, and high temperatures (104). The survivors must have been equipped with powerful antistress mechanisms: cellular envelopes, stress genes and proteins, and the ability to form ultraresistant structures such as spores and multicellular aggregates surrounded by a protective shield. It will be difficult to say whether these ideas are correct because it is not possible to accurately repeat what

happened long ago. Nevertheless, experimentation and comparative analyses of genes and proteins and other molecules and supramolecular structures, as well as of cells and supracellular structures, should shed light on the role of stress and antistress mechanisms in survival both in the past and today.

Along the same lines of reasoning, the participation of stress genes and proteins in development has been investigated. Associations between stress-gene induction and synthesis of Hsp and embryonic development have been reported (105–108). Also, the same associations have been observed in relation to the development of specialized organismal structures (23), which means that stress genes and proteins may participate in the process of cellular differentiation. This idea should not be too surprising because a good portion of the stress proteins, particularly chaperones and chaperonins, play a role in protein biogenesis, as described in several sections in the preceding text. Protein biogenesis must be an integral part of the processes of differentiation and development, which in many organisms includes histogenesis, namely, the formation of multicellular structures (tissues and organs). In this regard, it is noteworthy that recent data suggest that not all cells in a cloned population respond to stressors in the same fashion (109). The stress response of individual cells submitted to the same stressor varies even if all the cells belong to a single clone. There is cell heterogeneity in terms of stress response among cloned cells.

THERMOTOLERANCE AND THE FUTURE

One of the consequences of the stress response is thermotolerance. This term describes the situation sometimes observed after stress caused by heat shock and characterized by increased resistance to a temperature elevation. For example, an organism with a temperature optimum for growth of 37 °C that is capable of mounting a stress response when exposed to 45 °C but dies at 55 °C, will survive the latter temperature if it was previously heat-shocked at 45 °C. This phenomenon has been explained in detail elsewhere (110). It is a very interesting biological mechanism: it offers the opportunity for developing means to enhance resistance to stressors in cells of practical value. Areas of potential applications are environmental microbiology, sanitary engineering, medicine, and surgery, and so on as described previously (111). Remarkably, stress caused by a given stressor, for example, heat shock, will make the cell more resistant to heat shock and also to other stressors. This cross-tolerance will, for instance, allow the use of a relatively harmless compound that causes a mild stress response to generate tolerance to another, harmful, agent. Here again, the established terminology is not adequate. The term thermotolerance ought to be replaced by stress tolerance in as much as heat shock and other stressors can induce resistance to the same stressor but also to others.

Still another potential application of stress genes and the stress response in environmental microbiology will be in the field of monitoring and detection. Certain microbes could be engineered to carry stress genes, or their promoters with reporter genes, which will be induced when in contact with toxic compounds (111). These manufactured organisms can be placed where monitoring and detection of contamination is necessary, in soil for instance, and then collected and examined for the presence of signs of a stress response such as an increased transcription of the stress or reporter genes. In this regard, the future prospects are very promising. Other promising fields of applications pertain to plants (112,113), plant pathogens (19,114,115), and symbionts (11,16–18).

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STROMATOLITES. See AGGREGATES AND CONSORTIA, MICROBIAL; BIOMINERALIZATION BY BACTERIA

SUBAERIAL COMMUNITIES

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The subaerial microorganisms are perhaps the most obvious, but most overlooked, group of microorganisms known. By definition they are found on surfaces above the soil and water lines, exposed directly to air (1–3). Rich growths, in the form of green, red, brown, or black streaks and coatings on rocks, trees, masonry, the roofs of many homes, wooden structures of all types (especially fences, telephone poles, and window sills), metal signs, and older automobiles, are readily apparent to microbiologists and nonmicrobiologists alike. Yet, there is remarkably little known of the ecology of these organisms. In this article, I will attempt to summarize what is known and to indicate areas for future research, with special emphasis given to the photosynthetic segment of the community.

DIVERSITY OF SUBAERIAL COMMUNITIES

Habitats

Subaerial communities are usually defined by the nature of the substratum (3). Epilithic communities are those found on the upper surface of stones. These can be either naturally occurring or the result of human activity (monuments and buildings). Epiphytic communities are those found growing on plants and plant parts. These are sometimes divided into the corticolous community found on bark, the lignicolous community found on exposed wood or wooden objects, and the phyllosphere community associated with living leaves, although, strictly speaking, it is only the segment of the phyllosphere community inhabiting the phylloplane, or leaf surface that is subaerial. Other specialized habitats can also be identified, for example, the surfaces of frescos or oil paintings (see review by Cifferi (4) and the references therein), or leather objects (5), but the communities found there are not usually given special names.

Organisms

Techniques of Analysis. Ideally, the identification of subaerial organisms should be made by direct observations of the community. This would allow relative numbers of individuals and spatial relationships to be assessed. However, the identification of heterotrophic bacteria through microscopic examination is next to impossible without some prior knowledge of the microflora. The situation is somewhat better for the structurally more complex cyanobacteria and eukaryotic algae, especially diatoms and desmids. However, many of the genera found in subaerial habitats are either morphologically simple, so that the descriptions of even unrelated genera tend to overlap, or morphologically pleomorphic, so that their appearance changes with changes in environmental conditions. In order to make a positive identification, it is necessary to study the organisms in culture so that their complete life-history and their complete range of metabolic processes can be determined (6–8). Perhaps the development of genetic markers and probes for each of the important taxa, which could be used on field samples, will solve the problem (9–11). The standard methods used to study both heterotrophic and autotrophic microorganisms begin with enrichment cultures. Techniques for obtaining enrichment cultures from subaerial environments have been reviewed by Hirsch and coworkers (12) and Jacques

and Morris (13). The use of enrichment cultures brings with it another set of concerns. First, the types of organisms recovered depend to some degree on type of medium used and on the culture conditions. For example, Hirsch and coworkers (14) report that the use of malt extract medium resulted in higher fungal counts than Sabourad dextrose agar and that the use of oligotrophic media such as PYGV resulted in 10-fold higher bacterial colony counts and greater diversity than copiotrophic media. In addition, incubation of enrichment cultures in dim light usually results in a larger number of morphotypes among the heterotrophic population, possibly in response to organic compounds released by photosynthetic cyanobacteria and algae. Second, the organisms selected for in the enrichment are not necessarily the dominant forms in the habitat. Cox and Hightower (15) acknowledged this problem explicitly when they noted that it was possible (but unlikely) that none of the algae identified in their study formed part of the normal bark flora of Tennessee.

Rölleke and coworkers (16) tried to overcome some the drawbacks of the traditional methods of community analysis by applying denaturing gradient gel electrophoresis to PCR-amplified gene fragments coding for 16S rRNA from a medieval wall painting. They were able to demonstrate the presence of five bacterial species not previously isolated from wall paintings. At the same time, acinetobacters, which could be easily isolated, did not form a strong band in the DGGE pattern, suggesting that their role in the community is not as great as might be inferred from enrichment cultures. There remains the problem, recognized by the authors, that the DNA amplified might not originate from active members of the community (4,16).

The identification of subaerial cyanobacteria is complicated by the existence of competing taxonomic systems (17). Traditionally, cyanobacteria were studied by botanists and the taxonomy of cyanobacteria, following the general approach of classical botany, was based primarily on the morphological characteristics of field specimens (18,19). This approach, with some modifications to allow the use of data from cultures and molecular investigations (20–24), is still widely used by field biologists. During the 1970s and 1980s, Stanier, Rippka, and their colleagues, emphasizing that cyanobacteria are first and foremost bacteria, began a revision of the group on the basis of bacteriological principles, with an emphasis on the use of cultures and biochemical and molecular data. Although still in its early stages, this is the system used in the second edition of *Bergey's Manual of Systematic Bacteriology* (25).

Cyanobacteria and Eukaryotic Algae. Floristic and ecological studies have implicated members of well over 200 genera of algae and cyanobacteria as participating in subaerial communities (26). Many of these are also common constituents of the soil microflora (27–29); to date no concerted effort has been made to separate the two groups. Within the subaerial algae, the most successful are those that form symbiotic relationships with fungi in lichen associations; these are discussed in more detail elsewhere. Second in importance are sheathed cyanobacteria, most notably members of the genera *Chroococcus*, *Chroococcidiopsis*, *Gloeocapsa*, *Phormidium*, *Schizothrix*, *Scytonema*, *Stigonema*, and *Tolypothrix*. As a group the sheathed cyanobacteria reach their maximum development in the tropics, where they dominate on most surfaces (1,2,30–35). However, they can be found just about anywhere in the world where suitable surfaces are exposed to sufficient light and periods of flowing water of sufficient duration (36–48); many of the same species grow epilithically both in the tropics and in temperate regions (35). If the flow of surface water is not frequent enough, and the surface porous enough, they may retreat into the rock surface, living as endoliths (49–52). Third in importance is the so-called *Pleurococcetum*. This community is dominated by spherical green algae of the *protococcoid* genera: *Apatococcus*, *Desmococcus*, *Pleurastrum*, and so on. In the past, these organisms were commonly referred to the genus *Protococcus* Agardh, a name that, although still used colloquially, has been discarded for nomenclatural reasons (53,54). The members of this group are particularly difficult to identify, and the serious student should consult the recent monographs by Ettl and Gärtner (28) and Andreeva (29). The name of the association is derived from *Pleurococcus* Meneghini, a name that some consider synonymous with *Protococcus*; Gärtner and Ingolic suggest changing the name of the association to the more meaningful and taxonomically correct *Apatococcetum* (55). The association tends to be restricted to the drier rock surfaces and to tree bark and bare wood (36,37,43,56–58). The frequency of this community decreases in tropical regions, possibly because the increased rainfall and higher temperatures favor the expansion of cyanobacterial communities (1,2). Also common, but rarely dominating, in subaerial communities are members of the unicellular genera *Chlorella*, *Chlorococcum*, and *Stichococcus*, and the filamentous genus *Klebsormidium*.

Trentepohlia, a chlorophyte with branched filaments, is often found on tree bark or in association with epilithic cyanobacteria in temperate regions (36,37). Typically, it forms characteristic wispy orange growths that are easily identified without magnification. The frequency and size of these tufts increase in tropical regions, where *Trentepohlia* may rival the growth of the cyanobacteria (56). Its relatives *Cephaleuros*, *Phycopeltis*, *Printzia*, and *Stomatochroon* are also common in tropical regions. *Phycopeltis* and *Printzia* are free-living epiphytes and epiphylls (59); *Cephaleuros* and *Stomatochroon* are endophytes, with *Cephaleuros* growing below the cuticle of the twigs, leaves, or fruits of the host plant, and *Stomatochroon* inhabiting the stomata of the host (59). Epiphyllous cyanobacteria, most notably species of the heterocystous genera *Scytonema*, *Stigonema*, and *Hapalosiphon*, are also common in the tropics (60–62).

Subaerial diatoms, although a common and diverse group, are usually minor constituents of the community, either interspersed among other algae or growing epiphytically on bryophytes and lichens (63–67). Rich growths dominated by diatoms can occur, but they are usually restricted to the wettest surfaces (36,68,69). The implication is that diatoms, as a group, are not as well adapted to the subaerial environment, perhaps because of their need for dissolved silica (3), but this has not

been investigated in any detail. Common genera include the motile forms *Achnanthes*, *Hantzschia*, *Navicula*, and *Pinnularia*.

The division of a particular subaerial flora into smaller associations has also been attempted. For example, Golubic (37) in his classic study of the epilithic community in portions of the former Yugoslavia, was able to discern nine associations of epilithic algae and to arrange them into two parallel series on the basis of the moisture and light regimes. Similarly, Foerster (32), working the forests of Puerto Rico, was able to determine a number of associations of corticolous algae and to correlate them with perceived changes in the microhabitat. However, this practice raises a number of issues. First, there is the question of the effects of environmental conditions on the morphology of microalgae, in general, and of cyanobacteria, in particular. Second, there is the more general ecological question of what constitutes a meaningful microbial community. Because the fidelity of most species to any particular subaerial association is low, the defined associations typically reflect the presence or absence of one or two dominant forms. It is more likely, then, that we are tracing the boundaries of individual species, not associations; this is a useful exercise in itself, but should be recognized for what it is.

Nonphotosynthetic Bacteria and Fungi. Subaerial epiliths have primarily been studied in the context of the biodeterioration of stone, masonry, and paintings. One of the most obvious features of the group is its variability in space and time, (4,12,14,70–73). However, a few comments concerning the general composition can be made. Representatives of the genus *Thiobacillus* are commonly associated with regions of gypsum formation on limestone and stucco (71,72); other sulfate-cycling bacteria may also be found (4). Nitrifying bacteria from the genera *Nitrosovibrio*, *Nitrosomonas*, and *Nitrosospira*, as well as species of *Nitrosobacter* have been recovered from building stones with high concentrations of ammonia in their surface layers (74). Species of *Arthrobacter*, *Bacillus*, *Geodermatophilus*, *Micrococcus*, *Pseudomonas*, and *Sarcina* are also common (16,73,75). The predominant species on frescoes from underground tombs and grottoes are members of the Actinomycetales, especially *Streptomyces* and, to a lesser extent, *Nocardia*, (4,16,48). Common genera of epilithic fungi include *Alternaria*, *Aspergillus*, *Aureobasidium*, *Candida*, *Cladosporium*, *Fusarium*, *Mucor*, *Paecilomyces*, *Penicillium*, *Phoma*, *Rhizopus*, *Sporobolomyces*, and *Urocladium* (14, 71). Black yeasts (14,76) and members of the genus *Urocladium* (71) have been implicated in the blackening of ancient marble and limestone buildings.

Phyllosphere bacteria and fungi are often involved in processes detrimental to the host plant, and so are studied from a plant-pathology perspective. A complete review of this topic is beyond the scope of this article; I will try to restrict my comments to items related to the general ecology of microorganisms living in a subaerial environment. Those wishing for a more detailed coverage are referred to recent reviews by Kinkel (77) and Hirano and Upper (78). Plant-pathogenic members of the genera *Pseudomonas*, *Xanthomonas*, and *Erwinia* are among the most commonly investigated heterotrophic bacteria in the phyllosphere (78). However, a number of free-living bacteria can also be found including: nitrogen-fixing bacteria of the genera *Beijerinckia* and *Azotobacter*, (60,78,79,80), pink-pigmented facultative methylotrophs of the genus *Methylobacterium* (78), and common heterotrophs such as *Bacillus*, *Micrococcus*, and *Serratia* (78). Sundin and Jacobs (81) cite evidence that a majority of bacterial isolates from the phyllosphere are pigmented.

THE SUBAERIAL HABITAT

The subaerial habitat is considered by some to be one of the most extreme habitats colonized by microorganisms (78). As will be seen, moisture, light, and temperature conditions not only reach extreme values, they can fluctuate from benign to harsh within minutes or hours, putting an additional stress on the organisms as it tries to adapt.

Determining the exact conditions in the habitat is difficult. First, there is the matter of scale. As pointed out by Andrews (82) and Hirano and Upper (78), even the surface of a leaf presents a topography punctuated by peaks, valleys, and craters. Therefore, we should expect physical conditions to vary significantly from those measured by standard microclimate monitoring stations. Changes can also occur within the habitat over distances of one or two millimeters. These facts have prompted Friedmann and his colleagues to use the term *nanoclimate* when referring to the physical conditions within the lithic environment (83,84). Measuring the environmental parameters at the appropriate scale is difficult and may require the construction of special sensors (83,85). Extra care must also be taken to ensure that the act of measurement does not change what is to be measured. Computer models can be used to alleviate this problem by allowing us both to verify field measurements and to extend them without damaging the system (86,87). Second, conditions change not only over periods of minutes and hours, but over months and years, so that long-term measurements are necessary to gain a full understanding of the habitat. Although automated recording devices now allow us to construct continuous nanoclimate records over periods of years, even in remote areas (82,83), their use is still limited.

Moisture Conditions

It is commonly assumed that "Microbes, regardless of their habitat, are aquatic creatures" (88); see also the review by Brown (89). Therefore, the availability of moisture is considered to be the primary environmental factor determining the development of subaerial communities. The importance of the moisture regime in the subaerial environment can be seen in the development of tintenstrich communities, dark-colored subaerial communities that trace the boundaries of rain tracks on rocks, trees, and buildings (47).

Moisture can reach the habitat in at least three ways: from the air as dew fall, rain, snow, or spray; as surface runoff or subsurface seepage from meltwater or rainfall; or as water vapor absorbed directly from the air. Clearly,

it is not sufficient for a surface to be occasionally wetted or all surfaces would be covered by dense growths of microorganisms. The frequency and the duration of the wetting events are also important. This led Danin and Garty (90) to propose the cumulative imbibition time as the most important environmental parameter in lithic environments. The cumulative imbibition time refers to the total period of time when the organisms are turgid from the assimilation or imbibition of water; Büdel and Lange (91) suggest that a turgid state is a prerequisite for metabolic activity. A similar concept, the leaf wetness duration, is used in phytopathology, in which it is a key determinant of fungal infectivity (92). The frequency of wetting events depends primarily on the local climate: how often does it rain, during which seasons does it rain, and so on. It can also depend on the nature of the substratum; Danin and Garty found that small stones in the Negev Highlands cooled more quickly at night and were thus subjected to increased dewfall (90). The duration of the wetting event is much more complex, involving interactions between the local climate and the substratum. The water reaching the community can continue to flow past, can soak into the substratum, or can evaporate. The flow rate depends on the slope and texture of the surface; smooth, vertical surfaces will not retain water for long periods, whereas rough, horizontal surfaces allow some degree of puddling. The amount of water absorbed by the surface depends on its porosity and water holding capacity; its main effects are related to the rate of evaporation. The rate of evaporation depends on the relative humidity and temperature of the surrounding air, the strength and direction of the prevailing winds, the degree to which the substratum is warmed by sunlight, and the amount of water retained in the substratum (57,82,93,94). As an additional complication, porous materials such as wood or sandstone may retain significant amounts of moisture for long periods of time, but the water may be available to surface dwelling organisms only as water vapor. A more or less steady supply of water vapor at high concentrations may also be available to bark and leaf organisms as transpiration through lenticels and stomata; Burkhardt and coworkers (85) found evidence that at least some transpired water vapor could be recondensed on the leaf surface.

Unfortunately, data regarding the duration of periods of water availability in subaerial environments are almost completely lacking. Turner (95) suggested that water, at least in the form of water vapor is never limiting in the subaerial community on larch trees; his measurements indicated that the relative humidity within a few millimeters of the surface of the bark remained above 60%. Bell (51) reached a similar conclusion for moisture in the endolithic habitat of the semiarid Colorado Plateau. However, as will be seen, it is not clear that water vapor alone is sufficient for the maintenance of metabolic activity in subaerial communities. Liquid water from rain events may be available to tropical epiliths as little as a few days per year in the dry savannah to nearly every day in the rainforest zone (35). In the Negev Highlands, Danin and Garty estimated that rainfall contributes from 25 to 130 hours per year to the cumulative imbibition time, dewfall an additional 250 to 600 hours for those stones on which dewfall was common (90). Friedmann and his colleagues arrived at similar values, 500 to 1,000 hours of activity per year, for the endolithic community of the dry valleys region of Antarctica; here the availability of moisture was estimated on the basis of changes in the conductivity of the substratum (83,84).

Two additional features of the moisture regime should be mentioned. Hirano and coworkers (96) found that raindrop momentum apparently triggers the growth *Pseudomonas syringae* in the phyllosphere. The mechanism is unclear. Strains of *Ps. syringae* and a handful of other phyllosphere-inhabiting species, are also well known as an ice-nucleating agents (78). Although the gene associated with ice-nucleating activity is well characterized, the role that ice-nucleation plays in the biology of bacteria expressing the gene is not.

Temperature Conditions

The temperature in subaerial habitats can be significantly (20 °C or more) higher than the ambient air temperature. In temperate regions, temperatures can reach 45 °C in bark communities (97), 73 °C in epilithic communities (36), and in excess of 85 °C on rooftops (98). In the tropics (47,99,100) or hot deserts (101), temperatures can reach 60 °C on a daily basis. Furthermore, the amplitude of the diurnal variation in these habitats is in excess of 30°. These conditions are all more extreme than would be encountered in an aquatic habitat from the same region. If the organisms were active at these temperatures, some degree of thermophily might be expected. However, they are typically desiccated before the more extreme temperatures are reached. The exact temperature regime of any particular community depends on the amount of solar radiation striking the surface, which is a function of the latitude of the site, the orientation of the surface, and the degree of shading by the surroundings, and the nature of the community and the substratum (86). Because light materials reflect more sunlight, they do not warm to the same degree as dark materials. This may explain the grayish appearance of some epilithic lichens in hot deserts. At the other extreme, it has been suggested that white sandstones in Antarctica never warm up sufficiently to sustain life (86). Polar communities must also survive freeze-thaw cycles with periods of 24 hours or less (102,103).

Wind Conditions

Wind can have both negative and positive effects on subaerial communities. First, as mentioned previously, it increases the rate of evaporation, thereby reducing availability of moisture in the community and the cumulative imbibition time. It also has the potential to remove parts of the community by erosion. At the same time, the wind probably contributes the most to colonization of fresh surfaces by supplying new recruits; this may explain some the diversity found on Sri Lanka (1,2) and Puerto Rico (32). It can also reduce the more extreme temperature conditions by increasing the rate of heat exchange with the surrounding air. The relative importance of each of these effects should be determined at each site. It is commonly noted, however,

that subaerial organisms grow primarily on the leeward side of trees and stones (3,42,43,57).

Light Conditions

Subaerial communities in exposed habitats can be subjected to photosynthetic photon fluxes greater than 2,000 μmoles photons $m^{-2} s^{-1}$. This level of solar radiation will not only contribute to the development of extreme temperatures, it can damage the members of the community directly. Photosynthesis by freshwater algae is typically saturated with respect to light at irradiances below 1,000 μmoles photons $m^{-2} s^{-1}$ (104,105). There is a growing body of evidence that the light responses of terrestrial algae are similar. Subaerial strains of *Gloeocapsa* and *Stichococcus* exhibited light saturation in the laboratory at photosynthetic photon fluxes below 250 μmoles photons $m^{-2} s^{-1}$ (106); it should be noted, however, that these strains are maintained at relatively low fluxes. Ong and coworkers (107) were able to demonstrate photoinhibition in an edaphic population of *Trentepohlia*. Lüttge and coworkers (108) reported at least some degree of decreased photosynthesis in light-stressed field samples from terrestrial environments. Weber and coworkers (109) demonstrated that endolithic strains of *Chroococcidiopsis* develop below the surface of artificial substrata in response to high surface illumination. Even if light saturation and photodamage do not occur while the alga is active, the organisms are often exposed to the same level of irradiation while dry; under these conditions the energy absorbed by their pigments cannot be dissipated and damage to DNA from the absorption of ultraviolet light cannot be repaired (98,110–112).

The Cave Habitat

The walls and ceilings of caves provide a unique habitat for subaerial microorganisms, with conditions that differ considerably from those just described. The relatively constant temperature and typically high humidity create some of the most favorable conditions associated with subaerial habitats. In addition, seep-water from the ground above the cave provides a more stable source of liquid water than is found in exposed habitats. The result can be a rich growth of algae, often dominated by members of the cyanobacterial genera *Geitleria*, *Leptolyngbya*, and *Scytonema* at the entrance of the cave (113,114). The growth of subaerial algae has been reported on frescoes in underground catacombs (4,45,48,115). Because the amount of light available for photosynthesis drops off rapidly, the growth of algae is restricted to within a few meters of the mouth of the cave (116,117). However, because conditions are otherwise so favorable, the provision of even low levels of illumination for tourism can lead to the growth of photosynthetic communities at these so-called light oases (4,115,116,118).

ADAPTATIONS TO THE SUBAERIAL ENVIRONMENT

The Moisture Regime

By definition, all subaerial microorganisms experience intervals when they are not surrounded by liquid water. As the water content of a cell drops during these intervals, metabolism and growth stop. Under ideal conditions, the organism would then enter a state of cryptobiosis lasting until the moisture regime improves and metabolic processes take up where they left off. However, the situation is not so simple. Changes in the concentration of solutes during the drying process can lead to imbalances in the cell's metabolic processes. The shortage of the water molecules required for the maintenance of macromolecules and subcellular structures such as cell membranes can cause irreversible damage and the death of the cell (98,112). The ability to survive even brief periods of desiccation, then, is one of the fundamental attributes of subaerial microorganisms. Once desiccated, the individual cell is still subject to chemical and physical damage, which cannot be repaired until metabolism restarts. If the damage incurred while in the desiccated state is too great, the cell is unable to make repairs fast enough, and dies. At the same time, the association as a whole is subject to losses by erosion and predation that cannot be replaced until cellular reproduction restarts. Any features that would either prolong the periods of activity or help the cell to survive the periods of desiccation, then, would be adaptive. The actual mechanisms used are varied and only now are beginning to be understood (89,98,112,119); the major mechanisms are summarized below. Note that many of these adaptations serve multiple purposes. For example, as will be seen, the presence of a sheath can serve to retard water loss, maintain cell membrane integrity, and protect against photodamage.

Adaptations to Prolong the Period of Activity. Higher plants and terrestrial animals thrive in the subaerial environment because they form complex multicellular structures with an essentially water-tight surface. The interior cells remain in an aqueous environment and the period of activity is extended indefinitely. This option is not open to unicellular forms. However, it is possible for microorganisms to use extracellular structures to accomplish the same end, albeit not as efficiently. Examples include the development of mucilaginous sheaths (98,120–125), growth on or within particulate substrata (49,93,122,126), or the inclusion in a complex, pseudomulticellular structure such as an algal mat or bacterial consortium (1,2,127) or the thallus of a lichen (126,128,129). Any of these structures may serve as water reservoirs, may help retard evaporation and, possibly, may condense water at high humidities. The development of a mucilaginous sheath is a particularly common, if not universal, attribute of subaerial cyanobacteria.

A second, less widespread, adaptation to prolong the period of activity is the ability to absorb sufficient moisture to sustain cellular activity directly from the air. The ability of some subaerial algae to maintain photosynthetic activity using only water vapor was first noted by Fritsch (120,121), Zeuch (130), and Edlich (97). More recent work has confirmed this ability in the chlorophyte genera *Apatococcus* (129,131), *Trebouxia* (126,129), *Trentepohlia* (107), and, to a lesser extent, in subaerial strains referred to *Chlorella*, *Klebsormidium*, *Stichococcus* (125). Members *Apatococcus* and *Trebouxia* are able to

carry on photosynthesis when equilibrated with air with a relative humidity as low as 70%. This adaptation seems to be restricted to eukaryotes (111,125,126,132–135). However, only a few species of algae have been tested, with the major emphasis given to species forming lichen associations. Lange and coworkers (136) have some evidence that the desert cyanobacterium *Microcoleus sociatum* may be able to use water vapor at very high relative humidities. The physiological basis of the ability to use water vapor and the reason that this adaptation is restricted to eukaryotes are unknown. As mentioned previously, Büdel and Lange suggest that the ability of the cell to become turgid through the absorption of water vapor is a requirement (90). The presence of hydrophobic material in the cell wall has been suggested as a contributing factor (131), although the mechanism is unclear; it is possible the hydrophobic character is more of an adaptation to resist restarting metabolism too soon (98).

The ability of *Apatococcus* and other green algae to use water vapor may explain the abundance of this group on tree bark and bare wood in drier climates. As previously mentioned, much of the moisture in this habitat reaches the surface community as vapor. If cyanobacteria are unable to use water vapor, the habitat is free for colonization by any green alga with this ability.

As a final note, the ability to use water vapor contradicts the assertion made earlier that all microorganisms are essentially aquatic. In one sense, these organisms are the only group fully adapted to life without liquid water.

Adaptations to Survive the Desiccation Process. We just are beginning to understand the mechanisms by which subaerial microorganisms survive desiccation. First, it should be remembered that osmotic water stress, on the one hand, and matric water stress and desiccation, on the other, are not equivalent (98,112). At low levels of osmotic water stress, compatible solutes such as proline, glycerol, and sorbitol, may be sufficient to maintain cellular activity (88,119,137). However, at the extreme levels of desiccation experienced in subaerial habitats, something more seems to be required. Current theory suggests that molecules such as the disaccharides trehalose and sucrose replace the water required to stabilize proteins and membranes, thereby maintaining important subcellular structures. In the desiccated state, the accumulated sugars form an aqueous glass. In the glasslike state, all molecules are essentially immobilized and rates of reaction greatly reduced (see (98,112,138–140)). Extracellular polysaccharides may also play a role in stabilizing membranes and preventing the fusion of cells (98,112). It is interesting, in this respect, to note that Grilli Caiola and coworkers (120,141) found that cyanobacteria with intact, lamellated sheaths had greater viability than those without.

Adaptations to Survive the Desiccated State. The attainment of a true cytoplasmic glass should greatly enhance the survival of desiccated cells. However, the protection is not 100%. There is increasing evidence of high mortality rates in desiccated communities with the exact mortality rate species-dependent (103,124,142). The source of this mortality is unknown but oxygen-mediated and light-mediated damage to DNA, membranes, and proteins have all been implicated (see reviews by Potts (98,112), Smirnoff (143), and Whitelam and coworkers (144)). It is not surprising then that nearly all subaerial microorganisms possess some mechanism to at least reduce the damage caused by light. The sheaths of terrestrial and subaerial cyanobacteria often contain the yellow-brown pigment scytonemin; this is the cause of the dark colors associated with rich growths of subaerial cyanobacteria. The broad absorption peak of this pigment around 370 nm implies strongly that it may serve as protection against UV-light and high-energy visible light (98,145–149). Garcia-Pichel and Castenholz (146) found that scytonemin production was stimulated by high light and by UV-A radiation. Although stimulation of scytonemin by high light is probably the rule in the subaerial environment, Pentecost (150) found that in some instances, most probably during times of high growth rates, there is a negative correlation between the two.

Most of the eukaryotic subaerial algae, in contrast, are sheathless, and not all sheathed cyanobacteria produce scytonemin. Therefore, other mechanisms must be at work. Some cyanobacteria produce mycosporin-like amino acids in their cytoplasm; these can absorb some of the incident UV radiation (149,151). Many of eukaryotic forms, notably members of the genera *Chlorella*, *Chlorococcum*, and *Trentepohlia* have the potential to develop oil droplets colored by pigments such as astaxanthin or zeaxanthin. These seem to provide a degree of photoprotection (152–154). However, the development of these pigments is not necessarily related to light stress. Older cultures of some strains turn red or orange, probably in response to nutrient depletion (155,156). Not all subaerial algae have the ability to make these pigments. It has been suggested that in at least some desiccation tolerant algae there is a decoupling of the light-harvesting complex from the photosystem II reaction center in the desiccated state (111,144,157). In the desiccation tolerant strain *Nostoc commune* UTEX 584, there is evidence that phycobilisomes, used for light-harvesting, are degraded during short-term drying in the light (112). These changes in light-harvesting complexes should reduce photooxidative damage by reducing the rate at which active oxygen is formed. Other mechanisms used by algae to avoid light stress include the development of a complex community structure and organization, with more heavily pigmented cells at the surface (127); incorporation into the thallus of a lichen with melanin-producing mycobionts (87); and growth in hypolithic and endolithic habitats (87,101,122). Some subaerial diatoms form internal valves that may have an as-yet unknown role in desiccation resistance (158).

The nonphotosynthetic component of the community must also cope with the possibility of light-induced damage in the desiccated state. Many of the fungi are dark-colored, chiefly through the production of melanin and melanin-like compounds (14,71,76). Like scytonemin, these absorb light preferentially in the lower wavelengths and may provide some degree of protection from damage by UV radiation. Tests among phyllosphere-derived strains of

bacteria indicated that those forming pink or orange pigmentation were more tolerant of UV radiation (81). In this case, the pigments may serve to scavenge active oxygen after its formation (149); Garcia-Pichel (159) has calculated that smaller cells, less than 1 μm in radius, cannot use internal pigments as sunscreens, but must rely on external sheath-pigments.

Adaptations to the Temperature Regime

The temperature regime in the subaerial environment can be almost as severe as the moisture regime, with temperatures reaching the extremes for the particular region. How the organisms survive these extremes is an interesting, but almost untouched question. In hot environments it is usually assumed that the highest temperatures are reached while the organisms are dry, and that the desiccated state confers some sort of heat protection (122). Some preliminary results are available, however. Tripathi (99,100,160) has demonstrated the presence of heat-stable chlorophyll and proteins in subaerial cyanobacteria from habitats subjected to extreme (60° or higher) temperatures; he suggests that the stability of the proteins is related to a higher number of sulfhydryl groups in the heat-stable proteins. At the other extreme, in Antarctica, cryptoendolithic algae are able to maintain photosynthetic activity down to at least −8° (84), and at least one, *Hemichloris antarctica*, can withstand repeated cycles between −5° and +5°, with temperature changes on the order of 1.5°/min (161).

SIGNIFICANCE OF THE SUBAERIAL COMMUNITY

Colonization of Bare Surfaces

Subaerial organisms are the first to colonize rock outcrops and other bare surfaces. The initial inoculum is presumably brought in by wind (14,78,162), but transport by runoff in wet areas and by arthropods are also possible (14,162). Attachment of epiliths to the substratum has not been studied extensively. Garty (163) suggests that the microtopography of the rock surface, including holes and pits produced by previous inhabitants, plays a key role by providing favorable microenvironments. The actual attachment seems to be mediated through extracellular polysaccharides (97,164,165). Some have suggested that hydrophobic substances in the cell wall or extracellular matrix may also be involved; Potts (98) points out that, although hydrophobicity may be important in aquatic systems, its role in subaerial and terrestrial systems is unclear. Romantschuk (166) has reviewed the attachment mechanisms for plant-specific bacteria. Here the evidence suggests that in addition to extracellular polysaccharides, different types of proteinaceous filaments, including fimbriae and pili, are involved.

The subsequent development of the community strongly depends on local environmental conditions, especially the availability of moisture. Under laboratory conditions with regular supplies of moisture, visible growth appears in less than two weeks, and thick films form within two months (106,167). In natural settings, development can take months or years. Pentecost (44) investigated the process in the United Kingdom by clearing visible growth from two subaerial epilithic sites. The wetter site recovered completely within one year; the drier site still showed signs of the clearing after 10 years. This matches my own observations of recovery after herbivory or artificial disturbances in the southern United States. Freiberg (59,60) found visible development of nonpathogenic epiphyllous organisms on two- and three-month-old leaves in a tropical rainforest; visible colonies of cyanobacteria, including *Scytonema*, appeared later.

Productivity

The annual productivity of subaerial communities, and its significance to the local ecosystem is generally unknown. This is, in part, a result of the difficulty in monitoring the environment. Active subaerial communities change their structure and their activity over the course of a year (31,70,73,77,145,168). Therefore, in order to make reasonably accurate determinations of the productivity, it is necessary to either monitor photosynthesis in the field for long periods of time and under a variety of conditions, or monitor the environmental conditions in the habitat, and compare them with laboratory measurements of the photosynthetic response of the community or its members; only the latter has been attempted so far, and only rarely. Turner (95) estimated the productivity of the *Pleurococcetum* in Europe as 0.9 to 1.8 g Cm^{-2} $year^{-1}$. Most of this presumably provided food for wood lice and other invertebrates. Büdel (35), using measured values of photosynthesis over short intervals and regional climate data, estimated that tropical epiliths fix carbon dioxide at a rate of 8.4 g CO_2 m^{-2} $year^{-1}$ in the thorn bush savannahs, 27 g CO_2 m^{-2} $year^{-1}$ in the dry savannah, and 12 g CO_2 m^{-2} $year^{-1}$ in the humid savannah. When the area of rock surface is taken into account, these values represent sizable fractions of the total productivity of the ecosystem of the semidesert and savannah ecosystems. Friedmann and coworkers (84) were able to make use of a five-year record of micro- and nanoclimate data to determine the total period of activity and the environmental conditions during the active period in the Antarctic ecosystem. These estimates were then coupled with laboratory data concerning the photosynthetic response of the cryptoendolithic community to arrive at a gross productivity of 1.2 g Cm^{-2} $year^{-1}$, with about half of the gross productivity consumed by respiration. Of the remaining 0.6 gm, almost all is thought to be lost to the environment through erosion and leaching.

Subaerial organisms are a source of food for many invertebrates including wood lice (95), mites (14), and terrestrial snails (14,169). Uneaten remains and chemical exudates are available for use by the soil community.

The Role of Subaerial Organisms in Biodeterioration

Epilithic communities, including lichens, are well known for their ability to weather bare rock outcrops (72,90,170,171), historic monuments (14,71,76,172) and buildings (39,45,74,173–176), glass (163), and frescoes (4). The basic mechanisms involved are the subject of several recent reviews (4,14,72,74,173). Briefly, two distinct processes occur. First, mechanical damage results

from the shrinkage and subsequent swelling of cells and sheaths because they are subjected to repeated wet and dry cycles. The forces exerted are thought to be sufficient to dislodge individual grains from the substratum. Second, the organisms are responsible for the production of acids that change the chemical makeup of the substratum. Fungi, in particular, are cited as producing a number of organic acids, including oxalate, formate, fumarate, lactate, and gluconate, which can demineralize the substrate, either through their activity as chelating agents or through altering the pH. The activities of sulfur-oxidizing (*Thiobacillus*) bacteria result in the production of sulfuric acid that can cause the conversion of calcium carbonate to calcium sulfate (gypsum); the increased activity of sulfur-oxidizing bacteria in response to increased sulfur dioxide in the atmosphere have been especially noted in the destruction of frescoes and limestone (4). Similarly, nitrifying bacteria, responding to higher levels of ammonium ions in the surface layers, can produce nitric acid (74). We should not forget the importance of these processes in the history of life on Earth. It is now recognized that the advent of terrestrial vascular plants was preceded by a long period during which the land was colonized by microorganisms and nonvascular plants. These organisms set the stage for the later radiation of vascular plants by contributing to the production of a true soil containing organic matter (177–179). It is distinctly possible that some of these original terrestrial microbes were also subaerial (177).

PROSPECTS

In this entry, I have tried to emphasize the adaptations displayed by subaerial organisms to what is in many respects an extreme environment, at the expense of some equally interesting topics in their biology. This ubiquitous group of extremophiles displays a number of unique traits, namely, desiccation resistance or resistance to photooxidative damage, which we have just begun to appreciate and investigate. The identification and characterization of the molecular bases of some of these is already beginning to bear fruit. For example, the characterization of ice-nucleating activity in *Pseudomonas syringae* led to the development of ice-minus strains with potential applications in agriculture (78). This and other, more distant applications, such as inclusion as components of the life-support system of spacecraft (106), or in terraforming other planets (180), are indicative of the potential of this young field.

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SUBSURFACE MICROBIAL COMMUNITIES: DIVERSITY OF CULTURABLE MICROORGANISMS

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Interest in the microbiology of the terrestrial subsurface has increased steadily since the early 1980s, when it was discovered that several comparatively shallow aquifers (<30 m below land surface) contained sizable populations of culturable microorganisms (see Reference 1 for a review of this research). During the past two decades, culturable microorganisms, especially bacteria, have been detected in saturated and vadose-zone subsurface environments that ranged in depth from near-surface to 2.7-km below land surface, and in ambient temperature from 8 °C to 65 °C. Moreover, bacteria have been cultured from a geologically diverse range of unconsolidated and consolidated subsurface materials. Unconsolidated materials yielding bacterial isolates include lacustrine (lake) sediments (2), paleosols (2–5), fluvial sands and gravels (2–4), deep mine-gallery clays (6), and Atlantic coastal plain sands and clays (7–13). Different types of subsurface rocks from which bacteria have been isolated include Triassic siltstones (14–16), basalts (and their associated clay-silt interbeds) (3), unwelded volcanic tuffs (17–19), granitic rocks (and associated groundwater) (20–22), and Cretaceous shales and sandstones (23,24). Substantial numbers of microbial isolates from these environments have been preserved, so that they can be studied and characterized; for example, the U.S. Department of Energy Subsurface Microbial Culture Collection (25) now contains more than 13,000 strains of aerobic and anaerobic bacteria.

Only a small percentage of the many bacterial strains that have been isolated from subsurface environments have been characterized in any detail. Yet, the characterization studies completed to date provide much information on the diversity and composition of the culturable microbial communities in certain types of subsurface environments and some insights on how the isolates are related to previously described bacterial species. The objective of this article is to review and summarize these studies, focusing primarily on the phylogenetic characterization of culturable subsurface bacteria by analysis of 16S ribosomal RNA gene sequences.

PHYLOGENETIC BACTERIAL DIVERSITY IN SELECTED TYPES OF SUBSURFACE ENVIRONMENTS

It is necessary to characterize either a large proportion or a good representative sample of the microorganisms isolated from a particular subsurface environment to develop a reasonably complete understanding of the species composition and diversity of the culturable microbial community living therein. Only a few subsurface microbial communities have been examined this thoroughly to date, possibly because it is tedious and expensive to do so when a large number of isolates must be examined.

Atlantic Coastal Plain Sediments at the Savannah River Site

Among the most extensively studied culturable subsurface, microbial communities are the aerobic and facultatively anaerobic chemoheterotrophic bacterial communities in Atlantic coastal plain (ACP) sediments, at the U.S. Department of Energy Savannah River Site (SRS) near Aiken, SC. The sediments at this site consist of sandy aquifers separated by periodic clay confining zones (26). The bacteria isolated from all 15 sample depths (ranging from 24 to 265 m) in borehole P24 at the SRS (26) have received the most attention, having been characterized morphologically (7), physiologically (8), and phylogenetically (27,28).

Morphological and physiological studies showed that the culturable heterotrophic bacterial communities at the SRS were quite diverse. For example, it was reported that a set of 1,112 isolates yielded 626 distinct patterns of response to 21 selected physiological tests (8). However, the actual nature of the diversity implied by these results (e.g., strain-level versus a much broader range of diversity) could not be understood without detailed phylogenetic information. To avoid unnecessary phylogenetic characterization of very similar strains, all of the 187 bacterial isolates from borehole P24 were prescreened with a restriction endonuclease analysis (REA) technique (27). This analysis placed 126 of the 187 isolates in 21 clusters of very closely related strains, based on their restriction fragment patterns. The other 61 isolates did not appear to be closely related to each other or to any of the strains that clustered.

Representative strains from each REA cluster were characterized phylogenetically by analysis of their 16S rRNA gene sequences (27,28). The genera to which these strains were assigned are listed in Table 1. When two or more strains from a single cluster were analyzed, they always appeared to be most closely related to the same genus. The strains within each cluster, then, were at least

Table 1. Taxonomic Assignments for Bacterial Strains Isolated from Atlantic Coastal Plain Sediments at the Savannah River Site[a]

Genus or Higher Taxonomic Unit	No of Strains Isolated
α-Proteobacteria	
Sphingomonas	6
β-Proteobacteria	
Comamonas	29
Alcaligenes	10
γ-Proteobacteria	
Acinetobacter	19
Pseudomonas	11
High-G + C gram-positive bacteria	
Arthrobacter	38
Terrabacter	10
Micrococcus	3

[a] On the basis of REA prescreening and phylogenetic analysis of 16S rRNA gene sequences; data condensed from Table 2, Balkwill and coworkers (22).

phylogenetically similar enough to be members of a single genus. The 126 isolates that clustered were assigned to a total of just five gram-negative and three gram-positive genera (Table 1). Moreover, 68% of these strains were placed in just three genera (*Arthrobacter*, *Comamonas*, and *Acinetobacter*). Therefore, the diversity detected in earlier morphological and physiological studies (7,8) did not appear to represent substantial genus-level diversity. Additional genera might be detected if the 61 isolates that failed to cluster during REA were sequenced and analyzed, because these isolates apparently were not closely related to each other or to those that clustered. Even so, most of the diversity detected by morphological and physiological analyses must be at the strain and species levels.

Morphogical, physiological, and phylogenetic studies of bacterial strains from the P24 borehole at the SRS all indicated that distinct groups of organisms had been isolated from different sample depths and geologic formations. In the phylogenetic studies, for example, 17 of the 21 REA clusters consisted entirely of strains that were detected at only a single depth. There were also distinct differences in the genera that were isolated from different depths and aquifers (27).

Saturated Sediments at the Hanford Site

The culturable aerobic (and facultatively anaerobic) chemoheterotrophic bacterial communities in deep saturated sediments at the U.S. Department of Energy's Hanford Site near Richland, WA are also among the subsurface microbial communities that have been characterized extensively. The sediments examined at this site ranged in depth from 173 to 220 m below land surface and were located within the Ringold formation (2,29). The types of material sampled included lacustrine sediments, paleosols (ancient buried soils), and fluvial sands and gravels. A total of 169 strains of bacteria were isolated from these materials on various plating media, and all of these were characterized phylogenetically, by analysis of their 16S rRNA gene sequences (27,30).

The major phylogenetic groups and bacterial genera to which the strains from the Hanford Site were most closely related (by analysis of 16S rRNA sequences) are detailed in Table 2. There appeared to be considerably more diversity among the Hanford isolates, than was detected among those from ACP sediments at the SRS (as mentioned earlier), although some of the difference might simply be due to the fact that only selected strains (representative of the 21 REA clusters) were characterized in the SRS study. In any event, the Hanford Site isolates could be assigned to six major taxonomic groups: the high- and low-G + C gram-positive bacteria; the alpha-, beta-, and gamma-subdivisions of the *Proteobacteria*; and the *Flexibacter-Cytophaga-Bacteroides* group. Moreover, the isolates were most closely related to at least 33 different bacterial genera within these groups. (A few isolates were not closely related to any genus in the current public 16S rRNA sequence databases and, thus, might represent novel genera.) Seventy percent of the isolates were gram-positive, and 60% of those were placed in the high-G + C group. *Arthrobacter* was by far the most frequently detected high-G + C gram-positive (40 of 73 strains), whereas all but one of the low-G + C isolates were strains of *Bacillus* or *Staphylococcus*. The most frequently noted gram-negative genera among the Hanford Site isolates were *Variovorax*, *Sphingomonas*, *Caulobacter*, and *Pseudomonas*. There was also a group of eight gram-negative isolates that were closely related to a 16S rRNA clone obtained from deep granitic groundwaters in the Stripa Mine (in Sweden) with direct molecular biological methods (31).

Table 2. Taxonomic Assignments for Bacterial Strains Isolated from Saturated Sediments at the Hanford Site[a]

Genus or Higher Taxonomic Unit	Number of Strains Isolated
Flexibacter-Cytophaga-Bacteroides group	
Flectobacillus[b]	1
Microscilla[b]	1
α-Proteobacteria	
Sphingomonas[b]	6
Caulobacter	5
Erythromicrobium	2
Azospirillum	1
Agrobacterium	1
Methylobacterium	1
Rhodobacter	1
Blastobacter	1
β-Proteobacteria	
Variovorax	8
Stripa Mine clone	8
Telluria[b]	3
Leptothrix	2
Comamonas	1
Rhodocyclus	1
Zooglea	1
γ-Proteobacteria	
Pseudomonas	4
Acinetobacter	2
Low-G + C gram-positive bacteria	
Bacillus[b]	25
Staphylococcus[b]	20
Alicyclobacillus	1
High-G + C gram-positive bacteria	
Arthrobacter	40
Micrococcus[b]	7
Rhodococcus[b]	5
Streptomyces	4
Rothia	3
Clavibacter	3
Mycobacterium	2
Frankia[b]	2
Gordona	2
Nocardioides	2
Dermatophila[b]	1
Aureobacterium	1
Genus affiliation uncertain	1

[a] On the basis of phylogenetic analysis of 16S rRNA gene sequences; data from Tables 3 and 4 in Balkwill and coworkers (22).
[b] Genus in 16S rRNA sequence databases to which strains in this group are most closely related; relatedness may not be sufficiently close in the case of one or more strains to be certain of genus assignment.

The Hanford Site isolates were similar to those from the SRS in that, the majority of them (74%) could be assigned to a relatively small number of genera (just 10 of the 33 genera detected at that site). Nevertheless, there were some clear differences between the culturable heterotrophic populations at these two sites. For example, no low-G + C gram-positive bacteria have been detected at the SRS yet, whereas *Staphylococcus* and *Bacillus* were among the most significant components of the culturable microflora at Hanford. In contrast, *Pseudomonas* and *Acinetobacter* were numerically predominant components of the culturable community at the SRS, but were detected only infrequently in the saturated sediments at the Hanford Site.

Vadose Zone Sediments at the Hanford Site

Balkwill and coworkers (32) recently characterized the culturable aerobic (and facultatively anaerobic), chemoheterotrophic bacterial communities in comparatively shallow vadose (unsaturated) zone sediments at the Hanford Site. The main purpose of this study was to compare the bacterial communities occurring at high- and low-recharge sites, to seek evidence for vertical transport of bacterial cells through the vadose zone. However, the information obtained during this study also facilitates a comparison of the culturable vadose-zone microbial communities to those occurring in the deeper, saturated sediments at the Hanford Site (as seen earlier). All of the isolates from several depths (ranging to 15 m below land surface) at one high-recharge and one low-recharge site were characterized in the vadose-zone study. Streptomycetes were identified by cell and colony morphological traits, and nonstreptomycete isolates were characterized phylogenetically by analysis of 16S rRNA gene sequences. The bacterial genera to which the isolates were assigned by morphological and phylogenetic analyses are listed in Table 3.

In general, the culturable bacterial community in the vadose-zone sediments was quite different from that isolated from the deeper saturated sediments at the Hanford Site. Overall genus-level diversity was far lower in the vadose zone, with 203 isolates falling into a total of only eight genera (as opposed to 169 isolates from the saturated sediments being assigned to at least 33 different genera; see in the preceding text). A large proportion (72%) of the vadose-zone isolates were found to be strains of *Streptomyces*, a genus that was only rarely isolated from the deep saturated sediments. Both culturable bacterial communities were mostly gram-positive, but the proportion of gram-positive strains was higher in the vadose zone (93% versus 70% for the saturated sediments). Moreover, all of the gram-positive bacteria isolated from the vadose zone were members of genera that either form spores (*Streptomyces* and *Bacillus*), or that, are known for having unusually good survival capabilities (*Arthrobacter*). The culturable vadose-zone communities at the Hanford Site, then, seem to be largely limited to a specialized group of bacteria that are well equipped to deal with very low moisture and nutrient levels.

Table 3. Taxonomic Assignments for Bacterial Strains Isolated from Vadose Zone Sediments at the Hanford Site[a]

Genus or Higher Taxonomic Unit	No. of Strains Isolated
α-Proteobacteria	
Azospirillum	6
Bradyrhizobium	3
Rhizobium	2
β-Proteobacteria	
Telluria	1
γ-Proteobacteria	
Pseudomonas	1
Xanthomonas	1
Low-G + C gram-positive bacteria	
Bacillus	37
High-G + C gram-positive bacteria	
Streptomyces[b]	146
Arthrobacter	6

[a]As determined by phylogenetic analysis of 16S rRNA gene sequences, unless otherwise noted; data from Table 1, Balkwill and coworkers (32).
[b]Determined by colony morphology, rather than by 16S rRNA sequence analysis.

Deep Mine Gallery Clays

Boivin-Jahns and coworkers (6) have characterized the culturable bacterial communities in deep mine gallery clays of the Boom formation at Mol, Belgium. The clays in this study were situated 224-m below land surface and were deposited by marine sedimentation 35 million years ago. Seventy-four bacterial strains were isolated from these materials and characterized phylogenetically by analysis of their 16S ribosomal RNA gene sequences. The genera or other taxonomic units to which the isolates were assigned by this analysis are listed in Table 4.

Table 4. Taxonomic Assignments for Bacterial Strains Isolated from Deep Mine Gallery Clays at a Site in Belgium[a]

Genus or higher taxonomic unit	Number of strains isolated
α-Proteobacteria	
Rhizobium/Agrobacterium[b]	1
β-Proteobacteria	
Alcaligenes	16
γ-Proteobacteria	
Pseudomonas	9
Acinetobacter	3
Moraxella	3
Low-G + C gram-positive bacteria	
Staphylococcus	17
Bacillus	4
High-G + C gram-positive bacteria	
Micrococcus	6
Arthrobacter	4
Corynebacterium	3
Nocardoides	2
Streptomyces[b]	2
Rhodococcus	1
Clavibacter[b]	1
Unidentified strains[c]	2

[a]As determined by analysis of 16S rRNA gene sequences; data condensed from Table 1, Boivin-Jahns and coworkers (6).
[b]Most closely related genus; actual genus-level identification uncertain.

Overall genus-level diversity in the mine gallery clays was lower than that noted in deep saturated sediments at the Hanford Site but higher than that detected in the other subsurface environments described earlier. The clay isolates could be assigned to eight gram-positive and six gram-negative genera. Gram-positive isolates were only slightly more abundant (55%) than gram-negative isolates at this site. *Staphylococcus* was the most frequently isolated gram-positive genus, whereas the most frequently noted gram-negative genera were *Pseudomonas* and *Alcaligenes*.

Deep Granitic Groundwater

Pedersen and Ekendahl (20) examined culturable microbial communities in the deep granitic groundwaters (maximum depth = 860 m) of southeastern Sweden. Most of the organisms that were detected by plating on a medium containing organic substrate were facultatively anaerobic, gram-negative, nonfermenting, heterotrophic bacteria. Seven isolates from the plate-counting medium were identified phenotypically and found to be members of just two bacterial genera: *Pseudomonas* and *Shewanella*. However, studies utilizing enrichment cultures showed that sulfate-reducing bacteria and microorganisms capable of growth on C-1 compounds and hydrogen (presumably methanogenic bacteria) were also culturable members of the granitic groundwater communities. The enrichment culture approach has also been used to survey physiological diversity in other subsurface microbial communities, including those in ACP sediments (9,11,12, and 33). Pedersen and coworkers (22) have since characterized the diversity of deep granitic microbial communities in more detail, using direct molecular methods that do not require culturing.

SPECIFIC GROUPS OF WELL CHARACTERIZED SUBSURFACE BACTERIA

Some characterization studies have dealt with specific groups of bacteria isolated from subsurface environments, as opposed to the composition and diversity of culturable subsurface microbial communities. In some cases, specific groups of subsurface bacteria have been characterized because they have unusual or potentially useful metabolic traits. In other cases, certain groups of isolates have been characterized, phylogenetically, to see how they are related to previously described bacterial taxa, especially to the most similar species known to live in topsoils and other surface environments. The primary objective of the latter studies has been to find out whether, and how subsurface bacteria differ from typical surface organisms.

Aromatic-Degrading *Sphingomonas* Isolates from Atlantic Coastal Plain Sediments

A small group of bacterial isolates from ACP sediments at the SRS (as mentioned earlier) were found to degrade a variety of aromatic compounds that frequently occur as environmental contaminants, including naphthalene, toluene, *p*-cresol, and xylenes (34). A preliminary phylogenetic analysis indicated that these isolates were strains of *Sphingomonas*, a genus that is differentiated from most other genera of bacteria by the presence of sphingolipids (35). Because aromatic-degrading *Sphingomonas* strains have been isolated from surface soils (36–39), it was of interest to see how similar the subsurface isolates are to these organisms. Surprisingly, an analysis of 16S rRNA sequences showed that the subsurface strains were not very closely related to the aromatic-degrading topsoil strains (40). In the resulting phylogenetic tree, the subsurface bacteria were assigned to a distinct and rather distant portion of the genus. They were most closely related to *Sphingomonas capsulata*, an organism that was isolated from a clinical source and that does not degrade aromatics. However, the subsurface bacteria did not actually cluster with any known *Sphingomonas* species, thus implying that they are novel species. A more detailed polyphasic taxonomic study involving analysis of cell lipids, determination of DNA-DNA reassociation values, and BOX-PCR DNA fingerprinting (40) confirmed that the five subsurface isolates examined in the study represented three new species: *Sphingomonas stygia*, *Sphingomonas aromaticivorans*, and *Sphingomonas subterranea*. The subsurface isolates, then, were phylogenetically rather distinct from the most physiologically similar topsoil isolates. More recently, it has also been shown that the genes for biphenyl and *m*-xylene degradation are located on plasmids in the subsurface strains, although the equivalent (and similar) genes are chromosomal in two topsoil *Sphingomonas* strains that are able to degrade aromatics (41). The subsurface strains, then, seem to differ in their evolutionary history from the otherwise similar topsoil isolates.

Arthrobacter Isolates from Saturated Sediments

Arthrobacter strains were one of the most frequently cultured bacterial forms in ACP sediments at the SRS and various types of deep saturated sediments at the Hanford Site (see in the preceding text). The phylogenetic and, in many cases, physiological characteristics of these microbes have been examined in detail in order to look for diversity among them and to determine how closely they are related to each other and to previously described species of *Arthrobacter* (27,30).

An analysis of 16S ribosomal RNA gene sequences for a group of *Arthrobacter* strains isolated from ACP sediments at the SRS produced the phylogenetic tree, as shown in Figure 1, in which the subsurface strains are separated into five phylogenetically distinct clusters (**A–E**). On the basis of the clustering and branching patterns of the established species of *Arthrobacter* that were included in the analysis for comparison, the subsurface clusters appear to represent distinct species and indicate definite species-level diversity among the subsurface strains. Additional diversity is also likely at the strain-level, as implied by the banding patterns seen during restriction endonuclease analysis (REA) of bacterial isolates from the SRS (as in the preceding text). None of the subsurface strains corresponded exactly to any of the previously described species of *Arthrobacter* included in the analysis, although two of them clustered with *Arthrobacter polychromogenes* and *Arthrobacter oxydans*, whereas two others clustered with *Arthrobacter atrocyaneus* (see

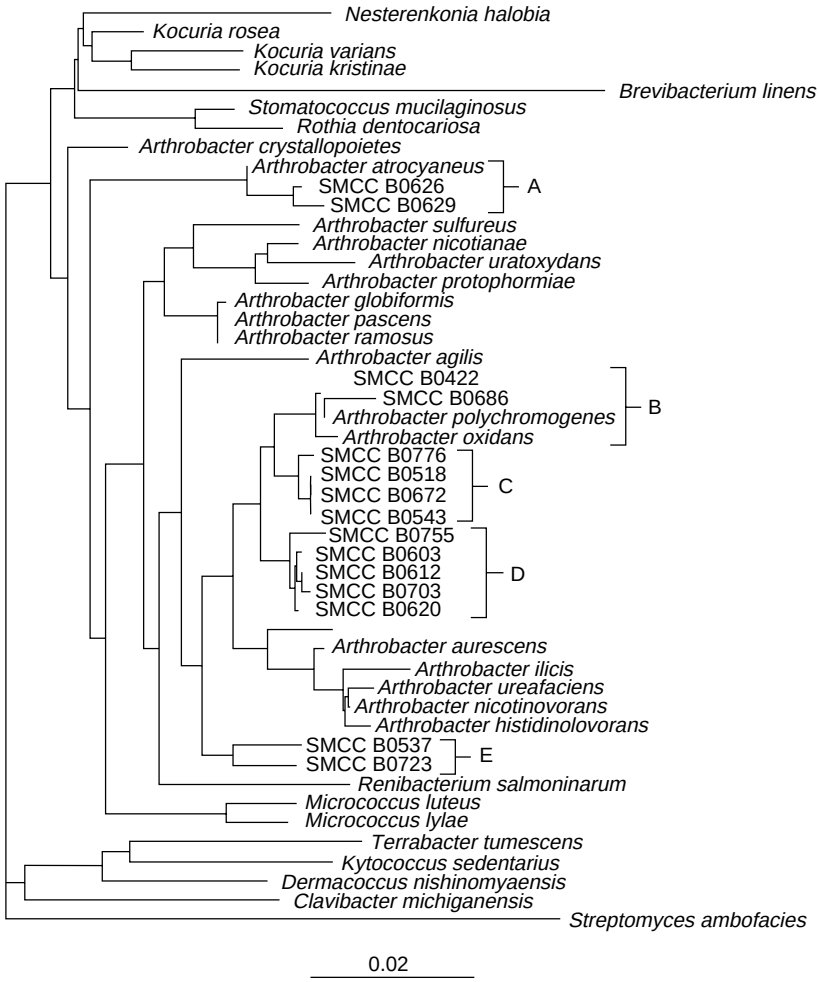

Figure 1. Phylogenetic tree produced by distance matrix analysis of 16S ribosomal RNA gene sequences for *Arthrobacter*-like isolates from the Savannah River site (designated by SMCC accession numbers), previously described species of *Arthrobacter*, and other high-G + C gram-positive bacteria that were included for comparison. *Streptomyces ambofaciens* was used as the outgroup. The subsurface strains fall into five distinct clusters (**a-e**), most of which are not closely associated with established *Arthrobacter* species. Scale bar represents 2 base substitutions per 100 bases.

Fig. 1). The other three clusters of subsurface strains were well separated from all of the established species of *Arthrobacter* (typically on independent branches of the phylogenetic tree), thereby indicating that they might contain novel species of this genus.

Similar results were obtained when 38 *Arthrobacter* isolates from deep saturated sediments at the Hanford Site were characterized (30). Phylogenetic analysis of their 16S rRNA gene sequences assigned these isolates to seven clusters that probably represented distinct species, thus indicating considerable species-level diversity among the subsurface strains. Five of the 38 subsurface isolates clustered with and, in some cases, were almost identical to established *Arthrobacter* species. The other 33 subsurface isolates, however, were well separated from all known *Arthrobacter* species in the phylogenetic tree and, as a result, may be novel species. As was the case with those from ACP sediments (as mentioned earlier), then, most of the *Arthrobacter* isolates from saturated sediments at the Hanford Site were phylogenetically distinct from species that are known to occur in topsoils or other surface environments. A detailed analysis of physiological traits (data not shown) indicated that the subsurface isolates and established species of *Arthrobacter* differed in some of their metabolic capabilities as well.

Acinetobacter Isolates from Atlantic Coastal Plain Sediments

Although some groups of subsurface bacteria have proven to be phylogenetically distinct from most similar (or physiologically equivalent) organisms that are found in surface environments, this is not the case for all subsurface isolates. Balkwill and coworkers (27) characterized 19 *Acinetobacter* isolates from ACP sediments at the SRS, by phylogenetic analysis of 16S ribosomal RNA gene sequences. In the resulting phylogenetic trees (not shown), the isolates were separated into four distinct clusters, implying approximately the same extent of species-level diversity that was detected earlier among subsurface *Arthrobacter* and *Sphingomonas* isolates. In this case, however, all but one of the subsurface isolates clustered with established *Acinetobacter* species, and most of them were nearly identical to those species in regard to their 16S rRNA sequences (sequence similarities >99.5%). The subsurface *Acinetobacter* isolates, then, did not differ noticeably from species and strains that have been isolated from surface environments.

Anaerobic and Thermophilic Metal-Reducing Isolates

Many of the bacteria cultured from terrestrial subsurface environments before the mid-1990s were aerobic or facultatively anaerobic mesophiles (mostly heterotrophs).

Strict anaerobes were detected or enumerated in most subsurface environments studied up to that point (using MPN assays and other methods), but there were few attempts to culture and isolate them. In recent years, though, anaerobic (both strict and facultative) and/or thermophilic forms have been cultured more frequently, as microbiologists have explored increasingly deeper and hotter environments. Several of the isolates obtained from such environments have been shown to reduce metals. This property is of interest to the U.S. Department of Energy and other agencies that are concerned about the fate of metal and radionuclide contaminants in the subsurface, especially in aquifers, in which the contaminants may migrate with the flow of groundwater. Microorganisms that can reduce metals could be significant in such environments, because many of the metals of interest (e.g., U, Tc, and Cr) are less soluble — and, therefore, less mobile — in their reduced forms. Selected examples of metal-reducing bacteria from deep subsurface environments are described later, to illustrate the diversity and metabolic characteristics of these potentially useful organisms.

Boone and coworkers (15) described a novel species of *Bacillus* — *Bacillus infernus* — that was isolated from a deep (2.7-km below land surface) soapstone within the Taylorsville Triassic Basin at a site in Virginia. *B. infernus* is a strict anaerobe (the only strict anaerobe in the genus *Bacillus* when it was first described) that can grow on formate or lactate with Fe(III), MnO_2, trimethylamine oxide, or nitrate (which is reduced to nitrite) as an electron acceptor. The organism also grows fermentatively on glucose. It is very slightly alkaliphilic (good growth at pH 7.8), halotolerant (growth up to 0.6 M Na^+), and thermophilic (optimum growth at 61.4°C). Geologic evidence suggests that microbes inhabited the Taylorsville Triassic Basin between 200 and 140 million years ago, when penetration of meteoric water into the basin was probably greatest. Since then, most of the groundwater flow has been preferentially funneled through the overlying permeable sediments. It is unlikely that any subsequent introduction of microbes has taken place because they would have to be transported through, approximately, 2.5 km of sedimentary rock with low porosity and permeability (14). There is a good chance then that *B. infernus* has survived in the deep subsurface for a very long time.

Kieft and coworkers (42) described a novel strain of *Thermus*, designated SA-01, that was isolated from groundwater in a South African gold mine. The groundwater was sampled from a horizontal borehole that was situated at a depth of 3.2 km and that penetrated 121 meters into the Witswatersrand Supergroup, a 2.9-billion-year-old formation composed of low-permeability shales and sandstones with minor volcanic units and conglomerates. The ambient temperature of the rock was approximately 60 °C. Strain SA-01 grows over a temperature range of 35 °C to 70 °C, and has an optimum temperature of 65 °C. It cannot grow fermentatively; an external electron acceptor is required for anaerobic growth. The organism can reduce soluble Fe(III), complexed with citrate or nitrilotriacetic acid (NTA). Only comparatively small quantities of hydrous ferric oxide are reduced unless the humic acid analog, 2,6-anthraquinone disulfonate, is added to the medium as an electron shuttle. Strain SA-01 is able to reduce Mn(IV), Co(III)-EDTA, Cr(VI), and U(VI) in the presence of lactate. It can also mineralize NTA to carbon dioxide and couple its oxidation to growth and the reduction of Fe(III). Strain SA-01 is the first *Thermus* isolate known to couple oxidation of organic compounds to the reduction of Fe, Mn, or S.

A novel strain of *Shewanella putrefaciens* (strain CN-32), with relatively versatile metal-reducing capabilities, has been isolated from Cretaceous period sandstone at a depth of 250 m in the Morrison formation of northwestern New Mexico (43,44). Strain CN-32 grows over a temperature range of 2.7 °C to 42 °C. It utilizes several organic acids and other simple organic compounds as sources of carbon. With lactate as the electron donor, it is able to reduce Fe(III), Co(III), Cr(IV), U(VI), and Tc(VII). Strain CN-32 can also reduce Fe(III) and Co(III) when complexed with chelating agents such as NTA or EDTA. This organism is of particular interest to researchers dealing with subsurface contamination at U.S. Department of Energy sites, where the movement of metals such as Cr(VI), U(VI), and especially Tc(VII) in the groundwater is a major concern. As noted earlier, microbially mediated reduction of these metals may limit their migration by reducing their solubility in the groundwater.

The aforementioned isolates and studies in which organisms were not necessarily isolated (45,46), may indicate that diverse populations of metal-reducing bacteria are widely distributed in the deep subsurface. For more information on the possible significance of these organisms on subsurface mineralogy and geochemistry, see Geochemical and Geological Significance of Subsurface Microbiology and Biomineralization by Bacteria, this Encyclopedia.

CONCLUSION

A wide variety of microorganisms (primarily eubacteria and archaea) have been cultured from terrestrial subsurface environments. These organisms are phylogenetically diverse, falling into dozens of different genera. Detailed studies on culturable microbial communities in selected subsurface environments indicate that most of the communities are quite diverse, although the numerically predominant forms often fall into a relatively small number of genera. Among the isolates that belong to a single genus, however, one usually sees a considerable amount of additional diversity at the species and strain levels. Given the diverse nature of many subsurface culturable communities, there is at least the potential for a wide range of microbially mediated chemical transformations (of organic and inorganic compounds) to take place in deep-earth environments.

Many of the bacteria cultured from the subsurface appear to be new species or, in some cases, novel genera, although some of them are phylogenetically indistinguishable from previously described species that were isolated from surface environments. Several subsurface

isolates that have been examined in detail have been shown to differ from the most closely related surface species in their physiological and/or genetic characteristics, most likely indicating that the subsurface organisms have a distinct evolutionary history. It seems likely, then that microorganisms cultured from deep subsurface environments represent a significant source of new genetic information. Some of these microbes also have potentially valuable metabolic capabilities, such as ability to degrade toxic organic compounds, or to immobilize metals and radionuclides in groundwater. Therefore, they may not only influence the fate of contaminants in the subsurface, but might also have applications in the field of bioremediation.

Although much information has been derived from the study of microorganisms cultured from subsurface environments to date, it is recognized that the cultured strains probably represent only a small fraction of the total communities in these environments. Direct molecular biological methods have detected a broad variety of as-yet uncultured microbes in many natural environments including several in the subsurface. Hopefully, information from direct molecular techniques will eventually facilitate the culturing of a larger proportion of subsurface microorganisms and, thereby, enable scientists to study their potentially novel or unique characteristics.

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SUBSURFACE MICROBIOLOGY.

See GEOCHEMICAL AND GEOLOGICAL SIGNIFICANCE OF SUBSURFACE MICROBIOLOGY

SUBSURFACE SAMPLES: COLLECTION AND PROCESSING

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Microbiological data, interpretation, and conclusions from subsurface samples ultimately depend on the quality

and representative character of the samples. Subsurface samples for environmental microbiology ideally contain only the microbial community and geochemical properties that are representative of the subsurface environment from which the sample was taken. To that end, sample contamination by exogenous microorganisms or chemical constituents must be eliminated or minimized, and sample analyses need to begin before changes in the microbial community or geochemical characteristics occur.

The objective of this article is to present sampling methods and sample processing techniques for collecting representative samples from a range of subsurface environments. Factors that should be considered when developing a subsurface sampling program are discussed, including potential benefits, costs, and limitations; enabling researchers to evaluate the techniques that are presented and match them to their project requirements. Methods and protocols to address coring, sampling, processing, and quality assessment issues are presented in this chapter.

EVALUATING RESEARCH OBJECTIVES AND SAMPLE NEEDS

Subsurface sampling programs can be relatively expensive undertakings, and thus require considerable forethought and planning (1,2). Planning an effective and cost-efficient field sampling program starts with an assessment of the research program objectives, and subsequent determination of the samples required to test the research hypotheses or to provide survey data on extant subsurface microbial communities. This assessment process begins with the most obvious and intuitive questions and often evolves into more complex issues, such as how to determine whether the samples represent the environment from which they were collected. The sampling technologies and techniques that are ultimately employed will depend on the outcome of this assessment process.

Investigators can begin the assessment process by simply determining if they need solid phase lithologic samples (with attached microbial communities), liquid phase samples (with unattached or detached microbial communities), or both. The samples that meet the immediate needs of the survey or research project will typically be obvious. Consideration should also be given to potential longer-term objectives and sample or data correlations that, at present, may not be readily apparent. Simply stated, it is desirable to systematically collect an appropriate number of samples and as many different types of samples as is economically feasible because it is often difficult, if not impossible, to return to the sample location and collect additional materials. Thus, every effort should be made to ensure that sufficient sample numbers and volumes are collected to meet both planned and some level of unanticipated research needs.

Investigators should also determine whether contamination of field samples by exogenous microorganisms or other "contaminants" is of concern. In cases where analytical results may be biased by the presence of exogenous microorganisms or nonnative materials, it will be necessary to select sampling technologies and processing protocols that limit exposure of the sample to contaminants. This is most certainly the case in the vast majority of subsurface microbiological research programs, particularly where microbial ecology or community structure are key elements of the hypotheses being tested (1,3–11). However, not all subsurface microbial investigations are highly sensitive to contamination, as is the case in which specific microorganisms are stained or otherwise "tagged" and injected into the subsurface as tracers (1,12). If contamination is a concern, then investigators will typically need to implement quality control methods by which the degree and nature of the contamination can be estimated. Fortunately, a growing body of sample processing and sophisticated subsurface microbiological data is beginning to make it possible to assess the representative character of sample results by comparison with earlier results. An example of this type of assessment is discussed later.

Other questions that should be addressed include the number and volume of samples required, and whether samples require specific preservation both upon initial collection and during shipment. If samples are anaerobic in situ and the project requires data on culturable anaerobic microorganisms, steps must be taken to prevent exposure to oxygen during sample collection, processing, and shipping.

ASSESSING THE SAMPLING TARGET

While evaluating research objectives and sampling needs, investigators must simultaneously begin to assess the sampling target and address the questions of where they want to collect samples and why. In order to fully assess the sampling target, investigators must be knowledgeable of the information available on the field site geology, hydrogeology, and geochemistry.

The specific depths of sampling points or intervals must be specified early in the assessment process, since sampling depth is a key consideration in selecting a sampling technology. Sampling target depths are most commonly based on the hypotheses examined in relation to the site geology and the stratigraphic heterogeneity that is typical of most geological systems (4,7,11,13–15). To effectively pick precise target sample depths, investigators must have some knowledge of the site stratigraphy on a scale that represents the intrinsic chemical and physical heterogeneities that will impact the distribution of autochthonous microorganisms (16–18). Descriptions of nearby outcrops or borehole geophysical and core data from nearby wells can be invaluable for understanding the subsurface geology and in selecting sampling points or intervals (10,11,14). In addition to understanding stratigraphic relationships and correlative sediment types, the degree of consolidation exhibited by the targeted lithologies will have a major influence on the selection of sampling technologies and the quality control protocols that are employed (1,2). For example, deep indurated lithologies require drilling technologies that employ circulation fluids, whereas shallow unconsolidated sediments can ordinarily be sampled without the use of circulation fluids. Formations that are highly fractured, or that are subject to voids such as are typical in volcanic flow deposits or limestone karst environments, may require high viscosity circulation fluids or "drilling muds."

Circulation fluids significantly enhance the likelihood of sample contamination by providing an environment that is conducive to bacterial growth and thus transporting microorganisms from the surface (mud tanks) or from shallow depths to the sample target depth. Sampling under conditions utilizing drilling fluids requires more robust quality control protocols to improve sample quality and to evaluate the degree of potential contamination (19).

The degree to which the sample interval is saturated with pore fluids is also critical, as is some knowledge of the pore fluid chemistry. For example, dissolved oxygen concentrations will influence the selection of sample collecting and processing protocols to be employed, particularly in cases where obligate anaerobic bacteria, methanogens, or sulfate/iron–reducing bacteria are of interest to the research program, or where oxidation might otherwise alter the sample or have a deleterious impact on the autochthonous microbial community. In such cases, anaerobic sample processing may be required. Other extreme in situ conditions, including naturally high or low pH, or extreme pressures or temperatures, may also require special sample handling and preservation techniques.

Selecting Types of Subsurface Samples

Subsurface media comprise a remarkably diverse range of physical, geochemical, and microbiological characteristics. For example, lithified quartz sandstone at 1-km depth is extremely hard, typically consists of nearly pure SiO_2, and possesses a high permeability and relatively low microbial biomass. In contrast, a clay-rich soil at 1-meter depth exhibits complex chemistry and mineralogy, including a high organic carbon concentration, orders of magnitude lower permeability, and orders of magnitude higher microbial biomass and activity. Yet, both types of materials are sampled as a part of research projects on subsurface environmental microbiology and obviously require very different sample collection and sample processing approaches.

However, all subsurface samples possess solid phases consisting of minerals or naturally occurring glass and have some degree of porosity (space not occupied by solids), containing either liquid (commonly groundwater or petroleum) or gas (most commonly air, CO_2, or natural gas) or both. Microorganisms occur in the pores, either attached to solid phases or unattached in a liquid phase. Research suggests that orders of magnitude more microbes are attached to solid surfaces than are free-floating in a liquid phase (20), although making this determination is clearly difficult. Effectively separating fluid phases contained in pores from solid phases without modifying the ratio of attached to free-floating cells is virtually impossible. Nonetheless, microbiological analyses are routinely performed on both solid and fluid samples. Subsurface gas samples ordinarily are not characterized microbiologically because of the limited ability of a gas phase alone to provide physical support or habitat for cells. Sampling and microbial characterization of gases are therefore not discussed in this chapter. The kinds of sample materials typically encountered are discussed in the following section.

Condensed Subsurface Fluids

Groundwater. Groundwater is obtained most frequently by sampling wells and includes a wide range of dissolved and suspended constituents that are important sources of nutrients for microbial populations and indicators of microbial metabolic function (21). Before entering the bore of the well during sampling, groundwater resides in pores or fractures, and thus is actually pore water. Groundwater sampling in a conventional manner by pumping wells thus represents a volume-averaged sample of water-rich fluids occupying pores in the subsurface. Because groundwater samples contain a small, variable content of particulate and colloidal materials in addition to unattached microbes, sterile filtration can be used to separate and concentrate these materials from groundwater. Such samples are readily analyzed by standard culture methods or lipid analysis or DNA extraction and cloning techniques (see section on Groundwater Sampling).

Pore Water. Pore water refers to water-rich fluid samples obtained by removing (most commonly by centrifugation) the fluid contents from a solid sample after it has been extracted from the subsurface. Because of drainage that occurs during extraction of the solid sample, pore water ordinarily comes from a smaller size fraction of pores in the subsurface than does groundwater. Pore water samples originate from the volume of the solid sample, a much smaller volume (100 to 1,000 mL) than represented by a conventional groundwater sample that may interrogate several to hundreds of cubic meters.

Petroleum Hydrocarbons. Liquid petroleum hydrocarbons occupy pore space (usually at depths from a few hundred meters to greater than several thousand meters) instead of groundwater. Studies on the microbial populations in such fluids are limited but have demonstrated that microbial activity plays a very significant role in the evolution and formation of petroleum reservoirs. Samples are typically extracted by pumping. The petroleum equivalents of pore water samples ("porepetroleum" samples) are relatively unusual for microbial studies but, in principle, could be obtained from deep core samples.

Subsurface Solids

Sediment. Unconsolidated or poorly consolidated clastic (granular) sedimentary deposits formed in a variety of marine and nonmarine environments are sampled extensively for microbial studies. This is partly because they occur at relatively shallow depths, and partly because such sediments are typically the materials contaminated by industrial activities, and hence are the target for application of bioremediation of a wide range of contaminants. It also reflects the observation noted above that the bulk of microbial populations exist in the subsurface attached to solid materials, in this case sediment particles. Grain size of the particles ranges widely, from gravel size or larger to silt and clay sized. Typical unconsolidated sediments are in the sand to silt size (1.0 to 0.01 mm) range. Unconsolidated volcanic materials (many of which are essentially sedimentary in nature) are included in this group of materials.

Lithified Materials. From the perspective of sampling and sample processing, all lithified rock materials require similar treatment and are thus discussed as a group. A wide range of rock types and properties are included: all lithified sedimentary rocks, most igneous rocks, and metamorphic rocks. Material properties, such as compressive strength, porosity, and permeability vary significantly and strongly influence details of processing. Indeed, there is a continuum between unconsolidated sediments described earlier and fully lithified sedimentary rocks. Core samples most amenable to effective processing without cross-contamination or contamination with drilling fluids are actually those that are moderately lithified and with moderate to low permeability. Such samples are less likely to be impacted by exogenous materials (including drilling fluids) and can readily be carved with sterile tools, enabling removal of exterior parts of the core without generating dust particles.

What Sample Types Will Meet Project Needs?. All of the sample types listed earlier have been used to "probe" subsurface microbiology, no doubt with varying, but commonly unevaluated, degrees of success. Groundwater samples are thought to underrepresent both total subsurface microbial biomass and subsurface diversity because of the much lower biomass in groundwater compared to subsurface sediments (20). Furthermore, conventional groundwater samples interrogate a much larger volume of the subsurface than the typical sediment sample, making groundwater samples less precise in terms of spatial resolution. This is not always negative. For example, groundwater analyses combined with filtration have been successfully used to reveal remarkable diversity and key relationships between uranium concentration and specific clones of sulfate-reducing bacteria present (22). Emerging techniques may help reduce the need for subsurface sediment samples (see later), but for now, groundwater samples typically would be used mainly for exploratory sampling and surveys. Detailed definition of subsurface microbial communities requires sediment or lithified samples that are most productively used in combination with groundwater biogeochemistry information.

SELECTING A SAMPLING TECHNOLOGY

Once investigators have identified the types of samples required and have targeted sampling depths and intervals based on geology, hydrogeology, and geochemistry of the site, it is appropriate to select a sampling technology. In selecting the most appropriate sampling technology, three key elements to consider are sampling depth, degree of induration in the target interval, and budget. Shallow, friable lithologies can be sampled by a number of technologies, but as sample target depths get deeper, the numbers of available technologies decrease, while at the same time the relative costs associated with these sampling technologies increase, often dramatically (1,2). Various technologies for sampling the shallowest to deepest subsurface environments are described in the following sections, and each example presented is amenable to at least a minimum level of quality control to enhance sample integrity.

Shallow Sampling Technologies

Shallow sampling technologies can be employed from the surface to depths on the order of 10^1 to 10^2 meters below the ground surface. Shallow sampling may be as simple as scraping the face of a rock outcrop or a shallow backhoe trench, or employing manual or hand-held drilling equipment such as augers. These auger systems can be effective to depths up to approximately five meters below ground surface, but their application is limited to unconsolidated soils and sediments (without gravel or cobbles), Hand-held auger samples can be collected from the saturated zone in some subsurface environments, provided the sampling horizon and overlying materials are sufficiently cohesive to prevent collapse of the borehole wall. However, pore fluids may drain from the sample as it is retrieved. Sample sizes vary, but are typically on the order of 1.2 to 7.6 cm diameter $\times$ 15 to 30 cm length. Many small-scale auger systems offer sample liners or inserts to contain the sample and limit its exposure to contaminants. These simple auger systems are inexpensive and are available through numerous commercial soil and groundwater sampling equipment suppliers.

The single biggest advantage to sampling shallow, unconsolidated subsurface environments is that there are a number of sampling technologies, which are highly effective and that do not employ circulation fluids that can contaminate the sample. These sampling technologies include a variety of relatively inexpensive "direct-push" and percussion-type samplers. Direct-push technologies include cone-penetrometer testing (CPT) equipment, Shelby tubes, and pitcher barrels. While somewhat more costly than Shelby tubes or pitcher barrels, CPT samplers have the distinct advantage of providing real time subsurface characterization data before sampling with a variety of direct-push probes including gamma radiation, resistivity, oxidation reduction potential, seismic (with an external source), fluorescence, pore pressure, and tip or sleeve stress. Many of these probes, including the resistivity and tip/sleeve stress probes, can give detailed and highly accurate depictions of subsurface stratigraphy and thus facilitate selection of sampling points. CPT technology also allows for collection of both sediment/soil samples and groundwater samples. Sediment or soil samples are typically up to 3.8 cm in diameter $\times$ 60 cm in length, and can be captured inside plastic liners to minimize exposure to contamination. CPT groundwater samples can be collected in discrete intervals through screened samplers. These samples are collected via a dual tubing system in which one tube purges the sample chamber with pressurized gas, forcing the sample to the surface through the second tube. By using inert gases to purge the sample chamber, this system can maintain low dissolved oxygen concentrations in groundwater samples.

Disadvantages of CPT sampling technology are that it is typically limited to depths of 20 to 40 m below ground surface in unconsolidated soils or sediments, though in ideal conditions it can potentially reach greater depths. It

is also relatively expensive, typically $1,500 to $2,500 per day, depending on the applications employed, and most systems are operated from inside a 50-ton truck that can potentially restrict access to sites with limited space or difficult terrain.

Shelby tubes and pitcher barrels are usually conveyed on drill rods through hollow-stem augers. The borehole is first advanced to the sampling depth by adding sections, or "flights," of augers with a retrievable insert sealing the inside of the bottom auger flight. After augering to sample depth, the insert is retrieved and the Shelby tube or pitcher barrel is conveyed down the inside of the hollow augers to the exposed sample point at the bottom of the borehole. There, the samplers are pushed into the sediment by continuous pressure on the drill-string. After the sample is retrieved and the insert reinstalled, the augers can be advanced to the next sample point. Advantages of the Shelby tube and pitcher barrel sampling systems include their larger sample size, which is typically on the order of 0.6 m in length and a minimum of 5.0 cm in diameter, and the fact that the auger flights keep the borehole open as the sample is freely retrieved. These systems are readily available through most commercial drilling companies, and are somewhat less expensive than CPT technologies, with costs in the range of $1,000 to $1,500 per day. Auger rigs can also reach depths of up to 100 m under optimum conditions. Small auger rigs can be mounted on the back of all-terrain vehicles, but most are truck-mounted.

Another readily available and relatively inexpensive sampling technology that is also used in conjunction with hollow-stem augers or cable-tool drilling rigs is the split-spoon sampler. Instead of direct pressure on the drill-string, split-spoon samplers are driven into the sample interval by blows from a rig-powered weight or "hammer" at the top of the drill-string. Once the sampler is retrieved at the surface, both ends are removed, and the two halves of the sampler are laid open to expose the sample, which may or may not be contained inside a liner. Split-spoon samplers are more robust than Shelby tubes or pitcher barrels, and because they are driven into the sample horizon by hammer blows, they are capable of collecting samples from more compacted or dense samples. Split-spoon sample sizes are typically 60-cm long $\times$ 7.6 cm in diameter, though thick-walled split-spoons of 10-cm diameter have been used to collect microbiological samples (23).

Piston samplers have been designed to collect several kilograms of samples from unconsolidated saturated flowing sands (24). These samplers comprise a hollow sample barrel with an inner piston. As the sampler is advanced into the sample interval at the bottom of the borehole, the piston slides up the sample barrel and creates a vacuum that holds the sample in place as the sampler is retrieved at the surface. Piston samplers can be used with larger auger rigs as well as rotary drill rigs, but work well only in sand-sized unconsolidated materials with little or no gravel or cobble-sized materials.

A Geoprobe rig is a combination direct-push and percussion-type sampling technology that simultaneously pushes and hydraulically hammers a sample probe into the sample zone. Geoprobes can be mounted on small trucks or all-terrain vehicles, and are capable of collecting sediment samples that range from 61 cm $\times$ 3.2 cm to 1.2 m $\times$ 3.8 cm. They also have the ability to drive water samplers or piezometers. Geoprobe rates are typically in the $1,000 to $1,500 per day range, so are relatively inexpensive. However, they are generally limited to a maximum depth range of approximately 35 m below ground surface, although some larger models may penetrate more than 50 m in unconsolidated media.

Vibration or "vibra-coring" technologies also offer highly effective methods by which larger volumes of sample can be collected in unconsolidated sediments. Portable vibra-coring equipment that can be transported by a two-person crew is capable of collecting 1.8-m long $\times$ 7.6 cm diameter cores in liners. The vibrating head for these systems is attached to the outer casing that is then vibrated into the sediment. The casing is then retrieved with a manual "come-along" attached to an overhead tripod, and the inner liner is removed and capped. Vibra-core systems are economical at less than $1,000 per day, but they are not as commonplace as many of the other technologies that have been described, and they are limited to depths of 3 to 10 m in most soils or sediments.

The most sophisticated drilling technology that doesn't require circulation fluids is sonic drilling. A resonant sonic drilling head with counter oscillating roller bearings that oscillate at high velocities powers a sonic drilling rig, instead of simply vibrating through soil or sediment. This motion creates centrifugal force that translates into high frequency waves that deliver forces in excess of 280,000 foot-pounds down the drill-string (25). Sonic rigs are effective in unconsolidated sediments, semiconsolidated sediments, consolidated but friable sediments, course gravels, and even semi-indurated rock. Under optimum conditions, rotosonic drilling can advance through subsurface lithologies at rates up to several feet per second. This technology is capable of collecting core samples up to 3-m long and 8.9 to 20 cm in diameter, including 8.9-cm diameter cores in plastic liners. It can also be used to collect samples from angled boreholes. Sample quality is high, and sonic rigs have been used frequently in subsurface microbiological sampling programs (11). They are, however, relatively costly at rates of $3,500 to $4,000 per day, and they are typically limited to depths between 120 and 150 m, though a depth of 213 m was reached under optimum conditions (25). The dual-wall drill-string system allows downhole equipment to be conveyed where needed, such as packer systems that can isolate discrete water bearing intervals for sample collection. The vibration associated with sonic and vibratory drilling rigs has an as-yet unquantified impact on microbial communities. Possible effects include stimulation and redistribution of microbial communities; effects that are not thought to be significant, provided analyses are performed in a timely manner (see Section on Processing of Samples).

Shallow sampling, on the order of tens of meters, can be conducted with horizontal drilling technology, as well as in cases where restricted access might dictate horizontal or angled drilling. Samples are usually small, on the order of 5 $\times$ 30 cm, but these technologies require circulation fluids for their operation. Although not common, they are

nonetheless available from commercial horizontal drilling firms, at costs in the range of $1,000 per day or less.

Deep Sampling Technologies

Collecting samples from depths of several hundreds to thousands of meters requires that fluid media be circulated through the drill-string, and between the drill-string/borehole wall annulus (26). These circulating fluids serve different purposes, including cooling and lubricating the drilling assembly at the bottom of the hole, and preventing formation fluids under lithostatic pressure from flowing into the borehole. Their primary function, however, is to circulate rock fragments and dust, commonly referred to as "cuttings," from the bottom of the hole to the surface, thus keeping the borehole open. Circulation fluids vary in viscosity and include air, foam, water, and bentonite-based drilling "mud." The choice of which drilling fluid to use depends on a number of factors, including borehole wall stability, borehole depth, potential for formation fluid flow, and degree of induration. In highly indurated lithologies that maintain a stable borehole wall, low viscosity media, such as air or foam can be used, although higher viscosity fluids may be preferable for cooling the drill bit. As formations become more friable or exhibit a tendency to expand into the borehole, it may become necessary to use higher viscosity and higher density fluids or drilling "muds," which have a higher hydrostatic pressure in the borehole, and that build a dense "mud-cake" on the borehole wall. Boreholes of several hundreds to thousands of meters depth always require higher viscosity circulation fluids, since air loses both velocity and "carrying capacity" as it travels greater distances up the borehole annulus. Unfortunately for microbiological sampling, this fluid can serve as a source and incubator for microorganisms both from the surface and from shallower formations, particularly when fluid additives provide nutrient and energy sources. Similarly, fluids can alter geochemistry by adding, reacting with, or transporting contaminants throughout the borehole volume. Circulation fluids can also contaminate the sample zone at the bottom of the hole ahead of the sampling device, as well as during sample retrieval to the surface (1,2).

Fundamental research programs have employed more exotic and costly inert drilling fluids in order to minimize potential contamination by suspended microorganisms. Researchers at the Idaho National Engineering and Environmental Laboratory in Idaho Falls, ID used an air rotary rig to advance a borehole through interbedded basalt flows and sediment facies (27). Approximately three meters above each sample target, the circulating air was changed to 99.9% pure vaporized liquid Ar, to prevent the core sample from coming in contact with suspended allochthonous aerobic microorganisms. A research program operated by Pacific Northwest National Laboratory (Richland, Washington) attempted a similar approach at a research site in New Mexico, with liquid nitrogen flushed through an expansion unit before being circulated downhole. In the vast majority of situations, properly employed QA protocols and carefully selected sampling technologies, that are well suited to the subsurface geology are as effective, if not more effective, than these approaches at minimizing sample exposure to contamination and maximizing sample quality.

Sampling tools vary considerably for deeper sampling horizons. Both the programs described earlier employed a highly versatile sampling tool known as a core-punch, or "C-P" wire line coring assembly. Most sample core barrels are conveyed to the bottom of the borehole on the end of the drill-string, thus requiring the time-consuming and costly tasks of removing or "tripping" the drill-string out of the borehole, and then tripping back in, each time a core is collected. In contrast, a wire line core assembly can be quickly conveyed down the hollow center of the drillpipe on a wire line, and is locked in place inside the bottom-hole assembly. In soft friable sediments, the bottom "shoe" of the wire line coring assembly can extend beyond the drill bit into the sample zone, to minimize contact between the sample and the drilling fluids circulating through the drill bit. After the core has been collected, an "overlatch" mechanism is sent down the inside of the drill-string, on a wire line, that releases the locking mechanism and retrieves the sample. C-P core samples are 8.4 cm in diameter and 1.5-m long, and they are contained inside either stainless steel split core barrels or clear polycarbonate liners. Samples can be collected from depths of several hundred meters, and costs are typically in the range of $1,500 to $2,500 per day for the rig, equipment, and crew. Costs increase dramatically with multitruck operations. These sampling systems are provided by commercial drilling companies, and can be employed in conjunction with most circulation media. Wire line coring systems have been employed in a number of microbiological research programs, where rigorous quality control protocols were employed (3,4,7,27,28, and Fig. 1).

Deep sampling at hundreds to thousands of meters typically requires extremely large and costly drilling rigs (29), unless access to the formation is available via tunnels or mine adits. Investigators at the Nevada Test Site collected samples from tunnel faces that had been exposed by tunneling equipment, at depths from 50 to 450 meters (5,15,30,31). A comparison of samples collected from freshly exposed consecutive tunnel faces, and 4.7 cm $\times$ 75 cm cores drilled into each of the tunnel faces indicated, that contamination levels in the cores were higher due to the circulation fluid. Yet, the abundance, diversity, distribution, and distinct colony types were similar (5). However, opportunities to collect fresh sediment or rock samples from great depths by means other than drilling are relatively rare.

Drilling rigs that can reach depths of thousands of meters are of a scale in size that is typically found in the mineral exploration industry, with costs that can reach tens of thousands of dollars per day. As a result, sampling is generally done in partnership with mineral exploration ventures. Coring tools conveyed on the end of the drill-string can collect very large core samples on the scale of 15 cm $\times$ 10 m, and can core in highly consolidated or indurated rock. Many petroleum service companies offer coring technologies that can protect the core from fluid loss and exposure to drilling fluids by sealing the core in flexible membranes or gel coatings, and they can also maintain

Figure 1. A typical truck mounted, rotary wireline coring rig operating at the Shiprock, NM uranium mill tailings site. At the time of the photograph, the rig was being used for installation and operation of straddle packers for conducting stress tests, for estimating in situ permeability of Mancos Shale, underlying the permeable terrace gravels. Samples for microbiology were obtained from both alluvial gravel deposits and Mancos Shale. See color insert.

cores at the same lithostatic pressure, as that, from which they were collected. Such systems are designed to preserve petroleum contents of oil or gas reservoir rocks, and have been only rarely applied to microbiological sampling.

In addition to drill-string conveyed coring tools, petroleum service companies offer wire line conveyed coring tools that collect multiple small samples in the range of 6×2 cm from the borehole walls. These coring tools include rotary sidewall coring tools that are comprised of small-scale, hollow, rotary coring drill bits, which rotate 90 degrees from the suspended coring tool and core into the borehole wall. The bit and sample are then backed away from the borehole wall, and the bit rotates down into the suspended coring tool and the core is extruded into a holding chamber. Percussion sidewall cores are fired into more friable sample targets by a small explosive charge. The core "bullet" is attached to the core "gun" by a cable, and the sample is extracted as the gun is retrieved up the borehole. Unfortunately, samples collected by these methods can be highly contaminated, due to the fact that they are not protected from exposure to the circulation fluids. This is particularly true of percussion sidewall cores, which can fracture samples on impact. Nonetheless, these technologies were used successfully to collect microbiological samples from 2,630 m to 2,801 m below ground surface, in the Taylorsville Triassic Basin of Virginia (10,14,19).

Groundwater Sampling

Groundwater samples obtained from existing wells provide a relatively inexpensive means of interrogating the subsurface microbial community. The principal limitation of groundwater microbiology is that free-living microorganisms are primarily sampled, plus an unknown number of attached microbes are detached during groundwater extraction. Methods for extracting groundwater samples include bailers, suction systems, or pumps. Examples of pumps include peristaltic, positive displacement, bladder, inertial, or submersible pumps. Depth to the water table, flow rate desired, volumes required, and tests to be performed are major determinants of the water extraction system employed. Monitoring well construction involves placement of a screened zone at a depth that permits water to flow into the well, and at the same time prevents collapse of the borehole. Well construction also typically includes a sand pack to ensure that the well screen does not clog with fines. A sand pack is, sometimes, not used to eliminate the addition of foreign material to the subsurface. Screened zones vary in length from a fraction of a meter to several tens of meters or more. Conventional practice for monitoring wells is to minimize screen thickness (1 to 3 m) and place screens at multiple depths, if it is necessary to detect differences in target analytes as a function of depth. Because of the cost and low spatial resolution of this approach, a variety of systems have been developed for obtaining groundwater samples from specific depths within a well, including tubing-conveyed sampling probes and screens, dual or multiple packer systems, active multilevel sampler systems (e.g., Westbay), passive multilevel samplers (32,33), and inflatable sleeves with attached sampling ports. CPT technologies also permit groundwater sampling of discrete intervals during insertion of the penetrometer (see section on shallow sampling technologies).

Tradeoffs exist among these various approaches between high spatial resolution and available sample volume. For example, passive multilevel samplers (Fig. 2) have a maximum vertical spatial resolution of approximately 6 cm, but a sample volume of approximately 40 ml. At the other end of the spectrum, a 2-m screen zone in a permeable formation could produce a very large sample of 2,000 liters in a few to several hours of pumping, but the associated spatial resolution is on the order of several meters. Given the typical subsurface porosity of 15 to 30%, there is no way around this trade-off. It is tempting to assume that longer pumping times from closely spaced, down-well sampling ports will provide a large volume while maintaining a high spatial resolution. However, this is simply not the case because, as pumping proceeds, a larger and larger volume of the subsurface is sampled, both in a vertical and horizontal direction. Even in the case of the passive multilevel sampler, the actual horizontal resolution may be very large because the sampler integrates groundwater flow over a period of days to

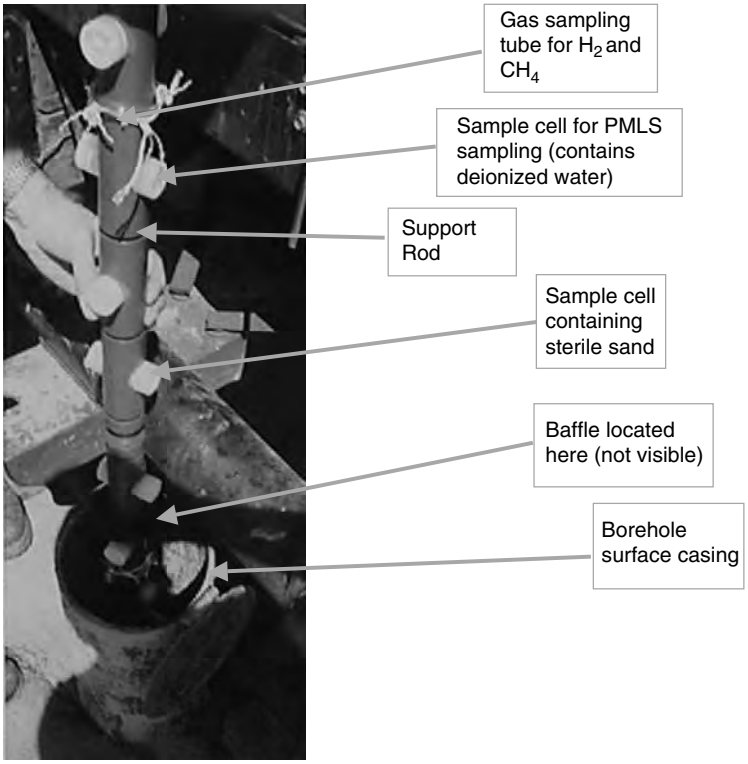

Figure 2. Photograph of the PMLS being inserted into a borehole. See color insert.

weeks. Regardless of the method used, care must also be taken to ensure that vertical flow or cross-connections are not smearing the microbial or geochemical signal in the groundwater sample.

The environmental conditions within and adjacent to a well may differ dramatically, from those in aquifers and subsurface formations. Consequently, it is imperative to purge water in and adjacent to the well bore before collecting samples for microbiological or geochemical analyses. Geochemical and microbiological properties of water associated with the well, reflect impacts from the well casing and installation materials, as well as altered permeabilities from installation procedures. Biofilms accumulate on well casings, well screens, and proximal subsurface materials. Biofilms and associated biogeochemical precipitates will detach with pumping, particularly at elevated pumping velocities. Ideally, three to seven well volumes should be purged before sampling. Monitoring of the pH, Eh, and turbidity of pumped waters can be utilized to determine when native formation water is discharged, as all monitored parameters become stabilized (34). Stabilization of the monitored parameters often occurs within three purge volumes, thereby eliminating excessive purging. Purging at too high of a velocity can compromise water quality by increasing turbidity of the water, altering concentrations of dissolved constituents, dewatering of pores, or drawing water from lower permeability areas of the screened zone (34). In addition, overpumping of relatively low permeability wells can result in lowering the water level in the well bore, below the water table in the surrounding formation. This causes cascading in the well, which among other effects noted earlier, will increase the dissolved oxygen in the sample. Accordingly, overpumping can alter both microbiological and chemical constituents of groundwaters.

Mined openings are an underutilized source of groundwater for environmental microbiological research. Pedersen has extensively studied groundwater from mined openings used for examining the feasibility of disposal of high level radioactive waste (35,36). Similarly, gold mines in South Africa have provided groundwater for microbiological studies (37). However, there are a very large number of mines worldwide where groundwaters have not been sampled for microbiological characterization. Care is required when sampling in such environments to avoid the effects of introduced oxygen such as acid mine drainage (unless, that is the objective of the study). Sampling flows that are isolated and have a sufficient flow rate to not be impacted by back-diffusion of oxygen are required to obtain samples that are representative of undisturbed (pre-mining) conditions (36).

When collecting groundwater samples for biogeochemical analyses, it is particularly important to use inert materials that can be autoclaved. If items cannot be autoclaved, disinfection is an option. For example, maintaining chlorine residuals for several hours, before and after use, provides effective disinfection. Collection of groundwater samples can be relatively straightforward. The simplest and most widely used approach, flushes and fills clean sterile glass bottles from tubing exiting the groundwater recovery system. Overflowing the bottles with continued flushing helps eliminate air in the bottle headspace. Flushing three volumes of water (after well purging) and removing air bubbles reduces oxidation of redox-sensitive constituents, and facilitates recovery of strict anaerobic

microorganisms. When using peristaltic, submersible, or suction pumps, care should be taken to eliminate air exposure resulting from pump cavitation or overpumping. These procedures are applicable for recovering bacteria, protozoa, or viruses (38).

Microbial concentrations in groundwater are typically between 10^3 and 10^5 cells per ml (20,39), so it may be desirable to concentrate the biomass for many analyses. Stevens and coworkers (39) used a sand-packed cartridge connected in-line to the water delivery system when selecting for bacteria that could attach to sand. Pedersen and Ekendahl (35) used cleansed slides in biofilm reactors to study microbial communities in deep bedrock. Kuwabara and Harvey (40) used hollow-fiber tangential-flow filtration to concentrate bacteria from groundwater for microscopic enumeration and lipid analyses. Fry and coworkers (41) also used hollow-fiber filtration to concentrate groundwater bacteria for nucleic acid probe and sequence analyses. Bacterial recovery efficiencies of greater than 90% can be achieved with hollow-fiber filtration, with somewhat less collection efficiency of smaller microorganisms. Importantly, tangential filters require effective disinfection and cleansing, before and after each use, to ensure bacteria are not entrained within the filter. Many of the available tangential filter systems can withstand repeated autoclaving while maintaining integrity of the filters even though the manufacturer may not recommend autoclaving. Filtration, using one to two liters of groundwater through Anodisc glass fiber filters, has also been successfully used for lipid and DNA analysis (22). One factor to consider in filtration is the sediment load in the groundwater. This is sometimes difficult to quantify, but is critical because sediment particles contribute to the total bacterial content of the sample. Samples that have apparently higher biomass may also contain higher sediment loading. Monitoring wells and other groundwater sources commonly vary a great deal in terms of the sediments they produce, depending on well construction, formation sediment or rock characteristics, and pumping rates.

QUALITY ASSURANCE

Sources of microbial and chemical contaminants that may compromise and adversely impact the quality of recovered subsurface materials include drilling fluids, groundwater-sediment slurry in the borehole, slough from overlying formations, and borehole sidewall materials. Contamination may occur before, during, and after coring and sampling operations. The most noticeable and common form of contamination associated with sediment or rock sampling is the intrusion of drilling fluids. Large numbers of bacteria and solute contaminants alter sediment and groundwater chemistry, as well as observed biogeochemical processes. Drilling fluid contamination also impacts sediment biogeochemical properties by altering pH, redox, and nutrient concentrations. Oxidation of sediments while drilling with air or gaseous drilling fluids is particularly problematic. The incorporation of multiple conservative tracers to mimic impacts of contaminants enables one to measure and quantify the extent of potential contamination, and then to evaluate sample integrity and quality. The discussion below focuses on tracers used to evaluate microbiological or geochemical contamination of cored subsurface materials.

Microbiological Tracers

(See TRACERS IN GROUNDWATER: USE OF MICROORGANISMS AND MICROSPHERES, this Encyclopedia)

"Serendipitous" bacterial tracers, representing microorganisms present and/or growing in drilling fluids can be present in high concentrations. Serendipitous histogical tracers observed in previrous projects includes *Gluconobacter* (42) or coliforms (43). Fluorescent microspheres are routinely used as bacterial surrogates. Microspheres are available in a range of bacterial-sized latex beads containing different fluors and charges. Microspheres are readily quantified using epiflorescence microscopy or flow cytometry. Some sediment/rock samples do contain materials that fluoresce at the same excitation wavelength as charged carboxylated latex 1-μm. YG microspheres are routinely employed as surrogate bacterial tracers during coring operations (28,44,45). Microspheres are typically deployed at/or near the interface between the coring tool and the formation by placing several ml of a concentrated solution containing $\sim 10^{10}$ microspheres in a Whirlpak bag, sometimes in concert with chemical tracers. The wire closures of the Whirlpak bag may then be wrapped around the base of a core catcher in the shoe of a coring tool. As cored materials enter the shoe of the coring tool, the bag ruptures, releasing the microspheres to be dispersed in and around the core. Intrusion of the microspheres into centermost portions of the core indicates a potential for coparticulates. Another approach for introducing biological and dissolved tracers is to add them to an aqueous solution that is placed inside the core-barrel liner.

Other approaches to estimate the level and extent of contamination include comparative biological analyses of core samples and drilling fluids. These analyses can include membrane lipid profiles, molecular-based probes for amplifiable extracted nucleic acid analyses, 16S rRNA sequencing (46), comparisons of clone libraries, and community level physiological profiles (19). Such community level measures can detect gross contamination, but may be ineffective for detecting low-level microbial or particulate contamination. Analyses of individual microbial isolates can also contribute to assessing sample integrity on a qualitative basis by comparing biochemical profiles, lipid profiles, or genetic fingerprinting between sample and drilling fluid isolates. Comparative approaches require caution because the microbiota of the contaminant source may be indigenous to the sampled subsurface (i.e., enriched in the drilling fluids during site operations).

Table 1 compares results gleaned by analyzing subsurface sediments independent of any tracer analyses. Accordingly, results may be compared to historical data sets to see if results derived from recent materials are representative of previous efforts. In Table 1, results from analyzing sediments obtained from 3–18 m depth of a semiarid U.S. site (designated site Z) are compared to

Table 1. Use of Historical Data as an Independent Indicator of Potential Issues Regarding Recovered Subsurface Materials*

Site Name and Description	Number of Sample Examined	Average Biomass (pmol/g)	Biomass Range (pmol/g)	Average Number of PLFA Quantified	Percent Samples with Branched PLFA	Percent with 2 to 10% Polyenoic PLFA	Percent with 10 to 50% Polyenoic PLFA	Percent with >50% Polyenoic PLFA
Oak Ridge National Laboratory shale sediments 3–30 m deep	6	7.8	3–16	16	100	17	17	0
Taylorsville Triassic Basin, 3 km deep limestones/shales	4	13	7–19	25	100	25	0	0
Hanford sediments 60 m deep heterogeneity study	19	12	6–34	21	100	52	32	0
Hanford Yakima barricade sediments 50–100 m depth	4	15	3–47	31	100	75	0	0
Hanford sediments 80–100 m depth	6	17	4–31	10	100	50	33	0
Semiarid site Z sediments, 3–18 m depths	30	48	3–283	14	47	10	30	57
Site Z samples of 3–30 pmol/g biomass with branched phospholipid fatty acids (PLFA) (bacterial)	6	19	7–32	17	100	0	83	0
Site Z samples of low biomass (3–30 pmol/g) without branched PLFA (eukaryote dominated)	16	12	3–27	6	0	0	19	81

*Summary of data from several sites examined over the past decade. The Hanford sediments were similar to site Z in that they are from semiarid low-biomass sites. All samples compared were from three m or greater depth, to insure they were below the soil root zone and contained three or more pmol of phospholipid fatty acid (PLFA)/g of sediment, to insure that the PLFA biomass was at least threefold above detection limits. Each profile used in this comparison also contained five or more individual PLFA, so as to indicate a minimal level of diversity characteristic of membrane lipids of microbial communities.

other analyses from the past decade. The three comparative data sets obtained from the U.S. Department of Energy lands near Hanford, Washington, are also from a semiarid climate. The Oak Ridge National Laboratory site in eastern Tennessee and the Taylorsville Triassic Basin site in Virginia represent other data sets exhibiting relatively low phospholipid biomass.

Samples within the compared data sets typically exhibited less than 50 pmol phospholipid fatty acids (PLFA)/g, which is indicative of communities corresponding to less than 10^6 microorganisms per gram of sediment. For comparative purposes only, those samples beneath the soil root zone (deeper than three m below land surface), and those samples containing three or more pmol PLFA/g (threefold greater than detection limits) were scrutinized. Interestingly, each of the selected samples exhibited five or more PLFA's, indicating diversity characteristic of microbial communities. The average PLFA concentration within a grouping ranged from 8–48 pmol PLFA/g. The average number of individual PLFA's quantified from each site ranged from 10-31, with site Z appearing to fit well within expected ranges.

Upon closer scrutiny the PLFA from Site Z appeared distinct for the other data sets, in that, less than half of the samples revealed branched fatty acids, typical components of many bacterial members Half of the samples from Site Z contained more than 50 mol% of polyenoic fatty acids typical of eukaryotes, such as fungi and the dominance of polyenoic fatty acids appeared in samples with lower levels of PLFA indicative of lower biomass in those samples. Two subsets of lower biomass samples (less than 30 pmol PLFA/g) from site Z samples were further scrutinized based on the presence of absence of branched fatty acids. Branched fatty acids are not required in many bacteria, but evidence of branched PLFA is typically observed in bacterial communities. In contrast, polyenoic membrane components are indicative of eukaryotes with the polyunsaturated 18-carbon fatty acid, 18 : 2w6 being typical of fungi. The subset of Site Z samples that contained branched PLFA appears somewhat typical of subsurface samples, in that, samples containing >10 pmol PLFA/g typically exhibit more than 10 PLFA's including branched PLFA's. Somewhat atypical was the observance that 83% of the samples exhibited significant percentages (>10%) of polyunsaturated PLFA. While many samples from a myriad of sites revealed polyenoic PLFA's greater than 10 mol%, and several samples exhibit insignificant polyenoics of less than 2 mol%.

The site Z subset containing detectable (3 or more pmol PLFA/g of sediment) but low biomass less than 30 pmol PLFA/g that did not show evidence of branched fatty acids consists of 12 samples with an average biomass typical of the other sets presented in in Table 1. It is obvious that the number of PLFA represented in this subset is unusually low, and all of the samples within that subset exhibited between five and seven PLFA's, indicating very little diversity. More importantly, each of the samples exhibited high levels of polyunsaturated PLFA's. Unlike any sediment samples previously analyzed, 13 of the 16 sample subset exhibited >50 mol% of polyenoic PLFA's. The remainder of the PLFA's included straight-chain saturated PLFA's and the 18 : 1w7cis fatty acid, which is a precursor to a common fungal lipid fatty acid, 18 : 2w6. Interestingly, the observed PLFA patterns are typical of fungi and other eukaryotes, but are atypical of subsurface sediments from other sites.

While one should not conclude that there was overgrowth of fungi in the samples recovered from Site Z, one should evaluate the PLFA and other sources of available information within the context of sample storage times/conditions, previous experience, and other lines of evidence from the investigation. Based on the evidence in Table 1, one should conclude that samples from Site Z require detailed scrutiny and comprehensive examination before they are considered representative of the subsurface environment from which they were derived. While it is possible that Site Z represents a unique and novel subsurface community, it may also be hypothesized that post-sampling activities adversely impacted sample quality, enabling atypical microorganisms to proliferate over time. By examining tracer information, drilling and sampling procedures, postsampling processing and storage activities, procedural QA/QC and blank tests, sediment and groundwater geochemistry, and handling and shipping processes, investigators can develop multiple lines of evidence providing explanations of observed phenomena; be it representative of native subsurface materials or resulting from postcoring alterations.

Geochemical Tracers

Chemical tracers should be conservative, nonreactive, readily quantified, inexpensive, and easily distinguished from native materials. Rhodamine and fluorescein dyes (2,47) have often been used to trace drilling fluids because their fluorescence is easily detected at low concentrations in the field. Fluorescent dyes do tend to adsorb to bentonite drilling muds, and are typically sensitive to oxidizing agents and pH. Bromide is routinely used as a nonreactive solute tracer because it is normally present at low concentration in groundwater, has low toxicity, and can be measured in the field using an ion-specific electrode. Potassium has also been used as an ionic tracer, but potassium sorbs to clays in the drilling fluids, and can be present at high background concentrations in some groundwaters. Sulfate can be used as an anionic tracer, but sulfate is biologically reactive under anaerobic conditions (i.e., sulfate reduction), and is present naturally and as a contaminant at many sites. Inert perfluorocarbon compounds have recently gained favor as inert chemical tracers; added either as gaseous or aqueous tracers. Despite their limited solubility in aqueous solutions, perfluorocarbons can be detected at extremely low concentrations by gas chromatography with electron-capture detection (48,49). Different perfluorocarbons such as perfluorohexane and perfluoromethylcyclohexane can be used at different intervals in the same borehole as a check on the potential for contamination from the previous core run (48). Perfluorocarbons are quite volatile so precautions should be taken whenever samples are processed in an enclosed space, such as a glove bag to prevent volatilization from, and cross-contamination of, samples. Solute tracers such as bromide can be used to trace the

intrusion of borehole slurry into cores. Bromide or other solute tracers as well as microbiological or inorganic particulate tracers can be prepared as a concentrated solution and added to the borehole using a bailer, placed into whirlpak bags for dispersal at the bit-sediment interface, or added to the drilling fluids. For example, ionic salt solutions or perfluorcarbons may be added directly to the mud tank during making of the drilling muds. In the case of ionic salts, a concentration of 100 to 500 mg per liter may be maintained in the circulating drilling muds throughout the drilling operations. In the case of perfluorcarbons, a standing concentration of a mg per liter may be sufficient for detection at a dilution of a millionfold. Alternatively, perfluorcarbons can be metered directly into drilling fluids via an HPLC pump minutes before coring, so that the tracer-containing fluids are uniform throughout the corehole before sampling. Metered and staged tracer additions are particularly useful in the subseafloor coring, where drilling fluids are for single use and not recirculated, and where constant addition over weeks of operations would be cost prohibitive.

State, local, and site regulatory agencies must be consulted before the introduction of microbiological or chemical tracers to subsurface environments. However, the advantages of a thorough tracer regimen cannot be overstated. Even when sampling the vadose zone, contamination from particulates may fall into the borehole from overlying sediments, from surface soil, or from corehole sidewall slough. Contaminating particulates may also enter with the drilling tools. A dry tracer mix consisting of glass beads (used as an inert carrier), fluorescent microspheres as a microbial surrogate, and tungsten carbide particles as a chemical tracer was developed for use during cable-tool coring (50). This mixture was placed at the bottom of the borehole using a bailer before each sampling event. Rotary drilling methods employing recirculating aqueous drilling fluids exhibit the greatest potential for introducing contaminants into cores as compared to hollow-stem augering or/and cable-tool coring methods. Due to the thousands of liters of drilling fluids required for rotary drilling, it is not economical to mix fluorescent microspheres directly into drilling fluids. In contrast, chemical tracers can be introduced directly into the fluid. Because of regular losses of drill muds over time, and dilution with groundwater, it is necessary to monitor and periodically adjust the concentration of tracers in the muds.

SAMPLE PROCESSING

Processing Overview

Regardless of care taken in sample acquisition, inappropriate sample processing or shipping from the point of collection to the point of analysis will readily compromise analytical results and thus resulting interpretations. In this section, we discuss techniques used for processing samples that will increase the probability that samples will retain their representative character (see introduction). The need for performing analyses on representative samples imposes rigorous constraints that are difficult both to meet and to assess. Nonetheless, at minimum, it is appropriate to match processing protocols with project objectives to maximize the likelihood that data from analyzed samples, appropriately and consistently represent subsurface microbiology and geochemistry.

It is neither desirable nor possible to provide prescriptive protocols to cover even a limited range of sample processing. Rather, the objective of this section is to present the sampling processing techniques that have been employed, so project managers and researchers can quickly match available techniques to their requirements while gaining an understanding of potential benefits, costs, and limitations. It is our experience that there is considerable room for improvement in sample processing techniques. Unfortunately, sample processing, commonly, is given limited consideration and resources. Areas for future technique development and evaluation are therefore identified.

Sample processing takes samples as they are obtained from the sampling device (e.g., core barrel or groundwater pump line), and converts them into samples provided to specific researchers or analytical laboratories for analysis or experimentation. In some cases, this is a very simple process such as placing a groundwater sample into a container meeting particular requirements. In other cases, this is a complex process in which a core sample is systematically dismembered in a glove bag under an inert atmosphere such as Ar or N_2 gas (Fig. 3). In the case of complex processing, specific objectives for sample preservation should be defined and traded off against each other, and against cost to arrive at a specific sequence of events that best meets sampling objectives. There are few, if any, absolutes in establishing a workable protocol. For example, if assessing the anaerobic microbial community is an important objective, then processing samples under anaerobic conditions (usually an inert atmosphere) is a requirement. The questions are, how anaerobic and how inert? It is well established that Ar or N_2 atmospheres in glove bags are not totally oxygen free, suggesting the need for a H_2-CO (or similar redox couple) atmosphere to maintain a strictly anaerobic atmosphere. However, H_2 is an electron donor for hydrogen utilizers, including fungi, and this creates the potential for undesirable changes in community structure as a result of exposure to H_2. Typically, the trade-off is to accept some loss of the anaerobic community due to O_2 toxicity, in order to avoid the potential impacts of H_2 exposure. However, each project needs to assess these types of trade-offs. In some projects, it may be desirable to include a separate glove bag in which strict anaerobic conditions are maintained for processing a subset of the samples (Fig. 3).

Sample Processing and Shipping Sequence

The sequence of sampling processing and shipping typically includes the following steps:

- Transport of samples from sampling point to processing facility
- In-field processing steps and requirements (subsampling, preservation requirements, and methods)
- Sample Shipping

Each of these steps is discussed next.

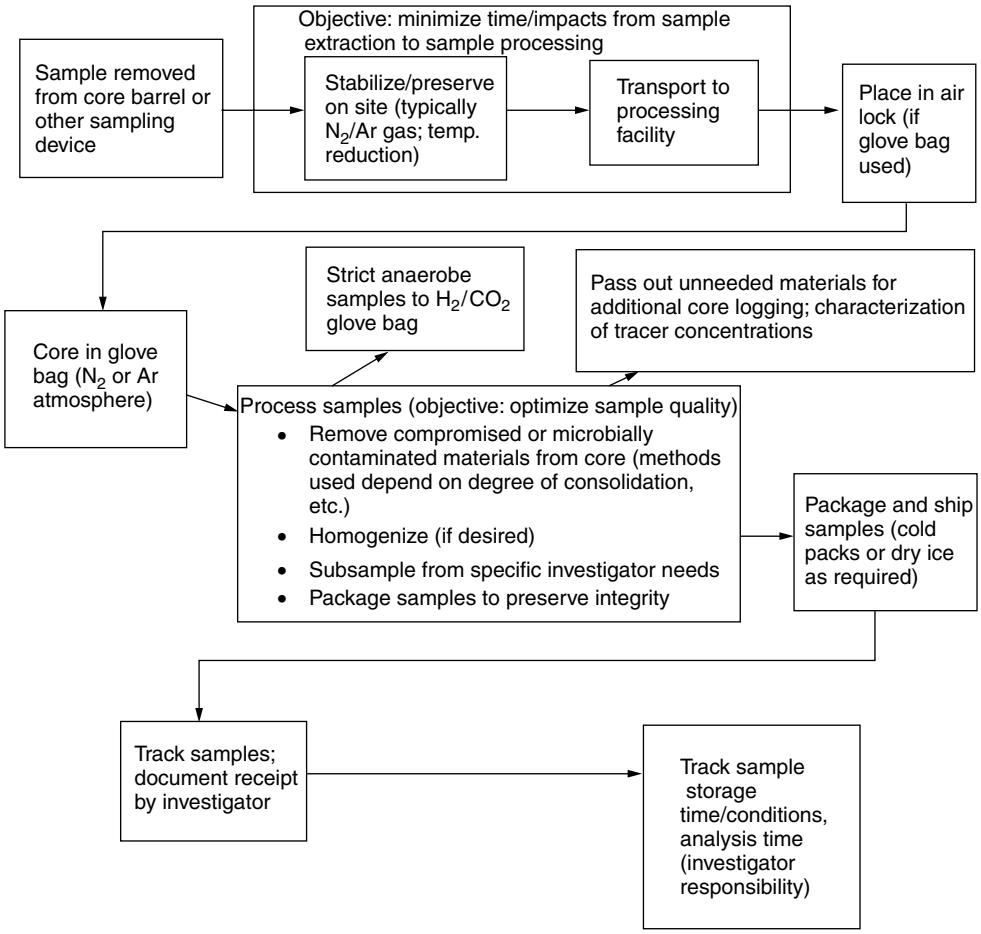

Figure 3. Typical core sample processing flow sheet.

Transport of Samples from Sampling Point to Processing Facility. Distance of the drilling rig, backhoe pit, wellhead, drill ship, or other point at which a sample is extracted from the subsurface may be a minimal or a significant distance from the point at which in-field processing occurs. It is highly desirable to minimize this distance and, for this reason, it is common to have one or more field processing trailers (or shipboard processing facilities) at a drill site to house glove bags and other on-site processing equipment. The objective here is to minimize the time involved in sample extraction, sample processing, and the initiation of analyses or experiment on those samples.

At the other end of the spectrum, entire core segments are preserved on-site by placing immediately in gas-tight PVC, flushing with an inert gas, and shipping to a laboratory for processing. This has the disadvantage of not permitting immediate preservation such as freezing (desirable for PLFA analyses and DNA extraction). Also, it extends the time between extraction from the subsurface and shipping of subsamples to individual investigators. The benefit is reduced cost of in-field processing, but it is necessary to make sure that this type of approach is consistent with the objectives of the sampling project. Some studies (51) have combined immediate emersion of samples in Ar and short transport of samples (two to five minutes) to an in-field processing facility. Future possibilities include controlling the well bore atmosphere or otherwise minimizing oxygen content from the time of sample extraction in the subsurface to the point of sample processing.

Another approach that minimizes field costs and transport time is to process samples in air but focus on immediate enclosure of samples in sterile containers with no headspace. While this is typically used with relatively inexpensive shallow sampling techniques (e.g., backhoe sampling) in relatively oxic environments, it has been shown that significant parts of anaerobic microbial communities survive this treatment. The keys to this and similar approaches that flush container headspace with Ar gas are rapid sample enclosure, maintenance of samples at near in situ temperatures, and same-day overnight shipping to ensure arrival at the receiving laboratory within 24 to 48 hours of sample collection.

To summarize, the objective is to minimize sample impact during transport from point of extraction to sample processing point. This is done mainly by minimizing time of transport, but also may involve controlling the atmosphere to which samples are exposed. This is linked closely to decisions on the degree of in-field processing. Few, if any, studies have systematically evaluated the effects of different approaches to this phase of sample collection, and we suspect that there is room for both assessment of and improvement to typical practice.

In-Field Processing Steps and Requirements. Sample processing should occur under an inert atmosphere to minimize chemical or biological alteration of sample materials. Exposure to air causes oxidation of reduced species such as Fe(II) and sulfides, inactivation of anaerobic and microaerophilic microorganisms, and drying of the sample. Stimulation of biological activities resulting from coring disturbances (38,50,52,53,54), is reduced by processing under inert, nutrient-limited conditions. Anaerobic chambers filled with argon or nitrogen gas, typically modified with a core transfer tube (Coy, Ann Arbor, MI) (4), are routinely used for sample processing (see 29 for pictures of glove bags and coring paring processes). Cores should be processed as soon as possible upon recovery to minimize perturbations, and to separate representative material from drilling fluids, slough, and contaminants. In practice, this means adjusting the number and size of glove bags to the maximum rate of core removal. Alternatively, it may be necessary to intentionally slow the rate of drilling and core removal, with an obvious increase in cost and the possibility of in well contamination of uncored samples if pressure head in the well exceeds the hydraulic head in the formation. On-site processing also provides opportunities for on-site evaluation of sample quality and allows opportunities for modifying site operations.

Core liners containing materials are placed into the transfer tube, which is then flushed and evacuated for three or more cycles with an inert gas to displace the oxygen within air. After three or more flush and evacuation cycles, materials are transferred to the processing chamber. Shorter and unconsolidated cores can often be extruded from core liners. Core liners may have to be scored or cut to retrieve tightly packed or consolidated materials. If liners require cutting, that may best be accomplished before their entering the transfer airlock. This creates greater exposure to oxygen but reduces processing time, because cutting core liners within processing chambers is difficult, slow, and may involve safety risks. Each project needs to assess the relative merit of each approach, committing necessary resources to ensure an effective and safe process. Upon exposure, cores can be examined and geologically logged and evaluated. If the core material appears disturbed, mixed, impacted with drilling fluid, or chemically compromised, the core is not suitable for microbiological or chemical analyses.

Removal of outer core material that has the greatest potential for being contaminated should be standard practice, especially for low-biomass subsurface materials. Drilling fluids are typically most concentrated at the ends and outermost edges of the core. These regions must be carefully pared with sterile tools. If a visible tracer such as rhodamine dye was used, the distance of penetration can be used as a guide for paring. Unconsolidated sediments are typically pared and subcored with sterile knives, spoons, spatulas, or chisels. Consolidated sediments or rocks may require use of chisels or hydraulic splitters to pare outer portions of a core from the innermost representative materials. Hydraulic core splitters can further be used to subsample pared sections. Crushing in a sterilized Plattner mill or a hammer mill can further disaggregate materials. Between uses, the hydraulic splitters should be thoroughly disinfected, removing both particulates and fluids.

Subsamples of drilling fluids, outer portions of the core, and representative materials from inside the core should be collected and analyzed for tracers. Tracers as well as contamination will not be distributed evenly along a core, thus making it necessary to sample multiple fractions of edges, ends, parings, and innermost samples from different locations along the length of the core. All tracer samples should be preserved in sealed containers. Methanol should be added to all perfluorocarbon-containing tracer samples, as they are volatile. These core and paring samples should be compared to samples of drilling fluids, borehole slurries, materials from the coring shoes, samples from the processing chamber, and numerous blanks and controls. Rigorous subsampling and analysis of tracers, as well as scrutiny of comparative measures and geochemical modeling, permit quantification of the extent of contamination and evaluation of sample quality.

Before sample disbursement and transport, samples can be homogenized (if desired), segregated, transferred into sterile Whirlpak® bags, and placed into sealable canning jars. To minimize exposure of samples to air during transport, jars should be flushed with oxygen-free nitrogen or argon gas via a cannula before sealing. This can be accomplished inside the anaerobic chamber. A novel approach for storing anaerobic samples during transport has been described by Cragg and coworkers (45) who used heat-sealable nylon-aluminum-polyethylene "wine bags." Subsamples can be placed in these bags with a commercially available O_2-absorbing system used for culturing anaerobic bacteria.

Sample Shipping. Sealed samples can be transported to laboratories or shipped by overnight express carrier, with cold packs to keep samples cool during transport. The inclusion of cold packs protects samples from elevated temperatures that can occur during transport. Analyses of macromolecules such as lipids and nucleic acids require that those samples be frozen immediately on-site using a standard $-20\,^{\circ}C$ freezer. Dry ice or liquid nitrogen can also be used. Samples must remain frozen during transport, enabling the preserved materials to be thawed and analyzed at a later time. Shipping containers are typically cardboard boxes lined with Styrofoam, but standard coolers also work well, especially for mason jars, which can be encased in foam and used repeatedly. Ideally, trial shipping runs are made with recording thermometers to ensure that samples remain at approximately $4\,^{\circ}C$ during shipping. It is important also to ensure that cold packs are not sufficiently cold and in direct contact with samples to actually freeze samples. This is a real possibility, especially during cooler times of the year, which also present the possibility of nighttime freezing of samples, if sample containers are left in vehicles overnight. For longer shipping times or extremely hot conditions, special shipping containers designed for maximum thermal performance are commercially available.

The importance of timely receipt and initiation of sample analysis cannot be overemphasized. If samples

are left on a hot loading dock for 24 hours after receipt or samples are not analyzed for days after receipt in spite of being stored under desired conditions, results will almost certainly be altered (17,52,53,55). Essentially, the entire investment in sample acquisition, processing, and shipping is placed at risk. Good communication between sample shipper and sample recipient is therefore essential. E-mailing or telephoning tracking numbers for each shipment to recipients serves to remind recipients of incoming packages and to enable them to track their own packages, if they fail to arrive as planned.

Quality Control for Sample Processing

Subsampling within a glove bag environment creates the possibility of cross-contamination from one sample to the next, and from compromised outer rinds of core samples to nominally pristine parts of core. Systematic evaluation of such contamination is rarely attempted. Anecdotal evidence suggests that indurated samples are more likely to be cross-contaminated than unconsolidated samples. This is attributed to creation of dust from splitting of solid samples within the glove bag. Placing sterile petri dishes in the glove bag during processing provides one indication of the level of dust contamination. Results from analysis of the petri dishes should be compared to those from analysis of core subsamples, to assess the proportion of the microbial community that could be attributed to in-glove-bag contamination. Additional particulate or coating tracers (including known microbial species) have been used with limited success. The concept is to coat the exterior of a core segment before processing. Detection of tracer in processed samples then provides a basis for estimating the effectiveness of the core paring and processing procedure. Cost, typically limits extensive use of this type of quality assurance procedure, suggesting that innovative approaches to this problem are needed.

FUTURE DEVELOPMENTS

Consistently collecting and analyzing the microbiology and associated geochemistry of subsurface samples is a difficult task that is clearly still in its infancy. There are major limitations on our understanding that derive from the limited data set on subsurface microbiology, and from limitations on our ability to more effectively collect and process subsurface samples. In this section, we discuss the most promising future developments.

1. Systematic Analysis of Sample Size and the Heterogeneous Distribution of Microbial Colonies from a Range of Rock Types and Subsurface Environments. A limited number of studies have attempted to assess in situ microbial heterogeneity, and even fewer have related microbial heterogeneity to sample size (56). A greater understanding of typical microbial heterogeneity in different rock types and subsurface environments would significantly improve our ability to estimate needed sample size in advance, to achieve a desired level of repeatability and uncertainty in resulting data. At the present, sampling sizes and requirements are largely driven by investigator needs for a given analytical technique. It is particularly important to note that homogenization alone is unlikely to address this problem because of both the difficulty in complete homogenization and because of unknown effects of homogenization on colony viability.

2. Use of Colonization Surfaces in Subsurface Environments. Placing colonization surfaces in the subsurface, usually in a well bore and allowing microbial populations to attach to the surfaces provides a microbial sample that is specific to depth and the microbial population present in the well bore and, to some unknown degree, the surrounding formation. Several approaches have been developed including the use of rock or mineral coupons, holders with sterile quartz sand or other granular materials, and collection of samples from the surfaces of devices placed in the well bore for other reasons. Techniques for systematically obtaining these samples and interpretation of microbial analyses of such samples are still under development (57), but, with appropriate control of groundwater circulation within the well bore and adjacent sand pack, this approach may yield a high spatial resolution.

3. More Effective, Partially Automated Hydraulic Paring Devices. Removal of contaminated outer rinds of core samples or other types of solid samples is commonly the rate-limiting step in sample processing. Therefore, it would be highly advantageous if this process could be accelerated, particularly for indurated samples such and shales and sandstones. Hydraulic paring devices are available, but they have not been adapted or optimized for use in a glove bag.

4. Rapid, Inexpensive, In-field Tracer Analyses. Field and sampling reality is that more tracers are deployed than are taken full advantage of in sample or paring analyses. At this point in the development of subsurface microbiology, there is a tendency to accept uncritically the results from subsurface samples, with or without tracers. Rapid and routine in-field analysis of particulate and chemical tracers is needed to make the cost of appropriate QA practices affordable.

5. Direct Linkage of Pore Water or Groundwater Geochemistry with Microbial Community Data on a Scale of a Few cm to a Few Tens of cm. Scale differences between bulk groundwater samples and individual microbial core samples are large, commonly exceeding an order of magnitude. New techniques are needed that will enable efficient linkage of pore water chemistry and microbiology on a fine scale. Combining passive multilevel samplers and microbial colonization surfaces offers considerable promise, but the degree to which formation microbial communities are reflected in well bore microbial communities is currently unknown. Research in this area is potentially difficult and expensive, but could yield very important advances.

6. Systematic Assessment of the Loss of Anaerobic Community Members as a Function of Limited Exposure to O_2 Under Field Conditions. Heterogeneity issues make it difficult to perform an assessment of anaerobic microbial community survival rates under varying concentration of O_2 (field conditions). Such an assessment is critical both to decision on processing protocols and interpretation of existing data.

7. Adaptation of Coiled Tubing Drilling Technology to Environmental and Subsurface Microbiological Sampling. The petroleum industry has developed drilling technology that uses coiled steel tubing and a steerable drilling head for drilling directional and multiple completion of oil production wells. New, compact designs that will provide improved control of drilling speed and direction, and minimization of drilling fluid impacts are under development (58). Adaptation of this technology to terrestrial environmental applications and exploration for life on mars is very attractive (59). For microbiological sampling, potential miniaturization of the drilling string and sampling devices is particularly advantageous, enabling collection of samples while drilling on a scale comparable to heterogeneities that commonly exist in sedimentary strata. A number of downhole sensors are under development, and that information could be directly associated with microbiological and geochemical samples obtained from a given depth (60).

Appropriate and effective sample collection and processing continues to be critical to the development of subsurface microbiology. Advances in the next 10 years will play a key role in advancing our understanding of the subsurface microbial world.

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SUBSURFACE SAMPLING.

See SAMPLING TECHNIQUES FOR ENVIRONMENTAL MICROBIOLOGY

SUBSURFACE SEDIMENTARY ENVIRONMENTS.

See MICROBIOLOGY OF DEEP HIGH TEMPERATURE SEDIMENTARY ENVIRONMENTS

SUBSURFACE SEDIMENTS.

See MICROBIAL STARVATION SURVIVAL IN SUBSURFACE ENVIRONMENTS

SULFATE REDUCING BACTERIA.

See BIOCORROSION: ROLE OF SULFATE REDUCING BACTERIA; SULFUR BACTERIA IN DRINKING WATER

SULFATE-REDUCING BACTERIA: ENVIRONMENTAL AND TECHNOLOGICAL ASPECTS

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Sulfate-reducing bacteria (SRB) are a unique physiological group of prokaryotes that couple growth with sulfate respiration. Because of the production of high levels of sulfide by sulfate-reducing organisms, they are members of the sulfidogenic group that converts thiosulfate, sulfite, or elemental sulfur to hydrogen sulfide. The anaerobic electron flow with sulfate as the terminal electron acceptor occurs with several genera in both the *Bacteria* domain and the *Archaea* domain. General information about the biology of these anaerobic sulfate-reducing bacteria can be found in recent monographs (1–3).

Table 1. Reactions in the Dissimilatory Reduction of Sulfate by Microorganisms (4)

	Reaction	$\Delta G^{0'}$
(1)	$SO_4^{2-} + ATP + 2H^+ \rightleftharpoons APS + PP_i$ (Activation)	+46 kJ/mol
(2)	$APS + H_2 \rightarrow HSO_3^- + AMP + H^+$ (Reduction)	−68.6 kJ/mol
(3)	$HSO_3^- + 3H_2 \rightarrow HS^- + 3H_2O$ (Reduction)	−171.7 kJ/mol
(4)	$SO_4^{2-} + 4H_2 + H^+ \rightarrow HS^- + 4H_2O$ (Overall Reaction)	−152.2 kJ/mol

The energetics of sulfate reduction does not favor the use of this compound as an electron acceptor because sulfate must be activated by adenosine triphosphate (ATP) before it can be reduced. As shown in Table 1, the equivalent of two moles of ATP are used for every mole of sulfate activated. Following activation by ATP, there are two sequential reactions in the eight-electron reduction system. The use of sulfate as a terminal electron acceptor is referred to as *dissimilatory sulfate reduction*, which is in contrast to the reduction of sulfate for biosynthesis of amino acids, and is designated as *assimilatory sulfate reduction*. Dissimilatory sulfate reduction is the only electron acceptor system used by microorganisms in which energy is required for electron-accepting activity. Perhaps this unique energy requirement accounts for sulfate reduction being less widely distributed than nitrate reduction.

INVOLVEMENT OF SRB IN BIOGEOCHEMICAL CYCLES

In addition to sulfate reduction, these organisms participate in reactions involving portions of several important nutrient and element cycles. As indicated in Figure 1, SRB interact with various aspects of the global cycles of nitrogen and carbon. It is increasingly apparent that SRB have an influence on the environmental cycling of iron, manganese, selenium and molybdenum. In addition, SRB affect the geochemical cycling of chromium, uranium, and mercury.

Carbon

The most common carbon sources that support growth of SRB are lactate and pyruvate; however, utilization of these carbon compounds is not universal because there are a few isolates that are unable to grow with lactate or pyruvate. Generally, strains of *Desulfovibrio* (*D.*) and *Thermodesulfobacterium* oxidize lactate or pyruvate with the accumulation of acetate, whereas other SRB have complete oxidation with CO_2 as the end product (5). The

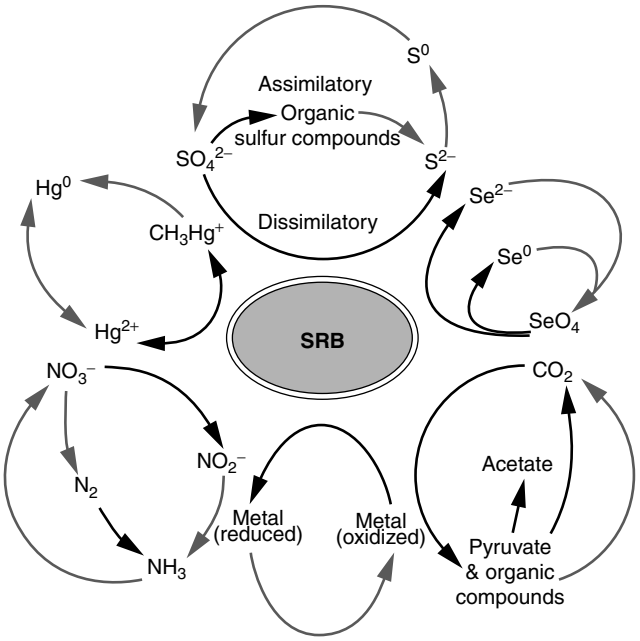

Figure 1. Interaction of SRB with geochemical cycles.

acetate-producing SRB may form syntrophic relationships with methanogens or other acetate-utilizing anaerobes. A number of SRB are autotrophic with growth on H_2 and CO_2 (5) and the metabolic pathways for CO_2 fixation have been well established (6).

The SRB have an extremely diverse metabolism in which about 100 different organic compounds will serve as electron donors resulting in growth. These carbon compounds include hydrocarbons, alcohols, monocarboxylic acids, dicarboxylic acids, amino acids, sugars, aromatic compounds, and complex organic compounds. The individual compounds and their utilization have been enumerated in previous reviews (5,6) and will not be listed here. Although there has been considerable interest in finding a sulfate-reducing bacterium that can oxidize methane, no pure cultures that have this feature have been obtained. Several strains of SRB can dismutate carbon compounds in the absence of sulfate where the organic compound is both the electron donor and acceptor. When the organic compound serves as an electron acceptor, it is an example of bacterial fermentation. With SRB, dismutation is seen with fumarate, malate, choline, and cysteine (5).

Several strains of SRB can metabolize recalcitrant compounds and these SRB have the potential for environmental remediation. Specific strains of SRB that detoxify organic compounds have been reviewed in considerable detail by Ensley and Suflita (7). Recent isolates of SRB will oxidize toluene (8), dehalogenate 2,4,6-tribromophenol (9), or reduce trinitrotoluene to triaminotoluene (10,11).

Sulfur

SRB can use a variety of sulfur oxy-anions in addition to sulfate for dissimilatory reduction. Although tetrathionate, trithionate, thiosulfate, and sulfite are reduced by SRB, these compounds do not accumulate in the environment, but are transiently formed because of SRB metabolism. *D. dismutans* and *Desulfobacter curvatus* can use sulfite and thiosulfate with one sulfur atom oxidized and one reduced. Recently it has been demonstrated that *Desulfobacter proprionicus* can disproportionate elemental sulfur (12). The disproportionation reactions are given in Table 2, and the electron-accepting activity of the sulfur atoms has been suggested to represent inorganic fermentation (13).

Table 2. Disproportionation of Sulfur Compounds Associated with SRB (13)

	Reaction	$\Delta G^{0'}$
(5)	$4SO_3^{2-} + H^+ \rightarrow 3SO_4^{2-} + HS^-$	−235.6 kJ/mol
(6)	$S_2O_3^{2-} + H_2O \rightarrow SO_4^{2-} + HS^- + H^+$	−21.9 kJ/mol
(7)	$4S + 4H_2O \rightarrow SO_4^{2-} + 3HS^- + 5H^+$	−56.2 kJ/mol*

*With SO_4^{2-} and HS^- at 1 mM and pH = 8.0.

The oxidation of sulfur compounds by SRB has only recently been considered important for their persistence in various environments. To accomplish the oxidation of reduced sulfur compounds, appropriate levels of molecular oxygen are required to function as the electron acceptor. Certain strains of *D. desulfuricans* will oxidize thiosulfate, sulfite, and hydrogen sulfide to sulfate with the reduction of oxygen (14). Growth will occur in these cells because coupled with the formation of sulfate is the production of ATP that is produced by the reverse of Reaction 1 in Table 1. Elemental sulfur is oxidized by *D. desulfuricans* to thiosulfate but not to sulfate. In a highly intriguing report, Dannenberg and coworkers (14) indicated that *D. proprionicus* oxidizes sulfide with NO_3^- or NO_2^- as the electron acceptor, resulting in the formation of NH_3. Certain strains of *D. desulfuricans* will oxidize sulfite and thiosulfate to elemental sulfur (S^0) with nitrite as the electron acceptor.

During oxygen limitation, *D. desulfuricans* establishes a syntrophic relationship with *Thiobacillus thioparus* (15). In this situation, *T. thioparus* produces S^0 and not sulfate as a product of sulfide oxidation. *D. desulfuricans* uses the S^0 as a dissimilatory electron acceptor with growth. Another mini-sulfur cycle occurs with the association of SRB with an anaerobic phototroph. As reviewed by Fauque (16) a stable syntrophic relationship is formed in which *Desulfovibrio* reduces sulfate to sulfide and the sulfide is oxidized to sulfate by *Chlorobium limicola*.

In addition to the use of inorganic sulfur compounds as electron acceptors, several strains of *Desulfovibrio* have been shown to use sulfonates (17). The sulfur atom in sulfonate was reduced to sulfide and the organic carbon fraction of the sulfonate molecule could serve as the electron donor. Not all sulfonates are utilized by the SRB, but those that are include isethionate (2-hydroxy-ethanesulfonate), cysteate (alanine-3-sulfonate), and sulfoacetaldehyde (acetylaldehyde-2-sulfonate). In a provocative report that examined SRB colonization in the gastrointestinal tract of mice, it was reported that SRB were most prevalent in those regions where sulfomucin

was abundant (18). Thus the sulfur atom of sulfomucin may also serve as the electron acceptor for SRB. Clearly, SRB are involved in many facets of the sulfur cycle and under appropriate environmental conditions many different sulfur compounds can be either electron acceptors or electron donors.

Nitrogen

Several strains of SRB will fix nitrogen (N) and grow with N_2 as the sole nitrogen source. Diazotrophy has been demonstrated by direct cultivation in N-free medium, by following $^{15}N_2$ incorporation, by measuring acetylene reduction, and by hybridization studies using the *nifH* gene from *Klebsiella pneumoniae* (19). Although the genes for N_2-fixation appear to be genomic and not plasmid-carried, many isolates do not have genes for N_2-fixation. The rates of N_2-fixation are sufficient to enable microorganisms to grow in N-limited environments; however, the impact of SRB on the global nitrogen cycle has not been thoroughly examined.

Some of the common strains of SRB can use nitrate in dissimilatory reduction. As reviewed by Moura and coworkers (20), the best-characterized system is that of *D. desulfuricans* ATCC 27774, which has a periplasmic nitrate reductase. Nitrite generated by the nitrate reductase is reduced to ammonia by a transmembrane nitrite reductase. The nitrite reductase in *D. gigas* will energize transmembrane transport of protons and establish sufficient proton motive force on the plasma membrane to generate ATP by anaerobic oxidative phosphorylation (21). This dissimilatory reduction of nitrite to ammonia is common in bacteria and may be widely distributed in SRB. Thus far SRB have not been demonstrated to produce N_2 from the reduction of nitrate.

Iron and Manganese

The reduction of iron and manganese also occurs with SRB, in part because of the copious production of H_2S. The potential for dissimilatory reduction of Mn(IV) and Fe(III) to be coupled with cell growth has been demonstrated with the growth of *Desulfotomaculum reducens* (22). An interaction of the sulfur and manganese cycles has been demonstrated with *D. desulfuricans*, which couples the oxidation of Mn(IV) with the reduction of S^0 (12). Iron sulfide is generally produced in the amorphous form; however, nanosized deposits of magnetic iron sulfide have been produced by SRB (23). It has also been reported that hematite (α-Fe_2O_3) dissolution can occur in the presence of *D. desulfuricans* owing to hydrogenase reduction and H_2S oxidation, resulting in ferrous sulfides in the form of pyrrhotite (24).

Selenium

Although some anaerobic bacteria can use selenate or selenite as an electron acceptor by growth-supporting respiration, this has not been demonstrated in SRB. Selenium is assimilated by SRB to produce a selenium-containing hydrogenase (5) and a formate dehydrogenase. Selenium is an essential trace element for SRB, but at high concentrations, selenium in the form of selenate, Se(VI), and selenite, Se(IV), are toxic to SRB. As previously reported (25), growth of *D. desulfuricans* is inhibited by Se(IV) at a lower concentration than by Se(VI), and resistance to Se(IV) and Se(VI) is achieved after previous exposure at sublethal concentrations. Selenate and selenite are structurally similar to sulfate and sulfite, respectively. Selenate inhibits Reaction 1 and selenite inhibits Reaction 3 in Table 1. Although the mechanism of reduction is not known, *D. desulfuricans*, and presumably numerous SRB, will reduce selenate or selenite to elemental selenium. Resistance and reduction of selenium oxy-anions are distinct mechanisms because the formation of Se^0 was not apparent with either Se(IV) or Se(VI) until *D. desulfuricans* entered the stationary phase (25).

Molybdenum

Molybdate has been commonly used to limit the growth of SRB by interfering with Reaction 1 shown in Table 1. However, molybdenum is also required by SRB for the various molybdoproteins that these organisms use (5). At sublethal concentrations of molybdate, several species of *Desulfovibrio* will reduce Mo(VI) molybdate to Mo(IV) with the production of molybdenum disulfide (26,27).

Uranium

The pursuit of SRB-mediated metabolism of metals was initiated with the report by Lovley and colleagues (28) demonstrating the conversion of soluble uranyl, as U(VI), to insoluble uranite, U(IV). Many strains of SRB can reduce U(VI) but currently only *Desulfovibrio* strain UFZ B490 (29) and *Desulfotomaculum reducens* (22) have been demonstrated to grow with dissimilatory uranium reduction. The mechanism for reduction of uranyl ion remains to be established.

Chromium

With the exception of *Desulfotomaculum*, SRB do not produce spores and, therefore, remain in the environment as a result of continuous growth. Overcoming metal toxicity is one of the apparent requirements for SRB to persist in the environment. Chromate, Cr(VI), and other strong oxidizing agents have been used to prevent bacterial growth in cooling towers. At sublethal concentrations, Cr(VI) is reduced by *D. vulgaris* to Cr(III) by a hydrogenase-cytochrome system (30). *Desulfotomaculum reducens* is one of the few SRB that can couple growth to dissimilatory reduction of Cr(VI) (22).

Mercury

SRB have an important role in the biogeochemical cycle of mercury. Although mercury salts in the environment are toxic, dimethyl mercury, $(CH_3)_2Hg$, is highly volatile and a potent neurotoxin. Many different bacteria, including SRB, methylate Hg^{2+} to produce methyl mercury, CH_3Hg^+, and at aerobic/anaerobic interfaces SRB are the principal group of bacteria that convert methyl mercury to dimethyl mercury. *D. desulfuricans* has been demonstrated to transfer the $-CH_3$ moiety from methyl cobalamine in the production of dimethyl mercury (31).

As reviewed by Hobman and coworkers (32), CH_3Hg^+ will react with H_2S, which is unstable, and HgS is formed along with dimethyl mercury. Demethylation of methyl mercury has been reported by Oremland and coworkers (33), and it is unresolved if this is due to the spontaneous degradation of $(CH_3Hg)_2S$ or an enzymatic process. Once HgS is formed, it remains in sediments until oxidation reactions produce Hg^{2+} and S^{2-}. It is important to note that under acidic conditions dimethyl mercury is unstable and slowly decomposes to CH_4 plus Hg^0.

DISTRIBUTION OF PHYSIOLOGICAL GROUPS OF SULFATE REDUCERS

SRB are broadly distributed in anaerobic areas of the earth, existing at temperatures that span from 82 °C to 4 °C, withstanding high pressures in water columns, and having varying salt requirements (Table 3). Although most SRB belong to the *Bacteria* domain, the only genus of the *Archaea* domain that is a sulfate reducer is the hyperthermophile *Archeoglobus*. Distinguishing the various species of SRB are cellular characteristics and metabolic activities including the unique electron donors used to support growth. There are a large number of new species isolated every year and a comprehensive listing of the SRB species is beyond the scope of this review. Specific metabolic and cellular characteristics associated with various SRB strains are given in recent reviews (2,3,13). In addition to SRB existing as single agents or in consortia with other bacteria, they are also found associated with different eukaryotes, and some of these associations are listed in Table 4. Numerous new species, and even genera, of SRB may be identified from ciliates, clams, or polychaete annelids. Of considerable interest is the presence of bacteria on the dorsal integument of the polychaete annelid, *Alvinella pompejana*, that grows along deep-sea hydrothermal vents. Through molecular techniques, the annelids have been found to harbor bacteria that contain sulfite reductase, which catalyzes Reaction 3 of Table 1; however, these bacteria have not been cultivated. When SRB have been found in the various regions of animals including humans, they have generally been one of several bacteria isolated. The only SRB that has been considered to contain some disease producing activity in humans is *Desulfovibrio fairfieldensis* (34) and additional studies are needed to clarify the virulence of this strain.

Table 3. Environmental Distribution of Sulfate-Reducing Bacteria Based on Temperature, Salinity, and Pressure Requirements

Physiological Group	Optimal Conditions for Growth	Habitat	Genus	Species	Reference
Hyperthermophile	82 °C	Marine hydro-thermal vents	*Archaeoglobus (A.)*	*fulgidus, profundus*	35
Thermophile	65–70 °C	Hot springs and warm oil field water	*Thermodesulfo-bacterium (Td.)*	*commune, mobile*	36
	55–65 °C	Soil and sediments	*Desulfotomaculum (Dm.)*	*geothermicum, knuznetsovii, nigrificans, thermoacetoxidans*	36
Mesophile	27–37 °C >0.2% salt required	Soil and sediments	*Desulfovibrio (D.)*	*vulgaris*, desulfuricans**	16
			Desulfobacterium (Dbm.)	*hydrogenophilus**	
			Desulfobulbus (Dsb.)	*elongatus*	
			Desulfococcus (Dc.)	*niacini*	
			Desulfonema (Dn.)	*magnum**	
			Desufosarcina (Ds.)	*variabilis*	
			Desulfomicrobium (Dsm.)	*aspheronum*	
			Desulfomonas (Dmn.)	*pigra*	
	Moderate salt (2.5%)	Marine sediments	*Desulfobacter (Db.)*	*postgatei*	37
			Desulfovibrio	*salexigens, Africanus*	
	Halophilic/halotolerant (5–17%)	Salt lake sediments and oil fields	*Desulfohalobium*	*retbaensi*	38
			Desulfotomaculum	*halophilum*	39
			Desulfovibrio	*gabonensis,*	40
				halophilus,	41
				senezii,	42
				vietnamensis	43
Psychrotolerant	4 °C	Oligotrophic lake sediments	*Desulfovibrio*	*cuneatus, litoralis*	44
Barotolerant	100–150 atm	Deep marine sediments	*Desulfovibrio*	*profundus*	45

*There are numerous species in these genera; only the most commonly found are listed.

Table 4. Interactions Between Sulfate-Reducing Bacteria and Eukaryotes

Sulfate Reducer	Eukaryotic Association	Reference
Unidentified SRB	Ectosymbiont on marine ciliates	46
Unidentified SRB	Epizoic SRB on shell of clam, *Artica islandica*	47
Unidentified SRB	Deep-sea polychaete annelid *Alvinella pompejana*	48
D. zosterae	Roots of macrophyte *Zostera marina*	49
Desulfovibrio	Root cortex of sea grass *Halodule wrightii*	50
D. intestinalis	Hindgut of flower termite, *Mastotermes darwinensis*	51
D. giganteus	Gut of soil termite, *Cubitermes speciosus*	52
D. termitidis	Hindgut of termite	53
Desulfotomaculum	Intestine and cecum of neonatal mice	18
D. desulfuricans	Bacteremia in dog	54
Desulfovibrio	Human colon	55
D. fairfieldensis	Brain abscess and blood of humans	34
Desulfovibrio	Pyrogenic human liver abscess	56
Desulfovibrio	Intracellular infection in ferrets with bowel disease	57
Desulfobacter, *Desulfovibrio*	Periodontal pockets of human mouth	58

PRESENCE OF SULFATE REDUCERS IN DEEP SUBSURFACE

The range of SRB is not limited only to marine and surface terrestrial environments, but SRB have been found in deep subterranean rock. When a sandstone core was extracted from the United Kingdom Continental Shelf oil field at the 1,000 m depth, it was found to contain SRB (59). The unique feature of these SRB was the formation of a stable community with other anaerobes. In another case, SRB were detected in a natural gas formation at a depth of 2,800 m where the bacteria were apparently trapped inside the pores that contained formation water (60). The localized presence of SRB at the interface of sandstone and shale layers from depths down to 247 m has also been detected (61). Although microbial activity is limited in shale because of restrictive pore size, electron-donating organic material from the shale could diffuse into the more permeable adjacent sandstone, helping to explain the presence of SRB and other microorganisms near the interfaces. It appears that SRB are distributed to great depths of the earth where cell survival and growth would be a tremendous accomplishment.

ACTIVITIES OF SULFATE REDUCERS IN BIOLOGICAL COMMUNITIES

Biofilms can form on any surface in contact with an aqueous environment. Also known as biofouling, microorganisms (including, but not limited to bacteria, fungi, algae, and protozoa) adhere to the surface by such means as extracellular polysaccharides or specialized adhesion structures, forming microcolonies. Other organisms can capitalize on this initial colonization and diverse microbial communities may develop. SRB are commonly found in biofilms or biomats in artificial aquatic settings, marine environments, and hypersaline lakes. The microbial diversity in biofilms increases with time, and SRB become established relatively late in biofilm development. Studies conducted using a combination of microsensors and molecular techniques indicated that a lag time exists between initial formation of a biofilm and sulfide production. Concomitant with the appearance of sulfate reduction is the detection of *Desulfobulbus* and *Desulfovibrio* (62).

Biofilms can be divided into three zones of aerobicity; permanently oxic, fluctuating oxic and anoxic, and permanently anoxic. On the basis of molecular detection methods, SRB have been found to be present in all three regions. The anaerobic zones provide an ideal environment for the growth of SRB, but a surprising number of SRB can be found living in oxic regions, particularly *Desulfobulbus* and *Desulfovibrio* species (62), and species similar to *Desulfonema* and *Desulfococcus* (63). The microenvironments in biofilms are typically heterogeneous, and it has been shown that SRB-containing biofilms have high sulfide flux in the interface between oxic and anoxic zones (62). The sulfide produced by SRB did not diffuse out of the biofilm; rather it was rapidly oxidized to SO_4^{2-} or other oxidized S compounds. These compounds, in turn, were reduced by SRB in a sulfur cycle that involves a very small pool of reduced sulfur compounds with a high turnover rate, resulting in a high sulfide flux and high SRB activity at the oxic/anoxic interfaces within the biofilm. It has long been known that SRB display a tolerance to molecular oxygen, and recently aerotaxis with oxygen-dependent growth has been demonstrated (64,65). Thus low levels of oxygen in biofilms would enhance SRB activities.

Another example of the cycling of sulfide occurs in root-associated biofilms that contain both SRB and *Beggiatoa*. As reviewed by Larkin and Strohl (66), both SRB and Beggiatoas exist in the transition zone between oxic and anoxic environments. There is a rather intricate relationship between rice roots, SRB, and Beggiatoas. In the absence of Beggiatoas, sulfide produced by SRB is toxic to rice. Oxygen excreted by plant roots supports the metabolism of the aerobic Beggiatoas and sulfide is oxidized to sulfate.

SRB are commonly found associated with plant roots in underwater environments. Although SRB have been detected on root surfaces, they also can be found deep in the root cortex. Intracellular colonization of roots by SRB has been demonstrated in rice plants (67), and in sea grass, in which SRB including *Desulfovibrio* species were found on all epidermal cells, and up to 60% of the deepest cortex cells (49). As observed in other biofilms, SRB-inhabiting plant roots are found in anoxic and oxic regions, and seem to be tolerant to oxygen exposure (49,68). SRB are not found alone on plant roots, but are generally part of microbial populations that include

acetogenic bacteria, methanogens, sulfide oxidizers, and other microorganisms that, with the plants, comprise complex communities (49,67,69).

Interspecies interactions are quite important to the formation and maintenance of biofilms. For example, subsurface biofilms containing SRB may form in the sandy soil of artificial golf courses and create an impermeable "black plug layer," so named because of the black sulfides produced and the inability of water and oxygen to cross the fouled zone. Current remediation strategies involve excavation and reconstruction of the affected area. Using bacteria isolated from a black plug layer it has been demonstrated that microbial consortia produce this layer, whereas individual species do not form black plug layers (70). In particular, SRB may interact with cyanobacteria to produce black globules found in such layers (71).

The metabolism of H_2 can be an important determinant for growth of anaerobes. Hydrogen transfer between species of *Desulfovibrio* and H_2-oxidizing anaerobes has been reviewed by Fauque (16). Certain SRB cannot grow with ethanol in the absence of sulfate owing to the accumulation of H_2; however, SRB will grow if H_2 is utilized by a methanogen, thereby serving as the electron acceptor. In the situation where H_2 is the electron source in the environment, anaerobes that are H_2-scavengers will be in competition for the H_2. Because the hydrogenase of SRB has greater affinity for H_2 than hydrogenase from methanogens, SRB can outcompete methanogens for H_2.

BIOGENIC HYDROGEN SULFIDE PRODUCTION

In some oil fields, there is considerable sulfide present, resulting in oils known as "sour oil," whereas oils from fields with no sulfide are "sweet oils." The souring of oil is generally a reflection of SRB converting sulfate to sulfide in hydrocarbon reservoirs. Frequently, this is found with offshore drilling operations in which seawater, which contains 28 mM sulfate, is used to balance pressures in the subterranean oil reservoirs. In cases where fresh water is pumped into the reservoirs, sulfate in the environment is dissolved, and similar problems result. Control of SRB to prevent souring frequently requires the addition of biocides that may include glutaraldehyde, chlorine, cetylpyridinum chloride, or alcohols. Many of the specific conditions and procedures used in the oil industry to inhibit the growth of SRB have recently been reviewed (72,73) and will not be enumerated here.

Appreciable quantities of H_2S can be produced by SRB and can accumulate to toxic levels. Perhaps one of the best examples of H_2S accumulation is seen in the coastal bottom waters of the Baltic Sea (74). Nitrogen and phosphorus accumulated because of additions to the Baltic Sea from agriculture and papermills. Eutrophication occurred and produced anoxic conditions in benthic sediments where only H_2S-tolerant organisms survive.

In some instances biologically produced hydrogen sulfide is used to immobilize toxic metals. As reviewed by Barton and Tomei (75), SRB have been effective in the removal of Cd^{2+}, Cu^{2+} and Ni^{+} from mine and smelter wastes. In Budel-Dorplein, The Netherlands, there is a large commercial production for removal of Zn^{2+} from groundwater, which uses SRB to produce hydrogen sulfide that precipitates Zn^{2+} as ZnS. SRB may be useful for the immobilization of heavy metals in acid-mine drainage because SRB remain metabolically active in bioreactors at pH 3.25 (76).

APPLICATIONS OF SULFATE-REDUCING BACTERIA

Because of the numerous activities of SRB, there has been some interest in technologies for the application of enzymes and cells for industrial development. The potential for use of hydrogenase and electron carriers from SRB for commercial H_2 production by biophotolysis has been reviewed by Barton and Tomey (75). The hydrogenases from SRB are unique because they are not denatured by exposure to oxygen and have been immobilized by a variety of support media. Clearly there is the potential to use several of the enzymes from SRB for remediation of the environment or for development of bioprobes for monitoring of environmental processes. With respect to control of environmental pollution, SRB have a broad role in treatment of commercial effluents. The precipitation of heavy metals in various waste streams as sulfides has already been presented. (See Biogenic hydrogen sulfide production). Technologies have been developed for the treatment of waste streams from distilleries and fish canneries, and for the treatment of wastewater containing methanol, furfural, and molasses (75).

SRB are being evaluated for various applications in energy technologies. Although SRB cannot utilize sulfur compounds in bitumen (77), *D. desulfuricans* has been found useful for removing sulfur dioxide from flue gases with the formation of hydrogen sulfide (78). It has been suggested that SRB have been involved in tar-sand deposits (79) and that SRB may be used to increase oil production in existing wells. SRB of several taxonomic groups have been reported to synthesize alkanes using lactate as the carbon source with an atmosphere of H_2:CO_2 at a ratio of 9:1 (80).

THE ROLE OF SULFATE REDUCERS IN BIOCORROSION

Corrosion attributed to microorganisms can occur in any environment where biofilms develop. The term *biocorrosion* has been used to describe corrosion of metals, alloys, composites, or any material in contact with biofilms or microbial mats that suffers increased rates of corrosion or decomposition as a result of biological activity. Recently, however, the more general phrase "microbially influenced corrosion" (MIC) has been replacing "biocorrosion" in the literature, and this reflects the limitations of our understanding of specific corrosion processes associated with microbial activity; in some cases biofilms have even been shown to inhibit rather than accelerate corrosion (81). The role of SRB is significant in commonly encountered examples of iron and mild steel corrosion. There are, however, reports of SRB involvement (either acceleratory or inhibitory) in the corrosion of stainless steel and some transition metals (82,83).

The role of SRB has been addressed in reviews of MIC (81,82). In basic terms, MIC occurs at the surface of materials that are exposed to microbial activity in an aqueous environment. SRB-mediated biocorrosion can involve enzymatic (hydrogenase) reduction and hydrogen sulfide oxidation. On a microscopic scale, an electrochemical cell can form on metal surfaces under anaerobic conditions. It then becomes possible for localized cathodes and anodes to form on the metal because of differential aeration cells in the biofilm. Corrosion consists of metal dissolution at one site coupled with electron uptake by an acceptor at a cathodic site (82). In the cathodic depolarization model of iron and corrosion, SRB containing hydrogenase remove accumulated cathodic hydrogen from the surface of the iron, which in turn forces the dissolution of iron at the anodic site. Accelerating the process is the cathodic reduction of microbially produced H_2S, yielding S^{2-} as a product, and the corresponding acceleration of the anodic reaction because of the formation of iron sulfide, FeS (81). Another role for SRB in MIC of iron and mild steel involves active electron transport where bacterial cytochrome and hydrogenase siphon electrons directly from the metal and accelerate H_2 production (84). The most comprehensive review concerning the role of SRB and hydrogenase in corrosion of ferrous metal is provided by Laishley and colleagues (85).

The biological and chemical processes associated with corrosion were recently reviewed and Odom (72) indicates that the magnitude of the ferrous corrosion problem is such that in an industrial country it accounts for about 4% of the gross national product. There are about a million miles of concrete sewer conduits in the United States, and concrete may be degraded by a process attributed to SRB (86). The anaerobic sediment and wastewater in the conduits contain SRB, which results in the copious production of H_2S. As H_2S reaches the upper portion of the conduit, S^0 is produced as a result of chemical reaction between H_2S and atmospheric oxygen. *Thiobacillus* and other sulfur-oxidizing bacteria will oxidize the hydrogen sulfide and S^0 to sulfuric acid, which promotes decomposition of the concrete conduit.

Several methods have been used to evaluate the effectiveness of biocides in preventing microbial colonization on ferrous surfaces. The American Petroleum Institute (API) recommends a procedure for screening biocides against SRB that requires 28 days, but Zhou and King (87) have developed a bactometer method based on impedance microbiology principles that requires only one day. Another test for microbial activity in biofilms is the microcalimetric method, which provides the capability to measure the efficacy of biocides (88).

DETECTION OF SULFATE REDUCERS

The difficulty in culturing SRB resulted in a need for quick tests to detect these organisms in oil fields, and this has encouraged the development of numerous presumptive tests. A lactate–sulfate medium containing appreciable levels of iron was developed to detect SRB, and it has been recommended for use by the API (89). The formation of a black precipitate owing to FeS is used to indicate the presence of SRB in the inoculum. The medium for this test is commercially available. An amphimetric test for growth of SRB was developed (90); however, the instrumentation needed did not lend itself to performing a large set of samples in a single oil field. Laishley and coworkers (91) observed that most species of SRB have hydrogenase and developed a rapid test that uses reduction of methyl viologen to indicate the presence of SRB. This hydrogenase test kit is commercially available (Caproco (1987) Limited, Edmonton, Alberta, Canada). An immunoassay was developed using an antibody against ATP sulfurylase, the enzyme associated with Reaction 1 of Table 1 (92). The antibody reaction is performed in microtiter plates similar to standard enzyme-linked immunosorbent assay (ELISA) tests. Kits for the antibody are commercially available. Another test developed to detect only a few hundred *Desulfovibrio* is the fluorimetric detection of sulfite reductase, the enzyme of Reaction 3 in Table 1 (93). With the addition of alkali to SRB, a specific fluorescence associated with the sulfite reductase provides for the rapid detection of 10^3 SRB cells/ml. A radiometric test has been used to quantify SRB in estuaries (94). Where SRB produce radiolabeled hydrogen sulfide from $Na_2^{35}SO_4$, the amount of radiolabeled sulfide produced is used to indicate the number of cells present. All of these tests can be compared to standards or to actual numbers resulting from viable counting of SRB as established by Gibson and coworkers. (95). Detection of deep subterranean SRB has been accomplished by Krumholz and colleagues (61) using a modified ^{35}S method. The faces of rock core samples from as deep as 300 m were pretreated with radiolabeled sulfate and then placed in contact with oxidized silver foil. The $H_2^{35}S$ produced was trapped by foil as $Ag_2^{35}S$, which could be detected by radio-image analysis. This method not only gives a quantitative measure of sulfate reduction, but also yields two-dimensional distribution of the activity within the rock.

More definitive identification of SRB is sometimes required, and several varieties of molecular identification methods have been developed that can be especially useful when confronted with mixed cultures. The use of ribosomal RNA sequences for identification of microorganisms has found widespread application, and several techniques can be used to detect the presence of SRB, and in some cases individual SRB species. It is possible to use radio- or fluorescently-labeled oligonucleotide probes designed specifically for 16S or 23S rRNA sequences for in vitro or in situ hybridization studies to identify SRB (96). Table 5 lists some of the 16S rRNA sequences useful for molecular identification of some strains of bacteria.

Oligonucleotides specific to 16S or 23S rRNA sequences may also be used as polymerase chain reaction (PCR) primers for selectively amplifying and sequencing rRNA from mixed samples for identifying or classifying microorganisms (99). Molecular methods based on the physiology of SRB have been used to detect SRB. Minz and colleagues (63) have used the sequencing of physiology-linked genes (e.g. dissimilatory sulfite reductase) to indicate the presence of SRB in heterogeneous microbial mats. For mixed microbial communities a method has been developed that uses total genomic DNA as a probe for

Table 5. Specificity, Target Sites, and Sequences of 16S rRNA Oligonucleotide Probes

Specificity	Target Position*	Sequence	Reference
All organisms (Prokaryotes and Eukaryotes)	907–928	5′-CCGTCAATTCCTTTGAGTTT-3′	62
Bacteria (not including *Archaea*)	341–357	5′-CCTACGGGAGGCAGCAG-3′	62
Most δ-proteobacteria, and a few gram-positive bacteria	385–402	5′-CGGCGTCGCTGCGTCAGG-3′	96
Desulfovibrio spp. and some members of the following genera: *Geobacter, Desulfomonas, Desulfuromonas, Desulfomicrobium, Bilophila*, and *Pelobacter*	687–702	5′-TAACGGATTTCACTCCT-3′	62
Desulfobacterium spp., *Desulfobacter* spp., *Desulfococcus multivorans, Desulfosarcina variabilis, Desulfobotulus sapovorans*	804–821	5′-CAACGTTTACTGCGTGGA-3′	62
Desulfococcus multivorans, Desulfosarcina variabilis, Desulfobotulus sapovorans	814–831	5′-ACCTAGTGATCAACGTT-3′	97
Desulfovibrio spp.	687–702	5′-TACGGATTTCACTCCT-3′	97
Desulfobulbus spp.	660–679	5′-GAATTCCACTTTCCCCTCTG-3′	97
Desulfobacterium spp.	221–240	5′-TGCGCGGGACTCATCTTCAAA-3′	97
Desulfobacter spp.	129–146	5′-CAGGCTTGAAGGCAGATT-3′	97

*Position in the 16S rRNA of *Escherichia coli* (98).

detection of individual species, using a "reverse genome" approach (100). A feature of this technique is that it allows economical analysis of mixed samples and can accurately describe SRB present.

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SULFATE REDUCING BACTERIA IN PETROLEUM RESERVOIRS.

See PETROLEUM RESERVOIRS, INFLUENCE, ACTIVITY AND GROWTH OF SUBSURFACE MICROFLORA IN

SULFATE REDUCTION IN MARINE SEDIMENTS.

See SEDIMENTS: SULFATE REDUCTION IN MARINE SEDIMENTS

SULFUR BACTERIA IN DRINKING WATER

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Microorganisms of interest in the drinking water environment appear relegated to three categories, namely, biological indicators, primary and opportunistic waterborne pathogens, and nuisance organisms. Certain sulfur bacteria are rightfully included in the group of nuisance organisms and may potentially contribute directly or indirectly to several economic problems in potable water systems, including microbially influenced corrosion, tastes and odors, turbidity, chlorine demand, and in the case of groundwater wells, clogging and deterioration of well screens and casings. In addition, sulfur bacteria may facilitate or assist formation of biofilms, within which the retention and protection of coliform bacteria and pathogenic organisms from antimicrobial agents, for example, chlorine residuals, is possible.

Chemically, sulfur may exist in valence states, ranging from +6 (highest oxidation state) to −2 (lowest oxidation state). Thus, sulfur, like nitrogen, can undergo chemical transformation on several valence levels, and specific microorganisms have evolved in response to the favorable thermodynamic properties of such reactions. As a group, the sulfur bacteria occur in both aerobic and anaerobic environments, and are represented by the nutritional modes of photolithotrophy (principal energy provided by light with reducing power obtained from molecular hydrogen or reduced, inorganic sulfur compounds), chemolithotrophy (both energy and reducing power supplied by reduced, inorganic chemical compounds), and mixotrophy (carbon derived from organic sources and energy and reducing power supplied by inorganic chemical compounds, most notably, reduced sulfur species). Phototrophic and lithotrophic sulfur bacteria, typically, utilize carbon dioxide as their major carbon source; however, many chemoorganotrophic (carbon, energy, and reducing power acquired from preformed organic matter) bacteria, not labeled sulfur bacteria as such, contribute to the genesis of sulfide through desulfuration of sulfur-containing organic matter, most notably, proteins.

SULFUR CYCLE

A Series of Oxidation-Reduction Reactions

The major mineral form of sulfur is sulfate. Sulfate may be present in both ground and surface waters and derived from natural and artificial sources. The principal forms of sulfur reaching the earth from atmospheric deposition, is sulfate and sulfur dioxide; the latter originating in a major way from fossil fuel combustion. Other important sources of sulfate in aquatic systems are mineral deposits. Groundwaters may contain high concentrations of sulfate, owing to aqueous dissolution of certain minerals, for example, barite ($BaSO_4$), epsomite ($MgSO_4 \cdot H_2O$), gypsum ($CaSO_4 \cdot 2H_2O$), weathering of sulfidic minerals, for example, pyrites (FeS_2), and, in coastal regions, salt water intrusion. Surface waters are typically subject to direct catchments of acidic, sulfur-bearing precipitation and anthropogenic pollution, deriving from mining and mineral processing, paper and pulp production, textile manufacturing, and tanneries (1).

Examination of the microbiologically mediated sulfur cycle (Fig. 1) reveals several biogeochemical niches for physiological groups of the sulfur bacteria. Broadly speaking, these niches may encompass sulfur oxidation (linked and nonlinked to energy conservation) and sulfur reduction. Lentic water bodies may provide the appropriate conditions for interactive behavior between photosynthetic sulfur bacteria, sulfur-oxidizing bacteria, and sulfate-reducing bacteria; an arrangement referred to as a "sulfuretum." In the context of potential problems for potable water facilities, activities of sulfate- (sulfur) reducing bacteria are most conspicuous.

The Sulfur Bacteria

Sulfur bacteria are of interest in potable water systems, principally for their potential to lend an aesthetically displeasing quality to the water or to, directly or indirectly, impact the stability of the physical facilities involved in water treatment and delivery. Currently, there are no known public health concerns related to the sulfur bacteria. Natural aquatic habitats in which the sulfur bacteria may be found are morphometrically diverse. Since both surface and groundwaters serve as sources of potable waters, various forms of sulfur bacteria and their influences on the chemical quality of the water may be encountered at some level of treatment. A brief overview of the relevant groups of sulfur bacteria is presented in the following section.

Photosynthetic Sulfur Bacteria. The photosynthetic sulfur bacteria are a morphologically diverse group and are

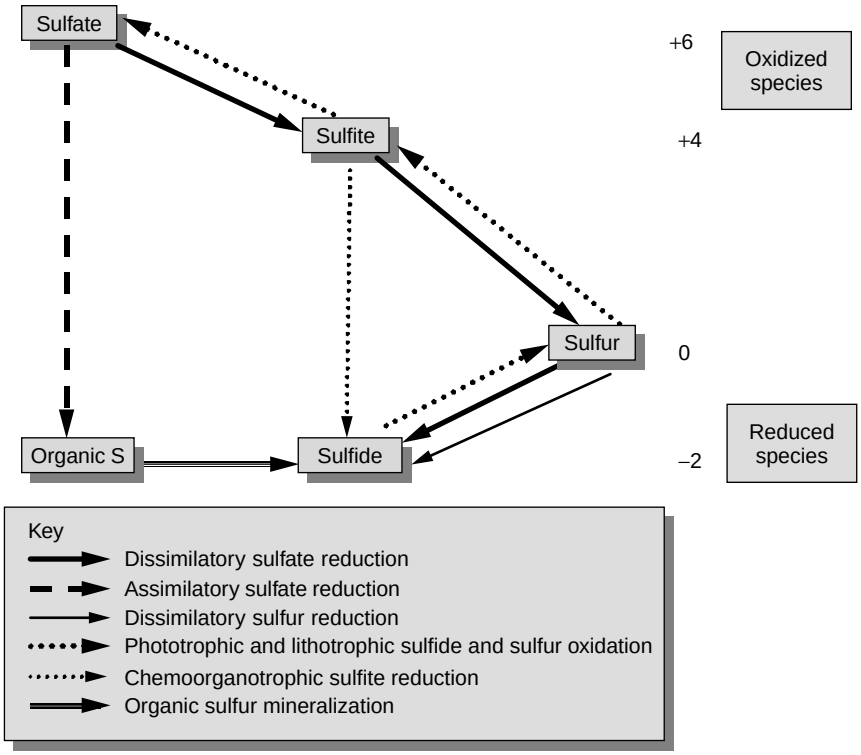

Figure 1. Microbiological sulfur cycle. Sulfur species are aligned with valence state of sulfur, shown in vertical scale at the right of diagram.

delineated according to pigmentation, as green (brown) sulfur, purple sulfur, and purple nonsulfur bacteria. The green bacteria are obligately anaerobic, and all groups function anaerobically when in the photosynthetic mode. Major requirements for the bacteria during photosynthesis are radiant energy (sunlight) and a source of reducing power, which for the green and purple sulfur bacteria is typically molecular hydrogen and reduced sulfur compounds, for example, hydrogen sulfide (H_2S). Phylogenetically, green sulfur bacteria are distinct from the purple sulfur bacteria; the former occupying Kingdom VII and the latter Kingdom I (γ subgroup of Proteobacteria) of the Domain Bacteria. On the contrary, many of the purple nonsulfur bacteria, which are represented in the α- and β-subgroups of the Proteobacteria and typically, derive reducing power from certain organic acids and ethanol, are capable of using low concentrations of hydrogen sulfide to meet this requirement. In addition, this group is classically characterized by a photoheterotrophic metabolism of several organic compounds as carbon sources while obtaining energy from a radiant source (Table 1).

Photosynthetic bacteria thrive in static waters, at precise levels in the water column, where they may absorb specific wavelengths of electromagnetic radiation and obtain reducing power. When reduced sulfur forms are the source of reducing power, zero valence sulfur is a by-product, which typically is transiently stored within the cells of the purple bacteria, or externally precipitated by the green bacteria. Worldwide, biogenic sulfur deposits, especially of aquatic origin, are the result of the precipitation of elemental sulfur during acquisition of reducing power by photosynthetic sulfur bacteria. Unlike aerogenic microorganisms, for example, cyanobacteria that are capable of deriving reducing power from the splitting of water, photosynthetic sulfur bacteria depend for reducing power on the by-products of putrefactive and sulfate-reducing microorganisms located in aquatic sediments and deep oxygen-deficient water strata. Green sulfur bacteria are more efficient in light scavenging than the purple bacteria, hence they may be found in deeper water strata. Stratified, raw water impoundments, reportedly may attain hypolimnial levels of sulfide up to 10 mg/L in midsummer (2) through sulfate reduction and desulfuration of organic matter in the sediments. Hence, photosynthetic sulfur bacteria of deep lakes and reservoirs serving as raw water supplies could potentially assist in the improvement of water quality through the removal of odor-producing hydrogen sulfide. In fact, purple sulfur bacteria have been credited with the transformation of sulfide species in wastewater lagoons and in industrial wastes (3). As may be the case with certain algae and cyanobacteria, photosynthetic sulfur bacteria are not known to be toxigenic and their contribution to turbidity in water, if any, would probably pale in comparison with the aerogenic photosynthetic populations stimulated by cultural eutrophication.

Sulfur-Oxidizing Bacteria. Sulfur-oxidizing bacteria, frequently referred to as *colorless sulfur bacteria*, are well distributed in the natural environment. A dominant genus of the sulfur-oxidizing bacteria is *Thiobacillus*; a morphologically unspectacular group of rod-shaped bacteria with species relegated to the α-, β-, and γ-subgroups of Proteobacteria. *Thiobacillus* species occupy a wide pH range with respect to pH optima for growth, hence appear in many different environments, for example, acidic mine

Table 1. Characteristics of Some Representative Genera of Major Groups of Photosynthetic Sulfur Bacteria and Their Potential Relationship to Potable Waters

Bacteria and Genera	Descriptive Features	Residence in Potable Water System	Biogeochemical Features	Economic Relevance
Green, sulfur	Anoxygenic, nonmotile	Deep, lacustrine water bodies	H_2S oxidized with sulfur precipitated external to cells	Minor
Chlorobium	Straight or curved rods; nonvesicular			
Pelodictyon	Network of branched rods; gas vesicles			
Purple, sulfur	Anoxygenic, morphologically diverse; motile and nonmotile; vesicular and nonvesicular	Meromictic, deep water bodies	H_2S oxidized with sulfur precipitated internal to cells	Minor
Chromatium	Ellipsoid or rod-shaped; motile; nonvesicular			
Thiopedia	Plates of tetrad spheres; nonmotile; nonvesicular			
Purple, nonsulfur	Morphologically and metabolically diverse; fix N_2	Environmentally ubiquitous	Principally photoheterotrophic with small, fatty acids and amino acids as carbon sources	Possible turbidity associated with bloom episodes
Rhodospirillum	Spirillum-shaped cells; motile			
Rhodopseudomonas	Rod-shaped cells, motile; budding			

drainage, sulfur springs, soils, wastewaters, and seawater (Table 2). Sulfur-oxidizing bacteria transform several reduced species of sulfur including sulfide, elemental sulfur, di-, tri-, and polythionates, and thiosulfate to sulfite and sulfate. Hydrolysis of the end-products of sulfur oxidation in the absence of alkaline cations produces sulfurous/sulfuric acids, thus lending a corrosive nature to the surrounding. Acidic weathering of concrete sewer pipes by the combined action of sulfate-reducing and sulfur-oxidizing bacteria remains a serious economic liability (4).

Among troublesome microorganisms in ferruginous (iron-bearing) groundwater systems are the filamentous iron and sulfur bacteria. Accelerated growth of these organisms in the summer months exacerbates the problem of meeting peak water demands in communities. Microbial biomass collected at the surface of well screens and casings severely reduce the well yields in the short-term and enhances corrosion and subsequent deterioration of the well components over time. Prominent filamentous sulfur bacteria in freshwater environments are species of *Beggiatoa* and *Thiothrix*; both of which belong to the γ-subgroup of Proteobacteria. The latter genus is often identified in suspended growth forms of biological wastewater treatment as the etiologic agent of a condition of poor biomass settleability known as filamentous activated sludge bulking (5). Filamentous sulfur bacteria are aerobic but thrive under microaerophilic conditions, and have been observed to reduce partially oxidized sulfur compounds, for example, thiosulfate, to sulfide in the absence of oxygen. Axenic isolates of *Thiothrix* spp. have demonstrated chemolithotrophic (6,7), chemoorganotrophic (8,9), and mixotrophic (8,9) behaviors. Thus, filamentous sulfur bacteria are endowed for survival under a variety of physiological and nutritional conditions.

Sulfate (Sulfur)-Reducing Bacteria. Of the various groups of sulfur bacteria, those members that are capable of utilizing oxidized and partially oxidized sulfur species as

Table 2. Characteristics of Some Representative Genera of Major Groups of Sulfur-Oxidizing Bacteria and Their Potential Relationship to Potable Waters

Bacteria and Genera	Descriptive Features	Residence in Potable Water System	Biogeochemical Features	Economic Relevance
Unicellular, sulfur-oxidizing	Aerobic and facultatively anaerobic; obligate and facultatively lithotrophic; obligately acidophilic and neutrophilic species	Iron, and concrete surfaces as may pertain to surface water (pipes, valves, fittings) distribution and storage and groundwater procurement (well screens and casings)	Aerobic and anoxic oxidation of reduced inorganic sulfur species to sulfate with potential for sulfuric acid formation	Aid the corrosion and deterioration of facilities; acidify water; solubilize heavy metals
Thiobacillus	Rod-shaped cells; motile		One species (*Thiobacillus ferrooxidans*) oxidizes ferrous iron to ferric iron in addition to inorganic sulfur species	
Filamentous, sulfur-oxidizing	Extended length filaments; lithotrophic, mixotrophic, and heterotrophic species	Locales of high sulfide levels, for example, active regions of sulfate reduction, that is, certain biofilms and aquatic sediments; sulfur springs	Oxidation of sulfide; disproportionation of thiosulfate to sulfur granules and sulfate	Clogging of well screens; biofilm resident
Thiothrix	Filaments in rosettes; cells contain sulfur granules; specialized cells (gonidia) show twitching motility			
Beggiatoa	Filaments exhibit gliding motility; cell contain sulfur granules			

ultimate electron acceptors have received greatest attention as contributors to undesirable events in both water and wastewater treatment facilities. As previously noted, well water systems are especially susceptible to anaerobic corrosion, and sulfate-reducing bacteria are chief mediators of iron dissolution through electron transport ultimately to sulfate in the groundwater. Furthermore, the end-product of the reduction of sulfate is the odorous and noxious hydrogen sulfide. At circumneutral pH values, approximately 50% of free, dissolved sulfide exists as hydrogen sulfide and 50% as the bisulfide (HS^-). In the anaerobic biogenic corrosion cycle, sulfide readily reacts with soluble ferrous iron released from the corroding metal to form ferrous sulfide, which may precipitate on base metal. Ferrous sulfide is a relatively poor passivating (protective) agent for the metal surface and may even augment electron conductance by catalyzing the cathodic reduction of protons to hydrogen, thereby promoting the corrosion event. In addition, metal sulfide particles and iron-impregnated biomass may be dislodged from the well screen through pumping of the groundwater. Remnants of these materials, particularly where little treatment of the water other than disinfection takes place, can offer both disagreeable tastes and odor and discoloration to the finished water. Accelerated rates of sulfide production in the presence of ferrous iron can produce deep discoloration and a condition known as *black water*, which is not to be confused with another form of "black water" stemming from the presence of substantial oxidized manganese.

Sulfate-reducing bacteria are obligately anaerobic thriving best at oxidation-reduction potentials in the vicinity of −100 to −200 mV (pH 7). Several morphological forms of sulfate-reducing bacteria (Table 3) exist and their primary sources of carbon and energy are the metabolic end-products of organic matter fermentation, most notably lactic and pyruvic acids and molecular hydrogen. The ability to use acetic acid varies among sulfate-reducing bacteria, resulting in two groups of the bacteria on the basis of this metabolic feature. The phylogenetic placement of sulfate- and sulfur-reducing bacteria is the δ-subgroup of the Proteobacteria, with the exception of *Desulfotomaculum*, which belongs in Kingdom II (gram-positive bacteria). Of the many genera of sulfate-reducing bacteria, *Desulfovibrio* and *Desulfotomaculum* are predominant in freshwater systems.

Sulfate-reducing bacteria may contribute to the form and stability of biofilms (10) through elaboration of extracellular (matrix) polysaccharides (11). Although ferrous sulfide formation may occur in the aqueous phase, it has been shown that the biofilm can entrap the iron sulfide, bring it in contact with susceptible surfaces, and facilitate corrosion (10). Further augmenting the corrosion-promoting action of the biofilm is the spatial heterogeneity (patchiness) of immobilized microbial aggregates on the

Table 3. Characteristics of Some Representative Genera of Major Groups of Sulfate (Sulfur)-Reducing Bacteria and Their Potential Relationship to Potable Waters

Bacteria and Genera	Descriptive Features	Residence in Potable Water System	Biogeochemical Features	Economic Relevance
Sulfate-reducing	Morphologically diverse; obligate anaerobes; both mesophilic and thermophilic species; some mixotrophic and lithotrophic species	Aquatic sediments; distribution system and well screen and casing biofilms	Reduce sulfate and partially reduced sulfur species to hydrogen sulfide; dinitrogen fixation by some species	Major contributor in anaerobic corrosion of iron and copper pipes; taste and odor
Desulfovibrio	Gram-negative; nonsporing; curved rods; motile	Acetate not oxidized		
Desulfotomaculum	Gram-positive; spore-forming; rod-shaped; motile	Acetate not oxidized		
Desulfobacter	Gram-negative; nonsporing; rod-shaped, may be motile	Acetate oxidized		
Sulfur-reducing	Obligately anaerobic; nonsporing; rods or vibrios; motile	Found in habitats similar to those of sulfate-reducing bacteria	Zero valence sulfur and some partially reduced sulfur species are reduced but not sulfate; acetate oxidized	
Desulfuromonas	Gram-negative; straight rods		Some species exhibit reductive dechlorination.	

metallic substratum and the thickness of the anoxic zone within the aggregates. Irregularities of this sort in thickness and surface distribution facilitate a discontinuity in biofilm structure (12). Of primary importance to sulfate-reducing bacteria are the chemical and nutritional gradients that prevail in the depths of the biofilm, especially, pertaining to dissolved oxygen and metabolic end products (certain organic acids and hydrogen) formed by the fermentative members of the film community. Sulfate-reducing bacteria demonstrate sensitivity both to the presence of dissolved oxygen and the end-products of sulfate reduction, particularly, hydrogen sulfide. In mixed culture, sulfate-reducing bacteria derive benefit from chemoorganotrophs capable of creating an anaerobic environment through oxygen consumption and the formation of utilizable electron donors.

POTABLE WATER SYSTEMS

Drinking Water Chemical Composition

The concentration of sulfate in water supplies varies widely depending on the source (Table 4). Groundwater quality is largely related to hydrological and mineralogical characteristics of the subsurface. Concentrations of mineral ions in groundwater are often related to the depth and geology of the aquifer and the drainage capacity of pertinent soils; poorly drained soils, that is, soils with high clay content promote slow movement and long contact times of percolating waters resulting in greater yield of inorganic solids to groundwater. The effect of depth is exemplified by the comparative data in Table 4 for groundwater C versus groundwaters D and E. Typically, groundwaters contain appreciably greater levels of total dissolved solids than surface waters; however, surface waters are greatly susceptible to adulteration by anthropogenic activity. For example, agricultural drainage to the Colorado River appreciably elevated the concentrations of dissolved salts (Table 4). The Federal Safe Drinking Water secondary maximum contaminant limit (SMCL) for sulfate is 250 mg/L. It is estimated that only about three percent of finished waters in the United States exceed the SMCL for sulfate; however, some community drinking waters in South Dakota may contain up to 1,350 mg sulfate/L, with private wells reaching 2,000 mg sulfate/L (13). In the case of some surface waters with low sulfate concentrations, introduction of metallic sulfate coagulants in water purification schemes to treat turbidity will actually increase the sulfate concentration over background levels. Interestingly, however, results of controlled kinetic studies on sulfate-reducing bacteria (14) support the possibility that these organisms could thrive in distribution systems even at very low concentrations of constantly available sulfate. Natural systems have been characterized wherein dissolved sulfate was nearly nonexistent but sulfate-reducing activity was possible owing to mineral reservoirs of sulfate that slowly diffused into the water phase (15). It appears that sulfate reduction within mature biofilms may proceed at rates unimpeded by sulfate diffusion limitations (15). Therefore, sulfate-reducing bacteria seem suited for persistence and activity within biofilms of water distribution systems.

Table 4. Selected Chemical Constituents of Specific Surface and Groundwater Supplies[a,b]

Parameter (mg.L)	Water Source				
	A[c,d]	B[e,d]	C[f]	D[g]	E[h]
Sulfate	149	32	1,800	31	310
Sulfide	–[i]	–	40	ND[j]	ND
Iron	ND	ND	3.4	0.47	3.8
Total dissolved solids	432	232	2,900	445	882
Hardness (as $CaCO_3$)	207	95	2,180	391	730

[a,b]Ref. (37,38).
[c]A: Metropolitan Water District of Southern California Diemer water filtration plant effluent derived from a blend of Northern California (State Project) and Colorado River surface waters (2000).
[d]Metropolitan Water District data shown are average values.
[e]B: Metropolitan Water District of Southern California Mills water filtration plant effluent derived from Northern California (State Project) surface water (2000).
[f]C: Crawford County, Ohio deep well (350 feet), limestone stratum (1997).
[g]D: Crawford County, Ohio shallow well (32 feet), sandstone stratum (1997).
[h]E: Crawford County, Ohio, shallow well (60 feet), sand and gravel stratum (1997).
[i]–: No data.
[j]ND: Not detected.

It is difficult to separate chemical and microbiological discussions on the consequences of iron and sulfur in the groundwater environment. The phenomenon of "ferric clogging" (16), which was alluded to earlier as a detriment to maintaining well pumping yields, is generally thought to involve a number of bacterial genera including *Gallionella*, *Sphaerotilus*, *Siderocapsa*, *Toxothrix*, *Crenothrix*, *Clonothrix*, *Siderococcus*, *Thiobacillus*, and, most probably, some of the slime-producing manganese bacteria. Many of the aforementioned genera have not been characterized by axenic culture studies.

The presence of dissolved iron under sulfate-reducing conditions can actually reduce the odor accompanying dissolved hydrogen sulfide through iron sulfide precipitation if rates of formation exceed those of sulfate reduction (12). Usually, however, sulfate-reducing and sulfur-oxidizing bacteria, under gradients of anaerobic to microaerophilic regimes, act in well water systems to cooperatively promote corrosion of metal surfaces (Fig. 2), and by virtue of the presence of these bacteria and their acidic by-products, for example, acetic acid together with hydrolyzable sulfates and insoluble mineral products of chemical action, for example, iron sulfides, lend to rapid deterioration of screens and casings (16).

Distribution System

Potable water distribution systems are subject to invasion by microorganisms through connections with "open reservoirs; enclosed, unchlorinated reservoirs; new construction that disturbs the existing distribution system; main breaks (which will become an increasing problem as systems age); back pressure; dead ends in mains and stagnant water; living organisms that protect bacteria but that may release

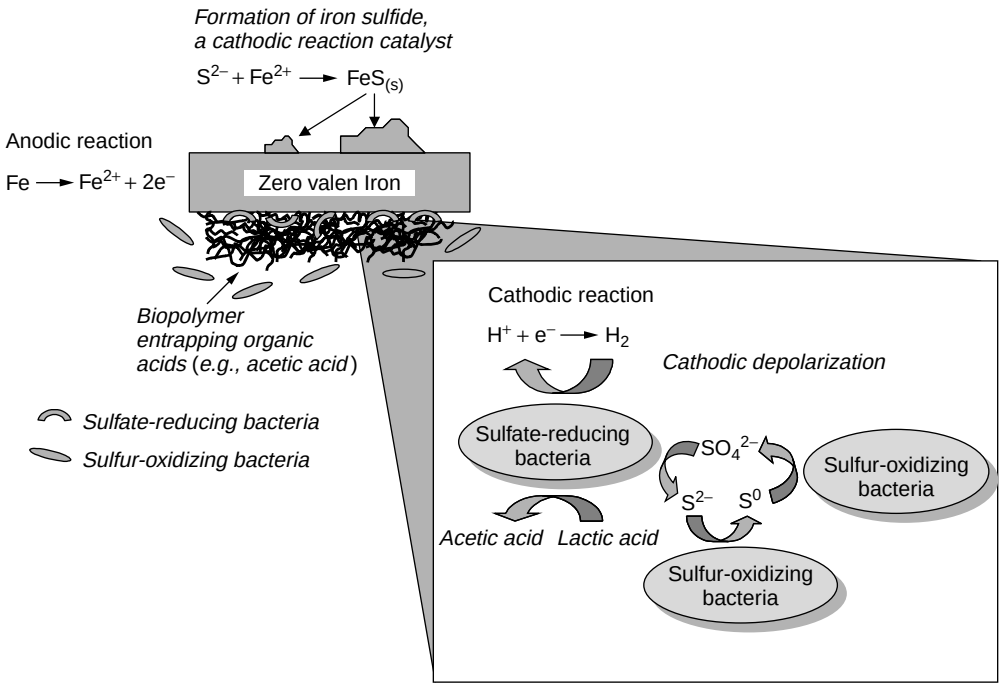

Figure 2. Anaerobic corrosion of iron with subsequent formation of sulfide. Sulfate-reducing bacteria are embedded in oxygen-deficient regions of the biopolymer close to the metal surface and promote the flow of electrons to protons by oxidizing hydrogen via. reduction of sulfate. In addition, acetic acid formed in the transformation of lactic and pyruvic acids create an aggressive environment within the biofilm. Sulfide may precipitate ferrous iron in the vicinity of the metal surface or be transformed to sulfate by the action of sulfur-oxidizing bacteria.

bacteria into the drinking water when mains are disturbed; and sewage cross-connections" (17). In addition, entry of bioavailable organic matter, use of deteriorative construction materials in piping and fittings, insufficient or intermittent maintenance of a protective disinfectant residual, and water temperatures in excess of 15 °C can augment the survival and development of microbial communities (18,19). The water treatment facility proper may include unit processes, for example, sand filters and activated carbon columns, that promote nonspecific bacterial proliferation and a source of organisms entering the distribution system. *Thiobacillus* spp. have been suggested to be capable of survival on sulfur-containing caulking compounds in water pipes (20). Despite the well-recognized ability of sulfate-reducing bacteria to develop on metallics used in potable water systems (21) and the documented presence of sulfate-reducing bacteria in corroding mains (22,23), understanding the role of these organisms in distribution system biofilms remains topical among the overall research needs in drinking water microbiology (24–26).

Potable water distribution systems carry oxygen-containing waters and the oxidation-reduction potential, especially in loop systems, may be too positive for the development of sulfate-reducing bacteria. Furthermore, some mains are constructed of noncorrosive materials, for example, asbestos cement and polyvinyl chloride. Nevertheless, cast iron ductile piping and metallic appurtenances are in major use in modern distribution systems and are susceptible to biofilm formation and corrosion. In these regimes, the sulfate-reducing bacteria exist deep in occlusions formed on the surface of piping and where the presence of dissolved oxygen and a disinfectant residual is minimal or absent (27). These occlusions appear in the form of iron tubercles and biofilms and set up chemical gradients, which can promote corrosion reactions. Continued development of tubercles over time may result in severe reduction of water flow through the pipe. Microscopic examination of tubercles has revealed the presence of bacteria of varied morphologies including (28) or not including (23) filamentous bacteria. Sulfate-reducing bacteria were detected in 80% of iron tubercles sampled from cast iron water distribution piping in Columbus, Ohio, and their presence correlated strongly with background heterotrophic pour plate counts (29). A cultural enumeration of microbial groups, including sulfur bacteria, present in source (untreated) water and a distribution system transporting very low temperature and soft (treated) water is presented in Table 5 (30). Recovered from iron tubercles in larger numbers than sulfate-reducing bacteria were sulfide-forming chemoorganotrophs, for example, *Clostridium* spp. and species of *Enterobacter* and *Klebsiella*, which reduce sulfite and thiosulfate, respectively (30). Chemoorganotrophs must have a supply of preformed organic matter for sustained existence. Even sulfate-reducing bacteria apparently utilize specific by-products of anaerobic fermentation, for example, lactate, as the primary electron donor in respiration as opposed to cathodically generated hydrogen (31), thus underscoring the importance of bioavailable organic compounds for respiration by these organisms. It has been suggested that corrodible pipes carrying dissolved organic matter are at greater risk of deterioration by sulfate-reducing

Table 5. Microbial Composition of Water Samples and Pipe Corrosion Deposits

	Untreated Water (Aug. 1989)		Untreated Water (March 1990)		Treated Water March (1990)		Corrosion Tubercles	
	20 °C	8 °C	20 °C	8 °C	20 °C	8 °C	20 °C	8 °C
Aerobic SPC*	2.2×10^4	1.5×10^5	3.2×10^3	9×10^1	20	ND	2.9×10^7	2.0×10^4
Anaerobic SPC*	2.0×10^1	ND	1.0×10^1	ND	<1	ND	3.0×10^4	ND
Total coliforms†	570	350	200	75	<1	ND	5.0×10^4	ND
Fungal SPC‡	4.8×10^4	2.1×10^2	3.5×10^3	2.0×10^2	3	ND	3.0×10^3	7.8×10^2
Iron-reducers§	540	240	70	49	<0.3	<0.3	>24,000	430
Sulfate reducers↑↓	280	130	<3	<3	<0.3	<0.3	>24,000	110
Sulfate reducers↑↓	120	93	4	4	<0.3	<0.3	460	210
Thiosulfate reducers¶	540	170	240	79	9.3	1.5	920	540
Iron oxidizers¶	54	24	7.9	7	<0.3	<0.3	75	64
Presence/absence								
*T. thiooxidans***	P	P	P	P	P	P	P	P
T. thioparus††	P	P	P	P	P	P	P	P

Source: Table reproduced with permission (Ref. 30)

*cfu/ml (water) or, cfu/g (corrosion tubercle), 7 d incubation at 20 °C, 10 d incubation at 8 °C.

†cfu/ml (water) or, cfu/g (corrosion tubercle) 48 h incubation at 35 °C, 10 d incubation at 8 °C.

‡cfu/ml (water) or, cfu/g (corrosion tubercle) 7 d incubation at 20 °C, 10 d incubation at 8 °C.

§Organisms/ml (water) or, cfu/g (corrosion tubercle), by 5-tube MPN, using dilutions of 1.0–0.0001 ml in B_{10} broth, 14 d incubation.

↑↓Organisms/ml (water) or, cfu/g (corrosion tubercle), by 5-tube MPN, using dilutions of 1.0–0.0001 ml in Butlin's broth + either 1% (v/v) Na_2SO_4, Na_2SO_3 or $Na_2S_2O_3$, 14 d incubation.

¶Organisms/ml (water) or, cfu/g (corrosion tubercle) by 5-tube MPN, using dilutions of 1.0–0.0001 ml in modified Winogrudsky's broth, 14 d incubation.

**Presence/absence, using Starkey's medium for sulfur oxidizers + elemental sulfur, pH 3.

††Presence/absence, using Starkey's medium for thiosulfate oxidizers, pH 6.

ND = not determined.

action than ones that transport waters essentially free of bioavailable organic matter (32).

Home Water System

Difficulties attributed to bacterial reduction of sulfur species, especially taste and odor, water discoloration, and metal corrosion, may occur in the household water pipes, fixtures, and appurtenances. Corrosion not only destroys the metallic parts directly but also permits release of heavy metal ions, for example, copper, into the drinking water. The hot water outlet of the household water heater has been identified as a site of sulfate-reducing bacterial activity in situations in which malfunction or intentional lowering of the thermostat results in sublethal temperatures to the bacteria. Also implicated as a contributing factor to sulfide generation is the magnesium rod sometimes inserted in the water heater to act as a sacrificial anode. Electrons released from the rod can combine with protons to produce hydrogen, which serves as an energy source for the bacteria with consequent reduction of sulfate to hydrogen sulfide. Aluminum and zinc rods have been employed in place of magnesium to reduce the rate of electron flow.

CONTROL OF SULFUR BACTERIA

Concerns about the presence of sulfur bacteria in potable water systems are principally directed at sulfate-reducing bacteria. Attempts to curtail the activities of these organisms in drinking water require procedures applicable to the specific task at hand.

Wells

A popular method aimed at eliminating general bacterial populations, including sulfur bacteria, from individual and community well water systems is shock chlorination. Typically, sufficient chlorine (Cl_2) is added to the well to produce a concentration of 200 mg Cl_2/L; a concentration that is arrived at by determining the volume of water in the well and the concentration of chlorine in the disinfectant employed. The volume of water in the well is estimated from the diameter of the well casing and the depth of the well. Shock chlorination is practiced for newly constructed wells and well systems that have undergone repair or were subject to flooding. Before shock chlorination, systems should be thoroughly cleaned and pumped to remove dislodged suspended matter from the well screen and casing. The disinfectant should remain in contact throughout the system for at least 12 to 24 hours followed by backflushing to remove all chlorine from the well and distribution system. It is important to note that sulfide species exert a chlorine demand. Therefore, a determination of soluble sulfide should be made and allowance of 2 mg Cl_2/L made for each 1 mg sulfide/L. In addition, other chlorine demand may exist in the form of reduced iron and manganese and organic matter.

As previously noted, sulfate-reducing bacteria are suppressed in contact with dissolved oxygen. Increased aeration or ozonation of well water systems has been recommended in this regard.

Public Distribution Systems

As is the case for microorganisms in general, controlling the proliferation of sulfur bacteria in water distribution networks is a matter of prevention rather than remediation. Since major detriments that may involve sulfur bacteria are likely to be associated with the presence of biofilms and tubercles, preventive measures to preclude formation of such deposits on the walls and joints of piping should be exercised. Particularly important in this regard are (*1*) maintenance of uninterrupted water flow and positive pressure at all times, (*2*) maintenance of a protective disinfectant residual, and (*3*) maintenance of dissolved oxygen, particularly, as pertains to suppression of sulfate-reducing bacteria. The hydraulic features of the distribution system are important because soil–water infiltration and accompanying bacterial and organic matter contamination of the potable water, may occur under low pressure and extensive formation of microbial films are promoted in low water flow (stagnating) systems (33). The type of disinfectant employed in water purification may factor in the establishment of a microbial consortium in the distribution system through, for example, alteration of complex organic matter to a bioavailable form (chlorine, ozone), inability to act as a protective or persistent residual (ultraviolet radiation, ozone), rapid dissipation in reactions with organics and metals (chlorine), and ineffective biocidal action at low concentrations (chloramine; 34).

CONCLUSION

Notwithstanding the well-recognized contribution of sulfate-reducing bacteria to the anaerobic corrosion of metals, the role of these organisms and those of the sulfur bacteria in all phases of potable water production remains to be elucidated. In addition, much of the available literature reporting the existence of filamentous sulfur bacteria among microbial residents of biofilms and tubercles in water systems may be questioned with respect to the accuracy of the identification of the organisms and the absence of quantitative data on their existence. Curiously, modern detailed investigations directed at revealing and characterizing the microbial composition of biofilms in distribution systems, generally, have not included specific efforts to detect sulfur bacteria(34). Oligonucleotide probes have been employed in the analysis of the microbial composition of biofilms recovered from three locations in a German water distribution system and it was found that the γ-subgroup of the Proteobacteria, to which certain filamentous sulfur bacteria belong, was a very minor percentage of the total cells; δ-probe was not used(35). Application of modern microbiological analytical techniques to the specific determination of the types and roles of sulfur bacteria in areas of biofilm and tubercle formation is needed and until the results of such studies become available, the extent to which these nuisance organisms are a liability to the water industry will be based largely on circumstantial evidence.

Acknowledgment
The assistance of Dr. Richard A. Royer in the preparation of the figures is appreciated.

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SULFUR CYCLE. See SEDIMENTS: SULFATE REDUCTION IN MARINE SEDIMENTS

SULFUR CYCLE IN SOILS

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GLOBAL SULFUR CYCLE

Sulfur is an essential element for the growth and activity of all living organisms. It is one of the ten major bioelements required by organisms in relatively high concentrations (i.e., $> 10^{-4}$ M). Sulfur also is an important source of metabolic energy for many bacteria. For example, certain chemoautotrophic (i.e., chemolithotrophic) bacteria obtain energy for cell growth and division by oxidizing reduced sulfur compounds.

Microorganisms are an important component of the global sulfur cycle. Sulfur is volatilized as hydrogen sulfide, carbonyl sulfide, dimethyl sulfide, and so on from

marine algae, marshlands, mud flats, plants, and soil, with dimethyl sulfide being the principal sulfur compound entering the atmosphere. Dimethyl sulfide is converted photochemically to methanesulfonic acid, which is degraded by a wide range of bacteria to carbon dioxide and sulfate (1). Plants primarily assimilate sulfate (2) that is then immobilized as sulfoquinovosyl diacylglycerol (SQDG) or volatilized as dimethyl sulfide (3). Microorganisms degrade SQDG releasing sulfate and closing the terrestrial sulfur cycle (Fig. 1) (4). However, most of the sulfur that cycles through the atmosphere is due to human activities. In fact, since the industrial revolution, increased burning of fossil fuels has almost doubled the rate of sulfur entering the atmosphere by approximately 1.5×10^{11}-kg sulfur per year. Soils subject to atmospheric pollution receive sulfur from the atmosphere largely in the form of dilute sulfuric acid. Thus, sulfate is the main sulfur ion entering soils from the atmosphere; smaller quantities of sulfite and bisulfite may also contaminate these soils. Atmospheric pollution deposits, consisting largely of soot, may also be locally important sources of reduced sulfur compounds particularly in areas adjacent to industrial plants, such as coking and steel works. Because sulfate is the major sulfur ion entering soil from atmospheric pollution, the major sulfur transformations will involve sulfur mineralization and sulfur reduction rather than sulfur oxidation. However, reduced sulfur compounds deposited from atmospheric fallout are rapidly oxidized.

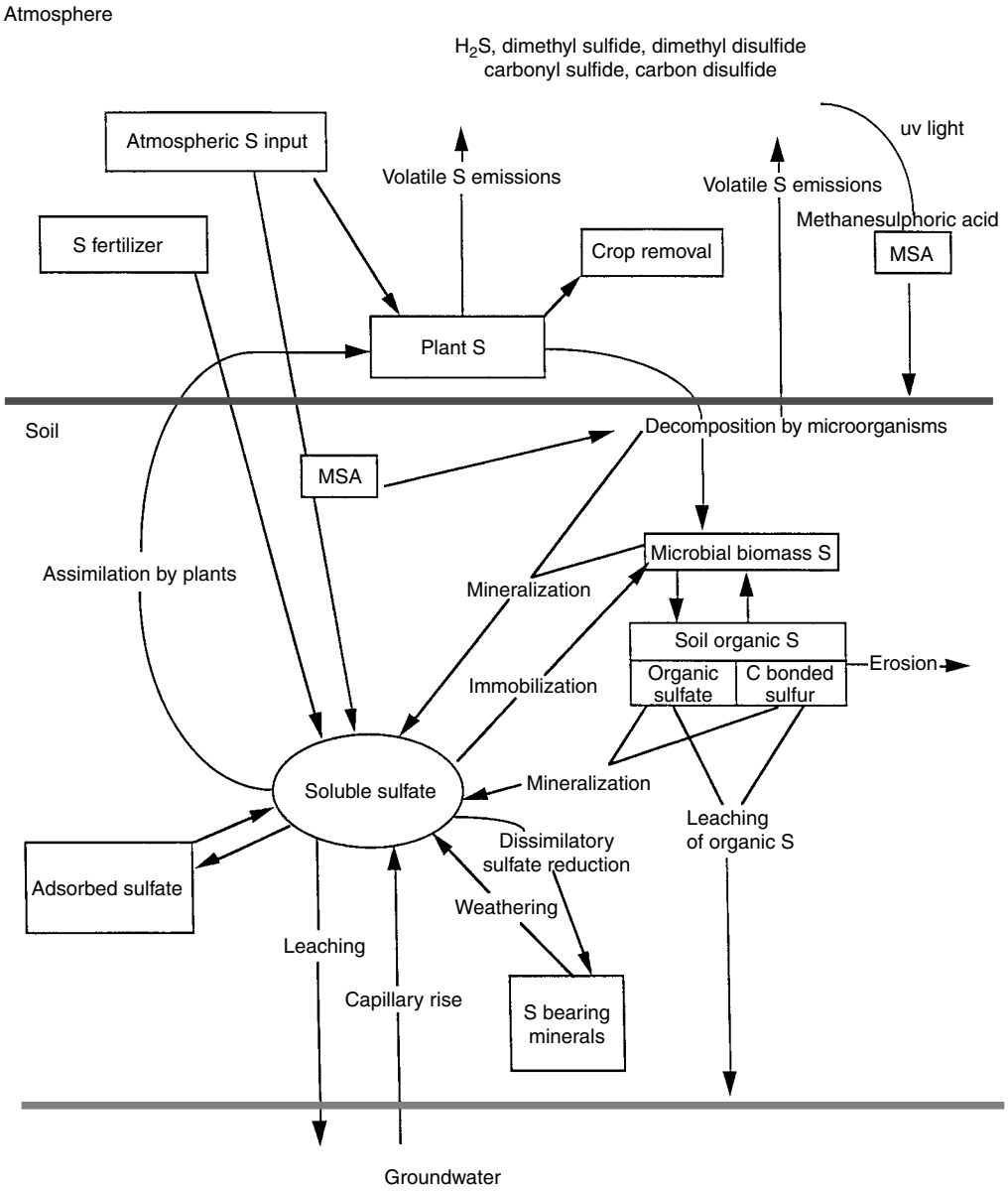

Figure 1. Terrestrial sulfur cycle. Modified from J. J. Schoenau and J. J. Germida, in R. W. Howrarth, J. W. B. Stewart, and M. V. Ivanov, eds., *Sulfur Cycling on the Continents: Wetlands, Terrestrial Ecosystems, and Associated Water Bodies-SCOPE 48*, John Wiley & Sons, Chichester, U.K., 1992, pp. 261–277.

Table 1. Amounts and Distribution of Sulfur in Some World Soils

Location	Type of Soil	Total Sulfur ($\mu g\ g^{-1}$)
Alberta, Canada	Forest	364–1,593
Brazil	Agricultural	43–398
British Columbia, Canada	Agricultural	214–438
	Forest	162–2,328
	Grassland	286–928
	Organic	1,122–30,430
Carolinas, U.S.	Tidal March	3,000–35,000
	Forest	539–1,344
Chilean	Agricultural	129–926
Eastern Australia	Agricultural	38–545
Florida, U.S.	Forest	31–104
Germany	Forest	74–526
Hawaii, U.S.	Volcanic	180–2,200
Illinois, U.S.	Forest	280–555
Iowa, U.S.	Agricultural	57–618
New Hampshire, U.S.	Forest	452–1,563
New York, U.S.	Forest	119–1,780
Nigeria, Africa	Agricultural	25–177
Quebec, Canada	Forest	200–1,003
Saskatchewan, Canada	Agricultural	88–760
Tennessee, U.S.	Forest	129–243

Source: adapted from E. A. Paul, and F. E. Clark, *Soil Microbiology and Biochemistry*, Academic Press, San Diego, Calif., 1989 and R. W. Howarth, J. W. B. Stewart and M. V. Ivanov, *Sulfur Cycling on the Continents, Wetlands, Terrestrial Ecosystems, and Associated Water Bodies-SCOPE 48*, John Wiley & Sons, Chichester, U.K., (1992).

The nature and quantities of the various sulfur pools in surface soils are the basis for sulfur cycling in terrestrial environments. Pedogenic factors such as climate, regional vegetation, and local topography influence these sulfur pools. For example, the total sulfur content of soils ranges from 0.002 to 10%, with the highest levels found in tidal flats, saline, acidsulfate, and organic soils. To a large extent, the impact of pedogenic factors on sulfur pools in soil can be attributed to alterations in pH, cationic nutrients, and microbial activity (5,6). The comparison of sulfur values of soils from diverse geographic areas illustrates the importance of pedogenic factors in total sulfur concentrations Table 1 (7,8).

Nature and Forms of Organic and Inorganic Sulfur in Soil

Organic sulfur constitutes more than 90% of the total sulfur present in most surface soils. However, because of the polymeric nature of many organic sulfur compounds in soil, their chemical structure is not yet clear. Organic sulfur is grouped into two broad categories — organic sulfates and carbon-bonded sulfur (C–S). Organic sulfates (R–O–S) include sulfate esters (C–O–S), sulfamates (C–N–S) and sulfated thioglycosides (N–O–S). Organic sulfates constitute 30 to 75% of total organic sulfur in soil. C–S includes the sulfur present in amino acids, proteins, polypeptides, heterocyclic compounds (e.g., biotin and thiamin), sulfinates, sulfones, sulfonates, and sulfoxides (Fig. 2). A large portion of C–S present in soil is yet to be identified; however, in some cases, the C–S of amino acids may constitute up to 30% of the organic sulfur in soil. Carbon-bonded sulfur is more stable than organic sulfates and not liable to chemical hydrolysis. One particular sulfonate of interest is the sulfolipid, SQDG, which is one of the most abundant sulfur-containing organic compounds after the amino acids, glutathione, cysteine, and methionine (3). A variety of photosynthetic and nonphotosynthetic organisms use SQDG as a low phosphate replacement of anionic lipids under phosphate-limiting conditions.

Inorganic forms of sulfur account for less than 25% of the total sulfur in most agricultural soils. Sulfur exists in

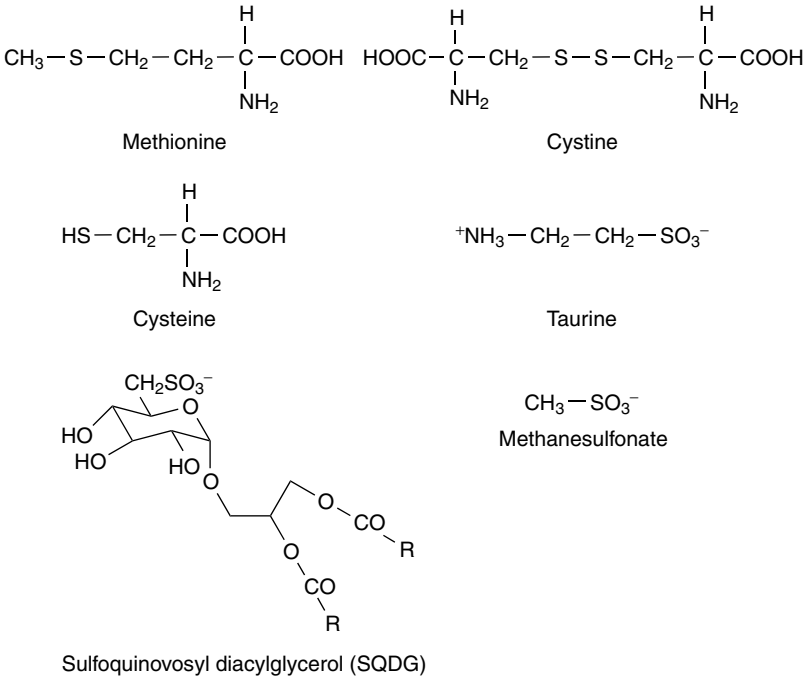

Figure 2. Examples of important organic sulfur compounds containing C–S bonds in the terrestrial environment.

Table 2. Important Forms of Sulfur and Their Oxidation States

Compound	Formula	Oxidation State(s) of Sulfur
Sulfide	S^{2-}	−2
Polysulfide	S_n^{2-}	−2,0
Sulfur[a]	S^0	0
Hyposulfite (dithionite)	$S_2O_4^{2-}$	+2
Sulfite	SO_3^{2-}	+4
Thiosulfate[b]	$S_2O_3^{2-}$	−1,+5
Dithionate	$S_2O_6^{2-}$	+6
Trithionate	$S_3O_6^{2-}$	−2,+6
Tetrathionate	$S_4O_6^{2-}$	−2,+6
Pentathionate	$S_5O_6^{2-}$	−2,+6
Sulfate	SO_4^{2-}	+6

[a]Occurs in an octagonal ring in crystalline form.
[b]Outer *S* has a valence of −1 inner *S* has a valance of +5.
Source: Adapted from A. Vairavamurthy, B. Manowitz, G. W. Luther III, and Y. Jeon, *Geochim. Cosmochim. Acta* 57, 1,619–1,623 (1993).

a number of forms with a wide range of oxidation states (Table 2) (9). Sulfide, S^0, sulfite, thiosulfate, tetrathionate, and sulfate are the main forms of inorganic sulfur in agricultural soils. Sulfate is the most common form of inorganic sulfur found in well–aerated agricultural soils. In contrast, sulfides account for less than 1% of total sulfur, and measurable quantities of thiosulfate and tetrathionate are usually detected only in soils treated with sulfur fertilizer or those receiving pollutants. The adsorption of inorganic sulfur to soil is highly pH-dependent, with little or no sorption of sulfate observed at pHs greater than 6.0.

MICROBIAL TRANSFORMATIONS OF SULFUR (SEE ALSO SULFUR CYCLE IN THE MARINE ENVIRONMENT, this Encyclopedia)

Microbial transformations of sulfur are the result of organisms metabolizing sulfur-containing compounds as a source of sulfur for their own anabolic needs, or else, sulfur- containing compounds are used as electron acceptors or donors. This activity regulates the fluxes of sulfur between different sulfur pools in soil (inorganic sulfate, labile organic sulfur, and resistant organic sulfur pools). It can also lead to losses of sulfur from soil by converting complex organic sulfur compounds into mobile forms that may be lost by leaching or by volatilization to the atmosphere. Much of the organic sulfur present in soils is not readily available for assimilation by microorganisms or plants. In fact, it has been shown that organic sulfur added to soil must first be processed by microorganisms to sulfate before plants can assimilate it (2). This sulfur is rapidly immobilized into the microbial biomass as various forms of inorganic and organic sulfur and then remineralized as predators consume microbes. However, the mineralization of immobilized sulfur is not complete and a small fraction of the sulfur enters the "resistant" pool of organic sulfur present in soil (10). Organic sulfur present in the resistant pool is normally found in large (> 5, 000 Da) polymers that must be depolymerized before the sulfur can be mineralized (11). The depolymerization of these polymers is not energetically favorable and many of these polymers are protected from microbial attack by their close association with clay in soil aggregates. Thus, there is a continuum of organic sulfur complexes in soils ranging from very old, stable (e.g., organic sulfur found in soil humus) fractions to very young, short-lived (e.g., organic sulfur found in the amino acid cysteine) fractions. The microbial biomass is the engine for the conversion of passive fractions into active fractions (and vice versa) (Fig. 3) (12).

Assimilation and Mineralization of Sulfur

Biological mineralization and immobilization are processes that occur concurrently and exhibit a strong relationship with the soluble sulfate pool in the soil. Immobilization occurs as a result of the microbial assimilation of nutrients that are then rendered unavailable for further plant or microbial uptake until the cell dies and is "remineralized." Immobilization of sulfur may also involve precipitation as metal sulfide, especially pyrite, as in salt marshes. Soil factors that influence the growth and activity of microorganisms (such as pH, temperature, and moisture) will also affect the rate of sulfur transformations because microbes mediate these transformations. To estimate or predict the available sulfur status of soils, it is necessary to understand the factors that influence these processes.

Assimilation (immobilization)

Microbial assimilation and conversion of inorganic SO_4^{2-} into organic sulfur through the assimilatory sulfate reduction pathway leads to temporary immobilization of sulfur from plant or microbial availability. This process involves ATP sulfurylase and two energy-rich sulfate nucleotides, that is, APS (adenosine 5′-phosphosulfate) and PAPS (3′-phosphoadenosine-5′-phosphosulfate). The overall reaction of SO_4^{2-} incorporation into amino acids is depicted in Figure 4 (13). Typically, the addition of inorganic SO_4^{2-} to soil leads to its quick incorporation into the organic sulfur fractions via microbial assimilation. The presence of an energy source (e.g., the presence of labile organic matter or addition of easily degradable carbon sources such as glucose) increases sulfur assimilation. Later, much of this accumulated sulfur is found in the fulvic acid fractions, especially as organic sulfates.

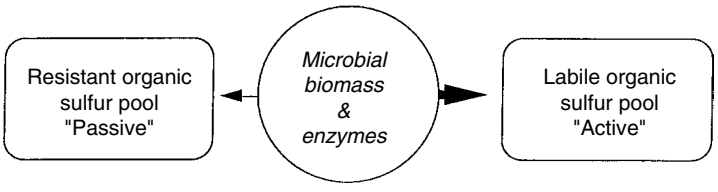

Figure 3. Microbially mediated cycling of organic sulfur between the passive and active sulfur pools in soil. The relative flux of sulfur between pools is reflected in the arrows depicting microbial conversion (12).

Microbial assimilation of aromatic sulfonates is controlled by the sulfate stimulation-induced (SSI) operon. The SSI genes are upregulated in the presence of aromatic sulfonates, but repressed by the presence of sulfate, cysteine, or thiocyanate (14). Aromatic sulfonates are extracellularly bound and transported across the cell membrane. Once inside the cytoplasm, the C–S bond is cleaved by a monooxygenase, and the sulfite produced enters the sulfite assimilation pathway at the level of sulfite reductase as depicted in Figure 4 (13). In contrast, the use of aromatic sulfonates as a source of carbon and energy is substrate-induced and involves dioxygenases that are typically encoded on plasmids.

The use of alkane sulfonates as sources of cellular sulfur is mediated by either α-ketoglutarate-dependent dioxygenases, used only for taurine or $FMNH_2$-dependent monoxygenases, used for methanesulfonate and other alkanesulfonates. Taurine desulfurization involves a dioxygenase, which is only used with taurine, that introduces molecular oxygen into the sulfonate and α-ketoglutarate that degrades to aminoacetaldehyde. This reaction, mediated by *TauD* is specific for taurine, with no desulfonation activity seen for alkane sulfonates less than C6. In contrast, the sulfonate monooxygenase *SsuD*/*MsuD* is used for a variety of alkane and aromatic sulfonates, with only a single molecular oxygen being introduced into the sulfonates (Fig. 5).

Most of the sulfur accumulated by microorganisms is in the form of amino acids in proteins; however, microorganisms also accumulate sulfate-esters, sulfonates, vitamins, cofactors, and so on. Some microorganisms such as fungi accumulate large amounts of sulfate-esters in particular. This is important because organic sulfates (e.g., sulfate-esters; thioglucosides) are considered to be the most labile form of organic sulfur in the soil, and may comprise up to 30 to 70% of the organic sulfur in surface soils. The relative proportion of fungal biomass to bacterial biomass in the soil (ca. 2 : 1) underscores the potential importance of microorganisms accumulating ester-sulfur compounds.

The assimilation of sulfate-esters occurs through a variety of enzymes that can be classified as alkaline sulfatases with pH optima of 8.3 to 9.0 or as acid sulfatases with pH optima of 6.5 to 7.1. These arylsulfatases contain an active glycine site. This glycine is hydrated and attacks the sulfate ester, breaking the sulfur–oxygen bond and forming a brief covalent bond with the SO_3 group (15). The sulfite enzyme complex decomposes to regenerate the enzyme and sulfate (15). Almost all arylsulfatases are repressed by the presence of inorganic sulfate indicating that these enzymes are involved in the assimilation of sulfur from sulfate esters (14). In contrast, only two alkylsulfatases, lithocholate sulfate sulfatase (16), and cholinesulfatase (17) are involved in sulfur assimilation with the remainder of the known alkylsulfatases providing carbon and energy to the organism. Novel arylsulfatases are postulated for those organisms that are able to anaerobically assimilate sulfur from sulfonates but do not possess the typical arylsulfatases (18).

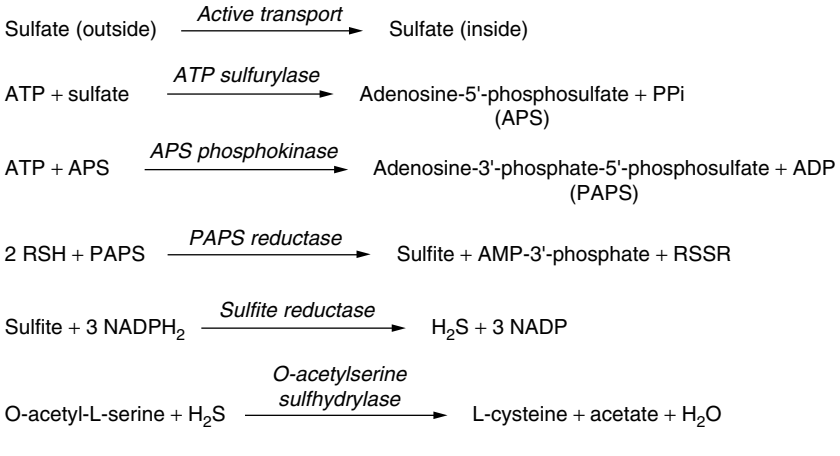

Figure 4. Assimilatory reduction of sulfate and formation of cysteine. RSH stands for thioredoxin; its reduced form is regenerated from the oxidized form (RSSR) through reduction by $NADPH_2$ [13].

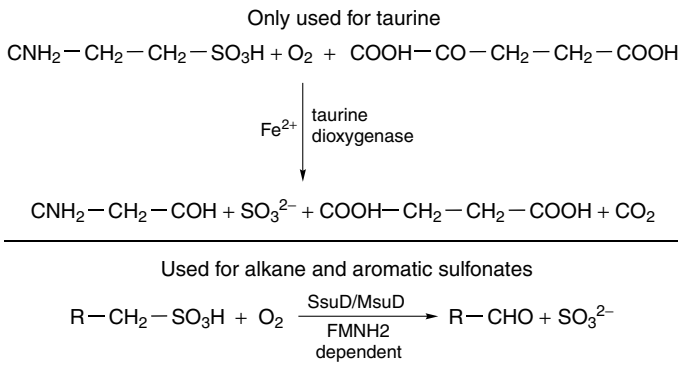

Figure 5. Two different pathways of organosulfonate assimilation. The sulfite produced by both systems is used in the typical cysteine pathway.

Mineralization

Mineralization of organic sulfur in soil is mediated by soil microorganisms. The various known pathways of sulfur mineralization are summarized in Figure 6 (19). Carbon–bonded sulfur is mineralized either through oxidative (aerobic) decomposition or desulfurization (anaerobic) processes, whereas various sulfatases are involved in the mineralization of sulfate–esters. The mineralization process may be direct (i.e., biological mineralization), involving viable microbial cells, or indirect (i.e., biochemical mineralization), involving extracellular sulfatase enzymes. In the case of direct mineralization, elements (e.g., nitrogen, and sulfur) in direct association with carbon are mineralized as microorganisms oxidize the organic carbon compounds to obtain energy. Heterotrophic soil microorganisms decompose organic sulfur compounds to grow, and as the C–S bond is broken, the sulfur is released, usually as sulfide. Because this process involves actively growing microorganisms, their requirement for sulfur may meet or even exceed the sulfur supplied by the substrate. Thus, the net mineralization of sulfur by this process may not be reflected by increases in the sulfate-sulfur pool in soil. In the case of indirect mineralization, those elements that exist as sulfate-esters are hydrolyzed by intracellular or extracellular enzymes. This process, also known as enzymatic mineralization, occurs mainly outside the cell and may be regulated by end-product inhibition (i.e., the level of SO_4^{2-}). Other pedogenic factors such as percentage of organic carbon and soil type have a significant influence on indirect mineralization. For example, activities of arylsulfatases in four different soil types varied between 79 and 308 μg *p*-nitrophenol $g^{-1}h^{-1}$ (20). Furthermore, the dependence on the reaction rate of sulfur concentrations also varied between soils indicating that the enzymes themselves may also differ between soil types (21). Direct mineralization is controlled by the microbial need for carbon and energy sources, whereas indirect mineralization is controlled by factors influencing enzyme synthesis, activity, and kinetics.

Mineralization is generally measured as net mineralization, that is, the amount of SO_4^{2-}-sulfur accumulated during the period under study or the differences between gross mineralization and assimilation. Thus, for higher net mineralization to occur, the mineralization-assimilation process has to be driven toward mineralization. A breakeven point for mineralization and immobilization can be calculated based on C : S ratio of the substrate, the decomposer organisms, and the yield coefficient. For example, if we consider the decomposition of crop residues, net mineralization will generally occur with a C : S ratio of 200 or less, whereas net sulfur immobilization occurs when the ratio is greater than 400 : 1. Because microbial activity is the driving force for these two processes, they are significantly influenced by all factors affecting microbial metabolism; energy and nutrient supply, C : S ratio, abundance of organic sulfur, water availability, pH, temperature, and redox potential (Eh).

For example, actively growing plants may significantly increase sulfur mineralization in soils. Plants supply energy sources to the rhizosphere in the form of root exudates, which increases microbial growth and activity, thus increasing sulfur mineralization. However,

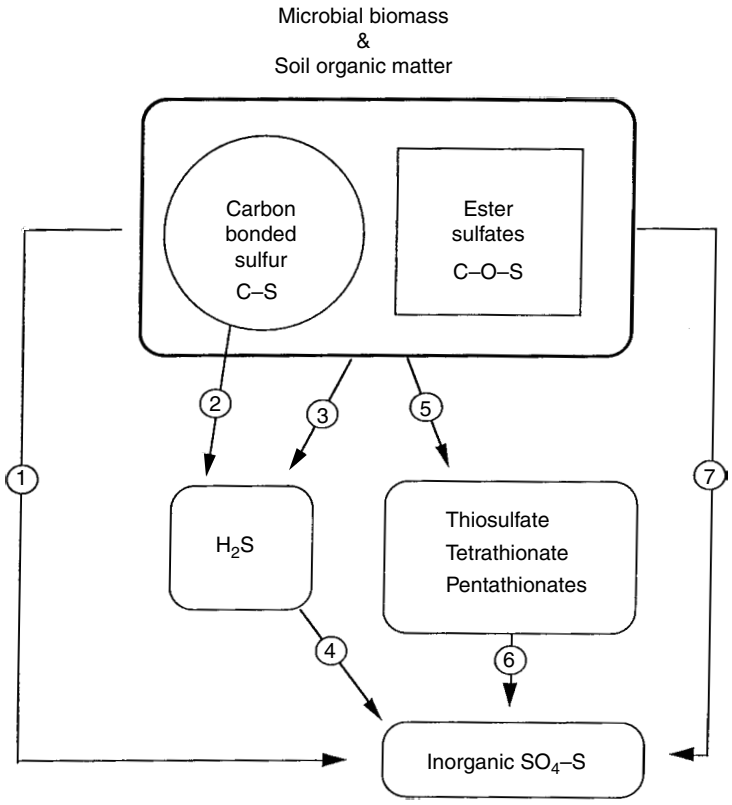

Figure 6. Conceptual pathways for the mineralization of organic sulfur compounds in soil: (1) biological (direct) mineralization during the oxidation of carbon as an energy source; (2) hydrolysis of cysteine by cysteine desulfhydrolase; (3) anaerobic mineralization (desulfurization) of organic matter; (4) biological oxidation of hydrogen sulfide and sulfite; (5) incomplete oxidation of organic sulfur into inorganic sulfur compound; (6) biological oxidation of tetrathionate to sulfate through sulfide; and (7) biochemical (indirect) mineralization when sulfate esters are hydrolyzed by sulfatases (19).

the reassimilation of inorganic sulfates released by the growing microorganisms may not result in an increase in the "plant available" sulfur pool and may even result in reduction of the "plant-available" sulfur pool when the microbial demand exceeds the rate of sulfur mineralization (2). A good example of this can be seen in a study by Ghani and coworkers (22) in which the effect of glucose amendments on the mineralization of sulfate was compared. These authors found that 50% less sulfate was mineralized by an active microbial biomass when compared with a more dormant community. The mineralization and/or assimilation balance can also be shifted by the amount of available sulfate in the soil (6). Soils that have low amounts of available sulfur rapidly assimilate radiolabeled sulfate that is added to it. However, in the presence of added sulfate, little of the biomass is labeled with the radioisotope tracer indicating that the microbial community is not assimilating the added sulfate (10).

The direct mineralization of aromatic sulfonates can occur by two major pathways and results in the release of sulfite from the aromatic sulfonate as shown in Figure 7. In the first pathway (Fig. 7a), sulfate is removed from the aromatic ring before cleavage by the action of a dioxygenase. This type of pathway has been established in an *Alcaligenes* sp. and *Comamonas testosteroni* strain during the metabolism of *p*-toluenesulfonate (23,24). Alternatively, the sulfate can be removed after ring cleavage by a hydrolytic reaction (Fig. 7b) as is typified by the pathway elucidated in *Hydrogenophaga palleronii* SI and *Agrobacterium radiobacter* S2 (25).

Direct mineralization can also occur under anaerobic conditions. Initially, the ability of organisms to use organic sulfonates as a terminal electron acceptor was discounted as experimental error. However, in recent years it has become evident that a wide range of bacteria can use sulfonates as terminal electron acceptors and some strains can ferment these compounds (26–28). The fermentation of sulfonates results in the release of thiosulfite, whereas the use of sulfonates as terminal electron acceptor releases sulfide as an end-product. It appears that the use of sulfonates or sulfates as terminal electron acceptors occurs by two discrete regulatory pathways with cells accustomed to using sulfate as a terminal electron acceptor, preferring sulfate to sulfonate and vice versa. In addition to their use as terminal electron acceptors, some sulfonates can serve as electron donors with nitrate acting as an electron acceptor (29). This unique reaction performed by an *Alcaligenes* sp. links the sulfur cycle to the nitrogen cycle, with taurine acting as an electron donor. Sulfate and nitrogen gases are produced as a result. The prevalence of sulfonate-using denitrifying bacteria remains to be seen.

MICROBIAL OXIDATION OF INORGANIC SULFUR COMPOUNDS

The abiotic oxidation of reduced sulfur compounds occurs to a limited extent in soils, however microbial reactions are clearly the dominant mechanisms for sulfur oxidation. For example, the biological oxidation of sulfide in soils apparently takes place via the following sequence of reactions although some of the products may result from abiotic side reactions

$$S^0 \rightarrow S_2O_3^{2-} \rightarrow S_4O_6^{2-} \rightarrow SO_4^{2-}$$

Many different microorganisms are important for the oxidation, reduction, and cycling of sulfur in the soil and other ecosystems (Table 3) (30). In the case of sulfur oxidation, the microorganisms can be divided into the following groups

- chemoautotrophs (lithotrophs) such as members of the genus *Thiobacillus*
- photoautotrophs, including species of purple and green sulfur bacteria (photosynthetic bacteria)
- chemoheterotrophs (organotrophs), including a wide range of bacteria and fungi

The chemoautotrophs and heterotrophs are largely responsible for oxidizing sulfur in most aerobic, agricultural soils.

Many chemoautotrophic bacteria (e.g., thiobacilli) are capable of oxidizing reduced inorganic sulfur compounds. The biochemistry of sulfur oxidation by thiobacilli

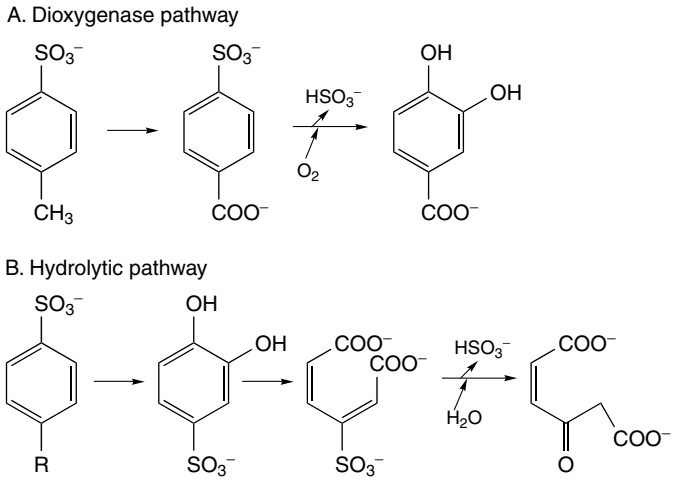

Figure 7. Two different mechanisms, dioxygenase (**a**), or hydrolytic (**b**) desulfonation of aromatic sulfonates during microbial utilization on these compounds for carbon and energy.

Table 3. Sulfur-Using Bacteria Occurring in Soil and Aquatic Habitats

Group	S–Conversion	Habitat Requirements	Habitat Example	Examples of Genera
Heterotrophs that use oxidized S species as electron acceptors	$SO_4^{2-} \rightarrow HS^-$ $S_2O_3^{2-} \rightarrow HS^-$ or S^o $S^o \rightarrow HS^-$ $SO_3 \rightarrow HS^-$	anaerobic; organic substrates available; light not required	anoxic sediments and soils	*Desulfomonas* *Desulfovibrio* *Desulfotomaculum* *Desulfomonas* *Campylobacter*
Obligate and faculative autotrophs that use reduced S as an energy source	$HS^- \rightarrow S^o$ $S^o \rightarrow SO_4^{2-}$ $S_2O_3^{2-} \rightarrow SO_4^{2-}$	H_2SO_2 interface; light not required	mud; hot springs; mine drainage	*Thiobacillus* *Thiomicrospira* *Achromatium* *Beggiatoa*
Phototrophs that use reduced S an electron donor	$HS^- \rightarrow S^o$ $S^o \rightarrow SO_4^{2-}$	Anoxia; H_2S; light	shallow water; anoxic sediments; metalimnion or hypolimnion; anoxic water	*Chlorobium* *Chromatium* *Ectothiorhodospira* *Thiopedia* *Rhodopseudomonas*
Heterotrophs that can disproportionate thiosulfate or sulfonates	$S_2O_3^{2-} \rightarrow SO_4^{2-} + HS^-$ $R\text{-}SO_3 \rightarrow S^0 + SO_4^{2-}$	Anoxia	sediments sewage	*Desulfovibrio sulfodismutans* *Desulfonispora thiosulfatigenes*
Heterotrophs that use organic–S compounds as energy sources or that hydrolyze esters	org S$\rightarrow HS^-$ org S$\rightarrow$ volatile org S ester $SO_4 \rightarrow SO_4^{2-}$	Source of organic–S compounds	sediments; soils; water column	Many
Microorganisms that use SO_4^{2-} or H_2S in biosynthesis	$SO_4^{2-} \rightarrow$ protein $HS^- \rightarrow$ protein $SO_4^{2-} \rightarrow$ DMSP*	nonspecific	sediments; soils; water column	Many

*DMSP, dimethylsulfoniumpropionate.

Source: Adapted from R. B. Cook and C. A. Kelly, in R. W. Howarth, J. W. B. Stewart and M. V. Ivanov eds., *Sulfur Cycling on the Continents, Wetlands, Terrestrial Ecosystems, and Associated Water Bodies-SCOPE 48*, John Wiley & Sons, Chichester, U.K., 1992, pp. 145–188.

growing in vitro has been extensively reviewed, and detailed information can be found elsewhere (31,32). For acidophilic thiobacilli, the most common sequence of reactions involved in sulfur oxidation is as follows

$$S^0 \rightarrow SO_3^{2-} \rightarrow SO_4^{2-}$$

A variety of thiobacilli, ranging from obligate acidophilic chemoautotrophs to facultative chemoautotrophs (thiobacilli that grow autotrophically with reduced inorganic sulfur compounds as energy sources, but are also capable of heterotrophic growth) to mixotrophs (thiobacilli able to utilize mixtures of inorganic and organic compounds simultaneously), can be isolated from natural habitats. The thiobacilli differ in their physiological characteristics and in the reduced sulfur compounds used as energy sources (Table 4) (33–35). The majority of these thiobacilli are obligate aerobes, although some such as *Thiobacillus denitrificans* can grow anaerobically using nitrate as a terminal electron acceptor (36). Other species of thiobacilli use electron donors such as ferrous iron (*T. ferrooxidans*) and thiosulfate (*T. thioparus*) in addition to sulfur. Although thiobacilli can oxidize sulfur to plant-available SO_4^{2-} in some soils, it is evident that this process is also mediated by many different heterotrophic soil microorganisms including the genera: *Thiomicrospira*, *Thiosphaera*, *Thiovulum*, *Beggiatoa*, *Thiothrix*, *Thioploca*, *Thiodendron*, *Thiobacterium*, *Macromonas*, *Achromatium*, *Thiospira*, *Paracoccus*, *Hyphomicrobium*, *Alcaligenes*, *Pseudomonas*, and *Hydrogenobacter*. Some bacteria can oxidize thiosulfate or sulfite and couple this to the reduction of nitrate to ammonia (37).

The view that thiobacilli play the dominant role in sulfur oxidation in soils is largely based on the observation that these bacteria achieve rates of sulfur oxidation in culture far greater than those achieved by heterotrophs growing under the same conditions. The fact that most thiobacilli are facultative or obligate chemoautotrophs means that they are able to oxidize sulfur independent of the supply of available carbon. Marked increases in numbers of thiobacilli generally follow the addition of reduced forms of sulfur to some soils, supporting the concept that populations of thiobacilli are important oxidizers of the added sulfur. However, no consistent correlation has been found between sulfur-oxidation rates and the incidence of thiobacilli, except that rates of sulfur oxidation are generally low in soils that lack these organisms and are accelerated in soil inoculated with thiobacilli. It is probable that in many

Table 4. Characteristics of the Species of Genus *Thiobacillus*

Species	Electron Donor	Electron Acceptor	Facultative Heterotroph	Facultative Anaerobe	pH Optimum
T. thiooxidans	H_2S,S^0,$S_2O_3^{2-}$	O_2	−	−	2.2
T. ferrooxidans	S^0,$S_2O_3^{2-}$, Fe^{2+}	O_2	+	−	3.0
T. neapolitanus	S^0,$S_2O_3^{2-}$	O_2	−	−	6.6
T. kabobis	S^0	O_2	−	−	ND
T. tepidarius	$S_2O_3^{2-}$	O_2	−	−	7.0
T. thioparus	S^0,$S_2O_3^{2-}$, NCS^-	O_2, NO_3^-	−	+	6.9
T. denitrificans	S^0,$S_2O_3^{2-}$, $S_4O_6^{2-}$	O_2,NO_3^-	−	+	7.0
T. intermedius	$S_2O_3^{2-}$	O_2	+	−	ND
T. novellus	$S_2O_3^{2-}$	O_2	+	−	8.4
T. acidophilus	ND	O_2	+	−	3.0
T. organoparus	ND	O_2	+	−	ND
T. versutus	$S_2O_3^{2-}$	O_2, organic-C	+	+	8.2
T. perometabolis	$S_2O_3^{2-}$, S^0	O_2	+	−	ND

ND- no data.

Source: Adapted from J. J. Germida and H. H. Janzen., *Fertil. Res.* 35, 101–114 (1993), A. E. Konopka, R. H. Miller, and L. E. Sommers, in M. A. Tabatabai ed., *Sulfur in Agriculture*, American Society for Agronomy, Madison, Wis., 1986, pp. 23–56, J. G. Kuenen and R. F. Beudeker, *Phil. Trans. Royal Soc. London Ser. B.* 298, 473–497 (1982).

soils, the initial oxidizers of reduced sulfur compounds will be heterotrophic organisms until the pH is reduced sufficiently to permit oxidation by chemolithotrophs. In addition, there is good evidence that consortia of heterotrophs and auototrophs working together bring about the oxidation of sulfur in agricultural soils. Recent work has shown that there is a dynamic cycling between oxidized and reduced forms of sulfur in the rhizosphere of rice paddies (38). Plant roots provide electron donors, that is, energy sources necessary for the sulfate-reducing bacteria to reduce sulfate to sulfide. This process is aided by the low-oxygen conditions surrounding the root system and the development of anoxic microsites such as the formation of iron sulfide aggregates. Sulfur-oxidizing bacteria such as *Thiobacillus* quickly oxidize the reduced sulfur back to sulfate and the cycle is complete.

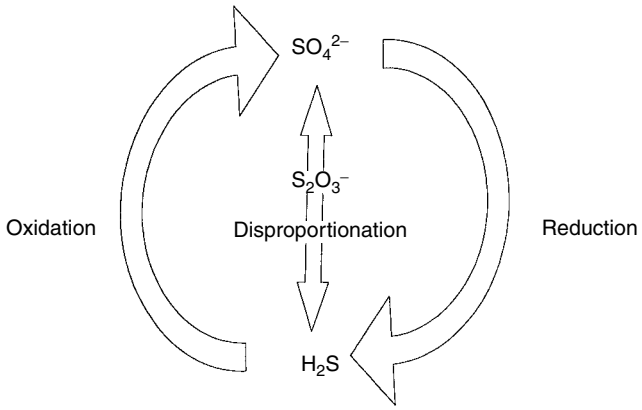

Figure 8. The thiosulfate shunt in anoxic sediment (41).

Thiosulfate Shunt

In the late 1980s, it was discovered that bacteria can disproportionate thiosulfate or sulfite (39,40). A disproportionation reaction, also called fermentation, takes place when one portion of the molecule acts as an electron donor and another part acts as an electron acceptor. In the case of an isolate of *Desulfovibrio sulfodismutans*, this results in the following reactions

$$S_2O_3^{2-} + H_2O \rightarrow SO_4^{2-} + HS^- + H^+$$

$$4SO_3^{2-} + H^+ \rightarrow 3SO_4^{2-} + HS^-$$

This discovery was quickly followed up by the realization of the importance of this disproportionation reaction to the global sulfur cycle and was termed *the thiosulfate shunt* (Fig. 8) (41). Its effects on sulfur flow in sediment is dependent on the oxygen status of the sediment, with sulfate predominating in the upper oxic sediment layers and sulfide in the lower anoxic layers. Similar results have been found for sulfonates, with the bacteria fermenting taurine and disproportioning the sulfonate to sulfate and sulfide. In fact, this group of bacteria has been proposed as a new genus *Desulfonispora*, because there appear to be a number of isolates capable of disproportionating organic sulfonates (28,42). This shunt has been found to predominate in marine sediment, (43) but its importance in upland terrestrial systems is not yet clear. However, this process has been found to account for approximately 60% of the thiosulfite transformation in oxic sediments, and thus one could expect the presence of this process to occur in terrestrial systems as well.

Other Sulfur Bacteria

Other bacteria may also oxidize sulfur compounds. The gliding sulfur oxidizers include those bacteria whose cells are arranged in trichomes that show a gliding motion on the substrate. The most important members of this group in relation to sulfur oxidation in soils are species of *Beggiatoa*, the bacteria that participate in sulfide oxidation in the root zone of rice. All strains of *Beggiatoa* deposit sulfur in the presence of H_2S. (see SOIL BACTERIA, this Encyclopedia) Phototrophic bacteria, such as *Chromatium*

and *Chlorobium* also play an important role in sulfide oxidation in rice paddy soil, but not in aerobic agricultural soils. A number of nonfilamentous, chemolithotrophic sulfur- oxidizing bacteria, such as *Sulfolobus*, *Thiospira*, or *Thiomicrospira*, have also been isolated from special habitats, but the importance of these bacteria in sulfur oxidation in soils is yet to be determined. The activity of different groups of sulfur-oxidizing bacteria might be predicted on the basis of the relative turnover rates of inorganic sulfur compounds and organic substrates.

Biogenesis and Oxidation of Metal Sulfides

Metal sulfides may be formed through biotic or abiotic reactions. In both cases the metal sulfide results from the interaction between a metal ion and a sulfide ion:

$$M^{2+} + S^{2-} \rightarrow MS$$

Many sulfate-reducing bacteria, such as *Desulfovibrio* spp. or *Desulfotomaculum* spp. are involved in the biogenesis of sulfides of antimony, cobalt, cadmium, iron, lead, nickel, and zinc. The extent of metal sulfide genesis depends on many factors, the most important of which is the relative toxicity of the metal ion. In nature, this toxicity is probably reduced when the metal ions are adsorbed on clays or complexed with organic matter. The formation of metal sulfides during the mineralization of organic sulfur compounds is also possible, although little is known about this phenomenon.

The oxidation of metal sulfides in soil involves both chemical and microbial processes and, as a result, is a more complex process than the oxidation of S^0. Chalcocite (Cu_2S), chalcopyrite ($CuFeS_2$), galena (PbS), pyrite (FeS_2), and nickel sulfide (NiS) are just a few examples of metal sulfides that are subject to microbial transformations. For example, the biological oxidation of pyrite (FeS_2) can follow one of two pathways. The first follows a series of oxidation steps described in equations 1 to 4. The second mechanism of pyrite oxidation involves thiosulfate as an intermediate, but in the end, requires the oxidation of elemental sulfur to sulfate by *Thiobacilli* (45). The reader is invited to two excellent reviews for further details of this area (46,47). These biotic oxidations are responsible for the formation of acid mine drainage and acid soil formation in surface mine spoils. (see ACID MINE DRAINAGE, this Encyclopedia) First, ferrous sulfate is formed as the result of an abiotic oxidation step:

$$2FeS_2 + 2H_2O + 7O_2 \rightarrow 2FeSO_4 + 2H_2SO_4 \qquad [1]$$

This reaction is then followed by the bacterial oxidation of ferrous sulfate generally by *T. ferrooxidans:*

$$4FeSO_4 + O_2 + 2H_2SO_4 \rightarrow 2Fe_2(SO_4)_3 + 2H_2O \qquad [2]$$

This reaction occurs chemically but can be accelerated 10^6 to 10^8 times by thiobacilli. This bacterial oxidation of ferrous ion plays an important role in the bioleaching of metal sulfides in the environment because it cycles the iron between +2 and +3 and the iron is then free to abiotically oxidize sulfide minerals (Fig. 9) (48).

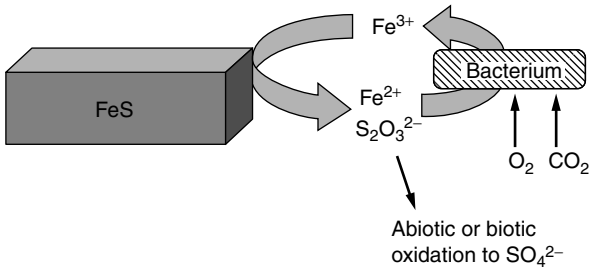

Figure 9. Indirect mechanism by which bacteria oxidize pyrite (48).

Subsequently, ferric sulfate is reduced and pyrite oxidized by a strictly chemical reaction.

$$Fe_2(SO_4)_3 + FeS_2 \rightarrow 3FeSO_4 + 2S^0 \qquad [3]$$

$$2S + 6Fe_2(SO_4)_3 + 8H_2O \rightarrow 12FeSO_4 + 8H_2SO_4 \qquad [4]$$

The elemental sulfur produced is finally oxidized by *T. thiooxidans* and *T. ferroxidans*, and the acidity produced helps the whole process to continue.

$$2S^0 + 3O_2 + 2H_2O \rightarrow 2H_2SO_4$$

Note the net production of 10 molecules of H_2SO_4 during the process.

Although several sulfur-oxidizing thiobacilli and heterotrophs can be isolated from acid sulfate soils in which pyrite is being oxidized, they appear not to play an important role in the process, with the exception of *T. ferroxidans*. The biological oxidation of sulfides and other reduced sulfur compounds can have severe consequences for the environment. For example, acid mine drainage contaminates several thousand miles of streams in the Appalachian coal mining region of the United States. (see ACID MINE DRAINAGE, this Encyclopedia)

MICROBIAL REDUCTION OF INORGANIC SULFUR COMPOUNDS

Bacterial Sulfate Reduction

The reduction of sulfate to hydrogen sulfide is mediated mainly by anaerobic bacteria, typically referred to as sulfate-reducing bacteria (SRB). This process may be significant in anaerobic soils (i.e., waterlogged soils), but is usually not important in well-aerated agricultural soils except in anaerobic microsites. Nevertheless, sulfate reduction is a major component of the sulfur-cycle in soils exposed to waterlogging or periodic flooding, especially where readily decomposable plant residues are present.

Microorganisms reduce oxidized sulfur compounds by either an assimilatory or dissimilatory process. Assimilatory sulfate reduction is used by organisms to meet their sulfur requirements (see preceding section). In dissimilatory sulfate reduction, bacteria use sulfate as a terminal electron acceptor, and large quantities of hydrogen sulfide (H_2S) are excreted. Dissimilatory sulfate reduction

Table 5. Dissimilatory Sulfate-Reducing Bacteria

Genera	*Desulfobacter*, *Desulfobulbus*, *Desulfococcus*, *Desulfonema*, *Desulfosarcina*, *Desulfovibrio*, *Desulfomicrobium*, *Desulfomonas*, *Desulfobacterium*, *Desulfococcus*, *Desulfomonile*, *Desulfotomaculum* - Gram positive, endospore former *Thermodesulfobacterium* - thermophilic *Archaeoglobus*–archaeal thermophilic
General characteristics	Anaerobes Grow at mildly acid to mildly alkaline pH Generally mesophilic, but some species thermophilic Some species can use alternative electron acceptors such as nitrate or oxygen.
Substrates	Most SO_4^{2-} reducers will also reduce sulfite and thiosulfate Some species reduce elemental S Organic matter utilization varies with genus and species As a group, capable of completely oxidizing fatty acids from C1–C18, lactate, pyruvate, low-molecular-wt. alcohols, and some aromatic compounds
Habitats	Anaerobic sediments of freshwater, brackish water and marine environments, thermal regions, marine and animal intestines; water logged soils

Sources: Adapted from P. A. Trudinger, in M. A. Tabatabai ed., *Sulfur in Agriculture*, American Society for Agronomy, Madison, Wis., 1986, pp. 1–22 & H. F. Castro, N. H. Williams, and A Ogram, *FEMS Microbiol. Ecol.* **31**, 1–9 (2000).

is a strictly anaerobic process. In this case, it is carried out by bacteria such as *Desulfovibrio* spp., *Desulfomonas* spp., and *Desulfotomaculum* spp. (Table 5) (49,50) These bacteria utilize end-products of other fermentations such as lactate, malate, and ethanol as electron donors.

When a soil is flooded, electron acceptors become reduced in an ordered sequence: first oxygen, followed by nitrate, nitrite, manganic, and ferric compounds, and lastly sulfate and carbon dioxide. Although the reduction of one compound does not have to be completed before another is reduced, oxygen and nitrate must be removed before the reduction of ferric and sulfate ions can occur. Because of this reaction sequence, there are generally sufficient ferrous ions available to react with any H_2S produced, and as a result, free H_2S is rarely liberated from soils. Sulfate reduction increases during the period of soil submergence and following the addition of organic matter. Sufficient organic substrates to stimulate the process are also liberated from seeds and from roots into the rhizosphere, with the result that in paddy soils, blackening caused by ferrous sulfide deposits often occurs in the root region. (see FLOODED SOILS, this Encyclopedia) There is evidence, however, that rice roots can aerate the soil sufficiently such that ferric iron is observed on the root surface. In general, the rate of sulfate reduction increases with decreasing Eh, with the optimum being a function of soil pH, around −300 mv at pH 7. Sulfate reducing bacteria are active in habitats such as soil, sediments, polluted water, oil-bearing strata, and shales. Their activity may be beneficial or detrimental to the surrounding environment and have serious economic consequences.

Recent work has identified alternative metabolic strategies for SRB that live in environments low in sulfate. Sulfate reducing bacteria can live in close association with methanogens (51). In environments, rich in organic substrates but low in electron acceptors such as sulfate, SRBs transfer the reducing equivalents produced by degradation of organic compounds, to methanogens living in close proximity Fig. 10 (51). The methanogens then use this hydrogen to produce methane and keep the hydrogen partial pressure in the appropriate range that makes the extracellular transfer of hydrogen a feasible alternative for SRB (52). Alternatively, SRB can use a variety of sulfonic acids such as isoethionate or cysteate as terminal electron acceptors. The pathway used by SRB differs from that used for the reduction of sulfate in that it does not appear to use ATP sulfurylase or APS reductase (53). The ability to use alternative electron acceptors among the SRB may be limited to only a few specialized strains and the relevance of these alternative electron acceptors to field situations is still not clear (54). In the presence of sulfate, SRB revert to their normal metabolic processes because the use of sulfate as an electron acceptor is metabolically the most efficient compared with the alternatives.

Sulfate-reducing bacteria also cause significant environmental problems because of the production of the corrosive hydrogen sulfide as well as the methylation of mercuric ions. For example, SRB are a major cause of corrosion of underground iron pipes, causing damage between $1.6 and $5 billion in the United States in 1990. Turf managers find that SRB can produce a black layer in golf course putting greens by using the organic matter in root exudates to reduce soil sulfates to ferrous sulfides. The methylation of the inorganic mercuric ion by SRB is one of the mechanisms by which flooding of previously dry soil severely affects the soil. Aerobic soils in which SRB were not dominant may become flooded and eventually anaerobic. Consequently, SRB become active and methylate mercuric ion present in the soil (now

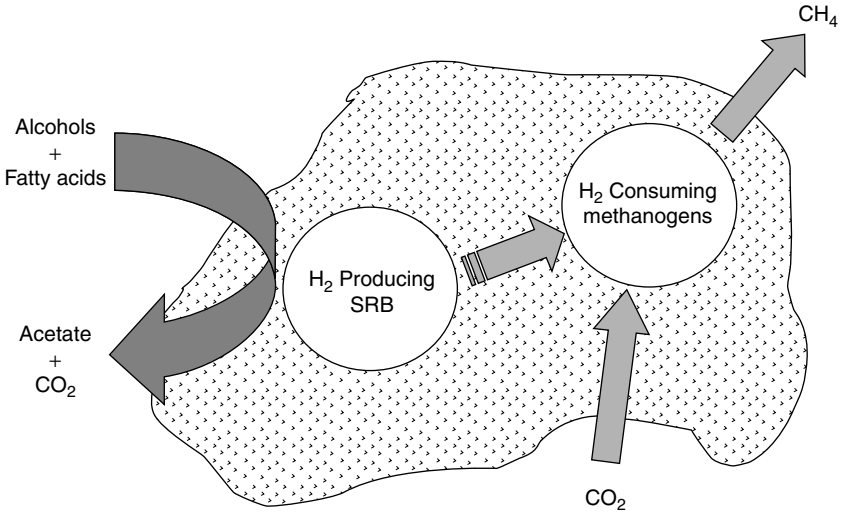

Figure 10. Close association of hydrogen-producing sulfate-reducing bacteria and hydrogen-consuming methanogens allows sulfate-reducing bacteria to be active in the absence of sulfate [51].

sediment) (55). Even when sulfate is depleted, SRB can continue to methylate mercuric ion by transferring hydrogen to methanogens (56). This methylated mercuric ion is toxic and bioaccumulates in the food chain. Mercury is methylated as a side reaction of normal SRB metabolic processes and is not a detoxification reaction (57,58).

CONCLUSION

The cycling of sulfur through aquatic, terrestrial, and atmospheric ecosystems is similar to that of other elements such as carbon and nitrogen, and is influenced by natural and anthropogenic processes. Sulfur exists in a number of oxidation states as inorganic and organic compounds that undergo a number of biotic and abiotic transformations. A large variety of microorganisms mediate the biotic transformations of sulfur and influence its bioavailability and toxicity. Understanding how key processes in the sulfur cycle respond to environmental factors (e.g., construction of models of mineralization and volatilization processes that include temperature, moisture, substrate, and microbial composition response functions) will help us predict accurately the impact of changes, either induced by humans or natural, on sulfur fluxes in all components of the biosphere.

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SULFUR OXIDIZING BACTERIA.

See SULFUR BACTERIA IN DRINKING WATER

SURFACES.

See ACTIVITY AND CARBON TRANSFORMATIONS IN BIOFILMS

SUSPENDED BIOFILMS.

See MICROBIAL FLOCS SUSPENDED BIOFILMS

SUSTAINABLE AGRICULTURE.

See INSECTICIDES, MICROBIAL

SUSTAINABLE AGRICULTURE: ROLE OF MICROORGANISMS

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The natural processes that sustain life on earth are threatened by anthropogenic influences such as increasing human populations, resource consumption, social instability, and environmental degradation (1,2). These natural processes, especially microbial processes, can be managed to reduce the negative impact of these anthropogenic influences. Global climate change, depletion of the protective ozone layer, reduction in species biodiversity, loss of productive agricultural lands, and degradation of soil, water, and air are among the most pressing concerns associated with our technological search for a higher standard of living for an ever-growing human population. Past management of agriculture and other ecosystems to meet population and development needs has taxed the resiliency of soil and natural processes to maintain global balances of energy and matter (3,4). To preserve and protect the world

for future generations, the focus must be on ecosystem sustainability and development of production systems that rely less on nonrenewable, petrochemical-based resources; more renewable resources from the sun and soil must be relied on for food, fiber, and energy needs; and the ecological intensification needed to meet the increased future food demand must be achieved (5). Microorganisms can play a critical part in remedial efforts.

As strategies are developed to attain sustainable systems, the dilemma is to balance the immediate goal of economic viability and survival of the land manager with the long-term efficient and wise use of resources for a safe and clean environment (6). To this end, strategies that include soil microorganisms to better use natural supplies of energy and nutrients and reduce reliance on nonrenewable fossil fuels and petrochemicals can help achieve this balance.

HIGH-INTENSITY AGRICULTURE AND SOCIETY

In the twenty-first century, population growth rates and standard of living expectations will more than double the need for food supplies to feed a population twice its current size. While the human population is increasing, agricultural lands are shrinking, with little new land being placed into production. Degradation and loss of productive agricultural lands are our most pressing ecological concerns, rivaled only by environmental problems caused by humans, such as global climate change, depletion of the protective ozone layer, and serious declines in biodiversity (7). Under our current industrial model for food production, achieving the required agricultural production to support increasing food needs will require increased inputs, further stressing our natural and nonrenewable resources and increasing the potential for environmental pollution (8,9).

Excessive cultivation for seed bed preparation, pest control, and continuous cropping systems have, over the years, led to erosion of valuable topsoil and degradation of organic matter reserves (10). An estimated 40% of the earth's arable land has been degraded by adverse anthropogenic impacts such as soil erosion, atmospheric pollution, extensive soil cultivation, overgrazing, land-clearing, salinization, and desertification (11). Agriculture is considered the most widespread contributor to nonpoint source water pollution in the United States (12). In addition, nitrate nitrogen is a major water contaminant in North America and Europe. The principal sources of nitrate nitrogen are conversion of native land to arable use, animal manures, and fertilizers. Human alterations of the nitrogen cycle have doubled nitrogen inputs to terrestrial ecosystems over the past 30 years, resulting in large increases in the transfer of nitrogen from land to the atmosphere and to rivers, groundwater, estuaries, and coastal oceans (13–15). Land management practices can influence atmospheric quality through changes in the soil's capacity to produce or absorb important atmospheric gases such as carbon dioxide, nitrous oxide, and methane, and many are governed by microbial processes (16,17). The present threat of global climate change and ozone depletion, through elevated levels of greenhouse gases and altered hydrological cycles, can be ameliorated through a better understanding of the influence of land management on soil processes and adequate remediation strategies, particularly microbial aspects (18).

If soil parameters are to be used as indicators of sustainability of land management practices, they must respond to changes in management. As stated in Doran and Parkin (19), "indicators should be sensitive enough to reflect the influence of management and climate on long-term changes in soil quality but not be so sensitive as to be influenced by short-term weather patterns." Soil microorganisms are useful indicators of sustainable land management because they are (*1*) sensitive to variations in management, (*2*) well correlated with soil function, and (*3*) useful for elucidating ecosystem processes (20). The challenge before us is to use soil microbial analyses that can aid decision-making processes and are easily accessible to land managers.

SOIL — AN ESSENTIAL LINK IN THE CYCLE OF LIFE

Soil is a dynamic, living, nonrenewable resource whose condition is vital both to the production of food and fiber and to global balance and ecosystem function (21). The number of living organisms in one teaspoon of fertile soil (10 g) can exceed nine billion, which is 1.5 times the human population of the earth. Microorganisms have been isolated from all environments including hot springs and ice flows in Antarctica. Like water, soil is a vital natural resource essential to civilization but, unlike water, soil is nonrenewable on a human timescale. Soils form slowly, averaging 100 to 400 years per centimeter of topsoil, through the interaction of climate, topography, living organisms (microorganisms, animals, plants, and humans), and mineral parent material over time (22,23). Soils are composed of different sized inorganic mineral particles (sand, silt, and clay); reactive and stable forms of organic matter; a myriad of living organisms such as earthworms, insects, bacteria, fungi, algae, nematodes, and so on; water; and gases including O_2, CO_2, N_2, NO_x, and CH_4. The physical and chemical attributes of soil regulate soil biological activity and interchanges of molecules or ions between the solid, liquid, and gaseous phases, which influence nutrient cycling, plant growth, and decomposition of organic materials. The inorganic components of soil play a major role in retaining cations through ion exchange and nonpolar organic compounds and anions through sorption reactions. Essential parts of many global cycles occur in soil, including carbon, nitrogen, phosphorous, and sulphur, and water cycles. Large portions of these cycles are microbial processes. Soil functions as a filter for a large portion of our groundwater supplies, and soil organic matter is a major pool for carbon, nitrogen, phosphorus, and sulfur. Soil organisms in their constant search for food and energy sources are continually altering the cycling rate and availability of these elements.

The sun is the basis for most life on earth and provides radiant energy for heating the biosphere and for the photosynthetic conversion of carbon dioxide and water into food and oxygen sources for consumption by animals and other organisms. Most living organisms use oxygen to

metabolize these food sources, capture their energy, and recycle heat, carbon dioxide, and water to the environment to begin this cycle of "life" again.

$$\text{(Radiant)} \xrightarrow{\text{Photosynthesis}} \text{(Food)}$$

$$6CO_2 + 6H_2O + \text{Energy} \qquad C_6H_{12}O_6 + 6O_2$$

$$\text{(Heat)} \xleftarrow[\text{Decomposition and combustion}]{} \text{(Fuel)}$$

A simplified version of this "Equation of Life" is depicted in Figure 1.

The amount of carbon dioxide in the atmosphere is small and represents less than 0.04% of all gases in the atmosphere. If all the combustion and respiration processes on earth were halted, the plant life of the earth would consume all available carbon dioxide within a year or two. Thus, there is a fine balance between carbon dioxide production and utilization in the biosphere. Decomposition processes in soil play a predominant role in maintaining this balance, both in recycling carbon to the atmosphere as carbon dioxide and in the formation of soil organic matter, which serves as a sink for atmospheric carbon. These processes are brought about by a complex web of organisms in soil, each playing unique roles in the physical and chemical breakdown of organic plant and animal residues. Decomposition is a critical responsibility of the microbial world. In the food web, almost 95% of all material must cycle through the microbial portion (24). Together, fungi, actinomycetes, and bacteria possess a vast array of biochemical pathways that are capable of mineralizing any naturally occurring organic material. The physiological diversity of this decomposer community, however, enables continued activity over a wide range of conditions, an essential attribute in a soil environment that is continually changing. Soils play a major role in transforming sunlight and stored energy and recycling matter through plants and animals. Decomposition processes, as mediated by organisms in soil, play a predominant role in completing this cycle of life. This capability is essential for the completion of biogeochemical cycles and continuation of life on earth. The capacity to degrade natural organic compounds is also the key to the use of microorganisms for bioremediation of organic pollutants such as pesticides, solvents, and propellants made by humans. Many of these compounds can be degraded to some extent, using the same biochemical pathways used to degrade natural organics. These microorganisms, as recyclers, provide building block nutrients to plants and C as CO_2 to the atmosphere. Thus, the thin layer of soil covering the surface of the earth and the microbes contained within it are the major interface between agriculture and the environment and represent the difference between survival and extinction for most land-based life (21). The health and quality of soil and thus the functioning of the microorganisms with the soil are integral in directing agricultural sustainability (25), environmental quality (26), and plant, animal, and human health (27).

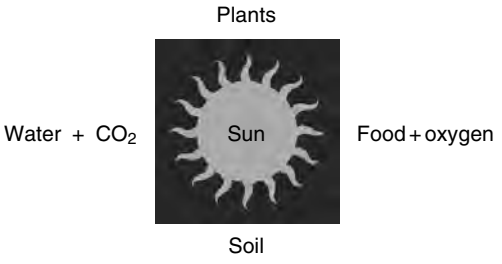

Figure 1. Soil — an essential link in the cycle of life.

SOIL HEALTH AND SUSTAINABLE MANAGEMENT

Interest in the quality and health of soil has been stimulated by renewed awareness that soil is vital to production of food and fiber, global ecosystems function, and the maintenance of local, regional, and global environmental quality (28,29). Soil plays a key role in the health of humans by being the medium for food production, serving as a major interface with the environment, affecting the quality of the drinking water, and quality of air breathed. Soil health and quality assessments can be used to predict the sustainability of systems or management practices (30). Soil health or quality can be broadly defined as the capacity of a living soil to function within natural or managed ecosystem boundaries, sustain plant and animal productivity, maintain or enhance water and air quality, and promote plant and animal health (21,31). Soil quality and health change over time because of natural events or human impacts such as management and land-use decisions. Sustainable practices will result only when the multiple functions of soil are included in decisions. Management strategies that consider only single functions such as crop productivity but not environmental impact or vice versa will not be successful. Indicators of soil quality and health should be designed to adequately describe ecosystem processes through physical, chemical, and biological properties. These indicators also need to be sensitive to management and climatic variations, accessible, and easy to use (32). As illustrated in Figure 2, soil quality is conceptualized as the major linkage between the strategies of conservation management practices and achievement of the major goals of sustainable agriculture (30,33). Soil quality or health assessment and the direction of the change of select indicators with time can be the basic indication of sustainable land management.

Profitability and environmental protection are both goals that need to be attained if sustainability is to be achieved. For example, one framework for determining the sustainability of hill country agriculture in the Philippines employs indicators that consider both the satisfaction of farmer needs for productivity, profitability, stability, and viability, and those needed for conservation of soil, water, and air resources (34). In this example the threshold values, or trigger values, for sustainability were identified relative to the average local conditions for crop yield, profit, risk, soil depth, organic matter content, and so on. Modifications of this framework for assessment of sustainability can be expanded to include other needs of society and environmental conservation (35). In reviewing

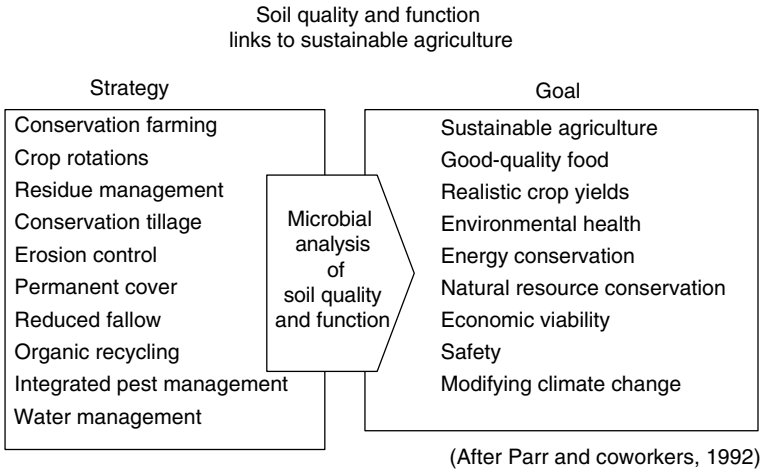

Figure 2. Soil quality and function links to sustainable agriculture.

microbiological indicators of soil quality, Stenberg (36) presented a similar approach developed by a research network in Europe to assess soil quality and sustainability. However, this approach emphasized the shortfall of results from a desired optimum instead of defining minimum thresholds, normalized for local conditions.

MICROBES AND SUSTAINABLE AGRICULTURE

Soil microorganisms are key to sustainability because they are responsible for a variety of functions that impact soil dynamics and agroecosystem health (Table 1; 37). The biological component of the soil is responsible for soil humus formation, cycling of nutrients, pesticide degradation, and building soil tilth and structure along with a myriad of other functions (38,39). Knowledge of microbial functioning is limited, and presently, fully manipulating soil microbial communities has not been successful. By increasing knowledge of microbes and microbial ecology, the ability to manage ecosystems for optimum sustainability is enhanced.

Biodiversity

Microorganisms are responsible for biochemical processes that are needed to sustain life; however, microorganisms can also provide us with information on life processes and evolution (Table 2). They provide sources of genetic material and function as indicators of environmental change (40). Knowledge of soil microorganisms may increase our understanding of biological interactions and aid in conservation and restoration biology. Over 100,000 microbial species may exist, but only a fraction of them are actually identified (41). There is a vast wealth of unknown genetic potential in the biodiversity found in the soil. Soil microbial diversity is thought to overshadow diversity found within higher life-forms (42,43). Little is known about life on earth; less than 10% of microbial species have been identified (41). This untapped potential of genetic material in soil is ready for discovery. Sustainability may in part be tied to the changes in this diversity. Also, the status or health of a system may be assessed by the diversity of that system. The functional and even taxonomic diversity within a given population of microbes may be helpful to determine changes with disturbance or cultural practices. Microbial diversity decreased in chemically or heavy metal–stressed soils (44,45). Diversity may play a critical role in an ecosystem's resilience or ability to buffer the effects of extreme disturbances (46) so that monitoring diversity as an indicator of change or in response to a stress may yield valuable information. However, meaningless changes in diversity and species number should not be misleading. Species composition may not be as defining as

Table 1. The Functions of Soil Microbes in Agriculture Systems

Decomposition of organic residues with release of nutrients
Formation of beneficial soil humus by decomposing organic residues and through synthesis of new compounds
Release of plant nutrients from insoluble inorganic forms
Plant growth enhancement
Improved plant nutrition through symbiotic relationships
Transformation of atmospheric dinitrogen to plant-available nitrogen
Improvement of soil aggregation, aeration, and water infiltration
Antagonistic action against insects, plant pathogens, and weeds (biological control)
Pesticide degradation

Table 2. Justification for Investigations of Soil Microbial Ecology (from ASM, 1994)

Microbes are an important source of knowledge about life strategies and limitations
Microbes are key to understanding evolutionary history
The untapped diversity of microorganisms is a resource for new genes and organisms of value to biotechnology
Microbes and microbial diversity patterns can be used as indicators of environmental changes
Microbial communities are key to understanding biological interactions
Microbes play a key role in conservation and restoration biology of higher organisms
Microbes are critical to sustainability

the flux of species within a system, and the functioning of individual species within that system. The diversity within the soil system allows for exploration of greater genetic potential and understanding functional diversity changes with management.

Soil Organic Matter

The microbial processes that have the greatest impact on sustainability are those processes involved in residue decomposition and organic matter formation. Decomposition processes, as mediated by organisms in soil, play a predominant role in supplying key elements. The processes of soil humus formation, cycling of nutrients, and building soil tilth and structure (38,39) are distributed among a large number of different genera and species. Soil organic matter is the basis of soil quality and soil health. Organic matter consists of organic components, decomposition products, and the soil biomass. The labile fractions of soil organic matter are rapidly turned over, whereas the more biologically resistant fractions and chemically recalcitrant fractions form soil organic matter. Organic matter increases water-holding capacity and nutrient availability, improves soil physical properties, and acts as a cementing agent to bind soil particles together. Organic material can provide sites for bacterial and fungal growth and thus control colonization and community development. Incorporation of crop residues, crop rotation, and addition of animal and green manures can increase soil organic matter.

Residue management is critical to soil organic matter dynamics and is a major factor in determining soil fertility and productivity and in preventing soil erosion (47,48). The residues remaining after harvest can serve as a cover to protect the soil. With practices such as conservation and no-till drilling, more residue is left on or near the soil surface, giving more cover, conserving organic matter, reducing erosion, and often increasing yield. More soil moisture is retained for crop growth, with no-tillage than with conventional tillage (49). The residue and the remaining relatively undisturbed roots serve as a source of food for microbial activity, with significantly higher microbial biomass in no-tilled soils compared to conventionally tilled soils (50). Decomposing roots provide nutrients for microorganisms and also result in the formation of particulate organic matter, a labile fraction of organic matter, most susceptible to loss with intensive cultivation (51). Residue management is critical to nutrient-cycling dynamics in cropping systems, and management strategies that consider microbial processes will lead to sustainability.

When wheat decomposition rates in conventional and no-till systems were compared by evaluating carbon and phosphorus losses, more carbon and phosphorus were lost from fields under conventional tillage than from those under no-till management (52). It was concluded that no-till management, over a period of time, leads to a significant accumulation of carbon and phosphorus, whereas in conventional systems, carbon and phosphorus are lost during the decomposition. Residue retention and decomposition have greater consequences than merely soil protection and yield potential. Recent interest in changes in the global carbon cycle and nutrient cycling (53,54) have increased the focus on determining the potential for agricultural systems to store carbon. Consequently, agricultural soils are recognized not only for their continued potential for producing food and fiber but also as a major interface with the environment, affecting the air and drinking water. These studies have demonstrated a potential for biological management of agricultural cropland soils to sequester carbon and help mitigate the greenhouse effects, atmospheric carbon dioxide, and global climate change (55). Proposals to provide economic incentives to farmers to build soil organic matter and offset industrial emissions of carbon dioxide into the atmosphere heralds a new era in which environmental quality is recognized as a potential novel product of agriculture.

Nutrient Cycling

Microbial processes are integral to the breakdown of organic material and nutrient cycling. Microorganisms are primary decomposers and are critical in the soil food web. It has been estimated that more than 90% of all nutrients pass through this group of organisms to higher trophic levels. Soil microbes constitute a large dynamic source and sink of nutrients in all ecosystems and play a major role in plant litter decomposition and nutrient cycling (51,56,57). Most natural and xenobiotic compounds can be broken down or at least transformed by soil microorganisms.

Certain bacteria have the enzymes necessary for dinitrogen fixation. The symbiotic relationship between bacteria and legumes is one of the most widely studied and applied plant–bacterial interactions (58). The bacterium *Rhizobium* forms nodules on the roots of the legume plant, takes nitrogen from the air, and transforms it to plant-available nitrogen (i.e., NH_4^+, NO_3^-). The plant provides nodules and photosynthate for the bacteria, whereas the bacteria give the plant the nitrogen it needs. Inoculation of legumes with nitrogen-fixing *Rhizobium* can add appreciable amounts of nitrogen to the soil.

Carbon and nitrogen cycling was studied in a comparison of three agricultural systems for corn production (59). One system was the traditional corn and soybean rotation, with high inputs of fertilizer and pesticide use. The other two systems consisted of organic practices and relied on lower inputs of nonrenewable resources in which either manure or legumes were used as "renewable" nitrogen sources. Over a 10-year period, the three systems produced similar yield and were similar in profitability. Their carbon and nitrogen cycling were, however, significantly different. Organic nitrogen increased dramatically in the manure-based system and somewhat in the legume-based system, and was reduced or unchanged in the conventional system. Increasing rotation diversity, altering the carbon to nitrogen ratio of the crops selected, and addition of manure can alter the carbon and nitrogen storage without compromising yield and farm profit. At the Rothamsted Experimental Station in the United Kingdom, similar results have been found (60). Wheat yields on manured fields were equal to NPK-fertilized plots, but the soil organic matter and total soil nitrogen contents increased dramatically, further indicating the benefit of manure-based systems to soil carbon and nitrogen storage.

Plant Growth

Microorganisms are also responsible for many transformations in soil directly related to plant nutrition and health. Although potentially harmful effects from soil microorganisms include plant diseases, production of plant-suppressive compounds, and competition for nutrients, the majority of soil organisms are beneficial to plant growth (38). Specific microorganisms can be manipulated to produce beneficial effects for agriculture; for example, rhizobia to increase plant available nitrogen (58); mycorrhizal associations to enhance nutrient uptake (61); or biological control of plant pests to reduce chemical inputs (62,63). Beneficial soil bacteria can enhance plant performance by increasing mineral solubilization, the production of hormones, and the suppression of harmful pathogens.

Plant species can influence microbial activities and in turn can be greatly influenced by soil microbes. Microbial communities in agroecosystems change with management history. In continuous cropping systems, cycling of pathogens and antagonists of pathogens, and changes in crop yields can often be seen because of the alteration in disease pressure over time. An example of this is the decline of a disease in wheat called "take-all," which results from a change in the soil microbial community. After several years in continuous wheat, the microbial community shifts to favor growth of antagonists of the pathogen *Gaeumannomyces graminis* var. *tritici*, which in turn results in decline of the disease (64). Crop rotation is a critical strategy used in sustainable systems to enhance beneficial microbial species and interactions, interrupt devastating cycles of pathogens, and reduce weed populations. Legumes in crop rotations supply symbiotically fixed nitrogen to the system, aid in maintaining proper water status, and reduce pathogen loads. The benefits of crop rotations to plant growth have been attributed, in part, to changes in the bacterial community composition (65,66). In other studies, continuous monocropping also caused changes in the soil community. The pathogen load was higher and barley growth lower in grains grown in monoculture compared to multiple-crop rotation (67). The populations and aggressiveness of pathogens were altered with crop rotation, indicating the effect of management on microbial numbers and function (68).

Mycorrhizae are nonpathogenic fungi that form symbiotic associations with plant roots. These beneficial mycorrhizal fungi can enhance plant growth by increasing nutrient (62) and water uptake (69). Mycorrhizal fungi have been found growing in association with 90% of terrestrial plants examined (70). Mycorrhizal associations are most important in stressed environments, phosphorus-deficient soils, eroded sites, and acidic or reclaimed lands. This association may be key in plant productivity and nutrient cycling (71,72). Wheat plants inoculated with vesicular arbuscular mycorrhizae (VAM) and subjected to water stress had greater grain yield and biomass than uninoculated plants (73). In Pacific Northwest soils, mycorrhizal fungi lessened the severity of water stress in winter wheat. Plant growth increases were especially notable in soil from eroded ridge tops (58). Vesicular arbuscular mycorrhizae species, abundance, and spore distribution are affected by tillage and crop inputs (74,75). In a comparison of a conventional corn and soybean system and low-input systems, lower levels of VAM fungi were found in the conventional farmed system when compared to the low input with cover crops versus green and animal manures (74). *Glomus occultum* numbers were higher under no-till, whereas other *Glomus* species were more abundant under conventional tillage in a corn and soybean rotation (75). The interaction involving mycorrhizal fungi and rhizobia may further affect the host plant by reducing the need for nitrogen and phosphorus inputs (72). These interactions are specific, further illustrating the complexity of the plant–microbe interaction and the changes in diversity of various microbial groups that can affect plant growth or impact other soil features.

Microorganisms may influence plant competition either through mutualistic or antagonistic relationships. Rhizosphere microorganisms may affect plant growth directly (76) or indirectly by their effects on each other and on soil physical and chemical properties. The effect of microorganisms on competitive plant interactions can be manipulated (77,78). Both bacteria and fungi can be used to control insects, pathogens, and weeds either by lowering the populations of the pest or by reducing the pest's impact (79). An example of the practical application of soil bacterial diversity is the emerging area of biological control of plant pathogens (62). Bacteria have the potential to be used in biological control, which is the suppression of one pest by using its natural pest or antagonist. Bacteria and fungi that produce different types of antibiotics can be used to control many plant pathogens (62).

Weed management may also benefit from the use of soil microorganisms (63,80). Investigations of plant-suppressive bacteria have led to changes in crop management practices (76) and show potential for biological weed control of several weed species (63,81). Research on weed-inhibitory microorganisms has led to the concept of managing soils for weed-suppressiveness and utilizing the community, rather than just one organism of microorganisms for suppression of plant species (80). Biocontrol is critical to sustainable agricultural systems, but a greater understanding of soil microbes, their ecology, and the identification of practical indicators of system performance is required before biological control can be successfully implemented. The wealth of genetic material contained within the soil can potentially enhance sustainability practices, and investigations into this natural resource will also benefit other areas of science.

Pesticide Degradation

The fate of pesticides in soil is a microbially mediated process whose rate depends on abiotic factors as well. Many pesticides are readily degraded, whereas others tend to be more recalcitrant. The degradation process is dependent on the soil and environmental conditions and on the presence of specific bacteria that possess the active degradative enzymes (82). Also, the degradation rate is often greater in soils with higher soil organic matter content, and inorganic nitrogen has been shown to enhance degradation of atrazine (82). Although chemical

degradation has been shown, the presence of microbial populations are critical to pesticide dissipation (83). The microbial processes can be managed for reduction of herbicides as potential pollutants.

Soil Structure

Good aggregation reduces erosion and improves water infiltration and soil aeration. Stable aggregates reduce sealing of the soil surface because of increased infiltration. Once grassland is plowed, however, aggregate stability and soil microbial processes decrease rapidly. Microbes play a major role in the formation of soil structure (84,85). Hyphal threads produced by fungi and actinomycetes help bind soil particles together. Bacteria and fungi produce extracellular polysaccharides that also bind soil particles together, assisting in building soil structure. Humic materials from microbial action form organic matter or clay complexes. This action reduces erosion, allows for good water infiltration, and maintains adequate aeration of the soil. Soil aggregation influences the distribution of both pores and water (86). Soil aggregation can be increased by the addition of residues, resulting in additional microbial activity (87). The available carbon and nitrogen pools regulate microbial biomass, decomposition rates, and production of polysaccharides (88). Carbohydrate content and microbial biomass influence soil macroaggregate stability.

Crop rotation and cover crops can influence aggregation because crop diversity leads to a carbohydrate fraction that is more varied and fosters a more diverse microbial community (89). The ability of fungi and bacteria to influence aggregation varies with species and is substrate-dependent (90). Limited availability of nitrogen in the soil solution reduces biomass while increasing polysaccharide production, leading to greater aggregation (46). Soil stability may be managed by addition of different amendments to stimulate microbial activity (91).

Soil aggregate stability increases with conservation management (92). No-till and chisel-plow soils had greater microbial biomass and carbohydrate carbon per unit total organic carbon than conventionally tilled comparisons, which suggests labile carbon sequestration in conservation management systems. Relatively high ratios of microbial biomass carbon:total organic carbon, and carbohydrate carbon: total organic carbon, found in no-till and chisel-plow soils compared to conventional soils, suggested a sequestration of labile carbon in conservation management systems. Similarly, indicators of labile carbon, for example, particulate organic matter, were higher in no-till when compared to conventional tillage (92). Light fraction organic matter and macroorganic matter measurements were the most responsive carbon indicators to changes in soil management in eastern Canadian soils (93). These are favorable attributes to study when evaluating soil quality changes with alterations in short-term management.

DISTURBANCE AND SOIL MICROORGANISMS

Microorganisms are sensitive to variation or disturbances in their environment, such as tillage or addition of toxic compounds and pollutants (46). This sensitivity may allow them to function as early indicators of changes in soil health and quality. Microbial populations and their functions change rapidly as their environment is altered. These changes may contribute to the understanding of soil quality and the development of sustainable agroecosystems (94,95). Sustainability may be connected to a soil's resilience or the ability of the soil to recover after disturbance. Reduced tillage and increased crop rotations and management to improve soil health can improve soil resilience (46). In addition, crop rotations longer than three years, decreased disease-related problems (96), which can also increase crop resilience. The impact a stress may have on soil health will depend on the type of stress.

In agricultural soils, tillage may affect the general biological status, whereas pesticides or other stressors may affect only individual functional groups (97). The microbial community after minimum tillage practices will be vastly different from that after tillage for seedbed preparation (92,98–100). Tillage greatly alters the soil structure and components supporting growth of the bacterial population by altering the soil's physical and chemical properties. In no-till agricultural systems, microbial activities are stratified with depth, with the greatest microbial activity occurring near the no-till surface; in the tilled system, activities were more evenly distributed throughout the plowed layer (99). Fungi, protozoa, and several bacterial and fungal feeding groups increased in no-till situations, whereas total bacterial numbers were not affected by tillage (101). The composition of the microbial community influences the rate of residue decomposition and nutrient cycling in both no-till and conventionally tilled systems (92). Fungi dominated residue decomposition in the no-till system, whereas in conventionally tilled systems, the bacterial component was found to be responsible for a greater portion of the decomposition of residue. These studies illustrate the alteration of the makeup of the microbial communities and possibly the diversity of basic microbial groups, with changes in management systems. In comparisons of conventional and conservation tillage practices used on former conservation reserve program (CRP) lands, fatty acid methyl ester analyses indicated that no-till practices maintained the microbial community structure most similar to those found in 10 years of undisturbed grass (102). However, the benefits accrued during CRP can be lost in a relatively short time when these lands are plowed for conversion to row cropping (103). Sustainable systems will likely need to adopt low-disturbance conservation tillage practices to ensure ecological continuity of microbial and biological communities. Further, establishing the relationships between microbial communities, soil health, and soil resilience will lead to a greater understanding of microbial indicators of sustainability.

STRATEGIES FOR SUSTAINABLE MANAGEMENT

In defining sustainable agricultural management practices, Doran and coworkers (104) stressed the importance

of holistic management approaches that optimize the multiple functions of soil, conserve soil resources, and support strategies for promoting soil quality and health. They initially proposed use of a basic set of indicators to assess soil quality and health in various agricultural management systems. The microbial-oriented indicators, some of which are the most sensitive, include respiration, enzyme analyses, and organic matter. Although many of these key indicators are extremely useful to specialists (i.e., researchers, consultants, extension staff, and conservationists), many of them are beyond the expertise of the producer (105). Also, it is possible that the measurement of soil quality and health will do little to improve the sustainability of the system under which the soil is managed. In response to this dilemma, Doran and coworkers (21,106) presented strategies for ensuring sustainable management, which included generic indicators of soil quality and health. These are measurable by and accessible to anyone and are not overly time-consuming to conduct, as given in Table 3. Note that soil organic matter serves as a primary indicator of soil quality and health for both scientists and farmers and is controlled by microbial processes (107). Soil organic matter is also a major source of food for soil microorganisms. Consequently, whenever organic matter is increased by management, soil microbes benefit, especially in low organic matter soils.

Scientists make a significant contribution to sustainable land management by translating scientific knowledge and information on soil function into practical tools and approaches by which land managers can assess the sustainability of their management practices (108,109). Specifically, assessment of soil quality and health is needed to identify problem production areas, make realistic estimates of food production, monitor changes in sustainability and environmental quality, and assist government agencies in formulating and evaluating sustainable agricultural and land-use policies (Fig. 3). Use of a given approach for assessing or indexing soil quality is fraught with complexity and precludes its practical or meaningful use by land managers or policy makers (110). However, the use of simple indicators of soil quality and health that have meaning to farmers and other land managers will likely be the most fruitful means of linking science with practice, in assessing the sustainability of management practices (111,112). For example, Gajda and coworkers (113) have demonstrated the utility of simplified procedures for soil quality, such as particulate and total soil organic matter by weight loss-on-ignition, in assessing the sustainability of conventional and alternative management systems in the Central Great Plains of the United States.

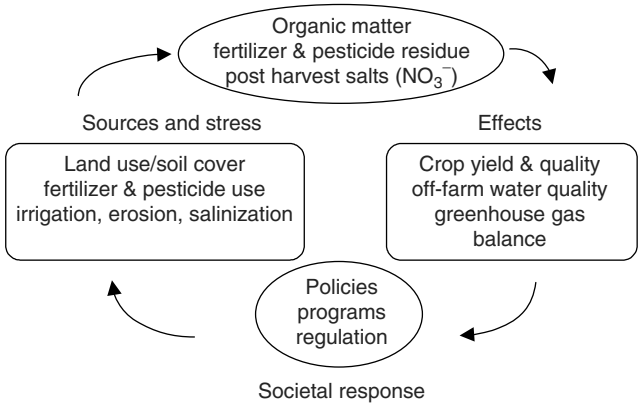

Figure 3. Soil condition and quality.

Table 3. Strategies for Sustainable Agricultural Management and Proposed Indicators of Crop Performance and Soil and Environmental Health

Sustainability Strategy	Indicators for Producers
Conserve soil organic matter through maintaining soil C&N levels by reducing tillage, recycling plant and animal manures, and/or increasing plant diversity, where C inputs > or = C outputs as microbial respiration	Direction and change in organic matter levels with time (visual or remote sensing by color or chemical analysis); Specific OM potential for climate, soil, and vegetation; Soil water storage
Minimize soil erosion through conservation tillage and increased protective cover (residue, stable aggregates, cover crops, green fallow)	Visual (gullies, rills, dust, and so on.) Surface soil properties (topsoil depth, organic matter content and texture, water infiltration, runoff, ponding, % cover)
Balance production and environment through conservation and integrated management systems (optimizing tillage, residue, water, and chemical use) and by synchronizing available N&P levels with crop needs during year	Crop characteristics (visual or remote-sensing of yield, color, nutrient status, plant vigor, and rooting characteristics) Soil physical condition and compaction Soil and water nitrate levels Amount and toxicity of pesticides used
Better use renewable resources through relying less on fossil fuels and petrochemicals and more on microbial processes, renewable resources and biodiversity (crop rotations, legumes, manures, IPM, and so on.)	Input/output ratios of costs and energy Leaching losses/soil acidification Crop characteristics (as listed above) Soil and water nitrate levels

In his article entitled "The Greening of the Green Revolution," David Tilman concluded that the uncertainties of sustaining a high-intensity agriculture have renewed the search for practices that can provide sustainable yields with fewer environmental costs (114). Since the beginning of agriculture, farmers had countered declines in fertility caused by agriculture by manuring fields, alternating crops that increase fertility (such as nitrogen-fixing legumes) with other crops. In a 15-year research trial in eastern Pennsylvania, Drinkwater and coworkers (70) demonstrated that organic management systems, employing animal manure and legumes for nitrogen supply, were equally as profitable as higher input conventional systems. The organic management systems tended to be more environmentally benign with lower leaching losses of nitrogen and higher levels of organic carbon and nitrogen stockpiled in soil. The organic management practices enhanced soil health and supported the major strategies for agricultural sustainability of conserving soil organic matter, minimizing erosion, balancing production with environmental needs, and making better use of renewable resources. Research has clearly demonstrated that initial yield reductions with organic management are more than offset by environmental benefits of soil organic matter buildup and synchronization of nitrogen availability with crop needs during the growing season. Research is needed to help agricultural managers assess the sustainability of agricultural management using indicators of soil quality and health to which they have access.

CONCLUSION

The multifaceted and changing nature of sustainability is difficult to define but is aptly captured by an Iowa farmer's simple definition of sustainable agriculture as, "an agriculture that sustains the people and preserves the land." Soil microorganisms play a major yet quiet role in these actions. Agricultural input alternatives that reduce reliance on nonrenewable fossil fuels and petrochemicals, ensure productivity, and maintain the quality of air, water, and soil resources are needed. Soil health and quality indicators, and the changes in those indicators and the microbes associated with those processes, can be a major link between the strategies of conservation management practices and achievement of the major goals of sustainable agriculture.

Microorganisms are key to the integrated functioning of nutrient cycles and decomposition, soil structure, and plant growth in agricultural systems. Research is needed to increase our understanding of the ecology and functioning of microbial communities, their response to management practices, their impact on soil health and quality in agroecosystems, and their role in sustainability. Investigations of residue management, organic matter formation, nitrogen and carbon cycling, soil structure maintenance, and biological control will assist in discovering system approaches that are both profitable and environmentally friendly. The challenge ahead is to gain an understanding of the wealth of microbial life in the soil and the resiliency of the biological community to maintain a quality ecosystem, thus fostering sustainability.

One of the greatest challenges for researchers is in "translating science into practice" through identifying indicators of system performance that are useful to farmers and land managers in assessing the economical, ecological, and environmental components of sustainability. Because so many processes are microbially mediated, defining the role of microorganisms is complex and challenging as many functions are unknown at this time. Management strategies need to optimize the microbial component of soil using practices such as crop rotation for greater diversity and tighter cycling of nutrients, reduction of soil disturbance to maintain soil organic matter and reduce erosion, and development of systems that make greater use of renewable biological resources. Crop rotation, legume companion crops, and animal manuring practices can reduce reliance on nonrenewable fossil fuels and petrochemicals. Ultimately microbial processes and functioning, indicators of soil health, and strategies for sustainable management must be linked to the development of management systems that foster reduction in the inputs of nonrenewable resources, maintain acceptable levels of productivity, and minimize impact on the environment. A healthy soil through soil management will result in a healthy soil microbial population efficient in cycling and balanced in its microbial diversity, which in turn supports a sustainable agricultural system.

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SUSTAINABLE SOLID WASTE MANAGEMENT.

See LANDFILLING OF MUNICIPAL SOLID WASTES: MICROBIOLOGICAL PROCESSES AND ENVIRONMENTAL IMPACTS

SYMBIOTIC NITROGEN FIXATION.

See PERIPHYTON

T

TEXTILE INDUSTRY AND BIOAEROSOLS.

See Bioaerosols in Industrial Settings

THERMOPHILES.

See Extremophiles: Life in Extreme Environments; Hyperthermophiles

THERMOPHILES: ANAEROBIC ALKALITHERMOPHILES

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Several excellent reviews on mesophilic and aerobic alkaliphiles and their habitats have been published in the past and the reader is referred to these extensive publications (1–8). This article deals only with a more recently observed group of bacterial and archaean extremophiles, the anaerobic alkalithermophiles, covering them on the order of their habitats from which they have been isolated. It is assumed that alkaline water bodies had existed early on when life evolved, although there is no unequivocal agreement on whether the Hadean Ocean really was alkaline (9–12). Zavarzin (13) suggested that the microbial communities in alkaline environments, especially in alkaline soda lakes, are remnants of communities from the early Proterozoic era when "epicontinental soda lakes were the centers of nascent terrestrial biota." If, as the author of this article believes, life originated under thermobiotic conditions, this statement should be even more true for the alkalithermophiles. Today alkaline environments, more mesobiotic than thermobiotic in nature, can be found all over the world. Although most of the earth's surface is covered by slightly alkaline ocean water (usually around pH 7.5 to 8.5), relatively few alkaliphilic microorganisms have been isolated from this environment. In particular, only two anaerobic alkaliphilic thermophiles have been isolated from marine hydrothermal locations. One reason could be that many of the vent fluids are more neutral or even acidic and the temperature gradient is very steep, that is, the mixing of the hot vent fluid with the cold alkaline ocean water does not produce a thermobiotic alkaline marine environment. Alkaline environments differ greatly in their geographic distribution, origins, water and geochemistry, stability, and their extent (large bulk environments vs. microniches in an otherwise neutral milieu) (1,2,14 and references).

Besides various natural environments, there are various anthropogenic ones that include niches, such as those related to the storage of waste products or in waste streams from industrial processes using NaOH or $Ca(HO)_2$ or producing alkaline compounds such as amines, as products. Examples include waste from olive and other food-processing plants, waste streams from NaOH-using pulp processes, alkaline bleaching, extraction of metals in mining processes, alkaline electroplating, production of cement caused by the formation of $Ca(OH)_2$, waste storage tanks kept alkaline in attempts to "prevent" or at least reduce microbial growth (such as the radioactive waste tanks from the production of weapons grade plutonium or storage tanks of spent fuel rods), and in some cases, around manure disposal sites in high-density livestock facilities caused by heavy ammonia formation. Natural habitats include those where bauxite is found or more generally where calcium-silicate minerals are weathering under the formation of $Ca(OH)_2$, alkaline hot springs (geothermally heated), various soda lakes and soda deserts that can reach pH values above 11.5, and most importantly for this article, can have thermobiotic temperatures around 45 °C (sun heated) or more than 100 °C geothermally heated) or contain hot springs such as the Mono Lake in California, the Lower, Middle, and Upper Alkaline Lakes (California and Nevada), and the soda lakes in the East African Rift Valley that are heated by both the sun and volcanic activity.

DEFINITION OF "EXTREME"

The term *extremophiles* means simply microorganisms that love extreme conditions. When using such an anthropogenic term, one has to keep in mind that extreme is defined by what is outside of the norm and the norm, defined by default, is what is suitable and comfortable to humans and our microbial "pets," *Escherichia coli*, and their inherent biochemistry and physiology. In other words, for the various so-called extremophiles, the optimum growth conditions for *E. coli* are extreme and stressful and in some instances, even outside the conditions for survival, for example, halophiles, will undergo lysis in the absence of high salt concentrations.

MESOPHILIC AND THERMOPHILIC ALKALIPHILES HAVE BEEN STUDIED ONLY RECENTLY

The study of true alkaliphiles is more recent than for most other extremophiles, except for the hyperthermophiles growing above 100 °C. The isolation of one of the first alkaliphilic microorganisms was reported by the Dutch microbiologist Vedder in 1934 (15) who isolated them from human and animal feces. More systematic study started in the early 1970s when Horikoshi and coworkers searched for alkali-stable proteases (16), which lead to the well-known application of the alkali-stable and alkaliphilic protease from *Bacillus* now sold by Novo Industries for use in laundry detergents. However, this does not mean that alkaliphilic microorganisms were not used in the past. One example is the centuries-old Asian practice of dying cloth blue using the indigo plant. This process used

a mixture of alkaliphilic microorganisms, some of which were finally isolated in 1960 (17) and in 1999 (18) The moderate thermophilic (T_{opt}50 °C; T_{max}55 °C) *Clostridium isatidis* (18) was isolated from a fermenting wood vat prepared as was done in the medieval times in Europe. Although its pH_{opt} is around 7.2, it grows in the pH range from 5.9 to 9.9 and thus, it is a pH-tolerant thermophile. Alkalithermophiles (also called *thermophilic alkaliphiles*) were unknown until a few years ago when the research group of the author, similar to Horikoshi years ago, searched for alkali-stable proteases, which were active and stable at elevated temperatures (above 60 °C) for laundry detergents to be used for high-temperature washing cycles. One reason for the lack of serious attempts to search for alkalithermophiles in the past was that most of the hot springs in the world are acidic, whereas most alkaline lakes are mesobiotic. Furthermore, it was assumed that microorganisms could not exist under the combined condition of high temperatures and alkaline pH because of hydrolysis of the protein and ester bonds. However, as reported in this entry, such microorganisms indeed exist. This entry summarizes the microbial diversity and their habitats from which they were isolated. Similar to the numerous and more thoroughly studied mesophilic alkaliphiles, the alkalithermophiles are widely distributed in thermobiotic and mesobiotic environments with slightly alkaline to acidic bulk-pH values. The author is convinced that, so far, only the tip of the iceberg of the diversity within this group has been observed (19).

ALKALITHERMOPHILES

Alkalithermophiles belong to the category of extremophiles. They are extreme in two or even three properties, that is, requiring or at least tolerating elevated temperatures (thermophilic), alkaline pH (alkaliphilic), and some of them because of the nature of alkaline (soda) lakes, requiring high salt concentrations (halophilic). Several of the described alkalithermophiles are halotolerant to various degrees, but to date no obligately halophilic anaerobic alkalithermophile has been validly described (i.e., published in the *International Journal of Systematic and Evolutionary Microbiology* directly or in one of the validation lists therein). Table 1 gives simplified definitions (as used by the author) for the terms *thermophilic, alkaliphilic*, and *halophilic* using the cardinal temperatures, pH values, and salt concentrations for growth.

Several of the thermophilic anaerobes and a few aerobes can grow up to $pH^{25\,°C}$ 8.5 [the superscript denotes the temperature at which the pH was determined; see text and (19)] or even 9.0, [e.g., the well known *Thermoanaerobacter* (basonym *Clostridium*) *thermosaccharolyticum*, *Thermoanaerobacter* (basonym *Clostridium*) *thermohydrosulfuricus*, or some strains (biotypes) of the archeaon *Methanothermobacter* (basonym *Methanobacterium*) *thermautotrophicus* (see following text]; however, because their pH optima are below pH 8.0, only some are briefly mentioned in this article, which is primarily concerned with anaerobes with pH optima for growth above $pH^{25\,°C}$ 8.0 and T_{opt} above 50 °C.

THERMOPHILIC "SUPERBUGS" AMONG VALIDLY DESCRIBED SPECIES OF EXTREMOPHILES

Because anaerobic alkalithermophiles have to cope with more than one extreme condition, it is useful to compare them to the "champions," that is, to the thermophilic microorganisms that exhibit the most acidic or alkaline pH_{opt} (Table 2).

The upper temperature limit for isolated microorganisms is held by an anaerobic archaeon, *Pyrolobus fumarii*, with a T_{max} of 113 °C and a T_{opt} of 106 °C (20). The highest values for a (eu)bacterium are below 100 °C, for example, *Aquifex pyrophilus*, with a T_{opt} of 85 °C and T_{max} of 95 °C (21). It is evident from the examples in Table 2 that not all extreme environments and growth conditions are equally populated by bacteria and archaea and that it appears that some groups are more able to adapt than others to the extreme conditions. It is important, however, to keep in mind that we probably know less than 3% of the

Table 1. Simplified Definitions of Alkaliphiles, Thermophiles, and Halophiles

Thermophiles	T_{min} —	$T_{opt}>50$ °C	$T_{max}>60$ °C
Extreme thermophiles	T_{min} usually >60 °C	$T_{opt}>75$ °C	$T_{max}>85$ °C
Alkalitolerant microorganisms	pH_{min}*	$pH_{opt}<8.5$*	$pH_{max}>9.0$*
Alkaliphiles		$pH_{opt}\geq 8.5$*	$pH_{max}\geq 10.0$*
Facultative	$pH_{min}<8.0$*		
Obligate	$pH_{min}>8.0$*		
Halotolerant Microorganisms	$[NaCl]_{min}$** 0	$[NaCl]_{opt}<1\%$	$[NaCl]_{max}>3\%$
Halophiles:**			
Facultative (haloversatile)	$[NaCl]_{min}>0$	$[NaCl]_{opt}>1\%$	$[NaCl]_{max}>10\%$
obligate,	$[NaCl]_{min}>1\%$		
moderate	$[NaCl]_{min}>2\%$	$[NaCl]_{opt}>3\%$	$[NaCl]_{max}>5\%$
extreme	$[NaCl]_{opt}$ usually >10%	$[NaCl]_{opt}>15\%$	$[NaCl]_{max}>20\%$

*The values depend on the temperature at which the pH was determined and the pH meters were calibrated. Depending on the medium, temperature, and the pH range, the differences can be as large as 1 pH unit (see Fig. 2 in cit. 19). Since this is frequently not stated, the presented data from the literature are difficult to compare. In the presence of elevated salt concentrations the pH value is even more difficult to measure. The above values are the ones usually used by the author, however, various definitions exist and there is no uniformous agreement among scientists.

**A more detailed definition is given by Grant et al. (3).

Table 2. The Most Acidophilic and Alkaliphilic Thermophiles Among Validly Published Species*

	Acidophile	pH_{opt}	T_{opt}	Alkaliphile	pH_{opt}	T_{opt}
Archaea						
Aerobic	*Picrophilus oshimae*	0.7	60	*Aeropyrum pernix* (facultative)	7.0	93
	torridus			*Pyrobacculum* spec.	3.0	100
Anaerobic	*Sulfuricoccus mirabilis*	2.0	72	*Thermococcus alcaliphilus*	9.0	85
	yellowstonii	2.5	62			
Methanogens	*Methanococcus igneus*	5.7	88	*Methanothermobacter thermoflexus*	8.1	55
(Eu)bacteria						
Aerobic	*Sulfobacillus acidophilus*	2.0	50	*Bacillus palidus*		
				Sphaerobacter thermophiles	8.5 55	63
Anaerobic	*Thermoanaerobacterium aotearoense*	5.2	62	*Clostridium paradoxum*	$10.3^{(25\,^{\circ}C)}$ $9.3^{(58\,^{\circ}C)}$	55

*Validly described species are species published in the *International Journal of Systematic and Evolutionary Microbiology* (former *International Journal of Systematic Bacteriology*) either directly or included in the table of microorganisms published outside of the Journal.

estimated existing microorganisms and that this distribution depends on the presently culturable microorganisms. This distribution could have changed by the time this article has been printed. Novel microorganisms are presently described at an elevated rate because of renewed interest in biodiversity and the easiness of obtaining 16S rDNA sequence data as a tool for discriminating isolates and for demonstrating that unknown microorganisms exist in a given environment (environmental 16S rDNA sequences). The problem frequently is to find the right conditions to grow the "not yet cultured" microorganism under laboratory settings.

An elegant way to show the diversity of the alkalithermophiles with respect to temperature and pH is to plot the optimum growth temperature versus the optimum pH (Fig. 1). The majority of isolated bacteria through all categories are neutrophiles and grow optimally between pH 6.5 and 7.5 (not shown in Fig. 1 for clarity). An exception is the obligately aerobic archaea, which with one or two exceptions, grow optimally at the acidic pH ranges between 1 and 2.5. The data in Figure 1 suggest a phenomenon that also has been seen among strains of a species, that is, the more alkaline the pH optimum for a bacterium is, the lower is its temperature optimum. This leads to the occurrence of a "white spot" in the upper left side in this diversity map (Fig. 1) created by the void of observed microorganism with optimum growth temperatures above 65 °C and optimum $pH^{25\,^{\circ}C}$ values for growth above 9.0.

BIOGEOGRAPHY AND BIOGEOCHEMISTRY

Alkalithermophiles require alkaline conditions and elevated temperatures for growth. Does this mean they can only be found in very distinct niches where both conditions are obviously provided, such as alkaline hot springs, alkaline (soda) lakes with geothermal or solar heat sources?

The more general question is an old one, already asked in a broader sense by Beijerinck as cited by Baas-Becking (37) saying, "Everything is everywhere, but the milieu selects." In other words, the question from the view of biogeography is, are indeed all bacteria everywhere (i.e., ubiquitous) and the subsequent question from the view of biogeochemistry is then whether only the geochemistry and environmental conditions determine which of the microorganisms are prevalent in a given habitat. Presently, this question cannot be answered even for the small percentage of microorganisms that have been described because of lack of data. On the other hand, one can speculate that most bacteria are somewhat "ubiquitous" because many microorganisms, especially when adsorbed to dust particles, can withstand various states of dryness and thus are distributed worldwide by wind. Ash particles from volcanic eruption such as Mt. Helena have traveled around the globe within a few days, and after big sandstorms in the Sahara, the sand can be collected on the west coast of the United States. Tiny water droplets containing microorganisms can also be distributed by strong air movements. The question, "How often does this occur?" remains to be answered through more sophisticated studies. As example, the author has isolated from antarctic snow thermophiles also found in steam vents from the volcano Mt Erebus, miles away from the site where the snow was collected (unpubl. results). So, theoretically, "all" bacteria should be more or less distributed around the globe. But the question then is whether the receiving environments will support the survival of the "seeded" microorganisms and, furthermore, whether these organisms can compete successfully against the existing microbial population.

Returning to the original question with respect to alkaliphiles and specifically to the anaerobic alkalithermophiles, Horikoshi and Aiba (38) showed that one could isolate mesophilic, aerobic alkaliphiles from soils with neutral and acidic bulk pHs. The same is true for the anaerobic alkalithermophiles (19). Although only a few anaerobic alkalithermophiles have been validly described,

	pH_{opt}	pH-range	T of pH-det.* * (°C)	T-range (°C)	T_{opt} (°C)	Habitat or Location of Isolation	Ref.
Methanogenic Archaea							
Methanohalophilus zhilinae	9.2				45	Natrono Lake in **Wadi el Natrun** (Egypt)	22
*Methanothermobacter thermautotrophicus***	8.0			35–75	60		23
'*Methanothermobacter thermoflexus*'	8.1				54.5	Sewage Digestor	24
Anaerobic Archaea							
Thermococcus alcaliphilus	9.0	6.5–10.3	25	73–95	85	Shallow marine hydrothermal vent in Vulcano, Italy	25
Thermococcus fumicolans	8.5	4.5–9.5		73–103	85	Deep-sea hydrothermal vent at North Fiji Basin	26
Anaerobic Bacteria, (Gram-type positive)							
Clostridium paradoxum	10.1 9.3	7.0–11.0	25 55	30–63	56	Sewage sludge, aerobic and anaerobic, wide spread	27
Clostridium thermoalcaliphilum	9.8 9.2	7.0–11.0	25 50	27–57.5	51	Sewage sludge, anaerobic, Atlanta, GA, U.S.A.	28
Anaerobranca gottschalkii	9.5	6.0–10.5	25	30–65	52	Hot spring in terrestrial alkaline lake; Lake Bogoria, Kenya	29
Desulfotomaculum alcaliphilum	8.7	8.0–9.5	50		53	Cow/pig manure in the Moscow area, Russia	30
Anaerobranca horikoshii	8.5	6.9–10.3	60	34–66	57	Hot springs (restricted) Yellowstone NP (U.S.A.)	31
Thermosyntropha lipolytica	8.5 8.0	7.1–9.5	25 60	52–70	63	Hot spring in terrestrial alkaline lake; Lake Bogoria, Kenya	32
Thermobrachium celere	8.2	5.4–9.5	66	43–75	66	Ubiquitous in sediments, manure compost, hot springs	33
Caloramator indicus	8.1	6.2–9.2		37–75	63	Artesian aquifer, (India)	34
Thermoanaerobacter ethanolicus	8.0	4.5–9.8	25	35–78	69	Hot springs, slightly alkaline or acidic, Yellowstone NP (U.S.A.)	35
Thermoanaerobacterium thermosaccharolyticum	8.0	5.8–8.8	25	20–67	58	Ubiquitous, canning industry	36
Anaerobic Bacteria, Gram-type negative							
'Thermopallium natronophilum'	9.5	?–10.5	25	?–78	70	Hot spring in terrestrial alkaline lake; Lake Bogoria, Kenya	5

*See text for correct pH determination with pH meter calibrated with electrode equilibrated at the elevated temperature.

**Only the strains that were originally described as *Methanobacterium thermoalcaliphilum*, now belonging to *M. thermautotrophicus*.

Figure 1. Anaerobic Alkalithermophiles and Anaerobic Alkali-tolerant Thermophiles

a great variety of distribution patterns have been observed: a species can be found

1. only in one very restricted location (= narrow biogeography),
2. only in one type of environment but at different geographic locations (= narrow and restricted biogeochemistry), and
3. apparently ubiquitously distributed (= relaxed biogeography and biogeochemistry). However, for nearly half of the known anaerobic alkalithermophiles, only one or two strains have been described and no reports have been made of failed attempts to isolate them from other locations or other environments. Thus, their distribution cannot be discussed. The presently known anaerobic alkalithermophiles and alkali-tolerant thermophiles with pH_{opt} of and above 8.0 will be discussed later in order of these four groups.

SOME COMMENTS ON METHODS FOR STUDYING ANAEROBIC ALKALITHERMOPHILES

The cultivation of the anaerobic thermophiles in the laboratory requires a few precautions, which have been

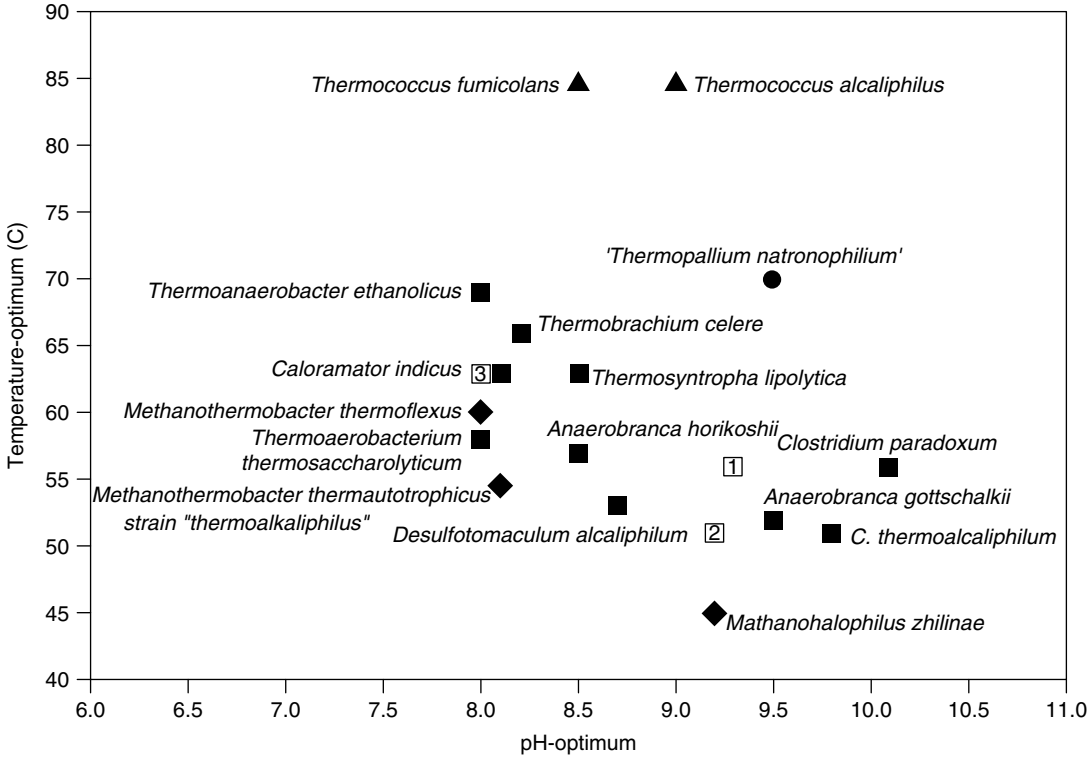

Figure 2. Distribution of anaerobic alkalithermophilic alkali-tolerant thermophilic bacteria and archaea in an optimum growth temperature versus optimum growth pH chart. Keep in mind that the optima are some times very broad, and thus an intermediate value has been chosen for clarity (see text and Table 3 for details). Furthermore, in several instances, it is not clear how authors had determined the pH, and thus a direct comparison of the published pH_{opt} data is not always proper (see text) ■, □ gram-type positive anaerobic bacteria; □ pH values determined at the actual growth temperature: 1 *Clostridium paradoxum*; 2: *Clostridium thermoalcaliphilum*, 3: *Thermosyntropha lipolytica*; • gram-type negative anaerobic bacteria; ♦, ⅄ anaerobic archaea. with ♦ for methanogens.

described in detail previously (39). The precautions used when working with mesophilic alkaliphiles are naturally also to be observed for thermophilic ones. In addition, at high pH and elevated temperatures, substances other than agar must be used to solidify growth media; these include products such as Gelrite or Phytogel (both Sigma, St. Louis) or silica gel (39–41). As already indicated earlier, one problem encountered when comparing reports on alkali- or acidophilic extremophiles is that in the descriptions no indications are given as to how the pH was determined or that the reported pH was measured at room temperature or with a pH meter containing a temperature probe without proper calibration of the pH meter. Thus, it is very difficult to compare the published pH data. Depending on the medium used, the differences at pH 10 and 60 °C can be around one whole pH unit. Around pH 7.0, the error is usually relatively small, about +/− 0.2 pH units. The proper way is to determine the pH with a pH meter and electrode calibrated with standards kept at the elevated temperature and their value accordingly corrected. The author suggested indicating how the pH was measured by a superscript, for example, $pH^{65\,^{\circ}C}$ when determined correctly at 65 °C or $pH^{25\,^{\circ}C}$ when measured at 25 °C (19). Another problem can be caused by the instability of bicarbonate at temperatures above 50 °C forming carbon dioxide and carbonate, but on the other hand, when carbon dioxide is provided in the gas phase beyond the equilibrium for the given temperature, it will be adsorbed into the alkaline medium, again potentially changing the pH, especially if the medium is slightly buffered. The distribution of the $CO_2/HCO_3^-/CO_3^-$ species is described by the Henderson-Haselbach equation. At high salt concentrations one appropriate method is to analyze the salt components and theoretically calculate the pH (42–45). Knaus and coworkers (44) and Mesmer (45) discuss the use of cells without liquid junction to minimize the salt effect. Other typical problems include chemical reactions mimicking biological activities. For example, when studying alkaliphilic iron-reducing thermophiles, it is important to run proper substrate controls because at the elevated temperatures many compounds (including glucose and other sugars and carbohydrates with reducing ends) reduce iron (III) fast in the growth media. Furthermore, at elevated temperatures and alkaline pH, many substrates will undergo increased modifications with increased pH values or react with other compounds in the medium forming potentially inhibitory products. One other point to be aware of is that many antibiotics are not stable at elevated temperatures. Peterrandel and coworkers (31)

investigated only neutral and slightly acidic pH values at 50 and 70 °C but demonstrated that some antibiotics quickly lose their activity totally, whereas some others yielded the breakdown products that were more potent than the parental compounds. Unfortunately, these data are missing for the alkaline growth conditions. If an antibiotic, however, is bacteriocidal for the bacteria or archaea in question, regardless of the antibiotic's stability, it will still work for enrichments or discrimination against resistant strains as long as no spores are present, which could later germinate when the antibiotic has become ineffective.

ANAEROBIC ALKALITHERMOPHILIC BACTERIA AND THEIR HABITATS KNOWN THUS FAR

Anaerobic Alkali-Tolerant Thermophiles/Alkalithermophiles Found in Only One Location

Anaerobranca horikoshii **(Firmicutes).** All strains isolated so far originated from only one relatively small, geothermally heated area near Old Faithful Geyser, Yellowstone National Park. Nine strains of this alkalithermophile were isolated and described by Engle and coworkers (29). No similar strains could be isolated from other sites in Yellowstone National Park or from samples collected from geothermally heated locations in New Zealand, Italy, or Japan despite using the same or similar isolation procedures. One of the peculiarities is that this little-researched geothermal field contains slightly acidic and slightly alkaline hot springs intermingled and just a few yards apart from each other. *Anaerobranca horikoshii* was isolated from both, the acidic and the alkaline pools. Recently, a new species, *Anaerobranca gottschalkii* (46), was isolated from the alkaline lake Bogoria in Kenya (pH 9 to 10, temperature 50 to 80 °C), from which two other anaerobic alkalithermophiles were isolated so far (for description see following text).

Anaerobranca horikoshii is gram-type positive. This species was the first published anaerobic thermophile exhibiting truly branched cells. Cells are flagellated and usually show a sluggish tumbling motility. The G + C mol% is 33 to 34 mol%, which places it in the middle range of the low G + C gram-type positive branch. Spore formation has not been observed, but characteristic sporulation genes are present (27). Its growth characteristics reflect the two types of pools from which it was isolated: $pH^{60\,^{\circ}C}$ range is 6.5 to 10.3; optimum 8.5 to 8.8 (strain dependent), and temperature range for growth is 30 to 66 °C; optimum approximately 57 °C. The bacterium is proteolytic and in contrast to *A. gottschalkii* (46) does not show any growth with carbohydrates. The fermentation products from yeast extract are mainly acetate carbon dioxide and hydrogen. Interestingly, this bacterium can also use fumarate as an electron acceptor forming succinate. In the absence of fumarate, however, 1 mM succinate is growth-inhibiting.

[this term was introduced to differentiate between "gram" as taxonomic term (i.e., gram reaction or gram-staining result) and the use as systematic term (gram type). Both terms can be different for one bacterium, as for example, is seen for many clostridial species that systematically belong to the gram (type)-positive (i.e., absence of LPS) Firmicutes branch but stain negative at all growth phases; (47,48)].

Anaerobic Thermophiles/Alkalithermophiles Found Only in One Type of Environment But at Various Geographic Locations

Clostridium paradoxum **and** ***Clostridium thermoalcaliphilum*** **(Firmicutes).** *Clostridium paradoxum* is presently the most alkaliphilic thermophile among the validly published microorganisms growing optimally above 50 °C (28,33). Its exclusive habitat is sewage sludge and it has been found in samples from sewage plants on three different continents. One strain was isolated from a New Zealand hot spring, which, however, was contaminated with wastewater in the past ((19), and unpubl. results). The temperature range for the growth of *C. paradoxum* is around 30 to 63 °C with an optimum at 56 °C and for *C. thermoalcaliphilum* between 27 to 57.5 °C with an optimum at 51 °C. For both, the pH range is between $pH^{25\,^{\circ}C}$ 7.0 and 11 with optimum at $pH^{25\,^{\circ}C}$ 10.1 ($pH^{55\,^{\circ}C}$ 9.3) and $pH^{25\,^{\circ}C}$ 9.8 ($pH^{50\,^{\circ}C}$ 9.2), respectively (28,33). The pH in the sludge at the Athens sewage plants and similarly at a plant in California reveals that the temperature does not exceed peak values of 40 °C and $pH^{25\,^{\circ}C}$ of 7.8. None of the plants from which samples were obtained had recorded peak $pH^{25\,^{\circ}C}$ values above 7.8 and/or peak temperatures above 40 °C. Because the bulk temperatures and pH values of the investigated sewage plants exhibited values that are at the lower growth limit of this bacterium, one would not expect to find this bacterium in relative high numbers. Enumerations, however, that did not discriminate between *C. paradoxum* and the physiologically very similar bacterium *C. thermoalcaliphilum*, yielded between 100 and 10,000 colony forming units per milliliters of sewage sludge. Also, surprisingly, the bacterium was found in similar numbers in the samples from the anaerobic digesters and from the aerobic (activated) sludge. The authors were not able to isolate these bacteria either from the samples of the discharge water leaving the sewage plant in Athens Georgia, or from the samples of the river sediment collected between 100 m and 3 km downstream of the plant's discharge point. *Clostridium thermoalcaliphilum* was only characterized by strains from the sewage plant in Atlanta, Georgia, and is apparently less abundant than *C. paradoxum* but should be present in most other sewage plants. Unfortunately, no 16S rRNA probes have been made and used for elucidating the distribution. Although spores could not be detected in *C. thermoalcaliphilum* cultures, and the culture is not transferable after three to four days from cultures tubes stored between 4 and 50 °C, it contains representative sporulation genes (27). In contrast, in a two-day-old culture, up to 99% of *C. paradoxum* cells exhibited sporulation. Cells that start to sporulate become very motile. After an additional one to two days, the cells lyse and release the spores, which form aggregates in a matrix of carbohydrate-like material (28). We assume that this feature is responsible for the spores becoming trapped in the slime and then attached to particles in the sludge and thus retained in the sewage plant. Surprisingly, it appears that this occurs to 100% because, as mentioned earlier,

no viable cells (or spores) could be recovered from outside the sewage plants except from the stored dewatered sludge dredged out of the settlement ponds. The relatively high numbers of colony-forming units determined in agar shake-roll tubes (40) suggests that these bacteria, especially *C. paradoxum*, must be able to grow under the bulk neutrophilic and mesobiotic conditions of the sewage plant. The author assumes that two features contribute to the relatively high numbers of *C. paradoxum*–like bacteria in the sewage systems: (*1*) The occurrence of microniches with elevated temperatures and alkaline pH as a result of various microbial activities (production of metabolic heat during high microbial metabolism of fast degradable substrates and release of ammonium ion from amines and amino acid–rich waste components) and (*2*) the short doubling times of 16 (*C. paradoxum*) and 19 minutes (*C. thermoalcaliphilum*), which allow these bacteria to take advantage of temporary microniches (28,33).

Both bacteria are peptidolytic and glycolytic, using sugars such as glucose, fructose, sucrose, maltose, cellobiose, and xylitol, depending on the strain. None of the strains grew with xylose or ribose. Main differences between *C. paradoxum* and *C. thermoalcaliphilum* are in sporulation (as discussed earlier) and in their cell wall types despite only about 2% difference in the evolutionary distance based on the 16S rDNA sequences. *Clostridium paradoxum* has the meso-diaminopimilic direct type cell wall and exhibits a combination of the restriction and septation mechanism during cell division, whereas *C. thermoalcaliphilum* has the L-Orn-D-Asp type of cell envelope and the regular septation mode of cell division. Cells of both gram-type positive species stain gram-negative because of a relatively thin cell wall (28,33), but LPS is absent as demonstrated by the LPS-polymyxin B test (48). Growth of both microorganisms is enhanced in the presence of supplemented sodium (the base medium contains around 5 mM Na^+). In the presence of 100 mM K^+, 20 mM Na^+ are required for growth. The optimum sodium-ion concentrations for *C. paradoxum* were 50 to 100 mM and 50 to 500 mM at pH 9.6xs and 7.5, respectively, and in the presence of 100 mM KCl, the optimum concentration for Na^+ shifted to 150 to 300 mM. However, under all conditions, 1 M inhibited growth completely, whereas *C. thermoalcaliphilum* could tolerate up to 1.25 M (7.3 %) NaCl and thus can be classified as a moderate halotolerant alkalithermophile. *Clostridium paradoxum* did not sporulate below 10 mM Na^+ and motility was also sodium-ion and pH dependent. The analyzed sewage fluids contained between 0.5 and 1.5 mM K^+ and 3 to 3.5 mM Na^+, values clearly far below the optimum of the purified cultures, and should not allow *C. paradoxum* to sporulate. However, the sewage samples contained spores because *C. paradoxum* could be easily isolated from pasteurized (85 °C for 20 minutes) samples (unpubl. results). Obviously, other compounds in the sewage must substitute for the low Na^+ concentration.

Both species are sensitive to several common antibiotics at $pH^{25\,°C}$ 7.5 and 10.5. A comparison of the phospholipid fatty acids showed that *C. thermoalcaliphilum* had a similar profile to that of *C. paradoxum* cells grown at pH 7.5 but was different for the profile from cells grown at $pH^{25\,°C}$ 10.5. The main difference at the two pHs was the abundance of *i*17 : 0, but under all conditions both the species had *i*15 : 0 as the main fatty acid. In summary, from the earlier analysis and from the analysis of the fatty acid composition of *Caloramator gottschalkii* (46) and *Tb. celere* (36), no unusual or, for anaerobic alkalithermophiles, specific fatty acid pattern has emerged.

Ubiquitous Anaerobic Alkali-Tolerant Thermophiles/Alkalithermophile

***Thermoanaerobacterium* (basonym *Clostridium*) *thermosaccharolyticum* and its Tartrate-Using Biotype '*Clostridium tartarivorum*' (Firmicutes).** *Thermoanaerobacterium thermosaccharolyticum* ($pH^{25\,°C}$ optimum around 7.8 to 8.0, $pH^{25\,°C}_{max}$ of 8.5 to 8.8; T_{opt} of 55 to 60 °C, T_{max} 67 °C) is an alkali-tolerant thermophile and among those it is a truly ubiquitous, spore-forming, saccharolytic anaerobe found in both mesobiotic and thermobiotic soil and sediment worldwide ((49) and Wiegel unpubl. results). This bacterium is well known as the can-swelling ("hard swelling") agent in the canning industry because it can produce carbon dioxide to pressures of several atmospheres from various carbohydrates including starch and pectin. For the reason that spores of this species survive frequently in sugar-refining and vegetable-canning processes, industrial research has focused on its formation of heat-resistant spores (D_{10}-time four minutes at 121 °C) (50). However, it should be mentioned here that these spores are not the most heat-resistant spores: spores of *Moorella thermoacetica* strain 4 have a D_{10}-time of nearly 2 h at 121 °C (51). The previously described alkali-tolerant species, '*C. tartarivorum*', which uses tartrate, is now recognized only as a different strain (biotype) of *Th. thermosaccharolyticum* (52). Fermentation products from glucose include the typical products also observed with many other anaerobic thermophiles such as ethanol, butyric acid, acetate, lactate, carbon dioxide and hydrogen. Whether mainly ethanol or butyric acid is formed depends on the strain and applied growth conditions (53). Propan-1,2-diol can also be a major product from glycerol.

This is the only species among the anaerobic thermophiles for which a plasmid has been clearly isolated and characterized (54).

***Thermobrachium celere* (Firmicutes).** *Thermobrachium celere* is the second truly ubiquitous alkali-tolerant anaerobic thermophile described so far; however, it is able to grow at more alkaline conditions ($pH^{25\,°C}_{max}$ 10.1) than *Th. thermosaccharolyticum* ($pH^{25\,°C}_{max}$ 8.8). *Thermobrachium celere* grows in a $pH^{66\,°C}$ range from $pH^{66\,°C}$ 5.0 to 9.3 for *Th. thermosaccharolyticum* with an optimum $pH^{66\,°C}$ between 8.1 and 8.6 and in the temperature range from greater than 37 to 75 °C (no growth at 77 °C) with an optimum around 60 to 67 °C. As the species name indicates, one of its main characteristic features is a fast growth rate. For the type strain JW/YL-NZ35, the doubling time under optimum growth conditions is 10 minutes in batch and continuous culture ((36) and unpubl. results). For other strains, it is between 10 and 25 minutes. The type strain was isolated from a mixed water sediment sample of a hot spring receiving human wastewater from a bathhouse in

Ohinimutu, New Zealand. It is the same spring from which the only nonsewage sludge-derived strain of *C. paradoxum* was isolated. The in situ temperatures of the sampled site ranged from 56 to 75 °C and the pH ranged from 7.4 to 7.8. The mixed sample had a pH of around 7.7. Many other strains were isolated from various thermobiotic samples including samples from several pools in Yellowstone National Park and a slightly acidic pool in the thermal area in Larderello (Toscani, Italy) with a water pH of 4.8 and a temperature of 60 to 65 °C. Interestingly, this sample had been stored for more than five years at 4 to 7 °C before the enrichment was done, and no spores or heat-resistant (15 minutes 85 °C) forms observed for any of the strains from this species. Again, however, Brill and Wiegel ((27); unpubl. results) demonstrated that characteristic sporulation genes are present in this species. In contrast to the cells in the sediment samples, in which the cells survived for several years, grown cultures tended to lyse when stored at or below room temperatures within a few days or months depending on the strain tested. The mesobiotic sources for isolation included sediments from the Platte River in Argentina (pH 5.0) and wet soil samples from its floodplain meadows (pH 6.8) and River Elbe in Brunsbuettel (Germany) (pH 7.0). Both rivers are regarded as highly polluted by industrial and urban sources. We also isolated a strain from the alkaline Mono Lake in California, ($pH^{25\,°C}$ 9.0, ambient sediment at the edge of the Lake). Several strains were also isolated from anthropogenic environments such as horse manure compost at the University of Georgia (pH 7.8; ambient to 55 °C). Many other strains that appear physiologically similar, exhibit similar protein profiles, and thus are assumed to be strains of this species were isolated from many other mesobiotic (United States) and thermobiotic places (e.g., Iceland, Thailand, Fiji Island), however their 16S rDNA sequence has not been determined. All strains tested required yeast extract for growth. The typical fermentation products for thermophilic glycolytic/peptolytic anaerobes, acetate, carbon dioxide, hydrogen and ethanol and the less common product among thermophilic anaerobes, formate, are formed from some hexoses and pentose. However, no lactate was found as a fermentation product. Interestingly, neither pyruvate nor lactate could serve as substrate. Thus, this bacterium neither competes with lactate/pyruvate-using sulfate reducers for substrates, nor does it become involved in the lactate-based substrate-product-substrate food web. Considering the wide distribution and different environmental conditions of the samples from which they originated, it is not surprising that a wide morphological variability among the strains is observed. No microaerophilic growth or growth in oxidized media was observed with any strain tested, characterizing this as an obligate anaerobic bacterium. However, short exposures to oxygen does not kill this bacterium.

The type strain JW/YL-NZ35 contained structures resembling defective bacteriophages. However, we were not able to isolate any lytic or lysogenic phage particles from any of the samples (including sewage sludge) or strains (D. Byrer, dissertation, 2001; University of Georgia). It is interesting to note here that no virus has been described for any of the anaerobic thermophilic Firmicutes, despite several attempts to isolate them. Only one plasmid has been characterized thoroughly and made available (54). The few descriptions of other detected plasmid in anaerobic thermophiles could not be reproduced in the author's laboratory, nor has their confirmation been reported in the literature.

Bacterial Species That Comprise Only One or Two Strains Isolated From One Location But Without Published Attempts to Isolate Them From Other Areas

The old fashioned tradition of having several strains isolated from more than one location for the description of novel microorganisms has vanished, lately with the increased description of anaerobic extremophiles. Thus, for several of the alkalithermophiles, only one or two isolates from the same location have been described. To the author's knowledge, neither accounts of additional isolations of the novel strains of these species nor reports of failed attempts have been published. Thus, at this time, it is not clear to which of the aforementioned categories these species belong. In a few instances, relatively similar environmental 16S rDNA sequences have been obtained. The possible conclusions that the occurrence of relatively similar environmental 16S rDNA sequences demonstrates that additional strains or at least physiologically very similar strains exist elsewhere, however, is to be taken with great caution. In the laboratories of the author (55), and from others, it has been observed in several instances that similar 16S rDNA (>98% similarity) sequences do not necessarily indicate a similar physiology. Thus, environmental sequences are not considered in this article and the readers are referred to the gene banks for their own analyses and conclusions.

Species from Geothermally Heated Environments

Lake Bogoria

Anaerobramca gottschalkii. *Anaerobramca gottschalkii* (46) is with a $pH^{25\,°C}$ range from 6.0 to 10.5 (optimum $pH^{25\,°C}$ 9.5) and 30 to 65 °C (optimum at 50 to 55 °C), a true alkalithermophile and was isolated from the inlet of Lake Bogoria (East African Rift Valley, Kenya). It is one of the three anaerobic thermophiles isolated so far from this alkaline lake, which contains hot springs and has only recently been studied in some depth. In contrast to the other alkalithermophiles described earlier, this isolate grows well on several monosaccharides including glucose, ribose, mannose, fructose, and polysaccharides such as starch, xylan, pullulan, and cellulose. Besides being glycolytic, it is also proteolytic, growing on peptone, tryptone, and yeast extract. This species requires NaCl and is slightly halophilic. Under optimum growth conditions, the doubling time of 48 minutes is significantly longer than the doubling times reported for the earlier discussed alkalithermophiles from mesobiotic environments. Prowe and coworkers (32) studied energy transduction in this halophilic, alkaliphilic, anaerobic thermophile and concluded that the uptake of amino acids required Na^+ and that leucine uptake occurred by an obligately Na^+-symport. Using inside-out membrane vesicles, they

obtained a stimulation of the ATPase activity by Na^+ and Li^+ ions, which suggests that the primary mechanisms of energy transduction depended on the cycling of Na^+ ions. The anaerobic thermophilic communities have not been studied in most of the saline alkaline lakes. Because one expect that alkalithermophiles are involved in the other breakdown reactions of autotrophically produced biomass or using autotrophic substrates in the sediment, it is very likely that more and physiologically different species will be described in the future.

Thermosyntropha lipolytica (Firmicutes). Thermosyntropha lipolytica, a nonmotile, slightly curved rod-shaped and gram-stain negative bacterium, is the second species that has been isolated only from samples of Lake Bogoria (Kenya). Three different strains, showing different lipolytic patterns, have been isolated from different samples, but all came from the same sampling site. Because this bacterium uses only the fatty acids and not the glycerol from the lipids, it is obligately syntrophic (i.e., requiring a hydrogen-utilizing partner) when growing on lipids. Linear saturated and unsaturated fatty acids with 4 to 18 carbons are used apparently because of the presence of at least two different lipases/esterases (unpublished observations). As to be expected, evennumbered acids yield mainly acetate and oddnumbered fatty acids also yield additionally 1 mol propionic acid per fatty acid degraded. Although hydrogen (100%, vol/vol, gas phase) is not inhibitory during growth on yeast extract, however, a low-hydrogen partial pressure is required during growth on lipids because H_2 = producing the fatty acid degradation is thermodynamically unfavorable (positive delta $G^{o'}$). Thus, the reaction needs to be "pulled" by maintaining a low-hydrogen partial pressure through interspecies hydrogen-transfer by either methanogens reducing carbon dioxide-moieties to methane or sulfate reducers reducing sulfoxy anions to H_2S. Free fatty acids above 1 mM are inhibitory and therefore the equimolar addition of Ca^{++} ions is necessary for maintaining growth. The lipase activity of the *Ts. lipolytica* in the three tested strains was constitutive (we are presently investigating the lipase(s) in more detail); thus, growth is inhibited by the fatty acids when grown in pure culture in the presence of lipids such as olive oil because of the detergent action of the free fatty acids. *Thermosyntropha lipolytica* is the first thermophilic syntrophic bacterium described and its 16S rDNA sequence places it in the phylogenetic neighborhood of the mesophilic syntrophs *Syntrophospora* and *Syntrophomonas*. Similarly, to the mesophilic syntrophs, crotonate serves as a good substrate to purify this thermophile. Growing on yeast extract under optimum conditions the doubling time is around one hour. The $pH^{25\,^\circ C}$ range is between 7.1 and 9.5 with an optimum between 8.1 and 8.9 ($pH^{60\,^\circ C}$ 7.6–8.1). The temperature range is 52 to 70 °C with an optimum at 60 to 66 °C. Among the tested sugars, only ribose and xylose, but not glucose, fructose, mannose, arabinose, raffinose, galactose, sucrose xylan, or starch are used. Betain and pyruvate serve as good growth substrates. The main organic fermentation product from glucose was acetate (production of carbon dioxide and hydrogen was not analyzed). Thiosulfate could serve as an electron acceptor, as probably could elemental sulfur, but not sulfate (56).

Surprisingly, *Ts. lipolytica* is relatively sensitive to elevated salt concentrations. It tolerates 0.5% (wt/vol) NaCl, but not 1% and growth is totally inhibited by 2% (~350 mM).

The presence of this novel type among the anaerobic thermophiles in the alkaline lake is not surprising taking into account the high concentration of primary organic matter producers such as cyanobacteria and algae, which die off and become part of the anaerobic sediment (1,5), requiring the presence of microorganisms to degrade the large volume of lipids so produced.

'Thermopallium natronophilum' (Thermotogales). Jones and coworkers (5) and Meijer and coworkers (35) described the most alkaliphilic one among the extreme thermophiles, *'Tp. natronophilum'*, the third species isolated only from Lake Bogoria. It grows optimally at 70 °C (T_{max} 78 °C) at pH 9.5 (pH_{max} 10.5). To date, it is the only gram-type negative (LPS present in the cell envelope) among the alkalithermophiles and the only extreme thermophile among the true alkaliphilic anaerobic bacteria, which are the main reasons that this bacterium is mentioned here despite that it has not been validly or formally published. The other reason for mentioning this species here is that its discovery starts to make the "white spot" on the map of the diversity of alkalithermophiles (Fig. 1) smaller and shows that probably there are many more alkalithermophiles to be isolated. *Thermopallium natronophilum* is a heterotrophic bacterium with a sheetlike outer structure ("toga"), characteristic of *Thermotogales*, although the temperature range is somewhat lower than for other members of the *Thermotogales*. *Thermopallium natronophilum* has a distinct requirement for carbonate.

Yellowstone National Park

***Thermoanaerobacter ethanolicus* (Firmicutes).** *Thermoanaerobacter ethanolicus* (57) is the type species of the genus *Thermoanaerobacter*, a genus that so far only contains thermophilic species. Most are neutrophiles and have been isolated from various mesobiotic and anthropogenically and geothermally heated thermobiotic environments. From the same and similar environments, physiologically similar species of the related genus *Thermoanaerobacterium* have been isolated (58). This genus also contains only thermophilic anaerobes. Besides the differences in the 16S rDNA, the main taxonomic difference between these two genera of the Firmicutes is that generally the latter forms sulfur granules from thiosulfate, whereas the former reduces it all the way to H_2S (with the exception of *Thermoanaerobacter 'sulfurigignens'*, Dashti and Wiegel, unpublished results).

Thermoanaerobacter ethanolicus is a somewhat special case among the alkali-tolerant thermophiles and facultative alkalithermophiles. It exhibits an extremely broad flat $pH^{25\,^\circ C}$ optimum between 5.5 and 8.5 and thus falls under both definitions for a facultative alkaliphile and alkali-tolerant microorganism. Usually, a value in the middle of a broader optimum, is given, but for the type strain JW 200 practically no difference in the doubling time was

observed between pH 5.5 and 8.5. Although several of the *Thermoanaerobacter* species can grow at pH values around pH 9.0, all other species have a distinct, although frequently broad, pH_{opt} in the neutral pH range of 6.5 to 7.5. So far only one true (by 16S RDNA sequence analysis) *T. ethanolicus* strain, the type strain JW 200, has been published (57). For the second strain JW 201, so far, no 16S rDNA sequence has been obtained; the strain was assigned to this species by the authors on the basis of the similar physiology. Both were isolated from hot springs at Yellowstone National Park, strain JW 200 from the famous Octopus Spring area with a slight alkaline pH 8.8 and strain JW 201 from the slightly acidic (pH 5.5) Dragon's Mouth in the Sulfur Caldron area. For the other widely studied and published strain, *T. ethanolicus* 39E ((59) and references cited therein, (60)), also isolated from the Octopus Spring (Yellowstone Natl. Park), the 16S rDNA sequence reveals that it should be reclassified in its own species. It exhibits a more narrow pH optimum. A strain apparently similar to strain 39E (61) was isolated as a contaminant from the culture-collection culture of *Clostridium thermocellum* JW 20 [originally isolated from Louisiana cotton bales; (62)].

Thermoanaerobacter ethanolicus is a glycolytic thermophile requiring, as do most of the *Thermoanaerobacter* and *Thermoanaerobacterium* species, yeast extract, peptone, or tryptone for growth and for significant metabolic activity of resting cells (could not be substituted with amino acids). The most interesting physiological features is that similar to yeast, it can ferment glucose to ethanol at ratios of up to 1.9 mol ethanol per used glucose. In addition, it also produces ethanol from other hexoses and pentoses, such as xylose, which most yeast cannot ferment. Its industrial application is hampered by the lack of a genetic system to develop a robust industrial strain. The flagellated rods frequently exhibit uneven cell division leading to coccoid cells dispersed among chains of typical rod cells (57). At the time of isolation and characterization, no spores or heat-resistant forms were detected; however, according to the definition of Brill and Wiegel (27), *T. ethanolicus* is not asporogenic (= absence of sporulation genes) because it contains representative sporulation genes and thus can also be called *cryptic spore formers* (58). In the past, when spores were occasionally observed in long time subcultured *T. ethanolicus* cultures, they were attributed to contamination with the sporulating and ubiquitous *T. thermohydrosulfuricus* (found in mesobiotic and thermobiotic environments, including dust from the inside of buildings). The detection of representative sporulation genes in this bacterium suggests that it may indeed form spores on rare occasions, similar to what has been observed in *Thermoanaerobacter brockii* (63).

With respect to the growth response (doubling time) to temperature, *T. ethanolicus* exhibits a biphasic curve. This property is shared by several other *Thermoanaerobacter* and *Thermoanaerobacterium* species and some other bacterial (e.g., *Geobacillus stearothermophilus*) and archaeal species (e.g., *M. thermautotrophicus*), which grow in an extended temperature range from about 30 upto 78 °C, that is, a temperature span above 35 °C. It has been interpreted that these microorganism contain additional sets of genes for the rate-limiting steps either for the lower or the elevated temperature range (64). This enables these microorganisms to thrive in the upper mesobiotic and in the full thermobiotic temperature range, for example, in the hot water of the pools and in the cooler runoff waters and along the temperature gradient in the sediments. It is not known whether the doubling of the genes and obtaining genes (64) by horizontal gene transfer is a more recent adaptation to expand their habitats, for example, a response to changing temperatures around hot springs and geothermally heated environments or whether it may represent an adaptive step early during evolution (34). Although formulated as a hypothesis based on physiological observation, this question might be answered in the near future when more than one of the genome sequences for microorganisms with this feature have been published and thoroughly been analyzed and compared [e.g., from bacteria such as *T. ethanolicus, T. thermohydrosulfuricus, G.* (basonym Bacillus) *stearothermophilus* and archaea such as *M. thermautotrophicus*]. In contrast to *G. stearothermophilus* that only at temperatures above 50 °C can grow anaerobically with the concomitant formation of ethanol, the fermentation balance for *T. ethanolicus* does not change with the growth temperature.

Thermobiotic Artesian Spring

Caloramator indicus (Firmicutes). This alkali-tolerant thermophile described by Chrisostomos and coworkers (30) was isolated from deep-seated, nonvolcanically heated water of an artesian aquifer in the Surat District of the Indian Gujarat State. The aquifer samples had a temperature of 60 and 65 °C and exhibited no mat formations at the borehole. The in situ measured pH was 7.7 to 7.8. Although phenotypically similar strains were isolated from a parallel sample, this species has not been isolated from other locations. On the other hand, it is physiologically very similar to the ubiquitous *Tb. celere*; thus, it is speculated that additional strains could also be found in other geothermally heated environments (such as the Australian deep-seated aquifer) and may even be found in mesobiotic environments as well (see in the preceding text for the discussion on biogeography and biogeochemistry). Phenotypically similar isolates could be obtained from the same sample even after two years' storage, however, similar to observations with *Tb. celere* strains, cultures of the isolated strains grown to the stationary phase in laboratory media tended to lyse easily within several weeks, especially in media with pH values around 7.2 compared to pH 8.1.

The nonmotile rod-shaped cells of *C. indicus* stain gram-negative despite the typical gram-type positive cell wall. Filamentous cells up to 100 μm in length were frequently observed; however, no branched cells, as observed with *Tb. celere*, were reported. Also, no heat-resistant spores were observed. Growth on tryptone-yeast extract glucose agar plates did not occur (no training on soft agar was attempted), and thus isolation was by end point dilution series. The type strain IndiB4 grows in a temperature range of about 38 to 75 °C with an optimum between 60 to 65 °C and within a pH range of 6.0 to above pH 9.2 (the exact pH_{max} has not been published) classifying this

species as alkali-tolerant thermophile. Under optimum growth conditions, the doubling time for the type strain is 20 minutes; thus, it is the fastest growing alkali-tolerant thermophile to be isolated only from thermobiotic water. *Caloramator indicus* ferments carbohydrates including starch, dextrin, sucrose, cellobiose, glucose, and fructose, but not cellulose and dextran. The main fermentation products from glucose are ethanol, acetate, lactate, carbon dioxide, and hydrogen. In addition, branched fatty acids were observed, probably formed from the required addition of yeast extract or trypticase peptone. Similar to *Tb. celere*, storage granules have been observed.

Animal Feces

Desulfotomaculum alcaliphilum (Firmicutes). This spore-forming alkalithermophile with a $pH^{50\,^{\circ}C}$ 8.6 to 8.7 and a T_{opt} 50 to 55 °C was isolated from mixed cow/pig manure samples (Moscow region). Although manure can have alkaline pH values because of production of ammonia, these samples had pH values ranging from pH 6.0 to 70 (65). Most of the thermophiles isolated, so far, from manure have their pH_{opt} in the neutral range but are able to grow well above $pH^{25\,^{\circ}C}$ 8.5, for example, *Clostridium thermobutyricum* with a pH-range from $pH^{25\,^{\circ}C}$ 5. 8 to 9.0 and a $pH^{25\,^{\circ}C}_{opt}$ of 7.0 (66) and *M. thermoacetica* (25). However, this sulfate reducer is obligately alkaliphilic because it is not able to grow below $pH^{50\,^{\circ}C}$ 8.0. Neither did it grow in the absence of sulfoxy species in yeast extract–containing glucose-peptone medium. The bacterium requires low NaCl concentrations; no growth was obtained without the addition of NaCl to the mineral media in which Na_2S and Na_2CO_3 were replaced by potassium salts. As the strain was not isolated from an alkaline natrono lake, it is not surprising that the optimum salt concentration is low, that is, 0.1% to 1% NaCl. Five percent NaCl slowed growth down, whereas 7% totally inhibited growth, values that are typical for many of the anaerobic neutrophilic heterotrophic thermophiles isolated from geothermally heated springs (58). Furthermore, this alkaliphile requires obligatory carbonate (the range of optimum concentrations has not been given). However, this strain could tolerate (with extended lag times) 5% NH_4Cl and grew without any inhibition at 1.5%, surely an advantage for this organism's existence in manure.

To date, *D. alcaliphilum* is the only alkalithermophilic sulfate reducer and is described as an obligate anaerobe. The growth substrates used are typical for sulfate reducers: hydrogen and acetate (also used as carbon source), lactate, pyruvate, formate, and ethanol. The addition of vitamins is required for growth. Sulfate, thiosulfate, and sulfite can serve as electron-acceptors, but not sulfur or nitrate.

ARCHAEAL SPECIES AND THEIR KNOWN HABITATS

Among the archaeal alkalithermophiles and alkali-tolerant thermophiles are species belonging to the heterotrophic *Thermococcales* branch within the *Crenarchaeota* and to the methanogenic branch within the *Euryarchaeota*. *Thermococcus alcaliphilus* represents the species among the alkali-tolerant and alkalithermophiles with the highest optimum growth temperature of 85 °C, although growing optimally at pH of 9.0. So far, there are no reports of (extreme) thermophilic methanogens from any environment growing optimally above pH 9.0. Again, similar to many of the earlier discussed bacteria, only one strain of each of the archaeal species discussed later was isolated and characterized from one location. Thus, at this time one cannot make any conclusions about the biogeography of the archaeal alkalithermophiles, except that as a group they have been isolated from a variety of geothermally and anthropogenically heated environments similar to the alkalithermophilic bacteria.

Geothermally Heated Environments

Hyperthermophilic Heterotrophs

***Thermococcus alcaliphilus* and *Thermococcus fumicolans* Among.** the alkalithermophilic anaerobes growing optimally at or above $pH^{25\,^{\circ}C}$ of 8.5, these two species exhibits the highest T_{opt} (85 °C) for growth. *Thermococcus fumicolans* grows in a temperature-range of 73 to 103 °C and a relatively wide pH range of 4.5 to 9.5 with a pH_{opt} of 8.5 (22), whereas *Tc. alcaliphilus* has a $pH^{25\,^{\circ}C}_{opt}$ of around 9.0 and a pH range from 6.5 to 10.5 but exhibits a lower T_{max} of around 95 °C (26,67). Thus, both are hyperthermophilic, facultative alkaliphiles. Interestingly, again the species with the higher T_{max}, *Tc. alcaliphilus* (103 °C), has the lower pH range and optimum. Both species are marine isolates exhibiting a NaCl optimum around 2 to 4%. *Thermococcus fumicolans* was isolated from a deep-sea hydrothermal vent in the North Fiji Basin, whereas *Tc. alcaliphilus* came from a shallow hydrothermal vent at Vulcano Island in Italy. Both are irregular cocci, typical for the genus, and are proteolytic, that is, they grow best with complex media including yeast extract, peptone, and casein using sulfur or polysulfide as electron acceptors and forming H_2S as reduced product. Neither uses hydrogen but are not inhibited by its presence. *Thermococcus alcaliphilus* cannot use casamino acids or any sugar (so far tested), whereas *Tc. fumicola* can grow on a mixture of 20 amino acids. Although final cell yields are not affected, in the presence of polysulfide, the doubling time of *Tc. alcaliphilus* was 1.3 h, whereas in its absence it was 4 h. In the absence of sulfur compounds, the final product was hydrogen. Optimum growth conditions required 0.2% yeast extract and 6 mM polysulfide or sulfur. Besides 25 mol H_2S, other products per 10^8 cells were 15 mol acetate, 3.6 mol propionate, 1.9 mol isobutyrate, traces of isovalerate and carbon dioxide and ammonium (both not quantified).

Sun-Heated Alkaline Environments

Thermotolerant Methanogens

Methanohalophilus zhilinae. Although it is only a thermotolerant archaeon (T_{opt} 45 °C), the gram staining positive, methanol and methyl amine-using, coccoid archaeon *M.* (proposed to be changed to *'Methanosalsus') zhilinae* deserves to be mentioned here as the most alkaliphilic methanogen able to grow at elevated temperatures. The type strain WeN5 was isolated from the

sediment of the alkaline natrono Bosa Lake in the Wadi Natrun area (Egypt) with a water pH of 9.7 (68). At the same time, the authors isolated four other similar alkaliphilic methanogens; however, they were rod-shaped and mesophilic with a T_{opt} of 31 and 37 °C, respectively. An additional *Mh. zhilinae*, strain Z-7,936, (91% DNA : DNA homology to the type strain) was isolated from the alkaline soda lake Magadi (Kenya) (24). It grows optimally at pH_{opt}^{RT} up to 9.5 when grown on methanol and pH 8.5 when grown on methyl amines, however, this second strain is a mesophile with a T_{opt} around 37 °C and a T_{max} below 48 °C. It is inhibited by 30 mM methyl amine and ammonium and by 1 atm of hydrogen in the gas phase. Interestingly, the thermophilic type strain is also inhibited by 1 mM sulfide when growing on methyl amines, however, most of the alkaline lakes have concentrations of sulfides below this value (5).

Anthropogenic Niches

Methanogen

'Methanothermobacter thermoflexus' and Methanobacterium thermoalkaliphilum (sic). Now Strains of *Methanothermobacter thermautotrophicus*. The most alkaliphilic methanogen able to grow optimally above 50 °C is *M. thermoflexum* (sic) (69), which is to be renamed *Mt. thermoflexum* (23) (however, this species is not yet validly renamed according to the rules). This autotrophic, formate-using thermophile was isolated from the sludge of an anaerobic digester operating at elevated temperature and using wastewater containing methacrylates. This archeaon, in the presence of 150 mg/L Na_2S, and 1–30 g/L NaCl, grows with a doubling of 3.5 hours at 55 °C and pH 7.9 to 8.2. Such doubling time is typical for thermophilic methanogens but is long compared with one of the fastgrowing *C. paradoxum*. A very similar pH range has been observed for a strain isolated from cow manure and originally described as *M. thermoalcaliphilum* (70) (pH_{opt} 7.5 to 8.5; T_{opt} 58 to 62 °C) but later identified by 16S rDNA sequence and other characteristics as a strain (biovar) of the type species of *Methanothermobacter* (basonym *Methanobacterium thermautotrophicus* with its type strain delta H also isolated from sewage sludge (71). The type species with the type strain delta H, however, has a higher T_{opt} (around 68 °C) and a lower pH_{opt} of around 7.5. The typical *Mt. thermautotrophicus* strains can be easily isolated from many different mesobiotic (including lake sediments in which bulk temperature does not exceed 20 °C) anaerobic sediments and sewage digesters all over the world. It is regarded as a truly ubiquitous microorganism. However, no other similar alkaliphilic strains have been reported, although one can speculate that similar strains are present in manure of cows in other countries.

POSSIBLE CORRELATION BETWEEN THE SHORTEST OBSERVED DOUBLING TIMES FOR A SPECIES AND THE TYPE OF HABITAT FROM WHICH THE ALKALITHERMOPHILES WERE ISOLATED

From the few presently isolated and validly described anaerobic alkalithermophiles and alkali-tolerant thermophiles, it appears that bacteria isolated from mesobiotic and more pH-neutral habitats tend to exhibit unusually short doubling times of less than 20 minutes, whereas those isolated from thermobiotic environments, that is, geothermally heated environments or from alkaline habitats exhibit usually longer doubling times above 40 minutes. Among the archaea, all alkalithermophiles and alkali-tolerant thermophiles were isolated from thermobiotic environments and all exhibit doubling times longer than one hour. As only a few species of alkalithermophiles have been isolated and described, this trend might be just anecdotal. On the other hand, it could be that the organisms living in environments with a neutral bulk pH and mesobiotic temperatures can only thrive in these environments if they are able to react quickly ("instantly") to transient alkaline pH and elevated temperatures. The transient-elevated temperatures and alkaline pH values are thought to occur mainly in microniches created by microbial metabolism as discussed in the preceding text. It is futile to discuss whether the microorganisms have adapted to such environments or whether these environments have selected these type of microorganism until many more alkalithermophiles from various environments have been isolated, characterized, and thoroughly compared.

CONCLUSION

During the last eight years, several anaerobic alkalithermophiles and alkali-tolerant microorganisms have been isolated. They belong to several physiological types and to different systematics groups. The majority of isolated strains belong to the gram-type positive bacteria with proteolytic or glycolytic metabolisms, but the present group also contains one sulfate reducer, one gram-type negative bacterium, an extreme thermophilic member of the *Thermotogales*, two hyperthermophilic archaea, and three moderate thermophilic methanogenic archaea. The highest T_{opt} (85 °C) among the anaerobic alkalithermophiles is observed for the archaeal *Tc. fumicolans* (T_{max} 103 °C; pH_{opt} 8.5; pH_{max} 9.5) and *Tc. fumicolans* (T_{max} 95 °C; $pH_{opt}^{25\,°C}$ 9.0; $pH_{max}^{25\,°C}$ 10.3). The highest pH optimum and maximum is exhibited by the gram-type positive *C. paradoxum* with a $pH_{opt}^{25\,°C}$ 10.1 (pH_{opt}^{55C} 9.3) and a $pH_{max}^{25\,°C}$ of 11.0 (pH_{max}^{55C} 10.3). The various species differ from being isolated only from one location (restricted biogeography) to being truly ubiquitous. The habitats include anthropogenically and geothermally heated environments with alkaline but also acidic pH values, and mesobiotic (bulk property) neutrophilic environments such as river and lake sediments, and animal feces. It is expected that the diversity will increase when more alkalithermophiles will be isolated through intensified studies on the ecology and diversity of these microorganisms and investigation of additional alkaline habitats.

The anaerobic alkalithermophiles described till date obviously do not represent the most alkaline nor the most thermophilic microorganisms, however, they are the most thermophilic alkaliphiles (the most alkaliphilic thermophiles). But they contain some of the fastest growing bacteria known to date such as *Tb. celere* with a doubling time of 10 minutes. The alkalithermophiles

represent an interesting but relatively little investigated group of microorganisms, which should receive much more attention in the near future.

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THERMOPHILES, DIVERSITY OF

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Thermophiles are heat-loving organisms that thrive in naturally and artificially heated environments. For the purposes of this review, we define thermophiles as organisms capable of growing best above 45 °C, and hyperthermophiles are thermophiles that grow best above 80 °C. Thermophiles are found in all three domains of life, the *Bacteria*, *Archaea*, and *Eukarya* (1; Fig. 1). Among the

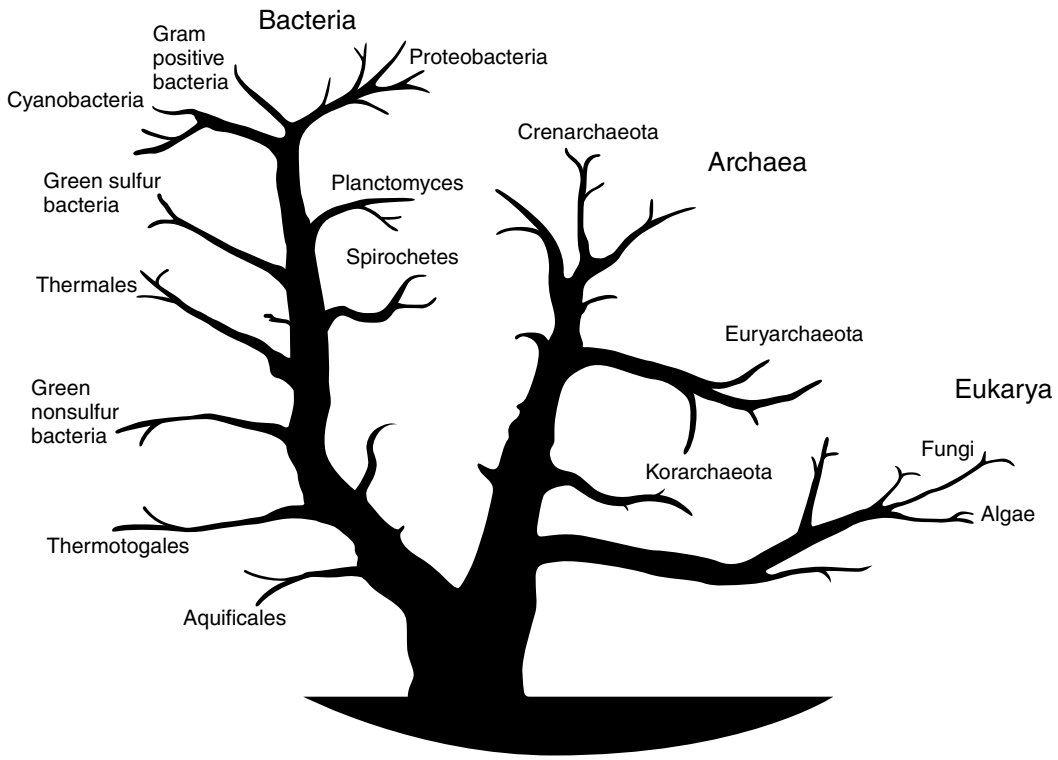

Figure 1. Stylized phylogenetic tree of all life showing the major thermophilic lineage in the three domains of life. See alrlab pdf. edu for photographs of many thermophiles.

Eukarya only a few algae and fungi are thermophilic. In contrast, thermophiles are widely distributed amongst the *Bacteria* and occupy many primary lineages in the *Archaea*. The diversity of thermophiles is reflected by their ability to grow in chemically diverse, high-temperature environments. In geothermally heated pools at Yellowstone National Park in Wyoming, U.S.A., colorful microbial mats are produced by photosynthetic thermophiles, such as *Cyanidium caldarium*, a moderately thermophilic alga, with an upper temperature limit for growth of 57 °C, and *Synechococcus*, a cyanobacterium with an upper temperature limit for growth of about 75 °C (2). In man-made, self-heating coal refuse piles, one finds *Thermoplasma acidophilum*, a cell wall-less *Archaea* that grows at temperatures from 40 to 62°C (3). Far removed from Yellowstone and coal mines, and a few thousand meters beneath the ocean's surface, hyperthermophiles, such as the archaeum *Pyrolobus*, grow at deep-sea vents at temperatures as high as 113 °C (4). These and other thermophiles share in common high temperatures for growth. In the past 20 years, the discovery of new species of thermophiles has grown exponentially and has challenged our preconceptions of the limits and origins of life.

ECOLOGICAL NICHES OF THERMOPHILES

Thermophiles are found in a wide variety of ecological niches. These include artificially heated environments, such as coal refuse piles and water from geothermal power plants (5,6). Solar-heated soils, fermenting compost piles and anaerobic sewage sludges are also good sources of thermophiles. By far, most of the thermophiles isolated are from geothermal environments, such as terrestrial hot springs, shallow marine and deep-sea vents, and deep subsurface ecosystems (7).

The ecological niches available for thermophiles in geothermally heated environments are diverse and reflect the unique physical forces at work in these environments. For instance, deep subsurface aqueous environments are heated by the Earth's core, with an average temperature increase of about 25°C per kilometer within the crust (8,9). Microbial growth in deep subsurface ecosystems is dependent upon either compacted organic material in sedimentary rock or the slow and modest energy-yielding chemical reactions that are continuously at work between the inorganic elements of rocks and minerals in the Earth's crust (9).

Deep-sea hydrothermal vents are, in part, surface expressions of hot deep subsurface environments (see HYDROTHERMAL VENTS: BIODIVERSITY IN DEEP-SEA HYDROTHERMAL VENTS and HYDROTHERMAL VENTS: PROKARYOTES IN DEEP-SEA HYDROTHERMAL VENTS). The geological forces creating deep-sea hydrothermal vents are similar for shallow marine and terrestrial vents. As a result of plate tectonics, nascent oceanic crust forms at mid-oceanic spreading centers and moves away in opposite directions until it subducts below the less dense continental crust. Generally, volcanically active regions occur along these mid-oceanic spreading centers or plate boundaries. The exceptions are hot spots, which are volcanically active regions, such as Yellowstone, Iceland and Hawaii that are far-removed from plate boundaries. These are caused by plumes of hot lava rising through the Earth's mantle. The near-surface lava at mid-oceanic spreading centers and hot spots superheats water in the Earth's crust, ultimately convectively forcing it to the surface. The reduced, hot emerging hydrothermal fluid is the culmination of a hydrological cycle that began with seawater or groundwater percolating through rocks in the Earth's crust. Before it emerges at the vent, the superheated fluid reacts with the surrounding rock, losing elements and ions, such as Mg^{2+} and SO_4, and adding minerals and gases, such as Fe^{2+}, Mn^{2+}, carbon dioxide, methane, and hydrogen sulfide. In deep-sea hydrothermal vents, when the seawater emerges, it mixes with cold oxygenated seawater, causing many of the iron-sulfur minerals to precipitate, forming a "smoky "plume characteristic of "black smoker "vents. At these depths (>2, 000 m), water can remain liquid at temperatures up to 400 °C because of the increased pressure. Just as was the case for microbial growth in the deep subsurface, energy from sunlight is not available at deep-sea vents. Microbes are therefore dependent upon the abundant supply of inorganic chemicals associated with the hydrothermal fluid. Organic carbon is also available as a result of the high productivity in these "oases "of the deep-sea. Shallow marine vents can be distinguished from deep-sea vents, as light energy from sunlight in addition to chemical energy from hydrothermal fluids is available for microbial growth in these shallower environments.

Terrestrial hot springs also occur as a result of plate tectonics. These are distinguishable from deep-sea hydrothermal vents and shallow marine vents because groundwater, and not seawater, percolates through the crust. Terrestrial thermal springs may have pH values ranging from 1 to 10, depending on the subsurface hydrological and geochemical reactions. For example, thermal fluid rich in hydrogen sulfide gas will react with water to produce sulfuric acid. These thermal springs are very acidic and, if located in limestone formations, the fluid dissolves the surrounding rock and produces the characteristic low pH mudpots. Microorganisms thrive in these acidic thermal mudpots, which may have pH values less than 4.

PHYSIOLOGICAL DIVERSITY

The metabolic and physiological diversity of thermophiles is a direct expression of their ability to inhabit the wealth of ecological niches thus far discussed. Metabolically, there are three general strategies by which microbes obtain their carbon and energy sources for growth — photoautotrophy, chemolithoautotrophy, and heterotrophy (or chemoorganotrophy) — and thermophiles employ them all. Photoautotrophs use energy from sunlight and fix carbon dioxide, chemolithoautotrophs use energy from inorganic molecules and fix carbon dioxide, and heterotrophs use organic molecules as both energy and carbon sources. In addition, some microbes are photoheterotrophs, obtaining energy from sunlight and using organic carbon molecules as a carbon source. Heterotrophs and photoautotrophs are

found in all three domains of life. Chemolithoautotrophy is restricted to the bacterial and archaeal domains.

In addition to the variety of metabolic strategies for gaining energy and organic carbon, thermophiles are physiologically adapted to environments that are extreme in more than temperature. Thermoacidophiles inhabit hot and extremely acidic environments, such as sulfur- and iron-rich terrestrial acidic hot springs and coal refuse piles. Thermoacidophiles not only tolerate extremely low pH, they require it to grow. At the other end of the pH scale, thermophilic alkalophiles that grow up to pH 10 have been isolated from some thermal environments. Many of the thermophiles are resistant to heavy metals. Some of the thermophiles from deep-sea hydrothermal vents are barophilic (piezophilic) or barotolerant, able to grow at the high pressures from which they were isolated. Additionally, they may be halophilic, specifically adapted to high saline conditions.

In summary, thermophiles are found in virtually any environment where high temperatures occur. Thermophiles are diverse, capable of growth as chemolithoautotrophs, photoautotrophs, or heterotrophs and they can occupy extremes in pH, salinity and pressure. This metabolic and physiological diversity is a reflection of the diverse environmental settings and ecological niches in which thermophiles are found.

PHOTOTROPHS

One dramatic difference between deep-sea and deep subsurface high-temperature environments and shallow marine and terrestrial hydrothermal environments is the prevalence of photosynthetic communities in the latter. Phototrophic thermophiles include members of both the eukaryal and bacterial domains. Two general mechanisms of photosynthesis are used by thermophiles, namely oxygenic and anoxygenic photosynthesis. Oxygenic photosynthesis occurs when water is the electron donor and in which the by-product of photosynthesis is oxygen. This is characteristic of thermophilic algae (*Eukarya*) and cyanobacteria (*Bacteria*). Thermophiles that use *an*oxygenic photosynthesis, in which molecules, such as hydrogen and hydrogen sulfide serve as the electron donors, and oxygen is not evolved as a by-product of photosynthesis, is restricted to several lineages within the Bacteria. The mechanisms by which these anoxygenic photoautotrophs and photoheterotrophs photosynthesize differ considerably from each other and will be discussed in the following sections.

Oxygenic Phototrophs

Within the *Eukarya*, thermophilic representatives are thus far restricted to the algae and fungi. The photosynthetic organelle in algae is the chloroplast, and based on small subunit rRNA (16S rRNA) sequence comparisons, chloroplasts are closely related to the cyanobacteria (10). Both algae and cyanobacteria use water as the source of electrons, and light energy splitting water and generating oxygen: $2H_2O + h\nu \rightarrow O_2 + 2H_2$. Oxygenic photosynthesis is characterized by two distinct photosystems — photosystem I and photosystem II — each of which absorbs sunlight at different wavelengths. Chlorophyll *a* and phycocyanin, the sunlight-absorbing pigments within cyanobacteria and eukaryotic algae, give these phototrophs their characteristic blue-green color.

Algae are very prevalent in terrestrial thermal springs at temperatures below 45 °C. They produce the very characteristic bright yellow, orange, and reddish-brown mats (such as produced by *Zygogonium*) on the edges of many thermal pools. At the higher temperatures, cyanobacteria prevail, except in acidic thermal springs, like Nymph Creek, in Yellowstone National Park. At pH less than 4.5 and temperatures between 35 and 57 °C, the single-celled alga, *Cyanidium caldarium* forms spectacular blue-green communities with the thermophilic fungus, *Dactylarium gallopava*. These communities have been likened to the symbiotic relationships that exist with lichens. *Cyanidium* has been found in many hot springs around the world, including hot springs in Japan, Costa Rica, New Zealand, and the Azores.

Thermophilic cyanobacteria are generally the dominant phototroph in near-neutral thermal springs, present up to 73 °C. Some examples of thermophilic cyanobacteria include the filamentous gliding *Oscillatoria*, the globally distributed *Mastigocladus* and the kidney-shaped rods of *Synechococcus*. *Synechococcus* cells are abundantly distributed in the top 1-mm thick photic zone of the extensive microbial mats in Yellowstone Park (11). Laboratory isolates of *Synechococcus lividus* exhibit motility in response to light intensity, but no diel shifts in vertical distribution because of light intensity have been detected in situ (11). *Synechococcus lividus* dominates Yellowstone microbial mats throughout the light-deficient winter months, possibly because the ecological role of *S. lividus* is like that of trees in a climax forest community, dominating biomass even when conditions are not optimal (11). Furthermore, cyanobacterial mats, such as those dominated by the filamentous cyanobacterium, *Phormidium*, have been used as models for studying stromatolite (laminated mineralized structures that can be biogenic in origin) formation.

Anoxygenic Phototrophs

The oxygenic phototroph *Synechococcus* occurs in close association with anoxygenic phototrophic *Bacteria*, such as the green nonsulfur bacterium, *Chloroflexus*. During anoxygenic photosynthesis, chemicals that are abundant around terrestrial and shallow marine vents, such as dissolved hydrogen sulfide $H_2S_{(}aq)$, molecular hydrogen (H_2) or organic compounds are used as electron donors and light serves as the energy source. Anoxygenic phototrophs are found in three different lineages in the bacterial tree. They differ from each other in their pigments and modes of photosynthesis. These lineages are the green nonsulfur bacteria (e.g., *Chloroflexus*), the green sulfur bacteria (e.g., *Chlorobium*) and the purple sulfur or Proteobacteria (e.g., *Chromatium*).

Although not all members of the green nonsulfur bacteria are phototrophs or thermophiles, *Chloroflexus aurantiacus* is commonly found in the lower layers of

mats containing the thermophilic oxygenic phototroph, *Synechococcus*. *Chloroflexus aurantiacus* is a filamentous, gliding bacterium that grows optimally at 55 °C, with an upper temperature limit of 65 to −70 °C (12). The long, slender filaments of *C. aurantiacus* are thought to provide stability to microbial mats. *Chloroflexus aurantiacus* is an unusual phototroph in that it exhibits considerable metabolic diversity. Under aerobic conditions and without light, *C. aurantiacus* grows chemoorganotrophically. In the presence of light, *C. aurantiacus* grows best photoheterotrophically on a wide variety of sugars, amino acids and organic acids, but can also grow photoautotrophically with hydrogen sulfide or molecular hydrogen as electron donors.

Based on 16S rRNA phylogenetic analyses, *C. aurantiacus* and its close allies form the deepest branching group of photoautotrophs (1). This phylogenetic placement of *C. aurantiacus* is of particular interest because *C. aurantiacus* converts carbon dioxide to organic carbon by the unique hydroxyproprionate pathway. Unlike the Calvin cycle and the reverse citric acid cycle, the hydroxyproprionate pathway involves the conversion of two molecules of carbon dioxide to glyoxylate. Phylogenetic analysis of conserved photosynthetic light harvesting pigments placed the green sulfur bacteria in close relationship with the green nonsulfur bacteria (13), However, based on 16S rRNA phylogenetic analysis these two groups form distinct unrelated lineages. Both of these groups have bacteriochlorophyll *c*, yet they possess different photosynthetic pathways. A common member of the green sulfur thermophiles, *Chlorobium tepedium*, can be found in high-sulfide hot springs where temperatures have cooled below 50 °C. These anoxygenic phototrophs produce S^0 as a by-product of photosynthesis, which is deposited outside the cell.

Chlorobium is often found in close association with another anoxygenic phototroph, the purple sulfur proteobacterium, *Chromatium tepedium*. These organisms form photosynthetic mats in high sulfide, near neutral pH thermal springs (temperature ~50 °C). *Chlorobium* can generally withstand higher H_2S concentrations than *Chromatium*, and the different organisms stratify within a microbial mat accordingly. Additionally, *Chromatium* is generally absent from thermal springs dominated by *Chloroflexus* because the latter is more thermotolerant. Like *Chlorobium*, *Chromatium* produces elemental sulfur during phototrophic growth on hydrogen sulfide, however, *Chromatium* deposits the sulfur within the cell.

CHEMOLITHOAUTOTROPHS

The oxygenic and anoxygenic phototrophs dominate many of the terrestrial thermal springs. However, at temperatures at which photosynthesis has not been reported and in thermal springs where the chemistry inhibits photosynthesis, chemolithoautotrophic and chemoorganotrophic thermophiles proliferate.

The abundance of inorganic electron donors and acceptors in hydrothermal vents, provides a wide range of inorganic energy sources for chemolithoautotrophic thermophiles. Many chemolithoautotrophs are facultative, using organic compounds as energy sources if they are available.

Chemolithoautotrophic *Bacteria*

By far most of the thermophiles that have been isolated from the bacterial domain, are heterotrophs. However, two deeply diverging lineages with the *Bacteria*, the *Aquificales* (14) and "*Desulfurobacteriales* " (15) are primarily chemolithoautotrophic thermophiles. The *Aquificales* obtain energy from hydrogen oxidation using the "knallgas" reaction ($H_2 + O_2 \rightarrow 2H_2O$). These organisms are all microaerophiles, as they tolerate and grow on low levels of oxygen. Many of the *Aquificales* can also use alternate electron donors (other than H_2), such as elemental sulfur and thiosulfate ($S_2O_3^{2-}$), and they can use alternate electron acceptors (instead of O_2), such as nitrate (NO_3^-).

Members of the *Aquificales* are widespread in thermal environments. They have been isolated from shallow marine vents (*Aquifex pyrophilus*), deep-sea vents ("*Perseponella*"), terrestrial thermal springs (*Calderobacterium*, *Hydrogenobacter*), and heated compost (*Hydrogenobacter*). Many of these thermophiles form visible filamentous biomass in terrestrial thermal springs, and in some cases have evaded cultivation and isolation. One such organism forms the pink filaments ("streamers") from Octopus Spring, Yellowstone National Park. After years of effort this organism was only recently isolated and named *Thermocrinis ruber*.

The story of the isolation of *T. ruber* is an example of how environmental 16S rRNA gene analysis can place as yet uncultured microbes in a phylogenetic tree, thereby providing a guide for enrichment culturing approaches. The dominant 16S rRNA gene sequence obtained from DNA extracted from the pink filament community in Octopus Spring was closely related to other members of the *Aquificales*, (16). From this information, Huber and coworkers (17) speculated that, like their close relatives, the pink filaments were hydrogen oxidizers. Indeed, they were successful in isolating *T. ruber* from Octopus Spring under growth conditions favorable to H_2 oxidizers.

Closely related to the *Aquificales*, yet a distinct lineage, is the recently isolated sulfur-reducing chemolithoautotroph, *Desulfurobacterium thermolithotrophum*. This is the first S^0-reducing chemolithoautotroph to be isolated within the domain *Bacteria* (15). For *D. thermolithotrophum*, molecular hydrogen always serves as the electron donor. The sulfur compounds used as electron acceptors include elemental sulfur, thiosulfate, and sulfite (SO_3^{2-}).

The gram-positive *Bacteria* have many representatives that are thermophilic (see following text), but few that are also chemolithoautotrophs. *Ammonifex degensii* and *Thermoterrabacterium ferrireducens* are both facultative chemolithoautotrophs, and capable of growing heterotrophically. The former is a nitrate reducer, whereas the latter reduces iron.

Within the *Proteobacteria*, *Thermothrix azorensis* is a beta proteobacterium with a physiology very reminiscent of the *Aquificales*. This thermophile is capable of growing by oxidizing compounds, such as sulfur and thiosulfate.

It is likely that as we explore diversity of more thermal environments, more chemolithotrophic *Bacteria* will be isolated. For example, the bacterial diversity reported by Hugenholtz and coworkers (18) from a single hot spring in Yellowstone National Park, Obsidian Pool, revealed 54 distinct phylotypes (16S rRNA gene sequence types), perhaps some of these lineages are also chemolithoautrophs?

Chemolithoautotrophic *Archaea*

There are two major kingdoms within the *Archaea*: the Euryarchaeota and the Crenarchaeota. A proposed third kingdom, the Korarchaeota, was proposed based on 16S rRNA sequences obtained from Obsidian Pool in Yellowstone National Park (19). Until recently, it was thought that all members of the Crenarchaeota were thermoacidophiles, requiring high temperature and low pH for growth. But low-temperature members of the Crenarchaeota have been identified in temperate regions, revealing that the Crenarchaeota are more physiologically diverse than previously thought. The Euryarchaeota include the methane-producing methanogens, salt-loving halophiles and many of the thermophilic *Archaea*. For a review on the metabolism of many of these *Archaea*, see Schonheit and Schafer, 1995 (20).

Perhaps some of the best studied chemolithoautotrophic members of the Crenarchaeaota are *Pyrodictium*, *Pyrolobus* and *Acidianus*. *Pyrodictium occultum* is a hyperthermophilic sulfur reducer that was isolated from shallow marine vents and grows optimally at 105 °C. In addition to a high optimal growth temperature, *P. occultum* exhibits an unusual morphology. The cells of *P. occultum* are disk-shaped, and they grow moldlike on sulfur crystals in a huge network of hollow, proteinaceous fibers. The sulfur reducer *Pyrolobus fumari* is best known for it high temperature growth characteristics. This hyperthermophile grows best at 106 °C (up to 113 °C) and survives autoclaving at 121 °C for one hour (4). *Pyrodictium* and *Pyrolobus* grow optimally in moderately acidic conditions (pH 5.5), whereas their relative *Acidianus brierleyi* thrives at pH of 2.0. (21). *Acidianus brierleyi* is a facultative anaerobe that grows aerobically by sulfur oxidation, producing sulfuric acid (H_2SO_4), or anaerobically by sulfur reduction coupled with hydrogen oxidation producing hydrogen sulfide (H_2S). Unlike *Acidianus*, its close relative, *Sulfolobus* is an obligate aerobe that grows by sulfur oxidation in the presence of oxygen, producing sulfuric acid. Both *Acidianus* and *Sulfolobus* can oxidize iron (Fe^{2+} to Fe^{3+}), making them good candidates for high-temperature leaching of copper and iron ores. Another member of this group, *Metallosphaera*, can oxidize sulfide ores, such as pyrite, chalcopyrite, and sphalerite, producing sulfuric acid and mobilizing heavy metals in the process (22,23).

In the Euryarchaeota, the sulfate reducer, *Archaeoglobus fulgidus* grows chemolithoautotrophically with sulfate as an electron acceptor (5), whereas *A. profundus* can only grow chemoorganotrophically with sulfate as an electron acceptor (24). *Archaeoglobus* is unusual because it also produces small amounts of methane and possesses coenzyme F_{420}. Coenzyme F_{420} is typically unique to the methanogenic Archaea. It absorbs light at 420 nm and fluoresces light blue, providing an easy method for preliminarily identifying methanogens. The presence of F_{420} in the methanogens and in *Archaeoglobus* suggests that sulfate reduction may represent a transitional metabolism in *Archaea* between sulfur respiration and methanogenesis.

Examples of thermophilic chemolithoautotrophic methanogens are *Methanococcus*, *Methanothermus*, and *Methanopyrus*. These methanogens reduce carbon dioxide and oxidize molecular hydrogen ($CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$) to produce methane. *Methanococcus* and *Methanopyrus* have both been isolated from deep-sea vents (25,26), whereas *Methanothermus* has only been isolated from terrestrial thermal springs in Iceland (27). *Methanopyrus kandleri*, grows optimally at 98 °C and up to 110 °C (25).

In summary, the thermophilic chemolithoautotrophs described in this section illustrate only a sample of the many metabolic pathways possible within this metabolic group, and more will no doubt be discovered. Additionally, many of the same microorganisms or their close relatives can also grow chemoorganotrophically.

HETEROTROPHS

Chemolithoautotorphs, like photoautotrophs, are the basis of the carbon food chain. These organisms produce organic carbon from inorganic carbon (primarily CO_2) that is then a source of carbon for heterotrophs. Thermophilic heterotrophs can grow either aerobically or anaerobically, by respiration or by fermentation of organic compounds. Many of these thermophiles use complex organic mixtures containing peptides and sugars.

Heterotrophic *Eukarya*

Within the *Eukarya*, about 30 species of approximately 50,000 species of fungi are thermophilic heterotrophs and almost all are strict aerobes (28). With few exceptions, thermophilic fungi cannot grow anaerobically, although the sexual stage of at least one species, *Talaromyces (Penicillium) duponti*, is initiated under anaerobic conditions (28). An example of a thermophilic fungus is *Dactylaria gallopava*, mentioned previously in conjunction with *Cyanidium caldarium*, the thermoacidophilic algae that forms extensive mats at Yellowstone National Park. *Dactylaria gallopava* relies on low molecular weight organic compounds excreted by *C. caldarium* as an energy and carbon source (2).

Compost piles are an excellent source for thermophilic eukaryotes. As composts are piles of organic plant matter consisting primarily of cellulose, an important step in the degradation of the organic matter is cellulose degradation. *Chaetomium thermophile* releases simple sugars as it degrades cellulose that the noncellulolytic thermophilic heterotrophs like *Thermomyces lanuginosus* can consume. Other noncellulolytic thermophilic heterotrophs grow on xylan, which is also present in the cell wall of plants (28).

Heterotrophic *Bacteria*

Compost piles are also a source of novel thermophilic Bacteria. At temperatures of 65 to 69 °C, obligately heterotrophic bacteria, such as *Bacillus stearothermophilus*, thrive (29). The genus *Bacillus* belongs to the gram-positive *Bacteria*, a group with many heterotrophic thermophilic representatives isolated from diverse warm environments. For example, *Bacillus thermoaerophilus* was isolated from sugar beet factories (30) and *B. sporothermodurans* was obtained from milk-processing plants (31). *Clostridium thermopalmarium* (32) and *B. thermoamylovorans* (33) were isolated from palm wine, a traditional alcoholic beverage from tropical countries where palm trees are common. The heterotrophic iron reducer, *B. infernus*, was obtained from samples collected 2.7 km below the Earth's surface. *Bacillus infernus* ferments glucose or oxidizes formate or lactate when using iron (Fe^{3+}) or manganese dioxide as an electron acceptor (34). *Thermoterrabacterium ferrireducens*, another iron-reducing thermophilic gram-positive bacterium was isolated from Calcite Springs at Yellowstone National Park (35). *Moorella glycerinia* was also isolated from the same environment and is capable of growth solely on glycerol.

Whereas *Bacillus* relatives grow well within composts from 65 to 69 °C, heterotrophs, such as *Thermus thermophilus*, can thrive in composts with internal temperatures above 70 °C (36). *Thermus aquaticus*, a close relative of *T. thermophilus*, is found in geothermally heated environments throughout the world and even in hot water heaters (37). It forms filaments up to 200 μm in length (Fig. 2a). *Thermus aquaticus* may be best known as the source of *Taq* polymerase, the DNA polymerase used in the polymerase chain reaction (PCR) that has revolutionized the field of molecular biology.

Another filamentous chemoorganoheterotroph isolated from terrestrial vents is *Isosphaera pallida*, which may appear salmon-colored because of the presence of carotenoids (38). *Isosphaera pallida* is a member of the poorly studied Planctomyces group, which reproduce budding and produce stalks. The importance of this group was recently exemplified when a nonthermophilic Planctomycete was implicated in the anaerobic oxidation of ammonia via the "anammox" reaction ($NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O$) (39). One might expect to find similar unusual organisms associated with high-temperature ecosystems.

Spirochetes have also been identified in thermal environments (Fig. 2b,c) and grow anaerobically by fermentation of complex organic compounds. Microscopic examination of samples from a New Zealand thermal spring revealed that thermophilic spirochetes grew best from 45 to 50 °C (40) although they were also prevalent at 50 to 58 °C. Thermophilic spirochetes have also been obtained from Hunter Hot Spring, Oregon, U.S.A. (41) and Furnas, Azores (Aguiar and Reysenbach, unpublished results, Fig. 2b,c).

The only hyperthermophilic and heterotrophic lineage within the *Bacteria*, is the *Thermotogales*. These organisms are characterized by having a characteristic sheath, or "toga," which surrounds the cell (Fig. 2d). The genus,

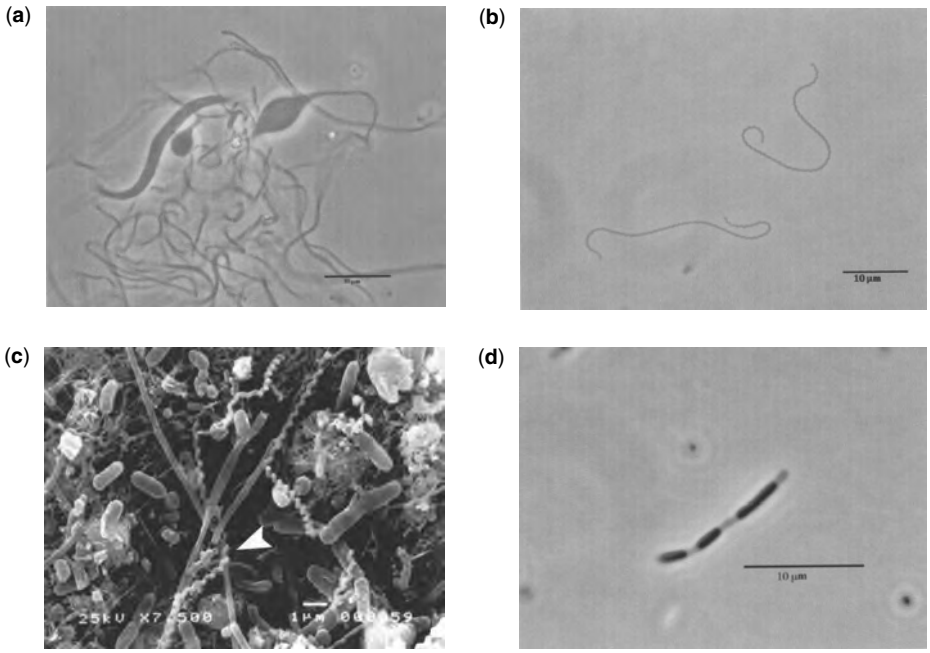

Figure 2. (**a**) A phase contrast micrograph of a filamentous *Thermus* sp. isolated from Yellowstone National Park (courtesy C. Takacs). (**b**) A phase contrast micrograph of a thermophilic spirochete isolated from Furnas, Azores, Portugal (courtesy P. Aguiar). (**c**) Scanning electron micrograph of a microbial mat community from a hot spring from Furnas, Azores, Portugal. Note the spirochete. Arrow points to a spirochete (courtesy P. Aguiar). (**d**) Phase contrast micrograph of a deep-sea hydrothermal vent Thermotogales isolate from Guaymas Basin, Mexico. Note the "toga" around the dark cells (courtesy M. Kendall). See color insert.

Thermotoga has been isolated from deep-sea vents, terrestrial vents, and oil production wellheads (see PETROLEUM RESERVOIRS, MICROBIAL DIVERSITY IN) (42–45). Depending on the species, *Thermotoga* grows best anaerobically from 65 to 80 °C. Although *Thermotoga* is a fermentative chemoorganoheterotroph, all species of *Thermotoga* grow faster when they utilize thiosulfate as a terminal electron acceptor (44). The reduction of thiosulfate produces hydrogen sulfide, (also a by-product of sulfate-reducing bacteria) a potential hazard and corrosive agent of oil pipelines (46).

Heterotrophic *Archaea*

Perhaps the best-studied hyperthermophiles are the euryarchaeal heterotrophic sulfur-reducing *Thermococcales*. Three genera are represented by this order: *Thermococcus*, *Pyrococcus*, and *Paleococcus*. More new species have been described that belong to the genus *Thermococcus* than almost any other hyperthermophilic genus, and these species are distinguished primarily by the organic compounds they are able to ferment. For example, *Thermococcus chitonophagus* uses chitin, the second most abundant natural polymer on Earth, as its sole carbon and energy source (47). Although there are thermophilic *Bacteria* that use chitin as an energy and carbon source (48), *Thermococcus chitonophagus* was the first archaeum to do so, and the first such thermophile isolated from deep-sea vents (47). Although this group is primarily marine, a few terrestrial isolates have been obtained from terrestrial hot springs in New Zealand.

Closely related to the *Thermococcales* are the methanogens. As mentioned in the preceding text, methanogens may grow chemolithoautotrophically or chemoorganoheterotrophically. The latter occurs through the reduction and oxidation of methyl-containing compounds (e.g., $4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O$) or through the cleavage of acetate to methane and carbon dioxide ($CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$). *Methanosarcina thermophila*, isolated from a 55 °C anaerobic digester, is an example of a chemoorganoheterotrophic methanogen that is capable of either reaction.

An unusual heterotroph within the Euryarchaeota is *Thermoplasma*, a cell wall-less thermoacidophile that grows best at pH 2.0. *Thermoplasma* is an aerobic chemoorganoheterotroph that can also grow by sulfur respiration, producing large amounts of hydrogen sulfide. These organisms have been found in terrestrial springs (*T. volcanium*, 49) and hot coal refuse piles (*T. acidophilum*, 3). An extreme thermoacidophile related to *Thermoplasma* is *Picrophilus oshimae*, which grows best at pH 0.7. *Picrophilus* is an obligate aerobe and chemoorganoheterotroph. Unlike *Thermoplasma*, *Picrophilus* cannot grow by sulfur respiration (50).

Among the Crenarchaeota, many of the members of the thermoacidophilic *Sulfolobales* grow only chemoorganoheterotrophically (e.g., *Sulfurisphaera ohwakuensis*, 49). Another crenarchaeal heterotroph, *Thermofilum*, grows in culture only in association with *Thermoproteus* or when supplemented with a high polar lipid fraction obtained from *Thermoproteus* (52). Both *Thermofilum* and *Thermoproteus* are moderately acidophilic, with an optimal pH of 5.0.

Like *Thermofilum*, *Pyrobaculum organotrophum* is capable of respiration using organic compounds as the electron donors, where sulfur compounds, such as elemental sulfur are the electron acceptors. A close relative, *P. aerophilum* uses nitrate or oxygen instead of sulfur compounds as the electron acceptors. *Pyrodictium abyssi*, however, ferments organic compounds, such as starch and glycogen, producing end products, such as carbon dioxide, butanol, isovalerate, acetate, and isobutyrate.

An example of a halophilic thermophile within the Euryarchaeota is *Holoarcula quadrata*, which grows best at 50 to 53 °C and requires a minimum of 3 M NaCl to grow (53). *Holoarcula* is a chemoorganoheterotroph, and it exhibits an unusual morphology of predominantly flat and square cells.

CONCLUSION

Thermophiles are found among all domains of life, and are metabolically and physiologically diverse and include photoautotrophs, chemolithoautotrophs, and chemoorganoheterotrophs. Both anoxygenic and oxygenic photosynthesis occur in terrestrial and shallow marine environments. Chemoorganoheterotrophs use carbon sources that include complex biomolecules, such as cellulose and chitin, simple sugars, such as glucose, and simple organic acids, such as acetate. Many of these heterotrophs are facultative chemolithoautotrophs, capable of using molecules, such as hydrogen, thiosulfate, pyrite and sulfur as electron donors and sulfur, thiosulfate, sulfate, sulfite, nitrate, ferric iron, and oxygen as electron acceptors.

It is generally believed that early Earth offered a hot and anoxic environment to its inhabitants, and thermophilic chemolithoautotrophs are likely to have been among the first organisms to inhabit Earth. Many of the deepest lineages in the phylogenetic tree of life are thermophiles, which suggests that perhaps they are relatives of the ancestors of early life. With such a vast array of genetic, metabolic and physiological diversity, and their biogeochemical and evolutionary significance, thermophiles are testimony to the resiliency of life and the contribution of microbes to nutrient cycling on Earth.

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THERMOPHILIC ALKALIPHILES.

See THERMOPHILES: ANAEROBIC ALKALITHERMOPHILES

TNT BIODEGRADATION.

See MICROBIAL DEGRADATION OF EXPLOSIVES

TOXICITY OF METALS TO MICROORGANISMS.

See METALS: MICROBIAL PROCESSES AFFECTING METALS

TOXICITY OF ORGANIC SOLVENTS TO MICROORGANISMS

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Some hydrocarbons have been present in the environment for a long time as a result of natural biosynthesis (1,2). Additionally, the production and use of various hydrocarbons as organic solvents is widespread in industry. Because of this widespread industrial use, the amount of solvents present in the environment has increased since the early industrial revolution. Their toxic and even carcinogenic effects make them a potential threat to human health. However, some groups of microorganisms are able to transform organic solvents, often leading to complete mineralization of these compounds (3–5). Although many microorganisms degrade organic solvents at low concentrations, various solvents can become toxic at slightly higher concentrations (6,7). Hydrophobic organic solvents are toxic to living organisms because they accumulate in cell membranes and cause disruption. The toxicity of a compound correlates with the logarithm of its octanol/water partition coefficient ($\log P_{OW}$). Substances with a log P value between 1 and 5 are, in general, toxic for whole cells. Such hydrocarbons can only be degraded at low concentrations and, consequently, stay often as persistent pollutants in the environment. Therefore, toxic

effects of hydrocarbons and other solvents on microorganisms can cause problems in bioremediation, waste gas, and wastewater treatment (8).

In addition, the toxicity of organic solvents plays not only a decisive role in environmental microbiology but also in the biotechnological production of fine chemicals (9). The use of organic solvents also has several advantages in the application of whole cell systems (10–12). Solvents can increase the available amount of poorly water-soluble substrates for the cells. When organic solvents are used as a second phase, the products of a biotransformation can be extracted continuously from the aqueous reaction system. This enables not only the reduction of inhibitory effects caused by the product but also a much easier recovery with positive effects on the costs for the downstream-processing. Solvents or structurally similar compounds are also useful as substrates for some applications. However, problems resulting from the toxicity of hydrophobic organic solvents to whole cells are still an important drawback for the application of these compounds in biocatalysis (11,13) and in environmental biotechnology in general.

In recent years, different bacterial strains that adapted to organic solvents have been isolated and characterized. These strains grow in the presence of a second phase of solvents previously believed to be lethal. For the applications mentioned in the preceding text, an understanding of the effect of hydrophobic solvents on microorganisms and the adaptation of solvent-tolerant microorganisms is of great economic importance. This entry focuses on effect of hydrophobic solvents on microorganisms and the adaptation mechanisms found in microorganisms growing in the presence of such compounds.

EFFECTS OF SOLVENTS ON MICROORGANISMS

Most microorganisms tolerate water-miscible solvents such as lower alcohol's and acids. In addition, it has been established that very lipophilic natural solvents including some hydrocarbons are not toxic for whole cells. However, many of the organic solvents used in petrochemical operations occupy a position between the water-soluble alcohol's and acids and the lipophilic compounds. Solvents of intermediate hydrophobicity, such as aromatic solvents, are very toxic to cells. These have been widely used as antimicrobial agents (14,15) and food preservatives, disinfectants, tools for the permeabilization of cells, and as narcotic agents (16–19).

The antimicrobial action of a solvent correlates with its hydrophobicity, as expressed by the logarithm of the partition coefficient of the compound in a mixture of *n*-octanol and water (log P_{ow}). Organic solvents with log P_{ow} values between 1 and 5, such as toluene, are highly toxic for microorganisms. This influence of hydrophobicity on toxicity can be found in different solvent classes, for example, aromats, alcohols, phenols, alkanes (20–24). Table 1 lists classes of solvents and their respective hydrophobicities and toxicity's, expressed in terms of their log P_{ow} values and their midpoint cytotoxicity values (LD_{50} values). It should be emphasized that the toxic effects of solvents, and their dose-response relationships,

Table 1. Hydrophobicity and Toxicity of Several Solvent Classes

Solvent Class	Compound	Log P[a]	LD_{50}[b] (mmol l^{-1})
short-chain alcohols	methanol	−0.76	501.1
	ethanol	−0.28	223.9
	1-butanol	0.9	64.5
weak acids	acetic acid	−0.23	60.0
aromatic alcohols	phenol	1.5	7.94
	4-chlorophenol	2.4	1.29
	2,4-dichlorophenol	3.2	0.144
	2,4,5-trichlorophenol	4.1	0.016
	pentachlorophenol	5.1	0.0067
Aromatic solvents	benzene	2.1	17.38
	toluene	2.6	3.58
	styrene	2.9	3.15
	ethylbenzene	3.2	0.9
	tetraline	3.9	0.75
Alkanes	hexadecane	8.8	>1,000

Source: (Modification of table presented by H. J. Heipieper et al.., *Trends Biotechnol.* **12**, 409–415 (1994)).

[a] Data from A. Inoue and K. Horikoshi, *J. Ferment. Bioeng.* **71**, 194–196 (1991) and C. Laane et al., in C. Laane et al.., eds, *Biocatalysis in Organic Media*, Elsevier Science Publishers, Amsterdam, The Netherlands, 1987 pp. 65–84.

[b] LD_{50} values measured with *Pseudomonas* cells.

are similar for a variety of microorganisms. Thus, the toxicity of hydrophobic solvents is caused by general, nonspecific effects, and no metabolic or chemical reactions are associated with the toxic effects.

It has been established that there is a systematic relationship between values of log P in the range between 1 and 5 and the partitioning of solvents in membrane buffer systems (19,23,24). The absolute values of partition coefficients are approximately eight times lower in membranes than in octanol. These can be estimated by the following equation from Sikkema and coworkers (24):

$$\log P_{M/B} = 0.97 \cdot \log P_{O/W} - 0.64$$

where M = membrane, B = Buffer, P_{ow} = octenol/water coefficient $P_{M/B}$ = membrane/buffer partition coefficient.

Hence, the log P value is a suitable parameter that describes the accumulation of these solvents in membranes. However, two aspects have to be taken into consideration. First, in addition to hydrophobicity, the molecular structure of a compound also influences its solubility in membranes. Amphipatic molecules will dissolve relatively well in membranes because their structure is similar to that of the phospholipids in the membranes. Consequently, such compounds are relatively more toxic than others with the same log P value. This explains the relatively high toxicity of aromatic structures with hydrophilic substituents, particularly phenols. Second, the composition of the membrane will also influence the partitioning of a compound into the membranes. So far, this has only been demonstrated with artificial membranes. The partition coefficient of lindane is, for instance, 50 times higher in liposomes

of dimyristoylphosphate (C14 : 0) than in liposomes of distearoylphosphatidylcholine (C18 : 0) (27). In artificial membranes, the position of a solvent partitioning into the membranes depend on the molecular structure (28). Therefore, changes in the membrane structure can affect the preference of solvents entering the membrane.

The accumulation of the solvent toluene into bacterial membranes can be made visible by electron microscopy (16,29). This demonstrates that the membrane in which the solvents accumulate is the main target of the toxic effect. This of course does not rule out additional sites of toxic action because they may be caused by the specific properties of a molecule.

Although no individual analytical technique is able to determine fully the effects of solvents on a membrane, several mechanisms of membrane toxicity have been reported.

First, the accumulation of organic solvents leads to a permeabilization of the cell membranes. In *Escherichia coli*, it was observed that potassium ions and ATP are released after treatment with phenol (30). For toluene, the leakage from the cell of macromolecules such as phospholipids, proteins, or even RNA, has been demonstrated (31,32). This permeabilization was due to a considerable damage to the cytoplasmic membrane, whereas the outer membrane was still intact (16). For artificial membranes the presence of the solvent tetraline increased proton permeability (19,24). Other studies with bacterial and artificial membranes revealed a passive flux of protons and other ions across the membrane because of the presence of solvents (19,24,33–37). This flux of ions dissipates the proton motive force (Δp), and affects both the proton gradient (ΔpH) and the electrical potential ($\Delta\psi$) (24,36).

The second mechanism of the membrane toxicity of organic solvents is to diminish the energy status of the cell. The effect of solvents on the energy transduction of membranes was tested in liposomes reconstituted with cytochrome *c* oxidase. The presence of the solvent tetraline caused decreases in the proton gradient ΔpH and the electrical potential $\Delta\psi$ of 80% and 50%, respectively (24). It has been established that the dose-response curves for a range of solvents were very similar if the dissolved membrane concentration of the solvent is considered to be the dose. Intact cells show a similar effect of solvents on ΔpH and $\Delta\psi$ (19).

The decrease of the proton motive force is not the only reason for lower energy levels of cells in the presence of organic solvents. The presence of solvents also leads to impaired ATP synthesis because of a partial inhibition of ATPase and of other proteins engaged in the energy transducing process (38,39).

Third, besides the proteins engaged in energy transduction, the accumulation of solvents into a membrane also affects the function of other proteins embedded in the membrane. In *E. coli*, toluene leads to a total inactivation of the galactose permease system (31) and in *Saccharomyces cerevisiae*, the proton-potassium translocation is blocked (35).

Fourth, the fluidity of the membrane, an important aspect of membrane structure, is affected by organic solvents (24). The effect of organic solvents on the fluidity was measured through the use of fluorescent probes. The probe 1,6-diphenyl-1,3,5 hexatriene (DPH), partitions into the hydrophobic inner parts of cell membranes, whereas a trimethylamine derivative of this compound (TMA-DPH) anchors its hydrophilic group in the headgroup region of the bilayers. Solvents partitioning into the membrane will affect the polarization of the probe located at the site entered by the solvent. Several solvents with a logPo/w between 1 and 5 affected the polarization of both probes (24). Thus, the whole membrane was fluidized. An increased fluidity of membranes results in changes in stability, structure, and interactions within the membrane (40,41).

Fifth, membrane-active compounds can affect the hydration characteristics of the membrane surface (42) and the thickness of the membrane (43). Swelling of the membrane can be investigated through the use of liposomes labeled with the fluorescent fatty acid, octadecyl rhodamine β-chloride. An expansion of the membrane will lead to a dilution of the fluorescent probe in the membrane, which can be recorded as a reduction in fluorescence self-quenching. Swelling of the membrane is monitored by plotting relative fluorescence against the amount of solvent added to the liposomes. This was tested for several solvents with logP values between 2 and 5 (24). For all solvents tested, the surface area of the membrane increase linearly up to a concentration of 0.5 μmol mg^{-1} of solvent in the membrane. At that concentration, the maximum increase in the membrane swelling was reached. This maximum level corresponds to approximately one solvent molecule per two phospholipid molecules.

It can be concluded that once a solvent has dissolved in a membrane, it will disturb the integrity of the membrane and hence its function as a barrier, as matrix for enzymes and as energy transducer. For membranes of living cells, these effects are difficult to quantify in view of the complex and heterogeneous nature of the protein-containing bilayer.

ADAPTATION OF MICROORGANISMS TO ORGANIC SOLVENTS

Despite the general toxic effects of organic solvents, some microbial strains can adapt to high concentrations of otherwise toxic organic solvents. This surprising observation was first made by Inoue and Horikoshi in 1989 for a *Pseudomonas putida* strain, IH-2000, which grows in the presence of a second phase of toluene (44,45). This strain was able to survive the presence of toluene as a second phase but was not able to metabolize the toluene. Soon afterward, other researchers confirmed this initial observation. Other *P. putida* strains were shown to grow in a two-phase solvent-water system containing toluene or other solvents. These strains were isolated on xylene, styrene, or toluene (46–49). They all were able to grow in the presence of a second phase of various solvents such as xylene, styrene, and toluene, but benzene as a second phase was not tolerated. Some of the strains were able to metabolize the solvents, others had to use alternative carbon and energy

sources. All solvent-tolerant *P. putida* strains were all isolated from a normal soil environment. Similarly, other solvent-tolerant species of *Pseudomonas* (*P. aeruginosa, P. fluorescens, P. mendocina*) were also isolated (50–54). Attempts to isolate solvent-tolerant strains from more extreme environments, such as the deep sea, resulted in the isolation of solvent-tolerant members of other genera. A *Flavobacterium* was reported to grow in the presence of a second phase of benzene (5%) (55), and apparently was even more tolerant of solvents than the strains belonging to the genus *Pseudomonas*.

Solvent-tolerant gram-positive strains have also isolated been isolated from deep-sea environments. Strains belonging to the genus *Bacillus* that survived a second phase of benzene were described (56,57). The authors attributed this remarkable property of benzene tolerance to the source from which the strains were isolated. A benzene-tolerant *Rhodococcus* strain was isolated from a contaminated site in Australia, with benzene as the growth substrate (58). More recently, it was shown that gram-positive bacteria tolerating benzene and/or toluene can also be isolated from normal soil environments.

Another way to obtain solvent-tolerant strains is to increase the resistance of nontolerant strains by mutations. Mutant strains with enhanced solvent-tolerance properties were obtained with *P. putida* PpG1 (59) and *P. aeruginosa* PAO1161 (60). *Escherichia coli* K12 could also be mutated to yield strains that are more solvent-tolerant (61).

Table 2. Strains Growing in the Presence of a Second Phase of Toxic Organic Solvents

Strain	Solvents Tolerated	Reference
Pseudomonas putida IH-2000	Heptanol, toluene	44
Pseudomonas putida Idaho	Dimethylphthalate, toluene	46
Pseudomonas aeruginosa ST-001	Heptanol, toluene	51
Pseudomonas putida S12	Dimethylphthalate, toluene	47
Flavobacterium DS-711	Benzene, toluene	55
Bacillus DS-994	Benzene, toluene	56
Pseudomonas aeruginosa LST-03	Toluene	52
Pseudomonas putida DOT-T1	Toluene	48
Pseudomonas LF-3	Toluene	64
Pseudomonas mendocina LF-1	Dimethylphthalate, toluene	54
Pseudomonas mendocina K08-1		
Rhodococcus strain 33	Benzene	58
Bacillus thuringiensis R1	Toluene	65
Pseudomonas putida GM73	Toluene	49

Source: (Modification of table presented by S. Isken and J. A. M. de Bont, *Extremophiles* **2**, 229–238 (1998)).

However, the tolerance of a particular strain to a solvent is not always tested for in a clear-cut way. The medium composition, the cultivation conditions, and the history of the inoculum have an effect on the ability of an organism to grow in the presence of a solvent. Strains that can grow in the presence of a second phase of the toxic organic solvent toluene have been compiled in Table 2. From the Table it is obvious that the potential for solvent resistance is much higher in gram-negative than in gram-positive strains. This observation is in agreement with the observation that gram-negative bacteria appear to be less sensitive to lipophilic compounds than gram-positive bacteria (26,62,63). This fact possibly may be explained by the presence of the additional outer membrane in gram-negative bacteria. Indeed, for all microbial strains tested, growth can be detected in the presence of a second phase of a solvent as long as the log P of the solvent is less a strain-dependent value (Table 3).

ADAPTATION MECHANISMS

Since the first solvent-tolerant strain was isolated, research started to uncover the mechanisms responsible for this unique ability. Already, in the first paper dealing with solvent-tolerant bacteria, changes in the membrane composition were predicted to play a crucial role in the mechanisms contributing to solvent tolerance (44). This was likely as the membrane is the main target of the toxic action of solvents. Indeed, several adaptive changes in the structure of the membrane have been observed in reaction to the accumulation of organic solvents in the membranes of microorganisms. Mechanisms involved in solvent tolerance, as considered by various researchers, are shown in Figure 1.

Adaptation on the Level of the Cytoplasmic Membrane

Adaptations on the level of the cytoplasmic membrane have often been studied with less toxic solvents such as ethanol (68,69). In the cytoplasmic membrane, changes at the level of the lipids and proteins have been observed. These adaptations reestablish the stability and fluidity of the membranes once it is disturbed by the solvents (28). In principle, several mechanisms are possible here and may vary from strain to strain. Mechanisms described so far are (*1*) the length of the fatty acid chains, (*2*) the degree of saturation of the fatty acids, (*3*) *cis*/*trans*-isomerization of unsaturated fatty acids, (*5*) composition of phospholipid headgroups, and (*5*) dynamics of the phospholipid turnover.

The organisms that exhibit the greatest resistance to alcohols, *Lacotobacillus homohiochii* and *Lactobacillus heterohichii*, react to the presence of ethanol by altering the composition of their membranes. They increase the chain length and decrease the amount of *cis*-unsaturated fatty acids in their membranes (68).

In nearly all micoorganisms, the presence of solvents causes a shift in the ratio of saturated to unsaturated fatty acids. Generally, cells react to the presence of organic solvents by increasing the amount of saturated fatty acids in the membrane (70–74). Alterations in the degree of

Table 3. Growth of Various Strains in the Presence of a Second Phase of Organic Solvents

Solvent	Dodecane	Decane	Nonane	Octane	Cyclooctane	Hexane	Propylbenzen	Cyclohexane	Ethylbenzene	Xylene	Styrene	Toluene	Benzene
Log P	7.0	6.0	5.5	4.9	4.5	3.9	3.7	3.4	3.2	3.1	2.9	2.6	2.1
Pseudomonas putida S12	+	+	+	+	+	+	+	+	+	+	+	+	−
Pseudomonas putida PPO200	+	+	+	+	+	+	+	+	+	+	+	−	−
Pseudomonas fluorescens IFO3507	+	+	+	+	+	+	+	+	−	−	−	−	−
Escherichia coli IFO3806	+	+	+	+	+	+	+	−	−	−	−	−	−
Alcaligenes faecalis JCM1474	+	+	+	+	+	−	−	−	−	−	−	−	−
Agrobacterium tumefaciens	+	+	+	+	−	−	−	−	−	−	−	−	−
Bacillus subtilis AHU1219	+	+	+	+	−	−	−	−	−	−	−	−	−
Saccharomyces uvarum ATCC26602	+	−	−	−	−	−	−	−	−	−	−	−	−

Source: (Data from A. Inoue and K. Horikoshi, J. Ferment. Bioeng. 71, 194–196 (1991) and S. Isken et al., *Appl. Environ. Microbiol.* **65**, 2,631–2,635 (1999)).

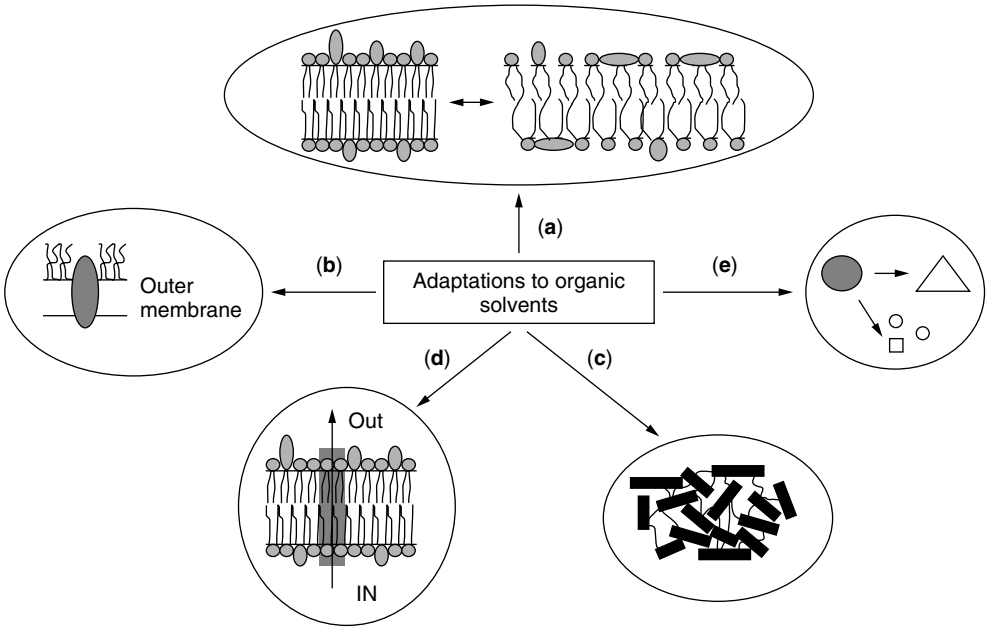

Figure 1. Schematic presentation of adaptation mechanisms that protect cells against the toxic effects of organic solvents. (**a**) Changes in the structure of the cytoplasmic membrane. (**b**) Modification of the LPS or porines of the outer membrane. (**c**) Reduction of cell wall hydrophobicity. (**d**) Active export of the solvents. (**e**) Transformation of the solvent. The scheme is a modification of the one presented by J. Sikkema et al., *Microbiol. Rev.* **59**, 201–222 (1995) (67).

saturation of the fatty acids change the fluidity of the membrane and in this way compensate for the effects caused by solvents. This stabilization of the membrane fluidity is known as "homeoviscous adaptation" (75). Changes in the fatty acid composition of membrane lipids are the most important reaction of bacteria against membrane-active substances. Most bacteria synthesize fatty acids by the well-established anaerobic pathway (76) and are only able to change their membrane fluidity by de novo synthesis of membrane lipids with a different ratio of

saturated to *cis*-unsaturated fatty acids during growth. As a result, bacteria are not able to perform a postbiosynthetic modification of their membrane fluidity.

One exception to this restriction is the isomerization of *cis*- to *trans*-unsaturated fatty acids in strains of the genera *Pseudomonas* and *Vibrio* (76,77). These bacteria are able to change their membrane fluidity by isomerizing *cis*-unsaturated fatty acids to the *trans*-isomers. At least, some representatives of the genus *Pseudomonas* convert their *cis* into the corresponding *trans* unsaturated fatty acids after the addition of toxic compounds (77,78). This conversion also takes place in nongrowing cells. Furthermore, cells are still able to accumulate *trans* fatty acids when the biosynthesis of fatty acids was inhibited by cerulenin (79,80). The isomerization of the double bond is a special mechanism of this bacterium to adapt to high concentrations of toxins under conditions not allowing growth and de novo synthesis of lipids. The benefit of this reaction lies in the steric differences between *cis* and *trans*-unsaturated fatty acids. Unsaturated fatty acids in the *cis*-configuration with their bent steric structures (a nick in the acyl-chain) result in a membrane with a relatively high fluidity. In contrast, the long extended steric structure of the *trans*-configuration is able to insert into the membrane structures similar to saturated fatty acids which are also mostly in all-*trans*-conformation.

The *cis* to *trans*-isomerization is catalyzed by an energy-independent enzyme (81,82). The enzyme responsible for the *cis*/*trans*- isomerization in nonsolvent-tolerant strains has been purified (83,84). The enzyme is periplasmic and acts in vivo both on phospholipids and on free fatty acids (85). In vitro, the purified isomerase acts only on free unsaturated fatty acids (84). Holtwick and coworkers (82) obtained isomerase-negative mutants of *P. putida* P8. A DNA fragment that complemented the mutation was isolated and cloned. The DNA sequence showed no significant homologous regions when the deduced amino acid sequence was compared with other proteins. Close to the N terminus of the predicted polypeptide of the *cis*/*trans*-isomerase gene (*cti*) a cytochrome c-type heme-binding motif was found (83). As a consequence a possible mechanism of heme-catalyzed *cis*/*trans*-isomerization of unsaturated fatty acids is discussed currently.

To prove that the *cis*/*trans*-isomerization is necessary for the survival of solvent-tolerant strains, studies were performed with mutants lacking the ability to perform this isomerization (86). A transposon mutant of the solvent-tolerant *P. putida* DOT-T1 was both solvent-sensitive and unable to perform the isomerization (87). However, the *cis*/*trans*-isomerization is unlikely to be the only necessary adaptation mechanism to organic solvents because strains are known that can perform the isomerization and are still solvent-sensitive (73,87). The *cis*/*trans*-isomerization has also been reported as a response to starvation (88) and in the presence of antibiotics (89) or heavy metals (90). This indicates that the *cis*/*trans*-ratio may be part of a general stress response of microorganisms.

Apart from the fatty acid composition, the headgroups of lipids are also altered during solvent-adaptation. In solvent-tolerant *P. putida* strains the relative amount of diphosphatidylglycerol (cardiolipin) increases during the adaptation to the solvent toluene (28,87). Such a change in the headgroups had also been found in *E. coli* mutants with an increased resistance to solvents (91). *Pseudomonas putida* Idaho was shown to adapt differently. The amount of phosphatidylethanolamine increases in this solvent-tolerant strain (92). In general, the regulation of the headgroup composition is said to control the phase preference of the lipids. In this way, the effect of solvents on the fluidity, the volume, and the density of the lipids is compensated (28).

Next to changes in membrane composition, the dynamics of biosynthesis of membrane compounds may play an important role in solvent tolerance. Pinkart and White (92) demonstrated that in the solvent-tolerant strain *P. putida* Idaho the rate of phospholipid synthesis increases after exposure to xylene. The total amount of phospholipids increases in this strain. A solvent-sensitive control strain, *P. putida* MW1200, has a much lower turnover of lipids and a reduction of the phospholipid content after exposure to xylene. Therefore, it is likely that *P. putida* Idaho is better equipped to repair damaged membranes than the solvent-sensitive strain.

Apart from changes in the composition of the cytoplasmic membrane and in the dynamics of the formation of phospholipids, alterations in the protein content have been observed as a response to solvents (68,93,94). In addition, lipid soluble compounds were shown to play a role in adaptation to solvents. *Zymomonas mobilis* increases the amount of hopanoids as response to ethanol (95) and in *Staphylococcus aureus*, the tolerance to oleic acid correlates with carotenoid production (96).

Adaptation on the Level of the Outer Membrane

As mentioned earlier, gram-negative bacteria are less sensitive to solvents than gram-positive organisms. However, no differences between gram-positive and gram-negative bacteria were observed with regard to the critical concentration of molecules dissolved in the cytoplasmic membrane (63). Hence, the differences in solvent tolerance must be based on other alterations.

In contrast to the gram-positive bacteria, the gram-negative bacteria have an additional outer membrane. The outer membrane was shown to be engaged in promoting solvent tolerance. Ions, such as Mg^{2+} or Ca^{2+}, stabilize the organization of the outer membrane and contribute to a higher resistance of solvent-tolerant *Pseudomonas* strains toward toluene (28,45,48). After adaptation to toluene solvent-tolerant *P. putida* S12 cells become less hydrophobic (28). Recently, it was shown that a reduction of the cell hydrophobicity correlates with changes in the LPS content (97). Indeed, the LPS composition of solvent-tolerant *P. putida* Idaho changes as result of the presence of solvents (73).

Apart from changes in the LPS, the porins that are embedded in the outer membrane have been related with solvent tolerance. On the one hand, mutants of *P. putida* Dot-T1 lacking the porin Ompl are hypersensitive to solvents, possibly because of the missing stabilization of the envelope integrity by Ompl (87). On the other hand, the absence of the porin Ompf in *P. aeruginosa* (98) leads to a higher tolerance toward solvents. Such an increase

in solvent tolerance because of the absence of a porin was also obtained in *E. coli* (96). The authors of these studies suggested that organic solvent molecules were able to pass through the porins. Therefore, mutants lacking these porins have a higher tolerance to solvents. Later, this theory was disproved for *E. coli* (99). The organic solvent tolerance was shown to be independent of Ompf levels in the membrane. Earlier found relations between solvent sensitivity and the level of Ompf were caused by variations in transcriptional activators.

Adaptation on the Level of the Cell Wall

Bacteria with hydrophobic cell walls were shown to have a higher affinity for hydrophobic compounds (100,101). Therefore, cell wall modifications that lower the hydrophobicity of the cell may provide a higher tolerance to solvents.

Adaptation Caused by Biodegradation of Solvents

Resistance to antibiotics is often based on the degradation of these antimicrobial compounds into less or nontoxic products. Many of the toxic solvents studied up to now can be degraded by microorganisms. Therefore, tolerance could be mediated by degradation of the solvents. The benzene tolerance of *Rhodococcus* strain 33 may partly depend on this mechanism because this strain was shown to be an effective degrader of benzene (58). Because many organic solvents can be utilized as carbon source this may be the case for other strains. Additionally, biodegration was predicted to play a role in tetraline-tolerance in *E. coli* (102). However, many of the solvent-tolerant strains described so far are able to cope with a broad range of solvents up to a second phase which often cannot be biodegraded at all by these strains. *Pseudomonas putida* S12 is, for instance, tolerant for both styrene and toluene. Of these solvents, however, only styrene can be metabolized (47). Hence, degradation may mediate the resistance of some strains to specific solvents, but it cannot be the main mechanisms contributing to the tolerance to a broad range of solvents.

Adaptation Caused by Active Excretion of Solvents

Metabolism of toxicants is the only mechanism that decreases the amount of toxins in the cell or membrane actively. An alternative method is to decrease the concentration of the toxin by removing it from the cell by active excretion. Such efflux systems are well known for lipophilic cytotoxic agents, such as antibiotics (103–106). Many of the energy-driven export systems play an important role in drug resistance because they are able to pump out a wide range of compounds having no common chemical structure. The only common feature is that most of these compounds are charged amphiphilic molecules. It was shown that genes coding for the proteins engaged in such an export can be induced by structurally unrelated hydrophobic compounds (107). In several strains the adaptation to organic solvents results in an increased resistance to various chemically and structurally unrelated antibiotics (89). Therefore, research started to determine whether organic solvents can be actively excluded from a bacterial membrane. Indeed, we demonstrated that the amount of toluene accumulated in *P. putida* S12 is dependent on energy originating from the proton motive force (108,109). This strain was not able to transform the toluene. Therefore, it was concluded that the amount of toluene in the cell was kept at a relative low level by the action of an active efflux system. Since then, active efflux as a mechanism contributing solvent tolerance has also been observed in other *Pseudomonas* strains (49,87,110). The gene responsible for the export of toluene in *P. putida* S12 was identified (111) and showed homology with other well-known export systems responsible for the active efflux of antibiotics out of the cell. The homology of this gene called *srpABC* with proton-dependent efflux pumps, such as the *acrAB* operon in *E. coli*, implicates again that the solvent efflux is dependent on the proton motive force. The participation of the multidrug efflux pump AcrAB, encoded within the *acrAB* operon, in the solvent tolerance of *E. coli* was demonstrated by White and coworkers (112). Rojas and coworkers (113) demonstrated that the presence of several solvent efflux pumps can be necessary to provide efficient tolerance to toluene in *Pseudomonas* strains.

OLE OF GENERAL STRESS RESPONSE

The large number of adaptation mechanisms described in the preceding text suggest that solvent tolerance is not mediated by one mechanism only. It is likely that a combination of different mechanisms is necessary to provide solvent tolerance. A kind of cascade of fast (urgent), midterm, and long-term mechanisms are working together to reach a full adaptation to toxic solvents. This includes the presence of a general stress response system (114,115), such as the one known for heat shock, which is induced or activated by the solvents. Indeed, the induction of a large number of proteins by toxins was demonstrated in *E. coli* in the presence of pollutants (116) or the uncoupler 2,4-dinitrophenol (117) leads to the induction of 39 or 53 different proteins, respectively. When *Clostridium acetobutylicum* initiates the solvent transformation, various known heat-shock proteins are expressed (118). In *P. putida* Kt2442 the expression of approximately 100 proteins is affected by the presence of 2-chlorophenol (119).

As mentioned earlier, the adaptation to a solvent does not only enhance the resistance to other solvents (80), but also to heavy metals (90) and antibiotics (89) in the solvent-tolerant strain *P. putida* S12. Such a correlation was earlier found in *E. coli* in which the overexpression of stress-response genes enhanced tolerance to various environmental factors (120–123). The question remains whether genes, which mediate solvent resistance and whose functions are still unknown, like *osta* (124), can be embedded in the cascade system of stress response.

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TOXICITY TESTING IN SOIL, USE OF MICROBIAL AND ENZYMATIC TESTS

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The economic well-being of human society is dependent on the productivity and sustainability of arable soils. Soil is the ultimate receptor of, and the incubation chamber for, decomposing organic material and recycling nutrients back to plants, as well as detoxification of organic pollutants. Soil quality can improve or deteriorate depending on several factors. If mismanaged, the soil can work against us, for example, it can pollute the air by emissions of nitrous gases and pollute water by leaching of nitrogen and other plant nutrients or pesticides. We are becoming more aware of the frequent exposure of soils to anthropogenic substances and that there is a delicate balance between maintaining good soil quality and achieving production goals. Therefore, there is an urgent need for tools to measure toxicity in soils (Fig. 1), to interpret toxicity data, as well as to develop a strategy for evaluating soil quality (1).

Our aim is to give a short overview of the soil microbial ecosystem and some important factors pertaining to soil formation, and against this background to discuss what kind and quality of information soil tests should deliver. Examples of useful microbial and enzymatic tests are given. Finally, we discuss strategies and problems in screening and testing the effects of chemicals and heavy metals, the assessment of soil quality, and the monitoring of long-term changes in both naturally managed and polluted soils.

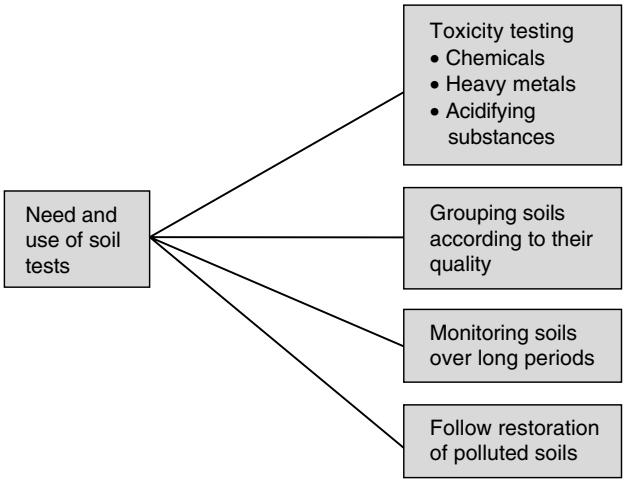

Figure 1. The need of various soil tests to be able to assess soil quality.

THE SOIL MICROBIAL ECOSYSTEM

The soil ecosystem gains most of its energy from dead organic materials, for example, plant and animal residues. During the mineralization of these residues, carbon dioxide and inorganic nutrients such as nitrate, phosphate, and sulfate are released so that plants can utilize them again. The microbial biomass itself represents a major pool of readily available nutrients, which is continuously shunted into the growth cycles of macro- and microphytes. Consequently, soils that host a high level of microbial biomass are capable not only of storing more nutrients, but also have the potential of cycling more nutrients through the ecosystem.

As microorganisms are adapted to survive under extreme conditions, they are present in soil both in large numbers and under almost all environmental conditions. Many catabolic and anabolic functions are widely distributed among soil micropopulations, and groups of taxonomically diverse organisms can therefore replace one another in the decomposition cycles. The microbial community carries out the majority of decomposition processes in soil and is stimulated by the activities of soil invertebrates, especially saprotrophs feeding on decaying organic matter and microbivores grazing the microflora. Furthermore, microorganisms are irreplaceable in the biological transformation and degradation of synthetic organic compounds and natural waste materials.

Soil microorganisms are critical in creating and maintaining good soil structure, which is important for proper soil aeration and the formation of humus and particle aggregates. Filamentous fungi and actinomycetes, in particular, entrap soil particles to form aggregates. In addition, bacteria produce extracellular metabolites, for example, polysaccharides, lipids, and proteins, which function as gums and cementing agents that stabilize the aggregates. Soil texture and structure, in combination with a variation in moisture levels, can drastically affect the aeration status, thus influencing the distribution of physiological groups in the microbial community.

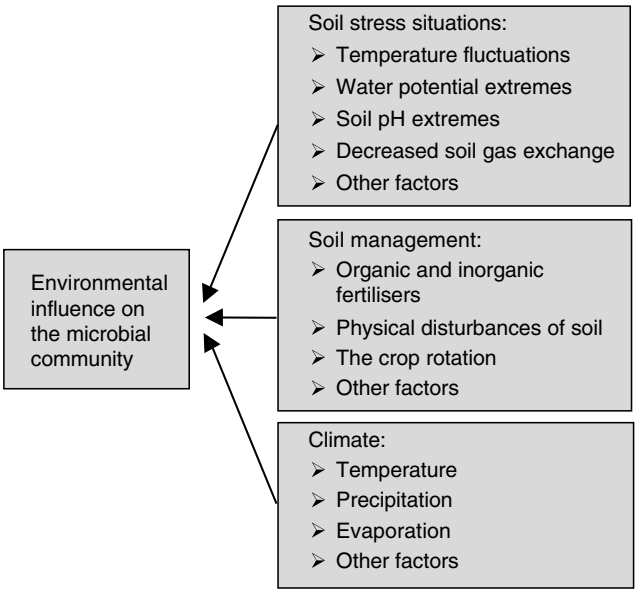

Figure 2. The environmental factors influencing soil microbial communities.

In all terrestrial ecosystems, microorganisms are more or less continuously exposed to changing environmental conditions. This means that the microbial community in soil is exposed to stress situations owing to both soil management and climatic conditions (Fig. 2).

SOIL QUALITY

In agriculture, soil quality is an integral part of agricultural sustainability, and is influenced by a number of degrading and conserving forces (2). Soil is a complex system created by a number of factors that cannot be easily influenced, such as geology, topography, and climate (Fig. 3). Soil quality is controlled by physical, chemical, and biological components. Soil quality factors that can be influenced are humus content; the number, composition, and activity of microorganisms; the degree of base saturation; nutrient status; and others. A number

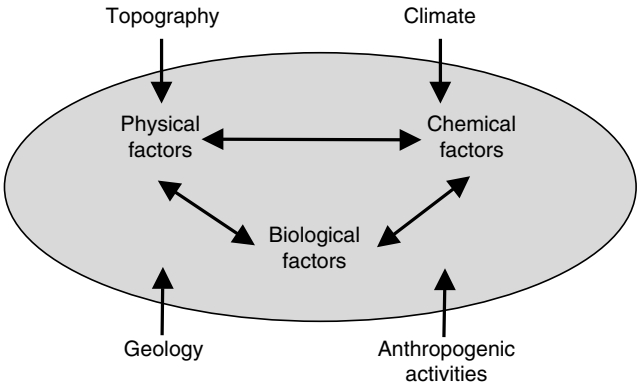

Figure 3. Soil is a complex structure created by influences of geology, topography, and climate as well as anthropogenic activities.

of short-term practices, such as soil cultivation or use of fertilizers and pesticides, are generally not included as soil quality factors. Neither are unintended deposition of chemicals, heavy metals, or acidifying substances considered soil quality factors. Many attempts have been made to define soil quality (3–5). All definitions have in common the capacity of a soil to function effectively both at present and in the future. As suggested by Doran and Parkin (3), soil quality can be defined as "the capacity of a soil to function within ecosystem boundaries to sustain biological productivity, maintain environmental quality, and promote plant and animal health." Such a definition implies that good soil quality is relative, and must be individually defined for each soil ecosystem (see SOIL QUALITY: THE ROLE OF MICROORGANISMS, this Encyclopedia).

SOIL TEST DATA — A COMPREHENSIVE PICTURE

With the development of new methods for assessing soil biomass and microbial or enzymatic activity, it is important to have a clear idea of what kind of information the test should deliver. It is important to consider whether to choose a test for the evaluation of a soil property or to test the effect of a specific substance. Furthermore, when interpreting data the basic test design, that is, the possibilities and limits of the test, must be considered. This work would be simplified if apparently different tests and test data could be treated in a common frame, that is, to use the same basic concept of kinetics. The kinetic discussion in the following text refers to tests of potential activities, that is, tests performed under optimized environmental conditions so that only the amounts of organisms and enzymes are rate limiting.

In its simplest form, a quantitative soil test assay uses the assumption that no product is present at time zero. After a certain time of incubation the first and only sample is withdrawn for analysis of the product. A constant product formation rate must be assumed to allow calculation of a process rate from such limited data. A more accurate way is to also establish the initial concentration of the product. Even if in most cases the product formation rate of some tests is constant, the above test strategy has some uncertainty in the rate and, hence, should be viewed as semiquantitative information. The uncertainty will increase not only with time of incubation, but also when an unknown substance to be tested is added. A better experimental design is to take a reasonable number of samples during the test so that a straight-line relation can be established by linear regression. Moreover, many samples give more accurate data, that is, random errors in sampling and analysis cancel each other. The linear product formation can be described by the following general formula:

$$p = p_0 + Et \tag{1}$$

where p and p_0 are the amounts of product at time t and $t = 0$, respectively, and E is the enzyme activity (Fig. 4a). When referring to the enzyme activity of a specific microbial process showing no growth during the test, E can be replaced by, for example, K, a rate constant for potential microbial activity (Fig. 4b).

The complexity increases when nonlinear test data are generated because nonlinear processes, by necessity, demand a larger number of sampling points to establish the shape of the curve. Several means of linearization of such data have been proposed. Perhaps the most common way is to make only a very short incubation and to subjectively fit a straight line to the nonlinear data. A biased estimate will probably be achieved in this way. As a nonlinear product formation rate is most likely owing to de novo synthesis of enzymes, with or without cell growth, the use of antibiotics with inhibitory effect on protein synthesis has been proposed to maintain linear rates.

Another strategy is to analyze the phenomenon as a nonlinear process. Such a strategy generates more information of the process tested for. In most test situations enzyme activity, as well as growth of microorganisms, must be considered. These two properties are described in the formula

$$p = p_0 + \frac{r}{\mu}\left(e^{\mu t} - 1\right) \tag{2}$$

where r is the initial microbial activity and μ the specific growth rate (6). Thus, by assuming a constant amount of enzymes without cell growth (i.e., zero-order kinetics with respect to cell growth), a constant product formation rate (K), as discussed earlier, will explain the straight-line result of a test (Fig. 4b). Assuming a substrate saturated enzyme system with cell growth (i.e., first-order kinetics with respect to cell growth), both the initial rate of product formation (r) and specific rate of cell growth (μ) will explain the nonlinear curve of some tests (Fig. 4c).

For general soil processes such as respiration, it is not likely that all microorganisms react to a substrate in the same way. It has been suggested that mineralization of glucose is performed by two main groups of microorganisms. The first group grows exponentially as a result of substrate addition, whereas the other group increases its respiration activity to a higher rate without multiplication. In a further modification of formula (2), the activity of the nongrowing group, denoted K, was introduced (7), resulting in the new formula:

$$p = p_0 + \frac{r}{\mu}\left(e^{\mu t} - 1\right) + Kt \tag{3}$$

By applying this formula (Fig. 4d), a deeper understanding of underlying structures in the soil ecosystem can be achieved (8).

From the preceding discussion it is evident that results from different tests permit different degrees of interpretation and understanding. Many enzymatic tests used today are simplifications chosen to get rapid and inexpensive tests. One reason for this is that authorities and the chemical industry have urged the development of these kinds of tests. Another reason is that the awareness of the enormous task ahead with so many untested chemicals necessitates the use of simple tests. On the other hand, the consequence of using such simple tests might be that important pieces of information are overlooked. Thus, a contradiction can be seen between using soil tests as research tools or as practical environmental indicators.

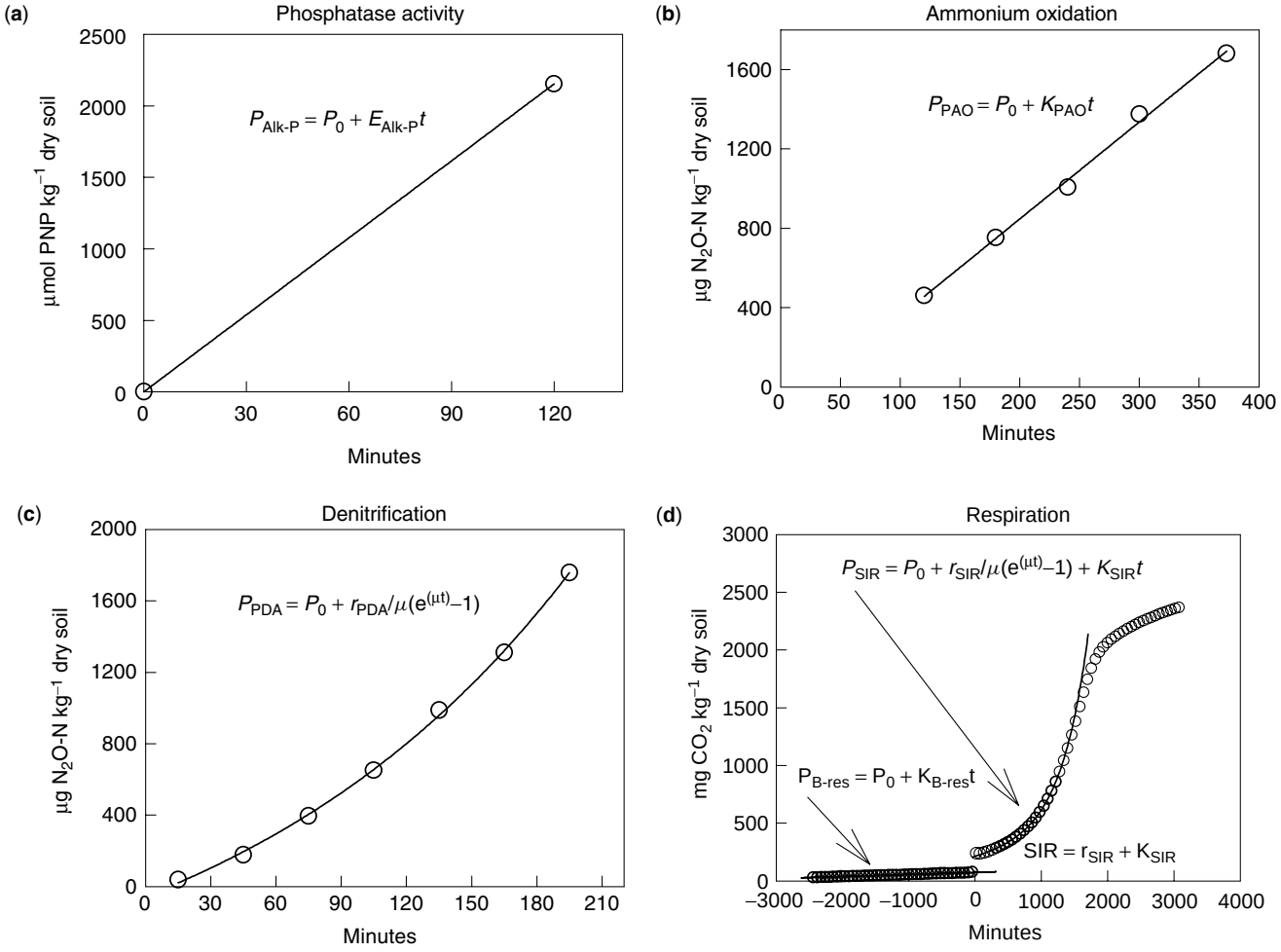

Figure 4. Examples of microbiological test data and a kinetic approach to evaluating product formation rates: (**a**) rate of phosphatase activity (P_{Alk-P}), (**b**) potential NH_4^+ oxidation rate (P_{PAO}), (**c**) potential denitrification rate (P_{PDA}), and (**d**) substrate induced respiration rate (P_{SIR}) and basal respiration rate (P_{B-res}). Gray circles are data not included in the regression analysis.

The conclusion is, however, that both types are needed and their development must go hand in hand.

MICROBIAL AND ENZYMATIC TESTS

Commonly, microbial soil tests are grouped into biomass estimations, activity measurements, and assays of soil enzymes. The distinction between activity and enzymatic assays is not straightforward as several enzymes may simultaneously be located intracellularly, on cell surfaces, and also be actively exuded as free enzymes into the bulk soil. In addition, "dead" or dormant cells can be regarded as being in between these two states. Another common practice is to relate tests to the general biogeochemical cycles of carbon, nitrogen, phosphorus, and sulfur. To be more precise, we have chosen to classify the tests according to different functional levels within ecosystems: (*1*) basic microbial soil functions (biomass and processes performed by virtually all groups of microbes), (*2*) specific microbial soil functions (performed by a more or less well-defined group of microbes), and (*3*) general microbial growth. A review of some important tests that are commonly used for assessing the effects on the soil ecosystem is given later. Several manuals with detailed information on soil test performance (9–11) and soil toxicity testing (9,12) have been produced.

Basic Microbial Soil Functions

Biomass. (see BIOMASS: SOIL MICROBIAL BIOMASS, this Encyclopedia) The level of total soil biomass has often been regarded as a fertility indicator as a microbial biomass is correlated with nutrient turnover. Moreover, a high biomass contains a reserve of valuable nutrients. Historically, the total biomass of soil microorganisms has been determined by viable plate counts or direct counts by microscopy. The former method seriously underestimates the biomass whereas the latter is tedious and time consuming. By combining the microscopic technique with immunological techniques or molecular genetic probing techniques, specific groups of soil bacteria can be enumerated. Also, various techniques for indirect estimation of the microbial biomass have been developed,

of which the chloroform fumigation–extraction (CFE) techniques of biomass carbon, nitrogen, or phosphorus are the most widely used (13–16). Another method for indirect measurement of biomass is the substrate-induced respiration (SIR) technique (16,17). After a period of 7 to 10 days of preincubation the soil sample is amended with glucose in surplus. To ensure no nutrient limitation, ammonium and phosphate can also be added. Immediately after substrate addition, the respiration pattern is recorded for at least 8 hours. The maximum response in CO_2 production or O_2 consumption before start of cell growth is proportional to the amount of biomass. Several techniques exist for the determination of carbon dioxide produced, such as titration, gas chromatography, or infrared gas analysis. One convenient respirometer to use is a Respicond III (Nordgren Innovations AB, Umeå, Sweden). It is based on the capture of carbon hydroxide in potassium hydroxide and measurement of the resulting decrease in conductivity of the KOH solution. The respirometer can measure the respiration of 96 soil samples two times every hour. A computer program allows the calculation of SIR. In addition, data can be exported to a computer program capable of nonlinear regression analysis. By fitting data to formula (3) above, the total biomass can be split into the amount of growing (r) and nongrowing (K) bacteria, respectively.

Basal Respiration. Soil respiration is the degradation of organic forms of carbon. For organisms to access the carbon, several enzyme systems must be involved, resulting in the release of various nutrients bound in the organic matter. Thus, the ultimate products of respiration, or carbon mineralization, are carbon dioxide, water, and various nutrients. Owing to competition for energy among fast-growing microorganisms, the most available carbon fractions, such as simple sugars and amino acids, will be degraded first, leaving the more recalcitrant structures of hemicellulose and lignin to slow-growing organisms. In an undisturbed soil, respiration is dominated by the degradation of more complex forms of carbon and is often referred to as basal respiration. The simplest way to measure basal respiration is to preincubate a soil sample to allow the initial flush of carbon dioxide caused by sample manipulation to level off. Thereafter, carbon dioxide production is measured in a closed system by absorption of carbon dioxide in an alkaline KOH solution. By titration, the remaining potassium hydroxide can be determined and the amount of carbon dioxide produced can be calculated. By using the respirometer described earlier, basal respiration can conveniently be monitored during a 48-hour period before the glucose addition for determination of SIR. The respirometer method gives very accurate estimates as many data points per assay are generated.

Nitrogen Mineralization. Nitrogen mineralization is the process during which organic bound nitrogen is enzymatically degraded to the mineral form ammonium ion (18). In soil, the process is performed by many diverse bacteria, both aerobic and anaerobic. Thus, the mineralization process is more or less independent of the oxygen status. To degrade organic macromolecules, microorganisms have to excrete extracellular enzymes, many of which are unspecific. Owing to the importance of nitrogen in crop production, many methods have been proposed to estimate the nitrogen mineralization capacity. One technique is to make a standardized aerobic incubation of a soil sample over several weeks or months. During the incubation period, nitrogen that is soluble in water is leached, either at the end or repeatedly at specified intervals. Alternatively, many replicates are started and destructive extractions with a KCl solution are made. The leachates/extracts are analyzed for ammonium and nitrate, and eventually nitrite. This technique estimates net mineralization because virtually all microbial nitrogen transformation processes might occur during the incubation. Moreover, if the effect of a test substance is to be evaluated, it is impossible to determine which group of organisms involved in nitrogen transformation are affected. Another commonly used technique to measure nitrogen mineralization capacity is the slurry assay (19) performed under anaerobic conditions with water as the only additive. The incubation period is 7 to 14 days. The advantage of an anaerobic slurry assay is that problems with determination and maintenance of an optimum soil water content is avoided. Substrate limitations because of restricted diffusion are also minimized. Moreover, nitrification is inhibited and assimilation is retarded by the anaerobiosis, as well as by uncontrolled losses of gaseous nitrogen. Thus, only ammonium has to be analyzed. Ammonium can effectively be analyzed by the indophenol blue method on a spectrophotometer provided with a flow cuvette system. The net mineralization capacity is calculated as the difference in ammonium content at the start and end of incubation.

Specific Microbial Soil Functions

Nitrification. Autotrophic nitrification is the two-step process by which ammonia is first oxidized to nitrite and then further to nitrate (20). In this aerobic process nitrifying bacteria gain energy for growth and reducing capacity to fix carbon dioxide. Nitrification ability is restricted to only a few bacterial species, all within the family *Nitrobacteriaceae*. Owing to their complicated metabolic machinery, nitrifying bacteria are sensitive to various environmental disturbances and thus can be used as organisms indicative of low levels of stress. Another reason for using nitrification in a test system is its important role in the biogeochemical cycle of nitrogen. Two basic nitrification assays are used: (*1*) assessing only the first step by analysis of nitrite after a short incubation period and (*2*) assessing the full nitrification pathway by analysis of ammonium and/or nitrate after a longer incubation period. The former assay has become increasingly popular because of its rapidity and simplicity (21). In the assay a soil slurry is generated by adding an optimum concentration of ammonium dissolved in a buffer (pH 7.2). The second step in the nitrification pathway is blocked with chlorate. The product, nitrite, can easily be analyzed colorimetrically. To increase the analysis capacity, the use of an automated

spectrophotometer technique is recommended. As NH_4^+ oxidizing bacteria have long generation times (>10 hours), the rate of product formation will be constant when short incubation periods are used. This means that data can be evaluated by linear regression. The NH_4^+ oxidation test is now in its final revision in becoming an ISO standard (ISO DIS 15685).

Denitrification. Biological denitrification is the process by which nitrogenous oxides, mainly nitrate and nitrite, are reduced to the nitrogen gases nitric oxide, nitrous oxide, and anitogen (20). Most denitrifiers prefer oxygen as the terminal electron acceptor and therefore reduce nitrogenous oxides only under anaerobic conditions. The complex pathway of denitrification is not fully understood, but is thought to consist of more than 26 genes and to be regulated both at the enzyme and gene levels by a number of environmental factors. Moreover, the steps in the denitrifying pathway have different sensitivities to various kinds of disturbances. Denitrification is a functional trait found within many taxonomical and physiological groups of bacteria. The genera *Pseudomonas*, *Alcaligenes*, and *Bacillus* are thought to be the most frequently found denitrifiers in soil. However, the list of denitrifiers is increasing with the introduction of molecular biology techniques for identification and determination of taxonomic relationships. Thus, denitrifiers can be viewed as representatives of a broad range of soil microbial populations. Denitrification is not just a process where nitrogen is lost to the atmosphere but can also indicate easily available organic carbon, as most denitrifiers are organotrophic and mineralize organic matter both under aerobic and anaerobic conditions. A common way to characterize denitrification in soil is to determine the potential denitrifying activity (PDA). In this method a soil slurry is incubated anaerobically with additions of an optimum amount of nitrate and an easily available carbon and energy source, such as glucose (22). At the start of the incubation, acetylene is added to block the last step in the denitrification pathway, the reduction of nitrous oxide to nitrogen. The accumulated product, nitrous oxide, is then analyzed on a gas chromatograph (GC) equipped with an electron capture detector (ECD). In this assay, problems with substrate diffusion have been eliminated and thus only the amounts of denitrifying enzymes will be rate limiting. The use of chloramphenicol (CAP) has been suggested to lock the enzyme concentration at its initial test concentration. The result will be a prolonged initial phase of linear product formation. Unfortunately, CAP seems to affect not only the synthesis but also the activity of denitrification enzymes (22,23). Recently, it has been demonstrated that it might be possible to use low concentrations of CAP ($<0.1\ g\,L^{-1}$) without negative effects (24), however, we recommend omitting CAP. The resulting nonlinear data can be evaluated by fitting data to formula (2). A manifold system for changing gases in the assay vessels improves the method. Such systems are not commercially available and must therefore be constructed in the laboratory.

Nitrogen Fixation. Diazotrophy, that is, the capacity to biologically reduce atmospheric dinitrogen gas into ammonia and further incorporate the nitrogen into cell biomass, is restricted to bacteria (20,25). Nitrogen-fixing bacteria are a diverse group of prokaryotes. Some of these bacteria are members of the bacterial family *Rhizobiaceae* and fix nitrogen in root nodules in a symbiotic relation with legumes. A second large group of nitrogen fixers are organotrophic and heterotrophic bacteria in the soil ecosystem. A third group, originally classified as algae, are the photosynthetic cyanobacteria (blue green algae). The actual fixation is performed by the oxygen-sensitive nitrogenase enzyme system. This system is complex and is under the control of at least 21 different genes which are regulated both on enzyme and gene level by a number of environmental factors such as ammonium, nitrate, and oxygen (see NITROGEN FIXATION IN SOILS — FREE-LIVING MICROBES, this Encyclopedia). Owing to both the economical and ecological importance of nitrogen fixers, many methods have been proposed to monitor their activities. The most commonly used technique, the C_2H_2 reduction technique, takes into account the higher affinity with the nitrogenase to acetylene than with the normal substrate nitrogen (26). When acetylene is reduced by the nitrogenase the end product ethylene is formed. As ethylene can be produced by other soil organisms, the endogenous production must be estimated by the CO blockage method to get an accurate estimate of nitrogenase activity (27). In the assay of heterotrophic N_2 fixation, a soil sample is preincubated with glucose for 48 hours, after which the sample is placed in a gastight chamber and injected with acetylene (28). After 24 to 48 hours the concentration of ethylene is analyzed on a GC equipped with a flame ionization detector (FID). Assuming a linear product formation, the rate of C_2H_2 reduction is calculated. For the determination of cyanobacterial nitrogen fixation, a moistened soil sample is incubated under permanent light for two to four months (29). During the light incubation period, ethylene production is assayed at regular intervals by placing the sample in a gastight chamber, which is injected with acetylene. Soil is incubated for one to four hours before C_2H_4 concentration is analyzed. The result is reported as the mean value of ethylene formed at each determination. The reproducibility of both the heterotrophic and the cyanobacterial nitrogen fixation methods is not very high, as both methods depend on a period of enrichment of the bacteria before the actual activity assay is performed.

Phosphatase Activity. (see SOIL ENZYMES, this Encyclopedia) Depending on the organic matter content, the total amount of organically bound phosphorus in soil varies from a little more than zero up to nearly 90% of the total soil P fraction. As phosphorus is an essential nutrient, not only for plants but also for all living organisms, a deficit of free mineral phosphate is the normal status of the soil ecosystem. Moreover, the deficiency is amplified by the formation of insoluble P complexes owing to pH and the mineral content of metals such as iron, aluminum, and calcium. The mineralization of phosphorus by phosphatases is therefore probably rate limiting for the activity of many soil organisms. Phosphatases are a group of enzymes of plant root and bacterial origin that hydrolyze esters and anhydrides of phosphoric acids. Even if microbial phosphatases

dominate in soil, they cannot be attributed to specific groups of bacteria. Phosphatases occur intracellularly in the periplasmic space of gram-negative bacteria, adsorbed to cell surfaces or as extracellular enzymes adsorbed to humic or mineral surfaces. Groups of phosphatases found in soil are phosphomonoesterases, phosphodiesterases, phosphotriesterases, and polyphosphatases, of which the activity of the three latter groups is rarely assayed. A parallel to the nitrogen mineralization assay would be just to analyze the content of phosphate after a mineralization period of naturally occurring or added phosphate esters. Owing to the influence of assimilation processes and the occurrence of more or less firmly chemically precipitated phosphate in soil, this technique is unreliable. The most widely used phosphatase assay is that of acidic or alkaline phosphomonoesterases (30). There are strong indications that acidic phosphatases are largely extracellular. Alkaline phosphatases are known to occur at high levels in the periplasmic space but can also be stabilized outside the cell. The phosphomonoesterase activity is determined as the release of *p*-nitrophenol after the addition of a buffered (pH 6.5 or 11) solution of the artificial substrate *p*-nitrophenyl phosphate. *p*-Nitrophenol is a yellow colored product that can be analyzed colorimetrically. As the substrate is added in excess to form a soil slurry, the product formation will be dependent on the amount of active enzymes. Assuming no product at the start, the rate is calculated as the increase in product after one hour of incubation. To estimate the basic phosphatase activity the soil should be preincubated at constant moisture for at least six weeks prior to the test.

Other Enzymes. The metabolism of living cells involves a vast number of different enzymes. Some are strictly intracellular whereas others are exposed on the outer cell surface or even exported to the environment. The role of excreted enzymes is to hydrolyze substances that are too large to be taken up and transported through the cell membrane. Other exoenzymes are involved in the detoxification of chemicals harmful to the cell. The mineralizations of carbon, nitrogen, phosphorus, and sulfur are complex processes each involving many different enzymes, both general and binding specific. Methods have been developed for measuring the activity of more than 50 soil enzymes (31). Usually, the enzyme assays are carried out by adding to a soil sample a solution with an optimum concentration of a specific substrate and then carrying out an incubation for 1 to 24 hours. As temperature, pH, and other environmental conditions are also optimized, it is the potential activity that is measured. Some commonly assayed exoenzyme activities of the carbon metabolism are: amylase and cellulase; nitrogen metabolism: proteinase, peptidase, and urease; and sulfur metabolism: sulfatase. A group of enzymes involved in internal redox reactions of the cell are the dehydrogenases. As these are general and ubiquitous enzymes, they are thought to be good indicators of the overall metabolic status of the microbial system. Most often enzyme assays are carried out using an artificial substrate that becomes colored when the target group is cleaved off, or the product can be colored by a developing procedure. The process can thus be measured with a spectrophotometer.

By setting up a system capable of monitoring many enzymes, an entire enzyme profile of a soil sample can be determined. Likewise, by modifying the SIR method to involve respiration with not only glucose but also a larger set of carbon sources, a substrate profile can be generated. A simple system for establishing substrate profiles is the Biolog system (Biolog, Inc., Hayward, California). The system consists of a 96-well microtiter plate containing different sole carbon sources and nutrients, and a control. Microbial utilization of the substrate leads to formation of colored products. The substrate utilization pattern can be read in a microtiter-plate reader. Enzyme or substrate profiles can be used to follow changes in the metabolic diversity of a soil ecosystem.

Microbial Growth

The ability to grow and divide is a fundamental property of the cell. This process involves enzymes for generating energy and building blocks for the new cells, as well as enzymes for duplicating the genetic pool. This is a complex process susceptible to various disturbances. Unfortunately, techniques to follow microbial multiplication in soil, such as counting bacteria by culture-dependent methods or by microscopic techniques, are too insensitive to give reliable results. However, by combining methods for measurement of product formation with kinetic theories, bacterial growth can be calculated. Data from SIR and PDA have successfully been used for estimation of the specific growth of both aerobic bacteria (7,8) and denitrifying bacteria (22), using glucose as the carbon and energy source. Moreover, the specific growth rate is a sensitive indicator of toxicity, for example, of silver (32). Advantages of this approach are that no separate assays need to be performed, and growth rates can be derived from ordinary SIR and PDA assays, thus making such assays cost-effective.

SCREENING AND TESTING OF CHEMICALS AND METALS

In addition to the approximately 100,000 chemicals used currently in society, a substantial number of new chemicals are introduced every year. The effects of most of these chemicals on the soil ecosystem are not known. The addition of any potentially toxic compound to a soil ecosystem may be a serious threat to microbial functions and hence to the sustainability of the soil. Ideally, it would be possible to screen many chemicals, heavy metals or complex substances to differentiate between those that are harmful and those that are not. In a second step, acceptable values for doses or concentrations to the soil ecosystem should be determined in dose-response tests. In all soil toxicity testing the selection and handling of the soils to be used as standard soils is crucial for the outcome of the tests.

Standard Soils

The properties of a soil influence the bioavailability of chemicals, thus leading to varying degrees of bioaccumulation, degradability and toxicity in different types of soil.

Several ideas have been presented to deal with the problem of determining acceptable concentrations of bioavailable chemicals in soils. One obvious way is to use a wide range of soils representing different properties. Soils ranging from low to high levels of organic material, clay to sand ratios, and pH values are generally chosen. Sometimes only soils representing the two ends of the spectrum with respect to each variable are tested to generate information on worst-case situations. Another strategy is to identify a set of soils representing the most common types within a specific region. However, with the latter strategy only the most common situations can be simulated, and no information on the boundaries for risks or use will be achieved. Another weakness of this strategy is that transport of soils may alter their properties, and hence lower the quality and reliability of the test results. A third strategy is to specify general criteria for the types of soil to be used in testing. The Organization of Economic Cooperation and Development (OECD) agreed on a type of soil for testing the side effects of pesticides on soil microflora (33) and that any soil/sediment fulfilling specified criteria could be used. The criteria include specified ranges in pH, organic content, sand content and that a certain fraction of the total organic content is microbial biomass, to ensure sufficient biological activity. If soil criteria are set correctly, it should be possible to find suitable soils within a convenient distance from the test laboratory.

To be able to conduct a large number of tests with both reliable and reproducible results, proper handling of the soil is important. As the soil microbial environment constantly changes during the season, one strategy is to collect a large batch of soil to be used for a period of a year. The soil batch should be homogenized by mixing and sieving the fresh soil before further storage. If the soils are collected under wet conditions, it might be necessary to allow them to dry gently to about 50 to 60% of their water holding capacity. This is best done under a constant temperature of $+4\,^{\circ}C$. Many protocols also stipulate soil storage at this temperature. As many microoorganisms are active even at low temperatures, changes in microbial populations and organic matter content will gradually occur during storage. Therefore, the best method of conserving a soil is to freeze it at $-20\,^{\circ}C$ (34), at least for soils from the temperate part of the world.

Screening of Chemicals

For rapid screening of a high number of substances, high-capacity microbiological assays must be used. The SIR and PAO are two such tests. In addition, they represent microbial indicators of low and high sensitivity, respectively, and so complement one another. Numerous tests and procedures have been used for screening purposes. In the screening procedure, one or a few concentrations of the test substance are added in a series of assays. Typically the highest dose chosen is 10 to 100 times higher than the expected field rate or soil contamination level, to ensure that substances also with low toxic effects are identified. Given later are some examples of the screening of pesticides, heavy metals, and sewage sludge.

The acute toxicities of 54 pesticides on nitrification and denitrification in soil were surveyed in a screening test by assessing effects on PAO, PDA, and μ_{PDA} (35). The results revealed that 35% of the substances tested had a significant effect on PAO, 23% on PDA, and 26% on μ_{PDA}. For nitrification, all observed effects were inhibitory, whereas for denitrification both stimulatory and inhibitory effects were observed.

The effects of a large number of pesticides, for example, fumigants, herbicides, and insecticides, on various microbial and enzymatic activities in soil were tested (36–38). The microbial activities tested were ammonification, nitrification, denitrification, and sulfur oxidation. The enzyme activities tested were amylase, dehydrogenase, invertase, nitrogenase, phosphatase, and urease. The results showed that most pesticides had a significant effect on most of the activities tested. However, the recovery of these processes was rapid and the authors concluded that soil microbes can tolerate chemicals used for control of soil pests.

The effect of 16 heavy metals at four rates was tested on 16 microbial indicators (39). The enzymes involved in carbon-cycling were the least affected, whereas various enzyme activities related to cycling of nitrogen, phosphorus, and sulfur showed a considerable decrease in activity. Arylsulfatase and phosphatase were the most sensitive enzymes.

The effect of sewage sludge on PDA and acidic phosphatase activity in soil was tested using sludge amounts comparable with normal field applications. Three different sludges influenced PDA differently at increasing rates, from inhibition through no effect to stimulation (Fig. 5). The same sludges all stimulated phosphatase activity at higher application rates.

Dose-Response Testing

The effects of various substances on specific processes in soil can be quantified by assessing the effect concentration (EC). The concentration required to cause an inhibition of 50% is thus designated EC_{50}, whereas the highest dose causing no effect is designated no effect concentration (NOEC). Many similar terms have been used in various contexts for expressing dose effects (Table 1). Various mathematical models have also been suggested to describe data and to calculate cardinal effect concentrations. Both sigmoidal dose-response curve models (40) and Michelis-Menten kinetic models (41,42) have been used. Our experience is that if a sufficient number of concentrations are used, ranging from concentrations with no effect to concentrations causing inhibition, semilogarithmic models for the effect range can be used to calculate various EC values as well as the NOEC. Because often too few concentrations are tested, making it difficult to use mathematical models to calculate EC values, the highest concentration tested showing no effect is used as the NOEC value.

In Fig. 6 the results of a dose-response test to assess the effect of the organic solvent toluene on PDA is shown. In similar dose-response tests, the effects of the fungicide mancozeb was tested on PAO and PDA (35), and the effects of Cr(VI) were tested on seven microbial indicators (42). In all three studies it was concluded that denitrification is a sensitive test.

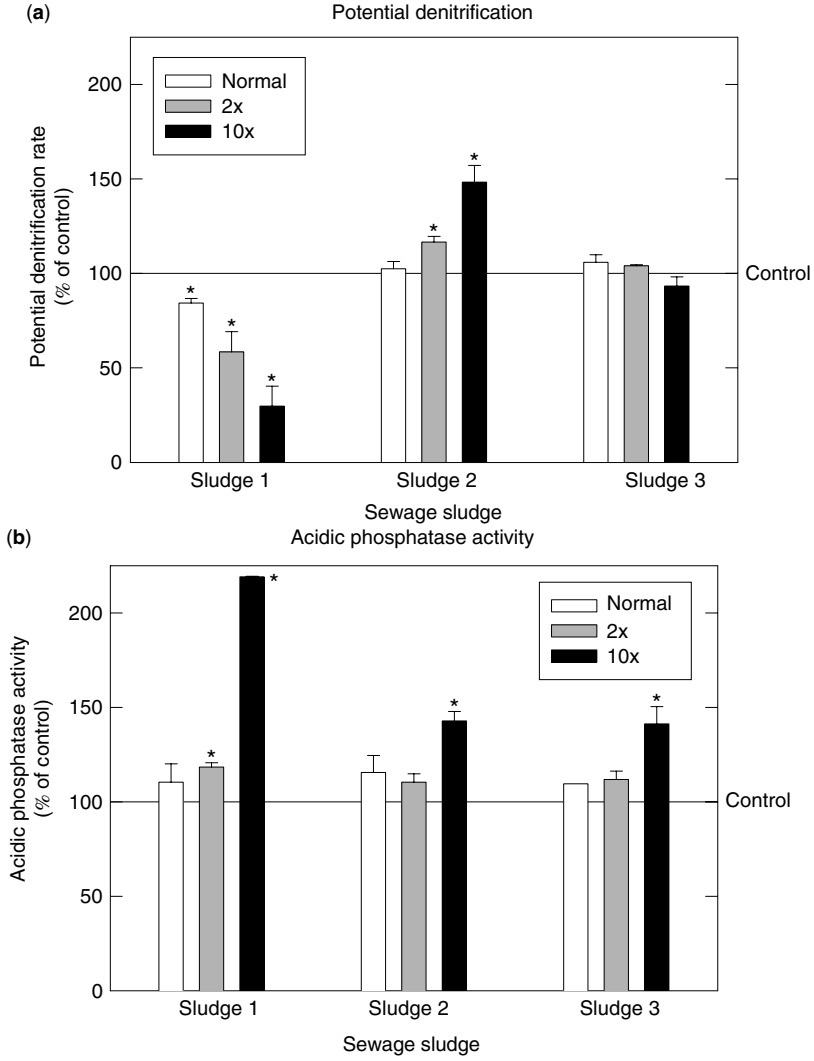

Figure 5. Effect of the addition of three different sewage sludges, at three rates each, on potential denitrification and phosphatase activity in soil (normal rate 4 ton ha^{-1} yr^{-1}, double rate, and 10 times the normal rate). Asterisks indicate significant differences ($p < 0.05$) to control.

Table 1. Commonly Used Definitions and Abbreviations in Expressing Results of Soil Microbial Toxicity Results

Abbreviation	Definition
BEC	Bound Effect Concentration. The effect of the biologically available amount of a chemical or metal.
NOEC	No Observed Effect Concentration. The highest test concentration at which the substance has no observed effect on the test species.
NEC	No Effect Concentration.
PNEC	Predicted No Effect Concentration. Environmental concentration that is regarded as a level below which the balance of probability is that an unacceptable effect will not occur.
PEC	Predicted Environmental Concentration. Estimated environmental concentration based on either measured or calculated data.
LOEC	Lowest Observed Effect Concentration. The lowest test concentration at which a chemical is observed to have a "statistically significant" effect on test organisms.
LEC	Lowest Effect Concentration.
EC_X	Effective Concentration. The concentration which affects X% of a test population after a specified exposure time. The EC_{50} usually relates to effects other than lethality in 50% of the test organisms. The effect concentration may refer to other percentages such as 10%, 90%, e.g., EC_{10} and EC_{90}, etc.

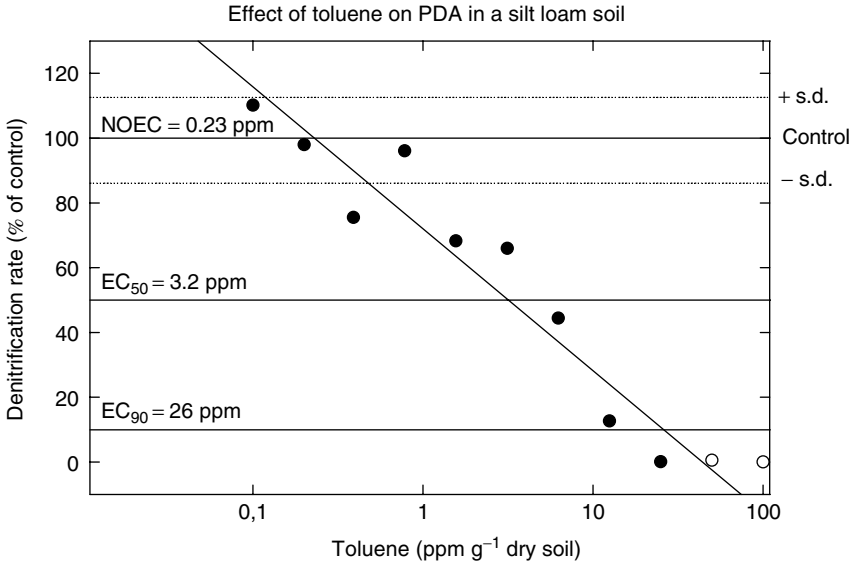

Figure 6. Relative rates of potential denitrification activity (PDA) in soil at different concentrations of toluene. Horizontal lines are mean value ± standard deviation of the control without toluene ($n = 3$). Solid symbols are rates included in the linear regression line from which the dose-response calculations of NOEC, EC_{50}, and EC_{90} were made.

Some Considerations

The data for toxicity evaluations used by environmental protection agencies and authorities to prepare guidelines are based on results derived from a wide variety of laboratory test methods and investigations of field trials or polluted areas. In addition, data are scarce for many chemicals. Reviews of data for pesticides (43) and heavy metals (44,45) have been presented. A consequence of the lack of a standardized test system is that the reliability of the database for different substances varies widely, and hence the quality of the guidelines.

INTERNATIONAL GUIDELINES

The OECD has acted to standardize test protocols and laboratory standards for investigation and evaluation of chemicals. They have published the "OECD Guidelines for Testing of Chemicals" (46) and "Principles of Good Laboratory Practice-GLP" (47). Two guidelines for toxicity testing in soil have been approved: (*1*) Soil Microorganisms, Nitrogen Transformation Test (TG 216, Original Guideline, adapted 21st January 2000), and (*2*) Soil Microorganisms, Carbon Transformation Test (TG 217, Original Guideline, adapted 21st January 2000).

In the OECD nitrogen transformation test, soil is amended with powdered plant meal and either treated with the test substance or left untreated (control). A minimum of two test concentrations is recommended. At the start and after a period of incubation, samples of treated and control soils are extracted with an appropriate solvent, and the quantities of nitrate in the extracts are determined. The rate of nitrate formation in treated samples is compared with the rate in the controls, and the percentage deviation of the treated from the control is calculated. In the carbon transformation test, soil is either treated with the test substance or left untreated (control). After incubation and sampling at the same time intervals as for the nitrogen transformation test, samples of treated and control soils are mixed with glucose, and SIR are measured. Respiration rates are expressed as carbon dioxide released or oxygen consumed. Results from both the nitrogen and carbon transformation tests, with multiple concentrations, are analyzed using a regression model, and the EC_X values with 95% confidence limit are calculated, that is, EC_{50}, EC_{25}, and/or EC_{10}.

Both OECD tests are tests on net soil processes, so include a multitude of microbial transformation processes. Therefore, the test cannot be viewed as very sensitive. If on the other hand, a negative effect is detected then the test substance can be expected to have a serious negative impact on the microbial soil community.

In many countries, national soil toxicity test guidelines similar to those described in the preceding text have been issued, for example, by the U.S. Environmental Protection Agency (U.S.EPA) and the German Biologische Bundesanstalt (BBA). Also, international organizations such as the European and Mediterranean Plant Protection Organization (EPPO) are developing guidelines for the environmental risk assessment of plant protection products (48,49). Another international organization, the International Organization for Standardization (ISO), is acting on standardization of test routines and has also issued standards for soil tests (50,51).

It can be concluded that despite the imminent danger of chemicals to our environment, surprisingly few international test guidelines have been approved. The reason for this is probably conflicting aims of testing between a multitude of potential users. Moreover, the time from proposing a test until its becoming accepted means that the development of new tests is far behind the research front in soil microbiology.

TESTING EFFECTS ON SOIL QUALITY

Taking a soil sample to the laboratory for testing effects of specific substances is relatively easy, but the natural soil ecosystem is exceedingly complex (Fig. 3). To use a soil in an effective but also sustainable way, we must

have an understanding of the ecosystem from which it is possible to establish criteria for normal levels and ranges of microbial activities. Not until such baseline knowledge has been established will it be possible to tell if a soil system is affected by an anthropogenic chemical. In the present situation our task is to collect and systematize information from essential soil indicators, and from this growing database try to describe and understand intrinsic relations in the soil system. This must be done for a range of both undisturbed as well as polluted systems. Two important prerequisites for the success of such a work are good soil sampling design and proper sample handling (2,34).

Integrated Functional Structures Evaluation

The interactions between, and within, the physical, chemical, and biological compartments of the soil ecosystem are numerous. Therefore, a description of the soil and its various functions unavoidably generates large amounts of data. To be able to handle and interpret such large data sets, methods to simplify and sort out the important signals must be used. One approach is multivariate data analysis (52), for example, principal components analysis (PCA) or factor analysis (FA). Such methods can be viewed as variable reduction techniques that facilitate interpretation and understanding of complex relations (53).

PCA has been used for grouping soils into fertility classes based on their characteristics (54), and for determining factors controlling aerobic nitrogen mineralization in different soils (55). By use of PCA, changes were revealed in microbial community patterns 20 years after addition of heavy-metal contaminated sewage sludge (56). Changes in the phospholipid fatty acid composition of bacterial cell membranes and metabolic fingerprints determined by the Biolog system indicated similar effects of the heavy metals. On the other hand, observed changes in seven microbial indicators could not be related to any effect of several heavy metals and xenobiotics in sewage sludge that had been applied for 16 years (57).

The relations of 29 different biological, chemical, and physical variables in two sets of soil data, also representing two scales, was explored by use of PCA (58). The first set consisted of samples taken in a single arable field whereas the second set consisted of bulk soil samples from 26 very diverse agricultural fields scattered all over Sweden. The main conclusion was that despite the very large difference in scales, the data sets displayed more similarities than dissimilarities regarding functional structures. Three main variable groups were found in both data sets. In the first group, organic matter variables were tightly clustered together. The second group consisted of pH dependent variables, whereas the third group consisted of various heterotrophic microbial activities.

MONITORING LONG-TERM CHANGES IN SOIL QUALITY

Another use of microbial soil tests is to monitor changes in soil over the long term. The aim of a monitoring program is to collect information over a long enough period to be able to detect when drastic changes occur or, when progress in reclamation or remediation has been made. The political willingness to undertake the long-term costs for starting and running a monitoring program must be well anchored. Therefore, in reality the number of soil samples as well as the number of indicators must be kept at a minimum. However, too few variables do not generate enough information. Also, a monitor program must have a readiness to respond to unseen future demands.

Soil Indicators of Minimum Data Sets

The use of minimum data sets (MDS) has been proposed to describe soil quality. The indicators included in an MDS should relate to functions such as productivity, water regulation, the soil as an environmental filter, and higher animal health. In addition to several physical and chemical variables, the following biological indicators have been proposed as part of an MDS: microbial biomass (carbon and nitrogen), potential mineralizable nitrogen and soil respiration (5,59). All three variables can indicate the nutrient status and thus crop productivity of the soil. The nitrogen-supplying capacity of a soil is a good predictor of yields within arable systems. In addition, the three microbial variables may indicate the potential for degradation of anthropogenic substances. An MDS including only three biological properties, though manageable, is probably insufficient to define the soil-microbial ecosystem. To increase the information the additional microbial variables PAO and PDA have been suggested (2). As opposed to the other biological variables, PAO is not directly coupled to organic matter and is also sensitive to environmental stress. Apart from being dependent on high-quality organic matter, PDA probes the response of microbes to anaerobic conditions such as created by soil compaction.

Near-Infrared Reflectance (NIR)

Near-infrared reflectance (NIR) spectroscopy has been successfully used to analyze a wide variety of organic products. As NIR offers a high-speed, low-cost, method as well as being a nondestructive analysis, its use has spread to a wide area of applications. When used for analysis of low organic matter soils, a close correlation of NIR spectra with total carbon and total nitrogen have been found (60). NIR together with multivariate regression has also been used to model both basal respiration and SIR in forest soils (61). In our own work, NIR spectra modeled by PCA showed close correlations not only with organic carbon, magnesium and pH but also with acidic phosphatase activity and PDA. Moreover, NIR has been demonstrated to predict crop uptake of nitrogen (62). It is not likely that NIR directly measures biological activities, but rather gives an integrated index through its correlation with soil properties that can be measured by NIR.

NIR seems to be a suitable method for presurveying potential locations for a soil-monitoring network. On the basis of the results, a stratified sampling design can be worked out. NIR should also be included in the actual analysis program because it is able to capture, directly or indirectly, important signals from the biological, physical, as well as chemical compartments of soil.

Monitoring Programs

A national benchmark program was established in Canada with 23 sites to monitor changes in agricultural soil quality resulting from land use and management practice (63). Besides collecting extended baseline data sets of various soil properties, the program includes collection of continuous monitoring data at intervals ranging from daily to every 5 or 10 years, depending on their sensitivity. In France, a similar program has been set up with 11 sites (64). The objective of the program is to detect early changes in soil quality and to identify their causes. Neither of these programs includes biological soil indicators, which is probably due to lack of a standardized methodology combined with the perception that biological soil indicators are unpredictable. However, the importance of including microbiological soil indicators in soil monitoring has been stressed (2). The German Special Working Group on "Basic Information for Soil Conservation" proposed the following microbial indicators to be analyzed in a system of permanent soil monitoring sites: microbial biomass, basal respiration, and the enzyme activity of protease, catalase, glucosidase, and arginine ammonification. In a project commissioned by the Swedish Environmental Protection Agency, the inclusion of microbial indicators in a future Swedish soil-monitoring program is recommended (2).

IMPORTANT REMARKS ON SOIL TESTING

A strategy for soil testing is that the test criteria used should guarantee the protection of the structure and function of the soil ecosystem (65). Ideally, adequate data for the effects of a toxic compound should be obtained in the ecosystem of concern. As not all organisms and functions in an ecosystem can be tested against all possible compounds and combinations, extrapolations are often made from simple laboratory tests to the ecosystem. Moreover, in addition to the vast number of nontested substances, a great number of methodological problems and difficulties in interpretation of data remain to be dealt with. Not until many of these problems have been solved can an overall strategy for soil toxicity testing and soil quality testing be laid out. Some important problems and suggested strategies to overcome them are considered in the following.

Problems

Choice of Test Soil. Different physicochemical soil conditions change the bioavailability of compounds. For example, the availability and toxicity of most metals increase with reduced pH and organic matter content, whereas for selected metals, for example, Mo, the solubility decreases with decreasing pH. This means that physical and chemical data for substances to be tested are important when choosing a soil for an individual test program. If physicochemical data are not available, soils with "extreme" characteristics, for instance, low and high pH, are the best choice. The test results form a reasonable basis for determining if a compound constitutes no risk or requires further testing.

Combination of Tests. The sensitivity of different tests, that is, end points, is not the same for all organisms and compounds. Depending on the end point chosen, more sensitive parameters, and hence lower effect-levels, may be neglected. It is not obvious whether sensitive tests or tests with a coarse resolution are the best general choice. The best strategy is to combine several methods, for example, respiration or biomass, and nitrification tests.

Addition of Test Substance. The common practice of using carrier solvents or the addition of soluble salts, often just before the start of the test, may lead to a greater bioavailability of the pollutants in laboratory experiments than in the field situation. Under field conditions, part of the pollutant becomes not only sorbed and unavailable, but may also be transformed to other forms. In laboratory studies, after mixing the dissolved chemical into the soil, the solvent is allowed to evaporate before start of the assay. This procedure is questionable because many organic solvents themselves have been proven to affect the microorganisms and their activity. Alternatively, the test substance can first be mixed into an inert carrier such as sand and then blended with the soil. A third way is to use an ultrasonic bath to dissolve and distribute as much as possible in an aqueous suspension. Eventually, mild leaching with water should be included to rid the soil of water-soluble remains immediately after adding the test substance. In subsequent dose-response tests, a leached and unleached soil should be tested in parallel.

Test Level. Laboratory-derived NOEC and LOEC values do not necessarily represent "true" no-effect concentration (NEC) or lowest effect concentration (LEC) values (Table 1). An alternative approach for the future, as pointed out by Hoekstra and van Ewijk (66), is the use of bound effect concentrations (BEC). Furthermore, it is difficult to assess if protection of the functioning of an ecosystem is required that all species are protected, or if some loss is accepted. Structure and function of an ecosystem are often uncoupled and thus toxicity data for single species cannot be used to predict safe levels for ecosystems with a great level of safety. Thus, the obvious strategy must be to test at the level of ecosystem, community, or population, rather than at the level of species or individual, although the recent rapid development in molecular biology allows for such studies.

Evaluation and Interpretation. The normal situation today is that toxic compounds are discharged or present in the environment in mixtures. Therefore, more attention must be paid to interactive or synergistic effects among compounds. The strategy must be, in addition to making field observations, to design laboratory tests to study some commonly found mixtures. Microbial tests might even be used instead of some of the very complicated and expensive chemical analyses of organic environmental chemicals.

In soil toxicity testing, sometimes the tested chemical is observed to have a stimulation effect on microorganisms. Stimulatory effects are a difficult phenomenon to deal with, as stimulation does not always mean a positive effect, but can be a stress response of the microorganisms.

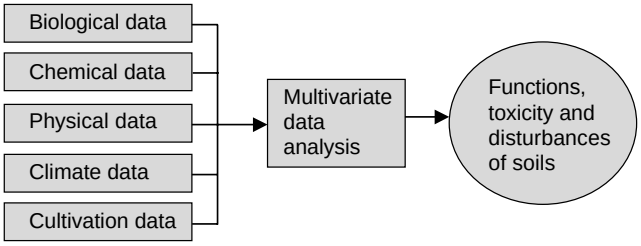

Figure 7. Strategy for integrated evaluation of data leading to a picture of the function of the soil or an indication of the potential outcome of disturbances in the soil.

Another explanation of stimulatory effects is that resistant organotrophic bacteria proliferate on the biomass of the organisms killed by the compound. Therefore, one must be cautious when such observations are made, and not automatically interpret increased levels of microbial activity as benign. Instead, stimulatory effect should stimulate further investigation on possible negative effects.

Loss or lowering of a microbial activity can be temporary and does not necessarily mean that the genetic base of the microbial community has been lost or diminished greatly. However, some genetic capacities will undoubtedly be lost owing to anthropogenic activities. The consequences of such losses are difficult to foresee, the only thing known is that there is no way back. Thus, future recommendations for toxicity testing in soil must include genetic diversity testing as an integral part of a test system.

Soil Quality Testing. It is obvious that no single measurement of a soil factor, whether it be biological, chemical, or physical, will give a comprehensive picture of the quality of a specific soil (1). Because of the complexity of the soil (Fig. 3), evaluation of a single parameter provides only a small part of the answer as to whether the soil has a low or high quality status. Therefore, a strategy for integrated evaluation of a soil quality is needed. A number of indicators that reflect biological, chemical and physical components of a soil would be used in such a strategy. Climatic conditions as well as data concerning cultivation measures, previous and present, would also be included. Appropriate statistical tools are needed to evaluate the complex data sets associated with these variables. Principal component analysis (PCA), a multivariate statistical analysis, is such a tool (Fig. 7).

CONCLUSION

The vast number of chemicals used in society today urge for simple and rapid tests to assess toxic effects on the soil ecosystem. Several microbial methods for soil toxicity testing and soil quality testing exist today. Surprisingly, few of these tests have, though, reached the level of becoming international standards. By using such tests for screening purposes and dose-response testing it is possible to identify chemicals, and concentrations of these chemicals, that could be allowed without seriously threatening the productivity and sustainable use of soils.

A general conclusion, however, is that we have a long way to go before acceptable safety levels can be defined for our soil ecosystems. Much work remains before we will be able to detect and understand the meaning of anthropogenically induced changes in microbial functions. At present, not even generally accepted simple test programs for microbial soil toxicity exist.

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TOXICITY TESTING IN WASTEWATER TREATMENT PLANTS USING MICROORGANISMS

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INTRODUCTION

Process waters released by industry must be treated in a wastewater treatment prior to discharge into receiving waters. Traditionally, discharge limits were focused on biochemical oxygen demand (BOD) and suspended solids concentrations. Nowadays, there is a great concern over the impact of toxic industrial pollutants on wastewater treatment processes and on receiving waters. To avoid damage to the environment, two approaches can be taken to control industrial discharges (1):

1. Biomonitoring of the mixing zone where the effluent mixes with the recipient water. Biological surveys or biosurveys can be used to assess the impact of toxic chemicals on the resident aquatic communities and require the use of a reference site. These surveys are, however, expensive, and the results obtained are difficult to interpret. Some believe that they have a limited value as regards the evaluation of the impact of effluent discharges (2).
2. Another approach is the use of whole effluent toxicity (WET). The advantages of toxicity testing of complex effluents are (1):

- There may be numerous toxicants in complex effluents. Even sophisticated chemical analyses detect only a fraction of these chemicals.
- Toxicity assays give an indication of the deleterious effects of chemicals and encompass antagonistic or synergistic interactions between chemicals.
- Toxicity testing aided by fractionation can help identify the toxic chemical within the complex effluent.

There are regulations concerning the discharge of effluents into receiving waters in many industrialized countries such as the United States, the United Kingdom, France, Japan, and Germany (3). For example, in the United States, effluent discharge to surface waters is enforced under the National Pollutant Discharge Elimination System (NPDES) of the Clean Water Act and is administered by the U.S. Environmental Protection Agency (EPA), which may delegate its authority to state agencies. Under the NPDES process, permits are issued to dischargers, who must comply with the industry sector minimum standards. NPDES sets two types of performance criteria (3,4): (*1*) *Technology-based criteria*, which deal with the ability of a wastewater treatment plant to remove specific pollutants; (*2*) *Water quality-based criteria*, which specify that a wastewater treatment plant must not discharge effluents "in toxic amounts" into receiving waters. Toxicity must be reduced sufficiently to a level that is not deleterious to the biota in the receiving water.

The main criteria for an ideal toxicity test are ecological relevance, reproducibility, rapidity, simplicity, and low cost. However, no test fulfills all of these criteria. For example, microcosms are ecologically relevant but do not have a good reproducibility or low cost. It is thus recommended to use a battery of tests that covers several trophic levels. For example, in Germany, an effluent biotesting battery includes a fish test, a 24-hour *Daphnia* test, an algal assay using *Scenedesmus subspicatus*, and a bioluminescence inhibition assay using a marine bacterium (1).

The goals of toxicity testing in wastewater treatment plants are:

1. to test any deleterious effect toxic chemicals in wastewater may have on the wastewater microorganisms, leading to a reduction in treatment efficiency. These screening tests should also be useful for indicating the source of the toxicants entering the wastewater treatment plant.
2. to assess from a toxicity standpoint the removal of organic and inorganic toxicants following their passage through a wastewater treatment plant.
3. to assess the toxicity of effluents and their impact on receiving waters.
4. if the effluents are toxic, there may be a need to perform a toxicity reduction evaluation (TRE) to identify the problem toxic chemical(s).

The most common test organisms used in biological monitoring are fathead minnow (*Pimephales promelas*) and daphnid (*Daphnia magna, Ceriodaphnia dubia*) toxicity tests. We will, however, focus on microbial- and enzyme-based toxicity tests and examine their applications to wastewater treatment.

SURVEY OF MICROBIOTESTS FOR MONITORING TOXICITY IN WASTEWATER TREATMENT PLANTS

Microbiotests, also called microscale toxicity tests, are based on the inhibition of the activity of enzymes, bacteria, algae, fungi, and protozoa (5–10). The microbiotests (microbe- and enzyme-based tests) most suitable for toxicity testing in wastewater treatment plants are shown in Figures 1 and 2. These tests are simple, rapid, and relatively inexpensive; require low sample volume; can be miniaturized; and can be made portable for field applications. The advantages of microbiotests are further displayed in Table 1 (7). We now describe these toxicity tests and discuss their application to municipal and industrial wastewater treatment plants.

Enzyme Assays

Enzymes serve as catalysts of biological reactions in animal, plant, and microbial cells. Toxicity tests based on the

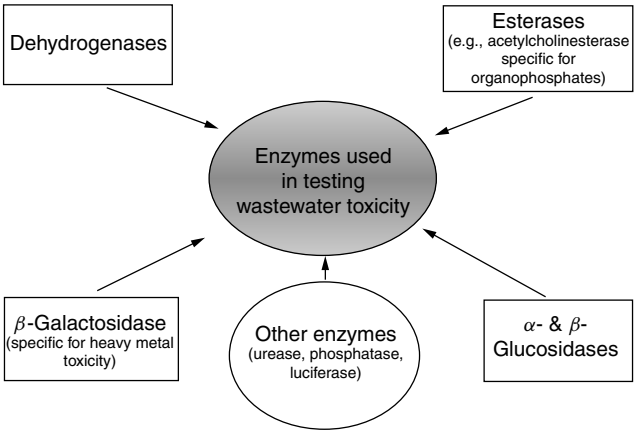

Figure 1. Enzymatic tests used in the determination of wastewater toxicity.

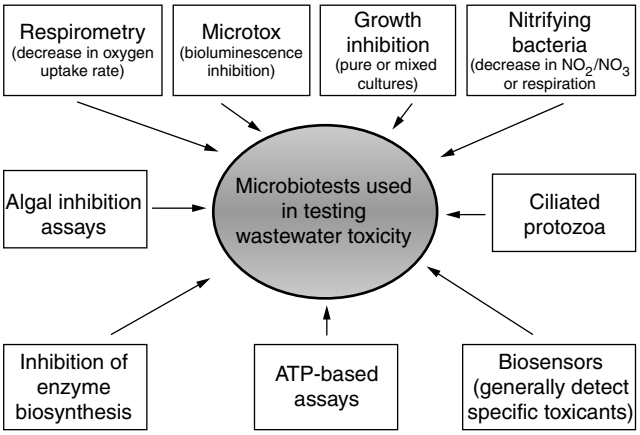

Figure 2. Microbiotests used in the determination of wastewater toxicity.

Table 1. Some Features of Microbiotests

Feature	Explanatory Remark
Inexpensive *or* cost efficient	Cost is test-dependent and can vary from a few dollars to several hundred dollars in Canadian currency
Generally not labor-intensive	As opposed to steps involved in undertaking fish bioassays, for example
High sample throughput potential	When automation technology can be applied
Cultures easily maintained or maintenance free	Freeze-drying technology can be applied
Modest laboratory and incubation space requirement	As opposed to a specialized laboratory essential for fish bioassays, for example
Insignificant post-experimental chores	Owing to disposable plastic ware, which is recycled instead of having to be washed for reuse, as in the case of large experimental vessels
Low sample volume requirements	Often, a few milliliters suffice to initiate tests instead of liters
Sensitive/rapid responses to toxicants	Short life cycles of microorganisms enable end point measurements after just minutes or several hours of exposure to toxic chemicals
Precise/reproducible responses	High number of assayed organisms, increased number of replicates, and error-free robotic technology are contributors to this feature
Surrogate testing potential	Microbiotests are adequate substitutes for macrobiotests in some cases
Portability	Cases where microbiotests are conveniently amenable to being applied in the field

Source: From Blaise, 1991; with permission from the publisher.

inhibition of the activity of a wide range of enzymes have been suggested for assessing chemical toxicity in aquatic environments, including wastewater treatment plants. The enzymes include dehydrogenases, ATPases, esterases (particularly acetylcholinesterase), phosphatases, urease, luciferase, β-galactosidase, protease, amylase, and α- and β-glucosidase (11,12). Some of these enzymes are specific to particular toxicants. Hence, acetylcholinesterase is specifically inhibited by organophosphates and carbamate pesticides and has been used to monitor surface waters for these chemicals. β-galactosidase and urease are relatively insensitive to organic toxicants and respond mostly to heavy metals (19,21).

Dehydrogenases have been frequently utilized as target enzymes in toxicity testing in wastewater treatment plants. These enzymes are assayed by using specific oxidoreduction dyes such as triphenyl tetrazolium chloride (TTC), 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT), nitroblue tetrazolium (NBT), 3-4,5-dimethyl thiazole-2-yl 2,5-diphenyl tetrazolium bromide (MTT), or resazurin (11). These oxidoreduction dyes are routinely used to determine microbial activity in surface waters, soils, sediments, and other environmental samples. Lehnard (13) was one of the first to demonstrate the usefulness of dehydrogenase activity (DHA) as a basis for assessing the effect of toxicants on biological treatment of wastewater. An INT reduction assay was used to measure the activity of filamentous bacteria in activated sludge following exposure to chlorine and hydrogen peroxide (14,15) (see FILAMENTOUS BULKING IN ACTIVATED SLUDGE, CONTROL OF and ACTIVATED SLUDGE — FOAMING). Botsford (16) proposed a toxicity test based on the inhibition of the reduction of MTT by *Rhizobium meliloti* used as the indicator organism. A resazurin reduction assay was considered for measuring mercury toxicity in activated sludge (17). DHA-based toxicity tests are often well correlated with those based on oxygen uptake in wastewater systems.

In vivo microbial enzyme activity has been measured in the flow stream of bioreactors containing a fixed bed of immobilized microorganisms. The test water is pumped through the bioreactor, and enzyme activity is determined by injecting a synthetic substrate through the reactor (12,18). This semi–on-line system should be potentially useful for the determination of toxicant impact on microorganisms in water and wastewater treatment plants.

Several enzymes (α-glucosidase, phosphatase, acetylcholinesterase, β-galactosidase, urease) were screened for their sensitivity to heavy metals and organic toxicants. β-galactosidase and urease were found to be the most sensitive to heavy metals but were not affected by organic toxicants. This led to the idea of developing microbial kits for the specific detection of heavy metal toxicity. A urease toxicity assay (UTA) was developed and was found to be quite sensitive to metals (19) but did not respond to organic toxicants. However, this test performed poorly as regards wastewater effluents. Since ammonia is the end product of urease activity, the high levels of ammonia in wastewater and other environmental samples interfere with the assay. An attempt was made to immobilize urease to avoid the ammonia interference. However, immobilized urease generally is less sensitive to metals than free urease (19).

A crucial finding was the selective response of β-galactosidase activity to toxic chemicals. This enzyme was relatively insensitive to organic toxicants while being sensitive to cationic metals (20) and provided the basis for the development of a toxicity test that is selective

for heavy metals. Subsequently, two kits, MetPAD™ (21) and MetPLATE™ (22), were developed for assessing metal toxicity in environmental samples. Both toxicity test kits are based on the specific inhibition of the activity of β-galactosidase in an *E. coli* strain by heavy metals. MetPAD™ is a semiquantitative test with the enzyme assay performed on a supplied pad, whereas MetPLATE™ is a quantitative test, allowing EC_{50} determination in a 96-well microtitration plate. These tests were used to determine metal toxicity in surface waters, wastewater effluents, landfill leachates, ashes, soils, sediments, biosolids, and metal-hyperaccumulating plants (23). Heavy metal toxicity of wastewater effluents was assessed using MetPAD™ assays. Five of eight samples displayed toxicity related to heavy metals, which were also detected with chemical analysis (21). Sample toxicity was removed following passage through a cation exchange resin, confirming that the test was indeed specific for heavy metals (24). MetPLATE™ was also useful in detecting metal toxicity in industrial effluents (22).

Microbial Assays

Microtox

Microtox is based on the inhibition of a bioluminescent marine bacterium, *Vibrio fischeri* (formerly known as *Photobacterium phosphoreum*) by toxic chemicals (25–27). Microtox is a 5- to 15-minute bacterial test that can be extended to 60 minutes. It has been used to test the toxicity of thousands of pure chemicals as well as complex domestic wastewater effluents, industrial effluents, fossil fuel process water, surface waters, stormwater, groundwater, and solids which include among others, soils, biosolids, solid wastes, and sediments. It has also been used to test the interactions between chemicals. Several countries (e.g., Canada, France, Germany, Italy, Mexico, Spain, Sweden, the United States) have adopted Microtox for screening toxicity of environmental samples, including wastewater effluents. Comparative studies of Microtox with other toxicity tests (fathead minnow, rainbow trout, daphnids) showed a correlation coefficient r varying from 0.4 to more than 0.9 (27). Conflicting results were obtained when comparisons were made to examine the relationship between toxicity data obtained through Microtox and *Ceriodaphnia dubia* tests. Some reported that *C. dubia* was much more sensitive than Microtox to wastewater effluent toxicity (28,29), whereas others (30–32) suggest that Microtox can serve as a surrogate test for the determination of wastewater toxicity. Sherry and coworkers (33) used a battery of toxicity tests to assess the acute and chronic effects of refinery effluents. Only Microtox and a submitochondrial particle test detected acute toxicity in these refinery. Others have found that Microtox was much less sensitive to a refinery effluent than rat liver mitochondria (34). Microtox was also used to evaluate the toxicity of effluents from the textile, chemical, pharmaceutical, tank cleaning, forest products, food production, and olive oil industries (35–37). Brown and coworkers (38) used a luminescence-based *Ps. fluorescens* assay (*lux* gene introduced into *Ps. fluorescens 10586s/pUCD607*) to monitor the toxicity of a paper mill effluent. The toxicity test based on bioluminescence inhibition was more sensitive than a test based on the respiration of the same bacterial strain (39,40). In a limited number of tests, this bacterium was found to be more sensitive to Cd than Microtox. Inhibition of other bioluminescent bacterial species (*Vibrio harveyi, Photobacterium leiognathi*) was used as an end point in testing industrial wastewater (41).

There have been several developments concerning the use of bioluminescence inhibition or induction as the end point. Mutatox® is a genotoxicity assay based on the induction of bioluminescence in the marine bacterium *Photobacterium leignathi* in response to the presence of DNA-damaging agents (42,43). Another development is the 22-h Microtox chronic toxicity test (44), which is based on growth inhibition of *Vibrio fisheri*. Following testing of 14 wastewater treatment plant effluents, Sweet and coworkers (45) reported a good correlation between chronic Microtox and *Ceriodaphnia dubia* bioassays.

ATP-Based Assays

ATP is the primary high-energy molecule that is formed following catabolic reactions such as oxidative phosphorylation in heterotrophs, photophosphorylation in phototrophs, or oxidation of inorganic chemicals by chemotrophs. It is a good indicator of viability of microbial, plant, and animal cells. ATP assay is relatively simple and consists of measuring light emission, using a luminometer, following reaction of cell ATP with firefly luciferin. The reaction is catalyzed by the luciferase enzyme (11). ATP assays have been used to measure live biomass in environmental samples and in engineered systems such as water and wastewater treatment plants. Some 30 years ago, investigators considered ATP assays for assessing the operating conditions in wastewater treatment plants (46–48), and this has led to their use in toxicity testing. Thus, an ATP-based toxicity assay is quite useful in monitoring the impact of industrial discharges on wastewater treatment plants. Bitton and Koopman (11) suggested the necessity to monitor the potential effect of toxicants on the firefly luciferase itself. This has led to the development of the ATP-TOX system, which is a 5-hour toxicity test that is based on inhibition of both ATP (growth inhibition) and the enzyme luciferase that drives the production of bioluminescence in some marine bacteria such as *Vibrio fisheri* (49,50). Several bacterial strains were examined for their sensitivity to toxicants. *E. coli* PQ37 was found to be the best suited for the ATP-TOX system. This test was found to be about as sensitive to metals as Microtox but was clearly less sensitive to organic toxicants. The test detected toxicity in field samples of river water and sediments in Canada (51,52), but no studies are available on its application to wastewater samples.

Growth Inhibition Assays

Assays based on growth inhibition have long been used by microbiologists to assess the sensitivity of

microorganisms to antibiotics. Growth inhibition assays are quite appropriate for determining the impact of industrial discharges on pure bacterial cultures (e.g., *Pseudomonas fluorescens*) or mixed cultures of wastewater microorganisms. These assays consist of measuring the changes in microbial numbers caused by the presence of a given toxicant generally within a 24-hour incubation period. These changes are often measured with a spectrophotometer or by measuring ATP as performed in the ATP-TOX assay discussed earlier (50). Recently, a 22-hour Microtox assay based on the growth inhibition of *Vibrio fisheri* was proposed as a chronic toxicity test (44). The 96-hour algal toxicity test is also considered a chronic toxicity test (see algal assays section).

Assays Based on De Novo Enzyme Biosynthesis in Bacteria

Toxic chemicals can also decrease the de novo biosynthesis of inducible enzymes in bacteria. A commercial toxicity assay, based on the inhibitory effect of chemicals on β-galactosidase biosynthesis, has been developed and marketed as Toxi-Chromotest (53). This test was successfully applied to wastewater treatment plants and revealed a decrease in toxicity in a large plant in Jacksonville, Florida (54). It was, however, less sensitive than assays with *Daphnia pulex, Ceriodaphnia dubia*, and Microtox (54,55). A monitoring program for nuclear and hydroelectric facilities in Ontario, Canada, has shown the low sensitivity of Toxi-Chromotest, which displayed a poor correlation with the *D. magna* acute test (56).

The effect of toxicants on the de novo biosynthesis of other inducible enzymes (tryptophanase in *E. coli* and α-glucosidase in *Bacillus licheniformis*) was also investigated (57,58).

Bacterial Biosensors

Another advantage of using bacteria as test microorganisms in toxicity monitoring is their amenability to genetic manipulations, which would allow their use for targeting specific toxic chemicals. The construction of a bacterial biosensor involves the inclusion of both sensing and reporter elements. There is a wide variety of sensing elements that detect different classes of toxicants or stresses. The most convenient reporter system used in bacterial biosensors are the *lux* genes from *Vibrio fischeri* (59). The *lux* system contains seven genes, five of which are generally used in the reporter system. *Lux A* and *lux B* code for the enzyme luciferase, whereas *lux C, D*, and *E* code for an aldehyde (60).

Target compounds detected by bacterial biosensors include heavy metals (61–64) and hydrophobic organic compounds (65,66). Bacterial biosensors using *Alcaligenes eutrophus* and designed to specifically detect the bioavailability of individual or mixtures of heavy metals contain a reporter gene (*luxCDABE*) under the control of genes involved in bacterial resistance to metals (61,62). An *E. coli arsB: luxAB* luciferase gene fusion strain has been used for the specific detection of arsenic and responded to the presence of chromated copper arsenate traditionally used for wood preservation. This biosensor had a detection limit of 10 μg/L arsenic (67).

Others have constructed *E. coli*–based biosensors, which are less specific but are able to detect different categories of stresses, including heat shock, oxidative (peroxide and oxygen radicals), or DNA-damaging stresses (68,69). The use of a panel of five of such *E. coli* constructs showed oxidative (oxygen radicals) and DNA-damaging stresses in industrial wastewater (59).

Nitrifying Bacteria

In the nitrogen cycle, nitrification probably is the process most sensitive to the deleterious effect of toxicants. Nitrification is driven by chemoautotrophic bacteria such as *Nitrosomonas*, which oxidizes NH_4 to NO_2, and *Nitrobacter*, which oxidizes NO_2 to NO_3. Both bacteria are sensitive to a wide range of chemicals but, although *Nitrosomonas* is more sensitive to toxicants than *Nitrobacter*, the latter was the most used in toxicity testing. Toxicity tests are needed to monitor the risk of nitrification inhibition in wastewater treatment plants in order to meet standards for ammonia discharge (see ACTIVATED SLUDGE — MICROBIOLOGY OF NITROGEN REMOVAL). These tests are based on measuring the rate of formation of NO_2 and/or NO_3, NH_4 removal rate, or respiration inhibition.

Gernaey and coworkers (70) used a rapid (14-minute) test based on respiration inhibition of a nitrifying sludge culture. The test was quite sensitive to organic toxicants (EC_{50} of 2.67 mg/L for phenol and 0.51 mg/L for 3,5-dichlorophenol) but not as sensitive to metals (EC_{50} of 173 mg/L for Cu^{2+} and 8.3 mg/L for Cd^{2+}). Due to its relatively short response time, this method is amenable to on-line operation in a treatment plant. Others have considered fixed-film nitrifying bioreactors to measure respiration inhibition by toxicants (71). Hayes and coworkers (72) developed Amtox^{TM}, an on-line monitor based on inhibition of cultures of nitrifying bacteria immobilized in a polyvinyl alcohol (PVA) matrix. The bacterial monitor is preceded by a carbon removal stage. Amtox^{TM} was used in a large wastewater treatment plant and revealed low chronic inhibition followed by peaks of acute inhibition caused by the incoming wastewater.

Nitrobacter was also used in the toxicity identification evaluation (TIE) of wastewater from a forest product processing industry. Resin acids and unsaturated fatty acids were shown to be responsible for the observed toxicity (73).

Algal Toxicity Assays

Algal assays are generally conducted as flask or 96-well microplate assays. Comparative studies have shown that the results obtained with both systems are comparable. Microplate-based toxicity tests using microalgae (e.g., *Selenastrum, Chlamydomonas, Scenedesmus, Chlorella, Anabaena*) as test organisms have been proposed as cost-effective and relatively simple assays for toxicity testing (74,75). These tests generally are based on inhibition of algal growth or ATP content and sometimes inhibition of esterase activity (76). The tests can be carried out with

unialgal cultures or with a battery of algal cultures to obtain more ecotoxicological information (32,77). To avoid culture maintenance, some have proposed to cryopreserve the algal cells (78). An Algaltoxkit is now being marketed to facilitate the use of algal assays by wastewater treatment plants operators and others interested in environmental monitoring for toxic substances. The kit is also based on inhibition of algal growth, as measured spectrophotometrically at 670 nm. The algal seed is provided as cells immobilized in alginate, which eliminate the need to maintain algal cultures in the laboratory (79,80).

Algal tests have been recommended as standard procedures for determining the toxicity of environmental samples in several countries. They also have been used for the determination of toxicity in domestic and industrial wastewaters (32,80–83) and sanitary landfill leachates (84). Algal toxicity assays are discussed in more details by Blaise (this encyclopedia).

Ciliated Protozoa

Ciliated protozoa are unicellular protista that are widely distributed in the environment and play a crucial role in ecological food chains. They serve as a link between the microbial and the zooplankton communities. Ciliates found in activated sludge plants (see PROTOZOA IN ACTIVATED SLUDGE) are quite sensitive to metals such as Cd, Cu, and Hg (85). Protozoan grazing on bacteria (i.e., bacterivory) is an important process implicated in the treatment of domestic wastewater. Such grazing has been shown to be significantly reduced in the presence of toxicants (e.g., heavy metals). For example, *Aspidisca costata* grazing on bacteria in activated sludge is reduced in the presence of cadmium (86). The wide distribution of protozoa and their significant role in food chains make them desirable test organisms to be used in toxicity testing in the environment. The ciliate genera most used in toxicity tests are *Tetrahymena, Paramecium, Colpidium, Colpoda*, and *Euplotes*, and the general parameters selected most to evaluate the response of ciliates to toxicants are growth inhibition, survival, respiration, ingestion rates, and chemoattraction inhibition tests (87). A relatively recent toxicity test is based on measuring the impact of toxicants on the rate of ingestion of fluorescent latex microspheres by ciliates (88). However, this test necessitates the use of relatively expensive beads and a fluorescent microscope. Another rapid test is the inhibition of the chemotactic response of ciliates to yeast extract, which serves as an attractant. This test helped evaluate the toxicity of an effluent from the metal-plating industry but did not perform as well with a paper mill effluent because the ciliates were found to be attracted to it (89,90).

Ciliates are ecologically important organisms, and their response to toxicants in complex effluents deserve to be evaluated in a battery of toxicity tests. However, there is a need for standardized procedures regarding their use as test organisms in toxicity testing.

Respirometry

The rate of oxygen uptake by microorganisms is a useful parameter used to measure the acute effect of toxic chemicals and other insults to microorganisms (91). The earliest approach for measuring respiration rates in wastewater is via manometry (e.g., Warburg-type manometer). Electrolytic respirometers avoid, however, the drawbacks of manometers (e.g., oxygen limitation) by including reoxygenation by electrolysis. Dissolved oxygen can also be directly measured via titrimetric methods, oxygen electrodes that are sometimes incorporated in flow-through devices, or electrodes incorporating immobilized microorganisms. Biosensors using pure microbial cultures (e.g., *Bacillus subtilis, Clostridium butyricum, Trichosporon cutaneum*) or mixtures of activated sludge microorganisms have all been considered for biochemical oxygen demand (BOD) estimation in wastewater.

Toxicity tests based on respiration inhibition generally are carried out over time periods ranging from minutes to days in the presence of substrates such as wastewater, synthetic wastewater, or pure organic compounds (e.g., glucose, amino acids). The test biological component can be a pure culture of bacteria or mixed cultures as found in wastewater and activated sludge. Mixed cultures are used in the BOD inhibition method. The method varies according to the type of information needed to be drawn from the respiration inhibition test. On-line measurement of oxygen uptake can be useful for detecting toxic shocks in activated sludge systems (92). Rodtox (Rapid Oxygen Demand and TOXicity), an on-line device that measures the endogenous and exogenous respiration rate of microorganisms in an activated sludge sample taken from the plant under investigation, was useful in pinpointing the source of toxic input to the Antwerp, Belgium, wastewater treatment plant (93).

CONCLUSION

Microorganisms are suitable alternate or complementary tools for carrying out toxicity testing in environmental samples. They offer numerous advantages (see Table 1) that make them attractive for toxicity assessment in wastewater and effluents from municipal and industrial treatment plants. We have seen that several approaches have been taken to achieve this goal. The nine types of microbiotests described in this review can give a reasonable account of the impact of toxic chemicals on wastewater treatment plants and receiving waters. Biosensors are not "typical" toxicity tests but include a biological component that indicates the presence of a given toxicant in the sample. Enzymes, especially the dehydrogenases, have also been considered in assessing wastewater toxicity. Some of the enzymes respond to selective categories of toxicants. An example is the inhibition of β-galactosidase activity in MetPAD/MetPLATE kits that selectively detect heavy metal toxicity.

A useful application of microbiotests is their use in toxicity identification evaluation (TIE) and toxicity reduction evaluation (TRE) proposed by the U.S. EPA (94,95). TIE and TRE entail a series of tests that determine the source of effluent toxicity, the specific causative toxicant(s), and the effectiveness of pollution control measures to reduce effluent toxicity. These fractionation schemes generate more than 50 fractions per sample, which can be conveniently and rapidly tested using microbiotests.

A battery-of-tests approach has been suggested because no single microbiotest can detect all the categories of environmental toxicants with equal sensitivity. Some of the microbial toxicity tests (e.g., Microtox, Toxi-Chromotest, MetPAD/MetPLATE) are now commercially available and are utilized in wastewater treatments facilities by environmental toxicologists.

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TOXIGENIC MOLDS.

See AIRBORNE TOXIGENIC MOLDS

TOXINS, MICROBIAL.

See BIOTERRORISM

TOXOPLASMA GONDII

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Infection with the protozoan parasite *Toxoplasma gondii* is one of the most common parasitic infections afflicting man and other warm-blooded animals (1). It has been found worldwide from Alaska to Australia. Nearly one-third of humanity has been exposed to this parasite (1). In most adults, it does not cause serious illness, but it can cause blindness and mental retardation in congenitally infected children and devastating disease in those with depressed immunity. It is a common cause of abortion in goats and sheep.

CLASSIFICATION

T. gondii is a coccidian parasite with cats as the definitive host, and warm-blooded animals as intermediate hosts (2). It is among the most important of parasites of animals. It belongs to:

Phylum: Apicomplexa; Levine, 1970
Class: Sporozoasida; Leukart, 1879
Subclass: Coccidiasina; Leukart, 1879
Order: Eimeriorina; Leger, 1911
Family: Toxoplasmatidae, Biocca, 1956

There is only one species of *Toxoplasma*, *T. gondii*.

Coccidia, in general, have complicated life cycles. Most coccidia are host-specific, and transmitted by a fecal-oral cycle. *Toxoplasma gondii* has adapted transmission by a fecal-oral cycle, by carnivorism, and transplacentally (1,2).

STRUCTURE AND LIFE CYCLE

The name *Toxoplasma* (toxon = arc, plasma = form) is derived from the crescent shape of the tachyzoite stage (Fig. 1). There are three infectious stages of *T. gondii* namely, the tachyzoites (in groups), the bradyzoites (in tissue cysts), and the sporozoites (in oocysts).

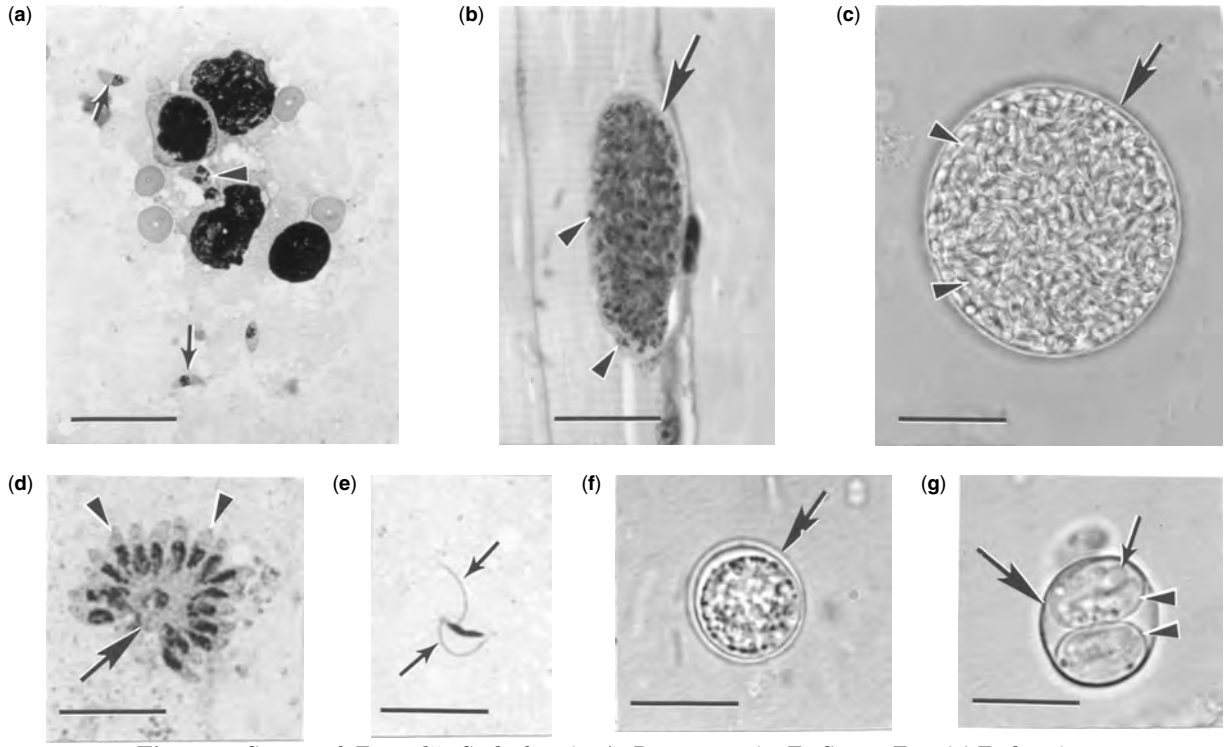

Figure 1. Stages of *T. gondii*. Scale bar in A–D = 20 μm, in E–G = 10 Fm. (**a**) Tachyzoites in impression smear of lung. Note crescent-shaped individual tachyzoites (arrows) dividing tachyzoites (arrowheads) compared with size of host red blood cells and leukocytes. Giema stain. (**b**) Tissue cysts in section of muscle. The tissue cyst wall is very thin (arrow) and encloses many tiny bradyzoites (arrowheads). Hematoxylin and eosin stain. (**c**) Tissue cyst separated from host tissue by homogenization of infected brain. Note tissue cyst wall (arrow) and hundreds of bradyzoites (arrowheads). Unstained. (**d**) Schizont (arrow) with several merozoites (arrowheads) separating from the main mass. Impression smear of infected cat intestine. Giemsa stain. (**e.**) A male gamete with two flagella (arrows). Impression smear of infected cat intestine. Giemsa stain. (**f**) Unsporulated oocyst in fecal float of cat feces. Unstained. Note double-layered oocyst wall (arrow) enclosing a central undivided mass. (**g**) Sporulated oocyst with a thin oocyst wall (large arrow), 2 sporocysts (arrowheads). Each sporocyst has four sporozoites (small arrow) that are not in complete focus. Unstained.

The tachyzoite is often crescent-shaped and is approximately the size (2×6 μm) of a red blood cell (Fig. 1a). Its anterior end is pointed and its posterior end is round. It has a pellicle (outer covering), several organelles including subpellicular microtubules, mitochondrium, endoplasmic reticulum, a Golgi apparatus, apicoplast, ribosomes, rough-surfaced endoplasmic reticulum, a micropore, and a well-defined nucleus. The nucleus is usually situated toward the posterior end or in the central area of the cell.

The tachyzoite enters the host cell by active penetration of the host cell membrane and can tilt, extend, and retract as it searches for a host cell. After entering the host cell, the tachyzoite becomes ovoid in shape and becomes surrounded by a parasitophorous vacuole. *Toxoplasma gondii* in a parasitophorous vacuole is protected from the defense mechanisms of the host. The tachyzoite multiplies asexually within the host cell by repeated divisions in which two progeny form within the parent parasite, consuming it (Fig. 1a). Tachyzoites continue to divide until the host cell is filled with parasites.

After a few divisions, *T. gondii* form another stage called *tissue cysts*. Tissue cysts grow and remain intracellular. Tissue cysts vary in size from 5 to 70 μm (Figs. 1b,c). Although tissue cysts may develop in visceral organs, including lungs, liver, and kidneys, they are more prevalent in muscular and neural tissues (Fig. 1b), including the brain (Fig. 1c), eye, skeletal, and cardiac muscle. Intact tissue cysts probably do not cause any harm and can persist during the life of the host.

The tissue cyst wall is elastic, thin (<0.5 μm), and may enclose hundreds of the crescent-shaped slender *T. gondii* stage known as *bradyzoites* (Fig. 1c). The bradyzoites are approximately 7×1.5 μm. Bradyzoites differ structurally only slightly from tachyzoites. They have a nucleus situated toward the posterior end, whereas the nucleus in tachyzoites is more centrally located. Bradyzoites are more slender than tachyzoites and less susceptible to destruction by proteolytic enzymes.

All coccidian parasites have a resistant stage in their life cycle, called *oocyst*. Oocysts of *T. gondii* are formed only in cats, not only domestic cats, but probably all members of the Felidae (Fig. 2). Cats shed oocysts after ingesting any of the three infectious stages of *T. gondii*, namely, tachyzoites, bradyzoites, and sporozoites (3–5). Prepatent periods (time to the shedding of oocysts after initial infection) and frequency of oocyst shedding vary according to the stage of the *T. gondii* ingested. Prepatent periods are three to ten days after ingesting tissue cysts and 21 days or more after ingesting tachyzoites or oocysts (3–5). Less than 50% of cats shed oocysts after ingesting tachyzoites or oocysts, whereas nearly all cats shed oocysts after ingesting tissue cysts (4).

After the ingestion of tissue cysts by cats, the tissue cyst wall is dissolved by the proteolytic enzymes in the stomach and small intestine. The released bradyzoites penetrate the epithelial cells of the small intestine and initiate the development of numerous generations of asexual and sexual cycles of *T. gondii* (3). *Toxoplasma gondii* multiplies profusely in intestinal epithelial cells of cats (entero-epithelial cycle) and these stages are known as *schizonts* (Fig. 1d). Organisms (merozoites) released from schizonts form male and female gametes. The male gamete has two flagella (Fig. 1e) and it swims to and enters the female gamete. After the female gamete is fertilized by the male gamete (Fig. 1e), oocyst wall formation begins around the fertilized gamete. When oocysts are mature, they are discharged into the intestinal lumen by the rupture of intestinal epithelial cells.

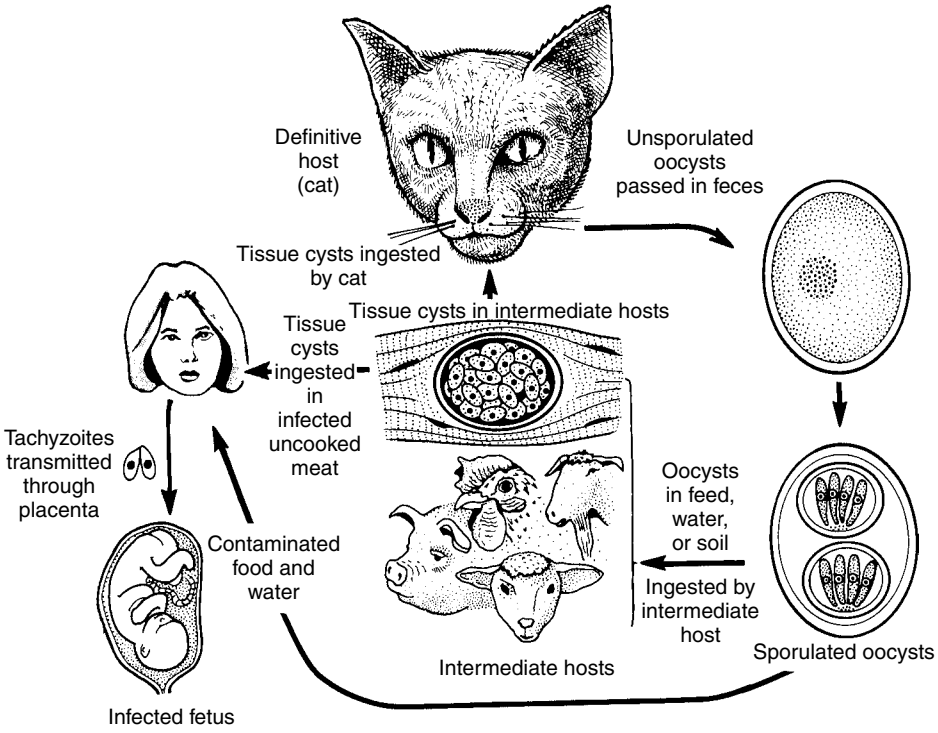

Figure 2. Life cycle of *T. gondii*.

In freshly passed feces, oocysts are unsporulated (noninfective). Unsporulated oocysts are subspherical to spherical and are 10×12 μm in diameter (Fig. 1f). They sporulate (become infectious) outside the cat within one to five days, depending on aeration and temperature. Sporulated oocysts contain two ellipsoidal sporocysts (Fig. 1g). Each sporocyst contains four sporozoites. The sporozoites are 2×6 to 8 μm in size.

As the entero-epithelial cycle progresses, bradyzoites penetrate the lamina propria of the feline intestine and multiply as tachyzoites. Within a few hours after the infection of cats, *T. gondii* may disseminate to extraintestinal tissues. *Toxoplasma gondii* persists in intestinal and extraintestinal tissues of cats for at least several months, and possibly for the entire lifespan of the cat.

CULTIVATION, MOLECULAR BIOLOGY, AND GENETICS

Toxoplasma gondii has not been grown in cellfree media. It can be cultivated in laboratory animals, chick embryos, and cell cultures. Mice, hamsters, guinea pigs, and rabbits are all susceptible, but mice are generally used as hosts because they are more susceptible than the others and are not naturally infected when raised in the laboratory on commercial dry food that is free of cat feces.

Toxoplasma gondii tachyzoites will multiply in many cell lines in cell culture. Although most strains of *T. gondii* can develop tissue cysts in cell cultures, the yield is lower than that produced by infection in mice. Feline entero-epithelial stages of *T. gondii* have not yet been cultivated in vitro. Oocysts can be obtained by feeding tissue cysts from infected mice to *T. gondii*–free cats.

The *T. gondii* nucleus is haploid except for the zygote, in the intestine of the cat (6). Sporozoites result from a miotic division followed by mitotic divisions, and genetic segregation seems to follow classical Mendelian laws. The total haploid genome contains approximately 8×10^7 base pairs. There is also a 36- kb circular mitochondrial DNA. Nine chromosomes have been identified by pulsed field gel electrophoresis. Unlike many other microorganisms, *T. gondii* is very stable with very little mutation. Although minor differences exit among different isolates of *T. gondii* so far analyzed, at present there is no definitive way to classify or type the different isolates of *T. gondii* because they are all morphologically identical and all cross-react immunologically. On the basis of antigens, isoenzymes, antigens, and DNA characteristics, all *T. gondii* isolates can be broadly grouped into three types (types I,II,III) (7–10). Although type I strains have been isolated from patients with symptoms, a definitive association of virulence with the type has not been established. Variations among the three types are being recognized (11).

HOST–PARASITE RELATIONSHIP

Toxoplasma gondii can multiply in virtually any cell in the body. How *T. gondii* is destroyed in immune cells is not completely known (12). All extracellular forms of the parasite are directly affected by antibodies, but intracellular forms are not. It is believed that cellular factors, including lymphocytes and lymphokines, are more important than humoral factors in the immune-mediated destruction of *T. gondii* (12).

Immunity does not eradicate infection. *Toxoplasma gondii* tissue cysts persist several years after acute infection. The fate of tissue cysts is not fully known. Whether bradyzoites can form new tissue cysts directly, without transforming into tachyzoites, is not known. It has been proposed that tissue cysts may at times rupture during the life of the host. The released bradyzoites may be destroyed by the host's immune responses. The reaction may cause local necrosis accompanied by inflammation. Hypersensitivity plays a major role in such reactions (12). After such events, inflammation usually again subsides with no local renewed multiplication of *T. gondii* in the tissue, although occasionally there may be a formation of new tissue cysts.

In immunosuppressed patients, such as those given large doses of immunosuppressive agents in preparation for organ transplants and in those with AIDS, rupture of a tissue cyst may result in the transformation of bradyzoites into tachyzoites and renewed multiplication. The immunosuppressed host may die from toxoplasmosis unless treated. It is not known how corticosteroids cause relapse, but it is unlikely that they directly cause rupture of the tissue cysts.

Pathogenicity of *T. gondii* is determined by the virulence of the strain and the susceptibility of the host species. Strains of *T. gondii* may vary in their pathogenicity in a given host. Certain strains of mice are more susceptible than others and the severity of infection in individual mice within the same strain may vary. Certain species are genetically resistant to clinical toxoplasmosis. For example, adult rats do not become ill, whereas young rats can die of toxoplasmosis. Mice of any age are susceptible to clinical *T. gondii* infection. Adult dogs, like adult rats, are resistant, whereas puppies are fully susceptible to clinical toxoplasmosis. Cattle and horses are among the hosts more resistant to clinical toxoplasmosis, whereas certain marsupials and New World monkeys are the most susceptible to *T. gondii* infection (1). Nothing is known concerning genetically determined susceptibility to clinical toxoplasmosis in higher mammals, including humans.

INFECTIONS IN HUMANS

Toxoplasma gondii infection is widespread among humans and its prevalence varies widely from place to place. In the United States and the United Kingdom, it is estimated that about 16 to 40% of people are infected, whereas in Central and South America and continental Europe, estimates of infection range from 50 to 80% (1). Most infections in humans are asymptomatic, but at times, the parasite can produce devastating disease. Infection may be congenitally or postnatally acquired. Congenital infection occurs only when a woman becomes infected during pregnancy, and the severity of the disease may depend on the stage of pregnancy when the woman becomes infected. Infections

acquired during the first trimester are more severe than those acquired in the second and third trimester (13,14). Although the mother rarely has symptoms of infection, she does have a temporary parasitemia. Focal lesions develop in the placenta and the fetus may become infected. At first, there is a generalized infection in the fetus. Later, infection is cleared from the visceral tissues and may localize in the central nervous system. A wide spectrum of clinical diseases occurs in congenitally infected children (13). Mild disease may consist of slightly diminished vision, whereas severely diseased children may have the full tetrad of signs: retinochoroiditis (inflammation of inner layers of eye), hydrocephalus (big head), convulsions, and intracerebral calcification. Of these, hydrocephalus is the least common but most dramatic lesion of toxoplasmosis. By far the most common sequel of congenital toxoplasmosis is ocular disease (13,14).

The socio-economic impact of toxoplasmosis in humans suffering and the cost of the care of sick children, especially those with mental retardation and blindness, are enormous (15,16). The testing of all pregnant women for *T. gondii* infection is compulsory in some European countries including France and Austria. The cost benefits of such mass screening are being debated in many other countries (14).

Postnatally acquired infection may be localized or generalized. Oocyst-transmitted infections may be more severe than tissue cyst–induced infections (1,17–21). Enlarged lymph nodes are the most frequently observed clinical form of toxoplasmosis in humans (Table 1). Lymphadenopathy may be associated with fever, fatigue, muscle pain, sore throat, and headache. Although the condition may be benign, its diagnosis is vital in pregnant women because of the risk to the fetus. In a British Columbia outbreak, of 100 people who were diagnosed with acute infection, 51 had lymphadenopathy and 20 had retinitis (18,19).

Table 1. Frequency of Symptoms in People with Postnatally Acquired Toxoplasmosis

	Patients with Symptoms (%)	
Symptoms	Atlanta Outbreak[a] (35 Patients)	Panama Outbreak[b] (35 Patients)
Fever	94	90
Lymphadenopathy	88	77
Headache	88	77
Myalgia	63	68
Stiff neck	57	55
Anorexia	57	NR[c]
Sore throat	46	NR
Arthralgia	26	29
Rash	23	0
Confusion	20	NR
Earache	17	NR
Nausea	17	36
Eye pain	14	26
Abdominal pain	11	55

[a] From Teutsch et al., (20).
[b] From Benenson et al., (17).
[c] Not reported.

Encephalitis is the most important manifestation of toxoplasmosis in immunosuppressed patients because it causes the most severe damage to the patient (1). Infection may occur in any organ. Patients may have headache, disorientation, drowsiness, hemiparesis, reflex changes, and convulsions, and many become comatose. Encephalitis caused by *T. gondii* is now recognized with great frequency in patients treated with immunosuppressive agents.

Toxoplasmosis ranks high on the list of diseases that lead to the death of the patients with acquired immunodeficiency syndrome (AIDS); approximately 10% of AIDS patients in the United States and up to 30% in Europe are estimated to die from toxoplasmosis (22) Although in AIDS patients, any organ may be involved, including the testis, dermis, and the spinal cord, infection of the brain is most frequently reported. Most AIDS patients suffering from toxoplasmosis have bilateral, severe, and persistent headache that responds poorly to analgesics. As the disease progresses, the headache may give way to a condition characterized by confusion, lethargy, ataxia, and coma. The predominant lesion in the brain is necrosis, especially of the thalamus (23).

INFECTION IN ANIMALS OTHER THAN HUMANS

Toxoplasma gondii is capable of causing severe disease in animals other than humans (1). Toxoplasmosis causes great losses in sheep and goats. *Toxoplasma gondii* may cause embryonic death and resorption, fetal death and mummification, abortion, stillbirth, and neonatal death in these animals. Disease is more severe in goats than in sheep. Outbreaks of toxoplasmosis in pigs have been reported from several countries, especially Japan. Mortality in young pigs is more common than mortality in adult pigs. Pneumonia, myocarditis, encephalitis, and placental necrosis have been reported to occur in infected pigs. Sporadic and widespread outbreaks of toxoplasmosis occur in rabbits, mink, birds, and other domesticated and wild animals.

DIAGNOSIS

Diagnosis is made by biological, serological, or histological methods or by some combination of these. Clinical signs of toxoplasmosis are nonspecific and are not sufficiently characteristic for a definite diagnosis. Toxoplasmosis, in fact, mimics several other infectious diseases.

Detection of *T. gondii* antibody in patients may aid diagnosis. There are numerous serological procedures available for the detection of humoral antibodies; these include the Sabin-Feldman dye test, the indirect hemagglutination assay, the indirect fluorescent antibody assay (IFA), the direct agglutination test, the latex agglutination test, the enzymelinked immunoabsorbent assay (ELISA), and the immunoabsorbent agglutination assay test (IAAT). The IFA, IAAT, and ELISA have been modified to detect IgM antibodies (2,14). The IgM antibodies appear sooner after infection than the IgG antibodies, and the IgM antibodies disappear faster than IgG antibodies after recovery (14).

The finding of antibodies to *T. gondii* in one serum sample only establishes that the host had been infected at some time in the past. It is best to collect two samples from the same individual, the second sample two to four weeks after the first. A 16-fold higher antibody titer in the second sample indicates an acute infection. A high antibody titer sometimes persists for months after the infection. A rise may not be associated with clinical symptoms for, as indicated earlier, most infections in humans are asymptomatic. The fact that titers persist in infected people after clinical recovery complicates the interpretation of the results of the serological tests. Establishing the recency of infection in pregnancy is of clinical importance with respect to medical intervention to minimize damage to the fetus, and there is not a single test that can achieve this at the present.

Toxoplasma gondii can be isolated from patients by inoculation of laboratory animals and tissue cultures with secretions, excretions, body fluids, tissues taken by biopsy and tissues with macroscopic lesions, taken postmortem. Using such specimens, one may not only attempt isolation of *T. gondii*, but may search for *T. gondii* microscopically or for toxoplasmal DNA by the use of the polymerase chain reaction (24).

As just noted, diagnosis can be made by finding *T. gondii* in the host tissue removed by biopsy or at necropsy. A rapid diagnosis may be made by the microscopic examination of the impression smears of lesions. After drying for 10 to 30 minutes, the smears are fixed in methyl alcohol and stained with one of the Romanowsky strains, the Giemsa stain being very satisfactory. Well preserved *T. gondii* are crescent-shaped (Fig. 1a). In the sections, the tachyzoites usually appear round to oval. Electron microscopy can aid diagnosis. Tachyzoites of *T. gondii* are always located in vacuoles. Tissue cysts are usually spherical, lack septa, and the cyst wall can be stained with a silver stain. The bradyzoites are strongly periodic acid Schiff (PAS) positive. The immunohistochemical staining of parasites with *T. gondii* antiserum can aid in diagnosis.

TREATMENT

Sulfadiazine and pyrimethamine (Daraprim) are two drugs widely used for the therapy of toxoplasmosis (25). Although these drugs have a beneficial action when given in the acute stage of the disease process, when there is active multiplication of the parasite, they usually will not eradicate infection. It is believed that these drugs have little effect on subclinical infections, but the growth of tissue cysts in mice has been restrained with sulfonamides.

Certain other drugs, diaminodiphenylsulfone, atovaquone, spiramycin, and clindamycin are also used to meet toxoplasmosis in difficult cases.

EPIDEMIOLOGY

As noted earlier, toxoplasmosis may be acquired by the ingestion of oocysts or by the ingestion of tissue-inhabiting stages of the parasite. The contamination of the environment by oocysts is widespread because oocysts are shed by cats, not only the domestic cat, but by other members of the Felidae as well (1,2). Domestic cats are probably the major source of contamination because oocyst formation is greatest in the domestic cats, and they are extremely common. Widespread natural infection of the environment is possible because a cat may excrete millions of oocysts after ingesting as few as one bradyzoite or one tissue cyst and many tissue cysts maybe present in one infected mouse (3,26). Sporulated oocysts survive for long periods under most ordinary environmental conditions. They can survive in moist soil, for example, for months and even years (1). Oocysts in soil do not always stay there because invertebrates such as flies, cockroaches, dung beetles, and earthworms can mechanically spread these oocysts and even carry them onto food.

Although a few cats may be shedding *T. gondii* oocysts at any given time (as few as 1%), the enormous numbers shed and their resistance to destruction assures widespread contamination. Under experimental conditions, infected cats can shed oocysts after reinoculation with tissue cysts (27). If this occurs in nature, it would greatly facilitate the spread of oocyst. Congenital infection can also occur in cats, and congenitally infected kittens can excrete oocysts, providing another source of oocysts for contamination. Infection rates in cats are determined by the rate of infection in the local avian and rodent population because cats are thought to become infected by eating these animals. The more oocysts in the environment, the more likely prey animals would be infected, and this in turn would increase the infection rate in cats.

Infection in humans is probably most often the result of the ingestion of tissue cysts contained in undercooked meat (1, 28, 29). Infection by *T. gondii* is common in many animals used for food including sheep, pigs, and rabbits. Prevalence is very high (80%) in bears in the United States. Infection in cattle is less prevalent than is infection in sheep or pigs. *Toxoplasma gondii* in tissue cysts survive in animals used for food for years.

Cultural habits of people may affect the acquisition of *T. gondii* infection. For example, in France, the prevalence of antibody to *T. gondii* is very high in humans. Although 84% of pregnant women in Paris have antibodies to *T. gondii*, only 32% in New York City and 22% in London have such antibodies (1). The high incidence of *T. gondii* infection in humans in France appears to be related, in part, to the French habit of eating some of their meat raw. In contrast, the high prevalence of *T. gondii* infection in Central and South America probably is a result of the high levels of contamination of the environment by oocysts (1). However, it should be noted that the relative frequency of the acquisition of toxoplasmosis from eating raw meat and that caused by the ingestion of food contaminated by oocysts from cat feces is very difficult to determine and as a result, statements on the subject are at best controversial.

There is little, if any, danger of *T. gondii* infection by drinking cow's milk and, in any case, cows milk is generally pasteurized or even boiled, but drinking goat's milk without boiling it has led to infection (1). Raw hens' eggs, although an important source of *Salmonella* infection, are extremely unlikely to transmit *T. gondii* infection.

Transmission by sexual activity, including kissing, is probably rare and epidemiologically unimportant (1).

Transmission may occur through blood transfusions and organ transplants. Of these means, transmission by transplantation is most important. Toxoplasmosis may actually arise in two ways in people undergoing transplantation, namely, from the implantation of an organ or bone marrow of an infected donor into a nonimmune immunocompromised recipient and from the induction of disease in an immunocompromised, latently infected recipient. The tissue cysts in the transplacental tissue or in the latently infected are probably the source of the infection. In both cases, the cytotoxic and immunosuppressive therapy given to the recipient is the cause of the induction of the active infection and the disease (1,12).

Reservoirs and Detection of *T. gondii* in the Environment

As mentioned earlier, humans become infected by ingesting tissue cysts in undercooked or uncooked meat or by ingesting food and water contaminated with oocysts from infected cat feces. There are no tests at the present time to determine the source of infection in a given person. All the evidence is based on epidemiological surveys. For example, in certain areas of Brazil, approximately 60% of the six- to eight-year-old children have antibodies to *T. gondii* linked to the ingestion of oocysts from the environment heavily contaminated with *T. gondii* oocysts (30). Oocysts of *T. gondii* survive even in harsh environments for months. Infection in aquatic mammals indicates contamination and survival of the oocysts in seawater (31). The largest outbreak of clinical toxoplasmosis in humans was epidemiologically linked to the drinking of water from a municipal water reservoir in British Columbia, Canada (32). This water reservoir was thought to be contaminated with *T. gondii* oocysts excreted by cougars (*Felis concolor*) (33,34).

Oocysts can be detected by an examination of cat feces. Concentration methods (e.g., flotation in high-density sucrose solution) are often used because the number of *Toxoplasma gondii* oocysts in cat feces may be too few to be detected by a direct smear (Fig. 3). For definitive identification, *T. gondii* oocysts should be sporulated and then bioassayed in mice to distinguish them from other related coccidians (1).

For epidemiological surveys, detection of *T. gondii* oocysts in cat feces is not very practical; at any given time, only 1% of cats are found shedding oocysts because oocysts are shed for only a short period (one to two weeks) in the life of the cat (1). Determining serological prevalence is a better measure of exposure of cats to *T. gondii* infection than detection of oocysts. It is a fair assumption that cats that are seropositive have already shed *T. gondii* oocysts. In an epidemiological survey on *T. gondii* infection on pig farms, *T. gondii* oocysts were detected in only 5 of 274 (1.8%) samples of cat feces, 2 of 491 (0.4%) samples of feed, and 1 of 79 (1.3%) samples of soil on some farms; 267 of 391 (68.3%) cats had antibodies to *T. gondii* (35).

Although *T. gondii* has been isolated from soil, there is no simple method for use on an epidemiological scale.

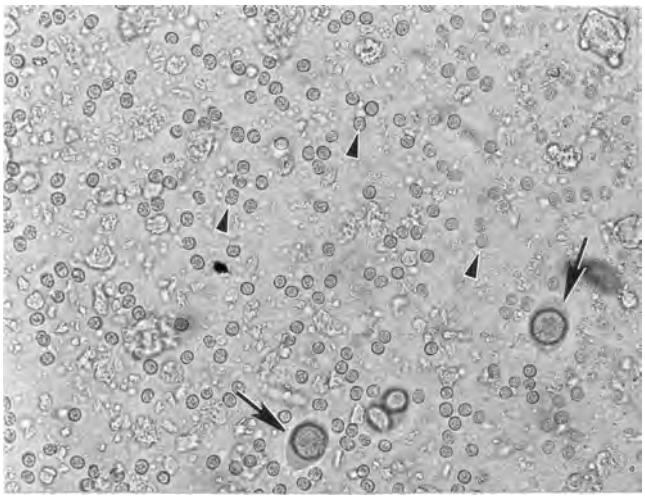

Figure 3. *Toxoplasma gondii* oocysts in sugar fecal float of an infected cat. Note many spherical *T. gondii* oocysts (arrowheads). Also note oocysts of *Isospora felis* (arrows), which are often present in cat feces. *Isospora felis* is the most common coccidium of cats. Its oocysts are about four times the size of *T. gondii* oocysts and they are often in a different focus than *T. gondii* oocysts.

Bioassay of soil samples in pigs and chickens may be more useful than the direct determination of oocysts in soil; pigs can be infected by feeding on as few as one oocyst (36). In a study of feral chickens, *T. gondii* was isolated from 54% of 50 chickens by bioassay in mice (37). Because feral chickens on small farms feed on the ground, finding *T. gondii* in chickens is a good indicator of infection in the environment. Although attempts to recover *T. gondii* oocysts from water samples in the British Columbia outbreak were unsuccessful, methods to detect oocysts were reported (32). At present, there are no commercial reagents available to detect *T. gondii* oocysts in the environment.

As stated earlier, among food animals, infection is more prevalent in sheep, goats, pigs, and rabbits than in cattle, horses, and water buffaloes (1). Virtually, all edible portions of an animal can harbor viable *T. gondii*. In one study, viable *T. gondii* was isolated from 17% of 1,000 adult pigs (sows) from a slaughter plant in Iowa (38). *Toxoplasma gondii* infection is also prevalent in game animals. Among wild game, *T. gondii* infection is most prevalent in black bears and in white-tailed deer. Approximately 80% of the black bears are infected in the United States (39), and about 60% of the raccoons have antibodies to *T. gondii* (35,39). As raccoons and bears scavenge for their food, infection in these animals is a good indicator of the prevalence of *T. gondii* in the environment.

The number of *T. gondii* in meat from food animals is very low. It is estimated that as few as one tissue cyst may be present in 100 grams of meat. Therefore, without using a concentration method, it is not practical to detect this low level of *T. gondii* infection. Therefore, digestion of meat samples in trypsin or pepsin is used to concentrate the *T. gondii* in meat (40). Digestion in trypsin and pepsin ruptures the *T. gondii* tissue cyst wall releasing hundreds of bradyzoites. The bradyzoites survive in the digests for several hours. Even in the digested samples, only a few

T. gondii are present and their identification by direct microscopic examination is not practical. Therefore, the digested material is bioassayed in mice (40). The mice inoculated with digested material have to be kept for six to eight weeks before *T. gondii* infection can be detected reliably; this procedure is not practical for mass scale samples. The detection of *T. gondii* DNA in meat samples by PCR has been reported (41) but there are no data on specificity and sensitivity of this method to detect *T. gondii* in meat samples. A highly sensitive method using a real-time PCR and fluorogenic probe was found to detect *T. gondii* DNA from as few as four bradyzoites (42). This method is now being tested to detect *T. gondii* in meat samples obtained from slaughtered animals.

PREVENTION AND CONTROL

To prevent infection of human beings by *T. gondii*, the hands of people handling meat should be washed thoroughly with soap and water before they go to other tasks (1,29). All cutting boards, sink tops, knives, and other materials coming in contact with uncooked meat should be washed with soap and water also. Washing is effective because the stages of *T. gondii* in meat are killed by the contact with soap and water (1).

Organisms of *T. gondii* in meat can be killed by exposure to extreme cold or heat. Heating the meat throughout to 67 °C kills the tissue cysts in it (43). *Toxoplasma gondii* in meat is killed by cooling to −13 °C (44). *Toxoplasma* in tissue cysts is also killed by exposure to 0.5 kilorads of gamma irradiation (45). Meat of any animal should be cooked to 67 °C before consumption, and tasting meat while cooking or while seasoning should be avoided. Pregnant women, especially, should avoid contact with cats, soil, and raw meat. Pet cats should be fed only dry, canned, or cooked food. The cat litter box should be emptied everyday, preferably not by a pregnant woman. Gloves should be worn while gardening. Vegetables should be washed thoroughly before eating because they may have been contaminated with cat feces. Expectant mothers should be aware of the dangers of toxoplasmosis (46). At present, there is no vaccine to prevent toxoplasmosis in humans.

CONCLUSION

Infection by the protozoan parasite *T. gondii* is widely prevalent in humans and animals. Although it causes asymptomatic infection in immunocompetent adults, *T. gondii* can cause devastating disease in congenitally infected children and those with depressed immunity. To prevent human infection, all meat should be cooked well before consumption and gloves should be worn while gardening to prevent exposure to soil contaminated with *T. gondii* oocysts excreted in cat feces.

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TRACE ELEMENTS IN MARINE MICROORGANISMS.

See INORGANIC NUTRIENT USE BY MARINE MICROORGANISMS

TRACE GASES SOIL

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MICROBES AND THE ATMOSPHERE

When we consider interactions between the atmosphere and microorganisms, we tend to think about atmospheric effects on microorganisms, particularly with the distinction of aerobes versus anaerobes. Alternatively, perhaps, we think on the geological timescale about evolution of cyanobacteria and creation of an aerobic Earth. We tend not to think about the critical role of ongoing microbial activities in controlling the day-to-day composition of the atmosphere. Current microbial activities have limited impact on the bulk composition of the atmosphere: the 99.96% of the atmosphere that is N_2, O_2, or argon. However, the last 0.04% of the atmosphere, the "trace gases" are absolutely critical to the atmosphere's chemistry and radiation balance.

With the exception of the inert gases (helium, argon, etc.) and some halogenated gases, such as chlorofluorocarbons (CFCs), all the trace gases are produced by and/or consumed by the biota, many by microorganisms in both marine and terrestrial ecosystems (Fig. 1). For many of these gases, microbial processes function as the major sources to the atmosphere. For some of the gases, microorganisms act as important sinks as well. Thus, to understand the dynamics of the Earth's atmosphere and global biogeochemical cycles fully, it is necessary to have an understanding of the microbial processes that control atmospheric gases. In the remainder of this entry, I will give a very brief overview of atmospheric chemistry, and then discuss the primary groups of gases, their budgets in the atmosphere, the organisms responsible for their production or consumption, the mechanisms by which they are produced, and aspects of the microbial ecology that control the process dynamics.

ATMOSPHERIC CHEMISTRY

The atmospheric concentrations and roles of trace gases are described in Table 1. Atmospheric chemistry differs dramatically between the lower atmosphere (troposphere) and the upper atmosphere (stratosphere). In the troposphere, atmospheric chemistry is dominated by oxidation reactions carried out largely through the action of hydroxyl radical (OH•). Hydroxyl radical acts as the atmosphere's general cleaning agent. For example, it reacts with CH_4, other hydrocarbons, CO, and even some halocarbons, oxidizing them to carbon dioxide (1). SO_2 is oxidized to SO_4^{2-}, whereas NO and NO_2 are oxidized to NO_3^-. Through their reaction with OH•, trace gases may affect the dynamics of other gases even though they may not react directly with each other. For example, it is thought that the increase in CH_4 leads to a reduction in OH•, and thereby to an increase in the lifetime of other gases in the atmosphere (2). Tropospheric oxidation chemistry is also important in the production and destruction of tropospheric ozone and in the production of smog.

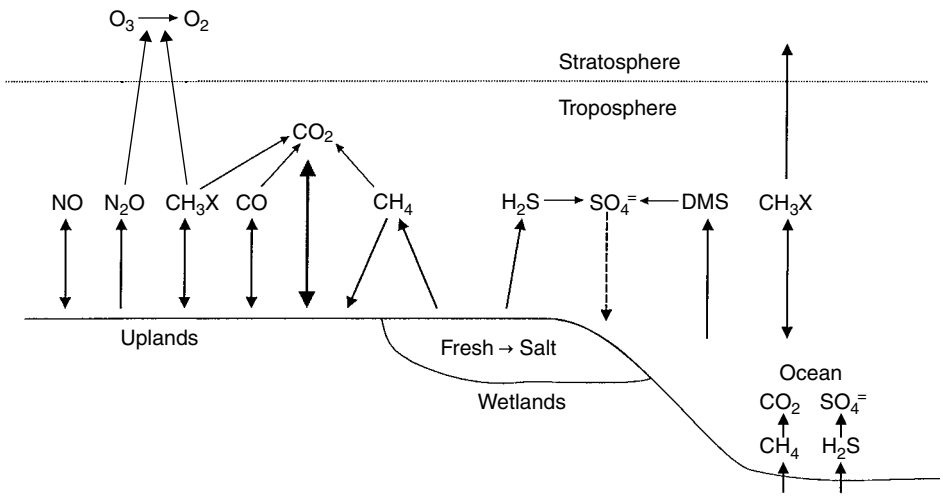

Figure 1. Overview of global trace gas dynamics.

Table 1. Characteristics of Important Atmospheric Trace Gases (Data from Refs. 2 and 3)

Compound	Current Concentration	Rate of Increase	Atmospheric Lifetime	Global Warming potential[a]	Other Effects
CO_2	370 ppm	0.4%	~100 years	1 (by definition)	
CH_4	1.8 ppm	0.6%	12 years	56	Oxidation chem.
CO	50 to 150 ppm		1 to 4 months	No direct	Oxidation chem.
CFC-11	0.27 ppb	0%	50 years	4,900 direct, net ca. 2,000	Strong ozone depleter
CH_3Cl	0.6 ppb	~0	1.5 years		Weak ozone depleter
CH_3Br	0.012 ppb	~0	1.3 year		Weak ozone depleter
N_2O	320 ppb	0.8 ppm/year	120 years	280	Ozone depleter
NO/NO_x	Variable		1 to 10 days		Oxidation chem.
Dimethyl sulfide	6 to 200 pg/L		Hours to days		Cloud formation
H_2S			Hours to days		Cloud formation

[a] 20-Year time horizon.

In the stratosphere, atmospheric chemistry is dominated by photochemical reactions, made possibly by high-energy ultraviolet (UV) radiation. The availability of UV allows the breakdown of chemicals that are inert in the troposphere. For example, N_2O is resistant to attack by OH•, but can react with an energized oxygen atom, $O(^1D)$, that is produced when ozone (O_3) is cleaved by UV. The product of N_2O breakdown reaction is two NO molecules. CFCs are also inert in the troposphere, but can be cleaved either directly by UV or through reaction with $O(^1D)$. The breakdown of CFCs in the stratosphere produces chlorine and ClO radicals. Chlorine catalyzes ozone destruction through the following reaction sequence:

$$Cl + O_3 \longrightarrow ClO + O_2$$

$$O_3 \longrightarrow O + O_2$$

$$ClO + O \longrightarrow Cl + O_2$$

$$\text{Net: } 2O_3 \longrightarrow 3O_2$$

Thus chlorine is regenerated through the reaction cycle and can cause the destruction of hundreds or thousands of ozone molecules before being "quenched" by reaction with another free radical. NO acts through a similar reaction pathway in which NO and NO_2 replace chlorine and ClO.

Many trace gases absorb infrared radiation emitted by the Earth, trapping it in the atmosphere and thereby contributing to the "greenhouse effect." The natural greenhouse effect is enough to warm the Earth by roughly 33 °C (1). Most of this is caused by water vapor and clouds, which are short-lived and variable. The long-lived trace gases, on the other hand, contribute more than 8 W/m^2 to the atmosphere (enough to cause several degrees of warming). This is an increase of 2.45 W/m^2 from preindustrial levels, and if the increase in tropospheric ozone is included, this total increase is closer to 3 W/m^2 (4). This extra energy input to the lower atmosphere is known as radiative forcing. The climate system responds to this forcing in multiple ways, including simply warming up, and through evaporating water, thus increasing humidity and rainfall. Whereas many trace gases impose a direct, positive radiative forcing on the atmosphere, some gases can have an indirect negative forcing as well. This occurs through two mechanisms. The first is through catalyzing ozone depletion. Ozone is a strong greenhouse gas, and so the gases that catalyze its destruction reduce radiative forcing (4). If the reduction in ozone is great enough, a gas may have a net cooling effect on the atmosphere (e.g., CCl_4 and some brominated compounds). The second mechanism for indirect negative radiative forcing is through stimulating cloud production. Clouds are reflective (they have a high albedo) and so they reduce energy inputs to the Earth's surface, thus cooling it. Sulfur gases, in particular are important in this. They are oxidized to SO_4^{2-}, which forms particles that act as cloud condensation nuclei (CCN). The radiative forcing caused by sulfate aerosol stimulation of cloud production is estimated currently at around -0.5 to -0.8 W/m^2 (5). The role of trace gases in atmospheric oxidation chemistry interacts with their role in radiative forcing. The overall radiative forcing caused by a greenhouse gas increases with its lifetime in the atmosphere. Therefore, if a gas reacts with OH•, it will increase the atmospheric lifetimes of other gases, and thereby increase their contribution to the overall greenhouse effect. Reducing OH• will also reduce the rate at which sulfur gases are oxidized to SO_4^{2-}, reducing the supply of CCN, also possibly increasing global warming.

CARBON DIOXIDE

The main role of carbon dioxide in the atmosphere is as a greenhouse gas. Whereas carbon dioxide is a much weaker absorber of IR radiation than most of the other gases on a molecule-by-molecule basis, it is present in much greater concentrations. Thus, carbon dioxide accounts for around 60% of the total radiative forcing caused by trace gases (4).

The biology of carbon dioxide is fundamentally different from that of other trace gases, because it is central to the metabolism of just about every living organism on Earth. It is produced by almost all heterotrophs (from bacteria to animals) as the final product of catabolism. There are only a few fermentative bacteria that do not produce carbon dioxide (e.g., lactic acid bacteria, which produce lactic acid as a sole product). It is consumed by every autotroph. Of the carbon dioxide consumed from the atmosphere, about 60% is accounted for by marine algae, whereas the remainder goes into terrestrial plants (1).

Nonphotosynthetic autotrophs are insignificant players in the carbon dioxide cycle. Because carbon dioxide is cycled by such a wide variety of organisms, and because so many specific processes are involved in its production, there is little point in even attempting to discuss the specifics of the microbiology that control its dynamics in the atmosphere.

METHANE

Importance

Methane has several important roles in the atmosphere. First, it is a greenhouse gas. It is roughly 20 to 60 times as powerful as carbon dioxide (depending on the time interval considered; 2). Beyond that, however, CH_4 is important in atmospheric oxidation chemistry. Methane is an important sink for OH•. Increasing CH_4 is thought to be partly responsible for reducing atmospheric OH• concentrations (2). Methane is also reasonably stable in the troposphere (lifetime of 12 years), and thus some fraction diffuses up into the stratosphere. When it is oxidized photochemically in the stratosphere, it produces H_2O. This is one of the few sources of stratospheric water vapor, which is required for the formation of polar stratospheric clouds: a central component in ozone depletion and the formation of the ozone hole (2). Thus, CH_4 is indirectly involved in ozone depletion.

Budget

Methane is increasing in the atmosphere at an average rate of 0.6% per year, though the rate decreased somewhat during the early 1990s (2). This small net increase, however, results from the difference in large production and consumption fluxes. The current budget estimates for CH_4 are presented in Table 2. The total estimated emission of CH_4 to the atmosphere is roughly 400 Tg CH_4-C/year. Of this only 120 Tg CH_4-C/year (29%) is thought to be from entirely natural sources (mostly wetlands). The remainder comes from various anthropogenic sources, of which 60% is still microbially produced (e.g., methanogenesis in rice paddies and cattle).

The bulk of atmospheric CH_4 is consumed by photochemical oxidation. Only about 20 to 25 Tg CH_4-C/year of atmospheric CH_4 is consumed by microorganisms (6% of total consumption), mostly by soil bacteria (Table 2). However, this is only a fraction of the total CH_4 that is consumed by microorganisms. A significant amount of the CH_4 produced in sediments and soils is consumed before it ever reaches the atmosphere. The total, gross amount of CH_4 that is actually produced on Earth is probably closer to 900 Tg CH_4-C/year, compared to the actual emissions of 400 Tg CH_4-C/year (6). Total consumption is 520 Tg CH_4-C/year or roughly 60% of the CH_4 produced on Earth. Thus the greatest importance of methane oxidizers is to prevent CH_4 emissions, rather than to consume atmospheric CH_4.

Table 2. Sources and Sinks of CH_4 (Data from Ref. 2)

Source	Estimated Flux (Tg C/year)	Sink	Estimated Flux (Tg C/year)
		Microbial	
Natural wetlands	86	Microbial consumption in soil	22.5
Rice paddies	45		
Landfills/ sewage	67.5		
Ruminant animals	64		
Termites	15		
Marine	3.75		
Freshwater	7.5		
		Nonmicrobial	
Geological	7.5	Oxidation by OH•	334
Fossil fuel related	75	Reaction in stratosphere	30
Biomass burning	30		
Total sources	401	Total sinks	386
		Atmospheric increase	22.5

Organisms and Physiology

Production. Methane is produced predominantly by a group of anaerobic archaea, known as the methanogens (7). These organisms form several closely related branches within the Euryarchaeota. Methane is produced by two dominant pathways.

$$CO_2 + H_2 \longrightarrow CH_4 \text{ (CO_2 reduction)}$$

$$CH_3COOH \longrightarrow CH_4 + CO_2 \text{ (acetate fermentation)}$$

Some methanogens can use other C-1 compounds, such as methanol, methylamine, and formate. However, these pathways are not dominant in nature. The pathway of carbon dioxide reduction begins with the reduction and condensation of carbon dioxide with methanofuran (MFR) to produce formyl-MFR. The pathway then proceeds through a cycle that involves a number of other specific coenzymes. The final step (cleavage of methyl-co-M), in which CH_4 is released, also restarts the cycle by activating carbon dioxide (7). The other pathways of methanogenesis feed into this cyclic pathway by reacting with co-M to form methyl-co-M.

There are organisms other than the true methanogens that produce CH_4, but these are generally considered to be "minimethane producers" because they produce CH_4 as a minor side product of their normal metabolism, rather than as a primary metabolic pathway. These organisms have been studied very poorly, and the full biochemistry involved is not clear (7). They are not thought to be important in global CH_4 cycling.

It is important to note that because methanogens only act on simple compounds, they rely on an extensive food web to supply them with substrates. Polymeric material, such as proteins, cellulose, chitin, and other

compounds, must first be depolymerized to produce soluble monomers. These are then fermented by a wide suite of bacteria to produce H_2, CO_2, and acetate, which the actual methanogens act on. In a number of cases, the fermenters and the methanogens live in very close association. As the free energy available from fermentation is only negative when H_2 concentrations are very low, any build up of H_2 can cause fermentation to shut down (8). By living in close association with fermenters, therefore, methanogens maintain their substrate supply.

Consumption. The bulk of CH_4 consumption is thought to be carried out by the methanotrophs, a group of gram-negative aerobic bacteria (9). There are two primary groups (Types I and II) that are distinguished primarily by the structure of their internal membrane structures. Both types use the same basic pathway of CH_4 oxidation. The first, key enzyme in the pathway is methane monooxygenase (MMO), which converts CH_4 to CH_3OH. There are two forms of this enzyme: a soluble form and a membrane-bound form, known as particulate MMO or pMMO. pMMO is found in all methanotrophs, whereas only a small number of species are known to make the soluble form (9). pMMO appears to be evolutionarily closely related to ammonia monooxygenase, which is found in nitrifiers (10). Methane and ammonia are very similar in size, shape, and structure, and as a result, both enzymes can oxidize both substrates, though each is more selective for its "proper" substrate (11). In methanotrophs, carbon is assimilated from formaldehyde, though there are two distinct pathways that do this. Both of these pathways involve condensing formaldehyde with more complex carbon compounds. Type I methanotrophs generally use the ribulose monophosphate cycle in which formaldehyde is combined with ribulose-5-phosphate. Type-II methanotrophs generally use the serine cycle, in which formaldehyde reacts with tetrahydrofolate to form methylene tetrahydrofolate, which then reacts with glycine to form serine.

Whereas methanotrophs have appeared to be the dominant consumers of atmospheric CH_4 in nature, some other organisms have some ability to oxidize CH_4 as well. Predominant among them are the ammonia oxidizers. Their ability to oxidize CH_4 results from the similarity between CH_4 and NH_3 mentioned earlier. Several studies (12) have suggested that nitrifiers may be important CH_4 consumers in some ecosystems, but the significance of this process is not known.

In marine sediments, where large amounts of CH_4 are produced and consumed, most of the consumption appears to be carried out under anaerobic conditions by a process known as sulfate-dependent methane oxidation. The organisms responsible for the process are not well known, but the process appears to require syntrophy between methane-oxidizing archaea and sulfate-reducing bacteria (13). The specific organisms responsible for the methane oxidation have not been isolated.

Ecology

Production. There are several levels of control over CH_4 production. The first level is redox, and it acts largely as an on/off switch. Methane is produced under only anaerobic conditions (generally redox < -100 mV). Thus, it occurs at high rates only in flooded ecosystems (e.g., wetlands, sediments, animal guts, and a few other situations such as landfills). Draining a wetland rapidly shuts off CH_4 production. Furthermore, the addition of any alternate electron acceptor that will raise the Eh of the system can also shut down CH_4 production. Methane production is commonly limited by high concentrations of SO_4^{2-} such as occur in marine sediments, salt marshes, and even freshwater wetlands experiencing SO_4^{2-} deposition (14). In freshwater wetlands, NO_3^- inputs will reduce methanogenesis.

The effect of environmental controls on CH_4 production can be complicated, and it is often difficult to evaluate where in the whole fermentative pathway they act. For example, in a study of methane production in peats from northern Canada, Valentine and coworkers (15) ascribed temperature effects to the fermentation process but pH effects directly to the methanogens. Some studies have found extraordinarily strong temperature responses for CH_4 production with Q_{10} values greater than 20 (16). This too is likely to reflect responses at multiple stages in the food web, as low temperature inhibits H_2 production, shuts down carbon dioxide reduction, and forces shifts in the specific methanogenic pathways involved. Thus the overall temperature effect may result from multiplicative responses of each group of organisms rather than just the effect on any one.

After redox, the most important control on CH_4 production is substrate quantity. Whereas H_2 and acetate availability directly limit the rate of methanogenesis, their availability is controlled entirely through the fermentative food web, and so the availability of substrate to methanogens is ultimately controlled by the quality of organic substrates entering the soil or sediment. High-quality inputs are decomposed more rapidly and so provide a greater supply of substrates to the methanogens. Because of the importance of the quality of substrates entering an ecosystem, the nature of the plant community is an important factor in controlling CH_4 production rates in terrestrial ecosystems.

Consumption. Methane consumption behaves quite differently in saturated environments (wetlands, sediments), where there is a local source of high concentrations of CH_4, than in unsaturated environments (soils), where the only source of CH_4 is the atmosphere. In saturated terrestrial systems, the primary control on the rate of CH_4 consumption is the availability of O_2. In sediments and saturated soils, methanotrophs tend to occur at very high densities at the anaerobic–aerobic interface (17). In unsaturated systems, because the atmosphere is the source of CH_4 gas, diffusion is the primary control on the rate of CH_4 consumption (18). Thus, soil structure and moisture are the critical controls on consumption, that is, the porosity and their portions that are filled with water.

In upland, unsaturated systems, methane consumption appears to be sensitive to many chemical and physical perturbations. Factors that have been observed to inhibit consumption include plowing, compacting, fertilizing, and

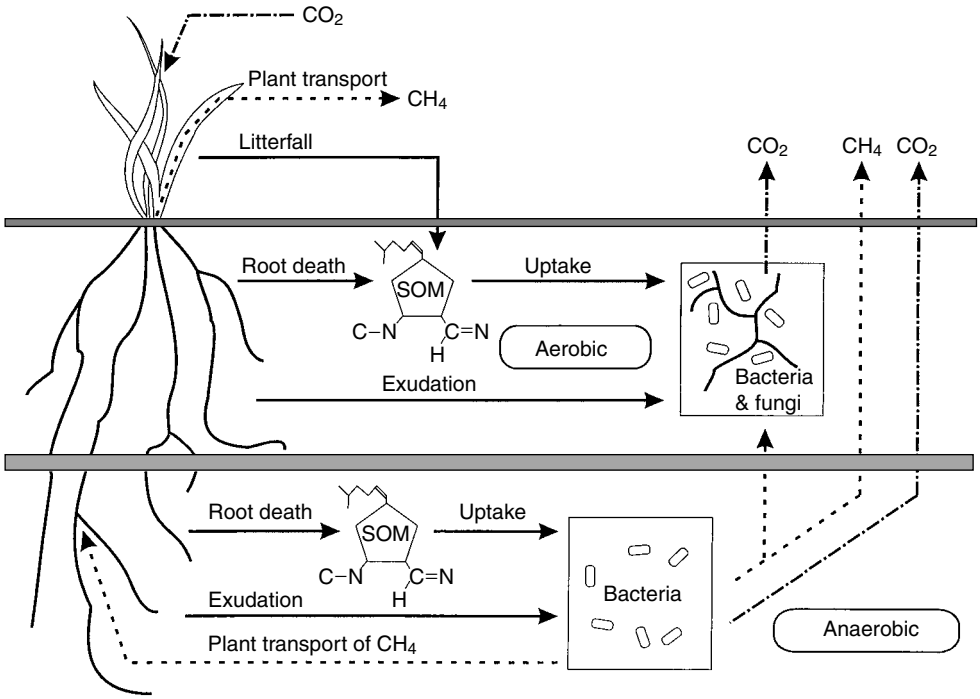

Figure 2. Carbon fluxes in a wetland ecosystem (from 24).

adding salts (19,20). Many physical disturbances may reduce porosity and thus the ability of CH_4 to diffuse into the soil. Nitrogen additions, on the other hand, appear to inhibit CH_4 consumption primarily by competitive inhibition at the enzyme level, as CH_4 and NH_3 are very similar in size and structure (19). The specific mechanisms of some of the other factors are not well understood.

Control on Overall Flux. The actual rate of net CH_4 efflux from a system depends on the balance of CH_4 production and consumption. In nonvegetated sites, this is controlled primarily by the rate of CH_4 production in the anaerobic zone and the diffusion rate of that CH_4 through the aerobic zone where it is consumed. In vegetated systems, the pattern of control is more complex (Fig. 2) because plants dominate CH_4 dynamics. Not only do they supply the carbon and the energy for CH_4 production, but the supply of carbon can also control O_2 consumption and hence the extent of anaerobiosis in the soil. Additionally, wetland plant roots contain aerenchyma, which are air-filled passages within the roots that provide O_2 for root metabolism. Aerenchyma transport O_2 into the soil, producing an aerobic zone around each root, which can allow extensive rhizosphere CH_4 consumption. Aerenchyma also allow CH_4 to diffuse from the deep soil to the atmosphere and avoid the CH_4 consuming zone entirely. Plant transport is typically the dominant efflux mechanism in vegetated wetland systems, though the ability of wetland plants to transport CH_4 varies greatly. Thus, whereas CH_4 flux correlates well with total plant productivity at the global scale (21), at the plot scale, total flux may have little to do with the rate of CH_4 production and may be dominated by the ability of the plants present to transport CH_4 out of the soil (22). The balance of efflux versus consumption varies tremendously among systems, with consumption ranging from as low as 10 to 15% of produced CH_4 in some systems to greater than 90% in others (23).

CARBON MONOXIDE

Importance

Carbon monoxide is one of the most important and complex of the biogenic trace gases (2). It is the most important trace gas in terms of controlling atmospheric oxidation chemistry, as reaction with CO is the major sink for OH• (CO consumes about six times as much OH• as CH_4). Whereas CO is itself not a greenhouse gas because it is too short lived, it has strong indirect effects on the greenhouse effect through its reactivity. For example, it has been estimated that 10 to 37% of the increase in atmospheric CH_4 concentrations has been caused by reduced consumption because of increased competition with CO for OH• (25). Finally, tropospheric CO is strongly involved in tropospheric production of O_3, which is a greenhouse gas (4), but which can also be toxic.

Budget

Carbon monoxide, perhaps surprisingly, cycles through the atmosphere in quantities larger than any trace gas other than carbon dioxide (800 to 1,200 Tg CO-C/year). However, its turnover time is rapid (100 days) and so its average concentration is low; roughly 100 ppb. Because of its rapid turnover and low concentration, the budget for CO has large uncertainties. The major source is the oxidation of organic chemicals (including CH_4) in the atmosphere,

Table 3. Sources and Sinks for CO (Data from Refs. 2 and 26)

Source	Estimated Flux (Tg C/year)	Sink	Estimated Flux (Tg C/year)
	Biotic		
Terrestrial	26 to 69	Soil uptake	6.5 to 270
Oceanic	8.6 to 86		
	Abiotic		
Technological	130 to 240	Reaction with OH•	600 to 1,115
Biomass burning	130 to 300	Stratospheric loss	~43
Atmospheric oxidation	260 to 690		
Total sources	**780 to 1,160**	**Total sinks**	**900 to 1,300**

and much of this results from anthropogenic activity, including industry and biomass burning (Table 3). There are direct microbial sources, but they are rather minimal in the global budget. The largest sink is the chemical reaction with hydroxyl radical in the atmosphere, which accounts for 80% or more of the total sink. Soil microbes are smaller sinks, and estimates of their importance vary greatly. Prather and coworkers (2) in the Intergovernmental Panel of Climate Change 1994 assessment estimated microbial consumption of between 110 and 275 Tg CO-C/year; more recent studies on the other hand, argue that the number may be only in the range of 7 to 20 Tg CO-C/year (26).

Organisms and Physiology

The biological processes affecting CO dynamics are poorly understood. CO is produced as part of organic matter breakdown, apparently by a wide range of organisms. Potter and coworkers (26) simply model it as a fixed proportion of total decomposition. Microbial consumption of CO occurs by a variety of processes, with oxidation to carbon dioxide being the dominant one. CO can also be reduced to CH_4 under anaerobic conditions, but diffusion into saturated systems limits the activity of the anaerobic pathway. Aerobic organisms that can consume CO include carboxydotrophs, methanotrophs, ammonia oxidizers, and some fungi. In the case of carboxydotrophs, they can actually grow on CO and rely on a CO dehydrogenase enzyme that oxidizes CO to CO_2. Methanotrophs and ammonia oxidizers oxidize CO as a side reaction of methane/ammonia monooxygenase.

Ecology

CO production by microbes appears to be controlled by the same suite of factors that control overall microbial activity, as would be expected for a process that appears to be a side reaction. Thus, organic matter content, temperature, and moisture all appear to be important factors (26). The most general control on consumption of atmospheric CO by microorganisms is simply diffusion of the gas to the microbes (26). Any factor that controls diffusion kinetics becomes a control on CO consumption by soils. The dominant such factors are soil texture (porosity) and water content. This is similar to the controls on CH_4 consumption, except that while CH_4 is generally consumed in deeper soils (20 to 50 cm), CO oxidation appears to occur in the surface horizon (27). Thus the key control is diffusion into the immediate surface soil rather than through the soil profile. Consumption rates are greatest in moist soils with adequate porosity and are lower in soils that have a heavy texture or are saturated. Thus, consumption in aquatic, wetland, and tundra systems is limited. Moist, but drained upland soils (forests, grasslands, and agricultural soils) of the tropics are the dominant sinks for CO (26). The secondary controls will depend on which specific groups of microbes are responsible for the activity, and are not well understood.

HALOGENATED HYDROCARBONS

Importance and Budget

Halogenated compounds (chlorocarbons and bromocarbons) are important in the atmosphere (Table 1). They are all strong infrared absorbers and so can act as greenhouse gases with direct global warming potentials that are typically several thousand times that of carbon dioxide (Table 1). They are also involved in ozone depletion as their photochemical breakdown in the stratosphere releases chlorine and bromine radicals that catalyze ozone destruction. Thus, the net effect of these gases on global warming is less than their innate IR absorption would suggest. In fact, brominated compounds, which are potent catalysts of O_3 breakdown (4), act to cool the atmosphere.

The atmospheric effects of the different chlorocarbons vary with their chemical structure. An important distinction is whether the carbons are completely halogenated (e.g., CFCs) or contain hydrogen (including hydrochlorofluorocarbons; HCFCs). CFCs are very inert chemically; they do not react well with OH•, and so have very long lifetimes in the troposphere. They are only active in photochemical reactions, in which high-energy UV can split off the halogens (2). Thus, they become reactive in the stratosphere, where the generation of halogen radicals catalyzes O_3 destruction. The HCFCs, on the other hand, are much more susceptible to chemical reaction with OH•, and thus more likely to be consumed in the troposphere. Thus, while they do contribute to ozone depletion, they are much less effective than the CFCs. Early workers assumed that halocarbon cycling through the atmosphere was almost entirely dominated by anthropogenic emissions and chemical consumption. More recent work, however, has shown that microorganisms are involved in both the production and consumption of halogenated compounds (2,28,29).

Organisms, Physiology, and Ecology

Microorganisms appear to be responsible for producing substantial amounts of several halogenated methane derivatives, particularly CH_3I, CH_3Br, and CH_3Cl, though compounds with multiple halogens are not unknown (2). Natural sources of some of these compounds are

substantially larger than the anthropogenic sources for most of these gases. For example, despite recent concern about the use of methyl bromide as an agricultural fumigant, natural sources of this gas are an order of magnitude greater than the total anthropogenic sources (0.3 Tg versus 0.05 Tg; 30). The same pattern appears to be true for CH_3Cl as well.

The main organisms responsible for the halocarbon production appear to be marine algae, though terrestrial fungi are also capable of producing halocarbons (30). The biochemistry involved in halomethane production is unclear. There are several possible pathways involved in the production of these compounds, but there is strong evidence that at least some large fraction is the result of biosynthesis, rather than from untargeted side reactions. For example, algae are known to contain iodoperoxidases, and a methyltransferase has also been identified that methylates Cl ions (30). The reason why microorganisms produce these compounds remains unclear.

It is not surprising that, since microorganisms are capable of producing halocarbons, they are also capable of consuming them. There has been relatively little research on microbial consumption of halocarbons, but clear evidence exists that it does occur (28). For example as much as one-third of the methyl bromide that is applied to soil as a fumigant may be oxidized in situ (31). Atmospheric methyl bromide is also consumed in soils through microbial activity, and the measured rates are enough to account for 25% of the total global consumption of CH_3Br (28). The organisms involved may be methanotrophs or nitrifiers, as both methane and ammonia monooxygenase are capable of oxidizing CH_3Br. Organisms that are capable of consuming CH_3Br also appear able to consume other methyl halides (29). As for the other atmospheric gases, the primary control on the consumption of halocarbons is diffusion into the soil. Thus, soil structure and moisture content are the critical variables.

NITROGEN GASES: NITROUS OXIDE AND NITRIC OXIDE

Importance

There are two nitrogen-containing gases that are produced by microbial processes: nitrous oxide (N_2O) and nitric oxide (NO). These gases can both be produced by several processes. Essentially all microbial processes that involve oxidizing or reducing nitrogen have the potential to produce one or both gases, though for most processes they are minor side products. The dominant sources of both N_2O and NO are nitrification and denitrification. As both gases are produced in the same pathways, they will be discussed together.

N_2O is a long-lived species in the atmosphere, with an average turnover time of 160 years (Table 1). It is stable in the troposphere, and its primary pathway for destruction is through reaction with $O(^1D)$ in the stratosphere (2). As a result of its lifetime and chemistry, N_2O is a powerful greenhouse gas (a global warming potential 280 times that of CO_2), and it is directly involved in ozone depletion.

NO, on the other hand behaves very differently in the atmosphere. NO is extremely reactive chemically, and so its lifetime in the atmosphere is short, ranging from less than 1 day up to several days (Table 1; 2). It is strongly involved in atmospheric reactions, including the production and destruction of tropospheric ozone. At low concentrations, NO catalyzes O_3 removal, whereas at high concentrations (as occurs in polluted air), NO catalyzes O_3 production (1). Because of its short lifetime, the role of NO in atmospheric processes tends to be local to regional, rather than global.

Budgets

The "likely" values of N_2O and NO fluxes are shown in Tables 4 and 5, respectively. The best-known value in the N_2O budget is its rate of atmospheric increase (3.9 Tg/year) as this can be measured reasonably accurately. There is substantial error in all the other terms, particularly the source estimates, because these are extrapolated from a limited set of field measurements. Several points, though are clear: the major sources to the atmosphere are the oceans, tropical forests, and agriculture. Whereas nitrification accounts for a substantial proportion of the total N_2O emitted (particularly in drier biomes), denitrification accounts for the bulk of emitted N_2O. The only substantial sink for N_2O is reaction in the stratosphere. The budget for NO is less well known, because it has a short lifetime in the atmosphere. However, the largest source is combustion, particularly of fossil fuels (24 Tg N/year). Soil emissions are the second largest source, with estimates ranging from 12 to 20 Tg N/year. The main source of NO is nitrification. In soils that become wet enough to stimulate denitrification, NO diffusion out of the soil is reduced and NO is more likely to be consumed

Table 4. Sources and Sinks of N_2O (Data from Ref. 2)

Source	Estimated Flux (Tg N/year)	Sink	Estimated Flux (Tg N/year)
	Microbial		
Oceans	1 to 5	Soil consumption	Low
Temperate forest	0.1 to 2.0		
Temperate grassland	0.5 to 2.0		
Tropical forest	2.2 to 3.7		
Tropical savanna	0.5 to 2.0		
Crop agriculture	1.8 to 5.3		
Feed lots	0.2 to 0.5		
	Nonmicrobial		
Industrial	0.7 to 1.8	Stratospheric reaction	9 to 16
Biomass burning	0.2 to 1.0		
Total sources	**10 to 17**	**Total sinks**	**9 to 16**
		Atmospheric increase	**3.1 to 4.7**

Table 5. Sources and Sinks of NO (Data from Refs. 2 and 32)

Source	Estimated Flux (Tg N/year)	Sink	Estimated Flux (Tg N/year)
	Microbial		
Temperate forest	0.1		
Temperate grassland	0.6		
Tropical forest	2.6		
Savanna	7.7		
Chaparral	2.0		
Crop agriculture	7.2		
	Nonmicrobial		
Fossil fuel combustion	24	Atmospheric oxidation	24 to 64
Biomass burning	8		
Lighting	5		
Total production	**57**.2	**Total sinks**	**24 to 64**

before it escapes. The vast bulk of the NO emitted to the atmosphere is consumed by reaction with OH• and O_3 in the troposphere.

Organisms and Physiology

Nitrification. In nitrification, NO and N_2O are produced by NH_4^+ oxidizers. These are a moderately diverse group of obligately aerobic, chemoautotrophic bacteria. Whereas most genera are found in the β-proteobacteria, some lineages are found in the χ-proteobacteria (33). Despite this, the biochemistry and enzymology of nitrification are quite similar in both sets of lineages (34). Ammonia is oxidized by the enzyme ammonia monooxygenase to produce hydroxylamine. Hydroxylamine is then oxidized to NO_2^-. Nitrous oxide is produced by ammonia oxidizers reducing NO_2^- to N_2O. Nitrite reduction is linked to NH_3 oxidation and occurs predominantly under low O_2 conditions (35). Nitrite reduction and N_2O production appears to be a minor side reaction in most natural ecosystems (accounting for a few percent of the NH_4^+ oxidized), but in sewage treatment systems and some other specialized circumstances it may account for a significant fraction of the total products (36).

Denitrification. Denitrification is broadly distributed across the bacteria (37). Almost all known denitrifiers are aerobic bacteria that switch to denitrification when O_2 becomes limiting. Most denitrifiers are chemoheterotrophs and use organic carbon as an electron donor, with representative organisms covering most branches of the Proteobacteria, as well as some gram-positives, such as several species of *Bacillus*, and even some archaea. Denitrification is not, however, limited to the heterotrophs, as there are known denitrifiers among the sulfur oxidizers (e.g., *Thiobacillus denitrificans*), which uses reduced sulfur as an electron donor. This broad distribution of denitrification is likely because in most cases, it is a "backup" physiology that uses most of the same enzymatic machinery as aerobic respiration and only diverts electron flow at the terminal stage to NO_3^- (or other nitrogen oxides) instead of O_2 (37). Denitrification follows a multistep pathway in which NO_3^- is successively reduced to NO_2^-, NO (this functions as an intermediate, but its exact chemical status is unclear), N_2O, and finally to N_2. Most denitrifiers can carry out all the steps in the process, though there are many isolates that do not have (or have lost) nitrous oxide reductase (38).

Ecology

Controls on nitrogen-containing trace gas production by microbes have commonly been described by the "leaky pipe" analogy of Firestone and Davidson (39). The controls can be separated into those that control the overall rate of nitrification or dentrification (flow through the pipe) and those that control the fraction of product released as NO or N_2O (size of the leaks).

The two most general controls on both nitrification and denitrification are O_2 and nitrogen availability. Ultimately, low nitrogen availability (as NH_4^+) limits nitrification, and thus the supply of NO_3^- to denitrifiers as well (unless NO_3^- is transported in). Oxygen, on the other hand, acts as an on/off switch for both processes. Nitrification requires O_2 for ammonia monooxygenase, whereas denitrification is repressed by O_2.

Nitrification. Because of their great demand for NH_4^+ (they require it for both energy and biomass production), nitrifiers compete poorly against other microorganisms for NH_4^+. Thus, nitrification is invariably low in environments that are poor in available NH_4^+. In terrestrial systems, nitrification is generally rapid in agricultural soils and in many tropical soils. Tropical soils are often phosphorus limited, and so nitrogen is often present in excess. Grasslands sometimes have substantial rates of nitrification, whereas temperate and high-latitude forests are often nitrogen limited and so have low nitrification rates. In many aquatic systems nitrification occurs, even when NH_4^+ is dilute. The supply of carbon is often so limited that nitrifiers are able to compete for the available NH_4^+.

Nitrifiers are able to exist and function under many circumstances that would appear to be difficult for them, as long as adequate NH_4^+ is available. Thus in most NH_4^+-rich environments, NO_3^- dominates the inorganic nitrogen pools even if other factors might appear to be unfavorable for nitrification. One such factor is low pH. Low pH often leads to reduced nitrification rates, and many workers have concluded that nitrifiers are intolerant of low pH. This conclusion comes from both pure culture studies, where it has been very difficult to isolate acid tolerant nitrifiers, and field studies where nitrification is often observed to be limited in low pH environments. However, low pH environments are typically low in available NH_4^+, and thus would be expected to have low nitrification rates. If NH_4^+ levels are increased in such ecosystems, such

as occurred when a watershed was clear-cut at Hubbard Brook, New Hampshire, nitrification rates were very high, despite very low soil pH (40). Removing plants increased available NH_4^+ levels to the point that nitrifiers could compete. It is likely that part of the effect of pH is because nitrifiers act on NH_3, rather than NH_4^+, and at low pH the equilibrium is strongly to the side of NH_4^+ and so NH_3 is likely to be in particularly low supply.

Another factor that has often been discussed as a possible control on nitrification in terrestrial systems is toxic effects of specific plant chemicals, a phenomenon known as allelopathy (41). However, most of the effects of plant chemicals can be explained by changes in nitrogen mineralization and the supply of NH_4^+ to nitrifiers (42). Given recent results, it seems likely that specific toxicity of plant chemicals to nitrifiers is uncommon (43).

In nitrification, O_2 is the critical factor that controls the amount of nitrified nitrogen that is released as NO or N_2O. Since the trace gases are produced by NO_2^- reduction, this happens more rapidly when O_2 is limiting. However, because the process is aerobic, as O_2 drops so does the overall rate of nitrification. Thus, there is an optimum level of O_2 for producing trace gases that balances the rate of the process with the fraction of side products. This optimum has been found to be around 1% O_2 (44).

Denitrification. The primary control on denitrification is O_2 availability, as the process only occurs under anaerobic conditions (though one organism has been isolated that can denitrify aerobically-*Thiosphora pantotropha*; 45). Once conditions have become anaerobic, NO_3^- availability is generally the most important factor limiting denitrification rates, though carbon availability has been shown to limit rates in some circumstances. Denitrification functions rather differently from many other anaerobic processes because the organisms involved (facultative anaerobes) can live aerobically and denitrification can occur at relatively high redox potentials. Thus, denitrification can switch on rapidly when conditions become anaerobic. There is no need for an extended period of anaerobiosis for developing a substantial population of denitrifiers. Additionally, denitrification can occur in anaerobic microsites in an otherwise aerobic environment (46). So in a soil environment, denitrification usually occurs in pulses following rain events, when soil aggregates and organic matter fragments become wet enough to develop anaerobic microsites. In one classic study, Parkin (47) subdivided a soil core into progressively smaller components and showed that 85% of the total denitrification activity was associated with one small fragment of decaying plant material (Fig. 3). There are ecosystems where carbon and NO_3^- are more continuously available, where denitrification may function as more of a bulk process. One such important system is aquatic sediments where there is a constant downward diffusion of NO_3^- into the anaerobic zone. Another type of system is riparian areas along streams. In riparian zones, groundwater carries NO_3^- back to the soil surface in an area where high water contents induce anaerobiosis and root and litter inputs provide large amounts of available carbon (48). Riparian areas can therefore be very important as buffer zones to reduce NO_3^- runoff from agricultural lands. On the other hand, this same process may release a lot of N_2O to the atmosphere.

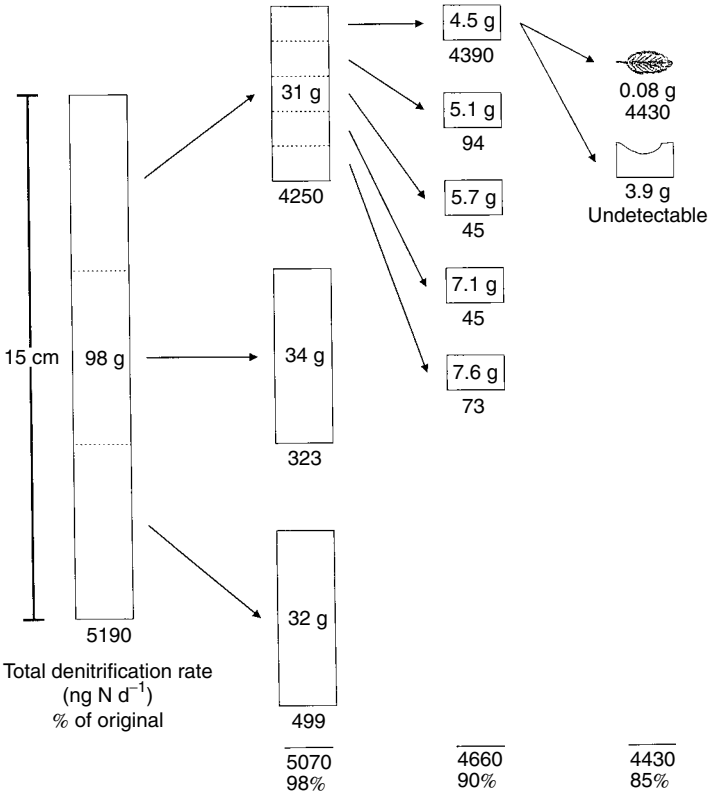

Figure 3. Microsite nature of denitrification. A single soil core was progressively subdivided into smaller and smaller components. The numbers at the bottom of the columns are the sum of the amounts of denitrification measured in each component and the rate percentage measured on the intact core. Ultimately 85% of the total denitrification in the core was associated with a single piece of decomposing plant material (from 47).

The controls on the proportion of NO and N_2O that are released as side products in denitrification are complex. In general, whereas N_2O can be the dominant product, NO rarely accounts for more than a few percent of the total product mix. As for nitrification, O_2 is an important factor in controlling the product mix. As O_2 increases, the fraction of products that remains in a more oxidized form (NO and N_2O) increases. However, as O_2 increases, the rate of denitrification decreases, producing an optimum where decreasing rates of denitrification balance the increasing fraction of product as NO or N_2O. Coincidentally, this balance is at roughly 1% O_2 (49). The balance of NO_3 and carbon availability is also important in controlling NO and N_2O production. When carbon is more available than NO_3^-, the NO_3^- will tend to be used "efficiently," that is, completely reduced to N_2. However, when NO_3^- is present in abundance, it is used less efficiently and more is released as N_2O and NO (39). A final factor that controls the proportion of N_2O released is pH. Nitrous oxide reduction is inhibited at low pH (38), and so N_2O can become the only significant product of denitrification.

SULFUR GASES

Importance and Budget

Both inorganic and organic sulfur gases are important in sulfur cycling and atmospheric chemistry. These gases are produced by different organisms under different conditions and in different ecosystems. The total emissions of sulfur gases to the atmosphere are shown in Table 6. The single largest source of sulfur to the atmosphere is industrial SO_2, but marine emissions of organic sulfur compounds, particularly dimethyl sulfide (DMS) are probably second in importance. All of the sulfur gases that are emitted to the atmosphere react with hydroxyl radicals and other oxidants to produce SO_2 and ultimately SO_4^{2-}. This is the dominant pathway for consumption of atmospheric sulfur gases (1); there are no significant microbial sinks for any of the sulfur gases.

Organic Sulfur Gases

The major organic S gas that is emitted to the atmosphere is DMS. The primary atmospheric source of DMS is marine algae, though smaller amounts are also produced by higher plants (such as *Spartina* spp.). It is produced as an enzymatic cleavage product of dimethylsulfonium propionate (DMSP). DMSP appears to serve primarily as an osmoregulatory agent in most of the major groups of marine algae. The major producers appear to be dinoflagellates, but other algae, including Chlorophyta, Rhodophyta, Phaeophyta, and others, also produce DMSP (3). DMSP is also produced by marine Cyanobacteria. The conversion of DMSP to DMS is accelerated by bacterial breakdown of senescent algae or by grazing. These various processes that cause release

Table 6. Sources for Sulfur Gases (Data from Refs. 3 and 50). Essentially all S gases are oxidized in the atmosphere

Biotic Source	Estimated Flux (Tg S/year)	Abiotic Source	Estimated Flux (Tg S/year)
		DMS	
Oceans	20	Biomass burning	??
Salt marshes	0.6		
Swamps	0.8		
Soil and plants	0.2 to 4.0		
		Other organic gases	
Oceans	0.7 to 1.7	Biomass burning	0 to 1
Salt marshes	0.3		
Swamps	4.8		
Soil and plants	0.8 to 3.5		
		H_2S	
Oceans	0 to 15	Biomass burning	0 to 1
Salt marshes	0.8 to 0.9		
Swamps	11.7		
Soil and plants	3 to 41		
		SO_2	
		Biomass burning	2.5
		Volcanoes	8
		Industry	67
Total biotic sources	43 to 104	Total abiotic sources	77.5
Total atmospheric oxidation		120.5 to 181.5	

of DMS can produce concentrations in surface seawater of up to several hundred nanograms per liter. This creates a strong DMS source to the atmosphere.

There is the potential for a strong negative feedback between DMS production and local climate: elevated temperatures increase DMS production and emission, which in turn increases cloud formation, reducing temperatures (1). However, increased cloudiness will also reduce light levels, reducing phytoplankton growth and DMS production, in turn producing a negative feedback on cloudiness. These feedbacks may produce a complex regulation between local marine climate and phytoplankton growth and DMS production. The full extent to which to this potential feedback loop actually functions, however, remains nuclear (51).

Other organic sulfur gases are released to the atmosphere, including dimethyl disulfide, carbon disulfide, and carbonyl sulfide. These gases together account for only around 9 Tg S, or about 10%, of total global S emissions to the atmosphere (3). These gases are produced by a variety of mechanisms, including as simple byproducts of the microbial decomposition of sulfur-containing organic molecules.

Hydrogen Sulfide

The second major source of sulfur to the atmosphere is H_2S. H_2S is produced under anaerobic conditions by dissimilatory reduction of sulfur. This process is a form of anaerobic respiration in which sulfur compounds are used as terminal electron acceptors. This process is carried out by a diverse group of strictly anaerobic bacteria, including members of the archaea, the gram-positive bacteria (genus *Desulfotomaculum*), and the gram-negative bacteria (52). These organisms have been divided into several major groups based on their physiologies. One fundamental distinction among the organisms is whether they are capable of reducing SO_4^{2-} to H_2S, or whether they can only reduce elemental sulfur (e.g., *Desulfuromonas*). Among sulfate reducers, there is a large group of bacteria that use acetate as their primary carbon source (including *Desulfobacter, Desulfosarcina*, etc.).

Sulfur reduction only occurs in low redox environments, such as under constantly flooded conditions, and where SO_4^{2-} or elemental sulfur concentrations are high. As the process is carried out by heterotrophs, organic carbon must be available. The most widespread environment where these conditions occur is marine sediments. Sulfur reduction may dominate the biogeochemistry that occurs in these sediments. However, sediments are not a large source of H_2S to the atmosphere, because much of the H_2S that is produced is oxidized in the aerobic surface sediments or in the water column, and more may precipitate out as pyrite or other metal sulfides. The largest actual biogenic sources of H_2S to the atmosphere are terrestrial (3). In terrestrial ecosystems highly reducing conditions exist close enough to the atmosphere that a substantial portion of the H_2S produced is released to the atmosphere. Salt marshes, in particular, are hot spots of H_2S emissions, because of the large inputs of SO_4^{2-} into a saturated environment. However, the small area of salt marshes globally reduces their importance in the overall global H_2S cycle.

Whereas microbes are not largely involved in removing sulfur gases from the atmosphere, H_2S consumption by autotrophic sulfur oxidizers is critical in limiting the amount of sulfur gases that are emitted to the atmosphere in the first place. In marine sediments and salt marshes, a large amount of energy is recycled through the oxidation/reduction cycle of H_2S/SO_4^{2-} and the rates of H_2S oxidation are very high. The role of sulfur oxidizers is thus analogous to the importance of methanotrophs in the global CH_4 cycle: neither group consumes a large fraction of the gases that make it into the atmosphere but both groups are critical to limiting what makes it into the atmosphere in the first place.

CONCLUSION

Microorganisms (including archaea, bacteria, fungi, and algae) are central to controlling the chemically and radiatively active components of the atmosphere, the trace gases, as shown here. The organisms involved in trace gas dynamics are diverse, and the processes involved are complex. Whereas many environmental and biotic factors interact in controlling trace gas dynamics, this entry has focused on single-factor controls. In fact, many of these factors can interact with each other to produce behaviors that are more complex than would be expected from single-factor analysis (53). Additionally, the specific composition of the microbial communities (rather than just the presence of a functional group such as methanotrophs) may affect the rates and controls of trace gas fluxes (54). Whereas we have an adequate understanding of the large-scale biogeochemistry of most trace gases, our understanding of the microbiology and microbial ecology controlling them is still somewhat limited.

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TRACERS IN GROUNDWATER: USE OF MICROORGANISMS AND MICROSPHERES

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Microorganisms have been deliberately injected into groundwater environments for well over a hundred years. Reasons for the additions include increasing oil recovery from less permeable zones, increasing rates of organic contaminant biodegradation, a better understanding of the hydrologic properties of aquifers, particularly those characterized by a high degree of physical heterogeneity, a better understanding of the factors controlling the transport and (or) survival of pathogenic, genetically engineered, or other nonindigenous microorganisms in groundwater systems, and assessments of the vulnerability of an aquifer to microbial (pathogen) contamination. This chapter will focus on addition and recovery studies in which microorganisms are used as tracers in order to gain information about subsurface microbial transport behavior, biorestoration potential, geohydrology, or vulnerability to microbial contamination of groundwater systems. Because of the importance of microbial-sized microspheres to the overall understanding of subsurface microbial attachment and transport behavior and of the hydrologic properties of different types of aquifers, the uses of microspheres as microbial-sized tracers are also discussed.

HISTORY OF MICROBIAL TRACERS IN GROUNDWATER

The first published studies involving the use of microorganisms as tracers in injection and recovery studies of

groundwater systems occurred in the latter part of the 19th century. In the earliest studies, pigment-producing bacteria were used to delineate flow paths in karst and fractured-rock aquifers (1,2). During the following century, other types of microorganisms were employed, including enteric viruses, bacteriophages, yeast, and protozoa. However, the use of human and animal viruses as tracers in groundwater studies has been limited because of safety and water quality considerations. There are a number of reviews focusing on the use of microorganisms as groundwater tracers. The interested reader is referred to Keswick and coworkers (3) and Gerba (4) for brief descriptions of microbial tracer studies conducted before 1981, to Harvey (5) for a history of microbial tracers up to 1997, to Hötzl and coworkers (6) for descriptions of studies in which microbial tracers are used in a number of geohydrologic studies involving granular and karstic aquifers, and to Rossi and coworkers (7) for a discussion of the use of viruses in groundwater studies.

Bacteriophages

Following their first use as a surface water tracer as described by Wimpenny and coworkers in 1972 (8), bacteriophages (bacteria-specific viruses), often referred to simply as *phages*, were employed widely as tracers in groundwater applications. A modest number of phage tracer experiments involving groundwater systems were conducted in the 1970s and 1980s. Most notable were transport studies involving the use of an Aerobacter aerogenes phage (9), phage T4 in a gravel aquifer in New Zealand (10), three unspecified phages, having *Escherichia coli* K12, *Serratia marcescens* and *Enterobacter cloacae* as their respective hosts, in a chalk aquifer in the United Kingdom (11,12), phages f2 and P22H5 in a karst aquifer in Greece (13), and MS2 in a sandy aquifer in Arizona (14). A protocol for using phages as groundwater tracers in granular aquifers is depicted in Figure 1.

Many of the bacteriophages commonly used as tracers in groundwater applications are coliphages, that is, they have *E. coli* as their host. Although there are many RNA- and DNA- containing coliphages (15), only a few (e.g., MS2, T4, T7, ϕX174, and f2) were used in tracer applications involving subsurface media. In addition to the aforementioned list of coliphage tracers, it is noted that the popular tracer phage, PRD1, which can also infect a strain of *E. coli*, has *Salmonella typhimurium* as its primary host. Numerous injection and recovery studies were conducted in the 1990s that involved comparing coliphage and conservative solute tracers in a variety of groundwater environments. The tracer test sites included sandy aquifers in Cape Cod, Massachusetts (16–18), and Ontario (19), highly fractured till in Ontario (20), fractured saprolyte in Tennessee (21), a floodplain aquifer in Montana (22), an alluvial aquifer in New Zealand (23,24), and a dune recharge site in the Netherlands (25). In addition to their widespread use with conservative tracers in injection and recovery experiments, coliphages are often used as indicators or tracers of fecal contamination in groundwater environments, including recent groundwater studies in the United States (26,27), Spain (28), Finland (29), and Israel (30). As noted by Edberg and coworkers (31) and Leclerc and coworkers (32), there are a number of problems associated with the uses of coliphages as tracers of groundwater contamination and the future of such uses remains unclear. A major problem is the lack of a strong relationship between bacteriophage recovery from groundwater and enteric gastroenteritis outbreaks attributed specifically to enteric viruses. A detailed discussion of the use of coliphages as indicators of human enteric viruses in groundwater is provided by Snowdon and Cliver (15).

Recently, various marine phages have been used with conservative solutes as groundwater tracers to understand better the geohydrology in karst (7), heterogeneous (periglacial) granular (7,33), and fractured-granite (33) aquifers in Switzerland. Unlike coliphages and phages of other bacterial inhabitants of mammalian intestinal tracts, marine phages are not a regulatory or water quality concern because their hosts are harmless. Also, the background concentration of marine phages in freshwater aquifers should be zero, even in groundwater contaminated by domestic or agriculture wastes. Comparisons of the transport characteristics of different marine phages with coliphages, indicate that at least one marine phage, H40, may have superior transport characteristics to the coliphage MS2 (33). Recent genetic studies show that marine phages are closely related to coliphages, although their life histories can be quite different (34). Although marine phages have not been widely used as groundwater tracers outside Switzerland, the authors anticipate that their use in injection and recovery experiments will spread. Conversely, phages commonly associated with contaminated freshwater environments were used to trace septic tank contamination in the Florida Keys into the marine environment (35).

All tracer experiments employing phages have the drawback that the standard plaque assays do not account for the total mass of the phage in the sample. This is because the phages are subject to an inactivation rate (rate of loss of host infectivity). Although the inactivation rate often is described as a simple function of temperature, there is increasing evidence that inactivation is more complicated and in some cases, loss of infectivity in groundwater systems may be poorly calculated by mean groundwater temperature. This is because the inactivation rates are subject to change, depending on whether the phage is attached to surfaces or unattached. Also, the intersurface forces that come into play when phages interact with solution-solid and water-air interfaces can cause inactivation. One method of assessing the true mass movement of phages in the aquifer involves radiolabeling the protein capsid with S^{35} or genetic material therein with P^{32} (17,18). However, the hazards and regulatory issues involving the use of radiolabeled phages restrict its use to a small number of controlled groundwater study sites.

Bacteria

The choice of bacteria in the first (nineteenth century) injection and recovery microbial tracer studies was made primarily because of their ease of detection in collected groundwater samples, rather than their transport or survival characteristics. The fact that the bacteria

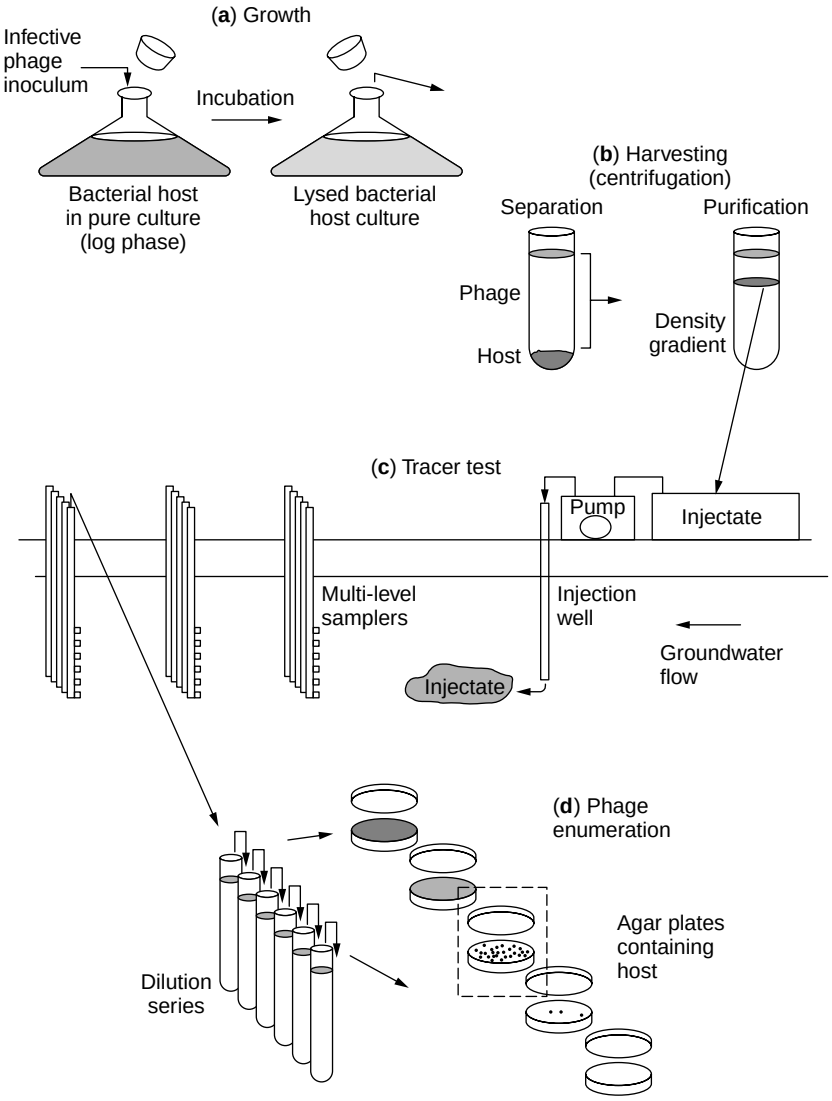

Figure 1. Protocol for the use of bacteria-specific viruses (bacteriophages) as tracers in injection and recovery tests in granular aquifer sediments: (**a**) Growth involves infecting the host bacterium during log phase growth in nutrient broth. (**b**) Purification of the phage involves initial separation of host cell debris from the new phage by centrifugation, followed by density gradient centrifugation of the supernatant. (**c**) Natural-gradient transport study involves a pulse injection of the purified phage and a conservative solute tracer upgradient from an array of multilevel samplers that are in rows perpendicular to the direction of groundwater flow. (**d**) Enumeration of phages in groundwater samples involves a dilution series and enumerating plaques (clearing zones) in agar plates containing a cloudy suspension of the bacterial host.

employed in the early studies formed brightly colored (red or yellow) colonies on solid media greatly facilitated differentiation of the tracer bacterium from the indigenous bacteria in the aquifer. There was a dearth of similar injection and recovery experiments through the first seven decades of the twentieth century, although a number of microbial tracer studies focused on tracking bacterial indicators of fecal contamination downgradient from known sources (36,37). Such studies provide very limited information about subsurface microbial transport because of difficulties in defining the source. A notable exception involved a bacterial tracer study in which known quantities of specific pigment-producing bacteria, including *S. marcescens*, were introduced into an Alaskan aquifer and tracked downgradient (38).

A resurgence in injection and recovery tests involving bacterial tracers occurred in the 1970s and 1980s. A number of bacterial tracer studies published during that period employed antibiotic resistance as a mechanism to facilitate differentiation of the bacteria of interest from other bacteria in groundwater samples (39,40). More definitive labeling of bacteria with a DNA-specific fluorescent (fluorochrome) stain, that is, DAPI (4′,6-diamidino-2-phenylindole) (41) and a radioisotope (P^{32}) (42) facilitated bacterial tracer experiments in the late 1980s. Other fluorochromes possessing a variety of wavelengths of excitation and emission are available for labeling bacteria and were considered in tracer applications. However, unlike DAPI, (43,44), several of the more common fluorochromes, that is, ethidium bromide (2,7-diamino-10-ethyl-9-phenyl-phenanthridinium bromide), acridine orange (3,6-bis(dimethylamino)acridinium chloride), and Hoechst 33258 (2′-(4-hydroxylphenyl)-5-(4-methyl-1-piperazinyl)-2,5′-bi-1-H-benzimidazole) can alter the attachment behavior of bacteria in porous media (44), thus limiting their use in groundwater tracer applications.

In the 1990s, microbial tracer studies involved tracking nalidixic acid-resistant (45) and antibiotic-resistant (46) *E. coli* and antibiotic-resistant *Pseudomonas fluorescens* (47) through the subsurface. Also, the first bacterial tracer tests were conducted in which bacteria

that were labeled with a stable isotope (C^{13}) (48) and a vital fluorescent stain, 5-(and 6-)-carboxyfluorescein diacetate, succinimidyl ester (CFDA/SE) (49), were injected into a sandy aquifer in Virginia. Also, a bacterial tracer was developed based on the ability of some naturally occurring and engineered bacteria to facilitate the formation of ice in small water droplets at approximately 5 °C (50). One such ice nucleation active (INA) bacterium, *Pseudomonas syringae*, was employed as a readily identifiable bacterial tracer in a tracer experiment involving a shale-saprolyte aquifer in Tennessee (21). A discussion of methods to label bacteria apriori for subsequent groundwater tracer studies is provided by Harvey and Harms (51) and Deflaun and coworkers (52). Colwell and coworkers (53) discuss the advantages of using a cyanobacterium as an easily quantified particulate tracer in the terrestrial subsurface. Beeman and Suflita (54) describe the use, as tracers, of sulfate-reducing bacteria serendipitously present in drilling muds to help assess to what degree subsurface core sample are compromised by the inadvertent introduction of non-indigenous microorganisms through drilling and recovery operations.

Yeast and Protozoa

Uses of yeasts and protozoa in groundwater tracer applications have been limited. The large size of yeasts and protozoa may preclude their use in many aquifer studies because of problems relating to correspondingly high sedimentation (settling) rates. Although the first use of eukaryotic microorganisms as tracers in groundwater studies is not clear, Reichert (55) mentions the use of "yeast bacteria" in aquifer tracer studies conducted in the early 1900s. In 1975 and 1976, hydrologic studies were conducted in Texas in which 3 μm Baker's yeast (*Saccharomyces cerevisiae*) were employed as particulate groundwater tracers along with conservative halide (chloride, bromide, and iodide) tracers. The purpose was to delineate secondary permeability features within a heterogeneous granular aquifer interdispersed with clay and partly cemented with calcium carbonate (56). Baker's yeast had the advantages of being easily obtainable in large (kg) quantities and being easily identified and counted. The first use of a protozoan tracer in an injection and recovery experiment occurred in 1991. In order to better understand the transport behavior of groundwater protists, the nanoflagellate, *Spumella guttula* (Kent) was isolated from a sandy aquifer in Massachusetts, grown up in liquid media, labeled with the vital stain hydroethidine, and injected back into the aquifer along with bromide (57). A subsequent experiment was conducted in 1994, in which *S. guttula* was grown in aquifer sediments under low pH and carbon conditions, labeled with DAPI, and again injected into the aquifer with a conservative tracer. Although the smaller (2 to 3 μm), porous-media-grown nanoflagellates were advected through the sandy sediments (58), their delicate nature and the difficulties in obtaining large numbers limit their usefulness as groundwater tracers.

Microspheres and Spores

Well-characterized, microbial-sized microspheres (minute beads consisting of organic polymers) were first employed as particulate surrogates for microbial groundwater tracers starting in the 1980s. In 1986, bacteria-sized (0.2, 0.5, 0.6, 0.7, 0.9, 1.4 μm) microspheres that differed in surface properties were injected into and recovered from a sandy aquifer in Cape Cod, Massachusetts to gain information about the physicochemical controls of subsurface microbial transport (Table 1; 41). Microspheres

Table 1. Use of Microbial-Sized Microsphere Tracers in Granular Aquifers (in Progress)

Factor aquifer	Microsphere	Size μm	Test		RF[b] %	RF[c]	Ref.
			Type	Distance[a]			
Heterogeneity							
silt/sand/gravel/pebbles	Polystyrene	2	FG[d]	1–4	0.2–0.5	NC[e]	62
well-sorted sand	Carboxylated	2	NG[e]	2–4	0.5–1.0	NC[e]	57
well-sorted sand	Carboxylated	0.7	NG	6	1.0–1.7[f]	NC[e]	59
Charge							
well-sorted sand	Uncharged latex	0.5	NG	7	1.0	0.05	41
well-sorted sand	Polyacrolein	0.8	NG	7	1.3	3.11	41
well-sorted sand	Carboxylated	0.5	NG	7	1.4	0.04	41
Size							
well-sorted sand	Carboxylated	0.2	NG	7	1.4	0.01	41
well-sorted sand	Carboxylated	0.9	NG	7	1.4	0.06	41
well-sorted sand	Carboxylated	1.4	NG	7	1.1	0.12	41

[a] meters.
[b] RF is retardation factor (ratios of the respective velocities of the peak concentrations of the microorganisms and conservative solutes).
[c] RB is relative breakthrough, determined by dividing the numerical integration of the microspheres abundance versus time by that of the conservative tracer.
[d] NG is natural-gradient.
[e] NC = not calculated.
[e] FG is forced-gradient.
[f] Range of values correspond to the three different depths, that is, 8.8, 9.0, and 9.5 m below land surface.

in the 0.8, 1.2, and 2.1 μm size class were also injected into and recovered from a fractured-granite aquifer at the Chalk River site in Ontario (42). A number of injection and recovery experiments employing virus-, bacteria-, and (or) protist-sized microspheres in a variety of geohydrologic environments were conducted in the 1990s. These tests included injections of both bacteria-sized (0.7 μm) (16,59) and protozoa-sized (57) (2 to 6 μm) microspheres at the Cape Cod site, bacteria-sized (0.2 to 1.0 μm) microspheres in a fractured-rock sites in Northern California and New Hampshire (60), bacteria-sized microspheres in marine sediments (61), virus-sized (100 nm) microspheres in a weathered shale saprolyte in Tennessee (21), and protist-sized (2, 5, and 15 μm) microspheres in a heterogeneous granular aquifer in Idaho (62).

Microbial-sized microspheres sometimes are injected into the subsurface to determine the degree by which the aquifer material, subsequently recovered from the same site, has been compromised by drilling and recovery activities. This microsphere tracer application involves determination of the number of log unit reductions between the abundance of microspheres added to the drilling fluid or borehole and the microsphere abundance in the recovered core material. Examples of this use of microspheres as subsurface tracers for quality-control purposes include studies involving deep aquifer sediments in South Carolina (63) and subsurface sediment and basalt in Idaho (64).

Various types of spores have been used as particulate tracers in groundwater systems. Many of these studies involved *Lycopodium clavatum* (clubmoss) spores that are typically approximately 30 μm and are stained apriori for easy identification in groundwater samples. The first published use of *L. clavatum* spores as groundwater tracers occurred in 1926 (65). However, stained spores of *L. clavatum* have largely been restricted to hydrologic studies involving karst aquifers (66,67). This is because the relatively large sizes of *L. clavatum* spores render them a less attractive choice in many fractured-rock and granular aquifers. Kass and Reichert (68) describe methodologies for using spores as groundwater tracers, including preparation, sampling, and quantification. Endospores of the bacterium, *Bacillus subtilis* were used in several injection and recovery studies involving heterogeneous granular aquifers in New Zealand (24). The small (<1 μm) size and high resistance to environmental conditions make bacterial endospores better candidates as groundwater tracers in many granular and fractured groundwater systems.

HYDROLOGIC STUDIES

The search for definitive, quantifiable particulate tracers to understand better the hydrology of different types of aquifers provided the impetus for the first uses of microorganisms as groundwater tracers. It should be noted that particulate and solute tracers can exhibit very different transport behaviors in the same aquifer. Many of the hydrologic studies employing microbial tracers that were conducted during the last few decades used both particulate and conservative solutes. In the process of assessing and comparing the breakthrough curves of both microbial and solute tracers, important information has been acquired, not only about the hydrology of aquifers, but about how microorganisms being transported through different groundwater environments are affected by chemical and physical heterogeneities.

Table 2 lists the retardation factors (ratio of the velocity of the microorganisms to that of the conservative tracers) for microbes being advected downgradient in injection and recovery tests. Results are compared for a number of different types of subsurface media. The retardation of microorganisms relative to a conservative tracer is a very complex phenomenon, but is strongly affected by the degrees of physical and chemical heterogeneity. In fractured media, the peak concentration of microorganisms appearing downgradient often significantly precedes that of a conservative tracer. For example, it is reported that the velocities associated with peak concentrations of microbial tracers exceeded those of a conservative tracer by two or more factors of ten in aquifers characterized by high heterogeneity and fracture-flow, that is, weathered-shale saprolyte (21) or clay-rich till (69). This leads to calculated retardation factors for microorganisms that are substantially less than one. The specific mechanism for apparently faster transport of the peak concentrations of microorganisms relative to those of conservative tracers remains unclear, although volume/pore-size exclusion of the microorganisms, hydrodynamic retardation of the conservative solute, and immobilization of the trailing microbes can contribute to this phenomenon. Studies performed in highly heterogeneous granular aquifers in New Zealand and Switzerland indicate that the appearance of the peak abundance of microbial tracers can substantially precede that of conservative tracers, although substantially less so than in aquifers dominated by fracture flow.

A number of studies involving relatively homogeneous well-sorted granular media in Massachusetts suggest that the transport of viral-, bacterial-, and protozoan-tracers can often occur concomitantly with that of the conservative tracer (16,41,59,71), that is, the retardation factor is unity. This suggests that the microbial and conservative tracers are taking the same flow paths and accessing the same pore volumes. Studies involving karstic aquifers indicate that peak concentrations of microbial tracers and conservative solutes can also appear coincidently (6,7). However, such observations involving karst undoubtedly depend on the physical nature of the aquifer. Finally, substantial retardation of microorganisms advecting through granular media may be expected under conditions of unsaturated flow (72). This is because microorganisms, unlike conservative solutes, become trapped at air-water interfaces that move much more slowly than the bulk flow of water.

Microspheres were extensively used, in lieu of and in addition to microbial tracers, to gain information concerning the hydrology of different types of aquifers. Data concerning selected studies involving the injection and recovery of microbial-sized microsphere tracers that have resulted in information about how microorganisms move through granular aquifers are summarized in Table 1. Results of these studies indicate the roles of

Table 2. Retardation in Transport of Microorganisms Relative to Conservative Solutes for In situ Tracer Experiments Involving Different Types of Subsurface Media

Media Type	Microorganism(s)		Test		RF[a]	Ref.
	Species	Type	Type	Distance		
Fractured						
Shale saprolyte (Tennessee)	MS2, PRD1	phage	NG[b]	35 m	0.002	21
Clay-rich till (Ontario)	MS2 & PRD1	phage	NG	4 m	0.01	69
Granite (Ontario)	*Escherichia coli*	bacterium	FG[c]	13 m	0.1	42
Granite (Switzerland)	Hb, H40, MS2	phages	FG	1.2 m	0.4–0.7	33
Layered basalt (Hawaii)	*Bacillus* sp	bacterium	FG	27 m	0.6	5
Granular, highly heterogeneous						
Periglacial gravel/cobbles (Switzerland)	H4, H6, H40	marine phages	NG	10–64 m	0.2–0.9	33
Sand/gravel/clay/carbonates (Texas)	*Saccharomyces cerevisia*	yeast	FG	1.5 m	0.7	56
Alluvial gravel (New Zealand)	*Bacillus subtilis* endospores	bacterium	NG	20–90 m	0.9	70
Karst						
Jura limestone (Switzerland)	H40 & H6	marine phages	NG	2.5 km	1.0	7
Lurbach System karst (Austria)	P22H5	phage	NG	5 km	1.0	6
Granular, Relatively Homogeneous						
Well-sorted sand & gravel	Indigenous community	bacteria	FG	1.7 m	1.0	41
(Massachusetts)	Indigenous community	bacteria	NG	6.7	1.0	70
	PRD1	phage	NG	13	1.0	16
	Spumella guttula (Kent)	nanoflagellate	NG	1.0	1.0[e]	58
Granular, unsaturated						
Tujunga loamy sand (California)	MS-2	phage	UF[d]	0.3 m	254	71

[a]Retardation factor (ratios of the respective velocities of the peak concentrations of the microorganisms and conservative solutes).
[b]NG is natural-gradient.
[c]FG is forced-gradient.
[d]UF is unsaturated flow.
[e]Flagellates grown in liquid broth exhibited retardation factors of up to 4.7 (31) at the same field-site location.

heterogeneity in aquifer structure, of microbial size, and of microbial surface charge upon the retardation and relative breakthrough/immobilization of microorganisms, as they are advected downgradient. Microspheres with similar surface chemistries and buoyant densities, but different sizes, were important in determining the effect of microbial size during transport through granular (41) and highly fractured (60,73) aquifers. Microspheres were also useful in determining the suitability of applying the clean-bed colloid-filtration theory (74) in multi component models describing microbial transport in a well-sorted sandy aquifer (41,71) and in determining whether or not 2 to 15 μm sized bacteria-containing encapsulations could be efficiently dispersed in a heterogeneous granular aquifer (41).

PUBLIC HEALTH STUDIES

In the United States, subsurface transport of pathogens continues to be responsible for a majority of the outbreaks of waterborne disease. The interested reader is referred to Macler and Merkle (75) for discussions of what is currently known about groundwater pathogens and their control. Valuable information was gathered about how pathogens behave in the subsurface by using microbial tracers, including the use of nonpathogenic surrogates in groundwater injection and recovery experiments. More specifically, microbial tracers have been useful tools for delineating the likelihood of pathogen transport pathways from known sources of subsurface microbial contamination and have provided a better delineation of how pathogens move in different types of media. Microbial tracer information has been valuable to regulators, environmental engineers, public health officials, and environmental microbiologists.

Future experiments should focus on the use of tracer microorganisms to assess the vulnerability of various aquifers to microbial contamination. Of particular concern is microbial contamination resulting from ongoing and planned aquifer storage and recovery, deep well injection of treated domestic wastewater, artificial recharge, bank filtration, and deployment of decentralized waster water treatment systems (e.g., septic tanks). The utility of a microbial tracer for aquifer vulnerability studies depend, in part, on how well the tracer represents the survival and transport characteristics of the pathogens of concern in groundwater. Macler and Merkle (75) list the contaminant groundwater viruses of concern as Coxsackie, Echo, Norwalk, Hepatitis A & E, Rota, Enteric adeno, Calici, and Astro viruses. They also list the bacterial pathogens of concern as *E. coli*, *Salmonella* spp., *Shigella* spp., *Campylobacter jejuni*, *Yersinia* spp., *Legionella* spp., *Vibrio cholera* and the protozoan pathogens as the obligate parasites *Cryptosporidium parvum* and *Giardia lamblia*. However, it would be difficult, at best, to find a microbial tracer that represents the transport and survival of more than a few of the aforementioned pathogens.

Phages have often been the microbial tracer of choice, largely because they are harmless, easy to assay

using standard plaque assays, and exhibit good survival characteristics (3). Phages are found in a number of habitats, including marine and freshwater environments, wastewater, soils, plants, animals, and food (7). It is clear that phages are an important and frequently abundant component of a number of aquatic ecosystems (76,77). For some marine systems, phage abundances can be as high as 10^7to10^8 per mL (78). However, phage tracers can be differentiated easily from the often abundant indigenous phages in aqueous systems because of their high degree of host specificity, often involving a single bacterial host. Their small size (10 to 200 nm) also contributes to their suitability as a tracer because it results in negligible settling, good penetration into smaller porosity, and a low propensity for clogging of granular media. Although a number of phages, particularly coliphages, were used as tracer viruses in public health studies, a number of recent studies focused specifically on PRD1 and (or) MS2. Schijven (79,80) describes the extensive series of experiments and modeling efforts concerning the use of PRD1 and MS2 to assess the efficacy of groundwater environments in the Netherlands to remove harmful viruses. Unfortunately, important questions remain about how well PRD1 and MS2 represent the pathogenic viruses of interest in groundwater applications. At the time of this writing, detailed in situ studies were yet to be conducted to determine the suitability of many other phages as groundwater tracers or as surrogates for other viruses.

BIOAUGMENTATION/BIORESTORATION STUDIES

Recently, experiments were conducted in which microorganisms were added to subsurface media and used as tracers to gain useful information for the growing field of in situ bioremediation. Bioaugmentation involves the addition to the contaminated subsurface of specialized or genetically engineered microorganisms (GEMs) to affect a measurable degree of biodegradation. The majority of studies supporting bioaugmentation involved laboratory microcosms. This is, in part, because regulatory concerns have restricted the uses of GEMs in the field. However, critical information needed to predict the potential of GEMs to disperse and survive in contaminated subsurface environments must come from in situ experiments. This is because many of the complex and interrelated processes operative on the field-scale are not represented well in lab-scale studies. One of the first microbial "tracer" applications in support of future biorestoration procedures involved the injection of *Pseudomonas* sp. strain B13, a 3-chlorobenzoate metabolizing bacterium, into the aquifer in Cape Cod, MA. The bacterium was subsequently recovered from aquifer sediments 14 months later (81) suggesting that the survival time in an aquifer for a nonindigenous, genetically altered bacterium may be long enough to enhance aquifer remediation, at least under some circumstances. Although 3-chlorobenzoate was not present around the zone of injection, the genes required to affect degradation of that compound served as a key to its identification and quantification in aquifer samples. In a subsequent in situ bioremediation study, the bacterium *Pseudomonas stutzeri* strain KC that degrades carbon tetrachloride (CT) under denitrifying conditions was injected into a granular, CT- and nitrate-contaminated aquifer in Michigan (82). The bacterium not only served as its own microbial tracer, based on its DNA sequences and tendency to form distinctive "fried-egg" shaped colonies on solid media, it substantively enhanced the degradation of CT within the aquifer.

Another "tracer" application of bacteria for contaminated subsurface environments involves the use of specific GEMs as "sentinels" or indicators of whether or not specific contaminants are present and how the contaminants are distributed. One study involved a controlled, intermediate-scale field release of a bioluminescent bacterium *P. fluorescens* Hk44 in a vadose zone contaminated with polyaromatic hydrocarbons (PAHs). The bacterium harbored an introduced lux gene fused with a naphthalene-degradative pathway, allowing the bacterium to bioluminesce in the presence of naphthalene (83). The bioluminescence at depth then was measured by employing fiber-optic-based biosensor devices. The use of lux-based bioreporter bacteria such as *P. fluorescens* Hk44 as tracers for subsurface contaminants is a promising new tool in aquifer biorestoration.

Another recent subsurface bioaugmentation study involving the addition of a GEM included the addition to atrazine-contaminated soil of killed, stabilized *E. coli* genetically engineered to overproduce atrazine chlorohydrolase (84). Results supported the tractability for using bacteria engineered with "overexpressing" catabolic genes to increase significantly in situ biodegradation rates. However, many technical problems regarding the use of GEMs in subsurface bioaugmentation lie ahead. Recently, the field-usage of GEMs was permitted at a number of locations. Consequently, future additions of GEMs to contaminated aquifers and vadose zones are likely to be forthcoming in order to gain more information about the feasibility of using different recombinant bacteria to enhance biodegradation in a variety of subsurface environments. In these types of experiments, the GEMs can serve both as microbial tracers, because their engineered DNA sequences can be traced in space and time, and as agents of bioremediation.

CONCLUSION

In conclusion, microorganisms and microbial-sized microspheres are used as tracers in groundwater-related studies in many ways. The first injection and recovery studies in which microorganisms were used to delineate groundwater flow in karst have evolved into much more complex experiments involving both conservative and particulate tracers and well-instrumented sites in many different geohydrologic environments. The use of more sophisticated labeling methods, such as the use of recombinant DNA, is allowing for more efficient differentiation and quantification of microorganisms introduced into freshwater aquifers. In turn, the increasing number and technical sophistication of injection and recovery studies employing microbial tracers will further enhance our knowledge of groundwater systems, particularly karstic, fractured rock, and heterogeneous granular aquifers, and of the potential

fate of pathogens in different types of groundwater environments. Studies that employ microorganisms as tracers also facilitate new bioaugmentation technologies that will be used in future aquifer restoration projects.

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TRANSPORT OF BACTERIA THROUGH SOILS.

See Wetlands: Biodegradation of Organic Pollutants

TRANSPORT OF PATHOGENS IN SURFACE WATERS.

See Source Water Protection: Microbiology of Source Water

TROPHIC STATE AND ALGAL COMMUNITY STRUCTURE.

See Eutrophication and Algae

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UNBALANCED GROWTH CONDITIONS.

See STORAGE POLYMERS: ROLE IN THE ECOLOGY OF ACTIVATED SLUDGE

URANIUM CYCLING.

See METAL (U, Fe, Mn, Hg) CYCLING

USE OF COLD-ADAPTED MICROORGANISMS IN BIOTECHNOLOGY

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In most environments, more than 99% of the prokaryotic microorganisms (generically referred to as *bacteria*) observed under a microscope are unculturable, yet they represent considerable genetic diversity, as determined by molecular techniques (1,2). Given that most of earth's inhabitable environments are permanently cold [$<20\,^{\circ}C$; $<5\,^{\circ}C$ in the volumetrically dominant ocean (3)], a significant fraction of the unculturable microbial life is likely to be cold-adapted — uniquely evolved to exploit the resources present in their vast and chemically diverse aquatic and terrestrial environments. In fact, native consortia of cold-adapted bacteria often catalyze reactions at rates comparable to their mesophilic counterparts inhabiting warmer climes (4). Knowledge of the genetics and physiology of isolates in culture and biogeochemical information on native consortia in cold environments indicate that the probability of these diverse uncultured microorganisms expressing novel processes or products of biotechnological interest is high. This article provides a review and prospectus of molecular-genetic technologies, catalytic proteins or enzymes, and bioremediation strategies as they pertain to the biotechnological application of cold-adapted microorganisms.

Although the terms "psychrophilic" (cold-loving) and "psychrotolerant" (cold-tolerant, also synonymous with "psychrotrophic") have been used in the microbiological literature for decades to describe varying degrees of cold adaptation (3), their specific meanings in research articles are often unclear or misunderstood. Here we refer to those microorganisms capable of growth at temperatures below $15\,^{\circ}C$ (the lower threshold for mesophiles) as "cold-adapted." Cold-adapted thus includes bacteria that require low temperatures ($\leq 15\,^{\circ}C$) for optimum growth, originally defined by Morita (3) as psychrophiles, and those that prefer warmer temperatures (usually $>20^{\circ}C$) but can still grow slowly at temperatures as low as $0\,^{\circ}C$, which are considered psychrotolerant. For catalytic proteins and their functions at low temperatures, we use the term "cold-active," regardless of the thermal class of the enzyme-producing organism.

UNDERLYING PRINCIPLES AND MOLECULAR GENETIC TECHNOLOGIES

Recombinant Gene Cloning

Bacteria have evolved a variety of strategies at the molecular level to adapt to cold temperatures (5,6). Structural changes in cellular membranes that allow maintenance of membrane fluidity and permeability, for example, have long been understood to play a crucial role in growth at low temperatures (7). Structural changes in proteins that allow enzymatic activity in the cold, and thus low-temperature biotechnology applications of the proteins, are the subjects of intensive investigation. An ability to explore such adaptations at both the molecular and organismal level is expanding rapidly as powerful new tools have become available. The new field of proteomics, taking advantage of two-dimensional gel electrophoresis (8), can give insight into gene regulation and optimum expression conditions for cold-active enzymes. The still rapidly developing technology of recombinant gene cloning, however, has already played an important role in understanding the structure-function relationships of bacterial enzymes and in enhancing the production of commercially important enzymes for the food, detergent, and pharmaceutical industries (9). In fact, more than 50% of the industrially important enzymes are produced from recombinant expression systems (10).

Typical gene cloning techniques begin with the generation of random or specific DNA fragments from the organism (or environmental DNA sample) under study, followed by the joining (ligation) of these fragments to a DNA-containing vector (such as a plasmid or virus). The vector, complete with its foreign DNA, is replicated in great quantity after introduction into a suitable host (typically *Escherichia coli*). The DNA fragments of interest are then selected by methods depending on the genetic system used and the desired gene product. Screening methods can include activity assays, when the host system has also allowed for efficient transcription and translation of the gene, or approaches that begin with reisolation of the DNA and analysis of its size and sequence.

Recombinant DNA technology thus not only gains access to the direct nucleotide sequence of the gene of interest, but also potentially yields the gene product in large quantities. For proteins encoded by cloned genes to make up 10 to 50% of the total protein produced by the host *E. coli* cells is not uncommon (11). Furthermore, the expression of genes from the uncultivated vast

majority of microorganisms is absolutely dependent on recombinant DNA technology and identification of suitable expression systems. Examples of success with this approach for uncultivated cold-adapted microorganisms are already evident: recombinant technology has allowed for the cold-active DNA polymerase from the uncultivated psychrophilic archaeon, *Cenarchaeum symbiosum*, to be cloned, sequenced, and expressed in *E. coli* using genomic DNA prepared from a sample of marine sponge tissue (12).

In many cases, however, researchers have experienced difficulties expressing recombinant catalytic proteins derived from extremophiles (whether cold- or heat-loving) in mesophilic hosts such as *E. coli*. These enzymes may require conditions for activity and stability that are not conducive to growth of a mesophilic host. Relatively few genes from cold-adapted organisms have been expressed successfully in *E. coli*; gene expression is normally achieved in large quantities only when *E. coli* is grown at suboptimum temperatures (13–15). Discovery of a host expression system using a cold-adapted bacterium, in which the growth rate of the host at lower temperatures is higher than that of *E. coli*, is expected to enhance the ability to express cold-active enzymes efficiently. Developing a cold-active expression system should also circumvent the problem of expressed proteins becoming insoluble in the host cell because of suboptimum temperature, and thus interfering with efficient protein expression.

Genetic Compatability

Given the highly developed genetic systems in mesophiles and relative lack of comparable systems in cold-adapted bacteria, fundamental genetic compatibility between the two thermal classes of microorganisms has been instrumental to successful genetic engineering in low-temperature applications. Such compatability has been demonstrated by the successful exchange of whole plasmids, implying shared translational functions and regulatory motifs (16). For example, antibiotic resistance has been conferred from *E. coli* to Antarctic psychrotolerant bacteria, *Moraxella* spp. (17), and the capacity to degrade toluene, from a mesophilic strain of *Pseudomonas putida* to a psychrotolerant strain of the same species (18). The latter example emphasizes the potential of genetic engineering for enhancing bioremediative processes in natural cold environments (16). Indeed, known plasmids commonly encode catabolic functions that include the degradation of environmental contaminants such as naphthalene, chlorobiphenyl, and chlorobenzoate in addition to toluene (19). Plasmids can also be designed to encode multiple degradative steps that otherwise would require complex bacterial communities. For example, a *P. putida* strain (with unspecified thermal characteristics) was designed for the degradation of polyhalogenated compounds to nontoxic products that otherwise required both aerobic and anaerobic bacterial populations (20). The mere physiological capability thus engineered, however, does not necessarily result in expression in situ, nor can the designed bacterium be assumed to compete successfully with the natural flora.

Plasmid transfer may also occur in the opposite direction, from cold-adapted microorganisms to mesophiles. For example, a nonconjugative plasmid encoding resistance to chromium and ampicillin was transferred by electroporation and transformation from a psychrotolerant, cryptoendolithic Antarctic bacterium into other cryptoendolithic bacteria and *E. coli*, with successful conferral of resistance (21). A shuttle vector has recently been constructed from plasmids of the Antarctic bacterium *Psychrobacter* sp. and *E. coli* that allows genetic manipulation in both organisms (22). Taken together, these findings suggest the ready potential to express recently discovered novel plasmids, predominantly of unknown function, from cold marine environments (23,24). That the replicative burden of carrying such plasmids is maintained in a large percentage of the in situ population (24 to 28% in the study cited) implies a selective advantage associated with each plasmid, perhaps pertinent to biotechnologically useful chemical processes in these environments. Some studies suggest that the frequency of catabolic plasmids within a population increases with the degree of pollution (19), implying mechanisms for in situ and interspecific transfer at cold temperatures and an innate bioremediative capacity (25–29). Some of these plasmids have broad host-range replicons (30), suggesting a potential promiscuity of interest both to biotechnological and environmental applications. Concomitant with such benefits, however, are the implied risks of unintentional transfer of introduced plasmids into the community at large, with unknown and unpredictable consequences (31–33).

The fundamental genetic compatibility of cold-adapted organisms with mesophiles has also been emphasized by recent analyses of cold-shock responses in both groups. Cold shock refers to the synthesis of a novel suite of more than a dozen proteins in response to a rapid decline in temperature (34). Regulation of cold-shock genes at the transcriptional level (35) may be of particular interest from the perspective of genetic engineering. Numerous experiments involving mesophiles (*E. coli*), psychrotolerant bacteria (*Lactococcus lactis* MG1,363, *Yersinia enterocolitica*) and a psychrophile (*Colwellia maris* ABE-1) have indicated enhanced transcription of cold-shock genes during temperature downshift (36–42). Particular advances in understanding such cold-shock regulation have emerged through recent research on the psychrophile *C. maris* ABE-1 and its two genes for the enzyme isocitrate dehydrogenase, one of which is preferentially expressed at low temperatures. This low-temperature gene appears to be controlled transcriptionally with a cold-inducible promoter (a specialized DNA sequence preceding the gene and required for transcription) and a transcription silencer (regulating the promoter activity) (42). Deletion of the promoter prevents low temperature-dependent expression, whereas deletion of the silencer results in 20-fold overexpression of the gene at low temperature.

Fusion of a promoterless gene of biotechnological interest to a cold-inducible promoter should allow preferential expression of that gene with lowered temperature. This type of expression has been demonstrated in *E. coli* using a promoterless, non-cold-shock gene for chloramphenicol acetyltransferase (36). In an environmental or industrial

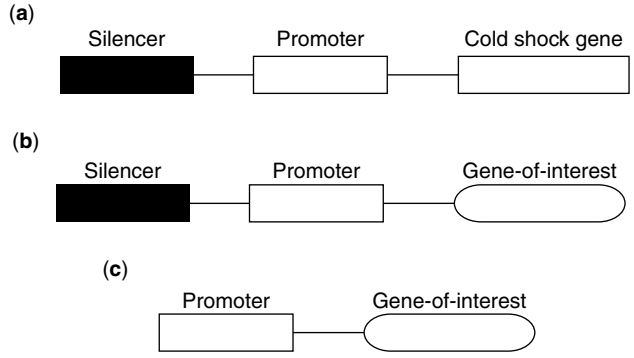

Figure 1. Schematic depiction of the potential exploitation of (**a**) cold-shock regulation, such as in *C. maris* ABE-1 (42), for (**b**) preferential expression of a gene of interest at low temperature or (**c**) mass production of the desired gene product at low temperature.

setting, this approach could provide a means to switch engineered genes — and with them, associated metabolic processes — on or off as a function of temperature. Deletion of a silencer, such as that in *C. maris* ABE-1, also suggests a simple mechanism to overexpress a given gene product specifically at low temperature (Fig. 1). Because the cold-shock response to decreases in temperature is relative to the normal growth temperature of the organism, exploitation of cold-shock genes could proceed at a variety of low temperatures by selecting an organism with the appropriate thermal characteristics. Because proper gene expression involves other factors, including structural stabilization of DNA and RNA and perhaps specialized RNA polymerases that may require additional regulatory components, such manipulation of intrinsic regulatory mechanisms may be more likely to succeed than de novo engineering.

Further advances in understanding and manipulating gene expression in cold-adapted bacteria can be expected with the establishment of appropriate genetic systems. Development of the psychrotolerant *Photobacterium profundum* strain SS9 system, which includes conjugal transfer of broad host-range plasmids and transposon mutagenesis (43), is encouraging. The linked goals of elucidating genetic regulation in psychrophiles and enhancing biotechnological exploitation of them should be facilitated further by completion of the first whole-genome sequences for psychrophilic microorganisms, anticipated for the Archaeon *Methanogenium frigidum* (44) and the Bacterium *Colwellia psychroerythraea* 34 H (45,46).

Viruses as Genetic Tools

Although viruses, specifically bacteriophage, are a common genetic tool for the manipulation of mesophilic bacteria, their use with cold-adapted microorganisms for biotechnological purposes represents a new field of endeavor. The potential for developing a low-temperature viral transduction system to facilitate genetic engineering work with psychrophiles (5,16) is supported by the number of viruses already isolated for cold-adapted bacteria, predominantly relevant to the food industry (47–53). Given high concentrations of viruses in the vast and cold ocean (54), appropriate phage-host systems may be much more abundant than realized. The typical specificity of a virus for a host species or strain (55) and the growing evidence for transduction in the natural environment (56–61) together suggest that targeted modification of a bacterial species may eventually be possible by in situ transduction (see Fig. 2). Viruses in nature are likely to influence bacterial community diversity and dynamics: they may select against dominant species, increase or decrease growth rate depending on the type of viral infection (62,63), regenerate nutrients through lysis to support growth of unsusceptible bacteria (64), and help maintain disadvantageous phenotypes in a population despite selective pressure (59). These properties, along with the specificity of viruses, may prove useful tools for stimulation or inhibition of select members of indigenous communities (Fig. 2) — a bioremediative strategy that may be more promising than the introduction of cultured organisms (65,66) discussed later.

Shortly after the discovery of bacterial viruses, d'Herelle proposed their use in the treatment of pathogenic bacterial infection (67), an idea subsequently elaborated in Sinclair Lewis's famous novel *Arrowsmith* (68). The discovery of antibiotics distracted researchers from further pursuit of this concept, but the growing specter of antibiotic resistance has renewed interest in the possibility. A number of characteristics of viral infection, including the viral replicative ability and specificity, make phage therapy attractive if sometimes problematic (69). Broad-scope antibiotics select resistant bacteria regardless of species; moreover, such resistance is frequently transferable. Resistance to a lytic phage, on the other hand, although likely to develop, will be restricted to the host species and has a reasonable probability of selecting for less virulent pathogens by selecting

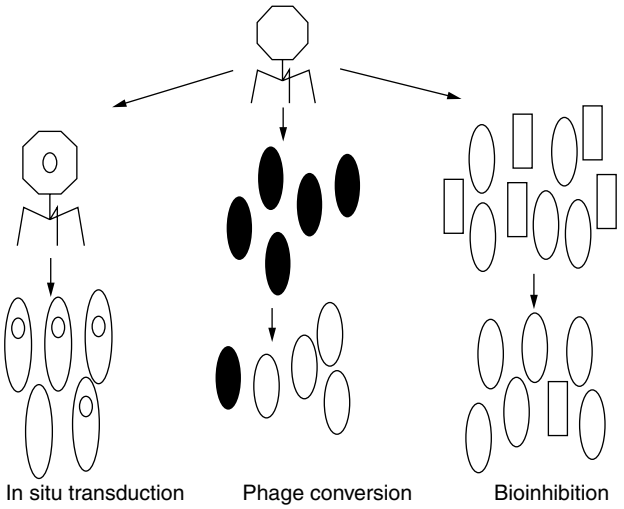

Figure 2. Potential exploitation of viruses for environmental bioremediation: transfer of foreign DNA (interior circles) encoding desired trait to select bacteria (ovals) in a natural community (in situ transduction); display of altered phenotype by infected bacteria (phage conversion); or shift in community structure from the natural dominant to desired bioremediator (bioinhibition).

against expression of antigenic surface receptors (70–72). Low-temperature phage therapy could be particularly practicable for applications in aquaculture, agriculture, and food storage. One study to consider such applications demonstrated that viruses could increase the shelf life of milk products an additional week at 7 °C by extending the lag phase of cold-adapted bacterial contaminants (53). The isolation of viruses specific to bacteria causing fish and meat spoilage (48,52) suggests additional applications of such an approach. Some bacterial pathogens or food-spoilers are psychrotolerant (*Listeria monocytogenes*) or psychrophilic (*Clostridium gasigenes*) (73) and could be controlled or attenuated by similar means. Numerous cold-adapted bacteria are known pathogens of fish (74) or fish eggs (75) and can cause serious fish diseases such as cold-water vibriosis (76). Antibiotic treatment in such cases is unsatisfying because of the likely development of resistance, whereas vaccination may injure fish, making alternative treatment possibilities desirable.

Other applications of cold-active viruses will involve their own genetic potential, because their encoded proteins and enzymes are also likely to be cold-active. Useful viral genes may be easier to find and express than their bacterial counterparts: viral genomes are much smaller than bacterial genomes; viruses replicating within a host may result in a high copy number (burst size); and the phenomenon of phage conversion may transform the bacterial phenotype to reflect viral genes (Fig. 2). For example, studies of viruses infecting bacteria that produce exopolysaccharides indicate that the viruses may encode the polysaccharase activity (77), the expression of which facilitates the spread of the virus through the biofilm. An analogous but cold-active enzyme could be employed to degrade or control biofilm formation in low-temperature industrial processes.

COLD-ACTIVE ENZYMES

For bacteria to grow in the cold, they must possess enzymes capable of catalyzing chemical reactions with high specific activity at low temperatures. Such enzymes generally have higher specific activities (in the range of 0 to 30 °C), lower temperature optima for activity (45), and greater lability at warmer temperatures than enzymes derived from mesophilic or thermophilic organisms (78,79). The realization that cold-active enzymes offer novel tools for biotechnological purposes (6) has sparked interest and increasing research in such promising areas as the expression and structural features of cold-active enzymes, especially as such knowledge can be applied to goal-oriented enzyme engineering.

Early approaches to enzyme research involved growing the enzyme-producing wild strain to sufficient cell density, obtaining an extract with the desired enzyme activity, purifying the native enzyme to homogeneity, and determining the amino acid composition and sequence (left pathway in Fig. 3). Note that the primary sequence of a cold-active enzyme was only determined in this way relatively recently (80). From the amino acid sequence, the nucleotide sequence of the gene encoding the enzyme

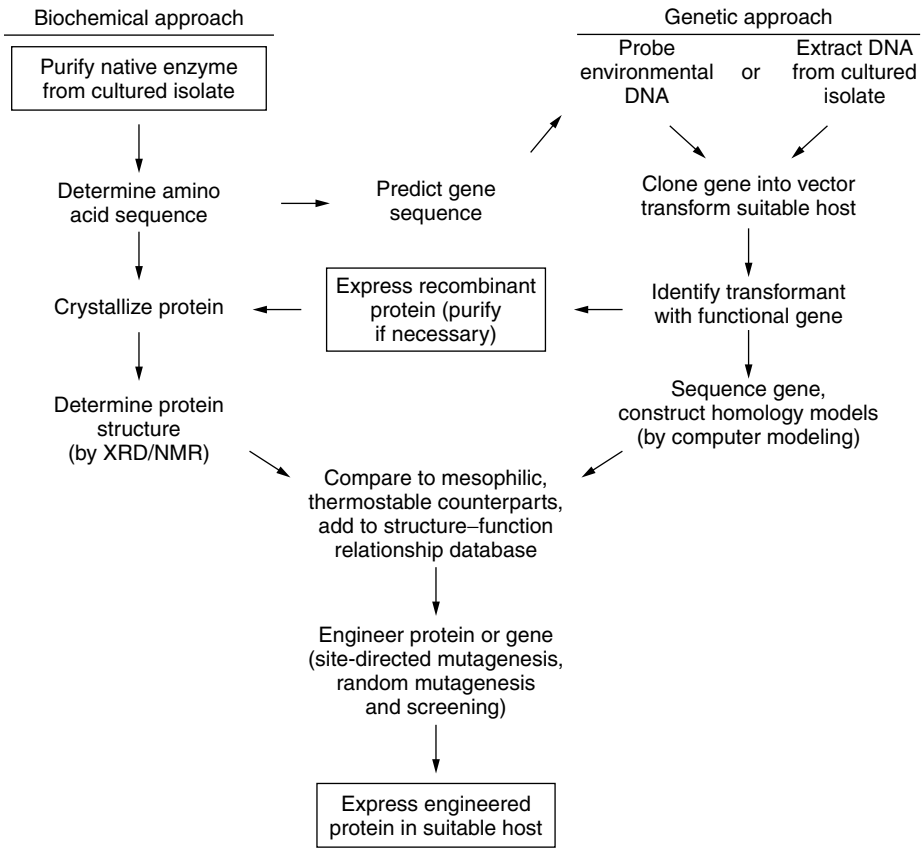

Figure 3. Traditional biochemical approach to studying enzyme structure and function (left), recombinant genetic approach (right), crossing and shared pathways, and steps (boxes) in which enzyme can be produced for biotechnological purposes.

Table 1. Cold-Active Enzymes That Have Been Cloned and Sequenced[a] from Prokaryotic Microorganisms

Enzyme	Enzyme Source	Publication Date (Reference)[a]
Lipase	*Pseudomonas* sp. B11-1	1988 (82)
	Moraxella sp. TA144	1991 (83)
	Psychrobacter immobilis	1993 (84)
Lactate dehydrogenase	*Bacillus psychrosaccharolyticus*	1990 (85)
Esterase	*Pseudomonas* sp. LS107d2	1992 (86)
α-Amylase[b]	*Alteromonas haloplanktis*	1992 (87)
Triosephosphate isomerase[b]	*Moraxella* sp. TA137	1993 (88)
	Vibrio sp. strain ANT-300	1995 (89)
Isocitrate dehydrogenase	*Vibrio* sp. ABE-1	1993 (39)
Subtilisin	*Bacillus* sp. TA41	1994 (90)
	Bacillus sp. TA-39	1997 (91)
β-Galactosidase	*Arthrobacter* sp. B7	1994 (92)
	Arthrobacter sp. B7	1995 (93)
	Carnobacterium piscicola BA	(1999) (94)
	Planococcus sp. SOS orange	2000 (95)
Pyruvate kinase	*Bacillus psychrophilus*	1995 (96)
Malate dehydrogenase[b]	*Vibrio* spp. strain 5,710	1996 (97)
	Photobacterium sp. SS9	1997 (98)
3-Isopropylmalate dehydrogenase	*Vibrio* sp. 15	1997 (99)
Citrate synthase[b]	*Eubacterial isolate* DS2-3R	1997 (14)
DNA polymerase	*Cenarchaeum symbiosum*	1997 (12)
β-Lactamase	*Psychrobacter immobilis* A5	1997 (100)
Protease[b]	*Pseudomonas fluorescens* CY091	1998 (101)
Elongation factor 2	*Methanococcus burtonii*	1998 (102)
Alanine dehydrogenase	*Shewanella* sp. Ac10	1999 (103)
	Carnobacterium sp. St2	1999 (103)
Glucanase	*Fibrobacter succinogenes* S85	1999 (104)
Serine alkaline protease	*Shewanella* strain Ac10	1999 (105)
DNA ligase	*Pseudoalteromonas haloplanktis*	2000 (106)
RNA helicase	*Methanococcoides burtonii*	2000 (107)
Phosphatase I	*Shewanella* sp.	2000 (108)
Glutamate dehydrogenase	*Psychrobacter* sp. TAD1	2000 (109)
Catalase	*Vibrio rumoiensis* S-1^T	2000 (110)
Phosphoglycerate kinase	*Pseudomonas* sp. TACII18	2000 (111)
Metalloprotease	*Pseudomonas* sp. TACII18	2000 (112)
Pectate lyase	*Pseudoalteromonas haloplanktis* ANT/505	2001 (113)

[a] Ordered by publication date to underscore newness of the field.
[b] Crystalline structure also now available (see Table 2).

can then be predicted. Determining the entire amino acid sequence of a protein is a substantial and not always straightforward process; determining the amino acid sequence from the encoding gene sequence instead is far easier (11). The development of recombinant techniques has made the latter approach feasible in many cases (right pathway in Fig. 3), obviating many of the problems associated with earlier biochemical approaches to the study of enzyme structure and function. The databases building from each approach, biochemical and genetic, now serve the other to maximize the research effort (cross pathways in Fig. 3).

Although the number of genes for cold-active proteins cloned and sequenced is relatively small compared with those of mesophilic and thermostable counterparts, it is growing rapidly (Table 1). Only very recently have researchers attained direct structural determinations of protein crystals for cold-active enzymes (Table 2), held by many to be the best approach to investigate the structure–function relationships of an enzyme. In the meantime, much of the research on structural aspects of cold-active enzymes has been the result of computer-based predictions of protein folding (homology models) based on gene sequences and the known three-dimensional structures of homologous enzymes optimized to warmer temperatures. Although this modeling approach is widely accepted, it can give only limited information critically dependent on the accuracy of computer programs (81). Often activity in the cold is due to unique structural modifications that simply cannot be predicted from gene-homology models. As

Table 2. Available Crystalline Structures for Cold-Active Enzymes[a] from Prokaryotic Microorganisms

Enzyme	Enzyme Source	Publication Date (Reference)[a]
Protease	*Pseudomonas aeruginosa*	1997 (114)
α-Amylase	*Alteromonas haloplanktis*	1998 (115)
Triosephosphate isomerase	*Vibrio marinus*	1998 (116)
Citrate synthase	*Eubacterial isolate* DS2-3R	1998 (35)
Malate deyhdrogenase	*Aquaspirillium arcticum*	1999 (117)

[a]Ordered by publication date to underscore newness of the field.

more structures for cold-active enzymes are solved, direct comparisons to homologous proteins optimized to warmer temperatures may yield a better understanding of the structure–function relationships that allow for activity in the cold.

Molecular Features Allowing for Cold Activity

Understanding molecular features that confer activity in the cold contributes to effective engineering and use of biotechnologically relevant enzymes. Molecular adaptations to temperature have been deduced largely from comparisons of mesophilic and highly thermostable enzymes. Although the active sites and overall three-dimensional structure of homologous enzymes tend to be conserved, a general relationship between overall enzyme flexibility, stability, and specific activity appears to exist.

For example, enzymes derived from thermophilic organisms have displayed high stability under conditions that denature mesophilic enzymes, but relatively low specific activity at moderate temperatures (118,119). This enhanced stability has been attributed to the relatively rigid, less flexible structure of thermostable enzymes. The most frequently observed features thought to contribute to this structural rigidity include:

1. increases in the number of salt-bridges;
2. increases in hydrophobic residues;
3. increases in ionic interactions and hydrogen bonding; and
4. reductions in the active site of the enzyme (120,121).

Cold-active enzymes, as inferred from high specific activities and thermolabilities, appear to be characterized by an increased flexibility of the overall polypeptide chain, enabling greater accessibility to the active site and helping to counteract the effects of low temperature (122). The recent availability of structural determinations of cold-active enzymes has enabled researchers to directly test the hypothesis that cold-active enzymes have a more flexible structure than their warmer counterparts. Preliminary trends to explain cold activity include:

1. decreased number of salt-bridges;
2. more polar and fewer hydrophobic residues;
3. fewer hydrogen bonds, aromatic interactions, and ion pairs; and
4. extended surface loops (79,123,124).

At first glance, the structural features conferring flexibility in cold-active enzymes appear to oppose those rendering enhancing stability in thermostable enzymes. An in-depth look at available gene sequences and protein structures, however, suggests that the relationship between stability, activity, and flexibility is more complex. When a small number of amino acids were introduced into a wild-type structure (via site-directed or random mutagenesis) in some recent studies (91,125), the thermostability and catalytic activity of the cold-active enzymes were both enhanced. Enzyme adaptation to temperature will not be summarized easily by a continuum of amino acid substitutions affecting overall structural flexibility along a temperature spectrum; cooperative local effects are likely to prove critical.

The first suggestion of the important role of local flexibility in cold activity came from comparative investigations of the crystalline structures of a cold-active salmon trypsin and a mesophilic bovine trypsin (126). The reduced stability of the cold-active trypsin appeared to derive from weakened interactions between local domains (regions) of the molecule, not differences in overall flexibility between the two structures. These weaker interdomain interactions were suggested to ease motion during catalysis, thus explaining increased catalytic efficiency of the cold-active trypsin. In a comparative study of the crystalline structure of cold-active citrate synthetase and a thermostable homolog (81), greater differences in flexibility between the large and small domains of the cold-active enzyme were considered to lead to greater relative movement and thus activity in the cold. Although maximal flexibility in domains directly involved in the catalytic reaction (the active sites) appears critical to cold activity, rigidity in areas not directly involved in catalysis is also important (127), as exemplified by thermal unfolding studies of cold-active and mesophilic phosphoglycerate kinases (128), in which the stability of a presumed noncatalytic domain actually exceeded that of the corresponding domain in the mesophilic homolog. Thus, activity in the cold may be a result of local flexibility and relative interdomain movement in enzymes, as opposed to overall flexibility.

Protein Engineering

Despite advances in deducing possible molecular adaptations from the first structures for cold-active enzymes, little progress has been made in determining specific mechanisms affecting enzyme activity. Often structures available for comparison are derived from divergent organisms that, as a result of genetic drift and differential selective pressures, may contain numerous amino acid differences not related to cold activity (129). Simply comparing the gene sequences of cold-active enzymes to existing structures of homologous enzymes is not intuitively satisfying and can be misleading. Alteration of the amino acid

sequence of a protein of interest in a controlled and documentable manner, known as protein engineering, has thus emerged as a useful tool for examining structure–function relationships.

Protein engineering can be achieved either through rational protein design using site-directed mutagenesis or through random mutagenesis, which includes directed evolutionary approaches. In site-directed mutagenesis, prior knowledge of enzyme structure is needed to make rational mutations in the encoding gene. Mutations can be in the form of replacement, deletion or insertion of one or more nucleotides into the gene sequence. Directed evolution, on the other hand, does not require that mutations be targeted; mutations are introduced randomly throughout the protein via sequential rounds of mutagenesis each followed by selective screening. Selecting for only adaptive mutations decreases the interference of neutral evolution. Directed evolution has been used in a variety of studies to generate specific enzymatic properties while simultaneously determining the mutations that led to them (125).

In addition to elucidating structure–function relationships, the application of protein engineering technologies to modify enzyme structures and give enhanced properties for biotechnological purposes appears to have unlimited potential. These methods have already been successful in engineering cold-active enzymes, in which both stability and activity were improved simultaneously (91,125). Such properties can be beneficial economically in industrial processing, resulting in higher rates of reaction with less enzyme loss because of denaturation. Also, enzyme immobilization is widely used in industrial applications to stabilize and retain enzyme activity (130,131). These benefits must be balanced, however, against diffusional limitations and decreased specific activity of some enzymes when immobilized to a surface because of limited accessibility of the active site (132). Given recently obtained knowledge of the importance of interdomain movement for cold activity in enzymes, protein engineering can now be used to substitute specific amino acids in the remaining stable regions known to bind preferentially to the support matrix. This approach should enable enzyme immobilization although allowing the catalytic domain to retain flexibility and remain exposed in solution, perhaps enhancing catalytic activity. In many cases, industrial processes target the production of artificial compounds using heat or other nonenzymatic means to generate them. Protein engineering may aid in the development of enzymes with novel catalytic properties that could replace or improve the efficiency of rates of production of such compounds.

Applications of Cold-Active Enzymes

Enzymes are ideally suited to catalyze the production or breakdown of specific compounds, both because of their high substrate specificity and greater ease of control, relative to an introduced organism, for example. Enzymes from extremophiles already play a significant role in a variety of applications — the annual market for thermostable enzymes is $250 million U.S. dollars (133). Although the economical value of cold-active enzymes has yet to be realized, the potential for use of their novel properties in a variety of applications is great. Growing interest in cold-active enzyme catalysis is evident through the establishment of such projects as "COLDZYME" in the European Union, aimed at coordinating research efforts to better understand the molecular basis of cold-activity in proteins and their potential biotechnological uses. Some of these applications are outlined later.

Food Industry. Enzymes have been involved in the preparation of foods for recorded history. Traditionally, cold-active enzymes have been associated negatively with food spoilage. The beneficial applications, however, that take advantage of their high catalytic activity at low temperatures and ease of inactivation are numerous. Cold-active β-galactosidases that work optimally at neutral pH can be used in milk to degrade lactose rapidly at cold temperatures, enhancing the digestibility and sweetness but minimizing the growth of contaminants (5), whereas β-galactosidases optimized to more acidic pH can be used to degrade lactose in acid-whey, a product of cottage cheese manufacture. The increased sweetness and fermentability of the resulting breakdown products, glucose and galactose, expand the potential to reuse whey as a sweetener for beverages, syrups, and so on., thus decreasing the demand for sweeteners while mitigating the cost of waste treatment (134). Cold-active lipases and proteases can be used to accelerate the ripening of cheese at low temperatures, whereas proteases may act as efficient meat tenderizers. In the juice industry, pectinases may be used to enhance juice yield and clarification, subsequently inactivated by a relatively small increase in temperature, resulting in a less "cooked" taste for the juices. In baking, enzymes such as amylases, proteases, and xylanases may be used to reduce the dough fermentation time by acting directly on starch, gluten, and hemicellulases (133). Although generally regarded as having hydrolytic properties, these enzymes can catalyze reverse reactions under certain conditions, thus synthesizing organic compounds (9). Cold-active enzymes may prove very lucrative in such processes because the amount of energy required to drive the reverse reaction of a cold-active enzyme will be relatively low as a result of their inherently low activation energies. Furthermore, cold-active enzymes may even replace some chemical preservatives in foods by acting to disrupt microbial cells, deplete metabolites, or degrade enzymes needed for contaminant growth (135,136). Although they are not technically enzymes, ice-nucleating proteins expressed by a number of cold-active bacterial species can serve as a template to trigger ice-crystal formation at subzero temperatures (-2 to $-5\,^{\circ}$C) at which water would otherwise remain liquid and supercooled. These proteins could be used to improve ice-cream manufacture, and freeze-texturing or freeze-drying of foods at relatively warm temperatures, thus decreasing energy consumption (137).

Detergent Industry. Hydrolytic enzymes such as proteases, lipases, α-amylases, and cellulases already comprise significant ingredients in many common detergents; the uses of proteases in laundry detergents accounts for approximately 25% of the global sale of enzymes (9). The benefits of using cold-active enzymes as detergent additives, thus allowing sufficient hydrolysis to take place at

cold temperatures, include reductions in energy consumption and the fabric degradation characteristic of warm- and hot-water washing. Along with energy constraints, the increasing use of synthetic fibers, which can only tolerate moderate wash temperatures, is also increasing the demand for cold-water washing. Other more specific uses of cold-active enzymes can also be projected. For example, cellulases can increase the softness of cotton garments that become frayed over time. Use of a cold-active enzyme for this purpose is particularly attractive because the enzyme could be inactivated during the wash cycle by a small increase in temperature so as to halt further degradation of the fabric (133).

Molecular Biology. Many of the enzymes presently used in molecular biotechnology were developed to work at moderate temperatures to facilitate research with *E. coli* cells and tissue cultures. Recent interest in working with and expressing components of cold-active systems in a variety of molecular applications creates the need for enzymes with higher activities at lower temperatures (see Table 3). The radioactive end-labeling of nucleic acids by T4 polynucleotide kinase, for example, is a useful tool in determining the structure of nucleic acids. Before kinase can act, however, removal of the existing phosphates at the 5′ termini of oligonucleotides must be completed by alkaline phosphatase (APase), whose activity must then be eliminated. Use of a cold-active APase would aid in this process (138) because of its thermolability at relatively moderate temperatures that do not affect nucleic acids. Presently, commercially available DNA ligases have poor activity below 15 °C, which results in long incubation times at these temperatures, thus increasing the risk of residual nuclease activity degrading the DNA and interfering with the ligation reaction. The application of a cold-active ligase would result in shorter incubation times, helping to alleviate nuclease degradation of DNA (106). Other enzymes directly involved with molecular processes such as a cold-active restriction endonuclease (139) and a DNA polymerase (12) also demonstrate great potential as tools for molecular research and biotechnology.

BIOREMEDIATION

As the global human population has grown, dependence on hydrocarbons for heating and transportation, on yields of large-scale agriculture boosted by the use of pesticides and herbicides, and on large-scale industrial processes to provide goods deemed essential or desirable has also increased. The scale of these activities and limited attention to containment have resulted in widespread pollution by a variety of chemicals. Although many of these compounds and their congeners may occur naturally, human exploitation and manipulation of such resources, even with the best of intentions, has resulted in amplification of chemical concentrations well above natural background levels, in some cases representing serious threats to ecosystem and human health. These problems are accentuated in polar environments, because many chemicals produced and released at low latitudes are transported by planetary circulation systems (atmospheric and oceanic) and deposited in cold high-latitude regions that otherwise would be pristine (140–146). Developing applications of cold-adapted microorganisms (or cold-active enzymes) to bioremediation, defined as the use of organisms (through their degradative enzymes) to remove hazardous pollutants from the environment (147),

Table 3. Examples of Demonstrated and Proposed Applications[a] of Cold-Active Heat-Labile Enzymes to Genetic Engineering

Enzyme	Application	Publication Date (Reference)
Alkaline phosphatase	Radioactive end-labeling of nucleic acids: enzyme rapidly inactivated with heat, allowing its removal for subsequent reaction steps	1984 (138)
DNA polymerase	DNA synthesis: DNA amplified enzymatically at cold temperatures	1997 (12)
Restriction endonucleases	Sequence-specific cleavage of DNA, including possible new specificity with blunt or single-stranded ends: enzyme activated at low temperatures, then inactivated by heating	1997 (139)
DNA ligase	DNA recombination: enzyme activated at low temperatures nonpermissive to competing nucleases, then inactivated by warmer temperatures still below that of DNA denaturation	2000 (106)
Reverse transcriptase	Reverse transcription of RNA into DNA: enzyme activated at low temperatures nonpermissive to competing nucleases could make RT-PCR more quantitative and possibly more economical	(proposed here)
Essential enzymes	Heat-controlled inactivation of organisms: deliberate design of strains with essential but heat-labile enzymes could allow more efficient exploitation of the organism and protection if inadvertently released	(proposed here)

[a] Ordered by publication date to underscore newness of the field.

thus emerges as an important endeavor in the broader field of environmental biotechnology. The concept of bioremediation currently includes both the manipulation of environmental conditions to enhance native microbial activities (biostimulation) and the addition of laboratory-cultured organisms with demonstrated and sometimes unique degradative abilities (bioaugmentation). Recent findings from both laboratory and field studies (addressed later) reveal the potential of cold-adapted microorganisms and their enzymes to provide solutions to the bioremediation of organic contaminants — hydrocarbons, substituted hydrocarbons, and other xenobiotics — present in cold environments.

Hydrocarbon Degradation

While some hydrocarbon compounds represent a direct threat to human and ecosystem health as a result of leaking underground storage tanks, oil spills, and chronic smaller-scale spills at transfer stations, many microorganisms have the ability to utilize hydrocarbons as sources of energy for metabolism and growth. The biodegradation of naturally occurring aliphatic (open-chained) hydrocarbons is almost exclusively an oxidative process, beginning with the addition of oxygen to the first or second carbon in the molecule, as mediated by the enzyme monoxygenase (148). The final product is acetyl CoA, which shunts into the citric acid cycle, the common central pathway for respiration in aerobic organisms. Unsaturated aliphatic hydrocarbons, characterized by a terminal double bond that is susceptible to anoxic decomposition, may also be oxidized by sulfate-reducing and other anaerobic bacteria.

Aromatic hydrocarbons (benzene-ring derivatives) comprise one of the most common classes of organic contaminants in sediments; many of these compounds, especially the polycyclic (multi-ringed) aromatic hydrocarbons or PAHs, are known carcinogens (149,150). Their strong hydrophobicity explains not only their sorption to sediment particles and organic materials, and thus concentration in freshwater and marine sediments, but also why such hydrocarbons tend to bioaccumulate in organisms, thus toxifying aquatic food webs. Many aromatic hydrocarbons can be degraded microbially under aerobic or anaerobic conditions, whereas PAHs with four or more fused rings are believed to be largely recalcitrant to biodegradation. Although microorganisms are generally understood to derive nutrition from materials in solution, direct action on particle-sorbed PAHs has been implicated (151). Biosurfactants and bioemulsifiers may play an important role in increasing the bioavailability, and thus biodegradability, of these strongly hydrophobic compounds (152–154).

Bioremediation in the Cold. Numerous studies on the potential for biological remediation of hydrocarbon contamination in cold environments have led to the conclusion that an indigenous fraction of hydrocarbon-degrading bacteria, present in virtually all environments, rapidly becomes enriched when a pollution event occurs. The hydrocarbon-degrading fraction of the population may be as small as 0.1% in pristine environments, yet constitute up to 100% of viable organisms in contaminated environments (155). Factors determining this enrichment effect, and thus the rate and extent of hydrocarbon degradation, include temperature, nutrient concentrations, aeration, bioavailability of the pollutant, and history of contamination of the site. The overall amount of hydrocarbons subject to indigenous microbial action after a spill depends on abiotic processes that contribute immediately to hydrocarbon losses from the system. The most volatile compounds are lost through evaporation, whereas other constituents of hydrocarbon mixtures may be transformed by photo-oxidation. Contamination can also be removed physically through wind, waves, and currents; numerous studies have reported that up to 30% of hydrocarbons are lost shortly after they enter an environment (156).

The specific effects of low temperature on the rate and extent of microbial degradation of hydrocarbons thus take both biological and nonbiological forms. Rates of microbially mediated processes are often observed to reduce by a factor of two for every 10 °C reduction in temperature (157,158), a phenomenon commonly known as the Q_{10} effect. Low temperatures make the target compounds more viscous, reduce evaporation of toxic short-chain alkanes, and increase water solubility (159), affecting the extent of abiotic loss and the degree to which a hydrocarbon mixture is available to microorganisms for degradation. The combined effects serve to delay the onset of hydrocarbon degradation.

Despite these commonly cited negative effects of low temperature, the indigenous genetic potential for hydrocarbon biodegradation in a variety of cold environments remains clear (Table 4), spurring continued interest in the potential for in situ attenuation and mineralization of contamination in cold climes. For example, of 135 psychrotolerant strains of bacteria isolated from pristine and contaminated sites all over Canada, including soils, sediments, lake water, seawater, and groundwater, most of the isolates were able to mineralize toluene, naphthalene, dodecane, and hexadecane at 5 °C (160). In the wake of the *Exxon Valdez* oil spill in Alaska, the genotype of the culturable hydrocarbon-degrading population was observed to change over time and reflect the hydrocarbon mixture to which it was exposed (161). In fact, the natural attenuation of many types of hydrocarbons has been demonstrated in cold environments, but over extended periods of time (162–164). Evaluations must be made on a case-by-case basis (147), but natural attenuation or in situ bioremediation may be most appropriate for spills in areas posing less immediate threats to ecosystem or human health.

Biostimulation. To enhance the rate of hydrocarbon degradation in situ at low temperatures, the addition of either oleophilic (oil-loving) or water-soluble fertilizers (biostimulation) has been a common and effective approach (Table 5). The most publicized in situ tests of biostimulation occurred after the *Exxon Valdez* oil spill in Alaska, where seawater temperatures fall between 10 and 17 °C. Improvements in the aesthetics of contaminated beaches, and increased rates of hydrocarbon

Table 4. Examples of the Degradation of Organic Pollutants at Low Temperatures by Cultured Microorganisms

Environmental Sources	Temperatures) (°C)	Organisms	Findings	Reference
Antarctic soil	−3.2, 3.1, 25, 37	Variety of isolates	Hydrocarbon-degrading strains isolated, need for adaptation to target compound if environment pristine recognized	165
Antarctic soil	16	*Rhodococcus* spp.	Psychrotolerant strains isolated from contaminated soil, able to degrade alkanes (hexane through eicosane)	166
Sub-Arctic coastline	10 to 17	Variety of isolates	Numerous plasmid-containing strains isolated, able to mineralize alkanes and aromatic hydrocarbons; composition of contaminants reflected in population genotype	161
Sub-Arctic and Arctic soil and sediment, sea-, fresh- and groundwater	5, 23	Variety of isolates	135 psychrotolerant strains isolated, most able to mineralize toluene, napthalene, dodecane, hexadecane; potentially novel pathways for toluene degradation, possible gene for degrading short and longer chain alkanes	160
Sub-Arctic coastline	5, 25	*Pseudomonas* spp.	Three plasmid-containing strains isolated, able to degrade alkanes (C_5-C_{12}), toluene and napthalene; both *nah* and *alk* plasmids carried by given strain	167

Table 5. Examples of Hydrocarbon Degradation at Low Temperatures by Native Microbial Consortia, With or Without Addition of Chemicals (Biostimulation)

Source or Type of Compounds	Environment	Temperatures (°C)	Findings	Reference
Arabian Light crude oil	Sub-Antarctic coastline	1 to 8	Degradation in winter low, due to effects of cold and initial state of native microbial consortia	164
Arabian Light diesel oil	Antarctic sea ice	−1.4 to −1.7	Degradation potential enhanced by addition of nutrients, number of hydrocarbon-degrading bacteria increased dramatically	172
Prudhoe Bay crude oil	Sub-Arctic coastline	10 to 17	Degradation enhanced by addition of oleophilic fertilizers, nutrients (inopol EAP22); number of hydrocarbon-degrading bacteria increased	168,169,173
JP-5 jet fuel, toluene	Arctic aquifer	4 to 6	Mineralization at low temperatures similar to mesophilic rate at 20 °C	162
JP-5, gasoline, diesel fuel	Arctic soils	∼10	Degradation enhanced by addition of lowest level of nutrients (N:P of 100 : 45)	174
Petroleum hydrocarbons, dodecane, phenanthrene	Arctic tundra	7, 15, 22	Mineralization substantial at 7 °C, not inhibited by heavy metals or high hydrocarbon concentrations, enhanced by nutrient additions and by inocula of indigenous or nonindigenous oil-degrading bacteria (see also Table 6)	171
Diesel oil	Alpine soils	10, 25	Degradation enhanced by addition of fertilizers	175

mineralization (complete degradation to carbon dioxide) compared with untreated plots, were reported (168–170) (note that distinguishing biological degradation from physical removal was not trivial and was later questioned (65)). From a study of Arctic permafrost, in which microbial communities were observed to degrade hydrocarbons actively at 4 to 7 °C, the availability of adequate nutrients was essential to obtaining appreciable rates of hydrocarbon degradation (171).

Bioaugmentation. The approach of adding microorganisms or groups of microorganisms with demonstrated biodegradative abilities (bioaugmentation; Table 6) has also been used in an attempt to improve both the rate and extent of hydrocarbon degradation. While several tests have been performed (65,149,176,177), few have yielded anything beyond a short-term increase in degradative activity or end-products significantly different from those generated by native organisms (171). Among the suspected shortcomings of this approach are the preferential use of other compounds by the added organisms and unsuccessful resource competition with native microorganisms. Bioaugmentation may be worth pursuing in particularly harsh or cold environments, where

Table 6. Examples of Hydrocarbon Degradation at Low Temperatures by Native Microbial Consortia, With or Without Addition of Microorganisms (Bioaugmentation)

Source or Type of Compounds	Environment	Temperatures (°C)	Findings	Reference
Alkanes, phenanthrene	Antarctic coastline	10, 25	Degradation of n-alkanes and phenanthrene with inocula from contaminated lake and beach sand	179
Diesel fuel, n-alkanes	Sub-Arctic soil	0, 5, 25	Mineralization of diesel fuel greater at 5 °C with inoculum of *Rhodococcus* sp. strain Q15	180
Diesel oil	Alpine soils	10	Degradation enhanced slightly with inoculum of cold-adapted bacteria, enhanced more by nutrient addition	176
Weathered diesel oil	Alpine soils	10, 25	Degradation inhibited by nutrient addition with inoculum, highly stimulated by nutrients without inoculum	177
Diesel oil	Alpine soils	10	Degradation temporarily enhanced by inoculum of oil-degrading bacteria, greatest effect with native consortium alone and fertilizer added	178

reduction in the lag time to biodegradation may be of greater importance than rate of removal (178).

Pollution History and Microbial Adaptation. The negative effects of cold temperature may also be overridden by pollution history as a factor in determining the rate and extent of hydrocarbon biodegradation, underscoring the importance of indigenous microbial adaptations to chronic pollution. For example, a lake subjected to chronic contamination had indigenous microorganisms that were able to degrade hydrocarbons at very low temperatures (0 °C), whereas organisms from pristine sites were unable to utilize hydrocarbons at that temperature (181). Other studies have also documented this phenomenon (160,179), including a case in which soils were challenged and rechallenged with the herbicide Napropamide (an aliphatic amide; see more on substituted hydrocarbons later). The time to 50% disappearance of the initial dose was observed after 25, 45, and 75 days at 25, 15 and 5 °C, respectively. After the second challenge, the degradation rate was at least five times more rapid, even at the lowest temperature tested (50% disappearance observed after 4, 7, and 15 days at 25, 15 and 5 °C, respectively). Given a high degree of adaptability when challenged with organic pollutants, diverse natural degradative abilities, and biostimulation as an effective strategy for enhancing in situ rates of degradation, efforts to harness cold-adapted microorganisms and native microbial consortia in the remediation of low temperature environments may prove both efficient and cost-effective. Because as much as 30% of initial contamination can remain in the form of asphalts (hydrocarbon residue) after microbial degradation has run its natural course, the challenge is to fine-tune existing methods and develop new strategies that foster breakdown of even the most recalcitrant materials currently released into the enivronment or produced by long-term weathering.

Degradation of Substituted Hydrocarbon and Other Xenobiotic Compounds

In addition to fuel and heating oils, the more complex or substituted (halogenated, alkylated, brominated) aliphatic and aromatic hydrocarbons, (which are used for such purposes as pesticides, herbicides, industrial solvents, wood preservatives, and dielectric fluids), represent large classes of environmental pollutants. Although more than a thousand of these types of xenobiotic compounds are produced and used widely for pest control and various industrial processes, relatively few studies document their degradation in low temperature environments (Table 7). Microorganisms known to degrade these compounds at moderate temperatures are diverse; some can utilize them as both carbon and energy sources and some can mineralize them to carbon dioxide and water. The typical first step in the degradation of a variety of chlorinated (halogenated) aliphatic and unsaturated hydrocarbons is reductive dechlorination under anoxic conditions, although aerobic degradation of some halogenated aliphatic compounds is known to be mediated by methanotrophic and ammonia-oxidizing bacteria.

Low temperature research on the microbial degradation of substituted hydrocarbons and other xenobiotics has taken pathways similar to those for fuel and heating oils (e.g., effects of biostimulation, importance of adaptation time), but with a difference in focus on the basis of the anaerobic steps often involved (Table 7). The need for anaerobic conditions has thus spurred studies of the effects of alternative electron acceptors, in which such "nutrients" become the test stimulant, and gradients in environmental temperatures. A range of both electron acceptors and temperatures occur in the sediments in which many of these hydrophobic pollutants accumulate.

For example, in studies of the degradation of polychlorinated biphenyl compounds (PCBs) in Hudson River sediments in winter, significant PCB removal occurred at

Table 7. Examples of the Degradation of Substituted Hydrocarbons and Other Xenobiotics at Low Temperatures by Native Microbial Consortia or Mixed Cultures

Source or Type of Compounds	Environment	Temperatures (°C)	Findings	Reference
Polychlorinated biphenyls (PCB)	Temperate sediment	4	Greater than 50% loss of Aroclor 1,242 after five months	182
	Temperate sediment	4–66	Best PCB dechlorination rates between 12 and 34 °C, little to no loss at 4 °C.	183
	Arctic soil	7, 37, 50	Greater PCB removal by cold-adapted (compared to mesophilic) bacteria at low temperature	184
Polychlorinated pesticide (DDE)	Temperate sediments	10	Dechlorination under sulfidogenic and methanogenic marine conditions	185
Trichloroethane	Temperate groundwater	10	Degradation by mixed cultures of methane-oxidizing bacteria in presence of methane	186
Trichloroethylene	Temperate groundwater	10, 12, 24	Degradation by ammonia-oxidizers, more effective than by methanotrophs	157
Chlorobenzene, tetrachlorethylene	Temperate groundwater	4, 10, 20	Degradation more effective by native consortia than with inoculum[a]	187
Chlorophenol	Temperate groundwater	4–17	Degradation rates high, over 99.9% removed at lower temperatures	188
Brominated phenols	Sub-Arctic sediments	4	Degradation of phenols and benzoic acid under sulfidogenic, methanogenic, and iron-reducing estuarine conditions	189
Phenol	Industrial wastewater	10	Degradation rate at low temperature 15–40% of mesophilic rate; useful in cold regions where temperatures fluctuate	190
Ethylene glycol, propylene glycol	Temperate soil	−2 to 25	Degradation at all temperatures with no lag time	191
Aliphatic herbicide Napropamide	Temperate soil	5, 15, 25	Degradation rate dependent on microbial adaptation, adapted consortia suitable for bioaugmentation[a]	192

[a]See Table 6 for other examples of bioaugmentation.

4 °C in the upper 15 cm of the sediment, but no loss was detected in the lower horizons after a 5-month study (182). Lower chlorinated compounds were observed to degrade in the shallower aerobic layers of the upper sediment, whereas the more highly chlorinated biphenyl and chlorinated aliphatic compounds were degraded mainly under reducing conditions deeper in the sediment (187). In a year-long study of the degradation of a tetrachlorobiphenyl over a range of temperatures (4 to 66 °C), seven different reaction pathways were observed, each dependent on the temperature of incubation (183). In the case of brominated phenols, enrichments with the electron acceptors required to fuel iron-reducing, sulfate-reducing or methanogenic microorganisms (Fe(III), SO_4^{2-} or HCO_3), obtained from cold (4 °C) marine and estuarine habitats, indicated a hierarchy of degradation rates in reverse order, with methanogenic conditions providing the highest debromination rates (189); removal of bromobenzoate, but not bromophenol, isomers required a significant adaptation period. Clearly, the actions of multiple and diverse native microbial consortia may be required under equally diverse in situ conditions to remove some xenobiotics effectively.

When a xenobiotic compound of known human health hazard (192) proves particularly recalcitrant in situ, as observed for chlorophenols (CPs) in cold groundwater aquifers (the temperature of the average North American aquifers is 10 to 13 °C), or is only partially degraded, leaving toxic intermediates behind, as observed for the solvent trichloroethylene (TCE), more aggressive engineering strategies may be in order. Ex situ treatments in the form of aerobic, fluidized bed reactors have been shown effective at removing >99.9% of total CPs from groundwater at temperatures as low as 4 °C (188). Although TCE is known to be degraded by methanotrophs aerobically (and anaerobically by reductive dechlorination), laboratory biostimulation with ammonium has been shown to be effective at reducing contamination with TCE at 12 °C, suggesting that field biostimulation with ammonium, an inexpensive and nontoxic chemical, may be an important remediation technique for TCE-contaminated aquifers. Nevertheless, observations of toxic intermediates persisting in studies of TCE degradation (186) suggest that TCE-degrading microorganisms may be prime targets for genetic engineering to overcome the toxic-intermediate

bottleneck and position them for use in ex situ reactors to achieve complete TCE degradation.

Finally, studies of the deicing-fluids used routinely in the winter to control ice formation on the wings of aircraft (191) reveal both the power of native cold-adapted microbial consortia as bioremediators in spite of the potentially constraining effects, biological and abiotic, of very cold temperatures on contaminant degradation. The principal organic constituents of these deicing fluids are ethylene, propylene, and diethylene glycols; they are sprayed on the aircraft before departure and thus wash off the runway onto surrounding soils. These glycol products, whether presented singly or in mixtures, have been shown to be mineralized to carbon dioxide in these soils when incubated at temperatures as low as $-2\,^{\circ}C$ (191). No adaptation period was required: biological transformation of the test chemicals began immediately after addition to the soil and regardless of exposure to very high concentrations. Rates of degradation ranged from 2.3–4.5 to 19–27 to 66–93 mg kg^{-1} soil day^{-1} at -2, 8 and 25 °C, respectively, translating to Q_{10} values well in excess of the expected value of two, if biological reactivity were the primary limiting factor. The suggestion that even mild warming significantly increases the bioavailability of these energy-rich compounds (a form of biostimulation) on top of the typical increase in biological reactivity portends well for positive seasonal trends in the degradation of such compounds by native microbial consortia, without aggressive treatment of the contaminated environment.

SOME FUTURE APPLICATIONS OF COLD-ADAPTED MICROORGANISMS

Fatty Acids for Aquaculture and Medicine

As part of their strategies to maintain membrane fluidity at low temperatures, many cold-adapted microorganisms produce a diversity of polyunsaturated fatty acids (PUFA) (193,194). Some PUFAs, particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are important supplements in aquaculture feeds (194). They are also essential elements in the human diet, required for retinal and neurological development in infants and helpful in treatment of rheumatoid arthritis, ulcerative colitis, skin lesions, psoriasis, coronary artery disease and some cancers (195). Use of genetic engineering tools to achieve overexpression of PUFA production for fish or human consumption represents a potential biotechnological application of cold-adapted microorganisms. Studies of both PUFA production and the genetic regulation of the process in the psychrotolerant *P. profundum* strain SS9 (43) demonstrate the potential.

Enzymes for Agriculture

Studies with cyanobacteria have indicated that transfer of the gene encoding a fatty acid desaturase enhances the ability of the transgenic organism to tolerate low temperatures, without effecting its photosynthetic activity (196,197). Potentially, such manipulation could be used to stabilize the thylakoid membranes (loci for photosynthetic activity) of commercial plants at cold temperatures. To our knowledge, a cold-active desaturase enzyme has not yet been identified, but the recent isolation of psychrophilic polar oscillatorians provides an excellent opportunity to search for one specifically compatible with cyanobacteria (198).

Degradative Pathways for Detoxification

Studies with some xenobiotic compounds have indicated that microbial degradation of the parent compound leads to more toxic and lethal intermediate compounds. Elucidation of these pathways, and how they may change with temperature, is needed to design organisms capable of degrading toxic intermediates to benign forms in low-temperature applications. Temperature-dependence of degradation pathways has already been observed for a tetrachlorobiphenyl compound, with each of seven different dechlorination pathways being favored by a given temperature (183). Meanwhile, the use of genetic engineering to achieve removal of toxic intermediates has been validated for mesophilic conditions. The presence of a genetically engineered strain of a *Pseudomonas* sp., designed to degrade mixtures of chloro- and methyl-aromatics toxic to other organisms, enabled a functional laboratory-scale sewage treatment plant; in its absence, all protists and metazoans were killed by the xenobiotics and culturable bacteria were reduced by three orders of magnitude (199).

Reporter Genes for Bioremediation

Some compounds or microbial processes of bioremediative interest are not amenable to widespread analysis in cold (or warm) environments because of detection procedures and costs. Genes with expression products that can be readily assayed have been engineered to serve as affordable "reporters" for other genes and products more difficult to measure. Typically, the gene of interest is fused with promoterless versions of a reporter gene, such that expression of the latter allows a quantitative measure of the linked expression of the former. Common reporter systems include *lacZ* gene fusions, in which β-galactosidase activity is assayed, and *lux* gene fusions, in which bioluminescence is assayed. For example, environmental concentrations of naphthalene and salicylate can be quantified quickly and inexpensively by measuring the expression of associated degradative genes with a *lux* fusion in specially designed bacteria (200). *Lux* reporters have also been used to monitor genetically engineered bacteria in field-release studies (201). For cold-specific applications, a more promising development may be that of an ice-nucleation (*lnaZ*) reporter system using the relevant gene encoded on a plasmid of broad host range from the psychrotolerant plant pathogen, *Pseudomonas syringae* (202). Assaying ice-nucleation is easy and metabolically inexpensive (203). The *lnaZ* system is also reported to be 2 to 3 orders of magnitude more sensitive than *lux*-based systems and 5 to 6 orders of magnitude more sensitive than *lacZ*-based systems (202). The combination of high sensitivity and low metabolic cost recommends the *lnaZ* reporter system for monitoring oligotrophic (nutrient-poor) environments,

measuring contaminants at low concentrations, and evaluating targeted processes in the cold. Analogously, specific cold-active enzymes, or cold-inducible promoters, could also be developed into bioreporter systems to study processes at cold temperatures in the laboratory or the environment.

Genetic Engineering for Metal Remediation

Although progress in understanding and harnessing microbial capabilities for degrading organic contaminants in cold environments is central to bioremediation, heavy metal contamination represents an additional and substantial societal challenge. The availability of cold-adapted microorganisms naturally or genetically engineered for metal remediation could be useful in detoxifying the greater than three trillion liters of radioactively contaminated (inherently cold) groundwater in North America (204). Exploitation of *Deinococcaceae* for this purpose appears promising. The *Deinococcaceae* in general have an incomparable ability to withstand and correct DNA damage, most famously due to radiation exposure (205), but the exemplar of the family, *Deinococcus radiodurans*, also tolerates exposure to heavy metals, actively reducing Fe(III), Cr(VI), U(VI) and Tc(VII) (206). *Deinococcus radiodurans* R1 has also proven readily amenable to genetic manipulation by transformation, which, along with its resilience to toxic metals and radiation, and the availability of a complete genome sequence (207), makes it an ideal candidate for genetically engineered metal remediation (204). Other work indicates that the expression systems pioneered in *D. radiodurans* can be used in its thermophilic relative, *Deinococcus geothermalis* (208). The promise of cold-adapted *Deinococcaceae*, perhaps with a similar genetic pliability, stems from a recent report that gene sequences most closely related to *D. geothermalis* have been found in Antarctic snow, where general microbial activity was detected at subzero temperatures (209). A cold-adapted *Deinococcus* would be an obvious candidate not only for heavy metal remediation but also for exploitation in outer space or on other planets, although the practicalities and philosophical implications of the latter are staggering.

CONCLUSION

The widespread availability of cold-adapted microorganisms in vast portions of the global environment stands in contrast to the limited attention given to their fundamental features and capabilities. Biotechnological interests provide a strong impetus to change this situation. Fundamental research, however, has informed such interests and even generated them. With expanding molecular-genetic and protein engineering methodologies, it will continue to do so. Solid progress in the linked exploration of fundamental and biotechnological aspects of cold-adapted microorganisms can be expected in the realms of enzyme production and environmental bioremediation, due in large part to supporting knowledge and databases in these fields of endeavor at warmer temperatures and to significant starts at cold temperatures. The future lies in creative applications of cold-specific genetic systems, especially viral-bacterial systems, in the exploration of genome-based information, and in pure discovery, as the vast numbers of uncultivated cold-adapted microorganisms and their metabolic capabilities come into view.

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USE OF MICROSCOPIC ALGAE IN TOXICITY TESTING

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Microalgae comprise a crucial component of the aquatic food web owing to their oxygen production and because they constitute a vital food source for other trophic levels. Their ubiquity in all water bodies ensures energy flow in the food chain and hence the stability and well-being of aquatic ecosystems. This in turn sustains other terrestrial and avian life-forms that are (in)directly dependent on healthy aqueous environments for their survival (Fig. 1). In short, microalgae are essential to support all life-forms on our planet including mankind. As contaminant-mediated phytotoxicity, through diverse anthropogenic activities, can harm or kill phytoplankton and ultimately disrupt the functional and structural homeostasis of species living in aquatic habitats, it is important for environmental agencies to apply tools and strategies that will enable proper assessment of toxicity stresses imposed on phototrophic organisms.

After about more than three decades of work by scientists to address the various issues of ecotoxicity,

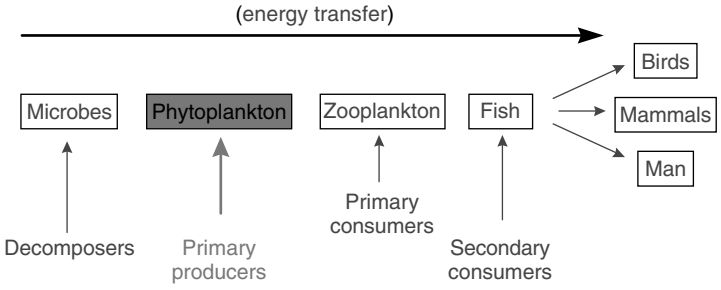


Figure 1. Hierarchy of trophic chains. See color insert.

it is now well recognized that microalgae are useful indicators of environmental quality, particularly because they have been shown to be sensitive to a wide spectrum of both inorganic (1–3) and organic (4,5) pollutants, as well as to complex environmental samples (e.g., toxic industrial effluents and contaminated sediments) containing mixtures of these (6). Because of other attractive features of microalgae as discussed later in this review article, simple (early warning) laboratory-based screening procedures have been developed that provide relevant information for sound environmental decision-taking with respect to pollutional events detected by microalgae as well as by other types of bioindicators.

The field of biological testing with microalgae continues to grow as new technologies and knowledge pave the way for breakthroughs yielding innovative tools and/or improvements for continuing environmental appraisal. In a historical context, it has gone from initial enrichment studies (e.g., determining the fertility potential of water bodies), to toxicity studies (e.g., establishing the toxic potential of contaminants) and to interaction/uptake studies (e.g., metabolic influence of microalgae on the genotoxic potency of industrial effluents). Examples of how microalgae have been profitably used in this evolving field will be presented herein with emphasis being placed on the more simple techniques available for conducting laboratory phototrophic bioassays. Recent developments related to (*1*) the incorporation of phytotoxicity tests within a bioassay battery approach, (*2*) direct sediment toxicity assessment with microalgae, and (*3*) eliminating the need for continuous laboratory cultivation of microalgae by preserving cells in a protective matrix will also be highlighted.

Finally, the reader must be reminded that the field of phototrophic ecotoxicology is very broad, and that even this review on laboratory-based studies only presents an overview, which is far from being exhaustive. Another important complementary domain concerns the strategies and approaches employed to study the ecotoxicity of natural algal communities. As it is beyond the scope of this paper, interested readers are encouraged to consult specific papers on this topic (7–10).

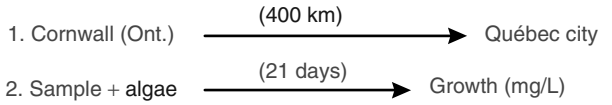
EUTROPHICATION STUDIES

Whereas toxicity tests with microalgae are the focus of this chapter, it is nevertheless important to bring to the reader's attention that they evolved from techniques employed in earlier eutrophication studies undertaken during the 1970s (11–13). As an example, Keighan (12) collected samples of surface waters from the mouth of the major tributaries discharging to the St-Lawrence River between the city of Cornwall (Ontario) and Quebec City (Quebec), spanning some 400 km of river, to determine the degree to which they could contribute to the fertility potential of this major waterway (Fig. 2). In this biological test, 40 mL of each sample were placed in 125 mL Erlenmeyer flasks to which was added a standardized inoculum of microalgal cells (1,000 cells/mL of sample). After an incubation time of 21 days, algal growth in each flask was first estimated by enumerating cells with an electronic particle counter. Afterward, growth was finally reported in mg (dry weight)/L with a predetermined conversion factor linking cell counts with dry weight measurements. With this simple test based on algal growth potential (AGP) of surface water samples, tributaries could be categorized in terms of their eutrophication potential by comparing their specific AGP to a three-class water quality index (Fig. 2). This would in turn trigger decision making so that corrective actions might be taken to protect the aquatic environment from auxinic pollutants (e.g., nitrogen- and phosphorus-containing fertilizers used in agriculture) known to stimulate phytoplankton growth. Although the data are not shown in this article, Keighan (12) used the same biotesting approach to characterize the enrichment level of 36 major industrial effluents discharging to the same portion of the St-Lawrence River, in which he demonstrated AGPs ranging from 1.7 to 183 mg (dry weight)/L.

TOXICITY STUDIES

Chronic and Acute Exposure Tests

As mentioned in the preceding section, toxicity assays with microalgae evolved from the earlier eutrophication studies flask tests. Chronic exposure tests of 21 days (12), 7 to 8 days (14) or 4 days (1), all seeking to measure algal growth inhibition resulting from chemical insult, were first developed and applied. Eventually, chronic tests were standardized at an exposure time of three days by standardization organizations, such as the Organization of Economic Development and Cooperation (OECD) and the International Standards Organization (ISO), as experience and new knowledge demonstrated that no loss of microalgal sensitivity toward contaminants occurred with this shorter exposure time. Three-day chronic tests also have the advantage of eliminating or alleviating potential problems or interferences resulting from more prolonged times of exposure (e.g., pH shifts, carbon dioxide depletion, contaminant bio- or phototransformation, algal adaptation), which can alter toxicity responses and thus confound experimental results and complicate interpretation (15). Whereas chronic exposure tests clearly outrank tests of shorter duration in numbers, acute exposure tests of only several hours have also been developed with the intent of rapidly measuring phototrophic responses to toxicant



Major tributaries	Growth potential (mg/L)	Water quality index
6 rivers	2.9–4.9	< 5 = good
12 rivers	5.5–9.9	5–10 = doubtful
15 rivers	10.7–47.7	> 10 = deteriorated

Figure 2. Enrichment level characterization of the St-Lawrence River (12). See color insert.

challenges (16–19). Because of their sensitivity, acute procedures employing motility inhibition (16) or enzyme inhibition (17) parameters as assessment end points can prove particularly useful to detect quick changes that toxic chemicals can exert on primary producers.

Experimental Testing Conditions and Toxic Effects Measurements

Undertaking toxicity tests with microalgae is relatively uncomplicated and simply requires a basic set of experimental conditions to be met (Fig. 3). An inoculum is obviously necessary, which will vary according to the intended test exposure period. Physiologically active (logarithmic phase) cells are employed for testing and inoculum size is selected such that algae will continue to be in an active state of growth at the end of the exposure period. For example, an 8-day exposure assay might call for an algal inoculum of 1,000 cells/mL to be introduced into test flasks, as this will yield sufficient cell numbers ($>10^6$ per mL in control flasks) at the end of the test to measure a growth inhibition effect resulting from the toxicity of the sample under investigation (14). For example, if the average growth in control flasks and test flasks is determined to be 2×10^6 cells/mL and 1×10^6 cells/mL, respectively, after 8 days, it will be evidently clear that a 50% growth inhibition effect has resulted owing to sample toxicity. In contrast, an acute exposure assay of only four hours based on detecting a biochemical change such as intracellular ATP (adenosine triphosphate) demands a much more important inoculum to start off with, because an inoculum of 1,000 cells/mL is well below the detection limit of ATP measurement (20). Hence, 10^6 cells/mL might be a more appropriate starting inoculum in this instance, as it will not only guarantee measurement of the ATP content of control algae, but also that of sample-exposed algae, which may display a reduction of ATP owing, once again, to toxic effects. A defined algal growth medium (AGM), composed of macro- and micronutrients is also called for to promote adequate growth of algal cultures maintained in the laboratory (13). It should be noted that medium composition is important as it has been shown to influence toxicity results (21).

In chronic toxicity tests based on growth inhibition end points, spiking all experimental flasks with a specific concentration of AGM is essential, as it ensures that algae will reach a specific biomass at the end of test exposure (e.g., 2×10^6 cells/mL for control algae after 8 days). If algae in sample-exposed flasks do not attain a similar growth yield, the experimenter can then be certain that reduced growth results from sample toxicity and not from a dearth of nutrients. A specific light source (e.g., cool-white fluorescent lighting with quantal flux between 60 and 100 $\mu E\,m^{-2}\,s^{-1}$) and incubation temperature (e.g., $24 \pm 2\,^{\circ}C$ for some green algae) will complete the essential requirements for optimum cell division. Depending on the testing procedure, a photoperiod (e.g., a 16-hour light period followed by an 8-hour dark period) may be called for, as can (manual or programmed) shaking or nonshaking of experimental vessels during the exposure period. Modern algal toxicity tests are now generally performed under continuous illumination (i.e., without a photoperiod), as this favors faster cell division in view of the fact that (shorter) 3-day chronic tests are now recommended. It also ensures asynchronous growth (algal cells are not at the same cell cycle stage at the same time and therefore do not divide in synchrony), which more closely mimics environmental situations.

- Inoculum:	Exposure	Cells/mL
	8d	1,000
	3d	10,000
	4h	10^6
- Defined growth medium (Macro- and micro-nutrients)[a]		
- Illumination ($\mu E.m^{-2}.s^{-1}$)[b]		
- Temperature ($24 \pm 2°C$)		
- Photoperiod: + (synchronous growth)		
– (asynchronous growth)		
- Agitation (+/–)		

(a) consult reference (13)

(b) Quantal flux units: consult reference (26)

Figure 3. Basic experimental conditions for undertaking toxicity tests with algae. See color insert.

Microalgal Test Species

Several species of algae can be called upon for the conduct of toxicity tests and include representatives of freshwater chlorophytes (green algae), xanthophytes (yellow-brown algae) and cyanophytes (blue-green algae) (4,15). Of these three groups, chlorophytes including the genera *Selenastrum*, *Chlorella*, *Scenedesmus*, *Chlamydomonas*, and *Monoraphidium* have been extensively used and, in particular, the green alga *Selenastrum capricornutum* (Prinz) has been frequently employed in biological testing, particularly in North America. Its popularity is attributable to its attractive characteristics (unicellular, nonmotile, nonpolymorphic, nonagglomerating), which make it easily enumerable in the laboratory with the help of electronic particle counters. Further incentives for the use of this alga include its recognized sensitivity to heavy metals (18,22), organic contaminants (4,22) and complex samples, which may contain mixtures of these (6,23,24), and to the fact that it is a representative bioindicator because it is found in both oligotrophic and eutrophic aquatic systems. As can be seen from Figure 4, *S. capricornutum* is a crescent-shaped unicellular alga, whose dimensions vary between 3 and 5 microns (width) and 10 to 15 microns (length). Studies undertaken with this chlorophyte surpass those reported with all other microalgae to the point where it could certainly merit to be acknowledged as the "*Escherichia coli* of phytotoxicity testing". It is also known as *Pseudokirchneriella subcapitata*, particularly in Europe, whereas the name *S. capricornutum* is more prevalent in North America. Although the material presented herein focuses primarily on work performed with this particular alga because of the author's personal experience, it should be noted that similar studies can clearly be undertaken with other unicellular algae as well. Unlike their freshwater counterparts, phytotoxicity tests conducted with marine unialgal species are scarce. Several species of

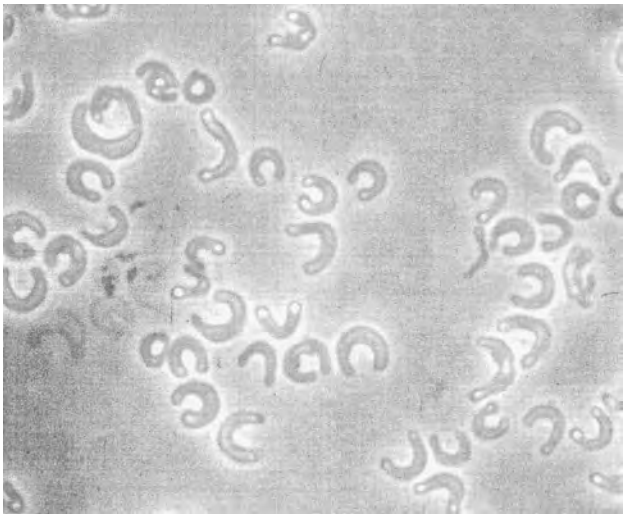


Figure 4. The freshwater chlorophyte, *S. capricornutum*, seen under phase contrast microscopy at a magnification of 400*x*. See color insert.

marine diatoms, however, have been appraised in short-term exposure tests to screen for toxic effects of metals, organics, or surface seawater samples (17,25).

Quantifying Algal Toxicity of Liquid Samples

Determining the sample concentration at which a 50% effect occurs in relation to control algae is a typical way of reporting toxicity when conducting microalgal toxicity tests. Table 1 gives an example of actual data generated in our laboratory with the heavy metal Zn^{2+}. Here, growth inhibition is termed the *assessment end point* and the 72h-IC50 (the sample concentration causing a 50% reduction in the growth of the algal population following a 72-hour exposure period) is termed the *measurement end point*. Essentially, algal cells in each experimental container (or microplate well, as discussed in the following text) are first enumerated with the help of an electronic particle counter. Next, the mean cell yield of control algae and of sample-exposed algae are determined at the end of exposure from which growth inhibition percentages can be calculated for each sample test concentration. Percentage inhibition values (*y*-axis) and sample concentration values (*x*-axis) can then be plotted graphically and a line of best fit drawn around the data points (Fig. 5). Finally, another line (. . .) is drawn perpendicularly from the 50% inhibition point on the *y*-axis to the regression line and again perpendicularly downwards until it meets the *x*-axis. This point of encounter will identify the 72h-IC50 for Zn^{2+}. Computer software is available, of course, to reduce and plot phytotoxicity data, as well as to determine appropriate measurement end point values (26).

Miniaturization of Unialgal Toxicity Tests

Whereas the initially developed algal toxicity assays conducted with glass (Erlenmeyer) flasks in the late 1970s are scientifically sound, they suffer from the fact that they are labor-intensive and do not lend themselves to a high capacity for sample throughput. This dearth in cost-effectiveness sparked a genuine desire in several research groups to find more practical alternatives for phytotoxicity testing. As a result, 96-well polystyrene

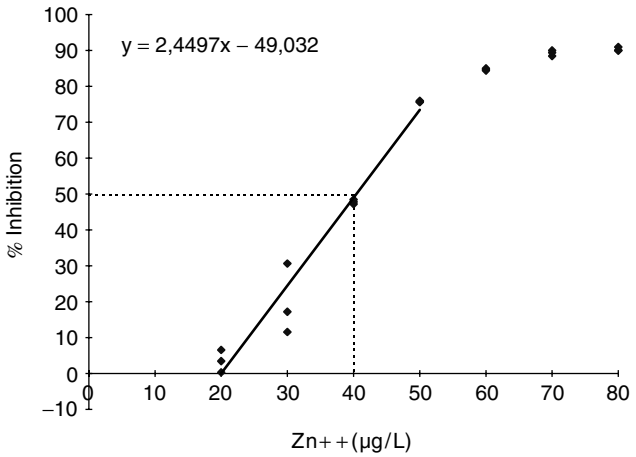


Figure 5. Concentration-response curve indicating percentage growth inhibition of *S. Capricornutum* after a 72-hour exposure to a range of Zn^{2+} concentrations. Here, the 50% effect concentration or IC50 = 40 µg/L of Zn^{2+}. See color insert.

Table 1. Phytotoxicity Data Generated for Zn^{2+} (Test Concentrations were Prepared with a $ZnSO_4$ Solution) with the Environment Canada *S. capricornutum* Microplate Biological Testing Procedure (26)

Zn^{2+} Test Concentrations (µg/L)	Algal Concentration (Cells × 10^5)	Mean Algal Concentration (Cells × 10^5)	Growth Inhibition in Relation to Control Algae (%)
0 (control)	9.54, 8.4, 9.35, 8.05, 8.47, 9.9 ($n = 6$)	8.95	—
20	8.63, 8.35, 8.94 ($n = 3$)	8.64	3.5
30	6.19, 7.41, 7.90 ($n = 3$)	7.16	20
40	4.62, 4.56, 4.68 ($n = 3$)	4.62	48.4
50	2.06, 2.11, 2.11 ($n = 3$)	2.09	76.7
60	1.27, 1.27, 1.31 ($n = 3$)	1.28	85.7
70	0.85, 0.95, 0.82 ($n = 3$)	0.87	90.3
80	0.81, 0.79, 0.73 ($n = 3$)	0.78	91.3
Calculated 72h-IC50 = 40.4(34.9–46.1)[a] µg/L			

[a]95% Confidence intervals.

microtitration plates (or "microplates") — for which varied applications began in the field of clinical microbiology (27) — were soon discovered, tested, validated and employed by environmental research groups for the conduct of algal bioassays (15). Microplate-based tests confer many advantages for undertaking biological tests with a variety of microorganisms, among which are small sample volume requirement, elimination of postexperimental washing of glassware and automation potential for test initiation (23). Whereas experimental configurations for algal microplate assays can be varied, they are usually tailored to meet specific testing objectives (e.g., acute or chronic testing with one or more species) or to offset potential interferences (e.g., toxicant volatility). As as example, Figure 6 displays a typical experimental disposition that was initially used to evaluate the phytotoxicity of herbicides (22) and which is now recommended by Environment Canada to perform standardized toxicity testing with *S. capricornutum* (26). As a phenomenon commonly associated with 96-well microtitration plates is the so-called "edge effect," whereby the evaporation rate of the circumferential wells tends to be greater than that of the centrally located wells, experimental dispositions tend to exclude peripheral wells from testing because they increase variability among replicates. Hence, for chronic tests of one day or more, experimental designs are usually built around the 60 internal wells, as seen in Figure 6. Whereas peripheral wells are not directly employed for testing, they are nevertheless always filled with water to increase humidity inside the microplate and, in this sense, they contribute positively to the overall experimental protocol.

Because the use of microplate tests — with a few notable exceptions — essentially came after that of the earlier flask tests, some researchers felt it important to demonstrate that microplate phytotoxicity assays did indeed reproduce the results of their glassware counterparts. Displaying agreement between the two methodologies would indicate microassay reliability and obviously enhance microtesting acceptance by the scientific community. In this perspective, several studies were undertaken to establish interprocedural compatibility. These comparative investigations conducted with varied liquid samples (metals, herbicides, phenol, effluents) essentially pointed out that microplate and flask methodologies gave concordant results insofar as generation of phytotoxicity data with microalgae is concerned. Each of these studies also presented evidence that microplate tests are reliable because of their repeatability and reproducibility (15).

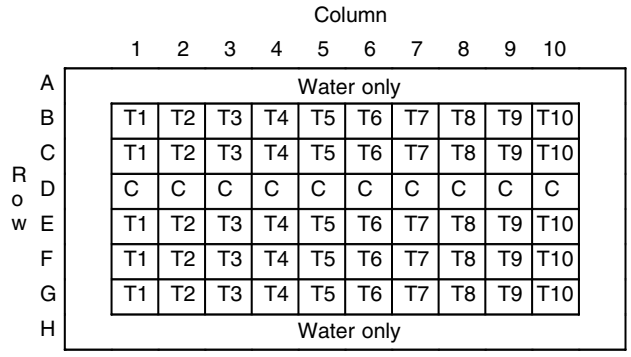


Figure 6. A typical microplate experimental configuration for toxicity testing. There are five replicates per concentration with T1 and T10 being the highest and the lowest concentrations, respectively. There are 10 centrally located control (C) wells (22).

Applications with Algal Toxicity Tests

Two examples of toxicity testing applications with microalgae are given so readers can appreciate their usefulness in contributing to the knowledge base of contaminant-related toxic effects. In a first study (6), the toxicity responses of *S. capricornutum* were appraised following an 8-day exposure to 46 effluent samples of the Pulp and Paper sector — a major industrial force in the province of Quebec, Canada (Fig. 7). Chronic 8-day IC50 values were first determined for each of the effluents, whose wastewater treatment was either absent or present based on primary, primary and secondary, and the Best Practicable Technology (BPT) installations. The BPT mill was the only one, at the time, exemplifying optimum treatment capabilities for this industrial sector. Individual 8-day-IC50s were then transformed into toxic units (TU), summed up for each of the effluent treatment categories and an average TU value calculated for each (see Fig. 7). Results show that the algal bioassay yielded responses that were able to readily discriminate effluent toxicity on the basis of treatment application. Phytotoxicity test results also demonstrate the efficiency of combined primary and secondary treatment coupled to BPT capacity in markedly reducing the toxic potential of these liquid wastes, which were at the time unquestionably toxic toward primary producers.

In a second study aimed at improving understanding of the potential impact of acute chemical exposure on microalgae, *S. capricornutum* was exposed for four hours to selected chemicals, after which (control and chemical-exposed) algal cells were removed from their contaminated medium and allowed to grow in normal algal growth medium for 96 hours (18). Significant growth inhibition in relation to controls resulting after 96 hours would thus indicate a toxic effect that had manifested itself during the initial four-hour exposure. This microplate-based *exposure-recovery* assay was able to identify chemicals posing low acute exposure risk to algae. Furthermore,

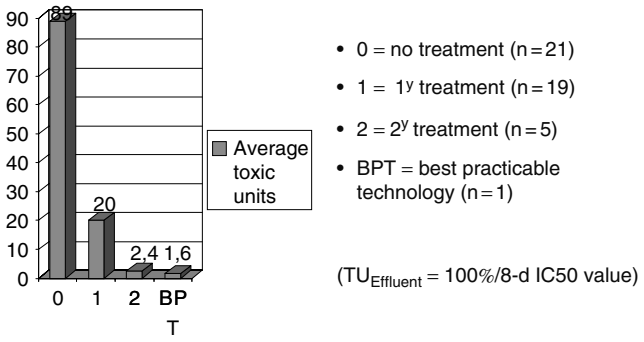


Figure 7. Toxicity responses of *S. capriconutum* exposed to pulp and paper effluents having different waste treatment facilities (6). See color insert.

Table 2. Time-Related Phytotoxicity Effects of Chemicals Based on Four Hours Acute (*A*) and 96 Hours Chronic (*C*) responses of *S. capricornutum* (18)

Chemical	4h-IC50 (*A*) (mg/L)	96h-IC50 (*C*) (mg/L)	*A*/*C*[a]
DCP[b]	112	102	1.1
Hg^{2+}	0.05	0.02	2.5
Cu^{2+}	0.14	0.05	2.8
Zn^{2+}	8.0	0.05	160
TB[c]	>100	0.102	>980

[a] Acute to chronic ratio.
[b] Dichlorophenol.
[c] Tebuthiuron.

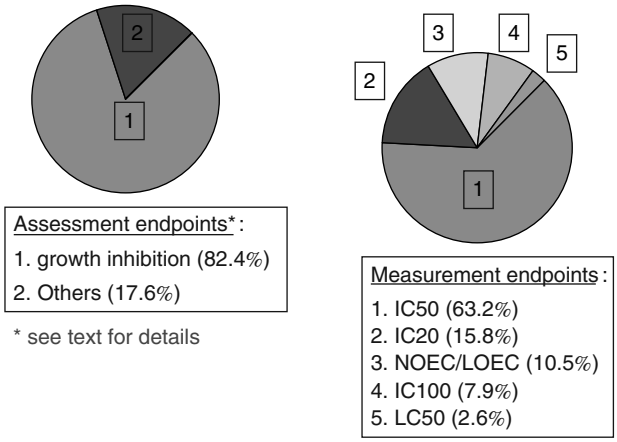


Figure 8. Endpoints for testing liquids with microplate-based phytotoxicity procedures. See color insert.

when comparing Acute (4h-exposure/96h-recovery IC50s) to Chronic (96h-IC50s) end point values, A/C ratios could identify those chemicals capable of rapid (DCP, Hg^{2+}, Cu^{2+}) and less rapid (Zn^{2+}, TB) phytotoxic effects (Table 2). DCP (dichlorophenol), for example, is a fast-acting chemical in terms of phytotoxicity, as it yields similar acute and chronic IC50s. In contrast, TB (tebuthiuron), an efficient substituted-urea class herbicide, is nontoxic to *S. capricornutum* after four hours of exposure, but very toxic after 96 hours of exposure. Clearly, combining results of time-related phytotoxicity data can give valuable insights into chemical mechanisms of toxicity and prove useful in the area of contaminant risk assessment.

End Points Measured and Types of Samples Evaluated

End points determined after testing aqueous samples with microplate-based phytotoxicity procedures are varied but some are clearly favored over others (Fig. 8). For example, intracellular ATP, esterase activity and motility have thus far been sparingly employed to report assessment end points, but growth inhibition (determined by cell counts, ATP, absorbance, fluorescence, and visual observation) has distinctly been the most popular choice. In terms of measurement end points, IC50s have been most frequently determined, but IC20s, IC100s, and NOECs/LOECs have also been computed (15).

Types of liquid media assessed with phytotoxicity tests are quite varied, as recalled in Table 3. Microalgal assays are essentially applicable to any sample that is in liquid form or "liquefiable," such as a sediment sample from which an extract would first be prepared or a hydrophobic compound, which may also require the use of a solvent carrier to insure adequate dissolution during test exposure. In such cases, the experimenter must verify that the solvent concentration employed is not phytotoxic per se and that sample/solvent interactions will also not be at play to affect the toxicity results. Whereas small-scale biological tests undertaken at all

Table 3. Examples of Liquid Test Samples Assessed with Phytotoxicity Tests and Corresponding End Points Determined

Medium Investigated	Assessment End Point and Reference (in Parentheses)
Contaminants	
Metals in solution	ATP energy loss (18); growth inhibition (28)
Organics in solution	Visual reporting of the lowest sample concentration indicating total growth inhibition at end of exposure (4); cell growth recovery (18); esterase inhibition (17)
Herbicides	Growth inhibition (22,29); cell mortality by flow cytometry measurement (30)
Complex Samples	
Oil dispersants	Growth inhibition (31)
Organic extracts	Absorbance as indicator of biomass inhibition (32)
Groundwater	Absorbance as indicator of biomass inhibition (33)
Surface waters	Esterase inhibition (19); absorbance as indicator of biomass inhibition (33)
Effluents	Growth inhibition (23); fluorescence as indicator of biomass inhibition (34); growth inhibition (24)
Solid waste leachates	Growth inhibition (35,36)
Sediment elutriates	^{14}C uptake inhibition (37)
Sediment pore waters	Growth inhibition (38)

levels of biological organization (e.g., tests with microbes, algae, microinvertebrates, fish) have thus far focused on assessing the toxic potential of liquids, some microbial assays have recently been developed to specifically address the toxicity of solid media, such as whole sediments. One such assay, conducted with microalgae, is described further on.

INTERACTION STUDIES

A third and more recent area of investigation involving microalgae was born when pioneering research groups demonstrated that vegetal systems could detoxify (39), activate (40,41), or bioaccumulate (39,42) genotoxicants. Such contaminants expressly exert their aggression toward the hereditary material of cells (i.e., DNA) and potential consequences both at the cellular (e.g., DNA damage, mutations, chromosomal breakage) and organismic/population (e.g., decreased reproductive capacity, developmental disruption, cancer formation) levels can be far-reaching.

The influence that microalgal metabolism can have on the genotoxic status of complex industrial effluents is illustrated in Table 4. In this study (43), a specific biomass of *S. capricornutum* was allowed to interact with the effluent samples for four hours. After this period, the genotoxic status of each effluent, from which the algae have first been removed by centrifugation, was determined with the SOS Chromotest (a genotoxicity screening assay). Four-hour postexposure genotoxic activity of each effluent was then compared with that determined prior to algal interaction to determine whether detoxication, activation and/or uptake has occurred.

Results of these algal exposure experiments revealed a variety of interactive patterns, as seen in Table 4, ranging from no effect (effluent samples 1 and 2) to de-activation (effluent sample 3), activation (effluent samples 6 and 7) and uptake (effluent sample 9). This particular study, including others in this (still) fledgling field of phytotoxicology, demonstrates that the fate of genotoxicants in mixtures, such as complex effluents or surface waters contaminated with these, is difficult to predict and that phytoplankton may play an important role in altering the potency of such contaminants released to aquatic systems. Microalgae therefore comprise an important "metabolic reservoir", because of their ubiquity, capable of intimate links with discharged xenobiotics. Further investigations on the chemical biotransformation and/or bioaccumulation properties of microalgae to more fully understand environmental risk appear justified (44).

PHYTOTOXICITY TESTING WITHIN AN INTEGRATED BATTERY OF BIOLOGICAL TESTS TO ASSESS THE POTENTIAL HAZARD OF INDUSTRIAL EFFLUENTS

Because toxic effects of chemicals can affect different trophic levels with varying intensity, it is now recognized that relevant batteries of bioassays should be employed for comprehensive appraisal of the hazards represented by specific contaminants and complex point source discharges (24). One example of an integrated bioassay battery approach developed to serve the purposes of environmental management is the Potential Ecotoxic Effects Probe (PEEP) index which enables the assessment and comparison of the toxic potential of industrial effluents (45).

This effluent assessment index relies on the use of an appropriate suite of multitrophic bioassays (decomposers, primary producers, and consumers) allowing the measurement of various types (acute, chronic) and levels (lethal, sublethal) of toxicity. At the time of its conception, this index integrated the results of selected small-scale screening bioassays (*Vibrio fischeri* Microtox^R test, *S. capricornutum* growth inhibition microtest, *Ceriodaphnia dubia* lethality, and reproduction inhibition tests, *E. coli* genotoxicity SOS Chromotest), and took

Table 4. Influence of Microalgal Metabolism on the Genotoxic Status of Complex Industrial Effluents (Adapted from Harwood et al., 1989) (43)

Source	Genotoxic Activity[a] Before and After a Four-Hour Interaction with Algae		Genotoxic Activity[a] of Algal Extracts After their Interaction with Effluent Samples	Remarks
	Before	After		
Sample 1: Organic chemical manufacturing effluent	−	−	−	No influence of algal metabolism
Sample 2: Organic chemical manufacturing effluent	+	+	−	No influence of algal metabolism
Sample 3: Pulp and Paper effluent	++	+	−	Decrease in genotoxicity owing to algal metabolism
Sample 6: Organic chemical manufacturing effluent	+	++	−	Increase in genotoxicity owing to algal metabolism
Sample 7: Inorganic chemical manufacturing effluent	−	+	−	Increase in genotoxicity owing to algal metabolism
Sample 9: Organic chemical manufacturing effluent	+	+	+	Uptake by algae of genotoxic components from effluent

[a] Genotoxic activity was quantified with the SOS Chromotest detection system (43).

into account the persistence of toxicity (meaning that biotests were performed on an effluent *before* and *after* a 5-day biodegradability procedure), (multi)specificity of toxic impact (number of bioindicators affected by an effluent), as well as toxic loading (effluent flow in m^3/h). The resulting PEEP index number is reflected by a $\log_{10}$ value that can normally vary from 0 to 10. The structure of the mathematical formula generating PEEP values is simple and "user-friendly" in that it can accommodate any number and type of bioassays to fit particular needs (Fig. 9a).

The PEEP formula can best be understood by means of an example showing actual toxicity data generated with an effluent sample collected in 1991 from a Pulp and Paper mill located in the province of Quebec, Canada. Measurement end point effluent concentration data calculated in % v/v are first transformed into toxic units (Fig. 9b). These values are informative per se in terms of the toxicity characteristics of the effluent under investigation. For example, it is clear that this wastewater sample is highly phytotoxic (183.8 toxic units observed following microalgal testing of the neat effluent sample), but that most of this toxicity is not persistent (5.7 toxic units remaining after the effluent sample has been subjected to a biodegradability procedure). It also illustrates the fact that effluent toxicity is trophic-level dependent and that bioassays should be conducted with different organisms to properly circumscribe the full hazard potential that complex discharges can represent with respect to aquatic biota. Interested readers are urged to consult the original PEEP article (45) for details on various conceptual aspects and further information on the significance of bioassay data generated with this managerial tool, which are outside the scope of the present article. Once toxic units are calculated for all bioassays, they are integrated in the *toxic print* portion of the PEEP formula, which is multiplied by effluent *flow* datum ($Q = 3,213$ m^3/h). The product of *toxic print* and *flow* yields the *toxic load* of the effluent. The resulting PEEP index value of 5.8 is then simply the $\log_{10}$ of the calculated effluent sample *toxic load*. The value of "1," inserted into the PEEP formula just ahead of the *toxic print*, insures that the inferior scale of the PEEP index will commence at "0" for effluents that are nontoxic (i.e., those in which toxicity responses are absent for all of the bioassays and which yield a $T_i$value $= 0$).

This approach has the advantage of combining information on (*1*) the biodegradability/persistence of effluent toxicity (indicative of its possible fate in receiving waters), (*2*) the trophic levels targeted by effluent toxicity (indicative of the ecological scope of impact), and on (*3*) the flow characteristics of the effluent (indicative of toxic loading released to the environment). The integration of these concepts into a PEEP scale or index is innovative in that it brings together factors of relevant ecotoxicological importance into a simple, practical and useful management tool that identifies the hazardous potential of industrial effluents via a bioanalytical screening strategy. Its effectiveness in predicting the overall hazard potential of wastewaters was revealed for 77 priority effluents investigated under the first two St-Lawrence Action Plans (46). In quantifying the toxicity of industrial discharges, the PEEP index unambiguously points a finger at the most problematic ones requiring priority attention in terms of cleanup action, such that environmental protection effectiveness can be achieved. It is evident by observing the range of PEEP values determined for the industrial effluents studied that some effluents are irrefutably more noxious than others (Fig. 10). As with other scales that reduce complex information into single synthetic values (e.g., species diversity indices in ecology, Richter scale for earthquakes), the effluent PEEP index produces values that can be easily understood by environmental managers, industrialists and the general public. In light of the specific

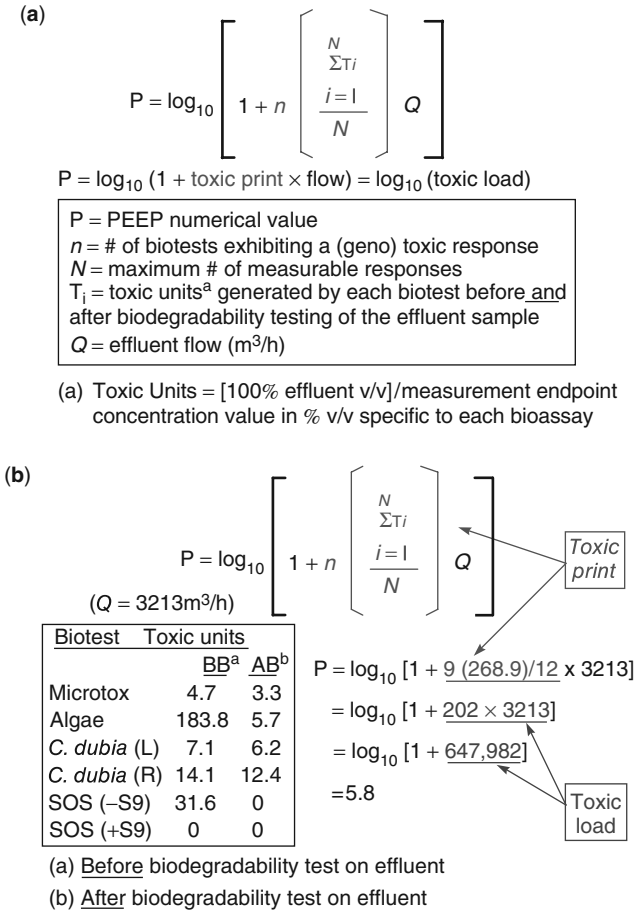


Figure 9. (**a**) PEEP index formula and (**b**) PEEP index calculation for actual toxicity data obtained for a Pulp and Paper effluent. See color insert.

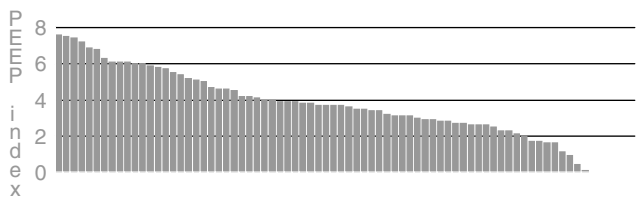


Figure 10. PEEP values for 77 Canadian-based effluents investigated under two St-Lawrence River Action Plans (1988 to 1998). See color insert.

focus of this article, it must be emphasized once again that incorporating a microalgal biotest within an integrated strategy such as the PEEP index is essential to appraise the degree of hazard that point source discharges can represent with respect to primary producers.

TESTING SOLID MEDIA: AN ALGAL SOLID PHASE ASSAY (ASPA) TO ASSESS FRESHWATER SEDIMENT TOXICITY

Because microalgae and other organisms can be adversely affected by toxicity resulting from sediment resuspension owing to both natural (e.g., flood scouring) and man-made activities (e.g., dredging, open water deposition), direct contact microassays that can react to both readily soluble contaminants and to those either adsorbed or absorbed are important to develop and apply. Whereas there are numerous microbiotests to assess liquid media (38,47), few are presently adapted to properly measure the toxic potential of solid media and there is clearly a gap to fill in this respect in terms of microscale bioassay development. Presently available direct contact tests are mostly conducted with bacteria (48–52), although a new algal solid phase assay (ASPA), has recently been developed with the green alga *S. capricornutum* to evaluate the degree of toxicity of freshwater sediments (53). In this assay, the capacity of cell esterases to cleave the (nonpolar) stain fluorescein diacetate (FDA) and liberate fluorescein, a polar and fluorescent by-product, is the criterion that allows us to determine the extent to which algae have been intoxicated after a 24-hour exposure to serial dilutions of test sediment. As illustrated in Figure 11, algal cells insulted by direct contact with sediment contaminants will be less fluorescent than normal cells either because loss of esterase activity will have transformed less FDA into fluorescein (F) and/or because any produced fluorescein will tend to leak out of algal cells owing to damaged membranes that make cells more porous. After an exposure period of algae with test sediment conducted in tubes under defined conditions (Fig. 12), individual cell fluorescence (measured from a total of 2000 cellular events in each experimental test tube) can then be precisely quantified with the help of a flow cytometer, an instrument that measures specific optical properties of cells in motion.

The resulting toxicity end point (IC50) relates to both esterase inhibition and cell membrane integrity. In applying ASPA on a series of naturally contaminated sediments sampled from various locations in the Québec portion of the St-Lawrence River, the assay was able to correctly discriminate sediments on the basis of their contamination level (53). As an example, Table 5 illustrates this by showing that ASPA toxicity responses spanned more than two orders of magnitude (i.e., IC50 of sample 4/IC50 of sample 1 = >10/0.056 = >179) after assessment of four representative sediment samples. Validation studies by generating data with additional sediments are continuing for ASPA. Whereas cost considerations clearly inhibited large application of flow cytometry in the 1980s for environmental R&D, increasing

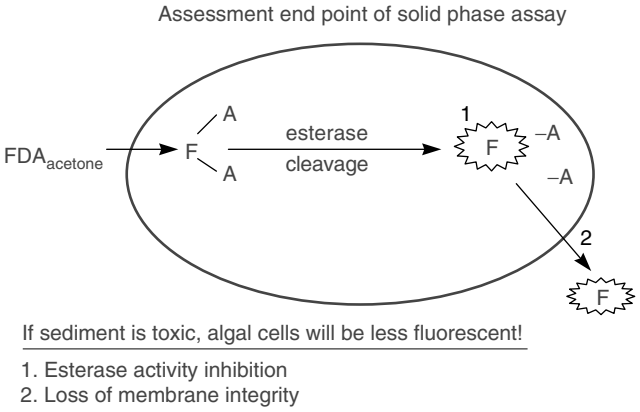


Figure 11. Principle of the algal solid phase assay (ASPA): see text for details. See color insert.

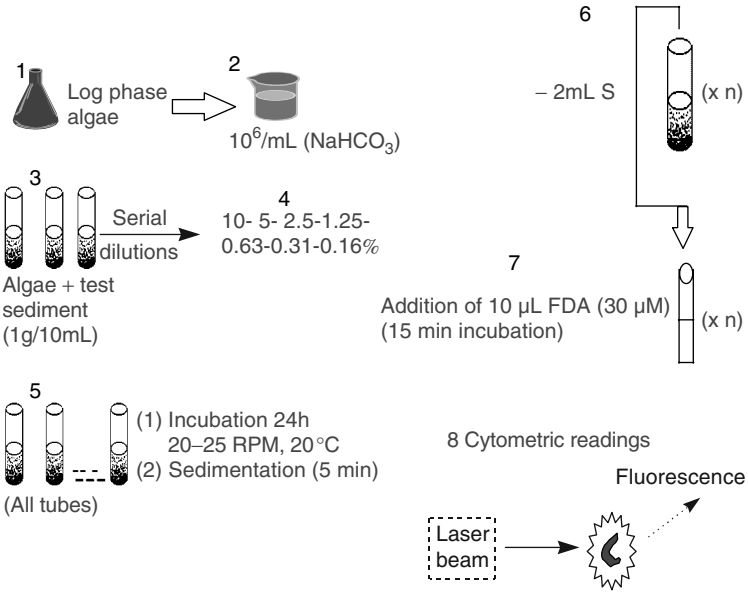


Figure 12. Algal solid phase assay: a culture of exponentially growing (log phase) algae (1) are used to prepare an algal concentration of 10^6 cells/mL in $NaHCO_3$ (2); test sediment tubes contain a starting concentration of 10% test sediment (3) from which additional serial dilutions are prepared (4); all tubes are stoppered with Parafilm and exposed under specified conditions (5); a 2-mL volume of supernatant (containing essentially postexposure algae) is withdrawn from each tube (6) and dispensed into a smaller tube compatible for flow cytometry measurements; to check for esterase activity, an FDA solution is micropipetted into each small tube and allowed a 15-minute interaction time with algae (7); FDA-induced fluorescence, indicative of cell viability, is then quantified by flow cytometry (8). See color insert.

Table 5. Algal Solid Phase Assay (ASPA) Responses to Four Sediment Samples (53)

Sample Origin	IC50 (% Wet Weight)	Confidence Intervals (95%)
Ile aux Corbeaux	0.056	0.019 to 0.10
Montréal harbor	0.24	0.097 to 0.57
Hamilton harbor	1.66	1.42 to 4.41
Lake St-François	>10	>10

demand for this versatile technology, coupled with contemporary commercialization of more cost-effective and compact (portable) flow cytometers, should stimulate the use of ASPA-like procedures by interested laboratories for a variety of environmental studies.

PRESERVING MICROALGAE FOR BIOLOGICAL TESTING

As a final section to this review on phytotoxicity testing, it is of interest to mention the advantages that readily available biological reagents can confer to the field of small-scale toxicology. Eliminating the need, therefore, for continuous culturing of indicator organisms employed for undertaking biological tests is an attractive venture, as it markedly reduces labor and increases cost-effectiveness for laboratories involved in toxicity studies. Lyophilization of luminescent bacteria (54) and the use of cryptobiotic (dormant) stages of microinvertebrates (55) are two outstanding examples of the ways in which toxicity testing can be made more user-friendly by precluding the need for constant maintenance of laboratory cultures. Microalgae have also inspired a significant amount of research in the quest for an appropriate storage technique, which would make these cells culture-free (e.g., agar preservation and cryopreservation). Immobilization of exponentially growing microalgae in an alginate bead matrix has, in particular, shown promise as an effective means of preserving cells for prolonged periods (56). More recently, a validation study aimed at standardizing an alginate-immobilized microalgal reagent with *S. capricornutum* confirmed that cells retained full viability for several months and that comparative toxicity testing with laboratory-cultured algae of the same species yielded concordant results (28). The major steps involved in the immobilization and de-immobilization of *S. capricornutum* are schematized in Figure 13. Perhaps not readily evident to the reader is that alginate-immobilized algae can be rapidly de-immobilized in 30 minutes, after which they are immediately available as inoculum for initiating toxicity testing. Immobilization of microalgal cells is an interesting breakthrough that further facilitates and enhances the use of phytotoxicity testing for assessment of xenobiotics (28,56–58).

CONCLUSION

Initially used as eutrophication indicators, microalgae are also now being employed for toxicity and interaction studies, as this review has shown. Simple algal bioassays, thanks to ingenuity, miniaturization and new instrumental technologies, can be profitably applied to enhance our knowledge of the ecotoxicity of environmental contaminants. In this respect, they have been (and continue to be) invaluable for screening, ranking and prioritizing the potential hazards of chemicals and varied complex media, alone and/or as key adjuncts to batteries of bioassays undertaken at different trophic levels. Phytotoxicity testing is an essential cog of the various tools and strategies (e.g., other bioassays, chemical analysis, field surveys), which collectively contribute to ecotoxicological knowledge to ensure rational decision making. In this sense, it is unquestionable that phytotoxicity data, generated with microalgae as bioindicators, will continue to serve the interests of aquatic environmental programs worldwide.

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Table 4: Elsevier Publishers, Amsterdam, Biomedical Division.
M. Harwood, C. Blaise, and P. Couture, 1989. Algal interactions with the genotoxic activity of selected chemicals and complex liquid samples. *Aquatic Toxicol.* **14**, 263–276 (1989) (Table II: adaptation of selected data).

Table 5: National Water Research Institute, Environment Canada, Burlington, Ontario, Canada.
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Figure 6: Wiley-Liss, a division of John Wiley & Sons Inc., New York.
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Figure 10: Ann Arbor Press, Ann Arbor, MI.
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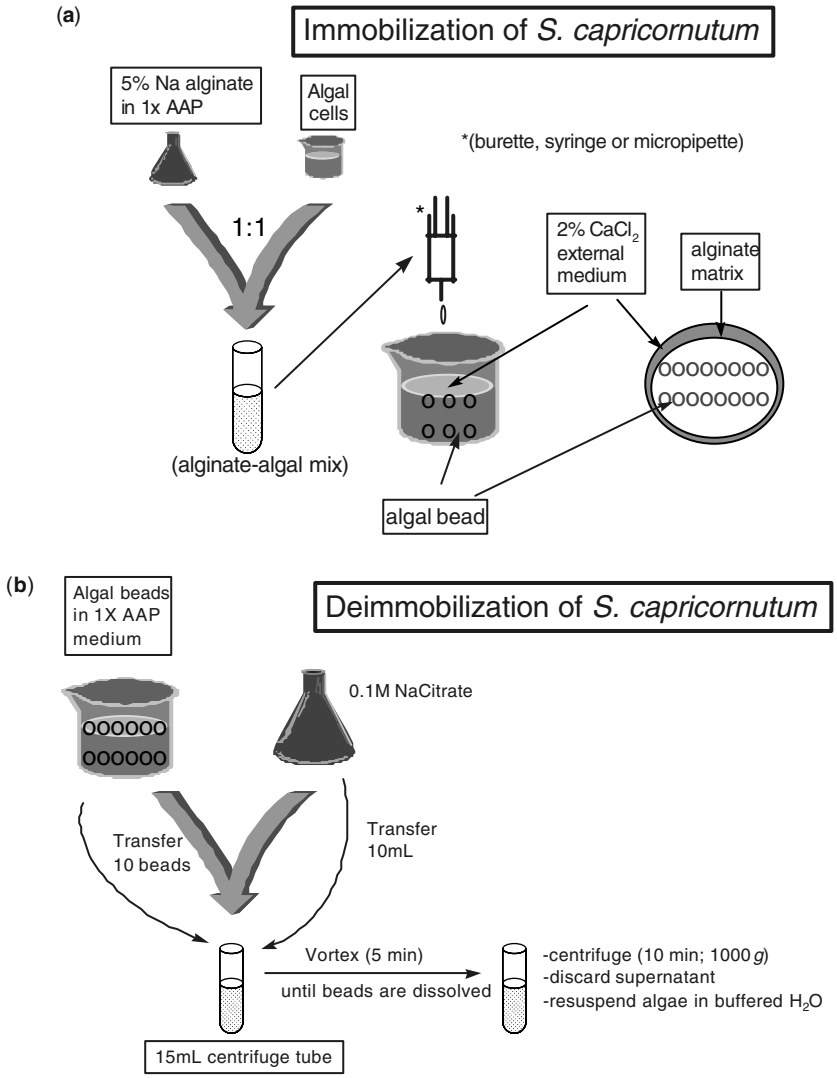

Figure 13. (**a**) Immobilization and (**b**) de-immobilization of *S. capricornutum* preserved in an alginate matrix. (**a**) Algal cells are mixed in sodium alginate in equal portions. Drops of the mixture are allowed to fall into a beaker containing a $CaCl_2 \cdot 2H_2O$ solution, during which algal beads are formed. (**b**) Algal beads are added to a sodium citrate solution to free algae from their alginate matrix in preparation for testing (see references 56 and 58 for details). See color insert.

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UV DISINFECTION — THEORY TO PRACTICE

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Ultraviolet (UV) light is electromagnetic energy that is located in the electromagnetic spectrum at wavelengths between those of X-rays and visible light. UV is further broken down into UV-A, UV-B, UV-C, and vacuum UV. These types of light reside from about 40 to 400 nm in wavelength. The UV light effective in inactivating bacteria and virus resides in the UV-B and UV-C ranges of the spectrum (200 to 310 nm). Maximum effectiveness for inactivation of most bacteria and virus species occurs around 265 nm.

It is a fundamental premise of photochemistry that reactions cannot occur if the reactant species does not absorb energy in the spectral range of the incident light. Therefore, for UV to be an effective disinfectant some critical biochemical moiety of the given organism must absorb energy in the appropriate range. The photochemical interactions between UV light and living organisms, termed *photobiology*, was well explained by considering the adsorption spectra of DNA DNA, and thus living cells, such as *Escherichia coli*, adsorb significant amounts of light energy in the range of 200 to 300 nm with a peak around 260 to 265 nm. Further investigation indicated that thymine bases on the nucleic acids (DNA and RNA) were particularly reactive to UV light and would form dimers (thymine = thymine double bonds). These dimers then chemically inhibit transcription and replication of nucleic acids thus rendering the organism sterile. Once

the organism is sterile it cannot cause infection — thus effective disinfection has occurred.

It has been found that many organisms are capable of repairing the thymine dimers and restoring the original nucleic acid structures using enzymes. This repair is termed *photoreactivation* if it occurs in the presence of light or *dark repair* if light is absent. As a result of this repair phenomenon, the strategy in UV disinfection has been to provide a high enough dosage that enough nucleic acid damage occurs to prevent effective repair.

This classical explanation of the germicidal mechanisms of UV irradiation holds extremely well in cases where continuous wave, monochromatic, moderate to low energy UV light is used — which has been the standard case for the past 30 years. More recently however, continuous or pulsed wave, polychromatic, high intensity UV lamp technologies have been developed and applied to water and wastewater treatment. Continuous wave, medium pressure mercury vapor lamps are becoming increasingly popular in higher flow water and wastewater applications owing to overall cost savings. Pulsed UV technology, which relies upon a high energy xenon arc (or flash) lamp has shown promising results in recent drinking water treatment research at the bench, pilot, and full-scale. Medium pressure lamps emit significant amounts of energy at 222.5, 253.7, 265, and 296.5 nm (with lower emissions at several other wavelengths in the germicidal range). Pulsed UV lamps emit high-energy bursts (pulses) of polychromatic light in the range of 185 to 800 nm.

It is theorized that these higher intensity, polychromatic emissions may do significantly more biochemical damage (similar to that of ionizing radiation) to not only nucleic acids but also to cellular proteins and enzymes. Related work suggests that these high-intensity sources may enhance the formation of secondary radical reactions giving rise to germicidal chemical species, such as hydroxyl radicals, ozone, or hydrogen peroxide.

Biological techniques intrinsically allow accounting for the most factors that influence UV dose. These tests are usually performed in the same water that is treated by an on-line UV system or proposed system so that the water matrix effects are inherent in the test results. However, laboratory irradiation of cultures is independent of hydrodynamic effects, such as short circuiting or dispersion that can be present in flowing systems. Bioassay procedures examine a microorganism's response when exposed to a specific UV dose, known as the organism's *UV susceptibility*. For most microorganisms, there exists a first order relationship between UV dose and log inactivation.

One particular bioassay method used in the wastewater industry, NSF 55, uses *Bacillus subtilis* spores. Under controlled laboratory conditions, early experiments showed that a 4 log inactivation of spores was achieved at a UV dose of about 31 mW s/cm^2. Because drinking water applications will likely require a slightly higher UV dose, obtaining a sufficiently large initial population of spores to measure substantial inactivation is a problem. For this reason, other organisms that are more resistant to UV may be required to measure these higher UV doses. One such organism is MS-2 Coliphage, which is an F-specific single-stranded RNA virus. MS-2 is about 20 nm in diameter and can be used as a viral surrogate.

Organic matter typically found in colored surface water supplies will exert a UV absorbance at 254 nm (A/L) of between 0.100 and 0.800 cm^{-1} ($\alpha = 0.23$ to 1.84). Treated surface waters and typical groundwaters have significantly lower absorbances, generally ranging from 0.005 to 0.500 cm^{-1} ($\alpha = 0.01$ to 0.12).

Waters with lower absorbance will traditionally require less UV energy to achieve the same disinfection as waters with high absorbance. In a flowing system, UV dose is inversely proportional to system throughput. For a given UV system, water with lower energy requirements can increase throughput proportionally over water that requires more UV energy. As a general rule, when the value of α for a given water matrix exceeds 0.43 ($A/L > 0.187$ cm^{-1} or $\%T < 65\%$) pretreatment to reduce UV interference (or attenuation) will be required to insure successful UV disinfection.

The negative impacts on UV disinfection effectiveness of turbidity (particles or suspended solids) in the water quality matrix is also well documented. Unfortunately, measurement of UV absorbance (or $\%T$) does not always account for the turbidity effects. Turbidity causing particles or suspended solids can increase UV absorbance but often it has a minimal effect on absorbance measurements or α. Nevertheless, turbidity can still reduce disinfection effectiveness by shielding microorganisms from the UV. Therefore, it is also wise to measure turbidity when assessing water quality matrix effects on UV disinfection. In most cases, if the turbidity is maintained below 1 NTU the effects on UV disinfection efficiency will be minimal. To maintain low turbidities, surface waters will require filtration and some groundwaters will require iron and/or manganese pretreatments that include filtration.

BACKGROUND

Conventional UV disinfection is a physical disinfection process that uses commercially available lamps, which emit UV light in the wavelengths of 200 to 300 nm. These so-called, *germicidal wavelengths*, cause damage to DNA and RNA by dimerizing bases, such as thymine and uracil. The damaged nucleic acids prevent replication thus rendering the potential pathogen sterile and unable to cause infection. UV disinfection has had widespread use in the wastewater treatment field throughout North America. However, it has long been believed that UV was incapable at inactivating protozoan cysts or oocysts, such as *Giardia lamblia* cysts and *Cryptosporidium parvum* oocysts at cost effective dosages (1,2). Recent findings in North America that conventional, continuous wave UV disinfection can inactivate *C. parvum* as well as many other human pathogens of concern in drinking water at cost effective dosages has spurred tremendous excitement and research throughout the United States and Canada.

Pending regulations, focus on simultaneously protecting the public health from microbial risks such as those

from *Giardia*, *Cryptosporidium* and viruses and the potential chemical health risks from disinfection by-products has posed a difficult problem for the drinking water industry. In the United States negotiations are underway between all stakeholders to adopt the Stage 2 Microbial and Disinfection By-Product regulations. Concerns exist that if tighter *Cryptosporidium* inactivation requirements are imposed then drinking water systems will need to shift to more costly technologies, such as long ozonation $C \times T$ times or membrane filtration. In addition, if disinfection by-product (DBP) regulations for bromate or individual brominated organics are imposed then ozone may not be an optimal solution. The potential that UV disinfection processes could be used to inactivate microbes of concern while reducing or as a minimum not adding to DBP formation has caused many drinking water organizations to focus on bench, pilot, and full-scale research for applying UV to surface drinking water disinfection requirements.

EFFECTIVENESS OF UV DISINFECTION

Drinking water disinfection requirements have been driven in recent years by concerns about the risks from *G. lamblia, C. parvum*, and human enteric viruses. In the United States, the 1986 Surface Water Treatment Rule (SWTR) mandated a 3-log removal/inactivation of *Giardia* and a 4-log removal/inactivation of viruses. More recently, the Interim Enhanced SWTR added a 2-log removal of *Cryptosporidium* through optimized filtration requirement. Figure 1 shows the effectiveness of UV for inactivation of *C. parvum* oocysts and *Giardia* cysts. These data reflect the recent understanding that UV effectiveness for inactivating protozoan cysts or oocysts must be evaluated using animal infectivity or cell tissue culture methods (3). The *Cryptosporidium* data show that a UV dose of 10 mJ/cm^2 inactivated up to 4-log of oocysts in most cases. Finch and Belosevic (4) observed a tailing effect in their results but concluded that a UV dose of 10 mJ/cm^2 would reliably achieve a 2-log inactivation. By contrast older literature suggested that doses of hundreds or thousands of mJ/cm^2 would be required to inactivate protozoan cysts (2). It is also interesting to note that the *Cryptosporidium* data based on animal infectivity generated by Bukhari and coworkers (3) and by Finch and Belosevic (4) corresponds well with the *Cryptosporidium* data based on cell tissue culture generated by Linden and Sobsey (5) and Mofidi and coworkers (6).

The *Giardia* data suggest that greater than 2-log inactivation of cysts can be achieved at a dose of 10 mJ/cm^2. Finch and Belosevic (4) working with *G. muris* cysts noted a tailing effect, which was not observed by Linden and Sobsey (5) who were using *G. lamblia* cysts. In either case, the data sets both demonstrate that a UV dose of 10 mJ/cm^2 or less will effectively inactivate at least 2-log of *Giardia* cysts.

The effectiveness of UV for inactivating virus is well documented but it is important to note that there is a wide variety of virus strains that may be of concern in drinking water and their relative susceptibility to UV can vary widely. Table 1 summarizes the effects of UV on the major virus strains currently of interest to the drinking water industry. These data are compiled from work done by the authors on a variety of water qualities and data available in the current literature. These data suggest that Adenovirus, a double stranded (DS) DNA virus, may be the most resistant to UV inactivation. This finding would be consistent with earlier reports that Adenovirus has the capability to enzymatically repair damage to the DNA. Aside from Adenovirus data, which is currently undergoing further study and verification, it has been generally accepted that the surrogate MS-2 phage is a conservative indicator of human enteric virus inactivation by UV. As a result, it has been proposed by USEPA that a UV dose of 40 mJ/cm^2 would be granted a 2-log virus

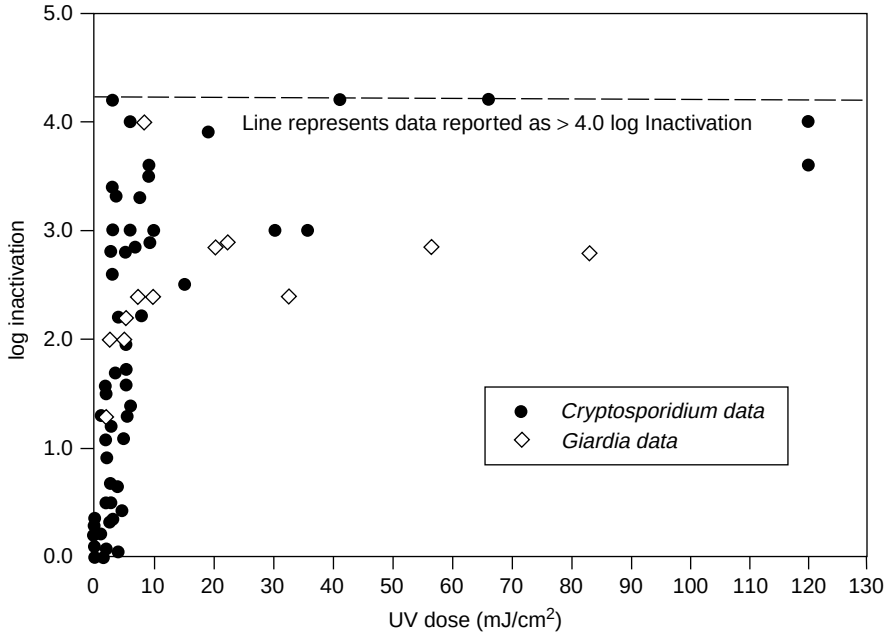

Figure 1. UV dose-response data for *C. parvum* oocysts in filtered surface water samples. Data reported by: Bukhari and coworkers (3), Finch and Belosevic (4), Linden and Sobsey (5), and Mofidi and coworkers (6).

Table 1. UV Effectiveness for Virus Inactivation

Virus	UV Dose (mJ/cm^2) for Inactivation of			Reference
	2-log	3-log	4-log	
Adenovirus 40	59	90	121	7
Adenovirus 41	50	80	—	7
Coxsackievirus B5	14	21	—	8
Hepatitis A HM-175	45	11–13	16–22	3
Poliovirus Type 1	8–11	15–19	23–29	3
Reovirus Type 1	36	—	—	9
Rotavirus WA	25–32	35–46	50–70	3
Rotavirus SA-11	19	25	36	10
Surrogate: MS-2	25–39	38–63	50–93	3

inactivation credit. If future work supports the Adenovirus data then the UV dose for 2-log credit would likely be increased to about 60 mJ/cm^2.

FACTORS EFFECTING UV SYSTEM PERFORMANCE

Water quality conditions can effect all disinfection processes. In the case of UV these effects are broken into direct effects and indirect effects. Direct effects include water quality constituents that attenuate or block UV light and thus reduce the moles of photons of UV light, which reach the nucleic acid of the target pathogen. Indirect effects are water quality conditions which can effect lamp performance, foul UV lamp quartz sleeves and UV sensors or cause organisms to surface-associate with particles or aggregate (clump) thus making them more resistant to UV.

Direct effects can be the result of dissolved solutes, such as iron, sulfites, natural organic matter (color or TOC), and synthetic organic compounds or the result of particles (turbidity) and suspended solids. It is well documented that UV disinfection is not directly effected by pH and temperature. Snicer and coworkers (11) performed a study funded by the American Water Works Associated Research Foundation (AWWARF) in which 30 groundwaters, 15 conventionally treated surface waters and 18 synthetic waters were examined to determine which water quality parameters directly affected the ability of UV to inactivate human enteric viruses and the surrogate MS-2 bacteriophage virus. An example of these data is shown in Figure 2. The figure displays the unadjusted applied UV dose versus the resulting microbial inactivation. The study showed that the parameters, which significantly affected UV performance, were the dissolved iron concentration, the UV absorbance measured at 254 nm (resulting from dissolved organic matter or iron) and the nature and type of particles. The effects of dissolved solutes can be accounted for by using the UV absorbance measurements to adjust the UV dose provided to the water. However, the degree to which the UV dose can be increased to account for background absorbance is limited by practical space and cost considerations.

Effects of particles (often measured by turbidity) or suspended solids on UV performance are a function of their nature, type, and concentration as well as their interaction with the target organism. Experiments looking at the effects of three different particles on the ability of UV to inactivate MS-2 are shown in Figure 3. In these studies, samples of wastewater effluent (representing amorphous biological particles); settled conventional drinking water (representing amorphous inorganic (alum) floc); and

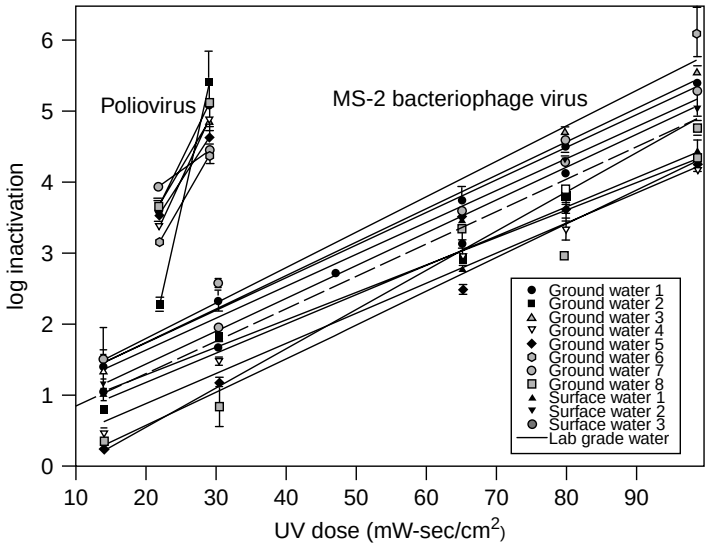

Figure 2. Effects of differing water qualities on UV performance. From Ref. 13.

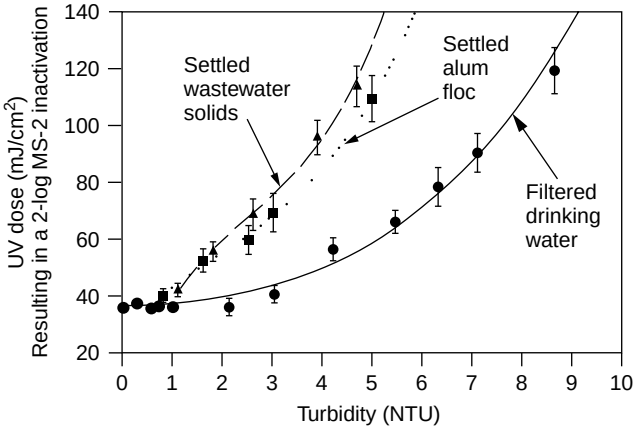

Figure 3. Effects of particle (turbidity) type and concentration on UV performance. From Ref. 13.

conventional filter effluent that had breakthrough of discrete inorganic claylike particles were collected and spiked with MS-2. After 8 hours of mixing these samples were subjected to batch bench-scale collimated beam UV tests to determine the dose required to inactivate 2-log of MS-2. These data show that amorphous solids have a much more dramatic effect on UV performance. Further, the particles passed through conventional filtration did not significantly effect performance until levels above 3 NTU were obtained. These data suggest that the type and nature of particles will be important. In addition, these data suggest that placing UV post filters rather than prior to filters is the optimal location in a conventional drinking water plant. Additional work is in progress to determine how the nature of particles, which may contain high levels of organic matter or algae, will effect UV.

These types of particles are often encountered in unfiltered water supplies and in conventional filter plant effluent that are treating highly colored waters or algal laden reservoirs.

The most significant indirect effects of water quality parameters were found to be sleeve and sensor fouling by dissolved iron, hardness, or minerals and the effects of water temperature on lamp stability. Iron and minerals were found to significantly increase the rate of UV lamp sleeve fouling especially in medium pressure UV lamp systems because of their increased operating temperatures. Water temperature was found to significantly effect low-pressure and low-pressure high-output UV lamps systems likely because these lamp systems typically operate at internal temperatures of 40 to 60 °C. Treating surface drinking water supplies in northern climates with UV is one of the first applications in which waters entering the UV system may be at temperatures as low as 0.5 °C. At these lower temperatures the UV lamps may have unstable output or take longer to reach a stable operating level. Therefore, designers must account for these effects when selecting lamp types, quartz sleeve designs, and operating procedures. Temperature effects on medium pressure UV lamp systems were not found likely owing to the fact that MP UV lamps operate at internal temperatures of 400 to 600 °C.

IMPORTANT CONSIDERATIONS IN UV SYSTEM DESIGN

As shown in Figure 4, there are several factors, which must be considered when designing UV systems. One critical factor is minimum UV dose, which is affected by UV lamp design (type, number, and orientation), UV reactor hydraulics, and water quality parameters. As previously discussed, the target organism and the degree of inactivation desired will also strongly influence the minimum design dose because organisms vary in their resistance to UV. The importance of water quality has also been demonstrated in this paper.

UV sensors are critical to the day-to-day operation and performance verification of UV systems. Concerns over UV sensor reliability remains the largest obstacle to UV acceptance by regulatory officials and water utility operators. The author has found that the precision, accuracy and stability of UV sensors vary widely. An initial survey of UV sensors in place at over 100 wastewater UV disinfection facilities found that 80% of them were unreliable and hence not used. In wastewater UV systems day-to-day performance can be verified by measuring effluent coliform organisms however, in drinking water treatment there is no acceptable surrogate that is always present in the water and UV sensors will be critical. Increased emphasis by UV manufacturers on developing reliable sensors has led to improvements. Recent AWWARF research performed by the author has shown that reliable UV sensors for low-pressure systems are now in use. However, effective sensors for medium-pressure UV systems are less common. All sensors must be properly maintained and calibrated frequently (at least quarterly) to produce meaningful results.

CONCLUSION

UV disinfection systems have been found to effectively inactivate *Giardia* cysts, *Cryptosporidium* oocysts, and human enteric viruses at cost-effective dosages. This result combined with earlier findings that UV does not increase disinfection by-products or contribute to regrowth problems in distribution systems makes it an attractive technology for meeting emerging water quality regulations in the United States. UV system design and performance is a function of lamp design and reactor hydraulics as well as key water quality parameters, such as UV absorbing constituents, particles, and solids, and constituents that can foul UV quartz sleeves and sensors. UV sensors are critical to the day to day performance monitoring of UV systems and the sensors must be carefully maintained and calibrated frequently to insure reliability.

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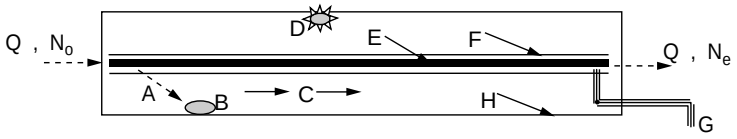

Factor Index:
A. Minimum UV dose - f (lamp and reactor hydraulic design)
B. Target organism and desired kill - (dose-response varies)
C. Water quality Matrix - f(Fe, Mn, pH, TOC, turbidity/particles)
D. UV sensores- critical to performance monitoring
E. UV lamp type (LP, MP, LPHO, flash)
F. Sleeve material (quartz, teflon, coated quartz) and Cleaning
G. System electronics (ballasts), Instumentation and controls
H. Reactor type/material (channel vs. pressure vessel - SS vs. PVC)

Figure 4. UV reactor schematic and key design factors. From Ref. 13.

and Laurel Passantino have contributed greatly to our current knowledge of UV disinfection.

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V

VADOSE ZONE MICROBIOLOGY

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The vadose zone extends from land surface to the water table. In regions with high precipitation, it may be a few meters thick or less, whereas in desert regions, it may extend to depths of hundreds of meters. Generally speaking, the vadose zone is the part of the subsurface that is unsaturated with respect to water; however, it can contain intervals of perched water, that is, zones of water saturation immediately overlying relatively impervious rocks or fine-grained sediments. Apart from these perched water zones, the vadose zone can be considered to be synonymous with the unsaturated zone.

Vadose zone microbiology is important for a number of reasons. First, because aquifers are a source of drinking water, vadose zone processes are important for potential mitigation of groundwater contamination by anthropogenic chemicals and pathogenic microorganisms. Second, although deep vadose zones with very little moisture recharge represent one of the most extreme environments for microorganisms, they are of interest from the standpoint of defining ultimate limits on microbial existence and whether microorganisms may over long periods negatively impact the storage of nuclear waste buried in such vadose zones. This article will summarize the major physical, chemical, and microbiological aspects of the vadose zone, special considerations for microbiological studies in the vadose zone, and the practical and basic science themes involving vadose zone microbiology.

PHYSICAL AND CHEMICAL CONDITIONS

As an unsaturated porous medium, the vadose zone contains three phases: solid, liquid, and gas. The water of the liquid phase exists as thin, discontinuous water films coating the solids. The amount of water in the unsaturated zone is a major factor controlling the abundance and diversity of microorganisms, but it almost never poses a direct desiccation stress on microbes in the subsurface. Even in extremely arid regions of the world, the vadose zone below the surface soil layer is relatively moist. Water content in surface soils varies spatially and temporally between saturated conditions and extreme desiccation. In contrast, the moisture content below the rooting zone fluctuates more slowly and over a much narrower range. The water potential in unsaturated porous media is negative with respect to pure water and is influenced mainly by the matric potential, which is caused by adsorption and capillary effects at the solid/liquid interface. The matric water potential of the vadose zone beneath the soil layer is generally greater than (i.e., less negative than) -0.1 MPa. At 20 °C, this corresponds to a water activity (a_w) of approximately 0.999. Thus, the vadose zone presents no more of a direct water stress than a typical liquid growth medium. However, water is an important controlling factor because the thin, discontinuous water films of the vadose zone limit the flow and transport of dissolved nutrients and of microorganisms. At -0.1 MPa matric water potential in a porous medium, the average water film thickness is about 2.9 μm (1). Advection and diffusion of solutes and mobility of microorganisms are severely decreased as the water film thickness decreases. Water films may also be discontinuous, further restricting solute transport. Therefore, vadose zone moisture distribution causes extremely static conditions. Microbes experience a very limited nutrient flux, and limited motility further constrains their ability to gain access to nutrients. This nutrient limitation is especially pronounced in the thick vadose zones of arid and semiarid environments, where the organic carbon input from overlying surface soils is typically less than in more mesic regions, and where rates of groundwater recharge are considerably less than in areas with greater annual precipitation.

Rates of groundwater recharge through the vadose zone vary with climate, topography, vegetation, and subsurface hydraulic conductivity. Diffuse recharge rates (i.e., recharge of directly infiltrating precipitation in areas not influenced by runoff, land surface water) range from tens of micrometers to 80 mm yr^{-1} in arid and semiarid regions of the United States and may be as high as 500 mm yr^{-1} in high-precipitation regions of the eastern United States (2,3). Under the unsaturated conditions of the vadose zone, the bulk of groundwater recharge flows primarily through the rock or sediment matrix, that is, through small pore throats and very narrow fractures; whereas the majority of saturated flow (that can occur in the vadose zone following heavy precipitation events or beneath surface water) is via large fractures and porous material with large pore throats.

The gaseous composition of the vadose zone reflects the makeup of the overlying atmosphere and also microbial activities. Oxygen is generally present throughout the vadose zone; however, it may be in lower concentration than in the overlying atmosphere and may be depleted within microhabitats such as the centers of soil aggregates, biofilms in regions high in organic matter, and in regions that are heavily contaminated by organic carbon compounds. The vadose zone is sometimes called the "zone of aeration," indicating the general availability of oxygen. Gaseous products of microbial metabolism such as CO_2, CH_4, H_2, NH_3, H_2S, and volatile fatty acids may be present

in elevated amounts in the vadose zone, especially where organic carbon supports high microbial activities.

Organic carbon can occur in the vadose zone in the solid, liquid, or gas phases. Solid-phase organic carbon can be entrained with the rocks or sediment at the time of burial, as in the case of paleosols (buried soils). Dissolved organic carbon can be transported downwards from overlying surface soils. This tends to consist of relatively recalcitrant compounds that are not degraded by surface soil microorganisms and that are generally present in vadose zone pore waters in very low concentrations. Gaseous organic carbon compounds, for example, volatile fatty acids generated by fermentative microorganisms, may occur, especially in proximity to saturated zones containing residual organic carbon.

MICROBIOLOGICAL PROPERTIES

Primary Controlling Factors

Microbiological properties in the vadose zone are strongly controlled by nutrient availability, which is in turn controlled primarily by the climate (Fig. 1) and differential water flow. In high precipitation climates, abundant vegetation and high recharge result in relatively large inputs of nutrients. Conversely, in arid climates plant communities are very sparse and recharge is low, resulting in extremely small nutrient input. Large- and small-scale features of the geologic media such as contacts between strata and lenses, discontinuous strata, fractures, and differences in the size and connectivity of pores result in partial or total bypass of moisture and soluble nutrients to regions of the vadose zone. For these reasons, vadose zone microbiological properties can be highly variable from one location to another and with respect to the scale of measurement at a given location.

Abundance

At a given location, microbial populations are generally lower than in surface soils and underlying saturated zones. Direct microscopic counts vary from 10^4 to 10^7 cells g^{-1}. Direct counts are typically lowest in low recharge vadose zones. Very low percentages of the direct count cells can be cultured in vadose zone materials. Heterotrophic plate counts are orders of magnitude higher in shallow vadose zones ($<10^1$ to 10^3 CFU g^{-1} to 10^4 to 10^6 CFU g^{-1}) than in deep, low recharge vadose zones ($<10^1$ to 10^2 CFU g^{-1} to $<10^1$ to 10^4 CFU g^{-1}) (4–15).

The median percentage of microscopically visible cells (after staining with the dye acridine orange) that could be cultured and the median percentage of viable cells (estimated by measuring microbial phospholipid fatty acid extracted from the sediment) that could be cultured were 0.0003% and 0.01%, respectively, for deep vadose zones with low recharge. The equivalent values were approximately three orders of magnitude higher for deep vadose zones from the eastern coastal plain where

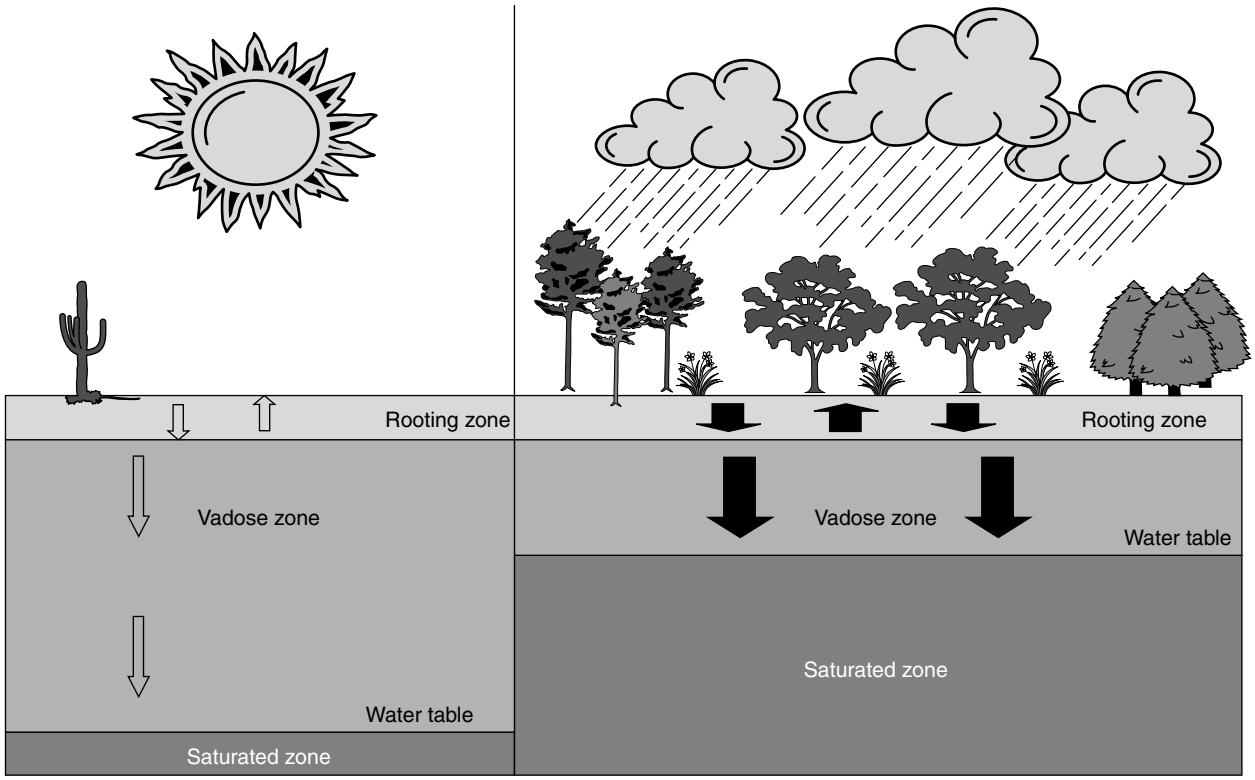

Figure 1. Contrasting moisture movement in the vadose zone in a dry arid climate with transient ponding (left panel) and wet humid climate (right panel). Arrow width represents magnitude of moisture flux with dashed arrows representing very low fluxes. Downward and upward arrows show recharge and evapotranspiration, respectively.

recharge is much higher (16). The lower percentages for the acridine orange cells that could be cultured was probably because of the presence of intact dead cells as, in a separate study, the DGA:PLFA ratio (diglyceride fatty acid [indicative of dead cells] to phospholipid fatty acid [indicative of viable cells]) increased with increasing depth from the surface to 37-m deep (11).

At contaminated vadose zone sites, microscopic counts, and heterotrophic plate counts are typically orders of magnitude higher than at uncontaminated sites (7,10). The highest densities are obtained when contaminants are present at relatively high but not toxic concentrations. Under these conditions, microscopic counts and heterotrophic plate-counts approach or exceed those found in uncontaminated aquifers and surface soils.

Activity

Actual in situ rates of metabolic activities in subsurface environments are generally extremely slow and are best estimated using geochemical modeling approaches (17–19). In aquifers with relatively high hydraulic conductivity, this usually entails modeling microbially mediated changes in groundwater chemistry along a flow path (20); however, this is not possible in vadose zone environments.

Rates of in situ microbial activities in a vadose zone sediment have been estimated based on the disappearance of organic matter since the time of sediment burial. It was estimated that 0.3×10^4 g C m^{-2} had been leached from the sediment and that 4.7×10^4 g C m^{-2} had been biologically degraded in the 7-m-thick vadose zone sediment. Assuming a spatially and temporally constant rate of biodegradation during 11,500 years since the end of the last glaciation, they estimated the in situ rate of microbially mediated carbon mineralization activity to be 0.6 g C m^{-3} yr^{-1}. This corresponds to a rate of CO2 production of 8.0×10^{-4} mmoles CO_2 m^{-3} pore water yr^{-1}. In a separate study, CO_2 concentrations were measured in the same sediments and a mass-balance approach used to estimate rates of microbial activity. They estimated relatively low rates of metabolic activity in most of the depth interval between the surface soil and the water table and a higher rate of 7.1 mg C m^{-3} yr^{-1} in a zone immediately above the water table (termed the "capillary fringe"). This is approximately 9×10^{-3} mmoles $CO_2 m^{-3}$ yr^{-1}. The higher rate in the capillary fringe is consistent with the idea that electron donors may diffuse up from the saturated zone and mix with oxygen in the unsaturated zone to stimulate microbial activities. The water table can also fluctuate up and down at the capillary fringe, thus redistributing nutrients by advection.

Although the rates of metabolic activity estimated by these studies are slow relative to most other types of environments, much slower rates of microbial activity have been estimated for thick unsaturated zones in arid and semiarid environments. Rates of CO_2 production in deep vadose were estimated to be less than 1×10^{-10} mmoles CO_2 m^{-3} yr^{-1} (18). The faster rates that were estimated in the former studies are explained by the fact that these are shallower vadose zones in a moderately high precipitation region beneath pastureland (i.e., abundant vegetation and relatively large nutrient inputs).

Transport

Microbial transport and movement in the vadose zone is key to successful bioremediation success and protection of groundwater from pathogens. Many physical, chemical, and biological factors affect transport and movement of microorganisms in the vadose zone, however physical factors unique to the vadose zone play a major role in controlling transport (Table 1). Given a particular subsurface location, microbial transport is much more limited if the system is unsaturated. This is because the presence of air in the pores results in films and/or pendular rings of water. At a relatively wet −0.1 MPa matric (−1 bar) water potential in a porous medium, the average water film thickness or *effective* pore diameter for the microorganism is about 2.9 μm^3 (1). In addition, in laboratory experiments bacteria appear to have a propensity to adsorb to air-water interfaces, adsorption increases with decreasing saturation, and adsorption may be irreversible in some cases (22–24). If microorganisms are not adsorbed to the porous media or to the air-water interface, they may move chemotactically or by water advection at such relatively wet matric potentials. However, active (via chemotaxis) and passive movement (via advection) are unlikely at matric potentials below approximately −0.05 MPa (25). As the volumetric water content (and water-filled porosity and matric potential) decreases the films and pendular rings become more discontinuous both on the surface of individual particles and at the intersections of particles, thereby increasing the tortuosity and eventually separating the system into a myriad of isolated microcompartments. Fine-textured porous materials may retain a network of continuous films at much more negative matric potentials than coarser materials, however, the water films are thinner and microbial transport is inhibited by adsorption and/or filtration.

Laboratory investigations of microbial transport under unsaturated conditions have typically been performed in homogenous sand columns or in simulated porous media with high concentrations of bacteria and/or nutrients.

Table 1. Factors Affecting Microbial Transport in the Vadose Zone. Factors Unique to the Vadose Zone Are in Bold

Physical
Pore size distribution, pore connectivity, local water flux, **water film thickness, connectivity of water, adsorption of cells to air-water interface, transient saturated flow**
Chemical
Organic and inorganic coatings sponsor adsorption and/or attachment, nutrient concentrations affect growth rate and detachment
Biological (cell characteristics)
Size, shape, hydrophobicity, motility, attachment behavior, detachment behavior

Bacterial transport in agricultural soil microcosms has been relatively well studied; however, soils are structurally and chemically different from the vadose zone. Although these laboratory and field studies do not accurately represent most vadose zones, transport processes in soil can be extrapolated to the vadose zone. In soils, microbial transport occurs primarily by preferential (saturated or near-saturated) flow within soil channels or macropores (26–30) and matrix flow during transient saturated conditions (31). Likewise, transport of microorganisms in the vadose zone probably occurs primarily within preferential flow pore networks or fracture systems during transient saturated or near-saturated flow following storm events or rapid snowmelt, or underneath transient surface water channels and streams. This conclusion is supported by a study of the distribution of indigenous microorganisms in vertical (4- to 15-m deep) unsaturated flow paths at 5 semiarid field sites with either high recharge accompanied by rare saturated flow, high recharge via solely unsaturated flow, or low unsaturated recharge (4). This study found several lines of evidence suggesting transport of microbial cells at the high-recharge sites with rare saturated flow and lack of evidence for microbial transport at the other sites. Saturated flow in preferential flow paths is common in shallow vadose zones with high precipitation climates but rare in deep vadose zones in arid and semiarid climates.

Microbial transport and colonization can be major processes in contaminated vadose zones if the contaminant(s) supports growth and is present at high but nontoxic concentrations. In addition, contaminants are typically delivered to the vadose zone by a transient saturation event and/or lengthy periods of elevated pore saturations, which enhances transport and colonization as described in the preceding text. Cells have been shown to be transported between saturated pores by gas bubbles generated by fermentation (32), and a similar process may occur in the vadose zone during bioventing and in the capillary fringe during biosparging (see Bioremediation section).

Heterogeneity

A number of processes discussed in the preceding text, several of which are reiterated in this paragraph have potential for generating a high degree of microbiological spatial heterogeneity at a given vadose zone site. Differences in nutrient flux (in pristine and contaminated systems) in regions that experience preferential flow, only matrix flow, or only diffusion are likely to generate large differences in number, type, and activity of microorganisms. Interfaces between sedimentary layers often generate localized regions of greater saturation where microorganisms may exhibit increased numbers or activity due to improved access to sediment-associated nutrients. Finally, low water contents in coarser porous media results in a highly fragmented moisture distribution where diffusion within each pore-scale water compartment is the only process supplying nutrients to trapped microorganisms (in the absence of any gas phase transport of electron donors and macronutrients). Under these conditions, chemistries in the individual subpore water compartments can be very different as a result of the heterogeneous distributions of minerals and sediment-associated nutrients at this scale. Thus, microbes may survive in rare favorable microsites and die from extreme nutrient deprivation over geologic time periods in the majority of the microsites, giving rise to heterogeneity (33). These and other heterogeneity-promoting processes are likely to impact the distribution of specific microbial functional types and specific contaminant-degraders to a greater extent than the microbial population as a whole. Although spatial heterogeneity of some soil microbiological processes has been examined in detail (34–36), little research has been devoted to systematic examination of microbial spatial heterogeneity in the vadose zone and its impact on contaminant transformation. Similarly, the presence and distribution of specific microbial functional types, such as different types of strict anaerobes (in high recharge vadose zones with high nutrient concentrations) and chemolithotrophs, has rarely been addressed.

The impact of water flux, and by extension nutrient flux, on microbial distribution was studied by comparing sites receiving 0.015 μm, 0.1 to 10, and 200 mm average annual recharge. Approximately 10^4 viable cells g^{-1} were present in multiple samples from the three sites, based on phospholipid fatty acid analysis performed on 75-g samples. The spatial distribution of microorganisms capable of metabolism were examined by performing several 50-sample transects (5 to 10 cm sample spacing) at each site, and 1-g aliquots from a 15-g homogenized sample were assayed for ^{14}C-glucose mineralization (with postincubation acid treatment to recover dissolved ^{14}C-CO_2) and ^{3}H-acetate incorporation into membranes in 2- to 3-month unsaturated incubations. Microbial activity was not detected in 60 to 70% of the samples from the low-recharge sites compared to 0 to 25% at the 20-cm recharge site (37). Samples from the low-recharge sites were 4- to 8-fold more likely to have detectable activity in silts than in the lower water content sands. Because the silt and sand had similarly low organic carbon content, this result is probably attributable to greater water connectivity at the pore scale in the

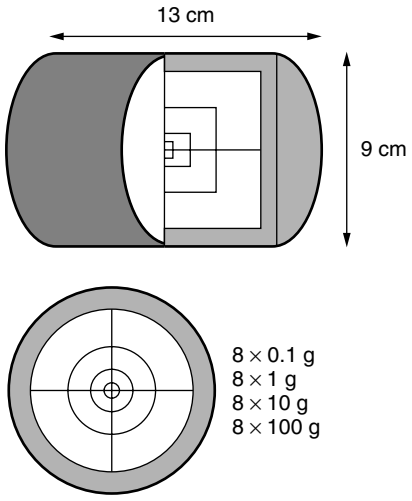

Figure 2. Longitudinal sectioning and sampling of an intact core section for multiple-scale assays of microbial activity.

silt and thus greater survival on account of improved diffusion of nutrients to isolated microorganisms. The large percentage of samples lacking detectable activity with the highly sensitive ^{3}H-acetate incorporation assay at the low-recharge sites indicates that large volumes

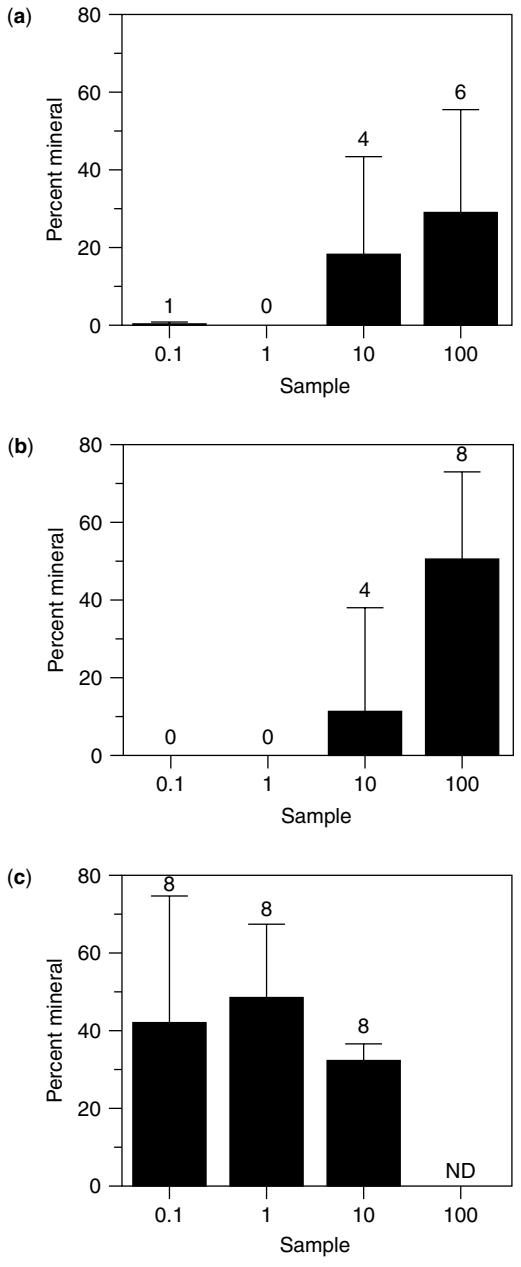

Figure 3. Microbial activity (mean and standard deviation) in eight replicate samples, obtained as shown in Figure 2, from cores at different sites. Values above bard indicate the number of replicates in which activity was detected. Panel A: average annual recharge (AAR) of 15 micrometers, 60 day aerobic incubation. Panels B and E: AAR between 100 micrometers and 1 mm, 60 day aerobic and anaerobic incubations, respectively. Panels C and F: AAR of 20 centimeters, 3 day and 26 day aerobic and anaerobic incubations, respectively. Panel D: surface soil B horizon in mesic climate, 7 day aerobic incubation (for purpose of comparison). ND = assay not done.

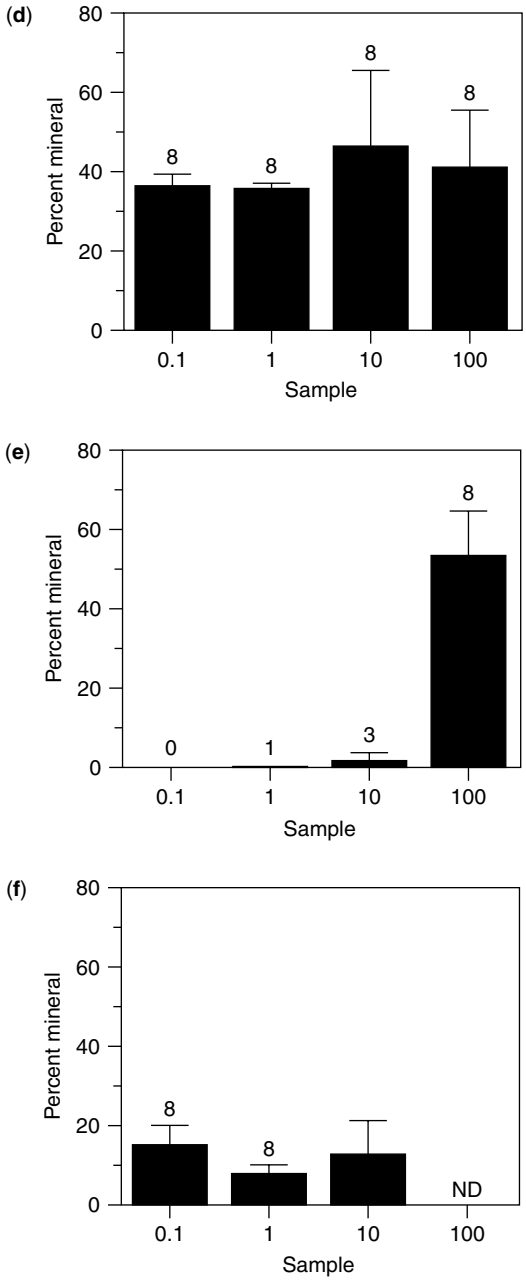

Figure 3. (*Continued*)

of sediment (several tens of cubic cm) may have very few if any microorganisms capable of metabolism. This conclusion was supported by activity assays of 0.1-, 1-, 10-, and 100-g samples ($n = 8$ at each scale) removed without sample homogenization from the same core (Fig. 2). The assay system was designed and validated to prevent scale-dependent artifacts. In the low-recharge sites, activity was rarely, if ever, detected in the small samples and the frequency of detection of activity increased with increasing sample size (38). In contrast, at the 20-cm recharge site, activity was detected and approximately equal in all eight replicates for all sample sizes (Fig. 3). Thus, microorganisms capable of metabolism were very patchy

at the low recharge sites and homogeneously distributed (at the scales of resolution examined) at the high-recharge site. The patchiness of activity in these transect and multiscale studies using easily metabolized substrates mirrors that observed for denitrification in soils (34–36) and suggests that the distribution of specific contaminant-degraders in uncontaminated low recharge vadose zones will be extremely patchy.

SPECIAL CONSIDERATIONS FOR VADOSE ZONE MICROBIAL STUDIES

Sampling Access

There is generally a greater number of options for vadose zone sampling than for saturated zone sampling. In addition to using traditional drilling and coring techniques, one can also access the vadose zone via sediment and rock outcrops, by excavation, and through caves and tunnels. Drilling and coring methods have been reviewed in detail elsewhere (39,40), including methods for sampling in mines and tunnels (41).

Drilling and coring techniques have the advantage that they can be continued into the underlying saturated zone to obtain multiple depth intervals at a single site. Cores can be obtained from shallow unsaturated, unconsolidated sediments using relatively simple drilling technology such as a hollow-stem auger and a split-spoon sampler (142). Hollow stem auger techniques can be used to depths of approximately 100 m. Cable-tool drilling is a simple and ancient technology that has been used with good results to depths of 90 m in unsaturated, unconsolidated sediments (43), and much deeper in a saturated zone study (44). Hollow-stem auger and cable tool drilling have the advantage that they do not require drilling fluids, which can be sources of chemical and microbiological contamination of core samples. Deep, hard-rock vadose zones require more aggressive approaches such as the rotary drilling and wire line coring, as exemplified by the air-rotary, diamond drill bit method (45,46).

Tracers should be used whenever possible to quantify contamination from the drilling process (16,17,20,47) When drilling fluids are used, the tracers can be added to the drilling fluid; for drilling/coring methods not requiring fluids, the tracers can be added directly to the borehole at intervals during the drilling and coring process. Tracers are subsequently quantified in the outer portions of cores and in the interior subcores that are sampled for microbiology. Ideally, tracers should include conservative solute tracers (e.g., bromide) and particulate tracers that serve as surrogate bacterial tracers, for example, fluorescently-labeled, carxboylated microspheres. Tungsten carbide has also been used as a particulate chemical tracer during vadose zone sampling (7).

Rock and sediment outcrops (and also roadcuts) of vadose zones can be directly accessed for physical, chemical, and microbiological analyses. The advantage of this sampling approach is that it allows direct observations and sampling of spatial patterns over a wide area; the major disadvantage is that exposure at the surface leads to physical, chemical, and microbiological alterations of the vadose zone materials. Physical weathering, desiccation, chemical oxidation, and colonization by allochthonous microorganisms can contribute to alteration of vadose zone rocks and sediments at outcrops and roadcuts (13,14). These near-surface alterations can be avoided if one drills or excavates laterally far enough into the formation. In a study in a dry climate, moisture content was measured during horizontal coring into a roadcut. Moisture loss extended 2 m into the formation, yet laboratory measurements of microbial activity and culturability stabilized 30 cm into the formation (F. J. Brockman, unpublished data). The extent of this near-surface alteration of the vadose zone at outcrops in undoubtedly highly variable and dependent on texture, degree of fracturing, and recharge rates.

Caves, mines, and tunnels can provide relatively easy access to vadose zone environments. For example, extensive sampling was performed at a proposed nuclear waste repository by excavating rock in tunnels (8,9,22,23). However, as with surface outcrops, the exposed rock surfaces are subject to desiccation and airborne contamination. These effects can be minimized when a freshly mined area is sampled (21). Surface rock is aseptically removed exposing fresh rock surface that is then collected for microbiology. This approach can be combined with the use of tracers such as Br^- (23) or fluorescent microbeads to preserve intensity of samples (49a). The tracers are applied to the rock surface after a layer of surface-contaminated rock has been removed; subsequently the surface is further excavated to remove fresh rock, and that fresh rock is subsequently sampled. Tracers are quantified in rock collected from the surface and at depth; tracers should not be detectable in sample collected from the rock face or should be no more than three orders of magnitude less than that collected at the surface. This approach was used to ensure integrity of volcanic tuff samples collected in the Exploratory Shaft Facility of the proposed Yucca Mountain high-level nuclear waste repository at the Nevada Test Site (21).

Airborne contaminants within the mine can be assessed by exposing agar growth media in petri dishes during the sampling process and subsequently incubating the plates. Because fungi are common airborne contaminants but are uncommon within deep vadose zone samples, they have been used as serendipitous tracers for microbial contamination of rock samples (18). An alpine miner, a mechanical device with multiple high-speed carbide steel bits capable of excavating large volumes of rock quickly, has been used to sample a 27 m^3 volume of volcanic tuff (9,23). By stopping the alpine miner at intervals.

Unsaturated Incubation Conditions

Laboratory incubations are often used to measure rates of potential microbial activity (e.g., rates of $^{14}CO_2$ production from ^{14}C-labeled substrates, enzymatic activities, etc.) in vadose zone rocks and sediments. These laboratory microcosm assays are useful for comparisons of relative microbial abundance and activities among environmental samples, and they can also be used to test for the presence of a particular metabolic activity, for example, ability to degrade a specific contaminant compound. However,

the data should be considered to estimate *potential* microbial activity, not actual in situ rates of metabolic activity. Even when exogenous substrates are not added to samples, the laboratory microcosm approach yields activities that are considerably faster than in situ rates (14,15). This is because the process of sample collection, handling, and storage can stimulate microbial activities by redistributing nutrients, moisture, and microorganisms such that nutrient availability is increased (4). This stimulation can occurs even when intact cores are used (33).

Simulating vadose zone conditions in laboratory microcosms requires special care. Saturated slurries generally do not represent vadose zone conditions. Addition of solutes to adjust the water potential to that of the vadose zone is inappropriate because the effects of solute water potential and the effects of specific ions on microbial activities are different from the effects of matric water potential. However, the total water potential in a porous medium can be set by equilibration in a sealed container with an atmosphere that has had its relative humidity set by a solution of known solute water potential. Sample handling can partially desiccate vadose zone samples (10,21). Moisture can be added back when it has been lost from a sample, but these moisture changes can alter microbial community structure and activities. Amendment with nutrient solutions will increase moisture contents and water activities, so this should be minimized by using small volumes of more concentrated solutions.

PRACTICAL IMPORTANCE OF VADOSE ZONE MICROBIOLOGY

Wastewater Treatment by Artificial Recharge

Artificial recharge of wastewater is a low-cost method for disposing of and treating wastewater, while restoring water to underlying aquifers. This process, also known as soil-aquifer treatment and infiltration-percolation treatment, is an especially attractive option for water conservation in arid and semiarid regions, where heavy reliance on groundwater can contribute to aquifer depletion. The wastewater can be from industrial and/or domestic sources or from agriculture, for example, runoff from concentrated animal feeding operations (50). The wastewater can be treated, partially treated, or untreated. Application can be by injection, spraying on the surface, or through surface infiltration basins. In some arid and semiarid regions where water conservation is an especially vital issue, treated wastewater is reused as irrigation water for agriculture and the water subsequently recharges underlying aquifers (51,52). The expectation is that wastewater contaminants (chemical and biological) will be removed during infiltration through the vadose zone before it reaches underlying groundwater. This can include mineralization of organic contaminants, immobilization of inorganic contaminants (e.g., heavy metals), transformation of inorganic contaminants (e.g., nitrate) to harmless products, and sorption and inactivation of pathogens.

Nitrogen contamination of aquifers, especially in the form of nitrate, is a major concern when wastewater is treated by artificial recharge. Nitrification results in oxidation of ammonia to nitrate in the vadose zone (53). Nitrate removal requires denitrification to nitrogen. In studies of artificial recharge of wastewater runoff from concentrated animal feeding operations through playa lakes in Texas, denitrification activity in the vadose zone was sufficient in most cases to prevent significant contamination of the underlying aquifer with nitrate (50). In soil-aquifer treatment pilot projects in Phoenix, Arizona, 30 to 70% N removal from wastewater treatment plant effluent was reported during percolation through the vadose zone (54). The efficiency of N removal was dependent on the hydraulic loading rate (recharge rate) and the organic C content of the effluent. Recharge rates <1 m/day were most effective. Intermittent recharge, that is, alternating flooding and drying periods, appeared to favor denitrification. Phosphate removal was 40 to 80%, also depending on hydraulic loading. Organic carbon was reduced to very low levels, as indicated by near-zero biological oxygen demand (BOD) after passage through the vadose zone.

The effectiveness of pathogen removal from wastewater during artificial recharge depends, in part, on the rates of infiltration and the thickness of the vadose zone. Column studies have shown greater than or equal to 90% removal of bacteriophages (used as surrogates for human enteric viruses) during unsaturated flow through 4.5 m of vadose zone sediment at a high infiltration rate of 15 m day^{-1}, and much more efficient removal at 1 m day^{-1} (55). Transport rates for pathogenic microbes are generally directly related to water content. Virus concentrations in effluent from 1-m long saturated laboratory soil columns have been reported to equal influent concentrations within two pore volumes. Comparatively, the effluent virus concentrations from similar columns under unsaturated conditions were shown to be 5 to 27% the influent concentration (56). The same study found little adsorption of viral particles to the soil material, indicating a substantial inactivation process. The inactivation rate of viruses is dependent on environmental conditions (57). Likewise, adsorption, dispersion, and advection may be important factors for virus removal (55,57,58) under certain types of vadose zone chemical and hydrological conditions.

Efficient removal of indicator bacteria (coliforms and fecal streptococci) has also been demonstrated in column experiments and field measurements (59–61). Helminth eggs have also been shown to be eliminated during wastewater treatment by artificial recharge (Guessab and coworkers 1993). However, in some cases, sorption and/or inactivation of microorganisms during artificial recharge in the field has been disappointing, a finding that is usually attributed to high pore water velocities, especially as a result of preferential flow through heterogeneous systems (62,63).

Bioremediation

The vadose zone, with its various physical, chemical, and biological processes, can be important in protecting groundwater from surface contaminants. These processes, under favorable conditions, can slow the migration of contaminants by sorption to soil particles, entrapment of gas and liquid phase contaminants in the pore space,

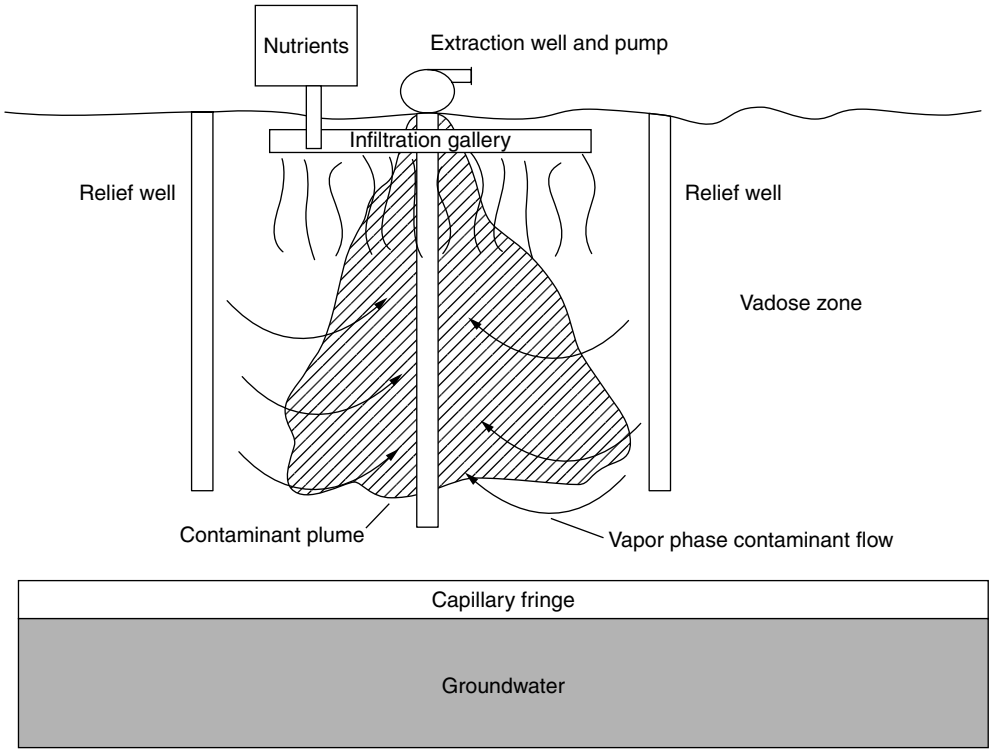

Figure 4. Schematic of bioventing showing air being extracted from the vadose zone and air (oxygen) being pulled through the unsaturated contaminant plume. Soluble nutrients such as nitrogen and phosphorus can be supplied through an infiltration galley or a slotted horizontal pipe to further increase the number of biodegrading microorganisms.

transformation, degradation, and even mineralization of the contaminant. To some extent, this process progresses without human intervention, and has been shown to reduce the mass, toxicity, mobility, or volume of contaminants in the vadose zone and groundwater (64). To enhance these natural processes, engineered remediation processes have been developed that exploit the physical, chemical, and biological process intrinsic to vadose zone environments. Reviews of current technologies and innovations have been written (65–69).

The first commercial application of bioremediation was in 1972 to treat a gasoline pipeline spill in Pennsylvania (70,71). As the use of bioremediation matured, it became apparent that a major limiting factor in the process was oxygen availability. Nutrient limitation was also problematic. Dick Raymond patented the use of water amended with oxygen and nutrients to promote biodegradation in 1974 (70). Hydrogen peroxide was also used as an effective source of oxygen, but had limitations when used in unsaturated vadose zone environments. Soil vapor extraction (although initially used to physically extract vapor phase contaminants) was found to have a significant biological component in the early 1980's (70).

The process, called bioventing when focused on stimulating biological activity takes advantage of the unsaturated nature of the vadose zone and its gas-filled pore space. The process is similar in design to soil vapor extraction in that air is moved through the contamination area either through air injection or soil gas extraction. In the case of soil vapor extraction, this air movement is used to carry the contaminant vapor to the surface for removal/recovery. In the case of bioventing, the air movement is meant to deliver oxygen and other nutrients to indigenous microbes, in turn stimulating biotransformation, biodegradation, or mineralization of the contaminant (Fig. 4; 72). Bioventing has developed into one of the most cost-effective petroleum hydrocarbon remediation processes (EPA webdoc, 1995). As of 1995, the EPA and U.S. Airforce had more than 150 successful bioventing remediation sites (web doc, 1995).

Volatile organic compounds are common vadose zone and groundwater contaminants. Although chlorinated solvents are a serious threat to groundwater at many sites, petroleum hydrocarbon contamination is the most common. These sites usually occur from underground leaking fuel storage tanks, refineries, military airfields, and petroleum spills. As these contaminants enter the vadose zone, they partition into one (or more) of four phases including nonaqueous-phase liquid (NAPL), soil solids, soil water, and interstitial air (Yu, 1995). This partitioning within the vadose zone effectively slows the migration of the contaminant toward the groundwater.

Sites contaminated with toxic metals and highly structured polychlorinated biphenyl compounds remain a challenge in terms of engineered bioremediation strategies.

Radioactive/Hazardous Waste Repositories

Deep vadose zone environments in arid and semiarid environments have been targeted as potential sites for

storage of hazardous waste materials, including radioactive waste. Examples of such sites are (*1*) Yucca Mountain, Nevada, which is a proposed site for a high-level nuclear waste repository, (*2*) the Waste-Isolation Pilot Project (WIPP) Site in southeastern New Mexico, where transuranic waste is now being deposited within a 700-m deep unsaturated Triassic salt deposit, and (*3*) various underground storage facilities within the Hanford Site in south-central Washington state, where nuclear weapon-production-related wastes are stored. These sites have been chosen in part for their low recharge rates. Low recharge rates mean that should a spill occur within the repository, the flow of contaminants to underlying aquifers will be relatively slow. In the case of the Yucca Mountain site, ongoing studies are being performed to predict the fate of the contaminants over a period of 10,000 years or more (See also NUCLEAR WASTE RESPOSITORY IN YUCCA MOUNTAIN: MICROBIOLOGICAL ASPECTS, this Encyclopedia) Predicting the fate and transport of contaminants is always challenging, but the task is especially difficult when one attempts to factor in the effects of microorganisms. The problem has been separated conceptually into two areas, the "near field" and the "far field." The near field refers to the area in the immediate proximity to the stored waste; the far field refers to the more distal, unsaturated regions of Yucca Mountain that separate the near field from the underlying aquifer (150 m below the repository) and the land surface. Microorganisms are a concern in both the near and far fields. In the near field, microbially mediated corrosion of containment vessels can lead to waste spills. Once a spill has occurred, transport through the surrounding vadose zone can be influenced by microbial activities. Hersman (73) reviewed the possibilities for microbial influence on the fate and transport of spilled radionuclides. Microbes may retard transport of radionuclides through sorption, biodegradation of organic-radionuclide complexes, oxidation/reduction reactions, and pH changes; or they may accelerate transport by production of chelates, oxidation/reduction reactions, and pH changes. Unfortunately, there are no current models that can predict with any accuracy which of these processes will predominate.

The volcanic tuff at Yucca Mountain has been shown to harbor native microbial populations (21). Culturable cells were measured at 10^1 to 10^3 cfu g^{-1}; total cells were estimated from phospholipid fatty acid (PLFA) concentrations to be 5.9×10^3 to 2.2×10^5 cells g^{-1}. Although these are relatively low numbers relative to aquifer environments, the activities of these microorganisms could be significant when integrated over time and distance (10,000 years and 150 m, respectively, at Yucca Mountain). Moreover, the numbers of microorganisms in the repository environment can be greatly increased by tunnel-boring and construction activities. Microbial biomass (estimated from PLFA data) was found to be 10^2 to 10^4-fold greater in sand deposited on the tunnel floor than in pristine tuff (74). This stimulation of microbial activities by repository construction activities could increase corrosion problems in the near field, and possibly influence microbial activities in the far field. When the waste is actually deposited at Yucca Mountain, there will be radiation flux in the near field that will probably be damaging to some microorganisms. However, Pitonzo and coworkers (75) found that many indigenous microorganisms are capable of surviving gamma irradiation at the dose rate that is expected within the repository (1.6 Gy min^{-1}) by entering a viable but nonculturable state. Microbial threats to repository performance should also be considered at other sites. Microbes have been isolated from halite at the WIPP site (76), and Hanford Site vadose zone sediments are known to harbor metabolically active microorganisms (7,10).

The Vadose Zone as an Extreme Environment

On the basis of estimates of 10^4 viable cells g^{-1} and a growth yield of approximately 3.6%, calculated average generation times for microorganisms in the vadose zone has been calculated to be in the range of 3×10^3 to 3×10^5 yrs (77). Considering the ages of vadose zone sediments, this means that many vadose zone populations have undergone fewer than 100 cell division since the time of burial. Clearly these vadose zone microbes are in long-term starvation survival mode rather than active growth. This long-term starvation survival is especially remarkable when one considers that many vadose microorganisms appear not to be spore-formers, and thus are surviving nutrient deprivation as quiescent vegetative cells. The low nutrient fluxes and concomitantly slow rates of metabolic activity indicate that deep vadose zone environments are among the most nutritionally extreme in the entire biosphere. Low concentrations of dissolved organic carbon combine with extremely slow rates of groundwater recharge make for infinitesimal nutrient fluxes. Microbes that are sequestered in these relatively static, diffusion-dominated vadose zone sediments and rocks are among the most nutritionally challenged in the biosphere.

In some vadose zone environments with very low recharge rates, the microbial communities may date from the time of geologic deposition. One can constrain the age of the microbial community by determining the age of the groundwater (youngest possible age of the community) and the age of the geologic material (oldest possible age). This approach has been applied to thick vadose zone loess sediments in semiarid eastern Washington state, where recharge rates are relatively slow (5,11, Balkwill and coworkers 1998). Pore water ages were estimated from groundwater recharge rates that were measured using the chloride mass balance method. A diversity of bacterial types were cultured in vadose sediments with pore water ages of 15,000 and 30,000 years (4). Viable microorganisms (evidenced by phospholipid fatty acids, mineralization of organic substrates, and plate counts) were found in loess sediments with pore water ages as great as 1,200 years and sediment ages approaching one million years) (11). In this case, the microbial communities may be as young as the pore waters; however, microbial transport through sediments is generally greatly retarded, and so the age of the communities may be much older than 1,200 years. Age of the communities does not mean age of individual cells. The microbes in these communities have probably undergone

occasional cell division; however, as noted above, this is probably a very infrequent event given the low nutrient fluxes. Similarly, spore-forming and non-spore-forming bacteria and were cultured PLFAs indicative of approximately 10^4 cells/g measured in sediments for which the age of the communities was estimated to be between 12,000 years (time since last significant recharge event in the form of postglacial flooding) and four million years (sediment age) (5). Although these microbes are not nearly as ancient as those reported to have been recovered from Dominican amber (78) and Permian halite (79), they do support the concept of bacteria persisting in deep vadose zones for thousands of years or more with little access to exogenous energy sources. Whether these vadose zone microbes have special adaptations for survival under vadose conditions has yet to be determined.

Comparisons of closely related surface and subsurface bacteria for their abilities to survive in unsaturated sediments for more than a year showed no significant differences between surface and subsurface strains (80); however, laboratory survival studies conducted over a one-year time frame are probably not adequate to discern differences in microbial abilities to survive for geologically relevant lengths of time. Further studies of vadose zone rocks and sediments may reveal microbial strains that exhibit adaptations for long-term survival under the low-nutrient flux conditions that are typical of deep vadose zone sediments.

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VIABLE BUT NOT CULTURABLE (VBNC).

See BOTTLED WATER, MICROBIOLOGY OF; *CAMPYLOBACTER JEJUNI* AND OTHER ENTERIC *CAMPYLOBACTER*; CHOLERA

VIABLE BUT NOT CULTURABLE (VBNC) MICROORGANISMS

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Bacteria can grow rapidly, yet there are some circumstances where it appears that they should grow, where they grow slowly, or not at all. There are many interesting cases where resources are present, so seemingly the cells could grow, but they do not, presumably for some more important reason than that of just increasing the number of independent propagules. Therefore, they forego a growth opportunity. An especially interesting case has been called the "viable but not culturable" (VBNC) (1) state in which cells appear to be alive but do not immediately produce colonies and is the topic of this article. However, there are a number of other cases that will be briefly covered here or in the article on Oligotrophism because they are or may be related to VBNC and more explicitly provide aspects of the VBNC way of life. These and key references for them are

- being an obligate oligotroph (2)
- being in a state of "shutdown" metabolism (3)
- substrate activated death (SAD) caused by a substrate in nongrowth medium (4)
- lactose death caused by lactose to an organism with multiple *lac* operons (5)
- quiescent cells intermittently growing under almost starvation conditions (6)
- SOS response after DNA damage (7)
- osmotic rupture when pumps are too effective (8)
- oxidative stress causing an abundance of oxidative damage to the DNA (9,9a)
- blockade of nucleic acid and protein synthesis by inhibition of wall enlargement (10)
- streptomycin killing (11)
- destruction by a lysogenic bacteriophage of the host cells (12)

- suicide of individuals for the 'good' of the community (12)
- sporulation/germination (13)

The last is quite familiar and common in gram-positive organisms. Sporulation results when nutrient is temporarily missing in the medium, there is a special division that protects the organism and results in a nongrowing cell (i.e., the spore). Spores may later germinate and resume vegetative growth. For this case, the reason for stopping and starting growth is clear, it has to do with a special strategy to respond to specific starvation. Sporulation allows the bacterium to remain indefinitely in a state in which metabolism is essentially nil under conditions where they are much more resistant to environmental stresses than is the vegetative cell. In VBNC and in many other cases, the rationale for decreased growth is not so obvious and has consequently been the subject of many arguments and much discussion. It is usually pointed out that the VBNC strategy gives long-term selective advantage. However, I believe the aforementioned phenomena have similar advantages and disadvantages, but all may backfire, for example, when resources are available but missed and not utilized while cells are in the spore or other quiescent state.

THE CONCEPT OF THE VIABLE BUT NON CULTURABLE (VBNC) CELL

The phenomenon of VBNC was formulated by Xu and coworkers (1) and became of more general interest to microbiologists with the appearance of the seminal review by Roszak and Colwell (14) in 1987. They itemized many observations that the viable count of a culture declined before (or much faster) than the total count in the test tube. Possibly the first convincing observation of this phenomenon was that of Fliermans and Schmidt (15) who used both autoradiography and immunofluorescence to study *Nitrobacter* in natural systems. However, this work only shows that some cells of the *Nitrobacter* strain are metabolically inactive. Recent work in the VBNC area has been carried out with *Vibrio cholerae* by the Colwell group (16) and *Vibrio vulnificus* by Oliver and his collaborators (18,18a). Bogosian presented very significant studies with the latter organism and with enteric bacteria that argue very effectively against the reality of the VBNC State (19,20).

The VBNC Phenomenon

To demonstrate the VBNC state it is necessary to follow a growing culture under defined conditions that cause cells to enter this special state; usually these conditions are starvation or a lowering of the growth temperature (18). If there is subsequently a decrease in colony count, but little decrease in total count with time, and if evidence can be produced to show the cells are capable of metabolic activity, then the term VBNC is deemed by some workers to be appropriate. Other workers prefer the term VBC and others such as Kell and coworkers (19) would prefer ABNC to signify "active, but not culturable." "Dormant" is an inappropriate term because it means "sleeping" and although sleeping people have some signs of life they can be easily awakened. The activities of cells in the presumed VBNC state that have been followed experimentally are few and of limited scope.

It should be noted that a good deal of the objection to the validity, reality, and legitimacy of VBNC state has appeared in the recent literature mainly as very short articles or letters. The terminology used in the VBNC field is confusing, not clearly specified, and facts are often not well documented. Kell and coworkers (19) have dealt especially critically with the terminology and logic. In order to obtain an appreciation of this field the original review by Roszak and Colwell (14) and the reviews by Oliver (18) and Barer and Harwood (20) are particularity cogent.

Rationale for the Existence of the VBNC State

There need not be only one unique reason for the VBNC state. There many reasons for some of the related conditions in the long list given that may apply to VBNC. The reason why single-cell organisms do not grow could come from five major directions:

Firstly, when the failure to grow (or death of a cell) is actually favorable for the growth of the species or colony. The classical case (24,25) has to do with preventing a pathogen, such as a lysogenic bacteriophage from infecting a cell; the cellular response is to commit suicide presumably to protect the neighboring cells.

Secondly, the cell may have adopted a strategy that is purposefully an intentionally irreversible process. This would be equivalent to one exhibited by many plants and animals in aging and leaving reproductive propagules, but not themselves.

Thirdly, by a species having specialized to interact with another species for their mutual good. In this third case it may be impossible for one organism to grow without the other. One can think of plants and their pollinators or many other pairs of organisms coexisting commensally, mutualistically, or symbiotically. For example, many trees must be rooted in a mycorrhizal environment.

Fourthly, a cell may have adopted a strategy that has the negative aspect that puts it at risk of being sensitive to environmental factors, such as too much of a nutrient, too much of a strong oxidant, etc. It has been suggested that oligotrophic organisms may take up osmolytes when available and, then unfortunately swell and burst (26). For cases in this latter category, the microbiologist often is the cause of the problem. If the experimenter were to adequately take these possibilities into consideration and feed only smaller amounts at a time, it should be possible to culture such cells without killing them.

Fifthly, the cell may have entered a physiological state in which it has specialized for movement and not for growth, as in the phenomenon dubbed "the shutdown cell" (3) as exhibited by *Rhodomicrobium* and *Caulobacter*. Some of the other phenomena listed earlier are special and additional reasons will be given in the following text.

Sixthly, there are other aspects that might cause the appearance of not growing when growth seems possible. One has to do with the property of organisms of being able to exhibit different sets of properties in

different environments. There are many situations in which pathogenic bacteria greatly alter their metabolism when they enter a host and later return to their original behavior when no longer in an active pathogenesis mode. The strong implication is that the cells can exhibit more than one quite different appearance and behavior to the outside world, dependent on their circumstance. Conversion between these states fluctuates more rapidly from one to another compared to ordinary gene mutations, but much more slowly than adaptive responses. It has become clear in the last few years that bacteria have some genes that have been modified in the past so that they are capable of reversible mutations at rapid rates (24,25). This can occur, for example, when a tandem array of repeats whose unit length is not a multiple of three is present. Tandem repeats can be gained or lost through copying errors or by failure in repairs and thus changes the frame of protein translation and consequently alters the protein product of the gene. This situation creates a circumstance in which the process of forward and back mutation are rapid and equally possible.

Methods of Study

Roszak and Colwell (14) enumerated some 40 methods and techniques that have been employed in the literature to distinguish living from dead cells. Kell and coworkers (19) and Barer and Harwood (23) gave more methods. Methods in use to detect various metabolic activities were also reviewed by Oliver (18). His list, as do some of the others, starts with the Kogure's Direct Viable Count (DVC) method (26). For this test an agent is added to block some limited aspect of replication, usually this is nalidixic acid that blocks DNA gyrase. The important aspect is that it does not stop other processes directly. It does, however, prevent the next rounds of cell divisions from occurring although both protein synthesis and wall synthesis would continue. Then if a cell elongates during a short experimental period when cell division and cell separation cannot occur, it is concluded that the cell has a broad variety of metabolic processes that can actually function. These would be needed for cytoplasmic and wall growth. With nalidixic acid the observation of cytoplasmic growth is not confused by the occurrence of cell division. If elongation takes place the cell is not dead, but if the cell does not elongate, it is assumed to be dead. Although this is a most powerful approach, it could give false-negative responses because the control mechanisms of the presumptive VBNC cell might block elongation processes. On the other hand, if the VBNC state blocks DNA synthesis or cell division uniquely, the cells will have already elongated to their limit before the nalidixic acid is added. It should be noted that cells engaged in the SOS response to ultraviolet radiation would meet this test (see following text).

A second common method uses a tetrazolium dye. When cells have metabolic ability and sufficient available reducing power via the electron transport system to reduce the colorless oxidized form of the dye to the colored formazan, the test is positive. Reduction of the tetrazolium requires integrity of the cell, an active redox enzyme system plus an available reductant, but does not require a capability for macromolecular synthesis.

A third test, like the second test, requires that the cell's integrity has been maintained so that the cells are still intact. The basis of this test is to identify cell-shaped objects that have nucleic acids. Acridine orange (AO) and/or 4′,6-diamidino-2-phenylindole (DAPI) are used. The DAPI stain binds selectively to double-stranded DNA, whereas the former binds to the more abundant single-stranded RNA. In addition however, AO binds to double-stranded DNA, but it then fluoresces a different color. Consequently, this dye can provide additional useful information because cells that are actively growing have much more RNA than DNA and become stained orange instead of green.

A fourth method involves measuring the level of ATP as a measure of biomass; ATP content has been presumed in microbial ecology for 50 years to be a valid measure of viable cellular biomass. Existence of cellular ATP implies that the cell envelope is intact and that the energy-trapping machinery is functioning to some fraction of that during normal growth.

Many complaints have been registered that none of these tests in any sense measures viability because viability means to be alive and to be able to reproduce. Being alive requires the combination of many special properties, not just one. Obviously, after long periods of incubation, any of the methods cited would carry more weight in the assignment of VBNC. This is because it is reasonable to assume that after months or years a dead organism would decompose or be decomposed on account of autolysins or other hydrolytic enzymes or be converted into a hollow shell and not have any of the properties mentioned earlier.

Of the set of techniques listed earlier, the first, demonstrating the ability of a cell to elongate, in the presence of nutrients and of an agent that blocks cell division is believed to be the most effective in providing evidence that the cell has many of the aspects of life. A cell that passes this test was almost certainly alive before the nalidixic treatment, even if it is not able to grow indefinitely afterward. The Kogure test should probably be the first test performed. Oliver's laboratory has used it (28,29) very critically to demonstrate that resuscitation was not obscured by growth of residual living bacteria. On the other hand, Weichart and Kjelleberg (30) had earlier thought that instead of resuscitation, regrowth could be the explanation of cultures passing the test.

THE REALITY OF THE VBNC PHENOMENON

One might reasonably assume that a "viable, but not culturable" organism is actually culturable, and that the microbiologist has not yet discovered the secret. Barrer and Harwood (20) designate this state AYU for "As Yet Uncultured." Our ignorance in this regard applies to a majority of the organisms given the VBNC appellation and to most organisms that exist in this world.

Sometimes regeneration of apparently nonculturable organisms has been obtained. Now the problem is finding out how common the return from the VBNC state is.

Doubters (37–43) claim that there was a rare colony-forming organism present that had multiplied and that most of the presumptive VBNC cells are really dead and thus the measurement has not been properly carried out. Believers hold that the switch from vegetative to a cell apparently incapable of division and vegetative growth is a highly rational strategy for long-term survival of the species. This leads me to a detour into some of my own research to show that many organisms are capable of charting and following a strategy that appears to be contradictory to their immediate success.

The Problem of Seeming Irrational Self-Choice

Bacteria in exponential growth, particularly if they have been cultured in this state for many generations, should be growing as fast as possible, limited only by their genotype and the resources in their medium. This is the philosophy we inherit from Malthus and Darwin. In ecological or evolutionary terminology such typical organisms should have maximized their inclusive fitness. But there are many organisms that do not seem, to us humans, to obey this rule.

Insights into the fundamental nature of bacterial growth emerged in the 1950's from pioneers such as Sir Cyril Hinshelwood, Aaron Novick, Leo Szilard, Seymour Cohen, Herbert Kubitschek, Elio Schaechter, Jacques Monod, Ole Maaløe, and Allan Campbell. The essential concept was that of balanced growth. The way to study bacterial physiology, it was believed, was to examine steady state growth so that the organisms were increasing exponentially in number. Subsequently, while measurements were being made the cells were not changing in properties. The proportion of cellular amounts measured during balanced growth made it possible to calculate the rate of biochemical reactions (see 39). The best way that cultures in the balanced state can be obtained readily is by using a chemostat. Much understanding of microbial physiology resulted from studies of such cultures.

Not only was basic microbial physiology elucidated, but the considerably more complex ways in which organisms interact with their environment were discovered. As an example, let me describe an early experiment (35) of my own that has left me puzzled ever since, but emphasizes the versatility and commitment of bacteria and their ability to "choose" and adopt a particular strategy. A chemostat culture of *Escherichia coli* ML 308 was under study; it was limited by glucose in a minimal medium and caused to grow with a doubling time of 10 hours. This was subcultured into a sample of the reservoir medium. Because of continued bacterial action, the culture medium in the reactor (growth chamber) had a thousand times lower glucose concentration than was in the reservoir. Therefore, this subculturing constituted a shift-up in the glucose concentration of a thousandfold. It took six hours to achieve the characteristic (steady state) doubling time of 45 minutes in the glucose medium. This was the control part of the experiment. At this time I had on hand in the laboratory (27) a constant temperature growth apparatus within a double-beam spectrophotometer linked to a computer. With it the cell biomass could automatically be measured every two seconds, very precisely and accurately. The output of the spectrophotometer was sent to the computer that could estimate the growth rate on-line to a precision of 1% in a three-minute measuring period. Surprisingly, when the same chemostat culture was "shifted-up" into Luria broth, a rich medium with yeast extract and tryptone as a source of amino acids, supplemented with the same high level of glucose, and made up in the minimal salts medium that had been used for the chemostat culture, the results were much different. In less than a minute the cells had shifted to the definitive doubling time of 25 minutes. This growth rate was then quite stable during further growth.

This clearly demonstrates the ability to the organisms to select or be directed into different courses. It poses the highly important question of what "biology" allowed the organism "to decide" that conditions had become "just" satisfactory and therefore cautious acceleration was appropriate. How did the organism "decide "that conditions had become very good and that an immediate acceleration of growth rate should take place to the maximum allowed either by the enriched medium or by some internal limitation? Conversely, in the absence of the added yeast extract and tryptone why were the organisms unable to exploit the resource, and in anthropomorphic terms, why were they "suspicious" of the sudden increase in the glucose concentration? Why did they not immediately grow with the 45-minute doubling time that was then possible? They certainly could synthesize all the necessary amino acids. The biosynthetic enzymes must have been present in excess, but subject to a feedback inhibition that could be rapidly relieved. Instead, these organisms only slowly adapted to exploiting fully their new environment that now had a surfeit of glucose. On the other hand, the cells had been growing for many generations in a minimal medium, in which there would be no signals to elicit the production of the many permease systems needed to take up the amino acids, peptides, and cofactors that became available after the LB broth was added. Note that the shift-up in growth rate in the LB broth was accomplished faster than a gene could be transcribed and translated. Gene regulation could not be the answer, because to become functional, additional time would be required for insertion of new transport proteins into the membrane and allowing them to gain the right conformation. So, how is it that the increased growth rate was achieved in a few seconds in the enriched medium in LB?

An anthropomorphic (teleonomic) explanation that might come to mind is that the bacteria had existed in the chemostat in an environment in which the glucose concentration had fluctuated widely depending on the cell's position at each instant of time relative to the inflow tube. In the past I argued against this because of experiments showing that the bacteria could store 18 seconds worth of glucose (and products) and this result suggested that the cells were not being starved for a part of the time under the culture conditions used (27). Still it is possible in spite of the effective stirring used that the cells have a regulatory circuit that had been turned on by the fluctuations in the very small limiting glucose concentrations in the

environment that caused the cells to be 'suspicious' of even a massive increase. (Note: that all other components were present in excess and their amounts could fluctuate only infinitesimally. For pedagogic reasons over the years, I have descriptively called this phenomenon the "New York" effect, because if you stand in Times Square and offer people five dollar bills gratis, they will not accept the bills because they know that there must be a "catch").

With still more anthropomorphic speculation, one could believe that the cells "hoped" that good times would come and therefore invested resources in uptake systems even in a glucose-only chemostat. This is similar to the idea that I have proposed to account for the maintenance of more than enough protein synthesis machinery in slowly growing cells (35). This is a supposition and belief that I have expressed in a number of publications, but it is not a fact.

The Limitations of the Chemostat

Several workers for various reasons have questioned whether the chemostat is truly a continuous culture. I have voiced these ideas (35) as has Chesbro (personal communication) and Poindexter (36). The latter has gone so far as to rename the usual chemostat culture as only being a "perpetual" culture and not being a "continuous culture." This fluctuation of the environmental concentration is not a problem with the biology of the bacteria but more with the apparatus used and the way the experimenter uses it. The problem lies in the mixing. Mixing must be adequate so that none of the organisms of the culture perceive a fluctuation in their environment. The substrate would be more uniform during actual experiments, if there were faster stirring, if more and smaller drops were used, or if a limiting substrate was chosen that had a higher K_m for uptake. It could be best tested by supplying a nonutilizable substrate with the main fluid flow and with a second inflow add a slow input of an enzyme that converts that substrate into a nutrient utilizable by the bacteria. The mechanical mixing of the original substrate and the enzyme is very easy to achieve as they are not being depleted by the bacteria and their concentrations remain essentially constant. The feeding of the bacteria in this case is equivalent to a chemostat that had many quadrillions of inflow tubes distributed all over the culture volume. I hope that such types of systems will be tried in physiology laboratories. Only then will we know how continuous the fermenters and chemostats in use, actually are.

OTHER CASES OF ORGANISMS 'CHOOSING' NOT TO GROW MAXIMALLY

Some of the categories from the list presented at the beginning of this article are discussed in this section. Those selected have been chosen because they are not adequately discussed elsewhere.

Relationship of VBNC with Oligotrophs

From the list provided in the introduction, the one case most clearly related to the VBNC phenomenon is that of oligotrophy. Although this topic is covered under Oligotrophic Bacteria, in this Encyclopedia, there are some additional concepts presented here because of their contrast with the VBNC state.

Some organisms are usually found in environments in which nutrients are chronically in low concentration or generally scarce. To use the terminology of Poindexter (40), these would be oligotrophs as distinct from copiotrophs. Oligotrophs are characterized by their inability to grow at high levels of nutrients, not solely by an exceptional ability to use low concentrations of substrates. In fact, an ability to use a substrate has a lower limit imposed not by biology but by the physics of diffusion (32–34). For example, when cultured in the chemostat and diluted into a very low glucose minimal medium, the *E. coli* took up glucose at such a fast rate that it could be calculated that if they had no outer membrane their uptake would be the theoretical maximal rate limited by physical diffusion. Therefore, no gram-negative organism of the same size could conceivably do better than *E. coli*. This is relevant here because this organism is not considered an oligotroph, but is considered a drudge; that is, that it is a single-minded workaholic copiotroph.

The essential point for the present topic is that oligotrophs, particularly when freshly isolated do not grow rapidly in a sufficient medium. In fact, they may die. Although many cases are known in which they can be slowly adapted to rapid growth, it must be assumed that in their usual state they are destroyed or inhibited by too rich a medium, if suddenly applied. On the other hand, in contrast with the VBNC state, they do grow when fed, although initially slowly.

Those organisms that are isolated as oligotrophs behave in a more extreme way than do the glucose-grown chemostat cells described earlier, on dilution into the higher, but usual culturing levels of glucose. *Escherichia coli* cells from slow chemostats change their oligotrophic behavior gradually in a period of hours, but the usual obligate oligotrophs do not change their behavior in days or weeks (although in months they may). Also, while the *E. coli*, described earlier, may respond immediately to a rich environment and grow fast, obligate oligotrophs do not grow. The word "obligate" implies that the oligotrophic state is permanent. Morita (40) and Gottschal (41) feel that there is no such thing as a truly obligate oligotroph; that is, a bacterium that is permanently in the state of an inability to cope with high substrate concentrations. On the other hand, I wish that someone would find, grow, and document the existence of an oligotroph in a state that is truly permanent. Such an organism would be a worthy subject of study because it would mirror the behavior of organisms before the division of life-forms into the three Domains. At that time cells were almost always chronically starved because anoxygenic photosynthesis and methanogenesis were rare or nonexistent and oxygenic photosynthesis and respiration based on oxygen had not as yet been invented. Therefore, because the available mechanisms to trap energy and fix carbon should not have been very common or efficient, there was nearly continuous famine of energy and of compounds of intermediate oxidation/reduction state

because organic molecules would have only been derived extraterrestrially or made very slowly abiotically.

Pheromone and Quorum Sensing Interactions

Cold-shock proteins can be produced that block DNA replication in vivo (42). Additionally, dormancy of *Micrococcus luteus* can be related to a proteinaceous factor (43) secreted by the organisms. There is now extensive literature concerning *E. coli's* response to starvation (44). Extensive work in several laboratories has demonstrated the existence of mechanisms that aid in the long-term starvation of this organism (and there would be every reason to suspect that such mechanisms would be quite general). Consequently, there may be evolutionary reasons, not appreciated now, for a mechanism involving social interaction; still it is difficult to appreciate conceivable reasons for not growing when it is presumably possible.

Classical Oligotrophs, *Caulobacter crescentus* and *Cycloclasticus oligotrophus* RB1

Caulobacter crescentus is often cited as the prime (and first mentioned) example of an extreme oligotroph. After it was brought into laboratory and repeatedly cultured, it became adapted to extensive and rapid growth on high nutrients and consequently grew to a quite high cell density at a fairly high growth rate; now it should not be called an oligotroph. It is in this form that it is studied in the molecular biology laboratory (45). Jeanne Poindexter informed me (personal communication) that *crescentus* is not a typical *Caulobacter*. Many other caulobacters are difficult to study or to grow by most bacteriologists, not to mention molecular biologists.

Another potential case for an obligate oligotroph was *C. oligotrophus* RB1 (46). This small, marine organism was isolated as a pure strain as an obligate oligotroph by an MPN (most probable number) technique. However, further study showed that it could be grown to higher titers with aromatic compounds such as carbon substrates (47). For more discussion, see OLIGOTROPHIC BACTERIA, this Encyclopedia.

Relationship with the "Shutdown" Cell Concept and a Cell with a Compact Nucleoid

Dow, Whittenbury, and Carr (3) showed tremendous insight in their studies of *Rhodomicrobium vanneilii* and other prosthecate bacteria. These organisms, like the more well-studied *C. crescentus*, undergo a differentiation process as part of every cell cycle. This means that, on division, one daughter cell becomes flagellated and has the important function of searching for a new habitat, the other persists as a sessile vegetative cell that remains localized and proceeds to grow and divide. The authors' insight was that the swarmer is metabolically a "shutdown" cell — and synthesizes only very few proteins and in a real sense is largely metabolically inert; it appears that growth is not its activity, but motility is. So it is similar to the VBNC case in many ways. For these two prosthecate organisms the major role of the shutdown organism is to move until it finds a new environment with an adequate nutrient supply. This can be correlated with the fact that the nucleus of these cells is very compact and moves faster in a velocity gradient centrifuge (45). This compaction of the chromosomal DNA is by virtue of a higher degree of coiling [Koch, in preparation], and may also be associated with more attachments of the nucleus to the envelope.

The compaction of the nucleoid in this special phase of growth is a consistent pattern and is a pattern followed by quite diverse and widely taxonomically distributed bacteria. Generally, under such a situation messenger RNA synthesis is largely stopped, subsequently the nucleoid compacts and, in so doing, expels water. For example, this happens during sporulation of *Bacillus* and other cells. This may protect the genome from certain kinds of damage, such as free radicals generated by the "indirect" irradiation of cell water. This density shift also happens in starved *E. coli* (Baldwin and Koch, in preparation). It has been observed that either the cell or the isolated nuclei have a greater density in this kind of resting stage.

For the major purposes of this review, the important facts are that the "shutdown" cell does not need the ribosomal and the enzyme content that vegetative cells must have and that its genome acquires a smaller volume, and therefore these cells with a compacted, denser nucleoid can have a reduced size and the chromosome may be only available for limited or special transcriptions.

Substrate Activated Death (SAD)

Postgate observed in the 1960s that cells that had been grown with a certain carbon source died more rapidly if incubated in a medium not supporting growth but containing that particular substrate (4). If the cells have not been accustomed to the carbon source in question then this effect did not occur. Later work by Calcott (52), who had worked earlier with Postgate, showed that SAD was correlated with cyclic AMP (cAMP) depletion inside the cell. Another peculiarity of cAMP metabolism is that internal concentrations of cAMP are in the μM range, whereas cellular effects depend on supplementing media with cAMP in the mM range. It also should be noted that cAMP is effective in regulating the phosphotransferase system (PTS) (49,51).

It is possible that lack of cAMP regulates some transport properties and leads to starvation for resources. This leads to the possibility that this internal starvation is the actual cause of three responses in the initial list: oligotophy, the "shutdown cell," and VBNC. This possibility calls for measurement of the intracellular cAMP levels in the relevant situations.

Lactose Death

When cells are grown in a lactose-limited chemostat, the selection pressure for improved uptake activity is strong. This leads to the selection of constitutive mutants of the *i* gene of the lactose operon; these strains continuously form the permease, and in a lactose-limited chemostat they take over the chemostat. Besides these constitutive regulatory mutations, other mutations that have extra copies of the constitutive *lac* operon accumulate. The result is that the cells have very high levels of galactoside permease in their

cytoplasmic membranes. When such cells are cultured in normal batch minimal medium with the usual amount of lactose (>0.1%), they die (5).

We (Burt and Koch, unpublished) studied selected *E. coli* cultures isolated from human infections that behaved similarly. Initially they were of clinical interest because these cell would be suspected of being pathogenic *Shigella*, but on further analysis it was found that they were actually Lac-negative *E. coli*. We determined that these *lac*-negative strains actually had the *lac* operon intact except for the y-gene (or permease gene). The regulatory elements of the operon were intact, including the i, o, and p genetic elements and also the transacetylase. Our conclusion was that the galactoside permease itself was the cause of death. This was consistent with the finding of Dykhuisen and Hartl (5). In the presence of too much of a galactoside, the destruction of the cell occurred whether the supplied galactoside was metabolizable or not. How the killing process occurs has never been adequately explained.

Quiescent Cells in Low-Dilution Rate Chemostats

Koch and Coffman (6) found that when *E. coli* cells were cultured at low-dilution rates in the chemostat, some cells at any time were in a state in which they were not synthesizing protein for hours at a time. A fuller discussion of this phenomenon is given in Koch (27).

In these experiments, it was shown that all cells in batch and chemostat cultures growing with doubling times of up to 13 hours had a uniform and rapid response to an inducer in the formation of β-galactosidase. On the other hand, cells in chemostat cultures growing more slowly, were heterogeneous with respect to enzyme synthesis. The obvious interpretation is that under these conditions, a particular cell may be temporarily quiescent in protein synthesis, but be accumulating reserves for later growth. At various times each cell appeared to self-activate and synthesize proteins at the normal rate until it used its reserves. The amino acid step time was found to be that characteristic of cells from a faster growing chemostat culture or from cells growing in an a large amount of glucose in medium of otherwise the same composition (6). For a 24-hour chemostat culture under our conditions, only about one-third of the cells were inactive at any time, but all cells were active over a 3-hour period. The cause of this heterogeneity could not be because of inadequacy of mixing in the chemostat culture because such mixing effects are at most for a few seconds. The plausible explanation of the different behaviors of individual cells in the culture is that at the time of assay there are variations in the internal energy resources available to individual organisms. Consequently in a slow dilution rate chemostat, the time needed for accumulation and the time needed to utilize the stockpile of amino acids once synthesis start, will determine the proportion of cells engaged in a period of protein synthesis and its duration.

The suggestion then is that chronic starvation can block synthesis of proteins and other macromolecules. However, if the low level of resource is available then slowly the resource will be accumulated to an adequate level. It is possible that an individual cell can sense this and switch its strategy and start and stop macromolecular synthesis. It may utilize the resource until depletion stops biosynthesis and forces it to return to a quiescent period.

The SOS Response

The SOS phenomenon was discovered incidentally to ultraviolet light irradiation studies of *E. coli* K12 (7). This bacterium happened to bear a prophage and UV illumination of the bacteria induced excision of the prophage, the destruction of the cell, and the production of a large burst of virus. This lysogenic virus, now called lambda, uses a protective device of the cell (the SOS system) for its own purposes. Presumably, the prokaryotes had developed the SOS system to protect themselves from UV damage to their DNA. The observation of virus induction led to the finding and dissecting of the SOS system (7). Damage that forms single-stranded regions of DNA causes the *recA* gene product to bind to these regions and subsequently act as a "co-protease" with the LexA protein. This causes the LexA protein to cleave itself. Cleaved LexA is no longer able to repress the synthesis of more than 20 bacterial, plasmid, and viral genes. One of the consequences of the induction of this system is that the lambda C1 repressor is cleaved causing the excision of the viral genome and promoting the lytic cycle of the virus. This was the original event, mentioned earlier, leading to the finding and understanding of the SOS phenomenon. One gene induced by the cleavage of LexA protein relevant for this review is *sulA*. Ordinarily the background level of the SulA protein is small because it is rapidly degraded by the protease Lon. When there is sufficient extra SulA present, because of derepression, it inhibits FtsZ, whose action is needed for the cell division process. For the present review, it can be imagined that a similar system might prevent cell division indefinitely until a reversing signal becomes developed.

This SOS system is a possible prototype for VBNC. Except for the fact that with the SOS phenomenon the inhibition of the two aspects of growth, cell division, and chromosome replication, is only several hours long (7). In the SOS and VBNC processes, a system is triggered by an environmental signal that needs a qualitatively different signal to reverse the blockade and to signal the start vegetative growth.

Osmotic Rupture

This section deals with death due to the microbiologist's action or due to lethal shift-up events that the bacteria are not able to overcome. External compounds can frequently be concentrated many thousandfold by active transport systems (35). So cells not only need to have effective active transport systems, but they also need ways to control the overactivity of the transport systems (49,50). To prevent the internal concentration of a given compound of osmolytes from becoming dangerously high, the transport mechanism must be attenuated by feedback inhibition caused by the accumulated internal pool of substance. Alternatively, some other safe way to eliminate the danger of the incoming compound is needed. Speculative hypotheses presented previously (23,23a) presume that

protective pathways exist to dispose of a surfeit of internal pools in copiotrophs and in facultative oligotrophs. Several possible ways are: by reversal of the uptake system and secretion of the excess nutrient, excreting a metabolite derived from it into the medium, or inhibiting the uptake system by something like a two-component system. The presumption is that none of these processes is successfully executed by strictly obligate oligotrophs.

Consider the problems that a cell would encounter while revising its growth strategy from one appropriate for an environment with adequate resources to a strategy designed for a chronic low level of nutrients or for an indefinite time in the essential absence of some essential nutrient. Evidently an important part of the shift in adapting the cell to a slow growth-rate state is that functional mechanisms to extrude or passively leak small compounds from the cytoplasm must now be blocked so scarce resources can be frugally conserved. Once the cell has converted to the strategy appropriate for the oligotrophic state a new problem arises. The cell now faces the problem that a surge of internal resource may cause damage due to the osmotic pressure difference across the cell envelope. Such a surge would follow introduction of a moderate level of a resource if the cell had an efficient active uptake system for it. Possibly this might occur in seconds, much faster that synthesis of controlling or enzymatic proteins can take place. This may, therefore, be the reason that some cells from natural environments may be killed by high concentrations of a substance in the medium and subsequently may not grow at all. Of course, many kinds of organisms may adapt over time, particularly if gradually exposed to this type of challenge.

This may be analogous to the phenomenon of substrate-activated death (SAD). When osmotically sensitive cells are subcultured by investigators who give them what the investigator deems to be low levels of resources, the result of transport may result in an excess of internal materials resulting in flooding of the cell's pools with a lethally high concentration of osmolytes.

Oxidative Stress

A critical point has been made (9,9a) that oxidizing radicals can come from the environment and also be made as a by-product and side reaction to the oxidation of metabolic intermediates and resources. These may destroy cells and account for the cells that cannot be resuscitated. They could also account for the response of the cell in altering their physiology so that they were less susceptible to oxidizing radicals.

Action of a Rigid and Nonenlarging Wall in Blocking Cytoplasm Accumulation

Streptococcus mutans is a gram-positive coccus that has a very thick wall. In the presence of the penicillin types of inhibitors, cell wall growth stops (10), but the cells do not lyse or die.

Contrast this with the well-known behavior of typical gram-negative rods; under these conditions "rabbit ear" structures form. These are the results of the sacculus rupturing in the middle of the rod-shaped cell where division is scheduled to occur. Such structures are the result of the old poles and sidewall remaining intact, whereas the nascent cross wall, which ordinarily forms very rapidly, does not stay intact. Most gram-positive cells will do the same. As this does not happen with *S. mutans*, it is reasonable to assume that this failure to be killed in the presence of penicillin type antibiotics is because its wall's strength is too high and does not rupture under the heightened turgor pressure. Although DNA, RNA, and protein synthesis initially continue, they soon halt. I suggest that the osmotic pressure rises so high in this case that water and perhaps potassium salts are extruded as a response to high pressure. This is on the assumption that these are the only major molecules that can escape the cell and this change in the cytoplasmic ionic environment leads to the inhibition of macromolecular synthesis; that is, the cell becomes metabolically inactive and dormant.

Thus, some aspects of oligotrophy, the "shutdown" cell, and VBNC could possibly be explained by a cellular change that eliminates or blocks the penicillin binding proteins (PBP) that are essential for wall growth. On this hypothesis, the result is the indirect blockade of some metabolic processes but later (maybe some months later) the change is reversed to allow wall enlargement.

Complete Elimination of the Function of a Factor Needed for Protein Synthesis

There are many antibiotics that function by stopping protein synthesis. Streptomycin is a well-studied antibiotic and much has been said about its mechanism of action. It is highly positively charged and, therefore, binds to phosphate residues of nucleic acids. Additionally, it binds to a protein component of the ribosome; namely, S12. These properties should make it block growth while it is present and thus it should be a bacteriostatic agent. However, interestingly, although one would expect that the cell would recover when the antibiotic is removed, this is not so. Instead, the cells die. One suggestion for this is that the antibiotic irreversibly binds to and inactivates a factor needed for elongation (Robert Harvey, personal communication). This blockade would be absolute and irreversible when all molecules of the factor needed for protein synthesis have been inactivated. Then restarting protein synthesis after the antibiotic has been removed is not possible.

This type of process may be the prototype of the way that the VBNC state cells have stopped macromolecular synthesis. Restarting could be because of some special trigger that bypasses the factor's action in a way that only needs to be temporary and need not even be highly effective.

STILL OTHER KINDS OF LETHAL PROCESSES

There are processes other than osmotic and oxidative stress that may kill. See (24) for a list and (21,22) for a description of a chromosomal gene that can kill; also see the large literature on addiction genes that enable low copy number plasmids to cause the death of their host if not transmitted to a newborn cell. These will not be further considered here.

THE DIFFERENT KINDS OF BACTERIAL DEATH

The microbiologist's functional definition of a living or a dead bacterium is whether it can produce a colony or clone under conditions that usually support indefinite growth. A lethal injury is something that damages the cell so that a colony cannot result, but within this definition, it can happen that a few, but not continued, divisions may occur. It should be noted that the usage of the term "living" in microbiology differs from the common usage where an animal or person is not thought to be dead just because it stops reproducing. But colony formation is the operational definition in microbiology; we will have to use it, but there is much more to be said.

Although a lethal event ultimately leads to death, it may be that following this event, that another event occurs or is triggered, which is the actual proximate cause of irreversible death. Thus in the SOS case, DNA may be damaged in a potentially lethal way, but the actual lethal event is the failure to repair the DNA correctly and in a timely fashion before the defect is permanently encoded in double-stranded DNA. Throughout the history of microbiology, a good deal of attention has been focused on the destruction of organisms with heat sterilization, disinfectants, antibiotics, chemotherapy, radiations, virus infections, bacteriocins, host immunity mechanisms, etc. Some of these events are the primary, but some are secondary death processes. Although many examples could be given, I will cite only the finding of Evelyn Witkin (54) on cells treated with ultraviolet light. This causes "lethal" mutations. She found that if good growth conditions are provided, more cells will fail to give colonies than if the cells are incubated temporarily under poor or nongrowing conditions. She showed that given time, repair may undo the damage under these conditions and the repaired cells will grow when nutrient conditions are provided later. As in the other cases, it is easy to see a parallel in the microbial choices of the slower, decreased, halted processes for more precise growth.

CONCLUSION

There is good reason why there ought to be a VBNC state. There is good reason to object to some experimental studies purporting its existence. There is good reason to believe that many of the cases in nature depend on special signals from other organisms, such as the role of *Acanthamoeba castellanii* with *Legionella pneumophilia* (55). There is good reason to extend the studies being highly critical and designing more critical experiments; such as Ref. (53,55–60).

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VIABLE COUNTS IN ENVIRONMENTAL SAMPLES.

See METHODS FOR FLOW CYTOMETRY AND CELL SORTING

VIRAL AEROSOLS

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Viruses cause a variety of diseases in human and animals (1,2) and air can play an important role in the spread of many viral diseases. In general, such spread of viruses is rapid and difficult to prevent and control (3).

SOURCES OF VIRAL AEROSOLS

Activities such as sneezing, coughing, flushing toilets, and changing diapers, as well as shaking, homogenization, and sonication of virus-containing materials can generate infectious aerosols. Preventing the generation of, and avoiding exposure to, such aerosols are particularly important in laboratories and other settings where infectious material is handled (4,5).

When any virus-containing liquid is ejected into air, larger droplets settle out rapidly in the immediate surroundings, whereas "droplet nuclei" (usually <5.0 μm in diameter) become airborne and can be transported over long distances. Windblown carriage of animal pathogenic viruses has been documented to result in outbreaks of disease several kilometers downwind from the source (6–9).

Inhalation of air containing viral aerosols can lead to their retention in the respiratory tract, and airborne spread has been clearly documented for a variety of viral infections of humans (10,11) and animals (11). Figure 1 shows how susceptible hosts can be exposed to airborne viruses. However, infection through the inhalation and retention of droplet nuclei is generally regarded as true airborne spread. Infectious viruses (11) or their nucleic acids (12) have been detected in field samples of air. Air may also play a role in the spread of viral infections of the gut (13) through the capture of airborne viruses in the upper respiratory tract and their subsequent ingestion (14).

STUDY OF VIRAL AEROSOLS

Study of airborne viruses requires stringent safety precautions (15) and equipment (16) that may not be readily available commercially. Suspensions with relatively high titers ($>10^7$ units/mL) of viable viral particles are also essential for such studies because virus infectivity can be reduced during generation and collection of aerosols and by dilution of the aerosolized material in air. Basically, one needs the following equipment and procedures for the study of viral aerosols. Please refer to other published studies for further details in this regard (11,17–23).

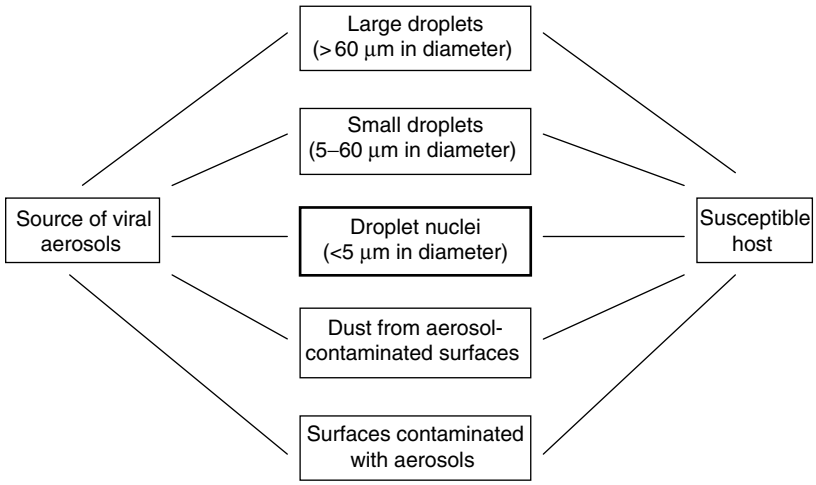

Figure 1. Direct or indirect exposure of susceptible hosts to viral aerosols (12).

Aerosol Generation

The study of viral aerosols requires the generation of particles that are small enough (<5 μm in diameter) to remain suspended in air. Several nebulizers are commercially available for this purpose (11,17,23,24). Nebulizers for the aerosol delivery of therapeutic drugs also produce particles in the respirable range (25) and can be adapted for generating viral aerosols. The size distribution of the aerosolized particles and the stability of the virus in them will depend to a large extent on the nature and composition of the medium used for suspending the virus to be aerosolized (11).

A change in the level of infectivity of the aerosolized virus is usually caused by a combination of: (*1*) virus inactivation during the process of nebulization, (*2*) dilution of the aerosolized particles in air, (*3*) settling out of a proportion of the aerosolized particles (physical decay), and (*4*) the actual loss in virus infectivity in air (biological decay). An accurate determination of the rate of biological decay makes it necessary to incorporate a "physical tracer" in the virus suspension to be sprayed. To be useful, such a tracer must be safe for the virus while being resistant to breakdown during aerosolization and the aging of aerosols. The best tracer is a radioisotope incorporated into the virus particles because such virus particles can act as their own tracers. Another but perhaps a less satisfactory way is to simply add a radioisotope to the virus suspension to be aerosolized.

Uranine, a fluorescent dye, added to the virus suspension, has been found to be as effective as a radiolabeled virus (26); the dye is harmless to viruses and cell cultures and is also much less expensive and safer to use than radioisotopes. One possible drawback of the use of such dyes may be their potential to enhance the photosensitivity of viruses (17). Bacterial spores, which can readily withstand nebulization and are relatively resistant to biological decay, can also be used as tracers (17,23).

Virus suspensions to be aerosolized often contain proteins that tend to froth during the process of nebulization, and an addition of a suitable antifoam agent to such suspensions is necessary to address this problem (26).

Retention and Aging of Aerosols

To investigate the influence of various environmental factors on the stability of aerosolized viruses, a custom-built rotating stainless steel drum (23,27,28) is generally used to store the aerosols in a dynamic state (Fig. 2). The drum, which is usually housed in a sealed chamber, is continuously rotated mechanically along its axis at a predetermined rate (e.g., 4 rpm) to keep the aerosolized material from settling out. The air inside the drum can be preconditioned to the desired relative humidity (RH) level and temperature. Thorough air flushing of the drum is required between experiments.

Ultrafine (<1 μm in diameter) natural (spiderweb) and artificial (tungsten) threads can be used as supports for small virus-containing particles. Such anchored particles are still subject to the influence of various environmental factors around them (17,23,28). Despite the limitations (23), ultrafine threads are the best means to study the influence of atmospheric chemicals (24) and light and irradiation (29,30) on airborne viruses. Ethylene vinyl

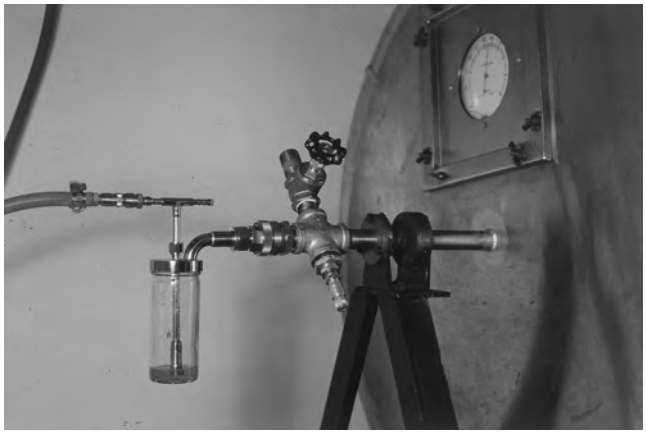

Figure 2. Stainless steel drum for the aging of aerosols. A Collison nebulizer is ready to spray a viral suspension into a 300-L stainless steel drum on the right.

acetate fibers (2–3 μm in diameter) have been found to be as suitable as spider threads to study the airborne survival of bacteria (31), but their suitability for working with viral aerosols remains to be investigated.

Collection and Sizing of Aerosols

Most commercially available aerosol collectors (15,19–21,32) have been designed primarily for working with bacteria and fungi and are not suitable for dealing with airborne viruses. All-glass impingers (AGIs) are often the devices of choice for collecting viral aerosols (11,23) in relatively small volumes (2–3 liters L/minutes) of air. The air to be sampled is drawn into the impinger, where it passes through a tube with a limiting orifice and impacts on the surface of the collecting fluid containing an antifoaming agent (26). Use of preimpingers with AGI sampling improves the collection of airborne viruses (11). The volume of air sampled must be replaced with fresh air to avoid creating a vacuum in the aerosol-holding device such as a rotating drum.

In the field, commercial large volume air samplers (LVAS), which can easily process 10 m^3 of air per minute, can be used to recover airborne viruses (11,17,23,33). The collection efficiencies of various LVAS vary considerably depending on the type of virus, the type and volume of air sampled, the nature of the collection fluid, and the rate of sampling. The use of acidic buffer-moistened cartridge filters to recover polioviruses aerosolized during the flushing of toilets has been reported (34).

The Andersen six-stage sieve sampler (35) can be used to determine the size distribution of viral aerosols by replacing the agar-based medium in the sampler's petri plates with tryptose phosphate broth containing 3% gelatin (26). After sampling, the collection medium can be liquefied by holding the plates at 37 °C for 1 hour and poured out for virus titration and measurements of the physical tracer levels.

Experimental Exposure to Viral Aerosols

To determine the susceptibility of humans or animals to airborne viruses or to assess the minimal infective dose of viruses by the respiratory route, experimental subjects are exposed to standardized clouds of viral aerosols under controlled conditions. The Henderson apparatus (36) is used for the aerosol exposure of small animals such as mice where individual animals are placed in cylindrical holders, with only their nostrils protruding, and exposed to airborne viruses.

Devices for working with larger animals such as cattle (37) or pigs (38) also have been described. In the design and performance of any such aerosol challenge studies it is extremely important to avoid the exposure of the experimental animal to the test virus by means other than inhalation. Chung and coworkers (39) have reported the development of a low-cost wind tunnel for exposing human subjects to aerosols of more than 2 μm in diameter. Particles generated by a Collison-type nebulizer and introduced into the tunnel by using an aerosol injector are uniformly distributed to expose human subjects; the device is yet to be tested with viral aerosols.

AIRBORNE SURVIVAL OF HUMAN AND ANIMAL PATHOGENIC VIRUSES

How long a given virus can remain infectious in air depends on the nature of the virus and the medium in which it was suspended before becoming airborne, ambient temperature, RH, atmospheric gases, lighting, and irradiation (11). The absence of standardization of the experimental protocols and wide variations in the system of reporting the results make direct comparisons of the findings from different studies extremely difficult. Table 1 is a summary of published information on the airborne survival of selected human and animal pathogenic viruses (11,40).

CHALLENGE WITH VIRAL AEROSOLS

Several studies have challenged human or animal hosts with artificially generated viral aerosols (11). Because of the difficulties inherent in the design and performance of such experiments, their findings should be interpreted with the following limitations in mind: (*1*) often the amount of challenge virus inhaled was either not determined or may have been too high to be relevant to levels encountered in nature, (*2*) exposure of test subjects by means other than through inhalation could not be excluded because of flaws in the experimental design, (*3*) many reports failed to specify important parameters such as RH and air temperature in such experiments, and (*4*) in the absence of standard procedures for aerosol challenge studies, it is virtually impossible to compare the findings of different investigations.

The limitations enumerated earlier point to the need for much developmental work before accurate and reliable information on the behavior of viral aerosols could be generated.

COLLECTION OF NATURALLY OCCURRING VIRAL AEROSOLS

When compared to studies with experimentally aerosolized viruses, collecting infectious viruses from naturally occurring aerosols is even more challenging. The devices currently available for the purpose are generally noisy, bulky, expensive, and somewhat inefficient. As is true for many other types of environmental sampling, it is often too late to look for the suspected virus in air in an outbreak investigation. Even if it were feasible, regular monitoring of air for viruses is not recommended because of the limited significance of the findings. However, there are certain situations where sampling of air for naturally occurring viral aerosols can be extremely valuable. This is exemplified by the studies of bat caves for airborne rabies virus (65).

Air is probably the main vehicle for the spread of viral infections such as measles and influenza. Viruses, which normally spread through other means and vehicles, have been found to spread through air in rare instances. The airborne spread of rabies is a case in point (33). But it must be noted that any virus, which can survive aerosolization, has the potential for airborne transmission.

Table 1. Summary of Published Information on the Ability of Selected Human and Animal Viruses to Survive in and Spread Through Air

Virus	Cause Of	Comments
Foot-and-mouth disease virus	Foot-and-mouth disease in animals	The virus has been studied extensively for its capacity to spread through air (11), and several airborne outbreaks of the disease documented (8). Sheep breathing air from cabinets housing virus-infected pigs contracted the disease (41).
Hantaviruses	Respiratory and kidney infection	Infections in those handling these viruses or animals carrying them now well documented (2,42–44); the most likely means of exposure was by inhalation of infectious aerosols. Artificially generated aerosols of Hantaviruses can infect rats (45).
Influenzaviruses	Influenza	The behavior of aerosolized influenzaviruses has been studied in some detail (11). Recent studies in this regard have focused on challenging vaccinated animals with viral aerosols to study the protective effect of immunization.
Lassavirus	Hemorrhagic fever	Survival of Lassa fever virus in artificially generated aerosols was favored at low (30%) RH levels, and even at 32 °C the virus survived long enough to permit its dispersal by air (46). Experimental aerosol exposure of monkeys and guinea pigs could infect and kill them.
Newcastle disease virus	Newcastle disease in poultry	Convincing evidence for the airborne spread of the virus and air filters in poultry houses highly effective in preventing outbreaks of the disease (47). A vaccine strain of virus used as a model to study the airborne behavior of enveloped viruses (29,48) and to field test protective equipment and methodologies for the rapid collection and identification of viruses in air.
Norwalk virus	Acute diarrhea	Airborne spread strongly suggested from patterns of spread in outbreaks (14,49–52). Contamination of air may occur by aerosolization of virus during vomiting (53,54). Aerobiology cannot be studied because no animal models or cell culture systems available thus far.
Papilloma viruses	Warts and cancer	Reports of warts in the respiratory tract of laser therapists (55) suggest exposure to the viruses in the smoke from vaporized warts (56). A PCR-based study (57) showed widespread contamination of the facial area of the therapists and the operating room environment with viral DNA released during laser treatment and electrocoagulation of human warts and neoplasia.
Pseudorabies virus	Infection of nervous and respiratory systems in animals	Even though this enveloped virus is relatively fragile, its capacity to spread through air parallels that of foot-and-mouth disease virus (8), a picornavirus. The virus in air survived the best when RH was 55% and the air temperature was 4 °C (58); the half-life of the virus under these optimum conditions was less than one hour.
Retroviruses	Cancer and AIDS	Little is known about their airborne spread nor is there any evidence that they can spread by air. However, infectious HIV-1 detected in aerosols from certain types of surgical power tools (59), and the blood-containing aerosols were in the respirable range (60). Potential for airborne spread of HIV-1 and other retroviruses exists where high-titered suspensions of such viruses are handled.
Rhinoviruses	Common cold	The half-life of a human rhinovirus was nearly 14 hours at 20 °C with an RH level of 80%, clearly indicating its potential to survive and spread through air (61).
Rotaviruses	Acute diarrhea	Airborne spread strongly suggested from patterns of spread (62–64). Survival in air favored by lower levels of RH (26).
Varicella-Zoster Virus	Chickenpox and shingles	Spreads by air. Its DNA found in air samples from rooms housing patients with chickenpox or shingles (12) showing that PCR technology may prove useful in studying the airborne spread of viruses.

CONCLUSION

Despite decades of studies, our knowledge of the aerobiology of viruses remains somewhat rudimentary. This is while the potential for the airborne spread of many human and animal pathogenic viruses continues to increase because of the increasing use of recycled air (66,67), presence of ever-larger numbers of immunosuppressed and transplant patients, and changing lifestyles (68). We also need safer and more economical methods for removal and/or inactivation of infectious viruses from recycled air.

Airborne dissemination of nonviable viruses or their components can lead to problems in laboratories using PCR or other such techniques, and presently available containment facilities may not be quite adequate to address this issue (69). Aerosol generation by surgical tools (11,59,67,70,71) and laboratory equipment (72) suggests that the potential of any such technology should be assessed at the design stage and corrective measures taken. Effective means are needed for protecting animals and humans in case of deliberate or accidental release of viral aerosols (23).

Rapid inactivation (30) and higher levels of dilution of vertebrate viruses in open air suggest that they may be less likely to spread outdoors. However, the airborne survival and transport of such viruses over long distances has been documented (73,74), thus showing their potential to cause outbreaks at distant locations (6–8). Perhaps long-distance transport of viruses may explain the peculiar transcontinental movement of outbreaks of rotaviral infections (75).

Airborne viruses continue to be a threat to human and animal health in spite of the sophisticated design and efficient protective functioning of the respiratory system (60). Much of the research thus far has documented outbreaks of airborne viral infections and identified to some degree the factors that influence virus survival in air. Currently greater emphasis is needed on reducing generation of infectious aerosols in indoor and outdoor settings and on enhancing the removal and/or inactivation of viruses (20) and other infectious agents in air. Many human and animal pathogenic viruses continue to be considered as potentially useful bioweapons and recent accounts (76) of their production and stockpiling testify to this. The details of past and possibly ongoing work in this regard may never become available to the general scientific community.

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VIRAL DISEASES. See ENTEROVIRUSES: BASIC BIOLOGY AND DISEASES; HEPATITIS VIRUSES (HAV-HEV); HUMAN CALICIVIRUSES: BASIC VIROLOGY AND EPIDEMIOLOGY; ROTAVIRUSES

VIRAL DISINFECTION

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Disinfectants do not necessarily kill all microorganisms, but are intended to reduce the number of disease-causing microorganisms to a safe level. Disinfectants used for the inactivation of viruses are termed viricides. To limit the transmission of pathogenic viruses at home, in childcare and health care facilities, in water and wastewater, and on living surfaces, physical or chemical viricides are applied. Human pathogenic viruses may be present in an infectious state in water supplies, body fluids, food products; or on inanimate objects (fomites) such as tables, countertops, children's toys, etc. Thus, viricide practices include the disinfection of the home, office, water and wastewater treatment plants, childcare centers, health care facilities, and industry. The action of viricides includes destruction or blocking of viral capsid proteins important for host cell attachment and/or destruction, or fatal mutation of viral nucleic acid. Chemical disinfectants include chlorine and its related compounds, alcohols, detergents, and phenolics. Physical disinfectants include heat, ultraviolet light, and radiation. Important chemical and physical disinfectants, disinfection kinetics, mechanisms of viral inactivation and factors important in disinfectant effectiveness are discussed in the following sections.

APPLICATIONS FOR THE INACTIVATION OF VIRUSES

One of the most important uses of disinfectants is for the inactivation of pathogenic viruses in drinking water. Drinking water disinfection has been practiced in the United States since the early 1900s to circumvent the outbreak of waterborne disease (1). Examples of chemical and physical disinfectants employed in water treatment

Table 1. Disinfectants Applied for Water Treatment Purposes

Chlorine
Chloramines
Ozone
Chlorine dioxide
Potassium permanganate
Ultraviolet light

are listed in Table 1. In drinking water treatment, disinfection is the final barrier between viral pathogens and drinking water consumers as viruses, because of their small size, can bypass filtration processes. In addition to drinking water, appropriate disinfection of wastewater is also critical because receiving waters may be used for drinking, recreation, shellfish harvesting, and irrigating food crops that require minimal processing before consumption.

Viricides are widely used in health-related fields for the inactivation of viruses on tables, benchtops, floors, medical instruments, and bodily fluids to prevent transmission to susceptible individuals. An excellent example of the important use of viricides is the disinfection of blood and blood products. To secure clean blood supply, pathogenic viruses such as human immunodeficiency virus (HIV), hepatitis B and C and other bloodborne viruses must be inactivated using physical or chemical disinfectants. Moist heat disinfection, specifically autoclaving, is one of the most popular methods in the health-related industry for the sterilization of biohazardous materials and medical instruments. Even in the home, disinfectants are used to reduce the number of microorganisms on the countertops and floors of kitchens and bathrooms, on laundry and dishes. Commercial products often contain chlorine, ammonia, and detergents for the reduction of microbes in domestic settings. Also, detergents are used for the removal and inactivation of viruses from the hands or other parts of the body. Classes of commonly used disinfectants, their modes of action and associated applications are listed in Table 2.

VIRUS STRUCTURE AND REPLICATION

Viruses have a much simpler makeup compared to bacterial and animal cells because they lack ribosomes, cannot multiply independently, do not respire or undergo any energy generating processes, their genome may be composed of either deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) and may be single- or double-stranded, and the nucleic acid itself may be infectious. Moreover, viruses are much smaller in size, ranging from 0.02 μm to 0.3 μm.

The structure of the viral particle consists of an outer protein shell, called a capsid. The capsid is composed of protein subunits, termed capsomeres that are arranged in two main types of symmetry: cubical (icosahedral) and helical. The capsid and the nucleic acid constitute the nucleocapsid. For lipid viruses, the capsid is surrounded by a lipid membrane, which is acquired from the host cell on release. The capsid may also surround a protein core that encloses the viral nucleic acid. The viral genome consists of either DNA or RNA, and may be double- or single-stranded. Viruses cannot replicate independently; instead they must infect a host cell and utilize the host cell's metabolic machinery for their replication. For animal viruses, the key steps for their replication include (*1*) attachment to the host cell by specific viral proteins; (*2*) penetration of the host cell by either endocytosis or fusion; (*3*) uncoating of the viral particle; (*4*) replication of viral nucleic acid; (*5*) synthesis of viral proteins important in replication of the nucleic acid and/or capsid proteins;

Table 2. Classes of Some Commonly Used Disinfectants, Their Modes of Action, and Associated Applications

Viricide	Mode of Action	Disinfectant Applications
Physical Disinfectants		
Moist heat (autoclaving, steam, and boiling)	Denaturation of proteins and nucleic acid	Health field: Sterilization of health-related supplies/equipment
Radiation Ionizing (gamma)	Nucleic acid destruction	Sterilization of pharmaceuticals and health supplies/equipment
Nonionizing (UV)	Pyrimidine dimer formation	Same as mentioned earlier and wastewater treatment
Chemical Disinfectants		
Chlorine and related compounds (chlorine, chlorine dioxide, and ozone)	Oxidation of protein and nucleic acid	Water, wastewater, industrial, and household disinfection applications
Metal ions (copper and silver)	Many mechanisms: damage to proteins and nucleic acid	Pools, spas, hot tubs, and home-faucet purification units
Photoantimicrobials (Titanium dioxide and phenothiazines: methylene blue, toluidine blue, azure B, and others)	Damage to nucleic acids	Wastewater, blood, and blood products

(*6*) assembly of the viral particle; and (*7*) release of the mature viral particle from the host cell via budding or lysis.

VIRAL INACTIVATION BY DISINFECTANTS

Because of the simple structure of viruses, the ways in which disinfectants inactivate viral particles are limited (Table 3). Disinfectant action may include the destruction, blocking, or altering of capsid host cell receptors so that host cell attachment cannot occur. Chemical viricides, which are strong oxidizers, for example, ozone, can destroy capsid proteins rendering the virus noninfectious. Also, changes in pH can produce conformational changes in viral proteins that may either aid in disinfectant action by exposing proteins involved in host cell attachment or internalize them leaving them unavailable for disinfectant interaction (2). Adsorption of molecules onto viral capsid proteins may effectively block attachment to receptors on a host cell. Immobilization of a viral particle onto a surface is another example of disinfectant action. In the case of aluminum, viruses adsorb onto it's surface and when released, are rendered noninfectious (3). Destruction or fatal mutations in the viral genome are other ways by which viricides may inactivate viral particles. To react with the viral nucleic acid, the disinfectant must be able to diffuse through the capsid, destroy the capsid ejecting the nucleic acid or prior dissociation of the nucleic acid must have occurred. For RNA viruses, destruction of their nucleic acid is critical because previous studies have demonstrated the ability of susceptible cells to uptake viral RNA and produce infectious viral particles (4).

Table 3. Possible Mechanisms of Viral Inactivation by Viricides

Viral capsid	Blocking of viral host cell receptors
	Destruction of capsid proteins
	Conformational change of viral host cell receptors
Viral nucleic acid	Fatal mutations
	Complete destruction

Table 4. Factors Affecting Viral Disinfection

Factors	Effects/Concerns
Temperature	Higher temperature = increased inactivation rate
	Lower temperature = decreased inactivation rate
pH	Affects ionic species of disinfectant
	Affects conformational form of virus (capsid proteins)
	May induce clumping or aggregation
Organics	Consumes oxidizing chemical disinfectants (chlorine and related compounds)
	Particulate organics: shields from disinfectant action
Salts	Increase capsid stability, thus increasing viral resistance (heat inactivation)
	Increase viral inactivation rate (chlorine)
Aggregation	Protects inner particles from disinfectant action
Different viral types	Varying susceptibilities among closely related viral types to same disinfectant
	Use of surrogates or indicators may not be useful to describe closely related viruses
Laboratory vs. naturally occurring viruses	Laboratory strains may not represent the resistance of naturally occurring viruses to disinfectants

FACTORS AFFECTING PHYSICAL AND CHEMICAL DISINFECTION

The effectiveness of a disinfectant to inactivate a viral particle is dependent on many physical and chemical characteristics of the virus, disinfectant and the medium in which the virus and disinfectant are in contact (Table 4). Temperature influences the rate of viral inactivation, at which higher temperatures tend to have higher inactivation rates. Temperature may also affect the stability of some viruses. The pH of the disinfecting medium may affect the ionic species of chemical disinfectants such as chlorine and chlorine dioxide. In addition, changes in pH above or below the isoelectric point (IEP) of a virus, may alter the conformation of disinfectant target sites on the capsid, reducing or diminishing interaction with the disinfectant (5). The IEP is the pH at which the viral particle has no charge. Viral particles are either negatively or positively charged, depending on whether the pH of the suspending medium is above or below the IEP of the virus. Concentrations of salt have been reported to significantly increase the viral inactivation rate by disinfectants such as chlorine (6). However, a decrease in thermal inactivation rate has been observed in the presence of salt, which may increase capsid stability (6,7).

Clumping or aggregation of viral particles protects the inner particles from the action of disinfectants. The enteric viruses are shed clumped and associated with cell debris in the feces of infected individuals (8). Several studies have demonstrated the increased resistance of enteric viruses, which are aggregated or debris-associated compared to dispersed viral particles (9–11). Viral aggregation may be caused by changes in virion hydrophobicity; where pH changes induce virion hydrophobicity or by the presence of salts (5,12).

Dissolved organic matter and particulates are other important constituents that may lower viricide effectiveness. Dissolved organic matter reacts with disinfectant, either oxidation or adsorption, reducing the concentration available to react with the microorganisms. For most disinfectants, particulate matter (organic or inorganic) may consume the disinfectant and protect or shield any embedded or adsorbed microorganisms. Particulate matter may also scatter light (ultraviolet light or sunlight), decreasing its intensity. However, UV research conducted with

inorganic particulates varies, because some studies conducted with viruses associated with inorganic particles were inactivated similarly to unassociated viruses (10).

Individual virus properties may affect the efficiency of disinfection. In general, the resistance of viral groups is in the following order from least to most resistant: lipid viruses < large nonlipid viruses < small nonlipid viruses (13). Viruses have been shown to have varying susceptibilities to disinfectants even within one population or between different viral types (5,14,15). Use of surrogates or indicators that are supposed to represent similar viruses may not be adequate, due to the differences in resistance between closely related viral types. The viral particle's state of hydration has also been shown to affect disinfection kinetics, in which hydrated viral particles are less resistant than nonhydrated particles (5). Further, laboratory viral strains have been shown to be less resistant to chlorine compared to chlorinated drinking water isolates (9,16). However, for some viral types, greater resistance of the chlorinated drinking water isolates was not observed (9). Finally, like all microorganisms, viruses are more sensitive to some disinfectants compared to others; for example, viruses are more susceptible to inactivation by ozone than chlorine.

Multiplicity Reactivation

If a disinfectant produces random damage to a viral particle, many of these particles, under the appropriate conditions, may aggregate or clump. Infectious viral particles may be produced if there is at least one functional copy of each damaged unit (protein or nucleic acid), and these viral components are able to enter a susceptible host cell. In 1942, Luria first postulated this phenomenon, termed multiplicity reactivation (5). Sharp (1982) demonstrated an increase in titer following chlorine disinfection of clumped versus dispersed echovirus. Differences in titer between these two treatments ranged by a factor of 10-3,000. Because of multiplicity reactivation, a disinfectant must effectively destroy the viral capsid proteins important in attachment to the host cell, fragment the nucleic acid, and not produce conditions conducive to clumping or aggregation of these viral components (5).

Disinfection Kinetics

Viral inactivation by chemical and physical disinfectants proceeds at specific reaction rates, are controlled by kinetics and affected by a number of factors including disinfectant concentrations, temperature, pH, and the presence of interfering substances (17). Developed on the basis of experimental data, kinetic models have been used in the formulation of disinfection design criteria for water treatment (18). Specifically, these models attempt to predict the complex interactions of different microorganisms, from bacteria to viruses, with different disinfectants, which have different cellular targets and modes of action (18). Consequently, because of these complex interactions, predicting microbial inactivation is difficult.

There are many kinetic models that have been used to predict the interaction of disinfectant with microorganisms, but probably the best known is the Chick-Watson model. This model combines Chick's rate law and the Watson function. According to Chick's rate law, the number of microorganisms destroyed per unit time is proportional to the number remaining for a given disinfectant concentration. Thus, Chick's law assumes that the reaction between a chemical disinfectant and a microorganism follow first-order kinetics. In this law, however, the effect of varying disinfectant concentrations is not taken into consideration, so Watson derived an empirical logarithmic function called the Watson function. The incorporation of Watson's function into Chick's rate law produces the Chick-Watson pseudo first-order rate law (18) (Table 5). More often however, first-order kinetics are not observed for microbial inactivation by disinfectants, instead various types of inactivation kinetics are observed as demonstrated in Figure 1. Curve 1 illustrates first-order or exponential inactivation kinetics, which usually describes microbial UV light inactivation. Curve 2 represents a shoulder curve which may be caused by one or more of the following, ineffective dispersion of the microorganisms or disinfectants, delays in disinfectant diffusion to critical sites on the microorganism, and

Table 5. Examples of Kinetic Models Used for Prediction of Microbial Inactivation by Water Disinfectants

Model Name	Kinetic Equation[1] $\mathrm{Ln}\,(N_t/N_0) =$
Chick's Law	$-k\mathrm{t}$
Watson's Function	$\mathrm{C}^n\mathrm{t}$
Chick-Watson	
Disinfectant demand-free conditions	$-k\mathrm{C}^n\mathrm{t}$
Disinfectant demand conditions	$-k/\mathrm{k}' = n(\mathrm{C}_0{}^n\text{-}\mathrm{C}_f{}^n)$
Efficiency Factor Hom	
Disinfectant demand	$-k\ \mathrm{C}_0{}^n\mathrm{t}^m[(1\text{-}\exp(\text{-}n\mathrm{k} = \mathrm{t}/m)/(n\mathrm{k} = \mathrm{t}/m)]^{\mathrm{m}}$

Source: Adapted from L. L. Gyurek and G. R. Finch, J. Environ. Eng. 124, 783–793 (1998).
[1]Refer to Table 6 for term definitions.

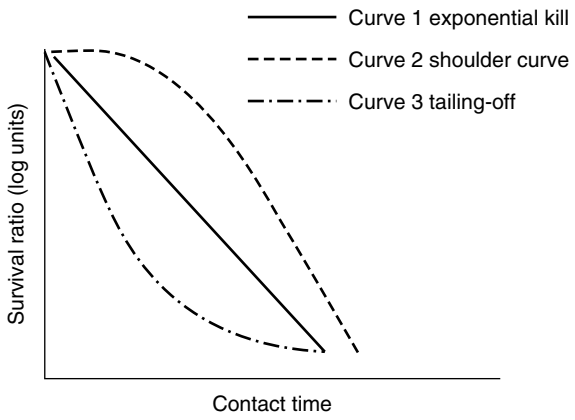

Figure 1. Typical microbial survival curves.

disinfectant interaction of multiple targets necessary for microbial inactivation (18,19). Curve 3 displays the effects of clumping or aggregation, presence of different microbial populations having varying disinfectant resistance or increased disinfectant decay (5,18,19). While the Chick-Watson can only accurately predict inactivation kinetics represented by Curve 1, other kinetic inactivation models have been mathematically proposed to describe curves that deviate from first-order kinetics. Examples include the Hom, Hom-Power, Efficiency Factor Hom, and the Selleck models. Definitions for all kinetic model constants are presented in Table 6. These models have been shown to provide a statistically improved fit to the bench-scale inactivation process over the Chick-Watson model (18).

The *Ct* concept was derived from the Watson's function, where C represents the concentration of the disinfectant and t is the time required to inactivate a predetermined percentage, usually 99%, of the population at specific pH and temperature conditions (20). The higher the *Ct* value, the more resistant the microorganism is to inactivation by the disinfectant, whereas a lower value would indicate decreased resistance. By comparing these values, evaluation of the effectiveness of pathogen inactivation by various chemical disinfectants can be determined. In addition, differences between different groups of microorganisms and variability within groups may be ascertained. The Ct method is used by the water treatment industry to help predict the amount of disinfectant needed to inactivate pathogenic microorganisms occurring in the water supply (18). For chlorine and it's related compounds, the Ct is measured in milligrams per liter multiplied by the time in minutes (mg/L * minutes). For UV light, the UV dose is measured in milliwatts multiplied by seconds per centimeter squared (mWs/cm^2).

Table 6. Kinetic Model Parameters and Definitions

Parameter	Definition	Comments
k	Disinfectant decay constant	Units usually 1/minutes first-order decay assumed rate assumed to be independent of the microorganisms present
T	Time required to achieve a given level of inactivation	Units usually in minutes
N	Coefficient of dilution	Represents the average number of molecules to have combined with the microorganism necessary to cause inactivation
k, m	Microbial inactivation constant	Specific for the microorganism and set of conditions
C, C_0, C_f	Concentration of disinfectant, at time zero and final time of inactivation experiment	Units depend on disinfectant

Physical Disinfectants

Thermal Inactivation. Inactivation of pathogens by heat is one of the most widely used disinfection or sterilization procedures in the medical (autoclave sterilization) industries. Moist and dry heat methods are described in Table 7. To describe the inactivation of viruses or other microorganisms by heat, three concepts have been devised. The thermal death point (TDP) is used to define the lowest temperature in which all microorganisms are killed/inactivated in 10 minutes. Secondly, the thermal death time (TDT) is defined as the minimum amount of time in which all microorganisms are inactivated at a specific temperature. The DRT, (D value) or decimal reduction time is the time in which 90% of the studied microorganisms are killed at a given temperature. Any of these concepts may be used to determine and compare the heat resistance of one microorganism to another.

The type of heat sterilization, moist or dry heat, and the conditions (moisture content, organic matter, and solute concentration) of the medium to be sterilized may affect the efficacy of viral heat inactivation. For example, with increasing moisture, the effectiveness of heat to inactivate microorganisms also increases. Dry viral preparations require higher temperatures and longer times than viral suspensions. Many microorganisms are quickly killed by moist heat because hydrogen bonds, important for protein structure, are quickly broken. This is especially true for moist heat methods such as autoclaving. Boiling, another moist heat method, may not be as effective as autoclaving for the inactivation of all viruses. Dry heat inactivates microorganisms through oxidation and is typically not as effective as moist heat (longer time and higher temperatures required). Enteric viruses have been shown to increase resistance to heat inactivation in the presence of cationic salts. This increased resistance may be caused by stabilization of the viral capsid. Other substances that may increase viral resistance to heat may include the presence of sugars which decrease water activity. Finally, the presence of organic matter or particulates may also increase resistance to heat inactivation (21).

Ionizing Radiation. Gamma rays and high-energy electron beams are an effective form of radiation because the ionization of water and other substances effectively cause mutagenic effects. In fact, gamma irradiation is one of

Table 7. Moist and Dry Heat Methods

Moist Heat	
Boiling	100 °C, 10 minutes
Autoclaving	121 °C, 15 pounds of pressure per square inch, 15 minutes
Dry Heat	
Incineration	Burning to ashes
Hot air sterilization	170 °C, 2 hours

the most frequently used methods for the sterilization of large batches of small volume items used in the medical field, such as needles and catheters. Free radicals, especially the hydroxyl radical, produced through the ionization of water, interact with viral proteins and nucleic acid. Because damage to the nucleic acid may cause incorrect transcription of pertinent genes, ionization can be lethal. This is especially true for disinfection with high doses of radiation in which multiple hits may occur. The absence of oxygen and water and the presence of organic matter all contribute to increased resistance to ionizing radiation. Viruses are the most resistant to ionizing radiation compared to other microorganisms possibly because of their small size (21). Determination of the effectiveness of gamma irradiation to inactivate different viruses is by comparison of their D values. The D value is the dose of radiation needed for 90% inactivation. The D values for selected viruses in different media are listed in Table 8.

More widely used than ionizing radiation, ultraviolet (UV) radiation or ultraviolet light exposes substances to a germicidal wavelength of 254 nm. This wavelength is very close to the absorption maxima of nucleotide bases where the pyrimidines, thymine (T), cytosine (C) and uracil (U), undergo photodecomposition at a much higher rate than the purines (adenine and guanine). The most important class of photoproducts formed by photodecomposition are the 5, 6-cyclobutyl dipyrimidines, more commonly referred to as pyrimidine dimers (25). Three types of dimers may be formed that include T-T, C-C and T-C. Of these, T-T dimers are produced more often because their formation has a greater quantum yield than either C-T or C-C dimers (25). It is the formation of these dimers that inactivates microorganisms by blocking replication of the nucleic acid (26). Protein damage due to UV absorption at the germicidal wavelength is much lower than nucleic acids; therefore it is not as important in microbial inactivation compared to the formation of pyrimidine dimers (25).

First-order kinetics involving UV inactivation of microorganisms, including viruses, has been reported in the majority of studies. In addition, double-stranded (ds) DNA viruses have been shown to be more resistant than single-stranded (ss) RNA viruses, and varied resistance among closely related viruses has been reported (15,21,27). Turbidity of suspending medium, aggregation, or clumping and UV intensity can affect the effectiveness of UV disinfection. Table 9 demonstrates the differences in resistance between closely related viruses.

Table 8. Gamma Irradiation of Selected Viruses Suspended in Sludge, Ice, and on Lettuce and Strawberries

Virus (medium virus was irradiated in/on)	D Value	Reference
Bovine herpesvirus-1		
(frozen with cell debris)	4.72 (kGy)	22
(frozen without cell debris)	7.31 (kGy)	
Hepatitis A virus		
(surface of strawberries)	2.72 (kGy)	23
(surface of lettuce)	2.97 (kGy)	
Poliovirus		
(sludge)	350 (krad)	24
Adenovirus		
(sludge)	150 (krad)	24

Table 9. UV Light Inactivation of Selected Enteric Viruses

Virus	Dosage mWs/cm^2	Reduction (%)	Reference
Poliovirus 1	5.0	90	28
	7.7	90	29
Coxsackievirus B1	15.6	90	30
Coxsackievirus B5	15.0	99	31
Coxsackievirus A9	35.7	99.9	30
Echovirus 1	1.3	99	30
Echovirus 11	1.4	99	30
Adenovirus 40	54.3	90	32
Adenovirus 41	23.6	90	33

Chemical Disinfectants

Chlorination and Chlorine-Related Compounds. For more than 100 years, chlorine has been used as a disinfectant for the reduction of disease-causing microorganisms in water (1). In fact, chlorine and related compounds are applied for the treatment of water more frequently than any other water disinfectant (34). In water treatment, chlorine gas is added to water resulting in two reactions, hydrolysis

$$Cl_2 + H_2O \leftrightarrow HOCl + H^+ + Cl^- \quad (1)$$

$$HOCl \leftrightarrow H^+ + OCl^- \quad (2)$$

(Eq. 1) and ionization (Eq. 2), in which HOCl is hypochlorous acid and OCl^- is the hypochlorite ion. Free available chlorine refers to the amount of unbound HOCl and OCl^- present in water, whereas total available chlorine refers to both bound and unbound forms. The concentration of HOCl and OCl- present in a water sample is governed by pH, and is described in Figure 2. Furthermore, HOCl is a much stronger oxidant, and therefore better disinfectant, compared to other forms of chlorine (Table 10). Because of chlorine's strong oxidizing properties, it not only reacts with pathogenic microorganisms, but also other easily oxidizable substances present, including metals such as iron and manganese, organic matter, hydrogen sulfide and ammonia. These reactions reduce the chlorine forms to the unreactive chloride ion (1). This effect, where disinfectant is consumed by water constituents, is commonly referred to as disinfectant demand.

Table 10. Estimated Effectiveness of Residual Chlorine Forms

Chlorine Form	Estimated Potency Compared to HOCl
Hypochlorous acid (HOCl)	1
Hypochlorite ion (OCl^-)	0.010
Monochloramine (NH_2Cl)	0.007
Dichloramine ($NHCl_2$)	0.013
Trichloramine (NCl_3)	Possibly more effective than $NHCl_2$

Source: Adapted from AWWA, 1995 (39).

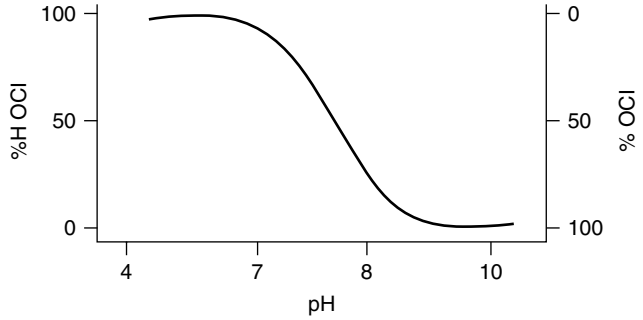

Figure 2. Distribution of HOCl and OCl in water as a function of pH. Adapted from Bitton 1994 (27).

Table 11. Inactivation of Selected Enteric Viruses by Chlorine

Virus	°C	pH	Chlorine Dose (mg/L)	Estimated Ct (% Reduction)	Reference
Poliovirus 1	5	6	0.5	1.05 (99%)	39
	5	10	0.5	10.5 (99%)	39
Hepatitis A	5	6	0.5	2.3 (99.99%)	11
Hepatitis A cell-associated	5	6	0.5	29 (99.99%)	11

However, when chlorine combines with ammonia, chloramines are formed in three sequential reactions:

$$NH_3 + HOCl \rightarrow NH_2Cl(\text{monochloramine}) + H_2O \quad (3)$$

$$NH_2Cl + HOCl \rightarrow NHCl_2(\text{dichloramine}) + H_2O \quad (4)$$

$$NHCl_2 + HOCl \rightarrow NCl_3(\text{nitrogen trichloride or trichloramine}) + H_2O \quad (5)$$

in which the di- and trichloramines are eventually oxidized to nitrogen gas. These three competing reactions are affected by pH, temperature, contact time, the ratio of chlorine to nitrogen and most importantly, the initial chlorine and ammonia nitrogen concentrations. Continual application of chlorine will eventually increase the chlorine to nitrogen ratio to 7.6 : 1 and higher, at which chloramines will be oxidized to nitrogen gas, nitrate, and other end products and a free chlorine residual appears. This point in the ammonia-chlorine interaction is termed the breakpoint (1). Ammonia-chlorine reactions are important in wastewater treatment, in which naturally occurring concentrations of ammonia convert applied free chlorine to chloramines. Because chloramines are considerably weaker disinfectants, chlorine is applied until the breakpoint reaction is reached (1).

The mechanism by which chlorine inactivates viral pathogens is controversial. Viral inactivation may be caused by interaction with the viral capsid proteins and/or the nucleic acid. In some studies, viral RNA had a greater affinity for chlorine than the capsid, whereas other researchers observed that the virus became noninfectious before damage to the capsid occurred (35,36). Furthermore, depending on the concentration of chlorine, studies have indicated that nucleic acid damage may occur at low (0.8 mg/L) concentrations, whereas high (>0.8 mg/L) chlorine concentrations may result in damage to both the nucleic acid and protein capsid (37). Varied resistance among closely related viruses has been reported. Differences in chlorine resistance and Ct values of selected viruses are shown in Table 11.

Chlorine dioxide (ClO_2) is a gas that is highly soluble in water at low temperatures. However, chlorine dioxide in water is extremely volatile and is lost to the atmosphere readily. Unlike chlorine, chlorine dioxide produces fewer trihalomethanes (THM) and does not combine with ammonia. Thus, for disinfection of substances that contain high concentrations of organic matter, chlorine dioxide may be a better alternative than chlorine. In the food industry, chlorine dioxide has been used successfully for the disinfection of the recycled water containing high concentrations of organic matter. In the health industry, chlorine dioxide gas is effective for the sterilization of packaged medical products. For drinking water treatment, chlorine dioxide destroys phenols that cause tastes and odors, and removes iron and manganese (1).

As a viricide, ClO_2 is a very effective, especially under conditions of high pH (40). At these high pH values, ClO_2 is a better viricide than HOCL for feline calciviruses and adenovirus (Thurston, unpublished data). Like chlorine, differences in the mechanisms of inactivation have been reported. It appears that both viral proteins and nucleic acid are damaged (21,26).

Bromine and Iodine. Bromine and iodine are not used for the disinfection of drinking water because of a combination of expense and possible adverse health effects. Free bromine(Br_2) forms trihalomethanes, reacts quickly with organics reducing disinfectant concentration and residual, produces a medicinal taste to water, and production of bromine is costly (1). Consequently, Br_2 applications are restricted to disinfection of water in cooling towers, small swimming pools, hot tubs, and spas. Bromine chloride (BrCl), an interhalogen compound, has been recommended as an alternative to chlorine for controlling biofouling of industrial (cooling towers) equipment. Further, BrCl has been suggested to be a better disinfectant compared to Br_2 (1). Viral inactivation by low (0.3 to 5 mg/L) concentrations of bromine have been suggested to occur by either prevention of viral attachment to the host cell, by damage to the protein coat, or by prevention of uncoating of the viral particle. However, at higher bromine concentrations, the protein capsid was degraded (40).

Iodine is also applied for small-scale water treatment. Iodine was first used by military troops for the sterilization of water in World War I. More recently though, iodine-containing quaternary resins have been placed in portable and home water disinfection systems. For treatment

purposes, iodine has been shown to be effective in herpes eye infections. Iodine at high pH values (above pH 8) has increased viricidal effectiveness because of the predominant form, hypoiodous acid, which has stronger oxidizing power over other forms of iodine (41). Although some authors report structural damage to the viral capsid, more recent evidence suggests uptake of iodine and subsequent destruction of the inner components of the viral particle, including the nucleic acid (21,42).

Metal Ions. Several metals may produce a viricidal effect including aluminum, copper, silver, mercury, and others. Because of the low toxicity of silver and copper, they are used for the disinfection of pools, spas, and home-faucet water treatment systems. Although, the rate at which metal ions inactivate viruses is slow compared to oxidizers such as chlorine, they are advantageous because the duration of their activity is much longer (22).

Metal ions can inactivate viral particles in a number of ways. Metal ions may adsorb to the virus capsid, blocking receptor sites. Once absorbed, the metal ion may be reduced, and the possible generation of hydroxide radicals may cause multihit damage on the viral capsid. Other damaging reactions to the viral capsid include decarboxylation and hydrolysis. In the case of metallic aluminum, viral binding to the surface of the metal was shown to render poliovirus noninfectious (5,14). Other work with aluminum demonstrated degradation of poliovirus RNA without damage to the viral capsid (43). Damage to the viral nucleic acid may occur if the nucleic acid is free in solution, or if the metal ion is able to diffuse through the capsid. Degradation of the phosphate backbone, intercalation, and disruption of base-pairing are ways in which viral nucleic acid may be damaged by metal ions (5,14).

CONCLUSION

The effects of viricides bear immeasurable importance for the prevention of viral transmission and thus, prevention of viral diseases. With an increasing immunocompromised population, viricide research directed at improving the safety of the blood, water, and food supply is essential. Currently, a great deal of information is lacking or controversial regarding the effectiveness or mechanisms of disinfectant viral inactivation. Disparities between viral disinfection studies make conclusions regarding the effectiveness and mechanisms of various disinfectants difficult. Future research must be conducted under similar conditions to allow comparison of results between different research groups and investigations should aim to determine the effectiveness of viral disinfectants under conditions natural to their use.

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VIRUS SURVIVAL IN SOILS

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The potential for pathogens in wastewater to contaminate potable water supplies is dependent on a number of factors including the physical characteristics of the site (e.g., soil texture), the hydraulic conditions (e.g., wastewater application rate, wetting/drying cycles), the environmental conditions (e.g., rainfall, temperature) at the site, and the characteristics of the specific pathogens present in the reclaimed water. The factors that influence the survival and transport of pathogens in the subsurface have been the subject of a number of reviews (1–5). The factors that affect the survival of viruses in soil will be discussed in detail in the following sections of this article.

TEMPERATURE

Temperature is probably the most important factor influencing virus inactivation in the environment, including soils (6). Lefler and Kott (7) found that it took 42 days for 99% inactivation of poliovirus in sand at 20–25 °C, whereas more than 175 days were required at 18 °C. Poliovirus was found to persist for more than 180 days in saturated sand and sandy loam soils at 4 °C, whereas no viruses could be recovered from the soils incubated at 37 °C after 12 days (8). Hurst and coworkers (9) studied the survival of poliovirus at three temperatures: 1, 23, and 37 °C in loamy sand. They also found that the inactivation rate was significantly correlated ($p < 0.01$) with incubation temperature, noting faster inactivation rates at the higher temperatures.

The exact mechanism whereby temperature inactivates viruses in soils has not been determined, but several theories have been proposed. The inactivation may be due to denaturation of the viral capsid (6); however, it has been shown that the RNA released during thermal denaturation remains infective, as it is more resistant to heat inactivation (10). Dimmock (11) suggested that different mechanisms might be at work, depending on the temperature. At low temperatures (less than 44 °C), the rate of virus inactivation corresponds with the inactivation rate of the viral RNA. However, at high temperatures (greater than 44 °C), the rate of virus inactivation exceeds that of the inactivation rate or the viral RNA, and is associated with structural changes in the viral capsid. Kapuscinski and Mitchell (12) suggested that temperature itself may not be responsible for virus inactivation, but may merely control whether other inactivation mechanisms can occur.

MICROBIAL ACTIVITY

There are conflicting reports regarding the role of microorganisms in virus inactivation; many of these have been reviewed (12,13). Sobsey and coworkers (14) found that the inactivation rates of poliovirus and reovirus in eight different soil suspensions were almost always greater under nonsterile conditions compared with sterile conditions. Hurst and coworkers (9) reported that virus inactivation rates were similar in nonsterile anaerobic soils and sterile soils at 1, 23, and 37 °C. Under aerobic conditions, poliovirus and echovirus inactivation rates were more rapid in nonsterile soil preparations than sterile at 23 and 37 °C. At very low temperatures such as 1 °C, inactivation rates were similar in both sterile and nonsterile soils.

Further studies by Hurst (15) investigated the influence of the oxygen status of the soil, and conditions of sterility on the survival of poliovirus 1 in soil. These studies showed that the virus inactivation rate was significantly affected by the presence of aerobic microorganisms. Two- to threefold increases in virus inactivation rates were found in the presence of aerobic microorganisms. This observation was made at all three incubation temperatures, 1, 23, and 37 °C. Anaerobic microorganisms did not have any significant effects on the virus survival in this study.

Several mechanisms by which microorganisms may influence virus inactivation have been suggested (16). Proteolytic enzymes released by certain bacteria were capable of destroying the capsid of coxsackievirus A9, but polioviruses were unaffected (17). Some microorganisms produce substances, which render viruses more susceptible to inactivation by photodynamic processes (18) or enzymes (19). Other compounds produced by microorganisms such as humic acids, tannins, phenols, and ascorbic acid may act as oxidizing or reducing agents, which lead to virus inactivation (16). Studies by Lipson and Stotzky (20) on the adsorption of reovirus to clay particles suggested that microorganisms may enhance virus inactivation in two ways. One is by the production of a soluble metabolic product that is virucidal; the other is by interfering with virus adsorption to soil particles.

SOIL MOISTURE CONTENT

The moisture content of the soil also influences virus persistence in soils. Although some investigators have

observed no difference in inactivation rates in dried versus saturated sand (7), the majority of the reports have indicated a difference. Bagdasaryan (21) observed that several enteroviruses, including poliovirus 1, coxsackievirus B3, and echoviruses 7 and 9, could survive for 60 to 90 days in soil with 10% moisture, as compared with only 15 to 25 days in air-dried soils. In another study, 99% inactivation of poliovirus occurred in one week, as the soil moisture content was reduced from 13 to 0.6%; however, 7 to 8 and 10 to 11 weeks were required for the same amount of inactivation in soils with 25 and 15% moisture content, respectively (22).

Hurst and coworkers (9) found that the moisture content affected the survival of poliovirus in loamy sand. The inactivation rate increased as the moisture content was increased from 5 to 15%, then decreased as more liquid was added. It was noted that the fastest inactivation rate occurred near the saturation moisture content of the soil (15 to 25%). The slowest inactivation rates were observed at the lowest moisture contents, 5 and 10%.

In another study, it was observed that virus inactivation rates were greater in more rapidly drying soils. In a field study of virus inactivation during rapid infiltration of wastewater, it was shown that allowing the soils to periodically dry and become aerated, enhances virus inactivation (23). Using radiolabeled viruses, Yeager and O'Brien (8) showed that the viruses are inactivated during the drying process, rather than becoming irreversibly bound to the soil particles.

Other studies by Yeager and O'Brien (24) suggested that the mechanisms responsible for poliovirus inactivation are different in moist and drying soils. In moist soils, under both sterile and nonsterile conditions, the viral RNA was recovered in a degraded form, suggesting that the nucleic acid was degraded prior to its release from the capsid. In dried, nonsterile soils, complete loss of viral infectivity was observed, and the RNA was recovered in degraded form. In contrast, the nucleic acid was recovered intact from sterile dry soils.

pH

The effect of pH on virus survival in soil has not been extensively studied. It has been suggested that pH indirectly influences virus survival by controlling adsorption onto soil particles which, in turn, affects virus survival (25). A few investigators have studied the direct effects of pH on virus persistence. Sobsey (26) reported the results of a study using simian adenovirus SV-11, in which it was shown that inactivation was more rapid at pH 5.0 and 6.0 than at pH 4.0 and 7.0.

The stability of five enteroviruses including hepatitis A virus, coxsackievirus A9, coxsackievirus B1, poliovirus 1, and echovirus 9, was studied under conditions of extreme acidity at room temperature (27). Of the viruses studied, hepatitis A virus was the most resistant to inactivation. In order to eliminate the possibility, that attachment to cellular material was responsible for the high resistance to inactivation, the studies were repeated with highly purified hepatitis A virus. The purified virus was found to be even more resistant to inactivation, remaining infectious for eight hours at pH 1.

Salo and Cliver (19) found that in the aqueous environment, pH affects different viruses in different ways. While poliovirus 1 was inactivated more rapidly at pH 3 and 9 than the near-neutral values of 5 and 7, coxsackievirus A9 was inactivated more rapidly at pH 5 than the extremes of 3 and 9. Murphy and coworkers (28) observed that mouse encephalomyelitis virus survival was longer in neutral soils than in soils with the pH adjusted to 3.7 or 8.5. Hurst and coworkers (9) studied the survival of poliovirus 1 and two bacteriophages, MS2 and T2, in nine soils with pH values ranging from 4.5 to 8.2. They found that virus inactivation was significantly correlated ($p < 0.05$) with soil saturation pH, with longer survival at the lower pH values.

The exact mechanism whereby pH causes virus inactivation has not been fully elucidated, but one investigator (29) found that the inactivation of adenovirus 2 was accompanied by an increase in sensitivity to DNase and the loss of structural proteins from the virus capsid. Results obtained by Salo and Cliver (19) also suggested that virus inactivation involves alterations in the virus capsid. These investigators found that the RNA of the inactivated virus particles became sensitive to ribonuclease at all pH levels tested (pH 3–9), and at pH 5 and 7 the RNA was hydrolyzed in the absence of ribonuclease.

Burge and Enkiri (30) found that the adsorption of bacteriophage ΦX174 to five soils showed a significant negative correlation with soil pH. The adsorption of poliovirus 1 and reovirus 3 to eight soils suspended in wastewater at pH values from 3.5 to 7.5 was generally greater at the lower pH values (14). The adsorption of the encephalomyelitis virus to montmorillonite clay was greatest at pH 5.5 (31). At pH 9.5, the adsorption was less, but the poorest adsorption was observed at pH 3.5. In an extensive study of poliovirus adsorption by 34 minerals and soils, no significant correlation was found between substrate pH and virus adsorption (32). The investigators reported that although adsorption by most of the neutral and acidic materials was strong, the variation in percentage of virus bound in the alkaline materials was so great that no significant correlation could be detected.

Loveland and coworkers (33) studied the effects of pH, on the reversibility of virus attachment to mineral surfaces. Their experiments on the attachment of PRD1 to quartz and ferric oxyhydroxide-coated quartz indicated that the isoelectric point of the mineral surface controls attachment. The attachment of PRD1 was observed at pH values 2.5 to 3.5 units above the isoelectric point of the mineral surface. Below this pH, the attachment of PRD1 was found to be complete and irreversible, while above this pH, the adsorption was minimal and reversible.

The mechanism(s) whereby pH affects virus adsorption to soil particles has been explained in terms of the electrochemical nature of the virus and soil surfaces (25,26,34). The outer surface of the enteric viruses is made of protein; therefore, the surface charge is influenced by the ionization of the carboxyl and amino groups in the capsid. The isoelectric point (pI) of many enteric viruses is below

7 (34), thus, at neutral pH, most viruses are negatively charged. Most soils are also negatively charged at neutral pH, and virus adsorption is not favored due to the mutual repulsion. If the pH of the environment is decreased, the surface charge of the virus will become positive (or less negative) due to increased ionization of the amino groups and decreased ionization of the carboxyl groups. While soils also become less negatively charged at lower pH values, soil pI values are generally lower than those of viruses, thus they may still have a net negative charge at acidic pH levels. This results in an electrostatic attraction between the virus particle and the soil, which leads to adsorption. In reality, the effect of pH on virus adsorption is not so clear-cut. There are many complicating factors that can interfere with the mechanism discussed earlier. One is that a given virus may have more than one isoelectric point: poliovirus 1 (Brunhilde strain) has isoelectric points at pH 4.5 and 7.0 (34). The factors responsible for passage from one form to another are unknown at this time. Other soil factors such as cations and humic and fulvic acids may also influence the net surface charge of viruses.

SALT SPECIES AND CONCENTRATION

The presence of certain chemicals may render a virus more or less susceptible to inactivation. Burnet and McKie (35) found that bacteriophage inactivation at 60 °C was partially prevented in the presence of 0.002 to 0.01 M $CaCl_2$, or $BaCl_2$. However, when the concentration was increased to 0.15 M or greater, thermal inactivation was increased. Enhanced stabilization of poliovirus at temperatures ranging from 4 °C to 50 °C in the presence of high concentrations (1 M) of $MgCl_2$ has been reported (36). Echoviruses have also been found to possess this property (37). Cords and coworkers (38) found that many type A coxsackieviruses were inactivated more rapidly in low ionic strength media than in high ionic strength media.

Thurman and Gerba (39) studied the effects of modifying soil with several metals on the survival of MS2 and poliovirus. The addition of aluminum metal, magnesium oxide, and magnesium peroxide had a significant negative effect on the inactivation rate of MS2 when compared with unmodified control soils. The addition of unrefined substances such as zeolite, bauxite, limonite, and glauconite (which contain multivalent cations and oxides of iron and aluminum) did not have a significant negative effect on the inactivation rate of MS2. Rather, they seemed to have a protective effect, as indicated by the lower inactivation rate as compared with the unamended control soil. Exposure of poliovirus to the aluminum-amended soil also resulted in significantly higher rates of inactivation, as compared with controls. Further experiments by these investigators indicated that contact between the aluminum and virus was necessary for inactivation of the viruses. They postulated that a combination of electrodynamic van der Waals interactions and electrostatic double layer interactions promoted virus adsorption to the surface of the aluminum, where inactivation of the virus could then take place.

The role of the soil cation exchange capacity (CEC) in virus adsorption has also been investigated. Burge and Enkiri (30) found that the CEC of four of five soils was correlated significantly ($p < 0.05$) with virus adsorption. In contrast, Goyal and Gerba (40) did not find a significant correlation between soil CEC and adsorption of 15 different viruses. Additionally, no correlation was found between virus adsorption and total phosphorus, or total and exchangeable iron, calcium, and magnesium.

Experiments to determine the effects of water quality on virus retention in soil columns were conducted by Sobsey and coworkers (41) using poliovirus 1, echovirus 1, and hepatitis A virus. Little difference in the amount of virus adsorption was noted, when the columns were dosed with either groundwater or primary wastewater effluent. Dosing with simulated rainwater resulted in considerable elution of all three viruses from the Corolla sand columns at 5 °C. Elution of the three viruses from columns of FM loamy sand was also observed, although the effects were more pronounced for echovirus 1 than for the other two viruses.

VIRUS ADSORPTION TO SOIL

The adsorption of viruses to soils and other surfaces may prolong or reduce survival, depending on the properties of the sorbent. The survival of poliovirus and reovirus was not always prolonged in soil suspensions, as compared with soil-free controls in a study using eight different soil materials (14). Murray and Laband (42) found that poliovirus adsorption onto some inorganic substances, such as CuO, results in decreased infectivity of the virus. These investigators suggested that van der Waal's interactions between the virus and the particle surface induced spontaneous disassembly of the virus.

More commonly, however, adsorption to soils has been found to prolong virus survival. Jorgensen and Lund (43) compared the survival of viruses in groundwater to survival in soil-groundwater mixtures. Coxsackievirus B3, adenovirus 1, and echovirus 7 were seeded into groundwater and groundwater-soil mixtures, and incubated at 4 to 7 °C. Their studies showed that the viruses survived for longer periods of time in the soil-groundwater mixtures than in the groundwater alone. Their results indicated that the majority of the viable virus particles were contained in the soil fraction of the mixture, rather than in the groundwater. A similar study on the survival of hepatitis A virus in several soils showed that, in all cases but one, virus survival was prolonged in the presence of soil as compared with freely suspended in groundwater (41). These studies suggest that the protective effects of soil are the result of adsorption to the solid surface.

The mechanisms whereby adsorption to a solid surface prolongs or reduces virus survival have not been elucidated. However, Gerba and Schaiberger (44) have suggested several possibilities, including interference with the action of virucides, increased stability of the viral protein capsid, prevention of aggregate formation, and adsorption of enzymes and other inactivating substances.

AGGREGATION OF VIRUS PARTICLES

The formation of aggregates influences virus survival in natural waters. It has been suggested that this is because virus particles within the aggregates are highly resistant to destruction by environmental factors (6). It has been shown that aggregation renders virus particles more resistant to inactivation by chemical disinfectants such as bromine (45), free chlorine, and monochloramine (46). Although there are no definitive reports on the effects of aggregation on virus survival in soil, the results of studies in aqueous media would suggest that aggregates would survive longer in soils than would monodispersed viruses (26).

SOIL PROPERTIES

The influence of soil properties on virus survival is probably related to the degree of adsorption. Hurst and coworkers (9) suggested that the correlation between pH and virus survival was probably mediated through its influence on virus adsorption. A positive correlation between virus survival and soil exchangeable aluminum, and a negative correlation with resin-extractable phosphorus were also attributed to influencing virus adsorption to the soil.

Sobsey and coworkers (41) compared the rate of inactivation of hepatitis A virus in five different soil types including clay soil, clay loam, loamy sand, sand, and organic muck. The survival was greatest in the clay soils, in which at least eight weeks were required to inactivate 99% of the infectious viruses.

VIRUS TYPE

As is obvious from the previous discussions, different viruses vary in their susceptibility to inactivation in the subsurface environment. Sobsey and coworkers (14) found that the rates of inactivation of poliovirus and reovirus in eight soils were different. Hurst and coworkers (9) also showed that the inactivation rates of seven different viruses varied even when incubated under the same conditions. In a comparative study of the survival of poliovirus, echovirus, and coliphage MS2 in several different groundwater samples, no significant difference ($p < 0.01$) was found overall (47). There were, however, differences in the inactivation rates of the viruses in individual water samples. In a recent report on the survival of hepatitis A virus (HAV), it was shown that this virus, generally, survived longer than poliovirus and echovirus at 25 °C (41).

Lefler and Kott compared the survival of poliovirus 1 and f2 coliphage in dry sand (7). Poliovirus survived for at least 77 days at room temperature. The f2 phage survived considerably longer, possibly for as long as one year under dry conditions. On the basis of the previous discussions, it is possible that the survival times of both viruses would be greatly increased if water were added to the soil.

The survival of human rotavirus strain Wa was compared to that of poliovirus 1, which is commonly used as a model for virus behavior in the environment (48). Inactivation experiments were conducted using five water samples (lake water, creek water, groundwater, nonchlorinated secondary effluent, and tap water) at 20 °C. The inactivation rates for the two viruses were significantly different from one another in all water samples except the tap water. This led to the conclusion that poliovirus is not a good model for the behavior of human rotavirus in water. The authors proposed that human rotavirus strain Wa can be used as a model for the persistence of other rotaviruses in water, as it survived for longer than has been reported for simian rotavirus SA11 in similar environmental conditions. Reported virus inactivation rates are given in Table 1.

ORGANIC MATTER

The influence of organic matter on virus survival has not been firmly defined. In some studies, it has been found that proteinaceous materials present in wastewater may have a protective effect on viruses; however, in others no effect has been observed (6). Lefler and Kott (7) found that poliovirus survived longer in sand columns saturated with oxidation pond effluent than distilled water. They suggested that the prolonged survival was probably due to the protective effect of proteinaceous materials in the effluent. Hurst and coworkers (9) did not find a significant correlation between virus survival and percent soil organic matter.

Humic and fulvic acids in soils may cause loss of virus infectivity and prevent adsorption (51). The infectivity could be partially restored by treatment with 3% beef extract, pH 9. Sobsey (26) observed a similar phenomenon: poliovirus infectivity was reduced in the presence of humic acid. At least some of the infectivity could be restored when the samples were filtered, probably by disruption of the humic acid–virus complexes.

Dissolved organic matter has generally been found to decrease virus adsorption by competing for adsorption sites on soil particles. In their study of poliovirus adsorption by 34 minerals and soils, Moore and coworkers (32) found that organic matter showed a strong negative correlation ($p < 0.001$) with virus adsorption. Bitton and coworkers (52) suggested that the organic material in secondary effluent interfered with virus adsorption to a sandy cypress dome soil. Powelson and coworkers studied the effects of natural humic material and wastewater sludge–derived organic matter on the transport of MS2 bacteriophages in unsaturated soil. (53). The transport of MS2 was found to be higher in a loamy fine sand column that had been treated with the organic material, than in a parallel column that had not been treated. In a series of experiments, Bales and coworkers (54,55) studied the effects of hydrophobic organic material on the attachment of bacteriophages MS2 and PRD1 and poliovirus 1 to silica beads. These investigators found that even very small amounts of hydrophobic organic material (>0.001%) can retard the transport of viruses in porous media.

Several investigators have found that organic material can act not only as a competitor for virus adsorption

Table 1. Reported Virus Inactivation Rates in Soils

Virus	Medium	Sand %	Silt %	Clay %	pH	Inactivation Rate log10/d^{-1}	Reference
Poliovirus 1	Dune sand					0.029	49
Poliovirus 1	Pomello sand	89	8	3	7.1	0.019	50
Poliovirus 1	Rubicon sand	92	4	4	7.1	0.007	50
Poliovirus 1	Flushing meadows sand	89	8	3	7.8	0.02–0.027	50
Poliovirus 1	Pomello sand	89	8	3	7.1	0.016	50
Poliovirus 1	Rubicon sand	92	4	4	7.1	0.009	50
Poliovirus 1	Ponzer muck				3.6	0.071	41
Poliovirus 1	Corolla sand	98.1	0.8	1.1	8.3	0.041	41
Poliovirus 1	Flushing meadows	89	8	3		0.032	41
Poliovirus 1	Bentonite	0.5	0.5	99		0.026	41
Poliovirus 1	Ponzer muck				3.6	0.095	41
Poliovirus 1	Flushing meadows	89	8	3		0.071	41
Poliovirus 1	Cecil clay	46	18.9	35.1	4.6	0.032–0.036	41
Poliovirus 1	Kaolinite	0.5	0.5	99		0.041	41
Poliovirus 1	Corolla sand	98.1	0.8	1.1	8.3	0.095	41
Poliovirus 1	Bentonite	0.5	0.5	99		0.048	41
Poliovirus 1	Kaolinite	0.5	0.5	99		0.029	41
Poliovirus 1	Clarion loam					0.163–0.406	28
Hepatitis A virus	Ponzer muck				3.6	0.018	41
Hepatitis A virus	Kaolinite	0.5	0.5	99		0.009–0.029	41
Hepatitis A virus	Cecil clay	46	18.9	35.1	4.6	0.018	41
Hepatitis A virus	Corolla sand	98.1	0.8	1.1	8.3	0.027	41
Hepatitis A virus	Cecil clay	46	18.9	35.1	4.6	0.018	41
Hepatitis A virus	Bentonite	0.5	0.5	99		0.006–0.018	41
Hepatitis A virus	Corolla sand	98.1	0.8	1.1	8.3	0.036	41
Hepatitis A virus	Ponzer muck				3.6	0.018	41
Hepatitis A virus	Flushing meadows	89	8	3		0.018–0.036	41
Hepatitis A virus	Corolla sand	98.1	0.8	1.1	8.3	0.018	41
Echovirus 1 V239	Rubicon sand	92	4	4	7.1	0.007	50
Echovirus 1 V239	Pomello sand	89	8	3	7.1	0.019–0.022	50
Echovirus 1 V239	Flushing meadows sand	89	8	3	7.8	0.026–0.027	50
Echovirus 1	Cecil clay	46	18.9	35.1	4.6	0.057	41
Echovirus 1	Ponzer muck				3.6	0.057	41
Echovirus 1	Cecil clay	46	18.9	35.1	4.6	0.095	41
Echovirus 1	Corolla sand	98.1	0.8	1.1	8.3	0.048	41
Echovirus 1	Kaolinite	0.5	0.5	99		0.026	41
Echovirus 1	Bentonite	0.5	0.5	99		0.029–0.057	41
Echovirus 1	Flushing meadows	89	8	3		0.032–0.048	41
Echovirus 1	Corolla sand	98.1	0.8	1.1	8.3	0.036	41
Echovirus 1	Kaolinite	0.5	0.5	99		0.036	41
Echovirus 1	Ponzer muck				3.6	0.048	41

sites, but also as an eluting agent, that is, it can cause sorbed viruses to desorb from the soil (34). Pieper and coworkers (56) studied the effects of wastewater-derived organic matter on the transport of bacteriophage PRD1 in a sand and gravel aquifer on Cape Cod, MA. They found that the removal of the virus was greater in an uncontaminated area (83%), than in an area that had been contaminated by secondary wastewater effluent (42%). They concluded that the difference in removal was due to the higher content of organic matter in the contaminated zone (2.0–4.4 mg/l vs. 0.4–1.0 mg/l dissolved organic carbon), which blocked the adsorption of the PRD1. The attachment of the viruses to the soil surfaces was reversible, as demonstrated by the recovery of viruses upon addition of linear alkylbenzene sulfonates (LAS) to the system. In the contaminated zone, 87% of the attached PRD1 bacteriophages were remobilized. Jin and coworkers (57) also found that addition of a solution of 1.5% beef extract, which is high in organic matter, could remobilize ΦX174 bacteriophages from Ottawa sand.

Widespread use has been made of this property of organic matter, to remove viruses from filters in order to detect them in environmental samples. Mucks and other soils with high organic matter content are poor adsorbers of viruses (58), and may not be suitable for wastewater application sites (26).

In contrast to the other studies, Powelson and coworkers (59) found that the type of effluent (primary, secondary, and tertiary) had no significant effect on the transport of three viruses (poliovirus 1, MS2 and PRD1) through columns of alluvial coarse sand.

HYDRAULIC CONDITIONS

The rate at which water or effluent is applied to the soil affects the amount of virus removal or adsorption to the soil particles. Several investigators have shown that the amount of virus removal increases as the application rate decreases. Lance and Gerba (60) found that increasing the application rate from 0.6 to 1.2 m day^{-1} caused an increase in the number of virus particles in the column effluent. Increasing the application rate above 1.2 m day^{-1} up to 12 m day^{-1} did not, however, cause any further increase in virus movement through the columns. Vaughn and coworkers (61) also found that application rate had a large influence on the movement of poliovirus through a coarse sand-fine gravel soil. They speculated that the formation of a surface mat of wastewater solids might have been responsible for greater virus removal at the lower application rates.

MECHANISMS OF VIRUS SORPTION

The mechanisms of virus sorption to solid surfaces have been summarized in several review articles (34,62,63,64,65). Sorption of viruses has been studied with various types of viruses and sorbents (Table 2). It is difficult to draw general conclusions because the experimental conditions differ from study to study. Virus sorption depends on the chemical composition of the liquid phase and the nature of the solid surface. Key variables influencing sorption are pH and ionic strength of the solution, presence of compounds competing for sorption sites, functional groups, and isoelectric points of virus and sorbent. Depending on the type of virus and sorbent, sorption is dominated by electrostatic interactions, van der Waals forces, hydrophobic effects, and covalent binding to surfaces.

Table 2. Virus Adsorption to Soils

Virus	Soil Texture			pH	Surface Area (m^2/g)	CEC (meq/100g)	Organic Matter (%)	Adsorption Coefficient (ml/g)	Reference	Comments
	Sand (%)	Silt (%)	Clay (%)							
Rotavirus SA11	13	16	53	4.9	15	53	1.4	124	40	buffer
Rotavirus SA11	77	10	13	8.2	38	4.2	0.27	1.1	40	buffer
Rotavirus SA11	92	4	4	5.5	18	5.6	0.4	3.2	40	buffer
Rotavirus SA11	48	13	39	4.5	84	32	0.3	9999	40	buffer
Rotavirus SA11	26	20	54	7.1	203	71	4.2	48.5	40	buffer
Rotavirus SA11	59	13	28	8	52	23	1.4	21.7	40	buffer
Poliovirus 1 LSc	26	20	54	7.1	203	71	4.2	19	40	buffer
Poliovirus 1 LSc	77	10	13	8.2	38	4.2	0.27	4.58	40	buffer
Poliovirus 1 LSc	92	4	4	5.5	18	5.6	0.4	1.27	40	buffer
Poliovirus 1 LSc	13	16	53	4.9	15	53	1.4	15.67	40	buffer
Poliovirus 1 LSc	59	13	28	8	52	23	1.4	99	40	buffer
Poliovirus 1 LSc	48	13	39	4.5	84	32	0.3	999	40	buffer
Echovirus 1	59	13	28	8	52	23	1.4	0.12	40	buffer
Echovirus 1	26	20	54	7.1	203	71	4.2	0.27	40	buffer
Echovirus 1	92	4	4	5.5	18	5.6	0.4	3.55	40	buffer
Echovirus 1	77	10	13	8.2	38	4.2	0.27	0.15	40	buffer
Echovirus 1	48	13	39	4.5	84	32	0.3	332.33	40	buffer
Echovirus 1	13	16	53	4.9	15	53	1.4	9	40	buffer
Coxsackievirus B3	59	13	28	8	52	23	1.4	32.33	40	buffer
Coxsackievirus B3	40	24	36	8	105	30	0.78	32.33	40	buffer
Coxsackievirus B3	92	4	4	5.5	18	5.6	0.4	2.7	40	buffer
Coxsackievirus B3	77	10	13	8.2	38	4.2	0.27	0.54	40	buffer
Coxsackievirus B3	13	16	53	4.9	15	53	1.4	4.88	40	buffer
Coxsackievirus B3	26	20	54	7.1	203	71	4.2	11.5	40	buffer
Coxsackievirus B3	48	13	39	4.5	84	32	0.3	249	40	buffer
Polivirus 1	46	19	35	4.6				99	41	ground water @ pH7
Polivirus 1	96.1	0.8	1.1	8.3				0.724	41	primary effluent @ pH7
Polivirus 1	46	19	35	4.6				99	41	primary effluent @ pH7
Polivirus 1	98.1	0.8	1.1	8.3				0.515	41	secondary effluent @ pH7
Hepatitis A Virus	98.1	0.8	1.1	8.3				0.064	41	ground water @ pH7
Hepatitis A Virus	98.1	0.8	1.1	8.3				0.064	41	secondary effluent @ pH7
Hepatitis A Virus	46	19	35	4.6				99	41	primary effluent @ pH7
Hepatitis A Virus	46	19	35	4.6				99	41	ground water @ pH7
Hepatitis A Virus	98.1	0.8	1.1	8.3				0.124	41	primary effluent @ pH7
Echovirus 1	46	19	35	4.6				99	41	ground water @ pH7
Echovirus	98.1	0.8	1.1	8.3				0.0309	41	secondary effluent @ pH7
Echovirus	98.1	0.8	1.1	8.3				0.042	41	primary effluent @ pH7

Sorption usually increases with increasing cation concentration in solution, particularly in the presence of divalent cations (52,54,66,67). The enhanced sorption is attributed to the decrease of the thickness of the electric double layer (34). The pH determines the net charge of viruses and sorbents and is therefore a dominant factor (40,54,66). Hydrophobic effects may also play a major role. Bales and coworkers (54) showed that sorption of MS2 was dominated by hydrophobic factors. Bales and coworkers (54) also demonstrated increased sorption with increased temperature. This temperature effect might be due to the endothermic unfolding of protein structures at the interface or due to increased sorption rate at higher temperatures (54).

THE ROLE OF THE GAS–LIQUID INTERFACE

It is well known that certain proteins and viruses are sorbed and inactivated at the gas-liquid interface. Adams (68) and Zittle (69) presented excellent reviews on this topic. Shaklee and Meltzer (70) observed that shaking a pepsin solution in the presence of an air–liquid interface resulted in degradation of pepsin, whereas in the absence of the air–liquid interface, that is, when the bottles were completely filled with liquid, no degradation occurred. Shaklee and Meltzer (70) also observed that bubbling air or carbon dioxide through the solution results in denaturation of pepsin, and that the denaturation rate increased with decreasing pH of the solution. These early observations have been confirmed and extended in other studies (Table 3).

The mechanisms of denaturation or inactivation are closely related to sorption of viruses and proteins at the gas–liquid interface. Proteins spread at the gas–liquid interface and form a monomolecular surface film (71,72,73). The spreading at the interface results in denaturation of proteins, and the sorbed proteins become insoluble in water (71,72,74). Unfolding of the globulax protein structure due to interfacial forces causes the denaturation (68,73,75). The unfolding of the molecular structure is an endothermic process (69). When proteins desorb from the interface, the native state cannot be regained anymore. Due to the significant change in physicochemical properties of the molecule, protein sorption at gas–liquid interfaces has been considered as an irreversible process (73,74,76). The inactivation of viruses is presumably an analogous process to denaturation of proteins. The forces at the interface alter the virus structure in a way that affects the infectivity (68).

Under static conditions, proteins form a monomolecular film at the interface, and once the interface is saturated, dissolved protein molecules cannot reach the interface anymore. Shaking a solution destroys old and creates new interface, unfolded proteins at the interface coagulate, and new native proteins may be sorbed, resulting in increased denaturation or deactivation (68,71).

Sorption of proteins at the gas–liquid interface is in principle the same mechanism as sorption on solid surfaces. On solid surfaces, proteins seem to sorb in monolayers and do not desorb easily. However, in contrast to the gas–liquid interface, when the chemistry of the solution (pH, ionic strength, addition of macromolecules competing for sorption sites) is changed, proteins and viruses desorb from the solid surface and regain their native configuration. It has been argued that the unfolding at a solid–liquid interface does not lead to denaturation or inactivation because the interface is rigid, and therefore it

Table 3. Virus Inactivation and Protein Denaturation at the Gas-Liquid Interface (Modified from Yates and Coworkers) (77)

Compound	Observation	References
Bacteriophage	Inactivation by violent shaking	78
Proteins	Protein unfold at interface and form monomolecular film, film is insoluble, stirring creates new interface area	71
Proteins	Spreading of protein at gas-liquid interface results in denaturation of protein, the proteins become insoluble	72
Proteins	Proteins form surface film, coagulum formed when solution is shaken, rate of denaturation dependent on size of bottle, shaking intensity but not concentration	73
Influenza A virus	Inactivation by bubbling air through virus solution	79
Equine encephalitis virus	Inactivation by shaking in buffered saline solution and by bubbling gas through solution, inactivation increased as pH reduced form 7 to 5	80
Bacteriophage T1,.., T7	Inactivation by shaking and bubbling air through solution, inactivation dependent on pH, presence of gelatin prevents inactivation	68
Enzymes, proteins	Unfolding of macromolecules largely determined by interfacial energy, sorption follows Langmuir isotherm	75
Proteins	Irreversible binding of proteins to interface	76
Viruses	EMC virus not affected, Bacteriophages T3,T5 only little affected, MS2 and Semliki Forest virus inactivated by bubbling air or nitrogen gas through solution, inactivation prevented by adding peptone and apolar carboxylic acids, rate of inactivation dependent on salt concentration, more sorption at higher salt concentration	81
Proteins	Denaturation at interface due to unfolding, insoluble coagulum formed when solution is shaken	74

is more likely that the proteins can return to their original state (69).

CONCLUSION

There are a number of factors that have been shown to affect the length of time that viruses remain in an infective form in the soil environment. The magnitude of the effect varies among viruses, and interactions among the factors undoubtedly occur. To date, it has not been possible to quantitatively describe the relationships between the rate of virus inactivation and these factors. This has severely hampered the development of mathematical models that can be used to independently predict the length of time that viruses may pose a potential public health risk in contaminated soils.

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VIRUS TRANSPORT IN THE SUBSURFACE.

See MODELING OF VIRUS TRANSPORT AND REMOVAL IN THE SUBSURFACE

VIRUS TRANSPORT THOUGH SOILS.

See MODELING OF VIRUS TRANSPORT AND REMOVAL IN THE SUBSURFACE

VIRUSES AND PROTOZOAN PARASITES IN FOOD, INCLUDING METHODOLOGY

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All the pathogens to be considered in this entry have been described biologically, and the methods of final detection for each are given in other sections of this Encyclopedia. This article discusses the selection and processing of food samples to enable final laboratory detection of viruses and protozoa. As water and shellfish are covered in other entries, they are not discussed in this article. Before describing methods for extracting viruses and protozoa from foods, it is important to consider why testing needs to be done. There are at least four possible occasions for detecting viruses and protozoa in food: (*1*) investigation of outbreaks, (*2*) validation of processes as critical control points in hazard analysis-critical control point food safety systems, (*3*) routine quality control testing, and (*4*) random surveys. Applications (3) and (4) are of limited value and are not discussed in detail. Application (2), appropriately done, should allow processing of foods without testing them for the presence of these agents in the finished product; thus, methods of quantification, either of the starting level of the pathogen, the level of survivors, or both, are needed. Application (1) receives the most attention despite the fact that incubation periods of the illnesses caused by these agents are often so long that it is very doubtful that appropriate food samples will still be available for testing. On a positive note, the pathogen that is being sought is usually known in the cases of applications (1) and (2), so specialized methods are appropriate. In the following sections, the relative emphasis on one or another pathogen or on one or another food depends considerably on the available, limited epidemiological record.

FOOD VEHICLES OF VIRUSES AND PROTOZOA

Viruses and Protozoa Among Agents of Food-Borne Disease

Human enteric viruses and parasites have been increasingly recognized worldwide as important causes of

food-borne diseases. Among food-borne viral diseases, hepatitis A and acute viral gastroenteritis associated with Norwalk virus, rotaviruses, and other small, round gastroenteritis viruses are most common (1,2). Food-borne outbreaks are usually associated with consumption of raw or undercooked shellfish contaminated by fecal materials and ready-to-eat food items contaminated by food handlers (1,3).

Parasitic agents of food-borne illness belong to intestinal protozoa, tissue protozoa, and tissue helminths, with protozoa being especially prominent in the United States. Clinically significant protozoa include amoebae (*Entamoeba histolytica*), flagellates (*Giardia*), and coccidia (*Cryptosporidium*, *Cyclospora*, and *Toxoplasma*). Although few food-borne protozoal diseases were reported before the mid-1980s, these have since been more commonly reported, due to increased awareness and improved diagnosis and detection methods. *Entamoeba histolytica* has been a recognized food-borne agent for long, especially in communities with poor personal hygiene and drinking water supplies (4). *Cryptosporidium parvum* and *Giardia lamblia*, the only human-pathogenic species in the respective genera, cause the most common human enteric parasite infections in the United States (5,6). *Cyclospora* has recently been associated with the consumption of contaminated fruits (7,8). *Toxoplasma gondii* is of particular concern because it affects both pregnant women and the fetus. It causes the third most deaths among all food-borne pathogens (following *Salmonella* and *Listeria*) (9). Other intestinal protozoa that are transmissible via food, such as *Isopora*, *Microsporidia*, and *Sarcocystis*, and intestinal helminths, such as *Ascaris*, *Anisakis*, *Taenia*, and *Trichinella*, will not be discussed.

Food-associated outbreaks caused by viruses and protozoa are much less commonly reported than those caused by bacteria. As many cases of such diseases are not reported because important information or samples may be inaccurate or unavailable, and because of the lack of sensitive methods for the detection of these agents, the significance of viral and parasitic agents in food-borne transmission is believed to have been underestimated. A report cited viral gastroenteritis as the most common food-borne illness in Minnesota from 1984 to 1991 (10). The same report ranked human enteric viruses as fifth and sixth among identified causes of food-borne disease in the United States. It was indicated that 10% of the 4,617 outbreaks of food-borne disease of unconfirmed etiology reported from 1973 to 1987 met at least two of the clinical criteria for the outbreak of acute viral gastroenteritis (11,12).

In a review of food-borne diseases in the United States (selected years 1952–1982), only one incident of protozoal disease was recorded — five cases of *E. histolytica* infection in 1967. In 1973–1987, only 1.9% (140 out of 7,458) food-borne outbreaks in the United States were attributed to parasites (11). *Trichinella spiralis* accounted for 128 of those outbreaks, *G. lamblia* for 5, and other parasites for 7. In 1993–1997, only 0.7% of the food-borne outbreaks were determined to be parasite-caused, among them four were caused by *G. lamblia* (13). Intestinal protozoa have also been implicated as important causes of sporadic intestinal illness in the United States. According to a recent survey conducted over a portion of the country comprising 6% of the total population, *C. parvum* alone ranked fourth among identified agents (following *Campylobacter*, *Salmonella*, and *Shigella*) (14).

Viruses and protozoa present a range of transmission patterns that influence the likelihood that one or the other may be present in food and needs detection. Viruses known to be transmitted via foods are all human-specific and are shed only in feces (except for a few that are also shed in vomitus (1)). In contrast, the potential food-borne protozoa present several host patterns: *Cyclospora cayetanensis* and *E. histolytica* are essentially human-specific; *C. parvum* and *G. lamblia* are shed in feces and are transmissible from person to person but are more often harbored by nonhuman species (with some possible exceptions); and *T. gondii* is associated with cat feces or with undercooked meat and is probably not transmissible from person to person under any circumstance. These properties are significant to find how food may become contaminated with the pathogens of concern. In contrast to the situation in areas where night soil fertilization is practiced, contamination of foods by human-specific viruses and protozoa in most developed countries results from contact with the unwashed hands of infected persons or with water that has been inadequately treated after being used to carry human feces. The possibility of *Cryptosporidium* or *Giardia* contamination of vegetables via manure or of *T. gondii* in garden soils from buried cat feces may lead to very different associations of the pathogens with the food. No food other than bivalve mollusks is known to be able to concentrate pathogens from the environment; and none of these pathogens can multiply in soil, water, or even cells of unsusceptible animals. Therefore, a viral or protozoan contaminant of food can only retain its infectivity until the food is eaten. If the final test method to be applied is capable of distinguishing between infectious and inactivated pathogens, the sampling and sample processing methods must be selected so as not to inactivate pathogens that were infectious at the moment of sampling.

Peroral Infectious Dose. There is a general perception that a test for a food-borne pathogen is adequately sensitive if, and only if, it is capable of detecting a single infectious dose or less in a serving-sized sample of the food. Test sensitivity is governed by the size of the food sample, the efficiency with which the pathogen is extracted from the food and concentrated to a volume that can be tested, and the sensitivity of the test that is finally applied.

This approach of defining adequate sensitivity is inherently reasonable but is complicated by some further considerations: (*1*) There are no generally agreed-upon units of measure for most of these agents. Protozoan cysts and oocysts can generally be counted on a microscope, but the counting techniques do not ordinarily include a viability-infectivity criterion. It is not generally feasible to count viral particles, so those that can be quantified in cell culture are usually enumerated in plaque-forming units, which may each be equivalent to approximately 100 particles. (*2*) Distribution of the agent in the food is likely to be very heterogeneous, whereby one serving may

contain little or none, while another contains at least enough to cause an infection. (*3*) Although it is usual to speak of infectious doses, what is meant often is a dose that causes illness. The likelihood that infection and illness will result from a given infectious dose is a function of many host factors, such as age, pregnancy, and impaired immunity caused by immune suppressants (say, following an organ transplant), cancer chemotherapy, or HIV infection (10). Although the aspiration to have a test that will detect one infectious dose or less per serving of food is reasonable, the selection of methods is ultimately arbitrary and incapable of scientific validation by means that are currently at hand. A peroral infectious dose of virus may well comprise 100 to 1000 physical particles, whereas 30 or less oocysts of *C. parvum* have infected human volunteers. The polymerase chain reaction (PCR), for example, can often detect smaller copy numbers of nucleic acid than these, so the onus is on sampling and processing to collect the equivalent of an infectious dose from a relatively larger sample of food and then concentrate the pathogen from the extract to harvest its nucleic acid in a volume that the PCR will accommodate. And because positive PCR results are often obtained with nonviable organisms, one would wish that the process included some means of selecting only viable agents for PCR.

Selected Food-Associated Outbreaks. A few reported outbreaks are summarized here as a basis for the selection of foods for which sample-processing methods are described. Again, mollusks and water are not included because they are covered elsewhere.

Hepatitis A

- The staff of a hospital in St. Louis, Missouri, suffered an outbreak of 28 cases of hepatitis A in 1962 (15). A worker who was apparently infected by the virus had thawed and reconstituted frozen orange juice in a manner that evidently led to contamination. She did not become ill, but her husband, who was not at the hospital, developed hepatitis at the time of the outbreak.
- Outbreaks involving 82 cases in Arkansas and 58 cases in Texas were traced to a single worker in 1978 (16). The man prepared sandwiches and salads in vegetarian health food restaurants. He was visibly ill while working at the second location.
- Food from a single bakeshop in the United Kingdom transmitted hepatitis A to 50 people in several villages in 1989 (17).
- Lettuce, apparently handled by an infected worker, transmitted hepatitis A to 110 people in Missouri in 1990 (18).
- Caviar, illegally imported from Latvia, was the apparent vehicle of hepatitis A virus that infected four people in Denmark in 1994 (19).

Norwalk-Like Virus

- In 1982, Norwalk virus was transmitted to some 3,000 people in the area of Minneapolis and St. Paul, Minn., by butter cream frosting prepared by a baker's assistant, who was ill, and later applied on a great number and variety of pastries (20).
- A small, round virus in ice, made with contaminated water, that was used to cool drinks served at a college football game and a fundraising gathering caused gastroenteritis in approximately 5,000 people in 1987 (21).
- Residents (155 illnesses, 2 deaths) and employees (28) who ate shrimp dishes at a retirement facility in the area of San Francisco Bay, California, were sickened by the Snow Mountain agent, a small, round, structured virus (SRSV) that is antigenically distinct from the Norwalk virus (22).
- Norwalk-like viruses in school lunches from a preparation center that served nine schools affected 3,236 students and 117 teachers in Toyota City, Japan (23).
- Freshly cut fruit served in a Hawaiian cruise ship transmitted gastroenteritis virus to 238 people in 1990 (24), and in another Hawaiian cruise ship in 1992, approximately 200 people served with contaminated ice were infected (25).

Cryptosporidium

- Fresh, hand-pressed apple cider, served at a school agricultural fair in central Maine in 1993, transmitted *Cryptosporidium* to an estimated 160 people, many of whom passed on the infection to others (26). Apples were collected from the ground in an orchard where calves grazed.
- Chicken salad, prepared by a woman operating a licensed day care home (including diaper changing), was the apparent vehicle in a Minnesota outbreak of cryptosporidiosis that affected an estimated 50 people in 1995 (27).
- Drinking unpasteurized apple cider, from apples that may have been contaminated by well water during washing, led to 10 or more cases of cryptosporidiosis in Connecticut and New York in 1996 (28).
- Green onions, probably contaminated in the field, may have been the source of *Cryptosporidium* that infected 54 people who attended a banquet in Spokane, Washington, in 1997 (29).

Giardia

- Salmon and a cream cheese dip, served at a school in Minnesota, led to 29 cases of giardiasis in 1979 (30). The probable source of contamination was a woman who helped prepare the food and had changed the diaper of her 12-month-old grandson, an asymptomatic shedder of *Giardia* cysts.
- In a 1988 outbreak, cold noodle salad served at a picnic transmitted *Giardia* to 13 of the 16 people who attended (31). Spices were added by hand to the noodles by a neighbor who became ill the next day; her children, who did not attend the picnic, were shedding *Giardia* cysts.
- An outbreak following a family party, in which 9 of the 25 persons became ill, was associated with the

consumption of a fruit salad (32). The woman who prepared the fruit salad had a child in diapers and a pet rabbit, both of whom were shedding *Giardia*.

- In 1990, 21 of the 108 persons who attended a church dinner in New Mexico were infected. Lettuce, onions, and tomatoes were implicated epidemiologically; however, no obvious contamination was found (33).
- An outbreak among insurance company employees in November 1990 comprised 18 laboratory confirmed cases and 9 suspected cases of giardiasis (34). Case-control studies implicated raw vegetables, sliced by a food worker infected with *G. lamblia*, which were served in the employee cafeteria.

Cyclospora

- The first reported food-borne outbreak of *Cyclospora* in nontravelers occurred in 1995 in eastern Florida. At that time, the outbreak was erroneously associated with strawberries from California (35).
- In 1996, 1,465 cases of cyclosporasis were reported in 20 states of the United States and in Canada. Some 55 events were identified as being a part of this outbreak, whose suspected vehicle was Guatemalan raspberries (36–38).
- There were 25 outbreaks in nine states in 1997, involving 1,450 cases associated with raspberries, basil, and lettuce (39–41). In some outbreaks, implicated raspberries were imported from Central Guatemala (42,43). In two events in restaurants in Florida, no raspberries were served and infection was associated with eating mesclun (a mixture of baby leaves of various types of lettuce (40)). In the outbreak in Northern Virginia, Washington, D.C., and Baltimore (39), consumption of fresh basil (believed to have been contaminated in the field) from a specific company was identified as the probable vehicle of *Cyclospora* transmission.
- In 1998, few cases were identified in the United States; but there was an outbreak in Canada in which 192 cases of cyclosporiasis in 13 clusters were associated with eating fresh raspberries from Guatemala (44). Canada permitted the import of raspberries from Guatemala that spring, but the United States did not.

Toxoplasma. Food-borne infection in man is generally uncommon and reports of food-borne toxoplasmosis are relatively few, owing to the often asymptomatic nature of infections in immunocompetent hosts. Having cats (that may eat infected birds or rodents) as pets is a major factor in the maintenance of *T. gondii* as a food-borne pathogen. On the other hand, livestock that eat feeds or hay contaminated by the feces of *T. gondii*-infected cats develop infections that eventually result in tissue cysts in edible parts of the animals. Although cooking and freezing readily kill the tissue cysts, eating raw or undercooked meat, such as pork, beef, mutton, and horsemeat, is a possible route of infection.

- It was demonstrated in an outbreak that eating inadequately cooked venison might represent an important vehicle of human toxoplasmosis infection (45).
- A family outbreak of toxoplasmosis in Australia was thought to be associated with the consumption of a homemade meat dish with raw lamb (46).
- In another family outbreak, drinking unpasteurized goat's milk was associated with the illness and *Toxoplasma* was isolated from the milk (47).
- In September 1993, there were 17 cases of symptomatic acute toxoplasmosis acquired by the ingestion of raw mutton offered during a party (48).
- Two outbreaks of acute toxoplasmosis involving eight adult patients in Korea were linked to eating undercooked pig organs. The first outbreak was owing to the consumption of a meal consisting of raw spleen and liver of a wild pig, whereas the second outbreak was owing to eating a meal consisting of the raw liver of a domestic pig (49).

Entamoeba. Amoebiasis is caused by the parasite *E. histolytica*, which is estimated to infect about half a billion people worldwide and cause about 100,000 deaths per year; but is now rare in the United States. Transmission by food and water was described in the 1940s, especially as a result of shedding by food handlers. In recent times, food handlers are suspected of causing many scattered infections. For example, in a limited outbreak of symptomatic intestinal and extraintestinal amebiasis within an Italian family, the infection was believed to be transmitted by a Philippine housemaid, who was an asymptomatic carrier of *E. histolytica* infection acquired in her native country (50). However, there have been no large outbreaks of food-borne disease.

It is apparent that a great variety of foods may be contaminated by viral and protozoal pathogens. Foods that are contaminated and then not cooked before being eaten (e.g., fruits and some vegetables) are relatively frequent vehicles.

PROBLEMS IN TESTING FOR VIRUSES AND PROTOZOA IN FOODS

Food-associated outbreaks of viral or protozoal disease are most often recognized on the basis of diagnosis in those who are ill, followed by case-control studies that implicate a food. Detection of the pathogens in the suspect foods is rare. This is partly because the incubation periods of hepatitis A and many protozoal illnesses are so long that food samples are often unavailable by the time testing is considered. Gastroenteritis caused by the Norwalk-like viruses has a short incubation period, but laboratory diagnosis of these illnesses is seldom attempted in the United States because the laboratory results do not influence the course of treatment of those who are ill.

Viruses or parasites are likely to be present in a food sample in small numbers. Failure to confirm a viral or parasitic etiology of a food-borne outbreak is therefore partially due to the lack of adequate methods to detect the causative agent. In the past few years, improved diagnostic methods have been developed, some of which are applicable to detection of these pathogens

from suspected food samples. For human enteric viruses, earlier methods were based on infectivity assays in established cell cultures and enzyme immunoassays; however, cell culture methods are not available for detection of the hepatitis A and Norwalk-like viruses, and enzyme immunoassays are relatively insensitive. For protozoa, detection procedures have included microscopic methods, such as wet mount and trichrome staining, modified acid-fast, and auto-fluorescence of *Cyclospora* oocysts, and immunofluorescence methods employing pathogen-specific antibodies. More recently, the ability to extract viral and parasitic nucleic acid and amplify by reverse transcription-polymerase chain reaction (RT-PCR) or direct PCR from clinical samples has increased the usefulness of nucleic acid-based molecular methods. With the theoretical potential to detect a single copy of the viral or parasitic genome, PCR-based methods are the most sensitive detection methods available today and are highly specific. Not only are PCR-based approaches increasingly applied in diagnosis and pathogen identification, but sequence analysis approaches for genetic analyses of isolated pathogens have also become increasingly feasible and affordable.

Assuming that molecular methods are the best available for detection of food-borne viruses and protozoa, there is a need to increase the efficiency of food sample preparation so that the results can be better and more accurately interpreted. Unlike bacterial agents, which can be enriched in specific growth medium, both viruses and parasites are environmentally inert and cannot replicate in food, water, or environmental samples. On the other hand, food-transmitted human enteric viruses and parasites are environmentally stable, can withstand many methods used to inactivate bacterial pathogens and commonly practiced food storage and processing conditions. Therefore, virtually any kind of food could serve as a vehicle for their transmission. Detection of food-borne bacteria often relies on enrichment culture and selective medium to increase numbers of the suspected pathogen and to differentiate it from nonpathogenic microflora. The lack of an equivalent to the bacterial enrichment in detection of viral and parasitic agents makes it extremely important to have a suitable method for the concentration and preparation of viruses and parasites, so that maximal sensitivity is obtained when a food sample is assayed. At present, emphases are being placed on cost- and time-effective concentration methods to improve recovery and detection limits and to prevent inhibition of the detection methods by food-related compounds.

Food-borne viral and parasitic pathogens are more frequently transmitted via contaminated water, and a number of concentration techniques, most of which are applicable to liquid foods, have been developed for the recovery of such agents from water (see other sections describing waterborne viruses and parasites). Therefore, efficient methods to recover and detect viruses and protozoa from solid foods are the greater challenges. As food contaminants, viruses and parasites differ from bacterial pathogens in that the bacteria are often inside food, whereas viruses and protozoa are usually surface contaminants. The exceptions to this norm are virus-contaminated shellfish, in which viruses are found in the edible intestinal portion, and *T. gondii* cyst-containing meats. Therefore, getting virus and protozoa out or off food, with minimal inclusion of the foodstuff, is a major concern of sample preparation.

Much of the difficulty in implementing (RT)-PCR for the analysis of food samples lies in the problems encountered during the preparation of template DNAs or RNAs from food matrices. Frequently, concentrated samples are directly used for (RT)-PCR. However, during the sample concentration steps, undefined food components are often concentrated as well and they might be inhibitory to (RT)-PCR. Substances in concentrated food samples may also interfere with other detection methods, such as immunofluorescence assays (IFAs) and tissue culture assays. Therefore, one emphasis on improving detection efficiency is to get rid of inhibitory materials present in the food samples. Currently evaluated measures include the separation of viral and parasitic agents from such inhibitory substances by the application of magnetic beads coated with antibodies, a technique known as immunomagnetic separation (IMS) (51,52). IMS-(RT)-PCR has been used for the capture of various pathogens, including *Giardia*, *Cryptosporidium* (53–55), hepatitis A virus, and other human enteric viruses (56–60). Alternatively, the inhibitory effects may be reduced by the use of additives. For example, detection of enteric viruses in shellfish was found to be obstructed by the presence of RT-PCR inhibitors and such inhibitors can be removed by guanidinium isothiocyanate extraction (60,61).

EXTRACTION AND CONCENTRATION OF VIRUSES FROM FOOD

Because generally there are no specific techniques available to detect viral and parasitic pathogens in foods, methods are adapted from procedures that have been proven useful in detecting such agents from clinical samples. Detection of the viral and parasitic food-borne pathogens from foods is usually more difficult than their detection in clinical samples because the number of pathogens present in food is much lower; and detection efficiencies are widely variable, depending on the contamination level and concentration techniques.

The choice of extraction method depends not only on the type of pathogens to be tested, but also on the type of foods from which the pathogens are to be detected. Generally speaking, methods used for extraction of parasitic agents are not appropriate for extraction of viruses, and vice versa. This is mainly due to their difference in physical characteristics: during gravity centrifugation, protozoa are in the sediment, whereas viruses are in the supernatant; in filtration, the pore size of the filter is usually smaller than the size of a protozoa but larger than the size of a virus particle, so protozoa are retained on the membrane, whereas viruses are in the filtrate.

Sampling and Sample Size

Steps in processing a sample for virus testing generally comprise liquefaction (if the food is not already a liquid),

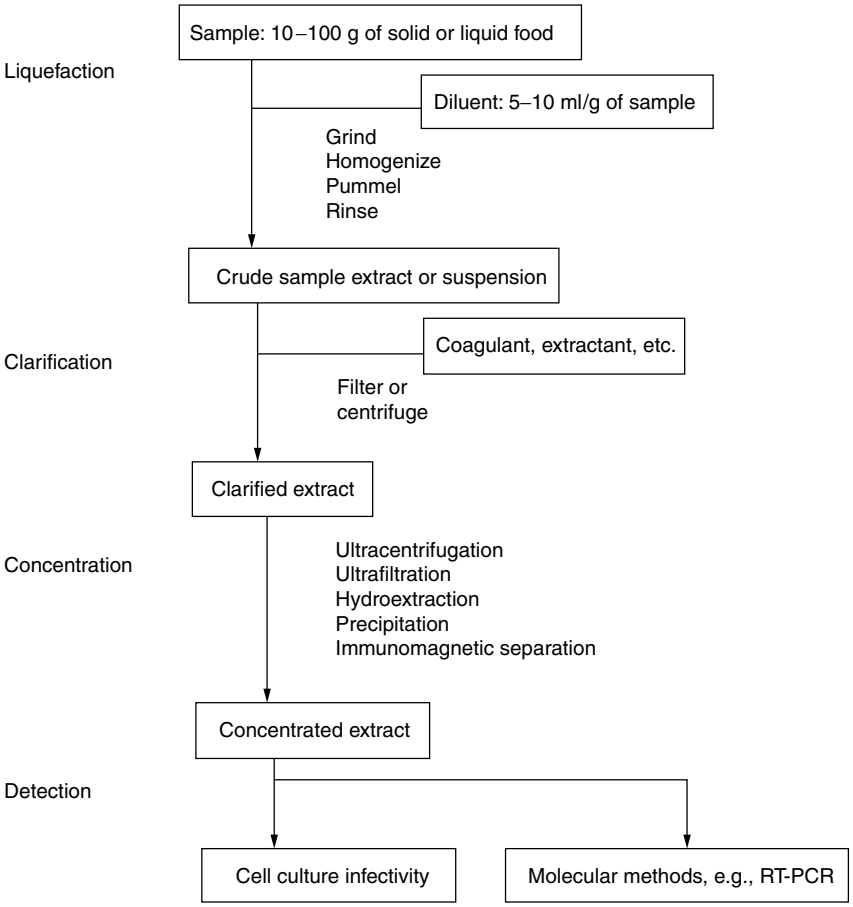

Figure 1. Extraction and concentration of viruses from food — flow diagram.

clarification, and concentration (Fig. 1). The size of a sample for virus extraction might be chosen on the basis of the probable serving size of the food and the desirability of being able to detect a human infectious dose therein. In fact, sample sizes have been more usually based on expediency, with quantities of 10 to 100 g being usual. It may be desirable to make a composite sample, assuming that enough is known of the probable distribution of the virus in the food to make such a decision.

Liquefaction

If contamination is likely to be limited to the surface of the food, larger samples may be processed, using methods that recover virus with minimal inclusion of the food in the resulting suspension. Published methods for recovering viruses from the surfaces of fruits and vegetables (62–68) are fairly old. Devices such as the Stomacher® and Pulsifier® probably deserve testing in these applications. Methods for recovering viruses from milk have also been described (68,69).

If the virus contaminant is thought likely to be within a solid food, the sample will have to be liquefied. This has most often been done with a high-speed homogenizer (70), taking precautions against overheating, although a sonic apparatus might also be used. Laboratories that process small numbers of samples might even resort to a mortar and pestle with sterile sand. The liquid in which the food is to be suspended is usually an alkaline buffer, at a ratio of 5 to 10 ml per gram of sample. Because the food solids will need to be removed from the sample suspension, it is important not to reduce them to a size that complicates this. The most common approach is to keep the pH of the suspension at or above neutrality, to discourage adsorption of the virus to the food solids.

Clarification

Clarification is intended to remove food solids from the sample suspension to enable concentration of the remaining fluid and, ideally, to remove substances that are toxic to cell cultures or that may inhibit RT-PCR test procedures. Bacteria are often removed at this stage as well. Clarification may be done by filtration or centrifugation, perhaps aided by the polycation sewage flocculent, Cat-Floc® (Calgon Corp., Pittsburg, Pa.), at a final concentration of 0.1 to 0.5% (67,71,72). Freon TF® (1,1,2-trichloro-1,2,2-trifluoroethane) (E. I. DuPont de Nemours, Wilmington, Del.) extraction has also been used for this purpose (70).

If the suspension has been sufficiently clarified, it may be possible to remove bacteria at this point by filtration at 0.22 to 0.45 μm porosity, taking care that the virus is not adsorbed to the filter (73). Alternatives to filtration include treatment with antibiotics, chloroform, or ether, in instances where the sample extract is still somewhat turbid.

Concentration

Concentration of the sample is intended to reduce the sample volume to the amount required for testing by the chosen method. Cell cultures usually accommodate a volume of about 0.02 ml per square centimeter of monolayer surface, although volumes as large as 1.4 ml cm^{-2} may be tested under some circumstances (74). Volumes to be tested by RT-PCR are not likely to exceed 1 ml — usually much less.

Early concentration methods employed hydroextraction (dialysis against polyethylene glycol) (70,75), preparative ultracentrifugation, or ultrafiltration (67,71). These are relatively expensive and labor-intensive. More recently devised methods are based on precipitation of the virus by adsorption of the virus to the precipitated protein under acidic conditions (76). The selection of the protein to be used for this purpose is critical, as some may inhibit expression of the virus in cell culture. Polyethylene glycol can also be used to precipitate viruses from food extracts, for the purpose of concentration (77–83). Other methods of final concentration and of removing substances inhibitory to RT-PCR entail addition of the cationic detergent cetyltrimethylammonium bromide (83–86) or of Pro-Cipitate, a protein-precipitating agent that has also been used successfully to remove inhibitors of RT-PCR (77,79,87).

EXTRACTION AND CONCENTRATION OF PROTOZOA FROM FOOD

Most of this section will necessarily deal with the recovery of *Cryptosporidium* and *Giardia*, inasmuch as methods for other protozoa are exceedingly limited. A number of methods have been developed for the concentration of protozoa from feces and in environmental samples. Traditional methods to concentrate parasitic agents from water samples (including surface, drinking, and irrigation water) are based on membrane filtration or cartridge filtration, and subsequent centrifugation of the retained materials (88,89). For the concentrations of smaller sample volumes, simple centrifugation or the flocculation technique can also be used (90). On the other hand, *Cryptosporidium* and *Giardia* in stool specimens are frequently concentrated via gradient centrifugation (Sheather's sucrose, sodium chloride, Percoll's solution, etc.). It is possible to process fluid foods suspected of being contaminated with (oo)cysts in a similar manner to concentration from water samples, whereas solid foods must be done in other ways.

Extraction from Liquid Foods

Contaminated apple juice (cider) and raw milk (and some milk products) are identified examples of potential vehicles of *Cryptosporidium* and *Giardia*. Although it is possible to use simple centrifugation or filtration-centrifugation to concentrate (oo)cysts in a similar manner to extraction from water samples, the presence of other food components makes it necessary to add extra processing steps for the extraction of (oo)cysts from liquid foods (Fig. 2). Deng and Cliver (91) investigated oocyst recovery from milk and milk products using a Sheather's sucrose flotation-based procedure. While low fat milk and thawed ice-cream were directly centrifuged, an ether-extraction step was added

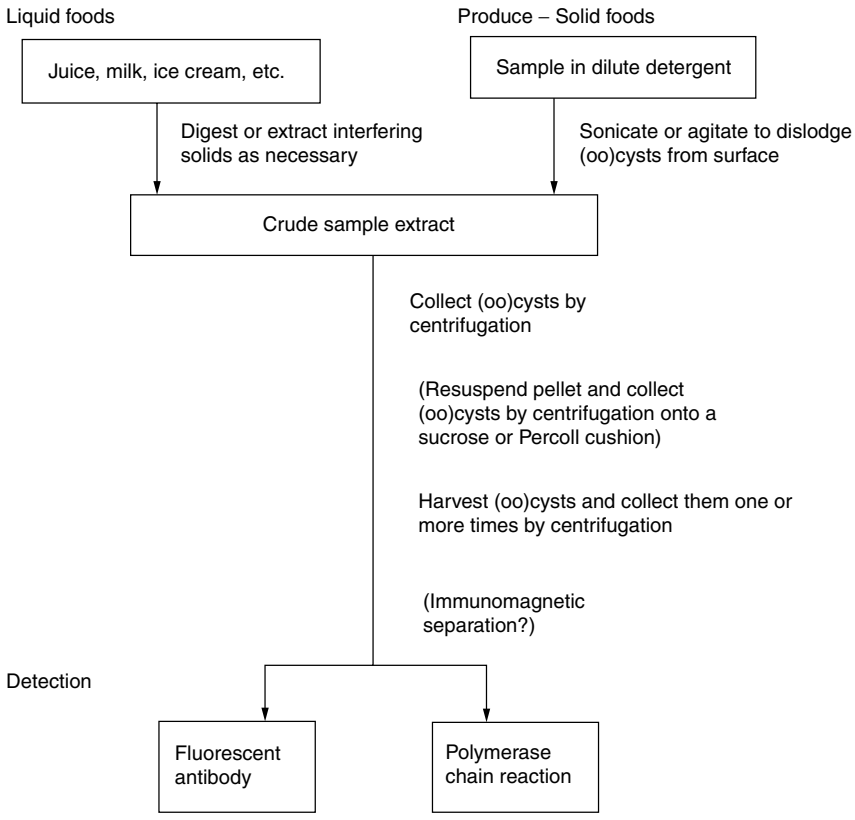

Figure 2. Extraction and concentration of protozoa from food — flow diagram.

before centrifugation for yogurt made from low fat milk. Following centrifugation, the pellet was overlaid over a sucrose solution, with specific gravity 1.20 $g\,ml^{-1}$, and centrifuged for 10 minutes at $1,000 \times g$. The interlayers containing oocysts were pooled, washed, and further centrifuged. The average respective recoveries were 82% from 100 ml of low fat milk, 61% from 100 g of yogurt made from 1% fat milk, and 62% from 100 g of ice cream.

A problem encountered with such filtration-centrifugation and gradient-centrifugation procedures is that not only the protozoa, but also other types of particles, are concentrated. In addition to decreasing extraction efficiency, the presence of such food components could be inhibitory to IFA and PCR-based procedures, so additional treatment may be necessary to improve the ultimate detection efficiency.

Laberge and coworkers (92) developed a PCR assay for the detection of *C. parvum* oocysts from raw milk. The raw milk samples were treated with trypsin and Triton X-100 at 50 °C, to remove milk proteins and other inhibitory substances before the samples were processed by simple centrifugation and examined by PCR. This treatment enabled the detection of 1 to 10 oocysts in 20 ml of experimentally contaminated raw milk.

An alternative to get rid of inhibitory food components is to use specific IMS. Deng and Cliver (93) compared efficiencies of different methods for extraction and detection of *C. parvum* oocysts from apple juice. When an IMS step was added following Sheather's sucrose flotation, higher detection efficiencies were consistently attained. In a similar study, PCR following IMS was able to detect 10 oocysts in 100 ml of apple juice or low fat milk (94). In such a procedure, the preconcentrated (by simple centrifugation or gradient centrifugation) sample reacts with *C. parvum*-specific antibody and is then incubated with paramagnetic beads coated with a secondary antibody against the *C. parvum*-specific antibody. The magnetic beads-oocyst complex is then separated from other food components by means of a magnetic field.

Extraction from Produce

Vegetables, including cabbage, tomato, lettuce, cucumber, carrot, and radish, can play an important role in the spread of *Cryptosporidium*, *Giardia*, *Cyclospora*, and *Entamoeba*. Fruits, especially raspberries and blackberries, have been associated with a number of outbreaks of cyclosporiasis. In nonindustrialized countries, the use of excreta as fertilizer is a common cause of contamination in the field. In addition, water used for irrigation, from rivers that receive industrial, domestic, and agricultural wastewater — especially during the rainy season — could be highly contaminated. Salad mix could also be contaminated when prepared by an infected food handler.

Methods for extraction of protozoa from produce are relatively simplistic. Protozoa are usually recovered by the rinse solution method (95). This involves dislodging (oo)cysts from the surfaces of the vegetables or the fruits by washing with a detergent-containing saline solution and sedimentation or centrifugation of the organisms from the cleaning water. The concentrate can then be used for conventional staining, IFA, or PCR-based assays. Such methods have been used in a few surveys on risk of protozoa-contaminated vegetables (96–99). In the U.S. Food and Drug Administration procedure (97), a 1-kg portion of fruit or vegetable is divided into 200-g subportions and sonicated for 10 min in a sonic cleaning bath with 1.5 l of detergent solution containing 1% sodium dodecylsulfate and 0.1% Tween 80. The sample is then thoroughly drained and the wash water is collected and centrifuged for 15 minutes at $1,500 \times g$. When the sediment contains a larger quantity of extraneous matter, it is further concentrated by layering over Sheather's fluid and centrifuged for 15 minutes at $1,500 \times g$.

While the extraction procedure is fairly straightforward, unsatisfactory efficiencies have been reported. This problem is mainly associated with the inability to wash (oo)cysts off produce surfaces, as they appear to stick to the surface, especially on rough fruit and leaf surfaces. Another reason for the lack of efficiency is that only a small percentage of the crop or batch can be sampled. To overcome these, methods capable of efficiently dislodging protozoa from food-contact surfaces without dislodging food solids are required. Alternatively, it may be appropriate to sample irrigation waters or to develop a large batch-rinse technique and sample the rinse water (99).

Extraction from Solid Foods

For *Cryptosporidium*, *Giardia*, *Cyclospora*, *Entamoeba*, and not for *Toxoplasma*, contaminated foods are usually ready-to-eat foods contaminated by the hands of a food worker. When the contaminating pathogen resides on the surface of food, the key point is to dislodge the organism from the food surface without disturbing the food material; one might try shaking the food and diluent in a plastic bag, or use a Pulsifier®. Under other conditions where the whole sample has to be tested, the sample is usually homogenized and can be processed similarly to oocyst extraction from fecal and soil samples. If suspended food solids and other materials may interfere with large volume collection and the detection process based on microscopic examination, IFA, and PCR, IMS could be applied. It should be pointed out that because an antibody is not yet available for *Cyclospora*, IMS does not yet have an application with this agent.

In the case of *Toxoplasma*, recent epidemiological studies have associated acute toxoplasmosis in pregnancy with ingestion of both raw meats and ready-to-eat cured meat (100). Detection of tissue cysts in these foods would probably proceed approximately as in histopathology.

Entamoeba histolytica cysts can be recovered from contaminated food by methods similar to those used for recovering *Giardia* cysts and *Cryptosporidium* oocysts from feces. Filtration is probably the most practical method for recovering cysts from liquid foods, while flotation and sedimentation could be used to recover cysts from solid foods. It is important to differentiate *E. histolytica* cysts from cysts of other, nonpathogenic amoebae (101).

VIABILITY (INFECTIVITY) CRITERIA IN TESTING

Viruses and protozoa in the environment and foods may be inactivated by a number of agents, including heat, UV

irradiation, chemical action, or other biological factors. Therefore, when detecting viruses and protozoa from a food sample, it is important to consider the viability of the virus particles and (oo)cysts and hence their infectivity to humans. This should also be taken into consideration while new methods and procedures are developed for detection of viral and parasitic pathogens from foods.

Viruses

Traditionally, viruses have been detected by their effects on cells. The cell culture propagation procedure, namely *plaque assays* and *cytopathogenicity assays*, can be used to enumerate viable polioviruses, adenovirus, and to a lesser extent, rotaviruses. However, they are ineffective for the detection of hepatitis A virus (HAV), Norwalk virus, and other SRSVs. Only few laboratory-adapted HAV strains can effectively propagate in cell lines and cause cytopathic effects (CPEs), so as to permit quantitative assay. Wild-type HAV strains usually do not grow well in cell culture or require long incubation periods (many days or even weeks). In addition, most HAV strains do not cause CPE, so a plaque assay is unavailable. There is at least a possibility that HAV, and perhaps other food-borne viruses, do replicate silently in cell cultures. If this is so, detection of viral products, such as replicative intermediate forms of RNA or viral coat protein, in the cells may serve as an infectivity-dependent criterion of detection. There has been little or no success in developing cell or tissue culture systems capable of supporting the growth of Norwalk and related SRSVs. Cell culture propagation assays are readily available for poliovirus, and it has been suggested that vaccine strains of poliovirus may serve as indicators of the presence of other pathogenic viruses for which assay procedures are unavailable.

One major limitation of molecular-based methods in virus detection is that they cannot differentiate infectious from noninfectious viral particles. During inactivation, their capsid proteins that protect the genomic structure could be damaged. While such viruses are not infectious due to the loss of functions in host recognition and cell invasion, their RNA may remain intact for an extended period and may be amplified by RT-PCR. For example, rotavirus lost its infectivity after being exposed to ethanol, but its RNA could be amplified by RT-PCR (102). Similarly, heat and detergents can effectively destroy the infectivity of poliovirus without any significant effect on its detection by RT-PCR (103). In another study, HAV maintained in sterile artificial seawater was amplified by RT-PCR for 232 days, even though it was infectious in cell culture for only 35 days (104). Heat-inactivated HAV is also readily detected by RT-PCR — heating HAV at 99 °C for 5 minutes, which inactivates the virus, is a routine means of freeing its RNA for RT-PCR (57).

To overcome this, a few methods combining RT-PCR and antigen capture (AC) of virus particles have been developed, especially via the use of magnetic beads coated with antibodies (56–60,105,106). Because such methods detect the presence of viral genomes that are packed in capsid viral proteins, and not just the presence of proteins or of nucleic acids, they are more informative and provide a better correlation with viral infectivity than detection methods based on RT-PCR or antigen capture alone. Alternatively, some noncytopathic human enteric viruses could be detected via direct in vitro amplification of their nucleic acids (77). However, it is to be noted that such methods are unable to differentiate infectious viral particles from noninfectious ones with unchanged antigenicity and intact genome.

Protozoa

In the cases of protozoa, the morphology of parasites, surface antigen, or nucleic acids could be preserved even though (oo)cysts are inactivated. Therefore, traditional light microscopy, IFA, or PCR cannot distinguish between viable and nonviable (oo)cysts. However, unlike viruses, viability of protozoa could be assessed in vitro by different means, including in vitro excystation, vital dye staining, tissue culture assay, and RT-PCR to detect messenger RNAs that are present only in viable cells.

Giardia. Usually, the viability of *Giardia* cysts is determined in vitro by excystation and by fluorescein diacetate (FDA) and propidium iodide (PI) staining. In the excystation method, viable cysts can excyst to produce trophozoites that are morphologically distinct and can be propagated in artificial medium. In the FDA/PI staining procedure, nonviable cysts will be stained fluorescent red because PI cannot penetrate the compact membrane of a viable cell. FDA, on the other hand, is a much smaller molecule that can pass through the cell membrane freely and, only in viable cells, is metabolized from nonfluorescent to another form with green fluorescence. Both methods are indicative of cyst infectivity in animals (107). As the in vitro excystation is not included in routine detection procedures due to the rather complicated excystation induction treatment, Dowd and Pillai (108) developed a viability test in conjunction with indirect fluorescent antibody staining in which a *Giardia*-specific antibody is used for organism identification while PI is included as a viability indicator.

Mahbubani and coworkers (109) proposed a RT-PCR procedure to discriminate live and dead cysts by measuring the giardin mRNA level before and after the induction of excystation. Abbaszadegan and coworkers (110) developed a method to amplify mRNA of heat-shock protein after heat shock. A similar procedure was developed for the detection of viable *Giardia* cysts from water samples (111) and it may be used for food samples as well.

Cryptosporidium. Similar to *Giardia* cysts, the viability of *Cryptosporidium* oocysts can be assessed by in vitro excystation and by vital dye staining (112). Despite the fact that both methods, when compared to animal infectivity assay, overestimate oocyst viability, they have been widely used in investigating oocyst survival under environmental conditions and inactivation by different modes (113,114). They have also been included in various detection procedures.

The method developed by Dowd and Pillai (108) is also useful for *Cryptosporidium* detection, in which a *Cryptosporidium*-specific antibody is used for organism

identification and PI serves as a viability indicator. The Environmental Protection Agency method (EPA method 1663, 1999) combines DAPI/PI staining with direct fluorescent antibody staining so that oocyst viability could be more accurately determined.

A number of PCR-based methods capable of differentiating live from dead oocysts have been developed; all of them include an excystation treatment step. Filkorn and coworkers (115) combined a DNA digestion step and an oocyst excystation protocol prior to PCR. Because digestion with DNase I eliminated free DNA released from disintegrated oocysts and sporozoites, after excystation only the DNA from intact viable oocysts was available for amplification. Wagner-Wiening and Kimmig (116) developed a similar procedure in which an excystation protocol was applied before DNA extraction so that only DNA of sporozoites released from viable oocysts was amplified. In an IC-PCR procedure developed by Deng and coworkers (54), a simple heat-treatment was used to extract DNA from sporozoites and partially excysted oocysts following excystation treatment.

In addition to excystation-PCR-based methods, an RT-PCR procedure was developed to detect the mRNA heat-shock protein (111). Recently, it was suggested that mRNA of betatubulin could be a marker of oocyst viability (117).

Progress in in vitro cultivation of *Cryptosporidium* made it possible to detect oocysts based on their ability to cause cell infection. Owing to sensitivity considerations, such methods are currently integrated with PCR (118,119). Because the ability of oocyst to cause infection in a cell line more precisely indicates oocyst infectivity to humans, such methods are more informative than methods using excystation or membrane permeability as a viability criterion.

Cyclospora, Toxoplasma, and Entamoeba. Because cyclosporiasis is often (or strictly) associated with consumption of produce, whenever oocysts are detected from such a food item it provides sufficient information on the possibility of that particular food item as a vehicle of *Cyclospora*. However, sporulation and excystation can be used for viability determination. There are now a few PCR procedures available for detection of oocysts, so methodologies similar to those being employed for *Giardia* and *Cryptosporidium* could be useful.

For *Toxoplasma*, tissue cysts in raw meat should always be viable. For oocysts from contaminated water, an aeration procedure (so that oocysts will sporulate) followed by inoculation of sporulated oocysts into 25 to 30 g mice and examination of mouse tissue sections has been described. Another approach to detect cysts from raw meat or milk entails feeding it to a cat and looking for oocyst shedding.

INTERPRETATION OF RESULTS

It seems clear that both the procedures for processing food samples described here and the detection methods for viruses and protozoa that are described in other sections are complex and demanding. Further, none of the procedures has really been standardized, in the sense of having undergone interlaboratory comparative testing and receiving a sanction by some professional organization. Government agencies and many nongovernment laboratories have devoted a great deal of effort to developing more sensitive methods for detecting food-borne viruses and protozoa. As it is still difficult to obtain samples for testing, most of the experiments have been performed with experimentally contaminated foods.

A well organized laboratory that uses a full set of positive and negative controls can give credence to any positive test result, except that the agent detected may not have been infectious when the sample was collected (unless special criteria are applied). However, negative results probably prove little — either because the sample processing and testing methods were inadequate or because the sample tested was not representative of the entire lot of food, only some of which was contaminated. Diagnostic testing of ill persons and epidemiological investigation techniques seem likely to be critical for years to come.

Meanwhile, there is an abiding effort to identify indicators or surrogates for viral (or even protozoal) pathogens, other than bacterial indicators of fecal contamination. Indicators, found in a field sample, should convey a high probability that human enteric virus is also present. Surrogates would be used to evaluate unit processes that were supposed to eliminate viral contaminants from food. Bacteriophages are undergoing testing in these roles; although none has yet proven either an optimum indicator or surrogate, they are certainly the brightest hope in sight. Unfortunately, indicators of protozoal contamination are not in prospect; surrogates, such as *Cryptosporidium* species that do not infect humans, are a possibility if there were specific antiprotozoal processes to be evaluated.

CONCLUSION

This section has addressed processing of food samples, other than shellfish, for recovery of viruses or protozoa that would be detected by methods described in other sections. The most likely application of such methods would be in investigation of outbreaks that had been identified on the basis of diagnoses of the same illness in several human patients. Given the long incubation periods of some of these illnesses, representative food samples might well not be available for testing by the time the outbreak was recognized. The methods surveyed include steps to separate the pathogen from the food into a fluid suspension, remove food solids and other substances that might interfere with detection, and reduce the volume of the sample to what the detection method can accommodate. Ideally, the overall sensitivity of the test should allow detection of one peroral, human infectious dose or less per serving of food.

Recovery methods might also be applied in validating food processes that were designed to inactivate viruses or protozoa, using the pathogens themselves or carefully

selected surrogates. Most studies by which the methods described were developed entailed testing of experimentally contaminated foods, which means that little has been learned of the location or distribution of the pathogens in accidentally contaminated foods. Given the sporadic, infrequent incidence of these pathogens in foods, this situation is unlikely to change in the near future.

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VIRUSES: ASTROVIRUSES. See ASTROVIRUSES

VIRUSES: CALICIVIRUSES. See HUMAN CALICIVIRUSES: BASIC VIROLOGY AND EPIDEMIOLOGY

VIRUSES, CONCENTRATION AND DETECTION.

See ENTEROVIRUSES IN WATER: CONCENTRATION AND DETECTION

VIRUSES: ENTEROVIRUSES.

See ENTEROVIRUSES: BASIC BIOLOGY AND DISEASES

VIRUSES: HEPATITIS VIRUSES.

See HEPATITIS VIRUSES (HAV-HEV)

VIRUSES IN DRINKING WATER AND GROUNDWATER

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VIRUSES

Viruses are the smallest and most basic of known life-forms. They consist only of nucleic acid and a protein shell (or capsid), with no reproductive machinery. Their simple genetic system consists of either single-stranded or double-stranded DNA or RNA. Because of their inability to autonomously reproduce, they must take over a living cell and use the cell's reproductive mechanism in order to replicate and continue their parasitic life. After replication, and the subsequent death of the host cell, viral particles are spread to neighboring cells, resulting in the infection of the individual.

VIRUSES IN THE ENVIRONMENT

Viruses that replicate in animal cells and are subsequently shed from the gastrointestinal tract are known as enteric viruses. More than 140 different enteric viruses are known to infect humans, which are principally transmitted by the fecal-oral route. Enteric viruses are excreted in the feces of infected individuals and may directly or indirectly contaminate water intended for drinking. These viruses are excreted in high numbers per gram of feces of infected individuals and are commonly isolated in domestic wastewater, even after disinfection. Once in the environment they can survive for long periods of time, even months under the right conditions. The following groups of viruses have been identified as sources of waterborne disease outbreaks or that have the potential to cause outbreaks; enteroviruses, rotaviruses, Hepatitis A & E viruses, caliciviruses, adenoviruses, astroviruses, reoviruses, and others. These viruses infect the gastrointestinal and/or respiratory tracts and are capable of causing a wide range of illness including diarrhea, fever, hepatitis, paralysis, meningitis, and heart disease. Despite causing a variety of illness in human, many of viral infections are remarkably asymptomatic (with no clinical symptoms). However, viral multiplication in an infected individual occurs, and virus particles are shed in the environment.

Groundwater of the United States may be subjected to fecal contamination from a variety of sources, including sewage treatment plant effluents, on-site septic waste treatment discharges, land runoff from urban, agricultural, and natural areas, and leachates from sanitary landfills. Under proper conditions, viruses have been observed to travel more than 100 meters through the subsurface (1). Because of their smaller size, viruses (23 to 80 nm) are transported further in groundwater than bacteria (0.5 to 3 μm) or protozoan parasites (4 to 15 μm). Thus, the occurrence of viruses in groundwater in the absence of coliforms may not be surprising. Information on the occurrence of viruses in groundwater is largely limited to studies in which land application of domestic wastewater has been practiced (2), outbreak investigations (3), or when viruses have been purposely added (1). Surveys on viruses in groundwater have been limited because of the need to concentrate large volumes and the expense of using animal cell culture. However, the development of molecular based methods for virus detection in groundwater allows for application of less expensive methods to detect a wide range of viral contamination (4,5).

Evidence for a possible route of fecal contamination of surface and groundwater is provided by the detection of enteric viruses in either surface or groundwater, and the continued occurrence of viral waterborne disease. During early 1970s, there have been several outbreaks of viral gastroenteritis. In Michigan, restaurant patrons who drank unchlorinated well water became ill within 30 hours (45). Poliovirus 2 was recovered when 2.5 gallons of water were concentrated by ultra centrifugation. In a migrant labor camp in Dade County, Florida Hepatitis A outbreak was reported during March 1975. Water samples from that camp yielded hepatitis A, echovirus, and poliovirus. Mosley (1967) summarized 50 Hepatitis A outbreaks attributed to contaminate drinking water. Craun and coworkers (1976) reported 13 outbreaks of waterborne Hepatitis A that affected 351 people during 1971 to 1974. The Environmental Protection Agency (EPA) and Centers for Disease Control (CDC) have maintained a database of disease outbreaks in the United States since 1971 (6). The database is formulated on the responses to a voluntary reporting by state and local public health officials, but a large number of waterborne outbreaks may not be recognized or reported (7). Between 1971 and 1994, 650 drinking waterborne outbreaks of disease and 569,754 cases of illness were reported in the United States, with 58% of these outbreaks associated with groundwater sources and 33% associated with surface water sources (6). Eight percentage of all reported outbreaks (6) were due to enteric viruses (hepatitis A virus, Norwalk virus, and rotaviruses). It is suspected that many waterborne disease outbreaks for which no etiological agent was identified (about 47% of all reported outbreaks) may be caused by viruses because of the limitations of current detection

methodology and the failure to collect and analyze clinical specimens for viruses. Analysis showed that viruses caused 10% of the groundwater outbreaks, whereas 4% of the surface water outbreaks were viral (8).

Examination of waterborne outbreak data for public groundwater systems (community and noncommunity) for 1971 to 1994 showed that three types of source water contamination were involved: untreated water, disinfected water, and filtered systems (Table 1). Eighty-one percent of these 356 groundwater outbreaks were related to source contamination with 45% attributed to untreated water and 35% to improperly disinfected water. Community systems using groundwater experienced 113 outbreaks (Table 2), whereas noncommunity systems using groundwater experienced 243 outbreaks (Table 3). Thirty-one and sixty-two percentage of outbreaks in community systems were traced to distribution system and source water contamination, respectively. The vast majority (89%) of outbreaks in noncommunity systems were associated with source water contamination (7% were related to the distribution system). Among recognized causes of groundwater viral outbreaks in public water systems during 1971 to 1994, hepatitis A virus was associated with 8 community, and 9 noncommunity systems; whereas Norwalk virus was identified as the etiologic agent in 4 community, and 12 noncommunity outbreaks (8).

Table 1. Cases of Waterborne Disease Outbreaks, Public Water Systems Using Water Sources, 1971 to 1994

Type of Contamination	Total Number of Outbreaks	Percentage of Total
Source contamination	287	81%
Untreated groundwater	158	45%
Disinfected groundwater	126	35%
Filtered groundwater	3	1%
Distribution system contamination	50	14%
Inadequate control of chemical feed	8	2%
Miscellaneous, unknown cause	11	3%
Total	356	100%

Source: Tables 1 to 3 compiled by Gunter Craun and Rebecca Calderon, EPA,RTP,NC.

ENTERIC VIRUSES IN WATER

The enteroviruses (poliovirus, Coxsackie A and B viruses, echovirus) can cause a variety of illnesses ranging from gastroenteritis to myocarditis and aseptic meningitis (9). Many studies have documented the presence of enteroviruses in both raw and (occasionally) treated drinking water (1,10), wastewater (11) and sludge (12). Enteroviruses in the environment pose a public health risk because these viruses can be transmitted via the fecal-oral route through contaminated water (12), and low numbers can initiate an infection in humans.

Table 2. Cases of Waterborne Disease Outbreaks in Community Water Systems Using GroundWater Sources, 1971 to 1994

Type of Contamination	Outbreaks in Community Systems	Percentage of Total
Source contamination	70	62%
Untreated groundwater	31	27%
Disinfected groundwater	36	32%
Filtered groundwater	3	3%
Distribution system contamination	35	31%
Inadequate control of chemical feed	5	4%
Miscellaneous, unknown cause	3	3%
Total	113	100%

Table 3. Cases of Waterborne Disease Outbreaks in Noncommunity Water Systems Using Groundwater Sources, 1971 to 1994

Type of Contamination	Outbreaks in Noncommunity Systems	Percentage of Total
Source contamination	217	89%
Untreated groundwater	127	52%
Disinfected groundwater	90	37%
Filtered groundwater	0	0%
Distribution system contamination	15	7%
Inadequate control of chemical feed	3	1%
Miscellaneous, unknown cause	8	3%
Total	243	100%

Note: The source water could not be identified for 21 community and 6 noncommunity systems experiencing outbreaks; therefore, these systems are not included in Tables 1 to 3.

Rotaviruses are the leading cause of acute infantile gastroenteritis and diarrhea-related infantile death (13). The virus has also been associated with diarrhea outbreaks among the elderly and among immunocompromised patients (14). Rotavirus group A has been documented as a cause of waterborne outbreaks in humans (15). The virus has been isolated from humans, monkeys, cattle, sheep, mice, cats, dogs, other mammals, and from chickens and turkeys. The organism has been detected in fresh water and sewage (16). Although the various strains of rotavirus are usually associated with a specific species, reports have documented infections through interspecies transmission of the virus; including a human infection by a bovine strain of rotavirus (17). Additionally, recent work has shown that an isolated rotavirus surface protein, alone, can produce diarrhea in mice (14). Rotavirus infections (from all sources) are responsible for an estimated 3.5 million cases of diarrhea and 20 deaths in infants and young children in the United States every year (18). There are an estimated 500,000 physician visits and 50,000 hospitalizations yearly related to rotavirus from all source of transmission including water and food. In addition, rotavirus accounts for 30 to 50% of hospitalizations in the United States for diarrhea in children younger than five years (19).

Hepatitis A virus (HAV) is an important waterborne virus because of the severity of the disease it may cause in susceptible individuals. HAV is the cause of acute infectious hepatitis and was the first enteric virus identified as associated with a waterborne disease outbreak in the United States (20). The virus was shown to survive more than four months at both 5 °C and at 25 °C in water, wastewater, and sediments (20). Hepatitis A is a major cause of acute gastroenteritis, and its symptoms may be the most serious of those caused by the enteric viruses. In one survey, hepatitis virus was identified as the causative agent in more than 20% (68 of 322 outbreaks) of waterborne disease outbreaks in the United States from 1946 through 1980, for which a causative agent was identified (21).

The calciviruses and the Small Round Structured Virus (SRSV) groups have been implicated or suspected in several outbreaks of acute diarrheal illness. These groups are composed of members such as Norwalk virus, Snow Mountain agent, Hawaii, Taunton, Parramatta, and other viruses that are as yet unnamed (22). Shared morphological and genomic characteristics of several of these viruses, including: possession of a single-stranded RNA genome, a single protein capsid, and shared genome organizational similarities, have led to calling these viruses the Norwalk Group of viruses, with the Norwalk virus as its most typical member.

ENVIRONMENTAL WATER SAMPLING PROGRAM

Sampling program and strategies are the heart of any successful environmental microbial monitoring, which include site selection criteria, environmental sampling method, sample size, frequency of sampling and the microbial detection methodologies. A properly designed monitoring program helps in understanding the source water vulnerability and variability to microbial pathogens. The source characterization also assists water suppliers and regulators to implement strategies to better protect public health from waterborne pathogen outbreaks.

Site Selection

For the virus occurrence in groundwater sources, a proper sample collection plan is of paramount importance, which aims at gathering groundwater samples from different geographic locations and with a variety of physical and chemical characteristics.

Initially, groundwater sources can be selected at random from sites volunteered by water utilities personnel agreeing to participate in the project. Sites known to be either under the influence of surface water, which has no well log records available, or considered to be poorly constructed should be excluded. In addition, sites can be selected on the basis of their geologic characteristics to match the actual national profile for groundwater sources. The objective of such selection criteria is to ensure that the samples closely represent the various geologic formations throughout the sites used for groundwater production.

Sampling Kit

To provide consistent sampling procedures, identical sample kits should be assembled. Each kit should contain all equipment needed to collect a sample, including all hoses and connectors, a filter and a filter housing, protective gloves, reusable ice packs, sample bottles, a sample data sheet, and a detailed written protocol. The kits also need to include a flowmeter and an in-line flow-restricting device to limit the filtration rate to 4 gallons per minute. A typical sample kit is illustrated in Figure 1. In addition, to ensure a consistent water-sampling procedure a short VHS video can be professionally produced illustrating all of the details of the procedure.

Figure 1. Sampling kit. Filter Housing containing a 1MDS filter; a hose with a brass quick-connect, a backflow prevention valve and a flow restrictor; two additional hoses with a quick-connect tap connection; a brass quick-connect tap connection; pH meter; thermometer; water meter; three ice packs; two one-liter presterilized bottles; surgical gloves; sample data sheet; sample collection protocol; aluminum foil sheets; return address label with shipping instructions; and training video.

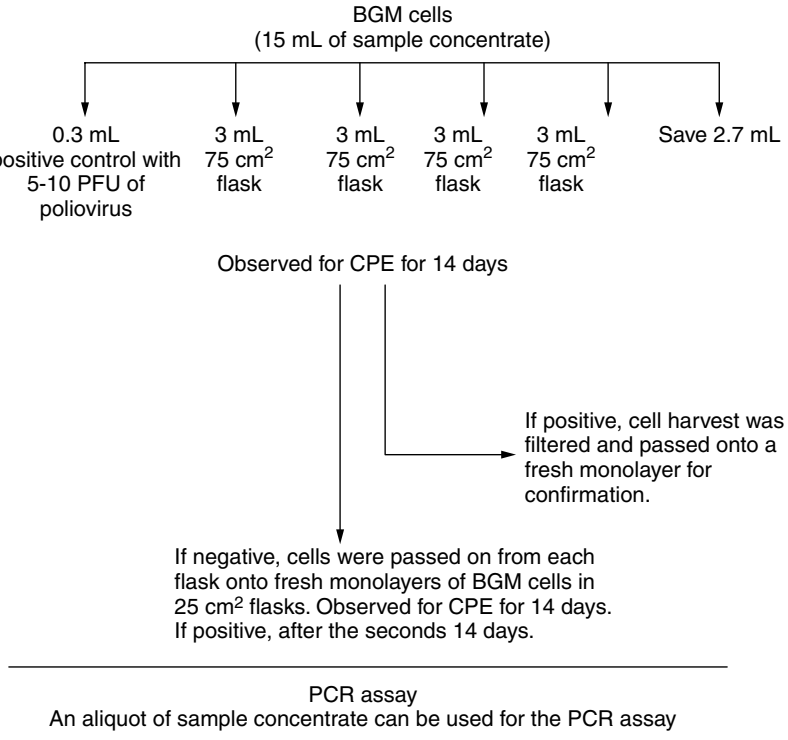

Figure 2. Cell culture assay protocol.

CONVENTIONAL CELL CULTURE ASSAY

Conventional methodology for the detection of enteric viruses from the environment relies on a few established cell lines. The Buffalo Green Monkey (BGM) kidney cell line is the most commonly used for the detection of enteroviruses in the environment (23). This cell is preferred over others, including primary cells, because it provides high sensitivity to natural isolates of enteroviruses (24). Its sensitivity can be further enhanced by pretreatment of the cells with enzymes or other substances (25). Unfortunately, the use of other cell lines is required to detect other groups of enteric viruses (26). This can greatly increase the cost and time of the sample analysis. Although the cell culture assay can detect infectious viruses in environmental samples, without additional tests, no determination can be made as to the particular strain of virus present in the sample. Additionally, the length of time needed to detect infection in the cell culture can vary greatly, from a few days to several weeks, depending on the type and number of viruses present. Figure 2 summarizes a cell culture assay protocol. Figure 3 shows an uninfected and infected BGM cell monolayers.

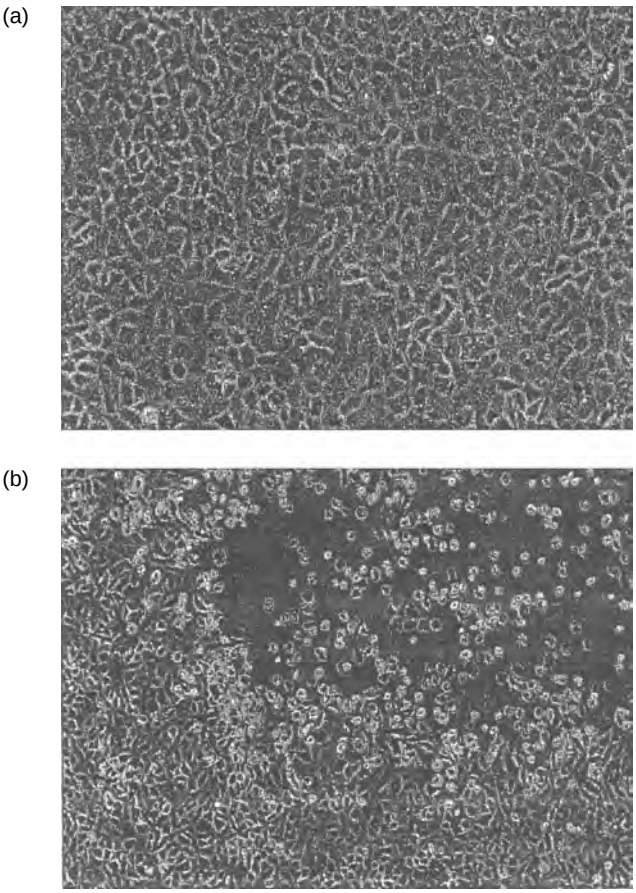

Figure 3. The figures show uninfected BGM cell culture monolayer (**a**) and an infected BGM cell monolayer exhibiting cytopathogenic effect (CPE) (**b**).

THE POLYMERASE CHAIN REACTION TECHNIQUE

Since its invention, PCR has become one of the most widely used biochemical assays. The speed, specificity, and low cost of the procedure has led to its use in fields such as criminal and pathological forensics, genetic mapping, disease diagnosis, systematics and evolutionary studies, and environmental science.

PCR can be used to amplify, to detectable levels, nucleic acids associated with low levels of pathogens in water

samples. PCR assays must be able to detect viruses after concentration from large volumes (100 to 1,500 L) of water (27). This is usually accomplished by a filter-adsorption and elution method, resulting in a concentrate containing viruses, and organic and dissolved solids. Compounds, such as humic substances, once concentrated, can interfere with the activity of the enzymes used in PCR assay.

PCR is a process in which target DNA, polymerase enzyme, and the DNA subunits are combined in a test tube and subjected to the temperature changes needed for the DNA duplication to occur. By repeating this process many times, a large amount of DNA is generated. This reaction, termed the Polymerase Chain Reaction (28,29), or PCR, can, under ideal conditions, generate millions of copies of a single DNA molecule in just 20 to 30 repetitions of the temperature cycle — each cycle requiring only a few minutes. The PCR assay, however, selectively amplifies only a portion of the target DNA for diagnostic applications. For analysis of RNA viruses in water, an enzyme called a reverse transcriptase is used to first translate the RNA to DNA. Then DNA can then be processed using the PCR technique. The combined reverse transcriptase and PCR assay is abbreviated RT-PCR.

The advantages of PCR are numerous. When compared with techniques such as cell culture for the detection of viruses, the time required for the assay can be reduced from days or weeks to just a few hours. Both the initial and recurring costs for PCR analysis are much less than cell culture techniques, and the technique is easily performed. Additionally, PCR can be used to identify a specific pathogen found in water. Standard PCR, however, cannot be used to detect the infectious state of an organism — only the presence or absence of pathogen-specific DNA or RNA.

Comparison of Virus Assays Methodologies

Both the cell culture assay and the RT-PCR assay attempt to detect viruses in the sample. However, they are not directly comparable. The cell culture assay can only detect infectious viruses, whereas the RT-PCR assay can potentially detect both infectious and noninfectious viruses. Additionally, the RT-PCR assay is designed to detect specific viruses, on the basis of their RNA sequence. Conversely, the cell culture assay can potentially detect any infectious virus capable of causing infection in the cultured cells. For instance, BGM cells used in the cell culture assay could possibly show infection from mainly enteroviruses and possibly rotaviruses or other viruses, whereas the RT-PCR assay does not result in this kind of ambiguity. Additionally, each assay differs in level of sensitivity and the effect of inhibitory or toxic substances in the sample and the equivalent sample volume examined.

The use of cell culture and PCR for virus detection differs significantly in several ways. For cell culture, the minimum detection level of viruses in a sample is, by definition, one PFU per unit volume — a quantity of virus particles that may range from just a few or many more — at least some of which must be infectious. In addition, when a sample tests positive for viral infectivity using cell culture, the infectious agent is not necessarily known. The BGM cell line, routinely used for enterovirus assays, is susceptible to infection by many viruses, including reoviruses such as rotavirus, a pathogen often present in environmental samples in numbers greater than enterovirus (30). Cell culture protocols do not detect all human viruses present in the environment. Norwalk virus, for instance, has yet to be successfully grown in cell cultures, and therefore environmental samples cannot be assayed for this pathogen. Finally, because each environmental sample is unique, little is known regarding possible sample components that may inhibit the viral infectivity in culture. Cell culture, however, does offer the advantages of isolating an infectious viral pathogen, and is widely accepted as the standard method for viral detection in water.

Conversely, RT-PCR is potentially a much more sensitive assay, in that it is possible to detect as little as a single molecule of RNA. The technique can detect less than one PFU of a virus (because some virus particles may not be infectious), and PCR can detect both infectious and noninfectious viruses. The two techniques also differ in the amount of time and cost required. Most cell culture protocols (including the procedure used in the Information Collection Rule) call for a 14-day initial passage and for a 14-day secondary passage of the sample on cells, followed by a seven-day confirmation passage of putative positive samples. To test for different viruses, multiple cell lines must be used.

Given its increased sensitivity and ability to detect an intact virus particle (5), PCR analysis would be expected to reveal more positive results than cell culture analysis. Because either cell culture analysis or PCR can only reveal a "snapshot" of the quality of the groundwater being sampled, PCR would be a desirable rapid initial screening tool, in that the presence of noninfectious or nonintact viruses would suggest that a groundwater supply may be subject to contamination and thus the potential for health risk. The most sensitive method of detection would be the most desirable, even without the ability to confirm infectivity of the sample contamination.

Although the RT-PCR and cell culture methods assayed different equivalent volumes of the original sample (5 liters versus 600 liters respectively), the difference in the assays' sensitivities must be considered while comparing the test results to understand the practical application for water analysis. The minimum detection level of viruses using a cell culture assay is one plaque forming unit (PFU) per tested sample volume. Because hundreds of virus particles may be required to produce a single PFU, assay methods such as PCR that detect virus particles directly will result in significantly greater sensitivity. The minimum detection limit of viruses using RT-PCR is one virus particle, and it has been shown that PCR can consistently detect 10^{-2} PFU of virus (4). Because the RT-PCR assay is approximately 100 times more sensitive, assaying 100 times less sample by RT-PCR approximately equates to the volume assayed by cell culture.

A Case Study

Groundwater samples collected from 448 sites throughout the United States (Fig. 4). Out of 448 sites, 25 sites were sampled more than once (average of three to four times).

Figure 4. Location of wells sampled are indicated in blue (shaded).

A total of 539 samples were collected for the study. Each sample was assayed for virus infectivity using cell culture assay, the presence of viral nucleic acid using RT-PCR, bacteriophage using three hosts, total coliforms, enterococci, *Clostridium perfringens*, total organic carbon, various metals and minerals.

Virus Cell Culture Assays

Twenty-one sites (4.8% of 442) and a total of 22 samples (4.1% of 529), tested positive for viral infectivity by exhibiting cytopathic effect (CPE) in Buffalo green monkey (BGM) kidney cells.

Virus RT-PCR Assays

Sample concentrates were assayed for viral RNA by the RT-PCR method. Four different pairs of primers, specific for enterovirus, rotavirus, hepatitis A virus, or Norwalk virus were used. Each sample was also assayed twice with each primer pair — once after having been seeded with a positive control virus, and once unseeded. The seeded reactions were performed to determine whether the sample would inhibit the RT-PCR reaction. The sample was deemed inhibitory if no PCR product was found in the seeded reaction. These samples were recorded as "unknown "with respect to the RT-PCR assay results (Table 4).

Comparative Results

Cultural methods used for various microorganisms include cell culture assays for infectious enteroviruses, double layer agar assay for bacteriophage, and membrane filtration for bacterial indicator. All these assays were performed on each sample. Besides, cultural PCR assays were used for the detection of four commonly found enteric viruses in drinking water. The target organisms for this project are indicative of fecal contamination of source

Table 4. Microbial Occurrence Results of Sites Sampled

Microorganisms Tested	N	Positive	Negative	Unknown
Viruses CC				
Infectious enteroviruses	448	21	427	0
Viruses RT-PCR				
Enterovirus	448	68 (15.2%)	360 (80.3%)	20 (4.5%)
Rotavirus	448	62 (13.8%)	363 (81.0%)	23 (5.1%)
Hepatitis A virus	448	31 (6.9%)	399 (89.1%)	18 (4.0%)
Norwalk	317	3 (0.9%)	309 (97.5%)	5 (1.6%)
Combined (any virus)*	448	141 (31.5%)	302 (67.4%)	5 (1.1%)**
Bacteria				
Total coliform	445	44 (9.9%)	401 (90.1%)	0
Enterococci	355	31 (8.7%)	324 (91.3%)	0
Clostridium	57	1 (1.8%)	56 (98.2%)	0
Combined (any bacteria)***	445	67 (15.1%)	378 (84.9%)	0
All (three bacteria)****	445	0 (0%)	445 (100%)	0
Bacteriophages				
E. Coli C	444	18 (4.1%)	426 (95.9%)	0
E. Coli C-3000	444	48 (10.8%)	396 (89.2%)	0
Salmonella WG-49	440	42 (9.5%)	398 (90.5%)	0
Combined (any host)*****	444	92 (20.7%)	352 (79.3%)	0
All (three hosts)******	444	1 (0.2%)	443 (99.8%)	0

N = Number of sites/wells; Norwalk virus, Enterococci, and *Clostridium* assay begun latter in the course of the study.
CC = Cell Culture; RT-PCR = Reverse Transcriptase Polymerase Chain Reaction.
*Sample tested positive for any of the viruses using RT-PCR.
**For some samples the RT-PCR assay was inhibited for all of the four viruses.
***Site tested positive or negative for one or more of the bacteria mentioned in the table.
****Site tested positive or negative for all of the bacteria mentioned in the table.
*****Site tested positive or negative for one or more of the bacterial host mentioned in the table.
******Site tested positive or negative for all of the bacterial host mentioned in the table.

Table 5. Comparison Between Total Coliform, Enterococci, and Bacteriophage and RT-PCR Results

	RT-PCR Positive (positive for any* virus)	RT-PCR Negative	RT-PCR Unknown
Total [1]coliform positive	19	25	0
Total coliform negative	120	276	5
[2]Enterococci positive	9	22	0
Enterococci negative	86	236	2
[3]Bacteriophage positive	34	55	3
Bacteriophage negative	105	245	2
[4]Total cultural positive	55	99	3
Total cultural negative	86	203	2

[1]For Coliform a total of 445 sites were compared in this table. Three samples were excluded for which one assay results was undetermined.
[2]For Enterococci a total of 355 sites were compared for in this table. Samples excluded for which one assay result was undetermined.
[3]For Bacteriophage a total of 444 sites were compared in this table. Samples excluded for which one assay results were undetermined.
[4]For total cultural all 448 sites were compared with either one of cell culture, enterococci, total coliform, clostridium, and bacteriophage.
*Enterovirus, Rotavirus, Hepatitis A, and Norwalk.

water. A total of 32.6% samples were positive by all cultural assays and 31% (141/448) were positive by RT-PCR (Table 4).

Virus Assays Compared With Other Potential Indicator Assays

One objective of the study was to evaluate other biological and physical attributes of groundwater as potential indicators of virus presence. Often these assays are simpler to perform and are less expensive and more familiar to water utility personnel. To this end, the relationship between virus assay results and the results of assays for total coliforms, enterococci, and bacteriophage were examined. In addition, total cultural assays (cell culture, bacterial, and bacteriophage assays) were examined with the RT-PCR results (Table 5).

Physical Characteristics

The physical characteristics of each well were gathered through a questionnaire for the study (Fig. 5). Turbidity ranged between 0.0 and 85 NTU, temperature ranged between less than 1 to 35 °C, TOC ranged between 0.123 and 85.37 mg/L, and pH ranged between 4 and 9.6. The cell culture and RT-PCR samples that tested positive were analyzed for the distance to a source of contamination. Septic tanks, sewer lines, and wastewater plants were identified as sources of contamination. The total number of samples tested in categories <150 ft distance, 151 to

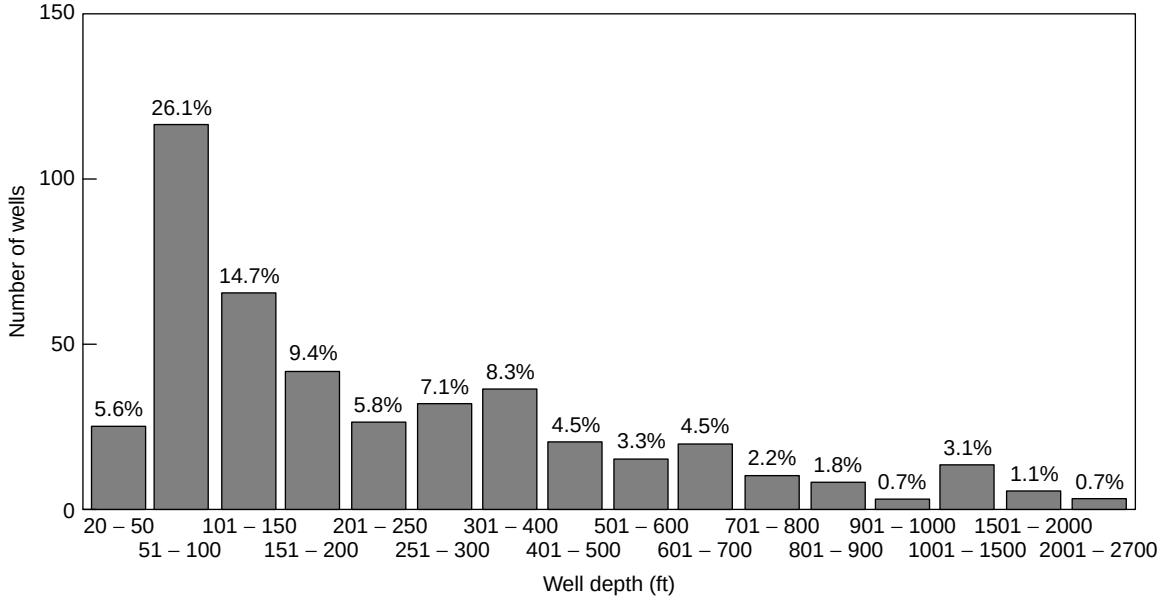

Figure 5. Number of wells sampled at different depths. Note: Over 75% of the wells ranged from 51 to 500 feet in depth.

<550 ft distance, and >550 ft to unknown distance were 147, 154, and 147, respectively. The majority of positive results from all three categories have a sewage source less than 150 feet away (all three in the 41 to 47.6% range) and 80% of sites that tested positive had a sewage source closer than 550 feet.

Analysis of Results by Geologic Formation

Geologic formations are classified into two basic groups; bedrock or unconsolidated. Table 6 categorizes the 448 wells by classification and by subgroups. The positive viral and bacterial assay results within those classifications are summarized in Table 7. The bedrock situated wells

Table 6. Comparison of Samples Collected at each Geology Deposit with the Percentage Production at National Level

Geology/Deposit	Number of Sites Collected	% Sites Collected	% Production at National Level*
Unconsolidated	287	64.1	69.5
Alluvial Sand & Gravel	180	62.7	32.7
Coastal Plain	31	10.8	17.1
Fluvial/Eolian	0	–	2.4
Glacial Valley	19	6.6	2.3
Glacial Outwash	44	15.3	13.8
Glacial Valley &/OR Outwash	0	–	1.2
Other	8	2.8	—
Unknown	5	1.7	—
Bedrock	122	27.2	30.5
Carbonates	46	37.7	18.1
Sandstone & Conglomerate	40	32.8	8.3
Siltstone	11	9.0	0.2
Plutonic Igneous & Metamorphic	10	8.2	0.6
Limestone	4	3.8	
Volcanic	8	6.6	3.4
Unknown	3	2.5	—
Unknown	39	8.7	—
Total	448	100	100

*On the basis of U.S. Geological Survey Circular 1081, Estimated Use of Water in the United States In 1990 (31).

Table 7. Biological Assays by Geologic Formation

Formation	All Sites (wells)		Cell Culture Positive Sites		Enterovirus RT-PCR Positive Sites		Positive Sites for any Bacteria		Positive Sites for Bacteriophage	
	N	%	N	%	N	%	N	%	N	%
Bedrock	122	27.2	4	19.0	22	32.4	18	26.9	23	25.0
Igneous/metamorphic	10	8.2	0	–	0	–	0	–	0	–
Sedimentary/carbonates	46	37.7	3	14.3	11	16.2	7	38.9	14	15.2
Sedimentary/ sandstone	40	32.8	1	4.8	6	8.8	7	38.9	5	5.4
Siltstone	11	9.0	0	–	2	2.9	2	11.1	3	3.3
Volcanic	8	6.6	0	–	3	4.4	1	5.6	1	1.1
Limestone	4	3.8	0	–	0	–	1	5.6	0	–
Unknown	3	2.5	0	–	0	–	0	–	0	–
Unconsolidated	287	64.1	15	71.4	38	55.9	41	61.2	54	58.7
Alluvial	180	62.7	9	42.9	24	35.3	24	58.5	34	37.0
Coastal plain	31	10.8	2	9.5	2	2.9	5	12.2	6	6.5
Glacial outwash	44	15.3	1	4.8	7	10.3	7	17.1	8	8.7
Glacial valley	19	6.6	1	4.8	2	2.9	4	9.8	2	2.2
Abandoned mine	3	1.0	0	–	0	–	1	2.4	0	–
Other	5	1.7	0	–	0	–	0	–	1	1.1
Unknown	39	8.7	2	9.5	8	11.8	8	11.9	15	16.3
Totals	448		21		68		67		92	

% = The percentages refer to the portion of the total for the assay category column. For example, in the column "Cell culture positive," 4 positive samples were from bedrock formations, which represents 19.0% of the 21 positive cell culture assays.
None of the assays — cell culture, RT-PCR, bacterial, or bacteriophage — showed any significant trends when the percentages of positive assays are compared with the percentages of the actual samples within each geologic formation category.

represented a slightly lower percentage of the positive biological assays 25.9% compared with 27.2% of the wells, whereas the unconsolidated sites showed a lower percentage of the total number of positive assays (60.9%) than their percentage of all sites (64.1%). Within the bedrock group, 63 sites were positive, 30 of those sites (47.6%) were from the sedimentary/carbonate group, whereas this subgroup represented only 37.7% (46/122) of the total bedrock geology (Table 7). Within the unconsolidated formation, in which 148 sites were positive, 92 of those sites were the alluvial subgroup representing 62.2% of the positive sites (92/148), whereas for all unconsolidated sites, this formation represented only 62.7% (180/287) (Table 6). This would seem to indicate that these two particular formations — unconsolidated/alluvial and bedrock/sedimentary-carbonate — are more likely to test positive for biological indicators and thus may represent a higher risk of fecal contamination.

Repeat Sampling

A total of 25 sites were sampled more than once with an average frequency of three to four repeats. Thirteen sites were negative for all the assays. The remaining 12 sites tested positive for one or more of the assays. When the results were analyzed considering all the 539 samples (448 sites) to determine a relationship between virus occurrence in groundwater and any of the indicators, no such relationship was observed. However, the twelve sites tested positive for repeat sampling at different occasions were positive for pathogens by cell culture and/or PCR. Only two sites tested positive for pathogens but were negative for indicators. The other ten sites that tested positive for pathogen also tested positive for bacteriophage and/ or bacteria. However, when only repeated sampling positive sites were examined, it was observed that if a site was positive for any of the microorganisms, often there was again a positive site in one of the subsequent samples by one of the assays (Table 8).

Seasonality

Seasonal trends can be seen for some of the viruses tested by PCR. Enteroviruses have seasonal peaks in summer and early fall (31). Rotavirus infections peak from late autumn to early spring and start in the Southwest regions of the country moving to the Northeast states by Spring (13,19,32). Hepatitis A virus infections do not show a seasonal pattern (33). Studies revealed a seasonal trend for the detection of virus, with a higher number of positives being observed in summer months and in the early Fall (Fig. 6). No clear trend was observed for the cultural methods (Fig. 7). Figures 8, 9, and 10 represent the sample temperature trend for PCR positive and cultural assays.

The objective of the analysis was to examine whether there was any relationship between PCR and cultural methods in certain temperature group. The number of samples in each temperature group was not the same. The number of sample in 11 to 15 °C and 16 to 20 °C temperature groups was 232 and 100 respectively, which are higher than all other temperature groups. No direct comparison was made among the temperature groups. However, within all temperature groups a similar trend was observed between the PCR positive hits and cultural methods (cell culture, bacteriophage, and bacteria). At the 11 to 15 °C temperature range, the number of positive samples by PCR was much higher than all other temperature group. This temperature range has the highest number of positive samples by the cultural methods, suggesting that indicator organisms or PCR may be used to predict the presence of human pathogens in groundwater.

As a result of the study, a comprehensive database on virus occurrence in the untreated source water of

Table 8. Multiple Hits for Repeated Sampling

			Viruses					Indicators			
Samples in Each Site Tested Positive	# A Site Sampled**	# A Site Tested Positive***	Cell Culture	Entero-virus	Rota-virus	HAV	WG49	*Entamoeba coli* C-3000	*Escherichia coli* C	Coliform	Entero-cocci
IL-4,5 and IL-5,4	2	2	1	1	0	0	1	1	0	2	ND
O-IA-2 and O-IA-6	2	2	1	2	0	0	0	2	0	1	1
IN-8 and O-IN-2	2	2	0	1	0	0	0	0	0	1	0*
O-NJ-11 and NJ-6	2	2	0	0*	1*	0	0	0	0	2	0
OH-3 and OH-4	2	2	1	1	0	0	0	0	1	0	0*
AZ-1,3 and AZ-3,1	4	2	2	1	0	0	1	1	0	0	ND
PA-3, O-PA-13, O-PA-14, O-PA-18 and O-PA-26	17	5	0	2	1	0	1	0	1	3	0*
MA-1 and MA-10	2	2	0	0*	0	1	0	1	1	0	0*
MO-2 and 0-MO-7	2	2	0	1	0	0	0	1	0	0	0*
ID-2,4 and ID-4,2	2	2	1	2	0	0	0	0	0	0	ND
VA-1 and VA-2	2	2	0	1	1	0	0	1	0	0	1*
MO-1 and O-MO-19	10	2	1	0	1	0	0	0	0	2	1

Shading indicates more than one test was positive for a site; The number in the table denotes the number of replicates tested positive for a particular assay.; WG49, *E. coli*-3000, and *E. coli* C are three different hosts used for the enumeration of bacteriophage.; Enterovirus, rotavirus, and hepatitis A virus were assayed by PCR. ND = No data for all replicates; *Denotes no data for one replicate; **Number of times that site was sampled; ***Number of times that site tested positive.

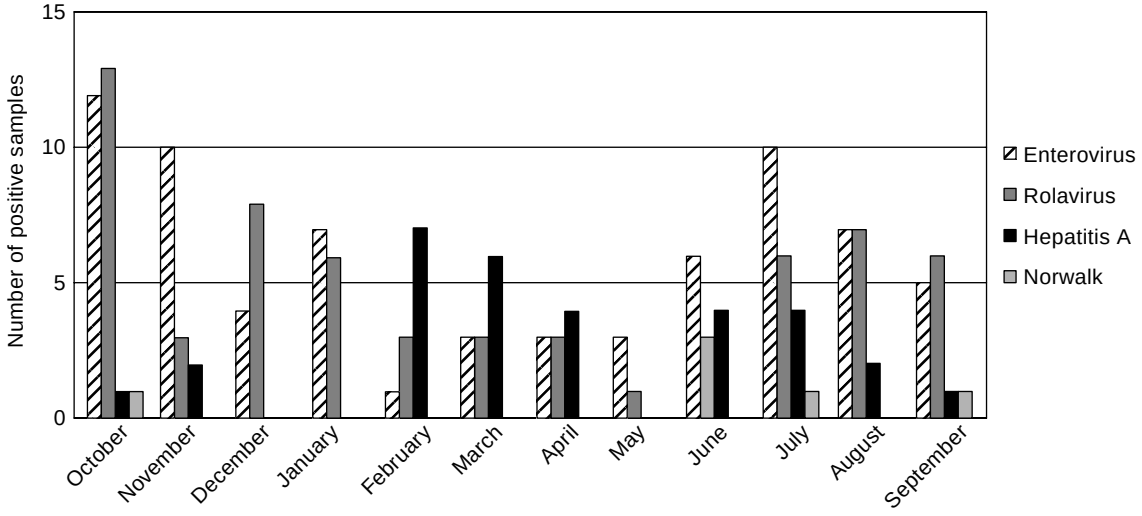

Figure 6. Seasonal trends for PCR positive samples.

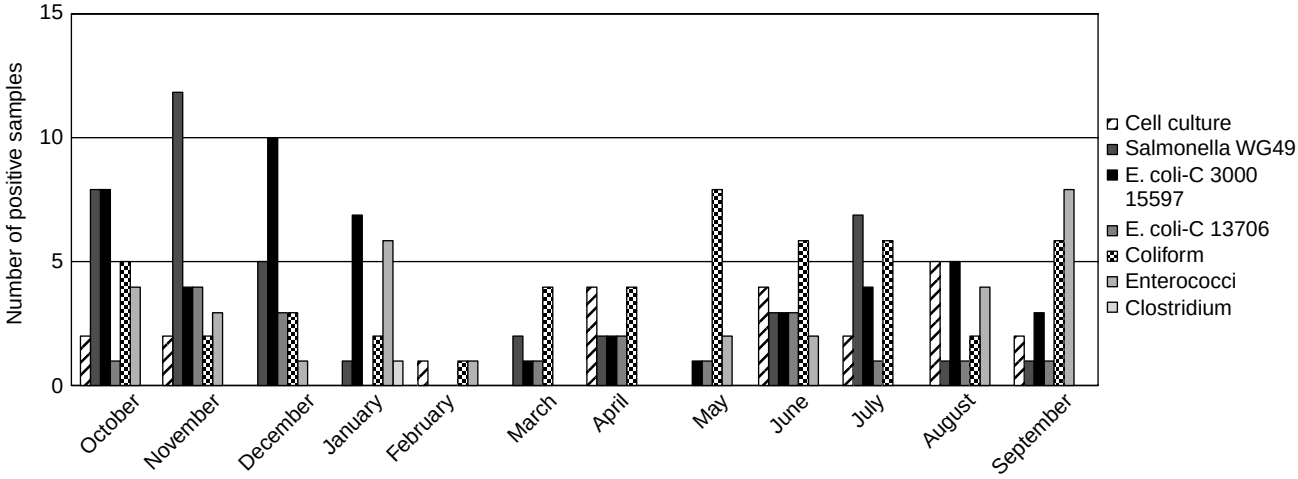

Figure 7. Seasonal trends for positive cultural methods.

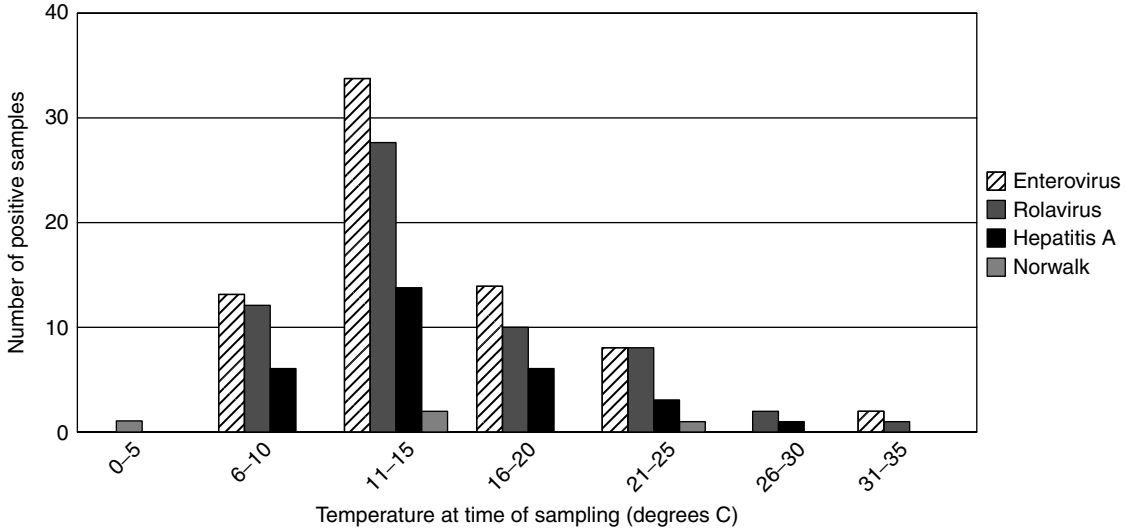

Figure 8. Sample temperature trends for PCR positive samples.

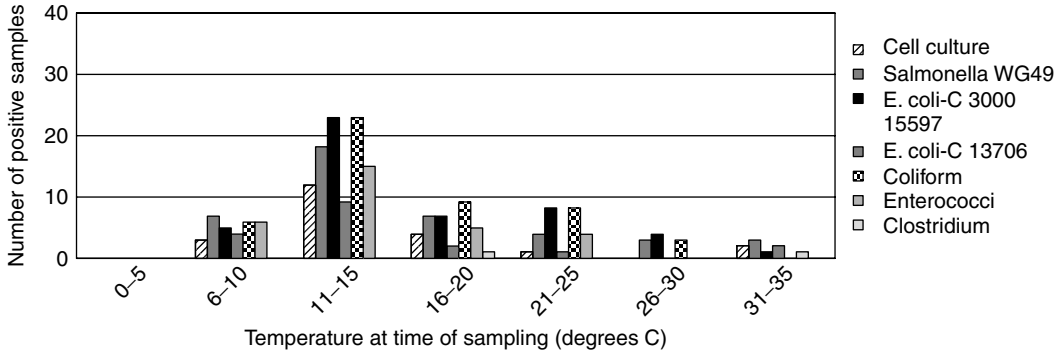

Figure 9. Sample temperature trends for positive cultural methods.

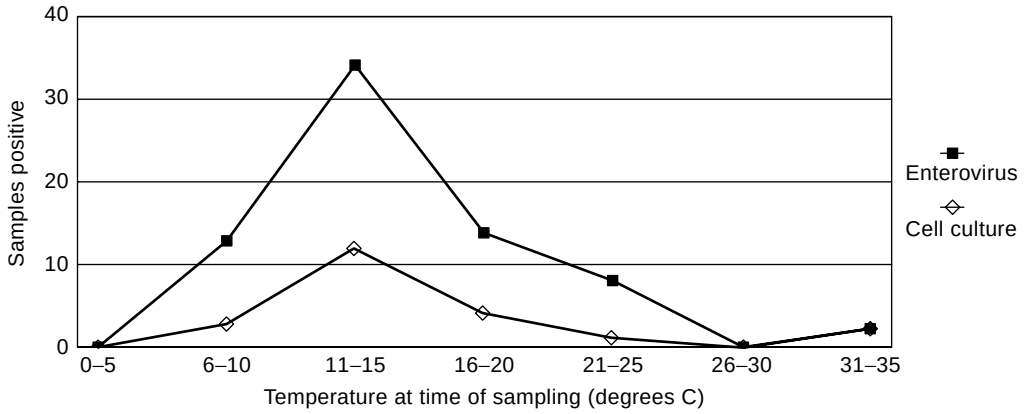

Figure 10. Sample temperature trends for cell culture and enterovirus PCR positive samples.

public groundwater systems at the United States was established. In addition, various water quality parameters and the occurrence of microbial indicators in groundwater and their possible correlation with the presence of human viruses were investigated. The representativeness of the study for groundwater sites in the United States as a whole was established by the U.S. EPA after conducting a comparative study between this study and national database for nitrate data (34), which is the only comprehensive groundwater database available in the United States. Nitrate data for 216 sites from this study were compared with 216 sites in the U.S. Geological Survey database (35). Analysis of variance statistical tests conducted to compare the mean log nitrate concentrations in this study versus the U.S. GS data indicated that at a 95% confidence level, there was no significant difference (36). The U.S. EPA report concluded that the samples appropriately represented wells in the United States with low nitrate concentration.

PCR Assay Compared with Cell Culture

Analyses using the PCR assays suggest a greater viral contamination (Table 4). It is important to note that PCR assay is indicative of the presence of viral nucleic acid, and not necessarily the infectious viral particle. Therefore, the PCR results should be interpreted as indicative of the possibility of virus transport within the aquifer and a potential risk of disease rather than an absolute public health problem. It is noted that the total of the cultural indicators (coliforms, enterococci, *Clostridium*, bacteriophage) were positive in 32.6% of the samples tested, whereas 31.5% of the total samples were positive for RT-PCR, suggesting that the general overall indication of fecal contamination of groundwater sources did not substantially differ between the molecular technique (PCR) and the cultural indicators.

PCR revealed a greater level of viral contamination than did the cell culture assay, and that could be because of several reasons: (*1*) greater sensitivity of the PCR method for the detection of viruses in water samples, (*2*) not all enteric viruses cause CPE in BGM cell line, so, non-CPE forming viruses were not detected by cell culture assay, (*3*) the ability of PCR to detect a wider variety of viruses than the cell culture method, and (*4*) the possibility of detecting noninfectious viral nucleic acids by PCR.

Repeat Sampling

Male-specific bacteriophages are similar to human enteric viruses (37) in size, shape, survival and transport behavior in the environment. In addition, their removal during coagulation is similar to enteroviruses (38). Correlations between the presence of F-specific RNA bacteriophages and enteric viruses in fresh water have been reported (39). It appeared that very often when a well is positive for any

of the biological assay tested, it is very likely to be positive in future sampling for at least one of the assays. Although no direct correlation among the assays existed, it indicated the vulnerability of the wells to microbial contamination. These results suggest that microbial indicators such as coliforms, enterococci, or bacteriophage may be useful for monitoring those groundwater sources, despite the lack of a 1 : 1 correspondence to viral occurrence.

Seasonality

Seasonal trends were observed for enterovirus and rotavirus tested by PCR. An increased rate of PCR positive results were observed in the summer months and in the early Fall. No clear trend was observed for the cultural methods (Fig. 7). Enteroviruses have seasonal peaks in summer and early fall (31,40). Rotavirus infections peak from late autumn to early spring and start in the Southwest region of the country moving to the Northwest states by spring (13,19,32). A similar pattern was observed for the PCR results. PCR confirms the presence of virus genome regardless of it's status of infectivity. Figures 8 and 9 represent the sample temperature trend for PCR-positive and cultural assays. The temperature grouped data showed a similar trend between PCR and cultural methods (Fig. 10). These patterns suggest the validity of PCR assay as a tool for microbial monitoring.

Although no waterborne outbreaks were documented during the study, the high level of indicators of fecal contamination does suggest a mechanism by which illness could result in members of the community. It has been reported that annually 3 to 6% of community water systems in a six state area exceeded total coliform levels during a four-year period (41). Between 0.3 and 1.3% of the systems contained *Escherichia* or fecal coliform bacteria. However, because the Total Coliform Rule requires samples to be collected from the distribution system, these rates may not reflect the occurrence of bacterial indicators in the source water. Total coliform occurrence rates in private water systems (which are typically not disinfected) ranged between 15 and 59% (41) and are consistent with the findings of the study.

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VIRUSES IN FOOD. See VIRUSES AND PROTOZOAN PARASITES IN FOOD, INCLUDING METHODOLOGY

VIRUSES IN THE MARINE ENVIRONMENT

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Viruses are now known to be integral components of marine biological and biogeochemical systems. They are extremely abundant in both the water column and in sediments, and they play several roles in system function. They are important in the mortality of prokaryotes and eukaryotes, and in the process act as catalysts of nutrient regeneration and recycling. Because of their host specificity and density dependence, they have a tendency to selectively attack the most abundant organisms, thus "killing the winner" of competition and fostering diversity. They also are mediators in the exchange of genetic material between organisms, a critical factor in evolution and also relating to spread of human-engineered genes. Although these processes are only now becoming understood in any detail, there is little doubt that viruses are significant players in marine systems and aquatic environments in general.

Lately, the relatively increased interest in marine viruses can be viewed as part of the recent great expansion of general interest in marine microorganisms. The "microbial loop" as a major part of marine ecosystems became a topic of wide general interest starting about 25 years ago. The discovery of high bacterial abundance as learned by epifluorescence microscopy of stained cells, with counts typically 10^9 per liter in the plankton first prompted attention in this area (1–3). With such high abundance, it became important to learn how fast they were dividing, and this was determined by development and application of methods examining the frequency of dividing bacteria (4) and bacterial DNA synthesis (5,6). These methods showed that bacterial doubling times were on the order of a day, and it quickly became apparent that bacteria were consuming a significant amount of dissolved organic matter, typically at a carbon uptake rate equivalent to about half of the total primary production (7) (see also PROTOZOA IN MARINE AND ESTUARINE WATERS, this Encyclopedia). Given that bacteria are too small to sink out of the water column and that their abundance stays relatively constant over the long term, there must be mechanisms within the water to remove bacteria at rates similar to the bacterial production rate. Originally it was thought that protists were the only "sink" for bacterial production. This followed from the observations that heterotrophic protists of the types known to consume bacteria are ubiquitous in seawater, that they are capable of growth at typical natural bacterial abundance, and they are apparently capable of controlling bacterial abundance near natural levels (7). However, evidence suggested that protists are not the only agents controlling bacteria. In the late 1980s, careful review of several studies showed that the best estimates of grazing by protists often fell short of balancing the best estimates of bacterial production, suggestive of additional loss processes (8). About that same time, data began to accumulate that viruses may also be important as a mechanism of removing bacteria. By now the evidence is fairly clear that this is so, and it will be outlined below. This entry will briefly summarize much of what we know about how viruses interact with marine microorganisms, including general properties, abundance, distribution, infection of bacteria, mortality rate comparisons with protists, biogeochemical effects, effects on species compositions, and roles in genetic transfer and evolution.

WHAT ARE VIRUSES AND WHAT DO THEY DO?

Viruses are small particles, usually about 20 to 200 nm long, and consist of genetic material (DNA or RNA, single or double stranded) surrounded by a protein coat (some have lipid as well). They have no metabolism of their own and function only via the cellular machinery of a host organism. As far as is known, all cellular organisms appear to be susceptible to infection by some kind of virus. From culture studies, it has been learned that a given type of virus usually has a restricted host range, most often a single species or genus, although some viruses infect only certain subspecies and less than 0.5% may infect more than one genus (9). Viruses have no motility, and contact the host cell by passive diffusion. They attach to the cell usually via some normal exposed cellular component, such as a transport protein or flagellum. There are three basic kinds of virus reproduction (Fig. 1): (*1*) lytic infection, in which the virus attaches to a host cell, injects its nucleic acid that causes the host to produce numerous progeny viruses, and then bursts the cell to

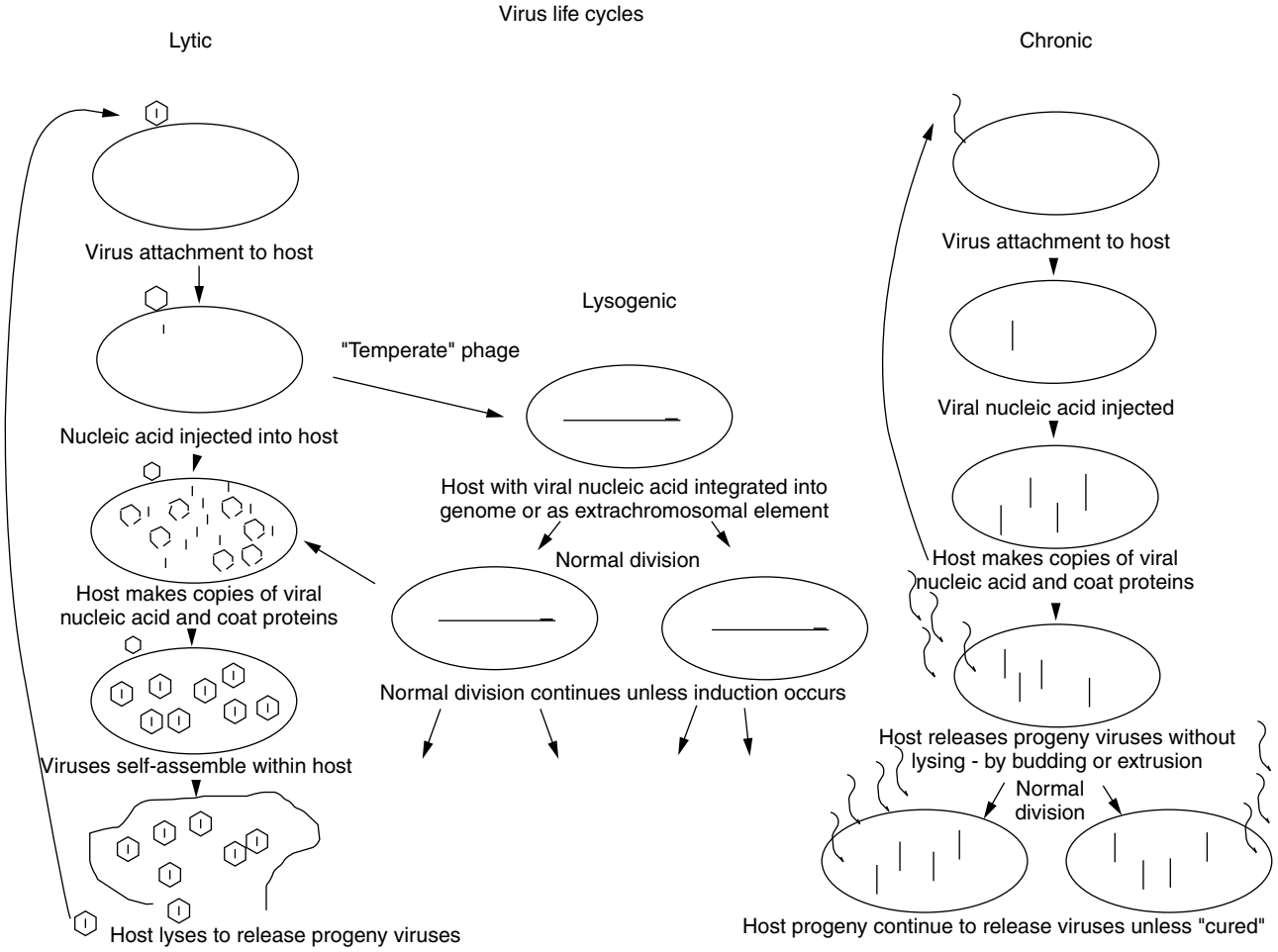

Figure 1. Virus life cycles.

release the progeny and begin the cycle again; (*2*) chronic infection, in which the progeny virus release is nonlethal and the host cell releases them by extrusion or budding over several generations; and (*3*) lysogeny, in which after injection, the viral genome becomes part of the genome of the host cell and reproduces as genetic material in the host cell line unless an "induction" event causes a switch to lytic infection. Induction is commonly caused by DNA damage, such as from UV light or agents like mitomycin C. Viruses or viruslike particles (VLP) may also be involved in killing cells by mechanisms that do not result in virus reproduction (9); also see reference (10).

DISTRIBUTION OF VIRUSES

In order to determine how viruses are distributed, it is necessary first to develop techniques to observe and enumerate them. Because of their small size, near or below the resolution limit of light microscopy (ca. 0.1 μm), the only way to observe any detail of viruses is to use electron microscopy. Sample preparation requires concentrating the viruses from the water onto an electron microscopy grid (coated with a thin transparent organic film). Because viruses are denser than seawater this can be done by ultracentrifugation, typically at forces of at least $100,000 \times g$ for a few hours (11–13); note that under ordinary gravity, forces like drag and Brownian motion prevent viruses from sinking. To be observable the viruses must be made electron-dense, and this is done by staining with heavy metals like uranium salts. The viruses are recognized by their size, shape, and staining properties (usually electron-dense hexagons or ovals, sometimes with a tail; Fig. 2), and counted. Typical counts are on the order of 10^{10} viruses per liter in surface waters, with abundance patterns similar to those of heterotrophic bacteria (see following text). Recently it has been found that viruses can also be stained with nucleic acid stains like diamidino-2- phenylindole (DAPI), YoPro, or SYBR Green I, and observed and counted by epifluorescence microscopy (14–17). Epifluorescence viewing of viruses is illustrated in Figure 3, a micrograph of SYBR Green-I stained bacteria and viruses. It graphically illustrates the high relative virus abundance. Epifluorescence microscopy of viruses is possible even though the viruses are below the resolution limit of light because the stained viruses are a source of light and appear as bright spots against a dark background (just as stars are visible at night despite their small apparent size). Such epifluorescence counts are reported to be similar or even higher than

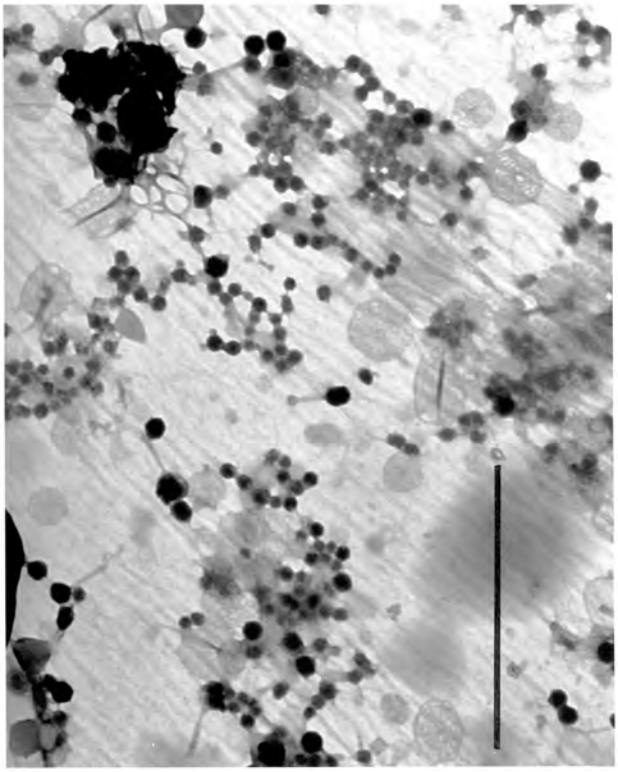

Figure 2. Electron micrograph of viruses and other microbes from Santa Monica Bay, California. The viruses are the dark small hexagonal or oval particles, some with tails. The scale bar is 1 μm.

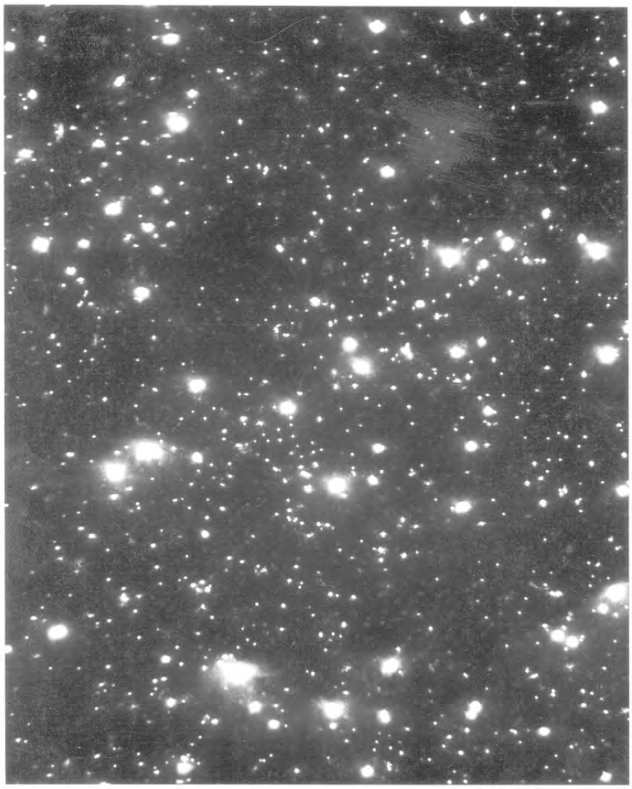

Figure 3. Epifluorescence micrograph of prokaryotes and viruses from 16 km offshore of Los Angeles, stained with SYBR Green I (17). The viruses are the very numerous tiny dots, and the prokaryotes are the larger dots. See color insert.

transmission electron microscopy (TEM) counts from seawater, especially at higher abundance levels (16,17); counts with SYBR Green tend to be about 30% higher than with TEM (17). Possible reasons for the higher epifluorescence counts may be that the TEM counts may miss unexpectedly shaped viruses or ones obscured by other dark-stained material in the TEM preparations. Particular benefits of the epifluorescence methods are rapidity, ability to work in the field (e.g., on board ship), and lower cost. However, such methods do not yield data on virus size or morphology, unlike TEM.

Electron or epifluorescence microscopy reveals the total, recognizable, virus community, and it is reasonable to ask what kinds of viruses make up this community. It is commonly assumed that most of the total virus community is made up of bacteriophages (viruses that infect bacteria). This is assumed because viruses lack metabolism and have no means of actively swimming from host to host (and depend on random diffusion), so the most common viruses would be expected to infect the most common organism, and heterotrophic bacteria are generally the most abundant organisms in the plankton, often by far (18). Data from field studies also show a strong and robust correlation between viral and bacterial abundances, whereas the correlations between viruses and chlorophyll are weaker and often not statistically significant (19,20). This suggests that the majority of viruses are bacteriophages rather than those infecting phytoplankton or other eukaryotes. On the other hand, it is known that viruses infecting culturable cyanobacteria (*Synechococcus*) are also quite common and sometimes particularly abundant in seawater, exceeding 10^5 per milliliter in some cases (21–23). Similarly, viruses infecting some common culturable eukaryotic picoplankton, such as *Micromonas pusilla*, have been found to be sometimes quite abundant as well, occasionally near 10^5 per milliliter in coastal waters (24). These studies have been done with most probable number (MPN) methods, looking for agents from seawater that lyse pure cultures when tested at various dilutions. Overall, the data suggest that most viruses from seawater infect nonphotosynthetic bacteria, but viruses infecting prokaryotic and eukaryotic phytoplankton also occur regularly and can make up a significant fraction of the total. As a final note, we are only now learning about the distribution of archaea in seawater, and that they apparently constitute a significant fraction of the total countable prokaryotes, especially in deeper waters (25,26). Whereas most of this entry discusses "bacteria," it must be considered that this practical, generic term includes the archaea plus bacteria for the purposes of general discussion. Thus, many of the viruses we observe may infect archaea, particularly in deeper ocean waters.

Viral abundance has been examined in several locations and habitats worldwide. Counts come from all sorts of planktonic environments — coastal, offshore, temperate, polar, tropical, and deep sea (Table 1, Fig. 4). Within the

Table 1. Typical Counts of Viruses from Various Marine Planktonic Environments. See also Figure 4

Location, Depth[a], Season[b]	10^8 Viruses per Liter	Method[c]	Reference
North Atlantic, spring	150	TEM	11
Raunefjord (Norway)	100	TEM	11
Raunefjord, late winter	5	TEM	27
Raunefjord, spring	20–100	TEM	27
S. California nearshore	111–282	TEM	20
S. California offshore	13–124	TEM	20
S. California offshore, 50 m	4–57	TEM	20
S. California offshore, 900 m	25	TEM	20
Bering and Chukchi Seas	20–360	TEM	29
Northern Adriatic Sea	50–380	TEM	30
Gulf of Mexico, U. Texas Pier	104	TEM	16
Gulf of Mexico, offshore	3–57	TEM	16
Gulf of Mexico, offshore	3–82	Yo-Pro	16
S. California, 190 km offshore	135	TEM	17
S. California, 190 km offshore	170	SYBR	17
Equatorial Pacific	53	FCM	31
Mediterranean Sea	23	FCM	31

[a]Near-surface unless otherwise noted.
[b]Summer unless otherwise indicated.
[c]TEM is ultracentrifugation directly onto TEM grids for counting, without prior concentration steps. Yo-Pro and SYBR are epifluorescence of Anodisc-filtered samples with those stains. FCM is flow cytometry with SYBR Green stain.

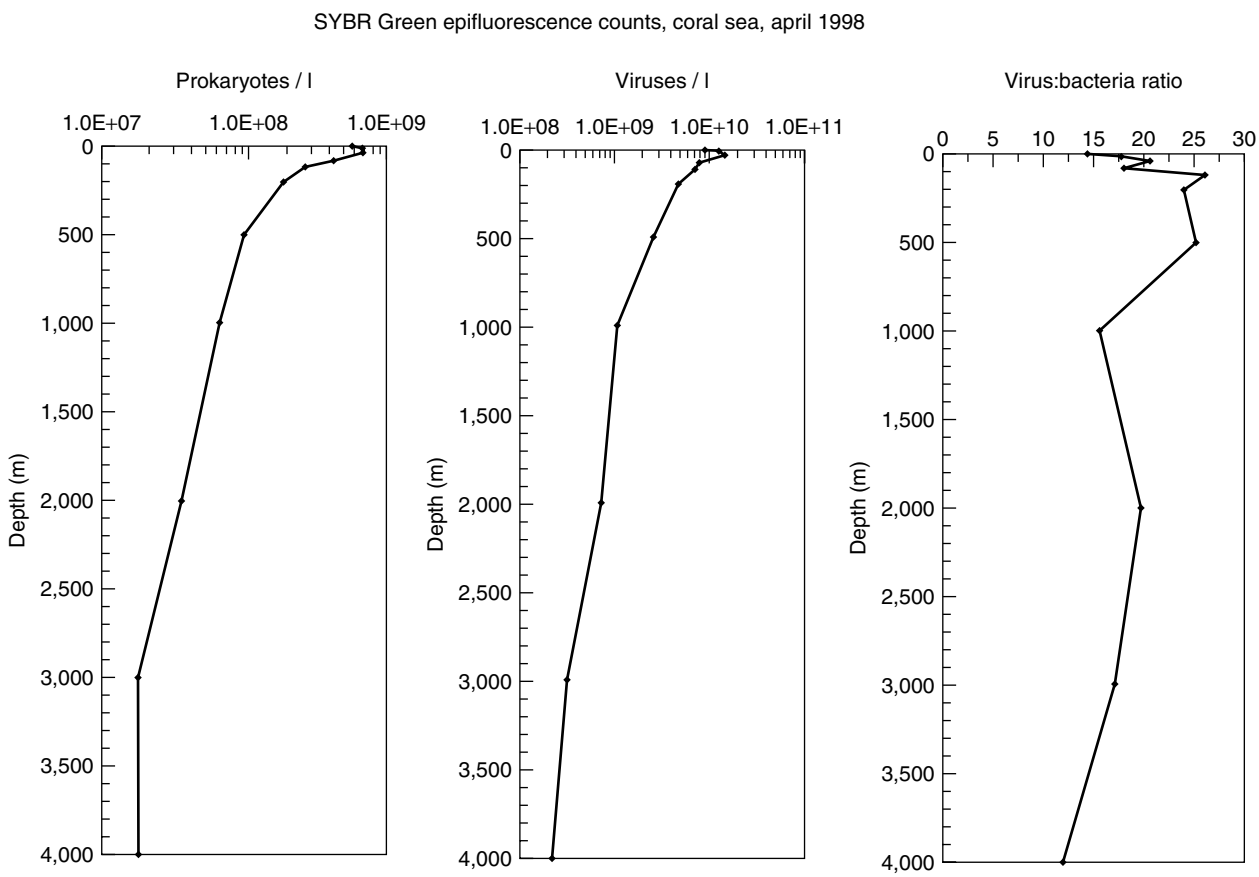

Figure 4. Depth profile of total prokaryote (bacteria + archaea) counts, total viral counts, and virus: bacteria ratios from the Coral Sea (April 1998), as determined by epifluorescence microscopy of SYBR Green stained samples (17). Note the log scales.

Table 2. Typical Counts of Viruses from Near-Surface Sediments

Location	10^8 Viruses per cm^3	Method[a]	Reference
Florida Bay, U.S.A.	1–5	TEM	32
Lac Gilbert, Quebec, Canada	8–40	TEM	33
Bering Sea, Alaska, U.S.A.	0.3	TEM	29
Chesapeake Bay, U.S.A.	3.6	Yo-Pro	34
Mediterranean Sea — Sporades Basin and Ierapetra Trench	10–20	SYBR	35

[a]TEM is ultracentrifugation directly onto TEM grids for counting, without prior concentration steps. Yo-Pro and SYBR are epifluorescence of Anodisc-filtered samples with those stains.

plankton, typical virus abundance is 1 to 5×10^{10} per liter in rich nearshore surface waters, dropping to about 0.1 to 1×10^{10} per liter in the euphotic zone of offshore low-nutrient areas, and also decreasing with depth, by about an order of magnitude. A typical deep offshore profile is shown in Fig. 4. Onshore-offshore gradients are also evident, similar to those of the bacteria (19,20). As may be expected, seasonal changes are also common, with viruses following general changes in phytoplankton, bacteria, etc. (27) (e.g., see Raunefjord data in Table 1). One study indicated that virus abundance can sometimes be remarkably dynamic, changing drastically in time frames of minutes to hours (28). This has been interpreted as synchronized release from some hosts followed by rapid decay of many of the viruses. Given the relative youth of this part of the field, many parts of the world have not been investigated with regard to virus abundance, and the data are patchy. Also, there are a few measurements of total virus abundance in sediments, and the abundance there is orders of magnitude higher, on a per unit volume basis, than in the water column. Typical counts are in the range of 10^8 to 10^9 per cm^3 of sediment, with a report from Alaska waters a slightly lower (Table 2).

Counts are often also compared as virus:bacteria ratios. In plankton, this ratio is typically 5 to 25, and commonly is close to 10, even as abundance drops to low levels in the deep sea. It is unknown what factors keep this ratio in such a relatively narrow range, but it does suggest a link between these organisms, and also some reasonably tight regulatory mechanisms. Correlations to chlorophyll and other parameters have been shown in some studies, and it appears to depend on the scale examined. Given that bacteria and chlorophyll are often correlated (36), a strong possibility is that the relationship to chlorophyll is primarily a general relationship with the trophic status of the water rather than a specific relationship.

The virus:bacteria ratio has been reported to be much more variable in marine sediments, ranging from 2 to 5 in some Mediterranean sediments (35), 1 to 32 in a Canadian Lake (33), to 29 to 85 in Chesapeake Bay (34). This variation may indicate fundamental differences in processes between plankton and sediments, and between different kinds of sediments. However, because it may be more difficult to count viruses and bacteria in sediments, especially those attached to particles, there is a possibility that the variations in the ratio is in part because of a variable undercount of the viruses and/or bacteria with current techniques.

VIRAL ACTIVITIES

Viruses do not have activity on their own, so when the topic of viral activity is raised, the primary concern is usually lytic infection. Although this is an important topic and will be the main issue for discussion that follows, it should also be realized that lysogeny (where the viral genome resides in the host's genome — see Fig. 1) is common. Evidence of lysogeny is usually from culture studies or direct induction with DNA-damaging agents (37). Lysogens (bacteria harboring integrated viral genomes) can readily be found and isolated from seawater, and lysogeny, which is linked to genetic transfer in a variety of bacteria, probably has important impacts in microbial population dynamics and evolution. However, the natural induction rate seems low under ordinary conditions, and lysogenic induction appears to be responsible for only a tiny fraction of total virus production in marine systems. Evidence for this conclusion comes from two kinds of studies: (*1*) Growth of "seawater cultures" of natural marine bacteria inoculated into filtered seawater and grown under simulated in situ conditions shows rapid growth of bacteria but no appearance of viruses (as would be expected from lysogenic induction) even after several days, as long as the initial filtered seawater was cleared of viruses by filtration through a membrane with pore size of 0.02 μm.. However, when the filtered seawater is prepared by filtration through a 0.2 μm filter and thus starts with many viruses, the virus abundance increases after a few days of bacterial growth, suggesting that infection from the viruses in the inoculum is occurring (38). (*2*) When lysogen abundance is estimated from artificial induction experiments (e.g., with mitomycin C), calculation of the maximum likely number of viruses released from lysogens under optimal conditions usually represents only a few percentage of the total estimated virus production rate (37,39).

At this time, we know essentially nothing about chronic infection in the ocean, but it is often presumed to be low. Release of filamentous (or other kinds of budding) viruses

from native marine bacteria has not been noted in TEM studies, nor have significant numbers of free filamentous viruses. However, these may be hard to recognize or differentiate from other filamentous objects like cilia and bits of cells, and filamentous or nondistinct viruses may partly explain why epifluorescence methods (looking for tiny particles containing densely packed nucleic acids) usually count more viruses than TEM methods. Therefore it may turn out that chronic infection is more common than currently thought.

One of the main issues regarding viral activity in seawater is the effect on bacterial mortality via lytic infection. Several studies have been done recently on this topic, and they tend to converge on the conclusion that viruses cause approximately 10 to 50% of total microbial mortality, depending on location, season, etc. These estimates are fairly robust, having been by determined several independent ways, as described in the following text.

Percentage of Infected Bacteria

This is based on the idea that infection is visible by TEM (i.e., assembled viruses within host cells) only at the last step before lysis. Observations from ocean waters ranging from the relatively rich coastal Long Island Sound to the oligotrophic Sargasso Sea showed that about 1 to 4% of the bacteria and cyanobacteria are visibly infected (40), and subsequent measurements from other habitats have similar observations (41). Although this percentage sounds low, it represents only the final stage of infection, which apparently covers the last 10 to 20% of the infection cycle as observed with pure cultures (42). The total infection rate has been calculated from the visibly infected fraction via a relatively simple model (40,42), and the final interpretation has been that the percentage of total mortality because of viruses is approximately 5 to 10 times the percentage visibly infected, or about 5 to 40%.

Viral Decay

If virus production is stopped but viral decay continues, then one may estimate the virus production rate that would be needed to maintain observed levels. Virus production is linked to mortality of hosts via lysis, and the burst size (number of viruses released per lysed host cell) is the conversion factor. Heldal and Bratbak (43) used this concept by treating seawater with cyanide to stop production, but allow virus destruction processes to proceed. Rate measurements from Norwegian coastal waters suggested virus turnover times on the order of a few hours. This method has sometimes implied rapid bacterial mortality considerably in excess of bacterial production (44), suggesting a substantial overestimate of mortality or underestimate of production.

Viral DNA Synthesis

Steward et al. (45) adapted a method that had been used to measure bacterial production with tritiated thymidine, and applied it to estimate viral production. The idea is that the appearance of nuclease-resistant label in viral size fraction after incubation with tritiated thymidine or ^{33}phosphate is indicative of viral DNA or RNA synthesis, and can be used to calculate viral production. An empirically derived conversion factor is used to calculate the production rate. This approach has been applied in Southern California (46,47) and also the Arctic. The results from these environments show a range of results, with viruses typically causing 5 to 50% of the total ascribed mortality of bacteria. Although some of the data may suggest higher percentages in richer coastal waters, there was no consistent pattern of the variation in this percentage with trophic status. The sensitivity level of this method was found to be most suitable for rich coastal environments.

Disappearance of Bacterial DNA in the Absence of Protists

An interesting approach to investigate bacterial mortality, by means of measuring the decay of labeled DNA, was developed by Servais et al. (48,49). In this approach, cellular DNA in natural communities is pulse-labeled with ^{3}H thymidine such that all the added tracer is taken up in a matter of hours. The subsequent decline of labeled DNA is thought to track bacterial mortality, on the presumption that DNA is not destroyed in healthy living cells. When protists are removed by size fractionation, the decline in labeled DNA has been considered an estimate of virus-caused mortality. Results from this approach in Southern California coastal waters have found that protist-free mortality is about half the total, and this has been interpreted as implying a significant viral impact (47).

Fluorescent Virus Tracers

This method is based upon the same idea as so-called *isotope dilution studies*, namely that one can measure both the production and loss rate of a substance if some of the substance can be tagged and the proportion of tagged and untagged can be monitored over time. In this case, fluorescently labeled viruses are made by concentrating native viruses from seawater and staining with SYBR Green I. These are added back to seawater that is incubated under simulated in situ conditions. Over time, the amount of added labeled viruses (no extra staining) and total viruses (stained just prior to counting with SYBR Green) are counted. Production of viruses adds unstained ones to the system, reducing the proportion of stained ones. However, removal of viruses takes away both stained and unstained ones, and this should reduce the number of stained viruses but not change the relative proportions. By means of calculations analogous to those used for isotope dilution studies, one can use these results to calculate simultaneously the production and decay rates of viruses (50). This method has measured virus turnover times in Southern California nearshore and offshore waters of about 1 to 2 days, estimated to cause the majority of the total bacterial mortality (50).

COMPARISON TO MORTALITY FROM PROTISTS

Given that the earlier thinking was that protists are the primary cause of bacterial mortality in marine

planktonic systems, it is reasonable to ask how the impact of viruses on bacterial mortality compares to that of protists. A few studies have addressed this question directly, by different approaches. One study, by Weinbauer and Peduzzi (51) used multiple correlation analysis of abundances of bacteria, viruses, and flagellates to conclude that virus-induced mortality of bacteria could occasionally prevail over flagellate grazing, especially at high bacterial abundances. However, a more direct approach is to use rate measurements. A few studies have compared virus-caused mortality with other causes directly and balanced total mortality and loss rates with independent estimates of bacterial production. One study used three virus methods (frequency of infected bacteria, virus production estimated with tritiated thymidine, and size fractionated disappearance of labeled bacterial DNA) and two protist methods (removal of fluorescently labeled bacteria, size fractionated disappearance of labeled DNA) simultaneously with California coastal waters, finding that the total mortality balanced production (thymidine and leucine incorporation methods) within 30%, and that the other methods agreed remarkably well; that study concluded that viruses were responsible for about 40% of the total mortality (47). Another study examined viral processes by two methods, frequency of infected bacteria and viral incorporation of labeled phosphate, and separately estimated protist-caused mortality in the Bering and Chukchi Seas (Arctic), finding that viruses and protists were responsible for a similar amount of bacterial mortality, with protists dominating in some water samples and viruses in others (29). In the latter study, the total mortality estimates typically fell short of balancing production estimates, often by greater than 50%. Those authors also concluded that the viral effect is probably larger in eutrophic than in oligotrophic waters. A freshwater study (52) made the comparison between virus-caused and grazing mortality of bacteria in the epilimnion (aerobic), metalimnion (boundary), and hypolimnion (anaerobic) layers of Lake Plussee in Germany. They found that mortality from viruses was strongly dominant in the hypolimnion and metalimnion (where protists do poorly because of oxygen deprivation), and that viruses were responsible for up to 30 to 50% of the mortality in the epilimnion, similar to marine plankton results.

Overall, the consensus is that viruses often are responsible for a significant fraction of bacterial mortality in marine planktonic systems, typically in the range of 10 to 40%. In some waters, viruses may dominate bacterial mortality, whereas in others they may have little impact on it. Some evidence suggests that the impact is usually higher in richer coastal systems than relatively nutrient-poor ones. But there are not enough studies to back up broad generalizations or address the reasons behind any patterns. An obvious factor would be the host abundance, because when hosts are rarer, the viruses are more likely to be inactivated before diffusing to a suitable host. Host abundance is a combination of total cell abundance (lower in oligotrophic systems) and quantitative species compositions, and one must also factor in the appropriate host range for the viruses. Of these three, we only really know the total abundance at this time. One may speculate that the bacteria in oligotrophic systems are probably more diverse than eutrophic systems (analogous to zooplankton and phytoplankton (53)), and this, plus lower bacterial abundance, would tend to work against viruses in oligotrophic waters. At this time, however, quantitative evidence on natural species diversity in most marine systems is not available. Also, there is a possibility that unknown factors, such as broadened host range, may compensate for changes in host diversity.

ROLES IN FOOD WEB

Our ideas about the roles of microorganisms in marine food webs has been revised considerably following the initial discovery of high bacterial abundance and productivity, and it is now well established that a significant fraction of the total carbon and nutrient flux in marine systems passes through the heterotrophic bacteria via the dissolved organic matter (7,54). How do viruses fit into this picture? Given the accumulated data that viruses can be major agents in the mortality of bacteria, one must ask how this mechanism may alter our view of matter and energy flux in the system. Focus has been given to three pertinent features of viruses, (*1*) small size, (*2*) composition, and (*3*) mode of causing cell death, which is to release cell contents and progeny viruses to the surrounding seawater.

Viruses and the cellular debris produced when a host cell lyses consist of readily used protein and nucleic acid, plus all other cellular components, in a nonsinking form that is operationally defined as DOM. This is composed of dissolved molecules (monomers, oligomers, and polymers) plus colloids and cell fragments. Recent publications have shown that the marine DOM pool contains readily detectable remains of bacteria, such as membrane porins and peptidoglycan (55,56). Whereas such fragments could conceivably be the result of grazing by protists, it seems probable that at least the more labile components (e.g., proteins) are free in the water as a result of viral lysis of bacteria, because a protist may tend to digest them rather than release them.

What becomes of this material released by lysis? The most likely assumption about its fate is immediate or eventual availability to bacteria (27,40,54). The release and availability of the lysis products to bacteria has recently been confirmed experimentally (57,58) although a few percentage of the viruses may be grazed directly by heterotrophic flagellates (59). If the cell lysed is a bacterium, then uptake by other bacteria represents a semiclosed trophic loop, whereby bacterial biomass is consumed mostly by other bacteria. Because of respiratory losses and inorganic nutrient regeneration associated with utilization of dissolved organic substances, this loop has the net effect of oxidizing organic matter and regenerating inorganic nutrients (Fig. 5) (27,40,54). This bacterial-viral loop essentially "robs" production from protists that would otherwise consume the bacteria (8), and sequesters the biomass and activity into the dissolved and smallest particulate forms. The net effect has been illustrated by a model, showing that a food web with 50% of bacterial mortality from viruses has 27% more bacterial respiration

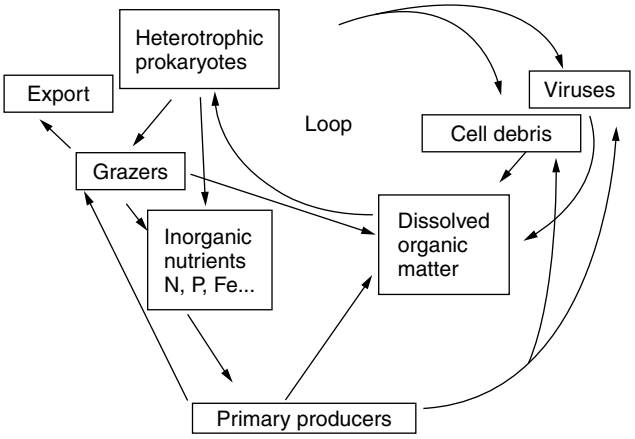

Figure 5. Bacterial-viral loop within the microbial food web. Arrows represent transfer of matter.

and production, and 37% less bacterial grazing by protists, culminating in a 7% reduction in macrozooplankton production, compared to the same system with no viruses (54). That original steady state model had only bacteria being infected and all the viral matter being consumed by bacteria (54). A modification of that model, now including a small amount of viral infection of phytoplankton (7% loss) and also flagellate grazing of 3% of the virus production, has essentially the same net effect of increasing bacterial production and respiration (by 33%) and reducing protist and animal production (60).

Sequestration of materials in viruses, bacteria, and dissolved matter leads to better retention of nutrients in the euphotic zone in virus-infected systems because more material remains in these small nonsinking forms (54). In contrast, reduced viral activity leads to more material in larger organisms that either sink themselves or as detritus, transporting carbon and inorganic nutrients to depth. This may be particularly important for potentially limiting nutrients like nitrogen, phosphorus, and iron, which are relatively concentrated in bacteria compared to eukaryotes. Thus, viral activity has the potential effect of helping to maintain higher levels of biomass and productivity in the system as a whole.

Lysis of organisms and release of their cell contents to the water have other potential geochemical effects, because of the chemical and physical nature of the released materials and the location in the water column where the lysis occurs. For example, polymers released from lysed cells contribute to small-scale viscosity of seawater that influences many biological and microscale physical-chemical processes, and may facilitate aggregation and sinking of material from the euphotic zone (61–63). On the other hand, viral lysis of microorganisms within sinking aggregates may effectively "dissolve" the particles, converting some sinking particulate matter into nonsinking dissolved material and colloids at whatever depth the lysis occurs (61). This contributes to the dissolution of sinking organic matter and its availability to free-living bacteria in the ocean's interior, as discussed by Cho and Azam (64).

The role of viruses in producing long-lived DOM is not currently known. This would probably mostly be from cell lysis products, because viruses themselves are protein and nucleic acid that are thought to be relatively labile, whereas bacterial cell walls and some other components are probably more recalcitrant to degradation. If a large fraction of the lysis products turn out to be relatively nonlabile materials, this would alter the quantitative aspects of conclusions made above, but most of the qualitative aspects would be the same. A change may be that any long-lived DOM could remain in the system a long time or be transported to deep or distant locations, to eventually fuel bacterial production.

EFFECTS ON SPECIES COMPOSITIONS

Viruses are density-dependent and generally species- or genus-specific. This combination means dominant bacteria are most susceptible to infection, and rare ones least so. Lytic viruses can only get ahead when the average time to diffuse from host to host is shorter than the average time they remain infectious. Thus, when a species or strain becomes more densely populated, it is more susceptible to infection, making viral infection work in opposition to competitive dominance (65).

This may have direct relevance to solving Hutchinson's "Paradox of Plankton," which asks how so many different kinds of phytoplankton coexist on only a few potentially limiting resources, when competition theory predicts one or a few competition winners (66). Although there have been several possible explanations for this paradox (67), viral activity may also help solve it, because as stated above, competitive dominants become particularly susceptible to infection whereas rare species are relatively protected. It may be expected that the same principle applies to bacteria in addition to phytoplankton, although we know much less about bacteria diversity.

At this time there is relatively little experimental evidence regarding viral control of species compositions. This is hardly surprising, given that we are now only starting to understand the species distributions and dynamics of marine bacteria. However, there are some data showing shifts in overall bacterioplankton community composition on scales of weeks to months (68,69). These reports indicate that the dominant members of the microbial community shift significantly over time. However, the cause of those shifts is not known. One report did correlate community composition with viruses. Waterbury and Valois (23) examined the relative abundance in temperate Atlantic waters (near Woods Hole, Massachusetts and also offshore) over several seasons of various culturable strains of *Synechococcus* cyanobacteria and viruses that infect them. They reported that although they calculated only a small percentage of *Synechococcus* were lysed daily, from less than 1 to about 3% per day, the culturable cyanobacteria tended to be resistant to the co-occurring phage. They concluded that the cyanobacteria affected the species or strain composition of the community more than the total abundance.

Thingstad and Lignell (70) have modeled the potential factors controlling aquatic microbial systems, regarding biomass and species compositions. Their models include growth limitation by organic carbon, inorganic phosphate,

or nitrogen (inorganic or organic), and cells losses include grazing by protists and viral lysis. Even when bacterial abundance is assumed to be controlled by protist grazing, the models have the robust result of showing that viruses control the steady state diversity of the bacterial community, whether bacterial growth rate limitation is by organic or inorganic nutrients. Thus, both empirical and theoretical analyses indicate a major role of viruses in regulating patterns of diversity.

RESISTANCE

The development of host resistance to viral infection is a common occurrence. Such resistance, whereby bacteria mutate to resist the viral attack, is well known from nonmarine experiments that usually involve highly simplified laboratory systems (71). It seems sensible to ask about its occurrence in nature. In the natural marine world, this phenomenon was cited to explain the results of Waterbury and Valois (23), discussed in the preceding text.

Along those same lines, Olofsson and Kjellenberg (72) have suggested that because of the development of resistance, significant mortality from marine viruses would be expected as a transient effect only as new virulent virus strains emerge. Suttle and coworkers (73) responded that in dynamic, diverse and sparse natural populations, other factors may control strain compositions more than resistance. It makes sense that natural systems with many species and various trophic levels have far more interactions than the simple laboratory systems. Obviously, a species with a large fraction of mortality for one type of virus benefits from developing resistance, and this must occur in nature. But resistance is not always a good thing. First, it often confers some competitive disadvantage via loss of some important receptor (9,71). Even complete resistance to viral attachment, without any receptor loss, if that were possible, would not necessarily be an advantage. For a bacterium in an oligotrophic environment whose growth may be limited by nitrogen, phosphorus, or organic carbon, unsuccessful infection by a virus (e.g., stopped intracellularly by a restriction enzyme, or with a genetic incompatibility) may be a significant nutritional benefit to the host organism, because the virus injection of DNA is a nutritious boost rich in carbon, nitrogen, and phosphorus (40)! Even the protein coat, remaining outside the cell, is probably digestible by cell-surface-associated proteases (74). In this situation, one may even imagine bacteria using "decoy" virus receptors to lure viral strains that cannot successfully infect them. Given favorable distributions of various types of bacteria and viruses, the odds could be in favor of the bacteria, and if an infectious virus (i.e., with a protected restriction site) occasionally gets through, the cell line as a whole may still benefit from this strategy.

Another consideration that works against resistance relates back to the system model results, showing that the heterotrophic bacteria as a group benefit substantially from viral infection, boosting their production significantly by essentially taking carbon and energy away from larger organisms. Recall also that viruses boost the entire system biomass and production by helping to maintain nutrients in the lighted surface waters. However, these arguments would require invoking some sort of group selection theory to explain how individuals would benefit from not developing resistance (i.e., why not "cheat" by developing resistance and letting all the other organisms give the group benefits of infection?). Nevertheless, for whatever reasons, resistance of native communities to viral infection may be common but cannot be close to complete because there is continued ubiquitous existence of viruses approximately 10 times as abundant as bacteria and with turnover times on the order of a day. Simple mass balance calculations, typically reported in several papers cited earlier on viral decay, show that significant numbers of hosts must be infected and releasing viruses all the time. For example, with a typical lytic burst size of 50 and viral turnover time of one day, maintenance of a 10-fold excess of viruses over bacteria requires 20% of the bacteria lyse daily. Whether lack of comprehensive resistance is due to frequent development of new virulent strains, rapid dynamics, or patchiness in species compositions, or to a stable coexistence of viruses and their hosts has yet to be learned. All these are possible and they are not mutually exclusive.

GENETIC TRANSFER

Viruses also can be agents of genetic transfer between microorganisms, through two processes. The more direct process is known as transduction, in which viruses package some of the host's own DNA into the phage head (sometimes with active phage genes and sometimes without, depending on the virus type) and then inject it into another potential host. Transduction in aquatic environments is frequent enough to measure, and has been demonstrated (75). Although transduction usually occurs within a restricted host range, a recent report by Chiura (10) indicates that some marine bacteria and phage are capable of transfer across a wide host range. A second mechanism whereby viruses mediate genetic transfer is by causing the release of DNA from lysed host cells that may be taken up and used as genetic material by another microorganism. This latter process is called transformation. Although the extent of these mechanisms in natural systems is unknown at this time, they could have important roles in population genetics, by homogenization of genes within a potential host population, and also on evolution at relatively long time scales. Horizontal gene transfer is an integral component of microbial evolution, and the genomes of modern-day microbes contain numerous genes that have obviously been transferred from other species (76). On shorter time scales, this process is of interest in the possible dissemination of genes that may code for novel properties, whether introduced to native communities naturally or via genetic engineering.

CONCLUSION

There is now substantial evidence that viruses are important players in the control of marine microbial

systems. A major effect is on mortality of bacteria and phytoplankton, with the expected result of stimulating bacterial activity at the expense of larger organisms and also stimulating the entire system via aiding retention of nutrients in the euphotic zone. Other important roles include influence on species compositions and possibly also genetic transfer. Much of this work is still in its infancy, and a great deal has yet to be done.

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VIRUSES IN WASTE STABILIZATION PONDS.

See WASTEWATER STABILIZATION PONDS

VIRUSES: ROTAVIRUSES.

See ROTAVIRUSES

VOLCANIC TUFFS: DEEP SUBSURFACE MICROBIOLOGY OF

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Much of the impetus behind subsurface research is fueled by the fact that alterations of subsurface environments can be attributed to the presence or activity of bacteria (1). In addition to the role that microbes play in biogeochemical cycling, subsurface bacteria are known to be important in processes varying from the souring of oil (2) to the degradation of toxic materials (3–5). To successfully prevent or alternatively encourage microbial activity in these situations and to effectively monitor the results of remediation or other projects in the subsurface environment, an understanding of basic microbial ecology is essential. The Department of Energy (DOE) developed a Deep Subsurface Microbiology Subprogram in 1986 (a part of the Subsurface Science Program) in an effort to better understand the microbial ecology of subsurface environments. They envisioned the use of microbes for cleanup efforts at contaminated DOE facilities (6). In the first phase of the program, several boreholes were drilled at the Savannah River Site (SRS), South Carolina, with the specific intent of studying microbiota and the sediment in which they were entrained. New drilling technologies, utilizing conservative tracers for quality assurance, were subsequently developed and employed at two western Department of Energy (DOE) facilities, The Hanford Reservation (HR), Washington, and the Idaho National Engineering Laboratory (INEL) in Idaho Falls, Idaho (7,8). The Nevada Test Site (NTS), was selected as an additional site for characterization, providing an opportunity to sample without the necessity of drilling. Our research team investigated the microbiology of volcanic tuffs at the NTS. The data summarized or referenced herein describe the investigations of mined volcanic tuff materials and their endolithic microbial communities.

The NTS is located approximately 60 miles northwest of Las Vegas, in the southern tip of Nevada. At the NTS, 26 km of tunnel systems from 50 to 450 m depth were accessible, and importantly, samples of rock (volcanic tuff) could be collected from the sides of the tunnels without drilling. Drilling is technologically complex and time consuming, thus expensive, and drilling fluids are a known source of contamination. This site afforded the opportunity for sampling in three-dimensions on large (km) and comparatively small (m) scales. Because large amounts of rock could be obtained, it also allowed for microbiological, geochemical, and geological analyses as well as investigations of perturbation phenomena. Additionally, Rainier Mesa serves as a structural analog to Yucca Mt., the nation's proposed high-level nuclear waste repository, and therefore, information obtained from research on Rainier Mesa could serve to enhance microbial research efforts in the Yucca Mt. Project (See NUCLEAR WASTE RESPOSITORY IN YUCCA MOUNTAIN: MICROBIOLOGICAL ASPECTS, this Encyclopedia).

PILOT INVESTIGATION

Of primary concern in the first NTS sampling effort, was whether viable microorganisms would be recovered from the volcanic tuffs of Rainer Mesa. In a pilot study (9), culturable organisms were recovered using several media from one water sample and three rock samples, one from each of four locations within Rainier Mesa 12 n tunnel system (approximately 400 m deep). Purified isolates were

morphologically and physiologically characterized and it was demonstrated that different types of microbes were isolated from the four locations within one tunnel system. Further, it became obvious that conventional identification techniques such as API-rapid NFT strips (21 physiological tests), BIOLOG (96 physiological tests) identification plates, and MIDI (fatty acid methyl ester) analysis either failed to provide identifications that were acceptable or the systems did not agree as to the identification of the isolates. The pilot study led to three roads of investigation: (*1*) microbial characterization of the subsurface tunnel systems within Rainier Mesa, (*2*) the determination of microbial community change during sample storage, with special emphasis on changes that occur in the culturable community, and (*3*) the possibility that endolithic microorganisms have been isolated from the surface for long periods.

SAMPLE COLLECTION

The extensive tunnel systems within Rainier Mesa at the NTS provided a unique opportunity to study the microbial ecology of the deep subsurface without the need for drilling. Samples were collected by chipping into existing rock faces of tunnels with sterile tools to create a fresh sampling face. Rock was then aseptically chipped into sterile containers for rapid transport to the laboratory in coolers containing ice (10). Research described later and that of others has demonstrated the importance of immediate analysis to recover microbial communities representative of the in situ environment (11–16).

An Alpine miner (Fig. 1) was a valuable tool that aided the recovery of representative subsurface samples. The miner removed cubic meters of rock in minutes, and thus allowed sampling deeper into rock faces without the concern of contamination by drilling fluids. Others have shown that temperatures in excess of 40 °C may decrease recovery of viable microbes (17), and because the miner removed cubic meters of rock in minutes, the production of excessive heat was also avoided. While comparing coring methods with use of the Alpine miner, coring was found to be more technically challenging, although similar abundances and types of microorganisms were recovered from cores when compared to rock samples recovered with the Alpine miner (18). The recovery of large volumes of material from the tunnels permitted extensive geological/hydrogeological analysis of rock material (19). Samples could also be taken in a three-dimensional sampling scheme such as the one used in analysis of the microbiota within a 21-m^3 rock section (20). In this work, a statistically designed sampling array was selected for the analysis of 19 samples within the rock section (Fig. 2). Microbial distribution on a vertical scale had been described by others involved in drilling operations, but work describing lateral heterogeneity relied on samples taken within the same geological formation but within different boreholes that may have been quite distant from each other (7,21,22). Other investigations of lateral heterogeneity have been reported (23).

Sampling outcrops of Rainier Mesa proved to be a viable method of obtaining samples for microbiological

Figure 1. The Alpine miner was used to create passageways within the subsurface of Rainier Mesa, Nevada Test Site (**a**). The carbide bits on the rotating heads allowed rapid removal of relatively large volumes of rock without the necessity of drilling fluids or the production of excessive heat (**b**). After removal of large portions of rock, the Alpine miner was backed out of the alcove, and hand sampling was conducted with sterile implements (**c**).

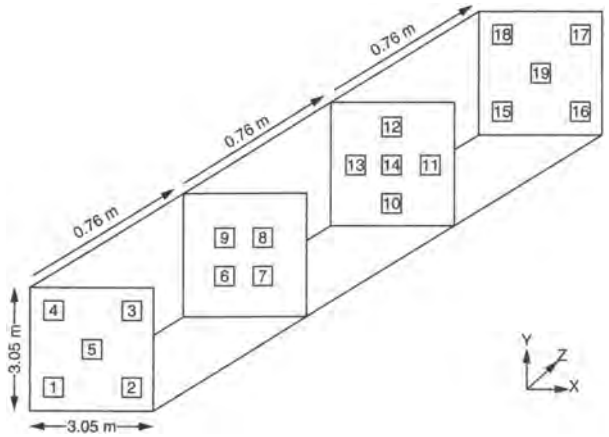

Figure 2. Sampling design showing three-dimensional array from which 19 samples were collected within a 21 m^3 section of subsurface rock.

analysis on a vertical scale at the NTS (Fig. 3). Although samples could be procured in a directly vertical axis within the tunnels, the sample range was limited by the height of the tunnel itself. When sampling between geological formations was desired over a larger vertical range, exposed outcrops on the exterior of Rainier Mesa were sampled for comparison with those collected within tunnel systems (24). Additionally, because tunnel systems were mined at various locations and elevations within Rainier Mesa, comparisons could also be made between tunnel systems. Because rock strata within Rainier Mesa are nearly horizontal (25), an elevational gradient also served as a chronosequence of depositional history. Others had reported the recovery of representative material for microbiological analysis by hand sampling into outcrops (14,26,27) or drilling horizontally into surface sandstone using sterile water as a lubricant (17).

Because sampling by mining was a relatively untried approach to sample acquisition in the subsurface, a series of controls were developed to insure that material

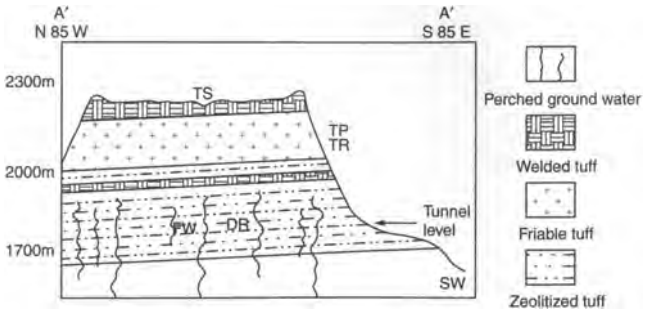

Figure 3. Cross section of Rainier Mesa depicting layering of volcanic rock and sampling locations. TS (topsoil), TP (top paleosol), TR (top rock), FW (fracture water), DR (deep rock), and SW (spring water). (See Table 2 for geological description of samples).

representative of pristine rock was obtained. Table 1 lists types of measures undertaken to ensure quality sampling.

MICROBIAL CHARACTERIZATION OF TUNNEL SYSTEMS

Strategies for Surveying Indigenous Microbial Communities

Samples of volcanic tuff were obtained from tunnels, U12 b, U12 g, U12 n, and U12 p to conduct a large-scale survey of microbial distribution (1 m–2 km) and from a variety of rock strata, including those within saturated and vadose zones (Fig. 3; 10). Physical and geological information is provided in Table 2. As a part of this tunnel survey, several different plating and enrichment media, and incubation temperatures were employed to recover maximal microbial diversity. Researchers have demonstrated that media composition affects the recovery of heterotrophic microbial communities, and specifically that minimal media may allow better recovery of microorganisms from oligotrophic environments (33–36). Fluorescent direct counting techniques and the determination of total biomass by phospholipid extraction (and subsequent determination of liberated PO_4^{3-}), were used to determine the abundance of nonculturable organisms in the tunnel characterization research (10).

A second experiment was conducted to describe the distribution of microbiota in three-dimensions on a smaller scale, specifically within a 21 m^3 section of rock (20). Analyses of bacterial isolates that were recovered from the rock section included MIDI analysis (fatty acid methyl ester/gas chromatography). An important advantage of MIDI analysis was its ability to cluster isolates into groups of related organisms such that the distribution and diversity of microbes within the rock section could be discerned, even if they could not be individually named. Microbiology and geology/geochemistry were compared as a part of this sampling effort to determine if hydrogeological/geochemical factors might be predictive of microbiological abundance or diversity (19).

Another investigation was initiated because geochemical/geological data indicated that the zeolitized rock beds of Rainier Mesa allowed negligible water flow, except in fractures, and thus probably limited microbial transport to depth in the recent past. Further, initial characterization of organisms recovered from zeolitized rock in the pilot study suggested that some isolates were capable of long-term starvation survival (37). These organisms demonstrated patterns of survival similar to those reported by others investigating long-term survival in marine and soil environments (38–40). Thus, microbiota were sampled in a vertical gradient as described earlier. Additionally, fracture water and outflow spring water were compared (24), as well as the microbiota occurring within and away from a free-flowing fracture. The hypothesis was that if surface microbiota had been rapidly transported to depth in fracture water, similar microbial communities would likely

Table 1. Integrity of Mined Rock Samples from Rainier Mesa

Approach	Justification	Reference
Swab cultures of rock surfaces	To compare surface microbiota to that found deep within the tunnel rock	9
Fungal monitoring	A biomarker of surface contamination and microbial migration potential through the rock matrix	20,28,29
Morphological and physiological profiling of recovered microbiota	Comparison of surface and rock matrix microbial properties	18,19,24
Fall-out plates for collection of airborne bacteria	Microbial contamination that may have been introduced with dust particles	9
Fluorescent microbeads as tracers	To determine if surface microbiota were transferred into the deeper rock during sampling	30,31
Investigations of microbial transport through zeolitized rock cores	To determine the potential for microbial movement through zeolitized tuff	25,32
Sampling and analysis of sterile rock material	Rock samples were either innately devoid of culturable microbiota or were purposely sterilized to monitor aseptic laboratory handling	10
Isotopic measurements of pore water	To determine pore water evaporation and microbial isolation	19

Table 2. Physical and Geological Characteristics of Tuff Samples from Rainier Mesa Tunnel Systems

Sample	Temp. (°C)	Depth of Overburden Surface (m)	Gravimetric Moisture (%)	Sample Type	Distance to Portal (km)	Reference
B	17	50	5	Vitric, Oak Spring, Tunnel Bed 5 (fine grain)	0.1	10,27
P1	24	250	4	Vitric, Oak Spring, Paintbrush Tuff (coarse grain, silicified)	0.41	10,27
P2	24	250	6	Vitric, Oak Spring, Paintbrush Tuff (coarse grain, friable)	0.57	10,27
N	18	390	23–25	Fine to coarse grain, tunnel bed 4 k	1.8	10,19
G1	16	450	11	Vitric, Oak Spring, Tunnel Bed 4h (zeolitized grading to vitric)	1.3	10,27
G2	15	430	22	Vitric, Oak Spring, Tunnel Bed 5 (zeolitized grading to vitric)	1.6	10,27
DR	15	400	13	Zeolitized tuff	N/A	24
FW	16	400	100	South Drift Tunnel μ12n, fracture water	N/A	24
SW	16	0	100	Spring water outflow from Rainier Mesa	4.8 from Rainier Mesa	24
TR	25	2,050 m elevation, 250 m above subsurface samples	6	Friable Tuff, Rainier Mesa surface	Exposed outcrop	24
TP	26	2,060 m elevation, 260 m above subsurface samples	9	Paleosol, Rainier Mesa surface	Exposed outcrop	24
TS	26	2,200 m elevation, 400 m above DR	3	Surface soil from Rainier Mesa	Exposed outcrop	24

N/A = unknown

be recovered. Tritium dating of fracture water suggested travel times of 6–30 years to reach N tunnel depth (25). Investigation included comparison of most probable number determinations (MPN) utilizing several media, total cell enumerations, and the comparison of culturable microbiota (24).

Microbial Distribution

It is now commonly accepted that a wide range of microbial types inhabit the deep subsurface and that they do not necessarily decrease in abundance or diversity with depth (7,21,41). Likewise, this holds true in the subsurface of Rainier Mesa. We recovered diverse microbial communities from depths to 450 m, containing up to 1×10^4 culturable heterotrophic organisms per gram of rock (10). Within Rainier Mesa, microbial communities appear to be distributed in a heterogeneous manner as has been described for other terrestrial subsurface environments (14,21,22,26,42–44). In our studies heterogeneous distribution was demonstrated to be as great between samples taken 1 m apart as those taken from diverse tunnel systems up to 10 km apart.

We demonstrated that different bacterial types were recovered from clay, water, and rock samples taken in very close proximity to one another, some samples less than a meter apart (10,20,24). Other researchers have also demonstrated differences between the type of microbes recovered from geological formations with various physical characteristics (21,26,43). However, while comparing recoverable microbiota to geological and/or hydrological properties of the terrestrial subsurface environments, few predictive relationships have been found. Fredrickson and coworkers (45) found lower abundances of microorganisms associated with clay layers as compared with sandy strata. In research conducted at Hanford, sediment texture and organic carbon were related to microbial abundance (26,45). Few predictive relationships were found in our investigations of Rainier Mesa, although 1,700 correlations between microbial, hydrogeological, and geochemical parameters were investigated (19). One of the few significant correlations was between levels of nitrate and bacteria positive for nitrate reduction (19). This seemed counterintuitive because bacteria capable of reducing nitrate were present in rock strata with high nitrate levels. It is probable that these microbial populations were limited by some other parameter. For example, the lack of an available carbon source would prevent use of the nitrate as an electron acceptor or as a nitrogen source.

Lack of trends or correlations between microbiology and hydrogeological/geological parameters provided evidence for the isolated nature of subsurface microbial communities in the zeolitized rock beds of Rainier Mesa [zeolitized rock has negligible water permeability (32)]. First, the lack of spatial trends in the distribution of microbiota indicated that no horizontal or vertical transport of bacteria was likely. One would expect more homogeneity in bacterial colonization if connections between rock pores would have

allowed for water, nutrient, and microbe transport. Experimental work involving determination of transport rates of bacteria through tuffaceous cores, including zeolitized tuff, supports this hypothesis (32).

Perhaps microorganisms recovered from the deep subsurface of Rainier Mesa were dormant bacterial cells; bacteria that colonized and adapted to the environmental conditions of the rock matrix in a time past, but were not actively growing/metabolizing at the time of sampling. Pore water in zeolitized tuff beds, sampled at 400 m depth in Rainier Mesa, has been age-dated to at least 250,000 years ago, whereas free-flowing fracture water is much younger (9). These bacteria may exist as dormant vegetative forms that are not currently influencing or being influenced by their geochemical environment. Other subsurface environments may also contain organisms that have been isolated from the surface for extensive periods (27,40,46).

Microbial Diversity

Heterotrophic bacteria have been recovered most commonly within Rainier Mesa, although anaerobic (47,48), microaerophilic (45), phototrophic (49), and lithotrophic (45) bacteria have been recovered from other subsurface environments. Other microorganisms, including fungi, protozoa, algae (50), and viruses (51), that have been recovered from other subsurface environments were not cultured from Rainier Mesa samples. Most probable number enrichments were used to attempt to recover phototrophic, sulfate-reducing, sulfur-oxidizing, iron-oxidizing, and nitrifying bacteria, but a number of these bacterial types were below the level of detection ($<$10/g) under the culture conditions used (10). Chemolithotrophic and sulfate-reducing bacteria have been recovered from a similar subsurface environment, the volcanic tuffs of Yucca Mountain, a proposed nuclear waste repository in southern Nevada (52,53).

The total numbers of bacteria and biomass estimates based on the extraction of phospholipids indicate that only a fraction of the microbial community was recovered with our standard culture conditions on R2A agar (DIFCO) with incubation at 24 °C for at least 14 days (10,20,24). Other media and incubation temperatures were used in initial experiments (as well as MPN enrichments) to enhance the recovery of greater bacterial diversity: malt extract agar, starch casein agar, starch, casein agar containing rifampicin, 0.1% R2A, and incubations at ambient rock temperature (16–18 °C), 24 °C, or elevated temperature treatments (60 °C for 1 hour, or 80 °C for 3 min.) followed by standard incubation to select for the recovery of spore-forming organisms. Bacterial incubation on R2A agar at 24 °C provided the best recovery (highest abundances and greatest diversity), and was subsequently used for all remaining investigations. R2A has been used by other microbiologists to investigate oligotrophic environments (34,54–57). Other researchers in the DOE Subsurface Science Program have also used a complex, but low nutrient medium (1% PTYG).

Most of the heterotrophic bacteria that have been isolated from the deep subsurface of Rainier Mesa have not been identified with a high degree of certainty by any identification system. This is not surprising as the databases used, and most in existence, were developed for the identification of bacteria of clinical importance. We have found that phylogenetic identification (partial sequencing of 16s rRNA gene and comparison of sequences to known databases) is the most definitive means of identifying bacteria to the genus level. Previous work in the subsurface of SRS relied on physiological characteristics generated from API-Rapid Nonfermenting test strips [subsequent to these investigations, much has been published about phylogenetics of subsurface microbial isolates (58–62)]. This method of comparing bacteria has limited utility in some instances because many bacteria that have the same API profile are different by genetic methods (63). However, use of the MIDI (Microbial Identification System, Co.) is a more time/cost efficient method of determining the identification of strains, and the relatedness of isolates to both known strains and to each other. This latter ability of the MIDI system has proved invaluable in describing culturable heterogeneity of microbial distribution, which would have been impossible if species identification of all or even most of the isolates had been necessary.

The bacterial genera recovered from Rainier Mesa fell into several broad groups based on fatty acid methyl ester (FAME) profiles, which clustered at Euclidean distances $\leq$45, and include *Methylobacterium*, *Micrococcus*, *Arthrobacter*, *Bacillus*/*Xanthomonas*, *Nocardiodes*, unmatched (2 groups), Acinetobacter/Gordona, and *Pseudomonas*/*Hydrogenophaga*/*Acidovorax* clusters (11–13,20). The most numerically abundant cluster was the *Arthrobacter* and mixed gram-positive bacterial groups, which contained 33% of all isolate types. The 16s rRNA gene of several isolates from the unmatched groups have been partially sequenced (62). Comparison of the sequences to then current databases indicated that these bacteria were related to known bacterial genera, including *Arthrobacter*, *Terrabacter*, *Rhodobacter*, *Mycobacterium*, *Bacillus*, and *Sphingomonas*, but it is still not clear why these bacterial types did not cluster with known organisms. Perhaps more extensive sequencing or comparison to updated databases will be required to document whether these organisms are very different, although related to common species. Perhaps a more detailed knowledge of their physiological and FAME profiles will shed light onto the classification of these organisms. Preliminary work along these lines suggests that some of the previously unidentified organisms belong to the genus *Terrabacter*, but are most likely species not described as yet.

The MIDI growth regime required incubation of cultures on trypticase soy agar (TSA) for 24 hours as a standard procedure because bacteria are known to change the composition of membrane lipids based on their growth stage/dormancy stage, temperature and nutritional conditions (64,65). While some of the bacterial types recovered on the low nutrient media could be cultured on nutrient-rich media after initial isolation, some could not, and these may truly represent oligotrophic organisms (56). Unfortunately, classification of these organisms by growth characteristics or by MIDI analysis remains a formidable task unless new libraries can be

created that are based on data developed from the use of low nutrient media.

Of the bacterial diversity that was recovered, representative colony types were selected for further characterization. It has become important to test the validity of selecting a representative colony type for analysis on the basis of colony morphology. An experiment was conducted to determine the extent of diversity within a single visual morphotype to determine if much of the potential diversity was ignored by selecting only one isolate from many, which looked identical. Although diversity is lost while using an approach such as the selection of a representative colony by morphotype (66,67), isolates from the NTS that appeared similar on initial isolation plates were all related at the genus level; most to the species level or closer (68). Thus, the selection criteria used in the initial (and subsequent) site characterization undoubtedly underestimated total recoverable diversity, but did provide a method for the selection of microbiota for study that could be feasibly analyzed with available resources and allow comparison between sampling sites. This was necessary in order to reduce the number of isolates to a manageable collection of strains, and has been used by other subsurface researchers (21,43).

STORAGE-RELATED PHENOMENA

For research of storage-related phenomena, particular advantages of sampling in Rainier Mesa at the NTS included, the proximity to the laboratory and the large volumes of rock that could be obtained. Because we realized that storing samples before analysis would affect the recovery of microbiota and distort descriptions of in situ bacterial communities, we became interested in learning more about storage-related phenomena as they pertained to protocols for the handling and transport of subsurface material. Investigations of the magnitude of community shifts during storage included (*1*) the time course of microbial change, (*2*) the effects of temperature and sample perturbation on community structure, and (*3*) the impact of the physical characteristics of sample materials (e.g., rock, clay, moisture content) on community change. Further, we were interested in ecological aspects of community change. Which microbial types were active in community change? What characteristics of microbial types appearing after different storage times allowed them to be recovered or detected in higher numbers during storage? Were changes in the abundance of culturable organisms due to the outgrowth or resuscitation of specific bacterial types? Results of investigations concerning these questions are presented in this section, while ecological aspects of storage phenomena are presented in the following section on microbial survival.

An understanding of microbial change in stored samples is of paramount importance to subsurface researchers and those interested in bioremediation. To adequately determine relationships between microbes and their environments, in situ samples need to be analyzed with minimal perturbation and delay. The microbial communities in samples stored, even for a short period can change, and this will distort an understanding of the native microbial community. The simple act of sampling irrevocably changes the community because the biotic and abiotic microenvironments associated with each organism are altered. For example, when rock is removed in small chips from the walls of the tunnels, potential changes in water, temperature, and exposure to oxygen could result (13).

One of the effects of perturbation is that microbial activity can be stimulated (11–14,69,70). This effect may be of great utility to bioremediation efforts that intend to activate in situ subsurface communities. We have observed that bacterial communities change in response to sample storage, especially increasing in culturable bacterial numbers without the addition of nutrients. Others have also demonstrated that the activity of microbial communities is stimulated by unknown aspects of sample storage or handling (14,71). Nutrients or moisture may not be limiting in some subsurface environments, but rather their bioavailability may be (69). Perturbations created during sampling may serve to redistribute moisture, nutrients, and bacteria, thus providing substrates for enhanced microbial activity. The increase in abundance observed after storage may in part be due to the outgrowth of microbes that were physically located in microenvironments, especially those in nontransmissive environments such as the zeolitized tuffs of Rainier Mesa where nutrient flux is believed to be minimal.

Evidence suggests that several mechanisms of change occur in subsurface samples, including the outgrowth of specific microbial types and the resuscitation of dormant organisms (11–13). To determine which microbial types changed in abundance throughout storage, dendrograms (see Fig. 4) were used to compare organisms recovered before and after storage. Additionally, physiological characterization and growth rates of isolates recovered at different times throughout storage were compared to determine if specific bacterial types recovered after sample storage had a competitive advantage; either the ability to use a wider array of carbon substrates or faster doubling times (12). Phospholipid fatty acid methyl ester (PLFA) analysis was employed to determine entire community shifts in phospholipid composition as compared to changes in culturable communities. PLFA analysis was then a relatively new technique that was increasingly utilized to characterize environmental samples. The types and abundances of fatty acids recovered in a chloroform/methanol extraction were used to determine microbial abundance, inclusive of viable, culturable, and dead cells, as well as the presence of specific microbial groups based on signature fatty acids (11–13).

When samples must be stored before microbiological analysis can be initiated, storage at a cool temperature is a standard procedure in ecological studies (72,73). A temperature of 4°C has commonly been employed to inhibit the outgrowth of organisms, whereas colder temperatures have been avoided to prevent membrane disruption associated with freezing. Research conducted on Rainier Mesa samples demonstrated that microbial diversity decreased in many types of samples stored at 4°C. Larger numbers of culturable organisms were recovered, but fewer types of organisms were evident

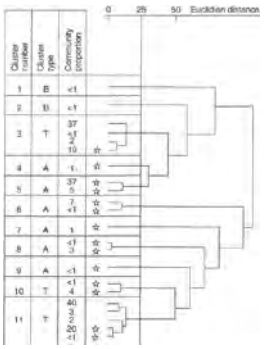

Figure 4. An example of a dendrogram that was used to compare organisms recovered before and after storage. Dendrograms were created from comparison of fatty acid methyl ester analyses of individual isolates. A Euclidean distance of ≤ 25 approximates relatedness at the genus level. The stars represent organisms recovered after one week of sample storage. Cluster type designations describe whether organisms were isolated only before (**b**), only after (**a**), or at both time points (T) of sample storage.

after storage. The time course of microbial change was as soon as 24 hours after sampling and change continued in the community throughout 45 days (12). Others have noted changes up to several months, but short-term changes were not reported (14,74,75). The temperature of sample storage affects the magnitude of microbial change. For samples stored at 24 °C, more dramatic increases in abundance and community shifts were observed during shorter periods than at 4 °C. Culturable counts remained constant in samples stored at freezing temperature (−20 °C), indicating that this temperature inhibited increases in microbial abundance. However, it should be noted that the types of bacteria recovered throughout storage at −20 °C demonstrated that the microbial community underwent structural shift although bacterial numbers remained relatively constant (12).

The magnitude of microbial change appears to be dependent on the type of material sampled. The largest increase in culturable counts has been observed in water samples, and in some cases, a direct relationship to the magnitude of change and moisture content has been demonstrated (15). Brockman and coworkers (14) demonstrated that paleosol samples with a higher moisture content exhibited a greater change, and while comparing volcanic rock samples from NTS, a similar trend was observed among samples of a similar rock type. However, a clay sample with a 35.1% moisture content exhibited less change than rock samples containing 4–10% moisture, and therefore, moisture was not the only factor that contributed to the magnitude of change.

The addition of moisture to homogenized rock led to increased abundance and decreased diversity in a shorter time than with unamended samples (11). In one experiment, rifampicin was added to inhibit the outgrowth of bacteria in stored samples, and in comparison to a control without rifampicin, microbial change was reduced. However, experiments such as these were not continued because the initial recovery of microbial diversity was diminished with rifampicin addition. Other physical properties of samples are no doubt important as well as moisture content: nutrient concentrations, permeability, levels of physical disruption, and so on. Others have shown that samples stored in 5 to 10 g intact pieces demonstrated less increase in abundance during storage as compared with crushed or ground rock samples (14,15). However, it is important to note that the shift in the types of bacteria recovered was just as great in the intact sample from Rainier Mesa as compared to the replicate sample that was ground and homogenized in our experiments.

LONG-TERM SURVIVAL OF SUBSURFACE BACTERIA

Dormant, Dead, and Viable but Nonculturable (VBNC) Microbial Biomass

The numbers of cultured bacteria obtained from Rainier Mesa samples as compared to measurements of total cell numbers or total biomass, suggest that not all microbial diversity was recovered during routine sample analyses. If the environments sampled were static and ancient as evidence suggests, it would not be surprising to find large numbers of dormant, dead and/or VBNC bacterial cells.

Dormancy is often associated with cellular survival under conditions of low nutrient availability (38–40,56,65,76). Endolithic Rainier Mesa isolates demonstrated starvation-survival patterns indicative of long-term survival (37,38,40). Measurements of diglyceride fatty acids (DGFA), indicators of dead biomass, suggested that large numbers of dead cells were present in the rock microbial community (12).

VBNC bacteria may be described as cells that are nonculturable under the current growth regime, injured or dormant (76,77). Experiments described in the following section provide evidence for the existence of VBNC bacteria in Rainier Mesa samples. Different cell types were recovered by the use of various growth media and incubation regimes. Obviously then, any one count of culturable bacteria recovered under a single set of conditions would underestimate the total number of viable bacteria in the sample and may include organisms that were VBNC under another set of conditions. This was demonstrated when heterotrophic bacteria were cultured from turbid but negative MPN tubes designed to enrich for chemolithotrophic bacteria. For example, ammonia and sulfur-oxidizing media were negative for chemical conversion but heterotrophic bacteria could be cultured from them after six weeks incubation. Heterotrophic cells were recovered on R2A agar from MPN tube inocula, and yet they were morphologically and physiologically different from cells recovered from the same samples when they were initially plated on R2A. This suggests that the new cell types were capable of growth under the conditions provided (R2A with 24 °C incubation) but only subsequent to certain unknown conditions having been met. Perhaps they were injured or dormant cell types that required resuscitation before becoming culturable. An alternative hypothesis is that perhaps outgrowth of these organisms from very small populations (during MPN tube incubation)

was required before sufficient cell numbers were available for detection by plating.

Some storage-related phenomena suggest that dormant cell types were present in deep subsurface rock as well. Not all heterotrophs were recovered with initial R2A plating. Following storage of rock samples, new bacterial types were recovered under the same culturing conditions. Some unknown process (es) may have allowed some cell types to be detected after sample storage, but not during initial isolation. One possibility is that rare bacterial types outgrew during sample storage (or in MPN tubes) and that death or lack of growth of the previously more dominant bacterial types caused an observable community shift.

Several important factors make it appear as if alternative events to outgrowth might have occurred. First, samples were stored at a restrictive growth temperature without the addition of nutrient sources. These factors should have limited microbial activity and growth. Second, some, but not all of the bacteria recovered after storage exhibited enhanced growth rates and enhanced ability to use a variety of carbon sources when compared to those recovered upon initial plating (11). Those bacteria with lower growth rates or less nutrient flexibility would have been less likely to scavenge nutrients and outcompete the initially recovered microbiota. Perhaps these bacterial types were relatively abundant in the original sample, just initially VBNC.

In summary, some cells recovered from the NTS rock samples may have been dormant bacterial populations that were resuscitated due to some aspect of sampling or sample handling. Reports of vegetative dormant bacterial populations or VBNC bacterial cells existing in natural environments have increased and include bacteria from soil and aquatic environments (74,77–82). Factors that have been demonstrated to awaken dormant cell populations or resuscitate injured cells include the use of nonselective media (79,80), and temperature (83,84) or osmotic shifts (85). Changes in all of these parameters probably occurred in NTS rock samples due to the rearrangement of water, nutrients or bacteria, during perturbation and storage of samples.

CONCLUSION

Microbiota recovered from the deep subsurface of Rainier Mesa represent a diverse set of aerobic heterotrophic microorganisms. Cells were found primarily in the vegetative form. Some were related to common surface soil genera, whereas some are not known as yet. The numbers, types, and distribution of organisms suggests that more microbial diversity could have been recovered from this deep subsurface environment. We found evidence for the existence of dormant, dead, and VBNC bacteria in subsurface tuffs of Rainier Mesa. Subsequent research at a structural analog site supported these findings in that rDNA isolated, cloned and sequenced from the subsurface at Yucca Mt. included many noncultured genera. The nature of cell physiological status is currently under investigation by many laboratories. It has implications for health, bioremediation and microbial ecology in general.

The NTS provided a unique opportunity to investigate heterogeneity at many levels. We discovered that microbial communities located within meters of each other were as different from one another as those located kilometers apart. Heterogeneity and the effects of sample storage and perturbation on subsurface endolithic bacteria are important to those interested in recovery of representative microbiota from natural environments and for those interested in bioremediation of the deep subsurface. These studies will also aid in the design of the nation's high-level nuclear repository, which is proposed to be built in the volcanic tuffs of the NTS.

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WASTEWATER AND BIOSOLIDS AS SOURCES OF AIRBORNE MICROORGANISMS

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It is well known that municipal wastewater and wastewater residuals contain a variety of pathogenic (disease-causing) microorganisms (1). When sewage from a large population is collected and treated in a centralized location or when reclaimed wastewater or wastewater residuals are recycled and applied to agricultural fields, the public health must be protected by reducing public exposure to these concentrated sources of pathogens. One potential pathway for transmission of these pathogens to the public is the aeromicrobiological pathway (2). The aeromicrobiological pathway includes aerosolization of the microbial pathogens from the wastewater source, followed by atmospheric transport (usually associated with wind currents), and eventual deposition possibly in the lungs of a susceptible human. (See: Bioaerosols: Transport and Fate, this Encyclopedia).

HISTORY OF AEROMICROBIOLOGY AND WASTEWATER

The initial link between wastewater and disease transmission dates back to John Snow (3), who showed that a cholera epidemic sweeping London was linked to the use of drinking water contaminated with sewage. He noticed that cholera occurred much more frequently in customers of the Southwark and Vauxhall Water Company. This water company drew its potable water from the lower Thames. At the time, London's sewage system emptied directly into the Thames, upstream of where Southwark and Vauxhall withdrew their water. Snow, eventually convinced London officials to remove the pump handle from a well used by a district in London, where there were an increased number of cholera cases. On removal of the pump handle, which prevented use of the well, there was a significant decrease in the number of new cholera cases. This essentially confirmed that cholera was spread via water contaminated with sewage.

Soon after Snow (3) published his epidemiological breakthrough related to waterborne transmission of cholera, another scientist, Louis Pasteur, initialized a new field of biological study. Pasteur was able to show, that invisible airborne particles termed *germs* were responsible for mysterious fermentative reactions in his laboratory experiments. These unexplained fermentations were up to that point considered by many other scientists to be a spontaneous generation of life (4). This field of biology has come to be termed *aeromicrobiology* or the study of the aerosolization, transport, and deposition of microorganisms.

Bringing the work of John Snow and Louis Pasteur together, Horrocks (5), evaluated aerosols generated in sewers, concluding that bacteria originating from sewer pipes could be launched into the air by the action of bursting bubbles. This may have been one of the first aeromicrobiology studies related to wastewater, providing initial interest in the potential for the spread of airborne disease, originating from sewage. Unfortunately, interest in this branch of science was temporarily quenched in 1917, when investigations by the Committee on Standard Methods for the Examination of Air (6) indicated that only in special cases, such as in association with concentrated animal feeding operations (CAFOs), were wastewater sources of aerosols a potential public health concern. According to Fair and Wells (7), this report may have suppressed the interest in wastewater bioaerosols for more than 20 years. In fact, only very few studies related to aerosolization of microorganisms from wastewater sources were actually performed until the 1970s, when appropriate and efficient air sampling methodologies and renewed interest truly developed.

AEROSOLS ORIGINATING FROM WASTEWATER TREATMENT PLANTS

Control of domestic wastewater began toward the middle of the 1800s, when diseases such as cholera were becoming increasingly common, and it was recognized that many such diseases were transmitted by sewage-contaminated drinking water. This knowledge prompted the initiation and development of the best management practices for the collection, treatment, and disposal of wastewater. Modern sewage treatment saw its beginnings at the turn of the twentieth century, when processes were developed to reduce the organic content found in domestic wastes, preventing fouling of clean water supplies. Now, more than 15,000 wastewater — treatment plants process, approximately, 150 billion liters of wastewater per day in the United States alone. Wastewater treatment facilities are large concentrated sources of sewage. For this reason, most of the research on wastewater bioaerosols has focused in and around them.

Following the 1907 report of Horrocks and the 1917 advisory of the Committee on Standard Methods for the Examination of Air, Fair, and Wells (7) examined the concentration of bacteria in the air around aeration basins and trickling filters at a wastewater treatment facility. It was suspected that aeration basins and trickling filters were the primary sources of microbial aerosols. They isolated coliform bacteria at 71 CFU/m^3 over the source and up to 35 CFU/m^3 downwind, concluding that a definite public health risk existed, related to aerosolization of the etiological agents of respiratory and skin diseases. Other than this, no other comments related to public health risks were made, except to indicate that dilution of aerosolized microbes during aerosolization and transport should be taken into account during risk assessment.

Randall and Ledbetter (8), in a comprehensive study, used Andersen six-stage air samplers (Andersen 2000

Inc. Atlanta, Ga.) and All Glass Impingers (AGI-30; Ace Glass Inc., Vineland, N.J.) to measure bacteria air pollution around activated sludge units. They isolated what they described as potentially pathogenic bacteria from air around the activated sludge units, noting that many of these organisms were able to travel and remain viable over considerable distances. *Klebsiella pneumonia* and *Escherichia coli* were isolated in many of their sampling runs, raising public health questions related to the role of sewage treatment plants in the spread of these pathogens. *Escherichia coli* and *K. pneumonia* were recovered up to 31-m downwind of the source. Randall and Ledbetter also concluded that the airborne bacterial populations increased dramatically, when passing over sludge treatment units (8 CFU/m^3 upwind and 1,170 CFU/m^3 downwind); respiratory tract pathogens were more prevalent in these aerosols than enteric pathogens, and respiratory pathogens were thought to be better able to survive aerosolization better than enteric pathogens. They indicated that activated sludge units represented definite health hazards, and that the best indicator of bacterial air pollution originating from sewage would be *Klebsiella* spp.

Napolitano and Rowe (9) compared the bioaerosol emission rates of activated sludge and trickling-filter sewage plants, and evaluated the effect of various meteorologic conditions on aerosolization of coliforms. They concluded that activated sludge facilities yielded higher concentrations of coliforms than trickling filter facilities. The sludge aeration tanks were identified as the source that contributed most to the airborne contamination at these facilities. They also noted that half of the airborne microbes were associated with particles greater than 5 μm in size, indicating that the primary route of exposure to these airborne microbes would be deposition in the nasal-pharynx followed by swallowing, while those particles smaller than 5 μm were potential "health hazards" that would penetrate into the lungs.

Adams and Spendlove (10) found that trickling filter sewage treatment plants were "exceptional" sources of aerosolized microorganisms. Using Andersen samplers, they detected that substantial numbers of coliforms were aerosolized from the trickling filters, and that their airborne concentrations were affected by the size of the source and the wind velocity. It was also noted that high wind velocities, high relative humidity, low solar radiation (darkness), and low temperatures allowed for the greatest recoveries of coliforms. At night, coliforms were recovered up to 1,287 m from the source.

Kenline and Scarpino (11) also evaluated the numbers and types of bacteria emitted into the air from activated sludge and extended aeration sewage treatment plants. Using a back calculation, they estimated emission rates from these sources to be 440 CFU/m^2/s and 120 CFU/m^2/s respectively. It was also noted, that the concentrations of culturable, airborne bacteria were primarily depleted by dilution and inactivation. Like Randall and Ledbetter (1966), it was also noted that there were relatively high concentrations of *Klebsiella*, *Aerobacter*, and *Escherichia* in the aerosols.

Goff and coworkers (12) considered coliforms to be an adequate indicator for the evaluation of aerosols, originating from trickling filter sewage treatment plants. Using Andersen samplers, samples were taken from 5-m to 5-km downwind of the source. At 15-m downwind, an average of 347 coliforms/m^3 were recovered, whereas at 700-m downwind the average was 90 coliforms/m^3. As in the study by Adams and Spendlove (10), Goff and coworkers stated that the conditions most conducive to high aerosol emissions were low solar radiation, high relative humidity (30 to 60%), and medium wind velocities 2.6 to 4.4 m/s. Finally, it was concluded that *E. coli* could be used as an indicator of bacterial air pollution, originating from sewage plants.

Pereira and Benjaminson (13) used Andersen samplers inside an aeration tank chamber, inside the exhaust stack for the aeration tank, and 300-m downwind of this source. *Mycobacterium*, *Klebsiella*, and *Streptococcus*, which were described as potentially pathogenic, were the major genus isolated from the air, in and around the sewage facility. *Klebsiella* was the most prevalent genera identified, and could be found in the air at all sampling locations. The effect of ozonation on the viability of airborne microorganisms was also evaluated because the plant in which they were sampling practiced ozonation of the aeration tank chamber for odor control, but they were unable to detect any reductions in the concentrations of bacteria. It was concluded, that there existed a possible health hazard (mycobacterial disease) for sewage treatment workers and highly susceptible populations, such as young children, the elderly, and the infirm, who might reside in areas where the atmosphere would be contaminated by the gaseous effluent of the sewage treatment plants.

Fannin and coworkers (14) investigated two activated sludge and two trickling filter plants, screening the air for coliphage (viruses-infecting bacteria). Using a multislit impinger, they evaluated viruses originating from wastewater treatment plants. Coliphage were recovered at distances between 2 and 15 m, and the highest concentration obtained was 6.67×10^{-1} PFU/m^3. It was noted that there was a correlation between the airborne coliphage concentration and the source strength (concentration of coliphage in the sewage). In addition, the relative humidity correlated with the recovery efficiency, with high relative humidity allowing for significantly higher coliphage recoveries. Fannin and coworkers (15), in a second study, used large-volume air samplers (LVAS) for viruses and Andersen samplers for coliform bacteria, but were unable to recover animal viruses although coliphage were recovered at average concentrations of 3.0×10^{-1} PFU/m^3. Coliform bacteria were also recovered at average concentrations of 2.1×10^2 CFU/m^3 and a correlation between increased wind velocity and increased coliform recoveries was seen. A correlation was also seen between high relative humidity and increased recovery of airborne coliphage, and it was noted that coliphage survived longer in the atmosphere than did coliforms. Finally, the potential densities of airborne animal virus was estimated by creating a ratio between coliphage in the air and coliphage in the sewage, compared to the

animal virus concentration in the sewage. These predicted concentrations of airborne animal viruses were considered a public health concern, especially if the viral aerosols could be shown to reach large susceptible populations.

Cronholm (16), using two-stage Andersen samplers, found that activated sludge tanks were a source of aerosolized bacteria, whose concentrations and makeup were related to the individual plants effluent quality. Interestingly, the relationship as stated, was that the plant with the highest quality effluent produced the most bacterial aerosols from it's activated sludge tanks, whereas the plant with the lowest quality effluent produced the least aerosolized bacteria during activated sludge treatment. It was noticed, that five species of bacteria (*Enterobacter agglomerans*, *Enterobacter cloacae*, *K. pneumonia*, *E. coli*, and *Shigella flexnerii*) predominated in the bioaerosols. He went onto evaluated deposition of enteric bacteria on foliage around the sewage treatment plant, and on the basis of his findings, reasoned that there was definitely a cause for concern, especially considering that nearby residents kept vegetable gardens well within the distance of aerosol dispersion from the activated sludge tanks. During the course of this investigation, the effects of *Klebsiella* aerosols on mice were also evaluated, and it was determined that the 50% lethal dose for mice was 1.4×10^8 organisms. Mice were then directly exposed to aerosols originating from the sewage treatment plant, but no adverse health effects were observed. Viable *Klebsiella* spp. and other bacteria were isolated from the lungs of these mice; however, showing that wastewater bioaerosols containing viable pathogens had deposited into their lungs. From these results, it was concluded that humans with compromised immunological defenses would be at risk from exposure to aerosols around sewage treatment plants.

Fannin and coworkers (17), using multistaged impaction samplers and large-volume scrubbers, evaluated aerosols around an activated sludge wastewater treatment plant, before and after these facilities began operation. In these control samples, bacteria were detected around the site, but after operation, the levels of indicators and total counts dramatically increased. Coliphage were the indicator organisms detected in the highest concentrations downwind of the sources, enteric viruses were recovered only at night, and fecal streptococci were overall the most effective indicator of fecal air contamination. They did admit, however, that background levels of fecal streptococci were high enough to raise questions regarding their origins. Finally, it was also noted that the sensitive detection of indicators would not indicate densities of pathogens, rather it would only measure the potential for airborne fecal contamination, possibly serving as a measure of exposure for epidemiological studies.

Laitinen and coworkers (18) evaluated the relationship between bacterial counts and endotoxin concentrations in air surrounding wastewater treatment plants. Using Andersen samplers (R2A) for airborne bacteria, and sterile glass fiber filters for airborne endotoxin, they collected samples within 1 m of various potential aerosol sources in the treatment plant. The results of this investigation showed no correlation between endotoxin levels and viable or total direct counts of bacteria. Airborne endotoxin levels ranged from 0.6 to 310 ng/m^3, and most samples exceeded the occupational exposure limit proposed by regulatory agencies (30 ng/m^3 in eight hours). It was calculated, that this concentration of endotoxin in the air would equal an airborne bacterial concentration of 7,200 CFU/m^3.

Sawyer and coworkers (19) evaluated the bacterial aerosol emission rates from municipal wastewater aeration tanks. Bacteria were detected over aeration tanks at concentrations of 2,068 total CFU/m^3, 410 total coliforms/m^3, 112 fecal coliforms/m^3, and 24 fecal streptococci/m^3. Emission rates were calculated from these data using two methods: (*1*) Conventional ($E = C \cdot V$ where, E is the emission rate, C is the concentration of the organisms, and V is the velocity of the air rising off the surface) and (*2*) Empirical ($E = C/(X/T)$ where X is the cross-sectional area of the tower and T is the sampling duration). Using the conventional calculation, the average emission rates (CFU/m^2/s) for total bacteria, total coliforms, fecal coliforms, and fecal streptococcus were 1.61, 0.20, 0.06, and 0.02 respectively. The empirical formula provided rates of 2.18, 0.27, 0.07, and 0.03 respectively. The benefits of the emission rate calculation they suggested were to be found when conducting mathematical modeling, related to bacterial aerosols originating from such a source.

Carducci and coworkers (20), using the Surface Air System, took samples at 2-, 20-, and 30-m downwind of the plants aeration tanks over a period of 12-months. It was noticed that there was a seasonal variation in the levels of coliphage, with the highest monthly means occurring in the summer months (12–13 PFU/m^3) at 2 m from the aeration tanks (4 PFU/m^3 at 20 m). These concentrations declined in winter and spring. Enteric virus samples were positive only in the months of August through November. They suggested that the occurrence of enterovirus was out of phase with the occurrence of phage; however, careful evaluation of their data might suggest that some environmental factor (relative humidity, solar radiation, temperature etc.) played a role in the lack of enteroviruses recovered during summer months. They concluded however, that coliphage were not useful indicators of enteroviruses in aerosols. Carducci and coworkers (21), in a second study, evaluated fecal streptococci, coliphage, and total bacterial levels, and found that both total bacteria and fecal streptococci in aerosols showed a positive relationship with the presence of animal viruses in aerosols, but coliphage did not. It was also noted that if viruses were detected in the sewage, they were also found in the air samples. Viruses were recovered only in summer and fall. It was noticed that viral contamination showed a greater capacity for spread than bacteria or bacteriophages, and appeared to remain viable in the air longer. They concluded that reoviruses were highly stable in air, and would probably serve as an indicator of viral presence in aerosols.

One of the overriding similarities in the studies related to aerosolization of microorganisms around wastewater treatment facilities was the focus was on airborne bacteria, particularly coliforms. Heterotrophic bacteria or *Klebsiella* spp. may have been better indicators than coliforms

when evaluating microbial air contamination, but even as recently as 1993, coliforms were still considered as the indicator bacteria of choice. In most studies, significant proportions of the airborne bacteria were associated with particles less than 5 μm, and were thus able to penetrate the lungs. This ability to penetrate the lungs was considered by most to be the most significant health risk. However, many of these studies also considered particles greater than 5 μm to be health hazards, as they would potentially deposit in the nasal-pharynx, where they would subsequently be swallowed. After being swallowed, these airborne particles could potentially enter the intestinal tract where subsequent infection could result. In several of these early studies, potentially pathogenic organisms were detected, and their isolation, linked to a public health risk.

BIOAEROSOLS ASSOCIATED WITH SPRINKLER IRRIGATION USING WASTEWATER

Because of the need for conservation and recycling of limited natural resources, especially in arid regions, it is becoming increasingly common for communities to reuse wastewater and wastewater residuals. For instance, since 1970, Aurora, Colorado, has used reclaimed domestic wastewater to irrigate the local golf courses and city parks. In 1980, the average cost of this water reuse system was $0.43/1,000 gallons compared with $0.78/1,000 gallons that the city of Aurora would have spent for traditionally supplied water. The use of treated municipal wastewater effluent for such purposes, offers a cost-effective opportunity to recycle and dispose wastewater while conserving available freshwater supplies. Water reclamation can also provide an alternative to disposal; in areas where surface water disposal is now banned, or where surface waters are unable to assimilate the nitrogen, carbon, and phosphorus contaminants that remain in most treated wastewater effluent discharges. Today, the majority of reclaimed water goes toward practices such as the landscape irrigation of golf courses, parks and lawns, industrial processing, air-conditioning, fire fighting, water table management, toilet flushing, and construction usage.

Merz (22) investigated aerosolization from sprinkler irrigation of wastewater, applied to a golf course, and found coliforms 41-m downwind of the sprinkler. This was the only published U.S. field study at the time that addressed the hazards from wastewater irrigation aerosols. Hickey and Reist (23) reported that Merz recovered coliforms, 41-m downwind of the sprinkler. In addition, all of them found coliforms or *E. coli* downwind of sewage spray irrigation sites. The majority of these reports estimated that coliforms could be carried 400 to 650-m downwind under moderate wind velocities (5–7 m/s). Coliforms are generally found downwind of sewage spray irrigation sites (22,24–28).

Sorber and coworkers (29) used Andersen samplers for bacteria, and an electrostatic precipitator (LEAP sampler (Environmental Research Corp., St. Paul, Minn) to sample for viruses downwind of a spray irrigation site. They were able to detect up to 330 coliform-like bacteria/m^3 at 47 m. Of these bacteria, up to 47% were assigned to the genus *Klebsiella*. They modeled the transport of the aerosolized bacteria, and estimated that it would take anywhere from 518 to 1,800 m to reduce the increased concentration of the total aerobic bacterial aerosol population to 5 organisms/m^3 above the background bacterial airborne concentrations. A 3-log decrease in airborne bacterial concentrations was found in these experiments when chlorination of the source water was implemented. It was concluded, that terminal disinfection (chlorination) of the wastewater source when properly performed, was more practical and economical than the use of buffer zones between the sprinkler aerosol source and the public. The typical buffer zone used in most cases was only about 100 m and it was felt that this distance, would result in only a very limited reduction of the concentration and viability of airborne microorganisms.

Katzenelson and Teltsch (30) recovered coliforms at a distance of 70 m using an AGI-30 and up to 350 m using an Andersen sampler. It was noted that with low wind velocity around spray irrigation site, a homogenous concentration of coliforms was formed, whereas at higher wind velocities, the concentration of aerosolized coliforms was higher but recoveries were erratic because of increased turbulence. They also pointed out that the health risk to agricultural workers and neighboring settlements from the use of wastewater spray irrigation was virtually neglected. They indicated that adequate source disinfection would probably be the most effective way of reducing this risk. Finally, it was calculated that an individual working at a distance of 100 m from a wastewater irrigation sprinkler line would inhale approximately 36 coliform bacteria in 10 minutes.

Parker and coworkers (31) sampled downwind of spray fields, where food-processing wastes were being used for irrigation, and used an area source model to perform predictions related to the downwind dispersal of the microbial aerosols. Coliforms were recovered (32 coliform-bearing particles/m^3) up to 1,493 m from the source. It was estimated, that it would take between 5 and 30 Km before the bacterial concentrations within aerosol clouds were reduced to background levels.

Teltsch and Katzenelson (32) used Andersen stacked sieve air samplers and large volume aerojet-general liquid scrubbers to evaluate the airborne concentrations of enteric bacteria and viruses associated with wastewater spray irrigation. It was discovered that if the concentration of coliforms in the wastewater was greater than 10^3 CFU/ml, then they were also recovered in air samples. Echovirus 7, was detected at a distance of 40 m from the sprinklers, and a correlation was noted between the aerosol densities with respect to relative humidity. They noted that 50% of the bacteria sampled were associated with particles smaller than 7 μm, which constituted a health hazard as they were in the respirable size range. They also went onto suggest that the larger particles were also of health significance because they could be caught in the upper respiratory tract and swallowed.

Teltsch and coworkers (33) developed a model equation, using inactivation kinetics to estimate downwind concentration of live microorganisms as well as the inactivation coefficient of an *E. coli*. Using radiolabeled *E. coli* as

tracers, they provided realistic information on the ability of *E. coli* to survive the aerosolization process. Results showed that there was a much greater inactivation during the afternoon, than in the morning. The bacterial decay rate was between 8.8×10^{-3}/sec in the morning, and 6.6×10^{-2}/sec in the afternoon, and higher relative humidity promoted survival of aerosolized bacteria.

Teltsch and coworkers (34) evaluated aerosolized pathogenic microorganism concentrations downwind of wastewater spray irrigation fields to evaluate the suitability of coliforms as indicators of airborne microbial contaminants. Coliforms were consistently recovered up to 60 m from the source, and were detected even at 120 and 200 m in concentrations of 501 and 26 coliforms/m^3, respectively. *Salmonella* spp. were also detected in 5 out of 26 samplings, at concentrations up to 5.4×10^{-2} CFU/m^3, but only at 40 m. Serotyping of the *Salmonella* identified them as *S. anatum*, *S. hadar*, *S. ohio*, and *S. infantis*. Enteroviruses (poliovirus type 2, echoviruses type 1, 25, and 17, coxsackievirus B1, and two uncharacterized viruses) were seen in 7 out of 17 samples at distances ranging between 40 and 100 m, in concentrations up to 1.4×10^{-1} PFU/m^3. It was concluded that the survival of coliforms in the air was lower than the survival of either enteroviruses or *Salmonella*, thus coliforms did not fulfill the first requirement of a good indicator organism. They also noted that the ratios of *Salmonella* and coliforms in the wastewater source and the air did not correlate, and that coliforms died out long before enteroviruses. Thus, they concluded that the use of coliforms as indicators was not an accurate assessment of the true presence, or persistence of bacterial or viral pathogens.

Bausum and coworkers (35), using Andersen, AGI-30, and LEAP samplers evaluated the use of f2 bacteriophage as a possible tracer, to aid in the mathematical modeling of the aerosol dissemination of enteroviruses, concluding that coliphage f2 was a very suitable indicator organism as it possessed a very high stability in aerosols, and could be recovered at distances up to 563-m downwind of the source.

Overall, the findings related to the aeromicrobiology or spray irrigation were similar to those seen in association with wastewater treatment. Multiple studies evaluated potential airborne fecal contamination indicators, and coliforms were once again considered as the indicator of choice. However, one study did finally prove, conclusively that coliforms were not adequate airborne pathogen indicators because they did not fulfill at least three of the major requirements for an efficient pathogen indicator. It can also be seen that, like aeromicrobiology studies around wastewater treatment plants, the focus of the more recent studies on spray irrigation also shifted from bacteria to viruses.

BIOAEROSOLS ASSOCIATED WITH LAND PLACEMENT OF BIOSOLIDS

Sludge (more properly termed "biosolids"), which is a by-product of the municipal wastewater treatment processes, contains large concentrations of organic matter as well as phosphorus, nitrogen, and other nutrients that can, following adequate treatment such as composting, be applied to farmland. The practice of land application onto agricultural land not only improves the physical properties and agricultural productivity of soils, but also serves as a logical alternative to unproductive and expensive disposal options, such as incineration or landfilling. Land application of municipal wastewater, treated wastewater, and biosolids, has been practiced since the beginning of modern wastewater treatment, in the mid-nineteenth century. Now, with restrictions placed on certain sludge disposal practices such as ocean dumping and landfill disposal, public wastewater treatment utilities have begun to view the agricultural use of sludge as a cost-effective alternative. In the United States, about 37% of sludge is surface applied to soil for several beneficial purposes, including agricultural fertilization, turf grass production, and reclamation of surface mining areas; 37% is placed in landfills; 16% is incinerated; and the remainder is surface disposed by other means (Committee on the Use of Treated Municipal Wastewater Effluents and Sludge in the Production of Crops for Human Consumption and coworkers, 1996).

Edmonds and Littke (36), using AGI-30 samplers, evaluated the aerosolization of coliforms generated by the placement of sludge (dewatered sewage) into a forest clear-cut. It was noted, that the highest bacterial aerosolization occurred after dry-weather periods when air temperature, solar radiation, and wind velocity were high. This was believed to be due to surface drying of the biosolid material, which promoted aerosolization enhanced by the action of wind currents. They concluded that the highest concentrations of indicator could not only be detected in the first few weeks following placement of biosolids but could also be detected months afterward.

Pillai and coworkers (37), using AGI-30 samplers, evaluated the occurrence of pathogen indicators during land placement onto agricultural land. A number of sampling locations were evaluated, including the hopper loading site, which represented a site with significant mechanical agitation of the biosolid material, the current application sites in the fields undergoing application, the population interfaces, which were on the edge of nearby towns, and old application sites, which were fields where biosolids had been applied several months before sampling. A number of indicator organisms such as thermotolerant *Clostridium* spp., bacteriophage, coliforms, fecal streptococci, and H_2S producers were evaluated. It was found that none of the samples tested contained *Salmonella* spp., and that H_2S producers and *Clostridium* indicators were detected only at the hopper loading site. Molecular methods were then used to link the aerosolized *Clostridium* spp. to those found in the biosolid material. They used ribosomal fingerprinting of the clostridial 16S-23S intergenic region, but were unable to develop any clear genetic link between airborne and biosolid derived isolates. They concluded that under the environmental and placement conditions described during the sampling runs, there was little health risk to populations located downwind of the biosolid application sites. Dowd and coworkers (38), using similar methods, also evaluated hopper loading sites and field sites undergoing placement. At the hopper loading sites, the most prevalent indicators recovered in air samples were H_2S producers, which were detected in 93% of the samples,

followed by *Clostridium* spp., which were detected in 73% of the air samples. At the biosolid application sites, the most prevalent indicators recovered were bacteriophage, followed again by *Clostridium* spp. The mean concentrations of thermotolerant clostridia were 6.8×10^2 CFU/m^3, and the coliphage were recovered at mean concentrations of 1.1×10^3 PFU/m^3. They also concluded that H_2S producers, and fecal streptococci, in addition to the coliphage and clostridia, were much better indicators of fecal air contamination than either total or fecal coliforms.

Dowd and Pillai (39) evaluated clostridium molecular fingerprinting as a method for evaluating the origins of fecal contamination during land placement of biosolids. Using the polymerase chain reaction (PCR), they evaluated a total of 60 clostridial isolates; 30 isolates recovered from air samples taken during land placement of biosolids, and 30 isolates taken directly from biosolid material. A total of 15 different banding patterns were found among the 60 *Clostridium* spp. isolates. A total of 10 aerosolized clostridial isolates from one sampling run showed similar banding patterns, even after restriction in digestion to four of the biosolid derived *Clostridium* spp. isolated on the same day. During the second day of sampling, a number of clostridial isolates were again shown to have genetic similarity with biosolid-derived isolates.

Dowd and coworkers (40) evaluated airborne microbial concentrations, including total heterotrophic bacteria, enteroviruses, coliforms, and *Salmonella* spp., during biosolid-enhanced remediation of mine tailings. In this study, the biosolid material was classified as "excellent quality" or highly treated biosolids. Biosolids were placed onto the surface of the mine tailings by large bottom dumpers. In air samples taken before biosolid placement, airborne heterotrophic concentrations were below detection limits in most of the air samples taken around the mine-tailing sites. During biosolid placement and up to six months after placement, these concentrations were at least 3 logs higher than the initial control samples. No pathogen or pathogen indicators were present in aerosols, but extensive genetic characterization of the airborne heterotrophic bacteria was performed. They used PCR amplification and sequencing of the small subunit ribosome of the bacterial isolates, followed by computer database homology comparison to identify the organisms to the genus level. The most prevalent bacteria associated with mine tailings were *Streptomyces* spp., whereas for the biosolid material the most prevalent isolates were *Bacillus* spp. Air samples taken after placement of the biosolids contained a wide variety of organisms but the most prevalent isolates were *Bacillus* spp. Several of the airborne *Bacillus* spp. were shown to be genetically similar to those found in the biosolid material.

Wastewater treatment facilities generate the highest concentrations of coliform bacteria. The first use of molecular tools in studies on wastewater aerosols were performed on aerosols originating from wastewater residuals being applied to soil. The benefits of molecular methods can be seen in their ability to trace aerosols to their actual source, and their ability to identify the genus and species of airborne bacteria. In these studies, the pathogen indicator issue is once again prevalent, and a new airborne fecal contamination indicator (thermotolerant clostridia) is suggested. Only one study attempted to isolate animal viruses without success, but bacteriophage were once again detected arising from biosolid sources.

MISCELLANEOUS SOURCES OF WASTEWATER AEROSOLS

Darlow and Bale (41), Bound and Atkinson (42), and Newsom (43) evaluated the microbial aerosols generated by toilets. In each case, bacterial or viral aerosols were generated. Darlow and Bale (41) indicated that these aerosols remained airborne in significant concentrations for at least 12 minutes after the flush. Bound and Atkinson (42) demonstrated the generation of aerosols, originated by flushing of toilets that contain coliforms. Gerba and coworkers (44) showed that even after the original toilet flush, indicators remained in the bowl water in significant concentrations and were aerosolized once again with subsequent flushing, and that if toilet bowls were seeded with organic matter and the concentrations of *E. coli* typically found in feces, number of *E. coli* launched into the air by flushing was up to 6.6×10^4 CFU/m^3. Similarly, when seeded with coliphage, they found that up to 2.8×10^3 PFU were ejected. It was also found by Gerba and coworkers, that *E. coli* would remain viable after settling on various surfaces in the bathroom area (e.g., faucets, floor, door handle, etc.) Thus, they could be transmitted to hands resulting in potential self-inoculation. It was noted that at the concentration of the indicator organisms seen in feces, even after the initial flush of the toilet, subsequent flushing could launch potential infective doses.

Milner and coworkers (45) evaluated the dispersal of *Aspergillus fumigatus* from sewage sludge composting piles, and found that the aerosolization of microorganisms was very dependent on the amount of mechanical agitation created by manipulation or mechanical handling of the compost. A maximum actinomycetes recovery of 1.5×10^5 actinomycetes particles/m^3 was found. They suggested the use of noncellulosic bulking agents, extended periods of storage before disturbances, and partial enclosure of composting piles to reduce the production of such aerosols and decrease health risks.

Adams and coworkers (46) evaluated the production of bacterial aerosols that were generated, by cooling towers that used wastewater effluent as makeup water. Sampling air coming from one of the cooling tower exhaust pipes, they found up to 1.2×10^3 bacteria/m^3 of air. Characterization of these organisms identified *Pseudomonas* spp., enterobacteria, and *Bordetella* spp. to be the most prevalent bacteria. It was concluded that chlorination or coagulation of the effluent would be beneficial to reduce potential health effects caused by these bacteria.

Wheeler and coworkers (47) investigated the production of aerosols generated by sludge suction tankers used to remove sewage from portable latrines and septic tanks. They found that suction tankers generated relatively low levels of aerosolized bacteriophage, although actual results indicated that up to 1.0×10^6 PFU were expelled during

operation over a three minute time period. This appears to be a relatively high concentration, especially if the organisms were actual pathogens such as the Norwalk agent. They concluded that because of the low degree of carry over, there would be no significant hazards to human health from aerosols generated by the vacuum handling of normal sewage suction devices.

Health Significance of Wastewater Aerosols

Spendlove (48) detailed problems related to aerosols that arose in industry, agriculture, and municipal processes. This report briefly mentions aerosols arising from wastewater, citing studies that described application of wastewater to land, studies that revealed microorganisms in aerosols as a result of spray irrigation, and aerosols associated with wastewater treatment facilities. Apart from reviewing the cited papers, his comments were that "the possible public health danger of aerosolized sewage organisms to people surrounding areas can be visualized when one considers the great number and variety of pathogens, which may be present in raw sewage." In addition, he felt at the time that spray irrigation had not adequately been evaluated and that there was a significant health hazard associated with aerosolization of pathogens in the vicinity of wastewater treatment plants.

Sorber and Guter (49) investigated the health and hygiene aspects of spray irrigation. After investigating aerosol contamination studies, they also stated that bacterial aerosols were more prevalent and remained viable longer, and thus traveled further, when wind velocity and relative humidity was high, and temperature and solar radiation was low. They mentioned that evaporation rates showed that a 50-mm water droplet evaporates in 0.31 seconds when aerosolized at 50% humidity and 22 °C. The resultant nuclei containing microorganisms was more likely to remain both airborne and better protected from environmental insults. It was also recognized that most of the aerosol studies related to wastewater had been centered on coliforms, and that the most frequently isolated coliform, when characterizations were performed, was of the genus *Klebsiella*. Because *Klebsiella* are known respiratory pathogens they thought that specific testing for this genus should be performed in lieu of indicators. They then discussed the infectivity mechanism of aerosols, as related to particle size. In summary: (*1*) particles 2 to 5 μm are captured in the upper respiratory tract, moved upward by cilia action and swallowed, entering the intestinal tract where infection can occur; (*2*) particles 1 to 2 μm can be deposited directly into the alveoli of the lung where infection can occur; (*3*) particles less than 1 μm slowly decrease in the probability of alveolar deposition until 0.25 μm (the size of the smallest enteric viruses not associated with airborne particles), when deposition in the alveoli again becomes more likely; and (*4*) particles greater than 5 μm are deposited in the upper nasal-pharynx where they are likely to be swallowed entering the intestinal tract. Thus, mechanisms exist for respiratory and intestinal pathogens to be transmitted by means of aerosolization. It was indicated that the minimal infective doses (TCD_{50}) for viruses were relatively low and ranged from 1.0 to <790 viruses for influenza, coxsackieviruses (A21 and B4), adenovirus, rhinovirus, measles, and respiratory syncytial virus. They stated that at an activated sludge aeration basin, it was estimated that 40 to 60% of biological aerosols would penetrate the lungs and 6 to 13% would penetrate the alveoli. Finally, on the basis of these figures they also calculated that a man working within five feet of the aeration tank would inhale a viable *Klebsiella* particle every two breaths. Overall, Sorber and Guter concluded, that the risk from aerosolization of pathogens associated with wastewater irrigation could be significant.

Katzenelsen and coworkers (50) published results of an epidemiological study in which they studied the incidence of enteric diseases in more than 200 agricultural settlements. Of these Kibbutzim (communal settlements), 77 were practicing spray irrigation with wastewater that had been partially treated by pond oxidation, whereas remaining communities did not use wastewater for irrigation. It was found that there was an increased prevalence of shigellosis, salmonellosis, typhoid fever, and infectious hepatitis (two to four times higher) in those communities practicing wastewater spray irrigation. They suggested that aerosols were probable pathways for transmission of these diseases, in addition to alternate mechanisms such as vector (clothing and bodies) transmission. They concluded that their results helped strengthen the hypothesis that a relationship existed between wastewater irrigation and enteric diseases.

Shuval and Fattal (51) performed an epidemiological study related to spray irrigation at Kibbutzim in Israel that used two types of settlements. The first settlement used effluent for irrigation during two consecutive years, followed by two years in which only noneffluent water sources were used. The second group of settlements consisted of a total of 23, 12 of which used effluent sources and 11, which used only noneffluent water sources for irrigation. Results suggested that increased disease incidence did not occur at those settlements practicing wastewater irrigation when compared with those settlements that did not. Some indications of excess enteric disease incidence in the age group zero to four years were noticed, however, in those kibbutzim that used effluent water sources for irrigation. It was felt, that such groups of previously unexposed people were the most susceptible to change in health status. They suggested that further analysis of the data was required, and thus they were unable to draw any realistic conclusions.

Johnson and coworkers (52) investigated aerosolization of bacteria and viruses associated with wastewater, and sought to identify health effects attributed to living near wastewater treatment plants. Monitoring of the environment and the health of persons near a new activated sludge treatment plant before and after its initial operation was performed; and a survey was conducted on the incidence of respiratory, gastrointestinal, eye, ear, and skin diseases, and symptoms of persons living within 5 km of the plant just before and after it's initial operation. They also monitored the environment using LEAP samplers, and collected blood, feces, urine, throat swabs, sputum, and hair samples from the test populations over time. Microbiological analysis of aerosols

was consistent with previous studies, showing an increase in airborne pathogens and indicators after the plant began operation. In clinical studies of infection, they found significant increases in throat swab isolates of *Streptococcus*-beta and *Staphylococcus aureus* after initial operation of the plant. Interestingly, they noted a slight increase in the incidence of parasites, such as *E. coli*, *Giardia lamblia*, and *Trichomonas*, isolated from feces after operation of the plant commenced. Picornavirus isolation significantly increased in the operational periods of the plant. Serology showed a significant, or borderline significant, increase in antibody titers to Echovirus 29, Echovirus 13, Echovirus 33, and Poliovirus 1, but they were unable to link any of the viral antibody reactions to exposure to aerosols. The health survey revealed significant increases in the reported incidence of skin disease, gastrointestinal syndromes (diarrhea, nausea, vomiting, and general weakness), and of pain in the chest that occurred when breathing deeply. These increases occurred primarily in the downwind areas up to 2 km from the plant. Finally, they also noticed a significant increase in sore throat and fever above 103 °F, especially in young children. No absolute conclusions were made related to any definitive health problem or lack thereof.

Camann and coworkers (53) performed a health-related investigation, concurrent with aerosol monitoring at an elementary school located 370 to 470 m from the aeration basin of an advanced wastewater treatment plant. Using local school attendance records and information derived from field monitoring, they sought to evaluate health aspects of the wastewater treatment plant's proximity to the elementary school. They hypothesized that adverse health effect related to the plants operations, would result in a higher absenteeism at the elementary school in the years following start-up of the wastewater treatment facility. Microbiological screening of the aerosols was very comprehensive, and showed elevated levels of total coliforms (12.2 CFU/m^3), fecal streptococci (4.2 CFU/m^3), Mycobacteria (19.1 CFU/m^3), and coliphage (1.5 PFU/m^3). Camann and coworkers on review and analysis of school absentee records noted that even before operation of the treatment facility, the school, when compared to other "control" schools, had a higher rate of absenteeism. After initial operation, the experimental school did show a slight decrease in absentee rates compared with the absentee rates seen before the plant began operation, and also in comparison with absentee rates at control schools that were not located in the vicinity of a wastewater plant. Following continued operation of the wastewater treatment facility, however, the experimental school actually showed a statistical increase in attendance, when compared with rates at the school before plant operation and rates in control schools. Conversely, it was noted that first and second grade students at the school showed higher absenteeism during numerous periods after the treatment plant began operation, as compared with both previous records and the control schools. They concluded that an appreciable increase in the concentration of airborne fecal streptococci and mycobacterium was observed at the school when the wind was blowing from the direction of the treatment plant, but little effect on the health of the students at the school was observed.

Scarlett-Kranz and coworkers (54) performed a health survey among municipal sewage and water treatment workers. They submitted a questionnaire to wastewater treatment workers as the experimental group and drinking water treatment workers as a control group. They found that sewage workers reported a significantly higher frequency of headache, dizziness, sore throat, skin irritations, and eye irritations when compared with the drinking water treatment workers. They postulated on the routes of transmission, and indicated that many of the reported health problems could best be explained by aerosol exposure.

Clark and coworkers (55) evaluated exposure of newly hired employees at a wastewater facility, and compared their level of antibodies to Rotavirus, Norwalk agent, and *Prototheca wickerhamii*. They noted a slightly increased incidence of gastrointestinal disease among the recently hired wastewater workers. A serological survey found a fourfold or greater antibody titer level increase in response to rotaviruses in 21 out of 30 of the inexperienced workers and two out of five of the experienced workers. There was also an increase in the antibody titers to Norwalk agents in a small number of the inexperienced workers that was not seen in any of the control groups analyzed. On further analysis of the results from the study, a positive correlation was seen that related these increases to exposure to specific aerosol sources at the plant, indicating that the aerosols may have increased exposure to these agents. On adjusting for covariables such as race and economic status, the only significant increase in titer related to aerosols was the Norwalk agent. They concluded by stressing the importance of minimizing contact with wastewater and associated aerosols as well as continued promotion of basic hygiene practices among wastewater workers.

Fattal and coworkers (56) investigated the occurrence of viral antibodies in an agricultural settlement during a viral disease outbreak. They screened sera from 777 individuals living in agricultural settlements. These individuals were grouped according to the type of exposure. Some were exposed to aerosols from wastewater irrigation at nearby towns, some exposed to other sources of wastewater aerosols, including wastewater originating from the settlement itself, and some were not exposed to wastewater. The investigation was conducted immediately following a national outbreak of echovirus type 4. Antibodies against echovirus type 4 were consistently and significantly present in high titers from children exposed to wastewater aerosols from nearby towns. It was also noted that this increase in antibody titer against echovirus type 4 was not seen among wastewater irrigation workers. They noted that there was the possibility of unusually high levels of contact infection in populations exposed to wastewater from distant urban populations, possibly related to the ongoing epidemic. They suggested that more effective wastewater treatment would result in a greater reduction in the concentration of pathogens in the effluent, which would be prudent from a public health standpoint.

Melbostad and coworkers (57) evaluated various health related symptoms, and their correlations to sewage worker

exposure to bacterial aerosols. Airborne levels of total bacteria (direct microscopic counts, which were divided into spherical and rod-shaped bacteria), endotoxins, and hydrogen sulfide were evaluated, and compared to the results of questionnaires that assessed health related symptoms, which manifested during and after the airborne measurements were taken. They found no correlation between endotoxin levels and any of the parameters screened. The aerosol exposure levels of workers reporting a symptom (headache and tiredness) were correlated to exposure to rod-shaped bacteria and total bacteria levels. It was concluded that higher exposure to rod-shaped and total bacteria were found among sewage workers with respiratory symptoms, headache, tiredness, and nausea when compared with workers not reporting such symptoms.

Laitinen and coworkers (58), using a health questionnaire, an Andersen sampler, and glass fiber filters, performed a health related survey that monitored symptoms, airborne culturable bacteria, and endotoxin levels. They performed these extensive air contaminant sampling runs at various phases of the wastewater treatment process, including influent pumping stations, influent screens, sedimentation basins, biofilter towers, aeration basins, and sludge treatment beds. Endotoxin levels ranged from less than 0.01 to 350 ng/m^3. The highest mean concentrations of endotoxin occurred in the sludge treatment area, followed by the aeration basin, and the highest airborne bacterial counts were found in the sludge treatment area. The questionnaire on work-related symptoms revealed that 6 out of 16 workers reported positive for symptoms, including fever and chills, or eye and nasal irritation (four workers), and cough or cough with phlegm (three workers). Two workers suffered from all of the symptoms evaluated. Other symptoms were headache, fatigue, and gastrointestinal symptoms. They concluded that there was an association of the symptoms, particularly eye and nasal irritation that was correlated to bacterial and endotoxin exposure. It was also admitted that for solid conclusions to be drawn, a larger study would be needed. Gregersen and coworkers (59) described an outbreak of Pontiac fever (nonpneumonic legionellosis) in wastewater workers. These workers, who had been involved in repairing a sludge decanter over a 10-day period, became ill with fever and flulike symptoms characteristic of Pontiac fever. While repairing the inoperable decanter, a second decanter was centrifuging sludge and suspected of releasing aerosols containing *Legionella pneumophilia*. The workers wore only B2 filter masks (charcoal) not meant for aerosol protection. Clinical tests showed that the workers tested positive for *Legionella* sero-conversion. In addition to a positive antibody response, *L. pneumophilia* was found in the sludge. It was concluded that the workers on exposure to aerosols from a sewage sludge decanter contracted Pontiac fever.

Dowd and coworkers (60) combined actual field sampling, mathematical transport modeling, and dose-response modeling to evaluate potential health risks associated with land application of biosolids. Actual airborne levels of *Salmonella* spp. and indicator virus were used to back-calculate the release rates of bioaerosols from the sources, which included an area source and a point source. They evaluated the concentrations of bacterial and viral pathogens predicted to be viable at various distances downwind of the source, entered these predicted concentrations into two dose-response models, and calculated the predicted percentage of exposed individuals that would inhale an infective dose. They found a 3% chance of infection from Coxsackie B3 at 100 m after only one hour of exposure, during low wind conditions. The risk at this distance from the source climbed to 47% after 24 hours of continual exposure. They also indicated that only half of the persons infected with *Salmonella* would possibly become ill, with an even a lower percentage of clinical illness attributed to Coxsackie B3 exposure. When considering bacteria, the potential risk was increased slightly more than tenfold, resulting in a 60% chance of infection after one hour of exposure 100-m downwind of the source. No significant probability or risk of infection was associated with aerosolization and transport of viruses or bacteria, to a nearby population center that was located some 3 km away from the source. It was mentioned that health risks to workers at the biosolid application site was evident although considering the lack of compromised immune status, probably evident in individuals within this limited population, the risk would be considerably less than predicted.

CONCLUSION

The concern over the aerosolization of pathogens from wastewater treatment plants, and the related public health issues date back to almost 100 years. It has been shown that a significant increase in the airborne concentration of microorganisms occurs in association with wastewater sources, and in many studies the presence of aerosolized pathogens has been shown. Another similarity in the studies reviewed has been the search for an effective airborne indicator organism. It appears that coliforms, *Klebsiella* spp., fecal streptococci, thermotolerant clostridia, and coliphage have been the preferred indicators. Unfortunately, no one indicator has been proved to be adequate in all cases for all pathogens that might actually be present. Almost 100 years after the initial studies of wastewater treatment facilities, we now see that viruses may be the concern of interest rather than bacteria.

In at least one case, wastewater aerosols have been proved to be the source of a potentially fatal disease (Pontiac fever), and in numerous cases these wastewater aerosol sources can be linked epidemiologically to various disease symptoms or syndromes. Workers at sewage treatment plants who are exposed to high concentrations of aerosolized microorganisms tend to exhibit transient symptoms such as eye, skin, and respiratory irritation, headaches, or just fatigue, which may be related to the high concentrations of aerosolized microorganisms or endotoxins they are exposed to during normal activities. Yet, it is unlikely that such workers would develop clinical symptoms, except in the most extreme cases. The other possibility for seeing increased disease manifestation in a population exposed to its own wastewater aerosols is in relation to enteric or respiratory pathogens,

which have acute symptoms, and against which little long-term immunity develops (e.g., protozoa such as *Cryptosporidium parvum*, a few viruses such as Norwalk virus, and certain bacteria such as *L. pneumophila*).

To prevent aerosolization of pathogens during the reuse of wastewater or during actual wastewater treatment, best management practices or preventive procedures can include disinfection of wastewater before application to land, aiming sprinklers at lower angles toward the ground to minimize production of aerosols, the maintenance of adequate buffer zones between aerosol sources and the public, physical barriers such as vegetation between sources and the public, and advanced treatment of wastewater and biosolids. When considering wastewater treatment plants, the best management would be to cover aeration basins, inlet streams, and trickling filters. In addition, workers at all wastewater related sites should be encouraged to wear approved protective respirators, especially in enclosed areas where sewage or sludge is aerated. In addition to adequate breathing protection, workers should also wear protective clothing that is removed before leaving work and laundered on-site (not at the workers home), they should practice liberal hand-washing before and after leaving high-risk work areas, especially before eating, and they should even be required to shower before leaving work. These procedures would help prevent contact spread of pathogens to the worker's families, and subsequently to the public.

Hickey and Reist (28) concluded that there are four basic alternatives for future action related to the issue of pathogen aerosolization in association with wastewater: (*1*) conclude that no health hazard exists from viable wastewater aerosols on the basis of present evidence; (*2*) continue to seek further evidence on the aeromicrobiological pathway spread of pathogens from wastewater to humans; (*3*) continue to perform epidemiological studies to link aerosols originating from wastewater sources to disease occurrence; or (*4*) concede that a health risk exists and initiate best management practices for aerosol control. These alternatives are still representative of conclusions forthcoming from this review 30 years later. It can also still be concluded that: (*1*) microbial aerosolization from aerated wastewater process to areas where plant workers and nearby residents are located has been adequately demonstrated; (*2*) aerosols originating from these sources contained a variety of pathogenic bacterial genera and animal viruses; (*3*) health risks have not been adequately confirmed; (*4*) future studies should be both epidemiological and aeromicrobiological in nature; (*5*) health risks arising from bacterial, protozoan, and viral respiratory and intestinal pathogens, as well as, allergic responses should be investigated; (*6*) best management practices should be implemented to prepare for outbreak situations; and (*7*) further research is needed related to aerosol monitoring and development of improved methodologies, especially for animal virus recovery.

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WASTEWATER STABILIZATION PONDS

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Waste stabilization ponds (WSPs) are an extremely effective, natural form of wastewater treatment. They combine simplicity, robustness, low cost, and a very high degree of disinfection. WSPs are usually designed as one or more series of anaerobic, facultative, and maturation ponds, with the former two being mostly responsible for the removal of organic matter (biological oxygen demand, BOD), helminth eggs, and *Vibrio cholerae*, and the latter for the removal of fecal bacteria and viruses. Sometimes anaerobic ponds may not be used (Fig. 1), and sometimes (depending on the required microbiological quality of the final effluent) maturation ponds may not be needed (Fig. 2).

WSPs are a very common form of treatment, with over 7,000 systems in the United States of America, over 2,500 in France, and 1,100 in Germany, for example. They are also common in tropical and subtropical regions where the warm climate leads to increased efficiency and, consequently, lower land area requirements.

The process and physical design of WSPs is described in detail in several engineering design manuals and reviews (1–10).

BACTERIAL POPULATIONS

Little work has been done on the nonfecal bacterial communities in WSPs. In anaerobic ponds, the same

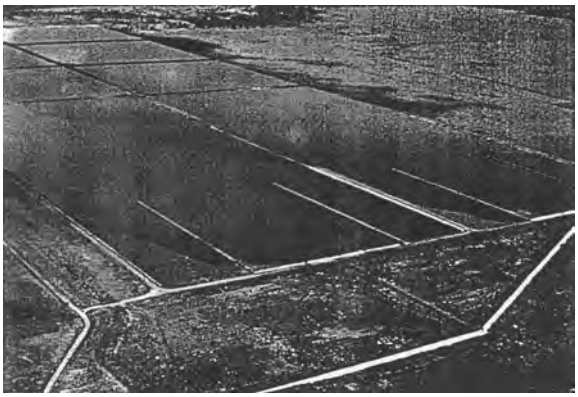

Figure 1. Phase I of the Dandora WSP, Nairobi, Kenya, comprising two series of a facultative and three maturation ponds. Phase II comprises six additional series to the right of Phase I, and Phase III will comprise anaerobic ponds at the head of the eight series.

Figure 2. Two series of anaerobic and facultative ponds at Braslândia in the Federal District, Brazil.

bacterial community is present as in other anaerobic reactors (see BIOSOLIDS: ANAEROBIC DIGESTION OF), that is, the obligately anaerobic acidogenic, hydrogenic bacteria and methanogens, together with sulfate-reducing bacteria. It is the odor-generating potential of the latter group that is of most practical concern with WSPs; however, if the volumetric BOD loading is below 400 $g/m^3\,d$ (11) and if the sulfate concentration in the raw wastewater is below 500 mg SO_4^{2-}/L (12) odor release is very unlikely to reach nuisance levels.

In facultative and maturation ponds, the normal heterotrophic bacteria of aerobic wastewater treatment are present. As these bacteria are facultatively anaerobic, they are well suited to the fluctuating dissolved oxygen conditions in facultative ponds. Anaerobic photosynthetic bacteria are also present in facultative ponds and, to a lesser extent, in maturation ponds (see Photosynthetic bacteria).

The oxygen required by the heterotrophic bacteria for the bio-oxidation of organic matter in facultative and maturation ponds comes mainly from algal photosynthesis, although some comes into the pond via surface reaeration. The carbon dioxide needed in algal photosynthesis comes mainly from the bacteria, although again some enters the pond via its surface from the air. There thus exists a mutualistic relationship between the algae and heterotrophic bacteria in facultative and maturation ponds (Fig. 3).

Cyanobacteria (blue-green algae) are often present in facultative and maturation ponds, but in only small numbers, and they are not a significant component of the pond flora, except in extremely lightly loaded ponds, such as the last maturation ponds in a series comprising a large number (6–8) of these (13).

ALGAL POPULATIONS

Facultative and maturation ponds contain high concentrations of algal biomass, and they are consequently colored dark green. The algae are extremely important in the operation of WSPs: they provide most of the oxygen needed for bio-oxidation and they create most of the conditions required for fecal bacterial die-off (high pH, high dissolved oxygen — see Removal of Excreted Pathogens). Their one commonly perceived disadvantage, that they contribute to a high effluent concentration of suspended solids, is less of a disadvantage in practice as many regulatory agencies permit WSP effluent samples to be filtered prior to BOD analysis, and so exclude the algal BOD contribution because "algal BOD" is very different in nature from "wastewater BOD." In Europe, for example, WSP effluents are required to have less than or equal to 25 mg filtered BOD per liter and less than or equal to 150 mg suspended solids per liter (14).

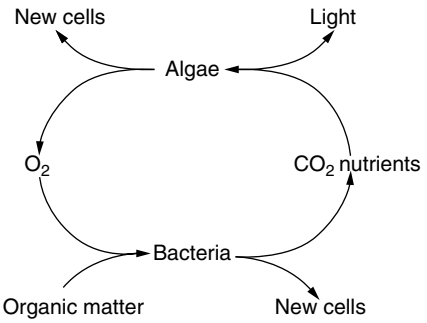

Figure 3. Algal–bacterial mutualism in facultative and maturation ponds.

The algae that tend to predominate in the turbid waters of facultative ponds (see Table 1) are the motile genera (such as *Chlamydomonas, Pyrobotrys*, and *Euglena*), as these can optimize their vertical position in the pond water column in relation to incident light intensity and temperature more easily than nonmotile forms (such as *Chlorella*, although this is also fairly common in facultative ponds) (Fig. 4). The concentration of algae in a facultative pond is usually in the range of 500 to 2,000 μg chlorophyll *a* per liter, but depends on temperature and organic loading (Fig. 5).

As a result of the photosynthetic activities of the pond algae, there is a diurnal variation in the concentration of dissolved oxygen. After sunrise, the dissolved oxygen level gradually rises, in response to photosynthetic activity, to a maximum in the mid-afternoon, after which it falls to a minimum during the night when photosynthesis ceases and respiratory activity consumes oxygen. The position of the oxypause (the depth at which the dissolved oxygen concentration reaches zero) similarly changes, as does the pH because at peak algal activity carbonate and bicarbonate ions react to provide more carbon dioxide

Table 1. Examples of Algal Genera Present in Waste Stabilization Ponds

Algae	Facultative Ponds	Maturation Ponds
Euglenophyta		
Euglena	+	+
Phacus	+	+
Chlorophyta		
Chlamydomonas	+	+
Chlorogonium	+	+
Eudorina	+	+
Pandorina	+	+
Pyrobotrys	+	+
Ankistrodesmus	A	+
Chlorella	+	+
Micractinium	A	+
Scenedesmus	A	+
Selenastrum	A	+
Carteria	+	+
Coelastrum	A	+
Dictyosphaerium	A	+
Oocystis	A	+
Rhodomonas	A	+
Volvox	+	A
Chrysophyta		
Navicula	+	+
Cyclotella	A	+
Cyanophyta		
Oscillatoria	+	+
Anabaena	+	+

Note: +, present; A, absent.

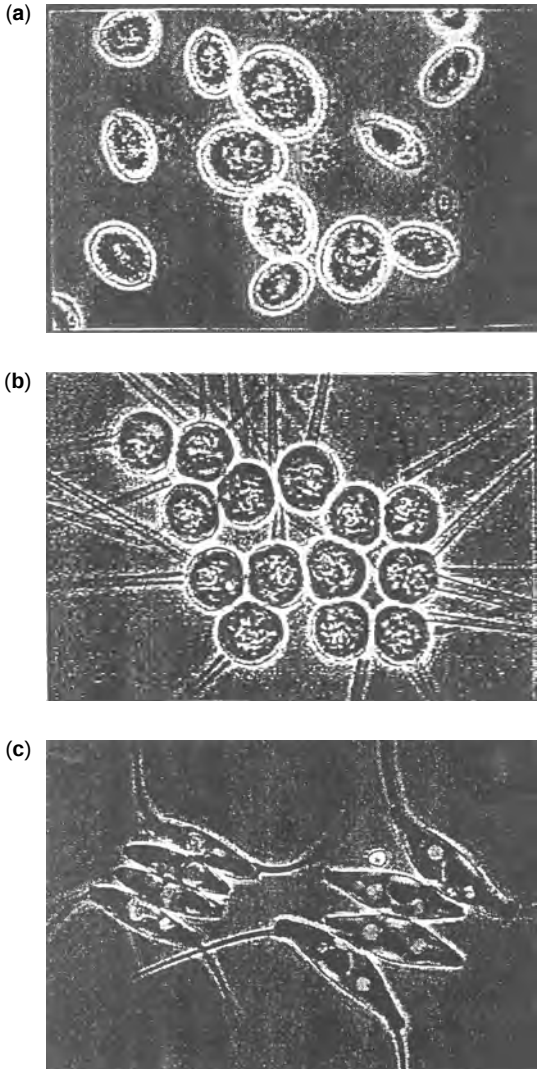

Figure 4. Typical WSP algae: (**a**) *Chlamydomonas* sp. from an anaerobic pond; (**b**) *Micractinium* sp. from a facultative pond; and (**c**) *Scenedesmus* sp. from a maturation pond.

for the algae, so leaving an excess of hydroxyl ions with the result that the pH can rise to above 9, which kills fecal bacteria (see Photosynthetic bacteria).

The wind has an important effect on the behavior of facultative ponds, as it induces vertical mixing of the pond liquid. Good mixing within the upper aerobic layer ensures a more uniform distribution of BOD, dissolved oxygen, bacteria, and algae and hence a better degree of waste stabilization. In the absence of wind-induced mixing, the algal population tends to stratify in a narrow band, some 20-cm thick, during daylight hours. This concentrated band of algae moves up and down through the top 50 cm of the pond in response to changes in incident light intensity, and causes large fluctuations in effluent quality (especially BOD and suspended solids) if the effluent take-off point is within this zone (see Photosynthetic bacteria).

Maturation ponds usually show less vertical biological and physicochemical stratification and are well oxygenated throughout the day. Their algal population is more

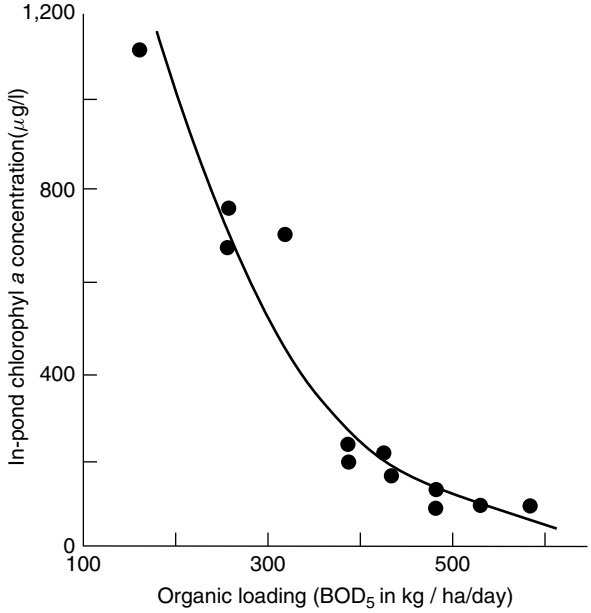

Figure 5. Variation in algal biomass (as chlorophyll *a* concentration) with organic loading in facultative ponds in northeast Brazil at 25 °C.

diverse than that of facultative ponds (Table 1), with nonmotile genera tending to be more common; algal species diversity increases progressively along the pond series.

Ammonia and Sulfide Toxicity

Free ammonia (NH_3) and hydrogen sulfide (H_2S) are both toxic to pond algae. Both dissolved gases inhibit photosystem II, the site of oxygen production. With ammonia the toxicity increases with increasing pH, and with hydrogen sulfide it increases with decreasing pH, as the proportion of these dissolved gases increases with these shifts in pH.

Abeliovich and Azov (15) found that with algae from high rate algal ponds ammonia concentration of 2 mM (28 mg/L) seriously inhibited photosynthesis at pH greater than 8, but Pearson and coworkers (16) found that facultative pond algae were much more tolerant of ammonia, up to 5 mM (70 mg/L) at pH 8 to 9 (Fig. 6).

Sulfide is toxic at lower concentrations than ammonia, with 0.25 μM (8 mg/L) seriously inhibiting photosynthesis at pH 7 to 9 (Fig. 7). *Chlamydomonas* is most resistant to sulfide, and this explains why it may be found as a thin surface film in anaerobic ponds.

PHOTOSYNTHETIC BACTERIA

The obligately anaerobic purple and green sulfur photosynthetic bacteria, mainly members of the Chlorobiaceae, the Chromatiaceae, and the Ectothiorhodospiraceae, are commonly found in facultative ponds and, to a lesser extent, in maturation ponds. Bacteriochlorophyll absorbs light of a longer wavelength than algal chlorophyll, and so the photosynthetic bacteria are often found below the algae in facultative ponds, especially primary facultative ponds,

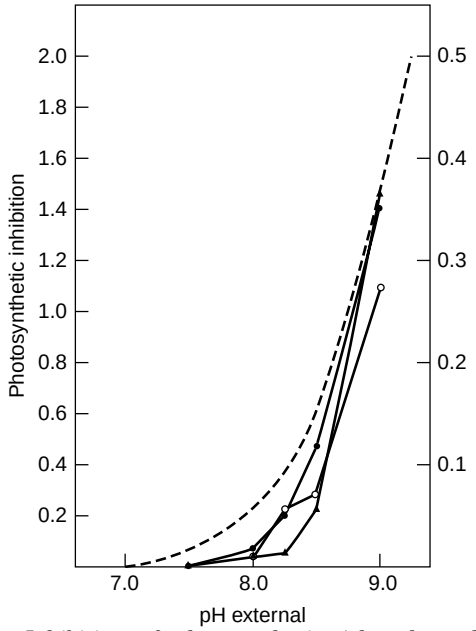

Figure 6. Inhibition of photosynthesis (plotted as $\log_{10}$ of reciprocal percentage activity) by ammonia at different external pH values for *Euglena* (▲), *Chlorella* (◯), and *Scenedesmus* (●), with pNH_3 shown as —.

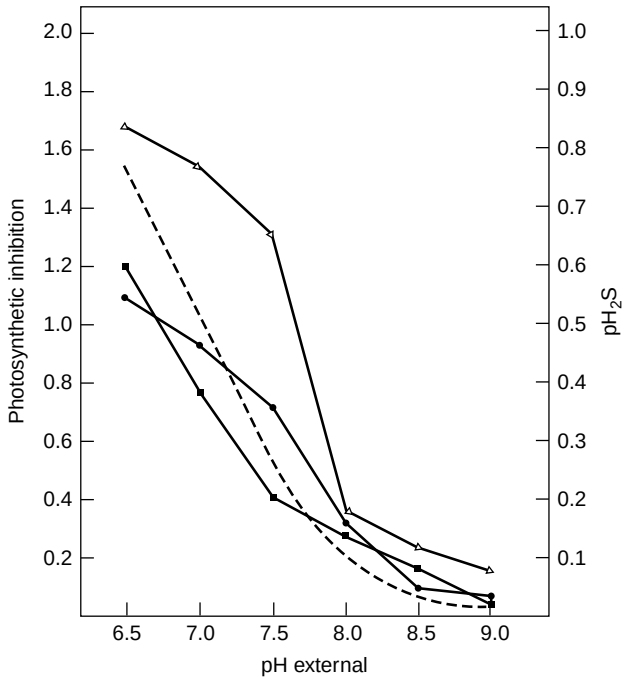

Figure 7. Inhibition of photosynthesis (plotted as $\log_{10}$ of reciprocal percentage activity) by sulfide at different external pH values for *Euglena* (△), *Chlorella* (■), and *Scenedesmus* (●), with pH_2S shown as —.

in which they fix carbon dioxide and oxidize hydrogen sulfide:

$$21H_2S + 10CO_2 + 2NH_3 \xrightarrow{\text{light}}$$

$$2(C_5H_8O_2N) + 21S + 16H_2O$$

The sulfur so formed is deposited within the cell by members of the Chromataciae (Fig. 8), and outside the cell by the Chlorobiaceae. It can also be oxidized to, for example, sulfate:

$$21S + 30CO_2 + 6NH_3 + 36H_2O \xrightarrow{\text{light}}$$

$$6(C_5H_8O_2N) + 21H_2SO_4$$

Both the Chromatiaceae and the Chlorobiacae can utilize simple organic compounds (e.g., acetate) for photoheterotropic sulfide oxidation:

$$H_2S + 5CH_3COOH + 2NH_3 \xrightarrow{\text{light}}$$

$$2(C_5H_8 - O_2N) + S + 6H_2O$$

$$S + 15CH_3COOH + 2NH_3 \xrightarrow{\text{light}}$$

$$6(C_5H_8O_2N) + 21H_2SO_4 + 14H_2O$$

Thus, the photosynthetic sulfur bacteria act as a "sulfide filter" protecting the pond algae from the toxic effects of sulfide (see Algal Populations). However, when facultative ponds are overloaded, the toxic effects of sulfide become too much for the algae, resulting in overgrowth of photosynthetic bacteria, so turning the pond from green to pink or purple. This condition may be permanent or, if the pond is only slightly overloaded, intermittent.

Also present in facultative ponds are the purple nonsulfur bacteria, the Rhodospirillaceae. Apart from adding color to the ponds, these contribute little to pond activities.

REMOVAL OF EXCRETED PATHOGENS

Helminth Eggs

The human intestinal nematode eggs (i.e., the eggs of *Ascaris lumbricoides*, the human roundworm, *Trichuris trichiura*, the human whipworm, *Ancylostoma duodenale* and *Necator americanus*, the human hookworms) are removed by settling owing to their relatively high sedimentation velocities and the quiescent conditions in WSPs. This occurs mainly in the first two ponds in the series (i.e., in the anaerobic and secondary facultative ponds, or primary facultative and first maturation ponds). The percentage egg removal (R) can be estimated from the following equation (1):

$$R = 100[1 - 0.14\exp(-0.38\theta)] \quad (1)$$

where θ is the mean hydraulic retention time (= volume/flow) in the pond, days.

This equation is equally valid for egg removal in anaerobic, facultative, and maturation ponds. For design purposes, the following Equation (2), which corresponds to the lower 95% confidence limit of Equation (1), can be used:

$$R = 100[1 - 0.41\exp(-0.49\theta + 0.0085\theta^2)] \quad (2)$$

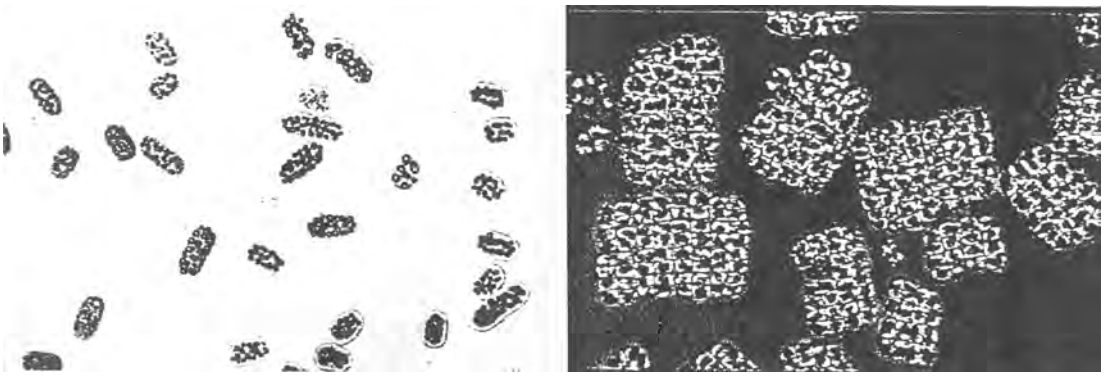

Figure 8. *Chromatium* sp. (left) and *Thiopedia* sp. (right) with intracellular sulfur granules.

Table 2. Geometric Mean Bacterial and Viral Numbers[a] and Percentage Removals in Raw Wastewater (RW) and the Effluents of Five Waste Stabilization Ponds in Series (P1–P5)[b] in Northeast Brazil at a Mean Mid-Depth Pond Temperature of 25 °C

Organism	RW	P1	P2	P3	P4	P5	Percentage Removal
Fecal coliforms	2×10^7	4×10^6	8×10^5	2×10^5	3×10^4	7×10^3	99.97
Campylobacter	70	20	0.2	0	0	0	100.00
Salmonellae	20	8	0.1	0.02	0.01	0	100.00
Enteroviruses	1×10^4	6×10^3	1×10^3	400	50	9	99.91
Rotaviruses	800	200	70	30	10	3	99.63

[a] Bacterial numbers per 100 mL, viral numbers per 10 L.
[b] P1 was an anaerobic pond with a mean hydraulic retention time of 1 day; P2 and P3–P5 were secondary facultative and maturation ponds, respectively, each with a retention time of 5 days.

It is thus possible to design a WSP series specifically for egg removal to comply with the recommendation of the World Health Organization (18) that treated wastewater used for crop irrigation should contain no more than one egg per liter, and also with the recommendation of Blumenthal and coworkers (19) that this level should be reduced to 0.1 egg per liter when children under 5 years of age are exposed or when conditions favor egg survival.

Protozoan (Oo)cysts

The cysts of *Giardia lamblia* and *Entamoeba coli* and the oocysts of *Cryptosporidium parvum* are smaller than helminth eggs, and hence their removals are lower. For example, Grimason and coworkers (20) found a minimum retention period in Kenyan pond systems of 37 days was required to ensure complete removal of *Giardia* cysts and *Cryptosporidium* oocysts; a similar time was found necessary in French ponds (21).

Viruses

Little work has been done on viral removal in WSPs. It is thought that they are removed because of adsorption onto solids, including algae, followed by sedimentation of the solids and dead algae. Oragui and coworkers (22) reported removals of 99.9 and 99.6% for enteroviruses and rotaviruses, respectively, in a series of ponds with an overall retention time of 21 days at a temperature of 25 °C (Table 2).

Bacteriophages have been used as surrogates for viral removal. Vorkas and Lloyd (23) found that phages of *Erwinia* spp., *Pseudomonas* spp., and *Serratia* spp. could be used as indicators of viral transport and removal in WSPs. They also found the phage die-off was accelerated by in-pond pH values greater than 8.5 and by sunlight-mediated effects as found by Curtis and coworkers (24) for fecal bacterial die-off in WSPs, although these factors are yet to be evaluated for human viral pathogen die-off in WSPs.

Bacteria

There is considerable information on the removal of fecal indicator (principally fecal coliform) bacteria in WSPs, but much less on the removal of bacterial pathogens. Oragui and coworkers (22) found that Campylobacter and salmonellae were removed to undetectable levels when the fecal coliform numbers had been reduced to 7,000 per 100 mL in a series of WSPs with an overall retention time of 21 days at 25 °C (Table 2). Oragui and coworkers (25) reported the complete removal of *Vibrio cholerae* in a series of WSPs after 11 days retention when the fecal coliform count was 6×10^4 per 100 mL (Table 3).

Bacterial Removal Mechanisms. The factors responsible for fecal bacterial removal in WSPs are time and temperature (26), high pH (27,28), the combination of high light intensity and high levels of dissolved oxygen (24)

Table 3. Geometric Mean Numbers of *Vibrio cholerae* and Fecal Coliform Bacteria in Raw Wastewater (RW) and a Series of 10 Waste Stabilization Ponds (P1–P10)[a] in Northeast Brazil at a Mean Mid-Depth Pond Temperature of 25 °C

Sample	*V. cholerae* (per liter)	Fecal coliforms (per 100 mL)
RW	485	2×10^7
P1	28	8×10^6
P2	8	3×10^6
P3	3	1×10^6
P4	8	5×10^5
P5	3	2×10^5
P6	0	6×10^4
P7	0	4×10^4
P8	0	8×10^3
P9	0	3×10^3
P10	0	

[a]P1 was an anaerobic pond with a mean hydraulic retention time of 1 day; P2 and P3–P10 were facultative and maturation ponds, respectively, each with a retention time of 2 days.

and, for the removal of *V. cholerae*, sulfide levels above 3 mg/L (25).

Time and Temperature. Marais' (26) Equation (3) for the removal of fecal coliform bacteria in WSPs assumes that it can be reasonably well represented by first-order kinetics in a completely mixed reactor; his equation for a single pond is:

$$N_e = \frac{N_i}{(1 + k_B\theta)} \tag{3}$$

where N_e and N_i are the numbers of fecal coliforms per 100 mL of pond effluent and influent, respectively, and k_B is the first-order rate constant for fecal coliform removal in a completely mixed reactor, day^{-1}.

The value of k_B is strongly dependent on temperature. Marais' Equation (4) for this is:

$$k_B = 2.6(1.19)^{T-20} \tag{4}$$

where T is the pond temperature, °C (for design purposes T is taken as the mean air temperature of the coldest month in the case of river discharge, wastewater-fed aquaculture, or discharge into shellfish growing areas, or of the coolest month in either the irrigation season or, in the case of coastal discharge, the bathing season). Marais derived Equation (4) from an analysis of the fecal coliform data from maturation ponds in the United States of America at temperatures of 2 to 21 °C (29).

The value of k_B depends not only on time and temperature, but also on organic loading: the higher the loading, the lower the algal biomass (Fig. 5) and therefore the lower the pH and dissolved oxygen concentration, and thus the lower the value of k_B (see next section).

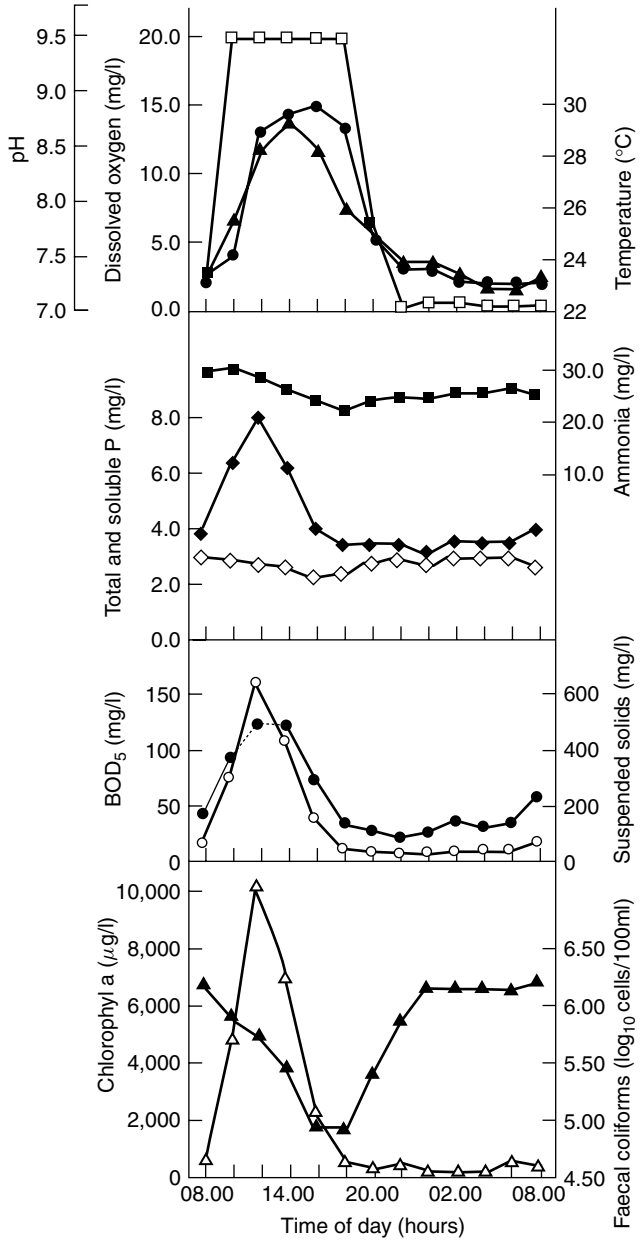

Figure 9. Facultative pond effluent variations in (a) dissolved oxygen (□), pH (▲), water temperature (●); (b) ammonia (■), total (♦) and soluble phosphorus (◇); (c) suspended solids (○), biological oxygen demand (BOD_5) (●); (d) chlorophyll *a* (△), fecal coliforms (▲). Dissolved oxygen was measured in the pond 5 cm below the surface near the effluent. Dotted lines indicate underestimated data owing either to instrument sensitivity or (with BOD) an insufficient dilution range.

For a series of anaerobic, facultative, and maturation ponds, Equation (3) becomes:

$$N_e = \frac{N_i}{(1 + k_B\theta_{an})(1 + k_B\theta_{fac})(1 + k_B\theta_{mat})^n} \tag{5}$$

where N_e and N_i are now the numbers of fecal coliforms per 100 mL of the final effluent and raw wastewater, respectively, and n is the number of maturation ponds.

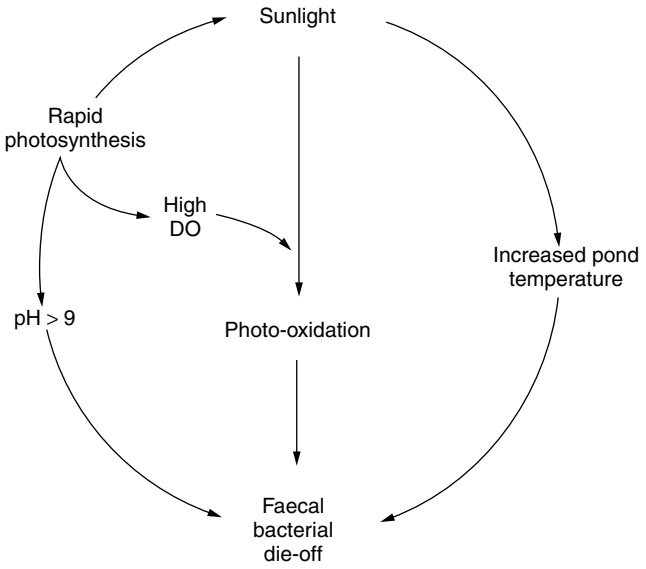

Figure 10. Conceptual mechanisms for fecal coliform die-off in WSPs.

Although Equation (5) assumes the same value of k_B is valid for each type of pond (which is, of course, unlikely), it is nevertheless a reasonable representation for design purposes, when used in conjunction with Equation (4), of fecal coliform removal in the pond series as a whole (30).

High PH and High Light Intensity with High Dissolved Oxygen. Figure 9 shows the diurnal variation of fecal coliform bacteria, algal biomass (as chlorophyll *a*), organic matter (as BOD), suspended solids, ammonia, total and soluble phosphorus, pH, dissolved oxygen, and temperature in the effluent of a facultative pond in northeast Brazil (31). There are peaks of BOD, suspended solids, total phosphorus, pH and dissolved oxygen, which are coincident with the algal biomass peak, and a fecal coliform trough nearly coincident with the algal peak. This shows that algal effects such as increased pH and increased dissolved oxygen determine fecal coliform die-off. Laboratory work shows that high pH is indeed an important factor in fecal bacterial die-off: at pH values greater than 9 fecal coliforms die-off very quickly (28). Curtis and coworkers (24) showed that high dissolved oxygen alone was ineffective in promoting fecal bacterial die-off, but its conjunction with high visible light intensities (light of wavelengths greater than 425 nm) was extremely effective, especially at high pH. The ability of light of wavelengths in the range 425 to 700 nm to damage fecal coliforms depended strongly on high dissolved oxygen levels and the presence of dissolved humic substances (24). A conceptual model for fecal bacterial die-off in WSPs is shown in Figure 10.

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WASTEWATER TREATMENT AND BIOAEROSOLS.

See BIOAEROSOLS IN INDUSTRIAL SETTINGS

WASTEWATER TREATMENT AND GIARDIA.

See *GIARDIA*: DETECTION AND OCCURRENCE OF IN THE ENVIRONMENT

WASTEWATER TREATMENT MICROBIOLOGY

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> . . . when we compromise the air, the water, the soil and the variety of life, we steal from the endless future to serve the fleeting present

Most microbiological research revolves around human health. Yet only 1% of the world's bacteria (Three major lines of primary descent, archae, bacteria, and eukaryotes have now been distinguished using molecular methods (1). In this article, the domains of archae and bacteria are referred to as bacteria) are pathogens. The rest are decomposers and nutrient recyclers that are essential to our very existence. They control the biological wastewater treatment processes that help prevent us from compromising the natural environment.

Advanced wastewater management relies extensively on the bacterial growth kinetics in biological nutrient removal (BNR) processes (2) Reliable design, control and operation of these systems depends on quantitatively predicting the growth kinetics of the bacteria in the process. What is the potential (maximum) bacterial growth rate? How will bacteria spend the energy generated during substrate oxidation? Will they grow and divide (i.e., generate more sludge) or will they expend more energy on cell maintenance (i.e., generate more metabolic products such as carbon dioxide and less sludge) rather than on growth? Measuring and predicting bacterial responses to changes in the wastewater treatment environment is essential for our fundamental understanding and the ultimate improvement of treatment processes.

Only recently have methods been developed with the ability to directly measure the dynamics (growth–kinetics) of bacterial communities or populations in open treatment processes. Existing methods cannot separate bacterial growth from metabolic activity. The chemical transformations that the bacteria cause are seen reflected in changes to substrate and product concentrations (e.g., RBCOD, COD, BOD_5, O_2, CO_2, ATP, $NADH_2$, NH_4, NO_3, CH_4). However, the ability to observe and measure bacterial dynamics is left in the void between substrate use and product formation (Fig. 1).

Historically, engineers considered biological wastewater treatment processes from a hydraulic perspective. They progressed to modeling the kinetics of microbial decomposition of substrates (3) in which influent (substrate input) is converted to a product (output) in reactions that are catalyzed by microorganisms (4). However, (micro)biologists have revealed sufficient intracellular detail to show that the blackbox in Fig. 1 consists of a complex series of reactions mediated by a dynamic and interactive microbial consortia (5–10).

As a microbial ecologist interested in water quality and wastewater research, I am concerned about the relationships of bacteria with each other, with other organisms, and with their environment. Their importance is not so much in their identity but rather the chemical and physiological changes that their growth kinetics cause to the wastewater environment. However, without the proper tools to study bacterial dynamics in situ we cannot begin to understand the ecology of the bacterial communities in the wastewater environment. Hence, this article aims to describe techniques that have the ability to directly measure in situ bacterial community and population growth kinetics (dynamics). The long-term view is to use such methods to fill the void of knowledge ("Black box," Fig. 1) between substrate use and product formation and the relationship between metabolism and growth.

In the measurement of bacterial growth kinetics Meadow and Pirt (12) aptly observed, "There is probably no other technique whose principles are so often ignored with the result that experiments are seriously limited if not meaningless." Along with biodiversity and physiology, quantifying the growth kinetics of bacterial communities in situ will bring about major advances in our understanding of the ecology of microorganisms.

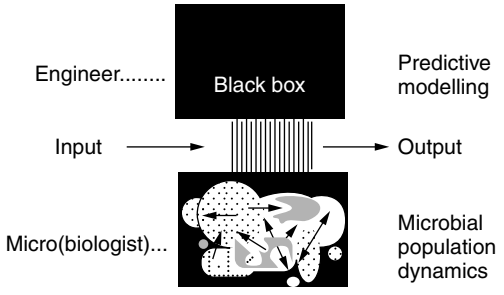

Figure 1. Linking engineering and (micro)biological approaches to quantify the dynamics of microorganisms in wastewater treatment processes. (After V. K. Bhupathiraju, M. Hernandez, P. Krauter, and C. L. Alvarez, *J. Hazard. Mater.* **June 67**, 299–312 (1999b).

A quantitative approach to the dynamics of bacterial communities is essential, rather than the traditional descriptive and qualitative approaches (13). Quantitative measures of microbial growth kinetics are necessary to predict the fate of compounds in both natural (14) and engineered environments (15,16).

DISTINGUISHING METABOLISM FROM GROWTH

Bacterial metabolism is geared primarily toward growth of cells rather than just increasing the size of existing cells. Fundamental to understanding the dynamics of bacterial communities in wastewater treatment processes is recognizing that bacterial growth and metabolism describe bacterial physiology at two different levels of complexity. The approach that will be developed here differentiates growth from overall metabolic activity. Growth, generally, is an increase in the number of cells in a population.

Metabolism, metabolic activity, or just activity are all encompassing terms that are used to describe all the chemical reactions in the cell; they are divided into catabolic and anabolic reactions (17,18). These reactions include biochemical processes that involve the generation of energy, so they are often measured in terms of the rates of respiration or the energy intermediates, such as ATP. This includes reactions that are responsible for cell maintenance, growth, transport, biosynthesis, synthesis of exopolymers and motion. On the other hand, bacterial growth is the result of anabolic reactions that involve the ability of cells to divide (12). Cell metabolism and the relationship between bacterial use of substrates in wastewater, respiration, and the generation of new cell biomass can be seen in Figure 2.

Bacterial growth is the increase in the number of individuals in a population (18,20). In the literature, bacterial growth is often used synonymous with activity. However, the techniques for measuring growth refer to bacterial division; bacteria oxidize and reduce matter to produce new bacterial biomass (Fig. 2). Activity implies metabolic activity.

In the past, the measurement of growth rate of bacterial populations was confined to the ability to culture the organism in the laboratory. Plate counts (colony forming units — CFU) is a method that has been used to determine the number of living cells in wastewater samples. However, the method has long been recognized as not representing the number of bacteria in situ for natural and engineered environments — "the great plate-count anomaly" (21).

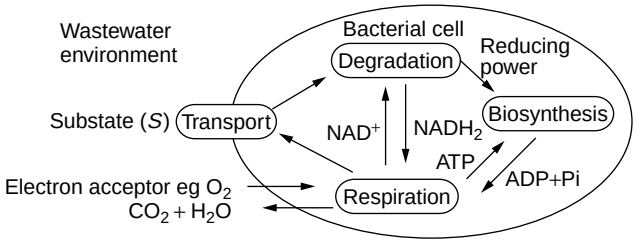

Figure 2. Metabolism and cell synthesis — the material and energy flow of a bacterial cell in a wastewater environment (After T. D. Brock, *Symp. Soc. Gen. Microbiol.* **41**, 1–17 (1987).

These methods are erroneous estimates of in situ growth because the artificial growth conditions of the culture system substantially influences bacterial growth kinetics, morphology, and physiology (20,22). At best 1% of bacteria in a natural environment can be cultivated on an artificial media (23–25) and around 15% in activated sludge (Amann, and coworkers, 1995).

Redox dyes are also used to measure cellular activity. Dehydrogenase activity is used to indicate living bacterial biomass on the basis that the electron transport chain is functioning. The oxidized form of redox dyes, such as INT (2-(4-iodophenyl)-3- (4-nitrophenyl)-5-phenyl tetrazolium chloride) and CTC (5-cyano-2,3-ditolyl tetrazolium chloride) are almost colorless and nonfluorescent. When they are incubated with environmental samples, they are taken up and reduced to formazan by dehydrogenase activity in the cellular electron transport chain. The redox dye subsequently becomes insoluble and the cells with respiratory activity fluoresce (26–28). Similar methods have been used for counting actively respiring bacteria in municipal wastewater treatment plants, and in groundwater and seawater samples (29,30). These methods do not, however, measure bacterial growth kinetics.

Metabolic activity mistakenly has also been assumed to be directly related to bacterial growth on the basis that cellular activity could be used to estimate biomass (31). These methods have relied on measuring concentrations of ATP or $NADH_2$, dissolved oxygen or the activity of dehydrogenase enzymes (31–33). Droste and Sanchez (32) critically evaluated these techniques and prefaced their work with the statement that the direct measurement of the active mass was impossible. They concluded that ATP concentrations offered the best-available estimate of activity. However, the concentration of ATP and $NADH_2$ are also involved in catabolic and anabolic processes of the bacterial cell (34), not just those of cell multiplication. These methods cannot be used as a growth rate measurement because they depend on the metabolic state of the bacterial cell.

ATP and $NADH_2$ methods have almost been abandoned by water treatment specialists as a measure of bacterial growth because the results are so difficult to interpret. The dehydrogenase activity methods are hindered by the complex wastewater environment, whereas DNA methods are tedious and unreliable (35). Currently, respirometric methods are used to estimate bacterial growth rates in wastewater treatment systems (36–38). However, the existing respirometric methods often lead to false interpretation of results (40). There is no clear consensus on how the growth kinetics should be measured or what the results from respirometric assays really mean (16,41).

Identifying the structure and dynamics of bacterial populations in biological nutrient removal (BNR) processes has involved the use of molecular techniques, such as nucleic acid homology, RNA sequencing, and RNA probes (21,42,43). These methods are not without their conceptual difficulties, for example, clearly distinguishing species and relating the ecological roles of the bacterial species that have been identified (44). However, these

techniques have considerable potential for quantifying the biomass of individual species or groups of bacteria.

Amann and coworkers (21) believe that once the problems associated with the use of in situ fluorescent-dye probes have been overcome, the method could be used to measure the growth rate of bacterial species. However, in situ probing methods still rely on quickly visualizing changes in bacterial numbers in open ecosystems, an almost impossible task amidst rapid losses of bacterial biomass through processes such as dilution, grazing, and viral lysis (45–47).

Current methods in wastewater treatment research rarely directly measure in situ bacterial growth rates; the methods that are used are very different from those used in other fields. Most notable are those used in studies of the microbial ecology of natural aquatic environments (48–51). Brock (14,52), along with these other microbial ecologists, has emphasized the need to develop tools to quantify the growth kinetics of bacterial populations in situ.

Major advances have been made in methods used to directly measure bacterial biomass and growth kinetic in fields outside wastewater research that are only now being recognized by the industry (53). Bacterial growth rates have been measured in situ in natural environments using the thymidine assay. In marine, freshwater, soil, and clinical research, the thymidine assay is the most widely accepted in situ method used to measure cellular growth kinetics (54–63).

Engineered wastewater environments are comparatively simple and better suited to use with the thymidine assay because they are engineered trophic ecosystems built on bacterial growth (division)—"Bacteria constitute the major part of the biomass and they are the basic trophic level in all stages of biological sewage treatment. . ." quote from Pike (64). This next section is concerned with the historical development and principles of an *in situ* measure of bacterial growth kinetics.

DIRECTLY MEASURE BACTERIAL GROWTH KINETICS

Historical Development

For a long time now, the thymidine assay has been validated in natural complex ecosystems as the preferred method for measuring bacterial growth kinetics for in situ studies of aquatic microbial ecology. This assay originated in clinical research long before it became a quantitative tool for use in aquatic environments.

Bacterial growth is the creation of new bacterial biomass (in the form of proteins, carbohydrates, lipids, RNA and DNA) that culminates in cell division, once DNA synthesis is completed. Growth is marked by the synthesis of new bacterial DNA and by cell division. Hence, bacterial growth rates can be measured by the rate at which new DNA is synthesized. In the thymidine assay, radiolabeled thymidine is introduced into the cell's environment. Each time the cell divides the new DNA molecules are labeled. The rate of cell growth is determined from the rate at which thymidine is incorporated into the bacterial DNA.

As early as 1965, Roodyn and Mandel (65), measured the rate at which *Bacillus cereus* divided with the thymidine technique. Thymidine metabolism and the theoretical basis of the thymidine assay was originally based on studies of the growth of mammalian cells (54).

Then the key issues identified for applying the method were

- the need for an isotope-dilution method to determine endogenous dilution of dTTP;
- correlating concentrations of exogenous thymidine with endogenous dTTP;
- estimating rates of DNA synthesis with rates of thymidine incorporation.

It was not until the early 1980s, that these same issues were addressed by microbial ecologists in their application of the method.

Brock was the first to use radioactively labeled thymidine with autoradiographic techniques to indicate bacterial metabolic activity in natural environments (66,67). Later, his review comments underpinned the direction of the thymidine growth assay in environmental research (14). He emphasized the need for studies that incorporated methods for measuring microbial growth rates in nature, that is in situ, as opposed to laboratory experiments. He also pointed out that only viable bacteria take up and incorporate thymidine into dividing bacterial DNA. On this basis, others used the thymidine assay for detecting bacterial metabolic activity in soil and lake sediments (68,69). The thymidine assay was first applied to the marine environment as a measure of the rates at which unattached bacteria were turned over by bacteriovores (70).

The breakthrough in quantitatively measuring bacterial growth rates came when the rates of incorporation of thymidine into DNA were used to estimate rates of bacterial cell division using a conversion factor for bacterioplankton (71,72) and for marine sediments (73,74). Theoretical factors were used to convert the rate of thymidine incorporation into the rate of synthesis of new bacterial cells. The conversion factor was based on the average size of a bacterial DNA molecule and its number of thymine residues (i.e., mol %[G+C]). The theoretical factor was validated with empirical estimates that were made by measuring increases in bacterial numbers (72,75). By the end of 1982, the thymidine method had been identified, as having the ability to measure the production of new cells in situ. In time, the paper of Fuhrman and Azam (72) became the most cited—a classic citation (76).

Subsequently, the specific activity of the added exogenous thymidine was shown to be significantly diluted by intracellular and extracellular sources of DNA precursors. This caused significant underestimates of bacterial growth rates. This dilution problem was overcome in the marine environment by applying an isotope dilution method (77). The same methods were latter applied to bacterioplankton of eutrophic lakes (78).

Subsequently, the method has been validated as an absolute measure of growth rate by comparing it with other methods such as ^{3}H-leucine and P^{32} incorporation and the frequency of cellular division (49,77,79–82). From 1982, microbial ecologists used the thymidine

assay in trophic studies of aquatic ecosystems. Estimates of bacterial production are determined from bacterial growth rates measured with the rate of thymidine incorporation (55,83–92).

Since 1975, aquatic microbial ecologists have thought long and hard about the best quantitative in situ measures of bacterial growth and production. They have come to accept and use the thymidine assay because it appears to measure growth

- of only bacteria
- of bacteria in balanced or unbalanced growth states
- in situ
- at a molecular level of sensitivity
- independently of bacterial biomass and mortality.

In contrast to this plethora of research, the wastewater treatment industry has struggled, and continues to struggle to confidently measure key bacterial growth kinetic parameters (16,40,41,93). The wealth of experience and knowledge associated with the development of methods to measure bacterial biomass, production, and growth kinetics in clinical and aquatic research has not yet been transferred into wastewater research.

To design, apply, and interpret the incorporation of thymidine into any new environment requires a knowledge of factors that influence thymidine incorporation. This is especially true for biological wastewater treatment processes in which the bacterial populations are complex and two to three orders of magnitude larger than those in natural systems (55,64). The wastewater industry can now capitalize on the experiences that have led to the development and application of the thymidine assay in natural aquatic environments, avoiding the "pit-falls."

Principles and Biochemical Pathways

In wastewater treatment, the measurement of a bacterial species growth kinetics is just as important as its identity. The basis of bacterial physiology is metabolic activity that is geared to maximize rates of growth (94). Quantifying growth is important because the survival of a bacterial species in any environment depends on how fast it grows.

When radioactively labeled thymidine ([methyl-^{3}H] thymidine) is added to wastewater, it is actively transported into the cell. The DNA of newly synthesized bacterial cells are radioactively tagged, whereas the DNA of nongrowing cells are not labeled (56). The bacterial growth process and radioactive labeling of the newly synthesized DNA is illustrated in Figure 3. The significance of the thymidine assay is that it is able to quantitatively measure the bacterial growth process in situ, that is without changing the wastewater environment (95).

For a long time the incorporation of thymidine has been used as a measure of DNA synthesis. Even today, Cleaver's (54) paper is still the most comprehensive review of thymidine metabolism. In principle, exogenous radioactively labeled thymidine is rapidly and efficiently incorporated into bacterial DNA as the bacteria replicate. The advantage of using thymidine as a DNA precursor

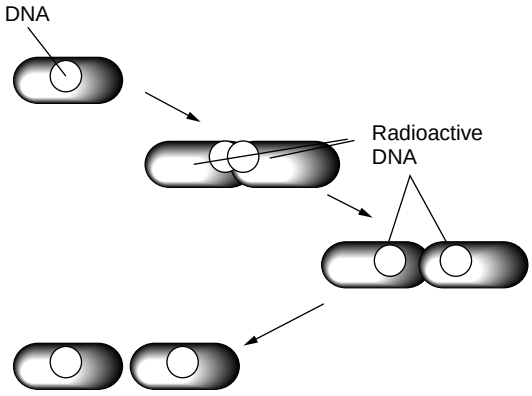

Figure 3. The bacterial growth process in the presence of [methyl-^{3}H] thymidine.

is that it is only used by bacteria for DNA synthesis; nongrowing cells do not synthesize DNA. Thymidine is not involved in other metabolic pathways, as is ATP (96). Thus, the rate at which thymidine is incorporated into bacterial DNA can be directly correlated to the rate of cell division. The method is a sensitive measure of bacterial growth rate at a molecular level. However, the quantitative use of the method requires an understanding of the biochemistry of thymidine metabolism (54).

After thymidine is actively transported across the bacterial cell membrane, it is phosphorylated to deoxythymidine monophosphate (dTMP) in a reaction catalyzed by thymidine kinase or it is catabolized by a phosphorylase to thymine (Fig. 4). The catabolism of thymine is important because it ultimately results in the formation of a radioactively labeled free methyl radical (Fig. 4) that enters the general metabolic pathways of the cell. Eventually, nonspecific label will be incorporated into macromolecules other than DNA. Some loss of thymidine along this catabolic pathway is not critical, as long as thymidine is added in excess. However, this catabolism makes it necessary to separate the radioactively labeled DNA from other macromolecules and keep incubation times short. Another advantage of using thymidine as the DNA precursor is that uracil, which is not radioactive is an intermediate in the catabolic process (Fig. 4). Hence there is no direct route for the rapid radioactive labeling of RNA.

The salvage pathway is the route through which exogenous [methyl-^{3}H] thymidine is incorporated into bacterial DNA. Once thymidine is phosphorylated to dTMP (Fig. 4), it is further phosphorylated in a series of thymidylate kinase catalyzed reactions to deoxythymidine diphosphate (dTDP) and then triphosphate (dTTP); eventually it is incorporated into DNA via DNA polymerase as shown in Fig. 5 (97). These kinases are labile in the absence of thymidine (98). When bacteria are dividing in the presence of thymidine the newly synthesized DNA becomes radioactively labeled (Fig. 3).

In the de novo pathway, dTMP is synthesized from a series of anabolic reactions within the bacterial cell. Deoxy uridine monophosphate (dUMP) is methylated to dTMP via the enzyme thymidylate synthetase. Both the de novo and salvage pathways share the one dTMP pool. The ensuing kinase reactions, leading to the synthesis of

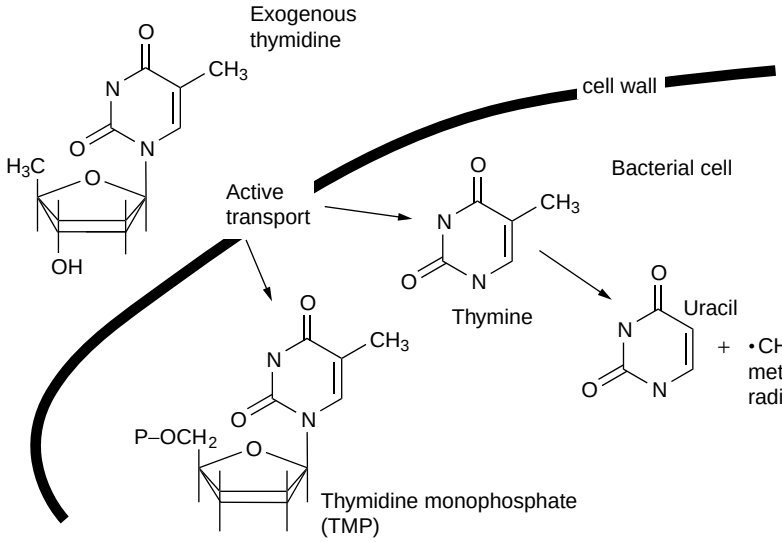

Figure 4. Thymidine uptake, phosphorylase, and kinase activity. The incorporation of exogenous thymidine into bacterial DNA is via deoxythymidine monophosphate (dTMP). Phosphorylase may also catabolize thymidine to form thymine and ultimately a nonradioactive uracil molecule with a radioactive free radical.

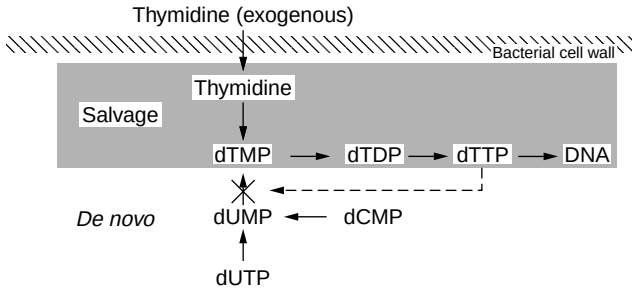

Figure 5. Salvage and de novo pathways of thymidine incorporation into DNA. At high thymidine concentrations, deoxythymidine triphosphate (dTTP) will allosterically feedback inhibit the de novo biosynthetic pathway, preventing endogenous dilution of thymidine. The abbreviated phosphorylated nucleotides are given in the text.

DNA are the same as for the salvage pathway. Because both pathways contribute to the same dTMP pool, the de novo pathway internally dilutes the exogenous supplies of [methyl-^{3}H] thymidine that are incorporated in DNA. This is referred to as isotope dilution (77).

Bacterial growth rates can underestimate growth if isotope dilution is not eliminated or measured. De novo sources of dTMP can be eliminated by taking advantage of the end-product, dTTP, inhibition. dTTP is a specific and potent allosteric inhibitor of the thymidylate synthetase in the de novo pathway (99). At appropriately high concentrations of exogenous thymidine (i.e., low thymidine specific activities) endogenous isotope dilution can be inhibited. Appreciating the intricacies of these biosynthetic pathways was, and still is, an important part of the historical development and application of the thymidine assay to natural and engineered aquatic environments.

Key Factors to Consider — "Traps for New Players"

Thymidine and thymine generally have no fate other than their incorporation into DNA. They are also rapidly incorporated into DNA. Thymidine has an advantage over thymine such that it is accumulated in the bacterial cell and is more rapidly and efficiently converted into the dTTP pool (96,97,56). Following are the key factors that must be considered when applying the thymidine assay as a quantitative measure of bacterial growth in wastewater treatment processes.

Isotope Dilution — Eliminate or Account. Bacteria can also synthesize thymidine internally via the de novo biosynthetic pathway and contribute to large intracellular pools of dTMP. When exogenous thymidine is incorporated into the dTMP pool, via the salvage pathway, it can be diluted substantially by the de novo source (Fig. 5). Both these pathways contribute to a common pool of thymine — DNA precursors. However, the de novo pathway contribution will dilute the radioactively labeled thymine that is incorporated into DNA (97), artificially lowering the estimate of bacterial growth. This isotope dilution must be dealt with before the assay can be applied to the wastewater environment.

Isotope dilution experiments are designed to take this dilution into account (77,78). These experiments are important to any new environment in which the thymidine assay is applied. The thymidine assay can be manipulated to take advantage of the fact that, at sufficiently high concentrations of thymidine, a corresponding increase in dTTP causes feedback inhibition of the de novo pathway (Fig. 5). The use of high concentrations of thymidine (i.e., low specific activity [Ci.mmol^{-1}]) of [methyl-^{3}H] thymidine have been shown to remove the effect of isotope dilution. The amount, however, must be determined for each new environment (89,100). The concentration of thymidine can be adjusted to either eliminate or minimize isotope dilution.

Thymidine Transport and Kinase Activity. There are two key underlying assumptions of the thymidine assay, that is, the bacteria being studied have the capacity to:

1. actively transport thymidine across the bacterial cell membrane; and

2. have an active thymidine kinase to catalyze the phosphorylation of thymidine to dTMP (Fig. 4).

Initially it was thought that nearly all bacteria were able to meet both these criteria (54). Subsequently, however, several workers have found that although most heterotrophic bacteria will transport and incorporate [methyl-^{3}H] thymidine, some cultured isolates do not (77,101). For the genus *Acinetobacter*, one important difference from many other bacteria is its inability to incorporate thymine or thymidine into DNA. This genus is physiologically identified by its unique lack of both thymidine kinase and phosphorylase (102).

Also, some sulfate-reducing bacteria have been observed not to incorporate thymidine (103). Some cultures of methanogens have also been shown not to incorporate thymidine (104,105), although some of this evidence is contradictory (56). Johnstone and Jones (106), for example, demonstrated a methanogen that did not incorporate thymidine into DNA, whereas others have shown that the same species did have such ability (107). Others have concluded that thymidine does not measure bacterial growth in some aquatic environments (108). However, these authors did not allow for the influence of isotope dilution, their conclusions are left in doubt.

Not taking into account the factors that influence thymidine incorporation was the most likely cause of these inconsistencies. The true test of thymidine's ability to measure bacterial growth rates will come from in situ experiments that take into account isotope dilution.

Overall, there is compelling evidence that most bacteria do incorporate exogenous thymidine. Thymidine measurements of growth rate are consistent with other measures of growth such as the frequency of dividing cells and the incorporation of tritiated leucine (49,82,109,110). In applying the thymidine assay to any new environment, the investigator needs to be aware that not all the actively dividing bacteria may have the ability to incorporate [methyl-^{3}H] thymidine into bacterial DNA.

Nonspecific Labeling of Macromolecules. Thymidine assays are deliberately short to minimize the radioactive labeling of macromolecules other than DNA (see Thymidine catabolism; Fig. 4). Preliminary kinetic studies are important to determine the optimum time that thymidine can be incubated with environmental or wastewater samples. As we have seen earlier, thymidine catabolism leads to the formation of a radioactively labeled methyl radical or forms tritiated water that enters general metabolic pathways (56). These catabolic products label macromolecules other than DNA, such as proteins (101). It is important to determine an incubation time that is sufficient to radioactively label DNA while minimizing the labeling of these other macromolecules.

Isolating bacterial DNA from the wastewater treatment environment can also be used to eliminate nonspecific labeling. There is an extensive repertoire of molecular methods for extracting and isolating DNA for qualitative studies of bacterial DNA (111). For wastewater research needs, however, it is necessary to consider methods that quantitatively recover the DNA.

Probably one of the most controversial and most lively debated subjects was the factor used to convert the rate of thymidine incorporation into the rate of bacterial growth. The calculation of bacterial growth rates requires an estimate of the number of cells produced per mole thymidine incorporated into DNA (80). The factor is controversial because it assumes an average mol %[G+C] and an amount of DNA per cell (i.e., average genome size) for complex bacterial communities (72).

Two approaches have been used to validate these conversion factors:

- empirically, from alternative growth measurement, and
- theoretically, using an average mol %[G+C] and DNA content of a bacterial cell.

Generally, there is good agreement between both these factors (72,80,112). Across the literature, factors have ranged from 1 to 60×10^{18} cells per mole of thymidine. However, reviewers of conversion factors tend to agree on a value of 2×10^{18} cells per mole of thymidine (51,57,80,92,96). This is the value used in this thesis research (K_{tdr}). The accepted mean literature conversion values used here for calculating bacterial growth rates should be checked by determining the K_{tdr} conversion factor for each new wastewater treatment system. Because the microbial physiology depends on both the type of bacteria and the environmental conditions, more accurate measures of growth rate conversion factors require in situ measurements of the conversion factor (114).

Moriarty (96) emphasized the need to consider isotope dilution and ensure that only radioactively labeled DNA is isolated when determining K_{tdr} conversion factors. This has been more recently corroborated by others who pointed out that empirical and theoretical conversion factors do not converge unless exogenous thymidine concentrations are greater than 10 nM (115,56). At lower concentrations, isotope dilution occurs.

MEASURING BACTERIAL GROWTH IN ACTIVATED SLUDGE

As early as 1942, Monod (Monod's 1942 Ph.D. thesis, in French, has never been translated into English. However, Panikov, 1995 has empathically interpreted some of his work in the context of Jacques Lucien Monod's life (1910–1976) and times; it is worthy reading.) viewed the quantitative measurement of bacterial growth as a tool for studying bacterial physiology and biochemistry. The original chemostat research was carried out by Monod (116–118), and Novick and Szilard (119). However, others (120) are credited with succinctly and mathematically predicting the steady state concentrations of bacteria and substrates on the basis of Monod's and Novik's work.

Herbert and coworkers (120), started their description of the kinetics of bacterial growth from the "familiar exponential growth curve":

$$\mu = \frac{dx}{dy} \cdot \frac{1}{x} \tag{1}$$

where x is the concentration of bacteria at time t. They described bacterial growth rate as the rate of increase in the number of bacteria (dx/dt); whereas μ was called the specific growth rate as it was the growth rate divided by the number of bacteria. Thus, μ takes into account the standing biomass.

They point out that Monod (116) first showed the simple relationship between μ and the concentration of the limiting substrate (Fig. 6; Eq. 2).

$$\mu = \mu_{\max}\left(\frac{S}{K_s + S}\right) \tag{2}$$

where S is the concentration of the substrate-limiting growth, $\mu_{\max}$ is the maximum specific growth rate of the bacteria when the substrate is not limiting growth, and K_s is a saturation constant (the concentration of substrate at half $\mu_{\max}$). This relationship is the basis of most mathematical models that are used to simulate the average growth and removal kinetics of wastewater treatment systems (3,40,121–124).

A knowledge of bacterial specific growth rates in the wastewater treatment environment is essential for designing, operating, and evaluating the state of the process; as Monod said, growth kinetic measurements give a measure of bacterial physiology and biochemistry. Reliable design of wastewater treatment processes depends on simulating the growth of the bacterial population that converts wastes to products and biomass (3,122). Yet, in the monitoring and control of treatment processes, there have been no direct measurements of bacterial growth kinetics (125). The methods the industry uses to control treatment processes are respiratory-based (38,126), however, these can lead to false and confusing results (16,39,93).

Directly measuring bacterial growth rates alongside metabolic activity allows advances in the knowledge of the key microbial processes in wastewater treatment systems. Being able to distinguish bacterial growth from metabolic activity will enable researchers to observe how the bacterial cell allocates its resources between cell maintenance and growth. For example, under the wastewater treatment process operating conditions, are bacterial cells likely to divide and create more biomass (sludge) or to allocate more energy to cell maintenance (decrease the mass of sludge)? Achieving the latter is a major objective of wastewater treatment processes (127–129).

Reliable design and operation of biological wastewater treatment systems demand robust models of the biological-degradation processes. However, methods to directly measure key stoichiometric and kinetic parameters have not been readily available. Pollard and Greenfield (100) show how to directly measure bacterial numbers, biomass, and growth rate parameters in activated sludge systems. The method is simple, as shown in Fig. 7 but does require an understanding of the factors that impact on the biochemical pathways of thymidine incorporation into bacterial DNA. They measured volumetric bacterial growth rates via the thymidine growth assay, whereas the number of bacteria and biomass were determined with epifluorescent microscopic techniques. Bacterial-specific growth rates (μ) were subsequently determined by dividing the volumetric growth rates by the concentration of bacteria. They compared the community growth kinetics in the different compartments of an activated sludge treatment process (Fig. 8). Bacterial cell counts were converted to biomass in terms of COD with a factor derived here; $20 \pm 4 \times 10^{-11}$ mgCOD.cell^{-1} as described by Von Münch and Pollard (130).

In the aerobic compartment, measurements of bacterial-specific growth rate, concentration of bacteria and biomass were 0.5 d^{-1}, 1.1×10^{10} cells mL^{-1}, 2×10^3 mg COD L^{-1}, respectively. Bacterial growth, numbers, and biomass were quantified simply and specifically in the different compartments of a biological nutrient removal

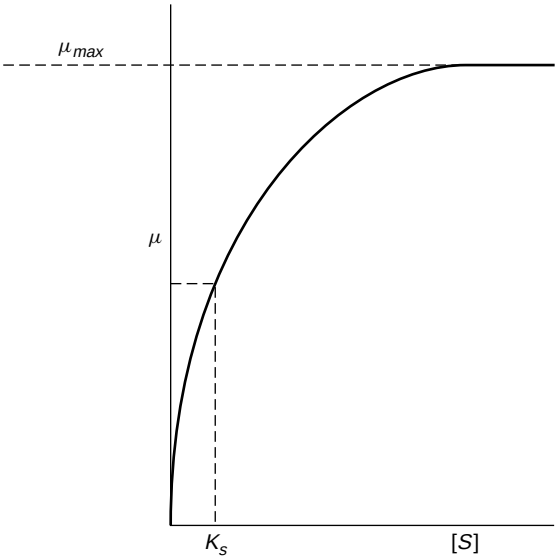

Figure 6. Relationship between bacterial specific growth rate & substrate.

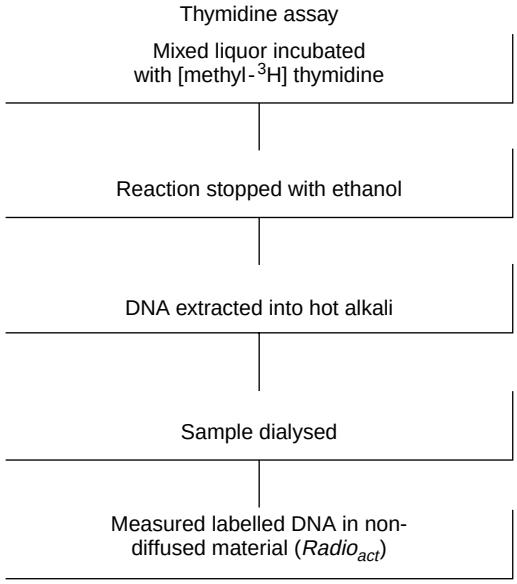

Figure 7. Flow diagram of the thymidine assay used for in situ measurement of volumetric bacterial growth rates.

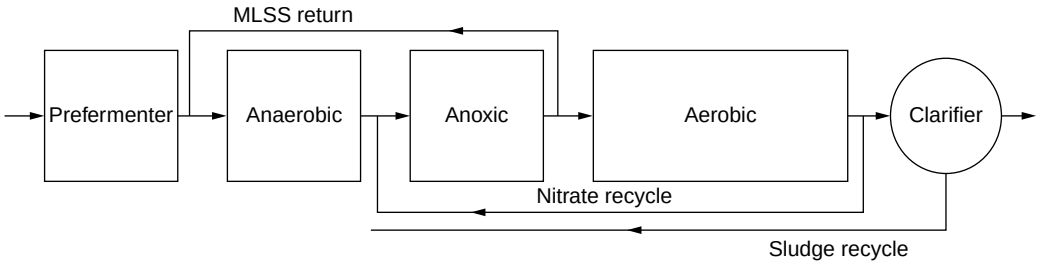

Figure 8. Schematic of UCT configured high-rate biological nutrient removal (BNR) treatment of domestic wastewater at Brendale, Pine Rivers Shire Council, QLD Australia.

process. This represents the first time these very powerful tools have been applied to directly observe the growth kinetics of bacteria in wastewater treatment processes.

Calculating Volumetric Bacterial Growth Rates

To calculate bacterial growth rates in the different stages of a biological nutrient removal (BNR) treatment process (Fig. 8), they converted the radioactivity incorporated into bacterial DNA in each compartment into the increase in the number of new bacterial cells. The radioactivity incorporated into DNA ($Radio_{act}$; dpm.mL^{-1}) was converted to μCi ($C_{dpm}/\mu Ci = 2.2 \times 10^6$ dpm.μCi^{-1}). Knowing the specific activity (SA; μCi nmol^{-1}), the rate thymidine was incorporated into new DNA ($r_{v,tdr}$), during the incubation time (t), was:

$$r_{v,tdr}[\text{nmol tdr} \cdot \text{mL}^{-1} \cdot \text{h}^{-1}] = \frac{Radio_{acr} \cdot f_{dil}}{C_{dpm/\mu Ci} \cdot SA \cdot t} \qquad (3)$$

A conversion factor (K_{tdr}) was used to determine the rate of formation of new cells from the rate of incorporation of labeled thymidine ($r_{v,tdr}$). Similar values for this factor have been derived both empirically and theoretically (80). Reviews of the literature suggest an average value for K_{tdr} of 2×10^9 cells nmol tdr^{-1}.

Volumetric bacterial growth rates ($r_{v,x}$; cells mL^{-1}.h^{-1}) were then simply calculated from the radioactivity (dpm.mL^{-1}) incorporated into DNA with the following equation:

$$r_{v,x} = r_{v,tdr} K_{tdr} \qquad (4)$$

Determining Specific Growth Rates

Henze and Harremoes (131), referred to volumetric biological growth rate ($r_{v,x}$) with dimensions−mass per unit volume per unit time, for example, Kg COD (biomass).m^{-3}.d^{-1}. The rate of incorporation of thymidine is also a measure of volumetric bacterial growth rate but it is dimensioned by the number of cells mL^{-1} h^{-1}.

Bacterial-specific growth rate is described as the volumetric increase in cell numbers divided by the concentration of bacteria (Eqn. 5). Thus, bacterial-specific growth rates (μ) were determined by dividing the volumetric bacterial growth rates by the concentration of bacteria and was multiplied by 24 to convert h to d:

$$\mu[d^{-1}] = r_{v,x} \cdot \frac{1}{C_{cells}} \cdot 24 \qquad (5)$$

A major advance of the thymidine growth assay is that it is a direct measurement of growth kinetics. Volumetric bacterial growth rate, ($r_{v,x}$), is measured directly and independently of the initial concentration of bacterial cells C_{cells}. The method allows a level of sensitivity to changes in bacterial growth rate that is immediate and that has not been previously possible for open wastewater treatment systems (132).

In biological wastewater treatment processes, the bacterial cell concentration (C_{cells}) is not measured directly, although direct DNA-staining methods and epifluorescent microscopy methods are available (130,133,134). The conventional approach is to measure biomass in terms of the particulate chemical oxygen demand (COD) or volatile suspended solids; VSS (36,135). With these measures, however, bacteria are not distinguished from the particulate material (130) that is enmeshed in the flocs of wastewater (136). Other methods have relied on measuring bacterial metabolic activity, for example, ATP and respiration rates (35,137). These methods are not reliable because they depend on the physiological (metabolic) state of the bacteria (34,138,139).

Fluorescent DNA−staining methods are well-accepted quantitative techniques that have been used for determining the number of bacteria in aquatic environments (140). The value of using these microscopic methods is that the bacterial biomass measurement is direct and specific. The methods are independent of extracellular material, abiological material, and eukaryotic microorganisms, such as protozoans.

When interpreting specific growth rate measurements, remember that within the wastewater bacterial community C_{cells} includes dormant (not dividing) and/or slowly growing bacteria. Thus, μ (Eqn. 5) is a weighted average of the specific growth rate of the bacterial populations. Only recently has the direct measurement of bacterial biomass been distinguished from cellular activity (Vollersten, 2001).

Pollard and Greenfield (100) measured the volumetric bacterial growth for the prefermenter overflow, secondary clarifier, aerobic, anaerobic, and anoxic compartments of a BNR municipal treatment process. The kinetic parameters and state variables bacterial growth and numbers, are compared in Figure 9. It is important to recognize the difference between growth and specific growth rate measurements (Eq. 4 *cf* Eq. 5).

Literature values for maximum specific growth rates measured in activated sludge range from 2 to

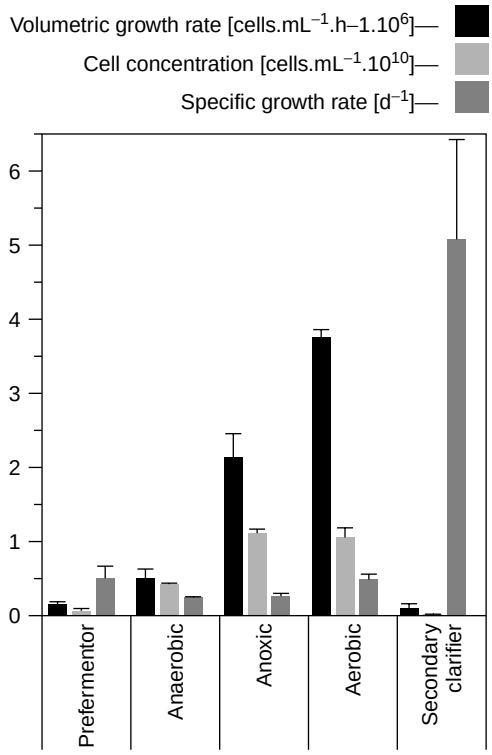

Figure 9. Growth kinetics in the different compartments of the BNR processes (After Pollard and Greenfield, 1997).

6 d^{-1} (3,40,141). These kinetic values are often used in modeling the activated sludge process. These are maximum rates determined with batch culture assays. For the activated sludge used in the study of Pollard and Greenfield (100), the direct measure of specific growth rate was 0.5 d^{-1} (Fig. 9; aerobic compartment). This in situ specific growth rate measurement was under conditions that were not at saturating substrate concentrations (117). This rate also agrees with those measured under operating conditions of municipal activated sludge treatment processes in which bacterial-specific growth rates have been determined as 0.6 d^{-1} or greater using respirometric-based methods (141,142).

RESPIRATION VERSUS GROWTH KINETICS

In biological wastewater treatment, predicting the ability of the bacterial community to degrade and assimilate influent nutrients is the basis of the mathematical models that are used to design and operate the process. For effective models, the kinetic parameters must accurately mirror the degradative ability of the bacterial community in situ.

Respirometry-based methods, such as the batch assay, derive bacterial growth rates from rates of substrate oxidation that rely on respiration measurements. They assume a constant coupling between catabolic and biosynthetic (anabolic) processes. However, physiological stress from the sudden increases in substrate or the presence of toxic compounds in industrial wastewater, for example, may stimulate respiratory activity at the expense of bacterial growth (143–145). Consequently, only using respiration as a measure of bacterial growth may be misleading when growth becomes uncoupled from catabolism (146). Ignoring the effects of toxins, such as aromatic hydrocarbons, on the viability of bacterial populations can lead to significant underestimates of the growth rate of the bacterial community in the original treatment environment (147).

In BNR systems, the rate of nutrient removal is a function of bacterial metabolism, of which bacterial growth rate is a part. Bacterial growth and metabolism are terms that describe bacterial physiology differently. Metabolic activity describes bacterial catabolic and anabolic reactions, and includes reactions that are responsible for cell maintenance and growth. Bacterial growth is the ability of the cell to divide, that is, to create new biomass, all other processes are generally referred to as cell maintenance (148).

Batch reactor assays are used to estimate maximum rates of substrate oxidation and growth for both heterotrophic and autotrophic bacteria (36). The importance of this assay to the measurement of bacterial growth is well recognized (16,40,93,149–151). Volumetric substrate consumption rate ($r_{v,s}$) and volumetric bacterial growth rate ($r_{v,x}$) are related by the yield constant (Y) (135):

$$r_{v,s} = -\frac{1}{Y} r_{v,x} \tag{7}$$

The standard approach is to assume that the relationship between substrate consumption and specific growth rate (μ) and concentration of bacteria (X) follows simple Monod (117) kinetics and so is described as follows:

$$r_{v,x} = \mu \cdot X \tag{8}$$

$$\mu = \mu_{max} \cdot \frac{S}{K_s + S} \tag{9}$$

Using this system model, it can be expected that the bacterial populations will grow at their maximum ($\mu = \mu_{max}$) when supplied with excess substrate ($S \gg K_s$), where K_s is the half-saturation constant; this is the basis of the batch assay.

In a domestic activated sludge batch assay, the saturating substrate condition is achieved by adding excess raw sewage to a smaller amount of mixed liquor from the full–scale aerated reactor. The bacterial growth rate is then inferred from the initial oxygen uptake rate (OUR) measurement (36,40). However, the method has recently been thoroughly reviewed and shown to have several shortcomings (16). At high substrate to biomass ratios ($S_o/X_o > 20$), the batch assay will measure the maximum rate of substrate oxidation, but it does not represent the growth rate and physiological state of bacteria in the original treatment environment in situ.

The greater the batch reactor S_o/X_o ratio, the greater the change in the bacterial community away from that of the original wastewater environment, the more likely it is that the measured kinetics will reflect the characteristics of the fastest growing bacteria, or changes in the physiological state of the bacterial community. Hence,

lower S_o/X_o ratios (<2) are preferred in batch assays. Under these conditions, bacterial growth rate is assumed not to be altered from that of the original treatment environment (39,93). The thymidine growth assay is an alternative approach that allows studies of the dynamics of the diverse interactive microbial consortium in the batch reactor environment.

Batch assays are used to estimate maximum specific growth rates. This measure is fundamental to the modeling of biological nutrient removal processes. However, ignoring its potential and shortcomings can lead to meaningless data and wasted research. Pollard and coworkers (152) used the thymidine assay to directly measure the specific growth rate of the bacterial community alongside a respirometric method (oxygen uptake rate; OUR), soluble substrate losses (SCOD) and bacterial biomass increases. They describe how and when bacteria allocate energy between new cell synthesis and cell maintenance, their results are shown in Figure 10.

The changing bacterial-specific growth rate measurements (μ_{tdr}) (Fig. 10) conflicted with this conventional model of the batch assay that assumes that the initial constant OUR represented a constant specific bacterial growth rate.

Ekama and others (36,124) adopt a standard Monod kinetic model of bacterial growth for the batch reactor, which dictates that during the initial period of excess substrate, the bacteria grow at their maximum rate. The thymidine measurement of the dynamic behavior of the bacteria in the batch reactors challenge the fundamental theory of the batch assay in which, in reality,

- μ is not constant, nor at its maximum
- the biomass yield varies

Simultaneously measuring respiration and in situ bacterial growth rates, using the thymidine assay and a direct measure of bacterial numbers, gave more information about how the cell allocated its energy resource from the initial oxidation of substrate (Fig. 11).

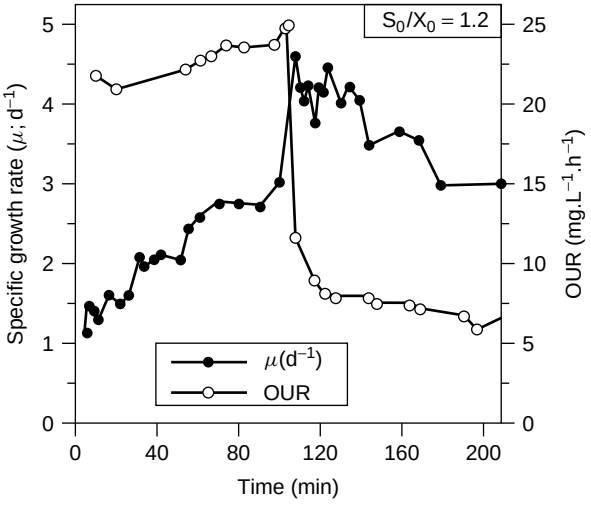

Figure 10. Municipal batch reactor μ compared with OUR at $S_o/X_o = 1.2$. The specific growth rates were derived from volumetric bacterial growth rate and the concentration of bacteria (After R. D. Robarts and R. J. Wicks, *Limnol. Oceanogr.* **34**, 213–222 (1989).

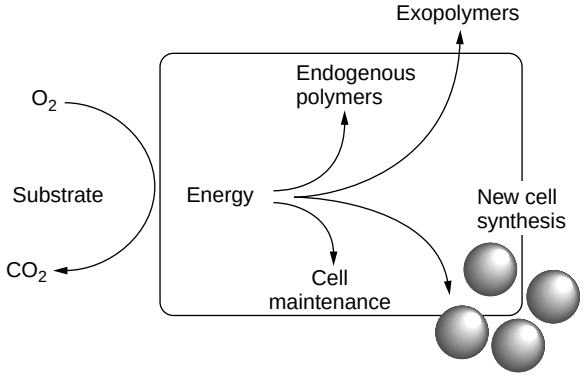

Figure 11. Energy flow, growth, and cell maintenance. The initial oxygen uptake rates (OUR) of batch reactors are related to the energy generated in the first substrate oxidation step. On its own, the OUR measurement does not indicate how the cell used this energy. (After R. D. Robarts and R. J. Wicks, *Limnol. Oceanogr.* **34**, 213–222 (1989).

For the bacterial community in municipal activated sludge, bacterial-specific growth rate increased, whereas the rate of substrate oxidation remained relatively constant. The characteristics of the bacterial community within the batch reactors does not represent those of the original treatment environment. Inferring growth kinetic parameters for continuously operated flow-through processes, using the closed environment of a batch reactor, does not reflect the degradative potential of the original wastewater treatment system.

Researchers have expressed concern over the ability of batch assay to reflect the conditions of the original treatment environment. However, it has been widely accepted because of the lack of alternative methods. Although the thymidine assay is more complex, combined with respirometric measures, it can provide far more information about bacterial behavior and energy flow in the original treatment environments. Methods to determine kinetic parameters for the biological models no longer need be restricted to respirometry.

BIOFILMS

Biofilms are found on almost any wet surface, transforming and removing soluble compounds from solution (153–155). The importance of biofilm bacterial communities is well recognized and topical (156). New Scientists described them as "Chic, urbane, sophisticated, and sometimes deadly — such are the inhabitants of the world's weirdest metropolis" (157).

Historically microbiologists' concepts of bacterial behavior have been built on laboratory isolates, far removed from their natural habitat. However, in naturally complex environments, bacteria rarely grow as single species. Rather, they grow in communities "cheek-by-jowl in slime cities," helping each other to exploit substrates through synergistic relationships (157,158).

Biofilm bacteria are held in an attached matrix that absorbs and metabolizes dissolved compounds. Most microbial metabolic activity occurs in these structured bacterial communities at the solid–liquid interface (156,159). The wastewater industry has exploited the efficiency of attached bacterial growth to remove pollutants from wastewater in fixed-film processes (135,160–162).

In the treatment of wastewater, constructed wetlands can be considered as large-scale, essentially fixed-film bioreactors (163). However, the literature that describes these processes has largely ignored the in situ growth-kinetics of the attached bacterial communities (164, Mitsch and Gosselink, 1993; Duncan and Groffman, 1993). To understand the kinetics of the bacterial communities in the biofilm's microenvironment requires a quantitative measurement of the growth kinetics of the biofilm bacteria. However, only recently have tools been available to directly measure the growth kinetics of biofilm bacteria in fixed-film treatment processes using radioactively labeled thymidine incorporation. The significance of applying the thymidine assay here is that biofilm bacterial growth kinetics can be measured without disturbing the biofilm.

For example, the surface biofilm on plant stems in constructed wetlands can be assayed by immersing the tissue in solutions of the thymidine without disturbing the biofilm as shown in Figure 12.

The measurements of in situ biofilm bacterial growth kinetics both displayed, and accommodated, the inherent heterogeneity of the complex wetland ecosystems. Biofilm bacterial metabolic activities, using the redox dye CTC (Bhupathiraju and coworkers, 1999a&b; Sherr and coworkers, 1999; Flood and coworkers, 1999) and growth rates could be measured simultaneously. The thymidine assay showed that the redox dye, CTC, does not inhibit bacterial growth rate. With CTC as a measure of biofilm bacterial respiratory activity, biofilm metabolic activities and growth kinetics can be measured simultaneously.

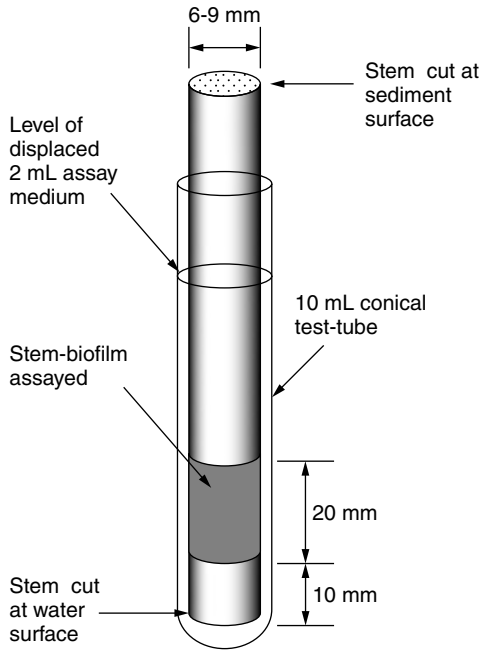

Figure 12. Biofilm on wetland plant-stem-section incubating in medium with radioactively labelled thymidine and/or the redox dye, CTC (for details see Pollard and coworkers, 1995; Flood and coworkers, 1999).

IN SITU GROWTH KINETICS OF SINGLE SPECIES

Measuring the growth kinetics of bacterial species (i.e., a population) within a microbial community is fundamental, yet it seems an almost impossible objective given the complexity of bacterial communities in situ. However, if we are to understand how microbial communities function and mediate the changes in water quality parameters, quantifying the individual species growth kinetics is essential. We need to know the fraction and populations of the community that are metabolically active and growing (Pinhassi, and coworkers, 1999; Gasol and coworkers, 1999 and Sherr and coworkers, 1999; Ducklow, 2000; Vollersten and coworkers, 2001).

We have seen how to estimate bacterial community growth kinetics with the thymidine method. However, this method does not indicate the fraction of the populations in the microbial community that are not dividing. For specific growth estimates, the thymidine growth measurement is averaged over all the populations in the community when bacterial numbers are estimated with fluorescent dyes. Fluorescent DNA stains can be used to measure the number of bacteria (Nobel and Fuhrman, 1998) and respiratory dyes used for estimates of whether cells are living or dead (Gasol and coworkers., 1999; Sherr and coworkers, 1999; Vollersten, 2001).

The assumption used here is that if the bacterial cell is intact and stains positive for DNA material, it may not be dividing but it mostly likely maintains the potential to divide. However, there are inconsistencies in the literature that report the percentage of bacterial cells that contain DNA and that are also metabolically active (Zweifel and Hagstraom, 1995; Choi and Sherr, 1996).

We have in situ methods for identifying bacteria using DNA or RNA based fluorescently labeled probes that target important bacterial species or groups of bacteria in wastewater. Individual bacterial species can then be identified in situ. Molecular studies of bacterial communities focus on identifying bacteria and/or changes in the community structure between different environments (Lee and Fuhrman, 1991 (Telang, and coworkers, 1994; Lambert, and coworkers, 1993; Raskin, and coworkers, 1995; Sevior and Blackall, 1998; bond and Banfield, 2001). However, there is little attention given to quantitative methods to measure their in situ growth rate.

For at least 25 years, fluorescent DNA probe methods have been available for counting bacteria in natural aquatic environments (Hobbie, and coworkers, 1977). The accuracy and precision of the methods are well documented (Lebaron, and coworkers, 1994; Gasol, and coworkers, 1999), and they can be applied quantitatively to engineered wastewater environments (von Münch and Pollard, 1997; Vollersten and coworkers, 2001). There is a reason these methods have not been used to measure bacterial growth rates in situ.

Molecular techniques have considerable potential for identifying and counting individual species or groups of bacteria in wastewater. Currently, these methods are thought to have the potential for measuring in situ species growth rates. This assumes that increases in the number of a bacterial species can be used as a measure of growth rates in situ, for example, the use of fluorescently labeled oligonucleotide hybridization probes combined with flow cytometry (Amann, and coworkers, 1995; Veal, and coworkers, 2000) and to a some degree, real-time PCR (Becker, 2000). However, these approaches are limited to measurements of bacterial numbers/biomass at a point in time, and the methods rely on observing in situ increases of bacterial numbers amidst an array of processes that are continually removing the bacterial biomass.

Monitoring increases in bacterial numbers with fluorescent probes could not distinguish such losses from gains. The net change in biomass may be zero, as in a chemostat, for example, where $dx/dt = 0$. The sensitivity of this approach to biomass change is limited to the time required to detect an increase in a population. This may be on the order of hours. What might be measured as a slow-growing bacterial population may well be growing rapidly in the presence of a ravenous protozoan community (Caron, and coworkers, 1993; Van Loosdrecht and Henze, 1999) or being removed from the system by dilution or death through viral lysis (Wommack and Colwell, 2000).

The thymidine assay is well accepted as an in situ measure of bacterial growth rate that is independent of bacterial biomass concentration. However, this measurement of the growth rate is an average value of the community not for a single species. The scope of the assay could be significantly expanded with the ability to measure species growth rates in situ. The thymidine growth assay may be coupled to current advanced molecular methods that identify bacteria. The combined technique then is able to quantify species growth rate in situ.

The principle has been demonstrated in laboratory cultures and in a municipal activated sludge treatment process (Pollard, 1998). The DNA of all dividing bacteria are radioactively labeled with [methyl-^{3}H] thymidine, thereby creating a mixture of radioactive DNA molecules (^{3}H-Tdr-DNA). The target DNA from an isolate of interest was subsequently immobilized on a membrane. When the ^{3}H-Tdr-DNA mixture is incubated with the immobilized-membrane DNA, the complementary (homologous) radioactively labeled DNA molecules reassociated. Only those bacteria that were dividing in the initial [methyl-^{3}H] thymidine incubation were detected on the membrane strip (Pollard, 1998).

This approach is sometimes referred to as a reverse gene probe method because the membrane-bound DNA is of the known bacterial species (Voordouw, and coworkers, 1991; Ehrmann, and coworkers, 1994), whereas it is the unknown community in the Southern blot method. In a conventional hybridization preparation, such as that of the Southern blot method (Southern, 1975), a radioactively labeled or fluorescently tagged DNA probe of a known bacterial species is synthesized. DNA extracted from an environmental sample is immobilized on a membrane. The membrane is then probed for the presence of its complementary single-strand of DNA.

Others have used a similar reverse gene probe approach to identify changes in bacterial community structure in natural environments (Lee and Fuhrman, 1990; 1991; Voordouw, and coworkers, 1991). They used "nick" translated gene probes of environmental samples, qualitatively observing changes in bacterial populations.

Further advances may be made on measurements of single species growth rates using the thymidine assay. The in situ measurement of the specific growth rate (μ_{sp}) of a single species is possible. A molecular probe could be designed and applied to identify and count the number of a bacteria species, whereas its growth rate is measured with the thymidine assay. For example, Rosselló–Mora and coworkers (1995) designed a 16S rRNA–oligonucleotide probe for *Z. ramigera* and counted the number of bacteria in activated sludge. Pollard (1998) quantified the growth rate of the same species in activated sludge. If both methods were performed simultaneously the specific growth rate of *Z. ramigera* could be determined by dividing the species growth rate by the number of these bacteria. Simultaneously applying these two powerful molecular-based methods has enormous potential. Synergistic,

Table 1. The Thymidine Assay Combined with Different Molecular Methods to Determine Key Kinetic Parameters in situ. The Measurement of these Parameters can be Used to Simulate Treatment Processes. The Specificity of the Dynamic Measurement Increases (Top to Bottom) from a Community Level to the Specific Growth Rate of a Bacterial Population

Thymidine Assay Combined With		In Situ Growth Kinetic Parameter
No other assay	⟶	Community average–volumetric growth rate (average of populations)
General fluorescent DNA probe; AO and DAPI	⟶	Community specific growth rate
DNA-DNA hybridisation	⟶	Population volumetric growth rate
DNA-DNA hybridisation & species–specific DNA probes	⟶	Population specific growth rate

Increasing specificity

Table 2. Summary of Stoichiometric and Growth Kinetic Parameters Determined in this Research. Specific Growth Rate Measurements Generally Agreed with Alternative Indirect Measurements

Wastewater System	Volumetric Growth Rate 10^9.cells.mL^{-1}.d^{-1}	Numbers 10^{10}.cells.mL^{-1}	Specific Growth Rate μ (Tdr/AODC) [d^{-1}]	Specific Growth Rate μ^* (Indirect determin) [d^{-1}]	Remarks and References Regarding Alternative–Indirect Measurement of μ^*
Biological nutrient removal					
— prefermentor	0.2 ± 0.1	0.04 ± 0.01	0.4 ± 0.3	—	100
— anaerobic	0.7 ± 0.1	0.40 ± 0.04	0.2 ± 0.1	—	100
— anoxic	3.0 ± 0.6	1.1 ± 0.1	0.3 ± 0.1	—	100
— aerobic	5.4 ± 0.1	1.1 ± 0.2	0.5 ± 0.1	>0.6, 4 to 6#	142, 141
— clarifier	0.2 ± 0.1	0.004 ± 0.0001	5.1 ± 1.4	—	# μ_{max} range; 3; 40
Sequencing batch reactor					
Aerobic period	1.8 ± 0.3	0.4 ± 0.2	0.4 ± 0.1		
Industrial (Phenol) waste	35 ± 2	3.8 ± 0.3	1.3 ± 0.1	2.8	144
Population dynamics (municipal activated sludge)					
Zoogloea ramigera	0.7	0.11 ± 0.02†	0.7	—	†10% of all sludge populations (186)
Batch reactors					
Municpal	0.7 to 4.5	1 to 2	1.2 to 4.5	4.5	152
				4.1	Empirically determined from increases in cell numbers
Industrial	0.26 ± 0.03	1.35 ± 0.03	0.02 ± 0.003	2.8	Determined from initial OUR in batch (144)
Anaerobic Digesters					
Acidogenic (fermentor)					132
— lab-scale (CSTR)	2.1 ± 0.2	0.3 ± 0.1	0.8 ± 0.2	1.7	Hydraulic residence time
— full-scale	1.5	0.07 ± 0.01	2.2	3	Hydraulic residence time
UASB (methanogenic)					
— 1.5 m depth	0.40 ± 0.02	0.11 ± 0.01	0.35 ± 0.02	—	132
— 3 m depth	3.2 ± 0.10	0.7 ± 0.1	0.4 ± 0.1	0.4	188
Biofilms (Fixed-films)					
Fluidised–bed					
— solids	0.20 ± 0.02	1.9 ± 0.3	0.25 ± 0.06	Hydraulic residence time	
— liquid	0	0.04 ± 0.007	0	—	
Macrophytes of constructed wetlands				0.01 to 45	
Inlet high–P	0.014 ± 0.020	0.06 ± 0.02	2.1 ± 0.8	—	Marine epiphytic bacteria; 189
Outlet high–P	0.015 ± 0.003	0.07 ± 0.02	2.3 ± 0.7	—	165
Inlet low–P	0.020 ± 0.002	0.09 ± 0.03	3.3 ± 1.3	—	167
Outlet low–P	0.004 ± 0.001	0.05 ± 0.01	1.4 ± 0.6	—	165, 167

symbiotic, competitive, and diauxic growth, and inhibition, may now be studied in the wastewater environment in a truly ecological fashion.

CONCLUSION

Today's advanced wastewater management extensively uses biological treatment. These processes rely on bacterial growth to remove/transform pollutants. However, few of the wastewater bacterial populations can be cultured outside the treatment environment, hence little is known about their behavior in situ. Methods used to monitor microbial activity in wastewater do not measure bacterial growth rates in situ. Measuring bacterial biomass alone cannot account for the continued removal of bacteria through dilution, death, viral lysis, and protozoan grazing.

The thymidine assay has been shown to directly measure the average volumetric growth rate. The significance in applying this approach to wastewater research is that it is a measure of the in situ volumetric bacterial growth rates at a molecular level of sensitivity independent of the bacterial biomass. It is a mean measure of the slow to the most rapidly growing bacteria. On its own, the thymidine assay is limited because high volumetric growth rate measurements may have resulted from either a few bacteria growing rapidly or many bacteria growing slowly. However, combining this measurement with other in situ measures of bacterial biomass expanded the versatility of the method significantly (Table 1).

The versatility of the thymidine assay when combined with other molecular methods is summarized in Table 1. The key growth kinetic parameters that can be derived are shown in order of increasing bacterial specificity.

Combining measurements of volumetric bacterial growth rate and respiratory activity give more information about how the bacterial populations allocates the energy generated from substrate oxidation in the wastewater environment.

Table 2 shows a summary of stoichiometric and growth kinetic parameters for a range of treatment processes. Specific growth rate measurements are compared to alternative indirect measurements. These other methods determined specific growth rates on the basis of respirometry (OUR), increases in cell numbers (AODC), and hydraulic residence time. Generally, the thymidine measurements of specific growth rates agreed with the values from these alternative techniques (Table 2).

Respirometric methods were not always the same as those determined with the thymidine assay. Estimates of μ in the OUR batch assay using phenolic wastewater (Batch reactor — Industrial; Table 2) were substantially higher than those determined by the thymidine assay. This was most probably because the respirometric method measured rates of oxygen uptake that was due to the toxic affects of phenol while inhibiting bacterial growth.

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WASTEWATER TREATMENT, MICROBIOLOGY OF.

See Activated Sludge — Molecular Techniques for Determining Community Composition; Algal Turf Scrubbing: Potential Use for Wastewater Treatment; Parasitic Protozoa: Fate in Wastewater Treatment Plants; Toxicity Testing in Wastewater Treatment Plants Using Microorganisms; Wetlands and Readbeds for Wastewater Treatment; Wastewater Stabilization Ponds

WASTEWATER TREATMENT-SEPTIC TANK SYSTEMS.

See Septic Tank Systems

WATER ACTIVITY.

See Desiccation by Exposure to Space Vacuum or Extremely Dry Deserts: Effect on Microorganisms

WATER DISTRIBUTION SYSTEMS.

See Biodegradable Dissolved Organic Carbon in Drinking Water; Coliform Bacteria — Control in Drinking Water Distribution Systems; Controlling the Microbial Quality of Drinking Water in Distribution Systems

WATER FUNGI AS DECOMPOSERS IN FRESHWATER ECOSYSTEMS

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This article provides an overview of fungi in freshwater habitats. We have organized it according to the major ecological functions that fungi assume in these systems,

rather than using taxonomic delineations as the guiding thread. On the basis of studies on nutritional strategies and systematics, fungal diversity, distribution and dispersal patterns we thus focus on the roles of fungi as decomposers, symbionts, and parasites. For each of these functions we will present one or more examples from freshwater systems. A final section is devoted to anthropogenic effects of fungi in freshwater habitats. This structure implicates that several systematic groups will be discussed repeatedly in different contexts.

The objective of the article is not to provide a complete review of the topic; the diversity of systems and organisms, and the rudimentary knowledge on some of these, prevents coverage of all potentially important aspects. Rather, our overview is meant to serve as a directory that draws attention and provides access to areas of freshwaters ecology where fungi are likely to play crucial roles, many of which still are poorly recognized at present.

ORGANOOSMOTROPHY AS A KEY FEATURE

Two modes of nutrition are realized among heterotrophic organisms. Animals and Protozoa take up particles by means of phagocytosis, a nutritional mode known as phagotrophy, whereas other heterotrophic eukaryotes and prokaryotes rely on dissolved low-molecular weight compounds. Organic particles and dissolved organic carbon of higher molecular weight are first broken down and cleaved extracellularly into small units before they are assimilated by these organisms. This mode of nutrition is referred to as organoosmotrophy.

A GLIMPSE ON FUNGAL SYSTEMATICS

The eukaryotic organoosmotrophs occurring in freshwaters are phylogenetically diverse, McLaughlin and coworkers (1). Although traditionally considered fungi, major groups bear no relationship even at the highest taxonomic level and have consequently been assigned to different Kingdoms (2,3). This classification is well supported by both ultrastructural and molecular evidence and is now generally accepted. Representatives of the Fungi *sensu stricto* include the Chytridiomycota, Zygomycota, Ascomycota, and Basidiomycota, which collectively are referred to as Eumycota (1). The Microsporidia have recently been added to this group (2,3). Deuteromycota — also known as Fungi Imperfecti, anamorphic, mitosporic, or conidial fungi — are asexual forms that are mostly related to the Ascomycota or, less frequently, to the Basidiomycota and thus belong to the Eumycota as well. They comprise the Hyphomycetes and Coelomycetes (4,5).

Chytridiomycota, Deuteromycota, Ascomycota, and probably also Microsporidia are the predominant groups of Fungi in freshwaters, although members of the other groups may also be present. Basidiomycota are notably rare. In addition, a major group of phylogenetically distinct organoosmotrophs is prominent in freshwater environments. These organisms, previously known as Oomycota, bear a much closer relationship to heterokont algae than to the true Fungi (1). Together with the Hyphochytrea, or Hyphochytriomycota, another unique group of fungal-like organisms with about 25 species known at present, the oomycetes are thus placed in the Kingdom Chromista (3).

This and other taxonomic delineations and terms used in this article are adopted from the authoritative treatise edited by McLaughlin and coworkers (1) with some modifications following Cavallier-Smith (3). A host of other names and systems pervades the mycological literature, reflecting differences in opinion but also the fundamental progress in the systematics of these organisms in recent years. The term fungi, in particular, has given rise to much debate. For the purpose of this article, we will thus use Fungi with a capital letter to refer to Fungi sensu stricto, that is, to the Eumycota. For convenience, we will also refer to fungi, spelled in lower case, to designate the entire range of organisms traditionally studied by mycologists. It must be emphasized, however, that this usage is simply one of convenience, implying no phylogenetic relationship whatsoever.

DIVERSITY, DISTRIBUTION, AND DISPERSAL

Current world estimates of the number of Fungal species range from a few hundred thousand (6) to 1.5 million (7), 72,000 of which are known to science (8). These numbers illustrate that Fungi have been greatly underexplored, particularly compared with plants and animals, although in terms of species numbers, effects on other organisms, and significance in ecosystem functioning they may well be one of the most important groups of eukaryotes that have evolved on Earth. However, only about 1% of the species known at present have been recorded from freshwater habitats. Wong and coworkers (9,10) estimated their number as some 600, although a more extensive literature survey and inclusion of the Microsporidia would certainly yield a much higher number. Nevertheless, and even if the proportion of known to extant species is lower than the 5% estimated for fungi in general, the total number of species occurring in freshwater habitats would be distinctly lower than 20,000.

As with other microorganisms, many species of fungi occurring in freshwaters have a widespread, often cosmopolitan distribution (11–15) although in the Ascomycota notable differences exist between genera of temperate and tropical regions (Ho & Hyde 1997). The number of recorded species is also lower in the tropics than in temperate climates. However, this discrepancy may simply reflect the less intense investigations carried out in tropical areas rather than a real underrepresentation of freshwater fungi at low latitudes.

Freshwater fungi are adapted to life in aquatic environments most obviously by the development of effective mechanisms for colonization and dispersal in a viscous medium such as water. Dispersal may occur via either motile or nonmotile spores. The former are typical of the Oomycetes, Hyphochytrea, and the Chytridiomycota. Nonmotile spores characterize the aquatic Ascomycota and Deuteromycota. Many of these nonmotile spores have elaborate shapes, appendages, or wall ornamentations, or are surrounded by various sheaths, all of which

have been interpreted as adaptations to dispersal and/or attachment (16). Wong and coworkers (9,10–22) give a more complete account of these putative adaptations.

DECOMPOSERS

Mycelial Growth and the Availability of Resources

Mycelial growth is a characteristic feature of filamentous fungi, which ultimately has major repercussions on the functions of these organisms in freshwater ecosystems. Growth in ramifying filaments allows to assimilate nutrients over a large surface area relative to body volume, and facilitates internal translocation of substances (e.g., organic carbon compounds, inorganic nutrients, oxygen) over appreciable distances. The older hyphae are rigid and permit a high turgor pressure to be exerted at the plastic and extensible hyphal tips (Money 1994), favoring penetration of hard surfaces and subsequent spreading within the substrate underneath. As a result, filamentous fungi do not typically grow on top of surfaces in biofilms as is often erroneously assumed. They are rather fully embedded in their substrate. Hyphae could in fact be viewed as self-extending digestive tracts that have been turned inside out. Clearly, therefore, mycelial growth is an effective adaptation to exploiting solid coarse-particulate organic substrates such as plant and animal tissues, and it is not surprising that the mycelial growth form has evolved independently in different lineages of both prokaryotic (Actinomycetes) and eukaryotic (Oomycota and Eumycota) organoosmotrophs.

The organic substrates most suitable for exploitation by filamentous fungi occur in greatest abundance where aquatic systems are intimately linked with their terrestrial surroundings. Streams, for example, that are fully shaded by a riparian canopy typically receive some 400 to 800 g of coarse-particulate organic matter m^{-2} yr^{-1}, the bulk of it in the form of leaf litter, twigs, and small branch wood derived from the streamside vegetation (Weigelhofer and coworkers, 1994; 23,24,25). This sort of allochthonous matter is of utmost importance for stream food webs and total system functioning (Wallace and coworkers 1997). Inputs of large branch wood and whole trees are more episodic and therefore more difficult to quantify but likewise are a key feature of pristine forest streams (26,27) and other water bodies, permanent or temporary, with tall woody vegetation in their perimeter (e.g., swamps and many river floodplains).

In marshes and littoral zones of lakes, coarse-particulate organic matter is produced mainly by emergent macrophytes. These macrophytes may form extensive, often monospecific stands in freshwater marshes, littoral zones of lakes, floodplains, and river deltas. Common plants occurring in these areas are members of the sedges (*Carex* spp.) and other Cyperaceae (e.g., *Cladium*, *Cyperus*, *Eleocharis*, *Scirpus*), rushes or Juncaceae (*Juncus*), grasses or Poaceae (e.g., *Phragmites*, *Glyceria*) and cattails or Typhaceae (*Typha* spp.). Emergent macrophytes are often highly productive (28,29), rivaling crop production in intensive farming. Hocking (30,31), as extreme examples, estimated the annual aboveground plant production of *Phragmites australis* and *Juncus effusus* at almost 10 kg/m^2. Because little of this production is normally removed by herbivorous animals during the growing season (28), large amounts of plant matter senesces and hence becomes available as a habitat, carbon, and energy source for fungal decomposers. Thus, large amounts of plant litter characterize aquatic habitats at the interfaces with terrestrial ecosystems. These are the environments in which, owing to their lifestyle, one would expect filamentous fungi to be competitive decomposers of organic matter.

Although most of the coarse-particulate organic matter in freshwaters is plant litter, animal carcasses may sometimes be an important component as well. An impressive example comes from streams along the Pacific coast of North America, where some salmon species suffer mass mortality following upstream migration and spawning in the headwaters. Historically, the massive mortality of these fishes may have resulted in the deposition of 1.1 kg of dry fish body mass per m of stream length (32). The role of fungi in the decomposition of salmon carcasses is, however, unknown and will not be further considered here. Instead, we will first give a brief account of the fungi colonizing decomposing leaves and wood in streams, and subsequently address the fungi associated with emergent macrophyte tissues decomposing in lentic water bodies under both submerged and standing-dead conditions. Fungi no doubt act as decomposers of a variety of other litter types in many other freshwater systems, but for the most part much specific information is not currently available (33).

Leaf Litter Decomposers in Streams

The fungi associated with leaves decomposing in streams have received considerably more attention than any other fungal decomposers in aquatic ecosystems (33–42). Both Oomycota and Chytridiomycota are prevalent in streams and are regularly found in association with decomposing leaf material (34,35,43). Hallet & Dick (11,44) and others (Park & McKee, 1978; 45,46) have postulated a role in leaf decomposition for the Oomycota, but significant involvement in the process has not been convincingly demonstrated to date. Likewise, the sexual stages (teleomorphs) of ascomycetes are rarely observed on leaf litter in stream habitats (18). Terrestrial hyphomycetes are common inhabitants of the phylloplane before leaves fall into streams (34,35,37). Although the importance of these Fungi in decomposition is not certain, but most evidence suggests that their activity is limited at the low temperatures prevailing during and after autumn leaf fall in temperate streams (Suberkropp and coworkers 1976; 34–36,43,47–50).

The fungi most active in the leaf decomposition process in streams are the so-called aquatic hyphomycetes or Ingoldian fungi (20,34–51). They are found nearly invariably associated with leaves decomposing in stream habitats. Aquatic hyphomycetes possess elaborate enzyme complements for degrading plant polymers (33,38–40,52) with the possible exception of lignin, and produce these enzymes when growing on

submerged leaves in culture (33,53–55). The ability to produce pectinases (pectin-degrading enzymes) is especially noteworthy and probably instrumental in the degradation of leaves (39–42,52–54,56). Aquatic hyphomycetes cause mass loss of leaves when grown in pure culture (41–43,49,53,55,57–60) and sporulate (i.e., reproduce) abundantly under submerged conditions both on pieces of agar and submerged leaf litter collected from the field. The dynamics of spore concentrations in stream water tend to correlate with the seasonality of leaf inputs, suggesting that the life cycle of aquatic hyphomycetes is tied closely to the seasonal availability of leaves as their main carbon and energy source. Increases in spore concentrations in stream water of several orders of magnitude may occur during (and shortly after) periods of bulk leaf fall (34,35,40–42,61).

Aquatic hyphomycetes are considered to be well adapted to the stream environment (9,10,34,35,38,40). Colonization of leaf litter by aquatic hyphomycetes is initiated by the landing of spores on submerged leaf surfaces. It is favored by the distinct shapes of their spores, often tetraradiate or sigmoid, interpreted as a morphological adaptation for attachment in flowing waters (20,21). Efficient trapping of tetraradiate and sigmoid spores on submerged surfaces has indeed been demonstrated experimentally (62). Additional explanations have been proposed (Bandoni, 1974; 38,63). Apart from efficient impaction of surfaces, aided by spore morphology, aquatic hyphomycetes are adapted to colonization in flowing water environments by the fast attachment after settling (*Lemonniera aquatica* within 30 min), aided by mucilage secretion and apressorium formation, and germinate within two to six hours in most species (19). In addition, the germination potential of the spores is high (64). Aquatic hyphomycete spores survive gut passage in stream invertebrates (65,66) and transitory residence in moist terrestrial environments remarkably well (67,68).

Once a spore has germinated, the growing hyphae extend inside the leaf matrix, thereby degrading the leaf tissue and producing significant amounts of biomass within a few weeks of leaf colonization. Many species show a rapid growth at the low water temperatures prevailing in the season following leaf fall in temperate climates (49,69,70). Instantaneous growth rates are highest at the initial stages of colonization and subsequently decline (58,71). Maximum growth rates of $0.2\ d^{-1}$ have been measured under field conditions (58,71,72) although typical rates are an order of magnitude lower (51). The high growth rates result in the rapid accumulation of considerable biomass ((71,73) overview in Gessner (51)), which greatly outweighs the biomass of bacteria associated with leaves (41,42,72,74–76). More than 15% of the total detrital mass (leaf + fungal mass) may be accounted for by fungi (71,73), even when apart from signs of tissue degradation the presence of fungi is not obvious to the naked eye.

On the basis of an average fungal growth efficiency of 35% (60) and estimated fungal production on standardized leaf packs submerged in streams, it has been calculated that fungi may account for 8 to 66% of overall leaf mass loss (74,75). Leaf decomposition rates are correlated with both fungal biomass accumulation and sporulation activity, as well as with concentrations of the recalcitrant leaf constituent lignin (73,77) and dissolved nutrients in stream water (78) (Sridhar). Taken together, these results suggest that fungi are key mediators in controlling leaf decomposition rates as a function of litter quality and environmental conditions (33,41,42).

The only available estimate of secondary fungal production in streams has been published by Suberkropp (79). His figure of 34 g dry mass $m^{-2}\ yr^{-1}$ is well in the range of production estimates for stream macroinvertebrates (24), demonstrating the quantitative importance of fungi for total system metabolism. Model calculations based on litter inputs to streams and decomposition rates and fungal biomass dynamics confirm that the magnitude of Suberkropp's (79) estimate is realistic (51). Significantly, the production of $34\ g\ m^{-2}\ yr^{-1}$ was achieved in a stream characterized by low concentrations of dissolved nutrients (flow-weighted annual average of 2.3 μg soluble reactive $P\ l^{-1}$ and <38.9 μg dissolved inorganic $N\ l^{-1}$) and a riparian vegetation that delivers primarily recalcitrant leaves (*Fagus grandifolia and Quercus* spp.). Both factors are unfavorable to fungal productivity (33,73,77,80).

Fungal biomass production (growth) within the leaf matrix is closely followed by the production of conidiophores (spore generating structures) and release of spores. Aquatic hyphomycetes may thus complete their full asexual (anamorphic) life cycle within the stream and, by doing so, virtually swamp their habitat with propagules during periods when resources tend to be most available. As leaf decomposition proceeds, sporulation rates first increase rapidly and subsequently decline equally abruptly, giving rise to pronounced temporal pattern of reproductive output. Sporulation early in the life cycle is typical in aquatic hyphomycetes (39,40,76,81–83). In aquatic hyphomycetes, this trait has been interpreted as an adaptation to the highly ephemeral nature of leaf accumulations in streams; leaves not only decompose rapidly in this environment (Bärlocher (81,82) has created the picture of a sinking archipelago) but also run the risk of being swept downstream when discharge increases (39–42). Maximum sporulation rates can reach 7×10^6 conidia per g of litter dry mass per day (34,35,55,73,84), resulting in spore concentrations in stream water that may exceed 20,000 per liter (20). How many of these are able to establish new colonies is unknown, as is the proportion used as food by invertebrates. Nevertheless, a simple model calculation testifies to the potential importance of this organic matter pool: If a concentration of 20,000 spores l^{-1} and a conservative individual spore weight of 0.3 ng is assumed (83), a stream with a discharge of $100\ l\ s^{-1}$ (say about 4 m in width) would transport some 50 g of organic matter per day in the form of aquatic hyphomycete spores. For comparison: This number exceeds the total annual production of bacterioplankton (pelagic bacteria) in many marine and freshwater environments.

As leaves decompose, community composition of the associated — sporulating — aquatic hyphomycetes changes. Distinct patterns of community shifts are not always apparent (34,35), partly because they may be masked by differences in successional speed of

individual leaves (samples) coupled with the pronounced sporulation dynamics during decomposition (cf. Chamier and coworkers (85)). As a result, large numbers of replicate samples would be required to discern a clear picture. Recurrent successional patterns have nevertheless been observed in several instances (34,35,86,87). Community shifts result, however, from changes in relative abundance of species rather than veritable true species replacements (86,70). Some species, such as *Flagellospora curvula*, have been identified as early colonizers, whereas others start to sporulate consistently at later decomposition stages (34,35,86). *Heliscella stellata* is a good example of the latter group (87). Some species tend to persist throughout, however, and others cannot be clearly assigned to a particular stage because their temporal occurrence has varied among studies (34,35). *Tetrachaetum elegans*, for example, normally recognized as an early colonizer (34,35,86), was dominant in all but the first sample in the study by Chamier & Dixon (54). Reasons for these discrepancies are potentially complex and largely obscure at present. They might be elucidated in the future as progress is made in understanding the ecological requirements of individual species, their metabolic capacities, life-history patterns including potential sexual stages, genetic and phenotypic variability, and their competitive and trophic interactions within decomposer communities.

Superimposed on successional changes during decomposition are seasonal shifts in species composition. According to Suberkropp's (70) temperature threshold hypothesis, species such as *Lunulospora curvula* and *Triscelophorus monosporus* become dominant on leaves as stream water temperature exceeds the threshold of 16 to 18 °C for any longer period of time. Conversely, *L. curvula* and associate species are replaced by cold-water assemblages when temperature drops below 5 °C. Either one assemblage persists in the intermediate temperature range between 5 and 15 °C. This temperature threshold hypothesis is supported indirectly by observations in both permanently warm (88) and cooler streams (70,86) where no seasonal species replacement occurs. In addition, prolonged colonization required by one sort of leaves compared with another coincided with a decrease in water temperature and a concomitant disappearance of *L. curvula* (34,35). Species such as *L. curvula* and *T. monosporus*, which in temperate streams may dominate at the higher summer temperatures (70,89), are often important components of aquatic hyphomycete assemblages year-round at lower latitudes (Sridhar & Kaveriappa 1989; 38,88) Some dominant cool-water species characterizing winter assemblages (e.g., *Alatospora acuminata*), in contrast, are less common in tropical and subtropical streams (34,35). These patterns of seasonal occurrence are partly, but not fully, congruent with both the growth and sporulation responses to temperature of pure cultures grown on either agar plates or leaves (34,35,69,70,83,90).

What is the spatial configuration of aquatic hyphomycete communities in leaves and what are the driving forces determining it? The spatial arrangement of species on leaves has been mapped in two meticulous studies (85,91). Their results indicate that substantial spatial overlap of individuals occurs in a given leaf (39,40), provided the areal resolution considered in these studies (36 mm^2 and 4 mm^2, respectively) is in the range of hyphal extension in leaves (colonies in culture can be much larger). This overlapping spatial association, together with the similar enzymatic complements of different species (39,40,52) and the persistence of some communities after transplantation to environments with a different species pool (15,70,78), suggests that there is potential for interspecific competition to influence community structure. In line with this idea, some aquatic hyphomycetes may inhibit the growth of other fungi associated with submerged leaves and wood in streams when grown on culture media (92,93). However, these standard tests for fungal interaction have mostly failed to indicate strong competitive abilities of those species typically associated with deciduous leaves rather than wood (92). Hyphal interactions among aquatic hyphomycete species likewise are weak or absent in culture conditions (94); however, whether the outcomes of these culture experiments are pertinent to the markedly different growth conditions in submerged leaves is currently unknown. When Bärlocher & Kendrick (43) inoculated leaves with mixed-species assemblages, rates of leaf decomposition were significantly higher compared with the best performing component species. This results indicates that positive interactions within the communities — that is, facilitation rather than competition — might have prevailed overall. The synergistic effect known as overyielding (95), if true, implies that aquatic hyphomycete species diversity may enhance the rate of an important ecosystem process. In view of the current trend of accelerated species losses worldwide, this is an issue of tremendous theoretical and practical interest at present (96). Aquatic hyphomycetes on decomposing leaves may constitute an excellent general model system for examining relationships between biodiversity and ecosystem functioning.

Interactions of aquatic hyphomycetes with detritivorous/mycophagous stream invertebrates are much better understood than interactions within the fungal assemblages in leaves (40). When given the choice, most stream detritivores (shredders) select partly degraded leaves colonized by fungi (15,40,97) and also prefer pure fungal mycelium to (uncolonized) leaves. Craneflies (*Tipula* spp.) are an exception (98–100). These preference patterns tend to be correlated with the nutritional quality of leaves as judged from the effects of food type on survivorship, growth rate, and fecundity of shredders (15,39,40,97,101,102). It appears, thus, that the choice of colonized leaves for food is instrumental in determining shredder fitness.

The enhancement of leaf palatability and nutritional quality as a result of microbial colonization has been termed conditioning (40,103–105). Highly selective feeding on pure mycelium and fungal patches on otherwise uncolonized leaves (and leaves coated by mycelium) indicates that the conditioning effect is caused by the presence of fungal biomass (34,35,39,40,102,106). Feeding experiments with artificially modified leaves (e.g., treatment with NaOH, HCl, organic solvents, fungal enzyme extracts) that lack fungal growth, have established that chemical-structural changes of the leaf matrix contribute further to the conditioning effect (107), although the

biomass effect appears to be greater (40,102). As fungal establishment proceeds, leaves become increasingly palatable and nutritionally enhanced, up to a point at which leaves are said to be fully conditioned. Subsequently, leaf quality and palatability declines (40,108,109). The timing of full conditioning appears to correspond approximately to the point at which fungal biomass concentrations are at a maximum and about 50% of the initial leaf mass has been lost.

The exact outcome of the trophic interactions between leaves, fungi, and detritivores are complex. The preferences exhibited by shredders depend not only on the degree of conditioning, but also on environmental conditions, the leaf species and fungal species involved, with different shredder taxa showing distinct responses (40). Fungal identity may affect leaf palatability to the extent that preferences of detritivores for different leaf species are reversed. However, although unsaturated fatty acids have been invoked as feeding stimulants in last, but not earlier, instar larvae of the detritivorous caddisfly, *Clistoronia magnifica* (110), it is still entirely unclear which physical and/or chemical cues exactly trigger the selection behavior of stream detritivores shredders that feed on decomposing leaves (39,40,111).

What, in turn, are the effects of detritivore feeding on fungal community structure on leaves? Bärlocher (81,112) analyzed aquatic hyphomycete communities on leaves confined in fine-mesh bags and thus protected from detritivore feeding with communities on accessible leaves in coarse-mesh bags, and found that fungal species richness was reduced when shredders had access. However, Howe & Suberkropp (113), in a similar experiment, found no evidence suggesting an impact of detritivores on aquatic hyphomycete communities in their study stream.

In conclusion, then, aquatic hyphomycetes act both as effective decomposers of leaves in streams, and as mediators of organic matter flow in stream food webs (40). The temporal and spatial patterns of community structure are complex, and the major driving forces shaping the communities at scales ranging from the individual leaf piece to continents are for the most part poorly understood. Apart from environmental factors such as temperature (70) and water chemistry (41,42,114–116), trophic and competitive interactions, and perhaps also synergistic effects, may play critical roles.

Wood Decomposers in Streams

A significant portion of allochthonous material entering freshwaters consists of twigs, branches, and logs (23,24,117). The amounts stored in streams in a given physiographic and forest setting is highest in the headwaters and decreases with increasing stream size (26,27). More than 100 g of pieces greater than 10 cm in diameter may be stored per m^2 of stream bed (26). Wood plays multiple roles in stream ecosystem structure and functioning (26,27). It influences channel morphology, retains organic matter and dissolved nutrients, provides habitat, and serves as an indirect or direct carbon and energy sources for microbial decomposers, invertebrates, and fish (18,26,27,117–119). Probably, it also plays important roles in other freshwater systems, such as forest ponds or littoral zones of lakes, but wood in those systems has been barely studied (120). Because of its persistence even when large fluctuations in discharge occur, wood has been suggested to have a reservoir function for aquatic hyphomycetes, ensuring initial inoculation following fresh leaf inputs in autumn (121). In addition, wood might play a role in the long-distance dispersal of aquatic hyphomycetes (121), although to date conclusive data to support this idea are lacking.

The decay of wood is slow and therefore often thought to have a less distinct impact on stream energy budgets than the decomposition of leaves. Webster and coworkers (25), for example, found no evidence for significant mass loss of large experimental logs submerged in a stream over a period of eight years, and dendrochronological evidence indicates that the complete decay of large pieces may take decades or even centuries (26,27,122). In part this long duration appears to be due to the fact that wood decay in freshwaters is largely a surface phenomenon (123), which is in contrast to both terrestrial and marine ecosystems. Nevertheless, when the amount of wood stored in a stream or other freshwater habitat is large, even a slow decay and transformation would have an appreciable effect on overall ecosystem metabolism.

Wood clearly is an important habitat for Fungi in freshwaters. Shearer (website) reports 275 species of Ascomycota and, in 1992, an additional 46 species of aquatic hyphomycetes (review). More than 100 species of hyphomycetes have been added since (119,124–130). This brings the total number of lignicolous Fungi in freshwaters to more than 400, even if one takes into account that some of the ascomycetes may have anamorphs on wood (Shearer, website; (131)). The hyphomycetes associated with twigs and logs are commonly found also on submerged leaves (121), which has fostered the idea that lignicolous Fungi in freshwaters show little substrate preference (132,133,134). However, in contrast to the hyphomycetes, only a small number of ascomycetes from submerged wood have been observed on leaves, implying a close if not specific relation of these ascomycetes to the woody substrate (18).

The communities of Fungi on wood submerged in streams undergo successional changes that involves species replacements (Yuen and coworkers 2000; 132,134,135). This is in contrast to the simple changes in the relative abundance of species that are observed on leaves. The differences between substrates appears to be due to the longer persistence of wood compared with leaves, and the abrasion of wood surfaces and scraping by invertebrates, constantly creating opportunities for new fungal colonization (121). *Nectria* species such as *N. lugdunensis* (Ascomycota) and its anamorph, the aquatic hyphomycete *Heliscus lugdunensis* Sacc., as well as *Pythium* and some other oomycetes have been identified as early successional species (132,133,135). They are thought to take advantage of the relatively labile components of wood (soluble sugars, starch, and proteins) but not to attack cellulose or lignin, which make up the bulk of their substrate (132,136). As a result, these species cause relatively little mass loss. The early successional species are later replaced by other fungi. It is

not clear, however, whether the species shifts are caused by changes in substrate quality or by seasonal changes in environmental conditions (18), although a number of observations point to the first hypothesis (see in the following text)

At least two patterns of wood exploitation can be distinguished among wood-colonizing fungi in freshwater habitats (121). The so-called soft-rot fungi are characterized by their ability to form distinctive cavities in lignified cell walls and to cause extensive weight-loss of wood. These constitute the second group of colonizers, which are better adapted to the wood habitat than the early successional species. Polysaccharide components of the cell walls are degraded preferentially, but there may be some ability to attack lignin as well (137). Soft rot is the most common type of fungal wood decay in both permanent and alternating water-logged conditions. Most species known to cause soft rot are Ascomycota or Deuteromycota (136,138–142)). Species of the other group cause only minor weight-loss and form no soft-rot cavities (121,136,138,142). This group is thought to take advantage of the relatively labile wood components (soluble sugars, starch, and proteins) and not to attack cellulose or lignin, which make up the bulk of their substrate (132,136). These Fungi are typically early successional species, such as *Nectria* spp. The typical brown-rot and white-rot fungi pervading logs and stumps in terrestrial environments are notably absent from water-logged wood.

The species appearing later during succession often show antagonistic behavior in culture experiments (92,143). In general, they are also more inhibitory to potential invaders and resistant to invasion than their counterparts on leaves (92). Growth inhibition over a distance has been demonstrated in several ascomycetes, including *Chaetosphaeria*, *Massarina*, *Ophioceras*, and *Pseudohalonectria* (143,144) (Yuen and coworkers 2000). This is consistent with the discovery of antibiotics in some of these fungi (93). Furthermore, wood panels inoculated with some single fungal strains, or combinations of two, before placing them in a stream for three months (145) were colonized by significantly lower number of species than uninoculated control panels. Thus, at least in some established cases, species or species combinations can clearly prevent invasion of new arrivals. Competition may even occur intraspecifically, as is suggested by the slower wood mass loss observed when a single fungal isolate was inoculated on two opposite sides of a wood block compared with a control where only one side of the block was inoculated (139). Taken together, the results of these experiments and observations suggest, thus, that apart from a possible deficiency of labile wood constituents for those fungi unable to degrade recalcitrant constituents (such as cellulose), competitive interactions might be involved in determining successional patterns of fungi on submerged wood (18). On the other hand, the enhanced wood decay by species mixtures compared with single-species inoculations (139), and the increased resistance to invasion when certain species combinations are present (145), suggests that positive interactions (synergisms, facilitation) may also be effective in these communities.

Many freshwater fungi, including wood colonizers, possess a wide range of enzymes for degrading plant polymers, and differences in enzymatic capabilities among species appear to be small (121,136,138). Lignin, however, can only be degraded by a relatively small number of species, and even in those cases the available evidence of ligninolytic activity is not conclusive. It may be indicated, for example, when lignocellulose is mineralized in the absence of organisms other than fungi. However, in culture experiments with aero-aquatic hyphomycetes, Bergbauer & Newell (146) found that the destructive capabilities of lignocelluloses varied with experimental conditions, calling for caution in the extrapolation of such results to field situations. Similarly, it is known that the extracted lignins often used in agar plate assays are modified forms of the native lignin (121), and additional difficulties have been encountered when assessing lignolytic activity using agar plate techniques (147).

The dynamics of enzyme activities on wood panels submerged in streams have been followed in several studies (148–151). Enzyme activity is detected already during the first days of colonization, reflecting rapid microbial colonization, growth, and degradative activity. Activity of β-glucosidase, a key enzyme in cellulose degradation, increase rapidly following wood submergence and subsequently remains high (148,149,152). Phenoloxidase, which is involved in lignin degradation, show different patterns. Peaks in activity may first increase and subsequently decline (151) or increase continuously (148,152). Although these enzyme activities may be due to organisms other than fungi, scanning electron micrographs of wood biofilms and bacterial direct counts by epifluorescence microscopy have shown that microbial colonization on wood is primarily fungal (Tank & Winterbourn, 1995) (153).

The limited information available on the biomass of fungi associated with submerged wood also suggests that fungi are an important component of the microbial decomposer assemblage (148,151,153,154). In surface scrapings or small pieces of wood, concentrations of ergosterol, a measure of fungal biomass, increase following submergence of wood in streams (148,151,152,153). On areal basis, concentrations can even be much higher than on leaves (148), indicating that fungal growth on wood can indeed be extensive.

Tank & Webster (153) observed an interaction between leaf litter availability and wood biofilm processes in small forest streams. Experimental exclusion of leaf litter input enhanced microbial respiration, extracellular enzyme activity and fungal biomass on wood logs, with biomass being as much as seven times higher than in a reference stream, implying competition between leaf- and wood-associated microorganisms for nutrients in these streams (153). This is in accordance with the observation that nutrient addition to wood enhances microbial mineralization activity (123). Whether these effects are actually caused by fungi or other microorganisms is currently unknown.

Decomposers in Freshwater Marshes

Fungal communities associated with emergent macrophyte plant litter in freshwater marshes, littoral zones

of lakes, floodplains, and river deltas represent a diverse assemblage of decomposer microorganisms, comprising a number of both taxonomically and ecologically distinct groups (18,155–165). The well studied ingoldian fungi (aquatic hyphomycetes), which are the dominant assemblages on submerged leaf and wood litter in streams, are notably scarce in macrophyte-dominated wetlands.

A unique feature of many emergent wetland plants, which is often overlooked, is the absence of leaf or shoot abscission following senescence and death. Frequently, leaves, leaf sheaths, and culms of emergent macrophyte remain attached to the parent plant in a aerial standing position for long periods of time, and are heavily colonized by terrestrial decomposer fungi well before their eventual collapse into water surface sediments. Growing evidence has established that fungal assemblages inhabiting this litter are adapted to the harsh environmental conditions experienced in the aerial standing-dead phase (Kuehn and Suberkropp 1998, Kuehn and coworkers 1998) (166), suggesting that these microorganisms can contribute significantly to plant litter decay before its entry into the aquatic environment. As a consequence, decomposer fungi associated with plant matter in wetlands are exceedingly more ecologically diverse, ranging from terrestrial taxa, which inhabit standing litter, to aquatic forms, which inhabit plant litter after its entry into the aquatic milieu.

In 1889, Saccarodo reported 168 fungal taxa that were known to be associated with the common reed, *Phragmites australis*, an emergent macrophyte with a worldwide distribution. Our recent compilation of data from the literature now suggests that nearly 600 species have been recorded from this single plant, despite the fact that relatively few comprehensive surveys have been conducted (156–158,167,168). Ascomycetes (42%) and hyphomycetes (30%) were among the most common taxa associated with decaying plant litter, with coelomycetes (22%) and basidiomycetes (6%) occurring less frequently. However, recent increases in reports and new species descriptions (10,169–172) suggest that many more species have yet to be discovered on *P. australis* and probably other wetland plants as well.

Distinct fungal communities have been observed on both aerial standing-dead shoots and fallen litter of emergent macrophytes (157,158). Often different plant parts, such as leaf blades, leaf sheaths, culms (nodes and internodes), rhizomes, and roots, harbor different fungal taxa (155–158), possibly resulting from the varying amounts of recalcitrant compounds found in emergent macrophyte tissues and the environmental conditions of decay. Prior studies have documented a spatial distribution of fungal taxa associated with standing-dead shoots of emergent macrophytes (155,173). Apinis and coworkers (157), reported that ascoma of *Massarina arundinacea* were frequently observed to occupy standing-dead culms of *P. australis* in a region just above the water surface. It was believed that this fungal species was responsible for the weakening of reed culms near the water surface, thus making culms more susceptible to toppling by wave-action.

Besides spatial differences, distinct temporal differences in fungal taxa associated with particular plant components may also occur (174). Often, one sees a succession of differing fungal species as plant litter decomposition proceeds (155–158,175). Pugh and Mulder (155) found that leaves still attached at the parent plant often have an inhabitant fungal community that is different from that on submerged leaves. Their studies revealed a distinct succession in fungal taxa during decomposition of *Typha latifolia* leaf litter. Leaves of *Typha* proceeded through early stages of decay, which were similar to terrestrial plants, with primary colonization by typically leaf surface fungal taxa such as *Aureobasidium pullulans*, *Cladosporium herbarum*, *Phoma typharum*, and *Epicoccum nigrum*. This was followed by a secondary phase in which ascomycetous fungi (i.e., *Leptosphaeria* spp.) were the dominant decomposers. Final stages of leaf decay following toppling of plant matter into the water were dominated by predacious fungal taxa (i.e., *Arthrobotrys* and *Dactylaria* spp.).

Recent studies by Kuehn and coworkers (176) support the idea of a potential shift in fungal taxa between an aerial (terrestrial) and submerged litter decay phase. Kuehn and coworkers (176) observed a rapid decline in fungal biomass and production associated with decaying leaf litter of the emergent macrophyte *Juncus effusus* following its movement from a standing-dead to a submerged litter decay environment. This decrease was followed by an increase in fungal biomass and production after a period of submerged litter decay. These results suggest that fungal taxa associated with standing-dead leaf litter cannot adapt and survive in an aquatic decay environment following shoot collapse (176,177).

In *P. australis* and related plants, leaf blades begin to die from the bottom to the top. As this process proceeds, ergosterol contents of leaves, a measure of associated fungal biomass, increase from nondetectable to several hundred μg per g of leaf dry mass (178). Based of an average ergosterol conversion factor of about 5 μg/g fungal biomass (179) and a growth efficiency of 35% (60), these ergosterol concentrations suggest that some 5% of the standing-dead mass can be fungal, rather than foliar, and that half of the leaf mass lost during the standing-dead phase is accounted for by fungal assimilation (178).

Active mineralization of lignocellulose from freshwater macrophytes is demonstrated in a few studies (180,181). Lignocellulose of *Carex* leaves is degraded by aquatic hyphomycetes and aero-aquatic hyphomycetes (180). Fungal decay of thatched *Phragmites* stems causes soft-rot cavities and a rot causing a thinning of secondary wall from the lumen side toward the external cell walls of the lignified cells (181), these observations are comparable to the depolymerization of macromolecules by *Spartina* fungi by two different types of soft rot (182).

CONCLUSION

The taxonomic affiliation and ecological functions of fungi in freshwater environments are diverse. Functions range from the decomposition of a variety of organic matter in various freshwater systems to regulatory functions of population dynamics of both freshwater animals and algae. The latter aspects have not been covered

here. However, parasitism (Powell 1993, Canter-Lund & Lund 1995, Holfeld 1998, 2000, Van Donk & Bruning 1992, Srivastava 1987, Diéguez-Uribeondo and coworkers 1995), commensalism (Lichtwardt 1986, Misra 1998) and symbiotic mutualism (Wigand & Stevenson 1994, Wigand and coworkers 1998, Miller & Sharitz 2000) occur. Fungal parasitism on both animals and plants is common. In some cases, the impact of fungal parasites is so strong that species once abundant have been nearly complete wiped out/eradicated by an invasive fungus (Canter-Lund & Lund 1995, Holfeld 2000). The dramatic spread and effects of the crayfish disease of the European crayfish, *Astacus astacus*, by the oomycete, *Aphanizomenon*, is a case in point.

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WATER SAMPLING.

See Sampling Techniques for Environmental Microbiology

WATER TREATMENT PLANTS: PROTOZOA IN SPENT FILTER BACKWASH WATER.

See Occurrence of Protozoa in Spent Filter Backwash Water

WEATHERING.

See Biodeterioration of Mineral Materials

WEATHERING, MICROBIOL

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Most natural materials are susceptible to some kind of microbial attack often called microbial weathering or bioweathering. In general, all microorganisms may contribute to microbial weathering. Thus, in actual cases, the contribution of bacteria, algae, fungi, and lichens has to be taken into account. The higher plants may contribute due to root growth and indirectly by the excretion of substances that serve as substrates for microorganisms. However, the latter is not the subject of this article. The materials either serve microorganisms as substrate, meaning as energy and carbon source like organic compounds, for example, cellulose, or as substrata-like inorganic compounds, for example, mineral materials or metals, mainly as a growth support (for attachment) and/or for a removal of metabolic endproducts. The microbiological degradation of organic substances, called *biodegradation*, has to be distinguished from the term *biodeterioration*, which describes the attack of mineral materials such as natural stone, mortar, and concrete. For example, the reaction of biogenic sulfuric or nitric acid with the binding material calcium silicate and/or calcium carbonate/hydroxide to become neutralized is regarded as biodeterioration. In the

case of metal corrosion by biogenic hydrogen sulfide, this compound reacts with metal ions to form insoluble metal sulfides and, thus, the inhibitory sulfide (inhibitory for the growth of sulfate reducing bacteria) becomes removed from the solution. In both examples, the microorganisms excreting the deleterious compounds find improved living conditions in their environment allowing for further and/or more rapid growth. The energy, however, has to come from an external source.

It should be mentioned that a few artificial compounds, termed xenobiotics do not fit into this scheme. These compounds are not biodegradable nor susceptible to any strictly microbiological attack. Thus, they need additional chemical and/or physical steps to allow for degradation.

The mechanisms that interact jointly in microbial weathering are highly complex. Forces resulting from physical, chemical, biological, as well as fields such as materials sciences, mineralogy, geology, electrochemistry, and others have to be taken into account if a causal explanation is to be given. These explanations, although difficult to derive, are essential for an appropriate selection of materials and/or for protective measures. An important strategy to understand the processes involved includes simulation experiments. However, it always must be considered that microbial weathering is an ongoing, natural process. Throughout the history of the Earth, it is the process that causes soil formation by weathering/deterioration of rocks (together with physical and chemical forces). Without the involvement of microorganisms, this planet would still lack higher forms of life.

MICROORGANISMS INVOLVED IN BIODETERIORATION

Algae and Phototrophic Bacteria

Growth of photolithoautotrophic algae and cyanobacteria occurs independently of any supply of organic substrates. Therefore, they belong to the primary colonizers of building materials such as natural stone and mortar (1). Although algae are primarily found in relatively moist areas, cyanobacteria are also present in dry parts of buildings (2,3). Because phototrophic microorganisms accumulate organic substances on stone material, they may support proliferation of chemoorganotrophic microorganisms. Above this, biofilms of algae and cyanobacteria may exert mechanical stress on the material (2), may change the water characteristics of the material (3), and may contribute to discoloration (4).

Chemoorganotrophic Bacteria

Building materials exposed to the atmosphere are soon colonized with chemoorganotrophic bacteria, because many chemoorganotrophic bacteria can live under oligotrophic conditions (4,5). On strongly alkaline materials such as mortar and concrete, the colonization is suppressed as long as the pH at the surface remains above nine. It is difficult to estimate the weathering potential of chemoorganotrophic bacteria, because diverse genera with different metabolic activities inhabit building materials. Acidogenic and nonacidogenic representatives of chemoorganotrophic bacteria were isolated (6). Because high numbers of airborne chemoorganotrophic bacteria are deposited on aboveground buildings (7), it is impossible to distinguish between actively growing and adsorbed bacteria (8). Nevertheless, chemoorganotrophic bacteria are likely to cause adverse effects on building materials by carbon dioxide production, biofilm formation, and discoloration (4).

Fungi

Depending on the presence of chemoorganotrophic bacteria, fungi seem to be secondary colonizers of building materials (9). Again, the colonization of mortar and concrete is largely prevented as long as the pH at the surface remains above nine. Acidogenic and nonacidogenic fungi were isolated (6,10). Acidogenic fungi are able to attack mineral materials (11–13) and evidence for a mechanical attack by fungi was reported recently (14). Formation of melanins by some genera of fungi contributes to discoloration (15). Fungi isolated from building stones produced a similar spectrum of organic acids, as found in samples from buildings (6,8). Thus, in situ cation chelation by microbially produced organic acids may be the main effect of fungal weathering. Furthermore, in natural stone, filamentous fungi are involved in biogenic iron and manganese oxidation (16).

Chemolithotrophic Bacteria

Chemolithoautotrophic bacteria gain their energy from the oxidation of inorganic compounds. Carbon dioxide is their main carbon source.

Endolithic nitrifying bacteria are the main representatives of the chemolithoautotrophic microflora in building stones and have been identified in deteriorated mortar and concrete (8,17). The microbial conversion of ammonium to nitrate, called nitrification, is a two-step process catalyzed by nitrifying bacteria. Ammonia oxidizers produce nitrous acid that is used by nitrite oxidizers as a substrate, and then finally oxidized to nitrate:

$$NH_4^+ + 1\frac{1}{2}O_2 \rightarrow NO_2^- + H_2O + 2H^+ \text{ ammonia oxidizers}$$

$$NO_2^- + \frac{1}{2}O_2 \rightarrow NO_3^- \text{ nitrite oxidizers}$$

$$NH_4^+ + 2O_2 \rightarrow NO_3^- + H_2O + 2H^+ \text{ nitrification}$$

Nitrifying bacteria are involved in biodeterioration of natural stone mortar and concrete causing a biogenic nitric acid attack and an accumulation of nitrates (8,9,17). The weathering potential of nitrifiers has been demonstrated extensively in laboratory simulation experiments (see the following text).

Chemolithoautotrophic sulfur-oxidizing bacteria such as thiobacilli grow on reduced sulfur compounds including hydrogen sulfide, elemental sulfur, thiosulfate, and polythionates. During oxidation of reduced sulfur compounds to sulfate, protons are released. Thus, as nitrifying bacteria (18), thiobacilli have a strong weathering potential as a result of the production of sulfuric acid (sulfuric acid attack). Thiobacilli are regularly detected in sewage systems, in which they cause serious damage to concrete, mortar, and brickwork (19), whereas they

are seldom found in historical buildings (9). Neutrophilic and moderately acidophilic thiobacilli such as *Thiobacillus intermedius*, *Thiobacillus neapolitanus*, and *Thiobacillus novellus* seem to be the primary colonizers of concrete, contributing, together with nitrifiers, to the consumption of the alkaline reserve of neutral or alkaline mineral materials (19,20). After a slightly acidic pH has been reached, settling and proliferation of strongly acidophilic thiobacilli like *Acidithiobacillus thiooxidans* occurs, with all the disastrous consequences of final pH-values as low as zero (21–23).

MICROBIAL MECHANISMS CONTRIBUTING TO BIOWEATHERING

Although almost all microorganisms contribute in one way or other to the biodeterioration of mineral materials, the number of mechanisms appears to be quite limited. Known mechanisms can be summarized under seven different categories. In this context, it must be mentioned that microorganisms are generally not interested in deterioration of mineral materials per se, but favor environmental conditions permitting them to grow. This is an important difference compared with biodegradation processes occurring with organic materials because these, after breakdown, also serve as nutrients. However, in the case of mineral materials, the microbial process of deterioration/weathering "simply" serves as an improvement of the environmental conditions for growth purposes. Microorganisms can grow on and in mineral materials. These modes are called epi- or endolithic respectively. Especially the latter mode confers some kind of protection to microorganisms in harsh climates. The surrounding mineral material reduces the danger of desiccation, freezing, or solar radiation. Thus, endolithic life-forms occur, for example, in the arctic regions or in deserts. To be able to endolithically grow in mineral materials, cavities need to be generated for the "uptake" of microbial cells if suitable pores are not available. For this purpose, either chemically/physically preformed cavities may be used or the microorganisms may form them by their own action, that is, by the excretion of organic or inorganic acids able to dissolve the material, or by mechanical forces.

Inorganic Acids

As an end-product of their metabolism, several specialized groups of bacteria produce strong acids, namely, nitric acid (8,9,18) and sulfuric acid (18,19). These acids are produced and excreted in the course of microbial metabolism, if nitrogenous or sulfurous compounds such as amino acids, ammonia, and urea, or sulfides, and sulfur are available in the environment. The acids may react with acid-susceptible materials such as carbonates, hydroxides, and silicates in effect dissolving them. Often, the latter not only acts as the binding material in natural stone but they also perform a similar role in concrete and artificial stone. The dissolution of the binding material results in the weakening of the structure and a loss of filler material such as grains of quartz and so on. In the case of nitrous and nitric acid, the dissolution of the binding material results in the production of soluble compounds (nitrites or nitrates), which are easily washed away. The reaction products of sulfuric acid mostly have a low solubility and generally remain at the site of formation. For this reason, gypsum crusts are often detected and seem to indicate that only sulfuric acid was involved in the weathering process.

$$2HNO_3 + CaCO_3 \rightarrow H_2O + CO_2 + Ca^{2+} + 2NO_3^-$$

$$H_2SO_4 + CaCO_3 \rightarrow H_2O + CO_2 + CaSO_4$$

The two equations give a simplified example of this kind of attack.

The third inorganic acid, which is often forgotten or ignored, is carbonic acid, H_2CO_3. This acid, although considered chemically to be weak, can also react with binding substances of mineral materials and transform them, for example, from hydroxides or silicates to carbonates. Although the latter still remain in place and function as binding agents, the pH is considerably lowered from 12 to 14 to pH8, which is the optimal value for growth. As a consequence in the case of reinforced concrete, the steel used for reinforcement is no longer protected against corrosion. It starts to corrode with water and oxygen access, losing its reinforcing function, and because of the increased volume of the ferric oxides, exerts a swelling attack on the surrounding concrete.

Organic Acids

Almost all microorganisms may excrete organic acids in the course of their metabolism because of metabolic imbalances (12,24). The excreted organic acids are chemically quite diverse. Acidic compounds may occur from all stages of microbial metabolism, namely, glycolysis or similar catabolic pathways, anaplerotic sequences, amino acids, uronic acids, nucleic acids, and so forth. In addition to imbalanced growth, some acidic compounds are excreted deliberately. This concerns, for example, the acidic compounds in the extracellular polymeric substances (EPS), surrounding a cell as a slime layer. Here, amino acids, uronic acids, nucleic acids, and fatty acids have been detected (25).

Although these acids may be taken up again in a later stage of metabolism, the acids can react chemically with the environment and form salts by reaction with susceptible materials. In general, their action is comparable to that described earlier for inorganic acids, besides the fact that many acids may form complexes with (heavy) metals. In this way, for example, citric or oxalic acids additionally contribute to the dissolution of inorganic materials. This complexation may have a distinct advantage for the excreting microorganism by the consequently increased availability of trace metals for growth. In this context, it may be discussed whether the excretion deliberately occurred to ensure or at least increase the trace metal supply of these microorganisms. Fungi are especially known to excrete organic acids in the course of their metabolism. Recent research regarding the EPS of bacteria indicated that the structural organic acids play a pivotal role in metabolism by affecting substrate degradation and allowing surface adhesion due to complexed metals (see BIOLEACHING).

Salt Attack

Most mineral materials have a porous structure. These pores have sizes ranging from submicron up to the millimeter. The pores are filled with water and gases. Although the large pores loose their water content and dry out during periods of desiccation, the micron- and submicron-type of pores resist desiccation more effectively. This becomes even more pronounced if the pores are colonized with microorganisms. The latter excrete in the course of their metabolism various types of metabolic compounds, for example, (*in*)organic acids that after reaction with the (susceptible) mineral material may form a salt. Salts are in general hydrophilic compounds that therefore bind water molecules (hydratation). Because of this effect, molecules are formed, which are too large for the available pore space. Consequently, a swelling attack in such a pore is observed. Because of the significant physical forces of hydratation, the pore system cracks. It must be kept in mind that the formation of the salt and the hydratation are purely physical effects, whereas the formation of the original compound, such as nitrates, sulfates, and organic acids is a result of microbial metabolism. This example shows how difficult it is to distinguish between chemical, physical, and biological deterioration mechanisms.

Salt attack may cause the destruction of large surface areas of affected materials. This is called scaling. It can cause a loss of surface layers, or in the case of wall paintings, the removal of pigments from the underlying stone or concrete. As a consequence of biogenic sulfuric acid corrosion, the gypsum thus formed may undergo a series of hydratation reactions, causing the formation of ettringite. The latter compound is also known as "concrete-bacillus." Because of the hydratation, it contains a large volume ($3CaO \cdot Al_2O_3 \cdot 3CaSO_4 \cdot 32H_2O$), and seriously endangers concrete structures. Another effect of biogenic salts must also be mentioned. Besides the formation of large crystals (hydratation), salts are generally hydrophilic compounds and bind water molecules. Thus, the presence of salts result in the presence of water in the pore system of a mineral material. This water will not evaporate as easily as pure water. Thus, porous systems containing salts have an increased water content. This does not cause problems per se. However, in the case of freeze-thaw attacks, which are usual in moderate climates during autumn, winter, and spring, this water may form ice crystals, which according to their increased volume tend to exert a swelling attack that is similar to the effect of salts on porous materials.

The Role of Biofilms in Bioweathering

Most microorganisms (>90%) grow attached to surfaces. In this respect, porous mineral materials offer optimum substrata (26,27). In the course of this attached growth, microorganisms metabolize available nutrient sources, excreting intermediates and end-products of (in)organic nature, and protect themselves against dryness, predation, toxic substances and so on by the excretion of EPS. EPS, as explained earlier, consist of many different compounds, namely, sugars, lipids, amino acids, and nucleic acids as well as some complexed heavy metals. Because of this composition, the EPS function as nutrient scavengers and, as a result of their hydrophilic nature, effectively bind water in place. The system consisting of EPS, living or dead microbial cells, and (in)organic detritus is called biofilm. This biofilm is found in the case of mineral materials on the surface and in the pore system. Especially pores with a diameter between 0.5 and 10 μm are optimally suited for microbial growth. This is because these pores are large enough to allow the invasion of microbial cells and their internal growth, resulting in a totally filled pore. The latter becomes impossible for pores with an excessively large diameter. As a consequence of this biofilm formation, the same effects arise as have been described earlier for the salt attack: an increased water content in a porous system, an increased salt content, and susceptibility to a freeze-thaw attack. Finally, a biofilm tends to clog the pore system of a porous, mineral material. This means that, on the one hand, the pore water will not evaporate and therefore remains within the system and on the other hand, the blocked pores do not allow the application of stone "consolidants" because they cannot penetrate into the pores. However, for an application of these compounds to be effective, a minimum invasion of several centimeters up to the structurally sound stone is required. If this cannot be achieved, the application often causes more harm when applied than when not applied. Cases in which because of a biofilm, whole facades were lost as a result of imperfect penetration, scale formation, and a subsequent freeze-thaw attack are known.

Additional effects of biofilms may be aesthetic effects like discoloration, increased heat absorption, and reduced porosity.

Discoloration

Aesthetic effects are a major concern for porous mineral materials. Materials exposed to light and the open atmosphere tend to be colonized by phototrophic microorganisms such as cyanobacteria, algae, and lichens, all of which are colored. Thus, these surfaces become discolored, at first appearing green and very often becoming almost black, with the appearance of being dirty. Additionally, dust particles from the atmosphere like soil and/or soot are included in the EPS, contributing to the impression of dirt. In the case of bright stones, a considerable change of the optical impression may result. Because of this effect, many buildings, especially in an industrially polluted atmosphere (including traffic as a source) have turned black. Other effects are a surface growth of fungi on historical glass, which causes it to become turbid or change in color because of metal extraction. The black surface also causes an increased absorption of solar light, thereby increasing the temperature of the affected areas. Furthermore, the increasing differences between day and night temperatures and the gradient between inside and outside becomes steeper than with uncolored surfaces. As a consequence, the physical stress on the materials is increased.

Noxious Compounds

Ammonia (NH_3) is increasingly introduced into the biosphere as a result of the increase of manure production

from livestock breeding, processes for NO_x-removal in combustion processes (power plants, traffic), and the use of mineral fertilizers for farming. The biological degradation of ammonia and precursors like urea or amino acids during the process of nitrification results in the generation of nitrous and nitric acids, the effects of which have been described earlier under inorganic acids. Nitrogen oxides, summarized as NO_x, comprise NO, NO_2, and N_2O. The first two compounds arise in the course of combustion processes and can be seen as anhydrides of nitrous and nitric acid, respectively. There is some evidence that NO and NO_2 may contribute to biodeterioration of building stone by serving as substrates for nitrifying bacteria. The latter compound, N_2O, is produced in the course of denitrification and an effect of this compound on mineral material degradation is not evident.

Methane (CH_4) is a natural component of the atmosphere. Besides, it is produced by microorganisms that reduce (CO_2) under anaerobic conditions, for example, in rice fields, and it is the main component of natural gas, which is increasingly pumped off the ground for industrial and private use. Methane-degrading bacteria, besides emitting carbon dioxide, tend to produce EPS, thereby exerting negative effects on mineral materials. However, the degradation of methane itself seems to be nonproblematic (9).

Hydrogen sulfide (H_2S) is the most dangerous compound for mineral materials (19,22). It may be of chemical or biological origin (sulfate reducing bacteria). Under aerobic conditions, sulfur-oxidizing bacteria transform H_2S into sulfuric acid, the effects of which have been described earlier. Furthermore, H_2S may react with reinforcement steel and cause its deterioration as a result of metal sulfide formation.

Organic Solvents

Many microorganisms excrete organic solvents in the course of metabolism. These include compounds such as formic acid, acetic acid, butyric acid, lactic acid, ethanol, butanol, and many others. Not all of these compounds directly contribute to the weathering of mineral materials; however, they may have a negative impact in the case of a combination of minerals with organic materials.

Exoenzymes

It has been known for a long time that microorganisms excrete extracellular enzymes into the environment. Actually, the first biotechnologically based processes such as degradation of starch for beer and bread production are based on exoenzymes; however, this applies to the degradation (cleavage) of organic macromolecules into small, dissolved units, which, sensu strictu, does not play a role in the weathering of mineral materials. Exceptions are combinations of mineral materials with organic compounds and the problem of steel reinforcement corrosion. The latter may be an effect of exoenzymes causing ennoblement of stainless steel and, consequently, biocorrosion.

MATERIALS, PROPERTIES, AND EXAMPLES OF COMPLEX WEATHERING PROCESSES

Microbial processes contribute to the natural weathering of rocks and to soil formation in all its stages. Mineral materials are not only all naturally occurring rocks of magmatic origin (like granite, basalt), sedimentary rocks (like limestone, sandstone, even clay), metamorphic rocks [like marble, slate, etc. of inorganic origin (pure minerals included) used by humans], but also a large number of artificial, that is, processed or synthesized materials such as concrete, ceramics (mainly of clay origin but also oxide ceramics and special materials), and glasses. Detailed descriptions of these materials are given in appropriate textbooks. The term "microbial weathering" is generally used with reference to materials such as natural stone, for example, found at historical buildings. However, other construction materials such as concrete and mortar, brick, ceramic materials, as well as special artificial materials such as glass are also susceptible to a microbial attack. If buildings or other constructions are affected, the process becomes "unwanted."

The usual meaning of weathering is the partial or even total physical destruction of materials, but in some cases a physically minor alteration of an item may cause a total disfunction of a valuable instrument (e.g., optical glass) or may endanger the reliable function of a technical system. Thus, deterioration and degradation are closely related to weathering, because all these categories are based on similar microbiologically influenced processes.

Bacterial oxidation processes occurring on sulfidic ores and other technically useful treatments of materials based on microbially provoked dissolution processes are discussed in more detail under the topic "Bioleaching," but they should be mentioned here as well, because the biogenic sulfuric acid causes a very intensive weathering of susceptible rocks, with subsequent, in many cases, deleterious effects.

The mechanisms involved and effective in microbial weathering are best understood when case studies are discussed considering the materials' characteristics.

Natural Stone

With respect to the topic "microbial weathering" not only different kinds of construction materials (building materials) are relevant, but even naturally or even artificially formed massive items consisting of rocks, stones, or other naturally occurring raw materials should also be considered. On a large-scale, construction materials are especially used for buildings, however, mineral products are also applied for road and rail road construction.

Almost all attack and damaging mechanisms involved in microbially influenced deterioration of materials may act on mineral materials (see in the preceding text). The mechanism that plays the major role in a specific case, cannot be decided ex cathedrae, because an enormous multitude of possibilities exists.

Numerous examples from human history show that the major role in the choice of construction materials was at first their availability on-site (or nearby) and the

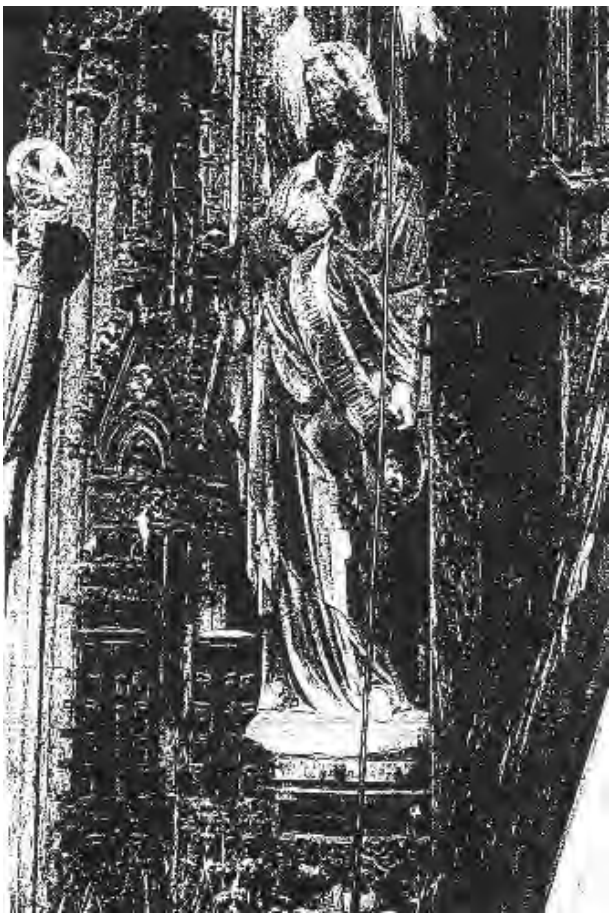

Figure 1. A sandstone sculpture from the dome of cologne, Germany, after exposure to the natural weathering process for nearly 100 years (by Dr. Wolff Köln).

ease of exploitation and processing. Different kinds of sandstone (Fig. 1), calcar, volcanic tuff, sand, and clay, all of them relatively susceptible to weathering, were the most frequently used materials. Accordingly, extremely hard materials, such as basalt and granite, were only rarely used.

Regarding microbial weathering of mineral materials, the physical, and chemical characteristics are of great importance. The combination of properties may be decisive in the susceptibility of a given material. Additionally, the properties of potential reaction products or compounds accumulating on or in the material may play a key role.

The properties of mineral materials to be considered are physical (porosity, capillarity, specific surface, physisorption, capillary condensation, thermic dilatation, thermic conductivity, susceptibility to freeze-thaw attack, color, and color-related radiation absorption), chemical (hydrophilicity, hydrophobicity, nutrient content, chemosorption of potential nutrients from air, reactivity toward potential chemical attack by acids, for example, nitric, sulfuric, carbonic acid, hydrogen sulfide, presence of complex forming ions and compounds, hydratation processes), and mineralogical (composition, structure, crystallography, homogeneity), with respect to the manifold weathering processes.

Thus, the weathering of a material depends strongly on the combination of its properties (chemical and mineralogical composition, microscopic and macroscopic structure, porosity) and on the specific environmental conditions (climatic — temperature and temperature changes, humidity, air movement; specific chemical composition of the whole surrounding environment — air, water, solids) than only on the properties or on the climatic/environmental conditions alone.

As a first example, two chemically similar but structurally different materials such as muschelkalk and marble shall be compared. The first one is a porous, structurally inhomogenous material showing a relatively high specific surface area. This enables processes like capillary condensation, capillary suction, and diffusion. In such conditions microorganisms find ecological microniches, where proper survival conditions can be maintained irrespective of extreme external atmospheric/climatic conditions. Humidity is usually available due to capillary condensation and capillary suction. High concentrations of metabolic or reaction products that are harmful to the microorganisms may be easily lowered/reduced by good diffusion (porosity and available humidity even in a dry climate). The chemical properties derived from the main component, calcium carbonate, are less important in this case, because they are similar to marble. However, marble is a crystalline, dense rock, showing nearly no porosity except for some cracks at the interface of crystallites. Thus, on marble a microbial population can settle only at the (external) surface of the material and must face considerably adverse growth conditions. Consequently, the muschelkalk will be populated to a significantly greater depth than marble. Also, only a few, very specialized organisms occur on marble, whereas muschelkalk will show a large variety and an increasing number of organisms.

A second example refers to structurally similar, but chemically different materials. Sandstones are always (more or less) porous materials that favor capillary and diffusion processes. However, significant differences arise with respect to the microbial population as a result of distinct chemical composition. In the case of acidic sandstone, possessing none or only little alkalinity in the form of calcium carbonate, acid-producing but acid-sensitive organisms such as nitrifiers are unable to proliferate. On the contrary, in sandstones with a high acid-neutralizing potential, nitrifiers will find optimum growth conditions, in which microbial weathering by the production of nitric acid is likely to occur (9).

These examples illustrate how properties and conditions influence the proliferation of certain microorganisms. This however, does not mean that other organisms, which are insensitive to the factors mentioned earlier, may not be present and that they act according to their specific metabolism in a different way. Especially chemoorganotrophic bacteria are almost always present, because they include a large number of different organisms, which are less demanding with respect to growth requirements, and contribute in a more or less evident way to the weathering process (excretion of organic acids). For instance, excretion and accumulation of organic metabolic products,

as present in biofilms (dead or alive), may alter some physical properties and the status of the materials such as diffusion-rates, gradients of temperature, humidity, concentrations of oxygen, salts etc.

Synthetic Stones (Concrete, Mortar)

Reduced nitrogen compounds such as ammonia/ammonium ions serve as energy sources for neutrophilic ammonia-oxidizing bacteria forming nitrite, which is further oxidized to nitrate also by neutrophilic nitrite-oxidizing bacteria. Hydrogen sulfide, elemental sulfur, or thiosulfate are oxidized by neutrophilic and acidophilic thiobacilli forming sulfuric acid. During these biooxidation processes hydrogen ions are released, which cause a strong acid attack on acid-sensitive compounds. These aerobic microorganisms inhabit the surface or pore system of materials and cause a direct dissolution of binding agents (calcium carbonate, calcium hydroxide, calcium silicate) in concrete and mortar. Carbonic acid may also dissolve carbonate. Calcium ions and the anions may diffuse in the pore system according to specific conditions.

Indirect microbial weathering is caused by secondary phenomena such as crystallization of salts (because of concentration by evaporation or precipitation reactions). Calcium ions formed at a certain site migrate to another site and, by a subsequent reaction with sulfate, compounds such as gypsum may form. Gypsum may be formed as well from biogenic sulfuric acid via its (neutralization) reaction with calcium carbonate or hydroxide (e.g., in concrete or mortar). Immense mechanical stress is caused by crystal growth within a pore resulting in additional deterioration (Fig. 2). The material subjected to this kind of mechanical deterioration is not necessarily involved in the primary microbial process. A combination of material properties and specific site conditions are necessary to cause these processes.

Synthetic cement-based stones (concrete) contain several calcium compounds such as (free) calcium hydroxide, calcium silicates, and/or aluminosilicates. During aging, besides internal structural changes (hardening, especially in the early stage within 28 days), carbonatization (formation of calcium carbonate) may occur. This process depends on factors such as cement content and quality, density and porosity, as well as environmental conditions. Besides the mechanical strength, chemical properties change. Cement- and/or lime-based and even non-cement mortars show changing chemical and physical properties, beginning at the surface. The changes continue with time and progress into the bulk of the material. Gradients appear, for instance, for pH. Such construction materials are subjected to a succession of continuously and progressively changing bacterial populations located in the beginning at the surface and progressively penetrating deeper and deeper into the material. The microorganisms cause primary changes by excreting metabolic products. Although on fresh concrete, survival and proliferation of organisms is impossible because of the very high pH (up to 12), as fast as the conditions change (carbonatization), the microorganisms settle in microniches. Finally, they invade large areas within the material (formation of extended biofilms). Concomitantly, depending on the actual pH, the consortium of neutrophilic organisms turns toward moderately acidophilic and finally strongly acidophilic bacteria. Values of pH as low as 0.8 occur on concrete. The consequence is a total destruction.

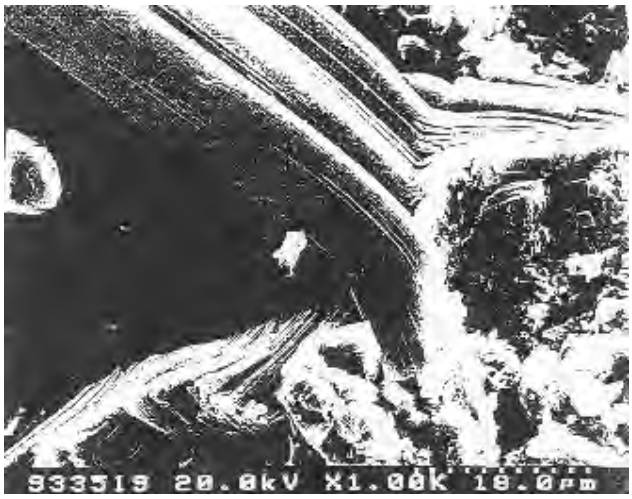

Figure 2. Gypsum crystals (ettringite) in a brick. Scanning electron microscope image showing the salt attack.

This scenario is valid for concrete and mortar. Attempts to avoid proliferation of strongly acidophilic thiobacilli by addition of (less expensive but weak) neutralizing agents (such as calcar, dolomite, marble) failed, because these neutral aggregates offer pH-neutral sites for other neutrophilic acid producers. These may prepare a proper microenvironment for a subsequent settlement and proliferation of strongly acidophilic species. Additionally, different species settle on different sites, forming distinct microniches according to their specific needs. Thus, nitrifiers and neutrophilic thiobacilli have been detected on visually sound sites with pH-values between six and eight. As soon as the neutralizing source (sacrificial aggregate) had been consumed, the pH decreased below five and the binding material was also attacked.

These processes may occur at relatively high rates. Mortar up to a depth of several centimeters was destroyed within two to three years in main sewage pipelines. Up to 5 cm of a plastic coated concrete was destroyed within two years in a sewage treatment plant, with a pH beneath the coating as low as 0.8.

A technically useful application of such processes has been developed (28) for a decontamination of radioactive concrete constructions in the nuclear industry. Spray application of a biofilm-like gel containing an inoculum of thiobacilli and elemental sulfur and the necessary trace elements allowed the extraction of a surface layer of concrete including acid-soluble radionuclides. After sufficient dissolution due to biogenic sulfuric acid production, a simple removal of the products is possible. Thus, high cost and hazardous work-intensive procedures may be replaced by biotechnological means.

Materials resistant to microbial weathering should be chemically resistant to the active agents involved. For example, different types of concrete show different resistance toward biogenic sulfuric acid produced by

thiobacilli. High calcium aluminate cements may give an acceptable, acid-resistant concrete for application in dangerous environments, for example, in sewage systems (29).

Prevention of weathering by coating of the materials may be an option for protection. Barriers against penetration of bacteria and against the active destructive agents may improve the lifetime of materials. Penetration of bacteria may be prevented only by microporous materials with a pore size maximum of 0.5 μm. However, at the surface of the coatings acid-producing bacteria may settle and proliferate. Thus, the structure of the coating must be resistant at the molecular or ionic level, because diffusion paths of molecular size are appropriate to allow for an acid attack on the underlying bulk material and a failure of the "protective" coating is the consequence.

A material used as coating, as filling material, as reinforcement, or as an addition for any other purpose (e.g., plasticizer) should not serve as a nutrient for microorganisms nor should it be susceptible to degradation by bacterial influence. Otherwise, only a limited service life will result.

An addition of inert filling materials such as glass fibers is of no use, when the bulk material is susceptible to microbial attack by any of the ways described earlier. Even an addition of specific biocides has only a limited effect because microorganisms living in microcolonies and microniches may survive the treatment. Biofilms are known to have a protective effect by creating a diffusion barrier toward the biologically "noxious" compounds. Even the effect of toxic heavy metals may be attenuated by the excretion of biogenic complexing agents such as oxalic or citric acid.

Clay Minerals

Clay is a mixture of mineralogically stable, finely grained aluminosilicate minerals resulting from primary rocks as the end-products of natural weathering. The grain size of clay particles is typically between 0.1 and 10 μm. Clays have a layered structure that allows the inclusion and exchange of cations. Depending on the stoichiometric ratio between the main cations (Si^{4+}, Al^{3+}, Mg^{2+}), the basic crystal lattice may be electrostatically balanced (neutral) as it is, for example, in the case of pyrophyllite $Al_2Si_4O_{10}(OH)_2$ and talcum $Mg_3Si_4O_{10}(OH)_2$. In the case of an isomorphic replacement of one cation with another one of usually lower valence (Mg^{2+}, Al^{3+}, Fe^{3+}, Ti^{4+}), the consequence is a net negative charge. Thus, additional cations must be included to compensate the electrostatic imbalance. This may happen with mono and divalent cations (H_3O^+, NH_4^+, Na^+, K^+, Mg^{2+}, Ca^{2+}). Depending on the exact structure of the basic lattice and on the nature of the additional cations, embedment of water by hydratation may occur. Thus, swelling and nonswelling clay minerals may be formed (30).

Clay minerals rarely occur as pure minerals. Usually, they occur with impurities or as mixtures with unaltered minerals such as quartz or feldspar, turmaline, rutile, zircon, or other weathering products of rocks such as iron oxides.

Clay minerals were used mainly for simple constructions, but valuable monuments have also been built using clay minerals during history in suitable climatic conditions. Historical buildings made of (mainly) clay with straw addition may withstand harsh climatic conditions for centuries. Organic carbon sources were often present due to animal feces added to the clay-mixture to improve adherence and coherence of the mixture. Studies regarding the microbial population in framework buildings revealed that deterioration of clay by microbial weathering is not the main cause of decay, although nitrifying bacteria occurred together with chemoorganotrophic bacteria and fungi in large cell numbers. Fixed ammonium caused by Lewis and Broensted acid sites on aluminosilicates (allowing for a strong chemosorption of ammonia) was detectable in clay samples, which proved to be accessible to nitrifiers in laboratory experiments.

A useful technical application of microbial weathering is known from ancient times. The Chinese discovered the improvement of clay quality and the transformation into high-quality kaolinite by a treatment with liquid manure for several years. A removal of reddish-brownish coloring iron compounds as a result of bacterial dissolution allowed them to obtain a raw material yielding a white "china" porcelain. Additionally, the kaolin became better processible because of its improved plasticity, exhibited an improved bending strength, and finally the fired product had an improved tensile strength. Thus, a very thin porcelain could be produced (30).

Ceramics

Brickwork (consisting of bricks and mortar) in a main sewer showed serious damage after 80 years of service. Chemical and microbiological analysis revealed that pH-gradients within the mortar joints and within the bricks themselves were correlated with species and cell counts of acid-producing microorganisms. Depending on the extent of the damage and on the specific conditions at those sites, specific consortia of coexisting microorganisms could be detected (20,31). Finally, the visual damages of the bricks could be attributed mainly to mechanical stress caused by a crystallization of gypsum needles within the pores of the bricks. The formation of calcium sulfate occurred in the carbonatized mortar as a result of the metabolism of thiobacilli (sulfuric acid excretion). As a consequence, the mortar was weakened because of a loss of its binding material, calcium carbonate. Calcium sulfate crystallized partly in the mortar, but it also partly diffused into the bricks and crystallized in the pores. In addition, thiobacilli were detected in the bricks, too; thus, sulfuric acid was formed in the brick as well. Dissolved calcium bicarbonate and/or calcium hydroxide (the deep layers of the mortar showed strongly alkaline pH) diffusing additionally from the mortar formed gypsum (crystals) with the biogenic sulfuric acid. Also, nitrifying bacteria were involved in the deterioration process. They obviously contributed to the dissolution of calcium carbonate by forming the water-soluble compound calcium nitrate, enhancing the amount/concentration of calcium ions diffusing toward the bricks.

In another case, glazed bricks (approximately 100 years old) exposed to the atmosphere showed severe weathering because of a high microbial biomass (fungi and bacteria) supported by the presence of photosynthetic microorganisms (filamentous algae and unicellular algae) (32).

Little information is available on microbial weathering of ceramic materials such as stoneware, which is supposed to be highly resistant toward inorganic and organic acids. Additionally, it is usually designed for heavy duty purposes (sewage, chemical industry, etc.) and fulfills demands regarding both corrosion and mechanical resistance. However, stoneware obtained by low-temperature firing may have remaining acid-susceptible components.

Glass (Historical and Recent Glassware)

Glass is a noncrystalline, amorphous material consisting of several oxides. Silicon dioxide, calcium oxide, and at least one alkali-oxide (sodium or potassium) are the most common components. Often, minor contents of phosphate, magnesium, aluminum, iron, and manganese are present, but heavy metals such as lead and sometimes copper are also present, which may represent major parts of crystal glass (lead glass) or ornamental glass. Special-purpose (modern) glass may contain as main or secondary components boron trioxide, barium, beryllium, lithium, or silver, but minor components or traces of many other elements are also deliberately used.

Distinct weathering processes occur, depending on the composition of the material: whether it was designed for a certain environment and used accordingly or whether it was exposed to other, inappropriate environmental conditions. Thus, weathering of glass in normal use (modern, recent, or even historical windows) (33–36) should be distinguished and differentiated from weathering under special conditions, for example, as in archaeological glassware (37).

Because numerous possibilities of different glass compositions exist, only general statements can be given. Organic compounds deposited from dust, fumes, vapors, or fingerprints may serve as nutrient source for chemoorganotrophic growth of microorganisms. Fungi seem to be the main organisms involved in a biological attack on glass materials. Excreted organic acids such as acetic, oxalic, citric, fumaric, and gluconic acid have been detected. Ion exchange of protons against metal ions in the glass matrix is the primary phenomenon, whereas alteration of the glass structure (gelification, stratification, fractures, and also pitting) may be the consequence. Differences between the tensile strength of the altered surface layers and of the sound, bulk glass mass may cause crack formation and propagation. Additionally, crystal growth and penetration of fungal hyphae can contribute to an enlargement of cracks and an exposure of new surfaces for further reaction. Dissolution of glass components may serve as the sole source for biologically essential elements for microorganisms such as iron, manganese, or phosphorus compounds. Precipitation of new solid phases, not always as crystalline minerals (biominerals), may produce color changes, reduce transparency, and alter optical performance. Biominerals such as (insoluble) calcium oxalate, lead phosphate, calcite, aragonite, and kalicinite have been identified so far. More complex, rather unknown compounds form under less controllable conditions, as found in archaeological glass samples. In these cases glass components, microbial or/and fungal metabolites, and soil components contribute to the formation of a phenomenon called “glaspest” (37).

Because nearly infinite glass compositions are possible, only very few model glass compositions have been studied so far with regard to biogenic weathering/deterioration under controlled conditions. Accordingly, simulation studies must be performed for any individual glass considering the specific environmental conditions for which it was designed. Extreme situations need to be considered in the case of glassware intended to be used in high-tech applications.

SIMULATION OF MICROBIAL WEATHERING

The colonization of a weathered material by microorganisms does not allow one to determine if microbial weathering is involved in the deterioration observed. Under natural conditions physical, chemical, and microbiological factors interact during weathering of a material. Because of this complexity a clear correlation between the presence of certain microorganisms and deterioration of the material is rarely possible. Because the weathering process under natural conditions is relatively slow, it may take years or even decades to observe measurable changes. Simulation experiments try to overcome the limitations of in-field investigations. Although approaches differ widely in their complexity ranging from simple inoculation-tests to complex environmental simulation, the general procedure of a simulation experiment can be divided into three steps (basically following Kochs postulates):

1. Quantification, isolation, and identification of microorganisms growing on the affected material.
2. Inoculation of pristine material samples with the microorganisms isolated and subsequent incubation under optimized and controlled conditions.
3. Characterization and quantification of the effects caused by the presence and/or the activity of microorganisms on the characteristics of the materials.

Simulation experiments are performed for different purposes. First of all they are used to demonstrate and quantify the weathering potential of a defined group or consortium of microorganisms and to study the mechanisms involved. In case of economically important materials such as concrete and metals, simulation of microbial weathering is often used as a standardized material-testing procedure. These tests aim at a differentiation between the susceptibility of different materials to microbial weathering and/or the colonization with microorganisms. They may include testing of countermeasures such as coatings, biocides, water repellents, and consolidants.

Although steps two and three of the procedure described earlier may appear to be the main parts of a simulation experiment, step one should not be neglected as long as the mechanisms are not fully understood. It is essential to

Figure 3. Various sandstone. Test specimen exposed to natural weathering.

correlate laboratory data with field data. Therefore, field surveys and field testing should be done in parallel with simulation experiments (Fig. 3).

Simulation aims at controlled and optimized experimental conditions. Usually, an acceleration of the microbial weathering process is achieved by the inoculation of test specimen with selected microorganisms and an incubation at conditions optimum for their growth. Optimum growth conditions are usually achieved by a high availability of substrate, a suitable temperature, and a high moisture content in the material and/or the atmosphere. The experimental time is a major influence on the results of a simulation experiment, because significant changes in the material, even under accelerated conditions, may occur only after months. Therefore, long-term experiments performed for months, a year, or even longer, are often necessary.

Significant changes of material's characteristics detectable after accelerated microbial weathering stand for the weathering potential exerted by the inoculated microorganisms. Parameters that may indicate microbial weathering are discoloration, hydrophobicity, porosity, mineral composition, water vapor diffusion, weight-loss, pH, material-loss, salt content, and loss of strength. To obtain proper results it must be ensured that the inoculated microorganisms actively grow or settle on the material and do not just survive without any significant activity. Furthermore, it is essential that changes in material's characteristics by microbial weathering are significant, when compared to noninoculated controls incubated under comparable conditions but kept free from microbial growth.

The accelerated conditions in simulation experiments can be termed "worst-case," a fact that makes them a powerful tool for the testing of materials. If a material resists microbial weathering and/or colonization under "optimum," but artificial conditions, it can be predicted as resistant to microbial attack/colonization under "suboptimum," but natural conditions. If sufficient data from case studies and/or exposure experiments are available, an acceleration factor can be calculated for a standardized test system allowing the estimation of a lifetime of the material (23,38,39).

A variety of simulation experiments have been performed to find out which microorganisms are the most relevant in microbial weathering of concrete, natural stone, and mortar.

The potential of filamentous fungi to weather mineral materials by the excretion of organic acids has been demonstrated several times by relatively simple, laboratory experiments (11,13,40). Mineral materials were immersed in nutrient-rich media, inoculated with fungi, and incubated. In a laboratory chamber experiment, the pitting of marble by slow growing dematiaceous fungi was demonstrated recently (14). After 10 months of incubation under alternating conditions of dryness and high humidity, inoculated cubes showed a weathering similar to that found on marble in Mediterranean regions. These experiments for the first time demonstrated that fungal weathering may also involve mechanical forces.

The weathering of natural stone and concrete by acid-producing thiobacilli and nitrifying bacteria has extensively been demonstrated in simulation experiments (41,42).

Microbially induced sulfuric acid attack of concrete by the activity of sulfur oxidizing thiobacilli causes severe problems in sewage pipes, which was well characterized and evaluated by simulation experiments (23,39,43,44). The microbiological weathering of concrete was accelerated 8 to 24 times (23,39). After three months, a visible deterioration of the mineral material appeared. With this test system, concretes of different composition and quality were differentiated according to their susceptibility to the biogenic sulfuric acid attack. In addition, the efficiency of coatings, paintings, and biocide treatments was tested. The microbiological attack was strongly influenced by interactions between the mineral surface and the organisms. Thus, the biological test cannot be replaced by a simplified chemical test (23,39).

In a test chamber, designed as a salt-spray-cabinet, the biogenic nitric acid attack on sandstone and concrete by nitrifying bacteria was demonstrated for the first time (45). After one year, the materials were differentiated according to their resistance to the microbial attack. The loss of calcium correlated with the weakening of the mineral structures. The conditions in the salt-spray-cabinet

resembled the ones found on wet concrete surfaces inside cooling towers, where high numbers of nitrifying bacteria were found.

In the case of historical buildings made from natural stone, weathering is a combination of physical, chemical, and microbial attack. Therefore, in a complex simulation experiment, biogenic nitric acid attack was reproduced in the presence of an artificial smog atmosphere (9,41). The air contained sulfur dioxide, nitric oxide, and nitrous oxide in concentrations 10- to 20-fold higher than the mean annual concentrations of these gases in urban environments in Germany. To differentiate between chemical and microbiological weathering, a special double-chamber-cabinet was used (41). By this approach interactions between chemical and microbiological weathering were demonstrated for the first time. Under optimum conditions a nitrifying biofilm developed on the calcareous Ihrlersteiner green sandstone. Cell numbers and activity of nitrifying bacteria increased during the experiment (lasting 40 weeks), indicating a positive effect of the smog atmosphere on these bacteria. The mean metabolic activities of ammonia oxidizers were 8 to 11 times higher and those of nitrite oxidizers 30 times higher than that of the mean values of samples from historical buildings. Compared with samples kept free of nitrifying bacteria, the formation of gypsum from sulfur dioxide was enhanced in the presence of actively nitrifying bacteria. At the same time the biogenic nitric acid attack was strongly reduced by chemodenitrification of nitric acid. Quantification of the weathering showed that the microbiologically induced nitric acid attack alone was eight times stronger than the chemical attack caused by the simulated smog atmosphere.

The latter results demonstrate the complexity of the situation, when different factors of weathering and their interactions are included in environmental simulation experiments. Therefore, most simulation experiments tend to be less complex and focus on microbial weathering alone (and may, thus, be misleading).

For algae, cyanobacteria, and chemoorganotrophic bacteria, clear evidence for microbial weathering from simulation experiments is still missing. Nevertheless, the colonization of a material with microorganisms does not necessarily imply physical and chemical degradation, but may initially be limited to less dramatic changes like discoloration and alteration of physical properties caused by the formation of biofilms. The aptitude of a material to be colonized by one or several groups of organisms without necessarily undergoing any biodeterioration is described by the concept of "Bioreceptivity" (46). In this sense, simulation/inoculation experiments can also be used to predict the susceptibility of materials that are to be colonized with certain microorganisms. From these data, guidelines for the selection of materials can be derived. In fact, the preferences of nitrifying bacteria to colonize certain types of natural stone as found in simulation experiments was confirmed in an extensive field survey concerning historical buildings in Germany. Another example of a good agreement between data from simulation experiments and observations on a building was observed during complex, long-term simulation experiments, which showed a strong colonization of hydrophobically treated natural stone. A similar observation was made at a historical building in Germany, where hydrophobically treated natural stone was significantly more colonized with fungi than nontreated material.

To conclude, it may be said that simulation experiments are essential to demonstrate and quantify the effects of microbial weathering and help choose the right material for certain applications. No other technique allows the inclusion of interactions between microorganisms and materials. Aside from the individualities of each experiment, its realization should follow the essential guidelines outlined here.

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WEATHERING: MINERAL WEATHERING AND MICROBIAL METABOLISM

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Weathering is the process by which primary minerals from igneous, metamorphic, and sedimentary rocks, formed under one set of geologic and geochemical conditions, are altered in form and composition to new sedimentary particles and rocks more consistent with the physical and chemical conditions of the weathering environment. Weathering plays a role in soil and ore formation, the maintenance of soil, freshwater, and ocean composition and productivity, and in moderation of atmospheric chemistry and global climate. Although weathering has traditionally been considered to be primarily a chemical and physical process, there is increasing realization that living organisms play a significant role in these supposedly abiotic processes. A number of field observations and laboratory experiments clearly demonstrate that microorganisms can and do play important roles in the physical and chemical alteration of rocks and minerals and are therefore responsible for many of the geologically and environmentally important consequences of the weathering process.

Historically, weathering processes have been divided into two categories:

- Physical weathering processes leading to the reduction in particle size with little or no impact on chemical or mineralogical composition; and
- Chemical weathering processes leading to both a reduction of particle size and to chemical and mineralogical alteration.

This division, however, is quite arbitrary because these processes co-occur in many environments and are interdependent. Physical processes contribute to chemical weathering by reducing particle size leading to the exposure of new chemically reactive surfaces. Chemical processes contribute to the enhancement of physical weathering by weakening the intergranular boundaries between minerals in rocks subject to abrasion. Plants and plant roots are known to play a significant role in both the physical and chemical weathering of rocks and minerals. In addition, there is increasing recognition that microorganisms and microbial metabolism play an equally important role in these processes, together with or in the absence of higher plants.

Although the term "weathering" initially was only used to describe those processes and reactions that occur on the exposed land surface, it is now recognized that significant chemical alteration of rocks occurs in geologic settings remote from the Earth's surface. As a result the use of this

term has been expanded to include all alteration of rocks, minerals, and synthetic solids that occur in the presence of the hydrosphere, atmosphere, and/or biosphere and at temperatures characteristic of the earth's surface (1).

Many of the important geologic and environmental consequences of weathering are the result of the chemical transformations involved in the weathering process (2). Chemical weathering is an important process in the creation of soils and the maintenance of soil productivity. Weathering reactions produce secondary mineral phases such as clays and oxyhydroxides and release trace nutrients such as phosphorus, potassium, and iron to soil solutions. Weathering reactions contribute to the evolution of ground- and surface-water compositions by consuming reactants and releasing ions to solution. Most environmentally relevant silicate minerals consume protons on dissolution and therefore contribute to the moderation of environmental acidity. The flux of weathering products from continents, both particles and solutes, contribute to the maintenance of seawater composition and productivity. There are a number of types of ore bodies, such as bauxite deposits, that result from mineral and rock weathering. Secondary porosity generated by mineral dissolution contributes to the success of petroleum migration and recovery, and it may also affect the transport of solutions and solutes through aquifers. The weathering of candidate matrices for the isolation of radioactive and other wastes may ultimately impact the long-term success of waste isolation strategies. Weathering has also been implicated in the alteration and deterioration of artwork, historical buildings, and monuments (3). Weathering reactions involving the rock-forming and silicate and aluminosilicate minerals are particularly important because of their abundance in nature and because they represent an important source of noncarbonate calcium that controls the long-term concentrations of atmospheric carbon dioxide and, therefore, regulates the global heat balance (4,5).

In this review, we consider the biogeochemistry of weathering reactions, focusing on the dissolution of feldspars, the most abundant class of primary aluminosilicate minerals. Microbially mediated mineral precipitation reactions, which are an important component of the overall weathering process, are discussed elsewhere in this Encyclopedia (6). We first consider the abiotic dissolution of feldspars and the mechanisms by which microorganisms and their metabolic products may affect the rates of feldspar dissolution reactions in the weathering environment. We then review the abundant experimental and field evidence for microbial weathering and consider the conditions under which microbes, microbial metabolism, and microbial metabolic products contribute to the weathering process. Finally, we briefly discuss some aspects of the global geochemical and environmental importance of the microbially mediated weathering process.

THE GEOCHEMISTRY OF WEATHERING

All rocks and minerals are subject to physical and chemical weathering under the right environmental circumstances. The earth's surface and the global environment represent a record of the physical and chemical alteration of primary igneous and metamorphic rocks and minerals. Although the details of the weathering process for different classes of rocks and minerals may be significantly different and depend on the chemistry, mineralogy, and petrology of each class, the fundamental trends in the geochemistry of weathering can be illustrated by reference to any one class of materials. In this review we illustrate these trends using feldspars; Ehrlich (7) reviewed microbial impacts on a wider range of mineral processes than is possible here.

Feldspars are framework silicates with the general composition of $(K,Na)_xCa_{(1-x)}Al_{(2-x)}Si_{(2+x)}O_8$, in which the charge of a three-dimensional framework of oxygen-sharing AlO_4^{-5} (aluminate) and SiO_4^{-4} (silicate) tetrahedra is balanced by the alkali and alkaline earth cations, K^+, Na^+, and Ca^{2+} (8). Within this mineral group, calcium and aluminum concentrations increase at the expense of (K,Na) and silicon concentrations to maintain charge balance. Feldspar composition can therefore be expressed by reference to the alkali and alkaline earth composition alone. This stoichiometric covariance allows for the examination of systematic trends in weathering behavior with changing mineral composition. Thus, this mineral series has often been used as an experimental model in many types of geochemical studies. Feldspars crystallize on cooling at igneous and metamorphic temperatures [750 –1,250 °C, depending on composition and pressure, (8)] and are metastable under many earth-surface conditions (8). A number of classes of trace elements occur in the feldspar matrix or as accessory minerals in the feldspar matrix (9). Some of these may have nutrient or trace-nutrient character and therefore may be important in maintaining ecosystem productivity (10). These elements may be released to solution as a consequence of the dissolution process.

When exposed to dilute aqueous solution in equilibrium with the atmosphere or CO_2-enriched soil, sediment, or aquifer waters, feldspars may dissolve congruently according to the reaction:

$$\begin{aligned}(K,Na)_xCa_{(1-x)}Al_{(2-x)}Si_{(2+x)}O_8(s) + 4x\ H_2O \\ + 4(2-x)H_2CO_3 \rightarrow (2+x)Si(OH)_4^\circ \\ + (2-x)Al^{3+} + x(K^+, Na^+) \\ + (1-x)Ca^{2+} + 4(2-x)HCO_3^-\end{aligned} \quad (1)$$

In many geochemical settings, however, secondary mineral minerals, often clays, may be the secondary products of incongruent dissolution:

$$\begin{aligned}2(K,Na)_xCa_{(1-x)}Al_{(2-x)}Si_{(2+x)}O_8(s) + (2+7x)H_2O \\ + (4-2x)H_2CO_3 \rightarrow (2-x)Al_2Si_2O_5(OH)_4(s) \\ + (2x)Si(OH)_4^\circ + x(K^+, Na^+) + (1-x)Ca^{2+} \\ + (4x)HCO_3^-\end{aligned} \quad (2)$$

These two representative reactions clearly indicate the fundamental geochemical consequences of the weathering process. The primary mineral (feldspar) is dissolved (or altered), acidity (in the form of H_2CO_3) is consumed,

secondary solids (such as kaolinite, $Al_2Si_2O_5(OH)_4(S)$, in this example) are produced, and major (such as K^+, Na^+, Ca^{2+}, and HCO_3^-) trace and nutrient elements (not shown in the simplified stoichiometry) are released into solution. The above stoichiometries represent an idealization of the weathering process in which a range of primary minerals, acids, and secondary products may be involved.

Reactions 1 and 2 are examples of acid hydrolysis (referred to as proton-mediated, proton-promoted, or acidolytic dissolution in the weathering literature), only one of the mechanisms by which weathering may occur. Alternative weathering mechanisms involving alkaline hydrolysis (sometimes referred to as hydroxyl-mediated, hydroxyl-promoted, or alkalinolytic reactions), ligands (ligand-mediated, ligand-promoted, or complexolytic reactions), or redox processes may also play a role in the weathering process (11,12). Acidic or alkaline attack of the mineral surface involves the adsorption of protons or hydroxyl groups to the mineral surface resulting in the disruption of the near-surface mineral structure (in the case of feldspar the Al−O−Al, Al−O−Si, or Si−O−Si bonds of the tetrahedral framework), and the release of mineral components to solution. Redox reactions, although important for some classes of iron-, manganese-, sulfur-, and uranium-bearing minerals, are not important in the weathering of feldspars and other similar rock-forming minerals, because these do not contain elemental components that are easily oxidized or reduced under earth-surface conditions.

As with all geochemical reactions, the rates of dissolution of primary minerals, the first step in the overall weathering process, are related to the:

- Chemical equilibria describing the composition and stability of the primary minerals and secondary products;
- Temperature of reaction;
- Accessibility of reactive mineral surfaces and the exchange of solutes and water from the mineral surface to the bulk reactive (and presumably mobile) solution; and
- Other characteristics of the geologic and geochemical setting in which the primary weathering reactions occur.

As a result, there is a great deal of variation in weathering rates observed in nature and in the laboratory, and it is difficult to a priori predict, in detail, the weathering rates in any specific context (13). For this reason, we focus this review on the trends observed in the geochemistry and biogeochemistry of weathering and not on specific rates of the weathering process that may be found in laboratory or field settings. Because the dissolution of primary minerals is a required first step for all subsequent geochemical transformations and elemental releases, we focus our discussion on this reaction step and the mechanisms by which microorganisms and microbial metabolism can affect the rates of this initial reaction.

Traditionally, geochemists believed that proton-mediated reactions were the dominant weathering mechanism because there is a strong dependence of dissolution rate on pH (14,15). The influence of organisms on the geochemistry of this "abiotic" weathering process was largely ignored. However, the increasing recognition that chemical weathering takes place largely in the soil zone where microbial respiration can lead to elevated levels of pCO_2, and therefore, high concentrations of carbonic acid, has increased the awareness of the potential role of biological processes on weathering. Recent studies, however, indicate that microbes and microbial metabolism may have additional effects on mineral weathering related to the production and excretion of organic metabolites and to the nutrient and energy requirements of the microorganisms (11,12). Organic metabolites participate in ligand-mediated weathering reactions. In addition, bacterial cell walls and their associated functional groups may serve as chelators and play a role in a process analogous to that of ligand-mediated dissolution (16).

THE BIOGEOCHEMISTRY OF WEATHERING

Recent research in the field of microbially mediated mineral dissolution does not question whether bacteria enhance mineral weathering rates. The largely microbial origin of soil carbon dioxide and the role of carbonic acid in the weathering process have clearly demonstrated this principle. The research, however, continues as a number of additional mechanistic questions remain. These include

- Is the proton-mediated mechanism the only important mechanism by which bacteria influence mineral weathering rates?
- If there are additional weathering mechanisms mediated by microorganisms, what are they and under what conditions are they important?
- Is there a relationship between types or amounts of bacteria or microbial metabolism and mineral weathering rates?
- Is direct contact between bacteria and reactive mineral surfaces a necessary component of microbially mediated weathering?
- What is the nature of the reactive interface between a microorganism and the mineral surface?
- Do bacteria derive a physiological or ecological benefit from the weathering process?
- Do bacteria play a role in the global weathering cycle that controls atmospheric chemistry and therefore global climate?

It is these research questions that will be addressed in this review.

Bacteria are ubiquitous in soils, sediments, sedimentary rocks and aquifers, the environments in which weathering reactions occur, at concentrations ranging from 10^5 to 10^9 cells/cm^3 (17). Natural consortia are diverse, viable, metabolically active, and able to grow on a wide range of substrates. Bacteria are found in free-living ("planktonic") forms and attached to mineral surfaces. As such, bacteria exist at the interface of the lithosphere and hydrosphere where weathering reactions occur. It is therefore not surprising that bacteria and microbial metabolism can both

affect (regulate?) and potentially benefit from weathering reactions in these environments.

Microbes in soils, sediments, and aquifers produce a variety of compounds that can potentially affect weathering rates. Bacteria can decrease pH as a result of respiration (organic carbon oxidation) or by the oxidation of other reduced species such as iron, sulfur, and nitrogen, thereby catalyzing mineral dissolution. Microorganisms can also increase hydroxyl-mediated mineral dissolution rates by increasing pH as a result of the production of alkaline compounds, such as NH_3, or by consuming acidic compounds such as CO_2.

In addition to inorganic acid production and consumption, microbes can also enhance or inhibit mineral weathering rates by the production of complexing organic ligands. Ligands can indirectly enhance mineral dissolution rates by complexing with the reaction products released to solution and altering the equilibria driving the dissolution process. This process serves to increase the carrying capacity of the reactive solution for reaction products. Alternatively, ligands may directly enhance ligand-mediated dissolution rates by adsorption to and reaction with the mineral surface in a process similar to that of proton- or hydroxyl-mediated dissolution. Ligands can also inhibit dissolution by complexing with or precipitating on the mineral surface, thus reducing the number of sites or the "activity" of sites available for the dissolution reaction. The ligands that participate in these ligand-mediated processes may be simple small molecules with a small number of reactive functional groups per molecule or polymers containing many functional groups per molecule. These ligands may be intentionally excreted or released on cell death. The functional groups on bacterial cell walls may act as complexing ligands and play a role in regulating primary-mineral dissolution (16). The relative importance of bacteria and microbial metabolites in dissolution enhancement and/or inhibition depends on mineral composition, solution pH, temperature, and the size, chemistry, and concentration of the complexing ligands in solution, in addition to other environmental factors that may control weathering reactions. Ligand production by microorganisms, however, often co-occurs with proton production (as in organic acid production by the partial oxidation of glucose) and therefore it is often difficult to distinguish between the microbial mediation of the "abiotic" hydrolytic reaction and the alternative or additional ligand-mediated mechanism of bacterial action either in the laboratory or in the field. Although the observational and experimental evidence that bacteria and microbial metabolic processes contribute significantly to the weathering process is extremely clear, the mechanism by which the bacteria affect this process is not always so obvious. The evidence of alternative mechanisms of microbial weathering results from experimental manipulations in the laboratory and the field.

Microbial production and excretion of complexing organic ligands can occur by several different mechanisms. In anoxic environments, fermentation of organic substrates can release soluble ligands such as formate, acetate, propionate, butyrate, and lactate (18). High concentrations of these compounds have been measured in anoxic and suboxic sediments (19,20). In oxic environments, both prokaryotes and eukaryotes can produce substantial concentrations of organic ligands, especially in organic-rich environments, such as soils. Although the concentrations of soluble organic ligands are often below detection in the bulk solutions of oligotrophic environments, they may nevertheless be produced and reach substantially higher concentrations in anoxic or suboxic microenvironments such as the interface between a cell and a mineral surface. In both oxic and anoxic environments, bacteria synthesize extracellular polymers that have complexing ability. Bacteria may also degrade particulate or dissolved organic substrates to produce ligands that affect the dissolution process. This latter process may be particularly important in petroleum-contaminated environments (21). Laboratory experiments have also demonstrated that significant production of organic ligands in oxic environments can occur when substrate utilization is limited by the lack of available accessory nutrients (22). This partial oxidation process may be important in both anoxic and suboxic environments when nutrient availability is limited.

EVIDENCE FOR MICROBIALLY MEDIATED MINERAL WEATHERING

There are three lines of evidence for the importance of microbially mediated mineral weathering in nature. The first involves the abiotic experimental evidence of mineral dissolution in solutions that mimic those found in microbial cultures and in nature. These experiments are normally compared to controls lacking one or more of the solutes in the experimental medium or by systematically varying one or more of the solute concentrations. The second line of evidence involves laboratory experiments in which metabolically active microbes are cultured in the presence of mineral or mineral-analog surfaces and these experiments are compared with various control experiments lacking organisms, minerals, nutrients, and/or organic substrates. The last line of evidence involves direct observation, primarily by scanning or transmission electron microscopy and other microanalytical techniques, of microorganisms and microbially produced textures on mineral and glass surfaces produced by experiment and/or collected in the field. Controls for these experiments include the original surface before experiment, unreacted samples of the experimental materials, or adjacent, but uncolonized surfaces of the reacted material. Each of these types of experiments has limitations, but each line of evidence supports the hypotheses that the rates of weathering reactions can be and are mediated by microorganisms and that there are components of microbially mediated weathering reactions that are distinct from the reactions that might occur in the absence of bacteria and bacterial metabolism. The three lines of evidence, taken together, clearly indicate that microbially mediated weathering reactions are important in a wide range of common earth-surface environments in which weathering occurs.

Abiotic Experiments

Abiotic experiments involve the reaction of mineral material in a solution mimicking that found in nature or in separate microbial culture experiments. Experimental variables include pH, ligand composition and concentration, temperature, and mineral composition. Typically, one of these parameters is varied, whereas others are held constant in a flow-through, batch, or column reactor. One problem with these types of experiments is that the concentrations of solutes used are very much higher than those found in nature. Therefore, it is difficult to extrapolate directly from experimental results to field conditions in which both reactive solute concentrations and mineral dissolution rates may be orders of magnitude lower than those used and found in the laboratory. This type of experiment, therefore, can indicate that microbial metabolites affect weathering rates and the conditions under which the microbiological effect may be significant, but it cannot determine whether this process is important in nature. Such experiments are very useful, however, because they allow for better control of reaction conditions and for more systematic and verifiable control studies. Studies of the concentrations of ligands and protons in microenvironments in which microbial metabolism and weathering occur and where local ligand concentrations may be higher than those found in the bulk solution are needed to support these experimental results. Recent measurements of pH in microenvironments have demonstrated that the pH in close proximity to microbial cells can be 1 to 4 pH units lower than that of the bulk solution (23,24).

The following discussion focuses primarily on feldspars, but similar results have been reported for other classes of rock-forming minerals. A recent review of the ligand-mediated dissolution of feldspar minerals in abiotic experiments (25) is summarized below.

pH Dependence

As in the case of "abiotic" proton-mediated dissolution experiments (14), the rates of (ligand + proton)-mediated dissolution have a strong pH dependence due largely to the effect of the protons. This result is not surprising because the initial steps in both proton- and ligand-mediated dissolution involve the interaction (adsorption) of protons or ligands in solution with the sites on the mineral surface. As proton concentration increases, the competition between protons and ligands for limited surface sites on the dissolving mineral surface will favor the protons. However, the competition between ligands and protons at near-neutral pH, where proton concentration is comparable or lower than that of the ligand, will favor the ligand and ligand-mediated dissolution. Experimental evidence in this near-neutral pH region, the pH of many natural settings in which weathering occurs, indicates that ligand-mediated dissolution rates can be up to 2 to 10 times higher than rates in the absence of ligands, which reflects the background rate of mineral hydrolysis in the absence of proton- (or hydroxyl-) mediation (Fig. 1; 26).

There is very little experimental evidence for ligand-mediated dissolution in basic solutions, but there are a number of reasons why ligand-mediated dissolution should be less important under alkaline conditions. In the first case, both mineral surfaces and ligands become deprotonated (or hydroxylated) in alkaline solutions. Under these conditions, both the ligand and the surface will be of similar anionic charge and therefore the initial adsorption of the ligand to surface sites, the important initial step in the dissolution reaction, will be electrostatically inhibited. In addition, as the concentration of hydroxyls increases with pH, competition between hydroxyls and ligands for surface sites will favor hydroxyls and the hydroxyl-mediated reaction, similar to the case of proton/ligand competition at lower pH.

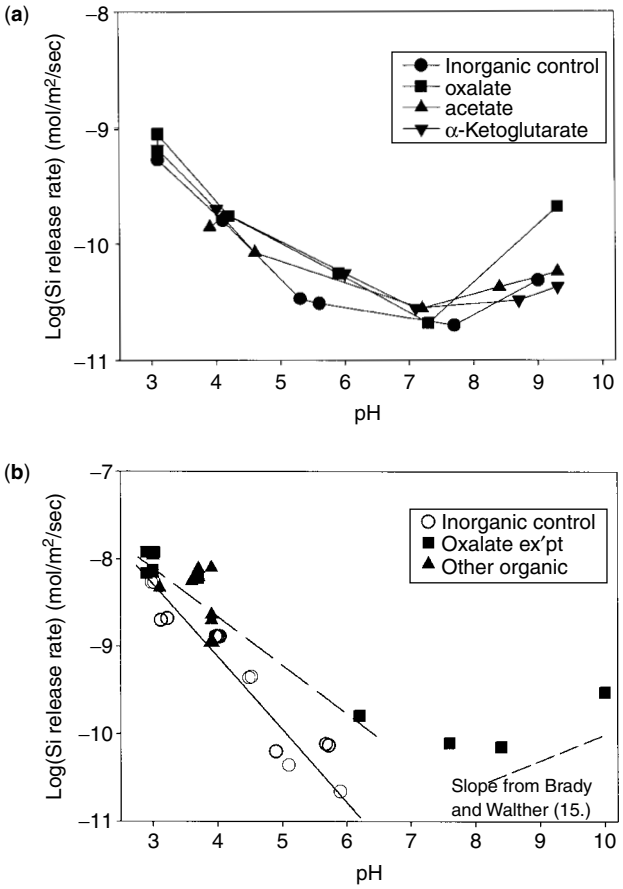

Figure 1. The pH dependence of feldspar dissolution. (**a**) Silicon release rates from two labradorite feldspars ($K_{0.05-0.06}Na_{0.46-0.48}Ca_{0.49}$). The rates of ligand-mediated dissolution follow the same pH dependence as for proton-mediated dissolution alone. Only for oxalate ion, is there evidence for dissolution enhancement at alkaline pH. (**b**) Si release rates from bytownite feldspar ($K_{0.01}Na_{0.23}Ca_{0.77}$). For this calcium-aluminum-rich member of the feldspar series, there is clear evidence of dissolution enhancement throughout the acid pH range with the relative rate of enhancement occurring closest to neutrality. There is clear difference in the slope of ligand-mediated dissolution in the acid pH range (- - -) from proton-mediated dissolution alone (——). At alkaline pH there is no apparent difference between the slope of the pH dependence of ligand-mediated dissolution and of hydroxl-mediated dissolution alone (15) suggesting that ligand-mediated dissolution is less important at alkaline pH. Experimental points taken from S. A. Welch, and W. J. Ullman, *Geochim. Cosmochim. Acta* **57**, 2,725–2,736 (1993).

Ligand Composition

Many low–molecular weight organic ligands that can be produced by bacteria under a variety of circumstances have been shown to enhance mineral dissolution rates over distilled water, dilute-salt, or dilute-acid controls over a range of pH (Table 1). Unfortunately, there are very few experiments in which the relative impacts of ligands of differing molecular weight, functionality, or thermodynamic properties on dissolution rates have been rigorously compared. In one such experiment (26), however, it was found that organic ligands with two or more functional groups (oxalate, salicylate, pyruvate, and citrate) that were capable of forming bidentate metal-organic complexes in solution, and therefore presumably at the mineral surface, had a greater effect on mineral dissolution rates per unit solute concentration than monofunctional ligands (propionate and acetate). Monofunctional ligands, in turn, had a greater impact on dissolution than distilled water, nitric acid, and hydrochloric acid controls. Multifunctional organic ligands were also found to enhance the dissolution of quartz (SiO_2) to a greater extent than monofunctional organic ligands (27). Difunctional aromatic ligands were found to enhance feldspar dissolution rates to a greater extent than more highly substituted aromatics in one study (28). These trends in the degree of dissolution enhancement by the ligand-mediated mechanism correlate weakly with the thermodynamic stability of Al-ligand complexes in solution (as a proxy for the association of ligands with aluminum sites at the feldspar surface). Other factors, however, may also be important including molecular weight and structure, size, and protonation constants of the ligand.

A study of the effect of biopolymers (as analogs of microbial exopolysaccharides) on mineral dissolution rates clearly indicates that polymer composition, functionality, and thermodynamic properties may play a significant role in the mineral weathering process (29). A number of polymers in this study had no effect on overall feldspar dissolution rates even up to concentrations sufficiently high (1 g/L) to change the viscosity of the solution. Alginate and polyaspartate, which are both charged polymers under the experimental conditions used, however, inhibited feldspar dissolution at the highest concentrations. In the case of poly-aspartate, only the solutions containing a high–molecular weight (26kD) form inhibited dissolution; a lower–molecular weight form (6kD) had no measurable effect on dissolution rates. An additional study of the effect of polymers on feldspar dissolution (30) demonstrated that acidic polymers could either enhance or inhibit dissolution rates, depending on pH and the degree of protonation of the acidic functional groups. At neutral pH, these polymers irreversibly adsorbed to the dissolving mineral and thus inhibited dissolution. However, these same polymers greatly enhanced dissolution rates under acidic conditions (Fig. 2). These authors further concluded that the orientation of the functional groups and therefore polymer structure in solution and on surfaces also influenced the degree of dissolution enhancement or inhibition.

There is some evidence for an effect of ligand/ligand competition, analogous to proton/ligand and hydroxyl/ligand competition, discussed earlier, on feldspar dissolution rates. Huang and Longo (31) found that the addition of oxalate to aqueous catechol solutions at 95 °C and pH = 8.5 reduced the effectiveness of dissolution enhancement compared with catechol alone. Because both ions enhance dissolution on their own but to a different degree, this intermediate level of enhancement suggests that competition for adsorption sites on mineral surfaces may affect the overall dissolution rate in mixtures of ligands such as would be expected to be found in nature. A similar observation was made for competition between gluconate and a bacterial exopolysaccharide (29). Further experiments with mixtures of ligands having differing effects on mineral dissolution rates will be needed to determine the extent to which ligands, protons, and other solutes interact at dissolving mineral surfaces and play competing roles in microbially mediated mineral weathering.

Table 1. Organic Ions That Have Been Shown to Enhance Dissolution Rates Under Some Experimental Conditions. A number of these are found in nature or are produced by bacteria. See (25) and references given therein

Aliphatic Monocarboxylic Anions	*Aliphatic Dicarboxylic Anions*
Acetate	Oxalate
Propionate	Succinate
	Malonate
Aliphatic Tricarboxylic Anions	*Amino Acid Anions*
Citrate	Glycine
	Aspartate
Aromatic Carboxylic Anions	*α-Hydroxy and α-Keto Carboxylic Anions*
Salicylate	Pyruvate
p-Hydroxybenzoate	Gluconate
Protocatechuate	α-Ketoglutarate
Caffeate	Tartarate
Gallate	
Vanillate	
Polymers	*Other Compounds*
Gum Xanthan	Catechol
Alginate	EDTA
Poly-Aspartate	Pyrocatechol Violet
Pectin	

Ligand Concentration

Experiments performed at ligand concentrations well above those observed in nature indicate that the rate of feldspar dissolution increases with increasing ligand concentration (when all other variables are held constant). At concentrations up to 1 to 2 mM, the increase can be approximated by a linear function (Fig. 3a). However, above this concentration, the effect of ligand additions appears to be reduced (Fig. 3b). Stillings and coworkers (32,33) suggest that the number of reactive sites on the mineral surface to which the ligand can adsorb and react limits the impact of ligands. Therefore, the overall effect of ligands on the dissolution process should be best represented by an adsorption isotherm with a dependence on free ligand activity with the overall dissolution rate being dependent on the concentration of adsorbed ligand (Fig. 3c). Although this argument and model appear to

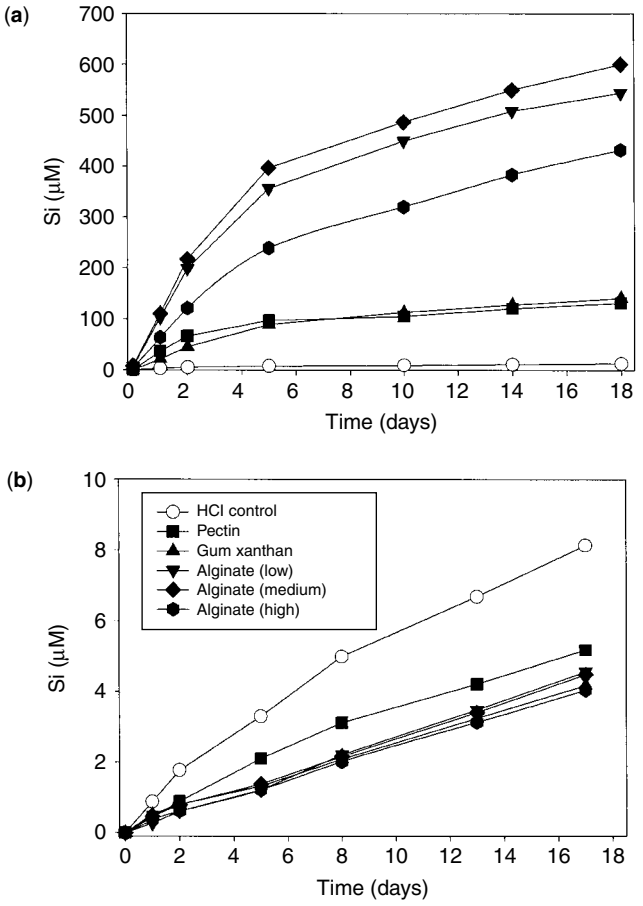

Figure 2. Polymer enhancement and inhibition. A number of the same polymers (pectin, gum xanthan, and alginate of three different molecular weights, low, medium, and high) that enhance feldspar dissolution rates in acidic solutions (**a**) inhibit dissolution at neutral pH (**b**). This difference in polymer impact may be related to changes in polymer charge, adsorption, or conformation with pH. See discussion in the text and in S. A. Welch, W. W. Barker, and J. F. Banfield, *Geochim. Cosmochim. Acta* **63**, 1,405–1,419 (1999).

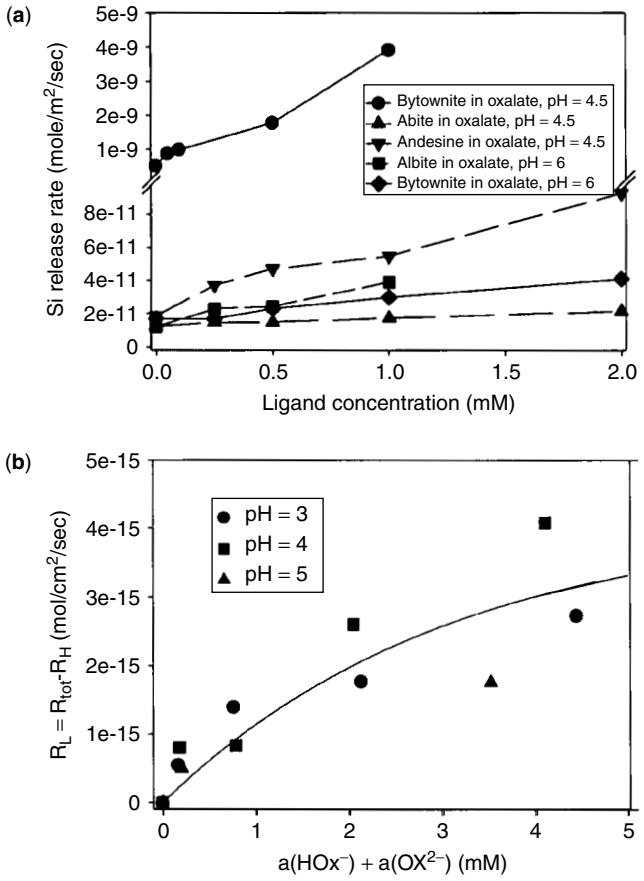

Figure 3. The concentration dependence of ligand-mediated dissolution. (**a**) At low concentration of a dissolution-enhancing ligand, the rate of feldspar dissolution is approximately linearly dependent on total ligand concentration. Examples of a number of experiments involving bytownite ($K_{0.01}Na_{0.23}Ca_{0.77}$), andesine ($K_{0.06}Na_{0.46}Ca_{0.49}$), and albite ($K_{0.09}Na_{0.88}Ca_{0.03}$) are shown. Data from W. J. Ullman and S. A. Welch Water-Rock Interactions, Ore Deposits, and Environmental Geochemistry, A Tribute to David Crerar, in R. Hellmann and S. A. Wood eds., Special Publication 7, The Geochemical Society, St. Louis, Missouri, 2001, (in press). and S. A. Welch and W. J. Ullman, *Geochim. Cosmochim. Acta* **57**, 2,725–2,736 (1993). (**b**) At higher ligand concentration, the dependence of dissolution rate on ligand concentration and activity is nonlinear, perhaps reflecting the saturation of reactive sites on the feldspar surface. (**c**) After taking into account ligand adsorption, the dissolution rate is more linearly proportional to the concentrations of bioxalate (HOx^-) and oxalate (Ox^{2-}) adsorbed on the mineral surface. Data for (**b**) and (**c**) taken from L. L. Stillings et al., *Chem. Geol.* **132**, 79–89 (1996), and L. L. Stillings, J. I. Drever, and S. R. Poulson, *Environ. Sci. Technol.* **32**, 2,856–2,864 (1998).

be well justified on the basis of observation, the linear model is probably sufficient for single ligand or single dominant-ligand experiments, particularly at low ligand concentrations ($\leq$2 mM). The isotherm model may be most useful at higher ligand concentrations and under conditions in which multiple ligands or mixtures of ligands and protons or ligands and hydroxyl groups are reacting with the mineral surface. Under these conditions, this model can incorporate some facets of competition between adsorbates and differences in the effect of adsorbates on dissolution rate. The ability of dissolved ligands to saturate the available reactive sites at the mineral surface is consistent with the idea that mineral dissolution rates are limited by the competition between different mechanisms of dissolution for a limited number of reactive surface sites. These results further suggest that the overall effect of bacteria on mineral dissolution rates in experimental or natural settings should not be a simple linear function of either bacterial numbers or microbial activity except, perhaps, at low abundances and activity.

Temperature

A large number of experiments have demonstrated that the rates of proton-mediated mineral dissolution increase with increasing temperature (34). Within the feldspar group, the temperature dependence of these reactions varies substantially with chemical composition, experimental methods, and pH. The assignment of Arrhenius activation energies, a commonly used exponential

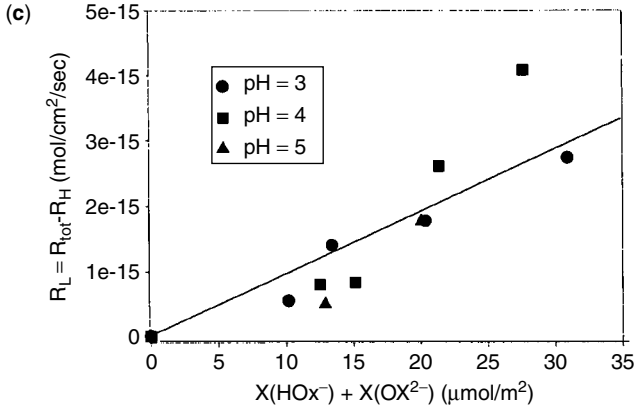

Figure 3. (*Continued*)

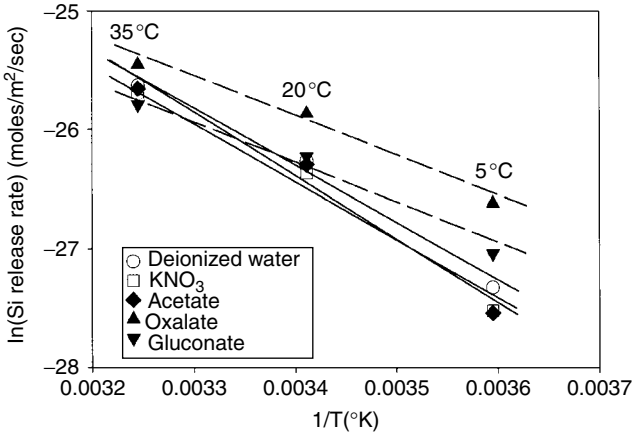

Figure 4. The temperature dependence of ligand-mediated dissolution of bytownite feldspar ($K_{0.01}Na_{0.23}Ca_{0.77}$) isclearly different for ligands that form strong complexes with aluminum in solution and presumably at the mineral surface (oxalate and gluconate; - - -) than for ligands and ions that do not form strong complexes (acetate, deionized water, and KNO3; ——). The apparent activation energy of ligand-mediated dissolution is approximately 3 Kcal/mol less than for the controls, indicating that ligand-mediated dissolution proceeds by a mechanism of dissolution distinct from proton-mediated dissolution. Experimental data from S. A. Welch and W. J. Ullman, *Chem. Geol.* **167**, 337–354 (2000).

expression for the temperature dependence of geochemical reactions, also depends on the kinetic interpretation of the reaction mechanism (34). In one experiment, the temperature dependence of ligand-mediated dissolution at low temperature (5 to 35 °C) and near-neutral pH (and using a pH-independent kinetic expression consistent with the experimental conditions) was evaluated (35). In this experiment, the temperature dependence of dissolution in solutions of acetate, an organic ligand that forms only weak complexes with Al^{3+} in solution and therefore, by analogy, with the Al sites at the feldspar surface, was found to be indistinguishable from that of distilled water and KNO_3 controls. The activation energy of dissolution in the presence of oxalate and gluconate, polyfunctional ligands that form stronger bidentate complexes with Al^{3+}, was significantly less (Fig. 4), clearly indicating the additional catalytic role that ligand mediation plays in the dissolution process under these conditions. What is surprising about these experiments is that the relative enhancement of dissolution due to bacteria and their metabolites was greater at the lower temperature. No similar experiments comparing the impact of ligand- and proton-mediated mechanisms on the temperature dependence of the dissolution of other rock-forming mineral are available, but the expectation is that the same trends will be observed for other mineral systems but with differing absolute values of the activation energies. Additional experiments are needed to determine the interplay between proton-, hydroxyl-, and ligand- mediated mechanisms on the temperature dependence of weathering reactions, under conditions relevant to the weathering environment, to predict the effect of microorganisms on mineral weathering at different temperatures and under different climatic regimes.

Mineral Composition

There are a number of independent lines of evidence that both protons and ligands attack the dissolving feldspar at the limited number of aluminum sites at the mineral surface. These include the correlation of the effectiveness of ligands in ligand-mediated dissolution and the stability of the Al-ligand complexes in solution (discussed earlier). In addition, the rates of dissolution of the calcium-aluminium-rich members of the feldspar series are greater than for the sodium-silicon-rich (Al-poor) members of the series for both proton-mediated and ligand-mediated dissolution (36,37). Furthermore, these observations are consistent with the observations of Goldich (38) that the persistence of feldspars in the soil environment is based on cationic dominance, following the stability sequence K > Na > Na(Ca) > Ca(Na) > Ca. Goldich concluded that feldspar persistence in weathering environments was the result of the thermodynamic stabilities of the minerals in this sequence, but the same result can be obtained independently on the basis of the composition-dependent kinetics of feldspar dissolution rates. Mineral structure and composition can also play a role in the temperature dependence of dissolution. For other classes of minerals, the effect of mineral structure on dissolution rates may dominate over compositional differences.

Biotic Experiments

There are substantially fewer mineral dissolution experiments that have been performed with live and metabolically active cultures than with abiotic solutions mimicking those found in these cultures. As in the case of the abiotic experiments, it is often necessary to perform these experiments under biological and chemical conditions very different than those expected in nature. As a result, these experiments do not alone constitute proof that microbially mediated weathering reactions are important in nature. Typically, higher concentrations of carbon substrate and nutrients are needed to keep cultures alive in the laboratory and to achieve significant experimental results. The microbial population in experimental cultures may also be substantially different than would be expected in nature in

terms of numbers, diversity, and metabolic activity. However, these experiments do provide additional evidence that microbial processes may be important in the weathering process and they give some indication of conditions under which microbial mediation of weathering reactions may be most important in natural settings.

In biotic experiments, it is often difficult or impossible to perform experiments under fully controlled conditions. Thus, the results of these experiments are most often of the qualitative or semiquantitative type. Problems associated with the simultaneous maintenance of both constant microbial numbers and activity as well as constant mineral dissolution rates have often restricted the types and length of experiments that can be performed. Even in relatively short experiments, the microbial culture may respond to the changing geochemical or biogeochemical conditions in the reactor and on the mineral surfaces (associated with dissolution) leading to changes in microbial abundance and metabolic activity. It is difficult to replicate these changes under controlled abiotic conditions or in the absence of mineral substrata. In longer experiments, the culture may also diverge from the original inoculum in both species composition and metabolic activity.

The earliest experiments in which microorganisms were cultured with minerals used a series of acid, oxalate, citrate, and other organic-acid-producing fungal strains isolated from rock surfaces and weathered stones (39). A number of mineral substrata, soils, and soil components were used in these experiments. In all cases examined, ions found in the mineral were released to the culture to a greater extent than in abiotic controls or biotic controls without minerals. The authors attributed this result to acid rather than ligand production following glucose metabolism by their experimental organisms. Subsequent work by this group demonstrated that bacterially produced gluconic acid could similarly solubilize mineral components (40), but there was no basis to assign this effect to proton- or the alternative ligand-mediated reactions. A number of similar qualitative experiments have been reported, principally in the soil science literature, since these earliest efforts. These clearly indicate that microbial processes can affect the solubility and dissolution rates of minerals, rocks, and soil substrata but do not clearly indicate the mechanism by which bacteria and fungi accomplish these results.

More recent experiments have attempted to more quantitatively describe the effect of bacteria and microbial metabolism on mineral dissolution, but many of these suffer from some of the same problems as the earlier qualitative experiments. Vandevivere and coworkers (41) surveyed a range of subsurface bacteria and noted that a number of strains significantly enhanced dissolution relative to bacteria-free controls in glucose-rich/nutrient-poor cultures. Although there was a significant decrease in pH in many of the cultures that enhanced mineral dissolution rates, there were some cultures that enhanced dissolution without acid production. Increases in gluconate, lactate, formate, and some unidentified ketoacids were found in the supranatant at the end of the experiments with acid-producing cultures. In one time-series experiment, microbial abundance, decrease in pH, and increase in gluconate concentration either preceded or co-occurred with the increase in mineral dissolution rate. Studies of building weathering (42) and experiments in which pH variations were minimized by automatic titration (43) similarly demonstrate that enhanced weathering of natural materials by bacteria (and fungi) can occur at neutral pH by the ligand-mediated mechanism.

Welch and Ullman (44) demonstrated that the degree of dissolution enhancement was related to the amount of gluconate production and the subsequent rate of gluconate utilization by a bacterium, and therefore, a function of temperature. Because the temperature dependence of gluconate production from the glucose substrate was larger than either that for gluconate consumption or mineral dissolution, the gluconate concentration attained higher levels at lower temperatures (5 °C) than at higher temperatures (35 °C). The overall rates of gluconate-mediated mineral dissolution were unexpectedly higher at these lower temperatures. In this experiment, however, higher gluconate concentrations co-occurred with lower pHs in the experiments at 5 °C (the buffering due to mineral dissolution was less than the rate of microbial acid production) and therefore it was not clear whether the enhancement of weathering was due to proton- or ligand-mediated processes, or both. At 35 °C, no acid or gluconate accumulated in the experimental cultures as gluconate was consumed (respired) as fast as it was produced and proton consumption by mineral dissolution kept up with metabolic acid production. As a result, the microbial effect on dissolution rate was negligible at 35 °C. Although these results are surprising, similar observations of higher accumulation of volatile fatty acids at lower temperatures have been observed in other settings (45). These results further suggest that microbially mediated weathering may be relatively more important in cold environments than previously thought and there is evidence of high rates of mineral weathering in polar regions (46,47).

Direct Observations

Some of the most interesting and direct evidence for the enhancement of weathering rates and alteration of weathering mechanisms by microorganisms and their metabolites comes from the direct imaging by light and/or electron microscopy and, more recently, by other microanalytical techniques (48,49) of natural and synthetic surfaces colonized by bacteria (Fig. 5). These surfaces may be collected from the field or produced in laboratory experiments; the results from laboratory experiments are subject to the same caveats as for the abiotic and biotic experiments described earlier. The contrasting textures found on surfaces colonized by bacteria and those found on bacteria-free surfaces, however, provide direct evidence of microbially enhanced weathering. Moreover, differences in the chemistry, mineralogy, and textures at these surfaces indicate that bacteria can both moderate the rates and modify the mechanisms of the weathering process.

Callot and others (50) found that two fungi enhanced the rates of etching of preformed microfractures in olivine ($Fe_xMg_{(1-x)}SiO_4$) and a borosilicate waste glass. In contrast to the abiotic controls, the fungal cultures

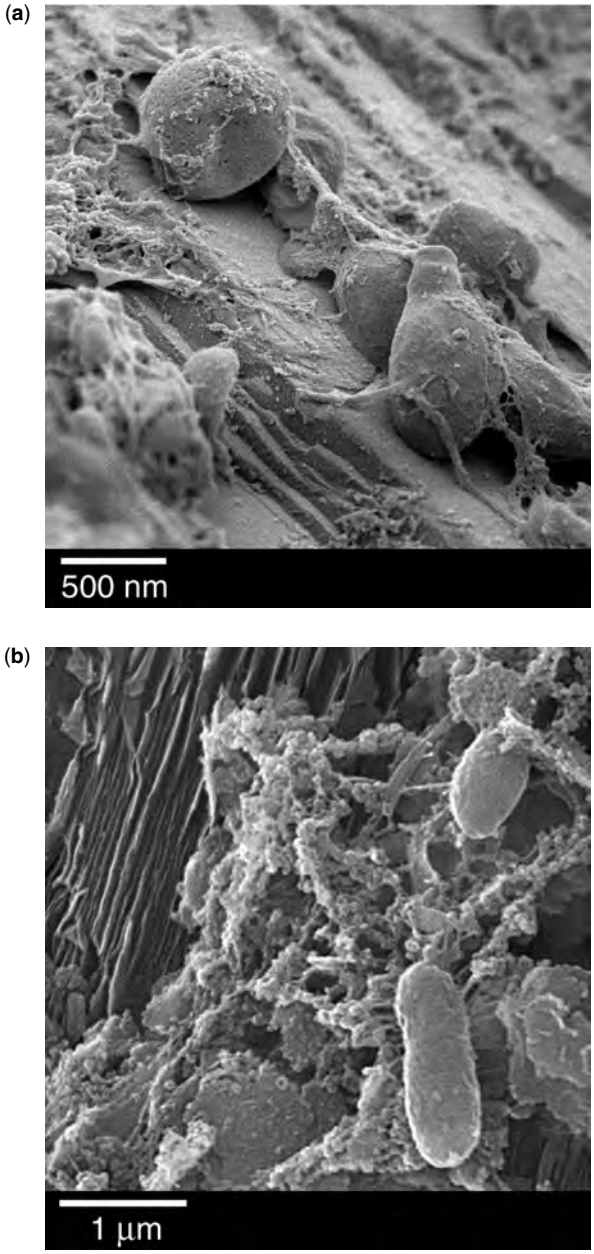

Figure 5. Bacteria and polysaccharides on feldspar surfaces. (**a**) Bacteria on a bytownite feldspar ($K_{0.01}Na_{0.23}Ca_{0.77}$) surface incubated in the laboratory showing bacteria oriented along a cleavage step. The cleavage faces appear rough and the cleavage steps are rounded indicating that dissolution has occurred on these surfaces adjacent to the attached bacteria. Image taken by W. W. Barker, University of Wisconsin. See (23) for the techniques used. (**b**) Bacteria on an anorthoclase feldspar incubated in the field. Striations on the upper left of the image are indicative of crystallographically controlled dissolution of the mineral adjacent to the attached bacteria. Image taken by J. R. Rogers, University of Texas, Austin. See (10) for the techniques used.

enhanced the rates of etching of the microfractures and did not leave a remnant leached-layer on the olivine and only a thin layer on the glass. These cultures also etched the olivine to a greater extent than the glass. In one experiment, one of the fungi was capable of producing siderophores, which strongly complex iron and make it more soluble and bioavailable. This fungus also significantly increased the rate of etching of a normally resistant fused-silica glass that contained no iron. These authors suggested that the increases in etching rate are not related to the production and haphazard excretion of organic acids, but rather that compounds were produced by enzymatic processes in the cell membrane for the express purpose of dissolving the mineral and that contact between the surface and the cell membrane was therefore required for increases in etching to occur. They further suggested that these organisms benefited ecologically by acquiring nutrition from the weathering process. These authors also identified a siderobacterium in a glacial melt-water pond that increased olivine-etching rates in this setting in the same fashion as their experimental fungi. It is intriguing to speculate that the differing temperature dependence of siderophore production and consumption at low temperature could combine to give particularly high levels of these dissolution-enhancing compounds and therefore mineral weathering rates in polar settings. This would be analogous to the high rates of gluconate-mediated dissolution at low temperature discussed earlier. Siderophores have also been implicated as important contributors to mineral weathering on the basis of observations made in a petroleum-contaminated field setting (51) and in the laboratory (52).

On the basis of a study of the naturally weathered basaltic glass, Thorseth and others (53) suggested that the variability in the observed textures and chemistry of the glass surface could only be explained by the existence of a range of chemical microenvironments on the weathering surface. On the basis of observations of cyanobacteria near the surface of the weathering basalt, they concluded that microbial mediation of weathering occurred on these surfaces through proton- and hydroxyl-mediation. The possibility of ligand mediation was not considered. Further experimental work by this group (54) demonstrated that textures and chemistries similar to those found in the previous observational study could develop in relatively short periods (400 days) in an evolving consortium of bacteria grown in an enriched nutrient medium. Thorseth and coworkers have also reported the existence of bacteria deep in basalts in oceanic settings and have inferred, based on of textural and chemical evidence, that microbial weathering of basalt plays a fundamental role in global-scale elemental cycling (55,56). Staudigel and others (57) have shown that substantial biofilms can form on glass surfaces exposed to natural seawater, even in the absence of high-nutrient and substrate levels, and that leaching of glass components from under these films is substantially higher than on biofilm-free surfaces under similar conditions. Staudigel also suggests that microbially mediated weathering of basalts, in which many trace chemical species are at substantially higher concentrations than in seawater, may control the oceanic cycles of a number of trace elements.

Bennett and coworkers have pioneered the use of in situ mineral microcosms to study the influence of bacteria and

microbial metabolism on mineral dissolution in organic-rich groundwater (10,58,59). In this type of experiment, well-characterized mineral and glass samples are inserted into aquifers or zones in aquifers having contrasting water chemistries and biological properties. After a period of time (months to years), these samples are recovered for the recharacterization of the surface and other analyses. This research group demonstrated that mineral dissolution occurred on surfaces even when the bulk groundwater solution was apparently saturated or supersaturated with respect to the dissolving mineral. This result is strong proof that the conditions in the reaction zone under and around bacteria attached to mineral surfaces are significantly different from the bulk solution and that, as suggested by Thorseth and coworkers, weathering occurs primarily in such microenvironments. This group also found that bacteria preferentially colonize and weather some surfaces compared to others, leading to an apparent reversal in the normal (Goldich) sequence of mineral stabilities in weathering environments (see previous text). On the basis of these results, they concluded that the bacteria are benefiting ecologically from the preferential attack of certain surfaces from which they derive needed nutrients (phosphorus, nitrogen, potassium, and/or iron) from the dissolving rock. The mobilization of nutrient elements by mineral dissolution may benefit not only the bacterium able to enhance dissolution rates, but also the whole microbial consortium, particularly in substrate-rich, nutrient-poor groundwater ecosystems in which Bennett and coworkers have worked.

In a high-resolution study of the natural weathering of rocks by lichens, Barker and Banfield (60) found that a polysaccharide gel between the lichen and mineral surfaces played a significant role in mineral leaching and the types of secondary minerals found after dissolution. Leaching did not require direct cell-to-mineral contact as suggested by Callot and others (see earlier discussion). Gradients in the composition and function of the polysaccharide layers with distance from the mineral surface contribute to the patterns of primary dissolution, ionic transport, secondary- product formation under lichen-covered surfaces. On the basis of these and subsequent analyses of the same rock, Barker and Banfield (61) suggested a layered model of the effect of lichens on the weathering of mineral surfaces, which may equally apply to the more general case of weathering by any surface-bound microorganism. In this model (Fig. 6), the production of protons and ligands takes place in a microorganism, which is attached to a mineral surface through a layer of exopolymer. This polymer layer reacts with the surface and secondary mineral phases are precipitated. Below this zone of contact, a third zone is found where the interstitial

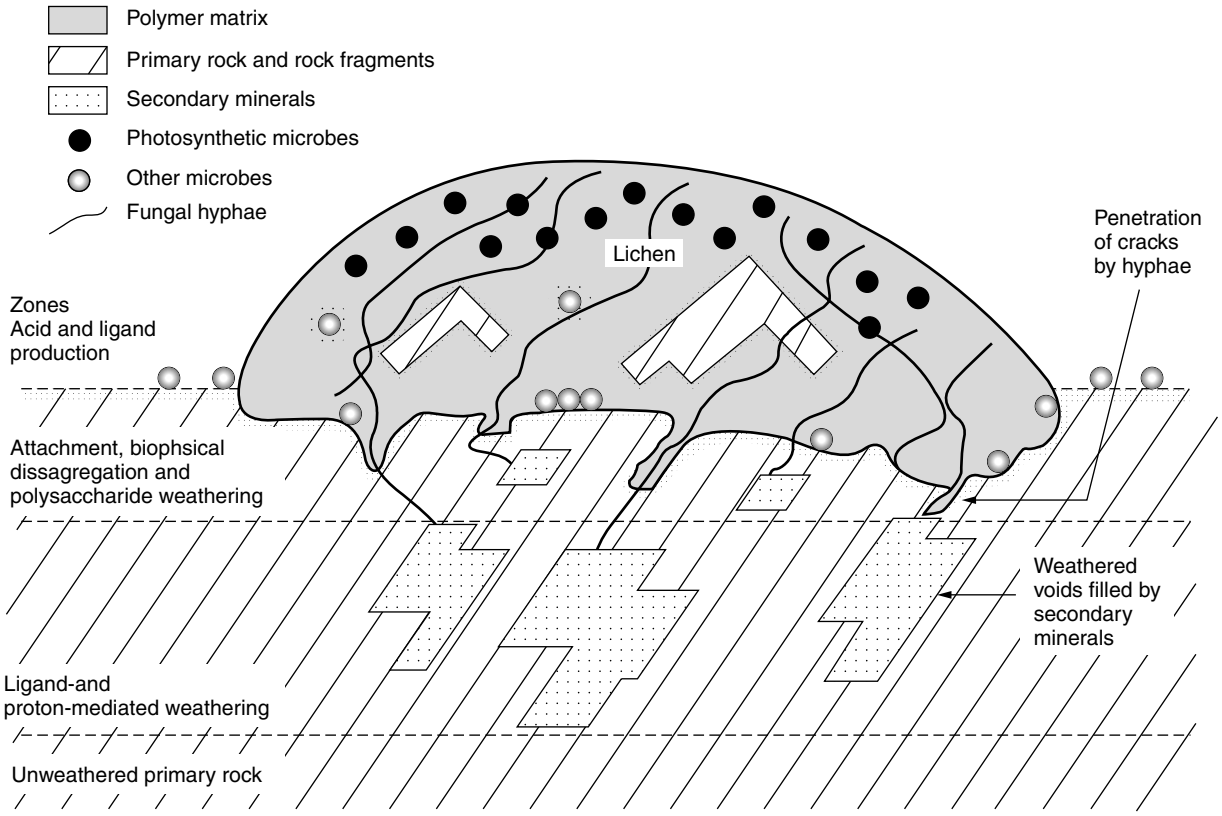

Figure 6. The layered model of Barker and Banfield (61) describing the characteristics of rock weathering by lichens. Although developed to describe the specific characteristics of lichen-mediated weathering, this model could be easily modified and expanded to three-dimensions to encompass the geometry of mineral weathering by other types of attached microorganisms over a range of field conditions.

pore sizes are insufficient to allow organisms or polysaccharides to penetrate, but where some of the soluble acid and ligands produced by the organisms in the upper layer contribute to ligand- and proton-mediated dissolution of the primary mineral. In this zone, secondary products may also be formed but below it the rock is essentially unweathered. In this model, the boundaries between these reaction zones are gradational in character, reflecting the continuity of geochemical and biochemical gradients in which these microbially mediated reactions occur. Although this model is described in a one-dimensional fashion, a similar three-dimensional model of the interfaces and interactions between patches of organisms and mineral surfaces may be more appropriate, given the observed variations in weathering textures and chemistry on two-dimensional surfaces.

GLOBAL GEOCHEMISTRY AND MICROBIALLY MEDIATED WEATHERING

The earlier evidence of the possible mechanisms of microbial mediation of mineral and rock weathering, the ability of these mechanisms to either enhance or inhibit the weathering process, and observational studies of weathered surfaces from the field and from laboratory experiments, document the importance of microbes and microbial metabolism on the weathering cycle. The weathering cycle plays an important role in producing and maintaining the climate and habitability of the earth-surface environment. Thus, microbial processes can be viewed as part of the feedback mechanism by which the regulation of global geochemical cycles occurs, particularly over geologic timescales. It is, therefore, instructive to briefly review the possible influence of microbial processes on two important and interrelated consequences of the global weathering cycle, the regulation of the global greenhouse effect, and the formation and maintenance of soils.

The regulation of atmospheric carbon dioxide concentrations and global climate is related to the weathering of calcium-, magnesium- and aluminum silicate minerals by the following geochemical reactions steps:

$$(Ca,Mg)SiO_3(s) + CO_2(g) + 2H_2O(l) \rightarrow (Ca,Mg)^{2+}(aq) + HCO_3^-(aq) + H_3SiO_4^-(aq) \quad (3)$$

$$(Ca,Mg)^{2+}(aq) + 2HCO_3^-(aq) \rightarrow (Ca,Mg)CO_3(s) + CO_2(g) + H_2O(l) \quad (4)$$

$$CO_2(g) + H_2O(l) \rightarrow H_2CO_3 \rightarrow 2HCO_3^-(aq) + H^+(aq) \quad (5)$$

$$H_3SiO_4^-(aq) + H^+(aq) \rightarrow SiO_2(s) + 2H_2O(l) \quad (6)$$

The overall reaction is often given as:

$$(Ca,Mg)SiO_3(s) + CO_2(g) \rightarrow (Ca,Mg)CO_3(s) + SiO_2(s) \quad (7)$$

In these reactions, $(Ca,Mg)SiO_3(s)$ represents the calcium, magnesium, and silicon components of primary rock-forming minerals (both silicate and aluminosilicate) subject to weathering on land surfaces (Reactions 3 and 7). $(Ca,Mg)CO_3(s)$ (Reactions 4 and 7) and $SiO_2(s)$ (Reactions 6 and 7) represent limestone and silica deposited primarily on the sea floor. The dissolution of limestone on continents (the reverse of Reaction 4) has no effect on the fixation of atmospheric carbon dioxide because Reaction 4 in the ocean removes the same amount of carbon dioxide as is added on the continent.

Reaction 3 is written as a proton-mediated dissolution reaction. However, any reaction that can dissolve a primary calcium-, magnesium-, or aluminosilicate mineral, or that can enhance mineral dissolution rates, can contribute to this process and therefore participate in the regulation of atmospheric composition and global climate. For example, the ligand-mediated dissolution of our model calcium silicate, magnesium silicate, or aluminum silicate mineral is fundamentally identical to Reaction 3 (using oxalate, $(COO)_2^{2-}$, as an example ligand):

$$(Ca,Mg)SiO_3(s) + 2H_2(COO)_2(aq) + H_2O \rightarrow (Ca,Mg)^{2+}(aq) + H(COO)_2^-(aq) + H_3SiO_4^-(aq) \quad (8)$$

when coupled to the biologically mediated reduction of CO_2 and H_2O to form $H_2(COO)_2(aq)$, the protonation and oxidation of $H(COO)_2^-$ (aq) to form CO_2 and H_2O, and Reaction 5. The role of microorganisms in the regulation of climate is therefore related to their contribution to the physical and chemical evolution of soils in which weathering predominantly occurs and their ability to increase the rate of weathering of the primary minerals that participate in carbon dioxide regulation.

In the absence of biological activity early in the evolution of the earth, the primitive "soils" would have consisted of physically and slowly produced proton-mediated weathering products and would have a composition similar to that of the primary rocks. Clays may have been present as a result of the abiotic weathering of the more reactive primary minerals. The pH in these soils would have been dependent on the concentration of atmospheric carbon dioxide, the environmental temperature, and the flux of rainwater through the soils. Therefore, the rates of weathering would have been substantially slower than at present and, as a result, atmospheric carbon dioxide levels would have been substantially higher in the early atmosphere than today (62). The retention of heat in the atmosphere due to carbon dioxide and water would have served to produce an environment warmer than today despite the lower solar heat flux during this period (63). The climate of the steady-state Earth during this period would have been substantially different from that subsequent to the microbial colonization of the continents, the development of soils, and the evolution of higher plants.

The initial colonization by autotrophic and heterotrophic microorganisms would have substantially altered the physics and chemistry of the soil, leading to substantial changes in weathering and carbon dioxide consumption rates and ultimately to a terrestrial climate more like the present. A number of mechanisms and factors could have contributed to the change in weathering patterns (64). These include:

- Residual organic matter from autotrophy would have allowed the soil to better maintain water levels adjacent to reactive mineral surfaces;

- The combination of autotrophy and heterotrophy would have transferred carbon dioxide from the atmosphere to the soil zone leading to a decreased soil pH and increased rates of proton-mediated respiration;
- Microbial production of organic acids and organic ligands would have allowed for ligand-mediated weathering even in the neutral pH range, leading to overall higher weathering rates;
- Microorganisms could have reduced pH to levels lower in the soil zone (by the oxidation of reduced compounds) than those resulting from carbon dioxide equilibria leading to even higher rates of weathering than could be otherwise sustained;
- The physical weathering of the primary rocks, leading to the exposure of new and reactive mineral surfaces for subsequent chemical weathering would have been increased because of freeze-thaw cycles and aided by microbial water retention and, ultimately, by the action of roots and fungal structures; and
- The physical stabilization of the soil by microorganisms and plants would allow sufficient time for the soil components to react with the available solutions.

The initial colonization of soils would have selected for a class of microorganisms that could survive in these primitive soils. These organisms would have had to be able to maintain their water content and to acquire nutrients from the soil material, by physical and chemical extraction. The primitive autotrophs would have had to extract the required nutrients from the mineral solids to produce organic material. Subsequently, organic compounds would have been excreted and respired to enhance the nutrient extraction efficiency, accelerate the rates of physical weathering and fluid transport through the soil zone, and stabilize the soil surface to inhibit further erosion. Thus, it is reasonable to conclude that the inhospitable terrestrial environment would have selected species that were capable of efficiently dissolving minerals and rocks. Microbial mediated weathering, therefore, should be regarded as an important common characteristic of this early terrestrial environment and we should therefore not be surprised that this characteristic ability of microorganisms remains common in the modern terrestrial ecosystem.

The primitive-soil organisms would have added organic matter to the soil, or at least to the surface of the soil, and by increasing the rate of clay production, would have contributed significantly to the ability of the soil to maintain its water content and solutes. This primitive soil would subsequently be able to support increased heterotrophic activity and the activity of larger plants such as those that appeared in the fossil record in the Silurian period and expanded rapidly by Carboniferous time. With colonization by these rooted plants would have come the deepening of the soil layer, the penetration of micro- and, eventually, macroheterotrophs into the soil zone, and the downward development of more modern soil structure. The high rates of organic matter production by the larger plants would have contributed to the higher levels of heterotrophic activity in soils and the maintenance of inorganic and organic acidity necessary for the continued extraction of nutrients and production of mineral particles to maintain soil productivity (65,66).

Thus, it is clear that microbially mediated weathering reactions do not reflect a new biogeochemical process, but rather are among the oldest and most fundamental of biogeochemical processes on the terrestrial surface where most weathering occurs. The role of microorganisms in the development and maintenance of soils is a fundamental geochemical process of global and paleohistorical consequence and remains as important today as in paleohistory.

CONCLUSION

Microorganisms and their metabolic activity can enhance mineral weathering rates and have played an important role in the global weathering cycle and climate moderation throughout geologic time. They accomplish this by a combination of mechanisms that include the regulation of pH and the production of complexing organic ligands in the soil zone, where most weathering occurs. The degree of enhancement of dissolution, the initial step in the sequence of weathering reactions, is dependent on the chemical composition and structure of the mineral substratum, pH, ligand composition and concentration, and temperature. Different primary reactants can affect dissolution rates in different ways, leading to either overall enhancement or inhibition of mineral weathering rates. For aluminum silicate minerals, such as feldspars, the affinity of protons and ligands for a limited number of aluminum sites on the mineral surface leads to a competition between reactive solutes and, therefore, mechanisms of dissolution. Mineral weathering can proceed by still other mechanisms including redox reactions and direct attack at other types of surface sites, in the absence of aluminum. Direct contact between microorganisms and the mineral surface, does not seem to be required for enhanced dissolution rates. However, the attachment of bacteria to surfaces and the formation of microbial microenvironments may create conditions that lead to much higher rates of mineral weathering than would be expected by the composition of the bulk interstitial solutions in soils and aquifers. The distribution of such microenvironments leads to the patchiness that has been observed on weathered surfaces of many types.

Microbially mediated weathering is not an anomalous process. Rather, it represents a fundamental geochemical process that responds to evolving environmental conditions, that evolved early in the history of the earth, and that continues to play an important role in the maintenance of the global environment.

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WETLANDS. See FLOODED SOILS

WETLANDS AND READBEDS FOR WASTEWATER TREATMENT

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Treatment wetlands are a class of natural systems for pollution control. Wetlands are ecologically intermediate between terrestrial and aquatic environments. They therefore occupy a treatment niche between land-based systems, such as infiltration and overland flow, and pond systems, such as facultative lagoons. Although wetlands share characteristics of these wetter and drier counterparts, they also possess unique features of their own. Constructed wastewater treatment wetlands imitate many of the characteristics of natural wetlands, but are designed and focused on water quality improvement as the main goal. Other wetland values, of aquaculture, flood protection, groundwater recharge, and wildlife habitat, are unavoidably present, but less important (1).

All variants of constructed treatment wetlands share common features. A shallow excavated basin, about one meter deep, serves to contain the wetland waters, soils, and biota (2,3) (Fig. 1). The berms of the wetland are formed out of removed soil. An inlet distribution system conveys the wastewater to the basin, and provides for uniform and complete distribution of the water to all portions of the inlet zone. At the outlet end of the wetland, uniform collection is provided, together with water level control and conveyance to the downstream recipient. The receiving water may be surface or groundwater, or another component of the treatment system. The interior of the basin is filled with a layer of either soil, or a porous sand or gravel media. Appropriate wetland plants are seeded or transplanted into the media, and allowed to develop to a mature ecosystem (1,4).

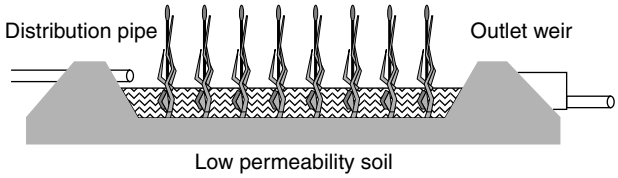

Figure 1. Treatment wetland schematic. See color insert.

Free water surface (FWS) systems (Fig. 2) are patterned after many types of natural wetlands (1,3). The soils used as initial rooting media are most often local topsoils, as a result of ready availability. The desire to maximize the treatment potential of a given land area leads to establishment of a continuously flooded and flowing water regime. Water depths are typically set within the range of 10 to 50 centimeter, which corresponds to the water tolerance of many emergent wetland macrophytes. Floating, submerged, or emergent vegetation may be initially emphasized, but some degree of natural invasion is inevitable (1). Constructed treatment wetlands are most frequently planted with tough emergent macrophytes, such as reeds (*Phragmites* spp.), bulrushes (*Schoenoplectus* spp.), and cattails (*Typha* spp.) because these species are tolerant of a wide variety of water chemistry and hydrology. However, the new wetland is often rapidly colonized by floating plants, such as duckweed (*Lemna* spp.), and by several forms of attached and free microscopic algae. Detritus from dead plant and animal parts forms after a growing season, and become substrates for decomposers. Very large and diverse microbial populations develop, principally on emersed surfaces of live and dead biomass, and sediments, and soils (1,3).

Subsurface flow (SSF) treatment wetlands are based on a water-saturated bed of porous media, for which there is no true analog in the realm of natural wetlands (4,5). A variety of soils, sands, and gravels may be employed, in either vertical or horizontal flow (Fig. 3). The plants are emergent macrophytes, of the same species used in FWS wetlands. In Europe, the plant of choice is the common reed (*Phragmites australis*), and the systems are called reed beds (4,5). The purposes of the media are basically twofold: a microbial substrate for treatment and prevention of exposure to the contaminated water. A portion of the underwater pore space becomes colonized with biofilms that perform many of the observed treatment functions. Comparison of Figures 2 and 3 shows that there are many important ecological distinctions between FWS and SSF systems. Water is not directly exposed to air and is out of potential contact with wildlife and humans. Thus, reaeration is poor, but contact health

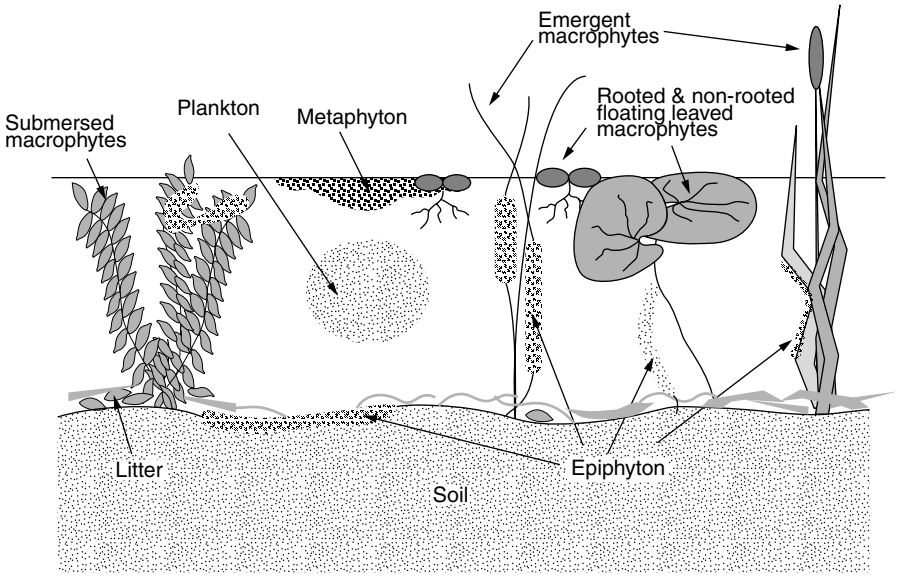

Figure 2. Major macroscopic components of a free water surface wetland.

problems are minimized. Volunteer vegetation is restricted to emergents, which are minimized by poor seedbed conditions. Litter is held above the water column, and decomposition processes have only minimum connection with the belowground water. Vertical intermittent flow may be used with SSF wetlands, thus providing episodic aeration of the drained portions of the bed (4,6).

Treatment wetlands have two unique types of local redox zonation (7). In the macroscopic vertical direction (Fig. 4), the top water layers are often oxygenated (Eh > 300 mv), but there is a sharp demarcation at the sediment–water interface, below which a nitrate reduction zone exists ($300 > Eh > 100$ mv). Below this lie successively more reducing zones: iron reduction ($100 > Eh > -100$ mv), sulfate reduction ($-100 > Eh > -200$ mv), and methanogenesis (Eh < −200 mv). Plants root about 30-cm deep, into one or more of the anaerobic zones, and must possess the ability for self-aeration to survive and respire. Wetland plants use airways (aerenchyma tissues in their stems) to oxygenate the rhizosphere (the immediate microzone surrounding roots and root hairs) and to vent gaseous waste products (8). Oxygen intake must be enough to support root respiration and to maintain an aerobic microzone adjacent to the root (Fig. 5) (8). The result is that aerobic and anaerobic environments exist in close proximity, allowing diffusion of dissolved constituents to and from these two redox environments.

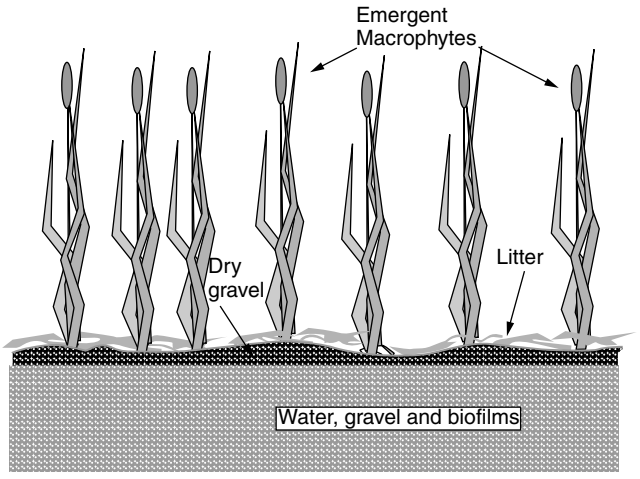

Figure 3. Major macroscopic components of a subsurface flow wetland.

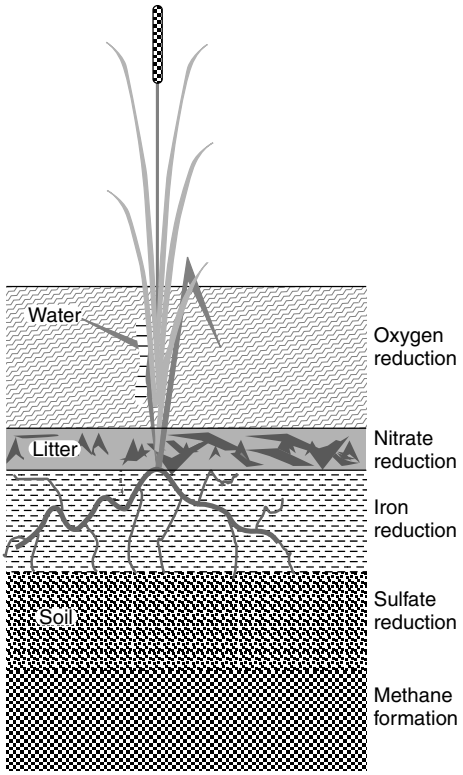

Figure 4. Vertical zonation of a FWS treatment wetland. Redox potential ranges from 300 mv in the water column, to −300 mv in the lower anaerobic zones.

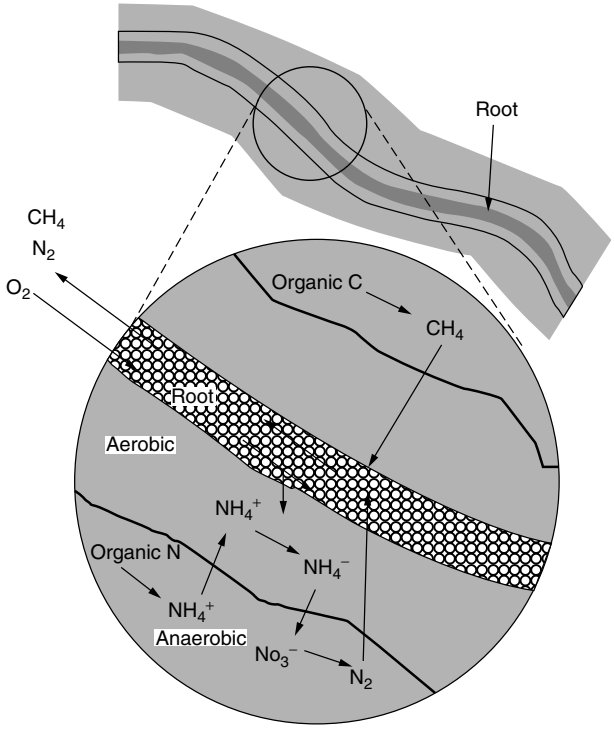

Figure 5. Pathways of carbon and nitrogen transformations in the immediate vicinity of a wetland plant root. (Adapted from Kadlec and Knight, 1996).

A common feature of horizontal flow treatment wetlands is the presence of longitudinal gradients in chemical and biological parameters in the direction of flow. Water flow is often a parallel network of flow paths from inlet to outlet, with varying path transit times (9). As water moves through the system, pollutant reductions and transformations occur, giving rise to longitudinal chemical gradients. Interactions between the vertical, mycorrhizal, and longitudinal spatial scales are provided by diffusional transport, and more importantly by transpiration water flows (10). Above water plant parts transpire water, which is extracted from the root zone, to the air to maintain their thermal balance. Pressure forced flow replenishes the root zone from adjacent and overlying waters.

Seasonal variations are present in many wetland parameters, including physical, chemical, and biological parameters. Water temperature has a critical influence on microbial processes. Solar radiation is a strong determinant of photosynthetic activity. When these environmental variables are combined with the spatial complexity, the resulting effects are beyond simple intuitive understanding. Mechanistic process models may be envisioned and coded, but the calibration data requirements cannot be met. Thus, the present state of performance modeling is limited to removal calculations, with enhancements for spatial and temporal variation, hydrology, and meteorology (1).

MICROBIALLY MEDIATED POLLUTANT REMOVAL

The wide variety of wetland microbial processes provides a unique environment for modifying water quality. Typically, compounds of carbon, nitrogen, sulfur, and iron are altered in the passage of water through wetlands because these are all intimately involved in biogeochemical cycling. The typical end-products of carbon processing are carbon dioxide, water, and methane.

Organic Matter Degradation

The carbon content of the incoming water is usually characterized by carbonaceous biochemical oxygen demand (CBOD), total organic carbon (TOC) or chemical oxygen demand (COD). But there are also both solid and water phase carbon sources in wetlands, in the form of decaying litter and sediments, and soluble hydrocarbons, including many types of organics. Aerobic zones in the wetland contain microbial populations that convert organic matter to carbon dioxide, water, ammonia, and other end-products. The wetland environment contains both aerobic and anaerobic zones, in varying proportions for different treatment wetland types. Methanogenesis has been identified as a major route for CBOD reduction for primary effluents fed to SSF wetlands but not for secondary effluents (11). However, soils of FWS wetlands have been shown to possess potential methane production rates that could consume all CBOD for secondary wastewaters (12).

Microbial populations in wetlands vary seasonally, and with vegetation type (13). Organic compounds in treatment wetlands originate from two sources: added wastewater and internal cycling of biomass, and both may be of importance to the observed performance of the wetland for carbon reduction. Specific organic compounds are oxidized by only a fraction of the aerobic consortia. For instance, only 3 of 30 studied strains of bacteria were found to grow in a glycol-rich environment, but both surface and subsurface flow wetlands remove glycol (14). This and other factors typically contribute to a lag phase in the consumption of individual organic chemicals, such as phenol (15). The result of the complex suite of bacterial carbon processes is the reduction of incoming excesses, down to but not below the natural background created by carbon cycling (1). This background is typically in the range of 5 to 10 mg/L BOD (1). Removal progresses with decreasing rates in the direction of water flow. Extremely wide ranges of BOD have been subjected to wetland treatment, with generally good success (Table 1).

Nitrogen Processing

The nitrogen cycle for wetlands is complex and contains a preponderance of microbially mediated transformations (Fig. 6). The sequential conversions of ammonification, nitrification, and denitrification are all important adjuncts to plant uptake and litter decomposition, the last also being bacterially controlled (1). The natural wetland nitrogen cycle is often shifted to different proportions of the sequential processes of Figure 6 by the introduction of excesses of any of the participating nitrogen species. Fixation is not generally observed in nitrogen-rich systems, but treatment wetlands receiving highly treated domestic effluents, including nitrification and denitrification in the upstream processes, may be deficient in ammonia and also possibly nitrate. By inference, fixation must be a component of the nitrogen supply in those wetlands (1).

Table 1. Example BOD Reductions in Treatment Wetlands

System	Type	Hydraulic Loading cm/d	BOD In mg/L	BOD Out mg/L	Reference
Benton, KY	FWS	6.2	26	10	17
Cannon Beach, OR	FWS	2.5	28	7	17
Listowel, ONT	FWS	2	56	9	18
Gustine, CA	FWS	4.2	356	30	17
Oregon State Univ.	FWS	3.9	677	275	17
Mandeville, LA	HSF	34	41	10	17
Hawkesbury, NSW	HSF	5.1	52	6	19
Viborg, Denmark	HSF	6.1	80	8	20
Oaklands Park, UK	VSF	15	246	14	21
Springe-Eldagsen, GR	VSF	2.7	289	8	22

FWS = **free** water surface, **HSF** = **horizontal** subsurface flow, **VSF** = **vertical** subsurface flow.

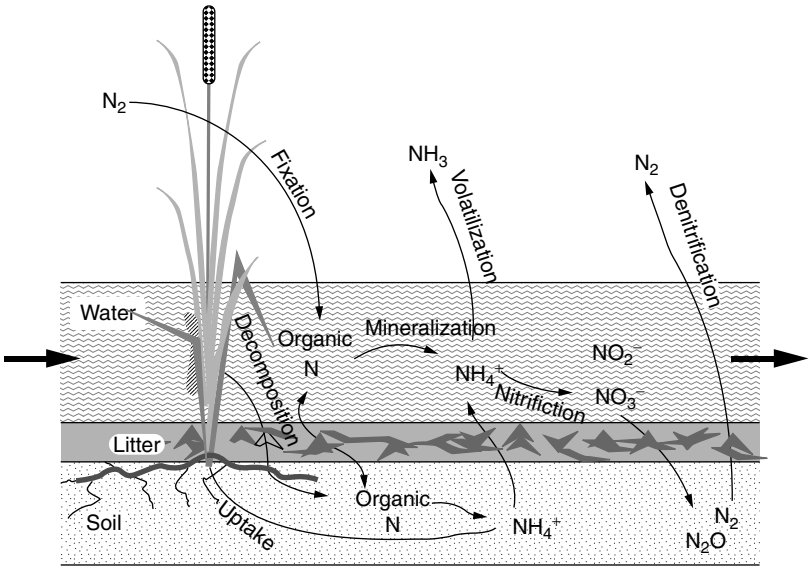

Figure 6. Wetland nitrogen processing. Almost all pathways are microbially mediated.

Mineralization, or ammonification, of organic nitrogen occurs in natural wetlands. For instance, about 50% of the gross mineralization was found to be recycled in a freshwater natural wetland (22). Treatment wetlands that receive large inputs of organic nitrogen produce large amounts of ammonium nitrogen via this pathway. Up to 1,000 gmN/m^2yr have been ammonified in wetlands treating potato wastewater containing 100 mg/L of organic nitrogen (23).

Ammonia reduction is a frequent treatment goal for wetlands. Plant uptake can use ammonia on a seasonal basis, but microbial nitrification is also important (1,4). Theoretically, the microbial reaction requires 4.57 mg of oxygen to nitrify 1.0 mg of NH_4-N, which often exceeds the capability of the wetland environment for aeration of the water column. Nitrification is accompanied by cell synthesis, which utilizes ammonia and reduces the oxygen requirement to 4.3 mgO_2/mgN (24). Wetland data indicate that oxygen transfer limits these processes, especially for horizontal flow reed beds (25). Nitrification competes with respiration for available oxygen, although denitrification supplies some of the joint requirement. Nitrifiers are found in treatment wetland substrates at 10^4 to 10^5 per dry gram in FWS wetlands, and 10^3 to 10^4 in horizontal SSF wetlands (17,26), with lesser numbers in winter.

Denitrification is one of the most efficient wetland processes. The carbon requirement of this reaction is 1.3 gmC/gmN, which must be supplemented by an additional 25% to support cell synthesis. In many treatment wetlands, the carbon source is available in the incoming wastewater (1,4). The wetland environment is ideal for denitrification, because a large renewable carbon source is available from the organic sediments, in an anaerobic zone close to the water column. The decomposition of plant and animal litter can provide up to 10,000 gm/m^2yr of dry detritus, of which about half is carbon that can support denitrification (27). Newly constructed wetlands do not have this source until the biomass cycle fully develops. Organisms are found predominantly on submerged stems and litter, and on the sediment–water interface. Populations in FWS wetlands number approximately 10^6 per gram, with lesser numbers

Table 2. Example Nitrogen Transformations in Treatment Wetlands

		Organic N mg/L	Ammonium mg/L	Nitrate mg/L	Total N mg/L
Washington potato wetland 1	In	91	73	1	165
24	Out	10	129	1	140
New Zealand primary meat	In	6	127	0.0	133
29	Out	1	108	4.4	113
NERCC, MN	In	9.4	74	0.02	83
30	Out	1.2	57	0.09	58
Washington potato wetland 4	In	13	26	43	82
24	Out	12	29	13	54
Grand Lake, MN	In	6.7	49	0.02	56
30	Out	4.2	37	0.02	41
Listowel wetland 4	In	11.8	12.2	0.2	24.2
18	Out	3.1	11.8	0.1	15.0
Phoenix, AZ wetland H2	In	1.48	1.72	2.73	5.91
31	Out	1.02	0.81	1.49	3.32

Grand Lake and NERCC are horizontal subsurface flow; others are free water surface.

in winter (18). Populations develop to larger numbers with continued exposure to oxidized nitrogen.

The end result of the set of nitrogen transformations is a complex removal and respeciation that often prevents simplistic interpretation (1). A wide variety of feed waters produce very different outflow concentrations (Table 2).

Sulfur Processing

Wetlands typically possess a very active, microbially mediated sulfur (S) cycle. Soils may contain up to 0.5% sulfur dry weight, as sulfates, sulfides, and organic and elemental sulfur. Many wastewaters contain abundant sulfates, as does the rain impinging on treatment wetlands. Many interconversions among the four oxidation states of sulfur occur in the different redox zones of wetlands, and most are microbially mediated (31). Depending on the chemical environment, soluble sulfides can form, which are very important in the precipitation of divalent metal cations, such as copper, cadmium, and lead (32). This reduction is favored by warm temperatures, pH > 5, and sulfate greater than about 30 mg/L (33).

Iron Processing

Wetland environments provide appropriate conditions for both iron oxidation and iron reduction. This feature has led to the implementation of hundreds of acid mine drainage treatment wetlands in the United States (34). There are strong interactions between iron processing and sulfur processing within wetlands. Pyrite and elemental sulfur may be oxidized to sulfate by microbial activity. However, the treatment mechanism is most simply described as sulfate reduction producing sulfides that remove dissolved iron to solid iron sulfide minerals (32).

In northern peatlands, ferrous iron in anaerobic groundwater is oxidized to insoluble ferric compounds called bog iron, a commercial ore deposit. Heterotrophic iron-oxidizing bacterial counts reach $> 10^6$ cfu per gram of surface soil in acid mine drainage-treatment wetlands.

Heterotrophic Microbial Activity

Addition of electron acceptors to methanogenic soils causes transition from methanogenesis to utilization of the alternate electron acceptor. In some instances, the transition is slow (days); in other instances it is essentially instantaneous. D'Angelo and Reddy (12) have developed a set of test protocols for wetland soils that assess the rates of various microbial processes under conditions that are not limited by electron acceptor supplies (i.e., NO_3^-, SO_4^{-2}, O_2, Fe^{+2}). Both consumption of the electron acceptor and evolution of CO_2 and CH_4 are measured after stable conditions are achieved. Test results for a northern natural FWS treatment wetland show that microbial consortia exist that can use the various electron acceptors (Table 3). The microbial processes, as assayed in the laboratory, are capable of consuming the incoming BOD, nitrate, and sulfate. Interestingly, ample supplies of either sulfate or ferrous iron in the lab tests do not initially inhibit methanogenesis, but after several days, inhibition is complete (12).

PATHOGEN OCCURRENCE AND REMOVAL

Bacteria

Pathogenic bacteria that enter with wastewater are reduced in treatment wetlands. Mechanisms may include natural die-off, adsorption, ultraviolet radiation kill, chemical toxicity, and grazing by protozoans (35–40). The wetland environment is inimical to many species of pathogenic bacteria, including *Escherichia coli*, *Pseudomonas aeruginosa*, fecal streptococci, and others (Table 4). Both reed beds and free-water wetlands are effective in achieving moderate reductions in numbers, but rarely cause complete elimination. Removals are generally in the range of 0.5 to 3.0 logarithms (base 10), or from 70 to 99.9% (1,4). Inspection of Table 1 shows that there is no difference in this range between FWS and SSF wetlands.

Fecal coliforms are a common indicator group used in regulation of water quality from treatment wetlands,

Table 3. Heterotrophic Activity in a Northern Treatment Peatland at Houghton Lake Michigan

Parameter		Natural Peatland Background Zone	Impacted Peatland Treatment Zone
Soil Parameters			
Total C	mg/gm	432	384
Total N	mg/gm	24.35	25.34
Total P	μg/gm	806	1,643
Bioavailable Fe	mg/gm	4.36	5.08
Microbial biomass C	μg/gm	732	1,164
Bulk density	gm/cc	0.114	0.079
Rates			
Methanogenesis			
Methane C emission	gm/m^{2}d	0.30	0.59
Carbon dioxide C emission	gm/m^{2}d	0.29	0.82
Wetland CBOD carbon loading	gm/m^{2}d		0.09
Oxygen consumption			
Oxygen consumption	gm/m^{2}d	6.09	10.69
Carbon dioxide C emission	gm/m^{2}d	1.55	3.18
Wetland CBOD loading	gm/m^{2}d		0.25
Nitrate reduction			
Nitrate N consumption	gm/m^{2}d	0.27	0.52
Carbon dioxide C emission	gm/m^{2}d	0.82	0.00
Wetland nitrate N loading	gm/m^{2}d		0.02
Sulfate reduction			
Sulfate S consumption	gm/m^{2}d	0.66	2.81
Carbon dioxide C emission	gm/m^{2}d	0.51	0.00
Wetland sulfate S loading	gm/m^{2}d		0.02

The wetland receives aerated lagoon effluent. Rates are for the top ten centimeters of wetland soil, brought to stable conditions in the laboratory (13). Electron acceptor supplies (i.e., NO_3^-, SO_4^{-2}, O_2, Fe^{+2}) were maintained nonlimiting.

Table 4. Removal of Various Pathogenic and Indicator Bacteria in Treatment Wetlands

Site	Pathogen or Indicator	Inlet (#/100ml)	Outlet (#/100ml)	Reduction ($\log_{10}$)	Reference
Free water surface (FWS)					
Carperby, UK	*Escherichia coli*	36,630	14,816	0.4	4
Slingsby, UK	*Escherichia coli*	16,644	2,343	0.9	4
Wetwang, UK	*Escherichia coli*	68,743	12,521	0.7	4
Mpophomeni, SA	*Escherichia coli*	160,000	3,400	1.7	41
Mpophomeni, SA	Fecal Streptococci	37,000	1,300	1.5	41
Cherry Burton, UK	Fecal Streptococci	650	136	0.7	4
Rathermon, Ireland	Fecal Streptococci	23,700	515	1.7	42
Waldo, FL	Fecal Streptococci	60,000	400	2.2	43
Cobalt, ONT	Fecal Streptococci	49	10	0.7	44
Glendale, AZ	Listeria monocytogenes	29,300	20,000	0.2	45
Glendale, AZ	*Clostridium perfringens*	1,630,000	1,310,000	0.1	45
Listowel, ONT	*Clostridium perfringens*	1,036	8	2.1	18
Listowel, ONT	*Clostridium perfringens*	13,148	12	3.1	18
Listowel, ONT	*Pseudomonas aruginosa*	10	2	0.8	18
Listowel, ONT	*Pseudomonas aruginosa*	662	6	2.1	18
Listowel, ONT	*Salmonella* spp.	10	0	1.5	18
Listowel, ONT	*Salmonella* spp.	228	1	2.4	18
Arcata, CA	*Salmonella* spp.	100	5	1.3	46
Listowel, ONT	*Yersinia entercolitica*	4,241	361	1.1	18
Listowel, ONT	*Yersinia entercolitica*	28,602	1,394	1.3	18
Subsurface flow (SSF)					
Holtby, UK	*Escherichia coli*	7,420	2,562	0.5	4
Nun Monkton, UK	*Escherichia coli*	10,046	1,984	0.7	4
Wiedersburg, Ger	*Escherichia coli*	6,000,000	30	5.3	47
Ettenbüttel, Ger.	*Escherichia coli*	70,000	200	3.5	47
Wiedersburg, Ger	Fecal Streptococci	110,000	12	4.0	47
Ettenbüttel, Ger.	Fecal Streptococci	50,000	70	2.9	47
Wiedersburg, Ger	*Clostridium perfringens*	90	6	1.2	47
Ettenbüttel, Ger.	*Clostridium perfringens*	900	300	0.5	47
Wiedersburg, Ger	*Campylobacter*	4,000,000	1,000	3.6	47
Ettenbüttel, Ger.	*Campylobacter*	9,000	60	2.2	47
Budds Farm, UK	*Salmonella* spp.	2,000	15	2.1	27
Budds Farm, UK	*Salmonella* spp.	6,800	25	2.4	27
Abbu Atwa, Egypt	*Vibrio cholerae*	530,000	390	3.1	27

Table 5. Fecal Coliform Bacteria Removal in FWS Wetlands

Site	System	Inlet (#/100ml)	Outlet (#/100ml)	Reduction (log_{10})	Reference	Note
Iron Bridge, FL	1	1	78	−1.9	Unpub. data	
Boggy Gut, SC	1	2	236	−2.1	17	
Brenton Cattle, IA	2	28	22	0.1	17	e
Tom Brothers, IN	1	29	28	0.0	17	
Brenton Cattle, IA	1	49	28	0.2	17	e
Purdue, IN	1	116	7	1.2	17	b
Purdue, IN	3	170	14	1.1	17	b
West Jackson Co., MS	1	239	674	−0.5	17	
Central Slough, SC	1	857	50	1.2	17	
Listowel, ONT	1	1,773	72	1.4	25	d,e
Listowel, ONT	2	1,773	573	0.5	25	d,e
Listowel, ONT	3	1,773	56	1.5	25	d,e
Arcata, CA	1	3,183	440	0.9	46	
Benton, KY	1	4,303	600	0.9	17	c
Benton, KY	2	4,303	111	1.6	17	c
Arcata, CA	2	12,500	316	1.6	46	
Brookhaven, NY	1	25,800	473	1.7	Unpub. data	e
Brookhaven, NY	2	25,800	898	1.5	Unpub. data	e
Lakeland, FL	1	27,000	115	2.4	Unpub. data	
Carolina Bays, SC	1	66,000	56	3.1	17	
Cobalt, ONT	1	159,301	1,087	2.2	44	
Pembroke, KY	1	166,000	270	2.8	17	
Sand Mt., AL	1	175,164	1,760	2.0	17	a
Sand Mt., AL	2	175,164	4,307	1.6	17	a
Sand Mt., AL	3	175,164	1,366	2.1	17	a
Listowel, ONT	4	228,292	141	3.2	18	d,e
Listowel, ONT	5	228,292	2,251	2.0	18	d,e
Oregon State, OR	1	749,436	59,088	1.1	17	c
Oregon State, OR	2	835,737	68,008	1.1	17	c
Oregon State, OR	3	835,737	52,874	1.2	17	c
Oregon State, OR	4	835,737	75,414	1.0	17	c
Oregon State, OR	5	835,737	74,851	1.0	17	c
Neshaminy, PA	1	1,290,600	5,600	2.4	Unpub. data	
Iselin, PA	1	1,303,840	2,113	2.8	50	
Waldo, FL	1	7,700,000	270,000	1.5	43	

Notes: a-Replicates, b-Variable loading, c-Different vegetation, d-Different geometry, e-Different wastewaters.

especially in the United States (48). In other countries, *E. coli* is the indicator of choice (4). The fecal streptococci group is sometimes used to confirm human fecal contamination, with a high ratio of fecal coliform to fecal streptococcus considered indicative of human origin (24). This test may not be reliable for wetlands as a result of the combined effects of differing removal rates and long wetland detention times. (See also FECAL CONTAMINATION, SOURCES OF, this Encyclopedia.)

Inflow densities of fecal coliforms for surface flow wetlands (Table 5) display a range of eight orders of magnitude, depending on the type of pretreatment before the wetland. Ultra-polishing wetlands, such as Iron Bridge, Florida, receive chlorinated effluent, with essentially no fecal coliforms. Lagoon effluents contain moderate levels of fecal organisms that enter the following wetlands. Animal lagoon wastewaters and minimally pretreated domestic wastewaters may bring 1 to 10 million cfu/100 ml into a treatment wetland. At high numerical input loadings, there is a trend of constant fractional removal for FWS systems. However, at low numerical loadings, there are lower fractional removals, and even additions of fecal coliforms for disinfected influents to the wetland. Replicated side-by-side wetlands (labeled "a" in Table 5) show fairly consistent results. Differences in vegetation (labeled "c") and geometry (labeled "d") have small effects.

At high loadings, SSF wetlands produce the same general reductions as FWS wetlands (Table 6). However, there are no low numerical loadings to be found in the literature, because disinfected effluents are not typically sent to SSF wetlands. Rather, the SSF option is often chosen for untreated wastewaters to prevent contact with the water during treatment. Consequently, some SSF wetlands receive over 10^8 fecal coliforms per 100 ml (Table 6). Replication, vegetation and geometry effects are small, as for FWS systems. However, higher loadings cause less reduction, as indicated for the Hamilton, New Zealand, wetlands (labeled "b" in Table 6).

The progression of removal creates gradients in numbers of organisms in the water along the flow direction (Fig. 7). This decline is sometimes, but not always, characterized by the logarithmic linearity associated with first-order reduction processes. First-order volumetric

Table 6. Fecal Coliform Bacteria Removal in SSF Wetlands

Site	System	Inlet (#/100ml)	Outlet (#/100ml)	Reduction (log_{10})	Reference	Note
Benton, KY	1	5,326	99	1.7	17	
Budds Farm, UK	1	11,000	120	2.0	27	d
Pretoria, SA	3	27,000	21	3.1	51	
Haughton, LA	1	27,500	1,750	1.2	52	
Budds Farm, UK	2	110,000	10,000	1.0	27	d
Phillips High School, TN	1	118,579	10	4.1	17	
Nun Moncton, UK	1	146,319	22,102	0.8	4	
Hardin, KY	1	176,424	1,162	2.2	17	a
Hardin, KY	2	176,424	263	2.8	17	a
Hamilton, NZ	1	189,000	8,800	1.3	53	b
Hamilton, NZ	2	189,000	15,000	1.1	53	b
Hamilton, NZ	3	189,000	39,000	0.7	53	b
Hamilton, NZ	4	189,000	45,000	0.6	53	b
Grand Lake, MN	1	166,000	730	2.4	30	f
NERCC, MN	1	450,000	201	3.1	30	a,f
NERCC, MN	2	450,000	332	3.3	30	a,f
Grand Lake, MN	1	230,000	136	2.2	30	g
NERCC, MN	1	219,000	1,407	1.5	30	a,g
NERCC, MN	2	219,000	1,966	1.4	30	a,g
Carlson, KS	1	300,000	30,000	1.0	54	
Beery, KS	1	400,000	10,000	1.6	54	
Brown, KS	1	400,000	30,000	1.1	54	
Abbu Atwa, Egypt	1	530,000	390	3.1	27	
Chmelna, CZ	1	1,258,925	79,433	1.2	31	
Richmond, NSW	1	1,288,250	18,621	1.8	55	c
Richmond, NSW	2	1,288,250	34,674	1.6	55	c
Richmond, NSW	3	1,288,250	28,840	1.7	55	c
Onsov, CZ	1	1,584,893	28,184	1.8	56	
Doksy, CZ	1	1,995,262	25,119	1.9	56	
Scott Co., KY	1	2,400,000	9,100	2.4	57	
Ondrejov, CZ	1	22,387,211	354,813	1.8	56	
Pretoria, SA	1	67,000,000	68,000	3.0	51	a
Pretoria, SA	2	67,000,000	27,000	3.4	51	a
Paris, KY	1	69,000,000	610,000	2.1	57	
Anderson Co., KY	1	130,000,000	330,000	2.6	57	
Kolodeje, CZ	1	141,253,754	141,254	3.0	56	

Notes: a-Replicates, b-Variable loading, c-Different vegetation, d-Different geometry, e-Different wastewaters, f-Summer, g-Winter.

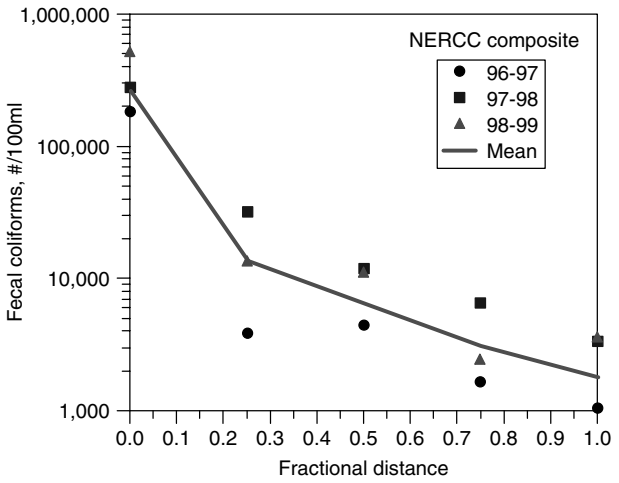

Figure 7. The reduction of fecal coliforms with travel distance in a subsurface flow wetland treating septic tank effluent at the Northeast Regional Corrections Center near Duluth, MN. Data are annual means for three years (30,58). See color insert.

removal rate constants are in the range of 0.2 to 2.0 d^{-1} at 20 °C (1,2). Temperature has been shown to be a factor in pathogen removal at some wetlands, but not others. For example, the NERCC SSF system of Figure 7 displayed a doubling of the removal rate for a 10 °C temperature increase while experiencing a relatively uniform level of incoming organisms (29,57,58).

The microbial populations in wetland waters demonstrate stochastic variability. There are also seasonal cycles in population densities. The cyclic portion of the population time series may be separated from the stochastic portion by detrending several years' data (Fig. 8). For the Grand Lake, Minnesota, SSF wetland, there was no large trend in inflow counts, but a simple sinusoidal trend was observed for densities in the wetland outflow (29). The distribution of individual fecal coliform counts, expressed as a fraction of the trend value, is a unimodal, nearly normal distribution (Fig. 8). The highest outlet values, corresponding to the poorest reduction, occurred in early spring; the lowest counts and best treatment occurred in late summer.

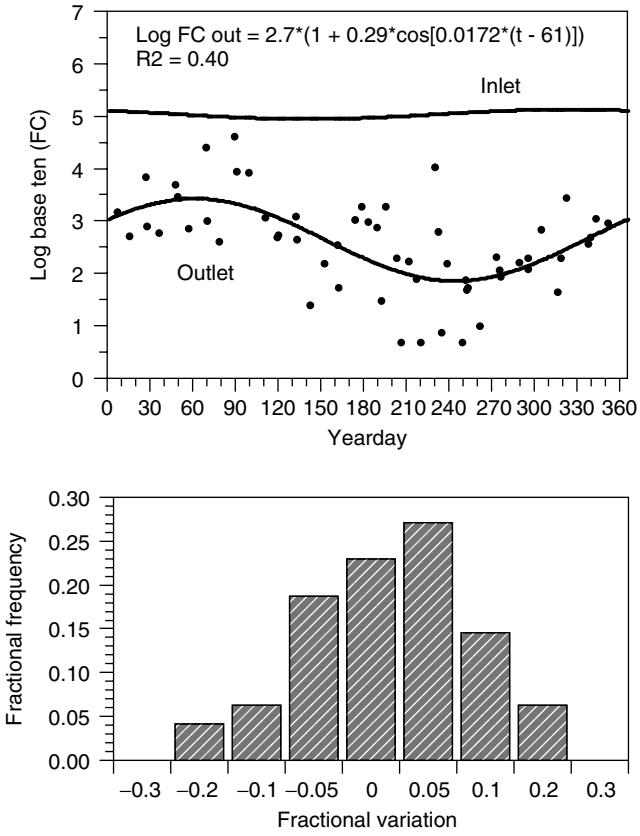

Figure 8. The reduction of fecal coliforms in subsurface flow wetlands treating septic tank effluent at Grand Lake, MN. Data are monthly for three years (30,58). Top: annual trend lines for organisms in the influent and effluent. Influent data points not shown. Bottom: histogram of the fractional stochastic residuals in organism counts. Variability is contained to ±20%. See color insert.

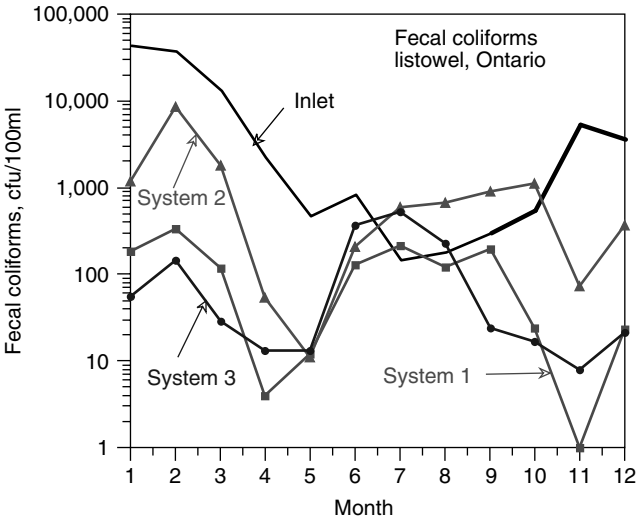

Figure 9. The reduction of fecal coliforms in surface flow wetlands treating lagoon effluent at Listowel, Ontario. Data are means for a given month for three years. The aspect ratio of System 2 was 17 : 1, and 84 : 1 for System 3 (18). See color insert.

Three parallel but geometrically different FWS wetlands at Listowel displayed a different and more complicated seasonal pattern for fecal coliforms (Fig. 9). The lagoon source water at Listowel showed an annual swing of three orders of magnitude in fecal coliform counts, with the highest values in winter, and the lowest in summer. The wetlands outflow count tracked the winter inflow count peak, but also displayed a late summer peak, which exceeded the inflow count for several weeks. Regrowth and desorption were considered possible explanations for the high summer values (17). A first-order reduction model cannot explain these observations because it does not provide for regrowth. Further, a temperature coefficient cannot be assigned, because other factors dominate.

Warm-blooded animals can contribute to populations of fecal organisms in wetlands. FWS wetlands can attract large numbers of birds, in particular, waterfowl and wading birds (1,59). FWS systems also attract rodents, such as muskrats (*Ondatra zibethica*), beavers (*Castor canadensis*) and nutria (*Myocastor copyus*) (1). As a consequence, natural and constructed wetlands have background counts of fecal organisms, in the absence of any introduced bacteria. Background for FWS wetlands has been observed to be in the range of 50 to 500 cfu/100ml (60,61). The Orlando Easterly wetlands in Florida are a well-studied example of this phenomenon (59). Fecal streptococci build in numbers along the flow path until reaching about 100 cfu/100 ml (Fig. 10). Interestingly, populations are lowest in late summer. Presumably, SSF wetlands would have a zero background of fecal organisms, because of the lack of animal interactions.

Viruses

Viruses enter wetlands used for domestic wastewater treatment. The enteroviruses: Polio types one, two,

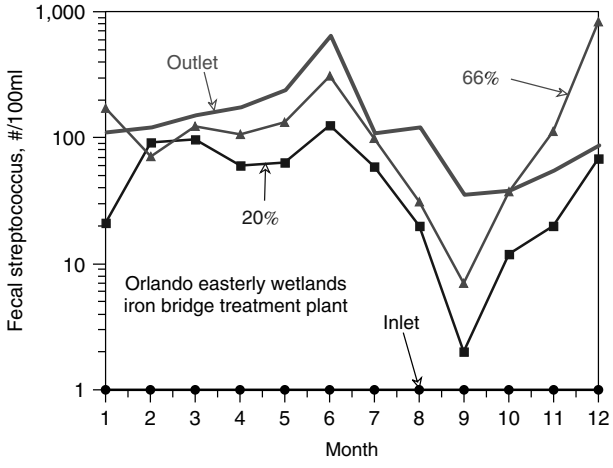

Figure 10. Time series of fecal streptococcus at various travel distances in a surface flow wetland treating tertiary, disinfected effluent from the Orlando Iron Bridge treatment plant. The 482 ha wetland receives zero organisms, but populations grow as the water moves through the system. Data are for 1994, but the seasonal patterns are also typical of other years. The percent labels refer to the fractional distance along the wetland flow path. See color insert.

Table 7. Virus Reduction in Treatment Wetlands. (*ND = Below* Detection)

Seeded virus	Vegetation	Virus	Inlet pfu/ml	Outlet pfu/ml	Reference
		Subsurface Flow			
Santee, CA	Bulrush 1W	FRNA phage MS2	9,420,000	8,021	63
Santee, CA	Bulrush 1W	polio type 1	5,154	11/12 ND, 0.2	63
Santee, CA	Bulrush 2Wa	FRNA phage MS2	528,000	1,183	63
Santee, CA	Bulrush 2Wb	FRNA phage MS2	5,070,000	1,639	63
Santee, CA	Bulrush 2W	polio type 1	2,323	8/10 ND, 0.3	63
Duluth, MN	Bulrush summer	somatic coliphage	1,451	23	58
Duluth, MN	Bulrush winter	somatic coliphage	1,000	100	58
Indigenous virus					
Santee, CA	Unplanted	F-specific phages	3,129	174	63
Santee, CA	Bulrush	F-specific phages	3,129	33	63
Abbu Atwa, Egypt	Phragmites	coliphage	94,000	300	65
Budds Farm, UK	Phragmites	coliphage	370	ND	65
Budds Farm, UK	Phragmites	coliphage	310	ND	65
Budds Farm, UK	Phragmites	enterovirus	470	ND	65
Budds Farm, UK	Phragmites	enterovirus	570	ND	65
Ettenbüttel, Ger.	Phragmites	coliphage	15,000	500	47
Wiedersburg, Ger	Phragmites	coliphage	700,000	500	47
		Free Water Surface			
Indigenous virus					
Houghton Lake, MI	Typha, Carex	reovirus + polio type 1	1,000	100	Unpubdata
Waldo, FL	Cypress new	bacteriophage	420	61	43
Waldo, FL	Cypress old	bacteriophage	57,000	120	43
Duplin County, NC	Typha, Sparganium	somatic coliphage	630,000	50,000	67
Duplin County, NC	Typha, Sparganium	somatic coliphage	50,000	8,000	67
Duplin County, NC	Typha, Sparganium	F + coliphage	16,000	3,000	67
Duplin County, NC	Typha, Sparganium	F + coliphage	3,000	320	67
Oxelösund, Sweden	Typha	coliphage	75,000	4,000	40
Glendale, AZ	Typha, Scirpus	coliphage	167,000	8,510	45

and three, Echo and Coxsackie; and Reovirus have all been reported in connection with treatment wetlands (43,62–65). Removals of both indigenous and seeded virus are typically one to three logs (Table 7). Biological, chemical, and physical processes are probably all responsible (62).

Suspended solids have been implicated as carriers of virus in wetland waters because of the initial rapid decline in counts along the flow direction (63). Initial first-order reduction rates of 0.06 to 0.18 hr^{-1} were followed by slower rates of about 0.01 hr^{-1} in phage seeding tests in a FWS wetland (63). Decay rates of 0.012 to 0.025 hr^{-1} were found for solid-free water in phage seed testing in a SSF wetland (62). No rapid initial rate was found for the solid-free testing. Somewhat higher removal rate constants were observed for indigenous phages in cypress wetlands, 0.035 to 0.075 hr^{-1} (43,62). These rates are comparable to other pollutant removal rates in wetlands, for easily degraded materials such as nitrate.

The influence of other factors of wetland design is as yet not known. Studies in northern climates under cold-water conditions are not available. Table 7 hints that SSF systems may be more efficient than FWS, but the evidence is not conclusive. Unvegetated SSF beds perform some treatment of virus, but the plants significantly improve removals. It thus appears that rhizosphere processes augment physical adsorption.

Protozoans

Treatment wetlands contain several genera of protozoa and metazoa, and wastewater addition may carry other species into the ecosystem (67) (Table 8). Some of these are human pathogens, such as *Giardia*, *Entamoeba*, and *Cryptosporidium*. Reductions to below detection have been reported for gravel reed beds, for cysts, or trophozoites of *Entamoeba* (37,39,68). Soil-based reed beds showed incomplete removal.

Some protozoa are known to graze on bacteria, thus providing bacterial removal by predation in the wetland (36,38). Twenty-two types of ciliates were found in four reed beds at Audlem, United Kingdom. Two community types were identified: one dominated by aerobic bacterivores (e.g., *Paramecium*) in gravel beds, and the other dominated by microaerophilic species (*Plagiopyla*, *Metopus*) in bare soil beds. Combinations also were found in planted soil beds. Rotifers, nematodes, and free-living amoebae were also observed, which may also have contributed to bacterivory. The numbers of these organisms declined along the flow direction, as did the numbers of bacteria, as indicated by *E. coli*. Ciliates alone

Table 8. Protozoa Found in Treatment Wetlands

	FWS *Typha* Thailand Ref. (68) %Frequency	FWS *Typha Ipomoea* Thailand Ref. (68) %Frequency		SSF *Phragmites Typha* Mexico City Ref. (69) Relative Frequency	SSF *Phragmites* UK Ref. (69) No. of Species
Flagellate			Free-Living Amoebae		
Monas	95	64	*Vahlkampfia*	24	4
Bodo	74	62	*Naegleria*	23	
Phagus	74	45	*Rosculus*	19	1
Rhyncomonas	42	38	*Platyamoeba*	17	
Euglena	21	12	*Hartmanella*	14	2
Paraphysomonas	21	5	*Vanella*	13	
Entosiphon	5	16	*Thecamoeba*	12	
			Acanthamoeba	11	5
Sarcordina			*Dactylamoeba*	10	
Arcella	59	41	*Mayorella*	6	1
Centrophyxis	58	38	*Guttulinopsis*	4	
Euglypha	47	47	*Acrasis*	4	
Vanella	29	12	*Cochliopodium*	3	
Myorella	8	10	*Glaeseria*	3	
Diffulgia	11	5	*Cashia*	3	
Actinophrys	9	2	*Vexillifera*	2	1
			Pocheina	2	
Ciliate			*Flamella*	1	
Colpidium	27	17	*Paratetramitus*	1	
Cyclidium	20	12	*Filamoeba*	1	
Vorticella	9	28	*Saccamoeba*	1	
Litonotus	9	5	*Dermamoeba*	1	
Chilodonella	8	5	*Echinamoeba*		1
Frontania	11		*Parvamoeba*		1
Colpoda	2	9	Pathogenic Protozoa		
Didinium	5	2	*Endolimax*	present	
			Entamoeba	present	
			Giardia lamblia	present	
			Iodamoeba bütschlii	present	

numbered about 600 per ml of water, and grazing at reported rates of 10 to 50 bacteria per ciliate per hour could have accounted for the observed *E. coli* reduction.

Parasite Eggs

Domestic wastewaters can carry propagules of a variety of parasites, particularly in tropical and subtropical climates. These helminths include *Ascaris* (roundworm), *Taenia* (tapeworm), and *Tricuris* (whipworm). The eggs are relatively large (ca. 50 μm), and heavy enough to settle effectively. Consequently, the solids trapping function of treatment wetlands provides removal. Protozoan predation has also been implicated in the removal of *Cryptosporidium* oocysts in constructed wetlands (39).

Both surface and subsurface flow systems have been found to be effective for reducing the numbers of eggs and cysts of parasites (36,68–71). *Ascaris lumbricoides* eggs were reduced from 120 to 220 per gram of solids at the inlet of a gravel–phragmites wetland microcosm, to nil at the outlet, in Mexico City (36). However, reductions were less, 120 to 240 per gram of solids at the inlet of a soil–phragmites wetland microcosm, to 40 at the outlet. Removal of *Ascaris*, *Taenia*, and *Tricuris* was complete in water hyacinth (*Eichhornia crassipes*) wetlands in Morocco (69).

Transect studies (37) showed large percentage reductions in five hours detention in a reed bed dosed with *Ascaris*, *Hymenolepis*, and *Toxocara*. However, longer detention did not remove all eggs, suggesting that some portions of the flow were not treated. Transect studies (70) also showed large percentage reductions in 1.2 to 3.5 hours detention in cross-ditched reed beds, for both Nematodes and Cestodes. Influent counts of four eggs/L were reduced to 0.2 to 1.0 eggs/L, with higher removals at longer retention times and higher temperatures.

Vectors and Wildlife Pathogens

Mosquitoes breed abundantly in wetlands, and can be the carriers of human pathogenic agents. Equine encephalomyelitis and St. Louis encephalitis viruses are among the arboviruses known to be transmitted by mosquitoes, such as *Culex tarsalis* (72). *Anopheles* spp. may carry the blood pathogen *Plasmodium vivax*, which causes malaria. Human health problems are thus associated with wetlands, including treatment wetlands. The incidence of malaria and encephalitis decrease with movement from the tropics to the subtropics to the north temperate climatic zones, and consequently the greatest concern is in warm regions. Treatment wetlands may be

better or worse than natural counterparts with respect to mosquito-borne diseases, but they do constitute additional breeding grounds on the landscape. Control measures include vegetation control, biological control with larvae-consuming mosquito fish, adulticiding and larvaciding. Mosquito-specific bacteria in the genus *Bacillus* may be aerially applied to control mosquito populations (73).

Wildlife pathogens are also potentially associated with treatment wetlands, as they are with natural wetlands. Avian cholera (caused by *Pasteurella multocida*) and avian botulism (caused by *Clostridium botulinum*) have been found in association with sewage lagoons and natural and treatment wetlands (74). Conditions that favor transmission are exposed mud flats, together with stressed and overcrowded bird populations (74). The nematode *Eustronglydes ignotus* has been identified as a potential threat to wading birds that feed and nest near nutrient rich sites. However, extensive research on the fish that are intermediate hosts have shown no potential vector in Florida wetlands treating agricultural runoff (75).

CONCLUSION

Treatment wetlands of many kinds are finding rapidly increasing application in numerous pollutant reduction projects. The wetland environment places water in close contact with soils and biota in both surface flow and subsurface flow designs and environments. The rhizosphere, with its active consortia of microorganisms, is accessible to contaminants and effective in promoting transformations. Large and diverse microbial populations are present, and contribute to biogeochemical cycling of nutrients and other substances. Stratification of redox potential leads to multiple functional zones that can communicate by diffusion. The rates of wetland microbial processing are high, and in many cases can absorb new chemical inputs and provide treatment.

Pathogenic organisms occur naturally in wetlands, and wastewaters may bring different and more numerous organisms into the treatment wetland. The wetland environment is hostile to human enteric pathogens, including viruses, bacteria, parasites, and their eggs. Thus, the technology offers a means to provide reduction in pathogenic organisms, but is not to be viewed as a means of total disinfection.

The challenge facing the researchers and practitioners is to understand the microbiology of these systems to the degree necessary to optimize their performance.

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WETLANDS: BIODEGRADATION OF ORGANIC POLLUTANTS

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Wetlands are complex ecosystems that link uplands to aquatic environments (1–3). They are distinguished from other ecosystems by their saturated soil conditions, oxidizing and reducing soil layers, organic matter accumulation, and colonization by vegetation and microbial communities adapted to these conditions. Many pollutant-removal processes are fostered under these conditions (1,4–6).

More than 10 million different organic compounds are known, and humans living in developed nations use over 70,000 of these on a day-to-day basis (7). In this entry, an organic pollutant refers to any chemical that contains at least one carbon-hydrogen linkage that is found at levels higher than background and has adverse effects on the environment. Organic pollutants include pesticides, explosives, dyes, phenols, and petroleum products contained in agricultural, domestic, and industrial effluents. Historically, many of these compounds were referred to as *xenobiotics* (meaning foreign to the biosphere) in an attempt to differentiate synthetic and naturally produced compounds. However, it has been suggested that this

term be discontinued because of the structural similarities between the so-called artificial and biosynthesized organic compounds (7).

The study of organic pollutants in wetlands is important because many constructed wetlands are being used to remediate polluted soil and water, and organic pollution of wetlands poses a threat to human health and wildlife (1,6,8,9). Wetlands receive organic pollutants from various sources, including (*1*) applications of pesticides to control aquatic weeds, algae, and mosquitoes, (*2*) agricultural and urban surface runoff, (*3*) landfill leachate and industrial chemical spillage, (*4*) residual pesticides in agricultural lands converted to wetlands, and (*5*) atmospheric fallout. According to a National Sediment Inventory (10), these sources contaminate 26% of U.S. rivers, streams, lakes, and coastal shoreline sediments at the Tier I level, that is, they pose the greatest health hazard to biota. Pollutants that cause the most concern include polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs), and pesticides, mercury, and other heavy metals. Many of these compounds bioaccumulate, and as a result there are over 2,500 fish-consumption advisories in 47 States (11). Organic pollutants interfere with many beneficial wetland functions, including water quality improvement and wildlife habitat utilization (12).

Systems of biotic and abiotic processes govern organic-pollutant removal in wetlands (Fig. 1). Biodegradation is the biologically catalyzed simplification of organic chemicals into its fundamental inorganic constituents, and is generally considered the main detoxification pathway in the environment (13). Biodegradation in wetlands depends on the chemical characteristics of the organic pollutant (water solubility, oxidation state, types of functional groups), and on biogeochemical conditions in the soil-water column (microbial consortia, pH, redox potential, temperature, salinity, nutrient and carbon availability, oxygen and other electron acceptor availability, vegetation type and amount) (14). Biogeochemical conditions can be highly dynamic in wetlands. Predicting organic-pollutant fate and impacts in these ecosystems requires an integration of several disciplines including soil microbial ecology, wetland science, chemistry, physics, engineering, botany, and ecotoxicology. These disciplines are integrated and applied in the field of bioremediation, which is the controlled and practical use of the biodegradative capacity of microorganisms to destroy chemical pollutants to establish safe concentrations (13).

This article covers biogeochemical principles, current biodegradation and fate research, and microbiological transformations of organic pollutants in wetlands. Finally, future directions regarding the study of organic pollutants in wetlands will be addressed. More detailed information about reaction mechanisms of organic pollutant degradation can be found in other entries of this Encyclopedia and other references (7,13,15–18).

WETLAND BIOGEOCHEMISTRY

Between 2 and 6% of the earth's surface is covered by wetlands, which include coastal wetlands (tidal freshwater, estuaries, and mangroves) and inland wetlands (marshes, peatlands, swamps, riparian wetlands, bogs, fens, potholes, pocosins, and floodplains) (2). Wetlands differ from other ecosystems in three main characteristics: periodicity and source of water inundation (i.e., hydrology), presence of hydric soils, and the presence of vegetation and microorganisms adapted to anoxic conditions (3). Hydric soils, as defined by USDA-NRCS and National Technical Committee for Hydric Soils, are... "soils formed under conditions of saturation, flooding, or ponding long enough during the growing season to develop anaerobic conditions in the upper part" (19). Hydric soils can be identified in the field by observing the presence of redoximorphic features, including organic matter accumulation at the surface, and soil zones with alternating dark and light colored areas in the subsurface (Fig. 2). The darker zones, made up of various shades of red, yellow, brown, and black, are indicative of iron and manganese oxides formed under oxidizing conditions; the lighter gray areas are indicative of reduced iron and manganese species formed under anoxic, reducing conditions. Soils containing both color indicators in close

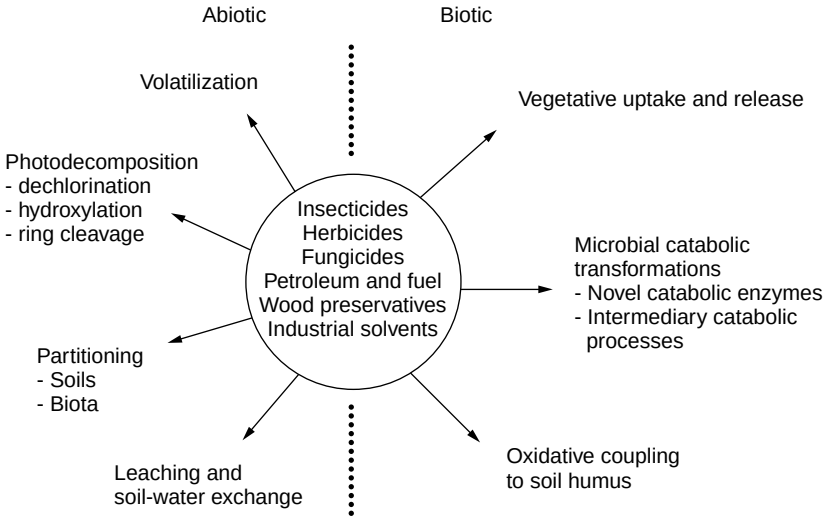

Figure 1. Fate pathways of organic pollutants in wetlands.

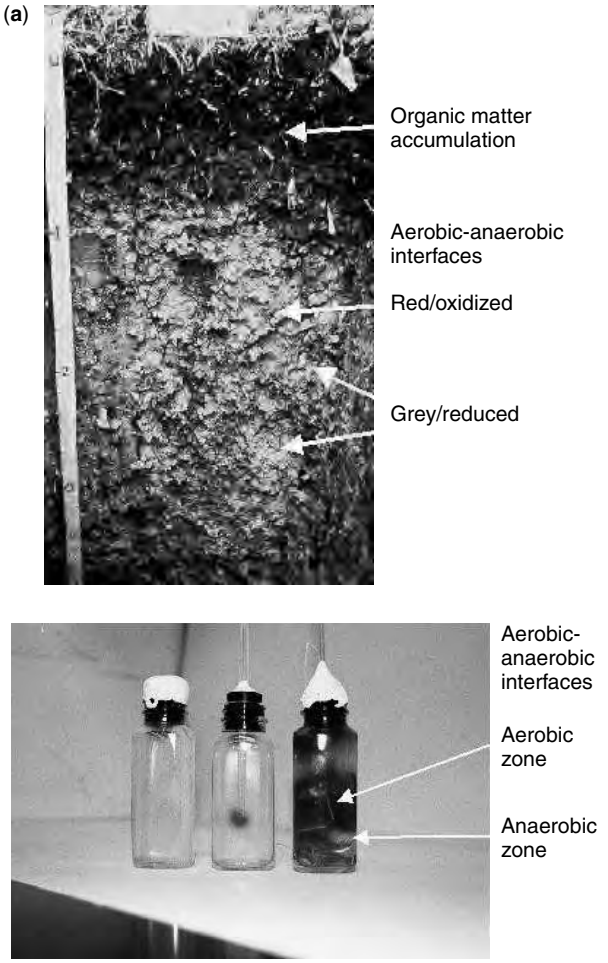

Figure 2. Development of aerobic and anaerobic interfaces in wetlands. (**a**) Wetland soil profile showing accumulation of organic matter in surface horizon, and adjacent red (oxidized) and grey (reduced) zones in the subsurface soil. (**b**) One day root oxygen loss by rice plants (*Oryza* sp.). Methylene blue dye in vessels turns from clear to blue upon exposure to oxygen. Left: severed root sealed from atmosphere using rubber stopper and sealing putty; Center: glass tube open to atmosphere; Right: intact rice plant with upper leaves exposed to atmosphere. See color insert.

proximity are characteristic of those that experience fluctuating water tables. In some wetlands, which are flooded for longer periods, soils can exhibit a rotten egg odor from hydrogen sulfide, which is generated from the activity of the anaerobic sulfate-reducing bacteria. Wetlands are extremely diverse and dynamic ecosystems, which significantly impact the types and extent of physical, chemical, and biological reactions that occur in these environments.

Perhaps, the two most important factors that distinguish wetland biogeochemical processes from those in other ecosystems are the presence of aerobic and anaerobic interfaces (20) and organic matter accumulation. Aerobic and anaerobic interfaces result from at least three wetland events, namely, hydrologic fluctuations (e.g., rise and fall of water table, flooding/draining), influx/consumption of electron acceptors (e.g., O_2, NO_3^-, Mn^{4+}, Fe^{3+}, SO_4^{2-}, and HCO_3^-), and oxygen transport by plants to the rhizosphere. The occurrence of these events can sometimes be visualized as macroscopic features and chemical gradients in the soil profile (Figs. 2 and 3). They are critical because they dictate the prevailing types of microbial communities and associated metabolic processes.

Wetland soils often undergo cyclic flooding and draining because of seasonal rainfall events. During the dry season, wetland soil pores (about 50% of the soil volume) may be largely filled with air (containing 10 to 21% oxygen). Under these conditions, aerobic microbial processes predominate. Microorganisms use oxygen for two main purposes: (*1*) as terminal electron acceptor during oxidation of organic matter and other energy substrates (electron donors) and (*2*) as a reactant in which oxygen atoms are added to organic molecules to facilitate degradation.

When soils are flooded, water displaces air in the soil pores. Because oxygen diffusion through water is 10,000 times slower than through air-filled soil pores, much of the soil profile rapidly becomes anoxic (devoid of oxygen) as a result of microbial activity (Fig. 3). Oxygen may be depleted in most of the soil in about a day, depending on the temperature and type and amount of materials present that foster oxygen-consuming processes (such as organic matter, sewage effluent, composts, and

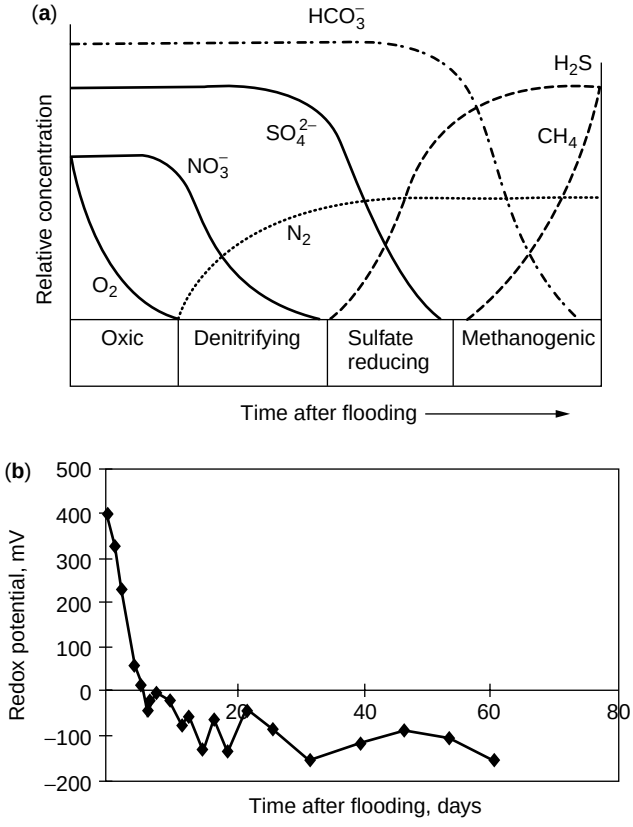

Figure 3. Temporal changes in aerobic and anaerobic processes in wetlands. (**a**) Sequential reduction of electron acceptors as a function of time after flooding, (**b**) Change in redox potential in the subsurface soil horizon after flooding a central Florida marsh soil.

NH_4^+). When oxygen is used up, facultative anaerobic bacteria (e.g., denitrifiers, manganic manganese reducers, ferric iron reducers) and obligate anaerobic microbial groups (e.g., fermenting bacteria, sulfate reducers, and methanogens) take over the transformation of organic matter and pollutants. There are very few examples of anaerobic fungi in wetlands. Anaerobic bacteria use alternate electron acceptors (NO_3^-, Mn^{4+}, Fe^{3+}, SO_4^{2-}, HCO_3^-, and simple organic substrates in the case of fermentation) to oxidize electron donors. Organochlorine and humic substances can also serve as terminal electron acceptors in the metabolism of some bacteria (21,22). These dominant electron acceptors can occur in distinctive patterns in the landscape and give rise to a hierarchy of terminal electron-acceptor processes (TEAPs). Electron acceptors are typically reduced sequentially over time, in accordance with their availability and the relative amount of energy derived from a particular electron acceptor/donor couple. For instance, thermodynamic principles predict that more energy would be gained from the oxidation of a substrate by aerobes using oxygen as compared with anaerobes using alternate electron acceptors. By gaining more energy, aerobes are able to more rapidly reproduce, synthesize new enzymes (including many types not produced by anaerobes), and degrade organic pollutants by outcompeting other groups for available resources. Sequential reduction of electron acceptors is often reflected as increases in the anaerobic decomposition products, including N_2O, NH_4^+, Fe^{2+}, Mn^{2+}, H_2S, and CH_4, and the appearance of redoximorphic features (e.g., change in soil color from red to gray and generation of rotten egg odor).

Sequential TEAPs are also reflected as changes in the redox potential (*Eh*) in the soil-water column (Fig. 3). Redox potential is the tendency of chemical pairs (i.e., electron acceptor and electron donor) to undergo a transfer of electrons, and can be measured in saturated soil using platinum and calomel reference electrodes and a voltmeter. The higher the *Eh*, the greater the tendency of a transfer of electrons from a donor molecule to acceptor molecule, and the greater amount of free energy available for the organism to grow and reproduce. In wetlands, *Eh* values, typically, range between +500 and −300 mV. At *Eh* values exceeding 300 mV, the presence of oxygen and aerobic metabolism are indicated. Lower *Eh* values indicate oxygen-depleted conditions and a shift to anaerobic metabolism, that is, fermentation and microbial utilization of alternate electron acceptors (Mn^{4+}, NO_3^-, Fe^{3+}, SO_4^{2-}, CO_2).

In wetlands flooded for long periods, *Eh* gradients are typically observed as a function of soil depth reflecting changes in the types of metabolic processes (Fig. 4). As expected, *Eh* values are usually greatest at or near the soil surface and in the plant rhizosphere because of oxygen introduction by several processes, including: (*1*) diffusion from the air to floodwater and soil, (*2*) algal production by photosynthesis in the water column, (*3*) burrowing and tunneling of macroinvertebrates in soils, and (*4*) diffusion and mass flow from aerial portions of the aquatic plants to the rhizosphere (Fig. 2b). The thickness of the aerobic zone may expand and contract up to several centimeters in a day because of diurnal fluctuations in light and temperature. Outside the aerobic zones, there is a shift to anaerobic microbial metabolism. Depending on the chemical composition of the water, most flooded wetland soils have several metabolic zones, which are defined by their TEAPs, namely, aerobic (O_2), denitrifying (NO_3^-), sulfate reducing (SO_4^{2-}), and methanogenic (CO_2) (Fig. 4). Freshwater systems are typically dominated by aerobic, iron-, nitrate-, and bicarbonate-reducing conditions, whereas marine systems are dominated by aerobic and sulfate-reducing conditions.

Aerobic and anaerobic interfaces also can be found in the rhizosphere of wetland plants (23,24) (Fig. 2b). Rice (*Oryza sativa*) and other wetland plants have a unique feature in that they transport atmospheric oxygen through the stem and roots. Some of this oxygen diffuses from the root to the adjacent soil layer. Besides oxidizing the reduced microzone around the root surface, the diffused oxygen also stimulates aerobic fungal and bacterial populations, and activity in the wetland plant rhizosphere (25–28). Root oxygen loss from wetland plants has been measured at rates of 13 mg O_2 $plant^{-1}$ day^{-1}, and was used for the oxidation of NH_4^+ and organic carbon substrates (29). Because the bulk soil around the

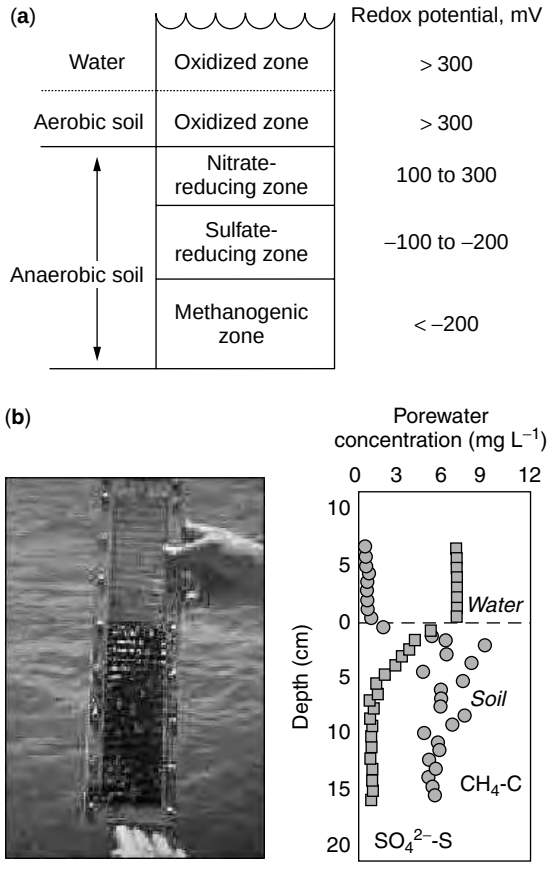

Figure 4. Spatial distribution of aerobic and anaerobic processes in wetlands flooded for extended periods. (**a**) Conceptual diagram showing electron acceptor reducing zones as a function of soil depth, (**b**) Distribution of sulfate reduction and methanogenesis in a central Florida wetland as determined using pore water equilibrators. See color insert.

rhizosphere is mostly anaerobic, the products resulting from aerobic processes to anaerobic zones, diffuse and are transformed by anaerobic microbial processes. This is an important mechanism for nitrogen removal by nitrification-denitrification in flooded soils planted with rice (30).

Aerobic–anaerobic interfaces impact potential rates of organic pollutant degradation. For example, biodegradation of compounds such as octane, benzene, toluene, ethylbenzene, and xylene, and PAHs derived from petroleum and creosote are enhanced under aerobic conditions compared with anaerobic conditions. For other compounds such as pentachlorophenol (PCP), rapid degradation can occur in either aerobic or methanogenic conditions, but is curtailed under denitrifying or sulfate-reducing conditions (31). These observations have obvious implications for the bioremediation of soil contaminated with organic pollutants.

Because wetlands contain redox gradients, there is an additional opportunity for organic pollutant biodegradation. For example, in surface-flow wetlands, pollutants are introduced into the aerobic surface layer where they can be oxygenated to intermediate metabolites and carbon dioxide. Oxygenated products can diffuse to the adjacent anaerobic layers as a result of concentration gradients, where anaerobic processes subsequently transform them. In subsurface-flow wetlands, where pollutants are introduced to anaerobic layers, the order of reactions is reversed. Enhanced transformations by sequential aerobic and anaerobic and diffusion processes have been demonstrated for urea (32) and methane (33). Sequential anaerobic followed by aerobic transformations were found to enhance degradation of the pesticide methoxychlor (34), and may beneficially degrade highly chlorinated compounds such as PCBs and PCP. Sequential biodegradative processes are expected to be more prevalent in wetlands compared with either uplands or permanently flooded aquatic systems because wetlands develop these aerobic and anaerobic interfaces.

The second factor that governs organic-pollutant biodegradation is organic-matter accumulation. Organic matter often accumulates in wetlands because plant and algal productivity are accelerated, stable humic substances are formed, and decomposition rates are slow. External loading may be significant in wetlands receiving domestic and other organic-rich wastes. Organic matter governs organic-pollutant fate through several mechanisms. First, it serves as a carbon and energy source to support the growth of aerobic and anaerobic heterotrophic bacteria and fungi. These groups are primarily responsible for the organic-pollutant biodegradation. Second, organic matter and its degradation intermediates (e.g., hydrogen, cellulose, proteins, fatty acids) facilitate biochemical reduction of organic pollutants in soil under anaerobic conditions. These compounds serve as electron donors for the reductive dehalogenation of organohalogens (e.g., polychlorinated biphenyls, chlorinated phenols, chlorinated ethenes, DDT, toxaphene, and dieldrin) and reduction of nitro groups of certain compounds (e.g., trinitrotoluene and RDX). An additional role of organic matter is sorption, which retards pollutant mobility, bioavailability, and toxicity. On the other hand, pollutant sorption to dissolved organic carbon may enhance mobility and transport out of wetlands (35). An often overlooked mechanism is the oxidative coupling of organic pollutants to humic substances, in which phenol- and aniline-based compounds covalently link to native humic substances (36). Finally, aerobic decomposition of organic matter at the soil surface and the rhizosphere of wetland plants consumes oxygen, causing a shift from aerobic to anaerobic microbial activity. The shift influences the types and extent of biodegradation and other fate processes, as will be discussed in the following sections.

ORGANIC POLLUTANT FATE IN WETLANDS

Several abiotic and biotic processes govern the fate of organic pollutants once they enter the wetlands (Fig. 1). In general, organic pollutants undergo similar processes as natural organic matter. Pollutants introduced into the water column may volatilize to the atmosphere (37), photodegrade (38–40), abiotically degrade (16,41), biodegrade, or interact with colloidal material and settle in the soil (42–63). In the soil and in the plant rhizosphere, organic pollutants may be transformed into intermediate compounds and carbon dioxide by aerobic and anaerobic microorganisms and plants. Organic pollutants may be stored for long periods in the wetland because of sorption and oxidative coupling in soil (64–69), or they may be returned to the water column by diffusion, or by resuspension events, or by the burrowing of macroinvertebrates (35,70). Aquatic plants, crustaceans, fish, and fish-eating birds may accumulate hydrophobic ("water hating") organic pollutants into their waxy and fatty tissues (71–73). Aquatic plants promote volatilization of organic pollutants into the atmosphere by increasing mass transport of constituents toward plant roots and shoots by transpiration, and by translocating chemicals through aerenchyma tissue to the atmosphere (74). Wetlands may attenuate different types of organic pollutants through a combination of some processes, thus reducing the threat to downstream aquatic environments (Tables 1–3).

The extent of many processes can be roughly estimated from the type of organic pollutant and prevailing environmental characteristics (Table 4). For example, a chemical with a high sorption coefficient to organic carbon [e.g., $K_{oc} > 10,000$ mg chemical kg^{-1} organic C $\times$ (mg chemical L^{-1} water)$^{-1}$] would be retained in organic-rich soils, and have limited mobility in the water column. Compounds containing certain types of functional groups are likely to undergo photodegradation, biodegradation, or abiotic reactions in wetlands. Wackett and Herschberger (7) suggested that predicting possible biodegradation routes for the vast number of organic pollutants could be greatly simplified by defining them in terms of their major functional groups. Microbial transformations have been documented in about 58 of the 94 functional groups so far. Environmental factors including organic carbon content of soil, seasonal light and temperature changes, water content, salinity, redox potential, pH, and microbial growth conditions play key

Table 1. Biotransformation of Pesticides in Wetlands and Wetland Microcosms

Pesticides	Sources	Wetland System/Substrate	Experimental Conditions	Rate[†] Sterile	Rate[†] Live	Mechanisms	Notes	Reference
Atrazine (2-chloro-4-ethylamino-6-isopropylamine-1,3,5-triazine)	^{14}C spiked soils	Estuarine sediment microcosm	Aerobic	ND[‡]	15–20 d	Dealkylation, hydroxylation	Sorption accounts for 10–85% loss	42
Atrazine	Spiked soils	Aquatic plant/gravel mesocosm	Aerobic	100% in 41 d	100% in 7–42d	ND; loss of parent compound	Acclimation and rhizosphere enhanced rates; toxicity to plants	43
Atrazine Alachlor (2-chloro-2′,6′-diethyl-N-(methoxymethyl) acetanilide)	Spiked wetland	Riparian forest wetland soils	ND	ND	94% (unspecified period) 59% (unspecified period)	Dilution; other undetermined processes	Dilution accounted for 53% of loss	44
2,4 D (2,4-dichlorophenoxyacetic acid) Atrazine	^{14}C spiked soils	Forested wetland soils	Aerobic	No loss	10–63% in 105 d 6–10% in 105 d	Mineralization	High N inhibits rates, especially in high N soils	45
Dicamba (3,6-dichloro-2-methoxybenzoic acid)	Spiked soil enrichment	Freshwater wetland soil enrichment	Methanogenic	No loss	100% in 30 d	Demethylation, reductive dechlorination	Five member consortia unable to mineralize ring	46
Dicamba Metribuzin (4-amino-6-(1,1-dimethylethyl)-3-(methylthio) 1,2,4-trazine-5-(4H-one)	Spiked soils	Riparian soil microcosms	Denitrifying, methanogenic	No loss	13 to >365 d	Demethylation, deamination	Acclimation enhanced rates	47
p-nitrophenol	^{14}C spiked soils	Everglades soil microcosms	Aerobic	No loss	10–30% in 75 d	Mineralization	High conc. inhibited rates; microbial inoculants enhanced rates	48
Metolachlor (2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2 methoxy-1-methylethyl) acetamide)	Spiked soils	Tidal soil microcosm	Aerobic		0.5% in 84 d	Mineralization	Leaching and sorption greater than mineralization	49

[†]Rates have units of half-life (d) or percentage removal over specified time.
[‡]Not determined.

Table 2. Biotransformation of Hydrocarbons in Wetlands and Wetland Microcosms

Hydrocarbons	Sources	Wetland System/Substrate	Experimental Conditions	Rate†		Mechanisms	Notes	Reference
				Sterile	Live			
Alkanes and Aromatics	Spiked with petroleum oil	Salt marsh plant/soil mesocosms	Diurnal flooding/draining	ND‡	ND	ND	Inorganic fertilizer enhanced plant growth and loss rates; microbial inoculants had no effect	50
Phenanthrene	^{14}C spiked water	Estuarine water microcosms	Aerobic	ND	8–35% in 12 d	Mineralization	Rates enhanced in bulk water compared with air-water microlayer	51
Phenanthrene	^{14}C spiked sediments	Salinity gradient sediment microcosms	Aerobic	No loss	24–384 ng/g/d	Mineralization	Increased salinity enhanced rates	52
Oil and grease	Petroleum and industrial wastes	Treatment wetlands	ND	ND	54–94% (unspecified period)	ND	Rates depend on pollutant-loading	9
Phenols					10–94% (unspecified period)			
Crude oil	Native contamination and spiked soils	In situ and salt marsh soil microcosms	Aerobic	20 d	8–9 d	Volatilization; mineralization	Fertilizer enhanced rates in lab, not in situ	53
Crude oil	Spiked soils	Marine habitats, incl. mangrove and salt marsh	Aerobic and methanogenic	10–37% in 14 d	5–30% in 14 d	Volatilization; mineralization	Oxygen-dependent mineralization; alkanes degraded faster than aromatics; forced aeration and fertilization suggested to bioremediate	54
Surfactants	Pond and laundromat wastewater	Aquatic plant/sediment microcosms	Aerobic	ND	0–40% in 30 d	Mineralization	Rhizosphere enhanced	55

†Rates have units of half-life (d), zero-order rates, or percentage removal over specified time.
‡Not determined.

Table 3. Biotransformation of Industrial Organohalogens and Other Organic Pollutants in Wetlands and Wetland Microcosms

Chlorinated Aliphatics and Aromatics	Sources	Wetland System/Substrate	Experimental Conditions	Rate[†]		Mechanisms	Notes	Reference
				Sterile	Live			
Polychlorinated dibenzo-p-dioxin	Native and spiked	Estuarine sediment microcosms	Anaerobic	No loss	16–54% in 90 d	Reductive dechlorination	Electron donors (esp H_2) enhanced rates	56
Pentachlorophenol	Spiked	Wetland soil microcosms	Varied electron acceptors/ donors and PCP levels	No loss	0–100% in 30 d	Reductive dechlorination	Aerobic, methanogenic conditions enhance rates; toxicity, denitrification inhibits	31
Pentachlorophenol	Native and ^{14}C spiked	Estuarine sediments	Varied pH/redox	ND[‡]	0.3–68% in 34 d	Mineralization	High redox and pH 6–8 enhance rates	57
Chlorine disinfection byproducts (THM, HAA, TOX)	Tertiary treated domestic waste	Treatment wetland	ND	ND	13–97% (unspecified period)	ND	Potential for chlorination of wetland dissolved organic C	58
Tetrachloroethane	Native and spiked	In situ freshwater tidal wetlands and microcosms	Methanogenic	79% in 35 d	100% in 16 d	Volatilization; Dichloroelimination; reductive dechlorination	50% loss by vol. in 1 d; toxic intermediates produced biotically and abiotically	59
Mono halogenated benzoates	Spiked	Estuarine wetland and river inocula	Denitrifying, aerobic, sulfate reducing	No loss	0–100% in 12 d	Dehalogenation; mineralization	Denitrifying conditions enhance rates with most compounds	60
Other								
TNT (2,4,6 trinitrotoluene)	^{14}C spiked	Submerged plant hydroponic mesocosms	Aerobic	No loss	90% in 12 d	Plant uptake, alkyl, aromatic oxidation, nitro reduction, conjugation	Significant extracellular release of toxic plant root metabolites	61
TNT (2,4,6 trinitrotoluene)	Contaminated groundwater and ^{14}C spiked	Aquatic plant/sediment mesocosms	Aerobic	No loss	0.04–0.2% in 7 d	Mineralization	Plant uptake, sediment sorption responsible for 2–80% removal	62
RDX (hexahydro 1,3,5 trinitro1,3,5 triazine)				No loss	0.5–10% in 13 d			
22 organic compounds (alcohols, alkanes, nitro and chlorophenols, surfactants)	Spiked	Forest wetland soil microcosms	Methanogenic	ND	0 to >75% in 56 d	ND	Compound dependant; nitrophenol toxicity inhibits rates	63

[†]Rates have units of half-life (d) or percentage removal in specified time.
[‡]Not determined.

Table 4. General Guide for Predicting Transformations, Partitioning, and Transport of Organic Pollutants Based on Chemical and Environmental Characteristics (16,75)

Pathway	Organic Pollutant Characteristics that Promote Process	Environmental Regulators
	Abiotic transformation	
P hotodecomposition	Chemicals with chromophoric functional groups (e.g., aromatic rings, C=C, C=O, C−NO_2, C−OH, C−NH_2 groups)	Light penetration depth, pH, photoquenching and photoactivation agents
Nucleophilic substitution	Chemicals with electron-deficient center atoms (e.g., S,P,C atoms bonded to electron withdrawing groups, e.g., oxygen, halogens, ester, ether, amide, phosphoester, thioester groups, etc.)	Nucleophile concentration (e.g., pH, sulfide, amines, etc.)
	Novel Catabolic Enzyme Reactions	
Oxidoreductases		
Monooxygenases and dioxygenases	Chemicals with electron-dense center atoms (e.g., carbon atoms of alkanes, alkenes, aromatic compounds)	Presence of O_2 (indicated by $Eh > 300$ mV); affected by organic substrates, nutrients
Phenoloxidases and peroxidases	Chemicals with phenolic and aniline based structures can oxidatively couple with humic substances	Presence of O_2; humic substance concentration
Reductases	Chemicals with electron-deficient center atoms (e.g., carbon atoms bonded to electron withdrawing groups, e.g., NO_2, halogens, etc)	Reductive dehalogenation typically occurs under anaerobic conditions (e.g., $Eh < 300$ mV); affected by supply of electron donors and acceptors
Hydrolases	Similar chemicals as for nucleophilic substitution	pH, water content, temperature
	Partitioning	
Sorption	Chemicals with elevated organic C normalized sorption coefficients [$K_{oc} > 10,000\ mg\,kg^{-1}$ OC $\times$ $(mg\,L^{-1}H_2O)^{-1}$]; ionic compounds can be held by exchange processes	Soil pH and organic carbon content (>1%); soils with high ion exchange capacity, metal oxide reactive surfaces
Bioaccumulation	Chemicals with elevated octanol: water partitioning coefficients [$K_{ow} > 500\ mg\,L^{-1}$ octanol $\times$ $(mg\,L^{-1}\ H_2O)^{-1}$]	Food chain characteristics
	Transport	
Volatilization	Chemicals with high Henrys Law constant [$K_h > 0.01$ atm $\times$ $(mol/L)^{-1}$]	Temperature, pH, wind speed, concentration in air and water
Mobility in water	Chemicals with high water solubility ($>30\ mg\,L^{-1}$)	Hydraulic conductivity, diffusion coefficient, concentration, and matric-potential gradients

roles in the fate of organic pollutants. Several models predicting organic pollutant fate according to these factors are available (16,75); however, they have not been validated in wetland ecosystems.

Experiments that examine the environmental fate of organic pollutants in wetlands typically involve monitoring the concentration of the parent compound, or metabolic products in contaminated microcosms, mesocosms, or in situ wetlands (Tables 1–3). Microcosm and mesocosm studies have an advantage in that environmental conditions can be more carefully controlled, so that the influence of individual factors on the rate and mechanisms of organic pollutant removal can be more effectively described. For example, microcosm studies that include sterile controls can distinguish between losses from biochemical and abiotic reactions. Microcosm experiments employing stable- or radioactive-carbon labeled organic pollutants can distinguish between partial transformations to intermediate by-products and mineralization to carbon dioxide. These studies are most useful when devising pollutant-amelioration strategies in wetlands. On the other hand, these studies do not reflect the complexity of the wetland soil-water-plant environment, and so may not accurately reflect the pollutant-removal potential of wetlands. To date, few studies have attempted to integrate different scale experiments to explain organic pollutant fate in wetlands.

The fate of selected agricultural pesticides including atrazine, 2,4 D, dicamba, metolachlor, and metribuzin have been evaluated in wetlands (Table 1) (42–49). Surface runoff is the dominant source bringing agricultural pesticides to wetlands. Recent evidence suggests, however, that air deposition from agricultural areas may be an important source (76). After deposition into wetlands, pesticides are lost at variable rates, depending largely on the type of compound and environmental conditions. For example, metolachlor was lost at a rate of 0.5% in 84 days in microcosms constructed with tidal

wetland soil (49), whereas atrazine was lost at a rate of 100% in 7 days from mesocosms constructed from aquatic plants and gravel materials (43). In both studies, abiotic processes (volatilization, leaching, sorption) were the dominant loss mechanisms. Mineralization played a less important role; however, rates depended strongly on environmental conditions. Factors showing positive effects on pesticide biodegradation included microbial acclimation, inoculation of contaminated samples with adapted microorganisms, presence of a plant rhizosphere, and nitrogen availability. Inhibitory factors included toxicity and high nitrogen concentrations. Several pesticides were transformed to intermediates by numerous degradative mechanisms. The fate and toxicity of pesticide metabolites to wetland biota is largely unstudied. Most pesticide-fate studies have been conducted under aerobic conditions, although several TEAPs occur in wetlands. Hence, it is not yet possible to adequately predict the fate or ecological consequences of many organic pollutants in wetlands.

Several hydrocarbon-fate studies have been conducted in both coastal and inland wetlands (Table 2) (9,50–55). This is probably attributed to the frequency of wetland contamination by this class of pollutant from oil spills and leaking underground storage tanks. Crude oil components include a mixture of long- and short-chain alkanes, and mono- and polyaromatic hydrocarbons (PAHs). Significant hydrocarbon loss has been observed, as with pesticides. In microcosm studies, rates of loss were between 5% in 14 days (54) and 50% in 8 days (53). A literature review of hydrocarbon losses in treatment wetlands revealed between 10 and 94% during one retention period, typically less than two weeks (9). Several variables and processes influenced hydrocarbon loss. Those with positive effects included nitrogen and phosphorus fertilizers, salinity, and oxygen. Amending contaminated soils with hydrocarbon-adapted microorganisms played a minimal role, suggesting that indigenous hydrocarbon degraders may have inhabited the soil (50).

Extensive hydrocarbon loading and inadequate wetland mixing may have deleterious impacts on loss rates. Toxicity may affect the degrading populations. Extensive loading may also influence the oxygen status of the wetland. For instance, high loading rates would rapidly consume oxygen in the wetland soil-water profile. Under anoxic conditions, hydrocarbon mineralization is highly restricted (54). Volatilization is also a major hydrocarbon-removal mechanism in wetlands, accounting for up to 37% loss in 14 days for volatile oil components (54).

The fate of many organohalide industrial chemicals including polychlorinated dioxins, chlorophenols, trichloromethane, trichloroethane, and chlorobenzoates have been investigated in diverse wetlands (Table 3). Sources bringing these chemicals to wetlands include industrial waste disposal, landfill leachate, domestic wastewater, accidental spillage, contaminated groundwater, and residual pesticides in agricultural lands converted to wetlands. Organochlorine compounds are also generated in wetlands with high amounts of natural organic matter that receive chlorine-disinfected, tertiary treated wastewater (58).

Rates and mechanisms of organochlorine loss are highly compound and site-specific. Rates of parent compound loss range from zero to 100% in 12 days (Table 3). The dominant mechanisms of parent compound transformation/removal included mineralization, reductive dechlorination, and volatilization. Reductive dechlorination involves the replacement of a Cl^- ion with two electrons and a proton (H^+), which is catalyzed by bacterial reductase enzymes or other biological reductants (discussed in following section). Although reductive dechlorination results in less chlorinated intermediates, these are sometimes as toxic or more toxic than the parent compound. The major factors controlling reductive dechlorination are electron donor and acceptor availability because these serve as sources and alternative sinks of electron equivalents. Organic matter amendments may help to promote reductive dechlorination in the environment. Other factors such as pH and toxicity also play major regulatory roles (31).

The fate of munition wastes, 2,4,6-trinitrotoluene (TNT), and RDX have been evaluated in planted wetland mesocosms under aerobic conditions (61,62) (Table 3). In these two studies, the predominant sources of these wastes were surface runoff and groundwater. Loss rates were up to 90% in 12 days, and were predominantly caused by sorption and plant uptake. Biodegradation, which occurred through a series of oxidation, reduction, and conjugation pathways, played a less important role over the course of these two studies. In addition to uptake, plants played a further role on TNT fate by excreting toxic-degradation intermediates into the root zone (61).

The various studies indicate that several wetland processes play major roles in decreasing organic-pollutant concentration from wastewater. These included dilution, vegetative uptake, volatilization, sorption, and biodegradation. Except for biodegradation, the other processes may not represent detoxification mechanisms because they simply transfer pollutants from one wetland component to another, which may be later released to the environment. Therefore, one must be cognizant of the difference between parent compound removal and detoxification by the wetland.

MICROBIOLOGICAL TRANSFORMATION REACTIONS

The purpose of this section is to provide an overview of the processes in the external and internal cell environment, leading to the degradation of organic pollutants in wetland soils (Fig. 5). More detailed description of individual processes can be found in other entries of this Encyclopedia and other reference materials (7,13,15–18).

Microbiological transformations of organic pollutants are important because many abiotic reactions, although thermodynamically favorable, occur extremely slowly because of kinetic limitations. Microorganisms accelerate reaction rates by employing enzymes, which are special proteins sometimes linked with nonprotein coenzymes, and metallic and organometallic cofactors. Enzymes often increase reaction rates by 10^8 to 10^{20} times. Enzymes increase reaction rates by bringing chemical reactants together, converting them to reactive species, and holding them in the correct position for specific types of chemical

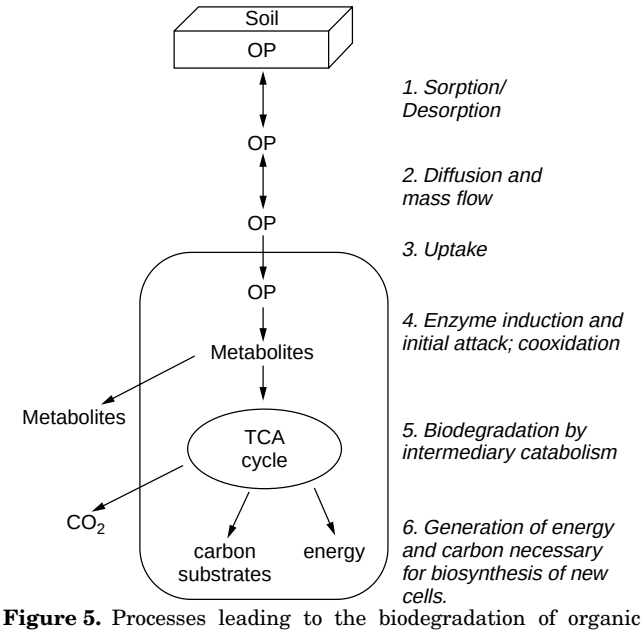

Figure 5. Processes leading to the biodegradation of organic pollutants (OP) in a soil type environment. [1 and 2. Desorption and migration of OP required for optimum microbial–OP interactions. 3. Microbial uptake of OP by passive and active transport mechanisms; 4. Induction of enzymes that either catalyze cooxidation (OP does not serve as carbon or energy source) or catalyze the production of common central metabolites that can enter into intermediary metabolic pathways. Key enzymes include those in oxidoreductase and hydrolase classes. 5. Biodegradation using common intermediary catabolic pathways, such as β-oxidation and the tricarboxylic acid (TCA) cycle. 6. Cell biosynthesis using energy and carbon generated from catabolic reactions].

reactions to take place. Because enzymes are regenerated after catalyzing a chemical reaction, they can repeat the process hundreds or thousands of times before losing activity. Most degradation enzyme reactions are intracellular, that is, they are conducted inside microbial cells.

Unfortunately, in wetlands and other soil environments, processes outside the cell may limit the overall degradation rate of organic pollutants (Fig. 5) (77). Perhaps the most important processes controlling microbial uptake and transformation of organic pollutants are sorption and desorption in the soil. Sorption is defined as physical and chemical interactions between a contaminant and the solid phase (e.g., soil minerals and organic matter) that reduce contaminant concentration in the dissolved pool. Sorption consists of several mechanisms that can be generally grouped into adsorption and absorption. Adsorption generally refers to two-dimensional surface phenomena and absorption consists of three-dimensional interactions between the soil (sorbent) and contaminant (sorbate). Sorption is the result of physical and chemical reactions with the solid phase including London-van der Waals forces, ion exchange, hydrogen bonding, ligand exchange, chemisorption, and hydrophobic interactions. The latter interaction occurs when hydrophobic contaminants get separated from the aqueous phase into the hydrophobic regions in the soil (e.g., organic matter). Combinations of adsorption and absorption phenomena occur in soil depending on the characteristics of the organic pollutant and soil. A common value used to quantitatively describe the extent of sorption is the equilibrium sorption coefficient, which is usually determined experimentally from sorption isotherms. For chemicals with high equilibrium sorption coefficients, greater amounts of chemical are associated with the solid phase compared with the dissolved phase. Desorption rates are typically slower for chemicals with higher sorption coefficients (78). Many microorganisms can enhance desorption by producing a variety of emulsifiers, or biosurfactants (7 and references therein). In addition, microbial organic–pollutant interactions can be dependent on the diffusive or convective migration of chemical toward the degrading microorganism. Sometimes these processes can limit overall degradation kinetics in contaminated environments.

In wetland environments, heterotrophic bacteria and fungi are primarily responsible for transforming or metabolizing organic pollutants. Metabolism refers to enzyme-catalyzed reactions occurring in living cells that synthesize new chemical structures for cellular growth (anabolism or biosynthesis) or break down chemicals to derive energy for cellular growth (catabolism). It is primarily through catabolism that organic pollutants are biodegraded in the environment.

Catabolism can be generally grouped into two categories, namely, central intermediary catabolism and peripheral (or novel) catabolic reactions. Intermediary catabolic pathways consist of core processes such as the tricarboxylic acid cycle and glycolysis, which are common to most living things, from human beings to microorganisms. Intermediary catabolic enzymes are typically constitutive, that is, they are always active in the living microbial cell. Peripheral catabolic reactions are rare processes, probably not ubiquitous in the microbial world, which organisms use to initiate organic-pollutant transformations. Typically, in the absence of the pollutant, a peripheral catabolic enzyme is not synthesized or its activity is repressed. Enzyme induction and derepression occur when the pollutant or another molecule is introduced. This may account for a lag period, or interval of time, before degradation is observed. Typically, a small subset of the total microbial population may be capable of carrying out the enzymatic reactions that initiate pollutant metabolism.

The main strategy used by organisms to degrade organic pollutants is to employ one or more catabolic enzymatic reactions, to yield common metabolites that feed into the existing intermediary catabolic pathways (Fig. 5). After desorption and migration of pollutants to microbial cells, peripheral catabolic reactions are often the biochemical rate-limiting steps to pollutant degradation, and will be the focus of the remainder of this section.

Enzymes are currently grouped into six classes by the Enzyme Commission (EC), including oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases (see Nomenclature Committee of the International Union of Biochemistry and Molecular Biology Enzyme Nomenclature, *http://www.chem.qmw.ac.uk/iubmb/enzyme/* and The University of Minnesota Biocatalysis/Biodegradation Database, *http://umbbd.ahc.umn.edu/index.html*). Enzyme classes are primarily distinguished by the type of

reactions that they catalyze. Of the six enzyme classes, the oxidoreductases and hydrolases are most frequently involved in novel catabolic pathways. The enzymes and their activity depend on the type of organism (e.g., fungi, bacteria, aerobe, anaerobe), type of compound (e.g., the types of functional groups), and environmental conditions (e.g., pH, temperature, availability of oxygen and other electron acceptors) (Table 4).

Oxidoreductases catalyze redox reactions in which hydrogen atoms, oxygen atoms, or electrons are transferred between molecules. Oxidoreductases are divided into subclasses, and are named based on the type of donor and acceptor molecule and the entity that is transferred. For example, dehydrogenases or reductases transfer hydride ions, oxidases transfer electrons to diatomic oxygen, peroxidases transfer electrons to hydrogen peroxide, and oxygenases transfer oxygen atom(s) from diatomic oxygen. Oxidoreductases are the largest class of enzymes, and act on an enormous variety of organic pollutants. Except for those catalyzed by the dehydrogenases (reductases), all oxidoreductase reactions, directly or indirectly, require oxygen and so occur in aerobic environments.

Some of the most important examples of oxidoreductases involved in the initial phases of pollutant degradation are the oxygenases (see OXYGENASE ENZYMES: ROLE IN BIODEGRADATION, this Encyclopedia) (Fig. 6). Oxygenases are enzymes that catalyze reactions between diatomic oxygen (O_2) and organic or inorganic substrates, which results in products that contain one or both of the oxygen atoms. Oxygen atoms are usually incorporated at locations in the molecule with the most readily available electrons, which often include aromatic rings, double bonds, alkyl groups, and nonbonded sulfur and nitrogen structures. Chemicals containing electron-deficient central atoms, such as highly chlorinated polychlorinated–biphenyls, -phenols, and -aliphatics, are often resistant to oxidation by oxygenases, peroxidases, and phenoloxidases (79,80). These compounds may be more readily transformed by dehydrogenases or reductases of the oxidoreductase class (see discussion that follows).

There are two main types of oxygenases, namely, monooxygenases and dioxygenases (Fig. 6). The monooxygenases incorporate one atom of oxygen into organic molecules, either as a hydroxyl group to one of the central atoms (hydroxylase-type monooxygenase), or an oxygen atom between a carbon–carbon bond (Baeyer Villiger monoxygenase). Dioxygenases insert two atoms of oxygen into organic molecules, either as two hydroxyl groups on carbons that lack hydroxyl groups (hydroxylase-type dioxygenase), or as two oxygen atoms of an aromatic ring already containing two hydroxyl groups (ring cleavage type dioxygenase).

Over a hundred different oxygenases are known and many are produced by heterotrophic bacteria and fungal species to facilitate the extraction of carbon and energy from organic substrates, such as lignin and cellulose in plant litter. Common examples of bacterial genera that transform organic pollutants using oxygenases include *Pseudomonas, Alcaligenes, Sphingomonas, Rhodococcus*, and *Mycobacterium*. Some oxygenases are relatively nonspecific with respect to the types of compounds that they transform; for example, naphthalene 1,2-dioxygenase reacts with at least 72 different substrates (81). In some cases, oxygenases intended for one compound also transform other organic pollutants. The term *cooxidation* applies to situations when the enzyme-producing organism does not gain carbon or energy from the reaction using the nonintended molecule. However, in an assemblage of several different microbial groups, other members may complete biodegradation of the cooxidation products. Two or more microbial groups acting together in a community in such a manner, is known as a *microbial consortium*.

By incorporating oxygen atoms, oxygenases chemically activate inert compounds and cleave cyclic rings (Fig. 6). Oxygenase reactions are essential early steps in aerobic mineralization of many compounds, consisting of only carbon and hydrogen, such as alkanes (e.g., octane in gasoline), alkenes, benzene, biphenyl, and PAHs. In the absence of oxygenase activity, these compounds are transformed much more slowly. Products of oxygenase reactions include alcohols, aldehydes, ketones, esters, epoxides, phenols, and carboxylic acids. Oxygenase reactions often lead to the replacement of halogen substituents and other functional groups of organic pollutants with hydroxyl groups. For example, both membrane-bound flavoprotein monooxygenases of *Flavobacterium* and *Arthrobacter* species and cytochrome P_{450} monooxygenase of *Mycobacterium* species para-hydroxylate pentachlorophenol (PCP)-producing tetrachlorohydroquinone (82–84). Oxygenase reactions often yield common metabolites that are biodegraded through intermediary catabolic steps, such as β-oxidation and the tricarboxylic acid cycle.

Other types of oxidoreductases, including peroxidases and phenoloxidases, are involved in the oxidative coupling reactions between certain types of organic pollutants and humic substances in the soil (36) (Fig. 6). These enzymes catalyze one-electron transfer from aniline-based and phenolic compounds to acceptor molecules of hydrogen peroxide and oxygen. One-electron oxidation of these organopollutants generates free radicals that react with other free radicals (e.g., from native humic substances) to form large molecular weight polymers. Polymerization of phenols can also be abiotically catalyzed by Mn(IV), Fe(III), and Al and Si oxides (85). Oxidative coupling is thought to be analogous to the humification process, and may benefit organisms by removing pollutants from the bioavailable pool (69,86).

Aerobic fungi and bacteria that produce oxidative-coupling enzymes have been discovered in wetland soils and plant rhizosphere, suggesting that oxidative coupling may be prevalent in the oxic zones of these environments (27,87,88). The production and activities of these enzymes may be promoted by manipulating the types of carbon substrates, pH, nitrogen, copper, manganese, and oxygen availability. Because of the requirement of oxygen (or hydrogen peroxide), oxygenase, peroxidase, and phenoloxidase reactions are restricted to the oxic soil–water interface and oxidized rhizosphere in flooded wetlands. On the basis of this understanding, it would be possible to control many oxidoreductase reactions by manipulating wetland hydrology.

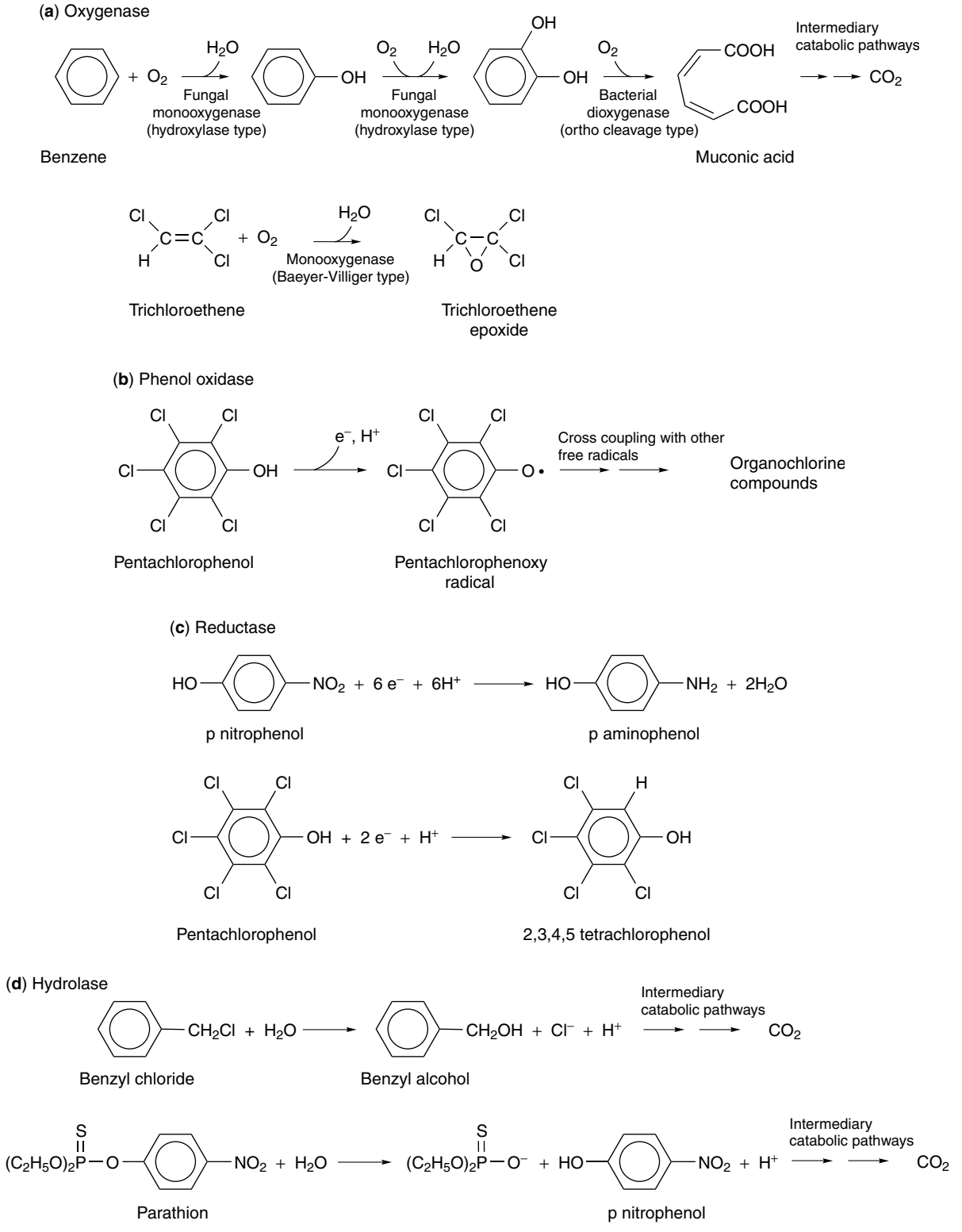

Figure 6. Some microbially mediated organic pollutant transformations by catabolic enzymes. (**a**) Oxygenases, (**b**) Phenol oxidase, (**c**) Reductases, and (**d**) Hydrolases.

Reductases are important examples of oxidoreductases involved in the peripheral catabolism of many organic pollutants (Fig. 6). These enzymes catalyze the transfer of hydride ($H:^-$) from donor to acceptor molecules. Donor molecules typically include NAD[P]H and other biological reductants generated from oxidation of organic compounds. Hydride atoms are usually incorporated at locations of the pollutant molecule containing oxidized (i.e., electron-deficient) central atoms, including those attached with electron-withdrawing functional groups. Examples of electron-withdrawing groups include hydroxyl, carbonyl, nitro, sulfoxide, sulfone, and halides.

Reductive dehalogenation is an important example of a reduction reaction that commonly occurs in anaerobic environments, including wetland soils (see Reductive Dehalogenation of Haloorganics, this Encyclopedia) (Table 3). Reductive dehalogenation involves the replacement of a halogen substituent of an organic pollutant with two electrons and a proton (Fig. 6). Electrons are, directly or indirectly, derived from the oxidation of organic substrates and hydrogen, and protons are derived from the dissociation of water. Dehalogenation reductases, known as *dehalogenases*, have been isolated from both microbial membranes and cytoplasm. Dehalogenases often contain reduced transition metal cofactors (e.g., Fe-S, heme, corrinoid groups) that are involved in electron transfer. Reductive dehalogenation has been observed for a multitude of alkyl halides (DDT, chloroform, PCE, TCE, lindane) and aryl halides (PCBs, chlorobenzoates, chlorophenols, chlorobenzenes).

Several organisms (e.g., *Desulfomonile tiedjei, Desulfitobacterium chlororespirans*, and others) gain energy for growth by coupling the oxidation of organic compounds to the reduction of organohalogens (89,90). This process is known as *halorespiration*, and is conceptually similar to the respiratory processes using oxygen or alternative electron acceptors (91,92). Many organohalogens have reduction potentials at par with Mn^{4+}, NO_3^-, and Fe^{3+}, and greater than SO_4^{2-} and HCO_3^-. Therefore, organisms conducting halorespiration gain a significant amount of energy from the reaction, and perhaps, outcompete other groups. Halorespiration is important because it means that the organohalide respiring bacteria can proliferate in contaminated environments, and so may facilitate the degradation process.

The presence of alternate electron acceptors such as O_2, NO_3^-, Fe^{3+}, and even other chlorinated organic pollutants typically inhibit reductive dechlorination of highly chlorinated compounds, presumably because they divert electrons away from the pollutant molecule (31). Also, less chlorinated compounds often do not undergo reductive dechlorination as rapidly as highly chlorinated ones, presumably because they are less effective electron acceptors (i.e., have lower reduction potentials). Hence, reductive dechlorination commonly results in the accumulation of toxic intermediates.

Several polyhalogenated organic pollutants are reductively dehalogenated by various metallo-porphyrins, including cytochrome P_{450} containing Fe^{2+}, vitamin B_{12}, and other corrinoids containing Co^{1+}, and cofactor F_{430} containing Ni^{1+} (93,94). The reduced metal ions donate one electron to alkyl halide (e.g., tetrachoroethene, carbon tetrachloride) and aryl halide (hexachlorobenzene) acceptors, resulting in dehalogenated products. Reduced metalloporphyrins are produced by many anaerobic bacteria, including acetogens, sulfate reducers, and methanogens. It is thought that these reactions do not generate significant amounts of energy for the microorganisms.

Reductive dehalogenation carried out by dehalogenases or metalloporphyrins frequently renders molecules more susceptible to aerobic and anaerobic catabolic breakdown. Because lesser halogenated products are amenable to aerobic degradation, it has been suggested that sequential anaerobic-aerobic microbial processes may be effective at detoxifying these chemicals. Wetlands provide a unique environment to promote these sequential reactions because they contain aerobic and anaerobic interfaces. For example, reductive dechlorination of an organohalide such as pentachlorophenol (PCP) to lower chlorinated congeners under anaerobic conditions (perhaps induced by soil flooding) could be subsequently oxidized to carbon dioxide under aerobic conditions (induced by soil draining). Alternatively, lower chlorinated congeners produced through reductive dechlorination in anaerobic soil layers in a flooded soil system may diffuse to aerobic zones (e.g., the soil–water interface or rhizosphere), and subsequently be metabolized to carbon dioxide. Regulating water table depth and flooding duration, establishing wetlands with macrophytes with high oxygen transport capacities, amending soil with electron donors, and controlling inputs of alternative electron acceptors can artificially control these processes.

In addition to the oxidoreductases described in the previous paragraphs, enzymes in the hydrolase class are extremely important in peripheral catabolic steps of many organic pollutants. Hydrolases catalyze the hydrolytic cleavage of ester, ether, anhydride, amide, and halogen bonds associated with organic pollutants such as organophosphates, phenoxyacetates, phenylureas, acid anilides, phenylcarbamates, and alkyl halides (Fig. 6). In hydrolytic reactions, the oxygen atom of water replaces hydrogen, chloride, phosphate, sulfate, and amino groups of the organic pollutants. The oxygen atom is usually inserted at similar positions in the molecule as the hydride atom catalyzed by reductases, that is, at electron-deficient central atoms. The products of hydrolytic reactions often resemble the oxidation products generated by oxygenases; however, the incorporated oxygen atoms are derived from water instead of diatomic oxygen. Hydrolytic reactions typically have wide specificity, are constitutive (i.e., always present in the cell), and are active in both aerobic and anaerobic environments. For many organic pollutants in anaerobic environments, hydrolysis is a critical mechanism because the addition of the oxygen atom activates the molecule for further catabolic breakdown, or facilitates expulsion of toxic compounds from the microbial cell. Many hydrolytic enzymes are excreted and catalyze organic pollutant reactions in the extracellular environment, resulting in by-products that may be taken up for microbial use. Heterotrophic aerobic and anaerobic microorganisms produce hydrolytic enzymes as a response to nutrients, suggesting that it

may be possible to manipulate this process through soil amendments (95,96).

CONCLUSION

Wetlands are effective treatment alternatives for conventional pollutants such as nutrients, organic carbon, and total suspended solids (1,8). In recent years, there has been increasing interest in wetland processing of organic pollutants, largely because of the extent of contamination from several point and nonpoint sources. Although excessive nutrient pollution causes eutrophication of wetlands and aquatic ecosystems, organic pollutants can result in additional acute and chronic toxicological effects (12). Organic pollution can be especially tragic because wetlands attract many fish and wildlife species. Documented cases of organic pollutant effects on wetland functioning include the disruption of the aquatic food chain (97–101), bioaccumulation of toxaphene and other chlorinated compounds in turtles, ducks, and fish (102–108), reproductive disorders in alligators from DDT contamination (106), and massive mortality of white pelicans from organochlorine pollution (110). These events emphasize the importance of taking a comprehensive, ecological-based risk assessment approach when attempting to use wetlands to treat organic pollutants (111). Wetland treatment of organic pollutants may be incompatible with the value of wetlands as wildlife habitats (112).

The dominant organic pollutant removal processes in wetlands are sorption to soil and sediment, volatilization through plants and from the water surface, uptake in the plant biomass, and biodegradation by soil microorganisms. Pollutant removal through these processes is highly variable, and depends on the type of compound and environmental conditions in the wetland, such as the extent of organic matter accumulation, nutrient availability, presence of oxygen and electron acceptors, and redox interfaces. However, one set of wetland conditions may be optimum to remove one type of compound, and inhibitory for the removal of another. Therefore, a successful bioremediation strategy must consider the type of wastes, and how mitigation actions might affect other biogeochemical processes in the wetland (e.g., fertilization effects on eutrophication).

Experiments using laboratory microcosms have proved invaluable in elucidating potential detoxification mechanisms in environmental samples. From these results, scientists and engineers have postulated and implemented several remediation strategies. However, several basic questions remain unanswered with respect to understanding organic pollutant biodegradation in wetlands: What microorganisms are involved in degradation? What are the mechanisms and regulators of these processes? What criteria should be used to define when wetland contaminants no longer pose an ecological risk? (113,114). Difficulties in addressing these issues are largely methodological ones. Traditional methods do not contend well with quantifying diverse biochemical processes occurring at microbial scales (1 to 100 μm) in the landscape. Encouragingly, several methods have recently been developed to improve our ability to characterize microbial populations and their activities in the environment (113). Examples of these new methods include extraction and analysis of genetic and other microbial biomarkers (e.g., DNA, mRNA, microbial lipids) and enzyme activities (e.g., laccases, reductases, oxygenases, hydrolytic enzymes). Selective chemical extractants (115) and biosensors (116,117) (genetically engineered microorganisms that respond to environmental pollutants) are being developed to quantify the bioavailability and toxicity of pollutants. These methods are yet to be applied in wetlands or other field habitats. Results obtained using these approaches will probably prove fruitful in establishing process-based remediation, and monitoring programs for contaminated environments in the next decade.

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WETLANDS, FUNGAL ACTIVITY IN.

See WATER FUNGI AS DECOMPOSERS IN FRESHWATER ECOSYSTEMS

WETLANDS, PERIPHYTON IN.

See PERIPHYTON

WHITE ROT FUNGI.

See PULP AND PAPER INDUSTRY: MICROBIOLOGICAL ASPECTS OF

WHITE ROT FUNGI IN BIOREMEDIATION.

See PESTICIDE DEGRADATION IN SOILS

WOOD DECOMPOSITION BY FUNGI.

See WATER FUNGI AS DECOMPOSERS IN FRESHWATER ECOSYSTEMS

WOOD DEGRADING FUNGI.

See FUNGI, FOR BIOTECHNOLOGY

WOOD PROCESSING AND BIOAEROSOLS.

See BIOAEROSOLS IN INDUSTRIAL SETTINGS

X, Z

XEROTOLERANT MICROORGANISMS

See Desiccation by Exposure to Space Vacuum or Extremely Dry Deserts: Effect on Microorganisms

ZOONOTIC TRANSMISSION OF PARASITE

See Cryptosporidium: basic biology and epidemiology

ZOONOTIC TRANSMISSION OF PATHOGENS

See Leptospirosis

INDEX

A
ABC bacteria, 2471
Abies, ectendomycorrhizal fungi, 2125–2126
Abrasion, biofilms vs., 547
Absicisic acid, 344
Absorption (See also Sorption), biofiltration, 590–591
Acacia, 2785
Acanthamoeba, 2594–2604
  Antarctic soils and, 1026
  biofilms and, 2342–2356
  bottled water and, 800
  desert environment and, 1579
  endosymbiosis and, 1123
  meningoencephalitis and, 1343–1350
  in source water, 2968, 2969–2978
  in spas and hot tubs, 2980–2984
  in wastewater/sewage, 2329–2337
  in water treatment systems, 1718
Acantharian, 2617
Acartia, 972
Acaryochloris, 964
Acaulospora, mycorrhizae, 2120–2124
Acclimation of microorganisms, 713, 2386
Accumulation vs. nonaccumulation, soil salinity, 2781
Acenaphthene, biodegradation, 2403–2416
Acenaphthylene, 696–712, 2403–2416
Acenapthalene, bioremediation, 696–712
Acetalglucosame, 458
Acetate, 2172
  activated sludge and, 741–748
  anaerobic granulation and, 235, 236
Acetic acid, 3147–3155
Acetobacter
  nitrogen fixation and, 2193–2201, 2938
  petroleum reservoirs and, 2430
Acetogenesis, acetogenic microbes
  activated sludge and, 741–748
  biofilms and, 83
  petroleum reservoirs and, 2430
  soil microorganisms and, 2892
Acetohalobium, 1514–1529
Acetoin, alkaliphiles, 228
Acetyl coenzyme A (CoA), 21–26, 683–692
Acetylcholine (ACh), 847
Acetylcholinesterase (AchE), 847
in wastewater/sewage, 3170
Acetylene reduction assay (See Nitrogen fixation in soils)
Achnanthes, 1053, 1054, 1055, 1057–1063
  periphyton and, 2357
  subaerial communities and, 3057–3065
Achromacia, 1337
Achromatium, 3111–3116
Achromobacter, 1200
  biodegradation and, 2386
  granular activated carbon (GAC) filters and, 1490
  heterotrophic bacteria and, 1541–1552
  rhizosphere and, 2717
Acid–base (AB) surface energies, hydrophobicity, 1600–1601
Acid–base levels (See pH levels)
Acid degradation in biodeterioration of mineral materials, 517
Acid mine drainage (See also Biomineralization), 828–832
  acidophilic chemolithotrophs, 677
  algae biotechnology and, 199–200
  biofilms and, 84
  bioleaching and, 633, 640, 642–650
  biomineralization and, 677
  bioremediation and, 709
Acid rain, 2679
Acidaminobacter, activated sludge, 744–748
Acidianus, 1235, 1629–1646, 3144–3147
  Archaea and, 298
  bioleaching and, 632–642, 645–650
Acidic lipids in biosurfactants/bioemulsions, 756–757
Acidification, nitrogen cycle, 2942–2943
Acidimicrobium
  activated sludge foaming and, 3–7
  bioleaching and, 645–650
Acidiphilium, bioleaching, 644–650
Acidithiobacillus
  biodeterioration of mineral materials and, 524
  bioleaching and, 632–650
  weathering, bioweathering and, 3366
Acidobacterium
  desert environment and, 1578
  flooded soils and, 1320
  soil microorganism and diversity in, 533, 538, 539
Acidophiles (See also Extremeophiles), 677, 1235, 2889, 3129
Acidophilic chemolithotrophs, 677
Acidothiobacillus, 881–882
Acidovorax, 3314–3319
  activated sludge and, 71
  granular activated carbon (GAC) filters and, 1490
Acineria, 2595–2604, 2606
Acinetobacter, 499, 757, 1196, 1197, 1855, 1302–1306, 3314–3319
  activated sludge and, 38, 71
  bacterial counts using, 27–29
  biodegradation and, 2407
  biofilms and, 566, 572, 579, 2342–2356
  bioremediation and, 715
  biosurfactants/bioemulsions and, 714–715, 750–770, 1129–1130
  desert environment and, 1578
  extracellular polymeric substances (EPS) and, 1230
  fluorescent studies of, 1835
  gene transfer and, 1413–1415, 1417
  granular activated carbon (GAC) filters and, 1490, 1569–1570
  in groundwater, 1671
  heterotrophic bacteria and, 1541–1552
  infectious airborne bacteria and, 1694–1695
  nosocomial infections and, 2236–2250
  resistance in, 607
  streams and, 3019
  subsurface microbial communities and, 2035, 3065–3071
  in drinking water, 944, 945
Acquired genes, 1446
Acrasiomycota, 2912
Acremonium
  in allergies and illness, 1367–1372
  cold-adapted microorganisms and, 881
  in drinking water, 945
Acridine orange (AO), 1676, 1776–1778, 1949, 2653, 3196
Acrylacylamidase, in soil, 2903–2910
Acrylonitrile, bioremediation, 707
Actinides, 671, 1902
Actinobacteria
  bacterioplankton and, 2524–2526

Actinobacteria (*continued*)
flooded soils and, 1320
soil microorganism and diversity in, 533
Actinocyclus, 1866–1869
Actinomycetes (See also Soil microorganisms), 1, 458, 2549, 2882, 2912
activated sludge foaming and, 1–7,26–43
airborne, 1
Antarctic soils and, 1027
biofilms and, 1083, 2342
desert environment and, 1577
filamentous forms of, in activated sludge, 1293–1306
granular activated carbon (GAC) filters and, 1490
halophiles and, 1506–1512
mycolic acid containing, 3
nonfilamentous, 4
soil microorganisms and, 2889
soil salinity and, 2775
in drinking water, 946
Actinopoda, 2605, 2618
Actinopolyspora, 1506–1512
Actinorhizal Frankia symbiosis, 2208–2209
Actinorhizal symbiosis (See Nitrogen fixation in soils)
Actinorhizosphere, 2714
Activated carbon (See also Granulated activated carbon)
activated sludge and, 44
biofiltration and, 590
biologically active carbon (BAC), 1489, 1491
granular activated carbon (GAC) filters in, 315, 1489–1496
Activated sludge, 738
aeration/oxygenation in, 46, 74–77, 739
aerobic processes in, 44, 46, 77, 79
algal blooms in, 77
amino acids and, 743–744
amoeba in, 2605
anaerobic ammonium oxidation (anammox) in, 11–14, 38, 71
anaerobic processes in, 1313–1314
anaerobic/aerobic storage in EBPR process and, 3011–3013
anoxic processes in, 77, 79, 1313–1314, 3011
astroviruses and detection, 334
ATV guidelines for, 49
bacteria counts in, 69, 70–71, 738–739
bacteriophages and, 360, 381
bacteriovorous ciliates in, 2611
balanced growth conditions in, 3007
Bardenpro reactor for, 77
bioaugmentation in, 40
biofilms and, 44, 51–52, 54, 58
biogas yield from, improving, 746–748
biological nutrient removal (BNR) and, 1313–1314
biological oxygen demand (BOD) in, 49, 50, 745–746
biomass in, 72, 74, 739, 1311, 1315
biosolids and, 738–750
bulking in, 1287–1306, 1306–1315, 2002, 3008–3011
capsular polymers in, 740
carbon dioxide and, 738
cell death and cell lysis in, 745–746
chemical oxygen demand (COD) for, 14–26, 49, 50
chemical pretreatment of, 747–748
ciliates in, 2605–2607, 2610–2611
clarification of, 59
community composition of, 26–43, 68–74, 75
completely mixed batch reactor (CMBR) for, 44, 75–76
completely mixed flow reactor (CMFR) for, 44, 45, 75–76
composting of, 738
configurations systems for, 75–77
contact stabilization in, 76–77
contact zones, contact times in, 1313
contaminants and, 44–45, 46
culturing, 69
Deep Shaft process in, 76–77
denitrification of, 34–38, 49, 58, 77–78
design parameters for, 1313–1314
dewatering of, 54, 59–60, 748
dissolved oxygen (DO) in, 1311
enhanced biological nutrient removal (EBNR) in, 70
enhanced biological phosphorus removal (EBPR), 15, 26, 32, 38–40, 62, 64–67, 78–80, 1311, 1313, 3005, 3011–3013
eutrophication in, 77
exopolymeric compounds in, 739–740
extracellular polymeric substances (EPS) in flocs of, 54
fatty acids in, 741, 744
fermentation in, 741
filamentous bacteria in (See also Filamentous bacteria; Filamentous bulking), 1–7, 69, 70, 1287–1287
flagellates in, 2605, 2611
floc formation in, 5–7, 44, 54–61, 68, 73, 75, 1288–1306, 1306–1315, 2002
foaming in, 1–7, 75, 1287–1306, 2002, 3008–3011
food chain within, 72
functional microorganism groups in, 32–40
G bacteria in, 40, 57, 61–68, 71
glycogen in, 73, 3005
growth rate of microorganisms in, 72
hepatitis viruses and, 1534
history of, 74–75
hydraulic residence time (HRT) in, 49
hydrophobic particles in, 44
hydroxyalkanoates in, 73
identifying microbes in, 70
improvements to, 50–51, 80
improving performance of, 73
International Water Association (IWA) model for, 49
kinetic (internal) induction and, 3007–3008
lipids in, 741
Ludzack Ettiger process for, 78
measuring bacterial activity in, 3342–3345
mechanical pretreatment of, 746–747
metabolic (external) induction and, 3006–3007
methane and methanogenesis in, 738, 740–748
mixed liquid suspended solid (MLSS) ratio in, 54
models of, microbiological basis for, 14–26, 14
Modified UCT process (MUCT) process for, 79–80
nitrification in, 49, 74, 76
nitrifying bacteria in, 34–35
nitrogen removal from, microbiology of, 8–14
nonaqueous phase liquids (NAPLs) in, 44
nucleic acids in, 741, 744
nutrient content of sludge following, 75, 1311
Opercularia in, 2611
oxic contact zone in, 1313
parasitic protozoa in, 2607
pathogenic protozoa in, 2607

pH levels and, 72, 1311
phases of operation in, 46–47
phosphorus removal in (See also Enhanced biological phosphorus removal), 38–40, 77
plant configuration and, 1312–1313
plug flow reactor (PFR) for, 44, 75–76
plug flow reactor with dispersion (PFDR) for, 44, 75–76
polyhydroxyalkanoates (PHA) in, 21–26, 78–80, 3005
polyphosphate accumulating bacteria (PAB), 64–67, 78
polyphosphates in, 73, 3005
polysaccharides in, 741–743
powdered activated carbon (PAC) and, 44
process of, 74–80
proteins in, 741
protozoa and, 2332, 2605–2612
purines in, 741
pyrimidines in, 741
return activated sludge (RAS) in, 75, 79
sampling in, 68–69
sequencing batch biofilm reactor (SBBR) for, 51–52
sequencing batch reactors (SBR) for, 43–54, 75, 2002
settling in, 59, 75, 1306–1315
simultaneous nitrification/denitrification (SND) in, 77
sizing of SBR plants in, 48–49
sludge biotic index (SBI) and, 2609–2611
sludge volume index (SVI) for, 1288
slurry systems for, 51–52
soil slurry sequencing batch reactor (SS-SBR) for, 51–52
solids retention time (SRT) and, 2006
specific bacterial growth rates in, 3344–3345
stabilization of, 738
storage polymers in, 3004–3014
structure of, 738–740
sugars in, 741–743
syntrophic degradation in, 744–745
temperature and, 1311
testate amoeba and, 2610
thermal pretreatment of, 747
total extended filament length (TEFL) in, 1307
toxic chemicals and heavy metals in, 72, 75
unbalanced growth conditions, 3006–3008
University of Cape Town (UCT) process for, 79–80
upflow anaerobic sludge blanket (UASB), 2002
viruses in, 70
volumetric bacterial growth rates in, 3344
waste activated sludge (WAS) in, 738
wastewater/sewage treatment plants and, 739
water in, 740
Wuhrmann process in, 77
zone settling velocity (ZSV) in, 1307
Activity testing, fluorescent probes and, 1783–1784
Acute exposure, toxicity testing, 3220–3221
Acute hemorrhagic conjunctivitis (AHC), 1151
Acute hemorrhagic cystitis, 93–100
Acute respiratory disease (ARD), 93–100
Acutiphycin, 976
Acylated homoseriine lactone (AHL), 567
Acyrthosiphon, 1114–1115
Adamsite (See Chemical warfare)
Adaptation
biochemical, 1195
evolution and, genetic adaptation, 1195
polluted environment and, 1194–1195
soil microorganisms and, 2057
Adenosine diphosphate (ADP), 1125
aerobic respiration and, 149–151
biomass estimation and, 2654
subsurface microorganisms and, 2022
Adenosine triphosphate (ATP), 89, 1125
activated sludge denitrification and, 9–14
aerobic respiration and, 149–151, 154, 157–158
alkaliphiles and, 138–139
assimilable organic carbon (AOC) and, 323
bacterioplankton and, 2521
biodegradation and, 2931
biofilms and formation of, 82, 83
bioluminescence and, 657–663
biomass estimation and, 2654
metal biodegradation and, 1878
oligotrophic bacteria and, 2273
soil microorganisms and, 2883–2884, 2889, 2948
subsurface microorganisms and, 2022
sulfate-reducing bacteria (SRB) and, 3087
wastewater/sewage treatment and, 3338–3339
Adenoviridae, 93
Adenovirus, 92–100, 2745
in spas and hot tubs, 2980–2984
in source water, 2969–2978
Adenylate energy charge (AEC), 2022
Adhesion (See also Biofilms), 113–132
acid–base interactions and, 104–105
adsorption and, 114
atomic force microscopy (AFM) and, 125, 126, 128–129
batch and packed bed systems to study, 118–119
biofilms and, 100–113, 560–561, 612–613, 628–629
bioleaching and, 635–636
boundary layers and, 114
Brownian motion and, 114, 115
centrifugation vs., 116–117
colloidal particles and, 127–128
conditioning films and, 101, 127, 938–939
deposition and, 114, 117–118
Derjaguin Landau Verwey Overbeen (DLVO) theory of, 102–104, 119–122, 124, 561, 2004–2005
diatoms and, 1052–1053
electrostatic interactions in, 103–104,
extracellular polymeric substances (EPS) and, 1224–1225
free energy of adhesion in, 101
heterogeneity of cell and substratum in, 128
hydrodynamic testing for, 117–118
hydrophilic microorganisms and, 100
hydrophobic microorganisms and, 100, 1599–1604
interfacial free energies in, 122–124
ionic strength and, 121–122
laminar flow and, 117
Lifshitz-van der Waals forces in, 101–104, 123, 124, 936, 938
macromolecule formation at interface in, 125–127
marine biotechnology and, 1855–1856
microsphere attachment and, 128

Adhesion (See also Biofilms), (*continued*)
pH levels and, 100, 104–105, 121–122
physicochemical aspects of, 119–124
Reynolds number and, 114
Robbins device for, 118
secondary minimum in, 104
sedimentation and, 114–116
shear stress and, 117
soil microorganisms and, 2062–2063, 2926
static systems for, 115–116
strength of, evaluating, 116–117
study of, methods for, 114–119
substratum interfaces and, 124–129
surface conditions vs., 124–129
surface modification vs., 120–121
van der Waals forces, 119–120, 124
Young equation in, 122–123
zeta potentials and, 100, 119
Adsorption (See also Adhesion; Sorption)
adhesion in biofilms and, 114
aggregates and consortia in, 162
bacteriophages and, into soil, 358, 389
biofiltration and, 590–591
conditioning films and, 937
enteroviruses and, 1169–1170
floc formation and, 57
home water treatment systems using, 1566
Aeolosoma, 1722
Aequorea (See also Green fluorescent protein), 559, 1341,1430, 1831
Aeration/oxygenation, 5–7, 74–77, 2017, 2266
Aerobacter
bioaerosols and, 3321
floc formation and, 2006
heterotrophic bacteria and, 1541–1552
Aerobic alkaliphiles, 133–140
Aerobic bacteria, bioaugmentation and bioremediation, 435
Aerobic bacteriochlorophyll containing (ABC) bacteria, 2471
Aerobic denitrification, activated sludge denitrification, 14
Aerobic endospores, 140–149
Aerobic processes
activated sludge and, 44, 46, 77, 79
aerobic storage in EBPR process and, 3011–3013
aggregates and consortia in, 161
biodegradation and, 2930–2932
explosives biodegradation using, 1987
flooded soils and, 1328
hydrocarbon degradation and, 2404–2411
hyperthermophiles and, 1633
oxygen and, 2277
petroleum reservoirs and, 2427
wetlands and, 3403–3405
soil microorganisms and, 2888
municipal solid waste landfills and, 1759–1762
Aerobic respiration, 149–160
adenosine diphosphate (ADP) in, 149–151
adenosine triphosphate (ATP) and, 149–151, 154, 157–158
chemiosmosis in, 149–151
conservation of energy in, 149–151
cytochromes, cytochrome c pathway in, 153
detoxification in, 151
diversity of respiratory chains and, 155–157
electrogenic protein complexes in, 150
electron transfer and charge separation in, 151, 153–154
F1F0 ATP synthase and, 157–158
Gibbs equation in, 149
heme copper oxidases in, 155
iron–sulfur centers in, 152–153
lipids and, 151–152
lithotrophs and, 151, 157
NADH in, 149–154, 156, 157
nitrifiers and, 151
oxidases in, 154–155
oxidative phosphorylation and, 149–151
oxygen sensing regulatory proteins in, 158
porphyrins in, 153
proton motive force (PMF) in, 150
pyrroles in, 153
Q loop in, 153
quinones and Q pool in, 151–153, 156, 157
regulation of, 158–159
succinate dehydrogenase and, 153, 154
Aeromicrobiology, 3320–3330
Aeromonas, 313, 386, 1091–1099
activated sludge and, 71
biocontrols and, 458
biofilms and, 1209, 2341–2356
bioplastics and, 684–692
bottled water and, 800
as cold-adapted microorganism, 872–885
fecal contamination and, 1271
gene transfer and, 1416
granular activated carbon (GAC) filters and, 1490–1492, 1569–1570
as heterotrophic bacteria, 1541–1552
neuston microbiology and, 2139
in source water, 2969
metal biodegradation and, 1885
Aerophitic microorganisms, 835–843
Aeropyrum, 1234, 3129, 1442, 1629–1646
Aerosol (See Bioaerosols)
Afipia, 1345
Aflatoxins, 182–189, 1375
Agar, 88, 192
Agaricus, 1384, 1983
Agent T (See Chemical warfare)
Aggregates and consortia, 160–181
anaerobic granulation and, 240–241
anoxic processes in, 166
bioaugmentation and bioremediation in, 437
biofilms and, 1213
biofouling and, 601–609
biogeochemical processes, 161, 165–166
carbon dioxide and, 175
characterizing structure and function of, 175–178
coagulation theory and, 162
community composition of, assessment methods for, 175–176
cyanobacteria and, 164–165, 166–168, 170–181
denitrification and, 166
dimethylurea (DMCU) and, 175
dissolved organic matter (DOM) and, 175
ecological roles of, 166
expopolysaccharides (EPS) and, 166
filamentous bacteria and, 164–165
floc formation and, 2001–2002
formation of, 162–166
hypersaline lakes and, 173
hyperthermophiles and, 1642
ice and, 174–175
microbial mats and, 161, 165–166, 170–173
microbial modification of, 164–165
nitrogen fixing bacteria and, 166, 167–181
oxygen and, 175
pH levels and, 175
photosynthesis and, 175

sea and lake ice, 164
sedimentation in, 164–165
soil microorganisms and, 2924–2925
stromatolitic mats and, 172–173
subtropical and tropical oceanic, 168–170
sulfate-reducing bacteria (SRB), 165–166
in surface waters, 928, 928
surficial and benthic habitats, 164
suspended and solid surfaces as microenvironment for, 161, 162–164
temperate marine ecosystems, 170–172
Agriculture, agricultural biotechnology
cold-adapted microorganisms and, 881
coliform bacteria and, 902
enzymes and, 3215
nitrogen fixation and, 2188–2211
rhizosphere and, productivity and, 2722–2723
sustainable, 3116–3126
Agrobacterium, 1385–1392, 3110
biocontrol use of, 425–455, 462, 2700, 2704
biodegradation and, 2396
biofilms and, 1209
bioluminescence in, 1831
floc formation and, 2006
genetically engineered microorganisms (GEMs) and, 1279
microbial insecticides using, 1710
rhizosphere and, 2717
soil salinity and, 2775, 2779–2789
stress response and, 3048
subsurface microbial communities and, 2035, 3067–3071
toxic solvents and, 3151
Agrocybe, explosives biodegradation, 1983
Agroterrorism (See also Bioterrorism), 181, 780–781
Ahermatypic corals, 1117
AIDS/HIV, 1690
activated sludge and, 70
algae biotechnology and, 195
amoebic meningoencephalitis and, 1343–1350
Cryptosporidium and, 652
heterotrophic bacteria and, 1545, 1548
Mycobacterium avium complex (MAC) and, 2114–2120
nosocomial infections and, 2242
Air-handling systems (See also Bioaerosols)
biofiltration and, 593–601
odor control in, 593–601
Air pollution, 181, 588, 782–795
Air sampling, 181
Air sparging, 695
Air stripping, 695
Air–water interface (See also Neuston microbiology), 3274–3275
neuston microbiology, 2133–2144
Airborne microorganism (See Bioaerosols)
Aircraft deicing fluid (ADF), biodegradation, 2930
Alaninebetaine, soil salinity, 2778–2789
Alasan, bioremediation, 715
Albacausuarina, 2124–2132
Alcaligenes, 1058, 1200, 2942, 3110, 3160
activated sludge and, 13–14, 35–38, 3006
bacterial counts of, 32
biocontrols and, 455
biodegradation and, 2386, 2387–2393
biofilms and, 572, 577, 579, 580, 581, 2342–2356
bioluminescence in, 1831
bioplastics and, 689–692
bioremediation and, 705
biosurfactants/bioemulsions and, 714–715, 750–770, 1129–1130
cold-adapted microorganisms in, 872–885
genetically modified microorganisms (GMMs) and, 1431
granular activated carbon (GAC) filters and, 1490
heterotrophic bacteria and, 1541–1552
metal biodegradation and, 1882, 1885, 1889, 1890, 1894
as microbial tracer in groundwater, 3172
municipal solid waste landfills and, 1766
subsurface microbial communities and, 3065–3071
toxic solvents and, 3151
in drinking water, 944
Alcalignin, 1882
Alcanivorax, biosurfactants/bioemulsions, 756
Alcoholic beverages
cold-adapted microorganisms and, 880
Alcohols, 929, 1760, 3147–3155, 3412
activated sludge and, 741–748
alkaliphiles and, 228
biofilms and, 85–86, 582
composting of, 916–917
enzymes and, for fuels, 1185–1186
fungi and production of, 1369, 1385
microbial protection from, 432
ALCOL model, biodegradable dissolved organic carbon (BDOC), 503
Aldehyde, 929, 1046, 3412
Aldehyde-based biocides, biofouling vs., 605
Aldolases, 928
aldoses, biofilms, 85–86
ALEXA dyes, 1676
Alexandrium, harm/in harmful algal blooms (HABs) and red tides, 2686–2693
Algae (See also Algal blooms), 189–205
algal solid phase assay (ASPA) in, 3227–3228
algal turf scrubbing (ATS) and, 213–219
biodeterioration of mineral materials and, 519–522, 524–525
biofilms and, 88, 612–613
bioluminescence in, 657–663
biomass of, 1190
bioremediation and, 196–200
biosorption in, 200
biotechnology, 196–200
blooms of (See Algal blooms)
blue-green (See Cyanobacteria)
cold-adapted microorganisms in, 872–885
culture technology for, 191–192
desert environment and, 1576, 1579
endosymbiosis and, 1115–1116, 1126–1128
eutrophication in, 1189–1194
food products from, 196, 1851, 1857, 2516
grazing vs., 1193–1194
halophiles, 1501–1512
harmful algal blooms (HABs), 2685–2693
high-rate algae ponds vs., 2333
loss functions in, 1193–1194
macrofouling from spores of, 615–616
nitrogen cycle and, 2183
nitrogen fixation and, 1191

Algae (See also Algal blooms), (*continued*)
nutrient concentrations and, 1190, 1191–1194
oxygen and, 2277
paleolimnology of, 2301–2306
periphyton, 2357–2367
Pfiesteria and toxic Pfiesteria complex (TPC), 2431–2447
pH levels and, 1191
phosphorus and, 1190
photoautotrophic, in low-light habitats, 836–843
photosynthetic pigments of, marine, 2453–2470
pigments in, 2306–2317
plankton and, 2502–2518
red tides and, 2685–2693
salt production and, 2805
sedimentation vs., 1193–1194
soil salinity and, 2775
solar radiation and, 1192–1193
streams and, 3020, 3021–3022
subaerial communities and, 3056–3065
temperature and, 1193
toxicity assays using, 3172–3173, 3219–3230
trophic state in, 1190–1191
in wastewater stabilization ponds (WSBs), 3331–3332
water turnover vs., 1193–1194
weathering and, 3364–3389
algal blooms (See also Harmful algal blooms and red tides), 204–213, 2685–2693
activated sludge and, 77
adsorption of by products from, 212
blue-green algae in, 206
causes of, 206–207
control of, 211–212
dinoflagellates in, 205
green algae in, 205
hepatatoxins in, 207
impact of, on water treatment, 207–210
nitrogen and, 206–207
phosphorus and, 206–207
preoxidation vs., 207–208
presedimentation vs., 207
yellow green or golden brown algae in, 206
Algal mats, nitrogen cycle, 2183
Algal solid phase assay (ASPA), 3227–3228
Algal toxicity assays, 3172–3173
Algal turf scrubbing (ATS), 213–219
Alginate lyase, 1211–1223
Alicyclobacillus, bioleaching, 645–650
Alkali environments, alkaliphiles, 219–231, 1235–1236, 1510, 3127–3140
aerobic, 133–140
alkalithermophiles, 3127–3140
ATP synthesis and, 138–139
auxotrophy and antibiotic resistance in, 137
Bacillus as, 220–231
cell wall makeup in, 137
cyanobacteria, 136
flagella and sodium ions in, 138
haloalkaliphiles, 135–136
lipid composition in, 137–138
membrane transport in, 138
pH regulation and, 138, 221
sodium/hydrogen antiporters in, 138
thermoalkaliphiles, 136
yeast and fungi in, 136
Alkaline degradation in biodeterioration of mineral materials, 517–518
Alkaline saline habitats, 1510
Alkalis, 1902
Alkalithermophiles, 3127–3140
Alkalitolerant microorganisms, 133–140
Alkaloids, 195, 975
Alkanes biodegradation, 697–713, 928, 2402–2404, 2411–2412, 3147–3155, 3412
Alkenes biodegradation, 2407–2408, 3412
Alklybenzes bioremediation, 713
Alkoxysiloxanes, in biodeterioration of mineral materials, 518
Alkylaromatics biodegradation, 1423–1429
Alkylbenzene composting, 916–917
Alkypyridines bioremediation, 707
Allergic bronchopulmonary aspergillosis (ABPA), 1363
Allergies and allergens (See also Bioaerosols; Spores and spore dispersal), 338
antibodies and, 1359–1366
enzyme linked immunoassay (ELISA) and, 1361
fungal, 1359–1383
immunoglobulin E (IgE) and, 1359–1366
major histocompatiblity class (MHC) and, 1360
radioallergosorbent (RAST) assay and, 1361
sensitization in, 1360, 1368–1369
T helper cells and, 1359–1366
volative organic compounds (VOCs) and, 1366–1383
Allochromatium, 2476
Allochthonous (See also Oligotrophic bacteria), 2268, 2347–2353, 2428
Allolevivirus, 364–374
Allorhizobium
endosymbiosis and, 1120
nitrogen fixation and, 2201–2211
soil salinity and, 2775, 2785
Alloxanthin, in paleolimnology, 2307–2317
Alnus, 2912
mycorrhizae and, 2124–2132
nitrogen fixation and, 2208, 2209
Alpha amylases, alkaliphiles, 223–224
Alphaglycerylglucose, 518
Alternaria, 1650, 1651, 1653
in allergies and illness, 1361–1366, 1367–1372
bioaersols and, 404–405
desert environment and, 1579
explosives biodegradation and, 1983
metal biodegradation and, 1885
in permafrost, 2369
spores and spore dispersal in, 405, 408, 409–510
subaerial communities and, 3057–3065
Alteromonas, 1610, 1851, 2550
Altrose, biosurfactants/bioemulsions, 756
Aluminum, 928, 1880–1893
Alvinella, 1118, 1609–1610, 1619–1620, 3090–3096
Amanita, 2124–2132
Amaricoccus, 61–63, 66
Ambient temperature catalytic oxidation, biofiltration, 587
Amebiasis, Entamoeba, 1136–1146
Americium, 1879–1893
Amidase, in soil, 2903–2910
Amides, 929
biomineralization and, 671
conditioning films and, 937–938
in surface waters, 928
Amidohydrolases, 1185
Amines, 671, 929
Amino acids, 2716
activated sludge and, 743–748
biofilms and, 85–86
microbial protection from, 432
soil salinity and, 2778–2789
Aminobenzene biodegradation, 2297–2300
Aminocyclopropane (ACC) biocontrols, 455
Aminohexanoate bioremediation, 693
Aminomethylcoumarin, 1676
Aminophenol biodegradation, 3413
Amitrole bioremediation, 705–712

Ammonia/ammonium, 1760
activated sludge denitrification and, 8–14, 20
activated sludge processing and, 24–26
anaerobic ammonium oxidation (anammox) in, 11–13, 24, 38, 71, 2146, 3145
anaerobic ammonium oxidizing bacteria in, 38
anoxi metabolism of, 9–11
bioaugmentation and bioremediation in, 435
biofilms vs., 582
biofiltration vs., 598
bioremediation and, 694, 707–708
heterotrophic ammonia oxidation, 13
in marine/ocean environment, 1698–1701
nitrification and, 2144–67, 2167–2176
nitrifying bacteria in, 34–35
nitrogen cycle and, 2176–2188, 2939
nitrogen fixation and, 2188–2221
oxidation of, 9
in wastewater stabilization ponds (WSBs), 3332
water treatment systems and, 2167–2176, 2167
weathering, bioweathering and, 3367–3368
Ammonia monooxygenase (AMO), 2145–2167
Ammonia oxidizing bacteria (AOB), 2144, 2167–2176
Ammonification, 2939
Ammonium nitrogen, activated sludge denitrification, 8–14
Amnesic shellfish poisoning (ASP), 2687
Amoeba (See also Protozoa), 2594, 2605
biofilms and, 2342
bottled water and, 800
desert environment and, 1579
Entamoeba and, 1136–1146
free-living, 1343–1350
heterotrophic bacteria and, 1540
lobose, 2616, 2617
marine/ocean environment and, 2616, 2617
meningoencephalitis and, 1343–1350
testate, 2610
Amoebobacter, 2527
Ampelomyces, 452, 454
Amphidinium, 1126, 2805–2809
Amphileptus, 2606
Amphipods, algal turf scrubbing (ATS), 215
Amphiphora, 1058, 2839
Amphora, 1053, 1055–1063
biofilms and, 612
salt production and, 2805–2809
Amplification fragment length polymorphism (AFLP), 443
Amplified rDNA restriction analysis (ARDRA), 299, 536–537, 2737–2738, 2920
Amylase, 223–224, 1177–1189, 2903–2910, 3170, 3207–3210
Amylopectin, 1181
Anabaena, 206, 207, 210, 961, 966, 972, 973, 980, 984–995, 1191, 1193
aggregates and consortia in, 167–168
meroplankton and, 1868–1869
metal biodegradation and, 1879
nitrogen fixation and, 2193–2201, 2201–2211, 2214–2221
paleolimnology of, 2302–2306
in permafrost, 2369
in wastewater/sewage, 3172
Anabolic processes, biofilms, 81
Anacystis, 972
algae biotechnology and, 196
cold-shock response and, 888–895
metal biodegradation and, 1879
Anaerobic ammonium oxidation (anammox) 11–13, 24, 38, 71, 2146, 3145
Anaerobic ammonium oxidizing bacteria, activated sludge, 38
Anaerobic bacteria
activated sludge foaming and, 6–7
bioaugmentation and bioremediation in, 435, 439
Clostridium and, 861–871
cyanobacteria and, 965–966
halophiles and, 1512–1529
sampling of, 827–828
subsurface microbial communities and, 3069–3070
Anaerobic granulation, 232–245
chemical oxygen demand (COD), 243
colonization and, 241–243
consortia in, 240–241
extracellular polymers (ECP) and, 238, 242–243
fermenting bacteria and, 239–240
filamentous bacteria in, 240
hydrophobicity in, 238, 241
layered structures in, 234–237
metabolic activity zones in, 238
methanogens and, 238
microbial composition of, 232–234
microcolonies in, 237–238
nonlayered structure in, 237
nuclei in, 238–241
sludge structure and, 234–238
species specific interaction in, 241–242
start-up procedures for, 243–244
syntrophy in, 240–241
volatile fatty acids (VFA) and, 235, 242–243
Anaerobic migrating bed reactor (AMBR), 232–245
Anaerobic oxidation, activated sludge denitrification, 10–14
Anaerobic oxidation of methane (AOM), 288–289
Anaerobic processes
activated sludge and, 21–26, 1313–1314
aggregates and consortia in, 161
anoxic storage in nitrogen removal processes and, 3011
biodegradation and, 2932–2933
explosives biodegradation using, 1983, 1985–1987
flooded soils and, 1319–1321, 1327–1328
granulation (See Anaerobic granulation), 232–245
hydrocarbon degradation and, 2411–2413
hyperthermophiles and, 1633
municipal solid waste landfills and, 1759–1762
nitrification and, 2146–2147
oxygen and, 2277
petroleum reservoirs and, 2427
in sediments and sedimentation, 2844–2845
soil microorganisms and, 2888, 2891–2893
sulfate-reducing bacteria (SRB) and, 3092
wetlands and, 3403–3405
Anaerobic respiration, 149
cold-adapted microorganisms, 872–885
Anaerobic sequencing bed reactor (ASBR), 232–245
Anaerobranca, 1236, 3130, 3132, 3134–3135
Anammox, 11–13, 24, 38, 71, 2146, 3145
Anatoxin, 976, 987–995, 2340
Ancylonema, 2835–2844
Ancylostoma, 3333–3334
Anaerobic respiration, 149
Anhydrobiosis (See also Desiccation), 245, 1030–1031
Animal and Plant Health Inspection Service (APHIS), 2704–2707

Animal feed, enzymes, 1188
Anisakis, in food, 3277–3287
Anisonema, 2841
Ankistrodesmus, 205
Annelida, 1117, 1722
Annotation, in genomic sequencing, 1442–1443
Anodic depolarization, sulfate-reducing bacteria (SRB), 472–475
Anodontia, 1606
Anolytes, biofouling, 604
Anopheles, 3399
Anoxic environments/processes
  activated sludge and, 77, 79, 1313–1314
  activated sludge and, 77, 79
  aggregates and consortia in, 166
  metabolism in, activated sludge denitrification and, 9–14
  protozoa and, 2596
Anoxygenic bacteria, 2892
Anoxygenic photosynthesis, 2280–2282
Antagonism, in biofiltration, 591
Antarctica
  cold-adapted microorganisms and, 1103
  cyclic climatic events and, 2558–2559
  endolithic microorganisms in, 1100–07
  hypersaline lakes in, 1510
  lichens in, 1101–1106
  marine/ocean environment of, 2557–2558
  mineral resources of, 1026
  nitrogen cycle and, 2182
  organic matter in soils of, 1025
  ozone hole and, 2564–2565
  permafrost and, 2367–2385
  physical characteristics of, 1023–1025
  psychrophilic bacteria and, 2639–2646
  salt content of soils in, 1027
  sea ice microorganisms, 2833–2844
  soil composition in, 1024–1025, 1104
  soil microbes in, 1023–1029
  solar radiation and, 1025, 1103
  temperatures in, 1103
  water and, 1024, 1104
"Antenna effect," biofilms, 560
Anthomedusa, 1116
Anthozoa, 1116
Anthracene biodegradation, 696–712, 917, 2404
Anthrax (See also Bioterrorism), 245, 771–782, 1689–1697
Antibiosis, biocontrols, 456
Antibiotics (See also Resistant bacteria)
  alkaliphiles vs., 137, 228
  bacteria-produced, 456–457
  biocontrols and, 456–458
  fungal produced, 457–458
  fungi and production of, 1369
  microbial protection from, 432
  microgravity and, 3001
  neuston microbiology and, 2139
  resistance to (See Heterotrophic bacteria; Nosocomial infections; Fecal contamination; Resistant bacteria)
  rhizosphere and soil microbes in, 347–348
Antibodies
  in allergies and illness and, 1359–1366
  fluorescent probes and, 1781
  rhizosphere and soil microbes in, 351
Anticancer agents, fungi production, 1369
Antifoam agents, for activated sludge, 6–7
"Antifreeze" Pseudomonas, 2694–2695
Antimicrobials, 195, 556–557
Antimony, 608, 671–681
Antioxidants and stress response, 3031
Antivirals, algae biotechnology, 195–196
Antonina, 1115
Anydrobiosis (See Freeze drying)
Apatococcetum, 3056–3065
Apatococcus, 3056–3065
Aphanizomenon, 206, 210, 961, 972, 980, 984–995, 1191
  aggregates and consortia in, 167–168
  paleolimnology of, 2302–2306
Aphanocapsa, 840–843, 984–995, 1507–1512
Aphanothece, 1507–1512
Aphantoxin, 976
Aplysiatoxin, 976
Aposymbiosis, 1113
Appendages, in soil microorganisms, 2885–2886
Apple scab spore dispersal, 413
Aquabacterium, 572
Aquaculture, 1850, 1856–1857, 1859, 3215
Aquareovirus, 2753–2773
Aquaspirillum
  activated sludge and, 3009
  nitrogen fixation and, 2193–2201
  psychrophilic, 2640–2646
Aquatic biofilms, 1216
Aquatic ecosystems (See also Marine/ocean environment; Surface waters)
  bioremediation and, 712–722
  Enterococci in, 1264–1278
  fecal contamination in, 1256–1264
  lipid biomarkers of and, 1821
  nitrification in, 2144–2167
  proteomes and proteins in, 306–307
  Streptococci in, 1264–1278
Aquifers, 245
  Atlantic Coastal Plain, 2028–2042
  bioaugmentation and bioremediation in, 440–441, 507, 695
  lipid biomarkers of, 1821
  microbial communities of, 1661–1673
  protozoa and, 2595–2596, 2599–2602
  sparging, 695
Aquifex, 1233, 1235, 1442, 1512–1529, 1629–1646, 3128–3140, 3143–3147
Aquificales, 1512–1529, 1614, 1629–1646, 3143–3147
Arabidopsis
  bioplastics and, 689–692
  genomic studies and, 1442
  microarray study of, 1973
Arabinases, 1187
Arabinose, 2716
Arachidonic acid (AA), cold-adapted microorganisms, 880–881
Arbuscular mycorrhizae (See also Mycorrhizae), 245, 2120, 2126
arbutoid mycorrhizae, 2126
Arbutus, 2124–2132,
Arcella, 2605
Archaea, 246–259, 2552
  biodeterioration of mineral materials and, 523
  bioleaching and, 634–642
  genomic studies and, 1440
  sulfate-reducing bacteria (SRB) and, 467–475
  Amplified ribosomal DNA restriction analysis (ARDRA), 299
  anaerobic granulation and, 236–245
  anaerobic oxidation of methane (AOM) and, 288–289
  autotrophism in, 266
  bacterial artificial chromosome (BAC) and, 299

bacterioplankton and, 2519
biocatalysis and, 259–276
biomass estimation and, 266, 2658
biomining and metal ion extraction using, 274
biotechnology using, 259–276, 290–291
cell envelope of, 248–256
cell wall structure of, 248
coenzymes and, 256–257
compatible solutes and, 273
culturing of, 246–247
denaturing gradient gel electrophoresis (DGGE) and, 278, 299
desert environment and, 1578
detection of, 246–259, 278–288
diversity of habitats for, 260–262, 276–285
ecophysiological attributes of, 288–280
Entner-Doudoroff pathway and, 262
enzymes and, 259–276
extraction and processing of, 252
fermentation and, 259–276
fluorescent in situ hybridization (FISH) and, 279, 301
Fourier transform infrared (FTIR) spectroscopy and, 257
gas chromatography (GC) and, 253–255
genome sequencing of, 263–266, 290–291, 1443–1446
in groundwater, 1671
halophiles as, 246–259, 262, 277–293, 297–298, 1512–1529
heteropolysaccharides in, 249
high performance liquid chromatography (HPLC) and, 252–253
hydrogen production and, 273–274
at hydrothermal vents, 278, 287–289, 293, 1611, 1622–1628
hyperthermophiles as, 246–259, 266, 277–293, 1629–1646
lipids and, 249–259, 271–272, 279–293
marine environment and, 276–283, 289–290
marine sediments containing, 285–287
metal biodegradation and, 1890
methane and, 256, 259–276, 296–297
methanochondroitin in, 248–249
methanogens as, 246–259, 262, 263, 277–293, 296–297, 1913–1914, 1916
molecular culturing techniques for, 247–248
nanotechnology and, 273
nitrogen fixation and, 2212–2221
noncultured approaches to sampling in, 299
oligotrophic bacteria and, 2268
origins and evolution of, 291
phospholipid ether liquid (PLEL) in, 252
photosynthetic pigments in, 2464
phylogenetics of, 259–260, 295
plankton and, 279–285, 288
proteins of, 263
pseudomurein in, 248
psychrophiles as, 246–259, 262
quinones and, 256
rhizosphere and, 300–301
seasonal variation in marine environment, 281–285
single-strand conformational polymorphism (SSCP), 299
small subunit ribosomal RNA (SSU rRNA) and, 278, 295, 301
soil habitats of, 293–305, 2880–2886
soil salinity and, 2776
stress response in, 3024–3034
sulfate-reducing (See Sulfate-reducing bacteria)
sulfolobales and, 298–299
sulfur dependent anaerobic methane oxidation and, 288–289
supercritical fluid chromatography (SFC) and, 255–256
surface (S) layers in, 248, 272–273
taxonomy of, 295
temperature gradient gel electrophoresis (TGGE), 299
terminal fragment length polymorphism (T-RFLP) and, 278, 299
thermoacidophiles as, 246–259, 261
thermophiles as, 246–259, 277–293, 3140–3147
thermoplasmales and, 297
thermoproteales in, 298
thin layer chromatography (TLC) and, 251
uncultured, accessing genomes of, 262–263, 299–301
Archaebacteria, 246–259
Archaeoglobales, 287, 1614, 1630–1646
Archaeoglobus, 1233, 1235, 1622–1628, 1630–1646, 3114–3116, 3144–3147
genetically altered, in biotechnology, 1448
hydrothermal vents and, 1611
metal biodegradation and, 1905
sulfate-reducing bacteria (SRB) and, 467–475, 3090–3096
Archezoa hypothesis, endosymbiosis, 1124–1125
Archianium, 1578
Arcobacter
diazotrophy in, 2541–2547
disinfection and, 1065
hydrothermal vents and, 1609–1610
Arctic and Arctic Ocean, 2556–2558
cyclic climatic events and, 2558–2559
permafrost and, 2367–2385
sea ice microorganisms, 2833–2844
Arctostaphylos, 2124–2132
Arden, Edward, 74
Ardern, 738
Arginine, 2899
Argon, 3183
Arhodomonas, 1507–1512
Armillaria, 2914
Aromatic hydrocarbons (See also Hydrocarbons), 435, 694, 2408–2410, 2412
Arrhenius equation, 1752, 2900
Arsenate, 608, 1878
Arsenic
algae biotechnology and, 196
biomineralization and, 671–681
bioremediation and, 694, 708
metal biodegradation and, 1888
microbial methylation of, 1885–1886
Arsenite bioremediation, 708
Arsenophonus, 1115
Artemia, 1031, 1513–1529, 2805–2809
Arthrobacter, 757, 1102–1106, 2549, 3314–3319
activated sludge and, 3009
biodegradation and, 1044, 1047–1048, 1996, 2387–2394, 3412
biofilms and, 572
biomineralization and, 675
biosurfactants/bioemulsions and, 714–715, 750–770, 1129–1130
cold-adapted microorganisms in, 872–885

Arthrobacter, (*continued*)
cold-shock response and, 892
desert environment and, 1578, 1580–1584
genetically engineered microorganisms (GEMs) and, 1425
granular activated carbon (GAC) filters and, 1490
identification of isolates in, 1964
metal biodegradation and, 1890, 1904
oligotrophic bacteria and, 2269, 2272
subaerial communities and, 3057–3065
subsurface microbial communities and, 2023, 3065–3071
Arthrobotyris, 455, 2913, 3360
Arthroconidia, 1647
Arthrospira, 170–172
Artificial neural network (ANN) (See also Neural networks), 1008–1009
Artificial recharge, vadose zone microbiology, 3242
Artificial substrata, periphyton, 2358
Artificial wetlands, 2333, 3389–3401
Aryl halide biodegradation, 1238–1252
Arylesterase, in soil, 2903–2910
Arylsulfatase, in soil, 2903–2910
Arylthioallkymaleimides, 605–606
Ascaris
in food, 3277–3287
in wastewater stabilization ponds (WSBs), 3333–3334
in water treatment systems, 1718
in wetlands for wastewater treatment, 3399
Ascomycetes, 406, 1359, 1394–1400
Ascomycota, 1367–1372, 1647, 2913–2914, 3353–3364
Ascomycotina, 2124–2132
Ascophyllum, 198, 199–200
Ascospores, 412–414, 1647
Asellus, asellidae, 1722–1723, 3021
Asenophonus, 1122
Aseptic meningitis, enteroviruses, 1151
Asparaginase, in soil, 2903–2910
Aspartame, 1185
Aspergillus, 182–189, 423, 1130, 1647, 1648, 1650, 1651, 1653–1654, 2649
in allergies and illness, 1359, 1362–1383
bioaerosols and, 3325
biocontrol use of, 2703, 2705
biodegradation and, 2386
biofilms and, 580, 1177, 2342–2356
bioleaching and, 635
cold-adapted microorganisms and, 880
composting and, 916
desert environment and, 1579
detection of, 1133
explosives biodegradation and, 1983
metal biodegradation and, 1886
mineral materials biodegradation and, 518
municipal solid waste landfills and, 1766
nitrification and, 2149
nosocomial infections and, 2245
radioactive waste degradation and, 2666–2674
rhizosphere and, 2718
soil microorganisms and, 2881
spores and spore dispersal in, 408, 412, 418, 421, 422
subaerial communities and, 3057–3065
in drinking water, 945
Asphaltene biodegradation, 696–712, 2403–2416
Aspidisca, 2608, 2595–2604, 3173
Assimilable organic carbon (AOC), 312–327, 485–507
adenosine triphosphate (ATP) and, 323
BDOC vs., 323–324
biodegradable dissolved organic carbon (BDOC) and, 485–507
biofilm formation rate (BFR) and, 324–325
biofilms and, 88
biological stability of, 323–325
concentration of, in water, 319–323
filtration vs., 321–322
growth measurement and calibration curves in, 318–319
heterotrophic plate count (HPC) for, 322–323
materials in contact with water and, 325
in source water, 2972
storage of water and, 322–323
tests for, 316–319
water treatment assessment of, 312–327, 1729
Assimilatory sulfate reduction, 3087, 3107
Associative symbiosis, nitrogen fixation as, 344, 2199–2200
Astaxanthin, 193, 1852, 2307–2317, 2307
Asterionella, 206, 210
Asterionellopsis, 2506
Asthma, fungal allergens, 1359–1366
Astraeus, 2124–2132
Astroviruses, 327–337
in source water, 2969–2978
in spas and hot tubs, 2980–2984
streams and, 3017
Atlantic Coastal Plain
aquifers of, 2028–2042
subsurface microbial communities of, 3065–3066
Atmosphere and microbes, 2061, 3183–3194
Atomic force microscopy (AFM)
adhesion in biofilms and, 125, 126, 125, 128–129
bioleaching and, 635
conditioning films and, 935–936
floc formation and, 2008
ATP-based assays, in wastewater/sewage, 3171
ATP binding cassette (ABC), 1208, 2781
ATPases, in wastewater/sewage, 3170
Atractella, photoautotrophic, in low-light habitats, 838–843
Atrazine biodegradation, biodegradation, 705–712, 921–922, 2289–2300, 2391–2396
Atriplex, 1511, 1577
Attachment, biofilms, 2088
Attachment system, filtration, for microbes, 2708
ATV guidelines, activated sludge, 49
Aulacoseira, 1864–1869
Aureobasidium, 121, 422, 2649, 3057–3065, 3360
Aureococcus, harm/in harmful algal blooms (HABs) and red tides, 2689–2693
Auricula, 1055
Auroeumbra, harm/in harmful algal blooms (HABs) and red tides, 2689–2693
Auto-associative neural networks, 1007
Autochthonous microorganisms, 1583, 2342–2343
Autotransporter pathway, 1208–1210
Autotrophs
activated sludge and, 18–26
Archaea and, 266
bacterioplankton and, 2521
biodeterioration of mineral materials and, 523

biofilms and, 81–82, 2341
hyperthermophiles and, 1633
nitrification and, 2144–2145, 2940–2941
Auxins, 344, 454
Auxotrophy, alkaliphiles, 137
Available dissolved organic carbon (ADOC), biofilms, 1086–1087
Aviadenovirus, 93
Axial filaments, 2885
Axinella, 539
Axisymmetric drop shape analysis, in biosurfactants/bioemulsions, 762
Azoarcus, 2282
bacterial counts of, 32
flooded soils and, 1328
nitrification and, 2149
nitrogen fixation and, 2193–2201
subsurface microorganisms and, 2035
Azobacter
aerobic respiration and, 151, 154
aggregates and consortia in, 164
nitrogen fixation and, 2212–2221
Azocarbonamide, 1186
Azogreen wheat, 351
Azolla, 973, 1119, 2196, 2201–2211, 2938
Azorhizobium, 2720
endosymbiosis and, 1120
nitrogen fixation and, 2201–2211
soil salinity and, 2775
Azospirillum, 345–354, 1118, 2720
flooded soils and, 1318
genetically modified microorganisms (GMMs) and, 1431
metal biodegradation and, 1880
nitrogen fixation and, 344, 2193–2201, 2938
resistant bacterial strains in, stress response, 349
soil salinity and, 2775, 2779–2789
subsurface microbial communities and, 3067–3071
Azotobacter, 347, 1924–1936, 2282, 2720
biofilms and, 1221
diazotrophy in, 2541–2547
floc formation and, 2006
nitrogen fixation and, 344, 2190–2191, 2193–2201, 2938
resistant bacterial strains in, stress response, 349
rhizosphere and, 2717
soil salinity and, 2775, 2779–2789
subaerial communities and, 3057–3065
Azpoarcus, nitrogen fixation, 2193–2201

B

Bacillariophyceae, 206
photoautotrophic, in low-light habitats, 837–843
photosynthetic pigments in, 2467
polar phytoplankton and, 2556
Bacilli, 2882
Bacillus, 339, 340, 499, 1031, 1233, 1235, 1236, 1895, 2054, 2549, 2601, 2698, 2723, 2942, 3129, 3145–3147, 3150, 3160, 3314–3319
activated sludge and, 35–38
aggregates and consortia in, 166
alkaliphiles and, 133, 134, 135, 137, 139, 220–231
alkalithermophiles and, 3127
bioaerosols and, 3325
biochips, biochip testing, 449
biocontrols and, 452, 455, 457, 462, 2700, 2703
biodegradation and, 1045
biodeterioration of mineral materials and, 518, 523
biofilms and, 82, 577, 579, 580, 1177, 1209, 1210, 1215, 2342–2356, 2960, 2962, 2963
bioleaching and, 635
bioluminescence in, 1831
biomineralization and, 673, 674
bioplastics and, 683–692
biosurfactants/bioemulsions and, 757–759, 766
bioterrorism and (See Anthrax)
cold-adapted microorganisms in, 872–885
cold-shock response and, 885–895
composting and, 915
desert environment and, 1578, 1580, 1583
desiccation and, 1033–1041
detection of, 1133, 1134
enzyme activity and, 2902
extracellular polymeric substances (EPS) and, 1229
flooded soils and, 1319
fluorescent studies of, 1834
gene transfer and, 1409, 1413–1415
genetically modified microorganisms (GMMs) and, 1431, 1447
genomic studies and, 1448
glutaraldehyde vs., 605
granular activated carbon (GAC) filters and, 1490
in groundwater, 1671
halophiles and, 1505–1512, 1505
high hydrostatic pressure (HHP) vs., 1559–1563
hydrothermal vents and, 1611, 1620–1628
infectious airborne bacteria and, 1689–1697
measurement of, using aerobic endospores, 143–149
metal biodegradation and, 1881, 1884, 1886, 1890, 1897, 1905, 1908
as microbial tracer in groundwater, 3198
microbial insecticides using, 1710, 1711–1712
microgravity and, 2998, 3002
municipal solid waste landfills and, 1766
nitrification and, 2146, 2149
nitrogen fixation and, 2214–2221
oligotrophic bacteria and, 2270
radiation and, 2987, 2992–2995
resistant bacterial strains in, stress response, 349, 607
rhizosphere and soil microbes in, 346
soil microorganism and diversity in, 533
soil salinity and, 2775, 2779–2789
stress response and, 3048, 3050–3051
subaerial communities and, 3057–3065
subsurface microbial communities and, 2024, 2032, 2035, 3067–3071
toxic solvents and, 3151
ultraviolet light and, 3231
viable but not culturable (VBNC) phenomenon and, 3251
in wastewater/sewage, 3172, 3173
in drinking water, 945, 946, 2266
in wetlands for wastewater treatment, 3400
Bacillus pertussis, biochips, biochip testing, 448
Bacillus thuringiensis, 417, 449, 450, 1279, 1434, 1437, 1448, 1710–1717, 2058, 2693, 2699, 2700, 2701
Back siphon, cyanobacteria, 943
Backwash water
biodegradable dissolved organic carbon (BDOC) and, 496
filtration and, 2707

Backwash water (*continued*)
protozoa remaining in, 2262–2267
BacTech process, for bioleaching, 647–648
Bacteria
activated sludge and 70–71,75
antibiotics produced by, 456–457
as biocontrol agents, 454–455
biodegradation and, 2386
biodeterioration of mineral materials and, 523
biofilms and, 551–559
bioluminescence in, 657–663
bottled water and, 795–801
cold-adapted microorganisms in, 872–885
desert environment and, 1577
hydrothermal vents and, 1620–1622
infectious airborne bacteria as, 1689–1697
lytic enzymes produced by, 458
in metal stressed environment, 1893–1901
as microbial tracers in groundwater, 3194–3202
microbial insecticides using, 1711–1712
photosynthetic pigments of, marine, 2453–2470
salt production and, 2805–2809
sea ice and, 2838
snow/in snow and ice environments, 2874
soil microorganisms and, 664–665
soil salinity and, 2775
in spas and hot tubs, 2979
streams and, 3017–3020
stress response and, 3034–3046
subaerial communities and, 3057–3065
in wastewater/sewage treatment, 3337
in wastewater stabilization ponds (WSBs), 3330–3331, 3334–3336
water purifiers vs., 1572–1573
weathering and, 3364–3389
Bacteria
bacterioplankton and, 2519, 2524–2526
bioleaching and, 634–642
halophiles and, 1514–1529
methanotrophic bacteria and, 1913–1914
nitrogen fixation and, 2212–2221
oligotrophic bacteria and, 2268
soil microorganisms and, 2880–2886, 2912
soil salinity and, 2776
stress response and, 3025
sulfate-reducing (See Sulfate-reducing bacteria)
thermophiles and, 3140–3147
Bacterial artificial chromosome (BAC), 299, 539
Bacterial contaminants in residential environment, 338–343, 338
baseline studies for, 338
bathrooms and toilets, 340
cross contamination studies in, 340–341
disinfection and, 341–342
fungal contamination and, 342
general living areas, 340
hazard analysis and critical control point (HACCP) concept, 342
home water treatment units and, 1573–1575
hygiene practices vs., 342
illness studies and, 341
kitchens and, 338–340
pathogens in, 340
sampling techniques for, 338
species found in, 338, 339
sponges, rags, fabrics, 340
viral contamination and, 342
Bacterial counts, 26–43, 2653
activated sludge and water treatment plants, 26–43, 69
aerobic endospores used in, 141–149
biofilms and, 565–566
flow cytometry and, 1948–1950
subsurface microorganisms and, 2031
wastewater/sewage treatment and, 3338
Bacterial phytostimulators (See Phytostimulators, bacterial; Rhizosphere)
Bacteriastrum, 2506
Bactericides, biofouling vs., 602–609
Bacteriochlorophylls, 2453–2470, 2473
Bacteriogenic iron oxides (BIOS), 1407
Bacteriome, 1114
Bacterioneustons, 2133, 2135–2140
Bacteriophages and coliphages, 363–374, 379–380, 384–385, 914, 2101
activated sludge and, 70, 360, 381
adsorption into soil of, 358, 389
aerosol samples and, 382
aggregation of virus particles and, 389
bacterioplankton vs., 2531–2532
Bacteroides fragilis-infecting bacteriophages as, 356–358, 378–379
biofilms and, 2342
biology of, 363–374
capsid of, 364
coliphages as, 363, 379–380, 384–385
colorimetric detection of, 381
detection methods for, 359–360, 374–384
ecology of, 385–387
enteric bacteria and, 384–392
enteroviruses and, 363–364, 384–385
F-specific RNA bacteriophages as, 356
fecal contamination and, 1257
gene amplification detection in, 383
genetics of, 363–374, 382–383
groundwater and, 375–377
heat treatment vs., 361, 387
inactivation of, 358–359
index use of, 356–358
indicator organism use of, 354–363
Information Collection Rule (ICR) protocol for, 376
ISO method for, 376–378
lambdoid phage, 370–373
lytic vs. lysogenic state in, 371–373
membrane filtration (MF) method for, 375–376
as microbial tracers in groundwater, 3194–3202
microbial activity vs., 387
molecular detection methods for, 382–383
MS2, 368–370, 387, 389–390
nucleocapsid of, 364
organic matter vs., 389–390
pathogen transport models and, 385
pathogenesis of, 366–367
persistence of, in environment, 387–390
pH levels and, 387–389
pollution index use of, 357–358
radiation vs., 360–361
removal and inactivation of, in nature, 358–359
RNA and DNA of, 367–373
salt levels vs., 389
sampling of, 376–381
sedimentation and, 358
in shellfish, 361, 382
soil properties vs., 389
soil recovery of, 381

somatic coliphages, 355–356, 377–378
in source water, 2974
structural units of, 364
taxonomy of, 364–366
temperatures vs., 387
U.S. EPA sampling methods for, 379–380
viral index use of, 357
virion of, 364
virus type vs., 389
wastewater treatment vs., 359
water treatment systems and, 359–360, 375–377
Bacterioplankton (See also Plankton and phytoplankton), 392, 2519–2539
abundance of, 2528–2530
adenosine triphosphate (ATP) and, 2521
Archaea in, 2526
autotrophs in, 2521
Bacteria of, 2524–2526
binary fission reproduction in, 2519
biodiversity of, 2524, 2526–2528
biomass of, 2520, 2528–2530
carbon fixation in, 2521–2523, 2533
cell wall in, 2519
chemoautotrophs in, 2521
chemolithoautotrophs in, 2522
colonies in, 2520
distribution of, spatial and temporal, 2526–2528
DNA/RNA analysis of, 2523–2524
ecological significance of, 2532–2534
electron use in, 2522
energy conservation in, 2521–2523
extreme environment and, 2526
flagella in, 2520
fluorescent in situ hybridization (FISH), 2524
food web and, 2534–2535
grazing in, 2531–2532
growth rate of, 2529–2531
habitats for, 2520–2521
heterotrophs in, 2521
light and solar radiation in, 2520
lipids in, 2519
metabolism in, 2526–2528
mortality in, 2531–2532
nitrogen cycle and, 2525, 2533–2534
nucleoid in, 2519
nutrients use by, 2520, 2521–2523, 2530–2531, 2532–2534
organic matter and, 2530–2531
oxygen use by, 2520, 2521–2522, 2530–2531
oxygenic photoautotrophy in, 2522
phosphorus cycle and, 2534
photoheterotrophs in, 2522
photolithoautotrophy in, 2522
photosynthesis in, 2521
polymerase chain reaction (PCR) and, 2523
predators of, 2531–2532
research questions/issues in, 2535–2536
salinity of water and, 2520
sixteen/16S RNA and, 2523–2524
taxonomy of, 2523–2524, 2526–2528
temperature and, 2520–2521, 2531
transduction and, 2532
viruses and bacteriophages vs., 2531–2532
Bacteriorhodopsins, 1505, 2464, 2470
Bacteriostatic filters, 1570–1572
Bacteriovorous ciliates, 2611
Bacterium, 2386
Bacterium salmonicida (See Aeromonas)
Bacteroides
cold-adapted microorganisms in, 872–885
coliform bacteria, 895–905
endosymbiosis and, 1122
fecal contamination and, 1256–1257
filamentous forms of, in activated sludge, 1302–1306
flooded soils and, 1320
halophiles and, 1506–1512
municipal solid waste landfills and, 1766
soil microorganism and diversity in, 533, 539
subsurface microorganisms and, 2035
Bacteroides fragilis-infecting bacteriophages, 356–358, 374–384
Baetis, 3020
Baeyer–Villiger monooxygenase (BVMO), 2290
Bailers, 2815
Balamuthia, meningoencephalitis, 1343–1350
Balantidium, 2712, 2980–2984
Balanus, biofilms, 614
Bank infiltration, 2014–2015
Bardenpro reactor for activated sludge, 77
Barron, George, 2914
Basaltic rock
lithotrophs and, 1826–1828
subsurface microbial communities and, 3065
Basidiomycetes, 1027, 2646–2647
Basidiomycota, 1367–1372, 1647, 2914, 3353–3364
Basidiomycotina, 2124–2132
Basidiospores, 412–414, 1647
Batch reactors for bioremediation, 695
Batch systems, adhesion in biofilms and testing, 118–119
Bathrooms, bacteria in home/residential environment, 340
Bathymodiolus, 1606, 1619
Bdellovibrio, 561, 1084
Beauveria
alkaliphiles and, 227–228
in allergies and illness, 1368–1372
microbial insecticides using, 1710
Beetles, bioluminescence, 657–663
Beggiatoa, 2284–2285, 3098–3104, 3111–3116
Archaea and, 286
filamentous forms of, in activated sludge, 1301–1306, 1310
hydrothermal vents and, 1610, 1618–1628
salt production and, 2805–2809
sulfate-reducing bacteria (SRB) and, 3091
Beijerinckia
nitrogen fixation and, 2193–2201
subaerial communities and, 3057–3065
Benchmark values, in water treatment systems, 1729–1730
Benthic algae (See Meroplankton)
Benthic-associated primary production (See also Primary production), 392, 2573
Benthic fauna, in sediments and sedimentation, 2852
Benthic habitats, aggregates and consortia, 164
Benthic pelagic coupling, nitrogen cycle, 2180–2188
Bentonite, activated sludge foaming, 6–7
Benzanthracene bioremediation, 696–712
Benzene biodegradation, 700, 714, 916–917, 1423–1429, 1763, 1994–2000, 2412, 2931, 3147–3155, 3405, 3412, 3413
Benzisothiazolinone (BIT), 605
Benzofluoranthene bioremediation, 697–712

Benzoperylene bioremediation, 697–712
Benzopyrene biodegradation, 697–712, 714, 917, 2403–2416
Benzothiophenes, desulfurization, 1047–1048
Benzyl alcohol biodegradation, 3413
Benzyl chloride biodegradation, 3413
Berkeleya, 2837–2844
Beta carotene, 2516
Beta cyclodextrin, 1185
Beta D glucans, 417
Betaine, 518, 2778–2789
Betula, ectendomycorrhizal fungi, 2125–2126
Bicarbonate, in activated sludge, 741–748
Bifidobacterium, 895–905, 1257
Biguanides, biofouling vs., 604–605
Bile acids, 1257
Binary fission, in bacterioplankton, 2519
Binary image enhancement, 1682–1683
Binuclearia, 1025
Bio Green, 881
Bioacidification, metal biodegradation, 1881
Bioactive barrier, 695
Bioactive zone, 695
Bioaerosol sampling (See also Bioaerosols), 392–404
  analysis of samples in, 400
  biochemical assay in, 402
  calibration of sampler in, 399–400
  collection time for, 399
  culturing in, 400–401
  data analysis for, 402
  efficiency of, 397–398
  electrostatic precipitation type, 397
  filtration type, 396–397
  gravity sampling in, 397
  identification in, 401
  immunoassay in, 401–402
  impaction type, 393–396
  liquid impingement type, 396
  microscopy in, 401
  number of samples in, 399
  polymerase chain reaction (PCR) in, 402
Bioaerosols (See also Bioaerosol sampling), 182, 392
  actinomyces, 1
  active dispersal mechanisms in, 405–407
  agricultural and outdoor settings, 404–416
  apple scab spores in, 413
  ascospores in, 412–414
  atmospheric transport of, 407
  bacterial pathogens, 182
  bacteriophages and, 382
  basidiospores in, 412–414
  bioassays of, 1130–1131
  biosensors and, 1132
  biotechnology and, 417–419
  bioterrorism and, 425, 780
  blue mold spores in, 414–415
  Buller's drop and, 406
  death rate of, 2110
  deposition of, 407, 428
  detection of, 1130–1136
  diffusion rates for, 427
  dispersal mechanisms in, 405–407, 425–427, 2108–2112
  dry air spora in, 408–414
  electrochemiluminescence in, 1132
  enzyme immunoassay (EIA) in, 1131
  enzyme linked immunosorbent assay (ELISA) in, 1131
  exponential decay in, 2108
  exposure recommendations for (NIOSH, OSHA, etc.), 420–423
  fate of, survivability and, 425, 428–432
  flow cyclometry (FCM) in, 1132
  food processing and, as byproduct of, 423
  fungi and fungal spores in, 182, 404–405, 407–415, 1359–1366, 1647–1661
  Fusarium spores in, 413–414
  gas chromatography MS in, 1132
  Gaussian plume dispersion model for, 2108–2110
  gram-negative bacteria, 429
  gram-positive bacteria, 429
  immunoassays in, 1131–1132
  industrial settings for, 416–425
  infectious airborne bacteria as, 1689–1697
  kinetic limulus assay with resistant/parallel line estimation (KLARE) in, 1131
  landfill spreading associated with, 3324–3325
  late blight of potato spores in, 414
  Legionella as, 1788–1806
  limulus amebocyte lysate assay (LAL) in, 1130–1131
  machining and, as byproduct of, 419–420
  mass spectrometry and, 1132
  matrix assisted laser desorption ionization mass spectrometry (MALDI MS), 1132
  microchip-based PCR for, 1134
  modeling of, 2107–2112
  moist air spora in, 412–414
  molds, 1647–1661
  molecular beacons and, 1134
  mycobacteria and, 416–417
  open air factor (OAF) and, 430–431
  organic dust toxic syndrome (ODTS) and, 422
  oxygen inactivation in, 430
  ozone vs., 430–431
  particle tracking model for, 2110–2112
  passive dispersal mechanisms in, 405
  plume height vs. dispersion of, 2110
  pollutants vs., 430
  polymerase chain reaction (PCR) in, 1132–1134
  protection of microbes in, 431–432
  regression decay in, 2108
  relative humidity vs., 428–430
  repair of microbes in, 431
  respiratory diseases and, 422
  rust spores in, 411–412
  sampling of, 392–404, 2818–2820
  saphrophytes and, 416
  smut spores in, 410–411
  solid waste management as source of, 3320–3330
  spores as (See also Fungi and fungal spores, above), 183–184
  sprinkler systems using wastewater, generation of, 3323–3324
  survival models for, 2108
  temperature vs., 431
  textile industry and, as by-product of, 422–423
  toxigenic molds, 182–189
  transport of, 425–432
  ultraviolet radiation vs., 431
  uredospores in, 411–412
  viruses in, 3255–3260
  waste treatment and, 420–421
  wastewater treatment as source of, 3320–3330
  wind speed vs. dispersion of, 2110
  wood processing and, as byproduct of, 421–422
  yeast spores, 405
Bioassays, 1130–31
Bioaugmentation and bioremediation (See also Biodecomposition), 434–442, 695, 2395–2397

activated sludge and, 6–7, 40
anaerobic bacteria in, 439
aquifer treatment using, 440
biofiltration using, in situ, 439–440
bioremediation and, 713
cold-adapted microorganisms and, 3212
constitutive degradative activity in, 435–436
desert environment and, 1584
energy enrichment for, 436–437
enhanced transport for, 436
hierarchy for, 434–435
improving organisms used in, 435
metal biodegradation and, 1886
as microbial tracer in groundwater, 3200
mixed cultures and consortia in, 437
organisms used in, 435
polybetahydroxybutyric acid (PHB) in, 436–437
in septic tank systems, 2862
survival of introduced bacteria in, 438–439
type 1, 437–439
type 2, 439–441
Bioavailability, 87–88, 725
Biocatalysis
Archaea and, 259–276, 271
cold-adapted microorganisms and, 881
enzymes and, 1185
Biochemical adaptation, 1195
Biochemical assay, 402
Biochemical fossils, 2307
Biochemical methane potential (BMP), 1760
Biochemical oxygen demand (BOD), 2972, 3168
Biochips (gene chips) (See also Microarrays), 443–450, 1448–1449
applications for, 447–449
classification of, passive vs. active, 444
detection by, 445–446
electrophoresis and, 445–446
hybridization techniques and, 446
micrototal analysis in, 446–447
nitrifying bacteria typing using, 448
oligonucleotide microarray analysis using, 449
reactions in, 445
rifampin resistance testing and, 448–449
sample preparation for, 444–445
Biocides
biofilms vs., 556–557
biofouling vs., 602–609, 625–626
enzymatic degradation of, 608
extracellular polymeric substances (EPS) vs., 1228
surface embedded, 606
Biocompatibility, bioplastics, 687–692
Biocontrols (See also Genetically modified microorganisms), 450–465
bacteria as, 454–455
biosurfactants/bioemulsions and, 766
cell wall lysis and, 458
combined usage of, 461–462
delivery of, 461
direct control through, 456–459
formulation of, 461
fungi as, 451–454, 1385
genetically modified microorganisms (GMMs) and, 1437
improvement of, molecular approaches to, 462–463
indirect control using, 459–460
integrated pest management (IPM) and, 462
mechanisms of, 456–461
microbial insecticides, 1709–1717
nematodes vs., 455–456
plant growth promoting rhizobacteria (PGPR) and, 454–455, 2722
regulation of, 2693–2707
selection of, 460–461
siderophores as, 454
Biocorrosion (See also Microbially induced corrosion), 464–475, 2423–2424
biofouling and, 621
cyanobacteria vs., 942–943
extracellular polymeric substances (EPS) and, 1227–1228
marine biotechnology and, 1855–1856
sulfate-reducing bacteria (SRB) and, 3092–3093
Biodegradable dissolved organic carbon (BDOC), 484–507
assimilable organic carbon (AOC) vs., 323–324, 485–507
biodegradable organic matter (BOM) estimation and 486–504
biofilm formation and, 88, 499–504, 572
bioreactors for, 487–488
dissolved organic carbon (DOC) and, 485–507
identification of, 485–486
measurement of, 485–493
nutrient levels in water and, 489
organic carbon transformation and, 494–499
parameters affecting, 489–491
particulate organic carbon (POC) and, 485–507
refractory dissolved organic carbon (RDOC) and, 487
regrowth of bacteria and, 499–504
removal of, 493–499
sand-attached bacteria and, 487
specific UV absorbance (SUVA) testing, 485
suspended bacteria and, 487
total organic carbon (TOC) and, 485–507
in water treatment systems, 1729
Biodegradable organic matter (BOM) estimation, 486–504
Biodegradation (See also Metal biodegradation), 475–484, 507–516, 1895, 2929–2930
accelerated degradation in, 2386
acclimation in, 2386
adenosine triphosphate (ATP) and, 2931
aerobic processes in, 2404–2411, 2930–2932
anaerobic processes in, 2411–2413, 2932–2933
aryl halides, 1238–1252
assessment of, 475–484
benzothiophenes and, 1047–1048
bioaugmentation and, 2395–97
biochemical adaption of microorganisms in, 1195
bioplastics and, 683–692
bioreactors for, 1942
bioremediation and, 478–482, 514, 2395–2397
biostimulation, 695, 714, 750, 2395–2397, 3211–3212
biosurfactants/bioemulsions and, 714–715, 750–770, 1129–1130
bromophenol utilization in, 512
carbon/energy cycle and, limitations to, 1872–1876
catabolic pathways and, 1197–1199, 1203–1204
catabolite repression in, mixed substrates, 1872
chemical warfare and, 846–847
chlorinated compounds, 3410
chlorinated methane and trichloroacetate utilization in, 511–512
chloroethene utilization in, 510–511

Biodegradation (See also Metal biodegradation), (*continued*)
chlorophenol utilization in, 512
cold-adapted microorganisms and, 3210–3215
cometabolic reductive dechlorination, 508–509
cometabolism in, 2386–2387
compost and, 914–927
contaminant levels vs., 2930
definition of, 475–476
dehalogenation in, 3414
desert environment and, 1583–1584
diauxic growth vs. mixed substrate growth in, 1870–1872
dibenzothiophene and, 1042–1049
dioxygenases in, 3412
enzymes and, 1875–1876
explosives, 1498, 1978–1993, 3410
fertilizer application to aid, 699, 714, 724, 2931
fuel oxygenates, 1994–2000
fungi and, 1390–1392, 2911–2912,
genetically engineered microorganisms (GEMs) and, 1420–1429
genetics of, 2387
growth kinetics and, 1874–1875
halogenated compounds and, 702, 703–704, 1195, 1238–1252, 1447, 3405–3410, 3414
halorespiration in, 3414
herbicides and, 2385–2393
hydrocarbons and, 694–697, 2402–2416, 3211–3215, 3405–3410
hydrogen peroxide and, 2932
IS1071 insertion element and, 1200
metabolic pathway evolution for, 1194–1207
metal (See also Metal biodegradation), 1878–1883, 1895, 3045, 3216
mineralization and, 2386, 2931
mixed microbial populations used in, 512–513
mixed organic pollutants and, 1869–1877
mixed substrate growth in, 1870
monitored natural attenuation (MNA) in, 1941–1942
monooxygenases in, 3412
municipal solid waste landfills and, 1759–1763
natural attenuation, 1238
neuston microbiology and, 2137–2139
nicotinamide adenine dinucleotide (NAD) and, 2931
nitrilotriacetate (NTA), 1871, 1875–1876
nitrogen and, 707–708, 2930, 2931, 2932–2933
nuclear wastes and, 250–260, 2661–2674, 3045
oil spills and (See Oil spills)
oxidative phosphorylation in, 2931
oxidoreductases in, 3412
oxygen and, 2930–2932
oxygenase enzymes in, 2288–2300
pesticides and, 2385–2402, 3121–3122, 3405–3410
petroleum and, 2402–2416
phosphorus and, 2930
plasmid borne degradation genes and, 2387
primary and ultimate, 2394–2395
protozoa and, 2594
in pulp and paper industry, 2646–2651
radioactive wastes and, 250–260, 2661–2674, 3045
reductases in, 3414
reductive dechlorination and dehalorespiration in, 509–510, 513–514
sampling for, 476–478
seagrass detritus and, 2829–2831
in septic tank systems, 2860–2861
soil adaptation and, 2386, 2923–2936
stress response and, 3043–3045
subaerial communities and, 3061–3062
subsurface microorganisms and, 2033
surfactants in, 714–715, 750–770, 1129–1130, 2411
terminal electron accepting processes (TEAP) in, 2932
toxic chemicals, 2650, 3153
triazine herbicides and, 2391–2393
water fungi and, 3353–3364
weathering and, 3364–3389
wetlands and, 3401–3417
wood and, 2646–2647
xenobiotics and, 1195
Biodesulfurization, hydrocarbons, 2414
Biodeterioration of mineral materials (See Weathering)
Biodiversity
bacterioplankton and, 2524, 2526–2528
desert environment and, 1577–1580
fungi and, 3354–3355
genomic studies and, 1450–1452
in groundwater, 1671–1672
hydrothermal vents, 1604–1628, 1625
marine biotechnology and, 1850
mycorrhizae and, 2127
permafrost and, 2367–2385
petroleum reservoirs and, 2426–2431
in sediments and sedimentation, 2848–2849
soil microorganisms and, 528–544, 2925–2926
subaerial communities and, 3055
subsurface microbial communities and, 2034–2036, 3314–3315
sustainable agriculture and, 3116–3126
thermophiles and, 3141–3142
wastewater/sewage treatment and, 3337
Bioemulsifiers, 750–770
Biofertilizers, 351
Biofilm Accumulation Model (BAM), 502
Biofilm formation rate (BFR), 1729
Biofilm reactor, 695
Biofilms (See also Adhesion; Biofouling; Conditioning films), 100, 551–559
abrasion vs., 547
accumulation of, 545
acetogenesis in, 83
actinomycetes and, 1083, 2342
activated sludge and, 44, 51–52, 54, 58
activity and carbon transformations in, 81–92
adenosine triphosphate (ATP) formation in, 82, 83, 89
adhesion and, 100–132, 560–561, 612–613, 628–629, 938–939
adsorption of macromolecules in, 937
aggregates and consortia in, 160–181, 1213
algae in, 88, 612–613
allochthonous pathogens in, 2347–2353
amides in, 937–938
amoebas in, 2342
analysis of, 624–625, 2006–2008, 2340
"antenna effect" in, 560

anthropogenic carbon sources for, 86–87
antimicrobials vs., 556–557
aquatic, 1216
architecture of (See Structure of)
assimilable organic carbon (AOC) and, formation rates (BFR), 88, 324–325
attachment of, 2088
autochthonous microorganisms in, 2342–2343
autotrophs in, 81–82, 2341
available dissolved organic carbon (ADOC) and, 1086–1087
bacterial counts for, 565–566
bacterial-fungal, 551–559
bacteriophages and, 2342
bioavailability concepts for, in carbon assimilation, 87–88
biocides vs., 556–557
biodegradable dissolved organic carbon (BDOC) and, 572, 499–504, 572
biodeterioration of mineral materials and, 516
biofilm formation rate (BFR) in, 1729
biofouling and, 601–609
biological weathering and, 517
blooms of, 2340
Brownian motion and, 936
carbohydrates in, 85–86
carbon dioxide and, 83–84, 87, 89–91, 1083
carbon metabolism in, 81–83, 559
carbon substrate sources for, DOC and POC, 84–87, 89
catabolic vs. anabolic reactions in, 81
catabolite repression in, 89
in caves and mines, 830–831, 842
cell signaling in, 567
cell walls and, 2959–2960
chain elasticity in, 937
characterization of, 553–554
charge neutralization in, 2004–2005
chemical weathering and, 516–517
chemoautotrophs in, 81–82
chemoorganotrophs in, 81–82
classification of, 81
coliform bacteria and, 907–909
colloidal particles and, 127–128, 2964–2965
colonization in, 611–612, 1083
composition of, 937–938
computer simulation of, 2089
conditioning films and, 101, 127, 559, 611, 928–941
confocal scanning laser microscopy (CLSM) to study, 558, 570
consortia and (See Aggregates and consortia)
control of, 556–557, 623–630
cooling systems and, 579
cultivation of, 563–564
cyanobacteria in, 83, 568–569, 1083
cytoplasm and, 2960
demethylation in, 2963
denaturing gradient gel electrophoresis (DGGE) and, 563
density of, 548–549
deposition of, 114, 938
Derjaguin Landau Verwey Overbeen (DLVO) theory of adhesion in, 102–104, 106, 119–122, 124, 561, 2004–2005
desorption limitation in, 88
detachment of, 544–550, 561, 2087–2088
detection of, 580, 624
development of, 551–553
diatoms in, 612–613, 1051–1063
direct viable count (DVC) in, 565
directed evolution and, 1176–1177
disinfection vs., 580–582, 625–628
dispersal of, 561, 606, 626–628
dissolved combined amino acids (DCAA) in, 85
dissolved components in, 2088
dissolved free amino acids (DFAA) in, 85
dissolved organic carbon (DOC) and, 569, 1086–1087
dissolved organic matter (DOM) and, 569
dissolved oxygen (DO) and, 2005–2006
disturbance of, ecological impact of, 1088
DNA analysis of, 565–566
ecological selection in, 546
electrostatic interactions in, 103–104, 120, 606, 936, 2004
environmental, 555–556
enzyme activity in, 1214–1221
equivalent spherical diameter (ESD) measurement in, 2006
eukaryotes and bacteria interaction in, 567–568
exogenous isolation and, 1410
exopolymers in, 561, 1083–1084
extracellular enzymes and, 560, 1207–1223
extracellular polymeric substances (EPS) in, 81, 89, 546, 548, 560, 569, 571, 628, 1081, 1083–1084, 1207–1223, 1408, 2000–2012, 2959, 2961, 2964
extracellular survival in, 2353
factors affecting, 2005–2006
facultative anaerobes in, 82
fecal pollution and, 2347–2350
fermentation and, 82
filamentous bacteria, 1083
floc formation and, 58, 89, 2000–2012
fluid in, 2089
fluorescence in situ hybridization (FISH) studies of, 570, 571
food industry and, 577–587
formation of, 554–556, 560–563, 611–613, 620–623, 936–937, 1082–1083, 2004–2005
fractionation of, 1213
free energy of adhesion in, 101
fungi in, 613, 2342
Gallionella in, 1406
gene exchange/gene transfer in, 567, 1408–1420
genetics of, 561–563
glycoproteins in, 937–938
gradients in metabolism of, 1085
gram-negative bacteria and, 2960
gram-positive bacteria in, 2959–2960
granular activated carbon (GAC) and, 1489–1496
grazing in, 2342
green fluorescent protein (GFP) markers for, 558–559, 1498
growth rate of, 545–546, 549, 558
hazard analysis and critical control point (HACCP) concept, 583
heterogeneity of, 549
heterotrophs in, 81–82, 2341
humic substances and, 86–88, 114
hydrogel matrix of, 81
hydrolysis in, 87
hydrophilic/hydrophobic forces in, 2004, 2005, 2007–2008
hyperthermophiles and, 1642
immobilization in (See also Adhesion), 100, 105–111
industrial, 555–556, 582–584, 619–632
intracellular survival in, 2353
iron and, 1085–1086

Biofilms (See also Adhesion; Biofouling; Conditioning films), (*continued*)
lectins and, 570
Lifshitz-van der Waals forces in, 101, 102–104, 123, 124, 936, 938
lipids in, 85–86, 2960
lipopolysaccharides and, 2960
lithotrophs in, 81–82
macrofouling and, algal spores in, 615–616
macrofouling and, invertebrate larvae in, 613–615
marine biotechnology and, 1855–1856
"marine snow" and, 89
marine/ocean environment and, 1081–1091
mass balance equations for, 2088–2089
mass transfer in, 2087
matrix of, 547–548
measuring transformations and activity in, 89–91
medical uses for, 554–555
metabolic activities of, 565–566
metals in, 2960–2963
methanogenesis in, 83, 87, 2963
methanotrophs in, 82–83
microautoradiographic studies of, 565
microbial mats and, 568–569
microbial transforms in substrates of, 88–89
microdomains in, 1215
microscopic analysis of, 2008
mineralization and biomineralization in, 82, 2961–2962
modeling of, 587, 2083–2094, 2340
Modified Robbins Device (MRD) to study, 557–558
nicotinamide adenine dinucleotide phosphate (NADPH) in, 83
nitrogen and, 1085–1086, 2005
nutrients in, for bacteria, 939–940
organic substances in, 2963–2964
oxygen and, 83–84, 1083, 2005, 2962
ozonation and, 87
particulate organic carbon (POC) and, 1086–1087
particulates in, 2088
pathogens in, 587, 621, 2339–2356
periphyton and, 2357–2367
pH levels and, 100, 104–105, 121–22, 1085–1086
phosphorus and, 1085–1086, 2005
phosphorylation in, 82–83
photoautotrophs in, 81–82
photoheterotrophic bacteria in, 1087–1088
photosynthesis in, 84
phototrophy and photoautotrophy in, 83
physiology of, 548–549
plankton and, 81, 82, 88, 114, 611–612, 1082–1083
polar interactions in, 937
polymer bridging in, 2005
polymerase chain reaction (PCR) to study, 558–559
polysaccharides in, 937–938
precipitation in, 2962
prevention of, 628–630
proteins in, 85–86, 937–938
pseudomonads in, 2343–44
in pulp and paper industry, 580, 2650
quorum sensing in, 549, 1220
redox and, 2960–2963
reduction and, 2962
remobilization in, 2965
resistance of bacteria in, 608
respiration in, 82–83, 565
restriction fragment length polymorphism (RFLP) in, 563
retention of microbes in, 111
"river snow" and, 570–571
riverine, 569–572
RNA analysis of, 565–566
rotating annular bioreactors (RAB) studies for, 570
saccharolytic enzymes vs., 606
Salmonella in, 2793
salt bridging in, 2005
sampling of, 624, 830–831, 2006–2008, 2814
settling in, 2007
shear rates and, 2341
sloughing in, 1083
sorption properties of, 587, 2958–2967
spalling, 517
steric forces in, 2004
stoichiometric matrix in, 2086–2087
stress and strain forces on, failure of, 547–548
structure and architecture, 553, 560–563, 1083–1084, 2341–2342, 2341
substratum properties for, 546, 938
sulfate-reducing bacteria (SRB) and, 82, 2962, 3091–3092
sulfur and, 82, 1085–1086
surface charge and, 2008
surface thermodynamics and, 2005
survival of, 2353–2354
suspended, 587
syntrophy and antagonism in, 83–84
terminal fragment length polymorphism (T-RFLP), 563
terrestrial, 1216
thickness of, 1083, 2088–2089
transfer processes in, 2087–2088
transformation processes in, 2086
transport processes in, 2087
turbulence vs., 547
van der Waals forces in, 119–129, 124, 552, 1083, 2004
ventilation and air handling systems and, 579–580
verocytotoxigenic E. coli (VTEC) and, 2349–2350
viable but nonculturable cells (VBNC) in, 565
viruses and, 2342, 2353
wastewater/sewage treatment and, 3346–3347
in drinking water, 560–575, 620–622, 945–947, 2960
weathering, bioweathering and, 3367
zeta potentials and, 100, 119
Biofilters, 587–593, 695
activated carbon in, 590
adsorption and absorption in, 590–591
ambient temperature catalytic oxidation in, 587
ammonia vs., 598
applications for, 588–589
bioaugmentation and bioremediation in, 439–440
bioodors and, 593–601
bioremediation and, 693
bioscrubbers in, 587
biotrickling filters in, 587, 782–795
chemical potential/chemical activity in, 592
chromatographic effect in, 590
compost as, 592, 914–927
compound specificity in, 589
degradation rates in, 590
destruction/removal efficiency (DRE) in, 587, 590
economics of, 589
granular activated carbon (GAC) filters and, 1491–1492

hydrogen sulfide vs., 598, 599–600
hydrophilic materials in, 591–592
hydrophobic materials in, 591–592
mechanism of, 589–591
media for, 591–592
microbial activity in, 598–599
moisture and, 592–593
organic media in, 591–592
physical potential (hydraulic head, water tension, suction) in, 592
POTWs vs., 598
synthetic media in, 592
temperature vs., 593
toxicity and antagonism in, 591
volatile organic compounds (VOCs) vs., 598
water films in, 590–591
Biofluffing, 695
Biofouling (See also Biofilms; Microbially influenced corrosion), 601–609
adhesion and, 612–613, 628–629
algae in, 612–613
analysis of, 624–625
bactericides vs., 602–609
biocides vs., 625–626
biofilm formation and, 611–613
colonization in, 611–612
conditioning films and, 611, 928–941
corrosion and, 621
countermeasures and control of, 623–630
detection of, 624
diatoms in, 612–613, 1051, 1058–1059
disinfection vs., 625–628
dispersants vs., 606
efficacy of treatment, factors affecting, 606–608
electrochemically activate water vs., 603–604
extracellular polymeric substances (EPS), 628, 1227–1228
formation or occurrence of, 620–623
fungi in, 613
heat exchangers and, 622–623
industrial, 619–632
macrofouling and, algal spores in, 615–616
macrofouling and, invertebrate larvae in, 613–615
marine biotechnology and, 1856
marine environment and, 610–619, 623,
microbially influenced corrosion (MIC) and, 623
molecular level, 611
oil industry and, 623
organic fouling in, 619
particle fouling in, 619
pathogens in, 621
prevention of, 628–630
removal or dispersal of, 626–628
resistance of bacteria in, 607–608, 625
saccharolytic enzymes vs., 606
sampling in, 624
scaling or mineral fouling in, 619
sequence of processes in, 610–611
ship hulls and, 623
sulfate-reducing bacteria (SRB) and, 3091
water treatment systems and, 602–609, 620–622
Biogas (See Activated sludge; Methane and methanogenesis)
Biogenic acids, bioleaching, 632–642
Biogenic trace gases (See Trace gases)
Biogeochemical cycles
aggregates and consortia in, 161, 165–166
flooded soils and, 1316–1318
nitrogen cycle and, 2180–2188
in sediments and sedimentation, 2844–2848
Bioherbicides, 1385
Biohydrometallurgy (See also Bioleaching), 632, 881–882
Bioindicator
protozoa and, 2630–2631
soil quality and, interpretation of, 2950–2951, 2953–2954
Bioinformatics, 1449–1450
Bioinsecticides, 1385
Bioleaching, 632–650, 695
applications for, 638–640
attachment of bacteria to mineral surfaces in, 635–636
BacTech process for, 647–648
biomining and, 638–639
biooxidation in, 648–649
BIOX process for, 647
chemoorganoheterotrophic microorganisms in, 635
chemotaxis in, 635–636
cold-adapted microorganisms and, 881–882
commercial practice of, 647–648
environmental problems and, 640
extracellular polysaccharide (EPS) and, 644
galvanic influence in, 637
heap vs. dump leaching in, 642, 648, 649
history of, 642–643
hydrogen sulfide pathway in, 637
iron and, 637–638, 643, 646
metal biodegradation and, 1881
metal sulfide dissolution in, microbiological, 635–638
metal sulfide oxidizing bacteria in, 634–635
microorganisms of, 634–647
minerals affected by, 633–634
polysulfide pathways in, 637
pyrite and, 638
sphalerite and, 637–638
thiosulfate pathways in, 636–637
weathering, bioweathering and, 3366
BIOLOG, 1961–1962
Biological fluidized bed, 695
Biological mat, in septic tank systems, 2858
Biological nutrient plants (BNR)
activated sludge and, 1313–1314
Microthrix parvicella and, 3
wastewater/sewage treatment and, 3337, 3338–3339
Biological oxygen demand (BOD)
activated sludge and, 49, 50, 745–746
algal turf scrubbing (ATS) and, 216
in wastewater stabilization ponds (WSBs), 3336
Biological plug, 695, 707
Biological pulping process, fungi, 2647
Biological soil crusts (BSCs), 1019–1023
Biological trickling filters, 74
Biological warfare (See Bioterrorism)
Biological weathering, 517
Biologically active carbon (BAC), 1489, 1491
Biologically controlled mineralization (BCM) (See also Biomineralization), 671
Biologically induced mineralization (BIM) (See also Biomineralization), 671
Bioluminescence, 657–663, 1830–1838
Biomarkers, lipids (See Lipid biomarkers)
Biomass, 2565
acridine orange and, 2653
activated sludge and, 22–26, 72, 74, 739, 1311, 1315
adenine nucleotides in, ATP, ADP, 2654
algal (See Plankton and photoplankton)

Biomass, (*continued*)
algal turf scrubbing (ATS) and, 213–219
Archaea and, 266, 2658
bacterial counts and, 2653
bacterioplankton, 663, 2520, 2528–2530
in biotrickling filters, 786–795
biochemical constituents of, 2654–2657
biofilms and, 81
cell wall analysis in, 2657
chloroform fumigation incubation/extraction (CFI/E) for, 2653
desert environment and, 1576, 1577, 1579
diamindino phenylindol (DAPI) and, 2653
DNA/RNA analysis of, 2656–2657
endolithic microorganisms and, 1104–1105
enzymes and, 1186
epifluorescence and, 2653
estimation techniques for, 2653
fatty acid methyl ester (FAME) and, 2656
fatty acids and, 2654–2656
flow cytometry and, 2653
fungal, 1397, 2657–2658
harmful algal blooms (HABs) and red tides, 2690
lipid biomarkers of, 1818–1819
marine biotechnology and, 1851
mass spectrometry for, 2656
metal bioadsorption by, 1879–1880
nucleic acid–based testing of, 2656–2657
periphyton and, 2357, 2359
phospholipid bound phosphate (PLP) and, 2654–2656
phospholipid fatty acids (PLFA) and, 2654–2656
phospholipids in, 2654–2656
photosynthetic pigment analysis in, 2657
plankton and phytoplankton, 2516, 2559–2560, 2653
polar phytoplankton and, 2559–2560
primary production (marine) and, 2566–2575
proteins in, cellular, 2654
protozoa and, 2628–2629
quantification of, 2652–2660
sea ice microorganisms and, 2841
soil (See also Soil microorganisms), 294–295, 663–670, 2895, 2948
subaerial communities and, 3061
subsurface microorganisms and, 1076, 1077–1078, 1080
toxicity testing and, in soils, 3158–3159
Biomaterials, 1850, 1853
Biomineralization (See also Mineralization; Sulfate-reducing bacteria), 670–681
biofilms and, 82
biologically controlled mineralization (BCM) and, 671
biologically induced mineralization (BIM) and, 671
bioremediation and, 679
carbonates, 672
cyanobacteria in, 672
freshwater lakes and rivers and, 677–678
geologic evidence of, 676–678
hydrothermal environment, 678
ionotrophy and, 672
iron and, 674–679
magnetotactic bacteria and, 678
manganese and, 675–676
redox transformations and, 673–675
significance of, 678–679
silicates, 672–673
stromatolites and, 678
sulfate-reducing bacteria (SRB) in, 672
Biomining (See also Metal ion extraction), 274, 682, 638–639
Biomonitoring
periphyton and, 2365
in wastewater/sewage, 3168
Bioodors
biofiltration and, 593–601
biotrickling filters, 782–795
Biooxidation, bioleaching, 648–649
Biopesticides (See Insecticides)
Biopile, 695, 914–927, 914
Biopitting, 518
Bioplastics, 682–692
Biopolaris, 1651
Biopolymers
biomineralization and, 671
bioplastics and, 682–692
marine biotechnology and, 1853
Bioprocess engineering, marine biotechnology, 1850, 1855–1856, 1858–1861
Bioprocessing, hydrocarbons, 2414
Bioreactors, 487–488, 1942
Bioremediation and detoxification (See also Biodegradation; Composting), 692–712, 1446–1447, 2402
acclimation of microorganisms in, 713
acid mine drainage and, 709
aerobic respiration and, 151
algae biotechnology and, 189–204
ammonium, 707–708
aquatic ecosystems and, 712–722
bacteria used in, 713
bioaugmentation and, 434–442, 713, 2395–2397
bioavailability of contaminants vs., 725
biodegradation and (See Biodegradation)
biofiltration and, 693
biomineralization and, 679
bioreactors for, 1942
biostimulation in, 695, 714, 750, 2395–2397, 3211–3212
biosurfactants in, 714–715, 750–770, 1129–1130
carbon and, 714, 724
case studies in, 734
closed loop, 695
cold-adapted microorganisms and, 882–883, 3210–3215
cold-adapted microorganisms and, 3215
commercial applications of, revenues from, 693
composting and, 693, 695, 914–927
concentration of contaminants vs., 725
ecology and, 693–694
electron acceptors for, 714, 724
encapsulation of microbial cells for, 730
enhanced, 712
ex situ, 695
explosives biodegradation and, 1988–1990
fertilizer application to aid, 699, 714, 724, 2931
fungi and, 1390–1392
genetic engineering and, 729–730
genetically engineered microorganisms (GEMs) in, 726, 729–730
groundwater pollution and, 699–700, 702–703, 705–708, 1447
halogenated compounds and solvents, 693, 701–705
heavy metals, 1855
herbicides and, 705–706
hydrocarbons and, 694–701, 713–719, 2414
inorganic contaminants and, 707–708
intrinsic, 695

lipid biomarkers of, 1822
marine bacterial isolate (MBI), 715
marine biotechnology and, 1850, 1855
marine/ocean environment and, 712–722
metal biodegradation and, 1886
metals and metalloids in, 708–709
methanogenesis, marine, 1921
methanotrophic bacteria in, 1936–1944
as microbial tracer in groundwater, 3200
military chemicals and, 706–707
mixed organic pollutants and, 1869–1877
moisture content (of soils) in, 723–724
monitored natural attenuation (MNA) in, 1941–1942
nitrogen compounds and, 707–708, 714, 724
oil spills and, 693–701, 713, 715–719
options for, 734–735
oxygen and, 714, 724
pesticide degradation and, 705–706, 2395–2397
pH levels and, 713–714, 724
phosphorus and, 714, 724
phytoremediation and, 709, 731–734
plant assisted microbial bioremediation, 1424–1425
polychlorinated biphenyl (PCBs) in, 726–735
pulsed, 695
rate monitoring in, 730–731
rhizofiltration and, 709
rhizoremediation, 695, 1424–1425
rivers and ponds, 705
soil contamination and, 700–707, 722–737
solubility of contaminants vs., 725
sorption characteristics of contaminants vs., 725
sulfate-reducing bacteria (SRBs) in, 708
surface water, 708,712–722
technology for, 694
temperatures vs., 713, 723
toxicity of contaminant vs., 724–725
vadose zone microbiology and, 3242–3243
vapors of halogenated compounds, 702
vapors of hydrocarbons and, 697–698
verification of, 478–482
wastewater/sewage treatment and, 693
xenobiotics, 693
Biorestoration (See Bioremediation)
Bioscrubbers, 587
Biosensors, 1132, 3172
Bioslurping, 695
Biosolids (See also Activated sludge), 420, 738–750
bioaerosols released from, 3320–3330
Biosorbents, metal, 750
Biosorption, algae biotechnology, 200
Biosparging, 695
Biosphere, soil biosphere, 294–295
Biostability of drinking water (See Assimilable organic carbon)
Biostimulation (See also Bioremediation), 695, 714, 750, 2395–2397, 3211–3212
Biosurfactants/bioemulsions, 714–715, 750–770, 1129–1130
acidic lipids in, 756–757
applications for, 763–766
biocontrols, 766
biodegradation and, 764
bioremediation and, 763–765
chemical production using, 766
critical micelle concentration (CMC) in, 751
emulsan in, 761
emulsion index for, 762–763
extracellular polymeric substances (EPS) and, 1129–1130
fengycin in, 757–759
flushing agent use of, 764
glucose lipids in, 756
glycolipids in, 752
iturin in, 757–759
lichenysin in, 759
lipoproteins in, 757–760
mannosylerythritol lipids in, 755–756
medical applications for, 765–766
neutral lipids, 756–757
phospholipids in, 756–757
polymeric, 760–762
rhamnolipids in, 752–753, 764–765
screening for activity in, 762–763
sophorolipids in, 753–755
surfactin in, 757–759
trehalose lipids in, 753
turbidity assay for, 763
Biosynthesis in cold-adapted microorganisms, 875–876
Biotechnology (See also Genetically engineered microorganisms), 1176–1189
algae in, 189–204
aquaculture and, 3215
Archaea and, 259–276, 290–291
bioaugmentation and, 3212
biodegradation and, 3210–3215
bioremediation and, 3210–3215
biostimulation and, 3211–3212
cloning and, 3203–3206
cold active enzymes and, 3206–3210
cold-adapted microorganisms and, 879–883, 3203–3219
enzymes in, 1176–1189
extracellular polymeric substances (EPS) and, 1229
field release of GEMs and, 1278–1287
fungi in, 771, 1384–1394
genetically modified microorganisms (GMMs) in, 1429–1440
intellectual property rights and, 1861
legal issues of, 1861
marine, 1850–1863
methanogenesis and, 771, 1920–1921, 1933
microbial insecticides using, 1710
petroleum reservoirs and, 2424–2425
plankton and phytoplankton, 2516
plant growth promoting rhizobacteria (PGPR), 351–352
protein engineering and, 3208–3209
psychrophiles and, 3203–3219
recombinant DNA and, 2693–2694
regulation of, 2693–2707
salt production using, 2804–2809
spores and spore dispersion in, 417–419
technology transfer issues and, 1861
Toxic Substances Control Act (TSCA) and, 1280
Biotechnology Science Coordinating Committee (BSCC), 2695–2707
Bioten, 1334
Bioterrorism (See also Bioaerosols; Toxicity testing), 771–782
aerosolization in, 780
agroterrorism and, 181, 780–781
anthrax in, 771–782
bioaersols and, 425
botulism in, 778

Bioterrorism (See also Bioaerosols; Toxicity testing), (*continued*)
brucellosis in, 775–776
characteristics of, 773–779
chemical weapons vs., 773–775
cholera and, 776, 853–861
combating, through detection and preparedness, 781
dispersion methods for, 779–789
dose response assessment in, 2745–2747
Ebola virus in, 777–778
glanders in, 771, 776
hemorrhagic fevers in, 777–778
history of, 771–773
Marburg virus in, 777–778
plague in, 776–777
ricin in, 778
sampling for, 781
smallpox in, 777
Staphylococcus aureus enterotoxin (B/SEB) in, 778
toxicity of, 773–775, 778–779
tularemia in, 777
Venezuelan equine encephalitis (VEE) in, 777
viruses for, 777–778
yellow rain in, 778–779
Biotoxins (See also Cyanobacteria; Harmful algal blooms and red tide)
Biotransformation, 695
Biotrickling filters, 587, 695, 782–795
Bioventing, 695
Biowall, 695
Bioweathering, extracellular polymeric substances (EPS), 1227–1228
BIOX bioleaching, 647
Biphenyl biodegradation, 2289–2300, 3412
Bipolaris, 408, 410, 1020–1023, 1650, 1654
Bis phthalate bioremediation, 707
Bisphenol, 605
Bivalvia, endosymbiosis, 1117
Bjerkandera, 1385, 2649
Black Sea, phototropism, 2476
Black water, 3099
Blackley, C.H., 404
Blastocladiella, 972
Blastoconidia, 1647
Blastomyces fungi, 1368
Blattabacterium, 1122
Bleaching samples, 1785–1786
Bleaching, biological, fungi, 2647–2648
Blister agents (See Chemical warfare)
Blood plasma sampling technique, 824
Blooms (See Harmful algal blooms and red tides)
Blue-green algae (See Cyanobacteria)
Blue mold, 414–415
Bodo, 2605, 2608, 2594–2604
Bodonids, 2619
Boletus, mycorrhizae, 2124–2132
Bombyx, microsporidia vs., 2065–2083
Bordetella, 1209, 3325
Boreal waters, nitrogen cycle, 2182
Borehole bioreactor, 695
Borellia, 1906
Bornholm disease, enteroviruses, 1152
Borodinella, 1109
Borrelia, 555, 1701, 1838–1849
Borreliosis (See Lyme borreliosis)
Botryococcus, 1854, 2302–2306
algae biotechnology, 199–200
Botrytis, 405, 412, 451
Bottled water, 795–802
artificial inoculation of, with bacteria, 799
bacteria in, 795–801
carbonated vs. noncarbonated, 799
Clostridium and, 866–871
contact lens cleaning and, 800
containers for, 798
dispensers for, 800
endotoxins in, 800
fluoridation of, 800
heterotrophic bacteria and, 1549–1550
mineral water and, 796
nomenclature of, 798
Pseudomonas and, 1549–1550, 2632–2639
storage of, 789–799
treatment of, 797–798
Bottom-up exploratory data analysis, 1005
Botulinum, botulism, 182–189
bioterrorism and, 778
Clostridium and, 862–871
high hydrostatic pressure (HHP) vs., 1559–1563
in wetlands for wastewater treatment, 3400
Bound effect concentration (BEC), 3163
Bound water, biofilms, 2960
Boundary layers, adhesion in biofilms, 114
Bovine astrovirus infection, 332
Brachiola, 2066–2083
Brachymonas, 32
Brackish water, aggregates and consortia, 166–168
Bradyrhizobium, 2720
biocontrol use of, 2703, 2704
endosymbiosis and, 1120
nitrogen fixation and, 2188–2211, 2188
soil salinity and, 2775, 2779–2789
stress response and, 3048
subsurface microbial communities and, 3067–3071
Bremia, 2895
Brevibacter, 753, 756, 1044, 1102–1106, 2387, 2394
Brevipalpus, 1122
Brewing and distilling, 1187, 1384
Brickwork, weathering, bioweathering, 3371–3372
Bridging, 2005
Brine fly, salt production, 2805–2809
Brine shrimp, salt production, 2805–2809
Bromine, 3184, 3266–3267
biofiltration and, 589
biofouling vs., 603
in spas and hot tubs, 2979
Bromophenol biodegradation, 512
Bromus, 1989
Bronopol, 605
Brown algae, 2467, 2504–2518
Brown rot fungi (See also Pulp and paper industry), 801, 1388–1389, 2646–2647
Brown tides (See also Harmful algal blooms and red tides), 801, 2689
Brownian motion
adhesion in biofilms and, 114, 115
aggregates and consortia in, 162
conditioning films and, 936
Brucella, 772, 775–776, 1689–1697
Brucellosis, 775–776, 1689–1697
Bryophtyes, 2504–2518, 3056–3065
Bryum, 1025
BTEX compound biodegradation, 699, 1994–2000, 2412, 2594, 2930, 2933
Buchnera, 1123
Buffering capacity, 1749
Bugula, 614
Building related disease (See Sick building syndrome)
Bulgaria, spores and spore dispersal, 406
Bulking, activated sludge, 24–26, 801, 1287–1306, 1306–1315, 2002, 3008–3011
Buller's drop, spores and spore dispersal, 406
Burkholderia, 1196, 1200, 1345

bioaugmentation and bioremediation in, 435
biocontrols and, 452, 455, 457, 2700
biodegradation and, 2289–2300
biofilms and, 1218, 2343–2356
bioremediation and, 703
bioterrorism and (See Glanders)
endosymbiosis and, 1120
flooded soils and, 1328
infectious airborne bacteria and, 1689–1697
nitrogen fixation and, 2193–2201
nosocomial infections and, 2236–2250
subsurface microorganisms and, 2035
Butane biodegradation, 2427, 2930
Butanediol, alkaliphiles, 228
Butanol, 3147–3155
Butoxyethanol bioremediation, 707
Butyrate
activated sludge and, 742–748
anaerobic granulation and, 236
Butyric acid, in activated sludge, 741–748
Butyrobetaine, soil salinity, 2778–2789

C

Cadmium biodegradation (See also Heavy metals), 197–200, 608, 1879–1893, 1894, 1902–1913, 2961
Caenorhabditis, 1442, 1834
Calcidiscus, 2508
Calciosolenia, 2508
Calcium, 1053, 1055, 1707, 1879–1893, 1902–1913, 2774
Calcium carbonate, weathering, 3364–3389
Calcium scaling, 1227–1228
Calcium silicate, weathering, 3364–3389
Caldarhaeol, Archaea, 279
Calderobacterium, 3143–3147
Caldophora, salt production, 2805–2809
Calibration in image analysis, 1684–1685
Caliciviridae, 327, 803, 1586–1598
Norwalk-like viruses (NLVs), 2221–2235
in source water, 2969–2978
in spas and hot tubs, 2980–2984
streams and, 3017
Caloneis, photoautotrophic, in low-light habitats, 838–843
Caloplaca, 1107–1112
Caloramator, 3130, 3136–3137
Caloranaerobacterazorensis, 1620–1628
Calothrix, 962, 963, 2193–2201, 2549
Calvin-Benson pathway, 1117
Calyptogena, 1606, 1619
Campylobacter, 338, 340, 803–811, 3111–3116
biofilms and, 2341–2356
Clostridium and, 869–871
diazotrophy in, 2541–2547
disinfection and, 1065, 1066
in food, 3277–3287
granular activated charcoal (GAC) filters and, 1571–1572
heterotrophic bacteria and, 1544
high hydrostatic pressure (HHP) vs., 1555–1563
hydrothermal vents and, 1609–1610
in source water, 2969–2978
in spas and hot tubs, 2980–2984
subsurface microorganisms and, 2025
in wastewater stabilization ponds (WSBs), 3334–3336
water treatment systems and, 3199
Canada, regulation of commercial microorganism use, 2706
Canadian Environmental Protection Act (CEPA), 2706
Cancers, fungi, 1375, 1377
Candida, 900
biodegradation and, 1871
biofilms and, 552–559
biosurfactants/bioemulsions and, 753, 755, 756
cold-adapted microorganisms in, 872–885
fecal contamination and, 1271
in groundwater, 1671
metal biodegradation and, 1885
municipal solid waste landfills and, 1766
nosocomial infections and, 2236–2250
in pulp and paper industry, 2649
subaerial communities and, 3057–3065
in drinking water, 945
Candidatus, 11–14, 38, 40, 1288–1306
Cantharellus, mycorrhizae, 2124–2132
Canthaxanthin, in paleolimnology, 2307–2317
Capillary array electrophoresis (CAE), 816–818
Capillary electrophoresis, 308, 445–446, 811–821
Capillary electrophoresis single-strand conformation polymorphism (CE-SSCP), 815
Capsicum, plant growth promoting rhizobacteria (PGPR) inoculation, 350
Capsid of bacteriophages, 364
Capsular polymers, activated sludge, 740
Capsules, soil microorganisms, 2884
Captured megaplasmids, 1446
Carbaryl composting, 921–922
Carbazole biodegradation, 696–712, 2297–2300
Carbetamide composting, 921–922
Carbohydrases, 1186
Carbohydrates (See also Nutrients and energy sources), 2716
anaerobic granulation and, 235
biofilms and, 85–86
diatoms and, 1053–1055
hyperthermophiles and, 1641–1642
municipal solid waste landfills and, 1760
in surface waters, 928, 928
Carbon/carbon cycling (See also Nutrients and energy sources), 928, 1706
activated sludge and, 15–26
bacterioplankton and, 2521–2523, 2533
biofilm transformations of, 81–92
bioremediation and, 714
cold-adapted microorganisms in, 872–885
denitrification and, 1013–1014
desert environment and, 1577, 1582
diatoms and, metabolism of, 1052
flooded soils and, 1317–1318
in groundwater, 1669–1671
hyperthermophiles and, 1633
in marine/ocean environment and, 1697–1709
mycorrhizae and, 2128
nitrogen fixation and, 2196–2198
oxygen and, 2278
phosphorus cycling and, 2450
primary production and use of, 2566–2575
protozoa and, 2622–2623
in sediments and sedimentation, 2844
in septic tank systems, 2862–2863
soil microorganisms and, 663, 666–670, 2059–2060, 2887
streams and, 3021
subsurface microbial communities and, 1075, 1455

Carbon/carbon cycling (See also Nutrients and energy sources), (*continued*)
sulfate-reducing bacteria (SRB) and, 3087–3088
Carbon dioxide, 1760, 2426, 3183, 3184–3185
activated sludge and, 738, 741–748
aggregates and consortia in and, 175
biofilms and, 83–87, 89–91, 1083
biotrickling filters, 782–795
in caves and mines, 823
desulfurization of fossil fuels, 1041–1051
halophiles and, 1505
heterotrophic bacteria and, 1542
hydrothermal vents and, 1611, 1618
hyperthermophiles and, 1633
marine biotechnology and, sequestration in, 1855
municipal solid waste landfills and, 1760
nitrogen cycle and, 2183–2184
oxygen and, 2277
plankton and phytoplankton, 2515
primary production and use of, 2566–2575
qCO_2 and, 2954–2956
rhizosphere and, 2721–2722
soil microorganisms and, 2057, 2061
subsurface microbial communities and, 1074–1075, 1461–1463
Carbon fixation, bacterioplankton, 2521
Carbon monoxide, 294, 1618
Carbon storage, activated sludge processing, 23–26
Carbon tetrachloride (CT) bioremediation, 434–442, 511–514, 701–712
Carbonaceous biochemical oxygen demand (CBOD), in wetlands for wastewater treatment, 3391
Carbonated waters, 799
Carbonates
bioleaching and, 632–642
biomineralization of, bacterial, 670–681
soil salinity and, 2774
Carbonic acid, weathering, bioweathering, 3366
Carbonic anhydrase, in marine/ocean environment, 1706
Carbonyl sulfide and soil biosphere, 294
Carbonyl biomineralization, 671
Carboxy cis muconic acid, biodegradation, 2295–2300
Carboxyl biomineralization, 671
Carboxylesterase, in soil, 2903–2910
Carboxylic acid, 928–929, 3412
Carcinogens, biodegradation, 507–516
Cardococcus, 63
Carnitine, 1185, 2778–2789
Carotenes/carotenoids, 1852, 2453–2470, 2473, 2512
algae biotechnology and, 193
alkaliphiles and, 228
cyanobacteria and, 964
in paleolimnology, 2307–2317, 2307
Carrageenan, algae biotechnology, 192
Carsonella, 1123
Carteria, 2835–2844
Cassia, nitrogen fixation, 2205
Casuarina, nitrogen fixation, 2209
Catabolic activity
biodegradation and, 1197–1199, 1203–1204
biofilms and, 81, 89
wetlands and, 3411–3415
Catalase, 432, 1181–1189, 2278, 2903–2910, 3207–3210
Catalysts, 2899
Catalytic properties of abiotic soil components, 1750
Catalyzed oxidation reduction reactions, subsurface microbial communities, 1454–1455
Catechol biodegradation, 2295–2300
Catechol oxidase, in soil, 2903–2910
Catenaria, 455
Catha, 454
Cation exchange capacity (CEC), 389
Cations, 241, 2099
Caulobacter
biofilms and, 90
granular activated carbon (GAC) filters and, 1490
oligotrophic bacteria and, 2268, 2271–2272
stress response and, 3048
viable but not culturable (VBNC) phenomenon and, 3247, 3251
Causarina, nitrogen fixation, 2202
Caves and mines (See also Acid mine drainage; Subsurface microbial communities), 835
aerophitic photoautotrophic microorganisms in, 835–843
air-filled, 822–825
anaerobic bacteria in, 827–828
biofilms in, 830–831, 842
blood plasma sampling technique in, 824
chasmoendolithic microbes in, 842
cold-adapted microorganisms in, 872–885
endolithic microbes in, 842
epiendolithic microbes in, 842
gases in, 823
"green sickness" of, 841–843
habitats in, 822–823
kick-plate boundary in, 829
microbes in, 823–825
microbiological sampling in, 821–835
minerals in, 823
on-site observations in, 827
permits and legal restrictions on, 832–833
pH levels in, 842
protection of, 832–833
rock sampling in, 829–830
safety in, 833
subaerial communities and, 3059
subsurface microbial communities and, 1076, 1078–1079, 3067–3068
surfaces in, 821
underground mines and, 828–832
volcanic tuffs, subsurface microbiology of, 3110–3119
water and moisture in, 821–835
water filled, 825–828
Cell–cell interactions, biofilms, 1056–1058
Cell membranes and stress response, 3031
Cell signaling in biofilms, 567
Cell sorting, 1944–1958
Cell surface hydrophobicity, activated sludge foaming, 5–7
Cell viability (See Laser scanning microscopy)
Cell wall structures
alkaliphiles and, 137
Archaea and, 248
bacterioplankton and, 2519
biofilms and, 2959–2960
biomass estimation and, 2657
cold-adapted microorganisms and, 874
desiccation and, 1029
lysis of, through biocontrols, 458–459
metal sorption to, 1879, 1895–1896
oligotrophic bacteria and, 2274–2275
plankton and phytoplankton, 2509

polar phytoplankton and, 2555
soil microorganisms and, 2882
toxic solvents and, 3153
Cellobiose, 756, 2899
Cellobiose dehydrogenase (CDH), 1387–1388
Cellubiase, 2899, 3021
Cellulase, 226, 1177–1189, 1388, 2903–2910
Cellulitis, Aeromonas, 1091–1099
Cellulomonas, 1583
Cellulose, 85–88, 682–693, 1210, 1760
Cellulosome, 1447
Cement, biodeterioration of mineral materials, 523–525
Cenarchaeum
Archaea and, 289–290
biotechnology and, 3204
soil microorganism and diversity in, 539
Centrifugal impactor type bioaerosol sampler, 395–396
Centrifugation, adhesion in biofilms vs., 116–117
Centropyxis, 2605
Cephaleuoros, 3056–3065
Cephalosporin, 1366, 1369, 1852
Cephalosporium, 2705
Ceramics, weathering, bioweathering, 3371–3372
Ceratium, 205, 1864–1869
Ceratocorys, 2507
Ceratocystis, 2648–2650
Cercocarpus, 2124–2132
Cercomonas, 1126, 2594–2605, 2608
Cercospora, 413
Ceriporiopsis, 1385, 2647
Ceroidaphnia, biomonitoring, 3169
Certaium, 2507
Cesium, 1879–1893
Chaetoceros, 1058, 2506
Chaetogaster, 1722
Chaetomium, 1650, 1654–1655, 3144–3147
in allergies and illness, 1367–1372, 1374–1383
biocontrols and, 457–458
composting and, 916
desert environment and, 1579
Chaetosphaeria, 3359
Chain elasticity, conditioning films, 937
Chalcopyrite bioleaching, 633
Chaperone machine, molecular (See also Heat shock; Stress response), 843, 3028, 3048, 3050, 3052
Chaperonins, 3028–3029, 3048, 3052
Chara, Charales, paleolimnology, 2304–2306
Charge separation, aerobic respiration, 151
Charophytes, 2504–2518
Chasmoendolithic microbes, 842, 1100–1112
Chattonella, 2467, 2508, 2686–2693
Cheilobus, 1721
Chelation, 517, 1881–1882, 2717
Chelatobacter, 1871, 1875
Chemical controls, biocontrol vs., 450–465
Chemical oxygen demand (COD)
activated sludge and, 14–26, 49, 50
algal turf scrubbing (ATS) and, 216
anaerobic granulation and, 243
in source water, 2972
in wetlands for wastewater treatment, 3391
Chemical potential/chemical activity, in biofiltration, 592
Chemical production
biosurfactants/bioemulsions and, 766
cold-adapted microorganisms and, 881
enzymes and, 1185
Chemical transformations in wastewater/sewage treatment, 3337
Chemical warfare (See also Bioterrorism), 844–852
biodegradation of, 846–847
blister agents in, 844
destruction of, 844–845
immobilized cell reactors for, 847
isopropyl methylphosphonate (IMPA) vs., 850
nerve agents in, 844
organophosphorus acid anhydrolases (OPAA) vs., 848
organophosphorus hydrolase (OPH)., 848–850
sequencing batch reactors (SBR) for, 847
stockpiles of, 844–845
toxicity characteristic leachate protocol (TCLP) analysis of, 846
VX agents in, 844
bioterrorism vs., 773–775
bioremediation and, 706–707
Chemical weathering, 516–517
Chemiosmosis in aerobic respiration, 149–151
Chemoautotrophs
activated sludge and, 71
bacterioplankton and, 2521
biofilms and, 81–82
hydrothermal vents and, 1606–1609
soil microorganisms and, 2889, 2891–2892
sulfur cycle and, 3110
Chemoheterotrophs, 71, 3110
Chemokines, in allergies and illness, 1360
Chemolithoautotrophs, 1075, 1455, 1824–1826, 2522, 3141–3144
Chemolithotrophs, 677, 1075, 1455
metal biodegradation and, 1880
weathering, bioweathering and, 3365–3366
Chemoorganoheterotrophic microorganisms
bioleaching and, 635
biodeterioration of mineral materials and, 525
cold-adapted microorganisms in, 872–885
Chemoorganotrophs
biofilms and, 81–82
weathering, bioweathering and, 3365
Chemosensing, diatoms, in biofilms, 1052–1053
Chemotaxis bioleaching, 635–636
Chemotrophs
biodeterioration of mineral materials and, 523
soil microorganisms and, 2887
Chemtax analysis, photosynthetic pigments, 2468
Chenodeoxycholic acid, alkaliphiles, 228
Chenopodium, 1435
Chironomids, algal turf scrubbing (ATS), 215
Chitin, 1210, 1447
Chitinase, 227–228, 458, 1211–1223
Chitinolytic enzymes, 458
Chitobiase, 3021
Chitobiosidase, 1212–1223
Chladysporium, 2342–2356
Chlamydia, 1345
genomic studies and, 1446
infectious airborne bacteria and, 1689–1697
Chlamydiamicrovirus, 364–374
Chlamydoconidia, 1647
Chlamydomonas, 206
algae biotechnology and, 196
sea ice and, 2835–2844
snow/in snow and ice environments, 2873, 2876
toxicity testing and, 3221–3230
in wastewater stabilization ponds (WSBs), 3331, 3332
in wastewater/sewage, 3172
Chloramines, 1069

Chloramphenicol (CAP), 3160
Chlorarachniophyta, 237–243, 2465, 2467
Chlorararchnion, 2467
Chlorbenzoates biodegradation, 1421–1429
Chlorbiaceae, 2471
Chlorella, 205, 1851, 1853
algae biotechnology and, 189–204, 196, 198
biodeterioration of mineral materials and, 519
endosymbiosis and, 1117
photoautotrophic, in low-light habitats, 840–843
polar phytoplankton and, 2556
sea ice and, 2835–2844
subaerial communities and, 3056–3065
toxicity testing and, 3221–3230
in wastewater stabilization ponds (WSBs), 3331
in wastewater/sewage, 3172
Chlorhexidine, 604–606
Chlorides, soil salinity, 2774
Chloridium, 2125–2126
Chlorinate volatile organic compounds (CVOCs), 434–442
Chlorinated compound biodegradation, 507–16, 916–917, 1421, 2289–2300, 2931, 3410
Chlorinated solvents, bioremediation, 713, 714
Chlorine, chlorination (See also Disinfection), 607, 1063–67, 1069, 2333–2334, 3184
activated sludge foaming and, 6–7
adenoviruses vs., 96
bacteriophages vs., 360
biodegradable dissolved organic carbon (BDOC) and, 498
biofilms vs., 582, 589
biofouling vs., 603, 625–626
enteroviruses and, 1172
in marine/ocean environment, 1707
ozone layer destruction and, 3184
in spas and hot tubs, 2979
viruses vs., 3265–3267
Chlorine dioxide, 1063–1067, 1069–1070
Chlorobactene, 2459, 2465
Chlorobenzene biodegradation, 1763
Chlorobenzoate biodegradation, 2932
Chlorobenzoic acid biodegradation, 1238–1252
Chlorobiaceae, 2455, 2458, 2459, 2465
flooded soils and, 1320
phototropism in, 2471, 2474, 2476, 2481, 2483
in wastewater stabilization ponds (WSBs), 3332–3333
Chlorobiphenol biodegradation, 1195
Chlorobiphenyl biodegradation, 1422–1429
Chlorobium, 2282, 3088–3096, 3111–3116, 3142–3147
biofilms and, 83
biomineralization and, 675
nitrogen fixation and, 2193–2201
in paleolimnology, 2312
phototropism in, 2474, 2479–2489
Chlorococcales, 1109, 2301, 2369, 3056–3065
Chloroethene biodegradation, 510–511
Chloroflexaceae, 83, 511, 2278, 2471, 3142–3147
Chlorofluorocarbons (CFCs), 3183, 3184, 3188–3189
Chloroform (CF), 434–442, 511–514
Chloroform fumigation incubation/extraction method (CFI/EM), 666–667
Chlorogloae, 840–843
Chlorogloeopsis, 962
Chloroherpeton, 2479–2489
Chloromethylisothiazolinone (CMIT), 605
Chloromonas, 838–843, 2873
Chlorophenol biodegradation, 512, 605, 921–922, 1421, 3147–3155
Chlorophtya, 2467, 2837–2844
Chlorophyll, 2453–2480, 2504, 2512
cyanobacteria and, 964
in paleolimnology, 2307–2317
Prochlorococcus, 2576–2593
soil biosphere and, 204
in wastewater stabilization ponds (WSBs), 3332–3333
Chlorophyta, 205
Antarctic soils and, 1025
periphyton and, 2357
photoautotrophic, in low-light habitats, 837–840
plankton and phytoplankton, 2503
polar phytoplankton and, 2556
Chloroplasts, plankton and phytoplankton, 2509–2510
Chlorosarcinopsis, 1109
Chlorotoluene biodegradation, 1421–1429
Chlorsalicylate biodegradation, 1421
Choanoflagellates, 2617, 2618–2619
Cholera, 776, 853–861, 3320
Cholesterol, 1257
Cholic acids, alkaliphiles, 228
Choline sulfate, soil salinity, 2778–2789
Chondrilla, 974
Chondromyces, desert environment, 1578
Chrionomidae, in water treatment systems, 1723
Chromate, 606–608, 1878, 1894
Chromatium/Chromatiaceae (See also Halochromatium), 2282, 2455, 2458, 2465, 3111–3116, 3142–3147
aggregates and consortia in, 173
biofilms and, 83
nitrogen fixation and, 2193–2201, 2214–2221
in paleolimnology, 2312
phototropism in, 2471, 2477–2482
salt production and, 2805–2809
in wastewater stabilization ponds (WSBs), 3332–3333
Chromatographic effect, biofiltration, 590
Chromium (See also Heavy metals), 671–681, 694, 708, 1883–1884, 1888–1889, 3089
Chromobacterium
granular activated carbon (GAC) filters and, 1490
heterotrophic bacteria and, 1541–1552
infectious airborne bacteria and, 1696
in drinking water, 945
Chromohalobacter, 1506–1512
Chromophytes, 2504–2518
Chromulina, 2317–2327
Chronic exposure, toxicity testing, 3220–3221
Chroococcales, periphyton, 2357
Chroococcidiopsis, 963, 964, 1102–1106
Antarctic soils and, 1025
desert environment and, 1579, 1582, 1584
photoautotrophic, in low-light habitats, 839–843
subaerial communities and, 3056–3065
Chroococcus, 963, 1507–1512
alkaliphiles and, 134
photoautotrophic, in low-light habitats, 840–843
subaerial communities and, 3056–3065
Chroomonas, photosynthetic pigments, 2467
Chrysene biodegradation, 694, 696–712, 2403–2416

Chrysochromulina, 2508
Chrysodidymus, 2317
Chrysolykos, sea ice, 2835–2844
Chrysomonads, 2619
Chrysophtyes (See also Paleolimnology), 861, 2301–2306, 2317–2327, 2504–2518
Chrysophycea, 206
in paleolimnology, 861, 2301–2306, 2317–2327, 2504–2518
photoautotrophic, in low-light habitats, 837–843
photosynthetic pigments in, 2467
polar phytoplankton and, 2556
sea ice and, 2837–2844
Chrysosphaerella, 2317–2327
Chrysosporium, in allergies and illness, 1368–1372
Chymosin, 1186–1187
Chymotrypsin, 2899
Chyseobacterium, 1302–1306
Chytridiomycota, 1367–1372, 2913, 3353–3364
Chytridium, 972
Chytrids, 2913
Ciguateratoxin, 976
Ciliated protozoa (See also Protozoa), 861, 2594, 2605–2607, 2610–2611, 2617
bacteriovorous, 2611
biodegradation and, 2626–2632
marine/ocean environment and, 2619–2620
Ciliophora (See also Protozoa), 2594, 2605
Cinetochilum, 2595–2604
Ciona, 614
Cisterns (See also Rainwater catchment systems), 861, 903, 2677–2680
Citrate, 88
Citric acid, 417, 632–642
Citrobacter
biomineralization and, 675
bioremediation and, 708
coliform bacteria and, 896–905, 906–914, 906
endosymbiosis and, 1118
explosives biodegradation and, 1985
granular activated carbon (GAC) filters and, 1491, 1571–1572
high hydrostatic pressure (HHP) vs., 1556–1563
infectious airborne bacteria and, 1694–1695
metal biodegradation and, 1890
municipal solid waste landfills and, 1766
nitrogen fixation and, 2193–2201
in drinking water, 944, 946
CL20, biodegradation, 1979–1993
Clades, 2524
Cladochytrium, 2552
Cladophora, 205
algal turf scrubbing (ATS) and, 215
paleolimnology of, 2305–2306
periphyton and, 2357, 2362, 2364
Cladosporium, 423, 1650, 1655, 3360
in allergies and illness, 1361–1366, 1367–1372, 1374–1383
bioaersols and, 404–405
cold-adapted microorganisms in, 872–885
desert environment and, 1579
spores and spore dispersal in, 405, 408–409
subaerial communities and, 3057–3065
in drinking water, 945
Cladothrix, 1401
Clarification
activated sludge and, 59
algal blooms vs., 209
water treatment systems and, high-rate, 2017
Clavibacter
biocontrol use of, 2705
genetically engineered microorganisms (GEMs) and, 1279
subsurface microbial communities and, 2035, 3067–3071
Clays
biofilms and, 88
soil microorganisms and, 2058–2059
weathering, bioweathering and, 3371
Clean Water Act (CWA), 2975, 3169
Clethrionomys, 2127
Climacodium, 2506
Climate change (See also Paleolimnology), 861, 3184
cyclic climatic events and, 2558–2559
desulfurization of fossil fuels, 1041–1051
greenhouse effect and, 3184
nitrogen cycle and, 2943
paleolimnologic studies of, 2312–2313, 2324–2325
periphyton and, 2365, 2380–2382
plankton and phytoplankton, 2515–2516
polar phytoplankton and, 2558–2559, 2564–2565
protozoa and, 2630–2631
rhizosphere and, 2721–2722
Clinorotation, simulated microgravity, 3000
Clitocybe, explosives biodegradation, 1983
Clone library screening, 2734
Cloning, 861, 2492–2494, 2524, 2732–2733
Archaea and, 247
cold-adapted microorganisms and, 3203–3206
soil genetic ecology, 2920–2921
stress response and, 3026
Clonothrix, 3101
Closed loop bioremediation, 695
Clostridia, 1236, 2551
Clostridiales, halophiles, 1515–1529
Clostridium, 136, 182–189, 861–871, 1233, 1854, 3129, 3102, 3130, 3131–33, 3145–3147
activated sludge and, 742–748
anaerobic granulation and, 232–245
bacteriophages and, 359
bioaerosols and, 455, 3324–3325
biodegradation and, 509
biofilms and, 82
bioremediation and, 706
biotechnology and, 3206
bioterrorism and (See Botulism)
cold-adapted microorganisms in, 872–885
cold-shock response and, 886–895
desert environment and, 1578
diazotrophy in, 2541–2547
endosymbiosis and, 1118
fecal contamination and, 1257, 1269
filtration and, 2709
gene transfer and, 1409
genetically altered, in biotechnology, 1447
halophiles and, 1521–1529
high hydrostatic pressure (HHP) vs., 1559–1563
hydrothermal vents and, 1622
metal biodegradation and, 1886
microbial insecticides using, 1714
municipal solid waste landfills and, 1760, 1766
nitrogen fixation and, 2193–2201, 2212–2221, 2938
oxygen and, 2278
psychrophilic, 2640–2646
rain/in rainwater roof catchment systems, 2680, 2682
sampling of, 2813
in source water, 2974
toxic solvents and, 3153
in wastewater/sewage, 3173

Clostridium, (*continued*)
water treatment systems and, 2015, 2266
in wetlands for wastewater treatment, 3400
Club root, 2913
Cluster analysis, 310–311, 1005–1006
Clyindrospermopsin, 987–995
Coagulation, 871
aggregates and consortia in, 162
algal blooms and, 208, 209
biodegradable dissolved organic carbon (BDOC) and, 494, 495
water treatment systems and, 2015–2018
Coal, 677, 1049
Coal gas biodegradation, 916–917, 2404
Coal tar bioremediation, 694, 2404
Coastal waters, nitrogen fixation, 2218
Cobalt (See also Heavy metals), 1879–1894, 2961
algae biotechnology and, 199
bioleaching and, 632–642
resistance of bacteria to, 608
soil microorganisms and, 2887
Coccachloris, 972
Cocci, 2882
Coccidioides, fungi, 1368
Coccidiosis, 1734–1737
Coccoliths, 2509, 2515
Cochaperones, stress response, 3030
Cochilobolus, 410, 2913
Cochlodinium, 195
Codakia, 1606, 1609
Codium, harm/in harmful algal blooms (HABs) and red tides, 2689–2693
Coelenterates, bioluminescence, 657–663, 1830
Coelenterazine, 1830
Coelomycetes, 3353–3364
Coenzymes, Archaea, 256–257
COG, 1450
Cohesion, extracellular polymeric substances (EPS), 1224–1225
Cold-active enzymes, 871, 3206–3210
Cold-adapted microorganisms (See also Psychrophilic bacteria), 871–885, 2639
agricultural biotechnology using, 881
in Antarctica, 2557–2558
aquaculture and, 3215
in Arctic Ocean, 2556–2558
bioaugmentation and, 3212
biocatalysis using, 881
biodegradation and, 3210–3215
biohydrometallurgy, 881–882
bioremediation using, 882–883, 3210–3215
biostimulation and, 3211–3212
biosynthesis in, 875–876
biotechnology and, 879–883, 3203–3219
cellular adaptations in, 874
cloning and, 3203–06
cold-shock proteins in, 874, 885–895
cold-shock response and, 874, 885–895
Csps and cold-shock response in, 888–892
cyanobacteria and, 963
cyclic climatic events and, 2558–2559
detergents and, 881
ecology of, 872–873
endothilic microorganisms and, 1103
enzymes and, 876–879, 3206–3210
extremeophiles and, 1232–1237
fatty acids and, 875–876, 888
food processing and, 879–881
genetic compatibility among, 3204–3205
growth characteristics of, 873
homeophasic theory in, 875
homeoviscous adaptation theory in, 875, 887
intracellular vs. extracellular enzymes in, 876
limits of adaptation in, 879
lipids in, 874–876, 887–888
membranes in, 874–876
metal degradation and, 3216
molecular biology and, 883
nucleic level cold-shock response and, 888
permafrost and, 2367–2385
phytoplankton of polar seas as, 2554–2565
polyunsaturated fatty acids (PUFA) in, 3215
protein engineering and, 3208–3209
proteins and, 874, 883, 888–893
psychrophiles as, 871–885, 2639–2646
psychrotrophs as, 871–885
ribosomal level cold-shock response and, 888
sea ice microorganisms, 2833–2844
snow and ice environments, 2872–2880
soil microorganisms and, 2889
subaerial communities and, 3061
subsurface microorganisms and, 2025
thermal regulation in, 875–876
wastewater/sewage treatment using, 882
Cold desert environment, soil microorganisms, 1023–1029
Cold extraction, 924
Cold-shock proteins, 874, 885–895
Cold-shock response, 874, 885–895
Cold-tolerant enzymes, Archaea, 268
Colds, 338
Coleophoma, 1579
Coleps, 2606
Coliform bacteria, 895–905
assimilable organic carbon (AOC) and, 312–327
bioaerosols and, 3327
biofilms and, 621, 907–909
bottled water and, 796
control of, 905–914
distribution case studies in, 909–913
fecal, 896–898
granular activated carbon (GAC) filters and, 1490–1491
heterotrophic bacteria and, 1541–1552
municipal solid waste landfills and, 1766
occurrence of, in U.S., 907
opportunistic, 898–899
pathogenic, 898–899
regrowth of (See Biodegradable dissolved organic carbon)
sampling for, 899
in source water, 2973
sources of, in drinking water, 906
Total Coliform Rule (TCR) and, 905, 906–907
in wastewater stabilization ponds (WSBs), 3334–3336
in drinking water, 895–914, 944, 946–951, 946
in wetlands for wastewater treatment, 3393–3397
Coliphage (See Bacteriophages; Somatic coliphages)
Colitis, Clostridium, 862–871
Collar, 2321
Collectotrichum, 405, 413
Collision frequency, kinetics of growth, 1741–1745
Colloidal particles, 928
adhesion in biofilms and, 127–128, 2964–2965
floc formation and, 2002
virus filtration and, 2097
Colonization
anaerobic granulation and, 241–243

bacterioplankton and, 2520
biofilms and, 1083
biofouling and, 611–612
heterotrophic bacteria and, 1546
subaerial communities and, 3061
Colorimetry, 381, 762
Colorless sulfur bacteria, 3097–3098
Colpidium, 2595–2604, 2607, 2626–2632, 3173
Colpoda, 3173
Coltivirus, 2753–2773
Colwellia
biotechnology and, 3204, 3205
cold-adapted microorganisms and, 875
hydrothermal vents and, 1610
psychrophilic, 2640–2646
Comamonas, 1200, 3110
filamentous forms of, in activated sludge, 1300–1306
spores and spore dispersion in, 420
subsurface microbial communities and, 2035, 3065–3071
Cometabolic reductive dechlorination, 508–509
Cometabolism, 914, 2386–2387
Commercial use of microorganisms (See Regulation of commercial uses)
Communicable diseases, 1690
Community analysis, subsurface microorganisms, 2030–2040
Community composition, lipid biomarkers, 1819
Community genome arrays (CGAs), 1977
Community level physiological profile (CLPP), 1580
Comparative genomics, 1450
Compatible solutes, Archaea, 273
Competition, biocontrols, 456
Complement fixation (CF), adenoviruses, 98
Completely autotrophic N removal over nitrite (CANON), 11–14
Completely mixed batch reactor (CMBR), 44, 75–76
Completely mixed flow reactor (CMFR), 44, 45, 75–76, 75
Complexation of heavy metals, 1894, 1902–1913
Composite transposons, 1200
Compost (See also Bioremediation), 914–927
activated sludge and, 738
biodegradation in, 914–927
biofiltration and, 592, 914–927
biopiling and, 914–927
bioremediation and, 693, 695, 914–927
cold extraction and, 924
cool down period in, 915
detoxification using, 916–917
enteroviruses and, 1173
explosives and, 922–924, 1988
fungi and, 2911–2912
halogenated compounds and, 919–920
humic substances in, 915
hydrocarbons and, 917–919
lipid biomarkers of, 1822
mesophilic microorganisms in, 915–916
microorganisms in, 915–916
military chemicals, 916–917, 922–924
municipal solid waste landfills and, 1759–1762
nutrients and, 915
organic matter and, 915–927
organic pollutants and, 924–925
pesticides and, 921–922
petroleum products and, 917–919
pH levels vs., 915
polycyclic aromatic hydrocarbons (PAH) and, 916–919
principles of, 915–916
soil contamination and, 921–922
Soxhlet extraction and, 924
stabilization and maturation period in, 915
temperature vs., 915–916
thermophilic microorganisms in, 915–916
total petroleum hydrocarbons (TPH) and, 916–919
Comprehensive microbial resource (CMR), 1449–1450
Computer simulation of biofilms, 2089
Concentration rate, 1008
Concrete, biodeterioration, 523–525, 3370–3371
Conditioning films (See also Biofilms), 101, 127, 559, 611, 928–941
adhesion and, 938–939
adsorption of macromolecules in, 937
amides in, 937–938
analysis of, 929–936
Brownian motion and, 936
chain elasticity in, 937
composition of, 937–938
contact angle analysis of, 930–932
deposition of, 938
electrokinetic analysis of, 933
electrospray ionization (EI) analysis of, 934
electrostatic interactions in, 936
ellipsometric analysis of, 935
filters for, 929
formation of, 936–937
Fourier transform infrared spectrometric (FTIR) analysis of, 934–935
Fourier transform Raman spectroscopic analysis of, 935
fragmentation analysis of, 932–933
glycoproteins in, 937–938
mass spectrometry (MS) analysis of, 933–934
mercury drop electrode analysis of, 932
near field optical microscopic analysis of, 935
nutrients in, for bacteria, 939–940
organic and inorganic components of, 928
polar interactions in, 937
polysaccharides in, 937–938
proteins in, 937–938
sampling of, 928–928
scanning probe microscopic (SPM)analysis of, 935–936
substratum surface and, modification by, 938
surface enhanced Raman spectroscopy (SERS) analysis of, 935
thickness of, 929
X ray photoelectron spectroscopic (ESCA) analysis of, 934
Confocal laser scanning microscopy (CLSM) (See also Image analysis), 56–57, 558, 570, 1335, 1338, 1416, 1675, 1772–1788
Conidia, 1647
Coniothyrium, 453, 454
Conjugaphyta, paleolimnology, 2302–2306
Conjugation, 1409–1413
Conjugative transposons, 1202–1203
Conjunctivitis, adenoviruses, 92–100
Connate water, 2418
Conservation of energy, aerobic respiration, 149–151
Conservation reserve programs, quality of soils, 2951–2952
Consortia (See Aggregates and consortia)
Constant denaturant capillary electrophoresis (CDCD), 814
Constants, kinetic, as measure of S, 1740–1741

Constructed Wetland Continuous Stirred Tank Reactor (CSTR), 695
Contact angle analysis/measurement (CAM), 2007–2008
  conditioning films and, 930–932
  hydrophobicity and, 1600–1601
Contact lens cleaning, bottled water, 800
Contact stabilization, in activated sludge, 76–77
Contact time, 495, 1069
Contact zones, activated sludge foaming, 5–7
Contagious diseases, 1690
Contrast, image, 1679
Conventional filtration, 2708
Conventional optical microscopy (COM), 2008
Cool down period in composting, 915
Cooling systems, biofilms, 579
Cooxidation, 2292
Copiotroph (See also Oligotrophic bacteria), 2268, 2271
Copper (See also Heavy metals), 1878–1894, 1902–1913, 2961
  algae biotechnology and, 196, 200
  cyanobacteria and, 969
  in marine/ocean environment, 1706
  municipal solid waste landfills and, 1763–1764
  resistance of bacteria to, 608
  soil microorganisms and, 2887
Coprinus, 916, 1983
Coprostannone, 1257
Coprostanol, 1257
Coprosterol, 1257
Coral bleaching, 1117
Coral reefs
  endosymbiosis and, 1116–1117
  nitrogen cycle and, 2183, 2219
Corbicula, 3019
Cordyceps, 2913
Corethron, 2506
Coriaria, 2209
Coronavirus, in spas and hot tubs, 2980–2984
Corrosion (See Biocorrosion; Microbially induced corrosion)
Corticium, 916
Corticoviridae/Corticovirus, 365–374
Cortinarius, 2124–2132
Corynebacterium
  activated sludge foaming and, 5–7, 5
  alkaliphiles and, 228
  biodegradation and, 1874, 2291–2300
  biofilms and, 2342–2356
  biosurfactants/bioemulsions and, 753, 756
  desert environment and, 1578
  granular activated carbon (GAC) filters and, 1490
  heterotrophic bacteria and, 1541–1552
  metal biodegradation and, 1885, 1890
  soil salinity and, 2779–2789
  subsurface microbial communities and, 3067–3071
  in drinking water, 945
Coryneform, adhesion in biofilms, 123
Cosmarium, 2304–2306
Cosmetics (See also Biosurfactants), 189–204, 952
Cosmic radiation, space microbiology, 2985
Coxiella, 772, 1689–1697
Coxsackievirus, 1152, 2743, 2745
  biodegradation and, 3289–3300
  soil/in soil, 3273–3276
  in source water, 2969–2978
  in spas and hot tubs, 2979, 2980–2984
  subsurface microorganisms and, 2025
  in wetlands for wastewater treatment, 3398
Crenarchaeota
  Archaea and, 246–259, 277, 280–285, 295, 299–301
  bacterioplankton and, 2526
  desert environment and, 1578
  halophiles and, 1515–1529
  nitrogen fixation and, 2212–2221
  tomato roots and, 301
Crenothrix, 676, 3101
Creosote biodegradation, 694, 2404
Cresol biodegradation, 605, 1763
Cretaceous shales and sandstones of Southwestern U.S., 2042–2047
Crinipellis, 2914
Critical micelle concentration (CMC), biosurfactants/bioemulsions, 751
Cropping systems, enzyme activity, 2907
Cross connections, cyanobacteria, 943
Crotonobetaine, soil salinity, 2778–2789
Crude oil (See also Hydrocarbons; Oil spills), 952
  biodegradation of, 2402–2404, 2930
  desulfurization of, 1048–1049
Crustacea
  endosymbiosis and, 1117
  hydrothermal vents and, 1619
  in water treatment systems, 1722–1723
Cry toxins, microbial insecticides using, 1712–1717
Cryconite, 963
Cryothecomonas, sea ice, 2835–2844
Cryptaulax, 2594–2604
Cryptobiosis, 2021
Cryptococcus, 1102–1106, 1890
  Antarctic soils and, 1025, 1027–1028
  cold-adapted microorganisms in, 872–885
  fungi and, 1368
  in permafrost, 2369
Cryptoendolithic microorganisms, 1100–1112
Cryptomonada, 2467
Cryptonectria, biocontrol use, 2705
Cryptoperidiniopsis, 2431
Cryptophtyes, 2453–70, 2505–2518
  periphyton and, 2357
  photoautotrophic, in low-light habitats, 837–843
  photosynthetic pigments in, 2467
  polar phytoplankton and, 2556
Cryptosporidium, 650–657, 903, 2598, 2079–2080, 2607
  activated sludge and, 72
  aquatic sediments and, 1252–1256
  biofilms and, 2341–2356
  bottled water and, 796
  Clostridium and, 868–871
  disinfection and, 360, 1067–1073
  filtration and, 2709
  flow cytometric studies of, 1951, 1952
  in food, 3277–3287
  granular activated carbon (GAC) filters and, 1493
  heterotrophic bacteria and, 1548
  high hydrostatic pressure (HHP) vs., 1561
  measurement of, using aerobic endospores, 141–149
  rain/in rainwater roof catchment systems, 2680
  sampling of, 2813
  in source water, 2968, 2969–2978
  in spas and hot tubs, 2979, 2980–2984
  streams and, 3021
  subsurface microorganisms and, 2025
  ultraviolet light and, 3231–3235
  in wastewater stabilization ponds (WSBs), 3334
  in wastewater/sewage, 2329–2337

water purifiers vs., 1572–1573
water treatment systems and, 1719, 1727, 2013–2019, 2262–2266, 3199
in wetlands for wastewater treatment, 3398–3399
Cryptostroma, 422
Crysophyceans, periphyton, 2357
Crystallolithus, 2508
Csps and cold-shock response, cold-adapted microorganisms, 888–892
CT concept, in disinfection, 1064–1065
Culex, in wetlands for wastewater treatment, 3399
Culturable subsurface microbial communities (See Subsurface microbial communities)
Culturable vs. unculturable organisms, 1113
Culturing
activated sludge and, 69
algae biotechnology and, 191–192
Archaea and, 246–247
astroviruses and, 333
bioaersol samples, 400–401
chloroform fumigation incubation in, 666–667
fungi and, 1648, 1652–1653
heterotrophic bacteria and, 1542–1544
hydrothermal vents and, 1611–1614
hyperthermophiles and, 1628–1633
plankton and phytoplankton, 2516
protozoa and, 2594
psychrophilic bacteria and, 2641–2642
ribosomal RNA targeted oligonucleotide probes and, 1339–1340
space microbiology and, 3000
subsurface microorganisms and, 1079
vadose zone microorganisms and, 3241–3242
Cunningham, D.D., 404
Cunninghamella, 459, 2410, 2649
Curvularia, 408, 410, 1579, 1650, 1655
Customblen, 699
Cutin, biofilms, 88
Cyanelles, 1126
Cyanidium, 838–843, 3141–3147
Cyanins, 1676
Cyanobacteria and blue-green algae, 136, 206, 952–995, 1853, 2453–2470, 2549
aggregates and consortia in, 161, 164–165, 166–168, 170–181
algae biotechnology and, 189–204
algal blooms and, 204–213
Antarctic soils and, 1025
bacterioplankton and, 2524–2526
biodeterioration of mineral materials and, 519–522, 524–525
biofilms and, 83, 568–569, 1083
biomineralization and, 672
cold-adapted, 963
cold-shock response and, 887–895
desert/in desert soils and biological soil crust (BSC), 1020–1023, 1020, 1576, 1579
disinfection of, 991–992
endolithic habitats for, 963–964, 1100–1112
endosymbiosis and, 1115, 1118–1119, 1126–1128
filamentous forms of, in activated sludge, 1312
floc formation and, 992
halophiles and, 1507–1512, 1513–1529
heterotrophy in, 966
hypersaline environment and, 963
meroplankton and, 1863–1869
metal biodegradation and, 1879–1880
microbial mats and, 568–569, 966–967
nitrogen and, 967–968, 2181, 2189
nitrogen fixation and, 2189, 2212–2221
oxidation vs., 992
oxygen and, 2277–2278
oxygenic photosynthesis in, 965–966
paleolimnology of, 2304–2306
pathogenic, 972–973
periphyton and, 2357, 2358
in permafrost, 2369
phosphorus cycling and, 969, 2450
photoautotrophic, in low-light habitats, 836–843
photosynthetic pigments in, 964–965, 2466
phototropism in, 2471, 2472
plankton blooms and, 970–972, 975–981, 989–990, 2503
polar phytoplankton and, 2555, 2556
predators of, 972–973
Prochlorococcus as, 2576–2593
research in, 980–981
safety and health guidelines for, in drinking water, 990–991
salt production and, 2805–2809
snow/in snow and ice environments, 2874, 2876
soil microorganisms and, 2890–2891
in source water, 2969–2978
subaerial communities and, 3056–3065
sulfur and, 969
symbiosis in, 973–975
toxins and, 975–980, 982–995
trace metals and, 969–970
ultraviolet resistance in, 963
vertical migration in water column by, 970–972
in wastewater stabilization ponds (WSBs), 3331
water treatment systems and, 982–995
Cyanobacterial mats (See Biofilms)
Cyanochlorontans, cyanobacteria (See also Blue-green algae), 952–982
Cyanoditolyl tetrazolium chloride (CTC), 1783
Cyanoginosins, 976
Cyanophages (See Cyanobacteria)
Cyanophora (See Cyanobacteria)
Cyanophtya (See Cyanobacteria)
Cyanospira, 134
Cyanothece, 961, 962, 963, 967, 972, 973
biodeterioration of mineral materials and, 522
nitrogen fixation and, 2214–2221
Cyanotoxins, 975–980, 982–995
Cyanoviridin, 977
Cyathomonas, 2594–2604
Cyclicidium, 2607, 2626–2632
Cycloalkanes biodegradation, 697–713, 2292–2300
Cycloclasticus, 2269, 3251
Cyclodextrin, 220, 224–225, 1185
Cyclohexane biodegradation, 2407
Cyclomaltodextrin /cyclomaltodextrin glucanotransferase (CGTase), 220, 224
Cyclonite bioremediation, 706–712
Cyclospora, 995–1001
disinfection and, 1067
in food, 3277–3287
high hydrostatic pressure (HHP) vs., 1561
in source water, 2969–2978
in wastewater/sewage, 2329–2337
in water treatment systems, 1719
Cyclosporiasis, 995–1001
Cyclosporin, 1366, 1369
Cyclotella, 206, 215
Cylindrospermopsin, 977

Cylindrospermopsis, 984–995, 1191
Cylindrospermum, 961, 979
Cymbella, 838–843, 1053, 1054
Cymodocea, 2549, 2823–2833
Cypovirus, 2753–2773
Cyprinus, 979
Cysteine, 605
Cystic fibrosis
  infectious airborne bacteria and, 1689–1697
  Mycobacterium avium complex (MAC) and, 2113–2120
Cystobacter, 1578
Cystoviridae/Cystovirus, 366–374
Cytoarchitectural requirements, theoretical analysis, 1745
Cytochrome c pathway in aerobic respiration, 153
Cytokines, in allergies and illness, 1360
Cytokinins, 344
Cytophaga
  activated sludge and, 71
  bacterial counts using, 31
  bacterioplankton and, 2524–2526
  biofilms and, 570, 1215, 1218
  cold-adapted microorganisms in, 872–885
  endosymbiosis and, 1122
  filamentous forms of, in activated sludge, 1302–1306
  flooded soils and, 1319, 1320
  heterotrophic bacteria and, 1541–1552
  psychrophilic bacteria and, 2641
  sea ice and, 2838
  soil microorganism and diversity in, 533, 539
  subsurface microorganisms and, 2035
Cytoplasm, 2884–2885, 2960
Cytoplasmic membrane, soil microorganisms, 2883–2884, 2883
Cytoplasmic polyhedrosis viruses (CPV), 1711

D

d'Herelle, Felix H., 363
Dactylaria, 3142–47, 3360
Dactylococcopsis, 2805–2809
Dairy products, enzymes, 1186–1187
Daldinia, 406
Daphnia, 2308–2317, 3169
Daramend bioremediation, 924, 1989
Dark adaption, sea ice microorganisms, 2840–2841
Dark field–bright field microscopy, 1675
Dark repair, 3231
Data analysis and modeling, 1002–1010
  auto associative neural networks in, 1007
  bottom up exploratory, 1005
  cluster analysis and, 1005–1006
  complexity and, modeling of, 1009
  content of information for, 1003
  data collection for, 1002–1003
  discriminant, 1007–1009
  exploratory, 1003–1007
  factor analysis and, 1006–1007
  Michaelis-Menten equation in, 1008
  neural networks in, 1007, 1008–1009
  regression, multiple regression, 1007–1008
  self organizing maps (SOMs) in, 1007
  Shannon's index and, 1003
  top down exploratory, 1005
  unweighted pair group average (UPGA) and, 1006
  Wards method of, in cluster analysis, 1006
Databases, genomic, 1449–1450
Datsica, nitrogen fixation, 2209
DDT biodegradation, 507–516, 703–712, 1238–1252, 1421, 2297–2300, 2933, 2963, 3415
Dead Sea, 1509
Death of bacteria, 3253–3254
Death rate, bioaerosols, 2110
Debromoaplysiatroxn, 976
Decarboxylase, in soil, 2903–2910
Decay, activated sludge processing, 23–26
Dechlorination
  cometabolic reductive, 508–509
  reductive dechlorination and dehalorespiration in, 509–510, 513–514
Deconvolution, image analysis, 1679–1681
Deep sea euryarcaheotic group (DSEG), 1611
Deep Shaft process, activated sludge, 76–77
Deferribacter, 1515–1529, 2429–2431
Defluvicoccus, 63
Degrading systems, quality of soils, 2952–2954
Dehalobacter, 511–513
Dehalococcoides, 511–513
Dehalogenation (See also Biodegradation), 507–516, 3414
Dehalorespiration, 435, 509–510
Dehalospirillum, 511, 513, 514
Dehydration (See Desiccation)
Dehydrogenase, 3170, 3207–3210, 3338
Deinococcus, 1102–1106, 1204, 2874, 3216
  bioremediation and, 1446
  desert environment and, 1582, 1584
  desiccation and, 1033–1041
  genetically altered, in biotechnology, 1446
  genomic studies and, 1442
  metal biodegradation and, 1883
  radiation and, 2987–2995
  radioactive waste degradation and, 2666–2674
Deleterious rhizobacteria (DRB), 2717, 2722
Deleya, 614, 2214–2221
Demethoxycylindrospermopisn, 977
Demethylation in biofilms, 2963
Demethylsulfonium propionate (DMSP), 3192–3193, 3192
Denatured gradient gel electrophoresis (DGGE), 28–29, 71, 247, 278, 299, 563, 1010, 1974, 2732, 2494–2496, 2735–2737, 2921
  activated sludge and, 71
  Archaea and, 247, 278, 299
  bacterial counts using, 28–29
  biofilms and, 563
  methanotrophic bacteria and study, 1931–1932
  microarray study of vs., 1974
  Spartina, 2545
Denaturing high performance liquid chromatography (DHPLC), 814
Denitrification (See also Nitrification; Nitrogen cycle), 2150, 2941–2942, 3189–3192
  activated sludge and, 8–14, 19–20, 34–38, 49, 58, 77–78
  aggregates and consortia in, 166
  algal turf scrubbing (ATS) and, 217–218
  anoxic storage in nitrogen removal processes and, 3011
  bacteria for, 1012–1013, 2891
  carbon and, 1013–1014
  controls of, 1013–1014
  desert soils and, 1022
  floc formation and, 58
  marine/ocean environment and, 1010–1019
  nitrogen cycle and, 2184
  oxygen and, 1013–1014, 2282–2283
  oxygen minimum zones (OMZs) vs., in marine environment, 1016

patterns of, in marine environment, 1016–1017
pH levels and, 1013–1014
Pseudomonas, 2632–39
quantification of, rates and, 1015–1016
sampling in, 1015–1016
in septic tank systems, 2860
temperature vs., in marine environment, 1016–1017
toxicity testing and, in soils, 3160
wetlands and, 3404
Dense nonaqueous liquids (DNAPLs), bioremediation, 436, 507–516
Deodorants, algae biotechnology, 195
Deoxythymidine diphosphate (dTDP), 3340–3341
Deoxythymidine triphosphate (dTTP), 3339–3342
Deoxyuridine monophosphate (dUMP), 3340–3341
Depolymerization, biodeterioration of mineral materials, 518
Deposition, adhesion in biofilms, 114
Derjaguin–Landau–Verwey–Overbeen (DLVO) theory of adhesion, 57, 102–106, 119–122, 124, 561, 2004
Derxia, nitrogen fixation, 2193–2201
Desert environment (See also Desiccation), 1019–1029
actinomycetes in, 1577
adaptations to, 1580–1583
algae in, 1576, 1579
amoebas in, 1579
Antarctica as, soil microbes and, 1023–1029
Archaea in soil of, 1578
autochthony in, 1583
bacteria in soil of, 1577, 1578
bioaugmentation in, 1584
biodegradation of pollutants in, 1583–1584
biological soil crusts (BSCs) and, 1019–1023
biomass of, 1576, 1577, 1579
carbon and, 1577, 1582
cold deserts, soil microbes in, 1023–1029
cyanobacteria in, 1576, 1579
desiccation and, 1029–1041, 1580–1582
diversity of microbes in, 1577–1580
dwarfing of bacteria in, 1582
endolithic microorganisms in, 1100–1112
environmental impact and conservation in, 1022
extracellular polysaccharides (EPS) and, 1577, 1581
extremeophiles and, 1232–1237
fungi in, 1576, 1577, 1578–1579
heat-shock response/proteins and, 1583
hot deserts, 1576–1586
hot deserts, endolithic microorganisms and, 1107–1110
microbial abundance in, 1577
nitrification and denitrification in, 1022
nitrogen and, 1577
nutrients and, 1576, 1582–1583
oxygen and, in soils, 1021–1022
pH levels and, 1576
phospholipid fatty acid (PLFA) profiles in, 1578, 1579
protozoa in, 1579
reclamation efforts in, plant-microbe symbiosis for, 1583
salinity of, 1576–1577, 1580–1582
soil microorganisms of, 1576–1586
solar radiation and, 1021–1022
spatial and temporal distribution of microbes in, 1577
temperature and, 1576, 1583
total organic carbon (TOC) and, 1577
water and, 1021–1022, 1576–1577, 1580–1582
Desert varnish, 963, 1103
Desiccation and dehydration, 1029–1041
aggregates and consortia in, ice embedded, 174
anhydrobiosis, 1030–31
damage by environmental agents and, 1033–1035
desert conditions and, survival under, 1039–1040
desert environment and, 1580–1582
DNA and, 1032–1041
dormancy and, 1029, 1030–1031
dynamic vacuum drying and, 1035–1036
enteroviruses and, 1174
extremeophiles and, 1232–1237
fluorescent probes and, 1335
freeze drying and, 1030, 1035, 1350–1359
fungi and, 1029, 1031
growth limits and, 1030
molecular events induced by, 1031–1035
panspermia theory and, 1029, 1037
removal of hydrate water in, 1032–1035
removal of liquid cell water in, 1031–1032
secondary reactions to, 1033–1035
space exploration and, 1035–1039
spores and, 1029, 1031, 1037–1041
subaerial communities and, 3060–3061
survival at, 1035–1039
Desmidaceae, 2301, 2302–2306, 2302
Desmids, snow/in snow and ice environments, 2874
Desmococcus, 840–843, 1107–1112, 3056–3065
Desorption limitation, biofilms, 88
Destruction/removal efficiency (DRE), biofiltration, 587, 590
Desulfacinum, petroleum reservoirs and, 2429–2431
Desulfobacter, 2849–2851, 3088–3096, 3114–3116, 3143–3147, 3193, 3414
biodegradation and, 511–514
biofilms and, 82
halophiles and, 1519–1529
nitrogen fixation and, 2214–2221
petroleum reservoirs and, 2429–2431
Desulfobotulus, flooded soils, 1326
Desulfobulbus, 2551, 3090–3096, 3114–3116
activated sludge and, 742–748
anaerobic granulation and, 232–245
flooded soils and, 1326
Desulfocella, halophiles, 1519–1529
Desulfococcus, 3090–3096, 3114–3116
Desulfofaba, psychrophilic, 2640–2646
Desulfofrigus, psychrophilic, 2640–46
Desulfohabdus, flooded soils, 1326
Desulfohalobium, 1514–1529, 3090–3096
Desulfolobus, 2429–2431, 2429
Desulfomaculum, 510, 2278, 2419, 3089–3096, 3111–3116, 3137, 3193
Desulfomicrobium, 3114–3116, 3090–3096
desulfurization and, 1047
in groundwater, 1671
halophiles and, 1519–1529
petroleum reservoirs and, 2429–2431
Desulfomonas, 3090–3096, 3111–3116

Desulfomonile, 509–510, 512, 513, 3114–3116, 3414
Desulfonatrobonovibrio, 134, 1519–1529
Desulfonema, 2851, 3114–3116
Desulfosarcina, 3090–3096, 3114–3116, 3193
Desulfosporosinus, biodegradation, 511
Desulfotalea, psychrophilic, 2640–2646
Desulfotomaculum, 1520–1529, 2054, 2848–2851, 3099–3104, 3114–3116, 3130
  biofilms and, 2962
  flooded soils and, 1326
  halophiles and, 1519–1529
  petroleum reservoirs and, 2429–2431
  sulfate-reducing bacteria (SRB) and, 467–475
Desulfovibrio, 1514–1529, 1672, 2285–2286, 2551, 2552, 2848–2851, 3087–3096, 3099–3104, 3111–3116
  anaerobic granulation and, 232–245
  biocontrols and, 455
  biodegradation and, 512, 1044
  biofilms and, 82, 2962
  biomineralization and, 672, 674
  diazotrophy in, 2541–2547
  flooded soils and, 1326
  genomic studies and, 1448
  in groundwater, 1671
  halophiles and, 1519–1529
  hydrothermal vents and, 1610
  metal biodegradation and, 1883, 1886, 1897, 1902, 1903, 1908
  nitrogen fixation and, 2193–2201, 2212–2221
  oxygen and, 2278
  petroleum reservoirs and, 2419, 2429–2431
  radioactive waste degradation and, 2666–2674
  sulfate-reducing bacteria (SRB) and, 467–475
Desulfurization of fossil fuels, 1041–1051
  benzothiophenes and, 1047–1048
  coal, 1049
  Desulfomicrobium and, 1047
  Desulfovibrio and, 1044
  diesel or crude oils, 1048
  enzymes and, 1046
  genes for, 1045–1046
  hydrocarbons and, 1046–47, 2414
  hydrodesulfurization (HDS) in, 1042
  microbial degradation and, of dibenzothiophene, 1042–1049
  monooxygenase system for, 1046
  oil–water emulsions and, 1047
  Sulfolobus and, 1045
  thermophilic microbes and, 1045
Desulfurobacterium, 1614, 1620–1628
Desulfurobolobus, Archaea, 298
Desulfurococcales, 1629–1646
Desulfurococcus, 298, 1233, 1234, 1611, 1624–1646
Desulfuromonas, 82, 511, 512, 2474, 3193
Detachment of biofilms, 2087–2088
Detergents, 1176
  alkaliphiles and, 220, 226
  alkalithermophiles and, 3127
  biofouling vs., 604
  cold-adapted microorganisms and, 881
  enzymes and, 1178–1180, 3209–3210
Detoxification (See Bioremediation and detoxification)
Deuteromycota, 1367–1372, 3353–3364
  in allergies and illness, 1359
  soil microorganisms and, 2913–2914
Dewatering of activated sludge, 54, 59–60, 748
Dextran sulfate, algae biotechnology, 195
Dextransucrase, in soil, 2903–2910
Diabetes, enteroviruses and, 1151–1152
Diacetylphloroglucinol (Phl), 457
Diadesmis, 840–843
Diadinoxanthin, 2307–2317, 2512
Diadochism, in minerals, 633
Diagenesis, subsurface microorganisms, 1074
Diamidinophenylindole (DAPI), 1068, 1676, 1776–1778, 1949, 2596, 2653, 3196
Diarrheal diseases and gastroenteritis, 93–100, 338, 2743, 3288–3300
  Aeromonas and, 1091–1099
  astroviruses, 327–337
  Campylobacter and, 803–811
  cholera and, 853–861
  Cryptosporidium and, 650–657
  Cyclospora and, 995–1001
  disinfection and, 1067
  Escherichia coli and, 2337–2339
  in food, 3277–3287
  Giardia and, 1469–1489
  human caliciviruses and, 1586–1598
  Isospora and coccidiosis, 1734–1737
  Norwalk-like viruses (NLVs) and, 2221–2235
  rotavirus and, 2743, 2753
  Salmonella and, 2789–2802
  Shigella and, 2865–2871
Diatomaceous earth, 204
Diatomae, 206, 2467
Diatomophyceae, harm/in harmful algal blooms (HABs) and red tides, 2685–2693
Diatoms, 204, 1051–1053, 2504–2518
  algal turf scrubbing (ATS) and, 215
  Antarctic soils and, 1025
  biofilms and, 612–613, 1051–1063
  biofouling by, 1051, 1058–1059
  calcium and, 1053, 1055
  carbon metabolism in, 1052
  cell–cell interactions in biofilms of, 1056–1058
  cell structure and taxonomy of, 1052
  dissolved organic carbon (DOC) and, 1061
  extracellular carbohydrate polymers in, 1053–1055
  extracellular matrix (ECM) and, 1054, 1058
  extracellular mucilage (ECM) in, 1053
  extracellular polysaccharide (EPS), 1060–1061
  footpads or footprints of, 1053–1055
  frustule associated components (FACs) and, 1054
  frustulins and, 1055
  harm/in harmful algal blooms (HABs) and red tides, 2685–2693
  meroplankton and, 1863–1869
  motility, adhesion, and chemosensing in, 1052–1053
  in paleolimnology, 2301–2306
  periphyton and, 2358
  photoautotrophic, in low-light habitats, 840–843
  photosynthesis in, 1060
  phycosphere of, 1056–1057
  salt production and, 2805–2809
  sedimentation and, 1059–1061
  snow/in snow and ice environments, 2874
  subaerial communities and, 3056–3065

surface energy of substratum and, 1055–1056
Diatoxanthin, 2453–2470
Diauxic growth vs. mixed substrate growth, 1870–1872
Diazinon bioremediation, 705–712, 921–922
Diazotrophic bacteria (See also Nitrogen fixation), 1063, 2541–2547, 2550–2551
Dibenzathrancene bioremediation, 696–712
Dibenzofurans biodegradation, 1421
Dibenzoparadioxin biodegradation, 2297–2300
Dibenzothiophene (DBT) biodegradation, 1042–1049
Dibenzothiophene biodegradation, 696–712, 2297–2300, 2403–2416
Dibromo chloropropane (DBCP) biodegradation, 2933
Dibutylphthalate (DBP), 882–883
Dicamba, composting, 921–922
Dichlorobenzene biodegradation, 1763
Dichlorodiphenyl chlororoethane (DDE) biodegradation, 1238–52
Dichlorodiphenyl trichloroethane (See DDT)
Dichloroethane (DCA) biodegradation, 434–442, 512–513, 919–920, 1763
Dichloroethylene (DCE), 434–442, 1937–44
Dichloromethane (DCM) biodegradation, 511–514, 919–920
Dichlorophenol, 3147–55
Dichlorophenoxyacetic acid (2,4D) biodegradation, 507–516, 1421, 2385–2393, 2931
Dichlorophenoxylacetate bioremediation, 703–712
Dichloroztriazin aminofluorescein (DTAF), 1676
Dictyocha, 2467, 2508
Dictyostelium, 1055, 1952, 2912
Didenium, 975, 2474, 2614
Didymella, spores and spore dispersal, 406, 413
Didymohelix (See also Gallionella), 1401
Dielectrophoresis, 820
Diesel fuel biodegradation (See also Hydrocarbons), 916–917, 1048–1049, 2930
Diethers, Archaea, 249–259
Diethylhexyl phthalate (DEHP), 1822
Dietzia, 3–7
Difference imagery, 1683–1684
Differential display (DD) technique, 538
Differential interference contrast (DIC) microscopy, 1675
Diffusion of bioaerosols, 427
Diffusion of gases through soil, 1749
Diffusion of solutes through soil, 1748–1749
Digester, 695
Digital cameras, 1676
Digitaria, nitrogen fixation, 2199
Diglyceride fatty acid (DGFA), subsurface microorganisms, 1079, 2030
Digoxigenin, 1334
Dihalogens, biofiltration, 589
Diisopropanolamine bioremediation, 707
Diisopropyl ether (DIPE) biodegradation, 1994–2000
Diisopropyl fluorophosphates (DTPs), 848
Dimefuron, composting, 921–922
Dimethyl sulfide (DMS), 3192
Dimethyldisulfide (DMDS), 2645
Dimethylhydrazine bioremediation, 706–712
Dimethyloxazoline (DMOX), psychrophilic bacteria, 2645
Dimethylsufonioacetate (DMSA), soil salinity, 2778–2789
Dimethylsulfide (DMS), 1701, 3192
Dimethylsulfoniopropionate (DMSP), 1701, 2778–2789
Dimethylurea (DMCU), 175
Dinitrogen, 8–14, 1010–1019, 2176–2188
Dinitrogenase (See also Nitrogen fixation), 1063, 2205
Dinitrotoluene (DNT) biodegradation, 1979–1993
Dinoaxanthin, in paleolimnology, 2307–2317
Dinobryon, 210, 2556
Dinoflagellates (See also Pfiesteria), 205, 2504–2518, 2617, 2618
  bioluminescence in, 1830
  harm/in harmful algal blooms (HABs) and red tides, 2685–2693
  meroplankton and, 1863–1869
  Pfiesteria and toxic Pfiesteria complex (TPC), 2431–2447
  photosynthetic pigments in, 2466
  salt production and, 2805–2809
Dinophyceae, 2466, 2685–2693
Dinophysis, 2461, 2507, 2686–2693
Dinophyta, 837–843, 2357
Dinoseb bioremediation, 705–712
Dioporeia, meroplankton, 1867–1869
Dioxin biodegradation, 507–516, 1238–1252, 1421, 2297–2300
Dioxin bioremediation, 713
Dioxygenases (See also Oxygenase enzymes), 1063, 2289–2300, 3412
Dipeptides, soil salinity, 2778–2789
Diphenyl, 605
Diplogaster, 1720
Diptera, in water treatment systems, 1723
Dipyridyl, microbial protection from, 432
Direct filtration, 2708
Direct viable count (DVC), biofilms, 565
Directed evolution in biofilms, 1176–1177
Disaccharides, 432, 1350–1351, 2716
Discaria, nitrogen fixation, 2208
Discoloration of materials, bioweathering, 3367
Discosphaera, 2508
Discriminant data analysis, 1007–1009
Disease resistance, plant, 2722–2723
Disinfection, 359–361, 1063–1073
  aerobic endospores and, in measuring effectiveness, 140–149
  algal blooms vs., 209
  algal turf scrubbing (ATS) and, 216–217
  analysis of, 1068–1069
  assimilable organic carbon (AOC) and, 313–327
  bacteria in home/residential environment and, 341–342
  bacteriophages and, 359–360
  biofilms and, 580–582, 625–628
  biofouling vs., 625–628
  bottled water and, 797–798
  bromine as, 3266–3267
  by-products (DBP) of, 1066
  chloramines in, 1069
  chlorine as, chlorine, 6–7, 96, 360, 498, 582, 589, 603, 607, 625–626, 1063–1067, 1069, 1172, 1707, 2333–2334, 2979, 3184, 3265–3267
  chlorine dioxide in, 1063–1070
  Clostridium, 867–871
  concentration in, 1069
  contact time and, 1069
  CT concept in, 1064–1065
  cyanobacteria and, 991–992
  diamidinopheylindole (DAPI) in, 1068
  enteroviruses and, 1172
  factors influencing, 1067–1069

Disinfection, (*continued*)
filtration and (See Filtration)
fluorescein diacetate (FDA) in, 1068
granular activated carbon (GAC) filters and, 1493–1494
heat/thermal, 361, 387, 1174, 3264
heterotrophic bacteria and, 1541–1552
home water treatment systems using, 1567
iodine as, 3266–3267
ionization as, 3264–3265
Legionella vs., 1803
metal ions as, 3267
monochloramine in, 1063–1066, 1069
nitrification vs., in water treatment systems, 2172–2175
ozonation in, 6–7, 87, 273, 430–431, 496–497, 603, 797–798, 1067, 1070–1071, 1172, 2334, 2979, 3262
propidium iodide (PI) in, 1068
protozoa vs., 1066–1073, 2333–2334
Pseudomonas vs., 2632–2639
rain/in rainwater roof catchment systems, 2681–2683
risk assessment and, 2749
sampling in, 1068–1069
solar powered, in RRCS, 2682–2683
in spas and hot tubs, 2979
synergistic effects of disinfectants in, 1071
ultraviolet light in (See also Ultraviolet radiation), 1071, 1173, 2334, 3230–3235
viability testing and, 1068–1069
viral, 3260–3268
virus transport and removal in subsurface, 2094–2107
in water treatment systems, 1729, 2266
Dispersants, biofilms vs., 606
Dispersion
of bioaerosols and, 2108–2112
of viruses, 2101
Dissimilatory sulfate reduction, 3087
Dissolved air floation (DAF), 2017, 2266
Dissolved combined amino acids (DCAA), 85, 928
Dissolved free amino acids (DFAA), 85, 928
Dissolved organic carbon (DOC), 485–507
biofilms and, 84–87, 89, 569, 1086–1087
conditioning films and, 928
diatoms and, 1061
in groundwater, 1665
landfill leachate and, 1768
protozoa and, 2596
streams and, 3017
subsurface microorganisms and, 1074
Dissolved organic material (DOM)
aggregates and consortia in, 175
biofilms and, 569
sea ice and, 2838
subsurface microbial communities and, 1455
Dissolved organic nitrogen (DON), 2176–2188
Dissolved inorganic phosphorus (DIP), 2448–2453
Dissolved organic phosphorus (DOP), 2448–2453
Dissolved oxygen (DO)
activated sludge and, 1311
biofilms and, 2005–2006
floc formation and, 2005–2006
in source water, 2972
Distillation, home water treatment systems using, 1567
Distribution system for water, 941–952
antibiotic resistant bacteria in, 944
back siphon in, 943
biofilms in, 945–947
bird habitation in storage tanks for, 950–951
breaks and repairs in, 943, 949
coliform bacteria in, 944, 946–951
corrosion and, 942–943
cross connections in, 943
granular activated carbon (GAC) filters and, 1494
heterotrophic bacteria and, 1546–1548
illegal taps in to, 949–950
macroinvertebrates in, 945
microbial flora in, 943–945
microorganisms in, 1725
monitoring microbial activity in, 947–948
opportunistic pathogens in, 944–945
pathogenic bacteria in, case studies in, 948–951
pigmented bacteria in, 945
pipes in, service life of, 942
pressure interruptions in, 949–950
quality control in, 941–952
quality management for, 943
sampling of, 2813
stability of water in, 947
sulfate-reducing bacteria (SRB) in, 3102
sulfur bacteria and, 3101–3103
turbidity and, 944
ultramicrobacteria in, 945
Disturbance of soils, 3122
Dithiocarbamates, 607
Ditylum, 2506
Diuron, diatoms vs., 1060
Diversity (See Biodiversity)
Diving techniques and equipment, in water-filled caves, 825–826
DNA chips (See Biochips)
DNA type microarray, 1969
DNA/RNA analysis and genetics, 1195, 1858, 2269
acquired genes and, 1446
amplified rDNA restriction analysis (ARDRA) in, 2737–2738, 2920
bacterioplankton and, 2523–2524
biodegradation and, 2387
biofilms and, 565–566
bioinformatics, 1449–1450
biomass estimation and, 2656–2657
capillary electrophoresis and, 811–821
captured megaplasmids in, 1446
catabolic pathways in, 1203–1204
clades, 2524
clone library screening in, 2734
cloning and, 2492–2494, 2524, 2732–2733
cold-adapted microorganisms and, 3203–3206
cold-shock response and, 888
community genome arrays (CGAs), 1977
comparative genomics and, 1450
composite transposons and, 1200
conjugation and, 1409–1413
conjugative transposons and, 1202–1203
denatured gradient gel electrophoresis (DGGE), 28–29, 71, 247, 278, 299, 563, 1010, 1974, 2494–2496, 2732, 2735–2737, 2921
desiccation and, 1032–1041
directed evolution and, 1176–1177
DNA rearrangement and, 1200
DNA-based phylogeny in, 2492–2496
enzyme inactivation and, 1196–1197
evolution and, 1195
exogenous isolation and, 1410

fecal contamination and, 1258
fingerprinting techniques in, 2734–2735, 2739
fluorescent probes and, 1333–1343
forward sample array hybridization, 2731–2732
functional gene arrays (FGAs), 1974–1996
functional genomics and, 1448–1449
gene activation and inactivation in, 1199–1200
gene capture in, 1200
gene chips and, 1448–1449
gene clusters, 2524
gene exchange in biofilms and, 1408–1420
gene expression and, using microarrays, 1971–1974
gene transfer techniques in, 1280, 1285
genetic engineering and, 1449
genetically engineered microorganisms (GEMs) (See Genetically engineered microorganisms)
genetically modified microorganisms (GMMs) in, 1429–1440
homologous recombination in, 1197–1199
horizontal gene transfer and, 1195, 1201–1203, 1446
hybridization in, 2922
identification of isolates in, 1960–1961, 1964–1965
insertion elements for, 1199
integrons and, 1201
IS1071 insertion element, 1200
knockout studies in, 1449
microarray analysis in, 1968–1979
microgravity and, 3001
mRNA detection in, 2499
mushroom culture and, 1384–1385
mutations, radiation induced, 2993
naked DNA uptake, 1203, 1280
oligonucleotide probe hybridization in, 2497–2499
oligotrophic bacteria and, 2273
orthologs and, 1450
phages and, 1202
phylogenetic oligonucleotide arrays (POAs), 1976–1977
phylogenetics, 2489–2501
phylotypes, 2524
plasmids and, 1201–1202
polymerase chain reaction (PCR), 1853, 2492–2494, 2730, 2733–2739
radiation effects and, 1986–1994
rearrangement and gene capture in, 1200
recombinant DNA in, 1176–1178
regulation of, 1204
regulatory functions in, 1197
replication and repair systems in, 1195–1196
replicative transposons and, 1201
retrotransfer in, 1201–1202, 1413, 1427
reverse transcription polymerase chain reaction (RT PCR) in, 1162–1164, 2500
ribosomal intergenic spacer analysis (RISA), 2738–2739
ribosomal RNA-based phylogeny in, 2491–2492
ribotyping, 2728–2741
RNA-based methods of phylogeny in, 2496–2499
sequencing in, 2491
shotgun cloning in, 2732–2733
single-strand conformational polymorphism (SSCP) in, 28–29, 299, 536–537, 813, 2737
soil genetic ecology, 2918–2923
soil microorganism and diversity in, 528–544
subsurface microorganisms and, 2035
subtractive hybridization and, 1451–1452
temperature gradient gel electrophoresis (TGGE), 299, 536–537, 818–819, 2735–2737
temporal temperature gradient electrophoresis (TTGE), 2735–2737
terminal fragment length polymorphism (T-RFLP) in, 28–29, 71, 177–178, 278, 299, 536–537, 563, 829, 1974, 2496, 2737–2738, 2921
transcription in, 1199–1200, 1637–1639, 2489–2491
transduction in, 1415–16
transformation in, 1413–1415
translation in, 1637–1639, 2489–2491
transposition and integration in, 1199
transposons in, 1280
viruses as gene transfer tools, 3205, 3308
wastewater/sewage treatment and, 3339–3342
Dobson units, 2565
Docosahexaenoic acid (DHA), cold-adapted microorganisms, 880–881
Dolomite, subsurface microbial communities, 1464–1466
Domestic microbiology (See Residential environment, bacteria in)
Dormancy, desiccation, 1029, 1030–1031
Dose response, toxicity testing, 2745–2747, 3162–3164
Doubling time, bacterioplankton, 2529
Drain fields, septic tank, 2855, 2858, 2859
Drancunculus, in water treatment systems, 1718
Drechsler, Charles, 2914
Drechslera, 405, 408, 410, 1651
Drinking water (See Water treatment systems)
Drop collapse test, biosurfactants/bioemulsions, 762
Drop shape analysis, biosurfactants/bioemulsions, 762
Drosophila
endosymbiosis and, 1121–1122
genomic studies and, 1442
stress response and, 3047
Drugs, biotechnology, 1447–1448
Dry air spora, 405, 408–414
Dryas, nitrogen fixation, 2209
Dual media filtration, 2707
Dunaliella, 1507–1529, 1851, 1853, 1854, 2508
algae biotechnology and, 189–204
salt production and, 2805–2809
Dust, bioleaching, 640
Dwarfing of bacteria, in desert environment, 1582
Dyes, microbial protection from, 432
Dynamic vacuum drying, 1035–1036
Dysentery (See also Diarrheal diseases), Shigella, 338, 2865–2871

E

Eadie–Hofstee transformation, 2901
Earthworm Toxicity Test, 725
Ebola virus, 777–778
Echinenone, in paleolimnology, 2307–2317
Echovirus, 389, 2743, 2745
bioaerosols and, 3327
biodegradation and, 3289–3300

Echovirus, (*continued*)
municipal solid waste landfills and, 1766
soil/in soil, 3271–3276
in source water, 2969–2978
in spas and hot tubs, 2979, 2980–2984
in wetlands for wastewater treatment, 3398
Ecocyc, 1450
Ecological selection in biofilms, 546
Ecology, bioremediation, 693–694
Ectenagena, 1606
Ectendomycorrhizal fungi, 2125–2126
Ecthyma gangrenosum, Aeromonas, 1091–1099
Ectoenzymes, 1210, 2451
Ectoine, soil salinity, 2778–2789
Ectomycorrhizal fungi (See Mycorrhizae)
Ectosymbionts or ectobionts, 1113
Ectothiorhodospira, 2482, 3111–3116
alkaliphiles and, 134
halophiles and, 1517–1529
phototropism in, 2471, 2472, 2477–2489
soil salinity and, 2777
in wastewater stabilization ponds (WSBs), 3332–3333
Ectothiospira, nitrogen fixation, 2193–2201
Edgeworthia, 227
EEO, 820
Effective concentration (ECx), 3163
Eicosapentaenoic acid (EPA), 880–881, 3215
Eimeriina, 650
Einstein unit, 2565
El Nino, 1040, 2558
Elaphomyces, 2124–2132
Eleagnus, nitrogen fixation, 2209
Electrical fields vs. biofilms, 606
Electrochemically activated water, biofouling vs., 603–604
Electrochemiluminescence, detection, 1132
Electrogenic protein complexes in aerobic respiration, 150
Electrokinetic analysis, conditioning films, 933
Electromagnetic radiation, soil microorganisms, 2061
Electron acceptors, bioremediation, 714, 724
Electron capture detector (ECD), 3160
Electron microscopy (EM), rotaviruses, 2765, 2767
Electron transfer, aerobic respiration, 151, 153–154
Electron transport system (ETS), 1783–1784, 2884
Electron use, bacterioplankton, 2522
Electronegative filters, enteroviruses and, 1156–1159
Electrophoresis (See Capillary electrophoresis)
Electroporation, freeze drying, 1355–1356
Electropositive filters, 1159–1160
Electrospray ionization mass spectrometry (ESI MS), 309, 820
Electrospray ionization (EI) analysis, conditioning films, 934
Electrostatic forces
adhesion in biofilms and, 103–104, 120
biofilms and, 2004
conditioning films and, 936
floc formation and, 57, 2004
hydrophobicity and, 1599
Electrostatic precipitators, bioaerosol samplers using, 397
Eliminases, 1210
Ellipsometry, conditioning films, 935
Elminius, biofilms, 614
Elongation factor 2, 3207–3210
Embden–Meyerhof (EM) pathway, hyperthermophiles, 1633
Embedment, fluorescent probes, 1335
Embellisia, desert/in desert soils and biological soil crust (BSC), 1020–1023
Emergency water supplies, heterotrophic bacteria, 1550
Emergent plants, marine/ocean environment and (See also Spartina), 2539–2549
Emericella, 1647
Emiliana, 1854, 2508
Empty bed contact time (EBCT), biodegradable dissolved organic carbon (BDOC), 495
Emulsan, biosurfactants/bioemulsions, 761
Emulsion index, 762–763
Emulsions, desulfurization, 1047
Encapsulation of microbial cells, bioremediation, 730
Encephalitis, 1343–1350, 3399
Encephalitozoon, 2065–2083, 2329–2337, 2969–2978
Endocellulase, 1211–1223, 3021
Endogenous respiration, activated sludge processing, 23–26
Endogone, mycorrhizae, 2124–2132
Endolimax, 1136
Endolithic microorganisms, 842, 1100–1112
Antarctica and, 1100–1107
biodeterioration of mineral materials and, 517–528,
biomass of, 1104–1105
cyanobacteria and, 963–964
extinction and fossilization of, 1106
growth of, 1105–1106
hot arid regions and, 1107–1110
Paleoclimate indicators and, 1108
weathering and, 1105
zonation and, 1104
Endomycorrhiza, 2120
Endonucleases, Archaea, 271
Endoplasm, 1115
Endorhizosphere, 2714
Endosphere, marine/ocean environment, 2548–2549, 2552
Endospores
aerobic, 140–149
high hydrostatic pressure (HHP) vs., 1559–1563
soil microorganisms and, 2886
Endosymbiosis, 1112–1130
algae and, 1126–1128
amoebas, free-living pathogenic, 1345, 1347
aposymbiosis in, 1113
Archezoa hypothesis and, 1124–1125
bacteriome in, 1114
culturable vs. unculturable organisms in, 1113
ectosymbionts or ectobionts in, 1113
endosymbionts or endobionts in, 1113
epixenosomes and, 1123
eukaryote cell origin and, 1123–1126
evolution and, 1123–1128
extracellular endosymbiont/extrasymbiont, 1113
horizontal or environmental infection in, 1118
host in, 1113
hydrothermal vents and, 1117–1118, 1606–1609
hyperparasitoids in, 1115
insects and, 1114–1115
intracellular endosymbiont/intracytobiont, 1113
Karyomastigont and, 1124
male-killing bacteria in, 1122
marine/ocean environment and, 1115–1116
methane rich waters and, 1117–1118
mitochondrial origin and, 1125

mutualism in, 1113
nitrogen fixation and, 1118–1120
obligate intracytobionts, 1113
obligate symbiosis in, 1123
parasitism in, 1113
parasitophorus vacuole or inclusion, 1113
parthenogenesis and, 1122–1123
pathogen emergence and, 1123
photoautotrophy and, 1115–1117
plastic origins and cyanobacteria in, 1126–1128
primary vs. secondary symbionts in, 1114–1115
reproductive parasites and, 1120–1123
Rhizobium/legume, 1119–1120
Serial Endosymbiosis Theory (SET), 1123–1126
sulfur rich waters and, 1117–1118
symbiont in, 1113
symbiosome, 1113
symbitotrophic systems in, 1113–1120
syntrophy in, 1113–1120
transovarial infection and, 1118
Endotoxins, 417, 421, 977, 1690
bioaerosols and, 3328
bottled water and, 800
Energy production, marine biotechnology, 1850, 1853–1855
Energy recovery from landfill gases, 1767
Energy sources (See also Methane/methanogenesis), 1921–1922
Enhalus, 2549, 2823–2833
Enhanced biological nutrient removal (EBNR), 70
Enhanced biological phosphorus removal (EBPR), 15–26, 38–40, 62–67, 78–80, 1311, 1313, 3005, 3011–3013
Enolic hydrogen, 929
Entamoeba, 1136–1146
biofilms and, 89
disinfection and, 1067, 1069
endosymbiosis and, 1125
in food, 3277–3287
high hydrostatic pressure (HHP) vs., 1561
in source water, 2969–2978
in spas and hot tubs, 2980–2984
subsurface microorganisms and, 2025
in wastewater stabilization ponds (WSBs), 3334
in wastewater/sewage, 2329–2337
in water treatment systems, 1718
in wetlands for wastewater treatment, 3398–3399
Entamoeba dispar, 1136–1146
Entamoeba histolytica, 1136–1146
Enteric adenoviruses (See Adenoviruses)
Enteric bacteria, bacteriophages and, 384–392
Enteric cytopathogenic human orphan (ECHO) viruses, 1147
Enteric viruses (See also Adenoviruses), 388, 1252–1256
Enterobacter, 499, 1345, 2596, 3102
bioaerosols and, 3322
biocontrols and, 455, 457, 462
biodegradation and, 511
biofilms and, 577, 580, 581, 2342–2356
bioluminescence in, 658–662
cold-shock response and, 885–895
coliform bacteria and, 895–914
disinfection and, 1065
endosymbiosis and, 1118
fecal contamination and, 1258, 1272
fluorescent studies of, 1835
genetically altered, in biotechnology, 1448
granular activated carbon (GAC) filters and, 1491, 1571–1572
heterotrophic bacteria and, 1545, 1550
infectious airborne bacteria and, 1689–1697
metal biodegradation and, 1883, 1885, 1886
as microbial tracer in groundwater, 3195
municipal solid waste landfills and, 1766
nitrogen fixation and, 2193–2201
O serogrouping, 1259–1260
rhizosphere and soil microbes in, 346
Salmonella and, 2789–2802
soil microorganisms and, 2896
in drinking water, 944, 946
Enterococci, 1264–1278, 1297
biofilms and, 556
cold-shock response and, 886–895, 892
gene transfer and, 1409
metal biodegradation and, 1889
municipal solid waste landfills and, 1766
nosocomial infections and, 2236–2250
in source water, 2974
Enterocytozoon, 2066–2083, 2329–2337, 2969–2978
Enterohemorrhagic E. coli (EHEC), 1146, 2338
Enteroinvasive E. coli (EIEC), 1146, 2339
Enteromorpha, macrofouling, 616
Enterotoxigenic E. coli (ETEC), 1146, 2339
Enterotoxin (B/SEB), bioterrorism, 778
Enteroviruses, 1146–1156, 1167–1175
bacteriophages vs., 363–364, 384–385
biodegradation and, 3289–3300
biofilms and, 2353
in spas and hot tubs, 2980–2984
streams and, 3017
in wastewater stabilization ponds (WSBs), 3334
Entner–Doudoroff (ED) pathway, 262, 1633
Entomopathogens (See also Insecticides, microbial), 1175, 1710
Entomopox viruses (EPV), 1711
Entomorphthora, microbial insecticides using, 1710
Entophysalis, 1507–1512
Entrophospora, mycorrhizae, 2120–2124
Environmental Directorate (DGXI), 2706–2707
Environmental genomics (See Soil genetic ecology)
Environmental impact of landfills, 1765–1769
Environmental infection, 1118
Environmental Protection Agency (EPA), 2695–2707, 3164, 3169
Environmental scanning electron microscopy (ESEM), 2008
Enzymatic toxicity tests (See Toxicity testing)
Enzyme electrophoresis, 309
Enzyme immunoassay (EIA), 97, 98, 333, 1131
Enzyme linked immunoassay (ELISA)
adenoviruses and, 97
in allergies and illness, 1361
astroviruses and, 333
detection of, 1131
nitrifying bacteria and, 2169
proteomes and proteins, 309
rotaviruses and, 2765, 2767–2769
Enzyme linked lectinsorbent assay (ELLA), 1061
Enzymes, 1176–1189, 1850, 1852
activity of, determining, 1210–1214
activity of, in biofilms, 1214–1216

Enzymes, (*continued*)
activity of, in municipal solid waste landfills and, 1760
activity of, in soil, 2906–2909
agriculture and, 3215
alkaliphiles and, 220, 221–223
Archaea and, 259–276, 1175
Arrhenius equation in, 2900
assays of, in soil, 2903–2904
ATP binding cassette (ABC) pathway in, 1208
autotransporter pathway in, 1208–1210
Baeyer Villiger monooxygenase (BVMO), 2290
bioaugmentation and bioremediation in, 435
biocide degradation and, 608
biodegradation and, 1875–1876
bioluminescence in, 657–663
biomass conversion, 1186
biotechnology and, 1176–1189
bleaching, fungal, 2647–2648
brewing and distilling, 1187
chemical synthesis and, 1185
cofactors and, 2900–2001
cold active, 268, 3206–3210
cold-adapted microorganisms and, 871–885
concentrations of, in soil, 2904–2906
cropping systems vs., in soils, 2907
dairy products and, 1186–1187
desulfurization and, 1046
detergents and, 1178–1180, 3209–3210
distribution of, in soils, 2907
Eadie–Hofstee transformation and, 2901
ecto-, 1210
environmental factors vs. activity of, 1216–1217
extracellular enzymes and, 1750
extracellular polymeric substances (EPS), 1207–1223
extremeophiles and, 1232–1237
in fat and oil processing, 1187–1188
in feeds for animals, 1188
fermentation, 1177–1178
fertilizer and, 2906
in fiberboard production, 1185
flooded soils and, 2906
fluorescent probes and, to trace activity of, 1784
food additives, 1186–1188
food processing and, 3209
formulation of, 1178
in fruits, juices, wines, 1187–1188
in fuel alcohols, 1185–1186
functions of, in biofilms, 1220–1221
fungal, 1369, 1385–1388, 2647–2648
genetically altered, in biotechnology and, 1447
halotolerant, 268
Hanes–Wolf transformation and, 2901
hydrogen peroxide generating, 1387
immobilization of, 2903
inactivation of, in genetic evolution, 1196–1197
industrial production of, 1176–1178
inhibition of, in soil, 2901–2902
ionic environment and, 2900–2901
leather processing, 1183
Linweaver–Burk transformation and, 2901
marine biotechnology and, 1853
market for, 1178
Michaelis–Menten equation and, 2901
molecular biology and, 3210
mycorrhizae and, 2129
nitrogen fixation and, 2189–2195
oil and gas drilling, 1185
organic solvent tolerant, 268
oxygenases, in biodegradation, 2288–2300
personal care products and, 1185
pesticides and, 2909
pH levels and, 2900, 2906, 2908–2909
phosphorus cycling and, 2450–2452
pressure tolerant, 269
in pulp and paper industry, 1183–1185
recombinant DNA in, 1176–1178
in recycling of paper, 1184–1185
regulation of, in biofilms, 1217–1220
secretion mechanisms in, 1207–1210
secretons in, 1209
soil microorganisms and, 2899–2910, 2949
sources of, in soils, 2902
in starches, 1180–1183
state of, in soils, 2902–2903
streams and, 3021
substrate concentration and, 2901
technical type, 1178–1186
temperature and, 2900
in textile processing, 1181–1183
thermostable, 267–269, 1852
tillage and residue management and, 2908
toxic metals and, 2909
in toxicity testing, 3155–3168
in wastewater/sewage, 3169–3171
weathering, bioweathering and, 3368
wetlands and, 3410–3415
Eo70, 3039–3040
Eosinophils, in allergies and illness, 1360
EPANET model, biodegradable dissolved organic carbon (BDOC), 502
Ephemera, streams, 3019, 3020
Ephydra, salt production, 2805–2809
Epibionts, hydrothermal vents, 1609–1610
Epichloe, spores and spore dispersal, 406
Epicoccum, 405, 408, 412, 1367–1372, 1650, 1655–1657, 3360
Epidemiological studies, municipal solid waste landfills, 1765
Epidermophyton, 1368, 1373–1383
Epiendolithic microbes, 842
Epifluorescence (EF), 1336–1338, 2340, 2653
Epifluorescence microscopy (See also Image analysis), 1189, 1675
Epilithic microorganisms (See also Subaerial communities), 517–528, 1189
Epiphytes, subaerial communities, 3056–3065
Episcopic differential interference contrast (EDIC), 2340
Epistylis, 2608
Epixenosomes, 1123
Epoxides, 3412
Epoxytrichothecenes, 1375
Equalization
filtration and, 2265–2266
image analysis and, 1679
Equivalent spherical diameter (ESD), floc formation and biofilms, 2006
Ergosterol, 1818–1824
Erwinia, 2723
cold-adapted microorganisms in, 872–885
detection of, 1134
soil salinity and, 2775
subaerial communities and, 3057–3065
in wastewater stabilization ponds (WSBs), 3334

Erysiphe, 454
Erythema migrans, 1838
Erythomicrobium, 2035, 2522
Escarpia, 1606
Escherichia coli, 314, 332, 339, 340, 386, 499, 539, 1132, 1345
adhesion in biofilms and, 123
aerobic respiration and, 155–157
alkaliphiles and, 223
bacteriophages and, 355–356, 374–384
bioaerosols and, 431, 3321, 3322–27
biochips, biochip testing sample, 444, 447, 449
biocontrol use of, 2703
biodegradation and, 513, 1874
biofilms and, 562, 567, 577, 581, 582, 621, 1209, 2341–2356, 2960
bioleaching and, 643
bioluminescence in, 658–662
biomineralization and, 674
biotechnology and, 3203–3204
bottled water and, 796, 799
Clostridium and, 868–871
cold-adapted microorganisms in, 872–885
cold-shock response and, 885–895
coliform bacteria and, 895–914
desert environment and, 1581
desiccation and, 1032–1035
disinfection and, 1065, 1066
enterohemorrhagic E. coli (EHEC), 1164, 2338
enteroinvasive E. coli (EIEC), 1164, 2339
enterotoxigenic E. coli (ETEC), 1164, 2339
fecal contamination and, 1257, 1269, 1272
flow cytometric studies of, 1951, 1952
fluorescent studies of, 1834, 1835
gene transfer and, 1409, 1413–1415
genetically modified microorganisms (GMMs) and, 1437
genomic studies and, 1446
granular activated carbon (GAC) filters and, 1489–1496
green fluorescent protein (GFP) studies for, 1498
heterotrophic bacteria and, 1541–1552
high hydrostatic pressure (HHP) vs., 1555–1563
identification of isolates in, 1964
immobilization of, 110
in marine/ocean environment, 1706
metal biodegradation and, 1880, 1886, 1890
microarray study of, 1971
as microbial tracer in groundwater, 3195
microgravity and, 2998, 3001
municipal solid waste landfills and, 1766
nitrogen fixation and, 2193–2201
nosocomial infections and, 2236–2250
O serogrouping, 1259–1260
oligotrophic bacteria and, 2269, 2270, 2272–2275
pathogenic, 899, 1164, 2337–2339
peroxide vs., 603
phosphorus cycling and, 2448–2451
pulsed field gel electrophoresis (PFGE), 1260–1261
radiation and, 2987
rain/in rainwater roof catchment systems, 2682
resistance in, 607–608
ribotyping of, 1261–1263
soil salinity and, 2781
in source water, 2968, 2969–2978
in spas and hot tubs, 2980–2084
spores and spore dispersion in, 421
streams and, 3019
stress response and, 3048, 3050–3051
subsurface microorganisms and, 2025
toxic solvents and, 3149, 3150, 3151, 3153
ultraviolet light and, 3230
verocytotoxigenic E. coli (VTEC) and, 2349–2350
viable but not culturable (VBNC) phenomenon and, 3249, 3250
in wastewater/sewage, 3171
in drinking water, 944, 945, 949, 2013, 3199
in wetlands for wastewater treatment, 3393–3397
Esterase, 1212–1223, 3021, 3170, 3207–3210
Esters, 928–929, 3412
Estrogen mimics, biodegradation, 507–516
Estuarines (See Marine/ocean environment)
Ethacrylate bioremediation, 707
Ethane, 2426
Ethanol, 699, 3147–3155
activated sludge and, 742–748
alkaliphiles and, 228
biodegradation and, 2410
Ethene (ETH) biodegradation, 510–514
Ether, 929
Etheylbenzene, 700
Ethyl alcohol (EtOH) biodegradation, 1994–2000
Ethyl ester, in surface waters, 928
Ethyl t-butyl ether (ETBE) biodegradation, 1994–2000
Ethylbenzene biodegradation, 714, 917, 1763, 1994–2000, 2412, 3147–3155, 3405
Ethylene, 344
Ethylene diamine tetraacetic acid (EDTA), 196–199
Ethylene glycol dinitrate (EGDN) biodegradation, 1979–1993
Ethylphenol biodegradation, 1763
Eubacteria, 744–748, 1440, 1443–1446
Eucalyptus, 2124–2132, 2649, 2911
Eucarya (See Eukarya)
Eudorina, 1863–1869
Eudorylaimus, Antarctic soils, 1026
Euendolithic microorganisms, 1100–1112
Euglena, 2508
algae biotechnology and, 196
endosymbiosis and, 1125
microgravity and, 3000
photoautotrophic, in low-light habitats, 837–843
in wastewater stabilization ponds (WSBs), 3331
Euglenophyta, 837–843, 2504–2518
Euglenozoa, 2465, 2466
Euglypha, 2605
Eukarya
Archaea and, 259
halophiles and, 1507–1512, 1514–1529
methanotrophic bacteria and, 1913–1914
microgravity and, 3000–3001
oligotrophic bacteria and, 2268
soil microorganisms and, 2880–2886, 2912
stress response and, 3025
thermophiles and, 3140–3147
Eukaryote cell origin, endosymbiosis, 1123–1126
Eumycota, 3353–3364
Eunotia, periphyton, 2364
Euoccidiida, 650
Euphotic zone nitrification, 2159
Euplotes, 2620, 2626–2632, 3173
European and Mediterranean Plant Protection Organization (EPPO), 3164

European Bioinformatics Institute (EBI), 1333
European Hygienic Equipment Design Group (EHEDG), 584
European Retrievable Carrier (EURECA), 1035, 1038–1041
European Union, regulation of commercial microorganism use, 2706
Eurotium, 1130, 1367–1372, 1647
Euryarchaeota
  Archaea and, 259, 277, 295
  bacterioplankton and, 2526
  halophiles and, 1515–1529
  nitrogen fixation and, 2212–2221
Eustigmatophyceae, photosynthetic pigments, 2467
Eustronglydes, 3400
Eutroph (See Oligotrophic bacteria)
Eutrophication, 1189–1194
  activated sludge and, 77
  nitrogen cycle and, 2942–2943
  paleolimnologic studies of, 2312–2313, 2324–2325
  periphyton and, 2364
  plankton and phytoplankton, 2516
  toxicity testing and, 3220
Evaporites, rock salts, 1510–1511
Evapotranspiration septic tank systems, 2856
Evolution, 1446
  endosymbiosis and, 1123–1128
  plankton and phytoplankton, 2504–2505
  subsurface microorganisms and, 1080
Ex situ bioremediation, 695
Exanthems, enteroviruses, 1151
Exguobacterium, alkaliphiles, 133
Exocellulase, 3021
Exoenzymes, bioweathering, 3368
Exogenous isolation, 1410
Exophialia, 136
Exopolymeric compounds
  activated sludge and, 739–740
  biofilms and, 561, 1083–1084
  floc formation and, 58
Exotoxins, Clostridium, 862–871
Expanded granular sludge blanket (EGSB), 232–245
Exploratory data analysis, 1003–1007
Explosives biodegradation, 922–924, 1421, 1498, 1979–1993, 3410
Exponential decay, 2108
Expopolysaccharides (EPS) (See Extracellular polymeric substances)
Exposure assessment, 2747–48, 3220–3225
Exserohilum, 410
Extinction, endolithic microorganisms, 1106
Extracellular endosymbiont/extrasymbiont, 1113
Extracellular enzymes, 1750
Extracellular matrix (ECM), diatoms, 1054, 1058
Extracellular mucilage (ECM), biofilms, 1053
Extracellular polymeric substances/polysaccharides (EPS), 109, 1223–1231, 1577
  accumulation of particles in, 1228
  activated sludge and, 54
  adhesion and, 1224–1225
  aggregates and consortia in, 166
  biocides vs., 1228
  biocorrosion and SRBs, 473–475, 1227–1228
  biodeterioration of mineral materials and, 518
  biofilms and, 81, 89, 546, 548, 560, 569, 571, 628, 1081, 1083–1084, 1207–1223, 1408, 2000–2012, 2959, 2961, 2964
  biofouling and, 628, 1227–1228
  bioleaching and, 644
  biosurfactants and, 1129–1130
  biotechnology and, 1229
  bioweathering and, 1227–1228
  calcium scaling and, 1227–1228
  cohesion and, 1224–1225
  composition of, 1223–1224
  diatoms and, 1053–1055, 1060–1061
  floc formation and, 2000–2012
  fluorescent probes for, 1778–1781
  heat transfer in, 1227
  identification of isolates in, 1960
  matrix architecture in, 1225–1227
  mechanical stability in, 1227
  microbially influenced corrosion (MIC) and, 1228
  sorption properties of, 1228–1229
  subaerial communities and, 3060
  viscoelastic properties of, 1227
  water retention in, 1227
  weathering, bioweathering and, 3366, 3367
Extracellular polymers (ECP), anaerobic granulation, 238, 242–243
Extremeophiles, 302–303, 1232–1237
  alkaliphiles as, 133, 219–231, 3127–3140
  alkalithermophiles as, 3127–3140
  Antarctic soil microbes as, 1023–1029
  Archaea and, 246–305
  bacterioplankton and, 2526
  cyanobacteria as, 136, 961–964
  fungi as, 136
  haloalkaliphiles as, 135–136
  halophiles as, 1501–1529
  hydrothermal vents and, 1604–1628
  hyperthermophiles and, 1628–1646
  in microbial mats in hypersaline lakes, 173
  neuston microbiology and, 2133–2144
  oligotrophic bacteria as, 2267–2277
  thermoalkaliphiles as, 136
  thermophiles as, 3127–3147
  vadose zone microbiology and, 3244–3245
  yeasts as, 136
Extrinsic allergic alveolitis, 1369, 1374
Exudates, rhizosphere, 2714, 2716–2717
Exxon Valdez (See Oil spills)
Eyespot, in plankton and phytoplankton, 2509

F

F-specific RNA bacteriophages, 356, 2102
F1F0 ATP synthase, 157–158
Fabrea, salt production, 2805–2809
Fabrics, bacteria in home/residential environment, 340
Factor analysis, 1006–1007
Facultative anaerobes, biofilms, 82
Faenia, 423
Fats and oil processing, enzymes, 1187–1188
Fatty acid methyl ester (FAME) analysis, 1096, 1258, 1820, 2644–2645, 2656, 1962
Fatty acids
  activated sludge processing and, 22–26, 741–748
  algae biotechnology and, 194–195
  anaerobic granulation and, 235
  biofilms and, 85–86
  biomass estimation and, 2654–2656
  bioplastics and, 683–692
  cold-adapted microorganisms and, 875–876, 888
  Microthrix parvicella and, 3
  psychrophilic bacteria and, 2639, 2644–2645

sea ice microorganisms and, 2840
in surface waters, 928, 928
Fecal contamination (See also Coliform bacteria; Escherichia coli), 896–898, 1256–1264
biofilms and, 2347–2350
Enterococci in, 1264–1278
in source water, 2968–2970, 2973, 2974
Streptococci in, 1264–1278
Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), 2695–2707
Feed, animal, enzymes, 1188
Fengycin in biosurfactants/bioemulsions, 757–759
Fermentation
activated sludge processing and, 22–26, 741
anaerobic granulation and, 239–240
Archaea and, 259–276
biofilms and, 82
enzymes and, 1177–1178
flooded soils and, 1319–1320
fungi and in, 1384
halophiles and, 1525
municipal solid waste landfills and, 1760
petroleum reservoirs and, 2429
Ferredoxin, biodegradation, 969, 1702, 2297–2300
Ferric hydroxide, bioremediation, 709
Ferrihydrite, biomineralization, 671
Ferritin, biomineralization, 671
Ferroglobus, 297, 1630–1646
Ferroplasma, bioleaching, 646
Fertilizer
enzyme activity and, 2906
biodegradation and bioremediation aided by, 699, 714, 724, 2931
bio-, 351
biofilms and, 86–87, 86
enzyme inhibition and, 2901
oligotrophic bacteria and, 2268
Fervidobacterium, 1233, 2419
FFSB cluster, soil microorganism and diversity, 533–534
Fiberboard production, enzymes, 1185
Fibrobacter, 2035, 2036
Fibrocapsa, 2467, 2686–2693
Fick's First Law of molecular diffusion, 84
Field and object parameters, image analysis, 1684
Field inversion capillary electrophoresis (FICE), 816
Field release of GEMs (See Genetically engineered microorganisms)
Fijivirus, 2753–2773
Filamentous and gliding green bacteria, 1287–1306, 1306–1315
activated sludge and, 1–7, 24–26, 69–70, 1287–1306, 1287
aggregates and consortia in, 164–165
anaerobic granulation and, 240
biofilms and, 1083
bulking in activated sludge and, 1287–1315
floc formation and, 1306–1315, 2002
foaming in activated sludge and, 1287–1306
Gordonia amarae like organisms (GALO), 3
hydrothermal vents and, 1610
Microthrix parvicella and, 3
Nostocoida limicola in, 4
phototropism in, 2471
Skermania piniformis as, 3–7
total extended filament length (TEFL) in, 1307
in drinking water, 945
Filtration (See also Biofiltration), 2707–2713
algal blooms vs., 208–209
Archaea and, 273
assimilable organic carbon (AOC) vs., 321–322
attachment system in, for microbes, 2708
backwashing of, 2707
bacteriophages vs., 375–376
bacteriostatic filters in, 1570–1572
bank infiltration and, 2014–2015
bioaerosol samplers using, 396–397
bioaugmentation and bioremediation in, biofilters and, 439–440
biodegradable dissolved organic carbon (BDOC) and, 495, 497–498
bio-, 439–440, 587–593
biotrickling filters, 782–795
Clostridium and, 2266
conditioning films and, 929
conventional, 2708
Cryptosporidium and, 2262–2266
direct, 2708
disinfection and, 2708
dual media, 2707
electronegative filters in, 1156–1159
electropositive filters in, 1159–1160
equalization in, 2265–2266
Giardia and, 2262–2266
granular activated carbon (GAC) in, 315, 1489–1496, 2707
granular high-rate, 2707–2713
hepatitis viruses and, 1534
heterotrophic bacteria and, 1541–1552
home treatment (See Home water treatment devices)
images, 1679
inline, 2708
membrane filtration (MF) method for, 375–376, 497–498
micro- and, 2334
microstrainers in, 2013
model of, 2709–2712
monomedia, 2707
nitrogenase in, 2211–2221
pathogenic microorganisms vs., 1315
performance of, 2709
pore size in, 2708, 2712
pretreatment of water, 2012–2013
protozoa and, 1315, 2262–2267, 2332, 2333
rate of, 2712
ripening period following backwashing of, 2707
roughing filters in, 2012–2013
sand filters in, 2333, 2707
sedimentation in, 2266
spent filter backwash water (SFBW), 2262–2267
transport in, 2708–2712
trickling filters in, 2332
trimedia, 2707
ultrafiltration and, 2266
viruses and, 2097
Fimbriae, 2885
Fingerprinting, genetic, 1316, 1887, 2734–2735, 2739
Fireflies (See Bioluminescence; Photinus)
Firmacutes, biodegradation, 510, 511–512, 2549, 2552
Firmibacteria, nitrogen fixation, 2214–2221
First-order rate constant, 1738
Fish
bioluminescence in, 657–663
cyanobacteria and cyanotoxins in, 975–980
Pfiesteria and toxic Pfiesteria complex (TPC), 2431–2447
Fisherella, 210
Fixatives, for fluorescent probe samples, 1335

Fixed bed bioreactor, 695
Fjords, phototropism, 2476
Flagellates (See also Protozoa), 1316, 2508, 2594, 2605, 2611, 2613, 2617, 2885
  alkaliphiles and, 138
  bacterioplankton and, 2520
  harm/in harmful algal blooms (HABs) and red tides, 2685–2693
  marine/ocean environment and, 2618–2619
  plankton and phytoplankton, 2509
Flame ionization (FID), 1820, 3160
Flanges, 2320
Flavenoids, algae biotechnology, 195
Flavin, desulfurization, 1046
Flavobacteria, 313, 499, 1200, 1501–1512
  activated sludge and, 71
  assimilable organic carbon (AOC) and, 316–317
  bacterioplankton and, 2524–2526
  bioaugmentation and bioremediation in, 436
  biodegradation and, 2386, 3412
  biofilms and, 566, 570, 572, 577, 580, 581, 2342–2356
  bioremediation and, 706
  cold-adapted microorganisms in, 872–885
  endosymbiosis and, 1122
  flooded soils and, 1319, 1320
  genetically modified microorganisms (GMMs) and, 1431
  granular activated carbon (GAC) filters and, 1490
  halophiles and, 1506–1512
  heterotrophic bacteria and, 1541–1552
  metal biodegradation and, 1885
  psychrophilic, 2641–2646
  rhizosphere and soil microbes in, 346
  sea ice and, 2838
  spores and spore dispersion in, 430
  streams and, 3019
  in drinking water, 945, 946
Flemming, Alexander, 1384
Flexibacter
  biofilms and, 90
  cold-adapted microorganisms in, 872–885
  filamentous forms of, in activated sludge, 1302–1306
  soil microorganism and diversity in, 533, 539
  subsurface microorganisms and, 2035
Floc formation, 1316, 2000–2012
  activated sludge and, 5–7, 44, 54–61, 68, 73, 75, 1288–1315, 2002
  aggregation in, 2001–2002
  algal blooms vs., 208
  anaerobic methanogenic granules in, 2002
  analyzing, 2006–2008
  biodegradable dissolved organic carbon (BDOC) and, 494
  biofilms and, 58, 89
  biological mechanisms of, 57–58
  charge neutralization in, 2004–2005
  chemical composition of, 2003
  clarification and, 59
  colloids and, 2002
  contact angle measurement (CAM), 2007–2008
  cyanobacteria and, 992
  denitrification and, 58
  density of, 2002, 2007
  Derjaguin–Landau–Verwey–Overbeen (DLVO) theory of adhesion, 2004–2005
  dewatering in, 59–60
  diffusional gradients and flow in, 2003–2004
  dissolved oxygen (DO) and, 2005–2006
  electrostatic forces in, 2004
  equivalent spherical diameter (ESD) measurement in, 2006
  extracellular polymeric substances (EPS) in, 2000–2012
  factors affecting, 2005–2006
  filamentous bacteria and, 1306–1315, 2002
  formation of, 2004–2005
  freshwater, 2000–2002
  hydrophilic/hydrophobic forces in, 2004, 2005, 2007–2008
  ideal composition of, 60
  influence of, on activated sludge, 59
  interfacial forces in, 2004
  marine snow, 2002
  marine/ocean environment and, 2002
  mechanisms of, 57
  microbes of, 56–57
  microbial adhesion to hydrocarbons (MATH) for, 2007–2008
  microscopic analysis of, 2008
  nitrogen and, 2005
  oxygen and, 2005
  pH levels and, 57, 58
  phosphorus and, 2005
  physicochemical factors of, 57, 2002–2003
  polymer bridging in, 2005
  porosity of, 2003, 2007
  salt aggregation test (SAT), 2007–2008
  salt bridging in, 2005
  salt production and, 2805–2809
  sampling, 2006–2008
  settling rates for, 59, 75, 2007
  solids retention time (SRT) and, 2006
  stability of, 58–59
  steric forces in, 2004
  structural properties of, 2002–2003
  surface charge and, 2008
  surface thermodynamics and, 2005
  van der Waals forces in, 2004
  water treatment systems and, 2015–2018
Flooded soils (See also Wetlands), 1316–1333, 3403–3404
  aerobic processes in, 1328
  anaerobic processes in, 1319–1321, 1327–1328
  biogeochemical cycling in, 1316–1318
  competition among microbes in, 1327–1328
  drainage of, 1323–1324
  enzyme activity and, 2906
  fermentation in, 1319–1320
  fungi and, 3359–3360
  homoacetogenesis in, 1320–1321
  hydrolysis in, 1319–1320
  iron cycling in, 1325–1326
  isotope effects in, 1326–1327
  methanogenesis in, 1319–1321
  microbial oxidation of methane in, 1321–1323
  nitrogen cycling in, 1324–1325
  organic carbon in, 1317–1318
  oxidant cycling in, 1323–1326
  oxidation of atmospheric methane and, 1323
  oxygen in, 1318
  plant/microorganism interaction in, 1328
  rhizosphere and, 1322–1323, 1324
  sequential reduction and redox gradients in, 1318
  sulfate-reducing bacteria (SRB) and, 3091–3092, 3114
  sulfur cycling in, 1326
  syntrophy in, 1320

vadose zone microbiology and, 3236–3246
Floodplains, 1316–33
"Flora of the lamps," photoautotrophes, 840–843
Flow cyclometry (FCM), 1132, 1944–1958, 2526, 2653
Flow-through samplers, 2813
Fluidized bed reactors, 695
Fluoranthene bioremediation, 696–712
Fluorene bioremediation, 694, 696–712
Fluorescein diacetate (FDA), 1068, 1212
Fluorescein isothiocyanate (FITC), 570, 1334, 1676
Fluorescence in situ hybridization (FISH), 812, 1333–1334, 1416
activated sludge bacteria counts using, 30–43, 62–63, 69
anaerobic granulation and, 235
Archaea and, 247, 279, 301
bacterial counts using, 30–43, 62–63, 69
bacterioplankton and, 2524
biofilms and, 570, 571
floc microbiology and, 56–57
methanotrophic bacteria and, 1931–1932
soil microorganism and diversity in, 529, 538
Fluorescence microscopy (See Laser scanning microscopy)
Fluorescent focus assay (FFA), rotaviruses, 2766–2767
Fluorescent probes, 1333–1343
activity testing using, 1783–1784
antibody identification using, 1781
attachment to, 1334
confocal laser scanning microscopy (CLSM), 1335, 1338, 1772–1788
confocal microscopy and, 1338
dehydration and embedding for, 1335
enzyme activity using, 1784
epifluorescence in, 1336–1338, 1336
extracellular polymeric substances (EPS), 1778–1781
fixation for, 1335
flow cytometry and, 1945–1958
fluorescent labels and, 1334
fluorescent proteins as reporters in, 1341–1342
green fluorescent protein (GFP), 558–559, 659, 1341–1342, 1412, 1496–1500, 1676, 1781, 1830–1838
hybridization techniques for, 1335–1336
labels for, 1676
laser scanning microscopy and, 1772–1788
luciferase, 1830–1838
as microbial tracer in groundwater, 3196
microenvironment testing using, 1784
molecular approaches to, 1339–1342
mounting and washing for, 1335
nucleic acid databases and, 1333–1334
red fluorescent protein (RFP), 1835–1836
reporter gene detection using, 1783–1784
ribosomal RNA targeted oligonucleotide probes and, 1339–1340
RNA targeting using, 1781, 1784
sample preparation for, 1334–1335
sectioning for whole cell hybridization and, 1334–1335
viability testing using, 1783
virus tracers and, 3305
Fluorescent proteins, as reporters, 1341–1342
Fluorescent recovery after photobleaching (FRAP), 1784
Fluoridation, bottled water, 800
Fluorochromes (See also Laser scanning microscopy), 1343,1676, 1786, 3196
Fluorometry, pigment analysis, 2462–2463
Flushing agents, biosurfactants/bioemulsions and as, 764
Flushing reactors, municipal solid waste landfills, 1769–1770
Foaming in activated sludge, 1–7, 75, 1287–1306, 1343, 2002, 3008–3011
Folliculitis, in spas and hot tubs, 2980–2984
Folsomia, 2915
Fomes, 1983, 2647
Fomitopsis, 2647
Food additives, 1176, 1850, 1852–1853, 2516
Archaea and, 259–276
enzymes and, 1186–1188
Food and Drug Administration (FDA), 2694–2707
Foodborne disease, 338–340
Aeromonas and, 1094–1099
astroviruses and, 331
biofilms and, 577–587
bioterrorism and, 779
Campylobacter, 803–811
Clostridium and, 862–871
cold-shock response and, 886–895
Cryptosporidium and, 650–657
Cyclospora and, 995–1001
Entamoeba and, 1136–1146
Enterococci in, 1264–1278
enteroviruses and, 1146–1156
Escherichia coli and, 2337–2339
Giardia and, 1469–1489
hepatitis viruses and, 1534
high hydrostatic pressure (HHP) vs., 1561
human caliciviruses and, 1586–1598
Norwalk-like viruses (NLVs) and, 2221–2235
protozoa and, 3276–3287
Pseudomonas, 2632–2639
risk assessment and, 2749
rotavirus, 2753–2573
Salmonella and, 2789–2802
Shigella and, 2865–2871
Streptococci in, 1264–1278
viruses and, 3276–3287
Food industry, 1343
algae as, 2516
aquaculture and, 1856–1857
bioaerosols and, as byproduct of, 423
biofilms in, 577–587
biofiltration and in, 588
cold-adapted microorganisms and, 879–881
coliform bacteria and, 901
contamination of food in, 1343
enzymes and, 3209
hepatitis viruses and, 1534
high hydrostatic pressure (HHP) vs. microbial inactivation, 1552–1563
marine biotechnology and, 1850, 1851–1853, 1857
preservation of foods and, 1343
protozoa and, 3276–3287
salting, halophiles and, 1511
viruses and, 3276–3287
Food sources
algae biotechnology and, 189–204
cyanobacteria and, 980
cold-adapted microorganisms and, 880–881
Food web
bacterioplankton and, 2534–2535
mycorrhizae and, 2127

Food web (*continued*)
periphyton and, 2360–2361
polar phytoplankton and, 2563–2564
protozoa and, 2620–2622, 2630
in sediments and sedimentation, 2844–2845
stream microbiology and, 3015–3024
viruses and, 3306–3307
Footpads or footprints, diatoms, 1053–1055
Foraminifera, 2613, 2616–2617
Forestry, mycorrhizae, 2129–2130
Formaldehyde, 605, 608, 625
Formaldehyde dehydrogenase (FdDH), 1927–1936
Formate, 741–748, 2172, 3062
Formate dehydrogenase (FDH), methanotrophic bacteria and, 1927–1936
Formation water, 2418
Formic acid, alkaliphiles, 228
Forward angle light scatter (FALS), flow cytometry, 1945
Forward sample array hybridization, 2731–2732
Forward scatter, flow cytometry, 1945
Fossil fuels (See also Hydrocarbons), 1343, 1850
desulfurization of, 1041–1051
Fossil infusorian, 1401
Fossilization
endolithic microorganisms and, 1106
in paleolimnology, 2301–2306
Fourier transform infrared spectrometry (FTIR), 257, 934–935
Fourier transform Raman spectroscopic analysis, 935
Fowler, Gilber, 74
Fractionation
activated sludge processing, 14–26
biofilms and, 1213
subsurface microbial communities and, isotopic, 1461
Fragillaria, 206
Fragmentation analysis, conditioning films, 932–933
Francisella (See also Tularemia), 432, 772, 777, 1123
Frankia, 1118
endosymbiosis and, 1119–1120, 1119
nitrogen fixation and, 2188–2211, 2939
plant growth promoting rhizobacteria (PGPR) inoculation and, 350
soil microorganisms and, 2912
Free energy balance, hydrophobicity, 1600–1601
Free energy of adhesion, 101
Free-living amoebas, meningoencephalitis, 1343–1350
Free water, 2418, 2960
Freeze drying, 1030, 1035, 1350–1359
artificial trehalose introduction in, 1355–1357
chemical permeability and electroporation in, 1355–1356
disaccharides in, 1350–1351
genetic biosynthesis of sugars in, 1356–1357
lipid phase transitions in, 1356
microbial protection from, 432
pore proteins and trehalose introduction, 1357
sugar glasses and stabilization in, 1351–1354
trehalose and, 1353–1357
Freshwater bodies (See Aquatic ecosystems; Surface waters)
Friedmanniella, 64, 1026, 1028
Frontia, 972
Frucose, 2716
Fructose, 1185, 1186
Fruit juices, enzymes, 1187–1188
Frustule associated components (FACs), diatoms, 1054
Frustulins, diatoms, 1055
Fucose, biosurfactants/bioemulsions, 756
Fucoxanthin, 2307–2317, 2453–2470, 2504, 2512
Fucus, algae biotechnology, 199–200
Fuel alcohols, 1185–1186
Fuel oxygenates, 1994, 2410
Full-cycle RNA approach, bacterial counts using, 29–34
Fulvic acid, in surface waters, 928
Fumarate, 3062
Fumonisins (See also Bioaerosols), 182–189, 1359, 1375
Funalia, 2649
Functional gene arrays (FGAs), 1974–1996
Functional genomics, 34, 1448–1449
Fungi (See also Bioaerosols; Mycorrhizae; Spores and spore dispersal), 338, 417, 1366–1400, 2910–2918, 3353–3364
actinomycetes and, 2912
activated sludge and, 71–72
airborne, 1647–1661
alkaliphiles and, 136
allergies and allergens of, 1359–1366
antibiotics produced by, 457–458
arbuscular mycorrhizae, 2126
arbutoid mycorrhizae and, 2126
Ascomycota in, 2913–2914
asexual vs. sexual forms, 1647
bacteria in home/residential environment and, 342
Basidiomycota in, 2914
beneficial chemicals from, 1369
as biocontrol agents, 451–454
bioaerosols and, 404–416
biodegradation and, 2386, 2911–2912, 3390–3392, 3412
biodeterioration of mineral materials and, 518–519, 524
biodiversity in, 3354–3355
biofilms and, 551–559, 2342
biofouling and, 613
biological bleaching and, 2647–2648
biological pulping process in, 2647
biomass estimation and, 2657–2658
bioremediation and, 1390–1392
biotechnology and, 1384–1394
brewing and distilling, 1384
brown rot, 1388–1389, 2646–2647
cellobiose dehydrogenase (CDH) and, 1387–1388
chytrids in, 2913
classification of, 1647
cold-adapted microorganisms in, 872–885
common species of, 1653–1661
conidia of, 1647–1648
control of spores from, 1370–1371
culturing of, 1648, 1652–1653
decomposition and, 1385–1389, 2646–2647, 3358–3359
desert environment and, 1576, 1577, 1578–1579
desiccation and, 1029, 1031
diseases caused by, 1366–1383
distribution of, 3354–3355
dry air spora in, 408–414
ectendomycorrhizal, 2125–2126
ectomycorrhizal, 2124–2132
enzymes produced by, 1385–1388, 2647–2648
explosives biodegradation using, 1983–1985, 1989
fermentation and, 1384
genetic manipulation of, 1389–1390

habitats of, 1648
hydrocarbon degradation and, 2405
hydrogen peroxide-generation from, 1387
hypersensitivity pneumonitis (HP), 1369, 1374–1375
hyphae and, 2646–2647
identification of, 1649–1653
infections caused by, 1368, 1372–1374
irritations caused by, 1369, 1375
leaf litter decomposers in, 3355–3358
lichens and, 2914
lignin degrading, 1385
lytic enzymes produced by, 458–459
marine/ocean environment and, 1394–1400, 2539–2540
marshes, decomposer fungi in, 3359–3360
methylation and, 1388
micro-, 1367
microbial insecticides using, 1710, 1711
microbial volatile organic compounds (MVOCs) and, 1372–1383
mildews and, 1647
moisture and, 1367–1368
monotropoid mycorrhizae and, 2126
municipal solid waste landfills and, 1766
mushroom culture and, 1384–1385
mycelial growth in decomposition, 3355
mycorrhizae and, 1385, 2120–2132, 2911–2912
mycotoxicosis and, 1369, 1378–1379
mycotoxins and, 1366–1383
nutrients and, 1648
organic dust toxic syndrome (ODTS) and, 1369, 1374–1375
organoosmotrophy, 3354
periphyton and, 2357–2367
pitch (wood sap) degradation in, 2648–2650
predatory role of, 2914–2915
pyroloid mycorrhizae and, 2126
residential environment and, 1372–1383
rusts, 411–412
safety in handling spores of, 1651
sampling of, 1369–1370, 1650–1651
sensitization to, 1368–1369
sick building syndrome (SBS) and, 1372
slime molds and, 2912–2913
smuts, 410–411
soft rot, 1389
soil microorganisms and, 664–665, 2910–2918
soil salinity and, 2775
solar radiation and, 1368
spore prints in, 1652
spores and spore dispersal in, 405–415, 1366–1383, 1647–1653, 3354–3355
staining of wood by, 2648–2650
streams and, 3021
subaerial communities and, 3057–3065
symbiosis in, 1367
taxonomy of, 3354
transmembrane redox potential in, 1388
trophic links between, 2914–2915
volatile organic compounds (VOCs) and, 1366–1383
in drinking water, 945
water fungi and, 3353–3364
water molds and, 2912
water purifiers vs., 1572–1573
weathering and, 3364–3389
wetlands and, 3412
white rot, 1385–1386, 2646–2647
wood degradation and, 1385–1389, 2646–2647, 3358–3359
yeasts and, 1647, 1661, 2913–2914
Zygomycota in, 2913
Fungi imperfecti, in allergies and illness, 1359
Fungicides, biocontrol vs., 450–465
Furan biodegradation, 1238–1252
Fusarium, 182–189, 1657
in allergies and illness, 1367–1372, 1378–1383
biocontrols and, 450, 451, 453, 454, 455, 460, 2705
bioterrorism and, 779
desert environment and, 1579
explosives biodegradation and, 1983
rhizosphere and, 2718
soil microorganisms and, 2913
spores and spore dispersal in, 405, 408, 413–414
subaerial communities and, 3057–3065
Fusobacterium, 555

G

G bacteria, activated sludge, 40, 57, 61–68, 71
Gaeumannomyces, 450, 2913, 3121
Galactanases, 1187
Galactic cosmic radiation (GCR), space microbiology, 2989–2991
Galactose, in biosurfactants/bioemulsions, 756
Galactosidase, 2903–2910, 3207–3210
Gallionella, 1401–1408, 3101
biofilms and, 572
biomineralization and, 676
metal biodegradation and, 1904
Galvanic reactions, bioleaching, 637
Gambierdiscus, 2686–2693
Gamma linoleic acid (GLA), cold-adapted microorganisms, 880–881
Gamma radiation, space microbiology, 2988–2989
Gammarus, 3021
Gangrene
Aeromonas and, 1091–1099
Clostridium and, 862–871
Ganoderma, 2647, 2914
Gas chromatography (GC), 253–255, 1132
Gas gangrene, Clostridium, 862–871
Gas liquid chromatography (GLC), 1096
Gas-liquid interface (See also Neuston microbiology), 3274–3275
Gas vesicles, halophiles, 1505
Gases, trace (See Trace gases)
Gasoline, 699, 916–917, 1994
Gastric ulcers, Helicobacter pylori, 1529–1530
Gastroenteritis (See Diarrheal diseases and gastroenteritis)
Gaussian plume dispersion model, bioaerosols, 2108–2110
Geitleria, 838–843, 3059
Gel electrophoresis for proteomes and proteins, 308
Gelidibacter, 2640–2646
Gelling agents, algae biotechnology, 192
Gene activation and inactivation, 1199–1200
Gene amplification, bacteriophages and detection, 383
Gene capture, 1200
Gene chips (See Biochips)
Gene clusters, 2524
Gene expression, 1971–1974, 2205
Gene probes (See also Laser scanning microscopy), 1974–1975, 2036
Gene transfer
biofilms/ in biofilms, 567, 1408–1420

Gene transfer (*continued*)
conjugation and, 1409–1413
exogenous isolation and, 1410
frequency of, in situ, 1416–1417
genetically engineered microorganisms (GEMs) and, 1280, 1285
horizontal gene transfer in, 1446
retrotransfer in, 1413
Reynolds number and, 1416–1417
transduction in, 1415–1416
transformation in, 1413–1415
to unknown organisms, 1416
viruses for, 3205, 3308
GeneChip system for proteomes and proteins, 310
General rate constant, kinetics of growth, 1741–1742
General stress resistance (GSR), 3038–3043
Genetic ecology of soil (See Soil genetic ecology)
Genetic engineering (See also DNA/RNA analysis), 1449, 1850
aquaculture and, 1856–1857
bioremediation and, 729–730
desulfurization and, microbial, 1045–1046
fungi and, 1389–1390
marine biotechnology and, 1856, 1858
trehalose production and freeze drying, 1356–1357
Genetic fingerprinting, 1887
Genetically engineered microorganisms (GEMs) (See also Genetically modified microorganisms), 726, 729–730, 1278–1287, 1420–1429
containment of, 1283–1284
ecosystem alteration from, 1282
field release of, 1278–1287
gene transfer techniques in, 1280, 1285
monitoring of, 1282–1285
persistence in environment, 1280–1281
plant assisted microbial bioremediation, 1424–1425
polymerase chain reaction (PCR) monitoring of, 1284–1285
propagation of, 1425–1426
regulation of, 2693–2707
retrotransfer in, 1427
rhizoremediation, 1424–1425
risk management for, 1283–1285
stability of, 1425–1426
survival of, 1425–1426
target microcosms and, 1426
Toxic Substances Control Act (TSCA) and, 1280
tracking of, 1425–1427
transfer of rDNA from Pseudomonas to other microorganisms and, 1426–1427
transport of, in environment, 1281–1282
xenobiotics and, 1420–1421
Genetically modified microorganism (GMMs) (See also Genetically engineered microorganisms), 1429–1440, 1429
biocontrols using, 1437
construction of, 1430–1431
containment of, 1430–1431
fate of, factors affecting, 1432
genetic impact of release of, 1436–1437
indigenous soil populations vs., 1434–1437
introduced soil populations of, 1431–1432
nitrogen fixation and, 1431, 1434–1437
plant growth and, 1434–1437
polymerase chain reaction (PCR) and, 1433–1434
reporter genes and, 1430–1431
sampling, 1432–1434
survival of, 1431–1432
tracking of, 1432–1434
Genetics (See DNA/RNA analysis and genetics)
Genome sequencing, 263–266, 290–291, 1442–141443
Genomics and genomic studies (See also DNA/RNA analysis), 1440–1453
acquired genes and, 1446
application of, 1443–1446
biodegradation of halogenated compounds and, 1447
bioinformatics applications for, 1449–1450
bioremediation and 1446–1447
captured megaplasmids in, 1446
comparative genomics and, 1450
databases of, 1449–1450
diversity of microbes and, 1450–1452
environmental modeling systems using, 1448
functional genomics and, 1448–1449
gene chips and, 1448–1449
genetic engineering and, 1449
in groundwater, 1671–1672
horizontal gene transfer in, 1446
hyperthermophiles and, 1637–1641
industrial applications for, 1447–1448
knockout studies, 1449
metal oxidation/reduction and, 1446–1447
microbial genome sequencing and annotation in, 1442–1443
nuclear magnetic resonance (NMR) in, 1449
open reading frames (ORFs) and, 1443
orthologs and, 1450
pathogenesis and, 1448
phylogeny and evolution in, 1446
physical gaps in, 1443
polymerase chain reaction (PCR) and, 1443
subtractive hybridization and, 1451–1452
unknown and conserved hypotheticals in, 1450
X ray crystallography in, 1449
Genotyping, rotaviruses, 2770–2771
Gentisic acid, biodegradation, 2295–2300
Geobacter
activated sludge and, 71
biomineralization and, 674
genetically altered, in biotechnology, 1447
metal biodegradation and, 1882, 1884, 1905
Geochemicals, subsurface microbial communities, 1453–1468
Geodermatophilous, 523, 1102–1106, 3057–3065
Geology, subsurface microbial communities, 1453–1468
Geometric increase in population, 1738
Geosiphon, 1118, 2120–2124
Geosmin, 210, 980, 2889
Geospirillum, metal biodegradation, 1905, 1908
Geostatics (See Soil distribution of microorganisms)
Geothermal environments (See also Hydrothermal vents)
biofilms and, 84
cyanobacteria and, 961–963
extremeophiles and, 1233–1235
high temperature subsurface environment, 2047–2057
Microthrix parvicella and, 3
thermophiles and, 3140–3147
Geothrix, 533, 1905
Geotoga, 1515–1529, 2420
Geotrichum, 916
Geovibrio, 1905

Gerdermatophilus, 1578
Germicidal wavelengths, 3231
Giardia, 903, 1469–1489, 2598, 2607
activated sludge and, 72
aquatic sediments and, 1252–1256
bioaerosols and, 3327
bottled water and, 796
Clostridium and, 868–871
disinfection vs., 360, 1065, 1066, 1067–1073
endosymbiosis and, 1124, 1125
filtration and, 2709
flow cytometric studies of, 1952
in food, 3277–3287
heterotrophic bacteria and, 1548
high hydrostatic pressure (HHP) vs., 1561
measurement of, using aerobic endospores, 141–149
rain/in rainwater roof catchment systems, 2680
sampling of, 2813
in source water, 2968, 2969–2978
in spas and hot tubs, 2979, 2980–2984
streams and, 3021
ultraviolet light vs., 3231–3235
in wastewater stabilization ponds (WSBs), 3334
in wastewater/sewage, 2329–2337
water purifiers vs., 1572–1573
water treatment systems and, 1719, 1727, 2013–2019, 2262–2266, 3199
in wetlands for wastewater treatment, 3398–99
Gibberelins, 344
Gibbs equation, aerobic respiration, 149
Giberella, 2913
Gigaspora, 455, 2120–2124
Glacial ice, 1024, 2873–2875
Glacieocola, 2640–2646
Glactosidase, 3170
Glanders, bioterrorism, 771, 776
Glass, weathering, bioweathering, 3372
Glasses, sugar glasses and stabilization, 1351–1354
Glaucocystophyta, 837–843
Glaucomys, 2127, 2595–2604
Gliocladium, 451–459, 461, 462, 1885, 2649
Gliotoxins, 1375
Global warming (See Climate change)
Globigerinoides, 1115–1116
Globodera, 455, 458
Gloeocapsa, 1102–1112
Antarctic soils and, 1025
biodeterioration of mineral materials and, 519
photoautotrophic, in low-light habitats, 840–843
subaerial communities and, 3056–3065
Gloeocystis, 840–843
Gloeosphera (See also Gallionella), 1401
Gloeothece, 2193–2201
Gloeotrichia, 1863–1869, 2302–2306
Glomus, 455, 1579, 2120–2124, 3121
Glucanase, 1187, 1188, 2903–2910, 3207–3210
Glucanotransferase, 1185
Glucoamylase, 1185, 1187
Gluconate, 3062
Glucosaminidase, 1211–1223
Glucose, 2716, 2899
activated sludge and, 742–748
anaerobic granulation and, 235–237
biofilms and, 88
flooded soils and, 1320
Glucose bacteria (See G bacteria)
Glucose isomerase, 1177–1189
Glucose lipids, biosurfactants/bioemulsions, 756
Glucose oxidase, 1185, 2903–2910
Glucosidase, 1211–1223, 2903–2910, 3021, 3170
Glucosylglycerol, soil salinity, 2778–2789
Glucuronidase, 1212–1223
Glutamate dehydrogenase (GDH), 1698–1701
Glutamate, soil salinity, 2778–2789
Glutaminase, in soil, 2903–2910
Glutamine, 2716
Glutamine synthetase (GS), 1698–1701
Glutaraldehyde, biofouling vs., 605
Glyceria, 2548
Glycerol, 518, 741–748
Glycidyl azide polymer (GAP), biodegradation, 1979–1993
Glycine, 2716, 2778–2789
Glycogen, 21–26, 40, 73, 2270, 3005
Glycogen accumulating organisms (GAOs), 40, 67
Glycolic acid, 607
Glycolipids, bioremediation, 715, 752, 1817–1824
Glycoproteins
Archaea and, 248
conditioning films and, 937–938
Glycosides, 2129
Glycosyl hydrolases, 269
Glyphosphate, bioremediation, 705–712
Gold, 1902–1913, 2961
algae biotechnology and, 199
biomineralization and, 671–681
metal biodegradation and, 1881
Golden-brown algae, 206
Gomphidius, 2124–2132
Gomphosphaeria, 972, 2805–2809
Goniomonas, 2594–2604
Gonyaulax, bioluminescence, 1830
Gordonia, 3314–3319
activated sludge and, 3–7, 3009
biosurfactants/bioemulsions and, 753
desulfurization and, 1048
filamentous forms of, in activated sludge, 1288–1306, 1312
Gordonia amarae-like organisms (GALO), 3–7
Grab samples, 2812
Gracilaria, 1857, 2689–2693
Gradients of metabolism, biofilms, 1085
Gram-negative bacteria, 1781–1783
bioaerosols and, 429
biodegradation and, 1996
biofilms and, 2960
G bacteria and activated sludge processes, 61–68
immobilization of, 109
infectious airborne bacteria and, 1694–1695
metal biodegradation and, 1895–1896
nosocomial infections and, 2236–2250
in permafrost, 2369
Salmonella and, 2789–2802
Shigella and, 2865–2871
soil microorganisms and, 2881–2886
soil salinity and, 2783–2786
Gram-positive bacteria, 1781–1783
activated sludge and, 38
alkaliphiles and, 133
bacterial counts using, 31
bioaerosols and, 429
biodegradation and, 1996
biofilms and, 2959–2960
genomic studies and, 1442
immobilization of, 109
nitrogen fixation and, 2214–2221
oligotrophic bacteria and, 2272
in permafrost, 2369
soil microorganisms and, 2881–2886
soil salinity and, 2781–2783
stress response and, 3048
subsurface microbial communities and, 3065–3071

Gram-positive bacteria, (*continued*)
viable but not culturable (VBNC) phenomenon and, 3253
Gram staining, 1781–1783
Granitic rocks, subsurface microbial communities, 3068
Granular activated carbon (GAC), 315, 1489–1495, 1541–1552, 1563–1575, 2707
Granular high-rate filtration, 2707–2713
Granular sludge (See Anaerobic granules)
Granulosis viruses (GV), 1711
Graphium, 422
Grass–bacteria association, nitrogen fixation, 2199–2200
Gravity (See Microgravity; Space microbiology)
Gravity sampling, bioaerosol samplers using, 397
Grazing
algae vs., 1193–1194
bacterioplankton and, 2531–2532
biofilms and, 2342
periphyton and, 2359, 2364
photosynthetic pigments in, 2455
plankton and phytoplankton, 2514
protozoa and, 2599–2601
sea ice microorganisms and, 2841
Great Salt Lake, 1509–1510
Green algae, 205, 2504–2518
paleolimnology of, 2302–2306
in permafrost, 2369
photoautotrophic, in low-light habitats, 837–843
photosynthetic pigments in, 2467
Green bacteria, 2470–2480, 2992–2993
Green fluorescent protein (GFP), 558–559, 659, 1341–1342, 1412, 1496–1500, 1676, 1781, 1830–1838
Green rust, subsurface microbial communities, 1464–1466
"Green sickness" of caves, 841–843
Green sulfur bacteria, 1515–1529, 2455, 2458, 3097–3104
Greenhouse effect (See Climate changes)
Greigite, biomineralization, 671
Groundwater
Atlantic Coastal Plain aquifers and, 2028–2042
bacteriophages and, 375–377
biodegradation of contaminants in, 507
bioremediation and, 699–700, 702–705, 707, 708, 1447
carbon transformation in, 1669–1671
Clostridium and, 865–871
coliform bacteria and, 903
dissolved organic carbon (DOC) in, 1665
diversity of microorganisms in, 1671–1672
environment of, 1665–1666
fecal contamination in, 1256–1264
Gallionella in, 1401–1408
genomic studies of, 1671–1672
halogenated compounds in, 702–703, 705
heavy metals in, 1768
heterotrophic bacteria and, 1541–1552
high temperature subsurface environment, 2051–2057
hydrocarbons in, 699–700
hydrogen dependency in, 1672
hydrologic studies and, 3198–3199
iron reducing bacteria (IRB) in, 1665, 1667–1669
isotope activity in, 1669–1671
leachate from landfills and, 1768
light nonaqueous phase liquids (LNAPLs) in (PICKUP), 507–516, 699
lipid biomarkers of, 1821
lithotrophs and, 1827
microbial communities of, 1661–1673, 1723–1724
microbial numbers in, 1666–1669
microbial tracers in, 3194–3202
military chemicals in, 707
nitrates, nitrates in, 708
nonaqueous phase liquids (NAPLs) in (PICKUP), 44, 699
oxygen in, 1665
pathogens in, 2025, 2971–2972
permafrost and, 2367–2385
pesticide contamination of, 706, 2398–2399
pH levels in, 1665
public health studies and, 3199–3200
sampling of, 1664–1665, 2815, 3073, 3077–3079
in source water, 2971–2972
subsurface microbial communities and, 1076, 1079, 1454–1468, 2025, 2028–2042, 3068
sulfate-reducing bacteria (SRB) in, 1665, 1667–1669
vadose zone microbiology and, 3236–3246
virus transport and removal in subsurface, 2094–2107
viruses and, 3288–3300
Growth factors, soil microorganisms, 2060
Growth inhibition assays, in wastewater/sewage, 3171–3172
Growth kinetics (See Kinetics of growth)
Growth rates, 1745
cold-adapted microorganisms and, 873
microgravity and, 2999–3000
protozoa and, 2615
Guinardia, 2506
Gunnera, 974, 975, 1118
Gutters and downspouts, in RRCS, 2677
Gymnodinium, 1058, 2431, 2507, 2686–2693
Gymnostoma, 2209
Gymodinium, 2805–2809
Gyrodinium, photosynthetic pigments, 2466

H

Haber–Bosch process (See Nitrogen fixation)
Haematococcus, 189–204, 1853, 1859
Haemophilus, 1413–1415, 1440, 1973
Hafnia, 1491, 1545
Halanaerobiales, 1515–1529
Haldule, 2551
Halfnia, 1766
Haliscomenobacter, 4–7, 1302–1306, 1310
Haloalkaliphiles, 135–136
Haloarcula, 135–136, 298, 1237, 1503–1512, 3146
Halobacteriaceae, 135–136, 246–259, 270, 295, 298, 1233, 1236–1237, 1501–1512, 2419, 2666–2674, 2776, 2806–2809
Halobaculum, 1503–1512
Halocarbons, 3183
Halocella, 1520–1529
Halochromatium, 1517–1529, 2477–2489
Halocins, 1505
Halococci, 135–136, 246–259, 270, 298, 1233, 1501–1512
Halodule, 2547, 2549, 2822–2833
Haloferax, 135–136, 1503–1512
Halogenated compounds and solvents, 3183, 3184, 3188–3189
biodegradation and, 507–516, 693, 701–705, 1195,

1238–1252, 1447, 3405–3410, 3414
biofilms and, 86–87
biofiltration and, 589
composting and, 919–920
groundwater pollution and, 702–703, 705
natural attenuation in, 1238
rivers and pond pollution, 705
soil contamination and, 702–705
wetlands and, 3405–3410
Halogenation, biofouling vs., 605
Halogeometricum, 1504–1512
Halomonas, 614–615, 1501–1512, 1610, 1619
Halomondacea, 246–259
Halonaerobium, 1514–1529
Halophila, 2549, 2822–2833
Halophiles, 1233–1237, 1501–1512, 3128
Archaea and, 246–259, 262, 277–293, 297–298
salt production and, 2805–2809
sea ice microorganisms, 2833–2844
soil biosphere and, 297–298
soil microorganisms and, 2889
soil salinity and, 2774–2789
subsurface microorganisms and, 2024–2025
Halophytophthora, 1394–1400
Halorespiration, 3414
Halorhabdus, 1504–1512
Halorhodopsins, 2470
Halorhodospira, 1513–1529, 2477–2489, 2477
Halorubrum, 1237, 1504–1512
Haloterrigena, 1504–1512
Halothermothrix, 1521–1529
Halotolerant enzymes, 268
Halotolerant microorganisms, 1501–1512, 1514–1529, 1514
Handling systems, biofilms, 579–580
Hanes–Wolf transformation, 2901
Hansenula, 136, 1871
Hantzschia, 840–843, 1025, 3057–3065
Hapalindole, 977
Hapalosiphon, 3056–3065
Haptophyta, 2467, 2556
Harmanella, 2594–2604
Harmful algal blooms (HABs) and red tides, 1529, 2002, 2340, 2565, 2685–2693
cyanobacteria and, 970–972, 975–980, 989–990
harmful algal blooms (HABs), 2685–2693
Pfiesteria and toxic Pfiesteria complex (TPC), 2431–2447
red tides and, 2685–2693
Hartmanella, 1123, 2342–2356, 2605
Hazard analysis and critical control point (HACCP), 342, 583
Hazard identification, in risk assessment, 2742
Hazardous wastes (See also Radioactive/nuclear wastes)
in municipal solid waste landfills, 1765
toxicity testing and, 3225–3227
vadose zone microbiology and, 3243–3244
Heap bioleaching, 642, 648, 649
Heat exchangers, biofouling, 622–623
Heat-shock response/proteins (hsp70) (See also Stress response), 1529, 1583, 3026–3028, 3024–3034, 3046–3055
Heat treatment/disinfection, 361, 387, 1174, 3264
bacteriophages vs., 361, 387
enteroviruses and, 1174
viruses vs., 3264
Heavy metals, 1529, 1878–1894, 2340
activated sludge and, 72
algae biotechnology and, 196–200
biodegradation and, 1855, 3402
bioleaching and, 648
biomineralization of, bacterial, 670–681
enzyme activity and, 2909
metal biodegradation and, 1886–1891
neuston microbiology and, 2137–2139
periphyton and, 2364–2365
resistance of bacteria to, 608
soil salinity and, 2774
sulfate-reducing bacteria (SRB) and, 3089–3090
toxicity testing and, in soils, 3162
Hebeloma, 2124–2132, 2914
Hedysarum, 2785
Heinrich sediments, 2314
Helicase, 3207–3210
Helicobacter
biochips, biochip testing, 447
biofilms and, 563, 1209, 2341–2356, 2351–2352
disinfection and, 1065
genomic studies and, 1451–1452
hydrothermal vents and, 1609–1610
phototropism in, 2471
in source water, 2969–2978
Helicobacter pylori, in gastric ulcer, 1529–1530
Heliothis, microbial insecticides using, 1710
Heliozoan, 2617
Heliscus, 3358
Helium, 3183
Helminthosporium, 410, 1579
Helminths, in wastewater stabilization ponds (WSBs), 3333–3334
Hemagglutination inhibition (HI), adenoviruses, 98
Heme copper oxidases, aerobic respiration, 155
Hemerocampa, microbial insecticides using, 1710
Hemiaulus, 2506
Hemicellulase, 1186
Hemicellulose, 85–86, 1760
Hemichloris, 1025, 1026, 1102–1106, 3061
Hemiselmis, 2508
Hemolysis of red blood cells, biosurfactants/bioemulsions, 762
Hemorrhagic fevers, bioterrorism, 777–778
Hepatitis, hepatitis viruses A and E (HAV/HEV), 95, 360, 1162, 1384, 1530–1540, 2743, 2744–2745, 2747
biodegradation and, 3288–3300
in food, 3277–3287
soil/in soil, 3271–3276
in source water, 2969–2978
in spas and hot tubs, 2979, 2980–2984
streams and, 3017
subsurface microorganisms and, 2025
Hepatotoxins, 207, 986–995, 2340
Hepatophyta, 837–843
Hepetosiphon, 1303–1306
Heptamethylnonane, biodegradation, 2407
Herbaspirillum, nitrogen fixation, 2193–2201
Herbicides
biocontrol vs., 450–465
biodegradation and, 693, 705–706, 916–917, 1421, 2385–2393
biofilms and, 88
biofiltration and, 591
groundwater pollution by, 706
regulation of, 2693–2707
soil contamination by, 706
Hermatypic corals, endosymbiosis, 1116–1117
Herpangina, 1150–1151
Herpes simplex, algae biotechnology, 195
Herpyzonema, 838–843

Heterduplex DNA analysis (HPA), 813
Heterobasidium, 451, 2646–2647
Heterococcus, 1102–1106
Heterocysts, 836–843, 2891
Heterodera, 455
Heterokonta, 837–843, 2912
Heterolobosea, 2912
Heteropolysaccharides, Archaea, 249
Heterorhabditis, biocontrol use, 2705
Heterosigma, 199–200, 2686–2693
Heterotrophic ammonia oxidation, activated sludge denitrification, 13–14
Heterotrophic bacteria, 1540–1552
  activated sludge and, 18–26
  algal turf scrubbing (ATS) and, 213–219
  antibiotic resistance in, 1545
  bacterioplankton and, 2521
  biodeterioration of mineral materials and, 523
  biofilms and, 81–82, 2341
  bottled water and, 1549–1550
  cultivation of, 1542–1544
  cyanobacteria and, 966
  genera of, 1541–1542
  indigenous bacteria as, 1544
  nitrification and, 2145–2146, 2941
  origins of, 1544–1545
  pathogenic, 1545
  phosphorus cycling and, 2450
  soil microorganisms and, 2889, 2891
  subaerial communities and, 3057–3065
  sulfur cycle and, 3110
  thermophiles and, 3141–3142, 3144–3146
  transient bacteria as, 1544
  in wetlands for wastewater treatment, 3393
Heterotrophic plate count (HPC), assimilable organic carbon (AOC), 322–323
Hexachlorobenzene (HCB) biodegradation, 1238–1252, 2963
Hexachlorocyclohexane (HCH) biodegradation, 1238–1252
Hexachlorodimethylsulfone, 605–606
Hexachlorophene bioremediation, 703–712
Hexacorallia, 1116
Hexadecane biodegradation, 2403–2416, 3147–3155
Hexahydrotrinitriotriazine (RDX) biodegradation, 706–712, 916–917, 923–924, 1979–1993, 3410
Hexanitrohexaazaisowurtzitane biodegradation, 1979–1993
Hexoses, 2716
High hydrostatic pressure (HHP) vs. microbial inactivation, 1552–1563
High Melting Explosive (HMX), 706–712, 923–924
High performance liquid chromatography (HPLC), 252–253, 1853, 2359, 2463
High-rate algae ponds, protozoa vs., 2333
High-rate clarification, 2017
High temperature subsurface environment, 2047–2057
Hippea, 1517–1529
Hippophae, 2209
Hirsutella, 1710
Histogram equalization, image analysis, 1679
Histoplasma, fungi, 1368
HIV (See AIDS/HIV)
Hoffman modulation contrast microscopy, 1675
Holoenzymes, 3039
Holophaga, 533, 539, 1320, 2549
Holosticha, 2595–2604
Home water treatment devices, 1550–1551, 1563–1575
Homeophasic theory, cold-adapted microorganisms, 875
Homeoviscous adaptation theory, cold-adapted microorganisms, 875, 887
Homoacetic acid, activated sludge, 741–748
Homoacetogenesis, flooded soils, 1320–1321
Homoanatoxins, 977
Homologous recombination, 1197–1199
Homoserine, 2716
Hopane biodegradation, 696–712, 2403–2416, 2403
Hopanoids, 1818–1824
Horizontal gene transfer, 1195, 1201–1203, 1446
Horizontal or environmental infection, 1118
Hormone herbicides, biodegradation, 2385–2393
Hormothamnin, 977, 1852
Host, 1113
Hot environments (See also Desert environment), 1576
  high temperature subsurface environment, 2047–2057
Hot tubs (See Spa and hot tub microbes)
Human bacterial flora, Clostridium, 861–871
Human caliciviruses, 1586–1598
Human health outcomes, in risk assessment, 2742–2744
Human rotavirus (HRV), 95
Humic acid, humic substances, 1750
  biofilms and, 86, 87, 88, 114
  composting and, 915
  floc formation and, 57
  in surface waters, 928, 928
Humicola (See also Thermomyces), 881, 3002
Humin, in surface waters, 928
Hyalella, 1065
Hyandtoinases, 1185
Hybridization, 2922
  activated sludge foam monitoring and, 4–7
  adenoviruses and, 97
  Archaea and, 247, 279
  astroviruses and, 333–334
  bacterial counts using, 29–31
  biochips, biochip testing and, 446
  fluorescence in situ (See Fluorescence in situ hybridization)
  fluorescent probes and, 1335–1336
  forward sample array, 2731–2732
  microarrays for, 1970–1971
  oligonucleotide probe, 2497–2499
  rotaviruses and, 2769–2770
  rRNA targeted probes for, 29–34
  subtractive, 1451–1452
Hydgenophaga, 1490
Hydraulic head, water tension, suction, biofiltration, 592
Hydraulic residence time (HRT), 49, 2857
Hydrocarbons
  aerobic degradation in, 2404–2411
  anaerobic degradation of, 2411–2413
  bacterial genera to degrade, 2404
  biodegradation of, 694–701, 713–719, 2289–2300, 2402–2416, 2930, 3211–3215, 3405–3410
  cold-adapted microorganisms in, 872–885
  composting and, 917–919
  desulfurization and, 1046–1047
  fuel oxygenate biodegradation, 1994–2000
  fungal genera to degrade, 2405
  high temperature subsurface environment and, 2055
  hydrophobicity and, 1599–1604
  marine sources for, 1853–1855

marine/ocean environments and, 715–719
microbially enhanced oil recovery (MEOR), 2421, 2424–2425
microbially influenced corrosion (MIC) and, 2423–2424
mousses of, for biodegradation, 2413
neuston microbiology and, 2137–2139
petroleum reservoirs and, 2416–2426
sampling of, 3073
soil contamination and, 700–735
sulfate-reducing bacteria (SRB) in, 2419–2420, 2422–2424, 2428–2429
in surface waters, 928
surfactants vs., 2411
toxic solvents and, 3147–3155
vapors of, bioremediation in, 697–698
wetlands and, 3405–3410
Hydrochlorofluorocarbons (HCFCs), 3188–3189
Hydrodesulfurization (HDS), 1042
Hydrodynamic testing, adhesion in biofilms, 117–118
Hydrodynamics of periphyton, 2363
Hydrogel matrix of biofilms, 81
Hydrogen (See also Nutrients and energy sources)
activated sludge and, 741–748
Archaea and to produce, 273–274
in groundwater, 1672
hydrothermal vents and, 1611, 1618
hyperthermophiles and, 1633
in marine/ocean environment and, 1697–1709, 1854–1855
municipal solid waste landfills and, 1760
subsurface microbial communities and, 1454–1455, 1464–1466
Hydrogen cyanide (HCN), 457
Hydrogen oxidizing soil microorganisms, 2890
Hydrogen peroxide, 582, 603, 1387, 2932
Hydrogen sulfide, 2427, 3193
biofiltration vs., 589, 598, 599–600
bioleaching and pathway in, 637
bioremediation and, 708
in caves and mines, 823
hydrothermal vents and, 1618
municipal solid waste landfills and, 1763–1764
phototropism in, 2473
sulfate-reducing bacteria (SRB) and, 3092
Hydrogenases, sulfate-reducing bacteria (SRB), 468–475
Hydrogenobacter, 3111–3116, 3143–3147
Hydrogenophaga, 439, 1996, 3314–3319
Hydrolases, 269, 1210, 2273, 2903–2910
Hydrologic studies, microbial tracers in groundwater, 3198–3199
Hydrolysis
activated sludge processing and, 22–26
biofilms and, 87
flooded soils and, 1319–1320
phosphorus cycling and, 2450–2452
Hydrophilic forces, 2004
Hydrophilic materials, biofiltration, 591–592
Hydrophilic microorganisms, adhesion in biofilms, 100
Hydrophobic materials, biofiltration, 591–592
Hydrophobic microorganisms, adhesion in biofilms, 100
Hydrophobicity, 1599–1604
acid–base (AB) surface energies in, 1600–1601
activated sludge and, 1–7, 44
anaerobic granulation and, 238, 241
assay methods to measure, 1599
biofilms and, 2004, 2005, 2007–2008
contact angle measurement for, 1600–1601
electrostatic forces in, 1599–1604
floc formation and, 57, 2004, 2005, 2007–2008
free energy balance in, 1600–1601
Lifshitz van der Waals forces and, 1600–1601
microbial adhesion to hydrocarbons (MATH) for, 1599–1600, 1601–1604, 2007–2008
microbial adhesion to solvents (MATS) for, 1600, 1601–1604
thermodynamic analysis of, 1600–1604
viruses in soil and, 2098
Hydrostatic pressure (See also High hydrostatic pressure), hydrothermal vents, 1626
Hydrothermal vents (See also Geothermal environments), 289, 1604–1628
abundance and distribution of microorganisms at, 1620
Archaea and, 278, 287–288
bacteria at, 1620–1622
bacterial mats and, 1618
biomineralization and, 678
carbon dioxide and, 1611
chemoautotrophs and, 1606–1609
crustaceans and, 1619
cultivation and isolation of microbes from, 1611–1614
deep sea euryarcaheotic group (DSEG) and, 1611
distribution of vent fields of, 1618
diversity of, 1625
endosymbionts and, 1606–1609
endosymbiosis and, 1117–1118
epibionts and, 1609–1610
fluid composition at, 1618
genera and species of, 1619
genetic elements and, 1625–1626
hydrogen and, 1611
hydrostatic pressures and, 1626
hydrothermal processes in, 1618
hyperthermophiles and, 1628–1646
inert surfaces of, 1618
invertebrate-associated bacteria of, 1619
low temperature habitats and, 1610–1611
marine benthic group (MGIII) and, 1611
marine crearchaeotic group I (MGI) and, 1611
marine euryarchaeotic group (MGII) and, 1611
marine hydrothermal vent groups (MHVGs), 1614
methanogenesis in, 1920
methanotrophs and, 1606–1609
minerals and, 1618
miscellaneous euryarchaeotic group (MEG) and, 1611
molluscs and, 1619
nonsymbiotic mesophiles in, 1618–1619
nutrients and, 1618
oxygen and, 1610
pH levels and, 1618
phylogenic analysis of, 1614
Polychaetes and, 1619–1620
prokaryotes in, 1617–1628
psychrophilic bacteria and, 1617–1628
subvent biosphere of, 1614–1615
sulfur oxidizing bacteria in, 1610
surrounding waters, mesophiles in, 1618
temperature and, 1619–1620
thermophiles and, 3140–3147

Hydrothermal vents (See also Geothermal environments), (*continued*)
thioautotrophs and, 1606
tungsten and, 1706–1707
Vestimentifera symbionts and, 1619
Hydroxides, biomineralization, bacterial, 670–681
Hydroxy carboxy muconic semialdehyde, biodegradation, 2295–2300
Hydroxy formylbenzothiophene (HFBT), 1043–1045
Hydroxyalkanoates, activated sludge, 73
Hydroxybenzoic acid, biodegradation, 2295–2300
Hydroxyl radicals, 2278, 3183
Hydroxylamine oxidoreductase (HAO), 9–14
Hydroxylaminobenzoate, biodegradation, 1423–1429
Hydroxylase, 435, 2290, 2297–2300
Hydroxylation, 2293–2300
Hydroxyls, biomineralization, 671
Hydroxymuconic semialdehyde, biodegradation, 2295–2300
Hydrozoa, 1116
Hydrurus, 2362
Hygiene, bacteria in home/residential environment, 342
Hymenobacter, 1026, 1028
Hymenogaster, 2124–2132
Hymenolepis, 3399
Hymenoscyphus, 2913, 2915
Hyocholic acids, 1257
Hyodeoxycholic acids, 1257
Hyperparasitoids, 1115
Hypersaline environments (See also Halophiles), 1512–1529
aggregates and consortia in microbial mats, 173
alkaline saline habitats and, 1510
Antarctic hypersaline lakes, 1510
Antarctic soils and, 1027
Archaea and, 262, 276–293
bacteriophages and, 389
cyanobacteria and, 963
Dead Sea, 1509
desert environment and, 1576–1577, 1580–1582
enteroviruses and, 1170
extremeophiles and, 1232–1237
fermentation in, 1525
Great Salt Lake, 1509–1510
halophiles and, 1501–1529
methanogenesis in, 1525
microbial ecology of, 1514–1515
organic matter in, 1513–1514
osmotic adaptations to, phototrophic bacteria, 2483–2486
pH levels in, 1513
purple and green phototrophic bacteria in, 2470–2480
salt deposits, evaporites, rock salts, 1510–1511
salted foods, 1511
solar lakes, sabkhas, pools and lagoons of Sinai, 1509
solar salterns, 1508–1509, 1511
Hypersensitivity pneumonitis (HP), fungi, 1369, 1374–1375
Hyperthermophiles, 1233–1235, 1628–1646
aerobic processes and, 1633
aggregates and consortia in, 1642
anaerobic processes and, 1633
Archaea and, 246–259, 266, 277–293
autotrophs as, 1633
biofilms and, 1642
carbohydrates and, 1641–1642
carbon and, 1633
carbon dioxide and, 1633
cultivation of, 1628–1633
ecology of, 1641–1642
Embden–Meyerhof (EM) pathway in, 1633
Entner–Doudoroff (ED) pathway in, 1633
genetic elements of, 1639–1641
hydrogen and, 1633
hydrothermal vents and, 1604–1628
interspecies interaction at high temperatures and, 1642
isolation of, 1628–1633
lithotrophs as, 1633
metabolism in, 1633–1637
nutrients and, 1633
pH levels and, 1628
polysaccharides and, 1641–1642
sulfate-reducing bacteria (SRB) and, 3090–3091
sulfur and, 1633
temperatures and, 1642
transcription and translation in, 1637–1639
tricarboxylic acid (TCA) and, 1633
tungsten and, 1706–1707
Hyperthermus, 1630–1646
Hyphae, 2646–2647, 2889
Hyphochytriomycota, 2912, 3353–3364
Hyphoderma, 2914
Hypholoma, 2915
Hyphomicrobium, 3111–3116
activated sludge and, 35–38, 71
biofilms and, 90, 572
identification of isolates in, 1958
metal biodegradation and, 1884, 1907
Hyphomonas, 90, 2549
Hyphomycetes, 3353–3364
Hysterangium, mycorrhizae, 2124–2132

I

Ice (See Snow and ice environments)
Identifying microbial isolates, 1958–1967
BIOLOG and, 1961–1962
DNA analysis and, 1960–1961, 1964–1965
extracellular polysaccharides (EPS) and, 1960
fatty acid methyl ester (FAME) analysis in, 1962
genetic analysis in, 1964–1965
most probable number (MPN) technique in, 1959
obtaining isolates for, 1959–1961
physiological/metabolic testing in, 1961–1962
structural components and, 1962–1964
viable but nonculturable (VBNC), 1960
visual traits and, 1961
Idiopathic pulmonary hemosiderosis (IPH), 185–186
Igneococcales, 1233, 1614, 1629–1646
Igneous rock
lithotrophs and, 1826
microbial communities of, 1661–1673
Image analysis, 1674–1688
acquisition of image in, 1677
analysis of image in, 1676–1677
applications for, 1685–1687
binary image enhancement in, 1682–1683
biofilms and, 2340
calibration of, 1684–1685
cameras for, video, digital, 1676
contrast in, 1679
deconvolution in, 1679–1681
difference imagery in, 1683–1684
field and object parameters for, 1684
filtration in, 1679
flow cytometry and, 1944–1958
fluorescent probes/labels in, 1676
histogram equalization in, 1679
microscopy in, 1674–1675
presegmentation processing in, 1677–1681
primary image in, 1677

processing of image in, 1676–1677
replication and sampling intensity in, 1685
resolution in, 1785
restoration of image in, 1785
segmentation/thresholding in, 1681–1682
shading corrections in, 1677–1689
Image processing systems, 1676–1677
Immobilization (See also Adhesion), 105–111
Archaea and, 273
biofilms and, 100
Derjaguin–Landau–Verwey–Overbeen (DLVO) theory of, 106
enzyme activity and, 2903
motile bacteria and, 110–111
polymers and, 109–110
remobilization in, 2965
rhizosphere and, 2719–2720
soil microorganisms and, 2887
substrata makeup vs. 106–109
sulfur cycle and, 3107–3108
Immobilized cell reactors, chemical warfare and, 847
Immune disorders (See also AIDS/HIV), 1152, 2065–2083
Immune electron microscopy (IEM), rotaviruses and, 2765, 2767
Immunity, viral, 2747
Immunoassay and, 401–402, 1131–1132
Immunofluorescence
activated sludge foam monitoring and, 4–7
adenoviruses and, 98
bacterial counts using, 27–29
proteomes and proteins, 309
Immunoglobulin E (IgE), in allergies and illness and, 1359–1366
Immunomagnetic capture (IMC), rotaviruses and, 2771
Immunosuppressive drugs, fungi and production of, 1369
Impaction type bioaerosol samplers, 393–396
In-line coagulation, water treatment systems and, 2018
In-well bioreactor, 695
Inanidrilus, 1118
Inclusions in soil microorganisms and, 2884–2885
Incubation, chloroform fumigation type, 666–667
Indenopyrene bioremediation and, 697–712
Indexes, 355, 356–358
Indicator organisms
bacteriophages as, 354–363
Bacteroides fragilis-infecting bacteriophages as, 356–358, 378–379
coliphages as, 379–380, 384–385
F specific RNA bacteriophages as, 356
somatic coliphages as, 355–356, 377–378
Indigo fermentation, alkaliphiles and, 219–220
Indolacetic acid, biodegradation and, 2385
Industrial biofilms, 555–556, 582–584, 619–632
Industrial biofouling, 619–632
Industrial environment
bioaerosols in, 416–425
biofiltration in, 588
biosurfactants/bioemulsions and, 765
Infections, nosocomial infections (NI), 2235–2250
Infectious airborne bacteria (See also Bioaerosols), 1689–1697
Burkholderia in, 1695–1696
gram-negative bacteria in, 1694–1695
infectious dose in, 1690–1691
Legionella, 1788–1806
Mycobacterium in, 1691–1693
Nocardia in, 1693–1694
prevention and control measures for, 1696
reservoirs and sources for, 1690
Rhodococcus in, 1694
transmission of, 1690
Infectious dose, 1690–1691
Infiltration percolation vs. protozoa, 2333
Influenza, 2743
Information Collection Rule (ICR) protocol, bacteriophages and, 376
Infrared spectrometry, conditioning films and, 934–935
Inipol EAP22, 699
Inline filtration and, 2708
Inoculation/infection
mycorrhizae and, 2129–2130
for nitrogen fixation and, 2202–2204, 2206–2207, 2209
Inocybe, 2124–2132
Inonotus, 2646–2647
Inorganic acids, bioweathering and, 3366
Inorganic contaminants, bioremediation and, 707–708
Inositol, microbial protection from, 432
Inoviridae/Inovirus, 366–374
Insecticides
biocontrol vs., 450–465
biodegradation and, 1421
biofilms and, 88
biopesticides, 351
biotechnology and, 1447–1448
composting of, 916–917
microbial, 1709–1717
regulation of, 2693–2707
Insects
bioluminescence in, 1831
endosymbiosis and, 1114–1115
resistance to microbial insecticides and, 1714–1717
viruses vs., 1711
in water treatment systems, 1723
Insertion elements, DNA, 1199
Insulin, 1384
Integrated Microbial Database (IMD), 1333
Integrated pest management (IPM), 462
Integration, genetic, 1199
Integrons and, 1201
Inteins, Archaea and, 271
Intellectual property, biotechnology, 1861
Interaction studies, toxicity testing and, 3225
Interfacial free energies, adhesion in biofilms and, 122–124
Interferon, 1384
International Water Association (IWA) model, activated sludge and, 14–26, 49
Interplanetary transfer of life and, 2994–2995
Interpro, 1450
Intracellular endosymbiont/intracytobiont, 1113
Intracytoplasmic membrane (ICM), activated sludge denitrification and, 9–14
Intrinsic bioremediation, 695
Invertebrates
algal turf scrubbing (ATS) and, 215–216
water treatment systems vs., 1718–1733
Iodamoeba, 1136
Iodine, 3266–3267
Iodophenyl nitrophenyl phenyltetrazolium chloride (INT), 1783
Ion exchange water treatment systems, 1567, 2018
Ionic environment

Ionic environment (*continued*)
enzyme activity and, 2900–2901
soil microorganisms and, 2060
Ionic strength, viral, 2099
Ionization, viruses vs., 3264–3265
Ionotrophy, biomineralization and, 672
Iosborneol, 980
Iron, iron cycle, 1878, 1882–1893, 1902–1913, 2961
algae biotechnology and, 200
bacteriogenic iron oxides (BIOS), 1407
biofilms and, 1085–1086
bioleaching and, 632–643, 646
biomineralization and, 674–79
bioremediation and, 714
cyanobacteria and, 969–970
flooded soils and, 1325–1326
Gallionella and, oxidation by, 1401–1408
hydrothermal vents and, 1618
in marine/ocean environment, 1701–1704
municipal solid waste landfills and, 1763–1764
nitrogen fixation and, 2213–2215
oxygen and, 2277, 2278, 2283–2284
petroleum reservoirs and, 2430
phytosiderophores in, 2717
soil microorganisms and, 2887
subsurface microbial communities and, 1454–1455, 1464–1466
sulfate-reducing bacteria (SRB) and, 3089
in surface waters, 928, 928
in wetlands for wastewater treatment, 3393
Iron bacteria, Gallionella as, 1401–1408
Iron oxidizing/reducing bacteria (IRB)
in groundwater, 1665, 1667–1669
in petroleum reservoirs and, 2430
soil microorganisms and, 2890
Iron salts, activated sludge foaming and, 6–7
Iron sulfide, sulfate-reducing bacteria (SRB) and, 471–475
Iron sulfur centers, aerobic respiration and, 152–153
Irrigated soils, 1316–1333
IS1071 insertion element, 1200
ISO, bacteriophages and sampling, 376–378
Isoalkanes biodegradation and, 2403–2416
Isobutane, 2427
Isobutyric acid, 228
Isochromatium, phototropism in, 2477–2489
Isolates, identification of, 1958–1967
Isomerase, 1177–1189, 3207–3210
Isomorphism, in minerals, 633
Isoparaffin biodegradation and, 2402–2404
Isoprenoids, bioplastics and, 682–692
Isopropyl methylphosphonate (IMPA), chemical warfare and, 850
Isopropylbenzyne bioaugmentation and bioremediation in, 435
Isorenieratene, in paleolimnology, 2307–2317
Isosphaera, 3145–3147
Isospora, 1722–1723, 1734–1737, 2329–2337, 3277–3287
Isothiazolone, 605, 606
Isotope dilution studies
flooded soils and, 1326–1327
in groundwater, 1669–1671
subsurface microbial communities and, 1456–1464
viruses and, 3305
wastewater/sewage treatment and, 3341
Isotope ratio mass spectrometer (IRMS), 90
Isotropic semivariance, soil microorganisms and, 2897
Isovaleric acid, alkaliphiles and, 228
Iturin in biosurfactants/bioemulsions and, 757–759
Ixodes, endosymbiosis and, 1115, 1123

J

Janua, biofilms and, 614
Jet fuel, composting of, 916–917
Juncus, 2541–2548
June Grass, harm/in harmful algal blooms (HABs) and red tides, 2689–2693

K

Karenia, harm/in harmful algal blooms (HABs) and red tides, 2685–2693
Karlodinium, 2431
Karsts (See Caves and mines)
Karyomastigont, 1124
KEGG, 1450
Keratin, 1447
Keto myxol methylpentoside, in paleolimnology, 2307–2317
Ketodeoxycholic acid, alkaliphiles and, 228
Ketone, 929, 3412
Ketoreductase, 1185
Kick-plate boundary, 829
Kinase, 3207–3210
Kinetic limulus assay with resistant/parallel line estimation (KLARE), 1131
Kinetics of growth
activated sludge processing, 17–18
ambient concentration of nutrients and, 1745–1746
Arrhenius equation for, 1752
bacterioplankton and, 2529
basic concepts of, 1738–1741
biological factors affecting, 1750–1752
buffering capacity in, 1749
catalytic properties of abiotic soil components and, 1750
collision frequency and, 1741–1745
community composition vs., 1751–1752
constants, as a measure of S, 1740–1741
diffusion of gases through soil and, 1749
diffusion of solutes through soil, 1748–1749
doubling time in, 2529
environmental factors vs., 1752
extracellular enzymes and, 1750
first order rate constant in, 1738
general rate constant in, 1741–1742
geometric increase in population in, 1738
growth and uptake parameters in, 1751
growth rates, 1745
leakage rates, 1745
limiting factors to, 1751
liquid diffusion coefficient in, 1749
Michealis–Menten plots in, 1740–1741, 1743
microbial processes in soil, 1748–1757
microgravity environment and, 2997–3001
mixed substrate growth, 1874–1875
models of, 1753–1755
Monod plots in, 1740–41, 1743, 1753–1754
nutrient accumulation and, 1743–1744
nutrient acquisition ability and, 1745
oligotrophic bacteria and, 2274
polar phytoplankton and, 2559–2563

population size and, 1750–1751
problems solved by, 1745–46
respiration data for, 1753
soil physical factors vs., 1748
sorption and, 1749–1750
specific affinity and, 1742–1743
specific bacterial growth rates in, 3344–3345
steady state in, 1738–1740
Stokes–Einstein equation in, 1749
substrate induced growth response (SIGR) in, 1750
substrate induced respiration (SIR) in, 1750
temperature and, 1752–1753
theoretical analysis of cytoarchitectural requirements and, 1745
3/2 power kinetics in, 1755
toxicity testing and, in soils, 3161
turnover time in, 2529
volumetric bacterial growth rates in, 3344
wastewater/sewage treatment and, 3337, 3339–3342
wastewater/sewage treatment and, single species, 3347–3350
water content/moisture vs., 1753
Kitchens, bacteria in home/residential environment and, 338–340
Klebsiella, 386, 2547, 2550, 2552, 3089, 3102
bioaerosols and, 3321, 3322, 3326
biofilms and, 580, 582, 621, 1209, 2341–2356
coliform bacteria and, 895–914
genetically engineered microorganisms (GEMs) and, 1282
genetically modified microorganisms (GMMs) and, 1431
granular activated carbon (GAC) filters and, 1489–1496, 1569–1570
heterotrophic bacteria and, 1541–1552
infectious airborne bacteria and, 1689–1697
municipal solid waste landfills and, 1766
nitrogen fixation and, 2191, 2193–2201, 2212–2221
nosocomial infections and, 2236–2250
in source water, 2968
in spas and hot tubs, 2980–2984
in drinking water, 944, 945, 946
water purifiers vs., 1572–1573
Klebsormidium, subaerial communities and, 3056–3065
Klyveromyces, 880
Knockout studies, genomic, 1449
Koch, Robert, 2269
Kochia, biodegradation and, 2396
Korarchaeota, Archaea and, 260, 287, 295
Krebs cycle, 1125
Kyoto Protocol, desulfurization of fossil fuels, 1041–1051

L

La Nina, 2558
Labeling, flow cytometry and, 1953–1955
Labyrinthula, 2823, 2825
Laccaria, 2124–2132, 2914, 2915
Laccase, 1182–1189, 1387, 2647, 2903–2910
Lachrymaria, 2342–56, 2835–2844
Lactamase, 3207–3210
Lactarius, 2124–2132
Lactase, 1187
Lactate, 742–748, 3062
Lactic acid, activated sludge and, 741–748
Lactobacillus
adhesion in biofilms and, 127–128
biofilms and, 82
cold-adapted microorganisms and, 880
cold-shock response and, 886–895
freeze drying and, 1353–1354, 1357
high hydrostatic pressure (HHP) vs., 1555–1563
in marine/ocean environment, 1701
toxic solvents and, 3150
Lactococcus
biotechnology and, 3204
flow cytometric studies of, 1950
fluorescent studies of, 1835
Lactone, 929
Lactoperoxidase, 1185
Lactose death, viable but not culturable (VBNC) phenomenon and, 3251–3252
Laetiporus, 2647
Lagoons, 2333, 2476
Lakes (See Aquatic ecosystems; Surface waters),
Lambdoid phage, bacteriophages and, 370–373
Lamellibrachia, 1606
Laminar flow, adhesion in biofilms and, 117
Lampenflora, 836–843
Lamprobacter, 2477–2489
Lampropedia, 38
Land effluent percolation, 74
Land farming, 695
Landfills (See Municipal solid waste)

Lanthanides, 671, 1902
Large subunit (LSU), 1333
Larix, ectendomycorrhizal fungi and, 2125–2126
Larvae, macrofouling and, invertebrates, 613–615
Laser scanning microscopy (LSM), 1772–1788
Last Universal Ancestor (LUA), oligotrophic bacteria and, 2268
Late blight of potato spores in, 414
Lathyris, bioremediation and, 725
Laxatoxin, 977
Laxus, 1118
Leachate, landfill, 1767–1768
containment sites for, 1768
dissolved organic carbon (DOC) in, 1768
groundwater contamination and, 1768
heavy metals in, 1768
treatment of, 1768–1769
Leaching, microbial, 2890
Lead biodegradation (See also Heavy metals), 197, 199–200, 607, 1763–64, 1879–1893, 1902–1913, 2961
Leaf litter decomposer fungi, 3355–3358
Leakage rates, 1745
Leather processing, enzymes and, 1183
Leccinum, 2124–2132
Lectins, 570, 1782
Legionella, 313, 1345, 1542, 1689–1697, 1788–1806
bioaerosols and, 3328
biofilms and, 563, 566, 579, 621, 1209, 2341–2356
disinfection and, 1065, 1066
endosymbiosis and, 1123
flow cytometric studies of, 1951
granular activated carbon (GAC) filters and, 1492
heterotrophic bacteria and, 1548, 1550
infectious airborne bacteria and, 1696
nosocomial infections and, 2245
in rainwater roof catchment systems, 2680
sampling of, 2813
in source water, 2968, 2969–2978
in spas and hot tubs, 2980–2984

Legionella, (*continued*)
in drinking water, 945, 3199
Legume rhizobial symbiosis, 1119–1120, 2202–2208, 2786–2787
Leishmania, endosymbiosis and, 1126
Lemna, 973
Lepidoptera toxin, biocontrol use of, 2700
Leptolyngbya
photoautotrophic, in low-light habitats, 839–843
in snow and ice environments, 2876
subaerial communities and, 3059
Leptoplina, endosymbiosis and, 1121–1122
Leptosphaeria, 406, 413, 3360
Leptospira, 1806–1817
in rainwater roof catchment systems, 2680
in spas and hot tubs, 2980–2984
Leptospirillum
bioleaching and, 632–642
biomineralization and, 675
metal biodegradation and, 1904
nitrification and, 2149
Leptospirosis, 1806–1817
Leptothrix, 1401, 2283
biofilms and, 572
biomineralization and, 675, 676
filamentous forms of, in activated sludge, 1291–1306
metal biodegradation and, 1884, 1897, 1904
Leptotrombidium, 1122
Leucaena, 2785
Leuconostoc, 1871
biofilms and, 1221
high hydrostatic pressure (HHP) vs., 1556–1563
Leucoprinus, 2914
Leucothrix, filamentous forms of, in activated sludge, 1302–1306
Leucythophora, 2649
Levansucrase, in soil, 2903–2910
Leveillula, biocontrols and, 454
Leviviridae/Levivirus, 364–374, 364
Lewisite (See Chemical warfare)
Lichenomycota, 2913–2914
Lichenothelia, 1107–1112
Lichens, 2891
Antarctic, 1101–1106
biodeterioration of mineral materials and, 522–523, 524
endolithic, 1100–1112
fungi and symbiosis and, 1367
in hot desert regions, 1107–1110
soil microorganisms and, 2914
subaerial communities and, 3056–3065
weathering and, 3364–3389
Lichenysin, biosurfactants/bioemulsions and, 759
Licmophora, sea ice and, 2837–2844
Licopene, in paleolimnology, 2307–2317
Life cycle, of protozoa, 2615
Lifshitz-van der Waals forces, 101–104, 123, 124, 936, 938, 1600–1601
Ligands, weathering, bioweathering and, 3380–3381
Ligase, 271, 3207–3210
Light nonaqueous phase liquids (LNAPLs), 507–516, 699
Lignin, 693, 1183, 1210, 1760, 2646–2647, 3021
biofilms and, 88
bioplastics and, 682–692
cold-adapted microorganisms in, 872–885
fungi vs., 1385–1386, 1385
Ligninase, 1385–1386
Lime softening (See also pH levels), 2017–2018, 2908–2909
enteroviruses and, 1173–1174
Limestone, biodeterioration of mineral materials and, 518–523
Limit of detection (LOD), 2811
Limit of quantitation (LOQ), 2811
Limonene biodegradation and, 2404
Limulus, bottled water and, 800
Limulus amebocyte lysate assay (LAL), 1130–1131
Linweaver–Burk transformation, 2901
Lipase, 226, 1177–1189, 1212–1223, 1852, 2649, 2903–2910, 3021, 3207–3210
Lipid phase transitions, freeze drying and, 1356
Lipids, 1210, 2489, 3021
activated sludge and, 741–748
aerobic respiration and, 149, 151–152
alkaliphiles and, 137–138
Archaea and, 249–259, 271–272, 279–293
bacterioplankton and, 2519
biofilms and, 85–86, 114, 2960
biomarker use of (See Lipids as biomarkers, below), 663, 1817–1824
cold-adapted microorganisms and, 874–876, 887–888
in surface waters, 928, 928
Lipids as biomarkers, 663, 1817–24
applications for, 1820–1822
aquatic environment analysis and, 1821
aquifer analysis and, 1821
biomass and, 1818–1819
bioremediation and pollution analysis using, 1822
community composition and, 1819
composting analysis, 1822
metabolic activity, 1819–1820
nutritional/physiological status and, 1819
procedures for using, 1820
soil analysis and, 1821
Lipoconjugates, 1817–1824
Lipopolysaccarides (LPS), 109, 417, 1210, 1818–1824, 2882
biofilms and, 2960
biomineralization and, 671
cyanobacteria and, 975
Lipoproteins, biosurfactants/bioemulsions and, 757–760
Lipoteichoic acid, 109
Liquid diffusion coefficient in, 1749
Liquid impingement type bioaerosol samplers, 396
Lirceus, 3020
Lissoclinium, 975
Listeria, 338, 340, 1345
adhesion in biofilms and, 123
biofilms and, 577, 579, 582
biotechnology and, 3206
cold-adapted microorganisms in, 872–885
cold-shock response and, 886–895
in food, 3277–3287
high hydrostatic pressure (HHP) vs., 1555–1563
microbial insecticides using, 1714
municipal solid waste landfills and, 1766
Lithified materials, sampling of, 3074
Lithocarpus, 2125–2126
Lithotrophs, 1824–1829
aerobic respiration and, 151, 157
basaltic rock and, 1826–1828
biofilms and, 81–82
chemolithoautotrophy in, 1824–1826
Gallionella as, 1401–1408
groundwater and, 1827
hyperthermophiles and, 1633
igneous rock and, 1826
prevalence of, 1828
significance of, 1828–1829
weathering and, 1827
Litonotus, 2606
Littorella, 2548
Live/Dead kits, 90, 1783

Liver diseases, Aeromonas and, 1091–1099
Lobose amoeba in, 2616, 2617
Lockett, William, 74, 738
Long Duration Exposure Facility (LDEF), 1038–1041
Loriella, 838–843
Lotus, plant growth promoting rhizobacteria (PGPR) inoculation and, 350
Low light environment (See Caves and mines)
Lowest effect concentration (LEC), 3163
Lowest observed effect concentration (LOEC), 3163
Loxodes, microgravity and, 3000
Luciferase/luciferin
  bioluminescence and, 657–663, 1830–1838
  in wastewater/sewage, 3170
Lucinacea, hydrothermal vents and, 1606
Lucinoma, hydrothermal vents and, 1606
Ludzack–Ettiger process, activated sludge and, 78
Lutein, in paleolimnology, 2307–2317
Lux operons, in bioluminescent research, 658
Lyase, 1210, 2903–2910, 3207–3210
Lycopene, 2459
Lycopersicon, plant growth promoting rhizobacteria (PGPR) inoculation and, 350
Lycopodium, 3198
Lyme borreliosis, 1838–1849
Lymnaea, 972
Lyngbya, 210, 963, 972, 980
  aggregates and consortia in, 164, 170–173
  desert environment and, 1579
  nitrogen fixation and, 2193–2201
Lyngbyatoxin, 977
Lysates, rhizosphere and, 2715
Lysine, 2716, 2899
Lysis, activated sludge processing and, 23–26, 745–746
Lysogenic state in bacteriophages and, 371–373
Lytic state
  biocontrols and, 458–459
  bacteriophages and, 371–373

M

Machining, bioaerosols and, as byproduct of, 419–420
Mackinawite, 2961
Macroalgae, 2302–2306, 2464
Macrocyctis, 1921
Macrofouling, 613–616
Macroinvertebrates, in drinking water, 945
Macromolecule labeling, wastewater/sewage treatment and, 3342
Macromonas, 3111–3116
Magnesium, 1879–1893, 1902–1913, 2774, 2961
Magnetite, 671, 1464–1466
Magnetotactic bacteria, 678
Major histocompatiblity class (MHC), in allergies and illness, 1360
Majusculamides, 1852
malaria, 3399
male-killing bacteria, 1122
Maleylpyruvic acid, biodegradation and, 2295–2300
Mallomonas, 210, 2317–2327, 2467
Maltase, 2899
Maltose, 756, 2716
Maltose sensitive hemagluttinin (MSHA), 1448
Maltotriose, biosurfactants/bioemulsions and, 756
Mamestra, biocontrol use of, 2701
Manganese, manganese cycle, 1878, 1902–1913, 2961
  biomineralization and, 675–676
  cyanobacteria and, 969
  hydrothermal vents and, 1618
  metal biodegradation and, 1884
  oxygen and, 2277
  soil microorganisms and, 2887
  subsurface microbial communities and, 1454–1455
  sulfate-reducing bacteria (SRB) and, 3089
  oxygen and, 2283–2284
Mannanase, 1178–1189
Mannitol, 518, 2778–2789
Mannose, biosurfactants/bioemulsions and, 756
Mannosylerythritol lipids, biosurfactants/bioemulsions and, 755–756
Mantoneilla, photosynthetic pigments in, 2468
Mapping, self-organizing maps in, 1007
Marburg virus, bioterrorism and, 777–778
Marichromatium, 2474, 2477–2489
Marine bacterial isolate (MBI), 715
Marine benthic group (MGIII), 1611
Marine biotechnology (See also Marine/ocean environment), 1850–1863
  adhesion in, 1855–1856
  aquaculture and, 1856–1857, 1859
  biocorrosion in, 1855–1856
  biofilms in, 1855–1856
  biofouling, 1856
  biomass protein from, 1851
  biomaterials and, 1853
  biopolymers and, 1853
  bioprocesses and, 1855–1856, 1858–1861
  bioremediation and, 1855
  carbon dioxide sequestration in, 1855
  ecology of, 1858
  energy production from, 1853–1855
  enzymes and, 1853
  genetic engineering and, 1856, 1858
  health and safety in, 1857–1858
  intellectual property and technology transfer, 1861
  legal aspects of, 1861
  natural products of, 1851–1853
  polysaccharides and, 1853
  wastewater/sewage treatment and, 1855
Marine crearchaeotic group I (MGI), 1611
Marine euryarchaeotic group (MGII), 1611
Marine hydrothermal vent groups (MHVGs), 1614
"Marine snow," 89, 2002
Marine/ocean environment
  actinopoda in, 2618
  aggregates and consortia in, 168–172
  ammonium in, 1698–1701
  amoebas in, 2616, 2617
  Antarctica, 2557–2558
  Archaea in, 276–293
  Arctic Ocean and, 2556–2558
  available dissolved organic carbon (ADOC) and, 1086–1087
  bacteriophages as pollution indicators in, 355
  bacterioplankton in, 2519–2539
  biofilms in, 1081–1091
  biofouling in, 623, 610–619
  biomass of, fungi in, 1397
  bioremediation and, 712–722
  blooms in (See Harmful algal blooms and red tides)
  calcium in, 1707
  carbon and, 1697–1709
  carbonic anhydrase and, 1706
  chlorine in, 1707
  ciliates in, 2619–2620
  Clostridium and, 864–871

Marine/ocean environment (*continued*)
cold-adapted microorganisms in, 872–885
colloids and, 2002
copper in, 1706
denitrification in, 1010–1019
diatoms and sedimentation in, 1059–1061
diazotrophy and diazotrophic bacteria and, 2541–2547, 2550–2551
dimethylsulfide (DMS) and, 1701
dimethylsulfoniopropionate (DMSP) and, 1701
dissolved organic carbon (DOC) and, 1086–1087
disturbance of biofilms in, ecological impact of, 1088
emergent plants and, 2539–2549
endosphere microbiota of, 2548–2549, 2552
endosymbiosis and, 1115–1116
Enterococci in, 1264–1278
eutrophication of algae in, 1189–1194
extracellular polymeric substances (EPS) in, 1081, 1083–1084
fecal contamination in, 1256–1264
ferrodoxin in, 1702
flagellates in, 2618–2619
floc formation and, 2002
foraminifera in, 2616–2617
fungi in, 1394–1400, 2539–2540
Giardia in, 1252–1256
glutamate dehydrogenase (GDH) in, 1698–1701
glutamine synthetase (GS) in, 1698–1701
green fluorescent protein (GFP) studies for, 1498
halophiles, 1501–1512
heterotrophic bacteria and, 1541–1552
hydrocarbons and, 715–719
hydrogen and, 1697–1709
hydrothermal vents, 1604–1628
inorganic nutrients in, 1697–1709
iron and, 1085–1086, 1701–1704
lipid biomarkers of, 1821
"marine snow" in, 89, 2002
meroplankton, 1863–1869
methanogenesis in, 1913–1923
molybdenum in, 1704–1705
nickel in, 1706
nitrates in, 1698–1701, 1702
nitrites in, 1698–1701
nitrogen, nitrogen cycle in, 1010–1019, 1085–1086, 1697–1709, 2176–2188
nitrogen fixation, 2211–2221, 2539–2554, 2828–2829
oil spills and, 693–701, 713, 715–719, 916–917, 2402–2416, 2931
oligotrophic bacteria and, 2271, 2272
osmotic balance and, 1707
oxygen and, 1697–1709, 2277–2288
oxygen minimum zones (OMZs) in, 1016, 2162–2163
particulate organic carbon (POC) and, 1086–1087
pH levels and, 1085–1086
phosphorus and, 1085–1086, 1190
phosphorus cycling in, 1697–1709, 2448–2453
photoheterotrophic bacteria in, 1087–1088
phyllosphere of, 2539–2540, 2824–2825
plankton and, 1082–1083, 2502–2518
plant microbe interactions in, 2539–2554
polar phytoplankton in, 2554–2565
potassium in, 1707
pressure at depth, 1626
primary production in, 2566–2575
Prochlorococcus in, 2576–2593
protozoa and, 1252–1256, 2613–2625
purple and green phototrophic bacteria in, 2470–2480
rhizosphere of, 2540–2548
salt bridges and, 1707
sampling of, 2812–2815
sea ice microorganisms, 2833–2844
seagrass communities in, 2550–2554, 2822–2833
Secchi depth in, 1190
sedimentary bacteria in, 2827–2828
sedimentation in, 1193–1194, 2540–2548
sodium in, 1707
solar radiation and, 1192–1193
stratification within, 715
Streptococci in, 1264–1278
submerged aquatic vegetation (SAV) in, 1190
submerged plants in, 2549–2552
subsurface microorganisms and, 1080
sulfate-reducing bacteria (SRB) and, 1697–1709, 2547–2548, 2551
sulfur and, 1085–1086, 1697–1709
temperature and, 1193
thermophiles and, 3140–3147
trace elements in, 1701–1707
tungsten in, 1706–1707
vadose zone microbiology and, 3236–3246
viruses and, 1252–1256, 3300–3310
water turnover vs. algae in, 1193–1194
zinc in, 1705–1706
Zostera and, 2550–2554, 2822–2833
Marinitoga, 1515–1529, 1620–1628
Marinobacter, 1610, 1619, 1741
Marinococcus, 1506–1512
Marinomonas, 2549
Marshes (See also Marine/ocean environment), 1316–1333
fungi and, 3359–3360
in sediments and sedimentation, 2851–2852
Marteilia, 1857
Martian microbiology, space microbiology and, 2985, 2995
Mass balance equations, biofilms and, 2088–2089
Mass spectrography (MS), 820
biomass estimation and, 2656
conditioning films and, 933–934
detection of, 1132
microarray study of vs., 1974
proteomes and proteins, 309–310
Mass transfer, in biofilms, 2087
Massarina, 3359, 3360
Mastadenovirus, 93
Mastigella, 1125
Mastigocladus, 961, 962, 3142–3147
Mastogloia, salt production and, 2805–2809
Mathanoignis, 1524–1529
Matrix assisted laser desorption ionization mass spectrometry (MALDI MS), 309, 1132
Matrix assisted laser desorption/ionization TOF mass spectrometry (MALDI TOFMS), 933–934
Matrix, biofilm, 547–548
Mattesia, 1710
Mayorella, 2594–2604, 2605
Mechanical filtration, 1566
Mecoprop biodegradation and, 2394
Medicago, 1434

Medical biofilms, 554–555
Medical biosurfactants/bioemulsions and, 765–766
Megaplasmids, captured, 1446
Melanin, 1879
Meloidogyne, 455, 456, 459
Melosira, 206, 210, 2837–2844, 2837
Membrane filters (See also Heterotrophic bacteria), 1863
  bacteriophages and, 375–376
  biodegradable dissolved organic carbon (BDOC) and, 497–498
Membrane inlet mass spectrometry (MIMS), denitrification and, 1015, 1017
Membrane transport, alkaliphiles and, 138
Meningitis, enteroviruses and, 1151
Meningoencephalitis, amoebic, 1343–1350
Mentylobacter, flooded soils and, 1322
Mercury (See also Heavy metals), 605, 1878–1883, 1890, 1894, 1902–1913
  algae biotechnology and, 197
  biodegradation and, 694, 709, 1424–1429, 1446–1447, 1878–1883, 3402
  microbial methylation of, 1886, 1909–1910
  resistance of bacteria to, 608
  sulfate-reducing bacteria (SRB) and, 3089–3090
Mercury drop electrode analysis, conditioning films and, 932
Merismopedia, 1107–1112
Meroplankton, 1863–1869
Mesocyclops, 1718
Mesodinium, 2620, 2626–2632
Mesophiles
  composting and, 915–916
  hydrothermal vents and, 1618
Mesorhizobium
  endosymbiosis and, 1120
  nitrogen fixation and, 2201–2211
  soil salinity and, 2775
Metabolism
  anaerobic granulation and, 238
  anoxic, 9–11
  bacterioplankton and, 2521–2523, 2526–2528
  biodegradation and metabolic pathway evolution in, 1194–1207
  cometabolic reductive dechlorination, 508–509
  hyperthermophiles and, 1633–1637
  identification of isolates in, 1961–1962
  lipid biomarkers of, 1819–1820
  marine biotechnology and, 1850
  microgravity and, 3002–3003
  mixed organic pollutant, 1869–1877
  oligotrophic bacteria and, 2273
  periphyton and, 2359
  permafrost and, 2379–2380
  petroleum reservoirs and, 2427
  in sediments and sedimentation, 2848–2849
  soil microorganisms and, 2951–2956
  subsurface microorganisms and, 1079–1080, 2033
  wastewater/sewage treatment and, 3338–2239
  weathering, bioweathering and, 3375–2289
Metabolites, alkaliphiles and, 228
Metagenome analysis, soil microorganism and diversity in, 539
Metal biodegradation, metal cycling, 708–709, 1875–1901, 1902–1913, 3045, 3216
  adenosine triphosphate (ATP) and, 1878
  arsenic methylation and, 1885–1886
  bioacidification and, 1881
  bioaugmentation and, 1886
  biofilms and, 2960–2963
  bioleaching of, 641–650, 1881
  biomass as bioadsorbents of, 1879–1880
  carbon and, 1878
  catalyzed mineral formation, byproducts of, 1897
  cation classification in, 1902
  chelation of, by organic acids and siderophores, 1881–1882, 2717
  chemolithotrophy and, 1880
  chromium as example of, 1883–1884
  cold-adapted microorganisms and, 3216
  complexation of heavy metals and, 1894, 1902–1913
  death of microbes and, metal overstress, 1898
  enzyme activity and, 2909
  heavy metals and, 1878–1893
  ion composition of, 1878
  iron cycling in, 1903–1907
  manganese as, 1884, 1907–1909
  mercury cycling and methylation in, 1886, 1909–1911
  methylation and volatilization of, 1884, 1894
  microbial processes affecting, 1878–1880
  municipal solid waste landfills and, 1763–1764
  nitrogen fixation and, 2213–2215
  passive mineralization and, 1896
  pH levels and, 1878, 1879, 1880–1882
  phytometallophores, 2717
  phytosiderophores in, 2717
  precipitation of minerals and, 1894–1897, 1898–1899
  proton release and, 1880, 1896–1897
  quinones in, 1883
  redox transformations of, 1882–1886, 1896–1897, 1902–1913
  selenium methylation and, 1884–1885
  solubilization of, by microbes, 1880–1882
  sorption to cell walls, 1879, 1895–1896
  sulfate-reducing bacteria (SRB) and, 1898–1899, 3092–3093
  survival of bacteria in, 1897–1898
  uptake and accumulation of, by living cells, 1878–1879
  uranium cycling in, 1902–1903
Metal chelants, microbial protection from, 432
Metal ion extraction, 259–276, 632–642
Metal ions, viruses vs., 3267
Metal oxidation/reduction, 1446–1447
Metal reducing bacteria, subsurface microbial communities and, 3070
Metal removal, extracellular polymeric substances (EPS) and, 1229
Metal stressed environment (See also Metal biodegradation), 1893–1901
Metal sulfide dissolution, microbiological, 635–638
Metal sulfide oxidizing bacteria, bioleaching and, 634–635
Metallogenium, 632–642, 676, 1904
Metalloids, bioremediation and, 708–709
Metalloprotease, 3207–3210
Metallosphaera, 298, 645–650, 1235, 3144–3147
Metallothionein, algae biotechnology and, 198

Metallurgy, biohydrometallurgy, cold-adapted microorganisms and, 881–882
Metalworking fluids (MWFs), bioaersols and, 419–420
Metanosarcina, 1233
Metaphyton, 2357
Metarhizium, 881, 1710
Metazoa
activated sludge and, 72
enteroviruses vs., 1171–1172
in snow and ice environments, 2874
Meteorites, space microbiology and, 2985, 2994–2995
Methane and methanogenesis (See also Methanogens), 1075, 1913–1923, 2426, 3185–3187
activated sludge and, 738, 740–748
anaerobic oxidation of methane (AOM) and, 288–289
Archaea and, 259–276, 296–297
biodegradation and, 507–516, 694, 2292–2300, 2931
biofilms and, 83, 87
cold-adapted microorganisms in, 872–885
endosymbiosis and, in ocean waters, 1117–1118
energy recovery from landfill gases, 1767
floc formation and, anaerobic methanogenic granules in, 2002
flooded soils and, production of, 1319–1321
halophiles and, 1525
hydrothermal vents and, 1618
marine/ocean environment and, 1913–1923
methanotrophic bacteria, 1923–1936
methylotrophs and, 1923–1925
municipal solid waste landfills and, 1761–1771
nitrification and, 2163–2164
in sediments and sedimentation, 2846
soil biosphere and, 294, 296–297
soil microorganisms and, 2057
Methane monooxygenase (MMO), 1924–1936, 1937–1944, 3186
Methanobacillus, 745
Methanobacteriales, 1630–1646, 1915–1923
Methanobacterium, 1233, 1672
activated sludge and, 742–748
anaerobic granulation and, 232–245
flooded soils and, 1321
genomic studies and, 1448
metal biodegradation and, 1885, 1895
petroleum reservoirs and, 2419, 2429–2431
Methanobrevibacter
activated sludge and, 742–748
anaerobic granulation and, 233–245
flooded soils and, 1321
Methanocalculus, 1524–1529, 1917–1923, 2429–2431
Methanocaldococcus, 1917–1923
Methanochondroitin, Archaea and, 248–249, 248
Methanococcales, 1233, 1523–1529, 1630–1646, 1915–1923
Methanococcoides, 1917–1923
Methanococcus, 1233, 1235, 1524–29, 1622–1628, 1630–1646, 1917–1923, 2292, 3129, 3144–3147
Archaea and, 278–293
hydrothermal vents and, 1611
nitrogen fixation and, 2193–2201, 2214–2221
petroleum reservoirs and, 2429–2431
Methanoculleus, 1321, 1917–1923, 2429–2431
Methanogenesis (See Methane and methanogenesis)
Methanogenium, 1917–1923
anaerobic granulation and, 233–245
biotechnology and, 3205
flooded soils and, 1321
psychrophilic, 2640–2646
Methanogens, 1233–1235, 3185–3187
activated sludge and, 741–748
alkalithermophiles and, 3138
anaerobic granulation and, 238
Archaea and, 246–259, 262, 263, 277–293
halophiles and, 1523–1529
petroleum reservoirs and, 2429
soil borne, 296–297
subaerial communities and, 3057–3065
soil microorganisms and, 2892
Methanohalobium, 1515–1529, 1917–1923
Methanohalophilus, 1513–1529, 1917–1923, 2419, 2429–2431, 3130, 3137–3138
Methanol, 699, 3147–3155
Methanol dehydrogenase (MDH), methanotrophic bacteria and, 1927–1936
Methanolacinia, 1917–1923
Methanolobus, 1524–1529, 1917–1923
Methanomicrobiales, 233–245, 1321, 1523–1529, 1917–1923
Methanoplanus, 1915–1923, 2429–2431
Methanoplasma, 1895
Methanopyrales, 1523–1529, 1630–1646, 1917–1923
Methanopyrus, 1233, 1235, 1524–1529, 1611, 1622–1628, 1630–1646, 1917–1923, 3144–3147
Methanosaeta
activated sludge and, 742–748
anaerobic granulation and, 232–245
Archaea and, 297
biodegradation and, 509
flooded soils and, 1321
metal biodegradation and, 1896
Methanosalum, 1524–1529
Methanosarcina, 1525, 1895, 1917–1923, 3146
activated sludge and, 61, 742–748
anaerobic granulation and, 233–245
Archaea and, 248–249, 295, 297
biodegradation and, 509
biofilms and, 1221
nitrogen fixation and, 2193–2201
petroleum reservoirs and, 2429–2431
stress response and, 3032–3033
Methanosarcinaecea, 1917–1923
Methanosarcinales, 1321, 1523–1529
Methanospirillum
activated sludge and, 742–748
anaerobic granulation and, 232–245
metal biodegradation and, 1896
Methanothermobacter, 1917–1923, 3128–3140
Methanothermus, 295, 1234, 1235, 1630–1646, 3144–3147
Methanothrix, Archaea and, 297
Methanotrophic bacteria, 1923–1944
bioaugmentation and bioremediation in, 435
biofilms and, 82–83
hydrothermal vents and, 1606–1609
Methlybacterium, 1936–44, 3057–3065
Methyl alcohol (MeOH), biodegradation of, 1994–2000
Methyl chlorophenoxyacetic acid (MCPA), biodegradation and, 2385–2393
Methyl ester, in surface waters, 928, 928

Methyl parathion, bioremediation and, 705–712, 705
Methyl t buytylether (MTBE), biodegradation of, 439, 699, 1994–2000, 2410
Methylactobacterium, 1120
Methylation
fungi and, 1388
metal biodegradation and, 1884, 1894
biofilms and, 2963
in sediments and sedimentation, 2846
Methylene bisthiocyanate (MBT), 605–607
Methylene chloride, biodegradation and, 511–514, 1763
Methylisothiazolinone (MIT), 605
Methylobacter, 1323, 1924–1936, 3314–3319
activated sludge and, 35–38
biodegradation and, 1996
biofilms and, 2342–2356
psychrophilic, 2640–46
Methylocaldum, 1322, 1924–1936
Methylocella, 1924–1936
Methylococcus, 1322, 1323, 1924–1944
Methylocystis, 1322, 1924–1944
Methylomicrobium, 1322, 1924–44
Methylomonas, 1322, 1323, 1672, 1924–1944
Methylophaga, nitrogen fixation and, 2214–2221
Methylophilus, in groundwater, 1671
Methylosinus, 1924–1944, 2292
bioaugmentation and bioremediation in, 435–436, 439
flooded soils and, 1322
nitrogen fixation and, 2193–2201
Methylospaera, 1322, 1924–1944, 2640–2646
Methylotrophs, 1923–1925
Methyltrimethylene isothiazolinone (MTI), 605
Methylvaleric acid, alkaliphiles and, 228
Metopus, 1125, 3398–3399
Metsulfuron, composting of, 921–922
Michaelis–Menten equation, 1008, 1740–1741, 1743, 2901
Michaelsarsia, 2508
Micractinium, in wastewater stabilization ponds (WSBs), 3332
Microalgae, cold-adapted microorganisms in, 872–885
Microanalytical assay systems, 819
Microarrays (See also Biochips), 1968–1979
Microautoradiographic studies
activated sludge and, 69
biofilms and, 565
floc microbiology and, 56–57
Microthrix parvicella and, 3
Microbial adhesion to hydrocarbons (MATH), 1599–1604, 2007–2008
Microbial adhesion to solvents (MATS), 1600–1604
Microbial consortium, 3412
Microbial genome sequencing and annotation in, 1442–1443
Microbial insecticides, 1709–1717
Bacillus thuringiensis and, 1710–1717
bacteria in, 1711–1712
Cry toxins and, 1712–1717
efficacy of, 1710–1711
entomopathogens in, 1710
fungi in, 1710, 1711
insect resistance to, 1714–1717
production technology for, 1710
protozoa in, 1711
safety and specificity of, 1710
sustainable agriculture and, 1717
viruses in, 1711
Microbial leaching, 2890
Microbial loop, protozoa and, 2630
Microbial mats, 568–569
aggregates and consortia in, 161, 165–166, 170–173
Archaea and, 286–287
cyanobacteria and, 966–967
cyanobacteria in, 568–569
Gallionella in, 1406
hydrothermal vents and, 1618
hypersaline lakes, 173
in ice, 174–175
nitrogen fixation and, 2218
in septic tank systems, 2858
Microbial tracers, in groundwater, 3194–3202
Microbial volatile organic compounds (MVOCs), 1372–1383
Microbially enhanced oil recovery (MEOR), 2421, 2424–2425
Microbially influenced corrosion (MIC), 464–475, 555–556, 608, 2423–2424
biofouling and, 602–609
genetically engineered microorganisms (GEMs) and, 1228
nuclear waste storage and, 2257–2259
sulfate-reducing bacteria (SRB) and, 3092–3093
Microbiocides, biofouling vs., 602–609
Microchip-based PCR, 1134
Micrococcus, 137, 339, 1102–1106, 1501–1512, 3314–3319
activated sludge and, 64
alkaliphiles and, 139, 225
biofilms and, 580, 1215, 2342–2356
biomineralization and, 674
cold-adapted microorganisms in, 872–885
desert environment and, 1578
granular activated carbon (GAC) filters and, 1490
heterotrophic bacteria and, 1544, 1545
in permafrost, 2369
psychrophilic, 2641–2646
rhizosphere and soil microbes in, 346
subaerial communities and, 3057–3065
subsurface microbial communities and, 2035, 3065–3071
viable but not culturable (VBNC) phenomenon and, 3251
in drinking water, 944, 945
Microcoleus, 963, 965, 970, 971, 1109, 1507–1512, 2286
aggregates and consortia in, 170–173
in desert soils and biological soil crust (BSC), 1020–1023, 1579
salt production and, 2805–2809
subaerial communities and, 3060
Microcolonies
anaerobic granulation and, 237–238
biofouling and, 601–609
Microcyclus, 1490
Microcystin, 977, 987–995, 2340
Microcystis, 206, 207, 210, 972, 979, 980, 984–995, 1191, 1193
Microdomains, 1215
Microelectrodes, biofilm measurement, 90
Microfiltration (See also Filtration; Ultrafiltration), 2334
Microfungi, 1367
desert environment and, 1579
municipal solid waste landfills and, 1766
Microgravity, space microbiology and, 2985, 2994, 2996–3004
Microhabitats, soil microorganisms and, 2058–2059
Microlayer in neuston microbiology and, 2134–2135
Microlunatus, 38, 64
Micromonospora, 1102–1106, 1578
Micromycetes, in permafrost, 2369
Microneustons, 2140

Micropruina, 64
Microscopic particulate analysis (MPA) of in source water, 2972
Microscopy, 1674
bioaerosol samples and, 401
conditioning films and, 935
confocal laser scanning (See Confocal laser scanning microscopy), 1675
dark field–bright field, 1675
differential interference contrast (DIC), 1675
epifluorescence in, 1336–1338, 1675
floc formation and analysis, 2008
Hoffman modulation contrast, 1675
laser scanning microscopy, 1772–1788
phase contrast microscopy, 1674–1675
rotaviruses and, 2765, 2767
soil microorganisms and, 666
Microsensors, rhizosphere and soil microbes in, 349
Microsphaera, 64
Microspheres
adhesion in biofilms and, 128
as microbial tracer in groundwater, 3197–3198
Microsporidia, 1719, 2065–2083, 3277–3287, 3353–3364
Microsporum, 1368, 1373–1383, 2365
Microstrainers, 2013
Microthrix, 2–7, 1288–1306, 1311, 1312
Micrototal analysis, biochip testing, 446–447
Microtox assay, in wastewater/sewage, 3171
Microviridae/Microvirus, 365–374
Mildews, 1647
Military chemicals (See also Chemical warfare; Explosives), 706–707, 916–917, 922–924
Milk products, 1187
Millerite, 2961
Mineral fouling, 619
Mineral water, 796
Mineralization
biodegradation and, 2386, 2931
biofilms and, 82, 2961–2962
protozoa and, 2622–2623
rhizosphere and, 2719–2720
in sediments and sedimentation, 2844
in septic tank systems, 2859
soil microorganisms and, 2887
sulfur cycle and, 3107, 3109–3110
toxicity testing and, in soils, 3159
Minerals, 928
Antarctic soils and, 1026
biomineralization, bacterial, 670–681
in caves and mines, 823
hydrothermal vents and, 1618
municipal solid waste landfills and, 1763–1764
soil microorganisms and, 663–664, 2060
subsurface microbial communities and, interactions with, 1464–1466
Mines (See Acid mine drainage; Caves and mines)
Mining, biomineralization and, 677
Minimum data sets (MDS), 3165
Miscellaneous euryarchaeotic group (MEG), 1611
Mitochondrial origin, endosymbiosis and, 1125
Mixed liquid suspended solid (MLSS) ratio, 54
Mixed organic pollutants, bioremediation and metabolism of, 1869–1877
Mixed substrate growth, 1870
Mixotrophy, protozoa and, 2621–2622, 2629–2630
Mixtricha, 1124
Model viruses, 2101
Modeling, 355, 1002–1010
bioaerosol, 2107–2112
biofilm, 2083–2094, 2340
filtration, 2709–2712
genomic studies and, 1448
kinetics of growth and, 1753
pesticide degradation in soil, 2397–2398
protozoa, in food web, 2623, 2624, 2623
radioactive waste disposal system, 2670–2671
virus transport and removal in subsurface, 2094–2107
weathering, bioweathering and, 3372–3374
Modified Robbins Device (MRD), biofilms and, 557–558
Modified UCT process (MUCT) process, 79–80
Moist air spora, 412–414
Moisture and water content
in allergies and illness and, 1367–1368
Antarctica, 1104
biofilms and, 2960
biofiltration and, 592–593
kinetics of growth and, 1753
municipal solid waste landfills and, 1762
nitrogen fixation and, 2198
permafrost and, 2367–2385
protozoa and, 2595
rhizosphere and, 2724–2725
soil microorganisms and, 1753, 2057, 2060–2061, 2925, 3268–3269
subaerial communities and, 3057–3058, 3059
subsurface microorganisms and, 1075
viruses and, 3268–3269
Mold
airborne toxigenic, 182–189
airborne, 1647–1661
blue mold spores in, 414–415
high hydrostatic pressure (HHP) vs., 1561
slime molds and, 2912–2913
soil microorganisms and, 2912
water molds and, 2912
Molecular biofouling and, 611
Molecular beacons, detection of, 1134
Molecular biology
cold-adapted microorganisms and, 883
enzymes and, 3210
Molecular chaperone machine, 3028, 3048, 3050, 3052
Molecular probes, activated sludge foam monitoring and, 4–7
Molluscs, hydrothermal vents and, 1619
Molybdenite, bioleaching and, 633
Molybdenum, 1882–1893
cyanobacteria and, 969
in marine/ocean environment, 1704–1705
nitrogen fixation and, 2213–2215
soil microorganisms and, 2887
sulfate-reducing bacteria (SRB) and, 3089
Monochloramine, 1069
Moniliella, 122, 454
Monitored natural attenuation (MNA) in, 1941–1942
Monitoring of water purity, aerobic endospores for, 141–149
Monitors, activated sludge foam monitoring and, 4–7
Monochloramine in, 1063–1066, 2172
Monoclonal antibodies, rhizosphere and soil microbes in, 351
Monod equation, 18, 314, 1740–1741, 1743, 1753–1754, 3343
Monoethanolamine bioremediation and, 707
Monohalogens and biofiltration and, 589
Monomedia filtration, 2707

Mononitrotoluenes biodegradation and, 1423–1429
Monooxygenases, 1046, 1996, 2289–2300, 2903–2910, 2941, 3412
Monoraphidium, toxicity testing and, 3221–3230
Monoterpenes, nitrification vs., 2158
Monotropoid mycorrhizae and, 2126
Monte Carlo analysis, in risk assessment, 2749
Montmorillinite, activated sludge foaming and, 6–7
Moraxella, 499, 1853
  biofilms and, 572, 2343–2356
  biotechnology and, 3204
  filamentous forms of, in activated sludge, 1302–1306
  granular activated carbon (GAC) filters and, 1490
  heterotrophic bacteria and, 1541–1552
  subsurface microbial communities and, 3067–3071
  in drinking water, 944
Morganella, explosives biodegradation and, 1985, 1987
Morita, 2269
Moritella, 2269, 2271, 2640–2646
Mortality and morbidity rates, in risk assessment, 2743–2744
Mortar, weathering, bioweathering and, 3370–3371
Mortierella, cold-adapted microorganisms and, 880–881
Most probable number (MPN) technique, 1959, 2030, 2150–2152
Motility
  diatoms and, in biofilms, 1052–1053
  microgravity environment and, 2997–3001
Mougeotia, paleolimnology of, 2304–2306
Mount Saint Helens, quality of soils and, 2951
Mounting specimens, fluorescent probes and, 1335, 1336
Mousses of oil, for biodegradation, 2413
mRNA detection, 2499
MS2 bacteriophages and, 368–370, 387, 389–390, 2101
Mucigel, rhizosphere and, 2715
Muconic acid, biodegradation and, 2295–2300
Mucor, 1187
  biofilms and, 580
  desert environment and, 1579
  explosives biodegradation and, 1983
  subaerial communities and, 3057–3065
Multilocus enzyme electrophoresis (MLEE), 309, 1096
Multiple antibiotic resistance (MAR), 1258–1259
Multiple exposure and immunity, viral, 2747
Multiple regression analysis, 1007–1008
Municipal solid waste (MSW) landfills, 1759–1771
  aerobic processes in, 1759–1762
  anaerobic processes in, 1759–1762
  bacterial populations in, 1764–1765
  bioaerosols released from, 3324–3325
  biochemical methane potential (BMP) in, 1760
  biodegradation and, 1759–1763
  BTEX compounds in, 1763
  carbohydrates in, 1760
  carbon dioxide and, 1760
  containment sites for leachate and, 1768
  decomposition and stabilization of, 1759–1762
  energy recovery from gases of, 1767
  environmental impact of landfills of, 1765–1769
  enzyme activity in, 1760
  epidemiological studies of, 1765
  fermentation in, 1760
  flushing reactors for, 1769–1770
  gas emissions from, 1766–1767
  hazardous wastes and, 1765
  health concerns and, 1765–1766
  hydrogen and, 1760
  inhibition in, 1763–1764
  leachate emissions from, 1767–1768
  leachate treatment and, 1768–1769
  methane production and, 1761–1771
  mineral/metal supplements added to, 1763–1764
  moisture and, 1762
  oxygen and, 1759–1762
  pathogenic microbes and, 1765–1766
  pH levels and, 1760, 1762
  protozoa in, 1765
  sulfate-reducing bacteria (SRBs) in, 1760
  sulfates and sulfides in, 1760
  sustainable waste management and, 1769–1770
  temperature of, 1762
  temperatures within, 1760
  toxic chemicals and, 1765
  viruses and, 1766
  volatile organic compounds (VOCs) and, 1765
  xenobiotic constituents in, 1762
Murein, 2881
Mushroom culture, 1384–1385
Mustard gas (See Chemical warfare)
Mutations, radiation induced, 2993
Mutualism in, 1113
Mycelia, in desert soils and biological soil crust (BSC), 1020–1023
Mycena, 916
Mycetozoa, soil microorganisms and, 2912
Mycobacteria, spores and spore dispersion in, 416–417
Mycobacteria, 313, 1201, 3314–3319
  activated sludge foaming and, 3–7
  bioaerosols and, 3321
  biochips, biochip testing, 447, 448–449
  biodegradation and, 2293–2300
  biofilms and, 563, 621, 2341–2356
  bioluminescence in, 657–663
  biosurfactants/bioemulsions and, 753
  detection of, 1133
  granular activated carbon (GAC) filters and, 1492
  heterotrophic bacteria and, 1548
  infectious airborne bacteria and, 1689–1697
  methanotrophic bacteria and, 1924
  microarray study of, 1974
  Mycobacterium avium complex (MAC), 1689, 1690, 1691–1693, 2112–2120
  nosocomial infections and, 2242
  rhizosphere and soil microbes in, 346
  soil microorganisms and, 2881
  in source water, 2968, 2969–2978
  in spas and hot tubs, 2980–2984
  spores and spore dispersion in, 419, 421
  in drinking water, 945
Mycobacterium avium complex (MAC), 1689, 1690, 1691–1693, 2112–2120
mycolic acid, 3–7, 109, 2881
Mycoplasma, 1449, 1895
Mycopolyspora, 1130
Mycorrhizae, 1099, 1385, 1579, 2120–2132, 2911–2912, 3121

Mycorrhizosphere, 2714
Mycosphaerella, 406, 1395–1400
Mycosporine, 2512
Mycotoxicosis, 1369, 1378–1379
Mycotoxins, 182–189, 779, 1366–1383
Myocarditis, enteroviruses and, 1152
Myonecrosis, Aeromonas and, 1091–1099
Myoviridae/Myovirus, 365–374
Myrica, 2209, 2912
Myrothecium, 779, 2701
Myxococcus, 1215, 1320
Myxolmethyl methylpentoside, in paleolimnology, 2307–2317
Myxomycete, 3000
Myxomycota, 2912
Myxophytes
  cyanobacteria (See also Blue-green algae), 952–982
  photoautotrophic, in low-light habitats, 836–843
Myxoxanthophyll, in paleolimnology, 2307–2317

N
N-Acetlyglytaminylglutamine amide (NAGGN), soil salinity, 2778–2789
Naegleria, 2605
  Antarctic soils and, 1026
  endosymbiosis and, 1123, 1126
  meningoencephalitis and, 1343–1350
  in source water, 2968, 2969–2978
  in spas and hot tubs, 2980–2984
  in wastewater/sewage, 2329–2337
  in water treatment systems, 1718
Nais, 1722
Naked DNA, 1203, 1280
Nalidixic acid, activated sludge foam monitoring, 4–7
Nanoclimates, 3057
Nanoflagellates, bacterioplankton vs., 2531
Nanofossils, 2021
Nanolayer, neuston microbiology, 2134–2135
Nanotechnology, Archaea, 273
Naphthalene biodegradation, 694, 696–712, 1763, 2409, 2298–2300, 2403–2416
Naphthene biodegradation, 2402–2404
Naphthenic acid, bioremediation, 696–712, 2403–2416
Naphthol, composting, 921–922
Nasonia, 1115, 1122
Nassula, 972
National Institutes of Health (NIH), 2693–2707
National Pollutant Discharge Elimination System (NPDES), 3169
Natrialba, 1504–1512
Natrinema, 1504–1512
Natrionella, 1514–1529
Natronobacterium, 135–136, 1504–1512
Natronococcus, 135–136, 223, 295, 1502–1512
Natronoincola, 1521–1529
Natronomonas, 1505–1512
Natronorubrum, 1505–1512
natural attenuation, 695, 1238, 1941–1942, 1989
Navicula, 206, 1055, 1058, 1061, 2506
  algal turf scrubbing (ATS) and, 215
  Antarctic soils and, 1025
  photoautotrophic, in low-light habitats, 838–843
  salt production and, 2805–2809
  subaerial communities and, 3057–3065
Near infrared reflectance (NIR), 3165
Necator, 3333–3334
Nectira, 3358
Neisseria
  biochips, biochip testing, 447
  bioterrorism and, 771
  gene transfer and, 1413–1415
Nematoda, endosymbiosis, 1117
Nematodes
  Antarctic soils and, 1026
  biocontrols and of, 455–456
  heterotrophic bacteria and, 1540
  in water treatment systems, 1718, 1720–1722
  in wetlands for wastewater treatment, 3400
Nematophthora, biocontrols, 455
Neoxanthin, in paleolimnology, 2307–2317
Neptunia, nitrogen fixation, 2202
Nerve agents (See Chemical warfare)
Nesterenkonia, 1501–1512
Neumann's equation of state, 2005
Neural networks, 1007–1009
  auto associative neural, 1007
  proteomes and proteins analysis, 310–311
Neurospora, 1886, 1983, 2987
Neurotoxic shellfish poisoning (NSP), 2687
Neurotoxins, 2340
  biodegradation of, 507–516
  cyanobacteria and, 986–995
Neuston microbiology, 2133–2144
  algal antibiotics vs., 2139
  bacterioneustons and, 2133, 2135–2140
  environmental stress and, 2136–2140
  Nevskia ramosa as, 2139–2140
  organic surface microlayer in, 2134–2135
  pH levels and, 2139
  phytoneuston and, 2133, 2140
  pleustons and, 2133
  precipitation (rain) and, 2139
  sampling of, 2133–2134
  solar radiation and, 2136–2137
  structure of habitat in, 2134–2135
  surface film in, 2134–2135
  surface microlayer in, 2134–2135
  surface nanolayer in, 2134–2135
  temperature and, 2139
  toxic substances and, 2137–2139
  ultraviolet radiation and, 2136–2137
  viruses and, 2140–2141
  zooneustons and, 2133
Neutral lipids, biosurfactants/bioemulsions, 756–757
Nevskia ramosa, 2139–2140
New Production Paradigm, nitrification, 2153
New Substance Notification (NSN), 2706
Newport Green, 1784
Nickel, 608, 1706, 1878–1893, 2961
Nicotinamide adenine dinucleotide (NAD), 1924–1936, 2887, 2931
Nicotinamide adenine dinucleotide (NADH)
  activated sludge and, 9–14, 742–748
  aerobic respiration and, 149–154, 156, 157
  biodegradation and, 2290–2296
  bioplastics and, 684–692
  desulfurization and, 1046
  methanotrophic bacteria and, 1927–1936
  wastewater/sewage treatment and, 3338–3339
Nicotinamide adenine dinucleotide phosphate (NADPH) in
  biodegradation and, 2290–2296
  biofilms and, 83
  bioplastics and, 684–692
  desulfurization and, 1046
  methanotrophic bacteria and, 1924–1936
  soil microorganisms and, 2887
Nictotinia, 414
Nigrospora, 412
Niphargus, 1724

Nitella, 2304–2306
Nitorsococcus mobilis, 32
Nitrate, 34, 2153–58
activated sludge denitrification and, 20
bioremediation and, 694, 707–708, 709
cyanobacteria and, 969
in marine/ocean environment, 1010–1019, 1698–1701, 1702
nitrogen cycle and, 2176–2188, 2936–2944
nitrogen fixation and, 2188–2221
oxygen and, 2277
primary production and use of, 2566–2575
water treatment systems and, 2167
Nitrate ester, biodegradation, 1421
Nitric acid, 3062
biogenic, in biodeterioration of mineral materials, 524
weathering and, 3364–3389, 3364
Nitric oxide, 3183, 3189–3192
activated sludge denitrification and, 8–14
nitrification and, 2161–2163
soil biosphere and, 294
Nitrification (See also Denitrification), 2144–2167, 2940–2941
activated sludge and, 8–14, 19–20, 49, 74, 76
ammonia monooxygenase (AMO) and, 2145–2167
ammonia oxidizing bacteria (AOB) in, 2144, 2167–2176
anaerobic, 2146–2147
anammox and, 2146
aquatic systems and, 2144–2167
autotrophic, 2144–2145
biochips, biochip testing for bacteria typing in, 448
denitrification and, 2150
desert soils and, 1022
disinfection vs., in water treatment systems, 2172–2175
distribution and abundance of nitrifers for, in aquatic environment, 2150–2152
distribution of, in water and sediments, 2153–2158
euphotic zone, 2159
factors affecting, 2158–2160, 2171–2174
heterotrophic, 2145–2146
identification and enumeration of nitrifiers for, 2169
indicators of, in drinking water, 2170–2171
inhibitory compounds vs., 2158
measurement of, 2152–2153
methane oxidation and, 2163–2164
monitoring of, 2174
monoterpenes vs., 2158
most probable number (MPN) of nitrifiers for, 2150–2152
New Production Paradigm and, 2153
nitrate and, 2153–2158
nitric oxide production during, 2161–2163
nitrifying bacteria in, 2144
nitrite oxidizing bacteria (NOB) in, 2144, 2167–2176
nitrogen cycle and, 2149–2150
nitrogen fixation and, 2188–2221
nitrous oxide production during, 2161–2163
oxygen and, 2160–2161, 2282–2283
oxygen minimum zones and, 2162–2163
pH levels and, 2168–2169
phylogeny of bacteria involved in, 2147–2149
prevention and control of, in drinking water, 2174–2175
rates of, in lakes, 2156–2158
rates of, in marine systems, 2154–2156
in septic tank systems, 2859
soil microorganisms and, 2949
solar radiation or light vs., 2158–2159
substrate concentration vs., 2160
temperature vs., 2158, 2168–2169, 2171–2172
toxicity testing and, in soils, 3159–3161
water treatment systems and, 2167–2176
wetlands and, 3404
Nitrifiers, nitrifying bacteria
activated sludge and, 18–26, 34–35
aerobic respiration and, 151
soil microorganisms and, 2889–2890
subaerial communities and, 3057–3065
in wastewater/sewage, 3172
Nitrilotriacetate (NTA), 1871, 1875–1876
Nitrite, 34
activated sludge and, 8–14, 20, 24–26, 38
bioremediation and, 707–708
in marine/ocean environment, 1010–1019, 1698–1701
nitrogen cycle and, 2176–2188, 2936–2944
nitrogen fixation and, 2188–2221
water treatment systems and, 2167
Nitrite oxidizing bacteria (NOB), 2144, 2167–2176
Nitrobacter, 2145–2167, 2168–2176, 2941
activated sludge and, 13–14, 20, 35, 739
aerobic respiration and, 157
biodeterioration of mineral materials and, 524
biofilms and, 2342–2356
flooded soils and, 1325
granular activated carbon (GAC) filters and, 1490
heterotrophic bacteria and, 1542
soil microorganisms and, 2890
in wastewater/sewage, 3172
Nitrobacteraceae, 2167, 3159
Nitrobenzoate, biodegradation, 1423–1429
Nitrocellulose (NC), biodegradation, 1979–1993
Nitrococcus, 13–14, 1325
Nitrogen and nitrogen cycle (See also Nitrogen fixation), 2149–2150, 2176–2188, 2936–2944, 3183, 3189–3192
activate sludge, removal of, 8–14, 15–26
algal blooms and, 206–207
algal turf scrubbing (ATS) and, 217–218
bacterioplankton and, 2533–2534
biodegradation and, 694, 707–708, 714, 724, 707–708, 2930, 2931, 2932–2933
biofilms and, 1085–1086, 2005
biofiltration and, 589
cold-adapted microorganisms in, 872–885
cyanobacteria and, 967–968
desert environment and, 1577
floc formation and, 2005
flooded soils and, 1324–1325
in marine/ocean environment and, 1697–1709
marine nitrogen cycle in, 1010–1019
mycorrhizae and, 2128
nitric oxide and, 3189–3192
nitrogen fixation in soil, 344
nitrous oxide and, 3189–3192
oxygen and, 2277, 2282–2283
petroleum reservoirs and, 2427–2428

Nitrogen and nitrogen cycle (See also Nitrogen fixation), (*continued*)
primary production and use of, 2566–2575
rhizosphere and, 2722
soil microorganisms and, 663, 667–670
subsurface microbial communities and, 1454–1455, 1461–1463
sulfate-reducing bacteria (SRB) and, 3089
in surface waters, 928, 928
in wetlands for wastewater treatment, 3391–3393
wetlands and, 3404
Nitrogen fixation (See also Rhizobium), 344, 454, 2188–2221
actinorhizal Frankia symbiosis in, 2208–2209
aggregates and consortia in, 167–181
algae and, 1191
alternative nitrogenases in, 2190–2191
associative symbioses in, 2199–2200
bacterioplankton and, 2525
carbon and energy sources for, 2196–2198
chemistry of, 2205
coastal waters and, 2218
cold-adapted microorganisms in, 872–885
combined nitrogen and, 2198
cyanobacterial mats and, 2218
dinitrogenase and, 2205
effective vs. ineffective nodules in, 2204–2205
endosymbiosis and, 1118–1120
energy costs of, 2194–2195
factors affecting, 2207–2208
gene expression and, 2205
genetically modified microorganisms (GMMs) and, 1431, 1434–1437
genetics of nitrogenase and, 2191
grass bacteria association in, 2199–2200
host species and, 2207
importance of, 2201–2202
inoculation/infection for, 2202–2204, 2206–2207, 2209
iron and, 2213–2215
legume rhizobial symbiosis in, 2202–2208
limiting factors for, 2196
marine/ocean environment and, 2211–2221, 2539–2554, 2828–2829
metals and, 2213–2215
methanotrophic bacteria and and, 1929–1930
microbial mats and, 2218
mineral nutrients and, 2208
moisture and, 2198
molybdenum and, 2213–1115
mycorrhizae and, 2120–2132
nitrogen cycle and, 2176–2188, 2936–2944
nitrogen limitation and, 2216
nitrogenase enzyme complex for, 2189–2195, 2211–2221
nodule development on roots and, 2204–2205
nonsymbiotic bacteria and, 2195–2200
nutrients and, 2199, 2208, 2213–2215
oxygen and, 2198, 2213–2215, 2282–2283
pH levels and, 2198–2199, 2207–2208
physiological prerequisites for, 2191–2194
phytoplankton and, 2216
prenodule formation in, 2208
rates of, measuring, 2215–2219
rhizosphere and, 2720
rotation of crops and, 2907
seagrass communities and, 2828–2829
sediments and, 2218–2219
soil biosphere and, 294
soil microorganisms and, 2057–2958, 2887
soil salinity vs., 2786–2787
subaerial communities and, 3057–3065
substrates for nitrogenase and, 2191
sustainable agriculture and, 3120
temperature vs., 2199
toxicity testing and, in soils, 3160
Nitrogen limitation, 2216
Nitrogen oxides, biotrickling filters, 782–795
Nitrogenase, 2189–2195, 2211–2221
Nitroguanidine (NQ), biodegradation, 1979–1993
Nitrophenol, biodegradation, 2931, 3413
Nitrosguanidine (NTG), alkaliphiles, 228
Nitrosobacter, 3057–3065
Nitrosococcus, 9–14, 57, 2147–2176
Nitrosocystis, 2147–2167, 2147
Nitrosolobus, 35
Nitrosomonas, 2145–2167, 2941
activated sludge and, 9–14, 20, 35, 57, 739
biodegradation and, 1447
biodeterioration of mineral materials and, 524
biofilms and, 2342–2356
genetically altered, in biotechnology, 1447
granular activated carbon (GAC) filters and, 1490
streams and, 3019
subaerial communities and, 3057–3065
in wastewater/sewage, 3172
Nitrosospira
activated sludge denitrification and, 9–14, 20, 35, 57
flooded soils and, 1325
subaerial communities and, 3057–3065
Nitrosovibrio
activated sludge and, 35
biodeterioration of mineral materials and, 524
subaerial communities and, 3057–3065
Nitrospira, 2147–2167, 2941
activated sludge denitrification and, 13–14, 20, 35
flooded soils and, 1325
soil microorganism and diversity in, 533
Nitrotoluene biodegradation, 706–712, 1423–1429
Nitrous oxide, 3183, 3184, 3189–3192
activated sludge denitrification and, 8–14, 20, 24–26
biofiltration and, 589
nitrification and, 2161–2163
soil biosphere and, 294
Nitzschia, 1055, 1058, 1061
algal turf scrubbing (ATS) and, 215
photoautotrophic, in low-light habitats, 838–843
salt production and, 2805–2809
sea ice and, 2835–2844
No effect concentration (NEC), 3163
No observed effect concentration (NOEC), 3163
Nocardia, 757
activated sludge foaming and, 1–7
biodegradation and, 2292–2300, 2409
biosurfactants/bioemulsions and, 753, 756
gene transfer and, 1409

infectious airborne bacteria and, 1690, 1693–1694
soil microorganisms and, 2881
subaerial communities and, 3057–3065
Nocardia amarae-like organisms (NALO), 3–7
Nocardiodes, 1027, 1855, 2035, 3067–3071, 3314–3319
Nocardiopsis, 227–228, 881, 1506–1512
N-Octodecane, activated sludge foam monitoring, 4–7
Nodularia, 164, 167–169, 961, 963, 984–995
Nodularin, 977, 987–995
Nomuraea, microbial insecticides using, 1710
Nonaqueous phase liquids (NAPLs), 44, 699
Nonculturable microorganisms, genomic studies, 1450–1451
Nonfilamentous microbes, activated sludge foaming, 1–7
Noninclusion viruses (NPV), 1711
Nonoxidizing biocides, biofouling vs., 604–609
Nonstarch polysaccharides (NSP), 1188
Nontuberculous mycobacteria (NTM), 1690, 1691–93
Norwalk-like viruses, 1586, 2221–2235, 2744–2745
bioaerosols and, 3327
biodegradation and, 3288–3300
in source water, 2969–2978
in spas and hot tubs, 2980–2984
streams and, 3017
Nosema, 1710, 2065–2083, 2701
Nosocomial infections (NI), 2235–50
Nostoc, 207, 966, 972, 975, 980, 984–995, 1107–1112
Antarctic soils and, 1025
desert environment and, 1579
in desert soils and biological soil crust (BSC), 1021–1023
mycorrhizae and, 2120–2124
nitrogen fixation and, 2193–2201
photoautotrophic, in low-light habitats, 840–843
in snow and ice environments, 2876
Nostochopsis, 964, 1109
Nostocoida, 4–7, 64, 1288–1306
Notholca, 1725
Nowakowskiella, 2552
Noxious compounds, weathering, bioweathering, 3367–3368
Nuclear magnetic resonance (NMR), 90, 1449
Nuclear wastes (See Radioactive and nuclear wastes)
Nucleases, oligotrophic bacteria, 2273
Nucleic acid (See also DNA/RNA analysis), 2489, 2716, 2731
activated sludge and, 741–748
biomass estimation using, 2656–2657
databases of, for fluorescent probes, 1333–1334
probes using, in soil contamination, 728–729
soil genetic ecology, 2919–2923
Nucleocapsid of bacteriophages, 364
Nucleoids, 2519, 2884
Nucleomorphs, 1127
Nucleopolyhderosis viruses (NPV), 1711
Nucleotides, biomass estimation, 2654
Nursery inoculation, using mycorrhizae, 2129–2130
Nutraceuticals, 1852–1853
Nutrient accumulation, kinetics of growth, 1743–1744
Nutrient cycling
bacterioplankton and, 2532–2534
protozoa and, 2629
rhizosphere and, 2719
soil microorganisms and, 663, 2946–2947
sustainable agriculture and, 3120
Nutrients and energy sources
acquisition ability, 1745
activated sludge and, 1311
algae and, 1190, 1191–1194
ambient concentration of, 1745–46
bacterioplankton and, 2520, 2521–23, 2530–2531, 2532–2534
coliform bacteria and, 900–901
composting and, 915
desert environment and, 1576, 1582–1583
fungi and, 1648
hydrothermal vents and, 1618
hyperthermophiles and, 1633
marine/ocean environment, 1697–1709
mycorrhizae and cycling, 2127
nitrogen fixation and, 2196–2198, 2199, 2208, 2213–2215
oligotrophic bacteria and, 2267–2277
periphyton and, 2362–2363
plankton and phytoplankton, 2503, 2512–2514
polar phytoplankton and, 2559–2560, 2562–2563
primary production and use of, 2566–2575
protozoa and, 2595, 2596, 2613–2615, 2622–2623
sea ice microorganisms and, 2837–2838
soil microorganisms and, 2057, 2059–2060, 2887, 2927
in source water, 2970
streams and, 3015
stress response and, 3034
vadose zone microbiology and, 3236–3246
wastewater/sewage treatment and, 3337
Nutritional/physiological status, lipid biomarkers, 1819
Nyquist sampling frequency, 1785

O

O serogrouping, fecal contamination, 1259–1260
Obligate intracytobionts, 1113
Obligate symbiosis, 1123
Oceanospirillum, 675, 1228, 1908, 2549
Ochratoxins, 182–189, 1375
Ochromonas, 2317–2327, 2508, 2805–2809, 2837–2844
Octadecabacter, 2640–2646
Octahydro tetranitro tetrazocine (HMX) biodegradation, 1979–1993
Octahydrotetranitrotetrazocine bioremediation, 706–712
Octane biodegradation, 3405, 3412
Oculina, endosymbiosis, 1117
Odentella, 2506
Odor control, biofiltration, 593–601
Odor in water
algal blooms and, 204–213
biotrickling filters vs., 782–795
Oedogonium, algal turf scrubbing (ATS), 215
Off-stream water storage, as pretreatment, 2013–2014
Oidium, 412, 454
Oil industry (See also Hydrocarbons; Petroleum)
biofouling and, 623
biomineralization and, 677
genetically altered microorganisms and, to increase productivity, 1448
microbially enhanced oil recovery (MEOR), 2421, 2424–2425
petroleum reservoirs and microbial activity in, 2416–2426
Oil spills, 693–701, 713, 715–719, 916–917, 2402–2416, 2931

Okenone, 2307–2317, 2458
Oligonucleotide microarray analysis, 449, 1969
Oligonucleotide probe hybridization, 2497–2499
Oligonucleotide probes, 1339–1340
Oligotrophic bacteria, 1027, 2267–2277, 3250–3251
Oliogchaeta, in water treatment systems, 1722
Olisthodiscus, 2467
Olpidium, 1385
Oocystis, 2837–2844
Oomycota, 2912, 3353–3364
OP nerve agents (See Chemical warfare)
Open air factor (OAF), bioaerosols, 430–431
Open reading frames (ORFs), 1443, 1971
Opercularia, 2610, 2611
Ophiobolus, 406
Ophioceras, 3359
Ophiostoma, 2648–2650
Ophyryoglena, 972
Optical tweezers, Archaea, 247–248
Optimum temperature for growth (OTG), 3025
Optodes, biofilm measurement, 90
Orbivirus, 2753–2773
Orbulina, 1116
Organic acids, 2716, 3062
  alkaliphiles and, 228
  biogenic, in biodeterioration of mineral materials, 524–525
  fungi and production of, 1369
  metal biodegradation and, 1881–1882
  weathering, bioweathering and, 3366–3367
Organic dust toxic syndrome (ODTS), 422, 1369, 1374–1375
Organic fouling (See also Biofouling), 619
Organic matter, 3120
  Antarctic soils and soils, 1025
  bacteriophages vs., 389–390
  bacterioplankton and, 2530–2531
  biodegradable dissolved organic carbon (BDOC), 484–507
  biofiltration and, 591–592
  composting and, 915–927
  functional groups in, 929
  permafrost and, 2374–76
  pesticide degradation vs., 2394
  primary production and use of, 2566–2575
  in sediments and sedimentation, 2844
  soil microorganisms and, 663–664, 669–670, 1513–1514, 3271–3272
  soil organic matter (SOM) and, 2945–2957
  subsurface microbial communities and, 1074, 1075, 1455
  sustainable agriculture and, 3120
  viruses and, 2099, 3271–3272
  in wetlands for wastewater treatment, 3391
  wetlands and, 3403
Organic solvent–tolerant enzymes, Archaea, 268
Organic solvents, bioweathering, 3368
Organic substances in biofilms, 2963–2964
Organic surface microlayer, neuston microbiology, 2134–2135
Organochlorines, composting, 916–917
Organoosmotrophy, 3354
Organophosphorus (See Chemical warfare)
Organophosphorus acid anhydrolases (OPAA), 848–850
Organophosphorus hydrolase (OPH), 848–850
Organotrophs (See Chemoheterotrophs)
Orientia, 1122
Ornithocercus, 975, 2507
Ortho compound biodegradation, 2289–2300
Orthoeovirus, 2753–2773
Orthologs, 1450
Orthoseira, 840–843
Oryza, 2548
Oryzavirus, 2753–2773
Oscillatoria, 206, 207, 210, 966, 967, 972, 979, 980, 984–995, 3142–3147
  aggregates and consortia in, 164, 170–173, 174
  algae biotechnology and, 199–200
  algal turf scrubbing (ATS) and, 215
  Antarctic soils and, 1025
  biomineralization and, 672
  desert environment and, 1579
  in desert soils and biological soil crust (BSC), 1020–1023
  nitrogen fixation and, 2193–2201
  in paleolimnology, 2308–2317
  photoautotrophic, in low-light habitats, 838–843
  salt production and, 2805–2809
Oscillaxanthin, in paleolimnology, 2307–2317
Osmolarity
  soil salinity and, 2775–2776
  stress response and, 3031, 3034
  viable but not culturable (VBNC) phenomenon and, 3252–3253
Osmoprotectants, 518, 2776–2781, 2783–2786
Osmosregulation
  in plankton and phytoplankton, 2511
  in marine/ocean environment, 1707
Osmotic rupture, viable but not culturable (VBNC) phenomenon, 3252–3253
Osmotic shock, 431
Oxalates, 670–681, 3062
Oxalic acid, 524, 632–642
Oxidants, flooded soils, cycling, 1323–1326
Oxidases, 154–155, 1210, 3021
Oxidation
  activated sludge denitrification and, 8–14, 46
  ammonia, 9
  anaerobic ammonium oxidation (anammox), 11–13, 24
  biofilms and, 2962
  cyanobacteria and, 992
  heterotrophic ammonia oxidation, 13
  subsurface microbial communities and, 1074–1075, 1454–1455
Oxidative phosphorylation, 149–151, 2931
Oxidative stress, 3037, 3253
Oxides, 632–642, 670–681
Oxidizing halogens, biofouling vs., 603
Oxidoreductases, 2903–2910, 3412
Oxoisocaproic acid, alkaliphiles, 228
Oxoisovaleric acid, alkaliphiles, 228
Oxolinic acid azide (OAA), 1273
Oxygen, 2277–2288, 3183
  activated sludge and, 739, 1311
  aerobic respiration and, 149–160
  aggregates and consortia in, 175
  analysis of, at microorganism scale, 2286
  anoxygenic photosynthesis and, 2280–2282
  bacterioplankton and, 2520, 2521–2522, 2530–2531
  biodegradation and, 2930–2932
  biofilms and, 83–84, 1083, 2005
  biofiltration and, 589, 591
  bioremediation and, 714, 724
  cyanobacteria and, 965–966
  denitrification and, 1013–1014
  desert soils and, 1021–1022

distribution of, in water and sediments, 2278–2279
floc formation and, 2005
flooded soils and, 1318
in groundwater, 1665
heterotrophic bacteria and, 1540
hydrothermal vents and, 1610
iron cycle and, 2283–2284
manganese cycle and, 2283–2284
in marine/ocean environment and, 1697–1709
municipal solid waste landfills and, 1759–1762
nitrification and, 2160–2161
nitrogen cycle and, 2282–2283
nitrogen fixation and, 2198, 2213–2215
petroleum reservoirs and, 2427
photorespiration and, 2279–2280
photosynthesis and, 2279–2283
phototropism in, 2474
primary production and use of, 2566–2575
protozoa and, 2595
rhizosphere and, 2724–2725
in sediments and sedimentation, 2844, 2847–2848
soil microorganisms and, 2057, 2061, 2887–2888
streams and, 3015
stress response and, 3034
subsurface microbial communities and, 1074–1075, 1454–1455, 1461–1463
sulfate reducing bacteria (SRB) and, 472–475, 2285–2286
sulfur cycle and, 2284–2285
toxicity of, 2278
vadose zone microbiology and, 3236–3246
wetlands and, 3403–3404
Oxygen inactivation, spores and spore dispersion, 430
Oxygen minimum zones (OMZs), 1016, 2162–2163
Oxygen uptake rate (OUR), wastewater/sewage treatment, 3345–3346
Oxygenase enzymes, 435, 2288–2300
Oxygenates, fuel, 1994
Oxygenic photoautotrophy, bacterioplankton, 2522
Ozonation, 1067, 1070–1071
activated sludge foaming and, 6–7
Archaea and, 273
biodegradable dissolved organic carbon (BDOC) and, 496–497
biofilms and, 87
biofouling vs., 603
bottled water and, 797–798
enteroviruses and, 1172–1173
protozoa vs., 2334
in spas and hot tubs, 2979
spores and spore dispersion in vs., 430–431
viruses vs., 3262
Ozone layer, 2564–2564, 2943, 3183, 3184, 3188–3189

P

Pachyshaera, 2508
Packaging materials, biofilms, 580
Packed bed systems, adhesion in biofilms and testing, 118–119
Paecilomyces, 1650, 1657–1658
alkaliphiles and, 228
in allergies and illness, 1367–1372, 1378–1383
biocontrols and, 453, 455
biofilms and, 580
cold-adapted microorganisms and, 881
subaerial communities and, 3057–3065
Paenibacillus, 818, 2723
biodegradation and, 1045
desulfurization and, 1048
nitrogen fixation and, 2193–2201
Paleoclimates, endolithic microorganisms as indicators (See also Climate changes), 1108
Paleococcus, 1611, 1624–1628, 3146
Paleoenvironment, permafrost, 2380–2382
Paleolimnology, 2301–2306
analytical methods used in, 2301–2302
chrysophyte structures in, 2317–2327
climate change studies using, 2312–2313, 2324–2325
eutrophication studies using, 2312–2313, 2324–2325
permafrost and, 2380–2382
pH level studies using, 2312–2313, 2321–2323
pigments as indicators in, 2306–2317
salt deposition studies using, 2323–2324
sileceous cell covering in, 2317–2320
silica deposition vesicles (SDV) and, 2320–2321
taxonomy of algae in, 2302–2305
Palisades, 2882
Pandorina, 206
Paneaolus, 916
Panspermia theory, desiccation, 1029, 1037
Pantoea, 420, 581, 1883
Paper products (See Pulp and paper industry)
Papilloma virus, in spas and hot tubs, 2980–2984
Papulaspora, 460
Paracoccus, 3111–3116
activated sludge and, 13–14, 35–38, 3007, 3009
aerobic respiration and, 156–157
granular activated carbon (GAC) filters and, 1490
nitrification and, 2146, 2149
Paraffin biodegradation, 2402–2404
Paralytic shellfish poisoning (PSP), 2687
Paralvinella, 1610
Paramagnetic beads and ribotyping, 818
Paramecium
biofilms and, 2342–2356
endosymbiosis and, 1117
microgravity and, 3000
soil microorganisms and, 2881
in wastewater/sewage, 3173
in wetlands for wastewater treatment, 3398–3399
Parapedinella, 2508
Paraphysomonas, 2317–2327
Parasites, 1113, 1857
biocontrols and for, 459
disinfection and, protozoan, 1066–1073
Entamoeba and, 1136–1146
Giardia and, 1469–1489
high hydrostatic pressure (HHP) vs., 1561
Isospora as, 1734–1737
male-killing bacteria in, 1122
nematodes, biocontrols and of, 455–456
parthenogenesis and, 1122–1123
protozoa as, 2327–2337, 2607
protozoan parasites, in aquatic sediments, 1252–1256
reproductive parasites and, 1120–1123
Toxoplasma gondii, 3176–3183
in water treatment systems, 1718–1719
in wetlands for wastewater treatment, 3399
Parasitophorus vacuole or inclusion, 1113
Parasponia, 1119–1120
Paratanytarsus, 1723
Parathion biodegradation, 1421, 2394, 3413
Parthenogenesis, 1122–1123

Particle fouling, 619
Particle tracking model, for bioaerosols, 2110–2112
Particulate organic carbon (POC), 485–507
  biofilms and, 1086–1987
  conditioning films and, 928
  streams and, 3021
Particulate phosphorous (PP), 2448–2453
Paspalum, 2199
Pasteur, Louis, 2269, 3320
Pasteurella, 3400
Pasteuria, 455
Pathogenesis, genomic studies, 1448
Pathogenic bacteria, 1850, 1857
  biofilms and, 621, 2339–2356
  cyanobacteria and, 972–973
  endosymbiosis and, 1123
  Escherichia coli as, 2337–2339
  groundwater and, 2025, 2971–2972
  heterotrophic bacteria and, 1541–1552
  hosts for, in water supply, 1719–1720
  infectious airborne bacteria as, 1689–1697
  nosocomial infections (NI) and, 2235–2250
  Pseudomonas, 2632–2639
  in septic tank systems, 2861–2862
  soil microorganisms and, 2058
  surface waters, 2971
  in drinking water, 944–945
  in wetlands for wastewater treatment, 3393–3400
  municipal solid waste landfills and, 1765–1766
Pathogenic Escherichia coli, 2337–2339
Pathogenic protozoa, 2607
Patina, biodeterioration of mineral materials, 518
Pavlova, 2508
Paxillus, 2124–2132, 2915
PCDF biodegradation, 1238–1252
Peat bogs, 3, 677
Pectinase, 227, 1188
Pediastrum, 2302–2306
Pedinellales, 2467
Pedomicrobium, 572, 675, 1884
Pelagococcus, 2467
Pelagophyceae, 2467
Pelobacter, 742–748
Pelodictyon, 2479–2489
Pelomyxa, 972, 1125
Peltoperla, 3020
Pendimethanol composting, 921–922
Penicillin, 417, 1366, 1369, 1384
Penicillium, 182–189, 423, 757, 1130, 1378–1384, 1650, 1651, 1658–59, 3144–3147
  in allergies and illness, 1359, 1361–1366, 1367–1372, 1374–1383
  biocontrols and, 454
  biodeterioration of mineral materials and, 518
  biofilms and, 580
  cold-adapted microorganisms in, 872–885
  desert environment and, 1579
  explosives biodegradation and, 1983
  metal biodegradation and, 1885–1886
  in permafrost, 2369
  spores and spore dispersal in, 408, 412, 421, 422
  subaerial communities and, 3057–3065
  in drinking water, 945
Peniophora, 451, 2649
Pennisetum, nitrogen fixation, 2199
Pentachlorophenol (PCP) biodegradation, 507–516, 703–712, 916–917, 1763, 3147–3155, 3413
Pentaerythritol tetranitrate (PETN) biodegradation, 1979–1993
Pentosanase, 1186
Peptidases, 1211–1223, 2903–2910, 3021
Peptides, cyanobacteria, 975
Peptidoglycan, 671, 2881
Peracetic acid, biofouling vs., 603, 625
Perchlorate bioremediation, 706–712
Perchlorethylene (PCE) biodegradation, 434–442, 510–514, 701–712, 2933
Peridinin, 2307–2317, 2459, 2504, 2512
Peridinium, 205, 206
Peripheral proteins, soil microorganisms, 2883
Periphyton, 2357–2367
  artificial substrata for, 2358
  biomass of, 2359
  biomonitoring using, 2365
  climate change and, 2365
  composition of, 2359
  distribution to different habitats, 2359–2360
  eutrophication and, 2364
  food web and, 2360–2361
  grazing rate on, 2359, 2364
  hydrodynamics of, 2363
  metabolism in, 2359
  metal pollution vs., 2364–2365
  nutrients and, 2362–2363
  pH levels and acidification vs., 2364
  sampling of, 2358
  solar radiation vs., 2362
  streams and, 3021–3022
  substrata for, 2363–2364
  surface waters, 2359–2360
  temperature vs., 2361–2362
  wetlands and, 2360
Periplasm, soil microorganisms, 2884
Perkinsus, 1857
Permafrost, 2367–2385
  cold-adapted microorganisms in, 872–885
  snow and ice environments, 2872–2880
Permeases, 2883
Peronospora, 407, 414–416
Peroxidases, 1210, 1212–1223, 1385, 1386–1387, 2647, 2903–2910, 3021
Peroxides, biofouling vs., 602–603
Perseponella, 3143–3147
Personal care products, enzymes, 1185
Pertussis, biochips, biochip testing, 448
Pesticides
  biocontrol vs., 450–465
  biodegradation of, 693, 705–706, 916–917, 921–922, 1238–52, 2385–2402, 3121–3122, 3402, 3405–3410
  biofilms and, 86–87
  bio-, 351
  cometabolism in, 2386–2387
  environmental factors affecting degradation of, 2393–2394
  enzyme activity and, 2909
  groundwater contamination by, 706, 2398–2399
  mineralization of, 2386
  modeling soil degradation of, 2397–2398
  organic matter vs. degradation of, 2394
  pH levels vs. degradation of, 2394
  primary and ultimate biodegradation of, 2394–2395
  regulation of, 2693–2707
  soil contamination by, 706
  temperature vs. degradation of, 2394
  triazine herbicides and, 2391–2393
  wetlands and, 3405–3410
Pet borne disease (See Zoonoses),
Petrogota, 1515–1529

Petroleum products (See also Hydrocarbons; Oil industry)
biodegradation of, 917–919, 2402–2416
composting and, 917–919
enzymes and, 1185
sampling of, 3073
soil slurry batch reactor for, 51–52
Petroleum reservoirs and their microbes, 2416–2426
acetogenic microbes in, 2430
aerobic processes in, 2427
allochthonous microbes in, 2428
anaerobic bacteria in, 2419–2420
anaerobic processes in, 2427
biodiversity in, 2426–2431
biotechnology and, 2424–2425
chemical treatments vs. microbial life in, 2421
fermentative microbes in, 2429–2430
gases in, 2426–2427
geophysical and geochemical characteristics of, 2426–2428
iron reducing bacteria in, 2430
metabolism in, 2427
methanogens in, 2429
microbial consequences of exploitation in, 2420–2424
microbially enhanced oil recovery (MEOR), 2424–2425
microbially influenced corrosion (MIC) and, 2423–2424
microbiology of, 2419–2420, 2426–2431
nitrogen in, 2427–2428
oil bearing rocks as microbial habitats in, 2418–2420
origins of, 2416–2418
oxygen in, 2427
pH levels in, 2427
phosphorus in, 2427–2428
pressure in, 2427
pressure of extraction vs. microbial life in, 2421
primary, secondary, and tertiary recovery from, 2420–2424
salinity of, 2427
souring and plugging of, 2422
sulfate-reducing bacteria (SRB) in, 2422–2424, 2428–2429
temperature and, 2427
temperature of extraction vs. microbial life in, 2421
thermophiles in, 2419–2420
water in (connate, free, formation), 2418–2419, 2421
Petrotoga, 2420
Pfam, 1450
Pfiesteria and toxic Pfiesteria complex (TPC), 2431–2447, 2502, 2621
harm/in harmful algal blooms (HABs) and red tides, 2686–2693
pH levels, 2427
acid mine drainage, 709
activated sludge processing and, 25, 1311
adhesion in biofilms and, 100, 104–105, 121–122
aggregates and consortia and, 175
algal blooms and, 209, 1191
algal turf scrubbing (ATS) and, 217–218
alkaliphiles and, 133–140, 138, 219–231, 3127–3140
alkalithermophiles, 3127–3140
bacteriophages and, 387–389
biodegradable dissolved organic carbon (BDOC) and, 494
biofilms and, 100, 104–105, 121–122, 1085–1086
biomineralization and, 671
bioremediation and, 713–714, 724
in caves and mines, 842
composting vs., 915
cyanobacteria, 136
denitrification and, 1013–1014
desert environment and, 1576
disinfection and, 1065, 1066
enteroviruses and, 1170
enzyme activity and, 2900, 2906, 2908–2909
extremeophiles and, 1232–1237
floc formation and, 57, 58
fungi, 136
in groundwater, 1665
haloalkaliphiles, 135–136
heterotrophic bacteria and, 1540
hydrothermal vents and, 1618
hyperthermophiles and, 1628
metal biodegradation and, 1879, 1880–1882
municipal solid waste landfills and, 1760, 1762
neuston microbiology and, 2139
nitrification and, 2168–2169
nitrogen fixation and, 2198–2199, 2207–2208
paleolimnologic studies of, 2312–2313, 2321–2323
periphyton and, 2364
pesticide degradation vs., 2394
photoautotrophes and, in low-light habitats, 842–843
protozoa and, 2595, 2596
rhizosphere and, 2723
soil microorganisms and, 1513, 2061–2062, 2889, 3269–3270
stress response and, 3034
subaerial communities and, 3062
thermoalkaliphiles and, 136
viruses and, 2098, 3269–3270, 3269
in wastewater stabilization ponds (WSBs), 3331–3336
water treatment systems and, 2017–2018
weathering, bioweathering and, 3379
in wetlands for wastewater treatment, 3393
yeast and, 136
Phacotus, 2305–2306
Phaeococcomyces, 136
Phaeocystis, 2556
Phaeolus, 2647
Phaeophorbides, 2456–2458
Phaeophtyin, in paleolimnology, 2307–2317
Phaeophycea, 837–843, 2467, 2504–2518
Phaeophytins, 2456
Phaeosphaeria, 1395–1400, 2539–2540
Phaeothaminophyceae, 2467
Phages (See Bacteriophages)
Phalacroma, 2507
Phanerochaete, 918, 1385, 1983–1985, 1987, 2396, 2647
Pharmaceuticals, 1850, 2516
algae biotechnology and, 189–204
biofiltration in, 588
marine biotechnology and, 1851–1852
Pharyngoconjuctival fever, adenoviruses, 92–100
Phase contrast microscopy, 1674–1675
Phaseolus, plant growth promoting rhizobacteria (PGPR) inoculation, 350
Phelbia, 453, 2649
Phellinus, 2647, 2914
Phenanthrene biodegradation, 694, 696–712, 917, 2298–2300, 2403–2416, 2403
Phenazines, 457
Phenol biodegradation, 605, 707, 916–917, 1763, 2295–2300, 2930, 3147–3155, 3412
Phenol oxidase, 1212–1223
Phenolic, 929
Phenylacetic acid, 228
Phenylalanine, 2899
Phenylamine, 228

Phenylmecury biodegradation, 1424–1429
Pheromones
biofilms and, 567
viable but not culturable (VBNC) phenomenon and, 3251
Phialophora, 2125–2126
Phlebia, 1385
Pho Regulon of E.coli, 2448–2451
Phoma, 3360
desert environment and, 1579
in desert soils and biological soil crust (BSC), 1020–1023
subaerial communities and, 3057–3065
Phormidium, 210, 963, 966, 972, 973, 980, 3142–3147
aggregates and consortia in, 170–174
Antarctic soils and, 1025
desert environment and, 1020–1023, 1579
salt production and, 2805–2809
in snow and ice environments, 2876
subaerial communities and, 3056–3065
Phosopholipids, biofilms, 85–86
Phosphatase, 1212–1223, 3021, 3207–3210
mycorrhizae and, 2129
phosphorus cycling and, 2451
soil, 2903–2910
toxicity testing and, in soils, 3160–3161
in wastewater/sewage, 3170
Phosphates
biomineralization of, bacterial, 670–681
phosphorus cycling and, 2450
primary production and use of, 2566–2575
in septic tank systems, 2860
subsurface microbial communities and, 1464–1466
Phosphodiesterase, in soil, 2903–2910
Phospholipid bound phosphate (PLP), 2654–2656
Phospholipid ether liquid (PLEL), Archaea, 252
Phospholipid fatty acid (PLFA), 1818–1824
biomass estimation and, 2654–2656
desert environment and, 1578, 1579
methanotrophic bacteria and, 1924–1936
sampling of, 2811
signature profile of, 1004
soil microorganism and diversity in, 538–539
subsurface microbial communities and, 1079, 2030, 3315
Phospholipids, 1818–1824
biomass estimation and, 2654–2656
biomineralization and, 671
biosurfactants/bioemulsions and, 756–757
microbial protection from, 432
in surface waters, 928
Phosphonium chloride, 605–606
Phosphorus and phosphorus cycling, 2448–2453
activated sludge and, removal of (See also Enhanced biological phosphorus removal), 15–26, 38–40, 77
algal blooms and, 206–207, 1190
algal turf scrubbing (ATS) and, 217–218
bacterial role in, 2450
bacterioplankton and, 2534
biodegradation and, 2930
biofilms and, 1085–1086, 2005
bioremediation and, 714, 724
carbon to phosphorus ratio of cells and, 2450
cyanobacteria and, 969
dissolved inorganic phosphorus (DIP) in, 2448–2453
dissolved organic phosphorus (DOP) in, 2448–2453
enzymatic hydrolysis of DOP in, 2450–2452
floc formation and, 2005
in marine/ocean environment, 1697–1709, 2448–2453
mycorrhizae and, 2128
particulate phosphorous (PP) in, 2448–2453
petroleum reservoirs and, 2427–2428
Pho Regulon of E. coli and, 2448–2451
phosphates and, 2450
primary production and use of, 2566–2575
sediments and, 2452
solubilizers of, in soil, 346
soluble nonreactive phosphorus (SNP) in, 2448–2453
soluble reactive phosphorus (SRP) in, 2448–2453
total dissolved phosphorus (TDP) in, 2448–2453
Phosphorus accumulating organisms (PAOs), 18–26, 57
Phosphorylation, 82–83, 149–151
Photinus, 657–663
Photoautotrophic microorganisms, 835–843, 2453–2470, 2565
biofilms and, 81–82, 1084
endosymbiosis and, 1115–1117
polar phytoplankton and, 2555
soil microorganisms and, 204, 2890–2892
sulfur cycle and, 3110
thermophiles and, 3141–3143
Photobacterium, 580, 3171, 3205
Photobiology, 3230
Photoheterotrophic microorganisms, 2463–2465
biofilms and, 1087–1088
bacterioplankton and, 2522
Photolithoautotrophy, bacterioplankton, 2522
Photolyase, 2987
Photon laser scanning microscopy (PLSM), 1773–1788
Photopseudomonas, 2168
Photoreactivation, 3231
Photorespiration, oxygen, 2279–2280
Photorhabdus, 658–662, 1448
Photosynthesis, 2471
aggregates and consortia in, 175
anoxygenic, 2280–2282
bacterioplankton and, 2521
biofilms and, 84
cold-adapted microorganisms in, 872–885
cyanobacteria and, 964–967
diatoms and, 1060
marine algae and bacteria, pigments for, 2453–2470
oxygen and, 2279–2283
plankton and phytoplankton, 2512
primary production (marine) and, 2566–2575
Prochlorococcus, 2576–2593
soil biosphere and, 204
in wastewater stabilization ponds (WSBs), 3332–3333
Photosynthetic pigments (See Pigments, photosynthetic)
Phototrophic purple and green bacteria, 2470–2489
Chlorobiaceae in, 2481, 2483
Chromatiaceae in, 2477–2482
ecology of, 2472–2476
Ectothiorhodospiraceae in, 2480, 2482
favorable conditions for, 2473
habitats for, 2474–2476
hydrogen sulfide and, 2473
isolates of, 2476–2481
light and, 2473–2474
oxygen and, 2474
phylogeny of, 2481–2483

purple nonsulfur bacteria in, 2480–2483
salt marshes and, 2475
salt requirements and osmotic adaptations of, 2483–2486
Phototrophs and photoautotrophy, 2471
biofilms and, 83
in permafrost, 2369
soil microorganisms and, 2887
weathering, bioweathering and, 3365
Phragmites, in wastewater treatment reedbeds, 3389–3401
Phragmonema, 838–843
Phthalate, 1822
biofilms and, 86–87
bioremediation and, 707
cold-adapted microorganisms and, 882–883
composting of, 916–917
soil slurry batch reactor for, 51–52
Phycobilins, 2453–2470
Phycobiliproteins, 193–194, 2453–2470
Phycobilisomes, 2461–2462
Phycocyanin, 1852
Phycocyanobilin, 2460
Phycoerythrine (PE), 1676, 2576–2593
Phycoerythrobilin, 2460
Phycopeltis, 3056–3065
Phycosphere, 1056–1057
Phycotoxins, 1857
PHYLIP, 812
Phyllosphere
emergent plants (See also Spartina), 2539–2549
marine/ocean environment and, 2539–2554
seagrass communities in, 2824–2825
submerged plants and, 2549–2552
Phylogenetic oligonucleotide arrays (POAs), 1976–1977
Phylogenetics (See also DNA/RNA analysis), 1446, 2489–2501
Archaea and, 259–260
capillary electrophoresis in, 811–821
cloning and, 2492–2494
denaturing gradient gel electrophoresis in (DGGE) in, 2494–2496
DNA-based, 2492–2496
mRNA detection in, 2499
oligonucleotide probe hybridization in, 2497–2499
polymerase chain reaction (PCR) in, 2492–2494
reverse transcription polymerase chain reaction (RT PCR) in, 2500
ribosomal RNA-based, 2491–2492
RNA-based methods of, 2496–2499
sequencing in, 2491
terminal restriction fragment length polymorphism (T RLFP) in, 2496
transcription in, 2489–2491
translation in, 2489–2491
Phylotypes, 2524
Phyma, 945
Phymatotrichopsis, 2913
Physarum, microgravity, 3000
Physical gaps, in genomic studies, 1443
Physical potential (hydraulic head, water tension, suction), biofiltration, 592
Phytane, 928, 928
Phytanyl ether lipids, 1818–1824
Phytate, 1188
Phytometallophores, 2717
Phytoneuston, 2133
Phytophthora, 407, 1395–1400
biocontrols and, 451, 458
biosurfactants/bioemulsions and, 766
soil microorganisms and, 2912
spores and spore dispersal in, 414
Phytoplankton (See Plankton and phytoplankton)
Phytoremediation, 709, 731–734, 1989–1990
Phytoreovirus, 2753–2773
Phytostimulators, bacterial, 343–354
PICCOLO software, 502
Picea, ectendomycorrhizal fungi, 2125–2126
Piceance Basin, 2045–2047, 2048–2057
Picocyanobacteria, 2526
Piconeustons, 2135–2136
Picornaviridae, 327, 363–364, 1530–1540, 3327
Picrophilus, 1233, 1235, 1625, 3129, 3146
Pigmented bacteria, photosynthetic pigments, 2489
algae biotechnology and, 193
biomass estimation and, 2657
Chemtax analysis of, 2468
concentration and extraction of, 2462
cyanobacteria and, 964–965
distribution of, 2463–2465
fluorometric analysis of, 2462–2463
heterotrophic bacteria and, 1541–1552
high performance liquid chromatography (HPLC) analysis of, 2463
marine algae and bacteria, 2453–2470
marine biotechnology and, 1852–1853
paleolimnology and, 2306–2317
photochemistry of, 2454
photosynthetic, in marine algae and bacteria, 2453–2470
psychrophilic, 2640–2646
purple and green phototrophic bacteria in, 2470–2480
spectrophotometric analysis of, 2462
taxonomy of, 2465–2468
in drinking water, 945
Pili, 2885
Piloderma, 2124–2132
Pimephales, biomonitoring, 3169
Pink snow, 2873
Pink water, 706–712
Pinnularia, 2839, 3057–3065
Pinus, ectendomycorrhizal fungi, 2125–2126, 2128
Pipecolate betaine, soil salinity, 2778–2789
Piptoporus, 2647
Pisolithus, 2124–2132, 2915
Pitch (wood sap) degradation, fungi, 2648–2650
Pithomyces, 408, 412
Plagioplya, 3398–3399
Plague, bioterrorism, 776–777
Planctomycetales
activated sludge denitrification and, 10–14, 38, 71
bacterial counts using, 31
nitrification and, 2146
in snow and ice environments, 2877
soil microorganism and diversity in, 533
Planetary catastrophe, subsurface microorganisms, 1080
Plankton and phytoplankton (See also Bacterioplankton; Harmful algal blooms and red tides; Prochlorococcus), 2502–2518
adaptations of, 2510–2514
Archaea and, 279–285, 288
bacterioplankton in, 2519–2539
biofilms and, 81, 82, 88, 114, 611–612, 1082–1083
biogeochemistry and, 2515–2516

Plankton and phytoplankton (See also Bacterioplankton; Harmful algal blooms and red tides; Prochlorococcus), (*continued*)
biomass of, 2516, 2559–2560, 2653
biotechnology and, 2516
blooms, 970–972, 975–981, 989–990, 2002, 2565
carbon dioxide and, 2515
cell shape in, 2508
cell size of, 2505–2508
cell wall in, 2509, 2555
characteristics of, 2555
chloroplasts in, 2509–2510
cholera and, 856–861
climate changes and, 2515–2516, 2564–2565
colonies of, 2509
culturing, 2516
cyanobacteria and, 970–972, 975–980, 980–981, 989–990, 2555
cyclic climatic events and, 2558–2559
distribution of, 2514–2515
endosymbiosis and, 1115
eutrophication and, 2516
evolutionary history and fossil record of, 2504–2505
eyespot in, 2509
flagella of, 2509
food web and, 2563–2564
grazing and, 2514
growth rates of, 2559–2563
invasions by, 2515
light or solar radiation and, 2502, 2512, 2555–2556, 2560–2562
meroplankton, 1863–1869
microbial attack on, 2514
morphology of, 2505–2510
nitrogen cycle and, 2181
nitrogen fixation and, 1191, 2216
nucleus of, 2510
nutrients and, 2503, 2512–2514, 2559–2560, 2562–2563
osmosregulation and, 2511
ozone hole and, 2564–2565
periphyton vs., 2357–2367
phosphorus cycling and, 2450
photoautotrophs as, 2555
photosynthesis and, 2512
phycosphere of, 1056–1057
phylogenetic relationships in, 2503–2504
polar marine, 2554–2565
primary production of, 2516, 2559–2563, 2566–2575
Prochlorococcus, 2576–2593
products from, 2516
pycnocline and, 2515
reproduction in, 2510
salinity of water and, 2502, 2511, 2563
seasonal succession in, 2515
sinking control, 2510–2511
soil biosphere and as, 205
storage products of, 2510
swimming in, 2511
systematics of, 2504
taxonomy of, 2556
temperature and, 2502, 2511, 2555–2556, 2563, 2564
toxins in, 2514
ultraviolet light and, 2560–2562
viruses vs., 3307–3308
Planktothrix, 972, 984–995
Planococcus, 1501–1512
Plant-assisted microbial bioremediation, 1424–1425
Plant growth (See also Mycorrhizae; Nitrogen fixation; Rhizosphere), 2713–2728
biocontrol and, 450–465
biotechnology and, 351–352
cold-adapted microorganisms and, 881
crop productivity and, 2722–2723
deleterious rhizobacteria (DRB), 2717, 2722
disease resistance and, 2722–2723
flooded soils and, 1328
genetically modified microorganisms (GMMs) and, 1434–1437
inoculation with PGPR and, 346, 350
mycorrhizae and (See Mycorrhizae)
nitrogen fixation and (See Nitrogen fixation)
phytostimulators for, 343–354
plant growth promoting rhizobacteria (PGPB), 2717, 2722
plant growth promoting rhizobacteria (PGPR), 343, 345–346, 349, 454–455, 2722
plant growth regulators (PGRs) in, 344–345
plant–microbe interactions in marine environment, 2539–2554
rhizosphere, 2713–2728
root adsorption and colonization of phytostimulators in, 348
soil microorganisms and, 663
submerged plants and, 2549–2552
sustainable agriculture and, 3121
wetlands and, 3405
Plant growth promoting rhizobacteria (PGPB), 2717, 2722
Plant growth promoting rhizobacteria (PGPR), 343, 345–346, 349, 454–455, 2722
commercial products for, 351–352
inoculation of soil with, 346, 350
Plant growth regulators (PGRs), 344–345
Plant Helper, 881
Plant–microbe interactions, marine/ocean environment, 2539–2554
Plant mucilage in rhizosphere, 2715
Plantae, 2465
Plasmid borne degradation genes, biodegradation, 2387
Plasmid incidence testing, subsurface microorganisms, 2036–2037
Plasmidophora, 2913
Plasmids in soil microorganisms, 1201–1202, 2884
Plasmodiophoromycota, 2912
Plasmodium, 1126, 3399
Plasmopara, 766
Plasticity of microorganism metabolism, 1002
Plastics, bioplastics, 682–692
Platyamoeba, 2595–2604
Platyhelminthes, in water treatment systems, 1718–1719
Plectonema, 972, 1107–1112, 2193–2201, 2282
Plectrovirus, 366–374
Pleistophora, 2066–2083
Pleuorotus, 1384
Pleuorsigma, 2839
Pleurastrum, 3056–3065
Pleurocapsa, 522, 672
Pleurochloris, 838–843
Pleurochrysis, 2508
Pleurococcetum, 3056–3065
Pleurodynia, enteroviruses, 1152
Pleurotus, 454, 2915
Pleustons, 2133
Plexura, 1851
Plug flow reactor (PFR), 44, 75–76
Plug flow reactor with dispersion (PFDR), 44, 75–76
Plugging, in petroleum reservoirs, 2422
Plume height vs. dispersion, in bioaerosols, 2110
Podophyra, 2606
Podosphaera, 454, 2606
Podovirus, 365–374

Pognophora, 1117, 1606
Poindexter, Jeanne, 2269
Point of entry (POE) water treatment devices, 1563–1575
Point of use water treatment devices, 1550–1551, 1563–1575
Polar interactions, conditioning films, 937
Polar lipids, 1817–1824
Polaribacter, 2640–2646
Polaromonas, 2640–2646
Poliovirus, 331, 363, 387, 388, 389, 1146–1156, 1170–1175, 2743, 2745
  bioaerosols and, 3327
  biodegradation and, 3289–3300
  in soil, 3271–3276
  in source water, 2969–2978
  in spas and hot tubs, 2979, 2980–2984
  subsurface microorganisms and, 2025
  water purifiers vs., 1573
  in wetlands for wastewater treatment, 3397–3398
Pollen, bioaerosols, 404–416
Polyacrylamide gel electrophoresis (PAGE), 308, 2765, 2769
Polyangium, desert environment, 1578
Polychaetes, hydrothermal vents, 1619–1620
Polychlorinated aromatic compound biodegradation, 1421
Polychlorinated biphenyls (PCBs), 507–516, 703–713, 726–735, 916–917, 925, 1238–52, 1421, 1447, 1763, 2289–2300, 2933, 3402, 3405
Polychlorinated dibenzodioxin (PCDD), 919–920, 1238–1252
Polychlorinated dibenzofurans (BCDF), 919–920, 919
Polyclonal antibodies, rhizosphere and soil microbes, 351
Polycyclic aromatic hydrocarbons (PAHs), 694, 713–735, 916–919, 2404–2416, 2404, 3211, 3402, 3405, 3412
Polyhexamethylen biguanide (PHMB) (See also Chlorhexidine), 604–605
Polyhydroxy, microbial protection from, 432
Polyhydroxyalkanoates (PHA), 21–26, 38, 78–80, 346–347, 682–692, 3005
Polyhydroxybutyrate (PHB), 21–26, 38, 64–67, 346–347, 436–437, 683–692, 1818–1824, 2270
Polykrikos, 2507
Polymerase chain reaction (PCR), 1853, 2492–2494, 2730, 2733–2739
  adenoviruses and, 97, 98
  Aeromonas assay using, 1096
  astroviruses and, 334
  bacterioplankton and, 2523
  bioaerosol samples and, 402
  biochips, biochip testing and, 443
  biofilms and, 558–559
  detection of, 1132–1134
  FISH vs., 1338–1339
  fungi and, 1370, 1389
  genetically engineered microorganisms (GEMs) and monitoring, 1284–1285
  genetically modified microorganisms (GMMs) and, 1433–1434
  genomic studies and, 1443
  granular activated carbon (GAC) filters and, 1492–1493
  methanotrophic bacteria and, 1931–1932
  microarray study of vs., 1974
  Norwalk-like viruses (NLVs) and, 2221–2235
  soil genetic ecology, 2919–2920
  soil microbe quantification using, 727
  viruses and, 3291–3299
Polymerase, 270–271, 1852, 3207–3210
Polymeric biosurfactants, 760–762
Polymers
  activated sludge foaming and, 6–7
  anaerobic granulation and, 241
  bioplastics and, 682–692
  immobilization of and, 109–110
Polymorphism
  bacterial counts using, 28–29
  capillary electrophoresis in, 811–821
in minerals, 633
Polyols, 518
Polyphenols, 195, 682–692
Polyphosphate accumulating bacteria (PAB), 64–67, 78–80
Polyphosphate accumulating organisms (PAO), 38
Polyphosphates, 3005
  activated sludge and, 73
  bioplastics and, 682–692
Polysaccharides (See also Extracellular polymeric/polysaccharide substances), 109, 458, 1210, 1852
  activated sludge and, 58, 741–743
  algae biotechnology and, 195
  Archaea and, 249
  biofilms and, 85–87, 114
  biomineralization and, 671
  bioplastics and, 682–692
  conditioning films and, 937–938
  floc formation and, 57
  hyperthermophiles and, 1641–1642
  marine biotechnology and, 1853
  mycorrhizae and, 2129
  in surface waters, 928, 928
Polysulfates, bioplastics, 682–692
Polysulfide, bioleaching, 637
Polytypism, in minerals, 633
Polyunsaturated fatty acids (PUFA), 1852–1853
  cold-adapted and psychrophilic microorganisms and, 880–881, 2639, 2644–2645, 3215
Polyvinylchloride (PVC), biofilms, 88
Polyvinylidene dichloride (PVDF), 621
Ponds (See Aquatic ecosystems; Surface waters)
Pontederia, 2548
Popillia, 1712
Populus, mycorrhizae, 2124–2132, 2911
Pore proteins, freeze drying and trehalose introduction, 1357
Pore size, in filtration, 2708, 2712
Pore water sampling, 3073
Poria, 2649
Porifera, 1117
Porins, 2883
Porphyra, 1857
Porphyridium, 189–204, 1853
Porphyrins in aerobic respiration, 153
Porphyromonas, 1213
Porthetria, 1710
Posidonia, 2823–2833
Posttreatment of water, algal blooms vs., 209
Potamogeton, 2550
Potassium (See also Nutrients and energy sources), 1707, 1879–1893, 1902–1913
Potato, late blight, 414
POTWs, biofiltration vs., 598
Powdered activated carbon (PAC), 44
Prasinococcus, 1107–1112
Prasinophyta, 2468, 2504–2518
Prasiola, 1025
PRD1 virus model, 2101
Precipitation, in biofilms, 2962
Precipitation (rain), neuston microbiology, 2139
Precipitation, of minerals/metals, 1894–1899
Predators

Predators (*continued*)
biocontrols and for, 459
fungi as, 2914–2915
protozoa as, 2620–2621
Predicted environment concentration (PEC)
Predicted no effect concentration (PNEC), 3163
Prefoldin, stress response, 3030
Presegmentation image processing, 1677–1681
Pressure (See also High hydrostatic pressure)
deep sea, 1626
petroleum reservoirs and, 2427
soil microorganisms and, 2061
stress response and, 3034
Pressure containers for deep sea sampling, 2814–2815
Pressure-tolerant enzymes, 269
Pretreatment of water, 2012–2015
Primary biodegradation, 2394–2395
Primary production, 2565, 2566–2575
Primary vs. secondary symbionts, 1114–1115
Primulin, 2596
PRINTS, 1450
Printzia, 3056–3065
Pristane, 928, 2403–2416
Pristionchus, 1721
Proacellus, 1722, 1724
Procatechuic acid, biodegradation, 2295–2300
Prochlorococcus (See also Cyanobacteria; Plankton and phytoplankton), 960, 2461, 2576–2593
bacterioplankton and, 2526
endosymbiosis and, 1126
in marine/ocean environment, 1701, 1704
photosynthetic pigments in, 2466
phototropism in, 2471
Prochloron (See also Cyanobacteria), 960, 964, 974, 975, 2471
Prochlorophytes, 952–982, 2471
Prochlorothrix (See also Cyanobacteria), 960, 2471
Productivity, benthic associated, 2573
Productivity, primary production in marine/ocean environment, 2566–2575
Prokaryotes
bioluminescence in, 1831
hydrothermal vents and, 1617–1628
oligotrophic bacteria and, 2267–2277
Proline, 518, 2778–2789
Propane, 435, 2427, 2930
Propidium iodide (PI), 1068
Propionate, 21–26, 236, 742–748
Propionibacter, 40, 2193–2201
Propionic acid, activated sludge, 741–748
Propionic acid phenothiazine (PPT), 1182
Propionigenium, 742–748
Propylene glycol biodegradation, 2930
Prorocecentrum, 2507, 2686–2693
PROSITE, 1450
Prosopis, 2785
Prosthecate bacterium, 2269, 2271
Prosthecochloris, 2474, 2479–2489
Prostheocbacter, 90
Protaspis, 2841
Proteaosome, stress response, 3031–3032, 3052
Proteases, 220–223, 269–270, 1177–1189, 1211–1223, 3021, 3170, 3207–3210
Protein engineering, 3208–3209
Proteinase, 2273, 2903–2910
Proteinchip array for proteomes and proteins, 310
Proteins, 109, 1210, 2489
activated sludge and, 741–748
algae biotechnology and, 193
Archaea and, 248, 263
biofilms and, 85–86, 114
biomass estimation and, 2654
biomineralization and, 671
cold-adapted microorganisms and, 874, 883, 872–885, 888–893
conditioning films and, 937–938
extremeophiles and, 1232–1237
floc formation and, 57, 58
heat-shock proteins and, 3049
microbial protection from, 432
oligotrophic bacteria and, 2270
oxygen sensing, in aerobic respiration regulation, 158
protein engineering and, 3208–3209
proteomes and proteins and, assessing presence of, 305–312
stress proteins and, 3028, 3030–3031, 3036–3039
in surface waters, 928, 928
viable but not culturable (VBNC) phenomenon and, 3253
Proteobacteria, 1204, 2036, 2545, 2549, 2552, 3143–3147
activated sludge and, 9–14, 34, 35, 38, 40, 61, 63, 70
anaerobic granulation and, 233–245
bacterial counts using, 31
bacterioplankton and, 2524–2526
biodegradation and, 510, 511, 512
biofilms and, 570, 571, 1218
endosymbiosis and, 1125
filamentous forms of, in activated sludge, 1299–1306
flooded soils and, 1319, 1320, 1322, 1325, 1326, 1328
fluorescent probes and, 1340
genomic studies and, 1442
halophiles and, 1506–1512, 1515–1529
hydrothermal vents and, 1606, 1609–1611, 1619, 1620
neuston microbiology and, 2139
nitrification and, 2167
nitrogen fixation and, 2214–2221
photosynthetic pigments in, 2470–2471
psychrophilic bacteria and, 2641
soil microorganism and diversity in, 533, 538
subsurface microbial communities and, 2035, 3065–3071
Proteolysis, stress response, 3031–3032, 3052
Proteomes, 305–312
aquatic environment and, 306–307
assessing presence of, 305–312
capillary electrophoresis for, 308
cluster analysis for, 310–311
electrospray ionization mass spectrometry (ESI MS) for, 309
enzyme electrophoresis for, 309
enzyme linked immunosorbent assay (ELISA) for, 309
gel electrophoresis for, 308
GeneChip system for, 310
immunofluorescence for, 309
Internet resources for assessment technology in, 311
mass spectrometry for, 309–310
matrix assisted laser desorption ionization mass spectrometry (MALDI MS) for, 309
multilocus enzyme electrophoresis (MLEE) for, 309
neural networks analysis of, 310–311
polyacrylamide gel electrophoresis (PAGE) for, 308
Proteinchip array for, 310
radioisotope analysis for, 308
Proteorhodopsin, 2471

Proteus
biodeterioration of mineral materials and, 518
cold-adapted microorganisms in, 872–885
desert environment and, 1578
microgravity and, 3000
nosocomial infections and, 2236–2250
in drinking water, 946
Protists, 2874, 3305–3306
Protochatechuate biodegradation, 1423–1429
Protococcus, 3056–65
Proton motive force (PMF) in aerobic respiration, 150
Protoperidinium, 2507
Prototheca, 842–843, 3327
Protozoa, 1857, 2594–2604
abundance of, 2615–2620
acantharian, 2617
actinopoda in, 2618
activated sludge and, 24–26, 72,75, 2332, 2605–2612
amoebas and, 2594, 2605, 2616, 2617
anoxic environments and, 2596
aquatic sediments and, 1252–1256
aquifers containing, 2595–2596, 2599–2602
artificial wetlands and, 2333
bacteriovorous ciliates, 2611
biodegradation and, 2594
bioindicator role of, 2630–2631
biomass of, 2628–2629
bodonids in, 2619
carbon and, 2622–2623
chlorine vs., 2333–2334
choanoflagellates in, 2617–2619
chrysomonads in, 2619
ciliates in, 2594, 2605–2607, 2610–2611, 2617, 2619–2620, 2626–2632
Ciliophora in, 2594
climatic change and, 2630–2631
control of, 2629
counts of, 2596–2597
culturing of, 2594
desert environment and, 1579
diamidinophenylindole (DAPI) counts and, 2596
dinoflagellate in, 2617, 2618
disinfection and, 1066–73, 2333–2334
dissolved organic carbon (DOC) and, 2596
distribution of, 2615–2620
ecological role of, 2620–2623
enteroviruses vs., 1171–1172
filter backwash water containing, 2262–2267
filtering vs., 2332, 2333
flagellates in, 2594, 2605, 2611, 2613, 2617–2619
food processing and, 3276–3287
food web and, 2620–2622, 2630
foraminifera in, 2616–2617
form and function of, 2613–2615
free-living, 2605–2606, 2613
grazing and, 2599–2601
growth rates of, 2615
heliozoan, 2617
high hydrostatic pressure (HHP) vs., 1561
high-rate algae ponds vs., 2333
indicators of sludge treatment performance in, 2608–2609
infiltration percolation vs., 2333
lagoons vs., 2333
Legionella, as host for, 1788–1806
life cycle of, 2615
lobose amoeba in, 2616, 2617
marine/ocean environment and, 2613–2625
measurement of, using aerobic endospores, 141–149
as microbial tracers in groundwater, 3194–3202
microbial insecticides using, 1711
microbial loop and, 2630
mineralization and, 2622–2623
mixotrophy and, 2621–2622, 2629–2630
models of, in food web, 2623, 2624, 2623
moisture and, 2595
municipal solid waste landfills and, 1765
neuston microbiology and, 2140
nutrients and, 2595, 2596, 2613–2615, 2622–2623, 2629
Opercularia in, 2611
oxygen and, 2595
ozonation vs., 2334
parasitic, 2327–2337, 2607
pathogenic, 2607
pH levels and, 2595, 2596
predatory role of, 2620–2621
prey role as, 2622
in rainwater roof catchment systems, 2681–2682
reproduction of, 2615
rhizosphere and soil microbes in, 348
role of, in subsurface, 2599–2601
salt production and, 2805–2809
sampling and enumeration of, 2596–2598, 2626–2627, 2813
Sarcomastigophora in, 2594
scutiocociliate, 2617
seasonal variations in, 2627–2628
sedimentation to remove, 2331–2332
sludge biotic index (SBI) and, 2609–2611
soil dwelling, 2594
soil salinity and, 2775
in spas and hot tubs, 2979
stabilization ponds for removal of, 2333
streams and, 3017, 3019, 3020–3021
subsurface dwelling, 2594
symbiosis and, 2621–2622
taxonomy of, 2594–2598, 2615–2620
tertiary wastewater treatment vs., 2333–2335
testate amoeba and, 2610
tintinnid ciliate, 2617
transport of, in subsurface, 2598–2599
trophic state vs. biomass in, 2628–2629
ultraviolet light vs., 2334
in wastewater stabilization ponds (WSBs), 3334
in wastewater/sewage, 3173
water purifiers vs., 1572–1573, 1572
water treatment systems vs., 1718–1733, 2601–2602
in wetlands for wastewater treatment, 3398–3399
Provannidae, 1606
Providencia, 1890, 1985
Prstachydrine, soil salinity, 2778–2789
Prymenesium, 2508
Prymnesiophyceae, 2467, 2685–2693
Prymnesiophytes, 2504–2518
Pseudoalteromonas, 1057
biofilms and, 615
fluorescent studies of, 1835
hydrothermal vents and, 1610
Pseudoanabaena, 210
Pseudocercosporella, 405
Pseudococcomyxa, 1107–1112
Pseudohalonectria, 3359
Pseudokirchneriella, 3221–30
Pseudomembranous colitis, Clostridium, 862–871
Pseudomonas, 313, 314, 339, 386, 499, 1061, 1132, 1196, 1200, 1201, 1203, 1501–1512, 1672,

Pseudomonas, (*continued*)
2549, 2632–2639, 2694–2695, 2722, 2723, 2725, 2942, 3111–3116, 3160, 3314–3319
activated sludge and, 35–38, 3009
adhesion in biofilms and, 118, 121, 123
aggregates and consortia in, 166
alkaliphiles and, 226
assimilable organic carbon (AOC) and, 316
bioaerosols and, 3325
bioaugmentation and bioremediation using, 436, 438–439
biocontrol use of, 2703, 2705
biocontrols and, 452–455, 457, 458, 460, 462, 2703, 2705
biodegradation and, 511–512, 693, 714–715, 1042–1047, 1421–1429, 1447, 1871, 1872, 1937–1938, 2289–2300, 2386–2393, 2394, 2396, 2408–2410, 2650, 2933
biofilms and, 82, 85, 89, 90, 561, 562, 566, 570, 572, 577, 579, 580, 581, 582, 614, 621, 1209, 1213, 1215, 1219–1220, 2341–2356, 2963
bioleaching and, 635
bioluminescence in, 658–662, 1831
biomineralization and, 674, 675
bioplastics and, 683–692
biosurfactants/bioemulsions and, 752, 763, 766
biotechnology and, 3204
bottled water and, 796, 797, 799–801, 1549–1550
cold-adapted microorganisms in, 872–885, 3215
cold-shock response and, 886–895, 892
desert environment and, 1578, 1580, 1583
desulfurization and, 1048–1049
explosives biodegradation and, 1989
extracellular polymeric substances (EPS) and, 1224
fecal contamination and, 1269, 1271, 1273
floc formation and, 2006
fluorescent studies of, 1835
gene transfer and, 1413–1415
genetically altered, in biotechnology, 1447
genetically engineered microorganisms (GEMs) and, 1279, 1421–1429
genetically modified microorganisms (GMMs) and, 1430–1440
granular activated carbon (GAC) filters and, 1489–1496, 1569–1572
green fluorescent protein (GFP) studies for, 1498–1499
heterotrophic bacteria and, 1541–52, 1544, 1545, 1548, 1549–151550
high hydrostatic pressure (HHP) vs., 1555–1563
hydrothermal vents and, 1610
identification of isolates in, 1964
immobilization of, 110
infectious airborne bacteria and, 1689–1697
metal biodegradation and, 1882, 1884, 1885, 1886, 1889, 1890, 1894
microarray study of, 1977
as microbial tracer in groundwater, 3196–3197, 3200
microbial insecticides using, 1710
microgravity and, 3002
municipal solid waste landfills and, 1766
nitrification and, 2149
nitrogen fixation and, 2193–2201
nosocomial infections and, 2236–2250
in pulp and paper industry, 2649, 2650
radioactive waste degradation and, 2667–2674
resistance in, 607–608, 625, 799
resistant bacterial strains in, stress response, 349
rhizosphere and, 345–354, 2717
soil microorganisms and, 2898, 2926
soil salinity and, 2775, 2779–2789
in source water, 2968, 2969–2978
in spas and hot tubs, 2980–84
spores and spore dispersion in, 421
stress response and, 3042
subaerial communities and, 3057–65
subsurface microbial communities and, 2023, 2035, 3065–3071
toxic solvents and, 3149, 3150, 3151, 3152, 3153
transfer of rDNA from, to other microorganisms, 1426–1427
in wastewater stabilization ponds (WSBs), 3334
in wastewater/sewage, 3172
in drinking water, 944, 945, 946
in wetlands for wastewater treatment, 3393–3397
Pseudomurein, 248, 2886
Pseudonitzchia, 2686–2693
Pseudopleurochloris, 2467
Pseudotrebouxia, 1025
Pseudotsuga, 2125–2126, 2127
Psittacosis, infectious airborne bacteria, 1689–1697
Psychrobacter, 2640–2646
biofilms and, 561
biotechnology and, 3204
cold-adapted microorganisms in, 872–885
fluorescent studies of, 1835
in permafrost, 2369
Psychroflexus, 2640–2646
Psychromonas, 2640–2646
Psychrophiles and psychrotrophs (See also Cold-adapted microorganisms), 871–885, 2639–2646, 3203–3219
Antarctic soils and, 1027
Archaea and, 246–259, 262
biotechnology and, 3203–3219
culturing of, 2641–2642
dimethyldisulfide (DMDS) and, 2645
dimethyloxazoline (DMOX) and, 2645
fatty acid methyl esters (FAME) and, 2644–2645
fatty acids and, 2644–2645
hydrothermal vents and, 1617–1628
polyunsaturated fatty acids (PUFA) and, 2644–2645
sampling of, 2641–2642
sea ice microorganisms, 2833–2844
snow and ice environments, 2872–2880
soil microorganisms and, 2889
temperature and, 2639, 2641, 2642–2645
Psychroserpens, 2640–2646
Pterosperma, 2508
Public health studies, using microbial tracer in groundwater, 3199–3200
Puccinia, 411–412, 454
Pullulanases, alkaliphiles, 225–226
Pullularia, 422
Pulmonary alveolar proteinosis, Mycobacterium avium complex (MAC), 2113–2120
Pulp and paper industry, 2646–2651

biodegradation and, 2646–2651, 2646
biofilms and, 580, 2650
biological bleaching in, 2647–2648
biological pulping process in, 2647
coliform bacteria and, 901–902
enzymes and, 1183–1185
fungal wood degradation in, 2646–2647
lipase and, 2649
pitch degradation in, 2648–2650
staining of wood in, 2648–2650
toxic effluent from, 2650
Pulsed bioremediation, 695
Pulsed field capillary electrophoresis, 815–816
Pulsed field gel electrophoresis (PFGE), 812, 1258, 1260–1261
Purifiers, water, 1567, 1572–1573
Purines in activated sludge, 741–748
Purple nonsulfur bacteria, 2470–2480
phototropism in, 2471–2472, 2480–2483
soil microorganisms and, 2992–2993
Purple sulfur bacteria, 2455, 2458, 2459, 2463, 2465, 3097–3104
Pycnocline, 162, 2515
Pycnoporus, 1387–1391
Pyochelin, 1882
Pyoluteorin (Plt), 457
Pyoverdin, 1882
Pyramimonas, 2508, 2835–2844
Pyrene bioremediation, 696–712
Pyrenophora, 410
Pyrite, 633, 638, 1049
Pyrmidines in activated sludge, 741–748
Pyrobaculum, 298, 1233, 1234, 1512–1529, 1629–1646, 1886, 3146
Pyrobotrys, 3331
Pyrococcus, 1233, 1235, 1512–1529, 1623–1628, 1630–1646, 1706, 1853, 3146
genomic studies and, 1446
halophiles and, 1525
hydrothermal vents and, 1611
Pyrodictium, 533, 1233, 1234, 1235, 1625–1628, 1630–1646, 3144–3147
Archaea and, 298
halophiles and, 1525
hydrothermal vents and, 1611
stress response and, 3026
Pyrodinium, 2507, 2686–2693
Pyrolobus, 287, 1233, 1611, 1625–1628, 1630–1646, 3128–3140, 3141–3147
Pyroloid mycorrhizae, 2126
Pyrophaeophorbide, 2456
Pyrophaeophytin, 2456
Pyrophosphatase, in soil, 2903–2910
Pyrrhophyta, 205, 2466, 2556
Pyrroles, 153, 1852
Pyrrolnitrin (Prn), 457
Pyrrolo quinolone qunione (PQQ), 1927–1936
Pyrsonympha, 1124
Pyruvate, 2172
Pythium, 1395–1400, 3358
biocontrols and, 451, 453, 457, 458, 460
biosurfactants/bioemulsions and, 766
soil microorganisms and, 2912
Pyxidicula, 2605

Q

Q fever, infectious airborne bacteria, 1689–1697
Q loop in aerobic respiration, 153
qCO2, quality of soils, 2954–2956
Quality control of drinking water, 2652
heterotrophic bacteria and, as indicators of, 1546
in rainwater roof catchment systems, 2683
in source water, 2972–2974
Quality of soil, 2944–2957
adenosine triphosphate (ATP) and, 2948
aggrading systems and, 2951
bioindicator interpretation of, 2950–2951, 2953–2954
biomass and, 2948, 3158–3159
chloroform fumigation incubation method (CFIM) and, 2948
conservation reserve programs and, 2951–2952
degrading systems and, 2952–2954
denitrification and, 3160
disturbance of soils and, 3122
enzyme activity and, 2949, 3161
long-term changes in and, monitoring, 3165
measurement of, 2947–2948
metabolism and, 2951–2956
microbial growth rates and, 3161
microbial processes and, 2949
minimum data sets (MDS) for, 3165
Mount Saint Helens and, 2951
mycorrhizae and, 3121
near infrared reflectance (NIR) in, 3165
nitrification and, 2949, 3159–3161
nitrogen fixation and, 3120, 3160
nitrogen mineralization and, 3159
nutrient cycling in, 2946–2947, 3120
organic matter and, 3120
pesticide biodegradation in, 3121–3122
phosphatase activity and, 3160–3161
plant growth and, 3121
qCO2 and, 2954–2956
respiration and, 2948–2949, 3159
rotation of crops and, 3122
sampling of, 2949–2950
soil organic matter (SOM) and, 2945–2957
soil structure and, 3122
sustainable agriculture, 3116–3126
toxicity testing, 3155–3168
Quantification of microbial biomass, 2652–2660
Quantitative structure activity relationships (QSARs), 1249–1250
Quaternary ammonium compounds, soil salinity, 2778–2789
Quiescent cells, viable but not culturable (VBNC) phenomenon, 3252
Quinones and Q pool, 929, 1818–1824, 2489
aerobic respiration and, 151–153, 156, 157
Archaea and, 256
metal biodegradation and, 1883
Quorum sensing, 549, 1220, 3251

R

Radiation and effect on cells, 360–361, 2985–2994
Radioactive and nuclear wastes, 250–260, 2250, 1879–1893, 1902–1903, 2661–2674
algae biotechnology and, 199–200
biodegradation and, 250–260, 1446, 3045
vadose zone microbiology and, 3243–3244
Yucca Mountain repository for, 250–260
Radioallergosorbent (RAST) assay, 1361
Radioisotope analysis for proteomes and proteins, 308
Radiolaria, 2613
Radon, 1377
Raffinose, microbial protection from, 432

Rags (cleaning material), bacteria in home/residential environment, 340
Rainwater roof catchment systems, 2674–2685
Ralstonia, 1200
  activated sludge and, 3006
  biodegradation and, 1875, 2295–2300, 2388
  biofilms and, 567
  bioplastics and, 684–692
  gene transfer and, 1416
  metal biodegradation and, 1882, 1888, 1890
  subsurface microorganisms and, 2035
Raman confocal microspectroscopy, floc formation, 2008
Raman spectroscopic analysis, conditioning films, 935
Random amplified polymorphic DNA (RAPD), 812, 816
Raphidophtya, 2467
Raphidophytes, 2504–2518
Rayleigh vs. open system isotope behavior, subsurface microbial communities, 1457–1461
Readily biodegradable substrate (RB COD), 19–20
Rearrangement, genetic, 1200
Reclamation, desert environment, plant-microbe symbiosis, 1583
Recombinant DNA (See also Biotechnology; enzymes), 1176, 2693–2694
Recombinant DNA Advisory Committee (RAC), 2694–2707
Recreational waters, coliform bacteria, 900
Recycling, enzymes, paper, 1184–1185
Red algae (See also Rhodophyta), 837–843, 2468, 2505–2518
Red Blood Cell Lysis assay, 725
Red fluorescent protein (RFP), 1835–1836
Red snow, 2873
Red tide (See Harmful algal blooms and red tides)
Redfield ratios, primary production (marine), 2566–2575
Redox
  biofilms and, 2960–2963
  biomineralization and, 673–675
  metals, microbial, 1882–1886, 1896–1897, 1902–1913
  soil microorganisms and, 2062
Redoxin, 2293
Reductase, 2297–2300, 3414
Reduction, in biofilms, 2962
Reductive dechlorination, 513–514
Reductive dechlorination and dehalorespiration, 509–510
Reedbeds, wastewater treatment, 2693, 3389–3401
Refractory dissolved organic carbon (RDOC), 487
Regression analysis, 1007–1008
Regression decay, 2108
Regrowth processes, bacterial, 499–504
  assimilable organic carbon (AOC) and, 312–327
  biodegradable dissolved organic carbon (BDOC) and, 499–504
  growth measurement and calibration curves in, 318–319
  Monod equation for, 314
  potential for, 314–315
Regulation of stress response, 3049–3051
Regulation of commercial uses of microorganisms, 2693–2707
Regulation of water systems, in source water, 2974–2975
Regulator functions, in evolution, 1197
Relative humidity, spores and spore dispersion vs., 428–430
Remediation (See Bioremediation)
Remobilization, biofilms, 2965
Remote assay systems, 819
Renibacterium, 1857
Renilla, 1830–1831
Rennet, 1187
Reovirus, 2980–2984, 3398
Replication and repair of genes, 1195–1196
Replication and sampling intensity, in image analysis, 1685
Replicative transposons, 1201
Reporter genes, 727–729
  cold-adapted microorganisms and, 3215
  fluorescent probes and for, 1783–1784
  genetically modified microorganisms (GMMs) and, 1430–1431
  rhizosphere and soil microbes in, 349–351
Reproduction
  bacterioplankton and, 2519
  plankton and phytoplankton, 2510
  protozoa and, 2615
Reproductive parasites, 1120–1123
Residential environment, bacterial contaminants, 338–343, 577–587
Residential environment, fungi, 1372–1383
Resiliency of ecosystems, mycorrhizae, 2127–2128
Resistant bacteria, antibiotic, 607–608, 625
  biochips, biochip testing testing, 448–449
  bottled water and, 799
  Pseudomonads, 799
  bioaerosols and, 432
  fecal contamination and, 1258–1259
  heterotrophic bacteria and, 1545
  multiple antibiotic resistance (MAR), 1258–1259
  nosocomial infections (NI) and, 2235–2250
  in drinking water, 944
Resistance of hosts to viruses, 3308
Resolution, image analysis, 1785
Respiration
  aerobic, 149–160
  biofilms and, 82–83
  kinetics of growth and, 1753
  rates of, in soil, toxicity testing and, 3159
  soil microorganisms and, 2948–2949
  in wastewater/sewage, 3173
  wastewater/sewage treatment and, 3345–3346
Respiratory diseases, 338
  airborne toxigenic molds and, 185–186
  bioaerosols released from wastewater and, 3320–3330
  bioaerosols and, 404–416, 422
  enteroviruses and, 1151
  fungal allergens and, 1359–1366
  Legionella, 1788–1806
  Mycobacterium avium complex (MAC), 2112–2120
Respiratory syncytial virus (RSV), 2743
Restoration of image, 1785
Restriction endonuclease analysis (REA), 1096
Restriction fragment length polymorphism (RFLP), 443, 812, 815, 1003
  biofilms and, 563
  fecal contamination and, 1258
  hydrothermal vents and, 1609
  rotaviruses and, 2765, 2771
Retention of microbes, biofilms, 111
Retinal, 2470–2480
Retrotransconjuant, 1413
Retrotransfer, 1201–1202, 1413, 1427
Return activated sludge (RAS), 75, 79

Reverse osmosis (RO), home water treatment systems using, 1566–1567
Reverse transcriptase polymerase chain reaction (RT-PCR), 1162–1164, 2500
  astroviruses and, 333–335
  methanotrophic bacteria and, 1931–1932
  rotaviruses and, 2765, 2770–2771
  viruses and, 3291–2199
Reynolds number, 114, 1416–1417
Rhabdochromatium, phototropism, 2477–2489
Rhabdosphaera, 2508
Rhamnogalacturonase, 1187
Rhamnolipids, 714–715, 752–753, 764–765
Rhapidophyceae, 2685–2693
Rhizobacterium, 1831
Rhizobiaceae, 2549
Rhizobium, 345–354, 1705, 2282, 2720, 2786–2787, 3120
  biocontrol use of, 2703, 2704, 2705
  bioremediation and, 733
  cold-shock response and, 886–895
  desert environment and, 1583
  endosymbiosis and, 1119–1120
  genetically engineered microorganisms (GEMs) and, 1279
  green fluorescent protein (GFP) studies for, 1499
  legume symbiosis with, 2786–2787
  metal biodegradation and, 1894
  nitrogen fixation and, 2188–2211, 2196, 2201–2211, 2212–2221
  resistant bacterial strains in, stress response, 349
  rhizosphere and soil microbes in, 351
  soil microorganisms and, 2929
  soil salinity and, 2775, 2779–2789
  subsurface microbial communities and, 3067–3071
Rhizoclonium, 215
Rhizoctonia, 451, 462, 766, 1983, 2914
Rhizofiltration, 709
Rhizoplane, 2714
Rhizopoda, 2605
Rhizopogon, 2124–2132
Rhizopus, 1648, 1650, 1659–1660, 3057–3065
Rhizoremediation, 1424–1425
Rhizosolenia, 975, 2506
Rhizosphere (See also Nitrogen fixation; Soil microorganisms), 731, 2713–2728
  actinorhizosphere in, 2714
  antibiotics in, 347–348, 351
  Archaea and, 300–301
  bacteria in, 2717–2719, 2880–2894
  bacterial phytostimulators in, 343–354
  biocontrol and, 450–465
  biodegradation and, 695, 1424–1429
  biodiversity of soil within, 528–544
  biotic and abiotic factors in, vs. PGPR, 349–350
  carbon dioxide use in, 2721–2722
  chelation in, 2717
  chemotaxis of bacteria in, 348–349
  commercial PGPR products for, 351–352
  crop productivity and, 2722–2723
  deleterious rhizobacteria (DRB), 2717
  diazotrophy and diazotrophic bacteria and, 2541–2547, 2550–2551
  disease resistance and, 2722–2723
  effect of, 2714–2716
  endo-, 2714
  endosymbiosis and, 1118–1120
  enzymes in, 2899–2910
  exudates in, 2714, 2716–2717
  flooded soils and, 1322–1323, 1324
  fungi in, 2910–2918
  genetically engineered microorganisms (GEMs) and, 1424–1429
  global warming and, 2721–2722
  immobilization in, 2719–2720
  inoculation with PGPR and, 346, 350
  lysates in, 2715
  marine/ocean environment and, 2540–2548
  microsensors and, 349
  mineralization in, 2719–2720
  motility of bacteria in, 348–349
  mucigel in, 2715
  mycorrhizae and, 1385, 2120–2132, 2714
  nitrogen fixation and, 344, 2188–2221, 2720, 2722
  nutrient cycling in, 2719
  oxygen in, 2724–2725
  pH levels and, 2723
  phosphorous solubilizers in, 346
  phytometallophores in, 2717
  phytoremediation and, 731–734
  phytosiderophores in, 2717
  plant assisted microbial bioremediation, 1424–1425
  plant growth promoting rhizobacteria (PGPB), 2717, 2722
  plant growth promoting rhizobacteria (PGPR), 343–346, 349, 454–455, 2722
  plant growth regulators (PGRs) in, 344–345
  plant mucilage in, 2715
  polyhydroxyalknoate (PHA) in, 346–347
  polyhydroxybutyrate (PHB) in, 346–347
  protozoa and, 348
  reporter genes and, 349–351
  research in, 2725–2726
  resistant bacterial strains in, stress response, 349
  rhizoplane in, 2714
  rhizoremediation, 1424–1425
  root adsorption and colonization of phytostimulators in, 348, 2718–2719
  root structures and, 2715–2716, 2718–2719
  rotation of crops and, 2907
  rRNA probes for rhizobacteria in, 351
  secretions in, 2715, 2716–2717
  siderophores in, 347
  soil microorganisms in, 2880–2894
  soil salinity and, 2775
  submerged plants and, 2550, 2822–2833
  sulfate-reducing bacteria (SRB) and, 2547–2548, 2551, 3091–3092
  temperature and, 2724
  tracking introduced phytostimulators, 350–351
  vadose zone microbiology and, 3236–3246
  water and moisture in, 2724–2725
  wetlands and, 3404–3405
Rhodamine stains, 1676
Rhodanese, in soil, 2903–2910
Rhodobacter, 3314–3319
  aerobic respiration and, 155, 157
  biomineralization and, 675
  metal biodegradation and, 1905
  nitrogen fixation and, 2212–2221
  phototropism in, 2478–2489
Rhodobium, 2478–2489

Rhodococcus, 1201, 1204, 2294–2300
activated sludge foaming and, 3–7
biodegradation and, 1045, 1996, 2392–2393, 2414
biosurfactants/bioemulsions and, 753, 756
desert environment and, 1583, 1584
desulfurization and, 1048–1049
explosives biodegradation and, 1987
filamentous forms of, in activated sludge, 1288–1306, 1312
infectious airborne bacteria and, 1694
molecular probes using, in activated sludge foam monitoring, 4–7
subsurface microbial communities and, 3067–3071
toxic solvents and, 3150, 3153
Rhodocyclus, 32, 40
Rhodoferax, 1300–1306, 2035, 2877
Rhodomicrobium, 675, 3247, 3251
Rhodomonas, 2508
Rhodopene, 2459
Rhodophyta, 837–843, 2357, 2453–2470, 2505–2518
Rhodopinal. in paleolimnology, 2307–2317
Rhodopseudomonas, 173, 2193–2201, 3111–3116
Rhodopsins, 2453–2480
Rhodospillum, 83
Rhodospira, 2473–2474, 2478–2489
Rhodospirillum (See also Rhodothalassium), 1517, 2455–2458, 2465
bioplastics and, 683–692
halophiles and, 1506–1512
nitrogen fixation and, 2193–2201
Rhodosporidium, 127–128
Rhodothalassium, 1517–1529, 2478–2489
Rhodothermus, 1853
Rhodotorula, 872–885, 1368
Rhodovibrio, 1517–1529, 2478–2489
Rhodovulum, 1517–1529, 1854, 2477–2489
Ribose, 2716
Ribosomal Database Project (RDP), 532
Ribosomal intergenic spacer analysis (RISA), 2738–2739
Ribosomal RNA (rRNA), 246–259, 443–450
Ribosomal RNA targeted oligonucleotide probes, 1339–1340
Ribosomes, 2270, 2884
Ribotyping (See also DNA/RNA analysis), 2728–2741
amplified rDNA restriction analysis (ARDRA) in, 2737–2738, 2920
biochips for, 443–450
capillary electrophoresis in, 811–821
clone library screening in, 2734
cloning in, 2732–2733
denaturing gradient gel electrophoresis (DGGE), 2732, 2735–2737, 2921
fecal contamination and, 1261–1263
fingerprinting techniques in, 2734–2735, 2739
forward sample array hybridization, 2731–2732
paramagnetic beads and, 818
polymerase chain reaction (PCR), 2730, 2733–2739
ribosomal intergenic spacer analysis (RISA), 2738–2739
shotgun cloning in, 2732–2733
single-strand conformational polymorphism (SSCP) in, 2737
temperature gradient gel electrophoresis (TGGE), 2735–2737
temporal temperature gradient electrophoresis (TTGE), 2735–2737
terminal restriction fragment length polymorphism (T-RFLP) in, 2737–2738, 2921
Ribulose monophosphate (RuMP) pathway, methanotrophic bacteria, 1927–1936
Rice fields (See also Flooded soils), 1316–1333, 2196, 2741
Richelia, 975, 2214–2221
Ricin, bioterrorism, 778
Rickettsia, 1122, 1123, 1125
Ridgea, 1619
Rifampin resistance, biochip testing, 448–449
Riftia, 1118, 1606–1617, 1619
Rimicaris, 1118, 1609–1610, 1619
Ripening period following backwashing, in filters, 2707
Risk assessment, 2741–2753
applications for, 2749–2750
characterization of risk in, 2748–2749
dose response assessment in, 2745–2747
exposure assessment in, 2747–2748
hazard identification in, 2742
human health outcomes in, 2742–2744
Monte Carlo analysis in, 2749
mortality and morbidity rates in, 2743–2744
multiple exposure and immunity in, 2747
outbreaks of disease and, 2744–2745
transmission methods in, 2744
virus exposure, 2741–2753
River snow, 570–571
Riverine biofilms, 569–572
Rivers (See Aquatic ecosystems; Surface waters)
Rivers Pollution Prevention Act of 1876, 74
RNA (See DNA/RNA analysis)
Robbins device, adhesion in biofilms, 118
Rock salts, 1510–1511
Rock sampling, 829–830
Rocks (See also Caves and mines; Subsurface microbial communities; Weathering and biodeterioration)
endolithic microorganisms in, 1100–1112
igneous, microbial communities in, 1661–1673
oil bearing, subsurface microbes and, 2418–2420
sampling of, 829–830, 3074
subsurface microbial communities and, 1076, 1453–1468, 1824, 3065–3071
weathering and, 3364–3389
Rods, 2882
Root colonization, by rhizobia, 2715–2719
Root zones (See Rhizosphere)
Rosculus, 2594–2604
Roseobacter, 2214–2221, 2522, 2524–2526, 2549
nitrogen fixation and, 2214–2221, 2214
Roseococcus, 2877
Roseospira, 1517–1529
Roseospirillum, 2475, 2478–2489
Rotating annular bioreactors (RAB), 570
Rotating biological contactor, 695
Rotation of crops, 2907, 3122
Rotavirus, 93, 95, 331, 360, 2743, 2745, 2753–2773
bioaerosols and, 3327
in food, 3277–3287
in soil, 3273–3276
in source water, 2969–2978

in spas and hot tubs, 2979, 2980–2984
streams and, 3017
in wastewater stabilization ponds (WSBs), 3334
Rotylenchulus, 456
Roughing filters, 2012–2013
Royal Demolition Explosive (RDX) bioremediation, 706–712, 916–917, 923–924
rRNA targeted probes, bacterial counts using, 29–34
Rubber, and bioplastics, 682–692
Rubisco, biofilms, 85–86
Rubrivivax, 1299–1306, 1996, 1997
Rubrobacter, 1578
Ruegeria, 2549
Ruminococcus, 742–748
Run-of-mine bioleaching, 649
Ruppia, 2550
Rural water supplies, heterotrophic bacteria, 1548
Russula, 2124–2132
Rusts, 411–412

S

S bacteriocytes, 1114
Saccharolytic enzymes, biofouling vs., 606
Saccharomyces
adhesion in biofilms and, 120–121
biosurfactants/bioemulsions and, 762
cold-adapted microorganisms in, 872–885
desiccation and, 1031–1032
enzyme activity and, 2902
freeze drying and, 1354–1355
genomic studies and, 1440
metal biodegradation and, 1886
microarray study of, 1973
as microbial tracer in groundwater, 3197
radiation and, 2987
soil microorganisms and, 2913
toxic solvents and, 3151
Safe Drinking Water Act (SDWA), 2974–2975
Salicylic acid biodegradation, 2295–2300
Salinicoccus, 1506–1512
Salinity (See also Halophiles; Hypersaline environments), 2565, 2774–2789
accumulation vs. nonaccumulation in, 2781
ATP Binding Cassette (ABC) transporters and, 2781
bacteria tolerant to, 2779
bacteriophages and, 389
bacterioplankton and, 2520
compatible solutes and osmoprotectants in, 2776–2781
efflux of compatible solutes in, 2783
enteroviruses and, 1170
gram-negative bacteria in, 2783–2786
gram-positive bacteria and, 2781–2783
nitrogen fixation vs., 2786–2787
osmolarity and, 2775–2776
osmoprotection in, 2783–2786
phases of osmotic adjustment to, 2780–2781
plankton and phytoplankton, 2502, 2511
polar phytoplankton and, 2563
Rhizobium–legume symbiosis in, 2786–2787
salt stress reactions in, 2777–2781
sea ice microorganisms and, 2839–2840
soil microorganisms and, 2774–2789, 3270
stress response and, 3034
symports and, 2781
uptake systems and, 2782–2783
viruses and, 3270
Salinivibrio, 1501–1512
Salix, mycorrhizae, 2124–2132
Salmo, 979
Salmonella, 74, 338, 340, 341, 386, 882, 901, 1431, 1857, 2789–2802
activated sludge and, 71
bacteriophages and, 356, 375–384
bioaerosols and, 3324, 3325, 3328
biofilms and, 577, 2793
bioluminescent tracking of, 658
bioterrorism and, 775
disinfection and, 1065
fecal contamination and, 1257, 1268, 1272
flow cytometric studies of, 1950
in food, 3277–3287
genomic studies and, 1446
granular activated carbon (GAC) filters and, 1492, 1571–1572
heterotrophic bacteria and, 1544
high hydrostatic pressure (HHP) vs., 1555–1563
identification of isolates in, 1964
metal biodegradation and, 1890
as microbial tracer in groundwater, 3195
municipal solid waste landfills and, 1766
radioactive waste degradation and, 2666–2674
in source water, 2968, 2969–2978
in spas and hot tubs, 2979, 2980–2984
stress response and, 3035
subsurface microorganisms and, 2025
in wastewater stabilization ponds (WSBs), 3334–3336
wastewater/sewage treatment and, 2789–2802
in drinking water, 946, 950, 1721, 2789–2902, 3199
Salt aggregation test (SAT), 2007–2008
Salt as soil contaminant, 700–701
Salt attack, bioweathering, 3367
Salt bridges, 1707, 2005
Salt deposits, 1510–1511
halophiles in, 2024–2025
studies of, in paleolimnology, 2323–2324
Salt lakes, phototropism, 2476
Salt marshes, 2475, 2851–2852
Salt production, 2802–2809
Sampling and analysis, 2809–2822
activated sludge and, 68–69
air filled caves and, 822–825
airborne specimens and, 2818–2820
anaerobes and, 827
bacteria in home/residential environment and, 338
bacteriophages and, 376–381
bailers for, 2815
bioaerosol sampling of analysis, 392–404, 1130–1136
biochips, biochip testing, preparation of, 444–445
biodegradable products, 476–478
biofilms and, 624, 830–831, 2006, 2814
bioterrorism and, 781
bleaching of, 1785–1786
blood plasma technique for, 824
chlorine, 828–829
coliform bacteria and, 899
conditioning films and, 928–929
containers for, underwater handling, 826
Cyclospora and, 999–1001
denitrification and, 1015–1016
disinfection and, 1068–1069
floc formation and, 2006
flow cytometry and, 1952
flow through samplers in, 2813
fluorescent dyes for, 828–829, 1334–1335
fungi, 1369–1370, 1650–1651

Sampling and analysis, (*continued*)
genetically modified microorganisms (GMMs) and, 1432–1434
grab, 2812
groundwater, 1664–1665, 2815, 3073, 3077–3079
igneous rock microbial communities, 1662–1665
limit of detection (LOD) in, 2811
limit of quantitation (LOQ) in, 2811
lithified materials and rocks, 3074
municipal solid waste landfills and, bacterial populations, 1764–1765
neuston microbiology and, 2133–2134
nitrate, 828–829
Nyquist sampling frequency, 1785
on-site observations in, 827
optimum temperature for growth (OTG), 3025
oxygen, 828–829
periphyton and, 2358
petroleum hydrocarbons, 3073
pore water, 3073
postcollection activities and, 827
pressure containers for deep sea environments and, 2814–2815
processing and, 3082–3085
protozoa, 2596–2598, 2626–2627
psychrophilic bacteria, 2641–2642
quality assurance in, 3079–3082
quantity of, 2811
rock, 829–830
rotaviruses and, 2765
sediment, 827, 2815–2818, 3073
shipping, 3082–3085
sites for, in caves, 826–827
size of, number of, 2811
soil, 665–666, 2815–2818, 2896, 2919–2923, 2949–2950
spore prints in, 1652
storage of samples in, 831–832, 2811–2812
subaerial communities and, 3055–3057
subsurface microbial communities, 1076–1079, 2030, 3071–3087
sulfate-reducing bacteria (SRB) and, 3093–3094
toxicity testing and, in soils, 3166
tracers and, 3079–3082
transport of samples in, 831–832, 2811–2812
vadose zone, 3241
viruses, waterborne, 3290
volcanic tuffs and, 3311–3312
water filled caves, 825–828
waterborne specimens and, 2812–2815
San Juan Basin, 2043–2047
SANCHO model, biodegradable dissolved organic carbon (BDOC), 502
Sand filtration, 2333, 2707
Sandstone, 2042–2047
bioweathering and, 518–523, 3368–3370
subsurface microbial communities and, 3065
Saphrophytes (See also Fungi), 416, 1367
Sapporo-like viruses (SLVs), 1587
Saprospira, 1302–1306
Sarcina, 3057–3065
Sarcocystis, 3277–3287
Sarcomastigophora (See also Protozoa), 2594
Sargam, 199–200
Sarin (See Chemical warfare)
Satratoxin H, 185
Satratoxins, 1378
Saturation rate, 1008
Saxitoxin, 977, 987–995
Scab, 2913
Scales, in paleolimnology, 2317–2327
Scaling or mineral fouling, 619
Scanning confocal laser microscopy (SCLM), 2008
Scanning electron microscopy (SEM), 234, 635, 643
Scanning probe microscopy (SPM), 935–936
Scanning tunneling microscopy (STM), 935–936
Scenedesmus, 205
algae biotechnology and, 196
biomonitoring and, 3169
sea ice and, 2837–2844
toxicity testing and, 3221–3230
in wastewater stabilization ponds (WSBs), 3332
in wastewater/sewage, 3172
Scerlocystis, 2120–2124
Schistosoma, 1718
Schizophyllum, 1397–1400
Schizosaccharomyces, 2987
Schizothrix, 210, 979, 980, 1507–1512
aggregates and consortia in, 172–173, 172
biodeterioration of mineral materials and, 519
in desert soils and biological soil crust (BSC), 1020–1023
subaerial communities and, 3056–3065
Schneeberg disease, 1377
Scleroderma, 2124–2132
Sclerotinia, 451, 458, 1116, 2913
Sclerotium, 451, 454
Scopulariopsis, 1886
Scotiella, 840–843
Scottnema, 1026
Scutellospora, 2120–2124
Scutiocociliate, 2617
Scyphosphaera, 2508
Scytonema, 972
aggregates and consortia in, 173
in desert soils and biological soil crust (BSC), 1021–1023, 1579
photoautotrophic, in low-light habitats, 838–843, 838
subaerial communities and, 3056–3065
Scytonemin, 2314
Scytophycin, 978
Sea ice microorganisms (See also Cold-adapted microorganisms; Marine/ocean environments; Psychrotrophs; Snow and ice environments), 2476, 2833–2844
Seagrass (See also Zostera), 2550–2554, 2822–2833, 2852
Seasonal trends
protozoa and, 2627–2628
viruses and, 3296–3299
Seaweeds, photosynthetic pigments, 2464
Secchi depth, 1190
Secondary maximum contaminant levels (SMCL), algal blooms, 204–213
Secondary minimum, adhesion in biofilms, 104
Secretions, rhizosphere, 2715, 2716–2717
Secretons, 1209
Sectioning for whole cell hybridization, fluorescent probes, 1334–1335
Sedimentary bacteria, marine, 2827–2828
Sedimentary organic matter (SOM), subsurface microbial communities, 1455
Sedimentary rocks, 2418–2420
subsurface microbial communities and, 3065–3066
high temperature subsurface environment, 2047–2057
Sediments and sedimentation, 2844–2853
adhesion in biofilms and, 114–116

aggregates and consortia in, 164–165
algae vs., 1193–1194
algal solid phase assay (ASPA) in, 3227–3228
anaerobic processes and, 2844–2845
bacteria in, marine environment, 2827–2828
bacterial competition within, 2845–2846
bacteriophages and, 358
benthic fauna and, 2852
biodiversity within, 2848–2849
biogeochemical cycling in, 2844–2848
carbon cycle and, 2844
Cryptosporidium in, 1252–1256
diatoms and, 1059–1061
disproportionation in, 2849
emergent plants and, 2540–2548
food web and, 2844–2845
Giardia in, 1252–56, 1252
Heinrich sediments and, 2314
heterotrophic bacteria and, 1540
macroorganism interaction with, 2851–2852
metabolism within, 2848–2849
methanogenesis in, 1919–1920, 2846
mineralization and, 2844
nitrogen fixation and, 2218–2219
organic matter and, 2844
oxygen and, 2278–2279, 2844, 2847–2848
phosphorus cycling and, 2452
phototropism in, 2474–2476
protozoa in, 1252–1256, 2331–2332
salt marshes and, 2851–2852
sampling of, 2815–2818, 3073
seagrass communities and, 2852
seasonal effects on actions of, 2847–2848
stoichiometry of, 2847
sulfate-reducing bacteria (SRB) and, 2844–2853
sulfur cycling and, 2844–2853
viruses and, 1252–1256
in water treatment systems, 2015–2018, 2266
zonation within, 2845–2846
Seeding of microbes, in septic tank systems, 2862
Segmentation, in image analysis, 1681–1682
Selanastrum, 3172, 3221–3230
Selectors, activated sludge foaming, 5–7
Selenihalanaerobacter, 1521–1529, 1521
Selenium
bioremediation and, 694, 708, 709
microbial methylation of, 1884–1885
sulfate-reducing bacteria (SRB) and, 3089
Selenite bioremediation, 708
Selenomonas, 744–748
Self-organizing maps (SOMs), 1007
Semiconductors, 633
Sensitization, in allergic response, 1360, 1368–1369
Separation distance in distribution of soil microorganisms, 2897
Separation membranes, Archaea, 259–276
Septic tank systems, 74, 2853–2864
Sequencing batch biofilm reactor (SBBR), 51–52
Sequencing batch reactor (SBR), 43–54, 75, 695, 847, 2002
Sequencing, genomic, 1442–1443, 2491
Serial Endosymbiosis Theory (SET), 1123–1126
Serine, 2716
Serotyping, rotaviruses, 2768–2769
Serpentinization, lithotroph, 1827
Serratia, 3196
bacteriophages and, 355
biocontrols and, 455
biofilms and, 562, 581
cold-adapted microorganisms in, 872–885
coliform bacteria and, 906–914
genetically modified microorganisms (GMMs) and, 1437
granular activated carbon (GAC) filters and, 1491, 1571–1572
heterotrophic bacteria and, 1541–1552
high hydrostatic pressure (HHP) vs., 1555–1563
as microbial tracer in groundwater, 3195
municipal solid waste landfills and, 1766
nosocomial infections and, 2236–2250
in pulp and paper industry, 2650
rhizosphere and soil microbes in, 346
spores and spore dispersion in, 430
subaerial communities and, 3057–3065
in wastewater stabilization ponds (WSBs), 3334
in drinking water, 945
Serum neutralization (SN), adenoviruses, 98
Sesbania, 1120, 2202
Setosphaeria, 410
Settling, 1725
activated sludge and, 59, 75, 1306–1315
aggregates and consortia in, 162
algal blooms vs., 209
biodegradable dissolved organic carbon (BDOC) and, 494
floc formation and, 2007
Sex pilus, 2885
Shale, 2042–2047
biodeterioration of mineral materials and, 518–523
subsurface microbial communities and, 3065
Shannon's index, 528, 1003
Shear rates, in biofilms and aggregates, 162, 2341
Shear stress, and adhesion, 117
Sheath flow, flow cytometry, 1945
Shellfish
amnesic shellfish poisoning (ASP), 2687
astroviruses and, 334–335
bacteriophages and, 361, 382
cyanobacteria and, 979, 989, 991
hepatitis viruses and, 1534
neurotoxic shellfish poisoning (NSP), 2687
paralytic shellfish poisoning (PSP), 2687
Salmonella in, 2796
toxins in, 1857, 2687
Shewanella, 1406, 1672, 1856
cold-adapted microorganisms in, 872–885
genetically altered, in biotechnology, 1446–1447
in groundwater, 1671
hydrothermal vents and, 1610
metal biodegradation and, 1882, 1884, 1903, 1905, 1908
microarray study of, 1974
psychrophilic, 2640–2646
subsurface microbial communities and, 3068, 3070
Shield, 2320
Shigella, 338, 2339, 2797, 2865–2871
bioaerosols and, 3322
bioterrorism and, 771
Clostridium and, 869–871
detection of, 1133
disinfection and, 1065
fecal contamination and, 1257
in food, 3277–3287
heterotrophic bacteria and, 1544
in source water, 2969–2978

Shigella, (*continued*)
in spas and hot tubs, 2979, 2980–2984
streams and, 3019
subsurface microorganisms and, 2025
in water treatment systems, 1721, 3199
Ship hulls, biofouling, 623
Shorea, 2125–2126
Shorelines (See Marine/ocean environment)
Shotgun cloning, 2732–2733
Shutdown cell concepts, in viable but not culturable (VBNC) phenomenon, 3251
Sick building syndrome (SBS), and fungi, 1372
Side angle light scatter (FALS), in flow cytometry, 1945
Siderite, 1464–1466
Siderocapsa, 572, 676, 3101
Sideroccoccus, 3101
Siderophores
alkaliphiles and, 228
biocontrols and, 454
metal biodegradation and, 1881–1882
rhizosphere and soil microbes in, 347
soil microorganisms and, 2887
Sigma factors, in stress response, 3040
Signal detection, bioluminescence, 1831–1832
Silica, 2515
Silica deposition vesicles (SDV), 2320–2321
Silicates
biodeterioration of mineral materials and, 517–518
bioleaching and, 632–642
biomineralization of, bacterial, 670–681
Silicium, 928
Silicoflagellates, 2467
Silicon
biodeterioration of mineral materials and, 517–518
hydrothermal vents and, 1618
Silicosis, in Mycobacterium avium complex (MAC), 2113–2120
Silver, 1879–1893, 1902–1913
biomineralization and, 671–681
resistance of bacteria to, 608
Silvex biodegradation, 2394
Simazine composting, 921–922
Simplot anaerobic bioremediation, explosives biodegradation, 1988–1989
Simultaneous nitrification/denitrification (SND), 77
Single cell detection, flow cytometry, 1951
Single reactor system for high ammonium removal over nitrite (SHARON), 11–14
Single-strand conformational polymorphism (SSCP), 28–29, 299, 536–537, 813, 2737
Sinking control, plankton and phytoplankton, 2510–2511
Sinorhizobium, 1434
endosymbiosis and, 1120
genetically engineered microorganisms (GEMs) and, 1279
nitrogen fixation and, 2201–2211
soil salinity and, 2775, 2779–2789
Siphoviridae/Siphovirus, 365–374
16S rDNA analysis, 824–825, 1334, 2729–2741
aggregates and consortia in, 175–176
Archaea and, 260, 279
bacterial counts using, 27–34, 62, 64, 69, 2523–2524
biofilms and, 563
endosymbiosis and, 1120
genomic studies and, 1450–1451
hydrothermal vents and, 1606, 1609
hypersaline environment and, 1508
microarray study of vs., 1974
phylogenetic tree-based on, 1441
soil genetic ecology, 2919–2923
Skeletonema, 198
Skermania, 3–7, 1288–1306
Skin diseases, 338, 2980–2984
Slime molds, 2912–2913
Slit impactor type bioaerosol sampler, 395
Sloughing, biofilms, 1083
Slowly biodegradable substrate (SB COD), 19–20
Sludge (See Activated sludge)
Sludge biotic index (SBI), protozoa, 2609–2611
Sludge volume index (SVI), 1288
Slurry phase process sabre, explosives biodegradation, 1988–1989
Slurry systems, activated sludge, 51–52
Small round structured viruses (SRSVs), 1587
Small round virus (SRV), 1587, 2980–2984, 3277–3287
Small subunit ribosomal RNA (SSU rRNA), 812, 1333, 2729–2741, 2919–2923
Archaea and, 278, 295, 301
soil microorganism and diversity in, 531–535
Smallpox, bioterrorism, 777
Smithella, 745
Smoker vents (See Hydrothermal vents)
Smuts, spores, and spore dispersal, 410–411
Snow algae, 872–873
Snow and ice environments, 2872–2880
aggregates and consortia in, 164, 174–175
Archaea and, 286–287
cold-adapted microorganisms in, 872–885
cyanobacteria and Gloeocapsa, 963
permafrost and, 2367–2385
phototropism in, 2476
sea ice microorganisms, 2833–2844
subsurface microorganisms and, 2025
Snow, John, 3320
Snow, marine, 2002
Soakaways, septic tank systems, 2856
Soda lakes, phototropism, 2476
Sodium, alkaliphiles, 138
Sodium alginate, algae biotechnology, 192
Sodium diethylithiocarbamate, 605–606
Sodium dodecyl sulfate (SDS), 626
Sodium, 1707, 1902–1913
Sodium/hydrogen antiporters, alkaliphiles in, 138
Soft rot fungi, 1389
Soil adaptation, 2386
Soil contamination and bioremediation (See also Quality of soil; Soil microorganisms), 700–707, 722–737, 921–922, 2927–2929
bioavailability of contaminants vs., 725
biosurfactants/bioemulsions and, 764–765
concentration of contaminants in, 725
encapsulation of microbial cells for, 730
genetic engineering and, 729–730, 729

genetically engineered microorganisms (GEMs) in, 726, 729–730
halogenated compounds and, 702–705
hydrocarbon, 700–735
metabolism of contaminants in, 726
military chemicals in, 707
monitoring of, 727–729
most probable number (MPN) quantification in, 727
nucleic acid probes in, 728–729
oil spills and bioremediation of (See also Oil spills), 698–701
pesticides and herbicide, 706, 2395–2397
phytoremediation and, 731–734
polychlorinated biphenyl (PCBs) in, 726–735
polymerase chain reaction (PCR) quantification in, 727
quantification of degrading microorganisms in, 726–727
rate monitoring in, 730–731
reporter systems, reporter genes in, 727–729
rhamnolipids in, 764–765
solubility of contaminants vs., 725
sorption characteristics of contaminants vs., 725
toxicity in, 724–725
Soil genetic ecology, 2918–2923
Soil microorganisms, 2057–2065, 2713–2728, 2880–2894, 2944–2957
accelerated degradation and, 2386
acclimation in, 2386
accumulation of storage materials in, 346–347
acetogens in, 2892
acidophiles and, 2889
actinomycetes in, 2882, 2889, 2912
adaptation of, 2057
adenosine triphosphate (ATP) and, 2883–2884, 2889
adhesion in, 2926
aerobic processes and, 2888, 2930–2932
aggregate formation in, 2924–2925
Amplified ribosomal DNA restriction analysis (ARDRA), 536–537
anaerobic processes and, 2888, 2891–2893, 2932–2933
analysis of distribution of, 2896–2898
Antarctic, 1023–1029
antibiotics in, 347–348, 351
appendages to, 2885–2886
Archaea and, 293–305, 2880–2886
Ascomycota in, 2913–2914
assessment of, 665–667
atmospheric composition ad, 2061
Bacillus thuringiensis and (See Bacillus thuringiensis), 2058
Bacteria and, 664–665, 2880–2894, 2912
bacterial artificial chromosome (BAC) and, 539
bacterial phytostimulators in, 343–354
bacteriophages and, 381, 389–390
Basidiomycota in, 2914
bioaugmentation and bioremediation in, 434–442
biocontrol and, 450–465
biodegradation and bioremediation by, 349–350, 2923–2936
biodiversity in, 294–295, 528–544, 2925–2926
biological factors affecting, 1750–1752
biological soil crusts (BSCs) and, 1019–1023
biomass of, 294–295, 663–670, 2895, 2948
biosphere and, 294
biotic and abiotic factors vs. PGPR in, 349–350
capsules and, 2884
carbon and, 663, 666–670, 2059–2060, 2887
carbon dioxide and, 2057, 2061
carbon monoxide and, 294
carbonyl sulfide and, 294
catalytic properties of abiotic soil components and, 1750
cell envelope in, 2881–2884
cell retention on surfaces, 2926
cell structure and function in, 2880–2886
cell wall in, 2882
characteristics of, 2063
chemoautotrophs in, 2889, 2891–2892
chemotaxis of bacteria in, 348–349
chemotrophs in, 2887
chloroform fumigation incubation method (CFIM) and, 666–667, 2948
chytrids in, 2913
clays and, 2058–2059
cold desert environment and, 1023–1029
cold-adapted microorganisms in, 872–885, 2889
colonization in, 2926–2927
commercial PGPR products for, 351–352
community composition of, 294, 528, 664, 665, 1751–1752
contaminants vs., 2927–2929
culturing of, 666–667
cyanobacteria in, 2890–2891
cytoplasm in, 2884–2885
cytoplasmic membrane in, 2883–2884
decomposition in, 2911–2912
denitrifying bacteria in, 2891
density and distribution of, 2925–2926
desert environment and, 1019–1029, 1576–1586
differential display (DD) technique for, 538
diffusion of gases through soil and, 1749
diffusion of solutes through soil, 1748–1749
distribution of, 665, 2894–2899
disturbance of soils and, 3122
DNA analysis of, 528–544
electromagnetic radiation and, 2061
electron transport and, 2884
endolithic microorganisms in, 1100–1112
endospores in, 2886
environmental factors vs., 1752
enzymes of, 2899–2910, 2949
Eucarya and, 2880–2886, 2912
extracellular enzymes and, 1750
factors affecting activity of, 2057–2065
fauna in, 664–665
FFSB cluster in, 533–534
"fingerprinting" of microbial community in, 536–537
flooded soils, 1316–1333
fluorescence in situ hybridization (FISH) studies of, 529, 538
functional gene analysis in, 535–536
fungi in, 664–665, 2910–2918
gas–liquid interface and, 3274–3275
genetically engineered microorganisms (GEMs) in, 726, 729–730
genetically modified microorganisms (GMMs) in, 1429–1440
gram-negative bacteria in, 2881–2886
gram-positive bacteria in, 2881–2886
green bacteria in, 2992–2993

Soil microorganisms, (*continued*)
green fluorescent protein (GFP) studies for, 1498
growth and uptake parameters in, 1751
growth factors for, 2060
habitat of, minerals and organic matter in, 663–664, 2888–2889
halophiles, 297–298, 2889
heterotrophs in, 2889, 2891
hot desert environment and, 1576–1586
humic substances and, 1750
hydraulic conditions and, 3273
hydrogen oxidizing bacteria in, 2890
immobilization and, 2887
inclusions in, 2884–2885
inoculation with PGPR and, 346, 350
interactions among, 2063
ionic composition and, 2060
iron and, 2887
iron oxidizing bacteria in, 2890
isotropic semivariance in, 2897
kinetics of growth in, 1748–1757
land use and, 2895
leaching in, microbial, 2890
lichens in, 2914
limiting factors to, 1751
lipid biomarkers of, 1821
lipopolysaccarides (LPS) in, 2882
metabolism of, 346, 2951–2956
metagenome analysis of, 539
methane and methanogens in, 294, 296–297, 2057, 2892
microbial activity analysis, at functional level, 537–539
microhabitats for, 2058–2059
microscopic study of, 666
mineralization and, 2887
minerals and, 2060
modeling, 1753–1755
molecular characterization of, 528–544
motility of bacteria in, 348–349, 2926
mycolic acid and, 2881
mycorrhizae and, 2120–2132, 2911–2912
nicotinamide adenine dinucleotide (NAD) in, 2887
nicotinamide adenine dinucleotide phosphate (NADP) in, 2887
nitric oxide and, 294
nitrification and, 2949
nitrifying bacteria in, 2889–2890
nitrogen and nitrogen cycle of, 663, 667–670, 2936–2944
nitrogen fixation (BNF) in, 294, 344, 2057–2058, 2188–2211, 2887, 2936–2944
nitrous oxide and, 294
nucleoids in, 2884
nutrients and energy sources for, 663, 2057, 2059–2060, 2887, 2927, 2946–2947
oil spills vs., 700–701
organic matter and, 663–664, 669–670, 3271–3272
osmosis and, 2775–2776
oxidation reduction potential, 2062
oxygen and, 2057, 2061, 2887–2888
pathogenic bacteria in, 2058
periplasm in, 2884
permafrost and, 2367–2385
pesticide degradation and, 2385–2402
pH levels and, 2061–2062, 2889, 3269–3270
phospholipid ester linked fatty acids (PLFA) studies in, 538–539
phosphorous solubilizers in, 346
photoautotrophs in, 2890–2892
phototrophs in, 2887
phylotype richness in, 528
plant growth and, 663
plant growth promoting rhizobacteria (PGPR), 343–345, 349, 454–455, 2722
plant growth regulators (PGRs) in, 344–345
plasmids in, 2884
polyhydroxyalknoate (PHA) in, 346–347
polyhydroxybutyrate (PHB) in, 346–347
population size and, 1750–1751
pressure and, 2061
protozoa and, 348, 2594
psychrophiles and, 2889
purple bacteria in, 2992–2993
qCO2 and, 2954–2956
quality of soil vs., 2944–2957
resistant bacterial strains in, stress response, 349
respiration and, 2948–2949
rhizosphere for, 2713–2728
ribosomes in, 2884
RNA analysis of, 528–544
root adsorption and colonization of phytostimulators in, 348
rRNA probes for rhizobacteria in, 351
salinity and, 700–701, 2774–2789, 3270
salt stress reactions in, 2777–2781
sampling of, 665–666, 2815–2818, 2896
in sediments, marine, 2540–48
separation distance in distribution of, 2897
Shannon's index and, 528
siderophores in, 347, 2887
single-strand conformational polymorphism (SSCP), 536–537
slime molds and, 2912–2913
small subunit ribosomal RNA (SSU rRNA) analysis of, 531–535
soil adaptation and, 2386
soil genetic ecology and, 2918–2923
soil organic matter (SOM) and, 2945–2957
soil structure and, 2924–2925, 3122, 3271
solar radiation and, 2061
sorption and, 1749–1750
spatial distribution of, 2063
staphylococci in, 2882
streptobacilli in, 2882
streptococci in, 2882
substrate induced growth response (SIGR) in, 1750
substrate induced respiration (SIR) in, 1750
subsurface microbial communities and, 1074–1081, 1453–1468, 3065–3071
sulfate-reducing bacteria (SRB) in, 2891
sulfolobales and, 298–299
sulfur and sulfur cycle in, 2887, 3104–3116
sulfur oxidizing bacteria in, 2890
surfaces of attachment for, 2062–2063
sustainable agriculture, 3116–3126
temperature and, 1752–1753, 2061, 3268
temperature gradient gel electrophoresis (TGGE), 536–537
terminal fragment length polymorphism (T-RFLP), 536–537
terrestrial cluster in, 533–534
thermophiles in, 2888–2889
thermoplasmales and, 297
thermoproteales in, 298

3/2 power kinetics, to model growth, 1755
toxic chemicals vs., 2927–2929
toxicity testing for, 3155–3168
trace gases and, 3183–3194
tracking introduced phytostimulators in, 350–351
vadose zone and, 3236–3246
viruses in, 3268–3276
water and moisture for, 1753, 2057, 2060–2061, 2925, 3268–3269
water molds and, 2912
xerophiles and, 2889
yeasts in, 2913–2914
Zygomycota in, 2913
Soil organic matter (SOM), 2945–2957
Soil sampling, 2815–2818
Soil slurry sequencing batch reactor (SS-SBR), 51–52
Soil vapor extraction, 695
Solar cosmic radiation (SCR), space microbiology, 2989–292991
Solar lakes, sabkhas, pools and lagoons of Sinai, 1509
Solar particle events (SPEs), 2989–2991
Solar powered disinfection system, for RRCS, 2682–2683
Solar radiation (See also Ultraviolet radiation)
algae and, 1192–1193
Antarctica, 1025, 1103
bacterioplankton and, 2520
desert environment and, 1021–1022
enteroviruses and, 1170
fungi and growth and, 1368
heterotrophic bacteria and, 1540
neuston microbiology and, 2136–2137
nitrification vs., 2158–2159
periphyton vs., 2362
phototropism in, 2473–2474
plankton and phytoplankton, 2502, 2512
polar phytoplankton and, 2555–2556, 2560–2562
sea ice microorganisms and, 2840–2841
soil microorganisms and, 2061
in source water, 2970
space microbiology and, 2985
subaerial communities and, 3059, 3060–3061
ultraviolet and (See Ultraviolet radiation)
in wastewater stabilization ponds (WSBs), 3336
Solar salt works, 2804–2809
Solar salterns, 1508–1509, 1511
Solemya, 1606
Solentia, 172–173
Solid waste management
bioaerosols released from, 3320–3330
Solid waste treatment (See Municipal solid waste landfills)
Solids retention time (SRT), 2006
Solubility, bioremediation, of contaminants, 725
Soluble methane monooxygenase (sMMO), 435–436
Soluble microbial products (SMP), activated sludge processing, 24–26
Soluble nonreactive phosphorus (SNP), 2448–2453
Soluble reactive phosphorus (SRP), 217–218, 2448–2453
Solvents biodegradation, 507–516, 693, 713, 714, 1447–1448, 3147–3155, 3368
Soman (See Chemical warfare)
Somatic coliphages (See also Bacteriophages), 355–356, 377–379, 2709
Sophorolipids, biosurfactants/bioemulsions, 753–755
Sorghum, nitrogen fixation, 2199
Sorption characteristics, 1749–1750
biofilm, 2958–2967
bioremediation and, of contaminants, 725
extracellular polymeric substances (EPS) and, 1228–1229
Sorting cells, flow cytometry, 1944–1958
SOS response, viable but not culturable (VBNC) phenomenon, 3252
Source water protection, 2967–2978
assimilable organic carbon (AOC) in, 2972
biochemical oxygen demand (BOD) in, 2972
chemical oxygen demand (COD) in, 2972
Clean Water Act (CWA) and, 2975
Clostridium in, 2974
coliform bacteria in, 2973
coliphages in, 2974
competition among microbes in, 2970
dissolved oxygen (DO) in, 2972
enterococci in, 2974
Escherichia coli in, 2973
fecal contamination in, 2973, 2974
fecal pathogen transport to, 2968–2970
groundwater and, 2971–2972
indicators of quality in, 2972–2974
microscopic particulate analysis (MPA) of, 2972
nonmicrobial indicators of fecal contamination in, 2974
nutrients and, 2970
pathogen sources in, 2968
protective strategies of microbes in, 2970–2971
regulation of, 2974–2975
Safe Drinking Water Act (SDWA), 2974–2975
solar radiation and, 2970
stormwater in, 2969–2970
stream microbiology and, 3015–3024
streptococci in, 2974
surface waters and, 2971
temperature of water and, 2970
total organic carbon (TOC) in, 2972
total oxygen demand (TOD) in, 2972
ultraviolet and, 2970
vadose zone microbiology and, 3236–3246
Souring, petroleum reservoirs, 2422
Soxhlet extraction, 924
Soygro, 351
Spa and hot tub microbiology, 2978–2984
Space microbiology, 2985–2996
antibiotic effectiveness in, 3001
clinorotation and simulated microgravity in, 3000
crew health and life support systems, 3001–3002
desiccation and, 1035–1039
early experiments in, 2998
eukaryotes and, 3000–3001
genetics and, 3001
interplanetary transfer of life and, 2994–2995
Martian life and, 2985, 2995
meteorites and, 2985, 2994
microgravity and, 2985, 2994, 2996–3004
mutations, radiation induced, 2993
photolyase and, 2987
radiation and effect on cells, 2985–2994
sporulation and secondary metabolites in, 3002–3003
subsurface microorganisms and, 1080
Spaherosporella, 2125–2126

Spaherotilus, 3101
Spalling, 517
Sparganium, 2548
Sparging, 695
Spartina, 1397, 2539–2554
Specific affinity, 1742–1743
Specific bacterial growth rates, 3344–3345
Specific UV absorbance (SUVA), biodegradable dissolved organic carbon (BDOC), 485
Specificity, microbial insecticides using, 1710
Spectrophotometric analysis, pigments, 2462
Spelaeopogon, 838–843
Spelunking (See Caves and mines)
Spent filter backwash water (SFBW), 2262–2267
Sphacelotheca, 411
Sphaerobacter, 3129
Sphaerotheca, 454
Sphaerotilis, 3009
  activated sludge foaming and, 4–7
  bacterial counts using, 27–29
  biomineralization and, 675
  filamentous forms of, in activated sludge, 1291–1306, 1310, 1312
  metal biodegradation and, 1897
Sphagnum, endosymbiosis, 1118
Sphalerite, 633, 637–638
Spheroidene, in paleolimnology, 2307–2317
Spheroidenone, in paleolimnology, 2307–2317
Sphingomonas, 1204, 3314–3319
  activated sludge and, 71
  biodegradation and, 2409
  granular activated carbon (GAC) filters and, 1490, 1569–1570
  subsurface microbial communities and, 2035, 3065–3071
Spiniferomonas, 2317–2327
Spirilla, 2882
Spirilloxanthin, 2459
Spirillum, 57, 166, 316
Spirobis, 614
Spirochaeta, 57, 1124, 1123, 1515–1529, 1515, 2885
Spirogyra, 205
Spiromicrovirus, 364–374
Spiroplasma, 1114–1115, 1122
Spirulina, 980, 1507–1512, 1853
  algae biotechnology and, 189–204
  alkaliphiles and, 134
  nitrogen fixation and, 2193–2201
  salt production and, 2805–2809
Spodoptera, biocontrol use, 2701
Sponges (cleaning material), bacteria in home/residential environment, 340
Spongospora, 2913
Sporangiospores, 1647, 2889
Sporcybe, 945
Spore prints, 1652
Spores and spore dispersal (See also Allergies; Fungi)
  active dispersal mechanisms in, 405–407
  aerobic endospores and, 140–149
  airborne toxigenic molds and, 183–184
  airborne, of fungi, 183–184
  apple scab spores in, 413
  ascospores in, 412–414
  Aspergillus, 418
  atmospheric transport of, 407
  basidiospores in, 412–414
  bioaerosols and, 404–416
  bioterrorism and, 779–789
  blue mold, 414–415
  Buller's drop and, 406
  deposition of, 407, 428
  desiccation and, 1029, 1031, 1037–1041
  diffusion rates for, 427
  dispersal mechanisms in, 405–407
  dry air spora in, 405, 408–414
  fungal, 404–415, 1359–1383, 1647–1653, 3354–3355
  Fusarium, 413–414
  high hydrostatic pressure (HHP) vs., 1559–1563
  late blight of potato, 414
  macrofouling, from algal, 615–616
  as microbial tracer in groundwater, 3197–3198
  microgravity and, 3002–3003
  moist air spora in, 412–414
  mycobacteria and, 416–417
  open air factor (OAF) and, 430–431
  oxygen inactivation in, 430
  ozone vs., 430–431
  passive dispersal mechanisms in, 405
  pollutants vs., 430
  relative humidity vs., 428–430
  rusts, 411–412
  sampling of, 1650–1651
  saphrophytes and, 416
  smuts, 410–411
  temperature vs., 431
  ultraviolet radiation vs., 431
  uredospores in, 411–412
Sporidesmium, 454
Sporobolomyces, 1367–1372, 2369, 3057–3065
Sporomusa, 1320
Sporotrichum, 2666–2674
Sprinkler type wastewater systems, bioaerosol release from, 3323–3324
Spriophyllum (See also Gallionella), 1401
Sprirogyra, 215, 2304–2306
Spumella, 2595–2604, 3197
Stabilization of activated sludge, 738
Stabilization and maturation period in composting, 915
Stabilization ponds, wastewater (WSBs), 3330–3337
  algae in, 3331–3332
  ammonia and sulfide toxicity in, 3332
  bacteria in, 3330–3331, 3334–3336
  biological oxygen demand (BOD) and, 3336
  helminth eggs in, 3333–3334
  light intensity and, 3336
  pathogen removal from, 3333–3336
  pH levels and, 3331–3332, 3336
  photosynthetic bacteria in, 3332–3333
  protozoa in, 2333, 3334
  temperature and, 3335–3336
  time of retention and, 3335–3336
  viruses in, 3334
Stachybotrys, 182–189, 1648, 1660
  in allergies and illness, 1367–1372, 1374–1383
  biofilms and, 555
  bioterrorism and, 779
  detection of, 1133, 1134
Stalk forming bacteria, Gallionella as, 1401–1408
Staphylococcus, 339, 340, 341, 499, 1629–1646
  adhesion in biofilms and, 123
  bacteriophages and, 355
  bioaerosols and, 3327
  biofilms and, 556, 562, 577, 580, 1215, 1218, 2342–2356
  bioluminescence in, 658–662
  bottled water and, 798
  fecal contamination and, 1269, 1273
  gene transfer and, 1409, 1413–1415
  heterotrophic bacteria and, 1545, 1548
  high hydrostatic pressure (HHP) vs., 1555–1563
  municipal solid waste landfills and, 1766

nosocomial infections and, 2236–2250
resistance in, 607
soil microorganisms and, 2882
in spas and hot tubs, 2980–2984
spores and spore dispersion in, 421
subsurface microbial communities and, 3067–3071
in drinking water, 944, 945
Staphylococcus aureus enterotoxin (B/SEB), bioterrorism, 778
Staphylothermus, 1234, 1235, 1611, 1625–1628
Starch degrading enzymes, 223–226, 1180–1183, 1210
Starches, bioplastics, 682–692
Starvation survival, 2019–2028, 3036–3037
oligotrophic bacteria, 2267–2277
stress response and, 3041–3045
subsurface microorganisms and, 1080
Staurastrum, 2304–2306
Stauroneis, 1054, 1058
Steady state, 1738–1740
Stemphylium, 412, 1579
Stenotrophomonas, 420, 454, 1987, 2236–2250
Steric forces, 2004
Sterols
biofilms and, 85–86
fecal contamination and, 1257
Stetteria, 1525, 1629–1646
Stichococcus, 1102–1106
biodeterioration of mineral materials and, 519
photoautotrophic, in low-light habitats, 840–843
subaerial communities and, 3056–3065
Stigeoclonium, 196, 215, 2362
Stigmatella, 1578
Stigonema, 519, 2835–2844, 3056–3065
Stoichiometry, in activated sludge processing, 17–18
Stoichiometric matrix, in biofilms, 2086–2087
Stokes–Einstein equation, 1749
Stone, bioweathering, 3364–3374
Storage of samples, 2811–2812
Storage polymers, 3004–3014
activated sludge studies in, 3010–3011
anaerobic/aerobic storage in EBPR process and, 3011–3013
anoxic storage in nitrogen removal processes and, 3011
balanced growth conditions in, 3007
bulking and foaming control with, 3008–3011
enhanced biological phosphorus removal (EBPR) and, 3005, 3011–3013
glycogen as, 3005
kinetic (internal) induction and, 3007–3008
metabolic (external) induction and, 3006–3007
mixed culture studies in, 3010
polyhydroxyalkanoates (PHA) as, 3005
polyphosphates as, 3005
pure culture studies in, 3009–3010
unbalanced growth conditions and, 3006–3008
Storage-related phenomenon, subsurface microbial communities, 3315–3316
Stored water supplies
heterotrophic bacteria and, 1541–1552
off-stream storage in, as pretreatment, 2013–2014
Stormwater, 2969–2970
Stramenopila, 1394–1400, 2912
Stream microbiology (See also Surface water), 3015–3024
Streptobacilli soil microorganisms, 2882
Streptococcus
adhesion in biofilms and, 127–128
bioaerosols and, 3321, 3327
biofilms and, 82, 555
bioluminescence in, 658–662, 1831
biosurfactants/bioemulsions and, 765
fecal contamination and, 1256, 1264–1278
filamentous forms of, in activated sludge, 1302–1306
fluorescent studies of, 1835
gene transfer and, 1409, 1413–1415
granular activated carbon (GAC) filters and, 1489–1496
high hydrostatic pressure (HHP) vs., 1556–1563
in marine/ocean environment, 1702
metal biodegradation and, 1889
microarray study of, 1973
municipal solid waste landfills and, 1766
soil microorganisms and, 2882
in source water, 2974
in spas and hot tubs, 2980–2984
viable but not culturable (VBNC) phenomenon and, 3253
water treatment systems and, 2013
Streptomyces, 423, 458, 1102–1106, 2062, 2282, 2723, 2889
alkaliphiles and, 223, 226
biocontrols and, 452, 455, 458
desert environment and, 1578, 1583
gene transfer and, 1409
genetically altered, in biotechnology, 1448
green fluorescent protein (GFP) studies for, 1498
metal biodegradation and, 1890
microbial insecticides using, 1710
microgravity and, 3002
nitrogen fixation and, 2191
soil microorganisms and, 2912
stress response and, 3048
subsurface microbial communities and, 3067–3071
Streptoverticillium, 880, 2912
Stress and strain forces, in biofilms, failure, 547–548
Stress proteins, 3028, 3030–3031, 3036–3039
Stress response, 3024–3034
antioxidants and, 3031
antistress mechanisms and, 3030–3031
Bacteria and, 3025, 3034–3046
bioremediation/biodegradation and, 3043–3045
built in protein stability and, 3031
cell membranes and, 3031
cell robustness and, 3038–3039
chaperones and, 3028, 3048, 3050, 3052
chaperonins and, 3028–3029, 3048, 3052
cloning and, 3026
cochaperones in, 3030
DNA repair in, 3039
early studies of, 3025–3026
envelope reinforcement in, 3039
Eo70 and, 3039–3040
Eucarya and, 3025
general stress resistance (GSR) and, 3038–3043
heat-shock protein (hsp70) and, 3026–3028, 3050
heat-shock proteins and, 3031, 3049
heat-shock, 3046–3055
holoenzymes and, 3039
manipulating robustness and, 3045

Stress response, (*continued*)
molecular chaperone machine and, 3028, 3048, 3050, 3052
molecular regulation of, 3039
multicellular structures and, 3032–3033
new perspectives on, 3052–3053
nutrients and, 3034
optimum temperature for growth (OTG), 3025
osmolarity and, 3031, 3034
oxidative stress and, 3037
oxygen and, 3034
pH levels and, 3034
posttranslational regulation of gene expression for, 3040–3043
prefoldin in, 3030
pressure and, 3034
proteaosome and, 3031–3032, 3052
protein repair in, 3039
proteolysis and, 3031–3032, 3052
regulation in, 3049–3051
salinity and, 3034
sensing stress and, 3041
sigma factors and, 3040
starvation survival and, 3036–3037, 3041–3042, 3043–3045
stress genes and, 3039
stress proteins and, 3028, 3030–3031, 3036–3039
temperature and, 3034, 3046–3055
thermotolerance and, 3053
toxic solvents and, 3153
two-component systems and, 3041–3042
viable but not culturable (VBNC) phenomenon and, 3252
Strobilidium, 2626–2632
Stromatolites, 983
aggregates and consortia in, 172–173
biomineralization and, 678
Strombidium, 2626–2632
Strontium, in algae biotechnology, 199
Structural units of bacteriophages, 364
Structure activity relationships (SARs), 1249–1250
Stygiolobus, 298, 1235, 1629–1646
Stylaria, 1722
Stylosanthes, 2202
Styrene bioremediation, 707, 3147–3155
Subaerial communities, 3055–3065
Subfossil algae, 2301–2306
Subgrouping, rotaviruses, 2769
Submerged aquatic vegetation (SAV), 1190
Submerged plants
marine/ocean environment and, 2549–2552
seagrass and Zostera, 2550–2554, 2822–2833
Substrata, periphyton, 2363–2364
Substrate
enzyme activity and, 2901
nitrification and, 2160
Substrate activated death (SAD), 3251
Substrate induced growth response (SIGR), 1750
Substrate induced respiration (SIR), 1750
Subsurface irrigation systems, septic tank, 2855
Subsurface microbial communities (See also Caves and mines; Soil microorganisms), 1074–1081, 1453–1468, 3065–3071
abundance of, 2034–2035
activity of, case study evidence of, 1455–1456
adenosine diphosphate (ADP) in, 2022
adenosine triphosphate (ATP) and, 2022
adenylate energy charge (AEC) and, 2022
Atlantic Coastal Plain aquifers and, 2028–2042
bacterial counts, 2031
basaltic rock and, 1826–1828
biodegradation in, 2033
biodiversity of, 2034–2036, 3314–3315
biomass of, 1076, 1077–1078, 1080
carbon and, 1075
carbon dioxide and, 1074–1075, 1461–1463
catalyzed oxidation reduction reactions in, 1454–1455
in caves and mines, 1076, 1078–1079
chemolithoautotrophs in, 1075, 1455, 1824–1826
chemolithotrophs in, 1075, 1455
cold-adapted microorganisms and, 2025
community analysis of, 2030–2040
cryptobiosis in, 2021
culturing of, 1079
diagenesis and, 1074
diglyceride fatty acid (DGFA) and, 2030
dissolved organic carbon (DOC) and, 1074
dissolved organic matter (DOM) in, 1455
distribution of, 1076
DNA analysis of, 2035
dolomite, 1464–1466
energy sources for, 1074–1075
factors affecting, 2033–2034
gene probes for, 2036
geochemical effects of, 1453–1468
green rust, 1464–1466
groundwater and, 1076, 1079, 1454–1468, 1827, 2025
halophiles in, 2024–2025
high temperature environment, 2047–2057
hydrogen and, 1464–1466
igneous rock aquifers, 1661–1673, 1826
iron and, 1464–1466
isotopes and, 1456–1464
isotopic fractionation and, 1461
lithotrophs in, 1824–1829
magnetite and, 1464–1466
in marine/ocean environment, 1080
measuring metabolic activity of, 1079–1080, 2033
methane production and, 1075
microbial fluid interactions in, 1454–1464
microbial mineral interactions in, 1464–1466
microbial reduction of oxygen, nitrogen, carbon dioxide by, 1461–1463
most probable number (MPN) in, 2030
nitrogen and, 1461–1463
nutrients and energy sources for, 2020–21, 2033–2034, 2045–2046
oil bearing rock formations and, 2418–2420
organic matter and, 1074, 1075 and, 1455
oxidation and oxygen in, 174–75, 1461–1463
permafrost and, 2367–2385
petroleum reservoirs and, 2416–2426
phosphates and, 1464–1466
phospholipid fatty acids (PFLA) in, 2030, 3315
phylogeny of, 3065–3068
planetary catastrophe and evolution in, 1080
plasmid incidence testing in, 2036–2037
pore size in media of, 1075–1076

protozoa and, 2594
Rayleigh vs. open system isotope behavior and, 1457–1461
rock and, 1076
sampling of enumeration of, 1076–1079, 2030, 3071–3087
in San Juan Basin, 2045
sedimentary organic matter (SOM) and, 1455
sedimentary rocks and, 2418–2420
siderite and, 1464–1466
spatial distribution of, 2037–2039
starvation survival in, 1080, 2019–2028
storage related phenomenon in, 3315–3316
sulfate-reducing bacteria (SRB) in, 1075, 1454, 1457, 2419–2420, 2422–2424, 3091
survival of, long term, 3316–3317
temperature and, 1075
transport in, 2039–2040
viable but not culturable (VBNC) phenomenon in, 3316–3317
virus transport and removal in subsurface, 2094–2107
vivianite and, 1464–1466
volcanic tuffs and, 3110–3119
water and, 1075
Subtilisin, 3207–3210
Subtractive hybridization, 1451–1452
Subtropical waters
aggregates and consortia in, 168–170
nitrogen cycle and, 2182–2183
Prochlorococcus, 2576–2593
Succinate, 21–26, 153, 154
Sucrose, 518, 2716
anaerobic granulation and, 235–237
biosurfactants/bioemulsions and, 756
soil salinity and, 2778–2789
Suctoria, 2606
Sudden infant death syndrome (SIDS), 186
Sugar glasses and stabilization, in freeze drying, 1351–1354
Sugar processing, heterotrophic bacteria, 1544–1545
Sugars, 2716
in activated sludge, 741–743
microbial protection from, 432
soil salinity and, 2778–2789
in surface waters, 928, 928
Suillus, 2124–2132, 2915
Sulfatase, 3021
Sulfate-reducing bacteria (SRB), 1898–1899, 2419–2424, 3087–3996, 3146, 3193
activity of, 3091–3092
adenosine triphosphate (ATP) and, 3087
aggregates and consortia in, 165–166
applications for, 3092
assimilatory sulfate reduction in, 3087
biocorrosion and, 464–475, 3092–3093
biodeterioration of mineral materials and, 524
biofilms and, 2962, 3091–3092
biofouling and, 3091
biomineralization and, 672
bioremediation and, 708
carbon and, 3087–3088
chromium and, 3089
detection of, 3093–3094
dissimilatory sulfate reduction in, 3087
distribution of, 3090–3091, 3102
flooded soils and, 3114
in groundwater, 1665, 1667–1669
halophiles and, 1519
heavy metals and, 3089–3090
hydrogen sulfide production and, 3092
iron and, 3089
manganese and, 3089
in marine/ocean environment and, 1697–1709, 2547–2548, 2551
mercury and, 3089–3090
metals and, 3092–3093
microbially influenced corrosion (MIC) and, 3092–3093
molybdenum and, 3089
municipal solid waste landfills and, 1760
nitrogen and, 3089
oxygen and, 2277, 2285–2286
petroleum reservoirs and, 2428–2429
in sediments and sedimentation, 2844–2853
selenium and, 3089
soil microorganisms and, 2891
subsurface microbial communities and, 1075, 2055, 1454, 1457, 3091
sulfur and, 3088–3089, 3113–3115
sulfur bacteria and, 3098–3100
uranium and, 3089
Sulfates and sulfides, 2042–2043, 2042, 2277, 3184
biogenesis and oxidation of, 3113
biomineralization of, bacterial, 670–681
municipal solid waste landfills and, 1760
oxygen and, 2277
soil salinity and, 2774
in wastewater stabilization ponds (WSBs), 3332
Sulfhydryls, biomineralization, 671
Sulfobacillus, 3129
bioleaching and, 644–650
biomineralization and, 675
metal biodegradation and, 1904
Sulfoindocyanine dyes, 1334
Sulfolane bioremediation, 707
Sulfolobales, 298–299, 1629–1646, 3146
Sulfolobus, 1233, 1235, 1629–1646, 3113, 3144–3147
Archaea and, 298
biodegradation and, 1045
bioleaching and, 632–642
metal biodegradation and, 1904
stress response and, 3026
Sulfonic acid, biodegradation, 2297–2300
Sulfonium compounds, soil salinity, 2778–2789
Sulfophobococcus, 1629–1646
Sulfuorococcus, 298
Sulfuorspirillum, 1609–1610
Sulfur and sulfur cycling, 3096–3100, 3104–3116, 3184, 3192–3193
biofilms and, 82, 1085–1086
biofiltration and, 589
biogenesis and oxidation of sulfides in, 3113
bioleaching and, 632–642
cyanobacteria and, 969
desulfurization of fossil fuels, 1041–1051
endosymbiosis and, in ocean waters, 1117–1118
flooded soils and, 1326
hyperthermophiles and, 1633
in marine/ocean environment and, 1697–1709
oxygen and, 2277, 2278, 2284–2285
in sediments and sedimentation, 2844–2853
soil microorganisms and, 2887
subsurface microbial communities and, 1454–1455
sulfate-reducing bacteria (SRB) (See Sulfate-reducing bacteria)
thiosulfate shunt in, 3112
in wetlands for wastewater treatment, 3393

Sulfur bacteria in drinking water, 3096–3104
Sulfur dependent anaerobic methane oxidation, 288–289
Sulfur dioxide, 589, 3183, 3192–3193
Sulfur mustard (See Chemical warfare)
Sulfur oxidizing bacteria, 1610, 2890, 3097–3098
Sulfuric acid
biogenic, in biodeterioration of mineral materials, 524
weathering and, 3364–3389
Sulfuricoccus, 3129
Sulfurisphaera, 1629–1646, 3146
Sulofolobus, 295, 675, 1233
Sunlight (See Solar radiation; Ultraviolet radiation)
Supercritical fluid chromatography (SFC), 255–256
Superoxide dismutase (SOD), 2278
Surface complexation precipitation theory (SCPT), 1406–1407
Surface embedded biocides, 606
Surface enhanced Raman spectroscopy (SERS) analysis, 935
Surface film, neuston microbiology, 2134–2135
Surface microlayer, neuston microbiology, 2134–2135
Surface nanolayer, neuston microbiology, 2134–2135
Surface waters
artificial wetlands and reedbeds in wastewater treatment, 3389–3401
bacterioplankton in, 2519–2539
biofilms in, 569–572
biomineralization and, 677–678
bioremediation and, 708, 712–722
carbon in, 928
caves and mines, 821–835
ciliates in, 2626–2632
Clostridium and, 864–871
cold-adapted microorganisms in, 872–885
coliform bacteria and, 900, 902–903
conditioning films and, 928–941
dissolved organic carbon (DOC) in, 928
Enterococci in, 1264–1278
fecal contamination in, 1256–64
floc formation and, 2000–2002
flooded soils and, 1316–1333
fungi in, 3353–3364
green fluorescent protein (GFP) studies for, 1498
halogenated compound pollution of, 705
heterotrophic bacteria and, 1540, 1541–1552
humic substances in, 928
hydrocarbons and, 715–719
lipid biomarkers of, 1821
meroplankton and, 1863–1869
microorganisms in, 1724
nitrification and, 2156–2148
oil spills and, 715–719
organic inorganic matter in, 928
in paleolimnology, chrysophyte structures and, 2317–2327
particulate organic carbon (POC) and, 928
pathogens in, 2971
periphyton, 2357–2367
permafrost and, 2367–2385
Prochlorococcus in, 2576–2593
river snow in, 570–571
sampling of, 2812–2815
snow and ice environments, 2872–2880
source water using, 2971
stratification within, 715
stream microbiology, 3015–3024
Streptococci in, 1264–1278
vadose zone microbiology and, 3236–3246
Surfactants (See also Biosurfactants), 1–7, 714–715, 750–770, 2411
Surfactin, 757–759
Sustainable agriculture, 3116–3126
disturbance of soils and, 3122
microbial insecticides using, 1717
mycorrhizae and, 3121
nitrogen fixation and, 3120
nutrient cycling in, 3120
organic matter and, 3120
pesticide biodegradation in, 3121–3122
plant growth and, 3121
rotation of crops and, 3122
soil structure and, 3122
strategies for, 3122–3124
Sustainable waste management (See also Municipal solid waste landfills), 1769–1770
Swimming, plankton and phytoplankton, 2511
SwissProt, 1450
Symbiont, 1113
Symbiosis (See also Mycorrhizae; Nitrogen fixation; Rhizobium)
actinorhizal Frankia symbiosis in, 2208–2209
associative symbioses in, 2199–2200
cyanobacteria and, 973–975
desert environment and reclamation and, 1583
endosymbiosis, 1112–1130
fungi and, 1367
grass bacteria association in, 2199–2200
hydrothermal vents and, 1619
legume–rhizobial symbiosis in, 2202–2208, 2786–2787
mycorrhizae and, 2120–2132
nitrogen fixation and, 344, 2188–2221
plant–microbe interactions in marine environment, 2539–2554
protozoa and, 2621–2622
soil salinity and, 2775
Symbiosome, 1113
Symbitotrophic systems, 1113–1120
Symhyonema, 838–843
Symploca, 210, 980
Symports, soil salinity, 2781
Synchytrium, 2913
Synechococcus, 210, 972, 973, 2461, 2576, 3141–3147, 3307
aggregates and consortia in, 170–172, 173, 174
alkaliphiles and, 134
bacterioplankton and, 2526, 2532
biodeterioration of mineral materials and, 521
biofilms and, 83
biomineralization and, 672
cold-shock response and, 888–895, 888
in marine/ocean environment, 1701, 1704, 1706
phosphorus cycling and, 2451
photoautotrophic, in low-light habitats, 838–843
photosynthetic pigments in, 2464
salt production and, 2805–2809
Synechocystis, 969
aggregates and consortia in, 170–173
biodeterioration of mineral materials and, 519
phosphorus cycling and, 2451
radiation and, 2987
salt production and, 2805–2809
Synedra, 206, 210
Synthase, 3207–3210
Syntrophic degradation, in activated sludge, 744–745
Syntrophobacter, 234–235, 745, 1340
Syntrophomonas, 745
Syntrophospora, 745
Syntrophus, 509
syntrophy, 83–84, 240–241, 1113–1120, 1320
Synura, 206, 210, 2317–2327, 2317, 2467
Syracosphaera, 2508

Syringodium, 2549
SYTO statins, 1777, 1949
Sytox stains, 1783
Syurophyceae, 2467

T
T-helper cells, 1359–1366, 1378
Tabellaria, 210
Tabun (See Chemical warfare)
Taenia, 3277–3287, 3399
Talaromyces, 451, 453, 916, 3144–3147
Talc, activated sludge foaming, 6–7
Tamyl methyl ether (TAME) biodegradation, 1994–2000
Tartigrades, in snow and ice environments, 2874
Taste of water, vs. algal blooms, 204–213
Taxonomy
  bacterioplankton and, 2526–2528
  protozoa and, 2615–2620
Taylorsville Basin, 2053–2057
t-butyl alcohol (TBA) biodegradation, 1994–2000
Technetium, 671–681, 1879–1893
Technology transfer issues, 1861
Tectiviridae/Tectivirus, 365–374
Teichoic acid, 109
Teichuronic acid, 109
Teichuronopeptide (TUP), 221
Telluria, 455, 3067–3071
Tellurite, resistance of bacteria to, 608
Temperate marine ecosystems
  aggregates and consortia in, 170–172
  nitrogen cycle and, 2181–2182
Temperature
  activated sludge and, 1311
  algae and, 1193
  alkalithermophiles, 3127–3140
  Antarctic soils and, 1023–1025, 1103
  Arrhenius equation for, 1752
  bacteriophages vs., 387
  bacterioplankton and, 2520–2521, 2531
  biodegradable dissolved organic carbon (BDOC) and, 495
  biofiltration vs., 593
  bioremediation and, 713, 723
  composting vs., 915–916
  denitrification vs., in marine environment, 1016–1017
  desert environment and, 1576, 1583
  enteroviruses and, 1169
  enzyme activity and, 2900
  extremeophiles and, 1232–1237
  harmful algal blooms (HABs) and red tides, 2689–2690
  heat-shock response, 3046–3055
  heterotrophic bacteria and, 1541–1552
  high temperature subsurface environment, 2047–2057
  hydrothermal vents, 1604–1628
  hyperthermophiles and, 1642
  kinetics of growth and, 1752–1753
  municipal solid waste landfills and, 1760, 1762
  neuston microbiology and, 2139
  nitrification vs., 2158, 2168–2169, 2171–2172
  nitrogen fixation vs., 2199
  oil recovery from petroleum reservoirs and, 2421
  periphyton and, 2361–2362
  permafrost and, 2367–2385
  pesticide degradation vs., 2394
  petroleum reservoirs and, 2427
  plankton and phytoplankton, 2502, 2511
  polar phytoplankton and, 2555–2556, 2563, 2564
  psychrophilic bacteria and, 2639, 2641, 2642–2645
  rhizosphere and, 2724
  sea ice microorganisms and, 2839
  in septic tank systems, 2857
  snow and ice environments, 2872–2880
  soil microorganisms and, 1752–1753, 2061, 3268
  of source water, 2970
  spores and spore dispersion vs., 431
  stress response and, 3024–3034, 3046–3055
  subaerial communities and, 3058, 3061
  subsurface microorganisms and, 1075
  temperature gradient gel electrophoresis (TGGE), 299, 536–537, 818–819, 2735–2737, 2735
  temporal temperature gradient electrophoresis (TTGE), 2735–2737
  thermophiles and, 3127–3147
  viruses vs., 2099–2100, 3268
  of in wastewater stabilization ponds (WSBs), 3335–3336
  weathering, bioweathering and, 3381–3382
Temperature gradient gel electrophoresis (TGGE), 299, 536–537, 818–819, 2735–2737
Temporal temperature gradient electrophoresis (TTGE), 2735–2737
Tephrocybe, 2914
Teratogens, biodegradation, 507–516
Terbutryn composting, 921–922
Termamyl, 1181
Terminal electron accepting processes (TEAP), 2932, 3404
Terminal fragment length polymorphism (T-RFLP), 28–29, 71, 177–178, 278, 299, 536–537, 563, 829, 1974, 2496, 2737–2738, 2921
  Archaea and and, 278, 299
  biofilms and, 563
  soil microorganism and diversity in, 536–537
  bacterial counts using, 28–29
  microarray study of vs., 1974
  activated sludge and, 71
  aggregates and consortia in, 177–178, 177
Termites, endosymbiosis, 1118
Terpenoids, algae biotechnology, 195
Terrabacter, 40, 3065–3071, 3314–3319
Terrestrial biofilms, 1216
Terrestrial cluster, soil microorganism and diversity, 533–534
Tessaracoccus, 64
Testate amoeba, 2610
Tetanus, Clostridium, 862–871
Tetrachlorodibenzofuran bioremediation, 703–712
Tetrachlorodioxin bioremediation, 703–712
Tetrachloroethane biodegradation, 512–513
Tetrachloroethylene biodegradation, 701–712, 1763
Tetrachloromethane biodegradation, 511–514
Tetrachlorophenol biodegradation, 3413
Tetraclorodibenzodioxin (TCCD) biodegradation, 507–516
Tetracystis, 1109
Tetradichloroethene composting, 919–920
Tetragenococcus, 1506–1512
Tetrahydrofuran biodegradation, 1763
Tetrahydronaphthalene bioremediation, 696–712
Tetrahymena, 1123, 2342–2356, 3173
Tetrakishydroxymethylphosphonium sulfate (THPS), 605–606

Tetraline bioremediation, 696–712, 3147–3155
Tetramethyl rhodamine isothiocyanate (TRITC), 570, 1334, 1676
Tetranitromethylaniline (tetryl) biodegradation, 1979–1993
Tetraselmis, 2805–2809
Tetrasphaera, 4–7, 40, 64
Tetrodotoxin, 1857
Tevnia, 1609, 1619
Texas Red, 1334, 1676
Textile industry
  bioaerosols and, as by-product of, 422–423
  enzymes and, 1181–1183
Thalassia, 2549, 2552, 2822–2833
Thalassionema, 2506
Thalassiosira, 1706
Thallobacteria, nitrogen fixation, 2214–2221
Thanatephorus, 2914
Thauera, 32, 57
Thaumatomastix, 2841
Thelephora, 2124–2132
Thermaerobacter, 1611
Thermal conductivity gas chromatography (TCD-GC), 1015
Thermal programmed capillary electrophoresis (TPCE), 814
Thermal regulation in cold-adapted microorganisms, 875–876
Thermal springs (See also Geothermal environments; Hydrothermal vents), 226, 262, 278, 287–288
Thermanaerobiaceae, 1512–1529
Thermanaerovibrio, 744–748
Thermatoga, 2419, 3146
Thermatogales, 1233, 2420, 3138
Thermoacidophiles, 246–259, 261
Thermoactinomyces, 136, 220–223, 422, 423, 1578
Thermoalkaliphiles, 136
Thermoanaerobacter, 1233, 2419, 2429–2431, 3128–3140
Thermoascus, 916
Thermobrachium, 3130, 3133–3134
Thermococcales, 287, 1233, 1611, 1614, 1615, 1630–1546, 3137, 3146
Thermococcus, 136, 1233, 1234, 1235, 1236, 1512–1529, 1623–1628, 1630–1646, 1853, 3129, 3130, 3137, 3146
  Archaea and, 270, 295
  genomic studies and, 1446
  hydrothermal vents and, 1611
  petroleum reservoirs and, 2420, 2429–2431
Thermocrinis, 1629–1646, 3143–3147
Thermodesulfbacterium, 3114–3116, 3087–3096
  petroleum reservoirs and, 2419
  petroleum reservoirs and, 2429–2431
  sulfate-reducing bacteria (SRB) and, 467–475
Thermodesulforabdus, 2429–2431
Thermodesulfovibrio, 467–475
Thermodiscus, 1629–1646
Thermodynamic analysis
  floc formation and biofilms, 2005
  hydrophobicity and, 1600–1604, 1602
  oligotrophic bacteria and, 2267–2277
Thermofilium, 298, 1234, 1629–1646, 3146
Thermohalobacter, 1521–1529
Thermolysin, 1185
Thermomyces, 881, 916, 3144–3147
Thermopallium, 3130, 3135
Thermophiles, 1233, 3127–3147
  Archaea and, 246–259, 277–293
  chemolithoautotrophs as, 3141–3144
  composting and, 915–916
  cyanobacteria and, 961–963
  desulfurization and, 1045
  heterotrophs as, 3141–3142, 3144–3146
  hydrothermal vents and, 1604–1628
  petroleum reservoirs and, 2419
  photoautrophs as, 3141–3143
  soil microorganisms and, 2888–2889
  subaerial communities and, 3061
  subsurface microbial communities and, 3069–3070
  sulfate-reducing bacteria (SRB) and, 3090–3091
Thermoplasma, 1235, 1625, 1895, 3141–3147
  Archaea and, 295, 297
  biomass estimation and, 2658
  endosymbiosis and, 1124
Thermoplasmales, 287, 297
Thermoproteales, 298 1629–1646, 1629
Thermoproteus, 295, 298, 533, 1233, 1234, 1235, 1512–1529, 1629–1646, 3146
Thermosipho, 1515–1529, 1614, 1620–1628, 2420
Thermosphaera, 1629–1646
Thermostable enzymes, 267–269, 1852
Thermosyntropha, 234–235, 3130, 3131, 3135
Thermoterrabacterium, 3143–3147
Thermothrix, 3143–3147
Thermotoga/Thermatogales, 1233, 1235, 1512–1529, 1620–1646, 3145–3147
  genomic studies and, 1442
  halophiles and, 1515–1529
  hydrothermal vents and, 1614, 1620
  petroleum reservoirs and, 2420, 2429–2431
Thermotolerance, 3053
Thermus, 1233, 1620–1628, 1853, 3145–3147
  Archaea and, 270
  hydrothermal vents and, 1611, 1620
  nitrification and, 2146, 2149
  subsurface microbial communities and, 3070
Theta X174 bacteriophage, 2101–2102
Thickening agents, algae biotechnology, 192
Thielaviopsis, 457
Thin layer chromatography (TLC), 251
Thio compounds, biofouling vs., 605
Thioalkalicoccus, 2477–2489
Thioautotrophs, 1117, 1606
Thiobacillus, 757, 2284–2285, 2425, 3088–3098, 3101, 3102, 3110–3116
  aggregates and consortia in, 166
  biodegradation and, 1871
  bioleaching and, 634–650
  biomineralization and, 673, 675, 676, 677
  bioremediation and, 731
  desulfurization and, 1049
  flooded soils and, 1326
  hydrothermal vents and, 1610
  metal biodegradation and, 1880, 1881, 1882, 1902, 1904
  nitrogen fixation and, 2193–2201
  odor control and biofiltration for, 594–601
  radioactive waste degradation and, 2666–2674
  soil microorganisms and, 2889, 2890
  subaerial communities and, 3057–3065
  weathering, bioweathering and, 3366
Thiobacterium, 872–885, 3111–3116
Thiocapsa, 2281
  biofilms and, 83
  bioplastics and, 684–692

halophiles and, 1517–1529
phototropism in, 2474, 2475, 2476
Thiocarbamates, 605–606
Thiococcus, 2475, 2477–2489
Thiocyanomethylthiobenzothiazole, 605–606
Thiocystis, 2474
Thiodendron, 3111–3116
Thiodiglycol (TDG) (See Chemical warfare)
Thioflavicoccus, 2475, 2477–89
Thiohalocapsa, 1517–1529, 2477–2489
Thiohodovibrio, 2477–2489
Thiomicrospira, 1610, 3111–3116
Thiopedia, 2193–2201, 3111–3116
Thioplaca, 2284–85, 3111–3116
Thiorhodococcus, 2477–2489
Thiorhodospira, 2477–2489
Thiosphaera, 3111–3116
Thiospira, 3111–3116
Thiosulfate, bioleaching, pathways, 636–637
Thiosulfate shunt, 3112
Thiothrix, 825, 1288–1306, 1310–1316, 1610, 1618–1628, 3098–3104
Thiovolum, 1609–1610, 2284–2285, 2805–2809, 3111–3116
3/2 power kinetics, to model growth, 1755
Threonine, 2716
Thresholding, 1951
image analysis and, 1681–1682
Thyasira, 1606
Thymidine rates, wastewater/sewage treatment, 3339–3342
Ticks, Lyme borreliosis, 1838–1849
Tidal marshes (See also Marine/ocean environment), 715, 1316–1333
Tillage and residue management, enzyme activity, 2908
Tilletia, 410–411
Tilted glass slide test, for biosurfactants/bioemulsions, 762
Time of flight mass spectrography (TOF MS), 820
Time of flight secondary ion mass spectrometry (TOF SIMS), 933–934
Tindallia, 136
Tintinnid ciliate, 2617
Tipula, 3020
Titanium, biomineralization, 671–681
TNT (See Trinitrotoluene)
Toilets, bacteria in home/residential environment, 340
Tokophyra, 2606
Toluene biodegradation, 51–52, 696–712, 714, 917, 1421–1429, 1446, 1763, 1994–2000, 2296–2300, 2408, 2412, 2931, 3147–3155, 3405
Toluene orthormonooxygenase (TOM), 435, 437
Tolypella, 2304–2306
Tolypothrix, 840–843, 3056–3065
Tomatochoroon, 3056–3065
Tomentalla, 2128
Top-down exploratory data analysis, 1005
Torula, 412
Torulopsis, 753, 872–885
Total Coliform Rule (TCR), 905, 906–907
Total dissolved phosphorus (TDP), 2448–2453
Total extended filament length (TEFL), 1307
Total organic carbon (TOC), 485–507
desert environment and, 1577
granular activated carbon (GAC) filters and, 1491
in source water, 2972
in wetlands for wastewater treatment, 3391
Total oxygen demand (TOD), in source water, 2972
Total petroleum hydrocarbons (TPH), 916–919
Toxic chemicals, 2340, 3147–3155
activated sludge and, 72, 75
algae biotechnology and, 196–200
algal/in algal blooms, 204–213
biodegradation and, 724–725, 1421
biofiltration and, 591
composting of, 916–917, 916
enzyme activity and, 2909
municipal solid waste landfills and, 1765
neuston microbiology and, 2137–2139
nitrogen cycle and, 2942
in pulp and paper industry, 2650
resistance of bacteria to, 608
soil microorganisms vs., 2927–2929
Toxic flow, 3226
Toxic load, 3226
Toxic Pfiesteria complex (TPC) (See Pfiesteria)
Toxic pneumonitis, 1369
Toxic print, 3226
Toxic Substances Control Act (TSCA), 1280, 2695–2707
Toxicity characteristic leachate protocol (TCLP) analysis, 846
Toxicity identification evaluation (TIE), 3168
Toxicity reduction evaluation (TRE), 3168
Toxicity testing, algal, 3219–3230
algal solid phase assay (ASPA) in, 3227–3228
eutrophication and, 3220
exposures testing, 3220–3225
hazardous wastes, hazardous effluents and, 3225–3227
interaction studies using, 3225
species used in, 3221–3222
toxic flow in, 3226
toxic load in, 3226
toxic print in, 3226
toxicity testing, bioterrorism and, 773–775
Toxicity testing, in soils
biomass and, 3158–3159
bound effect concentration (BEC), 3163
denitrification and, 3160
dose response testing in, 3162–3164
effective concentration (ECx), 3163
enzyme activity and, 3161
heavy metals and, 3162
international guidelines for, 3164
long term changes in soil quality and, monitoring, 3165
lowest effect concentration (LEC), 3163
lowest observed effect concentration (LOEC), 3163
microbial growth rates and, 3161
minimum data sets (MDS) for, 3165
near infrared reflectance (NIR) in, 3165
nitrification and, 3159–3161
nitrogen fixation and, 3160
nitrogen mineralization and, 3159
no effect concentration (NEC), 3163
no observed effect concentration (NOEC), 3163
phosphatase activity and, 3160–3161
predicted environment concentration (PEC), 3163
predicted no effect concentration (PNEC), 3163
respiration rates and, 3159
sampling for, 3166
screening and testing of chemicals and metals in, 3161–3164
Toxicity testing, in wastewater, 3168–3175

Toxicity testing, in wastewater, (*continued*)
algal toxicity assays for, 3172–3173
ATP-based assays for, 3171
bacterial biosensors for, 3172
biochemical oxygen demand (BOD) in, 3168
biomonitoring in, 3168
de novo enzyme biosynthesis tests in, 3172
enzyme assays in, 3169–3171
growth inhibition assays in, 3171–3172
microbial assays and, 3171
Microtox assay in, 3171
nitrifying bacteria in, 3172
protozoa in, 3173
respirometry in, 3173
toxicity identification evaluation (TIE) in, 3168
toxicity reduction evaluation (TRE) in, 3168
urease toxicity assay (UTA) and, 3170
whole effluent toxicity (WET) in, 3168
Toxicology, algae biotechnology, 196–199
Toxins
bioterrorism and 778–779
cyanobacteria and, 975–980, 982–995
plankton and phytoplankton, 2514
Toxocara, 3399
Toxoplasma, 3176–3183
disinfection and, 1067–1073
in food, 3277–3287
in source water, 2969–2978
in water treatment systems, 1719
Toxothrix, 3101
Trace elements, in marine/ocean environment, 1701–1707
Trace gases, soil, 3183–3194
Trace metals, cyanobacteria, 969–970
Tracehophtyes, 2504–2518
Tracers
geochemical, 3081–3082
groundwater, microbial, 3194–3202
microbiological, 3079–3081
viral, 3305
Trachipleistophora, 2066–2083
Trametes, 454, 1385, 1983
Transcription, 1199–1200, 1637–1639, 2489–2491
Transduction, in bacterioplankton, 2532
Transduction, 1415–1416
Transfer processes, biofilms, 2087–2088
Transferases, in soil, 2903–2910
Transformation processes, in biofilms, 2086
Transformation, 1413–1415
Translation, 1637–1639, 2489–2491
Transmembrane soil microorganisms, 2883
Transmission electron microscopy (TEM), 234, 635, 2008
Transmission of disease, 1690, 1690–1691, 2744
Transovarial infection, 1118
Transport processes
biofilms and, 2087
filtration and, 2708–2712
genetically engineered microorganisms (GEMs) and, 1281–1282
subsurface microorganisms and, 2039–2040
virus transport and removal in subsurface, 2094–2107
Transport of samples, 2811–2812
Transposition, genetic, 1199
Transposons, 1200–1203, 1280
Trebouxia, 1025, 1026, 3059
Trees, mycorrhizae, 2129–2130
Trehalose
biosurfactants/bioemulsions and, 753
freeze drying and, 1353–1357
microbial protection from, 432
soil salinity and, 2778–2789
TrEMBL, 1450
Tremetes, 2647
Trentepohlia, 519, 3056–3065
Trepomonas, 2605
Triacylglycerols, in surface waters, 928
Triarthra, 1725
Triatoma, 1115
Triazine herbicides biodegradation, 2391–2393
Tribonema, 215
Tribophyceae, 2467
Tricarboxylic acid (TCA), 1633, 2717
Trichinella, 1561, 3277–3287
Trichloroacetate (TCA) biodegradation, 511–512, 2394
Trichlorobacter, 512
Trichloroethane (TCE) biodegradation, 434–442, 510–514, 701–712, 919–920, 1763, 2931, 3413
Trichloroethylene biodegradation, 434–442, 701–712, 1422–29, 1763, 1933, 1937–1944, 2594, 2933
Trichloromethane composting, 919–920
Trichlorophenol, 3147–3155
Trichocysts, 2509
Trichoderma, 462, 1648, 1660
in allergies and illness, 1367–1372, 1378–1383
biocontrols and, 451–463
biofilms and, 580, 1177
bioterrorism and, 779
cold-adapted microorganisms and, 881
fluorescent probes and, 1342
rhizosphere and, 2718
spores and spore dispersion in, 422
Trichodesmium, 967, 970, 978, 2282
aggregates and consortia in, 167–170
bacterioplankton and, 2525
in marine/ocean environment, 1705
nitrogen cycle and, 2181, 2182
nitrogen fixation and, 2214–2221
Trichoethecene, bioterrorism and (See Yellow rain)
Tricholoma, 2124–2132
Trichomonas, 1124–1125, 3327
Trichophyton, 1368, 1373–1383
Trichosporon, 3173
Trichosporum, 580
Trichothecenes, 182–189
Trickling filters, protozoan removal using, 2332
Tricodesmium, 961
Tricuris, 3333–3334, 3399
Triglycerides, 85–86, 1818–1824, 2649
Trihalomethanes (THMs), 495
Trilobus, 1720
Trimedia filtration, 2707
Trinitrobenzene (TNB) biodegradation, 1979–1993
Trinitroglycerine (TNG) biodegradation, 1979–1993
Trinitrotoluene (TNT) bioremediation, 51–52, 707–712, 916–917, 923–924, 1421–1447, 1498, 1979–1993, 3410
Trisaccharides, microbial protection from, 432
Triticum, 570, 1434
Tritirachium, 1368
Troglobitic species, 837–843
Trogloxenic species, 838–843
Trophic state
algae and, 1190–1191
protozoa and, 2628–29
Trophosome, in deep sea invertebrates, 1619

Tropical waters
aggregates and consortia in, 168–170
coliform bacteria and, 902–903
nitrogen cycle and, 2182–2183
Prochlorococcus, 2576–2593
Trypanosoma, 1126
Trypsin, 2899
Tryptophan, 2899
Tsuga, ectendomycorrhizal fungi, 2125–2126, 2127
Tsukamurella, 3–7, 753
Tube worms, hydrothermal vents, 1606–1617, 1619
Tuber, 2124–2132, 2128
Tubercidin, 978
Tuberculosis, infectious airborne bacteria, 1689–1697
Tuberculosis, biochip testing, 449
Tubifex, 1722
Tuffs, volcanic, microbiology, 3110–3119
Tularemia, bioterrorism, 777
Tungsten, in marine/ocean environment, 1706–1707
Tungstenite, bioleaching, 633
Turbidity
biosurfactants/bioemulsions and, 763
cyanobacteria and, 944
Turbulence, biofilms vs., 547
Turnover time, bacterioplankton, 2529
Tween 20, 626
Two-photon laser scanning microscopy (2PLSM), 2008
Two-stage soil (TOSS) bioremediation process, 1989
Twort, Frederick, 363
Typha, 2548
Typhoid fever, Salmonella, 2789–2802
Typhula, cold-adapted microorganisms, 881
Tyrosin, 2899

U
U.S. Department of Agriculture (USDA), 2695–2707
Ubiquity of microorganisms, 1002
Ulcers, Helicobacter pylori, 1529–1530
Ulocladium, 1367–1372, 1374–1383, 1650, 1651
Ulothrix, 215
Ultimate biodegradation, 2394–2395
Ultrafiltration, 273, 1534, 2266
Ultramicrobacteria, in drinking water, 945
Ultraviolet (UV) radiation, 3184, 3188, 3230–3235
adenoviruses vs., 96
aerobic endospores and, in measuring effectiveness, 140–149
bacteriophages vs., 360–361
Clostridium vs., 868–871
cyanobacteria and resistance to, 963
dark repair and, 3231
disinfection and, 1071, 3230–3235
effectiveness of, 3232–3233
enteroviruses and, 1170, 1173
factors affecting performance in, 3233–3234
germicidal wavelengths and, 3231
neuston microbiology and, 2136–2137
ozone hole and, 2564–2565
photoreactivation and, 3231
plankton and phytoplankton, 2512
polar phytoplankton and, 2560–2562
protozoa vs., 2334
in rainwater roof catchment systems, 2682
in source water, 2970
in spas and hot tubs, 2979
space microbiology and, 2985
spores and spore dispersion in vs., 431
subaerial communities and, 3060–3061
Ulva, 614, 2689–2693
Umbilicosphaera, 2508
Umezakia, 984–995
Uncinula, 454
University of Cape Town (UCT) process, activated sludge, 79–80
Unweighted pair group average (UPGA), 1006
Upflow anaerobic sludge blanket (UASB), 232–245, 2002
Upflow sludge bed filters (USBF), anaerobic granulation, 232–245
Upper mixed layer, 2565
Uracil, cold-adapted microorganisms, 883
Uranium (See also Heavy metals), 1879–1893, 1902–1913
bioleaching and, 639
biomineralization and, 671–681
bioremediation and, 708–709
sulfate-reducing bacteria (SRB) and, 3089
Urate oxidase, in soil, 2903–2910
Urea, 2939
Urease, 2902–2910, 3170
Urease toxicity assay (UTA), 3170
Uredospores, 411–412
Urocladium, subaerial communities, 3057–3065
Uronema, 2607
Uronic acid, 85–86, 928
Urotricha, 2626–2632
Ustilago, 410–411, 756

V
Vacuum drying, 1035–1036
Vadose zone microbiology, 3236–3246
Vahlkampfia, 1123, 1579, 2595–2605
van der Waals forces, 57
adhesion in biofilms and, 101–104, 119–120, 123, 124, 936, 938
biofilms and, 552, 1083, 2004
floc formation and, 57, 2004
hydrophobicity and, 1600–1601
Vanadium, biomineralization, 671–681
Vannella, 2595–2604
Varicella zoster, algae biotechnology, 195
Variola, bioterrorism and (See Smallpox)
Variovorax, filamentous forms, in activated sludge, 1300–1306
Vector borne diseases
bioterrorism and, 779–780
microsporidia and, 2065–2083
Venezuelan equine encephalitis (VEE), bioterrorism, 777
Ventilation systems
biofilms and, 579–580
biofiltration and, 588, 593–601
odor control and, 593–601
Venturia, spores and spore dispersal, 406, 413
Verocytotoxigenic E. coli (VTEC), 2349–2350
Verrucomicrobia, 533, 1320
soil microorganism and diversity in, 533
Vertebrates, in water treatment systems, 1723
Verticillium, 451, 455, 460, 461, 462, 2897, 2913
Vesicomya, 1606
Vesicular arbuscular mycorrhizae, 2120
Vestimentifera, 1117, 1619
Viability testing, fluorescent probes, 1783
Viable but nonculturable (VBNC), 565, 1416, 1432, 1960, 2271, 3246–3255, 3316–3317

Vibrio, 74, 853–861, 1057, 1501–1512, 1698, 1857, 2339, 2549
Antarctic soils and, 1027
bacteriophages and, 355
biofilms and, 562, 567, 1209, 1219
bioluminescence in, 658–662
biomass estimation and, 2656
biomineralization and, 675
bioterrorism and (See Cholera)
bottled water and, 799
cold-adapted microorganisms in, 872–885
cold-shock response and, 886–895
endosymbiosis and, 1117
fecal contamination and, 1273
genetically altered, in biotechnology, 1446, 1447
genomic studies and, 1442, 1443, 1445, 1448, 1451
heterotrophic bacteria and, 1541–1552
high hydrostatic pressure (HHP) vs., 1555–1563
hydrothermal vents and, 1610
identification of isolates in, 1960
metal biodegradation and, 1884
nitrogen fixation and, 2214–2221
phosphorus cycling and, 2451
in source water, 2969–2978
in spas and hot tubs, 2980–2984
subsurface microorganisms and, 2021, 2025
toxic solvents and, 3152
in wastewater stabilization ponds (WSBs), 3330, 3334–3336
in wastewater/sewage, 3171
in drinking water, 945, 949, 3199
Vibrionacea, 803
Video cameras, 1676
Vinyl acetate bioremediation, 707
Vinyl chloride (VC) bioremediation, 434–442, 510–514, 701–712, 919–920, 919
Vinyl chloride polychlorinated biphenyls (See Polychlorinated biphenyls, PCBs)
Violooxanthin, 2512
Virion of bacteriophages, 364
Viruses (See also specific viruses, e.g., astrovirus, poliovirus, etc.), 338, 363, 417
activated sludge and, 70
adsorption to soil of, 2097–2098, 3270, 3273–3274
advection and dispersion of, 2101
aerosol type, 3255–3260
aggregation of, bacteriophages and, 389, 3271
algae biotechnology vs., 195–196
aquatic sediments and, 1252–1256
bacteria in home/residential environment and, 342
bacterial infection by, 3304–3305
bacteriophage theta X174, 2101–2102
bacteriophages and as index of, 374–384, 357, 2101
bacterioplankton vs.., 2531–2532
bioaerosols and, 3221–3222, 3327
biofilms and, 2342, 2353
bioterrorism and, 777–778
bromine and iodine vs., 3266–3267
cations and, 2099
cell culture assay of, 3291
chlorine vs., 3265–3267
coliphages, 2974
decay of, 3305
disinfection of, 3260–3268
dispersion of, 2101
DNA synthesis in, 3305
dose response assessment in, 2745–2747
exposure assessment in, 2747–2748
F specific RNA bacteriophages and, 2102
filtration vs., 2097
fluorescent tracers for, 3305
food processing and, 3276–3287
food web and, 3306–3307
gas liquid interface and, 3274–3275
gene transfer using, 3205, 3308
groundwater and, 3288–3300
hazard identification in, 2742
heat/thermal disinfection vs., 3264
high hydrostatic pressure (HHP) vs., 1561
human health outcomes in, 2742–2744
hydraulic conditions vs., in soil, 3273
hydrophobicity of soils and, 2098
ionic strength in, 2099
ionization vs., 3264–3265
isotope dilution studies of, 3305
marine/ocean environment and, 3300–3310
metal ions vs., 3267
as microbial tracers in groundwater, 3194–3202
microbial activity vs., 2100, 3268
microbial insecticides using, 1711
model, 2101
modeling of transport and removal in subsurface, 2094–2107
mortality and morbidity rates from, 2743–2744
MS2 model, 2101
multiple exposure and immunity in, 2747
municipal solid waste landfills and, 1766
neuston microbiology and, 2140–2141
organic matter and, 2099, 3271–3272
outbreaks of disease from, 2744–2745
ozonation vs., 3262
particulate matter and soil vs., 2100
pH levels and, 3269–3270
plankton vs., 3307–3308
polymerase chain reaction (PCR) and, 3291–3299
PRD1 model, 2101
protists and, 3305–3306
in rainwater roof catchment systems, 2681–2682
removal of, through soil transport, 2102–2105
resistance of hosts to, 3308
respiratory syncytial virus (RSV), 2743
reverse transcription PCR (RT PCR), 3291–3299
risk assessment of environmental exposure to, 2741–2753
salinity and, 3270
seasonal trends in, 3296–3299
soil pH vs., 2098
soil survival of, 3268–3276
in source water, 2969–2978
in spas and hot tubs, 2979
streams and, 3016–3017
subsurface microorganisms and, 2025
taxonomy of, 364–366
temperature vs., 2099–2100, 3268
transmission methods in, 2744
unsaturated conditions and, 2100–2101
in wastewater stabilization ponds (WSBs), 3330, 3334
water and moisture for, 3268–3269
water purifiers vs., 1572–1573, 1572
water treatment systems and, 1160–1164, 3288–3300
waterborne diseases and, 2743
in wetlands for wastewater treatment, 3397–3398
Vitamins, 2716

Vitrification, freeze drying, 1351–1354
Vittaforma, 2066–2083, 2329–2337
Vivianite, for subsurface microbial communities, 1464–1466
Volatile fatty acids (VFA), 235, 242–243, 1760
Volatile organic compounds (VOCs)
biofiltration vs., 598
biotrickling filters, 782–795
composting and, 919–920
fungi and, 1366–1383
municipal solid waste landfills and, 1765
Volatilization, metal biodegradation, 1884, 1894
Volcanic rocks, subsurface microbial communities, 3065, 3110–3119
Volumetric bacterial growth rates, 3344
Volvox, 206, 1863–1869
Vorticella, 2342–2356, 2608, 2611, 2620
VX agents (See Chemical warfare)
VX ICB, chemical warfare, 850–851

W

Wall and gate, 695
Wall-less spirochete, psychrophilic, 2640–2646
Wallemia, 1130
Wards method, 1006
Washing, fluorescent probes, 1335, 1336
Waste activated sludge (WAS), 738
Waste management (See also Municipal solid waste landfills), 1769–1770
Waste treatment, odor control and biofiltration, 593–601
Wastewater/sewage treatment (See also Activated sludge), 26–31, 1850
activated sludge and, 26–31, 739, 2332, 3342–3345
adenosine triphosphate (ATP) in, 3338–3339
algae biotechnology and, 199–200
algal toxicity assays for, 3172–3173
algal turf scrubbing (ATS) and, 213–219
anaerobic granulation and, 232–245
anaerobic migrating bed reactor (AMBR), 232
anaerobic sequencing bed reactor (ASBR), 232
artificial wetlands and, 2333
ATP-based assays for, 3171
bacteria in, 3337, 3338
bacterial biosensors for, 3172
bacteriophages as indicators in, 354–363
bioaerosols released from, 420–421, 3320–3330
biochemical oxygen demand (BOD) in, 3168
biodiversity in, 3337
biofilms and, 86–87, 3346–3347
biofiltration in, 588
biological nutrient removal (BNR) processes and, 3337–3339
biomonitoring in, 3168
bioremediation and, 693
blue-green algae, 206
chemical transformations in, 3337
cold-adapted microorganisms and, 882
coliform bacteria, 895–905
Cryptosporidium in, 1252–1256
de novo enzyme biosynthesis tests in, 3172
dehydrogenase in, 3338
deoxythymidine diphosphate (dTDP) and, 3340–3341
deoxythymidine triphosphate (dTTP) and, 3339–3342
deoxyuridine monophosphate (dUMP) and, 3340–3341
dinoflagellates in, 205
disinfection in, 2333–2334
DNA/RNA analysis in, 3339–3342
enteric viruses in sediment from, 1252–1256
Enterococci in, 1264–1278
enteroviruses and, 1171–1172
enzyme assays in, 3169–3171
expanded granular sludge blanket (EGSB), 232
filamentous bacteria in, 1287–1306
filtration in, 2332, 2333
Giardia in, 1252–1256
green, 205
growth inhibition assays in, 3171–3172
hepatitis viruses and, 1530–1540
heterotrophic bacteria and, 1541–1552
high-rate algae ponds in, 2333
infiltration percolation in, 2333
isotope dilution in, 3341
kinetics of growth and, single species, 3339–3342, 3347–3350
lagoons in, 2333
macromolecule labeling in, 3342
marine biotechnology and, 1855
metabolism vs. growth in, 3338–3339
microbial assays and, 3171
microfiltration in, 2334
Microtox assay in, 3171
NADH and, 3338–3339
nitrifying bacteria in, 3172
nutrients and, 3337
odor control and biofiltration in, 593–601
oxygen uptake rate (OUR) in, 3345–3346
parasites in, 2327–37
principles and biochemical pathways in, 3340–3341
protozoa in, 1252–56, 2327–2337
Pseudomonas, 2632–2639
reedbeds for, 3389–3401
respiration in, 3173, 3345–3346
Salmonella and, 2789–2802
sampling of, 2813
sand filters in, 2333
sedimentation in, 2331–2332
septic tank systems and, 2853–2864
specific bacterial growth rates in, 3344–3345
sprinkler type systems, bioaerosol release from, 3323–3324
stabilization ponds (WSPs) in, 2333, 3330–3337
Streptococci in, 1264–78
tertiary treatments in, 2333–2335
thymidine rates in, 3339–3342
toxicity identification evaluation (TIE) in, 3168
toxicity reduction evaluation (TRE) in, 3168
toxicity testing in, 3168–3175
upflow anaerobic sludge blanket (UASB), 232
upflow sludge bed filters (USBF), 232
urease toxicity assay (UTA) and, 3170
vadose zone microbiology and, 3242
viruses in, 1252–1256
volumetric bacterial growth rates in, 3344
wetlands for, 3389–3401
whole effluent toxicity (WET) in, 3168
yellow green or golden brown algae in, 206
Water absorbing polymers (WAP), 239
Water (See Moisture and water content)
Water molds, 2912

Water pollution (See Aquatic ecosystems; Groundwater pollution; Marine/ocean environment; Surface waters)
Water purifiers, 1567, 1572–1573
Water treatment systems (See also Distribution system for water), 941, 2012–2019
adhesion of, 1052–1053
aeration in, 2017, 2266
aerobic endospores used to measure efficacy of, 141–149
Aeromonas and, 1091–1099
algal blooms and, 204–213
algal turf scrubbing (ATS) and, 213–219
ammonium in, 2167
annelida in, 1722
anolytes in, 604
antibiotic resistant bacteria in, 944
asellidae in, 1722–1723, 1722
assessing presence of microorganisms in, 1726–1727
assimilable organic carbon (AOC) and, 312–327, 1729
astroviruses and detection, 334
bacteriophages as indicators in, 354–363, 375–377
Bacteroides fragilis-infecting bacteriophages in, 356–358, 378–379
bank infiltration and, 2014–2015
benchmark values for, 1729–1730
biodegradable dissolved organic carbon (BDOC) and, 484–507, 1729
biofilm formation rate (BFR) in, 1729
biofilms and, 560–575, 620–622, 945–947
biofouling and, 601–609, 620–622
biologically active carbon (BAC), 1489, 1491
bottled water and, 795–802
bromine compounds and, 603
cell cell interactions in, 1056–1058
chemical composition of water in, 3100–3101
chemistry of water in, 606–607
chloramines in, 1069
chlorine compounds and, 603, 625–626, 1063–1067, 1069, 1172
chlorine dioxide in, 1063–1066, 1067, 1069–1070
chrionomidae in, 1723
clarification in, high-rate, 2017
Clean Water Act (CWA) and, 2975
Clostridium and, 866–871
coagulation process in, 2015–18
coliform bacteria in, 895–914, 944, 946–951
coliphages in, 379–380, 384–385
controlling microbial quality in, 941–952, 1727–1730
crustacea in, 1722–1723
Cryptosporidium in, 1719, 1727
CT disinfection concept for, 1065–1065
cyanobacteria and, 980, 982–995
cyanotoxins in, 982–995
deterioration of, microbiological, 1720
diamidinopheylindole (DAPI) in, 1068
diptera in, 1723
disinfection by products (DBPs) in, 1066
disinfection in, 359–361, 1063–1073, 1729, 2172–2175, 2266, 3260–3268
dissolved air floation (DAF) in, 2017, 2266
distribution systems for, 941–952, 1725
efficacy of treatment, factors affecting, 606–608
electrochemically activated water in, 603–604
electronegative filters in, 1156–1159
electropositive filters in, 1159–1160
Enterococci in, 1264–1278
enteroviruses and, 1156–1165
equalization in, 2265–2266
extracellular matrix (ECM) and, 1054, 1058
F specific RNA bacteriophages in, 356
fecal contamination in, 1256–1264
feeding, growth, and reproduction of microorganisms in, 1725–1726
filtration, 2012–2013
floc formation in, 2015–2018
fluorescein diacetate (FDA) in, 1068
Giardia in, 1719, 1727
granular activated carbon (GAC) filters in, 315, 1489–1496
groundwater immigration of microorganisms to, 1723–1724
growth of microorganisms in, 1724–1725
hepatitis viruses and, 1530–1540
heterotrophic bacteria and, as quality indicators, 1540–1552
home or point of use treatment devices for, 1550–1551, 1563–1575
in-line coagulation in, 2018
insects in, 1723
invertebrates in, 1718–1733
ion exchange in, 2018
isopoda in, 1722–1723
lime softening in, 2017–2018
macroinvertebrates in, 945
microorganisms in, 943–945
microsporidia in, 2079–2080
microstrainers in, 2013
monitoring microbial activity in, 947–948
monochloramine in, 1063–1066, 1069
Monod equation, regrowth of bacteria in, 314
nematodes in, 1718–1722
nitrate in, 2167
nitrification and, 2167–2176
nitrifying bacteria in, 2167–2176
nitrite in, 2167
nutrient levels in water of, 489
odor control and biofiltration in, 593–601
off-stream storage in, 2013–2014
oliogchaeta in, 1722
opportunistic pathogens in, 944–945
ozonation and, 603, 1067, 1070–1071, 1172–1173
parasitic invertebrates in, 1718–1719
pathogen hosts in, 1719–1720
pathogenic bacteria in, case studies in, 948–951
pH levels and, 2017–2018
phycosphere of, 1056–1057
pigmented bacteria in, 945
platyhelminthes in, 1718–1719
point of entry (POE) water treatment devices, 1563–1575
potential for bacterial regrowth in, 314–315
pretreatment in, 2012–2015
propidium iodide (PI) in, 1068
protozoa and, 1718, 33, 2262–2267, 2601–2602
protozoan parasites in, disinfection of, 1066–1073
Pseudomonas, 2632–2639
rainwater roof catchment systems, 2674–2685

raw and SFBW quality parameters for, 2265
regrowth of bacteria in, 313–319, 499–504
resistance of bacteria in, 607–608, 625
reverse transcription polymerase chain reaction (RT-PCR) for, 1162–1164
roughing filters and, 2012–2013
Safe Drinking Water Act (SDWA), 2974–2975
Salmonella and, 2789–2802
sampling of, 2813
sedimentation in, 2015–2018, 2266
settling of microorganisms in, 1725
somatic coliphages in, 355–356, 377–378
source water protection and, 2967–2978
spent filter backwash water (SFBW), 2262–2267
stability of water in, 947
Streptococci in, 1264–1278
substratum and, 1055–1056
sulfate-reducing bacteria (SRB) and, 3098–3100
sulfur bacteria in, 3096–3104
surface energy of substratum and, 1055–1056
surface water immigration of microorganisms to, 1724
survival of microorganisms in, 1725
Total Coliform Rule (TCR) and, 905–907
ultrafiltration and, 2266
ultramicrobacteria in, 945
ultraviolet disinfection and, 1071, 1173, 3230–3235
vertebrates in, 1723
viruses in, 1160–1164, 3260–3268, 3288–3300
Water turnover, algae vs., 1193–1194
Waterborne diseases, 2340
aerobic endospores and, as measurement of, 140–149
Aeromonas and, 1091–1099
bioterrorism and, 779
Campylobacter and, 803–811
cholera and, 853–861
Cryptosporidium and, 650–657
Cyclospora and, 995–1001
disinfection and, 1067
Entamoeba and, 1136–1146
Enterococci in, 1264–1278
enteroviruses and, 1146–1156
Giardia and, 1469–1489
hepatitis, 1530–1540
high hydrostatic pressure (HHP) vs., 1561
human caliciviruses and, 1586–1598
Isospora and coccidiosis in, 1734–1737
Legionella and, 1788–1806
leptospirosis, 1806–1817
microsporidia and, 2065–2083
Norwalk-like viruses (NLVs) and, 2221–2235
pathogenic Escherichia coli and, 2337–2339
Pfiesteria and toxic Pfiesteria complex (TPC), 2431–2447
Pseudomonas and, 2632–2639
in rainwater roof catchment systems, 2681–2682
risk assessment and, 2749
rotavirus and, 2753–2773
Salmonella and, 2789–2802
Shigella and, 2865–2871
source water protection and, 2967–2978
in spas and hot tubs, 2979–2981
streams and, 3017
Streptococci in, 1264–1278
virus transport and removal in subsurface, 2094–2107
viruses and, 92–100, 2743, 3288–3300
Waterborne viruses, adenoviruses as, 92–100
Watersheds, stream microbiology, 3015–3024
Weathering and biodeterioration, 516–528, 3364–3389
acid degradation in, 517
algae and, 519–522, 524–525
alkaline degradation in, 517–518
alkoxysiloxanes and, 518
autotrophic bacteria and, 523
bacteria and, 523
biofilms and, 516
biological weathering and, 517
biopitting in, 518
cement/in cement and concrete, 523–525
chelation in, 517
chemical weathering and, 516–517
chemoorganotrophic bacteria and, 525
chemotrophic bacteria and, 523
cyanobacteria and, 519–522, 524–525
depolymerization in, 518
endolithic organism and, 517–528, 1105
epilithic organisms and, 517–528
extracellular polymeric substances (EPS) and, 518, 1227–1228
fungi and, 518–519, 524
lichens and, 522–523
in limestone, shale and sandstone, 518–523
lithotrophs and, 1827
nitric acid and, biogenic, 524
organic acid and, biogenic, 524–525
osmotic protectants and, 518
oxalic acid and, 524
patina in, 518
in silicon and silicates, 517–518
spalling in, 517
sulfate-reducing bacteria (SRB) and, 524
sulfuric acid and, biogenic, 524
Wells, sulfur bacteria, control, 3103
Wetlands (See also Marine/ocean environment; Marshes; Surface waters), 1316–1333, 3389–3401, 3401–3417
aerobic processes in, 3403–3405
anaerobic processes in, 3403–3405
biodegradation in, 3401–3417
biogeochemistry of, 3402–3405
carbonaceous biochemical oxygen demand (CBOD) in, 3391
catabolic activity in, 3411–3415
chemical oxygen demand (COD) in, 3391
dioxygenases in, 3412
enzyme activity in, 3410–3415
flooded soils and, 3403–3404
fungi and, 3359–3360, 3412
halorespiration in, 3414
heterotrophic microbial activity in, 3393
hydrocarbons and, 3405–3410
iron processing in, 3393
meroplankton and, 1868–1869
microbial transformation reactions in, 3410–3415
monooxygenases in, 3412
nitrogen processing in, 3391–3393, 3404
organic matter and, 3391, 3403
organic pollutant fate in, 3405–3410
organohalogens and, 3405–3410
oxidoreductases in, 3412
oxygen and, 3403–3404
parasites in, 3399
pathogens in, 3393–3400
periphyton and, 2360
pesticides in, 3405–3410
pH levels and, 3393
plant life in, 3405

Wetlands (See also Marine/ocean environment; Marshes; Surface waters), (*continued*)
pollution concerns and, 3401–3402
protozoa in, 3398–3399
reductases in, 3414
rhizosphere of, 3404–3405
soil types in, 3402–3403
sulfur processing in, 3393
terminal electron acceptor processes (TEAPs) in, 3404
total organic carbon (TOC) in, 3391
vectors and wildlife pathogens in, 3399–3400
viruses in, 3397–3398
wastewater treatment and, 3389–3401
White-rot fungi, 1385–1386, 2646–2647
Whole effluent toxicity (WET), in wastewater/sewage, 3168
Whooping cough (See Pertussis)
Wilcoxina, 2125–2126
Wind
bioaerosols and, 2110
subaerial communities and, 3058–3059
Wine making, 1187–1188, 1384
WIT, 1450
Wolbachia, 1115, 1121–1122
Wolinella, 1609–1610
Wood degradation (See also Pulp and paper industry)
biofilms and, 88
fungal, 1385–1389, 2646–2647, 3358–3359
spores and spore dispersion and, as byproduct of, 421–422
Wuhrmann process, activated sludge, 77

X

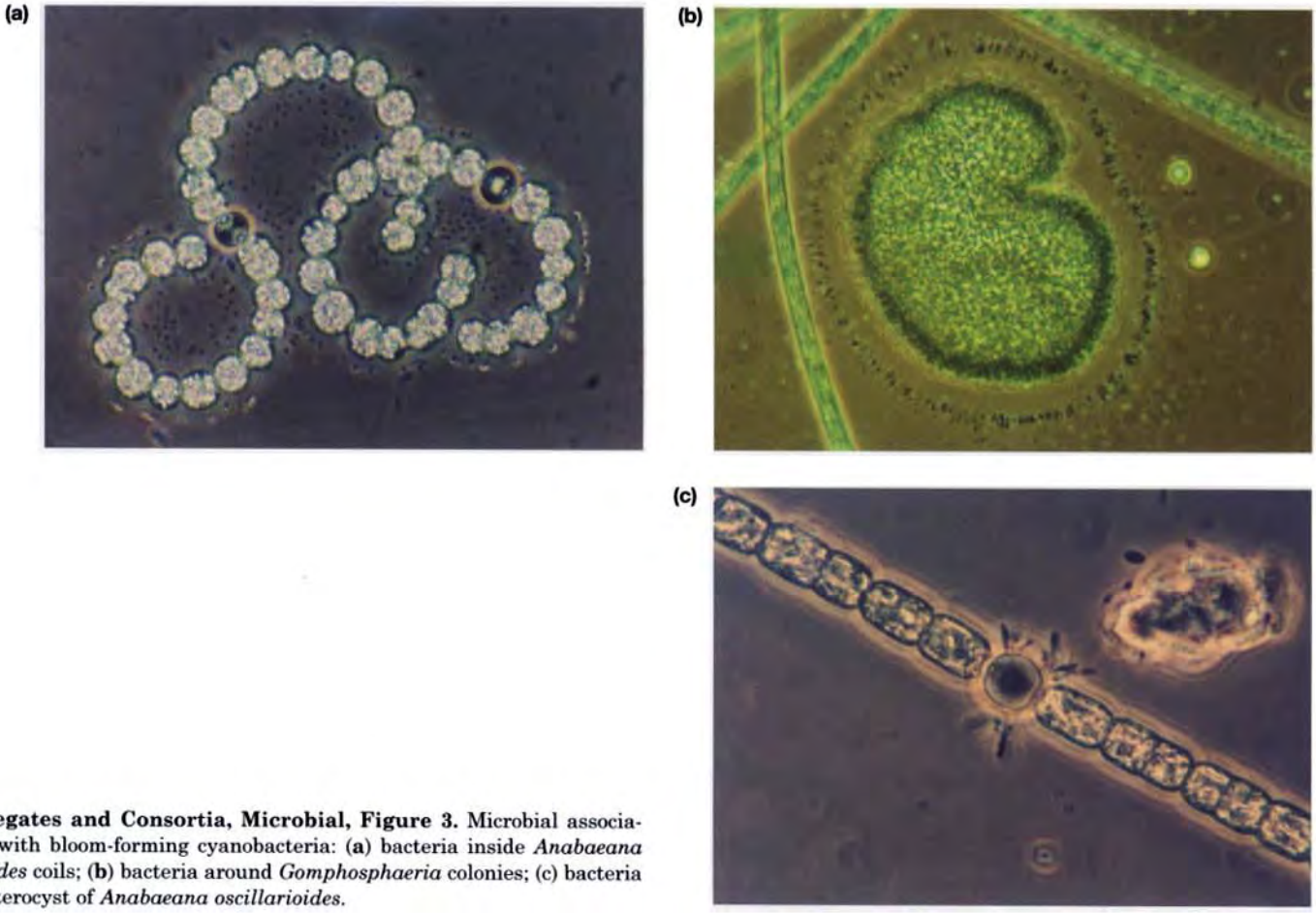
X-ray crystallography, 1449
X-ray photoelectron spectroscopic (ESCA) analysis, 934
X-rays, space microbiology, 2988–2989
Xanthan gum, bioplastics, 682–692
Xanthobacter, 1196, 1200
biodegradation and, 1938, 2292–2300
nitrogen fixation and, 2193–2201
Xanthomonas, 1889, 3314–3319
biocontrol use of, 2705
biofilms and, 1215, 2343–2356
cold-adapted microorganisms in, 872–885
extracellular polymeric substances (EPS) and, 1229
fluorescent probes and, 1340
genetically engineered microorganisms (GEMs) and, 1279
granular activated carbon (GAC) filters and, 1490
nosocomial infections and, 2236–2250
in pulp and paper industry, 2650
soil salinity and, 2775
subaerial communities and, 3057–3065
subsurface microbial communities and, 3067–3071
Xanthones, algae biotechnology, 195
Xanthophyceae
periphyton and, 2357
photoautotrophic, in low-light habitats, 837–843
photosynthetic pigments in, 2467
Xanthophylls, 2307–2317, 2453–2470
Xanthophytes, 2504–2518
Xenobiotics, 2058, 3401
biodegradation of, 1195, 1420–1421
bioremediation of, 693
in municipal solid waste landfills, 1762
Xenococcus, 519
Xenorhabdus, 658–662
Xerophiles (See also Desert environments; Hyperthermophiles; Thermophiles), 2889
Xiphinema, 1122
Xylan, 1210
Xylanase, 220, 226–227, 1183–89, 1447, 2648, 2903–2910
Xylene biodegradation, 700, 714, 917, 1763, 1423–1429, 1994–2000, 2296–2300, 2412, 3405
Xylose, 2716
Xylosidase, 1211–1223, 3021

Y

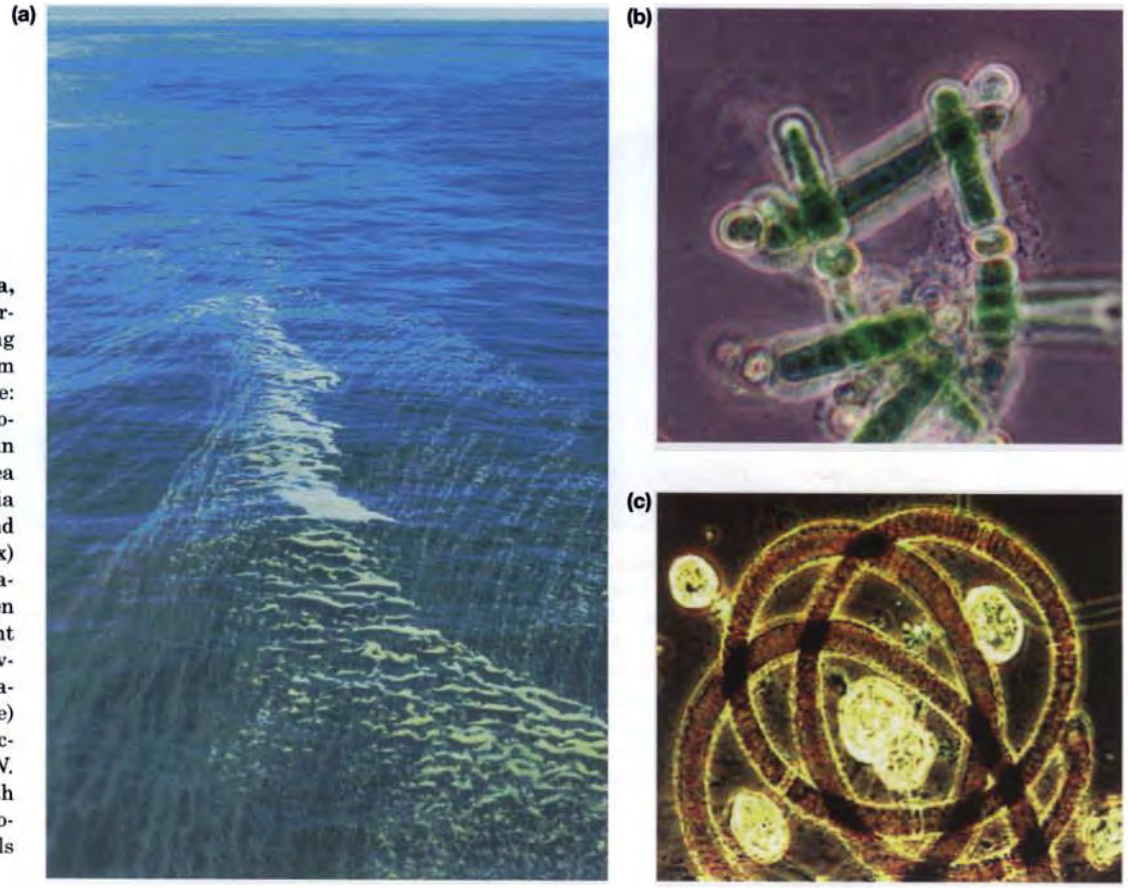
Yeasts (See also Bioaerosols; Fungi; Spores and spore dispersal), 405, 1647, 1661
alkaliphiles and, 136
Antarctic soils and, 1027–1028
biofilms and, 2342–2356
cold-adapted microorganisms in, 872–885
in groundwater, 1671
high hydrostatic pressure (HHP) vs., 1561
as microbial tracers in groundwater, 3194–3202
municipal solid waste landfills and, 1766
in permafrost, 2369
soil microorganisms and, 2913–2914
in drinking water, 945
Yellow-green algae, 206
Yellow rain, bioterrorism, 778–779
Yersinia, 338, 340
biofilms and, 577
biotechnology and, 3204
bioterrorism and (See Plague)
cold-shock response and, 886–895
detection of, 1133
disinfection and, 1065
granular activated carbon (GAC) filters and, 1492, 1571–1572
heterotrophic bacteria and, 1544
high hydrostatic pressure (HHP) vs., 1555–1563
municipal solid waste landfills and, 1766
in source water, 2969–2978
in spas and hot tubs, 2980–2984
water treatment systems and, 3199
Young equation, adhesion in biofilms, 122–123
Yttrium, 199
Yucca Mountain nuclear waste repository, 250–260

Z

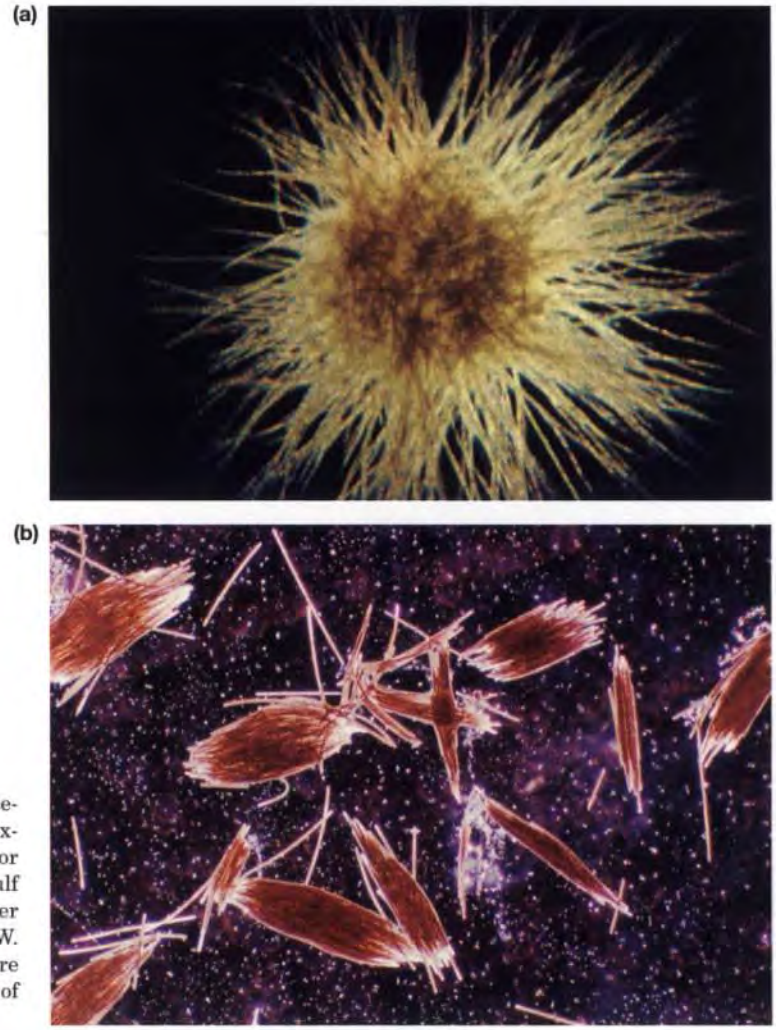
Zeaxanthin, 2307–2317, 2453–2470
Zeolite, activated sludge foaming, 6–7
Zeta potentials, adhesion in biofilms and biofilms, 100, 119
Zinc, 1878–1894, 1902–1913, 2961
algae biotechnology and, 196, 199–200
bioremediation and, 709
cyanobacteria and, 969
hydrothermal vents and, 1618
in marine/ocean environment, 1705–1706
municipal solid waste landfills and, 1763–1764
resistance of bacteria to, 608
soil microorganisms and, 2887
Zonation, endolithic microorganisms, 1104
Zone settling velocity (ZSV), 1307
Zoogloea
activated sludge and, 57, 3009
bacterial counts of, 31–32
biodegradation and, 2650
bioplastics and, 684–692
floc formation and, 2006
Zoomastigiophora, 2605



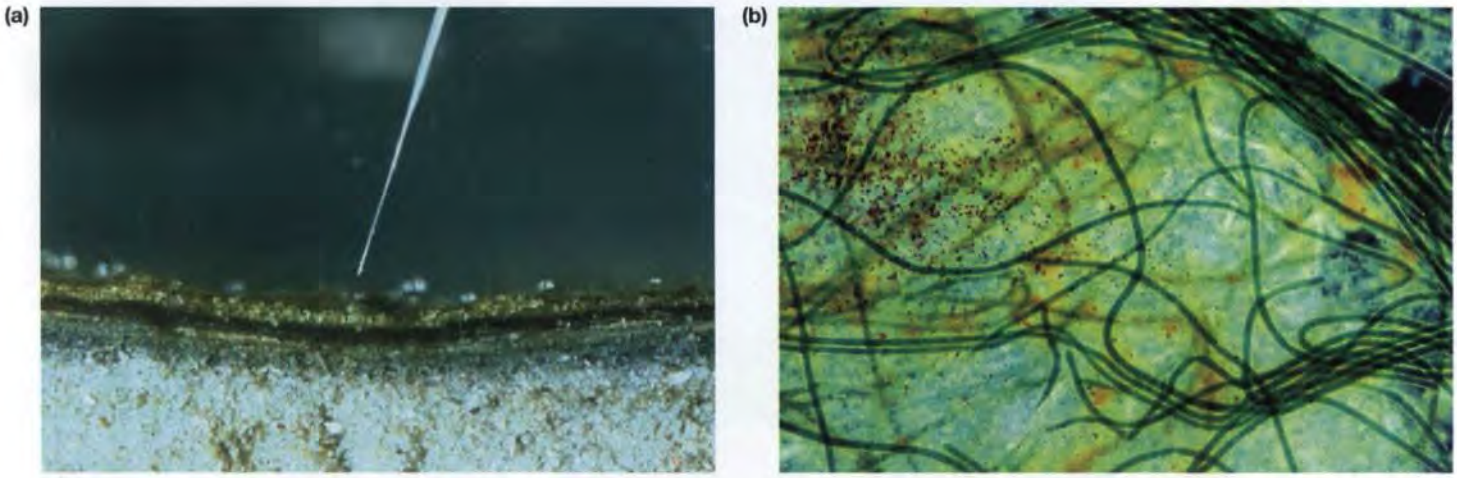
Aggregates and Consortia, Microbial, Figure 3. Microbial associations with bloom-forming cyanobacteria: (**a**) bacteria inside *Anabaeana spiroides* coils; (**b**) bacteria around *Gomphosphaeria* colonies; (c) bacteria on heterocyst of *Anabaeana oscillarioides*.



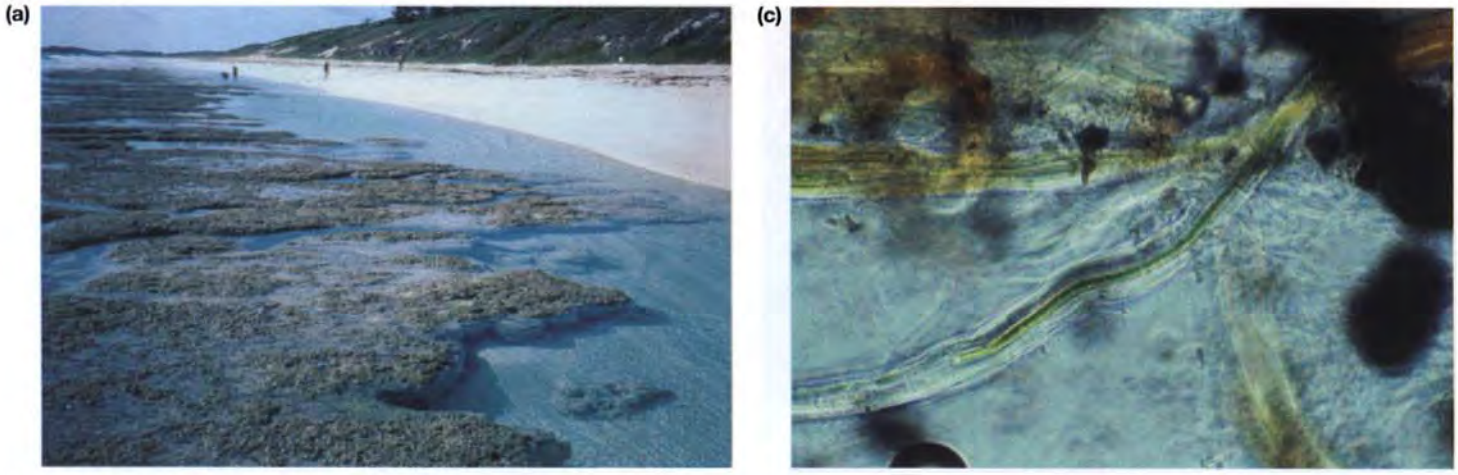
Aggregates and Consortia, Microbial, Figure 5. Observations of the N_2 fixing cyanobacterial bloom from *Nodularia* spp. Left-hand side: a *Nodularia*-dominated cyanobacterial bloom observed in the Gulf of Finland, Baltic Sea (photo courtesy of Pia Moisander). Upper right-hand side: photomicrograph (400x) of several *Nodularia* sp. filaments, showing the nitrogen fixing heterocysts (round, light green cells), neighboring heavily (green) pigmented vegetative (photosynthetically active) cells, as well as associated bacteria. Lower right: coiled *N. spumigena* aggregates with associated bacteria and protozoans present inside the coils (400x).



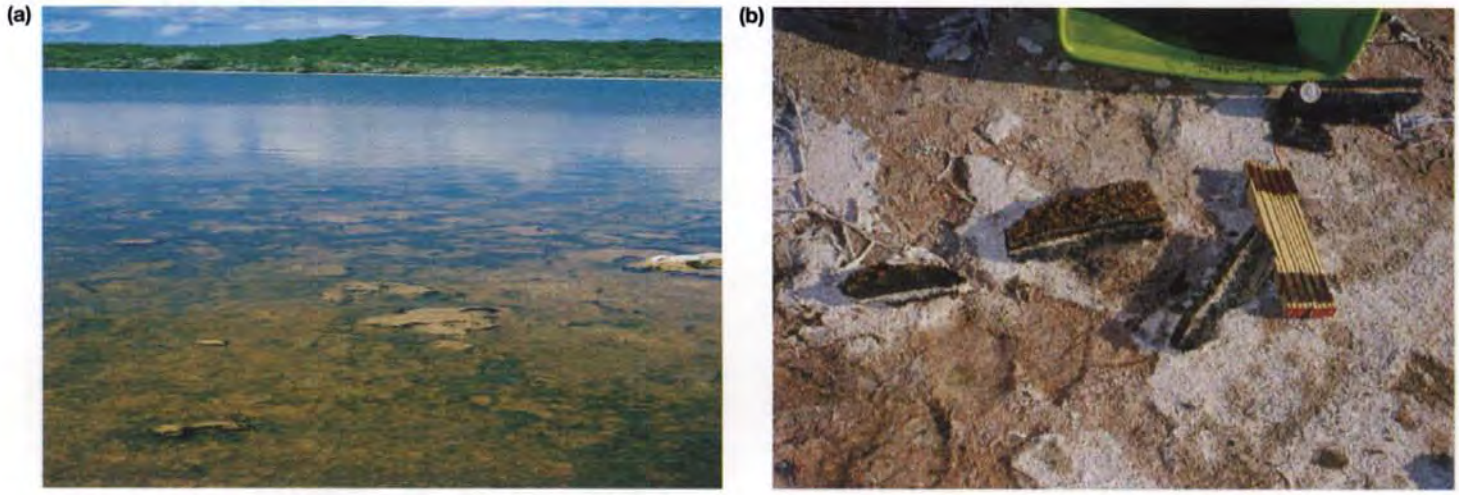
Aggregates and Consortia, Microbial, Figure 6. Surface-dwelling aggregates of the filamentous marine planktonic N_2 fixing cyanobacteria *Trichodesmium* spp. Upper frame: radial, or "puff"-shaped aggregates, sampled during a bloom in the Gulf Stream, W. Atlantic Ocean, off the coast of North Carolina. Lower frame: fusiform "tuft"-shaped aggregates sampled in the W. Atlantic Ocean near San Salvador Island, Bahamas. For more details on the N_2 fixing characteristics, and microbial ecology of these aggregates see Paerl (149).



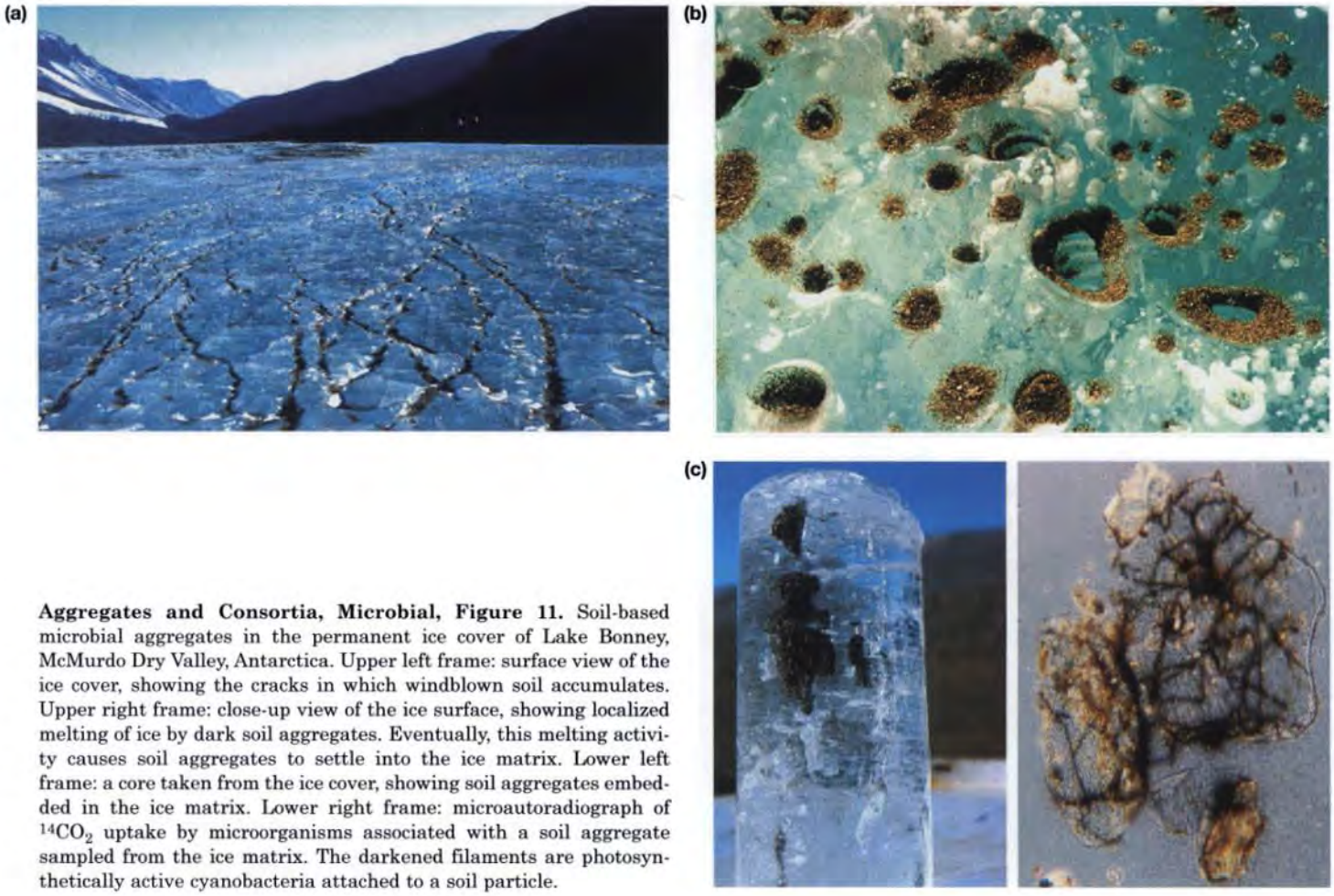
Aggregates and Consortia, Microbial, Figure 7. Laminated microbial mat obtained from the Bird Shoal, coastal North Carolina. As is the case with most marine intertidal mats, microalgal biomass and production is dominated by cyanobacteria. The cyanobacteria are concentrated in the upper, illuminated segment of the mat. The mat thickness is approximately 0.5 cm. Left frame: cross-section of the mat, showing distinct microbial laminations. Phototrophic cyanobacteria dominate the upper, euphotic zone of the mat. Below that is a layer composed of photosynthetic bacteria, cyanobacteria, and heterotrophic bacteria. The lowest layer contains obligate anaerobic heterotrophs, including sulfate-reducing bacteria. Methanogenic bacteria can also be found in this layer. The mat is situated on highly porous sands, which allow for rapid exchange of sweater and oxygen. Hence, the lighter oxygenated appearance of the lower sand layer. The tip (~100 μm diameter) of an oxygen microelectrode is shown for size reference. Right frame: photomicrograph of the mat matrix. Shown are nonheterocystous filamentous cyanobacteria (*Microcoleus chthonoplastes*) and a variety of bacteria, including microcolonies of purple photosynthetic bacteria (*Chromatium* sp.).



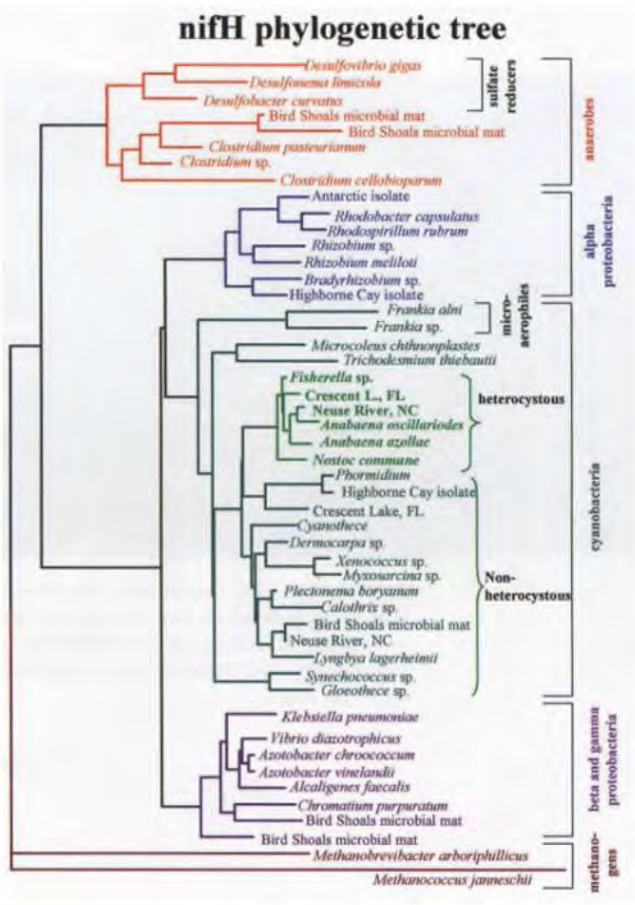
Aggregates and Consortia, Microbial, Figure 9. Stromatolitic mat communities on the Exuma Islands, Bahamas. Upper frame: view of the subtidal stromatolites situated off a beach at Highborne Cay, Exumas. Lower frame: cross-sectional view of a laminated ($CaCO_3$) stromatolite, showing the cyanobacteria-dominated mat residing on the surface of the stromatolite. Right frame: photomicrograph, showing the aggregated filamentous cyanobacteria (*Schizothrix* sp.) comprising a bulk of the surface mat.



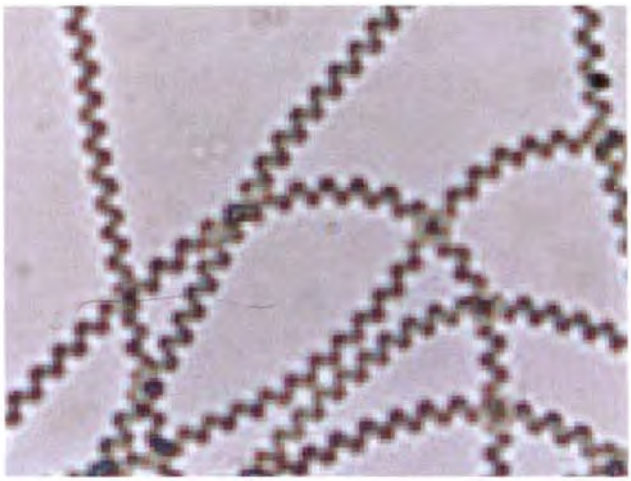
Aggregates and Consortia, Microbial, Figure 10. Left frame: hypersaline (100 to 160 psu) Salt Pond, located on San Salvador Island, Bahamas. The entire bottom of this lake is dominated by laminated mats dominated by filamentous, nonheterocystous cyanobacteria (*Microcoleus* sp., *Lyngbya* sp., and *Oscillatoria* sp.). Right frame: close-up side view pieces of laminated mat excised from the Pond.



Aggregates and Consortia, Microbial, Figure 11. Soil-based microbial aggregates in the permanent ice cover of Lake Bonney, McMurdo Dry Valley, Antarctica. Upper left frame: surface view of the ice cover, showing the cracks in which windblown soil accumulates. Upper right frame: close-up view of the ice surface, showing localized melting of ice by dark soil aggregates. Eventually, this melting activity causes soil aggregates to settle into the ice matrix. Lower left frame: a core taken from the ice cover, showing soil aggregates embedded in the ice matrix. Lower right frame: microautoradiograph of $^{14}CO_2$ uptake by microorganisms associated with a soil aggregate sampled from the ice matrix. The darkened filaments are photosynthetically active cyanobacteria attached to a soil particle.



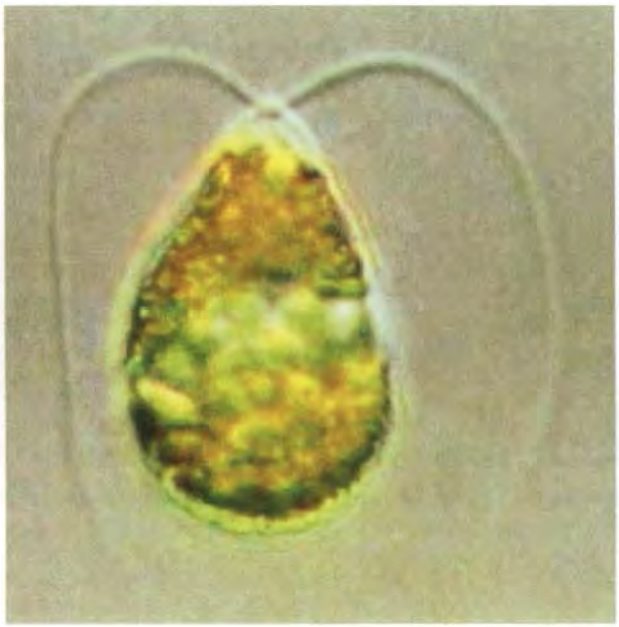
Aggregates and Consortia, Microbial, Figure 13. Phylogentic tree of nitrogen fixing microbial groups, many of which participate in consortial associations. The tree was based on analysis of a 326 base pair nucleotide fragment of the *nifH* gene, which encodes for the highly conserved dinitrogenase reductase subunit of nitrogenase, the N_2 fixing enzyme complex. The tree was constructed by the neighbor-joining method and bootstrap values greater than 50% are given above or beside the corresponding nodes.



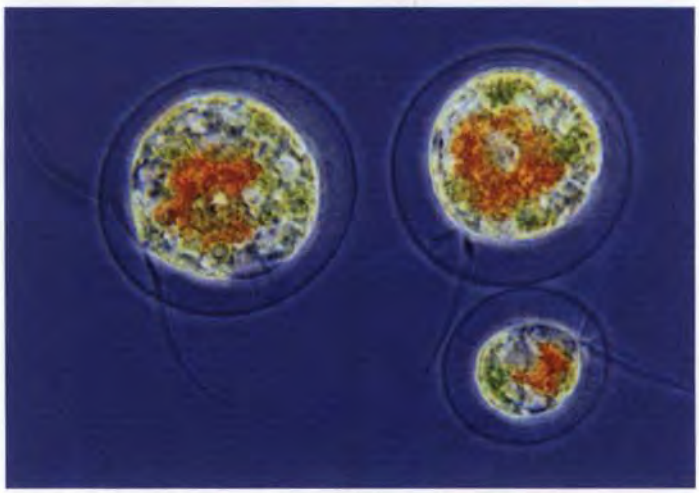
Algae Biotechnology, Figure 1. Cyanobacteria-Spirulina sp. *http://vis-pc.plantbio.ohiou.edu/algaeimage/pages/spirulina.html*

Algae Biotechnology, Figure 2. *Chlorella vulgaris, http://www.ifa.au.dk/~jabraham / algae.htm*

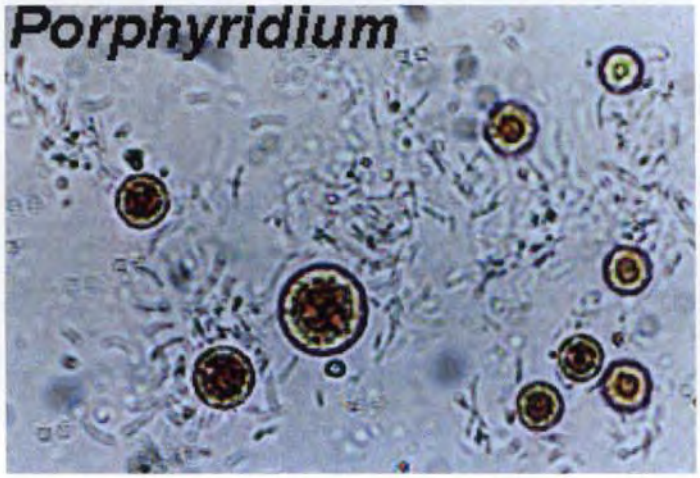
Algae Biotechnology, Figure 3. *Dunaliella, http://zobell.biol.tsukuba.ac.jp/~inouye/ino/g/chl/chl_pict.html*



Algae Biotechnology, Figure 4. *Dunaliella, http://zobell.biol.tsukuba.ac.jp/~inouye/ino/g/chl/chl\pict.html*



Algae Biotechnology, Figure 5. *Haematococcus, http://www.microscopy-uk.org.uk/mag/wimsmall/extra/haema.html*

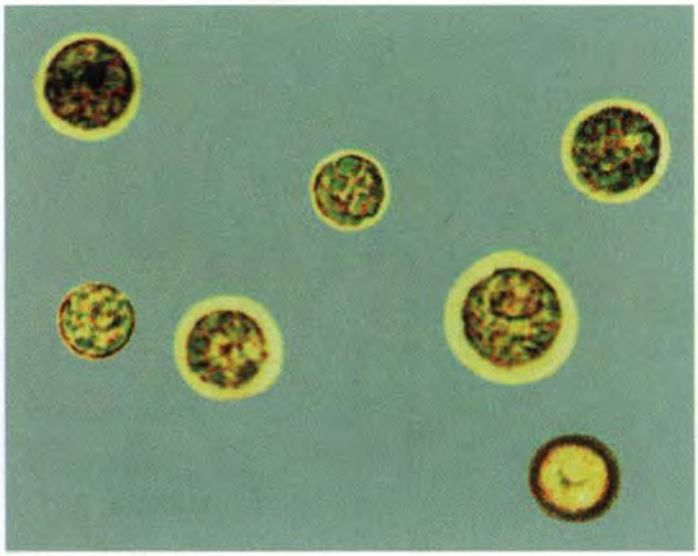


Algae Biotechnology, Figure 6. Rhodophyta-*Porphyridium purpureum http://vis-pc.plantbio.ohiou.edu/algaeimage/pages/Porphyridium.html*

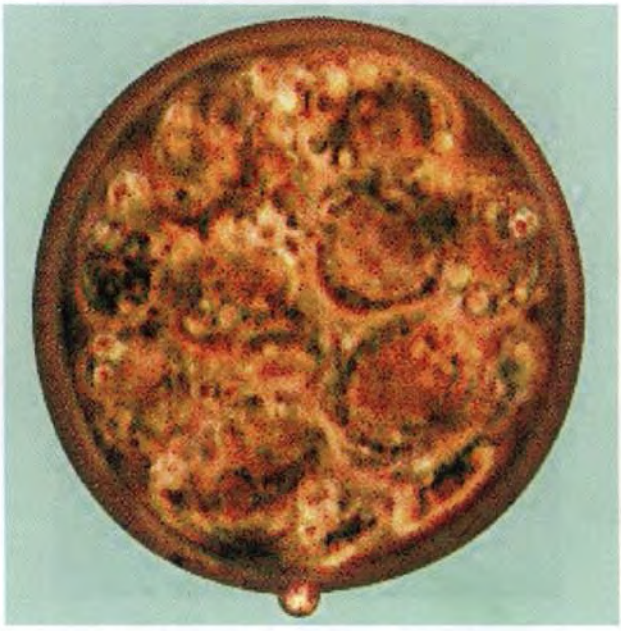
Algae Biotechnology, Figure 7. *http://members.nbci.com/_XMCM/rekel/ushn/helo/photo_daphnia.htm*

Algae Biotechnology, Figure 9. Astaxanthin, *http://www.bioprocess.is/product.html*

Algae Biotechnology, Figure 8. Provided by Dr. Lustigman & Dr. Lee.



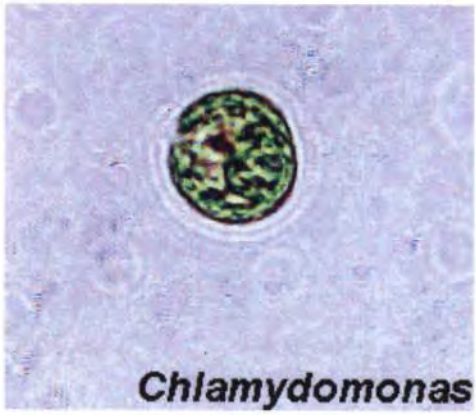
Algae Biotechnology, Figure 10. *Rhodella reticulata, http://www.cibnor.org/malgas/ifotrhr.html*



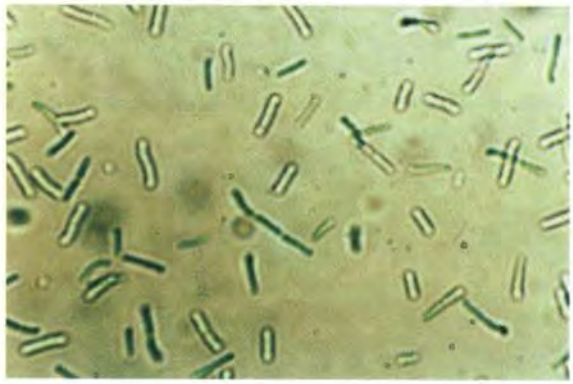
Algae Biotechnology, Figure 11. *Chlorococcum oleofaciens, http://www.cibnor.mx/malgas/efotcho.html*

Algae Biotechnology, Figure 12. Euglenophyta-*Euglena gracilis* *http://vis-pc.plantbio.ohiou.edu/algaeimage/pages/Euglena.html*

Algae Biotechnology, Figure 13. Chlorophyta-*Stigeoclonium* sp. *http://vis-pc.plantbio.ohiou.edu/algaeimage/pages/Stigeoclonium.html*

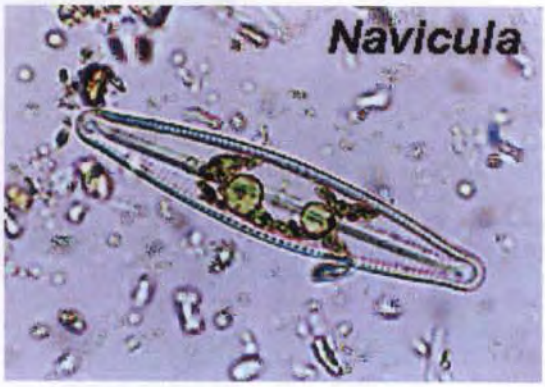


Algae Biotechnology, Figure 14. Chlorophyta-*Chlamydomonas* sp. *http://vis-pc.plantbio.ohiou.edu/algaeimage/pages/Chlamydomonas.html*



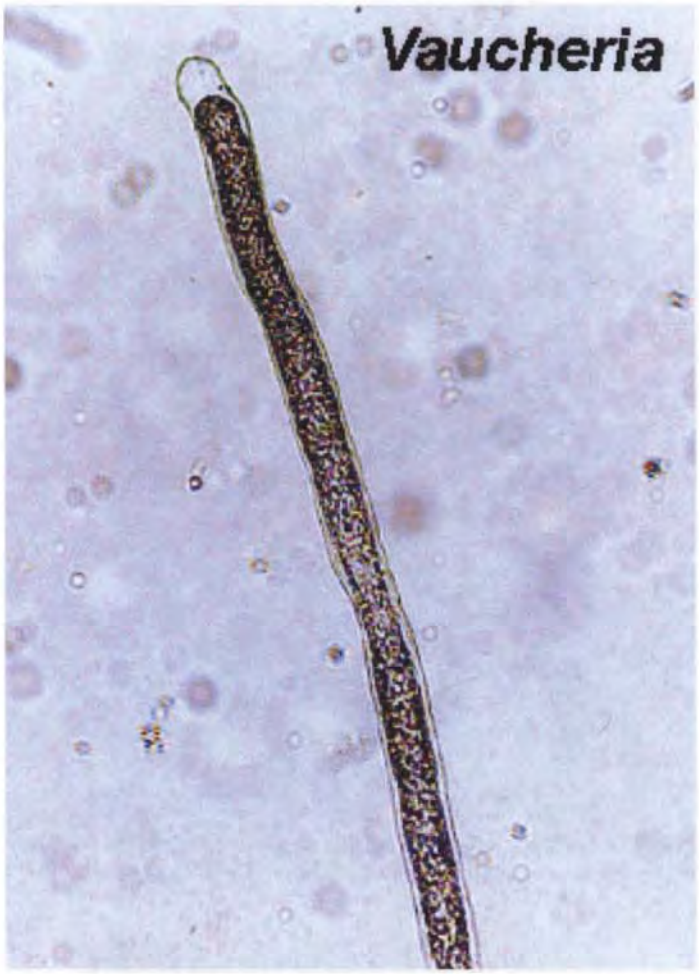
Algae Biotechnology, Figure 15. *Anacystis nidulans,* provided by Dr. Lee H. Lee.

Algae Biotechnology, Figure 16. Chlorophyta-*Scenedesmus* sp. *http://vis-pc.plantbio.ohiou.edu/algaeimage/pages/Scenedesmus.html*

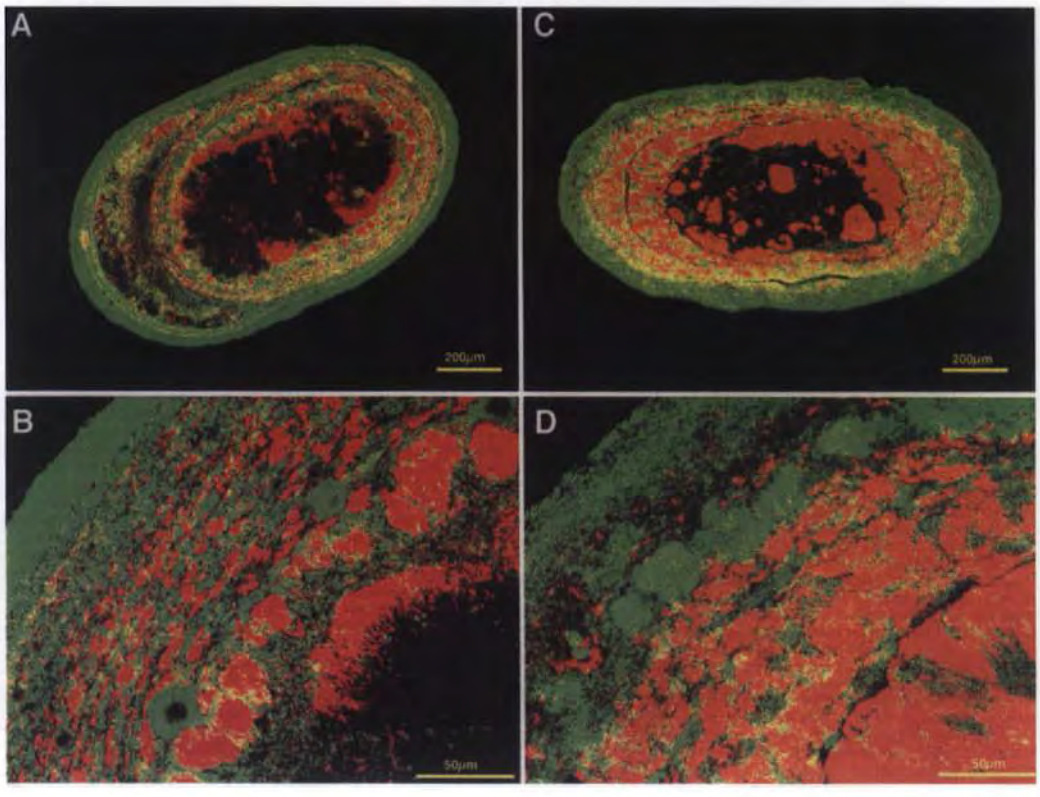


Algae Biotechnology, Figure 17. Bacillariophyceae-*Navicula* *http://vis-pc.plantbio.ohiou.edu/algaeimage/pages/navicula.html*

Algae Biotechnology, Figure 18. *Oscillatoria http://vis-pc.plantbio.ohiou.edu/algaeimage/pages/oscillatoria.html*



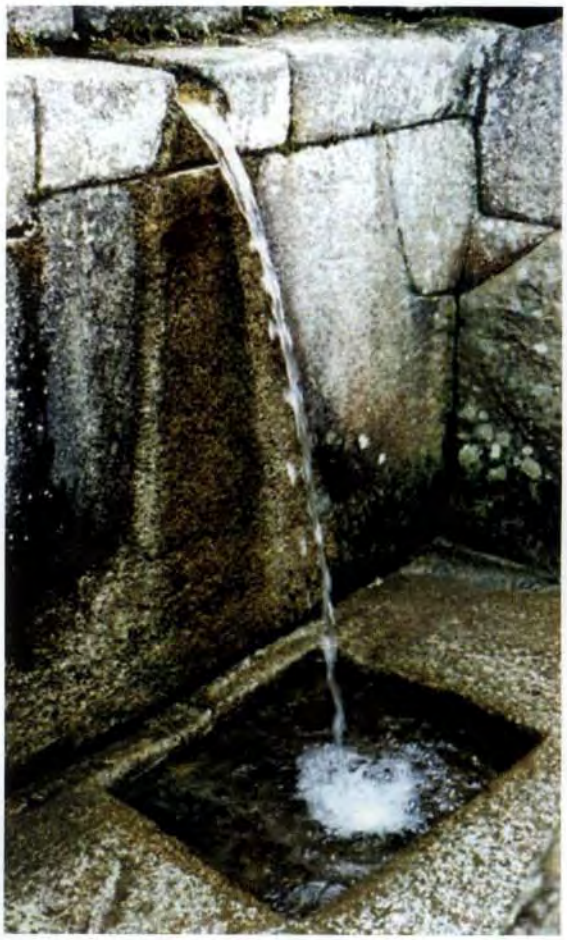
Algae Biotechnology, Figure 19. Xanthophyceae-*Vaucheria http://vis-pc.plantbio. ohiou.edu/algaeimage/pages/Vaucheria.html*



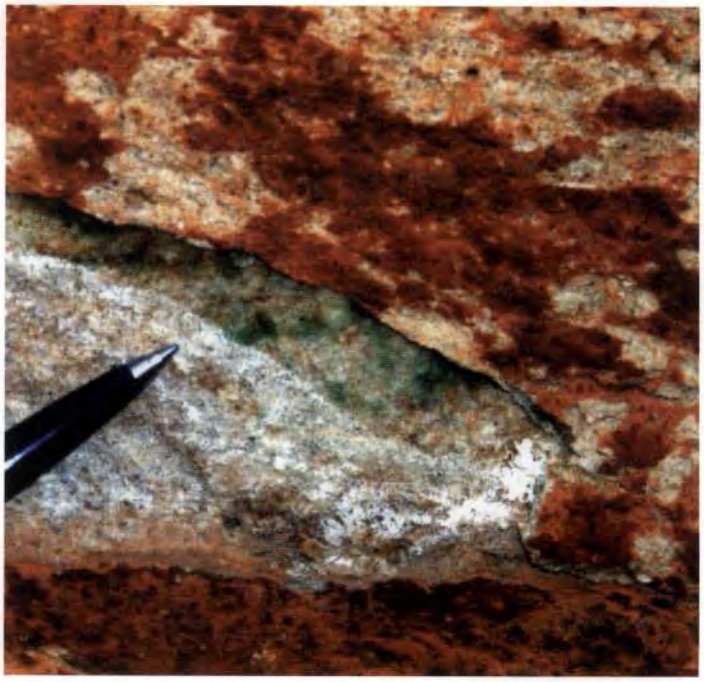
Anaerobic Granules and Granulation Processes, Figure 3. FISH of sections from mesophilic (**a** and **b**) and thermophilic (**c** and **d**) granules viewed by confocal laser scanning microscopy. The sections were simultaneously hybridized with a Cy-5-labeled bacterial probe (green) and a rhodamine-labeled archaeal probe (red). Reproduced with permission from Y. Sekiguchi et al., *Appl. Environ. Microbiol.* **65**, 1,280–1,288 (1999).

Biodeterioration of Mineral Materials, Figure 1. Discolored stone statue in Karlovy Vary, Czech Republic, showing growth of mainly mitosporic fungi and cyanobacteria.

Biodeterioration of Mineral Materials, Figure 2. Mayan building in Chichen Itza, Mexico, showing blackening of limestone by epilithic and endolithic fungi and cyanobacteria.

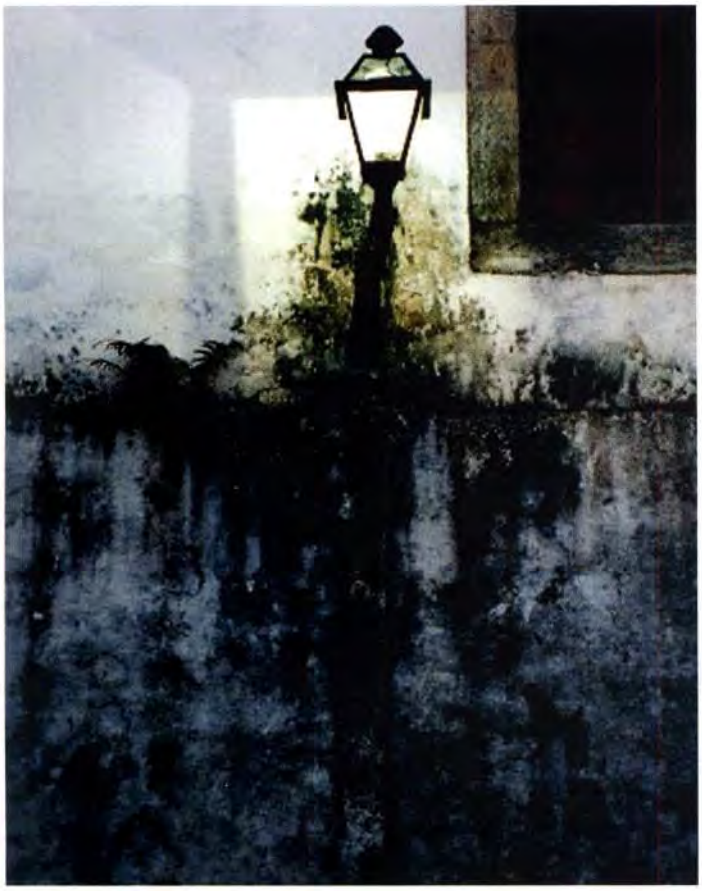


Biodeterioration of Mineral Materials, Figure 3. Fountain in Machu Picchu, Peru, showing intense growth of algae and cyanbacteria in the splash zone.

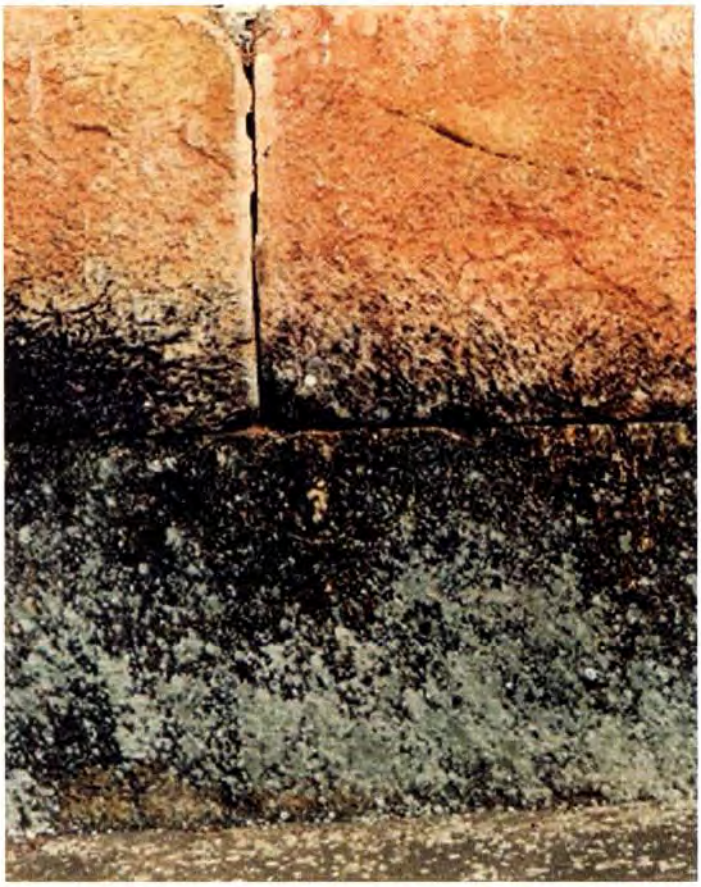


Biodeterioration of Mineral Materials, Figure 4. Spalled soapstone on the external wall of a church in Ouro Preto, Brazil. Cyanobacteria and actinomycetes were the major microorganisms detected below the detached layer.

Biodeterioration of Mineral Materials, Figure 5. Salting on the surface of mortar in the tomb of Sevilha, Necropolis of Carmona, Spain.



Biodeterioration of Mineral Materials, Figure 6. Intense algal growth behind a lamp attached to a building in Sao Joao del Rei, Brazil. Note the black growth of mitosporic fungi in the rain runoff area below the lamp.



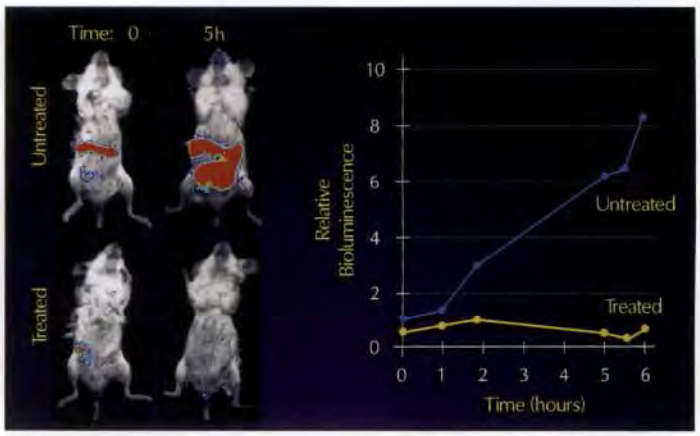
Biodeterioration of Mineral Materials, Figure 7. Growth of fungi (upper black layer) and lichen (lower pale green layer) on the quartzite course of a church in Tiradentes, Brazil.

Biodeterioration of Mineral Materials, Figure 8. Intense black discoloration caused by growth of mainly mitosporic fungi and cyanobacteria on the east-facing painted wall of a church in Ouro Preto, Brazil.

Bioleaching of Metals, Figure 1. The Rio Tinto, Spain, passing through the historic mining area. Note the ochre-colored water, which is reported to have dissolved ferric iron concentrations more than 2 g/L (1).

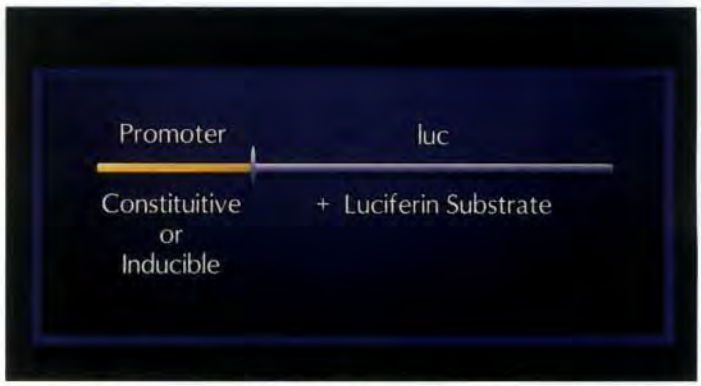
Bioleaching of Metals, Figure 2. A view of the Roman-era iron mines at Parys Mountain, Anglesey, Wales.

Bioleaching of Metals, Figure 6. One of the three biooxidation pretreatment heaps measuring approximately 1,000 by 500 feet, with ore stacked to 30 feet, containing 709,000 tons of low-grade refractory ore. Courtesy of Dr. J. Brierley, Newmont Mining Corporation.

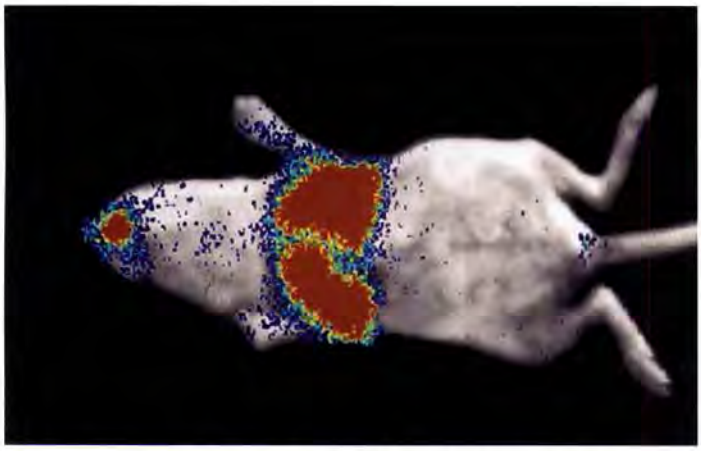


Bioluminescence Methodology, Figure 1. GI infection/Salmonella. The pseudocolor image is a color depiction of the intensity and quantity of photons with red being most intense and violet least intense. Untreated and treated mice are depicted at time zero (eight days after oral inoculation) with SL1344 lux *S. typhimurium,* but before treatment with ciprofloxacin. The same mice were imaged five and a half hours after treatment with ciprofloxacin (untreated control-received injection of phosphate-buffered saline). The graph shows relative bioluminescence intensity measured from the abdominal area at the time after treatment for both treated and untreated animals.

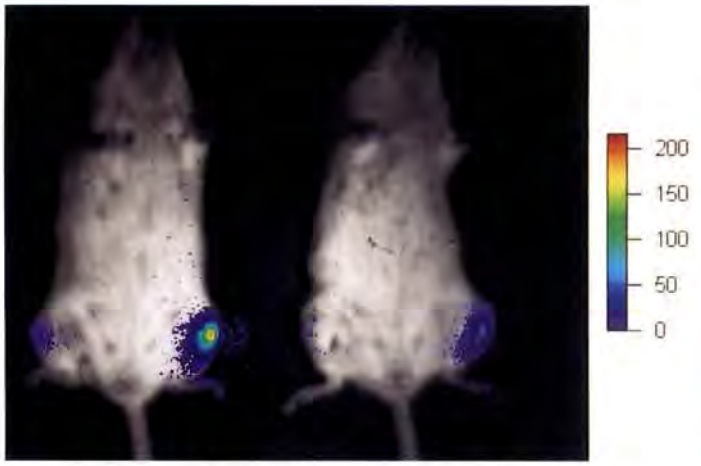
Bioluminescence Methodology, Figure 2. Prokaryotic Expression. For expression of light from prokaryotic cells, a construct is used that includes the *lux* operon from *P. luminescens* is an appropriate gram-negative or gram-positive plasmid vehicle. More recent constructs include transposons for chromosomal integration. Expression of the *luc* gene from the North American firefly may also be used in prokaryotes as long as the luciferin substrate is administered.



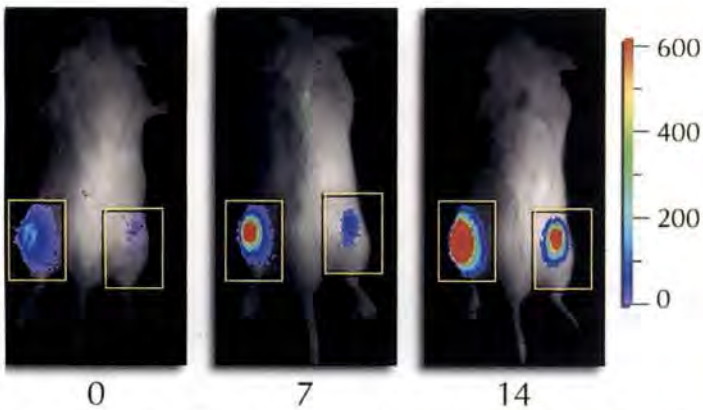
Bioluminescence Methodology, Figure 3. Eukaryrotic Expression. Expression of light from Eukaryrotic cells is accomplished with the pGL3 vector (Promega) containing the *luc* gene of the North American firefly regulated by an SV40 viral promoter. Luciferin is administered introperitoneally as the substrate for the enzymatic reaction.



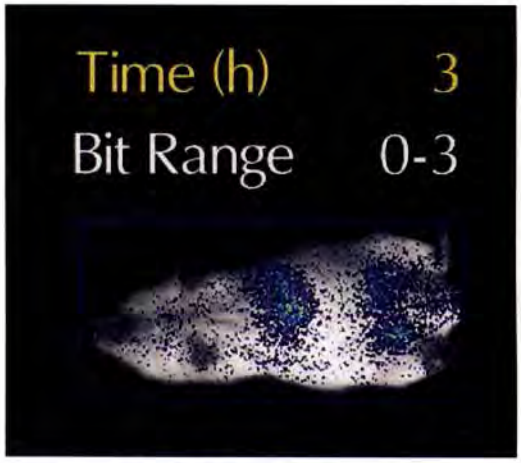
Bioluminescence Methodology, Figure 4. Lung Model. Neonatal mice (under two weeks of age) are susceptible to lung infection following oral inoculation with *S. typhimurium* SL1344 lux. A nasopharangeal infection is common in both adults and neonates from this mode of inoculation.



Bioluminescence Methodology, Figure 5. Soft tissue thigh model *Lux* tagged *E. coli* are injected at 10^4 bacteria/mL (right thigh) and 10^5 bacteria/mL (left thigh). Bacteria can then be quantified noninvasively with and without antibiotic treatment.

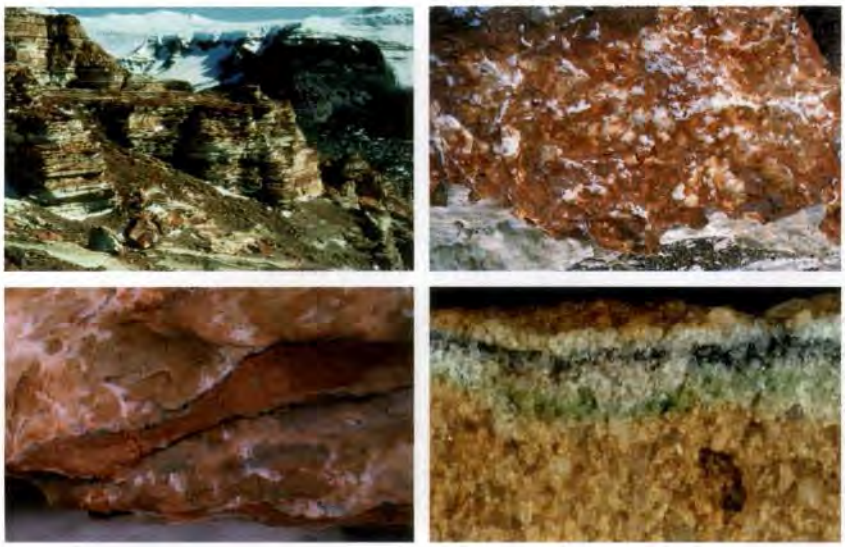


Bioluminescence Methodology, Figure 6. Labeled tumor cells. Labeled NIH 3T3 cells were injected with matrigel at zero time [10^4 cells (right thigh) and 10^5 cells (left thigh)] and mice were imaged between 7 and 14 days to monitor the increase in cell number, as demonstrated by the increase in red pixels of the pseudocolor image.

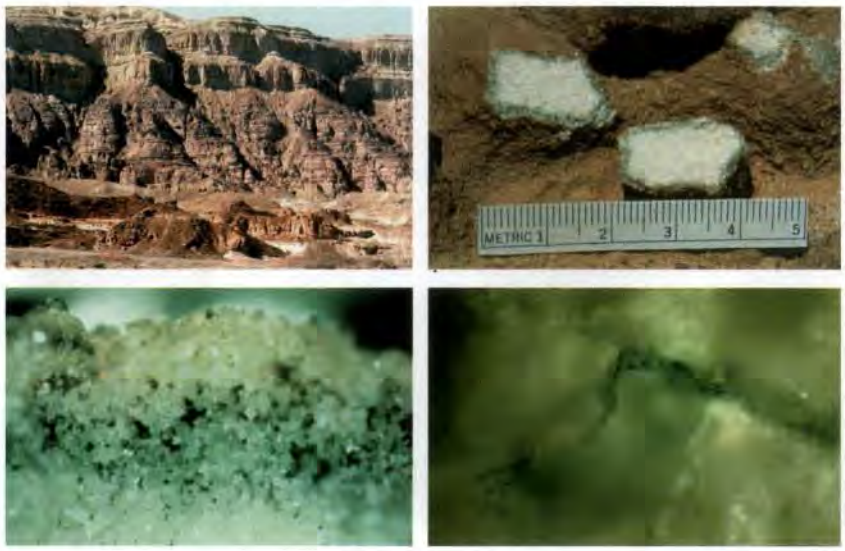


Bioluminescence Methodology, Figure 7. Transgenic animal exposed to heavy cadmium. A mouse line transgenic for a cadmium-regulated element, the hemooxygenase promoter, demonstrates in vivo monitoring of cadmium induction of *luc* in front of the *luc* gene. 10 mM cadmium was delivered IP and the mouse was imaged at zero and three hours. The three-hour time point with a camera sensitivity set at a bit range of zero to three is shown here.

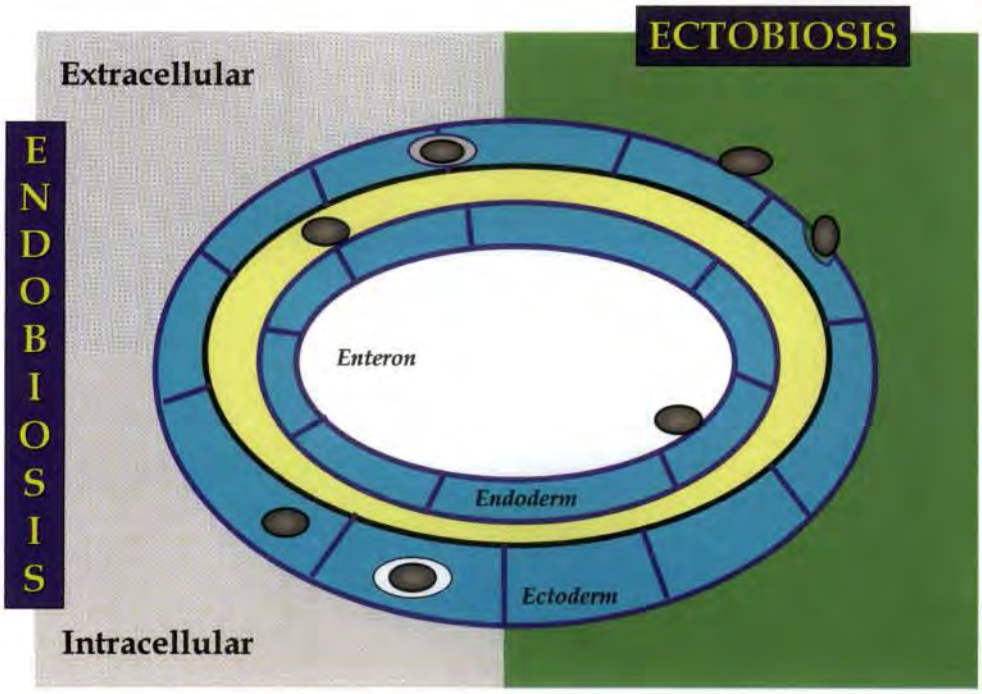
Desert Environments — Soil Communities in Cold Deserts, Figure 2. View in Wright Valley from the helicopter — a landscape typical of the Dry Valleys. Photograph by Wolf Vladimir Vishniac.



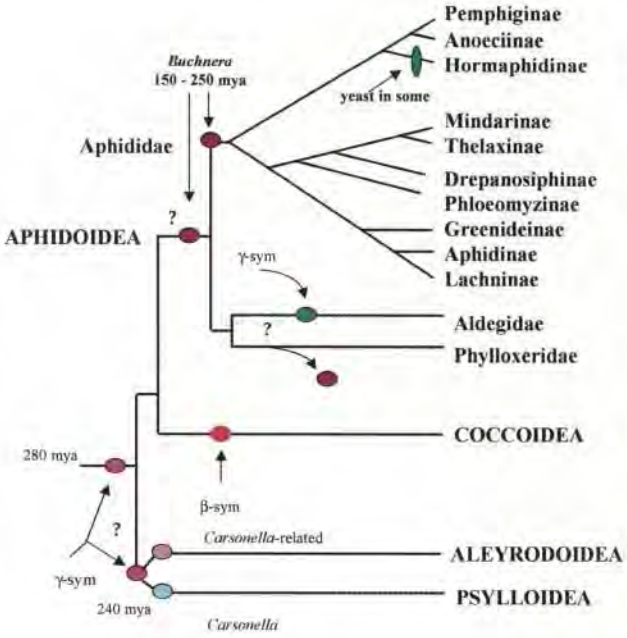
Endolithic Microorganisms in Arid Regions, Plate 1. Endolithic communities in cold deserts. Upper right. Sandstone cliffs in University valley, Ross Desert (McMurdo Dry Valleys), Antarctica. Photo: E. I. Friedmann. Upper left. Surface of colonized sandstone with the characteristic mosaic pattern caused by exfoliative weathering from Linnaeus Terrace, Ross Desert (McMurdo Dry Valleys), Antarctica. Photo: J. A. Nienow. Lower left. Broken sandstone boulder from the same locality colonized by cryptoendolithic microorganisms. Photo: J. A. Nienow. Lower right. Colonization by the lichen-dominated cryptoendolithic community: black zone — dark-pigmented fungi, both lichenized and nonlichenized; white zone — colorless (hyaline) fungi, lichenized with *Trebouxia,* the photobiont; green zone — *Hemichloris antarctica* and *Chroococcidiopsis.* Photo: E. I. Friedmann. (Reprinted with permission from Friedmann 1982 (*Science* **215**, 1,045-1,053). Copyright 1982 American Association for the Advancement of Science).



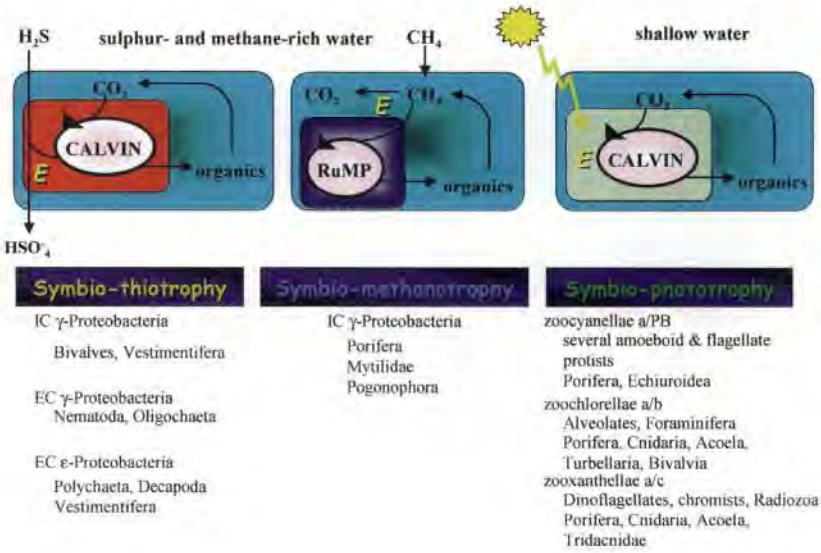
Endolithic Microorganisms in Arid Regions, Plate 2. Endolithic communities in hot deserts. Upper left. Limestone and sandstone (darker rocks in foreground) cliffs in Timna National Park, Negev Desert, Israel. Photo: E. I. Friedmann. Upper right. Broken sandstone from the same locality colonized by cryptoendolithic microorganisms. Photo: E. I. Friedmann. Lower left. Endolithic colonization of crystalline sandstone, Mitzpeh Ramon, Negev Desert, Israel. Green zone — *Chroococcidiopsis.* Photo: E. I. Friedmann. Lower right. Section of granite colonized by chasmoendolithic microorganisms, Sonoran Desert, Mexico. Photo: E. I. Friedmann.



Endosymbiosis in Ecology and Evolution, Figure 1. Terminology. Schematic drawing of a cross-section of a multicellular host to illustrate the relative position of symbionts. Ectosymbionts (at right) are positioned on the external surfaces of the host body, on the ectoderm, or on the intestinal cavity. Endosymbionts (at left) may be extracellular, or in other words, located between cells of tissues or within the host body fluids (e.g., the blood), or the endosymbionts may be harbored within the host cells, free into the cytoplasm, or enclosed in a membrane (symbiosome).

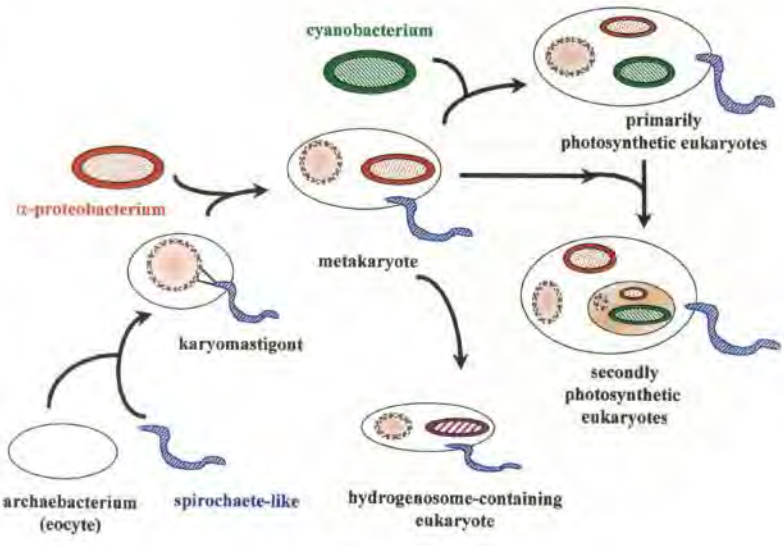


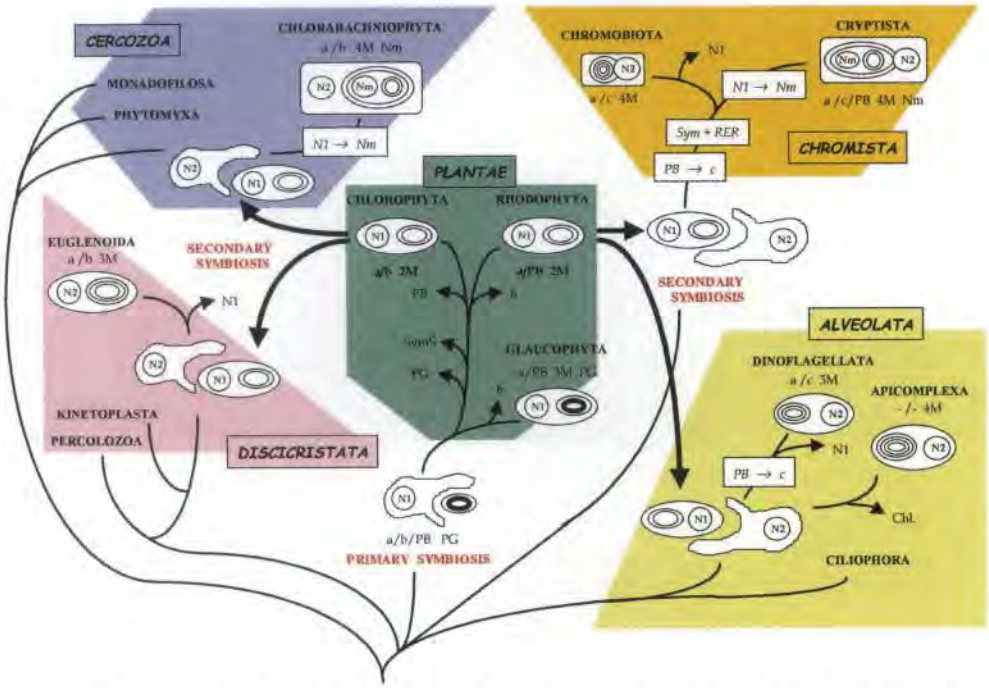
Endosymbiosis in Ecology and Evolution, Figure 2. Phylogeny of Sternorrhyncha and origin of P-symbionts. Dendrogramm showing the evolutionary history of the four superfamilies (in capitals) of the suborder Sternorrhyncha (order Homoptera). For the superfamily Aphidoidea the three families are indicated, and for the family Aphididae the subfamilies are also indicated. Circles indicate the origin of the infection by the primary symbionts, with the estimated time in million of years ago (mya). Extracellular yeasts have replaced *Buchnera* in the tribe Cerataphidini within the subfamily Hormaphidinae. Members of the family Aldegidae possess a γ-symbiont, but it is not known whether it belongs to *Buchnera*, whereas the family Phylloxeridae is lacking of symbiont, perhaps lost. Coccoidea harbor both γ- and β-symbionts, but the β-symbionts seem to be the P-symbionts. They form a monophyletic clade in molecular phylogenies based on 16S rDNA analyses, whereas the γ-symbionts appear polyphyletic. Members of the superfamilies Aleyrodoidea and Psylloidea harbor closely related γ -symbionts, perhaps evolving from a unique γ -symbiont infecting a common ancestor.



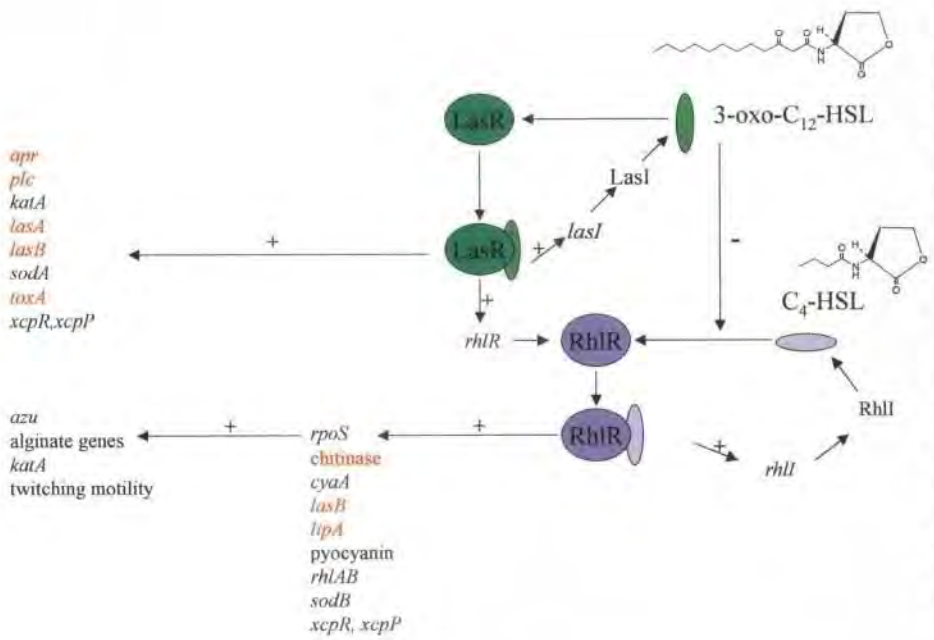
Endosymbiosis in Ecology and Evolution, Figure 3. Symbiotrophic animals.

Endosymbiosis in Ecology and Evolution, Figure 5. Serial endosymbiotic theory. The ancestral eukaryote cell is supposed to have originated from an archabacterium (eocyte), perhaps as a result of a merger with an eubacterium (spirochaete), as a motility symbiosis, or as a failed predation. A system of internal membranes has been developed to include all the genetic materials, giving rise to the nucleus, whereas the motility symbiosis reached a high integration, giving rise to the eukaryotic flagellum. The cell at this step is called a *kariomastigont*. At one moment a novel episode of endosymbiosis with an α-proteobacterium appears, giving rise to the mitochondrion. The cell at this step is called a *metakaryote*. Some metakaryotes adapt several times and independently to anaerobic habitats changing the mitochondrion into a hydrogenosome. Endosymbiosis with a cyanobacterium has allowed the emergence of photosynthetic eukaryotes, that form at the present the kingdom of Plantae. Successive episodes of secondary symbioses between a plant and a phagotrophic metakaryote originate the secondarily photosynthetic eukaryotes (euglenoids, chlorarachniophytes, chromist algae and some alveolates). Whereas the first steps of the evolution of the eukaryotic cell are still disputed, several theories have been proposed, the origin and evolution of mitochondria and plastids are now widely accepted.

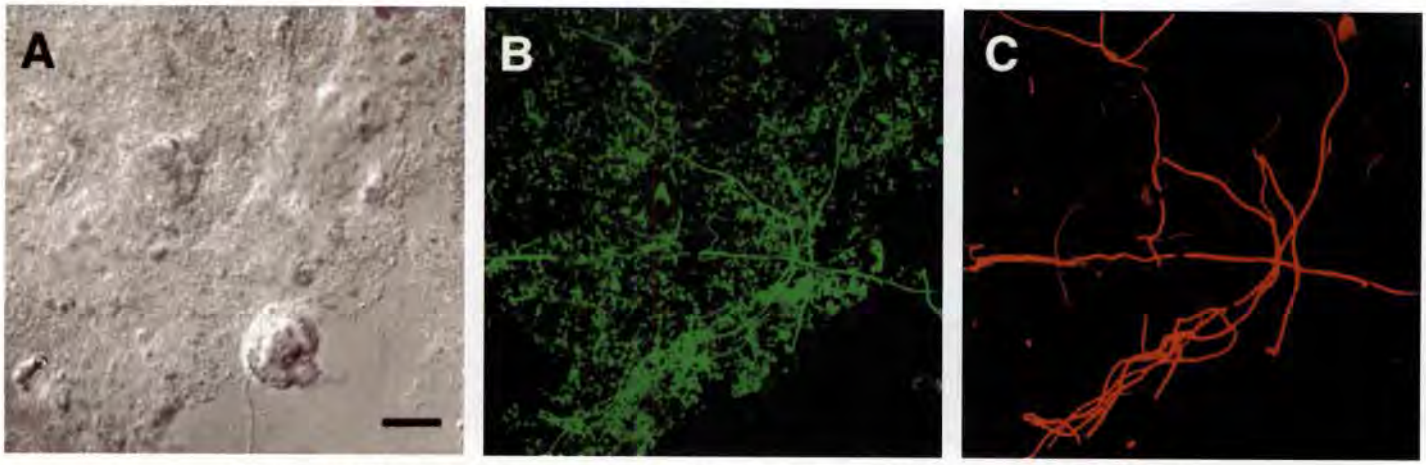




Endosymbiosis in Ecology and Evolution, Figure 6. Origin and evolution of the eukaryotic algae. The five lineages of eukaryotes containing photosynthetic members are indicated: Alveolata, Cercozoa, Chromista, Discicristata, and Plantae (according to Cavalier-Smith 1998 (111)), with the pigment composition (a, b, c and PB, for chlorophylls *a, b, c,* and phycobilins, respectively). The number of plastid membranes is also indicated (2 M, 3 M, 4 M, for two, three, or four membranes), as well as the eventual presence of peptidoglycan (PG), and the retention of the nucleus of the primary alga as nucleomorph (Nm). N1 = nucleus of the primary alga; N2 = nucleus of the secondary alga. In squares are indicated the major transitions: substitution of phycobilins with chlorophyll *c* (PB → c), degeneration of the primary alga nucleus N1 into a nucelomorph (N1 → Nm), fusion of the symbiosome membrane with the rough endoplasmic reticulum (Sym + RER). A single event of primary symbiosis between a phagotrophic eukaryote and a cyanobacterium has led to the establishment of the kingdom of Plantae, the only primarily photosynthetic eukaryotes. Lineages within the kingdom Plantae then diverged by differential losses of plastid pigments. Episodes of secondary symbioses (large arrows in bold) between a green alga or a red alga and some phagotrophic protists, have led to the euglenophytes and chlorarachniophytes, and to the dinoflagellates and chromist algae, respectively. The non-photosynthetic apicoplast of Apicomplexa seems to have originated from a green alga. In the Chromista, both Cryptista and Chromobiota harbor members without plastids, probably because of secondary loss. Cryptista and some Chromobiota (e.g., Eustigmatophycea) also possess phycobilins.

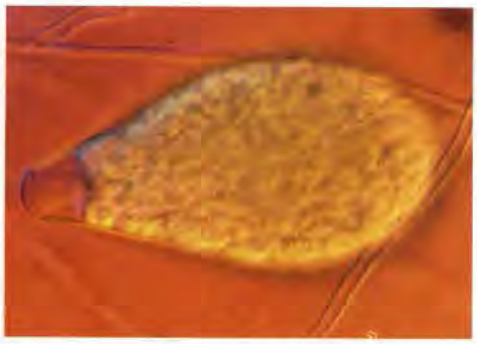


Extracellular Enzymes in Biofilms, Figure 2. Quorum-sensing regulation of gene expression in *P. aeruginosa*. A plus (+) sign indicates positive regulation, a minus (–) sign indicates negative regulation. The genes encoding extracellular enzymes are printed in red. The main regulatory system consists of the regulator LasR and the autoinducer synthetase LasI, which directs the synthesis of 3-oxo-C_{12}-HSL. The LasR-3-oxo-C_{12}-HSL complex positively regulates the expression of genes *aprA* (encoding alkaline protease), *plcN* (phospholipase C), *katA* (catalase), *lasA* (staphylolytic elastase), *lasB* (elastase), *sodA* (superoxide dismutase), *toxA* (exotoxin A), *xcpR* and *xcpP* (proteins of the type II secretion system), and *rhlR* (the regulator RhlR). The RhlR-C_4-HSL complex positively regulates the expression of genes *rpoS* (stationary-phase sigma factor), a chitinase gene, *lasB* (elastase), *lipA* (lipase), *rhlAB* (rhamnosyltransferase), *sodB* (superoxide dismutase), and *xcpR* and *xcpP* (proteins of the type II secretion system). The sigma factor RpoS itself positively controls the expression of genes *azu* (azurin), *katA* (catalase), and the genes involved in the biosynthesis of alginate and type IV pili required for twitching motility.

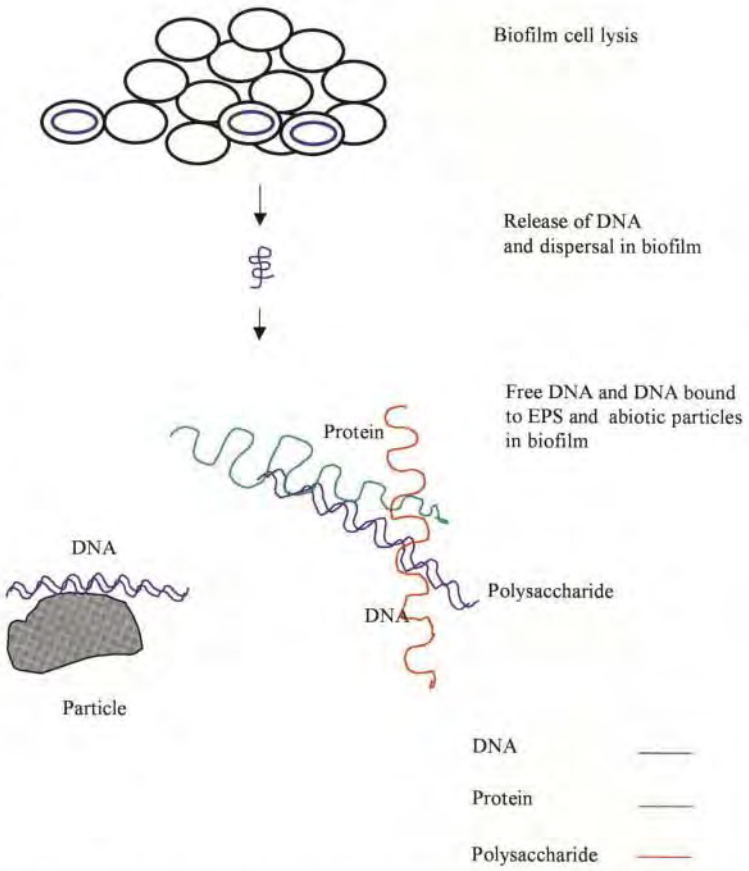


Filamentous Bacteria in Activated Sludge: Current Taxonomic Status and Ecology, Figure 4. In situ identification of Thiothrix/Eikelboom Type 021N in activated-sludge flocs using confocal laser scanning microscopy. (**a**) Transmission micrograph. (**b**) FISH using probe GAM42a specific for the γ-subclass of Proteobacteria. (**c**) FISH with probe G123T specific for Thiothrix/Eikelboom Type 021N (8). Bar represents 20 μm. Micrographs were recorded by Matthias Horn.

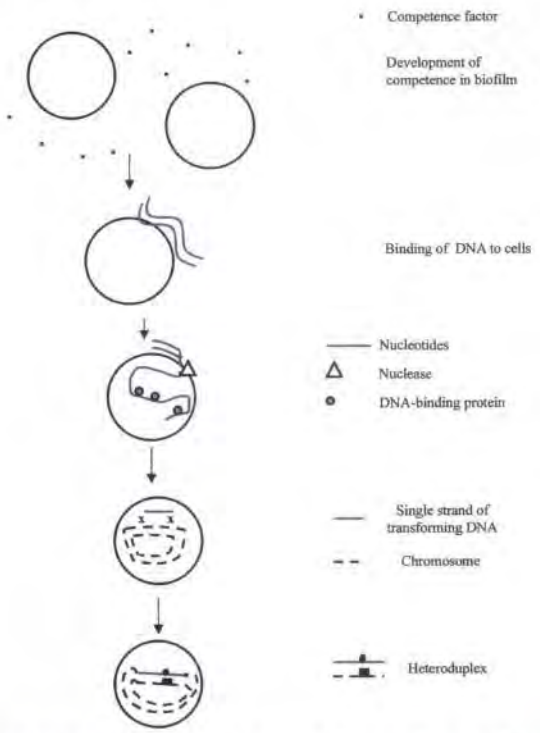
Fungi in Marine/Estuarine Waters, Figure 1. Typical deposit of ejected ascospores of two species of loculoascomycetes from naturally decaying smooth cordgrass leaf blades of Georgia saltmarshes (9). The smaller (about 7 μm wide), hyaline, 2-celled spores are of *Mycosphaerella* sp. 2 [Kohlmeyer and Kohlmeyer (31)] (Dothideales), and the larger (about 11 μm wide), dark, 4-celled spores are of *Phaeosphaeria spartinicola* (Pleosporales). The spores were explosively expelled at a rate of hundred per square centimeters per hour, onto a capture glass placed in front of a wetted decaying leaf, and many have germinated to hyphae. These two species are nearly always intimately mixed in the leaf-decay system, and may be mutualists (9,30,32).



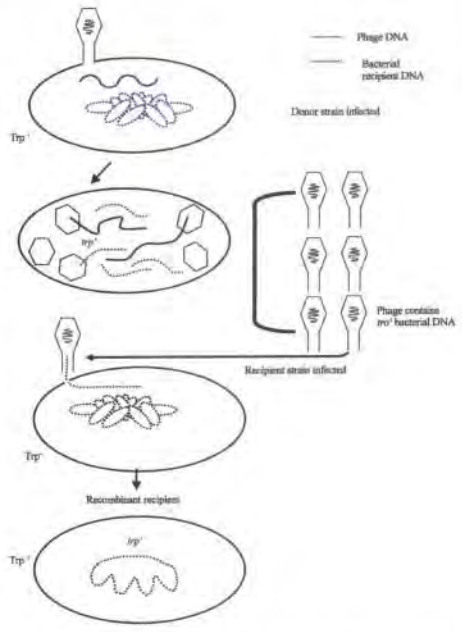
Fungi in Marine/Estuarine Waters, Figure 2. Zoosporangium of the cosmopolitan marine oomycote *H. vesicula* (about 40 μm wide at the widest point) (4,36). This is the "delicate" variety; see References 11 and 37 for photos of the "robust" variety. Species of *Halophytophthora* are common and rapid pervaders of fallen mangrove leaves (34,36,38,39). At full maturity, the inverted cone visible at the tip of the sporangium (left end) everts into a delicate, ephemeral release vesicle, and the biflagellate zoospores seen crammed against one another in the sporangium swim away through the vesicle.



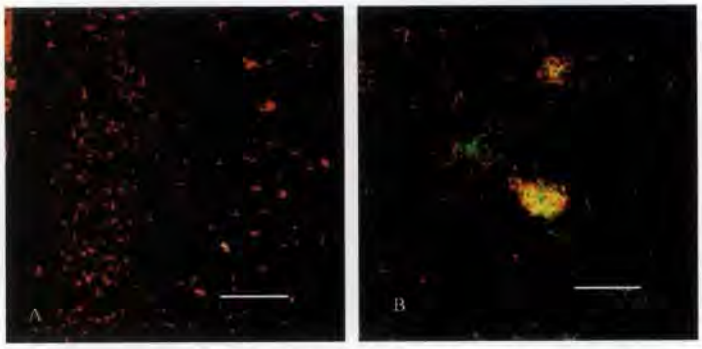
Gene Exchange in Biofilms, Figure 5. Conceptual view of the fate of DNA after cell lysis in a biofilm, depicting the first three steps in natural transformation (see text for details). The released DNA is either bound to biofilm constituents such as EPSs, cells, and abiotic particles or present as free DNA. See Figures 6 and 7 for uptake of DNA and internal processing by cells.



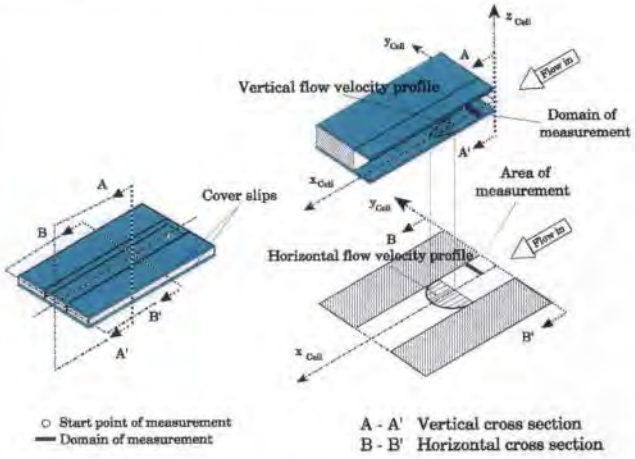
Gene Exchange in Biofilms, Figure 6. Uptake by and integration of transforming DNA into the chromosome of *Streptococcus pneumoniae*. Competence factors accumulate as the cells reach a high density. Double-stranded DNA binds to the cell and one strand is degraded. The remaining single strand replaces the strand of the same sequence in the chromosome, creating a "heteroduplex," in which one strand comes from the donor and one comes from the recipient. *Source:* Redrawn after Ref. 11 with permission from the American Society for Microbiology, © 1997.



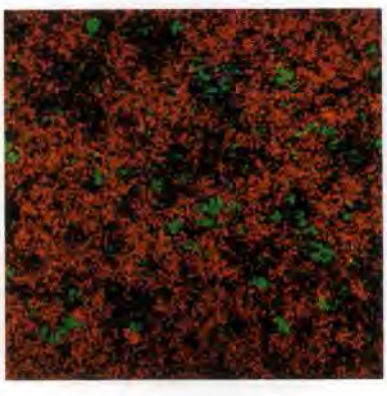
Gene Exchange in Biofilms, Figure 8. An example of generalized transduction. A phage infects a Trp^+ bacterium, and in the course of packaging DNA heads, the phage mistakenly packages some bacterial DNA containing the trp region instead of its own DNA into a head. In the next infection, this transducing phage infects the Trp^+ bacterial DNA instead of phage DNA into the Trp^- bacterium. If the incoming DNA recombines with the chromosome, a Trp^+ recombinant transductant may arise. Only one strand of the DNA is shown. *Source:* Redrawn after Ref. 11 with permission from the American Society for Microbiology, © 1997.



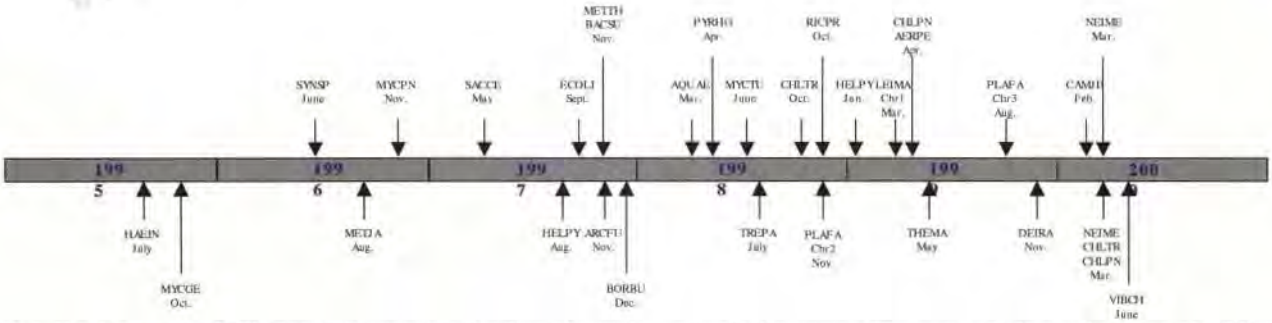
Gene Exchange in Biofilms, Figure 9. Transconjugant cells in a biofilm of *R. metallidurans* AE104 cultivated under nutrient-rich (**a**) and nutrient-poor (**b**) conditions. Recipient cells were grown as a confluent biofilm on microscope slides and exposed to high densities of donor and helper cells to allow transfer of the mob^+ plasmid, which carried the gene for the GFP. CLSM signals were collected consecutively and stored as gray scale images. AE104 cells were detected by FISH with a TRITC-labeled rRNA-directed oligonucleotide probe specific to the β-proteobacteria subgroup. With computer-assisted coloring, recipient AE104 cells were assigned the color red. Donor *E. coli* strain GM16 cells were detected based on the fluorescence emitted by GFP and are depicted in green. Transconjugants emitted both green light owing to GFP and red light owing to hybridization with the TRITC-labeled rRNA-directed oligonucleotide probe and are shown in yellow. Bar, 25 µm. *Source:* after Ref. 70; reprinted with permission from the American Society for Microbiology, © 1998.



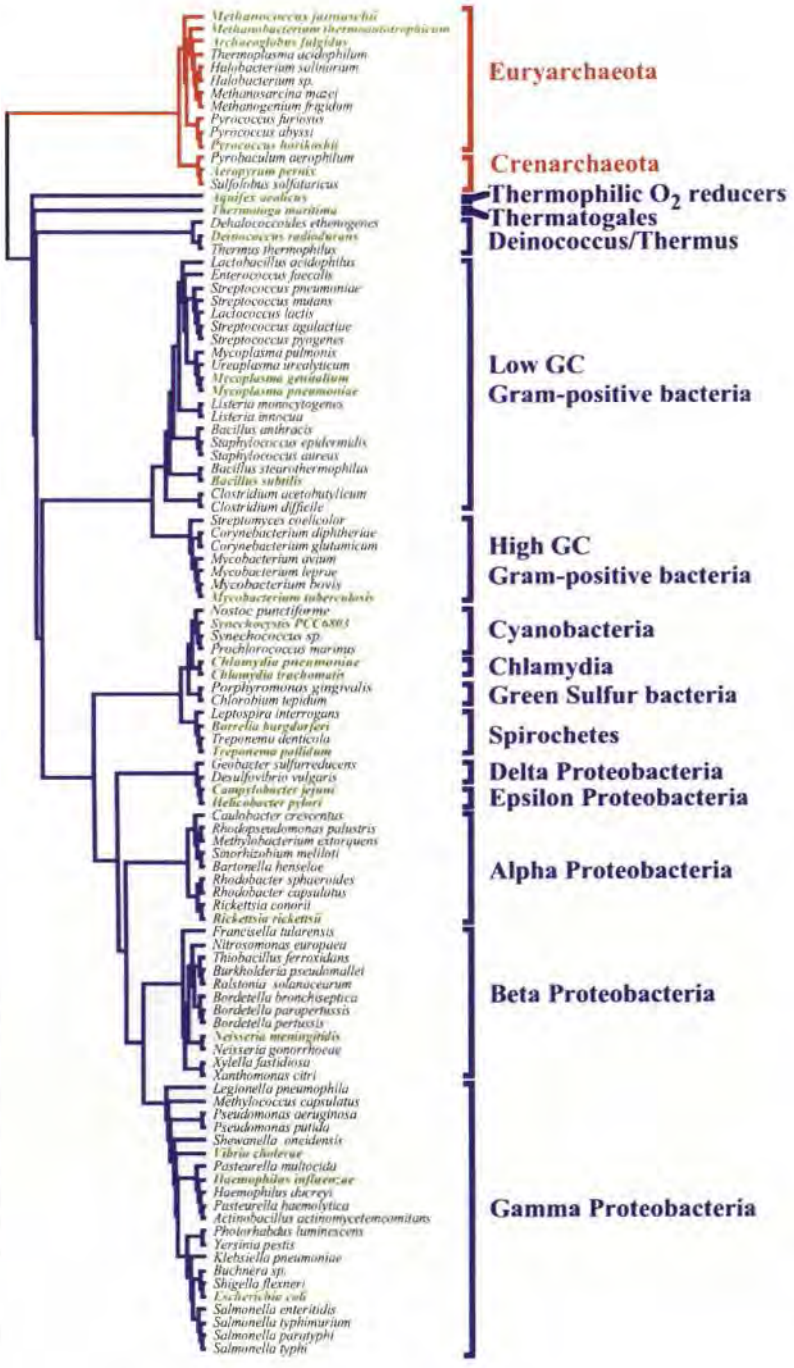
Gene Exchange in Biofilms, Figure 10. Schematic view of the flow cell indicating fluid velocity profiles and domain of measurement typically used to investigate gene tranfer events. *Source:* after Ref. 68; reprinted with permission from the American Society for Microbiology, © 1998.



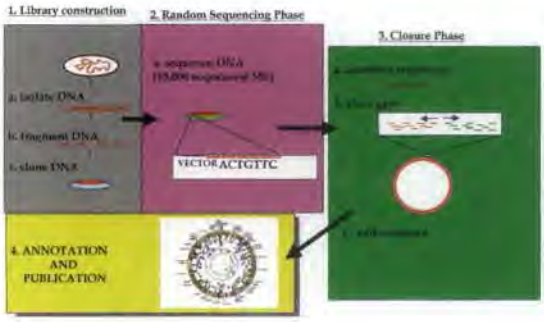
Gene Exchange in Biofilms, Figure 11. Transformant cells in an 18-hour biofilm of *Acinetobacter* sp. BD413 grown in a flow cell and exposed to 1 µg plasmid DNA/mL for 20 minutes. Cells were visualized with the general nucleic acid stain Syto 60 (Molecular Probes, Eugene, Oregon) and were detected with an LSM410 confocal laser scanning microscope (Zeiss, Jena, Germany). Transformants were detected by the emission of light owing to the GFP encoded by the plasmid, pGAR1.



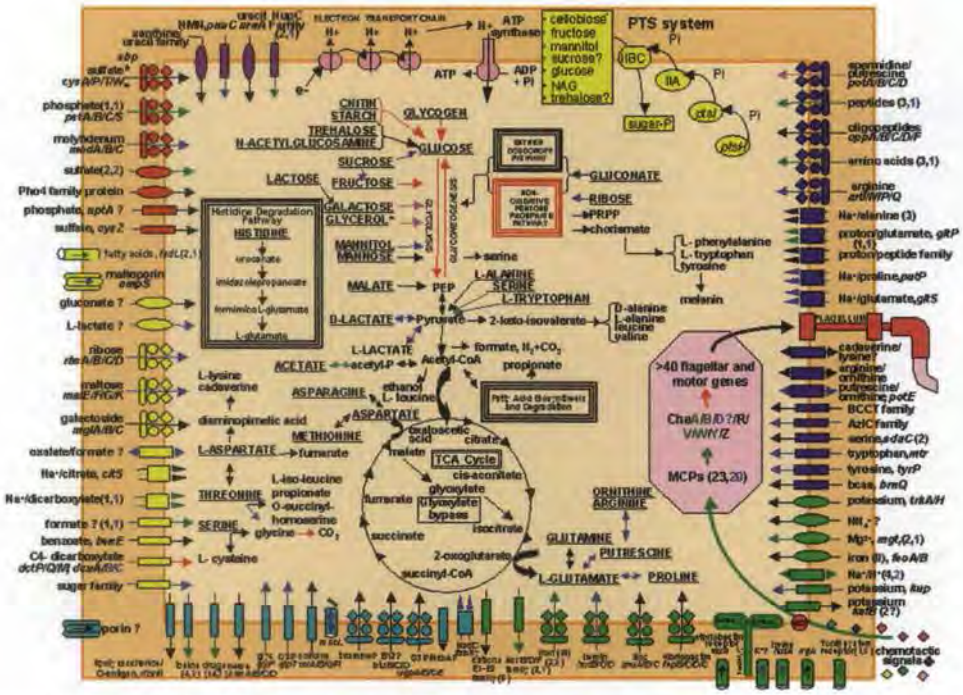
Genomics, Environmental, Figure 1. Sequencing timeline of complete microbial genomes. The arrows indicate the dates of publication for each of the completed genomes. The organism abbreviations are as follows: HAEIN, *Haemophilus influenzae*; MYCGE, *Mycoplasma genitalium;* SYNSP, *Synechocystis* PCC6803; METJA, *Methanococcus jannaschii;* MYCPN, *Mycoplasma pneumoniae;* SACCE, *Saccharomyces cerevisiae;* HELPY, *Helicobacter pylori;* ECOLI, *Escherichia coli;* METTH, *Methanobacterium thermoautotrophicum*; BACSU, *Bacillus subtilis;* ARCFU, *Archaeglobus fulgidus;* BORBU, *Borrelia burgdorferii;* AQUAE, *Aquifex aeolicus;* PYRHO, *Pyrcoccus horikoshii;* MYCTU, *Mycobacterium tuberculosis;* TREPA, *Treponema pallidum;* CHLTR, *Chlamydia trachomatis;* RICPR, *Rickettsia prowazekii;* PLAFA, *Plasmodium falciparum;* LEIMA, *Leishmania major;* CHLPN, *Chlamydia pneumoniae;* AERPE, *Aeropyrum pernix;* THEMA, *Thermotoga maritima;* DEIRA, *Deinococcus radiodurans;* CAMJE, *Campylobacter jejuni;* NEIMA, *Neisseria menigiditis;* VICHO, *Vibrio cholerae.*



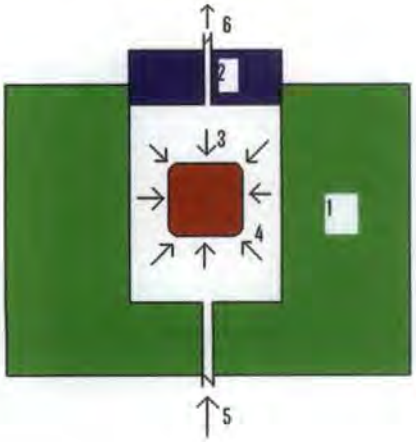
Genomics, Environmental, Figure 2. Phylogenetic tree based on 16S-rRNA sequences for each of the prokaryotic organisms whose complete genome sequence has been published (highlighted in green) and other organisms for which genome sequencing projects are underway. (*http://www.tigr.org/tdb/mdb/mdb.html*). The major prokaryotic phylogenetic groupings are indicated to the right of the tree. The *archaea* are indicated in red and the *eubacteria* are indicated in blue. The phylogenetic tree was derived from sequences from the Ribosomal Database project (RDP) (*http://www.cme.msu.edu/RDP/html/index.html*) using Phylip (52) for tree construction. In some instances where the rRNA sequence of the particular strain was not available, the rRNA of a close relative was substituted.



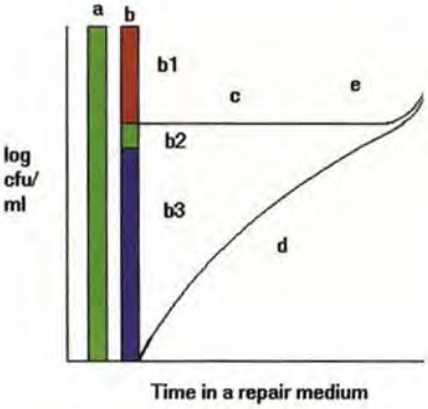
Genomics, Environmental, Figure 3. Strategy for random shotgun sequencing of microbial genomes. The first phase involves construction of small and large insert libraries with DNA from the organism of interest. The second phase involves the sequencing of random clones from the libraries to a predetermined level of coverage. The third phase involves assembling the random sequences into contiguous segments and subsequent gap closure. Finally, the completed sequence is annotated and published.



Genomics, Environmental, Figure 4. Reconstruction of transport and metabolism of *V. cholerae* on the basis of the annotated genome sequence. Pathways for energy production and the metabolism of organic compounds, acids and aldehydes are shown. Transporters are grouped by substrate specificity: cations (green), anions (red), carbohydrates (yellow), nucleosides, purines, and pyrimidines (purple), amino acids/peptides/amines (dark blue, and other (light blue). Question marks associated with transporters indicate a putative gene, uncertainty in substrate specificity, or direction of transport. Permeases are represented as ovals, ABC transporters are shown as composite figures of ovals, diamonds, and circles, porins are represented as three ovals, the large-conductance mechanosensitive channel is shown as a gated cylinder, other cylinders represent outer membrane transporters or receptors; all other transporters are drawn as rectangles. Export or import of solutes is designated by the direction of the arrow through the transporter. If a precise substrate could not be determined for a transporter, no gene name was assigned and a more general common name reflecting the type of substrate being transported was used. Gene location on the two chromosomes, for both transporters and metabolic steps, is indicated by arrow color: all genes located on the large chromosome (black), all genes located on the small chromosome (blue), all genes needed for the complete pathway on one chromosome, but a duplicate copy of one or more genes on the other chromosome** (purple), required genes on both chromosomes (red), complete pathway on both chromosomes (green). Gene numbers on the two chromosomes are in parenthesis and follow the color scheme for gene location. Substrates underlined and capitalized can be used as energy sources. Abbreviations: PRPP, phosphoribosyl-pyrophosphate; PEP, phosphoenolpyruvate; PTS, phosphoenolpyruvate-dependent phosphotransferase system; ATP, adeosine triphosphate; ADP, adenosine diphosphate; MCP, methyl-accepting chemotaxis protein; NAG, N-acetylglucosamine; G3P, glycerol-3-phosphate; GLYC, glycerol; NMN, nicotinamide mononucleutide. *Because *V. cholerae* does not use cellobiose, we expect this PTS system to be involved in chitobiose transport. **Complete pathways, except for glycerol, are found on the large chromosome.

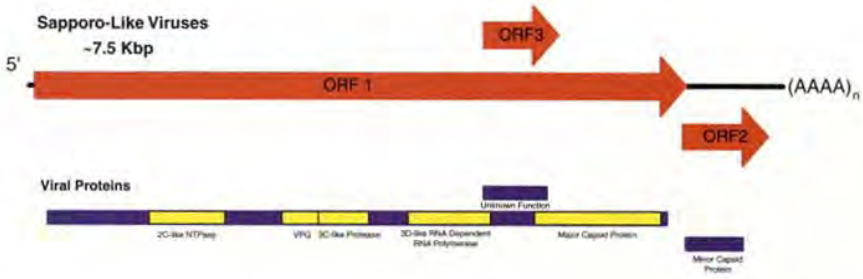


High Hydrostatic Pressure: Microbial Inactivation and Food Preservation, Figure 1. Schematic diagram of a high hydrostatic pressure vessel (7). The food package (4) is put in liquid (3) inside the chamber (1), which is closed at the top with a movable cylinder (2) with an opening and a valve (6) to remove air and excess liquid prior to pressurization. Once the chamber is closed, excess liquid is pumped through the inlet tube (5) that has a valve which closes when the desired pressure level is reached. The pressure is maintained constant during pressurization time, at the end of which the inlet valve (5) opens and the excess liquid is removed from the chamber by gravity. The liquid in the chamber can be heated by installing a heating coil around the vessel (7). The process is clean, energy efficient, and requires relatively less space compared with other food-processing methods.

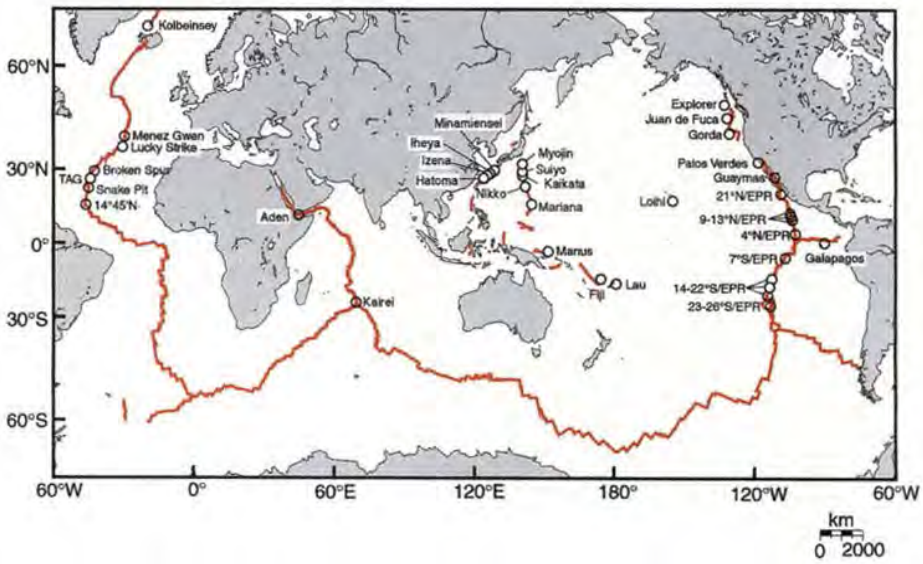


High Hydrostatic Pressure: Microbial Inactivation and Food Preservation, Figure 2. Schematic presentation of the effect of pressurization of a normal bacterial cell suspension (**a**) leading to production of three subpopulations (**b**), namely, dead cells (**b1**), normal cells (**b2**) and sublethally injured cells (**b3**). During subsequent incubation of the cells in a favorable environment for a period, the total survivor counts (b1+ = *hboxb2* = c) in a nonselective agar medium remain unchanged, suggesting no cell multiplication. However, the counts on selective agar medium increases rapidly (**d**) due to repair of injury and regain in resistance to selective environment; the rate of repair differs due to differences in the extent of injury among different cells. Cell multiplication starts following repair of injury as indicated by simultaneous increase in counts in both media (**e**).

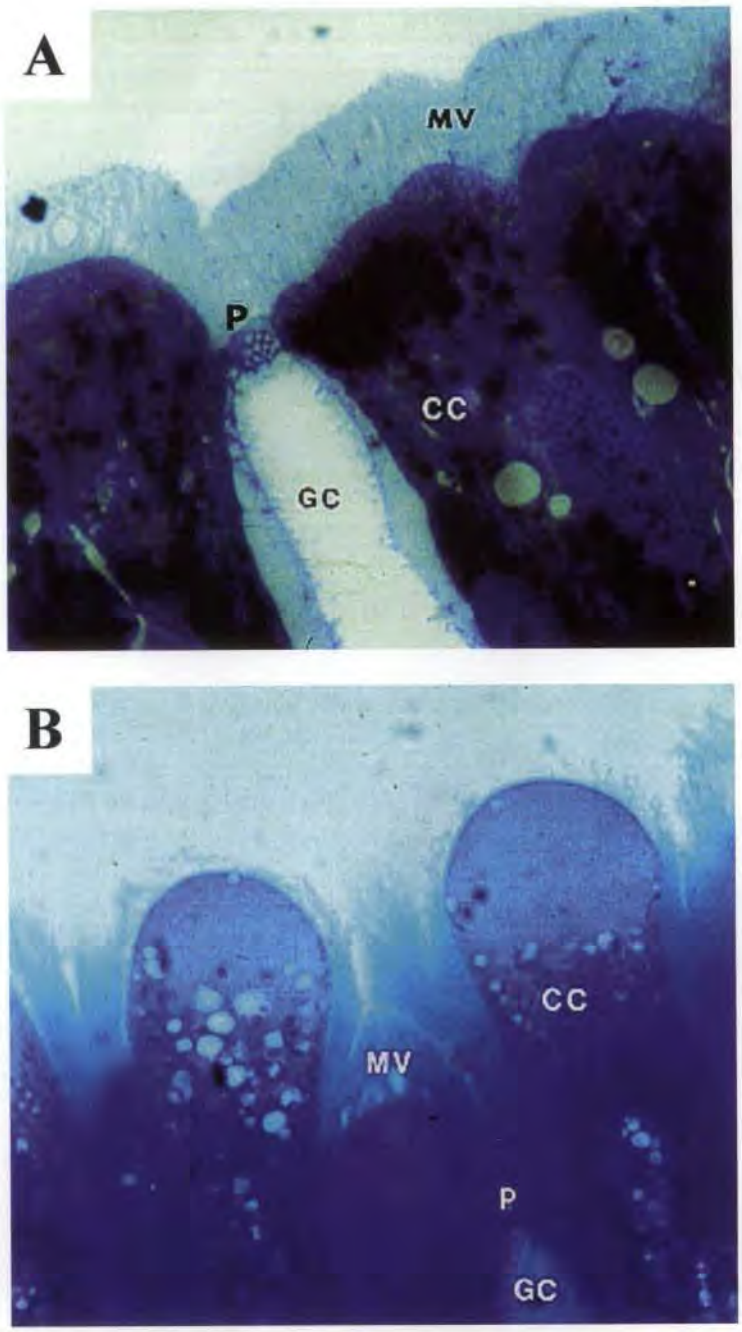
High Hydrostatic Pressure: Microbial Inactivation and Food Preservation, Figure 3. Scanning electron micrographs of *Leuconostoc mesenteroides* Ly cells (25,000 X). X (**a**) Unpressurized control. The cells are lenticular, about 1 × 1.5 mμ in size diplococcal stage with distinct constriction between the two cells. (**b**) Immediately after pressurization at 345 MPa for 5 minutes at 25°C. Pressurization caused more than 99% cell death, but the cells did not show a major visible change on the surface or on gross morphology. (**c**) Pressurized cells after 30 minutes incubation at 25°C after pressurization. Many cells were lysed (with the separation of cell wall between the two cells).



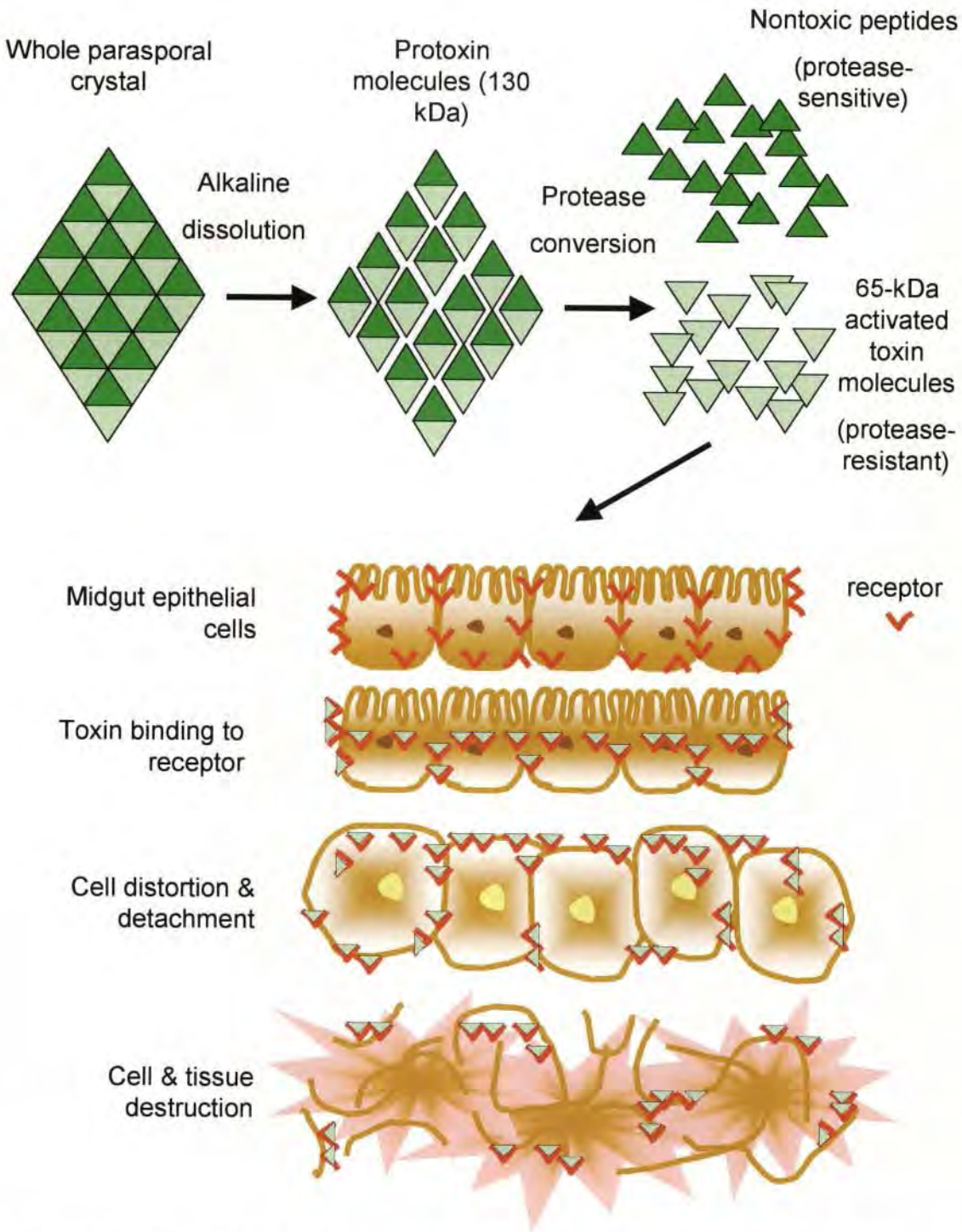
Human Caliciviruses: Basic Virology and Epidemiology, Figure 2. Comparision of the genomic organization of NLVs and SLVs. Open reading frames are indicated by red arrows, corresponding viral proteins are indicated in blue. Yellow highlighted regions represent individual proteins, presumably posttranslationally cleaved from the ORF1 polyprotein.



Hydrothermal Vents: Biodiversity in Deep-Sea Hydrothermal Vents, Figure 1. Location of map of deep-sea hydrothermal vent sites found so far.

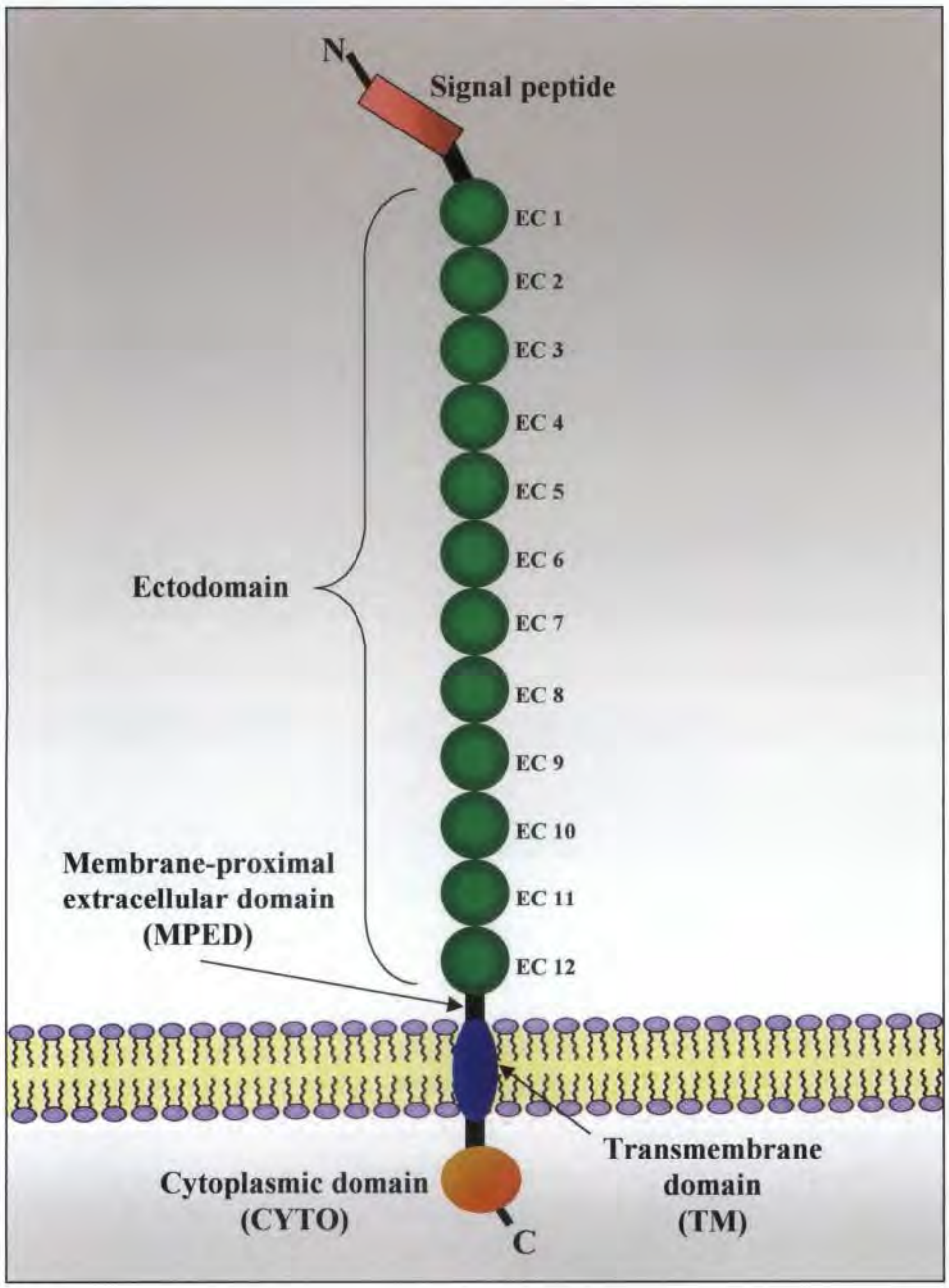


Insecticides Microbial, Figure 2. Effects of Cry1Ab toxin on *Manduca sexta* midgut epithelium. The midgut of insect larvae is a tube lined with columnar epithelial cells (CC). The apical ends of the midgut epithelial cells contain microvilli (MV) and this surface appearance is referred to as a *brush border.* Another type of cell that is present in the midgut is the goblet cell (GC). Goblet cells are intercalated with the columnar epithelial cells. Goblet cells contain a pore (P) at their apical ends through which enzymes and ions are secreted into the midgut. All cells that are facing the inner cavity of the midgut are attached to a basal membrane, which surrounds the tissue. The normal morphological features of the midgut epithelium change dramatically in about 1,530 minutes when a susceptible insect ingests Cry toxin. Micrographs of longitudinal sections of healthy (Panel A) and intoxicated (Panel B) midgut tissue from fourth instar *M. sexta* larvae show the morphological changes in the epithelial cells. On exposure to Cry1Ab toxin, the columnar cells swell and lose their microvillar structure, whereas the goblet cells do not appear to be affected, suggesting that toxin receptors are specifically expressed on the epithelial cells. Apical cell membrane distortion and total cell disruption occurs within one hour of intoxication, leading to devastation of the midgut tissue and death of the insect.

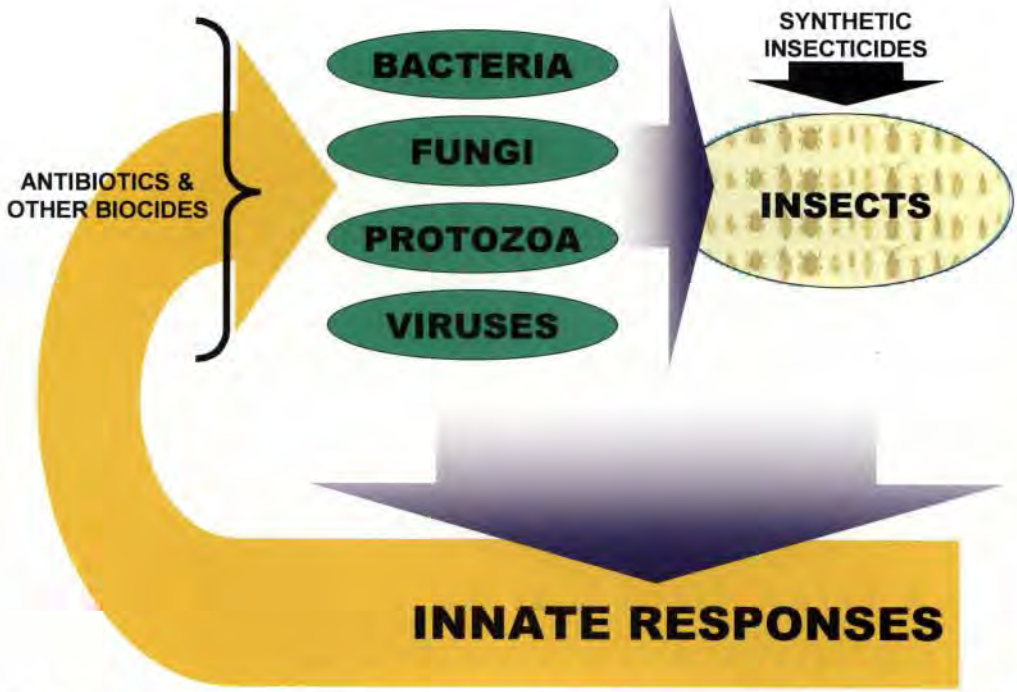


Insecticides Microbial, Figure 3. Activation of parasporal crystals and receptor mediated toxicity of Cry proteins. The entomopathogenic activity of *B. thuringiensis* mainly resides in the parasporal crystals that are composed of protoxin protein molecules. Following ingestion by a target insect, the protoxin is solubilized and converted to active toxin that binds to specific receptors on the surface of midgut epithelial cells. Both the specific physiological environment and the activity of proteolytic enzymes in the midgut of susceptible insects are involved in protoxin activation. Protoxin activation generates two polypeptide fragments, one that is hydrolyzed by midgut proteases and one (activated toxin) that is quite resistant to proteolytic attack in the target insect. Toxin binding to specific receptors on the epithelial cells brings about distortion, enlargement, and detachment of the cells, leading to devastation of the midgut tissue and death of the insect. On ingestion by a susceptible insect, the parasporal crystals are solubilized and the protein toxin is activated by alkaline conditions and the acitivity of proteolytic enzymes in the insect's gut. The toxicity of the activated toxin is dependent on the presence of specific receptor sites on the insect's midgut surface. Specific receptor-toxin interactions also determine the range of insect species killed by individual subspecies of *B. thuringiensis*. If the activated toxin attaches to receptor sites, it leads to destruction of the gut wall, causing the gut contents to leak into the body cavity and bloodstream.

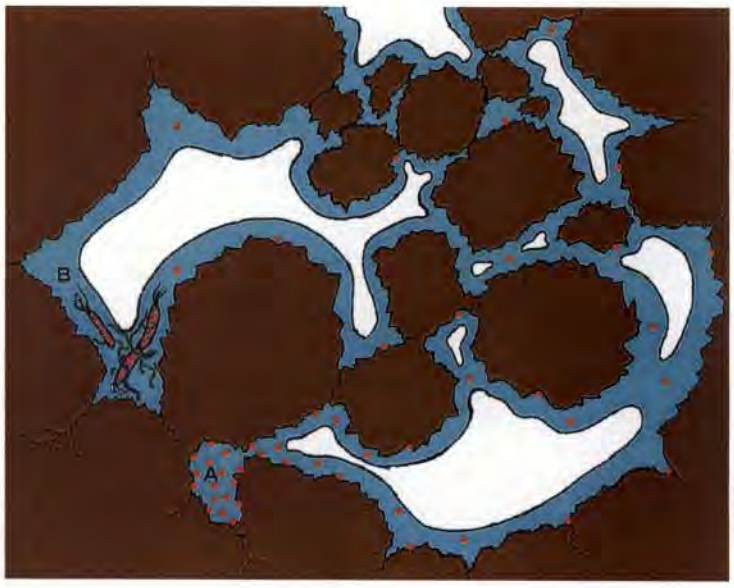
Insecticides Microbial, Figure 4. Effect of Cry1Ab toxin of *B. thuringiensis* on *M. sexta* larva. *Bacillus thuringiensis*-based insecticides are applied much like synthetic insecticides. Such insecticides are inactivated within a few days in most outdoor situations compared with the long-lasting presence of many chemical insecticides. Soon after ingesting whole cells of *B. thuringiensis* or activated toxin, susceptible insect larvae are poisoned and they stop feeding. If the appropriate amount of material is applied at the right time, the immediate and highly selective effect of *B. thuringiensis* is realized. *Manduca sexta* larvae feeding on a normal diet are large green in color. Larvae that have consumed Cry1Ab toxin turn black and usually die immediately or within a couple of days after sepsis. Vegetative cells of *B. thuringiensis* may multiply in the infected host, however, without producing abundant spores or parasporal crystals. The end result is that no infective units are released into the environment when a poisoned insect dies. Until the early 1980s, commercial *B. thuringiensis* products were effective only against caterpillars. However, strains that kill not only caterpillars but also several other types of pests have been identified and developed for commercial use. New isolates of *B. thuringiensis* with new Cry toxin variants will increase the spectrum of pests that can be controlled.



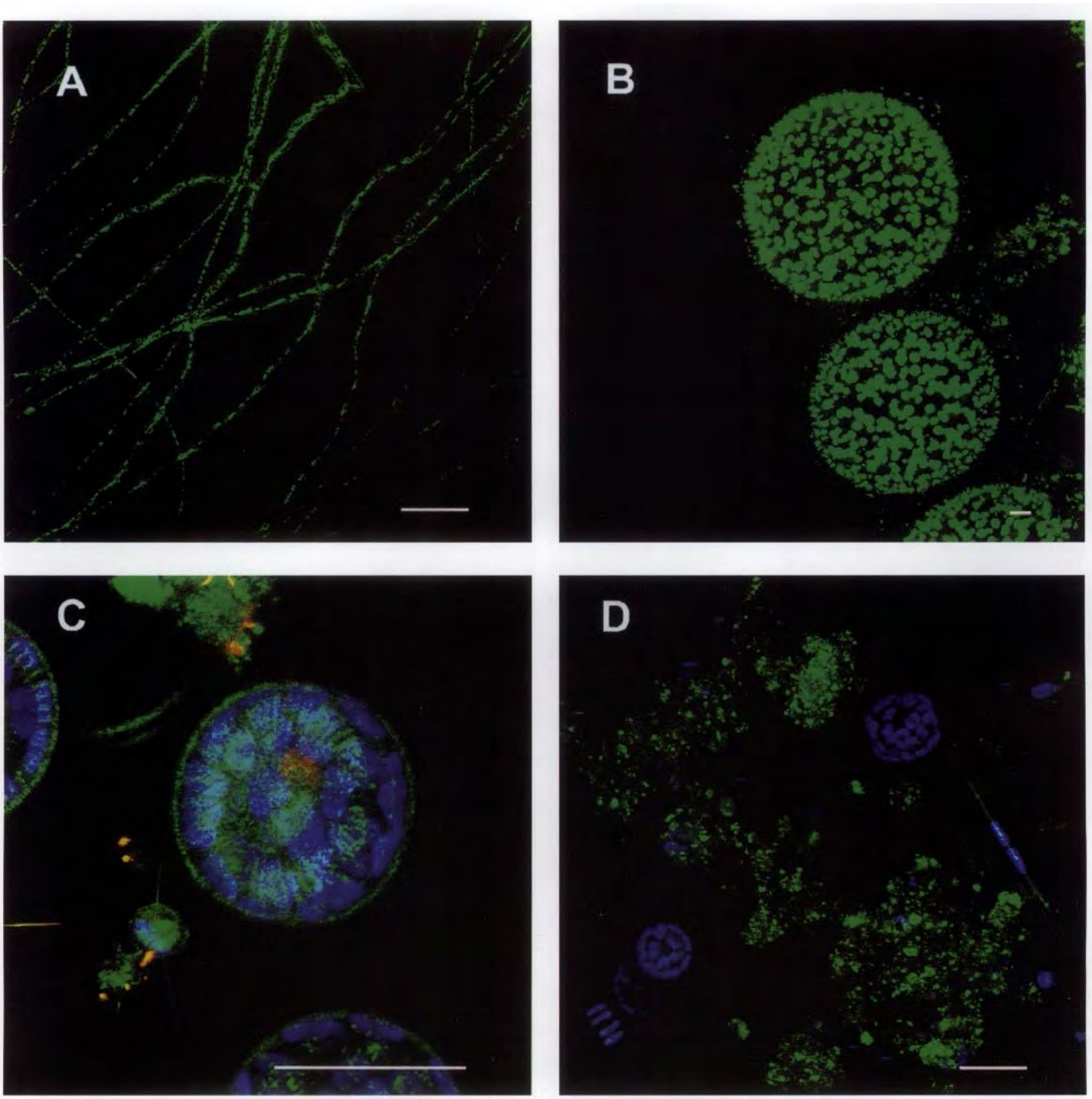
Insecticides Microbial, Figure 5. BT-R_1, the cadherin receptor of *M. sexta* for Cry1A toxins. On the basis of the predicted amino acid sequence, the BT-R_1 receptor (210 kDa) is composed of four domains: (*1*) ectodomain (EC), (*2*) membrane-proximal extracellular domain (MPED), (*3*) transmembrane domain (TM), and (*4*) cytoplasmic domain (CYTO). Downstream of a membrane signal sequence, the ectodomain consists of 12 cadherin repeats composed of β-sheets that are structured as ectodomain modules (EC1 through EC12). The ectodomain harbors putative molecular adhesion sequences. The structural features along with the protein-domain architecture indicate that BT-R_1 is a new type of heterophilic cadherin with a close relationship to protocadherins in the cadherin superfamily. The Cry1A toxins of *B. thuringiensis* bind to BT-R_1 with high affinity and specificity. Most probably, toxin binding adversely affects the structure and function of BT-R_1, consequently, compromising the integrity of the midgut epithelial cells that express the receptor molecule.



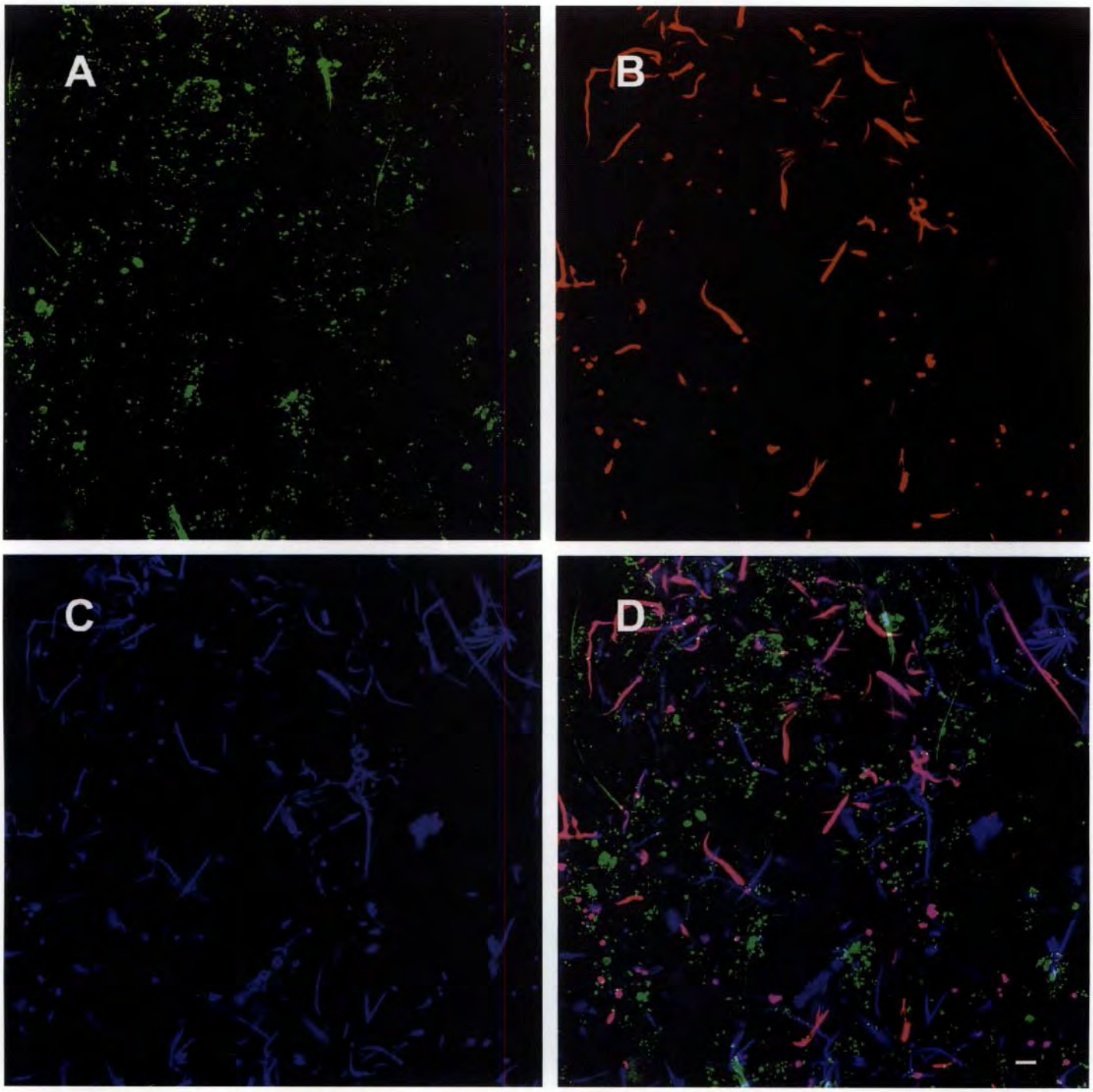
Insecticides Microbial, Figure 6. The relationship between innate responses and resistance to microbial insecticides. Innate cellular defense involves nonspecific responses found in organisms ranging from plants to humans. Invertebrates, including insects, do not produce antibodies and T cells to combat microbial challenges, as do vertebrate animals and humans. Instead, they rely primarily on innate responses to defend themselves against biological challenges. Constant contact of epithelial cells in the mucosal linings of animals harboring various microbes, and a large number of diverse antigens, has brought about the evolution of innate defense mechanisms. Innate responses are first-line cellular defenses and are pivotal to protecting an organism from potential invasion by various pathogens, while tolerating an indigenous microbial flora and a plethora of antigens commonly present in different food sources. Furthermore, information relays between innate defense reactions and adaptive protective devices now are appreciated as ancient evolutionary conserved immune responses. In fact, since the discovery of antimicrobial activities in the cabbage looper *Hyalophora cecropia,* studies of the fruit fly *Drosophila melanogaster* and mosquitoes have revealed notable conservation of innate defense mechanisms in both insects and mammals, especially in mucosal epithelia. Some innate defense mechanisms that guard against unwelcome microbes and antigens respond rapidly and promote adaptive functional changes, including the expression of those molecules that modulate various cell surface activities. The responses are triggered on the surface of epithelial cells on recognition of particular molecular motifs by certain surface receptors. Communication with signaling pathways promotes special physiological adaptations to the cell surface that somehow protect the cell against foreign invaders. Most often, these reactions sustain cellular adaptation on prolonged exposure to microbial activity or other stress conditions and eventually lead to resistance. however, the quick evolution of microbial pathogens is a potential for overcoming such resistance, generating natural competition involving continual challenges and responses that lead to resistance development among species. For instance, widespread commercial use of *B. thuringiensis* Cry toxins to control pest insects has produced insects that are resistant to this entomopathogen. Moreover, chemical insecticides stress insect population forcing many individuals to acquire an enhanced detoxification capacity and increased state of resistance, raising the potential for insects to become refractory to multiple insect-control agents. Therefore, it is crucual to implicate integrated pest management strategies with microbial insecticides and chemical-based pesticids to gain the greatest possible benefits.



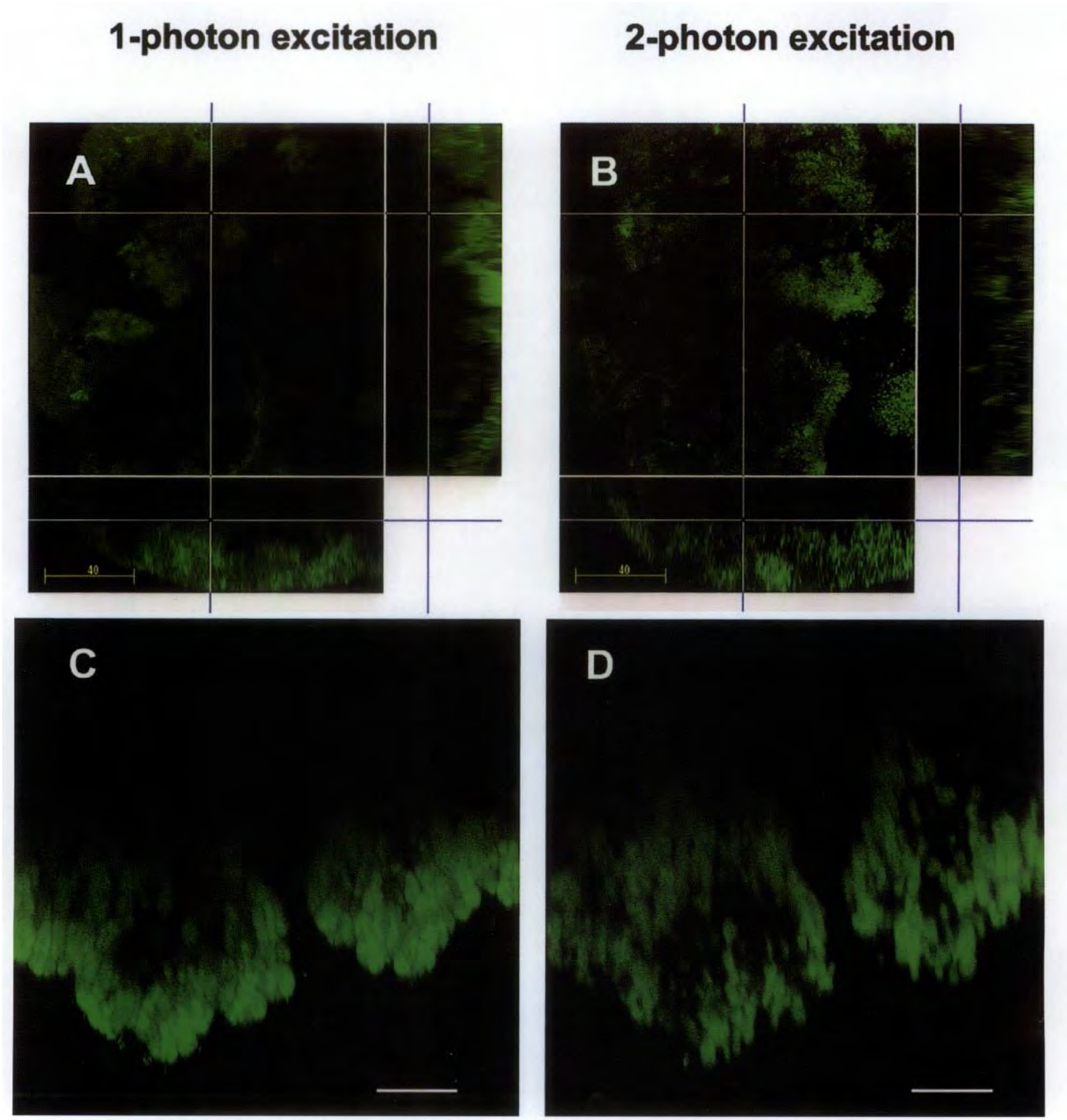
Kinetics of Microbial Processes and Population Growth in Soil, Figure 1. Diagram showing substrate molecules (red dots) diffusing down a concentration gradient from microspore A to bacterial colony B. The solid phase is shown in brown, the liquid phase in blue, and the gas phase in white. Note the relatively long distance that molecules must travel despite the short linear distance between A and B, because of the tortuosity of the water films. The substrate molecules are also shown adsorbing to soil surfaces, further slowing their movement.



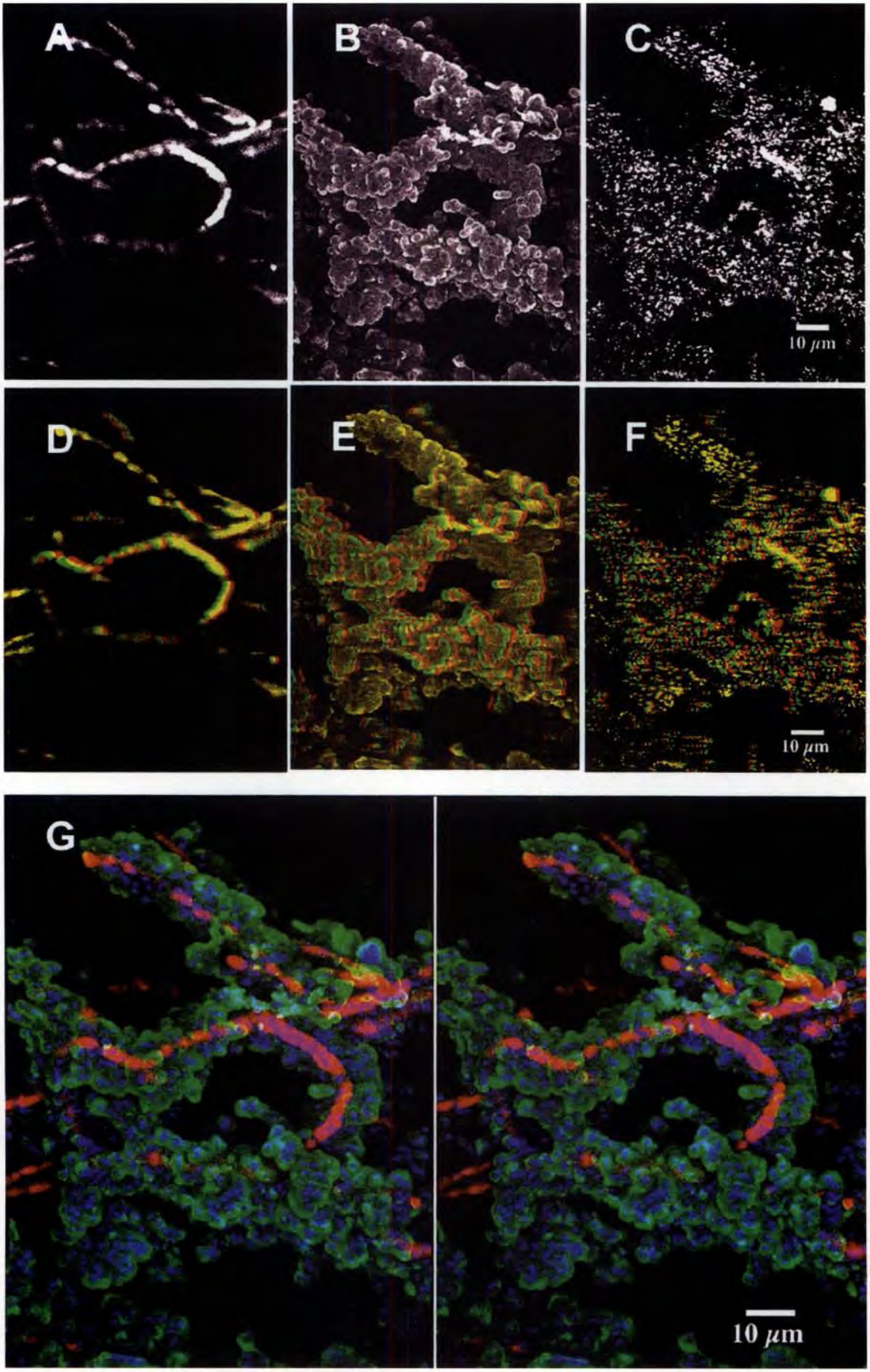
Laser Scanning Microscopy in Combination with Fluorescence Techniques for Biofilm Study, Figure 2. Reflection of diatoma, fungi, microbial mat, thiomargarita (plate with four images).



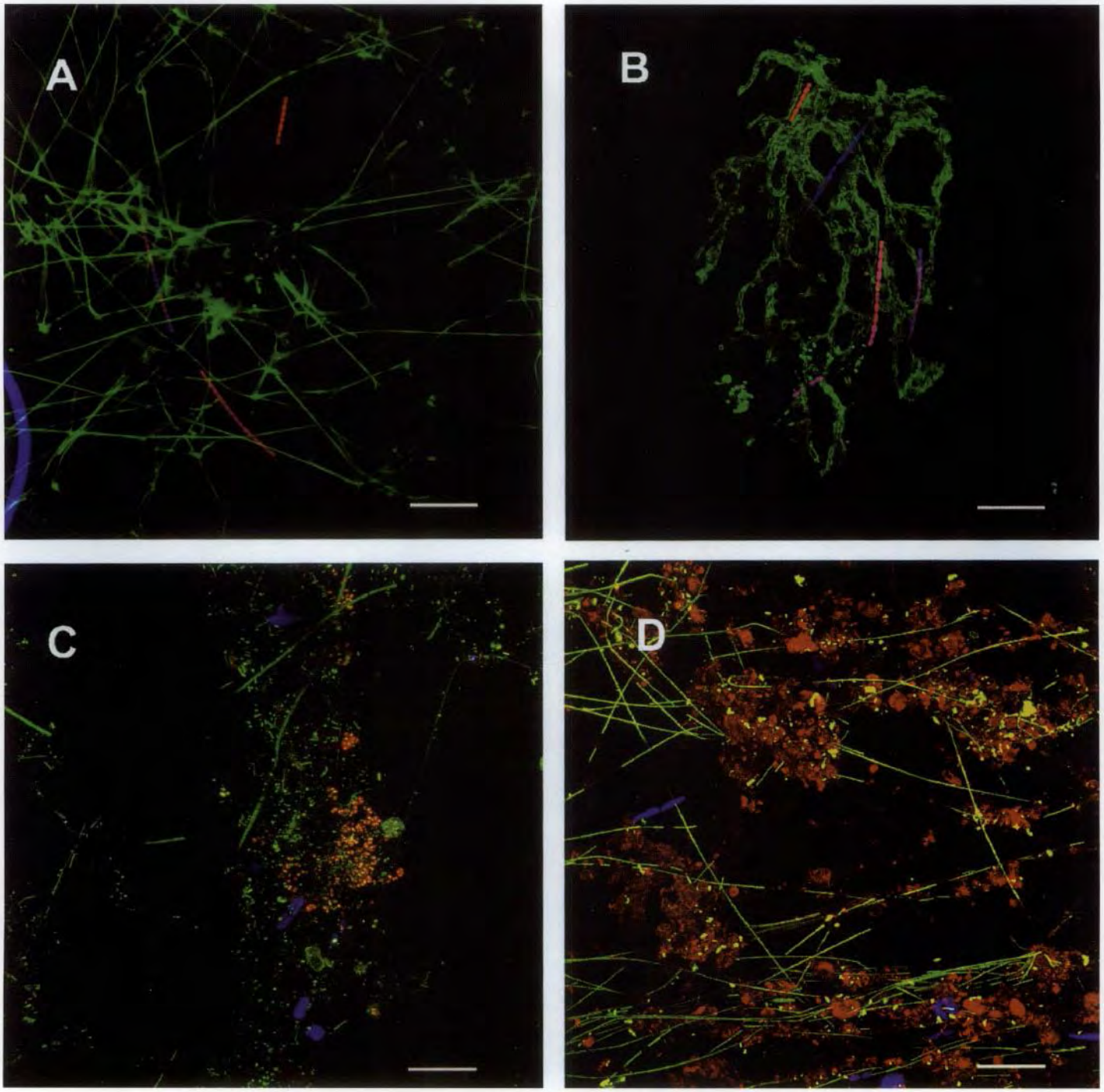
Laser Scanning Microscopy in Combination with Fluorescence Techniques for Biofilm Study, Figure 3. Autofluorescence with differentiation algae-cyanobacteria (plate with four images green-red-blue-overlay).



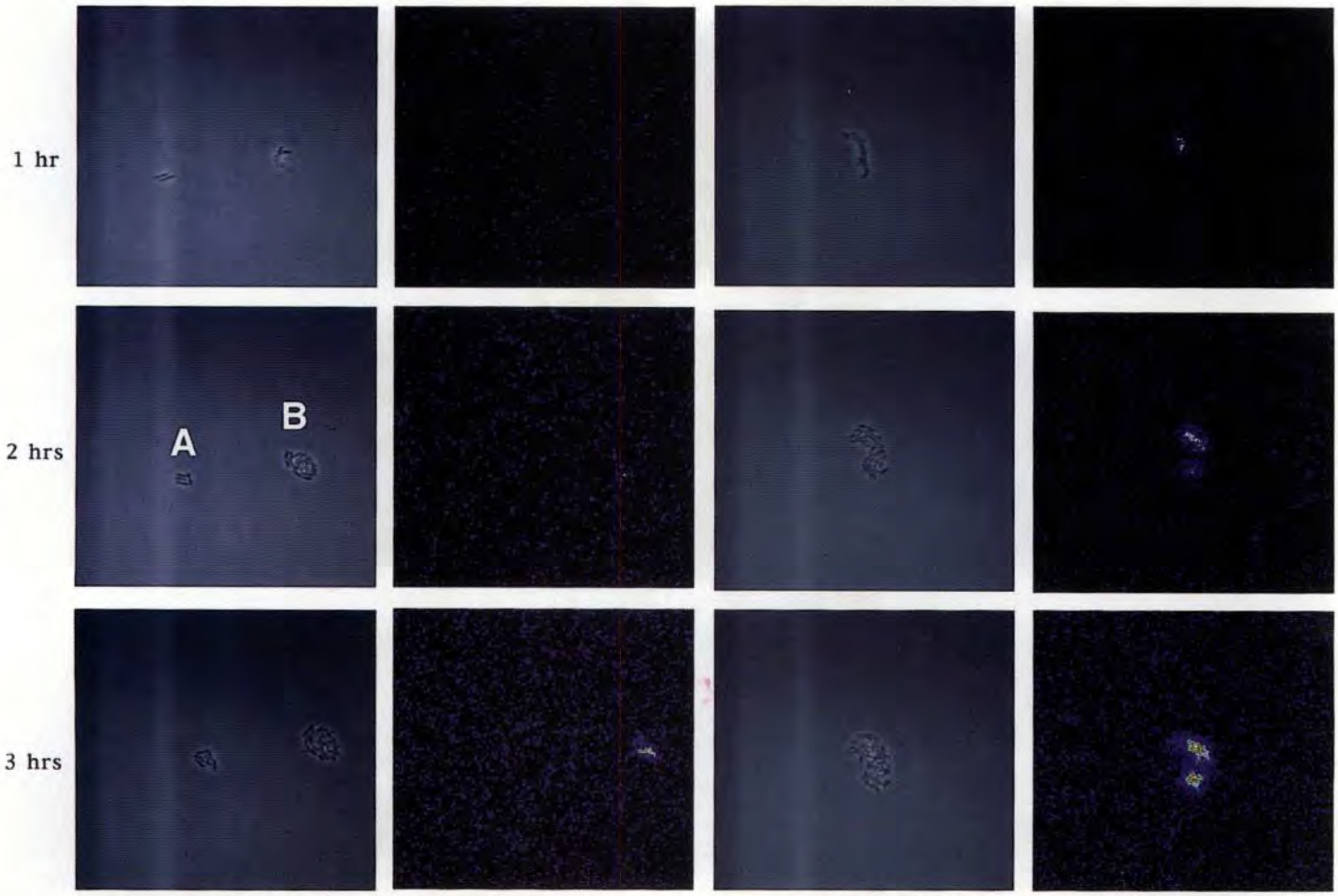
Laser Scanning Microscopy in Combination with Fluorescence Techniques for Biofilm Study, Figure 4. 2-photon (plate with 2/4 images 1p/2p).



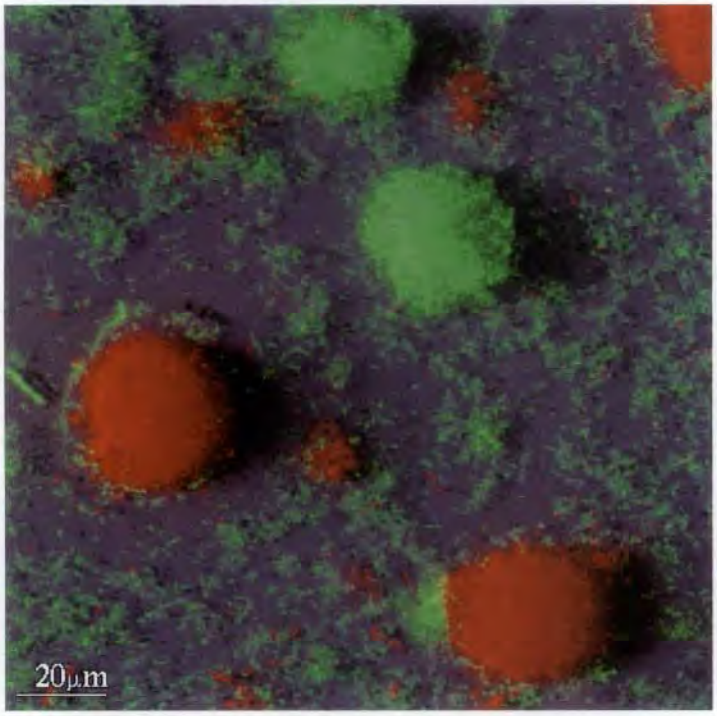
Laser Scanning Microscopy in Combination with Fluorescence Techniques for Biofilm Study, Figure 5. Stereo images (plate with red/green anaglyph and stereo pair).



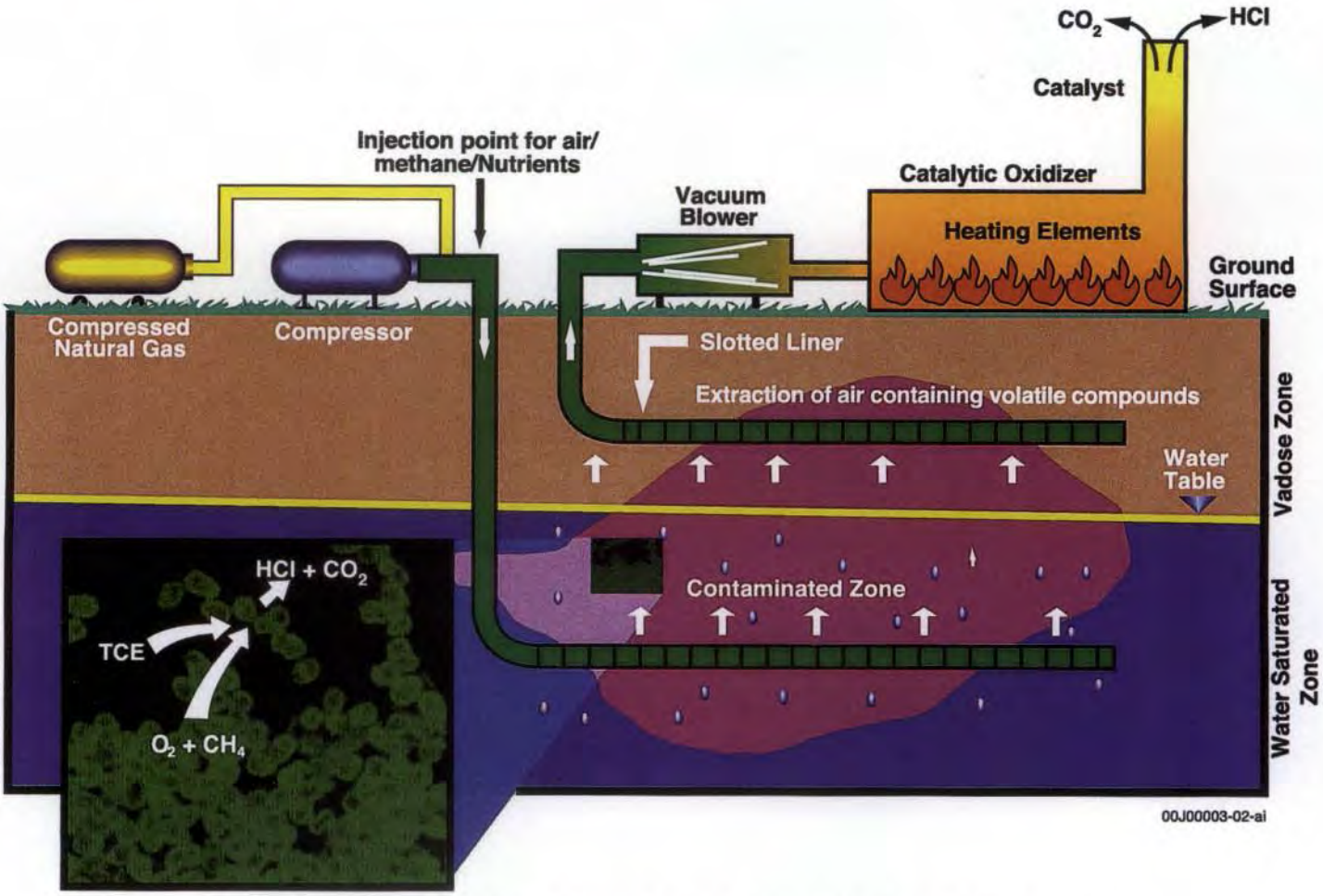
Laser Scanning Microscopy in Combination with Fluorescence Techniques for Biofilm Study, Figure 6. Bacteria and lectins 3/4 channels (plate with four images).



Luciferase and Green Fluorescent Protein as Bioreporters in Microbial Systems, Figure 1. Spatiotemporally resolved monitoring of toluene metabolism in genetically engineered *Psuedomonas putida* cells growing in a biofilm. Transmitted light images and photon-counting images (light production) of colonies in which a chromosomally integrated fusion between the toluene-degradation gene and luciferase (*tod-lux*) responds to the presence of toluene introduced at time zero. Three colonies were monitored over the course of three hours. The columns at the left show limited growth in two colonies (transmitted light images) and no induction (light production) from colony A, whereas colony B shows increased light emission over the three-hour monitoring period. The two right columns show marked growth and high light production from a third colony in the same experiment. This colony shows two distinct centers of light production (photon-counting image at three hours). The pseudocolor scale in the photon-counting images shows low light production in black and blue, with high light production in green through red/yellow.



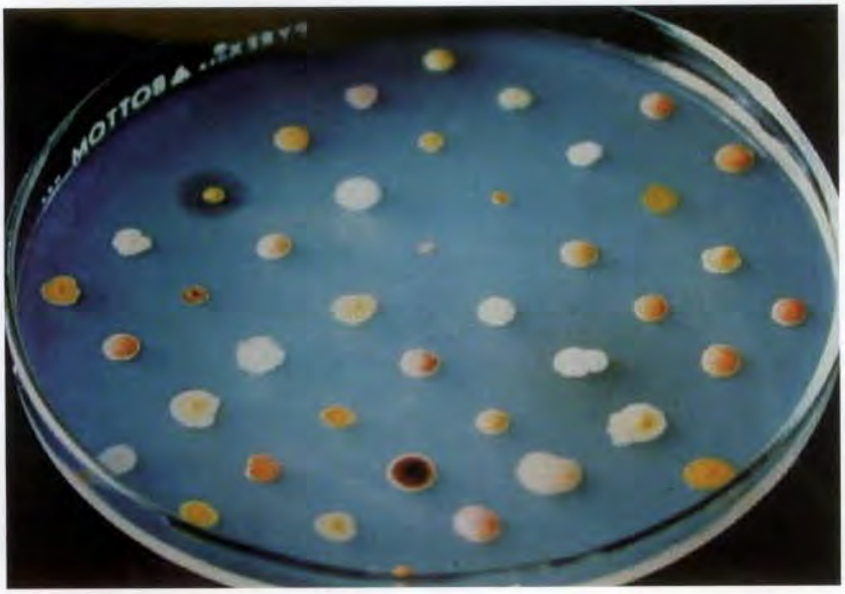
Luciferase and Green Fluorescent Protein as Bioreporters in Microbial Systems, Figure 3. *Psuedomonas* sp. B13 was transformed with either GFP or RFP (chromosomal integration of the fluorescent protein gene under control of a strong promoter), and the fluorescently labeled cells were inoculated simultaneously into an in vitro biofilm growth system. This confocal micrograph shows the biofilm five days of development. Note that the two bacterial types present in the inoculum, although isogenic except for the respective fluorescent protein gene, tend not to mix within the colonies.



Methanotrophic Bacteria: Use in Bioremediation, Figure 2. A side view of the horizontal wells in relation to the surface nutrient injection and extraction systems Modified from WSRC, Test Plan for In Situ Bioremediation Demonstration of the Savannah River Integrated Demonstration Project DOE/OTD TTP No.: SR 0566-01 (U), WSRC-RD-91-23, Westinghouse Savannah River Company, Aiken, SC, 1992.

Methanotrophic Bacteria: Use in Bioremediation, Figure 3. Concentrated groundwater methanotrophic bacteria on 0.2-μm filter labeled with fluorescent monoclonal-antibodies.

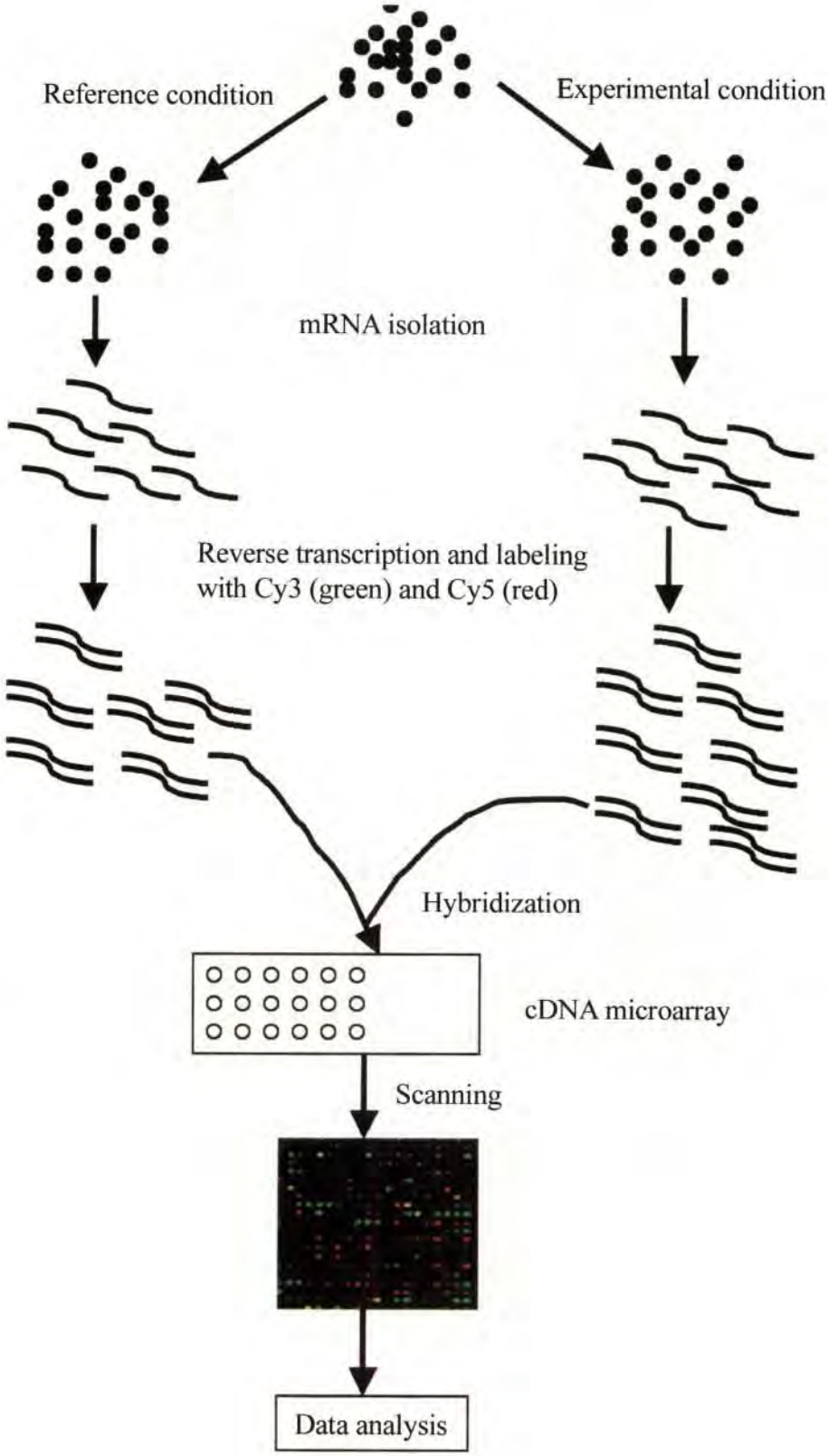
Methanotrophic Bacteria: Use in Bioremediation, Figure 4. In situ gaseous nutrient injection system at the Savannah River Site 70-acre nonradioactive waste disposal facility depicting the 400 ft and 600 ft horizontal wells. Insert is gas-nutrient pumping station.



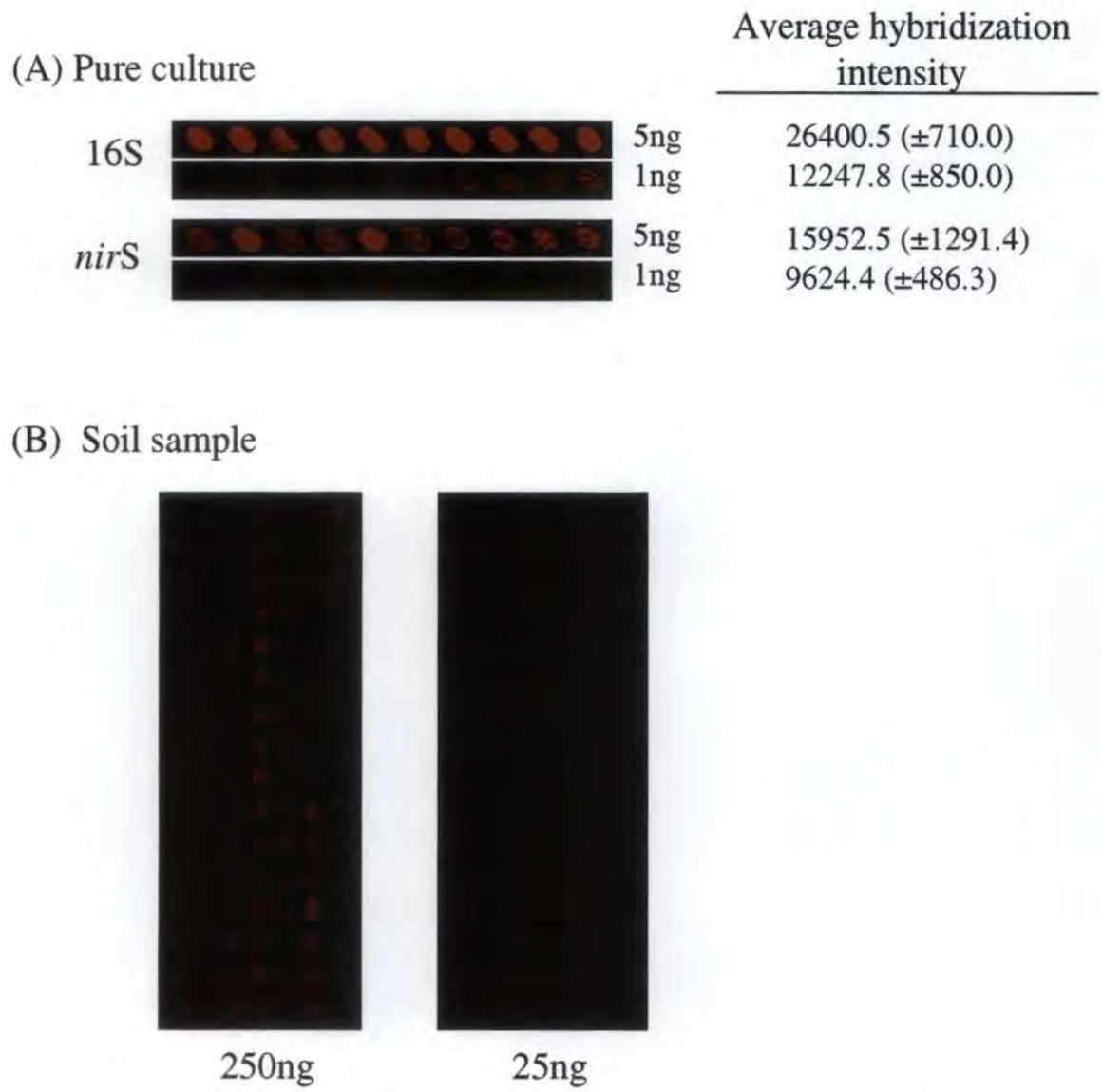
Methods for the Identification of Microbial Isolates, Figure 1. A bacterial garden of subsurface isolates applied to a grid after initial isolation from spread plates. Various colony morphologies and pigment production (e.g., dark colony surrounded by a light ring) can be useful during the identification process. Note: the colony surrounded by a dark ring demonstrates a physiological characteristic, such as agar degradation, that is potentially useful in identification.

Methods for the Identification of Microbial Isolates, Figure 3. Anaerobic chambers can be used to grow bacteria under defined atmospheric conditions. The gaseous composition can be manipulated to mimic natural environmental conditions.

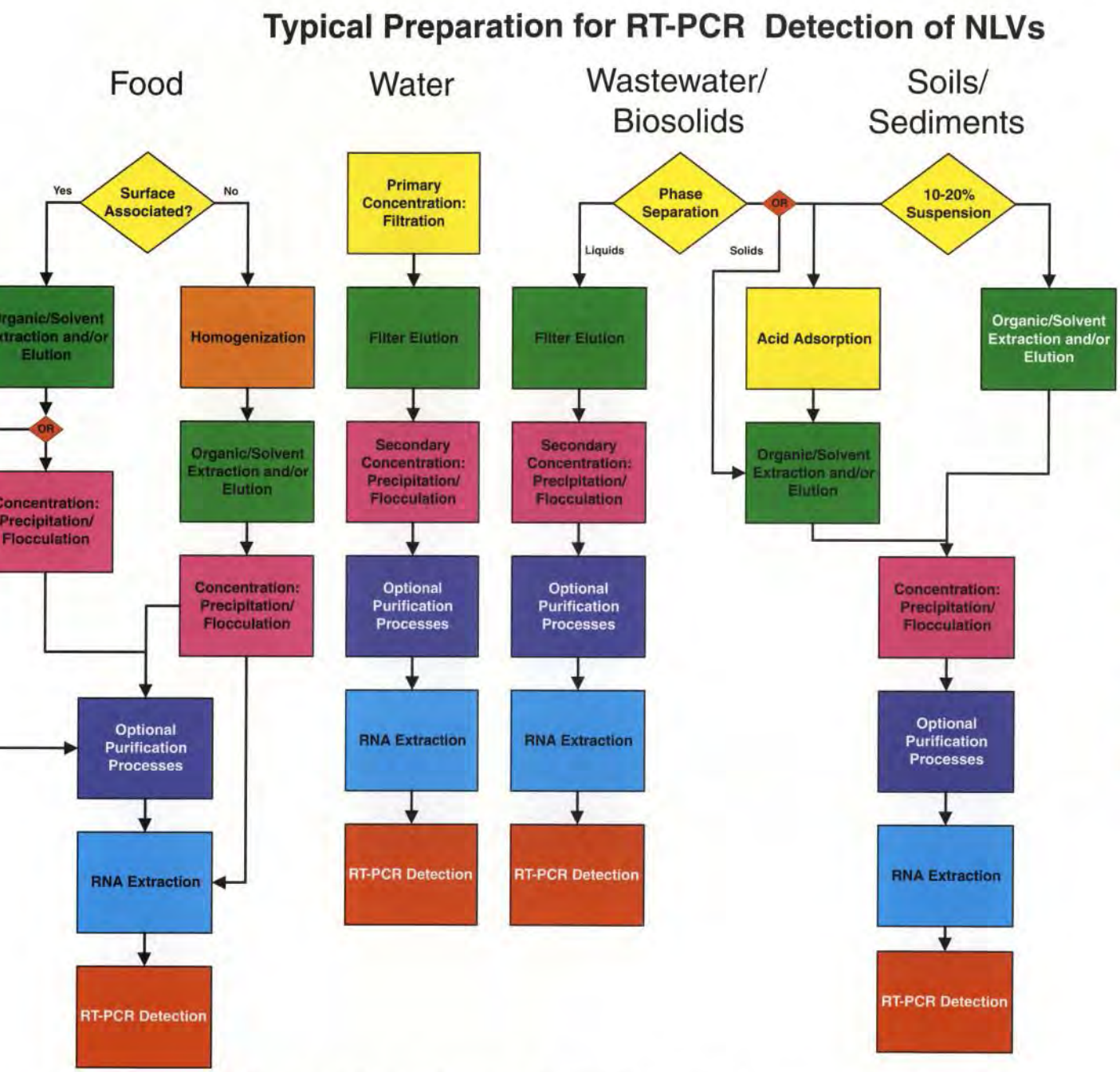
Methods for the Identification of Microbial Isolates, Figure 6. BIOLOG microtiter plates are used to create a metabolic fingerprint useful in the identification of bacterial isolates. Dark-colored wells indicate oxidation of individual carbon substrates.



Microarrays: Applications in Environmental Microbiology, Figure 2. General scheme of microarray experiments for monitoring gene expression.

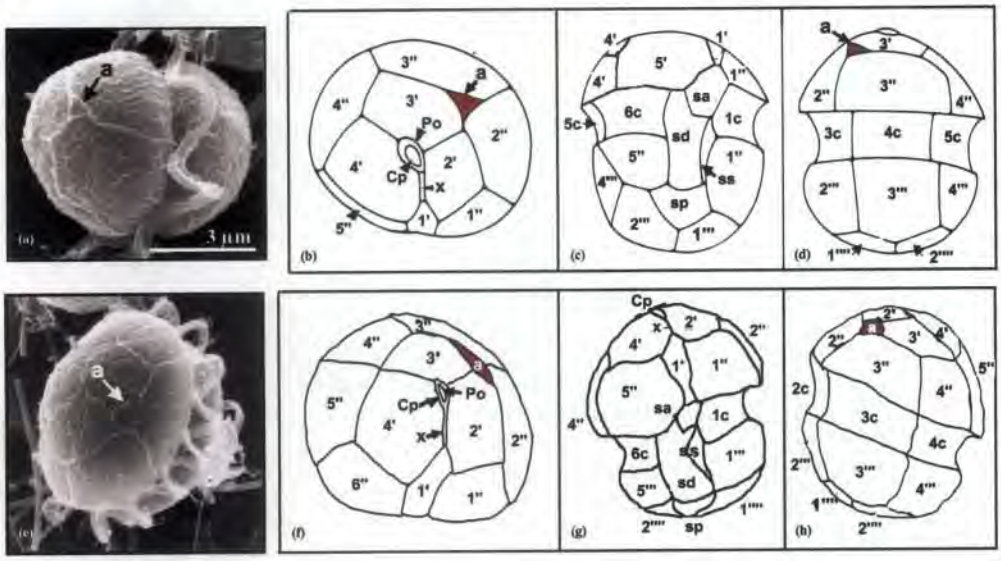


Microarrays: Applications in Environmental Microbiology, Figure 4. Array hybridization images showing the detection sensitivity with labeled pure genomic DNA and bulk community DNA from soil. (**a**) Genomic DNA from a pure culture of *nir*S-containing *Pseudomonas stutzeri* was labeled with Cy5 using a random primer labeling method. The target DNA was hybridized to nitrogen cycle microarrays at total concentrations of 1 and 5 ng. The average hybridization intensity at each target DNA concentration is presented. (**b**) Genomic DNA from surface soil was labeled with Cy5 as described in (**a**) and hybridized at total concentrations of 25 and 250 ng with the nitrogen cycle microarrays.

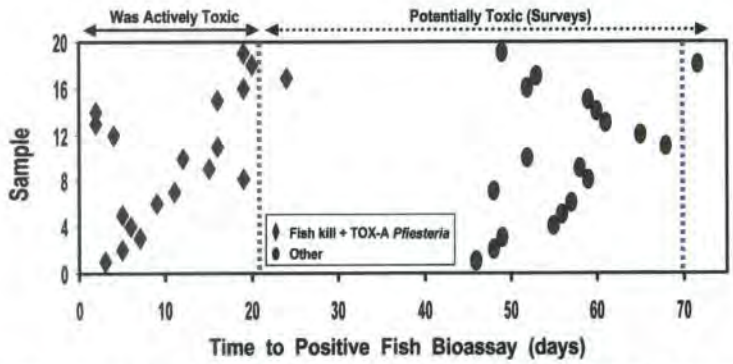


Norwalk-like Viruses: Detection Methodologies and Environmental Fate, Figure 1. Typical preparation of various samples for RT-PCR detection of NLVs. Corresponding steps between samples types are indicated by the same colored polygon.

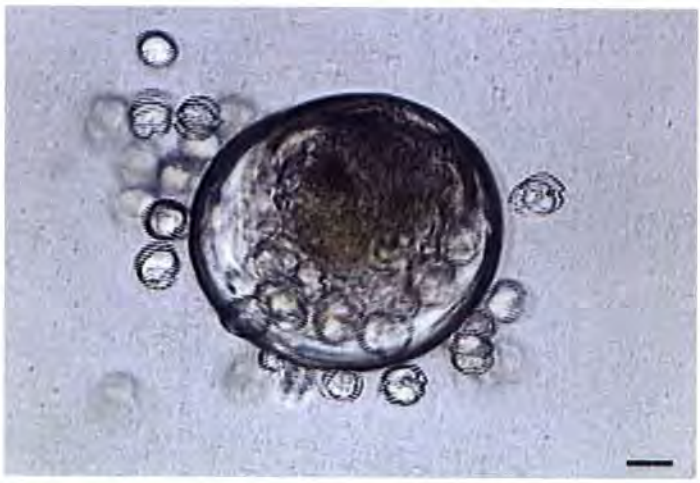
Pfiesteria, Figure 1. Light micrograph of a *Pfiesteria shumwayae* toxic zoospore (TOX-A functional type) with its peduncle (extended appendage used for attachment and feeding, arrow) attached to a larval sheepshead minnow (*Cyprinodon variegatus*), engaged in suctioning the contents from the tissue in active feeding (process called myzocytosis) (scale bar = 10μm). (From 1. J.M. Burkholder, H.B. Glasgow, and N.J. Deamer-Melia, Phycologia 40, (2001).



Pfiesteria, Figure 3. (**a**) Scanning electron micrograph of a suture-swollen zoospore of *Pfiesteria piscicida*, showing the three-sided anterior intercalary plate (**a**) (scale bar = 3μm); and (**b–d**) drawings of the plate structure of P. *piscicida* in (**b**) apical, (**c**) ventral, and (**d**) dorsal view (modified from 4. K.A. Steidinger et al., J. Phycol, 32, 157–164 (1996).); (**e**) scanning electron micrograph of a suture-swollen zoospore of *Pfiesteria shumwayae*, showing the four-sided anterior intercalary plate (**a**) (scale bar = 1μm; photograph by H. Glasgow, North Carolina State University); and (F-H) drawing of the plate structure traced from actual zoospores, including apical (**f**), ventral (**g**), and dorsal (**h**) views. (From 1. J. M. Burkholder, H. B. Glasgow, and N. J. Deamer-Melia, Phycologia 40, (2001).

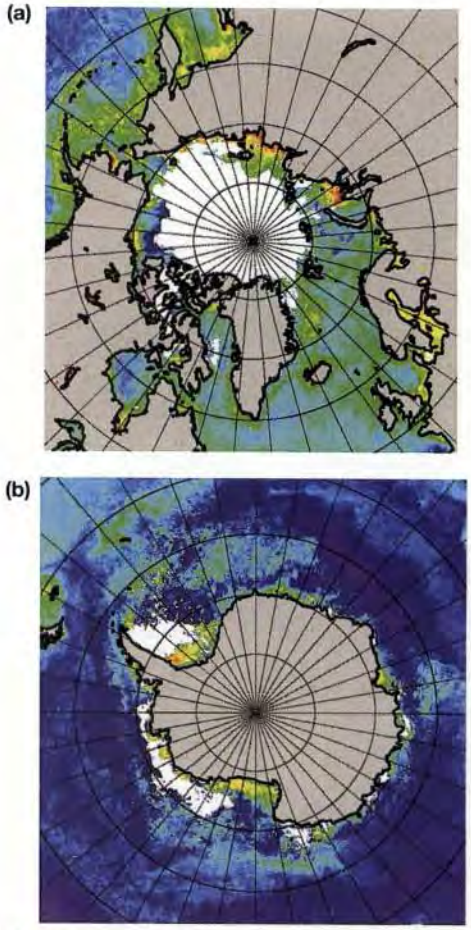


Pfiesteria, Figure 7. Comparison of the time interval required for positive fish bioassays (with fish-killing activity) for samples collected from estuarine fish kills in which actively toxic *Pfiesteria* spp.(TOX-A functional type) were implicated as primary causative agents (black diamonds; n = 20), versus samples that yielded toxic *Pfiesteria* in fish bioassays wherein the samples were taken during survey efforts in estuarine waters without diseased or dying fish (open ovals; n = 20)(8). The latter estuarine areas were interpreted to contain potentially toxic populations of TPC species (TOX-B functional type). In all cases, sample transport and other delays extended for 1 to 2 days; such handling is regarded as especially important for recently toxic populations, for example in efforts to implicate versus rule out involvement of actively toxic *Pfiesteria* from in-progress estuarine fish kills. Note that 19 of the 20 samples from events that we diagnosed as having involved actively toxic *Pfiesteria* were positive for fish-killing activity within 20 days (longest lag period, 1 sample within 24 days). To err conservatively, we consider that samples appropriately handled (with <3 days' lapse, including transport, following collection during an in-progress fish kill) should produce fish-killing activity within ≤21 days for actively toxic *Pfiesteria* to be implicated as a causative agent involved in the kill. Also note that samples that contained potentially toxic *Pfiesteria* populations that had not been recently in actively toxic mode toward fish did not show fish-killing activity until incubated with live fish for more than 6 weeks; 19 of the 20 samples from that set were ichthyotoxic by 10 weeks, with 1 sample requiring slightly longer. On the basis of well over 1,000 fish bioassays with estuarine samples, thus far we have not obtained toxic isolates of TPC species that have required more than 10.5 weeks to exhibit fish-killing activity, with two exceptions that required more than 12 weeks (8).

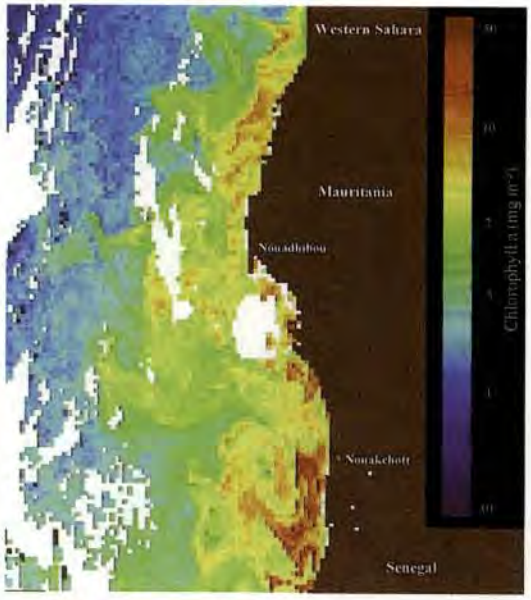


Pfiesteria, Figure 9. Actively toxic zoospores of *Pfiesteria piscicida* in the process of devouring an oyster pediveliger larva (scale bar = 10 μm), Note that many zoospores are swarming around the larva, and that some had used their peduncles to pry open the larva's shells to gain access to the soft tissue of the still-live organism, which were being rapidly consumed. The tissues of this larva were completely consumed by the zoospores within less than 30 minutes, except for the tough adductor muscle which was not attacked. (Photo from 36. J. Springer, Interactions Between Two Commercially Important Species of Bivalve Molluscs and the Toxic Estuarine Dinoflagellate, Pfiesteria piscicida, MS Thesis, North Carolina State University, Raleigh, N.C., 2000.)

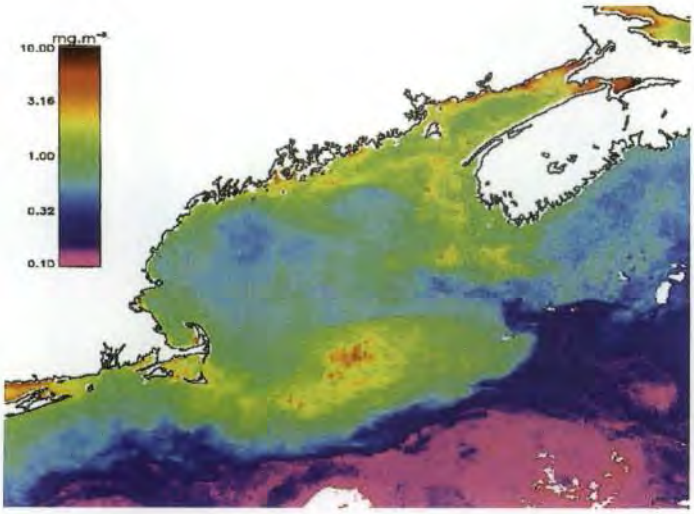
Pfiesteria, Figure 10. Focal lesion development resulting from exposure of tilapia (*Oreochromis mossambicus*, total length 5–7 cm) to actively toxic, clonal *Pfiesteria piscicida* in controlled laboratory trials, including (upper panel) Tilapia after 8–12 hr of exposure to 2.3 – 5.4 x 10^3 toxic zoospores/ mL (scale bar = 5 cm); and (lower panel) Oblique lateral view showing a deep, bleeding, ulcerated focal lesion posterior to the pectoral fin (scale bar = 1 cm).



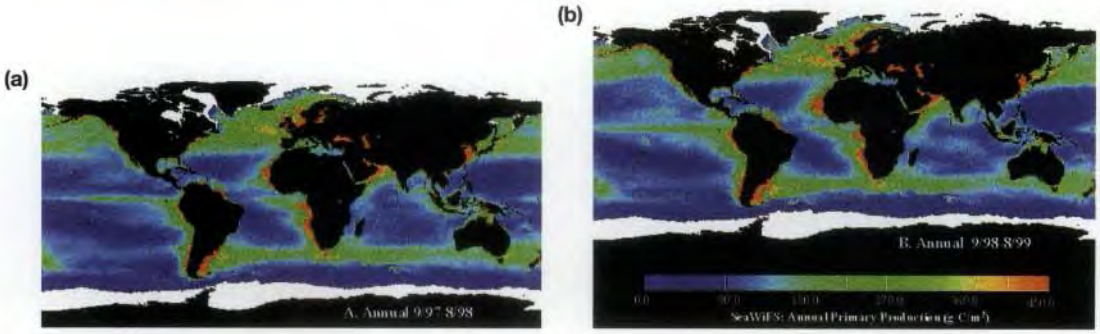
Polar Marine Phytoplankton, Figure 3. Satellite data showing polar projection maps of mean chlorophyll-a concentrations during summer in both polar regions. Highest chlorophyll-a concentrations are indicted by red, with decreasing concentrations as per the spectrum, with violet representing the lowest concentrations. The solid brown indicates areas where no data were obtained because of either ice or cloud cover.



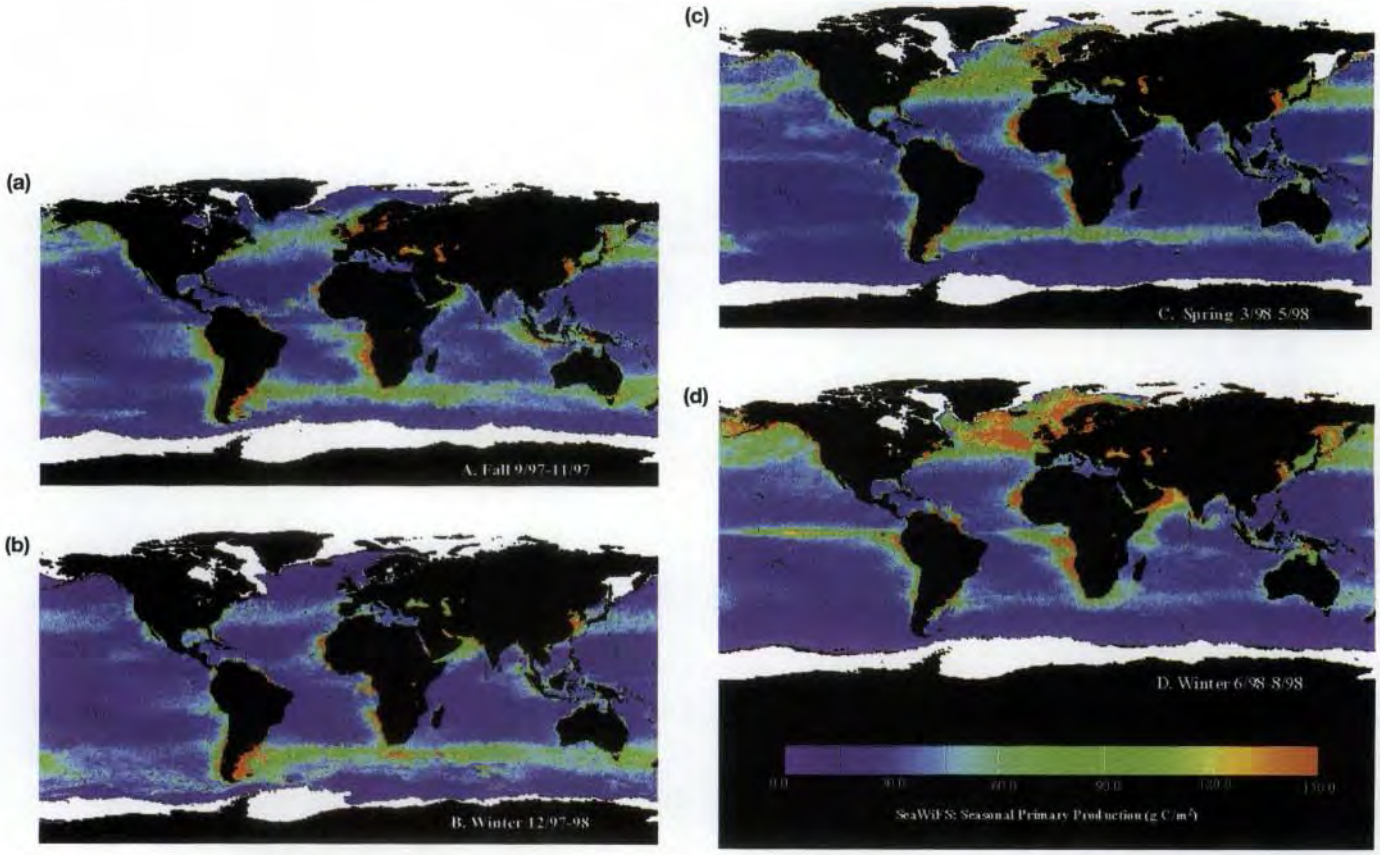
Primary Productivity in the Marine Environment, Figure 3. A SeaWiFS image of pigment concentrations off the coast of northwest Africa (March 22, 2001). Note the strong variations both offshore and along shore.



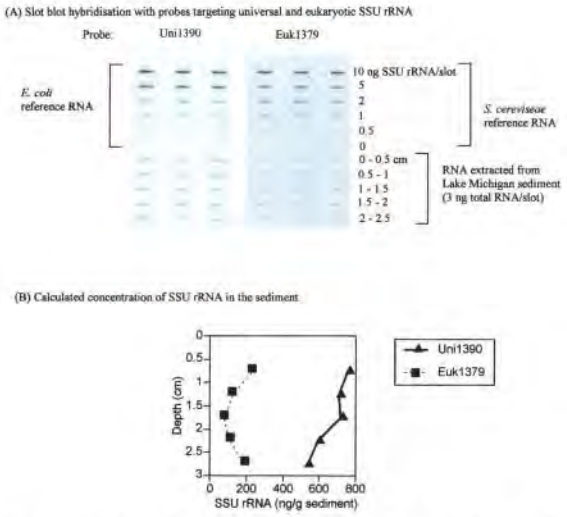
Primary Productivity in the Marine Environment, Figure 4. A SeaWiFS composite of pigment concentrations off the northeast coast of the United States (June 18–26, 1999). Note the presence of a large warm-core ring that substantially influences pigment concentrations and productivity.



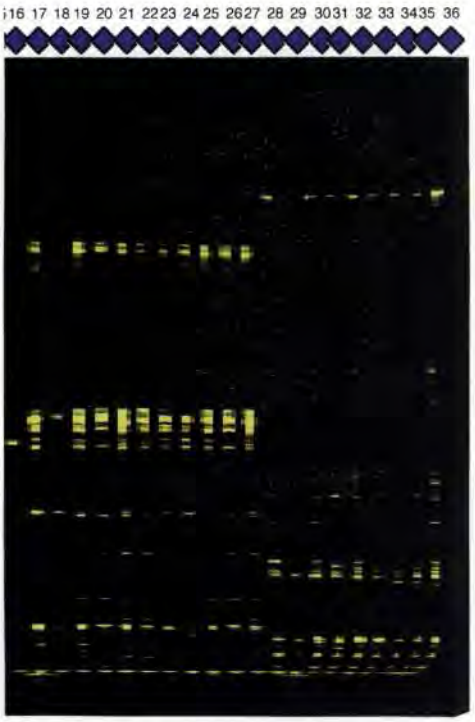
Primary Productivity in the Marine Environment, Figure 5. SeaWiFS annual composites of pigment concentrations throughout the ocean. (**a**) 1997–1998 and (**b**) 1998–1999. Strong spatial variations between years are observed, particularly in the equatorial pacific.



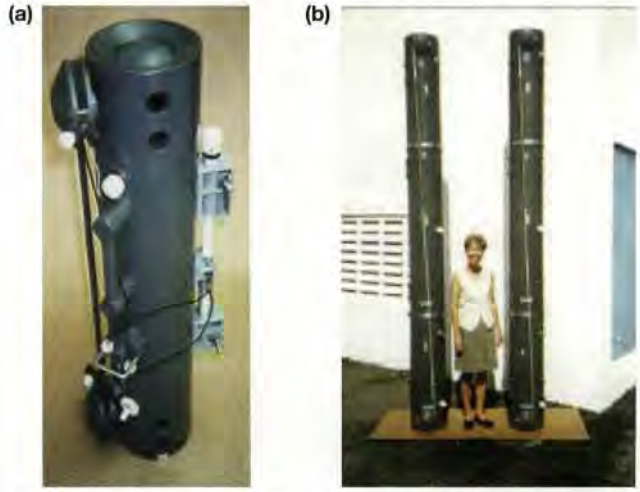
Primary Productivity in the Marine Environment, Figure 6. Estimates of primary productivity in (**a**) autumn, (**b**) winter, (**c**) spring, and (**d**) summer derived from satellite pigment estimates, incident irradiance, and the model of [M. Behrenfeld and P.G. Falkowski, *Limnol, Oceanogr.* **42**, 1–20 (1997)].



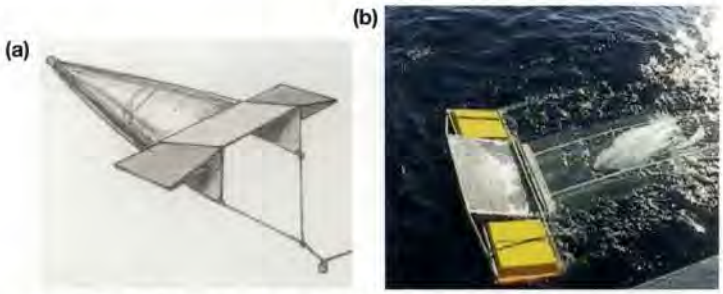
Ribotyping Methods For Assessment of In Situ Microbial Community Structure, Figure 2. Quantitative membrane forward-array hybridization. This experiment quantified the numbers of all microorganisms (Bacterial, Archaeal, and Eukaryotic) using a universal probe, and the numbers of eukaryotic microorganisms with depth in a freshwater lake sediment. The amount of probe bound was compared to the level of probe bound to standard quantities of Eukaryotic (*Saccharomyces cerevisiae*) and prokaryotic (*Escherichia coli*) SSU RNA. The quality of both the RNA and hybridization membrane are of crucial importance in such studies (17,23). Courtesy of Barbara J. MacGregor, Max Planck Institute for Marine Microbiology, Germany.



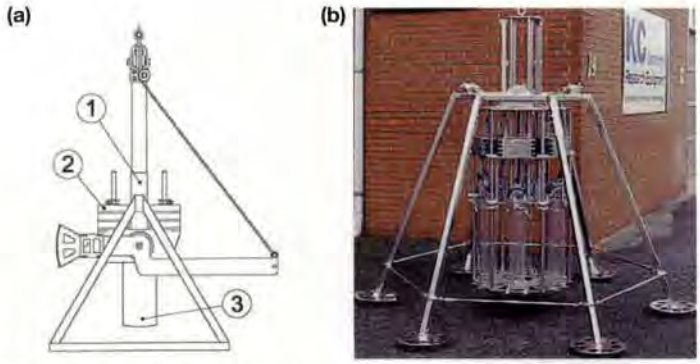
Ribotyping Methods For Assessment of In Situ Microbial Community Structure, Figure 6. T-RFLP analysis of bacterial communities. This digital image was generated by an ABI337 automatic sequencing apparatus and shows lanes 16–36 of a T-RFLP gel. Lane 16, the single band was produced from a pure culture of *Alcaligenes eutrophus*. Lanes 17–27 are profiles from DNA extractions from subsamples of a control soil, and lanes 28–36 are from a contaminated plot. The profiles of the control and contaminated soils are clearly different, demonstrating that the bacterial community has responded to the presence of the contaminant. Courtesy of Terrence L. Marsh, Department of Microbiology, Michigan State University, U.S.A.



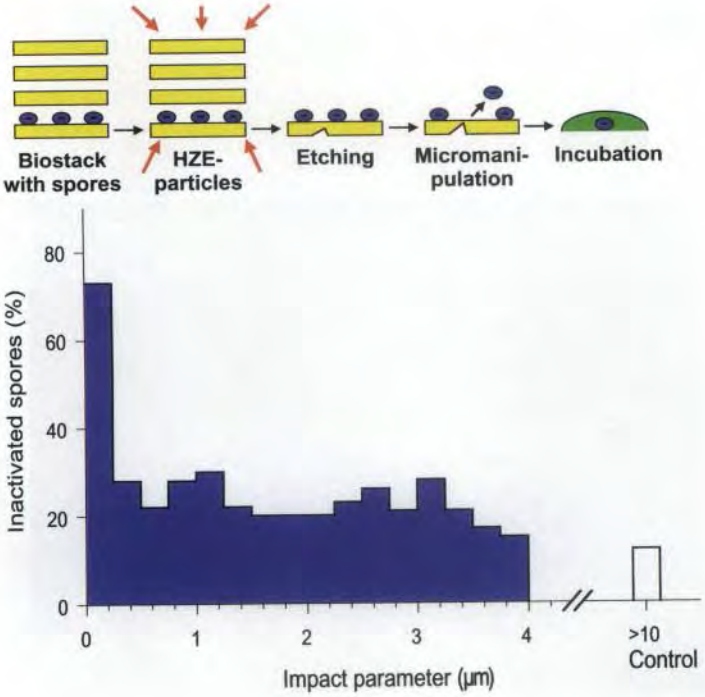
Sampling Techniques for Environmental Microbiology, Figure 2. Go-Flo sample bottle with closed top valve. Ball valves at each end are opened when the bottle reaches 1 atm pressure (about 30 ft) and are closed by a messenger (or electronically) when the bottle reaches the desired depth. Bottles typically range from 2.5 to 30L (**a**) but can be made as large as 200 L (**b**). Images and information generously provided by General Oceanics, Inc. (*http://www.General Oceanics.com*).



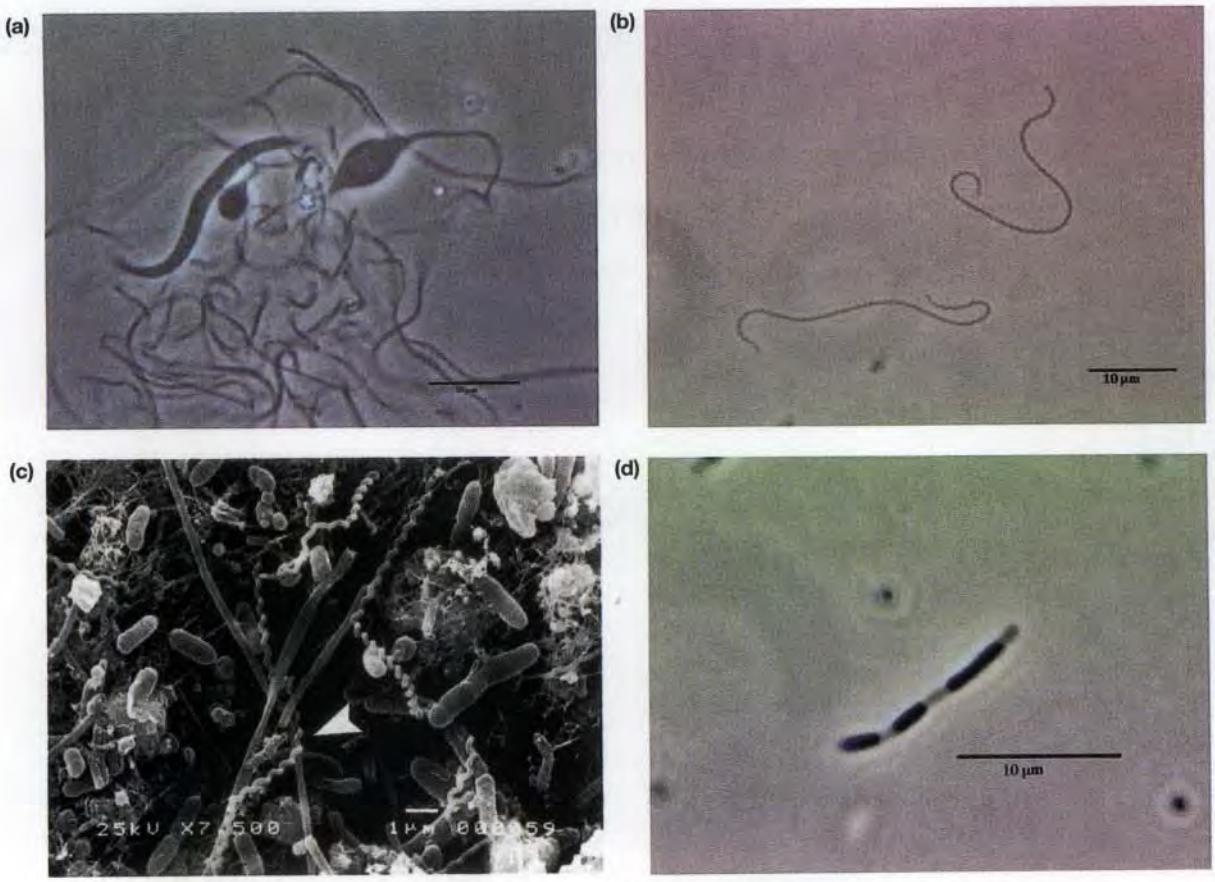
Sampling Techniques for Environmental Microbiology, Figure 3. Manta Net plankton sampler. (**a**) Diagrammatic representation. (**b**) Image of net in use. Other net designs are available for vertical hauls through the water column. Images courtesy of Ocean Instruments, Inc, San Diego, California (*http://www.oceaninstruments.com*).



Sampling Techniques for Environmental Microbiology, Figure 9. Examples of sediment corers. (**a**) Diagram of a box corer with the frame, weights, and sample tube enumerated, respectively. (**b**) Image of a multicorer with detachable legs, gyro-suspension, and polycarbonate sampling tubes. Both images were provided courtesy of KC Denmark (*http://www.kc-denmark.dk/*).

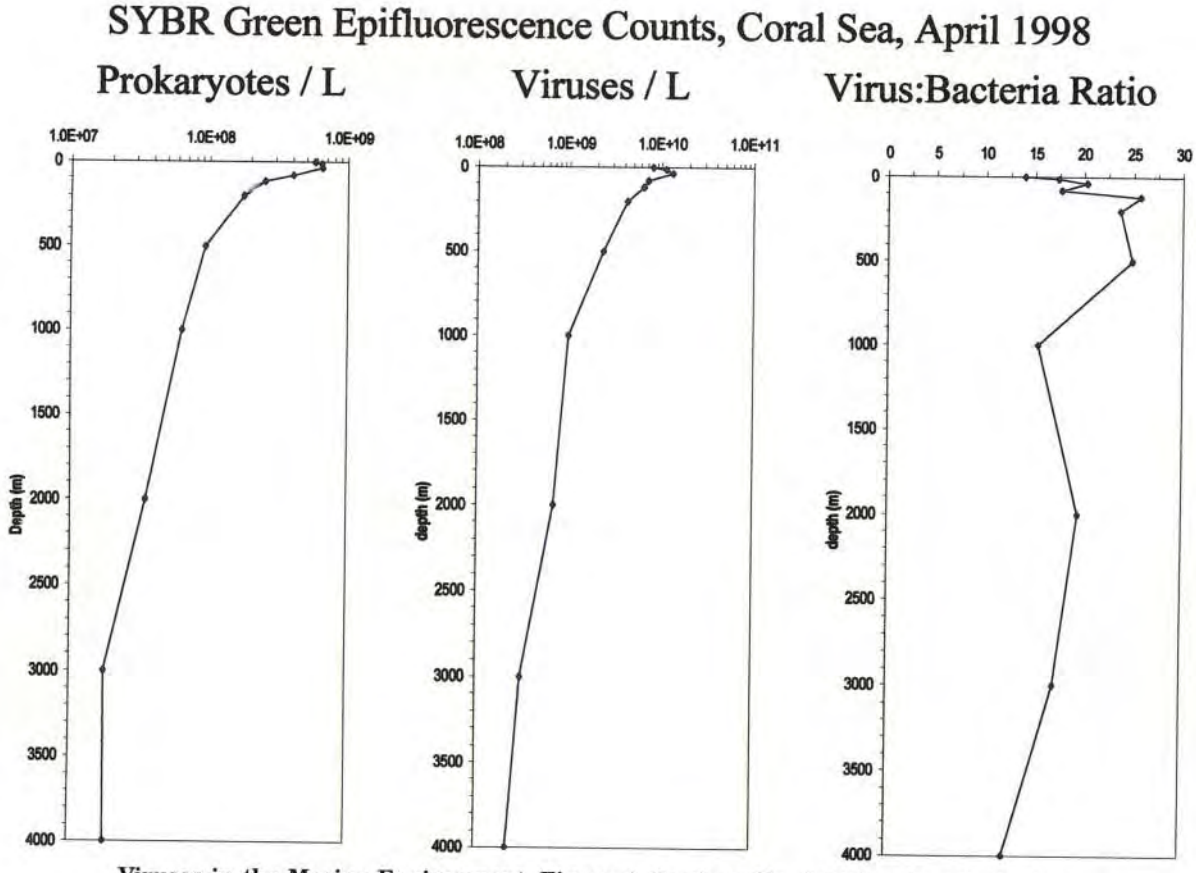


Space Microbiology: Effects of Ionizing Radiation on Microorganisms in Space, Figure 8. Results from Biostack experiments in space: inactivation probability of spores of *B. subtilis* as a function of the distance from the particles' trajectory (30).

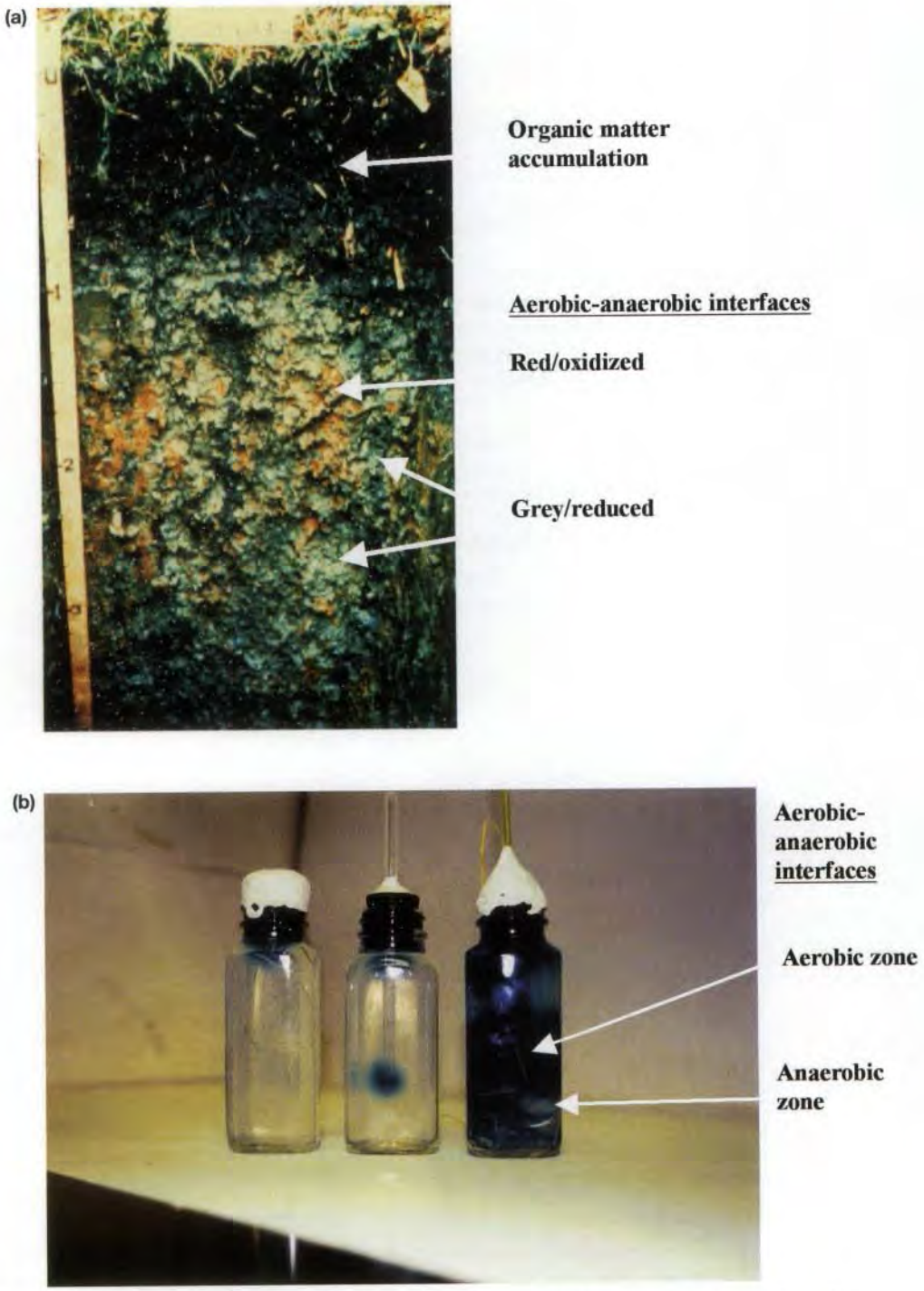


Thermophiles, Diversity of, Figure 2. (**a**) A phase contrast micrograph of a filamentous *Thermus* sp. isolated from Yellowstone National Park (courtesy C. Takacs). (**b**) A phase contrast micrograph of a thermophile spirochete isolated from Furnas, Azores, Portugal (courtesy P. Aguiar). (**c**) Scanning electron micrograph of a microbial mat community from a hot spring from Furnas, Azores, Portugal. Note the spirochete. Arrow points to a spirochete (courtesy P. Aguiar). (**d**) Phase contrast micrograph of a deep-sea hydrothermal vent Thermotogales isolate from Guaymas Basin, Mexico. Note the libear "Toga" around the dark cells (courtest M. Kendall).

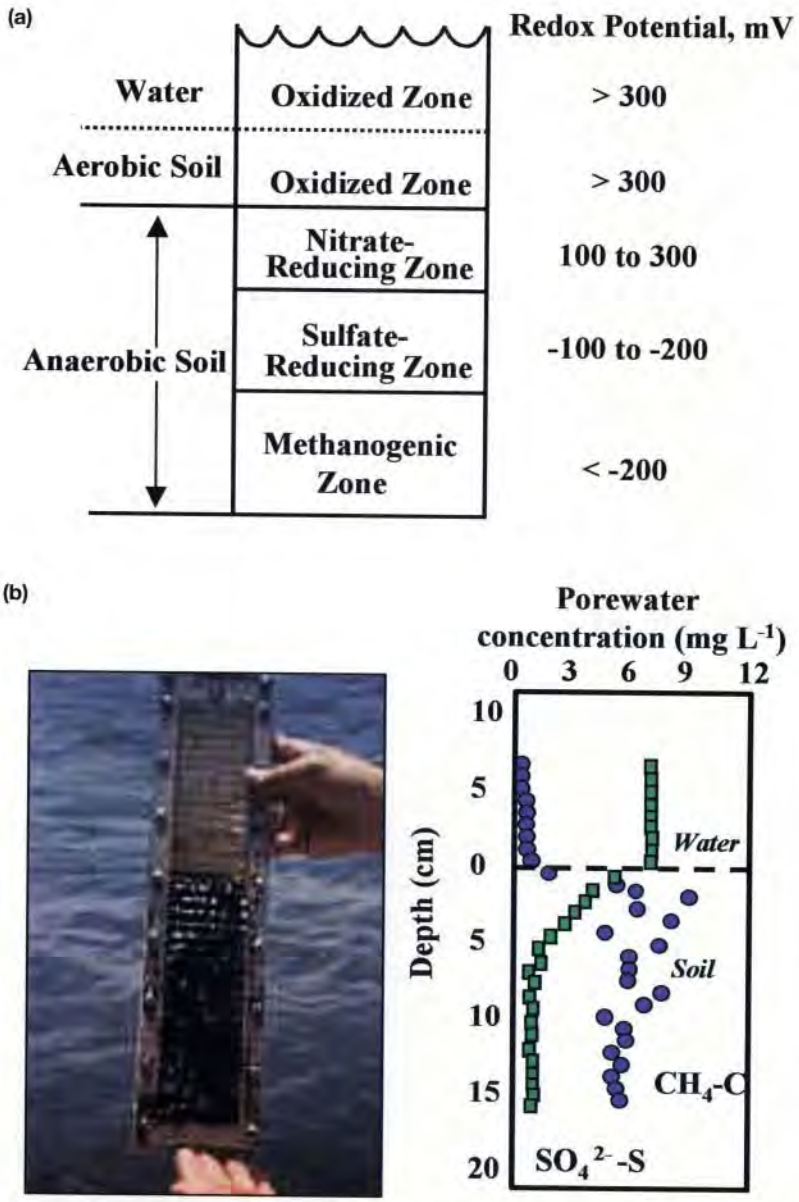
Viruses in the Marine Environment, Figure 3. Epifluorescence micrograph of prokaryotes and viruses from 16 km offshore of Los Angeles, stained with SYBR Green I (17). The viruses are the very numerous tiny dots, and the prokaryotes are the larger dots.



Viruses in the Marine Environment, Figure 4. Depth profile of total prokaryote (bacteria + archaea) counts, total viral counts, and virus: bacteria ratios from the Coral Sea (April 1998), as determined by epifluorescence microscopy of SYBR Green stained samples (17). Note the log scales.



Wetlands: Biodegradation of Organic Pollutants, Figure 2. Development of aerobic and anaerobic interfaces in wetlands. (**a**) Wetland soil profile showing accumulation of organic matter in surface horizon, and adjacent red (oxidized) and grey (reduced) zones in the subsurface soil. (**b**) One day root oxygen loss by rice plants (*Oryza* sp.). Methylene blue dye in vessels turns from clear to blue upon exposure to oxygen. Left: severed root sealed from atmosphere using rubber stopper and sealing putty; Center: glass tube open to atmosphere; Right: intact rice plant with upper leaves exposed to atmosphere.



Wetlands: Biodegradation of Organic Pollutants, Figure 4. Spatial distribution of aerobic and anaerobic processes in wetlands flooded for extended periods. (**a**) Conceptual diagram showing electron acceptor reducing zones as a function of soil depth, (**b**) Distribution of sulfate reduction and methanogenesis in a central Florida wetland as determined using pore water equilibrators.

Zooneustons, 2133
Zoonoses, 338
  Cryptosporidium and, 650–657
  Giardia and, 1469–1489
  leptospirosis and, 1806–1817
  Lyme borreliosis and, 1838–1849
  microsporidia as, 2065–2083
  Mycobacterium avium complex (MAC), 2112–2120
  Toxoplasma gondii, 3176–3183
Zoothamnium, 1117, 2620
Zostera, 2548, 2550–2554, 2822–2833
Zygnemataceae, Zygnema, 2302–2306
Zygogonium, 3142–3147
Zygomycetes, 2649
Zygomycota, 1367–1372, 1647, 2913, 3353–3364
Zygomycotina, 1359
Zygosphaera, 2508
Zymobacter, 1506–1512
Zymogenous (See Oligotrophic bacteria)